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Genetic Screens to Study GAA/TTC and Inverted Repeat Instability in *Saccharomyces cerevisiae*

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Abstract

Instability of trinucleotide and inverted repeats is a causative factor in the development of a number of neurological diseases, hereditary syndromes, and cancer. To understand the mechanisms that lead to repeat-induced genome destabilization it is important to identify factors that affect repeat metabolism. Here we present an approach that utilizes systematic and unbiased genome-wide screen in yeast *Saccharomyces cerevisiae* aimed to find genes that govern GAA/TTC and inverted repeat instability. These screens allowed for the identification of more than 30 mutants with increased fragility of both repeat motifs.

Keywords

Trinucleotide repeats; Inverted repeats; Genome instability; Double-strand breaks; Gross chromosomal rearrangements; Genome-wide screen

1 Introduction

DNA sequences that can adopt noncanonical secondary structures cause double-strand breaks (DSBs) and chromosomal rearrangements in humans and model organisms [1]. We have demonstrated that triplex-forming GAA/TTC repeats and long inverted repeats that form hairpin and cruciform structures induce chromosomal breakage in baker yeast *S. cerevisiae* [2–5]. One of the consequences of repeat-induced breaks is gross chromosomal rearrangements (GCRs). In the GCR reporters, initially developed in Dr. Kolodner's laboratory [6], the repeats are inserted next to the counterselectable marker *CAN1* on the left arm of chromosome V. DSBs at the fragile motifs lead to loss of the 40 kb telomere-proximal fragment and subsequent repair events restoring the telomere at the broken chromosome. These GCRs cause loss of *CAN1* and give rise to the growth of canavanine-resistant colonies. Depending on the nature and length of the repeats, the GCR levels are increased from 100- to 1000-fold due to the presence of the fragile motifs [3, 5, 7].

The GCR assay is also a convenient tool to monitor changes in the level of instability of the trinucleotide and inverted repeats. To systematically identify genes that control repeat metabolism we combined the repeat carrying GCR cassette marked with *hphMX* with mutant alleles marked with *kanMX*. We utilized three Open Biosystem mutant collections and employed a modified genome-wide approach developed by C. Boone and colleagues [8]. This analysis allowed us to test a complete set of 4786 deletion mutations (YKO collection) and ~800 essential genes, for which expression is either regulated by doxycycline (yTHC collection) or compromised due to mRNA perturbation (DAmP collection). As a result, we

have identified 33 mutants with augmented GAA/TTC fragility [4, 5]. Upon further examination, we found that the same mutants exhibited an increase in the large-scale expansions in the *URA3*-artificial intron assay [9] indicating the existence of common intermediates that can drive both types of instability. Moreover, the identity of mutants led us to discover the replication-dependent mechanism of GAA/TTC instability operating during S-phase and transcription-dependent breakage in nondividing cells. In the case of inverted repeats, genome-wide screen revealed that depletion or lack of components of the DNA replication machinery, the replication-pausing checkpoint pathway, proteins involved in Fe-S cluster biogenesis, the telomere maintenance complex or the Sgs1-Top3-Rrm1 dissolvasome augment fragility at the *Alu*-quasi-palindrome [4].

Below, we describe in details how we combined a GCR-based genome-wide screen approach for systematic identification of genes responsible for maintenance of repeat stability. Potentially, similar approach can be utilized (1) to identify not only hyperfragility but also hypofragility mutants; (2) to study instability of other secondary structure-forming motifs (e.g., CTG/CAG and CCG/CCG repeats); (3) to use other developed libraries based on the BY4730/BY4741 background (e.g., a collection of temperature-sensitive mutants [10]).

2 Materials

2.1 Media and Solutions

1. YPD: dissolve 10 g yeast extract, 20 g peptone and 20 g glucose in 950 mL water in a 2–3 L flask, adjust to 1 liter, add 17 g agar. Autoclave for 30 min, at 120 °C and pour ~35 plates after cooling media to 55 °C.
2. YPD + G418 and Hygromycin B: Autoclave 1 L of YPD medium, cool down to 55 °C, add 3 mL of G418 solution (100 mg/mL stock solution in water made from the powder) and 6 mL of Hygromycin B solution (50 mg/mL stock solution in PBS).
3. YPD + doxycycline: Autoclave 1 L of YPD medium, cool down to 55 °C, add 1 mL of doxycycline (2 mg/mL stock solution in water). Cover plates with foil to prevent exposure of plates to the light.
4. Sporulation medium: dissolve 20 g potassium acetate, 2.5 g Bacto yeast extract, 1 g glucose, 20 mg adenine sulfate, 20 mg L-arginine-Ha, 100 mg L-aspartic acid, 20 mg L-histidine-HCl, 100 mg L-glutamic acid, 30 mg L-isoleucine, 100 mg L-leucine, 30 mg L-lysine-HCl, 20 mg L-methionine, 400 mg L-serine, 50 mg L-phenylalanine, 200 mg L-threonine, 20 mg L-tryptophan, 30 mg L-tyrosine, 20 mg uracil, 150 mg L-valine in 950 mL water in a 2–3 L flask. Adjust pH to 7.0 with KOH and add water to 1 L. Add 17 g agar, autoclave for 30 min, at 121 °C and pour plates after cooling media to 55 °C.
5. SD –his, SD –arg, SD –trp, or SD –ura dropout: Prepare each of them as a mix of the following 16 compounds, omitting one required amino acid or base (e.g., SD –his is prepared by mixing 15 out of the 16 compounds, omitting histidine): 20 mg adenine sulfate, 20 mg L-arginine-HCl, 100 mg L-aspartic acid, 20 mg L-

histidine–HCl, 100 mg L-glutamic acid, 30 mg L-isoleucine, 100 mg L-leucine, 30 mg L-lysine–HCl, 20 mg L-methionine, 400 mg L-serine, 50 mg L-phenylalanine, 200 mg L-threonine, 20 mg L-tryptophan, 30 mg L-tyrosine, 20 mg uracil, 150 mg L-valine.

6. SD –his + cycloheximide: SD –his dropout, 6.7 g yeast nitrogen base with ammonium sulfate and without amino acids, 20 g glucose, 17 g agar. Autoclave for 30 min, at 120 °C, cool down to 55 °C, add 0.5 mL of cycloheximide solution (10 mg/mL stock solution in DMSO) and pour plates.
7. Canavanine: SD –arg dropout, 6.7 g yeast nitrogen base with ammonium sulfate and without amino acids, 20 g glucose, 17 g agar. Autoclave for 30 min, at 120 °C, cool down to 55 °C, add 3 mL/L (20 mg/mL canavanine stock solution in water) and pour plates.
8. 5-FOA: Filter-sterilize 7 g yeast nitrogen base with ammonium sulfate and amino acids, 1 g 5-fluoroorotic acid, 50 mg uracil, 20 g glucose dissolved in 500 mL water. Warm up solution to 50 °C. Autoclave 500 mL of 4% agar, cool down to ~60 °C and mix with prewarmed 5-FOA solution. Pour plates.
9. Galactose medium: Filter-sterilize 2% galactose, 1% yeast extract, 2% Bacto peptone.

2.2 Accessories

1. We used a simple template for patching yeast cells depicted in Fig. 1. For the best results in visual identification of the GCR-caused papillae on the plate, we suggest crossing the query strains with the mutant strains from the libraries by making 6 patches of the control and experimental strains (*see below*), per plate. Each patch should be approximately 0.7 cm wide.
2. To reduce the cost of the replica-plating experiments, we custom made 6" velveteen squares by cutting several yards of velvet fabric.

2.3 Yeast Strains and Plasmids

1. Query strains are derivatives of BY4742 from Open Biosystems (MAT α , *ura3*⁻, *leu2*⁻, *his3*⁻, *lys2*⁻). The genotype of the query strain, used in the screens is: MAT α , *ura3*⁻, *leu2*⁻, *his3*⁻, *lys2*⁻, *rpl28-Q38K*, *mfal* ::MPA1pr-HIS3, *V29617::hphMX*, *V34205::lys2::Fragile motif*. Fragile motifs inserted into *LYS2* were (GAA/TTC)₂₃₀, or the 100% and 94% homologous inverted *Alus*. For query strain construction, *see* Subheading 3.
2. The three collections of tester strains: yTHC (~860 strains), DAmP (~860 strains), YKO (4786 strains), were purchased from Open Biosystems (currently they are available from Dharmacon (<https://dhannacon.horizon-discovery.com>)). The yTHC collection is a set of strains where the expression of essential genes is under control of the doxycycline-regulatable TET promoter. The DAmP collection is a library of strains where the expression of essential genes is reduced by four to tenfold due to destabilization of their mRNA. The YKO

collection is a set of gene-disruption mutants. In all three cases, mutant alleles are labeled with the *kanMX* cassette.

3. *MATa* and *MATa* *lys5* mating type tester strains.

3 Methods

1. Query strain construction.
 - a. The *LYS2* fragment from the pFL39*LYS2* plasmid [11] was PCR amplified with primers canlys5 and canlys3 that carry homology to the *V34205* region (Table 1). The PCR product was transformed into the BY4742 strain to insert *LYS2* on the left arm of chromosome V telomere distal to *CAN1* (see Note 1).
 - b. The mating type of this strain was switched to *MATa* using the pJH132 plasmid (gift from Dr. James Haber) carrying the HO gene under control of the GAL1-GAL10 promoter. Transformants were selected on the SD –trp medium. Then, they were inoculated into 5 mL of galactose medium and shook for 2–4 h to induce HO expression and plated on YPD to get individual colonies. Colonies were patched on YPD and crossed with α - and a-specific *ade5* testers to identify *MATa* isolates. *MATa* clones that lost pJH132 were selected on 5-FOA media.
 - c. The *MFA1pr-HIS3* cassette was PCR amplified from y2454 [12] (gift from Dr. Kyungjae Myung) using primers mfa1-amp5 and mfa1-amp3 (Table 1) and inserted into the *MFA1* locus. Transformants were selected on SD –his medium.
 - d. The mating type was switched back, to *MATa* using pJH132 as described in 1.2.
 - e. The *hphMX* cassette was PCR amplified from the pAG32 plasmid [13] and inserted telomere proximal from *CAN1* on chromosome V at the position V29617 using km-5v and km-3v primers (Table 1).
 - f. We obtained spontaneous resistant colonies to the cycloheximide (YPD media supplemented with 5 mg/L cycloheximide). Selected clones were sequenced and found to contain the *rpl28-Q38K* mutation, identified in another study where cycloheximide was used as a selection drug [14].
 - g. The (GAA)₂₃₀ tract was moved to the *Bam*HI site of *LYS2* via *delitto perfetto* [15]. First, the CORE cassette (*URA3::kanMX*) was PCR amplified from the pCORE plasmid using primers lys2-core5 and lys2-core3. G418^RUra⁺Lys[–] transformants were used to verify insertion of CORE into *LYS2*. Second) (GAA)₂₃₀ tract was PCR amplified from the pYES-GAA228 plasmid [16] using primers 230PF and 230PR (Table 1). The resulting PCR fragment was used to replace CORE in *LYS2*. The correct replacements were verified in 5-FOA^R and G418^S clones by PCR using *LYS2*-specific primers: cpcr3 and s3393.

- h.** The 100% or 94% homologous inverted *Alus* were integrated into the chromosomal *LYS2* gene using plasmids pHS-I and p36-I via pop-in and pop-out method as previously described [11].
- 2.** Preparation of query and tester strains for the experiment. Patch MAT α query strains marked with hphMX (containing 100% or 94% homologous inverted *Alus* and (GAA)₂₃₀ tract) and 6 MATa tester strains carrying *kanMX* on YPD plates overnight in the following way. Streak out query strains for single-colonies and verify the presence of the unchanged (GAA)₂₃₀ tracts using colony PCR (this step is unnecessary in the case of strains with inverted repeats). Patch cells from single colonies in duplicates using template depicted on Fig. 1a. Defrost cells from the collections frozen in glycerol in 96 well plates on the bench, mix by pipetting using a multichannel pipette and spot 10 μ L of the cultures from 6 wells containing mutants to one side of the YPD plate as a column. Allow yeast to grow at 30 °C overnight and extend the original 6 spots to 6 rows of long patches by spreading the cells with flat-ended toothpicks (Fig. 1b). The overall scheme for combining mutant alleles marked with *kanMX* cassette and repeat-induced GCR assay is depicted in Fig. 2. Use the following protocol to carry out the screen (see Note 2).
 - 3.** Cross strains by replica-plating and incubate at 30 °C on YPD overnight.
 - 4.** Select diploids on medium containing 300 mg/L G418 and 300 mg/L hygromycin B.
 - 5.** Replica-plate diploids to sporulation medium and incubate for 5 days at 23 °C.
 - 6.** Transfer a mixture of diploids and asci containing MATa/MAT α haploid tetrads to SD –his plates supplemented with 5 mg/L cycloheximide. Incubate for 2 days. Since the query strain carries a mating type-specific reporter MFA1pr-*HIS3* and a recessive mutation *rpl28-Q38K* that provides resistance to cycloheximide, this procedure allows selecting only for MATa haploid progeny. The MFA1 promoter allows to eliminate MAT α haploids) while the presence of *rpl28-Q38K* prevents growth of MATa/MATa diploids that occur due to the gene conversion of mating type locus. To assure the selection of haploids, repeat this step.
 - 7.** Select MATa haploids harboring the chromosome V with repeats and mutated genes on medium containing G418 and hygromycin B.
 - 8.** In experiments involving YKO and DAmP collections, replica-plate cells to canavanine-containing plates. Strains from the yTHC collection incubate overnight on YPD medium containing 2 μ g/mL doxycycline and then replica-plate to cana-vanine-containing plates.
 - 9.** Evaluate the GCR-inducing capacity of tested mutants after incubation of yeast on canavanine media for 5 days. Record the number of revertants on canavanine plates on square patches resulting from the crosses of query strains with mutants and compare this amount with patches coming from the control crosses of repeat-containing strains with wild-type. Since the GCR rate induced by (GAA)₂₃₀ repeats ($\sim 2 \times 10^{-6}$) and 100% *Alu*-IRs ($\sim 2 \times 10^{-5}$) is ~ 10 and 100

times, correspondingly, higher than the mutation rate ($\sim 3 \times 10^{-7}$) at the *CAN1* locus in wild-type strains, the screen preferentially selects for GCR inducers or strong mutators (*see* Note 3).

4 Notes

1. Repeat tracks that can be amplified by PCR, such as GAA/TIC repeats, might be targeted next to *CAN1* directly using *delitto perfetto* approach [15], so insertion of *LYS2* can be omitted. Unlike trinucleotide repeats, inverted repeats cannot be amplified by PCR using currently available thermostable polymerases (K S. Lobachev, unpublished data). Thus, we had to use already available *lys2::IR* alleles which in turn necessitated *LYS2* integration.
2. Selection of diploids, induction of sporulation, selection of MATa progeny might be done in liquid cultures using robot systems that is utilized for replica pinning procedures (e.g., described in Costanzo and Boone [17]). However, the frequency of the repeat-induced GCRs is too low to readily detect changes in the number of canavanine-resistant papillae when pin-sized prints are replica-plated. Therefore, upon using robotic systems two last steps should be carried out similarly to what is described in this protocol by making approximately 0.7×0.7 cm patches of cells.
3. The validity of the effects detected in the identified mutants in the genome-wide screen should be verified by recreating the hyperfragile alleles in strains where the consequences of GCR events can be easily distinguished from mutations in *CAN1*. This step is also required to prevent erroneous annotations, unavoidable in large collections of yeast strains. We reassessed the effect of the identified mutants by recreating the hyperfragile alleles in strains with the *ADE2* gene inserted between *CAN1* and repeats that allows differentiation of GCRs from mutations based on the color of canavanine-resistant clones [4, 5].

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Fig. 1. Preparation of the query and tester strains for the screen. **(a)** Template for making plates with six patches of 0.7 cm wide. **(b)** Diagram for the recovery of the tester strains from collections and making streaks for crosses

Fig. 2. Methodology for genome-wide screens. (a) Flowchart of the screen (see detailed description in the Methods). (b) Relevant constructs in query strains for the discovery of the genotypes with increased inverted and GAA/TTC repeat fragility. The 100% or 94% homologous inverted *Alus* (*Alu*-IRs) and 230 copies of GAA/TTC repeats ((GAA)₂₃₀) were integrated next to *CAN1* on the left arm of chromosome V. The location of *HIS3* ORF under control of a MATa-specific promoter and *rpl28-Q38K* allele on chromosomes IV and VII, correspondingly, are shown. (c) Example of the screening plate. Columns are duplicates of the query strains containing repeats. Each row is one of the tester strains from the collections. The level of arm loss in TET-*PBL1*, TET-*TAD3*, TET-*BMS1*, TET-*SPT6*, is comparable to the GCRs observed in wild-type (not shown). Visible increase in the number of canavanine-resistant colonies at the intersections of the patch of the strain with down-regulated *RFC4* gene and patches of the strains with the GCR assay indicates that disruption of PCNA-loading RFC complex leads to destabilization of inverted *Alus* and GAA/TTC trinucleotide repeats (red and blue boxes, respectively)

Table 1

Primers used

Name	Sequence
canlys5	5' GTTCTTGTCTAATTAATTGTAACATAATTTATGCGCCGCCCAATTAATAAATAATTCGGCGG3', ^a
canlys3	5' GATACGATTACTCCAGTTCCTTTACAAGAAGCGCGCCCAATTTAATTATTGTACATGGACATATCATACGTAATGC3', ^a
mfal-amp5	5' GCACCTACTGCTACGGTTGG 3'
mfal-amp3	5' GTCGAAGCCGATGAACGAC 3'
Kim-5v	5' CAAATAATTTCATACATGCACATATAGTACTACATAGTCAAGAACAATATCATAAACAATTTATCGATGAATTCGAGCTCG3', ^b
Kim-3v	5' TCGGTAGAGCCAGCATCAGATGCAAAGCCATGCAAGAGACTGATATAAAGACTGTTATACC CGTACCGCTGCAGGCTCGAC3', ^b
lys2-core5	5' TGATATAATTATCCATAAATGGTGCGTTAGTTCACTGGGTTTATCCATATGCCAAAATTGAGGAGCTCGTTTGACACTGG3', ^c
lys2-core3	5' AAGA ACTTTGGCTTGCCGACGGCGGCTAAGCTCATAACATTT'GATAGTT'GAAATAACATTTTCCTTACCATL4AGITGATC3', ^c
230PF	5' GGTCGGT/ AGTT'CACTGGGTTTATCCATATGCCAAAATTGAGGGATCGCCCGGGCGATCGCAGAGACCTCAAAITTCGAT3', ^d
cper3	5' CGACGGCGGCTAAGCTCATAAC 3'
s3393	5' GTCAGGGCCAAGGATGAAG 3'

^aBold: homology to Chromosome V, italics: sequence annealing to *LYS2*^bBold: homology to the Chromosome V, italics: sequence annealing to *lphMX*^cBold: homology to *LYS2*, italics: sequence annealing to pCORE^dBold: homology to *LYS2*, italics: sequence specific to the flanking regions of the (GAA)₂₃₀ tract