ISOLATION AND CHARACTERIZATION OF GENES INVOLVED IN DNA DAMAGE RESPONSE AND TELOMERE MAINTENANCE IN *CAENORHABDITIS ELEGANS*

Julie Ann Boerckel

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Approved by:
Advisor: Shawn Ahmed
Reader: Greg Copenhaver
Reader: Bob Goldstein
Reader: Tom Petes
Reader: Jeff Sekelsky
ABSTRACT

JULIE ANN BOERCKEL: Isolation and Characterization of Genes Involved in DNA Damage Response and Telomere Maintenance in *Caenorhabditis elegans* (Under the direction of Dr. Shawn Ahmed, PhD)

Many cells, including somatic cells, are mortal, they divide a set number of times and then senesce or undergo cell death. However, germline and cancer cells have the ability to divide indefinitely and are thus considered immortal. In an attempt to better understand how cellular immortality is maintained I have been studying the pathways needed to maintain germline immortality in the nematode *C. elegans*. Several genes in various genome stability pathways have been identified to be exclusively activated in both cancer and germ cells suggesting they are important for cellular immortality. One pathway is the length maintenance of telomeres, the ends of chromosomes. Previously, members of the Rad9/Rad1/Hus1 DNA damage response complex were identified in *C. elegans* as being essential not only for germline immortality but also telomere maintenance. Based on these findings, two independent forward genetic screens were conducted to identify other genes involved in DNA damage response and telomere maintenance by first selecting for mutants that are hypersensitive to ionizing radiation (which causes double-strand DNA breaks) or resistant to hydroxyurea (which triggers S-phase cell cycle arrest) and then for the Mortal Germline phenotype, sterility after propagation for multiple generations. One mutant allele, *yp4*, was isolated as being resistant to hydroxyurea and is highly defective for cell cycle arrest in response to both ionizing radiation and hydroxyurea. Further characterization of
yp4 revealed that it is required for germline immortality and displays phenotypes indicative of progressive telomere shortening. Additionally, a reverse genetic screen identified hpr-17, which encodes a Rad17 homolog, as being the RFC clamp loader that facilitates the Rad9/Rad1/Hus1 complex activity in response to DNA damage and in telomerase-mediated telomere replication. Aside from the two genes identified to be involved in telomere maintenance, 15 radiation hypersensitive mutants were isolated that carry mutations in genes that are essential for germline immortality at 25°C, whereas 32 hydroxyurea resistant mutants were isolated that are essential for germline immortality at all temperatures. Further analysis of DNA damage response pathways revealed the mutants isolated represent a number of genes that play roles in cell cycle arrest and/or apoptosis. These mortal germline mutants suggest that at least two DNA damage response pathways, independent of telomerase, are required for maintenance of germ cell immortality.
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ABBREVIATIONS AND SYMBOLS

% Percent
°C Degree Celsius
# Number
9-1-1 Rad9/Rad1/Hus1 complex
ATP Adenosine Triphosphate
bp Basepair
C. elegans Caenorhabditis elegans
DAPI 4’,6-diamidino-2-phenylindole
DSB Double strand break
DDR DNA damage response
DNA Deoxyribonucleic Acid
dNTP Deoxyribonucleotide Triphosphate
EMS Ethyl methane sulfonate
Gy Gray
HU Hydroxyurea
IR Ionizing Radiation
kb Kilobase
MMS Methyl methane sulfonate
Mrt Mortal Germline
mM Millimolar
MRN Mre11/Rad50/NBS1 complex
N/D Not Determined
<table>
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<tr>
<td>NER</td>
<td>Nucleotide Excision Repair</td>
</tr>
<tr>
<td>NGM</td>
<td>Nematode Growth Medium</td>
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<td>NHEJ</td>
<td>Non-homologous end joining</td>
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<tr>
<td>p</td>
<td>p-value</td>
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<tr>
<td>PCNA</td>
<td>Proliferating Cell Nuclear Antigen</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>Rad</td>
<td>Radiation hypersensitivity</td>
</tr>
<tr>
<td>RFC</td>
<td>Replication Factor C</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>t-loop</td>
<td>Telomeric loop</td>
</tr>
<tr>
<td>ts</td>
<td>Temperature sensitive</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet radiation</td>
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<td>µl</td>
<td>Microliter</td>
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Chapter 1

INTRODUCTION

Telomeres protect the ends of chromosomes

The ends of chromosomes, telomeres, are essential for preventing terminal DNA loss. Telomeres typically consist of stretches of DNA composed of short repetitive sequences that are TG rich in the 5’ to 3’ direction toward the end of the chromosome. The sequence was first characterized in Tetrahymena thermophila as T₂G₄ (Blackburn and Gall 1978) and is conserved between species, for example in C. elegans and humans the sequence repeat varies by only one base, TTAGGC and TTAGGG, respectively (Moyzis, Buckingham et al. 1988; Wicky, Villeneuve et al. 1996). Telomere length, on the other hand, varies widely between organisms: S. cerevisiae telomeres are approximately 300 bp; C. elegans telomeres range from 2-5 kb; Arabidopsis telomeres are 2-4 kb; mouse telomeres are 40-150 kb; and human telomeres are 10-15 kb (Moyzis, Buckingham et al. 1988; Kipling and Cooke 1990; Starling, Maule et al. 1990; Richards, Chao et al. 1992; Wicky, Villeneuve et al. 1996). Despite the fact that telomeres are highly conserved chromosome structures, Drosophila relies on four non-long terminal repeat retrotransposons to maintain the ends of their chromosomes via transposition to a total length of approximately 14.5 kb (Levis, Ganesan et al. 1993). Regardless, telomeres protect the ends of chromosomes and are necessary elements for maintaining genome integrity.
Telomeres are replicated using the same DNA polymerase machinery that duplicates the rest of the genome, which results in an end replication problem: lagging strand synthesis results in a 3’ overhang when the RNA primer is removed (Figure 1.1) (Wright, Tesmer et al. 1997; Ohki, Tsurimoto et al. 2001). Progressive telomere shortening results and eventually the telomere degrades to a critical length which triggers a DNA damage response (DDR), resulting in cellular senescence or cell death (Harley, Futcher et al. 1990; Counter, Avilion et al. 1992). One way to combat progressive telomere shortening is through the extension of telomeres through de novo telomere repeat synthesis performed by a ribonucleoprotein called telomerase (Figure 1.1). Telomerase is a highly conserved reverse transcriptase consisting of a catalytic protein subunit and an RNA component (Greider and Blackburn 1989; Feng, Funk et al. 1995; Nakamura, Morin et al. 1997; Lue 1999; Masutomi, Yu et al. 2003; Meier, Clejan et al. 2006). The RNA component contains a short RNA sequence complementary to the 3’ telomeric repeats, which provides the necessary primer for telomere extension (Greider and Blackburn 1989; Feng, Funk et al. 1995; Lue 1999). Although the RNA component is essential for the function of telomerase, it has only been identified in Tetrahymena, yeast, mice and humans and is still unidentified in C. elegans and Arabidopsis (Greider and Blackburn 1989; Feng, Funk et al. 1995; Blasco, Lee et al. 1997; Lue 1999; Ritchie, Mallory et al. 1999). The lack of telomerase in yeast, nematodes, plants and mammals results in progressive telomere shortening and either cellular senescence or sterility (Lendvay, Morris et al. 1996; Blasco, Lee et al. 1997; Nakamura, Morin et al. 1997; Fitzgerald, Riha et al. 1999; Masutomi, Yu et al. 2003; Meier, Clejan et al. 2006). An example of this is in primary human cells and most human somatic
Figure 1.1: Telomeres shorten during DNA replication

A 3’ overhang remains after degradation of the RNA primer (represented by a dotted line) on the lagging strand during DNA synthesis of the telomere. If not lengthened, the end will shorten during progressive cell cycles as diagramed on the left side of the pathway. However, telomerase can extend the ends of the chromosome through de novo DNA synthesis as diagramed on the right side of the pathway. Black lines represent the parental strands and gray lines are the newly synthesized strand from DNA replication.
cells which have a limited proliferative capacity and in some cell types results from progressive telomere shortening (Hayflick and Moorhead 1961; Harley, Futcher et al. 1990). However, telomerase has been shown to be expressed at low levels in cycling human cells to maintain telomere length (Masutomi, Yu et al. 2003). Interestingly, the addition of telomerase can immortalize primary cells in culture resulting in their ability to divide indefinitely and in the extension of their lifespan (Kim, Piatyszek et al. 1994; Meyerson, Counter et al. 1997; Bodnar, Ouellette et al. 1998; Vaziri and Benchimol 1998). Telomerase is upregulated in most but not all cancer cells resulting in telomere length extension and immortality (Kim, Piatyszek et al. 1994; Meyerson, Counter et al. 1997; Bodnar, Ouellette et al. 1998; Vaziri and Benchimol 1998). In conclusion, telomerase is important in maintaining proper telomere length and is an essential pathway in cellular immortality.

*Formation of a T-loop helps protect telomeric ends*

Telomeres terminate in a G-rich 3’ overhang that can vary in length from chromosome to chromosome (Chai, Du et al. 2006). The overhang is a result of DNA replication of the lagging-strand that leaves a 3’ overhang or from processing of the blunt ended leading strand by either single strand degradation or the addition of nucleotides (Wright, Tesmer et al. 1997; Li, Lejnine et al. 1998; Ohki, Tsurimoto et al. 2001). The overhang can loop back and invade the double stranded telomeric DNA to form a telomeric loop (T-loop) that resembles a nicked Holliday junction intermediate (Figure 1.2) (Griffith, Comeau et al. 1999; Murti and Prescott 1999; de Lange 2002). Many proteins are involved in maintaining the structure of the t-loop. For example, Pot1 (protection of telomere 1) is a conserved single-stranded binding protein that binds the telomeric G-overhang
Figure 1.2: Telomeres form a t-loop to protect the ends of chromosomes from nucleolytic degradation

The telomeric 3’ overhang invades the double-stranded telomere DNA to form a T-loop which protects the ends of chromosomes as being recognized as DSB and triggering a damage response.

(Baumann and Cech 2001; Shakirov, Surovtseva et al. 2005). In fission yeast and humans there is one Pot1 gene and deletion results in immediate chromosome instability and rapid loss of telomeric DNA suggesting a role in telomere end capping (Baumann and Cech 2001; Veldman, Etheridge et al. 2004). Whereas, plants have two Pot1 homologs and a third that is alternatively spliced, and mice have two Pot1 genes, all of which perform distinct functions at the telomere (Shakirov, Surovtseva et al. 2005; Hockemeyer, Daniels et al. 2006; Wu, Multani et al. 2006). POT1 associates with TRF1 and TRF2 and acts in the same pathway for telomere capping (Loayza and De Lange 2003; Yang, Zheng et al. 2005). TRF1 and TRF2 both bind double-stranded telomeric DNA to form the t-loop (Chong, van Steensel et al. 1995; Bianchi, Smith et al. 1997; van Steensel and de Lange 1997; van Steensel, Smogorzewska et al. 1998; Griffith, Comeau et al. 1999; Ohki, Tsurimoto et al. 2001). TRF1 acts at telomeres to regulate telomere length whereas TRF2 is involved in telomere integrity (van Steensel and de Lange 1997; van Steensel, Smogorzewska et al. 1998). T-loops help to protect the chromosome ends from being recognized as double-strand breaks (DSB) by DDR proteins and from nucleolytic degradation.
Consequences of improper telomere length maintenance

If telomere length is not maintained by loss of telomerase activity or other proteins involved in telomere elongation, they progressively shorten resulting in cellular senescence or cell death (Harley, Futcher et al. 1990; Lendvay, Morris et al. 1996; Blasco, Lee et al. 1997; Fitzgerald, Riha et al. 1999; Hackett and Greider 2003; Shay and Wright 2005; Meier, Clejan et al. 2006). A short or uncapped telomere can be recognized as a DSB that triggers the DDR machinery which tries to repair the DNA by use of repair pathways such as non-homologous end joining (NHEJ) or homologous recombination, resulting in end-to-end chromosome fusions (Espejel, Franco et al. 2002; Jaco, Munoz et al. 2003; Riha and Shippen 2003; Heacock, Spangler et al. 2004). In yeast, plants and mammalian cells these fusions create dicentric chromosomes that are not segregated properly during cell division resulting in a breakage-fusion-bridge cycle (McClintock 1941; Watson 1972; Hackett, Feldser et al. 2001; McKnight, Riha et al. 2002; Shay and Wright 2005). However, in C. elegans, the chromosomes are holocentric and the fusions can be stably maintained and transmitted (Ahmed and Hodgkin 2000; Meier, Clejan et al. 2006). Interestingly, the shortening of only one telomere is required to trigger the breakage-fusion-bridge cycle resulting in chromosome rearrangements and genome instability in humans (Sabatier, Ricoul et al. 2005).

Since telomere shortening is progressive, results in senescence and can be a result of the lack of telomerase, telomere length maintenance has been associated with the aging of somatic cells partially due to the fact that most somatic cells lack a high level of telomerase activity (Harley, Futcher et al. 1990; Blasco, Lee et al. 1997; Rudolph, Chang et al. 1999). In
In contrast, telomere length in *C. elegans* has been shown to not correlate with lifespan (Ahmed and Hodgkin 2000; Raices, Maruyama et al. 2005; Meier, Clejan et al. 2006). The activation of telomerase can elongate telomeres, rescuing genome instability and leading to the immortalization of the cell line (Bodnar, Ouellette et al. 1998; Vaziri and Benchimol 1998). One example in nature is tumor cells that display an up-regulation in telomerase activity contributing to its immortality phenotype (Kim, Piatyszek et al. 1994; Meyerson, Counter et al. 1997; Shay and Wright 2005). Germ cells also have active telomerase, including the *C. elegans* germline, making them immortal from generation to generation (Mantell and Greider 1994; Hiyama, Hirai et al. 1995; Wright, Piatyszek et al. 1996; Eisenhauer, Gerstein et al. 1997; Meier, Clejan et al. 2006). In conclusion, telomere length is maintained primarily through the activity of telomerase and is important for cellular immortality.

**Several DNA damage response genes are involved in telomere maintenance**

Mammalian cells lacking TRF2 have uncapped telomeres that resemble DSBs and trigger responses by various DDR proteins such as 53BP1, γ-H2AX, Rad17, ATM and Mre11 (Takai, Smogorzewska et al. 2003). TRF2 also associates with known double-strand break repair proteins including Ku70 (Song, Jung et al. 2000), the Mre11/Rad50/Nbs1 (MRN) complex (Zhu, Kuster et al. 2000), the Werner (WRN) helicase (Opresko, von Kobbe et al. 2002; Machwe, Xiao et al. 2004) and ATM (Karlseder, Hoke et al. 2004). In diverse organisms, mutations in these genes and many other DDR genes results in telomere dysfunction and chromosomal abnormalities (d'Adda di Fagagna, Teo et al. 2004; Slijepcevic and Al-Wahiby 2005). In vitro experiments with synchronized cells have also
found DDR proteins to associate with the telomeres mostly during S phase when telomeres are being replicated. For example, the Rad9-Hus1-Rad1 (9-1-1) complex subunits and ERCC3 associate in late S/G2 phase; MRE11, ATM and ATR associate throughout S-phase; and RAD51 and RAD52 associate throughout the cell cycle (Verdun and Karlseder 2006). Whether their association with telomeres is a consequence of their role in DNA damage response and DNA replication or a specific role at telomeres is still unknown. Regardless, in vivo and in vitro studies in yeast, C. elegans and mammals do suggest possible roles for many DDR genes in various telomere maintenance pathways.

The Ku heterodimer (KU70/KU80) is essential for the canonical NHEJ double-strand break repair pathway. In S. cerevisiae, both yku70 and yku80 mutants display drastic telomere shortening but are viable (Boulton and Jackson 1996; Porter, Greenwell et al. 1996; Polotnianka, Li et al. 1998). However, double mutants with telomerase are inviable suggesting that yeast Ku functions in a telomere maintenance pathway that is separable from that of telomerase (Nugent, Bosco et al. 1998). Additionally, separation-of-function yeast Ku mutants demonstrate that the NHEJ and telomere maintenance pathways are regulated by different functions of Ku (Bertuch and Lundblad 2003). Although the yeast Ku70/80 heterodimer localizes to telomeres, it can be recruited to other sites in the genome in response to DNA strand breaks (Gravel, Larrivee et al. 1998; Martin, Laroche et al. 1999). Interestingly, C. elegans strains deficient for either Ku subunit do not display telomere defects (M. Lowden, unpublished). In mammalian cells, deletion of Ku or the DNA dependent protein kinase catalytic subunit (DNA-PKcs), which associates with Ku during NHEJ, results in profound telomere loss and inviability suggesting a role in telomere capping (Samper, Goytisolo et al. 2000; d'Adda di Fagagna, Hande et al. 2001; Espejel,
Franco et al. 2002; Espejel, Franco et al. 2002; Bailey, Brenneman et al. 2004; Jaco, Munoz et al. 2004; Myung, Ghosh et al. 2004). The Ku heterodimer and DNA-PKcs have been shown to bind human telomeres and this association is not significantly affected by DNA damage (Hsu, Gilley et al. 1999; Hsu, Gilley et al. 2000; d'Adda di Fagagna, Hande et al. 2001). Thus, Ku has a role in telomere maintenance functions that are independent of telomerase in yeast and mammalian cells. However, Ku can also facilitate telomerase activity in yeast via its interaction with the telomerase RNA (Stellwagen, Haimberger et al. 2003).

The MRN complex participates in DNA damage signaling and recombinational repair (D'Amours and Jackson 2002; Zhang, Zhou et al. 2006). In yeast, the MRN complex has been shown to play a role in telomerase-mediated telomere elongation as double mutants do not display a greater telomere loss compared to single mutants (Nugent, Bosco et al. 1998; Tsukamoto, Taggart et al. 2001). Targeting of catalytically active telomerase to the elongated telomeres rescued the senescence phenotypes of these mutants suggesting that the MRN complex’s role is in the recruitment of telomerase, not in its activation (Tsukamoto, Taggart et al. 2001). Additionally, the complex is recruited to telomeres in late S-phase and is required for the S-phase recruitment of ATR to telomeres (Takata, Tanaka et al. 2005). Interestingly Drosophila MRN mutants display telomere shortening and end-to-end chromosome fusions suggesting a role in end capping since Drosophila do not elongate telomeres via telomerase (Bi, Wei et al. 2004; Ciapponi, Cenci et al. 2004). Additionally, mammalian NBS1 deletion cells display shortened telomeres and chromosome instability resulting from breakage-fusion-bridge cycles and telomere loss was not found to correlate with the radiosensitivity of the MRN complex mutants, suggesting a separation of function.
for the two pathways of the complex (Bai and Murnane 2003). Mammalian MRE11 and RAD50 are associated with telomeres during interphase when they are capped and NBS1 is associated with telomeres and TRF2 in S-phase when they are replicated. This data agrees with data in yeast and Drosophila that the MRN complex is involved in both telomere replication and the telomere capping structure (Zhu, Kuster et al. 2000).

Werner syndrome results in premature aging in humans as a consequence of mutations that truncate the WRN RecQ-like helicase resulting in a lack of function. WRN is a 3’ to 5’ exonuclease that degrades 3’ recessed strands of double-stranded DNA or a DNA-RNA heteroduplex in mammalian cells (Huang, Li et al. 1998; Huang, Beresten et al. 2000). Cells from Werner’s patients display DNA repair defects and undergo premature senescence as a consequence of defects in telomere metabolism (Huang, Li et al. 1998; Wyllie, Jones et al. 2000). The shortened lifespan of Werner’s cells can be rescued by the addition of telomerase suggesting that the major cause of senescence is due to telomere dysfunction and not unrepaired DNA damage (Wyllie, Jones et al. 2000). In vitro studies show that TRF2 can physically interact with the C-terminal domain of WRN and telomeric duplexes, specifically in a D-loop structure that are prebound by Replication Protein A, and TRF2 can recruit and stimulate the helicase activity of WRN (Opresko, von Kobbe et al. 2002; Machwe, Xiao et al. 2004). Additionally, telomeric duplexes prebound with Pot1 can stimulate WRN to unwind telomeric forked duplexes and D-loop structures (Opresko, Mason et al. 2005). Thus, the exonuclease WRN may participate in the processing or resolution of the T-loop and abnormal telomeric DNA structures (Fouche, Ozgur et al. 2006).
Many DDR genes are direct targets of the ataxia telangiectasia mutated (ATM) kinase that is a transducer of the DNA-damage signal (Silverman, Takai et al. 2004). In yeast, deletion of ATM results in short, stable telomeres (Matsuura, Naito et al. 1999; Ritchie, Mallory et al. 1999). Additionally, in *S. cerevisiae* double mutants of *atm* and *tlc1*, the RNA subunit of telomerase, display delayed loss of viability compared to *tlc1* single mutants (Ritchie, Mallory et al. 1999). Similarly, *atm tert* (telomerase reverse transcriptase) double mutants in *Arabidopsis* also display an abrupt, early onset of genome instability (Vespa, Couvillion et al. 2005). Based on double mutant analysis in yeast and plants, ATM does not act in the same pathway as telomerase but rather in telomere capping. Additionally, studies in mammals also suggests a role in telomere capping based on ATM’s association with telomeres and its interactions with TRF2 which inhibits the autophosphorylation of ATM and thus its ability to trigger a damage response (Karlseder, Hoke et al. 2004; Verdun and Karlseder 2006). Mammalian ATM can be activated in response to telomere dysfunction as observed in *TRF2*<sup>−/−</sup>*Lig4*<sup>−/−</sup>*p53*<sup>−/−</sup> cells that result in telomeric DNA degradation and triggers various DNA damage response factors (Celli and de Lange 2005). In conclusion, ATM is important for telomere homeostasis through its ability to trigger a damage response to uncapped telomeres.

Lastly, the 9-1-1 PCNA (Proliferating Cell Nuclear Antigen)-like sliding clamp responds to DNA damage and triggers DNA repair, cell cycle arrest and apoptosis (al-Khodairy and Carr 1992; Rowley, Subramani et al. 1992; Caspari, Dahlen et al. 2000). In yeast deletion of 9-1-1 complex subunits results in short but stable telomeres (Dahlen, Olsson et al. 1998; Longhese, Paciotti et al. 2000; Nakamura, Moser et al. 2002). Chromatin immunoprecipitation analysis in *S. pombe* indicated that the 9-1-1 complex interacts weakly
with telomeric DNA (Nakamura, Moser et al. 2002). In contrast to yeast 9-1-1 complex mutants, *C. elegans* mutants deficient for the 9-1-1 complex subunits results in sterility after growth for multiple generations, a Mortal Germline (Mrt) phenotype, due to progressive telomere shortening that leads to telomere uncapping and end-to-end chromosome fusions (Ahmed and Hodgkin 2000; Hofmann, Milstein et al. 2002). The *C. elegans* 9-1-1 complex may act in the same telomere maintenance pathway as that of telomerase, given that double mutants of *trt-1*, the *C. elegans* reverse transcriptase subunit of telomerase, with *mrt-2*, a 9-1-1 complex subunit, display the same telomere shortening rate as the corresponding single mutants (Meier, Clejan et al. 2006). Mammalian cells deficient for HUS1 or RAD9 are inviable and exhibit chromosome abnormalities, end-to-end chromosome fusions and a dramatic reduction in telomere length (Francia, Weiss et al. 2006; Pandita, Sharma et al. 2006). Furthermore, members of the 9-1-1 complex interact with telomeric DNA *in vivo* and immunoprecipitate with telomerase activity (Francia, Weiss et al. 2006; Verdun and Karlseder 2006). Additionally, *in vitro* telomerase activity is reduced in cells lacking HUS1 or RAD9, suggesting that the mammalian 9-1-1 complex may be required for telomerase activity *in vivo* (Francia, Weiss et al. 2006). Studies in yeast, nematodes and mammals suggest a role in telomerase-mediated telomere replication for the 9-1-1 complex.

Many factors are involved in maintaining the integrity of telomeric DNA. Besides telomerase and telomere binding proteins, proteins initially identified as DDR genes have been implemented in telomere maintenance. For example, the Ku heterodimer and ATM are associated with the telomere independent of telomerase whereas the MRN and 9-1-1 complexes are involved in telomerase-mediated telomere replication. The MRN complex is also involved in telomeric end capping in addition to ATM that can also trigger a damage
response to uncapped telomeres. WRN is a helicase that aids in the processing and resolution of the t-loop. The roles of DDR genes at telomeres, though diverse, are functionally related to their roles at sites of DNA damage.

**Similar genes are activated in cancer and germ cells suggestive of roles in immortality**

Studies show that many genes are upregulated in cancer cells, some of which are also expressed in germ cells. Germ cells are important in the propagation of a species because their genomic content is passed on to the next generation and they are considered to be an immortal cell lineage. These genes that are upregulated in both human cancer and germ cells and not upregulated in more then two other non-germline normal tissues are known as cancer/testis antigens (Simpson, Caballero et al. 2005). Analysis of these genes revealed about half are located on the X chromosome, whereas the rest are dispersed among the autosomes (Simpson, Caballero et al. 2005). In humans it is predicted that approximately 10% of the genes on the X chromosome are cancer/testis genes (Ross, Grafham et al. 2005). Characterization of these genes has revealed that they are involved in various pathways that affect genome stability, for example: gankyrin contributes to unscheduled entry into the cell cycle and escape from cell cycle arrest and/or apoptosis (Li and Tsai 2002); SPO11 is involved in creating DSBs for recombination (Keeney, Giroux et al. 1997); PLU-1 is a transcription co-repressor that regulates gene expression in the germline (Tan, Shaw et al. 2003); and BRDT causes chromatin to compact following acetylation of histones (Pivot-Pajot, Caron et al. 2003). Additionally, telomerase is activated in most cancer cells and germ cells (Kim, Piatyszek et al. 1994; Mantell and Greider 1994; Hiyama, Hirai et al. 1995; Wright, Piatyszek et al. 1996; Eisenhauer, Gerstein et al. 1997; Meyerson, Counter et al. 2003).
Dissertation goals

Studies to understand cellular immortality have elucidated several pathways, including telomere maintenance and DNA damage response, to be essential to maintain immortality. Some genes play roles in both these pathways, including the 9-1-1 DNA damage response complex that has been shown in yeast, C. elegans and mammals to act at telomeres. However, experiments to understand the role of the complex in telomerase activity at telomeres are difficult in yeast because telomerase is still active thus telomeres do not reach critical lengths and in mammalian cells because the complex is essential and severe effects on telomere length preclude genetic pathway analysis with mutations that affect telomerase. However, such experiments can be conducted in C. elegans 9-1-1 complex mutants, as they are viable and display progressive telomere shortening phenotypes similar to those of telomerase mutants. Therefore, this phenotype can be utilized for the investigation of other genes involved in both DDR and telomere maintenance pathways in an attempt to better understand cellular immortality, which is the focus of this thesis. The aims include: a forward genetic screen to isolate mutants involved in DSB repair in response to IR and germline immortality (similar to mrt-2) and a reverse genetic screen utilizing RNAi to assay known genes involved in DDR and chromosome structure for their involvement in germline immortality; a second forward genetic screen for genes involved in...
S-phase cell cycle arrest that are essential for telomere maintenance; and a reverse genetic approach to determine if hpr-17, a RFC clamp loader, facilitates the function of the 9-1-1 complex at both DSBs and in telomerase-mediated telomere replication.
Chapter 2
IDENTIFICATION OF RADIATION HYPERSENSITIVE MUTANTS

Background and Significance

The Rad9/Rad1/Hus1 (9-1-1) complex responds to various forms of DNA damage, including ionizing radiation (IR) that results in DSB. The 9-1-1 complex subunits were initially identified in *S. pombe* in a genetic screen for radiosensitive mutants (Lieberman, Riley et al. 1989). Further investigation of the *rad1* mutant revealed that it was hypersensitive to IR and failed to arrest after exposure to both IR and HU (Rowley, Subramani et al. 1992). Rad1 associates with Hus1 and Rad9 to form a heterotrimeric ring structure that resembles the PCNA sliding clamp that is loaded onto double-stranded DNA (Volkmer and Karnitz 1999; Caspari, Dahlen et al. 2000; Venclovas and Thelen 2000; Lindsey-Boltz, Bermudez et al. 2001; Griffith, Lindsey-Boltz et al. 2002; Yang and Zou 2006). In yeast and mammalian cells, the 9-1-1 complex localizes to the nucleus, is phosphorylated, and forms foci at sites of DNA double strand breaks in response to DNA damaging agents (Volkmer and Karnitz 1999; Burtelow, Kaufmann et al. 2000; Caspari, Dahlen et al. 2000; Greer, Besley et al. 2003; Meister, Poidevin et al. 2003; Pandita, Sharma et al. 2006). In response to DNA damage, the 9-1-1 complex can phosphorylate other proteins such as Rad51 or Chk1 to initiate DNA repair, cell cycle arrest or apoptosis (Dang, Bao et al. 2005; Pandita, Sharma et al. 2006).
In *C. elegans*, homologs of the 9-1-1 complex have been identified from various screens (Ahmed and Hodgkin 2000; Boulton, Gartner et al. 2002; Hofmann, Milstein et al. 2002). Deficiency for *mrt-2*, *rad1* homolog, or *hus-1*, *hus1* homolog, by either mutation or RNAi results in cell cycle arrest defects in response to both IR and HU as well as defects in apoptosis (Ahmed and Hodgkin 2000; Boulton, Gartner et al. 2002; Hofmann, Milstein et al. 2002; Boerckel, Walker et al. 2007). In contrast to yeast and nematodes, mammalian cells deficient for 9-1-1 complex subunits fail to proliferate and display increased levels of chromosome abnormalities including chromatid breaks, aneuploidy, dicentrics and telomere loss. The essential phenotype of *rad9*, *rad1* and *hus1* mutants can be rescued with an additional mutation in either p21 or p53 and these double mutant cells are highly sensitive to IR or HU (Weiss, Enoch et al. 2000; Bao, Lu et al. 2004; Pandita, Sharma et al. 2006). Thus, the 9-1-1 complex plays an evolutionary conserved role in DNA damage response.

The 9-1-1 complex also plays a conserved role in telomere maintenance. Yeast cells deficient for the 9-1-1 complex subunits display short but stable telomeres (Dahlen, Olsson et al. 1998; Longhese, Paciotti et al. 2000; Nakamura, Moser et al. 2002). Mammalian 9-1-1 complex mutants display dramatic telomere loss and end-to-end chromosome fusions however, the cells are inviable (Bao, Lu et al. 2004; Francia, Weiss et al. 2006; Pandita, Sharma et al. 2006). *C. elegans* 9-1-1 complex mutants, *mrt-2* and *hus-1*, are viable and display progressive telomere shortening phenotypes that result in sterility at both 20°C and 25°C suggesting that they are essential for germline immortality (Ahmed and Hodgkin 2000; Hofmann, Milstein et al. 2002). Initially *mrt-2* was isolated from a screen for *mortal germline* (*mrt*) mutants, mutants that become sterile after propagation for multiple generations (Ahmed and Hodgkin 2000). Additionally, one other telomere replication
mutants were isolated from the screen, mrt-1 (B. Meier, unpublished). The frequency at which the two telomere replication mutants were isolated suggested that other genes with a similar phenotype may exist.

The Mrt and radiation hypersensitivity (Rad) phenotypes of mrt-2 are completely penetrant, allowing for easy selection. Based on these phenotypes a tiered screen was conducted to isolate other Rad genes required for telomerase. A forward genetic screen isolated 31 rad mutants from 906 EMS mutagenized lines. Of these, 15 displayed progressive sterility phenotypes at 25°C, a temperature-sensitive phenotype. The isolated mutant lines displayed varying defects for cell cycle arrest and apoptosis in response DNA damage. Additionally, a reverse genetic screen was conducted in an attempt to identify other genes involved in telomere maintenance. RNA interference (RNAi) was used to test 135 genes involved in various DDR pathways, chromosome stability and protein metabolism for their role in germline immortality. Both screens failed to identify any genes involved in telomere maintenance and DNA damage response, similar to mrt-2, suggesting that few genes that respond to IR/facilitate DSB repair are required for telomere maintenance in C. elegans. However, several IR-hypersensitive mutants were isolated that are required for germline immortality at high temperatures.
Materials and Methods

Strains

*C. elegans* strains were cultured at 20°C as described unless otherwise noted (Sulston and Hodgkin 1988). The following strains were used: *mrt-2(e2663), rad-5(mn159), rrf-3(pk1426)* and N2 Bristol wildtype.

EMS mutagenesis

NGM plates were staged with L4 larvae. Larvae were washed off with M9 and placed in EMS diluted in M9 to a final concentration of 50 mM for four hours. Larvae were transferred to fresh M9 and washed four times. Finally, larvae were placed on a fresh NGM plate at 20°C and 24 hours later were singled to fresh NGM plates.

DNA damage response assays

L1 assays were performed as previously described (Boerckel, Walker et al. 2007). Cell cycle arrest in response to hydroxyurea and IR and apoptosis assays were conducted as previously described (Boerckel, Walker et al. 2007).

RNAi of DDR genes

Bacterial strains containing RNAi cloning constructs were cultured from the Ahringer RNAi library (Kamath and Ahringer 2003; Kamath, Fraser et al. 2003). RNAi clones were induced with 1mM IPTG. Liquid cultures were concentrated and resuspended in S media and 75 µl was placed in a single microtiter dish well. Wildtype N2 Bristol strains and *rrf-3* RNAi hypersensitive strains were grown to near starvation on Petri dishes, washed
with M9 and 10 µl was transferred to a microtiter dish well containing resuspended RNAi bacteria. Dishes were incubated at 20°C or 25°C. Every week number of progeny compared to wildtype was analyzed and 5µl from each well was transferred to a fresh microtiter dish.

Results

Screen of mortal germline mutants for hypersensitivity to ionizing radiation

The Mrt and Rad phenotypes of mrt-2 were used in an attempt to identify other genes involved in DNA damage response and telomere maintenance in a screen of 906 EMS mutagenized F2 lines. Of these, 274 were initially isolated in a 25°C Mrt screen, 306 were characterized by their reduced number of progeny compared to wildtype (a phenotype characteristic of many DDR mutants) and the remaining 126 were uncharacterized, all of which originated from 3200 EMS mutagenized P₀ adults. Based on findings that mrt-2 and rad-5 mutants are completely sterile after 40 Gy IR whereas wildtype animals are not affected, this dose was used to select for the Rad phenotype (Figure 2.1 and data not shown). To determine which of the 31 rad mutants harbored mutations in genes involved in germline immortality all strains were outcrossed to wildtype two times by selecting for the Rad phenotype and tested for the Mrt phenotype at both 20°C and 25°C. Of the 31 mutants, 15 were Mrt at 25°C, five were temperature-sensitive sterile (Ts sterile) (sterile immediately upon exposure to 25°C) and 11 did not become sterile (Table 2.1). However, no strains became sterile at 20°C, as do mrt-2, hus-1 or other mutants that have been identified to be involved in telomere replication. To test if any of the 15 temperature-sensitive mrt mutants
Table 2.1: 31 *rad* mutants were analyzed for various DNA damage response phenotypes

<table>
<thead>
<tr>
<th>Strain</th>
<th>Brood Size</th>
<th>Irradiation hypersensitivity (Gy in which sterile)</th>
<th>Cell cycle arrest (Percent mitotic cells per germline arm: treated/untreated)</th>
<th>Apoptosis (average number of corpses)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>IR</td>
<td>HU</td>
</tr>
<tr>
<td>Wildtype</td>
<td>wildtype</td>
<td>100</td>
<td>33</td>
<td>24</td>
</tr>
<tr>
<td><em>mrt-2</em></td>
<td>wildtype</td>
<td>60</td>
<td>52</td>
<td>45</td>
</tr>
</tbody>
</table>

25°C mortal germine mutants

| yp21         | wildtype   | 60                                                 | 37     | 33     | 11.2                               |                                    |
| yp22         | wildtype   | 30                                                 | 47     | 33     | 4.3                                |                                    |
| yp23         | small      | 20                                                 | 46     | 52     | 6.3                                |                                    |
| yp24         | small      | 50                                                 | 53     | 30     | 2.4                                |                                    |
| yp25         | small      | 80                                                 | 59     | 47     | 4.3                                |                                    |
| yp26         | wildtype   | 40                                                 | 37     | 34     | 4.7                                |                                    |
| yp27         | wildtype   | 70                                                 | 28     | 26     | 3.1                                |                                    |
| yp28         | wildtype   | 30                                                 | 33     | 44     | 2.6                                |                                    |
| yp29         | wildtype   | 70                                                 | 55     | 26     | 4.6                                |                                    |
| yp30         | small      | 100                                                | 56     | 42     | 3.7                                |                                    |
| yp31         | wildtype   | 100                                                | 35     | 37     | 3.7                                |                                    |
| yp32         | wildtype   | 100                                                | 37     | 36     | 8.2                                |                                    |
| yp33         | wildtype   | 100                                                | 40     | 36     | 4.4                                |                                    |
| yp34         | wildtype   | 90                                                 | 50     | 42     | 3.3                                |                                    |

Temperature Sensitive

| yp36         | wildtype   | 20                                                 | N/D    | 46     | 5.2                                |                                    |
| yp37         | wildtype   | 60                                                 | N/D    | 100    | 0.5                                |                                    |
| yp38         | wildtype   | 20                                                 | N/D    | 36     | 4.4                                |                                    |
| yp39         | wildtype   | 20                                                 | N/D    | 38     | 4.8                                |                                    |
| yp40         | wildtype   | 20                                                 | N/D    | 48     | 5.6                                |                                    |

Non-mortal germine mutants

| yp41         | wildtype   | 70                                                 | N/D    | 21     | 2.5                                |                                    |
| yp42         | wildtype   | 70                                                 | N/D    | 29     | 3                                  |                                    |
| yp43         | wildtype   | 70                                                 | N/D    | 36     | 3.8                                |                                    |
| yp44         | wildtype   | 80                                                 | N/D    | 26     | 3.9                                |                                    |
| yp45         | wildtype   | 80                                                 | N/D    | 34     | 4.3                                |                                    |
| yp46         | wildtype   | 80                                                 | N/D    | 36     | 6.6                                |                                    |
| yp47         | wildtype   | 90                                                 | N/D    | 25     | 5.1                                |                                    |
| yp48         | wildtype   | 90                                                 | N/D    | 31     | 7.2                                |                                    |
| yp49         | wildtype   | 100                                                | N/D    | 32     | 3.7                                |                                    |
| yp50         | small      | 100                                                | N/D    | 22     | 3.3                                |                                    |
| yp51         | wildtype   | 100                                                | N/D    | 25     | 2.3                                |                                    |
harbored an allele relating to telomere replication, strains near sterility were assessed for end-to-end chromosome fusions. In wildtype germlines, six bivalent pairs of chromosomes can be visualized in the oocytes, whereas mrt-2 mutants display less then six bivalents near sterility (Figure 3.1 and 4.4). None of the 15 rad mrt mutants displayed chromosome fusions near sterility and therefore are unlikely to become sterile due to telomere replication defects. Regardless, the isolation of 15 rad mrt mutants suggested that multiple genes involved in various DDR pathways may play roles in germline immortality.

In an attempt to further characterize the 31 rad mutants, the IR dose at which they were completely sterile was determined. All the ts sterile mutants became sterile at extremely low doses of IR (Table 2.1). The extreme Rad phenotype and sensitivity to high temperature suggests that the ts sterile mutants may play an essential role in DSB repair by exogenous stress via IR and endogenous stresses caused by high temperature or for some other basic function such as DNA replication that is affected by both IR and high temperature. In contrast, the non-mrt mutants were all sterile at high doses of IR (>70 Gy) suggesting that these mutants may be more modestly defective for specific responses to DNA damage (Table 2.1). Finally, the mrt mutants became sterile over a range of IR doses (Table 2.1) and a radiation sensitivity curve was conducted to delineate their level of IR sensitivity (Figure 2.1). The mrt mutants represented a range of IR sensitivity suggesting they play varying roles in DDR relating to germline immortality at 25°C.

In conclusion, 31 rad mutants were isolated from an EMS mutagenesis screen. Of these, 15 are required for germline immortality at 25°C, 5 are essential for survival at high temperatures and 11 are not required for germline immortality. However, no mutants sterile at 20°C or involved in telomere replication were isolated, similar to mrt-2. Further analysis
Figure 2.1: The 15 rad mrt mutants are hypersensitive to IR over a range of doses

L1 radiation tests were conducted for each mutant strain in 10 Gy increments from 20 to 100 Gy. Total progeny was determined in a qualitative manner comparing to wildtype untreated total progeny. Relative brood size represents the average determined for three independent tests. Dose in which complete sterility, no progeny, occurred was determined for each line.

of the degree of sensitivity to IR grouped the mutants further. Interestingly the Mrt phenotypes segregated with the level of sensitivity to IR. These 31 mutants are likely to represent multiple genes involved in various DDR pathways and germline immortality at 25°C.

Several rad mutants are defective for cell cycle arrest and apoptosis in response to DNA damage

Hypersensitivity to IR measures a cell’s ability to respond to damage but does not suggest specific DDR pathways such as a DNA repair process or the induction of either cell cycle arrest or apoptosis. Cell cycle arrest and apoptosis responses can be measured in the germline of C. elegans which consists of two bilateral arms that begin with mitosis and progress through meiosis eventually developing into gametes (Figure 2.2A). Cell cycle
arrest in response to DNA damage can be observed in the mitotic portion of the germline. In wildtype germlines, mitotic cells enter cell cycle arrest in response to damaging agents such as IR or the ribonucleotide reductase inhibitor hydroxyurea (HU) that results in stalled replication forks, resulting in a reduction in the number of mitotic cells compared to untreated controls (Figure 2.2B). In contrast, mrt-2 mutants that are deficient for inducing cell cycle arrest, display reduced mitotic cell cycle arrest after treatment compared to wildtype (Figure 2.2B). The 31 rad mutant lines were tested for cell cycle arrest in response to both IR and HU. Eight of these were defective in inducing cell cycle arrest in response to IR at levels similar to mrt-2 (Table 2.1) (Figure 2.3). Five of the mrt mutants and three of the ts sterile mutants displayed cell cycle arrest defects in response to HU (Table 2.1) (Figure 2.3). Interestingly none of the non-mrt mutants displayed HU-induced cell cycle arrest defects suggesting this phenotype may be relevant to the subset of five mrt mutants that display defects in germline immortality.

An alternative to cell cycle arrest in response to DNA damage signaling is apoptosis. In the C. elegans germline, a low level of apoptosis naturally occurs in cells as they mature into oocytes and as a consequence about half of the cells that pass through the germline will undergo developmental apoptosis. In response to IR, apoptotic levels increase in wildtype germlines whereas mrt-2 mutants fail to induce apoptosis at higher than physiological levels (approximately 2 corpses at a given moment is observe in untreated wildtype germlines) (Figure 2.2C). One mutant, yp37 was completely defect for apoptosis whereas five other rad mutants were deficient for apoptosis induction (number of corpses less than three) and sixteen did not induce apoptosis at the same level as wildtype (Table 2.1) (Figure 2.3). All three types of rad mutant, mrt, ts sterile and non-mrt, displayed defects in apoptosis
Figure 2.2: Specific DNA damage response pathways can be observed in the germline of a *C. elegans* adult hermaphrodite

(A) A schematic of the germline of an adult hermaphrodite. Mitotic cells are located in the distal tip of the germline and develop into meiotic cells as they progress through the germline. The various cellular stages are labeled including mature oocytes which contain six highly condensed bivalent chromosome pairs.

(B) Cell cycle arrest in response to DNA damage occurs in the mitotic portion of the germline. DAPI stained wildtype and *mrt-2* germlines (shown is the mitotic portion of the germline until the transition zone starting at the distal tip, indicated by an arrowhead) with and without exposure to 25 mM HU are shown. Arrows point to nuclei.

(C) Apoptosis occurs in pachytene cells as they enter diakinesis and develop into oocytes. Apoptotic induction can be observed in wildtype germlines whereas *mrt-2* mutants do not induce apoptosis. Apoptotic cells are stained with Acrodine Orange.
Figure 2.3: DNA damage response defects are observed in all three rad mutant groups
The percent of rad mrt, ts sterile or non-mrt mutants defective for either IR induced apoptosis or HU cell cycle arrest is shown. Mutants slightly defective or completely defective for IR induced apoptosis are combined.

initiation suggesting that the apoptotic pathway is not essential for germline immortality (Figure 2.3).

RNAi of genes involved in various genome stability pathways did not reveal genes required for telomere replication

Previous studies in yeast, C. elegans and mammals have shown that many DNA damage response genes are important in telomere maintenance (d'Adda di Fagagna, Teo et al. 2004). In an attempt to identify more of these genes in C. elegans, a reverse genetic screen utilizing RNAi was conducted. RNAi involves the introduction of double-stranded RNA into the cell which is recognized by the RNAi machinery resulting in degradation of RNA products with the same sequence and a knock down in the expression of the targeted protein, mimicking a mutant phenotype (Fire, Xu et al. 1998; Kamath, Martinez-Campos et al. 2001). A list was compiled of genes involved in DNA repair, DDR, transcription/translation regulation and chromatin structure that have been identified in C. elegans through sequence homology, yeast two-hybrid studies and genetic screens (Boulton, Gartner et al. 2002; Pothof, van Haaften et al. 2003; Vastenhouw, Fischer et al. 2003; van
Haaften, Vastenhouw et al. 2004; van Haaften, Romeijn et al. 2006). In total 135 genes were chosen, most of which had not been previously published to result in an embryonic lethal RNAi phenotype and were represented in the Arhinger RNAi library (Table 2.2) (Kamath and Ahringer 2003; Kamath, Fraser et al. 2003) (MRC Gene Services). To conduct a Mrt RNAi screen using these genes, a Mortal Germline screen in liquid culture was developed in which C. elegans adults were fed the bacteria containing an RNAi construct and passaged every two generations. The screen was conducted at both 20°C and 25°C to identify genes required for germline immortality under all culture conditions. In addition, previous work with rad mrt mutants indicated that some DNA damage response gene mutations or knockdowns may be sterile at 25°C which could be represented in this list of RNAi clones. Seven of the 135 DNA damage response genes tested resulted in a Mrt phenotype at 25°C and only two became sterile at both 20°C and 25°C. The RNAi clones that induced the phenotype at 25°C were: mdf-1 is required for genome stability during mitosis; skr-15 is a kinetochore protein; mut-16 is required for maintaining low levels of transposon mobilization and RNAi; and M117.3 is a multifunctional chaperone. The two genes that became sterile at 20°C were bub-1, important in the mitotic spindle assembly checkpoint, and F11E6.1 that is involved in lysosome organization and biogenesis. When tested on agar plates, only three genes repeated the Mrt phenotype at 25°C, F11E6.1, mdf-1 and skr-15 and none repeated at 20°C. Additionally, when the nine genes were tested for telomere defects near sterility, none displayed chromosome fusions. Since three of the genes identified were kinetochore proteins that are important in chromosome segregation during the cell division, 21 other kinetochore genes
Table 2.2: Genes involved in various genome stability pathways that were tested for their involvement in germline immortality

<table>
<thead>
<tr>
<th>Sequence name</th>
<th>CGC name</th>
<th>Function</th>
<th>Sequence name</th>
<th>CGC name</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA repair and damage response</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H26D21.2</td>
<td>msh-2</td>
<td>Mismatch repair</td>
<td>Y76A2B.5</td>
<td></td>
<td>Uncharacterized</td>
</tr>
<tr>
<td>H26D21.1</td>
<td>hus-1</td>
<td>DDR</td>
<td>ZK520.3</td>
<td>dyf-2</td>
<td>Lifespan/dauer</td>
</tr>
<tr>
<td>K07G5.2</td>
<td>xpa-1</td>
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Chromatin structure

<p>| | | | | | |
| | | | | | |
| F47G6.4 | spe-15 | Myosin | Y66H1A.6 | hum-8 | Myosin complex |
| F02E9.4 | pqn-28 | Histone deacetylase | ZK1005.1 | pme-5 | Maintenance of chromatin structure |
| B0379.3 | mut-16 | RNAi transgene silencing | C50F4.11 | mdf-1 | Mitotic genome stability |</p>
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**Transcription/Translation regulation**

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**Other functions**

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<td>High temperature fertility/stress response</td>
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<td>cpt-2</td>
<td>Acyltransferase activity</td>
<td>Cyclin involved in G1</td>
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were tested for the Mrt phenotype (Table 2.2) (Maddox, Oegema et al. 2004). They were tested on RNAi plates in duplicate and in many cases only one of the two lines tested became sterile. Since the Mrt phenotype is mainly induced under various stresses it seems that they do not play a major role in germline immortality, possibly due to redundancy.

Interestingly, several highly studied DNA damage response genes, such as *atm-1*, *wrn-1*, *xpf-1*, *rad-51*, *blm-1*, *atl-1*, *mre-11* or *rad-50*, did not display a Mortal Germline phenotype despite reports in other organisms in their involvement in telomere maintenance and cellular immortality (Tsukamoto, Taggart et al. 2001; Opresko, von Kobbe et al. 2002; Zhu, Niedernhofer et al. 2003; Bi, Wei et al. 2004; d’Adda di Fagagna, Teo et al. 2004; Machwe, Xiao et al. 2004; Vespa, Couvillion et al. 2005; Eller, Liao et al. 2006). When deleted *rad-51*, *atl-1* and *mre-11* are lethal in *C. elegans*, however immediate lethality after RNAi depletion was not observed for these genes in this screen suggesting that RNAi is not
efficient for all pathways (Kelly, Dernburg et al. 2000; Alpi, Pasierbek et al. 2003; Garcia-Muse and Boulton 2005). In addition to this list of genes, *hus-1*, a member of the 9-1-1 complex, and *hpr-17*, the clamp loader of the 9-1-1 complex, depletion by RNAi did not confer a Mortal Germline at either 20°C or 25°C. In *C. elegans*, null mutants of either of these genes results in telomere shortening and an Mrt phenotype (Chapter 4) (Hofmann, Milstein et al. 2002; Boerckel, Walker et al. 2007). However modest Rad phenotypes have been observed in lines depleted of *mrt-2*, *hus-1* or *hpr-17* via RNAi (Boulton, Gartner et al. 2002). Genomic screens using RNAi to study specific groups of genes or different functions have shown that RNAi is not 100% effective for every aspect of how a gene functions (Fire, Xu et al. 1998; Tabara, Grishok et al. 1998; Asikainen, Vartiainen et al. 2005). Thus, RNAi might not be effective for identifying complete telomere replication defects. Additionally, RNAi of *trt-1*, the catalytic subunit of telomerase, does not result in a Mortal Germline or telomere fusions as a result of telomere shortening (B. Meier, personal communication). In conclusion, the RNAi screen identified genes involved in kinetochores and protein folding as potentially relevant to germ cell immortality but not telomere maintenance. Thus, the use of deletion mutants would be required to identify DNA damage response genes that are involved in telomere maintenance.

**Discussion**

In an attempt to identify more genes involved in DDR and telomere maintenance pathways, 274 25°C Mortal Germline mutants, 306 mutants with decreased number of progeny compared to wildtype and 126 uncharacterized EMS mutagenized F2 lines were screened for hypersensitivity to IR. Among them, 15 were found to co-segregate both Mrt
and Rad phenotypes suggesting that both phenotypes are a result of a mutation in the same gene. Upon further investigation none of the fifteen mutants became sterile at 20°C and none displayed telomere defects at 25°C, thus none of the mutants were similar to mrt-2. Additionally, five ts sterile mutants and eleven non-mrt mutants were isolated with the Rad phenotype. Other genes involved in germline immortality at 25°C but not 20°C have previously been identified, for example: a deletion mutant of him-6, which encodes the BLM helicase ortholog, displays a mutator phenotype and other genomic instability phenotypes, modestly shortened lifespan and a Mrt phenotype at 25°C (Grabowski, Svrzikapa et al. 2005); and pgl-1 is a RNA-binding protein present in germ granules that displays a partially penetrant Mrt phenotype at 25°C when deleted (T. Zucherro, unpublished) (Kawasaki, Shim et al. 1998). Further analysis of the 15 rad mrt mutants revealed that several displayed cell cycle arrest and apoptosis defects in response to DNA damage. Cell cycle arrest defects were seen in this category of mutants as well as in the Ts sterile group suggesting that this pathway may be relevant to germline immortality at high temperatures. In contrast, the results of this assay also suggested that the apoptotic pathway is not essential for germline immortality as apoptotic defects are observed in all three categories of germline mutants in agreement with previous studies showing the apoptosis does not increase in later generations and genes involved in the apoptotic pathway are not Mrt (data not show) (Ahmed 2006).

The screen isolated 15 / 274 (5.5%) mrt mutants to be Rad suggesting roles of DNA damage response in germline immortality at 25°C. It would be difficult to determine the number of genes represented by this number based on the observed phenotypes because point mutations are typically created by EMS which may lead to separation-of-function or partial loss-of-function mutations. Thus, some of the isolated mutants may be alleles of the
same gene despite displaying different Mrt or Rad phenotypes. However, predictions can be made as to the number of phenotypically similar mutants that lie within the three Mrt groups based on their Rad sensitivity, cell cycle arrest and apoptotic phenotypes. Within the mrt mutants, 11 different phenotypic groups of the 15 isolated mutants can be estimated. Despite four of the five ts sterile mutants becoming sterile at 20 Gy, they can be separated into two different groups, two display no other DNA damage response defects suggesting a role in DNA repair and the other two are defective for cell cycle arrest in response to HU. Finally, the non-mrt mutants can be placed into seven different phenotypic groups. Since the mutants display a range of phenotypes and can be separated in multiple groups it suggests DNA repair and cell cycle arrest may play a role in maintaining genome integrity at high temperature and thus germline immortality. Despite the analysis of these mutants, none display a range of defects similar to mrt-2 or hus-1 suggesting that the screen did not isolate some known DDR mrt mutants, therefore the screen not saturated.

In conclusion, results from both a reverse and forward genetic screen and DNA damage response assays suggest that many genes are involved in germline immortality. The lack of a mutant defective in telomere maintenance suggests that only a few genes that function in DSB repair are required for telomere maintenance (Ahmed and Hodgkin 2000). To better identify telomere maintenance genes and those essential for germline immortality, more refined screening is needed. Many 20°C, 25°C and liquid only mrt mutants have been isolated through other sterility screens. Initial analysis of 20°C mrt mutants isolated in an unbiased screen revealed that 35 of 78 tested were Rad agreeing with previous data that DDR plays a role in germline immortality (J. Boerckel, unpublished). Additionally, only five telomere maintenance mutants out of 7800 EMS mutagenized F2s were isolated in this
unbiased screen and none appear to be 9-1-1 complex subunits or other DDR genes (Y. Liu and J. Boerckel, unpublished). Despite the lack of telomere mutants, the study of the various mrt mutants will give insight into what pathways are essential for cellular immortality. This Rad screen selected for genes involved in DSB repair, since no other telomere replication mutants were isolated, it is possible that not very many genes fall into this category but several DSB repair genes are involved in germline immortality.

Acknowledgements

Thank you to Julie Ahringer and her lab for the creation of the RNAi library. Many members of the Ahmed lab were involved in the screens conducted. Yan Lui conducted the initial 25°C Mortal Germline screen of 3200 EMS mutagenized P₀ lines from which the 906 F2 lines for this study came. A former undergraduate in the lab, Sarah Mense assisted in many of the Rad testing. The RNAi liquid Mortal Germline screen was developed by a rotation student, Andrea Chaput, and myself. The DDR and kinetochore RNAi Mrt screens were conducted under my direction by an undergraduate, Swaroop Gonchikar. Additionally, thank you to former members of the Ahmed lab for discussion during the isolation and characterization of the mutants.
Background and Significance

Proper DNA replication is important to maintain the integrity of the genome. Many DNA lesions, such as DNA breaks caused by ionizing radiation (IR), intrastrand crosslinks caused by UV or nucleotide modifications, can result in a stalled replication fork. Additionally, inhibition of DNA polymerases, mutations in DNA replication machinery proteins or lack of dNTP pools can also trigger stalled replication forks. A conserved response from yeast to mammals relies on the Rad3-related kinase (ATR) to trigger a signaling cascade that enforces S-phase cell cycle arrest in response to stalled DNA replication. Genes involved in this process, when mutant, result in sensitivity to alkylating chemicals such as methyl methanesulfonate or the depletion of nucleotides resulting in stalled replication forks via hydroxyurea (HU). For example, yeast and mammalian cells carrying mutations in genes such as ATR, Rad53 and BLM (RecQ helicase) are unable to activate the S-phase DNA replication checkpoint in response to HU (Dart, Adams et al. 2004; Davies, North et al. 2004; Pichierri, Franchitto et al. 2004; Cordon-Preciado, Ufano et al. 2006).

Additionally, members of the Rad9/Rad1/Hus1 (9-1-1) complex are also involved in
the S-phase cell cycle checkpoint (Rowley, Subramani et al. 1992; Burtelow, Kaufmann et al. 2000; Caspari, Dahlen et al. 2000; Ahmed, Alpi et al. 2001; Dang, Bao et al. 2005; Boerckel, Walker et al. 2007). In *C. elegans*, *mrt-2*, Rad1 homolog, or *hus-1* mutants display a weak cell cycle arrest phenotype in response to HU treatment (Figure 4.3) (Boerckel, Walker et al. 2007). Additionally, these genes or proteins are also required for telomerase-mediated telomere replication (Ahmed and Hodgkin 2000; Hofmann, Milstein et al. 2002; Meier, Clejan et al. 2006). Other S-phase cell cycle checkpoint proteins, such as ATM, RPA and Mre11 have been shown to associate with telomeres in both yeast and mammals (Takai, Smogorzewska et al. 2003; Schramke, Luciano et al. 2004; Silverman, Takai et al. 2004; Dang, Bao et al. 2005). However, many of these genes in mammals are essential but some are not in *C. elegans*. The progressive telomere shortening phenotype observed in some *C. elegans* S-phase checkpoint mutants allows for a system to isolate and study genes involved in telomere maintenance (Ahmed and Hodgkin 2000; Hofmann, Milstein et al. 2002; Boerckel, Walker et al. 2007).

Since the hypersensitivity to IR (Rad) phenotype of *mrt-2* was not useful for finding telomerase mutants among 906 EMS mutagenized F2s screened, an alternative approach was taken utilizing the weak HU cell cycle arrest defect as a selection for S-phase checkpoint mutants like *mrt-2*. Thus, a two part screen was developed to isolate genes defective in S-phase cell cycle arrest, which could then be screened for a Mortal Germline (Mrt) and telomere maintenance defects. 334 out of 1160 EMS mutagenized P0 lines were isolated as initially being resistant to HU, 32 of which displayed a Mortal Germline phenotype at all temperatures. One of these 32 mutants, *yp4*, was determined to also be involved in telomere maintenance.
Materials and Methods

Strains

*C. elegans* strains were cultured at 20°C as described (Sulston and Hodgkin 1988). The following mutant strains were used: wildtype N2 Bristol, *mrt-2(e2663)*, *trt-1(ok410)*, *unc-29(e193)*, *dpy-5(e61)*, *unc-13(e51)*, *dpy-24(s71)*, *unc-4(e120)*, *unc-32(e189)*, *unc-17(e245)*, *dpy-20(e1282)*, *unc-60(e723)*, *dpy-11(e47)*, *ikb-1(nr2027)*, *pqn-28(tm1276)*, *F10G8.7(tm1981)*, *F10G8.7(tm2073)*.

EMS mutagenesis

NGM plates were staged to yield L4 larvae. Larvae were washed off with M9 and placed in EMS diluted in M9 to a final concentration of 50 mM for four hours. Larvae were transferred to fresh M9 and washed four times. Finally, larvae were placed on a fresh NGM plate at 20°C and singled 24 hours later.

HU screen

NGM plates containing isolated EMS mutagenized P₀ adults were allowed to starve to synchronize the F2 generation and a quarter of the plate (a chunk) was placed on a fresh NGM plate. Twenty hours later, the chunk was removed and the worms on the plate were exposed to 50 mM hydroxyurea. One week later, ~30 F3 L1 larvae, progeny of exposed F2 adults, were removed to a fresh NGM plate and allowed to recover. A single F4 L4 larva from any plates with F3 survivors was isolated to a fresh NGM plate, thereby ensuring that only independent HU-resistant mutations were recovered.
**Mortal Germline screen**

One F4 L4 larva from an F3 HU resistant parent was placed on a 30 mm NGM plate. Just before starvation the worms were washed with M9 buffer and 10 μl of worms in suspension was placed in a microtiter dish well containing 75 μl of OP50 bacterial culture resuspended in S Media. The microtiter dishes were placed at 20°C and every seven days (two generations) total progeny were quantified in comparison with wildtype, and 5 μl of each culture was transferred to a new microtiter dish containing fresh media. If a line became sterile in liquid, worms that had starved from four generations back was transferred to a NGM plate to test for repetition of the Mortal Germline phenotype. Every seven days, the total number of progeny was analyzed compared to wildtype and 6 L1 larvae were transferred to a fresh NGM agar Petri dish. If the sterility phenotype reproduced on agar plates, the line would be considered to have a Mortal Germline phenotype under all culture conditions.

**Mapping**

Strains containing visible phenotypes near the center of the chromosome were used for mapping the five autosomes (Figure 3.3). Backcrosses versus wildtype strains were used for mapping to the X chromosome. Marker / + males were crossed with mutant hermaphrodites and F2 non-Marker progeny were singled from F1 parents that segregated marker F2. F2 plates that did not segregate the marker phenotype in the F3 progeny were placed in the Mortal Germline screen. If all lines homozygous for mutations near a particular marker became sterile, the gene was deemed to be located at the center of the chromosome that carried the marker. Mendelian genetics predicts that a quarter of F2 lines will become
sterile due to random chromosome segregation if the gene is not located on the chromosome.
Backcrosses were also used in conjunction with the Rad phenotype to test if any of the *hu mrt* mutants that were sterile at 60 Gy IR were located on the X chromosome.

**DNA damage response assays**

L1 assays were performed as previously described (Boerckel, Walker et al. 2007). Cell cycle arrest response assays to HU and IR treatment and apoptosis induction assay to IR treatment were conducted as previously described (Boerckel, Walker et al. 2007).

**Outcrossing yp4**

After mapping *yp4* to the center of chromosome 1 in regards to the Mrt phenotype, markers located near the center were used for consecutive backcrosses to outcross *yp4*. *dpy-5,unc-29* doubles were used to outcross *yp4* three times. F2 progeny that no longer segregated the marker were grown out to sterility to check that they still harbored that *yp4* mutation. These lines were also tested for the HU cell cycle arrest defect. Backcrosses were then conducted using *unc-29,dpy-24* to further outcross *yp4* six more times. Again F2 progeny were tested for co-segregation of the Mrt and HU phenotypes.
Results

Identification of genes involved in S-phase cell cycle arrest that are essential for germline immortality

Hydroxyurea inhibits the ribonucleotide reductase which results in the depletion of dNTPs needed for DNA replication thus causing DNA replication forks to stall and inducing an S-phase checkpoint-mediated response. In response to HU exposure (25 mM), *C. elegans* wildtype animals induce cell cycle arrest in the mitotic cells of the germline (Figure 2.2B). In contrast, *mrt-2* mutants that are defective in DNA damage response do not induce mitotic cell cycle arrest at wildtype levels in response to HU (Figure 2.2B) (Ahmed, Alpi et al. 2001; Boerckel, Walker et al. 2007). Additionally, when *mrt-2* adults are subjected to high concentrations of HU (50 mM), some of their L1 progeny can recover after removal from HU and approximately 10% grow to adulthood, however, wildtype L1 larvae do not recover (data not shown). Based on this observation, a screen was developed to identify genes involved in S-phase cell cycle arrest by selecting for survivors in response to HU exposure. A mock screen was conducted to ensure that F2 homozygous mutants that are resistant to HU could be recovered from heterozygous F1 EMS mutagenized parents. One *mrt-2/+* hermaphrodite was placed on a NGM plate with 49 wildtype hermaphrodite to mimic the makeup of the F1 progeny from a P₀ EMS mutagenized line. When the mixed plates were put through the HU screen, *mrt-2* homozygous mutants were isolated in 15 out of 24 attempts, as confirmed by the Rad phenotype of *mrt-2* (note that no survivors were isolated from the other 9 attempts) (data not shown). Therefore, *mrt-2* mutants can be identified from large pods of worms based on their HU-resistance phenotype.
An EMS mutagenized P₀ *C. elegans* adult produces approximately 2800 F2 progeny on a 60 mm Petri dish, representing hundreds of homozygous gene mutations and allowing for many genes to be screened for a given phenotype on a single plate. 1160 EMS mutagenized wildtype P₀ animals were singled and their F2 progeny were exposed to HU. 334 (29%) mutant lines from independent P₀ were isolated based on their ability to recover after exposure to HU. The efficiency of the assay was analyzed by testing the level of resistance to HU of the mutants recovered versus wildtype and mrt-2. L4 larvae were exposed to 50 mM HU and the level of embryonic lethality and survival rate of L1 progeny larvae was assessed. As expected, wildtype lines displayed high levels of embryonic lethality and 100% L1 larval lethality, whereas mrt-2 mutants displayed ~20-30% L1 larval lethality, but did display embryonic lethality levels similar to wildtype. 21 *hu* mutants with wildtype number of progeny under normal growth conditions were also tested. All lines displayed ~10% L1 larval lethality and levels of embryonic lethality similar to *mrt-2* and wildtype. Additionally, five mutants (#12, 39, 120, 251 and 278) were more resistant than *mrt-2*, displaying less embryonic lethality, no larval lethality and a larger number of progeny. The results of this survival analysis test indicated that *mrt-2* is more HU resistant than wildtype to HU and that the mutants isolated in the screen were resistant to HU at a level similar to or greater than that of *mrt-2*.

To test if any of the S-phase defective mutants are also involved in germline immortality, the mutants were propagated to determine if they had a Mortal Germline phenotype. Of the 334 *hu* mutants, 32 (9.5%) were determined to be Mrt at 20°C and thus essential for germline immortality. The screen was conducted in liquid culture which can cause DNA damage due to hypoxia and osmotic stress, therefore all lines that became sterile
in liquid were checked for sterility on NGM agar plates, normal culture conditions. Fourteen
*hu* mutants only became sterile in liquid suggesting that some genes involved in S-phase cell
cycle arrest may promote germline immortality only under specific (stressful) culture
conditions. Another stress that can affect germline fertility is growth at high temperatures
(25°C). Of the 32 *mrt* mutants, seven were sterile immediately upon exposure to 25°C,
suggesting they may harbor a mutation in a gene essential for the S-phase checkpoint or any
of a number of other stress-response pathways. Telomere replication mutants, such as *mrt-2*,
a subunit of the 9-1-1 complex, and *trt-1*, the telomerase reverse transcriptase, become
sterile under all growth conditions, including 20°C, and near sterility display an increase in
embryonic lethality and a high incidence of males as a result of nondisjunction due to end-
to-end chromosome fusions (Ahmed and Hodgkin 2000; Meier, Clejan et al. 2006). End-to-end
chromosome fusions are visible near sterility in the oocytes of *mrt-2* and *trt-1* mutants,
in contrast to wildtype oocytes that display six highly condensed bivalents (Figure 3.1A). To
determine if any of the 32 *hu mrt* mutants are involved in telomere maintenance, all lines
near sterility were DAPI stained to assay for chromosome fusions. Only one mutant, *yp4* (#
279), displayed less than six bivalents suggesting a defect in telomere maintenance (Figure
3.1A). Also, one mutant, #289, displayed 8-11 DAPI positive spots per nucleus near sterility
characteristic of chromosome breakage or nondisjunction (Figure 3.1B). This mutant also
displays a high incidence of males that results from the loss of an X chromosome from XX
hermaphrodites, suggestive of chromosome nondisjunction.

In conclusion, a screen for mutants similar to *mrt-2* revealed only one out of 1160
EMS mutagenized P₀ lines that were defective for both S-phase cell cycle arrest and
telomere maintenance. Additionally, the screen found 334 lines that were resistant to HU, of
Figure 3.1: Two S-phase defective mutants display chromosome abnormalities near sterility

(A) Wildtype oocytes contain six highly condensed bivalent chromosomes. In contrast, telomere replication mutants such as \textit{mrt-2} and \textit{trt-1} display less than six bivalents near sterility, including the newly identified mutant \textit{yp4}.

(B) More than six DAPI spots are observed in \textit{hu mrt} mutant # 289 near sterility. All other \textit{hu mrt} mutants displayed six bivalents. Strains near sterility were DAPI stained and analyzed under fluorescence microscopy at 100x magnification. Chromosomes are encircled with a dashed line.

which 31 conferred an essential Mortal Germline phenotype suggesting a possible role for the S-phase DNA replication checkpoint in germline immortality.

\textit{Several of the hu mutants are also defective in responding to IR-induced double strand breaks}

S-phase cell cycle arrest can occur in response to various forms of DNA damage, such as double strand breaks or nicks caused by IR. To test if any of the mutants were hypersensitivity to IR they were exposed to an IR dose in which \textit{mrt-2} mutants are sterile.
(60 Gy) (Ahmed and Hodgkin 2000; Ahmed, Alpi et al. 2001). Of the 334 *hu* mutants, 67 (20%) were Rad, 36 were sterile at a dose in which wildtype is not affected (60 or 80 Gy), 16 were somewhat hypersensitive (sterile at 90 Gy or displayed a continual decrease in progeny compared to wildtype under all IR doses tested) and 15 displayed a slow growth phenotype in response to 60 Gy IR but were not sterile (Figure 3.2). 24 of the 32 *hu mrt* mutants were Rad (75%), of which nine were completely sterile at 60 Gy, eight were somewhat hypersensitive and seven displayed a slow growth phenotype (Table 3.1). Interestingly, analysis of 20°C *mrt* mutants isolated from an independent large scale screen found that 35 of 78 (45%) were Rad (J. Boerckel, unpublished). Though the Rad phenotypes initially observed in the *hu mrt* mutants have not been confirmed after outcrossing, the percentage recovered suggests that the HU screen enriched for mutants involved in double strand break repair.

![Figure 3.2: 67 of 334 hu mutants are hypersensitive to ionizing radiation](image)

**Figure 3.2: 67 of 334 hu mutants are hypersensitive to ionizing radiation**
The various *hu* mutants display different levels of sensitivity to IR based on the L1 Rad assay. Dose sterile represents mutants that produce no progeny at the indicated dose. Sensitivity is based on a marked decrease in progeny after IR treatment as compared to wildtype under the same conditions but do not become sterile at an earlier dose then wildtype. Slow growth describes are strains whose progeny have delayed development compared to wildtype treated lines and also display a slight decrease in the number of progeny.
Table 3.1: Some of the 32 *hu mrt* mutants also display other phenotypes

<table>
<thead>
<tr>
<th>Mutant number</th>
<th>Generation sterile at 20°C</th>
<th>Number of bivalents near sterility</th>
<th>Rad phenotype (Gy sterile)</th>
<th>Ts sterile</th>
<th>Phenotypes observed under normal culture conditions</th>
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<tr>
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<td>23</td>
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<td>60</td>
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<td>Males</td>
</tr>
<tr>
<td>12</td>
<td>15</td>
<td>6</td>
<td>60</td>
<td>No</td>
<td>Slow growth/mitotic tumors near sterility</td>
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<tr>
<td>39</td>
<td>13</td>
<td>6</td>
<td>60</td>
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<td>24</td>
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<td>60</td>
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<td>Unfertilized oocytes</td>
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<tr>
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<td>16</td>
<td>6</td>
<td>60</td>
<td>No</td>
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<td>60</td>
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<td></td>
</tr>
<tr>
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<td>6</td>
<td>60</td>
<td>No</td>
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<td>20</td>
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<td>80</td>
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<tr>
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<tr>
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<td>6</td>
<td>Slow growth</td>
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</tr>
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</tr>
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</table>
About half of the hu mrt mutants display other phenotypes

Besides the Mrt and Rad phenotypes of the 32 hu mrt mutants, some display other phenotypes in the absence of DNA damage that might be indicative of the pathways in which they act. Table 3.1 lists the various phenotypes for the hu mrt mutants. A high incidence of males and less progeny than wildtype suggest defects in chromosome segregation. Four mutants display a high incidence of males, including the mutant that has greater than six DAPI positive spots near sterility (#289) (Figure 3.1B). Four other mutants have less progeny than wildtype and also display a slow growth phenotype under normal culture conditions suggestive of chromosomal abnormalities. Additionally, six other mutants display the slow growth phenotype. One of these develops mitotic tumors in the germline near sterility suggestive of improper control of the transition from mitosis to meiosis. Laying unfertilized oocytes may indicate a problem with spermatogenesis, as observed in four of the mutant lines, including one that also has a high incidence of males, suggestive of chromosome non-disjunction. Finally, two lines display mutator phenotypes that may be a consequence of the EMS mutagenesis (i.e. an unlinked mutator locus which would segregate out after outcrossing) or of a mutator phenotype that occurs as a consequence of the mrt/hu mutation. In conclusion, 18 of the 32 hu mrt mutants displayed additional phenotypes that suggest problems in chromosome segregation or DNA repair defects, however, these observations are on unoutcrossed lines. To determine if these phenotypes are a consequence of the same mutation that results in the HU resistance and Mrt phenotype, the mutant lines should be outcrossed to wildtype several times selecting for the Mrt phenotype and then assaying for HU resistance and for the other observed phenotypes.
Ten of 32 hu mrt mutants map to the center of an autosome or the X chromosome

To try and determine the map positions of genes isolated in the screen, the mutants were mapped using the Rad phenotype to the X chromosome and using the Mrt phenotype to the centers of one of the five autosomes or the X chromosome. Initially, the nine hu mrt mutants that were sterile at 60 Gy IR were tested to see if they mapped to the X chromosome by selecting for the Rad phenotype in F1 progeny from a backcross with wildtype. The Rad phenotype of two mutants, #12 and #47, mapped to the X chromosome.

![Diagram showing the mapping of HU mutants to chromosomes](image)

**Figure 3.3: Ten hu mrt mutations map to the center of an autosome or the X chromosome**

The five autosomes and the markers used for mapping are indicated. Back crosses were used to map mutants to the X chromosome. The ten mutants that mapped to a chromosome based on their Rad or Mrt phenotype are indicated by the chromosome to which they mapped.
(Figure 3.3). The majority of essential genes in *C. elegans* are located near the center of autosomes one to three therefore these regions were first tested to determine the location of the mutations (Kamath, Fraser et al. 2003). Strains carrying mutations in genes that encode physical phenotypes (marker strains) located near the centers of the autosomes (1-5) were used for mapping the remaining 30 *hu mrt* mutants by selecting for the Mrt phenotype (Figure 3.3). If a mutation mapped to one of the chromosomes, all F2 lines selected from that cross should become sterile. Eight mutants mapped to a chromosome, four to autosomes and four to the X chromosome (Figure 3.3). Those that did not map to the center of an autosome carry mutations in genes located further on the arms of the autosomes, whose position can be mapped using other visible markers or by single nucleotide polymorphism (SNP) analysis (Wicks, Yeh et al. 2001; Davis, Hammarlund et al. 2005). Complementation tests between the six mutants that map to the X chromosome would have to be performed to determine how many genes are represented in this group. Two mutants mapped to the center of chromosome one, only one is a telomere replication mutant, *yp4*, thus it is likely that these two mutations define different *mrt* genes. The mapping created a one time outcrossed line for each of the 32 *hu mrt* mutants. To further test the efficiency of the screen, ten of the outcrossed mutant lines were tested to see if the HU cell cycle defect co-segregated with the Mrt phenotype. Six of the lines displayed a cell cycle arrest defect that was similar to *mrt-2* or was more defective (Figure 3.4) suggesting that the majority of the mutants recovered may play a role in HU induced S-phase cell cycle arrest. Further analysis of the mutants and complementation testing of mutants with similar phenotypes will reveal the number of genes isolated in the screen.
Ten outcrossed lines from mapping experiments were tested for HU cell cycle arrest defects. Three of the lines displayed cell cycle arrest defects similar to \textit{mrt-2} and three mutants were more defective than \textit{mrt-2}. The HU phenotype in these six mutants co-segregated with the Mrt phenotype.

One \textit{hu mrt} mutant, \textit{yp4}, has defects in telomere maintenance

The original purpose of the dual screen was to identify genes involved in HU induced cell cycle arrest and telomere maintenance, such as \textit{mrt-2} and \textit{hus-1} (Ahmed, Alpi et al. 2001; Hofmann, Milstein et al. 2002; Boerckel, Walker et al. 2007). Telomere maintenance mutants display progressive telomere shortening phenotypes that result in end-to-end chromosome fusions. Analysis of the \textit{mrt} mutants near sterility revealed that one mutant, \textit{yp4}, displayed end-to-end chromosome fusions near sterility (Figure 3.1) and southern blot analysis confirmed that progressive telomere shortening occurs in these mutants (Figure 3.5B). Interestingly \textit{yp4} does not become sterile until around F24, later than other telomere mutants identified suggesting that its role at telomeres may result in slower
Figure 3.5: *yp4* displays progressive telomere shortening

(A) Generation at which multiple lines of *mrt-2*, *trt-1* and *yp4* become sterile.

(B) Southern blot of two independently outcrossed lines (10 times) of *yp4* display progressive telomere shortening in early (F4-F8) and late generations (F20-F24). Telomere lengths are only shown for early generations (F4-F8) for *trt-1*. Southern blot analysis was performed using a (TTAGGC)$_n$ probe. Marker positions (kilobases) are shown to the left of the blot.

(C) Telomere shortening rates comparing *trt-1* to early and late generations of *yp-4*. Changes in telomere length were calculated for multiple telomeres per strain. Average rates of telomere shortening per generation are shown (±SD).
telomere degradation than complete telomerase defects, possibly independent of telomerase (Figure 3.5A). Initial calculations of the rate of shortening in two independent outcrossed lines demonstrated that indeed the initial rate of shortening was slower than trt-1 (Figure 3.5C). Interestingly, later generations of yp4 display a rate of telomere shortening similar to trt-1 (Figure 3.5C). Additionally, the initial length of the telomeres in yp4 is longer than what is seen in trt-1 and other telomere replication mutants (Figure 3.5A and 4.4B). A longer initial telomere length may be a result of the initial length in wildtype animals that were used for the mutagenesis screen or an extension in telomere length during HU exposure and would explain, in part, the delayed sterility phenotype. Southern blot analysis of early generations of various hu mrt mutants should be conducted to test if the initial telomere length is unique to yp4 or related to how the screen was conducted. Repetition and analysis of telomere shortening rates in more yp4 lines needs to be conducted to determine if the initial slower rate is significantly different then trt-1 and later yp4 generations. In conclusion, yp4 displays a unique telomere phenotype.

Although yp4 seems to play a different role at telomeres then mrt-2, it was tested to see if it acted in similar DDR pathways. Unlike mrt-2, yp4 did not become sterile at 60 Gy but did display a decrease in brood size compared to wildtype at all doses of IR tested (Figure 3.6A). Further analysis of yp4 revealed that it was modestly more defective for cell cycle arrest then mrt-2 in response to both HU and IR (Figure 3.6B). The HU cell cycle arrest phenotype co-segregates with the telomere phenotype as determined from analysis of lines outcrossed ten times. Finally, yp4 mutants induce apoptosis in response to IR at wildtype levels, unlike mrt-2 which is completely defective for this DDR (Figure 3.6C).
Figure 3.6: The telomere replication mutant, *yp4*, displays DDR defects

(A) *yp4* is sensitive compared to wildtype to ionizing radiation based on the L1 Rad assay but does not become sterile at moderate doses similar to *mrt-2*. Sensitivity is based on qualitative analysis of the number of progeny compared to untreated wildtype in response to increasing doses of IR.

(B) *yp4* is more defective for cell cycle arrest in response to 100 Gy IR and 25 mM HU than *mrt-2*. Error bars are ±SD.

(C) *mrt-2* mutants are defective for apoptosis induction in response to 120 Gy IR. However, *yp4* does induce apoptosis in response to IR similar to wildtype. Error bars are ±SD.
Thus, \( yp4 \) appears to play a different role than \( mrt-2 \) in response to DNA damage, one that is more specific to the response pathway that is triggered, mainly cell cycle arrest.

To determine what gene is mutated in \( yp4 \), mapping experiments with respect to the Mortal Germline phenotype were conducted. Initial mapping to the centers of the autosomes and the X chromosome revealed that \( yp4 \) is located on the center of chromosome one (Figure 3.3). Three-factor crosses were used to narrow down the location of the gene. All recombinants carrying the \( dpy-5 \) gene from a cross with \( dpy-5, unc-13 \) became sterile, mapping \( yp4 \) close to or to the right of \( unc-13 \). Located just to the right of \( unc-13 \) are two telomere replication genes, \( trt-1 \), the telomerase reverse transcriptase, and \( mrt-1 \) (Figure 3.7A) (Meier, Clejan et al. 2006) (B. Meier, personal communication). \( yp4 \) propagated in trans with \( trt-1 \) or \( mrt-1 \) complemented the Mortal Germline phenotype, therefore \( yp4 \) is neither \( trt-1 \) or \( mrt-1 \) (Table 3.2). Further mapping using \( unc-29,dpy-24 \) double mutants revealed that \( yp4 \) is located to the right of \( trt-1 \) and \( mrt-1 \), between \( unc-29 \) and \( dpy-24 \), +3.3 map units and +4.7 map units, respectively. Seven of the nine recombinants carrying \( dpy-24 \)

Table 3.2: The \( yp4 \) mutant is not \( trt-1 \) or \( mrt-1 \)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Approximate amount of progeny at F14</th>
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</thead>
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<tr>
<td>( mrt-1 / mrt-1 )</td>
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</tr>
<tr>
<td>( mrt-1 / yp4 )</td>
<td>wildtype</td>
</tr>
<tr>
<td>( yp4 / yp4 )</td>
<td>none</td>
</tr>
<tr>
<td>( trt-1 / trt-1 )</td>
<td>none</td>
</tr>
<tr>
<td>( trt-1 / yp4 )</td>
<td>wildtype</td>
</tr>
<tr>
<td>( yp4 / yp4 )</td>
<td>none</td>
</tr>
</tbody>
</table>

Complementation analysis was conducted in regards to the Mortal Germline phenotype. Marked \( trt-1 \) and \( mrt-1 \) strains were crossed with \( yp4 \) and trans heterozygotes harboring an allele of \( yp4 \) and either \( trt-1 \) or \( mrt-1 \) were propagated. Both genes complemented the \( yp4 \) mutation in regards to germline immortality. No progeny indicates sterility, Mrt phenotype.
and not unc-29 had Mortal Germlines placing yp4 around +3.6 map units. Several DDR genes are located between unc-29 and dpy-24, including claspin (+3.6 map units), ikb-1 (+3.9 map units) and F10G8.7 (+4.6 map units) (Figure 3.7A). Claspin is involved in the S-phase cell cycle checkpoint in response to DNA damage, a likely candidate based on the cell cycle arrest phenotype of yp4 and the predicted location from mapping. However, deletion mutants for claspin are lethal and sequencing of the yp4 mutant revealed no mutation in the claspin gene. Another gene, ikb-1 is involved in cell cycle control and interacts with mrt-2. A deletion mutant, nr2027, was obtained for ikb-1 (A. Aballay, personal communication). The deletion mutant displays a HU cell cycle arrest response similar to that of mrt-2 and is completely defective for apoptosis induction in response to IR as expected for a gene predicted to interact with mrt-2 (Figure 3.7B). Thus it is unlikely that yp4 is an allele of ikb-1. Finally, F10G8.7 encodes a homolog of ERCC-1, an endonuclease that is involved in nucleotide excision repair (NER) (Araujo and Wood 1999; Park and Choi 2006). Two deletion alleles, tm2073 and tm1981 were obtained (S. Mitani, personal communication). When exposed to HU, both deletion alleles were defective for the induction of cell cycle arrest at levels similar to that of yp4 (Figure 3.7B). Complementation analysis was conducted and yp4 / tm2073 and yp4 / tm1981 trans-heterozygotes were as defective for cell cycle arrest as the single mutants suggesting that they carrying mutations in the same gene (Figure 3.7B). To ensure that the lack of complementation did not result from haplo-insufficiency, yp4 / + and tm1981 / + heterozygotes were tested for HU cell cycle arrest and displayed wildtype levels of cell cycle arrest (Figure 3.7B). Initial sequence analysis of the majority of the exons of ercc-1 did not reveal a mutation in the gene. Further sequencing of the remaining coding sequence, introns, 3’ UTR and 5’ UTR needs to be conducted.
Figure 3.7: Mapping and identification of *yp4*

(A) A schematic of the right arm of chromosome 1 including physical markers used for mapping and candidate genes.

(B) The HU cell cycle arrest response assay for three candidate genes and complementation analysis between *yp4* and *ercc-1*. The F1 trans heterozygotes have the same level of HU cell cycle arrest induction as the single mutants. Additionally, the control heterozygotes have the same HU cell cycle arrest induction as wildtype confirming that both *yp4* and *ercc-1* are haplo-sufficient. Error bars represent ±SD.

Additionally, rescue and phenocopy by co-suppression-mediated gene silencing can be used to test if *yp4* is *ercc-1*. It is possible that *yp4* harbors a mutation in a gene nearby *ercc-1* that is affecting the regulation or transcription of the gene or that the results observed could be due to non-allelic non-complementation. Further analysis of both *yp4* and the deletion alleles of F10G8.7 need to be conducted to determine if F10G8.7 is the gene mutated in *yp4*. 
Discussion

A screen for genes involved in S-phase cell cycle arrest isolated 334 HU resistant mutant lines, of which 32 (9.6%) were found to be essential for germline immortality at all temperatures. A higher percentage of mrt mutants were isolated from the HU screen compared to an independent Mrt screen that identified 87 (1.1%) mrt mutants from 7800 EMS mutagenized F2 (Figure 5.1) (Y. Liu and J. Boerckel, unpublished). Thus, the HU screen seemed to enrich for mrt mutants. Analysis of the 32 hu mrt mutants revealed that 24 are defective in responding to DNA double-strand breaks, seven are immediately sterile at 25°C (possibly essential), one is involved in chromosome non-disjunction, and one is involved in telomere length maintenance. Additionally, ten mutants mapped to either the center of an autosome or the X chromosome. The screen did not isolate members of the 9-1-1 complex or hpr-17 suggesting that it is not saturated and complementation analysis between mutants with similar phenotypes will reveal the number of genes isolated from the screen. The phenotypes observed would suggest that S-phase defective mutants represent a group of DDR genes involved in germline immortality.

One out of the 32 hu mrt mutants displayed telomere replication phenotypes similar to mrt-2 and trt-1 (Figure 3.1 and Figure 3.5). Further analysis of the mutant, yp4, revealed that it is defective for cell cycle arrest in response to both HU and IR but is not defective for apoptosis induction. Preliminary data also suggests that yp4 may be hypersensitive to trimethyl-psoralen that creates DNA inter- and intrastrand crosslinks and UV which creates DNA intrastrand crosslinks (L. Shtessel, personal communication). Based on these phenotypes it is likely that the gene plays a role in detecting DNA damage and inducing a cell cycle arrest response which may or may not be in the same pathway as the 9-1-1
complex. Based on mapping and complementation analysis, *yp4* may harbor a mutation in F10G8.7, a homolog to ERCC1. ERCC1 interacts with XPF in nucleotide excision repair and forms a complex that is an endonuclease and makes a 5’ incision (Araujo and Wood 1999). Patients defective for XPF have symptoms of xeroderma pigmentosa that includes sensitivity to UV and the development of skin cancer. Mice defective for ERCC1 are also sensitive to UV. Additionally, ERCC1/XPF can cut DNA duplexes adjacent to a 3’ single stranded DNA flap (Zhu, Niedernhofer et al. 2003). The complex interacts with TRF2 which is involved in T-loop formation suggesting a role in capping (Zhu, Niedernhofer et al. 2003). Its endonuclease activity suggests that it could play a role in removing telomeric overhangs and deficiency in ERCC1/XPF results in the persistence of G strand overhangs (Zhu, Niedernhofer et al. 2003). Mouse cells deficient for XPF do not display telomere shortening and end-to-end chromosome fusions but do display extrachromosomal elements consisting of telomeric sequence observed by fluorescence in situ hybridization (Zhu, Niedernhofer et al. 2003). Analysis of *yp4* and the *ercc-1* deletion mutants near sterility would reveal if these same elements form in *C. elegans*. Thus, unlike *mrt-2* that is involved in telomerase-mediated telomere replication, *yp4* may play a role in removing the telomere overhang which would explain its slower time to sterility and initial slower progressive telomere shortening phenotype (Figure 3.5). Double mutant analysis of *yp4* with *mrt-2* or *trt-1* would determine if *yp4* acts in telomerase-mediated telomere replication. If *yp4* acts in a different pathway as *mrt-2* or *trt-1*, then the doubles should become sterile at a different rate than the singles. If *yp4* were to act in degrading the end of the telomere then a quicker rate of shortening may be observed as an additive affect with the loss of telomerase and telomere elongation. Alternatively, *yp4* may suppress the telomere erosion phenotype of *trt-1* which
could result in a slower rate or lack of telomere shortening in the double mutants compared to the single mutants. Additionally, further analysis of the yp4;mrt-2 double would reveal if it acted in the same DDR pathway as mrt-2.

The mrt-2 gene was isolated from a pilot screen for mortal germline mutants as one of sixteen mrt mutants (Ahmed and Hodgkin 2000). Additionally the screen isolated one other telomere replication mutant, mrt-1 (S. Ahmed, personal communication). In an unbiased large scale screen 87 of 7800 EMS mutagenized F2 lines were mrt under all conditions and 5 were telomere defective, none were alleles of the 9-1-1 complex, hpr-17, XPF or ERCC1 (Y. Liu and J. Boerckel, unpublished). In an attempt to identify more genes involved in both of these pathways, a two part screen was conducted that revealed only one telomere replication mutant out of 32 hu mrt mutants. The HU screen did not isolate any expected mutants, such as mrt-2, hus-1, rad-9 or hpr-17, nor did it identify XPF, the other essential subunit of the ERCC1 complex. Thus, the HU screen was not a terribly efficient screen in identifying genes involved in S-phase cell cycle arrest response. Approximately 34,800 F3 mutants from 1160 individual EMS mutagenized P0 were tested for resistance to HU, of which about 800 should harbor a homozygous mutation based on EMS mutagenesis rates (1 in every 4000). Analysis of mrt-2 HU resistance and the ability to recover the homozygous mutation when pooled in a wildtype population suggests a success rate of about 60%. Thus the screen is not effective in isolating all homozygous mutations that confer HU resistance and the number of F2 mutants analyzed is less then expected. Fortunately, the HU Mrt screen did identify a novel telomere mutant and was highly enriched for 20°C mortal germline mutants, suggesting a direct link between S-phase cell cycle arrest and germline immortality.
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Background and Significance

Telomeres, the ends of linear chromosomes, are typically composed of short repetitive sequences that are G-rich on the strand that runs 5’ to 3’ to the chromosome terminus (Blackburn and Gall 1978). Double-stranded telomeric DNA terminates in 3’ single-stranded overhangs that can loop back and invade the telomere duplex, forming a T-loop that may protect chromosome ends from degradation and suppress DNA damage signaling (Griffith, Comeau et al. 1999; Karlseder, Broccoli et al. 1999). Telomeric termini can shorten as a consequence of nucleolytic degradation, oxidative damage, or incomplete terminal replication by the lagging DNA strand synthesis machinery. The loss of telomeric sequence can be combated by telomerase, a ribonucleoprotein that adds telomere repeats to chromosome termini de novo. A number of proteins aside from the telomerase holoenzyme play roles in telomere maintenance (Collins 2006). Some of these proteins also respond to DNA damage, suggesting dual roles in preserving the integrity of the genome (d'Adda di Fagagna, Teo et al. 2004).

One example is the Rad9/Rad1/Hus1 (9-1-1) PCNA (Proliferating Cell Nuclear Antigen)-like sliding clamp which is a DNA damage response complex that also interacts with telomeres. The 9-1-1 complex was genetically identified in yeast as being required for
checkpoint control in response to DNA damage (al-Khodairy and Carr 1992; Rowley, Subramani et al. 1992; Caspari, Dahlen et al. 2000). Deletion of 9-1-1 complex subunits in yeast also results in short, stable telomeres (Dahlen, Olsson et al. 1998; Longhese, Paciotti et al. 2000; Nakamura, Moser et al. 2002). Chromatin immunoprecipitation analysis in S. pombe and mammals indicates that the 9-1-1 complex interacts with telomeric DNA (Nakamura, Moser et al. 2002; Francia, Weiss et al. 2006; Verdun and Karlseder 2006). Additionally, in vitro telomerase activity was impaired by a mutation of hus1 in mouse cells and by RNAi-mediated knockdown of hus1 or rad9 in human cells, suggesting that the mammalian 9-1-1 complex may play a direct role in telomere repeat addition (Francia, Weiss et al. 2006; Pandita, Sharma et al. 2006; Verdun and Karlseder 2006). However, mammalian cells deficient for RAD9, HUS1 or RAD1 are inviable and exhibit various chromosomal abnormalities including a dramatic reduction in telomere length (Weiss, Enoch et al. 2000; Bao, Lu et al. 2004; Francia, Weiss et al. 2006; Pandita, Sharma et al. 2006). These severe effects preclude genetic pathway analysis from the perspective of telomerase because they are strikingly different from the comparatively mild telomere attrition observed in telomerase mutants (Feng, Funk et al. 1995). In contrast, C. elegans 9-1-1 complex mutants are viable and display progressive telomere shortening phenotypes similar to those of telomerase mutants, allowing for genetic pathway studies to be conducted (Ahmed and Hodgkin 2000; Hofmann, Milstein et al. 2002; Meier, Clejan et al. 2006).

Structural analysis of the yeast and mammalian 9-1-1 complex revealed that it resembles the PCNA sliding clamp, which is a DNA polymerase processivity factor that is loaded onto single-stranded DNA by an RFC polymerase clamp loader (Venclovas and Thelen 2000; Griffith, Lindsey-Boltz et al. 2002; Bermudez, Lindsey-Boltz et al. 2003).
RFC clamp loaders are heteropentamers composed of four small constitutive subunits, RFC2-5, and one of four large RFC-like subunits. The canonical Rfc1 large subunit acts during DNA replication to load PCNA onto DNA at primer/template junctions in an ATP-dependent manner. Rad17 defines a second RFC complex that can load the 9-1-1 PCNA-like sliding clamp onto single-stranded DNA at sites of damage (Volkmer and Karnitz 1999; Griffith, Lindsey-Boltz et al. 2002; Bermudez, Lindsey-Boltz et al. 2003; Majka and Burgers 2003; Meister, Poidevin et al. 2003; Yang and Zou 2006). Yeast mutants deficient for Rad17 have short, stable telomeres, and Rad17 associates weakly with telomeric DNA (Dahlen, Olsson et al. 1998; Longhese, Paciotti et al. 2000; Nakamura, Moser et al. 2002). Double mutant analysis between Rad17 and members of the 9-1-1 complex revealed no additional telomere shortening as compared to the single mutants, suggesting that the 9-1-1 complex and Rad17 act in the same telomere maintenance pathway (Dahlen, Olsson et al. 1998; Longhese, Paciotti et al. 2000; Nakamura, Moser et al. 2002). A third RFC complex utilizes Ctf18 as a large RFC subunit and is essential for perinuclear positioning of telomeres during the cell cycle (Hiraga, Robertson et al. 2006). Finally, a fourth RFC complex contains Elg1 as the large RFC subunit that can load PCNA during DNA recombination and synthesis (Aroya and Kupiec 2005). Yeast elg1 mutants display an increase in telomere length that is dependent on telomerase and Ku (Smolikov, Mazor et al. 2004). Thus, three RFC subunits participate in both DNA metabolism and telomere maintenance: Rad17, Ctf18 and Elg1.

Given that Rad17 loads the 9-1-1 complex onto sites of DNA damage in yeast and mammals (Volkmer and Karnitz 1999; Bermudez, Lindsey-Boltz et al. 2003; Yang and Zou 2006), and given that subunits of the 9-1-1 complex are essential for telomere maintenance
in *C. elegans* (Ahmed and Hodgkin 2000; Hofmann, Milstein et al. 2002), we asked if the *C. elegans* Rad17 homolog, *hpr-17*, is required for the 9-1-1 complex to function during telomere replication. Analysis of strains deficient for *hpr-17* confirms that it functions in the same cell cycle arrest and apoptotic DNA damage response pathways as the *C. elegans* 9-1-1 complex subunits *mrt-2* and *hus-1*. Additionally, HPR-17 acts in the same telomere maintenance pathway as the 9-1-1 complex and facilitates telomerase-mediated telomere replication.

**Materials and Methods**

**Strains**

*C. elegans* strains were cultured at 20°C as described (Sulston and Hodgkin 1988). The following mutant strains were used: *hpr-17(tm1579), mrt-2(e2663), hus-1(op244), trt-1(ok410), unc-29(e193), dpy-18(e364), unc-64(e246), unc-11(e47), rol-6(e187), unc-52(e444), ypEx3 [hpr-17 rol-6(su1006)] and ypEx4 [hpr-17 rol-6(su1006)].* *hpr-17(tm1579)* was outcrossed ten times against the N2 Bristol wildtype strain by selecting for its radiation hypersensitivity phenotype. To construct double mutants, standard genetic crosses were conducted utilizing visible markers adjacent to or flanking each gene: *rol-6,unc-52* for *hpr-17*; *unc-11* for *hus-1*; *dpy-18,unc-64* for *mrt-2*; and *unc-29* for *trt-1*. Visible markers were selected against and the genotypes of all homozygous double mutant lines were confirmed via PCR.
Mapping and cosuppression

*hpr-17* mutant hermaphrodites were crossed with *rol-6,unc-52/+* males and F2 Rol- non-Unc recombinants were isolated. F3 progeny homozygous for each recombination event were propagated and tested for hypersensitivity to IR and propagated to determine if they displayed end-to-end chromosome fusion-mediated sterility. For cosuppression, PCR with primers hpr17cr (GCACACGAGACGATTTTTCAAGC) and hpr17cf (ATCGCCTCCAGCTTCCTCTCC) was performed using N2 genomic DNA as a template. Mixtures of 24 ng/ul of *rol-6* cosmid and 4 ng/ul of *hpr-17* PCR product were injected into N2 Bristol wildtype hermaphrodites. Rol and non-Rol siblings for two independent lines (*ypEx3* and *ypEx4*) were propagated in parallel and tested for IR hypersensitivity, progressive sterility and for the presence of end-to-end chromosome fusions.

IR hypersensitivity assays

Gamma irradiation was performed in a Shepherd Marc IV Cs137 irradiator at a dose rate of 430 Rad/min. For L1 assays, 6 L1 larva were picked to a single NGM plate and irradiated at the indicated dose. Plates were scored for complete sterility after one week. The L4 assay was performed as previously described (Ahmed and Hodgkin 2000).

Cell cycle arrest and Apoptosis

IR and HU assays were conducted as previously described (Ahmed, Alpi et al. 2001). Late L4 larvae were picked and either irradiated (100 Gy) or placed on NGM plates containing hydroxyurea (25 mM). After 14 hours of HU treatment or 12 hours post-IR, animals were stained with 4’,6-diamidino-2-phenylindole (DAPI) (Molecular Probes,
Eugene, OR). All mitotic cells from the distal tip to the transition zone of the germline were counted using a Nikon E800 fluorescence microscope at 100x magnification using a DAPI filter set. For apoptosis, L4 larvae were irradiated (120 Gy) and stained 24 hours post-IR with Acridine Orange (Molecular Probes, Eugene, OR) as described (Gartner, MacQueen et al. 2004). Corpses were counted using a green fluorescent protein filter set at 40x magnification.

Results

**hpr-17 mutants display DNA damage response defects**

The *C. elegans* Rad17 homolog, *hpr-17*, is predicted to encode a protein that is orthologous to Rad17 proteins ranging from yeast to mammals (data not shown). The HPR-17 protein contains an AAA ATPase domain (Figure 4.1) that is essential for its ability to bind to DNA in an ATP dependent manner (Venclovas and Thelen 2000; Lindsey-Boltz, Bermudez et al. 2001; Bermudez, Lindsey-Boltz et al. 2003; Majka and Burgers 2003).

**tm1579** is a 738 bp deletion in the *C. elegans hpr-17* gene, which contains one breakpoint in exon 3 and another breakpoint that lies 5 nucleotides from the beginning of exon 4 (Figure 4.1) (S. Mitani, personal communication). **tm1579** is predicted to confer an in-frame deletion that would eliminate 37 amino acids of the HPR-17 protein product, including a significant portion of the AAA ATPase domain resulting in a possible defect for conformational changes that facilitate loading of the 9-1-1 complex onto single-stranded DNA. Strains homozygous for the *hpr-17(tm1579)* deletion were viable but displayed a decrease in brood size and a weak high incidence of males (Him) phenotype (data not shown). XO males arise
as a consequence of loss of an X chromosome, suggesting a defect in chromosome stability (Hodgkin, Horvitz et al. 1979). Similar chromosome instability phenotypes have been observed for strains deficient for *C. elegans* 9-1-1 complex subunits (Ahmed and Hodgkin 2000; Gartner, Milstein et al. 2000; Ahmed, Alpi et al. 2001; Hofmann, Milstein et al. 2002), with which *hpr-17* is predicted to interact (Boulton, Gartner et al. 2002).

The *C. elegans* 9-1-1 complex mutants, *mrt-2* and *hus-1*, are highly sensitive to ionizing radiation (IR), which causes various forms of DNA damage including double-strand breaks (Ahmed and Hodgkin 2000; Hofmann, Milstein et al. 2002). A quantitative *C. elegans* assay for hypersensitivity to IR involves irradiation of L4 larvae, which contain many germ cells, and analysis of their progeny for embryonic lethality that occurs as a consequence of unrepaired DNA double-strand breaks and chromosome mis-segregation.

**Figure 4.1: The *C. elegans* hpr-17 gene**

*hpr-17* is located on chromosome II between *rol-6* and *unc-52*. Boxes represent exons in a schematic representation of the genomic structure of *hpr-17*. Also shown are the locations of the AAA ATPase motif and the *tm1579* mutation. Arrows indicate the location of PCR primers used to create the gene fragment used for cosuppression-mediated gene silencing. The *hpr-17* cosuppression PCR product includes the promoter region and five exons of a neighboring gene, F32A11.1. RNAi knockdown of F32A11.1 results in embryonic lethality and maternal-effect sterility (Maeda, Kohara et al. 2001; Sonnichsen, Koski et al. 2005). Although some lethality was observed, this is consistent with reports of other 9-1-1 complex subunits (Hofmann, Milstein et al. 2002), and the *hpr-17* cosuppression strains did not display maternal-effect sterility. Thus, expression of F32A11.1 is unlikely to be significantly affected in *hpr-17* cosuppression strains.

**Note:** This document is not converted to a structured format and may contain issues such as incorrect line breaks or missing content. The figure and text are intended to be read naturally, and any discrepancies or limitations in representation should be considered.
Figure 4.2: *hpr-17* mutants are hypersensitive to ionizing radiation

(A) Average levels of embryonic lethality (±SD) in response to different doses of IR for four independent lines of *hpr-17*, *mrt-2*, *hus-1* and their respective double mutants.

(B) Cosuppression lines, *ypEx3* and *ypEx4*, display the same levels of embryonic lethality as *hpr-17* in response to IR, whereas their non-Rol siblings, which lack the extrachromosomal array, mimic wildtype. Averages of two independent experiments ±SD are shown.

(C) Germline sterility in response to increasing doses of IR for *hpr-17*, *mrt-2*, *hus-1* single mutants and their respective double mutants. Ten plates with pools of L1 larvae were tested for sterility and averages for two independent mutant lines per strain are shown.
(Clejan, Boerckel et al. 2006). When subjected to moderate doses of ionizing radiation, mrt-2, hus-1 and hpr-17 strains displayed significant increases in levels of embryonic lethality when compared with irradiated wildtype controls (Figure 4.2A) (Ahmed and Hodgkin 2000; Ahmed, Alpi et al. 2001; Hofmann, Milstein et al. 2002). To determine if the observed radiation hypersensitivity (Rad) phenotype of the hpr-17 strain co-segregated with the hpr-17(tm1579) deletion, three-factor crosses were conducted using rol-6 and unc-52 mutations, which map to +0.9 and +23.4 on chromosome II, respectively, and flank the hpr-17 locus at +14.8 (Figure 4.1). 12 of 20 (60%) Rol-non-Unc recombinants displayed the Rad phenotype, agreeing with an expected recombination frequency of 65%. To further confirm that hpr-17 might be responsible for the Rad phenotype of strains carrying the tm1579 deletion, the hpr-17 gene was silenced in germ cells of the N2 Bristol wildtype C. elegans strain by cosuppression-mediated gene silencing. To accomplish hpr-17 cosuppression, extrachromosomal arrays carrying a high copy number of a PCR product corresponding to the promoter and first three and a half exons of hpr-17 (Figure 4.1) were created in a wildtype background (Dernburg, Zalevsky et al. 2000), which should eliminate endogenous hpr-17 transcripts via a process that depends on components of the RNAi machinery (Ketting and Plasterk 2000). Germlines of wildtype strains carrying either of two independent hpr-17 extrachromosomal arrays, ypEx3 or ypEx4, displayed Rad phenotypes that mimicked those of the hpr-17 deletion mutant (Figure 4.2B). Thus, cosuppression-mediated gene silencing of hpr-17 and analysis of the hpr-17 deletion tm1579 indicated that hpr-17 is required for responding to ionizing radiation in C. elegans, in agreement with a previously observed weak Rad phenotype induced by RNAi of hpr-17 and with a genome-
wide RNAi screen that suggested that hpr-17 may play a role in ensuring genome stability (Boulton, Gartner et al. 2002; Pothof, van Haften et al. 2003).

To determine if hpr-17 and subunits of the 9-1-1 complex act in the same pathway to facilitate the response to IR, double mutants were constructed. Progeny of IR-treated L4 larvae of hus-1 or mrt-2 single mutants and the hus-1;mrt-2 double mutant displayed levels of embryonic lethality that were not significantly different from one another, which agrees with data that these genes may physically interact (Boulton, Gartner et al. 2002), that the HUS-1 protein is mis-localized in mrt-2 mutants (Hofmann, Milstein et al. 2002), and with genetic and biochemical analysis of 9-1-1 complex subunits in yeast and mammals (al-Khodairy and Carr 1992; Rowley, Subramani et al. 1992; Volkmer and Karnitz 1999; Caspari, Dahlen et al. 2000; Griffith, Lindsey-Boltz et al. 2002; Bermudez, Lindsey-Boltz et al. 2003; Majka and Burgers 2003; Meister, Poidevin et al. 2003; Yang and Zou 2006). Furthermore, hpr-17 single mutants, and hpr-17;mrt-2 and hus-1;hpr-17 double mutants displayed similar levels of radiation hypersensitivity at the L4 stage, suggesting that they may act in the same DNA damage response pathway (Figure 4.2A). Additive or synergistic effects on IR sensitivity would have been expected if these mutations acted in different DNA damage response pathways. To further confirm these results, an irradiation assay was conducted using L1 larvae, an early C. elegans larval stage that harbors few germ cells. The dose at which L1 larvae of hpr-17, mrt-2 or hus-1 single mutants, as well as all respective double mutants, gave fully penetrate sterile phenotypes was 60 Gy (Figure 4.2C). Thus, independent radiation hypersensitivity assays using L1 or L4 larvae indicated that the 9-1-1 complex and the hpr-17 clamp loader act in the same DNA damage response pathway.
The 9-1-1 complex facilitates cell cycle arrest or apoptotic responses to damaged DNA that can be observed in adult *C. elegans* germlines, which are composed of mitotic cells and cells at various stages of meiosis and gametogenesis. In wildtype *C. elegans* hermaphrodites, at a given moment, germ cells in the late pachytene stage of meiosis display a low level of apoptosis, which increases 3- to 4-fold in response to high doses of IR (Figure 4.3A) (Gartner, Milstein et al. 2000). *mrt-2, hus-1* and *hpr-17* mutants failed to initiate an apoptotic response to gamma irradiation (Figure 4.3A, and data not shown) (Gartner, Milstein et al. 2000; Hofmann, Milstein et al. 2002), as previously suggested by a weak apoptosis defect observed upon RNAi of *C. elegans hpr-17* (Boulton, Gartner et al. 2002). The completely defective phenotype would be expected if *hpr-17* and the 9-1-1 complex function in the same apoptotic pathway. In wildtype germlines, cell cycle arrest occurs in mitotic germ cells in response to IR or to the ribonucleotide reductase inhibitor hydroxyurea (HU) (Gartner, Milstein et al. 2000; Ahmed, Alpi et al. 2001). The magnitude of a cell cycle

![Figure 4.3: hpr-17 is defective for apoptosis and cell cycle arrest in response to DNA damage](image)

(A) *mrt-2* and *hpr-17* mutants do not display an apoptotic response to IR. Each bar represents the average number of corpses counted for 20 germline arms ±SD.

(B) Levels of cell cycle arrest response are shown, as determined by comparing the number of mitotic cells in a germline arm with or without genotoxic stress (25 mM HU, black bar, or 100 Gy IR, gray bar). For all strains, the average is based on four independent experiments ± SD is shown.
arrest defect can be assessed by quantifying the relative change in the number of mitotic germ cells in response to genotoxic stress (Gartner, MacQueen et al. 2004), and additive effects can be observed in double mutants that function in different cell cycle arrest pathways. In response to IR or HU, *mrt-2*, *hus-1* and *hpr-17* single mutants and *mrt-2;hpr-17*, *hus-1;hpr-17* and *hus-1;mrt-2* double mutants displayed cell cycle arrest defects that were significantly different from wildtype (p < 0.01 and p < 0.04 for IR and HU treatment, respectively) but not from one another (Figure 4.3B). Thus, the effects of genotoxic stress on fertility, apoptosis and cell cycle arrest in strains deficient for *hpr-17* are consistent with the prospect that *hpr-17* acts in the same DNA damage response pathway as that of the 9-1-1 complex subunits *mrt-2* and *hus-1*.

**HPR-17 may facilitate telomerase activity at telomeres**

Aside from their role in responding to DNA damage, subunits of the *C. elegans* 9-1-1 complex are required to maintain telomere length (Ahmed and Hodgkin 2000; Hofmann, Milstein et al. 2002). As these mutants are propagated for multiple generations, fertility decreases as a consequence of telomere erosion and the formation of end-to-end chromosome fusions, which result in aneuploidy and sterility (Ahmed and Hodgkin 2000; Hofmann, Milstein et al. 2002). Propagation of *hpr-17* mutants for multiple generations resulted in progressive sterility (data not shown). In contrast to the normal complement of six bivalents observed in wildtype oocytes, chromosomes of late-generation *hpr-17* oocytes had fused together, as observed in late-generation *mrt-2*, *hus-1* or *trt-1* mutants (Figure 4.4A and data not shown)(Ahmed and Hodgkin 2000; Hofmann, Milstein et al. 2002; Meier, Clejan et al. 2006). Thus, fluorescence microscopy suggested that the progressive sterility
Figure 4.4: hpr-17 is required for telomerase-mediated telomere repeat addition

(A) Oocyte nuclei from DAPI-stained adult hermaphrodites. Wildtype displays six bivalent chromosomes (enclosed with a dashed white line) whereas hpr-17, trt-1 and mrt-2 mutants display chromosome fusions as a result of telomere dysfunction.

(B) Progressive telomere shortening was observed for successive generations of trt-1, hpr-17, mrt-2 or hus-1 single mutants and the indicated double mutants. Southern blot analysis was performed using a (TTAGGC)$_n$ probe. Marker positions (kilobases) are shown to the left of the blot.

(C) Telomere shortening rates. Changes in telomere length were calculated for multiple telomeres per strain. Average rates of telomere shortening per generation are shown (±SD).
observed in hpr-17 mutants may occur as a consequence of end-to-end chromosome fusions. When X-autosome end-to-end chromosome fusions are heterozygous, a dominant nondisjunction phenotype occurs that can be mapped to the left or right end of the X-chromosome (Ahmed and Hodgkin 2000; Meier, Clejan et al. 2006). To determine if the chromosome fusions visible in late-generation hpr-17 mutant lines were end-to-end fusions, late-generation hpr-17 hermaphrodites were crossed with wildtype males and F1 cross-progeny were identified that displayed a dominant chromosome loss phenotype. Two independent X-autosome fusions were isolated from hpr-17 mutants in this manner, both of which displayed breakpoints that mapped to the right end of the X chromosome (data not shown). In addition, the hpr-17 cosuppression lines ypEx3 and ypEx4 became progressively sterile and displayed chromosome fusions, as observed by DAPI staining (data not shown). Confirmation of the presence of end-to-end chromosome fusions was obtained by isolating an X-autosome chromosome fusion from ypEx4, whose X-linked fusion breakpoint mapped to the right end of the X chromosome (data not shown).

The above results indicate that strains deficient for hpr-17 become progressively sterile as a consequence of end-to-end chromosome fusions, which can result from progressive telomere erosion, as observed in C. elegans mutants deficient for telomerase-mediated telomere replication (Meier, Clejan et al. 2006). Southern blotting revealed that hpr-17 mutants displayed progressive telomere shortening (Figure 4.4B). Previously, the mrt-2 9-1-1 complex subunit was shown to act in the same telomere replication pathway as trt-1, the telomerase reverse transcriptase, as double mutants displayed the same rate of telomere shortening (Meier, Clejan et al. 2006). To show that hpr-17 acts in the same pathway as trt-1 and the 9-1-1 complex subunits mrt-2 and hus-1, double mutants were
constructed and DNA was prepared from both single and double mutants that were propagated for multiple generations (n > 4 independent strains for each genotype). Southern blot analysis of hpr-17, mrt-2 or hus-1 single mutants revealed rates of telomere shortening similar to those of trt-1 (p > 0.3) (Figure 4.4B, C). Additionally, double mutants of hpr-17 with mrt-2, hus-1 or trt-1 displayed rates of telomere shortening that were not significantly different than those of the single mutants (p > 0.6) (Figure 4.4B, C). Thus, hpr-17 acts in the same pathway as mrt-2 and hus-1 with respect to telomerase-mediated telomere elongation.

**Discussion**

In diverse organisms, Rad17 has been shown to play an important role in the cellular response to genotoxic stresses, such as UV, HU and IR (al-Khodairy and Carr 1992; Griffiths, Uchiyama et al. 2000; Boulton, Gartner et al. 2002; Heitzeberg, Chen et al. 2004). Mammalian cells lacking RAD17 and the 9-1-1 complex subunits fail to proliferate. Although this phenotype can be rescued with an additional deletion of p21 or p53 these suppressed mutations perturb DNA damage induced apoptosis and cell cycle arrest (Weiss, Enoch et al. 2000; Bao, Lu et al. 2004; Pandita, Sharma et al. 2006). Our genetic studies indicate that the C. elegans Rad17 homolog may function in the same pathway as the 9-1-1 complex with regards to repair of IR-induced DNA damage (Figure 4.2) and with regards to eliciting cell cycle arrest responses to either IR or HU (Figure 4.3). In addition, hpr-17 is fully required for an apoptotic response to IR (Figure 4.3) suggesting that it is likely to be required for apoptotic signaling in mammals.

Aside from its conserved role in responding to DNA damage, evidence from studies in yeast, nematodes and humans suggests that the 9-1-1 complex may play a variable role at
chromosome ends. Yeast 9-1-1 complex and Rad17 mutants display short telomeres, indicating that telomerase is functional but may be impaired. In contrast, *C. elegans* 9-1-1 complex and hpr-17 mutants display progressive telomere shortening phenotypes that result in sterility, suggesting a loss of telomerase activity in these mutants (Figure 4.4) (Ahmed and Hodgkin 2000; Hofmann, Milstein et al. 2002). Furthermore, our genetic studies indicated that hpr-17 acts in the same telomere maintenance pathway as both the 9-1-1 complex and telomerase *in vivo* (Figure 4.4). In agreement with our genetic evidence, biochemical analysis from mammalian cells has shown that members of the 9-1-1 complex and RAD17 can bind telomeric DNA and facilitate telomerase activity *in vivo* (Francia, Weiss et al. 2006; Verdun and Karlseder 2006). However, deficiency for the mammalian RAD17 and the 9-1-1 complex subunits causes immediate, severe rather than progressive effects on telomere length, suggesting that it may play an additional role at mammalian telomeres. Metaphase spreads of both human and mouse cells lacking RAD9, RAD1 or HUS1 display increased levels of chromosome abnormalities such as chromatid breaks, aneuploidy, dicentrics and telomere loss (Weiss, Enoch et al. 2000; Bao, Lu et al. 2004; Pandita, Sharma et al. 2006). Multiple abnormalities are observed within a single cell, thus, the chromosomal dysfunction observed in mammalian cells deficient the 9-1-1 complex or RAD17 may produce indirect effects on telomere stability perhaps as a consequence of recruitment of DNA damage response proteins that facilitate telomere capping to unrepaired sites of endogenous DNA damage (d'Adda di Fagagna, Teo et al. 2004). Alternatively, the mammalian 9-1-1 complex may play an additional telomerase-independent function in telomere stability (Francia, Weiss et al. 2006). We conclude that the 9-1-1 complex and its
RFC clamp loader, Rad17, may play a conserved essential role in facilitating telomerase activity in multicellular organisms.

The DNA damage response phenotypes of yeast, nematodes and mammalian cells deficient for the 9-1-1 complex and Rad17 suggest that they may be recruited to chromosome ends as a consequence of a telomeric structure that resembles damaged DNA. Given that \textit{mrt-2} is defective for repair of IR-induced double-strand breaks (Clejan, Boerckel et al. 2006), telomeric termini may trigger a double-strand break DNA damage response via \textit{hpr-17} and the 9-1-1 complex when they unfold during S-phase. This hypothesis is supported by the localization of other double-strand break repair proteins such as Ku and the MRN complex to telomeres (Song, Jung et al. 2000; Zhu, Kuster et al. 2000; Bertuch and Lundblad 2003). Further, the absence of TRF2 results in unprotected telomeres that trigger a DNA damage response via ATM, a double-strand break sensing kinase (Karlseder, Hoke et al. 2004; Celli and de Lange 2005). However, \textit{C. elegans mrt-2, hus-1} and \textit{hpr-17} mutants are also defective in initiating cell cycle arrest in response to HU (Figure 4.3), which results in stalled replication forks. The replication of highly repetitive telomeric DNA sequences may yield slipped DNA structures that cause replication fork arrest, which may be sufficient to recruit the 9-1-1 complex to telomeres during S phase (Fouche, Ozgur et al. 2006). Evidence for this hypothesis in mammalian cells shows that ATR, which primarily responds to replication-associated repair, is recruited to telomeric DNA in late S phase where it phosphorylates RAD17 (Verdun and Karlseder 2006). It is also possible that the unraveling of chromatin or telomere binding proteins at the T-loop, which resembles a recombination intermediate, might be sufficient to trigger a DNA damage response. In this context, deficiency for the 9-1-1 complex subunit \textit{mrt-2} has been shown to result in aberrant
homologous recombination events (Harris, Lowden et al. 2006). In addition, mrt-2 suppresses chromosome rearrangements that flank G-rich DNA tracts, which may resemble the G-rich strand of telomeric DNA in their ability to form non-canonical G quadruplex structures (Harris, Lowden et al. 2006). Once bound to telomeric DNA, the 9-1-1 complex may facilitate the recruitment of telomerase to chromosome ends, perhaps by modulating processing of the chromosome terminus or by acting to tether telomerase during telomere repeat addition, as might be expected for a protein complex homologous to the PCNA polymerase clamp.

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The work discussed in this thesis has been based on gaining a better understanding of (1) genes involved in double strand break repair and telomere maintenance/telomerase, and at the same time, (2) elucidating the nature of germ cell immortality using an unbiased genetic approach, including two forward genetic screens, one reverse genetic screen and one reverse genetic approach.

**Forward genetic screens**

Two independent forward genetic screens isolated a variety of mutants: one that is involved in DNA damage induced cell cycle checkpoint and telomere maintenance; 15 mutants that are essential for germline immortality at 25°C (Figure 5.1), some of which are involved in DSB repair; five mutants that are involved in DSB repair and are temperature-sensitive sterile; and 31 mutants essential for germline immortality at all temperatures, some of which are involved in the S-phase cell cycle checkpoint. The number of mrt mutants obtained in the screens suggests that by selecting for HU resistance first, I enriched for mrt mutants as compared to an unbiased Mrt screen and the Rad screen (Figure 5.1). Analysis of the mutants revealed that many of them are also involved in cell cycle arrest in response to HU or IR. Apoptosis does not seem to play a role in germline immortality as evidence by:
Figure 5.1: Comparison of efficiency of three forward genetic screens to identify mutants defective for germline immortality

Percent recovered is based on the number of EMS mutagenized lines tested. For the unbiased screen, 25°C and 20°C mrt mutants were identified and 5 out of 87 20°C mrt mutants were telomere defective. For the Rad screen, only 25°C mrt mutants were identified. For the HU screen, 20°C mrt mutants were identified and 1 out of 32 was telomere defective. 20°C mrt mutants were enriched among the hu mutants.

Mutants defective for apoptosis induction in response to DNA damage were obtained in both mrt and non-mrt mutants (Chapter 2); the lack of induction of apoptosis in mrt mutants near sterility (data not shown); and mutants defective for genes involved in the apoptotic pathway are not Mrt (Ahmed 2006). Thus, initial analysis of the various mrt mutants does suggest a link between some DDR pathways and germline immortality.

The main phenotype of interest is the Mrt phenotype because it is based on the loss of immortality in the germline which will in turn help us to understand the genes required to maintain cellular immortality. Outcrossing the isolated mutant lines several times in regards to their Mrt phenotype and then testing for co-segregation of the DNA damage response phenotypes may help to elucidate if the gene mutated is involved in both pathways. If the DDR phenotype does co-segregate then it would be the easier phenotype to use in complementation tests. Additionally, this information would allow for mutants to be placed in phenotypic groups and therefore fewer crosses would be needed to determine
complementation. The final proof would be the identification of a mutation in a gene and rescue of the phenotypes. Based on mutants that were not found identified in either the HU or IR screens, members of the Rad9/Rad1/Hus1 complex, hpr-17 or XPF (the binding partner of ERCC1), these screens are clearly not saturated. The HU screen did enrich for mrt mutants (32/334 compared to 15/906 EMS mutagenized F2 lines) whereas the Rad screen identified mutants that became sterile at 25°C (Figure 5.1). Thus, it can be concluded that two independent DDR pathways may be required for germ cell immortality; one under all conditions and one during growth at high temperature.

**Reverse genetic screens**

*Use of RNAi to isolate genome stability genes that are involved in germline immortality*

Studies have revealed that many genes that are expressed in cancer cells are also expressed in germ cells suggesting that some may play important roles in cellular immortality although many may be of the ‘house keeping’ capacity. Some of these genes define pathways involved in various aspects of genome stability. An RNAi-based Mortal Germline screen was conducted to identify genes involved in DDR/genome stability that are also essential for germline immortality in *C. elegans*. RNAi was used to allow for the testing of many genes. The screen found three genes cause a Mortal Germline at 25°C, whereas two genes became sterile at both 20°C and 25°C. Although the genes were involved in the mitotic spindle checkpoint, chromatin organization or protein folding, none play roles in DDR. Additionally, RNAi depletion of *hus-1* or *hpr-17* did not result in a Mrt phenotype. Although RNAi has been used to define many genes involved in development, it has also
been shown only to be highly effective for a subset of genes. For the 9-1-1 complex and *hpr-17*, however, RNAi based analysis of the DDR phenotypes of these genes revealed phenotypes weaker than those of strong loss-of-function mutants (Chapter 4) (Boulton, Gartner et al. 2002). Additionally, I have not been able to recapitulate the Rad phenotype of *mrt-2* or *hpr-17* deletion mutants with RNAi. Thus RNAi might not be as effective for many DDR genes which would in turn make it difficult to study other aspects of their functions, such as a role in germline immortality. Although time consuming, the best way to define genes involved in DDR and germline immortality, using reverse genetics, is to obtain deletion mutants, which are currently unavailable for many of these genes.

**HRP-17 is involved in DDR and telomere maintenance**

Since members of the RAD-9/RAD-1/HUS-1 (9-1-1) complex have been identified as being essential for germline immortality and telomere maintenance, a reverse genetic approach was taken to ask if HPR-17, an RFC clamp loader, interacted with the 9-1-1 complex to facilitate its role in telomerase-mediated telomere elongation. Analysis of the *hpr-17* deletion mutant revealed that it was essential for germline immortality and telomere maintenance. Genetic pathway analysis placed *hpr-17* in the same DDR pathway as the 9-1-1 complex, with respect to responding to IR induced DSBs, IR and HU cell cycle arrest responses and IR induced apoptosis. Additionally, *hpr-17* acts at telomeres as the clamp loader of the 9-1-1 complex and in the recruitment of telomerase which acts in telomere elongation.

The next step would be to show that the 9-1-1 complex and *hpr-17* bind telomeres and affect telomerase activity. One way to do this is to show direct binding through
immunoflourescence or chromatin immunoprecipitation. With \textit{mrt-2} antibodies currently available I have tried both of these assays but neither worked suggesting that the current \textit{mrt-2} antibodies are not good for either of these assays. Also, no antibodies are currently available for \textit{hus-1}, \textit{rad-9} or \textit{hpr-17}. Another approach would be to test telomerase activity in the mutants. A tagged \textit{trt-1} construct can be introduced through injection in to the 9-1-1 complex subunit mutants and \textit{hpr-17} mutant, immunoprecipitated and tested for the ability of the precipitate to extend telomeric oligos \textit{in vitro}. If the \textit{hpr-17} loads the 9-1-1 complex which then loads and/or activates telomerase then telomerase activity should be decreased compared to wildtype, as suggested by recent evidence in mammalian cells (Francia, Weiss et al. 2006).

\textbf{Identification and characterization of \textit{yp4}}

The \textit{hu mrt} mutant, \textit{yp4}, is unique in its involvement with telomeres. Initial characterization suggests that \textit{yp4} is involved in cell cycle arrest induction but not in the apoptotic response to DNA damage. The Mrt phenotype of \textit{yp4} is delayed compared to previously identified telomere replication mutants however, end-to-end chromosome fusions are observed near sterility suggesting that it is involved in telomere maintenance. Southern blot analysis revealed that the rate of telomere shortening in early generation \textit{yp4} mutants is slower then \textit{trt-1} but telomeres in later generation \textit{yp4} mutants shorten at the same rate as \textit{trt-1}. Interestingly the initial length of the telomeres is longer then previously observed telomere lengths of \textit{trt-1}, \textit{mrt-2} and \textit{hus-1}, possibly due to long telomere length in the wildtype strains used for mutagenesis or as a result of HU treatment. This can be tested by
observing initial telomere lengths of other *hu mrt* mutants and by comparing telomere lengths before and after HU treatment.

Mapping places *yp4* on chromosome one and complementation analysis suggests that it may harbor a mutation in the *ercc-1* homolog, which is a conserved endonuclease. Sequencing of the *ercc-1* gene in *yp4* is currently in progress but due to the size of the gene (15 kb) it has been broken down to first look for a mutation in the exons. Sequencing of the introns, the 3’ UTR and 5’ UTR will follow if no mutation is detected. Genetic mapping placed *yp4* about one map unit away from the location of the *ercc-1* gene, however, analysis of this region suggests that there is a lot of DNA in this portion of chromosome one (Wicks, Yeh et al. 2001). Also, the number of recombinants obtained from the mapping cross was small and more (i.e. 50 recombinants) need to be tested to obtain a more accurate genetic map location of *yp4*. Regardless, the genetic mapping does place *yp4* between *unc-29* and *dpy-24*, thus single nucleotide polymorphism mapping between these two genes can be utilized to further narrow down the region in which *yp4* is located. Two deletion alleles of *ercc-1*, *tm1981* and *tm2073*, are currently being passaged to determine if they have a Mrt phenotype and are at F24 where they are beginning to display a slight decrease in the number of progeny. If the two deletion alleles become sterile, they will be analyzed for the presence of end-to-end chromosome fusions and telomere shortening. Additionally, complementation analysis between *yp4* and *ercc-1* will need to be conducted in regards to the Mrt phenotype. Finally, rescue of the Mrt and cell cycle arrest phenotypes of *yp4* with the ERCC1 gene would also prove if this is the gene mutated in *yp4*.

If *yp4* is *ercc-1* then it may act at telomeres by processing the overhang which may lead to a slower rate of telomere shortening as observed in early generations of *yp4*. To
better understand the role \textit{yp4} plays at telomeres, double mutant analysis with \textit{yp4} and \textit{trt-1}, \textit{mrt-1} or \textit{mrt-2} can be conducted. If its role is independent of telomerase then the double mutants should display a different rate of telomere shortening. Additionally, the telomerase assay previously mentioned can be conducted to test for a requirement of \textit{yp4} for telomerase activity. An assay to analyze telomere overhang length has recently been developed in our lab which could be used to test if the overhang length is extended in \textit{yp4} and \textit{ercc-1} mutants (T. Lee, unpublished). Since XPF interacts with ERCC1 analysis of the mutant, \textit{xpf-1} for the Mrt and HU phenotypes should be conducted as well as double mutant analysis. Additionally, the double mutants of \textit{yp4;mrt-2} could be tested to determine if \textit{yp4} acts in the same pathway as the 9-1-1 complex in regards to cell cycle arrest in response to DNA damage. Whether or not \textit{yp4} acts in the same pathway as the 9-1-1 complex or telomerase, it is unique compared to previously identified telomere maintenance mutants.

\textbf{DDR genes and germline immortality}

These screens and studies of DDR genes have shown that at least two DDR pathways are involved in cellular immortality. Some are essential, as seen in the 20°C \textit{mrt} mutants, and some are required under circumstances that involve specific growth conditions, demonstrated by the 25°C \textit{mrt} mutants and those that become sterile in liquid only. Why would these genes be necessary to establish and maintain immortality? One thought may be that they play roles in repairing the DNA, to keep deleterious mutations from arising. However, most of the \textit{mrt} mutants studied in the screens do not show an increase in mutation rates that would be expected of unrepaired damage. Instead they all display a decrease in the number of progeny and gross morphology problems in the germline near
sterility. Near sterility, many of the mrt mutants possess germlines that are very small or absent suggesting stochastic proliferation defects. Additionally, many of the germlines display improper mitotic to meiotic transitions (often displayed as fewer or more of one of these types of cells) or general under-proliferation of germ cells suggesting improper cell cycle regulation or some consequence of too much DNA damage (either constantly occurring or unrepaired). This would agree with the finding that a subset of the 25°C mrt mutants and the hu mrt mutants are defective for initiating cell cycle arrest in response to DNA damage and the enrichment of mrt mutants obtained from the HU screen. Interestingly, many of the mrt mutants from these screens or other mrt screens have shown that the apoptotic pathway in regards to germ cell elimination is not affected, even in late generations.

Chromosome stability and segregation may also be affected in some of these mutants. An increase in the number of chromosomes in the oocytes near sterility is typically an indication of chromosome segregation defects, however this was observed in only one hu mrt mutant suggesting that it may not play a large role in germline immortality. Other phenotypes indicative of chromosome mis-segregation are an increase in embryonic lethality and high incidence of males near sterility, which was observed in many of the mrt mutants. Mis-segregation caused by improper pairing of the sister chromatids, either due to gross chromosomal rearrangements or defects in homologous recombination/forming a synaptinal complex, could lead to embryonic lethality. If the six bivalents were incorrectly paired but still segregated evenly then six bivalents would be visible in the oocytes. Fluorescent in situ hybridization analysis of the chromosomes would reveal if gross chromosomal rearrangements or improper pairing of chromosomes occurred.
The phenotypes observed in the mutants near sterility and under no stress conditions provide insight into possible explanations for their role in immortality. With time and further analysis the genes mutated and their involvement in germline immortality will be revealed, which in turn may help to better understand cellular immortality.


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