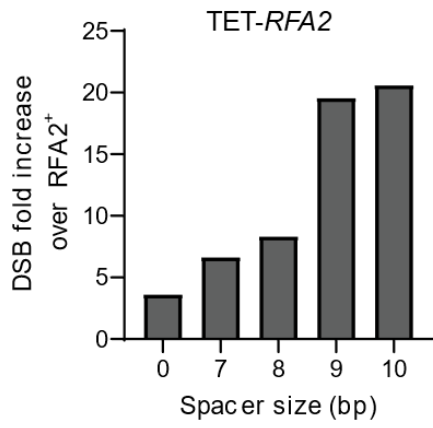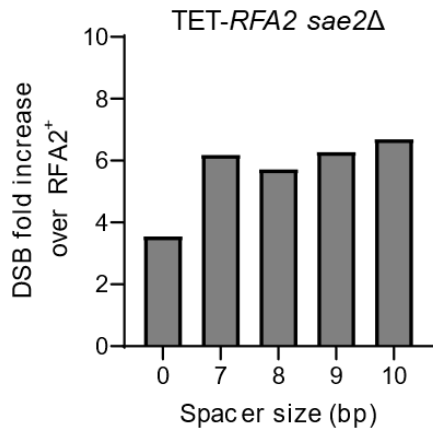**Figure S1.** Asymmetrical spacers inserted at the center of *Alu* palindromes. Related to figure 1.

The sequence of the spacer inserted into *Alu*-PAL to create *Alu*-QP used in this study is presented in red. The spacer consists of the *Sfa*NI (A) or *Bsm*I (B) restriction site (underlined sequence) plus random nucleotides.
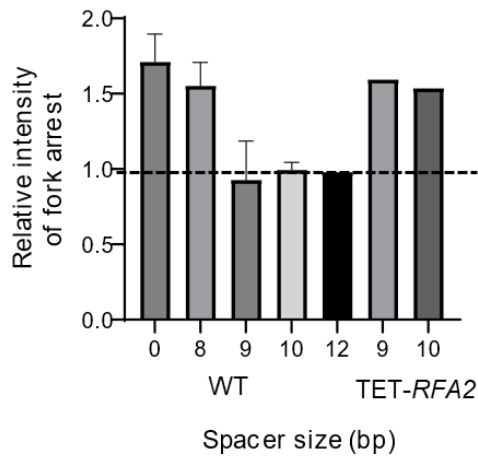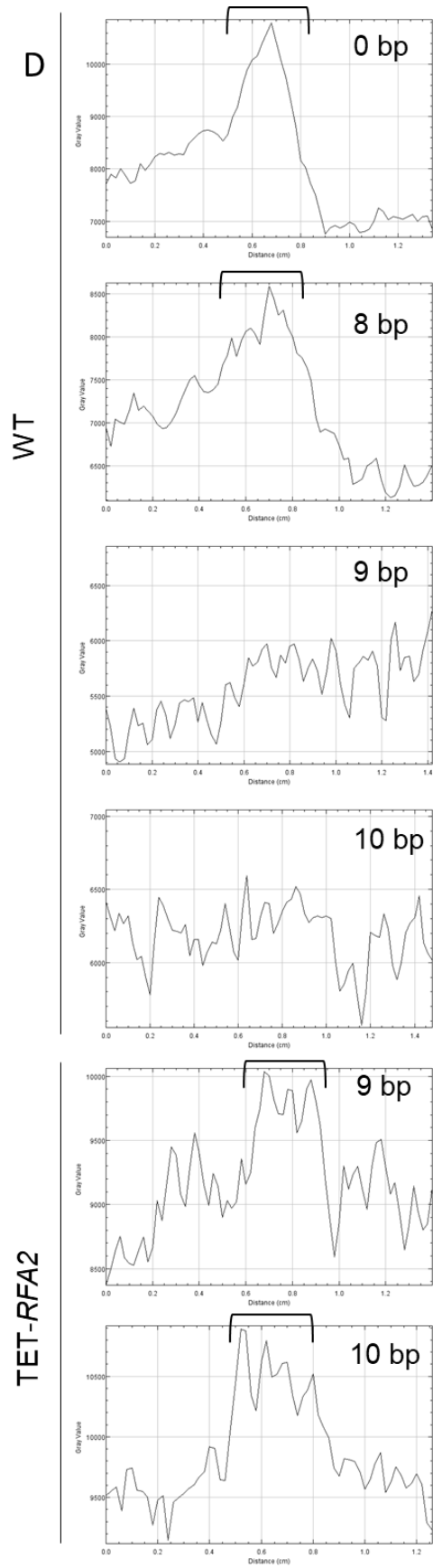
Figure S2. Effect of RPA downregulation on DSB formation at *Alu*-PAL and *Alu*-QP. Related to Figure 2.

A. and B. Data show the fold increase in DSB level in TET-*RFA2* relative to *RFA2⁺* in *WT* (A) and *sae2Δ* (B) strains. C. Quantification of the signal corresponding to site of fork arrest relative to the signal corresponding to site of unperturbed replication (downstream from the arrest). D. Densitometry analysis of the Y arc's long arm in the WT and TET-*RFA2* strains containing *Alu*-IR with indicated spacers. The presence of fork arrest is manifested as a spike in the relative signal. The bracket depicts the zone of replication arrest. Values are the mean ± standard deviation (SD).
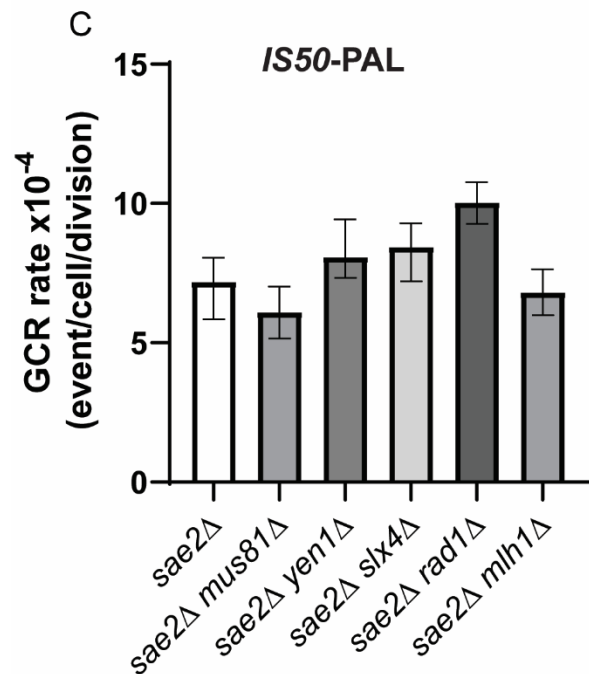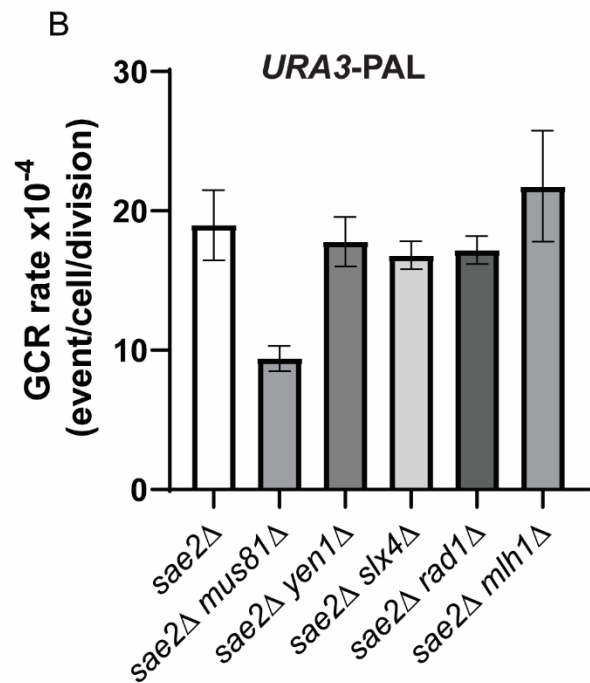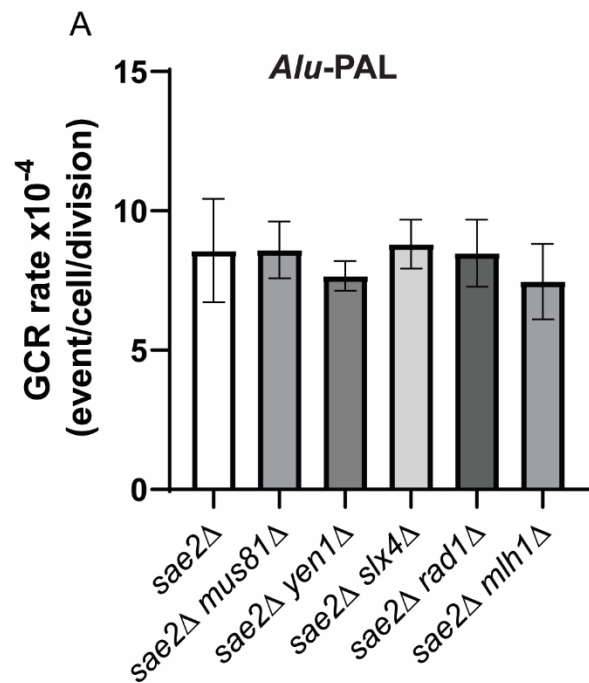
Figure S3. Effect of SSEs on palindromes-induced fragility. Related to Figure 3 and 4.

GCR rates were measured in *sae2Δ* strains deleted for either *MUS81*, *YEN1*, *SLX4*, *RAD1* or *MHL1*. A. GCR measurement at *Alu*-PAL. B. GCRs measurements at *URA3*-PAL. C. GCRs measurements at the transcribed palindrome *IS50*-PAL. Data are represented as the median value ± 95% confidence intervals.
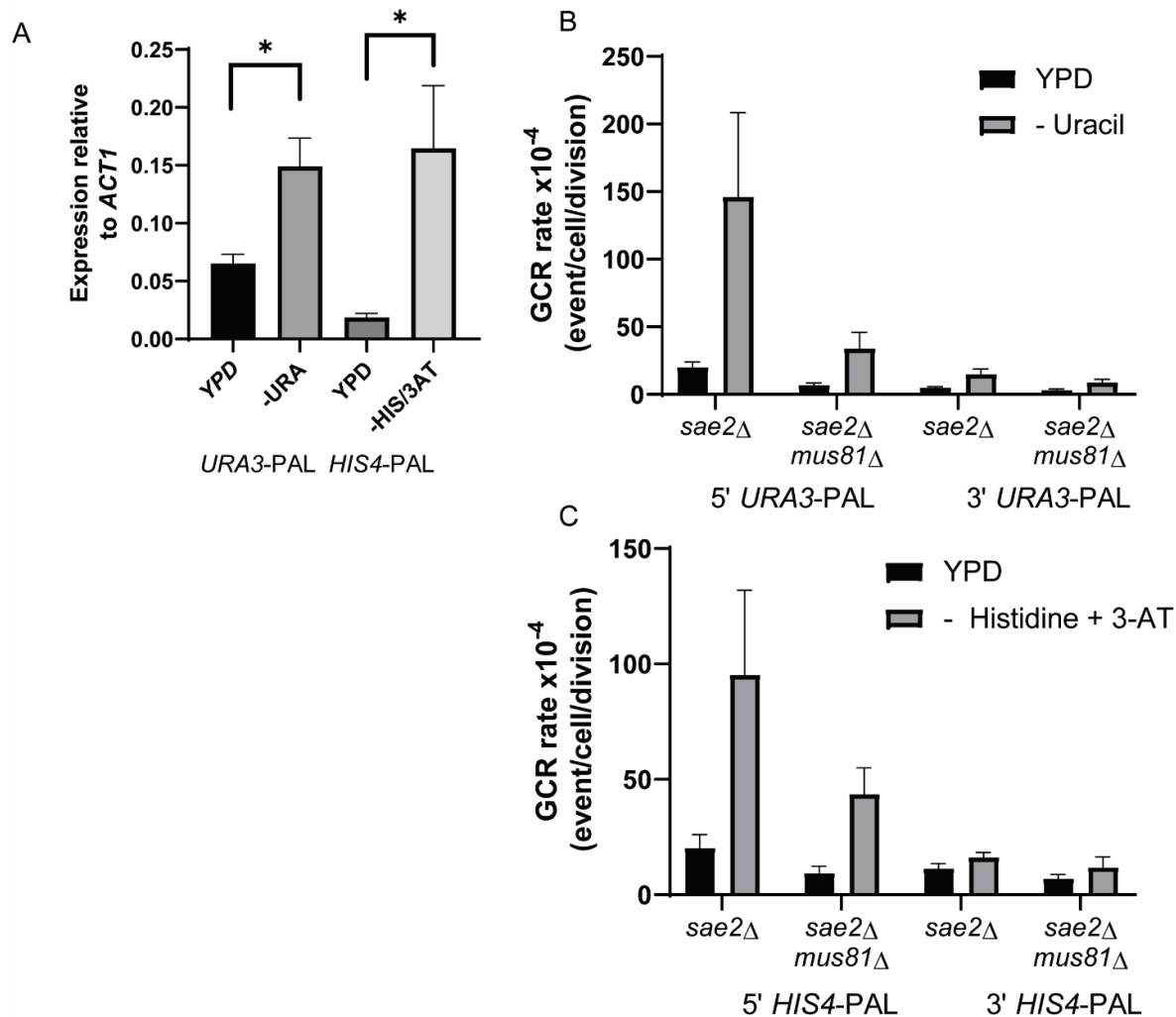
Figure S4. Effect Mus81 and transcription on GCRs induced at actively transcribed palindromes. Related to Figure 4.

A. Transcription level at *URA3* and *HIS4* measured by RT-qPCR. To analyze the effect of amino acid starvation on the transcription level, cells were cultured in YPD and uracil-depleted media to measure RNA transcript at *URA3*-PAL, and in YPD and histidine-depleted media supplemented with 3-AT to measure RNA transcript at *HIS4*-PAL. To determine the relative abundance, mRNA levels were normalized to *ACT1* and compared to the YPD control. To limit the impact of DSB formation and resection on the expression level of *URA3* and *HIS4*, the *sae2Δ mus81Δ* strain, in which the DSB level is minimal, was used. Data are the mean of 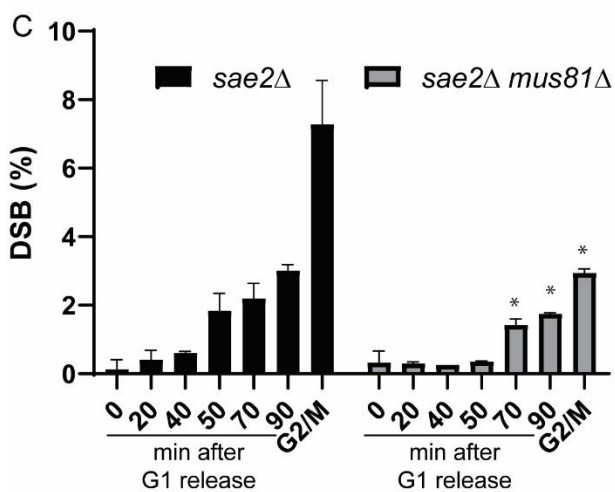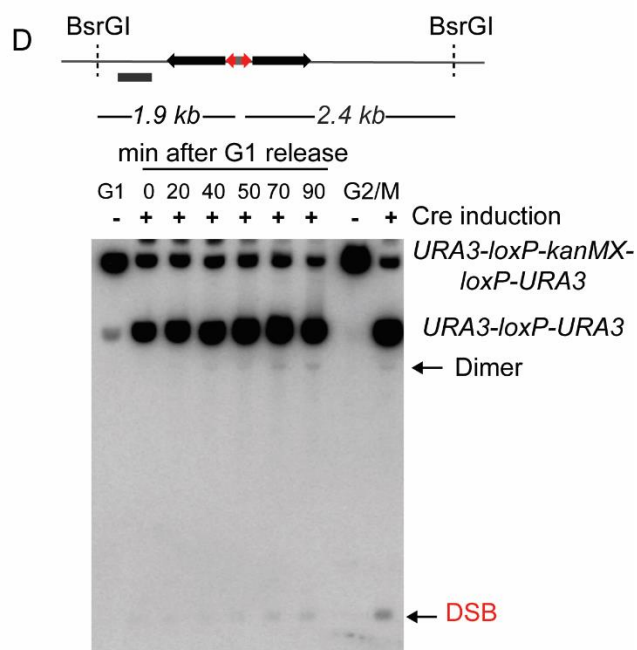at least 3 values ± standard deviation. Significance was estimated by two sample *t* tests. *p<0.004. B. GCR rates at 5' and 3' *URA3*-PAL in *sae2Δ* and *sae2Δ mus81Δ* in presence and absence of uracil in the growth media. C. GCR rates at 5' and 3' *HIS4*-PAL in *sae2Δ* and *sae2Δ mus81Δ* in presence of histidine or in media lacking histidine and supplemented with 3-AT. Data in B and C are represented as the median value ± 95% confidence intervals.

A

ATAACTTCGTATAAATGTATGCTATACGAAGTTAT   *kanMX*   ATAACTTCGTATAATGTATGCTATACGAAGTTAT

1 kb   *34 bp*   *1.5 kb*   *34 bp*   *1 kb*

URA3   *loxP*   *kanMX*   *loxP*   *URA3*

Cre expression

URA3   *loxP*   *URA3*

B

G2/M (Noco.)

90

70

50

40

20

G1 (a-Factor)

min after G1 release

400K   600K   800K

D

BsrGI   BsrGI

—*1.9 kb*— —*2.4 kb*—

min after G1 release

G1   0   20   40   50   70   90   G2/M

Cre induction

−   +   +   +   +   +   +   −   +

URA3-loxP-kanMX-loxP-URA3

URA3-loxP-URA3

← Dimer

← DSB

C

DSB (%)

■ *sae2Δ*   ▨ *sae2Δ mus81Δ*

0 20 40 50 70 90 G2/M   0 20 40 50 70 90 G2/M

min after G1 release   min after G1 release

E

cdc28-as1

DSB (%)

■ *sae2Δ*   ▨ *sae2Δ mus81Δ*

Nocodazole   1-NM-PP1

G2/M arrested cells

Figure S5. Conditional *URA3*-quasi palindrome to study DSB formation through the cell cycle. Related to Figure 5.

A. Diagram of the conditional *URA3*-QP system showing the *loxP* sequence. B. Representative diagram of DNA content analysis by FACS in G1 and G2/M arrested cells. Cells harboring the conditional *URA3*-QP were synchronized in G1 with alpha-factor. After Cre expression induction, cells were released into S phase. Progression through the cell cycle was analyzed at the same time points (minutes after G1 release) at which DSB analysis was performed. Cells were synchronized in G2/M with nocodazole. C. Quantification of DSBs in *sae2Δ* and *sae2Δ mus81Δ* presented in figure 5B. Timepoints after G1 release are indicated. G2/M corresponds to nocodazole arrested cells. Values are the mean ± standard deviation (SD). Significance was estimated by *t* test. * $p < 0.05$. D. Representative analysis of telomere-proximal DSB formation during the cell cycle in *sae2Δ*. Detection was carried out as described in Figure 5A-B. Genomic DNA embedded in agarose plugs was digested with *Bsr*GI. The position of the resulting quasi-palindrome (*URA3-loxP-URA3*) relative to the *Bsr*GI restriction site is represented. Southern hybridization was performed using a *LYS2*-specific probe located on the telomere-proximal side of the IR (black triangle) and revealed four different fragments. (i) a 5.8 kb fragment corresponding to the unbroken chromosome V containing the intact *URA3-loxP-kanMX-loxP-URA3*. This fragment is predominant when Cre is not expressed (Cre induction -). (ii) a 4.3 kb fragment corresponding to the unbroken chromosome V containing the *URA3-loxP-URA3* quasi-palindrome. This fragment becomes predominant upon Cre expression (Cre induction +). (iii) a 3.8 kb fragment corresponding to the dimer and (vi) a 1.9 kb fragment corresponding the break. E. Quantification of DSBs in *cdc28*-as1 *sae2Δ* and *cdc28*-as1 *sae2Δ mus81Δ* presented in figure 5C. Cells were arrested in G2/M with nocodazole or 1-NM-PP1. Values are the mean ± standard deviation (SD). Significance was estimated by *t* test. * $p < 0.05$.

Table S1. *Alu*-IR spacer length determines GCR rate

Alu-IR	GCR rate (x10⁻⁷)	
	WT	*sae2Δ*
Alu-PAL	7000 (5017-9943)[a]	7829 (6560-9459)
Alu-QP5	6375 (4549-8924)	ND[b]
Alu-QP6	7015 (4984-7732)	ND
Alu-QP7	4449 (2853-5390)	5549 (2993-5699)
Alu-QP8	3606 (4784-6584)	4603 (4062-5529)
Alu-QP9	1670 (1032-4064)	2141 (1660-2722)
Alu-QP10	577 (448-806)	2072 (1506-2746)
Alu-QP11	603 (256-1116)	1830 (1506-2010)

[a] Numbers in parenthesis correspond to the 95% confidence interval.

[b] Not determined.

Table S2. Effect of palindromic sequences on GCRs is not sequence dependent

Alu-IR (BsmI spacer)	GCR rate (x10⁻⁷)	
	WT	*sae2Δ*
Alu-QP8	2860 (2421-3358) [a]	3951 (2992-4346)
Alu-QP9	1974 (1561-2990)	3893 (3451-5490)
Alu-QP10	1095 (944-1520)	3288 (2520-3632)

[a] Numbers in parenthesis correspond to the 95% confidence interval.

Table S3. Strains used in this study

Strain number	Genotype*	IR as referred to in the text
KT849	lys2::Alu-core	
YKL719	lys2::Alu-SwaI	Alu-PAL
YKL3210	lys2::Alu-SfanI-12bp	Alu-QP12
YKL3243	lys2::Alu-SfanI-11bp	Alu-QP11
YKL3241	lys2::Alu-SfanI-10bp	Alu-QP10
YKL3361	lys2::Alu-SfanI-9bp	Alu-QP9
YKL3209	lys2::Alu-SfanI-8bp	Alu-QP8
YKL3206	lys2::Alu-SfanI-7bp	Alu-QP7
YKL3238	lys2::Alu-SfanI-6bp	Alu-QP6
YKL3304	lys2::Alu-SfanI-5bp	Alu-QP5
YKL4074	lys2::Alu-BsmI-10bp	Alu-QP10
YKL4070	lys2::Alu-BsmI-9bp	Alu-QP9
YKL4066	lys2::Alu-BsmI-8bp	Alu-QP8
YKL4062	lys2::Alu-BsmI-7bp	Alu-QP7
YKL733	lys2 ::Alu-SwaI Δsae2	Alu-PAL
YKL3290	lys2::Alu-SfanI-12bp sae2::hphMX	Alu-QP12
YKL3288	lys2::Alu-SfanI-11bp sae2::hphMX	Alu-QP11
YKL3286	lys2::Alu-SfanI-10bp sae2::hphMX	Alu-QP10
YKL3364	lys2::Alu-SfanI-9bp sae2::hphMX	Alu-QP9
YKL3282	lys2::Alu-SfanI-8bp sae2::hphMX	Alu-QP8
YKL3987	lys2::Alu-SfanI-7bp sae2::hphMX	Alu-QP7
YKL4090	lys2::Alu-BsmI-10bp sae2::hphMX	Alu-QP10
YKL4086	lys2::Alu-BsmI-9bp sae2::hphMX	Alu-QP9
YKL4082	lys2::Alu-BsmI-8bp sae2::hphMX	Alu-QP8
YKL4078	lys2::Alu-BsmI-7bp sae2::hphMX	Alu-QP7
YKL3994	lys2 ::Alu-SwaI rfa2::TET-RFA2:kanMX4	Alu-PAL
YKL4022	lys2::Alu-SfanI-11bp rfa2::TET-RFA2:kanMX4	Alu-QP11
YKL4018	lys2::Alu-SfanI-10bp rfa2::TET-RFA2:kanMX4	Alu-QP10
YKL4014	lys2::Alu-SfanI-9bp rfa2::TET-RFA2:kanMX4	Alu-QP9
YKL4010	lys2::Alu-SfanI-8bp rfa2::TET-RFA2:kanMX4	Alu-QP8
YKL4006	lys2::Alu-SfanI-7bp rfa2::TET-RFA2:kanMX4	Alu-QP7
YKL4026	lys2 ::Alu-PAL sae2::hphMX rfa2::TET-RFA2:kanMX4	Alu-PAL
YKL4058	lys2::Alu-SfanI-12bp sae2::hphMX rfa2::TET-RFA2:kanMX4	Alu-QP12
YKL4054	lys2::Alu-SfanI-11bp sae2::hphMX rfa2::TET-RFA2:kanMX4	Alu-QP11
YKL4050	lys2::Alu-SfanI-10bp sae2::hphMX rfa2::TET-RFA2:kanMX4	Alu-QP10
YKL4046	lys2::Alu-SfanI-9bp sae2::hphMX rfa2::TET-RFA2:kanMX4	Alu-QP9

YKL4042	*lys2::Alu-SfanI-8bp sae2::hphMX rfa2::TET-RFA2:kanMX4*	*Alu*-QP8
YKL4038	*lys2::Alu-SfanI-7bp sae2::hphMX rfa2::TET-RFA2:kanMX4*	*Alu*-QP7
YKL1261	*lys2::Alu-SwaI mre11::kanMX4*	*Alu*-PAL
YKL1369	*lys2::Alu-SwaI sae2::hphMX mus81::kanMX4*	*Alu*-PAL
YKL2610	*lys2::Alu-SwaI sae2::hphMX yen1::kanMX4*	*Alu*-PAL
YKL1395	*lys2::Alu-SwaI sae2::hphMX slx4::natMX*	*Alu*-PAL
KT1255	*lys2::Alu-SwaI sae2::TRP1 rad1::kanMX*	*Alu*-PAL
YKL2607	*lys2::Alu-SwaI sae2::hphMX mlh1::URA3*	*Alu*-PAL
YKL854	*lys2::IS50-PAL*	*IS50*-PAL
YKL918	*lys2::IS50-PAL sae2::TRP1*	*IS50*-PAL
YKL2613	*lys2::IS50-PAL sae2::TRP1 mus81::kanMX4*	*IS50*-PAL
YKL2619	*lys2::IS50-PAL sae2::TRP1 yen1::kanMX4*	*IS50*-PAL
YKL2629	*lys2::IS50-PAL sae2::TRP1 slx4::natMX*	*IS50*-PAL
KT1259	*lys2::IS50-PAL sae2::TRP1 rad1::kanMX*	*IS50*-PAL
YKL2616	*lys2::IS50-PAL sae2::TRP1 mlh1::URA3*	*IS50*-PAL
YKL776	*lys2::5'URA3-PAL*	*URA3*-PAL
YKL936	*lys2::5'URA3-PAL sae2::TRP1*	*URA3*-PAL
YKL1229	*lys2::5'URA3-PAL sae2::TRP1 mus81::kanMX4*	*URA3*-PAL
YKL2663	*lys2::5'URA3-PAL sae2::TRP1 yen1::kanMX4*	*URA3*-PAL
YKL1360	*lys2::5'URA3-PAL sae2::TRP1 slx4::natMX*	*URA3*-PAL
KT1263	*lys2::5'URA3-PAL-SwaI sae2::hphMX rad1::kanMX*	*URA3*-PAL
YKL2650	*lys2::5'URA3-PAL-SwaI sae2::TRP1 mlh1::URA3*	*URA3*-PAL
KT905	*lys2::3'URA3-PAL sae2::TRP1*	*3'URA3*-PAL
KT917	*lys2::3'URA3-PAL sae2::TRP1 mus81::natMX*	*3'URA3*-PAL
YKL2869	*lys2::5'HIS4-PAL sae2::TRP1 his4::natMX*	*5'HIS4*-PAL
KT925	*lys2::5'HIS4-PAL sae2::TRP1 mus81::kanMX4 his4::natMX*	*5'HIS4*-PAL
KT1218	*lys2::3'HIS4-PAL sae2::hphMX his4::natMX*	*3'HIS4*-PAL
KT1224	*lys2::3'HIS4-PAL sae2::hphMX mus81::kanMX4 his4::natMX*	*3'HIS4*-PAL
YKL1024	*lys2::URA3-loxP-kanMX-loxP-URA3 sae2::TRP1 leu2-3,112::GAL-Cre-EBD*	*Conditional URA3*-QP
YKL1053	*lys2::URA3-loxP-kanMX-loxP-URA3 sae2::TRP1 mus81::natMX leu2-3,112::GAL-Cre-EBD*	*Conditional URA3*-QP
YKL1109	*lys2::URA3-loxP-kanMX-loxP-URA3 sae2::TRP1 leu2-3,112::GAL-Cre-EBD cdc28::cdc28-as1*	*Conditional URA3*-QP
KT1383	*lys2::URA3-loxP-kanMX-loxP-URA3 sae2::TRP1 mus81::kanMX leu2-3,112::GAL-Cre-EBD cdc28::cdc28-as1*	*Conditional URA3*-QP

* All the strains share the same genetic background: *MATα bar1Δ his7-2 trp1Δ ura3Δ leu2-3,112 ade2Δ lys2Δ cup1Δ yhr054cΔ cup2Δ V34205::ADE2lys2::IR V29616::CUP1.*

Table S4. Primers used in this study

Name	Sequence	Purpose
ku-alu51	CCTCGGCCTCCCAAAGTGCTGGGATTACAGGCGTGAGCCACCGCGCCCGGCCGAGCTCGTTTTCGACACTGG	To insert CORE between Alu-IRs
ku-alu-31	CCTCCCAAAGTGCTGGGATTACAGGCGTGAGCCACCGCGCCCGGCCTCCTTACCATTAAGTTGATC	
Alu-SwaI	GCTGGGATTACAGGCGTGAGCCACCGCGCCCGGCCATTTAAATGGCCGGGCGCGGTGGCTCACGCCTGTAATCCCAGCA	To replace core with the spacer (blue sequence for perfect palindrome and red for quasi-palindromes)
Alu-SfaNI-12bp	AAAGTGCTGGGATTACAGGCGTGAGCCACCGCGCCCGGCCGCATCCCAACTGGGCCGGGCGCGGTGGCTCACGCCTGTAATCCCAGCACTTT	
Alu-SfaNI-11bp	AAAGTGCTGGGATTACAGGCGTGAGCCACCGCGCCCGGCCGCATCCAACTGGGCCGGGCGCGGTGGCTCACGCCTGTAATCCCAGCACTTT	
Alu-SfaNI-10bp	AAAGTGCTGGGATTACAGGCGTGAGCCACCGCGCCCGGCCGCATCAACTGGGCCGGGCGCGGTGGCTCACGCCTGTAATCCCAGCACTTT	
Alu-SfaNI-9bp	AAAGTGCTGGGATTACAGGCGTGAGCCACCGCGCCCGGCCGCATCACTGGGCCGGGCGCGGTGGCTCACGCCTGTAATCCCAGCACTTT	
Alu-SfaNI-8bp	AAAGTGCTGGGATTACAGGCGTGAGCCACCGCGCCCGGCCGCATCCTGGGCCGGGCGCGGTGGCTCACGCCTGTAATCCCAGCACTTT	
Alu-SfaNI-7bp	AAAGTGCTGGGATTACAGGCGTGAGCCACCGCGCCCGGCCGCATCTGGGCCGGGCGCGGTGGCTCACGCCTGTAATCCCAGCACTTT	
Alu-SfaNI-6bp	AAAGTGCTGGGATTACAGGCGTGAGCCACCGCGCCCGGCCGCATCGGGCCGGGCGCGGTGGCTCACGCCTGTAATCCCAGCACTTT	
Alu-SfaNI-5bp	AAAGTGCTGGGATTACAGGCGTGAGCCACCGCGCCCGGCCGCATCGGCCGGGCGCGGTGGCTCACGCCTGTAATCCCAGCACTTT	
Alu-BsmI-10bp	AAAGTGCTGGGATTACAGGCGTGAGCCACCGCGCCCGGCCGAATGCCAGTGGCCGGGCGCGGTGGCTCACGCCTGTAATCCCAGCACTTT	
Alu-BsmI-9bp	AAAGTGCTGGGATTACAGGCGTGAGCCACCGCGCCCGGCCGAATGCAGTGGCCGGGCGCGGTGGCTCACGCCTGTAATCCCAGCACTTT	
Alu-BsmI-8bp	AAAGTGCTGGGATTACAGGCGTGAGCCACCGCGCCCGGCCGAATGCGTGGCCGGGCGCGGTGGCTCACGCCTGTAATCCCAGCACTTT	
Alu-BsmI-7bp	AAAGTGCTGGGATTACAGGCGTGAGCCACCGCGCCCGGCCGAATGCTGGCCGGGCGCGGTGGCTCACGCCTGTAATCCCAGCACTTT	

S2870	CTGACGACTCTCAGTTTACCAATGT	Telomere proximal probe for Alu-PAL
O3138	GAGAGGGGTACGAACTTGGTAGCGTCTCA	
S3630	CTTCTACTCTTGACACTGAATACTAC	ARS-proximal probe
O3970	TCTACTGGAACCATATTCACGGA	
S2360	TTATCTACCAAACGGTGACTG	Telomere proximal probe for URA3-PAL
O2600	GTTTCAACCTCCTTTGGAAC	
URA3-rt5	GAACGTGCTGCTACTCATC	RT-qPCR at URA3-PAL
URA3-rt3	ACTCCAGTAATTCCTTGGTG	
HIS4-rt5	GTTGTTGTGGAAGAGAACGG	RT-qPCR at HIS4-PAL
HIS4-rt3R	TTGGTCGACAACTAGGGTGG	
ACT1-rt5	AACCGCTGCTCAATCTTC	RT-qPCR at ACT1
ACT1-rt3R	CAATACCGGCAGATTCCAA	