UbcD1, a Drosophila ubiquitin-conjugating enzyme required for proper telomere behavior

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The end-to-end association of chromosomes through their telomeres has been observed in normal cells of certain organisms, as well as in senescent and tumor cells. The molecular mechanisms underlying this phenomenon are currently unknown. We show here that five independent mutant alleles in the Drosophila UbcD1 gene cause frequent telomere-telomere attachments during both mitosis and male meiosis that are not seen in wild type. These telomeric associations involve all the telomeres of the D. melanogaster chromosome complement, albeit with different frequencies. The pattern of telomeric associations observed in UbcD1 mutants suggests strongly that the interphase chromosomes of wild-type larval brain cells maintain a Rabl orientation within the nucleus, with the telomeres and centromeres segregated to opposite sides of the nucleus. The UbcD1 gene encodes a class I ubiquitin-conjugating (E2) enzyme. This indicates that ubiquitin-mediated proteolysis is normally needed to ensure proper telomere behavior during Drosophila cell division. We therefore suggest that at least one of the targets of UbcD1 ubiquitination is a telomere-associated polypeptide that may help maintain proper chromosomal orientation during interphase.

[Key Words: Drosophila melanogaster; telomere behavior; ubiquitin-conjugating enzymes]

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Telomeres, the DNA–protein complexes at the ends of eukaryotic chromosomes, have long been recognized to have important roles in the protection, replication, and stabilization of chromosome ends (for review, see Harley and Villeponteau 1995; Zakian 1996). Telomeres are also known to influence the expression of adjacent genes, a phenomenon called telomere position effect (for review, see Shore 1995). In most organisms, telomeres are composed of long stretches of conserved, tandemly repeated simple DNA sequences whose addition to chromosome ends is catalyzed by the enzyme telomerase. This is not the case in Drosophila, where telomeres contain multiple copies of the telomere-specific retrotransposons HeT-A and TART instead of simple sequence repeats (for review, see Mason and Biessman 1995; Pardue 1995).

The possibility that telomeres may help organize the architecture of the interphase nucleus is appreciated less widely. Observations of both somatic and meiotic cells suggest that the positioning of telomeres within the nucleus is highly specific and dependent on interactions of telomeres with the nuclear envelope (for review, see Gilson et al. 1993; Dernburg et al. 1995). A nonrandom distribution of telomeres within the nucleus was first described by Rabl (1885) in amphibia. Rabl noticed that prophase chromosomes have a polarized arrangement, with the telomeres lying at one side of the nucleus and the centromeres clustered at the opposite side. Because this chromosome arrangement was very similar to that seen during the preceding anaphase, he concluded that chromosomes maintain their anaphase configuration throughout interphase. Similar conclusions were reached by Boveri (1909) in his classic studies on Parascaris chromosomes. He also observed that during both telophase and prophase the Parascaris telomeres are accommodated into nuclear evaginations. A Rabl orientation of chromosomes, with telomeres in close proximity to the nuclear envelope, has since been observed during prophase in somatic cells of a variety of organisms. Moreover, there is accumulating evidence that telomeres are associated with the nuclear envelope during interphase (for review, see Dernburg et al. 1995). It is currently unclear whether the relationship between telomeres and the nuclear envelope of somatic cells is a passive consequence of anaphase chromosome orientation or the result of an active process that maintains an ordered nuclear architecture during interphase.

At least during meiosis, dynamic processes appear to be of importance in establishing interactions between...
telomeres and the nuclear envelope. In meiotic cells of several organisms, telomeres are attached to a small region of the nuclear envelope, producing a specialized configuration of chromosomes called the "bouquet arrangement" (Digby 1919). This bouquet structure, which is thought to have a role in chromosome pairing and synapsis, first forms during zygotene by an active process whose molecular basis is not understood (Dernburg et al. 1995).

Abundant evidence indicates that telomeres not only interact with the nuclear envelope but also with each other. In some cases, telomeres are not just found at the periphery of interphase nuclei but are clustered tightly together there. For example, yeast cells immunostained with antibodies against RAP1, a telomere marker, exhibit fewer spots than the expected number of telomeres, suggesting telomere clustering (Klein et al. 1992). In some preparations from plants and certain insects, fibers connecting the telomeres of both homologous and non-homologous chromosomes have been observed (Hughes-Schrader 1957; Wagenaar 1969; Stack and Clarke 1973; for review, see Dernburg et al. 1995). The data are particularly convincing in the case of *Drosophila* polytene chromosomes, where these fibers hybridize in situ with a telomere-specific probe (Rubin 1978). Intriguingly, in rare preparations of somatic nuclei of the coccid *Chiysomphalus ficus* and of onion and other plant species, all of the chromosomes appear linked together at their ends to form a continuous chain or "spireme" (Hughes-Schrader 1957; Wagenaar 1969).

Normal mammalian cells do not exhibit end-to-end associations of chromosomes. However, such associations have been observed in senescent human fibroblasts (Benn 1976; for review, see Dernburg et al. 1995), in cells from patients with Thibierge-Weissenbach syndrome (Dutrillaux et al. 1978) or ataxia telangiectasia (Hayashi and Schmid 1975; Taylor et al. 1981), and, above all, in a variety of human tumors (for review, see Hastie and Allshire 1989; de Lange 1995). Telomeric fusions in senescent and tumor cells are particularly convincing in the case of *Drosophila*, all of the chromosomes appear linked together at their ends to form a continuous chain or "spireme"(Hughes-Schrader 1957; Wagenaar 1969).

Figure 1. Complementation analysis among *UbcD1* mutant alleles. (ms) Male sterile; (M^+) presence and (M^-) absence of mitotic defects; (m^-) presence and (m^+) absence of meiotic defects; (SL) semilethal; (LL) late lethal; (EL) early lethal. Note that the semilethal and late lethal combinations exhibit both mitotic and meiotic abnormalities.

In the course of genetic screens for mutations affecting the fidelity of chromosome segregation in *Drosophila*, we identified several mutants that exhibited peculiar cytological phenotypes, described in detail below, suggestive of defects in telomere behavior during mitosis and meiosis. Although these mutations affect different stages of the *Drosophila* life cycle, the complementation analysis summarized in Figure 1 shows that they are allelic. Because the gene defined by this complementation group encodes a *Drosophila* ubiquitin-conjugating enzyme (see below), we have called it *UbcD1*.

As shown in Figure 1, the mutations *UbcD1{eff1}*, *UbcD1{eff3}*, and *UbcD1{eff8}* (Castrillon et al. 1993, hereafter abbreviated as eff1, eff3, and eff8) are viable but male sterile and disrupt mitotic but not meiotic telomere behavior. *UbcD1{trel1}* (trel1) is also viable and male sterile, whereas *UbcD1{trel2}* (trel2) is semilethal with sterile male escapers; trel1 and trel2 affect both mitotic and meiotic telomeres. Each of these *UbcD1* mutations was associated with the insertion of a marked P element.

We generated new *UbcD1* alleles by remobilizing several of the P-element insertions in the locus. One of the resulting mutations, *UbcD1{A73A}* (A73A), is a deletion that removes almost the entire *UbcD1* transcripational...
unit. Another excisant line, *UbcD1Δ112* (*Δ112*), also disrupts a substantial, though molecularly uncharacterized, portion of the *UbcD1* gene. These deletions are lethal in homozygotes and in trans to each other, demonstrating that *UbcD1* is an essential gene. For *Δ73A* homozygotes, death occurs during embryogenesis. The weaker *Δ112* allele causes lethality during the pupal stage, and produces cytological defects in both mitotic and meiotic cells. Both *Δ73A* and *Δ112*, when in trans over *eff1*, *eff3*, or *eff8*, elicit abnormal telomere behavior during male meiosis. Homozygous or heterozygous females for all of the viable *UbcD1* mutant alleles are fertile.

**Molecular characterization of the *UbcD1* gene**

All of the original *UbcD1* alleles were obtained from mutagenesis experiments using the mobilization of marked P elements. Several of the mutations have been reverted to wild type by transposase-mediated excision of the P elements, demonstrating that the P-element insertions were the cause of the phenotypes that we have analyzed. To localize the corresponding gene, sequences from within the P elements were hybridized in situ to polytene chromosomes; all detected a single band of hybridization in polytene chromosome interval 88D (data not shown).

We were able to clone DNA sequences adjacent to the P-element insertion sites by plasmid rescue (see Materials and Methods). The structure of the genomic region in the vicinity of the *UbcD1* P-element mutant insertions is shown in Figure 2. Northern blots indicated that this region gives rise to three related transcripts. One of these transcripts is 2.1 kb in length and specific to the male germ line. It was observed in RNA from adult wild-type males but not in RNA isolated from either adult wild-type females or the adult agametic progeny of *tudor* females (Fig. 3A). This germ line-specific transcript was detectable in RNA from pupae and adult males but not earlier stages (data not shown). Two other *UbcD1* transcripts, 1.7 and 1.9 kb in length, are detected in RNA from both male and female wild-type flies, as well as in RNA from agametic flies (Fig. 3B). These RNA species.

Figure 2. Map and partial sequence of the *UbcD1* locus. (Top) Map of *UbcD1*, indicating the location of the transcripts, the P element-induced mutations used in this study, and the deletion *Δ73A*. The longest, 2.1-kb transcript is specific to the male germ line; two smaller transcripts (1.9 and 1.7 kb) are expressed broadly. Open areas of the transcripts represent exon regions that do not encode part of the *UbcD1* protein, solid areas are protein-coding regions of exons. Most of the hatched region of the transcriptional unit is composed of intronic sequences, but the details of intron/exon structure in this region have not been determined. Restriction enzyme recognition sites in genomic DNA: (B) BamHI, (H) HindIII, (P) PstI, (R) PspI, (H) XhoI. (Bottom) Partial DNA sequence starting from the 5' end of the coding strand of a near full-length cDNA representing the male germ-line-specific (2.1-kb) RNA. The downward arrow indicates the position of the 66-bp intron in this transcriptional unit. Right-pointing open arrows show the 5'-ends of the longest cDNAs obtained corresponding to the 2.1-, 1.9-, and 1.7-kb transcripts. Inverted triangles represent the sites of P-element insertions associated with *UbcD1* mutant alleles. The *effete* mutations are denoted with e; *trenino* mutations with t. The complete sequence of this cDNA has been submitted to GenBank under accession no. U68298.
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Figure 3. The UbcD1 gene encodes multiple transcripts. Autoradiography of Northern blots probed with UbcD1 cDNA clones. Sizes are indicated in kb. (A,B) DNAs were radiolabeled and used as probes against duplicate filter blots of poly(A)* RNA (5 µg per lane) from wild-type males (m), wild-type females (f), and from the agametic male and female progeny of tudor (5 vig per lane) from wild-type males (m), wild-type females (f), and used as probes against duplicate filter blots of poly(A)* RNA DNAs were radiolabeled {A,B} clones. Sizes are indicated in kb.

UbcD1 cDNA toradiograph of Northern blots probed with unrelated third chromosome locus required for spermatogenesis homozygous wild-type or mutant for the locus, probed UbcD1 scripts. (C) Filter blot of poly(A)* RNA (10 pg per lane) from flies the cDNA (base pairs 731-1226) common to all three tran­scripts or (B) a restriction fragment from a region of UbcD1 the jai, and females homozygous for a P[Z]-induced mutation in an The wild-type transcript pattern is seen in adult males as in B. 6-kb EcoRI genomic fragment that spans the entire transcrip­tion unit of the gene.

were found to be expressed throughout development and were especially abundant in early embryos (data not shown). We have obtained near full-length cDNA clones corresponding to each of these three transcripts. Restriction mapping and selected sequence analysis of these cDNAs and the corresponding genomic region has shown that the transcripts are related to each other [Fig. 2]. The bulk of the sequences are identical, but the transcripts differ at their 5' ends. The 2.1-kb male germ-line-specific transcript initiates upstream of the shorter RNAs. The genomic sequence encoding the unique part of this 2.1-kb transcript is interrupted by a 66-bp intron.

By sequencing the DNA flanking the insertions, we found that the original P-element insertions in UbcD1 all mapped to a 200-bp region near the 5' end of the transcrip­tional unit. All of the P elements lie within the second exon of the male germ-line-specific RNA, but all are upstream of the sequences representing the shortest (1.7 kb) RNA. The tre1 and tre2 insertions are within sequences encoding the 5' end of the 1.9-kb RNA; the positions of some of the remaining UbcD1 mutations with respect to the 1.9-kb RNA are ambiguous, as we do not know the exact 5' end of this transcript.

Northern blot analysis suggested that the male sterility of UbcD1 alleles was caused by disruption of the male germ-line-specific transcript. The male germ-line-specific transcript was absent or reduced greatly in abundance in adult male RNA from the viable but male-sterile UbcD1 mutant lines [Fig. 3C]. The constitutive transcripts in these RNA preparations were mostly unaffected, although their abundance appeared to be somewhat lower than in wild type.

All three UbcD1 RNA species give rise to the same protein product. Conceptual translation of the open reading frame ORF yielded a polypeptide belonging to the family of highly conserved ubiquitin-conjugating, or E2, enzymes. Database searches revealed that the identical ORF had been identified previously in a PCR screen for Drosophila homologs of yeast E2 genes [Treier et al. 1992]. The protein encoded by this Drosophila ORF was designated UbcD1. This protein is very similar in sequence (88% identity) to the E2 enzymes Ubc-2 of Caenorhabditis elegans, UbcH5A, UbcH5B, and UbcH5C of humans, and RnE2/2E and RnE2/4A from rat testis [Zhen et al. 1993; Sheffner et al. 1994; Jensen et al. 1995; Rolle et al. 1995; Wing and Jain 1995]. UbcD1 also exhibits similar but less marked similarity to the Saccha­romyces cerevisiae UBC4 and UBC5 proteins (81% and 82% identity) and to a family of five to six closely related Arabidopsis thaliana E2 enzymes [Treier et al. 1992; Gir­rod et al. 1993].

Mutations in the UbcD1 gene cause telomeric associations in larval brain cells

Examination of colchicine-treated brain squashes from larvae bearing various UbcD1 mutant allele combinations revealed a common cytological phenotype. In each case, we observed frequent telomere–telomere associations that result in a variety of aberrant chromosome configurations [Fig. 4; Table 1]. Two main types of telo­meric associations can be distinguished: double telomere associations [DTAs] conjoining one pair of sister chrom­atids with another pair, and single telomere associations [STAs] in which each of the two sister chromatids behav­es independently.

In the DTA class, the tips of two sister chromatids can fuse with two other telomeres of either the same chromo­some or of a different chromosome. In the former case, this results in a monochromosomal ring chromosome [Fig. 4e,h]; in the latter case, a dicentric linear chromosome is produced [Fig. 4b,c,e,g,h,i]. If more than one DTA occurs in a cell, polycentric rings and linear chromosomes carrying three or more centromeres are generated [Fig. 4d,f,i]. In a few cases, we observed polycentric chromo­somes involving most of the Drosophila genetic comple-
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Figure 4. Telomeric associations in larval brain cells of *UbcD1* mutants. (a–i) Colchicine-arrested metaphases; (j–m) anaphases from non-colchicine-treated preparations. (a–f, j–m) Aceto-orcein staining; (g–l) Hoechst 33258 staining. (a) Normal male metaphase; (b) a male metaphase with an XL-YS DTA dicentric (large arrow); (c) a female metaphase with two XL-autosome DTA dicentrics (arrows) and two attachments between autosomal sister telomeres (small arrows); (d) a female metaphase showing a *trenino* (little train) of chromosomes; only one chromosome of the complement is not attached to the *trenino*; (e) a male metaphase with an XL-autosome DTA dicentric (arrowhead) and a DTA ring autosome (arrow); (f) a female metaphase showing an STA involving chromosomes 2 and 3 (arrow) and a 3-4 dicentric DTA (arrowhead); (g) an XL-2-3 DTA tricentric (arrow) and a 2-3 DTA dicentric (arrowhead); (h) a normal anaphase; (i) an anaphase with a single bridge; (j) an anaphase with two resolved bridges; (k) an anaphase with bridges and an acentric fragment (arrow). Bar, 5 μm.

STAs were overall less frequent than DTAs (10% of cells exhibit STAs vs. 18% for DTAs). The most common type of STAs were those between sister chromatids [Fig. 4c]. STAs between nonsister chromatids, resulting in dicentric and polycentric linear configurations, were also observed frequently [Fig. 4h]. However, we never observed STAs producing monocentric ring chromosomes. The absence of these rings contrasts with the relatively high frequency of DTA rings and might reflect cell cycle-dependent variations in the distance between the opposite telomeres of the same chromosome [see Discussion].

Aceto-orcein squashes of colchicine-treated brains (Fig. 4a–f) additionally permit an evaluation of the relative frequencies with which the different telomeres are associated. The arms of the telocentric X and the submetacentric Y chromosomes are easily identifiable, whereas the large autosomes (chromosomes 2 and 3) are seen clearly as metacentrics. However, there are some limitations to this type of analysis. Chromosomes 2 and 3 cannot be distinguished from each other; the tiny fourth chromosomes are often difficult to visualize because of their small size. In addition, to minimize errors in scoring, we have restricted our analysis to DTAs, which are the most straightforward configurations. As shown in Table 2, our data indicate that all of the telomeres can participate in telomere–telomere associations, albeit with different frequencies. The telomeres of the major autosomes and of the left (long) arm of the X chromosome [XL] appear to have similar probabilities of involvement. These telomeres participate in DTAs more often than the XR, YL, YS, and fourth chromosome telomeres.

To ascertain whether the second and third chromosome telomeres were both involved in DTAs, we examined a limited sample of Hoechst-stained Δ112/Δ112 metaphases [Fig. 4g–i]. Hoechst staining permits unambiguous recognition of each chromosome of the *Drosophila melanogaster* complement [Gatti et al. 1994]. We examined DTAs involving 104 individual telomeres of the large autosomes. Of these, 57 were third chromosome telomeres and 47 second chromosome telomeres. The telomeres of the major autosomes therefore have similar probability of participating in telomeric associations.

The consequences of telomeric associations on subsequent steps in mitosis, we analyzed mitotic figures in non-colchicine-treated mutant brain squashes. This type of preparation allows the examination of both metaphase and anaphase figures. Metaphases showed frequent telomere–telomere attachments, indicating that colchicine has no effect on telomeric interactions in *UbcD1* mutants. Many anaphases exhibited single or multiple chromatin bridges that connected the two sets of chromosomes migrating to the opposite poles [Fig. 4k–m; Table 3]. However, three lines of evidence suggested that most telomere–telomere attachments that generate these bridges were resolved during anaphase. First, very few of these anaphases displayed lagging acentric fragments [Fig. 4m; Table 3]. Sec-
The Oregon-R wild-type strain was used as a control.

This class includes dicentric and polycentric STAs.

Table 1. Chromosome abnormalities in colchicine-arrested metaphases from UbcD1 mutant larval brains

| Genotype | No. of cells scored | STAs | Linear attachments<sup>c</sup> | Polyploid/hyperploid | Breaks<sup>a</sup> |
|----------|---------------------|------|-------------------------------|---------------------|-------------------|
|          |                     | su<sup>b</sup> | dic | 2 | 3 | 3+ | 1 | 2 | 2+ |                  |                  |
| tre1/tre1 | 669                  | 9 | 22 | 32 | 4 | 1 | 1 | 3 | 2 | 8 | 2 |
| tre1/Δ112 | 623                  | 45 | 48 | 34 | 2 | 0 | 2 | 0 | 0 | 15 | 1 |
| tre2/tre2 | 419                  | 22 | 20 | 62 | 13 | 3 | 13 | 1 | 1 | 1 |
| tre2/Δ73  | 1719                 | 77 | 70 | 242 | 34 | 13 | 64 | 10 | 1 | 13 | 11 |
| Δ112/Δ112 | 841                  | 73 | 37 | 160 | 23 | 12 | 22 | 7 | 4 | 21 | 19 |
| Control<sup>d</sup> | 481              | 3 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 2 | 0 |

<sup>a</sup>Sister unions (i.e., sister telomere attachments).
<sup>b</sup>This class includes dicentric and polycentric STAs.
<sup>c</sup>The number of chromosomes involved in linear multicentric figures is indicated below.
<sup>d</sup>The number of chromosomes involved in ring configurations is indicated below.
<sup>e</sup>Incomplete chromosome breaks (see text for explanation). Complete chromosome breaks are rare and in the range of controls.
<sup>f</sup>The Oregon-R wild-type strain was used as a control.

...ond, the frequency of metaphases with incomplete chromosome breaks [i.e., metaphases with a broken chromosome without the correspondingacentric fragment, or metaphases with a normal chromosome complement plus an extra acentric fragment] was relatively low (Table 1). These would be produced if anaphase bridges in the previous cell generation remained unresolved, therefore resulting in chromosome breakage, as occurs in l(1)nod<sup>DTS</sup> and lodestar mutant neuroblasts (Girdham and Glover 1991; M. Gatti and B.S. Baker, unpubl.). Third, the frequency of hyperploid and polyploid cells was also low (Table 1). A failure in the resolution of telomeric attachments would be expected to impair mitotic chromosome segregation, leading to the formation of hyperploid or polyploid daughter nuclei.

UbcD1 mutations disrupt male meiosis

Telomere–telomere attachments appear to take place during both meiotic divisions in Drosophila males mutant for UbcD1. In meiotic metaphase II figures we found many STAs (~20% in tre1) but we did not detect DTAs. Observations of metaphase I figures were less conclusive—no telomeric associations between chromosomes of different bivalents were found, whereas the tight pairing of the homologs did not permit a reliable scoring of DTAs and STAs within the bivalents. However, clear evidence that telomeric associations must occur in meiosis I as well as meiosis II was obtained from the analysis of meiotic anaphases and telophases. In UbcD1 mutants, 60%–80% of anaphase I and 40%–60% of anaphase II figures exhibited lagging chromatin along the two sets of migrating chromosomes (Fig. 5; Table 4). This type of meiotic abnormality was never seen in wild type. Mutant anaphases and telophases had completely normal spindles, indicating that the observed meiotic defects were not attributable to problems in spindle structure (Fig. 5).

To determine the nature of the lagging chromatin, we examined methanol/acetic acid-fixed anaphases stained with aceto-orcein. In tre1/tre1 mutants, 81% of anaphase I and 39% of anaphase II figures displayed apparently acentric chromosome fragments between the daughter nuclei (Fig. 6A, c and d; Table 5). A much lower proportion of these anaphases (4% of anaphase I and 13% of anaphase II cells) exhibited either intact lagging chromosomes (Fig. 6A,e) or nondisjunction (Fig. 6A,f).

To confirm that most of the lagging chromosomal ma-

Table 2. Involvement of individual telomeres in DTAs

| Sex | No. of telomeres<sup>a</sup> | Percent involvement in DTAs | STAs<sup>b</sup> | Linear attachments<sup>c</sup> | Polyploid/hyperploid | Breaks<sup>a</sup> |
|-----|-----------------------------|-----------------------------|-----------------|-------------------------------|---------------------|-------------------|
|     |                             |                             | su<sup>b</sup> | dic | 2 | 3 | 3+ | 1 | 2 | 2+ |                  |                  |
| F   | 1298                        |                             | 77.2 | 14.8 | 6.4 | — | — | 1.6 |                  |                  |
|     |                             |                             | (50.0) | (12.5) | (12.5) | — | — | (25.0) |                  |                  |
| M   | 559                         |                             | 77.0 | 5.9 | 3.4 | 4.6 | 4.3 | 4.5 | 6.25 | (6.25) | 4.5 | 25.0 |                  |                  |

The analysis was performed on the DTAs observed in the various mutant combination reported in Table 1. The expected frequencies are in parentheses.

<sup>a</sup>Total number of telomeres involved in DTAs.

Table 3. Effects of UbcD1 mutants on neuroblast anaphases

| Genotype | No. of anaphases scored | Normal | Irregular anaphases<sup>c</sup> | Percent aberrant anaphases |
|----------|-------------------------|--------|-------------------------------|----------------------------|
| tre1/tre1 | 90                      | 57     | 8 | 22 | 3 | 36.6 |
| tre1/Δ112 | 66                      | 40     | 3 | 18 | 5 | 39.4 |
| tre2/tre2 | 153                     | 100    | 17 | 34 | 2 | 34.6 |
| tre2/Δ73  | 223                     | 137    | 21 | 55 | 10 | 35.9 |
| Δ112/Δ112 | 83                      | 44     | 20 | 19 | 0 | 47.0 |
| Control<sup>e</sup> | 200             | 200    | 0 | 0 | 0 | 0 |

<sup>c</sup>(A) One chromatid bridge; (B) two or more chromatid bridges; (C) lagging acentric fragments between poles.
<sup>e</sup>Oregon-R.
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Figure 5. Lagging chromatin in meiotic anaphases and telophases I of UbcD1 mutant males. Shown are wild-type anaphases [a] and telophases [b] and anaphases [c] and telophases [d] from UbcD1 mutant testes. To visualize tubulin and DNA, testes were sequentially stained with antitubulin antibodies (green) and Hoechst 33258 (red). Note that the UbcD1 mutants have normal spindles with prominent central zones but exhibit lagging masses of chromatin. Bar, 10 μm.

Material consisted of acentric fragments, we performed fluorescent in situ hybridization [FISH] using probes specific for the pericentric regions of either the X or the third chromosome [see Materials and Methods]. The results of these experiments showed that in the large majority of cases, the centromeric regions of the X and third chromosomes had properly migrated toward the poles, indicating that the lagging chromatin was composed mainly of acentric fragments (Fig. 6B; Table 5).

In summary, the high frequency of metaphase II STAs, as well as the abnormalities seen in meiotic anaphases and telophases, strongly suggest that telomere–telomere attachments occur frequently in UbcD1 meiotic meta-

Table 4. Meiotic abnormalities in UbcD1 mutants

| Genotype   | Anaphase I+ | Anaphase II+ |
|------------|-------------|--------------|
|            | Total       | lag (%)      | Total    | lag (%)      |
| trel/trel  | 94          | 73           | 184      | 51           |
| tre2/tre2  | 67          | 70           | 45       | 40           |
| tre2/Δ112  | 87          | 62           | 48       | 40           |
| Δ112/Δ112  | 55          | 81           | 38       | 62           |
| Controlb   | 50          | 0            | 50       | 0            |

Meiotic anaphases and telophases were stained with anti-tubulin antibodies and Hoechst 33258 [see Fig. 5].

Mutations in UbcD1 do not affect female meiosis

Females homozygous or heterozygous for all the UbcD1 viable, male-sterile alleles are fertile. Therefore, we examined female meiosis by mating mutant females with males carrying a marked attached XY chromosome, allowing us to screen the progeny for phenotypes indicating XX and nullo-X nondisjunctive events in their mothers [see Materials and Methods]. Of a total of 2113 progeny from trel/trel females, we found no exceptions. In addition, no exceptions were found in 948 progeny from tre2/tre2 females and 1084 progeny from tre2/Δ112 females. We conclude that UbcD1 mutations do not affect female meiosis substantially.

Discussion

The role of UbcD1 in the ubiquitin-mediated pathway

Selective protein degradation by the ubiquitin–proteasome pathway has a crucial role in many cellular regulatory mechanisms, including those that govern the cell cycle [for review, see Jentsch 1992; Deshaies 1995; Hochstrasser 1995]. Ubiquitin-mediated proteolysis is a multistep process. Ubiquitin is first linked by a thiol ester bond to a ubiquitin-activating, or E1, enzyme. Activated ubiquitin is then transferred to a ubiquitin-conjugating, or E2, enzyme. Finally, this enzyme, often in cooperation with the accessory factor ubiquitin ligase, or E3, donates ubiquitin to a substrate protein. Repeated ubiquitination cycles lead to multiubiquitinated proteins that are degraded rapidly by the 26S proteasome. Most cells have several E2 enzymes and an unknown number of E3 factors, leading to the suggestion that the E2 and E3 factors may interact combinatorially to determine substrate specificity [Jentsch 1992; Chen et al. 1993; Hochstrasser 1995].

The UbcD1 gene encodes a class I E2 enzyme [Jentsch 1992; Treier et al. 1992]. Class I E2 proteins, such as the UbcD1 protein, contain only the 16-kD UBC domain common to E2 proteins [Jentsch 1992]. Class I E2 enzymes function poorly in vitro in the absence of an E3 factor [Hershko and Ciechanover 1992]. Other E2 enzymes contain additional sequences at either the carboxyl terminus [class II] or amino terminus [class III] and often carry out efficient ubiquitin conjugation without the addition of E3s [Jentsch 1992].

The UbcD1 protein exhibits a strong homology with the S. cerevisiae E2 enzymes UBC4 and UBC5, which perform overlapping functions along with UBC1. Deletion of the gene for one of these enzymes results in no evident phenotype; deletion of two of the three renders yeast sensitive to a variety of stresses; deletion of all three is lethal [Seufert and Jentsch 1990; Treier et al. 1992]. The Drosophila UbcD1-coding sequence has been transformed into yeast under the control of the yeast
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Figure 6. Abnormal meiotic chromosome segregation in UbcD1 mutant males. (A) Orcein-stained wild-type (a,b) and UbcD1 mutant testes (c–f). (a) A meiotic metaphase I in wild type; the chromosome ends are often closely associated because of the tight pairing of the homologs. This makes it very difficult to assess directly the presence of telomeric associations in UbcD1 mutants [see text for further explanation]. (b) A normal anaphase I; (c) a mutant anaphase I showing a broken chromatin bridge (arrow) and acentric fragments; (d) a mutant anaphase I with multiple lagging acentric fragments; (e) a mutant anaphase I with two apparently intact lagging chromosomes [X and fourth]; (f) a mutant anaphase I in which a pair of homologs has failed to disjoin properly. Bar, 5 μm. (B) In situ hybridization with the 359-bp satellite repeats (a, yellow signal) and the dodecasatellite DNA (b,c, red signals), which mark specifically the pericentric heterochromatin of the X and the third chromosome, respectively. (a,b) UbcD1 mutant meiotic anaphase I figures; (c) a UbcD1 mutant meiotic anaphase II. Note that in each case there is no hybridization on the lagging chromatin between the poles.

UBC4 promoter. This construct rescues a number of defects of yeast UBC4 UBC5 double mutants, restoring ubiquitin-mediated proteolysis of abnormal polypeptides (Treier et al. 1992). It is therefore quite likely that UbcD1 mediates ubiquitin-dependent proteolysis in flies.

Little is known about the substrates of class I E2 enzymes. UBC4 and UBC5 act synergistically with two other E2 enzymes to target the yeast MATα2 protein for degradation (Chen et al. 1993). Either the human UbcH5B and UbcH5C enzymes, or the related A. thaliana E2 enzyme AtUBC8, can in the presence of E6–E6AP complex (which acts as an E3 factor), mediate p53 ubiquitination (Sheffner et al. 1994; Rolfe et al. 1995).

Our cytological observations of phenotypes associated with five independent UbcD1 mutant alleles...

Table 5. Meiotic chromosome segregation in tre1/tre1 males

| Experiment, meiotic division | No. of anaphases scored | Regular | Lagging acentric fragments | Lagging chromosomes | Nondisjunction |
|-----------------------------|-------------------------|---------|----------------------------|---------------------|---------------|
| A, I                        | 91                      | 13      | 74                         | 2                   | 2             |
| A, II                      | 78                      | 38      | 30                         | 6                   | 4             |
| B, I                       | 61                      | 9       | 52                         | 0                   | —             |
| B, II                      | 57                      | 36      | 20                         | 0                   | 0             |
| C, I                       | 54                      | 23      | 30                         | 1                   | 1             |
| C, II                      | 41                      | 19      | 22                         | 1                   | 1             |

*(A) The analysis was performed by scoring aceto-orcein-stained meiotic squashes. (B and C) The anaphase figures were examined after FISH with either the 359-bp satellite DNA probe (B) that labels the pericentric heterochromatin of the X chromosome or with the dodecasatellite probe (C) that marks the pericentric heterochromatin of the third chromosome. In both B and C the anaphases with lagging acentric fragments and those with lagging chromosomes exhibit unlabeled and labeled lagging chromatin, respectively. In A the nondisjunction class includes any nondisjunctional events, whereas in B and C it solely refers to labeled chromosomes.
show clearly that UbcD1 is required for proper telomere behavior. However, at least in the male germ line, UbcD1 is likely to have additional functions. The leaky behavior during male meiosis, but are nonetheless sterile. This suggests that UbcD1 is also needed for ubiquitination of proteins required in postmeiotic stages of spermatogenesis.

The origin and fate of telomeric associations

Larval brain metaphases exhibit two types of telomere–telomere associations, DTAs and STAs. Neither kind of chromosomal configuration results from chromosome or chromatid exchanges because they are never accompanied by acentric fragments. Nevertheless, dicentric and ring DTAs are structurally similar to mutagen-induced chromosome exchanges generated during G1, whereas STAs have the same appearance as chromatid exchanges produced during S–G2 (Savage 1970). This suggests that DTAs arise from telomere–telomere fusions that occurred during G1 and that are replicated and maintained subsequently in S–G2. STAs are likely to be the consequence of associations between telomeres of chromosomes that have replicated already.

The formation of both DTAs and STAs requires the physical proximity of telomeres during interphase. Telomeres would be clustered at one side of the nucleus if chromosomes maintained their anaphase configuration in the subsequent interphase, as suggested by Rabl (1885). In D. melanogaster, a Rabl orientation of chromosomes is evident in both polytene salivary gland nuclei (Mathog et al. 1984; Hochstrasser and Sedat 1987) and embryonic nuclei (Foe and Alberts 1985; Hiraoka et al. 1990; Dernburg et al. 1996), though not in polytene chromosomes from the larval midgut (Hochstrasser and Sedat 1987), in imaginal discs, and neuroblast nuclei (Dernburg et al. 1996). To explain these findings, Dernburg et al. (1996) suggested that during the short embryonic interphases, chromosomes do not have sufficient time to relax from their anaphase configuration and, therefore, maintain a Rabl orientation throughout interphase. However, in later stages of development, when interphase is much longer, the chromosomes would have the time to assume a more compact arrangement within the nucleus.

Our observations on UbcD1 mutants suggest that the chromosomes of actively dividing brain cells maintain a residual Rabl conformation during early interphase (i.e., the G1 phase), but progressively lose it as these cells proceed through the cycle. That chromosomes continue to be arranged in a Rabl configuration for at least part of the interphase is suggested by the finding that, in both males and females, XL is involved in DTAs with a higher frequency than XR. Because XL is nearly the same length as the arms of the large metacentric autosomes, whereas XR is extremely short, in a Rabl configuration one would expect the tip of XL to be closer to the telomeres of the major autosomes than would be the tip of XR (Fig. 7). Moreover, two findings suggest that chromosomes relax from the Rabl orientation as brain cells proceed through the cycle. The observation that DTAs are more frequent than STAs can be interpreted as indicating that chromosome ends disperse when cells pass from G1 to S–G2. Similarly, the higher frequency of DTA rings compared with the STA rings may reflect cell-cycle-dependent variations in the Rabl conformation. During G1, the opposite telomeres of the same chromosome would be sufficiently close to interact to form DTA rings. However, the proximity of opposite telomeres would be reduced later in the cell cycle, preventing STA ring formation (Fig. 7).

Although our results show that telomeric associations must occur during both meiotic divisions, we believe these are restricted only to certain kinds of interactions. We did not detect interactions between telomeres of chromosomes in different bivalents during meiosis I, nor did we observe DTAs in meiotic metaphases II. The absence of telomeric associations between chromosomes in different meiotic bivalents is anticipated, because as early as prophase I, the bivalents are physically separated, which would prevent interactions between homologous chromosomes (Cooper 1965; Cenci et al. 1994). The failure of mutant metaphase II figures to show the presence of DTAs may reflect the fact that after the first meiotic division, secondary spermatocytes do not go through an S phase and are therefore unlikely to generate DTAs (see above).

Although most telomere–telomere associations in larval brain cells are resolved during anaphase, telomeric associations in male meiotic chromosomes fail to be resolved and give rise to chromosome breakage during anaphase. One explanation for this difference is that telomeres of meiotic chromosomes are connected more tightly than those of mitotic chromosomes. Alterna-
tively, these cells may differ in the mechanics or speed of anaphase chromosome movement and therefore the application of forces across telomere associations during anaphase A. This is conceivable because the meiotic spindle is much larger than the mitotic spindle (Cenci et al. 1994) and the maximum distance between the separating sets of anaphase chromosomes is higher in meiosis than in mitosis (10 μm in mitosis vs. 30 and 18 μm in the first and second meiotic division, respectively). Additional support for the latter hypothesis is the finding that although ~60% of mutant meiotic anaphases exhibit lagging acentric fragments, only 2% of these cells display chromatin bridges. This suggests that meiotic anaphase bridges persist for a shorter time than those found in somatic cells, implying that meiotic anaphase A is more rapid than mitotic anaphase in brain cells.

Interestingly, we found no evidence of chromosome loss and nondisjunction during meiosis of UbcD1 mutant females. One interpretation of these results is that telomere–telomere associations do occur in female meiosis but they are resolved before or during anaphase, as is the case for mitosis. Alternatively, female meiotic chromosomes might not undergo end-to-end associations. Ultrastructural studies on chromosome organization in Drosophila female pachytene nuclei support this second alternative. In these cells the pericentric regions of the chromosomes lie at one side of the nucleus but the telomeres are neither clustered in the typical bouquet arrangement nor obviously associated with the nuclear envelope (Carpenter 1975).

The basis for telomeric associations in UbcD1 mutants

Although our findings demonstrate that UbcD1 is required for proper telomere behavior, they do not prove that this enzyme interacts directly with telomeric proteins. For example, one could argue that telomeric associations might be the consequence of changes in cell-cycle timing, which cause chromosome end tangling. This is clearly not the case. The cytological phenotype of UbcD1 mutants is unique, in that none of the many Drosophila mitotic mutants, including those that cause more or less severe delays in the cell cycle, exhibit telomeric associations (i.e., see Gatti and Baker 1989). In addition, although telomeric associations have been observed in senescent and cancer cells, to the best of our knowledge, no one has reported that they can be induced by impairing proper progression of the mammalian cell cycle. Finally, and perhaps most importantly, Moazed and Johnson (1996) have found recently that the SIR4 protein of yeast telomeres binds the deubiquitinating enzyme UBP3, suggesting that telomeric protein ubiquitination is a general phenomenon.

Therefore, we propose that the telomeric associations we have observed in UbcD1 mutant mitoses and male meioses result from failure to degrade one or more telomere-associated proteins. We do not know the identity of these putative polypeptide targets of UbcD1. Telomeres in Drosophila are unique in their absence of short repeats and appear to be maintained by transpositions of particular retrotransposons to the chromosome ends [Mason and Biessman 1995; Pardue 1995]. Unfortunately, little is currently known about proteins that bind to these HeT-A and TART retrotransposons, or to components of subtelomeric heterochromatin.

There are three ways in which proteins could mediate telomere–telomere associations [Gilson et al. 1993]. The most straightforward is that dimers or other multimers of a telomere-binding protein might be able to associate simultaneously with two telomeres. This model has been proposed to explain protease-sensitive telomeric associations in ciliate macronuclei [Lippes et al. 1982]. Another possibility is that proteins may link telomeres indirectly, through a third element such as a component of the nuclear envelope. Finally, it is conceivable that telomere-associated proteins might facilitate DNA–DNA interactions between telomeres. The β subunit of the Oxytricha telomere protein and the RAP1 protein of yeast have the ability to help fold or stabilize simple repeat telomeric DNA into G-quartet structures that mediate telomere–telomere associations in vitro [Fang and Cech 1993; Giraldo and Rhodes 1994; for review, see Henderson 1995]. Although G quartets are unlikely to exist in Drosophila telomeres, protein-stabilized DNA–DNA interactions between either retrotransposons or other components of subtelomeric heterochromatin might explain the nucleic acid-containing fibers that link telomeres in polytene chromosomes [Rubin 1978].

Observations in many different kinds of cells indicate that the clustering and association of telomeres is a widespread aspect of nuclear architecture (see introductory section). We therefore believe that the most straightforward explanation of our results is that during at least some part of interphase in wild-type Drosophila cells, telomeres are normally associated directly or indirectly through UbcD1 targets. Action of the UbcD1 enzyme disrupts this association by prophase, where telomere–telomere interactions are not observed normally. Therefore, the metaphase and anaphase telomeric associations observed in UbcD1 mutants would be the consequence of the failure to degrade these protein targets of the ubiquitin pathway.

Materials and methods

Genetic strains

The UbcD1ter1 and UbcD1ter2 mutant alleles were isolated by a cytological screen of a collection of male sterile mutants generated in the laboratory at the Università di Roma “La Sapienza,” Italy. These mutants were obtained by transposition of the [lacW] element [Bier et al. 1989], according to the mutagenesis scheme described by Gatti and Goldberg (1991).

The mutations UbcD1eff1, UbcD1eff3, and UbcD1eff8 have been described previously as alleles of effete [Castillon et al. 1993], these have been renamed as alleles of the UbcD1 gene on the basis of the studies reported here. UbcD1eff1 and UbcD1eff3 represent insertions of a P{neo} element [Berg and Spradling 1991], UbcD1eff8 is caused by insertion of the P{lacZ, ry} element [MoDzik and Hiromi 1992]. All the UbcD1 mutant alleles were balanced over TM6c, which carries the dominant markers Sb and Tb. This allows unambiguous recognition of homozy-
gous mutant larvae and pupae based on their non-Tb phenotype. The tud mutation used to generate agametic flies has been described previously [Boswell and Mahowald 1985]. Marker mutations and balancer chromosomes are cataloged in Lindsley and Zimm [1992].

To analyze X chromosome segregation in UbcD1 mutant females, we crossed these flies with males carrying an attached XY marked with y and B*, as well as a normal Y chromosome. The progeny from these crosses were then scored for exceptional non-B* females and y B* males.

**P-element excision**

Mobilization of P-element insertions in UbcD1eff alleles was carried out as described by Eberhart and Wasserman [1995]. Many excisants of UbcD1eff and of other UbcD1eff alleles were precise excisions that reverted the mutant phenotype to wild type [data not shown]. Several imprecise excisions of UbcD1eff were obtained, including the deletions UbcD1effa and UbcD1effb described in the text. Similar procedures were used to mobilize the P[lacW] element in the UbcD1effd and UbcD1effe alleles are described by Gatti and Goldberg [1991]; again, many precise excisants that exhibited wild-type phenotypes as homozygotes were obtained.

**Nucleic acid manipulations**

Preparation of fly genomic DNA, agarose gel electrophoresis, library screening, Southern blotting, Northern blotting, hybridization, autoradiography, DNA sequencing, and sequence analysis were as described previously [Eberhart and Wasserman 1995]. All genomic clones were isolated from a λ-EMBL3 D. melanogaster genomic library provided by J. Tamkun [University of California, Davis]. cDNA clones for the male germ-line-specific UbcD1 transcript were isolated from a testis cDNA library [the gift of T. Hazelrigg, Columbia University, New York, NY] and from an adult male cDNA library [from T. Karr, University of Illinois, Urbana], both prepared in the λ Zap phagemid vector [Stratagene]. Plasmid clones of positive inserts were obtained by the in vivo phagemid rescue technique according to the manufacturer’s protocol. cDNA clones for embryonic UbcD1 transcripts were obtained from a 0- to 3-hr embryonic cDNA plasmid library [Brown and Kafatos 1988].

**Isolation of genomic DNA flanking P insertions**

Ten fly-equivalents of genomic DNA from UbcD1eff insert-bearing strains were digested with NheI and XbaI, SpeI and XbaI, or XbaI alone. Similar amounts of genomic DNA from the UbcD1transo mutations were cut with either EcoRI or PstI. The digested genomic DNA was then ligated 10–14 hr at 18°C at high dilution [five fly-equivalents/ml]; these conditions favor the circularization of fragments over their concatamerization. The products were collected by ethanol precipitation, redissolved in 10 µl of TE buffer, and one-tenth of the resuspended DNA was then used to transform high-efficiency XL-1 Blue cells [Stratagene].

**Cytological procedures**

Colchicine-treated metaphase chromosome preparations stained with either aceto-orcein or Hoechst 33258, and aceto-orcein stained anaphase figures from larval brains were obtained and analyzed as described by Gatti and Goldberg [1991] and Gatti et al. [1994]. Meiotic chromosome preparations were obtained from noncolchicine-treated larval or pupal testes. Testes were dissected in saline buffer [0.7% NaCl], transferred to a drop of aceto-orcein [Gatti and Goldberg 1991], and squashed gently.

Meiotic anaphases and telophases simultaneously stained with Hoechst 33258 and anti-tubulin antibodies were prepared as described by Cenci et al. [1994]. Tubulin and Hoechst 33258 fluorescence were recorded separately as gray-scale digital images using a cooled charge-coupled device [CCD; Photometrics] and the IP Lab Spectrum software. Images were then converted to Photoshop [Adobe] format, pseudocolored, and merged [for details, see Williams et al. 1995].

**In situ hybridization**

The probes used for in situ hybridization were the 359-bp satellite repeats, and the dodecasatellite DNA, which are localized specifically in the pericentric heterochromatin of the X and the third chromosome, respectively [Lohe et al. 1993; Carmena et al. 1993]. The 359 probe was amplified by PCR from whole genomic DNA [parameters from A. Demburg, Stanford University, Palo Alto, CA], whereas the dodecasatellite probe was synthesized as a 36-bp single-stranded oligomer. Both probes were labeled by primer extension incorporating biotin 11-dUTP. The slides were prepared by squashing gently in 45% acetic acid larval and pupal testes dissected in saline buffer [0.7% NaCl]. These slides were then frozen in liquid nitrogen; after removal of the coverslip they were air dried, denatured in 0.2 M HCl for 30 min at 37°C, and then dehydrated in ethanol at room temperature [Lohe et al. 1993]. Hybridization conditions, probe detection, and Hoescht 33258 staining are described by Williams et al. [1996].

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