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Supplemental information

Rapid, scalable, combinatorial genome engineering by marker-less enrichment and recombination of genetically engineered loci in yeast

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Supplementary data for

"Rapid, scalable, combinatorial genome engineering by Marker-less Enrichment and Recombination of Genetically Engineered loci (MERGE) in Yeast"

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This PDF file includes:

Supplemental Figures S1 to S9

Supplementary Figure S1- Related to Figure 1

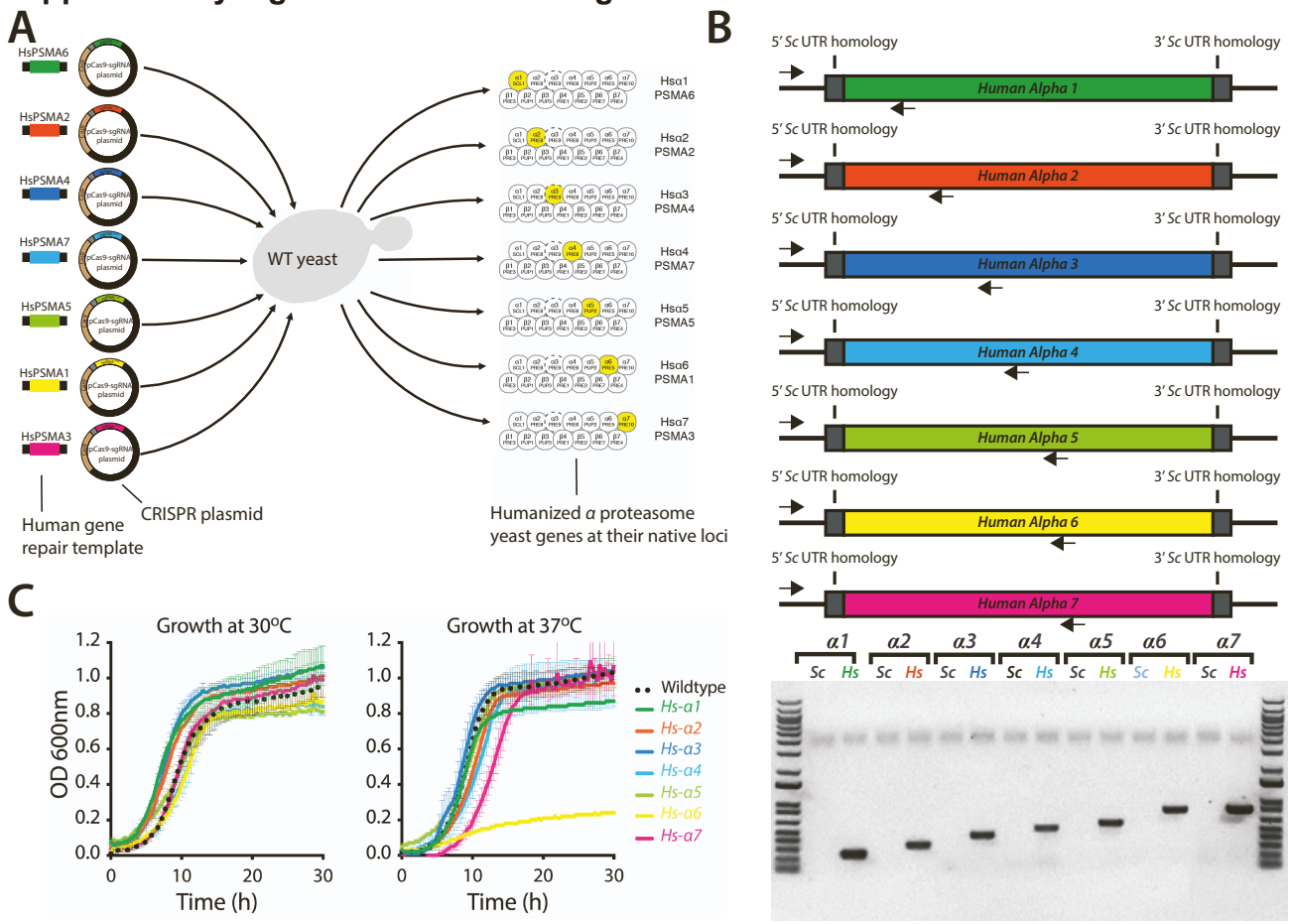


Figure S1. General scheme of CRISPR-Cas9-based gene replacement.
(A) For each gene, a 'knockout' (KO) plasmid (CRISPR plasmid) is constructed that expresses the Cas9 endonuclease and a sgRNA with a targeting sequence to the gene of interest. To replace the gene of interest, the CRISPR plasmid is co-transformed into yeast with a linear repair DNA, consisting of the replacing gene sequence (human ortholog) flanked by long homology sequences to the flanking region of the corresponding yeast gene. Cas9 and the sgRNA are co-expressed and target the gene of interest for cutting, allowing repair by homologous recombination (HR) with the provided repair DNA. **(B)** After obtaining transformants, sequencing across the junction reveals successful replacement (as shown for the human α-proteasome subunits replaced at the native yeast loci). PCR for the yeast locus (*Sc*) is negative, whereas for a human gene (*Hs*) is positive. **(C)** The growth of singly-humanized α subunits is not significantly impaired. Each curve represents an average of 4 replicates for each indicated strain, grown in YPD for 24 hours. No strains exhibit significantly impaired growth, though strains with human subunits α4, α5, and α6 show some extension of lag phase at 30°C and marginally slowed doubling times at 30°C. The behavior is similar at 37°C except in Hsα6, which exhibited a significant growth defect.

Supplementary Figure S2 - Related to Figures 1 & 3

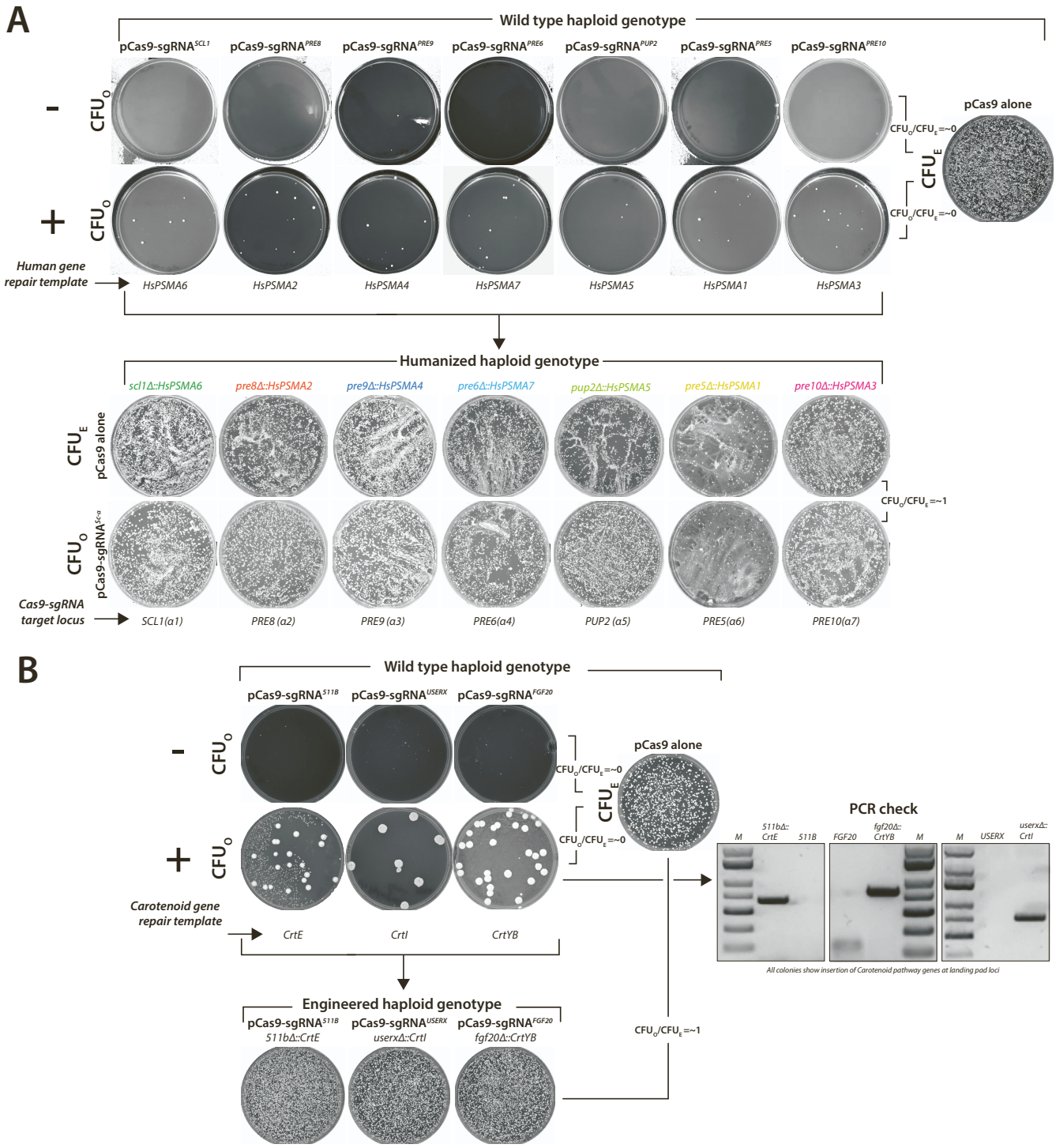


Figure S2. General scheme of CRISPR-Cas9-based gene replacement, CRISPR-mediated sensitivity and resistance of yeast loci.

(A) CRISPR plasmid (**pCas9-sgRNA^{locus}**) targeted to any yeast locus often leads to lethality with few surviving colonies (**CFU$_O$**). The corresponding transformation of a control plasmid (**pCas9 alone**) estimates the transformation efficiency (**CFU$_E$**). CRISPR plasmids targeting 7 yeast α-proteasome

genes show lethality (**CFU_O/CFU_E = ~0**) with or without the repair template. After humanizing the corresponding yeast loci, each single-humanized haploid strain shows resistance to further targeting by the corresponding CRISPR plasmid, respectively (**CFU_O/CFU_E = ~1**). **(B)** CRISPR plasmids targeted to landing pad loci (*511B*, *USERX* and *FGF20*) enabled editing by introducing carotenoid gene transcription units *CrtE*, *CrtI* and *CrtYB* (provided as PCR fragment repair templates). The engineered strains show resistance to further cutting by the corresponding CRISPR plasmids (**CFU_O/CFU_E = ~1**). PCR-based genotyping confirmed the integration of each carotenoid transcription unit for every colony tested.

Supplementary Figure S3 - Related to Figure 3

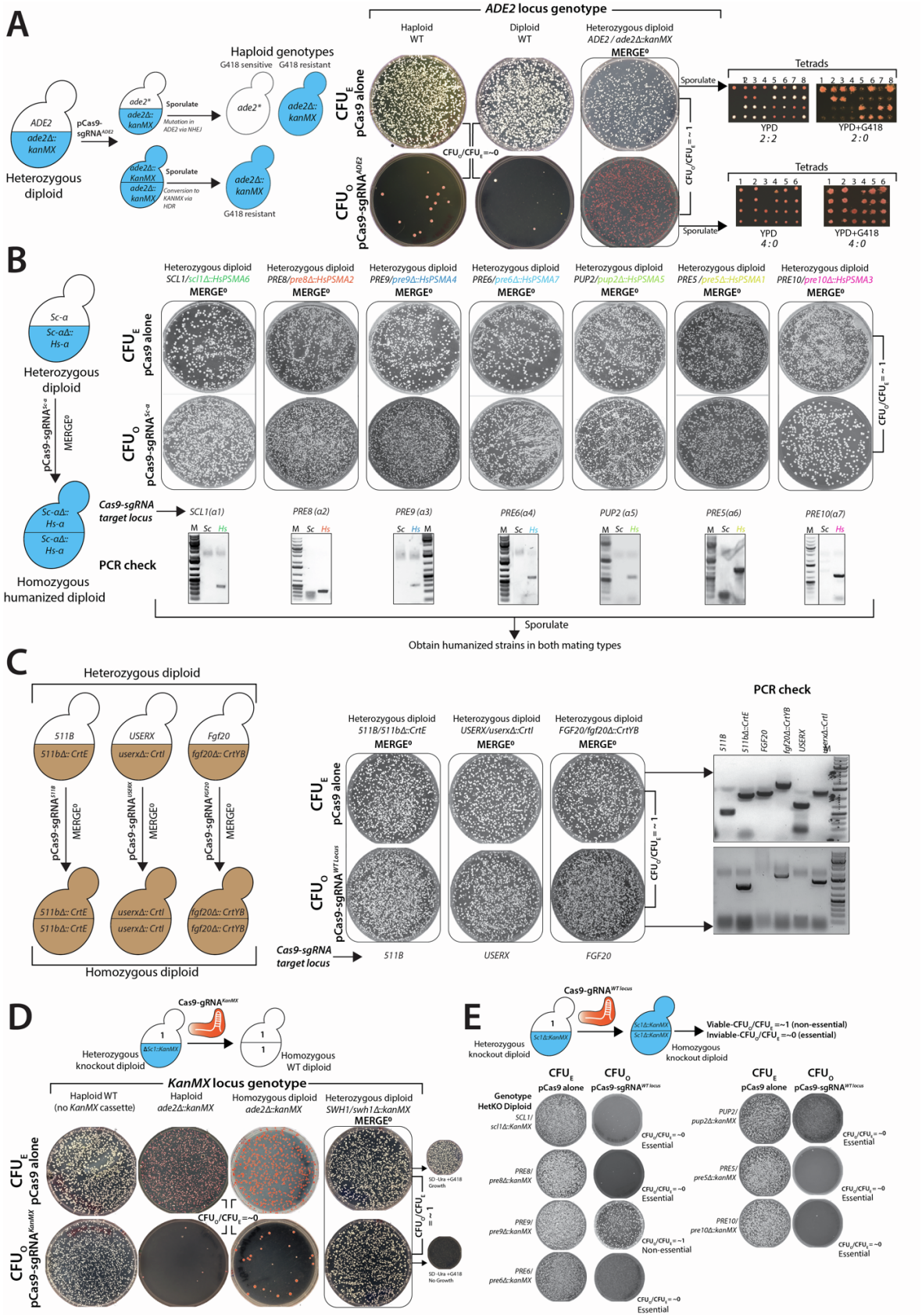


Figure S3. MERGE^0 enables the efficient conversion of single heterozygous to homozygous loci. **(A)** A schematic of possible genotypes after the *ADE2* locus in a heterozygous diploid *ADE2*/*ade2Δ::kanMX* strain is subjected to a CRISPR-Cas9-mediated DSB followed by sporulation. The repair via NHEJ could mutate the locus (*ade2**) or repair via HDR converts the locus to *ade2Δ::kanMX*; either scenario leads to a red colony phenotype. However, post sporulation, the prior scenario will show only 50% G418 resistant colonies compared to the latter outcome, which will exhibit 100% G418 resistance. CRISPR plasmid (**pCas9-sgRNA^{ADE2}**) targeted to the wild type *ADE2* locus quantifies the efficiency of **MERGE^0**. The transformation of **pCas9-sgRNA^{ADE2}** in the wild type haploid or diploid strains is lethal, with few surviving red colonies suggesting efficient ON-target activity (**CFU_O/CFU_E = ~0**). However, the transformation of **pCas9-sgRNA^{ADE2}** in heterozygous diploid *ADE2/ade2Δ::kanMX* strain shows no lethality (**CFU_O/CFU_E = ~1**). Instead, all surviving red colonies suggest efficient conversion to the knockout locus. Tetrad dissection of pooled cells from heterozygous diploid strains before **MERGE^0** (**pCas9 alone**) or after **MERGE^0** (**pCas9-sgRNA^{ADE2}**) shows that before **MERGE^0** cells appear as 2 : 2 (red : white) and 2 : 2 (G418-resistant : G418-sensitive) phenotype. However, after **MERGE^0,** cells show 4 : 0 (red : white) phenotype and 4 : 0 G418 resistance. **(B)** Schematics show that CRISPR plasmid targeted to one of the heterozygous alleles (wild type yeast gene) in a diploid strain permits efficient conversion to homozygous humanized loci. **MERGE^0** efficiently converts wild-type yeast loci α-proteasome genes to humanized loci (**CFU_O/CFU_E= ~0**). PCR-based genotyping verified the conversion to the CRISPR-resistant humanized loci for every colony tested. **(C)** Schematic shows how CRISPR plasmid targeted to the landing pad loci in a heterozygous diploid enables conversion to engineered carotenoid loci. **MERGE^0** converts heterozygous landing pad loci (*511B, USERX,* and *FGF20*) to engineered carotenoid loci (**CFU_O/CFU_E = ~1)**. PCR-based genotyping shows the heterozygous status of the loci before **MERGE^0** and conversion to engineered carotenoid loci after **MERGE^0**. **(D)** CRISPR plasmid targeting *kanMX* cassette (**pCas9-sgRNA^{kanMX}**) shows no OFF-target activity in a strain lacking the cassette (**CFU_O/CFU_E = ~1**). However, haploid or diploid strains harboring *kanMX* cassettes as the only allele show lethality (**CFU_O/CFU_E = ~0**), suggesting ON-target activity. However, the diploid strain heterozygous for the *kanMX* allele (*SWH1*/*swh1Δ::kanMX*) is viable (**CFU_O/CFU_E = ~1**), suggesting conversion to the wild type allele as demonstrated by the loss of G418 resistance after performing **MERGE^0**. **(E)** Alternatively, using hetKO strains and targeting CRISPR plasmid to the wild-type yeast locus enables a single-step gene essentiality assay in yeast. The remarkably high efficiency of **MERGE^0** converts every heterozygous yeast locus to homozygous null, therefore, allowing viability only if the gene is non-essential (as in the case of yeast proteasome α3 gene). All other essential loci show lethality after **MERGE^0**.

Supplementary Figure S4- Related to Figure 3

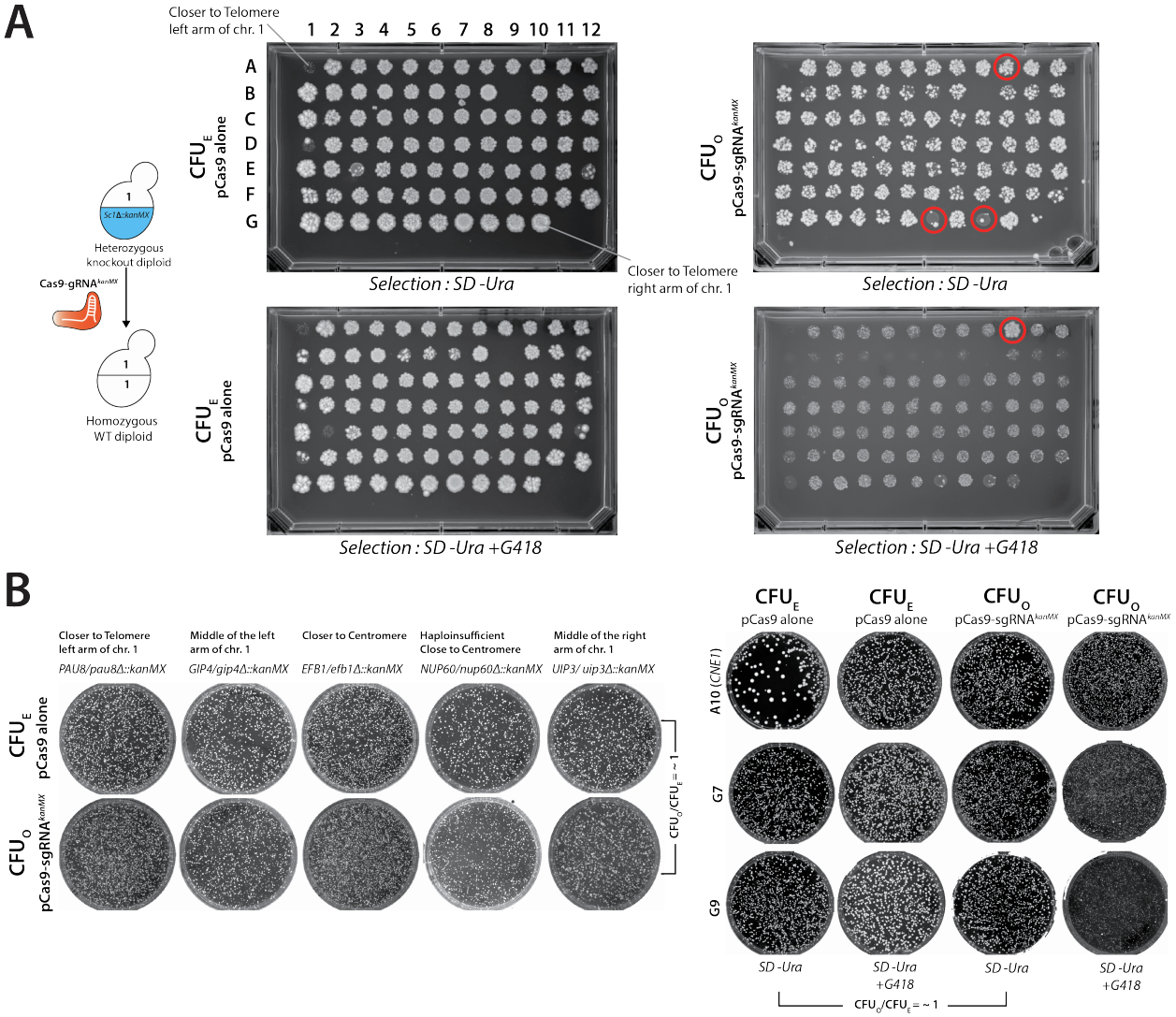


Figure S4. MERGE^0 efficiently converts yeast heterozygous to homozygous loci independent of the position on Chromosome 1.

(A) Schematic shows a CRISPR plasmid (**pCas9-sgRNA^{kanMX}**) targeted to the *kanMX* allele in a hetKO strain converts the locus to homozygous wild-type. The hetKO strains were arrayed as genes close to the left arm of a telomere through the centromere to the right arm of a telomere. The strains were transformed with **pCas9 alone** (**CFU$_E$**) or with **pCas9-sgRNA^{kanMX}** (**CFU$_O$**) and spotted on either SD-Ura or SD-Ura with G418 selection. Every genotype shows spots with a similar density of colonies showing no lethality due to the CRISPR plasmid (**CFU$_O$/CFU$_E$ = ~1**). The spots on SD-Ura with G418 selection show G418 resistance in the case of **pCas9 alone** transformants. However, all but one (A10 - *CNE1*) strain transformed with **pCas9-sgRNA^{kanMX}** lost the *kanMX* cassette resulting in G418 sensitivity. **(B) MERGE^0** mediated conversion of loci was tested on several unique hetKO genotypes on Petri plates, demonstrating agreement with 96-well spot assays. All tested strains show no lethality due to the CRISPR plasmid (**CFU$_O$/CFU$_E$ = ~1**). Ambiguous spots on a 96-well plate (A10, G7 and G9) were further analyzed on Petri plates. G7 and G9 spots show no lethality due to CRISPR plasmid

(**CFU_O/CFU_E = ~1**) and loss of *kanMX* cassette. While the A10 (*CNE1*) strain is resistant to the CRISPR plasmid, the cells retain G418 resistance. It is unclear why this behavior occurs.

Supplementary Figure S5 - Related to Figure 4

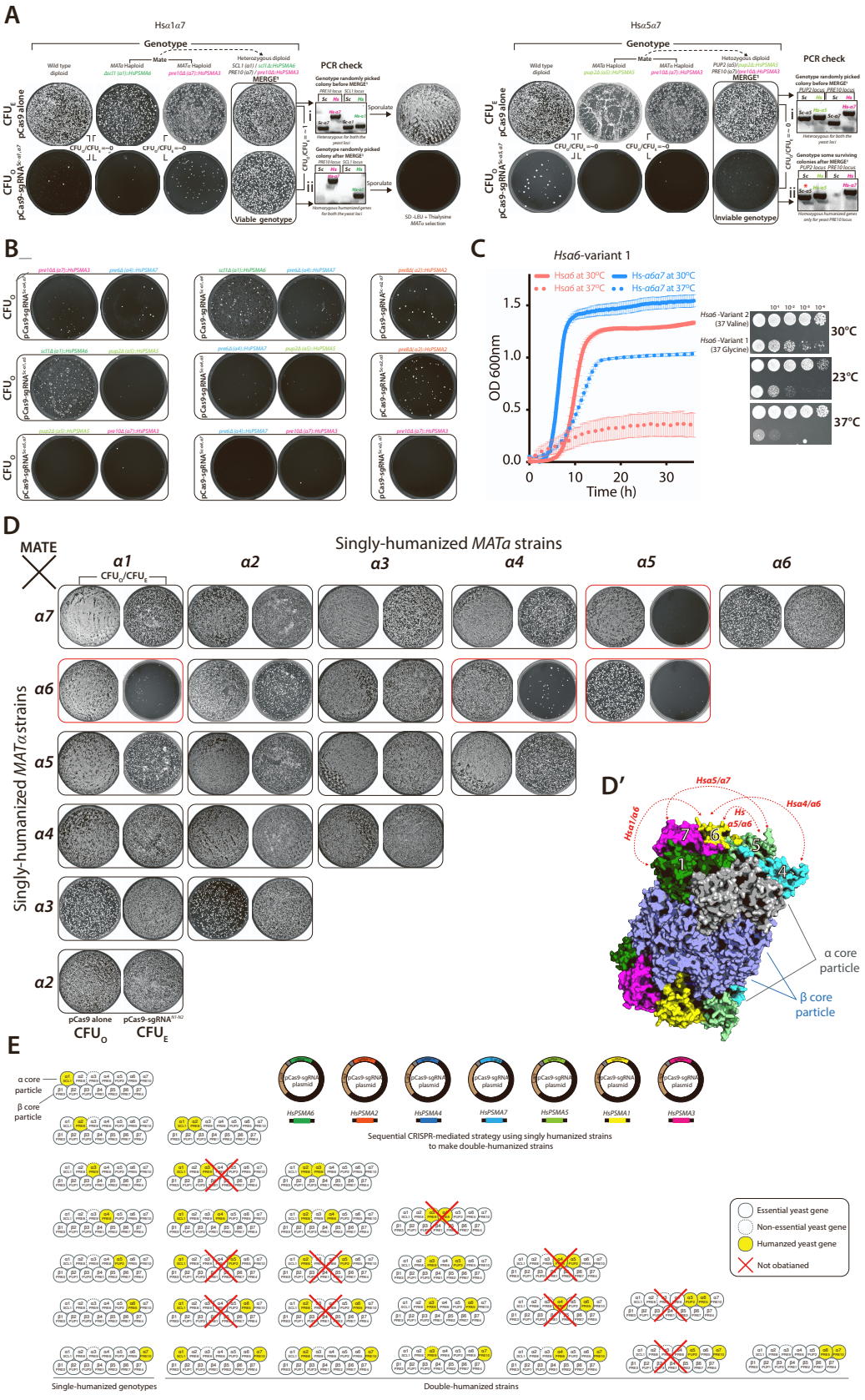


Figure S5. MERGE[1] is efficient at simultaneously combining two heterozygous to homozygous loci identifying compatible paired humanized genotypes.
(A) Double-sgRNA-CRISPR plasmid (**pCas9-sgRNA$^{Sc\text{-}\alpha1,\alpha7}$**) targeted to two wild type yeast loci (proteasome α1 and α7 genes) quantifies the efficiency of **MERGE[1]**. The transformation of **pCas9-sgRNA$^{Sc\text{-}\alpha1,\alpha7}$** in the wild type, and single-humanized Hsα1 or Hsα7 strains is lethal, suggesting ON-target activity (**CFU_O/CFU_E = ~0**). However, the transformation of **pCas9-sgRNA$^{Sc\text{-}\alpha1,\alpha7}$** in diploid heterozygous humanized strain shows no lethality (**CFU_O/CFU_E = ~1**). PCR-based genotyping of surviving colonies after **MERGE[1]** shows conversion of both yeast to the humanized loci compared to before **MERGE[1].** Alternatively, double-sgRNA-CRISPR plasmid (**pCas9-sgRNA$^{Sc\text{-}\alpha5,\alpha7}$**) targeting two wild type yeast loci (proteasome α5 and α7 genes) in a corresponding heterozygous diploid humanized strain showed lethality, suggesting incompatible combination (**CFU_O/CFU_E = ~0**). The transformation of **pCas9-sgRNA$^{Sc\text{-}\alpha5,\alpha7}$** in the wild type, and single-humanized Hsα5 or Hsα7 strains is lethal, with few surviving colonies, suggesting ON-target activity (**CFU_O/CFU_E = ~0**). PCR-based genotyping of surviving colonies after **MERGE[1]** shows the conversion of only one (α5) yeast to the humanized locus (**ii**) compared to before **MERGE[1]** showing heterozygous genotype at both loci (**i**). **(B)** All tested single-humanized strains transformed with double-sgRNA-CRISPR plasmids show lethality, demonstrating that selection only allows survival of viable paired genotypes. **(C)** A quantitative growth assay performed on a single-humanized Hsα6 strain (Variant 1, amino acid residue 37>Glycine) shows a temperature-sensitive (TS) phenotype at 37^{O}C (dotted red line) compared to growth at 30^{O}C (solid red line). The combined genotype of Hsα6,α7 partially rescues the TS phenotype at 37^{O}C (dotted blue line) compared to the growth at 30^{O}C (solid blue line). Spotted dilutions of single-humanized Hsα6 strains (Variant 1, amino acid residue 37>Glycine) and (Variant 2, amino acid residue 37>Valine) show Variant 2 with no growth defect at 30^{O}C, 23^{O}C and 37^{O}C compared to Variant 1. **(D)** Mating each single-humanized strain of opposite mating-types provided 21 heterozygous genotypes to test **MERGE[1]**. Heterozygous diploid humanized strains transformed with double-sgRNA-CRISPR plasmids specific to each yeast allele allow testing viability of all paired-humanized genotypes (**CFU_O**). If the genotype is permitted, the strategy enables survival similar to the pCas9 alone transformation (**CFU_E**). While the majority of combined double-humanized genotypes are viable (**CFU_O/CFU_E = ~1**), specific genotypes (Hsα1,α6, Hsα4,α6, Hsα5,α6 and Hsα5α7) were inviable, suggesting incompatibility. Other combinations, such as those involving the genotypes with Hsα2, showed a slow-growth phenotype. **(D')** Yeast proteasome structure (PDB-1RYP) with α and β cores show the humanized α subunits that are incompatible as paired genotypes (dashed red lines). Except for α5 and α6, the subunits are missing neighboring interactions within the α core. **(E)** A sequential strategy using single-humanized strains acquired several pairwise combinations of human α subunits. However, several other genotypes could not be obtained, revealing the drawback of the method. Comparatively, **MERGE[1]** is far more efficient in identifying incompatible paired genotypes.

Supplementary Figure S6 - Related to Figure 5

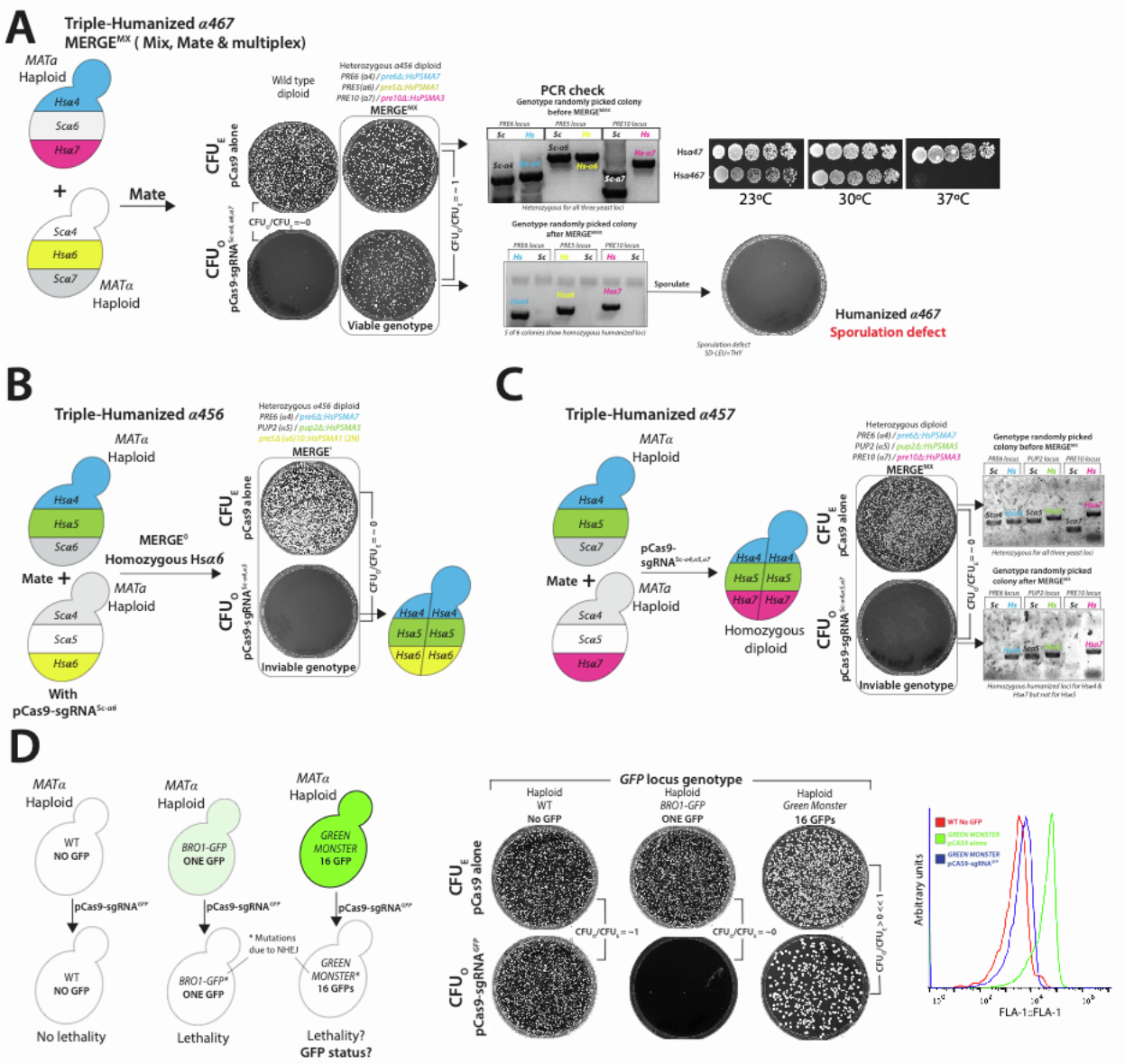


Figure S6. MERGE^{MX} (Mate and multiplex) targets and combines >3 yeast loci simultaneously. **(A)** Mating haploid yeast strains with 2 humanized loci (Hsα4,α7, *MAT*a) and 1 humanized locus (Hsα6, *MAT*α) generates diploid yeast with heterozygous alleles at three distinct loci. The transformation of **pCas9-sgRNA$^{Sc\text{-}\alpha4,\alpha6,\alpha7}$** in the wild type is lethal, suggesting ON-target activity (**CFU$_O$/CFU$_E$ = ~0**). However, the transformation of **pCas9-sgRNA$^{Sc\text{-}\alpha4,\alpha6,\alpha7}$** in a mated mix selected a diploid triple-humanized strain (**CFU$_O$/CFU$_E$ = ~1**). PCR-based genotyping of several colonies after **MERGE^{MX}** shows conversion of all yeast to the humanized loci (5 of 6). Spotted dilutions of humanized Hsα4,α7 and Hsα4,α6,α7 strains at 23°C, 30°C and 37°C show the triple-humanized strain with a temperature-sensitive phenotype at 37°C. The strain does not yield viable haploid spores observed as no growth on haploid-specific selection media (SD-LEU+Thialysine). (**B**) Double-humanized *Hs*α4,α5 (haploid *MAT*α) and single-humanized *Hs*α6 (haploid *MAT*a, harboring a **pCas9 alone** or **pCas9-sgRNA$^{Sc\text{-}\alpha6}$** vector) strains were mated, generating two distinct heterozygous diploid strains, one heterozygous for

three loci and another for two with homozygous Hsα6 locus. Each strain was transformed with double-sgRNA-CRISPR plasmid (**pCas9-sgRNA$^{Sc\text{-}\alpha4,\alpha5}$**) to perform **MERGE**[1]. The transformation caused lethality in both strains (**CFU_O/CFU_E =~0**). However, a few survivors in the context of heterozygous Hsα6 locus showed the conversion of two loci (Hsα4 and Hsα7), suggesting an incompatible triple-humanized genotype but viable double-humanized genotype harboring a heterozygous yeast-human α6 locus. **(C)** Mating Hsα4,α5 (*MAT*α) and Hsα7 (*MAT*a) strains yields triple-heterozygous diploid strain. The transformation of triple-sgRNA-CRISPR plasmid (**pCas9-sgRNA$^{Sc\text{-}\alpha4,\alpha5,\alpha7}$**) in the heterozygous strain caused lethality with few survivors (**CFU_O/CFU_E =~0**). Genotyping of the survivors showed the conversion of two 2 of 3 loci (Hsα4 and Hsα7), suggesting an incompatible triple-humanized genotype. **(D)** The schematic shows a CRISPR-based strategy targeting one or many genetic loci in yeast. Transformation of a CRISPR plasmid (**pCas9-sgRNA^{GFP}**) targeting a *GFP* cassette in a Green Monster (GM) strain tests the scalability. Transformation in the wild type (no *GFP*) strain should show no lethality, whereas strains harboring single or multiple *GFP*s should show a lethal phenotype. The **pCas9-sgRNA^{GFP}** transformation in wild type yeast showed viable cells (**CFU_O/CFU_E =~1**). In contrast, transformation in a strain harboring a single *GFP* cassette was lethal. While the Green Monster strain (*GFP* monster, 16 *GFP* cassettes) showed more viable cells than the single-*GFP* strain upon transformation with **pCas9-sgRNA^{GFP}** (likely due to recombination among GFP cassettes after DSB). Using flow cytometry, the pooled mixture of yeast cells that survive **pCas9-sgRNA^{GFP}** showed significantly reduced *GFP* expression compared to the pCas9 alone transformed cells, suggesting that nearly every *GFP* cassette was successfully targeted and mutated via NHEJ.

Supplementary Figure S7 - Related to Figure 6

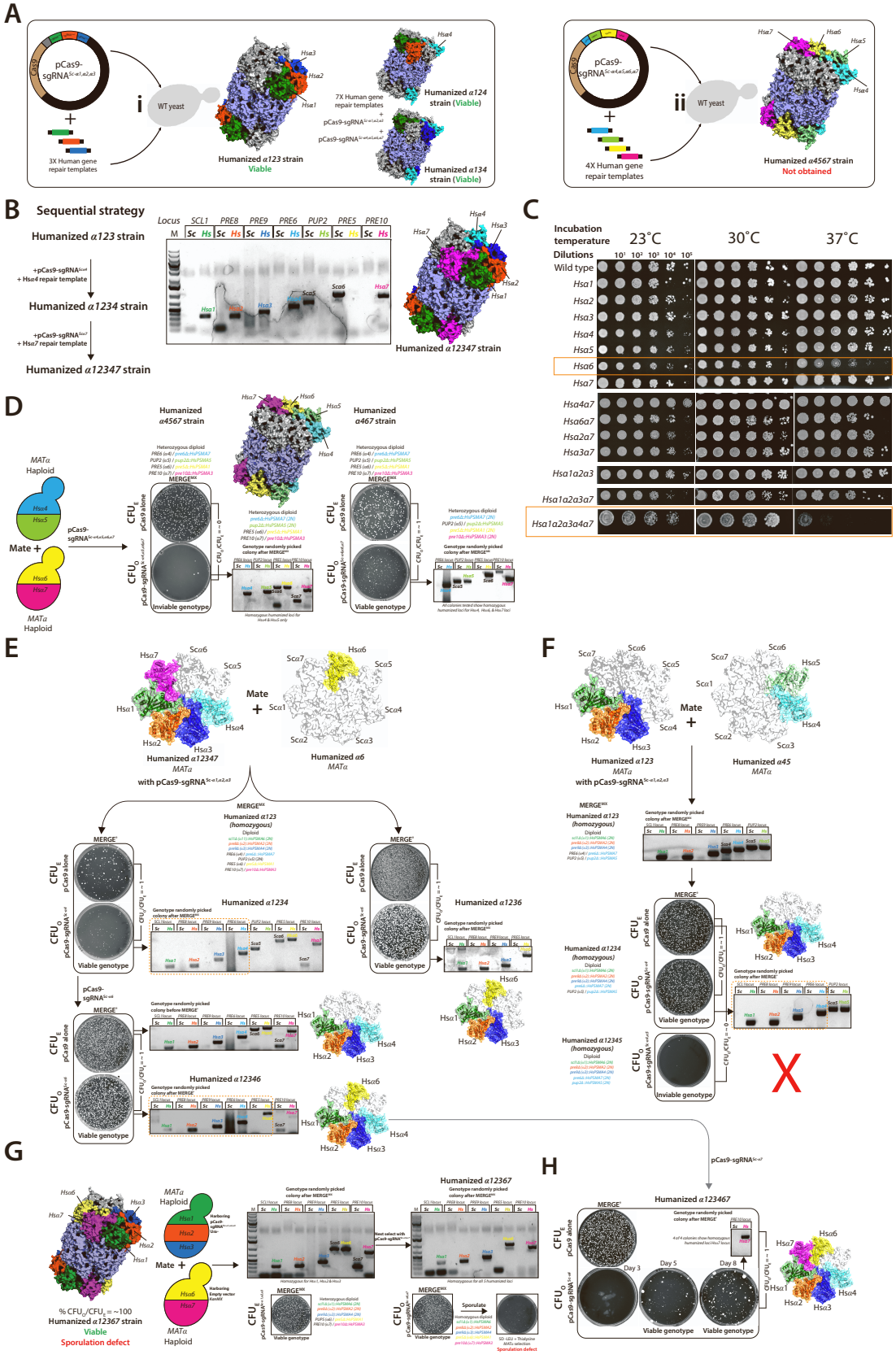


Figure S7. Compared to sequential strategy, MERGE efficiently tests several higher-order combinations (>3) of humanized yeast α-proteasome core

(A) Two multiple-sgRNA-CRISPR plasmids targeting yeast α subunits 1, 2, and 3 (**pCas9-sgRNA$^{Sc\text{-}\alpha1,\alpha2,\alpha3}$**), and α subunits 4, 5, 6, and 7 (**pCas9-sgRNA$^{Sc\text{-}\alpha4,\alpha5,\alpha6,\alpha7}$**) were transformed individually with the respective human gene repair DNA templates. A single colony with triple-humanized Hsα1,α2, α3 (**i**) was obtained. However, no clone for a quadruple humanized Hsα4,α5,α6,α7 (**ii**) could be obtained. A similar strategy also generated triple-humanized Hs,α1,α2,α4 and Hsα1α3,α4 genotypes. **(B)** Sequential strategy generated a quintuple-humanized Hsα1α2,α3,α4,α7 strain confirmed via locus-specific PCR. **(C)** Spotted dilutions of double-, triple-, quadruple-, quintuple- and sextuple humanized α-proteasome strains at 23°C, 30°C and 37°C show singe-humanized Hsα6 (variant 1) and quintuple-humanized Hsα1,α2,α3,α4,α7 strain with a temperature-sensitive growth phenotype. (**D**) Double-humanized haploid Hsα4,α5 (*MAT*α) and Hsα6,α7 (*MATa*) strains were mated. The resulting heterozygous diploid strain was transformed with quadruple-sgRNA-CRISPR plasmid (**pCas9-sgRNA$^{Sc\text{-}\alpha4,\alpha5,\alpha6,\alpha7}$**) to perform **MERGE^{MX}**. The transformation caused lethality with few survivors (**CFU$_O$/CFU$_E$ =~0**). The survivors showed the conversion of only 2 of 4 loci (Hsα4 and Hsα5), suggesting an incompatible quadruple-humanized genotype. However, the transformation of triple-sgRNA-CRISPR plasmid (**pCas9-sgRNA$^{Sc\text{-}\alpha4,\alpha6,\alpha7}$**) in this strain was viable (**CFU$_O$/CFU$_E$ =~1**). The genotyping of randomly picked colonies showed the conversion of 3 yeast to human loci (Hsα4, Hsα6, and Hsα7) with a heterozygous yeast-human *α5* locus. **(E)** Mating quintuple-humanized haploid Hsα1,α2,α3,α4,α7 [*MATa*, also harboring a triple-sgRNA CRISPR plasmid (**pCas9-sgRNA$^{Sc\text{-}\alpha1,\alpha2,\alpha3}$**)] and single-humanized Hsα6 (*MAT*α) strains simultaneously combined three yeast to humanized Hsα1,α2,α3 loci in a diploid yeast (**CFU$_O$/CFU$_E$ =~1**). The strain harbors heterozygous yeast-human alleles for *α4, α6,* and *α7* loci. Transformation of the diploid strain with **pCas9-sgRNA$^{Sc\alpha4}$** (left panel) or **pCas9-sgRNA$^{Sc\alpha6}$** (right panel) generated viable quadruple-humanized strains (Hsα1,α2,α3,α4 and Hsα1,α2,α3,α6) (**CFU$_O$/CFU$_E$ =~1**). Genotyping of randomly picked colonies confirmed the homozygosity of the humanized loci. Using the quadruple-humanized strain Hsα1,α2,α3,α4 as a background, the transformation of **pCas9-sgRNA$^{Sc\alpha6}$** yielded a viable quintuple-humanized (Hsα1,α2,α3,α4,α6) genotype (**CFU$_O$/CFU$_E$ =~1**) confirmed using locus-specific PCR. **(F)** Mating triple-humanized Hsα1,α2,α3 [haploid *MATa*, also harboring a triple-sgRNA-CRISPR plasmid (**pCas9-sgRNA$^{Sc-\alpha1,\alpha2,\alpha3}$**)] and double-humanized Hsα4,α5 (haploid *MAT*α) strains generated a heterozygous diploid strain for 5 loci. The resulting diploid strain simultaneously combined three yeast to humanized (Hsα1, Hsα2, and Hsα3) loci while harboring two yeast-human heterozygous loci (*α4* and *α5)* (**CFU$_O$/CFU$_E$ =~1**). The subsequent **MERGE^0** strategy (using **pCas9-sgRNA$^{Sc\alpha4}$**) generated a viable quadruple-humanized strain (Hsα1,α2,α3,α4) with heterozygous yeast-human α5 locus (**CFU$_O$/CFU$_E$ =~1**). The locus-specific PCR confirmed the homozygosity for most of the colonies. However, the subsequent CRISPR selection (using **pCas9-sgRNA$^{Sc\alpha5}$**) did not produce a viable strain (**CFU$_O$/CFU$_E$ =~0**), suggesting an incompatible quintuple-humanized (Hsα1,α2,α3,α4,α5) genotype. **(G)** Double-humanized haploid Hsα6,α7 (*MAT*α) and triple-humanized Hsα1,α2,α3 [*MATa*, also harboring a triple-sgRNA-CRISPR plasmid (**pCas9-sgRNA$^{Sc\text{-}\alpha1,\alpha2,\alpha3}$**)] strains were mated. **MERGE^{MX}** simultaneously combined three yeast to humanized Hsα1, Hsα2, and Hsα3 loci in the diploid background (**CFU$_O$/CFU$_E$ =~1**). The next transformation, using **pCas9-sgRNA$^{Sc\text{-}\alpha6,\alpha7}$** also homozygosed the Hsα6 and Hsα7 loci, generating a quintuple-humanized strain (**CFU$_O$/CFU$_E$ =~1**). However, post sporulation, the mix plated

on *MAT*α selection failed to produce any viable humanized haploid strains. **(H)** Using the quintuple-humanized diploid strain Hsα1,α2,α3,α4,α6 harboring a yeast-human heterozygous α7 locus as a background, the transformation of **pCas9-sgRNA$^{Sc\alpha7}$** yielded a viable sextuple-humanized (Hsα1,α2,α3,α4,α6,α7) genotype (**MERGE^0, CFU$_O$/CFU$_E$ =~1**) after incubation for 8 days. The genotype of the viable colonies confirmed the homozygosity and humanization of the α7 locus.

Supplementary Figure S8 - Related to Figure 6

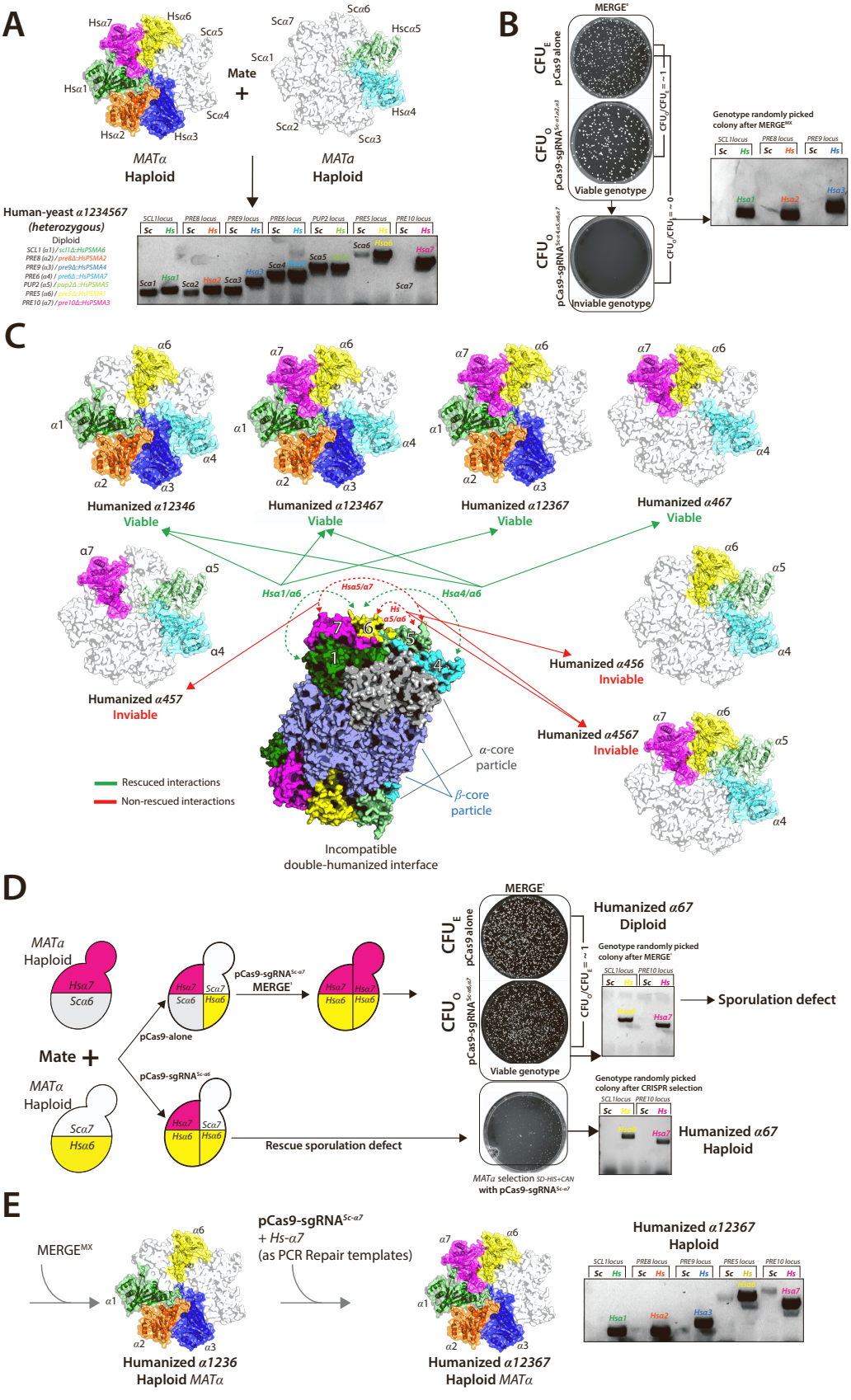
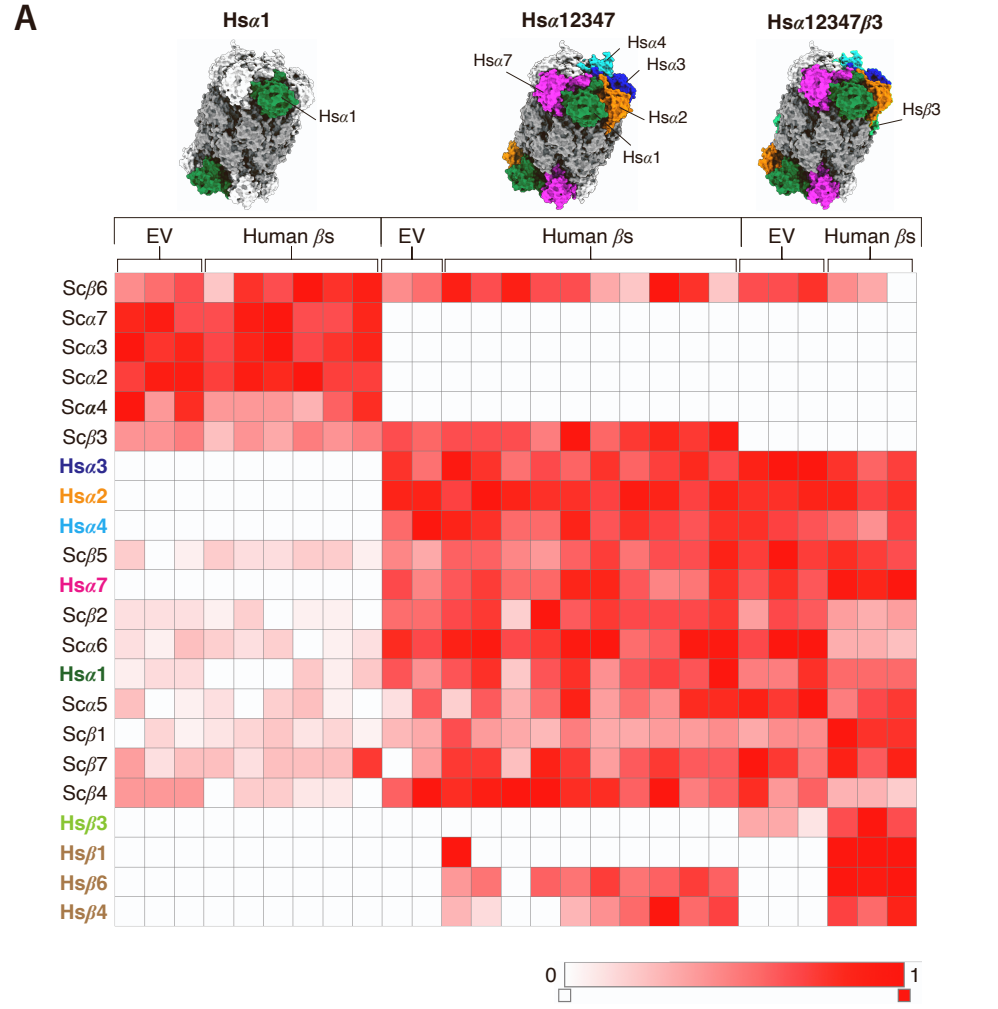
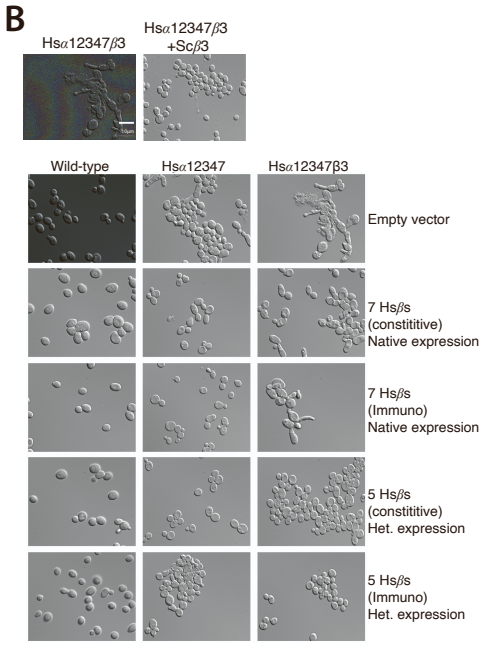


Figure S8. MERGE explores heptameric-gene humanized α-proteasome core in yeast revealing incompatibilities & alternative strategies to complement MERGE.
(A) Mating quintuple-humanized haploid Hsα1,α2,α3,α6,α7 (*MATα*) and double-humanized Hsα4,α5 (*MATa*) strains generated a diploid strain harboring yeast-human heterozygous alleles for 7 α-proteasome loci. Locus-specific PCR confirmed the heterozygous nature of the loci. **(B)** The resulting diploid strain was transformed with a triple-sgRNA-CRISPR plasmid (**pCas9-sgRNA$^{Sc\text{-}\alpha1,\alpha2,\alpha3}$**), resulting in a viable genotype homozygous for triple-humanized Hsα1,α2,α3 loci (**CFU_O/CFU_E =~1**) with yeast-human heterozygous alleles for α4, α5, α6, and α7 loci. The subsequent transformation of a quadruple-sgRNA-CRISPR plasmid (**pCas9-sgRNA$^{Sc\text{-}\alpha4,\alpha5,\alpha6,\alpha7}$**) in the diploid strain was lethal, suggesting an incompatible genotype (**CFU_O/CFU_E =~0**). **(C)** Inviable double-humanized genotypes, Hsα1,α6 and Hsα4,α6, are compatible when the neighboring interactions are restored in higher-order humanized strains (indicated as green lines). However, incompatible paired genotypes comprising Hsα5,α6 and Hsα5,α7 could not be rescued (marked as red lines). Proteasome core structures were generated using Pymol and PDB-1RYP. Colored structures show humanized α-proteasome subunits. **(D) & (E)** Strategies to generate viable humanized haploid yeast strains when the corresponding diploids manifest sporulation defects. **(D)** Mating single-humanized Hsα6 (*MATα*, with **pCas9 alone** or **pCas-sgRNA$^{Sc\text{-}\alpha6}$**) and Hsα7 (*MATa*) strains generated two diploid strains; one harboring yeast-human heterozygous allele at α6 locus and another with homozygous humanized α6 locus. **MERGE^0** (using strategy **pCas-sgRNA$^{Sc\text{-}\alpha7}$**) converted the heterozygous yeast-human α7 locus to a homozygous human allele at ~100% efficiency (**CFU_O/CFU_E =~1**). Locus-specific PCR confirmed the humanized homozygous alleles. However, the diploid strains cannot sporulate. By keeping the α7 locus as heterozygous rescues the sporulation defect. The haploid-specific selection (SD-HIS+CAN; *MATa*) allowed haploid yeast to grow, followed by CRISPR plasmid selection (using **pCas-sgRNA$^{Sc\text{-}\alpha7}$**) or simply genotyping the haploids to identify homozygous double-humanized Hsα6,α7 genotype. **(E)** The diploid quintuple-humanized Hsα1,α2,α3,α6,α7 strain shows a sporulation defect. However, using a sequential CRISPR strategy, a haploid quadruple-humanized Hsα1,α2,α3,α6 strain enables the generation of haploid quintuple-humanized Hsα1,α2,α3,α6,α7 strain to be used for subsequent **MERGE** experiments.

Supplementary Figure S9 - Related to Figure 6





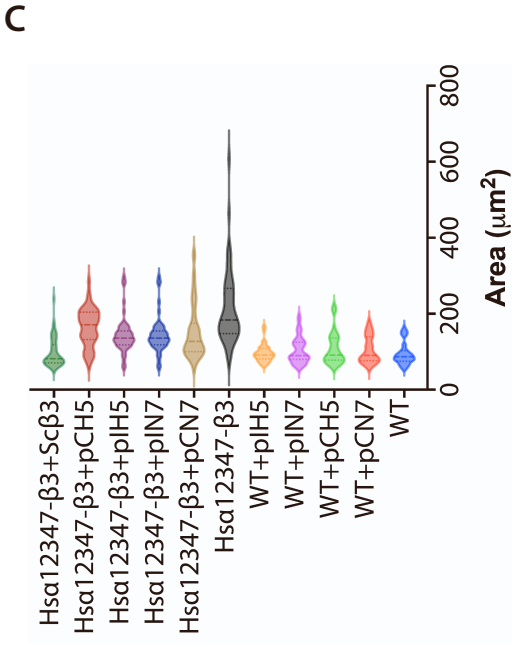


Figure S9. Humanized α-proteasome core yeast strains show stable expression of human *β*-core subunits.

(A) Heatmap showing abundance measurements for each of the yeast and human 20S proteasome core proteins from single-humanized Hs*α*1, quintuple-humanized Hs*α*1,*α*2,*α*3,*α*4,*α*7 and sextuple-humanized Hs*α*1,*α*2,*α*3,*α*4,*α*7,*β*3 strains harboring empty vector (EV) or plasmids expressing human *β*s (values for peptide abundances based on extracted ion-chromatography (XIC) are plotted as heatmap generated using MORPHEUS software; n=3). **(B)** Brightfield microscope images of humanized yeast cells harboring empty vector (EV), plasmids harboring 7 constitutive- (pCN7) and immuno-*β*s (pIN7) driven by native orthologous yeast gene promoters and terminators, and 5-functionally non-replaceable human constitutive-*β*s (pCH5) and immuno-*β*s (pIH5) driven by strong heterologous yeast promoters and terminators. **(C)** Humanized strains show varying cell sizes. Cell areas of humanized strains are represented as violin plots on the *X*-axis. Sextuple-humanized Hs*α*1,*α*2,*α*3,*α*4,*α*7,*β*3 strain shows elongated or abnormal cellular morphologies that can be rescued by the expression of the yeast *β*3 (Sc*β*3) or by the co-expression of several human *β*s. Significance comparisons were performed with wild type yeast determined by a standard *t*-test.