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Epigenetic Telomere Protection by Drosophila DNA Damage Response Pathways: A Dissertation

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Epigenetic Telomere Protection by *Drosophila* DNA Damage Response Pathways

A Dissertation Presented

By

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EPIGENETIC PROTECTION OF *DROSOPHILA* TELOMERES BY DNA DAMAGE RESPONSE PATHWAYS

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Interdisciplinary Graduate Program
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If you can see, look.
If you can look, observe.

From the *Book of Exhortations*

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Gratitude does not begin to describe it. Thank you Dr. Michael Brodsky, my advisor, for your guidance, support, and teaching during my time under your wings. You always managed to keep my plate full, yet left me hungry for more. I appreciate all the opportunities you have given me. I would also like to thank my committee members for their time: Nick Rhind, Tony IP, William Theurkauf, Paul Kaufman, and Kami Ahmad.

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ABSTRACT

Several aspects of *Drosophila* telomere biology indicate that telomere protection can be regulated by an epigenetic mechanism. First, terminally deleted chromosomes can be stably inherited and do not induce damage responses such as apoptosis or cell cycle arrest. Second, the telomere protection proteins HP1 and HOAP localize normally to these chromosomes and protect them from fusions. Third, unprotected telomeres still contain HeT-A sequences at sites of fusions. Taken together these observations support a model in which an epigenetic mechanism mediated by DNA damage response proteins protects *Drosophila* telomeres from fusion.

Work presented in this thesis demonstrates that the *Drosophila* proteins ATM and Nbs are required for the regulation of DNA damage responses similar to their yeast and mammalian counterparts. This work also establishes a role for the ATM and ATR DNA damage response pathways in the protection of both normal and terminally deleted chromosomes. Mutations that disrupt both pathways result in a severe telomere fusion phenotype, similar to HP1 and HOAP mutants. Consistent with this phenotype, HOAP localization at *atm,atr* double mutant telomeres is completely eliminated. Furthermore, telomeric sequences are still present, even at the sites of fusions. These results support a model in which an epigenetic mechanism mediated by DNA damage response proteins protects *Drosophila* telomeres from fusion.

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INTRODUCTION

Herman Muller first proposed the concept of telomeres almost 70 years ago when he made the observation that following irradiation, broken chromosome ends always fused together and resulted in gross chromosomal aberrations (Muller 1941; Muller 1954). He also noted that a broken chromosome end never fused to an unbroken end and suggested there is a "permanent distinct organelle" at the end of the chromosome that protects it from fusing and is required for genomic integrity (Muller 1941; Muller 1954). Muller's observations were not distinct to *Drosophila*; Barbara McClintock made similar observations in maize. She demonstrated that in maize endosperm, a chromosome broken during meiosis fuses with its sister chromatid following replication to form a dicentric chromosome (McClintock 1939). During anaphase the fused chromosomes form a bridge that is broken when the cell enters telophase. The cell continues this fusion-bridge-break cycle indefinitely or until the broken end is healed (McClintock 1941). McClintock demonstrated that in the embryo the broken chromosome could be healed, indicating that a chromosome could acquire a new telomere.

Telomeres were later shown to serve two essential functions. First, as Muller and McClintock initially suggested, telomeres protect genomic integrity by preventing the ends of chromosomes from fusing with each other (Zakian 1995; Blackburn 2001). Second, telomeres prevent the loss of terminal sequences due to the inability of polymerase to fully replicate the chromosome end (the end replication problem) (Watson 1972) . In addition, telomeres also help position the chromosomes within the nucleus and aid in chromosome segregation.

The model proposed by Muller and McClintock suggested that a cell must be able to differentiate between a double strand break and the end of a chromosome. If a telomere is recognized as a double strand break the results could be catastrophic; a DNA damage response could result in cell cycle arrest, apoptosis or repair. Repair could then result in a fusion. However, organisms have evolved a sequence specific mechanism to help solve the problem of distinguishing a chromosome end from a double strand break. In most organisms studied, telomeres are composed of short, tandem guanine rich repeats that are added by the reverse transcriptase, telomerase, after every round of replication (Zakian 1995; Blackburn 2001). In cells that do not express functional telomerase, the ends of chromosomes gradually shorten until a critical length is reached, at which point the telomere no longer exists and the cell undergoes senescence. Sequence-specific DNA binding proteins recognize the terminal repeat sequences and contribute to telomere elongation and protection (Cooper et al. 1997; Shore 1997; de Lange 2002; Karlseder 2003).

One problem with the model described above is that it does not take into account the role of DNA damage response proteins in telomere protection. There is abundant evidence from a variety of organisms indicating that DNA damage response proteins are integral components of telomeres (d'Adda di Fagagna et al. 2004). This creates a paradox because DNA damage response proteins are required for initiating cell cycle checkpoints, DNA repair and apoptosis at sites of double strand breaks while inhibiting the same responses at telomeres. Why DNA damage response proteins activate a damage response at double strand breaks but not at telomeres is an active area of research.

A second problem with the sequence specific model is that it does not explain the sequence-independent protection of *Drosophila* telomeres. *Drosophila* telomeres are replicated by a telomerase-independent mechanism (Mason and Biessmann 1995; Biessmann and Mason 2003; Melnikova and Georgiev 2005). The telomeric specific retrotransposons HeT-A and TART attach to the ends of *Drosophila* chromosomes following replication. However, HeT-A and TART sequences are not required for protection in *Drosophila*. Terminally deleted chromosomes, which lack all telomeric sequences, can be isolated and do not induce a damage response or result in fusions (Mason et al. 1984; Mason et al. 1997; Ahmad and Golic 1998). Furthermore, they can be stably maintained for several generations even though they continue to lose terminal sequences (average loss is 75bp per generation) (Biessmann and Mason 1988). The terminal sequences change after every round of replication indicating that telomere protection can be inherited via a sequence-independent or epigenetic mechanism. The results described in this thesis support an end recognition-model in which DNA damage response proteins recognize a DNA structure at the chromosome end and recruit or stabilize telomere protection proteins.

Mammalian and Yeast Telomere Structure and Maintenance

This section will provide an overview of the canonical sequence specific telomere maintenance mechanisms used by most eukaryotic organisms (see Figure 1.1). This section is broken into three parts that focus on eukaryotic replication, telomere specific

binding proteins, and telomere architecture. Emphasis will be on the role of the more extensively studied telomere binding proteins TRF1 and TRF2. Finally, a brief summary of yeast telomere binding proteins and their role in telomere maintenance will be provided.

Replication. Mammalian and yeast telomeres are composed of short, tandem guanine rich repeats as well as subtelomeric sequences (Zakian 1995; Blackburn 2001). The short repeat sequences range from 5-8bp (depending on the organism) and can extend for a couple hundred base pairs in yeast to several kilobase pairs in humans. The specialized reverse transcriptase, telomerase, which contains its own internal RNA template, extends the ends of chromosomes by adding the short repeat sequences after every round of replication (Figure 1.1A). In the absence of telomerase mediated elongation, yeast and mammalian telomeres can also be elongated by telomerase-independent mechanisms that rely on recombination (Biessmann and Mason 1997; Biessmann and Mason 2003; de Lange 2006).

Sequence Specific Telomere Binding Proteins. In mammals, the protein complex known as Shelterin regulates telomere elongation and protection (de Lange 2005). Shelterin consists of six protein components that are found exclusively at telomeres: TRF1, TRF2, POT1, RAP1, TIN2, and TPP1. TRF1 and TRF2 are sequence dependent DNA binding proteins, which directly bind telomeric double stranded DNA while POT1 is the only known protein to bind the

Figure 1.1.

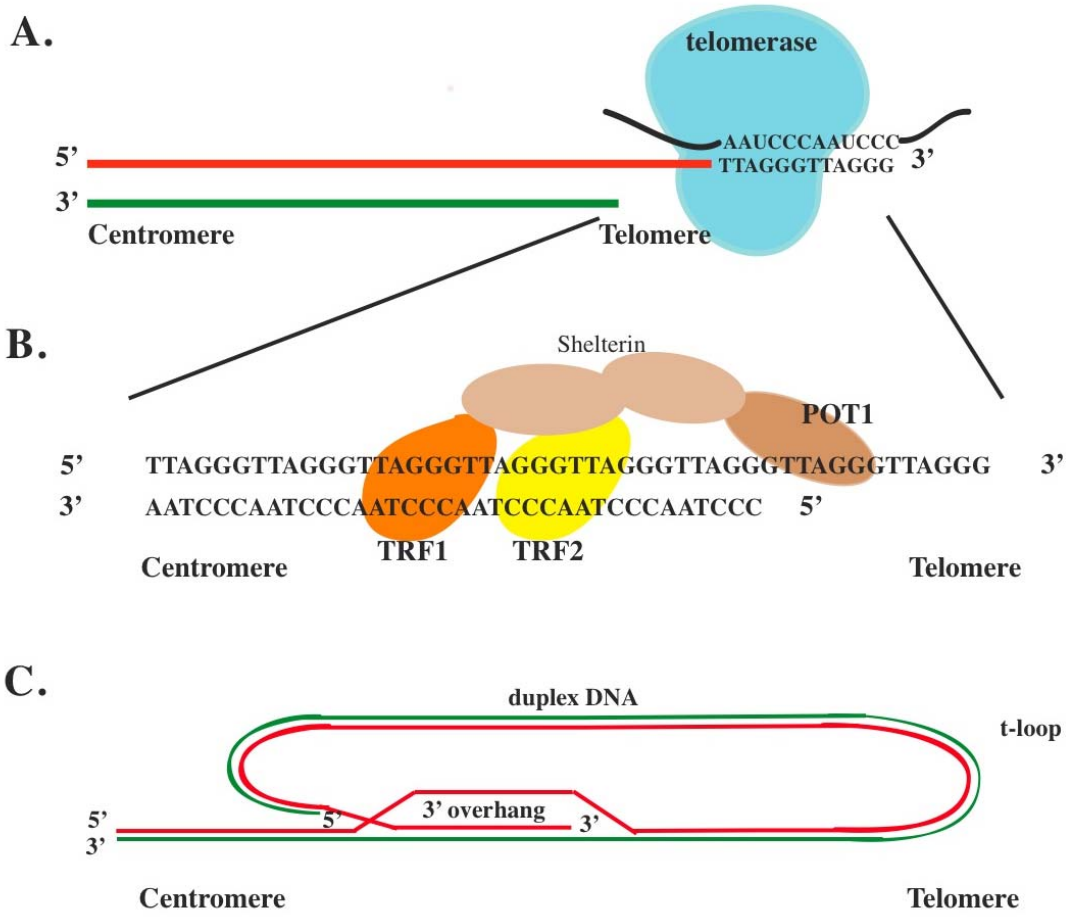


Figure 1.1. The canonical telomere maintenance pathway. (A) The reverse transcriptase, telomerase, which has its own RNA template, solves the end replication problem by adding short G-rich repeat sequences after every round of replication. (B) Sequence specific telomere binding proteins help regulate telomere elongation and telomere protection. (C) In mammals the 3' overhang loops back and invades the duplex DNA forming the t-loop. The t-loop hides the telomere from DNA repair enzymes and represents a second means of protection.

3'single stranded DNA overhang (Broccoli et al. 1997; Baumann and Cech 2001). The remaining proteins in the Shelterin complex are proposed to participate in protein-protein interactions that help form the stable complex (Figure 1.1B)(de Lange 2005).

TRF1 plays an important role in the regulation of telomere elongation. Over-expression of wild type TRF1 results in shortened telomeres while cells expressing a dominant negative form of TRF1 exhibit elongated telomeres (van Steensel and de Lange 1997). Taken together, these results suggest that TRF1 plays a role in the negative regulation of telomerase. Shortened telomeres have less telomeric DNA for TRF1 to bind resulting in increased telomerase access and subsequent elongation of the telomere. Therefore, when dominant negative TRF1 is expressed, only a small amount of endogenous TRF1 binds to the telomere resulting in enhanced telomerase access and increased elongation. When wild type TRF1 is over-expressed there is an abundance of TRF1 bound to the telomere, blocking telomerase access.

Like TRF1, TRF2 also plays a role in the negative regulation of telomere length (Smogorzewska et al. 2000). In addition, TRF2 is a key component of the mammalian telomere protection complex. Over-expression of a dominant negative TRF2, which disrupts its DNA binding activity, induces chromosome fusions in metaphase and DNA bridges in anaphase indicating that TRF2 is required for telomere protection (van Steensel et al. 1998). Genetic studies have demonstrated that these fusions are dependent on ligase IV and the NHEJ pathway (Smogorzewska et al. 2002). The TRF2 dominant negative phenotype closely resembles that of senescent cells except that the fusions still contain telomeric DNA (van Steensel et al. 1998). In addition, loss of TRF2

results in activation of p53- and ATM-dependent apoptosis indicating that unprotected telomeres can be recognized as damaged DNA (Karlseder et al. 1999; Smogorzewska et al. 2002). Finally, over-expression of TRF2 in senescent cells extends the lifespan and reduces the frequency of fusions (Karlseder et al. 2002). These observations suggest that TRF2 is also important for the protection of critically short telomeres.

The other components of the Shelterin complex all interact with either TRF1 or TRF2 and have been shown to contribute to the negative regulation of telomerase-mediated elongation and to telomere protection (de Lange 2005). Only tankyrase, a telomeric poly(ADP-ribose) polymerase has been demonstrated to positively regulate telomerase-mediated elongation by inhibiting TRF1 telomere access (Smith and de Lange 2000). Since TRF2 and TRF1 bind to sequence specific telomeric DNA, loss of terminal DNA results in a loss of binding sites. Therefore, it is not possible to distinguish unambiguously between the elongation and capping "protection" function of telomere-associated proteins in mammals.

Telomere Architecture. Mammalian sequence specific binding proteins also help organize the telomere into a higher order DNA structure known as a t-loop (telomeric loop) (Figure 1.1C) (Griffith et al. 1999; de Lange 2004). Following replication the telomere undergoes resection to form a single strand overhang of the 3' G-rich strand. The t-loop structure is the result of the single stranded 3' overhang folding back and invading the double strand DNA to form a loop. The proposed role of the t-loop is to hide the chromosome end, which resembles a double strand break, from DNA repair

proteins and nuclease degradation (de Lange 2002). Therefore, establishment of the 3' overhang is an important component in the protection of mammalian telomeres.

Yeast Telomere Binding Proteins. Like mammals, terminal-repeat binding proteins in yeast help regulate telomere function, indicating a conserved mechanism for telomere maintenance. Mutations in the fission yeast TRF homolog, Taz1, result in defects in both telomeric silencing and telomere length regulation (Cooper et al. 1997). Mutations in Taz1 alone do not cause telomere fusions, suggesting it does not have a direct role in telomere protection (Ferreira and Cooper 2001). Like Taz1, the budding yeast protein, Rap1, binds telomeric repeat sequences. Disruption of Rap1 results in an increase in the mean telomere length and a defect in telomere gene silencing (the reversible silencing of genes close to telomeres) (Lustig et al. 1990; Kyrion et al. 1993). In fission yeast, disruption of the Rap1 homolog, which does not directly interact with telomeric DNA results in impaired telomere length control, TPE (telomere position effect) and telomere clustering (Kanoh and Ishikawa 2001). Furthermore disruption of Rap1 in both budding and fission yeast results in NHEJ dependent telomere fusions (Miller et al. 2005; Pardo and Marcand 2005). Similar to fission yeast the human Rap1 homolog does not directly bind DNA but instead is recruited to telomeres by TRF2 and plays a role in the negative regulation of telomere length (Li et al. 2000; Li and de Lange 2003; O'Connor et al. 2004). Interestingly, t-loop structures have not yet been identified in yeast.

Drosophila Telomere Structure and Maintenance

Drosophila telomeres are not maintained by the canonical telomere maintenance mechanism described for mammals and yeast (see Figure 1.2). Therefore, this section will provide an overview of the unusual structure and maintenance of *Drosophila* telomeres and highlight the differences and similarities between *Drosophila* telomeres and other eukaryotic telomeres.

Drosophila Telomere Elongation. The failure of telomeric DNA from other organisms to hybridize to *Drosophila* telomeres and the recovery of terminally deficient chromosomes were the first indicators that *Drosophila* telomeres were different (Mason and Biessmann 1995). Subsequently, *Drosophila* were found to lack telomerase and the canonical short repeat sequences (Biessmann et al. 1990a). Instead, *Drosophila* telomeres are maintained by a telomerase-independent mechanism, despite serving an evolutionarily conserved function (Pardue and DeBaryshe 1999; Biessmann and Mason 2003). Sequence analysis of *Drosophila* telomeres revealed that in place of short G-rich repeats, *Drosophila* telomeres are composed of multiple copies of the telomere-specific non-LTR retrotransposons, HeT-A and TART (Figure 2A) (Biessmann et al. 1992; Levis et al. 1993; Mason and Biessmann 1995). Recently, a third retrotransposon with similarities to both HeT-A and TART, designated TAHRE (TART and HeT-A related), has been identified (Abad et al. 2004). These retroelements form randomly mixed arrays at the ends of chromosomes that can range from 10kb to 147kb long. HeT- A and TART elements are unique in that they

Figure 1.2.

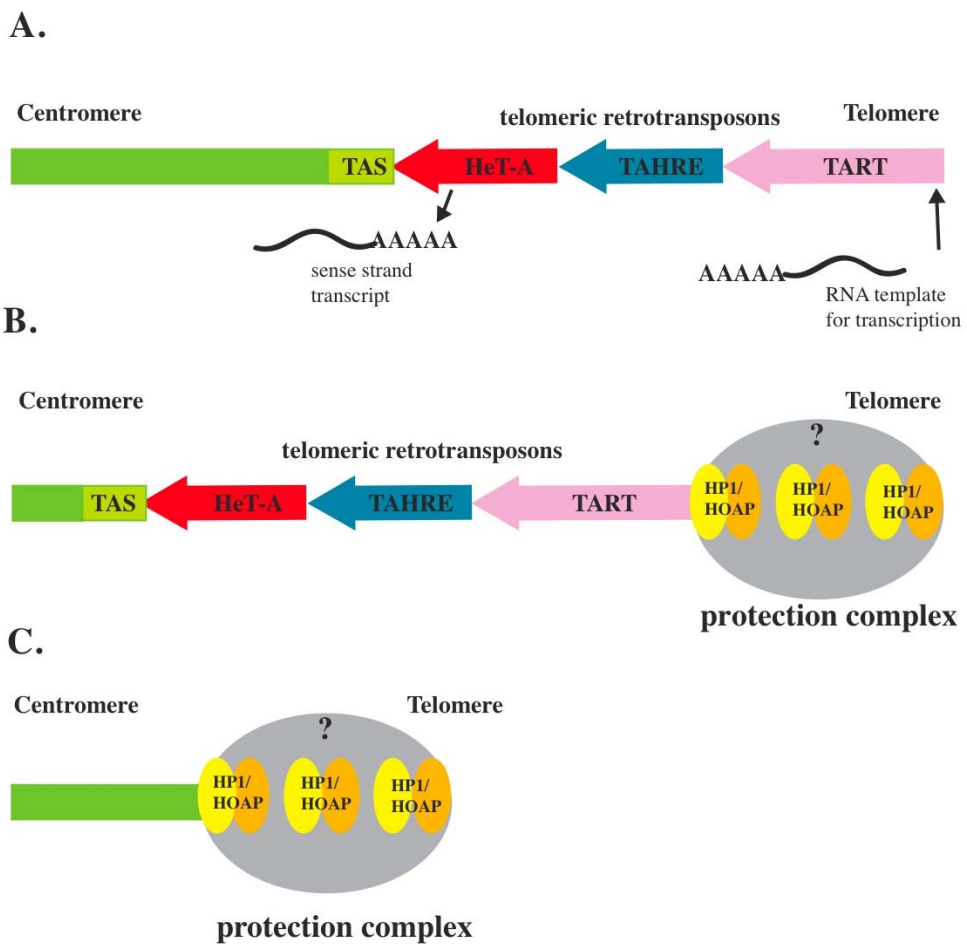


Figure 1.2. The *Drosophila* telomere maintenance pathway. *Drosophila* telomeres are maintained by a telomerase independent mechanism. (A) *Drosophila* telomeres are elongated by the addition of telomere specific non-LTR retrotransposons HeT-A, TART, and TAHRE. (B) The telomere-associated proteins HP1 and HOAP are required for telomere protection. (C) Terminally deleted chromosomes lacking all telomere different types of broken chromo indicating that the transposition of these elements does not associated sequence can be stably inherited and maintain a functional telomere protection complex.

transpose only to telomeres, although some elements have been found in other heterochromatic regions (mostly on the Y chromosome); none have ever been identified in euchromatin (Pardue and DeBaryshe 1999). Furthermore they can attach to many of these elements does not depend on a specific target sequence (Biessmann et al. 1990b).

Both TART and TAHRE are similar to other retrotransposons in that they encode both Gag and Pol proteins (Pardue et al. 2005). HeT-A however only codes for a Gag protein, suggesting that the reverse transcriptase activity is provided in trans, possibly by TART or TAHRE. HeT-A and TART are targeted to telomeres by the HeT-A encoded Gag protein (Rashkova et al. 2002; Rashkova et al. 2003). In addition to HeT-A, TART, and TAHRE, *Drosophila* telomeres also contain more proximally located telomere-associated sequences (TAS). In *Drosophila* the TAS appear to play an important role in telomeric gene silencing (Wallrath and Elgin 1995; Cryderman et al. 1999; Mason et al. 2000; Mason et al. 2003a; Mason et al. 2003b).

Although the mechanism of telomere elongation in *Drosophila* appears very different from other eukaryotes, there are actually several similarities (Pardue et al. 1997; Pardue et al. 2005). HeT-A and TART form head to tail arrays that resemble the repeat arrays found at the end of eukaryotic chromosomes that are elongated by telomerase. However, the retroelements are much more complex than the short repeat sequences. Therefore, *Drosophila* have much more diverse telomeric sequences compared to other eukaryotic telomeres. In addition, like mammals and yeast, HeT-A and TART are reverse transcribed directly onto the ends of the chromosomes. Transposition of HeT-A and TART requires the activity of a reverse transcriptase, mechanistically similar to

telomerase-dependent elongation. These similarities suggest that the telomeric retrotransposons are evolutionarily related to telomerase (Pardue et al. 1997; Pardue et al. 2005).

Although HeT-A and TART elements are found at the ends of almost all chromosomes they appear to be dispensable for chromosome stability. In yeast and mammals, loss of telomeric sequences leads to a senescent phenotype and chromosome fusions (Blackburn 2001; Maser and DePinho 2002). In contrast, *Drosophila* terminally deleted chromosomes, which lack the transposable elements and TAS, do not result in fusions or initiation of a damage response (Mason et al. 1984). Furthermore, terminally deleted chromosomes can be stably transmitted over several generations even though they continue to lose terminal sequences (Biessmann and Mason 1988; Ahmad and Golic 1998). Thus, unlike mammalian and yeast telomeres, the replication and capping (protection) function in *Drosophila* are separable, providing a useful system in which to study proteins involved in telomere maintenance.

Telomere length homeostasis in *Drosophila* is regulated by a variety of different proteins. Mutations in Heterochromatin Protein 1 (HP1) result in long telomeres and an increase in HeT-A transcription (Savitsky et al. 2002; Perrini et al. 2004), though the increase in transcription may be due to the increase in HeT-A copy numbers. Mutations in the RNAi components, *aubergine* and *spindle-E*, result in an increase in HeT-A and TART transcription and in HeT-A and TART attachment to broken chromosomes in the germline (Savitsky et al. 2006). The gene disrupted in the *Tel1* (yeast ATM homolog) mutant also plays a role in telomere length maintenance (Siriaco et al. 2002). Finally,

recent studies demonstrate that heterozygous mutations in the DNA repair proteins Ku70 and Ku80 result in long telomeres due to an increase in the frequency of HeT-A attachment and in the frequency of terminal DNA conversion (elongation using terminal DNA sequence from another chromosome) (Melnikova et al. 2005).

Similar to yeast and mammals, recombinatorial events such as gene conversion can contribute to telomere elongation in *Drosophila* (Mikhailovsky et al. 1999; Kahn et al. 2000). Conversion can occur between sequences on the same chromosome or between sequences of homologous chromosomes. Whether or not *Drosophila* telomeres have a similar 3' overhang and t-loop structure remains to be determined. In mammals the 3' overhang plays an important role in telomere protection, of interest will be if it has a similar function in *Drosophila*. A t-loop structure seems unlikely at *Drosophila* telomeres because they do not have the short repeat sequences required for t-loop formation.

Drosophila Telomere Protection Proteins. The sequence independent-protection of *Drosophila* terminally deleted chromosomes suggests that *Drosophila* telomere protection is regulated by an epigenetic mechanism. Consistent with this, no telomeric sequence (HeT-A, TART) specific DNA binding proteins (TRF2/TRF1 like proteins) have been identified in *Drosophila*. However, several telomere-associated proteins that are required for telomere protection in *Drosophila* are known. These proteins include Heterochromatin Protein 1 (HP1), Heterochromatin protein 1/ORC2 Associated Protein (HOAP), ubiquitin ligase, UbcD1, and the recently described transcription factor Without

children (Woc) (Cenci et al. 1997; Fanti et al. 1998; Cenci et al. 2003b; Raffa et al. 2005). This section will provide an overview of *Drosophila* telomere protection proteins. The main focus will be the role of the telomere-associated proteins HP1 and HOAP.

Heterochromatin Protein 1 (HP1). HP1 is best known for its role in gene silencing and transcriptional regulation. However the role of HP1 in telomere protection will be the focus of this thesis. The mechanism by which HP1 contributes to heterochromatin assembly is important for understanding how it functions to protect telomeres. Therefore, this section will provide background on the better-known roles of HP1 and then focus on the role of HP1 in telomere protection. Finally, results supporting a conserved role for HP1 in telomere protection in other organisms will be provided.

The Role of HP1 in Gene Silencing and Regulation. HP1 is a non-histone protein encoded by the modifier of position effect variegation *Su(var) 2-5* locus (Eissenberg et al. 1990). Position effect variegation (PEV) is the epigenetic phenomenon in which a normal expressing euchromatic gene is silenced when placed close to or in a heterochromatic region following a chromosomal rearrangement (Weiler and Wakimoto 1995). This phenomenon has been extensively studied in *Drosophila* using the white gene, a gene normally found in a euchromatic region on the X chromosome. The product of the white gene is cell autonomous and expression in all pigment cells is required for a uniformly red eye. When the white gene is placed in or near pericentric heterochromatin

the result is a variegating phenotype resulting from repression of white expression in some cells but not others. Genes next to telomeric heterochromatin can also exhibit repressed and variegated expression. This phenomenon is referred to as telomeric position effect (TPE) (Mason et al. 2000; Wallrath 2000).

Genes that influence PEV (heterochromatin formation) are known as either enhancers of variegation e(var)s or suppressors of variegation su(var)s. Heterozygous mutations in HP1 result in a less silencing of variegating genes at pericentric regions and at the fourth chromosome telomere, while over expression of HP1 enhances silencing of variegating genes indicating that PEV is sensitive to the dose of HP1 (Wallrath and Elgin 1995). The effect of HP1 on PEV is due to a direct role in heterochromatin assembly. In addition to its effects on centric heterochromatin HP1 also effects silencing of genes located in other regions of the chromosome. Experiments by Li et al demonstrated that tethering HP1 upstream of a reporter transgene inserted into euchromatic sites was sufficient to nucleate heterochromatin, indicated by silencing of the transgene (Li et al. 2003). Interestingly, a microarray study performed by Cryderman et al demonstrated that HP1 is also required for the expression of some euchromatic genes (Cryderman et al. 2005).

Consistent with a role in heterochromatin assembly, HP1 localizes predominantly to heterochromatic regions in addition to telomeres and several euchromatic sites (James et al. 1989; Fanti et al. 1998). The HP1 protein consists of two conserved domains, the chromo domain and the chromo shadow domain (Hiragami and Festenstein 2005). A hinge region separates the two domains. The chromo shadow domain mediates

interactions between HP1 and a variety of proteins. The chromo domain is required for binding to methylated lysine 9 of histone H3 (meK9H3), a histone modification found primarily in heterochromatic regions (Hiragami and Festenstein 2005).

Methylation of lysine 9 of histone H3 is mediated by the conserved methyl transferase Su(var)3-9. Similar to HP1, Su(var)3-9 exhibits dosage dependent effects on PEV (Schotta et al. 2002) . Studies in mammals and *Drosophila* demonstrate that Su(var)3-9 creates a binding site for the HP1 chromo domain and interacts with the HP1 chromo shadow domain (Bannister et al. 2001; Lachner et al. 2001; Schotta et al. 2002). In *Drosophila,* Su(var)3-9 co-localizes with HP1 at centromeric and telomeric sites but not at euchromatic sites. The localization of HP1 and Su(var)3-9 to heterochromatin is mutually dependent (Schotta et al. 2002). In HP1 mutants, Su(var)3-9 still associates with heterochromatin but localizes to additional euchromatic sites, indicating that HP1 may be required to restrict Su(var)3-9 binding. In *Su(var)3-9* mutants, HP1 is reduced at the chromocenter and an abundance of unbound HP1 can be observed in the nucleus. However fourth chromosome localization of HP1 and methylation of histone H3 is unaffected, suggesting that there may be another methyl transferase (Schotta et al. 2002).

The current model for HP1-mediated heterochromatin formation suggests a stepwise mechanism in which meK9H3 recruits HP1, which can in turn recruit the histone methyl transferase Su(var)3-9 (Maison and Almouzni 2004). Su(var)3-9 can in turn methylate adjacent lysines, creating additional binding sites for HP1 and propagation of heterochromatin along the chromosome. Human Suv39H1 can partially rescue the

silencing defects of a *Drosophila su(var)3-9* mutant, indicating a conserved mechanism for Su(var)3-9 and HP1 mediated heterochromatin formation (Schotta et al. 2002) .

Both HP1 and Su(var)3-9 localize to telomeres suggesting that they may also contribute to the maintenance of telomeric heterochromatin. Further support is provided by recent studies demonstrating that *Drosophila* telomeres are enriched in trimethylated lysine 9 of histone H3 (Cowell et al. 2002; Schotta et al. 2002). Furthermore, the role of HP1 and Su(var)3-9 in the epigenetic inheritance of chromatin modifications during cell division suggests that a similar activity may contribute to telomere protection. The role of Su(var)3-9 and methylation of histone H3 at *Drosophila* telomeres is just beginning to be revealed. Su(var)3-9 is not absolutely required for telomere protection because mutations in *Su(var)3-9* do not result in telomere fusions (Perrini et al. 2004). Moreover, Su(var)3-9 mutations also do not abolish meK9H3 at telomeres suggesting that another methyltransferase may compensate or may be required for methylation at telomeres. In contrast, studies have demonstrated that HP1 plays an important role in telomere protection in addition to telomere silencing and telomere elongation (Fanti et al. 1998).

The Role of HP1 in Telomere Protection. Analysis of *Su(var)2-5* mutant larval brain cells revealed a telomere fusion phenotype in which almost 100% of metaphase cells have at least one fusion with an average of 4-6 fusions per nuclei (Fanti et al. 1998). These fusions persist in telophase suggesting that they are covalent DNA-DNA linkages that cannot be resolved during anaphase. Supporting this assumption, *Su(var)2-5* mutants exhibit an increased frequency of polyploidy/aneuploidy and chromosomal

rearrangements indicating cells have undergone multiple rounds of fusion-bridge-break cycles.

Telomeric functions of HP1 include regulation of telomeric silencing, protection, and elongation. As mentioned, HP1 mutants have elongated telomeres, increased transcription of HeT-A/TART, and increased telomere fusions (Fanti et al. 1998; Cryderman et al. 1999; Savitsky et al. 2002). A recent study by Perrini *et al* shed light on how HP1 can mediate three separate aspects of telomere maintenance by utilizing separation of function alleles of *Su(var)2-5* (Perrini et al. 2004). Previous studies demonstrated that mutations in the HP1 chromo domain did not result in telomere fusions indicating that the chromo domain was dispensable for the function of HP1 in telomere capping (Fanti et al. 1998). Perrini *el al* (2004) demonstrated that the chromo domain is required for telomeric silencing and elongation. Mutations that disrupt the chromo domain also abolish meK9H3 at telomeres. These observations suggest that the telomeric silencing and elongation function of HP1 is mediated through its interaction with H3meK9. Furthermore, Perrini *et al* demonstrated that the telomere capping function of HP1 is due to its direct binding with DNA and is dependent on the hinge region of the HP1 protein. HP1 can bind both single and double strand DNA but has preference for single strand DNA suggesting that the structural design of *Drosophila* telomeres may contain a single strand 3' overhang similar to yeast and mammals (Perrini et al. 2004).

Heterochromatin and Orc2 Associated Protein (HOAP). HOAP was isolated from *Drosophila* embryo extracts as part of a complex with HP1 and ORC2 (Shareef et al.

2001). Analysis of the HOAP protein revealed that it contains an HMG domain and has DNA binding activity. Similar to HP1, mutations in HOAP cause a mild suppression of position effect variegation (Shareef et al. 2001) indicating that HOAP may also play a role in heterochromatin assembly. In contrast to HP1, HOAP localizes primarily to telomeres while only faint localization is detected at pericentric heterochromatin and euchromatic sites (Shareef et al. 2001; Badugu et al. 2003). Based on the localization of HOAP and its DNA binding activity, a likely assumption would be that HOAP binds telomeric DNA. However, there is no convincing data suggesting that HOAP binds HeT-A or TART telomeric DNA.

Caravaggio (*cav*), a mutant in the HOAP protein, was isolated from a mutagenesis screen designed to identify mutations affecting chromosome behavior (Cenci et al. 2003b). Cytological analysis of *cav* mutant larval brain cells revealed a fusion phenotype similar to that observed for HP1 mutants. Like HP1 almost 100% of metaphase cells have at least one fusion with an average of 4 fusions per nuclei. *cav* mutants also have a high frequency of chromosomal aberrations indicating multiple rounds of fusion-bridge-break cycles (Cenci et al. 2003b). The similar cytological phenotypes and the evidence that HOAP and HP1 are part of the same complex suggest that they might cooperate in telomere protection.

The HP1 and HOAP Complex. HOAP has been demonstrated to directly interact with HP1 although the significance of this interaction in telomere protection is still not clear. The chromo shadow domain and hinge region of HP1 mediate the interaction

between HOAP and HP1 (Badugu et al. 2003). Interestingly, HP1 is not absolutely required for HOAP telomeric localization since HOAP localizes to 70% of HP1 mutant mitotic telomeres not involved in fusions (Cenci et al. 2003b). The opposite experiment cannot be completed in a HOAP mutant because HP1 localization at mitotic telomeres cannot be unambiguously determined from its abundant localization to heterochromatin. Therefore, it is not known if HOAP is required for HP1 telomeric localization. Whether HP1 and HOAP are recruited independently to telomeres or if they are recruited together as a complex remains unclear. Interestingly, both HP1 and HOAP localize to terminally deleted chromosomes that lack all telomeric sequences and can protect these chromosomes from fusions (Figure 2C) (Fanti et al. 1998; Cenci et al. 2003b). The localization of HP1/HOAP to terminally deleted chromosomes indicates that they recognize something other than a specific DNA sequences.

UbcD1. Mutations in *UbcD1* result in telomere fusions without loss of telomeric DNA or telomere associated proteins (HP1) (Cenci et al. 1997). The fusions do not appear to be covalent DNA-DNA linkages because *UbcD1* mutants have a low frequency of acentric fragments and aneuploid cells. Therefore the fusions are most likely resolved during anaphase. Because the *UbcD1* gene encodes an ubiquitin conjugating enzyme, the fusions are proposed to be due to a failure to degrade some telomere associated-protein(s). Thus far, *UbcD1* telomeric targets have not been identified although one potential target has been ruled out. HP1 localizes normally to telomeres in *Ubdc1* mutants (Cenci et al. 1997).

Without Children (Woc). A genetic screen designed to isolate genes involved in telomere protection identified the zinc finger protein without children (Woc) (Raffa et al. 2005). The screen examined over 1600 third chromosome late lethal mutants for telomere fusion phenotypes. In total, nine independent genes were identified. Thorough cytological analysis of *woc* mutant larval brain cells revealed an average telomere fusion frequency of two fusions per metaphase. The *woc* mutant fusion phenotype is mild in comparison to the *su(var)2-5* and *cav* mutant fusion phenotype. Supporting the proposal that *woc* functions as a transcription factor (Wismar et al. 2000), the Woc protein localizes to many euchromatic bands in polytene chromosomes however, some diffuse Woc staining was observed at telomeres. Intriguingly, *woc* appears to act in a separate telomere protection pathway from other telomere protection proteins described thus far. HP1 and HOAP localize normally to *woc* mutant telomeres and *woc* double mutants have fusion frequencies equal to the sum of the single mutant frequencies (Raffa et al. 2005). Identifying transcriptional targets of Woc and other proteins that contribute to this pathway will be of great interest.

Conserved Functions for *Drosophila* Telomere Protection Proteins. Many of the functions described for *Drosophila* HP1 are conserved in other organisms. Mammals have three isoforms of HP1: alpha, beta, and gamma. Over-expression of HP1 alpha in *Drosophila* increases silencing of a white transgene at centric and telomeric heterochromatin indicating that human HP1 can participate in heterochromatin formation and gene silencing similar to *Drosophila* HP1 (Norwood et al. 2004). However, human

HP1 does not completely rescue the *Drosophila* HP1 mutant suggesting that in *Drosophila*, HP1 has other functions (Norwood et al. 2004). It is likely that one of the other HP1 isoforms may contribute to those functions in mammals.

Suv39h1 and HP1 homologs are both required for telomere function in mammalian cells. Over-expression of the alpha and beta isoforms of HP1 results in a decrease in telomerase binding at telomeres and a subsequent decrease in telomere length (Sharma et al. 2003). Expression levels of HP1 also affect telomere binding of Ku70, TRF2, and/or TRF1, three proteins known to localize to telomeres and regulate telomere length (Sharma et al. 2003). Primary cells from mice expressing a dominant negative Suv39h1 have elongated telomeres compared to wild type littermates (Garcia-Cao et al. 2004). Although these cells retain protective function (absence of telomere fusions), they have a reduction of meK9H3 and cbx proteins (homologous to HP1) at telomeres (Garcia-Cao et al. 2004). Collectively, these results suggest that in mammals, Suv39h1 plays an important role in telomere length regulation. Furthermore, these results indicate that telomere length in mammals is regulated by a chromatin mechanism.

Thus far data does not support an absolute requirement for HP1 in telomere protection in other organisms. Over-expression of HP1 results in a very small increase in telomere fusions compared to the frequency of fusions observed when TRF2 is disrupted (Sharma et al. 2003). In fission yeast, HP1 is not required for telomere protection but does regulate telomere length, telomere clustering, and telomeric gene silencing (Allshire et al. 1995; Ekwall et al. 1995).

No mammalian homolog of HOAP has been identified indicating that it might not be an evolutionarily conserved protein. Furthermore, the HOAP protein is encoded by a fast evolving gene (Cenci et al. 2003b), which makes sequence comparisons among different organisms difficult, in fact a homolog has not been described for any organism outside of *Drosophila*. The telomeric function of HOAP may be provided by one of the many telomeric binding proteins identified in mammals and yeast.

The DNA Damage Response

This section will provide an overview of the DNA damage response pathway based primarily on mammalian and yeast data. This section is divided into three parts the first part describes the canonical DNA damage response with emphasis on the sensor proteins involved in the activation of cell cycle checkpoints and apoptosis. The second section is a summary of the two primary DNA repair pathways. The final section highlights the human diseases that result from mutations in DNA damage response proteins. The Drosophila DNA damage response pathway will be described in a later section. The function of DNA damage response proteins at sites of damage is presumably critical for understanding how they function at telomeres. DNA damage response proteins are not only required for telomere protection but are also required to activate damage responses at dysfunctional telomeres.

The DNA Damage Response Pathway. DNA damage such as double strand breaks poses a considerable threat to a cell's genomic integrity and survival; unrepaired damage can lead to devastating mutations. Double strand breaks can arise spontaneously during normal cellular processes like replication or may be induced by a variety of exogenous damaging agents such as X-rays (Karagiannis and El-Osta 2004). Cells have evolved mechanisms to repair double strand breaks, to prevent cells with unrepaired breaks from undergoing cell division and transmitting damaged DNA to daughter cells (cell cycle checkpoints), or to eliminate cells with unrepairable damage (apoptosis). Collectively these mechanisms make up the DNA damage response pathway.

A cell's response to DNA damage is similar to other signal transduction pathways, in order to be effective, activation of the DNA damage response must be swift and precise. Following DNA damage a set of proteins is required to initially sense the damage, amplify the signal and transduce it to specific effector molecules in order to elicit the appropriate biological response (repair, arrest, or apoptosis). Mutations in genes required for the DNA damage response result in a group of human genetic disorders known as genomic instability syndromes (Taylor 2001).

One of the most well characterized sensors of DNA damage is the MRN complex. Components of this complex are some of the earliest proteins recruited to sites of double strand breaks (van den Bosch et al. 2003). The MRN complex consists of the nuclease Mre11, the structural maintenance of chromosomes protein Rad50, and NbsI. Structural and biochemical studies indicate that the MRN complex tethers the broken DNA ends together and processes the DNA for repair by homologous recombination. Nbs is also

required for several cell cycle checkpoints and is a known target of the kinase ATM following DNA damage (Iijima et al. 2004).

Central to the cellular response to DNA damage is a conserved family of protein kinases that is related to the P(I)3 kinases (Shiloh 2003). The P(3) like kinases include ATM (ataxia telangiectasia mutated), ATR (ATM and Rad3-related), and DNA-PKcs (DNA-dependent protein kinase catalytic subunit). All three kinases cooperate to regulate DNA damage responses. ATM and ATR both recognize and phosphorylate the same consensus sequence: serine or threonine followed by glutamate. To some degree, the activation of these kinases depends on the source and type of damage. ATM and DNA-PKcs are predominantly activated by double strand breaks, while ATR is activated primarily in response to single strand DNA lesions. However, the exact contribution of each kinase cannot be absolutely determined due to the high degree of complexity in the DNA damage cascade; ATM and ATR can phosphorylate many of the same substrates. Furthermore, the requirement for ATR during cell proliferation limits the analysis of DNA damage responses in its absence (Brown and Baltimore 2000).

Following DNA damage and activation, ATR and ATM can phosphorylate multiple substrates, including the checkpoint kinases Chk1 and Chk2. In addition, the p53 transcription factor can be activated by several of the upstream kinases. Phosphorylation of p53 can induce apoptosis, cell cycle arrest, or DNA repair.

DNA Repair. Eukaryotes have at least two major DNA repair mechanisms, nonhomologous end joining (NHEJ) and homologous recombination (HR) (Pastink et al.

2001; Valerie and Povirk 2003). HR is a precise mechanism in which the sister chromatid (or the homologous chromosome) is used as a template to repair the broken DNA; this process results in complete restoration of the original DNA sequence. In contrast to HR, NHEJ does not require any sequence homology and instead, the broken ends are directly ligated together (Hefferin and Tomkinson 2005). Because some processing of the ends is often required in order to make the ends compatible for ligation, repair by NHEJ often results in small insertions and deletions. Therefore, the NHEJ repair pathway is considered to be error prone. A cell's decision to repair by HR or NHEJ depends on several factors, including the phase of the cell cycle and an organism's developmental stage. For example, during late S and G2 phases of the cell cycle sister chromatids are in close proximity making HR more practical. Furthermore NHEJ plays an essential role in immunoglobulin gene rearrangement (V(D)J recombination) in mammals (Lieber et al. 2003).

Proteins involved in the NHEJ repair pathway have been identified through genetic and biochemical studies and include the DNA-PK complex and ligase IV (Lieber et al. 2003; Collis et al. 2005; Hefferin and Tomkinson 2005). The DNA-PK complex consists of the DNA end binding protein Ku, a heterodimer of Ku70 and Ku80, and the catalytic subunit DNA-PKcs. The current model of repair by NHEJ includes Ku70/80 acting to bridge the two DNA ends and then recruiting DNA-PKcs and its binding partner Artemis. DNA-PKcs and Artemis are required to process the ends for ligation by ligase IV. Although the NHEJ repair pathway is conserved in yeast, one distinction is that yeast

do not have a DNA-PKcs homolog. Another kinase may provide DNA-PKcs activity in yeast.

Genes required for HR were first identified by genetic experiments in yeast and belong to the Rad52 epistasis group (Pastink et al. 2001; Valerie and Povirk 2003). Genes in this group include the MRN complex, Rad52, and Rad51. In mammals, mutations in genes involved in HR are lethal suggesting that repair by HR is essential during development. In yeast, the contribution of NHEJ only becomes apparent when the HR pathway is inactivated (Hefferin and Tomkinson 2005). For example, in *S.cerevisiae* ligaseIV mutants are viable and only exhibit sensitivity to IR when in a Rad52 mutant background. Homologous recombination is the primary repair pathway in yeast and is critical during mammalian development.

Chromosome Instability Syndromes. Mutations in many of the proteins involved in the DNA damage response have been linked to a variety of distinct human diseases known as chromosome instability syndromes (Taylor 2001). Although these diseases result from the disruption or mutation of one particular gene they share some of the same clinical features, in addition to some unique clinical consequences. Furthermore, disruption of ATM and NBS1 telomeric functions may contribute to some of the disease phenotypes (Callen and Surralles 2004).

One of the first chromosome instability disorders to be identified was Ataxia-Telangiectasia (A-T), a progressive degenerative disease, characterized by the lack of muscle control (ataxia) and telangiectasias (dilated, superficial blood vessels) that appear

in the corners of the eye (Taylor 2001; Shiloh 2003). Individuals with this disease have several other clinical hallmarks including immunodeficiency, radiation sensitivity, high incidence of lymphomas, and premature aging. A-T is caused by mutations in the gene ATM (ataxia telangiectasia mutated), which encodes a protein kinase required for sufficient cellular response to DNA damage (see above). Many mutations affecting each of the 66 exons of ATM have been linked to A-T, with truncating mutations that reduce the function of the protein being the most common.

Patients with ataxia-telangiectasia like disorder (ATLD) share the same clinical features as A-T but with a less severe clinical course (Stewart et al. 1999). Originally patients with ATLD were misdiagnosed with A-T. However, later genetic analysis revealed that they did not carry mutations in ATM but instead had decreased levels of the Mre11 protein. As described above, Mre11 is found in a complex along with Nbs1 and Rad50 that is known to respond to double strand breaks. Mutations in the Nbs1 gene, which encodes the protein Nibrin, give rise to the human disease Nijmegen Breakage Syndrome (Matsuura et al. 2004). The most common mutation that results in the NBS disease is a 5 bp deletion in exon 6 that causes a frame shift. Hallmarks of this disease are similar to A-T and ATLD however patients are also plagued with microcephaly (reduced head size). Recently, mutations in the protein kinase, ATR (ATM and Rad3 related) have been identified in patients with Seckel Syndrome, a disorder that most closely resembles NBS because hallmarks include microcephaly and dwarfism but not chromosome instability or cancer (Alderton et al. 2004). The identification of mutations

in DNA damage response/repair genes that result in these related diseases strengthens the molecular connection between these proteins and the maintenance of genomic integrity.

DNA Damage Response Proteins and Telomere Maintenance

This section discusses the role of DNA damage response proteins in regulation of telomere length and telomere protection of both normal and unprotected (dysfunctional) telomeres. The results described in this section are primarily from yeast and mammalian genetic experiments. This section is divided into several parts. The first part is a summary of yeast and mammalian data supporting a role for DNA damage response proteins in the maintenance of normal telomeres. The role of DNA repair enzymes in telomere maintenance will be discussed in a separate section because repair proteins play counterintuitive roles at telomeres; they contribute to both telomere protection and to the fusion process. The third section describes the role of these same proteins at dysfunctional telomeres. The final section will describe results demonstrating the association and activity of damage response proteins with telomeres.

The Role of DNA Damage Proteins at Normal Telomeres. Genetic experiments in both yeast and mammals have demonstrated a conserved role for the P(I)3 like kinases and MRN complex in the maintenance of normal telomeres. Yeast have two members of the ATM family of (PI)3 like kinases, Tel1 and Mec1 in *S.cerevisiae* and Tel1 and Rad3 in *S. pombe*. In both budding and fission yeast, disruption of either *tel1* or *mec1* (*rad3*) results in moderately shorter but stable telomeres (Naito et al. 1998; Ritchie et al. 1999;

Chan et al. 2001). Simultaneous disruption of both kinases results in complete loss of telomeric repeat sequences and telomere fusions (Naito et al. 1998; Craven et al. 2002). In *S. pombe* the fusions can result in stable, circular chromosomes, which ultimately contribute to the viability of the mutant organism (Naito et al. 1998). Consistent with these results, a more severe phenotype is observed in *tel1 rad26* (Rad3 interacting protein) double mutants (Nakamura et al. 2002). Therefore in yeast, the upstream DNA damage protein kinases mediate parallel telomere maintenance pathways. The MRN complex (MRX in Budding yeast) also plays a role in telomere length and maintenance of normal telomeres in yeast (Tsukamoto et al. 2001; Ueno et al. 2003). Mutations in MRN components result in short telomeres similar to the Tel1 mutant phenotype. Double mutant analysis components of the MRN complex and *tel1* and *mec1* revealed that the MRN complex acts in the same telomere maintenance pathway as Tel1 (Ritchie and Petes 2000; Mieczkowski et al. 2003).

Similar to yeast, the upstream kinases and MRN complex also contribute to telomere maintenance in mammals. ATM deficient cells exhibit a modest increase in chromosome end-to-end fusions as well as accelerated telomere shortening (Pandita et al. 1995; Metcalfe et al. 1996; Pandita 2002). Inactivation of *Nbs1* in human cells by RNAi results in an increase in telomere associations (Zhang et al. 2005). To date a role for ATR in normal mammalian telomere maintenance has not been described. This could be due to the fact that ATR mutant mice are early embryonic lethal and mutant ES cells (embryonic stem cells) fail to proliferate and exhibit massive chromosome fragmentation

(Brown and Baltimore 2000; de Klein et al. 2000). Chromosome fragmentation and proliferation defects may mask any telomere defects.

DNA Repair Enzymes and Telomere Maintenance. The function of DNA repair proteins at telomeres is conserved from yeast to humans. Mutations in DNA-PKcs result in telomere fusions in metaphase and anaphase bridges but do not affect telomere length or telomerase activity in mice (Bailey et al. 1999; Goytisolo et al. 2001). This suggests a direct role for DNA-PKcs in telomere capping. In mice, loss of DNA-PKcs in telomerase deficient cells results in a faster rate of telomere repeat sequence loss and suppression of telomere fusions (Espejel et al. 2002). Taken together, these results indicate that NHEJ components are not only required to protect normal telomeres but are also required for the fusion of critically short telomeres.

In *S. cerevisiae,* mutations in the NHEJ proteins Ku70 and Ku80 result in defects in telomere silencing and short telomeres (Nugent et al. 1998). Because Ku is able to bind telomerase RNA, others have proposed that the short telomeres are a result of impaired telomerase regulation (Stellwagen et al. 2003). In contrast to budding yeast, fission yeast Ku70 and Ku80 are not required for telomeric position effect but do play a role in telomere length homeostasis (Baumann and Cech 2000; Miyoshi et al. 2003) (Manolis et al. 2001). Like the DNA damage sensor proteins, NHEJ components also associate with telomeric DNA, indicating a direct role for these proteins in telomere maintenance (Miyoshi et al. 2003).

In mammals mutations in the Ku70 and Ku80 DNA binding proteins result in telomere fusions in mouse cells, demonstrating a role for Ku in telomere protection (Bailey et al. 1999; Hsu et al. 2000). A similar phenotype was observed in human cells in which Ku80 function had been disrupted (Jaco et al. 2004) (Myung et al. 2004). Ku also plays a role in telomerase mediated telomere elongation. Mutations in human Ku80 result in shorter telomeres suggesting that like yeast, human Ku is required for telomerase regulation (Jaco et al. 2004; Myung et al. 2004). Finally, human Ku has also been demonstrated to interact with TRF1 further supporting a role for Ku in telomere length regulation (Hsu et al. 2000). The exact role of Ku in telomere length maintenance in mice is less clear although it is apparent that it has some function in the regulation of telomere elongation. Two separate studies using Ku-deficient MEFs describe opposite results. D'Adda di Fagagna et al. claim that telomeres become shorter in the absence of Ku while Jaco et al. claim that Ku80 mutant mice have longer telomeres. Differences in mouse strains could contribute to these conflicting results (d'Adda di Fagagna et al. 2001; Jaco et al. 2004).

The role of HR proteins in telomere regulation has not been extensively studied. Recent studies in mammals demonstrate that proteins required for repair by homologous recombination play a significant role in telomere maintenance. Deletion of Rad51D in both ALT (alternative lengthening of telomeres) and in telomerase positive cells results in shorter telomeres, apoptosis, and telomere fusions but normal TRF2 localization to telomeres (Tarsounas et al. 2004). Furthermore, Rad51D colocalizes with TRF2 at telomeres but does not directly interact with TRF2. These results suggest that Rad51D

acts in a separate telomere protection pathway from TRF2. In yeast, Rad51 plays a more significant role in telomere maintenance in the absence of telomerase (when elongation occurs by alternative mechanisms) (Le et al. 1999).

Disruption of the Rad51 interacting protein Rad54 results in significant loss of telomeric sequences without loss of telomerase, indicating a role for Rad54 in length maintenance (Jaco et al. 2003). Rad54 mutant MEFs also exhibit telomere fusions indicating a role in protection. Recently, a role for Rad9 in telomere maintenance has been described. Expression of a dominant negative form of Rad9 results in an increase in telomere fusion indicating a role for Rad9 in telomere protection (Pandita et al. 2006).

The Role of DNA Damage Response Proteins at Dysfunctional Telomeres. In addition to their role in the protection of normal telomeres, DNA damage response proteins also mediate apoptosis and cell cycle checkpoints in response to dysfunctional (unprotected) telomeres. Unprotected telomeres result in an ATM- and p53-dependent apoptotic response (Karlseder et al. 1999). Expression of dominant negative TRF2, but not ionizing radiation results in telomere dysfunction-induced foci (TIFs) (d'Adda di Fagagna et al. 2003; Takai et al. 2003). These foci colocalize with TRF1 and are composed of many DNA damage response proteins including MRE11, ATM, H2AX, Rad17 and 53BP1 (d'Adda di Fagagna et al. 2003; Takai et al. 2003). These results provide direct evidence that uncapped telomeres do resemble double strand breaks and are recognized by damage response proteins. The number of TIFs is diminished in A-T cells expressing dominant negative TRF2, suggesting that ATM is the main transducer of

the telomere dysfunction response (Takai et al. 2003). Some residual signal was observed at telomeres indicating that other proteins besides ATM, possibly one of the other P(I)3 like kinases can partially transduce the telomere dysfunction signal.

If damage response proteins contribute to normal telomere protection and are required for the response to uncapped telomeres, how then do telomeres prevent damage responses at capped telomeres and during S phase? Karlseder *et al* (2004) propose a model in which TRF2 blocks ATM-mediated DNA damage responses at telomeres without affecting responses elsewhere. Following irradiation, over expression of wild type TRF2 abrogates cell cycle arrest, induction of p53 and its targets, and autophosphorylation of ATM. Furthermore, the region of ATM that contains the damage induced autophosphorylation site was demonstrated to directly interact with TRF2 (Karlseder et al. 2004).

Another mechanism for how telomeres prevent a damage response at capped telomeres is based on results from a study in yeast that suggests the telomeric repeat sequence may act as an anti-checkpoint (Michelson et al. 2005). Induction of breaks adjacent to a telomeric repeat sequence results in an abbreviated checkpoint that depends on *mec1* and requires the MRN complex. A normal checkpoint is initiated when an induced break is greater than 0.6kb away from the repeat sequence. This data suggest that sequences near the telomere may not be repaired, and indicate a role for the repeat sequences in telomere protection (Michelson et al. 2005). Since all eukaryotic telomeres contain repeat sequences this function could be conserved in other organisms.

Telomeric Association and Activity of DNA Damage Response Proteins. In human cell, studies designed to find proteins that interact with TRF2 identified the MRN complex (Zhu et al. 2000). MRE11 and RAD50 colocalized with TRF1 and TRF2 throughout the cell cycle while NBS1 colocalized with TRF2 during S-phase only. Recently, Verdun et al demonstrated by chromatin immunoprecipitation that ATM, MRE11, and phosphorylated NBS1 and phosphorylated ATM associate with telomeric DNA during late S- and G2 phases (Verdun et al. 2005). These results suggest that the association of DNA damage response proteins with telomeres is cell cycle dependent. In addition DNA repair enzymes have also been demonstrated to localize to telomeres in mammals (Hsu et al. 1999; d'Adda di Fagagna et al. 2001)

Tel1 and Mec1 associate with telomeric DNA in a cell cycle dependent manner (Takata et al. 2004). Mec1 predominately associates with telomeres during S phase, which may be a result of its specificity for single stranded DNA. Thus the two kinases appear to be recruited to telomeres independently. The kinase activity of Mec1 is required for its association with telomeric DNA suggesting phosphorylation of substrates is part of its telomere function. In contrast, the kinase activity of Tel1 is not required for its telomeric association (Takata et al. 2004).

The *Drosophila* DNA Damage Response Pathway

Genetic experiments in *Drosophila* demonstrate that the DNA damage response pathway is conserved in flies. Like mammals, following DNA damage flies initiate

repair mechanisms, cell cycle checkpoints, and apoptosis. *Drosophila* p53 and Mnk (Chk2 homolog) mediate irradiation-induced apoptosis, and mutations in these two genes result in a defect in induction of apoptosis (but normal cell cycle arrest) following X-ray treatment (Brodsky et al. 2000b; Ollmann et al. 2000; Brodsky et al. 2004). Similar genetic experiments have demonstrated that damage-induced cell cycle arrest (and not damage-induced apoptosis) is mediated by Mei-41 (ATR) and mus304 (ATRIP) and the downstream checkpoint kinase Grps (chk1 homolog) (Hari et al. 1995; Ahmad and Golic 1999; Brodsky et al. 2000a; Brodsky et al. 2000b; Ollmann et al. 2000). Initially these two pathways appeared to be distinct, however results described here and results from others suggest that these pathways share some common mediators.

Microarray studies have demonstrated that *Drosophila* p53 regulates the induction of the proapoptoic genes *rpr*, *hid*, *skl* and components of the NHEJ repair pathway (Brodsky et al. 2003). Although the function of p53 in mediating damage-induced apoptosis is conserved in flies, there are some p53 functions that do not appear to be conserved (Brodsky et al. 2000a). Unlike mammalian p53, *Drosophila* p53 does not seem to play a role in the regulation of the G1 checkpoint. Another major difference between mammalian p53 and *Drosophila* p53 is its regulation. To date, no *Drosophila* Mdm2 homolog has been identified and there are no obvious candidates in the genome database (Sekelsky et al. 2000). Mdm2 is the major regulator of p53 protein levels in unstressed cells. Consistent with these observations, there is no rapid accumulation of p53 protein following DNA damage (Brodsky et al. 2003). Similar to mammalian p53,

Drosophila p53 is phosphorylated by Chk2 following DNA damage (Brodsky et al. 2003).

Currently, upstream activators of *Drosophila p53* and *chk2* are not known, but ATM is a probable candidate based on mammalian data. *Drosophila* encodes two damage response proteins in the ATM kinase family, Mei-41, which is most similar to ATR and CG6535/ATM, which is most similar to human ATM. *Drosophila* ATM likely plays a role in one or both of the DNA damage response pathways (Hari et al. 1995; Sekelsky et al. 2000; Laurencon et al. 2003; Brodsky et al. 2004). The role of *Drosophila* ATM in DNA damage responses and telomere protection will be the focus of Chapter 2. Other candidates in *Drosophila* likely to be upstream mediators of the DNA damage response pathways include the components of the MRN DNA repair complex. *Drosophila* has orthologs of all three components and studies have demonstrated that both Mre11 and Rad50 play a role in DNA repair and in the regulation of apoptosis. Mutations in Mre11 or Rad50 result in a high frequency of chromosome breaks and spontaneous apoptosis (Ciapponi et al. 2004; Gorski et al. 2004). In addition, Mre11 mutants exhibit a partial defect in cell cycle arrest following X-ray induction of DNA damage (Oikemus et al. 2006). The role of Nbs, the third component of the MRN complex, in the DNA damage response pathway is the focus of Chapter III.

Drosophila telomeres can be maintained by a sequence independent mechanism, which allows the elongation and protection processes to be studied separately. Our results demonstrating that *Drosophila* DNA damage response proteins are required for normal telomere protection are described in Chapters II and III.

CHAPTER II

DROSOPHILA ATM/TELOMERE FUSION IS REQUIRED FOR TELOMERIC LOCALIZATION OF HP1 AND TELOMERE POSITION EFFECT

Foreword

The work presented in this chapter has been published: Oikemus, SR., McGinnis, N., Queiroz-Machado, J., Tukachinsky, H., Takada, S., Sunkel, SE., Brodsky, MH. 2004. *Drosophila atm/telomere fusion* is required for telomeric localization of HP1 and telomere position effect. *Genes & Dev*. 18: 1850-1861.

Specific contributions are as follow: The *atm* cDNA rescue construct was created by Nadine McGinnis, Figure 2.1A. Analysis and quantification of HeT-A DNA at mitotic telomeres was contributed by Joana Queiroz-Machado, Figure 2.7A-E. Analysis of HeT-A sequences at telomeres of polytene chromosomes was performed by Nadine McGinnis, Figure 2.7F-G. Hanah Tukachinsky contributed to the analysis of anaphase bridges, Table 2.4.

Introduction

Telomeres are specialized DNA-protein structures required to replicate and protect the ends of eukaryotic chromosomes (Zakian 1995; Blackburn 2001). In most organisms, the reverse transcriptase telomerase prevents the loss of terminal sequences by adding short repeat sequences during S-phase. Sequence-specific DNA binding proteins that recognize telomere repeat sequences help regulate telomere length and protection (Cooper et al. 1997; Shore 1997; de Lange 2002; Karlseder 2003). DNA damage repair or signaling proteins also regulate telomere function (Chan and Blackburn 2002; Bertuch

and Lundblad 2003), possibly by recognizing DNA structures at telomeres that resemble damaged chromosomes.

A family of proteins related to the ATM kinase plays conserved roles in DNA repair and telomere function. Mutations in human ATM cause the inherited cancer predisposition syndrome ataxia telangiectasia (Shiloh 2003). Loss of ATM in humans or mice causes high levels of genomic instability and hypersensitivity to ionizing radiation. ATM phosphorylates multiple substrates, including the Chk2 checkpoint kinase and p53 transcription factor, which promote apoptosis, cell cycle arrest, and DNA repair following DNA damage. Two ATM-related kinases, ATR and DNA-PKcs, are also activated by DNA damage and cooperate with ATM to regulate the cellular response to DNA damage (Shiloh 2003). ATM also regulates telomere length and protection (Pandita et al. 1995; Metcalfe et al. 1996; Pandita 2002). ATM function is, at least partly, telomerase-independent as mice mutant for both telomerase and ATM have shorter telomeres and more anaphase bridges than single mutant mice and exhibit striking defects in stem cell populations and aging (Wong et al. 2003). Because of the intimate linkage between telomere length and telomere protection, it is difficult to determine if the targets of ATM are enzymes that extend or degrade telomere DNA or proteins that directly mediate telomere protection. Identification of these targets may help define telomerase-independent pathways that regulate telomere function.

Budding and fission yeast have homologs of ATM and ATR. While loss of one ATM-like kinase can affect telomere length and repression of gene expression near telomeres (telomere position effect), loss of both ATM and ATR in yeast causes rapid

shortening and fusion of yeast telomeres (Naito et al. 1998; Ritchie et al. 1999; Chan et al. 2001; Craven et al. 2002; Chan and Blackburn 2003). Assays that directly measure telomere protection demonstrate that these proteins prevent telomeres from being fused to double-strand DNA breaks or to other telomeres (DuBois et al. 2002; Chan and Blackburn 2003; Mieczkowski et al. 2003). As in mice, the yeast ATM-like kinases act, at least partly, in a telomerase-independent pathway (Ritchie et al. 1999; Nakamura et al. 2002; Chan and Blackburn 2003). Thus, in both yeast and humans, ATM can regulate telomere function independently of telomerase.

Analysis of telomere function in *Drosophila* has revealed that an epigenetic mechanism contributes to telomerase-independent protection of telomeres. *Drosophila* telomeres are composed of two non-LTR retrotransposons *Het-A* and *TART* as well as more proximal telomere-associated sequences (Biessmann et al. 1992; Karpen and Spradling 1992; Levis et al. 1993; Mason and Biessmann 1995; Cryderman et al. 1999; Fanti and Pimpinelli 1999). Terminally-deleted chromosomes, lacking all telomere-specific sequences, can be isolated following DNA damage (Mason et al. 1984; Tower et al. 1993; Mason et al. 1997; Ahmad and Golic 1998). Once obtained, the ends of these chromosomes are protected from end-to-end fusion; this property is stably inherited despite the gradual loss of terminal sequences due to the incomplete replication of chromosome ends by DNA Polymerase (Biessmann and Mason 1988). The heritable and sequence-independent protection of the ends of terminally-deleted chromosomes indicates that this aspect of *Drosophila* telomere function can be regulated by an epigenetic mechanism.

The *Drosophila* heterochromatin protein 1 (HP1) and heterochromatin protein 1/ORC2 associated protein (HOAP) are localized to telomeres and required for telomere function. HP1 proteins play an evolutionarily conserved role in heterochromatin function (Eissenberg and Elgin 2000; Grewal and Elgin 2002; Badugu et al. 2003; Kellum 2003). In *Drosophila*, HP1 is prominent at centromeric and telomeric heterochromatin, but is also observed at euchromatic sites (James et al. 1989; Fanti et al. 1998; Fanti et al. 2003). HOAP forms a complex with HP1 and is strongly localized to telomeres (Shareef et al. 2001; Badugu et al. 2003). Loss of either HP1 or HOAP leads to striking telomere fusion phenotypes, suggesting that chromatin structure plays a central role in telomere protection (Fanti et al. 1998; Cenci et al. 2003b). Chromatin-modifying proteins also regulate *Drosophila* telomere position effect (TPE) in *Drosophila*. TPE at the second and third chromosomes is sensitive to the dose of *Polycomb* group genes while TPE at the fourth chromosome or at a terminally deleted minichromosome is sensitive to the dose of HP1 (Cryderman et al. 1999; Donaldson et al. 2002; Boivin et al. 2003). These results demonstrate that regulation of chromatin structure by HP1 is required for both telomere protection and TPE.

Interestingly, terminally deleted chromosomes have normal levels of HP1-HOAP at their telomeres (Fanti et al. 1998; Cenci et al. 2003b). The sequence-independent localization of these proteins to telomeres suggests that a structural feature of telomeres, perhaps chromosome ends, helps establish or reinforce the localization of these telomere protection proteins. Gatti and colleagues have suggested that DNA damage detection

proteins may help recruit HP1-HOAP complexes to telomeres by recognizing chromosome ends (Cenci et al. 2003b).

Drosophila encodes two damage response proteins in the ATM kinase family, MEI-41, which is most similar to ATR, and CG6535/ATM, which is most similar to human ATM (Hari et al. 1995; Sekelsky et al. 2000; Laurencon et al. 2003; Brodsky et al. 2004). ATR/MEI-41 is required for ionizing radiation (IR)-induced cell cycle arrest, but not p53-dependent apoptosis (Hari et al. 1995; Ahmad and Golic 1999; Brodsky et al. 2000a; Brodsky et al. 2000b; Ollmann et al. 2000; Brodsky et al. 2004). Telomere fusions have not been described in mitotic cells lacking ATR/MEI-41 or its binding partner, ATRIP/MUS304 (Gatti 1979; Hari et al. 1995; Brodsky et al. 2000b).

In this study, we characterize the role of *Drosophila* ATM in telomere function. *Drosophila* ATM is required for viability and for eye, wing, and bristle development. We find high frequencies of telomere fusions and anaphase bridges in the absence of ATM. We demonstrate that chromosomes mutant for the *telomere fusion* (*tef)* gene (Queiroz-Machado et al. 2001) carry truncation mutations in ATM. In *atm/tef* animals, telomere fusions are accompanied by greatly elevated levels of spontaneous apoptosis during tissue growth. This apoptosis is suppressed by mutations in p53, suggesting that loss of telomere protection induces an ATM-independent, but p53-dependent, apoptotic signal. We demonstrate that ATM is specifically required for normal levels of HP1 and HOAP at telomeres, but not at centric heterochromatin. *atm* mutations suppress TPE, demonstrating that *atm* is required for normal telomere chromatin structure. *In situ* hybridization with telomere-specific sequences demonstrates that the telomere defects in

atm mutant cells are not due to loss of telomere sequences. These results support a model for telomere protection in which the recognition of DNA structures at chromosome termini by the ATM kinase provides a sequence-independent mechanism to help recruit telomere protection proteins.

Results

A *Drosophila* ATM homolog. The genomic structure of *atm/CG6535* is shown in Figure 2.1A. Based on cDNA sequencing and transgene rescue, we have identified a functional *atm* cDNA. Comparison of the predicted peptide sequence to mammalian checkpoint kinases indicates that it is the *Drosophila* homolog of *atm*. To characterize *atm* function, a deletion mutant, Δ356, was used that disrupts four kilobases of *atm*, including the start codon, and all of the adjacent gene *hsc70-4* (Bronk et al. 2001) (Fig. 2.A). Animals homozygous for this deletion and carrying a transgene containing the *hsc70-4* gene (Dasika et al. 1999) can be used to study the function of *atm* alone (Fig. 1A). We will refer to this combination as $atm^{\Delta 356}$. A second deletion, Δ16, includes *hsc70-4* but not *atm* and serves as an isogenic control (Bronk et al. 2001). As described below, we have also identified two mutations; atm^{tef} and atm^{Red31} that are predicted to truncated the ATM protein (Fig. 2.1A-C).

In contrast to Chk2 and p53, *Drosophila* ATM is required for normal development and viability. $atm^{\Delta 356}$ animals die shortly before or after eclosion with a rough eye phenotype, misshapen wings, and missing or abnormal bristles. $atm^{\Delta 356}$

produces similar defects in combination with a Deficiency, *Df(3R)PG4* that includes *atm* (Dasika et al. 1999). $atm^{\Delta 356}$/ atm^{tef} animals exhibit similar morphological phenotypes (Fig. 2.1D-I), but survive longer as adults. Defects were observed in 75% of bristles and 90% of wings (Tables 2.1 and 2.2). atm^{red31} homozygotes are also viable with rough eyes, but weaker wing and bristle phenotypes (not shown). Similar eye and bristle phenotypes have been previously observed in *Drosophila* following DNA damage or in mutant strains with high levels of genomic instability (Engels et al. 1987; Ahmad and Golic 1999; Brodsky et al. 2000b). Extensive aneuploidy is predicted to disrupt bristle morphology because bristles are sensitive to haploinsufficiency of ribosomal genes, which are present on all major chromosome arms (Ashburner, 1989). In our experiments, the specific bristles affected varied between individuals, consistent with aneuploidy in a variable subset of cells, rather than a specific defect in the pattern of bristle cell specification.

Table 2.1. *atm* mutant bristle phenotype

	# of bristles scored	# of normal bristles	# of reduced bristles	#of missing bristles
wild type	60	60 (100%)		
atm^{-}	60	16 (27%)	25 (42%)	19 (32%)

Five wild type (w^{1118}) and *atm* mutant ($atm^{\Delta 356}/atm^{\Delta 356}$) adult females were examined for bristle defects (12 bristles each). Bristles scored included orbitals (A,P), ocellar, verticals (A,P), and postverticals. Bristles were scored as defective if they were at least 50% shorter than the corresponding wild type bristle.

Table 2.2. *atm* mutant wing phenotype

	# of wings with no notches	# of wings with 1 notch	# of wings with 2 notches	# of wings with >2 notches
wild type	30/30(100%)			
atm-	3/31(10%)	11/31(35%)	14/31(42%)	4/31(13%)

Wild type (w^{1118}) and *atm* mutant ($atm^{\Delta 356}/atm^{tefu}$) adult wings were scored for notches in the wing margin.

To confirm that these phenotypes were due to loss of ATM, the Gal4-UAS system (Brand and Perrimon 1993) was used to demonstrate that an *atm* cDNA could rescue the observed *atm* phenotypes (Table 2.4). In addition, these experiments indicate that over-expression of *atm* does not disrupt normal development. Together, these results demonstrate that *Drosophila atm* has an essential role during normal development and identify a cDNA sequence sufficient to rescue ATM function.

Figure 2.1

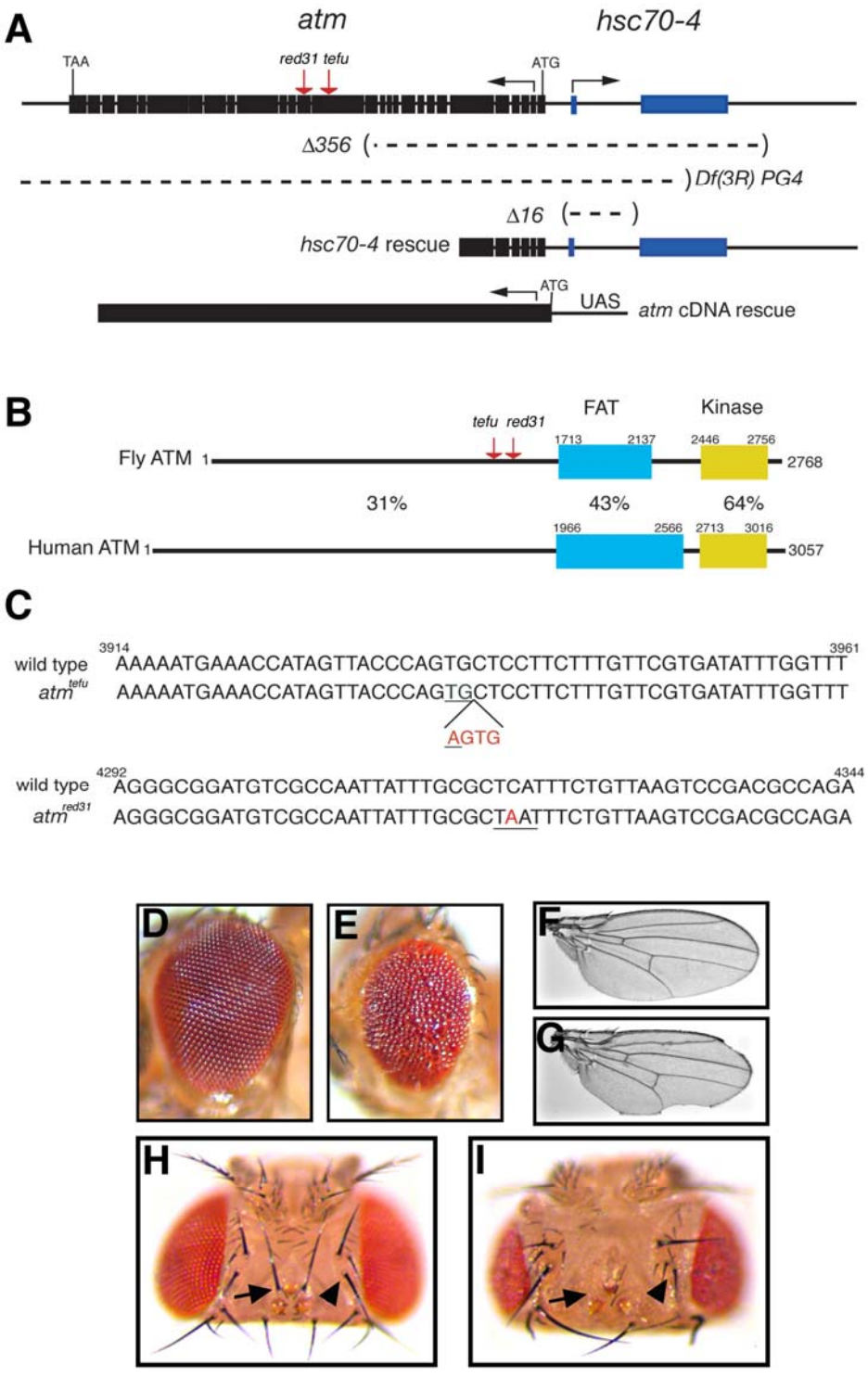

A
atm
hsc70-4
TAA
red31 tefu
ATG
Δ356
Df(3R) PG4
Δ16
hsc70-4 rescue
ATG
UAS
atm cDNA rescue
B
tefu red31
FAT
Kinase
Fly ATM 1
1713
2137
2446
2756
2768
31%
43%
64%
Human ATM 1
1966
2566
2713
3016
3057
C
wild type 3914 AAAAATGAAACCATAGTTACCCAGTGCTCCTTCTTTGTTCGTGATATTTGGTTT 3961
atm tefu AAAAATGAAACCATAGTTACCCAGTGCTCCTTCTTTGTTCGTGATATTTGGTTT
AGTG
wild type 4292 AGGGCGGATGTCGCCAATTATTTGCGCTCATTTCTGTTAAGTCCGACGCCAGA 4344
atm red31 AGGGCGGATGTCGCCAATTATTTGCGCTAATTTCTGTTAAGTCCGACGCCAGA
D
E
F
G
H
I

Figure 2.1. Molecular characterization of *Drosophila* ATM. (A) Diagram of *Drosophila ATM* gene structure and mutations. Black boxes indicate the exons of the *ATM* gene; blue boxes indicate the exons of the adjacent gene *hsc4*. Black arrows indicate the direction of transcription. Red arrows indicate the position of the ATM^{red31} and ATM^{tefu} point mutations. The ATM^{red31} chromosome also contains a large deletion. DNA deletions are represented by a dashed line. The *Δ356* deletion removes the entire *hsc4* gene and approximately 4kb of the 5' region of the *ATM* gene. *Df(3R)PG4* removes part of the *hsc4* gene, all of *ATM*, and an undetermined number of additional genes. *Δ16* removes only a portion of the *hsc4* gene. The *p[hsc4]* transgene rescues *hsc4* function and when used in combination with the Δ356 deletion results in a mutation in the *ATM* gene. (B) Schematic representation of ATM protein domains. The percent sequence similarity for the N-terminal region, FAT domain, and Kinase domain of *Drosophila* and human ATM proteins are shown. Numbers above the alignments indicate amino acid position. Red arrows indicate the position of the ATM^{red31} and ATM^{tefu} mutations in the ATM protein. (C) The *tefu* and *red31* point mutations. The *tefu* mutation is a four base pair insertion at cDNA nucleotide 3940, changing cysteine 1307 to a stop codon. The *red31* mutation is a single base pair change at nucleotide 4320, changing serine 1434 to a stop codon. Both the *tefu* and *red31* mutations are predicted to form truncated proteins lacking the conserved FAT and Kinase domains. (D) Wild type pharate adults have a characteristic pattern of bristles on the head with reproducible lengths and positions. (E) *ATM* pharate adults have many defective or missing bristles. Which bristles are affected varies among individuals.

ATM mutant tissues have high levels of spontaneous apoptosis. Because human ATM is required for damage-induced apoptosis, apoptosis was examined in animals mutant for *Drosophila atm*. Late third instar larvae were irradiated with 0 or 4000 rads of ionizing radiation (IR) and wing imaginal discs were stained for apoptotic cells using either the vital dye acridine orange (Fig. 2.2A-E) or an antibody against activated Caspase 3 (Fig. 2.2F-O). In these experiments, apical optical sections (Fig. 2.2F-J) reveal apoptotic cells dispersed among living cells with normal nuclear morphology while basal sections (Fig. 2K-O) highlight a mass of apoptotic cells with activated caspase staining and pyknotic nuclei, suggesting that these basal cells had progressed to late apoptotic stages. Untreated wild type discs exhibit very low levels of apoptosis (Fig. 2.2A, F, K). IR induces a substantial increase in the number of apoptotic cells throughout the wing disc (Fig. 2.2B, G, L). In contrast to wild type, untreated wing discs from *atm* animals exhibit extremely high levels of spontaneous apoptosis, particularly in the basal region of the disc (Fig. 2.2C, H, M). Because of the spontaneous apoptosis in *atm* discs, it is difficult to determine whether IR can induce increased apoptosis in the disc as a whole (Fig. 2.2D) or in basal sections of the disc (Fig. 2.2N). However, most cells in the apical region of *atm* wing discs are not apoptotic (Fig. 2.2I). In contrast to wild type discs (Fig. 2.2F, G), *atm* discs do not show a substantial increase in apical apoptotic cells following IR (Fig. 2.2H, I). Together, these results indicate that *atm* is required both to suppress spontaneous apoptosis during development and for normal induction of apoptosis in response to IR.

We considered the possibility that the spontaneous apoptosis in *atm* discs is due to activation of a DNA damage response pathway. Since *Drosophila p53* is required for DNA damage-induced apoptosis, the effect of a null mutation in *p53* (Rong et al. 2002; Brodsky et al. 2004) on the spontaneous apoptosis and lethality due to loss of *atm* was examined. Compared to *atm* single mutant animals, $atm^{\Delta 356}$, $p53^{1}$ double mutant animals exhibit substantially reduced levels of acridine orange staining (Fig. 2.2E), activated caspase staining (Fig. 2.2J, O), and pyknotic nuclei (not shown). Although most apoptosis is suppressed by removal of p53, the levels of spontaneous apoptosis in *atm*, *p53* mutant discs are still elevated compared to wild type (compare Fig 2.2A, F, K with 2.2E, J, O), revealing some p53-independent apoptosis. Loss of *p53* did not rescue *atm* lethality, as double mutant animals still died as pharate adults with missing or defective bristles (not shown). Thus, most of the spontaneous apoptosis in *atm* animals is p53-dependent, but suppression of that apoptosis is not sufficient to restore normal development or viability.

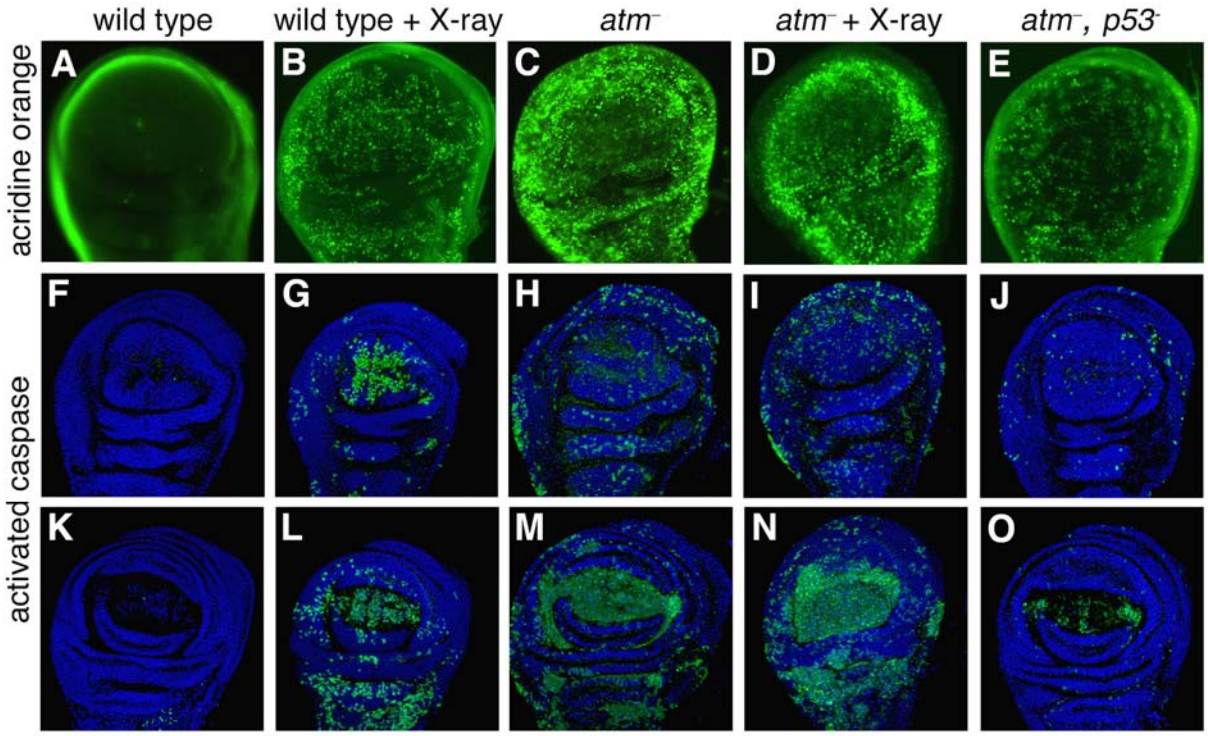


Figure 2.2. p53 –dependent apoptosis in *ATM* mutant wing discs. Third instar larval wing discs were stained with acridine orange (A-D) to detect apoptotic cells or DAPI (E-H) to visualize pyknotic nuclei associated with apoptosis. Discs are shown with anterior to the left. Optical cross sections of the DAPI stained discs (E-H) are shown below each disc with apical top and basal below. Apoptotic cells accumulate in the basal regions of these discs. Little apoptosis in untreated wild type discs (A, E). Increased apoptosis in wild type discs following irradiation (B, F). High levels of apoptosis in untreated *ATM* mutant discs (C, G). Suppression of apoptosis in untreated *ATM, p53* double mutant discs (D, H).

atm is not required for damage-induced cell cycle arrest. Because human cells lacking ATM are defective in cell cycle arrest following ionizing irradiation (IR), the role of *Drosophila* ATM in damage-induced cell cycle arrest was tested. The wing imaginal disc exhibits a G2/M DNA damage checkpoint following X-irradiation, which is dependent on *Drosophila* homologs of ATR and ATRIP (Hari et al. 1995; Brodsky et al. 2000b). Both wild type and *atm* wing discs exhibit a reduction in the numbers of mitotic cells at time points from one to eight hours following X-irradiation (Fig. 2.3A-G, data not shown). Thus, *Drosophila* ATM is not essential for G2 arrest following DNA damage.

Interestingly, higher magnification of the mitotic cells in unirradiated *atm* discs revealed anaphase bridges not observed in wild type discs (Fig. 2.3D, H). Anaphase bridges are typically the result of dicentric chromosomes entering mitosis. These results suggest that *atm* is required to prevent the formation of dicentric chromosome aberrations such as fusions or translocations.

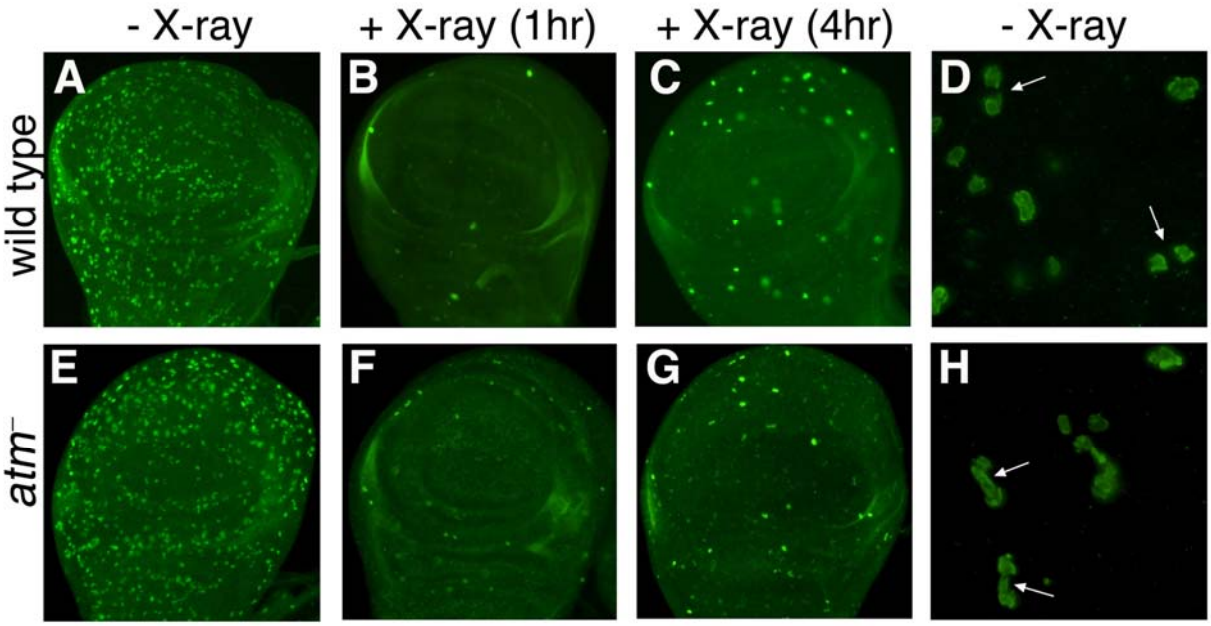


Figure 2.3. *Drosophila* ATM is not required for damage-induced cell cycle arrest. Third instar larval wing discs were stained with a mitosis-specific antibody against phospho-histone H3. The pattern of mitotic cells in wild type (A-D) and *ATM* discs (E-H) is shown following no irradiation (A, D, E, H), one hour (B, F), or 4 hours (C, G) after irradiation with 4,000 Rads X-rays. Mitosis is blocked in both wild and *ATM* mutant wing discs. At higher magnification, anaphase bridges (arrows) are seen in *ATM* mutant (H) but not in wild type discs (D).

atm is required to prevent telomere fusions and is mutated in the telomere fusion animals. To examine chromosome structure in *atm* animals, metaphase and anaphase chromosomes were prepared from wild type and *atm* larval neuroblasts. *atm* neuroblasts exhibit a high frequency of chromosome end fusions and anaphase bridges (Fig. 2.4A-L, Table 2.3, Table 2.4). The aberrations observed indicate that *Drosophila* ATM is required to protect telomeres from fusion and that these unprotected telomeres can undergo at least one round of a fusion-bridge-break cycle.

Table 2.3. Abnormal metaphase configurations in *atm*$^{-}$ larval brains

		Telomere Fusions					
				Double			
Genotype	Cells Scored	Normal	Single	Linear	Rings	Aneuploid/ Polyploid	%Metaphase with fusions
wild type	407	402	5	0	0	0	1
atm^{-}	445	87	158	277	48	67	80

Telomere fusions were scored in wild type (w^{1118}) and *atm* mutant ($atm^{\Delta 356}/atm^{\Delta 356}$) metaphases. Fusion categories are similar to those previously described (Fanti et al. 1998), for detail see Material and methods.

The telomere fusion phenotype observed in *atm* neuroblasts resembles the mutant phenotype of the previously described *telomere fusion* (*tef*) gene (Queiroz-Machado et al. 2001). *tef* maps near *atm* by meiotic recombination and fails to complement a chromosome with a deficiency (*Df(3R)red31*) in the cytological region 88B5-C. Although *atm* is in cytological region 88E3, *atm* mutations fail to complement both *tef* and *Df(3R)red31*. $atm^{\Delta 356}/tef$ and $atm^{\Delta 356}/Df(3R)red31$ trans-heterozygous animals exhibit

high levels of spontaneous apoptosis similar to the $atm^{\Delta 356}$ homozygous animals (data not shown). *tef* and *Df(3R)red31* also fail to complement *Df(3R)PG4*, which encompasses the *atm* locus. The phenotypes of $atm^{\Delta 356}$/*tef* animals can be rescued by expression of the *atm* cDNA and sequencing of *atm* revealed that both the *tef* and *Df(3R)red31* chromosomes contain stop mutations in *atm* (Fig. 2.1). To further demonstrate that the phenotype associated with the *Df(3R)red31* is not associated with the described deletion, the *atm* mutation was separated from the deletion by meiotic recombination. These results demonstrate that *atm* and *telomere fusion* are the same gene. There are some differences between the phenotypes previously described for homozygous *tef* animals and those described in this study; some of these may be due to a closely linked second mutation on the *tef* chromosome.

The effects of different alleles of *atm* on anaphase bridge frequencies were compared (Table 2.4). Animals homozygous for $atm^{\Delta 356}$ or heterozygous for $atm^{\Delta 356}$ and a deficiency that encompasses *atm* exhibited similar frequencies of anaphase bridges, suggesting that this allele is a null. Animals heterozygous for an *atm* deficiency and either *tef* or *Df(3R)red31* have similar frequencies of bridges. In addition, these allelic combinations also exhibit the high levels of spontaneous apoptosis seen in $atm^{\Delta 356}$ animals and are not required for G2 arrest following IR (data not shown).

Table 2.4. Anaphase bridges in *Drosophila atm* mutant neuroblasts

Genotype	Lethal Stage	Number of *Anaphases*	Number of *Anaphase Bridges*
w^{1118}	-	182	2 (1.0)
$atm^{\Delta 356}/hsc70\text{-}4^{\Delta 16}$	-	256	2 (0.8)
$atm^{\Delta 356}/atm^{\Delta 356}$	pupal	149	108 (72.5)
$atm^{\Delta 356}/Df(3R)PG4$	pupal	104	78 (75.0)
$atm^{tefu}/Df(3R)PG4$	pupal	108	86 (79.6)
atm^{red31},*Df(3R)red31/Df(3L)PG4*	pupal	142	104 (73.2)
GUS-*atm*, $atm^{\Delta 356}$/ ActinGal4,*P[hsc70-4^{+}];Df(3R)PG4*	-	150	3 (2.0)

The frequency of anaphase bridges was determined in *atm* mutant larval brains. Five or more brains were scored for each genotype. The percent of anaphase bridges for each genotype is shown in parenthesis.

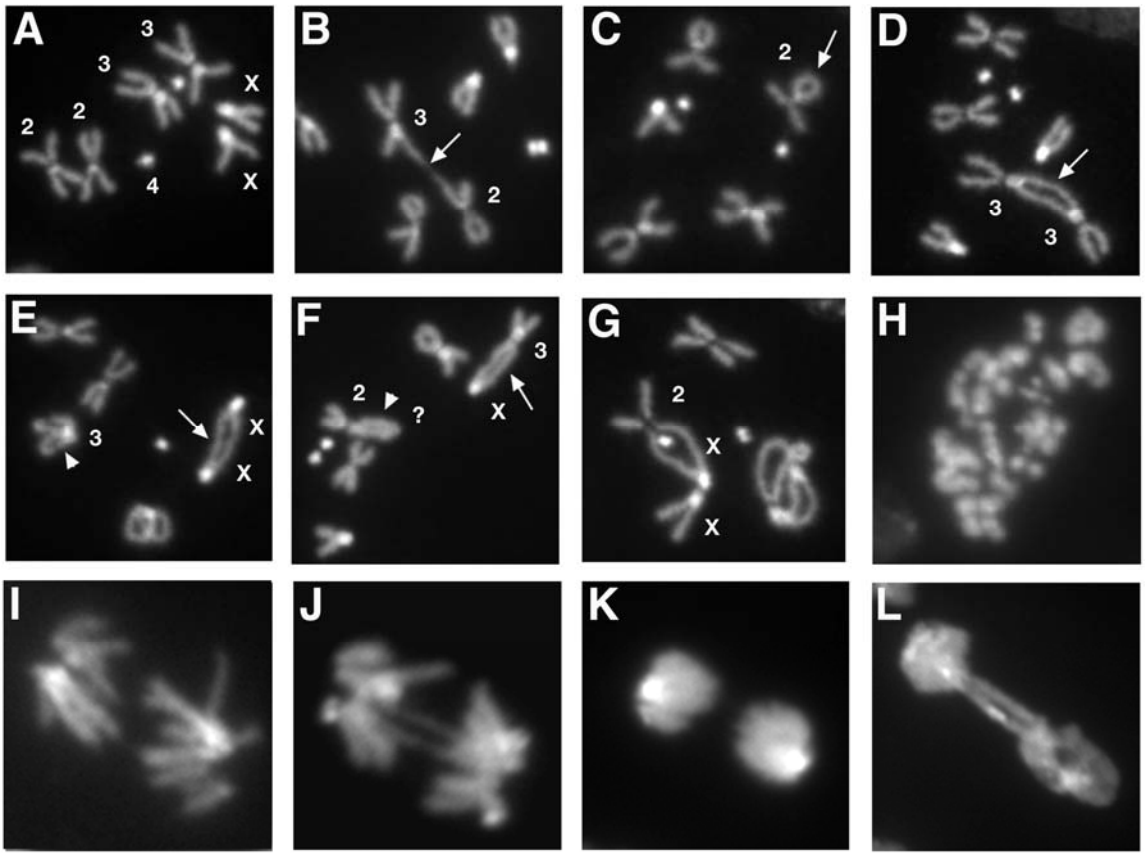


Figure 2.4. Telomere fusions in *ATM* mutant animals. Neuroblast squashes were prepared from wild type (A, I, K) and *ATM* mutant (B-H, J, L) third instar larval brains. In wild type metaphases there are no associations between telomeres (A). In *ATM* mutant metaphases, a variety of telomere associations (arrows) are observed including: single fusions between homologs (B), single fusions between sisters, double fusions between homologous (E, F) and heterologous (G) chromosomes. Ring chromosomes (E, arrowhead), possible chromosome translocations (F, arrowhead), and more complex rearrangements (G) or polyploidy (H) are also observed. Chromosome bridges are seen

in *ATM* mutant cells in anaphase (J) and telophase (L), but not in wild type cells (I, K). The frequencies of these abnormalities are shown in Tables 2.3 and 2.4.

atm mutations reduce HP1 and HOAP levels at telomeres and suppress telomere position effect. The phenotypes described above indicate that *Drosophila atm* is required to protect telomeres from fusion. HP1 and HOAP localize to the telomeres of polytene chromosomes, as well as other sites, and are required for telomere protection in mitotic cells (James et al. 1989; Fanti et al. 1998; Shareef et al. 2001; Cenci et al. 2003b). Immuno-staining was used to examine the distribution of HP1 and HOAP on wild type and *atm* polytene chromosomes (Fig. 2.5). Wild type and *atm* polytene chromosomes were prepared in parallel, all samples were treated with the same antibody solutions, and all images were captured using the same exposure times. For each genotype, chromosomes were prepared from ten different animals and immuno-stained with antibodies to both proteins. DNA was detected by DAPI staining. HP1 staining at the chromocenter, fourth chromosome, and several euchromatic sites is unaffected by loss of *atm* while HP1 staining is reduced at most *atm* telomeres (Fig. 2.5C, D, G, H, and Table 2.5). At the tip of chromosome 2R, similar levels of HP1 staining at an internal site (cytological position 60F, arrows in Figure 4 insets) can be observed in wild type and mutant chromosomes while HP1 is specifically reduced at the telomere of the mutant chromosome (asterisks in Figure 2.5 insets). Similar HP1 staining at wild type and mutant chromosomes was observed using a second, rabbit polyclonal HP1 antibody (data not shown). The normal levels of HP1 at sites other than the telomere indicates that the lack of telomere staining at *atm* chromosomes is not due to differences in chromosome

preparations or to global changes in chromatin structure in *atm* cells. Rather, *atm* is specifically required to recruit or maintain HP1 to chromosome ends.

Immuno-staining of the same chromosomes for HOAP revealed reduced staining at the telomeres of most *atm* chromosomes compared to wild type (Fig. 2.5A, B, E, F, Table 2.5). Similar decreases in HP1 and HOAP localization at telomeres were seen in $atm^{\Delta 356}$/ *Df(3R)PG4* (Fig. 2.5) and atm^{tef}/ *Df(3R)PG4* (not shown) animals, indicating that this phenotype is not allele-specific (data not shown). Quantification of the fluorescence intensity associated with HOAP and HP1 staining further demonstrates that there is a reproducible reduction at *atm* telomeres compared to wild type (Fig 2.5I, J). In contrast, HP1 staining at an internal chromosomal site (60F) is not reduced.

Table 2.5. Reduced levels of HP1 and HOAP at *atm* mutant salivary gland telomeres

	Total*	++	+	+/-	-
HP1					
wild type	156	128 (82)	28 (18)	0	0
atm^{-}	198	6 (3)	7 (4)	42 (21)	143 (72)
HOAP					
wild type	156	153 (98)	3 (2)	0	0
atm^{-}	198	6 (3)	111 (56)	74 (37)	7(4)

Wild type and $atm^{\Delta 356}$/*Df(3R)PG4* polytene chromosomes were prepared from ten different animals and immuno-stained with antibodies to both HOAP and HP1. From each squash, images of ten chromosomes spreads with most telomeres visible were taken using identical microscope and camera settings in order to compare staining intensities. Staining was scored as follows: ++, normal; +, reduced; +/-, strongly reduced; -, not detected. Percentages are shown in parentheses. *Total number of telomeres scored.

Because HP1 and HOAP are required to prevent telomere fusions in mitotic cells, but not salivary glands, localization of these proteins in wild type neuroblasts was examined. However, the wide spread distribution of HP1 on metaphase chromosomes prevents the unambiguous identification of telomeric HP1. HOAP staining was observed at the telomeres of both wild type and mutant neuroblasts, but the staining is too variable to conclude whether it is reduced as seen in salivary glands (not shown).

HP1 promotes heterochromatin formation, in part, by recruiting histone modifying enzymes. To probe if *atm* mutations alter chromatin at the telomeres mitotic cells, telomere position effect (TPE) at three telomeres was examined (Fig. 2.6). When the *white* reporter gene is placed adjacent to telomere-associated sequences (TAS), gene expression is silenced (Cryderman et al. 1999; Wallrath 2000). At each site tested, TPE is partially suppressed by mutations in *atm* (Fig. 2.6A-C, E-G). Previous studies have demonstrated that the TAS from the telomere of chromosome arm 2L is sufficient to silence *white* expression in transgenes inserted at non-telomeric sites (Kurenova et al. 1998). Unlike TAS in their normal location adjacent to telomeres, silencing by the non-telomeric TAS was not suppressed by *atm* mutations (Fig. 2.6D, F). These results indicate that the suppression of TPE is due to the specific action of *atm* on gene expression near telomeres.

In other organisms, attrition of telomeric sequences due to incomplete chromosome replication can eventually lead to failure to protect chromosome ends from fusion (Blackburn 2001; Maser and DePinho 2002). Although it is possible to recover terminal deletions that remove all telomere-specific sequences, these observations do not

rule out the possibility that telomere-specific sequences contribute to telomere protection or TPE at normal *Drosophila* telomeres. In fact, the number of telomere repeats has been shown to influence some forms of TPE (Mason et al. 2003a). To test if the telomere defects in *atm* animals could be due to loss of telomere sequences, fluorescent *in situ* hybridization was performed using a probe to the *Het-A* retrotransposon, which is specific to telomere DNA. Hybridization was performed with wild type and *atm* diploid and polytene chromosomes. In mitotic chromosomes from diploid neuroblast cells, the levels of *Het-A* hybridization are variable, but not significantly different between wild type and *atm* mutant cells (Fig. 2.7A-C). In polytene chromosomes *HeT-A* sequences are strongly detected at two telomeres of both wild type and *atm* chromosomes (Fig. 2.7F, G). Previous analysis of HP1 mutants demonstrated that telomere-specific sequences were still present at chromosome fusion sites (Fanti et al. 1998). In *atm* mutant cells, *Het-A* hybridization is also detected at sites of chromosome fusion (Fig. 2.7D, E). These results indicate that the reduction of telomeric HP1-HOAP and the fusion of telomeres in *atm* cells is not a direct or indirect result of telomere sequence loss.

Figure 2.5

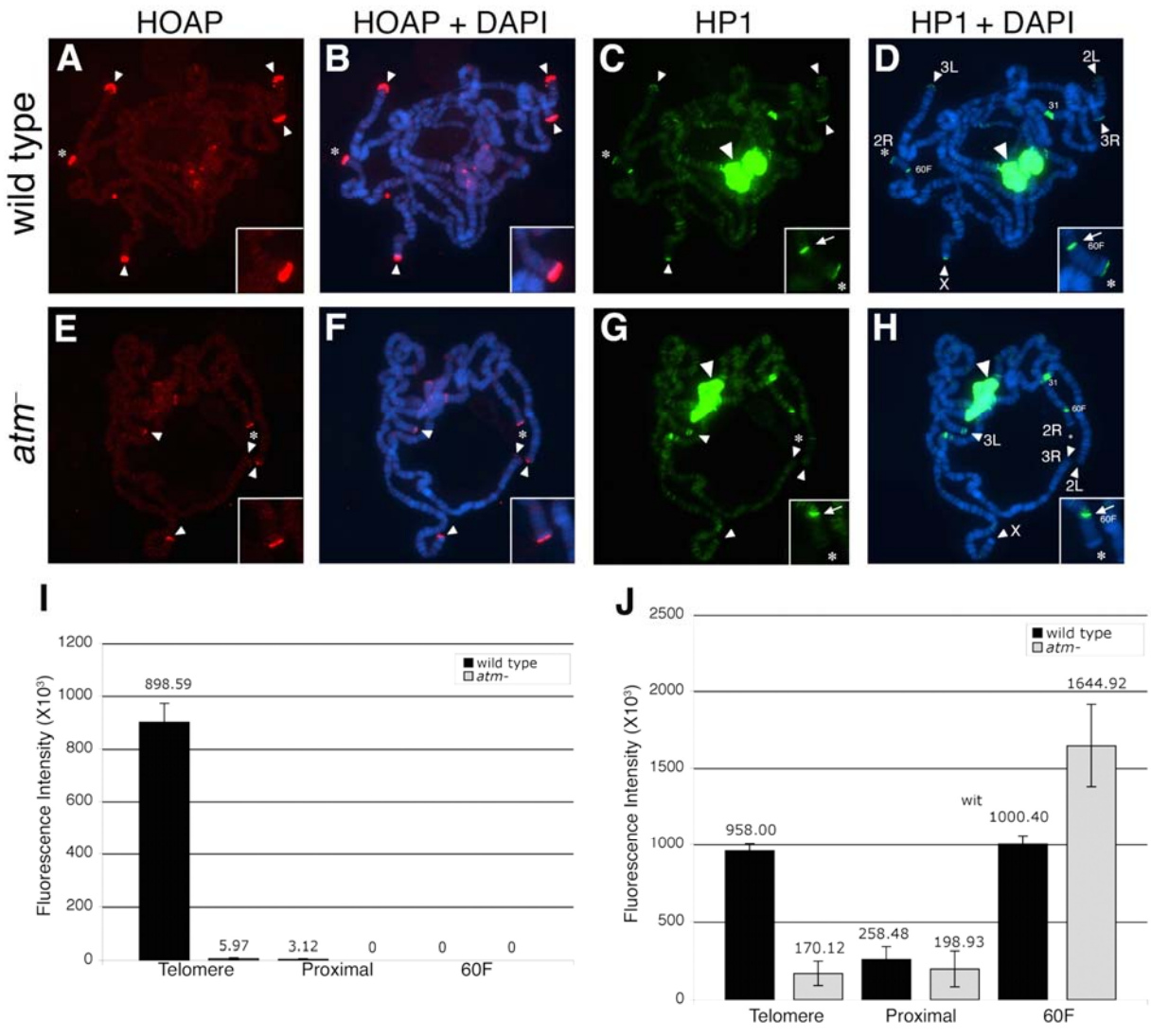
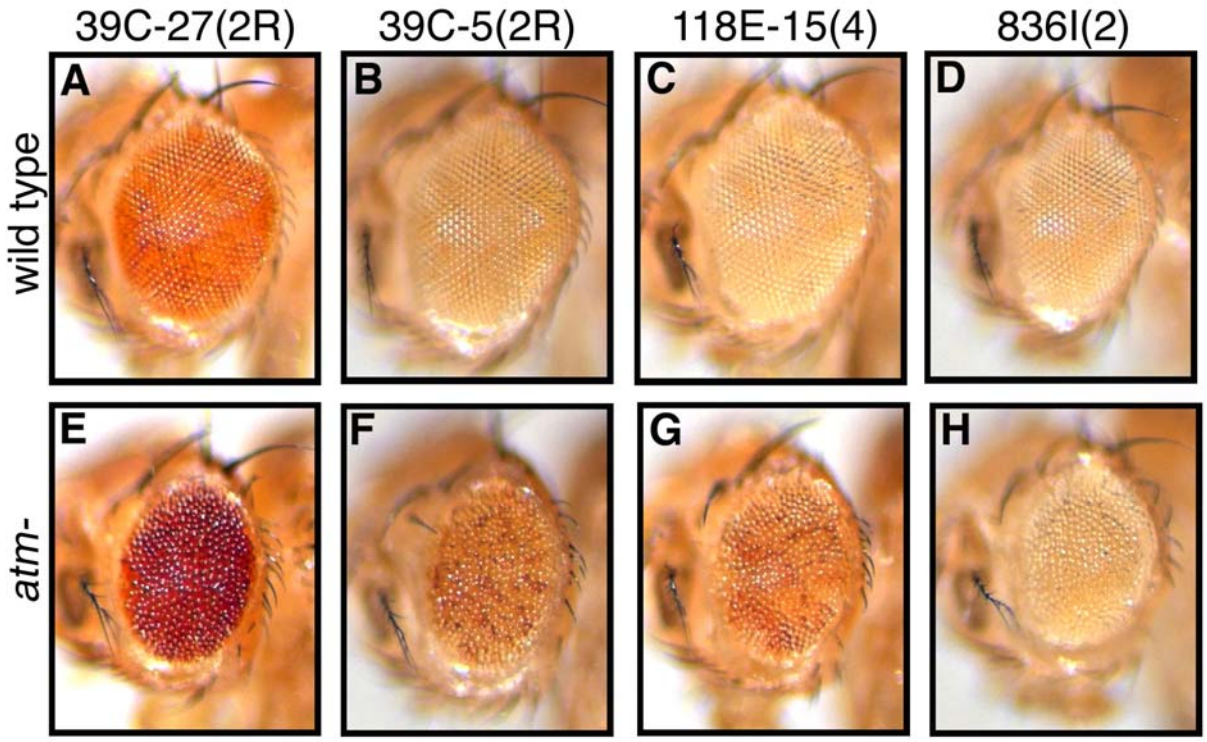

HOAP
HOAP + DAPI
HP1
HP1 + DAPI
wild type
atm⁻
A
B
C
D
E
F
G
H
3L
2L
2R
3R
X
I
J
Fluorescence Intensity (X10³)
wild type
atm-
898.59
5.97
3.12
0
0
0
958.00
170.12
258.48
198.93
wit
1000.40
1644.92
Telomere
Proximal
60F

Figure 2.5. Reduced HP1 and HOAP levels at *ATM* mutant telomeres. Wild type (A-D) and *ATM* mutant (E-H) polytene chromosomes were immunostained with antibodies against HOAP (A, B, E, F) and HP1 (C, D, G, H). Chromosomal DNA was stained with DAPI (B, D, F, H). All telomeres are marked with arrowheads except for 2L, which is marked with an asterisk. Insets show a higher magnification view of the telomere of chromosome 2L. Strong HOAP staining is observed at all wild type telomeres (A, B), but is significantly reduced at *ATM* telomeres. Wild type chromosomes have HP1 staining at telomeres, the chromocenter, the forth chromosome, and at euchromatic sites (C, D). *ATM* mutant chromosomes have reduced levels of HP1 at telomeres, but normal levels at other sites (G, H). Comparison of HP1 staining at the telomere of chromosome arm 2L and a nearby euchromatic binding site illustrates the specific loss of telomere staining on *ATM* chromosomes.

Figure 2.6


39C-27(2R)
39C-5(2R)
118E-15(4)
836I(2)
wild type
atm-
A
B
C
D
E
F
G
H

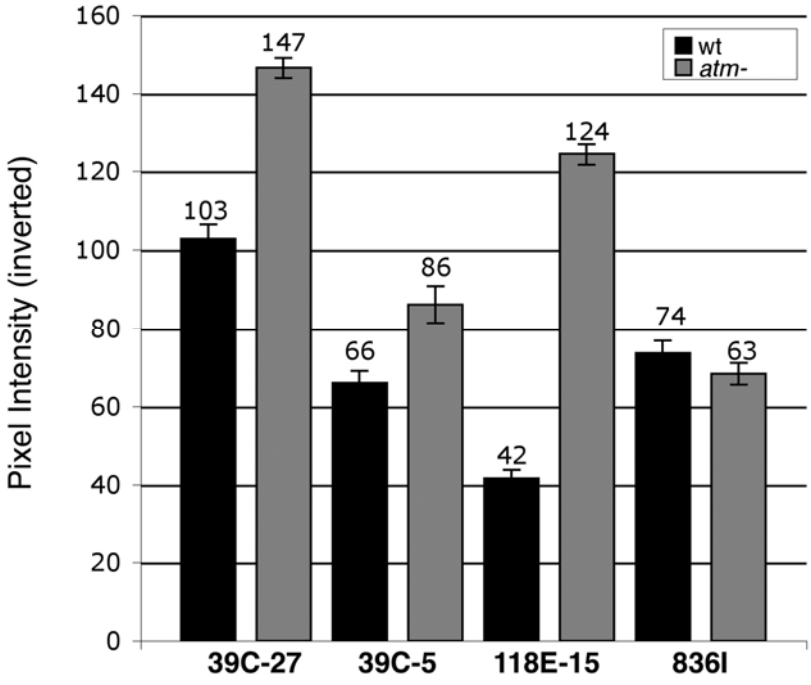

Pixel Intensity (inverted)
160
140
120
100
80
60
40
20
0
wt
atm-
103
147
66
86
42
124
74
63
39C-27
39C-5
118E-15
836I

Figure 2.6. *atm* mutations suppress telomere position effect. Amimals hemizygous for *white* transgenes inserted in the telomere associated sequence (TAS) of the right and left arms of chromosome 2 (lines 39C-27 and 39C-5) and in the telomeric region of chromosome 4 (line 118E-15) exhibit repression of the reporter gene, i.e. telomere position effect (A, B, C). A transgene containing a fusion of *white* to 6 kilobases of TAS sequence from chromosome 2L (line 836I) also represses *white* expression (D). Animals homozygous mutant for *atm* and hemizygous for a telomere insertion show derepression of the reporter gene (E, F, G). Repression of reporter gene expression by TAS at a non-telomeric position is not affected by *atm* mutations (H).

Figure 2.7

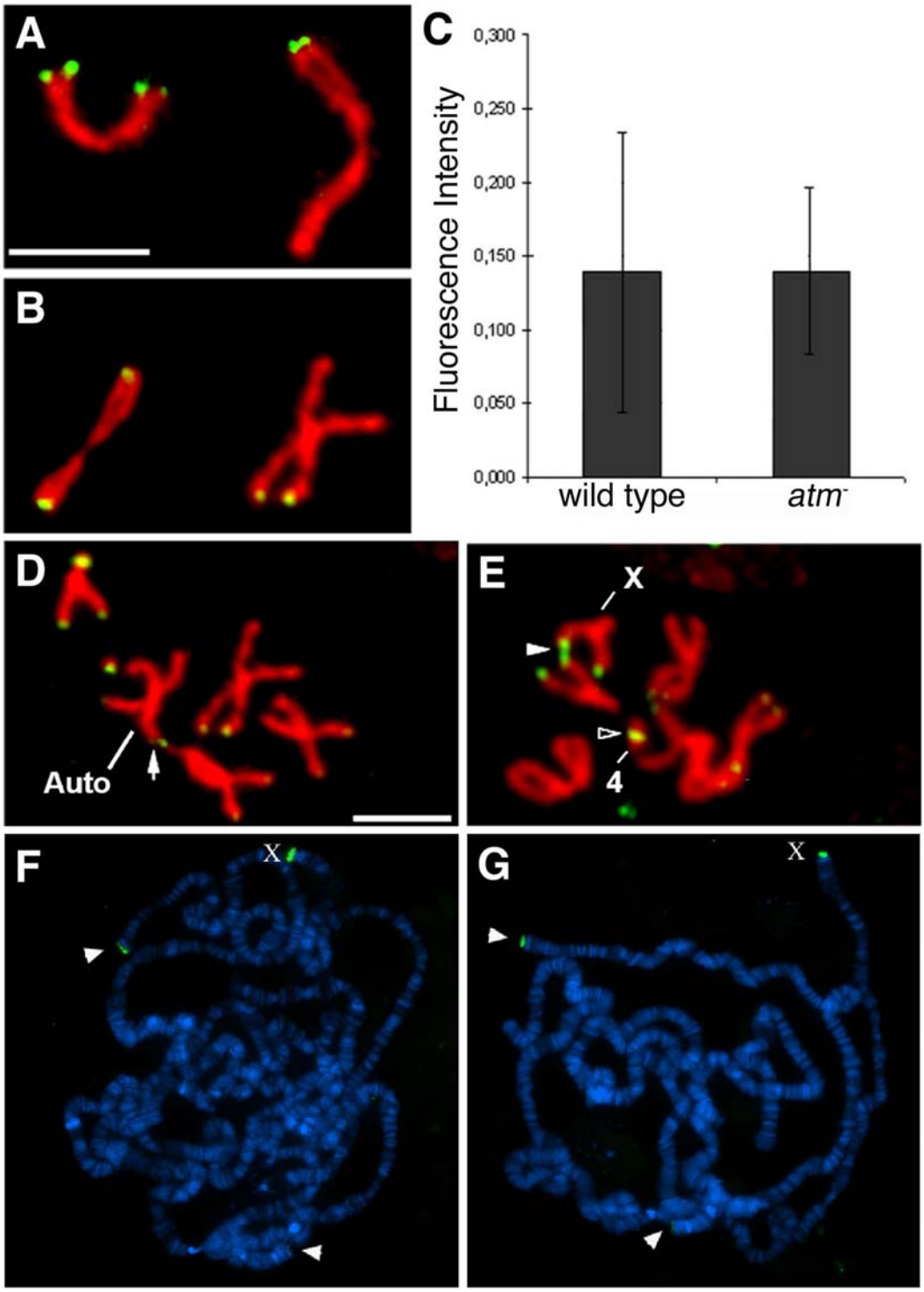

A
B
C
0,300
0,250
0,200
0,150
0,100
0,050
0,000
Fluorescence Intensity
wild type
atm-
D
Auto
E
X
4
F
X
G
X

Figure 2.7. Analysis of *Het-A* sequences in *ATM* cells. Wild type and *ATM* chromosomes were hybridized with a DNA probe to the telomere-specific retrotransposon *HeT-A* in mitotic and polytene cells. Mitotic chromosomes from control (A) and ATM^{tefu} (B) neuroblast cells were hybridized with a probe for *Het-A* (green) and the DNA was counterstained with propidium iodide (red). The fluorescent intensity of the *Het-A* signal at the telomeres of the two large autosomes was quantified in controls (n=31) and *tefu* (n=19) cells (C). The results do not show significant difference in intensity between controls and mutant cells suggesting that the mutant has not lost telomeric sequences. (D-G) *tefu* mutant cells showing telomere fusions that were hybridized with a probe for *Het-A*. (E) Cell showing telomere fusion of two autosomes (arrow) with *Het-A* sequences at the fusion site. (G) Cell showing fusion between two X chromosomes (arrowhead) in which the *Het-A* sequences extend along the entire fusion site. The cell also has the fourth chromosomes (back arrowhead) joined at the *Het-A* site. Polytene chromosomes from control (H) and ATM^{d356} (I) salivary gland cells were hybridized with a probe for *Het-A* (in green) and counterstained with DAPI (blue). *Het-A* hybridization to autosomal telomeres is marked with arrowheads. Staining at the X-chromosome telomeres is marked with an "X". Wild type and *ATM* chromosomes show similar levels of *Het-A* hybridization at three out of the five telomeres including the X chromosome (H, I).

Discussion

Both wild type and terminally deleted *Drosophila* chromosomes are protected from telomere fusion and are capped with the telomere protection proteins HP1 and HOAP (Biessmann and Mason 1988; Fanti et al. 1998; Cenci et al. 2003b). These results indicate that sequence-independent mechanisms can recruit and maintain telomere protection complexes to chromosome ends. Here, we demonstrate that *Drosophila atm/tef* is required to prevent chromosome end fusions, to regulate levels of HP1 and HOAP at telomeres, and to promote telomere position effect. We also find that *atm* is required for induction of apoptosis by ionizing radiation. Given the conserved role of ATM family proteins in recognizing DNA breaks, we suggest that *Drosophila* ATM protects telomeres by recognizing chromosome ends and recruiting chromatin-modifying proteins to those ends.

We have not, to date, directly detected ATM protein at *Drosophila* telomeres (unpublished results). Based on results in mammalian cells, it may be necessary to develop antibodies specific for activated forms of ATM to probe ATM activity at telomeres. However, several observations presented here indicate that *Drosophila* ATM acts at telomeres to prevent chromosome fusions. First, the chromosome rearrangements observed are consistent with a defect in telomere protection rather than translocations due to defective DNA repair or replication. Most chromosome fusions occur near the ends of chromosome arms and we demonstrate that the fused chromosomes still contain DNA sequences near chromosome ends. Second, we do not observe a high frequency of

acentric chromosome fragments during metaphase. In animals mutant for other damage signaling genes such as the *Drosophila* homologs of *ATR* and *ATRIP*, acentric chromosome fragments are often observed during metaphase, suggesting that these mutations cause a defect in DNA repair or replication that is not observed in *atm* animals (Gatti 1979; Brodsky et al. 2000b). Third, circular chromosomes do not undergo rearrangements in *tef* mutant animals (Queiroz-Machado et al. 2001), strongly indicating that chromosome fusions are due to fusion of existing chromosome ends rather than the creation of new chromosome breaks. Forth, we show that ATM is specifically required for localization of HP1 to telomeres but not centromeric or euchromatic sites. Finally, we demonstrate that *atm* suppresses silencing by telomere-associated sequences when they are adjacent to telomeres, but not when they are at euchromatic sites.

The telomere fusion defect seen in *atm* animals is consistent with a partial defect in telomere protection. While approximately 80 percent of *atm* metaphases contain a chromosome fusion, greater than 95 percent of metaphases from animals lacking HP1 or HOAP contain a fusion (Fanti et al. 1998; Cenci et al. 2003b). Furthermore, in some cells lacking HP1 or HOAP, nearly all telomeres appear to be fused; we have not observed this extreme phenotype in *atm* mutant nuclei. Consistent with a partial defect in telomere protection, we find that the levels of HP1 and HOAP at polytene telomeres are reduced, but not eliminated in *atm* animals. In mitotic cells, formation of repressive chromatin is also partially disrupted. Our interpretation of these results is that reduced and variable levels of HP1 at the telomeres of *atm* animals are sufficient to protect some, but not all telomeres from fusion. Our results also indicate that another pathway, possibly involving

other DNA damage response proteins, must contribute to HP1 and HOAP localization, TPE, and telomere protection.

The direct target of ATM at telomeres is unclear. The decrease in HP1 and HOAP levels at *atm* telomeres is not due to a loss of telomere sequences; wild type and *atm* chromosomes exhibit similar levels of a telomere-specific retrotransposon sequence as assayed by FISH and even sites of fusion retain this sequence. This result is consistent with previous demonstrations that the sequences at chromosome ends are not required for telomere protection or for telomeric localization of HP1 and HOAP (Biessmann et al. 1990a; Fanti et al. 1998; Cenci et al. 2003b). Instead, ATM is likely to affect the interaction of HP1 and HOAP with telomeres by regulating the formation of the HP1-HOAP complex or by modification of telomeric chromatin. Other proteins in the DNA damage response may act with ATM to maintain telomere protection. Although Chk1, Chk2, and p53 are targets of mammalian ATM during the DNA damage response (Shiloh 2003), *Drosophila* homologs of these proteins do not appear to be required for telomere protection since animals lacking one or more of these genes do not exhibit the high levels of apoptosis associated with loss of ATM (Brodsky et al. 2004). Mutations in homologs of other ATM targets such as NBS1 or SMC1 have not been described in *Drosophila*.

Recruitment of HP1 and HOAP by ATM is likely to alter chromatin structure at telomeres. HP1 plays a conserved role in heterochromatin structure, histone modification, and gene silencing (Eissenberg and Elgin 2000; Grewal and Elgin 2002). In *Drosophila,* both HP1 and HOAP are required for gene silencing at pericentric heterochromatin (Eissenberg et al. 1990; Eissenberg and Elgin 2000; Badugu et al. 2003). In addition, HP1

is required for gene silencing near fourth chromosome and terminally deleted telomeres, as well as repression of P-element transposition by subtelomeric P-element insertions (Ronsseray et al. 1996; Ronsseray et al. 1998; Cryderman et al. 1999; Donaldson et al. 2002). HP1 homologs are also associated with telomere function in other eukaryotes. In mammals, all three HP1 homologs are found at telomere and loss of histone H3 K9 methylases leads to reduced levels of HP1 homologs at telomeres as well as elongated telomeres (Garcia-Cao et al. 2004). In contrast, over-expression of mammalian HP1 homologs is associated decreased telomere length (Sharma et al. 2003). The fission yeast homolog of HP1 is not required for telomere protection, but does regulate telomere length, telomere clustering, and telomeric gene silencing (Allshire et al. 1995; Ekwall et al. 1995; Koering et al. 2002; Garcia-Cao et al. 2004). Interestingly, as in *Drosophila* telomere protection, some aspects of telomere function in fission yeast are controlled by an epigenetic mechanism (Sadaie et al. 2003). Together, these observations indicate that a requirement for HP1 in telomere function and chromatin structure is conserved, but that its precise role at the telomere may differ among organisms.

Regulation of telomere chromatin structure is also a conserved function of ATM-like kinases. Fission yeast Rad3 and budding yeast Mec1 are required for gene silencing at telomeres (Matsuura et al. 1999; Craven and Petes 2000) while mutations in human ATM are associated with altered nucleosomal periodicity at telomeres (Smilenov et al. 1999). The conserved role of ATM-kinases in telomere protection (see Introduction) and telomeric chromatin structure suggests that these functions might be linked. Our finding that *Drosophila* ATM is required for TPE and HP1-HOAP localization to telomeres

demonstrates one mechanism by which ATM can influence telomere chromatin. It is possible that in organisms that utilize sequence-specific binding proteins such as TRF2 to protect telomeres; regulation of telomeric heterochromatin by ATM and HP1 plays a minor role in protection of normal telomeres, but a more important role at short telomeres that cannot recruit sufficient levels of TRF2. Such a model could explain the synergistic telomere defects seen in cells lacking both telomerase and ATM (Ritchie et al. 1999; Tsukamoto et al. 2001; Chan and Blackburn 2003; Wong et al. 2003). The lack of obvious TRF2 homologs may explain why ATM and HP1 play such striking roles in the protection of *Drosophila* telomeres.

In addition to preventing chromosome end fusion by DNA repair enzymes, telomere protection is required to prevent activation of DNA damage responses including the induction of p53-dependent apoptosis and senescence (Chin et al. 1999; Karlseder et al. 1999; d'Adda di Fagagna et al. 2003; Takai et al. 2003). Our analysis of the cellular effects of ATM loss indicates that induction of p53-dependent apoptosis is a conserved consequence of unprotected telomeres in metazoans. Because these unprotected telomeres lead to anaphase bridges and chromosome breaks, p53 may be directly activated by unprotected telomeres or may be activated by subsequent chromosome breaks. ATM is required for the induction of apoptosis following IR. Since the spontaneous apoptosis in *atm* animals is, by definition, ATM-independent, a different pathway must be able to activate *Drosophila* p53 in response to unprotected telomeres. Similarly, loss of mammalian ATM reduces, but does not eliminate p53-dependent apoptosis in response to unprotected telomeres (van Steensel et al. 1998; Takai et al.

2003; Wong et al. 2003). Other DNA damage response pathways may activate *Drosophila* p53 in the absence of ATM.

In yeast, insects, and mammals, ATM-kinases are required to activate cellular responses to DNA ends generated by exogenous DNA damage, but also to suppress activation of these pathways by telomeres. Specific recognition of telomere sequences by telomere-repeat binding proteins provides one means to distinguish telomeric DNA ends from damage-induced DNA breaks. However, this mechanism is not sufficient to explain the epigenetic regulation of telomere protection in *Drosophila*. The requirement of ATM to recruit HP1 and HOAP to *Drosophila* telomeres suggests that recognition of chromosome ends contributes to chromatin-mediated telomere protection. This mechanism may help explain how terminally deleted chromosomes can be stably inherited without any telomere-specific sequences. Future studies should reveal which other damage response proteins help ATM protect telomeres, what their targets are at telomeres, and how these proteins distinguish between damage-induced DNA ends and the natural ends of linear chromosomes.

Materials and methods

Drosophila strains and sequence analysis. All animals were raised at 25°C. The deletion mutants, $atm^{\Delta 356}$ and $hsc70\text{-}4^{\Delta 16}$ have been previously described (Bronk et al. 2001). *Df(3R)PG4* and the transgenic rescue p[$hsc70\text{-}4^{+}$] strains were described by Hing et al (1999). The *tef* mutant was described previously (Queiroz-Machado et al. 2001). A strain carrying the *Df(3R)red31* chromosome was obtained from the Bloomington Stock Center. The $atm^{\Delta 356}$; p[$hsc70\text{-}4^{+}$] strain was created by S. Takada. The p[$hsc70\text{-}4^{+}$]*;* $atm^{\Delta 356}$, $p53^{1}$ strain was generated by recombination of the $atm^{\Delta 356}$ and $p53^{1}$ mutations (Rong et al. 2002). Sequence analysis was performed using genomic DNA obtained from *tef*/*Df(3R)PG4*, *Df(3R)red31*/*Df(3R)PG4*, and w^{1118} animals. *atm* genomic DNA was amplified by PCR and directly sequenced using gene specific primers.

Analysis of bristle phenotypes**.** Five wild type and five *atm* female pharate adults were dissected from their pupal cases. The following twelve bristles were scored on the head of each individual: orbitals (4), ocellar (2), verticals (4), and postverticals (2). Bristles were scored as shorter if they were at least 50 percent shorter than the equivalent wild type bristle.

Apoptosis and checkpoint assays. Apoptosis and cell cycle arrest was induced in developing wing discs by irradiation of late third instar larvae with 4000 rads X-rays in a Faxitron RX650 irradiator. Apoptotic cells were detected 4 hours following irradiation by

staining with the vital dye acridine orange (Abrams et al. 1993; Hay et al. 1994) or by fixation and staining with a polyclonal rabbit antibody raised against cleaved human Caspase 3 (Cell Signaling Technology) used at a 1:100 dilution. Mitotic cells were visualized one to four hours after irradiation using an anti-phospho-histone H3 antibody (Upstate Biotechnology) at a 1:500 dilution. Secondary antibodies were used at a 1:200 dilution. Fixation and staining was performed as described previously (Brodsky et al. 2000b).

Neuroblast preparations. Mitotic chromosome preparations were obtained from third instar larval brains according to Queiroz-Machado et al. (2001). Briefly, for metaphase spreads, brains were dissected in PBS, incubated in 0.05 mM colchicine for 30 minutes then transferred to a hypotonic solution (0.5% sodium citrate) for 10 minutes. Brains were fixed in 45% acetic acid for 1 minute and squashed gently in 60% acetic acid. The squashed brains were stained with 0.2ug/ml DAPI and mounted in vectashield. Anaphase spreads were obtained similarly with the following modifications: colchicine treatment was omitted; brains were incubated in hypotonic solution for 2 minutes. The steps that followed remained the same.

Immunostaining of polytene chromosomes**.** Polytene chromosomes were prepared for immunostaining as described by Shareef et al. (2001). Chromosomes were incubated with rabbit polyclonal anti-HOAP (gift from R. Kellum; 1:500/5%BSA) mouse monoclonal C1A9 anti-HP1 (Developmental Studies Hybridoma Bank; 1:50/5%BSA),

and rabbit polyclonal anti-HP1 (a gift from R. Kellum; 1:1000/5%BSA) antibodies at 4°C overnight, followed by a 2-hour incubation at room temperature with Cy3-conjucated sheep anti-rabbit and FITC-conjugated sheep anti-mouse (Jackson Immunoresearch Laboratories Inc; 1:200/5%BSA) secondary antibodies. For each genotype, salivary glands from 10 larvae were prepared and approximately ten chromosomes with good morphology and distinct telomeres were analyzed from each preparation. Stained chromosomes were analyzed using a Zeiss Axioskop 2 Plus microscope equipped with a Hamamatsu digital camera and Openlab software (Improvision). For immunostaining analysis the camera exposure times were held constant.

HP1 and HOAP fluorescence staining was quantified by performing z series scan on the tip of chromosome 2R using a Leica confocal microscope. Leica software was used to calculate the total fluorescence intensity for three regions of interest: the telomere, cytological band 60F, and a chromosome region approximately midway between the telomere and 60F (background). Five 2R telomeres were analyzed for each genotype.

in situ hybridization to polytene chromosomes. The DIG Labeling DNA Kit (Roche) was used to make a probe from 1 microgram of purified PCR product using a *Het-A* clone as a template (Danilevskaya et al. 1994). One microliter (ul) of probe in 20 ul of hybridization solution was used per slide. Salivary gland polytene chromosome squashes were prepared for hybridization by standard methods (Pardue 1994) except that the RNase treatment was not included. Slides were pre-hybridized at 58^0C in a moist

chamber for 1 hour in hybridization solution (0.6 mg/ml Fish Sperm DNA, 7.5mM $MgCl_2$, 3% 50X Denhardt's solution, 75mM $NaPO_4$ pH 7.0, 1M NaCl). Chromosome squashes were hybridized with the DIG-labeled probe overnight at 58^0C. Hybridized probe and DNA were detected using a 1:200 dilution in PBT of sheep anti-DIG FITC (Roche) and 0.5 ug/ml DAPI. Images were acquired as described for HP1 and HOAP staining above.

in situ hybridization to mitotic chromosomes. *in situ* hybridization to mitotic chromosomes was performed as described (Carmena et al. 1993). *Het-A* probe was labelled with biotin-14-dUTP using the Bionick Translation System (Gibco, BRL). Slides were mounted in Vectashield (Vector Laboratories, UK) containing 5 mg/ml of propidium iodide as a DNA counterstain. Quantification of the *Het-A* fluorescence intensity was performed using the Image J software. After selecting the area of *Het-A* signals, the average pixel intensity was determined and multiplied by that corresponding area.

Analysis of telomere position effect**.** To test the effect of *atm* mutations on telomere silencing, females carrying different telomeric *white*$^+$ insertions on the second chromosome and heterozygous for an *atm*$^{\Delta 356}$ mutation on the third chromosome were crossed to males heterozygous for the *atm*^{red31} or *atm*^{tef} mutation. Eye color was compared between homozygous *atm* mutant animals and wild type or heterozygous siblings. Images were taken using the same lighting conditions and exposure times using

a Leica dissecting microscope outfitted with a Zeiss AxioCam digital camera. Quantification of the results was performed using Adobe Photoshop. Images were inverted, a region of interest slightly smaller than the area of the eye was selected and the total luminosity was determined. Ten animals from each genotype were analyzed.

CHAPTER III

EPIGENETIC TELOMERE PROTECTION BY DROSOPHILA DNA DAMAGE RESPONSE PATHWAYS

Foreword

The work presented in this chapter has been published: Oikemus, SR., Queiroz-Machado, J., Lai, KJ., McGinnis, N., Sunkel, SE., Brodsky, MH. 2006. Epigenetic telomere protection by Drosophila DNA damage response pathways. *PLoS Genetics*. 2(5): 0693-0706.

Specific contributions are as follows: Identification and sequence analysis was done by Nadine McGinnis, Figure 3.1A. Analysis of chromosome break frequencies was performed by KuanJu Lai, Figure 3.5I. Analysis of HOAP staining and HeT-A sequences was contributed by Joana Queiroz-Machado, Table 3.2, Figure 3.6, Table 3.3, and Figure 3.7.

Introduction

The ends of eukaryotic chromosomes can be protected from end-to-end fusion by two distinct mechanisms. In most organisms, sequence-specific DNA binding proteins recognize telomere-specific sequences and protect telomeres from the activity of DNA repair systems (Karlseder 2003; de Lange 2005). However, genetic studies in *Drosophila* have demonstrated that telomeres can also be protected from end-to-end fusion by an epigenetic mechanism. The telomeric DNA of *Drosophila* chromosomes is composed of retrotransposons and repetitive telomere-associated sequences (Melnikova and Georgiev 2005). Terminal deletion chromosomes that completely lack these sequences can be

recovered and propagated (Mason et al. 1984; Biessmann and Mason 1988; Tower et al. 1993; Mason et al. 1997; Ahmad and Golic 1998). The telomeres of these chromosomes are protected from fusion and do not induce DNA damage responses such as cell cycle arrest or apoptosis. These observations demonstrate that a sequence-independent mechanism can protect *Drosophila* chromosomes from telomere fusion, and suggest that a similar mechanism contributes to protection of normal telomeres. The sequence-independent inheritance of telomere protection is conceptually similar to the epigenetic regulation of centromere function in which the function of a chromosomal domain is usually associated with a specific set of sequences, but can be stably transferred to alternative sequences (Karpen and Allshire 1997; Sullivan et al. 2001). Thus, *Drosophila* telomere protection can be grouped with centromere function and gene expression as processes that can be regulated by an epigenetic mechanism.

Two chromatin-associated proteins, HP1 and HOAP, are required for telomere protection and localize to the telomeres of both normal and terminally deleted chromosomes (Fanti et al. 1998; Badugu et al. 2003; Cenci et al. 2003b). The role of HP1 in the epigenetic inheritance of chromatin modifications during cell division (Maison and Almouzni 2004) suggests that a similar activity may contribute to telomere protection. Inheritance of chromatin modifications is often initiated or stabilized by specific chromosome features, such as binding sites for sequence-specific DNA binding proteins or repeat sequences at centromeres (Grewal and Rice 2004; Pirrotta and Gross 2005). The stable inheritance of terminally deleted chromosomes over many generations indicates

that a feature of telomeres other than telomere-specific sequences can recruit or maintain HP1 and HOAP at telomeres.

One signature of telomeres that might contribute to HP1 and HOAP recruitment is the chromosome end itself. Studies in yeast and mammalian cells have demonstrated that telomere protection requires proteins that act at broken chromosome ends during the cellular response to DNA damage; these include the ATM and ATR protein kinases and the Mre11/Rad50/NBS1 (MRN) DNA repair complex (d'Adda di Fagagna et al. 2004; Maser and DePinho 2004). Analysis of cells lacking telomerase and ATM suggests that ATM plays a particularly critical role in cells with short telomeres (Ritchie et al. 1999; Chan et al. 2001; Nakamura et al. 2002; Chan and Blackburn 2003). Such cells may be least able to utilize sequence-specific mechanisms for telomere protection. In both budding and fission yeast, the combined loss of the ATM and ATR pathways results in severe telomere protection defects (Naito et al. 1998; Ritchie et al. 1999; Craven et al. 2002; Chan and Blackburn 2003). In mammalian cell culture, acute inhibition of the MRN complex or of the ATM and ATR kinases also induces telomere fusions (Verdun et al. 2005). *Drosophila* homologs of most DNA damage response genes have been described (Figure 3.1). The *Drosophila telomere fusion (tefu)* gene is required to prevent fusions in proliferating cells and is encoded by the *Drosophila* homolog of *ATM* (Queiroz-Machado et al. 2001; Oikemus et al. 2004). Mutations in the *Drosophila* DNA damage response genes *tefu, mre11,* and *rad50* lead to partial loss of telomere protection and reduced recruitment of HP1 and HOAP to telomeres (Queiroz-Machado et al. 2001; Bi et al. 2004; Ciapponi et al. 2004; Oikemus et al. 2004; Silva et al. 2004; Song et al.

2004). Thus, a DNA damage response pathway contributes to the protection of *Drosophila* telomeres; however, HP1 and HOAP can also mediate some degree of telomere protection in the absence of this pathway (see discussion in Oikemus et al. 2004).

Here, we characterize the role of Nbs and the ATM and ATR DNA damage response pathways in the epigenetic protection of *Drosophila* telomeres. In humans, mutations in Nbs1 or ATM result in similar inherited syndromes (Shiloh 2003). In both mammals and yeast, Nbs1 forms a complex with Mre11 and Rad50 (the MRN complex) that acts in the ATM pathway in response to DNA damage and is required for DNA repair and telomere function (van den Bosch et al. 2003; Lavin 2004). We demonstrate that *Drosophila nbs* is required for *atm*- and *atr*-dependent DNA damage responses including DNA repair. *Drosophila mei-41* (the *ATR* homolog) and *mus304* (the *ATRIP* homolog) act in parallel to the *atm* pathway in telomere protection; cells lacking both pathways fail to recruit HOAP to the telomeres of mitotic chromosomes and exhibit a severe telomere fusion phenotype. The telomere fusion defect in *nbs* mutants suggests that it acts in both the *tefu* and *mei-41-mus304* telomere protection pathways and in the chromosome-joining step. We have taken advantage of the severe telomere fusion phenotype in cells lacking both pathways to test the role of DNA damage response pathways in the sequence-independent protection of *Drosophila* telomeres. Analysis of these cells reveals that loss of telomeric HOAP and telomere fusions are not due to loss of telomeric sequences. Furthermore, these DNA damage response pathways are also required to protect the telomeres of terminally deleted chromosomes, directly

demonstrating that the DNA damage response pathways are required for epigenetic regulation of telomere protection.

Results/Discussion

Drosophila Nbs is required for normal development. To identify genes that cooperate with *atm/tefu* in telomere protection, we characterized mutations in other *Drosophila* DNA damage response genes including *nbs*. Figure 3.1F lists several *Drosophila* DNA damage response genes and their mammalian homologs. Similar to *nbs* homologs in other organisms, *Drosophila nbs* encodes a protein with N-terminal FHA and BRCT domains and a short region of similarity to the Mre11 interaction domain encoded by human *Nbs1* (Figure 3.1A). To identify mutations in *Drosophila nbs,* we screened a collection of lethal mutations in the genetic region containing *nbs* (Leicht and Bonner 1988) for pupal lethality and excess apoptosis during wing development, phenotypes previously described for *Drosophila atm/tefu, mre11,* and *rad50* (Queiroz-Machado et al. 2001; Bi et al. 2004; Oikemus et al. 2004; Silva et al. 2004; Song et al. 2004). Two mutations with these phenotypes failed to complement each other, and their lethality was rescued by a transgene containing the *nbs* genomic region (Figure 3.1A; Materials and Methods). Sequencing of these mutations revealed that *l(3)67BDp^{1} (nbs^{1})* contains a 238–base pair (bp) deletion and 1bp insertion that disrupts the open reading frame while *l(3)67BDr^{1} (nbs^{2})* introduces a stop codon that truncates the reading frame at

amino acid position 685 (Figure 3.1A). Both of these mutations are predicted to eliminate the ability of Nbs to interact with Mre11.

Flies homozygous for the *nbs*^{1} mutation die as pharate adults with rough eyes and missing or abnormal bristles (Figure 3.1B–3.1E). In *tefu, mre11,* or *rad50* flies, this phenotype is accompanied by increased genomic instability and apoptosis (Queiroz-Machado et al. 2001; Bi et al. 2004; Oikemus et al. 2004; Silva et al. 2004; Song et al. 2004). *tefu,* but not *mei-41* or *mus304,* is also required for rapid induction (within 4 h) of additional apoptosis by X-irradiation (Brodsky et al. 2004; Oikemus et al. 2004). The developing wings of *nbs* mutant animals also exhibit high levels of spontaneous apoptosis compared to wild-type animals (Figure 3.1G, 3.1I, 3.1M, 3.1O, 3.1S, and 3.1U). X-irradiation of these discs does not induce the rapid, large increase in apoptosis observed in wild-type discs (Figure 3.1J, 3.1P, and 3.1V). These results suggest that *nbs* acts in the *tefu* DNA damage response pathway to regulate apoptosis. Consistent with this conclusion, *nbs tefu* double mutant animals also exhibit high levels of apoptosis and fail to induce further apoptosis following irradiation (Figure 3.2).

To determine whether the elevated spontaneous apoptosis in these discs requires *p53* or *mnk* (the *Drosophila Chk2* homolog), apoptosis was examined in *nbs p53* and *nbs mnk* double mutant discs. The *Drosophila p53* and *mnk* genes are required for induction of apoptosis by X-irradiation (Brodsky et al. 2000a; Ollmann et al. 2000; Xu et al. 2001; Sogame et al. 2003; Brodsky et al. 2004). Previously, the *Drosophila p53* gene was shown to be required for some, but not all, of the apoptosis observed in *tefu* mutant discs (Oikemus et al. 2004; Song et al. 2004). Apoptosis is substantially reduced in *nbs p53*

(Figure 3.1K, 3.1Q, and 3.1W) and *nbs mnk* (Figure 3.1L, 3.1R, and 3.1X) double mutant discs compared to *nbs* single mutants (Figure 3.1I, 3.1O, and 3.1U). Although *p53* has been implicated in a variety of stress response pathways, *Chk2* homologs appear to specifically function in DNA damage responses. Thus, these results suggest that the absence of *nbs* leads to apoptosis via activation of a DNA damage response. This response may be directly activated by unprotected telomeres or by chromosome breaks formed following telomere fusions. The regulation of this response must, however, differ from the regulation of apoptosis 4 h following X-irradiation, which requires wild-type *nbs* and *tefu* function.

Figure 3.1

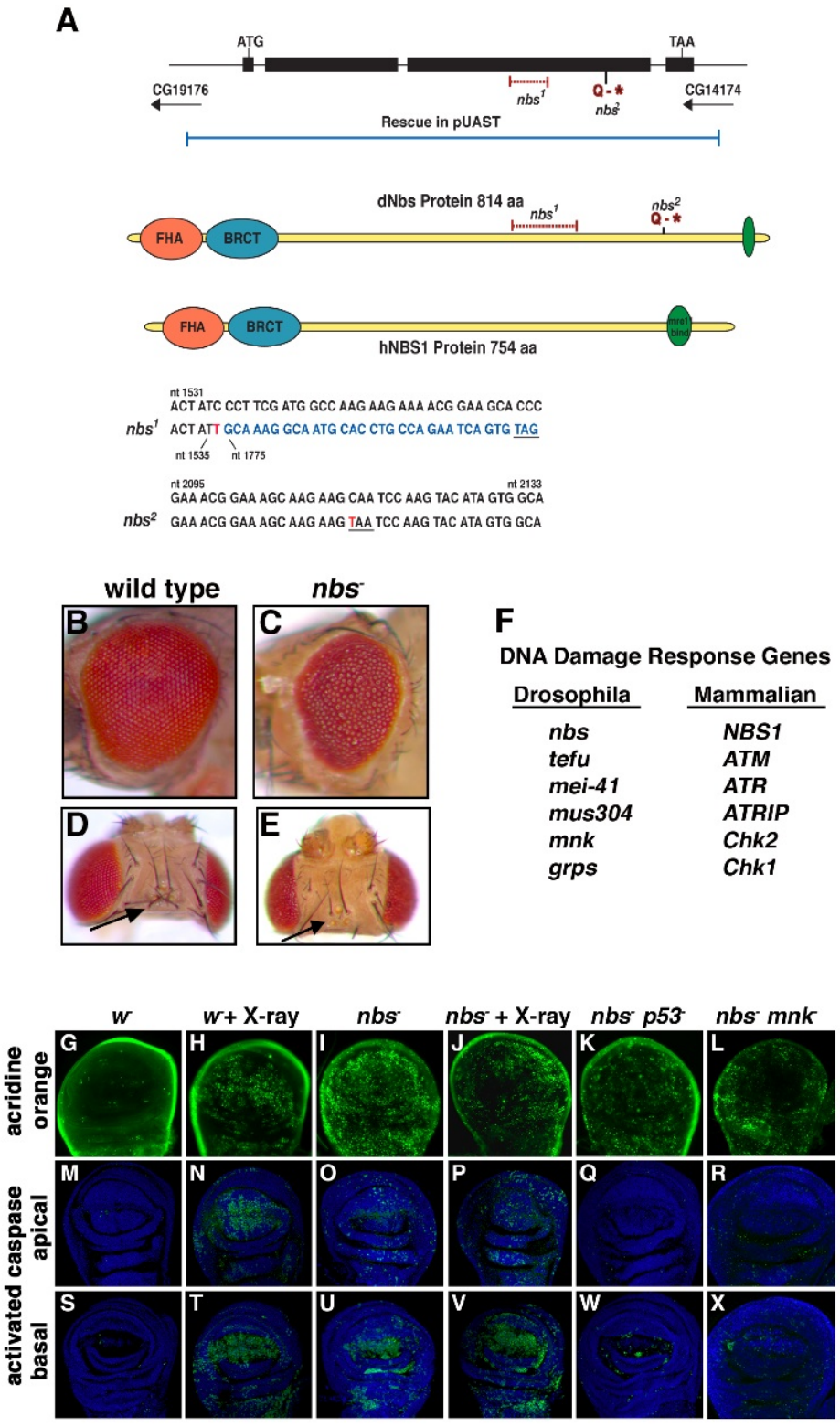

A
ATG
TAA
CG19176
CG14174
nbs1
Q - *
nbs2
Rescue in pUAST
dNbs Protein 814 aa
FHA
BRCT
hNBS1 Protein 754 aa
nt 1531
ACT ATC CCT TCG ATG GCC AAG AAG AAA ACG GAA GCA CCC
nbs1 ACT ATT GCA AAG GCA ATG CAC CTG CCA GAA TCA GTG TAG
nt 1535
nt 1775
nt 2095
nt 2133
GAA ACG GAA AGC AAG AAG CAA TCC AAG TAC ATA GTG GCA
nbs2 GAA ACG GAA AGC AAG AAG TAA TCC AAG TAC ATA GTG GCA
wild type
nbs-
B
C
D
E
F
DNA Damage Response Genes
Drosophila
Mammalian
nbs
NBS1
tefu
ATM
mei-41
ATR
mus304
ATRIP
mnk
Chk2
grps
Chk1
w-
w- + X-ray
nbs-
nbs- + X-ray
nbs- p53-
nbs- mnk-
acridine orange
activated caspase
apical
basal
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
X

Figure 3.1. Drosophila nbs is an essential gene required for DNA damage–induced apoptosis. (A) Upper: *nbs* gene structure. The *Drosophila nbs* gene is composed of four exons and encodes a protein (dNbs) with similar domain structure to the human NBS1 protein (hNBS1). *nbs* mutations are indicated in red. The P[nbs^{+}] genomic rescue construct is shown in blue. Middle: Nbs protein structure. *Drosophila* and human Nbs protein structures are depicted with the forkhead-associated (FHA) domain in orange, the BRCT domain in blue, and the Mre11-interacting domain in green. Lower: alignment of wild type and mutant *nbs* genomic DNA sequences. The nbs^{1} mutation is a 238-bp deletion and single bp insertion at nucleotide position 1,536 that results in a frameshift and a new stop codon (underlined). The inserted base is shown in red. Bases following the deletion are shown in blue. nbs^{2} is a point mutation at nucleotide 2,113 (shown in red) that introduces a stop codon at amino acid position 686 (underlined). (B–E) Pharate adult morphology of wild type (B) and (D) and nbs^{1}/nbs^{2} (C) and (E) animals. nbs^{-} pharate adults have a rough eye and missing bristle phenotype ([D] and [E], arrows). (F) *Drosophila* DNA damage, response proteins and their mammalian homologs. (G–X) p53- and Mnk-dependent apoptosis in *nbs* mutant wing discs. Wing imaginal discs from wild type and *nbs* mutant third-instar larvae were mock treated or X-irradiated (4,000 rads) and stained with acridine orange (G–L) or with an antibody against cleaved caspase 3 ([M–R], apical sections and [S–X], basal sections). Wild-type untreated discs have very low levels of apoptosis (G), (M), and (S). *nbs* mutant discs have high levels of spontaneous apoptosis (I), (O), and (U). Irradiation of wild-type wing discs induces high levels of apoptosis (H), (N), and (T). Irradiation of *nbs* mutant discs (J), (P), and (V) does

not greatly increase apoptosis beyond the elevated levels of spontaneous apoptosis (compare apical sections [N] and [P]). Apoptosis in *nbs* mutant discs is strongly suppressed by mutations in *p53* (K), (Q), and (W) and *mnk* (L), (R), and (X). The mutant alleles used in this figure and others are described in Materials and Methods.

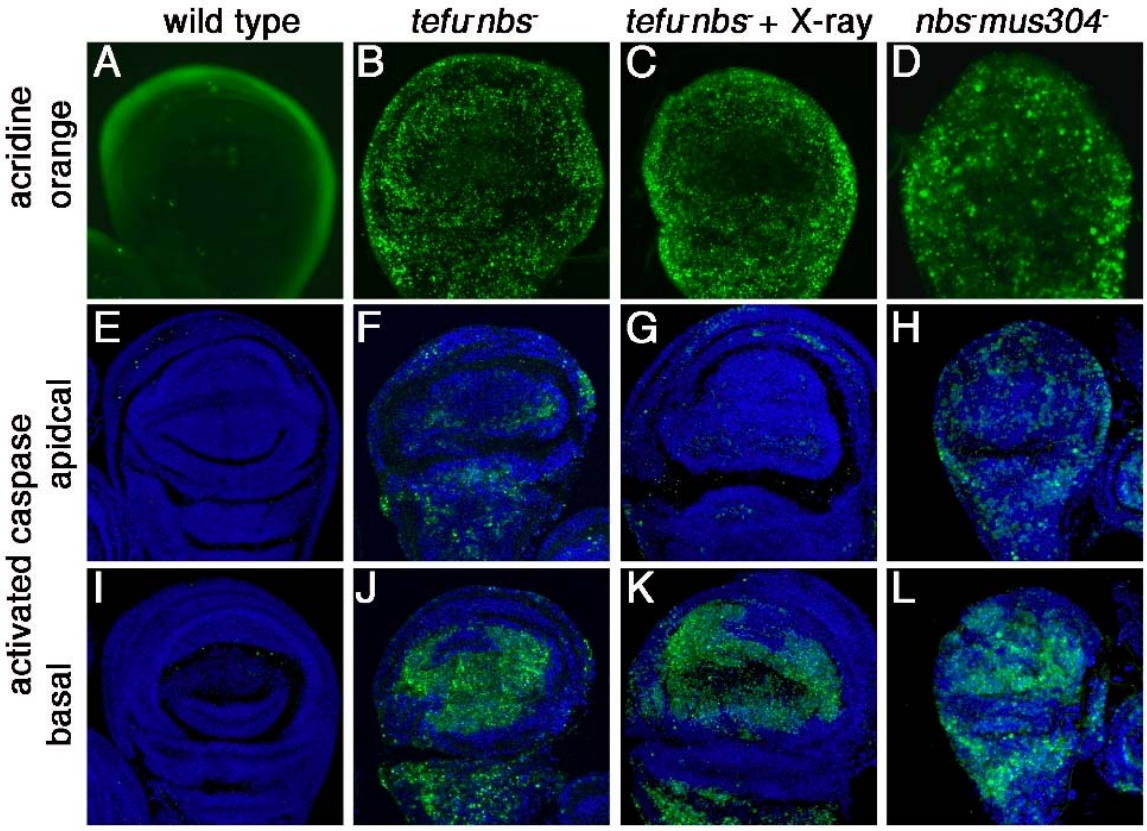


Figure 3.2. **Drosophila *nbs* and *tefu* act in the same damage-induced apoptosis pathway**. Third instar larval wing discs were stained with acridine orange or an antibody against activated caspase 3 in order to visualize apoptosis. Wild type untreated discs have very low levels of apoptosis (A, E and I). *tefu nbs* (B, F and J) and *nbs mus304* (D, H, and L) mutant discs have high levels of spontaneous apoptosis as seen in *tefu* and *nbs* single mutants. In addition, *nbs mus304* discs are small and misshapen compared to wild type discs. Irradiation of *tefu nbs* double mutant discs does not result in any further increase in apoptosis (C, G and K), similar to *tefu* or *nbs* single mutant discs. The mutant alleles used in this figure and others are described in the Materials and Methods.

DNA damage checkpoint and repair defects in *nbs* mutant cells. To further compare the function of *nbs* with *tef, mei-41,* and *mus304,* cell cycle arrest and double-strand DNA break repair were examined. Previous studies have demonstrated that *mei-41* is required for G2 arrest at both high (4,000 rads) and low (500 rads) doses of ionizing radiation (Hari et al. 1995; Brodsky et al. 2000b), whereas *tefu* is primarily required at low doses (Queiroz-Machado et al. 2001; Bi et al. 2005a), but not high doses (Oikemus et al. 2004; Silva et al. 2004; Song et al. 2004). Dose-response curves confirm that *mei-41* mutant discs fail to arrest in response to a range of irradiation doses whereas *tefu* mutant discs have a partial arrest phenotype at low doses, but not at 4,000 rads (Figure 3.3). Similar to *mei-41, nbs* is required for cell cycle arrest at all doses tested (Figure 3.3). These results demonstrate that *nbs* plays a *tefu*-independent role in cell cycle arrest and suggests that it acts in the *atr-atrip* pathway to mediate G2 arrest. A cell cycle arrest defect at low, but not high X-ray doses has been reported for *tefu* and *mre11* (Bi et al. 2005a); however, we observe that loss of *nbs* results in an arrest defect at high doses whereas loss of *mre11* results in a partial arrest at high doses (Table 3.1). *nbs mus304* and *nbs tefu* double mutants also exhibit a cell cycle checkpoint phenotype at high doses; however, the reduced number of mitotic cells and smaller discs indicates that mitosis has been severely disrupted in the double mutants, making direct comparisons to single mutants problematic (Table 3.1, Figure 3.4). We conclude that *nbs, mei-41,* and *mus304* are all essential for cell cycle arrest at high doses of X-irradiation whereas *tefu* is not.

Figure 3.3.

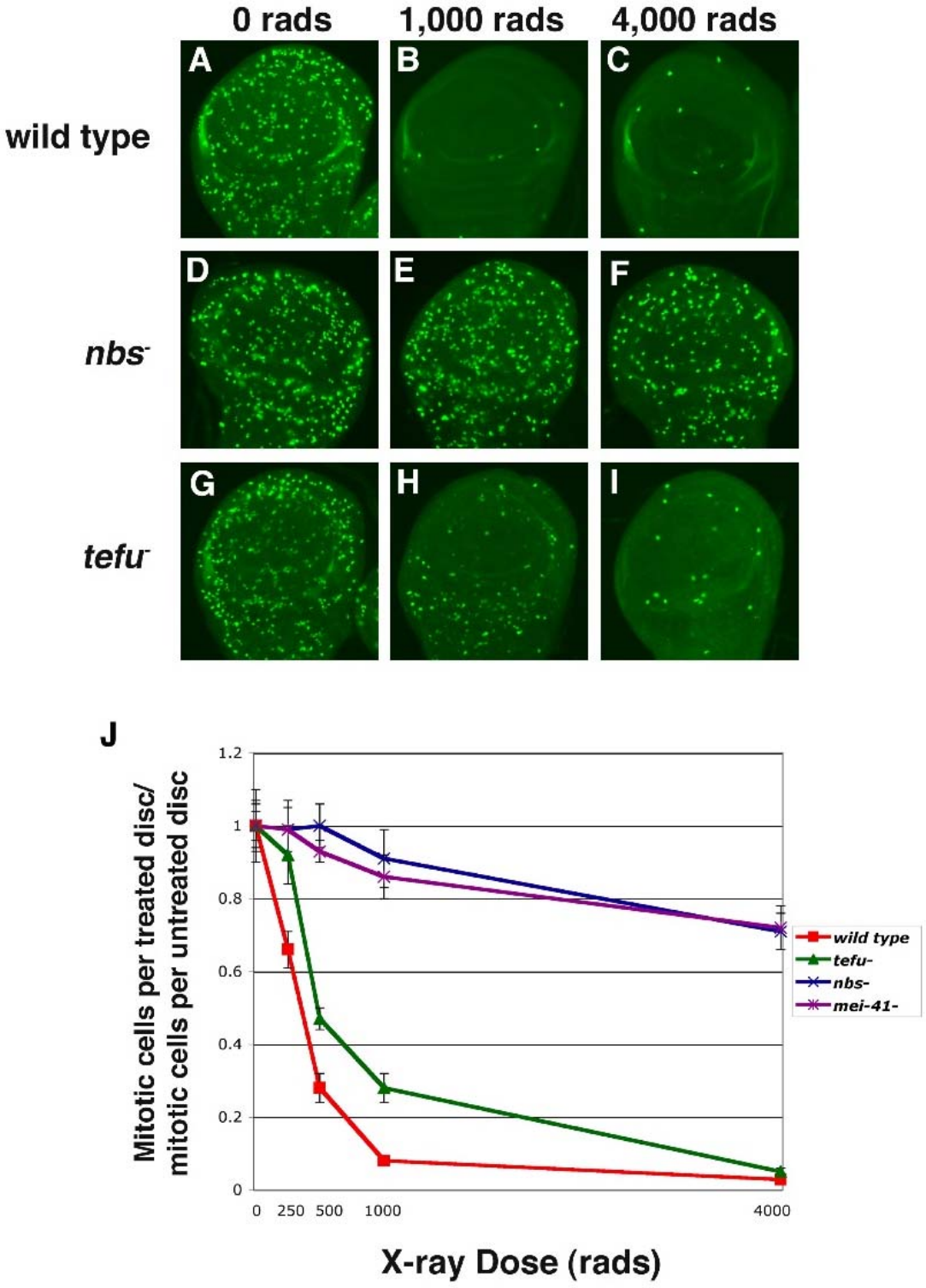

0 rads
1,000 rads
4,000 rads
wild type
nbs-
tefu-
A
B
C
D
E
F
G
H
I
J
Mitotic cells per treated disc/ mitotic cells per untreated disc
1.2
1
0.8
0.6
0.4
0.2
0
0
250
500
1000
4000
X-ray Dose (rads)
wild type
tefu-
nbs-
mei-41-

Figure 3.3. *Drosophila* Nbs is required for damage-induced cell cycle arrest. Third-instar larval wing discs were mock treated or treated with various doses (250, 500, 1,000, and 4,000 rads) of X-rays and then stained with an antibody against phosphorylated histone H3. (A–I) The pattern of mitotic cells in untreated and irradiated wild type (A–C), *nbs* mutant (D–F), and *tefu* mutant (G–I) larval wing discs are shown. At 1,000 and 4,000 rads, mitosis is blocked in wild-type wing discs (B) and (C) whereas *nbs* mutant discs fail to arrest (E) and (F). *tefu* mutant wing discs have a partial mitotic arrest following treatment with 1,000 rads (H). At 4,000 rads, mitosis is completely blocked in *tefu* mutant wing discs (I). (J) The ratio of mitotic cells in wild-type, *nbs, tefu,* and *mei-41* mutant wing discs following X-irradiation to the number of mitotic cells in untreated discs of the same genotype is shown. Error bars indicate the standard error of the mean.

Table 3.1. Average number of phosph-H3 positive cells per wing imaginal disc

Genotype	Average mitotic cells/disc untreated	Average mitotic cells/disc treated (4,000 Rads)
w^{1118}	348 (+/-19.9)	10 (+/-2.8)
mei-41^{-}	386 (+/-17.3)	278 (+/-22.6)
tefu^{-}	347 (+/-23)	18 (+/-2.1)
nbs^{-}	389 (+/-40.6)	276 (+/-20.9)
mre11	426 (+/-12.1)	169 (+/-15.2)
tefu^{-} *nbs*$^{-}$	257 (+/-14.5)	288 (+/-22.1)
nbs^{-} *mus304*$^{-}$	150 (+/-8.3)	139 (+/-7.1)

Five to ten discs were scored for each genotype and condition. The standard error of the mean is indicated in parenthesis.

Previous studies have demonstrated that *mei-41, mus304, rad50,* and *mre11* are all required for DNA double-strand break (dsb) repair in *Drosophila* (Brodsky et al. 2000b; Bi et al. 2004; Ciapponi et al. 2004; Gorski et al. 2004). The effect of *nbs* and *tefu* mutations on dsb formation and repair was examined in metaphase chromosomes from larval neuroblasts (Figure 3.5A–3.5C and 3.5I). As discussed in more detail below, dsbs can arise as an indirect result of telomere fusion followed by chromosome breakage during mitosis (see Figure 3.7E). Broken chromosomes generated by this mechanism will generally retain their centromere. To analyze breaks due to mechanisms other than telomere fusion, the number of acentric chromosome fragments was analyzed. In untreated cells, these fragments may reflect a role in preventing formation of breaks during DNA replication. Both *nbs* and *tefu* are required to prevent the spontaneous accumulation of dsbs during normal cell cycles (Figure 3.5B, 3.5D, and 3.5I). However, *nbs, mre11, mei-41,* and *mus304* mutant cells all have a more severe phenotype than *tefu* (Figure 3.5I). Analysis of double mutant cells suggests that *nbs* and *tefu* act in parallel to

mus304 to prevent accumulation of dsbs (Figure 3.5I). *nbs* cells also exhibit defective repair of X-irradiation–induced chromosome breaks (Figure 3.5C and 3.5I), consistent with the role of Nbs in the MRN DNA repair complex and with previous analysis of *Drosophila* Mre11 and Rad50 (Bi et al. 2004; Ciapponi et al. 2004; Gorski et al. 2004). Less severe dsb repair defects are seen following X-irradiation of *tefu, mei-41,* or *mus304* mutant cells (Figure 3.5I). Following irradiation, *nbs tefu* or *nbs mus304* double mutant cells do not exhibit a greater defect than *nbs* single mutants, suggesting that *nbs* acts in both the *tefu* and *mus304* pathways to mediate repair of induced DNA breaks.

In summary, *nbs* acts in both the *tefu* and *mei-41-mus304* DNA damage response pathways. Double mutant analysis indicates that *Drosophila nbs* acts in common genetic pathways with *tefu* and *mus304* during DNA repair (Figure 3.5). In addition, *nbs* has DNA damage response phenotypes in common with both *tefu* (defective induction of apoptosis, Figure 3.1) and *mei-41-mus304* (defective induction of cell cycle arrest at high doses of X-irradiation, Figure 3.3). Although Nbs1 homologs are best known for their roles in DNA repair and signaling in the ATM pathway (Shiloh 2003), human Nbs1 has also been reported to be required for signaling by the ATR pathway (Stiff et al. 2005). Thus, *nbs* has a conserved role in *ATM*- and *ATR-ATRIP*–dependent DNA damage responses.

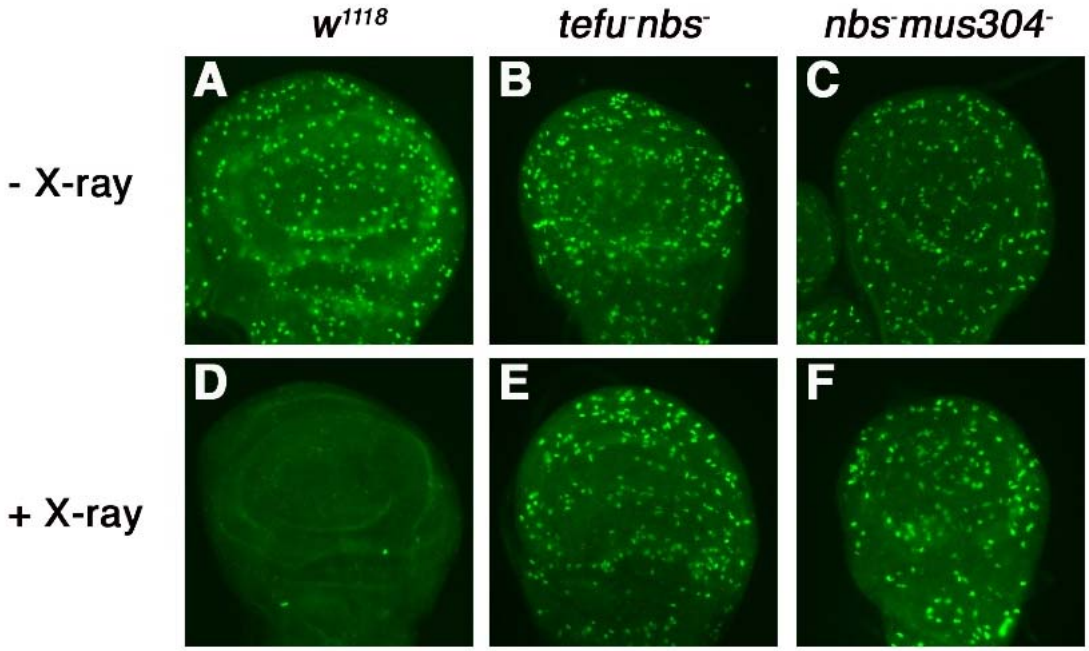


Figure 3.4. Double mutant animals exhibit a cell cycle arrest defect and a reduced number of mitotic cells. Third instar larval wing discs were mock treated or treated with 4,000 rads and then stained with an antibody against phosphorylated Histone H3. The pattern of mitotic cells was examined in wild type (A, C), *tefu nbs* (B, D) and *nbs mus304* (C, F) mutant larval wing discs. At 4,000 rads, mitosis is blocked in wild type wing discs (D). However, *tefu nbs* (E) and *nbs mus304* (F) mutant wing discs fail to arrest following irradiation. A direct comparison to the single mutants not possible due to the reduced size of the double mutant discs and corresponding reduced number of mitotic cells (Table 3.1).

Two DNA damage response pathways contribute to telomere protection. Metaphase larval neuroblasts were also used to examine the roles of different DNA damage response genes in telomere protection. Previous studies have demonstrated that *Drosophila tefu, mre11,* and *rad50* mutant cells have a partial defect in telomere protection (Queiroz-Machado et al. 2001; Bi et al. 2004; Ciapponi et al. 2004; Oikemus et al. 2004; Silva et al. 2004; Song et al. 2004). Consistent with these results, *nbs* mutant animals exhibit a high frequency of cells with one or more fusions (Figure 3.5). These fusions are observed during both metaphase (Figure 3.5B and 3.5J) and anaphase (Figure 3.5D and Table 3.2). Another group has also recently described a telomere fusion phenotype for nbs^{1} animals (Bi et al. 2005b). *nbs tefu* double mutant cells exhibit similar fusion rates as *tefu* single mutants, indicating that these genes act in a common telomere protection pathway (Figure 3.5E and 3.5J). These results are consistent with results in *Drosophila* and other organisms, indicating that ATM and components of the MRN complex act in a common telomere protection pathway (Nakamura et al. 2002; Bi et al. 2004; d'Adda di Fagagna et al. 2004). Downstream targets of ATM in the mammalian DNA damage response pathway include Nbs1 and the checkpoint kinases CHK1 and CHK2. The *Drosophila* homologs of these kinases are encoded by the *grp* and *mnk* genes and are required for DNA damage-induced apoptosis and cell cycle arrest (Brodsky et al. 2004; Song 2005). Both telomere protection and chromosome break repair are normal in *grp mnk* double mutant cells (Figure 3.5I and 3J), indicating that other targets of *Drosophila tefu* and *nbs* are responsible for their telomere protection and DNA repair functions.

Compared with mutations in the genes that encode the telomere protection proteins HOAP (Figure 3.5J, *cav*$^{-}$) and HP1, mutations in *tefu, nbs, mre11,* and *rad50* exhibit a significantly lower frequency of telomere fusions (Fanti et al. 1998; Queiroz-Machado et al. 2001; Cenci et al. 2003b; Bi et al. 2004; Ciapponi et al. 2004; Oikemus et al. 2004; Silva et al. 2004; Song et al. 2004), indicating that there may be a *tefu-nbs*–independent pathway for telomere protection. In mammals, the ATR checkpoint kinase is recruited to sites of DNA damage by ATRIP (Cortez et al. 2001; Itakura et al. 2004), and acts in parallel to the ATM kinase in the DNA damage response (Shiloh 2003). In budding and fission yeast, disruption of both *atm/atr* homologs results in loss of telomere protection (Naito et al. 1998; Craven et al. 2002). Mutations in *mei-41* or *mus304* do not result in telomere protection defects (Figure 3.5J). However, *nbs mus304, tefu mus304,* and *tefu mei-41* double mutant animals all show higher rates of telomere fusion than the corresponding single mutants (Figure 3.5F, 3.5G, 3.5H, and 3.5J) indicating that the *mei-41-mus304* pathway acts in parallel to a *tefu-nbs* pathway to mediate telomere protection. The higher fusion frequency in *tefu mei-41* double mutants compared with *tefu mus304* double mutants may indicate that there is a small amount of *mei-41* activity in the absence of *mus304.*

Figure 3.5

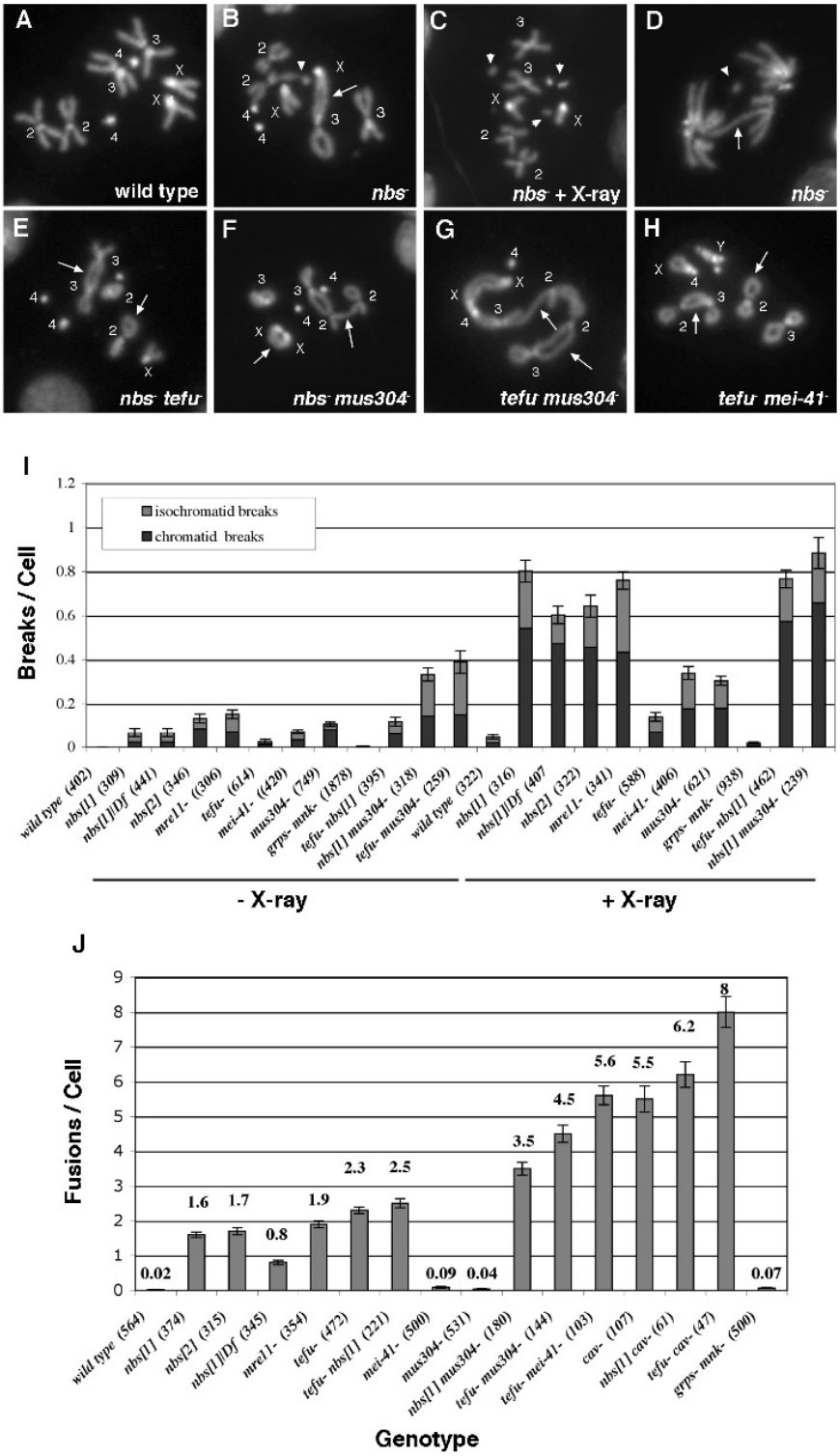

A
wild type
B
nbs-
C
nbs- + X-ray
D
nbs-
E
nbs- tefu-
F
nbs- mus304-
G
tefu mus304-
H
tefu- mei-41-
I
Breaks / Cell
isochromatid breaks
chromatid breaks
wild type (402)
nbs[1] (309)
nbs[1]/Df (441)
nbs[2] (346)
mre11- ((306)
tefu- (614)
mei-41- ((420)
mus304- (749)
grps- mnk- (1878)
tefu- nbs[1] (395)
nbs[1] mus304- (318)
tefu- mus304- (259)
wild type (322)
nbs[1] (316)
nbs[1]/Df (407
nbs[2] (322)
mre11- (341)
tefu- (588)
mei-41- (406)
mus304- (621)
grps- mnk- (938)
tefu- nbs[1] (462)
nbs[1] mus304- (239)
- X-ray
+ X-ray
J
Fusions / Cell
0.02
1.6
1.7
0.8
1.9
2.3
2.5
0.09
0.04
3.5
4.5
5.6
5.5
6.2
8
0.07
wild type (564)
nbs[1] (374)
nbs[2] (315)
nbs[1]/Df (345)
mre11- (354)
tefu- (472)
tefu- nbs[1] (221)
mei-41- (500)
mus304- (531)
nbs[1] mus304- (180)
tefu- mus304- (144)
tefu- mei-41- (103)
cav- (107)
nbs[1] cav- (61)
tefu- cav- (47)
grps- mnk- (500)
Genotype

Figure 3.5. *Drosophila nbs* acts with the *atm* and *atr-atrip* pathways to protect cells from telomere fusions and chromosome breaks. Mitotic chromosome spreads were prepared from wild type and mutant third-instar larval brains. Wild-type cells do not exhibit telomeric associations (A). *nbs* single mutant cells and *nbs tefu* double mutant cells exhibit DNA breaks (arrowheads) and telomere fusions (arrows) in metaphase (B), (C), and (E) and anaphase (D). Double mutant cells disrupting both the *atm-nbs* and *atr-atrip* pathways result in a more severe telomere phenotype, in which many telomeres are fused (F–H). The labels X, Y, 2, 3, and 4 refer to the relevant chromosome. The frequency of spontaneous (no X-ray treatment [−X-ray]) and damage-induced (treated with 100 rads X-rays [+ X-ray) chromosome breaks is elevated in mitotic cells from *nbs*- and other mutant cells (I). Both isochromatid (light shading) and chromatid (dark colored portion of the bar) breaks were counted as one break. The frequency of telomere fusions per cell is elevated in DNA damage response mutant cells (J). Double telomere associations were counted as two fusions. Individual genotypes are discussed in the Results section. The total number of cells scored for each genotype is in parenthesis. Error bars indicate the standard error of the mean.

DNA damage response genes regulate telomeric HOAP**.** The formation of telomere fusions requires two steps: (1) the failure of telomeric protein complexes, such as HP1-HOAP, to prevent telomeric DNA ends from being recognized as damage-induced ends, and (2) the subsequent ligation of unprotected telomeres by DNA repair systems. To probe the role of DNA damage response pathways in the first step, HOAP localization was examined in individual mitotic cells (neuroblasts). Previously, it was shown that the telomere protection proteins HP1 and HOAP are reduced at the telomeres of polytene chromosomes from *tefu* salivary gland cells (Oikemus et al. 2004), but that telomeric HOAP is not strongly reduced in mitotic chromosomes from neuroblasts (Bi et al. 2004; Oikemus et al. 2004); these results suggest that in the absence of *tefu,* neuroblasts utilize an alternative mechanism for HOAP localization. In contrast, both salivary glands and neuroblasts required *mre11* and *rad50* for normal HOAP localization (Bi et al. 2004; Ciapponi et al. 2004).

The frequency of neuroblast telomeres with HOAP staining and the intensity of staining at those telomeres were examined in wild type and mutant cells (Figure 3.6, Table 3.3). Measurements of fluorescence intensity can be used to demonstrate that HOAP levels at individual telomeres are reproducibly increased or decreased in different genotypes. (However, we note that there may not be a linear relationship between the percent change of fluorescence observed and the percent change of telomeric HOAP protein levels.) Most wild type, *tefu,* and *mus304* mutant metaphase cells are HOAP positive; between 77% and 94% of these cells had HOAP signals at chromosome ends (Figure 3.6, Table 3.3). Among the HOAP positive cells, between 66% and 72% of

telomeres stained for HOAP. The fluorescence intensity of HOAP staining was similar at the telomeres of each of the major chromosome arms in both wild type and *mus304* mutant cells (Figure 3.6G and 3.6H). However, the average intensity of HOAP staining was elevated in *tefu* mutant cells (Figure 3.6G and 3.6H), indicating that although HOAP is still recruited to telomeres, the mechanism regulating HOAP levels at telomeres may be perturbed. A more severe effect on HOAP localization was observed in *nbs* mutant metaphases, with only 44% of metaphases displaying HOAP signals and only 30% of the telomeres in those cells staining for HOAP (Table 3.3). This phenotype is similar to that reported for *mre11* and *rad50* mutant neuroblasts (Bi et al. 2004; Ciapponi et al. 2004). At the few HOAP-positive telomeres that are present in *nbs* cells, HOAP fluorescence staining intensity was elevated compared to wild type, similar to the HOAP staining at *tefu* mutant telomeres (Figure 3.6H). Together with the genetic data indicating that *tefu* and *nbs* act in a common telomere protection pathway, these results suggest that an alternative pathway can maintain HOAP levels at telomeres, but that this pathway is much less efficient in *nbs* mutant cells.

Since *mus304 nbs, mus304 tefu,* and *mei-41 tefu* double mutant cells have more severe telomere fusion phenotypes than *nbs* or *tefu* single mutants (Figure 3.6), the *mei-41-mus304* pathway is a clear candidate to recruit HOAP to telomeres in the absence of *tefu* or *nbs*. *mus304* single mutant animals do not exhibit a defect in either the frequency of HOAP-positive telomeres or the intensity of HOAP staining at those telomeres (Figure 3.6B and 3.6G and Table 3.3). In contrast, we were unable to detect telomeric HOAP staining in *mus304 tefu* or *mus304 nbs* double mutant cells (Figure 3.6E and 3.6F and

Table 3.3). Thus, the *mei-41-mus304* pathway partially compensates for the absence of *tefu,* limiting the severity of the *tefu* telomere fusion phenotype. Cells lacking both pathways exhibit loss of telomeric HOAP and a severe telomere fusion phenotype. In a report published while this manuscript was in preparation, Bi et al. also find that disruption of the *Drosophila atm* and *atr* pathways results in a high frequency of telomere fusions and loss of telomeric HOAP (Bi et al. 2005b).

Table 3.2. Percentage of telomeres with HOAP staining

Genotype	% Metaphases with HOAP [a]	% Labeled telomeres in HOAP positive metaphases [b]	% Fusions with HOAP [c]
w^{-}	82 (n=93)	72 (n=1272)	-
mus304^{-}	77 (n=56)	71 (n=412)	-
tefu^{-} [d]	94 (n=67)	66 (n=751)	27 (n=110)
nbs^{-}	44 (n=120)	30 (n=867)	20 (n=40)
tefu^{-} *mus304*$^{-}$ [e]	0 (n=69)	-	-
nbs^{-} *mus304*$^{-}$	0 (n=65)	-	-

[a] n=number of metaphases analyzed
[b] n=number of total telomeres analyzed
[c] n=number of chromatid fusions analyzed
[d] *tefu*^{1} */ Df(3R)PG4*
[e] *tefu*$^{\Delta 356}$ *mus304*^{D2}

These results support the model that the *Drosophila tefu* and *mei-41-mus304* DNA damage response pathways mediate telomere protection by recruiting or maintaining HOAP at telomeres. The more severe HOAP localization phenotype of *nbs* mutant cells compared with *tefu* cells indicates that *nbs* has a *tefu*-independent role in telomere protection. As described above, the common DNA repair and damage response phenotypes of *nbs* with *mei-41* and *mus304* indicate that *nbs* also acts in the *atr-atrip*

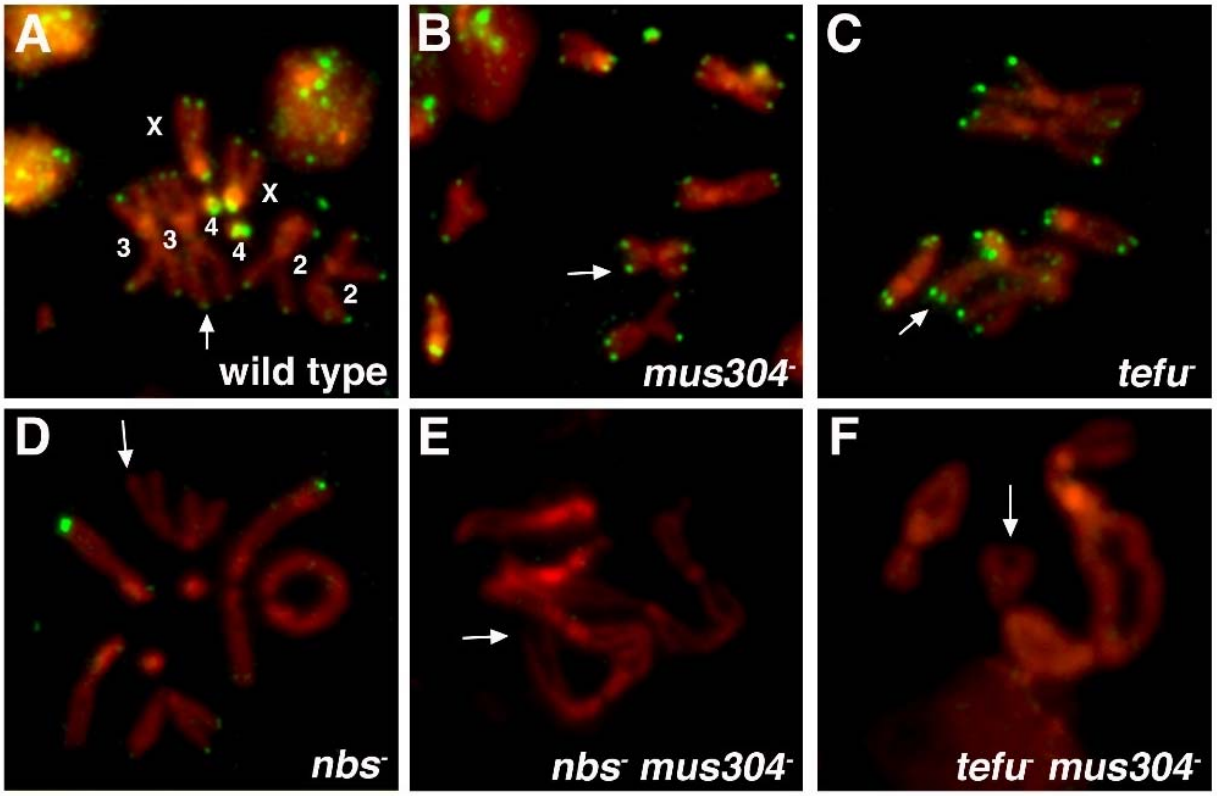
DNA damage response pathway. Thus, one explanation for the lower frequency of HOAP positive telomeres in *nbs* compared to *tefu* cells is that *nbs* mutations both disrupt the *tefu* telomere protection function and partially disable a compensatory telomere protection pathway mediated by *mei-41-mus304*.

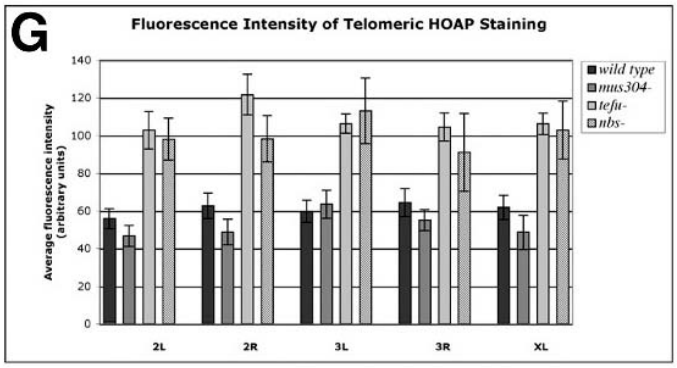
There is a good correlation between the levels of telomeric HOAP and the frequency of telomere fusion, except in *nbs, mre11,* and *rad50* mutants. In yeast and mammalian cells, some DNA repair genes are required to both maintain telomere protection and to promote joining of unprotected telomeres (Williams and Lustig 2003; d'Adda di Fagagna et al. 2004). The observed telomere fusion frequency in *nbs* mutant cells may reflect the combined effects of decreased telomere protection and inefficient fusion of unprotected telomeres. Although the loss of *nbs* has a more severe effect than *tefu* on telomeric HOAP (Figure 3.6), *nbs* and *tefu* mutant cells have similar telomere fusion frequencies (Figure 3.5). *nbs* mutations have a more severe effect on repair of DNA breaks (Figure 3.5), suggesting that *nbs* mutant cells may also have reduced joining of unprotected telomeres. Consistent with a role for *nbs* in fusion of unprotected telomeres, *nbs mus304* mutant cells have a lower telomere fusion frequency than *tefu mus304* cells, despite undetectable levels of telomeric HOAP in both genotypes. Similarly, *nbs cav* double mutant cells have a lower telomere fusion frequency than *tefu cav* double mutant cells (Figure 3.5J).

In summary, DNA damage response genes are essential for the telomeric localization of the protection protein HOAP. Analysis of DNA repair, telomere fusions and HOAP localization suggests that the telomere fusion frequency reflects a

combination of defective protection and reduced fusion of unprotected chromosomes. Although these results do not rule out the possibility that DNA damage response genes are also required for modification of HP1 and HOAP complexes at telomeres, they strongly suggest that recruitment or maintenance of these complexes to telomeres is critical for telomere protection.

Figure 3.6


A
X
X
3
3
4
4
2
2
wild type
B
mus304-
C
tefu-
D
nbs-
E
nbs- mus304-
F
tefu- mus304-


G
Fluorescence Intensity of Telomeric HOAP Staining
Average fluorescence intensity (arbitrary units)
0
20
40
60
80
100
120
140
2L
2R
3L
3R
XL
wild type
mus304-
tefu-
nbs-

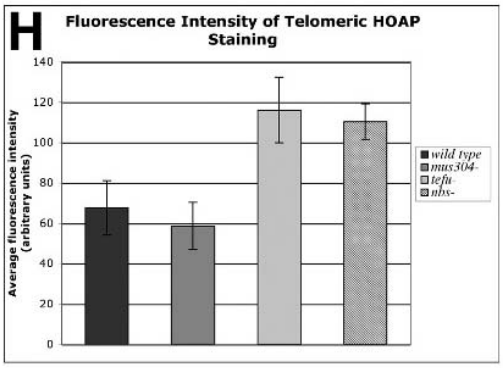

H
Fluorescence Intensity of Telomeric HOAP Staining
Average fluorescence intensity (arbitrary units)
0
20
40
60
80
100
120
140
wild type
mus304-
tefu-
nbs-

Figure 3.6. The *Drosophila tefu* and *mei-41-mus304* pathways are required for telomeric localization of HOAP. HOAP immunostaining of mitotic chromosomes prepared from wild type and mutant third-instar larval brains. Wild type, *mus304,* and *tefu* mutant mitotic chromosomes exhibit HOAP localization (shown in green) at most telomeres ([A–C] arrows). *nbs* mutant cells exhibit a decreased number of telomeres with HOAP signal ([D] arrow). No HOAP was detected at the telomeres of *nbs mus304* or *tefu mus304* mutant chromosomes ([E] and [F] arrows). Alleles examined in these experiments include (C) $tefu^{1}$*/ Df(3R)PG4* and (E) $tefu^{\Delta 356}$ $mus304^{D2}$. The frequency of HOAP-positive telomeres is shown in Table 3.3. The labels X, 2, 3, and 4 refer to the relevant chromosome. (G) The average fluorescence intensity of anti-HOAP immunostaining at the telomeres of chromosome arms 2L, 2R, 3R, 3L, and XL was determined for wild-type, homozygous $mus304^{-}$*,* $tefu^{1}$*/Df(3R)PG4,* or nbs^{-} animals. The average fluorescence intensity of the HOAP signal is higher in tefu and nbs mutant cells compared to wild type or mus304 mutant cells. (H) The average fluorescence intensity of HOAP staining at all telomeres from wild type and mutant cells. Error bars indicate the standard error of the mean.

DNA damage response pathways are required for epigenetic protection of telomeres. In many organisms, telomere-specific sequences are required to recruit proteins that prevent chromosome end fusion. Loss of telomere-specific sequences in cells that do not express telomerase or that are mutant for DNA damage response genes can result in telomere fusions. However, in Drosophila, the stable protection of terminally deleted chromosomes from telomere fusion suggests that a sequence-independent mechanism acts to protect the telomeres of normal chromosomes (Mason et al. 1984; Biessmann and Mason 1988; Tower et al. 1993; Mason et al. 1997; Ahmad and Golic 1998). Given the requirement of the tefu and mei-41-mus304 DNA damage response pathways for telomere protection, we propose that recognition of chromosome ends contributes to this epigenetic phenomenon. One prediction of this model is that cells lacking these pathways will exhibit telomere fusion without loss of telomeric DNA sequences such as HeT-A. HeT-A sequences should not be lost simply as a secondary effect of unprotected telomeres since telomere fusions in cells lacking HP1 function still retain these sequences (Fanti et al. 1998). A second prediction is that terminal deletion chromosomes lacking telomeric sequences will still fuse in the absence of the DNA damage response pathways. This observation would rule out the possibility that the epigenetic mechanism for protection of terminal deletions utilizes an alternative mechanism to recruit HP1 and HOAP that is independent of the DNA damage response pathways. These predictions can be evaluated in animals with the extreme telomere fusion phenotype associated with loss of both the tefu and mei-41-mus304 DNA damage response pathways.

To test the first prediction, the telomere-specific retrotransposon *HeT-A* was analyzed at individual telomeres of DNA damage response defective cells by fluorescence in situ hybridization. Measurements of fluorescence intensity can be used to demonstrate that *HeT-A* levels at individual telomeres are reproducibly increased or decreased in different genotypes. (However, we note that there may not be a linear relationship between the percent change of fluorescence observed and the percent change of telomeric *HeT-A* DNA.) Previously, telomere fusions in *tefu* mutant cells were shown to retain at least some *HeT-A* sequences (Bi et al. 2004; Oikemus et al. 2004). However, these studies only examined mutants with mild telomere fusion phenotypes and were less thorough than the analysis presented here. Because the number of *HeT-A* copies per telomere can vary between strains, particularly in strains with altered HP1 function (Savitsky et al. 2002), *HeT-A* signals at free chromosome ends in homozygous mutant animals were compared to chromosome fusion sites in the same cells and to free chromosome ends in an appropriate heterozygous parental strain (Figure 5, Table 2). *HeT-A* is still present at free telomeres and at chromosome fusion sites in *tefu, nbs, tefu mus304,* and *nbs mus304* homozygous mutant cells (Figure 3.7A–3.7F and Table 3.4). For each genotype, both the frequency and intensity of *HeT-A* staining at chromosome fusions is equal to or greater than that observed at the free chromosome ends (Table 3.4 and Figure 3.7G), indicating that loss of telomere-specific sequences does not correlate with telomere fusion in cells with defective DNA damage response pathways. Note that if a *HeT-A*–positive telomere fuses with another *HeT-A*–positive telomere, the intensity of

staining will increase; if it fuses with a *HeT-A*–negative telomere or a chromosome break, the intensity should be the same.

Different genotypes exhibit different relative intensities of *HeT-A* staining at chromosome fusions compared to free ends (Figure 3.7G). These differences may reflect different frequencies of telomere–telomere fusions versus telomere–break fusions or differences in the precise mechanism of telomere fusion. Nonetheless, the observation that the staining intensity at fusions is equal to or greater than the intensity at free chromosome ends demonstrates that loss of these sequences is not required for fusion in any of these genotypes.

Table 3.3. Percent chromosomes with HeT-A staining

Genotype	% Chromosome ends with HeT-A	% Chromosome fusions with HeT-A	% Chromosome ends with internal HeT-A signals
w^-	48.7 (n=624)	-	-
mus304⁻/ TM6	94.8 (n=211)	-	-
mus304⁻	91.0 (n=486)	-	-
tefu⁻/ TM6	40.4 (n=324)	-	-
tefu⁻ [(a)]	35.3 (n=241)	40.0 (n=58)	1.7 (n=241)
nbs⁻/TM6	88.8 (n=260)	-	-
nbs⁻	62.8 (n=290)	89.3 (n=56)	2.0 (n=290)
tefu⁻ mus304⁻/TM6	95.4 (n=518)	-	-
tefu⁻ mus304⁻	53.2 (n=111)	86.3 (n=80)	28.8 (n=111)
nbs⁻ mus304⁻/ TM6	95.5 (n=312)	-	-
nbs⁻ mus304 [(b)]	67.7 (n=198)	88.9 (n=54)	6.1 (n=198)

n= number of telomeres analyzed

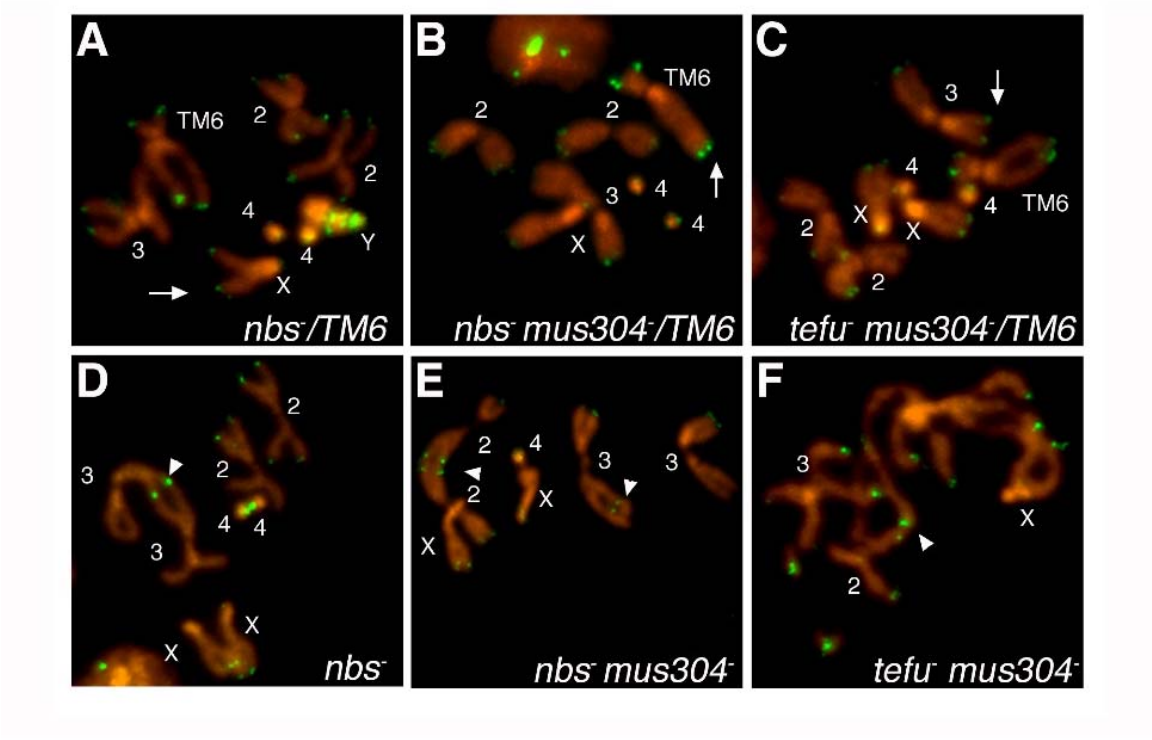
[a] $tefu^{1}$ / *Df(3R)PG4*

[b] $tefu^{\Delta 356}$ $mus304^{D2}$

The frequency and intensity of *HeT-A* staining was also compared at the free chromosome ends of mutant cells and the corresponding parental strain. The frequency of *HeT-A* staining at chromosome ends in homozygous *nbs, nbs mus304,* and *tefu mus304* mutant cells is lower than in cells from the corresponding heterozygous strains (Table 3.4). Although this decrease could reflect removal of telomeric sequences in homozygous mutant animals, two other factors are likely to contribute. First, defective DNA repair generates chromosome ends without telomeric sequences. As demonstrated above (Figure 3.5), several of these mutations result in high levels of spontaneous breaks. Second, progression of cells with telomere fusions through mitosis generates anaphase bridges and chromosome breaks via the fusion/bridge/break cycle. In one example (Figure 3.7E, arrowhead), an internal site of *HeT-A* (the original fusion site) is adjacent to a chromosome end without *HeT-A* (the break site). Chromosome ends with adjacent internal *HeT-A* sites are found in all mutant cells with telomere fusions (Table 3.4). The overall frequency of breaks resulting from fusion is underestimated by this analysis since some broken chromosomes will not include the original fusion site. Thus, chromosome breaks can account for the increased number of ends without *HeT-A* staining. However, at those chromosome ends that are *HeT-A* positive, the intensity of staining is equal to or greater than in the corresponding heterozygous cells (Figure 3.7G). Combined with the analysis of *HeT-A* staining at fusion sites described above, these results indicate that the fusion phenotype of single or double mutants in the DNA damage response pathways is not due to loss of telomeric sequences.

A second prediction of the end-recognition model for *Drosophila* telomere protection is that both normal and terminally deleted chromosomes will exhibit similar frequencies of fusion in cells lacking the DNA damage response pathways. The stable protection of terminally deleted chromosomes in wild-type cells suggests that the telomeres of normal chromosomes are also protected by sequence-independent mechanism; however, it is also possible that terminally deleted chromosomes acquire an alternative mechanism for telomere protection, and that the DNA damage response pathways must act in conjunction with a sequence-specific mechanism. To address this possibility, we examined fusion rates of a normal and a terminally deleted X chromosome in *tefu mus304* double mutant cells. Previous experiments have demonstrated that the telomere protection gene *UbcD1* is required to prevent fusion of terminally deleted chromosomes (Cenci et al. 2003a). In *tefu mus304* double mutant cells, a normal and a terminally deleted X chromosome fused to the sister or to heterologous chromosomes at a high frequency (Figure 3.8). The fusion frequency is similar, but lower, with a normal X chromosome ($p = 0.019$, two-tailed Fisher Exact Test); this difference may indicate that the terminally deleted chromosome is slightly less sensitive to the loss of DNA damage signaling pathways. Nonetheless, the frequent fusion of terminally deleted chromosomes in *tefu mus304* double mutant cells directly demonstrates that the DNA damage response pathways act in an epigenetic mechanism for telomere protection.

Figure 3.7


A
TM6
2
2
4
3
4
Y
X
nbs-/TM6
B
TM6
2
2
3
4
4
X
nbs- mus304-/TM6
C
3
4
4
TM6
2
X
X
2
tefu- mus304-/TM6
D
2
3
2
4
4
3
X
X
nbs-
E
2
4
2
X
3
3
X
nbs- mus304-
F
3
X
2
tefu- mus304-

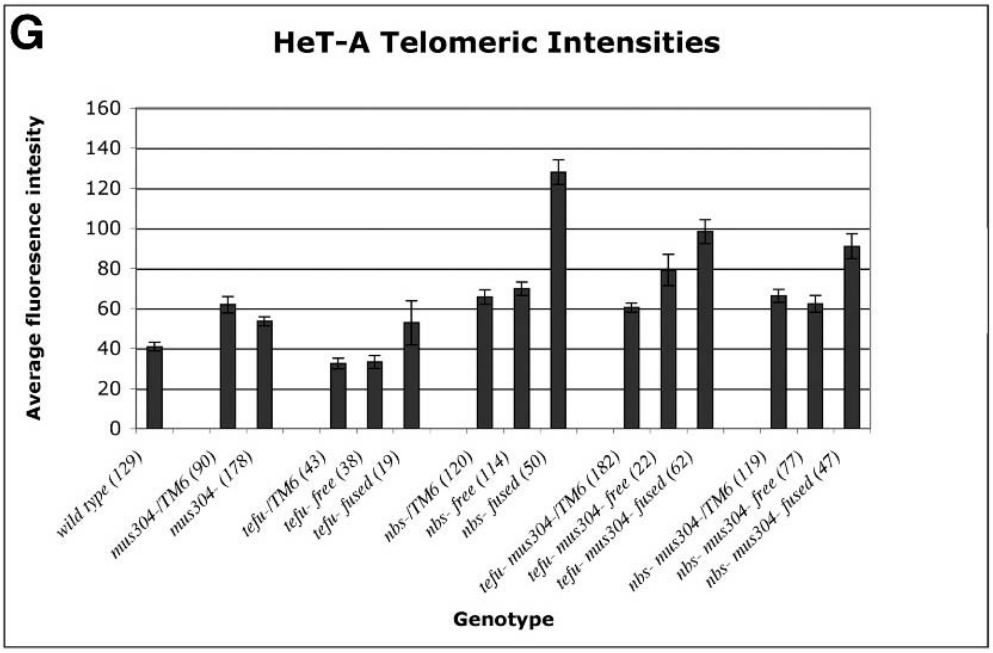
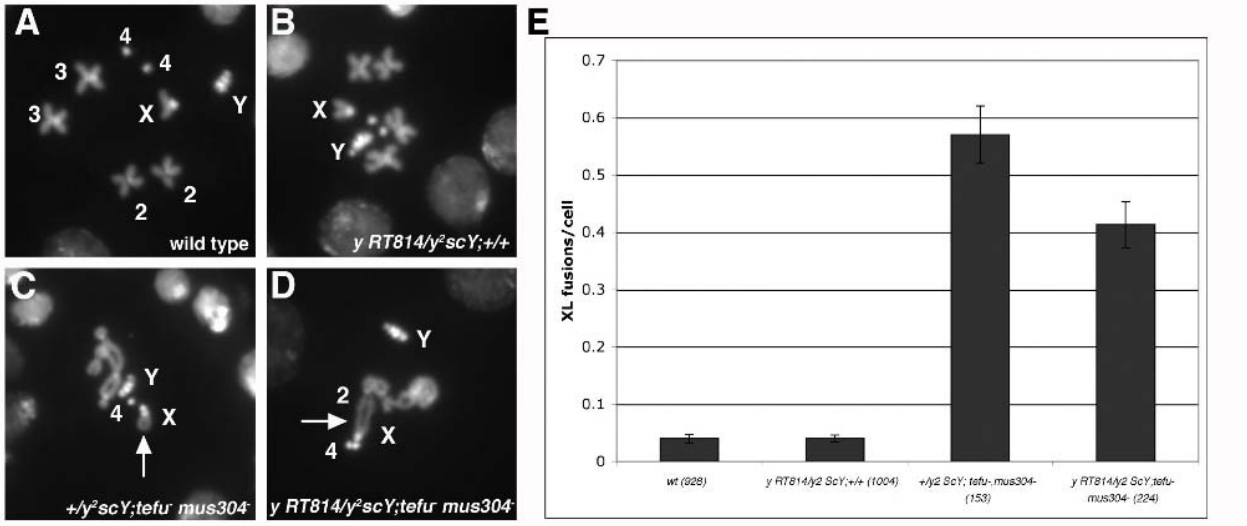
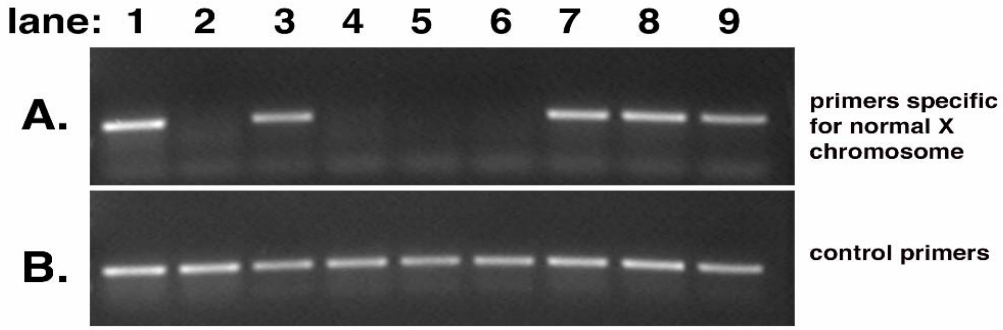

G
HeT-A Telomeric Intensities
Average fluoresence intesity
160
140
120
100
80
60
40
20
0
wild type (129)
mus304-/TM6 (90)
mus304- (178)
tefu-/TM6 (43)
tefu- free (38)
tefu- fused (19)
nbs-/TM6 (120)
nbs- free (114)
nbs- fused (50)
tefu- mus304-/TM6 (182)
tefu- mus304- free (22)
tefu- mus304- fused (62)
nbs- mus304-/TM6 (119)
nbs- mus304- free (77)
nbs- mus304- fused (47)
Genotype

Figure 3.7. Loss of telomeric sequences is not required for telomere fusions in cells lacking the *tefu* and *mei-41-mus304* DNA damage response pathways. (A–F) Fluorescent in situ hybridization of mitotic chromosomes from third-instar larval brains with a *HeT-A* probe. Telomeric *HeT-A* sequences are present at the free ends (arrows) and telomere fusion sites (arrowheads) of nbs^{1} (D) nbs^{1} $mus304^{D2}$ / nbs^{1} $mus304^{D1}$ (E) and $tefu^{1}$ $mus304^{D2}$ (F) homozygous mutant chromosomes. Similar levels of *HeT-A* hybridization are seen at the telomeres of the corresponding heterozygous chromosomes (A), (B), and (C). Internal sites of *HeT-A* adjacent to a chromosome end mark sites of telomere fusion followed by chromosome breakage ([E], Chromosome 3, arrowhead). Pairs of internal *HeT-A* sites mark sites where breakage was followed by a second fusion event ([E], Chromosome 2, arrowhead). The frequency of *HeT-A* positive telomeres is shown in Table 3.4. The labels TM6, X, Y, 2, 3, and 4 refer to the relevant chromosome. (G) The average fluorescence intensity of *HeT-A* signal at both free ends and fused telomeres of homozygous and heterozygous mutant animals was determined following fluorescent in situ hybridization. The fluorescence intensity of *HeT-A* signals is similar or greater in homozygous mutant cells compared with the corresponding heterozygous cell. For a given genotype, the fluorescence intensity of *HeT-A* signals is similar or greater at sites of chromosome fusions compared to free chromosome ends. Error bars indicate the standard error of the mean. The number of telomeres scored for each genotype is indicated in parenthesis.

Figure 3.8





lane 1: wild type
lane 2: *yRT814 / y² scY*
lanes 3-9: Identification of individuals with normal (3, 7-9) or terminally deleted (4-6) X chromosome

Figure 3.8. The *Drosophila tefu* and *mei-41-mus304* pathways act in an epigenetic telomere protection mechanism. Normal (A) and terminally deleted (B) X chromosomes are not fused in wild-type cells. Normal (C) and terminally deleted X chromosomes (D) are fused in *tefu mus304* double mutant cells. Both sister chromosome fusions (C) and non-sister fusions (D) are observed. High frequencies of X chromosome telomere fusions per cell are observed for normal and terminally deleted chromosomes in *tefu mus304* mutant cells (E). Error bars indicate the standard error of the mean. The number of cells scored for each genotype is in parenthesis. The labels X, Y, 2, 3, and 4 refer to the relevant chromosome. PCR analysis using primers specific for the wild-type *yellow* (y^+) gene (A) and control primers specific for both y^+ and y^2, an allele with a transposon insertion in the *yellow* gene (B) Genomic DNA was isolated from a w^{1118} male (wild-type control, lane 1) and from a *yRT814* / y^2 *sc Y* male (lane 2) that carries a terminally deleted X chromosome and a Y chromosome carrying a duplication of y^2 (lane 2). PCR analysis of genomic DNA isolated from individual *tefu mus304* male larvae from the cross, *yRT814*$^{/+}$*; tefu mus304*$^{D2/+}$ females crossed to $^{+/}y^2$ *sc Y; tefu mus304D1*$^{/+}$ males, identified mutants carrying the terminally deleted X chromosome (lanes 4, 5, and 6) and from those carrying a wild-type X chromosome (lanes 3, 7, 8, and 9).

Concluding Remarks

DNA damage response genes have evolutionarily conserved roles in telomere function. Unprotected telomeres are recognized by these pathways and elicit a variety of cellular responses including apoptosis and end-to-end fusion of chromosomes. However, these same pathways are also required to promote telomere protection. We demonstrate that the *Drosophila ATM* and *ATR-ATRIP* DNA damage response pathways act in an epigenetic mechanism to mediate telomere protection. Cells lacking both pathways fail to recruit the chromatin-associated protein HOAP to telomeres, and both normal and terminally deleted chromosomes undergo fusion at a high frequency. Furthermore, fusion of normal telomeres occurs without loss of telomere-specific sequences. Taken together, these results support an end-recognition model in which DNA damage response proteins recognize a DNA structure at the chromosome end, and recruit or stabilize the telomere protection proteins HP1 and HOAP at telomeres; in turn, these proteins act to prevent the ligation of chromosome ends by DNA repair enzymes and the activation of p53-dependent apoptosis (Figure 3.9). In other organisms, a similar epigenetic mechanism may act in conjunction with sequence-specific protection mechanisms or may be utilized to promote protection of critically short telomeres, which are least able to utilize sequence-specific binding proteins.

Figure 3.9

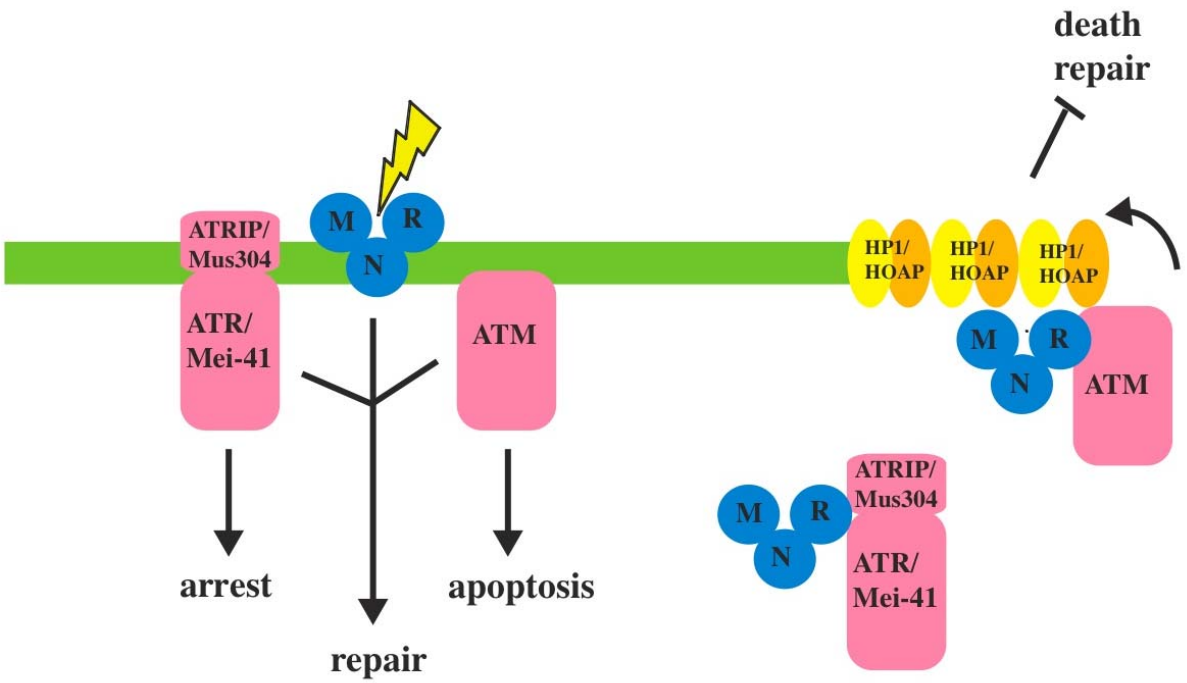


Figure 3.9. Model. The *Drosophila* DNA damage response proteins function at sites of double strand breaks to induce cell cycle arrest, DNA repair, and apoptosis. At telomeres Drosophila DNA damage response proteins function to recruit and/or maintain the telomere protection proteins, HP1 and HOAP in order to prevent ligation of telomere ends and activation of p53-dependent apoptosis.

Materials and Methods

Drosophila strains and crosses. All animals were raised at 25 °C. Mutations in *nbs* were identified from a collection of lethal mutations in the cytological region 67A-D (Leicht and Bonner 1988). To identify sequence alterations, genomic DNA from animals homozygous for $l(3)67BDp^{1}$ and $l(3)67BDr^{1}$ was amplified by PCR and sequenced. An *nbs* rescue construct was created by amplifying a genomic fragment containing the *nbs* transcript and 377 bp of upstream sequence and 102 bp of downstream sequence using the following primers: 5' GGCCAGATCTGGTCAGGTGAGACATGGGTTAC 3' and 5' GGCCGGTACCAGGAAACTGAATCCTCCTCC 3'. The genomic fragment was cloned into the BglI and KpnI sites of the pUAST vector (Brand and Perrimon 1993). Flies carrying the P[UAS-*nbs*] rescue transgene were created by P-element–mediated germline transformation. Transgene rescue was tested by crossing $^{+/+}$; $nbs^{1}/TM6BTb$ females to P[nbs^{+}]/*CyO;Df(3L)Ac1/TM6BTb* males and scoring non-balancer animals.

The w^{1118} strain was used as the wild-type stock. Where alleles are not otherwise indicated, the following alleles or allelic combinations were used: $mei\text{-}41^{D3}$; mnk^{p6}; grp^{fs1}; $mre11^{\Delta}$; $tefu^{\Delta 356}/tefu^{1}$; nbs^{1}; and $mus304^{D1}/mus304^{D2}$. Alleles are described in the text or at http://www.flybase.org. Double mutants were created by standard genetic crosses and confirmed by complementation analysis or PCR.

The terminally deleted X chromosome *y RT814* was originally generated by Dr. Jim Mason and was obtained from Dr. Maurizio Gatti. The deleted region includes the *yellow (y)* gene and all genes distal to it. In situ hybridization of polytene chromosomes

with *HeT-A* and *TART* probes was performed to confirm that the *y RT814* chromosome did not terminate with a retrotransposon. To examine this chromosome in *tefu mus304* double mutant cells, *y RT814*$^{/+}$*; tefu*$^{\Delta 356}$ *mus304*$^{D2/+}$ females were crossed to $^{+/}y^{2}$ *scY; tefu*$^{-}$ *mus304*$^{D1/+}$ males. Individual larval males carrying the terminally deleted X chromosome were identified by PCR analysis of the *yellow* gene using the following primers, which flank the *gypsy* transposon insertion in the y^{2} allele: 5' ATTGTGAATCATCGGTGACG 3' and 5' CATGCAGACAAAAATCCAGAAA 3'. Males with the deletion chromosome do not produce a PCR product because the X chromosome deletion removes the *y* gene and the Y chromosome carries the y^{2} allele (Figure S3A). A second pair of primers to a different region of the *y* gene were used as a positive control to amplify a product in animals with either a normal or terminally deleted X chromosome (Figure S3B): 5' CATGCAGACAAAAATCCAGAAA 3' and 5' ATTGTGAATCATCGGTGACG 3'. *tefu*$^{-}$ *mus304*$^{-}$ homozygous animals were identified by their small imaginal disc size and confirmed by their chromosome fusion phenotype. In all cases, the disc size and chromosome fusion phenotype matched. The high frequency of fusions and small disc size was confirmed to be specific for brains homozygous for *tefu*$^{-}$ *mus304*$^{-}$ and not for brains homozygous for *tefu*$^{-}$ and heterozygous for *mus304* (unpublished data).

Apoptosis and checkpoint assay. Late third-instar larvae were treated with 4,000 rads in a Faxitron RX650 (Faxitron X-ray Corporation, Illinois). Apoptotic cells were detected in wing imaginal discs 4 h after irradiation as described previously (Oikemus et al. 2004). Mitotic cells were visualized using a phospho-histone H3 antibody (Upstate

Biotechnology). Fixation and staining were performed as previously described (Brodsky et al. 2000b). The number of mitotic cells per wing disc was determined by flattening mounted wing imaginal discs and counting the number of phospho-H3–positive cells. The number of mitotic cells at each X-ray dose was normalized to the average number of mitotic cells in untreated discs. At least five discs were analyzed for each genotype and dose.

Fusion and break analysis. Late third-instar larvae were treated with 100 rads in a Faxitron RX650 using an aluminum shield to block lower energy wavelengths. Larval brains were dissected 2.5 h following irradiation, and chromosome spreads were prepared as described previously (Queiroz-Machado et al. 2001). Spontaneous and irradiation-induced breaks were quantified by counting chromatid and isochromatid breaks with acentric fragments**.**

Immunostaining of mitotic cells. Mitotic chromosomes were stained with a rabbit polyclonal anti-HOAP antibody (gift of R. Kellum, University of Kentucky). The HOAP antibody (1:200 dilution in 10% FBS, 1× PBS, 0.1% Tween) was pre-absorbed with fixed embryos overnight at 4 ºC. Neuroblast squashes were prepared as described (Queiroz-Machado et al. 2001) with the following changes: After hypotonic treatment, larval brains were fixed sequentially in formaldehyde solution (2% formaldehyde, 2% triton, 1× PBS) for 1 min and then in acetic acid/formaldehyde solution (2% formaldehyde, 45% glacial acetic acid in water) for 6 min. Slides were washed in PBST and incubated for 1 h at

room temperature in blocking solution (10% FBS, 1× PBS, 0.1% Tween). Slides were incubated with anti-HOAP antibody overnight at 4 ºC, then rinsed twice in PBST for 15 min and incubated for 1 h at room temperature with secondary antibody (anti-rabbit-Alexa 488, [Vector Laboratories, Burlingame, California, United States, diluted 1/2,000 in blocking solution). Finally, slides were washed twice in PBST for 15 min and mounted in Vectashield containing DAPI (Vector Laboratories). Chromosome preparations were observed using an Axiovert 200 Carl Zeiss microscope (Oberkochen, Germany), and mitotic figures were collected with the Axiovision 4.4 Zeiss software. Quantification of the HOAP fluorescence intensity was performed using the ImageJ software package (http://rsb.info.nih.gov/ij/).

Fluorescent in situ hybridization of *Drosophila* neuroblasts. In situ hybridization to mitotic chromosomes was performed as described (Carmena et al. 1993). *HeT-A* probe was labeled with Biotin-14-dUTP using the Bionick Translation System (GIBCO BRL, Rockville, Maryland, United States). Slides were mounted in Vectashield containing DAPI as a DNA counterstain (Vector Laboratories). Chromosomes were identified through their specific peri-centromeric banding pattern after DAPI staining. Chromosome preparations were observed using an Axiovert 200 Carl Zeiss Microscope, and mitotic figures were collected with the Axiovision 4.4 Zeiss software. Quantification of the *HeT-A* fluorescence intensity was performed using the ImageJ software package (http://rsb.info.nih.gov/ij/).

Accession Numbers. The National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) accession numbers for the genes and gene products discussed in this paper are ATM (472), ATR (545), ATRIP (11277), Chk1 (1111), Chk2 (11200), *grps* (34993), *mei-41* (32608), *mnk* (35288), *mus304* (40003), *nbs* (44259), NBS1 (4683), and *tefu* (41839).

CHAPTER IV

FUTURE DIRECTIONS

Analyses of specific experiments and results were given in the results and discussion sections of Chapters 2 and 3. Therefore, this chapter will focus on the future directions of the *Drosophila* telomere field and highlight specific questions that developed directly from the work presented in this thesis.

Which Proteins Mediate the Fusion of Unprotected Telomeres in *Drosophila*?

Which proteins mediate the fusion of unprotected telomeres in *Drosophila* still remains an unanswered question. NHEJ repair enzymes mediate fusion of unprotected telomeres in yeast and mammals (Smogorzewska et al. 2002; Miller et al. 2005; Pardo and Marcand 2005; Celli et al. 2006). Therefore, NHEJ repair proteins are likely candidates to mediate the ligation of unprotected telomeres in *Drosophila*.

Components of the NHEJ and HR repair pathways are conserved in *Drosophila* (Sekelsky et al. 2000). Studies in flies can take advantage of several unique techniques that permit targeted induction of a single double strand break (Romeijn et al. 2005; Beumer et al. 2006; Preston et al. 2006). Studies using these techniques have revealed some similarities and differences in *Drosophila* repair mechanisms compared to yeast and mammals. In addition these techniques allow the contribution of each repair pathway to be analyzed during different developmental stages and in the presence of defects in repair genes.

Similar to yeast, *Drosophila* do not have a DNA-PKcs ortholog, however they do have both subunits of the Ku complex, Ku70 and Ku80 and a ligase IV ortholog

(Sekelsky et al. 2000). Both Ku70 and Ku80 expression is regulated in response to irradiation in a p53-dependent manner (Brodsky et al. 2003), however, the exact contribution of the *Drosophila* Ku complex in DNA repair has not been extensively studied. A role for *Drosophila* Ku in the regulation of telomere elongation has been described using heterozygous *Ku* deficiencies (Melnikova et al. 2005). To date a role for Ku in *Drosophila* telomere protection has not been described. However, if the role of *Drosophila* Ku is similar to mammalian Ku then it is likely to contribute to both the telomere fusion event and telomere protection.

In mammals Ku70 plays a dual role in both protection of telomeres and in the joining of unprotected telomeres. Mutations in Ku70 result in a low frequency of fusions indicating a role in telomere protection (Bailey et al. 1999; Hsu et al. 2000). Furthermore, simultaneous induction of telomere fusions by disruption of TRF2 and deletion of Ku70 results in a decrease in telomere fusions (Celli et al. 2006), indicating a role for Ku70 in the fusion event of unprotected telomeres. Therefore, it will be interesting to determine whether *Drosophila* Ku suppresses the fusion phenotype of DNA damage response mutants and HP1 and HOAP mutants. However, a lack of established Ku mutants has hindered the testing of this hypothesis.

Several *Drosophila* ligase IV mutants have been described (Gorski et al. 2003; McVey et al. 2004). Interestingly, NHEJ in *Drosophila* is largely independent of DNA ligase IV. Ligase IV mutants are viable and exhibit a minor sensitivity to X-irradiation during embryogenesis compared to wild type animals. Studies by McVey *et al* have demonstrated that ligase IV is not required for the repair of double strand breaks induced

by p-element excision even in the absence of a functio nal homologous recombination repair pathway (McVey et al. 2004). Therefore, unlike yeast, even when the homologous recombination repair pathway is inactivated, ligase IV mutations do not appear to have a significant affect on repair by NHEJ.

The above findings suggest that *Drosophila* may have an alternative end joining mechanism that is not dependent on ligase IV or there may be an unidentified ligase that contributes to NHEJ. Recent studies have demonstrated that ligase IV may have a more significant role in the repair of double strand breaks in somatic cells (Romeijn et al. 2005). Consistent with these findings mutations in ligase IV can suppress the *atm* fusion frequency by 20% (Bi et al. 2004). This suggests that in *Drosophila* fusion of unprotected telomeres is partially dependent on ligase IV. What proteins are responsible for the ligase IV independent fusion events?

Preliminary results suggest that Mu2 and p53 may contribute to the fusion of unprotected telomeres (Brodsky Lab unpublished results). Analysis of larval brain cells from *atm p53* and *atm mu2* double mutants revealed a decrease in the frequency of fusions compared to *atm* single mutants. These results suggest a function for p53 and Mu2 in the fusion of unprotected telomeres. *mu2* mutations facilitate the recovery of terminal deficiencies at a high frequency indicating that these telomeres have acquired a new telomere (discussed in the Introduction) (Mason et al. 1984). Therefore, the normal function of Mu2 is to prevent the establishment of new telomeres, possibly at the sites of double strand breaks. A role for Mu2 in DNA repair in oocytes has been suggested (Mason et al. 1997).

Sequence analysis of the Mu2 protein revealed two c-terminal BRCT domains. BRCT domains bind phospho-proteins and are found predominantly in proteins involved in the DNA damage response (Manke et al. 2003; Yu et al. 2003). Preliminary sequence comparisons suggest that Mu2 is most similar to the mammalian 53BP1 (p53 binding protein 1) protein (Brodsky Lab, unpublished). 53BP1 was identified in a screen designed to uncover proteins that interact with p53 (Iwabuchi et al. 1994). In mammals 53BP1 localizes to H2AX containing damage-induced foci and plays a role in both the S phase and G2/M cell cycle checkpoints (Schultz et al. 2000; Rappold et al. 2001; Ward et al. 2003). 53BP1 is recruited to sites of DNA damage via an interaction with methylated lysine 79 of histone H3 (Huyen et al. 2004).

Interestingly a role for 53BP1 in NHEJ has recently been described. Nakamura et al. demonstrates that 53BP1 facilitates non-homologous end joining in chicken DT40 cells (Nakamura et al. 2006). If Mu2 plays a similar role in *Drosophila* then it is possible that the decrease in fusion frequency in *atm mu2* double mutants is a direct result of inactivation of NHEJ. Analysis of *lig4 atm mu2* triple mutants should reveal if *mu2* and *lig4* are acting in a similar pathway. However *mu2* mutations do not completely rescue the fusion phenotype suggesting that mutations in *mu2* only partially disrupt NHEJ. It is possible that another pathway contributes to the fusion of unprotected telomeres. It may be necessary to simultaneous disrupt several components of the NHEJ pathway in order to observe complete suppression of fusions at unprotected telomeres.

Preliminary results also demonstrated that mutations in *p53* could suppress the fusions in an *atm* mutant. Mutations in p53 can suppress the spontaneous apoptosis in

both *atm* and *nbs* mutants (Oikemus et al. 2004; Song et al. 2004; Oikemus et al. 2006). If Mu2 is the 53BP1 homolog it is logical to propose that Mu2 and p53 may cooperate in the fusion of unprotected telomeres. Analysis of more complex genotypes will provide valuable information for determining the exact roles of p53 and Mu2 in the mechanism of telomere-telomere ligation.

Drosophila appear to be more similar to yeast in the sense that HR appears to be the primary repair mechanism. Several genes belonging to the Rad52 epistasis group have been identified and studied in *Drosophila* and include Rad51, Rad54, Rad51D SpnB, SpnD, XRCC2 and the MRN complex (Sekelsky et al. 2000). However, in *S. cerevisiae* the MRN complex does contribute to repair by NHEJ (Daley et al. 2005). It is likely that *Drosophila* MRN also plays a role in the NHEJ and ligation of unprotected. Mutations in both *nbs* and *mre11* result in increased frequency of chromosome breaks and a lower frequency of telomere fusions compared to *atm* mutants (Bi et al. 2004; Ciapponi et al. 2004; Oikemus et al. 2004; Ciapponi et al. 2006; Oikemus et al. 2006). However, both *nbs* and *mre11* mutant telomeres have less telomeric HOAP compared to *atm* mutants (Bi et al. 2004; Ciapponi et al. 2004; Oikemus et al. 2004; Ciapponi et al. 2006; Oikemus et al. 2006). Taken together these results suggest that the *Drosophila* MRN complex functions in both telomere protection and in ligation of unprotected telomeres. Again, disruption of multiple repair proteins may be required to uncover the exact contributions of each protein.

Telomeric Localization of *Drosophila* DNA Damage Response Protein

It is not yet known if *Drosophila* DNA damage response proteins are stable components of the telomere cap complex or if they only transiently associate with exposed or critically short telomeres. In yeast, the telomeric association of ATM and ATR is cell cycle dependent (Takata et al. 2004). In human telomerase negative cells ATM, MRE11, and phosphorylated NBS1 associate with telomeres during late S and G2 when POT1 association with telomeres is at it's lowest and telomeres are more accessible (Verdun et al. 2005). In a separate study, MRE11 and RAD50 were demonstrated to associate with telomeres throughout the cell cycle while NBS1 was only observed during S phase (Zhu et al. 2000). Furthermore, inhibition of ATM and the MRN complex in the telomerase negative cells resulted in telomere dysfunction in the form of telomere-telomere fusions (Verdun et al. 2005). Verdun et al propose that the association of DNA damage response proteins with telomeres during G2 is necessary for proper telomere processing and formation of the t-loop. However, they do not show directly that the activity of these proteins is responsible for 3'overhang or t-loop formation.

Although a 3'overhang and t-loop structure has not been demonstrated for *Drosophila* telomeres, damage response proteins may still be required during specific phases of the cell cycle and for telomere end processing (or for modification of telomeric heterochromatin). The low expression of most DNA damage response proteins and lack of decent *Drosophila* antibodies against many of the DNA damage response proteins has

hampered progress on these studies. However, the creation of GFP tagged versions of these proteins would help to answer these questions.

In addition to the role of ATM and other DNA damage response proteins in the maintenance of normal telomeres, what about their role at unprotected telomeres? The role of *Drosophila* DNA damage response proteins at unprotected telomeres is still unclear. However, with sufficient antibodies the association of these proteins with telomeres in HOAP and HP1 mutants could be determined.

Telomeric Structure

The terminal structure of *Drosophila* telomeres is till a mystery. No higher order DNA structures similar to the t-loop have been formally described, although Perrini et al have reported observing a structure similar to the t-loop at telomeres of polytene chromosomes (de Lange 2006). Determining the chromatin modifications that are present at unprotected telomeres versus normal telomeres may provide clues to the terminal structure that is required to protect telomeres. Recent studies using indirect immunofluorescence to observe chromatin modifications at normal telomeres suggest that there are three distinct chromatin domains at *Drosophila* telomeres, the cap, the telomeric retrotransposons, and TAS (Andreyeva et al. 2005). These domains differ in histone modifications and chromatin-associated proteins. A more sensitive method, such as chromatin immunoprecipitation may be required to compare the differences in modifications directly at the chromosome terminus.

Recent studies have demonstrated that *Drosophila* telomeres are enriched for trimethylated lysine 9 of histone H3 (Cowell et al. 2002). However, mutations in the methyl transferase required for lysine 9 methylation do not result in a telomere fusion phenotype suggesting this modification is not important for telomere protection (Perrini et al. 2004). It also may be possible that *Drosophila* have another methyl transferase responsible for the methylation of meK9H3 at telomeres and required for telomere protection. In support of this a recent study by Bi *et al*.demonstrate that lysine 9 methylation is diminished at the telomeres of *atm* and *atm,atr* mutants compared to wild type (Bi et al. 2005b). The question then becomes how does *atm* contribute to histone modifications at telomeres (see section of ATM/ATR targets)?

In addition to meK9H3 another possible modification that could play a role in telomeric structure is methylation of lysine 79 of histone H3. In mammals this modification is required for the recruitment of 53BP1 to sites of double strand breaks (Huyen et al. 2004). The Grappa protein is the methylase responsible for this modification in *Drosophila* (Shanower et al. 2005). Initial studies indicate that *grappa* mutations can dominantly suppress silencing of transgenes inserted into telomeric heterochromatin but have no effect on silencing of transgenes inserted into centromeric heterochromatin. Furthermore, *grappa* mutants are pupal lethal and exhibit a rough eye phenotype (Shanower et al. 2005). This phenotype is similar to what is observed for *nbs* and *atm* mutants. Immunolocalization experiments demonstrated that methylation of lysine 79 of histone H3 was under represented at telomeres (Shanower et al. 2005). It is

possible is that this modification is indicative of unprotected telomeres or that a more sensitive detection method is required.

Another modification that may contribute to telomere maintenance is phosphorylation of histone H2A.v. Phosphorylation of the mammalian homolog of H2AX is known to occur at sites of double strand breaks and is one of the earliest events in the DNA damage response. In addition H2AX was observed at dysfunctional telomeres in mammalian cells (d'Adda di Fagagna et al. 2003; Takai et al. 2003). Additionally, ATM and ATR can phosphorylate H2AX. The ATM/ATR phosphorylation site is conserved in *Drosophila* H2Av (the *Drosophila* H2AX homolog) indicating a conserved function (Madigan et al. 2002). Moreover, Madigan *et al* demonstrated that H2Av is phosphorylated in response to DNA damage. H2Av mutants are pupal lethal and exhibit increased spontaneous apoptosis in imaginal tissue, a phenotype observed in DNA damage response mutants (Madigan et al. 2002). It is not known whether mutations in H2Av result in telomere fusions. Phosphorylation of H2Av at uncapped telomeres (HP1 or HOAP mutants) would indicate that a damage response similar to what is observed at dysfunctional mammalian telomeres also occurs at *Drosophila* telomeres. Phosphorylation of H2A.v could also be used as a direct readout for ATM and/or ATR activity at telomeres.

What are the ATM/ATR Telomere Targets?

ATM and ATR are kinases suggesting that a likely function at telomeres would involve phosphorylation of target proteins. However, it is possible that the telomeric function of these proteins does not require their kinase activity. In yeast the kinase activity of Mec1 (ATR) is required for it's telomeric association implicating it in a phosphorylation events at telomeres (Takata et al. 2004). The kinase activity of Tel1 is not required for its telomeric association but it is not known if the function of Tel1 at telomeres requires its kinase activity (Takata et al. 2004).

Results described in this thesis indicate that the telomeric targets of ATM and ATR are different from their DNA damage response targets. Mutations in the downstream checkpoint kinases Chk1(Grps) and Chk2(Mnk) did not result in telomere fusions indicating that they do not cooperate with ATM and ATR in the telomere protection pathway (Bi et al. 2005b; Oikemus et al. 2006). HP1 and HOAP are predictable telomeric targets of ATM/ATR. Sequence analysis of the HOAP revealed that it contains six ATM/ATR consensus phosphorylation sites (Bi et al. 2004). A recent study by Bi *et al* demonstrated that ATM does not phosphorylate HOAP in an in vitro kinase assay (Bi et al. 2005b). It is not clear whether HP1 contains any ATM/ATR consensus phosphorylation sites. However, it is still possible that ATM and ATR recruit HOAP and HP1 to telomeres through protein-protein interactions. In addition phosphorylation of other proteins by ATM and ATR may be required for the recruitment of HOAP and HP1 to telomeres.

Based on the end recognition model one plausible target of ATM and ATR are HDACs (histone deaceylases). In this model ATM and ATR would be required to recruit HDACs to telomeres, where they would be required for the creation of unmodified sites. The unmodified sites could then be methylated by Su(var)3-9 to create binding sites for HP1 and propagation of telomeric heterochromatin.

Supporting this hypothesis both mammalian ATM and ATR have been demonstrated to interact with HDACs. ATR was demonstrated to interact with HDAC2 (Schmidt and Schreiber 1999). Although the exact function of the interaction is not known several models have been proposed. ATM was demonstrated to interact with HDAC1; this interaction is increased following treatment with X-rays (Kim et al. 1999). Furthermore, studies in *Drosophila* have demonstrated that Su(var)3-9 can interact with HDAC1 and that deacetylation of histone H3 is required for efficient methylation by Su(var)3-9 (Czermin et al. 2001). Similar results were observed in mammalian studies (Vaute et al. 2002).

Drosophila encode five HDACs: HDAC1 (Rpd3), HDAC3, HDAC4, HDA6, and HDAC11. If HDACs are telomeric targets of ATM and ATR then a similar apoptotic and fusion phenotype would be expected in HDAC mutants. However, a phenotype may not be observed in single mutants due to compensation by one of the remaining HDACs. Treatment with HDAC inhibitors such as TSA (trichostatin A) may solve this issue however, not all *Drosophila* HDACs are sensitive to TSA (Barlow et al. 2001). Furthermore, if HDACs are targets of ATM and ATR and are required for recruiting HP1 and HOAP to telomeres then targeting HP1 to *atm* mutant telomeres should rescue the

telomeric fusion phenotype. This hypothesis could be tested using the HP1 tethering method described by Li *et al* (Li et al. 2003).

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