**Supplemental Figure Legends**

**Figure S1:** Maps of CRISPR/Cas9 plasmids and PCR fragments from the *NTS2* region of wild type and edited strains. A) Plasmid pML104 was modified by the insertion of three tandem sgRNA templates (grey arrows marked A, C, and D) or a single sgRNA template (F). B) The 762 bp wild type rARS region amplified by PCR primers (arrows) shows the relevant locations of PAM sites (A, B, C, D, and F), the ACS (green box) and a unique HaeIII site. Sizes (in bp) refer to the HaeIII cleavage products. Each of the Cas9-edited *NTS2* regions produces unique PCR fragment sizes and/or restriction fragment sizes. The ~100 bp insertions of *ARS1* or *ARS1max* are indicated by the inverted triangles.

**Figure S2:** Isolation and confirmation of Cas9 edited rDNA repeats. A) BY4741 was transformed with pML104 (control plasmid with only Cas9) or pACD (three sgRNA + Cas9) and the *rARSGC* repair template in either single stranded or double stranded form. B) Colony purification of eight transformants from the plates shown in A. Clones maintain their fast/slow growth properties. C) Characterization of PCR products of the *NTS2* region. Replacement of the rDNA origin with *rARSGC* produced the same size fragment as the wild type sequence but was sensitive to XmaI cleavage. The symbols below the XmaI cleavage gels summarize the events: **+** = un-edited sequence; Δ = deletion of all or part of the fragment targeted by PCR; ✔ = correct replacement; ? = incomplete digest or mixed PCR products. The clones rARSΔ-1 and rARSGC-1 are indicated with arrows.

**Figure S3:** Sanger sequence trace of rARSGC-1 (A) and rARSΔ-1 (B). A) The sequence of the Watson strand from BY4741 (Chr XII coordinates in bp), taken from the *Saccharomyces* Genome Database, is shown at the top with the ACS element and the A PAM site indicated with brackets. The sequence trace the *rARSGC* shows the successful replacement of both sequences. A blow-up of the region encompassing the GC-replacement illustrates no evidence of the original ACS remaining. B) The sequence of the Watson strand from BY4741 aligned to highlight the region of homology involved in the rARS deletion (gray box). The sequence trace confirms the breakpoint in the deletion and reveals that the rDNA repeats in rARSΔ-1 are a mixture of two sequences differing by 3 bp in their junctions.

**Figure S4:** Confirmation of *rARS* editing by PCR and restriction digestion. A) BY4741 was transformed with pACD and *rARS”wt”* repair template that has all four PAM site mutations. PCR fragment sizes reveal six potential deletion strains. Restriction digestion of the PCR products distinguishes unedited clones (+) from edited clones (✔) by differential digestion at the locations of the two PAM sites A and D. ApoI cleaves the edited A PAM site and HaeIII cleaves the unedited PAM site D. B) rARSGC-1was transformed with pF and *rARS1max* repair template. PCR and restriction digestion confirms that six of eight transformants were correctly edited (✔) while one retained the original GC insert (**+**) and one produced a mixed digestion pattern (**?**). C) BY4741 was transformed with pACD and either *rARS1* or *rARS1max* repair template. PCR and restriction digestion with BglII and BsaA1 distinguish the clones replaced by *rARS1* or *rARS1max*, respectively. In addition to unedited clones (+), correct transformants (✔) and apparent mixed clones (?), we recovered a deletion clone (Δ). Clones 1 and 2 of the *rARS1* transformants and clones 1-4 of the *rARS1max* transformants produced large colonies on the transformation plates. For future analysis of transformants, we only characterized small colonies.

**Figure S5:** CHEF gel analysis of BY4741 clones transformed with pML104. A) Ethidium bromide stain of the CHEF gel containing eight transformants shows little variation in chr XII size. B) and C) Southern blots of the CHEF gel in A probed sequentially with *CEN12* and *MAS1* show there is no prominent sub-chromosomal sized fragments from chr XII. The faint patterns below chr XII are cross hybridization to the other yeast chromosomes.

**Figure S6:** Chromosomal location of rDNA sequences in Cas9 transformants. CHEF gel Southern blots from Figures 2A and S5A were reprobed with an *NTS2* probe to look for translocation of rDNA sequences to other chromosomes or as extrachromosomal molecules.

**Figure S7:** CHEF gel analysis of *rARS”wt”* transformants. A) The ethidium bromide photograph is shown along with Southern blots using *MAS1* and 37S probes to assess chr XII size and rDNA repeat locations, respectively. Each transformant is categorized based on PCR and restriction digests shown in Figure S4A. B) Genomic DNA from the same 12 transformants was cleaved with FspI in agarose plugs. The rDNA copy number of each strain was deduced from the FspI fragment sizes on the Southern blot of the CHEF gel using uncut yeast chromosomes as size markers. C) FspI digests of *rARSΔ* and *rARSGC* haploids and heterozygous diploids.

**Figure S8:** Estimating 25S rRNA content of rARSGC-1 by comparative hybridization of a single copy gene, *ACT1*. A) Total nucleic acids were isolated from log phase cultures of BY4741 and rARSGC-1 and separated on an agarose gel. Lanes 2 and 4 contain nucleic acids from BY4741; lanes 3 and 5 contain nucleic acids from rARSGC-1. B) The gel was cut into two portions. The upper portion was Southern blotted and the lower portion was blotted as a northern. The Southern blot was probed for *ACT1* and the northern blot was probed for 25S rRNA. C) Quantification of total counts for replicate lanes were tallied and a ratio of rRNA/ACT1 for rARSGC-1 was normalized to BY4741.

**Figure S9:** Increase in chr XII size over 100 generations in three isolates with *rARS”wt”* (A) and one isolate of *rARS1max* (B).

**Figure S10:** Selection for suppressors of slow growth in turbidostat cuptures of one *rARSΔ* and three *rARSGC* clones. A) The growth rates calculated from the pump speeds for strains over the last ~five days of the turbidostat runs. B) CHEF gels for samples harvested on day 3 and on the days indicated in (A) by the black triangles. C) Southern blot analysis of changes in chr XII/rDNA over the course of the continuous growth.

**Figure S11:** Detecting chr II aneuploidy by CHEF gels. A) and B) The CHEF gels in Figure 3C were simultaneously hybridized with probes near CENs 2 and 9. C) Quantification of the ratio of chr II to chr IX hybridization signals were normalized to that of BY4741. Samples from the same DNA plugs for BY4741 and rARSGC-1 were included on both gels.

**Figure S12:** Monitoring chr XII size during serial passage of rARSGC-2 and rARSΔ-2. A) The ethidium bromide stained CHEF gel contains samples from the serial passage and four independent clones isolated at generation 90. B) Samples from alternate days of the same serial passage experiment were run under conditions to reveal subtle changes in rDNA sizes. The Southern blots were probed sequentially with *CDC45* to detect chr XII and then with *CEN2* and *CEN9*, simultaneously, to assess chr II copy number. C) and D) The Southern blot of the gel from A was probed sequentially with *CDC45* to detect chr XII and then with *CEN2* and *CEN9*, simultaneously, to assess chr II copy number. E) Quantification of the ratio of chr II/chr IX over the course of the continuous growth and in the gen-90 clones from a CHEF gel with samples collected every 10 generations.

**Figure S13:** Read-depth analysis from WGS for evolved rARSΔ-2 and rARSGC-1 clones. Read depth from WGS of genomic DNAs from populations on the last day of Run1 and Run2 turbidostat populations (T1-T4) reveals an increased copy number for chr IV or chr XII or a partial duplication of chr XII that includes the rDNA locus. Read-depth ratios of Chr IV or Chr XII were compared to ratios across ChrV, VI, VII and IX using the Wilcoxon-Rank Sum test. Ratios with a P value of >0.0001 are indicated by their relative copy number differences above each significantly amplified chromosome or chromosomal segment.

**Figure S14:** Analysis of clones transformed with pF and *rARS1* or *rARS1max*. A) CHEF gel analysis and Southern blotting of eight clones transformed with *rARS1max*. Notice the different hybridization patterns for the *MAS1* and 37S probes for clones 2, 4, and 7. B) CHEF gel analysis and Southern blotting of eight clones transformed with *rARS1*. Notice the different hybridization patterns for the *MAS1* and 37S probes for clones 2 and 5. C) 2D gel analysis of rARS1max-2 and rARS1max-4 that contain predominantly circular forms of the rDNA. The red circle highlights the absence of the 22.4 kb junction fragment between the rDNA locus and unique chr XII sequences.

**Figure S15:** CHEF gel analysis of Cas9 edited clones with circular rDNA. A) rARS1max-4 was restreaked and 12 individual colonies were analyzed to determine the stability of the extrachromosomal circular rDNA. The CHEF gel and Southern blot indicate that the ERSc are stably inherited. B) rARS1max-4 was serially passaged in liquid culture for 170 generations and analyzed by CHEF gel and Southern blotting. Samples at 10, 30, 50, etc. generations were examined. By 30 generations cells with stably integrated rDNA repeats had overtaken the culture.

**Supplemental Tables**

**Table S1: List of Primers, oligonucleotides, gBlocks and Sanger sequencing**

**Table S2: List of variant calls from WGS**

**Table S3. Variant filter parameters**

**Table S4. Summary of chromosomal/rDNA amplification events**