Checkpoint activation regulates mutagenic translesion synthesis

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Cells have evolved checkpoint responses to arrest or delay the cell cycle, activate DNA repair networks, or induce apoptosis after genomic perturbation. Cells have also evolved the translesion synthesis processes to tolerate genomic lesions by either error-free or error-prone repair. Here, we show that after a replication perturbation, cells exhibit a mutator phenotype, which can be significantly affected by mutations in the checkpoint elements Cds1 and Rad17 or translesion synthesis polymerases DinB and Pol. Cells respond to genomic perturbation by up-regulation of DinB in a checkpoint activation-dependent manner. Moreover, association of DinB with chromatin is dependent on functional Rad17, and DinB physically interacts with the checkpoint-clamp components Hus1 and Rad1. Thus, translesion synthesis is a part of the checkpoint response.

[Keywords: Checkpoint response; mutator phenotype; mutagenic translesion synthesis; DinB; Cds1; Rad17]

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Organisms, when faced with genomic stress, activate checkpoints to regulate cell-cycle transitions and facilitate DNA repair processes, or to induce apoptosis [Elledge 1996; Zhou and Elledge 2000]. Failure in these responses can result in the accumulation of mutations and cause genomic instability, which are predominant characteristics of cancer cells [Hartwell and Kastan 1994; Lengauer et al. 1998]. To achieve the checkpoint response, cells have sensors/transducers that detect genomic lesions and relay the signals to effector kinases that act on the targets [Zhou and Elledge 2000]. In fission yeast, Rad1, Rad3, Rad9, Rad17, Rad26, and Hus1, known as the checkpoint Rads, function as sensors/transducers for signals of replication perturbation and DNA damage [Rhind and Russell 1998; Caspari and Carr 1999]. Rad3 plays a central role in the checkpoint signaling pathway, forming a complex with Rad26 in vivo, and phosphorylating Rad26 in response to DNA damage and S-phase arrest [Edwards et al. 1999; O'Connell et al. 2000]. Rad1, Rad9, and Hus1 exist in a complex in vivo and have predicted structural homology to PCNA [Caspari et al. 2000; Venclovas and Thelen 2000; Melo and Toczyski 2002]. Rad17 contains homology to all five subunits of RF-C [Griffiths et al. 1995] and exists in a complex with Rfc2-5 [for review, see Caspari and Carr 1999; Melo and Toczyski 2002]. A fraction of fission yeast Rad17 binds to chromatin throughout the cell cycle, independent of the presence of other checkpoint proteins [Griffiths et al. 2000]. Increasing amounts of Rad17 bind to chromatin in response to DNA damage and remain tightly chromatin bound after replication arrest [Kai et al. 2001]. In budding yeast, Ddc1 (fission yeast Rad9 homolog) recognizes damage sites in a Rad24 (fission yeast Rad17 homolog)-dependent manner [Kondo et al. 2001; Melo et al. 2001], whereas inhibition of human Rad17 synthesis by siRNA leads to a reduction in the damage-induced binding of human Rad9 to chromatin [Zou et al. 2002]. These data have led to a hypothesis that the Rad17–Rfc2-5 complex senses genomic lesions and functions as a checkpoint-clamp loader, loading the checkpoint-clamp Rad1–Rad9–Hus1 complex onto chromatin. Once loaded onto chromatin, the checkpoint-clamp may recruit other checkpoint elements to activate the checkpoint response (Melo and Toczyski 2002). In addition to the checkpoint Rad proteins, the fission yeast checkpoint effector kinase Cds1 and its budding yeast counterpart Rad53 play a critical role in preventing accumulation of abnormal replication intermediates when replication is perturbed in order to restore the replication fork and to allow restart of DNA synthesis [Desany et al. 1998; Lindsay et al. 1998; Rhind and Russell 2000; Lopes et al. 2001]. Thus, checkpoint rad+ genes and cds1+ are essential in coordinating DNA replication and maintaining genomic stability.

Cells have also evolved a process that allows them to tolerate genomic lesions by either error-free or error-prone translesion synthesis [TLS]. In response to genotoxic stress, Escherichia coli has an SOS response that up-regulates genes to cope with the stress [Friedberg et al. 1995; Sutton et al. 2000; Goodman 2002]. Many of the gene products induced by the SOS response are involved...
in DNA repair. Of these, three SOS genes—umuC, umuD, and dinB—are exclusively responsible for the TLS process (Sutton et al. 2000; Goodman 2002). The UmuC protein associates with a proteolytically processed form of UmuD [UmuD'] and forms a UmuD/C complex [PolV]. In vitro, the UmuD/C complex bypasses certain lesions in a highly error-prone manner and has poor fidelity in copying undamaged DNA (Sutton et al. 2000; Goodman 2002). The gene product of dinB [PolIV] is a DNA polymerase that is devoid of 3’–5’ exonuclease activity, distributive in nature, and prone to frameshift and that frequently introduces –1 frameshift mutations (Wagner et al. 1999; Goodman 2002).

A large repertoire of eukaryotic genes known or predicted to encode translesional DNA polymerases has emerged in recent years (Woodgate 1999; Friedberg et al. 2000; Ohmori et al. 2001; Goodman 2002). Among these polymerases, Polξ is a B-family polymerase required for DNA damage-induced mutagenesis in budding yeast (Goodman 2002), which has the ability to bypass a cys–syn thymine–thymine (T–T) dimer (Nelson et al. 1996). Some of the other translesional polymerases are members of the Y-family or UmuC-super family, represented by UmuC, Rev1, DinB/PolIV/Polκ, Rad30/Polη/Eso1, and Polh (Ohmori et al. 2001; Wang 2001; Goodman 2002). Rad30/Polη performs translesion DNA synthesis of cys–syn T–T dimers with high fidelity. However, Rad30/Polη exhibits extremely low fidelity when acting on undamaged DNA or on DNA with noncognate substrates (Johnson et al. 1999; Masutani et al. 1999; Johnson et al. 2000; Matsuda et al. 2001; Washington et al. 2001; Goodman 2002). In fission yeast, the eso1+ gene encodes a dual function protein, with the N-terminal portion encoding the homolog of Rad30/Polη and the C-terminal region encoding the budding yeast Eco1 homolog (Tanaka et al. 2000). In vitro, human DinB/Polκ has a high misincorporation rate and can bypass abasic sites and N-2-acetylaminofluorene (AAF) adducts in a highly error-prone manner (Ohashi et al. 2000a,b). Polκ/DinB can also readily extend a misaligned undamaged primer terminus, causing –1 frameshift mutations (Washington et al. 2002). However, it bypasses thymine glycols with a preference for correct nucleotides incorporation (Fischhaber et al. 2002). Given the error-prone nature of these translesion synthesis polymerases in copying undamaged DNA and noncognate damaged-substrates (Friedberg et al. 2002; Goodman 2002), it seems likely these polymerases are a source of mutagenesis. Thus, understanding the regulation of the action of translesional polymerases will help to clarify how cells maintain normal genomic stability.

Studies of the E. coli SOS response have suggested that the umuCD gene products play two temporally separate physiological roles in the DNA damage checkpoint and TLS (Opperman et al. 1999). Little is known in eukaryotes about how TLS is regulated and its relationship with the checkpoint response. Genetic studies in budding yeast have shown that UV-induced mutagenic repair of irreparable lesions requires checkpoint function [Paulovich et al. 1998], whereas the activation of budding yeast S-phase checkpoint genes DUN1 or RFC5 correlates with an increase of mutation rate in the pol3-01 mutant (Datta et al. 2000). These studies suggest that when cells encounter an irreparable lesion, the checkpoint response may activate translesion synthesis to tolerate the lesion by mutagenic repair.

We have previously shown that a large repertoire of thermo-sensitive fission yeast replication mutants exhibits a mutator phenotype at the semipermissive temperature. The types of mutations are characterized by deletion of sequences flanked by homologous repeats and small sequence alterations comprised of base substitutions and single-base frame shifts (Liu et al. 1999). Replication perturbation caused by a semidisabled replication mutant could generate a single-strand gap, leading to a double-strand break. Subsequent repair by double-strand break repair, recombinational repair, or single-strand annealing could result in deletion of sequences flanked by homologous repeats (Paques and Haber 1999). In addition, a stalled replication fork caused by an impaired replication mutant could induce regression of the replication fork and formation of a Holliday junction (Paques and Haber 1999; Boddy et al. 2001). Resolution of the Holliday junctions could also cause a deletion mutator phenotype.

Small sequence alterations are often caused by polymerase slippage or misinsertions by mutations in DNA polymerases. Here, we use a fission yeast pola thermo-sensitive mutant, swi7-H4, to induce replication stress and a mutator phenotype at the semipermissive temperature. By analyzing the effect of the checkpoint proteins, Cds1 and Rad17, on the mutation rate and mutation spectra in the pola(swii7-H4) mutant, we find that Cds1 and Rad17 significantly affect its mutator phenotype. A mutation in cds1+ enhances the deletion mutations in the pola(swii7-H4) mutant, whereas mutations in rad17+ have a suppressive effect on both deletion and point mutations. Analysis of the deletion of three translesional polymerases in pola(swii7-H4) revealed that DinB, and Polξ to a lesser extent, contribute significantly to the induction of point mutations and single-base frame shifts. Further studies showed that activation of the checkpoint plays a significant role in regulating the translesional polymerase, DinB.

Results

Checkpoint effector kinase Cds1 affects the mutator phenotype in pola(swii7-H4).

We used a replicative polymerase mutant pola(swii7-H4) to induce replication stress and a mutator phenotype. This mutant exhibited an S-phase delay and activation of Cds1 kinase at its semipermissive temperature (30°C), indicating that the checkpoint is intact in pola(swii7-H4) (Fig. 1A,B). pola(swii7-H4) exhibited an elevated mutation rate compared with wild-type cells at the semipermissive temperature (Table 1). The mutant accumulated both deletions of sequence flanked by repeats and small sequence alterations such as base substitutions and
point mutations when compared with wild-type cells referred to as point mutations) and exhibited a 194-fold frameshifts (hereafter, all small sequence alterations are described as frameshifts).

Analysis of checkpoint function in Figure 1.

A. Progression of wild-type, pola[swi7-H4], pola[swi7-H4] rad17.K118E, and pola[swi7-H4] cds1.T8A cells. (A) Progression of wild-type, pola[swi7-H4], pola[swi7-H4] rad17.K118E, and pola[swi7-H4] cds1.T8A cells through G1 phase and then released into synthetic media containing nitrogen. The X-axis represents the relative DNA content, and the Y-axis represents cell number at the indicated time after release from nitrogen starvation. (B) Activation of Cds1 kinase in checkpoint mutants. Logarithmically growing pola[swi7-H4] and pola[swi7-H4] mutant cells in checkpoint mutant backgrounds were incubated at 30°C for 4 h, and the Cds1 kinase activity was assayed from the cell extracts as described in Materials and Methods. Levels of Cds1 used in the kinase assay were determined by the Cds1 immunoblot. The pola[swi7-H4] mutant is presented as swi7.

frame shifts (hereafter, all small sequence alterations are referred to as point mutations) and exhibited a 194-fold increase in deletion mutations and 16-fold increase in point mutations when compared with wild-type cells [Fig. 2].

Cds1, the checkpoint effector kinase, is thought to play a critical role in recovery from a stalled replication fork to restart DNA synthesis [Desany et al. 1998; Lindsay et al. 1998; Rhind and Russell 2000; Lopes et al. 2001; Tercero and Diffl ey 2001]. Ectopic expression of Cds1 effectively suppresses the mutation rate in pola[swi7-H4] to near wild-type level [Table 1], consistent with the notion that Cds1 somehow prevents accumulation of aberrant replication intermediates and promotes recovery of the replication fork.

Because deletion of cds1+ in the pola mutant cells is lethal [Bhaumik and Wang 1998], we tested the effect of a cds1 mutant, cds1.T8A, which retains Cds1 kinase activity and is only moderately sensitive to hydroxyurea (HU) treatment [Tanaka et al. 2001]. The double mutant pola[swi7-H4] cds1.T8A had a moderately compromised checkpoint, completing the cell cycle after 7 h [Fig. 1A] and had greater than 80% viability at the semipermissive temperature (30°C). In addition, Cds1 kinase activity was weakly induced in the pola[swi7-H4] cds1.T8A double mutant (Fig. 1B). However, pola[swi7-H4] cds1.T8A exhibited an overtly elevated mutation rate that was 160-fold higher than wild-type cells and 4.6-fold higher than the pola[swi7-H4] single mutant (Table 1). Moreover, the pola[swi7-H4] cds1.T8A double mutant accumulated a sixfold higher deletion mutations and a greater than twofold higher point mutations than the pola[swi7-H4] single mutant [Table 2]. These results support the notion that Cds1 prevents accumulation of abnormal replication intermediates that could lead to deletion mutations.

To confirm this notion, we analyzed another checkpoint rad mutant, rad26.T12, which has been shown to play a role in recovery from DNA damage [Al-Khodairy et al. 1994; Lindsay et al. 1998]. Similar to the effect of the cds1.T8A mutation, the mutation rate in the double mutant pola[swi7-H4] rad26.T12, was 147-fold higher than wild-type and 4.2-fold higher than the pola[swi7-H4] single mutant [data not shown]. These results indicate that checkpoint gene products involved in replication fork maintenance and recovery are important in preventing genomic instability in coordination with DNA replication.

Mutations in rad17+ and cds1+ have opposite effects on the pola [swi7-H4] mutator phenotype

Studies of fission yeast and human cells have shown that genomic perturbation enhances chromatin binding of Rad17, which is thought to initiate the checkpoint response by promoting the binding of the checkpoint clamp to chromatin [Kai et al. 2001; Zou et al. 2002]. We thus analyzed the effect of Rad17 on the mutator phenotype in pola[swi7-H4]. Because deletion of rad17+ in pola mutants is lethal [Bhaumik and Wang 1998], we analyzed the effect of three rad17 mutants: rad17.K118E, rad17.E198A, and rad17.S222A. These rad17 mutants contain mutations in the Walker domains of the Rad17 protein and exhibit a moderate checkpoint defect [Griffiths et al. 1995]. In contrast to the effect of the cds1.T8A mutation, mutations in rad17+ at all three alleles in pola[swi7-H4] suppressed the mutation rate compared with the pola[swi7-H4] single mutant, with rad17.K118E exhibiting the most significant effect [Table 1]. Both deletion and point mutations in pola[swi7-H4] were suppressed by the rad17.K118E mutation.
tation to near wild-type level (Fig. 2, left). The suppression is not owing to a further compromise of the checkpoint function of the double mutant, because at the semipermissive temperature, the double mutant \( \text{pol}/\text{H9251} (\text{swi7-H4}) \text{rad17.K118E} \) exhibited a delay in S phase similar to that of the \( \text{pol}/\text{H9251} (\text{swi7-H4}) \) single mutant (Fig. 1A), and an ability to activate Cds1 kinase activity (Fig. 1B). However, Cds1 was not autophosphorylated in the \( \text{pol}/\text{H9251} (\text{swi7-H4}) \text{rad17.K118E} \) double mutant (Fig. 1B), indicating that the \text{rad17.K118E} \) mutation in \( \text{pol}/\text{H9251} (\text{swi7-H4}) \) compromised some physiological function other than checkpoint signaling for cell-cycle arrest.

These experiments indicate that Rad17 and Cds1 act in opposite ways in coping with replication stress.

Translesional polymerases DinB and Polç are responsible in generating the point mutations accumulated in pola\{swi7-H4\} cells

The ability of mutations in \text{rad17} \) to suppress the mutator phenotype of pola\{swi7-H4\} implies that Rad17 must play a positive role in enhancing mutations in pola\{swi7-H4\} when replication is perturbed. One possibility is that Rad17 promotes translesion synthesis by using the specialized translesional DNA polymerases for mutagenic synthesis. To test this hypothesis, we constructed mutants containing a deletion of \( \text{pol}/\text{H9256} \) \) in \( \text{pol}/\text{H9251} (\text{swi7-H4}) \) (Table 2).

Deletion of \( \text{pol}/\text{H9256} \) \) in \( \text{pol}/\text{H9251} (\text{swi7-H4}) \) moderately reduced the mutation rate to a level 25-fold over wild-type cells compared with 35-fold in the \( \text{pol}/\text{H9251} (\text{swi7-H4}) \) single mutant. Deletion of the \text{rad30} \) domain of \( \text{eso1} \) \) individually and in combination with \( \text{pol}/\text{H9251} (\text{swi7-H4}) \) (Table 2). Deletion of \text{pol} \) in pola\{swi7-H4\} moderately reduced the mutation rate to a level 25-fold over wild-type cells compared with 35-fold in the pola\{swi7-H4\} single mutant. Deletion of the \text{rad30} \) domain of \( \text{eso1} \) \) in pola\{swi7-H4\} did not significantly change the mutation rate.

### Table 1. Effects of Cds1 and Rad17 on the pola\{swi7-H4\} mutation rate

| Genotype | Mutation rate \((\times 10^{-8})\) | C.I. | Fold increase over wild type | Mutation rate relative to \text{swi7} |
|----------|---------------------------------|------|-------------------------------|---------------------------------|
| wild type | 5.70 (4.98–6.12) | 1 | 1 | 5.70 (4.98–6.12) |
| Wild type +Cds1 | 3.98 (3.61–5.22) | 1 | 1 | 3.98 (3.61–5.22) |
| \( \text{swi7} \) | 198 (155–233) | 35 | 1 | 198 (155–233) |
| \( \text{swi7} +\text{Cds1} \) | 10.3 (7.59–12.5) | 2 | ↓19 | 10.3 (7.59–12.5) |
| \( \text{cds1.T8A} \) | 6.22 (4.55–7.71) | 1 | ↓1 | 6.22 (4.55–7.71) |
| \( \text{swi7 cds1.T8A} \) | 911 (796–1305) | 160 | ↑4.6 | 911 (796–1305) |
| \( \text{swi7 rad17.K118E} \) | 25.9 (20.3–30.1) | 5 | ↓7.6 | 25.9 (20.3–30.1) |
| \( \text{swi7 rad17.E198A} \) | 83.5 (64.7–103) | 15 | ↓2.4 | 83.5 (64.7–103) |
| \( \text{swi7 rad17.S222A} \) | 68.2 (59.2–72.5) | 12 | ↓2.9 | 68.2 (59.2–72.5) |
| \( \text{rad17.K118E} \) | 5.43 (4.68–6.33) | 1 | 1 | 5.43 (4.68–6.33) |
| \( \text{rad17.E198A} \) | 5.72 (4.42–6.59) | 1 | 1 | 5.72 (4.42–6.59) |
| \( \text{rad17.S222A} \) | 5.21 (4.41–6.29) | 1 | ↓1 | 5.21 (4.41–6.29) |

Mutation rates shown here are the average of three or more experiments. C.I. is the 95% confidence interval \([10^{-8} \text{ per cell division}]\). Fold increases or decreases in mutation rates are shown as ↑ or ↓, respectively. +Cds1 represents ectopic expression of Cds1 from pREP4l-cds1+.

Deletions of \( \text{pol}/\text{H9251} (\text{swi7-H4}) \text{rad17.K118E} \) were able to maintain the checkpoint at 30°C, it exhibited a cut phenotype at 36°C, indicating lack of a functional checkpoint at the elevated temperature.

These experiments indicate that Rad17 and Cds1 act in opposite ways in coping with replication stress.

Figure 2. Types of mutations in pola\{swi7-H4\} cells in rad17.K118E and in cds1.T8A backgrounds. Point mutations represent both base substitutions and single-base frameshift mutations. The rates of each mutation type were calculated by multiplying the rate of 5-FOA-resistant cells by the percentage of alterations that occurred as deletion or point mutations. Bar graphs show the types of mutation relative to wild-type. The pola\{swi7-H4\} mutant is presented as swi7.
rate. Deletion of \( \text{dinB}^+ \) in \( \text{pol}^+/\text{swi7-H4} \) reduced the mutation rate to 1.6-fold lower than that of the \( \text{pol}^+/\text{swi7-H4} \) single mutant (Table 2). The triple mutant of \( \text{pol}^+/\text{swi7-H4} \) with deletions of both \( \text{pol}^+ \) and \( \text{dinB}^- \) \( \text{pol}^+/\text{swi7-H4} \) \( \text{pol}^+ \text{dinB}^- \) exhibited a further decrease in mutation rate to a level twofold lower than the mutation rate of \( \text{pol}^+/\text{swi7-H4} \) single mutant. The triple mutants \( \text{pol}^+/\text{swi7-H4} \) \( \text{rad30}^\Delta \text{dinB}^- \) and \( \text{pol}^+/\text{swi7-H4} \) \( \text{rad30}^\Delta \text{pol}^\Delta \) exhibited a mutation rate similar to that of the double mutants \( \text{pol}^+/\text{swi7-H4} \) \( \text{dinB}^- \) and \( \text{pol}^+/\text{swi7-H4} \) \( \text{pol}^\Delta \), respectively (Table 2). These results indicate that both DinB and Pol\( \text{c} \) contribute to the elevated mutation rate in \( \text{pol}^+/\text{swi7-H4} \) when replication is under stress.

Analysis of the mutation spectra of these double and triple mutants further confirmed the contribution of DinB and Pol\( \text{c} \) to the mutator phenotype seen in \( \text{pol}^+/\text{swi7-H4} \) (Fig. 3). Deletion of \( \text{dinB}^- \) in \( \text{pol}^+/\text{swi7-H4} \) reduced the level of point mutation from 16.2-fold to 4.8-fold over that of the wild-type. In contrast, deletion of either \( \text{dinB}^- \) or \( \text{pol}^c \) in \( \text{pol}^+/\text{swi7-H4} \) did not affect the levels of deletion mutations in cells (data not shown). The double-mutant \( \text{pol}^+/\text{swi7-H4} \) \( \text{dinB}^- \) \( \text{pol}^\Delta \) had a comparable level of point mutations to that observed in \( \text{pol}^+/\text{swi7-H4} \) \( \text{rad17.K118E} \), which suppresses the point mutations (Fig. 3). The triple mutant \( \text{pol}^+/\text{swi7-H4} \) \( \text{dinB}^- \) \( \text{pol}^\Delta \) exhibited a further reduction of point mutation levels to near wild-type level (Fig. 3). The \( \text{pol}^+/\text{swi7-H4} \) \( \text{rad30}^\Delta \) double mutant had a similar point mutation level as the \( \text{pol}^+/\text{swi7-H4} \) single mutant. The triple mutant \( \text{pol}^+/\text{swi7-H4} \) \( \text{rad30}^\Delta \) \( \text{pol}^\Delta \) had similar levels of point mutations as \( \text{pol}^+/\text{swi7-H4} \) \( \text{pol}^\Delta \), whereas the \( \text{pol}^+/\text{swi7-H4} \) \( \text{dinB}^- \) \( \text{rad30}^\Delta \) triple mutant had similar levels of point mutations as the \( \text{pol}^+/\text{swi7-H4} \) \( \text{dinB}^- \) double mutant (Fig. 3).

Together, these results indicate that DinB and Pol\( \text{c} \) are responsible in generating the elevated mutation rate by accumulating point mutations when replication is perturbed in \( \text{pol}^+/\text{swi7-H4} \), with DinB contributing the most to the mutator phenotype.

**Up-regulation of DinB depends on checkpoint-activation in response to genomic perturbation**

The \( \text{rad17.K118E} \) mutation in \( \text{pol}^+/\text{swi7-H4} \) suppressed the mutator phenotype to a level similar to that of deletion of \( \text{dinB}^+ \) in \( \text{pol}^+/\text{swi7-H4} \) under replication stress (cf. Tables 1 and 2, Fig. 3). It is possible that the \( \text{rad17.K118E} \) mutation has a suppressive effect on the DinB function, resulting in a phenotype similar to that of \( \text{dinB}^+ \) deletion. These have prompted us to analyze how...
DinB is regulated in cells after genomic perturbation. The level of dinB+ transcripts from cells under replication stress and from cells treated with methyl methansulfonate (MMS) were compared to unperturbed wild-type cells by Northern analysis. Transcript levels of dinB+ were also compared with and without replication stress or DNA damage in the rad17.K118E mutant. The dinB+-transcript (∼1.7 kb) was up-regulated in response to replication stress and DNA damage. The rad17.K118E mutation did not affect the up-regulation of dinB+ transcript in response to either replication stress or DNA damage (Fig. 4A). Because the checkpoint response is somewhat intact and Cds1 can be activated by HU in the rad17.K118E mutant [Kai et al. 2001], the up-regulation of dinB+ transcript may be enforced by the residual checkpoint function in the rad17.K118E mutant. To test this possibility, we analyzed the dinB+ transcript expression in cells deleted for chk1+, cds1+, rad9+, rad17+, and rad3+ after MMS treatment (Fig. 4B). The dinB+ transcript was up-regulated in wild-type cells after MMS treatment and is moderately up-regulated in cells with cds1Δ. In contrast, MMS treatment failed to induce the expression of dinB+ transcript in cells with a deletion of chk1+, rad3+, rad9+, or rad17+ (Fig. 4B).

DinB protein levels from wild-type cells or pola(swi7-H4) mutant with or without the rad17.K118E mutation were analyzed with a chromosomal integrated myc-tagged dinB+. Similar to the induction of dinB+-transcript expression, DinB protein levels were up-regulated in response to replication perturbation induced by culturing pola(swi7-H4) at 30°C or by MMS treatment (Fig. 4C). To test whether up-regulation of DinB protein is dependent on checkpoint activation, we constructed cds1Δ, chk1Δ, rad9Δ, rad17Δ, and rad3Δ strains containing a chromosomal integrated dinB+-myc. The DinB protein level was up-regulated in wild-type cells and in cds1Δ cells after MMS treatment. However, in cells with a deletion of chk1+, rad9+, rad17+, or rad3+, the DinB protein level was not up-regulated after MMS treatment (Fig. 4D).

Together, these results indicate that both the transcript and the protein level of DinB are up-regulated in response to genomic perturbation and this up-regulation depends on checkpoint-activation.

Association of DinB with chromatin correlates to the Rad17 chromatin-binding ability

Although DinB protein is expressed in the rad17.K118E mutant in response to genomic perturbation (Fig. 4C),

Figure 4. Expression of DinB is dependent on checkpoint activation. [A] DinB transcript is up-regulated in response to damage and replication perturbation. Logarithmically growing wild-type and rad17.K118E cells were incubated in 0.025% MMS for 4 h. Replication perturbation was induced in pola(swi7-H4) and pola(swi7-H4) rad17.K118E cells by culturing for 4 h at 30°C. Forty micrograms of total RNA was prepared from each strain and analyzed by Northern blotting. [B] Up-regulation of DinB transcript is dependent on checkpoint activation. Wild-type and checkpoint mutants cells were MMS treated for 2 h as in A. Forty micrograms of total RNA was used for Northern analysis. His3+ was used as a loading control. [C] DinB protein level is up-regulated in response to damage and replication perturbation. Logarithmically growing wild-type:dinB-myc and rad17Δ:dinB-myc cells were MMS treated for 2 h as described in A. Replication in pola(swi7-H4):dinB-myc and pola(swi7-H4) rad17.K118E:dinB-myc cells was perturbed at 30°C as described in A. Cell extracts were prepared from the MMS-treated or replication-perturbed cells under denaturing conditions. DinB-myc was visualized by immunoblotting 40 µg of total cell extract protein with anti-myc antibodies. [D] Up-regulation of DinB protein is dependent on the checkpoint activation. Strains with dinB+-myc in checkpoint deletion mutant backgrounds were MMS treated for 2 h as above. Forty micrograms of cell extract protein from wild-type cells and mutant cells was analyzed by immunoblotting using the anti-myc antibody with α-tubulin as a loading control.
the rad17.K118E mutation in polα(swi7-H4) has a suppressive effect on the mutator phenotype (Table 1, Fig. 2). This suggests that the expressed DinB polymerase in the polα(swi7-H4) rad17.K118E double mutant was unable to perform mutagenic synthesis.

Our previous studies have shown that the rad17.K118E mutation compromised the ability of the Rad17 mutant protein to bind chromatin (Kai et al. 2001). This led us to speculate that the observed suppression of the polα mutator phenotype by the rad17.K118E mutant mutation is owing to failure of DinB to associate with chromatin. To test this possibility, we analyzed chromatin association of DinB in response to replication perturbation and DNA damage in the rad17.K118E mutant background (Fig. 5A). DinB-myc associated with chromatin after MMS treatment or replication perturbation induced by culturing polα(swi7-H4) at 30°C. However, DinB chromatin association was severely compromised in the rad17.K118E mutant background (Fig. 5A).

To test whether DinB chromatin binding requires a physical interaction with Rad17, a strain containing dinB'-myc and rad17'-HA at their respective genomic loci was constructed. DinB-myc failed to coimmunoprecipitate with the Rad17-HA protein after MMS treatment (Fig. 5C). Identical controls were performed as described above in DinB and Hus1 coprecipitation (Fig. 5C, lower panel). Because strain with an epitope tagged rad9' exhibited a mild checkpoint defect, coprecipitation of DinB and Rad9 was not performed.

These results suggest that in response to replication stress or DNA damage, the association of DinB with chromatin for mutagenic translesion synthesis is mediated by its interaction with the checkpoint-clamp loader containing a functional Rad17. Thus, a prerequisite of regulating DinB expression and chromatin association for mutagenic translesion synthesis is checkpoint activation.

**Discussion**

In this study, we used a DNA polymerase α mutant, polα(swi7-H4), to perturb DNA replication at the semi-permissive temperature (30°C). At the semi-permissive temperature, polα(swi7-H4) exhibited a delay of cell-cycle transition, activation of Cds1 kinase, and Cds1 autophosphorylation, indicating that the checkpoint is intact in the mutant (Fig. 1A,B). Despite having an intact checkpoint, polα(swi7-H4) exhibits an elevated mutation rate and accumulates both deletion mutations and point mutations in cells. The mutator phenotype of polα(swi7-H4), however, is significantly affected by mutations in cds1' and rad17'. Further analyses indicate that at 30°C, polα(swi7-H4) induces the expression of the translesional polymerase DinB in generating point mutations in a checkpoint activation dependent manner. Translesional polymerases are thought to function primarily in bypassing lesions on DNA template (Sutton et al. 2000, Goodman 2002). A replicative DNA polymerase is expected to generate misincorporations, single-stranded gaps on template, or mispaired primer termini, but not “lesions” on the template. Finding that a mutant of replicative polymerase induces the expression of DinB to generate point mutations is a novel mutation consequence found in replicative polymerase, in which an error-prone translesional polymerase is induced for mutagenic synthesis on a template without a lesion.

**How might mutations in rad17 affect point mutations in polα (swi7-H4)?**

Here, we demonstrate that in response to genomic stress, up-regulation of both the transcript and protein levels of DinB (Fig. 4) is dependent on rad3', rad9', and rad17' (Fig. 4). Because rad3', rad9', and rad17' are the checkpoint sensors/transducers required for activation of the checkpoint, these data indicate that checkpoint activation is a prerequisite for DinB expression in response to replication stress or DNA damage.

After replication perturbation or DNA damage, DinB is expressed in the rad17.K118E mutant (Fig. 4). How-
ever, the rad17.K118E mutation notably suppresses the point mutations in polα(sw7-H4) cells (Table 1; Fig. 2). The lysine\(^{118}\) [Lys\(^{118}\)] residue of Rad17 is localized in the Walker A domain of the Rad17 protein (Griffiths et al. 1995). Mutation of Lys\(^{118}\) to glutamate severely reduces the ability of the Rad17[118E] mutant protein to form a complex with Rfc2 and to associate with chromatin [Kai et al. 2001]. Furthermore, mutation at the equivalent ly-

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**Figure 5.** Chromatin association of DinB depends on the Rad17-chromatin-binding ability, and DinB interacts with checkpoint clamp components Hus1 and Rad1 in vivo. (A) Chromatin association of DinB in response to damage and replication perturbation depends on the Rad17-chromatin-binding ability. Logarithmically growing wild-type cells harboring dinB\(^{-}\)-myc and rad17/dinB\(^{-}\)-myc cells were treated with MMS as described in Figure 4. polα(sw7-H4);dinB\(^{-}\)-myc and polα(sw7-H4) rad17.K118EdinB\(^{-}\)-myc mutants were grown at 30°C to induce replication perturbation. Chromatin fractionation assay was performed as in Kai et al. (2001). Forty micrograms of total protein (total) and five volume equivalents of chromatin-bound protein (chr) were fractionated on SDS-PAGE, followed by immunoblotting. DinB-myc was visualized by using the anti-myc antibody. (B) DinB coprecipitates with Hus1. Crude cell lysates were prepared from 5 \times 10^8 wild-type cells harboring hus1\(^{-}\)-myc and dinB\(^{-}\)-HA at their endogenous chromosomal loci. Cells were treated with MMS as described in Figure 4, and polα(sw7-H4);dinB\(^{-}\)-HA:hus1\(^{-}\)-myc and polα(sw7-H4) rad17.K118EdinB\(^{-}\)-HA:hus1\(^{-}\)-myc cells were incubated for 4 h at 25°C or 30°C. (Bottom) Control cell lysates prepared from wild-type cells harboring an untagged dinB\(^{-}\) and hus1\(^{-}\)-myc or from cells with dinB\(^{-}\)-HA and hus1\(^{-}\)-myc treated with MMS were used as control. Cell lysates (300 µL) containing 1 mg protein were preincubated with 100 U of DNaseI and used for immunoprecipitation [IP] with the anti-HA antibody. The immunoprecipitates were immunoblotted with the anti-HA (left) or anti-myc monoclonal antibody (right). (C) Cocrplication of DinB with Rad1 requires a functional Rad17. Crude cell lysates were prepared from 5 \times 10^8 wild-type cells (rad17\(^{+}\)) and rad17.K118E mutant cells harboring dinB\(^{-}\)-myc and rad17\(^{-}\)-HA at their endogenous chromosomal loci. Cells were treated with MMS as above, and the immunoprecipitation was performed as in B. The immunoprecipitates were immunoblotted with the anti-myc (left) or anti-HA monoclonal antibody (right). (Bottom) Control immunoprecipitation from lysates of MMS-treated wild-type cells harboring untagged dinB\(^{-}\) and rad17\(^{-}\)-HA or rad17\(^{-}\)-HA and dinB\(^{-}\)-myc.
The physical interactions between DinB with Hus1 and Rad1 suggest that the checkpoint-clamp might recruit and secure the DinB protein on chromatin for effective mutagenic translesion synthesis. Alternatively, the chromatin-bound checkpoint-clamp could somehow help DinB to replace the replicative polymerase or to stimulate the DNA synthetic activity of DinB. In vitro, proliferating cell nuclear antigen (PCNA), together with replication factor C (RFC) and replication protein A (RPA), stimulates the DNA synthesis activity of human Polk[DinB] (Haracska et al. 2002). Given the predicted structural similarity between PCNA and the checkpoint-clamp, the physical interaction between DinB and the Rad1–Rad9–Hus1 checkpoint-clamp may be similar to that of the DinB–PCNA interaction in vitro, stimulating the DinB polymerase activity, or securely positioning DinB in the repair complex for mutagenic translesion synthesis.

Among the three fission yeast translesion synthesis polymerases analyzed in this study, DinB contributes most to the elevated mutation rate causing point mutations in cells, whereas Polζ contributes to a lesser extent when replication is perturbed (Table 2; Fig. 3). In vitro studies of human Polk have shown that Polk is a promiscuous extender of mispaired primer termini, and that both Polk and Polζ can extend mispaired primer ends efficiently on undamaged DNA. These in vitro findings have led to a proposed model: Polk or Polζ can readily incorporate a nucleotide to a mispaired primer terminus, and once the nucleotide is inserted, Polk and Polζ will dissociate from the primer terminus to allow Polδ to restart DNA synthesis (Washington et al. 2002). Given the low processive nature of polymerase α, the semidisabled polymerase α in polα[swi7-H4] could be expected to cause a stalled replication fork at the semipermissive temperature, frequently dissociating from the primer-template, and increasing the chances of forming mispaired primer-ends. Extension of the mispaired ends by DinB and/or Polζ would result in an elevated mutation rate by accumulating primarily point mutations in cells.

**How might mutations in cds1+ and rad17+ affect the deletion mutations in polα[swi7-H4]?**

Cds1 and its budding yeast homolog Rad53 are thought to be essential in preventing accumulation of aberrant replication intermediates and in promoting replication...
Checkpoint activation induces mutagenic synthesis

In response to genomic perturbations, the checkpoint is required to signal cell-cycle arrest to maintain genomic stability. Ironically, results of this study show that checkpoint activation can also induce genomic instability by activating the translesional polymerase for mutagenic translesion synthesis.

The mutagenic nature of translesional DNA polymerases operating on undamaged DNA is a troublesome source of genomic mutation. Knowledge of how these translesional polymerases are regulated is important for our understanding and, ultimately, prevention of genomic instability. Here, we have demonstrated that expression of the translesional polymerase DinB is regulated by checkpoint activation. Our results suggest that for DinB to associate with chromatin to perform mutagenic translesion synthesis in order to allow a stalled replication fork to restart, this process requires the ability of Rad17 to associate with chromatin. For DinB to be securely positioned on the chromatin, to enhance DinB polymerase activity, or to replace the replicative polymerase at the replication fork, an interaction between DinB and the checkpoint-clamp is required. Together, these findings suggest that mutagenic translesion synthesis by DinB and perhaps also some other translesional polymerases is a part of the checkpoint response. A model of how checkpoints respond to replication perturbations to arrest the cell cycle in order to maintain genomic stability and to activate translesional polymerases for mutagenic translesion synthesis is proposed (Fig. 6).

Materials and methods

General methods

All yeast media, YESS, Edinburgh minimal medium, and 5-fluoro orotic acid (5-FOA) medium were prepared as described in Moreno et al. [1991] and Liu et al. [1999]. Except where otherwise stated, all yeast strains were propagated at 23°C. All fission yeast genetic operations were performed as described in Gutz et al. [1974]. For the disruption of pol1- and the rad30- domain of eso1- , a one-step gene disruption method was used, followed by PCR-mediated generation of the kan gene flanked by 80-bp segments from the 5’ and 3’ regions of the gene to be disrupted [Bahler et al. 1998]. The DinB- deletion strain was kindly provided by Dr. A.M. Carr [Genome Damage and Stability Centre, University of Sussex, UK]. Epitope tagging of genes with either the myc- or HA-tag at their chromosomal loci was performed as described [Bahler et al. 1998]. The DinB- deletion strain was kindly provided by Dr. A.M. Carr [Genome Damage and Stability Centre, University of Sussex, UK]. Epitope tagging of genes with either the myc- or HA-tag at their chromosomal loci was performed as described [Bahler et al. 1998]. The DinB- deletion strain was kindly provided by Dr. A.M. Carr [Genome Damage and Stability Centre, University of Sussex, UK]. Epitope tagging of genes with either the myc- or HA-tag at their chromosomal loci was performed as described [Bahler et al. 1998].

Mutation rate analysis

Mutation rates on ura4- were calculated by fluctuation analysis by using the method of the median as previously described [Lea and Coulson 1940]. The reported mutation rates are the average of values obtained from at least three independent experiments using sets of 11 independent cultures. Significance levels between strains were determined by using the Mann-Whitney rank test with Analyse-it, a plug-in statistical package for Mi-
crosoft Excel. Unless specified otherwise, all comparisons had a “significant” p level of p < 0.005.

Mutation spectra analysis
Genomic DNA was isolated from 5-FOA+ colonies. The mutant ura4 gene was amplified, analyzed by 1.5% agarose gel electrophoresis, and classified as having either deletions or point mutations. The ura4 PCR products were also gel purified and sequenced to determine the mutations generated. Relevant genomic fragments were amplified by PCR. At least 100 5-FOA+ colonies from each strain were analyzed for the size of ura4+ PCR product by agarose gel electrophoresis, and 20 ura4+ PCR products were sequenced. The PCR reactions included the ura4+ primers (P0, AAGCTTAGCTACAAATCCCAC; and P8, AACGCCTAGGAAAACAAACGC). In addition to primer P0 and P8, the PCR products were sequenced using the following primers: P1, TTTCTTACCGTATTGTCCTAC; and P2, GGACCCTATCTCTGTGTTATC.

Immunoprecipitation and immunoblotting
Cell lysates were prepared as in Kai et al. (2001) with the addition of 300 mM NaCl and 1% TX-100 to the lysis buffer. Before immunoprecipitation, 100 U of DNaseI was incubated with each reaction, and immunoprecipitation was performed as described in Kai et al. [2001].

Cds1 kinase assay and chromatin fractionation assay
Cds1 kinase assays and chromatin fractionation of myc-tagged DinB was performed as in Kai et al. [2001]. Cds1 auto-phosphorylation was analyzed as in Tanaka et al. [2001].

Flow cytometry analysis
Cells were harvested, washed in water, and fixed in 70% ethanol. DNA content was measured by using a FACScan system, CellFIT cell cycle analysis, and LYSISII software (Becton Dickinson).

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