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Trajectory and uniqueness of mutational signatures in yeast mutators

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The acquisition of mutations plays critical roles in adaptation, evolution, senescence, and tumorigenesis. Massive genome sequencing has allowed extraction of specific features of many mutational landscapes but it remains difficult to retrospectively determine the mechanistic origin(s), selective forces, and trajectories of transient or persistent mutations and genome rearrangements. Here, we conducted a prospective reciprocal approach to inactivate 13 single or multiple evolutionary conserved genes involved in distinct genome maintenance processes and characterize de novo mutations in 274 diploid Saccharomyces cerevisiae mutation accumulation lines. This approach revealed the diversity, complexity, and ultimate uniqueness of mutational landscapes, differently composed of base substitutions, small insertions/deletions (InDels), structural variants, and/or ploidy variations. Several landscapes parallel the repertoire of mutational signatures in human cancers while others are either novel or composites of subsignatures resulting from distinct DNA damage lesions. Notably, the increase of base substitutions in the homologous recombination-deficient Rad51 mutant, specifically dependent on the Polɛ translesion polymerase, yields COSMIC signature 3 observed in BRCA1/BRCA2-mutant breast cancer tumors. Furthermore, “mutome” analyses in highly polymorphic diploids and single-cell bottleneck lineages revealed a diverse spectrum of loss-of-heterozygosity (LOH) signatures characterized by interstitial and terminal chromosomal events resulting from interhomolog mitotic crossovers. Following the appearance of heterozygous mutational signatures, the strong stimulation of LOHs in the rad27Δ/en1 and tsα1/prdx1 backgrounds leads to fixation of homozygous mutations or their loss along the lineage. Overall, these mutomes and their trajectories provide a mechanistic framework to understand the origin and dynamics of genome variations that accumulate during clonal evolution.

Significance

Deficiencies in genome maintenance genes result in increased mutagenesis and genome rearrangements that impact cell viability, species adaptation, and evolvability. The accumulation of somatic mutations is also a landmark of most tumor cells but it remains difficult to retrospectively determine their mechanistic origin(s). Here, we conducted a prospective reciprocal approach to inactivate evolutionary conserved genes involved in various genome maintenance processes and characterize de novo mutations in diploid S. cerevisiae mutation accumulation lines. Our results revealed the diversity, trajectory, complexity, and ultimate uniqueness of the clonal mutational landscapes. Some mutational signatures resemble those found in human tumors.

Author contributions: A.G.N. designed research; S.L., M.H., F.P., S.P., and S.B. performed research; S.L., M.H., F.P., S.B., S.P., and A.G.N. analyzed data; and S.L., F.P., S.P.J., and A.G.N. wrote the paper.

The authors declare no competing interest.

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Among all small variants, complex base substitutions were rare (7.8-fold increase) (Dataset S10). Similar to rad51, the SVs in rad27 reached a spontaneous frequency of $4.3 \times 10^{-5}$ per clone per passage. The deletions involved homeologically repeated regions located in cis but fewer (3/26 in rad27 instead of 11/17 in rad51) involved Ty/LTR elements (Dataset S8). The sixth class of mutational profile is represented by pif1, affecting various DNA metabolism functions (https://www.yeastgenome.org/), whose major feature is the rapid and complete loss of mitochondrial DNA (SI Appendix, Fig. S2B). Further, pif1Δ MA lines exhibit a slight increase of base substitutions (2.5-fold) (Fig. 1 and Dataset S10), consistent with the two- to threefold increase of spontaneous mutagenesis previously observed in WT cells lacking mitochondrial DNA (rho0) (24). Compared with our previous analyses of haploid mutants (23), the mutational spectrum and the overall frequencies of SNPs and small InDels per genome in haploid and diploid cells are similar (SI Appendix, Fig. S3), indicating no drastic effect of the ploidy variation.

To more broadly characterize all of the mutational landscapes, we also examined variation of mitochondrial DNA (mtDNA) and ribosomal DNA (rDNA) copy number. It was substantially variable in the WT and mutant parental strains with 0 to 103 mtDNA copies and 42 to 122 rDNA copies. In the MA lines, slight changes of mtDNA and rDNA copy number (∼1–2 copies) occurred from clone to clone (SI Appendix, Fig. S2B and C) compared with the parent. lig4 clones increased and mre11 clones decreased median copy number of mtDNA (34 and 40 copies, respectively). The study of additional MA lines issued from independent parental strains, preferentially with a variable amount of starting mtDNA, would be required to conclude if this is a mutant-specific effect, as observed in other yeast mutants (25), and determine its impact on mutational profiles. In summary, this set of mutator profiles illustrates a variety of mutator behaviors, leading to a considerable variety of mutational loads and mutational landscapes.

**Mutational Signatures**

The landscape of somatic mutations in tumor genomes has been correlated with distinct mutational processes via mathematical and statistical methods able to distinguish different mutational signatures (6, 12, 14, 26–28). It has allowed identification of 20 known mutational signatures (Fig. 2B). This recent advance is particularly valuable for human cancer studies (29), due to the wealth of data available, allowing a cataloging of COSMIC signatures (https://cancer.sanger.ac.uk/cosmic/signatures). Since we have not yet cataloged the COSMIC profile of rad51, we have shown the contribution of signatures 1, 3, 9, 18, and 30 (Fig. 2B) to the mutational landscape of our yeast mutants that yielded ≥500 SNP mutations (Fig. 2A) and the relative contribution of the COSMIC signatures (Fig. 2B).

**DNA Damage and Repair**

The major thioredoxin peroxidase that scavenges hydrogen peroxide in *S. cerevisiae* (30)—which yielded C>A and C>T mutagenesis previously observed in WT cells lacking mitochondrial DNA (rho0) (24). Compared with our previous analyses of haploid mutants (23), the mutational spectrum and the overall frequencies of SNPs and small InDels per genome in haploid and diploid cells are similar (SI Appendix, Fig. S3), indicating no drastic effect of the ploidy variation.

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Fig. 1. Mutational landscapes. (A) List of genes studied and their functions. (B) Experimental strategy to generate mutation accumulation lines. The WT diploid strains (BY/BY or SK1/BY background) were deleted for both copies (ΔΔ) of the potential mutator gene(s). Then, 4 to 16 independent clones of the WT and ΔΔ diploids were grown mitotically and derived for up to 100 single-cell bottleneck passages on YPD-rich medium at 30 °C (23). The genome of the resulting accumulation lines was individually sequenced by NGS and the reads were analyzed for detection of de novo mutations and genome rearrangements (SI Appendix, Materials and Methods and Fig. S1). (C and D) Mutational profiles in BY/BY and SK1/BY strains, respectively. N. of mutations: total number of de novo mutations detected in each strain, including single-nucleotide variants, small InDels, chromosome aneuploidies, and structural variants (large deletions/insertions). The SNPs and small InDels comprise both heterozygous (allelic ratio ~0.5) and apparently homoyzgous events (allelic ratio ~1.0). For each mutant, the class of mutator profile and number of clones, passages, and mutations are indicated. The mean number of mutations per clone normalized to the number of passages and the SE are shown. The mutational fold variation compared with the corresponding WT is shown in parentheses. The Mann–Whitney–Wilcoxon test was performed to compare each mutant with WT (ns, not significant; **P < 0.01).
profile predominantly involving C>A, C>G, and C>T changes exhibited signature 3 (Fig. 2B), consistent with its prominent role in homologous recombination (32). Also differently, our analyses of the base substitutions in the mutator DNA polymerase mutants pol1-L868M, pol2-M644G, and pol3-L612M (21) yielded the predominant signatures 8, 22, and 12, respectively (SI Appendix, Fig. S4). Altogether, these results outline the uniqueness of the base-substitution signatures to specific genes, and retrospectively inform on the molecular defects underlying the accumulation of mutations in specific tumors (6–15).

**Base Substitution in the Absence of Rad51 Specifically Requires Polζ.**

Two decades ago, the elevated mutagenesis of a rad51 mutant was found to decrease when cells were also mutated in REV3 (33), a gene now known to encode a component of the error-prone translesion synthesis (TLS) Rev1–Rev3–Rev7 Polζ complex (34). To further explore rad51 mutagenesis, we associated the rad51 deletion with each TLS polymerase deletion mutant and measured mutation frequencies with the sensitive CAN1 mutant assay (35). This revealed that rad51-enhanced mutagenesis was reduced essentially to WT levels in combination with rev1, rev3, or rev7 but remained unchanged with pol4 (Polδ) or rad30 (Polη) (Fig. 2C). Consistently, we did not find significant additive or synergic effects of combining rad51 with the rev1 rev3 pol4 rad30 quadruple mutant.

Since Rev3 carries the catalytic activity of Polζ while the Rev1 and Rev7 proteins might also serve as “recruitment platforms” involved in other related but distinct biological functions—the mammalian REV7 is involved in controlling DNA end resection and DNA damage responses via the Shieldin complex (36–38)—we also combined rad51 with the catalytically dead rev3-D1142A,D144A polymerase mutant (39). rad51-induced mutagenesis was reduced to the WT level (Fig. 2C), demonstrating a role for Rev3 TLS activity. Thus, Polζ appeared specifically involved in the default repair of DNA lesions in the absence of Rad51-dependent homologous recombination, most likely during replication. As Polζ is evolutionarily conserved (40), these results raise the possibility that Polζ is responsible for enhanced mutational loads observed in HR-deficient BRCAl/2 mammalian cells, as well as in patients with RAD51 mutations and Fanconi anemia-like phenotypes (41).
For comparison, we also combined rev3 with the other base-substitution mutators. We found no reduction of CAN1R cells in the tsα1 background, indicating that Rad52 foci accumulating in this mutant (42) result from a different lesion(s) from in the decrease (58%) of CAN1R setting. In contrast, the inactivation of tsα1 breaks and/or single-strand gaps similar to (Fig. 2 similar to thus partially contributing to the composite signature 8. Finally, default base-excision repair of apurinic/apyrimidinic sites (43), Rev3-independent base-substitution mutations may result from lesions arising in an HR-deficient context.

Occurrence of Homozygous de Novo Mutations. Beyond heterozygous mutations, we found some base substitutions and InDels with an allelic ratio of 1.0, implying loss of the wild-type allele. This mostly occurred in the msh2, tsα1, and rad27 diploids, representing 2.3, 6.6, and 13.4% of the total frequency of base substitutions and small-InDel mutations, respectively (Fig. 3A and Dataset S10). In such situations, various types of genomic events, distinguishable by the state of the homologous chromosomes, could be invoked (Fig. 3B). In msh2, 70/71 cases occurred in full diploid cells and resulted from two identical (18 cases) or two distinct (48 cases) InDels, located within the same homopolymer tract on the homologs (Fig. 3A and Datasets S3 and S6). It can be explained from the >1-nt length of these motifs and high rate of polymerase slippage within homopolymers during replication (44). In tsα1, the homozygous SNPs were also mostly found on chromosomes with two copies (54/60 cases) but all were located in nonrepeated nucleotide sequences (Fig. 3A and Datasets S3 and S4). This was rather similar in rad27, except that 22/163 cases were associated with a change of the local copy number (1 or >2). We hypothesize that along the lineages the heterozygous de novo mutations were rendered homozygous upon a subsequent LOH event.

Detection of LOH Signatures in Hybrid Yeasts. LOH can result from mitotic interhomolog recombination, short tract mitotic gene conversions, and/or break-induced replication (BIR) events that are difficult to detect in isogenic strains. To comprehensively detect LOHs, we generated additional WT and mutant MA lines with an allelic ratio of ∼1.0, associated or not with local or chromosomal copy-number variation.
Fig. 4. Detection of LOHs in the SK1/BY MA lines. (A) Total number of LOH regions per clone normalized per passage. (B) Examples of allelic profiles in WT, tsa1, and rad27 MA lines at final passage 25. The genotypes of the SK/BY polymorphisms are plotted on the 16 chromosomes. A minimum of ≥3 adjacent markers of the same parental genotype were retained to define the local haplotype (SI Appendix) being either heterozygous SK/BY (gray), homozygous SK (blue), or homozygous BY (red). Triangles indicate the location of heterozygous (black) or homozygous (purple) de novo mutations (SNPs, MNPs, complexes, and small Indels; SI Appendix, Materials and Methods). (C) Heatmaps of the genome-wide occurrence of homozygosity among SK1/BY tsa1 and rad27 clones. (D) Number of homozygous mutations originated from mitotic recombination or BIR (labeled REC), chromosome loss or deletion identified in the SK1/BY mutants, and being located or not in an LOH region. (E) Two-step occurrence of homozygous de novo mutations upon interhomolog mitotic recombination or BIR. (F) Percentage of interstitial and terminal LOH–REC tracts. N: total number of LOH events.
from the polymorphic SK1/BY diploid that carries >53,000 constitutive SNP markers, distributed on each chromosome with one marker every 218 bp on average (Dataset S1). Compared with the isogenic and hybrid WT, the mutant MA lines exhibited similar mutation frequencies and specific mutational landscapes (compare Fig. 1 C and D and SI Appendix, Fig. S2A) but revealed the presence of numerous LOH regions, robustly defined to involve ≥3 adjacent markers (Fig. 4A and SI Appendix, Figs. S5–S9). In WT and pif1, LOHs were rare (0.09 and 0.12 LOH per clone per passage, respectively) (SI Appendix, Figs. S7 and S8). It was modestly increased in rad51 (0.18 LOH per clone per passage), while many rose in tsal and rad27 (1.2 and 2.5 LOHs per clone per passage corresponding to a 12.7- and 27.3-fold increase, respectively). In numerous instances, these LOH events involved several chromosomes in the same clone (Fig. 4B and SI Appendix, Figs. S5A and S6A). Considering all of the clones, the LOHs covered a large fraction of the genome in tsal and almost all of the genome in rad27 (Fig. 4C). Regarding the occurrence of homozygous mutations, again this was most frequent in tsal and rad27 cells (Fig. 4D and Dataset S10). Notably, 16/22 in tsal and 95/114 in rad27 were located in LOH regions with two copies of the chromosome (Fig. 4 C and E and Datasets S3 and S4), consistent with the hypothesis that along the cell lineage, mutations arose as heterozygous and passively became homozygous as part of a subsequent overlapping LOH event (Fig. 4E). Among the remaining events, 2 cases in tsal and 10 cases in rad27 resulted from the occurrence of a de novo mutation on one homolog and an overlapping de novo deletion on the other homolog (Figs. 3B and 4D and Dataset S4), as frequently found in tumor cells that carry a germline susceptibility mutation and then acquire a secondary somatic deletion on the homologous chromosome (45). Thus, the highly mutagenic tsal and rad27 strains stimulated SNPs and LOH events, a dual signature that accelerates and enlarges the spectrum of genome modifications.

Distributions and Mechanisms of Interstitial and Terminal LOHs. In tsal and rad27, the majority of LOHs were interstitial (81 and 76%, respectively; Fig. 4F), with a length varying from 33 bp to 419 kb and 17 bp to 846 kb, respectively (Dataset S12). The remaining LOHs were terminal, with lengths varying from 659 bp to 1,079 kb in tsal and 55 bp to 1,079 kb in rad27 (Dataset S12). Globally, the interstitial LOHs are shorter than the terminal LOHs (SI Appendix, Fig. S10A), consistent with their origin resulting from gene conversion-like events and/or double cross-overs rather than a single cross-over. The LOH size ranges were similar to those observed in a previous study (46). In both mutants, the LOHs were from one or the other parental haplotype, with a slight BY vs. SK1 excess genotype (58 and 55%, respectively). Due to the extended polymorphism of the BY and SK1 genomes, this slight bias may result from intrinsic and emerging lethal allele incompatibilities when part of the genome becomes homozygous, a somatic manifestation of the spore inviability observed in the SK1/S288C haploid segregants (47, 48). The annotation of the LOH breakpoint regions did not localize to specific functional elements except in pif1, where they often were in proximity to an LTR/Ty region and/or the rDNA locus (SI Appendix, Fig. S10B). Thus, after only 25 single-bottleneck passages, the stimulation of LOH created mosaic diploid genomes (Fig. 4C and SI Appendix, Figs. S5A and S6A) that reached 4.7 to 28.9% homozygosity per clone in tsal, and 26.6 to 60.7% in rad27.

The formation of terminal LOHs is a hallmark of BIR (49, 50), whereas both terminal and interstitial LOHs can result from mitotic cross-over recombination and/or gene conversion. Since BIR specifically depends on the activity of POL32 and PIF1 (51–54), we examined the effect of deleting these genes in the tsal mutant (Dataset S1). Similar to tsal, the tsal pol32 and tsal pif1 SK1/BY MA lines displayed increased base substitutions (13.9 and 32.3-fold vs. WT, respectively) and LOHs (11.1- and 17.5-fold vs. WT, respectively). The absolute frequency of terminal LOHs, however, was not significantly reduced (0.22, 0.25, and 0.29 per clone per passage in tsal, tsal pol32, and tsal pif1, respectively) and the large excess of interstitial vs. terminal LOHs was retained (81, 72, and 81% in tsal, tsal pol32, and tsal pif1, respectively) (Fig. 4F). Thus, such LOHs result from stimulation of mitotic recombination, rather than BIR, explaining the synthetic lethality of the tsal rad51 double mutant (42). We examined the length of the terminal LOH in tsal, tsal pif1, and tsal pol32 (SI Appendix, Fig. S10A) and observed no significant difference between tsal and tsal pif1 but a significant increase of terminal LOH length in tsal pol32, suggesting a role of Pol32 in...
Fig. 6. Dynamics of de novo mutations and LOH formation in the tsa1 clone N and rad27 clone C lineages. (A and B) Trajectory and heterozygous (gray) vs. homozygous (purple) status of the 45 de novo mutations detected in tsa1 clone N and of the 90 de novo mutations detected in rad27 clone C, respectively, from passages 1 to 25. Green: mutation found in a three-copy-number region and exhibiting a 1/3 allelic ratio. The coordinates of the mutations (chromosome number, position, nucleotides in the parental BY reference, nucleotides in the mutant clone) are shown. Green star: heterozygous mutation that became homozygous; orange star: mutations eliminated in a single passage. Numbers in parentheses refer to chromosomes shown in C. (C) Examples of fixation and elimination of mutations upon LOH and of mutations associated with the occurrence or extension of a nearby LOH event.
the distribution of the initiating events although the annotation of the terminal LOH breakpoints in the three \textit{tsa1} strains is similar (\textit{SI Appendix}, Fig. S10C). The contribution of BIR to the stimulation of the \textit{rad27} LOHs could not be examined due to the synthetic lethality of the \textit{rad27 pol32} double mutant (55). Nevertheless, the synthetic lethality of \textit{rad27} (like \textit{tsa1}) with \textit{rad51} (55) suggests that \textit{rad27} LOHs also largely result from interhomolog mitotic recombination, albeit not necessarily stimulated by an identical initiating lesion(s).

**Conclusion**

During the evolutionary history of a cell lineage, more LOH-embedded genes may have been transiently mutated and reversible epigenetic events that may not leave long-term molecular signatures (2, 57, 58). Retrospectively, in contrast to recombination avoidance, weakening the WT allele could be beneficial to restore cell physiology. A similar scenario for a dominant mutation in a mutator gene will permit a wave of cell genetic diversification and its subsequent elimination, avoiding the accumulation of additional disadvantageous mutations (2, 30, 46 mutations, respectively) compared with 19 mutations on average in the other clones. This can be explained by the presence of the \textit{MLH1-D16I} homozygous allele from \textit{BY} and \textit{PMS1-818} homozygous allele from \textit{SK1}, previously reported to confer a mismatch repair-deficient phenotype in haploid strains (56). This case illustrates the occurrence of a secondary mutator phenotype occurring during the clonal drift.

Along the lineages, several \textit{de novo} heterozygous mutations (2/45 and 9/90 in \textit{tsa1} and \textit{rad27}, respectively) chronologically became homozygous in a single-bottleneck passage as a consequence of an overlapping LOH, whereas others (1/45 and 6/90 in \textit{tsa1} and \textit{rad27}, respectively) were eliminated in favor of the WT allele (Fig. 6A and B, Datasets S13 and S16, and Movies S1 and S2). This opposite outcome is explained by the occurrence of an overlapping LOH mediated by an interhomolog recombination event, followed by the segregation of the nonsister chromatids carrying both WT or mutant alleles in the daughter cells (Fig. 4E). Multiple fixations and eliminations of mutations, as well as extensions of LOH tracts, also occurred in a single passage (Fig. 6C). The biological impact of such a mutator phenotype is functionally important because during cell proliferation, stimulation of LOHs will allow the phenotypic expression of \textit{de novo} mutations when fixed but also erase \textit{de novo} mutations when fixed but also leave long-term molecular signatures (2, 30, 46 mutations, respectively) compared with 19 mutations on average in the other clones. This can be explained by the presence of the \textit{MLH1-D16I} homozygous allele from \textit{BY} and \textit{PMS1-818} homozygous allele from \textit{SK1}, previously reported to confer a mismatch repair-deficient phenotype in haploid strains (56). This case illustrates the occurrence of a secondary mutator phenotype occurring during the clonal drift.

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**Conclusion**

The mutation of genes controlling genome stability and/or the epigenetic deregulation of their expression contributes to create the genetic diversity on which Darwinian selection can act. Our study has illustrated the large variety of mutational profiles generated by genetic deficiencies in genome-stability genes and described the dynamics of \textit{de novo} mutations and genome rearrangements (fixation and disappearance) during vegetative growth. This knowledge suggests ways to mechanistically interpret tumor cell genome evolution and genetic sensitivity (6–11, 59, 60), as well as genome evolution in species (1–3). On the evolutionary scale, impaired function of genes such as \textit{RAD27}/\textit{PEN1} and \textit{TSX1}/\textit{PRDX1} may allow the generation of genetic diversity, including occasional beneficial mutations (or suppressors of less fit mutant states), while additional recombination-dependent changes may be beneficial to resolve burdens of allelic incompatibilities in polymorphic and hybrid species. In the future, extending analyses of mutomes in yeast should allow refinement of the mutator scope of additional genome maintenance genes and graph the complexity of the genes/pathways and their interactions (4). It will also likely suggest how related phenomena operate in other organisms such as \textit{Caenorhabditis elegans} (61) and engineered human cell lines (62) amenable to mutome analyses.

**Materials and Methods**

**Strains and Mutation Accumulation Lines.** Mutation accumulation lines were obtained from \textit{BY} or \textit{BY5K1} diploid mutants carrying homozygous deletions of the genes listed in Fig. 1A. Details of the strains are described in \textit{SI Appendix} and the complete strain genotypes are listed in Dataset S1. All strain constructions were checked by PCR, Southern blot, or Sanger sequencing. Proper gene deletions were confirmed by the lack of read coverage upon whole-genome sequencing of the parental and MA lines.

**Generation of Mutation Accumulation Lines.** The mutation accumulation lines were obtained as described in ref. 21. They were streaked 4 to 16 times on agar to obtain cell lines at the Wellcome Sanger Institute for help with sample submission, tracking, and mapping. Research in the S.P.J. lab is supported by Wellcome Strategic Award 206388/Z/17/Z, Wellcome Investigator Award 206388/Z/17/Z, Wellcome PhD Fellowship 098051 (to M.H.), Cancer Research UK Programme Grant C6946/A24830, and Wellcome WT203144 Institute Core Funding. We thank the Cancer Genome Project and DNA-sequencing pipelines at the Wellcome Sanger Institute for help with sample submission, tracking, library preparation, and sequencing.
