Supporting Information

Bezerra et al. 10.1073/pnas.1302094110

SI Text

1. Strains and Growth Conditions. *Escherichia coli* strain JM109 (*recA1 SupE44 endA1 hsdR17 gyrA96 relA1 thi* Δ*[Lac-proAB] F'[traD36 proAB-lacI lacZΔM15*) was used as a host for all DNA manipulations. *E. coli* cells were grown at 37 °C in LB broth medium or LB 2% agar (Formedium) supplemented with ampicillin (50 μg/mL; Sigma-Aldrich).

Candida albicans SN148 (arg4Δ/arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ ura3Δ::imm434/ura3Δ::imm434iro1Δ::imm434/iro1Δ::imm434) (1) was grown at 30 °C in 2% glucose, 1% yeast extract, and 1% peptone (YPD). *C. albicans* strains T0, T1, and T2 were grown in minimal medium lacking uridine (0.67% yeast nitrogen base without amino acids, 2% glucose, 2% agar, and 100 μg/mL required amino acids). Heterozygous $tRNA^{Ser}_{CAG}$ KO strains (T0KO1, T1KO1, and T2KO1) were grown in minimal medium lacking uridine and arginine, whereas homozygous deletion cells (T2KO2) were grown in medium lacking uridine, arginine, and histidine. Cells were grown aerobically at 30 °C unless indicated otherwise. All *C. albicans* strains used in this study are listed in Dataset S1, Table S3.

2. Plasmid Construction. The *C. albicans* plasmids used in this study were based on the integrating vector CIp10 (2). For expression of one copy of the mutant *Saccharomyces cerevisiae* $tRNA^{Leu}_{CAG}$ gene in *C. albicans* SN148, a DNA fragment containing the *S. cerevisiae* tRNA gene was cloned into SalI cloning site of CIp10 plasmid, yielding plasmid pUA701 (Fig. S1*A* and Dataset S1, Table S1). The mutant tRNA gene was amplified from pUA15 (3) using standard PCR protocols. The second copy of the gene was cloned into the ClaI cloning site of plasmid pUA701, yielding plasmid pUA705 (Fig. S1*C* and Dataset S1, Table S1).

The reporter system was constructed on the basis of the codon-optimized yeast enhanced GFP (*yE*GFP) gene (4) and assembled into CIp10, pUA701, and pUA705 in three different versions. First, a DNA fragment containing the *Ca*ACT1 promoter region, the *yE*GFP gene, and the *Sc*CYC1 terminator was amplified from pACT1-GFP (5) and inserted between the XbaI and BamHI cloning sites. This step originated plasmids $pUA702_{201\text{-}UUA}$, $pUA706_{201\text{-}UUA}$, and $pUA709_{201\text{-}UUA}$, respectively (Dataset S1, Table S1). Second, the *yE*GFP gene was altered by site-directed mutagenesis to change the codon UUA in position 201 to codon CUG, yielding plasmids $pUA702_{201\text{-}CUG}$, $pUA706_{201\text{-}CUG}$, and $pUA709_{201\text{-}CUG}$ (Dataset S1, Table S1). Third, the *yE*GFP gene was altered by site-directed mutagenesis to change the codon UUA in position 201 to codon UCU, yielding plasmids $pUA702_{201\text{-}UCU}$, $pUA706_{201\text{-}UCU}$, and $pUA709_{201\text{-}UCU}$ (Dataset S1, Table S1).

3. Strains Construction. ***3.1. Construction of T0, T1, and T2.*** To construct *C. albicans* T0, T1, and T2 strains (Dataset S1, Table S3), integration of different plasmids was targeted to the RPS10 locus of the parental SN148 strain.

pUA709 plasmid, bearing the fluorescent reporter system, was digested with StuI and integrated at the RPS10 locus in SN148 strain to generate strain T0.

pUA702 plasmid, bearing one copy of the mutant $tRNA^{Leu}_{CAG}$ gene, was digested with StuI and integrated at the RPS10 locus of the parental SN148 strain to generate strain T1.

pUA706 plasmid, bearing two copies of the mutant $tRNA^{Leu}_{CAG}$ gene, was digested with StuI and integrated at the RPS10 locus of the parental SN148 strain to generate strain T2.

All *C. albicans* SN148 transformations with the respective plasmid were performed according to the work by Walther and Wendland (6), and transformants were selected in minimal medium without uridine. The correct insertion of pUA709/pUA702/pUA706 was confirmed by PCR and DNA sequencing.

3.2. Construction of T0KO1, T1KO1, and T2KO1. To create *C. albicans* heterozygous tSCAG/tscagΔ strains, disruption of one copy of the endogenous $tRNA^{Ser}_{CAG}$ was carried out using an ARG4 auxotrophic marker cassette targeted with 500-nt flanking homology for homologous recombination as described in the work by Noble and Johnson (1). However, instead of using a fusion PCR procedure, the disruption fragment was assembled in an empty plasmid using regular cloning techniques.

$tRNA^{Ser}_{CAG}$ (*tScag*) 5′ and 3′ flanking sequences were amplified by PCR with primer pairs 1 and2 and 3 and 4, respectively. Primers 5 and 6 were used to amplify the ARG4 auxotrophic marker (Dataset S1, Table S2). The flanking sequences and the selectable marker were then cloned into pUA515 for the complete assembly of the disruption fragment. Primers 1–4 were used to amplify the *tScag::ARG4* fragment that was subsequently used in different transformations:

strain T0 was transformed with the *tScag::ARG4* cassette-originating strain T0KO1 (Fig. S2);

strain T1 was transformed with the *tScag::ARG4* cassette-originating strain T1KO1 (Fig. S2); and

strain T2 was transformed with the *tScag::ARG4* cassette-originating strain T2KO1 (Fig. S2).

Transformants were selected in minimal medium without uridine and arginine. Disruption of the first copy of the endogenous $tRNA^{Ser}_{CAG}$ was verified by PCR of the flanks surrounding the inserted marker. The absence of the $tRNA^{Ser}_{CAG}$ gene was also verified by Northern blot.

3.3. Construction of T2KO2. To create the *C. albicans* homozygous tscagΔ/tscagΔ strain, disruption of the second copy of the endogenous $tRNA^{Ser}_{CAG}$ was made using an HIS1 auxotrophic marker cassette targeted with 500-nt flanking homology for homologous recombination as described in the work by Noble and Johnson (1).

$tRNA^{Ser}_{CAG}$ (*tScag*) 5′ and 3′ flanking sequences were amplified by PCR with primer pairs 1 and 2 and 3 and 4, respectively. Primers 5 and 6 were used to amplify the HIS1 auxotrophic marker (Dataset S1, Table S2). The flanking sequences and the selectable marker were then cloned into pUA514 for the complete assembly of the disruption fragment. Primers 1–4 were used to amplify the *tScag::HIS1* fragment that was subsequently used in transformation:

the heterozygous strain T2KO1 was transformed with *tScag::HIS1* to produce the null mutant T2KO2 (Fig. S3).

Transformants were selected in minimal medium without uridine, arginine, and histidine. Disruption of both copies of the endogenous $tRNA^{Ser}_{CAG}$ was verified by PCR of the flanks surrounding the introduced markers. The absence of the $tRNA^{Ser}_{CAG}$ gene was also verified by Northern blot analysis.

4. Yeast Fitness Assays. For the determination of growth rates, *C. albicans* strains were grown overnight at 30 °C in liquid YPD medium as a preculture. For main cultures, fresh medium (YPD) was inoculated with cells from the preculture to an OD_{600} of 0.02. This culture was grown at 30 °C and 180 rpm until late

stationary phase. Growth rate was determined by recording the change in the natural logarithm of the OD at 600 nm of the culture at the indicated time points. At least three independent experiments were used for each strain.

Cell viability was assessed using the CFU assay. Cell density in liquid culture was determined by counting the number of cells using a Neubauer chamber. Then, the dilution factor necessary to dilute cells to 10^3 cells/mL was determined, and cells were dispersed by vigorous vortexing and diluted in sterile water; 0.1 mL diluted cells were plated onto an appropriate agar medium and incubated for 3 d at 30 °C. The colonies were counted, and plating efficiency was determined. The procedure was repeated at the indicated time points, and data are the result of three independent experiments with replicas.

5. Northern Blot Analysis. ***5.1. tRNA extraction.*** For total RNA extractions, 250-mL cultures grown overnight in minimal medium were harvested at an OD_{600} of 1.0–1.5, and the pellets were frozen overnight at −80 °C. Cells were resuspended in hot acid–phenol: chloroform 5:1 (pH 4.7) and buffer (10 mM Tris, pH 7.5, 10 mM EDTA, 0.5% SDS). Cell suspension was incubated for 1 h at 65 °C with repeated shaking every 10 min. The aqueous phase containing RNAs was separated from the phenolic phase by centrifugation at 8,000 × *g* for 30 min at 4 °C; then, it was transferred to a new tube and reextracted with fresh chloroform:isoamyl alcohol at 24:1. The aqueous phase was precipitated overnight at −20 °C with 3 volumes cold absolute ethanol and 0.1 volumes 3 M sodium acetate. Tubes containing RNAs were harvested at 8,000 × *g* for 30 min at 4 °C, and pellets were resuspended in 0.1 M sodium acetate, pH 4.5. Total RNAs were applied to a 20-mL diethylaminoethyl-cellulose column equilibrated with 0.1 M sodium acetate, pH 4.5. tRNAs were eluted with 0.1 M sodium acetate/1 M sodium chloride and precipitated with 2.5 volumes cold absolute ethanol; then, they were resuspended in 10 mM sodium acetate, pH 5.0 and 1 mM EDTA and stored at −80 °C (7).

5.2. Northern blot. Fractionation of total tRNAs was carried out on 12–15% polyacrylamide (40% Acril:Bis) gels containing 8 M urea (0.8 mm thick, 30 cm long). In each gel slot, 50 μg total RNA sample were loaded, and gels were electrophoresed at 500 V for 16 h. Fractioned tRNAs were localized by UV shadowing, and the portion of the gel containing tRNAs was cut and transferred onto a nitrocellulose membrane (Hybond N; Amersham) using a Semy-Dry Trans Blot (Bio-Rad). For hybridization, probes were prepared using 10 pmol dephosphorylated oligonucleotide and 4 μL γ-^{32}P-ATP (5,000 Ci/mmol; Perkin-Elmer) in 1× T4 kinase buffer, 10 mM spermidine, and 16 units T4 kinase (Takara). Labeling reactions were incubated at 37 °C for 1 h, and then, probes were extracted using phenol:chloroform:isoamyl alcohol. The hybridization protocol was performed as described in the work by Heitzler et al. (8). Membranes were prehybridized for 30 min at 53 °C in a hybridization solution [5× Denhardt's solution (1% Ficol, 1% Polyvinylpyrrolidone, 1% BSA), 6× saline sodium phosphate EDTA (SSPE) (3 M NaCl, 173 mM NaH_2PO_4, 25 mM EDTA), 0.05% SDS]. Membrane hybridizations were performed overnight in the above buffer using probes *GCGACACGAGCAGGGTTC* for detection of $tRNA^{Ser}_{CAG}$ and *GCGCCTCCGAAGAGATCA* for detection of mutant $tRNA^{Leu}_{CAG}$. Membranes were then washed four times (3 min each time) in 2× SSPE and 0.5% SDS at 53 °C, exposed overnight with intensifying screens, and developed using a Molecular Imager FX (Biorad).

6. Western Blot Analysis. ***6.1. Protein extraction and preparation.*** Cells from 10 mL overnight cultures (OD at 600 nm between 0.5 and 0.8) were harvested by centrifugation, washed two time in 1× PBS, and resuspended in 0.3 mL lysis buffer [50 mM PBS, pH 7.0, 1 mM EDTA, 5% Glycerol, 1 mM PMSF, EDTA-free protease inhibitors (Roche)]. The suspension was kept on ice and transferred to cryotubes, and 1 volume glass beads was added. The cell walls were digested using Precellys (5 cycles of 10 s in the beater and 2 min on ice). Then, tubes were centrifuged during 5 min at 10,000 × *g*, and supernatants were removed into new tubes. This suspension was spun down again for 10 min at 10,000 × *g*, and a clear protein extract was obtained. Protein quantification was carried out using the Pierce BCA Protein Assay Kit. Aliquots of about 30 μg extracted proteins were prepared in 15 μL 1× SDS gel loading buffer (50 mM Tris·HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and heated at 95 °C for 5 min immediately before running the gel.

Samples were fractionated on denaturing 15% SDS/polyacrylamide gel:water, acrylamide/bisacrylamide mix, pH 7.0, Tris base, SDS, ammonium persulfate, and tetramethylethylenediamine (TEMED); 15% resolving gels and 4% stacking gels were routinely prepared. After polymerization, gels were mounted in an electrophoresis apparatus filled with running buffer (25 mM Tris base, 250 mM glycine, pH 8.3, 0.1% SDS); 30 μg each of the samples were loaded into the wells. The gels were run at 80 V until the dye front moved into the resolving gel. Then, the voltage was increased to 150 V until the dye reached the bottom of the gel (about 4 h).

6.2. Western blot. After electrophoresis, samples were electroblotted onto a nitrocellulose membrane (Hybond ECL; Amersham) using the Bio-Rad wet transferring system. A nitrocellulose membrane, six paper filters with the same size, and the cushions were placed in transfer buffer (25 mM Tris base, 192 mM glycine, 12% methanol). The transfer system was mounted accordingly and added with transfer buffer. Blotting was allowed to run overnight at 4 °C. After blotting, membranes were peeled off from the gels and placed in 5% milk in tris-buffered saline (TBS) solution for 1 h. Then, the membranes were washed in 1× TBS and 0.1% Tween (TBS-T) and incubated for 1 h with an anti-GFP [full length (FL)] antibody (Santa Cruz) in an appropriate primary antibody solution in TBS-T. Membranes were washed three times with TBS-T during 10 min and incubated in the appropriate secondary antibody, the anti-rabbit IgG peroxidase from goat (Sigma). Incubation was carried out during 1 h in the dark. Finally, three additional washes in TBS-T during 10 min were carried out in the dark, and signal detection was performed using the Odyssey Infrared Imaging System (Li-Cor Biosciences). As an internal control, a monoclonal anti–α-tubulin antibody (Sigma) was used.

7. Epifluorescence Microscopy. *yE*GFP expression was visualized in *C. albicans* cells by epifluorescence microscopy. Strains were grown overnight in liquid medium at an OD_{600} of 2.0–2.5, and aliquots were spotted onto microscope slides. Fluorescence was detected using a Zeiss MC80 Axioplan 2 light microscope equipped for epifuorescence microscopy with the filter set HE38. Photographs were taken using an AxioCam HRc camera, and images were analyzed using ImageJ software. Mean fluorescence intensities (± SD) were quantified in individual *C. albicans* cells containing the reporter *yE*GFP leucine (Leu) -UUA_{201} (positive control), serine (Ser) -UCU_{201} (negative control), and Ser/Leu-CUG_{201} (reporter). *yE*GFP fluorescence (intensity/pixel) was determined for at least 1,000 cells in each case.

8. Phenotyping Assays. For the primary screen, the growth and colony morphology of two or more independent isolates of each strain were tested. *C. albicans* strains were grown overnight at 30 °C in minimal media without uridine and then serially diluted to 1,000 cells/mL. Approximately 50 cells were plated onto fresh agar plates and then allowed to grow at 30 °C for 7 d in a humidified incubator to prevent drying of the agar surface. Colonies were photographed using a dissecting microscope equipped with AxioCam HRc camera and Axio Vision Software from Zeiss.

For the phenotypic screens, a series of different conditions was tested according to a protocol established by Homann et al. (9), with a few alterations. Phenotyping media included nutritional cues,

temperature, antifungals, and a variety of environmental stressors (Dataset S1, Table S4). Standardization of the initial inoculum for each strain was done by growing cell cultures overnight in liquid medium at an OD_{600} of 0.5–1.0. Cell density in liquid culture was determined by counting the number of cells using a Neubauer chamber. Then, the dilution factor necessary to dilute cells to 10^8 cells/mL was determined, and cells were dispersed by vigorous vortexing and diluted in sterile water; 0.1 mL diluted cells were subjected to a series of 10-fold dilutions that were then transferred to 96-well plates. This format allowed the plating of strain dilutions with a 96-pin bolt replicator (Caliper) to each of the assay plates. The plates were incubated and photographed over the course of 1 wk using an AxioCam HRc camera and Axio Vision Software from Zeiss. All images were imported and processed using ImageJ software. On import, each spot of the assay ($A_{id\ stress}$) and control plates ($A_{id\ nostress}$) was measured, and a growth score was calculated (corresponding to the ratio between the area of the spot in the stress condition and the area of the same spot in the control situation). The growth score average of all of the spots corresponded to the score for that specific strain (GS_m) (Dataset S1, Table S6). Compared with the control strain T0 (GS_c), the scoring system classified the strength of the reduction or enhancement of growth relative to control.

GS_m = growth score mistranslating strain;

GS_c = growth score T0 control strain;

A_{id} = measured area for isolate i and spot dilution d;

n_{id} = set of all isolates in a given dilution for the strain under study;

$GS_m > GS_c$ = growth enhancement;

$GS_m < GS_c$ = growth reduction;

$$GS_m = \frac{1}{n_{id}} \sum_{n \in id} \left(\frac{[A_{id}]stress}{[A_{id}]nostress} \right), \text{ and}$$

$$GS_c = \frac{1}{n_{id}} \sum_{n \in id} \left(\frac{[A_{id}]stress}{[A_{id}]nostress} \right)$$

9. Human Dendritic Cell Response. ***9.1. Human dendritic cell preparation and stimulation.*** Peripheral blood mononucleated cells (PBMCs) were isolated from a buffy coat blood sample from six healthy donors from the Transfusion Unit of the Careggi Hospital (Florence, Italy). The experimental plan was approved by the local Ethical Committee of Azienda Universitaria Ospedaliera Careggi (Careggi Hospital, Florence, Italy), and written informed consent was obtained from all donors (approval document no. 87/10). The study conforms to the ethical guidelines of the 1975 Declaration of Helsinki, the international recommendation Dir. EU 2001/20/EC, and its Italian counterpart (DM 15 Luglio 1997; D.Lvo 211/2003; D.L.vo 200/2007). PBMCs were isolated by Ficoll–Hypaque density gradient centrifugation (Biochrom AG). Monocytes were obtained from low-density PBMCs by magnetic enrichment with anti-CD14 beads (Miltenyi Biotec) and cultured in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) (800 U/mL; Gentaur) and recombinant IL-4 (1,000 U/mL; Gentaur) for 6 d to allow for dendritic cell (DC) differentiation. Monocyte-derived DC activation was induced by fungal strains. *C. albicans* strains were cultured overnight in Saboraud medium at 28 °C. Cultures were then collected, washed two times with sterile water, and resuspended at 10^8 cells/mL. For the challenge, fungal preparations were added to the monocyte-derived DCs at a stimuli:DC ratio of 5:1.

9.2. Cytokine production assay. After 24 h of stimulation, supernatants were collected, and cytokine detection was performed. Milliplex map multiplex kit (Human Cytokine/Chemokine Panel I; Millipore) was used for detection of IL-1b, IL-10, and IL-12p70 according to the manufacturer's instructions.

10. Mice Infection Assays. For infection, female C57BL6 8- to 10-wk-old mice were purchased from Charles River (Calco). Mice were anesthetized by i.p. injection of 2.5% avertin (Sigma Chemical) before instillation of a suspension of 2×10^7 viable conidia/20 μL saline intragastrically. Fungi were suspended in endotoxin-free (Detoxi–gel; Pierce) solutions (<1.0 EU/mL as determined by the limulus amebocyte lysate method). Mice were monitored for fungal growth in exophagus, stomach, and colon (CFU/organ, mean ± SE), histopathology (Periodic acid–Schiff staining of sections of paraffin-embedded tissues), and patterns of cytokine production. Total and differential cell counts were done by staining with May–Grünwald Giemsa reagents (Sigma) before analysis. Photographs were taken using a high-resolution microscopy Olympus DP71 (Olympus).

11. Illumina Whole-Genome Sequencing and Data Analysis. For Illumina sequencing, genomic DNA was prepared and sequenced using manufacturer-supplied protocols and reagents as follows. One library per sample was constructed using Illumina DNA Sample Prep standard protocol with an insert size of 400–500 bp. Briefly, 5 μg high-molecular weight genomic DNA was fragmented by a Covaris sonication device. After sonication, DNA fragments were end-repaired and A-tailed. Adapters were then ligated by a 3′ thymine overhang. Finally, ligated fragments were amplified by PCR. The library was applied to an Illumina flow cell for cluster generation. Sequencing was performed on a Genome Analyzer IIx instrument using 150-bp paired-end reads.

Raw sequence data (146-bp paired-end reads with expected insert size of 400–500 bp) from each sample were trimmed by removing consecutive bases on both 5′ and 3′ flanks with base quality less than 20. Trimmed reads that did not pass filtering criteria for ambiguity (N content < 5%), complexity (score ≥ 10), length (50 bases or longer), and average base quality ≥ 20 were removed using bamtools (10) (Dataset S1, Table S7).

Remaining reads were mapped to the reference genome of *C. albicans* obtained from the *Candida* genome database (http://www.candidagenome.org/, assembly 21) using BWA, version 0.5.9 (11). Processing and filtering of mapped reads were done using Samtools, version 0.1.17 (12). After removal of duplicates, read pairs aligning to opposite strands or read pairs with predicted insert size that did not match actual size were removed. Additionally, read pairs were removed where one or both reads had low mapping quality (<20) or less than 95% sequence identity to the reference (Dataset S1, Table S8). Over all samples, about 49.4% of the read pairs remained after this filtering, covering, on average, 99.4% of the genome length for an average alignment depth of between 56.8 and 96.2 reads per base (Dataset S1, Table S8).

Mapped reads were analyzed using Samtools. The control strain used in this experiment, T0, is genetically slightly different from the SC5314, which has been used for the construction of the reference genome. We identified genomic regions not well-covered by the reference assembly and removed reads mapping to these regions. These regions were identified by reads covering genomic positions for which three or more allele types had been mapped with good quality as well as reads covering three or more biallelic genomic positions within 10-bp distance, where the least frequent allele type was represented by 20% or more of the reads for all three of the positions. On average, 0.4% of the length of the genome and 3.3% of the total number of remaining reads were removed this way (Dataset S1, Table S8).

Samtools was used to produce read pileups, detect single nucleotide variants, and call genotypes. Indels were not called. Bases with low base quality or read depth less than three or higher than two times the sample average coverage were called as unknown genotype. For the four samples containing the $tRNA^{Leu}_{CAG}$ gene, we analyzed the number and genomic class, such as coding, noncoding, or repeat region, of bases with different genotype compared with the control strain (Dataset S1, Tables S13 and

S14). All samples showed allele frequency spectra indicative of predominantly diploid characteristics with relatively low amount of possible aneuploidy. SNPs in coding regions were also analyzed for changes in codon use compared with the control strain. The same comparisons were done between strains T1 and T2 and between T2 and its derived strains T2KO1 and T2KO2.

1. Noble SM, Johnson AD (2005) Strains and strategies for large-scale gene deletion studies of the diploid human fungal pathogen *Candida albicans*. *Eukaryot Cell* 4(2):298–309.
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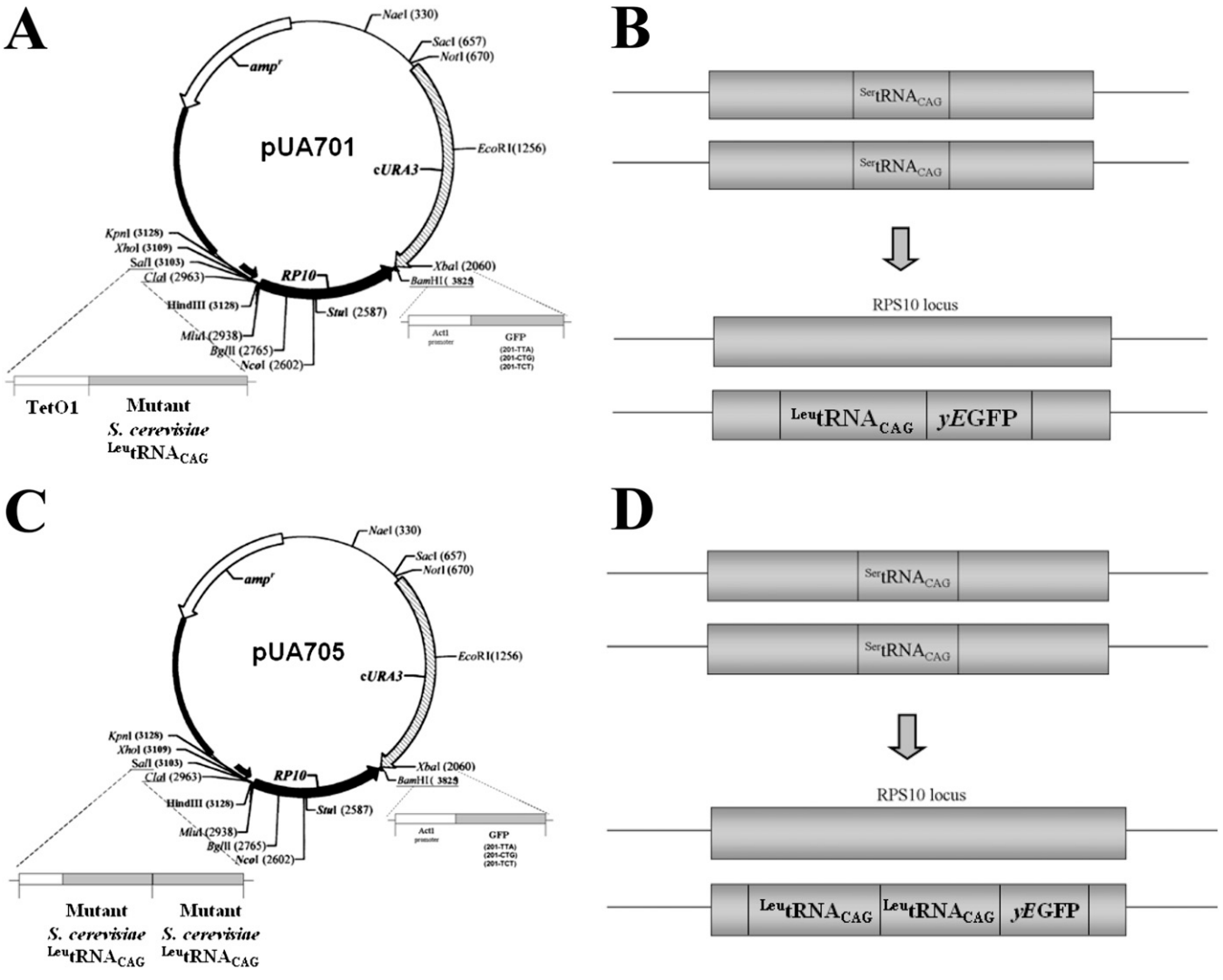

Fig. S1. Plasmids and strategy used to build ambiguous strains T1 and T2. (*A*) Plasmid carrying the fluorescent reporter system and one copy of the mutant $tRNA^{Leu}_{CAG}$ used for generating *C. albicans* T1 strain. (*B*) Integration of pUA701 was targeted to the RPS10 locus. This method produced a strain in which the $tRNA^{Leu}_{CAG}$ and the endogenous $tRNA^{Ser}_{CAG}$ competed for CUG decoding at the ribosome A site. (*C*) Plasmid carrying the fluorescent reporter system and two copies of the mutant $tRNA^{Leu}_{CAG}$ used for producing *C. albicans* T2 strain. (*D*) Integration of pUA705 was targeted to the RPS10 locus, which further increased the competition between the $tRNA^{Leu}_{CAG}$ and the endogenous $tRNA^{Ser}_{CAG}$.

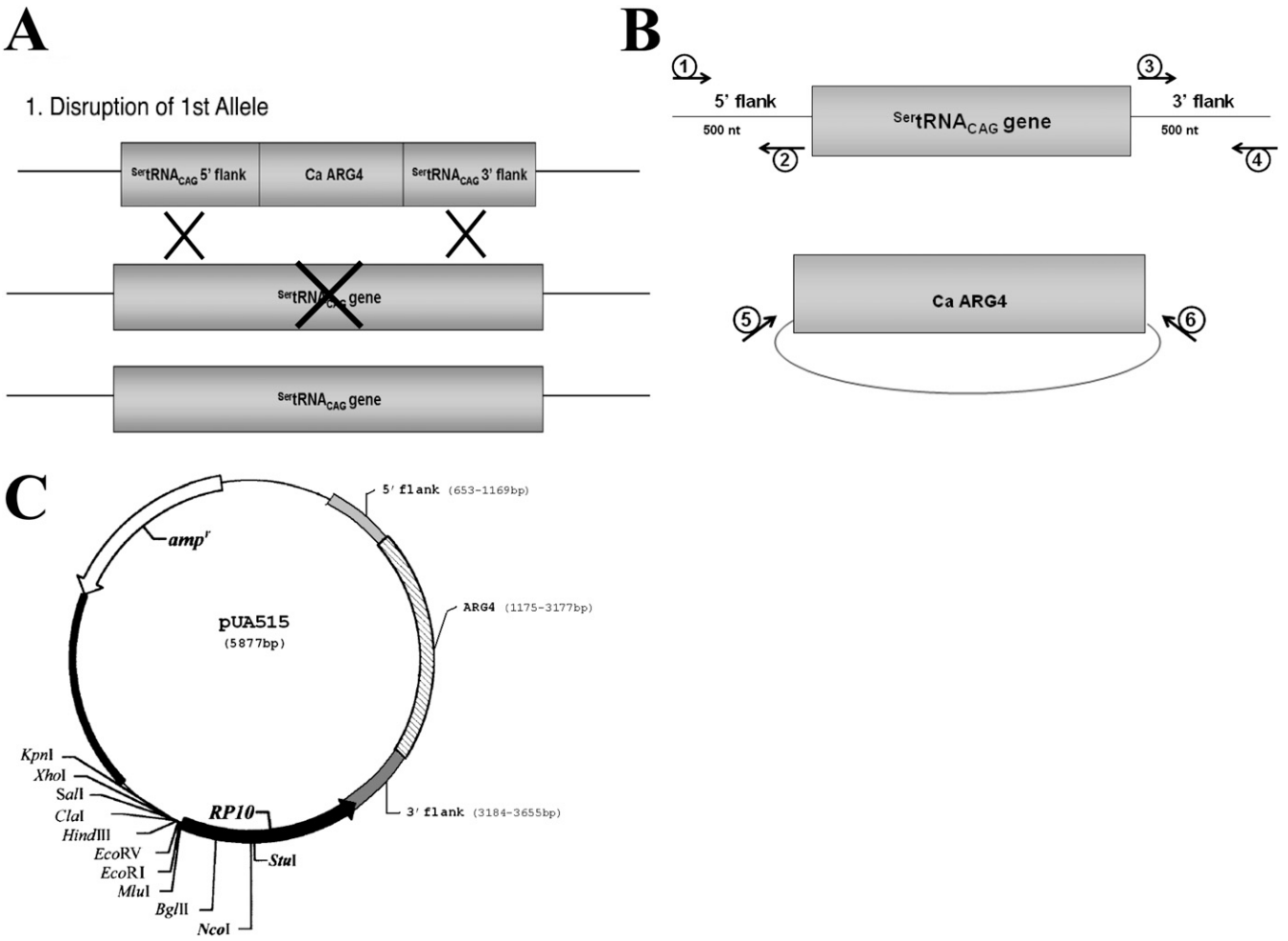

Fig. S2. Strategy used to disrupt one copy of the endogenous $tRNA^{Ser}_{CAG}$ from T0, T1, and T2 strains to originate T0KO1, T1KO1, and T2KO2 strains, respectively. (*A*) Deletion was carried out using an ARG4 marker cassette tagged with a 500-nt flanking homology for homologous recombination. (*B*) The $tRNA^{Ser}_{CAG}$ 5′ and 3′ flanking sequences were amplified by PCR with primers 1 and 2 and 3 and 4, respectively. Primers 5 and 6 were used to amplify the auxotrophic marker. (*C*) The flanking sequences and the selectable marker were cloned into an empty plasmid (pUA515) that allowed for the complete assembly of the disruption fragment. Primers 1 and 4 were used to amplify the *tScag::ARG4* fragment by standard PCR protocols. Strains T0, T1, and T2 were transformed with the *tScag::ARG4* cassette originating strains T0KO1, T1KO1, and T2KO1.

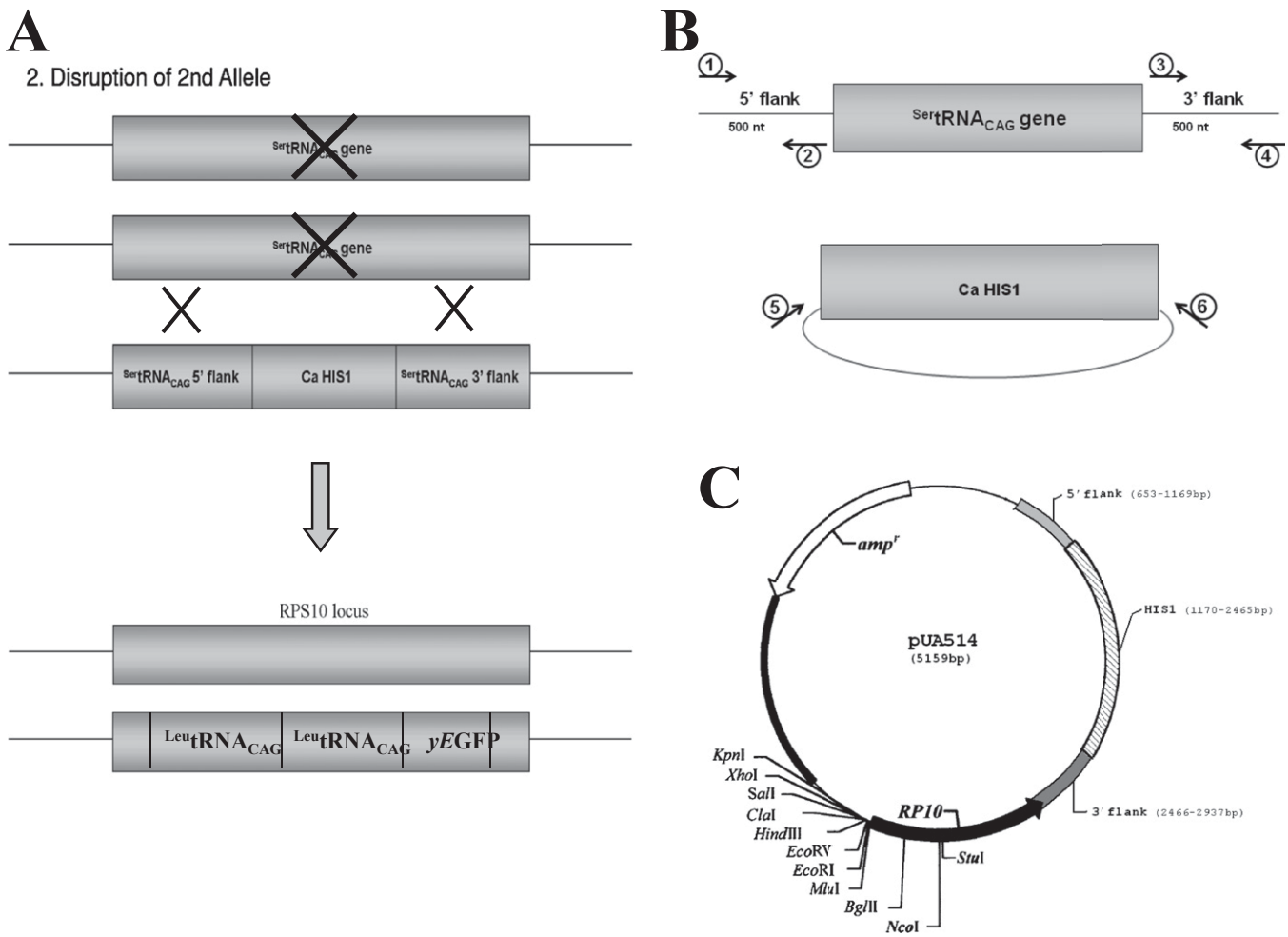

Fig. S3. Strategy used to disrupt the second copy of the endogenous $tRNA^{Ser}_{CAG}$ from the T2KO1 strain, producing the T2KO2 strain. (*A*) Deletion was carried out using an HIS1 marker cassette tagged with a 500-nt flanking homology for homologous recombination. (*B*) The $tRNA^{Ser}_{CAG}$ 5′ and 3′ flanking sequences were amplified by PCR with primers 1 and 2 and 3 and 4, respectively. Primers 5 and 6 were used to amplify the auxotrophic marker. (*C*) The flanking sequences and the selectable marker were cloned into an empty plasmid (pUA514) that allowed for the complete assembly of the disruption fragment. Primers 1 and 4 were used to amplify the *tScag::HIS1* fragment by standard PCR protocols. The heterozygote T2KO1 was transformed with *tScag::HIS1* to produce the null mutant T2KO2.

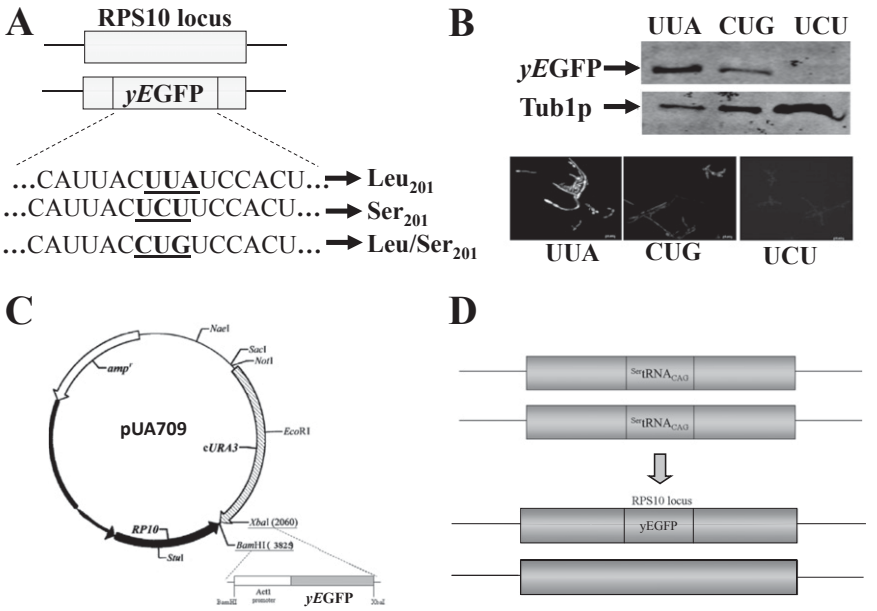

Fig. S4. Fluorescent reporter system used to quantify Leu insertion at CUG positions in vivo. (*A*) The system is based on the codon-optimized *yE*GFP gene. The Leu UUA codon at position 201 was mutated to the ambiguous CUG codon and a UCU Ser codon. Incorporation of Ser at this position inactivated *yE*GFP, whereas Leu misincorporation at the CUG position provided a functional fluorescent protein. (*B*) Western blot analysis of the three versions of the reporter was performed on equal amounts of protein using an anti-GFP polyclonal antibody. The molecular mass of the *yE*GFP bands is 27 kDa, which is in agreement with the theoretical predictions. No band was detected when Ser was inserted at position 201. Tubulin (50 kDa) was used as an internal control. The level of fluorescence was directly proportional to the amount of Leu inserted at the CUG-201 as measured using epifluorescence microscopy. (*C*) Plasmid carrying the fluorescent reporter system and no copy of the mutant $tRNA^{Leu}_{CAG}$ used for generating the *C. albicans* T0 strain. (*D*) Integration cassette pUA709 used to target the RPS10 locus. This approach allowed for the quantification of Leu misincorporation in a naturally ambiguous strain (T0).

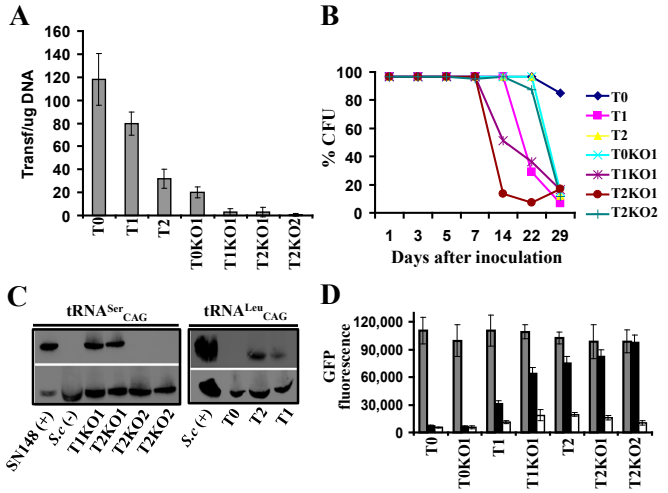

Fig. S5. Transformation efficiency and cell viability of the highly ambiguous and reverted strains. (*A*) Transformation efficiency of ambiguous clones was significantly lower than the control T0, indicating that increasing levels of Leu incorporation were toxic. (*B*) Ambiguous cells lost viability compared with control T0 cells. At day 14, strains T1KO1 and T2KO1 had only 50% and 15% of relative viability, respectively. Results are shown in CFU as a percentage of the cells plated. (*C*) Northern blot analysis of $tRNA^{Leu}_{CAG}$ and $tRNA^{Ser}_{CAG}$ expression on the newly constructed strains. Strains with one and two copies of the mutant *S. cerevisiae* tRNA showed the expected expression in the Northern blot. Strain T2KO2 presented no expression of the endogenous $tRNA^{Ser}_{CAG}$. (*Lower*) $tRNA^{Thr}_{UGU}$ was used as an internal control for both assays. (*D*) Mean fluorescence intensities were quantified in individual *C. albicans* cells containing the reporter *yE*GFP $^{Leu}TTA_{201}$ (gray bars), $^{Ser}TCT_{201}$ (white bars), and $^{Ser/Leu}CTG_{201}$ (black bars). GFP fluorescence (intensity/pixel) was determined for >1,000 cells in each strain.

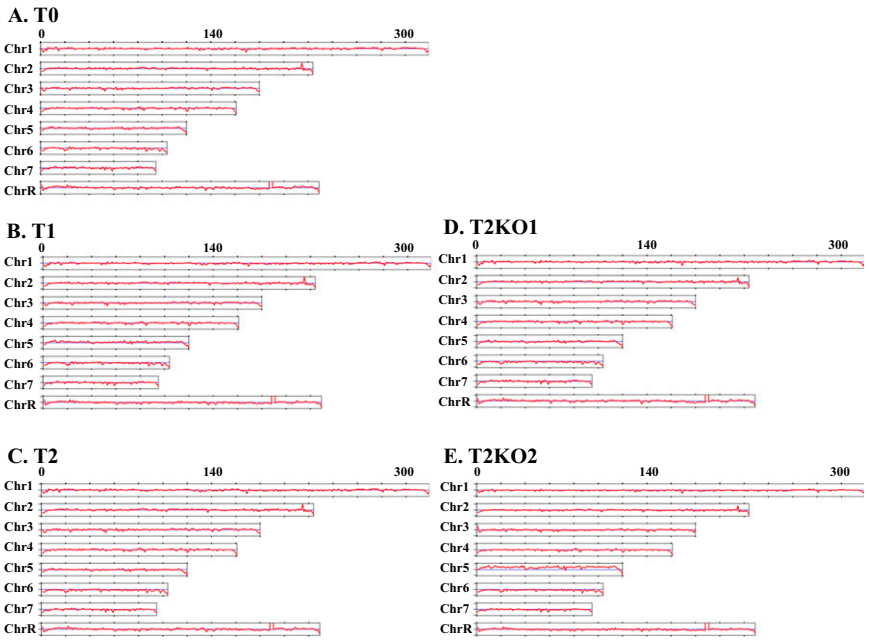

Fig. S6. Read coverage is relatively uniform across the genome. For each sample (*A*, T0; *B*, T1; *C*, T2; *D*, T2KO1; *E*, T2KO2), average read depth in a sliding window of 10 kb along each chromosome was normalized to the average depth for the whole corresponding sample. Apart from a short section on chromosome (Chr) R in all samples, the coverage is relatively uniform for all samples. The decreasing number to the chromosome ends is caused by numeric effects from the sliding window.

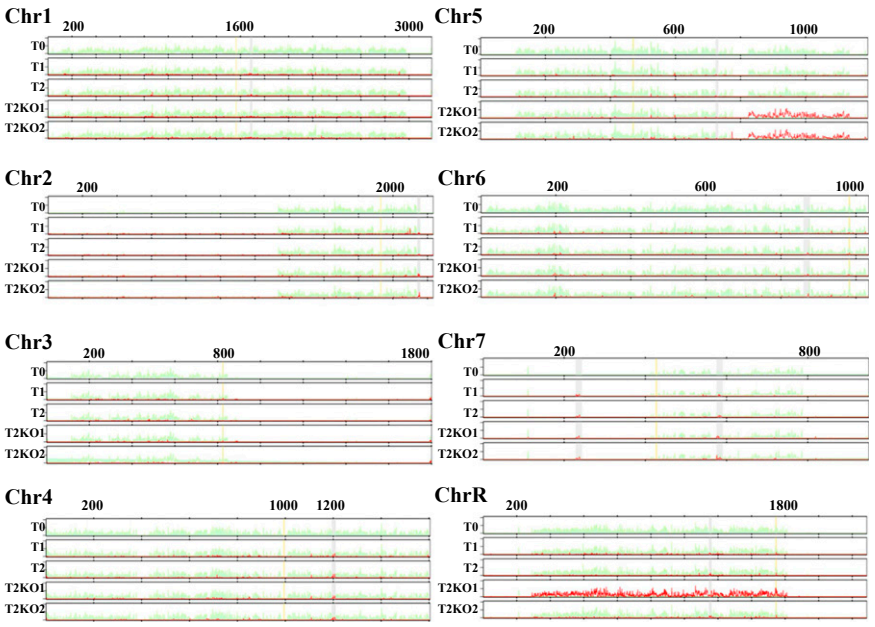

Fig. S7. The SNP density (SNPs per kilobase; green) varies along the Chrs but is essentially similar between the five strains (represented by tracks). There are homozygous stretches matching in all samples as well. The density of SNPs with loss of heterozygosity in a derived strain compared with T0 is plotted in red, showing a region on Chr5 in both T2KO1 and T2KO2 and the entire ChrR in T2KO1, where all heterozygote SNPs have changed to homozygous. Yellow and gray vertical lines indicate centromere and major repeat sequence (MRS), respectively.

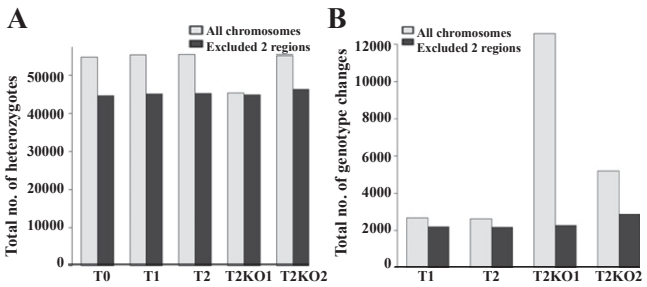

Fig. S8. (*A*) The five strains have essentially an unchanging number of SNPs (black bars) when discounting the loss of heterozygosity regions on Chr5 and ChrR. Total numbers of heterozygotes are shown in light gray, and only T2KO1, which is affected by the loss of heterozygosity on the entire ChrR, is different. (*B*) With an increasing degree of mistranslation, strains show an increasing number of genotype changes at polymorphic locations compared with T0 (black bars), which is heavily affected by including the loss of heterozygosity regions (gray bars).

Other Supporting Information Files

Dataset S1 (XLSX)