Complex Mutation Profiles in Mismatch Repair and Ribonucleotide Reductase Mutations Reveal Novel Repair Substrate Specificity of MutS Homolog (MSH) Complexes

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Abstract

Determining mutation signatures is standard for understanding the etiology of human tumors and informing cancer treatment. Multiple determinants of DNA replication fidelity prevent mutagenesis that leads to carcinogenesis, including regulation of free deoxyribonucleoside triphosphate (dNTP) pools and the repair of base mismatches. We utilized a targeted deep-sequencing approach to determine mutational signatures associated with mismatch repair (MMR) pathway defects. By combining *rnr1* and *msh* mutations to increase dNTP levels and alter the mutational load, we observed previously unreported specificities of Msh2-Msh3 and Msh2-Msh6. Msh2-Msh3 is uniquely able to direct repair of G/C single base deletions in GC runs, while Msh2-Msh6 specifically directs repair of substitutions at G/C dinucleotides. We also identified broader sequence contexts that influence variant profiles in different genetic backgrounds and found that there was not necessarily a simple additive relationship of mutation profiles in double mutants. Our results have implications for interpreting mutation signatures from human tumors, particularly when MMR is defective.

Introduction

Cancer is a genetic disease caused by mutation accumulation; DNA replication is an important source of mutation. Replicative polymerases, Polε and Polδ, minimize errors via a highly selective nucleotide binding domain to prevent deoxynucleotide triphosphate (dNTP) misincorporation and an exonuclease domain to proofread and remove errors (1-4). Together, these functions lead to error rates on the order of 10^{-7} (3,4). Appropriate levels and ratios of the four dNTPs are also essential for maintaining high fidelity polymerase function. This has been demonstrated in yeast using allosteric mutations in *RNR1* that alter dNTP pools in different ways. *RNR1* encodes the large subunit of the enzyme that catalyzes the rate-limiting step in dNTP synthesis, ribonucleotide reductase (RNR). Even a modest 2-fold increase above normal levels, as seen in *rnr1D57N*, increased nucleotide misincorporation by DNA polymerases and elevated mutation rates (5,6) (**Fig. 1A)**. More pronounced and skewed elevations in dNTP pools are generated in *rnr1Y285F* and *rnr1Y285A*, which increase dCTP and dTTP 3-fold and 20-fold, respectively(7). These increases in dNTP pool levels further compromise replication fidelity (7-10).

Nucleotide misincorporation and DNA slippage events in DNA replication result in DNA structures that are substrates for the mismatch repair (MMR) system, which functions as a spell-check, recognizing and directing the repair of errors in replication (11), thereby increasing the fidelity of replication by an additional 10-1,000 fold (2,3). Once recognized by a MutS homolog (Msh) complex, errors are targeted for excision. In most eukaryotes, two heterodimeric complexes bind errors at the replication fork: Msh2-Msh6 and Msh2-Msh3, which recognize a broad spectrum of mismatches and insertion/deletion loops (IDLs) with different specificities. The current model of post-replicative repair posits that Msh2-Msh3 recognizes, binds and directs repair of IDLs up to 17 nucleotides long (12,13), while Msh2-Msh6 targets mismatches and IDLs of 1-2 nucleotides. Msh2-Msh3 also has affinity for

some mismatches, especially C-C, A-A and (possibly) G-G (14,15). The synergistic effect on mutation rate seen in *rnr1D57N msh2Δ* and *rnr1D57N msh6Δ* (6) and in *rnr1Y285A msh2Δ* strains, (8,10)indicates that mismatch repair normally corrects the errors generated by DNA polymerases when dNTP pools are elevated (**Figure 1B)**.

MMR defects promote tumorigenesis. MMR is deficient in ~25% of sporadic cancers caused by increased rate of mutagenesis (16). Deficiencies in MMR genes *MSH2, MSH6, PMS2* and *MLH1* cause hereditary Lynch syndrome, which leads to a strong predisposition to cancers of the gastrointestinal tract, endometrial cancer and lymphomas, and are defined by microsatellite instability (17,18). MMR mutations are also implicated in breast and ovarian cancer(19,20). While Msh2-Msh3 has not been directly linked to Lynch syndrome, mutations in *MSH3* lead to cancer predisposition within and outside the gastrointestinal tract (21-26) as well as chemoresistant tumors (27-29). Loss of *MSH3* function leads to elevated microsatellite alterations of selected tetranucleotide repeats, which is distinct from microsatellite instability and has been associated with a number of different cancers, including up to ~60% of colorectal cancers (30). Therefore, while *MSH3* also plays a role in tumorigenesis, its role is distinct from that of Msh2-Msh6.

In mammalian cells, characterizing mutation signatures is a powerful tool to identify potential pathways that are implicated in tumorigenesis. However, this analysis rarely considers the likely presence of altered dNTP pools as a mitigating factor. Cancer cells have increased proliferative nature and thus elevated dNTP pools may be necessary to support accelerated DNA replication (31,32), which may, in turn, increase mutagenesis, promote molecular evolution and provide a selective advantage to the tumor. Notably, combining defects in both MMR and dNTP pool synthesis leads to synergistic increases in mutation rates and uncharacterized mutagenic potential(6). We sought to determine how altered dNTP pools influence the mutation signature or “mutation profile” with and without functional

MMR, with an eye to illuminating the interpretation of mutation signatures observed in tumors, i.e. to what extent do altered dNTP pools influence the mutation profile in MMR mutants. At the same time, the altered mutation profiles generated by *rnr1* alleles (33) allowed us to evaluate the role of Msh2-Msh3 and Msh2-Msh6 in directing repair of typically rare replication errors.

For this analysis, we paired selection for mutations at *CAN1* with next-generation sequencing (NGS), allowing higher throughput and increased sequencing depth compared with previous studies (33-35). We built mutation profiles from first principles, characterizing single mutants and then evaluating combinatorial effects on the mutation profiles of double mutants. We determined novel discrete substrates for Msh2-Msh6 and Msh2-Msh3 error recognition. We observed novel, distinct profiles in *rnr1* and *msh* backgrounds and non-additive mutation profiles when these mutations were combined.

Results

Deletion of *MSH* genes alters mutation profiles in distinct ways

To define the mutation profiles associated with specific MMR deficiencies, we individually deleted the *MSH2, MSH6* and *MSH3* genes, which encode subunits of the MMR recognition complexes (**Fig. 1**, **Table S1**), and used a targeted deep sequencing approach to derive mutation profiles from first principles (33). Deletion of *MSH2* effectively eliminates all MMR activity, retaining all errors made by replicative polymerases (36). We also removed only Msh2-Msh6 (*msh6Δ*) or Msh2-Msh3 (*msh3Δ*) to learn more about substrate specificity and repair efficiency of each complex. In general, *msh2Δ, msh3Δ* and *msh6Δ* mutation profiles were consistent with previous studies performed in yeast (**Fig. 2A**) (6,10,13,14,34,35,37), although previous studies focused primarily on *msh2Δ*. For *msh3Δ* and *msh6Δ* studies, sample sizes of less than 100 colonies were sequenced via Sanger sequencing (6,13,14). As a result, there was not sufficient statistical power to make conclusions about specific variant types. For *msh2Δ, msh3Δ* and *msh6Δ* backgrounds, our work significantly increased the number of colonies sequenced and the sequencing coverage. We found that deleting *MSH2* resulted in a relative decrease in single nucleotide variants (SNVs) as a result of increased deletions and insertions compared to wildtype, similar to previous observations (34,35). We particularly noted A/T single base deletions in *msh2Δ* (**Table S3**, **Fig. 2B, C, D)**. In *msh3Δ* cells there was an increase in G/C -1 bp deletions and complex deletions and insertions (> 1 base pair), consistent with the preference of Msh2-3 for binding larger IDLs (38,39). Nonetheless, over 25% of the mutations that accumulated in *msh3Δ* were SNVs (**Table S3**, **Fig. 2A)**, consistent with Msh2-Msh3 also playing a role in correcting misincorporation events (14,37). In contrast, the *msh6Δ* spectrum was dominated by SNVs, which represented >80% of the mutations (**Table S3**, **Fig. 2A,B)**. Although *msh6Δ* resulted in the highest accumulation of SNVs, the *types* of SNVs generated were similar for *msh2Δ* and *msh6Δ* (**Table S3**, **Fig. 2B).** The *msh3Δ* SNV

spectrum consisted of higher levels of CG>GC and TA>AT errors than *msh2Δ* and *msh6Δ*, despite the fact that these errors should be efficiently repaired by Msh2-Msh6 (40,41); TA>AT errors in *msh3Δ* were also higher than observed in wildtype.

Rates of canavanine resistance increase in *rnr1 msh*Δ

In assessing the mutation spectra in *msh* alleles, we are restricted to evaluating the relative contribution of Msh2-Msh3 and Msh2-Msh6 to repair of only those mutations that arise as a result of replication error. Because of the inherent proofreading activity of Polδ and Polε, this rate is low and certain mutations are very rare (42-44). This is particularly interesting, because dNTP pools are likely elevated in cancer cells (31,45) presumably including those caused by defects in MMR. To evaluate the impact of MMR on a broader range of mutations, we took advantage of three *rnr1* alleles, *rnr1D57N*, *rnr1Y285F* and *rnr1Y285A*, which we previously characterized (33). The mutation profile, SNVs, in/dels, and sequence contexts for mutation in each was altered from wild-type, and from each other. In *rnr1D57N*, which increases the pools of all four dNTPs by approximately two-fold, the overall frequency of SNVs was reduced, although the relative proportion of each SNV was similar. At the same time, there was a dramatic increase in G/C single base deletions (G/C-1) and insertions (G/C+1). In *rnr1Y285F*, which increases dCTP and dTTP approximately three-fold, there was a significant increase in SNVs compared to wild-type, primarily a result of an increased frequency of C/G→T/A mutations, and a significant increase in the proportion of G/C-1 events. In *rnr1Y285A*, which increases dCTP and dTTP ten- to twenty-fold, SNVs occurred at a frequency similar to wild-type but with increased C/G→T/A mutations and decreased C/G→G/C, C/G→A/T and T/A→G/C mutations. Therefore, we combined these *rnr1* alleles with *msh2Δ*, *msh3Δ* or *msh6Δ* to assess mutation profiles.

To ensure that the *rnr1 msh* double mutants displayed the anticipated increases in mutagenesis, we measured the forward mutation rates at *CAN1*. The relative mutation rates for *msh* deletions were consistent with previous observations (6,8,34,46), with *msh2Δ* exhibiting the highest mutation rate, *msh6Δ* exhibiting a lower but still increased mutation rate and *msh3Δ* exhibiting a mild mutator effect in this assay (**Table 1**). The *rnr1* mutation rates increased with the increasing dNTP levels displayed by each allele as expected (7), with *rnr1-Y285A* showing a 20-fold increase. When *rnr1D57N* and *rnr1Y285F* were combined with *msh* deletions, the mutation rates exhibited the same hierarchy (*msh2Δ* > *msh6Δ* > *msh3Δ*), but with higher rates than either of the relevant single mutants. In most cases the mutation rate of the double mutant approximated the product of the single mutant mutation rates. The *rnr1Y285F msh6Δ* strain had a particularly large increase, 85-fold wild-type, compared to the expected 19-fold given the mutation rates of the respective single mutants. Notably, *rnr1Y285A msh3Δ* exhibited a strong, synergistic mutator phenotype, indicating that the mutations generated in the presence of *rnr1Y285A* are substrates for Msh2-Msh3-mediated MMR. It is possible that the mutation rates for *rnr1Y285A msh2Δ* and *rnr1Y285A msh6Δ* are underestimates, due to the increased cell death observed with these strains.

Previous work (7,10) characterized *rnr1Y285F*, *rnr1Y285A* (+/- *MSH2)* in backgrounds that carried a silenced *pGAL-RNR1* gene (i.e. grown in the absence of galactose). We previously demonstrated that the mutation profiles of these strains were comparable to *rnr1Y285F* and *rnr1Y295A* without the *pGAL-RNR1* construct (33). They are included here, with and without *MSH* genes to provide a comparison with that previous work. In general, *pGAL-RNR1* did not impact the observed mutation profile (see below).

Synthetic genetic interaction of *rnr1Y285A* with MMR factors

The combined effect of *rnr1* alleles and *msh* deletions on mutation rates suggested that these genes both contribute to increased replication fidelity, and that therefore they might show synergistic effects on cell fitness. To test this possibility, we performed synthetic genetic array (SGA) analysis using each of the three *rnr1* alleles (**Fig. 3** and **Table S4-S10**). The *rnr1Y285A* query had the greatest number of genetic interactions, consistent with the Y285A mutation having the greatest effect on dNTP levels. The three *rnr1* alleles showed surprisingly little overlap in their genetic interactions (**Fig. 3A**), supporting the idea that different dNTP levels and pool balances stress cells in different ways. Both the *rnr1Y285A* screen and the *rnr1D57N* screen showed enrichment for the GO term 'DNA replication' (**Fig. 3B**) in addition to displaying unique enrichments for 'maintenance of DNA repeat elements' and 'DNA repair' (*rnr1Y285A*) and 'lagging strand replication' (*rnr1D57N*). Despite having interactions with several DNA replication genes, the *rnr1Y285F* screen did not show any statistically supported GO term enrichment. To further assess the functional properties of each *rnr1* allele genetic interactions, we applied spatial analysis of functional enrichment (SAFE) (47) to determine if any regions of the functional genetic interaction similarity yeast cell map (48) are over-represented for the negative genetic interaction gene sets (**Fig. 3C**). We found a statistically supported over-representation of the negative interacting genes in the DNA replication and repair neighborhood of the genetic interaction cell map for all three *rnr1* alleles, indicating that dNTP pool alterations impinge most dramatically on the DNA replication and DNA repair capacity of the cell. As with GO term enrichment, differences among the three *rnr1* alleles were also apparent in the SAFE analysis, with *rnr1Y285A* and *rnr1D57N* interactors being over-represented in the chromatin neighborhood, and *rnr1Y285F* and *rnr1Y285A* interactors showing more over-representation in the mitosis neighborhood than did *rnr1D57N* interactors.

Most notably, we found that almost the entire MMR pathway (*MSH2*, *MSH3*, *MSH6*, *MLH1*, *PMS1* and *EXO1*) was identified specifically in the *rnr1Y285A* screen and the 'mismatch repair' GO term was very strongly and specifically enriched in the *rnr1Y285A* screen (**Fig. 3C**), indicating a strong requirement for the mismatch repair pathway when dNTP pools are extremely unbalanced. We validated the specificity of the genetic interaction between MMR and *rnr1Y285A* by performing tetrad analysis of *msh6*Δ crosses with each of the three *rnr1* alleles (**Fig. 3D**). Fitness defects in double mutant colonies were only evident in the *rnr1Y285A* cross. In tetrad analysis, *msh6*Δ, and particularly *msh2*Δ showed stronger fitness defects when combined with *rnr1Y285A* than did *msh3*Δ (**Fig. 3E**), consistent with the reduced viability observed in mutation rate experiments. Our genetic interaction data indicate that all three *rnr1* alleles interface with DNA replication and repair pathways, and that *rnr1Y285A* might be expected to have a particularly dramatic effect in MMR deficient cells.

Mutation profiles are systematically genotype-specific

In our targeted NGS approach we extracted genomic DNA from pools of up to 2,000 canavanine resistant (Can^R) colonies (selected samples) for each different genetic background. These samples were subjected to next generation sequencing on the *CAN1* gene. The total variant frequency for all selected samples ranged from ~80% to >100%, with an average of 99.35%, while the total variant frequency in permissive (non-selected) samples was, on average 8.7% (**Fig. S1A**). We developed a permissive variant filter, based on the average frequency of individual variants in permissive samples (grown in the absence of selection) and the positions in which they occurred (33). The permissive filter did not have a large effect on mutation spectra across the different genotypes, consistent with the filter removing noise in our assay (**Fig. S1**).

NGS of a single gene provided enough sequencing depth to determine mutation spectra for 150 unique samples, representing over 30 different genotypes, and including biological and technical

replicates. *CAN1* was sequenced at an average depth of approximately 16,000 reads per base in *CAN1* per sample allowing for detailed characterization of mutation spectra. We characterized mutation events in two ways (**Table S3**): 1) the presence of a particular variant above background, i.e., the number of different C>A changes along *can1* ("unique counts") (Counts in **Table S3**) and 2) the frequency at which each of these unique variants occurred, i.e., the combined frequency of all C>A observed changes along *can1* ("sum of frequencies") (Freq. in **Table S3**). These analyses allowed us to determine whether different *types* of mutations occurred in a genotype-dependent manner, independent of frequency, and whether variant frequencies were altered in a significant way (Counts/Freq. in **Table S3**). For example, a decreased number for "unique counts" combined with unchanged or increased "sum of frequencies" would indicate that variant type is more localized, possibly indicating a mutational hotspot (e.g. *rnr1Y285A msh3Δ* in **Table S3**).

We used Spearman rank correlation and cluster analysis to determine the quantitative relationship between mutation profile and genotype. All unique variants for all genotypes were assessed in parallel, based on both the presence and frequency of unique variants, as described above. In general, biological replicates of the same genotype clustered because their mutational profiles were highly correlated (**Fig. S2**). Biological replicates from strains with lower mutation rates (e.g. wild-type, *msh3Δ*, *rnr1D57N*) were less well correlated than those from strains with higher mutation rates (e.g., *msh2Δ, msh6Δ, rnr1Y285A*), indicating that mutation events occurred more systematically (less stochastically) in these genetic backgrounds, consistent with specific mutation profiles. For example, the average correlation of all 7 wildtype biological replicates with all other samples was r_s = 0.192, while the average correlation within the 7 wildtype biological replicates was r_s = 0.191. In contrast, the *rnr1Y285A msh3Δ* biological replicates were much more highly correlated to one another (r_s = 0.527) than they were with all other samples (r_s = 0.264). Because of the observed positive correlations for

biological replicates within a genotype from each genetic background, we combined variants from all biological replicates within a genotype for the remainder of our analyses. Importantly, *rnr1Y285F* and *rnr1Y285F pGAL-RNR1* biological replicates were well-correlated, as were *rnr1Y285A* and *rnr1Y285A pGAL-RNR1*(**Fig. S2, box II, Fig. S3**), indicating little effect of the *pGAL-RNR1* construct on mutation profiles.

The distribution and cumulative frequency of variant types differed significantly between genotypes, indicating unique mutations profiles (**Table S3**). For instance, *msh6Δ* exhibited the highest proportion of unique events contributing to the mutation spectrum (Counts/Freq. = 1.62; **Table S3**). In contrast, *rnr1Y285F msh3Δ* and *rnr1Y285A msh3Δ* exhibited the lowest proportion of unique variants; the mutation spectra were instead dominated by G/C single base deletions, which occur at high frequencies (**Table S3**). Hierarchical cluster analysis using Spearman rank correlations based on the profile of unique variants between genotypes was consistent with genotype-specific mutation profiles (**Fig. 4A, Fig. S2)**, as was principal component analysis (PCA) based on unique variants (**Fig. 4B**). Overall, these results indicated that it is possible to distinguish between genotypes based on unique variant profiles observed from *can1* deep sequencing.

***rnr1* alleles combined with MMR deletion result in unique mutation profiles**

The *rnr1* alleles each exhibited a unique mutation profile, but these strains retained MMR, which modified that mutation profile based on MMR specificity and efficacy. We deleted *MSH2* in combination with the *rnr1* alleles, eliminating all MMR activity, to determine how altered dNTP pools affected replication fidelity at the level of the polymerases. When *msh2Δ* was combined with each *rnr1* allele, distinct mutation profiles were observed (**Table S3**, **Fig. 2A**). Although all mutation classes were observed in *rnr1Y285F* and *rnr1Y285A* alone, the profiles were biased toward G/C single base

deletions, CG>TA and CG>AT SNVs, and A/T single base deletions. Notably, the profiles in the double mutants (*rnr1Y285F msh2Δ* and *rnr1Y285A msh2Δ*) were almost completely dominated by these four types of errors (**Table S3**, **Fig. 2)**. These profiles indicated that polymerases generated these errors at extremely high frequencies, under even only modestly skewed elevations in dCTP and dTTP (*rnr1Y285F*) and began to saturate MMR activity and/or are poorly repaired by MMR.

Deleting *MSH6* in combination with *rnr1* alleles, so that only Msh2-Msh3-mediated MMR was present, also resulted in unique shifts in mutagenesis across *can1* (**Table S3**, **Fig. 2A)**. The effect was most dramatic in *rnr1Y285F msh6Δ* with a profile that was almost completely dominated by CG>TA and CG>AT SNV errors (**Table S3**, **Fig. 2B, C)**, indicating that Msh2-Msh3 is not efficient in correcting these mismatches. This effect was less apparent in *rnr1Y285A msh6Δ*, where the majority of the variant classes were still observed. However, in both the *rnr1Y285F msh6Δ* and *rnr1Y285A msh6Δ* double mutants there was almost a complete loss of the G/C -1 bp deletions that were observed at an increased frequency in *rnr1Y285F* and *rnr1Y285A* (**Table S3**, **Fig. 2C,D**), consistent with efficient repair of G/C -1 bp slippage events by Msh2-Msh3-mediated MMR. Strikingly, the opposite was observed in *msh3Δ* genotypes. In both *rnr1Y285F msh3Δ* and *rnr1Y285A msh3Δ*, there was a significant increase in G/C -1 bp deletions compared to either single mutant, indicating that Msh2-Msh6 was unable to correct this type of error. Notably, the mutation spectra of the *rnr1Y285F* and *rnr1Y285A* double mutants compared to their *pGAL-RNR1* counterparts were largely consistent (**Table S3**, **Fig. S3**). The most striking difference is an increased frequency in G/C -1 bp deletions in *rnr1Y285A-pGAL msh6Δ,* which is the opposite trend observed in the other *msh6Δ* double mutants. Unlike *rnr1Y285F msh3Δ* or *rnr1Y285A msh3Δ*, in which multiple independent locations across *CAN1* accumulated G/C -1 bp deletions at elevated frequencies, the mutation profile of all *rnr1Y285A-pGAL msh6Δ* replicates was dominated by two different G/C -1 bp deletions at discrete positions (32658 and 32608). These variants

occurred at frequencies greater than 10% in at least two *rnr1Y285A-pGAL msh6Δ* biological replicates and likely represent mutations that occurred early in the growth of liquid culture and were carried through subsequent rounds of replication.

Positions in *CAN1* that are susceptible to mutation vary by genotype

We identified positions within *CAN1* where particular variants occurred in a genotype-specific manner, with varying degrees of susceptibility. The most highly susceptible positions, defined as "hotspots", were positions where a variant occurred in at least 50% of replicates with a frequency 2-fold or greater above wildtype. The highly susceptible *CAN1* positions that we observed in *msh2Δ* overlapped hotspots previously observed (9,10); we noted additional susceptible positions, particularly in repetitive sequences (see below). We also noted positions that were moderately susceptible to mutation in *msh6Δ* and *msh3Δ* backgrounds, which had not been studied in this way previously. Moderately susceptible positions were defined as positions that occurred in more than 1 biological replicate with an average frequency above wildtype. Both *msh2Δ* (49 positions) and *msh6Δ* (96 positions) exhibited more susceptible positions than *msh3Δ* (18 positions), consistent with more stochastic mutations contributing to the mutation spectrum in the absence of *MSH3*. The *msh6Δ* sustained 13 hotspots, all SNVs, which occurred at elevated variant frequencies in 3 or more biological replicates and were not observed in wildtype. *msh6Δ* hotspots occurred almost exclusively in repetitive sequence contexts, including at dinucleotides and in runs 3bp or greater in length. A similar trend was observed in *msh2Δ*, which had a total of 11 hotspots, mostly deletions which occurred in repetitive runs, with few positions overlapping with *msh6Δ*. Thus, individual analysis of *msh2*Δ, *msh3*Δ, and *msh6*Δ revealed distinct genotype-specific mutagenic fingerprints.

Previous work did not detect hot spots in *rnr1Y285F* (8), and *rnr1D57N* has not been assessed (6). We detected susceptible and moderately susceptible positions in these backgrounds. As noted

with *msh* backgrounds, many of these susceptible positions in the *rnr1* backgrounds were associated with repetitive DNA sequences, particularly insertions and deletions.

The susceptible positions in *rnr1Y285A msh2Δ* identified in our study largely overlapped with those previously identified, which were defined as variants occurring at a rate >10-fold higher than wild-type (10,49). The increased sample sizes and depth of sequencing in our study also revealed new susceptible positions. One noteworthy position is a G>T at 32,940, which occurs in A-rich sequence (CCAA**G**AAAA) and is susceptible to mutation in *msh2Δ rnr1Y285A* and *rnr1Y285A-pGAL*. G>A SNVs also occurred in a variety of genotypes at this position, indicating decreased replication fidelity in this context. The variant frequency at this position increased synergistically in *rnr1Y285A-pGAL msh2Δ* and *rnr1Y285A msh2Δ* double mutants, occurring at a frequency at least 10-fold greater than most single mutants. The majority of susceptible positions in *rnr1Y285A msh2Δ* hotspots are also susceptible in *rnr1Y285A* but not *msh2Δ*, indicating that highly skewed/elevated dCTP and dTTP levels promoted specific errors to a level where MMR was invoked, and approached saturation of the MMR system. By contrast, *rnr1Y285A msh6Δ* and *rnr1Y285A msh3Δ* deletions showed more unique mutational fingerprints.

Insertions and deletions occur in distinct sequence contexts depending on genotype

In general, susceptible *CAN1* positions tended to be in or near repetitive sequences and it is well established that insertion and deletion events in particular occur more frequently in repetitive runs (4,50); our study found similar properties. Within *CAN1*, there are only short homopolymeric runs and A/T repeats are more abundant than G/C repeats: 266 AA/TT dinucleotides versus 160 GG/CC dinucleotides; 98 mononucleotide runs of 2 or more A/T bases versus only 32 G/C runs. These ratios are consistent with our observation that more A/T insertion and deletion events occurred in A/T

repeats than in G/C repeats in wildtype (**Fig. 2**). 96.1% of all A/T -1 bp deletions occurred in a repetitive run of 3 bp or greater. 89.6% of G/C deletions occurred in a repetitive run of 2 or more nucleotides. Notably, some G/C deletions occurred adjacent to repetitive runs in specific *rnr1* backgrounds. For example, a GΔ at position 31971 occurred in 4 out of 7 *rnr1D57N* biological replicates, between an A dinucleotide and C dinucleotide (ATAA**G**CCAA). CΔ deletions at positions 32398 (GAAA**C**GTAG) and 32630 (CCTT**C**GTTTT) were specific to *rnr1Y285A*. A GΔ at position 33018 was specific to *msh3Δ*; it is not flanked by a repetitive run but dinucleotides are nearby upstream and downstream (GGAT**G**TAAC).

Complex insertions and deletions (i.e. involving more than one nucleotide) were rare, but occurred at increased frequency in *msh3Δ* (**Fig. 5**). The majority of these complex events, especially insertions, were observed in a single biological replicate. The complex insertion of CT in the repetitive run stretching from positions 33206- 33215 (CTTAAG**CT**CTCTC) is noteworthy. It was observed almost exclusively in *msh2Δ* genotypes, and more frequently when paired with *rnr1* alleles. Increased CT insertion in *rnr1 msh2*Δ genotypes indicates that positions 33206- 33215 were particularly susceptible to mutation when dNTPs were elevated, even by a small amount as is the case in *rnr1D57N*. However, the CT insertion was very efficiently repaired by MMR, via either Msh2-Msh3 or Msh2-Msh6 directed repair as it was not observed in either *msh3Δ* or *msh6Δ*.

SNVs in trinucleotide context reveal unique mutation signatures

To gain mechanistic information about sequence context we analyzed SNVs and the nucleotides 3' and 5' of the variant. We determined the average number of times an SNV was observed in a particular triplet context per genotype, normalized by the number of times the triplet context occurs in *CAN1* (**Fig. 6)**. Notably, in all genotypes C→T changes (red bars, **Fig. 6**), particularly in G**C**C and G**C**G

sequence contexts, dominated. The proportion of G**C**C and G**C**G changes increased with altered dNTPs, most dramatically in *rnr1Y285F* and *rnr1Y285A* samples (note change in y-axis), which were highly correlated (r_s = 0.7284). The G**C**C and G**C**G sequence contexts are mutated in *rnr1Y285A*, as detected by whole genome sequencing (9) possibly as a result of increased extension after misincorporation of dTTP, which is in excess, superseding proofreading (51).

The wildtype SNV trinucleotide pattern was highly correlated with that of *msh3Δ* (r_s = 0.7351), *rnr1D57N msh3Δ* (r_s = 0.6752) and *rnr1D57N* (r_s = 0.6682). We noted that no C>T SNVs occurred in G**C**T context in these four genotypes, while they were observed in all *rnr1Y285A* and *rnr1Y285F* double mutant backgrounds, still at relatively low frequencies. This example shows an error that was specific to skewed increases in dCTP and dTTP and was efficiently repaired by either Msh2-Msh3 or Msh2-Msh6.

We also observed a decrease in the number of unique SNVs that occurred in trinucleotide context when *rnr1Y285F* or *rnr1Y285A* alleles were paired with *msh2Δ, msh3Δ,* or *msh6Δ*. Calculation of Spearman correlation coefficients of each SNV in unique trinucleotide context revealed that these genotypes are also highly correlated to one another, with *rnr1Y285F/A msh6Δ* or *msh2Δ* double mutants showing the highest correlation values (**Fig. S4**). For example, the spectrum of SNVs in trinucleotide context of *rnr1Y285A msh2Δ* was most highly correlated to *rnr1Y285F-pGAL RNR1 msh2Δ* (0.8616)*, rnr1Y285F msh6Δ* (0.8595), *rnr1Y285F msh2*Δ (0.8458)*, rnr1Y285F pGAL-RNR1 msh6Δ* (0.8197), *rnr1D57N msh6Δ (0.7686), rnr1Y285A msh6Δ* (0.7662) and *rnr1Y285A pGAL msh2Δ* (0.7640)*,* descending in value. We observed a complete loss of C>G SNVs in all trinucleotide contexts in *rnr1Y285A msh3Δ, rnr1Y285A msh6Δ,* and *rnr1Y285F msh6Δ* backgrounds, as noted above (**Fig. 2)**. The C>G variant rarely occurred in *msh2Δ* backgrounds, indicating that the replicative polymerases rarely generate these errors. It is noteworthy that double mutant profiles often showed distinct changes in

the SNV signatures, compared to either single mutant. For example, *rnr1Y285A* sustained a number of C>G mutations in multiple trinucleotide contexts, which were also observed in *rnr1Y285A msh2Δ,* but these were completely absent in *rnr1Y285A msh3Δ* and *rnr1Y285A msh6Δ*. Thus, there were combinatorial effects on the mutation spectra of specific MMR deletions in the presence of *rnr1* alleles, and different *rnr1* alleles paired with different MMR deletions result in distinct and unique mutational fingerprints.

Extended sequence context reveals sequence specificity of Msh2-Msh3 and Msh2-Msh6 error recognition

The depth of sequence within *can1* in single and double mutants allowed us to evaluate potential synergistic (combinatorial) effects of *rnr1* and *msh* alleles on mutation spectra. We performed hierarchical cluster analysis of all unique errors in our entire sample set to evaluate potentially larger sequence contexts that could drive distinct mutational signatures (**Fig. 7)**. Genotypes were clustered based on the types of variants that were differentially enriched as a function of genotype, using Pearson correlation (Spearman correlation analysis yielded similar results) (**Fig. 7**). It is worth noting that overall, clusters were similar to those observed in **Fig. 4**, based on variant frequencies, which suggests that unique variants were the main drivers of genotype-specific mutation profiles. We identified differentially enriched variants and performed motif analysis (12 base window) to determine whether broader sequence context influenced the occurrence of these variants, indicating the same biological mechanism.

Differentially enriched, genotype-specific variants occurred in distinct sequence contexts. Some of the clusters with the most pronounced differential enrichment between genotypes were SNVs that

occurred in CC dinucleotide context (**Fig. 7**). CC/GG dinucleotides bordered by G on either the 5' or the 3' side were frequently mutated in the presence of *rnr1* alleles, particularly *rnr1Y285F* and *rnr1D57N,* but not with *msh* alleles (**Fig. 8)**. MMR status, (especially *msh3Δ* or *msh6Δ*) in combination with these *rnr1* alleles appeared to reverse this bias with negative enrichment. The majority of SNVs that occurred in other CC dinucleotide contexts were positively enriched in *msh2Δ* and *msh6Δ* samples, but negatively enriched in *msh3Δ* (**Fig. 8 II, III, & IV)**, indicating that Msh2-Msh6 was uniquely required to recognize and direct repair of misincorporation events in the CC dinucleotide context. CC dinucleotides are frequently mutated in different types of cancer, including human lung cancer (52,53). A defect in Msh2-Msh6-mediated MMR or alteration in free dNTP levels in these cancers may result in synergistic increases in mutagenesis.

We also noted that *msh3Δ*, *rnr1Y285F* and *rnr1Y285A* single mutants showed an increase in G/C single base deletions within specific sequence contexts (**Fig. 9**). The frequency at which these errors occurred increased synergistically in the *rnr1Y285F msh3Δ* and *rnr1Y285A msh3Δ* double mutants (**Fig. 9).** This variant was neutral or negatively enriched in all other genotypes, consistent with apparent specificity for G/C single base deletions in *msh3Δ* backgrounds (**Fig. 2, Table S3**). Some G/C errors along *CAN1* were more specific to *msh3Δ* while others were more specific to *rnr1Y285F* and/or *rnr1Y285A*. Msh2-Msh3 was important for recognizing and directing repair of G/C single base deletions that occurred in homopolymeric runs bordered by G/C rich sequence on the 5' side of the run (**Fig. 9A-C**). G/C runs bordered by A/T nucleotides were more prone to mutagenesis when dCTP and dTTP were increased (**Fig. 9D-F)**. The frequency of these variants directly bordered by A/T increased synergistically when *MSH3* was deleted, but not when *MSH6* was deleted, indicating Msh2-Msh3 has specificity in directing repair of G/C -1 deletions in repetitive G/C context.

Discussion

Utilizing a *CAN1* selection-based deep sequencing approach (33), we characterized mutation spectra in over 30 single and double mutant genetic backgrounds. The sequencing depth afforded by our approach allowed us to expand our understanding of mismatch repair substrate recognition as well as the impact of altered dNTP pools on replication fidelity, particularly by combining mutations in *RNR1* with deletions in MMR genes. By using *rnr1* backgrounds that alter the *type* and *frequency* of mutations sustained, we revealed previously unrecognized specificities for the MMR initiation complexes, Msh2-Msh3 and Msh2-Msh6. The combinatorial effects that we find highlight the importance of studying mutation signatures in different genetic contexts.

Different mechanisms of mutagenesis result from distinct elevations in dNTP levels

In *rnr1D57N msh2Δ* the mutation rate increased 74-fold above wildtype and 3-fold above *msh2Δ*, yet the mutation spectrum of *rnr1D57N msh2Δ* is closely related to *msh2Δ* (**Fig. 3**). The same is true of *rnr1D57N msh3Δ* and *rnr1D57N msh6Δ;* their mutation spectra are most closely related to those of *msh3Δ* and *msh6Δ*, respectively, despite high increases in mutation rates (**Fig. 3, Table 1**). Therefore, the elevated dNTP pools in *rnr1D57N,* which resulted in a mutation spectrum similar to wildtype (33), did not affect the type of mutation generated. The low frequency variants that accumulate in *rnr1D57N* were effectively repaired by MMR, and even the absence of MMR did not result in an overt fitness defect in *rnr1D57N* (**Fig. 3**). We conclude that the balanced dNTP increases in *rnr1D57N* alter mutagenesis but not mutation spectrum, irrespective of the presence or absence of MMR.

Similar to *rnr1D57N*, the *rnr1Y285F* allele has a modest effect on mutation rate, but in contrast, *rnr1Y285F* yields a distinct mutation spectrum especially when paired with MMR deletions. In fact, the

rnr1Y285F spectrum closely resembles that of *rnr1Y285A*, despite *rnr1Y285A* having a higher dNTP skew and higher mutation rate. We conclude that even modest skewed increases in dNTPs (*rnr1Y285F*) result in distinct error accumulation, likely due to both a decrease in selectivity of the replicative polymerases, and to an increase in efficient mismatch extension at the expense of proofreading (8,9). **Fig. 10A** illustrates an example of two different positions in the *rnr1Y285A* background that are mutated at increased frequencies by this mechanism.

Interestingly, the increase in G/C deletions in both *rnr1Y285F* and *rnr1Y285A* can be explained by limiting levels of dGTP (**Fig. 10B)**. dGTP levels are limiting in both yeast and mammalian cells and notably led to distinct patterns of mutagenesis when the concentration decreases further, relative to the increase in the other 3 nucleotides, in *rnr1Y285F* and *rnr1Y285A*. Notably, *rnr1* alleles that cause increases in dGTP are also found to be extremely mutagenic (54), highlighting the importance of maintaining the proper level and relative abundance of dGTP.

New insights into MMR specificity

In assessing mutation spectra and profiles, researchers are limited by the number of errors generated by replicative (and other) DNA polymerases. By altering dNTP pools, we altered the frequency and types of replication errors that occurred, unveiling new specificities in the types of errors that Msh2-Msh3 and Msh2-Msh6 recognize. While G/C SNVs are common in wild-type backgrounds, G/C-1 deletions are relatively rare, making it difficult to determine the relative efficiencies of MSH complexes in repairing this type of error. However, elevating the dNTP pools to any extent increased the ratio of G/C-1:A/T-1 deletions and allowed us to assess MMR function at these sites. In particular, the meta-analysis of our dataset showed that the majority of mutations in *rnr1Y285F*, *rnr1Y285A*, and *msh3Δ* genetic backgrounds, specifically single base deletions, occurred in

G/C rich contexts (**Fig. 9, 10B**). The double mutants *rnr1Y285F msh3Δ* and *rnr1Y285A msh3Δ* exhibited mutation profiles that were completely dominated by G/C-1 deletions. Therefore Msh2-Msh6 was not able to compensate for the loss of Msh2-Msh3 for repair of G/C-1 deletions, despite the fact that both complexes have been implicated in directing repair of single base deletions (55).

It has been previously shown that there are few strand specific effects from altering dNTP levels(10), but numerous studies have indicated that MMR is more efficient on the lagging strand (56,57). It is also known that polymerase δ mutants deficient in proofreading (*pol3-L612M*) accumulate significantly more G/C deletions (4). This leads us to hypothesize that Msh2-Msh3 may have greater specificity for lagging strand DNA repair. Our targeted sequencing approach could be applied on strains with *CAN1* in the reverse orientation to further explore leading versus lagging strand DNA repair specificity of Msh2-Msh3 and Msh2-Msh6.

Similarly, SNVs at GG/CC dinucleotides were more prevalent in *rnr1D57N* and *rnr1Y285F* backgrounds than in wild-type (**Fig. 8**). We demonstrated that mutations in these G/C-rich patches were enriched in *msh2Δ* and *msh6Δ* backgrounds, but depleted in *msh3Δ,* indicating that Msh2-Msh6 directs more efficient repair of mutations in GG/CC dinucleotides than does Msh2-Msh3. GG dinucleotides are mutated as a signature mutation in colorectal cancer (58); cancers which are defined by defects in MMR. Mutations at CC dinucleotides are also found in human lung cancer (52,53) and could be generated due to defects in MMR and elevations in dNTP levels in combination. Notably, RNR subunit overexpression and deletion of *MSH6* in combination caused a synergistic increase in lung carcinogenesis in a mouse model(6). Our sequence context data provide new insight into MMR substrate specificity and sequence context information that influence the relative efficiency of MMR directed by either Msh2-Msh3 or Msh2-Msh6.

Notably, pairing elevations in dNTP levels with MMR deletions led to increased mutation rates and distinct mutation spectra. However, when measuring the rate of canavanine resistance in *rnr1Y285A msh2Δ* and *rnr1Y285A msh6Δ* backgrounds, we observed substantially lower colony numbers under permissive conditions, indicating reduced fitness even under conditions when all cells should be able to grow. Reduced fitness of *rnr1Y285A msh2Δ* and *rnr1Y285A msh6Δ* was also seen in SGA analysis and in tetrad dissections, consistent with the phenomenon of error extinction where the threshold of mutation rate that allows wild type cellular fitness is surpassed (59-61). We expect that the fitness defects we observe in the absence of selection would also reduce the number of cells that are able to grow under the selective conditions of mutation rate experiments, resulting in mutation rates that are underestimates (**Table 1**).

Implications for understanding human cancer mutation signatures

We observed distinct similarities between mutation spectra in specific sequence contexts and particular COSMIC signatures, most notably signatures 6 and 15. Signature 6 most commonly occurs in colorectal and uterine cancers and is associated with defective MMR. It is possible that elevations in dCTP and dTTP contribute to this mutation signature in human cancers leading to synergistic increases in these types of mutations in the absence of certain MMR genes. COSMIC signature 15 is characterized by C>T SNVs within NpCpG sequences in lung and stomach cancers with unknown origin. The origin of these cancers could be in part, due to defects in MMR coupled with skewed increases in dCTP and dTTP, consistent with the increased frequency of these SNVs at dinucleotides observed in *rnr1Y285F/A msh6Δ* backgrounds.

The *msh2Δ, msh3Δ* and *msh6Δ* mutation spectra all had features of MMR deficient human tumor samples (62). Notably, the *msh6Δ* spectrum in our study closely resembles that of *Msh6*$^{-/-}$ in a

HAP1 cell line (63). The percentage of substitutions and IDLs in the *msh6*Δ mutation spectrum is consistent with what is seen in a *Msh6*$^{-/-}$ cell line. While T>C variants did not dominate the *msh6Δ* mutation signature in yeast as it did in the *Msh6*$^{-/-}$ cell line, the overall frequency and proportion of T>C changes did increase significantly. These data indicate that mutation signatures developed through defined mutations and deletions in *S. cerevisiae* will be broadly applicable to mammalian systems. Mutation signatures from *C. elegans* also resemble human cancer signatures, albeit with some minor discrepancies (55,64). We note that *C. elegans* lacks a *MSH3* homolog, which is present in both yeast and humans (65). Our results highlight the importance of considering altered dNTP pools and combinatorial effects of genetic backgrounds, when defining the source of tumor mutation signatures.

Materials and Methods

Strains and plasmids

All strains in this study were derived from the W303 *RAD5+* background (**Table S1**). Strains containing *rnr1Y28F/A* in the *pGAL-RNR1* background and integration plasmids to re-create these strains (7) were kindly provided by Andrei Chabes. To integrate *rnr1* alleles at the endogenous *RNR1* locus in the absence of *pGAL-RNR1*, we created a new set of *rnr1* integration plasmids. *rnr1* alleles were made as previously described (see other paper). Mismatch repair genes were deleted by amplifying *msh2Δ::kanMX, msh6Δ::kanMX,* and *msh3Δ::kanMX* chromosomal fragments from deletion collection strains utilizing primers A and D specific to each locus (**Table S2**). PCR products from these strains were used for transformation to replace the endogenous *MSH* gene with the *kanMX* cassette, which confers resistance to the drug, G418. Transformants were selected on YPD plates containing G418 and deletions were confirmed by PCR.

Measuring mutation rates at *CAN1*

Mutation rates were measured at the *CAN1* locus as previously described (Xu et al. 2008). Briefly, strains were grown on complete media (YPD) until colonies reach 2 mm in size. Colonies were then suspended in 100 ul of 1x TE (10 mM Tris-HCl, pH 7.5; 1 mM EDTA) and diluted 1:10,000. 20 ul of the undilute colony suspension was plated on SC-ARG + Canavanine and 100 ul of the 10-4 dilution was plated on SC-ARG. The plates were incubated at 30C until colonies reached ~1 mm in size. Colonies were counted and mutation rates were calculated though FluCalc fluctuation analysis software (66). Isolates were independently analyzed at first and then grouped by genotype.

Synthetic Genetic Array Analysis

The genetic screens were performed using SGA technology (67). Briefly, query strains carrying each of the *rnr1* alleles were crossed to an ordered array of all the viable yeast deletion strains and an array of temperature sensitive alleles of yeast essential genes. Diploid cells were transferred to a sporulation-inducing medium, after which the germinated spores were selected for the simultaneous presence of the gene deletion and the *rnr1* allele. Colony size was quantified as a measure of fitness, and SGA scores and p-values were calculated as described in (67). SGA scores from deletion mutants and ts mutants were merged by scaling the ts screen scores according to the SGA scores of the deletion mutants that are present in the ts allele array. A z-score was calculated for all the genes in each screen, and a cutoff of z=-2 was applied to identify negative genetic interactions. The SGA data is presented in Tables S4-S10.

Gene Ontology enrichment analysis

GO term analysis was performed using the GO term finder tool (http://go.princeton.edu/) using a P-value cutoff of 0.01 and applying Bonferroni correction, querying biological process enrichment for each gene set. GO term enrichment results were further processed with REViGO (68) using the "Medium (0.7)" term similarity filter and simRel score as the semantic similarity measure. Terms with a frequency greater than 15% in the REViGO output were eliminated as too general.

Spatial Analysis of Functional Enrichment

Network annotations were made with the Python implementation of Spatial Analysis of Functional Enrichment (SAFE) ((47); https://github.com/baryshnikova-lab/safepy). The yeast genetic interaction similarity network and its functional domain annotations were obtained from (48).

Sample preparation and analysis pipeline

A detailed description of sample preparation and the analytical pipeline used for data analysis can be found in (33).

Hierarchical Cluster Analysis

Hierarchical Cluster Analysis was performed on data that was grouped by biological replicates and the number of times the different classes of variants occurred within a genotype. Data was combined on the average of unique counts of variants from all the biological replicates within a genotype. The different classes of variants include 6 classes of single nucleotide variants (SNVs), single base A/T or G/C insertions and deletions, complex insertions and deletions, as well as mononucleotide variants (MNVs) and replacements (Replac.). MNVs and replacements are complex errors identified in CLC. MNVs are dinucleotide SNVs, where two neighboring nucleotides are both mutated, ex: CC> AT. Replacements are complex insertions or deletions, where the deleted or replaced base is a variant. Two examples include AAC > G and C > AT. Both MNVs and replacements are extremely low frequency events and rarely occur in our data set; neither had a significant impact on clustering.

The data was condensed based on genotype by combining biological replicates and adding the total number of times a variant was seen in the total number of replicates. This analysis does not take frequency into account and instead totals how many unique types of variants occur in a sample. If the same variant occurred in multiple biological replicates within a genotype it was counted as such. Spearman correlation coefficients were calculated across all samples. Spearman correlation was chosen because we did not consistently observe linear relationships between variant frequencies in our data set, although Pearson correlation yielded nearly identical results. Correlation analysis was plotted in RStudio using the ggplots and ggplot2 libraries.

Principal Components Analysis (PCA)

PCA was performed on the number of times a unique variant was observed within a genotype. PCA was plotted using the "factoextra" package in RStudio, with a singular value decomposition (SVD) approach using the prcomp() function.

Determining SNV in trinucleotide context

A detailed description can be found in (33).

Cluster Analysis to look at correlation between genotypes

To identify variants specific to a particular genotype and to eliminate frequency bias from variants that occurred early on in the growth of a particular sample, we condensed unique variants based on the number of biological replicates sequenced for that genotype. If a particular variant occurred in 4 out of 4 biological replicates it was represented as 1, if it occurred in 3 out of 6 replicates it was represented as .5. This gives an unbiased approach to score for the probability that a particular variant was present in a particular genotype, without considering the absolute frequency at which that variant occurred. These data were clustered on rows (or unique variants), after applying a row sum cutoff of greater than 2. This cutoff eliminates low frequency variants which are less likely to be driving the differences in mutation spectra that we observe. By clustering the data only on unique variants, it allows us to see groups of different *types* of variants in specific sequence contexts that are potentially diagnostic for a particular genotype.

CC Dinucleotide cluster analysis

A python script using a sliding window approach was used to identify all reference positions containing CC dinucleotides within *CAN1*. Our dataset was then subset to include only variants that occurred in these dinucleotide CC positions. Of the 138 CC dinucleotide contexts across *CAN1*, 110 (~80%) were mutated, compared to 857/1,711 base pairs or ~50% of the base pairs in *CAN1.* Unique CC run variants were clustered based on the number of times that variant occurred in a given genotype, while accounting for (normalizing by) the number of biological replicates sequenced for each genotype, as described above. Heatmaps were plotted using the pheatmap library in RStudio and motif enrichment was performed using Berkeley web logos (69).

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Figure Legends

Figure 1. A cartoon depiction of decreased replication fidelity at the replication fork. (**A**) Elevated dNTP pool levels lead the replicative polymerases to favor synthesis over error-checking, leaving behind more mutations. Mutagenic replication is represented in red. (**B**) MMR repairs errors that are left behind during replication. Msh2-3 and Msh2-6 recognize and bind errors, initiating repair. Shown

here, Msh2-3 binds a small deletion loop, while Msh2-6 recognizes a base-base mismatch based on known specificities.

Figure 2. Mutation Spectra are distinct across genotypes. **(A)** The relative distribution of SNVs, deletions, insertions, MNVs and replacements by genotype. **(B)** The SNV spectra normalized relative total variants. **(C)** The deletion spectra normalized relative to total variants. (D) The insertion spectra normalized relative to total variants.

Figure 3. Genome-scale screens for synthetic fitness defects with *rnr1* alleles.

(**A**) The overlap of the *rnr1* negative genetic interactions for the three SGA screens is plotted as a Venn diagram. The number of genes identified in each screen is indicated, as is the *rnr1* allele for each screen. (**B**) GO-term enrichments for the negative interacting genes from each *rnr1* screen are tabulated. The –fold enrichment for each term is indicated. Note that *rnr1Y285F* did not display any statistically supported enrichment. (**C**) Spatial analysis of functional enrichment. On the left, the yeast genetic interaction similarity network is annotated with GO biological process terms to identify major functional domains (48). 13 of the 17 domains are labeled and delineated by coloured outlines. On the right, the network is annotated with negative genetic interactions from each *rnr1* SGA screen. The overlays indicate the functional domains annotated on the left. Only nodes with statistically supported enrichments (SAFE score > 0.08, $p < 0.05$) are colored. (**D**) Tetrad analysis of *rnr1* x *msh6*Δ crosses. Ten tetrads were dissected for each cross, and colonies were imaged after 3 days. Each column of 4 colonies is the 4 spores from a single meiotic ascus. Genotypes are indicated by circles (*msh6*Δ) and squares (*rnr1*). (**E**) Tetrad analysis of *rnr1Y285A* x *msh2*Δ and *rnr1Y285A* x *msh3*Δ crosses. Genotypes are indicated by circles (*msh2*Δ or *msh3*Δ) and squares (*rnr1Y285A*).

Figure 4. Distinct genotypes share unique features. (**A**) Hierarchical cluster analysis using Spearman Rank correlation on all the distinct genotypes in our study. Data was clustered based on the unique counts of the 14 different classes of variants that occurred in each genotype. The histogram shows the distribution of correlation coefficients across samples. (**B**) Principal components analysis performed on all unique variants from biological replicates within a genotype.

Figure 5. Larger deletions are relatively rare across genotypes. The most unique > 1bp deletions and insertions occur in *msh3Δ*. The number of unique insertions greater than 1 base pair (peach) and the number of unique deletions greater than 1 base pair (turquoise) within a genotype are plotted.

Figure 6. The average number of each SNV as it occurs in unique triplet nucleotide context differs by genotype. Bars are colored according to the six different types of SNVs. (**A**) The 16 different triplet contexts are lettered for display purposes. The variant change (C>A, turquoise bar) occurs at the middle nucleotide marked X in each triplet context. The same triplet context is repeated for each possible variant in the following panels. **(B)** Wildtype and single *rnr1* alleles. **(C)** Genotypes with *MSH2* deleted **(D)** Genotypes with *MSH3* deleted. **(E)** Genotypes with *MSH6* deleted.

Figure 7. Variants that occur in unique sequence contexts cluster together Hierarchical cluster analysis of all unique variants within our study by genotype (A) A heatmap displaying Pearson correlation enrichment value for a given variant between genotypes, with notable clusters boxed in black. (B) 12 base window motif enrichment on sequence contexts surrounding the notable clusters.

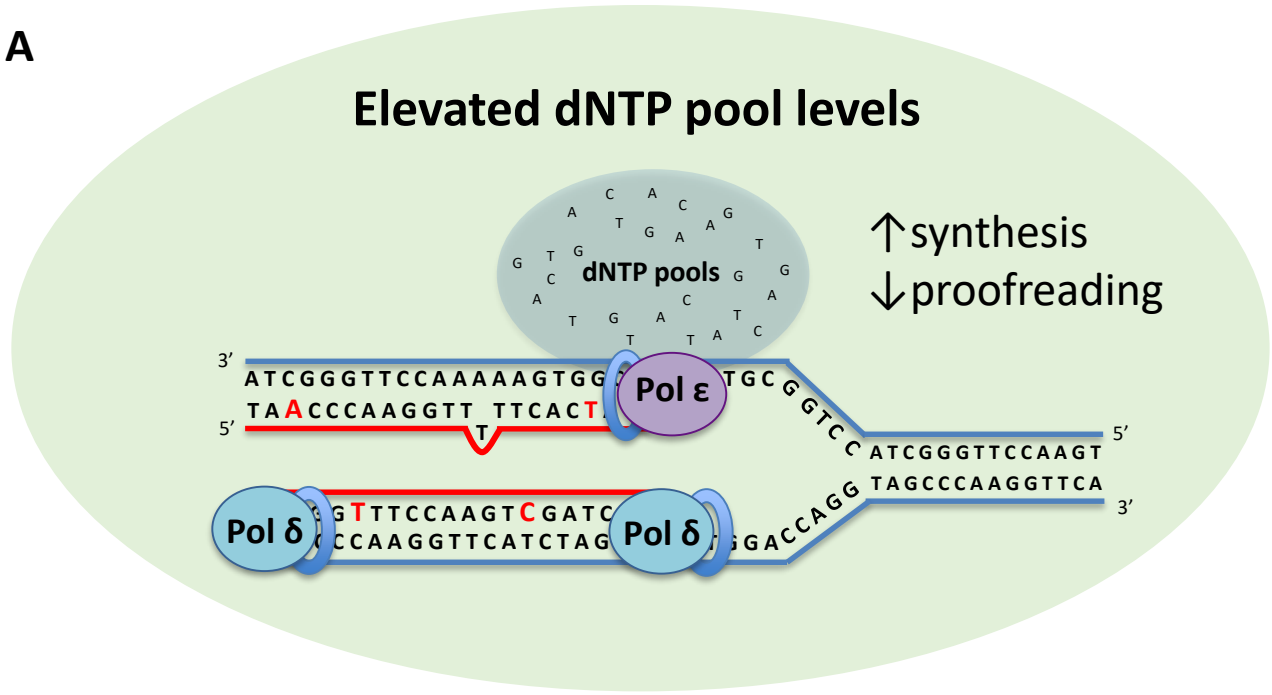
(C) The type of variant observed in the center of sequence context from B. (D) Summary of genotypes which show negative or positive correlation in a given cluster.

Figure 8. SNVs occur in C-C dinucleotide sequence contexts with differential enrichment between genotypes. (A) Hierarchical cluster analysis of all SNVs that occur at CC dinucleotides. Clusters of interest are boxed in black, labeled by roman numerals. (**B**) Motif enrichment of a 12 base window surrounding the mutated nucleotide was performed using Berkley web logos. The mutated base is at the 7th nucleotide position in all logos, indicated by the black arrow. (**C**) The most predominant type(s) of SNV in the cluster are displayed. (**D**) A summary of genotypes that show negative or positive correlation in a given cluster.

Figure 9. G/C single base deletions that show synergistic increases in variant frequency in *rnr1Y285A msh3Δ* genotypes. The deleted base is starred. (**A**) Motif enrichment of a 12 base window surrounding two G deletions (starred nucleotide) that are specific to genotypes with *msh3Δ*. (**B**) The variant frequency from biological replicates in wildtype, *rnr1Y285F/A* and *msh3Δ* genotypes for the single base deletion that occurred at position 31979 is plotted. (**C)** The variant frequencies across replicates for the G deletion at 32272. In both cases (**B** and **C**) there are very few events in the single mutants, but significant frequencies in double mutant backgrounds. (**D**) Motif enrichment for two C deletions that are specific to genotypes with *rnr1Y285F* and *rnr1Y285A*, but increase synergistically in double mutants with *msh3Δ* in combination. (**E)** The variant frequencies across replicates for the G deletion at 32747. (**F**) The variant frequencies across replicates for the G deletion at 32658. In **E** and **F**, this event occurs in *rnr1Y285A*, but not *rnr1Y285F* or *msh3Δ*. It occurs at increased frequencies in double mutant backgrounds.

Figure 10. Mechanisms of mutagenesis for the incorporation of errors specific to either Msh2-Msh6 or Msh2-Msh3 repair. The mutated base is represented in red. (**A**)**.** Two examples of sequence context surrounding CC dinucleotides from **Fig. 7B**, where mis-insertion is due to the nucleotide in excess in *rnr1Y285F/A* backgrounds. These errors are efficiently repaired by Msh2-Msh6. This specificity becomes apparent when *msh6Δ* is paired with *rnr1Y285F/A* alleles. (**B**) Two examples of sequence context from **Figs. 8A** and **8D**, where mis-alignment events occur due to the severely limiting amount of dGTP in *rnr1Y285F/A* genetic backgrounds. The run where the deletion occurred is underlined in black. These single base G/C deletions are efficiently repaired by Msh2-Msh3, but not Msh2-Msh6, a previously unidentified specificity of the repair complex.

Table 1. Canavanine mutation rates		
Genotype	**Mutation Rate [95% Confidence Intervals]**	**Fold Change**
Wildtype (n=115)	2.7×10^{-7} [2.3×10^{-7} – 3.2×10^{-7}]	1
msh2Δ (n=24)	6.7×10^{-6} [5.8×10^{-6} – 7.8×10^{-6}]	24.8
msh3Δ (n=32)	7.8×10^{-7} [6.1×10^{-7} – 9.7×10^{-7}]	2.9
msh6Δ (n=24)	1.8×10^{-6} [1.4×10^{-6} – 2.2×10^{-6}]	6.7
rnr1D57N (n=91)	7.3×10^{-7} [6.3×10^{-7} – 8.3×10^{-7}]	2.7
rnr1D57N msh2Δ (n=24)	2.0×10^{-5} [1.8×10^{-5} – 2.3×10^{-5}]	74.1
rnr1D57N msh3Δ (n=24)	2.2×10^{-6} [1.7×10^{-6} – 2.7×10^{-6}]	8.2
rnr1D57N msh6Δ (n=24)	5.6×10^{-6} [4.7×10^{-6} – 6.6×10^{-6}]	20.7
rnr1Y285F (n=42)	7.5×10^{-7} [5.8×10^{-7} – 9.5×10^{-7}]	2.8
rnr1Y285F msh2Δ (n=24)	2.9×10^{-5} [2.6×10^{-5} – 3.2×10^{-5}]	107.4
rnr1Y285F msh3Δ (n=24)	3.7×10^{-6} [3.0×10^{-6} – 4.5×10^{-6}]	13.7
rnr1Y285F msh6Δ (n=24)	2.3×10^{-5} [2.0×10^{-5} – 2.6×10^{-5}]	85.2
rnr1Y285F pGAL-RNR1 (n=24)	7.8×10^{-7} [5.7×10^{-7} – 1.0×10^{-6}]	2.9
rnr1Y285F pGAL-RNR1 msh2Δ (n=24)	1.5×10^{-5} [1.3×10^{-5} – 1.8×10^{-5}]	55.6
rnr1Y285F pGAL-RNR1 msh6Δ (n=12)	1.6×10^{-5} [1.3×10^{-5} – 1.9×10^{-5}]	59.3
rnr1Y285A (n=46)	5.5×10^{-6} [4.7×10^{-6} – 6.2×10^{-6}]	20.4
rnr1Y285A msh2Δ (n=33)	2.5×10^{-5} [2.1×10^{-5} – 3.0×10^{-5}]	92.6
rnr1Y285A msh3Δ (n=49)	6.2×10^{-5} [5.7×10^{-5} – 6.6×10^{-5}]	229.6
rnr1Y285A msh6Δ (n=33)	1.0×10^{-5} [8.9×10^{-6} – 1.2×10^{-5}]	37.0
rnr1Y285A pGAL-RNR1 (n=22)	1.5×10^{-5} [1.2×10^{-5} – 1.7×10^{-5}]	55.6
rnr1Y285A pGAL-RNR1 msh2Δ (n=24)	1.3×10^{-5} [1.1×10^{-5} – 1.5×10^{-5}]	48.2
rnr1Y285A pGAL-RNR1 msh3Δ (n=36)	7.3×10^{-5} [6.8×10^{-5} – 7.9×10^{-5}]	270.4
rnr1Y285A pGAL-RNR1 msh6Δ (n=30)	1.0×10^{-5} [8.7×10^{-6} – 1.2×10^{-5}]	37.0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Pol δ
Pol δ
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5'
5'
3'
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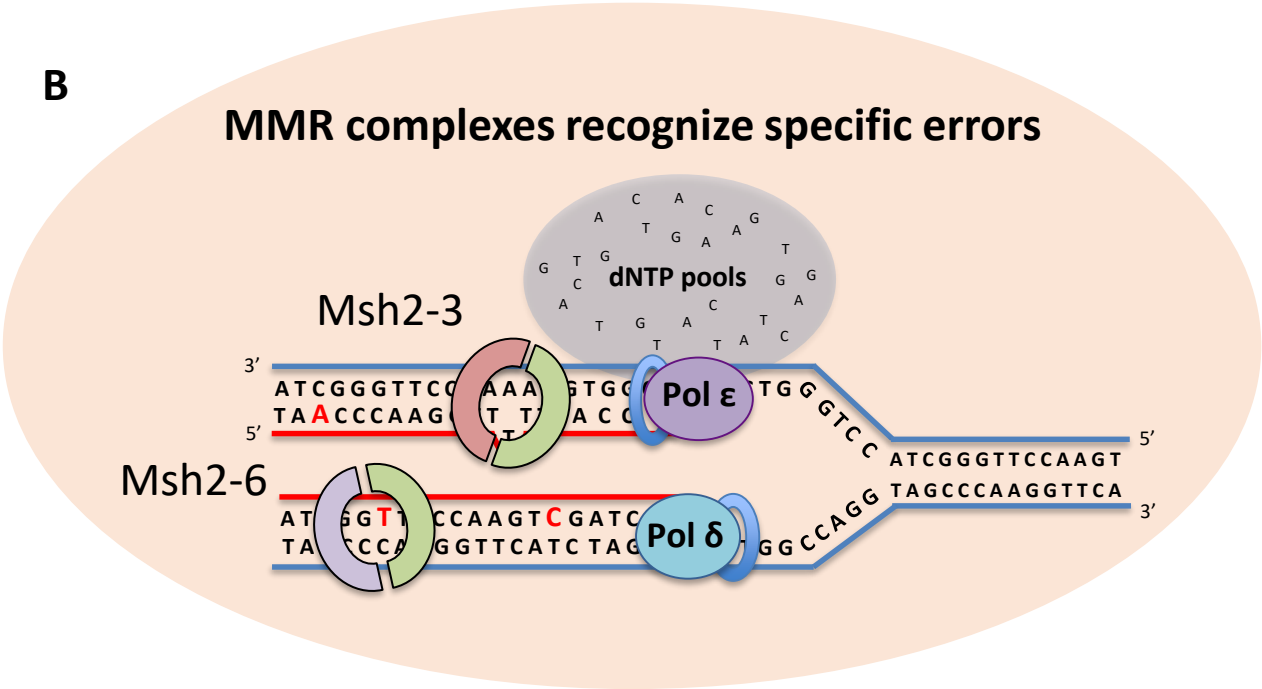
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dNTP pools
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Pol δ
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5'
5'
3'
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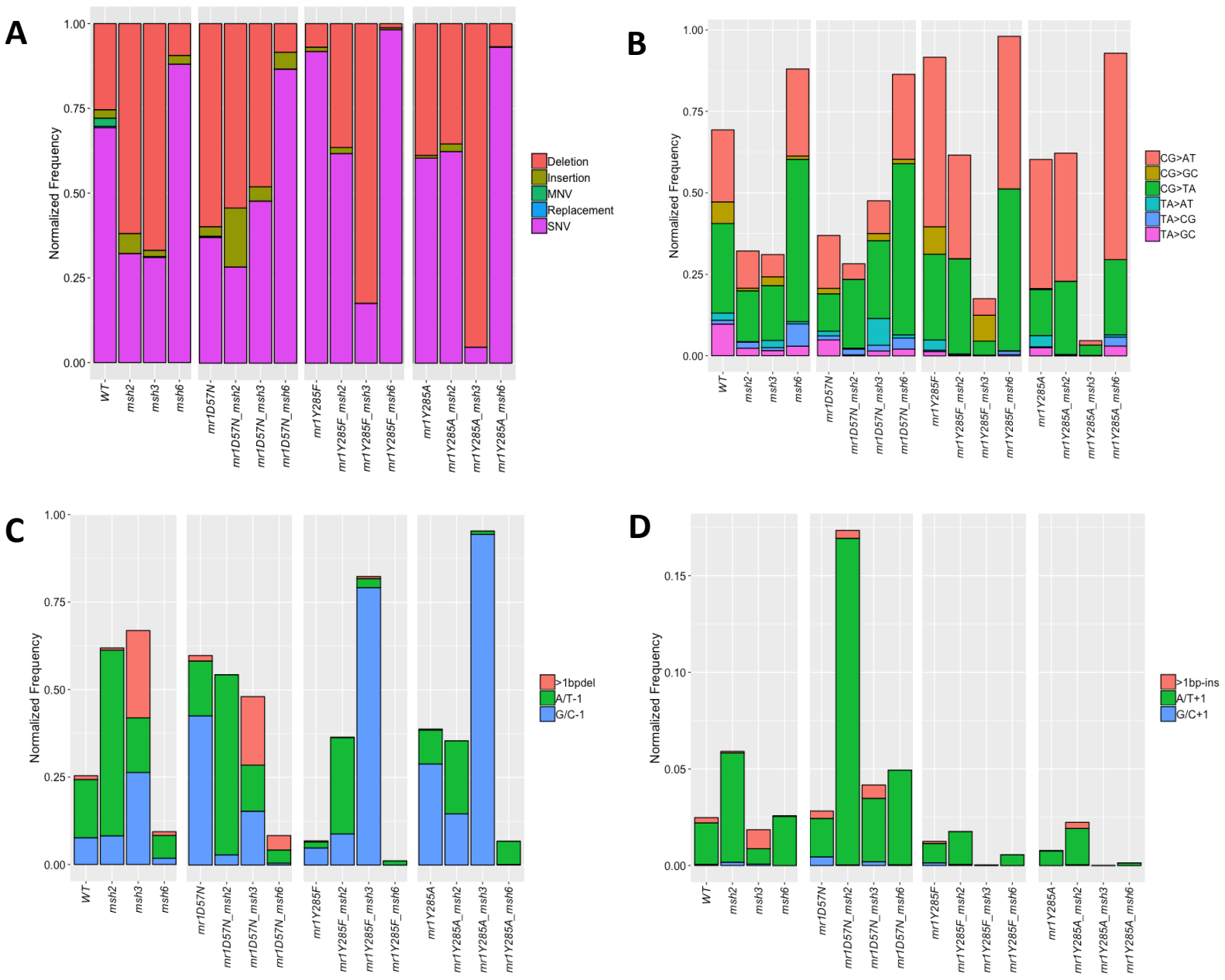
TAGCCCAAGGTTCA

Figure 1.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mr1D57N_msh3-
mr1D57N_msh6-
mr1Y285F-
mr1Y285F_msh2-
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CG>GC
CG>TA
TA>AT
TA>CG
TA>GC
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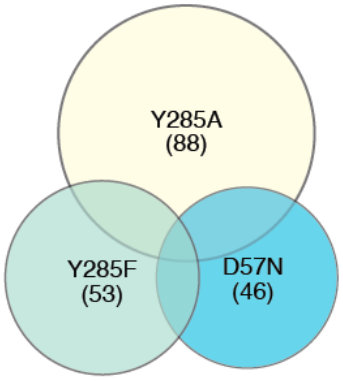
A/T+1
G/C+1

Figure 2.

A



B

rnr1Y285A

GO term ID	Description	Enrichment
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GO:0043570	maintenance of DNA repeat elements	14
GO:0006260	DNA replication	6
GO:0006281	DNA repair	4

rnr1D57N

GO term ID	Description	Enrichment
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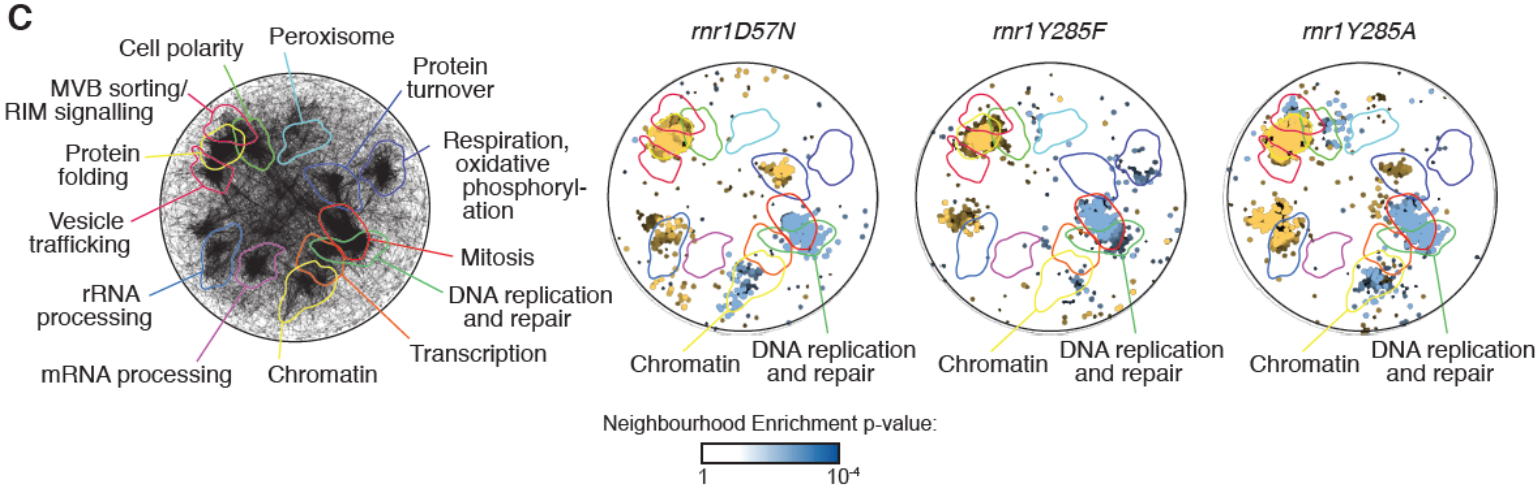
GO:0006260	DNA replication	7

C



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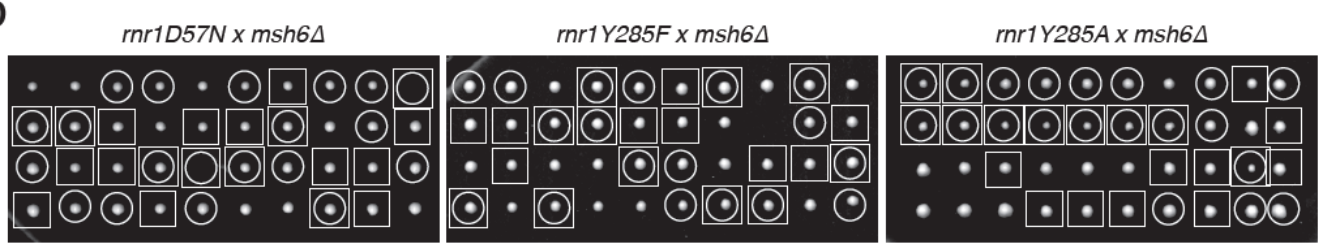

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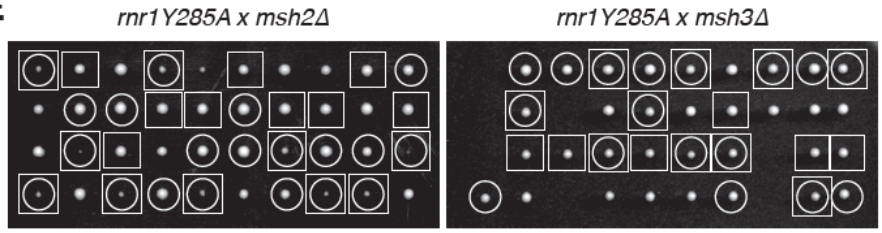

Figure 3.

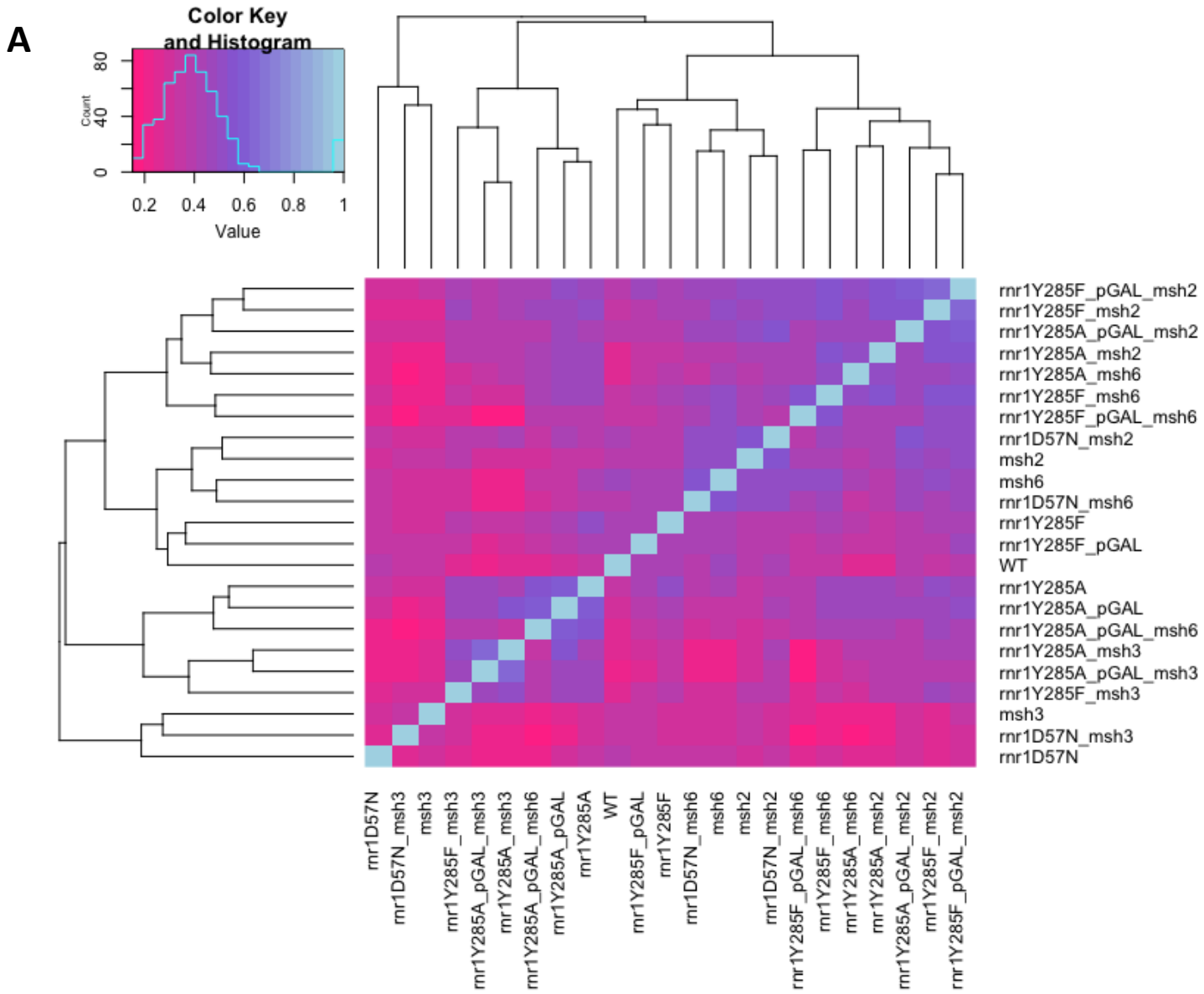
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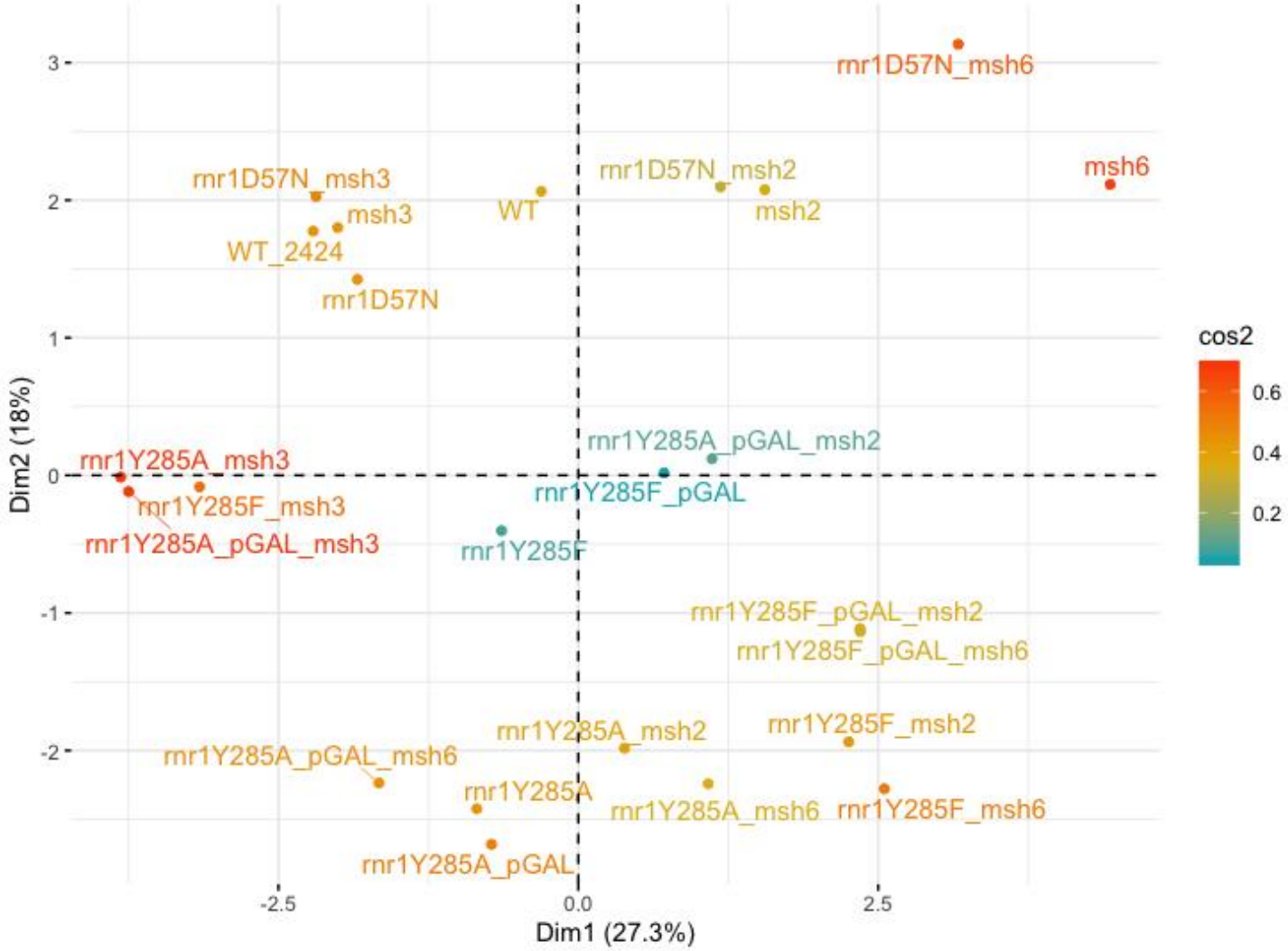
Figure 4.

B



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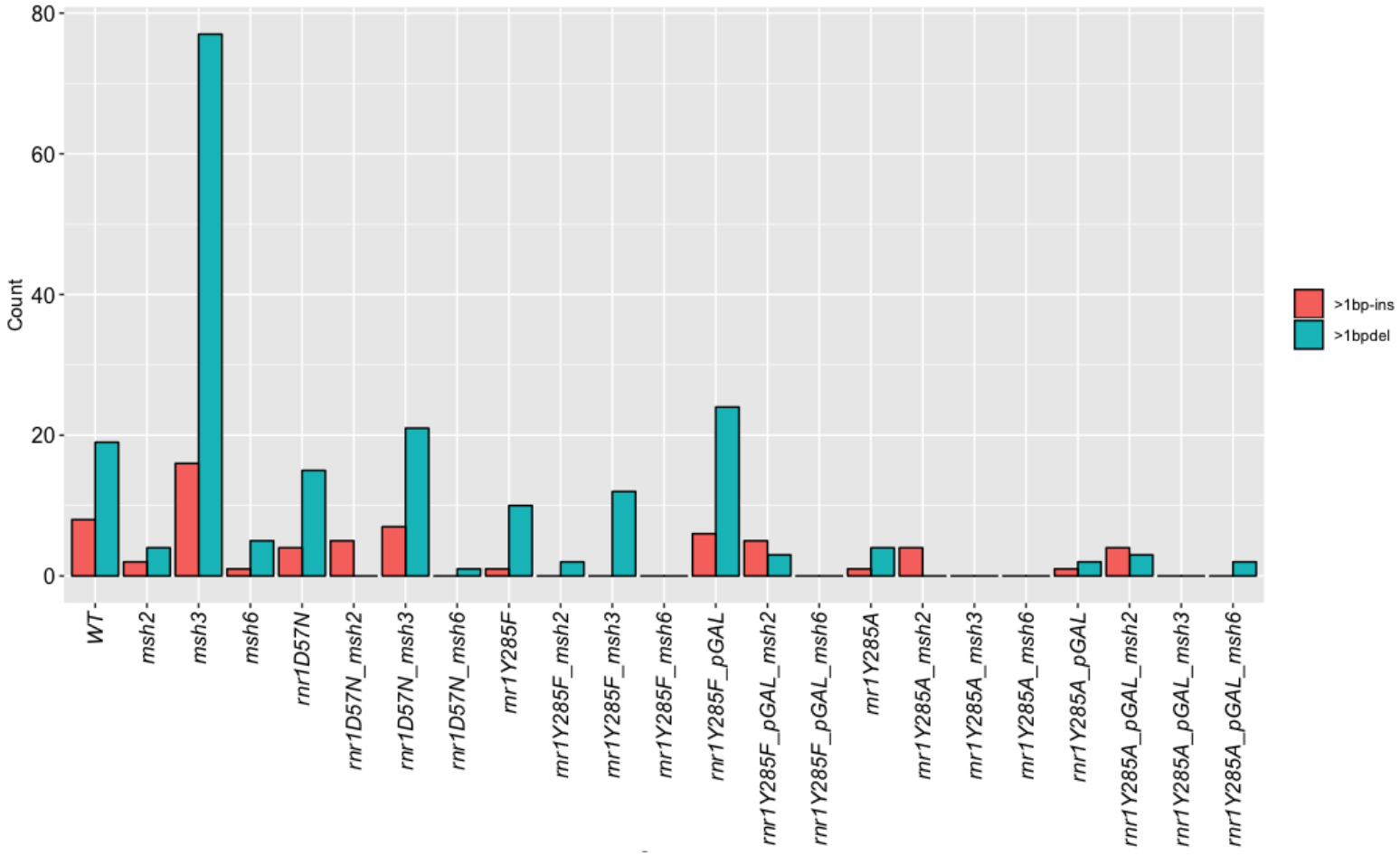
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rnr1Y285F_pGAL
rnr1Y285F_pGAL_msh2
rnr1Y285F_pGAL_msh6
rnr1Y285A
rnr1Y285A_msh2
rnr1Y285A_msh3
rnr1Y285A_msh6
rnr1Y285A_pGAL
rnr1Y285A_pGAL_msh2
rnr1Y285A_pGAL_msh3
rnr1Y285A_pGAL_msh6

Figure 5.

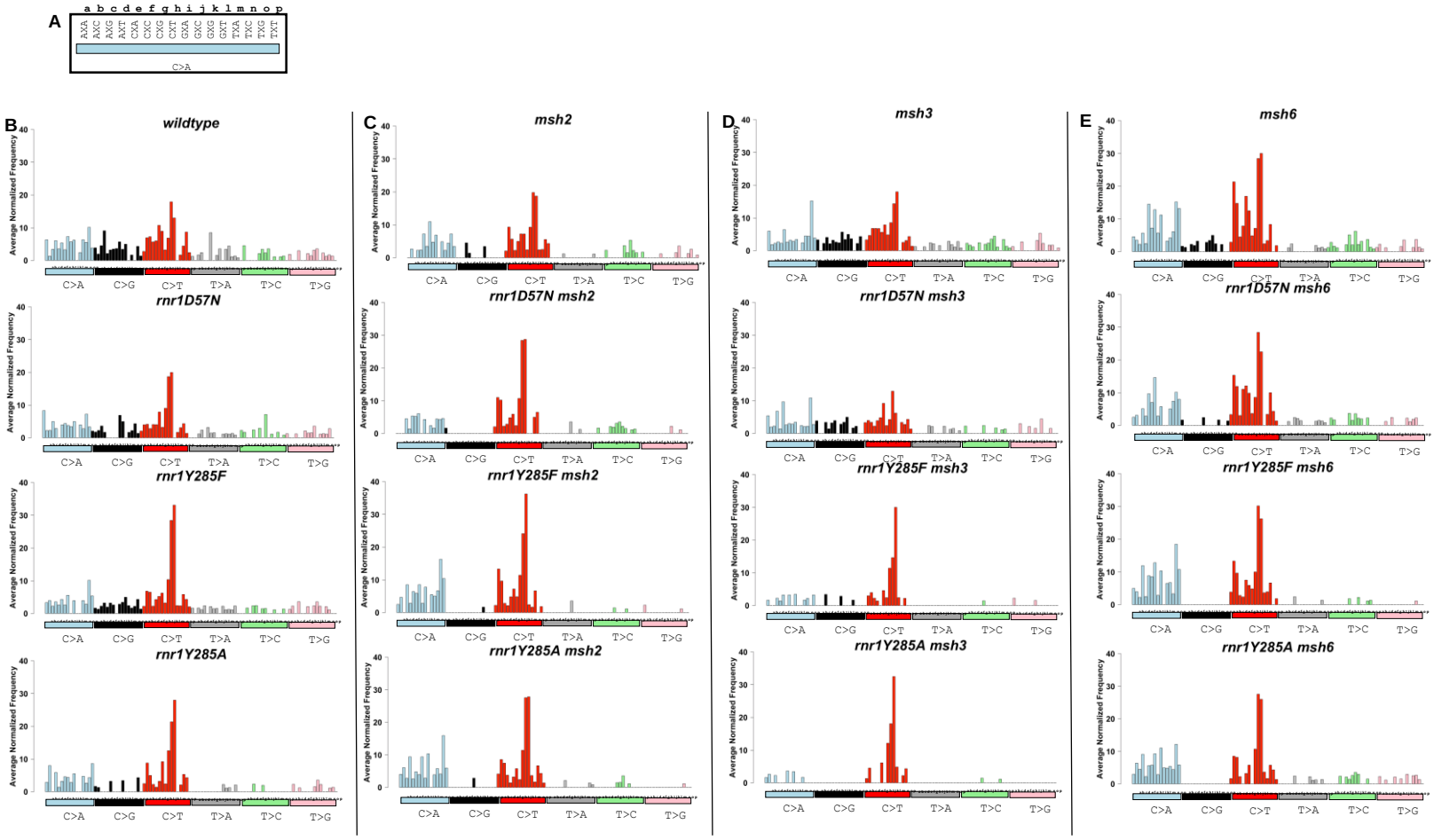

A
a b c d e f g h i j k l m n o p
AXA AXC AXG AXT CXA CXC CXG CXT GXA GXC GXG GXT TXA TXC TXG TXT
C>A
B
wildtype
rnr1D57N
rnr1Y285F
rnr1Y285A
C
msh2
rnr1D57N msh2
rnr1Y285F msh2
rnr1Y285A msh2
D
msh3
rnr1D57N msh3
rnr1Y285F msh3
rnr1Y285A msh3
E
msh6
rnr1D57N msh6
rnr1Y285F msh6
rnr1Y285A msh6
Average Normalized Frequency
C>A C>G C>T T>A T>C T>G

Figure 6.

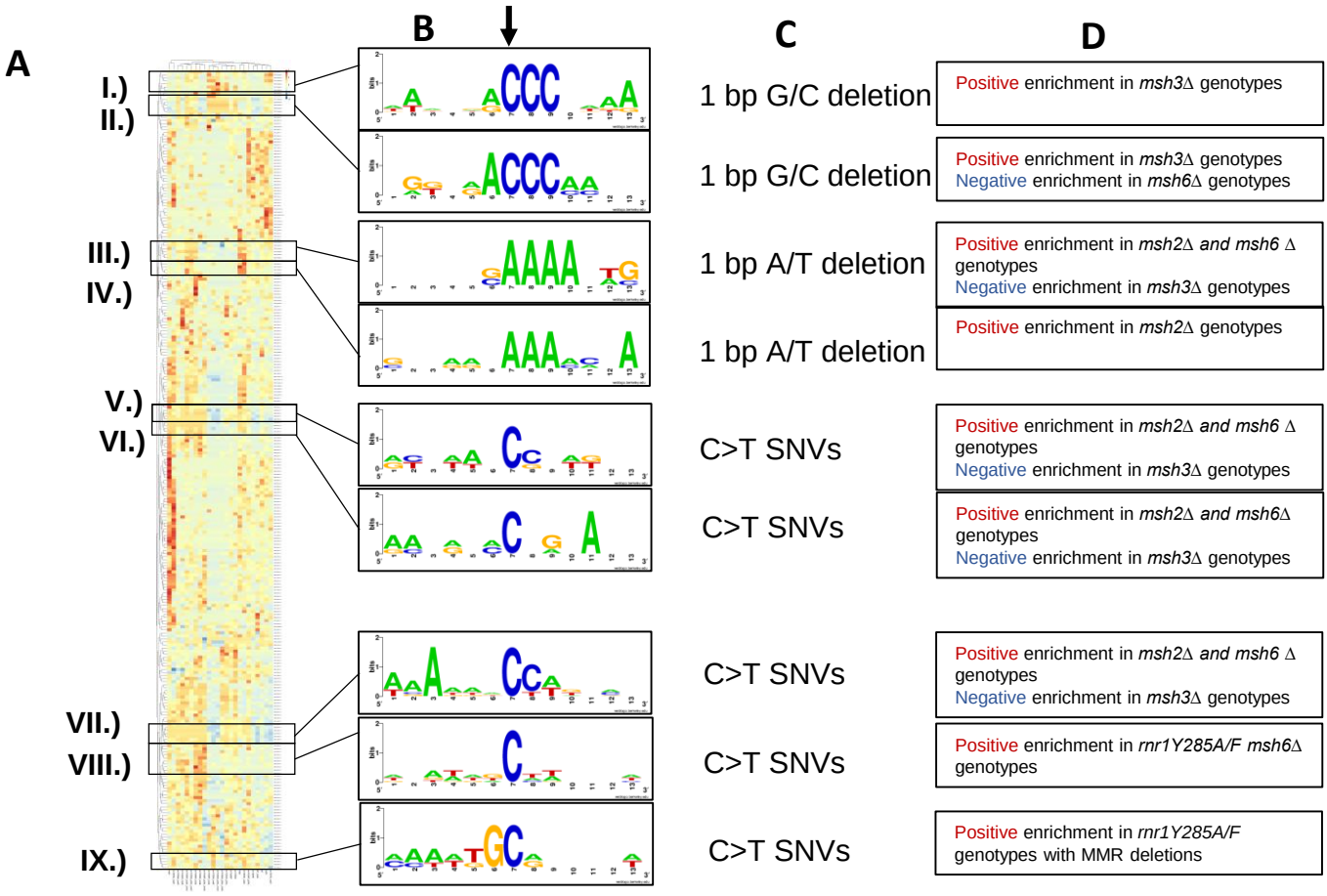

A
B
C
D
I.)
II.)
III.)
IV.)
V.)
VI.)
VII.)
VIII.)
IX.)
1 bp G/C deletion
Positive enrichment in msh3Δ genotypes
1 bp G/C deletion
Positive enrichment in msh3Δ genotypes
Negative enrichment in msh6Δ genotypes
1 bp A/T deletion
Positive enrichment in msh2Δ and msh6 Δ genotypes
Negative enrichment in msh3Δ genotypes
1 bp A/T deletion
Positive enrichment in msh2Δ genotypes
C>T SNVs
Positive enrichment in msh2Δ and msh6 Δ genotypes
Negative enrichment in msh3Δ genotypes
C>T SNVs
Positive enrichment in msh2Δ and msh6Δ genotypes
Negative enrichment in msh3Δ genotypes
C>T SNVs
Positive enrichment in msh2Δ and msh6 Δ genotypes
Negative enrichment in msh3Δ genotypes
C>T SNVs
Positive enrichment in rnr1Y285A/F msh6Δ genotypes
C>T SNVs
Positive enrichment in rnr1Y285A/F genotypes with MMR deletions

Figure 7.

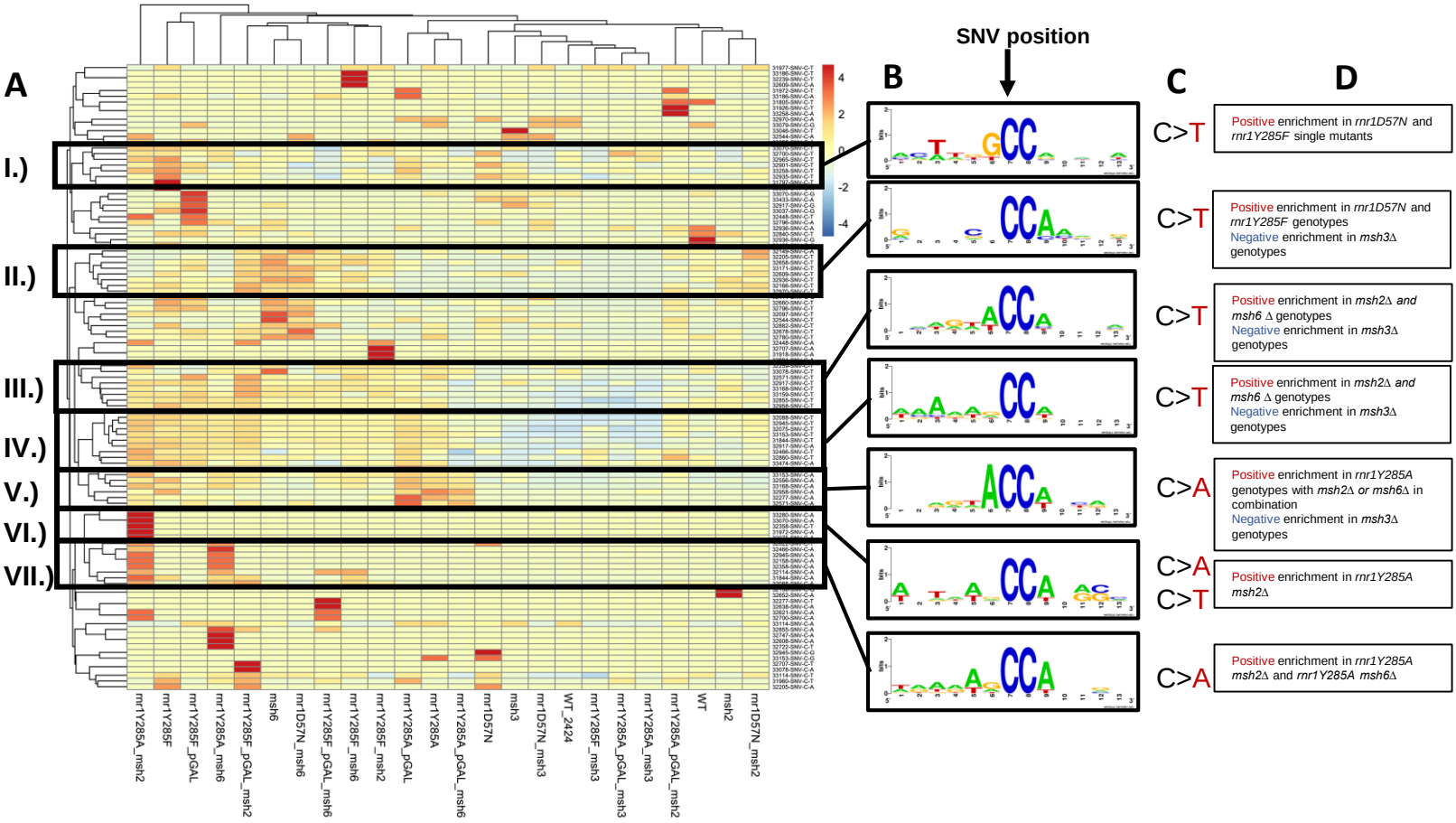

A
B
C
D
SNV position
I.)
II.)
III.)
IV.)
V.)
VI.)
VII.)
4
2
0
-2
-4
rnr1Y285A_msh2
rnr1Y285F
rnr1Y285F_pGAL
rnr1Y285A_msh6
rnr1Y285F_pGAL_msh2
msh6
rnr1D57N_msh6
rnr1Y285F_pGAL_msh6
rnr1Y285F_msh6
rnr1Y285F_msh2
rnr1Y285A_pGAL
rnr1Y285A
rnr1Y285A_pGAL_msh6
rnr1D57N
msh3
rnr1D57N_msh3
WT_2424
rnr1Y285F_msh3
rnr1Y285A_pGAL_msh3
rnr1Y285A_msh3
rnr1Y285A_pGAL_msh2
WT
msh2
rnr1D57N_msh2
C>T
Positive enrichment in rnr1D57N and rnr1Y285F single mutants
C>T
Positive enrichment in rnr1D57N and rnr1Y285F genotypes
Negative enrichment in msh3Δ genotypes
C>T
Positive enrichment in msh2Δ and msh6 Δ genotypes
Negative enrichment in msh3Δ genotypes
C>T
Positive enrichment in msh2Δ and msh6 Δ genotypes
Negative enrichment in msh3Δ genotypes
C>A
Positive enrichment in rnr1Y285A genotypes with msh2Δ or msh6Δ in combination
Negative enrichment in msh3Δ genotypes
C>A
C>T
Positive enrichment in rnr1Y285A msh2Δ
C>A
Positive enrichment in rnr1Y285A msh2Δ and rnr1Y285A msh6Δ

Figure 8.

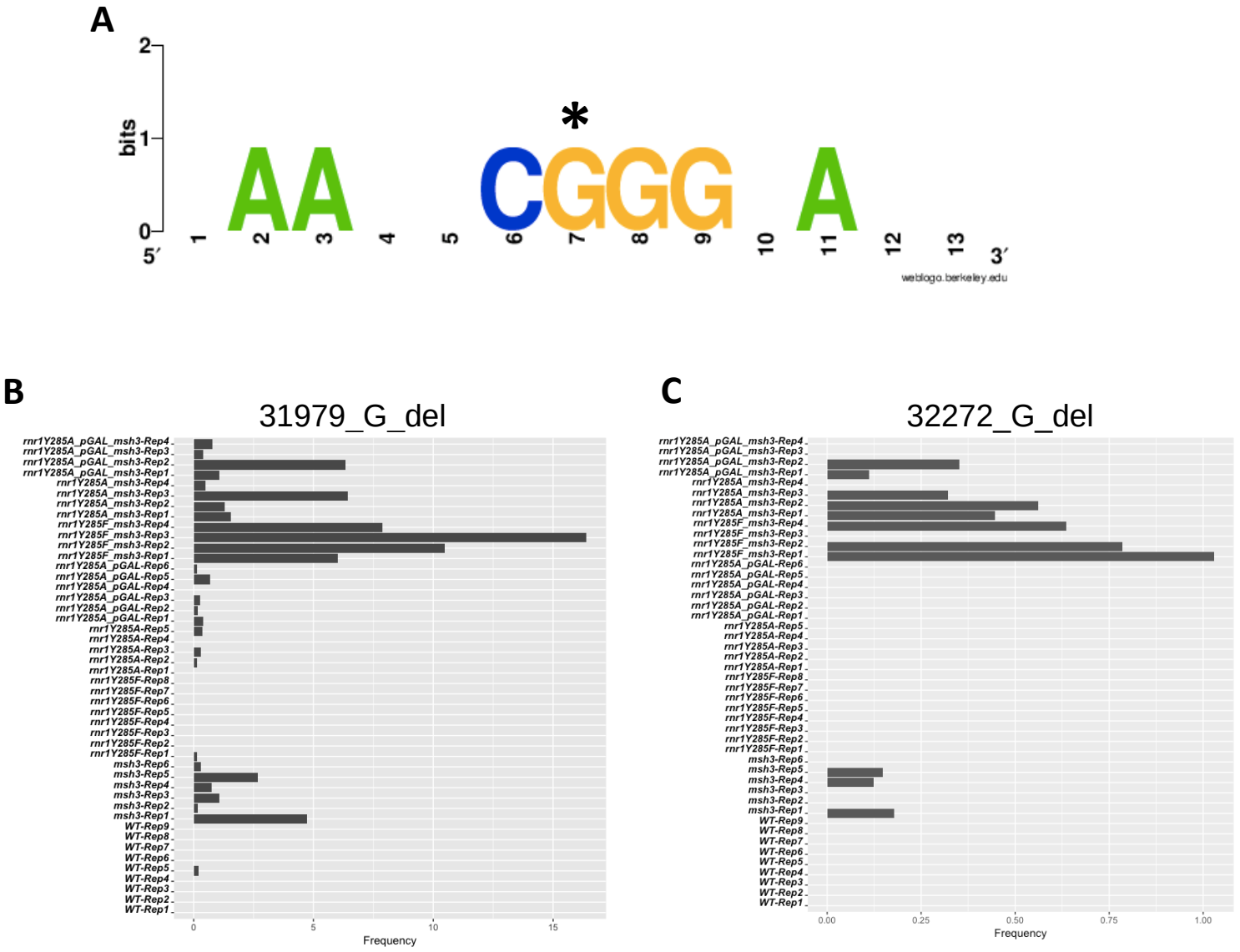
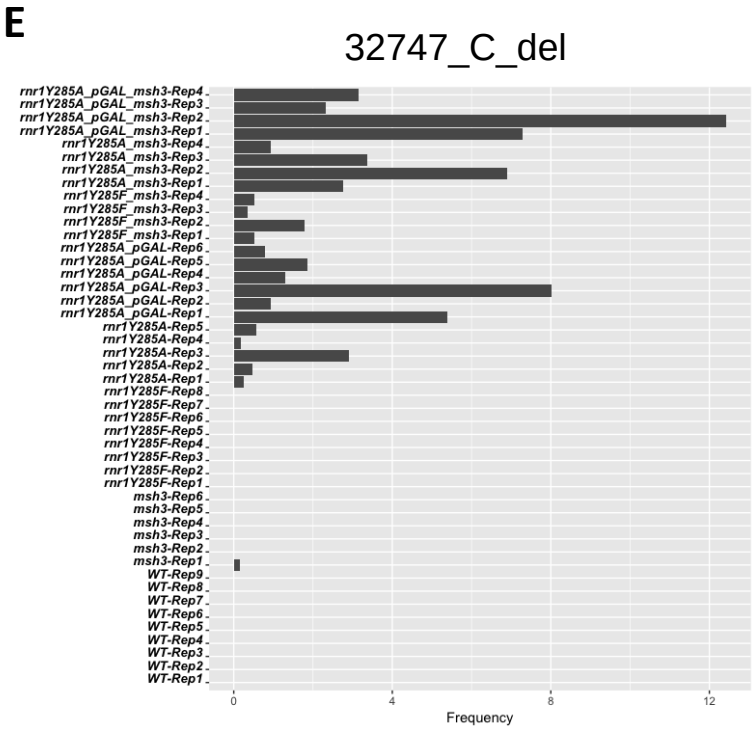

A
bits
2
1
0
5′
1
2
3
4
5
6
7
8
9
10
11
12
13
3′
*
AA CGGG A
weblogo.berkeley.edu
B
31979_G_del
Frequency
0
5
10
15
C
32272_G_del
Frequency
0.00
0.25
0.50
0.75
1.00

Figure 9.



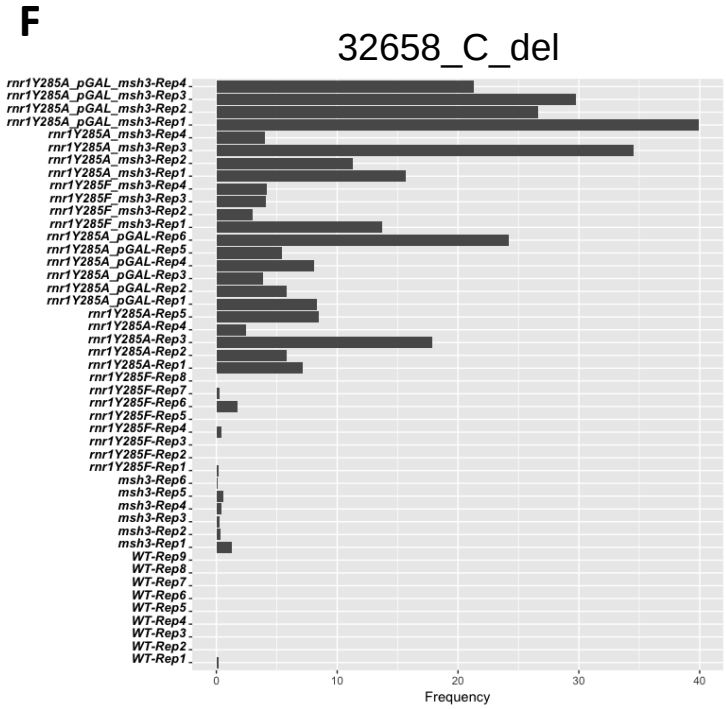


Figure 9. (continued)

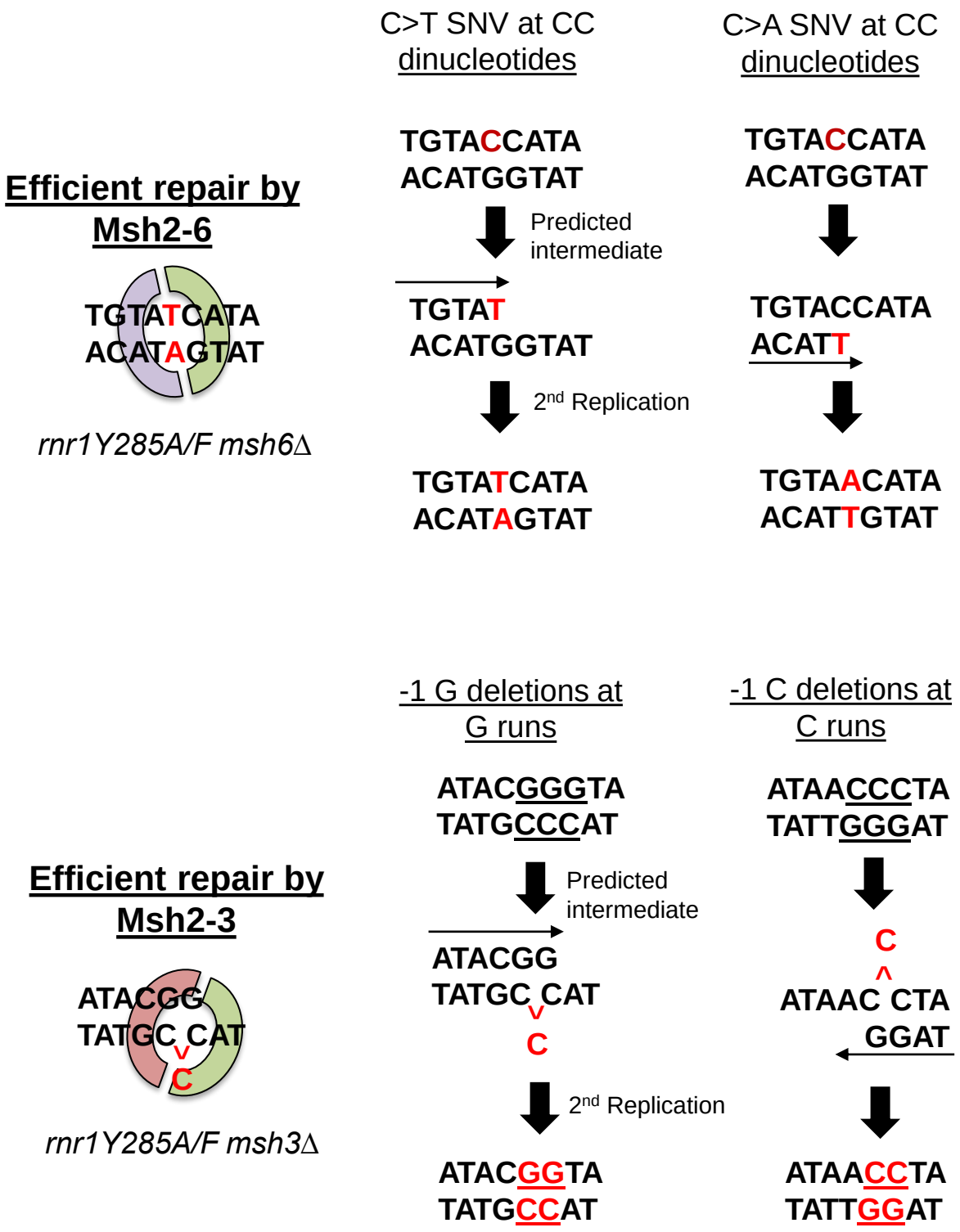

A
C>T SNV at CC dinucleotides
C>A SNV at CC dinucleotides
Efficient repair by Msh2-6
TGTATCATA
ACATAGTAT
rnr1Y285A/F msh6Δ
TGTACCATA
ACATGGTAT
Predicted intermediate
TGTAT
ACATGGTAT
2nd Replication
TGTATCATA
ACATAGTAT
TGTACCATA
ACATGGTAT
TGTACCATA
ACATT
TGTAACATA
ACATTGTAT
B
-1 G deletions at G runs
-1 C deletions at C runs
Efficient repair by Msh2-3
ATACGG
TATGC CAT
C
rnr1Y285A/F msh3Δ
ATACGGGTA
TATGCCCAT
Predicted intermediate
ATACGG
TATGC CAT
C
2nd Replication
ATACGGTA
TATGCCAT
ATAACCCTA
TATTGGGAT
C
ATAAC CTA
GGAT
ATAACCTA
TATTGGAT

Figure 10.