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# Consequences of a telomerase-related fitness defect and chromosome substitution technology in yeast *synIX* strains

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1 **Summary**

2

3 We describe the complete synthesis, assembly, debugging, and characterization of a synthetic  
4 404,963 bp yeast chromosome, *synIX*. Combined chromosome construction methods were used to  
5 synthesize and integrate the left arm of *synIX* (*synIXL*) into a strain containing previously described  
6 *synIXR*. During *synIX* strain characterization, we identified and resolved a bug related to *EST3*, a  
7 gene involved in telomerase function, producing a *synIX* strain with near wild-type fitness. To  
8 facilitate future synthetic chromosome consolidation and increase flexibility of chromosome transfer  
9 between distinct strains, we combined chromoduction, a method to transfer a whole chromosome  
10 between two strains, with conditional centromere destabilization to substitute a chromosome of  
11 interest for its native counterpart. We found that both *synIX* transfer via chromoduction and wild-  
12 type *IX* destabilization were efficient methods. We observed that wild-type *II* tended to co-transfer  
13 with *synIX* and was co-destabilized with wild-type *IX*, suggesting a potential gene dosage  
14 compensation relationship between these two chromosomes.

15

16 **Keywords:** *Saccharomyces cerevisiae*, *synIX*, megachunk assembly, chromosome debugging,  
17 *EST3*, chromoduction, chromosome substitution, centromere destabilization, transcriptomics

18

## 19 Introduction

20

21 The Synthetic Yeast Genome Project (Sc2.0) marks a key milestone in the development of  
22 “designer” eukaryotic genomes. This global effort seeks to produce a modified version of the ~12  
23 Mb *Saccharomyces cerevisiae* genome from the bottom up that retains native strain fitness while  
24 eliminating repetitive elements to improve genome stability and adding custom features to endow  
25 new genome functionalities. These changes include deletion of tRNA genes and introduction of  
26 loxPsym sites (nondirectional loxP sites that, being symmetric, can recombine with each other in  
27 two distinct orientations) downstream of non-essential genes, enabling genome rearrangement via  
28 the SCRaMbLE (Synthetic Chromosome Rearrangement and Modification by LoxP-mediated  
29 Evolution) system and subsequent study of how different DNA sequence changes affect  
30 organismal fitness and function.<sup>1-4</sup> More broadly, by establishing a set of common design principles  
31 for all sixteen yeast chromosomes, Sc2.0 aims to provide a blueprint for future eukaryotic genome  
32 engineering endeavors. As part of this project, each chromosome in the yeast genome has been  
33 redesigned and built from synthesized blocks of DNA. Following the previous construction of  
34 several synthetic chromosomes, we report the assembly and characterization of synthetic  
35 chromosome *IX* (*synIX*), coinciding with completion of the assembly phase of the Sc2.0 project as  
36 a whole.<sup>5-17</sup>

37

38 Our group initially assembled a circular version of the right arm of the chromosome, *synIXR*, as a  
39 proof-of-principle for feasibility of the Sc2.0 project,<sup>6</sup> and subsequently incorporated that  
40 chromosome arm into an otherwise wild-type and linear version of chromosome *IX*.<sup>2</sup> Here, we  
41 describe the construction and characterization of the left arm of chromosome *IX*, *synIXL*, and  
42 consequently, the completion of *synIX* in its entirety. As with many of the other completed Sc2.0  
43 chromosomes and in accordance with the Sc2.0 project objectives, *synIX*-containing strains,  
44 following a “debugging” process to resolve any deficiencies in fitness across multiple growth  
45 conditions, behave similarly to the ancestral BY4741 yeast strain with respect to growth fitness and  
46 transcriptional profiling.

47

48 Over the past decade, fitness defects have emerged during completion of many of the Sc2.0  
49 chromosome strains. We attribute these growth issues, or “bugs,” to our incomplete understanding  
50 of *Saccharomyces cerevisiae* and its genome during the project’s initial design phase. Identifying  
51 and resolving such problems may improve future genome engineering efforts and help us  
52 anticipate and avoid potential complications. More specifically, bugs in previous Sc2.0 strains have  
53 commonly arisen from design choices (sequence additions, deletions, or alterations) that  
54 unexpectedly affect protein or RNA levels. For example, PCRTag recoding (intended to alter DNA  
55 sequence without changing the resulting amino acid sequence, producing a watermark to  
56 distinguish synthetic and wild-type sequences) has lowered protein expression or interfered with  
57 transcription factor binding sites in other synthetic chromosome strains.<sup>7,9,11,12</sup> Addition of loxPsym  
58 sites and removal of introns as part of synthetic chromosome design have also affected RNA  
59 expression of genes in Sc2.0 strains, altering strain fitness.<sup>7,11</sup> In the case of *synIX*, we identify and  
60 characterize a fitness defect related to a sequence adjacent to the *EST3* gene, one of the  
61 components of yeast telomerase holoenzyme that is involved in yeast telomere replication.<sup>18-20</sup> We  
62 show that this was the consequence of a specific intentional design step, namely removal of a

63 tRNA gene and associated repetitive DNA sequences. Deletion of a tRNA gene, Ty1 retroelement,  
64 and the DNA between them led to reduced average telomere length and impaired the *synIX*  
65 strain's ability to grow at higher temperature (37°C), a likely consequence of significant Est3p  
66 reduction. Reintroduction of part or all of this sequence dramatically improved strain fitness, with  
67 associated partial or complete restoration of telomere length and Est3p levels depending on  
68 sequence composition and variable effects on the strain's transcriptional profile.

69  
70 The ultimate goal of Sc2.0 is to create a high-fitness yeast strain with a fully synthetic genome. A  
71 previously described chromosome consolidation method that relies on chromosome  
72 endoreduplication followed by sporulation becomes laborious and time-consuming as the synthetic  
73 chromosome number increases in the strain.<sup>2,21</sup> Here we present a strategy which combines  
74 chromoduction of the chromosome of interest and subsequent loss of the native counterpart to  
75 accelerate consolidation. This approach takes advantage of a karyogamy defect that arises when  
76 either parent in a yeast mating has a *kar1-1* mutation.<sup>22</sup> Such crosses yield heterokaryons  
77 consisting of nuclear material from one yeast strain plus a mixed cytoplasm of both parental  
78 strains. In rare cases, whole chromosomes can transfer between strains following a *kar1-1* x *KAR1*  
79 cross.<sup>23</sup> While chromoduction occurs more frequently with smaller chromosomes than with larger  
80 ones,<sup>23</sup> prior work has often employed a selection strategy to identify chromoductants in which a  
81 particular chromosome of interest has been transferred.<sup>24,25</sup> Conversely, whole chromosome  
82 destabilization can be achieved by activating a *GAL* promoter immediately upstream of yeast  
83 centromeres.<sup>7,26</sup> Here, we demonstrated the feasibility of synthetic chromosome transfer using  
84 *synIX*, and produced a final haploid yeast strain in which *synIX* has replaced wild-type  
85 chromosome *IX*. This represents a promising proof-of-principle for targeted transfer of  
86 chromosomes among yeast strains; indeed, this method is in active use to perform the final  
87 consolidation of Sc2.0 chromosomes into a single yeast strain.<sup>21</sup>

## 88 89 **Results**

### 90 91 **Design and assembly of *synIX***

92 The design of *synIX* adheres to the same principles used for the Sc2.0 project.<sup>2</sup> The designed  
93 sequence for *synIX* contains a variety of modifications, including deletion of ten tRNA genes  
94 (relocated to a separate tRNA neochromosome) and 11,632 bp of repetitive DNA, recoding of 54  
95 stop codons from TAG to TAA, 436 bp of restriction enzyme sites, and 7,943 bp of PCRTags  
96 (recoded DNA sequences designed to distinguish between synthetic and native DNA without  
97 altering the resulting amino acid sequence), and addition of 142 loxPsym sites downstream of  
98 nonessential genes that allow for the Cre-mediated SCRaMbLE evolution system (**Figure 1A**).<sup>1-4</sup>  
99 These changes reduced the total size of chromosome *IX* from a wild-type length of 439,885 bp to a  
100 synthetic length of 404,963 bp.

101  
102 Following the synthesis of *synIXR*,<sup>6</sup> we began construction of *synIXL* using a strain containing a  
103 linear, partially synthetic chromosome *synIXR* in which the left arm was in a native state  
104 (yLM461).<sup>2</sup> Assembly of *synIXL* proceeded in several stages (**Figure 1B**). Initially, we assembled  
105 750 bp building blocks from synthetic 60-70 bp oligonucleotides through polymerase cycling  
106 assembly (PCA) in the context of the Build A Genome undergraduate class (**Figure S1A**).<sup>5,6</sup> We

107 then combined these building blocks to form 2-4 kb minichunks. In later stages of the project, as  
108 DNA synthesis technology improved and costs dropped, we obtained the remaining minichunks  
109 directly from a DNA synthesis vendor.

110  
111 We divided *synIXL* into nine 30-60 kb megachunks, each made up of a series of minichunks that  
112 overlapped adjacent ones. To assemble the first six megachunks (megachunks A-F), we  
113 transformed component minichunks into our entry strain one megachunk at a time, including an  
114 auxotrophic marker added to the final minichunk of each region (**Figure 1C**). Through yeast  
115 homologous recombination, each assembly step overwrote the wild-type chromosome segment  
116 and auxotrophic marker attached to it with the synthetic segment and a new auxotrophic marker in  
117 accordance with the Switching Auxotrophies Progressively for Integration (SwAP-In) method,<sup>2,6</sup>  
118 enabling selection of strains containing newly integrated DNA (**Figure 1C, Table S1**). After each  
119 round of integration, the PCRTag watermarking system allowed identification of colonies  
120 containing desired synthetic DNA and lacking corresponding wild-type *chrIX* DNA (**Supplementary**  
121 **File 1**).

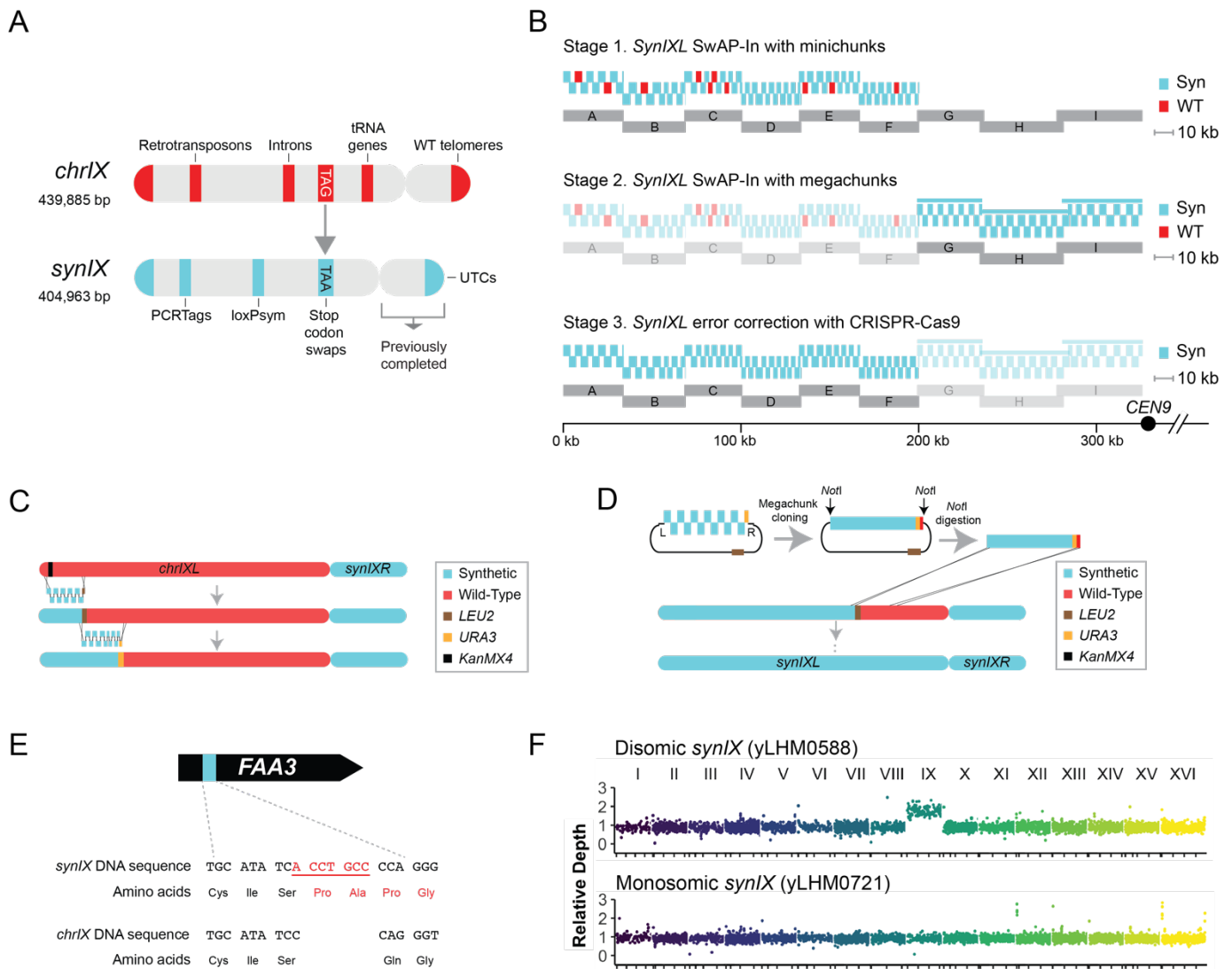
122  
123 The minichunk integration approach used in the assembly of megachunks A-F of *synIXL* left some  
124 unwanted patches of wild-type *chrIX* sequence (**Figure 1B, top**). To improve the efficiency of  
125 synthetic DNA incorporation and to avoid such wild-type DNA patches, later portions of *synIXL*  
126 used the “megachunk cloning” integration strategy.<sup>12</sup> For these sections of the chromosome, we  
127 first assembled minichunks into 40-60 kb megachunks (megachunks G-I) as extrachromosomal  
128 plasmids in yeast, with an assembly success rate of approximately 20-45% (**Figure 1D, Table S2**),  
129 and subsequently transformed them into *E. coli* for plasmid extraction. Following sequence  
130 verification of the megachunks, these larger assembled pieces were released from their plasmid  
131 backbones using restriction enzyme digestion, and delivered directly into our semisynthetic (A-F)  
132 *synIX* strain for SwAP-In. We verified successful integration of the three megachunks via  
133 PCRTagging analysis and whole genome sequencing. All three megachunks constructed in this  
134 manner were integrated into the *synIX* strain in their entirety and lacked novel mutations (**Figure**  
135 **1B, middle**).

136  
137 To convert the wild-type DNA patches seen in megachunks A-F of *synIXL* to synthetic, we used a  
138 CRISPR-Cas9 editing strategy to selectively target *synIX* at residual *chrIX* PCRTags within each  
139 patch (**Figure S1B**) and repair these sequences with appropriate synthetic donor DNAs.<sup>9,10</sup>  
140 Following replacement of ten minichunks over seven rounds of CRISPR-mediated editing, we  
141 obtained a version of *synIX* that contained all the synthetic minichunks included in the  
142 chromosome’s design (**Figure 1B, bottom, Table S3**).

143  
144 We continued CRISPR-mediated editing of this draft strain to correct point mutations, small  
145 deletions, and duplications in *synIX*, as detailed in **Table S4**. Additionally, we discovered a  
146 discrepancy that stemmed from inaccuracies in the wild-type yeast reference genome sequence  
147 that had originally been used for synthetic chromosome design. In *synIX*, the designer sequence  
148 for the non-essential *FAA3* gene contained an extra seven base pairs missing from the most  
149 recent version of the wild-type reference sequence, resulting in a frameshift (**Figure 1E**).<sup>27,28</sup> A  
150 previous genetic screen linked the *faa3Δ* null allele to decreased vegetative growth;<sup>29</sup>

consequently, we restored proper *FAA3* function in our *synIX* strain by deleting the unwanted sequence via CRISPR-mediated editing, reestablishing the normal reading frame of *FAA3* (Figure S2).

During this editing process, we unexpectedly identified whole-chromosome disomy of *synIX* (Figure 1F, top). To restore the normal karyotype of the *synIX* strain, we integrated a *URA3-pGAL* cassette upstream of *CEN9* into one *synIX* copy,<sup>26</sup> induced chromosome destabilization and loss via growth in media containing galactose, and then selected against the *URA3* marker to identify strains with a single copy of *synIX* (Figure S3). Whole genome sequencing confirmed that the resulting *synIX* strain contained approximately equal read depth for each chromosome, as expected for a monosomic haploid yeast strain (Figure 1F, bottom). There were no overt phenotypic differences between the disomic and monosomic strains, suggesting that the disomy was an inadvertent consequence of CRISPR-mediated editing rather than selection for a second copy of *synIX* due to haploinsufficiency. This monosomic strain was used for further debugging.



**Figure 1.** Design and assembly of *synIX*. (A) Diagram of the differences between wild-type (WT) *chrIX* and *synIX*. Designer features specific to *synIX* include the addition of PCRTags and loxPsym sites, the recoding of TAG stop codons to TAA, the replacement of wild-type telomeres with universal telomere caps (UTCs), and the removal of tRNA

171 genes (relocated to a tRNA neochromosome), introns, retrotransposons, and subtelomeric repeats. **(B)** Schematic of  
172 the *synIXL* construction process, reflecting the synthetic and (unwanted) WT composition of chromosome IX in the  
173 *synIX* strain after each stage of assembly. Stage 1: SwAP-In with minichunks (A-F). Stage 2: SwAP-In with pre-  
174 assembled megachunks (G-I, opaque with blue lines). Stage 3: Error correction with CRISPR-Cas9 (primarily in  
175 highlighted megachunks A-F). Blue: syn minichunks, red: WT minichunks, gray: megachunks. Red patches in stages 1  
176 and 2 reflect segments of DNA that were not replaced by their expected synthetic MC counterparts during SwAP-In  
177 with minichunks in stage 1 (A-F); appropriate synthetic sequences were integrated at these sites during stage 3. **(C)**  
178 Minichunk integration strategy for assembling megachunks A-F of *synIX* in yeast (used for stage 1 in **(B)**). A kanMX  
179 marker (black) was integrated into the left end of wild-type *chrIX* (red). Individual minichunks (blue, synthetic)  
180 comprising one megachunk were then co-transformed into the in-progress *synIX* strain and used to overwrite the wild-  
181 type *chrIX* sequence (red) via homologous recombination. Alternating auxotrophic markers (*LEU2*, brown, and *URA3*,  
182 orange) were used and overwritten at each step, allowing for selection of the new marker and counterselection against  
183 the previous round's marker. **(D)** Megachunk plasmid assembly and integration approach for building and integrating  
184 megachunks G-I in *synIX* (used for stage 2 in **(B)**). Minichunks (blue) were assembled in yeast, and an auxotrophic  
185 marker (here, *URA3*, orange) was added to the end of each assembly, followed by a segment of *chrIX* homology. The  
186 megachunk assemblon overwrote the prior round auxotrophic marker (brown, *LEU2*) and wild-type DNA (red) by  
187 homologous recombination. **(E)** Schematic depicting a frameshift mutation in the *FAA3* gene in the *synIX* design. The  
188 synthetic sequence included seven base pairs not present in the S288C wild-type yeast reference genome. The added  
189 bases, shown in red, cause a frameshift, with the resulting *synIX* amino acid sequence (red) varying from the expected  
190 *chrIX* amino acid sequence (black, bottom). **(F)** Coverage plots showing read depth along each yeast chromosome in  
191 disomic *synIX* strain yLHM0588 (top) and monosomic *synIX* strain yLHM0721 (bottom). X axis: Yeast chromosome  
192 (not to scale). Y axis: Relative depth based on number of reads at each position divided by average read depth across  
193 the sixteen yeast chromosomes.

## 194 195 **Debugging of *synIX***

196 After correcting the above *synIX* sequence issues, we found that this strain, coined  
197 yeast\_chr09\_9\_1 (also referred to as yLHM1192), grew more poorly than the parental BY4741  
198 strain under high-temperature (37°C) conditions on both dextrose-containing (YPD) and glycerol-  
199 containing (YPG) media (**Figure 2A**). A similar phenotype was observed in additional strain  
200 isolates equivalent to yLHM1192 (**Figure S5**). Following a pooled sequencing bug mapping  
201 method,<sup>9</sup> we backcrossed our *synIX* strain to a wild-type strain to generate a heterozygous diploid  
202 yeast strain for sporulation, and subsequently dissected tetrads. The goal of this process was to  
203 obtain spores containing a randomized mixture of recombinant *synIX* and *chrIX* DNA as a  
204 consequence of meiotic recombination (**Figure S4A**). By conducting whole genome sequencing of  
205 healthy and sick spores, we identified a region spanning from approximately 15-20 kb upstream of  
206 *CEN9* to 5 kb downstream of *CEN9* that appeared to be associated with our *synIX* fitness defect  
207 (**Figures 2B, S4B**). Consistent with this region's involvement in *synIX*'s growth deficiency, our  
208 *synIX* strain initially exhibited a marked decline in fitness on YPD at 37°C following the integration  
209 of megachunk I, which contained the synthetic sequence corresponding to this portion of the  
210 chromosome (**Figure S6**). To determine the gene responsible for the bug within this ~20-25 kb  
211 region, we assembled the wild-type version of each of the genes in this region into a *CEN* plasmid  
212 for complementation (**Figure S7**). The strains containing a plasmid with *EST3* showed a  
213 pronounced improvement in fitness on both YPD and YPG at 37°C compared with the buggy *synIX*  
214 strain (**Figures 2B, S7**).

215  
216 To investigate which modifications of the synthetic *EST3* gene might contribute to this fitness  
217 defect, we replaced sections of the synthetic region with corresponding wild-type sequences in our  
218 *synIX* strain and monitored strain fitness. Replacing the coding sequence of *EST3* was insufficient



219 to restore fitness on YPD or YPG at 37°C (**Figure 2C, yLHM1429**); however, restoring both a  
220 deleted segment containing an aspartyl tRNA gene and a nearby Ty1 long terminal repeat (LTR)  
221 element dramatically improved *synIX* strain fitness on both YPD and YPG at 37°C (**Figure 2C,**  
222 **yLHM1430 and yLHM1431**). To better assess the contributions of these *EST3*-adjacent elements  
223 to strain fitness, we next introduced smaller modifications to *EST3*'s upstream region in the *synIX*  
224 strain, including different combinations of the aspartyl tRNA gene, nearby Ty1 LTR, and an  
225 intervening single-copy “gap” DNA sequence with no known function that separates the tRNA gene  
226 and Ty1 LTR in the native *chrIX* sequence (**Figure 3A, Table S5**). We found that all of these edits  
227 improved strain growth fitness (**Figure 3A**). Similar phenotypes were observed in additional strains  
228 generated for each modification, and did not noticeably change following extended strain  
229 passaging (**Figure S8**).

230  
231 As *EST3* is involved in telomere maintenance, we hypothesized that *EST3*-associated changes in  
232 fitness might correlate with changes in average strain telomere length. Consequently, we next  
233 assessed telomere length of our *synIX* and variant strains via Southern blotting with a probe  
234 specific for telomeric repeats (**Figure 3B**). Following enzymatic digestion of yeast gDNA with *XhoI*,  
235 a smear of telomeric fragments from chromosome ends containing Y' elements can be seen at the  
236 bottom of each lane; these fragments were used to estimate average telomere length, with other  
237 telomeric fragments appearing at larger sizes in each lane. As expected, yLHM1192's telomeres  
238 were markedly shorter than those seen in the wild-type BY4741 strain. Interestingly, however, the  
239 telomeres in the modified *synIX* strains split into two length groups; three groups of strains,  
240 yLHM1504 (containing the entire tRNA, intervening “gap” sequence between the tRNA and Ty1  
241 with no known function, and Ty1 sequence), yLHM1506 (containing the tRNA and gap sequences,  
242 but not the Ty1 sequence), and yLHM1601 (containing a tRNA with a transcription-inactivating  
243 point mutation<sup>30-33</sup> and the aforementioned gap sequence) had telomeres similar in length to or  
244 slightly longer than wild-type yeast, while two other groups of strains, yLHM1505 (containing just  
245 the gap sequence and Ty1 LTR) and yLHM1591 (containing just the gap sequence), had  
246 telomeres that were shorter than BY4741, but slightly longer than yLHM1192.

247  
248 Next, we wanted to assess the role of these upstream *EST3* modifications on strain fitness and  
249 telomere length independent of any other *synIX*-associated strain changes. To do this, we  
250 introduced each of our synthetic modifications upstream of *EST3* (from yLHM1192, yLHM1504-  
251 1506, yLHM1591, and yLHM1601) to wild-type yeast, while otherwise maintaining the native *chrIX*  
252 sequence (**Figure 4A**). As in our *synIX* strains, the yLHM1192-equivalent wild-type edited strains  
253 showed a decline in fitness on YPG plates at 37°C, while the other modified strains showed fitness  
254 similar to wild-type BY4741 (**Figure S9**). Fitness did not change appreciably following extended  
255 strain passaging (**Figure S9**). We again examined average telomere length of these strains via  
256 Southern blot (**Figure 4B**). As with the synthetic strains, we saw three groups of telomere lengths:  
257 wild-type (BY4741, as well as the strains with the yLHM1504, yLHM1506, and yLHM1601-  
258 equivalent modifications), shortened (strains with the yLHM1192-equivalent modifications), and  
259 intermediate (strains with the yLHM1505-equivalent and yLHM1591-equivalent modifications).

260  
261 We next sought to understand why all of our modifications improved strain fitness, but only some  
262 fully restored telomere length to wild-type levels. We hypothesized that differences in *EST3*

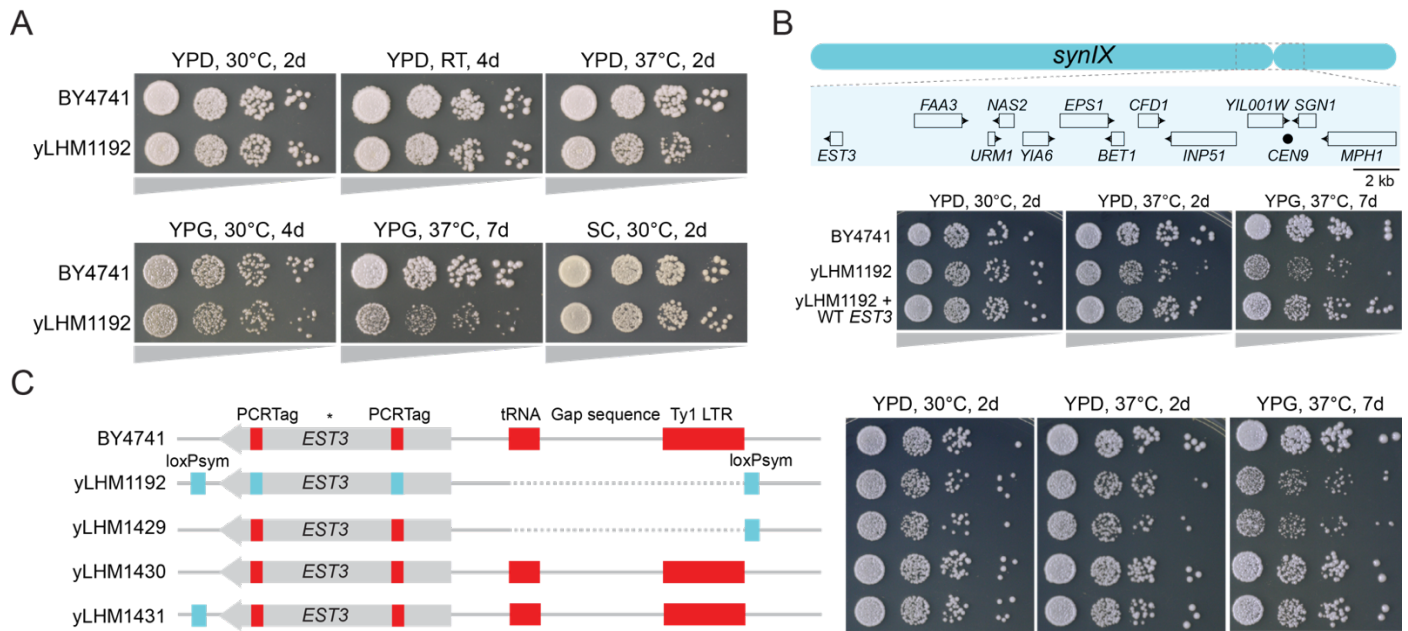
263 translation and/or transcription might result in variable telomere length across strains. To examine  
264 Est3p expression in our modified *synIX* strains, we tagged the 3' end of *EST3* with a 3X FLAG tag  
265 and monitored protein levels after strain growth at 37°C (**Figure 5A, Table S6**). FLAG-tagged  
266 synthetic strains were crossed to an untagged wild-type strain of opposite mating type (BY4742) to  
267 complement any other recessive *synIX*-related effects on strain growth while allowing for  
268 monitoring of Est3p production from the synthetic locus. Consistent with the telomere length data,  
269 the yLHM1504-, yLHM1506-, and yLHM1601-derived strains expressed higher levels of FLAG-  
270 tagged Est3p than did the yLHM1192-, yLHM1505-, and yLHM1591-derived strains (**Figure 5A**). A  
271 follow-up comparison of the yLHM1192-, yLHM1505-, and yLHM1591-derived strains revealed  
272 higher Est3p levels in the yLHM1505-derived and yLHM1591-derived strains than in the  
273 yLHM1192-derived strains, although all three strains had markedly lower Est3p levels than did  
274 wild-type yeast (**Figure 5B**). We saw neither longer nor shorter isoforms of FLAG-tagged Est3p  
275 associated with the low-abundance strains (**Figures 5B, S10**). Similar results were observed in  
276 FLAG-tagged heterozygous diploid strains generated by crossing FLAG-tagged versions of the  
277 wild-type *EST3* variant strains from **Figure 4A** and **Figure S9** to untagged BY4742 and monitoring  
278 Est3p levels (**Figures S11A, S11B**).

279  
280 To further elucidate what might contribute to the variations in Est3p levels in our engineered *synIX*  
281 strains, we examined RNA sequencing data to look for changes in RNA expression patterns. Of  
282 note, strand-specific RNA sequencing data revealed differences in the pattern of *EST3* transcripts  
283 in the wild-type and *synIX* strains at both 30°C and 37°C (**Figures 5C, 5D, S12A, S12B**). In  
284 particular, while both strains showed sequencing read depth throughout the *EST3* coding region,  
285 the *synIX* strain contained transcripts upstream of the expected *EST3* transcription start site that  
286 were absent from the wild-type strain. Importantly, given that these transcripts were transcribed  
287 from the same strand as native *EST3*, some *EST3* transcripts might have had an extended 5' UTR,  
288 predicted to encode upstream AUG codons that would not produce functional Est3p. In our  
289 modified *synIX* strains, we saw two classes of upstream transcript profiles (**Figures 5E, 5F**).  
290 yLHM1504, yLHM1506, and yLHM1601, the three strains with wild-type-length or slightly longer  
291 telomeres, showed a relative reduction in RNA sequencing reads directly upstream of *EST3*  
292 compared with strand-specific total reads aligned to the upstream plus coding sequence of *EST3*  
293 (**Figures 5E, 5F, S13A, S13B**). By contrast, yLHM1505 and yLHM1591, the two strains with  
294 intermediate-length telomeres, still showed substantial transcript read depth upstream of *EST3*.  
295 The wild-type strains with modifications upstream of *EST3* yielded a similar transcriptional pattern  
296 (**Figures S14A, S14B**).

297  
298 Nanopore direct RNA sequencing, which evaluates full-length transcript patterns, revealed a  
299 distinct population of *EST3*-spanning transcripts in yLHM1192 containing at least one out-of-frame  
300 AUG upstream of the expected *EST3* transcription start site (**Figure 5G**). Many of these transcripts  
301 started 250-500 bp upstream of the AUG that normally gives rise to the first methionine of  
302 functional Est3p (**Figures S15**). We hypothesize that such “non-translatable” upstream transcripts  
303 fail to yield properly translated Est3p, contributing to reduced telomerase function and decreased  
304 strain fitness (**Figure S16**). By contrast, while yLHM1505 and yLHM1591 contained a few non-  
305 translatable transcripts spanning *EST3*, most of the transcripts upstream of *EST3* that were  
306 observed in these strains terminated prior to the expected *EST3* transcription start site (gray in

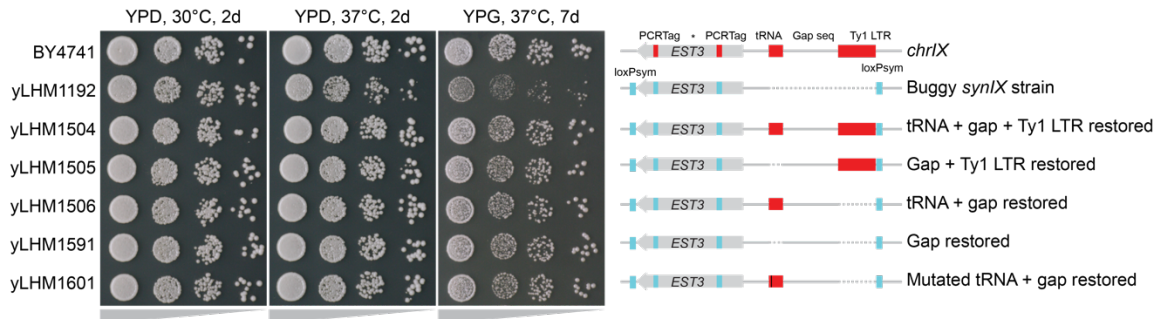
307 **Figure 5G**). yLHM1192, yLHM1505, and yLHM1591 all had relatively fewer full-length, presumably  
 308 translatable *EST3* transcripts than did the tRNA-containing strains (yLHM1504, yLHM1506, and  
 309 yLHM1601).

310  
 311 Taken together, these results point to a model in which upstream initiated transcripts are unable to  
 312 produce Est3p, and only reads initiating at or near the native transcription start site produce  
 313 functional *EST3* transcripts and resulting protein. The original *synIX* strain (yLHM1192) appears to  
 314 yield almost no functional Est3p, while variant strains produce either low (yLHM1505 and  
 315 yLHM1591) or near-wild-type (yLHM1504, yLHM1506, and yLHM1601) Estp3 levels. However,  
 316 strains with even a low level of functional Est3p and a moderate reduction in telomere length  
 317 appear to surpass a threshold for maintaining normal strain fitness. Thus, our three classes of  
 318 Est3p levels and telomere length yield only two fitness profiles; healthy strains and sick ones.

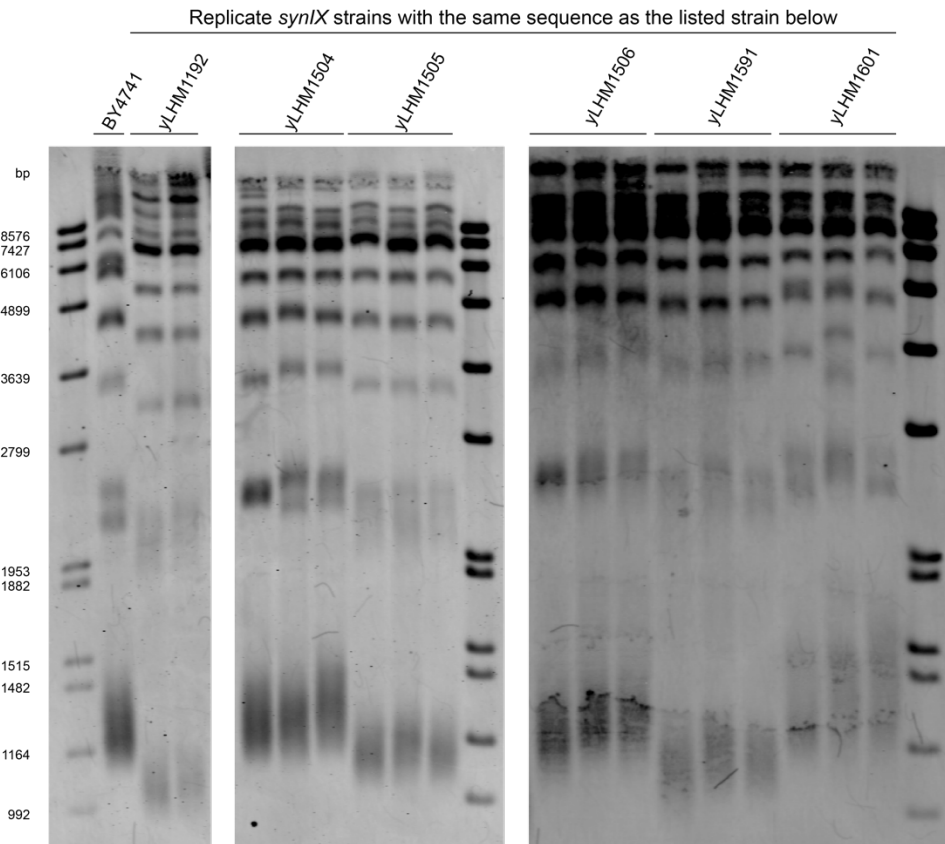


320  
 321  
 322 **Figure 2**. Identifying and mapping an *EST3*-related bug. **(A)** Spot assays comparing the growth of BY4741 (wild-type)  
 323 to yLHM1192 (synthetic) across a variety of growth conditions. Each column represents a tenfold dilution. RT: Room  
 324 temperature, approximately 22°C. **(B)** (Top) Schematic of region near *CEN9* identified as containing the gene  
 325 responsible for a *synIX* fitness defect. The unhealthy *synIX* strain (yLHM1192) was transformed with plasmids  
 326 containing transcription units for each of the genes shown. (Bottom) Spot assays comparing the growth of BY4741 and  
 327 yLHM1192 to yLHM1192 transformed with a plasmid containing the *EST3* coding sequence plus 500 bp of upstream  
 328 sequence and 200 bp of downstream sequence. Each column represents a tenfold dilution. **(C)** Spot assays  
 329 comparing growth of strains with different combinations of synthetic and wild-type features in and upstream of *EST3*.  
 330 Each column represents a tenfold dilution. Schematics on left illustrate wild-type (red) and synthetic (blue) features  
 331 present in each strain. The asterisk (\*) marks the site of a programmed +1 ribosomal frameshift during *EST3*  
 332 translation.

A

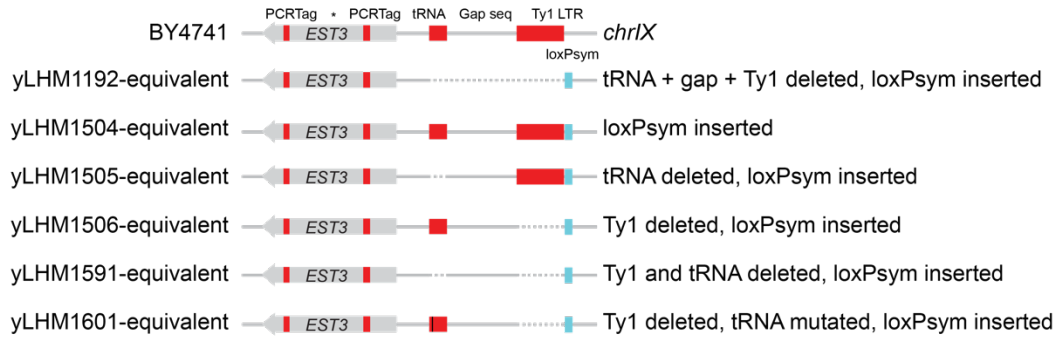


B

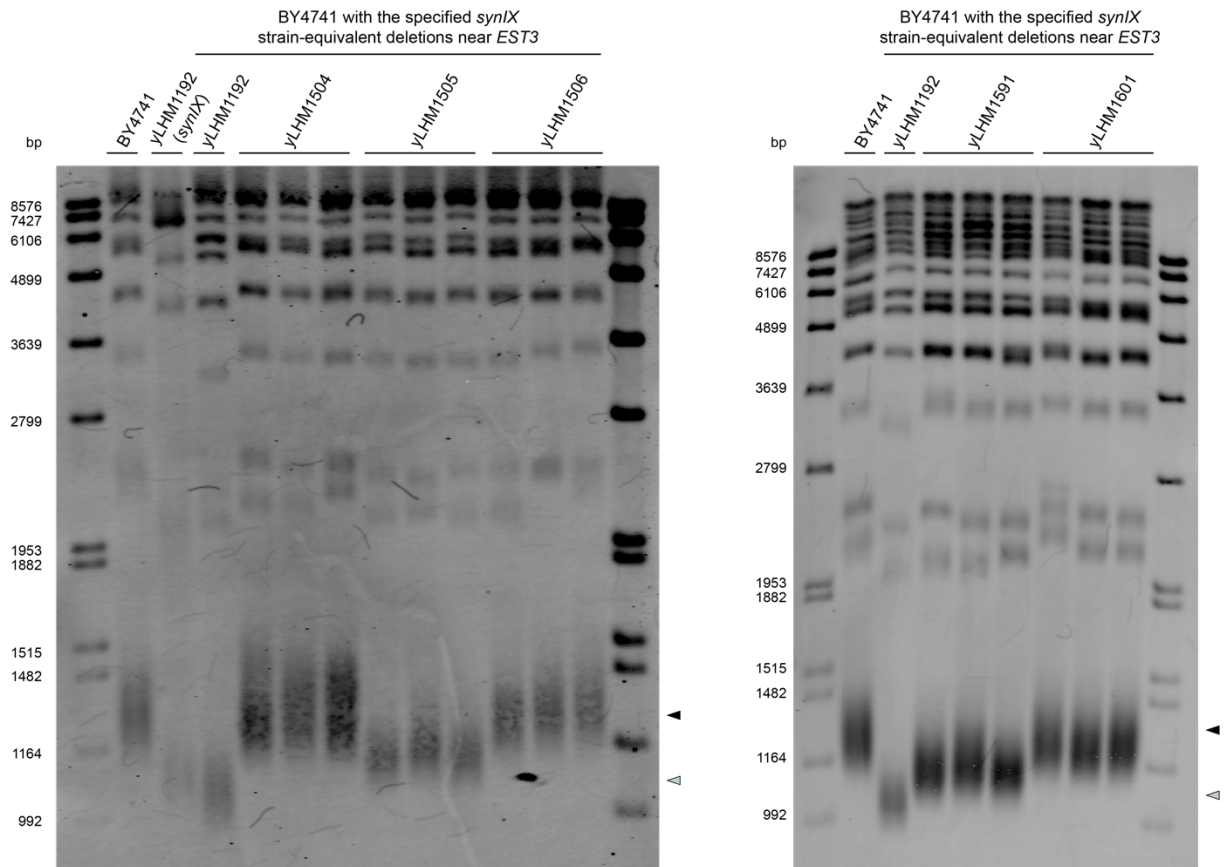


334  
 335  
 336 **Figure 3.** Modifying *synIX* strains upstream of *EST3* alters fitness and telomere length. (A) Spot assays and  
 337 accompanying schematics of the region upstream of *EST3* for strains BY4741 (wild-type *chrIX*), yLHM1192 (synthetic),  
 338 and variant strains derived from yLHM1192. Wild-type PCRTags, tRNA gene (Asp), and Ty1 long terminal repeat  
 339 (LTR) are depicted in red, synthetic PCRTags and loxP sites in blue, mutation in tRNA<sup>ASP</sup> gene as a vertical black  
 340 line. Each spot assay column represents a tenfold dilution. (B) Southern blot of *XhoI*-digested yeast gDNA derived  
 341 from BY4741 (wild-type) and replicate strains with modifications equivalent to yLHM1192 (synthetic) and variant strains  
 342 (yLHM1504, yLHM1505, yLHM1506, yLHM1591, and yLHM1601, as depicted in (A)). DNA was probed with a  
 343 digoxigenin-labeled fragment specific for telomeric repeats. Black arrow (right): Average size of Y'-containing telomeric  
 344 fragments for BY4741. Gray arrow (right): Average size of Y'-containing telomeric fragments for yLHM1192. Left and  
 345 middle panels: Left and right halves of one Southern blot, cropped to remove one lane in the middle. Right panel: Right  
 346 side of a second Southern blot, cropped to remove left-hand ladder, BY4741, and yLHM1192 lanes.

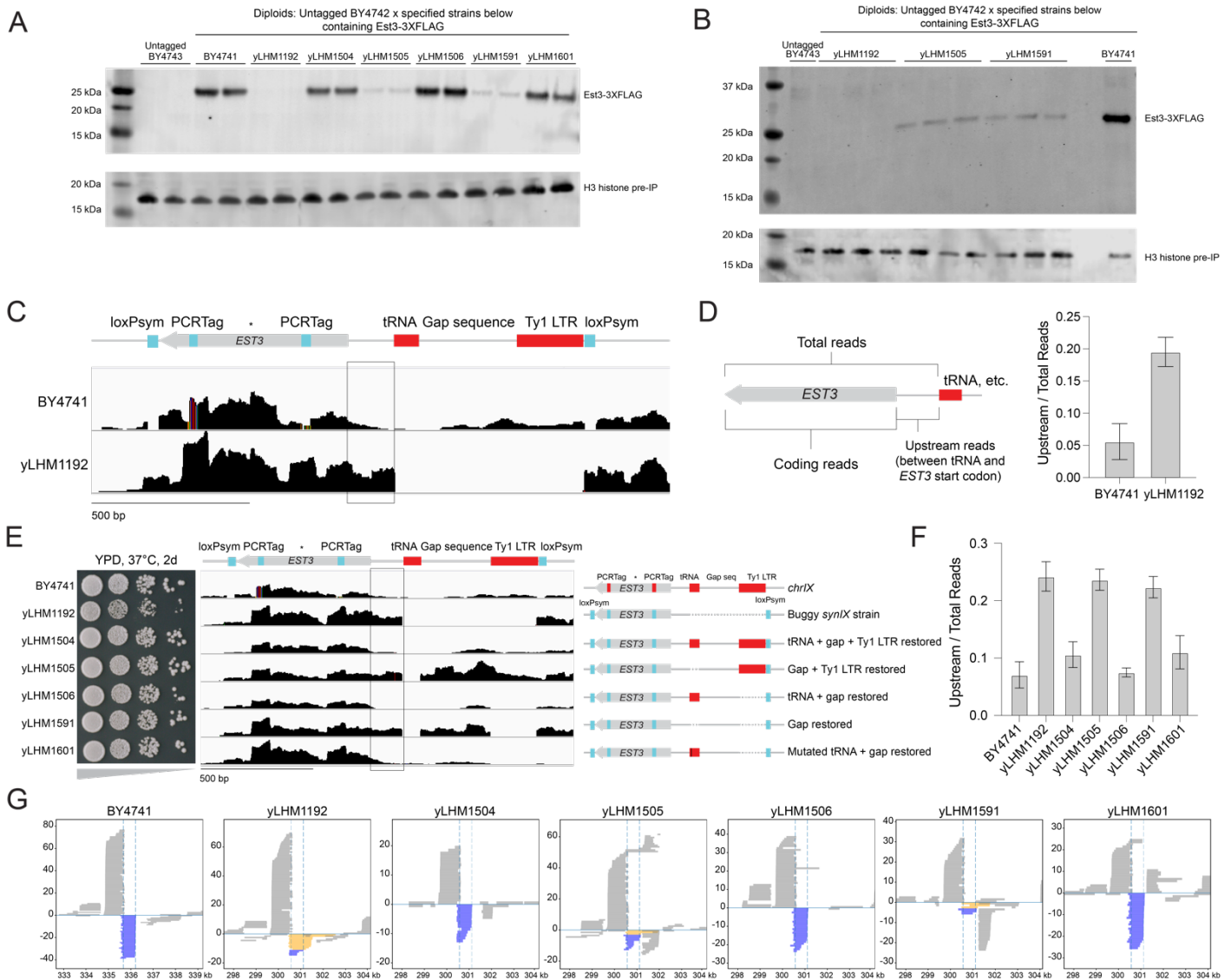
A



B



347  
 348  
 349 **Figure 4.** Modifying wild-type yeast strains upstream of *EST3* alters telomere length. **(A)** Schematics of the region  
 350 upstream of *EST3* for strains BY4741 (wild-type *chrIX*), yLHM1192-equivalent (synthetic modification matching  
 351 yLHM1192 in an otherwise wild-type yeast strain), and variant strains derived from the yLHM1192-equivalent strain.  
 352 Wild-type PCRTags, tRNA gene (Asp), and Ty1 long terminal repeat (LTR) are depicted in red, loxPsym sites in blue,  
 353 mutation in tRNA<sup>Asp</sup> gene as a vertical black line. **(B)** Southern blot of *XhoI*-digested yeast gDNA derived from BY4741  
 354 (wild-type), yLHM1192 (synthetic), and replicate strains with synthetic-equivalent modifications made upstream of  
 355 *EST3*, as depicted in **(A)**. DNA was probed with a digoxigenin-labeled fragment specific for telomeric repeats. Black  
 356 arrow (right): Average size of Y'-containing telomeric fragments for BY4741. Gray arrow (right): Average size of Y'-  
 357 containing telomeric fragments for BY4741 + yLHM1192-equivalent deletion strain.  
 358



**Figure 5.** Dissecting the effects of *EST3*-adjacent features on *EST3* protein and RNA expression. **(A)** Immunoblotting for immunoprecipitated (IP) Est3p (top) and H3 histone pre-IP loading control (bottom). Est3p was C-terminally tagged with 3X FLAG in each synthetic strain, and diploids were generated via mating with wild-type BY4742. Two independent colonies were grown in YPD at 37°C for each strain. Right-most lane (second ladder) was cropped out of image. **(B)** Immunoblotting for immunoprecipitated (IP) Est3p (top) and H3 histone pre-IP loading control (bottom) for low-expressing Est3p strains plus controls. Est3p was C-terminally tagged with 3X FLAG in each synthetic strain, and diploids were generated via mating with wild-type BY4742. Three independent colonies were grown at YPD at 37°C for each yLHM1192-, yLHM1505-, and yLHM1591-derived diploid strain. Right-most lane (second ladder) was cropped out of image. **(C)** Strand-specific RNA sequencing alignments for the reverse strand of BY4741 and yLHM1192 (strand from which *EST3* transcription is expected, i.e., “reverse strand reads”) grown at 37°C in YPD to a composite reference containing both synthetic and native *IX* features. Schematic on top illustrates wild-type *chrIX* (red) and synthetic (blue) features. Black box on the RNA sequencing alignment plot highlights region between the expected *EST3* start codon and the upstream tRNA. The asterisk (\*) marks the site of a programmed +1 ribosomal frameshift during *EST3* translation. **(D)** Quantification of reverse strand reads mapping directly upstream of *EST3* (between tRNA and start codon, corresponding to black box in **C**) as a fraction of the total reverse strand *EST3* coding and upstream reads for each strain shown in **C**. Read counts based on alignment to native (BY4741) or synthetic (yLHM1192) reference sequence. Error bars represent standard deviation of three biological replicates. **(E)** Spot assays and strand-specific RNA sequencing alignments for the reverse strand reads aligning to the *EST3* region for samples grown at 37°C on YPD plates or in YPD liquid media. Strains: BY4741 (wild-type *chrIX*), yLHM1192 (synthetic), and variant strains derived from yLHM1192. Wild-type PCRTags, tRNA gene (Asp), and Ty1 long terminal repeat (LTR) are

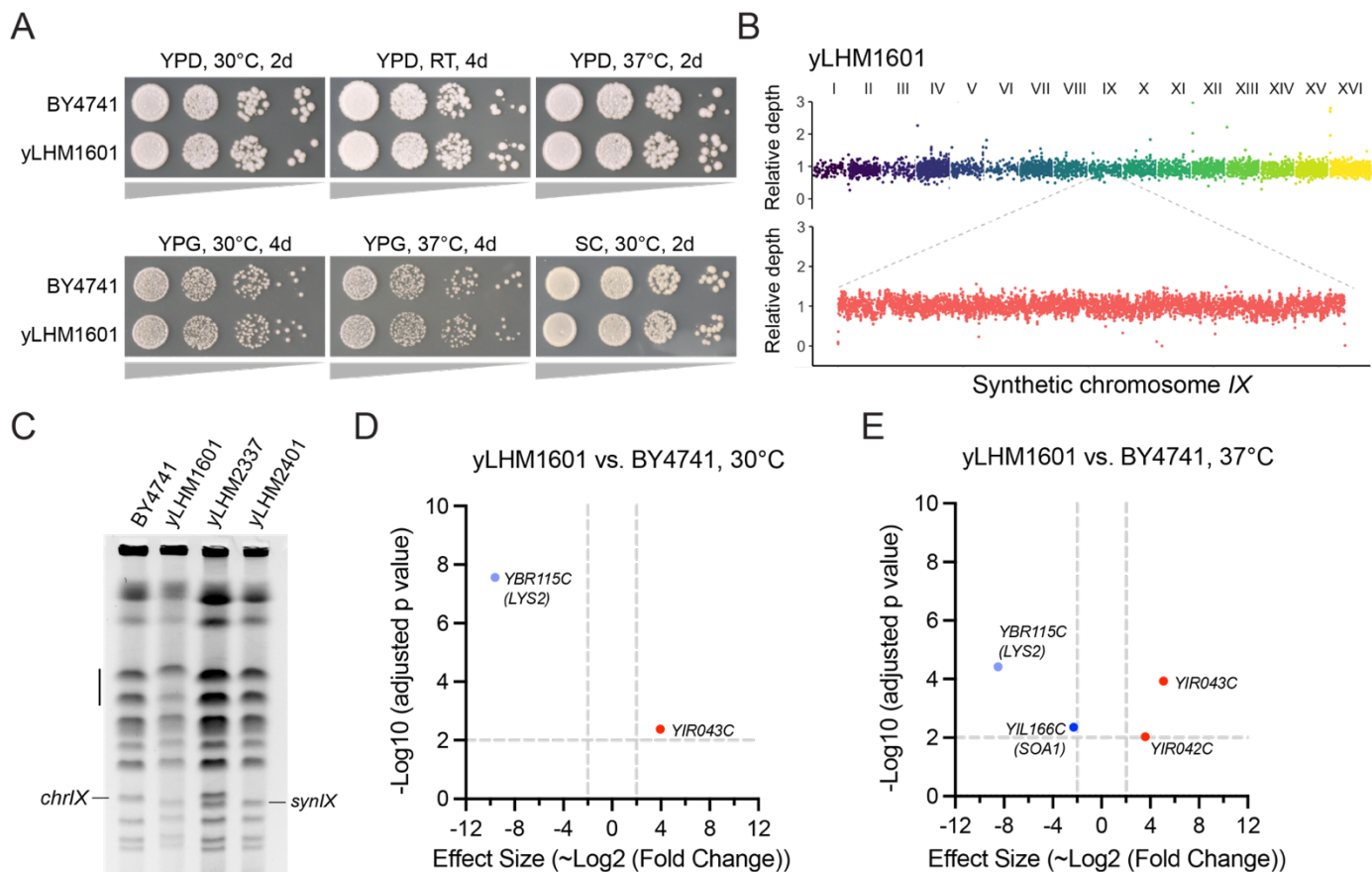
381 depicted in red, synthetic PCRTags and loxPsym sites in blue, mutation in tRNA<sup>Asp</sup> gene as a vertical black line. Black  
382 box on the RNA sequencing alignment plot highlights region between the expected *EST3* start codon and the  
383 upstream tRNA. The asterisk (\*) marks the site of a programmed +1 ribosomal frameshift during *EST3* translation. (F)  
384 Quantification of reverse strand reads mapping directly upstream of *EST3* (between tRNA and start codon,  
385 corresponding to black box in E) as a fraction of the total reverse strand *EST3* coding and upstream reads for each  
386 strain shown in E. Read counts based on alignment to native (BY4741) or synthetic (yLHM1192) reference sequence.  
387 Error bars represent standard deviation of three biological replicates. (G) Nanopore direct RNA sequencing reads  
388 aligned to the *EST3* region ( $\pm 3$ kb). Strains: BY4741, yLHM1192, and variant strains derived from yLHM1192, all grown  
389 at 37°C in YPD liquid media. X axis: Chromosome coordinate. Y axis: Number of reads. Forward strand reads are  
390 above the Y axis, reverse strand reads below the Y axis. Dotted lines indicate the boundaries of the *EST3* coding  
391 region. *EST3* transcripts are expected to lie on the reverse strand. Yellow reads: “Non-translatable” reads that contain  
392 at least one AUG upstream of the expected *EST3* start codon plus a minimum of 20 bp of 5' UTR sequence. Blue  
393 reads: Reads mapping to *EST3* that start no more than 20 bp upstream of the first AUG upstream of the expected  
394 *EST3* start codon, including “translatable/functional” reads spanning the entirety of *EST3* and reads starting  
395 downstream of the *EST3*-initiating AUG.

### 397 **Characterization of the healthy *synIX* strain**

398 We ultimately proceeded with the strain in which a mutated version of the deleted tRNA gene and  
399 the adjacent gap sequence were reintroduced upstream of *EST3* (yLHM1601 in **Figure 5E**). The  
400 tRNA sequence contained a point mutation previously shown to block tRNA transcription,<sup>30-33</sup>  
401 allowing functional tRNA relocation to a tRNA neochromosome<sup>15</sup> while maintaining Est3p levels  
402 similar to those of wild-type yeast (**Figure 5A**). This strain is most consistent with Sc2.0’s design  
403 principles of maximizing strain fitness while eliminating repetitive elements and tRNA genes in  
404 particular. This final *synIX* version, coined yeast\_chr09\_9\_2, grew similarly to wild-type yeast  
405 across a variety of media types and temperature conditions, as did additional sequence-matched  
406 replicate strains (**Figures 6A, S17, S18, S19**). The final strain largely matched the expected  
407 synthetic reference sequence, with slight deviations from the original design reflecting changes  
408 made during bug fixing and other minor alterations during the assembly process (**Table S7**). The  
409 final strain also showed even coverage across *synIX* and normal chromosomal copy number  
410 (**Figure 6B**). *SynIX* migrated slightly faster on pulsed-field gel electrophoresis (PFGE) than did  
411 native *IX* (**Figure 6C**). We also observed an unusual banding pattern among a couple of the higher  
412 bands in yLHM1601, possibly related to an expanded repetitive sequence near the telomere of  
413 chromosome *XVI*. This difference did not appear to affect strain fitness, and disappeared following  
414 *synIX* transfer to an alternative matched-background wild-type yeast strain via the chromosome  
415 substitution method discussed in the next section (**Figure 6C, lanes 3 and 4**).

417 Transcriptional analysis of the completed *synIX* strain (yLHM1601, or yeast\_chr09\_9\_2) identified  
418 a small number of differentially expressed genes compared to the wild-type strain (**Figures 6D, 6E,**  
419 **S20A, S20B**). Several hits found on *synIX* were located in or near telomeres, with some of these  
420 genes showing upregulation and other showing downregulation. On the left arm of *synIX*, *SOA1*  
421 (*YIL166C*) showed decreased expression in the *synIX* strain at 37°C (~5x lower than in BY4741).  
422 Several of the other genes originally found between *SOA1* and *TEL09L* in native chromosome *IX*  
423 were deleted in the *synIX* design. Consequently, we hypothesize that *SOA1* expression may be  
424 decreased due to increased silencing associated with its new position near *TEL09L*, as has been  
425 observed on other synthetic chromosomes where genes moved closer to the telomeres due to  
426 subtelomeric DNA deletion.<sup>12</sup> Conversely, right arm subtelomeric genes *YIR042C* and *YIR043C*

427 showed expression increases at 37°C (~12x increase for *YIR042C*) and both 30°C and 37°C (~15x  
 428 and ~34x increases for *YIR043C*), respectively. Unlike *SOA1*, *YIR042C* and *YIR043C* were  
 429 already located very close to *TEL09R* in native yeast chromosome IX. As a result, it is possible that  
 430 the universal telomere caps introduced to *synIX* per Sc2.0 design principles failed to silence  
 431 nearby genes to the same degree as the native telomeres, since the earliest version of the UTC  
 432 used in this case lacked the X sequence used at the left telomere and elsewhere in the project.  
 433 Since the X sequence, a known subtelomeric anti-silencing region,<sup>34</sup> is present in the native  
 434 *TEL09R*, its absence could explain the selectively elevated expression of these *TEL09R*-adjacent  
 435 genes.  
 436



437  
 438  
 439 **Figure 6.** *SynIX* characterization. **(A)** Spot assays comparing the growth of BY4741 (wild-type) to yLHM1601  
 440 (synthetic) across a variety of temperature conditions. Each column represents a tenfold dilution. RT: Room  
 441 temperature, approximately 22°C. **(B)** DNA sequencing coverage plot for *synIX* strain (yLHM1601). X axis: Yeast  
 442 chromosome (not to scale), or coordinate along *synIX*. Relative depth (Y axis) based on reads mapped to the  
 443 yeast\_chr09\_3\_55 reference sequence divided by the average read depth across the sixteen yeast chromosomes  
 444 (top) or *synIX* (bottom). **(C)** Pulsed-field gel showing chromosomes from BY4741 (*chrIX*), yLHM1601 (*synIX*),  
 445 yLHM2337 (BY4742-background strain with copies of *chrIX* and *synIX*), and yLHM2401 (yLHM2337 after loss of *chrIX*)  
 446 strains. Vertical black line indicates the location of a potential discrepancy in band intensity for larger chromosomes  
 447 between BY4741 and yLHM1601; *synIX* was moved to an alternative strain (yLHM2337) with larger bands resembling  
 448 BY4741 in intensity, and *chrIX* was subsequently lost from this strain (producing yLHM2401) via the strategy  
 449 described in the next section. **(D-E)** Volcano plots of differentially expressed genes obtained from RNA sequencing data for *synIX*  
 450 strain (yLHM1601) vs. BY4741 measured at 30°C **(D)** and 37°C **(E)** in YPD. Upregulated genes in yLHM1601 are  
 451 depicted in red (chromosome IX), downregulated genes in medium blue (chromosome IX) or light blue (other  
 452 chromosomes). Transcript counts are based on alignment to the S288C reference transcriptome. Auxotrophic gene  
 453 *LYS2* was present in BY4741, but not yLHM1601. Fold change cutoff is 4, adjusted p-value cutoff is 0.01. Three



454 biological replicates were used for each strain. For corresponding unfiltered plots of RNA sequencing data depicting all  
455 measured genes, see **Figures S21A** and **S21B**.

### 457 **Chromosome substitution of *synIX***

458 Here, we sought to combine chromoduction with subsequent loss of the corresponding native  
459 chromosome from the recipient strain to seamlessly move *synIX* between yeast strains while  
460 maintaining normal karyotype in the final strain of interest (**Figure 7A**). As a proof-of-concept, a  
461 disomic *synIX* strain was used as the donor, as an extra copy of *synIX* was deemed likely to  
462 produce higher chromosome transfer efficiency.<sup>24</sup> A *kar1-1* mutant strain was used as the recipient  
463 strain for blocking karyogamy during mating. Notably, the recipient strain also contained two  
464 mutations, *can1* and *cyh2*, as recessive selectable markers to ensure robust exclusion of donor  
465 cells or potential diploid cells on canavanine- and cycloheximide-containing medium. To facilitate  
466 selection of recipient strains containing *synIX* transferred from the donor strain following strain  
467 crossing, we deleted the *LYS12* gene on the recipient strain's wild-type chromosome *IX* (**Figure**  
468 **S21A**). This allowed facile selection for lysine prototrophy to ensure *synIX* was present in the  
469 chromoductants. Additionally, to enable eventual loss of the wild-type *chrIX* from the recipient  
470 strain, we subsequently knocked in a *URA3-pGAL* cassette upstream of *CEN9* in the recipient  
471 strain for *chrIX* destabilization and counterselection (**Figure S21B**).<sup>26</sup>

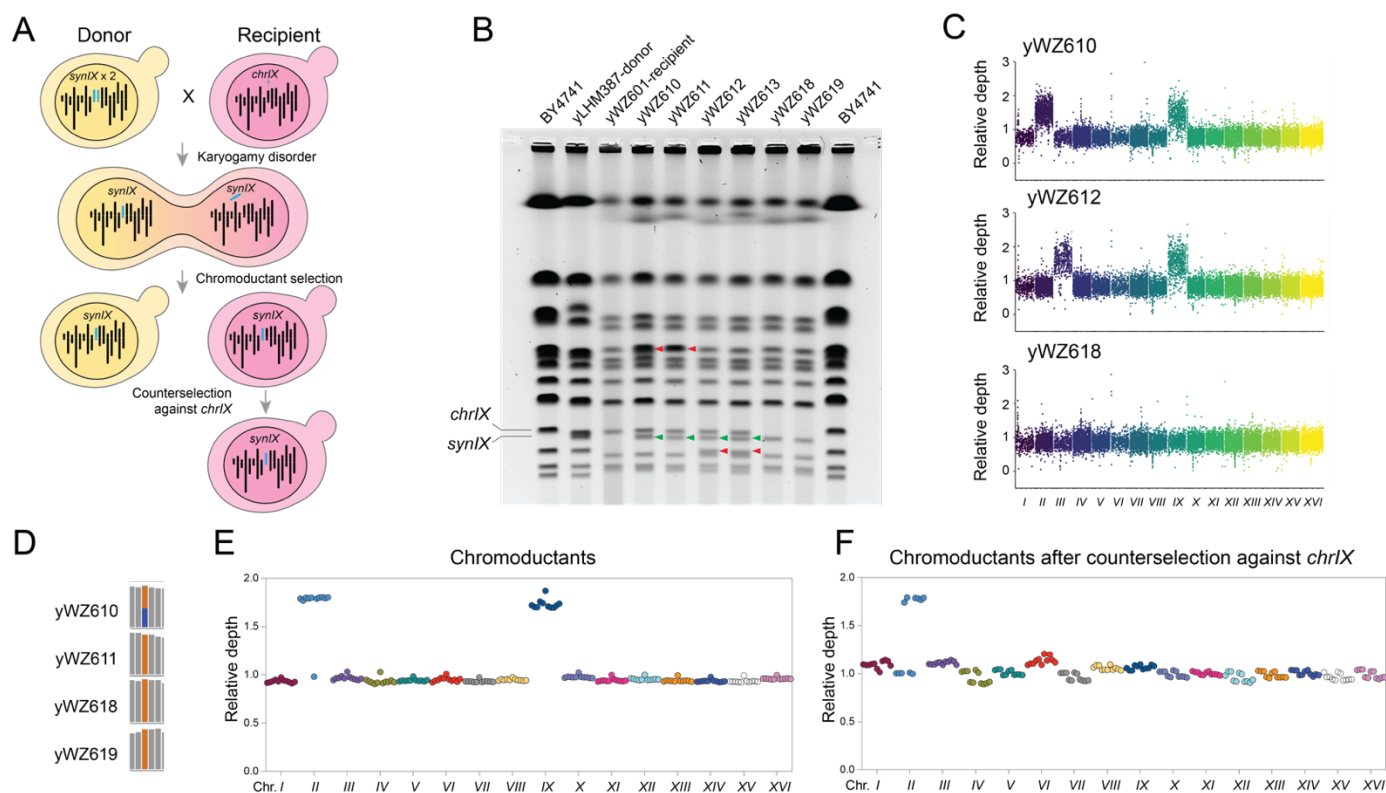
472  
473 After modifying the recipient strain, we mated the donor and recipient strains (yLHM0387 and  
474 yWZ601, respectively) and selected for recipient cells that had acquired at least one copy of *synIX*  
475 via chromoduction on SC–Lys–Arg+canavanine+cycloheximide plates. A PCRTagging assay was  
476 performed using selected PCRTags across *chrIX* and *synIX*. Four chromoductants (yWZ610-613)  
477 containing both *chrIX* and *synIX* PCRTag amplicons were isolated from 14 candidates (**Figure**  
478 **S21C**). Disomic chromoductants stably maintained both *chrIX* and *synIX* after restreaking onto  
479 fresh lysine-deficient plates. Genotyping validation of the four disomic chromoductants suggested  
480 that yWZ612 and yWZ613 had additionally acquired the *HIS4* gene on chromosome *III* from the  
481 donor strain (the recipient strain was *his4Δ0*), suggesting chromosome *III* was co-transferred along  
482 with *synIX* (**Figure S21D**).

483  
484 We next sought to destabilize *chrIX* in the disomic chromoductants to generate haploid strains with  
485 a single copy of *synIX*. We grew the disomic chromoductants (yWZ610, yWZ611) in galactose-  
486 containing media to activate transcription of the *GAL* promoter, destabilizing *CEN9* and  
487 consequently leading to *chrIX* loss during mitosis.<sup>26</sup> PCRTagging analysis of the resulting  
488 chromoductants (yWZ618, yWZ619) showed that only *synIX* was present (**Figure S21E**). Lastly,  
489 we subjected a series of yeast strains we generated during chromoduction to a PFGE assay. As  
490 expected, 1) *synIX* was slightly smaller than *chrIX* in both BY4741 and the recipient strain, 2) both  
491 *synIX* and *chrIX* were present in disomic chromoductants, 3) chromosome *III* was co-transferred  
492 with *synIX* in yWZ612 and yWZ613, and 4) *chrIX* was lost in chromoductants following galactose  
493 induction and counterselection against *chrIX* (yWZ618, yWZ619) (**Figure 7B**). However, we were  
494 surprised to observe that two of our chromoductants, yWZ610 and yWZ611, contained an extra  
495 copy of chromosome *II* in addition to copies of both *chrIX* and *synIX* (**Figure 7B**). Whole genome  
496 sequencing of these chromoductants confirmed these PFGE results (**Figure 7C**). However, further  
497 examination of SNPs differing between the donor and recipient chromosome *II* revealed that

498 yWZ610 contained one donor *II* and one recipient *II*, while yWZ611 contained two copies of  
 499 recipient *II* (**Figure 7D**). This finding suggested that in yWZ611, even though chromosome *II* was  
 500 not co-transferred with *synIX*, the yeast still endoreduplicated chromosome *II*, perhaps to balance  
 501 the gene dosage perturbation caused by *synIX* chromoduction. Interestingly, during the inactivation  
 502 of *chrIX* in the yWZ610 and yWZ611 strains, chromosome *II* was simultaneously lost as well  
 503 (**Figure 7B**, yWZ618 and yWZ619).

504  
 505 To investigate whether the co-transfer and simultaneous loss of chromosome *IX* and *II* was  
 506 coincidental, we next isolated another 11 disomic chromoductants from a repeated chromoduction  
 507 experiment. Interestingly, whole genome sequencing revealed that 10 of the 11 disomic  
 508 chromoductants gained an extra copy of chromosome *II* (**Figure 7E**). Following counterselection  
 509 against *chrIX*, four disomic chromoductants lost chromosome *II*, while six chromoductants  
 510 remained disomic (**Figure 7F**). To rule out the possibility that this phenomenon was specific to our  
 511 disomic *synIX* donor strain, we performed chromosome substitution using one alternative disomic  
 512 *synIX* donor strain and three monosomic *synIX* donor strains. As in earlier experiments,  
 513 chromosome *II* was frequently co-transferred with *synIX*, and the extra copy of chromosome *II*  
 514 often disappeared after culturing strains in galactose (**Table S8**). In conclusion, we successfully  
 515 transferred *synIX* from different donor strains to a *kar1-1* recipient strain via chromoduction. More  
 516 in-depth diagnosis of the chromoductants revealed that chromosome *II* was preferentially and  
 517 consistently co-transferred with *synIX*, and then was frequently lost during counterselection against  
 518 *chrIX*.

519



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524

**Figure 7.** Chromosome substitution of *synIX*. **(A)** Chromosome substitution occurs in two steps, chromoduction, in which *synIX* is transferred to a recipient strain, followed by counterselection against wild-type *chrIX* to remove the native version of the chromosome and restore normal chromosome copy number in the chromoductant strain.

525 Schematic diagram of substitution steps. Black bars, wild-type chromosomes. Blue bars, *synIX*. Recipient strain  
526 harbors *ade2* mutation, and thus forms pink colonies, for easily distinguishing chromoductants. (B) Pulsed-field gel  
527 electrophoresis of donor strain, recipient strain, disomic chromoductants, and chromoductants after counterselection  
528 against *chrIX*. BY4741 serves as control. *ChrIX* and *synIX* chromosomes indicated with black arrows. Green triangles  
529 indicate *synIX* in disomic chromoductants. Red triangles indicate the extra chromosome II or chromosome III in  
530 disomic chromoductants. (C) Whole genome sequencing read depth plots of disomic chromoductants (yWZ610,  
531 yWZ612) and *chrIX* counterselected (i.e., fully substituted) chromoductants (yWZ618). X axis: Sixteen yeast  
532 chromosomes, not drawn to scale. Y axis: Relative read depth, calculated as number of reads at each position divided  
533 by average read depth across the sixteen yeast chromosomes. (D) Plot of SNPs on chromosome II in chromoductant  
534 strains before and after counterselection against *chrIX*. Recipient strain SNP highlighted in orange, donor strain SNP in  
535 blue. Strains: yWZ610: chromoductant strain with one copy of chromosome II from the donor strain and one from the  
536 recipient strain (mixed SNP population); yWZ611: chromoductant strain with two copies of chromosome II from the  
537 recipient strain (endoreduplicated II); yWZ618: strain derived from yWZ610 after counterselection against *chrIX*, with  
538 one copy of II harboring recipient strain SNP; yWZ619: strain derived from yWZ611 after counterselection against  
539 *chrIX*, with one copy of II harboring recipient strain SNP. (E) Relative depth plot for chromosome substitution strains  
540 prior to counterselection against *chrIX*. X axis: Sixteen yeast chromosomes, not drawn to scale. Y axis: Relative read  
541 depth. (F) Relative depth plot for chromosome substitution strains after counterselection against *chrIX*. X axis: Sixteen  
542 yeast chromosomes, not drawn to scale. Y axis: Relative read depth.

543

## 544 Discussion

545

546 We successfully conclude the synthesis, debugging, and characterization of *synIX*. *SynIX*'s  
547 developmental history reflects advances in DNA synthesis technology over more than a decade.  
548 When the project first began, each segment of DNA was painstakingly assembled by  
549 undergraduates from oligonucleotides into minichunks, which in turn were integrated iteratively into  
550 the yeast genome to overwrite native chromosome IX. While such methods enabled successful  
551 incorporation of much of the synthetic sequence of interest, drawbacks included the inadvertent  
552 retention of wild-type patches and introduction of new point mutations. Newer approaches,  
553 including direct minichunk synthesis and megachunk cloning and sequencing prior to integration,  
554 greatly improved assembly efficiency and reduced construction error rates. Ultimately, we  
555 produced a yeast strain with a fully synthetic version of chromosome IX that displays near wild-type  
556 fitness.

557

558 While debugging *synIX*, we identified a fitness defect related to deletion of DNA sequence  
559 upstream of the *EST3* gene containing an aspartyl tRNA gene, a Ty1 LTR, and an intervening  
560 single copy DNA sequence upstream of the *EST3* gene. Strains with this deletion showed a  
561 reduction in telomere length as well as reduced Est3p levels and many RNA species mapping  
562 upstream of *EST3*'s normal transcription start site. Reinserting all or part of the deleted sequence  
563 was sufficient to restore normal strain growth fitness. However, only a subset of the edited strains,  
564 specifically those containing the tRNA gene, showed a reduction in transcriptional activity  
565 upstream of *EST3* and a restoration of normal or near-normal Est3p levels and normal telomere  
566 length. We hypothesize that the tRNA sequence normally provides a buffer against production of  
567 aberrant transcripts upstream of *EST3*, and thus, the transcripts that are produced in tRNA-  
568 containing strains more effectively give rise to functional, full-length Est3p. Indeed, there is  
569 considerable evidence that tRNAs suppress transcription from nearby RNA polymerase II  
570 promoters in a process referred to as tRNA-mediated gene silencing.<sup>33,35,36</sup> Consistent with this  
571 idea, nanopore RNA sequencing data suggests that a subset of transcripts include one or more

572 AUGs upstream of *EST3*, and thus presumably fail to undergo proper translation; strains with an  
573 abundance of such transcripts also appear to have fewer full-length, translatable transcripts and  
574 lower Est3p levels. Other changes made upstream of *EST3*, such as reinsertion of the gap  
575 sequence between the tRNA and the Ty1 LTR or Ty1 LTR itself, seemingly trigger small  
576 improvements in Est3p levels vs. those found in strains with the original *synIX* design. These  
577 strains have intermediate length telomeres, longer than those seen in the original *synIX* design but  
578 shorter than those seen in native yeast, and maintain stable fitness after repeated passaging. We  
579 speculate that the changes introduced to these strains may be sufficient to maintain Est3p  
580 production and consequent telomere length above a threshold level needed for normal strain  
581 fitness, despite not fully returning either to native levels.

582  
583 Interestingly, a similar occurrence of abnormal transcriptional patterns resulting from tRNA deletion  
584 and *loxPsym* site insertion was previously observed at the *HIS2* locus in *synVI*.<sup>7</sup> In this case, the  
585 authors suggested that a “cryptic start site” upstream of *HIS2*, generated by the altered sequence  
586 in this region, might yield a subset of transcripts incapable of producing functional His2 protein, or  
587 that this transcription might impair normal *HIS2* promoter activity. It would be useful to examine  
588 whether common principles underlie multiple Sc2.0 bugs following tRNA deletion.

589  
590 Finally, we demonstrated a chromosome substitution technique that combines selective  
591 chromoduction, or movement of a desired chromosome between yeast strains, with subsequent  
592 destabilization and loss of the corresponding native chromosome. During this process of  
593 chromosome substitution, we found that certain chromosomes appeared to co-transfer with *synIX*  
594 at higher frequencies than did others. Specifically, we saw multiple instances of transfer of  
595 chromosome *II* or chromosome *III* alongside *synIX*. These extra chromosomes often disappeared  
596 following the destabilization of *chrIX*. Prior work suggested that smaller chromosomes might be  
597 more likely to transfer during chromoduction than larger ones, and that co-transfer of multiple  
598 chromosomes commonly occurs.<sup>23</sup> However, as chromosome *II* is not a small chromosome, it  
599 seems unlikely that chromosome size fully explains the specific co-transfer of chromosome *II*.

600  
601 It is likely that an extra copy of chromosome *II* plays a role in balancing a gene dosage imbalance  
602 arising from the transient extra copy of chromosome *IX* introduced by chromoduction. In one of the  
603 chromoductant strains, analysis of SNPs from the recipient and donor strains’ copies of  
604 chromosome *II* revealed that both copies of the chromosome came from the recipient strain. The  
605 presence of an endoreduplicated chromosome *II* is consistent with the hypothesis that there is  
606 selective pressure for a higher dosage of chromosome *II* fitness in the presence of two copies of  
607 chromosome *IX*, and that the co-migration of chromosomes *II* and *synIX* in the other  
608 chromoductant strains may not have occurred by chance. Moreover, multiple donor strains,  
609 including strains disomic and monosomic for *synIX*, showed co-transfer of chromosome *II*  
610 alongside *synIX*. It may be valuable to ascertain the mechanisms that dictate which chromosomes  
611 tend to co-transfer between strains during chromoduction.

612  
613 Ultimately, chromosome substitution has numerous applications, including transfer and  
614 consolidation of chromosomes from other wild-type yeast strains, engineered strains, and non-  
615 laboratory strains. Existing work profiling collections of hundreds or thousands of yeast strains has

616 shed light on yeast diversity and evolution;<sup>37-39</sup> chromosome substitution will facilitate further  
617 probing of how changes on individual chromosomes or combinations of chromosomes affect strain  
618 fitness and function. Similarly, this technology could assist in analyzing why genomic changes in  
619 SCRaMbLEd yeast strains and other engineered strains produce particular phenotypic results.<sup>1-4</sup>  
620 More immediately, chromosome substitution is already in use to bring the initial phase of Sc2.0 to a  
621 close via the consolidation of all of the synthetic chromosomes into a single strain, marking a key  
622 eukaryotic genome engineering milestone.<sup>21</sup>

623  
624  
625

## STAR Methods

### Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Digoxigenin Recombinant Rabbit Monoclonal Antibody (9H27L19)	Invitrogen	Thermo Fisher Scientific Cat# 700772, RRID:AB_2532342
Monoclonal ANTI-FLAG® M2 antibody produced in mouse	Sigma-Aldrich	Sigma-Aldrich Cat# F1804, RRID:AB_262044
Rabbit anti-Histone H3 antibody	Abcam	Abcam Cat# ab1791, RRID:AB_302613
IRDye 800CW Goat anti-Mouse IgG1-Specific	LI-COR Biosciences	LI-COR Biosciences Cat# 926-32350, RRID:AB_2782997
IRDye 680RD Goat anti-Rabbit IgG	LI-COR Biosciences	LI-COR Biosciences Cat# 926-68071, RRID:AB_10956166
<b>Bacterial and viral strains</b>		
EPI300 <i>E. coli</i> strain	Lucigen	EC300150
TOP10 <i>E. coli</i> strain	Boeke lab collection	
<b>Chemicals, peptides, and recombinant proteins</b>		
Zymolyase 20T	US Biological	Z1000
Zymolyase 100T	US Biological	Z1004
EDTA-free protease inhibitor	Roche	11873580001
Lithium acetate dihydrate	Sigma-Aldrich	L6883
Polyethylene glycol	Sigma-Aldrich	81188
Herring sperm DNA	Promega	D1816
Potassium acetate	Fisher	BP364
Zinc acetate dihydrate	Sigma-Aldrich	Z0625
(S)-(+)-Camptothecin	Sigma-Aldrich	C9911
D-Sorbitol	Sigma-Aldrich	S1876
6-Azauracil	Sigma-Aldrich	A1757
Hydroxyurea	Sigma-Aldrich	H8627
Methyl methanesulfonate	Sigma-Aldrich	129925
Methyl 1-(butylcarbonyl)-2-benzimidazolecarbamate (Benomyl)	Sigma-Aldrich	381586
Cycloheximide	Sigma-Aldrich	01810
L-Canavanine sulfate	Sigma-Aldrich	C9758
Hydrogen peroxide	Millipore	88597
ULTRAhyb™ Ultrasensitive Hybridization Buffer	Invitrogen	AM8669
SSPE Buffer 20× Concentrate	Sigma-Aldrich	S2015
Intercept (TBS) Blocking Buffer	LI-COR Biosciences	927-60001
TWEEN 20	Sigma-Aldrich	P1379
Triton™ X-100	Sigma-Aldrich	X100
2-Mercaptoethanol	Sigma-Aldrich	M6250
GoTaq Green Master Mix	Promega	M7123
RNase A	Thermo Fisher Scientific	EN0531
<i>NotI</i> -HF	NEB	R1389L
<i>BbsI</i> -HF	NEB	R3539L
<i>AfeI</i>	NEB	R0652L
<i>XhoI</i>	NEB	R0146L

Amino-terminal FLAG-BAP™ Fusion Protein	Sigma-Aldrich	P7582
Dynabeads™ M-270 Epoxy	Invitrogen	14302D
Dynabeads™ Oligo(dT) <sub>25</sub>	Invitrogen	61005
SuperScript™ IV Reverse Transcriptase	Invitrogen	18090010
Agencourt RNAClean XP beads	Beckman Coulter	A63987
<b>Critical commercial assays</b>		
PureLink™ HiPure Plasmid Midiprep Kit	Invitrogen	K2100-15
Zyppy Plasmid Miniprep Kit	Zymo Research	D4037
Fungi/Yeast Genomic DNA Isolation Kit	Norgen	27300
Qubit dsDNA HS Assay Kit	Thermo Fisher Scientific	Q32854
Qubit dsDNA BR Assay Kit	Thermo Fisher Scientific	Q32850
NEBNext Ultra II FS DNA Library Kit	NEB	E7805
Zymo-Spin I Columns	Zymo Research	C1003
PALL 60207 Biodyne B High Sensitivity and Low Background Nylon Transfer Membrane, 0.45 µm Pore Size, 30 cm W x 3 m L Roll	Pall Corporation	60207
RNeasy Mini Kit	QIAGEN	74106
Qubit RNA BR Assay Kit	Thermo Fisher Scientific	Q10210
QIAseq Stranded Total RNA Library Kit	QIAGEN	180745
QIAseq FastSelect -rRNA Yeast Kit	QIAGEN	334217
NextSeq 500/550 High Output Kit v2.5 (75 Cycles)	Illumina	20024906
NextSeq 500/550 High Output Kit v2.5 (150 Cycles)	Illumina	20024907
MasterPure Yeast RNA Purification Kit	Lucigen	MPY03100
Agilent RNA 6000 Nano Kit	Agilent	5067-1511
Qubit RNA HS Assay Kit	Thermo Fisher Scientific	Q32852
Direct RNA Sequencing Kit	Oxford Nanopore Technologies	SQK-RNA002
Flow Cell Priming Kit	Oxford Nanopore Technologies	EXP-FLP001
MinION Flow Cell (R9.4.1)	Oxford Nanopore Technologies	FLO-MIN106D
<b>Experimental models: Organisms/strains</b>		
<i>Saccharomyces cerevisiae</i> : BY4741	Jef Boeke's laboratory	N/A
All other strains used in this study are listed in Table S1	N/A	N/A
<b>Oligonucleotides</b>		

PCRTag primers are listed in Supplementary File 1	N/A	N/A
<b>Recombinant DNA</b>		
See Tables S2-S6 for plasmids used in this study	N/A	N/A
<b>Software and algorithms</b>		
GeneDesign	40	
R	R Core Team	<a href="https://www.R-project.org/">https://www.R-project.org/</a>
Rstudio v1.3.1093	Rstudio	<a href="https://www.rstudio.com">https://www.rstudio.com</a>
Integrated Genomics Viewer (IGV) v2.7.2	Broad Institute <sup>41</sup>	<a href="http://software.broadinstitute.org/software/igv/">http://software.broadinstitute.org/software/igv/</a>
Image Studio Lite	LI-COR Biosciences	<a href="https://www.licor.com/bio/image-studio/">https://www.licor.com/bio/image-studio/</a>
GraphPad Prism v9.4.0 for macOS	GraphPad Software	<a href="http://www.graphpad.com">http://www.graphpad.com</a>
Trimmomatic v0.39	42	<a href="http://www.usadellab.org/cms/index.php?page=trimmomatic">http://www.usadellab.org/cms/index.php?page=trimmomatic</a>
FastQC v0.11.4	43	<a href="http://www.bioinformatics.babraham.ac.uk/projects/fastqc/">http://www.bioinformatics.babraham.ac.uk/projects/fastqc/</a>
Bowtie 2 v2.2.9	44	<a href="http://bowtie-bio.sourceforge.net/bowtie2/index.shtml">http://bowtie-bio.sourceforge.net/bowtie2/index.shtml</a>
ggplot2 v3.3.5	45	<a href="https://ggplot2.tidyverse.org/">https://ggplot2.tidyverse.org/</a>
Seqtk v1.3	N/A	<a href="https://github.com/lh3/seqtk/">https://github.com/lh3/seqtk/</a>
Kallisto v0.46.0	46	<a href="https://pachterlab.github.io/kallisto/">https://pachterlab.github.io/kallisto/</a>
STAR v2.5.2a	47	<a href="https://github.com/alexdobin/STAR/">https://github.com/alexdobin/STAR/</a>
SAMtools v1.9	48	<a href="http://samtools.sourceforge.net/">http://samtools.sourceforge.net/</a>
BEDtools v2.26.0	49	<a href="https://github.com/arq5x/bedtools2/">https://github.com/arq5x/bedtools2/</a>
sleuth v0.30.0	50	<a href="https://pachterlab.github.io/sleuth/">https://pachterlab.github.io/sleuth/</a>
<b>Other</b>		
Resource website for Sc2.0	N/A	<a href="https://syntheticyeast.github.io/">https://syntheticyeast.github.io/</a>

626

627

628

**RESOURCES AVAILABILITY**

629

**Lead contact**

630

631

Further information should be directed to and will be fulfilled by the lead contact Jef D. Boeke (jef.boeke@nyulangone.org).

633

634

**Materials availability**

635

636

All unique/stable reagents generated in this study are available from the lead contact with a completed Materials Transfer Agreement.

638

639

**Data and code availability**

640

641

Data: All data reported in this paper will be shared by the lead contact upon request.

643

Code: This work did not generate any code.

644

645

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

647

648

649

650



## 651 **Experimental Methods**

### 652 **SynIX design**

653 Design sequences for *synIX* were developed using BioStudio, according to the same guidelines  
654 used for designing the other synthetic chromosomes for Sc2.0. Designer sequences  
655 yeast\_chr09\_3\_54 and yeast\_chr09\_3\_55 were used as references for subsequent steps in the  
656 project. Version yeast\_chr09\_3\_56 was developed after completion of yeast\_chr09\_9\_02, and was  
657 used as a reference for final strain verification.  
658

### 659 **Yeast media**

660 Yeast strains were cultured in rich medium (YPD) or synthetic complete (SC) medium with  
661 appropriate components “dropped out” e.g., SC–Ura lacks uracil. Yeast transformations were  
662 performed using lithium acetate protocols.<sup>51</sup> YPG plates used for growth assays contained 3%  
663 glycerol in place of dextrose as a carbon source. For expanded spot assays, the following media  
664 types were used: pH 4 and pH 8: pH of 2X YEP + dextrose adjusted using HCl and NaOH,  
665 respectively before adding agar; camptothecin (Sigma-Aldrich, C9911): 0.1 µg/mL, 0.5 µg/mL, or  
666 1.0 µg/mL in YPD; sorbitol (Sigma-Aldrich, S1876): 0.5 M, 1.0 M, 1.5 M, or 2.0 M in YPD; 6-  
667 azauracil (Sigma-Aldrich, A1757): 100 µg/mL in SC medium; hydroxyurea (Sigma-Aldrich, H8627):  
668 0.2M in YPD; MMS: methyl methanesulfonate (Sigma-Aldrich, 129925), 0.05% in YPD; benomyl  
669 (Sigma-Aldrich, 381586): 15 µg/mL in YPD; cycloheximide (Sigma-Aldrich, 01810): 10 µg/mL in  
670 YPD liquid medium for 2 h followed by plating to YPD; H<sub>2</sub>O<sub>2</sub> (Millipore, 88597): 1 mM in YPD liquid  
671 medium for 2 h followed by plating to YPD. For chromoduction experiments, SC–Lys–Arg or SC–  
672 Leu–Arg plates were used with cycloheximide (10 µg/mL) and canavanine (12 µg/mL) added. For  
673 chromosome destabilization, strains were grown in YPD with 2% galactose and 1% raffinose  
674 added. Sporulation medium was prepared using a 50x base consisting of 50 g potassium acetate  
675 and 0.25 g zinc acetate dihydrate in 100 mL H<sub>2</sub>O. Final 1x sporulation media was prepared from 2  
676 mL of 50x sporulation medium base plus 300 µL of 10% yeast extract, 200 µM uracil, 2 mM leucine  
677 300 µM histidine, and H<sub>2</sub>O to 100 mL.  
678

### 679 **Minichunk assembly**

680 Minichunks were originally assembled according to the following process, similar to the one  
681 previously described for *synIII*:<sup>5</sup> First, approximately 70-mer oligonucleotides with 20-mer overlaps  
682 were designed using GeneDesign software<sup>40</sup> and assembled by members of the Build A Genome  
683 class at Johns Hopkins University using polymerase cycling assembly (PCA) to produce 750 bp  
684 building blocks. Building blocks were verified by sequencing. Overlapping building blocks with  
685 approximately 40 bp overlaps were then assembled into 2-4 kb minichunks by direct homologous  
686 recombination in yeast. Minichunks were recovered from yeast into *E. coli*, and the recovered  
687 minichunk plasmids were verified by sequencing.  
688

689 Later minichunks were ordered directly from DNA synthesis vendors with flanking *NotI* or *BbsI*  
690 sites, verified by sequencing, excised from the vectors in which they were built via restriction  
691 digestion or, for those containing internal *NotI* or *BbsI* sites, amplified by PCR, and integrated  
692 directly into the *synIX* strain or used for megachunk assembly.  
693

## 694 **Megachunk assembly and verification**

695 Unlike the subsequent chromosomes, *synIX* was initially designed in separate “left and right”  
696 halves, which were subsequently merged. During the design process, *synIXL* was originally  
697 divided into megachunks A-F and V-Z. Following the integration of megachunks A-F as described  
698 in the “Minichunk integration by SwAP-In” section, the minichunks comprising megachunks V-Z in  
699 the original *synIX* design were reapportioned into three new megachunks, designated G, H, and I.  
700 Each megachunk plasmid was designed with flanking *NotI* sites for eventual excision of the  
701 assembled megachunk, 13-16 minichunks, a terminal selectable marker (*LEU2* or *URA3*, for use  
702 with SwAP-In), and homology arms between the first and last minichunks and the plasmid  
703 backbone. Overlaps between segments ranged from approximately 100-800 bp. Minichunks were  
704 released from their backbone plasmids or amplified by PCR, and were co-transformed with linkers  
705 and megachunk backbone plasmid into wild-type yeast (BY4741). Following plasmid assembly in  
706 yeast by homologous recombination and two days of growth on appropriate selective medium  
707 (SC–Leu or SC–Ura) at 30°C, single colonies were isolated and assessed by PCR to detect the  
708 presence or absence of each desired assembly junction. Colonies containing all desired junctions  
709 were recovered from yeast by phenol extraction and isopropanol precipitation, and subsequently  
710 electroporated into EPI300 *E. coli* cells (Lucigen, EC300150). Megachunk plasmids were extracted  
711 from *E. coli* using the PureLink™ HiPure Plasmid Midiprep Kit (Invitrogen, K2100-15).  
712 To verify the sequences of the recovered megachunk plasmids, DNA sequencing libraries were  
713 prepared using 300 ng of plasmid DNA as input for the NEBNext Ultra II FS DNA Library Kit (NEB,  
714 E7805). DNA libraries were sequenced via Illumina NextSeq 500 using 36 bp pair-end reads.  
715 Following sequencing, reads were first processed using Trimmomatic v0.39<sup>42</sup> and FastQC  
716 v0.11.4<sup>43</sup> aligned to an appropriate reference sequence using bowtie2 v2.2.9,<sup>44</sup> and visualized  
717 using IGV v2.7.2.<sup>41</sup>

## 719 **Minichunk integration by SwAP-In**

720 Minichunks were released from their backbones by restriction enzyme digestion, and the  
721 minichunks corresponding to one megachunk in the *synIX* design sequence (approximately 11-16  
722 minichunks) were cotransformed into the partially completed *synIX* strain. Integration began at the  
723 left telomere of chromosome *IX* and proceeded in a stepwise fashion towards the centromere. At  
724 each step, the rightmost minichunk integrated contained either a *URA3* or a *LEU2* auxotrophic  
725 marker. These markers were alternated at each step according to the principles of Switching  
726 Auxotrophies Progressively for Integration (SwAP-In),<sup>2,6</sup> and colonies containing the new marker  
727 and lacking the previous strain’s marker were identified by replica plating. Strains were assessed  
728 by PCRTag analysis after each round of integration.

## 730 **Megachunk integration by SwAP-In**

731 Sequence-verified megachunks were released from their assembly backbones via *NotI* digestion.  
732 Approximately 1 µg of digest product was transformed into the semisynthetic *synIX* strain, and  
733 cells were grown for two days at 30°C on appropriate SC dropout plates to select for the desired  
734 integration product. As with minichunk integration by SwAP-In, alternating *LEU2* and *URA3*  
735 auxotrophic markers were used each round, enabling selection of colonies containing the desired  
736 new integration marker and a lack of the previous round’s integration marker, as determined by  
737 replica plating. Colonies were assessed by PCRTag analysis, and colonies containing the desired

738 PCRTags of interest were used for subsequent megachunk integration rounds. After integration of  
739 all three megachunks introduced by this method, yeast strain sequences were verified by whole  
740 genome sequencing, as described in the “Whole genome sequencing” section. To remove the  
741 *URA3* marker introduced during megachunk I integration, a PCR product with homology arms  
742 flanking *URA3* was transformed into the *synIX* strain, and colonies lacking the *URA3* marker were  
743 obtained following growth on 5-FOA medium.

#### 744 745 **PCRTag analysis**

746 Cells from a single yeast colony were scraped from a plate using a pipette tip and resuspended in  
747 30  $\mu$ L of 20 mM sodium hydroxide in a 96-well PCR plate. The PCR plate was sealed and placed  
748 in a thermal cycler using the following boiling cycle: 3 cycles of 98°C for 3 m and 4°C for 1 m. 1  $\mu$ L  
749 of boiled product and 0.5  $\mu$ M each primer were used in a 5  $\mu$ L reaction using the GoTaq Green  
750 PCR system (Promega, M7123). An acoustic liquid handler (Labcyte, Echo 550) transferred  
751 primers and DNA template. Samples were run through the following protocol in a 384-well thermal  
752 cycler: 95°C for 5 m, 30 cycles of 95°C for 30 s, 55°C for 90 s, and 72°C for 60 s, and a final  
753 extension step of 72°C for 7 m. Samples were visualized following electrophoresis on a 1%  
754 agarose in 1X Tris-Taurine-EDTA gel containing ethidium bromide using the ChemiDoc XRS+  
755 System (Bio-Rad).

#### 756 757 **Growth assays**

758 A single yeast colony for each strain was inoculated into 5 mL YPD and incubated at 30°C  
759 overnight with rotation. For assays monitoring strain growth across multiple passages, overnight  
760 cultures were diluted 1:1000 in YPD and incubated at 30°C overnight with rotation until reaching  
761 the desired passage number. Overnight yeast culture was diluted to  $A_{600}=0.15$  into 6 mL of fresh  
762 YPD liquid medium and grown at 30°C with rotation until  $A_{600}$  reached 0.5-0.6. Cultures were  
763 serially diluted in tenfold increments in water, with a target OD of approximately 0.1 in 100  $\mu$ L total  
764 volume for the first row of the wild-type control strain used to determine the starting culture volume  
765 in water for each sample. 5  $\mu$ L (YPD and YPG plates) or 10  $\mu$ L (SC plates) of each dilution was  
766 spotted to each plate. Plates were grown at appropriate temperatures (room temperature of  
767 approximately 22°C on YPD, 30°C on YPD, YPG, and SC, and 37°C on YPD and YPG) and  
768 photographed daily. For additional media types (**Figures S18 and S19**), plates were prepared as  
769 described in the “Yeast media” section. Strains were serially diluted in tenfold increments of water  
770 as described above, and 5  $\mu$ L of each dilution was spotted to each plate. Plates were grown at  
771 30°C and photographed daily.

#### 772 773 **Plate reader growth assays in liquid culture**

774 Three single yeast colonies per strain of interest were resuspended in 1 mL YPD in a 2 mL deep  
775 well plate and incubated for 24 h at 30°C with shaking at 800 rpm. Saturated cultures were diluted  
776 1:100 in 200  $\mu$ L YPD in a glass bottom 96-well plate with lid (Greiner Bio-One, 655892) and loaded  
777 into a Cytation 5 cell imaging multimode reader (Agilent). The following program was run using the  
778 Cytation 5 Gen5 software: 32 h at 37°C with continuous shaking, with  $A_{600}$  measured every 10 m.  
779 Data analysis and visualization were conducted using Microsoft Excel and GraphPad Prism 9.

## 781 **Genome editing using CRISPR-Cas9**

782 A two-plasmid system with one guide RNA (gRNA) plasmid and one Cas9 plasmid was used for  
783 most editing.<sup>52</sup> The Cas9 coding sequence was assembled together with a *TEF1* promoter and  
784 *CYC1* terminator in a pRS415 plasmid backbone (with *LEU2* marker). This plasmid was pre-  
785 transformed into yeast strains prior to the introduction of the gRNA plasmid and donor DNA. Guide  
786 RNA target sequences were selected by identifying 20 bp upstream of the desired protospacer  
787 adjacent motif (PAM) sequence (NGG). gRNAs were cloned into a 2-micron plasmid (pRS426, with  
788 *URA3* marker) under the control of a *SNR52* promoter using Gibson assembly. A modified version  
789 of the gRNA plasmid was used for editing two loci simultaneously. In this case, a second gRNA  
790 was cloned into the plasmid under control of a *RPR1* promoter and *RPR1* terminator. gRNA  
791 sequences were confirmed by Sanger sequencing (Genewiz). To edit the *synIX* strain,  
792 approximately 50 ng of gRNA plasmid and 200-300 fmol (for minichunk patch replacement) or 1  
793 pmol (for point mutation fixing) of donor DNA were transformed into yeast cells containing the Cas9  
794 plasmid. Cells were grown on dropout medium to select for the presence of both the Cas9 and  
795 gRNA plasmids, and editing was confirmed using either PCRTag analysis or PCR followed by  
796 Sanger sequencing (Genewiz).

## 798 **Sporulation and tetrad dissection experiments for bug mapping**

799 The *synIX* strain yLHM1192 was crossed to a wild-type yeast strain (BY4742 with a *URA3-pGAL-*  
800 *CEN9* cassette integrated near the centromere, also known as yLHM0539) to generate a  
801 heterozygous diploid (yLHM1233) with one copy of wild-type chromosome *IX* and one copy of  
802 *synIX*. To prepare strains for sporulation, a single colony of each strain was inoculated into 5 mL  
803 YPD and incubated at 30°C overnight with rotation. Overnight cultures were diluted to an OD of ~1  
804 in YPD and grown to an OD of ~4, washed five times with water, and resuspended in 2 mL of 1X  
805 sporulation medium. Strains were incubated at room temperature for 7-10 days with rotation, and  
806 monitored for the presence of tetrads. For tetrad dissection, 100 µL of these resuspended yeast  
807 cells in sporulation medium were washed and incubated with 25 µL of 0.5 mg/mL zymolyase in 1M  
808 sorbitol for 8 m. 200 µL of 1M sorbitol was added to the cells, and 10 µL of the resulting mixture  
809 was added to a YPD plate. Tetrads were separated and picked using a dissection microscope  
810 (Singer Instruments). Spores were grown for 2-3 days on YPD until visible colonies emerged.  
811 These strains were used for growth assays as described in the “Growth assays” section to classify  
812 each spore as healthy (similar fitness to the wild-type parent) or sick (similar fitness to the *synIX*  
813 parent). Strains from eighteen four-spore tetrads (72 strains) were prepared for whole genome  
814 sequencing, as described in the “Whole genome sequencing” section below.

## 816 **Plasmid cloning and transformation for *synIX* bug mapping**

817 Wild-type genes of interest plus approximately 500 bp of upstream flanking sequence and 200 bp  
818 of downstream flanking sequence were amplified from BY4741 by PCR. Genes were cloned into  
819 plasmid pRS413 linearized with *AfeI* by Gibson assembly and transformed into TOP10 cells.  
820 Colonies were grown up in 2 mL LB + 75 µg/mL carbenicillin and miniprep using the Zyppy  
821 Plasmid Miniprep Kit (Zymo Research, D4037). Plasmid insert sequences were verified by Sanger  
822 sequencing. Plasmids were subsequently transformed into the *synIX* strain and grown for two days  
823 on SC–His plates. Yeast colonies containing each plasmid of interest were isolated, grown in SC–

824 His liquid medium, and used for growth assays (as described in the “Growth assays” section) to  
825 assess their relative fitness compared to the starting *synIX* strain.

### 826 **Selective destabilization and loss of one copy of *synIX* from disomic strain**

827 A cassette containing a galactose-inducible centromere and a *URA3* marker (*URA3-pGAL-CEN9*)  
828 with homology arms matching *synIX* was amplified by PCR and transformed into the *synIX* strain.  
829 After two days of growth at 30°C on SC-Ura plates, single colonies were purified and assessed by  
830 junction PCR for the insertion of the *URA3-pGAL-CEN9* fragment. Strains were then grown in YEP  
831 + 2% galactose + 1% raffinose for two days and diluted to 5-FOA plates to obtain single colonies  
832 lacking *URA3*. Strains were single-colony purified and prepared for whole genome sequencing to  
833 assess chromosome copy number.  
834

### 835 **Whole genome sequencing**

836 A single yeast colony was inoculated into 5 mL of YPD liquid medium at 30°C overnight with  
837 rotation. Overnight cultured yeast cells were harvested by centrifugation at 3000 x g for 3 m, and  
838 yeast genomic DNA was extracted using the Fungi/Yeast Genomic DNA Isolation Kit (Norgen,  
839 27300). Genomic DNA concentration was determined using the Qubit dsDNA HS Assay Kit  
840 (Thermo Fisher Scientific, Q32854). DNA sequencing libraries were prepared using 300 ng of  
841 yeast genomic DNA as input for the NEBNext Ultra II FS DNA Library Kit (NEB, E7805). DNA  
842 libraries were sequenced via Illumina NextSeq 500 using either 75 bp or 36 bp pair-end reads.  
843 Following sequencing, reads were first processed using Trimmomatic v0.39<sup>42</sup> and FastQC  
844 v0.11.4,<sup>43</sup> aligned to an appropriate reference genome using bowtie2 v2.2.9,<sup>44</sup> and visualized using  
845 IGV v2.7.2.<sup>41</sup> Chromosome coverage plots were prepared in Rstudio version 1.3.1093 using the  
846 ggplot2 v3.3.5 package.<sup>45</sup>  
847

### 848 **Southern blot analysis**

849 A single yeast colony was inoculated into 10 mL of YPD medium at 30°C overnight with rotation.  
850 Overnight cultured yeast cells were harvested by centrifugation at 3000 x g for 3 m, and yeast  
851 genomic DNA was extracted using the MasterPure™ Yeast DNA Purification Kit (Lucigen,  
852 MPY80200) according to the manufacturer’s instructions, with an RNase A treatment step. DNA  
853 was quantified using the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, Q32850). 2 µg of  
854 DNA were digested with *XhoI* at 37°C overnight. Digests were electrophoresed on a 1% Tris-  
855 Borate-EDTA gel in 0.5X Tris-Borate-EDTA running buffer for 10 h at 50V. Following  
856 electrophoresis, the gel was washed in depurination solution (0.25 M HCl) for 15 m, denaturation  
857 solution (0.5 M NaOH, 1.5 M NaCl) for 60 m, and neutralization solution (0.5 M Tris, 1.5 M NaCl,  
858 pH 7.5) for 30 m. Chromosomes were transferred by capillarity to a nylon membrane (Pall  
859 Corporation, 60207) using 10X SSC buffer (20X SSC: 1.5 M NaCl, 0.3 M sodium citrate, pH 7) for  
860 18 h. After transfer, the membrane was washed in 2X SSC buffer for 5 m and baked at 80°C for 30  
861 m. Prehybridization was performed for 60 m in ULTRAhyb™ Ultrasensitive Hybridization Buffer  
862 (Invitrogen, AM8669) at 55°C with rotation. For hybridization, probes were prepared by labeling a  
863 ~360 bp PCR fragment of universal telomere cap sequence from plasmid pJS160 using the PCR  
864 DIG Probe Synthesis Kit (Roche, 11636090910) with oligos 5’-  
865 GCTATACGAAGTTATTAGGGTAGTGTG-3’ and 5’- CTGCAGGTCGACTCTAGAGGATC-3’,  
866 according to the manufacturer’s instructions. The following thermocycler conditions were used: 1  
867 cycle of 95°C for 2 m, 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 40 s, and a final  
868 elongation step of 72°C for 7 m. Probes were purified using Zymo-Spin I Columns (Zymo  
869

870 Research, C1003) and denatured at 95°C for 5 m. Hybridization was performed at 55°C overnight  
871 with rotation in 10 mL ULTRAhyb™ Ultrasensitive Hybridization Buffer (Invitrogen, AM8669) using  
872 500 ng of probe per experiment. The blot was washed twice with 2X SSPE buffer (20X SSPE  
873 buffer: 0.02 M EDTA and 2.98 M NaCl in 0.2 M phosphate buffer, pH 7.4; Sigma-Aldrich, S2015)  
874 for 5 m at room temperature, twice with 2X SSPE + 1% SDS for 30 m at 55°C, and twice with 0.1X  
875 SSPE for 15 m at 55°C. Blot was incubated overnight in 1:1 Intercept (TBS) Blocking Buffer (LI-  
876 COR Biosciences, 927-60001): 1X Tris-Buffered Saline (TBS) with 0.1% TWEEN 20 (Sigma-  
877 Aldrich, P1379) (1X TBST) and 1% SDS at room temperature. The next day, the membrane was  
878 washed with 1X TBST for 5 m and incubated for 1 hour at room temperature with rabbit anti-DIG  
879 antibody (working concentration 1:2500 in 1:1 Intercept (TBS) Blocking Buffer: 1X TBST;  
880 Invitrogen, 700772). The primary antibody solution was washed out three times with 1X TBST for  
881 15 m at room temperature. The membrane was incubated in goat anti-rabbit IgG secondary  
882 solution (LI-COR Biosciences, 926-68071, used at 1:10,000 in 1:1 Intercept (TBS) Blocking Buffer  
883 (LI-COR Biosciences, 927-60001): 1X TBST with 1% SDS) for 1.5 h. The blot was washed three  
884 times with 1X TBST for 15 m at room temperature plus one time with 1X TBS for 30 m at room  
885 temperature. A LI-COR Odyssey Instrument was used to develop the blot images.<sup>53</sup>  
886

### 887 **RNA extraction, sequencing, and analysis for short read sequencing**

888 A single yeast colony was inoculated into 5 mL of YPD liquid medium at 30°C overnight with  
889 rotation. Overnight yeast culture was diluted to  $A_{600}=0.15-0.2$  into 6 mL of fresh YPD liquid medium  
890 and grown at 30°C or 37°C with rotation until  $A_{600}$  reached 0.8-1.0. Yeast cells were harvested from  
891 4 mL of culture, and total RNA was extracted using the RNeasy Mini Kit (QIAGEN, 74106)  
892 according to the manufacturer's instructions. RNA was quantified using the Qubit BR RNA Assay  
893 Kit (Thermo Fisher Scientific, Q10210). 1 µg total RNA was used as input for the QIAseq Stranded  
894 Total RNA Library Kit (QIAGEN, 180745) plus QIAseq FastSelect -rRNA Yeast Kit (QIAGEN,  
895 334217). RNA libraries were sequenced via Illumina NextSeq 500 using 75 bp paired-end reads.  
896 For applications requiring similar numbers of reads per sample, downsampling was performed  
897 using seqtk v1.3. Samples were first processed using Trimmomatic v0.39<sup>42</sup> and FastQC v0.11.4.<sup>43</sup>  
898 Processed reads were aligned to the S288C transcriptome and a custom *synIX* transcriptome (in  
899 which chromosome *IX* transcripts were replaced with their synthetic versions) using Kallisto  
900 v0.46.0<sup>46</sup> and STAR v2.5.2a.<sup>47</sup> STAR-aligned results were further processed using SAMtools  
901 v1.9<sup>48</sup> and BEDtools v2.26.0,<sup>49</sup> and visualized in IGV v2.7.2.<sup>41</sup> Kallisto pseudoalignment data was  
902 further processed in Rstudio version 1.3.1093 using the sleuth v0.30.0<sup>50</sup> and ggplot2 v3.3.5<sup>45</sup>  
903 packages. For volcano plots, we calculated effect scores (approximately log<sub>2</sub> fold change values),  
904 tested results for significance using Wald's test, and corrected for multiple testing with the false  
905 discovery rate adjusted p-value using the Benjamini-Hochberg method. The following genes  
906 deleted from the *synIX* design were removed from the plots in **Figure 4D** and **Figure 4E**: YIL173W  
907 (VTH1), YIL171W (part of HTX12 pseudogene), YIL170W (part of HTX12 pseudogene), YIL169C  
908 (CSS1), YIL167W (SDL1, blocked reading frame), YIL082W, YIL060W, YIL059C, YIL058W,  
909 YILWTy3-1, YILCdelta3. The following dubious ORFs, retrotransposable elements, and  
910 subtelomeric genes were also removed: YBL100C, YGR296C-B, YHL050W-A, YHR219C-A,  
911 YIL020C-A, YJR029W, YLL066C, YML133W-B, YMR046C, YNL339W-B, YNL339C, YOR277C,  
912 YPL283W-B, YPR158W-B, YPR204C-A, YBLWdelta4, YCLWdelta3. Unfiltered versions of the  
913 volcano plots are depicted in **Figure S20**.

## 916 **RNA extraction, sequencing, and analysis for nanopore direct RNA sequencing**

917 Methods recently described by us were used to perform nanopore sequencing to analyze RNA  
918 isoforms.<sup>54</sup> Total RNA was extracted from 50 mL flash-frozen cell pellets grown to mid-log ( $OD_{600}$   
919  $\sim 0.65-0.85$ ) using the MasterPure™ Yeast RNA Purification Kit (Lucigen) including a Dnase I  
920 treatment step. RNA (diluted 1:10) quality and concentration were measured by Agilent 2100  
921 Bioanalyzer with the Agilent RNA 6000 Nano Kit and Qubit™ RNA High Sensitivity Kit (Thermo  
922 Fisher Scientific), respectively. Poly(A) mRNA was enriched from 50  $\mu$ g total RNA on 132  $\mu$ L  
923 Dynabeads oligo(dT)<sub>25</sub> beads. The Direct RNA Sequencing Kit (SQK-RNA002, Oxford Nanopore  
924 Technologies) was used to generate libraries from 500 ng poly(A) RNA. An optional reverse  
925 transcription was performed at 50°C for 50 m using SuperScript™ IV Reverse Transcriptase  
926 (Invitrogen) in between the ligation of the RTA and RMX adaptors. Following reverse transcription  
927 the RNA:cDNA was cleaned up with 1.8 volumes of Agencourt RNAClean XP beads and washed  
928 with 70% ethanol. Following RMX ligation only one volume of beads was used in the clean-up,  
929 and WSB (SQK-RNA002) was used in the wash steps. Direct RNA libraries (typically 150-200 ng)  
930 were loaded onto primed (EXP-FLP001) MinION flow cells (FLO-MIN106D, R9 version) in RRB  
931 buffer and run on the GridION with MinKNOW 5.2.2 for up to 72 h. Nanopore long reads were  
932 base-called, trimmed of adapter sequences, and filtered for quality, retaining only those with the  
933 best alignment scores for multi-mapping reads, as previously described.<sup>54</sup> For TSS distributions,  
934 long-reads that overlapped at least 75% of the annotated gene were used.

## 936 **Construction of FLAG-tagged *EST3* strains**

937 A DNA fragment containing a six-glycine linker plus a 3X FLAG tag (DYKDHDG-DYKDHD-  
938 DYKDDDDK) was designed for integration at the 3' end of *EST3*, in-frame with the rest of the  
939 protein and inserted just before the *EST3* stop codon. The segment containing the tag was flanked  
940 by 70-100 bp homology arms matching the yeast genome sequence adjacent to the desired  
941 insertion site. Genome editing using CRISPR-Cas9 in yeast (as describe above) was used to cut  
942 the genome near the end of the *EST3* coding sequence and insert the tag of interest. Colony PCR  
943 and Sanger sequencing (Genewiz) were used to confirm in-frame insertion of the tag.

## 945 **Immunoblot analysis**

946 A single yeast colony was inoculated into 5 mL of YPD liquid medium at 37°C overnight with  
947 rotation. Overnight culture was diluted to  $A_{600}=0.15-0.2$  into 50 mL of fresh YPD liquid medium and  
948 grown at 37°C with rotation until  $A_{600}$  reached 0.8-1.0. Yeast were harvested by centrifugation, and  
949 pellets were frozen overnight at -80C. Pellets were subsequently resuspended in 500  $\mu$ L of lysis  
950 buffer (20 mM HEPES pH 7.4, 1% Triton™ X-100 (Sigma-Aldrich, X100), 2 mM  $MgCl_2$ , 500  $\mu$ M  
951 NaCl, and 1X protease inhibitor (Roche)). Cell suspension was added to MP Biomedicals FastPrep  
952 Lysing Matrix C and subjected to the following shaking program at 4°C using a FastPrep-24 5G  
953 (MP Biomedicals): 7 cycles of 15 s shaking at 6.0 m/second + 30 s rest between cycles. Samples  
954 were centrifuged at 13000 x g for 10 m at 4°C, and 30  $\mu$ L of supernatant was transferred to a new  
955 tube and mixed with 10  $\mu$ L of 4X LDS loading buffer (Invitrogen, NP0007) (pre-immunoprecipitation  
956 sample). The remaining supernatant was transferred to pre-equilibrated FLAG M2 (Sigma F1804)-  
957 conjugated M-270 Dynabeads (Invitrogen, 14302D) for immunoprecipitation.<sup>55</sup> Samples with beads  
958 were incubated on ice for 60 m, washed 3 times with lysis buffer, and eluted at 70°C with shaking

959 at 700 rpm in 50  $\mu$ L 1.1X LDS loading buffer (Invitrogen, NP0007) in lysis buffer for 10 m. 2-  
960 mercaptoethanol (1.43 M final) was added, and samples were heated to 95°C for 5 m and loaded  
961 onto an SDS-PAGE gel for immunoblotting. Mouse anti-FLAG M2 antibody (Sigma F1804, used at  
962 1:5000) and rabbit<sup>54</sup> anti-histone H3 antibody (Abcam, 1791, used at 1:5000) were used for  
963 blotting proteins of interest overnight at 4°C. Goat anti-mouse IgG1 (LI-COR Biosciences, 926-  
964 32350, used at 1:20,000) and goat anti-rabbit IgG (LI-COR Biosciences, 926-68071, used at  
965 1:20,000) were used for blotting FLAG and H3 primary antibodies, respectively, during a one-hour  
966 incubation at room temperature. A LI-COR Odyssey Instrument was used to develop the blot  
967 images.<sup>53</sup>

### 969 Pulsed-field gel electrophoresis

970 A single yeast colony was inoculated into 7 mL of YPD liquid medium and grown at 30°C for two  
971 days with rotation. Approximately 40 mg of yeast cells were harvested by centrifugation. To  
972 prepare plugs, 16  $\mu$ L of zymolyase solution (25 mg/mL zymolyase 20T in 10 mM KH<sub>2</sub>PO<sub>2</sub> pH 7.5)  
973 and 360  $\mu$ L of 0.5% melted low melting point agarose (Bio-Rad, 1620137) in 100 mM EDTA pH 7.5  
974 were added to the cell pellet and mixed by pipetting. 90  $\mu$ L of this suspension was added per well  
975 of a BioRad plug mold, and the plugs were cooled for 30 m at 4°C. Solidified plugs were then  
976 transferred into 15 mL tubes containing 1 mL of digestion buffer (0.5 M EDTA, 10 mM Tris, pH 7.5),  
977 mixed by inversion, and incubated at 37°C with rotation overnight. The next day, 400  $\mu$ L of  
978 proteinase K solution (5% sarcosyl and 5 mg/mL proteinase K in 0.5 M EDTA, pH 7.5) were added  
979 to the tube. Samples were incubated at 50°C for five h, and then washed once with H<sub>2</sub>O and three  
980 times with TE (2 mM Tris, 1 mM EDTA, pH 8.0). Plugs were stored at 4°C until ready for gel  
981 loading. Half of one plug per sample was embedded per well of a gel consisting of 1% low melting  
982 point agarose in 0.5X Tris-Borate-EDTA buffer. Samples were separated in running buffer of 0.5X  
983 Tris-Borate-EDTA by clamped homogeneous electric field gel electrophoresis using the CHEF  
984 Mapper XA Pulsed-Field Electrophoresis System (Bio-Rad), as previously described.<sup>56</sup> The  
985 following program was used: auto-algorithm with size range of 200 kb to 2.5 Mb, temperature of  
986 14°C, voltage of 6 V/cm, included angle of 120°. After electrophoresis, gels were stained using 5  
987  $\mu$ g/mL ethidium bromide in water for 30 m, destained in water for 30 m, and then imaged using the  
988 ChemiDoc XRS+ System (Bio-Rad).

### 990 Chromosome substitution and conditional centromere destabilization

991 For initial experiments, disomic *synIX* strain yLHM0387 was used as the donor and yWZ601 as the  
992 recipient for *kar1-1*-mediated chromoduction. For follow-up experiments, disomic strain yLHM0604  
993 and monosomic strains yLHM0721, yLHM1591, and yLHM1601 were used as donors and yWZ602  
994 as the recipient for *kar1-1*-mediated chromoduction. yWZ601 and yWZ602 were derived from  
995 DMy044<sup>24</sup> following deletion of *LYS12* from wild-type chromosome IX and insertion of a *URA3-*  
996 *pGAL* promoter cassette upstream of *CEN9*.<sup>26</sup> Strains were grown independently on YPD plates,  
997 and subsequently mated on YPD plates via replica plating. After 14 h of growth, these plates were  
998 replica plated to SC–Lys–Arg plates with 12  $\mu$ g/mL canavanine and 10  $\mu$ g/mL cycloheximide  
999 added. Plates were grown until individual colonies emerged; if a patched formed instead, patches  
1000 were replica plated to the same selective medium and grown for additional time until individual  
1001 colonies emerged. Single colonies were restreaked to fresh selective plates and analyzed by



1002 PCRTagging, pulsed-field gel electrophoresis, and whole genome sequencing. To lose wild-type  
1003 chromosome *IX*, strains were grown in YPD overnight, diluted 1:1000 in YEP + 2% galactose  
1004 medium for 24 h, and plated on 5-FOA medium to select for strains that had lost wild-type  
1005 chromosome *IX*. Single colonies were genotyped by PCRTag analysis and further characterized by  
1006 pulsed-field gel electrophoresis and whole genome sequencing. For chromosome substitution to  
1007 move *synIX* from yLHM1601 to recipient strain yLHM2231 (derived from BY4742), a LEU2 marker  
1008 was integrated into *synIX* near the end of megachunk H, in minichunk 101, generating strain  
1009 yLHM1754. The same protocol as listed above was followed using donor strain yLHM1754 and  
1010 recipient strain yLHM2231, except after mating and 14 h of growth, plates were replica plated to  
1011 SC–Leu–Arg (rather than SC–Lys–Arg) plates with 12  $\mu\text{g}/\text{mL}$  canavanine and 10  $\mu\text{g}/\text{mL}$   
1012 cycloheximide added.

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1014

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1018

1019 **Competing Interests**

1020 Jef Boeke is a Founder and Director of CDI Labs, Inc., a Founder of and consultant to  
1021 Neochromosome, Inc, a Founder, SAB member of and consultant to ReOpen Diagnostics, LLC  
1022 and serves or served on the Scientific Advisory Board of the following: Logomix, Inc., Sangamo,  
1023 Inc., Modern Meadow, Inc., Rome Therapeutics, Inc., Sample6, Inc., Tessera Therapeutics, Inc.  
1024 and the Wyss Institute. Joel Bader is a founder of Neochromosome, Inc., consultant to Opentrons  
1025 Labworks, Inc. and advisor to Reflexion Pharmaceuticals, Inc.

1026

1027 **Author contributions**

1028 J.D.B., S.C., and W.Z. conceptualized the study. W.Z., S.C., L.H.M., and J.D.B. designed the  
1029 experiments. J.D.B. and J.S.B. designed *synIX*. The Build A Genome class members constructed  
1030 building blocks. J.S., E.C., H.M., and K.Z led Build A Genome class research. V.S., N.A., and S.R.  
1031 constructed minichunks and incorporated them for megachunks A to F. L.H.M. designed,  
1032 constructed, and delivered megachunks G through I and performed error correction, bug mapping,  
1033 bug correction, and functional studies of *synIX*. L.H.M. and W.Z. developed and evaluated  
1034 chromosome substitution method. L.H.M., A.L.H., J.C., V.F., E.L., and G.S. performed sequencing  
1035 and data analyses. L.H.M., W.Z., and J.D.B. wrote the manuscript. L.A.M., H.J., J.L., M.S.T., and  
1036 W.R.B. provided advice. S.C., J.D.B., J.S.B., and L.M.S. provided lab supervision. All authors  
1037 reviewed and edited the manuscript.

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