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# Reducing DNA Polymerase α in the Absence of Drosophila ATR Leads to P53-Dependent Apoptosis and Developmental Defects

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ABSTRACT

The ability to respond to DNA damage and incomplete replication ensures proper duplication and stability of the genome. Two checkpoint kinases, ATM and ATR, are required for DNA damage and replication checkpoint responses. In Drosophila, the ATR ortholog (MEI-41) is essential for preventing entry into mitosis in the presence of DNA damage. In the absence of MEI-41, heterozygosity for the *E(mus304)* mutation causes rough eyes. We found that *E(mus304)* is a mutation in *DNApol-α180*, which encodes the catalytic subunit of DNA polymerase α. We did not find any defects resulting from reducing Polα by itself. However, reducing Polα in the absence of MEI-41 resulted in elevated P53-dependent apoptosis, rough eyes, and increased genomic instability. Reducing Polα in mutants that lack downstream components of the DNA damage checkpoint (DmChk1 and DmChk2) results in the same defects. Furthermore, reducing levels of mitotic cyclins rescues both phenotypes. We suggest that reducing Polα slows replication, imposing an essential requirement for the MEI-41-dependent checkpoint for maintenance of genome stability, cell survival, and proper development. This work demonstrates a critical contribution of the checkpoint function of MEI-41 in responding to endogenous damage.

EUKARYOTIC cells constantly experience exogenous DNA damage from the environment as well as endogenous damage that occurs during DNA metabolism and replication. An inability to respond to either type of damage can result in genomic instability and loss of genetic material. To maintain genomic stability, cells have developed mechanisms for responding to DNA damage and/or incomplete replication. Maintenance of genome stability can be accomplished by coupling replication and repair with cell cycle regulation via the DNA damage checkpoint pathway. In this pathway, sensors recognize incomplete replication and/or DNA damage and then stimulate a variety of responses, including phosphorylation of downstream transducers. These transducers then activate or inactivate effectors that directly affect cell cycle progression, resulting in cell cycle arrest, presumably to allow time to complete replication or repair the damage (reviewed in Sancar *et al.* 2004).

ATM (for *a*taxia *t*elangiectasia *m*utated) and ATR (for *AT*M and *R*ad3 related) are two kinases that mediate the DNA damage checkpoint in response to incomplete replication and DNA damage. These kinases are highly conserved and required for $G_1$-S, intra-S, and $G_2$-M checkpoint responses (reviewed in Sancar *et al.* 2004; reviewed in Shiloh 2003). ATM and ATR function upstream of conserved transducers of the checkpoint response, Chk1 and Chk2. In mammals, ATM primarily phosphorylates Chk2 in response to damage that results in double-strand breaks (DSBs) (Canman *et al.* 1998). In contrast, ATR primarily activates Chk1 in response to incomplete replication and/or damage that results in single-strand DNA (Cliby *et al.* 1998; Wright *et al.* 1998; Unsal-Kacmaz *et al.* 2002; Das and Dashnamoorthy 2004). Although there is some functional overlap of these kinases and the transducers of the checkpoint response, the ATR/Chk1 pathway is primarily responsible for the intra-S checkpoint (Boddy *et al.* 1998; Chen and Sanchez 2004; Helt *et al.* 2005; reviewed in Sanchez *et al.* 1996; Sancar *et al.* 2004).

Many studies have characterized DNA damage response pathways using exogenous sources of damage, such as hydroxeurea, UV, ionizing radiation (IR), and alkylating agents. However, it is presumed that the most common type of damage that a cell must respond to is endogenous, such as lesions that occur during replication and regular DNA metabolism (Lindahl 1993; Bishop *et al.* 2000; Frosina 2000). Evidence from other organisms indicates that orthologs of ATR have important roles in responding to endogenous damage. Cells from ATR-Seckel syndrome patients with a mutated form of ATR demonstrate elevated genome damage and chromosome breaks following replication stress (O'Driscoll *et al.* 2004) and ATR-deficient mouse cells also accumulate

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spontaneous chromosomal breaks (Brown and Baltimore 2003). Similarly, *Saccharomyces cerevisiae* mutants lacking the ATR ortholog Mec1 have elevated rates of gross chromosomal rearrangements (Cobb *et al.* 2005) as well as spontaneous DNA breaks that map to replication slow zones (Cha and Kleckner 2002). These results demonstrate the need to further understand how ATR responds to endogenous damage that occurs during DNA synthesis.

The role of ATR in response to endogenous damage has been investigated in multiple organisms by examining interactions between checkpoint proteins and components of the replication machinery, especially DNA polymerase α (Polα) (reviewed in Foiani *et al.* 1997). Initiation of replicative DNA synthesis begins with formation of an RNA primer by primase. Polα forms a complex with primase and is responsible for synthesizing the initial DNA extension from the primer. Thus, Polα is required to initiate both leading-strand and lagging-strand synthesis; however, Polα is required continuously for lagging-strand synthesis, since every Okazaki fragment initiates with an RNA primer. In *S. cerevisiae*, Polα is stabilized in a Mec1-dependent manner after treatment with the replication inhibitor hydroxyurea (HU) (Cobb *et al.* 2003), and decreasing expression of the catalytic subunit of Polα by 90% in a *mec1* mutant results in increased genomic instability (Lemoine *et al.* 2005). In *Schizosaccaromyces pombe*, temperature-sensitive mutants of *pol*α cause activation of Chk1 (D'Urso *et al.* 1995; Bhaumik and Wang 1998). In *Xenopus laevis*, uncoupling of helicase and polymerase activity during replication results in Polα-dependent activation of Chk1 (Byun *et al.* 2005; Cortez 2005). These results reveal a conserved genetic interaction between DNA Polα and the ATR-mediated damage response.

Drosophila ATR, encoded by *mei-41*, is the primary kinase required for the checkpoint response after DNA damage during all phases of the cell cycle (Hari *et al.* 1995; Sibon *et al.* 1999; Brodsky *et al.* 2000; Garner *et al.* 2001; Jaklevic and Su 2004; Bi *et al.* 2005; LaRocque *et al.* 2007). *mei-41* mutants are sensitive to a wide range of agents that damage DNA or inhibit DNA replication, including ultraviolet light, methyl methanesulfonate, IR, and HU (Boyd *et al.* 1976; Sibon *et al.* 1999). Sensitivity to this broad spectrum of damaging agents suggests that MEI-41-mediated checkpoints are essential in the response to many types of DNA damage throughout the cell cycle. As in mice, humans, and *S. cerevisiae*, *mei-41* mutants have an elevated frequency of spontaneous chromosome breaks (Gatti 1979; Baker *et al.* 1980; Banga *et al.* 1986).

To learn more about the role of the ATR-mediated cell cycle checkpoint in responding to replication defects, we genetically reduced Polα in *mei-41* mutants. This resulted in P53-dependent apoptosis, increased genomic instability, and P53-dependent morphological defects. Our data also suggest that cell cycle regulation by MEI-41 is the major component of this interaction, although loss of the Chk1- and Chk2-dependent checkpoint cannot completely account for the defects.

## MATERIALS AND METHODS

**Drosophila stocks and genetics:** Flies were maintained on standard medium at 25°. The *mei-41* mutant males were hemizygotes of $mei\text{-}41^{29D}$ (Laurencon *et al.* 2003). The cyclin mutations used were $CycA^{C8LR1}$ (Sigrist and Lehner 1997) and $CycB^{2}$ (Jacobs *et al.* 1998). The *lok* mutants were homozygous for $lok^{30}$ and the *grp* mutants were heteroallelic for $grp^{209}$ and $grp^{Z5170}$ (LaRocque *et al.* 2007). The *p53* mutants used were $p53^{5A\text{-}1\text{-}4}$ (Rong *et al.* 2002). Reductions in *Pol*α used the *E(mus304)* mutant chromosome (Brodsky *et al.* 2000). Recombinants of *E(mus304)* and $p53^{5A\text{-}1\text{-}4}$ were generated and verified using allele-specific PCR for both mutations and for presence of a rough-eye phenotype in *mei-41* mutants.

**Mapping mutations in *DNApol-α180*:** Recombination mapping between *ebony* (*e*) and *claret* was used to confirm the published location of *E(mus304)* (Brodsky *et al.* 2000) using the rough eyes in *mei-41* mutants as the phenotypic marker. Deficiencies of the area surrounding and including 89D-F were used to narrow the location of the region down to five genes: *E2f*, *CG31176*, *CG6353*, *CG15497*, and *DNApol-α180*. Two genes, *E2f* and *CG31176*, were excluded from consideration when *mei-41* mutants failed to have a rough-eye phenotype when heterozygous for these mutations. The *E(mus304)* chromosome was sequenced for changes in *pol*α. Using GFP selection, genomic DNA was prepared from single embryos homozygous for *E(mus304)* and PCR was performed using gene-specific primers. PCR reactions contained 10 mm Tris–HCl, pH 9.0, 50 mm KCl, 2.5 mm $MgCl_2$, 0.1% Triton X-100, 1.25 μm of each primer, 250 μm each dNTP, 2 μl of the genomic DNA prep, and *Taq* DNA polymerase in a 20-μl volume. PCR products were isolated using gel electrophoresis, purified, and sequenced directly. The mutation was confirmed by sequencing the opposite strand. Mutations found from the ethyl methanesulfonate (EMS) screen were confirmed this way as well.

**EMS mutagenesis:** One- to 3-day-old males were fed 25 mm EMS (Fluka Chemika) in 1% sucrose on cotton pads overnight. Males were then transferred to clean bottles for 1 day and then crossed to *mei-41/FM7* females in bottles. To avoid screening progeny resulting from mutagenesis of premeiotic germline cells, males were discarded after 5 days. $F_1$ male progeny mutant for *mei-41* were screened for rough eyes, indicative of a possible dominant autosomal mutation that interacts with the *mei-41* mutation. Mutations that mapped to chromosome *3* were crossed to *E(mus304)*, and those that failed to complement the homozygous lethality phenotype of *E(mus304)* were sequenced to find mutations in *DNApol-α180*.

**SEM imaging:** Adult fly heads were fixed in phosphate-buffered saline (PBS) and 4% paraformaldehyde. Samples were stored at 4° for several days before being dehydrated through a series of washes in increasing ethanol concentration, with a final rinse in 100% ethanol, and then prepped with assistance from the Microscopy Services Laboratory at the University of North Carolina-Chapel Hill. Samples were transferred in absolute ethanol to a Balzers CGD 020 critical point dryer (BAL-TEC, Balzers, Principality of Liechtenstein) and dried using liquid $CO_2$ as the solvent solution. Heads were mounted and sputter coated with gold:palladium alloy (60:40) using a Hummer X Sputter Coater (Anatech, Alexandria, VA). Specimens were viewed on a Cambridge Stereoscan S200 scanning electron microscope (LEO Electron Microscopy, Thornwook,

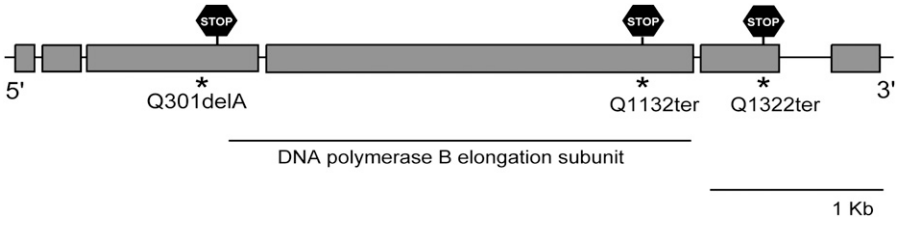


FIGURE 1.—*Enhancer of mus304* is an allele of *DNApol-α180*. *E(mus304)* was roughly mapped and predicted to be a mutation in *DNApol-α180* (see MATERIALS AND METHODS). Sequencing of this region confirmed a loss of an "A" in the third exon at codon 301, resulting in a frameshift and a premature stop 29 codons downstream. An EMS mutagenesis and screen for mutations conferring rough eyes to *mei-41* mutants resulted in two new alleles (see MATERIALS AND METHODS); both were nonsense mutations in glutamine codons. Shaded boxes are coding exons. Mutations are marked with asterisks.

NY) using an acceleration voltage of 20 kV and a working distance of 25 mm.

**Detecting apoptotic cells:** Imaginal discs were dissected from third instar larvae of appropriate genotypes in Ringer's solution and fixed for 45 min in 4% formaldehyde and PBS with 0.1% Triton-X (PBT). Discs were washed and blocked in PBT with 5% bovine serum albumin. Discs were incubated with 1:500 dilution of rabbit anti-human cleaved caspase-3 (Asp175) antibody (Cell Signaling Technology) in PBT overnight at 4°. Discs were incubated for 2 hr at room temperature with 1:1000 secondary goat anti-rabbit rhodamine-conjugated antibody (Molecular Probes, Eugene, OR) or secondary goat anti-rabbit fluorescein-conjugated antibody (Molecular Probes), stained with 10 μg/ml DAPI in PBT, and mounted with Flouromount-G (Southern Biotechnology Associates). Discs were visualized using TRIT-C and FIT-C filters of a Nikon Eclipse E800 fluorescent microscope. Quantification was performed on images of 7–14 wing discs of each genotype. Each disc was counted for the total number of caspase-positive cells per disc to obtain an average. Significance was computed using an unpaired *t*-test with Welch's correction using InStat statistical software.

**Genomic instability phenotypes:** Loss of heterozygosity (LOH) at *multiple wing hair* (*mwh*) was detected as described by BRODSKY *et al.* (2000). Briefly, wings of appropriate genotype were dehydrated in isopropanol and mounted in 1:1 methylsalicilate:Canada balsam (Sigma, St. Louis). Each wing was viewed at ×40 using the light filter of a Nikon Eclipse E800 fluorescent microscope and scored for *mwh* phenotype. A total of 10–20 wings were examined for each genotype to obtain an average rate of mitotic clones per wing. Standard deviations were determined on the basis of averages; significance was computed using an unpaired *t*-test with Welch's correction using InStat statistical software.

To detect increases in mitotic crossovers, unbalanced single males of appropriate genotypes heterozygous for *ebony* and *scarlet* (*st*) were crossed to *ru h th st cu sr e Pr ca/TM6B* females. Crossovers between *st* and *e* in the premeiotic male germline were scored in progeny of this cross. Over 3000 progeny were scored for each genotype. Significance was determined by analyzing a contingency table using chi-square approximation with Yates correction available through InStat statistical program.

## RESULTS

***Enhancer of mus304* is an allele of *DNApol-α180*:** A previously published study reported a spontaneous mutation that interacts genetically with *mei-41* and *mus304*, which encodes the ortholog of ATR-IP (BRODSKY *et al.* 2000). This mutation, referred to as *Enhancer of mus304*, is homozygous embryonic lethal (data not shown). However, heterozygosity for this mutation in *mei-41* or *mus304* mutants results in a rough-eye phenotype. The *Enhancer of mus304* mutation was mapped to region 93F on the third chromosome (BRODSKY *et al.* 2000). We further mapped *Enhancer of mus304* (see MATERIALS AND METHODS) to a region that includes *DNApol-α180*, which encodes the catalytic subunit of Polα. We sequenced the *DNApol-α180* coding region from the mutant chromosome and found a deletion of a single base pair in the third exon at codon 301 (Figure 1). This deletion results in a frameshift and a premature stop 29 codons downstream.

To confirm that the interaction with *mei-41* is due to a mutation in *DNApol-α180*, we conducted a mutagenesis screen to identify mutations that caused rough eyes when heterozygous in a *mei-41* mutant (see MATERIALS AND METHODS). Two new alleles of *DNApol-α180* were recovered (Figure 1). Both are nonsense mutations at glutamine codons (1132 and 1322). Heterozygosity for any of these alleles, or for a deletion of this region, confers a rough-eye phenotype to *mei-41* mutants. We conclude that reducing the dosage of *DNApol-α180* (hereafter referred to as *polα*) by half is sufficient to cause a developmental defect in *mei-41* mutants.

**Reducing Polα in *mei-41* mutants causes an increase in cell death:** The Drosophila compound eye comprises ~800 ommatidia, each of which has a precise number of cells in an identical arrangement, resulting in a smooth appearance. The correct number of cells results from a carefully orchestrated sequence in which some cells differentiate and others undergo cell death (reviewed in BONINI and FORTINI 1999). Because of this, eye development is highly sensitive to changes in cell survival, unlike other adult organs, such as the wing, whose cell number is largely dispensable for development (BAKER 2001). For example, overexpression of P53, which is required for DNA-damage-induced apoptosis, disrupts formation of an ordered array of ommatidia, resulting in eyes with a rough appearance (OLLMANN *et al.* 2000; LEE *et al.* 2003). Other mutations have also revealed a correlation between increased apoptosis and rough

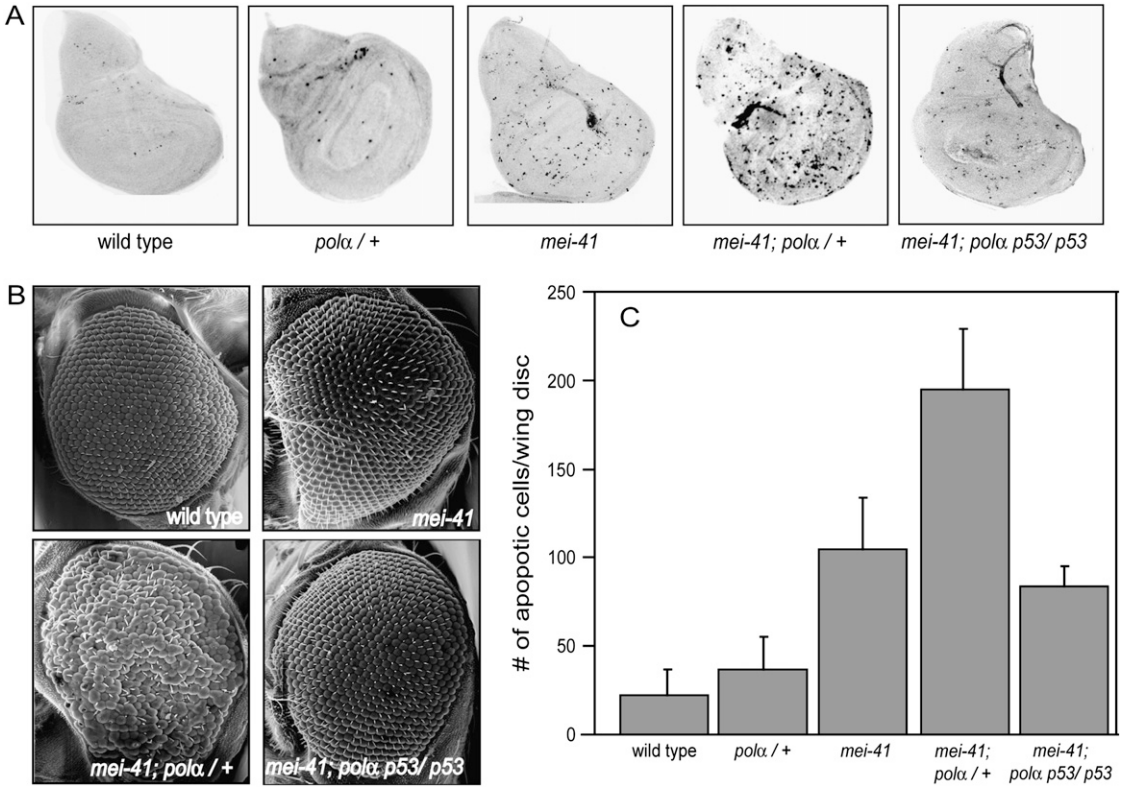


FIGURE 2.—Reducing Polα in *mei-41* mutants results in a variety of phenotypes. (A) Wing discs of third instar larvae were dissected, fixed, and stained with an antibody to cleaved human caspase 3, marking apoptotic cells. (B) As shown previously (BRODSKY *et al.* 2000), *mei-41; polα/+* mutants have a rough-eye phenotype that includes fused ommatidia and tissue loss. *mei-41* mutants are indistinguishable from wild type and are used for comparison. This rough-eye phenotype of *mei-41; polα/+* mutants was rescued by eliminating P53. (C) Quantification of apoptosis phenotype demonstrated in A. *mei-41* mutants had an increase in apoptosis compared to wild type ($P < 10^{-6}$), and this was quantitatively more severe when Polα was reduced ($P < 10^{-4}$ when compared to *mei-41*). Mutations in *p53* restored apoptosis to the levels seen in *mei-41* single mutants ($P = 0.19$ compared to *mei-41*).

eyes. Temperature-sensitive mutations in the *tefu* gene, which encodes Drosophila ATM, cause both rough eyes and increased apoptosis in imaginal discs (SILVA *et al.* 2004).

To determine whether *mei-41* mutants that are heterozygous for a *polα* mutation have increased apoptosis in proliferating imaginal disc cells, we quantified the number of apoptotic cells per imaginal wing disc, using an antibody raised against human-activated caspase-3, a conserved effector caspase that is cleaved and subsequently activated during apoptosis (reviewed in VAN LANCKER 2006). The human cleaved caspase-3 antibody also recognizes Drosophila cells undergoing DNA-damage-induced apoptosis (GIRALDEZ and COHEN 2003). The average number of apoptotic cells was increased fourfold in *mei-41* mutants compared to wild-type larvae ($P < 10^{-5}$; Figure 2C). A similar increase was also seen in *mus304* mutants ($P < 10^{-5}$; data not shown). Heterozygosity for a *polα* mutation did not increase apoptosis by itself ($P = 0.08$), but led to a further increase in *mei-41* mutants ($P < 10^{-5}$ for *mei-41; polα/+* compared to *mei-41* alone; Figure 2, A and C). Similar results were seen in other imaginal discs and when staining with the vital dye acridine orange (data not shown). These observations show that reducing Polα in *mei-41* or *mus304* mutants causes increased apoptosis in proliferating tissues. Most imaginal tissues can compensate for increased cell death through increased proliferation (HAYNIE and BRYANT 1977; JAKLEVIC and SU 2004), so development of most adult appendages appears to be unaffected. Patterning of the compound eye, however, is exquisitely sensitive to changes in cell survival; as a result, the rough-eye phenotype is a sensitive indicator of increased cell death.

**The increased apoptosis and rough-eye phenotypes of *mei-41; polα/+* mutants are P53 dependent:** As noted earlier, previous studies have suggested a correlation between P53-dependent apoptosis and eye development. Overexpression of P53 causes a rough-eye phenotype (OLLMANN *et al.* 2000; LEE *et al.* 2003), similar to the phenotype that we observe when Polα is reduced in *mei-41* mutants (BRODSKY *et al.* 2000; Figure 2A). We hypothesized that reducing Polα in *mei-41* mutants elicits a P53-dependent apoptotic response, leading to a rough-eye phenotype. To test this hypothesis, we eliminated P53 expression in these mutants. Loss of P53 in *mei-41*; *polα/+* mutants completely rescued the

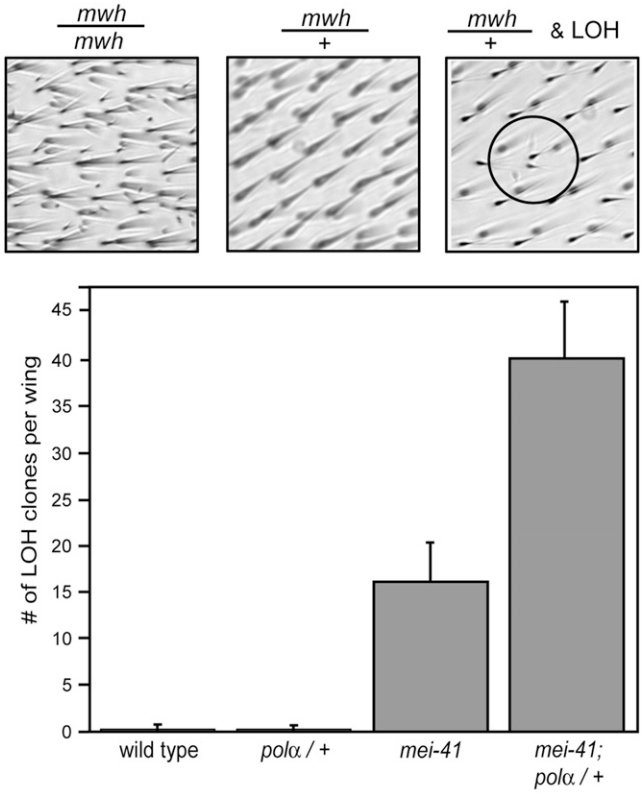


FIGURE 3.—Reducing Polα in *mei-41* mutants results in an increase of LOH. *mwh* mutant flies have multiple hairs from each hair cell of the adult wing, and *mwh*/+ flies are phenotypically normal. LOH at *mwh* will result in clones of cells with multiple hairs per cell (circled). LOH can occur through spontaneous mutation, gene conversion, deletion, or mitotic crossing over. Individual adult wings were scored for *mwh* clones. Bars represent the average number of clones per wing, and lines are the standard deviation based on 10–12 wings/genotype. Significance was determined by an unpaired *t*-test with Welch's correction.

rough-eye phenotype (Figure 2B) and restored the level of apoptosis to that seen in *mei-41* single mutants (Figure 2, A and C). Together, these data indicate that reducing Polα results in damage that elicits a MEI-41-dependent DNA damage response. In the absence of MEI-41, proliferating cells with reduced Polα undergo P53-dependent apoptosis, resulting in cell death and misregulated development of the adult eye.

***mei-41*; *polα*/+ mutants have increased genomic instability:** An inability to respond to spontaneous damage leads to increased genomic instability in *mei-41* and *mus304* mutants (BAKER *et al.* 1978; GATTI 1979; BRODSKY *et al.* 2000). One manifestation of genomic instability is increased LOH; both *mei-41* and *mus304* mutants have increased LOH at the *mwh* locus (BAKER *et al.* 1978; BRODSKY *et al.* 2000). We tested whether decreasing Polα in *mei-41* mutants results in a further increase in LOH frequency. We found an increase in LOH in *mei-41* mutants relative to wild type ($P < 10^{-5}$; Figure 3), as shown previously. There was no increase in

**TABLE 1**

**Mitotic crossovers between *ebony* and *scarlet***

| Genotype | *n* | % crossovers |
|---|---|---|
| Wild type | 3594 | 0 |
| *polα*/+ | 3721 | 0 |
| *mei-41* | 3825 | 0 |
| *mei-41; polα*/+ | 3073 | 0.16 (5)[a,*] |

* $P < 0.05$ compared to each other genotype.
[a] Number in parentheses is the number of progeny with crossover.

*polα*/+ mutants relative to wild type ($P = 0.83$), but heterozygosity for *polα* resulted in an increase in LOH in *mei-41* mutants ($P < 10^{-5}$, relative to *mei-41* single mutants).

LOH can result from many mechanisms, including chromosome loss, deletion, spontaneous mutation, and mitotic crossing over (reviewed in PÂQUES and HABER 1999). We quantified the frequency of mitotic crossovers between two markers on the third chromosome, *e* and *st*. Mitotic crossovers that occur in premeiotic germline cells are scored in progeny of males. As seen in wild-type, *mei-41* and *polα*/+ mutants completely lacked mitotic crossovers between these markers. In contrast, when Polα was reduced in *mei-41* mutants, there was a significant increase in the frequency of mitotic crossovers ($P < 0.05$; Table 1). This suggests that a subset, if not all, of the increased LOH observed at the *mwh* locus can be attributed to an increase in mitotic crossovers.

**Phenotypes manifested in *mei-41*; *polα*/+ mutants can be rescued by reducing mitotic cyclins:** We hypothesize that reducing Polα levels elicits a DNA damage response, due either to slowed and/or incomplete replication or to uncoupling of leading- and lagging-strand synthesis. We propose that this collective replication stress requires a MEI-41-dependent checkpoint response to regulate cell cycle progression, perhaps by giving enough time to complete replication before entry into mitosis. To test this hypothesis, we sought to bypass the requirement for MEI-41 by delaying entry into mitosis through other means. Reducing the maternal contribution of the mitotic cyclins, cyclin A and cyclin B, slows early embryonic cell cycle progression (EDGAR *et al.* 1994). Reducing cyclin A and cyclin B also bypasses the requirement for MEI-41 in regulating the midblastula transition during early embryonic development (SIBON *et al.* 1999) and rescues the sensitivity of mei-*41* mutants to *P*-element excision (LAROCQUE *et al.* 2007).

We attempted to rescue the rough-eye phenotype in *mei-41; polα*/+ mutants by reducing cyclin A and/or cyclin B. Cyclin B reduction partially rescued this phenotype, and reducing cyclin A (or both cyclin A and cyclin B) completely rescued the rough-eye phenotype, resulting in eyes that were indistinguishable from those of *mei-41* mutants or wild-type flies (Figure 4A). We then

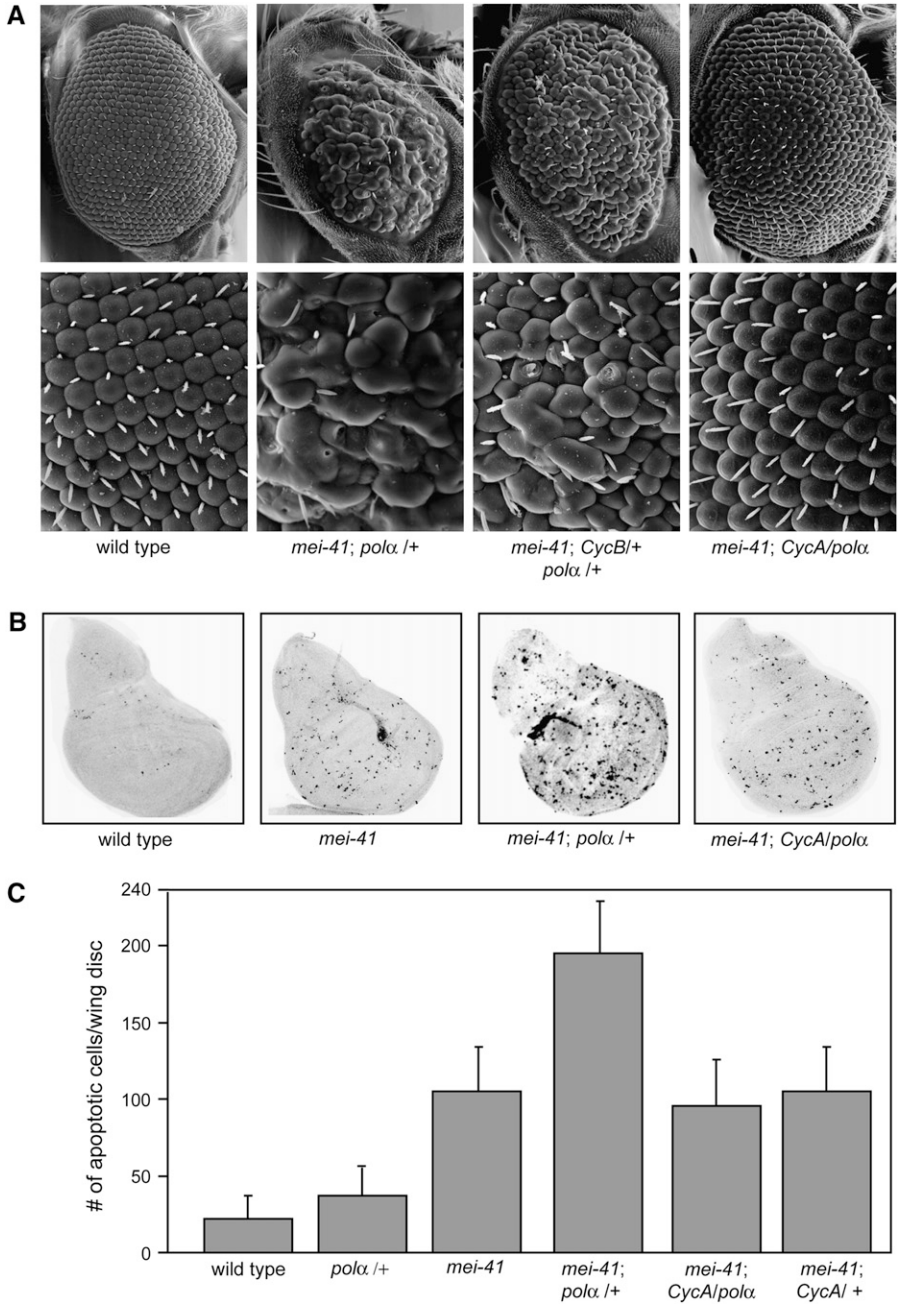


FIGURE 4.—Reducing mitotic cyclins rescues the rough-eye phenotype and apoptosis of *mei-41; polα/+* mutants. (A) The rough-eye phenotype of *mei-41; polα/+* mutants is partially rescued by reducing CycB and completely rescued when CycA is reduced. (B and C) The apoptosis phenotype observed in *mei-41; polα/+* mutants is rescued by reducing CycA. Samples were prepared and scored as described in Figure 2. Bars represent averages of 7–10 imaginal wing discs/genotype, and lines represent standard deviations. Significance was determined by an unpaired *t*-test with Welch's correction.

asked if we could rescue the increased-apoptosis phenotype by reducing mitotic cyclins. Similar to the rescue of the rough-eye phenotype, reducing cyclin A in these mutants rescued levels of apoptosis that were indistinguishable from *mei-41* single mutants ($P = 0.14$) or *mei-41; CycA/+* mutants ($P = 0.54$; Figure 4C). These data demonstrate that mitotic cyclin reduction is capable of suppressing both apoptosis and rough eyes, supporting the idea that reducing Polα elicits a damage response that requires the checkpoint function of MEI-41 to regulate cell cycle progression.

**Loss of the GRP/LOK-mediated checkpoint accounts for a degree of the phenotypes observed in *mei-41; polα/+* mutants:** Rescue of rough eyes and apoptosis by mitotic cyclin reduction suggests that cell cycle regulation contributes to the phenotypes that we have reported here. To further test this hypothesis, we examined the effects of Polα reduction on loss of Chk1 and Chk2, which have partially redundant roles in mediating the DNA damage checkpoint response in mammals (BODDY *et al.* 1998; CHEN and SANCHEZ 2004; HELT *et al.* 2005; reviewed in SANCHEZ *et al.* 1996; SANCAR *et al.* 2004). The Drosophila orthologs of Chk1 and Chk2 are encoded by *grp* and *lok*, respectively. Like *mei-41* mutants, *grp lok* mutants are completely defective in the replication and damage checkpoints (SU *et al.* 1999; YU *et al.* 2000; MASROUHA *et al.* 2003; BRODSKY *et al.* 2004; JAKLEVIC and SU 2004; DE VRIES *et al.* 2005; ROYOU *et al.* 2005; LAROCQUE *et al.* 2007).

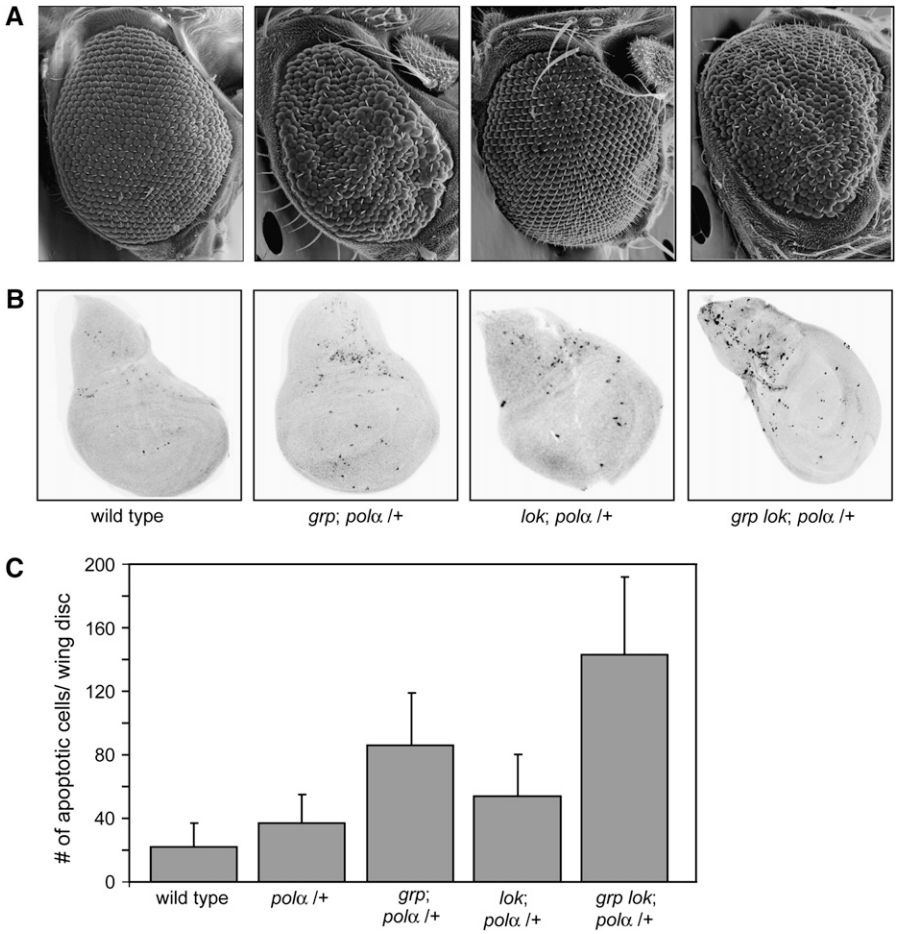


FIGURE 5.—Analysis of *grp* and *lok* mutants when Polα is reduced. (A) While eyes of *lok; polα/+* mutants are indistinguishable from those of wild-type flies, *grp* single mutants and *grp lok* double mutants have rough eyes when Polα is reduced. (B and C) *lok; polα/+* have only a slight increase in apoptosis compared to wild type ($P < 0.05$) whereas *grp; polα/+* and *grp lok; polα/+* mutants have a greater increase in apoptosis ($P < 10^{-4}$) but are not significantly different from each other ($P = 0.16$). Samples were prepared as described in Figure 2. Bars represent averages of 7–14 imaginal wing discs/genotype, and lines represent standard deviations. Significance was determined by an unpaired *t*-test with Welch's correction.

We first examined *grp* and *lok* mutants for the rough-eye phenotype conferred by heterozygosity for a *polα* mutation. The eyes of *lok*; *polα/+* flies were indistinguishable from those of wild-type flies. In contrast, *grp*; *polα/+* mutant males had a rough-eye phenotype (Figure 5A), but females had wild-type eyes. In *grp lok*; *polα/+* mutants, both males and females exhibited rough eyes; however, the phenotype was still not as severe as that of *mei-41; polα/+* mutants. We also measured the effects of *grp* and *lok* mutations on apoptosis. Neither the single mutants (*grp* or *lok*) nor the *grp lok* double mutants had the elevated levels of apoptosis observed in *mei-41* mutants (Figure 5B). However, *lok; polα/+* mutants had a slight increase in apoptosis compared to wild type ($P < 0.05$), and *grp; polα/+* and *grp lok; polα/+* mutants had a more substantial increase compared to wild type ($P < 10^{-4}$). Interestingly, the levels of apoptosis in these mutants were not as high as in *mei-41; polα/+* mutants ($P < 10^{-4}$). These data demonstrate that the Chk1/Chk2-meditated checkpoint function of MEI-41 plays an important role in response to reducing Polα; however, the intermediate phenotypes suggest that loss of this checkpoint cannot completely account for the severity of *mei-41; polα/+* mutants.

## DISCUSSION

We have shown here that genetically reducing Polα levels by only half in *mei-41* mutants results in increased P53-dependent apoptosis, rough eyes, and genomic instability, including elevated mitotic crossing over. Reducing mitotic cyclin levels rescues at least some of these phenotypes, supporting the idea that loss of MEI-41-dependent cell cycle regulation contributes greatly to the defects. However, the GRP/LOK-mediated checkpoint does not account for the severity of phenotypes observed in *mei-41* mutants. We suggest here that reducing Polα results in P53-inducing damage, such as incomplete replication, stalled replication forks, or uncoupling of leading- and lagging-strand synthesis. This "replication stress" requires the checkpoint function of MEI-41 to maintain developmental processes, cell survival, and genomic stability.

Reducing Polα alone does not cause any detectable defects, which suggests that the damage caused by reducing Polα in an otherwise wild-type background is relatively mild. It is possible that the defects that we observed are an additive effect of defects in *mei-41* mutants that we did not detect in *polα/+* flies. A more likely explanation is that MEI-41 function is exceptionally important in responding to the very low level of

endogenous damage that results from reducing Polα. This interpretation is consistent with our previous finding that *mei-41* mutants have reduced viability when a single *P* element is undergoing transposition during development (LaRocque *et al.* 2007).

If the damage that results from reducing Polα requires the checkpoint response of MEI-41, it should elicit a checkpoint response in animals that are wild type for *mei-41*. It is difficult to detect S-phase checkpoints in Drosophila tissues, but there has been one report of MEI-41-dependent decrease in BrdU incorporation into larval neuroblasts, following treatment with 1600 rad of IR (Jaklevic and Su 2004). We were unable to detect this decreased BrdU incorporation in *polα/+* larvae or in wild-type larvae after irradiation (data not shown). In contrast, irradiation induces a robust MEI-41-dependent delay of entry into mitosis (Hari *et al.* 1995). In imaginal discs, this $G_2$-M checkpoint is readily detected by staining with a marker for mitotic cells after irradiation with as little as 500 rad (Brodsky *et al.* 2000; Bi *et al.* 2005; LaRocque *et al.* 2007); we were unable to detect any effect of reducing Polα on the number of mitotic cells (data not shown). A likely explanation is that irradiation induces a burst of damage, resulting in rapid cessation of entry in mitosis that can be detected soon after treatment, whereas any damage resulting from genetic reduction of Polα would occur and be repaired throughout development. It might be possible to detect an increased steady-state level of MEI-41-dependent phosphorylation of checkpoint transducers or effectors, but this would depend on the level of damage resulting from reduction of Polα.

Reducing other components of the Polα complex did not result in rough eyes in *mei-41* mutants. These included the primase subunit (*DNAprim*), and the 50- and 73-kDa subunits of Polα (data not shown). We also reduced levels of other replicative polymerases and components of replication, using null alleles and/or deficiencies of *DNApol-δ*, *DNApolε*, *E2f*, and *mus209*, which encodes PCNA. None of these manipulations caused rough eyes in *mei-41* mutants. It is possible that there is an unknown function of Polα responsible for the interactions with MEI-41. Alternatively, whereas *pola-180* mutants are embryonic lethal, the reported lethal phenotypes of *DNAprim*, *E2f*, and *PCNA* null mutants include survival to at least first instar larvae (Royzman *et al.* 1997; Chen *et al.* 2000; Henderson *et al.* 2000). The 180-kDa catalytic subunit therefore may be the limiting factor of the primase complex, and reduction of this subunit, as observed with embryonic lethality, may have a more profound effect on replication than reducing other components of the replication machinery.

Previous work has demonstrated a link between increased apoptosis and rough-eye phenotypes (Ollmann *et al.* 2000; Lee *et al.* 2003; Silva *et al.* 2004). We therefore tested imaginal discs to see whether or not there was an increase in apoptosis that could presumably lead to rough eyes. We found a strong correlation between rough eyes and an increase in apoptosis: genotypes that had rough eyes also had an increase in apoptosis, and reducing the number of apoptotic cells also rescues rough-eye phenotypes (*mei-41; CycA/polα*). We directly tested whether eye development was dependent on P53-mediated apoptosis and found that eliminating P53 completely rescues both apoptosis and the rough-eye phenotype. Some genotypes that had an increase in apoptosis compared to wild type did not result in rough eyes: *mei-41* single mutants, *mus304* single mutants, and *grp; polα/+* mutant females (data not shown). Overall, however, we found a strong correlation between two dramatic phenotypes associated with mutants in cell cycle regulation and in reducing Polα.

Reducing mitotic cyclin levels rescued the rough-eye phenotype and increased apoptosis of *mei-41; polα/+* mutants. We propose that reducing cyclins slows cell cycle progression and therefore eliminates the need for MEI-41 checkpoint function to respond to damage induced by reducing Polα. We do not know the effects of cyclin reduction on cell cycle timing in proliferating cells of imaginal discs, but reducing cyclins does affect cell cycle timing during embryogenesis (Edgar *et al.* 1994; Crest *et al.* 2007) and ameliorates DSB repair defects of *mei-41* mutants (LaRocque *et al.* 2007). It is possible that mitotic cyclin reduction has no effect on response to DNA damage, but contributes to proper development by regulating developmentally controlled apoptosis. We cannot directly test this possibility, but our finding that the apoptosis and rough-eye phenotypes are P53 dependent supports the proposal that reducing Polα elicits a DNA damage response, since P53 is required for damage-induced apoptosis but not for developmentally regulated programmed cell death (Lee *et al.* 2003; Brodsky *et al.* 2004; Jaklevic and Su 2004; this study).

The interactions among *grp*, *lok*, and *polα* suggest varying contributions of GRP and LOK to the phenotypes reported here. The *lok; polα/+* mutants were indistinguishable from wild type in eye development and had only slight increases in apoptosis. In contrast, in *grp; polα/+* and *grp lok; polα/+*, there was a dramatic increase in apoptosis. These genotypes differed from one another in that only males had rough eyes in the *grp; polα/+* mutants, but both sexes had rough eyes in the *grp lok; polα/+* mutants. It is not clear why there is a difference between males and females in *grp; polα/+* mutants, as different genetic backgrounds show a similar discrepancy. It is possible that the severity of the defect in *grp; polα/+* mutants is near the threshold for causing rough eyes and that this threshold is lower in males than in females. Comparing all three genotypes (*grp* and *lok* single mutants and *grp lok* double mutants), however, we conclude that the phenotypic effects of reducing Polα can be attributed predominantly to the GRP-mediated checkpoint. Nonetheless, there does appear to be some

redundancy between GRP and LOK in these assays. Partial redundancy between Chk1 and Chk2 has been demonstrated in other organisms (Boddy *et al.* 1998; Chen and Sanchez 2004; Helt *et al.* 2005; reviewed in Sanchez *et al.* 1996; Sancar *et al.* 2004), as well as in the DNA damage checkpoint response in Drosophila (Xu *et al.* 2001; Brodsky *et al.* 2004; LaRocque *et al.* 2007) and in repair of DSBs induced through *P*-element excision (LaRocque *et al.* 2007).

Loss of both GRP and LOK did not produce defects as severe as those observed when MEI-41 was absent. We conclude that the GRP/LOK-mediated checkpoint cannot completely account for the defects seen in *mei-41*; *polα/+* flies. Studies in mammalian cells suggest a checkpoint-independent role for mammalian ATM kinases in DNA repair (reviewed in Jeggo *et al.* 1998; Lobrich and Jeggo 2005; Jeggo and Lobrich 2006; O'Driscoll and Jeggo 2006). We previously showed that *mei-41* mutants are more sensitive to *P*-element excision and have more severe defects in homologous recombinational repair compared to *grp lok* double mutants (LaRocque *et al.* 2007), and Jaklevic and Su (2004) found that *mei-41* mutants are killed by doses of IR that are not lethal to *grp* mutants, even though both mutants are defective in the IR-induced $G_2$-M checkpoint. Oikemus *et al.* (2006) found that both spontaneous and IR-induced chromosome breaks were increased in *mei-41* mutants but not in *grp lok* double mutants, suggesting that MEI-41 has a role in preventing chromosome breaks that is independent of GRP and LOK. Together, these studies strongly suggest that there is a role of MEI-41 that is independent of the GRP/LOK-mediated checkpoint in response to reducing Polα.

Despite numerous observations that MEI-41 has GRP/LOK-independent functions in response to DNA damage, it is possible that the MEI-41-mediated checkpoint is not completely eliminated in *grp lok* mutants and that there is an unidentified transducer of the checkpoint pathway. We and others have not been able to detect a $G_2$-M checkpoint after IR in *grp lok* mutants (Liu *et al.* 2000; Xu *et al.* 2001; Brodsky *et al.* 2004; LaRocque *et al.* 2007), consistent with studies in other model organisms that indicate that ATR/ATM-dependent DNA damage checkpoints are transduced entirely through Chk1 and Chk2 (Boddy *et al.* 1998; Chen and Sanchez 2004; reviewed in Sanchez *et al.* 1996; Sancar *et al.* 2004). We therefore favor the alternative hypothesis that MEI-41 has some role independent of its checkpoint function in response to damage caused by reducing Polα.

In conclusion, we have identified an interaction between regulators of the cell cycle and a component of replication machinery. These interactions are necessary for proper development of adult organs, maintaining genomic stability, and regulation of cell survival. This study reveals a checkpoint-dependent response when Polα is reduced, suggesting the importance for development and cell survival in responding to endogenous damage that occurs during normal DNA metabolism. Previous work in fungi and humans highlights a role for ATR orthologs in maintaining fragile site stability in response to slowing replication by aphidicolin treatment or genetically reducing Polα (Casper *et al.* 2002; Lemoine *et al.* 2005). Additionally, work in Xenopus has demonstrated that uncoupling of DNA polymerases from MCM helicase via aphidicolin treatment (Walter and Newport 2000), *cis*-platinum treatment, or UV irradiation (Byun *et al.* 2005) activates the ATR-dependent checkpoint. While most checkpoint studies rely on exogenously induced damage, our findings reveal the importance of an ATR-mediated checkpoint in responding to relatively mild endogenous defects. The results reported here demonstrate yet another conserved interaction between cell cycle checkpoint response and replication machinery, two cellular processes that are integral for genomic stability and cell survival.

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