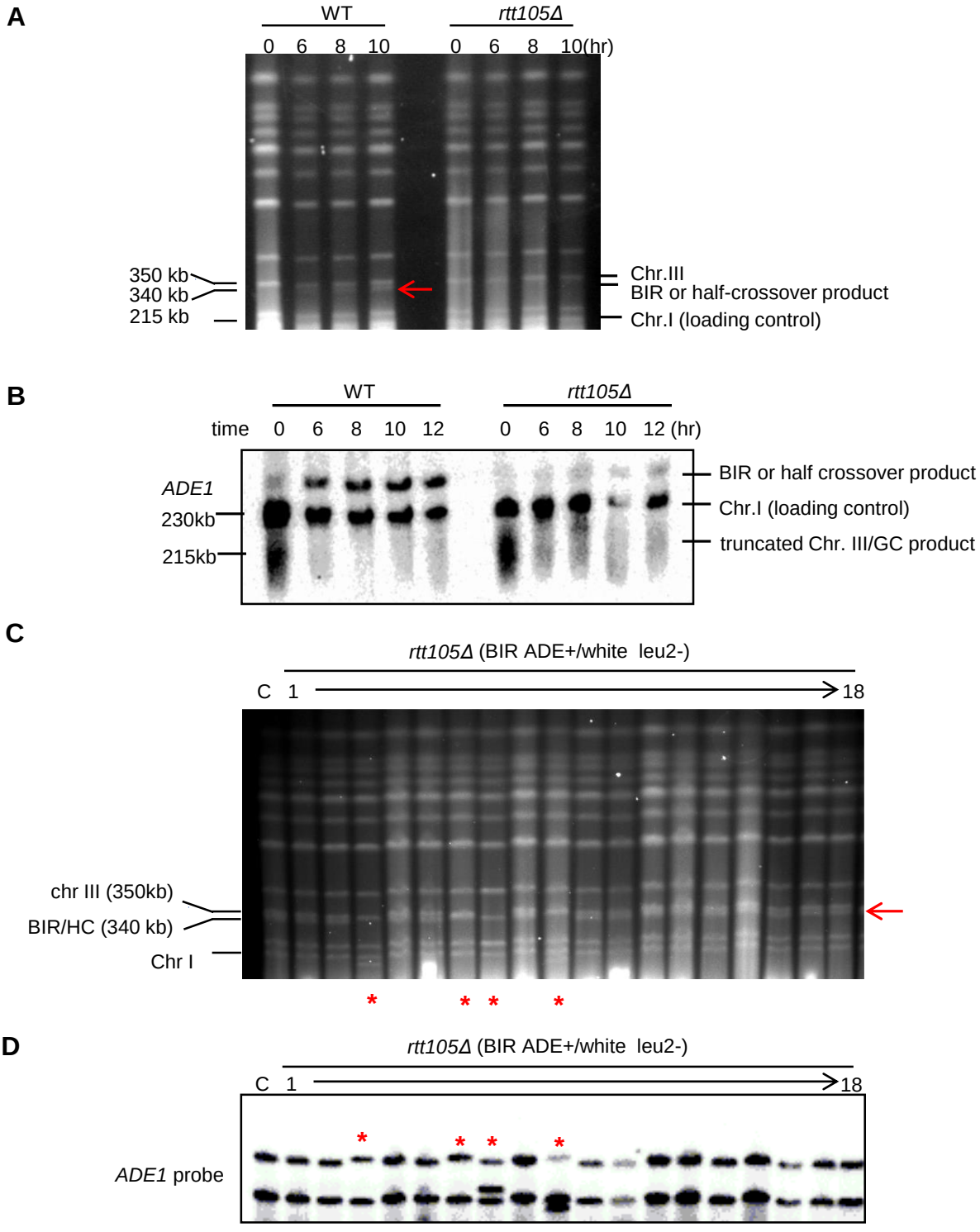
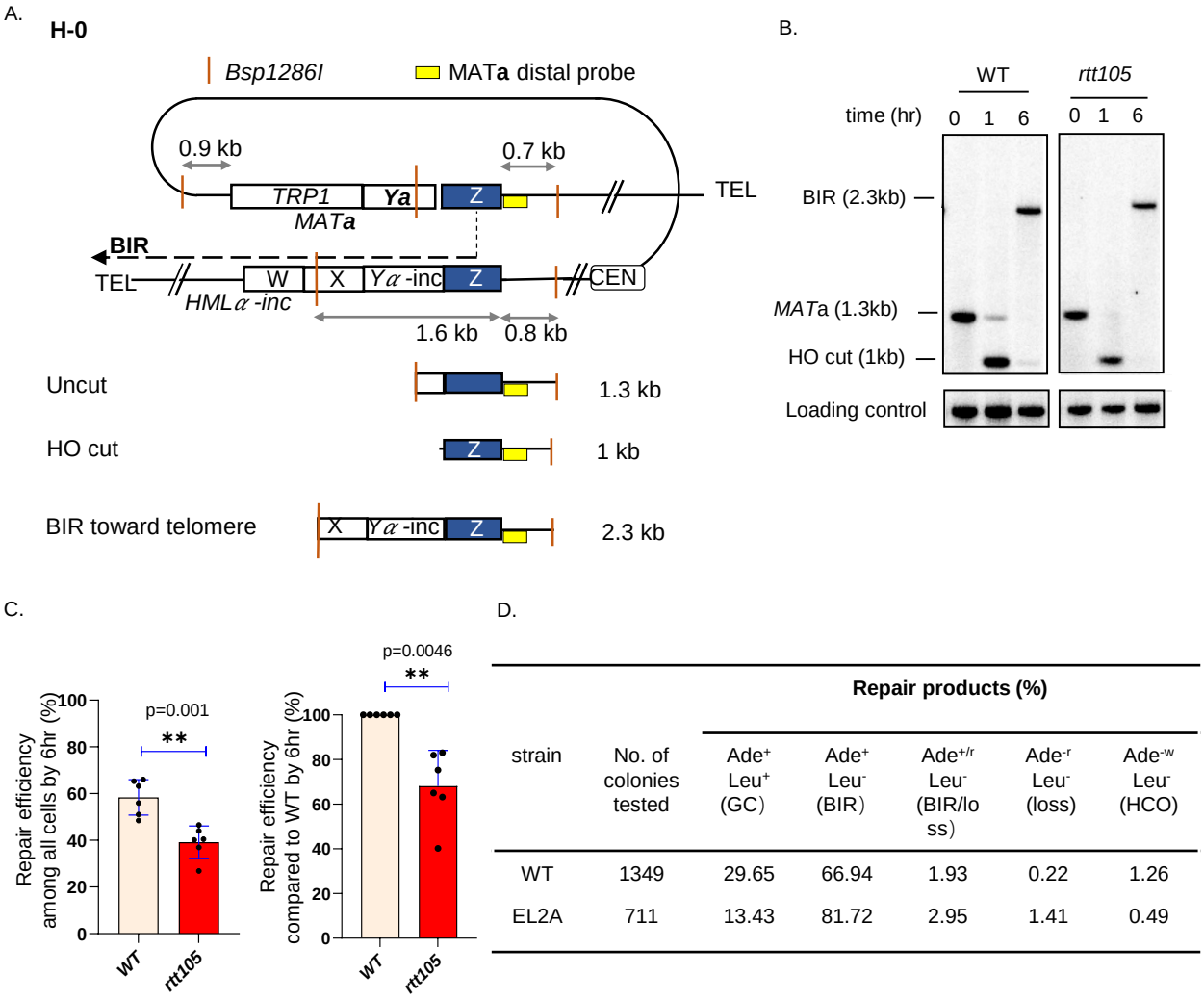


Fig. S1. Analysis of BIR kinetics and repair products. A, BIR kinetics revealed by pulsed-field gel electrophoresis (PFGE) for indicated cells. The BIR product is indicated by the arrow**.** B, Southern blot analysis of BIR kinetics for the WT and mutant cells. *ADE1* was used as probe. C-D, PFGE and Southern blot analysis of recombination products from Ade^{+}, NAT^{R} Leu^{-} colonies of *rtt105Δ* cells. ***** denotes recombination events associated with the rearrangement of the recipient chromosome.



D.

strain	No. of colonies tested	Repair products (%)				
		Ade^{+} Leu^{+} (GC)	Ade^{+} Leu^{-} (BIR)	Ade$^{+/r}$ Leu^{-} (BIR/loss)	Ade^{-r} Leu^{-} (loss)	Ade^{-w} Leu^{-} (HCO)
WT	1349	29.65	66.94	1.93	0.22	1.26
EL2A	711	13.43	81.72	2.95	1.41	0.49

Fig. S2. Analysis of BIR repair. A, Model showing the H-0 BIR system(intra chromosome BIR). B, Southern blot analysis of BIR kinetics for the WT and mutant cells in the H-0 system. Samples were collected 0, 1, and 6 hr after DSB induction. *MATa* was used as probe. C, Plot showing the quantification of BIR repair efficiency in B. Repair efficiency at 6 hr after DSB induction was calculated as the percentage of normalized pixel intensity of the BIR product band at 6 hrs compared to the normalized parental bands at 0 hr. D, Table showing repair outcomes for the WT or *rtt105-EL2A* cells in the allelic BIR strains (AM1003 background). GC: gene conversion, HCO: half-crossover. Loss: chromosome loss. Cells cultured in the pre-induction liquid media were plated on YEP-Galactose media to induce DSBs. Colonies formed were replica plated on Leu^{-} or Ade^{-} dropout media. The frequency for each category of repair outcome was calculated based on the percentage of colonies carrying markers specific for each repair outcome.

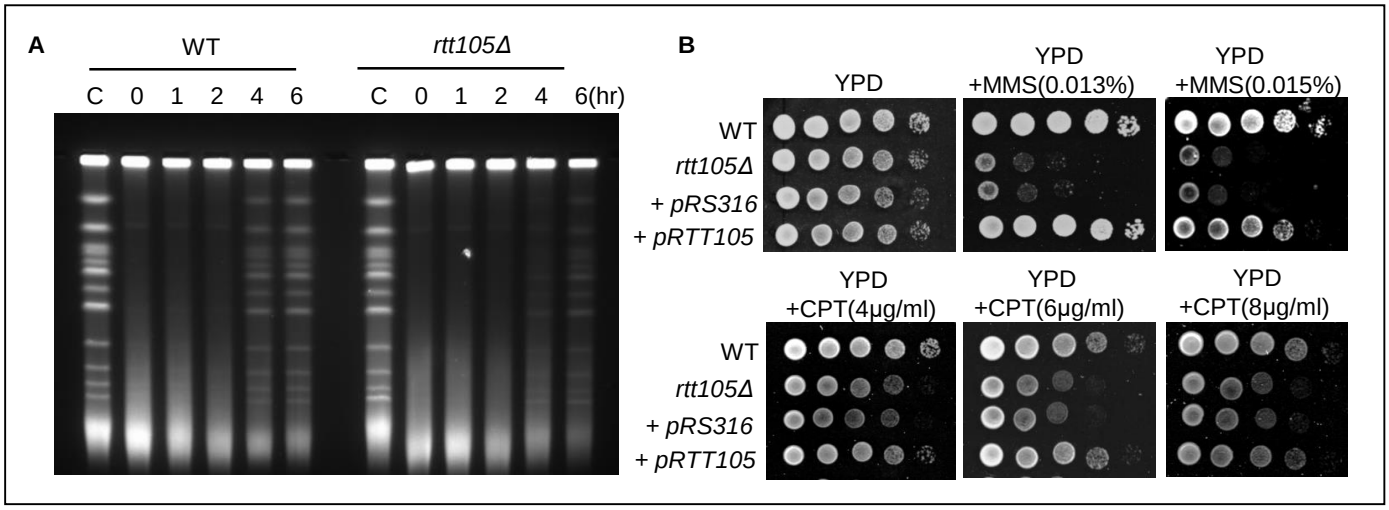
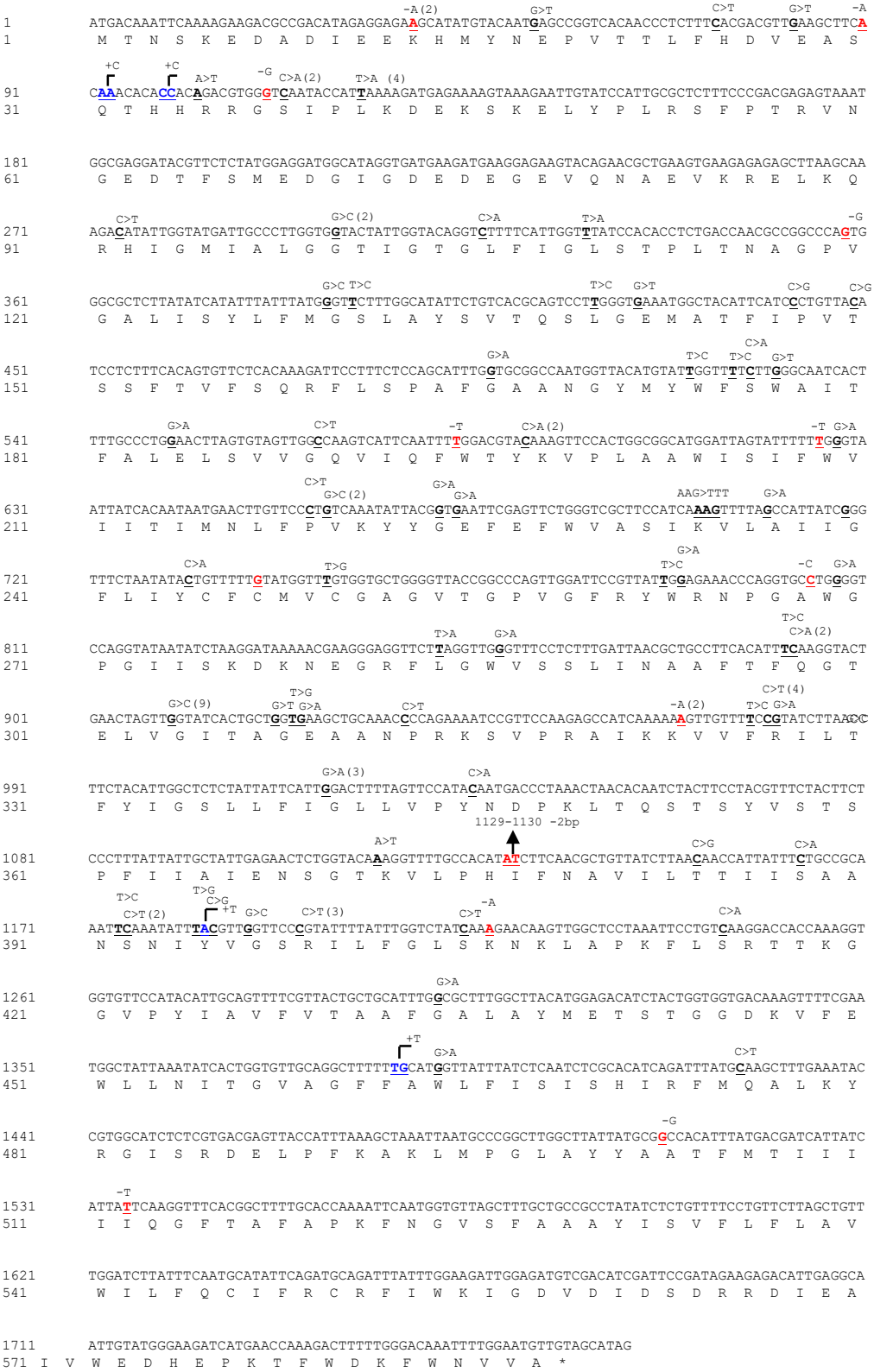
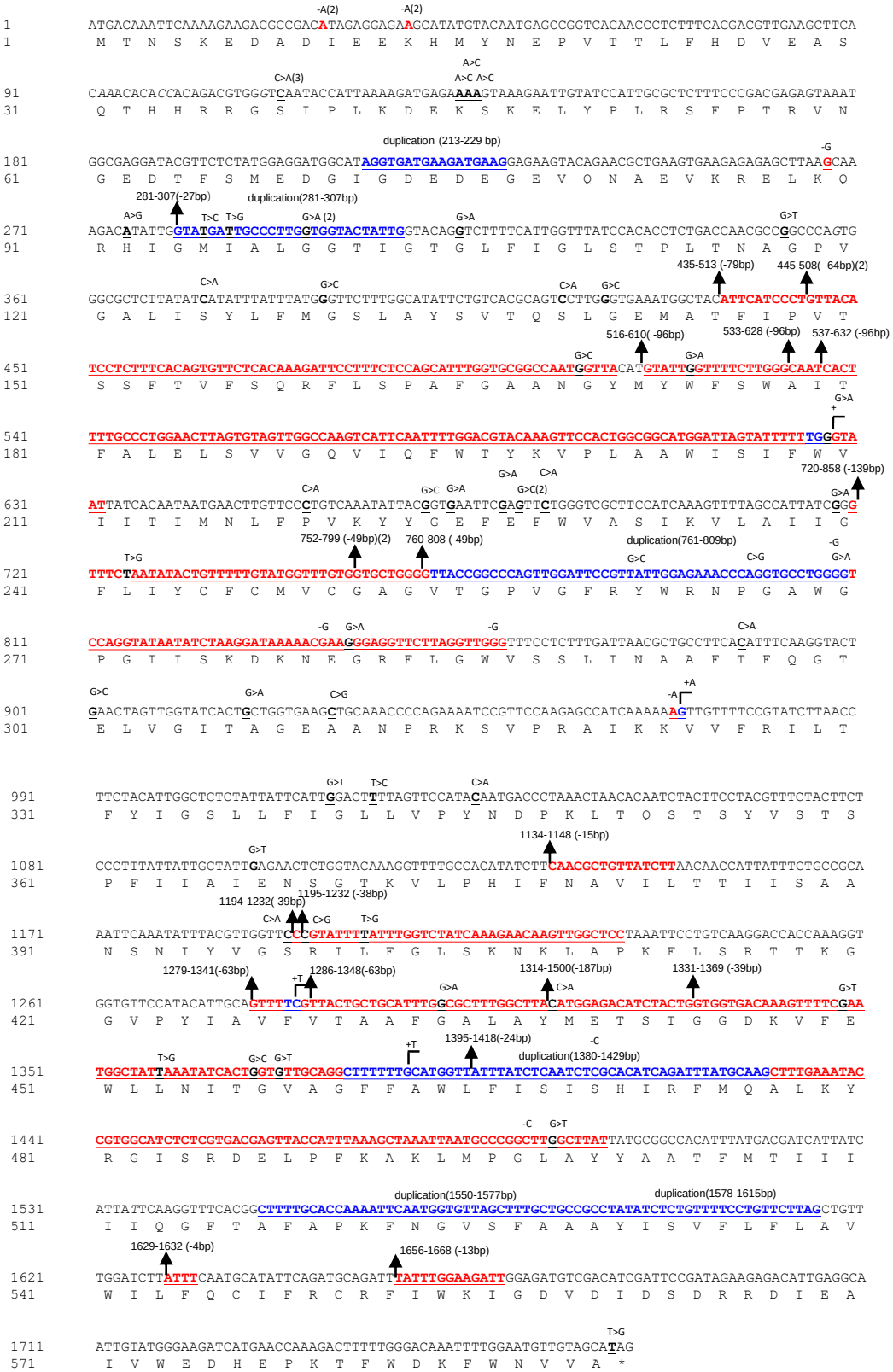


Fig. S3. Rtt105 promotes the DNA damage response and recovery. A, Analysis of chromosome integrity by PFGE. Cells were collected at different time points during the recovery from short MMS treatment (0.03%, 1hr). B, DNA damage sensitivity test for indicated strains at indicated drug concentrations.

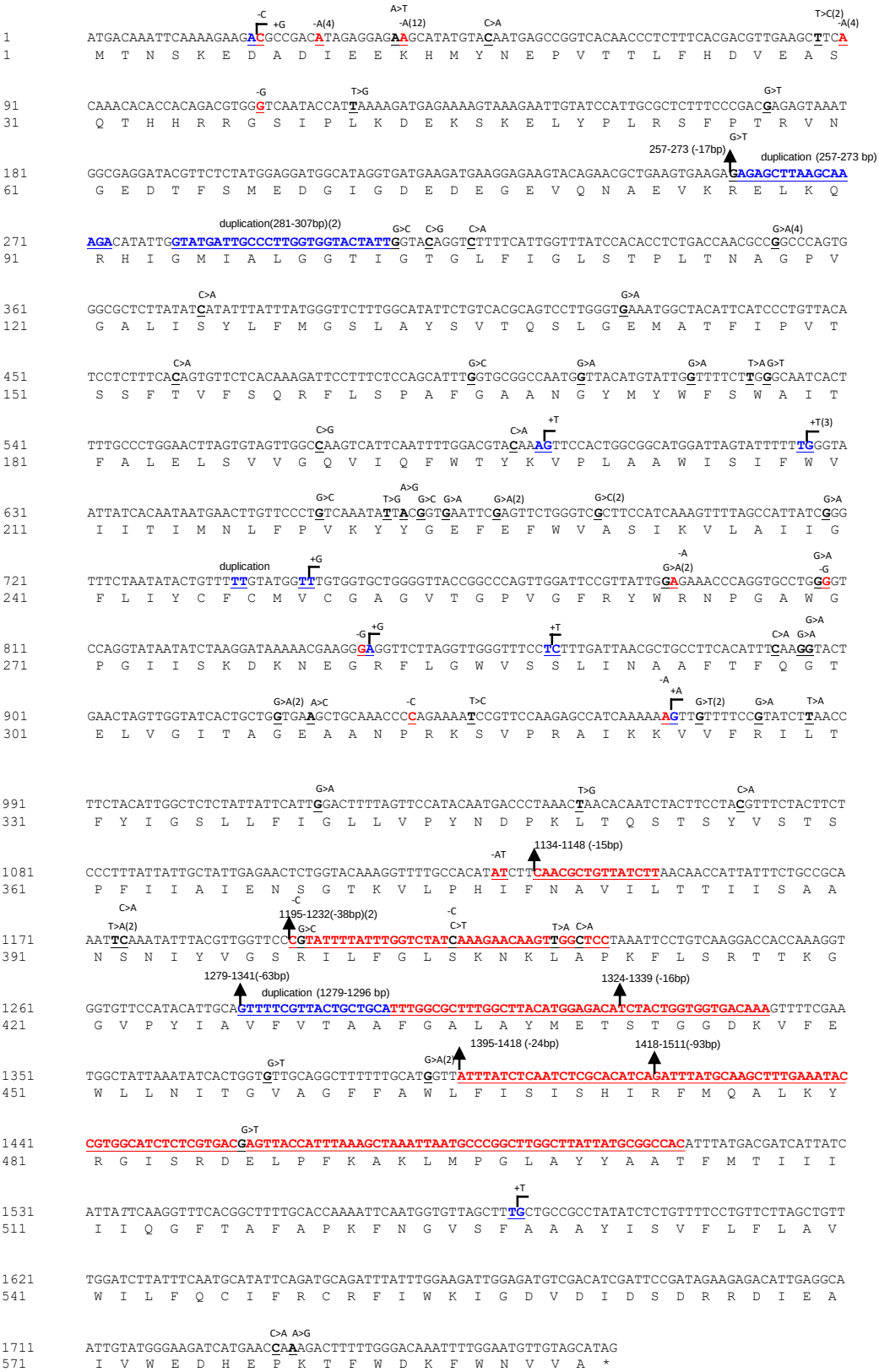
Mutation spectrum for CanR colonies derived from WT cells



Mutation spectrum for CanR colonies derived from *rtt105Δ* cells



Mutation spectrum for CanR colonies derived from *rtt105-EL2A* cells



Mutation spectrum for CanR colonies derived from *rfa1-V106A* cells

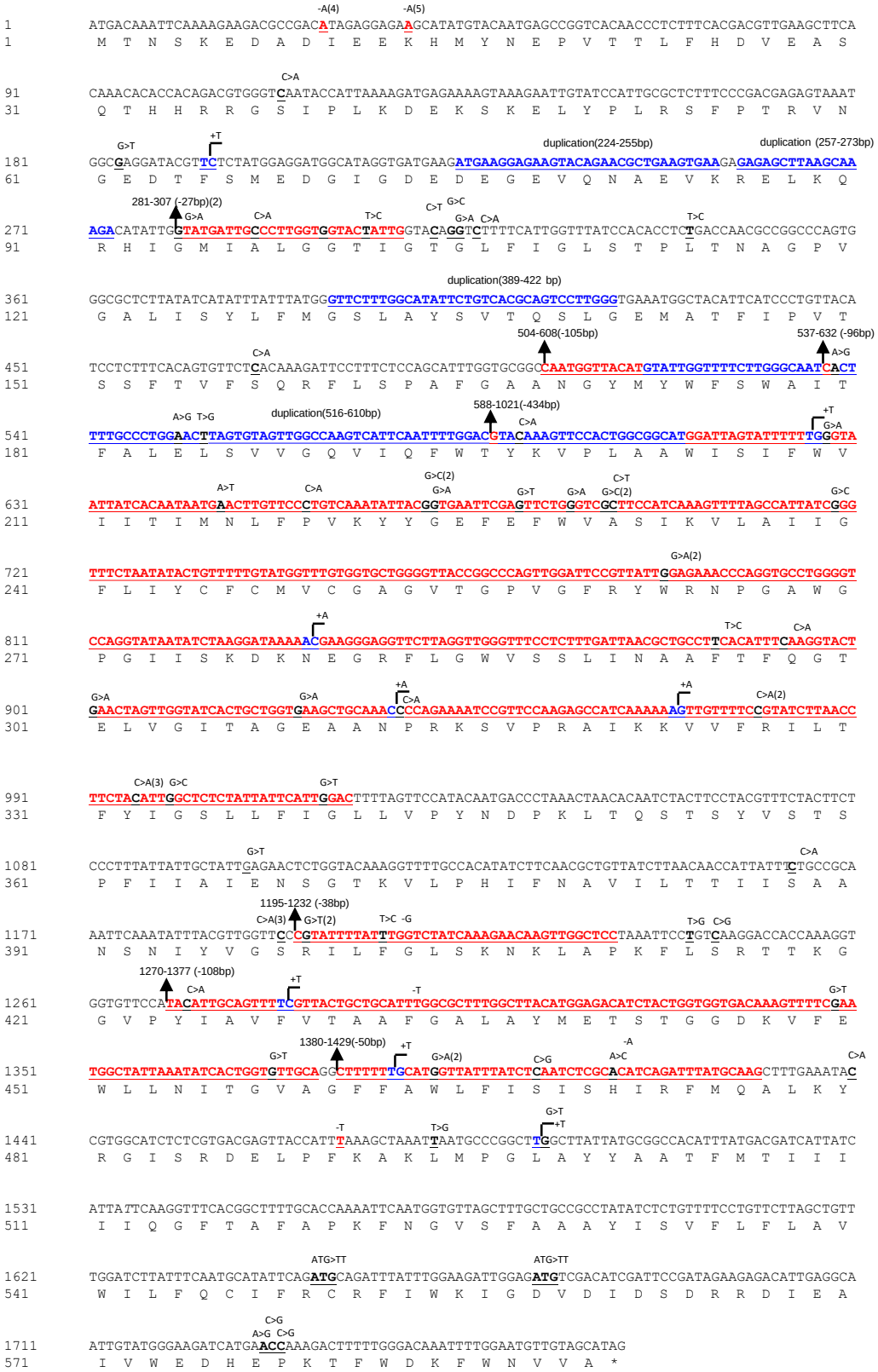


Fig. S4. Mutation spectrum of the *CAN1* gene for indicated yeast strains. CanR isolates were randomly picked and sequenced for the *CAN1* gene. Base substitution, deletion, insertion and duplication events are marked. Arrows mark the positions where large deletion events occurred. The exact positions and lengths of deleted or duplicated sequences are indicated. The deleted sequences are marked in red, while the duplicated sequences are marked in blue and underlined. The numbers in brackets represent the frequency of observation of the same event.

Fig. S5

Strain	Mutation type	Sequence	GC %	Position	Size (bp)	Micro-homology (bp)
rtt105	Deletion (22/89)	ACATATTGGTATGATTGCCCTTGGTGGTACTATTGGTACAGGT	40.6	281-307	27	5
		TTCTTGGGCAATCAC……TTTTTGGGTAATTAT	43.0	533-628	96	5
		GCAGTTTTCGTTACT…….CAAAGTTTTCGAATGGCT	45.7	1279-1341	63	7
		CCTGTTACATCCTCTT……CAATGGTTACATGTATTGG	43.7	445-508	64	7
		CCTGTTACATCCTCTT……CCAATGGTTACATGTATTGG	43.7	445-508	64	7
		GTGCTGGGGTTACCG……GGTGCCTGGGGTCCAGGTA	58.9	760-808	49	7
		GCTTTGGCTTACATGG……CCCGGCTTGGCTTATTATGCG	38.0	1314-1500	187	8
		TCTACTGGTGGTGAC…….AATATCACTGGTGTTGCAGG	39.1	1331-1369	39	7
		TCTACTGGTGGTGAC…….AATATCACTGGTGTTGCAGG	39.1	1331-1369	39	7
		TGCAGTTTTCGTTACT……GACAAAGTTTTCGAATGGCT	45.1	1286-1348	63	8
		ATCGGGTTTCTAATAT……TAGGTTGGGTTTCCTCTTTGA	50.0	720-858	139	7
		ATCTTATTTCAATGCATATTCAGATGCAG	0.0	1629-1632	4	2
		TGTGGTGCTGGGG……AACCCAGGTGCCTGGGG	58.5	752-799	48	5
		TGTGGTGCTGGGG……AACCCAGGTGCCTGGGG	58.5	752-799	48	5
		GGCTACATTCATC…….AATGGTTACATGTATTGGT	42.9	435-513	79	5
		TTACATGTATTGG……..CTGGCGGCATGGATTAGT	44.4	516-610	95	4
		TTACATGTATTGG……..CTGGCGGCATGGATTAGT	44.4	516-610	95	4
		GTTCCCGTATT…… TGGCTCCTAAATTC	39.0	1194-1232	39	3
		GTTATTTATCTCAA……ATCAGATTTATGCAAGCT	30.0	1395-1418	24	6
		TGCAGATTTATTTGGAAGATTGGAGA	22.2	1656-1668	13	5
		GGTTCCCGTATT……GTTGGCTCCTAAATTC	39.0	1195-1232	38	3
		GGCAATCACTTTTGCCC……TTTTTGGGTAATTATCACA	40.4	537-632	96	3
	Duplication (6/89)	GCATAGGTGATGAAGATGAAGGAGAAGT	44.4	213-229	17	5
		CAGGCTTTTTTGCA……TGCAAGCTTTGAAATAC	34.5	1380-1429	49	7
		GTGCTGGGGTTACCGGCCC….CCCAGGTGCCTGGGGTCCAG	58.9	761-809	49	7
		GTGTTAGCTTTGCTGC……CCTGTTCTTAGCTGTTTGGAT	43.2	1578-1615	38	6
		ACATATTGGTATGATTGCCCTTGGTGGTACTATTGGTACAGGTC	40.0	1550-1577	28	8
		ACGGCTTTTGCACCAAAATTCAATGGTGTTAGCTTTGCTGCCG	37.5	280-307	27	5
EL2A	Deletion (8/101)	TGGTTCCCGTATTTT…….CTCCTAAATTCCTGTCAAGG	36.0	1195-1232	38	4
		GTGAAGAGAGAGCTTAAGCAAAGACATATTGGTATG	38.1	257-273	17	4
		GTTATTTATCTCAATCTCGCACATCAGATTTATGCAAGC	30.0	1395-1418	24	6
		TGCAGTTTTCGTTACTGC…..GACAAAGTTTTCGAATGGCT	44.1	1279-1341	63	8
		ACATATCTTCAACGCTGTTATCTTAACAACCATTATTTCTGC	33.3	1134-1148	15	6
		CAGATTTATGCAAGCTT….GGCCACATTTATGACGATCATT	41.0	1419-1511	93	7
		GGAGACATCTACTGGTGGTGACAAAGTTTTCGAATGGCT	50.0	1324-1339	16	4
		TGGTTCCCGTATTTT….TGGCTCCTAAATTCCTGTCAAGGA	36.0	1195-1232	38	4
	Duplication (5/101)	GTGAAGAGAGAGCTTAAGCAAAGACATATTGGTATGATTG	38.1	257-273	17	4
		ACATATTGGTATGATTGCCCTTGGTGGTACTATTGGTACAGGTC	40.0	281-307	28	7
		CATTGCAGTTTTCGTTACTGCTGCATTTGGCGCTTTGGC	45.5	1279-1296	18	4
		ACATATTGGTATGATTGCCCTTGGTGGTACTATTGGTACAGGTC	40.0	281-307	28	8
		TGTTTTTGTATGGTTTGTGGTGCTGGG	0.0	738-739	2	2

Fig. S5 continued

Strain	Mutation type	Sequence	GC%	Position	Size (bp)	Micro-homology (bp)
V106A	Deletion (9/95)	TGGTTCCCGTATTTTAT.....GGCTCCTAAATTCCTGTCAAGGA	36.0	1195-1232	38	4
		GCAATCACTTTTGCCCT.....TTGGGTAATTATCACAATAATGAA	40.0	537-632	96	4
		GGTGCGGCCAATGGTTA.....GTTCCACTGGCGGCATGGATTAG	43.0	504-608	105	6
		ACATATTGGTATGATTGCCCTTGGTGGTACTATTGGTACAGGTC	40.0	281-307	28	8
		GGACGTACAAAGTTC.....AAAACGAAGGGAGGTTCTTAGGT	38.0	588-1021	434	6
		CAGGCTTTTTTGCATG.....GATTTATGCAAGCTTTGAAATACCGT	34.5	1380-1429	50	7
		AGGTGGTGTTCCATACATT.....AAATATCACTGGTGTTGCAGGCTT	43.0	1270-1377	108	4
		ACATATTGGTATGATTGCCCTTGGTGGTACTATTGGTACAGGTCTT	40.0	281-307	28	4
	Duplication (4/95)	TGATGAAGATGAAGGA.....ACGCTGAAGTGAAGAGAGAGCTTA	42.1	224-255	32	4
		TTACATGTATTGGTTTT.....CTGGCGGCATGGATTAGTATTTTTTG	44.4	516-610	95	7
		GTGAAGAGAGAGCTTAAGCAAAGACATATTGGTATGATTGCCCT	38.1	257-273	17	4
		TTATGGGTTCTTTGGCATATTCTGTCACGCAGTCCTTGGGTGAAA	50.0	389-422	34	8
pol32	Deletion (16/72)	TTACGTTGGTTCCCGTATTTTATTTGGTCTATCAAAGAACAAGTTG GCTCCTAAATTCCTGTCA	34.2	1195-1232	38	3
		ATGGGTTCTTTGGCATATT.....GGGAGGTTCTTAGGTTGGG	42.9	395-851	457	7
		AGATGAAGGAGAAGTACAGAACGCTGAAGTGAAGAGAGAG	46.2	230-255	26	5
		ATTCATCCCTGTTACATCCTCTTTCACAGTGTTCTC	45.5	443-453	11	5
		GGAGACATCTACTGGTGGTGACAAAGTTTTCGAATGGCTATTAA	50.0	1324-1339	16	4
		AAAGGTGGTGTTCCATA.....CATCTACTGGTGGTGACAAAGTT	59.7	1265-1336	72	7
		ACATATTGGTATGATTGCCCTTGGTGGTACTATTGGTACAGGT	44.4	284-310	27	8
		TTCATCCCTGTTACATCCTCTTTCACAGTGTTCTCACA	45.5	443-453	11	5
		TTCCTACGTTTCTACTTCTCCCTTTATTATTGCTA	37.5	1069-1076	8	4
		GTTCCCTGTCAAATATTACGGTGAATTC	0	662-663	2	1
		TACTATTGGTACAGGTCTTTTCATTGGTTTATCCA	38.9	308-325	18	5
		TTGGCTTATTATGC.....CCTGTTCTTAGCTGTTTGGATCT	39.2	1496-1615	120	3
		ACATATTGGTATGATTGCCCTTGGTGGTACTATTGGTACAGGTCTT	44.4	281-307	27	8
		ATCTTATTTCAATGCATATTCAGATGCAGATTTATTTGGAAGATTGG AGATGTCGACAT	33.3	1648-1662	15	2
		GGAGACATCTACTGGTGGTGACAAAGTTTTCGAATGGCT	50.0	1324-1339	16	4
		TGGTGGTACTATTGGTACAGGTCTTTTCATTGGTTTA	33.3	303-311	9	6
pol32 rtt105	Deletion (35/74)	GGGTTCTTTGGCATATTCTGTCACGCAGTCCTTG.....ATTCATCCC TGTTACATCCTCTTTCACAGTGTTCTCAC	35.9	411-449	39	4
		TAAGCAAAGACATAT.....GGGTTCTTTGGCATATTCTGTCACG	43.8	270-399	130	3
		TGCAGTTTTCGTTACTGC.....TGACAAAGTTTTCGAATGGCTA	46.0	1286-1348	63	8
		AAAGGTGGTGTTCCATA.....CATCTACTGGTGGTGACAAAGT	59.7	1265-1336	72	7
		CAGGCTTTTTTGCATGGT.....TTTATGCAAGCTTTGAAATACC	34.0	1384-1433	50	5
		GGTTCCCGTATTTTA.....GTTGGCTCCTAAATTCCTGTCAAGG	34.2	1195-1232	38	4
		ATTGCGCTCTTTCCCGACGA.....CCAGTGGGCGCTCTTATATCAT	48.1	164-369	206	8
		AAAGGTGGTGTTCCATAC.....GACATCTACTGGTGGTGACAAAG	45.8	1259-1330	72	7
		AAAGGTGGTGTTCCATAC.....GACATCTACTGGTGGTGACAAAG	45.8	1259-1330	72	7
		AAAGGTGGTGTTCCATAC.....GACATCTACTGGTGGTGACAAAG	45.8	1259-1330	72	7
		AAAGGTGGTGTTCCATAC.....GACATCTACTGGTGGTGACAAAG	45.8	1259-1330	72	7
		GGCCCAGTTGGATTCC.....GTGCCTGGGGTCCAGGTATAATATCT	54.8	773-814	42	4

Strain	Mutation type	Sequence	GC%	Position	Size (bp)	Micro-homology (bp)
pol32 rtt105	Deletion (35/74)	TGTATGGTTTGTGGTGCTGGGGTTACCGGCCCAGTTGG.....TTGGT ATCACTGCTGGTGAAGCTGCAAACCCCAGAAA	46.1	761-925	165	3
		AAGTTTTAGCCATTATCGGGTTTCTAATATACTGTT.....GATTCCGTT ATTGGAGAAACCCAGGTGCCTGGGGTC	44.3	720-789	70	2
		TCTACTGGTGGTGACAAA.....TTAAATATCACTGGTGTTGCAGGCT TTTTTG	39.1	1331-1369	39	7
		GGCTACATTCATCCCTGTTACATCCTCTTTCACAGTGTTCTCACAA AGATTCC	40.0	437-451	15	5
		TTTGGCATATTCTGT.....TATTGGTTTTCTTGGGCAATCACTTTTGC	44.4	400-534	135	4
		TTTGGCATATTCTGT.....TATTGGTTTTCTTGGGCAATCACTTTTGC	44.4	400-534	135	4
		CAAATTCAAATATTTACGTTGGTTCCCGTATTTTATTTGGTCTATCAA AGAACAAGTTGGCTCCTAAATTCCTGTCAAGGACCACCAAA	35.1	1196-1232	37	3
		ACTCTGGTACAAAGGTTTTGCCACATATCTTCAACGCT.....TATTT CTGCCGCAAATTCAAATATTTACGTTGGTTCCCG	38.1	1128-1170	42	2
		CATATTGGTATGATT.....ACAGGTCTTTTCATTGGTTTATCCACAC CTCTGACCAAC	42.2	281-325	45	6
		GGTTCCCGTATTTTAT.....AAGTTGGCTCCTAAATTCCTGTCAAG	34.2	1195-1232	38	3
		GGTTCCCGTATTTTAT.....AAGTTGGCTCCTAAATTCCTGTCAAG	34.2	1195-1232	38	3
		GGTTCCCGTATTTTAT.....AAGTTGGCTCCTAAATTCCTGTCAAG	34.2	1195-1232	38	3
		GGTTCCCGTATTTTAT.....AAGTTGGCTCCTAAATTCCTGTCAAG	34.2	1195-1232	38	3
		TGCAGTTTTCGTTACTG.....GTGGTGACAAAGTTTTCGAATGGCTA TTAAATATCAC	46.0	1279-1341	63	8
		CCTGTTACATCCTCTTT.....TGCGGCCAATGGTTACATGTATTGG	45.3	445-508	64	7
		TGTATTGGTTTTCT.....TGGCGGCATGGATTAGTATTTTTTGGGTAA TTATCACAATA	45.3	521-615	95	3
		CTCACAAAGATTCCT.....GACGTACAAAGTTCCACTGGCGGCATG GATTAGTAT	42.9	477-595	119	6
		ACTAACACAATCTACTTCCTACGTTTCTACTTCTCCCTTTAT..... TCCTGTTCTTAGCTGTTTGGATCTTATTTCAATGCATATTCAGA	39.6	1075-1620	546	-
		GTACAAAGTTCCACTGGC.....TCGCTTCCATCAAAGTTTTAGCCAT TATCGGGTTTCTAATAT	39.8	595-702	108	7
		CTACATTGGCTCTCTATTATTCATTGGACTTTTAGTTCCATACAA	33.3	1002-1019	18	6
		TTTGGCATATTCTGTCACG.....GGTTTTCTTGGGCAATCACTTTT	44.4	400-534	135	4
		CTGTTTGGATCTTATTTCAAT.....CAGATTTATTTGGAAGATTGGA	28.9	1625-1662	38	6
		CACGACGTTGAAGCTTCACAAACACACCACAGACGTGGGTCAA	46.4	79-106	28	5
	Duplication (1/74)	CATATTGGTATGATTGCCCT.....ACAGGTCTTTTCATTGGTTTATCCA CACCTCTGACCAA	42.2	277-321	45	6
rad59	Deletion (1/48)	TACGTTTCTACTTCTCCCTTTATTATTGCTATTGAGAACTCTGGTAC AAAGGTT	0	1090-1092	3	5
rad59 rtt105	Deletion (10/103)	TGCAGTTTTCGTTACTG.....GGTGACAAAGTTTTCGAATGGCTATT	45.7	1279-1341	63	8
		TTCTTGGGCAATCACTTTT.....TAGTATTTTTTGGGTAATTATCACA	43.0	533-628	96	5
		TTCCTACGTTTCTACTTCTCCCTTTATTATTGCTATTGA	37.5	1069-1076	8	4
		AAAGGTGTGGTGTTCCATAC.....GACATCTACTGGTGGTGACAAAG	45.8	1259-1330	72	7
		TGCATTTGGCGCTTTGGCTTA.....ATGCAGATTTATTTGGAAGATT	39.3	1302-1662	361	6
		ACCACCAAAGGTGGTGTTCCA.....CTACTGGTGGTGACAA	45.8	1259-1330	72	7
		GGCCAATGGTTACATGT.....CAATTTTGGACGTACAAAGTTCCA	40.2	507-593	87	3
		GTGGTGCTGGGGTTACCGGC.....TGCCTGGGGTCCAGGTATAA	58.0	760-809	50	7
		CAAAGGTGGTGTTCCATACATTG	33.3	1264-1266	3	2
		ACATTGCAGTTTTCGTTACT.....TCACTGGTGTTGCAGGCTTTTTT	42.4	1280-1378	99	6
	Duplication (1/103)	TCTTTGGCATATTCTGTCACGCAGTCCTTGGGTGAAATGGCTA	50	398-421	24	4

Fig. S5. Table listing the duplication or deletion events occurred between short repeats. The deleted or duplicated sequences are marked in red, while the flanking short homologies are marked in gray shadow. The GC content of the DNA sequences between the repeats are indicated. The sizes for the duplication, deletion or micro-homologies are indicated.

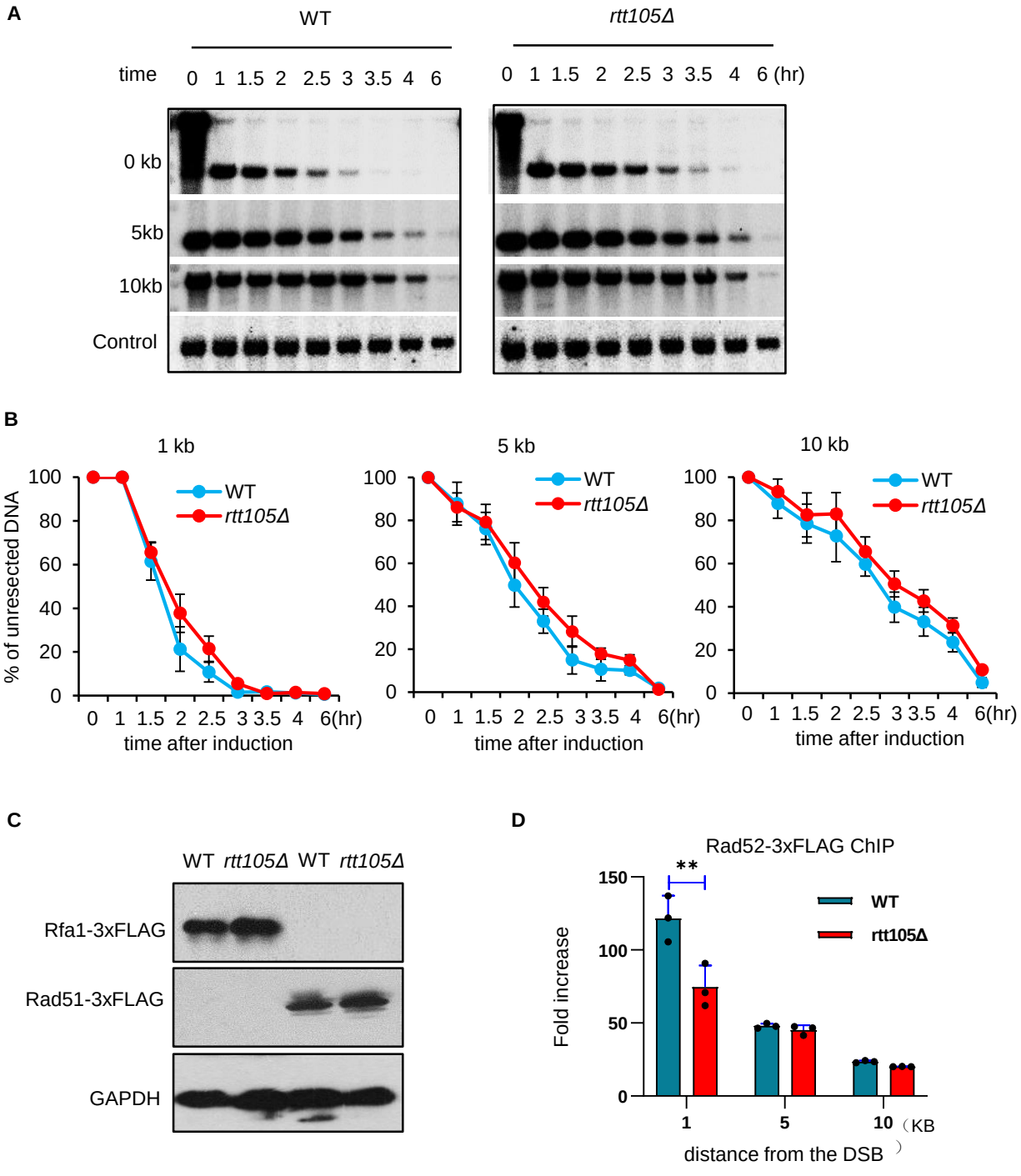


Fig. S6. The deletion of *RTT105* does not affect DSB resection or the protein levels of RPA and Rad51. A-B, Southern blot analysis and quantification of resection kinetics at indicated locations for the WT and *rtt105Δ* cells. Samples were collected at indicated time points after DSB induction. C, Western blot analysis of protein levels for RPA or Rad51 in the WT or *rtt105Δ* cells. D, ChIP analysis of Rad52-3xFLAG recruitment in WT or *rtt105Δ* cells. Error bar represents standard deviation from three independent experiments. ** p <0.01 (t-test).

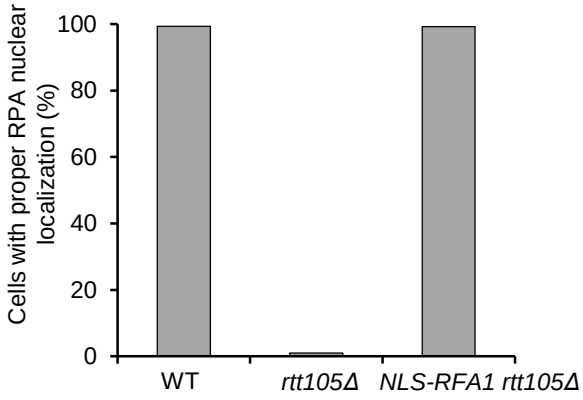


Fig. S7. Fusion of the NLS to the N-terminal of Rfa1 restores RPA nuclear localization in *rtt105Δ* cells. The plot shows the percentage of cells with normal RPA nuclear localization in indicated strains.

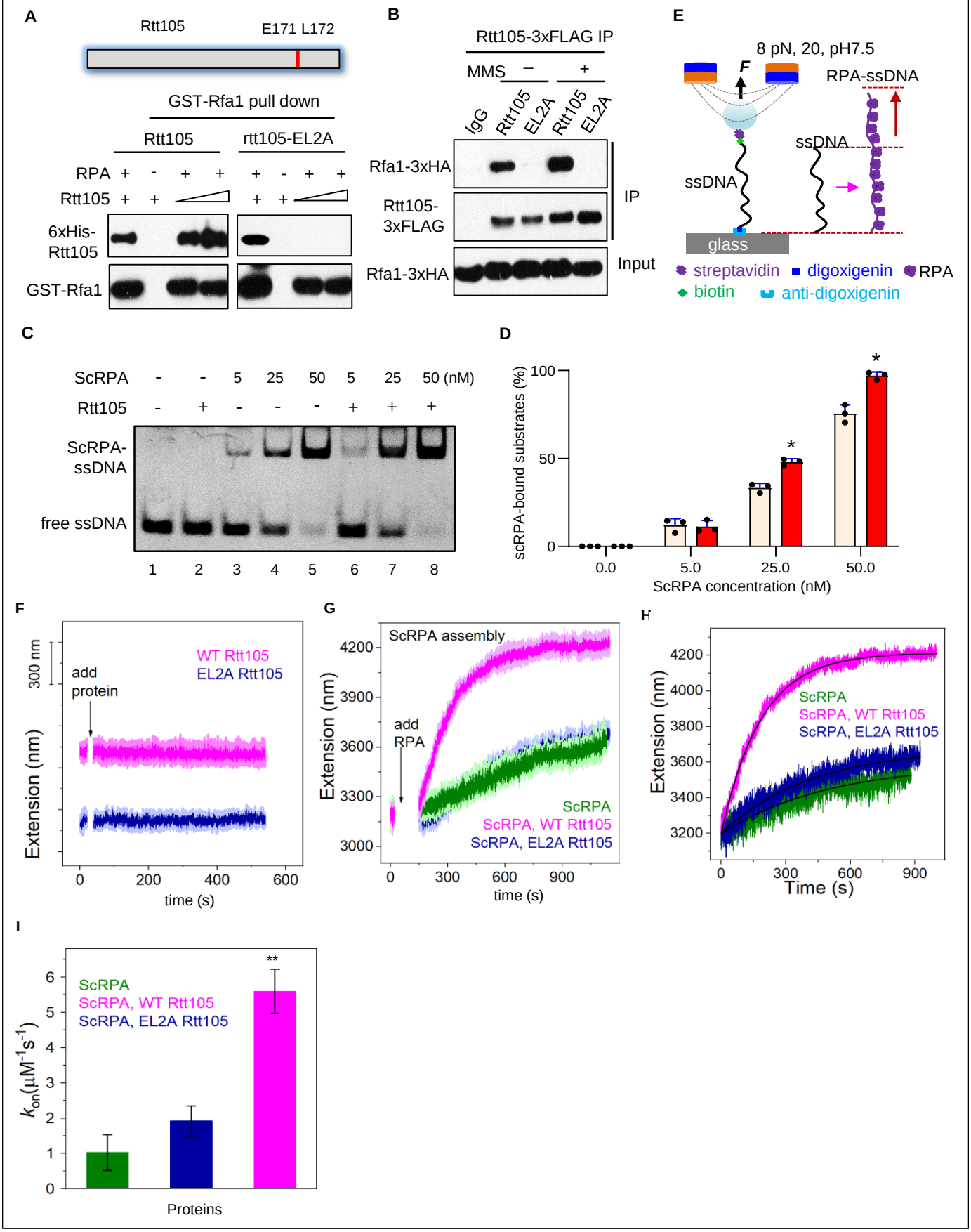

A
Rtt105
E171 L172
GST-Rfa1 pull down
Rtt105
rtt105-EL2A
RPA
Rtt105
6xHis-Rtt105
GST-Rfa1
B
Rtt105-3xFLAG IP
MMS
IgG
Rtt105
EL2A
Rtt105
EL2A
Rfa1-3xHA
Rtt105-3xFLAG
IP
Rfa1-3xHA
Input
E
8 pN, 20, pH7.5
F
RPA-ssDNA
ssDNA
ssDNA
glass
streptavidin
digoxigenin
RPA
biotin
anti-digoxigenin
C
ScRPA
Rtt105
ScRPA-ssDNA
free ssDNA
50 (nM)
1 2 3 4 5 6 7 8
D
scRPA-bound substrates (%)
ScRPA concentration (nM)
0.0
5.0
25.0
50.0
F
300 nm
add protein
WT Rtt105
EL2A Rtt105
Extension (nm)
time (s)
G
ScRPA assembly
add RPA
ScRPA
ScRPA, WT Rtt105
ScRPA, EL2A Rtt105
Extension (nm)
time (s)
H
ScRPA
ScRPA, WT Rtt105
ScRPA, EL2A Rtt105
Extension (nm)
Time (s)
I
ScRPA
ScRPA, WT Rtt105
ScRPA, EL2A Rtt105
Proteins

Fig. S8. Rtt105 interacts with Rfa1 and stimulates dynamic ScRPA assembly on ssDNA. A. GST pull-down assay showing the interaction between GST-Rfa1 and 6xHis-Rtt105 or 6xHis-rtt05-EL2A. The position of residues E171 and L172 that are required to mediate the Rtt105-RPA interaction is indicated. B. Immunoprecipitation showing the interaction between Rfa1-3xHA and WT or mutant Rtt105-3xFLAG proteins in indicated strains. C. EMSA showing the effect of Rtt105 on ScRPA binding on ssDNA. 20nM ssDNA (30nt) and 50 nM of 6xHis-Rtt105 were used for the experiment. D. Quantitation of the RPA-bound ssDNA in C. E. Monitoring the kinetics of RPA assembly on ssDNA in real-time by single-molecule twister analysis. F. Single-molecule twister analysis showing that neither the WT or mutant Rtt105 protein interacts with naked ssDNA. G. Rtt105 stimulates the assembly of ScRPA with ssDNA. The ScRPA-ssDNA filaments were assembled with ScRPA (50nM) with or without WT or mutant Rtt105 proteins (20nM). H. Plot showing the calculated binding curve (dark lines) overlaid to the curves monitored by MT. I. Plot showing the K_{on} value of ScRPA binding on ssDNA in the absence or presence of Rtt105 or rtt105-EL2A. Error bar means standard deviation from at least three independent experiments. * p <0.05 ** p <0.01 (t-test).

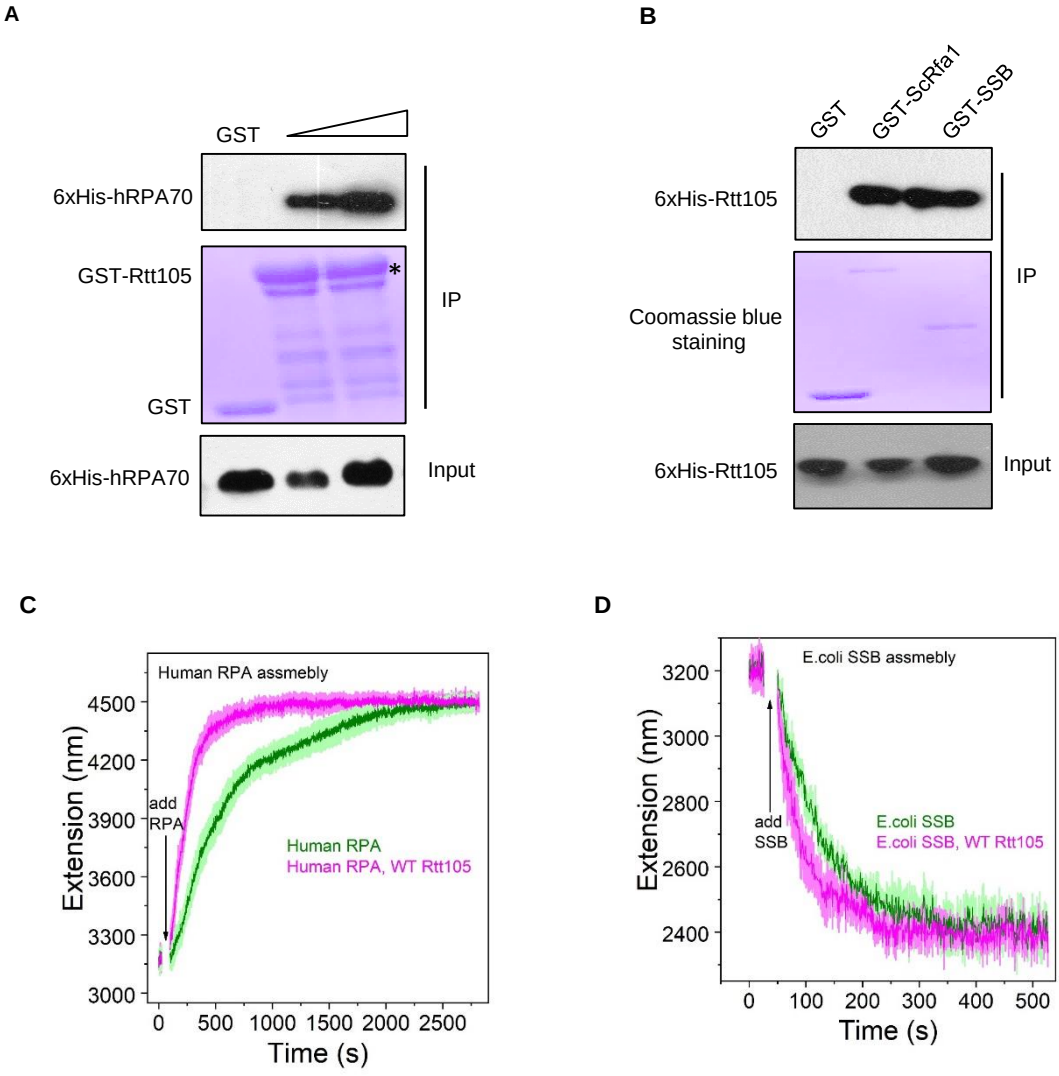


Fig. S9. Rtt105 interacts with human RPA and *E.coli* SSB and stimulates their assemblies on ssDNA. A. GST pull-down assay showing the interaction between GST-Rtt105 and 6xHis-hRPA70. B. GST pull-down assay indicating the interaction between 6xHis-Rtt105 and GST-ScRfa1 or GST-SSB. GST-tagged proteins were stained by Coomassie blue. C. Rtt105 (20nM) stimulates the assembly of hRPA (100nM) with ssDNA. D. Rtt105 (1nM) stimulates the assembly of SSB(1nM) with ssDNA.

Supplementary Table 1. Yeast strains

Strain name	Parental strain	Genotype	Source
JKM139		*MATa ho hml::ADE1 hmr::ADE1 ade1-100 leu2-3,112 trp1::hisG lys5 ura3-52 ade3::GAL::HO*	1
yLJB067	JKM139	*rtt105::KanMX*	This study
yXJ023	JKM139	*rtt105-E171A L172A-TRP1*	This study
yXJ215	JKM139	*rfa1-V106A-TRP1*	This study
yXJ208	JKM139	*pol32::KanMX rtt105::HPHMX*	This study
yXJ209	JKM139	*pol32::KanMX*	This study
yXJ306	JKM139	*rad59::KanMX*	This study
yXJ307	JKM139	*rad59::KanMX rtt105::HPHMX*	This study
yCW007	JKM139	*RFA1-3xFLAG-KanMX*	This study
yLJB108	JKM139	*RFA1-3xFLAG-KanMX rtt105::HPHMX*	This study
yCW005	JKM139	*RAD51-3xFLAG-KanMX*	This study
yXJ053	JKM139	*RAD51-3xFLAG-KanMX rtt105::HPHMX*	This study
yXJ315	JKM139	*Nup49-mCherry-TRP1 RFA1-YFP-HphMX*	This study
yXJ319	JKM139	*Nup49-mCherry-TRP1 rtt105-E171A L172A-KanMX RFA1-YFP-HphMX*	This study
yXJ317	JKM139	*Nup49-mCherry-TRP1 rfa1-V106A-YFP-KanMX*	This study
yXJ316	JKM139	*Nup49-mCherry-TRP1 rtt105::HPHMX RFA1-YFP-KanMX*	This study
yXJ318	JKM139	*Nup49-mCherry-TRP1 rtt105::HPHMX NLS-rfa1-YFP-KanMX*	This study
yXJ337	JKM139	*RTT105-KanMX NLS-RFA1-3xFLAG-NATMX*	This study
yXJ079	JKM139	*rtt105::HPHMX NLS-RFA1-3xFLAG-NATMX*	This study
yXJ081	JKM139	*rtt105::HPHMX NLS-RFA1-TRP1 RAD51-3xFLAG-KanMX*	This study
yLJB101	JKM139	*RTT105-3xFLAG-KANMX RFA1-3xHA-TRP1*	This study
yXJ020	JKM139	*RFA1-3xHA-TRP1 rtt105-E171A L172A-3xFLAG-KanMX*	This study
yXJ012	JKM139	*rtt105-E171A L172A-3xFLAG-KanMX*	This study
yLJB076	JKM139	*RTT105-3xFLAG-KANMX*	This study
yXJ005	JKM139	*RTT105-3xFLAG-KanMX mec1::NATMX tel1::LEU2 sml1::TRP1*	This study
yXJ006	JKM139	*RTT105-3xFLAG-KanMX sgs1::NATMX exo1::TRP1*	This study
yXJ007	JKM139	*RTT105-3xFLAG-KanMX mre11::NATMX*	This study
yXJ038	JKM139	*RFA1-3xFLAG-KanMX RTT105-3xHA-TRP1*	This study
yXJ236	JKM139	*rfa1-V106A-3xFLAG-KanMX RTT105-3xHA-TRP1*	This study
yXJ237	JKM139	*rfa1-L105AV106A-3xFLAG-KanMX RTT105-3xHA-TRP1*	This study
yXJ090	JKM139	*RFA1-3xFLAG-NATMX rtt105-E171A L172A-TRP1*	This study
yXJ226	JKM139	*rfa1-V106A-3xFLAG-KanMX*	This study
yXJ010	JKM139	*rtt105::KanMX + pRS316*	This study
yXJ011	JKM139	*rtt105::KanMX+pRS316-RTT105*	This study
yCW129	JKM139	*RAD52-13Myc-HPHMX*	This study
yXJ096	JKM139	*RAD52-13Myc-HPHMX rtt105::KanMX*	This study
yZSH176	JKM139	*yku70::KanMX*	This study
yXJ014	JKM139	*rtt105::HPHMX yku70::KanMX*	This study
yXJ176	JKM139	*yku70::NatMX rtt105-E171A L172A-TRP1*	This study
yXJ256	JKM139	*yku70::KanMX rfa1-L105A V106A-TRP1*	This study
yWY020	JKM139	*rtt105::HPHMX yku70::KanMX Rfa1-NLS-TRP1*	This study
yXJ320	JKM139	*Rfa1-NLS-TRP1*	This study
yXJ321	JKM139	*Rfa1-NLS-TRP1 rtt105::KanMX*	This study
yXJ073	JKM139	*NLS-RFA1-TRP1 rtt105::HPH*	This study
yXJ338	JKM139	*NLS-RFA1-TRP1 RTT105-kanMX*	This study

Strain name	Parental strain	Genotype	Source
tGI354		*MATa-inc arg5,6::MATa-HPH ade3::GAL::HO hmr::ADE1 hml::ADE1 ura3-52*	2
yLJB079	tGI354	*rtt105::KanMX*	This study
yXJ027	tGI354	*rtt105-E171AL172A-TRP1*	This study
yXJ322	tGI354	*RFA1-NLS-TRP1*	This study
yXJ318	tGI354	*RFA1-NLS-TRP1 rtt105::KanMX*	This study
yXJ213	tGI354	*rfa1-V106A-TRP1*	This study
yLJB156	tGI354	*NLS-RFA1-TRP1*	This study
yXJ339	tGI354	*rtt105::KanMX NLS-RFA1-TRP1*	This study
AM1003		*hmlΔ::ADE1/hmlΔ::ADE3 MATa-LEU2-tel/MATα-inc hmrΔ::HPH FS2Δ::NAT/FS2 leu2/leu2-3112 thr4 ura3-52 ade3::GAL::HO ade1 met13*	3
yXJ068	AM1003	*rtt105::KanMX*	This study
yXJ324	AM1003	*rtt105-E171AL172A-KanMX*	This study
GC1	BY4742	*MATα his3Δ200 ura3Δ0 met15Δ0 trp1Δ63/YAC(MFA1pr-HIS3 URA3 MET15 TRP1)*	4
yLJB081	GC1	*rtt105::KanMX*	This study
yWH378	yMV80	*rad51::URA3*	5
yXJ106	yMV80	*rtt105-E171A L172A-TRP1 rad51::URA3*	This study
yXJ234	yMV80	*rfa1-V106A-TRP1 rad51::URA3*	This study
yLJ162	yMV80	*rtt105::KanMX rad51::URA3*	This study
YAM033		*hoΔade3::GAL-HO HMLα-inc MATa hmr::ADE1 bar1Δ::ADE3 nej1Δ::KANMX ade1 leu2,3-112 trp1::hisG ura3-52 thr4 lys5*	6
NP477	YAM033	*WΔ,MATX XΔ::Cg-TRP1*	7
JL13	NP477	*rtt105::HPH*	This study

SI Appendix, Materials and Methods

Yeast strains and plasmids

Strains used in this study are derivatives of JKM139 (*ho MAT**a** hml::ADE1 hmr::ADE1 ade1-100 leu2-3,112 trp1::hisG' lys5 ura3-52 ade3::GAL::HO*), tGI354 (*MATa-inc arg5,6::MATa-HPH ade3::GAL::HO hmr::ADE1 hml::ADE1 ura3-52*), AM1003 (*hmlΔ::ADE1/hmlΔ::ADE3 MATa-LEU2-tel/MATα-inc hmrΔ::HPH FS2Δ::NAT/FS2 leu2/leu2-3112 thr4 ura3-52 ade3::GAL::HO ade1 met13) or* yMV80 (*ho hml ::ADE1 mata ::hisG hmr ::ADE1 his4::NatMXleu2-(XhoI- to Asp718) leu2::MATa ade3::GAL::HO ade1lys5 ura3-52 trp1*). All mutant strains were generated with standard genetic manipulation. Point mutants were confirmed by sequencing. Yeast strains used in this study are listed in Supplemental Table 1.

Fluorescence microscopy

Rfa1-YFP and Nup49-mCherry subcellular localizations in log phase yeast cells were examined using a ZEISS LSM 880 fluorescence confocal microscope carrying an Airyscan with a 63 x oil immersion objective lens and a YFP or RFP filter. Fluorescent images were captured and processed using ZEISS Blue Lite2 software. The percentage of cells with normal RPA nuclear localization were calculated from more than 200 cells.

Pulsed-field gel electrophoresis (PFGE)

Yeast growing cells (1.2 x 10^7 cells/ml) were treated with 0.03% MMS for 30 mins and then released into fresh YPD media to allow the recovery. Cells were harvested at the indicated time points. Chromosomal DNA plugs were prepared and separated on a 1% agarose gel using the CHEF DRII apparatus (Bio-Rad, parameter settings: initial switch time: 20s, final switch time: 150s, run time: 26-28h, volts/cm: 6V/cm)). Analysis of yeast chromosome integrity by pulsed-field gel electrophoresis was carried out as described by Maringele et al (8).

Mutation rate and spectra

The rate of accumulation of CanR mutations was determined as previously described (9) . Yeast cells from single fresh colonies were plated on SC arginine- dropout plates containing 60 mg/L canavanine. Mutation rate was determined by fluctuation analysis using the median method. To determine the mutation spectra for each strain, about 100 of fresh single colonies were patched on YPD plates and incubated at 30° C overnight followed by multiple replica plating to SC arginine- dropout plates containing 60 mg/L canavanine. This will allow to isolate single mutated colonies. Over 80 of individual colonies were cultured for each strain to extract genomic DNA, which is followed by PCR amplification and sequencing of the *CAN1* gene. The mutation spectra were characterized by analyzing the obtained sequences against the *CAN1* reference sequence.

Analysis of ectopic recombination, single-strand annealing and alt-EJ

To test the viability of DSB repair by ectopic recombination or SSA, cells were cultured in the pre-induction medium (YEP-Raffinose) overnight to log phase. Cells were then diluted and plated on YEPD or YEP-Gal plates followed by incubating at 30° C for 3 to 5 days. Viability (%)= (the number of colonies grown on YEP-Gal)/(the number of colonies grown on YEPD x dilution fold) x 100%. At least three independent experiments were performed for each strain.

The repair kinetics for ectopic recombination were monitored by Southern blot analysis as described (10, 11). The blot was exposed in a Phosphor screen. Signal on the screen was captured by scanning in an OptiQuant Cyclone Plus machine (Perkin Elmer). To measure the repair kinetics for ectopic recombination, we quantified and normalized the pixel intensity of target bands to that of corresponding parental bands on blots. The resulting values were further normalized to that of the control sample (uncut).

Analysis of 5'-end resection by Southern blot

Yeast cells were grown overnight in YEP raffinose medium (1% yeast extract, 2% peptone, 2% raffinose) to log phase. HO was induced when the cell density was ~1 × 10^7 cells/ml by adding 2% galactose. Samples were collected at 0, 1, 2, 4, 6, 8, 10 and 12 hr after galactose induction. Genomic DNA prepared with a standard phenol extraction method was digested with EcoRI followed by separated on 0.8% agarose gels. The restricted DNA was then transferred onto a Nylon hybridization membrane (GeneScreen). Southern blotting and hybridization with radiolabeled DNA probes was performed as reported(5, 12). Intensities of bands on Southern blots corresponding to probed DNA fragments were analyzed with the OptiQuant software (Perkin Elmer). Quantities of DNA loaded on gels for each time point were normalized using the *TRA1* DNA probe. DSB end resection beyond each EcoRI site for each time point was estimated as a percentage of the signal intensity corresponding to the EcoRI fragment of interest 1 hr after break induction.

Expression of recombination protein and GST pull-down assay

Protein expression and GST pull-down assay was conducted as described by Li *et al*.(13). 6xHis- or GST-tagged WT or mutated Rtt105 or Rfa1 and 6xHis-hRPA70, 6xHis-hRIPα or GST-tagged hRIPα, hRIPβ and hRIPδ recombination proteins were expressed in BL21 (DE3). Protein expression was induced by the addition of 1 mM IPTG at 0.8 OD600. Cells were cultured overnight at 16°C before harvest. After centrifugation at 4000 rpm for 20min, the cell pellets were collected and frozen at -80 °C until use. Cells was then resuspended in lysis buffer (20 mM Tris–HCl, pH 7.4, 50 mM NaCl, 0. 5 mM EDTA, 10% glycerol) and lysed by sonication. The lysate was clarified by centrifugation at 12,000 rpm for 30 min at 4°C. For the GST pull-down assay, GST-tagged WT or mutant Rfa1 or Rtt105 was immobilized on 30 µl of bed volume of glutathione agarose beads. After washing with lysis buffer, the resin was then incubated with His-tagged WT or mutant Rtt105 proteins at 4 °C for 4hrs on a rotator. The beads were washed extensively with wash buffer (20 mM Tris–HCl, pH 7.4, 200 mM NaCl, 0. 5 mM EDTA, 10% glycerol), and bound proteins were eluted by boiling the samples in 2xSDS loading buffer. The products were detected by Western blot or Coomassie brilliant blue staining of SDS-PAGE gels.

Protein purification

Purification of yeast RPA complex was performed as described by Binz et al (14). The full-length human RPA composed of three subunits Rfa1, Rfa2, and Rfa3 was expressed and purified according to the protocols described previously(9). For purification of 6xHis-Rtt105 and 6xHis hRIPα protein, cells were lysed by sonication in lysis buffer. Clarified lysate was incubated with Ni-NTA resin (Abclone) for 2 hrs at 4°C on a rotator. The beads were washed extensively with wash buffer containing 20 mM or 50 mM imidazole, followed by elution with wash buffer containing 100 mM imidazole. The eluate for these proteins was collected and dialyzed in 1x PBS overnight. For purification of Rtt105 without any tag, PGEX-6P-1-Rtt105 were transformed into E. coli strain BL21 (DE3). The expression of GST-Rtt105 was induced by adding 1 mM IPTG at 16°C for 16 h in 1 L culture. Cells were lysed and processed as described above. Recombinant GST-Rtt105 was immobilized on 2 mL of bed volume of glutathione agarose beads. After washing with lysis buffer, the resin was then incubated with the prescission protease at 4 °C for 12hrs on a rotator to cut the GST tag. Finally, the flow-through liquid was dialyzed and collected.

Electrophoretic mobility shift assay (EMSA)

To test the effect of Rtt105 on ScRPA assembly on ssDNA, 50 nM of 5'- biotin labeled ssDNA (30 nt, 5'-CGATAAGCTTGATATCGAATTCCGCAGCC-3') substrate was incubated with various amounts of ScRPA complex for 1hr at 4°C in 1× binding buffer (25 mM Tris-HCl, pH 7.5, 5 mM MgCl2, and 5% glycerol). The reaction mixture (20 µl in total) was loaded with 4 µl of 6x loading dye. The reaction products were resolved in a 6% native PAGE gel in cold 0.3×TBE buffer. The native PAGE were stained with GelRed. Signals were detected on a G-Box imager (Syngene). To test the effect of hRIPα on hRPA assembly on ssDNA, 20 nM of 5'-Cy5 labeled ssDNA substrate was incubated with various amounts of hRPA complex, and the fluorescent signal on the native PAGE or agarose gel was captured by scanning in a Typhon 9500 scanner. Band intensities were quantified with Image J.

Immunoprecipitation (IP)

Yeast cells culture (A600 ~ 1.0) with or without 0.1% MMS treatment (90 min) were collected and lysed on a bead beater in lysis buffer (100 mM HEPES, pH 8.0, 20 mM $MgCl_2$, 150 mM NaCl, 10% glycerol, 0.4% Nonidet P-40, 0.1mM EDTA plus protease and phosphatase inhibitors) with benzonase to digest DNA and RNA. The extract was clarified by centrifugation at 12,000 g for 10 min at 4°C, followed by incubating with protein G-agarose beads for 1 hr at 4°C to preclear non-specific binding. After centrifugation, the supernatant was incubated with anti-HA or anti-FLAG antibody at 4°C overnight with agitation. After the addition of protein G-agarose beads, the mixtures were incubated at 4°C for 3 hrs. Subsequently, the beads were washed with lysis buffer for five times (10 min each wash) at 4°C. Immunoprecipitated proteins were eluted by boiling beads in 2xSDS loading buffer for 5 min.

Western blotting

Whole-cell extracts were prepared using a trichloroacetic acid (TCA) method as previously described (15). Whole cell extracts, immunoprecipitated protein, or pull down samples were resolved on an 8% or 12% SDS-PAGE gel and transferred onto a PVDF membrane (Immobilon-P; Millipore) using a semi-dry method(Bio-Rad). Anti-HA and anti-FLAG antibodies were purchased from MBL and Sigma, respectively. Anti-mouse and rabbit IgG HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology. Blots were developed using the Western Blotting substrate (Bio-Rad).

Streptavidin pull-down assay

The effect of 6xHis-hRIPα on the binding of hRPA on ssDNA was also examined using streptavidin pull-down assay. 5'-biotinylated oligonucleotides (30 nt) (5'- CGATAAGCTTGATAT CGAATTCCGCAGCC-3') were immobilized on streptavidin MagBeads (GenScript) in TES buffer (10 mM Tris, 1 mM EDTA, 2 M NaCl, pH 7.5) for 30 min at room temperature. After an extensive wash with 1xPBS supplemented with 1 mM EDTA, the biotin-ssDNA-streptavidin beads were incubated with a gradient concentration of purified hRPA complex for 30 min at 4 °C. After wash with the binding buffer (25 mM HEPES, pH 7.5, 15 mM KCl, 150 mM NaCl, 1 mM EDTA, 0.05% TritionX-100, 0.5 DTT, 100 mg/mL BSA), purified 6xHis-hRIPα was added to each sample and incubated for 1 hr at 4 °C. Subsequently, the beads were washed with binding buffer, and the bound protein was eluted and detected by Western blot or Coomassie brilliant blue staining.

Single-molecule study

The 12.5 k-nt ssDNA was generated by one-sided PCR, and its two ends were labeled with digoxigenin and biotin groups, respectively. In MT experiments, the digoxigenin-labeled end of a single ssDNA molecule was anchored to the anti-digoxigenin coated glass surface in a flow cell. Then, a superparamagnetic microbead (M-270, Dynal beads) was attached to the biotin-labeled end of the anchored ssDNA molecule. A pair of permanent magnets was used to attract the microbead and thus exert a constant force to the anchored ssDNA molecule. The extension of ssDNA was determined to be the separation between the microbead and glass surface. The assembling buffer contained 100 mM NaAc, 10 mM $MgAc_2$, 1 mM ATP and 25 mM Tris-Ac pH 7.5. All experiments were performed at a constant force of 8 pN at 20 °C.

References

1. Lee, S.E., et al., *Saccharomyces* Ku70, mre11/rad50 and RPA proteins regulate adaptation to G2/M arrest after DNA damage. Cell, 1998. **94**(3): p. 399-409.
2. Ira, G., et al., Srs2 and Sgs1-Top3 suppress crossovers during double-strand break repair in yeast. Cell, 2003. **115**(4): p. 401-11.
3. Deem, A., et al., Defective break-induced replication leads to half-crossovers in Saccharomyces cerevisiae. Genetics, 2008. **179**(4): p. 1845-60.
4. Wahba, L., et al., RNase H and multiple RNA biogenesis factors cooperate to prevent RNA:DNA hybrids from generating genome instability. Mol Cell, 2011. **44**(6): p. 978-88.
5. Zhu, Z., et al., Sgs1 helicase and two nucleases Dna2 and Exo1 resect DNA double-strand break ends. Cell, 2008. **134**(6): p. 981-94.
6. Mehta, A., A. Beach, and J.E. Haber, Homology requirements and competition between gene conversion and break-Induced replication during double-strand break repair. Mol Cell, 2017. **65**(3): p. 515-526 e3.
7. Pham, N., et al., Mechanisms restraining break-induced replication at two-ended DNA double-strand breaks. EMBO J, 2021: p. e104847.
8. Maringele L & Lydall D (2006) Pulsed-field gel electrophoresis of budding yeast chromosomes. Methods Mol Biol 313:65-73.
9. Huang ME, Rio AG, Nicolas A, & Kolodner RD (2003) A genomewide screen in Saccharomyces cerevisiae for genes that suppress the accumulation of mutations. Proc. Natl. Acad. Sci. U.S.A. 100(20):11529-11534.
10. Ira G, Malkova A, Liberi G, Foiani M, & Haber JE (2003) Srs2 and Sgs1-Top3 suppress crossovers during double-strand break repair in yeast. Cell 115(4):401-411.
11. Zheng S*, et al.* (2018) Bre1-dependent H2B ubiquitination promotes homologous recombination by stimulating histone eviction at DNA breaks. Nucleic Acids Res 46(21):11326-11339.
12. Chen X*, et al.* (2012) The Fun30 nucleosome remodeller promotes resection of DNA double-strand break ends. Nature 489(7417):576-580.
13. Li S*, et al.* (2018) Rtt105 functions as a chaperone for replication protein A to preserve genome stability. EMBO J 37(17).
14. Binz SK, Dickson AM, Haring SJ, & Wold MS (2006) Functional assays for replication protein A (RPA). Methods Enzymol. 409:11-38.
15. Henricksen LA, Umbricht CB, & Wold MS (1994) Recombinant replication protein A: expression, complex formation, and functional characterization. J Biol. Chem. 269(15):11121-11132.