A product of the bicistronic *Drosophila melanogaster* gene *CG31241*, which also encodes a trimethylguanosine synthase, plays a role in telomere protection

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Summary

Although telomere formation occurs through a different mechanism in *Drosophila* compared with other organisms, telomere associations result from mutations in homologous genes, indicating the involvement of similar pathways in chromosome end protection. We report here that mutations of the *Drosophila melanogaster* gene *CG31241* lead to high frequency chromosome end fusions. *CG31241* is a bicistronic gene that encodes trimethylguanosine synthase (TGS1), which forms the m3G caps of noncoding small RNAs, and a novel protein, DTL. We show that although TGS1 has no role in telomere protection, DTL is localized at specific sites, including the ends of polytene chromosomes, and its loss results in telomere associations. Mutations of ATM- and Rad3-related (ATR) kinase suppress telomere fusions in the absence of DTL. Thus, genetic interactions place DTL in an ATR-related pathway in telomere protection. In contrast to ATR kinase, mutations of ATM (ataxia telangiectasia mutated) kinase, which acts in a partially overlapping pathway of telomere protection, do not suppress formation of telomere associations in the absence of DTL. Thus, uncovering the role of DTL will help to dissect the evolutionary conserved pathway(s) controlling ATM-ATR-related telomere protection.

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Introduction

Eukaryotic cells must cope with the problem of preserving chromosome ends and simultaneously eliminating free DNA ends arising from accidental DNA breaks. DNA double-stranded break (DSB) repair and chromosome end protection require seemingly opposing activities; nonetheless, the same pathways involving the ataxia telangiectasia mutated (ATM) and ATM and Rad3-related (ATR) kinases contribute to both processes (Bi et al., 2005; Ciapponi et al., 2006; Oikemus et al., 2006; Rong, 2008).

In contrast to most other eukaryotes, chromosome end protection in *Drosophila* takes place without a telomerase. In *Drosophila*, specific retrotransposon sequences accumulate at chromosome ends (Abad et al., 2004; Pardue and DeBaryshe, 2003), their presence, however, is neither necessary nor sufficient for end protection (Bi et al., 2004; Cenci et al., 2005; Fanti et al., 1998; Oikemus et al., 2004). Telomere associations in the fly arise from mutations affecting genes encoding the telomere-localized proteins HOAP (HP1/ORC-associated protein) and HP1 [encoded by *cav* and *Su(var)2-5*, respectively] (Cenci et al., 2003; Fanti et al., 1998), proteins involved in the ATM- and ATR-related pathways (including products of *tefu/atm*, *mre11*, *rad50*, *nbs1* and *mei-41/atr*, *mus-304*, respectively) (Bi et al., 2005; Bi et al., 2004; Ciapponi et al., 2004; Gorski et al., 2004; Oikemus et al., 2004; Silva et al., 2004; Song et al., 2004), and a few further genes, such as those encoding the ubiquitin-conjugating enzyme UbcD1 and the transcription factor Woc (Cenci et al., 2005; Cenci et al., 1997; Raffa et al., 2005). HOAP is the only *Drosophila* protein identified so far that is localized mostly at telomeres. However, the relationship between HOAP localization at chromosome ends and telomere protection is unclear, because although *cav*-null mutants by definition lack HOAP at chromosome ends and are fusigenic (Cenci et al., 2003), ATM-deficient mutants also have fusigenic telomeres despite the presence of HOAP at mitotic *tefu/atm* chromosome ends (Bi et al., 2004; Oikemus et al., 2006). Mutations affecting components of the ATR-related pathway themselves do not affect HOAP localization or induce telomere associations, whereas in combination with mutants of the ATM-associated pathways, they display a reduced level of HOAP at chromosome ends and significantly stronger telomere association phenotypes than *tefu/atm* mutants alone (Bi et al., 2005; Oikemus et al., 2006). Nonetheless, the involvement of the ATM and ATR kinases in telomere protection and DSB elimination is well documented in species as diverse as yeasts and mammals (Garber et al., 2005; Pandita, 2002; Vespa et al., 2005), suggesting a role for evolutionary conserved mechanisms for the protection of chromosome ends and genome integrity.

The *Drosophila melanogaster CG31241* gene is a complex transcription unit containing two open reading frames (ORFs) (Komonyi et al., 2005). The product of the downstream (d)ORF is the methyltransferase TGS1, which is involved in the formation of m3G cap of noncoding small RNAs. The 5′ region of the message

has an ORF with coding capacity for a protein of 178 amino acids, which we refer to here as DTL. Previously, we designated *CG31241* as *dtl* for *Drosophila* tat-like (Komonyi et al., 2005). Since the gene has two distinct products with different functions, we propose the use of *CG31241* when referring to the transcription unit and TGS1 and DTL for the products of the dORF and upstream (u)ORF, respectively. The DTL and TGS1 ORFs partially overlap. The unusual structure of the *CG31241* gene is also characteristic of the homologous genes of other *Drosophila* species and resembles the gene organization of retroviruses and retrotransposons.

We previously reported the identification of *Drosophila* TGS1 as a product of *CG31241* (Komonyi et al., 2005); here, we present evidence that DTL, another product of the same gene, has a function in the pathway controlling ATR-dependent chromosome end protection.

Results and Discussion

The product of the uORF of *CG31241* has a role in telomere protection

Mutants affecting both ORFs of *CG31241* are lethal, displaying a small disc phenotype, an elevated level of apoptosis, frequent telomere associations in larval neuroblasts and a lack of a TMG cap on snRNAs and snoRNAs (Fig. 1B-E) (Komonyi et al., 2005). To determine the contribution of the individual ORF products to the phenotype of *CG31241* mutants, we analyzed the rescue ability of transgenes encoding either the TGS1 or the DTL protein, or both proteins. Carriers of *P(967)*, *d192*, *d58* and *d189* alleles of *CG31241* (Fig. 1A) differ in respect to their lethal phases: *P(967)/d189* heterozygotes are pupa lethal, whereas *d58* and *d192* over *d189* result in lethality in larval phases. All phenotype features of each allele combination can be completely rescued by a transgene that carries the regulatory and coding regions of *CG31241* (Fig. 1A; Casper-DTL). These observations prove that the telomere associations and loss of TMG caps indeed result from the *CG31241* mutations, and suggest that *P(967)* is a hypomorph, whereas *d58*, *d192* and *d189* are null alleles of *CG31241*. A *CG31241* transgene with a deletion in the dORF (Fig. 1A; Casper-DTLd^{m}) did not change the lethality phases of *P(967)/d189* or *d192/d189* animals, and did not restore TMG cap formation, but in larval neuroblasts of transgene carriers there was no detectable increase in the level of telomere associations (Fig. 1E). By contrast, a *CG31241* transgene with stop codons inserted after the 150th amino acid codon

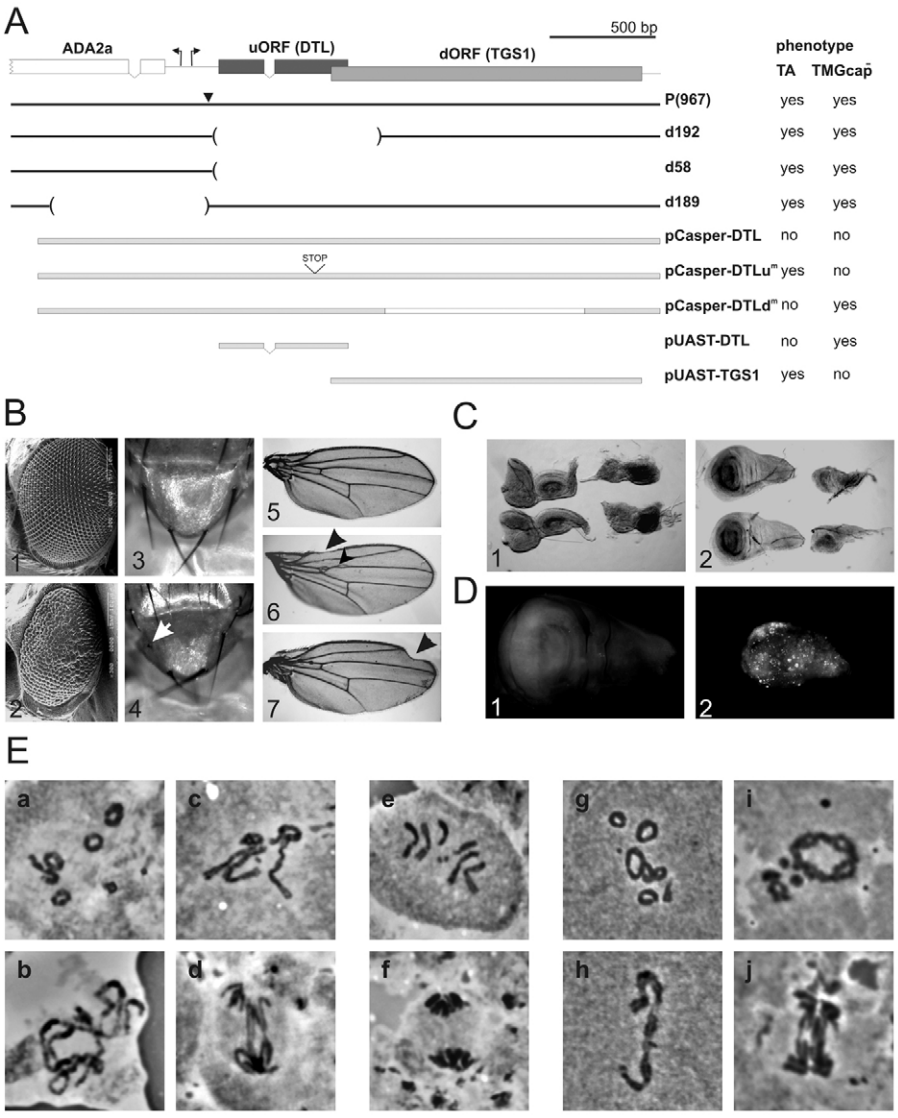

Fig. 1. (A) Structure of the *CG31241* gene, its alleles and transgenes with the associated phenotypes. Shaded boxes indicate the two ORFs of *CG31241* encoding DTL and TGS1. The gap in uORF(DTL) box indicates the position of an intron. *P(967)* is a P-element insertion in the 5′-untranslated region of *CG31241*. *d192*, *d58* and *d189* are null alleles for both DTL and TGS1 functions. Breaks enclosed by parentheses indicate the positions and extensions of deletions. Casper-DTL, Casper-DTLu^{m} and Casper-DTLd^{m} are transgenes that carry wild-type *CG31241*, a nonsense mutation in the DTL coding region and a deletion in the TGS1-coding region, respectively. pUAST-DTL and pUAST-TGS1 are cDNAs corresponding to the *CG31241* uORF and dORF, respectively. (B) Rough eye (2), missing thoracic bristles (4, arrow), abnormal development of veins (6, arrowheads), scalloped wings (7, arrowhead) are characteristic for animals carrying the Casper-DTLu^{m} transgene and the hypomorph *P(967) CG31241* allele. (1, 3 and 5 show corresponding controls). (C) In *CG31241*-null larvae, the size of imaginal discs (1, eye-antenna; 2, wing disc) is severely reduced compared with similar age controls (left, wild type; right, mutant). (D) Acridine orange staining of the imaginal discs indicates increased apoptosis in *CG31241*-null mutants. 1, wild type; 2, mutant wing disc. (E) Orcein-stained mitotic spreads of *CG31241* neuroblasts display telomere associations. Loss of both DTL and TGS1 functions in *d192/d189* cells (a-d), or DTL function in *P[DTLu^{m}]/+; d192/d189* cells (g-j), but not the loss of TGS1 function alone in *P[DTLd^{m}]/+; d192/d189* cells (e,f) causes defective mitotic chromosomes and frequent telomere associations.

in the uORF (Fig. 1A; Casper-DTLu^{m}) resulted in a delay in the lethality phase: *d192/d189 and P(967)/d189* animals carrying this transgene died at early pupa stage or hatched, respectively. Casper-DTLu^{m} carrier *P(967)/d189* adults eclosed and survived for a short time, exhibiting signs of locomotor defects and eye, wing and thoracic bristle abnormalities (Fig. 1B-D). We observed telomere associations in the larval neuroblasts of these animals (Fig. 1E); however, normal TMG caps were detectable on the snRNAs (data not shown) (Komonyi et al., 2005). *Act5*-driven expression of transgenes consisting of cDNA fragments corresponding to either the uORF or dORF of *CG31241* under the control of the UAST regulatory region provided rescue results identical to those described above; in short, the UAST-DTL transgene eliminated the telomere associations and the UAST-TGS1 transgene corrected the missing TMG cap phenotype. The two transgenes together completely rescued all *CG31241* mutant phenotypic features. Taken together, these data clearly show that the two ORFs of *CG31241* have distinct functions. The dORF of *CG31241* encodes TGS1, and the loss of methyltransferase activity results in larval lethality. The failure of rescuing *CG31241* mutants with the Casper-DTLu^{m} or the UAST-TGS1 transgene alone indicates that the uORF also carries an essential function. Mutations of the uORF result in small discs, an elevated level of apoptosis and frequent telomere associations, but have no effect on TMG cap formation. Since a transgene corresponding to an uORF cDNA completely rescued the telomere association phenotype of *CG31241* mutants, chromosome end fusions result solely from the loss of DTL protein encoded by the uORF.

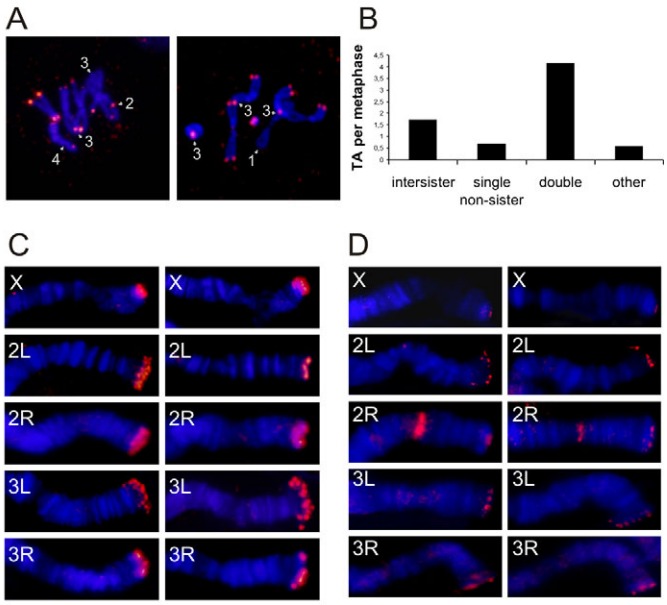

Fig. 2. HOAP and HP1 binding to *dtl* chromosomes and frequency of different telomere associations in *dtl* neuroblasts. (A) HOAP-specific staining of *CG31241* (*P[DTLu^{m}]/+; d58/d189*) mitotic chromosomes in larval neuroblasts. Chromosome end attachment types are indicated by arrows and numbers, indicating: intersister (1), single non-sister (2), double (3) and other (4) attachments. (B) Average frequencies for different types of telomere associations (TA) in *dtl*-null mutants (n>200). (C) Wild-type (left) and *CG31241* (*P[DTLu^{m}]/+; d58/d189*) (right) polytene chromosomes immunostained with HOAP-specific antibody (red) and DAPI (blue). (D) HP-1-specific staining of wild-type (left) and *CG31241* (*P[DTLu^{m}]/+; d58/d189*) (right) polytene chromosomes.

Characteristic mitotic figures observed in brain squashes of *CG31241* mutant third instar larvae are shown in Fig. 1E and Fig. 2A, and a comparison of the frequencies of different types of chromosome associations observed in *dtl* neuroblasts is shown in Fig. 2B. The most prominent cytological defects are chromosome end-to-end associations involving one, two or more chromosomes. Cells containing several circular chromosomes were frequently observed and often chromosomes were fused to form chromosome chains. Different chromosomes were involved at similar frequency in these abnormalities. Telomere associations, which include pairs of sister telomeres fused with another pair, were frequently observed. Chromosome bridges in anaphase cells indicate that the telomere associations present in *CG31241* mutants are not resolved in the late phases of mitosis, suggesting that they are DNA end fusions or strong protein-protein interactions.

The telomere-associated protein HOAP is present at fusigenic *CG31241* chromosome ends

In agreement with observations that the presence of specific sequences at chromosome ends does not ensure telomere protection in *Drosophila*, we demonstrated the presence of telomere-specific HeT-A transposon sequences at *CG31241* mutant chromosome ends by in situ hybridization (supplementary material Fig. S1).

Independently of telomeric DNA sequences nucleoprotein complexes containing the telomere-specific protein HOAP can assemble at *Drosophila* chromosome ends (Cenci et al., 2003). The gene encoding HOAP was identified by *caravaggio* (*cav*) mutations and it was found that approximately 99% of *cav* mutant metaphases contained chromosome end-to-end attachments (Cenci et al., 2003). We wondered therefore whether the DTL protein had a role in localizing the telomere-specific HOAP protein to chromosome ends. By immunostaining, we detected the presence of HOAP both at polytene and mitotic chromosome ends of *CG31241* mutants (Fig. 2A,C). Furthermore, HOAP-specific staining could be detected at mitotic chromosome fusions in *CG31241* mutant neuroblasts. Similarly, the localization of another telomere-associated factor HP1, whose absence also results in telomere associations (Fanti et al., 1998; Perrini et al., 2004), could be detected at least at polytene *CG31241* chromosome ends (Fig. 2D). A complete loss of either HOAP or HP1 from chromosome ends could not thus explain the telomere associations observed in *CG31241* mutant cells. To

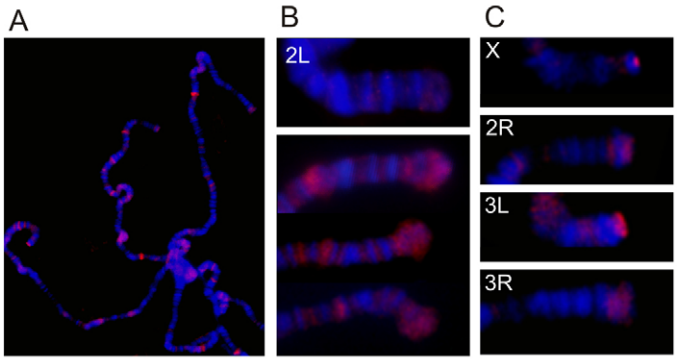

Fig. 3. Stained polytene chromosomes of wild-type L3 larvae ectopically expressing DTL-Flag. On each panel merged DAPI (blue) and Flag-specific antibody stained (red) images are shown. (A) Chromosome spread showing localization of DTL-Flag signals at specific interbands. (B) Enlarged 2L chromosome ends of wild-type (top) and DTL-Flag-expressing animals (bottom). (C) Chromosome ends of DTL-Flag-expressing animals.

determine whether the DTL protein is also localized specifically to chromosome ends, we ectopically expressed a Flag-labeled DTL from a transgene using an *Act5* promoter. The expression of this epitope-tagged protein in *CG31241* mutants, which also express a TGS1 transgene, rescued all observable *dtl* phenotype we studied, and resulted in the development of fully-fledged adults. Images of salivary gland polytene chromosomes of third instar DTL-Flag larvae immunostained with Flag-specific antibody are shown in Fig. 3. We observed DTL localization at selected interband regions on each chromosome arm and specific staining was also detectable at chromosome ends. We detected a Flag-specific signal only in transgene carriers and its localization was reproducible both at specific interbands and chromosome ends. Interestingly, the general distribution of DTL-Flag-specific signal was different to that obtained with either HOAP- or HP1-specific antibodies (data not shown), and at some chromosome ends DTL-Flag did not seem to be localized at the very end of the polytene chromosome (Fig. 3C). However, the fact that we detected the chromosomal distribution of DTL in animals ectopically expressing the protein should be taken into account in the interpretation of this result.

Interactions between DTL, ATM and ATR in telomere protection

In chromosome end protection, ATM acts in a common pathway with members of the MRN (Mre11-Rad50-Nbs1) complex and mutations affecting ATM or MRN subunits result in a phenotype showing strong telomere association (Bi et al., 2005; Bi et al., 2004; Ciapponi et al., 2004; Ciapponi et al., 2006; Oikemus et al., 2006). By contrast, mutations affecting ATR (*mei-41/atr)* and the gene encoding its associated factor ATRIP (*mus304*) do not induce telomere associations (Oikemus et al., 2006). A role of ATR in end protection, however, was revealed from observations that *mei-41/atr; tefu/atm* double mutants, as well as other combinations of mutants of the ATM- and ATR-associated pathways displayed significantly stronger telomere association phenotypes than *tefu/atm* mutants alone (Bi et al., 2005; Ciapponi et al., 2006; Oikemus et al., 2006). *CG31241* mutations, in combination with a *tefu/atm*-null mutation, caused lethality in an earlier phase than either mutation alone (Fig. 4A), and the number of anaphase chromosome bridges in neuroblasts of *tefu/atm CG31241* double mutants was as high or even higher than either mutation alone (Fig. 4B,C). Both the viability and telomere association phenotype of double mutants depend on the strength of the *CG31241* allele used. Thus, the genetic interaction indicates that DTL has a role in a telomere protection pathway other than the one in which ATM acts. DTL does not seem to have a role in ATM checkpoint control function, because, in contrast to *tefu/atm* mutants, *CG31241* mutants displayed mitotic arrest following low-dose X-ray irradiation (supplementary material Fig. S2).

In sharp contrast to ATM mutations, *mei-41/atr* mutations increased the viability of *CG31241* mutants, and *mei-41/atr; CG31241* double mutants had lower numbers of telomere associations than *CG31241* single mutants (Fig. 4D,E,F). The suppressor effect of *mei-41/atr* mutations correlated with the

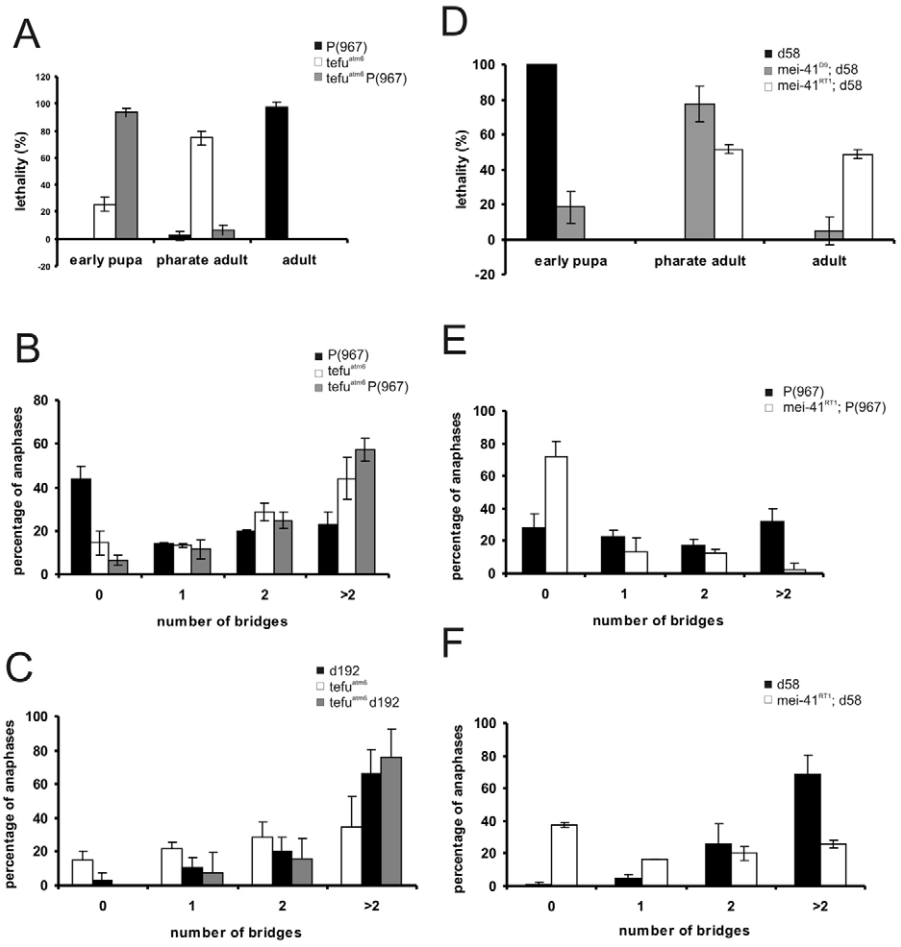

Fig. 4. Genetic interaction between *tefu/atm* and *mei-41/atr and CG31241* alleles. (A) Lethality phases of single and double mutants. (B,C) Frequency of anaphase bridges in t*efu/atm* and *CG31241* single and double mutant neuroblasts. Error bars indicate range; 150 or more anaphases were scored for each genotype, each obtained from three independent crosses. *P(967)* is a hypomorph, *d192* is a null allele of *CG31241*. *Mei-41/atr* mutations suppress *CG31241* phenotype with respect to both developmental arrest (D), and number of anaphase bridges (E,F). *d58* is null allele of *CG31241*, *mei-41*^{D9} and *mei-41*^{RT1} are weak and strong alleles of *mei-41/atr*, respectively. A detailed description of the crosses performed and the genotypes analyzed is given in Materials and Methods.

strength of the *mei-41/atr* allele used and was observable on both *CG31241*-null and hypomorph alleles. The different interactions of *mei-41/atr* and *CG31241* versus *tefu/atm* and *CG31241* mutants was surprising and indicate functional differences between the ATM- and ATR-related pathways. The observation that ATR mutations alone did not result in telomere associations, but telomere associations caused by *CG31241* mutant alleles were ATR dependent, indicates that DTL and ATR act in a common pathway in telomere maintenance. In the simplest case, DTL might be a negative regulator, acting downstream of ATR, however, a more complex relationship between the two factors can also be envisioned.

The molecular mechanism by which DTL participates in telomere maintenance is unknown. The unusual organization of the *CG31241* gene in that it also encodes TGS1, raises the question of whether DTL has a role in telomere maintenance through RNA modification. Although a role for TMG-cap-containing RNAs in both yeast and human telomere formation has been suggested (Jady et al., 2004; Seto et al., 1999), there is now data indicating that a specific RNA is involved in chromosome end protection in *Drosophila*. Our data do not suggest a role for TMG-cap-containing RNA(s) in *Drosophila* telomeres, and *CG31241* alleles that lose TGS1 function but retain DTL function do not display telomere associations.

DTL seems to be a *Drosophila*-specific protein. The putative products of CG31241-related genes of different *Drosophila* species show strong conservation in both the DTL and TGS1 ORFs. Although the putative DTL proteins have conserved amino acid blocks, they contain no domains or motifs identified in databases, except weak similarity to a region characteristic of prokaryotic helicases. Several putative phosphorylation sites were identified among the conserved amino acids of DTL proteins, but whether these have specific roles remains to be determined. Nonetheless, because the involvement of ATM- and ATR-related pathways in telomere protection seem to be conserved across the entire kingdom of eukaryotes, it is likely that flies also use these conserved pathways despite their different mechanism of telomere formation. Therefore, we believe that the identification of DTL and its interaction with ATR will facilitate studies to uncover the existence of similar functions in other organisms, which, in turn, could lead to novel insights into carcinogenesis.

Materials and Methods

D. melanogaster stocks and crosses

Tefu/atm mutant stocks were obtained from S. D. Campbell (Silva et al., 2004) other fly stocks were from the Bloomington and Szeged Stock Centers. Stocks were maintained at 25°C on standard cornmeal medium.

To generate *CG31241* alleles, we mobilized the P-element in line *l(3)S096713* by crossing to a transposase source [*TM3 ry^{RK} Sb^{1} Ser^{1} P(Δ2-3)99B/Df(3R)C7 ry^{506}*]. Imprecise excision lines were identified by scoring for loss of the mini-white marker and by genetic crosses based on their lethal phenotype over *Df(3R)P14*, which uncovers the CG31241 region. The breakpoints of deletion *d192* were determined by sequencing. The d189 (*CG31241^{d189}*) allele has been described (Pankotai et al., 2005). To analyze CG31241 mutant phenotype, *yw; CG31241/ TM3 Sb Ser y^{+}* females and males were crossed and the *yw; CG31241/CG31241* offspring larvae were selected based on their *y* phenotype. The wild-type *CG31241* transgene (pCasper-DTL), containing a 4.6 kb genomic fragment corresponding to the region between –926 to +3700 (with respect to the transcription initiation site of *CG31241* gene) and its derivatives pCasper-DTLu^{m} and pCasper-DTLd^{m} have been described (Komonyi et al., 2005). DTL and TGS1 cDNAs was generated by RT-PCR amplification on total embryonic RNA template, using primers as described (Komonyi et al., 2005). The cDNA fragments were inserted into pUAST insertional vector using restriction sites present in the primers to generate pUAST-DTL and pUAST-TGS1. For the construction of epitope-tagged DTL, an oligonucleotide encoding the Flag epitope was incorporated into the 5′-end of DTL cDNA. The structure of transgene constructs was verified by sequencing, and transgenic lines were established by embryo microinjection following standard protocol.

To compare the lethal phase and the frequency of telomere associations in *tefu/atm CG31241$^{P(967)}$* double and *CG31241$^{P(967)}$* and *tefu/atm* single mutants, we prepared the following crosses (for clarity in this section *tefu/atm* and *mei-41/atr* alleles are indicated using only the label with which the allele was first described): *yw; P[DTLu^{m}]; tefu^{atm6} d189/TM3y^{+}* crossed with *yw; +; tefu^{atm6} P(967)/TM3y^{+}*, *yw; P[DTLu^{m}]; tefu^{atm6} d189/TM3y^{+}* crossed with *yw; +; P(967)/TM3y^{+}* and *yw; P[DTLu^{m}]; tefu^{atm6} d189/TM3y^{+}* crossed with *yw; +; tefu^{atm6}/TM3y^{+}*. From these crosses, the *yw*; *P[DTLu^{m}]/+;tefu^{atm6} P(967)/tefu^{atm6} d189* (*tefu P(967)*) double mutant, the *yw; P[DTLu^{m}]/+; P(967)/tefu^{atm6} d189* (*P(967)*) single mutant and the *yw; P[DTLu^{m}]/+; tefu^{atm6}/tefu^{atm6} d189* (*tefu*) single mutant offspring were selected based on their yellow phenotype.

To compare the frequency of telomere associations in *tefu/atm* single, *d192* single, and *tefu/atm d192* double mutants, we performed the following crosses: *yw; P[DTLu^{m}]; tefu^{atm6} d192/TM3y^{+}* crossed with *yw; +; tefu^{atm6} d189/TM3y^{+}*, *yw; P[DTLu^{m}]; tefu^{atm6} d192/TM3y^{+}* crossed with *yw; +; d189/TM3y^{+}*, and *yw; P[DTLu^{m}]; tefu^{atm6} d192/TM3y^{+}* crossed with *yw; +; tefu^{atm6}/TM3y^{+}*. From these crossings the *yw; P[DTLu^{m}]/+; tefu^{atm6} d192/ tefu^{atm6} d189* (*tefu d192*) double mutant, the *yw; P[DTLu^{m}]/+; tefu^{atm6} d192/d189* (*d192*) single mutant and the *yw; P[DTLu^{m}]/+; tefu^{atm6} d192/ tefu^{atm6}* (*tefu*) single mutant offspring were selected based on their *y* phenotype.

To compare the lethal phase of *mei-41^{D9}; d58*, *mei-41^{RT1}; d58* double and *d58* single mutants, we prepared the following crosses: *mei-41^{D9}/Y; P[DTLu^{m}]; d189/TM6c Tb* crossed with *C(1)DX*, *y/Y; +; d58/TM6c Tb*, *mei-41^{RT1}/FM7a; P[DTLu^{m}]; d189/TM6c Tb* crossed with *w/Y; +; d58/TM6c Tb*, and *w; P[DTLu^{m}]; d189/TM6c Tb* crossed with *w; +; d58/TM6c Tb*. From these crosses the *mei-41^{D9}/Y; P[DTLu^{m}]/+; d58/d189* (*mei-41^{D9}; d58*) double mutant, the *mei-41^{RT1}/Y; P[DTLu^{m}]/+; d58/d189* (*mei-41^{RT1}; d58*) double mutant and the *w/Y; P[DTLu^{m}]/+; d58/d189* (*d58*) single mutant male offspring were selected based on their *Tb*+ phenotype.

To compare the frequency of telomere associations in *mei-41^{RT1}/atr; d58* double and *d58* single mutants, we prepared the following crosses: *mei-41^{RT1}/FM7a; P[DTLu^{m}]; d189/TM6c Tb* crossed with *w/Y; +; d58/TM6c Tb* and *w; P[DTLu^{m}]; dtl^{189}/TM6c Tb* crossed with *w; +; d58/TM6c Tb*. From these crossings the *mei-41^{RT1}/Y; P[DTLu^{m}]/+; d58/d189* (*mei-41^{RT1}; d58*) double mutant and the *w/Y; P[DTLu^{m}]/+; d58/d189* (*d58*) single mutant male offspring were selected based on their *Tb*+ phenotype.

To compare the frequency of telomere associations in *mei-41^{RT1}/atr; P(967)* double and *P(967)* single mutants, we prepared the following crosses: *mei-41^{RT1}/FM7a; P[DTLu^{m}]; d189/TM6c Tb* crossed with *w/Y; +; P(967)/TM6c Tb* and *w; P[DTLu^{m}]; d189/TM6c Tb* crossed with *w; +; P(967)/TM6c Tb*. From these crossings the *mei-41^{RT1}/Y; P[DTLu^{m}]/+; P(967)/d189* (*mei-41^{RT1}; P(967)*) double mutant and the *w/Y; P[DTLu^{m}]/+; P(967)/d189* (*P(967)*) single mutant male offspring were selected based on their *Tb*+ phenotype.

Cytology and polytene chromosome staining

We prepared neuroblast chromosome squashes of third instar larval brains and stained them with orcein as described (Gonzalez and Glover, 1993). For immunostaining mitotic chromosomes with anti-HOAP antibody, third instar larval brains were dissected in 0.7% NaCl, fixed for 7 minutes in 2% formaldehyde, 45% acetic acid and squashed in the same fixative. After washing in PBS containing 0.1% Tween (PBST) and blocking in PBST supplemented with 20% fetal calf serum (PBSTF), the chromosomes were incubated with anti-HOAP rabbit polyclonal antibody diluted 1:200 in PBSTF. As secondary antibody Alexa Fluor 555-conjugated goat anti-rabbit immunoglobulin (Molecular Probes) was used. The DNA staining marker DAPI (Sigma) was used at 0.1 μg ml^{-1} concentration. Following washes, the specimens were mounted in Fluoromount-G (Southern Biotech) and the images captured using a fluorescent microscope (Olympus BX51).

For immunostaining of polytene chromosomes, salivary gland squashes were prepared as described (Pile and Wassarman, 2002). We used anti-HOAP (provided by Rebecca Kellum, University of Kentucky, Lexington, KY), anti-HP1 (provided by Gunter Reuter, Martin Luther University Halle, Germany), M2 anti-Flag primary and Alexa Fluor 555-conjugated goat anti-rabbit secondary antibodies (Molecular Probes). Chromosomes were also stained with DAPI and mounted in Fluoromount-G (Southern Biotech). Third instar larval imaginal discs were stained with acridine orange as described (Pankotai et al., 2005).

In situ hybridization

In situ hybridizations were performed using biotinylated *HeT-A*-specific probes generated by PCR amplification and horseradish peroxidase detection according to standard protocols (Langer-Safer et al., 1982).

Cell cycle arrest analysis

To analyze DNA-damage-induced cell cycle arrest, we X-ray irradiated *w^{1118}, tefu^{atm6}/atm and yw; P[DTLu^{m}]/+; d192/d189* third instar larvae with 500 rad (150 kV; 0.5 mm Al filter; 1000 rad/minute) and after 60 minutes dissected the wing discs in PBS and fixed them for 20 minutes in PBS with 3.7% formaldehyde. Discs were washed three times in PBS, permeabilized in PBS with 0.3% Triton X-100 for 20 minutes, blocked in PBSTF for 30 minutes and immunostained with rabbit polyclonal anti-histone H3-PS10 antibody (Upstate; 1:500 dilution in PBSTF) overnight at 4°C.

As secondary antibody we used Alexa Fluor 555-conjugated goat anti-rabbit immunoglobulin at 1:500 dilution in PBSTF, for 60 minutes at 25°C. Discs were then washed six times in PBST and mounted in Fluoromount-G.

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