Supplementary Figure 1. *In vitro* recombination assay of purified Cre and Cre-EBD enzymes. 

a A 1432 bp linear DNA substrate containing direct repeats of the *loxP* site (triangles) is incubated with purified Cre and Cre-EBD recombinase under different time. Catalyzed by Cre enzyme results in production of a 748 bp circular product and a 684 bp linear product through intra-molecular excision. The quantity of DNA substrate and product were analyzed by agarose gel electrophoresis. Experiments were performed in triplicate, and representative images are shown. 

b Comparison of *K*ₘ and *k*ₖₐₜ between the Cre (white) and Cre-EBD (gray) enzymes; Data represent mean results ± s.d. from three independent experiments. Source data are provided as a Source Data file.
Supplementary Figure 2. Investigating the mortality rate of SCRaMbLEd synthetic yeast cells. The cell culture of haploid synthetic yeast strains harboring syn2369R (*synII, synIII, synVI* and *synIXR*) (blue), *synII* (red) and *ring_synII* (gray) undergoing GCE-SCRaMbLE in the presence of 1 mM OmeY were plated on synthetic complete (SC) medium at different timepoint. To better show the data points for three different groups at the same time points, the data points are slightly shifted from their original coordinates on X-axis. The survival rate is calculated by dividing the number of viable colonies by total colonies undergoing GCE-SCRaMbLE. The total colonies were normalized based on the unSCRaMbLEd group. Mortality rate is equal to 100% minus survival rate. Lines are second-degree polynomial curves of best fit, with $R^2$ values stated. Source data are provided as a Source Data file.
**Supplementary Figure 3.** The proportion of each type of recombination events including inversion (blue), deletion (gray) and duplication (red) under different conditions. **a** Synthetic yeast cells expressing Cre<sub>UAG14</sub> in the medium supplemented with varying concentration of OMeY (1, 2, 5 and 10 mM) by different ncAA concentration. **b** Synthetic yeast cells expressing Cre variants (Cre<sub>UAG5</sub> and Cre<sub>UAG14</sub>) in the medium supplemented with 1 mM OMeY. Source data are provided as a Source Data file.
Supplementary Figure 4. Comparison of the remaining chromosome content between diploid and haploid strains that were subjected to GCE-SCRaMbLE under the same condition. Y axis represents the remaining content of each synthetic chromosome including synII, synIII, synVI, right arm of synIX (synIXR) and circular form of synII (ring_synII). Different synthetic chromosomes synII (a), synIII (b), synVI (c), synIXR (d) and ring_synII (e) were analyzed. X axis represents the number of strains in each group (total 60). Haploid and diploid strains are labeled as blue and red respectively. Circle, square and rhombus represent 1 mM, 2 mM and 5 mM OMeY concentration respectively. Source data are provided as a Source Data file.
## Supplemental Table 1. Design of 23 groups of synthetic yeast cells for GCE-SCRaMbLE

| Group | Synthetic chromosomes | Ploidy  | Cre variants   | OmeY concentration |
|-------|------------------------|---------|-----------------|--------------------|
| 1     | synII synIII synVI synIXR | haploid | Cre\textsubscript{UAG5} | 1 mM               |
| 2     | synII synIII synVI synIXR | haploid | Cre\textsubscript{UAG14} | 1 mM               |
| 3     | synII synIII synVI synIXR | haploid | Cre\textsubscript{UAG14} | 2 mM               |
| 4     | synII synIII synVI synIXR | haploid | Cre\textsubscript{UAG14} | 5 mM               |
| 5     | synII synIII synVI synIXR | haploid | Cre\textsubscript{UAG14} | 10 mM              |
| 6     | synII                  | haploid | Cre\textsubscript{UAG5}       | 1 mM               |
| 7     | synII                  | haploid | Cre\textsubscript{UAG14}       | 1 mM               |
| 8     | synII                  | haploid | Cre\textsubscript{UAG14}       | 2 mM               |
| 9     | synII                  | haploid | Cre\textsubscript{UAG14}       | 5 mM               |
| 10    | synII                  | haploid | Cre\textsubscript{UAG14}       | 10 mM              |
| 11    | ring\_synII           | haploid | Cre\textsubscript{UAG5}       | 1 mM               |
| 12    | ring\_synII           | haploid | Cre\textsubscript{UAG14}       | 1 mM               |
| 13    | ring\_synII           | haploid | Cre\textsubscript{UAG14}       | 2 mM               |
| 14    | ring\_synII           | haploid | Cre\textsubscript{UAG14}       | 5 mM               |
| 15    | ring\_synII           | haploid | Cre\textsubscript{UAG14}       | 10 mM              |
| 16    | synII synIII synVI synIXR | diploid | Cre\textsubscript{UAG5}       | 1 mM               |
| 17    | synII synIII synVI synIXR | diploid | Cre\textsubscript{UAG14}       | 1 mM               |
| 18    | synII synIII synVI synIXR | diploid | Cre\textsubscript{UAG14}       | 2 mM               |
| 19    | synII synIII synVI synIXR | diploid | Cre\textsubscript{UAG14}       | 5 mM               |
| 20    | ring\_synII           | diploid | Cre\textsubscript{UAG5}       | 1 mM               |
| 21    | ring\_synII           | diploid | Cre\textsubscript{UAG14}       | 1 mM               |
| 22    | ring\_synII           | diploid | Cre\textsubscript{UAG14}       | 2 mM               |
| 23    | ring\_synII           | diploid | Cre\textsubscript{UAG14}       | 5 mM               |
Supplemental Table 2. List of strains and plasmids used in this study.

| Strain, plasmid | Description | Source or reference |
|-----------------|-------------|---------------------|
| **Strains:**    |             |                     |
| *S. cerevisiae* |             |                     |
| BY4741          | *MATa* ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 | 1 |
| BY4742          | *MATalpha* ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0 | 1 |
| synll           | *MATa* ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 synLYS2, synll | 2 |
| ring_synll      | *MATa* ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 synLYS2, ring_synll | This study |
| 2369R           | *MATalpha* ura3Δ0 leu2Δ0 his3Δ1 MET15 HO::SUP61 synLYS2, synll, synll, synVI, synIXR | This study |
| ring_synll Dip  | Diploid, ring_synll mating with BY4742 | This study |
| 2369R Dip       | Diploid, 2369R mating with BY4741 | This study |
| **E. coli**     |             |                     |
| BL21(DE3)       | F− _ompT hsdS2 (r−, m−) gal dcm (DE3) | Vazyme Cat number: C504-02 |
| **Plasmids:**   |             |                     |
| pET28a          | Km'         | MiaoLingPlasmid Cat number: P0023 |
| pRS415          | Ap′; LEU2.Shuttle plasmid, empty vector | 3 |
| pRS413          | Ap′; HIS3.Shuttle plasmid, empty vector | 3 |
| pXF231          | pRS415 LeuOmeRS pair | 4 |
| pXF220          | pRS413 carries CreUAG5 | This study |
| pXF221          | pRS413 carries CreUAG11 | This study |
| pXF238          | pRS413 carries CreUAG14 | This study |
| pXF239          | pRS413 carries CreUAG18 | This study |
| pSCW11-Cre-EBD  | Cre fused to EBD and controlled by daughter cell-specific promoter SCW11 | 5 |
| pLH_Scr18       | Km'; URA3. Carries terminator between LoxP before GFP | 6 |
| pLH_Scr19       | Km'; URA3. Carries GFP | 6 |

Ap′, ampicillin resistance; Km', kanamycin resistance.
**Supplemental Table 3.** List of primes used in this study.

| Primer Pair | Primer Sequence (5’-3’) | Description |
|-------------|--------------------------|-------------|
| Cre_F       | GCCGCGCGGCAGCCATATGTCAATTATCGACGGGTACAC | To amplify Cre for cloning into pET28a vector |
| Cre_R       | GTGCGGCCGCAACCTGTCAATCTTCCAGCAG | |
| Cre_EBD_R   | CGAGTGCGCGCGCAAGGCTTGACGGGAAAC | To amplify Cre-EBD for cloning into pET28a together with Cre_F |
| Vec_F       | TGAAGCTTCGGCAGCACTCAGCACC | For inverse PCR to amplify pET28a backbone |
| Vec_R       | GTCAATTGGACATATGGCTGCCGCGC | |
| Remove_EBD_F | GTAGAATGCCCTATTTGTTAGCTCTGTTGCG | To remove EBD on pSCW11-Cre-EBD |
| Remove_EBD_R | GATTAGTGCAATATCCAGGCTTATTCTCAATACGGCATCTTCCAGCTGGGACC | |
| Cre UAG5_F  | TATCGTACGTGTTATAGTCCATATACGCGACCAAATTG | To create Cre variant CreUAG5 |
| Cre UAG5_R  | CTGTACACTTTAAACCATATTATCTGAGGTTTAGAATGCTCAAATTG | |
| Cre UAG11_F | CCAATTACTGACGGTGACCATACCCAGGTGATTGTAACTTCGGAACAC | To create Cre variant CreUAG11 |
| Cre UAG11_R | GTGTTAAATGTCCCATATAGGCGGATTGCTTTCGCAACAA | |
| Cre UAG14_F | GACCGTGACCAAAAATTCTCGCATAGCGTCGATGCAACGAGT | To create Cre variant CreUAG14 |
| Cre UAG14_R | CCAATTACTGACGGTGACCATACCCAGGTGATTGCTTTCGCAACAA | |
| Cre UAG18_F | TGTGCCCATTCCAGGTTGATTAGACGGAGGTATGAGGTTCGCAAGAA | To create Cre variant CreUAG18 |
| Cre UAG18_R | TTTGCCCATTACCGGTGATTGAGGTATGAGGTTCGCAAGAA | |
| MAT_F       | AGTCACATCAAGATCCGTTTATGG | Mating type verification |
| MATa_R      | GCACGGAAATATGCGGACTCTCG | |
| MATα_R      | ACTCCACTTTCAAGTAAAGTTTG | |

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