Shared Genetic Pathways Contribute to the Tolerance of Endogenous and Low-Dose Exogenous DNA Damage in Yeast

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ABSTRACT DNA damage that escapes repair and blocks replicative DNA polymerases is tolerated by bypass mechanisms that fall into two general categories: error-free template switching and error-prone translesion synthesis. Prior studies of DNA damage responses in *Saccharomyces cerevisiae* have demonstrated that repair mechanisms are critical for survival when a single, high dose of DNA damage is delivered, while bypass/tolerance mechanisms are more important for survival when the damage level is low and continuous (acute and chronic damage, respectively). In the current study, epistatic interactions between DNA-damage tolerance genes were examined and compared when haploid yeast cells were exposed to either chronic ultraviolet light or chronic methyl methanesulfonate. Results demonstrate that genes assigned to error-free and error-prone bypass pathways similarly promote survival in the presence of each type of chronic damage. In addition to using defined sources of chronic damage, rates of spontaneous mutations generated by the Pol ζ translesion synthesis DNA polymerase (complex insertions in a frameshift-reversion assay) were used to infer epistatic interactions between the same genes. Similar epistatic interactions were observed in analyses of spontaneous mutation rates, suggesting that chronic DNA-damage responses accurately reflect those used to tolerate spontaneous lesions. These results have important implications when considering what constitutes a safe and acceptable level of exogenous DNA damage.

DAMAGE to cellular DNA that results from normal metabolic processes is classified as spontaneous, while that resulting from exogenous physical or chemical agents is considered induced. Spontaneous damage occurs at low, continuous levels and hence is chronic in nature. Though induced damage is typically delivered in a single, acute dose, it can also be chronic, with examples including daily exposure to solar radiation or cigarette smoke. Spontaneous and induced damage are dealt with by two general, evolutionarily conserved mechanisms: one that permanently removes the damage and one that temporarily bypasses the damage, allowing it to be tolerated (for a general overview, see Ciccio and Elledge 2010). Most damage removal occurs via conserved excision repair pathways, but a small number of damages can be directly reversed enzymatically (e.g., thymine–thymine

dimer reversal by photolyase). Damage removal by excision repair generates a single-strand gap that is filled using the complementary, undamaged strand as a template and hence is a high-fidelity process. Such repair only operates, however, when a relevant lesion is present in double-strand DNA. Damage that escapes repair and is encountered during replication can block the progress of DNA synthesis, leading to the formation of a single-strand gap opposite the lesion, replication fork collapse, cell-cycle arrest, and/or death. DNA-damage bypass/tolerance pathways thus become critically important during S and G2 phases, as these allow completion of replication and timely cell-cycle progression. Following bypass, the damage is once again present in duplex DNA and has another opportunity to be permanently removed via excision repair.

There are several distinct mechanisms of lesion bypass, and these are collectively referred to as DNA damage tolerance (DDT) pathways (reviewed by Friedberg 2005; Sale 2012). DDT can be subdivided into (1) error-free template switching that involves copying information from the undamaged sister chromatid and (2) error-prone bypass that is effected by specialized translesion synthesis (TLS) DNA polymerases. Though the exact nature of template

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Figure 1 Responses to acute and chronic DNA damage. The yellow lightning bolt and stars represent DNA damage, with single-strand gaps accumulating as a result of repair (A) or from blocked DNA replication (B). In B, black and red lines correspond to template and nascent DNA strands during replication, and dotted red lines represent DNA synthesized during template switching or gap filling. See text for further explanation.

switching remains elusive, current data suggest that it involves homologous recombination (HR) functions (Vanoli *et al.* 2010). TLS polymerases directly replicate over a template lesion, but do so with an elevated risk of introducing mutations; an incorrect nucleotide can be inserted either directly opposite the lesion or during coincident copying of adjacent, undamaged DNA. Whether insertion opposite the lesion is error-free or error-prone depends on the nature of the lesion and the particular TLS polymerase involved (reviewed by Waters *et al.* 2009).

Saccharomyces cerevisiae has served as a model for elucidating the genetic control and basic mechanisms of DDT in eukaryotes (reviewed by Boiteux and Jinks-Robertson 2013). Central to regulating DDT are post-translational modifications to PCNA, the sliding clamp that promotes processivity of replicative DNA polymerases and serves as a landing pad for numerous proteins involved in DNA metabolism (reviewed by Moldovan *et al.* 2007). Monoubiquitination (mono-Ub) of PCNA by the Rad6–Rad18 complex promotes TLS (Hoegge *et al.* 2002; Stelter and Ulrich 2003) by one of three yeast TLS polymerases: Pol zeta (ζ), Pol eta (η), or Rev1. Pol ζ works in concert with Rev1 and its activity generates >50% of spontaneous and 90% of induced mutations (Lawrence 1994). Whereas mono-Ub of PCNA promotes TLS, extension of the single Ub moiety into a polyubiquitin (poly-Ub) chain by Rad5 and Mms2–Ubc13 promotes template switching (Hoegge *et al.*

2002; Stelter and Ulrich 2003). Finally, sumoylation of PCNA by Siz1 promotes interaction with the Srs2 helicase (Pfander *et al.* 2005), which acts as an antirecombinase and prevents accumulation of inappropriate and potentially toxic HR intermediates (Krejci *et al.* 2003; Veaute *et al.* 2003). Defining the precise relationship between HR and template switching has been difficult, with members of the RAD52 epistasis group of HR genes being required for template switching (Zhang and Lawrence 2005; Gangavarapu *et al.* 2007; Vanoli *et al.* 2010), as well as for bypass via more canonical HR when template switching and/or TLS fails. Additionally, whether DDT normally occurs directly at a stalled replication fork or as a gap-filling process after fork passage, and which mechanism of DDT normally predominates, have been controversial. Though it is possible to limit expression of Rad18 to specific points in the cell cycle and thereby uncouple DDT from replication (Daigaku *et al.* 2010; Karras and Jentsch 2010), recent analyses suggest that template switching and TLS primarily occur during S and G2, respectively (Huang *et al.* 2013). The strong mutator phenotype observed when error-free DDT is eliminated suggests that TLS is normally the minor bypass/tolerance pathway (Broomfield *et al.* 1998; Minesinger and Jinks-Robertson 2005).

Ultraviolet (UV) light is a common mutagen used to define DNA-damage responses in yeast. Though acute doses are typically employed, recent studies have shown that cellular

Table 1 Yeast strains

Strain	Relevant repair genotype	Construction
SJR2646	<i>rev3Δ::hphMX4</i>	SJR1467 with PCR fragment amplified from <i>hphMX4</i>
SJR2717	<i>rad18Δ::hisG-URA3-hisG</i>	SJR1467 with <i>EcoRI</i> -digested pJJ239 (from L. Prakash)
SJR2733	<i>rad52Δ::kanMX6</i>	SJR1467 with PCR fragment amplified from pFA6a-kanMX6
SJR2774	<i>rad5Δ::hphMX4</i>	SJR1467 with PCR fragment amplified from <i>hphMX4</i>
SJR2815	<i>rad5Δ::hphMX4 rad18Δ::hisG-URA3-hisG</i>	SJR2774 with <i>EcoRI</i> -digested pJJ239
SJR2828	<i>rad5Δ::hphMX4 rad18Δ::hisG-URA3-hisG rev3Δ::kanMX6</i>	SJR2815 with PCR fragment amplified from pFA6a-kanMX6
SJR2830	<i>rev3Δ::hphMX4 rad52Δ::kanMX6</i>	SJR2646 with PCR fragment amplified from pFA6a-kanMX6
SJR2831	<i>rad5Δ::hphMX4 rad18Δ::hisG-URA3-hisG rad52Δ::kanMX6</i>	SJR2815 with PCR fragment amplified from pFA6a-kanMX6
SJR2923	<i>mph1Δ::kanMX6</i>	SJR1467 with PCR fragment amplified from pFA6a-kanMX6
SJR2929	<i>mms2Δ::kanMX6</i>	SJR1467 with PCR fragment amplified from pFA6a-kanMX6
SJR2939	<i>mms2Δ::kanMX6 rad52Δ::hphMX4</i>	SJR2929 with PCR fragment amplified from <i>hphMX4</i>
SJR2940	<i>mms2Δ::kanMX6 mph1Δ::hphMX4</i>	SJR2929 with PCR fragment amplified from <i>hphMX4</i>
SJR3005	<i>mms2Δ::kanMX6 mph1Δ::hphMX4 rad52Δ::hisG-URA3-hisG</i>	SJR2940 with <i>BamHI/EcoRI</i> -digested pSR136
SJR3036	<i>rad5Δ::hphMX4 rad52Δ::His3MX6</i>	SJR2774 with PCR fragment amplified from pFA6a-His3MX6
SJR3087	<i>rad5Δ::hphMX4 rad18Δ::hisG-URA3-hisG rev3Δ::His3MX6</i>	SJR2815 with PCR fragment amplified from pFA6a-His3MX6
SJR3088	<i>mph1Δ::kanMX6 rev3Δ::His3MX6</i>	SJR2923 with PCR fragment amplified from pFA6a-His3MX6
SJR3097	<i>mms2Δ::kanMX6 rev3Δ::His3MX6</i>	SJR2929 with PCR fragment amplified from pFA6a-His3MX6
SJR3110	<i>mms2Δ::kanMX6 mph1Δ::hphMX4 rev3Δ::hisG-URA3-hisG</i>	SJR2940 with <i>EcoRI/BamHI</i> -digested pPM292 (from L. Prakash)
SJR3124	<i>rad18Δ::hisG-URA3-hisG rad52Δ::hphMX4</i>	SJR2717 with PCR fragment amplified from <i>hphMX4</i>
SJR3132	<i>rad30Δ::loxP-URA3KI-loxP</i>	SJR1467 with PCR fragment amplified from pUG72
SJR3207	<i>rad5Δ::hphMX4 rad18Δ::hisG-URA3-hisG rev3Δ::His3MX6 rad52Δ::kanMX6</i>	SJR3087 with PCR fragment amplified from pFA6a-kanMX6
SJR3217	<i>rad30Δ::loxP-URA3KI-loxP rev3Δ::kanMX6</i>	SJR3132 with PCR fragment amplified from pFA6a-kanMX6
SJR3218	<i>rad30Δ::loxP-URA3KI-loxP rad52Δ::kanMX6</i>	SJR3132 with PCR fragment amplified from pFA6a-kanMX6
SJR3280	<i>rad30Δ::loxP-URA3KI-loxP rev3Δ::kanMX6 rad52Δ::hphMX4</i>	SJR3217 with PCR fragment amplified from <i>hphMX4</i>

All strains were derived from SJR1467 (*MATα ade2-101_{oc} his3Δ200 ura3ΔNco lys2ΔA746,NR*) by transformation. Plasmid *hphMX4* is described in Goldstein and McCusker (1999), plasmids pFA6a-kanMX6 and pFA6a-His3MX6 in Longtine *et al.* (1998), plasmid pSR136 in Freedman and Jinks-Robertson (2002), and plasmid pUG72 in Gueldener *et al.* (2002).

and genetic responses to chronic, low levels of UV damage are distinctly different from those observed following acute exposure (Hishida *et al.* 2009). This difference was presaged by early genetic studies demonstrating a decrease in induced, but an increase in spontaneous, mutagenesis in a *rad6* mutant (Lawrence 1994). Subsequent work demonstrated that *RAD5* is required for a separate pathway of spontaneous mutagenesis that can be detected in the absence of *RAD6/RAD18* (Liefshitz *et al.* 1998; Cejka *et al.* 2001; Minesinger and Jinks-Robertson 2005). In terms of survival following exposure to acute UV, early studies demonstrated that the nucleotide excision repair (NER) pathway is much more important for survival than is the *RAD6/RAD18* DDT pathway (Haynes and Kunz 1981). This hierarchy reverses, however, when cells are continuously exposed to a chronic, low dose of UV, with *RAD18*-mediated DDT being much more important for survival than NER (Hishida *et al.* 2009). In the absence of *RAD18*-dependent DDT, chronic damage was associated with the accumulation of single-strand gaps during replication and a subsequent G2 arrest. The critical role of NER for survival following an acute UV dose likely reflects the triggering of a G1 or G2 checkpoint by NER-generated gaps, which allows sufficient time to remove what would otherwise be a lethal load of damage during S or M phase, respectively (Novarina *et al.* 2011). In addition to genetic differences in response to acute vs. chronic UV exposure, dose-dependent differences are observed when yeast are continuously exposed to methyl methanesulfonate (MMS) (Huang *et al.* 2013). Based on genetic analyses, the transition from chronic to acute MMS exposure occurs at a drug concentration

of ~0.001%. Finally, NER is more important than HR for surviving an acute dose of formaldehyde, which causes DNA-protein crosslinks, while HR is more important during chronic formaldehyde exposure (De Graaf *et al.* 2009). A general framework of lesion repair/bypass that incorporates these observations is presented in Figure 1.

In the current study, the survival of yeast DDT mutants during chronic UV or MMS exposure was compared, and we find similar relationships and hierarchies between the genes examined. Importantly, analyses reveal a previously unappreciated role of TLS during the bypass of chronic UV. In addition to examining genetic responses to chronic damage, a Pol ζ-dependent mutation signature was used to examine the genetic control of error-free and error-prone DDT pathways that deal with spontaneous, replication-blocking lesions. These analyses reveal striking parallels between responses to spontaneous and chronic DNA damage in the mutagenesis and survival assays, respectively, indicating that chronic exposure to defined DNA-damaging agents provides an accurate and physiologically relevant reflection of how diverse types of endogenous DNA damage are tolerated.

Materials and Methods

Strain construction

All yeast strains were derived from strain SJR1467 (*MATα lys2ΔA746,NR ade2-101_{oc} his3Δ200 ura3ΔNco*) (Lehner *et al.* 2012). Mutant strains were constructed by one-step gene

Figure 2 Epistatic interactions between genes during growth in the presence of CLUV or CLMMS. (A–D) Ten-fold serial dilutions of cells grown to saturation in YEPD were spotted in parallel onto YEPD plates with or without the indicated concentration of MMS or in parallel onto YEPD plates that were shielded from or continuously exposed to the indicated UV dose. Plates were photographed after 2 days of incubation. In A, cells additionally were exposed in parallel to an acute UV dose. For each strain genotype, two independent isolates were tested and results were indistinguishable. It should be noted that the *rad52Δ* strain has a general growth defect.

disruption using PCR-generated cassettes containing a selectable marker. A complete list of strains is given in Table 1.

Mutation rates and spectra

Mutation rates were determined using at least 16 cultures, which were composed of at least 8 cultures from each of two independent strain isolates. Cultures were grown to saturation at 30° in nonselective YEPGE medium (1% yeast extract, 2% Bacto-peptone, 2% glycerol, 2% ethanol, and 250 mg/liter adenine). Appropriate dilutions were plated onto YEPD medium (YEP plus 2% dextrose) to determine total cell number and onto lysine-deficient synthetic glucose medium to select Lys⁺ revertants. Mutation rates were calculated by the Lea–Coulson method of the median using the FALCOR application (<http://www.mitochondria.org/protocols/FALCOR.html>). The mutation rate of a specific mutation type was calculated by multiplying its proportion in the corresponding spectrum by the total Lys⁺ reversion rate. Distributions of mutation frequencies between genotypes were compared using the nonparametric Mann–Whitney–Wilcoxon test in R (function: “pairwise.wilcox.test”).

To generate mutation spectra, DNA was extracted from purified Lys⁺ colonies isolated from independent cultures (http://jinks-robertsonlab.duhs.duke.edu/protocols/yeast_prep.html). The frameshift reversion window of the *LYS2* gene was amplified by PCR and sequenced by the Duke University DNA Analysis Facility (Durham, NC) using primer 5'-GTAACCGGTGACGATGAT.

Chronic, low-dose mutagen treatments

Cells were grown to near saturation for 2 days in liquid YEPGE medium with shaking at 30°. For chronic UV experi-

ments, 10-fold serial dilutions were spotted on YEPD plates; plates were covered with thin sheets of vinyl (cut from Staples heavy duty sheet protectors) and exposed to a chronic dose of UV (254-nm germicidal lamp) for 2 days at 30°. The UV dose was attenuated using wire mesh placed between the light source and plates. The experimental UV doses used were below the level of detection of a UV meter (UVX-25, Ultraviolet Products), but the 3× (highest) dose generated results phenotypically similar to those in other studies (Hishida *et al.* 2009, 2010). Control plates were treated the same, but covered with aluminum foil to prevent UV exposure. For MMS experiments, 10-fold serial dilutions were spotted on YEPD or YEPD + MMS plates and grown for 2 days at 30°. Fresh YEPD + MMS plates were prepared 18–24 hr before use.

Results

The relative importance of NER and DDT following acute vs. chronic UV exposure was confirmed in the haploid strain background used here. As shown in Figure 2A, eliminating NER by deleting *RAD14* resulted in much greater sensitivity to acute UV (5 J/m²) than did deleting *RAD18*, while a *rad18Δ* strain was much more sensitive to chronic UV than was a *rad14Δ* strain. Interactions between and hierarchies of genes specifically involved in DDT were then examined by growing mutants in the presence of a chronic, low dose of UV or MMS (CLUV and CLMMS, respectively) that had no effect on growth of the wild-type (WT) parent strain. In the case of CLMMS, serial dilutions of cells were plated on rich medium containing 0.00025–0.001% MMS. For CLUV experiments, serial dilutions of cells spotted onto rich medium were

Table 2 Genes used in genetic analyses

Gene	Protein function
<i>MMS2</i>	Forms a heterodimeric complex with Ubc13, a Ub conjugase that polyubiquitinates PCNA at K164. This modification of PCNA is required for error-free DDT.
<i>MPH1</i>	3' to 5' helicase involved in error-free lesion bypass.
<i>RAD5</i>	E3 Ub ligase that, together with the Mms2-Ubc13 complex, polyubiquitinates K164 of PCNA to initiate error-free DDT.
<i>RAD14</i>	Essential component of the nucleotide excision repair pathway.
<i>RAD18</i>	E3 Ub ligase that binds ssDNA and forms a complex with the Rad6 Ub conjugase. Rad6–Rad18 monoubiquitinates PCNA at K164, a modification required for both error-free and error-prone DDT.
<i>RAD30</i>	Pol η TLS polymerase.
<i>RAD52</i>	Required for error-free DDT and homologous recombination.
<i>REV3</i>	Catalytic subunit of the Pol ζ TLS polymerase.

continuously exposed to a low level of UV light, which was arbitrarily defined as a $1\times-3\times$ dose. The use of variable doses of UV or MMS allowed a dynamic range of growth inhibition to be monitored, allowing a more accurate assessment of possible genetic interactions. Genes relevant to the current study, along with a brief description of encoded protein function, are listed in Table 2.

Both subpathways of Rad18-dependent DDT are important during exposure to chronic damage

The DDT pathway regulated by the Rad6–Rad18 complex is composed of two subpathways: an error-prone pathway mediated by TLS polymerases and an error-free pathway that requires Rad5 and the Ubc13–Mms2 complex (reviewed by Boiteux and Jinks-Robertson 2013). The importance of error-free DDT for survival during CLUV treatment was previously inferred through analyses of *rad18* Δ and *rad5* Δ single mutants; loss of TLS alone (*rev1* Δ *rev3* Δ *rad30* Δ triple mutant) did not affect survival (Hishida *et al.* 2009). As observed in acute damage studies, deletion of *RAD18* conferred greater sensitivity to CLUV than did *RAD5* deletion, consistent with the role of Rad5 only in the error-free pathway of Rad18-regulated DDT. Because spontaneous-mutagenesis studies have implicated *RAD5* in *RAD18*-independent DDT as well (Liefshitz *et al.* 1998; Cejka *et al.* 2001; Minesinger and Jinks-Robertson 2005), however, we compared the CLUV sensitivity of *rad18* Δ and *rad5* Δ single mutants to that of a *rad18* Δ *rad5* Δ double mutant. If Rad5 acts only in the Rad18 error-free bypass pathway, then the double mutant should be no more sensitive than the most sensitive single mutant. As expected, a *rad18* Δ mutant was much more sensitive to $1\times$ CLUV than was a *rad5* Δ mutant. The combined effect of the deletions was additive/synergistic rather than epistatic, however, demonstrating that Rad5 has a Rad18-independent function when cells are exposed to CLUV (Figure 2B). A similar relationship was observed when the single- and double-mutant strains were grown in the presence of CLMMS.

In previous analyses, *rad5* Δ and *rad18* Δ mutants were indistinguishable in terms of triggering a checkpoint-dependent G2 arrest, suggesting that it is predominantly error-free DDT that promotes survival (Hishida *et al.* 2009). Because our data indicated that using *RAD5* to specifically

define error-free DDT can be potentially misleading, we reexamined the importance of the *RAD18*-dependent subpathways for tolerating chronic damage. This was done by deleting *MMS2*, which, like *RAD5*, is required during *RAD18*-dependent error-free bypass, and *REV3*, which encodes the catalytic subunit of Pol ζ and is required for most TLS. Although growth of neither the *mms2* Δ nor *rev3* Δ single mutant was impaired in the presence of CLUV or CLMMS, the *mms2* Δ *rev3* Δ double mutant was very sensitive to each, exhibiting sensitivity similar to the *rad18* Δ mutant (Figure 2C). We conclude that *RAD18*-dependent error-prone as well as error-free DDT is important for surviving multiple types of chronic DNA damage and that these subpathways are functionally redundant.

Homologous recombination is an alternative to error-prone DDT

An earlier study reported that deletion of *RAD52* alone, which is required for HR, did not confer CLUV sensitivity; in the absence of *RAD18* or *RAD5*, however, *RAD52* promoted survival (Hishida *et al.* 2009). Use of *RAD5* as a proxy for error-free DDT in these studies prompted us to reexamine the relationship between HR and the two *RAD18*-dependent bypass pathways. As shown in Figure 2D, a *rad52* Δ *rev3* Δ double mutant was very sensitive to CLUV or CLMMS, whereas neither single mutant exhibited sensitivity. By contrast, the *rad52* Δ *mms2* Δ double mutant, like the single mutants, had little sensitivity to either CLUV or CLMMS. These results suggest that Rad52-mediated HR primarily provides an alternative to Pol ζ -mediated damage tolerance and are consistent with a role for *RAD52* in Mms2-mediated error-free DDT. With regard to spontaneous lesion bypass, it should be noted that the *rad52* Δ strain exhibited a slight growth defect in the absence of exogenous damage, and this impairment was further exacerbated by deletion of *REV3*, but not by loss of *MMS2*.

MPH1 and MMS2 regulate separate pathways of chronic DNA damage bypass

Prior analyses using CLMMS implicated *MPH1* and *MMS2* in separate pathways of replication-associated lesion tolerance, each of which involves formation of recombination intermediates (Choi *et al.* 2010). At the levels of chronic MMS

Figure 3 Epistatic interactions between genes during growth in the presence of CLUV or CLMMS. See Figure 2 legend for details.

and UV damage used here, neither the single mutants nor the *mms2Δ mph1Δ* double exhibited growth inhibition (Figure 3A). In a *rev3Δ* background, however, a subtle effect on growth was detected when *MPH1* was deleted (Figure 3A), and the strong sensitivity of the *mms2Δ rev3Δ* double mutant was further enhanced by loss of *MPH1* (Figure 3B). Together, these data indicate that *MMS2* and *MPH1* regulate separate, Pol ζ-independent pathways for tolerating CLUV- as well as CLMMS-induced damage. The much greater sensitivity of the *mms2Δ rev3Δ* than the *mph1Δ rev3Δ* double mutant to chronic damage indicates that the *MMS2*-dependent pathway is more important than the *MPH1*-dependent pathway for tolerating the corresponding lesions.

The precise mechanisms of error-free DDT during replication have yet to be determined, but generally are assumed to involve a Rad52-mediated template switch to the undamaged sister chromatid (Branzei *et al.* 2008). Consistent with a role for HR in both *MPH1*- and *MMS2*-mediated alternatives to Pol ζ-dependent bypass, the sensitivity of the *rad52Δ rev3Δ* double mutant was similar to that of the *mms2Δ mph1Δ rev3Δ* triple mutant (Figure 3B). In addition, the *rad52Δ mms2Δ mph1Δ* triple mutant, like the *rad52Δ mms2Δ* double mutant, exhibited no sensitivity to chronic damage (Figure 3C).

***RAD5* and *RAD18* independently regulate Pol ζ-dependent bypass of chronic damage**

Data presented above indicate that *RAD5* has a *RAD18*-independent role in tolerating chronic damage (Figure 2B).

To investigate the roles of *RAD5* and *RAD18* during the error-prone bypass of chronic damage, we made use of a *rad52Δ* background, where Pol ζ plays a critical role in survival. The relationship between *rad18Δ* and *rad5Δ* observed in the absence of *RAD52* was the same as in its presence; deletion of *RAD18* conferred greater CLUV and CLMMS sensitivity than loss of *RAD5*, and the *rad52Δ rad18Δ rad5Δ* triple mutant was more sensitive than was the *rad52Δ rad18Δ* double mutant (Figure 4A). The *rad52Δ rad18Δ rad5Δ rev3Δ* quadruple mutant was no more sensitive than the *rad52Δ rad18Δ rad5Δ* triple mutant, indicating that Pol ζ-dependent bypass was eliminated upon deletion of *RAD18* and *RAD5*.

Our expectation was that the *rad52Δ rev3Δ* double mutant would be similar to the *rad52Δ rad18Δ rad5Δ* triple mutant in terms of chronic damage sensitivity, but it clearly was less sensitive (Figure 4A). Because *RAD18* regulates lesion bypass by Pol η as well as Pol ζ (McDonald *et al.* 1997), the gene encoding Pol η (*RAD30*) was deleted from appropriate mutant backgrounds. A *rad52Δ rev3Δ rad30Δ* triple mutant exhibited CLUV and CLMMS sensitivity similar to that of the *rad52Δ rad18Δ rad5Δ* triple mutant and greater than that of the *rad52Δ rev3Δ* double mutant (Figure 4B). In contrast to *REV3* deletion, however, loss of only *RAD30* did not enhance the sensitivity of a *rad52Δ* mutant (Figure 4C), indicating that Pol ζ is more important than Pol η for tolerating potentially toxic lesions generated by CLUV or CLMMS.

Figure 4 Epistatic interactions between genes during growth in the presence of CLUV or CLMMS. See Figure 2 legend for details.

46 large dels

54 large dels

The *lys2ΔA746* assay was made a more sensitive detector of events other than simple 1-bp insertions by deleting all runs >3N from the reversion window (Lehner *et al.* 2012). The resulting “no run” *lys2ΔA746,NR* allele was used in the current study and all analyses were done in an NER-proficient background. The spectrum of *lys2ΔA746,NR* reversion events obtained in WT is shown in Figure 5A. Two specific

Table 3 Pol ζ -dependent complex insertions in the *lys2 Δ A746,NR* assay

Genotype	Total Rate ($\times 10^{-10}$)	Complex insertions		
		Fraction	Rate ($\times 10^{-10}$)	Relative to WT
WT	15	11/169	1.0	1.0
<i>rad52Δ</i>	64	86/173	32	32
<i>rad52Δ rev3Δ</i>	22	0/78	<0.3	<0.3
<i>mph1Δ</i>	29	61/136	13	13
<i>mms2Δ</i>	38	43/130	13	13
<i>mph1Δ mms2Δ</i>	37	66/132	19	19
<i>rad52Δ mph1Δ mms2Δ</i>	42	88/184	20	20
<i>rad52Δ rad18Δ</i>	19	27/68	7.5	7.5
<i>rad52Δ rad5Δ</i>	25	26/90	7.3	7.4
<i>rad52Δ rad5Δ rad18Δ</i>	20	2/79	0.5	0.5

Spectra not shown in Figure 1 are presented in Supporting Information, Figure S1. The frequency distributions of cins in each genetic background are shown in Figure S2.

types of events are relevant: cins, which are Pol ζ dependent and account for $\sim 5\%$ of compensatory frameshift mutations (cins are shaded), and large deletions (dels), which have endpoints in 7- or 10-bp direct repeats and comprise $\sim 25\%$ of WT events. In the analyses that follow, the frequency distributions of Lys⁺ revertants obtained in different genetic backgrounds were compared using the Mann–Whitney–Wilcoxon test. To compare rates of cins or large dels between strains, a frequency distribution of the relevant event was obtained by multiplying the Lys⁺ frequency in individual cultures by the cins/dels proportion in the corresponding spectrum. The resulting frequencies were then compared by the Mann–Whitney–Wilcoxon test.

***MMS2* and *MPH1* reside in separate, *RAD52*-dependent pathways that counteract Pol ζ -dependent mutagenesis**

The CLUV and CLMMS data presented above demonstrate that Rad52-dependent HR processes, which are considered to be error-free, provide an alternative to Pol ζ -dependent DDT (Figure 2D). In accordance with these observations, the rate of *lys2 Δ A746,NR* reversion was elevated ~ 4 -fold in a *rad52 Δ* background (Table 3). This increase was accompanied by a strong enrichment for cins in the corresponding spectrum, where they accounted for $\sim 50\%$ of reversion events (Figure 5B). The rate of cins thus increased ~ 30 -fold upon deletion of *RAD52*, indicating that $>95\%$ of spontaneous DDT is normally error-free and HR dependent. When *REV3* was additionally deleted in the *rad52 Δ* background, the reversion rate was reduced to that of WT, and there were no cins detected among 78 Lys⁺ revertants sequenced (Table 3 and Figure 5C).

The chronic MMS and UV survival data are consistent with the interpretation that *Mms2* and *Mph1* are each relevant during HR-mediated template switching (Figure 3). To explore whether these proteins have similar roles during spontaneous damage tolerance, the corresponding genes were deleted singly or together from the WT or *rad52 Δ* background. Relative to WT, there was a 13-fold elevation in the cins rate in an *mph1 Δ* or *mms2 Δ* single mutant (Table 3; $P < 0.001$), and the cins rate was further increased in the *mph1 Δ mms2 Δ* double mutant ($P < 0.01$). Importantly, the

cins rate in the *mph1 Δ mms2 Δ* double mutant was not significantly different from that in the *rad52 Δ* single- or *rad52 Δ mph1 Δ mms2 Δ* triple-mutant background ($P = 0.25$ and $P = 0.59$, respectively). We conclude that the error-free, spontaneous lesion bypass pathways defined by *MPH1* and *MMS2* are distinct and that, as during chronic damage exposure, *RAD52* is relevant in each.

***RAD5* and *RAD18* define two pathways of Pol ζ -dependent bypass**

Separate *RAD18*- and *RAD5*-dependent pathways of Pol ζ -dependent bypass were previously inferred in the *CAN1* forward mutation assay (Liefshitz *et al.* 1998; Cejka *et al.* 2001) or using the cins signature in the *lys2 Δ A746* assay (Minesinger and Jinks-Robertson 2005). Here, a *rad52 Δ* background was used to reduce error-free bypass and thereby enhance dependence of survival on Pol ζ -dependent TLS, allowing us to focus on the roles of *RAD18* and *RAD5* during error-prone DDT in the *lys2 Δ A746,NR* assay. The large, 32-fold increase in cins observed upon deletion of *RAD52* was reduced to 7.5-fold (a 75% reduction) upon additional loss of either *RAD18* or *RAD5* (Table 3; $P < 0.001$). The overall Lys⁺ rates in the *rad52 Δ rad18 Δ rad5 Δ* triple- and *rad52 Δ rev3 Δ* double-mutant strains were indistinguishable ($P = 1$); there were only 3/79 and 0/78 cins in the corresponding spectra, respectively. Together, these data confirm that *RAD18* and *RAD5* define independent pathways of Pol ζ -dependent bypass. Furthermore, the 75% cins reduction in each single mutant suggests that *Rad18* and *Rad5* may sometimes work together to promote Pol ζ -dependent bypass. We previously observed a similar, dual requirement with regard to UV-induced, NER-dependent mutagenesis that occurs in nongrowing cells (Kozmin and Jinks-Robertson 2013).

***RAD52* and *REV3* limit the formation of large deletions**

We previously described large deletions (dels) among reversion events in the *lys2 Δ A746* and *lys2 Δ A746,NR* assays (Minesinger and Jinks-Robertson 2005; Lehner and Jinks-Robertson 2009). Although most of these events are 95 bp in size and have endpoints in 10-bp direct repeats,

5–10% have endpoints in 7-bp direct repeats and delete 131 bp. Here, we define these as type 1 and type 2 dels, respectively. As illustrated in Figure 6A, type 1 and type 2 events have a partially overlapping endpoint that is within the reversion window; the other endpoint is distinct and upstream of the window. Because large deletions are *REV3* independent, are dependent on the direction of replication fork movement through *LYS2* (Abdulovic *et al.* 2007), are elevated upon loss of the *Pol32* subunit of Pol δ (Minesinger and Jinks-Robertson 2005), and are not generated through double-strand breaks (Lehner *et al.* 2012), we previously suggested that they reflect direct-repeat-mediated realignment of a blocked 3' end during lagging-strand synthesis. Table 4 presents rates for total large dels, as well as those for type 1 and type 2 events individually. Because there were very few (fewer than five) type 2 events in most of the spectra analyzed, changes in this class are emphasized only in those genetic backgrounds where there was a clear proportional increase among total large dels.

Relative to WT, there was a significant, ~1.5-fold increase in large dels upon loss of *RAD52* ($P < 0.001$). In contrast to the similar increases in cins observed upon deletion of *MPH1* or *MMS2* (Table 3), their loss had different effects on large dels (Table 4). Relative to WT, the rate of large dels increased 2.7-fold in the *mms2* Δ background ($P < 0.0001$), but was unaffected in the *mph1* Δ background ($P = 0.25$). Interestingly, in the *mms2* Δ *mph1* Δ double- or the *rad52* Δ *mms2* Δ *mph1* Δ triple-mutant background, the rate of large dels was reduced to the level observed in the *mph1* Δ single mutant ($P = 0.62$ and $P = 0.82$, respectively). These data indicate that *RAD52* and *MMS2* limit the formation of large dels, and that the additional events detected in their absence depend on the presence of *MPH1*.

In the *rad52* Δ background, additional deletion of *RAD5*, *RAD18*, *RAD5* and *RAD18*, or *REV3* had little effect on the total rate of large dels ($P > 0.05$ in each case). The rate of type 2 events, however, was significantly elevated in the *rad52* Δ *rad5* Δ double, *rad52* Δ *rev3* Δ double, and *rad52* Δ *rad5* Δ *rad18* Δ triple mutants relative to the *rad52* Δ single mutant ($P < 0.001$, $P = 0.010$, and $P = 0.016$, respectively). These data suggest that the type 2 events reflect an alternative to Pol ζ -dependent bypass in this system, and are consistent with the cins data, suggesting that *RAD5* and *RAD18* promote separate pathways of Pol ζ -dependent bypass.

Discussion

Bypass/tolerance mechanisms fall into two general classes that together ensure the progression of replication in the presence of unrepaired damage as well as the subsequent filling of lesion-containing gaps left behind replication forks. In the work presented here, two complementary approaches were used to genetically dissect lesion-bypass pathways when low levels of damage are present and normal repair mechanisms and cell-cycle checkpoints are unperturbed. First, we assessed growth on solid medium when cells were

Figure 6 Large dels in the *lys2* Δ A746,NR frameshift-reversion assay. (A) Direct repeats at the endpoints of type 1 and type 2 dels are highlighted pink and blue, respectively. The hypothetical reversion window is demarcated by stop codons in the alternative reading frames and is surrounded by brackets; sequence highlighted gray indicates positions of homopolymer runs $>3N$ prior to their mutation. (B) Possible mechanisms of large del formation. Switches are shown at/near a replication fork, but additionally could occur behind the fork or after replication is complete. Although not illustrated, a template switch to the nascent strand of sister chromatid could also occur following replication fork regression.

continuously exposed to CLUV or CLMMS. Even though these agents produce very different lesions, the relationships between the genes examined were indistinguishable. In the second approach, a unique mutation signature (cins in a frameshift-reversion assay) produced by Pol ζ was used as a readout of bypass-pathway utilization in the absence of exogenous damage. Elimination of a gene whose product promotes Pol ζ -dependent bypass decreased its mutagenic footprint, while loss of a protein promoting alternate, error-free bypass increased the mutagenic signal. The similar epistatic relationships between genes in the reversion and growth assays suggest that low levels of UV or MMS accurately mimic spontaneous damage and can be used to assess the corresponding physiological responses, even though neither the nature nor the complexity of spontaneous lesions is known. Importantly, results revealed functional redundancies

Table 4 Rates of large dels in the *lys2ΔA746,NR* assay

Genotype	Type 1 + Type 2		Type 1 only		Type 2 only	
	Rate ($\times 10^{-10}$)	Relative to WT	Rate ($\times 10^{-10}$)	Relative to WT	Rate ($\times 10^{-10}$)	Relative to WT
WT	4.1 (46/169)	1.0	3.7 (41/169)	1.0	0.4 (5/169)	1.0
<i>rad52Δ</i>	6.3 (17/173)	1.5	5.5 (15/173)	1.5	0.7 (2/173)	1.6
<i>mph1Δ</i>	3.6 (17/136)	0.9	3.0 (14/136)	0.8	0.6 (3/136)	1.4
<i>mms2Δ</i>	11 (39/130)	2.7	9.9 (34/130)	2.7	1.4 (5/130)	3.2
<i>mph1Δ mms2Δ</i>	3.1 (11/132)	0.7	3.1 (11/132)	0.8	<0.3 (0/132)	<0.6
<i>rad52Δ mph1Δ mms2Δ</i>	3.2 (14/184)	0.8	2.5 (11/184)	0.7	0.7 (3/184)	1.5
<i>rad52Δ rad18Δ</i>	3.9 (14/68)	0.9	2.5 (9/68)	0.7	1.4 (5/68)	3.1
<i>rad52Δ rad5Δ</i>	7.6 (27/90)	1.8	5.3 (19/90)	1.4	2.2 (8/90)	5.0
<i>rad52Δ rad5Δ rad18Δ</i>	11 (44/79)	2.7	6.3 (25/79)	1.7	4.8 (19/79)	11
<i>rad52Δ rev3Δ</i>	15 (54/78)	3.7	10 (36/78)	2.8	5.1 (18/78)	11

The fraction of events in the corresponding spectrum is given in parentheses.

not evident in prior studies and clarify the hierarchies between the various bypass/tolerance pathways when DNA repair and checkpoint pathways are intact.

The seminal study of Hishida *et al.* (2009) documented distinct genetic responses to acute UV, where repair is most important for survival, and CLUV, where tolerance pathways are most important. In the absence of *RAD18*, single-strand gaps accumulated during replication and triggered a subsequent G2 arrest, allowing time for gaps to be filled by *RAD52*-mediated HR. *RAD5* was used to distinguish *RAD18*-mediated error-free and error-prone bypass, leading to the conclusion that TLS contributes little, if at all, to CLUV tolerance. Our use of *MMS2* to define error-free bypass revealed clear functional redundancy between the two *RAD18*-regulated subpathways; neither an *mms2Δ* nor *rev3Δ* single mutant was sensitive to CLUV, but the double mutant was exquisitely sensitive. Similar synergism with respect to CLMMS has been previously reported (Huang *et al.* 2013) and was confirmed here. *RAD18*-mediated error-free and error-prone pathways were interchangeable in terms of promoting growth, but epistasis analysis provided no information about the hierarchies or relative importance of these subpathways. This can be inferred, however, from our analysis of Pol ζ-dependent spontaneous mutagenesis. Deletion of *MMS2* strongly elevated the Pol ζ-dependent cins mutation signature, indicating that *RAD18*-dependent error-free bypass normally predominates. Based on prior studies, we suggest that this reflects temporal separation of bypass mechanisms during the cell cycle, with *MMS2*- and *REV3*-dependent bypass operating primarily in S and G2, respectively (Daigaku *et al.* 2010; Huang *et al.* 2013). This is consistent with *MMS2*-regulated template switching at a lesion-stalled replication fork in S phase (Choi *et al.* 2010) and with the strong accumulation of *Rev1* in G2/M (Waters and Walker 2006). It also implies that most damage is dealt with during S phase, with the small amount that remains being subsequently handled by TLS.

RAD52 is required during *RAD18*-mediated error-free bypass, for efficient strand invasion during HR and for strand-

annealing reactions (reviewed by San Filippo *et al.* 2008). *MPH1* was originally identified by virtue of the mutator phenotype observed in its absence (Schurer *et al.* 2004), and recently has been genetically implicated in HR-mediated sister chromatid interactions (Ede *et al.* 2011). *MMS2* and *MPH1* independently promote formation of X-shaped recombination structures on 2D gels when DNA replicates in the presence of MMS, and it was suggested that *MMS2* regulates template switching while *MPH1* promotes replication fork regression (Choi *et al.* 2010). Our observation that the CLMMS or CLUV sensitivity of a *rev3Δ* single or *mms2Δ rev3Δ* double mutant was further enhanced by deletion of *MPH1* is consistent with the separate roles of these proteins in promoting error-free bypass. The epistasis with *rad52Δ* likewise is consistent with a role for HR in both *MPH1*- and *MMS2*-regulated bypass mechanisms.

The CLUV or CLMMS sensitivity of double mutants indicated that *MMS2*-regulated bypass is a more robust alternative to Pol ζ-mediated TLS than *MPH1*-regulated bypass. In terms of the mutagenic Pol ζ-dependent signature in our frameshift reversion assay, however, loss of *Mph1* or *Mms2* had equivalent effects. Although one might consider these data to be contradictory, it must be emphasized that our mutagenesis assay is very selective because it only detects the subset of events that restore the correct *LYS2* reading frame. One intriguing aspect of the mutagenesis data concerns the roles of *MPH1* and *MMS2* in large dels, which are Pol ζ-independent events. Large dels were elevated in the absence of *MMS2*, and the elevation required *MPH1*. *Mph1* has helicase activity that has been implicated in the dismantling of recombination intermediates as well as in replication fork regression (reviewed by Whitby 2010). In the case of large dels, one possibility is that the helicase activity of *Mph1* is required to unwind a 3' end that is stalled within the first copy of the relevant direct repeat. Once unwound, the 3' end could then anneal to the second, complementary copy of the repeat on the same or different DNA strand (Figure 6B). Resumption of DNA synthesis would then delete the region between the repeats.

Efficient TLS requires Rad18-dependent mono-Ub of PCNA, with subsequent extension into a poly-Ub chain by Rad5 and Mms2–Ubc13 promoting error-free bypass; Rad5 in this context interacts with both Rad18 and Ubc13 (Ulrich and Jentsch 2000). Whether or not Rad5 additionally has a role in TLS, and whether this role is independent of Rad18 has been unclear. In early studies of Pol ζ -dependent induced mutagenesis, there was an absolute requirement for RAD6, but RAD5 was only necessary in specific reversion assays (Lawrence 1994). By contrast, in genetic studies of spontaneous Pol ζ -dependent mutagenesis, RAD5 and RAD18 were shown to regulate distinct TLS pathways (Liefshitz *et al.* 1998; Cejka *et al.* 2001; Minesinger and Jinks-Robertson 2005). More recently, a requirement of RAD5 for the Pol ζ -dependent bypass of a defined lesion engineered into a single- or double-stranded plasmid was reported (Pages *et al.* 2008; Kuang *et al.* 2013). Although the helicase and Ub-ligase activities of Rad5, which are required for error-free bypass, were not necessary for Pol ζ -dependent TLS in this context, a physical interaction with Rev1 was required. A specific model was proposed in which Rad5 interacts with Rad18, and then recruits either Ubc13 or Rev1 to promote error-free or error-prone bypass, respectively (Kuang *et al.* 2013). Consistent with a dual requirement for RAD18 and RAD5 during TLS, most (75%) of the Pol ζ -dependent cins in our frameshift reversion assay required both genes; the remaining events required only RAD18 or RAD5, consistent with each also independently promoting TLS. Separate contributions of RAD18 and RAD5 during TLS are further supported by the sensitivity of mutant strains to CLUV and CLMMS. In a *rad52 Δ* background where error-free bypass is disabled, RAD18 and RAD5 contributed independently to survival. With regard to the role of Rad5 in Pol ζ -dependent TLS, a consistent requirement for RAD5 has only been evident when damage levels are very low. The involvement of Rad5 in TLS might thus depend on whether damage is extensive enough to trigger a checkpoint response, as would be the case with acute doses. Finally, the clear increase in type 2 dels in *rad52 Δ rad5 Δ rad18 Δ* and *rad52 Δ rev3 Δ* backgrounds suggests that this class may reflect a specific alternative to Pol ζ -dependent bypass of the relevant lesion. Given that Pol ζ activity is largely confined to G2, type 2 dels might reflect a gap-filling process that occurs after the completion of bulk DNA synthesis. This distinctive signature could potentially be exploited to identify small molecules that inhibit Pol ζ activity.

The bypass pathways deployed by yeast to deal with persistent damage are diverse and functionally redundant, and together ensure successful genome duplication. The current study has revealed clear parallels between the genetic responses to endogenous DNA damage vs. that generated by chronic exposure to low doses of UV or MMS. Whether similar parallels will extend to additional agents likely depends on the specific types of lesions generated. Nevertheless, results reinforce the view that there likely is

no “safe” dose of external mutagens in terms of an ability to exert physiological effects.

Acknowledgements

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Supporting Information

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Shared Genetic Pathways Contribute to the Tolerance of Endogenous and Low-Dose Exogenous DNA Damage in Yeast

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A *mph1Δ* (N=135)

B *mms2Δ* (N=129)

C *mph1Δ mms2Δ* (N=133)

D *mph1Δ mms2Δ rad52Δ* (N=184)

E *rad52Δ rad18Δ*

F *rad52Δ rad5Δ* (N=90)

G *rad52Δ rad18Δ rad5Δ* (N=79)

Figure S1. *lys2ΔA746, NR* reversion spectra. See Figure 5 legend for details.

Figure S2. Frequency distribution of complex insertions (cins) in the *lys2ΔA746,NR* assay