A genetic screen pinpoints ribonucleotide reductase residues that sustain dNTP homeostasis and specifies a highly mutagenic type of dNTP imbalance

Tobias T. Schmidt\textsuperscript{1,2}, Sushma Sharma\textsuperscript{3}, Gloria X. Reyes\textsuperscript{1}, Kerstin Gries\textsuperscript{1}, Maike Gross\textsuperscript{1}, Boyu Zhao\textsuperscript{1,2}, Jui-Hung Yuan\textsuperscript{4}, Rebecca Wade\textsuperscript{4,5,6}, Andrei Chabes\textsuperscript{3,7} and Hans Hombauer\textsuperscript{1,*}

\textsuperscript{1}DNA Repair Mechanisms and Cancer, German Cancer Research Center (DKFZ), Heidelberg, D-69120, Germany, \textsuperscript{2}Faculty of Bioscience, Heidelberg University, Heidelberg, D-69120, Germany, \textsuperscript{3}Department of Medical Biochemistry and Biophysics, Umeå University, Umeå, SE-901 87 Sweden, \textsuperscript{4}Molecular and Cellular Modeling Group, Heidelberg Institute for Theoretical Studies (HITS), Heidelberg, D-69118, Germany, \textsuperscript{5}Interdisciplinary Center for Scientific Computing (IWR), Heidelberg, D-69120, Germany, \textsuperscript{6}Center for Molecular Biology of the University of Heidelberg (ZMBH), DKFZ-ZMBH Alliance, Heidelberg, D-69120, Germany, \textsuperscript{7}Laboratory for Molecular Infection Medicine Sweden, Umeå University, Umeå, SE-901 87, Sweden

* To whom correspondence should be addressed. Tel: +49 6221 42 3239; Fax: +49 6221 42 3237; Email: h.hombauer@dkfz.de
Table of contents:

Supplementary Experimental Procedures

Supplementary Figure S1. *RNR1* mutagenesis screen reveals mutations causing *exo1Δ*-dependent and -independent mutator phenotypes.

Supplementary Figure S2. Synthetic lethal interactions driven by specific *mr1* mutations in DNA replication- and DNA damage checkpoint-compromised backgrounds.

Supplementary Table S1. rNTP concentrations in strains expressing *mr1* mutant alleles on a centromeric plasmid.

Supplementary Table S2. dNTP concentrations in strains expressing *mr1* mutant alleles on a centromeric plasmid.

Supplementary Table S3. rNTP and dNTP concentrations in strains containing *mr1* mutant alleles integrated at the endogenous *RNR1* locus.

Supplementary Table S4. *CAN1* mutation spectra in strains carrying *mr1* mutant alleles.

Supplementary Table S5. *CAN1* mutation hotspots identified in strains carrying *mr1* mutant alleles.

Supplementary Table S6. *URA3* mutation spectrum in *mr1-I262V,N291D* mutant strain.

Supplementary Table S7. *S. cerevisiae* strains used in this study.

Supplementary Table S8. Plasmids used in this study.
Supplementary Experimental Procedures

**Media, strains and plasmids**

*S. cerevisiae* strains were grown at 30°C either in yeast extract-peptone-dextrose (YPD) media (1% Bacto yeast extract (Becton Dickinson), 2% Bacto peptone (Becton Dickinson) and 2% glucose), or in synthetic dropout (SD) media (0.67% Difco yeast nitrogen base without amino acids (Becton Dickinson), 2% glucose and supplemented with the appropriate amino acid dropout mix. For the CAN1 inactivation assay, plates were prepared in SD media lacking arginine (Arg), supplemented with 60 mg/L canavanine (Sigma). 5-FOA plates were done in SD media lacking uracil supplemented with 1 g/L 5-FOA and 50 mg/L uracil. Antibiotics were used at the following final concentrations: 200 µg/mL geneticin (Santa Cruz Biotechnology), 300 µg/mL hygromycin B (Thermo Fisher Scientific) and 100 µg/mL nourseothricin (clonNAT, Werner BioAgents). The DNA damaging agent hydroxyurea (HU) (H8627, Sigma) was used at 200 mM final concentration.

Specific rnr1 mutations were introduced at the chromosomal RNR1 locus by pop-in/pop-out strategy and the presence of the desired mutations, as well as the absence of unwanted mutations, were confirmed by sequencing.

For the RNR1 random mutagenesis screen we generated low-copy number RNR1-WT expression plasmids used to complement rnr1Δ mutant strains. For this, a 3.6 kb NotI-KpnI fragment from pHHB424 (containing WT-RNR1 including promoter and terminator) was subcloned into the NotI-KpnI digested pRS316 or pRS315 (21) generating pHHB560 and pHBB561, respectively (Supplementary Table S8).

The RNR1 random mutagenesis screen was done in HHY6555 (MATa ura3-52 leu2Δ1 trp1Δ63 hom3-10 his3Δ200 lys2-10A rnr1::kanMX4 exo1::hphNT1 lig4::HIS3 + pHHB560), which was complemented by pHHB560 (WT-RNR1-URA3) plasmid. To generate HHY6555, we inactivated the Lig4 gene (to prevent non-homologous end joining events) with a HIS3 cassette in RDKY5964 (MATa ura3-52 leu2Δ1 trp1Δ63 hom3-10 his3Δ200 lys2-10A) (19) and crossed with HHY1941 (MATa ura3-52 leu2Δ1 trp1Δ63 hom3-10 his3Δ200 lys2-10A exo1::hphNT1). In the resulting diploid strain one of the two RNR1 alleles was replaced by a kanMX4 cassette, amplified from pFA6a-kanMX4. The heterozygous diploid strain was transformed with pHHB560 (pRS316-RNR1) and sporulated to obtain HHY6555 (MATa ura3-52 leu2Δ1 trp1Δ63 hom3-10 his3Δ200 lys2-10A rnr1::kanMX4 exo1::hphNT1 lig4::HIS3 + pHHB560). HHY6124 (MATa ura3-52 leu2Δ1 trp1Δ63 hom3-10 his3Δ200 lys2-10A rnr1::kanMX4 exo1::hphNT1 + pHHB560) and HHY6551 (MATa ura3-52 leu2Δ1 trp1Δ63 hom3-10 his3Δ200 lys2-10A rnr1::kanMX4 + pHHB560), which were used for further analysis, were generated following a similar strategy.

To integrate rnr1 alleles into the RNR1 chromosomal locus, we first generated pHHB424, an integrative URA3 plasmid containing the WT-RNR1 gene. To generate pHHB424, the RNR1 gene (including promoter and 3’ UTR) was amplified from genomic DNA with primers 5’-CAG CTC AGT CAC ATG AGA C-3’ and 5’-GCG CAT CCT GGG AAT CTA-3’. The PCR product
was digested with KpnI and partially digested with BgII, gel extracted and cloned into KpnI and BamHI digested pRS306 (21), resulting in pHBB424, containing the WT-RNR1 gene including 786 nt before ATG and 135 nt after RNR1 STOP codon. Next, pHBB424 was used to generate derivative plasmids containing specific rnr1 mutations (by site-directed mutagenesis or subcloning) (Supplementary Table S8). Integrative plasmids were linearized before transformation with BgII (or Bsu36I for pHBB718 and pHBB752). To label chromosomally-integrated rnr1 mutant alleles, a HIS3 cassette was amplified from pRS303 (21) with primers 5'-GTC GAA TAA TTT AAC ATG AAC ATT TTA AGC TGT CCT TGT AAG AAG GCG AGC AGA TT  G TAC TGA GAG TGC ACC-3' and 5'-CAA TGT TGC CTA GAC CCC ATT TCG GGG CAG GGG GGA ATC TGT ATC ATG CTC CTT ACG CAT CTG TGC GGT ATT TC-3' and introduced 232 nt downstream of RNR1 STOP codon.

Construction of an rnr1 mutation library

To generate an rnr1 mutant library, the RNR1 gene was amplified from pHBB424 (pRS306-RNR1) using primers 5'-CGA TTC ATT AAT GCA GCT GGC-3' and 5'-GCA AGT GTA GCG GTC ACG C-3' with standard Taq polymerase (New England Biolabs), which lacks proofreading function, for 12 cycles under standard conditions in 52 independent reactions. PCR reactions were pooled and purified using Gel Extraction Kit (Qiagen). RNR1 PCR products together with a 6 kb DNA fragment, obtained after digesting pHBB561 with HindIII and NotI, followed by gel extraction, were co-transformed into HHY6555 for in vivo gap repair. Transformants containing the gap-repaired plasmids were selected by growth on SD plates lacking leucine (Leu) and replica plated on SD plates lacking Leu but containing 5-FOA, to select for the loss of pHBB560 (WT-RNR1-URA3) using plasmid shuffling.

Screening for mutator phenotypes, plasmid rescue and identification of rnr1 mutations

Colonies obtained after plasmid shuffling (Leu⁺ + 5-FOA₅) were replica-plated on SD media lacking threonine (Thr) or lysine (Lys) or lacking Arg but supplemented with canavanine, to screen for increased mutator phenotypes in the hom3-10, lys2-10A and CAN1 inactivation assay, respectively (16,24). Plates were incubated at 30°C for 3 days to allow growth on the mutator plates. Colonies resulting in increased papillation on at least two mutator assays (or growing in a cluster with many small canavanine₅ (Can₅) colonies) were re-tested for mutator phenotype. Clones that after re-testing still presented an increased mutator phenotype, were inoculated for DNA extraction followed by plasmid rescue after electroporation into Escherichia coli Top10F⁺.

Plasmids were prepared using Miniprep Kit (Qiagen) and transformed into competent HHY6214. Cells were grown on SD media lacking Leu, followed by plasmid shuffling on SD media lacking Leu, but containing 5-FOA. Transformants after plasmid shuffling were tested for mutator phenotype using all three mutator assays. Plasmids that reproducibly increased the mutator phenotype were sequenced to identify rnr1 mutation(s) (Table 1 and Supplementary Table S8). Plasmids conferring mutator phenotype in an exo1Δ background
were also transformed in an EXO1-WT background (HHY6551), selected on 5-FOA and tested for mutator phenotype.

Yeast cell lysates and immunoblotting

*S. cerevisiae* whole-cell protein extracts were generated as previously described (19) and were analyzed on 4-15% Mini-PROTEAN TGX precast gels (Bio-Rad) followed by immunoblotting. The following antibodies were used: rabbit polyclonal anti-Rnr1 (1:40,000, AS09576, Agrisera), rabbit polyclonal anti-Rnr2 (1:30,000, AS09575, Agrisera) and anti-Rnr3 (1:1,000, AS09574, Agrisera). The rat monoclonal YL1/2 antibody (YL1/2, 1:40,000, Sigma) was used to probe against tubulin and Rnr4. Tubulin was used as loading control. Horseradish peroxidase (HRP)-linked to donkey anti-rabbit IgG (NA934, GE Healthcare) and HRP-linked to goat anti-rat IgG (401416, Calbiochem) were used as secondary antibodies at a dilution of 1:10,000. Western blots were developed using Immobilon Western Chemiluminescent HRP substrate (Millipore) and imaged using a Fusion Solo S System (Vilber).

Live-cell imaging of Pms1-GFP foci

Exponentially growing cells were washed, resuspended and placed on agar pads, covered with a coverslip and sealed with valap (a 1:1:1 mixture of Vaseline, lanolin and paraffin by weight). Cells were imaged using a DeltaVision Elite imaging system (Applied Precision) based on an inverted microscope (IX71; Olympus) with a camera (CoolSNAP HQ2; Photometrics) and an UPlanFL N 100x (1.25 NA) oil immersion objective lens (Olympus). 20 Z stacks spaced 0.3 µm were deconvolved using SoftWoRx software and projected using the maximum intensity projection. Data is presented in box-plots with whiskers (showing the 25th and 75th percentile) and dots represent outliers. The line inside the box represents the median of cells with foci. Three independent biological replicates were analyzed per genotype. Mann-Whitman test was used to compare Pms1-4xGFP foci abundance in different genotypes.

Mutation rate analysis

The hom3-10 and lys2-10A frameshift reversion assays and the CAN1 inactivation assay were used to quantify mutation rates by fluctuation analysis, as previously described (16,24). For fluctuation analysis of plasmid-borne *rnr1* alleles, strains were grown in SD media lacking Leu to select for the mutant *rnr1* plasmid. Mutation rates were determined using two independent clones and at least 14 independent cultures. The 95% confidence intervals were calculated for all fluctuation tests.

**CAN1 and URA3 mutation spectra analysis**

*CAN1* mutation spectra analysis was performed as previously described (17). To determine the *URA3* mutation spectra caused by the *mr1-I262V,N291D* mutation, we generated two isogenic strains (HHY6634 and HHY6635) in which we replaced the *ura3-52* allele by a WT-
URA3 gene by transforming an URA3 cassette lacking the ATG, amplified from pRS306 with primers 5'-CGA AAG CTA CAT ATA AGG AAC-3' and 5'-TTA GTT TTG CTG GCC GCA TC-3'. Next, individual colonies were patched on YPD and replica plated on 5-FOA-containing plates. 5-FOA<sup>R</sup> clones were re-streaked on 5-FOA and used for genomic DNA isolation. The URA3 gene was amplified with Phusion High-Fidelity DNA polymerase (New England Biolabs) using primers 5'-GGG AAG ACA AGC AAC GAA AC-3' and 5'-GGA AAC GCT GCC CTA CAC-3' and sequenced with primers 5'-TCA TTA CGA CCG AGA TTC C-3' and 5'-GGA GCA CAG ACT TAG ATT GG-3' by GATC. Sequences were analyzed using Lasergene12 (DNASTAR) and mutations were annotated in the URA3 sequence. URA3 spectrum was compared to WT URA3 mutation spectrum reported by (42). Full CAN1 and URA3 mutation spectra for the reported strains are available upon request.
Supplementary Figure S1. RNR1 mutagenesis screen reveals mutations causing exo1Δ-dependent and exo1Δ-independent mutator phenotypes. (A) Schematic representation of the rnr1 mutagenesis screen. In brief, PCR-mutagenized RNR1 was co-transfected with a linearized plasmid (CEN6, ARSH4, LEU2) in HHY6555 for in vivo gap repair. To select for loss of the WT-RNR1-URA3 plasmid cells were replica-plated on media containing 5-FOA and subsequently screened for increased mutagenesis using three mutator assays (hom3-10 and lys2-10A frameshift reversion assays and CAN1 inactivation assay). (B) Qualitative patch-test analysis using lys2-10A (-lysine) frameshift reversion assay in strains expressing WT-RNR1 or rnr1 mutant alleles from a low-copy number plasmid in an EXO1-WT rnr1Δ plasmid shuffling strain (HHY6551). Increased number of colonies (relative to WT) is indicative of a mutator phenotype. rnr1 mutant alleles causing a strong mutator phenotype in an EXO1-WT background were highlighted in red. In all plates an exo1Δ rnr1Δ strain, complemented with mutant rnr1-G271S plasmid (exo1Δ + G271S), was used as "mutator control".
**Supplementary Figure S2.** Synthetic lethal interactions driven by specific *rnr1* mutations in DNA replication- and DNA damage checkpoint-compromised backgrounds. (A) Experimental outline used for the identification of genetic interactions (GD/SL) by plasmid shuffling. Low-copy number plasmids (ARS-CEN, LEU2) expressing WT-RNR1 or *rnr1* mutations were transformed into different *rnr1Δ* plasmid shuffling strains (left side) complemented by WT-RNR1-URA3 plasmid. Transformants were spotted in serial dilutions on Leu⁺ + 5-FOA media to eliminate the WT-RNR1-URA3 plasmid. (B) and (C) Representative images of yeast
cultures for the indicated genotypes (complemented by WT or mutant rnr1 plasmids), serially
dilated and spotted into Leu- +5-FOA containing media. Images were taken after 4 days at
30 °C.
### Supplementary Table S1. rNTP concentrations in strains expressing *rnr1* mutant alleles on a centromeric plasmid.

| Allele | CTP (pmol per 10^8 cells) | UTP (pmol per 10^8 cells) | ATP (pmol per 10^8 cells) | GTP (pmol per 10^8 cells) |
|--------|--------------------------|---------------------------|---------------------------|---------------------------|
| + WT-RNR1 | 2139 ± 165 (1.0) | 4249 ± 130 (1.0) | 13792 ± 870 (1.0) | 3232 ± 197 (1.0) |
| group 1 (no interaction or just with *pol3-01*) | | | | |
| + mr1-F15S | 2073 ± 50 (1.0) | 3926 ± 8 (0.9) | 14255 ± 40 (1.0) | 2753 ± 86 (0.9) |
| + mr1-I231T,T244A | 2105 ± 72 (1.0) | 3734 ± 36 (0.9) | 14223 ± 34 (1.0) | 2854 ± 81 (0.9) |
| + mr1-T244I,V278A | 2066 ± 7 (1.0) | 3810 ± 32 (0.9) | 14108 ± 8 (1.0) | 2888 ± 15 (0.9) |
| + mr1-T265A | 2122 ± 1 (1.0) | 3912 ± 197 (0.9) | 14480 ± 448 (1.0) | 2947 ± 42 (0.9) |
| + mr1-A283V,S425L | 2143 ± 37 (1.0) | 4050 ± 118 (1.0) | 13975 ± 383 (1.0) | 2610 ± 61 (0.8) |
| + mr1-A245V | 1930 ± 56 (0.9) | 4132 ± 119 (1.0) | 15154 ± 182 (1.1) | 3450 ± 37 (1.1) |
| + mr1-G271S | 2267 ± 39 (1.1) | 4218 ± 6 (1.0) | 15307 ± 11 (1.1) | 3674 ± 50 (1.1) |
| + mr1-Y285C | 2100 ± 105 (1.0) | 3904 ± 80 (0.9) | 14817 ± 173 (1.1) | 2907 ± 62 (0.9) |
| group 2 (interaction with *pol2-04 and pol3-01*) | | | | |
| + mr1-D226G | 2202 ± 61 (1.0) | 3913 ± 114 (0.9) | 14276 ± 518 (1.0) | 2870 ± 56 (0.9) |
| + mr1-D226V | 2096 ± 26 (1.0) | 3809 ± 33 (0.9) | 13770 ± 124 (1.0) | 2921 ± 47 (0.9) |
| + mr1-D226N,S117P | 2029 ± 70 (0.9) | 3728 ± 281 (0.9) | 13955 ± 1034 (1.0) | 3047 ± 81 (0.9) |
| + mr1-S242T | 2185 ± 14 (1.0) | 3849 ± 193 (0.9) | 14395 ± 462 (1.0) | 2915 ± 55 (0.9) |
| + mr1-R256H,Y779C | 2103 ± 60 (1.0) | 4101 ± 88 (1.0) | 13750 ± 58 (1.0) | 3005 ± 41 (0.9) |
| + mr1-G267C | 2182 ± 2 (1.0) | 4068 ± 8 (1.0) | 14103 ± 105 (1.0) | 2828 ± 15 (0.9) |
| + mr1-S269P | 1922 ± 35 (0.9) | 4154 ± 136 (1.0) | 14738 ± 1198 (1.1) | 3013 ± 233 (0.9) |
| group 3 (interaction with *pol2-04, pol3-01 and msh2Δ*) | | | | |
| + mr1-K243E | 2173 ± 9 (1.0) | 4177 ± 114 (1.0) | 14350 ± 222 (1.0) | 2703 ± 18 (0.8) |
| + mr1-J262T,M275I | 2075 ± 22 (1.0) | 3905 ± 54 (0.9) | 13932 ± 242 (1.0) | 2843 ± 52 (0.9) |
| + mr1-J262V,N291D | 2110 ± 15 (1.0) | 4432 ± 136 (1.0) | 15045 ± 131 (1.1) | 2970 ± 51 (0.9) |

rNTP concentrations (pmol per 10^8 cells) are the average of two biological replicates ± standard deviation with the fold increase over WT in parentheses.
**Supplementary Table S2.** dNTP concentrations in strains expressing *rnr1* mutant alleles on a centromeric plasmid.

| Allele                        | dNTP concentration (pmol per 10^8 cells) | dNTP concentration (pmol per 10^8 cells) | dNTP concentration (pmol per 10^8 cells) | dNTP concentration (pmol per 10^8 cells) |
|-------------------------------|-----------------------------------------|------------------------------------------|------------------------------------------|------------------------------------------|
| + WT-RNR1                     | 117 ± 17 (1.0)                          | 260 ± 17 (1.0)                           | 170 ± 20 (1.0)                           | 73 ± 4 (1.0)                             |
| group 1 (no interaction or just with pol3-01) |                                         |                                          |                                          |                                          |
| + mrt1-F15S                    | 866 ± 34 (7.4)                          | 1439 ± 129 (5.5)                         | 1125 ± 156 (6.6)                         | 461 ± 31 (6.3)                           |
| + mrt1-I231T,T244A             | 525 ± 17 (4.5)                          | 1065 ± 78 (4.1)                          | 287 ± 59 (1.7)                           | 256 ± 24 (3.5)                           |
| + mrt1-T244I,V278A             | 783 ± 12 (6.7)                          | 1377 ± 22 (5.3)                          | 352 ± 2 (2.1)                            | 255 ± 7 (3.5)                            |
| + mrt1-T265A                   | 436 ± 62 (3.7)                          | 833 ± 61 (3.2)                           | 205 ± 0 (1.2)                            | 164 ± 7 (2.3)                            |
| + mrt1-A283V,S425L             | 370 ± 17 (3.2)                          | 741 ± 61 (2.8)                           | 314 ± 19 (1.8)                           | 682 ± 49 (9.4)                           |
| group 2 (interaction with pol2-04 and pol3-01) |                                         |                                          |                                          |                                          |
| + mrt1-A245V                   | 524 ± 84 (4.5)                          | 1005 ± 105 (3.9)                         | 123 ± 21 (0.7)                           | 30 ± 7 (0.4)                             |
| + mrt1-G271S                   | 711 ± 106 (6.1)                         | 1426 ± 97 (5.5)                          | 343 ± 59 (2.0)                           | 101 ± 10 (1.4)                           |
| + mrt1-Y285C                   | 950 ± 76 (8.1)                          | 1662 ± 43 (6.4)                          | 166 ± 40 (1.0)                           | 74 ± 4 (1.0)                             |
| group 3 (interaction with pol2-04, pol3-01 and msh2Δ) |                                         |                                          |                                          |                                          |
| + mrt1-D226G                   | 521 ± 43 (4.5)                          | 896 ± 73 (3.4)                           | 204 ± 18 (1.2)                           | 238 ± 22 (3.3)                           |
| + mrt1-D226V                   | 565 ± 11 (4.8)                          | 945 ± 8 (3.6)                            | 200 ± 0 (1.2)                            | 235 ± 8 (3.2)                            |
| + mrt1-D226N,S117P             | 338 ± 44 (2.9)                          | 599 ± 66 (2.3)                           | 149 ± 4 (0.9)                            | 169 ± 23 (2.3)                           |
| + mrt1-S242T                   | 559 ± 30 (4.8)                          | 1033 ± 39 (4.0)                          | 87 ± 3 (0.5)                             | 358 ± 51 (4.9)                           |
| + mrt1-R256H,Y779C             | 1155 ± 80 (9.9)                         | 1771 ± 80 (6.8)                          | 121 ± 3 (0.7)                            | 303 ± 13 (4.2)                           |
| + mrt1-G267C                   | 548 ± 18 (4.7)                          | 1030 ± 28 (4.0)                          | 141 ± 7 (0.8)                            | 583 ± 39 (8.0)                           |
| + mrt1-S269P                   | 2135 ± 273 (18.3)                       | 3032 ± 338 (11.6)                        | 340 ± 70 (2.0)                           | 312 ± 2 (4.3)                            |
| group 4 (interaction with pol2-04, pol3-01 and msh2Δ and mutator in EXO1-WT) |                                         |                                          |                                          |                                          |
| + mrt1-K243E                   | 765 ± 36 (6.6)                          | 1331 ± 76 (5.1)                          | 259 ± 7 (1.5)                            | 968 ± 61 (13.3)                          |
| + mrt1-I262T,M275I             | 720 ± 50 (6.2)                          | 1163 ± 87 (4.5)                          | 168 ± 6 (1.0)                            | 536 ± 86 (7.4)                           |
| + mrt1-I262V,N291D             | 404 ± 35 (3.5)                          | 852 ± 22 (3.3)                           | 140 ± 14 (0.8)                           | 780 ± 16 (10.7)                          |

*dNTP concentrations (pmol per 10^8 cells) are the average of two biological replicates ± standard deviation with the fold increase over WT in parentheses.*
Supplementary Table S3. rNTP and dNTP concentrations in strains containing *rnr1* mutant alleles integrated at the endogenous *RNR1* locus.

### A

| Relevant genotype | CTP           | UTP           | ATP           | GTP           |
|-------------------|---------------|---------------|---------------|---------------|
| WT                | 2195 ± 18 (1.0) | 5449 ± 93 (1.0) | 11386 ± 363 (1.0) | 3473 ± 10 (1.0) |
| "overall increased" |               |               |               |               |
| *rnr1*-F15S       | 2110 ± 103 (1.0) | 5411 ± 111 (1.0) | 11773 ± 169 (1.0) | 3519 ± 42 (1.0) |
| *rnr1*-D57N       | 2167 ± 12 (1.0) | 5376 ± 212 (1.0) | 11754 ± 178 (1.0) | 3455 ± 64 (1.0) |
| "2 out of 4"      |               |               |               |               |
| *rnr1*-A245V      | 1997 ± 33 (0.9) | 5384 ± 152 (1.0) | 11725 ± 165 (1.0) | 3730 ± 4 (1.1) |
| *rnr1*-Y285C      | 2004 ± 34 (0.9) | 5322 ± 84 (1.0) | 11916 ± 77 (1.0) | 3702 ± 132 (1.1) |
| "3 out of 4"      |               |               |               |               |
| *rnr1*-S242T      | 2125 ± 14 (1.0) | 5804 ± 62 (1.1) | 11751 ± 96 (1.0) | 3246 ± 47 (0.9) |
| *rnr1*-R256H, Y779C | 2325 ± 22 (1.1) | 5094 ± 124 (0.9) | 11370 ± 251 (1.0) | 3468 ± 30 (1.0) |
| "3 out of 4 with extra high dGTP" | |               |               |               |
| *rnr1*-K243E      | 2283 ± 6 (1.0) | 5312 ± 803 (1.0) | 12946 ± 1484 (1.1) | 2953 ± 85 (0.9) |
| *rnr1*-I262V,N291D | 1907 ± 435 (0.9) | 5039 ± 333 (0.9) | 14892 ± 12222 (1.3) | 3152 ± 118 (0.9) |

### B

| Relevant genotype | dCTP           | dTTP           | dATP           | dGTP           |
|-------------------|---------------|---------------|---------------|---------------|
| WT                | 146 ± 18 (1.0) | 292 ± 27 (1.0) | 158 ± 17 (1.0) | 80 ± 7 (1.0) |
| "overall increased" |               |               |               |               |
| *rnr1*-F15S       | 972 ± 99 (6.7) | 1672 ± 116 (5.7) | 1151 ± 145 (7.3) | 522 ± 58 (6.6) |
| *rnr1*-D57N       | 521 ± 181 (3.6) | 984 ± 250 (3.4) | 640 ± 236 (4.0) | 294 ± 99 (3.7) |
| "2 out of 4"      |               |               |               |               |
| *rnr1*-A245V      | 1057 ± 71 (7.2) | 1712 ± 130 (5.9) | 69 ± 2 (0.4) | 54 ± 4 (0.7) |
| *rnr1*-Y285C      | 1304 ± 48 (8.9) | 2226 ± 62 (7.6) | 139 ± 2 (0.9) | 114 ± 2 (1.4) |
| "3 out of 4"      |               |               |               |               |
| *rnr1*-S242T      | 935 ± 44 (6.4) | 1596 ± 66 (5.5) | 133 ± 14 (0.8) | 762 ± 45 (9.6) |
| *rnr1*-R256H, Y779C | 481 ± 20 (3.3) | 784 ± 16 (2.7) | 80 ± 3 (0.5) | 221 ± 3 (2.8) |
| "3 out of 4 with extra high dGTP" | |               |               |               |
| *rnr1*-K243E      | 1796 ± 123 (12.3) | 2891 ± 292 (9.9) | 536 ± 7 (3.4) | 1656 ± 28 (20.8) |
| *rnr1*-I262V,N291D | 404 ± 88 (2.8) | 869 ± 10 (3.0) | 190 ± 1 (1.2) | 1365 ± 290 (17.1) |

rNTP (A) and dNTP (B) concentrations (pmol per 10⁸ cells) are the average of two biological replicates ± standard deviation with the fold increase over WT in parentheses.
**Supplementary Table S4.** CAN1 mutation spectra in strains carrying *rnr1* mutant alleles.

| Mutants sequenced | WT ‡ | *rnr1*-Y285C | *rnr1*-R256H,Y779C | *rnr1*-I262V,N291D |
|-------------------|------|--------------|---------------------|---------------------|
| 91                | 93   | 96           | 96                  |

| Mutations total | 92 (100) | 94 (100) | 96 (100) | 98 (100) |

| Base substitutions |     |     |     |     |
|--------------------|-----|-----|-----|-----|
| A-T → G-C          | 69  | 80  | 55  | 18  |
| G-C → A-T          | 6   | 14  | 9   | 2   |
| G-C → T-A          | 29  | 5   | 6   | 0   |
| A-T → C-G          | 3   | 20  | 6   | 1   |
| A-T → T-A          | 7   | 28  | 6   | 2   |
| C-G → G-C          | 6   | 4   | 11  | 0   |

| Transitions        | 24  | 23  | 26  | 15  |
|                   | 45  | 57  | 29  | 3   |

| Transversions      | 15  | 12  | 30  | 80  |

| One-base-pair frameshifts |     |     |     |     |
|---------------------------|-----|-----|-----|-----|
| ΔA/T                      | 5   | 9   | 25  | 79  |
| ΔG/C                      | 3   | 3   | 4   | 0   |
| +A/T                      | 6   | 0   | 1   | 1   |
| +G/C                      | 1   | 0   | 0   | 0   |

| Complex †             | 8   | 2   | 11  | 0   |

Mutation spectra analysis based on DNA sequencing of the CAN1 gene in independent CanISTR mutants, shown as the number of clones containing the indicated mutations, and in parenthesis as the percentage relative to the total.

* In few cases (about 1-2% of the sequenced clones) two simultaneous CAN1 mutations (more than 100 bp apart) were found. These mutations were included in the analysis and considered as independent mutational events.

† Includes: multiple mutations within ten nucleotides, insertions or deletions of more than one nucleotide and duplication events.

‡ CAN1 mutation spectrum of WT strain was taken from (17).
**Supplementary Table S5.** CAN1 mutation hotspots identified in strains carrying rnr1 mutant alleles.

| Position | Mutation | No of occurrences | Mutation rate (x10^-8) | Fold increase over WT | Predicted intermediate |
|----------|----------|-------------------|-------------------------|-----------------------|------------------------|
| **rnr1-Y285C**: 8.9 x dCTP, 7.6 x dTTP, 0.9 x dATP, 1.4 dGTP; CAN_R = 3.2 x 10^-7 (4) |
| 538      | A -> C   | 10 / 94           | 3.4                     | ≥36                   | 5'TCCCTTTTGGCGCC      |
|          |          |                   |                         |                       | TAGTGAAAAACGGG5'      |
| 680      | A -> T   | 10 / 94           | 3.4                     | ≥36                   | 5'TCGTTTTTGGG      |
|          |          |                   |                         |                       | AGCTCAAGACCCA5'       |
| 946      | T -> C   | 5 / 94            | 1.7                     | ≥18                   | 5'CCCAGAAAAATCCG      |
|          |          |                   |                         |                       | GGGTCTTTTTGGGC5'      |
| **rnr1-R256H,Y779C**: 3.3 x dCTP, 2.7 x dTTP, 0.5 x dATP, 2.8 dGTP; CAN_R = 9.5 x 10^-8 (1) |
| 964-969  | ΔA       | 6 / 96            | 0.6                     | 6                     | 5'CAAAAAAGTGGTTTTTTT  |
|          |          |                   |                         |                       | GTTTTCAACAATAAG5'     |
| 1381-1386 | ΔT     | 9 / 96            | 0.9                     | 9                     | 5'GTTTCAAGGCTTTTTTCC |
|          |          |                   |                         |                       | CAAAGTCGCCAAAAAC5'     |
| **rnr1-I262V,N291D**: 2.8 x dCTP, 3.0 x dTTP, 1.2 x dATP, 17.1 dGTP; CAN_R = 1.4 x 10^-5 (164) |
| 964-969  | ΔA       | 63 / 98           | 900                     | 9517                  | 5'CAAAAAAGTGGTTTTTTT  |
|          |          |                   |                         |                       | GTTTTCAACAATAAG5'     |

Mutations are shown relative to the coding strand. The predicted mutation is noted in red. Nucleotides incorporated after the mutation from dNTPs at higher concentrations than WT, are shown in green. dNTP levels are shown as fold over WT and CAN1 inactivation rate as median, with fold increase relative to WT in parentheses. A mutation hotspot is defined as a specific mutation found in more than 5% of all sequenced CAN_R clones in the indicated genotype. Mutation hotspots that are significant different to the WT control (Fisher’s exact test, Benjamini and Hochberg corrected p-value ≤ 0.05) are shown in bold.
Supplementary Table S6. *URA3* mutation spectrum in *rnr1-l262V,N291D* mutant strain.

|                        | WT‡ | *rnr1-l262V,N291D* |
|------------------------|-----|-------------------|
| Mutants sequenced      | 207 | 131               |
| Mutations total*       | 207 (100) | 100 (100)         |
| Base substitutions     |     |                   |
| A-T → G-C              | 4 (1.9) | 53 (53.0)        |
| G-C → A-T              | 42 (20.3) | 2 (2.0)         |
| G-C → T-A              | 68 (32.9) | 1 (1.0)        |
| A-T → C-G              | 11 (5.3) | 2 (2.0)          |
| A-T → T-A              | 22 (10.6) | 10 (10.0)      |
| C-G → G-C              | 20 (9.7) | 3 (3.0)          |
| Transitions            | 46 (22.2) | 55 (55.0)      |
| Transversions          | 121 (58.5) | 16 (16.0)      |
| One-base-pair frameshifts |     |                   |
| ΔA/T                   | 11 (5.3) | 25 (25.0)       |
| ΔG/C                   | 11 (5.3) | 1 (1.0)         |
| +A/T                   | 2 (1.0) | 0 (0.0)         |
| +G/C                   | 1 (0.5) | 0 (0.0)         |
| Complex†               | 15 (7.2) | 3 (3.0)        |

Mutation spectra analysis based on DNA sequencing of the *URA3* gene independent 5-FOA® mutants, shown as the number of clones containing the indicated mutations, and in parenthesis as relative percentage.

* In few cases (about 1-2% of the sequenced clones) two simultaneous *URA3* mutations (more than 100 bp apart) were found. These mutations were included in the analysis and were considered as independent mutational events.

† Includes: multiple mutations within ten nucleotides, insertions or deletions of more than one nucleotide and duplication events.

‡ *URA3* mutation spectrum of WT strain was taken from (42).
Supplementary Table S7. *S. cerevisiae* strains used in this study.

| Name       | Relevant genotype * | Reference         |
|------------|---------------------|-------------------|
| RDKY3686   | MAT ara3-52 leu2Δ1 trp1Δ63 hom3-10 his3Δ200 lys2-10A | Ref. 16       |
| RDKY5964   | MAT ara3-52 leu2Δ1 trp1Δ63 hom3-10 his3Δ200 lys2-10A | Ref. 19       |
| HHY6551    | RDKY5964 mrr1::kanMX4 + pHHB560 (pRS316-RNR1) | This study   |
| HHY6553    | HHY6551 dun1::hphNT1 | This study  |
| HHY6214    | HHY6551 exo1::hphNT1 | This study  |
| HHY6555    | HHY6551 exo1::hphNT1 lg4::HIS3 | This study   |
| HHY6556    | HHY6551 msh2::HIS3 | This study    |
| HHY6562    | HHY6551 pol2-04.natNT2 | This study  |
| HHY6566    | HHY6551 pol3-01.natNT2 | This study |
| HHY6570    | HHY6551 mrr3::hphNT1 | This study |
| HHY6572    | RDKY5964 mrr1::HIS3 | This study |
| HHY6574    | RDKY5964 mrr1-F15S::HIS3 | This study  |
| HHY6578    | RDKY5964 mrr1-D57N::HIS3 | This study |
| HHY6580    | RDKY5964 mrr1-S242T::HIS3 | This study |
| HHY6582    | RDKY5964 mrr1-K243E::HIS3 | This study |
| HHY6584    | RDKY5964 mrr1-A245V::HIS3 | This study |
| HHY6586    | RDKY5964 mrr1-R256H,Y779C::HIS3 | This study |
| HHY6588    | RDKY5964 mrr1-I262V,N291D::HIS3 | This study |
| HHY6596    | RDKY5964 mrr1-Y285C::HIS3 | This study |
| HHY1794    | RDKY5964 exo1::hphNT1 | Ref. 17    |
| HHY6598    | HHY1794 mrr1-F15S::HIS3 | This study |
| HHY6602    | HHY1794 mrr1-D57N::HIS3 | This study |
| HHY6604    | HHY1794 mrr1-S242T::HIS3 | This study |
| HHY6606    | HHY1794 mrr1-A245V::HIS3 | This study |
| HHY6608    | HHY1794 mrr1-R256H,Y779C::HIS3 | This study |
| HHY6610    | HHY1794 mrr1-I262V,N291D::HIS3 | This study |
| HHY6618    | HHY1794 mrr1-Y285C::HIS3 | This study |
| RDKY7588   | RDKY5964 pms1-4xGFP.kanMX6 | Ref. 19  |
| HHY6622    | RDKY7588 mrr1-F15S::HIS3 | This study |
| HHY6754    | RDKY7588 mrr1-D57N::HIS3 | This study |
| HHY6624    | RDKY7588 mrr1-S242T::HIS3 | This study |
| HHY6756    | RDKY7588 mrr1-K243E::HIS3 | This study |
| HHY6758    | RDKY7588 mrr1-A245V::HIS3 | This study |
| HHY6626    | RDKY7588 mrr1-R256H,Y779C::HIS3 | This study |
| HHY6628    | RDKY7588 mrr1-I262V,N291D::HIS3 | This study |
| HHY6632    | RDKY7588 mrr1-Y285C::HIS3 | This study |
| HHY6634    | RDKY5964 mrr1-I262V,N291D::HIS3 ura3-52::URA3 | This study |

* All strains derived from S288C. The genotype corresponds to the listed strain with the indicated modifications.
Supplementary Table S8. Plasmids used in this study.

| Name   | Relevant genotype | rnr1 base substitution (s) | Reference |
|--------|-------------------|---------------------------|-----------|
| pRS316 | amp’ CEN6 ARSH4 URA3 | none                      | Ref. 21   |
| pHHB682 | pRS316-RNR1 (amp’, CEN6, ARSH4, URA3) | none                      | This study |
| pRS315 | amp’ CEN6 ARSH4 LEU2 | none                      | Ref. 21   |
| pHHB651 | pRS315-RNR1 (amp’, CEN6, ARSH4, LEU2) | none                      | This study |
| pHHB649 | pRS315-m1-G8D,V278A (amp’, CEN6, ARSH4, LEU2) | c.23G > A, c.833T > C | This study |
| pHHB632 | pRS315-m1-F155S (amp’, CEN6, ARSH4, LEU2) | c.44T > C                 | This study |
| pHHB635 | pRS315-m1-D226G (amp’, CEN6, ARSH4, LEU2) | c.677A > G                | This study |
| pHHB648 | pRS315-m1-D226V (amp’, CEN6, ARSH4, LEU2) | c.677A > T                | This study |
| pHHB655 | pRS315-m1-S117P,D226N (amp’, CEN6, ARSH4, LEU2) | c.349T > C, c.676G > A | This study |
| pHHB650 | pRS315-m1-I231T,T244A (amp’, CEN6, ARSH4, LEU2) | c.692T > C, c.730A > G | This study |
| pHHB634 | pRS315-m1-S242T (amp’, CEN6, ARSH4, LEU2) | c.724T > A                | This study |
| pHHB628 | pRS315-m1-K243E (amp’, CEN6, ARSH4, LEU2) | c.727A > G                | This study |
| pHHB647 | pRS315-m1-T244I,V278A (amp’, CEN6, ARSH4, LEU2) | c.731C > T, c.833T > C | This study |
| pHHB651 | pRS315-m1-A245V,Q671R (amp’, CEN6, ARSH4, LEU2) | c.734C > T, c.2012A > G | This study |
| pHHB721 | pRS315-m1-A245V (amp’, CEN6, ARSH4, LEU2) | c.734C > T                | This study |
| pHHB630 | pRS315-m1-R256H,Y779C (amp’, CEN6, ARSH4, LEU2) | c.767G > A, c.2336A > G | This study |
| pHHB667 | pRS315-m1-R256H (amp’, CEN6, ARSH4, LEU2) | c.767G > A                | This study |
| pHHB668 | pRS315-m1-R256Q (amp’, CEN6, ARSH4, LEU2) | c.767G > A, c.768T > A | This study |
| pHHB642 | pRS315-m1-I2627T,M2751 (amp’, CEN6, ARSH4, LEU2) | c.785T > C, c.825G > A | This study |
| pHHB678 | pRS315-m1-I262V,N291D (amp’, CEN6, ARSH4, LEU2) | c.784A > G, c.871A > T | This study |
| pHHB677 | pRS315-m1-I262V,Q561L (amp’, CEN6, ARSH4, LEU2) | c.784A > G, c.1682A > T | This study |
| pHHB685 | pRS315-m1-I262V (amp’, CEN6, ARSH4, LEU2) | c.784A > G                | This study |
| pHHB637 | pRS315-m1-T269A (amp’, CEN6, ARSH4, LEU2) | c.793A > G                | This study |
| pHHB638 | pRS315-m1-G267C (amp’, CEN6, ARSH4, LEU2) | c.799G > T                | This study |
| pHHB641 | pRS315-m1-S269P (amp’, CEN6, ARSH4, LEU2) | c.805T > C                | This study |
| pHHB652 | pRS315-m1-G271S (amp’, CEN6, ARSH4, LEU2) | c.811G > A                | This study |
| pHHB653 | pRS315-m1-P274L,N468S (amp’, CEN6, ARSH4, LEU2) | c.821C > T, c.1397A > G | This study |
| pHHB1000 | pRS315-m1-P274L (amp’, CEN6, ARSH4, LEU2) | c.821C > T                | This study |
| pHHB636 | pRS315-m1-M275T (amp’, CEN6, ARSH4, LEU2) | c.824T > C                | This study |
| pHHB633 | pRS315-m1-T282A (amp’, CEN6, ARSH4, LEU2) | c.844A > G                | This study |
| pHHB676 | pRS315-m1-R212C,T282S (amp’, CEN6, ARSH4, LEU2) | c.61C > T, c.844A > T | This study |
| pHHB999 | pRS315-m1-T282S (amp’, CEN6, ARSH4, LEU2) | c.844A > T                | This study |
| pHHB654 | pRS315-m1-A283V,S425L (amp’, CEN6, ARSH4, LEU2) | c.848C > T, c.1274C > T | This study |
| pHHB679 | pRS315-m1-Y285C (amp’, CEN6, ARSH4, LEU2) | c.854A > G                | This study |
| pHHB876 | pRS315-m1-N291D (amp’, CEN6, ARSH4, LEU2) | c.871A > T                | This study |
| pRS306 | amp’ URA3         | none                      | Ref. 21   |
| pHHB424 | pRS306-RNR1 (amp’, URA3) | none                      | This study |
| pHHB718 | pRS306-m1-F155S (amp’, URA3) | c.44T > C                | This study |
| pHHB752 | pRS306-m1-D57N (amp’, URA3) | c.169G > A                | This study |
| pHHB669 | pRS306-m1-S242T (amp’, URA3) | c.724T > A                | This study |
| pHHB682 | pRS306-m1-K243E (amp’, URA3) | c.727A > G                | This study |
| pHHB736 | pRS306-m1-A245V (amp’, URA3) | c.734C > T                | This study |
| pHHB868 | pRS306-m1-R256H,Y779C (amp’, URA3) | c.767G > A, c.2336A > G | This study |
| pHHB933 | pRS306-m1-I262V,N291D (amp’, URA3) | c.784A > G, c.871A > T | This study |
| pHHB695 | pRS306-m1-Y285C (amp’, URA3) | c.854A > G                | This study |