

***C. elegans* RAD-5/CLK-2 defines a new DNA damage checkpoint protein**

Shawn Ahmed^{*S¶}, Arno Alpi^{¶¶}, Michael O. Hengartner[†]
and Anton Gartner[‡]

Background: In response to genotoxic stress, cells activate checkpoint pathways that lead to a transient cell cycle arrest that allows for DNA repair or to apoptosis, which triggers the demise of genetically damaged cells.

Results: During positional cloning of the *C. elegans rad-5* DNA damage checkpoint gene, we found, surprisingly, that *rad-5(mn159)* is allelic with *clk-2(qm37)*, a mutant previously implicated in regulation of biological rhythms and life span. However, *clk-2(qm37)* is the only *C. elegans clock* mutant that is defective for the DNA damage checkpoint. We show that *rad-5/clk-2* acts in a pathway that partially overlaps with the conserved *C. elegans mrt-2/S. cerevisiae RAD17/S. pombe rad1(+)* checkpoint pathway. In addition, *rad-5/clk-2* also regulates the S phase replication checkpoint in *C. elegans*. Positional cloning reveals that the RAD-5/CLK-2 DNA damage checkpoint protein is homologous to *S. cerevisiae* Tel2p, an essential DNA binding protein that regulates telomere length in yeast. However, the partial loss-of-function *C. elegans rad-5(mn159)* and *clk-2(qm37)* checkpoint mutations have little effect on telomere length, and analysis of the partial loss-of-function of *S. cerevisiae tel2-1* mutant failed to reveal typical DNA damage checkpoint defects.

Conclusions: Using *C. elegans* genetics we define the novel DNA damage checkpoint protein RAD-5/CLK-2, which may play a role in oncogenesis. Given that Tel2p has been shown to bind to a variety of nucleic acid structures in vitro, we speculate that the RAD-5/CLK-2 checkpoint protein may act at sites of DNA damage, either as a sensor of DNA damage or to aid in the repair of damaged DNA.

Background

DNA damage checkpoint genes encode a group of proteins whose function is (1) to physically detect DNA damage, (2) to transmit a signal that DNA damage is present, and then either (3) to elicit cell cycle arrest and DNA repair (which removes the damage), or (4) to elicit programmed cell death (which removes the compromised cell). The apoptotic response to DNA damage is found only in higher eukaryotes, including worms, flies, and mammals [1]. Proteins required for the DNA damage checkpoint are evolutionarily conserved and have primarily been identified through genetic analysis in yeast and biochemical studies in mammalian cells [2, 3].

Of the DNA damage checkpoint proteins identified thus far, it is unclear which protein actually senses DNA damage, although several candidates that interact with DNA are known. scDdc1p (*Saccharomyces cerevisiae* Ddc1p), scRad17p, and scMec3p form a complex that structurally resembles a PCNA sliding DNA clamp [4–8] and has recently been shown to associate to DNA close to double-

strand breaks in vivo, in a scRad24p-dependent manner [9, 10]. However, these proteins are not required for DNA damage-induced activation of the checkpoint protein kinase spRad3p, which is the *S. pombe* homolog of scMec1p and mammalian ATR [11]. Furthermore, the in vivo association of the scMec1p kinase with damaged DNA does not require scDdc1p, scRad17p, and scMec3p [9]. Together, these results suggest that the PCNA-like checkpoint protein complex is not required for sensing DNA damage [12–16]. Furthermore, although complexes of the PCNA- and RFC-like checkpoint proteins are likely to interact with DNA in response to DNA damage, purified complexes containing these proteins fail to bind to DNA in vitro, suggesting that they may be downstream of the initial DNA damage checkpoint signal [17]. Other DNA damage checkpoint proteins that are likely to interact with DNA include the MRE11/RAD50/NBS1 nuclease complex [18–20]. In addition to the above checkpoint proteins, studies in *S. cerevisiae* have also implicated DNA polymerase ϵ as an upstream component of checkpoint signaling that potentially acts as a sensor of DNA damage

Addresses: *MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, United Kingdom. †Institute for Molecular Biology, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland. ‡Max Planck Institute for Biochemistry, D-82152 Martinsried, Am Klopferspitz 18A, Germany.

Present address: §Department of Genetics and Department of Biology, Coker Hall, University of North Carolina, Chapel Hill, North Carolina 27599-3280, USA.

Correspondence: Anton Gartner
E-mail: gartner@biochem.mpg.de

¶¶These authors contributed equally to this work.

Received: 21 September 2001
Revised: 5 November 2001
Accepted: 7 November 2001

Published: 11 December 2001

Current Biology 2001, 11:1934–1944

0960-9822/01/\$ – see front matter
© 2001 Elsevier Science Ltd. All rights reserved.

during S phase [21, 22]. It is unclear, however, whether DNA polymerase ϵ , scMec1p/spRad3p/ATM/ATR, the MRE11/RAD50/NBS1 nuclease, some other checkpoint protein, or some combination thereof might be the primary sensor(s) of DNA damage. The DNA damage signal is relayed via the scMec1p/spRad3p/ATM/ATR kinases to CHK1 and CHK2 kinases, which cause cell cycle arrest via phosphorylation of key cell cycle proteins [23]. How DNA damage-induced apoptosis and DNA repair is regulated is less well understood.

We have recently shown that DNA damage-induced checkpoints occur in the *C. elegans* germline [24]. If wild-type *C. elegans* worms are irradiated, one observes programmed cell death of meiotic pachytene cells as well as a transient cell proliferation arrest of mitotic germ cells [24]. Radiation-induced germ cell death is dependent on the general cell death regulators *ced-3* and *ced-4* and is negatively regulated by *ced-9*. Moreover *egl-1* partially contributes to radiation-induced apoptosis [24]. Importantly, three DNA damage checkpoint mutants have been identified, *op241*, *rad-5(mn159)*, and *mrt-2(e2663)*, and these mutants are defective for radiation-induced cell death and cell cycle arrest [24]. *mrt-2* encodes the *C. elegans* homolog of budding yeast *RAD17*/fission yeast *rad1(+)* [25].

rad-5(mn159) is a *C. elegans* DNA damage checkpoint mutant that was identified in a screen for radiation-hypersensitive animals [26]. *rad-5(mn159)* worms are viable at 15°C or 20°C but show maternal-effect embryonic lethality at 25°C; *rad-5* is thus either an essential gene or is required for life at 25°C [26]. When grown at permissive temperatures, the *rad-5(mn159)* mutant has reduced brood sizes and is hypersensitive to agents like UV light, X-rays, and ethyl methane sulphonate, all of which damage DNA [26]. Since DNA damage fails to induce either cell cycle arrest or apoptosis in the germlines of *rad-5(mn159)*, the RAD-5 protein is required for the DNA damage checkpoint in *C. elegans* [24].

Here we show that the *C. elegans rad-5(mn159)* is allelic with *clk-2(qm37)*, a *C. elegans* gene that affects both biological rhythms and life span [27]. By epistasis analysis, we show that *rad-5/clk-2* mutants are defective for the *mrt-2* and *op241* DNA damage checkpoint pathway but that *rad-5/clk-2* mutants are also defective for the S phase replication checkpoint. Cloning of *C. elegans rad-5/clk-2* reveals that it is structurally related to budding yeast Tel2p, a protein that has been shown to bind DNA in vitro [28–30].

Results

rad-5(mn159) is allelic to *clk-2(qm37)*

To further characterize *rad-5(mn159)*, we refined its map position to the middle of chromosome III by a series of

three-factor crosses (Figure 1a). *rad-5(mn159)* displays a weak maternal-effect slow growth (Gro) phenotype, such that *rad-5 m+z-* homozygotes (*m*, maternal genotype; *z*, zygotic genotype) develop at wild-type rates, whereas *rad-5(mn159) m-z-* worms have slightly slower growth rates (Figure 1b) [26]. A maternal-effect Gro phenotype is rare in *C. elegans* and has been described for mutations in four other genes: *clk-1*, *clk-2*, *clk-3*, and *gro-1* [27]. We noticed that the *clk-2* and *rad-5* genes had similar map positions. In addition, the only *clk-2* mutant allele that has been identified, *qm37*, displays a maternal-effect embryonic lethal phenotype at 25°C, as seen with *rad-5(mn159)* [27]. To test the possibility that *rad-5(mn159)* and *clk-2(qm37)* might be allelic, we generated *rad-5(mn159)/clk-2(qm37)* transheterozygotes and found that they showed embryonic lethality at 25°C (Figure 1c). In addition, *rad-5(mn159)/clk-2(qm37)* heterozygotes grown at 20°C had a slow growth (Gro) phenotype that was intermediate between that of *rad-5(mn159)* (weak Gro) and that of *clk-2(qm37)* (strong Gro) (Figure 1b,c). Thus, *rad-5(mn159)* fails to complement *clk-2(qm37)* both for slow growth and for embryonic lethality at 25°C.

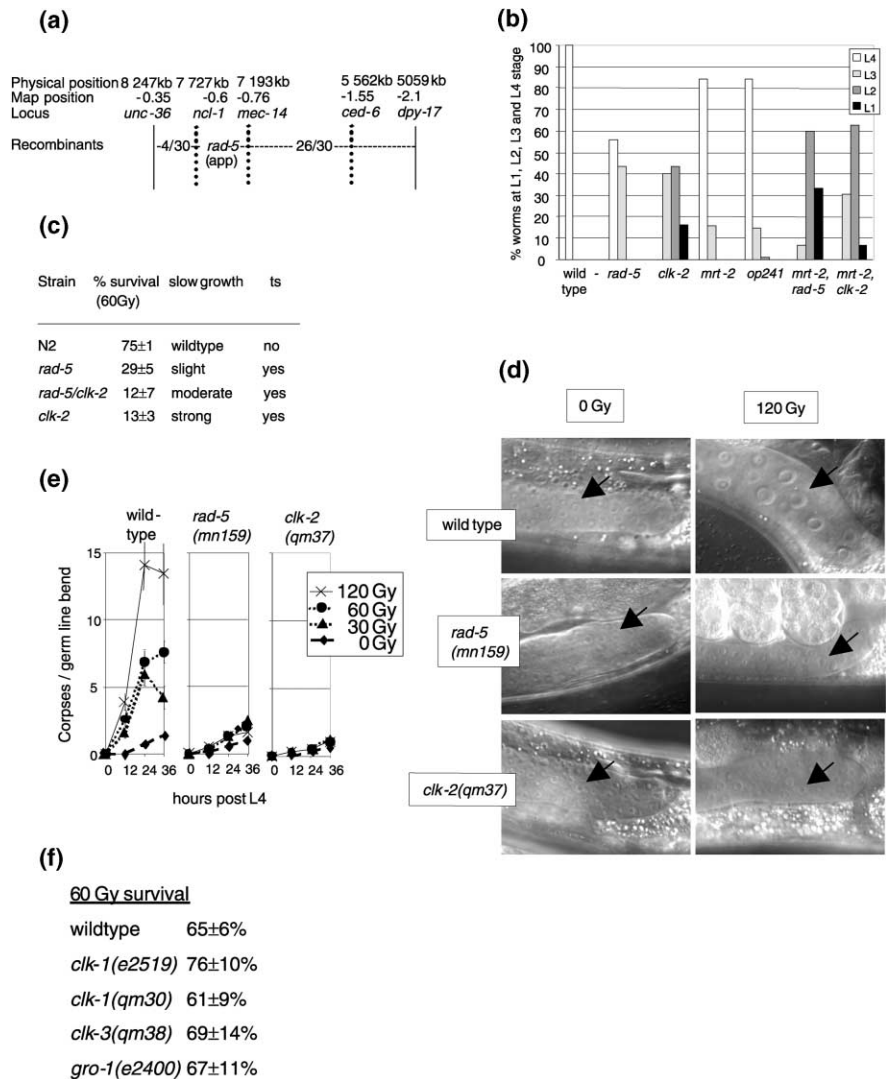
We next tested whether the *clk-2(qm37)* mutant displayed the DNA damage checkpoint defects seen with *rad-5(mn159)*. To measure radiation sensitivity, worms were irradiated at the L4 stage, and the survival rate of F1 embryos was determined by counting F1 larvae and dead eggs [24]. Indeed, *clk-2(qm37)* mutants showed reduced survival following irradiation, as did *rad-5/clk-2* heterozygotes (Figure 2c). In addition, *clk-2 qm37* was defective for DNA damage-induced cell cycle arrest (Figure 1d). In wild-type worms, mitotic cells in the distal arm of the *C. elegans* germline transiently halt cell proliferation after irradiation but continue to grow, as indicated by a decrease in cell number and enlargement of cellular and nuclear size. Checkpoint mutants such as *mrt-2(e2663)* and *rad-5(mn159)* are defective for this response. We observed that both *rad-5* and *clk-2* mitotic germlines continue to proliferate following irradiation (many small nuclei) (Figure 1d), as seen with the *mrt-2(e2663)* checkpoint mutant (data not shown). To further corroborate that *clk-2(qm37)* is checkpoint defective, we examined radiation-induced germ cell death in the meiotic part of the germline by scoring morphologically distinct apoptotic corpses under Nomarski optics as described previously [24]. Radiation-induced germ cell death is completely abrogated in *clk-2(qm37)* mutants (Figure 1e). Thus, the *clk-2(qm37)* mutant is defective for the DNA damage checkpoint and is allelic with *rad-5(mn159)*. Since both *rad-5* and *clk-2* mutant names have been previously published, we designate this gene *rad-5/clk-2* (*rad-5* being the first mutant published) [26, 27].

clk-2 is the only clock gene required for the DNA damage checkpoint

clk-2(qm37) mutants have a maternal effect Clock phenotype, which is defined by slow growth, slow defecation,

Figure 1

rad-5(mn159) is allelic with *clk-2 qm37*. **(a)** *rad-5(mn159)* mapping. *rad-5(mn159)*, whose map position was previously reported near -2 on chromosome III [26], was mapped more precisely using a multifactor cross with the strain WS711 *dpy-17(e164) ced-6(n1813) mec-14(u55) ncl-1(e1865) unc36(e251)*. Dpy-non-Unc and Unc-non-Dpy animals were picked in the F2 generation and scored for the temperature-sensitive lethality associated with *rad-5(mn159)*. The number of recombinants and the approximate map position of *rad-5(mn159)* are indicated. **(b)** Growth rates of various single and double mutants. Adult animals were allowed to lay embryos for 4 hr. After 48 hr at 20°C, 100% of wild-type animals are in the L4 larval stage. To estimate growth retardation, the proportion of various single and double mutants in the four larval stages, L1, L2, L3, and L4, was determined. Based on these data, we estimate that *rad-5(mn159)* reaches the L4 stage 6 hr later than wild-type, whereas *clk-2(qm37)* is retarded by ~18 hr. **(c)** Complementation analysis. Embryonic survival was scored as the percentage of surviving embryos laid after irradiation of mothers with 60 Gy of radiation. **(d)** Radiation-induced cell cycle arrest of mitotic germ cells was determined as described previously [24]. In brief, worms were irradiated at the L4 stage and checkpoint-induced cell cycle arrest defects were scored as a lack of mitotic germ cell enlargement. Mitotic germ cell nuclei are indicated by arrowheads. **(e)** To assay for radiation-induced germ cell death, worms were irradiated at the L4 stage with the indicated doses of X-irradiation, and apoptotic cell corpses were determined 12, 24, and 36 hr after irradiation using Nomarski optics [24]. **(f)** Radiation sensitivity of *clk* mutants. The radiation sensitivity of various *clk* mutants was determined as described in (c).



slow pharyngeal pumping, and slow movement [27]. Mutations in three other genes, *clk-1*, *clk-3*, and *gro-1*, also result in Clock phenotypes [27]. In addition, all known *clk* mutants have extended life spans [27]. We were curious to know if the checkpoint phenotype of *clk-2(qm37)* might be due to slow growth, and all *clk* mutants were tested for DNA damage checkpoint defects. We found that the germlines of *clk-1(e2519)*, *clk-3(qm38)*, and *gro-1(e2400)* all responded to radiation-induced DNA damage by inducing wild-type levels of cell cycle arrest in the mitotic germline and apoptosis in the meiotic germline (Figure S1 in the Supplementary material available with this article online; data not shown). Furthermore, the radiation sensitivity of *clk-1*, *clk-3*, and *gro-1* mutations is similar to that of wild-type (Figure 1f). Thus, the abnormal DNA damage checkpoint phenotypes of *rad-5(mn159)* and of *clk-2(qm37)* are

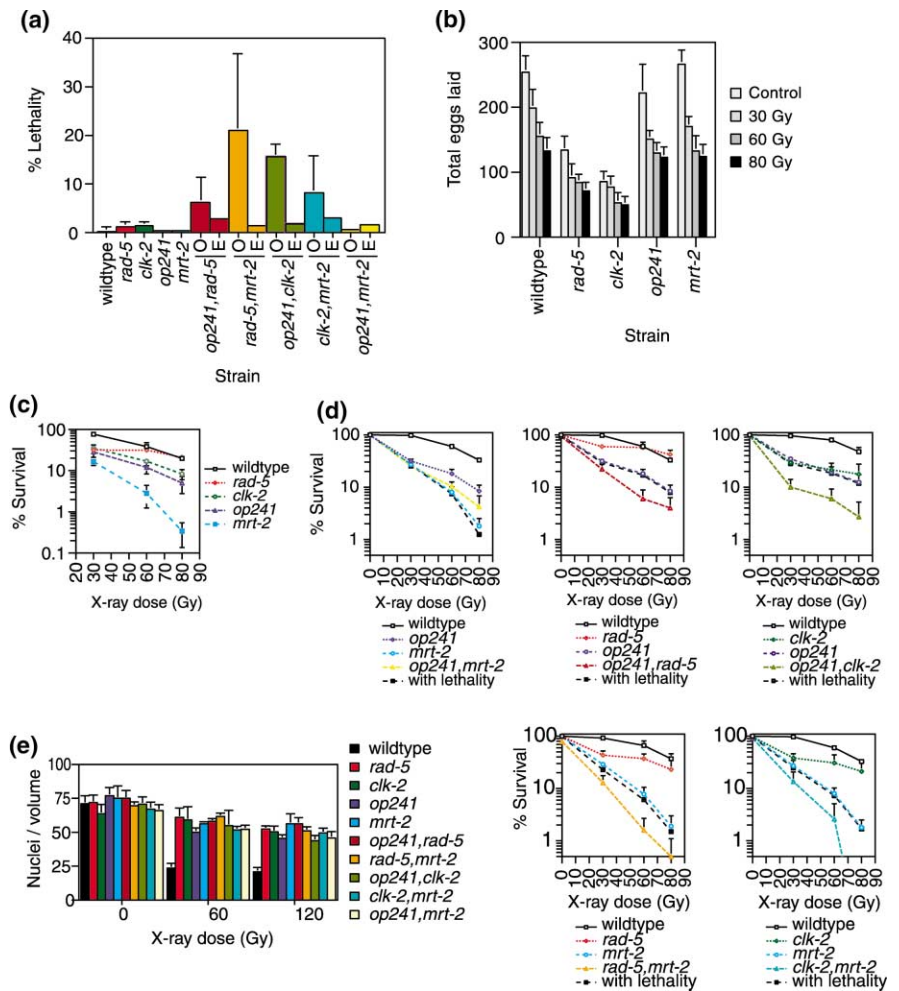
not simply a consequence of the slow growth phenotype of these mutants.

Epistasis between *rad-5/clk-2* and other checkpoint mutants

Three *C. elegans* DNA damage checkpoint mutants have been previously identified: *mrt-2(e2663)*, *op241*, and *rad-5(mn159)* [24, 25]. MRT-2 is a conserved DNA damage checkpoint protein and is homologous to *S. pombe* Rad1p and to *S. cerevisiae* Rad17p (a component of the Rad17p/Ddc1p/Mec3p PCNA-like complex) [25]. The *mrt-2(e2663)* splice junction mutation is likely to represent a severe allele of *mrt-2* [25]. In contrast, *rad-5(mn159)* and *clk-2(qm37)* are likely to be partial loss-of-function mutations, since they both cause temperature-sensitive embryonic lethality and are missense mutations (see below).

Figure 2

Epistasis analysis of *rad-5/clk-2* with *mrt-2* and *op241*. All experiments were performed at 20°C. **(a)** Synthetic lethality is observed in *rad-5* or *clk-2* double mutants. In double mutants, the expected lethality (based on adding the lethality of single mutants) is indicated by "E," whereas the observed lethality is indicated by "O." **(b)** Brood size drops by about 50% for all strains examined following high doses of irradiation. **(c)** X-ray hypersensitivity of single checkpoint mutants. To score for X-ray hypersensitivity, young L4s (grown at 20°C) were irradiated with 30, 60, or 80 Gy and allowed to lay eggs to score for embryonic lethality as described in the Materials and methods section. **(d)** X-ray hypersensitivity of various double mutant combinations was determined as described in the Materials and methods section. Although the same single mutant controls are used in several panels, these controls were part of a large experiment, and different graphs are used for clarity. **(e)** Radiation-induced cell cycle arrest was determined by scoring for the number of mitotic germ cell nuclei in a volume of 54,000 μm^3 12 hr after irradiation at the L4 stage. For each experiment, at least five germlines were scored.



Finally, *op241* is a mutation in an unknown checkpoint gene that maps to the left arm of chromosome I ([24]; R. Hofmann and M.O.H., unpublished data). Neither *mrt-2(e2663)* nor *op241* are temperature sensitive [24].

To establish the epistatic relationship between these DNA damage checkpoint mutants, we generated all double mutant combinations and assayed for embryonic lethality and for the extent of DNA damage-induced cell cycle arrest and apoptosis following ionizing radiation, which can damage DNA by causing double-strand breaks. Prior to construction of the double mutants, mutant strains were carefully outcrossed to eliminate the possibility of secondary mutations that could potentially affect radiation sensitivity (see Materials and methods). We found that *mrt-2(e2663)*, *op241*, *rad-5(mn159)*, and *clk-2(qm37)* single mutants displayed little embryonic lethality at 20°C and neither did the *op241;mrt-2(e2663)* double mutant (Figure 2a). In contrast, all double mutant combinations with either *rad-5(mn159)* or *clk-2(qm37)* showed an increase in embryonic lethality that was greater than the predicted

additive lethality for both single mutants (Figure 2a). In addition, the growth rates of all *rad-5(mn159)* or *clk-2(qm37)* double mutants were retarded in comparison with the respective single mutants (Figure 1b). The synergistic lethality of *rad-5/clk-2* mutations with the *mrt-2(e2663)* and *op241* DNA damage checkpoint mutations suggests that mutation of the *rad-5/clk-2* DNA damage checkpoint gene may result in endogenous DNA damage whose repair requires the *mrt-2* and *op241* gene products or vice versa.

The radiation sensitivity of germlines of single checkpoint mutants was examined by irradiating L4 larvae that have proliferating germlines and by then scoring for the survival of embryos that are generated from the irradiated germlines. Note that although *rad-5(mn159)* and *clk-2(qm37)* normally produce fewer eggs than the wild-type, the total number of eggs laid by irradiated worms drops to about half that of unirradiated controls for all strains examined (Figure 2b). In contrast, the amount of lethality among the eggs of irradiated strains varied significantly. When

single mutants were examined for X-ray sensitivity, *mrt-2(e2663)* was the most sensitive, *op241* was moderately sensitive, *clk-2(qm37)* was less sensitive, and *rad-5(mn159)* was the least sensitive (Figure 2c). The extensively outcrossed *rad-5(mn159)* strain used in this study was less sensitive to radiation than previously reported, because the original strain contains a second mutation that enhances radiation sensitivity [24]. These results agree with the possibility that *mrt-2(e2663)* is likely to be a strong allele (Figure 2c) and that *rad-5(mn159)* and *clk-2(qm37)* are temperature sensitive and therefore likely to be partial loss-of-function mutations (see below).

When the various double mutants were examined for their sensitivity to X-rays, the *op241;mrt-2(e2663)* double mutant was no more sensitive than either single mutant, indicating that these two mutations affect a single DNA damage checkpoint pathway. In contrast, germlines of double mutants that contained either *rad-5(mn159)* or *clk-2(qm37)* were always more sensitive to irradiation than the most sensitive single mutant, even when the added lethality of two single mutations was taken into consideration (Figure 2d). The enhanced sensitivity of *rad-5* and *clk-2* double mutants suggests that *rad-5/clk-2* might act in a pathway parallel to that of *mrt-2* and *op241*, thus helping to repair DNA damage independently of the *mrt-2* and *op241* checkpoint genes.

The radiation sensitivity experiments described above (Figure 2c,d) measure the survival of embryos generated from irradiated, proliferating germline nuclei. This assay is probably the most sensitive measure of DNA damage checkpoint regulation, accounting for the combined effects of the DNA damage checkpoint response, namely, cell cycle arrest, apoptosis, and DNA repair. To assess which of these factors might be responsible for the enhanced radiation sensitivity of *rad-5/clk-2* double mutants, radiation-induced cell cycle arrest was examined by scoring for the number of mitotic germ cell nuclei in a defined volume 12 hr after irradiation at the L4 stage (Figure 2e). Our experiments indicate that the cell cycle arrest response is equally strong in all single and double mutants (Figure 2e). Given that *rad-5(mn159)*, *clk-2(qm37)*, and the various double mutants are as defective as *mrt-2(e2663)* for DNA damage-induced cell cycle arrest (Figure 2e) and apoptosis (data not shown) [24], we conclude that these classical checkpoint responses do not fully account for the wild-type level of resistance of the germline to DNA damage. Thus, although *rad-5/clk-2* and *mrt-2* appear to function in two parallel checkpoint pathways in terms of total radiation sensitivity, the enhanced sensitivity is not due to enhanced defects in either cell cycle arrest or apoptosis but rather to defects in at least one additional parameter, possibly DNA repair. In addition, our data indicate that *rad-5/clk2* and *mrt 2* also function in a com-

mon pathway that regulates cell cycle arrest and apoptosis in response to DNA damage.

***rad-5/clk-2* mutants are defective for the S phase replication checkpoint**

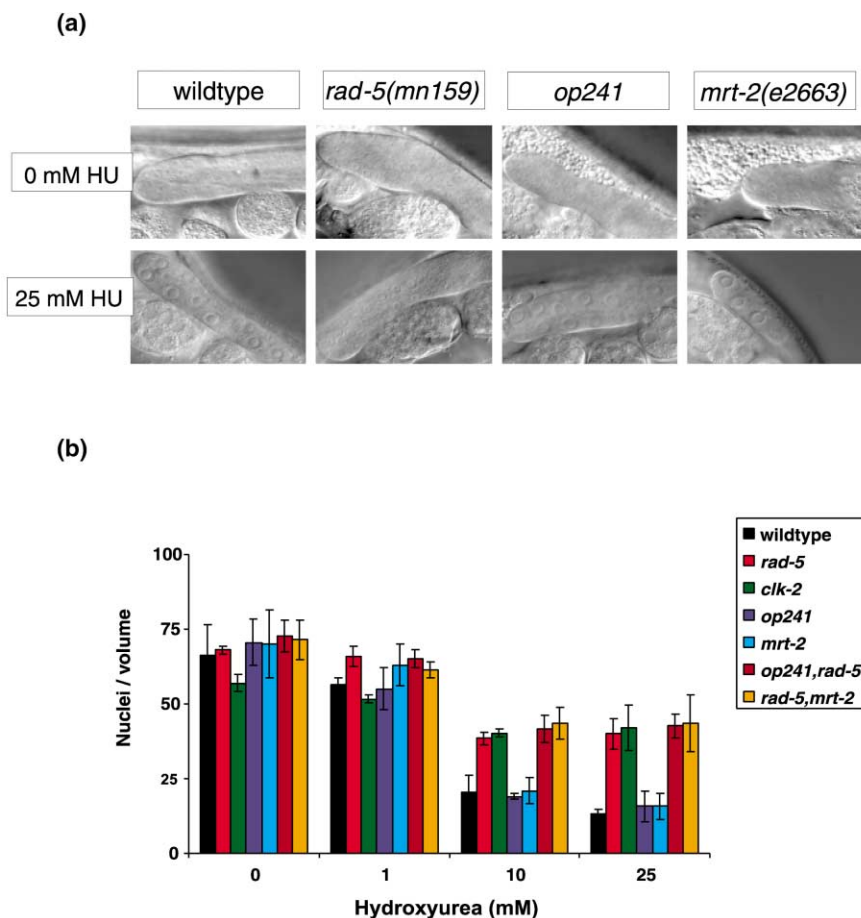
The S phase replication checkpoint is defined by hydroxyurea (HU), a drug that causes DNA replication arrest through inhibition of ribonucleotide reductase and the consequent depletion of dNTP pools [31]. A recent study demonstrated that HU treatment of *C. elegans* L4 larvae causes mitotic germ cell arrest and results in enlarged mitotic germ cell nuclei. In addition, the *mrt-2* DNA damage checkpoint protein was not required for this response [32]. Given that our double mutant analysis indicated that *rad-5/clk-2* functions in at least one checkpoint pathway that is independent of *mrt-2*, we tested to see if *rad-5/clk-2* might be involved in the S phase replication checkpoint (Figure 3a). We found that the HU-sensitive replication checkpoint is normal in *mrt-2* and *op241* animals but is severely compromised in *rad-5* and *clk-2* animals (Figure 3a,b). *rad-5* double mutants are equally resistant to HU as *rad-5* single mutants (Figure 3b). Thus, our data indicate that the RAD-5/CLK-2 DNA damage checkpoint protein is required in *C. elegans* both for the S phase replication checkpoint and for ionizing radiation-induced checkpoints that are specific for *mrt-2* and *op241* and are likely to occur at G1 and G2/M.

RAD-5/CLK-2 defines a new evolutionarily conserved protein

rad-5(mn159) mapped to an interval of 420 kb that is covered by 12 overlapping cosmids (Figure 4a). To determine the molecular identity of *rad-5/clk-2*, cosmid pools were injected. One cosmid pool stably rescued the *rad-5* ts lethality at 25°C. Injection of individual cosmids from this pool restricted the rescuing activity to a region unique to cosmid C07H6, which contains four genes (Figure 4b). We obtained five C07H6 transgenic lines that complemented the ts lethality of *rad-5(mn159)* and *clk-2(qm37)*, three of which also complemented the associated DNA damage checkpoint defect (Figure 4b). We were unable to phenocopy *rad-5* defects by either RNAi injection or by RNAi feeding, so we sequenced the four genes in the middle of cosmid C07H6 in both *rad-5(mn159)* and in *clk-2(qm37)* and found missense mutations only in C07H6.6. These mutations result in a G135C change in *rad-5(mn159)* and in a C772Y change in *clk-2 pm37* (Figure 4c). C07H6.6 and C07H6.8 are likely to be part of an operon (data not shown). Thus, a DNA fragment containing both genes was PCR amplified and used to rescue the temperature-sensitive lethality of both *rad-5(mn159)* and *clk-2(qm37)*, thus confirming the molecular identity of *rad-5/clk-2* (data not shown). Out of the four transgenic lines that rescued the temperature sensitivity of *clk-2(qm37)*, one line also rescued the checkpoint defect (Figure 4b). Rescue of the checkpoint phenotype was expected to be difficult, as this is a germline phenotype,

Figure 3

rad-5/clk-2 is defective in the S phase checkpoint. **(a)** Mitotic germ cells of *rad-5(mn159)* worms do not respond by S phase checkpoint activation upon HU treatment. Mitotic germ cells of HU-treated worms are shown. HU-induced cell cycle arrest was determined as described by MacQueen and Villeneuve [47]. In brief, worms at the L4 stage were plated on NGM plates containing 25 mM HU, and pictures of the mitotic part of the germline were taken as described in Figure 1d after 14 hr incubation on HU plates at 25°C. Similar results were obtained when the assay was performed at 20°C (data not shown). **(b)** Quantification of S phase defects. Worms were grown on NGM plates containing the indicated concentrations of HU starting from the L4 stage at 25°C. After 14 hr, the extent of HU-induced cell cycle arrest was determined as described for IR-induced cell cycle arrest (Figure 2e) by scoring for the number of mitotic germ cell nuclei in a volume of 54,000 μm^3 .



and injected, extrachromosomal DNA is often silenced in the germline of *C. elegans* [33, 34]. We thus conclude that *rad-5/clk-2* is encoded by C07H6.6. *rad-5/clk-2* is expressed throughout all developmental stages [35] (data not shown).

To determine whether *rad-5/clk-2* is evolutionarily conserved, PSI Blast (NCBI) was performed using the RAD-5/CLK-2 protein, which revealed a protein family with a single homolog in vertebrates, *Arabidopsis*, *Drosophila*, budding and fission yeast (Figure 5). After five rounds of PSI Blast, probability scores for *rad-5/clk-2* family members ranged from $1\text{e-}178$ to $1\text{e-}71$, whereas the highest score for an unrelated protein was 0.92. Equally, PSI Blast searches with partial RAD-5/CLK-2 sequences detected the same proteins (data not shown). RAD-5/CLK-2 is homologous to the *S. cerevisiae* Tel2p protein, which was identified in a genetic screen for budding yeast mutants with short telomeres. Telomeres of the *tel2-1* mutant shorten progressively for about 100 cell divisions and then stabilize [36]. In addition, Tel2p has been reported to bind to single-stranded, double-stranded, and four-stranded yeast telomeric DNA in vitro [29, 30]. We there-

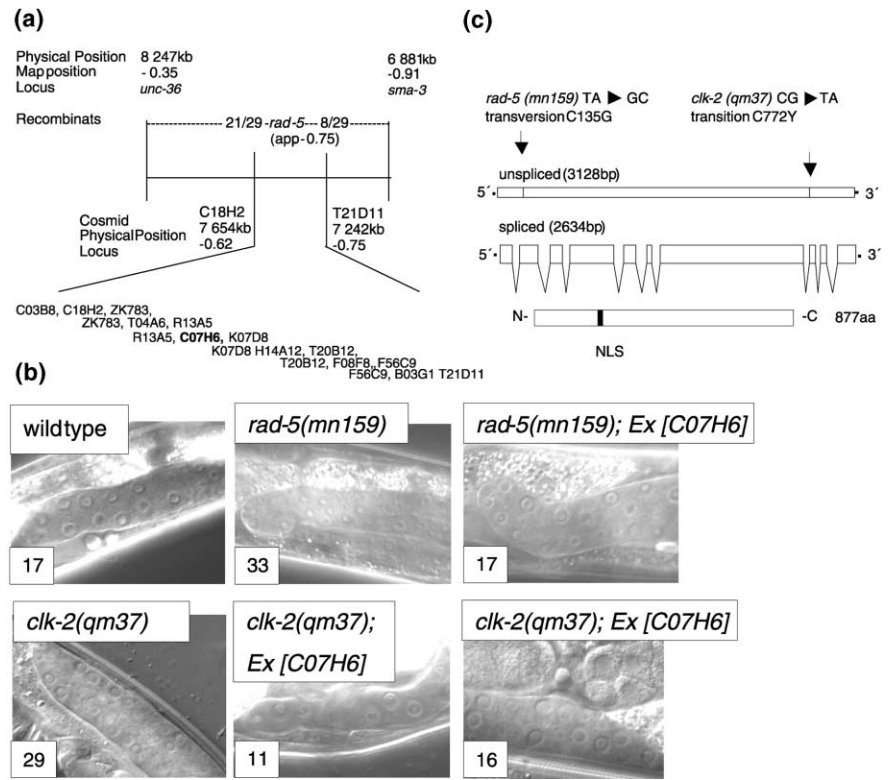
fore decided to examine telomere length in *rad-5(mn159)* and *clk-2(qm37)*.

Telomere behavior in *C. elegans* DNA damage checkpoint mutants

The MRT-2 checkpoint protein is thought to be required for telomere replication, because *mrt-2(e2663)* mutants display progressively shortened telomeres, late-onset end-to-end chromosome fusions, and late-onset sterility—phenotypes typical of telomerase-defective mutants in yeast and mouse [25]. In comparison with telomeres of N2 wild-type strains, telomeres of *rad-5(mn159)* and *clk-2(qm37)* strains tended to be slightly elongated, often containing a long telomeric restriction fragment found in *rad-5/clk-2* parental strains (data not shown). However, telomere length varies considerably in *C. elegans* strains and can fluctuate within single isogenic lines (S.A., unpublished data). Thus, crosses were performed between wild-type and *rad-5/clk-2* mutant worms, homozygous mutant and wild-type F2 progeny were picked from F1 heterozygotes, and independent F2 lines from the same F1 parent were analyzed in order to determine if *rad-5* or *clk-2* mutations affect telomere length (Figure 6a). Wild-type F2 lines

Figure 4

rad-5/clk-2 is encoded by C07H6.6. (a) To more precisely map *rad-5(mn159)*, three-factor mapping was done with an *unc-36 rad-5 sma-3* strain, which was crossed with the polymorphic CB4854 strain. Recombinant animals were picked, and the *rad-5(mn159)* map position was refined by analysis of single nucleotide polymorphisms between N2 and CB4854 [48]. The two most informative polymorphisms were ACA (N2)/T (CB4854) TTTTTTTTAC on Cosmid T21D11 and ATAACGTA (N2)/G (CB4854) ATAA on Cosmid C03B8 that unambiguously allowed placing *rad-5(mn159)* between T21D11 and C03B8. Cosmids were prepared using a Qiagen Plasmid isolation kit and injected at 2.5 ng/ul together with 50 ng/ul pRF-4[*rol-6(su1006)*]. One line transformed with cosmid pool R13A5, C07H6, KO7D8 rescued the ts lethal phenotype at 25°C. (b) Rescue of *rad-5(mn159)* and *clk-2(qm37)* checkpoint phenotypes by cosmid C07H6 and by a long PCR product encompassing C07H6.6 and C07H6.8 (Figure 4b, top right panel). N2 wild-type, mutant and transgenic worms were irradiated, and the status of the mitotic germline was scored after 12 hr. The number of mitotic germ cell nuclei is indicated. (c) Sequence analysis revealed a single point mutation in C07H6.6 in *rad-5(mn159)* and in *clk-2(qm37)*. The *rad-5/clk-2* cDNA sequence was determined by analyzing the apparently full-length EST yk447b4 (data not shown). A putative NLS (aa 290–307) was found using used InterPro search software.



from these crosses (*clk-2^{+/+}* sibs) had telomere lengths that were similar to those of their *rad-5/clk-2* mutant siblings, indicating that the *rad-5(mn159)* and *clk-2(qm37)* missense mutations have no immediate effect on telomere length (Figure 6a). In contrast, F2 lines of *mrt-2* all have discrete telomeric bands, suggesting tight regulation of telomere length in the absence of telomere elongation, a phenotype that is also seen in *Arabidopsis* telomerase-defective mutants [37].

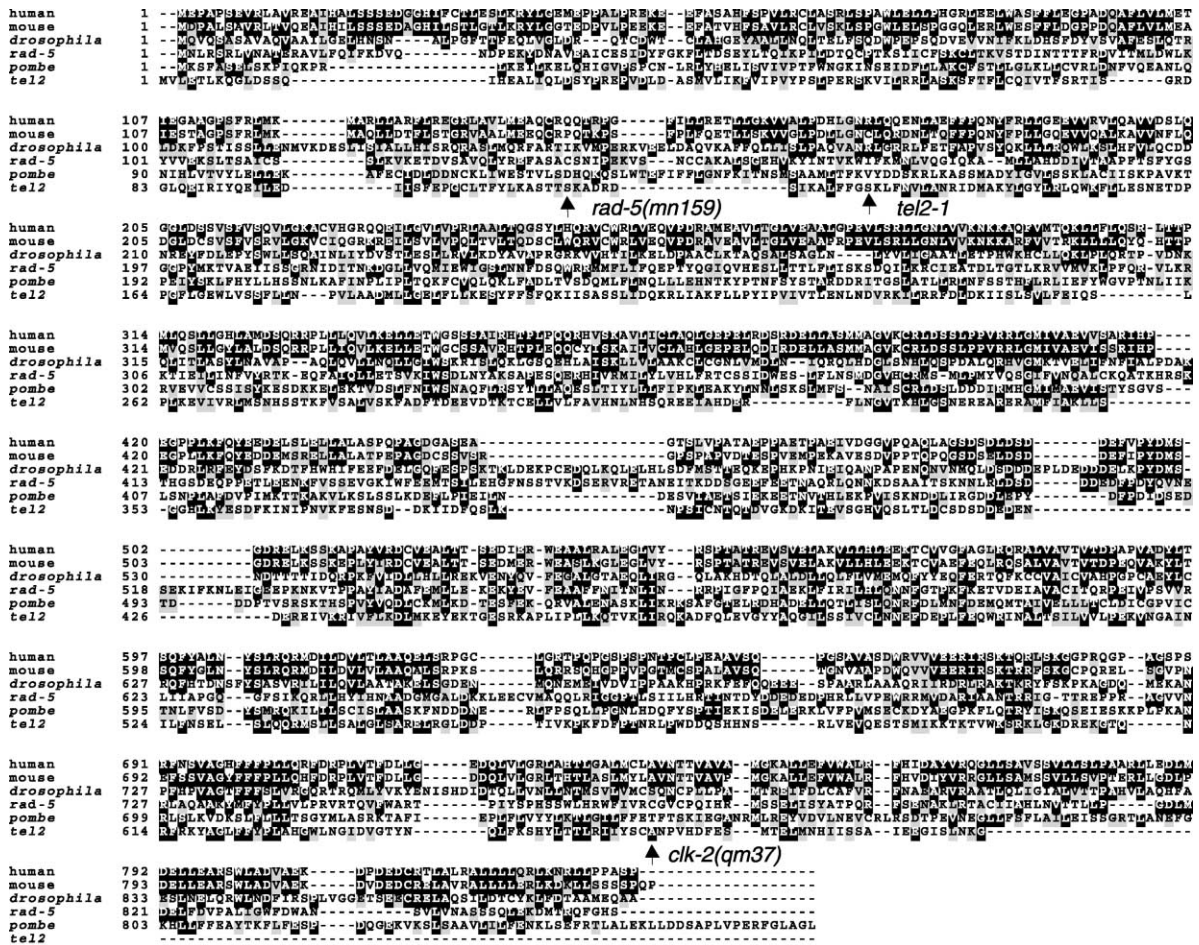
When telomere length of checkpoint-defective *C. elegans* strains was examined over several generations, N2 wild-type telomeres either stayed fairly constant in length (Figure 6b) or elongated slightly (in 3/6 experiments each) (data not shown), whereas *rad-5* and *clk-2* lines usually displayed slight telomere lengthening (in 5/6 experiments) (Figure 6b; data not shown). Note that the example shown in Figure 6b is misleading in the absence of further trials, as it suggests that wild-type telomeres remain the same length, whereas *rad-5* and *clk-2* telomeres elongate slightly with time. Wild-type telomeres often elongate as well (data not shown). *op241* telomeres showed little change in telomere length over time, whereas *mrt-2* telomeres shortened progressively (Figure 6b), as pre-

viously described [25]. We thus conclude that mutations of *rad-5/clk-2* have no significant effect on telomere length, which is in contrast to the telomere shortening observed in *S. cerevisiae tel2-1* mutants. Telomere length was also examined in the various double mutant combinations and shown to drift slightly in *op241, rad-5(mn159)* or *op241, clk-2(qm37)* strains (Figure 6c). Notably, telomeres of *op241, rad-5* double mutants shortened progressively (Figure 6c), although these double mutants did not display the discrete telomeric bands or mortal germline senescence that are seen for *mrt-2* telomerase-deficient mutants (data not shown) [25]. All double mutant combinations with *mrt-2* displayed progressive telomere shortening and mortal germline phenotypes, as is typical of *mrt-2* mutants (Figure 6c and data not shown) [25].

Checkpoint phenotypes of *S. cerevisiae TEL2*

Since *rad-5/clk-2* and *TEL2* are related by sequence, we were curious to know if Tel2p might be required for the DNA damage checkpoint in *S. cerevisiae*. We analyzed the single reported viable *tel2* allele, *tel2-1* [36], but did not observe enhanced sensitivity to X-irradiation, to methyl methanesulphonate, or to hydroxyurea, as we observed with a control DNA damage checkpoint mutant, *mec1-1*

Figure 5



RAD-5/CLK-2 has unique homologs in mammals, flies, and yeast and is related to budding yeast Tel2p. The multiple protein

alignment was performed using Clustal W 1.8.

(data not shown). We conclude that this partial loss-of-function *tel2-1* allele, which has a rather mild telomere shortening phenotype [36], does not display defects that might be expected for a yeast DNA damage checkpoint mutant.

Discussion

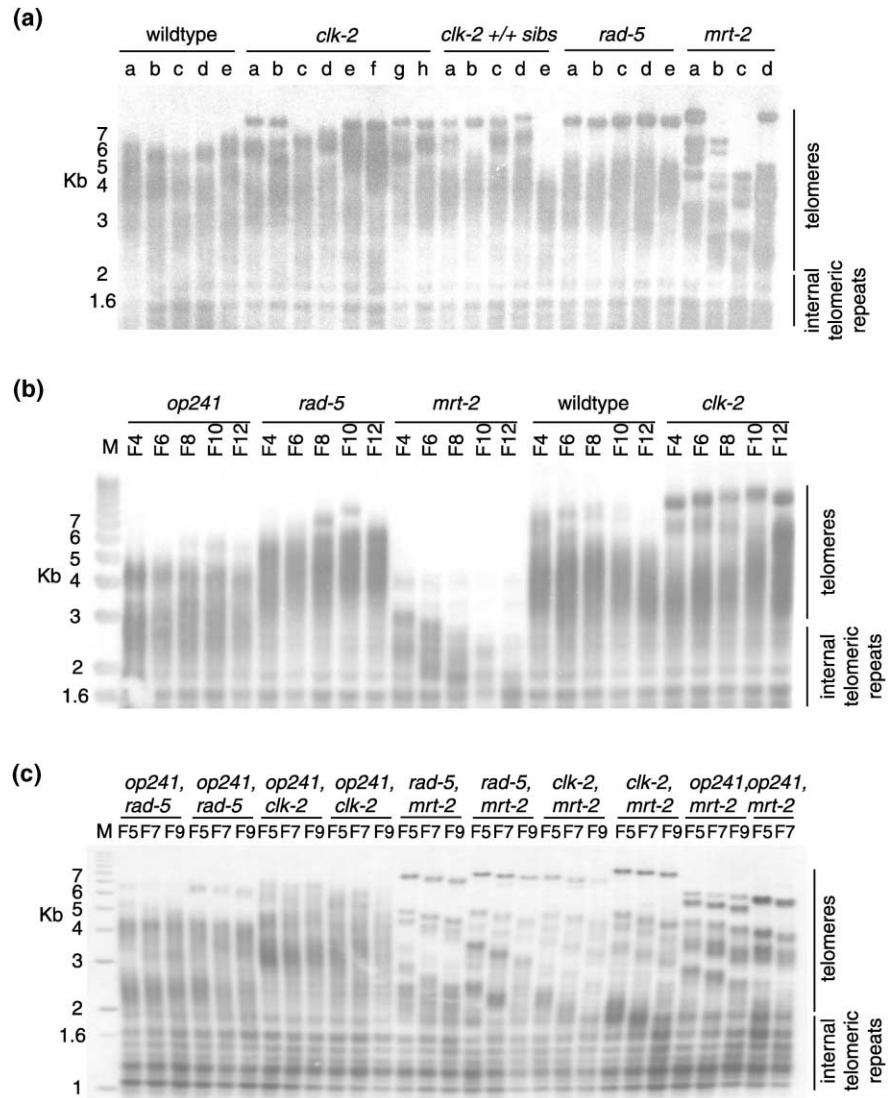
Using *C. elegans* as an experimental system, we have identified a novel DNA damage checkpoint protein that is structurally related to budding yeast Tel2p. Both *C. elegans rad-5(mn159)* and *clk-2(qm37)* alleles affect DNA damage checkpoint responses, have little effect on telomere length, and display a maternal-effect Gro phenotype. *clk-2(qm37)* displays stronger Gro and radiation sensitivity phenotypes than does *rad-5(mn159)* (Figure 1b), and *clk-2(qm37)* is also more sensitive to radiation (Figure 2d), indicating that it is a stronger allele. The two identified alleles are both missense mutations, and both of these are temperature-sensitive embryonic lethal. Both alleles

of *rad-5/clk-2* are therefore likely to be partial loss-of-function, suggesting that *rad-5/clk-2* is an essential gene. Since *rad-5/clk-2* is a DNA damage checkpoint gene, it seemed possible that the temperature-sensitive phenotype of *rad-5/clk-2* mutant worms might be due to an S phase defect at 25°C. However, developmental recordings of *rad-5(mn159)* or *clk-2(qm37)* embryos raised at 25°C failed to reveal any cell cycle defects in the first two cell divisions (data not shown) [38, 39]. In addition, telomere length in *rad-5(mn159)* and *clk-2(qm37)* animals grown to adulthood at 25°C was not different from that of wild-type controls (data not shown). It is possible that a subtle kind of lethal DNA damage accumulates at 25°C in these mutants. Alternatively, RAD-5/CLK-2 may be needed to organize late embryonic development [26] or may have another essential function.

Two recent papers report the cloning of *clk-2* [39, 40]. Given that *rad-5/clk-2* is related to budding yeast *TEL2*,

Figure 6

Telomere length of *C. elegans* checkpoint mutants. **(a)** Telomere length of different, freshly outcrossed F2 lines of *rad-5(mn159)*, *clk-2(qm37)*, *mrt-2(e2663)*, *op241*, and of *clk-2 (+/+)* wild-type siblings. To determine telomere length of single outcrossed F2 animals, these were allowed to produce progeny (F2 plates) needed for genomic DNA preparation. Due to the variation of telomere length in isogenic lines, several isogenic lines were analyzed (indicated by lower case letters) in single experiments. **(b)** Telomere length of various *C. elegans* checkpoint mutant strains over the course of several generations. Since telomere length can fluctuate even in wild-type *C. elegans* strains, six independent experiments were conducted, an example of which is shown. **(c)** Telomere length of various *C. elegans* checkpoint double mutants. Positions of telomeric and internal nontelomeric restriction fragments are indicated [49].



a gene that regulates telomere length, these reports have each tested telomere length in single *clk-2(qm37)* strains and have observed either elongated [39] or shortened telomeres [40] in comparison with wild-type. Given that telomere length is generally variable in *C. elegans* strains (S.A., unpublished data), it is not surprising that differences in telomere length might be observed between single *clk-2(qm37)* and wild-type strains from different laboratories. Furthermore, rescue of an elongated *clk-2* telomere phenotype by one laboratory resulted in telomeres that were shorter than normal [39], suggesting that cosuppression silencing of *rad-5/clk-2* may have occurred as a consequence of the rescuing extrachromosomal array [39]. Our analysis of wild-type and long-lived *clk-2(qm37)* F2 siblings that were derived from the same F1 parent revealed telomeres of similar lengths (Figure 6a), indicating that *clk-2(qm37)* does not have an immediate effect

on telomere length and that *clk-2* worms do not have extended life spans as a consequence of either long or short telomeres. Furthermore, we have followed telomere length of *clk-2(qm37)* and *rad-5(mn159)* mutant worms for many generations, and our data indicate that the *clk-2(qm37)* and *rad-5(mn159)* mutations do not significantly affect telomere length. If telomere length fluctuates slightly in these mutant strains over time, it also does so in wild-type strains.

Given that *rad-5/clk-2* is a DNA damage checkpoint gene, we decided to examine the single reported viable allele of the budding yeast homolog, *tel2-1*, but could not detect the enhanced sensitivity to DNA damaging agents (data not shown). However, as both *rad-5/clk-2* and *TEL2* are essential genes, the partial loss-of-function missense mutations that are available may not reveal all functions of

these genes, such as checkpoint defects in a *tel2* mutant or telomere defects in a *rad-5/clk-2* mutant. Several previous observations indicate that budding yeast Tel2p may have a checkpoint function. Tel2p has been shown to act in the same telomere length regulation pathway as that of the Tel1p checkpoint protein, which is related to the human ataxia telangiectasia-mutated DNA damage checkpoint protein [28]. In addition, the *tel2-1* mutant displays a weak chromosome loss phenotype, which is often seen with yeast DNA damage checkpoint mutants [41]. It is possible that a checkpoint defect for *tel2-1* may be revealed when combined with other yeast checkpoint mutants, as seen with *tel1* mutations [42, 43]. However, it is also possible that the *C. elegans* homolog of *TEL2*, *rad-5/clk-2*, may have acquired its checkpoint function during the evolution of multicellular organisms.

Our study reveals several DNA damage checkpoint functions for the conserved *rad-5/clk-2* checkpoint gene. Analysis of *rad-5/clk-2* double mutants using a variety of assays demonstrated synthetic effects with the *mrt-2* and *op241* checkpoint mutants (Figure 2). The enhanced radiation sensitivity of *rad-5/clk-2* double mutants may be solely due to an additional defect in the S phase replication checkpoint (Figure 3). Further, the failure to observe an S phase checkpoint defect in *mrt-2* mutants (Figure 3) [32] indicates that *mrt-2/scRAD-1/sprad17* family members may not be required for the S phase checkpoint in multicellular organisms. Similarly, *scrad17/sprad1* mutations are only weakly HU sensitive in fission and budding yeast [31, 44, 45].

Budding yeast Tel2p has been shown to bind a variety of nucleic acid structures in vitro, including single-stranded and unusual four-stranded DNA structures, which may resemble damaged DNA [29, 30]. Thus, the RAD-5/CLK-2 checkpoint protein may act at sites of DNA damage, either early in the pathway, as a primary sensor of DNA damage, or later in the pathway, to help repair damaged DNA. It is curious that DNA polymerase ϵ has been identified in yeast as an S phase checkpoint protein that may be a sensor of DNA damage. Double mutants between polymerase ϵ and canonical DNA damage checkpoint mutants show synthetic lethality and enhanced sensitivity to radiation [21, 22, 46], and we have observed similar synthetic phenotypes for *rad-5/clk-2*. However, DNA polymerase ϵ has only been shown to affect the S phase checkpoint, whereas *rad-5/clk-2* affects both the DNA damage checkpoint and the S phase replication checkpoint (Figures 2 and 3).

This study demonstrates that *C. elegans* genetics is useful for identifying novel DNA damage checkpoint proteins—proteins that are likely to have significance in mammals and, possibly, in oncogenesis. Further studies will be re-

quired to reveal the precise molecular functions of RAD-5/CLK-2 and its homologs.

Supplementary material

Supplementary material including additional methodological detail can be found with this article online at <http://images.cellpress.com/supmat/supmatin.htm>.

Acknowledgements

We are grateful to Michael Glotzer and his lab for housing A.G. and for physical and moral support during *rad-5* rescue experiments. We are furthermore grateful to Bettina Meier and to Ulrike Grüneberg for discussions and for help with yeast experiments; to Jonathan Hodgkin for helpful discussions and for providing *C. elegans* strains; to Yuji Kohara for EST clones; to the *Caenorhabditis* Genetics Center (which is supported by the National Institutes of Health National Center for Research Resources); to Kurt Runge and Tom Petes for yeast strains; and to Erich Nigg, Ludgar Hengst, Barbara Conradt, and Simon Boulton for comments on the manuscript. Work was supported by the Max Planck Society (Erich Nigg), by the Deutsche Forschungsgemeinschaft (DFG) grant 703/1-1 (A.G.), by NIH grant GM-52540 (M.O.H.), by the Medical Research Council, UK (S.A.), and by the Howard Hughes Medical Institute (S.A.). A.G. is grateful to Erich Nigg for providing generous start-up.

References

- Fraser A, James C: **Fermenting debate: do yeast undergo apoptosis?** *Trends Cell Biol* 1998, **8**:219-221.
- Rich T, Allen RL, Wyllie AH: **Defying death after DNA damage.** *Nature* 2000, **407**:777-783.
- Zhou BB, Elledge SJ: **The DNA damage response: putting checkpoints in perspective.** *Nature* 2000, **408**:433-439.
- Caspari T, Dahlen M, Kanter-Smoler G, Lindsay HD, Hofmann K, Papadimitriou K, *et al.*: **Characterization of *Schizosaccharomyces pombe* Hus1: a PCNA-related protein that associates with Rad1 and Rad9.** *Mol Cell Biol* 2000, **20**:1254-1262.
- Kondo T, Matsumoto K, Sugimoto K: **Role of a complex containing Rad17, Mec3, and Ddc1 in the yeast DNA damage checkpoint pathway.** *Mol Cell Biol* 1999, **19**:1136-1143.
- Burtelow MA, Roos-Mattjus PM, Rauen M, Babendure JR, Karnitz LM: **Reconstitution and molecular analysis of the hRad9-hHus1-hRad1 (9-1-1) DNA damage responsive checkpoint complex.** *J Biol Chem* 2001, **276**:25903-25909.
- Rauen M, Burtelow MA, Dufault VM, Karnitz LM: **The human checkpoint protein hRad17 interacts with the PCNA-like proteins hRad1, hHus1, and hRad9.** *J Biol Chem* 2000, **275**:29767-29771.
- Venclovas C, Thelen MP: **Structure-based predictions of Rad1, Rad9, Hus1 and Rad17 participation in sliding clamp and clamp-loading complexes.** *Nucleic Acids Res* 2000, **28**:2481-2493.
- Kondo T, Wakayama T, Naiki T, Matsumoto K, Sugimoto K: **Recruitment of mec1 and ddc1 checkpoint proteins to double-strand breaks through distinct mechanisms.** *Science* 2001, **294**:867-870.
- Melo JA, Cohen J, Toczyski DP: **Two checkpoint complexes are independently recruited to sites of DNA damage in vivo.** *Genes Dev* 2001, **15**:2809-2821.
- Edwards RJ, Bentley NJ, Carr AM: **A Rad3-Rad26 complex responds to DNA damage independently of other checkpoint proteins.** *Nat Cell Biol* 1999, **1**:393-398.
- Krause SA, Loupart ML, Vass S, Schoenfelder S, Harrison S, Heck MM: **Loss of cell cycle checkpoint control in *drosophila rfc4* mutants.** *Mol Cell Biol* 2001, **21**:5156-5168.
- Kim HS, Brill SJ: **Rfc4 interacts with Rpa1 and is required for both DNA replication and DNA damage checkpoints in *Saccharomyces cerevisiae*.** *Mol Cell Biol* 2001, **21**:3725-3737.
- Naiki T, Shimomura T, Kondo T, Matsumoto K, Sugimoto K: **Rfc5, in cooperation with rad24, controls DNA damage checkpoints throughout the cell cycle in *Saccharomyces cerevisiae*.** *Mol Cell Biol* 2000, **20**:5888-5896.
- Green CM, Erdjument-Bromage H, Tempst P, Lowndes NF: **A novel Rad24 checkpoint protein complex closely related to replication factor C.** *Curr Biol* 2000, **10**:39-42.
- Shimada M, Okuzaki D, Tanaka S, Tougan T, Tamai KK, Shimoda C, *et al.*: **Replication factor C3 of *Schizosaccharomyces***

- pombe**, a small subunit of replication factor C complex, plays a role in both replication and damage checkpoints. *Mol Biol Cell* 1999, **10**:3991-4003.
17. Lindsey-Boltz LA, Bermudez VP, Hurwitz J, Sancar A: **Purification and characterization of human DNA damage checkpoint Rad complexes.** *Proc Natl Acad Sci USA* 2001, **98**:11236-11241.
 18. D'Amours D, Jackson SP: **The yeast Xrs2 complex functions in S phase checkpoint regulation.** *Genes Dev* 2001, **15**:2238-2249.
 19. Grenon M, Gilbert C, Lowndes NF: **Checkpoint activation in response to double-strand breaks requires the Mre11/Rad50/Xrs2 complex.** *Nat Cell Biol* 2001, **3**:844-847.
 20. Usui T, Ogawa H, Petrini JH: **A DNA damage response pathway controlled by Tel1 and the Mre11 complex.** *Mol Cell* 2001, **7**:1255-1266.
 21. Navas TA, Sanchez Y, Elledge SJ: **RAD9 and DNA polymerase epsilon form parallel sensory branches for transducing the DNA damage checkpoint signal in *Saccharomyces cerevisiae*.** *Genes Dev* 1996, **10**:2632-2643.
 22. Navas TA, Zhou Z, Elledge SJ: **DNA polymerase epsilon links the DNA replication machinery to the S phase checkpoint.** *Cell* 1995, **80**:29-39.
 23. Dasika GK, Lin SC, Zhao S, Sung P, Tomkinson A, Lee EY: **DNA damage-induced cell cycle checkpoints and DNA strand break repair in development and tumorigenesis.** *Oncogene* 1999, **18**:7883-7899.
 24. Gartner A, Milstein S, Ahmed S, Hodgkin J, Hengartner MO: **A conserved checkpoint pathway mediates DNA damage-induced apoptosis and cell cycle arrest in *C. elegans*.** *Mol Cell* 2000, **5**:435-443.
 25. Ahmed S, Hodgkin J: **MRT-2 checkpoint protein is required for germline immortality and telomere replication in *C. elegans*.** *Nature* 2000, **403**:159-164.
 26. Hartman PS, Herman RK: **Radiation-sensitive mutants of *Caenorhabditis elegans*.** *Genetics* 1982, **102**:159-178.
 27. Lakowski B, Hekimi S: **Determination of life-span in *Caenorhabditis elegans* by four clock genes.** *Science* 1996, **272**:1010-1013.
 28. Runge KW, Zakian VA: **TEL2, an essential gene required for telomere length regulation and telomere position effect in *Saccharomyces cerevisiae*.** *Mol Cell Biol* 1996, **16**:3094-3105.
 29. Kota RS, Runge KW: **The yeast telomere length regulator TEL2 encodes a protein that binds to telomeric DNA.** *Nucleic Acids Res* 1998, **26**:1528-1535.
 30. Kota RS, Runge KW: **Tel2p, a regulator of yeast telomeric length in vivo, binds to single-stranded telomeric DNA in vitro.** *Chromosoma* 1999, **108**:278-290.
 31. Enoch T, Carr AM, Nurse P: **Fission yeast genes involved in coupling mitosis to completion of DNA replication.** *Genes Dev* 1992, **6**:2035-2046.
 32. MacQueen AJ, Villeneuve AM: **Nuclear reorganization and homologous chromosome pairing during meiotic prophase require *C. elegans chk-2*.** *Genes Dev* 2001, **15**:1674-1687.
 33. Ketting RF, Plasterk RH: **A genetic link between co-suppression and RNA interference in *C. elegans*.** *Nature* 2000, **404**:296-298.
 34. Dernburg AF, Zalevsky J, Colaiacovo MP, Villeneuve AM: **Transgene-mediated cosuppression in the *C. elegans* germ line.** *Genes Dev* 2000, **14**:1578-1583.
 35. Hill AA, Hunter CP, Tsung BT, Tucker-Kellogg G, Brown EL: **Genomic analysis of gene expression in *C. elegans*.** *Science* 2000, **290**:809-812.
 36. Lustig AJ, Petes TD: **Identification of yeast mutants with altered telomere structure.** *Proc Natl Acad Sci USA* 1986, **83**:1398-1402.
 37. Riha K, McKnight TD, Griffing LR, Shippen DE: **Living with genome instability: plant responses to telomere dysfunction.** *Science* 2001, **291**:1797-1800.
 38. Gonczy P, Echeverri G, Oegema K, Coulson A, Jones SJ, Copley RR, et al.: **Functional genomic analysis of cell division in *C. elegans* using RNAi of genes on chromosome III.** *Nature* 2000, **408**:331-336.
 39. Benard C, McCright B, Zhang Y, Felkai S, Lakowski B, Hekimi S: **The *C. elegans* maternal-effect gene clk-2 is essential for embryonic development, encodes a protein homologous to yeast Tel2p and affects telomere length.** *Development* 2001, **128**:4045-4055.
 40. Lim C-S, Mian S, Dernburg AF, Campisi J: ***C. elegans clk-2*, a gene that limits life span, encodes a regulator of telomere metabolism similar to yeast telomere binding protein Tel2p.** *Curr Biol* 2001, **11**:1706-1710.
 41. Klein HL: **Mutations in recombinational repair and in checkpoint control genes suppress the lethal combination of *srs2Delta* with other DNA repair genes in *Saccharomyces cerevisiae*.** *Genetics* 2001, **157**:557-565.
 42. Greenwell PW, Kronmal SL, Porter SE, Gassenhuber J, Obermaier B, Petes TD: **TEL1, a gene involved in controlling telomere length in *S. cerevisiae*, is homologous to the human ataxia telangiectasia gene.** *Cell* 1995, **82**:823-829.
 43. Morrow DM, Tagle DA, Shiloh Y, Collins FS, Hieter P: **TEL1, an *S. cerevisiae* homolog of the human gene mutated in ataxia telangiectasia, is functionally related to the yeast checkpoint gene MEC1.** *Cell* 1995, **82**:831-840.
 44. Paulovich AG, Margulies RU, Garvik BM, Hartwell LH: **RAD9, RAD17, and RAD24 are required for S phase regulation in *Saccharomyces cerevisiae* in response to DNA damage.** *Genetics* 1997, **145**:45-62.
 45. Lydall D, Weinert T: **G2/M checkpoint genes of *Saccharomyces cerevisiae*: further evidence for roles in DNA replication and/or repair.** *Mol Gen Genet* 1997, **256**:638-651.
 46. Feng W, D'Urso G: **Schizosaccharomyces pombe cells lacking the amino-terminal catalytic domains of DNA polymerase epsilon are viable but require the DNA damage checkpoint control.** *Mol Cell Biol* 2001, **21**:4495-4504.
 47. MacQueen AJ, Villeneuve AM: **Nuclear reorganization and homologous chromosome pairing during meiotic prophase require *C. elegans chk-2*.** *Genes Dev* 2001, **15**:1674-1687.
 48. Wicks SR, Yeh RT, Gish WR, Waterston RH, Plasterk RH: **Rapid gene mapping in *Caenorhabditis elegans* using a high density polymorphism map.** *Nat Genet* 2001, **28**:160-164.
 49. Wicky C, Villeneuve AM, Lauper N, Codourey L, Tobler H, Muller F: **Telomeric repeats (TTAGG)_n are sufficient for chromosome capping function in *Caenorhabditis elegans*.** *Proc Natl Acad Sci USA* 1996, **93**:8983-8988.