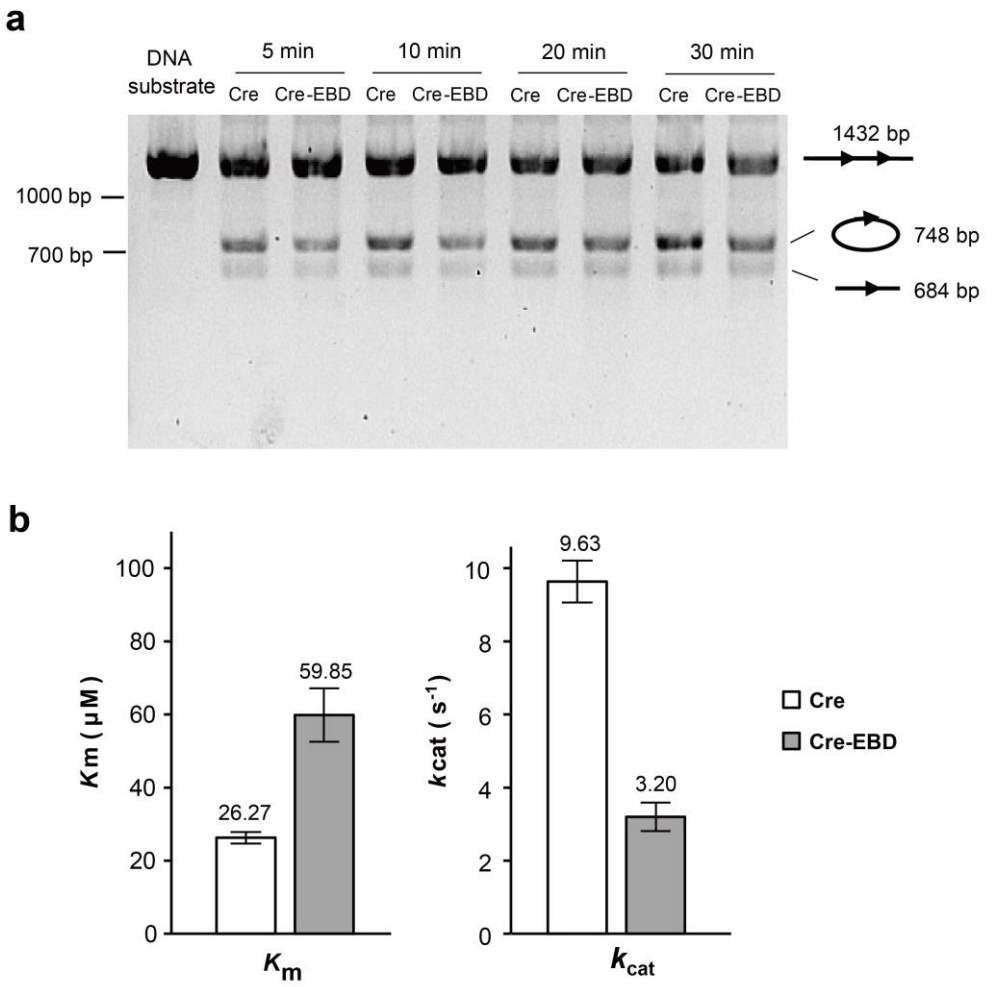
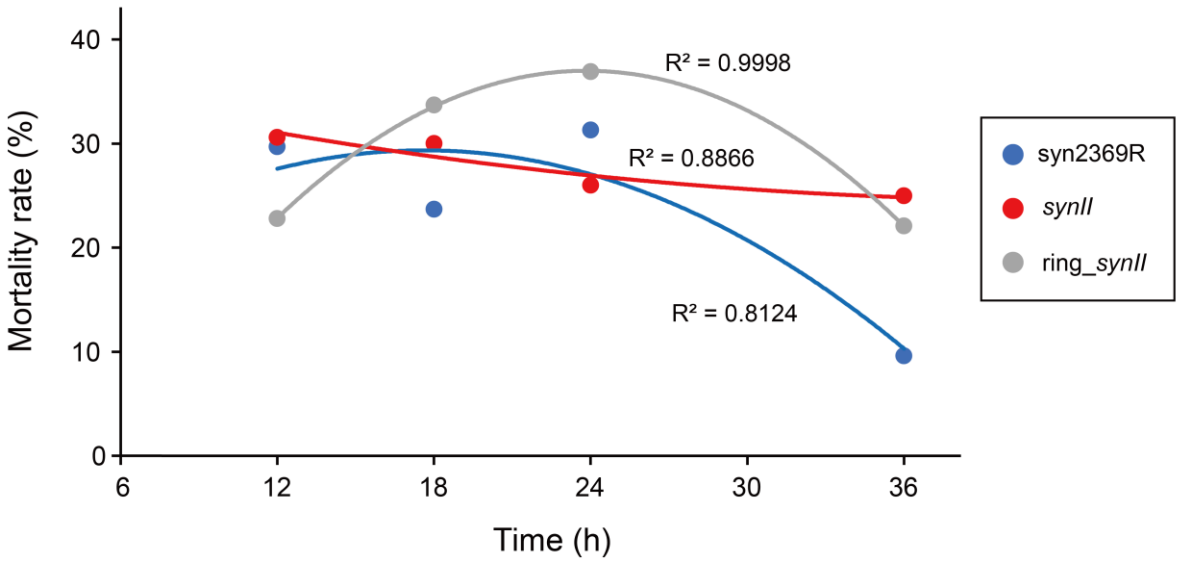
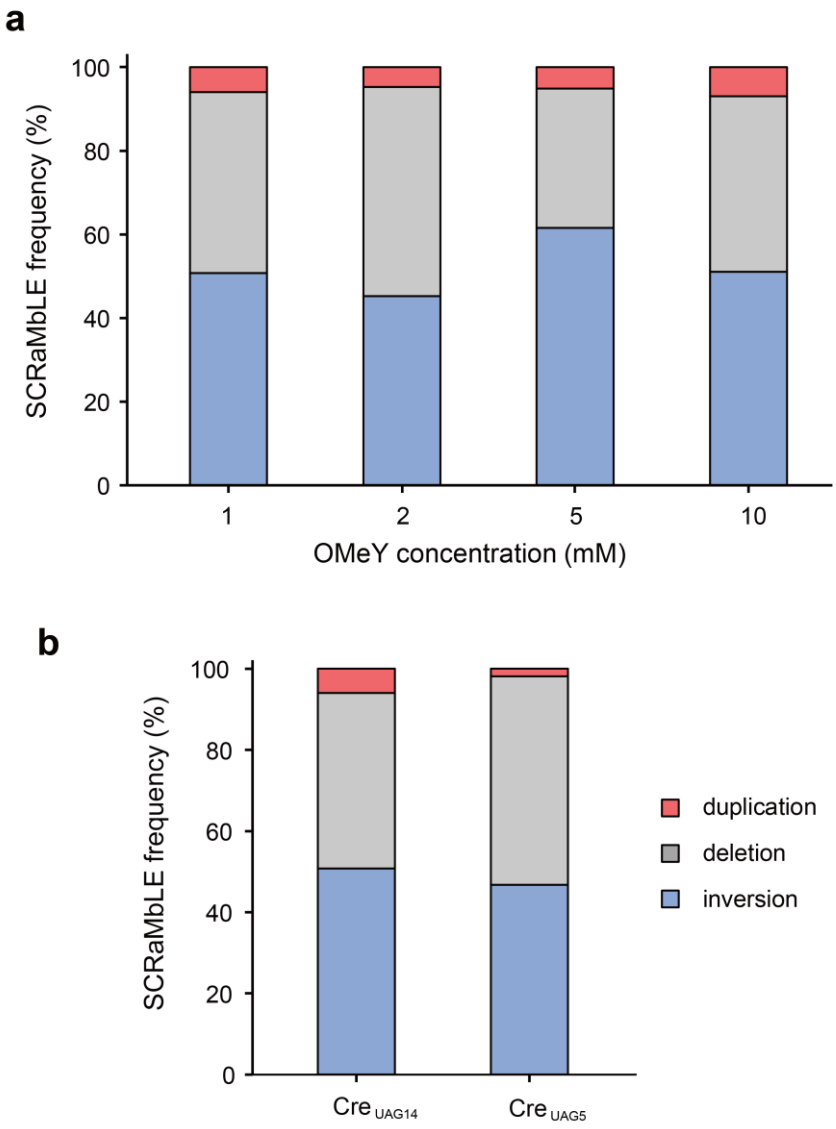


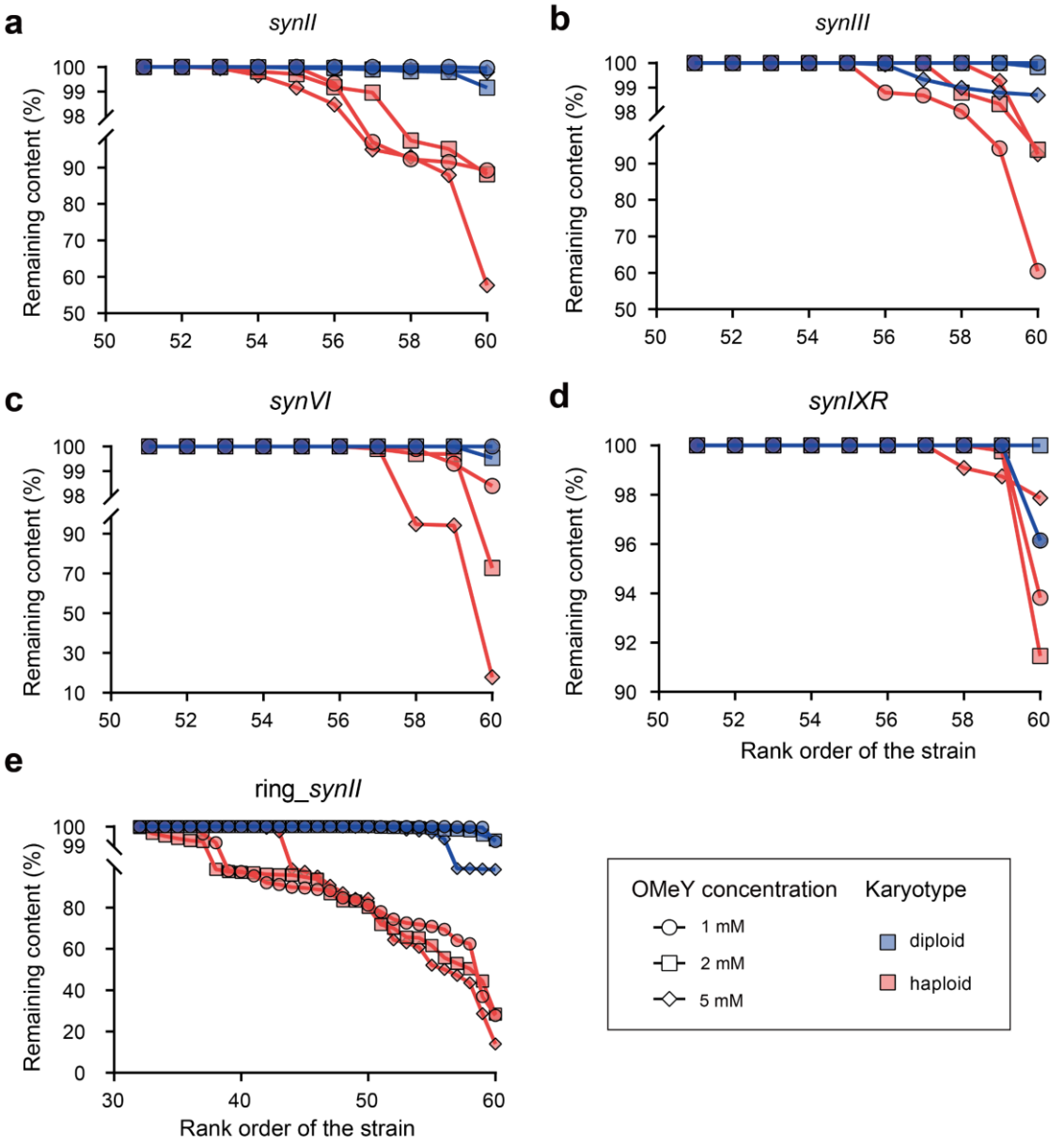
Supplementary Figure 1. *In vitro* recombination assay of purified Cre and Cre-EBD enzymes. **a** A 1432 bp linear DNA substrate containing direct repeats of the *loxP* site (triangles) is incubated with purified Cre and Cre-EBD recombinase under different time. Catalyzation of DNA substrate by Cre enzyme 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. **b** Comparison of *K*m and *k*cat between the Cre and Cre-EBD enzymes; Data represent mean results ± s.d. from three independent experiments.



Supplementary Figure 2. Investigating the mortality rate of SCRaMbLEd synthetic yeast cells. The cell culture of haploid synthetic yeast strains harboring syn2369R (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. 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.



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_{UAG14} 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_{UAG5} and Cre_{UAG14}) in the medium supplemented with 1 mM OMeY.



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.

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_{UAG5}	1 mM
2	*synII synIII synVI synIXR*	haploid	Cre_{UAG14}	1 mM
3	*synII synIII synVI synIXR*	haploid	Cre_{UAG14}	2 mM
4	*synII synIII synVI synIXR*	haploid	Cre_{UAG14}	5 mM
5	*synII synIII synVI synIXR*	haploid	Cre_{UAG14}	10 mM
6	*synII*	haploid	Cre_{UAG5}	1 mM
7	*synII*	haploid	Cre_{UAG14}	1 mM
8	*synII*	haploid	Cre_{UAG14}	2 mM
9	*synII*	haploid	Cre_{UAG14}	5 mM
10	*synII*	haploid	Cre_{UAG14}	10 mM
11	ring_*synII*	haploid	Cre_{UAG5}	1 mM
12	ring_*synII*	haploid	Cre_{UAG14}	1 mM
13	ring_*synII*	haploid	Cre_{UAG14}	2 mM
14	ring_*synII*	haploid	Cre_{UAG14}	5 mM
15	ring_*synII*	haploid	Cre_{UAG14}	10 mM
16	*synII synIII synVI synIXR*	diploid	Cre_{UAG5}	1 mM
17	*synII synIII synVI synIXR*	diploid	Cre_{UAG14}	1 mM
18	*synII synIII synVI synIXR*	diploid	Cre_{UAG14}	2 mM
19	*synII synIII synVI synIXR*	diploid	Cre_{UAG14}	5 mM
20	ring_*synII*	diploid	Cre_{UAG5}	1 mM
21	ring_*synII*	diploid	Cre_{UAG14}	1 mM
22	ring_*synII*	diploid	Cre_{UAG14}	2 mM
23	ring_*synII*	diploid	Cre_{UAG14}	5 mM

Supplemental Table 2. List of strains and plasmids used in this study.

Strain, plasmid	Description[a]	Source or reference
Strains:		
S. cerevisiae		
BY4741	*MATa* ura3Δ0 leu2Δ0 his3Δ1 met15Δ0	[1]
BY4742	*MATalpha* ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0	[1]
synII	*MATa* ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 synLYS2, *synII*	[2]
ring_*synII*	*MATa* ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 synLYS2, ring_*synII*	This study
2369R	*MATa* ura3Δ0 leu2Δ0 his3Δ1 synLYS2, *synII*, *synIII*, *synVI*, *synIXR*	This study
ring_*synII* Dip	Diploid, ring_*synII* mating with BY4742	This study
2369R Dip	Diploid, 2369R mating with BY4741	This study
Plasmids:		
pRS415	Ap^r; LEU2.Shuttle plasmid, empty vector	[3]
pRS413	Ap^r; HIS3.Shuttle plasmid, empty vector	[3]
pXF231	pRS415 LeuOmeRS pair	[4]
pXF220	pRS413 carries Cre_{UAG5}	This study
pXF238	pRS413 carries Cre_{UAG14}	This study
pSCW11-Cre-EBD	Cre fused to EBD and controlled by daughter cell-specific promoter SCW11	[5]
pLH_Scr18	Km^r; URA3. Carries terminator between LoxP before GFP	[6]
pLH_Scr19	Km^r; URA3. Carries GFP	[6]

Ap^r, ampicillin resistance; Km^r, kanamycin resistance.

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