Dosage Mutator Genes in *Saccharomyces cerevisiae*: A Novel Mutator Mode-of-Action of the Mph1 DNA Helicase

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ABSTRACT Mutations that cause genome instability are considered important predisposing events that contribute to initiation and progression of cancer. Genome instability arises either due to defects in genes that cause an increased mutation rate (mutator phenotype), or defects in genes that cause chromosome instability (CIN). To extend the catalog of genome instability genes, we systematically explored the effects of gene overexpression on mutation rate, using a forward-mutation screen in budding yeast. We screened ~5100 plasmids, each overexpressing a unique single gene, and characterized the five strongest mutators, MPH1 (mutator phenotype 1), RRM3, UBP12, PIF1, and DNA2. We show that, for MPH1, the yeast homolog of Fanconi Anemia complementation group M (FANCM), the overexpression mutator phenotype is distinct from that of mph1Δ. Moreover, while four of our top hits encode DNA helicases, the overexpression of 48 other DNA helicases did not cause a mutator phenotype, suggesting this is not a general property of helicases. For Mph1 overexpression, helicase activity was not required for the mutator phenotype; in contrast Mph1 DEAD-box function was required for hypermutation. Mutagenesis by MPH1 overexpression was independent of translesion synthesis (TLS), but was suppressed by overexpression of RAD27, a conserved flap endonuclease. We propose that binding of DNA flap structures by excess Mph1 may block Rad27 action, creating a mutator phenotype that phenocopies rad27Δ. We believe this represents a novel mutator mode-of-action and opens up new prospects to understand how upregulation of DNA repair proteins may contribute to mutagenesis.

KEYWORDS overexpression; mutator; genome-wide; forward mutation; Mph1

CANCER develops by accumulating stepwise genetic mutations in multiple genes that eventually lead to phenotypes such as uncontrolled proliferation and evasion of apoptosis (Vogelstein and Kinzler 2004; Hanahan and Weinberg 2011). Genome instability is an enabling characteristic of cancer, by increasing the likelihood of accumulating mutations in multiple driver genes (Hanahan and Weinberg 2011). Genome destabilizing mutations are thought to occur early during tumor development, reducing the fidelity of DNA transmission and repair, and thereby increasing the likelihood of accumulating multiple gene mutations (Negrini et al. 2010; Hanahan and Weinberg 2011). Genome instability can be classified under two distinct phenotypes: defects that increase mutation rate (mutator phenotype) and defects that increase the rate of aberrations to chromosome number or structure (chromosome instability or CIN). According to the mutator hypothesis, the high rate of genetic changes observed in cancer can be accounted for only by mutations that increase the mutation rate (Loeb 2011). For example, some deficiencies in DNA damage repair components will increase the spontaneous mutation rate, allowing the cells to acquire mutations that may offer a selective advantage and aid in the evolution and progression of tumors (Hanahan and Weinberg 2011).

The budding yeast *Saccharomyces cerevisiae* has been a valuable model system for delineating pathways involved in genome instability, and screens in yeast have identified mutator alleles that increase genome instability (Huang et al. 2003; Yuen et al. 2007; Stirling et al. 2012). A forward
mutation screen with the nonessential yeast deletion collection identified 33 genes whose null mutations resulted in a mutator phenotype (Huang et al. 2003), and 38 essential genes were identified in another screen (Stirling et al. 2014). Together, these screens and single gene studies generate a list of 127 yeast mutator alleles (Stirling et al. 2014). However, these studies were limited to studying loss-of-function (LOF, nonessential genes) or reduction-of-function (ROF, essential genes) alleles (Huang et al. 2003; Stirling et al. 2014).

As more cancer genomes are sequenced, it is becoming apparent that somatic copy number amplifications (SCNAs) are one of the most frequent genetic perturbations in cancer (Sanchez-Garcia et al. 2014). High recurrence of SCNAs suggests that some may contain cancer drivers (Sanchez-Garcia et al. 2014); however, a majority of recurrently amplified regions in tumor genomes (> 70%) do not contain known oncogenes or tumor suppressors (Zack et al. 2013). Since amplified regions often encompass multiple genes, identifying drivers remains a challenge (Zack et al. 2013), further emphasizing the need for methods to identify functionally relevant genes for tumor biology and progression within SCNAs.

To model potential effects on genome stability of gene amplification and/or overexpression, a genome-wide screen for an overexpression-induced mutator phenotype was conducted. For this purpose, we systematically overexpressed ~85% of the yeast open reading frames using an inducible promoter and assessed increases in the forward mutation rate. We identified 37 genes that we will refer to as dosage mutator (dMutator) genes, the majority of which are involved in DNA replication and DNA damage repair. The top five genes with the highest mutation rate were analyzed further. Overexpressing the DNA helicase MPH1 (mutator phenotype 1), the yeast homolog of human FANCM, led to the strongest dMutator phenotype, increasing the mutation rate by over 200-fold. Using a series of genetic and functional assays, we show that this phenotype is helicase- and translesion synthesis (TLS)-independent, and DEAH-box function- and RAD27-dependent. Thus, the mutator phenotype of MPH1 overexpression is distinct from that published for MPH1 in pDONR221 was performed using a Quick-Change kit (Stratagene, La Jolla, CA) following the manufacturer’s protocols. All clones were confirmed by sequencing. Genes were shuttled between vectors using Gateway Cloning (Life Technologies). Expression clones were obtained from the Lindquist Gateway Vector collection (Alberti et al. 2007).

**Dosage mutator screens and confirmations**

We used synthetic genetic array (SGA) technology to introduce a wild type CAN1 gene into the overexpression array (Tong et al. 2001). A query strain containing avt2Δ::KANMX was crossed to the yeast full-length expression ready (FLEX) array, each containing a plasmid with a single gene under the control of the GAL1 promoter (Douglas et al. 2012). The AVT2 gene is immediately adjacent to the CAN1 gene; thus, avt2Δ::KANMX provides a linked marker for selection of spores carrying the wild-type CAN1 locus. Following replica pinning steps, we generated an output array containing the avt2Δ::KANMX and the individual overexpression plasmids.

Cells were taken from the haploid selection plates and streaked to single colonies on haploid selection medium (SD-U-L-K+G418+Thialysine) and grown for 2 days at 30°C. Two induction steps were used to maximize the overexpression of genes from the plasmids. For the first induction, individual colonies were selected from the haploid selection plates and patched in duplicate onto medium containing galactose (SG-U-L-K+G418+Thialysine) and grown for 2 days at 30°C. Subsequently, for the second induction, cells from these patches were patched again into 1 cm × 1 cm patches on galactose-containing medium (SG-U-URA+G418). Patches were grown for 2 days at 30°C and cells were replica plated onto CAN selection plates (SD-R+ 50 μg/ml Canavanine). Plates were scored manually by counting colonies after incubating at 30°C for 2–3 days.

Direct transformations were used to validate our hits from the genome-wide mutator screen. Plasmids from the FLEX array were verified by DNA sequencing, and then transformed directly into a wild-type BY4741 strain, and single colonies were patched twice, in quadruplicate, onto galactose containing media (SG-U) for induction. Patches were then replica plated onto canavanine plates (SD-R+CAN) and scored as above.

**Fluctuation analyses**

Mutation rates per cell division were adapted from a previously described assay (Lang and Murray 2008). Briefly, four independent transformants from each strain were grown to saturation in synthetic complete medium lacking uracil and supplemented with galactose (SG-URA). Each saturated culture was diluted 1:10,000 into 24 wells of SG-URA and grown for 2 days at 30°C. Six random wells for each gene being tested were pooled and used to determine an average cell count using a TC20 cell counter (Bio-Rad, Hercules, CA). The remaining 18 wells were plated

**Materials and Methods**

**Yeast strains and plasmids**

Strains and plasmids used are listed in Supplemental Material, Table S2. Strain construction by homologous recombination at chromosomal loci was done using standard methods and confirmed by PCR (Longtine et al. 1998). Unless otherwise indicated, standard synthetic media lacking appropriate amino acids for plasmid selection with 2% galactose were used for mutation rate, and other assays. Site-directed mutagenesis of MPH1 in pDONR221 was performed using a Quick-Change kit (Stratagene, La Jolla, CA) following the manufacturer’s protocols. All clones were confirmed by sequencing. Genes were shuttled between vectors using Gateway Cloning (Life Technologies). Expression clones were obtained from the Lindquist Gateway Vector collection (Alberti et al. 2007).
onto plates containing medium supplemented with glucose and 50 μg/ml canavanine but lacking arginine (SD-ARG+Canavanine). Plates were incubated at 30°C for 2–3 days. Plates were scored for the frequency of CanR colonies. Rates were 0.01%, 25thecin (CPT), and hydroxyurea (HU) at concentrations of agents (DDAs) methyl methanesulfonate (MMS), camptothecin (CPT), and hydroxyurea (HU) at concentrations of 0.01%, 25 μg/ml, and 50 mM, respectively.

Serial spot dilutions

Strains were grown to saturation at 30°C in synthetic complete medium lacking uracil. Cultures were diluted to an OD₆₀₀ of 1 and plated in 10-fold serial dilutions onto plates supplemented with galactose and containing the DNA-damaging agents (DDAs) methyl methanesulfonate (MMS), camptothecin (CPT), and hydroxyurea (HU) at concentrations of 0.01%, 25 μg/ml, and 50 mM, respectively.

Growth curve and analysis

Strains were grown to saturation at 30°C in synthetic complete medium lacking uracil. Two microliters of the saturated culture was diluted into 200 μl of the appropriate medium containing galactose and DDAs (at concentrations specified above). OD₆₀₀ measurements were measured by a Tecan M200 plate reader at 30 min intervals for 72 hr at 30°C. Area-under-the-curve analysis was performed as previously described (Hamza et al. 2015).

Chromosome region specific effects

Strains containing URA3 located at different locations along chromosome VI (Lang and Murray 2008) were transformed with MPH1 overexpression plasmid marked with HIS3. Transformants were patched in quadruplicate onto SG-URA-HIS medium and incubated at 30°C for 2 days to induce overexpression. Patches were replica-plated onto SD-URA+5-FOA and incubated for 2 days.

Dependence on TLS pathway

TLS mutants, rev1Δ, rev3Δ and rad30Δ, from the yeast deletion collection (Winzezer et al. 1999) were transformed with the MPH1 overexpression plasmid and were patched onto SG-URA plates in triplicate. After 2 days incubation at 30°C, patches were replica plated onto SD-R+canavanine and incubated for 2–3 days at 30°C.

Data availability

Strains are available upon request. Table S2 contains all strains and plasmids used in this study.

Results

Systematic identification of dmutator genes using overexpression

To uncover genes whose overexpression results in an increased mutation rate, we performed a genome-wide screen in yeast. We screened an arrayed collection of yeast strains overexpressing ~5100 genes under the control of a galactose-inducible promoter for increased forward mutagenesis of the CAN1 marker (Hoffmann 1985; Douglas et al. 2012). Using SGA technology (Tong et al. 2001), we introduced a wild-type copy of the CAN1 gene into each of the yeast strains in the overexpression array, and screened for canavanine resistant (CANR) mutants in triplicate patches (Figure 1A). After screening ~5100 genes and confirming the primary hits with direct tests using sequence verified plasmids (see Materials and Methods), we generated a list of 37 dMutator genes whose overexpression increased the frequency of CANR mutants as compared to a vector alone control (Table 1). Approximately half of the dmutator genes (18/37) function in biological pathways such as DNA damage repair, DNA replication, or transcription, processes well known to influence genome instability (Saccharomyces Genome Database). For 12 of the genes, overexpression also increases chromosome instability (Duffy et al. 2016), highlighting the established considerable overlap between the mutator and chromosome instability phenotypes (Stirling et al. 2011).

Prior to this work, MLH1 was the only previously known dmutator gene (Shcherbakova and Kunkel 1999). Since it was not in the overexpression array, we tested directly the overexpression of MLH1 in our assay. Indeed, overexpression of MLH1 induced a dMutator phenotype under a galactose-inducible promoter (Figure 1B).

Determining mutation rate by fluctuation analysis

To determine the mutation rate of the dMutator genes, we used fluctuation analysis (Luria and Delbrück 1943; Lang and Murray 2008) and selected the top five dMutator genes, MPH1, UBP12, PIF1, RRM3, and DNA2, for further analysis. Overexpressing any one of these genes increased the mutation rate by at least 3-fold compared to a vector alone control (Figure 1C). Since four of these genes encode helicase activity, we reasoned that something common to ectopic helicase activity, we reasoned that something common to ectopic helicase activity could be driving mutagenesis. To assess this possibility, we directly retested 48 DNA helicases using the CAN1 mutation assay to determine whether they had been false negatives in the screen (Table S1). However, none of the additional helicases tested conferred a mutator phenotype when overexpressed, suggesting both that helicase activity alone is not a predictor of the dMutator phenotype and that the false negative rate of our assay was probably very low. Of the five identified dMutator genes, MPH1, a 3′–5′ DNA helicase and a sequence homolog of the human FANCM, resulted in a >200-fold increase in mutation rate (Figure 1C). While MPH1 deletion also results in a mutator phenotype (Entian et al. 1999), the mutation rates are dramatically different (Table 2).

Dosage mutator genes affect DNA metabolism

It is clear how the LOF or the ROF of a cellular protein may lead to a phenotype such as an increased mutator rate; however, it is not as clear how overexpression may affect this phenotype. Therefore, we wanted to further explore the mechanism behind the mutator phenotype for our top five dMutator genes. LOF alleles of two of the top five dMutator genes,
Figure 1 Dosage mutator (dMutator) screen workflow. (A) The overexpression array was mated to a query strain with avr2Δ::KanMX, which is immediately adjacent to the wild-type CAN1 gene using SGA. Following replica pinning steps, a haploid output array was generated where each strain contained both a unique gene overexpression plasmid and the wild type CAN1 gene. Haploid cells were streaked onto selective medium to obtain single colonies, and overexpression was induced by plating on medium containing 2% galactose. Cells were replica plated onto medium containing canavanine 48 hr postinduction to assess the mutator phenotype. (B) Top dMutator genes from our screen overexpressing the indicated genes with the vector alone control for comparison. MLH1 was tested as a positive control. Each gene was tested in triplicate and each patch represents an independent
PIF1 and MPH1, also increases the mutation rate (Entian et al. 1999; Huang et al. 2003), suggesting that, in these instances, overexpression may phenocopy LOF. To test this possibility, we chose to examine whether the phenotypic concurrence between overexpression and LOF will hold true for additional phenotypes.

Defective DNA repair is a well-established mechanism that leads to a mutator phenotype (Jackson and Bartek 2009), which often renders cells hypersensitive to DDAs. Thus, sensitivity to DDAs was an independent assay that could be utilized to compare directly the overexpression and LOF alleles of the dMutator genes. We tested the sensitivity of the top five dMutator genes to three DDAs—methyl methanesulfonate (MMS), hydroxyurea (HU) and camptothecin (CPT)—along with LOF alleles for mph1Δ, ubp12Δ, and rrm3Δ (Figure 2A and B). Direct comparison data were not collected for DNA2 transformant. For a complete list see Table 1. (C) Overexpression of MPH1, UBPI2, PIF1, RRM3, and DNA2 resulted in an increased mutation rate > 2-fold higher compared to a vector alone control. Mutation rates were quantified using fluctuation analysis. Error bars represent 95% confidence intervals with the average mutation rate shown above each bar.

| Table 1 Validated dMutator genes identified on solid media |
|-----------------------------------------------------------|
| ORF | Gene |
|-----|------|
| YBR234C | ARC40 |
| YER177W | BMH1 |
| YNL042W | BOP3 |
| YMR078C | CTF18 |
| YHR164C | DNA2A |
| YKL204W | EAP1 |
| YDR434W | GPI17 |
| YDR378C | LSM6 |
| YLR274W | MCM5 |
| YNL173C | MDG1 |
| YRR002C | MPH1 |
| YGR220C | MRPL9 |
| YKR087C | OMA1 |
| YPR162C | ORC4 |
| YML061C | PIF1 |
| YLR196W | PWPI |
| YML032C | RAD52 |
| YGL163C | RAD54 |
| YDR004W | RAD57 |
| YBR087W | RFC5 |
| YGL044C | RNA15 |
| YBR181C | RPS6B |
| YHR031C | RRM3 |
| YBR130C | SHE3 |
| YFL008W | SMC1 |
| YGL207W | SPT16 |
| YML010W | SPT5 |
| YHR041C | SSB2 |
| YGL097W | SRM1 |
| YLR005W | SSL1 |
| YCR042C | TAF2 |
| YOL006C | TOP1 |
| YLJ197W | UBP12 |
| YIL017C | VID28 |
| YDR248C | YDR248C |
| YGR126W | YGR126W |
| YHR122W | YHR122W |
| YMR167W | MLH1 |

| Table 2 Mutation rates of MPH1 overexpression and deletion |
|-----------------------------------------------------------|
| Mutation | 95% C.I. Upper | 95% C.I. Lower |
| Rate (10⁻⁹) | Bound (10⁻⁹) | Bound (10⁻⁹) |
| GAL-MPH1 | 224 | 251 | 199 |
| mph1Δ | 11.1 | 12.6 | 9.83 |

as it is an essential gene and pif1Δ does not grow on galactose (Figure 2A). Strains were determined to be “sensitive” or “not-sensitive” to DDAs based on relative percent fitness as measured by area-under-the-curve in liquid growth assays (P < 0.05) (Figure 2C). Overexpressing MPH1 sensitized cells to all three DDAs, whereas overexpressing UBPI2 and RRM3 increased sensitivity only to MMS and HU, or MMS and CPT, respectively (Figure 2C). Thus, the sensitivity profiles for MPH1, RRM3, and UBPI2 overexpression were distinct from the sensitivity profile of the corresponding deletes, suggesting different modes of action for overexpression-induced mutator phenotype.

We further tested the dMutator genes for the presence of increased DNA damage using Rad52 as a proxy. Rad52 is essential for homologous recombination and forms foci in response to double strand breaks (DSBs) leading to recombination events (Lisby et al. 2001; Symington 2002). The overexpression of one dMutator gene, RRM3, increased Rad52-foci (Figure 2, D and E). The mutator phenotype of the mph1Δ mutant is dependent on the TLS pathway (Entian et al. 1999; Schurer 2004). TLS allows bypassing of DNA lesions so replication can resume, and...
Figure 2. Overexpression of dMutator genes induces sensitivity to DDAs. (A) Serial spot dilutions of dMutator genes and respective deletion mutants on galactose, in the presence of the DDAs indicated. Deletion mutants were transformed with empty vector to enable tests on comparable growth medium. (B) Liquid growth curves of the dMutator genes and respective deletion mutants in galactose and in the presence of the indicated DDAs. (C) DDA sensitivities of dmutator genes and corresponding deletion mutants based on the liquid growth assays. Numbers represent relative percent fitness as measured by area-under-the-curve for each strain in triplicate. Red shading represents sensitive strains, and green shading represents strains that are not sensitive to drugs ($P < 0.05$). (D) Sample images of a reporter strain with Rad52-GFP was transformed with a plasmid overexpressing $RRM3$ or a vector alone control. (E) Quantified data for Rad52-foci in strains overexpressing dMutator genes and a $rrm3\Delta$. Data summarizes three independent experiments where > 100 cells were counted for each replicate.
involves several DNA polymerases that include Rev3 (DNA polymerase zeta), Rad30 (DNA polymerase eta), and Rev1 (deoxycytidyl transferase) (Johnson 1999; Friedberg 2002). A large portion of spontaneous mutations in the mph1Δ mutants arises by Rev3-mediated mutagenic bypass of DNA lesions using the error-prone TLS pathway (Scheller et al. 2000). Therefore, we tested if the mutator phenotype of MPH1 overexpression also relied on the TLS pathway. We transformed deletion mutants of the TLS polymerases REV1, REV3, and RAD30 with a plasmid overexpressing MPH1 and assayed for a mutator phenotype at CAN1. We did not observe a reduction in the frequency of CANR mutants in the individual TLS mutants (Figure 3A). We next generated double and triple mutants of TLS polymerases and quantified the mutation rate of MPH1 overexpression in these mutants. Removal of REV3 together with REV1 or RAD30 partially reduced the frequency of CANR mutants, and the simultaneous removal of all three TLS polymerases also reduced this frequency, although not more than the double mutants (Figure 3A). Thus, similar to the mph1Δ mutants, the mutagenesis induced by overexpression of MPH1 relies on Rev3-mediation mutagenic bypass; however, unlike the mutator phenotype of mph1Δ, mutations caused by MPH1 overexpression are only partially dependent on the TLS pathway.

**The dMutator phenotype of Mph1 depends on the DEAH-box but not on catalytic activity**

To further examine the dMutator phenotype, we next turned to the catalytic activities of MPH1. Mph1 possesses three conserved motifs: a DEAH-box (Scheller et al. 2000), an ATPase domain (Prakash et al. 2005), and a helicase domain (Kang et al. 2012). To determine whether catalytic activity (helicase and ATPase) and DEAH-box functions were necessary for the dMutator phenotype, we generated five point mutations that have been described previously. It has been shown that all these mutant proteins are expressed at similar levels and localize to the nucleus (Scheller et al. 2000). These include an ATPase mutant (K113Q), a helicase mutant (Q603D), and three DEAH-box mutants (D209N, E210Q, and H212D) (Banerjee et al. 2008). All of these mutations are predicted to impair the helicase activity, and one, D209N, to lack both ATPase and helicase activity but was proficient in DNA binding in vitro (Prakash et al. 2009).

The overexpression of all five single point mutants resulted in an overexpression mutator phenotype that was similar to that seen when wild-type MPH1 was overexpressed (Figure 3B). Therefore, similar to observations made with MPH1 overexpression-induced gross chromosomal rearrangement (Banerjee et al. 2008), the mutator phenotype of MPH1 overexpression does not appear to be due to the hyperactivation of the helicase or ATPase activities. However, while single DEAH point mutations had no effect, overexpressing double DEAH-box mutants (D209N, E210Q), (D209N, H212D), and (E210Q, H212D), or the triple DEAH-box mutant (D209N, E210Q, and H212D) with impaired Mg$^{2+}$ binding, ATP hydrolysis and/or NTP-dependent conformational change, and possibly protein stability or interaction defects (Scheller et al. 2000), lowered the mutation rate tremendously to near normal levels as compared to MPH1 (Figure 3B).
Mutations caused by MPH1 overexpression are localized to the telomeres

Overexpression of MPH1 also leads to the accumulation of single-stranded DNA (ssDNA) at telomeres (Luke- Glaser and Luke 2012). ssDNA can be more prone to damage since the nucleotides are more exposed to reactive species. Given that CAN1 was at the distal region of chromosome V, it seemed possible that the increased mutation rate at CAN1 is due to the presence of ssDNA. To determine whether gene position on the chromosome affected the mutation rate, we used strains in which URA3 was integrated at different locations on chromosome III (Figure 4A) (Lang and Murray 2008). Overexpressing MPH1 exhibited a mutator phenotype only when URA3 was located in the most telomeric regions (Figure 4B), while this phenotype is more generalized for other dMutator genes such as UBP12 and DNA2 (Figure S1, A and B).

The mutator phenotype of Mph1 is dependent on levels of Rad27 and Dna2

Since the dMutator activity of Mph1 depends on its DEAH box but not its specific catalytic activities, we reasoned that the mechanism may rely on competition for DNA binding with another DNA-binding protein. One ideal candidate is the flap endonuclease, Rad27. Both Mph1 and Rad27 are known to bind DNA flap structures and work in Okazaki fragment processing (Kang et al. 2009, 2012). One possibility is that when Mph1 is overexpressed it outcompetes the action of Rad27 from these structures, mimicking a RAD27 deletion. Alternatively, Mph1 has been shown to stimulate Rad27 in vitro, and this hyperactivity could be mutagenic. Also, consistent with coordinated action of Mph1 and Rad27 is that deletion of RAD27 has been reported to increase instability and ssDNA at telomeres (Parenteau and Wellinger 1999), and to cause a strong mutator phenotype (Tishkoff et al. 1997). Accordingly, while overexpressing RAD27 alone had no effect on the mutator phenotype, overexpressing RAD27 lowered the mutator phenotype caused by MPH1 overexpression (both qualitatively and quantitatively by fluctuation analysis) as compared to overexpressing MPH1 alone (Figure 4, C and D). Additionally, rad27Δ had a strong mutator phenotype that was not further enhanced by overexpression of MPH1 (Figure 4D). This epistatic relationship shows that MPH1 overexpression and RAD27 deletion work in the same mutagenesis pathway. Rad27 and Dna2 work coordinately in Okazaki fragment processing (Bae et al. 2001), and we also observed that overexpression of DNA2 reduced significantly the dMutator effect of MPH1, suggesting that lagging strand replication may be a target for mutagenesis by the MPH1 dMutator activity (Figure 4D).

Given that the mutator phenotype of MPH1 overexpression phenocopies the LOF mutation of RAD27, we tested whether this concordance was limited to the mutator phenotype or holds true for other phenotypes such as negative genetic interactions. Negative genetic interactions such as synthetic lethality takes place when the observed fitness defect of a double mutant is significantly less than that of the expected fitness based on the fitness of the two single mutants (Mani et al. 2008). Overexpression of MPH1 caused synthetic dosage lethality in strains lacking MUS81, ELG1, and MMS1, but had no phenotype in RAD1, CHL1, and EXO1 deletions (Figure 4E). This suggests that the mutator phenotype of MPH1 overexpression is only partially redundant with loss of rad27Δ. Interestingly, among those tested, mutations with specific functions in handling DNA replication stress (i.e., mus81Δ, elg1Δ, and mms1Δ) appear to be most negatively affected by MPH1 overexpression, possibly suggesting that the replication role of Rad27 is the relevant activity.

To further establish that MPH1 overexpression increases mutation rate by either outcompeting or squelching its functional partners or those of Rad27, we chose to examine the effect of removing, SGS1, MUS81, and SRS2 on the dMutator phenotype. SGS1 encodes another DNA helicase, which functions in parallel with Mph1 to regulate the choice of homologous recombination pathway to be used (Jain et al. 2016), the endonuclease Mus81 and the Srs2 helicase also influence repair pathway choice and recombination intermediate processing (Mazón and Symington 2013; Mitchel et al. 2013). Deletion of SGS1 or MUS81 led to small but significant decreases in the dMutator phenotype of MPH1 overexpression (Figure 4F), while deletion of SRS2 led to a > 5-fold decrease in mutation rate (Figure 4F).

Discussion

A genome-wide screen for genes that, when overexpressed, increased the mutation rate, identified 37 dMutator genes in yeast. The majority of these genes belong to biological processes, such as DNA repair, previously known to impact mutation rate, and ~30% of the dMutator genes also cause chromosome instability as seen previously for LOF and ROF mutations (Stirling et al. 2012). Incorporating the data from our screen with published data, a total of 210 genes are implicated in increasing the mutator phenotype in yeast (Huang et al. 2003; Stirling et al. 2012).

The five strongest dMutator genes, MPH1, UBP12, PIF1, RRM3, and DNA2, had mutation rates at least two times greater than wild type (Figure 1C). Since four of these genes possess helicase activity, we tested directly whether ectopic helicase activity is responsible for the dMutator phenotype by overexpressing a panel of other DNA helicases, and found that helicase activity itself did not predict the dMutator phenotype. We further characterized the top five dMutator genes by examining their sensitivities to DDAs, and their effects on DNA integrity. Cells overexpressing any one of the five dMutator genes were sensitive to DDAs, implicating defective DNA repair as one possible mechanism for the dMutator phenotype (Figure 2, A and B). However, overexpression of only one gene, Rrm3, induced higher than wild type levels of Rad52 foci (Figure 2E). Consequently, the dMutator phenotype of Rrm3 may be due to both increased DNA damage and defective DNA repair. When the DDA sensitivity profiles of
Figure 4  MPH1 dMutator phenotype is position-dependent and is epistatic to Rad27. (A) Schematic of absolute and relative positions of URA3 position along chromosome III. The black circle represents the centromere, and the black boxes represent the location of the URA3 gene relative to the centromere and the telomere. Numbers beside each schematic denotes the absolute position of URA3 on the chromosome. (B) Strains containing URA3 at positions indicated in (A) with a vector alone control or with the overexpression of MPH1. Each strain was assayed with four independent transformants. (C) The mutator phenotype of strains overexpressing RAD27 (URA marked), MPH1 (HIS marked), or both plasmids together along with the vector alone controls. (D) Mutation rates of strains overexpressing RAD27, MPH1, RAD27, and MPH1, as well as a rad27Δ strain overexpressing...
MPH1, RRM3, and UBP12 were compared directly to the corresponding LOF alleles. There was no concurrence between the profiles, implying different mechanisms of action (Figure 2C). In the case of MPH1, whose LOF allele also induces a mutator phenotype, the unique sensitivity profile to DDAs suggests that overexpression does not mimic deletion in this case (Veitia 2005). Taken together, these data suggest that dMutators are relatively rare, and, while they function in the same pathways as canonical mutators (such as DNA repair), overexpression causes mutations by different mechanisms as compared to LOF or ROF.

The differences in mechanism of action between overexpression and deletion were particularly pronounced for MPH1. MPH1 overexpression caused sensitivity to MMS, CPT, and HU, whereas MPH1 deletion caused sensitivity only to MMS (Figure 2, A and B). MPH1 overexpression also caused a higher mutation rate than the MPH1 deletion and, furthermore, the mutator phenotype caused by MPH1 overexpression was at least partially independent of TLS polymerases (Figure 3A and Table 2). We also showed that the MPH1 dMutator phenotype was independent of the helicase and ATPase activities of Mph1, but was abrogated by simultaneously mutating either two or three residues in the DEAH domain (Figure 3B). These DEAH mutations may also abolish binding to Mg2+, ATP hydrolysis, and/or the NTP-dependent conformational change, suggesting to us that proper DEAH-box function is the essential feature of its dMutator mechanism. Alternatively, the result of the multiple DEAH mutants may be impaired protein- or DNA-binding interfaces, which could arise due to conformational changes in the protein or because the protein has become unstable. Either way our analysis implicates noncatalytic functions of Mph1 in inducing CAN1 hypermutation.

RAD27 deletion causes a mutation rate comparable to MPH1 overexpression (Huang et al. 2003), and an increase in telomeric ssDNA (Parenteau and Wellinger 1999), as has been seen previously with MPH1 overexpression (Luke-Glaser and Luke 2012) (Figure 4B). Prompted by these similarities, we tested for genetic interactions between MPH1 and RAD27. Deletion of RAD27 was epistatic to MPH1 overexpression with respect to mutation rate (Figure 4D). This suggested that hypermutation in Mph1 overexpressing cells is mediated by impairing the Rad27 pathway. Consistent with this interpretation, overexpressing RAD27 or DNA2 [which works in parallel with Rad27 in Okazaki fragment processing (Bae et al. 2001)], together with MPH1 strongly reduced the mutation rate compared to MPH1 alone (Figure 4C). These data suggest that the relative stoichiometry of Mph1, Rad27, and Dna2 is critical to prevent mutations.

Several lines of evidence support defective replication as a mechanism for the dMutator phenotype of MPH1: (1) MPH1 overexpression recapitulated the genetic interactions between rad27Δ and DNA replication fork protection factors such as mus81Δ, mms1Δ, and elg1Δ (Figure 4E); (2) the position specific mutation rate increases we saw for URA3 at subtelomeric loci in Chr VI (Figure 4B) occur in late replicating regions of the chromosome (Lang and Murray 2011); and (3) removal of SRS2 strongly suppressed the dMutator phenotype of MPH1 overexpression (Figure 4F). Srs2 has a previously identified antimutator activity in deletions of MMS2, a ubiquitin-conjugating enzyme required for postreplicative repair (Broomfield and Xiao 2002). In addition, defects in Okazaki fragment processing caused by loss of RAD27 have been shown to activate postreplicative repair via the exposure of ssDNA (Parenteau and Wellinger 1999). Indeed, as with our data on MPH1 overexpression, these authors showed that mutation rates in rad27Δ cells were suppressed only partially by deletion of REV3 (Becker et al. 2015).

Together, these data lead us to propose a model where high levels of Mph1 lead to dysregulation of Okazaki fragment processing (Kang et al. 2009, 2012), signaling via Srs2 to the postreplicative repair and to damage-tolerance/TLS pathways, which together increase the frequency of mutations. This is particularly pronounced in late-replicating and/or subtelomeric regions, where both MPH1 overexpression or rad27Δ are known to enhance the exposure of ssDNA. While the enzymatic activity of Mph1 is not required for the dMutator effect, increasing the concentration of Rad27 or Dna2 in the cell can revert the dMutator phenotype. Therefore, we favor the view that competition for DNA flap binding by excess Mph1 may impair normal flap processing sufficiently to trigger mutations.

The human MPH1 homolog FANCN is a breast cancer susceptibility gene that is characterized by missense or nonsense somatic mutations in cancer cells (Kiiski et al. 2014; Peterlongo et al. 2015). There are also examples of FANCN overexpression in cancer that are of unknown significance (Cerami et al. 2012; Gao et al. 2013). Our study shows that the overexpression of DNA repair proteins, specifically Mph1, can cause imbalances in DNA transactions through competition to drive mutagenesis. Further analysis of the effects of gene overexpression on genome instability should reveal new mechanisms by which protein imbalances affect genome maintenance.

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MPH1 or a vector alone control assayed using fluctuation analysis. Error bars represent 95% confidence intervals, with the average mutation rate shown above each bar. (E) Synthetic dosage lethal interactions with MPH1 for mutations that are synthetic lethal with a RAD27-null mutant. (F) Mutation rates for strains lacking SG31, MUS81, and SRS2 overexpressing MPH1 or a vector alone control assayed using fluctuation analysis. Error bars represent 95% confidence intervals with the average mutation rate shown above each bar.
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Figure S1. Positional effects of the dMutator phenotype. (A) Schematic of absolute and relative positions of *URA3* position along chromosome III. The black circle represents the centromere and the black boxes represent the location of the *URA3* gene relative to the centromere and the telomere. Numbers beside each schematic denotes the absolute position of *URA3* on the chromosome. (B) Strains containing *URA3* at positions indicated in A with a vector alone control or with the over-expression of *UBP12, DNA2* and *RRM3*. Each strain was assayed with four independent transformants.
Table S1: DNA helicases tested for dMutator phenotype. (.xlsx, 40 KB)

Available for download as a .xlsx file at:
http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.116.192211/-/DC1/TableS1.xlsx
Table S2: Yeast strains and plasmids used in this study. (.xlsx, 38 KB)

Available for download as a .xlsx file at:

http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.116.192211/-/DC1/TableS2.xlsx