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Nucleus

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/kncl20

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Published online: 25 Oct 2011.

To cite this article: Grazia D. Raffa, Laura Ciapponi, Giovanni Cenci & Maurizio Gatti (2011) Terminin: A protein complex that mediates epigenetic maintenance of Drosophila telomeres, Nucleus, 2:5, 383-391, DOI: 10.4161/nucl.2.5.17873

To link to this article: http://dx.doi.org/10.4161/nucl.2.5.17873

Nucleus 2:5, 383-391; September/October 2011; © 2011 Landes Bioscience

Terminin

A protein complex that mediates epigenetic maintenance of Drosophila telomeres

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Key words: terminin, telomeres, telomere fusion, Drosophila

In most organisms, telomeres are extended by telomerase and contain GC-rich repeats. Drosophila telomeres are elongated by occasional transposition of specialized retroelements rather than telomerase activity, and are assembled independently of the sequence of the DNA termini. Recent work has shown that Drosophila telomeres are capped by a complex, we call terminin, which includes HOAP, HipHop, Moi and Ver; these are fast-evolving proteins that prevent telomere fusion, directly interact with each other, and appear to localize and function only at telomeres. With the possible exception of Ver that contains an OB fold domain structurally similar to the Stn1 OB fold, none of the terminin proteins is evolutionarily conserved outside the Drosophila species. Human telomeres are protected by the shelterin complex, which comprises six proteins that bind chromosome ends in a sequence-dependent manner. Shelterin subunits are not fast-evolving proteins and are not conserved in flies, but localize and function only at telomeres like the terminin components. Based on these findings, we propose that concomitant with telomerase loss Drosophila rapidly evolved terminin to bind chromosome ends in a sequence-independent fashion, and that terminin is functionally analogous to shelterin.

Introduction

Telomeres of all eukaryotes are nucleoprotein complexes that protect the extremities of linear chromosomes from degradation and fusion, and counterbalance incomplete replication of terminal DNA. When telomeres are not properly capped, they are not recognized as natural chromosome ends but are sensed as double stranded DNA breaks (DSBs), triggering the DNA damage response that arrests the cell cycle until the DNA lesion is repaired. Uncapped telomeres may also undergo inappropriate DNA repair, leading to end-to-end fusions that ultimately result in chromosome breakage.[1-3] Telomeres also cope with the inability of DNA polymerase to fully replicate the DNA of chromosome termini, often referred to as the end replication problem. In most organisms, this problem is solved by telomerase, a specialized reverse transcriptase that adds short GC-rich repeats to chromosome ends using an internal species-specific RNA template. In Drosophila, telomerase is absent[4] and telomeres are elongated by transposition of three specialized non-long-terminal repeat (LTR) retrotransposons, called *HeT-A, TART* and *TAHRE* (collectively abbreviated as HTT). These three elements transpose independently of each other and target individual telomeres at rates ranging from 10^{-2} to 10^{-4} per fly generation. Thus, most Drosophila chromosomes do not terminate with GC-rich repeats but carry HTT arrays of variable length (reviewed in refs. 5–7).

Several studies indicate that Drosophila telomeres are assembled independently of the HTT array and, more generally, independently of the sequence of the DNA termini (reviewed in ref. 8 and 9). In 1938 H.J. Muller observed that following X irradiation of males, terminal deletions could not be recovered. He concluded that chromosome ends are capped by special structures that he called telomeres and that are essential for chromosome stability and transmission.[10,11] However, subsequent studies showed that terminal deletions (TDs) can be recovered in several ways. For example, TDs were recovered following irradiation of females carrying a mutation in the mutator gene *mu2* and to lesser extent of wild type females.[12-14] These TDs can be transmitted for many generations without reacquiring HTT elements even if they undergo a progressive loss of terminal DNA due to the end replication problem.[15] It is now clear that TDs that do not end with HTT elements are capped by a neotelomere, which appears to have the same characteristics of the telomeres of intact chromosomes (reviewed in refs. 8 and 9). TDs with neotelomeres have also been recovered from mutational events occurred in the male germline. These events include X-ray induced breaks in the *Dp(1;f)1187* mini-chromosome,[16] the mobilization of a P element located near the telomere,[17] breakage of dicentric chromosomes generated by site specific recombination,[18,19] and induction of an enzymatic cut in an I-SceI site placed within a P element construct inserted near the telomere.[20] Collectively, these results demonstrate that the HTT elements are not required for fly telomere assembly and that virtually any DNA sequence has the ability to form the nucleoprotein complex that protects the ends of Drosophila chromosomes.

The structural features of the HTT elements, the molecular mechanisms underlying their transposition and their regulation

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Submitted: 07/01/11; Revised: 08/24/11; Accepted: 08/25/11
http://dx.doi.org/10.4161/nucl.2.5.17873

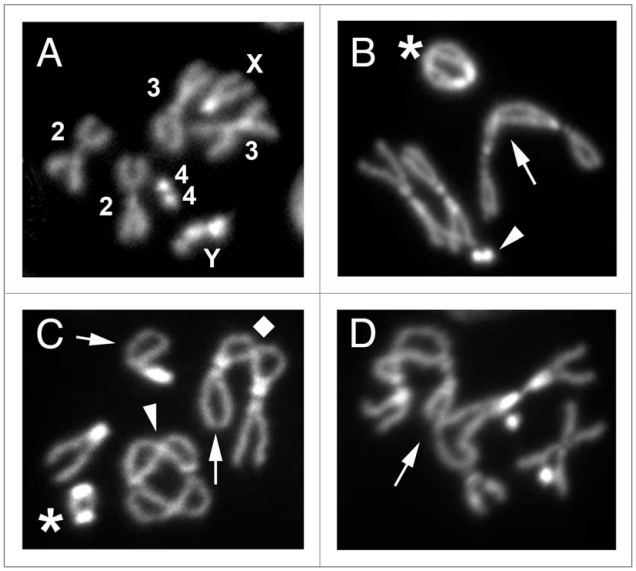

Figure 1. Mutations in genes encoding terminin components display frequent TFs. (A) wild type control metaphase. (B–D) Examples of TFs observed in larval brains from *ver* (B) and *moi* (C and D) mutants. Mutants in *cav, ver* and *moi* show two types of TFs: single TFs (STFs), in which a single telomere associates with either its sister or a nonsister telomere; and double TFs (DTFs), wherein a pair of sister telomeres joins with another pair. STFs and DTFs are likely to be generated during the S-G2 and the G_1 phase, respectively. In wild type cells, the frequency of TFs is less than 0.01/cell, whereas *cav, ver* and *moi* mutants exhibit approximately 5 TFs/cell, most of which are DTFs. (B) Metaphase containing 2–2 (arrow) and 4–4 (arrowhead) dicentric chromosomes and a dicentric ring involving both X chromosomes (asterisk), all generated by DTFs. (C) Metaphase showing a 4–4 DTA (asterisk), a 2–2 dicentric ring chromosome (arrowhead) and a 3–3 DTF (diamond); the XL and 3R telomeres exhibit sister union STFs (arrows). (D) Metaphase with a multicentric chromosome (arrow) containing 3–3 and XR-XR DTFs and 3 STFs involving both XL telomeres and individual telomeres of chromosomes 2 and 3 (the metaphases shown are from figures in ref. 31 and 32).

by small RNAs have been reviewed in recent excellent articles.[5-7] Here, we describe the genes/proteins required for Drosophila telomere capping, with a focus on terminin, a multiprotein complex that evolved after telomerase loss to bind fly telomeres in a sequence-independent fashion.

Telomere Capping Complexes in Organisms with Telomerase

In organisms with telomerase, telomeres associate with capping complexes that specifically bind the telomeric repeats generated by telomerase activity. In *S. cerevisiae,* telomeres are protected by the Rap1-Rif1-Rif2 complex that associates with the telomeric DNA duplex (dsDNA) through its Rap1 subunit, and by the Cdc13-Stn1-Ten1 complex (CST) that interacts with the 3' single stranded telomere overhang.[21,22] The three subunits of the CST complex all contain OB-fold domains and interact with each other to form an RPA-like complex that binds the 3' single stranded overhang via its Cdc13 subunit.[22-25]

Human telomeres terminate with a single stranded overhang of tandem TTAGGG repeats, which loops back invading the anterior TTAGGG duplex, thus creating a telomeric DNA loop (t-loop). A complex of six proteins, called shelterin, specifically associates with the TTAGGG repeats (reviewed in ref. 1). Three of the shelterin subunits directly interact with the TTAGGG repeats; TRF1 and TRF2 bind the TTAGGG duplex, and POT1 binds the 3' overhang. TRF1, TRF2 and POT1 are interconnected by TIN2 and TPP1, and TRF2 interacts with hRap1, a distant homolog of *S. cerevisiae* Rap1 with no DNA binding ability.[1] The shelterin subunits share three properties that distinguish them from the non-shelterin telomere-associated proteins: they specifically localize to telomeres; they are abundant at telomeres throughout the cell cycle; and their functions are limited to telomere maintenance.[1]

The CST and shelterin complexes are evolutionarily conserved, even if they vary in composition and architecture in different phyla. The Stn1 and Ten1 subunits of the CST complex are conserved in *S. pombe*, plants and humans, while shelterin-like elements are found in *S. pombe* and plants but not in *S. cerevisiae*.[26-29] *S. pombe* and plants have both shelterin-like and CST-like complexes, both of which are required for telomere protection. The two complexes are present also in humans and are thought to collaborate in telomere protection. However, the human CST complex does not share the shelterin properties and appears to have a relatively minor role in telomere capping.[28,29]

The shelterin and CST components of yeast, plant and mammalian telomeres interact with several conserved polypeptides required for telomere function. These polypeptides include many proteins involved in the DNA damage response and in DNA repair such as the ATM and Chk2 kinases, the Ku70/80 heterodimer, the MRE11/RAD50/NBS1 (MRN) complex, Rad51, the ERCC1-XPF and MUS81 endonucleases, the Apollo exonuclease and the RecQ family members WRN and BLM, which are mutated in the Werner and Bloom syndromes, respectively (reviewed in refs. 1, 2, 26 and 30). In addition, yeast and mammalian telomeres are enriched in proteins that are homologous to Drosophila HP1. All non-shelterin and non-CST proteins localize and function not only at telomeres but also elsewhere in the cell.[1,2,26,30]

Drosophila Telomeres are Protected by the Terminin Complex

The identification of Drosophila proteins required for telomere protection has mainly relied on the isolation of mutants that display frequent telomeric fusions (TFs) in larval brain cells (**Fig. 1**). The molecular characterization of the genes specified by these mutants identified ten loci that are required to prevent end-to-end fusion (**Table 1**). These are *Su(var)205* and *caravaggio* (*cav*) that encode HP1 and HOAP (HP1/ORC-associated protein), respectively;[33,34] *UbcD1* that encodes an E2 enzyme involved in protein ubiquitination;[35] the Drosophila homologs of the *ATM, RAD50, MRE11* and *NBS1* genes;[36-43] *without children (woc)* that specifies a putative transcription factor;[44] *modigliani (moi;* also called *DTL*) that encodes a nonconserved HOAP-binding protein;[31,45] and *verrocchio (ver)* that specifies an OB-fold containing protein structurally homologous to

Table 1. Drosophila genes required to prevent telomere fusion

Gene name	Protein name	Protein full name	Function outside of telomeres	References
cav	HOAP	HP1-ORC-Associated Protein	None known	34
hiphop	HiPHop	HP1-HOAP-interacting protein	None known	20
moi	Moi	Modigliani	None known	31, 45
ver	Ver	Verrocchio	None known	32
Su(var)205	HP1	Heterochromatin Protein 1	Heterochromatin regulation; transcription factor	33, 46
eff	UbcD1	Ubiquitin Conjugating Enzyme D1	E2 ubiquitin conjugating enzyme	35, 59
woc	Woc	Without Children	Transcription factor	44
mre11	Mre11	Meiotic recombination 11	DNA repair; Component of the MRN complex	36, 37
rad50	Rad50	Radiation sensitive 50	DNA repair; Component of the MRN complex	37
nbs	Nbs	Nijmegen breakage syndrome	DNA repair; Component of the MRN complex	41–43
tefu	ATM	Ataxia Telangiectasia Mutated	Kinase; DNA damage response	36, 38–41, 61
mei-41 (1)	ATR	Ataxia Telangiectasia Related	Kinase; DNA damage response	41–43
mus-304 (1)	ATRIP	ATR Interacting Protein	DNA helicase; DNA damage response	41, 43
armi (2)	Armi	Armitage	helicase; piRNA biogenesis	69
aub (2)	Aub	Aubergine	piRNA biogenesis	69

(1) Mutations in *mei-41* or *mus-304* do not cause TFs but genetically interact with mutations in *tefu*, so that *mei-41 tefu* and *mus-304 tefu* double mutants exhibit TF frequencies that are much higher than those seen in *tefu* single mutants. (2) Mutations in *armi* and *aub* cause TFs only during embryogenesis.

STN1.[32] An additional protein required to prevent telomere fusion, called HP1-HOAP interacting protein (or HipHop), was recently identified among the polypeptides that co-precipitate with HOAP.[20]

Mutations in *caravaggio* (HOAP), *modigliani* and *verrocchio* cause very high frequencies of TFs (~5 per cell), often producing multicentric linear chromosomes that resemble little "trains" of chromosomes. The genes specified by these mutations have been named to reflect this phenotype, as the three Italian trains that are dubbed with the names of these famous painters. The HOAP, Ver and Moi proteins directly interact with each other in GST pulldown assays; HOAP and Moi also bind HP1 but Ver does not. Immunolocalization experiments have shown that HOAP is specifically associated with the telomeres of both mitotic and polytene chromosomes. An analysis of GFP-Moi and Ver-GFP localization on polytene chromosomes showed that these proteins are exclusively enriched at telomeres where they precisely colocalize with HOAP and HP1. However, GFP-Moi and Ver-GFP could not be detected at mitotic chromosome ends, probably due to their very low abundance.[31,32] These results indicate that HOAP, Moi and Ver form a complex that accumulates only at telomeres of both interphase (polytene) and mitotic chromosomes. In addition, available data indicate that HOAP, Moi and Ver function primarily if not exclusively at telomeres. Thus the HOAP-Moi-Ver complex, which has been named terminin (after the name of Rome's train station), has the same properties as human shelterin and is likely to be a functional analog of shelterin.[31,32] HipHop directly interacts with both HOAP and HP1, although it is currently unknown whether it also binds Moi and Ver. HipHop specifically localizes at both mitotic and polytene chromosome telomeres and appears to function only at telomeres.[20] Thus, HipHop is likely to be an additional terminin component. Remarkably, HOAP, HP1 and HipHop all localize to the extremities of various types of terminally deleted chromosomes demonstrating that these proteins bind chromosome ends independently of the sequence of terminal DNA.[19,20,33,34] It should be noted that despite its direct interaction with HOAP, HipHop and Moi, HP1 should not be considered as a terminin component, because it does not localize exclusively at telomeres and has multiple telomere-unrelated functions (reviewed in refs. 46 and 47).

The structural and functional information on the terminin proteins is still rather limited; the architecture of the complex and the possible roles of its components are not well defined. Biochemical analyses showed that HOAP and HipHop are mutually dependent for their stability, as loss of one protein reduces the amount of the other.[20] In contrast, HOAP, Moi and Ver do not appear to be interdependent for stability, as loss of one protein does not destabilize the others. However, recruitment of terminin proteins to the telomeres is governed by precise dependencies: localization of Moi and Ver at chromosome ends requires HOAP, while Moi and Ver are mutually dependent for their association with telomeres. HOAP, HipHop and Moi do not exhibit structural features that help define their roles in telomere protection. BLAST searches did not detect HOAP, HipHop or Moi homologs outside the Drosophilidae insects. In addition, these proteins do not appear to contain known functional domains, although HOAP is thought to carry an HMG-like domain.[48] Previous studies showed that HOAP binds double stranded DNA of different sequence, although with different affinities.[48] Ver contains an OB-fold domain that shares structural similarity with the OB fold domain of Rpa2/Stn1 and binds single stranded DNAs of different sequence (reviewed in ref. 32; our unpublished results). Substitution of four critical amino acids in the Ver OB fold abolished the DNA binding ability of the protein (our unpublished results). When this mutated Ver version was expressed in flies,

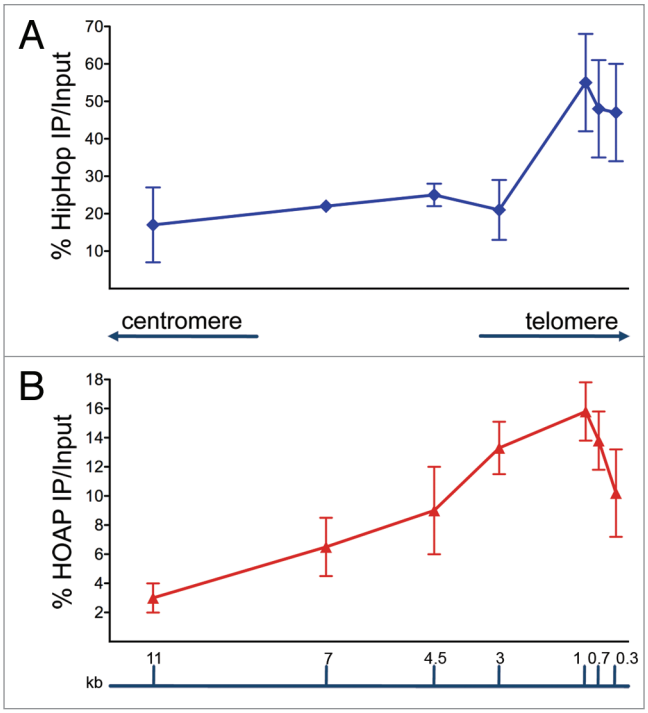

Figure 2. Distribution of HOAP and HipHop over the terminal DNA of a Drosophila chromosome ending with the *white* gene sequence (see text and ref. 20 for detailed explanation). Protein localization was determined by chromatin immunoprecipitation using anti-HOAP or anti-HipHop antibodies. Note that HOAP and HipHop exhibit similar distributions with the amount of each protein increasing from -11 to -1 kb from the end of the chromosome to drop in the terminal Kb (adapted from Fig. 6 in ref. 20; with permission of the authors).

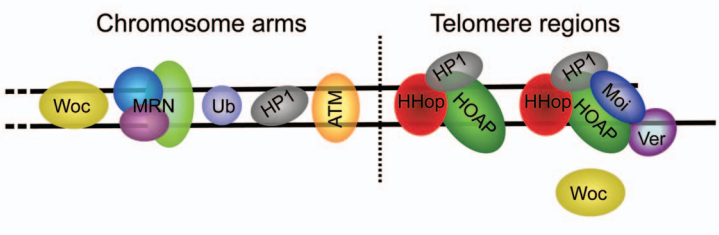

Figure 3. A tentative model for the molecular organization of Drosophila telomeres. We propose that the very ends of the chromosomes are capped by terminin, which includes HOAP, HipHop (HHop), Moi and Ver. HOAP and Ver bind double-stranded and single-stranded DNA, respectively, but it is currently unknown whether HipHop and HOAP directly associate with DNA. Moi and Ver would be absent from the telomeric DNA duplex proximal to the single stranded overhang; this duplex, however, would be associated with HOAP-HipHop. Drosophila telomeres are also enriched in HP1 and Woc, but these proteins are not terminin components because they associate with multiple polytene bands and play functions outside the telomeres. The UbcD1 (Ub) protein is enriched at the telomere region but appears to concentrate near the TAS rather than at the ends of the chromosomes (Cipressa and Cenci unpublished results). The MRN complex and the ATM kinase exhibit a rather uniform distribution along the Drosophila mitotic chromosomes and do not accumulate at polytene chromosome ends. Thus, UbcD1, Mre11, Rad50, Nbs and ATM were not included in the telomere region of the scheme, even if these proteins must function at telomeres, as mutations in the genes they specify cause telomeric fusions.

it was recruited at telomeres but was unable to prevent telomere fusion, suggesting that the DNA binding activity of the Ver OB fold is crucial for telomere protection.[32] It is not currently known whether Moi and HipHop can directly bind DNA. However, chromatin immunoprecipitation (ChIP) experiments using TD chromosomes terminating with the *white* gene sequence revealed that HOAP and HipHop are highly enriched within an 11-kb stretch of telomeric DNA. An analysis of the distribution of these proteins further showed their concentration increases from -11 kb to reach a peak at -1 kb but then drops in the terminal kb of telomeric DNA (**Fig. 2**). Although these results are rather fragmentary, they permit us to conceive a possible model for the interaction of terminin with telomeric DNA (**Fig. 3**). We would like to propose that HOAP and HipHop are primarily bound to the telomeric DNA duplex while Ver and Moi are associated with the single stranded overhang, which might span the terminal region of reduced HOAP/HipHop binding.

Although the terminin components are all required to prevent telomere fusion, they do not play identical roles at Drosophila telomeres. Previous work showed that HOAP-depleted telomeres trigger both the DNA damage response (DDR) and the spindle assembly checkpoint (SAC).[49,50] The SAC appears to be mediated by the BubR1 kinase, which accumulates at the uncapped telomeres in almost all *cav* mutant cells, but is never targeted to wild type telomeres. In the absence of Moi, Ver or HP1, telomeres have little or no ability to activate the DDR and trigger the SAC.[31,32,49-51] It is currently unknown whether HipHop depletion triggers the DRR and the SAC response. These results suggest that HOAP is crucial for protecting chromosome ends so as to prevent both telomere fusion and checkpoint responses. In contrast, Ver and Moi are not required to prevent checkpoint responses but are essential to hide chromosome ends from the DNA repair machineries that mediate telomere fusion. Interestingly, recent studies have shown that dysfunctional mouse telomeres also recruit BubR1, but it is unclear whether telomere-associated BubR1 can activate the SAC response.[52]

The Roles of Drosophila Nonterminin Proteins Required for Telomere Protection

Drosophila telomere capping is not only ensured by terminin but also by a number of proteins that do not share the terminin properties; namely, proteins that do not localize or function only at telomeres. To date, we know 7 nonterminin proteins required for telomere protection from fusion events: HP1a, UbcD1, Mre11, Rad50, Nbs, ATM and Woc.

The most characterized nonterminin telomere-capping factor is HP1a. Besides HP1a, the Drosophila genome harbors 5 additional HP1 paralogs (HP1b, HP1c, HP1d, HP1e and Umbrea/HP6), none of which has been unambiguously shown to be required for telomere protection (reviewed in ref. 47; our unpublished results). In polytene chromosomes, HP1a is enriched at the telomeres, the chromocenter, the fourth chromosome and many euchromatic bands.[33,53,54] Consistent with this localization pattern,

HP1a binds diverse proteins involved in a variety of processes including telomere capping, gene silencing, DNA replication and repair, the maintenance of proper chromosome structure, and transcriptional regulation (reviewed in refs. 46, 47, 55 and 56). In Drosophila, HP1a is not only required for telomere protection but it is also involved in the control of telomere length. In stocks heterozygous for lethal mutations in *Su(var)205* (which encodes HP1a) the telomeres are much longer than those of wild type flies due to a dramatic elongation of the HTT array. This is a likely consequence of an increased *HetA* and *TART* transcription, which has been observed in both *Su(var)205* heterozygotes and homozygotes.[57,58] We never observed telomere elongation in stocks heterozygous for mutations in *cav, moi* or *ver*. In addition, our real time RT-PCR experiments did not detect substantial increases in *HetA* and *TART* transcription in homozygous *cav, moi* or *ver* mutant larvae (our unpublished results). Thus, the extant observations suggest that terminin is not implicated in the control of Drosophila telomere elongation.

effete/UbcD1 was the first Drosophila gene shown to be required for prevention of telomere fusion.[35] *UbcD1* is an essential gene that encodes a highly conserved E2 ubiquitin conjugating enzyme implicated in several Drosophila cellular processes.[35] The UbcD1 protein associates with many polytene chromosome bands and is enriched at the telomere region of polytene chromosomes (Cipressa and Cenci G, unpublished observations). This suggests that failure to ubiquitinate one or more telomere proteins leads to fusigenic telomeres. However, we do not know whether UbcD1 interacts with terminin, and the telomere-associated target(s) of UbcD1 remain to be identified. Given that mutations in some of the proteasome components do not cause TFs (our unpublished results), we suspect that UbcD1-mediated ubiquitination of proteins involved in telomere protection is not required for their degradation but is instead a post-translational modification that ensures their proper capping function. Consistent with this idea, polytene chromosomes from *UbcD1* mutants and those from wild type controls display comparable telomeric concentrations of both HP1 and HOAP.[59] These results indicate that UbcD1 is required neither for HP1 or HOAP localization at telomeres nor for proteolysis of these proteins. However these findings do not exclude the possibility that either one or both of these proteins are ubiquitinated by UbcD1.

Studies performed in the last few years have shown that several proteins involved in DNA repair are also needed to prevent telomere fusion. Mutants in the *Drosophila mre11, rad50* and *nbs* genes die at late larval stages and exhibit both TFs and chromosome breakage in brain cells.[36,37,41-43] The Drosophila Mre11, Rad 50 and Nbs proteins are the fly orthologs of human MRE11, RAD50 and NBS1, which form the highly conserved MRN complex involved in both double-strand breaks (DSBs) repair and telomere maintenance. This complex mediates DSB repair by participating in both the homologous recombination (HR) and the nonhomologous end joining (NHEJ) pathways.[60] In humans, the MRN complex also associates with the TRF2 subunit of shelterin, facilitating telomerase recruitment and participating in detection and signaling of uncapped telomeres (reviewed in ref. 60). It is currently unknown whether the components of the Drosophila MRN complex interact with terminin. However, mutations in the *rad50*, *mre11* and *nbs* genes strongly reduce HOAP accumulation at mitotic telomeres. These mutations also inhibit HOAP and HP1 localization at polytene chromosome ends.[36,37,41-43] Consistent with these findings, mutations in *mre11* prevent Moi localization at polytene chromosome telomeres.[31] Collectively, these results strongly suggest that terminin recruitment to telomeres requires the wild type function of the MRN complex. However, even in the absence of MRN activity, mitotic chromosomes retain the ability to recruit low levels of HOAP[36,37,41-43] and, presumably, of the other terminin components. These low amounts of terminin are likely to provide a partial protection of telomeres, as suggested by the finding that *rad50*, *mre11* and *nbs* mutants display much fewer TFs than *cav* mutants.[34,36,37,41-43]

Another DNA repair protein involved in Drosophila telomere protection is the ATM kinase. Null mutations in the *tefu/atm* gene result in lethality at late larval stages and cause both chromosome breakage and TFs in larval brain cells.[36,38-40,61] *tefu* mutations do not substantially affect HOAP localization at mitotic telomeres, although they might reduce HOAP accumulation at polytene telomeres.[36,38] Thus, loss of Drosophila ATM does not appear to cause telomere fusion by preventing terminin localization at chromosome ends. Null mutants in the ATR-encoding *mei-41* gene or in the *mus-304* gene that encodes the ATR-interacting protein ATRIP are both viable and their larval brain cells exhibit chromosome aberrations but not TFs.[62-64] However, *tefu mei-41* and *tefu mus-304* double mutants fail to recruit HOAP at telomeres and have significantly higher frequencies of TFs than those observed in *tefu* single mutants.[41-43] This suggests that ATM and ATR/ATRIP have partially redundant roles in telomere protection and that failure to phosphorylate a common target leads to deprotected telomeres. The nature of this target is currently unknown and it does not appear to be HOAP (ref. 41; our unpublished results).

The mechanism by which the combined action of the MRN complex, ATM and ATR-ATRIP leads to terminin recruitment to telomeres is unclear. The MRN complex plays a central role in detection and repair of DNA double strand breaks and mediates recruitment of ATM at the site of DNA damage (reviewed in ref. 60). Although MRN preferentially associates with the ends of linear DNA molecules in vitro,[65] the Rad50, Mre11 and Nbs protein are uniformly distributed along Drosophila chromosomes (ref. 37; our unpublished results), making it unlikely that MRN directly tethers terminin to telomeres. Thus, it has been hypothesized that interactions of DNA ends with the MRN complex and ATM-ATR result in conformational changes that facilitate terminin recruitment at telomeres.[9,37,49]

Protecting Drosophila telomeres from fusion also requires the wild type activity of the *without children (woc*) gene. *woc* encodes a zinc finger protein that interacts with HP1c and functions both in transcriptional regulation and telomere capping.[44,66,67] Woc is enriched at polytene chromosome telomeres and co-localizes with all euchromatic bands that associate with the initiating form of Pol II.[44] We do not know whether Woc interacts with terminin. However, Woc localization at telomeres is not affected by

mutations in *Su(var)205, cav, atm* or *rad50,* and mutations in *woc* do not affect HOAP and HP1 localization at chromosome ends. In addition, *woc* mutations do not dominantly affect telomere length.[44] These results indicate that the Woc function at telomeres is independent of those played by HOAP, HP1, ATM or Rad50, and that Woc is a transcription factor with telomere-capping properties, as is also the case for yeast Rap1.[68]

The *armitage (armi)* and *aubergine (aub)* genes play a tissue-specific role in Drosophila telomere stability.[69] *armi* and *aub* mutants are viable but female-sterile, as they cause maternal-effect embryonic lethality. Both genes are involved in the biogenesis of piRNAs; *armi* encodes an RNA helicase and *aub* a piRNA-binding Argonaute-like protein (reviewed in ref. 70). In embryos from *armi* and *aub* homozygous mothers, there are frequent anaphase bridges that are probably caused by telomeric fusions. Consistent with this interpretation, chromatin immunoprecipitation studies showed that mutation in both genes disrupt telomere binding of HOAP in the embryo.[69] Mutations in *armi* and *aub* also reduced an embryonic subpopulation of piRNAs that share some homology with telomeric retotransposons, raising the possibility that these small RNAs may facilitate HOAP binding to the HTT array.[69] However, these piRNAs or other Armi-and Aub-dependent piRNAs (or the Armi and Aub proteins) must also facilitate HOAP binding to telomeres devoid of the HTT array, as diverse TD chromosomes are regularly transmitted during embryogenesis (see Introduction). It should also be noted that the Armi- and Aub-based mechanism of telomere protection is likely to be restricted to embryogenesis, as homozygous *armi* and *aub* individuals generated by heterozygous mothers are viable and do not exhibit TFs (our unpublished results).

Evolution of Drosophila Telomere Proteins

There is a general consensus that the crucial event that led the evolution of Drosophila telomeres was progressive loss of telomerase accompanied by the development of a regulated transposon-based mechanism for telomere elongation. It has also been suggested that the two mechanisms of telomere maintenance might have coexisted for some time.[5,7,71] This scenario is not difficult to envisage as in the silkworm *Bombyx mori*, which retains telomerase, there are two classes of retotransposons that specifically insert into the telomere regions.[72,73] It is logical to assume that the transition from a telomerase-driven to a transposon-driven telomere elongation mechanism resulted in a divergence of terminal DNA sequences, accompanied by a strong selective pressure toward the evolution of sequence-independent telomere-binding factors. In agreement with this idea, none of the shelterin or the CST components is conserved in flies, and none of the terminin proteins, with the possible exception of Ver, has obvious homologs in yeasts, mammals or plants.[20,31,32,34] Thus, we hypothesized that concomitant with telomerase loss, Drosophila lost the shelterin and the CST homologs that bind DNA in a sequence-specific fashion, and evolved terminin to bind chromosome ends independently of the DNA sequence.[31,32] Ver exhibits a very limited amino acid sequence homology with Stn1, but contains an OB fold domain that is structurally similar to the Stn1 OB fold.[32] We speculate that Drosophila evolved Ver exploiting an Stn1 ancestor similar to the RPA proteins that bind ssDNA with no sequence specificity. The origin of HOAP, Moi and HipHop is unknown, as no conserved proteins that share amino acid sequence homology with these terminin components have been so far identified.

It is conceivable that following telomerase loss selective pressure on terminin proteins was much stronger than that exerted on other telomere proteins not specifically involved in capping. Proteins involved in diverse cellular processes are indeed expected to have functional constraints that prevent them from responding to selection with the same high rate of amino acid substitutions as polypeptides that are solely involved in the protection of chromosome ends. Therefore, one would hypothesize that proteins directly and exclusively involved in telomere capping evolved more rapidly than the other telomere-associated proteins. This hypothesis is verified by two findings. First, all Drosophila nonterminin proteins required to prevent telomere fusion have clear mammalian homologs, most of which have been implicated in telomere maintenance (HP1a, ATM, MRE11, RAD50, NBS1).[31,32] Second, HOAP, Moi, Ver and HipHop all exhibit a very high rate of nonsynonymous substitution per nonsynonymous site, and are therefore fast-evolving proteins. In contrast, none of the nonterminin telomere proteins, including HP1, appears to be a fast-evolving polypeptide.[20,32,74] Consistent with these findings, sequence analysis of proteins from *D. melanogaster* and 11 recently sequenced species[75] revealed that the terminin components are substantially more divergent than non-terminin proteins (**Fig. 4**). In conclusion, the rate of nonsynonymous substitutions in HOAP, Moi, Ver and HipHop is so high and distinctive that we propose to use it as an additional identifying criterion for both the extant terminin components and for terminin proteins that might emerge from future screens.

Recent work has shown that the terminin complex can vary in composition in different Drosophila tissues. In male meiotic and postmeiotic cells, including the sperm nuclei, HipHop is substituted by the product of the *ms(3)k81* gene (henceforth referred as *k81*).[76,77] *k81* is a duplication of the *hiphop* gene, presumably originated through a retroposition mechanism.[78] Phylogenic analysis suggests that *k81* is a relatively young gene, as it is present only in the *melanogaster* subgroup of Drosophila species, although an independent *hiphop* duplication with male-biased expression was also found in *D. willistoni*.[76] K81 associates with the sperm telomeres and is specifically required for telomeric protection of male derived-chromosomes during embryogenesis; in embryos fathered by *k81* mutants, the paternal chromosomes display frequent TFs, ultimately leading to embryonic death.[76,77] The relationships between K81 and the other terminin proteins are unclear. In one study, K81 was found to colocalize with HOAP and HP1 in sperm nuclei of wild type males. HP1 and HOAP were absent from the sperm nuclei of *k81* mutants, suggesting that K81 is needed for the maintenance of capping complexes at the sperm telomeres.[76] In another study, mature sperm of wild type males were found to be devoid of both HP1 and HOAP signals, suggesting that the telomere-capping machinery of the sperm does not include HOAP and HP1.[77] It would be interesting

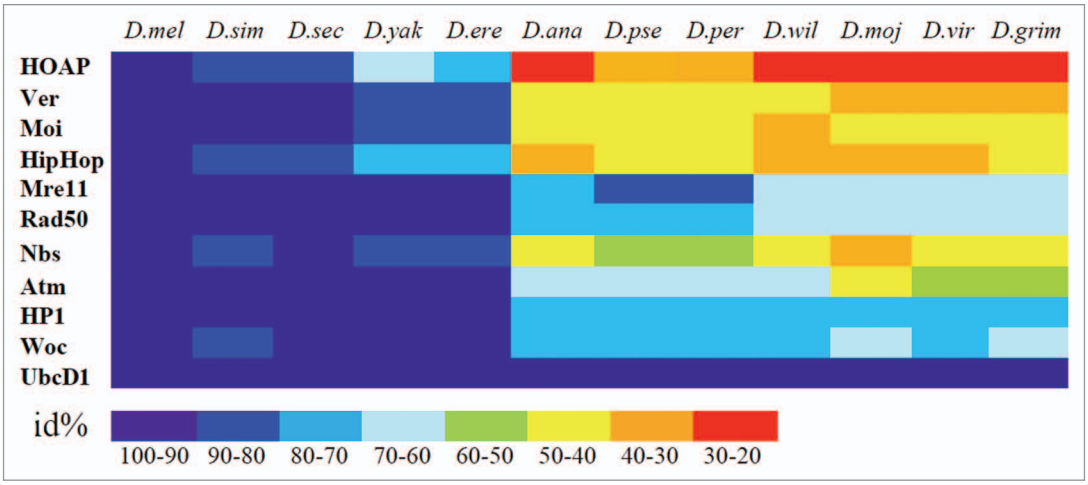

Figure 4. Identity percentages between the *D. melanogaster* proteins required for telomere capping and the homologous proteins form 11 Drosophila species (*D. mel, D. melanogaster; D. sim, D. simulans; D. sec, D. sechellia; D. yak, D. yakuba; D. ere, D. erecta; D. ana, D. ananassae, D. pse, D. pseudoobscura; D. per, D. persimilis; D. wil, D. willistoni; D. moj, D. mojavensis; D. vir, D. virilis; D. grim, D. grimshawi).* The identity percentage (id%; represented by the indicated color) is the percentage of identical matches between two amino acid sequences, calculated using the pairwise alignment EMBOSS Needle software.

to determine whether this machinery includes Moi and Ver and other proteins that, like K81, are required for telomere capping in sperm cells. Whatever the composition of the sperm telomere capping complex, the studies on K81 have clearly shown that during early embryogenesis there is substantial remodeling of the telomere structure; during this period K81 is substituted by HipHop and other telomere proteins are possibly recruited. It is also logical to speculate that the K81-HipHop transition period might be crucial for the formation of neotelomeres at the ends of TD chromosomes. It would be interesting to learn whether TD chromosomes, produced in males by means different from irradiation (see introduction), acquire a K81 cap in the sperm or are occasionally capable to escape from fusion events in the embryo and to recruit a normal capping complex during the K81-HipHop transition.

Conclusions and Perspectives

We have described the main characteristics of 15 Drosophila proteins that are necessary to protect telomeres from fusion events (**Table 1**). In a review published in 2005, we estimated that the Drosophila genome contains at least 40 genes required to prevent telomere fusion.[8] The current results do not alter this conclusion and we still believe that there are many Drosophila telomere-capping genes that await to be discovered. The molecular and genetic analyses of the genes so far identified have shown that Drosophila telomeres are capped by terminin, a complex composed of fast-evolving proteins that specifically bind the telomeres. An important issue that needs to be elucidated by future work is how terminin is recruited at the telomeres independently of the DNA sequence. We have learned that terminin recruitment requires the function of the MRN complex and the partially redundant activities of the ATM and ATR kinases. However the molecular mechanism underlying terminin recruitment remains elusive.

We have hypothesized that during the transition from a telomerase-based to transposon-based telomere elongation mechanism, Drosophila rapidly evolved terminin to bind chromosome ends independently of the DNA sequence. Consistent with this hypothesis, the Drosophila terminin proteins are not conserved in humans, while the shelterin proteins have no obvious Drosophila homologs. In contrast, Drosophila nonterminin and human nonshelterin telomere proteins are largely conserved from flies to mammals, and many of them play telomere-related functions in both organisms. These findings indicate that the main difference between Drosophila and human telomeres is in the protective complexes that specifically associate with the DNA termini. Thus, apart from the different mechanisms of elongation, Drosophila and human telomeres might not be as different as it is generally thougth. The conservation in humans of the nonterminin telomere proteins further suggests that the identification of additional proteins of this type may lead the discovery of novel components of human telomeres.

Acknowledgements

We are grateful to John C. Lucchesi for a critical reading of the manuscript. This work was supported in part by a grant from AIRC (Italian Association for Cancer Research) to M.G.

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