**The EMBO Journal (2012) 31,** 895–907 | © 2012 European Molecular Biology Organization | All Rights Reserved 0261-4189/12
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THE EMBO JOURNAL

# Endogenous DNA replication stress results in expansion of dNTP pools and a mutator phenotype

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**The integrity of the genome depends on diverse pathways that regulate DNA metabolism. Defects in these pathways result in genome instability, a hallmark of cancer. Deletion of *ELG1* in budding yeast, when combined with hypomorphic alleles of *PCNA* results in spontaneous DNA damage during S phase that elicits upregulation of ribonucleotide reductase (RNR) activity. Increased RNR activity leads to a dramatic expansion of deoxyribonucleotide (dNTP) pools in G1 that allows cells to synthesize significant fractions of the genome in the presence of hydroxyurea in the subsequent S phase. Consistent with the recognized correlation between dNTP levels and spontaneous mutation, compromising *ELG1* and *PCNA* results in a significant increase in mutation rates. Deletion of distinct genome stability genes *RAD54*, *RAD55*, and *TSA1* also results in increased dNTP levels and mutagenesis, suggesting that this is a general phenomenon. Together, our data point to a vicious circle in which mutations in gatekeeper genes give rise to genomic instability during S phase, inducing expansion of the dNTP pool, which in turn results in high levels of spontaneous mutagenesis.**
*The EMBO Journal* (2012) **31,** 895–907. doi:10.1038/emboj.2011.485; Published online 10 January 2012
*Subject Categories*: genome stability & dynamics
*Keywords*: deoxyribonucleoside triphosphates; DNA damage; DNA replication; mutagenesis; ribonucleotide reductase

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**Received: 1 May 2011; accepted: 9 December 2011; published online: 10 January 2012**

## Introduction

The integrity of DNA is constantly challenged by environmental and intracellular factors. Failure to safeguard the genome can result in genomic instability, a driving force in carcinogenesis. Conserved signalling cascades mediated by the Mec1 (human ATR) and Rad53 (human Chk1) kinases orchestrate a multifaceted response that enables yeast cells to cope with DNA damage and DNA replication stress. Mec1 and Rad53 facilitate many activities that promote genome integrity in the presence of DNA damage, including the activation of the Dun1 checkpoint kinase, which in turn mediates the upregulation of ribonucleotide reductase (RNR) activity (Zhou and Elledge, 1993). RNR catalyses the rate limiting step of deoxyribonucleoside triphosphate (dNTP) synthesis (Reichard, 1988). Yeast RNR is a tetrameric complex composed of a large and small subunit. Two Rnr1 polypeptides comprise the large subunit, and the small subunit contains Rnr2 and Rnr4 (Elledge and Davis, 1987, 1990; Chabes *et al*, 2000). By inhibiting the transcriptional repressor Crt1, Dun1 mediates the transcriptional induction of *RNR2* and *RNR4* (Elledge and Davis, 1990; Huang and Elledge, 1997), as well as *RNR3*, which encodes an alternative component of the large RNR subunit (Huang *et al*, 1998). Dun1 also triggers the degradation of Sml1 and Dif1, which inhibit RNR activity by directly interfering with catalysis and by nuclear sequestration of the small subunit, respectively (Zhao and Rothstein, 2002; Lee *et al*, 2008; Wu and Huang, 2008). Together, these Dun1 activities result in the expansion of intracellular dNTP pools, which promotes DNA repair and cell survival in the presence of genotoxic agents (Chabes *et al*, 2003).

In addition to the DNA damage response, a number of genes and pathways have been identified in yeast that when deregulated lead to spontaneous DNA damage and genomic instability. Normally, these pathways ensure the faithful execution of diverse DNA transactions including chromosome segregation, DNA repair, and DNA replication (Kolodner *et al*, 2002; Myung and Kolodner, 2002). Elg1 is a replication factor C (RFC) homologue that is believed to function in concert with the sliding clamp PCNA (Bellaoui *et al*, 2003; Ben-Aroya *et al*, 2003; Kanellis *et al*, 2003; Sikdar *et al*, 2009; Lee *et al*, 2010). Mutants in *ELG1* exhibit defects in S-phase progression and require intact replication fork restart, DNA repair, and checkpoint pathways for viability (Bellaoui *et al*, 2003; Kanellis *et al*, 2003), suggesting that lack of Elg1 causes replication stress. Additionally, absence of Elg1 is correlated with increased spontaneous DNA damage (Alvaro *et al*, 2007; Davidson and Brown, 2008), and with genomic instability phenotypes including increased rates of chromosome loss, recombination, gross chromosomal rearrangements, and mutagenesis (Bellaoui *et al*, 2003; Ben-Aroya *et al*, 2003; Kanellis *et al*, 2003). Here, we show that mutating both *ELG1* and *PCNA* together results in the accumulation of DNA damage that leads to upregulation of RNR

activity via a DNA damage response. Increased RNR activity results in greatly expanded dNTP pools in the double mutant, conferring resistance to inhibition of DNA replication by hydroxyurea (HU). However, the increase in dNTP concentrations adversely affects replication fidelity, contributing to spontaneous mutagenesis. These data, and similar phenotypes observed in distinct genome instability mutants in *RAD54*, *RAD55*, and *TSA1*, suggest a mechanism by which the response to DNA damage caused by endogenous replication stress could further confer a mutator phenotype.

## Results

### *Combining mutations in PCNA and ELG1 confers resistance to HU-induced DNA replication slowing*

Elg1-RFC was identified as a suppressor of genome instability and is proposed to function with the sliding clamp PCNA (Bellaoui *et al*, 2003; Ben-Aroya *et al*, 2003; Kanellis *et al*, 2003; Sikdar *et al*, 2009; Lee *et al*, 2010). The RFC-dependent function of Elg1 remains elusive as no PCNA-loading or unloading activity for Elg1 has been demonstrated despite their physical interaction (Kanellis *et al*, 2003; Lee *et al*, 2010). To gain further insight into the nature of the Elg1-PCNA function, we constructed double mutants of *elg1*Δ and PCNA, which is encoded by the *POL30* gene. As *POL30* is an essential gene, we constructed two hypomorphic *pol30* alleles, namely $pol30^{DAmP}$ and $pol30^{f}$. In $pol30^{DAmP}$, the 3′-untranslated region (3′UTR) immediately downstream of the *POL30* stop codon is disrupted, which usually results in destabilization of the mRNA, reducing the amount of protein translated (Breslow *et al*, 2008). $pol30^{f}$ encodes a C-terminally Flag-tagged PCNA. We assessed the steady-state expression of PCNA in wild-type cells, $pol30^{DAmP}$ and $pol30^{f}$ single mutants, and $pol30^{DAmP}elg1\Delta$ and $pol30^{f}$ *elg1*Δ double mutants. We found that both *pol30* single mutants exhibited reduced PCNA levels compared with wild-type cells with $pol30^{DAmP}$ mutants exhibiting a greater decrease (Figure 1A, lanes 3 and 4). Deleting *ELG1* in $pol30^{f}$ mutants caused a further reduction in PCNA abundance compared with $pol30^{f}$ alone (Figure 1A, lane 5 versus lane 3). This reduction was consistent with the observation that PCNA levels are modestly reduced in *elg1*Δ mutants alone (Figure 1A, lane 2). Additionally, $pol30^{f}$ conferred sensitivity to MMS (Supplementary Figure S1), suggesting that it is not a fully functional allele of PCNA. Therefore, the $pol30^{f}$ and $pol30^{DAmP}$ alleles reduced the levels of PCNA, and the $pol30^{f}$ allele additionally compromised PCNA function in MMS resistance.

We evaluated DNA synthesis by flow cytometry in wild-type, and single and double mutant cells, following release from arrest in G1 into S phase in the presence of HU. HU slows DNA synthesis by inhibiting the production of dNTPs by RNR (Krakoff *et al*, 1968; Alvino *et al*, 2007). After 120 min in HU wild-type cells remained in early S phase with predominantly unreplicated DNA as evidenced by the DNA content peak remaining near 1C (Figure 1B). The single mutants *elg1*Δ, $pol30^{DAmP}$, and $pol30^{f}$ displayed a small rightward shift in DNA content peaks, consistent with a small amount of DNA replication in the presence of HU (Figure 1B). However, double mutants $pol30^{DAmP}elg1\Delta$ and $pol30^{f}$ *elg1*Δ synthesized much greater amounts of DNA as indicated by the DNA content peaks approaching 2C after 120 min in HU (Figure 1B). This HU-resistant DNA synthesis, in which cells are refractory to the slowing of S phase that normally takes place in the presence of HU, was not the result of a checkpoint defect since deletion of *mec1* or mutation of *rad53* did not allow extensive DNA synthesis in the presence of HU (Supplementary Figure S2).

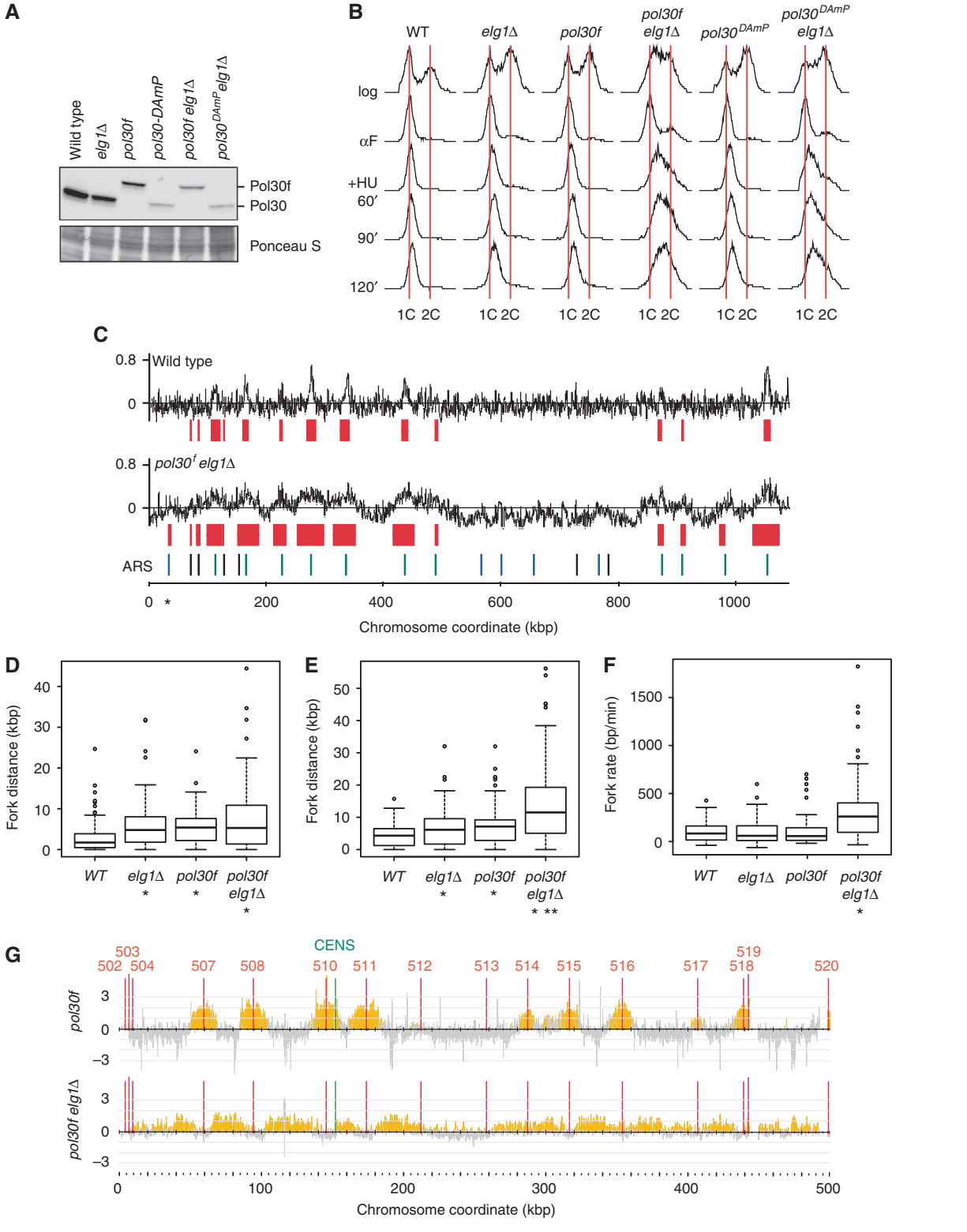
### *Increased DNA replication fork progression in HU in $pol30^{f}$ elg1Δ cells*

The HU-resistant DNA synthesis detected by flow cytometry could be the result of DNA replication or gene amplification. To distinguish these possibilities, we analysed DNA synthesis by comparative genome hybridization (CGH) on microarrays. Wild-type, *elg1*Δ, $pol30^{f}$, and $pol30^{f}$ *elg1*Δ cells were arrested in G1 and synchronously released in the presence of HU for 30 or 60 min. Genomic DNA was extracted, amplified, and hybridized to a whole-genome tiling microarray. Replicated regions were identified as peaks that exhibit increased copy number relative to G1 cells and overlapped confirmed replication origins (from the DNA Replication Origin Database; http://www.oridb.org; Nieduszynski *et al*, 2007; indicated as red tracks below the histograms in Figure 1C and

**Figure 1** *pol30 elg1*Δ mutants are resistant to inhibition of DNA replication by HU. (**A**) Western blot analysis of whole cell extracts prepared from the indicated logarithmically growing strains. Immunoblots were probed with anti-PCNA antibody. Ponceau S staining of the blot is shown in the bottom panel as a loading control. (**B**) Logarithmically growing cultures of the indicated strains were arrested in G1 with alpha factor (αF) and released into 0.2 M HU. At the indicated times, samples were fixed and DNA contents were analysed by flow cytometry. The positions of cells with 1C and 2C DNA contents are indicated. (**C**) DNA replication was measured in wild type and $pol30^{f}elg1\Delta$ by comparative genome hybridization on tiling microarrays after logarithmically growing cells were arrested in G1 with alpha factor and released into 0.2 M HU for 60 min. The log2 ratio of signal from each S phase (HU) sample relative to unreplicated (G1) DNA is shown for chromosome XV. Replicated regions, defined by identifying peaks that overlap replication origins, along chromosome XV are shown as red bars below each histogram. Confirmed replication origins annotated in oriDB are indicated, early-firing origins in green, late-firing origins in blue, and origins without timing data in black (McCune *et al*, 2008). Late-firing ARS1506.5 is indicated by an asterisk. (**D**) Replication fork distance distributions after 30 min in HU. The distance from the centre of each ARS to peak edge for 166 replication forks across the genome was measured and the result displayed as a boxplot. The median is indicated by the horizontal bar, the box spans the first through third quartiles, the whiskers extend to the last data points within 1.5 times the interquartile range, and outliers are plotted as circles. Median fork distance significantly greater than wild type ($P<0.01$, one-tail Wilcoxon rank-sum test) is indicated (*), as is median fork distance significantly greater than *elg1*Δ and $pol30^{f}$ (**$P<0.01$). (**E**) Replication fork distance distributions after 60 min in HU. (**F**) Replication fork rate distribution, between 30 and 60 min in HU. Fork rate was measured for 88 replication forks as the difference in fork distance between 60 and 30 min in HU, divided by 30 min. Fork rate in $pol30^{f}$ *elg1*Δ (260 bp/min) was significantly greater than wild type (84 bp/min; *$P=3\times10^{-9}$). (**G**) PCNA localizes to origin-distal regions in $pol30^{f}$ *elg1*Δ cells released from G1 into HU for 90 min. Enrichment of DNA fragments in the PCNA-bound fraction relative to the unbound fraction is shown along 500 kbp of chromosome V for $pol30^{f}$ (top) and $pol30^{f}$ *elg1*Δ cells (bottom). The signal intensity ratio on a log2 scale is shown on the *y* axis and the position along the chromosome is shown on the *x* axis. Positive signal represents occupancy by PCNA, and regions where the positive signal is statistically significant (Katou *et al*, 2006) over 300 bp are shown in orange. Replication origins are indicated.

Supplementary Figure S3). The distance travelled by 166 replication forks across the genome was computed by measuring the distance from the centre of each replication origin to the left or to the right edge of the peak coordinates of the peak overlapping that origin. The analysis included all forks that had a peak identified in all four strains at both time points, so that the same set of forks was compared across all experiments in analysing the fork distance distributions. We first noted that significant DNA synthesis occurred in wild-type cells in the presence of 0.2 M HU (Figure 1C) and that replication fork distance increased between 30 and 60 min (Figure 1D and E; Supplementary Figure S3B), in agreement with the previous observation that DNA replication significantly slows but does not terminate in



the presence of HU (Alvino *et al*, 2007). Analysis of the mutant strains revealed two important properties of the HU-resistant DNA synthesis. First, peak height did not exceed a log2 value of 1 indicating that any given locus doubled only once. Second, replicated regions were distributed throughout the genome and largely corresponded to regions containing early-firing replication origins. Together, these data indicate that the HU-resistant DNA synthesis we observed is *bona fide* DNA replication and not amplification of specific loci. Some replication peaks centered on late-firing origins (as defined by *CLB5* dependence; McCune *et al*, 2008) were evident, particularly in the *pol30$^f$ elg1*Δ cells, consistent with an advanced replication program in these cells (e.g., ARS1506.5; Figure 1C, *).

To compare the extent of replication from individual replication origins in wild type and each mutant, we measured the distance travelled by replication forks after 30 and 60 min in HU. The distributions of fork distances after 30 and 60 min in HU are shown in Figure 1D and E. The *elg1*Δ and *pol30$^f$* single mutants exhibited a significant ($P<0.01$) increase in fork distance relative to wild type and the *pol30$^f$ elg1*Δ mutant exhibited the largest fork distance, significantly greater than wild type at both 30 and 60 min and the single mutants ($P<0.01$) at 60 min. These data indicate that in the presence of HU, replication forks progress further from replication origins in *elg1*Δ, *pol30$^f$*, and *pol30$^f$ elg1*Δ mutants than in wild-type cells, with the greatest distance traversed by replication forks in *pol30$^f$ elg1*Δ cells.

Since it was formally possible that the wider replication peaks in the mutants resulted from earlier entry into S phase rather than an increase in the rate of DNA synthesis, we estimated the rates of replication fork progression in the wild-type and mutant cells (Figure 1F) by comparing the replication fork distances at 30 min with that at 60 min (Supplementary Figure S3B). For this analysis, we omitted forks that returned a rate $<0$, reasoning that these were not likely to be true replication peaks, or were forks at which significant replication was not occurring between 30 and 60 min, and again ensuring that the rates were derived from analysis of the same forks across all experiments. In the presence of HU, replication forks progressed at a median rate of 84 bp/min in wild-type cells (Figure 1F). The *pol30$^f$ elg1*Δ mutant exhibited a significantly higher rate of 261 bp/min ($P=3\times10^{-9}$). Thus, the more extensive DNA replication seen in the double mutant reflected an increase in replication fork progression rates. The fork rates for the single mutants were not significantly different from wild type during the time interval analysed. Together, the CGH data indicate that the HU-resistant DNA synthesis in *pol30$^f$ elg1*Δ mutants is the result of DNA replication at an increased rate, largely from early-firing replication origins.

Mutations in genes encoding DNA replication fork proteins can cause an uncoupling between the replisome component Cdc45 and sites of DNA synthesis (Katou *et al*, 2003). To determine the localization of replication fork proteins on chromosomes replicating in the presence of HU, we followed the genome-wide location of PCNA$^{Flag}$, using ChIP-on-chip analysis, comparing *pol30$^f$* and *pol30$^f$elg1*Δ cells. In *pol30$^f$* cells, PCNA was localized to origin proximal regions, whereas in *pol30$^f$elg1*Δ cells PCNA displayed a broader distribution with some enrichment at origin-distal regions (Figure 1G). These results suggest that replication forks progress further from initiation sites in *pol30$^f$ elg1*Δ double mutants than in *pol30$^f$* single mutants, consistent with our CGH analysis.

### *Mutations in ELG1 and PCNA cause an expansion of intracellular dNTP pools*

To determine how *pol30$^f$ elg1*Δ mutants are able to replicate significant fractions of the genome at HU concentrations that inhibit DNA synthesis in wild-type cells, we measured endogenous dNTP levels (Figure 2A). Normally, in yeast RNR activity is inhibited in G1 (Zhao *et al*, 1998; Yao *et al*, 2003; Lee and Elledge, 2006; Lee *et al*, 2008) resulting in low intracellular dNTP concentrations (Chabes *et al*, 2003; Koc *et al*, 2004). We found that *pol30$^f$* and *elg1*Δ single mutants exhibited increases in G1 dNTP concentrations of 2.4-fold and 3.1-fold over wild type, respectively. These dNTP stores, like those in wild-type cells, became depleted upon entry into S phase in the presence of HU (Figure 2A). Strikingly, *pol30$^f$ elg1*Δ mutants exhibited an average 8.3-fold increase in G1 dNTP levels compared with wild-type cells (Figure 2A). Even

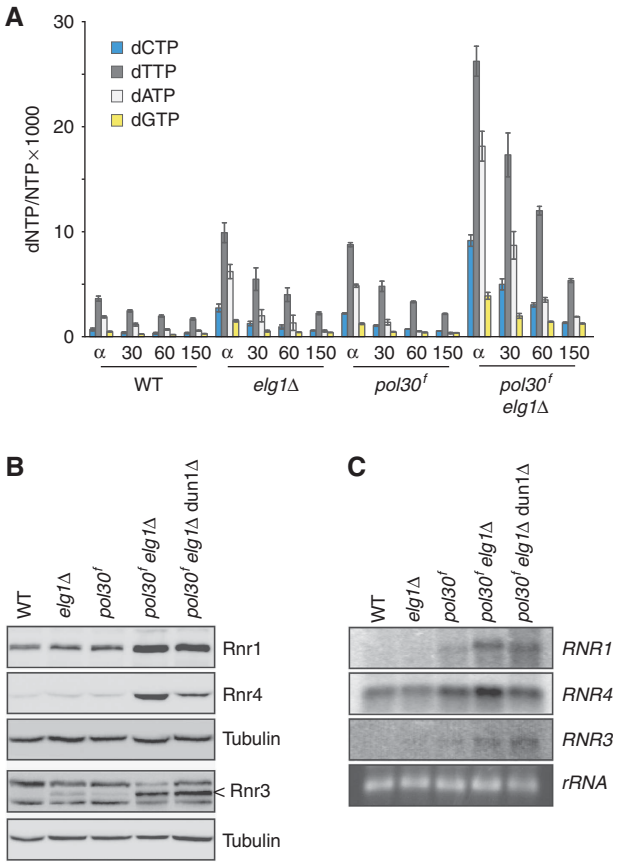



**Figure 2** RNR activity is upregulated in *pol30$^f$ elg1*Δ mutants. (**A**) Logarithmically growing cells were arrested in G1 with alpha factor (α) and released into 0.2 M HU. At the indicated times, samples were fixed and the level of each dNTP was measured in the strains shown. dNTP levels are expressed as dNTP:NTP ratios. (**B**) Western blot analysis of whole cell extracts prepared from cells that were arrested in G1 with alpha factor. Immunoblots were probed with anti-Rnr1, Rnr3, and Rnr4 antibodies to detect RNR subunits. Tubulin was used as a loading control and detected using an anti-tubulin antibody. (**C**) Northern blot analysis of RNA prepared from cells arrested in G1 phase. Blots were hybridized with probes for the *RNR1*, *RNR3*, and *RNR4* genes. The rRNA is shown as a loading control.

after 150 min in HU, dNTP concentrations were four times greater in the double mutant compared with wild-type cells. We also found that the natural dNTP pool asymmetry, where dTTP is most abundant, was maintained even while dNTP levels are dramatically higher. We conclude that *pol30$^{f}$*, *elg1*Δ, and to a much greater extent, *pol30$^{f}$elg1*Δ mutants exhibit an unscheduled expansion of the intracellular dNTP pools. This increase in dNTP pools occurs in the mutants in an otherwise unperturbed cell cycle (i.e., in the absence of HU), and facilitates DNA replication in the presence of HU in the subsequent S phase.

### *Expression of RNR subunits is upregulated in pol30$^{f}$ elg1*Δ *mutants*

To evaluate the mechanism by which dNTP pools were expanded in *elg1*Δ, *pol30$^{f}$*, and *pol30$^{f}$ elg1*Δ mutants, we examined the steady-state expression levels of Rnr1, Rnr3, and Rnr4 in these cells. While we found little increase in expression of RNR components in the single mutants, we observed elevated expression of Rnr1, 3, and 4 in *pol30$^{f}$ elg1*Δ double mutants in G1-arrested cells (Figure 2B). To determine whether this upregulation was transcriptional or translational, we also analysed the levels of mRNA from *RNR1*, *RNR3*, and *RNR4* in the G1 cells (Figure 2C). In all cases, the level of mRNA mirrored the level of protein, suggesting that the upregulation of RNR was occurring at the level of transcription or mRNA stability. We conclude that the expansion of dNTP pools in G1 in *pol30$^{f}$ elg1*Δ mutants is due to increased levels of RNR mRNAs and proteins.

### *Increased spontaneous DNA damage in pol30$^{f}$ elg1*Δ *mutants*

Elevated expression of *RNR* genes, particularly *RNR3*, is specifically indicative of activation of the DNA damage response (Elledge and Davis, 1987, 1989, 1990; Hurd *et al*, 1987; Yagle and McEntee, 1990; Zhou and Elledge, 1992; Huang and Elledge, 1997; Jia *et al*, 2002). To determine if *pol30$^{f}$ elg1*Δ cells accumulate spontaneous DNA damage, we examined the presence of γH2A, a marker of DNA damage (Downs *et al*, 2000), in these cells. We measured γH2A levels throughout an unperturbed cell cycle in both wild-type and *pol30$^{f}$ elg1*Δ cells. We found that *pol30$^{f}$ elg1*Δ mutants accumulated more γH2A compared with wild-type cells, with peak accumulations at 60 and 120 min (Figure 3A). The peaks of γH2A correspond to late S phase as deduced from analysis of cell-cycle position (Figure 3B; Supplementary Figure S4). Moreover, *pol30$^{f}$ elg1*Δ mutants exhibited a delay in S-phase progression, suggesting defects in DNA replication (Figure 3B). The γH2A signal decreased in both wild type and *pol30$^{f}$ elg1*Δ mutants as cells enter mitosis, at 80 min (Figure 3A and B; Supplementary Figure S4). However, γH2A was reduced to a lesser extent in *pol30$^{f}$ elg1*Δ mutants compared with wild type, suggesting the persistence of DNA damage following S phase, which is consistent with the observed mitotic delay in these cells.

### *Dun1 becomes activated during S phase in pol30$^{f}$ elg1*Δ *mutants*

The presence of elevated γH2A suggests an activated DNA damage response. We asked if *pol30$^{f}$ elg1*Δ mutants exhibit activation of Rad53, which can be gleaned from a diagnostic phosphorylation-dependent shift in Rad53 mobility on immunoblots (Pellicioli *et al*, 1999). Such a shift was observed when wild-type cells were treated with MMS (Figure 3A; Sanchez *et al*, 1996). We found that despite the presence of the γH2A DNA damage signal in S phase, *pol30$^{f}$ elg1*Δ mutants did not exhibit robust activation of Rad53 as evidenced by the lack of shift in Rad53 mobility (Figure 3A).

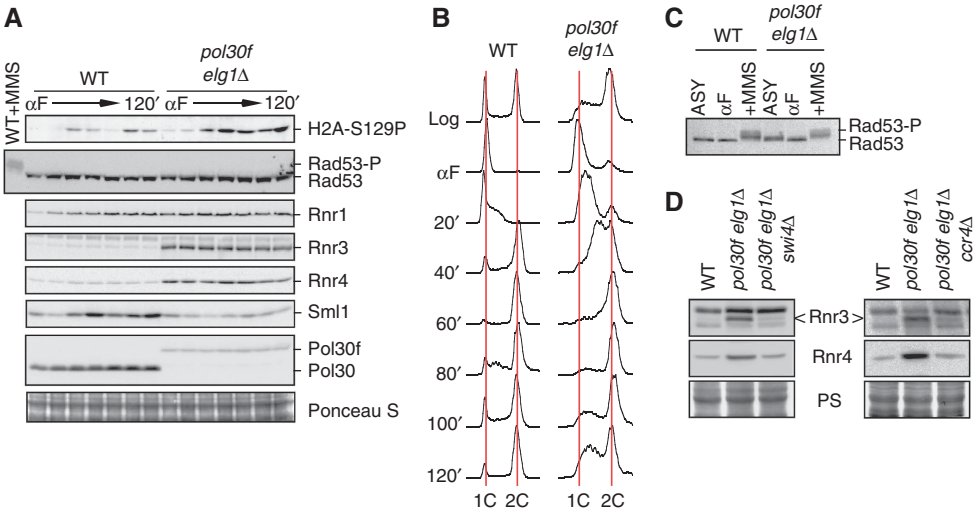



**Figure 3** *pol30$^{f}$ elg1*Δ mutants exhibit endogenous DNA damage (**A**) Logarithmically growing cells were arrested in G1 with alpha factor (α) and synchronously released into S phase. Samples were fixed every 20 min and extracts were fractionated on a western blot and probed with anti-γH2A, Rnr1, Rnr3, Rnr4, Sml1, and PCNA antibodies. Western blots were also probed with an anti-Rad53 antibody. Phosphorylation of Rad53 causes a shift in electrophoretic mobility (Rad53-P) and is a marker for checkpoint activation as seen in the control sample of asynchronously growing wild-type cells treated with MMS (lane 1). Ponceau S staining of the Pol30 blot is shown as a protein loading control. (**B**) Cells sampled from (**A**) were analysed by flow cytometry. The positions of 1C and 2C DNA contents are indicated. (**C**) The indicated strains were grown asynchronously (ASY), arrested in G1 with alpha factor (αF), or treated with MMS. Cells were fixed with TCA and whole cell extracts were analysed by western blot for phosphorylation-dependent Rad53 mobility shift using an anti-Rad53 antibody. (**D**) Western blot analysis of whole cell extracts prepared from the indicated strains following arrest in G1 with alpha factor. Immunoblots were probed with anti-Rnr3 and Rnr4 antibodies to detect RNR subunits. Ponceau S staining is shown as a protein loading control.

Absence of robust Rad53 activation was not due to an inability of *pol30$^f$ elg1*Δ mutants to activate Rad53. When the *pol30$^f$ elg1*Δ mutant was treated with MMS (Figure 3C) Rad53 activation was similar to that in wild-type cells.

Sml1 degradation is a more sensitive marker of checkpoint activation than is Rad53 phosphorylation (Barlow *et al*, 2008). We monitored Sml1 levels during an unperturbed cell cycle in wild type and *pol30$^f$ elg1*Δ mutants (Figure 3A). Both strains showed the typical decrease in Sml1 as cells enter S phase at 20 min (Figure 3A and B). In wild-type cells, Sml1 levels then increased as cells complete S phase, at 40 and 60 min, and then decreased again as cells enter the next cell cycle. By contrast, *pol30$^f$ elg1*Δ mutants showed only a slight increase in Sml1 at 60 and 80 min, and Sml1 never reached the peak levels seen in wild type (Figure 3A). The reduced levels of Sml1 following S phase were consistent with activation of the checkpoint in *pol30$^f$ elg1*Δ mutants, and correlated with the increase in γH2A that we observe. We conclude that the Mec1-Rad53-Dun1 pathway is activated in *pol30$^f$ elg1*Δ mutants following progression into S phase.

We also examined the effect of deleting *DUN1* in *pol30$^f$ elg1*Δ mutants. Interestingly, we found that Rnr3 levels in G1-arrested *pol30$^f$ elg1*Δ *dun1*Δ triple mutants were comparable to those in *pol30$^f$ elg1*Δ double mutants, and that Rnr4 levels were only partially diminished in the triple mutant (Figure 2B and C), suggesting that induction of RNR expression was occurring in a manner largely independent of *DUN1*. A *DUN1*-independent response to DNA damage resulting in transcriptional upregulation of *RNR2*, *3*, and *4* has been described (Huang and Elledge, 1997; Zaim *et al*, 2005). Consistent with the absence of consensus binding sites for the Crt1 repressor in the *RNR1* promoter (Huang *et al*, 1998; Zaim *et al*, 2005) and with a recent report (Tsaponina *et al*, 2011), expression of *RNR1* in G1 cells was also independent of *DUN1* (Figure 2B and C). We conclude that although the Mec1-Rad53-Dun1 pathway is activated in *pol30$^f$ elg1*Δ mutants, the increase we observed in *RNR1*, *RNR3*, and *RNR4* mRNA and protein levels in G1 in *pol30$^f$ elg1*Δ mutants is predominately independent of *DUN1*.

### *RNR is expressed at elevated levels throughout the cell cycle in pol30$^f$ elg1*Δ *mutants*

Since the checkpoint appeared to be activated during S phase in the *pol30$^f$ elg1*Δ mutant, we asked if expression of *RNR1, RNR3*, and *RNR4* was induced during normal cell-cycle progression of the *pol30$^f$ elg1*Δ mutant. We analysed expression of RNR protein subunits in wild-type and *pol30$^f$elg1*Δ cells following synchronous release from G1 (Figure 3A). As expected, in wild-type cells Rnr3 expression was barely detectable and Rnr4 was expressed at constitutively low levels, consistent with the absence of DNA damage. By contrast, expression of both Rnr3 and Rnr4 was elevated in *pol30$^f$ elg1*Δ cells, and these elevated levels were evident throughout the cell cycle. Rnr1 expression was similar in wild type and the *pol30$^f$ elg1*Δ mutant, with the exception of the G1 cells, which showed higher Rnr1 levels in the mutant (Figures 2B and 3A). Thus, like in G1 cells, Rnr3 and Rnr4 proteins were strongly induced in the *pol30$^f$ elg1*Δ mutant compared with wild type during normal cell-cycle progression. There was not, however, a correlation between peak RNR abundance and the DNA damage signal (γH2A) seen in

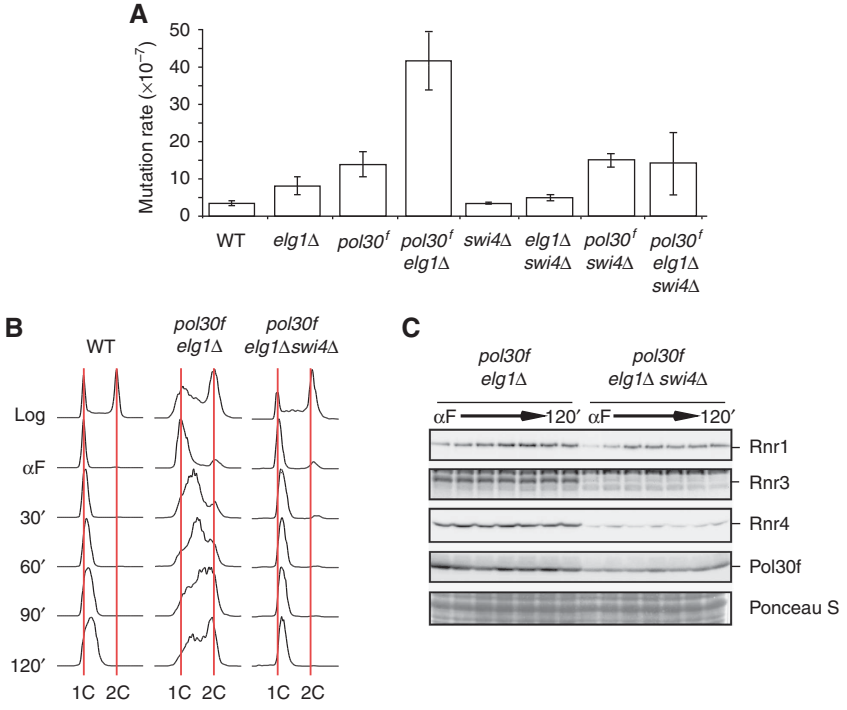



**Figure 4** *pol30$^f$elg1*Δ mutants exhibit a mutator phenotype. (**A**) The rates of forward mutation of *CAN1* cells to canavanine resistance were determined. The means and standard deviations of at least three independent fluctuation tests for the indicated strains are plotted. (**B**) DNA contents of wild-type, *pol30$^f$ elg1*Δ, and *pol30$^f$ elg1*Δ *swi4*Δ cells, either in logarithmic phase (log), arrested in G1 (αF), or released synchronously into the cell cycle for the indicated times in the presence of 0.2 M HU were analysed by flow cytometry. The positions of cells with 1C and 2C DNA contents are indicated. (**C**) Immunoblots of samples from (**B**) were probed with anti-PCNA and anti-Rnr1, Rnr3, and Rnr4 antibodies to detect RNR subunits. Ponceau S staining of the Pol30 blot is shown as a protein loading control.

S phase, as the RNR subunits were expressed at constitutively high levels throughout the cell cycle. We anticipate two possible explanations for this observation. One is that RNR is directly upregulated in the *pol30*$^{f}$ *elg1*Δ mutant and the second is that DNA damage is causing RNR upregulation but that stability of the RNR proteins and/or mRNAs results in little change when steady-state levels are examined. These possibilities can be distinguished by asking whether RNR upregulation in the *pol30*$^{f}$ *elg1*Δ mutant depends on known regulators of RNR DNA damage-dependent gene expression.

### *Upregulation of RNR activity in pol30*$^{f}$ *elg1*Δ *cells is a DNA damage response*

Since *pol30*$^{f}$ *elg1*Δ mutants exhibit DNA damage checkpoint activation in S phase as measured by phosphorylation of the Mec1/Tel1 target histone H2A and by destabilization of the Dun1 target Sml1, we asked if induction of RNR expression in *pol30*$^{f}$ *elg1*Δ mutants is a DNA damage response. To this end, we interrogated the role of two factors known to play a role in this process. We first tested the role of *SWI4*, which is important for HU-induced transcription of *RNR2* and *RNR3* (Ho *et al*, 1997), in induction of RNR expression in *pol30*$^{f}$ *elg1*Δ mutants. The upregulation of both Rnr3 and Rnr4 that was evident in *pol30*$^{f}$ *elg1*Δ was absent in *pol30*$^{f}$ *elg1*Δ *swi4*Δ (Figure 3D). Upregulation of RNR in response to replication stress can also be mediated by the transcription regulatory complex Ccr4-Not (Mulder *et al*, 2005), and this pathway is Dun1 independent (Woolstencroft *et al*, 2006). We found that deleting *CCR4* in *pol30*$^{f}$ *elg1*Δ mutants also abrogated induction of Rnr3 and Rnr4 (Figure 3D). Thus, eliminating two factors that are required for upregulation of RNR in response to DNA damage eliminated RNR upregulation in the *pol30*$^{f}$ *elg1*Δ mutant. We conclude that RNR upregulation in the *pol30*$^{f}$ *elg1*Δ mutant is a DNA damage response.

### *Increased mutagenesis in pol30*$^{f}$ *elg1*Δ *mutants*

Recent studies have demonstrated that elevated dNTP levels are required for tolerance of DNA damage in yeast, and that this occurs at the price of decreasing replication fidelity (Chabes *et al*, 2003; Sabouri *et al*, 2008). Additionally, proportional increases in dNTP levels, where the natural dNTP pool asymmetry is maintained, but the dNTP pool expands (as is the case in the *pol30*$^{f}$ *elg1*Δ mutant) are mutagenic in *E. coli* (Wheeler *et al*, 2005). We assessed the spontaneous forward mutation rate in wild-type, *elg1*Δ, *pol30*$^{f}$, and *pol30*$^{f}$ *elg1*Δ cells (Figure 4A). The *pol30*$^{f}$ and *elg*Δ single mutants exhibited 4.2-fold and 2.5-fold increases in mutation rate over wild type, respectively, whereas *pol30*$^{f}$ *elg1*Δ double mutants exhibited a 12-fold increase in mutation rate. The high mutation rate in *pol30*$^{f}$ *elg1*Δ double mutants correlated with higher dNTP concentrations in these cells compared with wild-type cells and the single mutants.

Since both PCNA and Elg1 have roles in DNA replication and might affect replication fidelity independently of dNTP levels, we introduced the *swi4*Δ mutation into the *pol30*$^{f}$ *elg1*Δ mutant. Deletion of *SWI4* abrogated the increased Rnr3 and Rnr4 levels in G1 in the *pol30*$^{f}$ *elg1*Δ mutant (Figure 3D) and during cell-cycle progression (Figure 4C). Importantly, deletion of *SWI4* was not acting by increasing the abundance of Pol30f (Figure 4C), and in fact caused reduced PCNA expression compared with the *pol30*$^{f}$ *elg1*Δ double mutant, consistent with the role of Swi4 in upregulation of transcription of DNA replication genes (Koch and Nasmyth, 1994). When we examined DNA replication in the *pol30*$^{f}$ *elg1*Δ *swi4*Δ triple mutant we found that deletion of *SWI4* also suppressed the HU-resistant DNA synthesis (Figure 4B) despite having only a modest effect on G1 transit and entry into S phase (Supplementary Figures S4–S6). In combination with the reduced RNR expression in the triple mutant, the inability of this strain to synthesize DNA in the presence of HU suggests that deletion of *SWI4* suppresses the expansion of dNTP pools that was observed in *pol30*$^{f}$ *elg1*Δ. When we measured mutation rate in the triple mutant we found that mutagenesis was suppressed from 12-fold wild type in *pol30*$^{f}$ *elg1*Δ to 4.2-fold in *pol30*$^{f}$ *elg1*Δ *swi4*Δ (Figure 4A). We conclude that the elevated dNTP level in *pol30*$^{f}$ *elg1*Δ cells makes at least a 2.9-fold contribution to the increased mutation rate. It is unlikely that Swi4 directly contributes to mutagenesis as *swi4*Δ single mutants exhibit a nearly wild-type rate of spontaneous mutagenesis (Figure 4A). Therefore, one biological consequence of the dNTP pool expansion in *pol30*$^{f}$ *elg1*Δ mutants is increased mutagenesis.

### *Increased dNTP pools and mutagenesis in distinct genome stability mutants*

The phenotypes we observe in *pol30*$^{f}$ *elg1*Δ could be specific to that genetic background, or could be more general phenomena relevant to a wide range of genome stability mutants. To test the possibility that upregulation of RNR, increased dNTPs, increased replication fork rate in HU, and mutagenesis occur in other genome integrity mutant backgrounds, we analysed these phenotypes in *tsa1*Δ, *rad54*Δ, and *rad55*Δ (Figure 5). Tsa1 is a peroxiredoxin important in cellular antioxidant defense. Deletion of *TSA1* gives rise to gross chromosomal rearrangements (Smith *et al*, 2004), increased mutagenesis (Huang *et al*, 2003), and has recently been shown to result in increased RNR activity (Tang *et al*, 2009). *RAD54* and *RAD55* encode recombination proteins, and mutants in these were identified in a recent screen for constitutive Rnr3 expression (N Thevakumaran and G Brown, unpublished observations). In all three mutants, we observed little change in bulk DNA synthesis, as measured by flow cytometry (Figure 5A), consistent with analysis of *elg1*Δ and *pol30*$^{f}$ single mutants (Figure 1B). Analysis by array CGH, however, revealed increases in replication fork distances (Figure 5B) that in the case of *tsa1*Δ and *rad55*Δ were significantly greater than wild type. Immunoblotting of extracts from G1-arrested cells revealed increased expression of Rnr3 and Rnr4 in *tsa1*Δ, *rad54*Δ, and *rad55*Δ relative to wild type (Figure 5C), indicating an active DNA damage response. Consistent with increased RNR expression, dNTP levels were increased in all three mutants (Figure 5D), as were mutation rates (Figure 5E). We conclude that upregulation of RNR, increased dNTPs, increased replication fork rate in HU, and mutagenesis occurs in different mutant backgrounds that impinge on genome integrity in different ways.

## Discussion

Replication stress caused by endogenous or exogenous agents can result in DNA damage in the form of double-strand DNA breaks during S phase. We find that DNA damage resulting from disrupting the activities of Elg1-RFC and PCNA elicits a

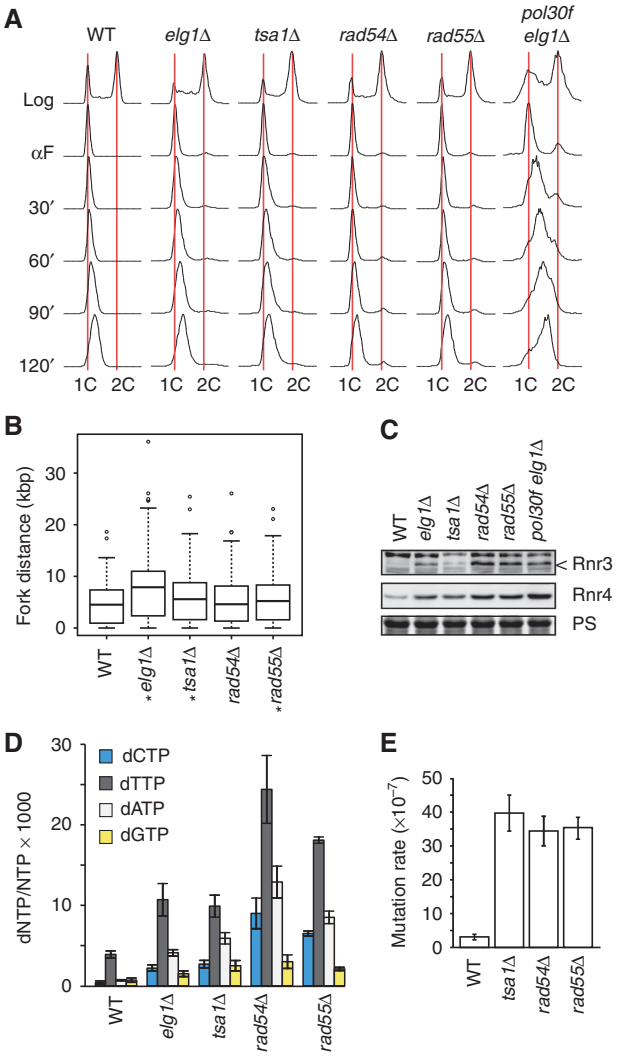



**Figure 5** Distinct genome integrity mutants exhibit increased dNTP levels and are mutators. (**A**) DNA contents of the indicated strains, either in logarithmic phase (log), arrested in G1 (αF), or released synchronously into the cell cycle for the indicated times in the presence of 0.2 M HU were analysed by flow cytometry. The positions of cells with 1C and 2C DNA contents are indicated. (**B**) Replication fork distance distributions after 60 min in HU. The distance from the centre of each ARS to peak edge for 164 replication forks across the genome was measured from aCGH data, and the results displayed as a boxplot. Median fork distance significantly greater than wild type ($P<0.01$, one-tail Wilcoxon rank-sum test) is indicated (*). (**C**) Western blot analysis of whole cell extracts prepared from the indicated strains following arrest in G1 with alpha factor. Immunoblots were probed with anti-Rnr3 and Rnr4 antibodies to detect RNR subunits. Ponceau S staining is shown as a protein loading control. (**D**) dNTP levels, expressed as dNTP:NTP ratios, were measured for the indicated strains following release of logarithmically growing cells from G1 into 0.2 M HU for 60 min. (**E**) The rates of forward mutation of *CAN1* cells to canavanine resistance were determined for the indicated strains. The means and standard deviations of three independent fluctuation tests are plotted.

DNA damage response that triggers the upregulation of RNR activity and consequently an expansion of intracellular dNTP pools. A similar response was observed in *rad54*, *rad55*, and *tsa1* mutants, suggesting that upregulation of RNR is a general response to genome instability. While the overproduction of dNTPs in response to endogenous DNA

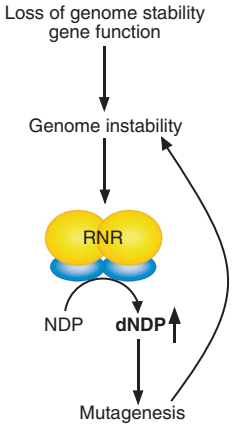



**Figure 6** Model of genome stability gene action in regulating dNTP levels. Mutation of genome stability genes results in DNA damage during S phase. The response to the DNA damage causes an upregulation of RNR and expansion of cellular dNTP pools, resulting in increased mutation frequency, which contributes to further genome instability.

damage most likely serves to promote tolerance of that damage, it also increases mutagenesis. We propose a model in which the response to endogenous DNA damage caused by loss of genome stability gene function results in a mutator phenotype (Figure 6), one of the hallmarks of oncogenic progression.

### *Spontaneous DNA damage in pol30$^{f}$ elg1Δ*

Our findings suggest that *pol30$^{f}$ elg1*Δ mutants accumulate significant levels of DNA damage. This observation is not surprising, as in the absence of Elg1 alone cells exhibit phenotypes that are indicative of the frequent occurrence of DNA damage. These phenotypes include large chromosomal aberrations, hyper-recombination, increase in spontaneous Ddc2 and Rad52 DNA repair foci, as well as a dependency on DNA damage checkpoint and repair pathways for fitness (Bellaoui *et al*, 2003; Ben-Aroya *et al*, 2003; Kanellis *et al*, 2003; Alvaro *et al*, 2007; Davidson and Brown, 2008). PCNA is important in the coordination of a myriad of DNA transactions, and thus plays a vital role in the maintenance of genome stability (Moldovan *et al*, 2007). Since Elg1-RFC and PCNA likely function together, it is not unexpected that spontaneous DNA damage may be exacerbated in the double mutant. In support of this notion, *pol30$^{f}$ elg1*Δ double mutants exhibit a marked increase in γH2A in comparison with *pol30$^{f}$* and *elg1*Δ single mutants (Supplementary Figure S7). Our data indicate that DNA damage in *pol30$^{f}$ elg1*Δ mutants arises during S phase (Figure 3A and B), suggesting that defects in DNA replication may be the source of this damage.

While the exact nature of the DNA damage in *pol30$^{f}$ elg1*Δ mutants is not known, it is likely distinct from the mutagenesis that results from increased dNTP levels. The increase in γH2A and Rnr3 in *pol30$^{f}$ elg1*Δ mutants (Figure 3A) suggests that the primary DNA damage events consist of double-strand DNA breaks or large single-strand DNA gaps. By contrast, the mutagenesis caused by increased dNTP pools consists predominantly of base substitutions with some small insertions or deletions (Chabes *et al*, 2003; Sabouri *et al*, 2008) and does not cause activation of the DNA damage response (Chabes and Stillman, 2007; Kumar *et al*, 2010), distinguish-

ing the damage caused by mutagenesis from that capable of eliciting a checkpoint response. We suggest that DNA breaks or gaps cause the checkpoint activation that we observe, which via an increase in dNTP pools results in point mutations and/or small insertions or deletions.

### *Upregulation of RNR occurs via a DNA damage response pathway*

When yeast cells are treated with genotoxic agents, the DNA damage response orchestrates the upregulation of RNR activity, resulting in an increase in intracellular dNTP concentrations, which promotes survival (Chabes *et al*, 2003), likely by facilitating DNA chain elongation in the presence of replication stress (Poli *et al*, 2012). Unperturbed *pol30$^{f}$ elg1*Δ mutants exhibit significant levels of DNA damage signal (Figure 3A and D), including phosphorylation of H2A serine 129, destabilization of Sml1, and *SWI4*- and *CCR4*-dependent *RNR3* and *RNR4* gene expression, suggesting that induction of RNR activity in these cells occurs through the activation of the DNA damage response. While formally possible, we disfavour scenarios in which increased dNTPs are upstream of DNA damage response activation, since increased dNTP pools alone do not elicit a DNA damage signal (Chabes and Stillman, 2007; Kumar *et al*, 2010). Recently, upregulation of *RNR* gene transcription has been observed in *pol30-8* mutants and proposed to arise from defects in histone assembly (Rossmann *et al*, 2011). We disfavour the possibility that global defects in histone assembly cause upregulation of RNR expression in *pol30$^{f}$ elg1*Δ cells as the *pol30-8* mutation specifically compromises physical interactions of PCNA with chromatin assembly factor CAF1 (Zhang *et al*, 2000), and *pol30-8* mutants exhibit genetic interaction profiles similar to those of chromatin assembly and silencing mutants, but distinct from PCNA mutants like *pol30$^{DAmP}$*, which reduce PCNA levels. It is also worth noting that suppression of RNR upregulation in *pol30$^{f}$ elg1*Δ mutants by deletion of *SWI4* further reduced PCNA levels and so was inconsistent with *pol30$^{f}$* directly causing upregulation of RNR transcription. Although the persistently elevated expression of Rnr3 and Rnr4 contrasted with the cell-cycle fluctuation of γH2A, we hypothesize that this results from a low rate of RNR turnover rather than RNR induction that is independent of DNA damage. We propose that upregulation of RNR activity in *pol30$^{f}$ elg1*Δ mutants occurs in response to the persistent DNA replication stress in these cells.

### *Elevated dNTP pools correlate with increased mutagenesis*

The level of dNTPs can be modulated in budding yeast by overexpression or mutation of *RNR1*. In these scenarios, in which increases in dNTPs are independent of DNA replication stress or spontaneous DNA damage, the increased dNTP levels correlate with increased spontaneous mutagenesis (Chabes *et al*, 2003; Sabouri *et al*, 2008). In our study, the extent of spontaneous mutagenesis in wild-type, *pol30$^{f}$*, *elg1*Δ, *pol30$^{f}$ elg1*Δ, *rad54*Δ, *rad55*Δ, and *tsa1*Δ strains correlated with the relative increase in dNTP levels in these cells (Pearson's $R = 0.80$; Supplementary Figure S8). The *tsa1*Δ mutant deviated from this correlation, displaying a higher mutation rate than expected for its dNTP level. This could reflect an additional role for Tsa1 in suppressing mutagenesis. High intracellular dNTP concentrations have been proposed to induce mutagenesis through misincorporation by replicative polymerases, by promoting mismatch extension, or possibly through an increase of DNA synthesis by error-prone polymerases (Chabes *et al*, 2003; Kumar *et al*, 2011). The *pol30$^{f}$ elg1*Δ mutant enters S phase with dNTP concentrations three-fold higher than peak levels observed in a normal S phase in wild-type cells (Chabes *et al*, 2003). Moreover, during S phase, dNTP levels are most likely further elevated as RNR activity continues to be upregulated through increased Rnr1 expression and Sml1 degradation (Figure 4A; Elledge *et al*, 1993), promoting replication errors.

Our data suggest that spontaneous activation of the DDR resulting in increased RNR activity is a general feature of mutations that cause genomic instability. We analysed mutants with defects in genome stability that are likely to be mechanistically distinct from the defects in *pol30$^{f}$ elg1*Δ double mutants. These included a deletion mutant of *TSA1*, which encodes a peroxiredoxin important in cellular antioxidant defense. The *tsa1*Δ mutation gives rise to gross chromosomal rearrangements (Smith *et al*, 2004) and increased mutagenesis (Huang *et al*, 2003), and has recently been shown to result in increased RNR activity and dNTP levels (Tang *et al*, 2009). Consistent with these data, we found that *tsa1*Δ exhibited increased expression of Rnr3 and Rnr4, increased dNTP pools, and increased mutagenesis, and also resulted in increased replication in HU. Rad54 and Rad55 function in homologous recombination, and deletion of *RAD54* or *RAD55* also resulted in increased RNR levels, increased dNTP levels by 5–6-fold, and increased mutagenesis by almost 10-fold. In further support of the hypothesis that upregulation of RNR activity is a general response to genetically induced replication stress that results in DNA damage, cells that harbour mutations in DNA polymerase α exhibit chronic expression of *RNR1–3* (Zhou and Elledge, 1992; Elledge *et al*, 1993). Loss of Ctf4, which is important in DNA replication and sister chromatid cohesion (Hanna *et al*, 2001; Gambus *et al*, 2006; Lengronne *et al*, 2006; Tanaka *et al*, 2009), results in constitutive DNA damage checkpoint activation (Gambus *et al*, 2009), increased cellular dNTP concentrations, and DNA replication in the presence of HU (Poli *et al*, 2012). Taken together, these data suggest that upregulation of RNR activity is a general response to genetically induced replication stress that results in DNA damage.

Could a similar response occur in human cells? Certainly, there are parallels between RNR regulation in yeast and humans. The large (RRM1) and small (RRM2) subunits of RNR in human cells are upregulated in response to DNA damage (Lin *et al*, 2004; Zhang *et al*, 2009), and the specialized small subunit RRM2B is upregulated in response to damage via p53 (Tanaka *et al*, 2000), although RRM2B might be primarily involved in providing dNTPs for mitochondrial DNA synthesis (Thelander, 2007). There is evidence of transcriptional regulation of RRM1 and RRM2 via E2F1 and the checkpoint kinase Chk1, and this resembles the transcriptional regulation of yeast *RNR* genes (Zhang *et al*, 2009). Yet, it is unclear at this point whether increases in *RNR* gene and protein expression result in increases in dNTP concentration as is observed in yeast. Additionally, whether dNTP pools increase in human cells following treatment with exogenous DNA damaging agents remains controversial (Kunz and Kohalmi, 1991; Hakansson *et al*, 2006). The dNTP pool is several-fold higher in transformed cells than in non-trans-

formed cells (Martomo and Mathews, 2002), suggesting that some kinds of oncogenic stress could result in dNTP pool expansion. Overexpression of RRM2 or RRM2B is mutagenic in mouse cells and promotes lung carcinogenesis (Xu *et al*, 2008), consistent with a link between dNTP metabolism, mutagenesis, and oncogenesis in mammals. It remains to be seen if endogenous DNA damage resulting from replication stress also results in dNTP pool expansion, and the degree to which this is mutagenic in human cells. Finally, even in the absence of an overt increase in total cellular dNTP pools, changes in RNR localization could cause mutagenic local increases in dNTP concentrations. Consistent with this possibility, RRM1 and RRM2 are recruited to sites of DNA damage in human cells (Niida *et al*, 2010).

Collectively, our data suggest that mutations that cause replication stress leading to genome instability force yeast cells into a vicious circle in which the cellular response to endogenous DNA damage induces mutagenesis which could then be a source of further genomic instability (Figure 6). The mutator phenotype theory of tumourigenesis posits that increased somatic mutation rate in pre-cancerous cells is necessary to account for the number of mutations found in many cancer cells (Loeb, 1991; Venkatesan *et al*, 2006). Although the necessity for a mutator phenotype (Bodmer *et al*, 2008; Loeb *et al*, 2008) and the means of acquiring it remain controversial, there is little doubt that all cancers contain mutations (Stratton *et al*, 2009) and that these contribute to carcinogenesis. Our data suggest that elevated dNTP pools could contribute to increased mutation frequency. In this respect, it is interesting to note recent observations that pre-cancerous lesions show evidence of DNA damage response activation (Bartkova *et al*, 2005; Gorgoulis *et al*, 2005) and that this activation can result from oncogene expression and the resulting replication stress (Bartkova *et al*, 2006; Di Micco *et al*, 2006). Models of oncogenesis that incorporate these observations propose that the DNA damage response to replication stress provides a barrier against malignancy by activation of p53 which regulates downstream senescence and apoptotic pathways (Halazonetis *et al*, 2008). Inactivation of this barrier, for example by p53 mutation, is selected for during tumour development (Halazonetis *et al*, 2008). One possibility is that activation of the DNA damage response could result in high dNTP levels and mutagenesis, and provide a mechanism for overcoming barriers to malignancy. Interestingly, recent data indicate that activating entry into S phase prematurely can cause DNA damage due to insufficient dNTPs for normal DNA synthesis (Bester *et al*, 2011). In this instance, DNA replication is initiating in a dNTP-poor environment, resulting in DNA breaks, and resembles studies in yeast in which instability is induced by deregulating the initiation of DNA replication (Tanaka and Araki, 2011). This contrasts with the scenario presented in our study, in which replication stress occurs without premature S phase entry and without depletion of the dNTP pool. Our data suggest that this distinct form of replication stress promotes a vicious circle in which S phase DNA damage feeds forward to activate dNTP synthesis and promote mutagenesis, providing a distinct mechanism by which DNA damage response components could become inactivated, and highlighting the importance of maintaining the intracellular dNTP pool at the optimum level.

## Materials and methods

### *Yeast strains and media*

All yeast strains used in this study were derivatives of BY4741 (Brachmann *et al*, 1998), and are listed in Supplementary Table S1. Strains were constructed using standard genetic methods. Non-essential haploid deletion strains marked with the kanamycin (G418) resistance gene were made by the *Saccharomyces* Gene Deletion Project (Winzeler *et al*, 1999). Deletion strains marked with nourseothricin (nat) resistance gene were constructed by switching the kanamycin resistance gene with the nourseothricin resistance gene as described previously (Tong *et al*, 2001). All strains harbouring a deletion of *ELG1*, except MBY45, were constructed by PCR transformation. Standard yeast media and growth conditions were used (Sherman, 1991).

### *HU and MMS sensitivity*

Strains were grown in YPD overnight, serially diluted, spotted onto plates and incubated at 30°C. HU and MMS (Sigma-Aldrich) plates contained 100 mM HU and 0.035% (v/v) MMS in YPD, respectively and were used within 24 h of preparation.

### *Cell synchronization and flow cytometry*

Yeast strains were grown at 30°C in YPD to an optical density at 600 nm of 0.3–0.4. Alpha factor was added to 5 μM. After 2 h of incubation, cells were harvested, washed once with YPD, and released from G1 arrest by resuspension in YPD or YPD with 0.2 M HU (Sigma-Aldrich). Cultures were sampled at the indicated times and processed for flow cytometry as described (Davierwala *et al*, 2005; Bellay *et al*, 2011). DNA content was measured using a Guava or a FACSCalibur flow cytometer. Data were analysed using FlowJo Flow Cytometry Analysis Software, Version 9.0. Histograms represent the cell-cycle distribution of the indicated samples. The *Y* axis of each graph has been scaled to represent the percentage of the maximum bin contained in that profile.

### *CGH on microarray*

CGH was performed, as described (Dion and Brown, 2009), with amplified genomic DNA from wild-type and mutant cells collected after the indicated times in 0.2 M HU. The control sample consisted of genomic DNA from wild-type cells arrested in G1. The analysis method is detailed in Supplementary data. A schematic diagram of the aCGH analysis work flow is presented in Supplementary Figure S4A, and the complete data set is in Supplementary Table S2. Raw microarray data can be obtained from Array Express (accession number E-MEXP-3458).

### *Chromatin immunoprecipitation*

Chromatin immunoprecipitation on microarray (ChIP-chip) experiments were performed as described previously (Katou *et al*, 2003, 2006). Cells were arrested in G1 with alpha factor and released into YPD containing 0.2 M HU for 90 min at 23°C. After crosslinking and DNA fragmentation, PCNA was precipitated and labelled. Bound and unbound DNA was hybridized to whole-genome tiling arrays (P/N 520055; Affymetrix, Santa Clara, CA). Arrays were washed and scanned as described previously (Lee *et al*, 2007). The analysis method is detailed in Supplementary data.

### *Whole cell extracts, immunoblotting, and northern blotting*

Cultures were collected, fixed with 10% trichloroacetic acid, and whole cell extracts were prepared as described previously (Pellicioli *et al*, 1999). Proteins were resolved on 7.5 or 12% SDS–polyacrylamide gels, transferred onto nitrocellulose membranes and subjected to immunoblot analysis. The immunoblots were probed with following antibodies: rabbit anti-Rnr1 (AS09 576; Agrisera AB, Sweden) and rabbit anti-Rnr3 (AS09 574; Agrisera AB) (Tsaponina *et al*, 2011), YL 1/2 (Abcam, Cambridge, MA) to detect Rnr4 (Kumar *et al*, 2010), rabbit anti-γH2A (Abcam), goat anti-Rad53 (yC-19; Santa Cruz), rabbit anti-Sml1, rat anti-tubulin (YOL1/34; Serotec, Indianapolis, IN), and rabbit anti-PCNA (kindly provided by Helle Ulrich, Cancer Research UK). Immunoblots were developed using SuperSignal ECL (Pierce Chemical, Rockford, IL). RNA was prepared using RiboPure, as described by the manufacturer (Ambion, Austin, TX) and fractionated on 1.2% agarose gels containing 1% formaldehyde. After transfer onto nylon membranes, transcripts were detected with $^{32}$P-labelled DNA probes.

### *dNTP analysis*

Following alpha factor block and release of cells into HU, samples for dNTP measurements were collected at the indicated times as described previously (Chabes *et al*, 2003). Quantification of dNTPs was performed as described (Kumar *et al*, 2010).

### *CAN1 forward mutation analysis*

Forward mutation to canavanine resistance was measured as described previously (Huang *et al*, 2002). Fluctuation tests were performed with five or nine parallel cultures, in triplicate, and the median value from each was used to calculate the spontaneous mutation rate by the method of the median (Lea and Coulson, 1949). *elg1*Δ strains analysed for *CAN1* mutation were constructed by direct gene replacement of *ELG1*, and therefore do not harbour the mutation at the *MSH3* locus that is found in strains constructed for the *Saccharomyces* gene deletion project (Lehner *et al*, 2007).

### *Supplementary data*

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

## Acknowledgements

We thank Charlie Boone for strains; Wasan Abada for strain construction; Rodney Rothstein for antibodies to Sml1; Olga Tsaponina for advice on Rnr1, 2 and 3 immunoblotting (Chabes *et al*, 2003); Helle Ulrich for antibodies to PCNA; Dan Durocher for comments on the manuscript; and Philippe Pasero, Armelle Lengronne, and Laure Crabbe for communicating results prior to publication. This research was supported by Canadian Institutes of Health Research grant MOP79368 (to GWB), Canadian Graduate Scholarships from the Natural Sciences and Engineering Research Council of Canada (to JAV and TLS), by Grants-in-Aid for Scientific Research (S) from MEXT (to KS), by the Swedish Foundation for Strategic Research, the Swedish Research Council, and the Swedish Cancer Society (to AC), and grants 1R01HG005084-01A1 and 1R01HG005853-01 from the National Institutes of Health (to CLM).

*Author contributions:* MBD and GWB designed the experiments. MBD, JO, TLS, JAV, NT, and GWB carried out the experiments. YK and KS designed and carried out the experiments in Figure 1G. AK, LM, and AC designed and carried out the experiments in Figures 2A and 5D. TX and CLM designed and carried out the analysis of aCGH data. MBD and GWB wrote the manuscript.

## Conflict of interest

The authors declare that they have no conflict of interest.

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