The Deadbeat Paternal Effect of Uncapped Sperm Telomeres on Cell Cycle Progression and Chromosome Behavior in *Drosophila melanogaster*

Takuo Yamaki,* Glenn K. Yasuda,[†] and Barbara T. Wakimoto*¹

*Department of Biology, University of Washington, Seattle, Washington 98195, and [†]Department of Biology, Seattle University, Washington 98122

ABSTRACT Telomere-capping complexes (TCCs) protect the ends of linear chromosomes from illegitimate repair and end-to-end fusions and are required for genome stability. The identity and assembly of TCC components have been extensively studied, but whether TCCs require active maintenance in nondividing cells remains an open question. Here we show that *Drosophila melanogaster* requires Deadbeat (Ddbt), a sperm nuclear basic protein (SNBP) that is recruited to the telomere by the TCC and is required for TCC maintenance during genome-wide chromatin remodeling, which transforms spermatids to mature sperm. Ddbt-deficient males produce sperm lacking TCCs. Their offspring delay the initiation of anaphase as early as cycle 1 but progress through the first two cycles. Persistence of uncapped paternal chromosomes induces arrest at or around cycle 3. This early arrest can be rescued by selective elimination of paternal chromosomes and production of gynogenetic haploid or haploid mosaics. Progression past cycle 3 can also occur if embryos have reduced levels of the maternally provided checkpoint kinase Chk2. The findings provide insights into how telomere integrity affects the regulation of the earliest embryonic cell cycles. They also suggest that other SNBPs, including those in humans, may have analogous roles and manifest as paternal effects on embryo quality.

KEYWORDS telomere maintenance; telomere capping; cell cycle checkpoint; paternal effect; sperm nuclear basic protein

TELOMERES are the natural ends of linear chromosomes and have distinct properties from broken ends. Multiple proteins are enriched predominantly, if not exclusively, at telomeres and form a capping complex that protects telomeric DNA from engaging in aberrant DNA repair activities. Protein components of the telomere-capping complex (TCC) vary among organisms, in part because species differ in telomeric sequences and whether telomeres are maintained by telomerase or alternative mechanisms (Raffa *et al.* 2011; Mason *et al.* 2015). Nonetheless, the TCC's essential functions are well conserved. Failure to assemble TCCs results in telomeric DNA degradation, telomere fusions, and genomic instability.

Mutations in at least 12 loci of *Drosophila melanogaster* lead to telomere fusions in neuroblasts. Their analysis has led

to the identification of telomere-enriched and telomere-exclusive proteins required for telomere elongation or TCC assembly, maintenance, or function (Cenci *et al.* 2005; Pimpinelli 2006). Absence of any one component results in telomere fusions but components have distinct activities (Pimpinelli 2006). For example, heterochromatin protein 1a (HP1a) binds modified histone H3-MeK9 and represses transcription of telomeric retrotransposons and telomere elongation. HP1a also binds DNA and this activity is required for its capping function. The TCC protein HOAP binds DNA and HP1a. Although HOAP is required for capping, it does not affect retrotransposon transcription or telomere elongation. A third protein, HipHop, binds both HP1a and HOAP. HOAP and HipHop are recruited to telomeres by DNA damage checkpoint/repair proteins. Interactions between HP1a, HOAP, and HipHop are required to form stable and functional TCCs (Gao *et al.* 2010). Similar to yeast and mammalian cells (Stewart *et al.* 2012), each round of DNA replication in *Drosophila* somatic cells provides the opportunity to assemble and maintain TCCs. However, a relatively unexplored question is how TCCs, once assembled, are stably maintained in the absence of DNA replication.

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We dedicate this paper to the memory of Gerold Schubiger, our mentor and friend.

¹Corresponding author: Department of Biology, University of Washington, Box 351800, Seattle, WA 98195-1800. E-mail: wakimoto@uw.edu

The male germ line provides unique opportunities to study telomere dynamics through mitosis, meiosis, and spermiogenesis, which is the postmeiotic period of spermatid differentiation. Telomere maintenance during spermiogenesis is particularly interesting because it is prolonged relative to other spermatogenic stages, lasting ~5.5 days in *D. melanogaster* (Lindsley and Tokuyasu 1980) and 3.4 weeks in humans (Amann 2008). Moreover, extensive chromatin remodeling occurs. Transformation of round spermatid nuclei to highly condensed sperm heads typically involves histone modification or nearly whole-scale histone replacement by sperm nuclear basic proteins (SNBPs) (Eirin-Lopez and Ausio 2009).

Several studies have been informative for revealing TCC composition in the *Drosophila* male germ line. In many species, TCCs contain HP1a, HOAP, and HipHop. However, in the melanogaster group species, HipHop has a testis-specific paralog called K81 (Dubruille *et al.* 2010; Gao *et al.* 2011). In *D. melanogaster*, K81 replaces HipHop in early meiosis and *k81* mutant males produce sperm lacking TCCs (Dubruille *et al.* 2010; Gao *et al.* 2011; Dubruille and Loppin 2015). The consequence is male sterility due to a perplexing paternal effect (Fuyama 1984). Most embryos of *k81* fathers arrest by midcleavage but a few survive to late embryogenesis as gynogenetic haploids with only the maternal genome (Fuyama *et al.* 1988; Yasuda *et al.* 1995).

Here we describe the *Drosophila* deadbeat (*ddbt*) gene and show that Ddbt is an SNBP that acts downstream of K81. Ddbt is recruited to telomeres and ensures that TCCs are maintained through spermiogenesis and transmitted to offspring. We provide evidence that uncapped telomeres delay the onset of the first embryonic anaphase and that the embryo's ability to deal with uncapped telomeres and newly generated breaks changes as it progresses to later cycles. Our findings provide new insights into *ddbt*'s and *k81*'s paternal effect defects and the impact of uncapped telomeres on cell cycle regulation in early embryos.

Materials and Methods

Drosophila mutations, transgenic strains, and fertility assays

The *ddbt*^{Z4344} and *k81*² mutations were isolated by Wakimoto *et al.* (2004) and Yasuda *et al.* (1995), respectively. Y. Rong provided the *gfp::k81*; *k81*² stock (Gao *et al.* 2011); W. Theurkauf provided the *grp*^{fs1} and *mnk*^{P6} stocks (Sibon *et al.* 1997; Brodsky *et al.* 2004); and A. Royou provided the *gfp::bubR1* stock (Royou *et al.* 2010). Other strains were obtained from the Bloomington *Drosophila* Stock Center.

We isolated the *ddbt*⁺ gene from BAC clone CH322-142G7 (BACPAC Resources) in a 3.84-kb *EcoRI*–*KpnI* fragment. This fragment contains 1.69 kb upstream and 1.45 kb downstream of the longest transcript annotated for the *CG34264* gene by the Berkeley *Drosophila* Genome Project. Because this fragment allowed for transgenic rescue of the *ddbt*^{Z4344} sterility (Table 1), we used it to construct all modified transgenes. To

make an *egfp::ddbt* transgene, EGFP coding sequence and a portion of the multiple cloning site encoding amino acids YSDLELKL was isolated from pEGFP-C3 (Clontech) and inserted in-frame and immediately upstream of the *ddbt* open reading frame. The *mCherry::ddbt* versions were identical except the *egfp* sequence was replaced with a PCR-amplified fragment containing *mCherry* coding sequences from pmCherry (Clontech). Sequences containing portions of the *Mst35Ba* and *Mst77F* genes and the *ddbt*^{R31D} mutation (CGC to GAC codon change) were created by custom synthesis (Genewiz) and replaced the corresponding sequence in *eGFP::ddbt*. *EcoRI*–*KpnI* fragments containing *ddbt*⁺ or modified versions were inserted into the pBDP transformation vector (Pfeiffer *et al.* 2008) (Addgene plasmid 17566). Transgenes were targeted to the *attP40* site by PhiC31 integrase (Groth *et al.* 2004), introduced into the *ddbt* mutant background, and tested for ability to rescue male fertility in two doses. A chromosome two carrying *mCherry-ddbt* and *gfp-k81* was created by recombination, then introduced into a double mutant strain to create the stock *w*; *P*{*w*^{+mC} *mCherry::ddbt*} *P*{*w*^{+mC} *gfp::k81*}; *ddbt*^{Z4344} *k81*²/TM6B, *Sb* Tb.

For fertility assays, we set up 10 crosses of single males to four wild-type *Sevelin* females and compared average progeny yields to those of sibling control males.

Comparative analysis of the *ddbt* gene and protein

Sequences were downloaded from FlyBase (<http://flybase.org>) or National Center for Biotechnology Information (<http://ncbi.nlm.nih.gov/nucleotide>). We used MacVector software for sequence analysis, PSIPRED v3.3 for protein secondary structure prediction (Buchan *et al.* 2013), Clustal Omega for multiple sequence alignment (Sievers *et al.* 2011), and PAML4 for *d_N/d_S* analysis (Yang 2007). A subset of FlyBase-reported protein annotations were corrected or modified to optimize matches among species (Supplemental Material, Table S1). Specifically, the *D. sechellia* protein required selecting alternative splice sites based on *D. melanogaster* and *D. simulans* annotations. For *D. persimilis*, we noted an error in the assembled genome sequence, so we report the predicted amino acid sequence after correction. For *D. grimshawi*, we propose the amino acid sequence based on similarity with *D. virilis* and *D. mojavensis* proteins. Newly annotated protein sequences were based on best fit with those of most closely related species. We did not detect a *D. willistoni* ortholog using tBLASTn or by careful inspection across 100 kb of the syntenic region. The tBLASTn searches also failed to identify orthologs outside of Schizophora. Selected pairwise comparisons in Figure 2A were depicted using format of Eisman and Kaufman (2013). Datasets used to derive the NFLR consensus motifs in Figure 1D consisted of 28 Ddbt proteins from 28 insect species and 17 *Mst35Ba/b* and 9 *Mst77F* proteins from 12 *Drosophila* species (*Drosophila* 12 Genomes Consortium *et al.* 2007) and were depicted using WebLogo (Crooks *et al.* 2004).

We used sequences from *D. melanogaster*, *D. simulans*, *D. sechellia*, *D. erecta*, and *D. yakuba* to test for positive selection of *ddbt* in the melanogaster species group. Clustal

Table 1 Transgenic rescue of male sterility

Male genotype	Relative fertility ^a
<i>ddbt^{Z4344} st/TM6</i>	1.00
<i>ddbt^{Z4344} st/Df(3L)7C</i>	0.00
<i>w; ddbt^{Z4344} e</i>	0.00
<i>w; P{w^{+mC} ddbt^{+13.8}}; ddbt^{Z4344} st/Df(3L)7C</i>	1.07
<i>w; P{w^{+mC} egfp-ddbt}; ddbt^{Z4344} st/Df(3L)7C</i>	0.90
<i>w; P{w^{+mC} mCherry-ddbt}; ddbt^{Z4344} e</i>	0.95
<i>w; P{w^{+mC} egfp-ddbt^{Mst358a}}; ddbt^{Z4344} e</i>	0.00
<i>w; P{w^{+mC} egfp-ddbt^{Mst77R}}; ddbt^{Z4344} e</i>	0.00
<i>w; P{w^{+mC} egfp-ddbt^{R31D}}; ddbt^{Z4344} e</i>	0.00

^a Based on average progeny yields from 10 single male crosses.

Omega yielded 109 aligned codons, which we used to estimate synonymous and nonsynonymous substitution rates as the d_N/d_S ratio and detect positive selection using the PAML4 CODEML program. We used a log likelihood ratio test for three comparisons of neutral and selection models (M1 vs. M2, M7 vs. M8, and M8a vs. M8). CODEML analysis was run with several different initial values of d_N/d_S to check for convergence. We calculated the $-2\Delta\ln L$ and determined P -values from a χ^2 distribution. We used Bayes empirical Bayes analysis implemented in PAML4 (Yang *et al.* 2005) to predict positively selected sites. Sites with posterior probability (P) of $>70\%$ and their location on *D. melanogaster* Ddbt are reported in Figure 2B and see Table 2.

Protein localization during spermatogenesis

We used protein localization methods of Wilson *et al.* (2006). For localization during spermatogenesis, a minimum of three males and five cysts from each male were examined to assess fluorescent signal intensity and variation. Antibodies used were: primary rabbit anti-HOAP (1:1000, a gift of W. Theurkauf; Klattenhoff *et al.* 2009), mouse anti-HP1 C1A9 (1:20, DSHB), rabbit anti-DsRed (1:500, Clontech), secondary goat anti-rabbit Alexa Fluor 568 (1:200, Molecular Probes, Eugene, OR), and goat anti-rabbit or anti-mouse DyLight 650 (1:1000, Thermo Scientific). Preparations were mounted in Vectashield (Vector Laboratories, Burlingame, CA) with the DNA dye DAPI. Images were captured on a DeltaVision Elite imaging system run by softWoRx package (Applied Precision) and equipped with a sCMOS camera (PCO) and a $\times 100$ /numerical aperture (N.A.) 1.4 oil immersion objective. Optical sections of 0.20 μm were acquired and deconvolved images were analyzed using National Institutes of Health ImageJ (Schindelin *et al.* 2012).

To compare location and intensity of mCherry-Ddbt foci to GFP-Ddbt or GFP-K81 foci in spermatids, we examined spermatids of experimental *w; P{w^{+mC} mCherry::ddbt}* *P{w^{+mC} gfp::k81}/+*; *ddbt^{Z4344} k81²* males and control *w; P{w^{+mC} mCherry::ddbt}/ P{w^{+mC} egfp::ddbt}; ddbt^{Z4344}* males. Reliable detection of mCherry-Ddbt expression required immunostaining, whereas EGFP-Ddbt and GFP-K81 could be detected by GFP fluorescence. Testes from three different males were used to examine the smallest focus from a total of 100 canoe stage nuclei for each genotype. Images were captured on the DeltaVision system, typically

with 15 0.20- μm sections. Deconvolved images were used to draw a line across each focus. Pixel intensities along the line were collected by ImageJ Plot Profile. Among foci, the number of pixels with signals above background levels varied from 6 to 8 pixels. To compare signal intensity distributions, location of peaks and degree of mCherry and GFP overlap, the ImageJ Coloc2 plugin was used to calculate the Pearson's coefficient for each focus. One hundred foci were analyzed and mean Pearson's coefficient and SDs are reported.

Analysis of ddbt-induced paternal effects

To characterize parental origin of defective chromosomes in *ddbt*-derived embryos, we collected embryos of mothers homozygous for *T(2;3) lt^{x13}* (Wakimoto and Hearn 1990) and fathers that were *ddbt⁺* or *ddbt^{Z4344}* for 45 min, then aged embryos for 45 min. Embryos were bleach dechorionated, devitellinized in octane and methanol (1:1), methanol fixed, then incubated for 5 min in 0.5% sodium citrate before they were transferred to poly-L-lysine-coated slides. They were treated briefly with 45% acetic acid, squashed under a coverslip, snap frozen, dehydrated in 95% ethanol, then rehydrated and mounted in Vectashield with DAPI.

For each timed series, we used crosses to obtain control (*ddbt⁺ st/TM6, Sb e*) and mutant (*ddbt^{Z4344} st/ddbt^{Z4344} e*) brothers from the same cross to minimize differences in genetic background. The brothers were mated in parallel to *Sevelin* females. We showed that 90.1% of the embryos ($n = 604$) produced by the control cross hatched but no embryos ($n = 690$) hatched from the experimental cross. Embryo processing involved dechorionation in bleach, devitellinization in a 1:1 mixture of octane and methanol, then fixation in methanol before DAPI staining and/or immunostaining as described by Rothwell and Sullivan (2000). Primary antibodies were mouse monoclonal anti- α -tubulin (1:250, Amersham, Piscataway, NJ), rabbit anti-CNN (1:1000, gift of T. Kaufman), rabbit anti-GFP (1:1000, Torrey Pines), rabbit anti-BubR1 (1:1000, gift of Claudio Sunkel), and mouse monoclonal anti- γ -H2AV (1:1000, Developmental Studies Hybridoma Bank). Fluorescent secondary antibodies were goat anti-mouse Alexa Fluor 488 (1:200, Molecular Probes), goat anti-rabbit Alexa Fluor 568 (1:1000, Molecular Probes), and goat anti-rabbit DyLight 650 (1:1000, Thermo Scientific). For DNA staining, embryos were mounted on slides in Vectashield with DAPI (Vector Laboratories) or stained with propidium iodide before mounting in SeeDB (Ke *et al.* 2013). Images were acquired on a Leica TCS SP5 II confocal microscope with $\times 40$ /N.A. 1.25 or $\times 63$ /N.A. 1.4 oil immersion objectives or $\times 63$ N.A. 1.2 water immersion objective, with 0.42- μm optical sections. Z-series stacks were assembled using ImageJ and figures were edited using Photoshop (Adobe).

For the time series, developmental stage and cell cycle phase were determined for each embryo with scoring blinded to genotypes. The data were used to compute descriptive statistics, including mean, standard deviation, and percentages of embryos in each stage and phase for each time period.

Figure 2 Conservation of Ddbt's central core. (A) Pairwise amino acid comparisons of orthologous proteins revealed a conserved central core. Representative examples compare *D. melanogaster* Ddbt (horizontal line) with the Hawaiian fruitfly *D. grimshawi* and the medfly *C. capitata* proteins. The protein was divided into seven subregions based on Figure 1 data and subregion lengths were compared. Numbers indicate if the *D. melanogaster* region has more (white), fewer or the same (black) number of amino acids. Percent similarity is depicted by height of the boxes, inclusive of darker colored boxes whose heights show percent identity with optimal alignment. (B) Location of positively selected amino acids on *D. melanogaster* Ddbt was based on analysis of five *melanogaster* subgroup species. Asterisks mark sites under positive selection at the noted posterior probability (P). See also Table 2 and Table S1.

binary outcome (e.g., synchronous or asynchronous phases within the embryo). To compare each binary outcome between time intervals, we created a logistic regression model. If a significant difference was observed, the Tukey–Kramer method of multiple comparisons was used to determine which time differed from its previous interval with respect to the outcome. For the number of combinations, we used a nonparametric Kruskal–Wallis test to examine any differences in time. We used the SAS version 9.4 statistical software package (SAS Institute, Cary, NC) and a significance level of 0.05 for all statistical tests.

To study the effects of maternal cytoplasm on *ddbt*-induced cell cycle arrest, we crossed mothers that were heterozygous or homozygous for the *chk1* mutation (*grp^{fs1}*) or the *chk2* mutation (*mnk^{P6}*) to males that were *ddbt^{Z4344 st/ddbt^{Z4344 e}}*. We used the *w¹¹¹⁸* strain as the control for maternal genotype (Brodsky *et al.* 2004) and processed embryos as described above.

Data availability

The *ddbt* mutant strains are available upon request. The newly annotated sequences noted in Table S1 have been submitted

to FlyBase. The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.

Results

A paternal effect gene, *deadbeat* (*ddbt*), encodes a sperm nuclear basic protein

We recovered the first *ddbt* mutation from a screen for recessive male sterile mutations (Wakimoto *et al.* 2004). We found that it belongs to a rare class of paternal effect mutations in which mutant males produce fertilization-competent sperm but no viable offspring. We mapped *ddbt* to the 61B-C cytogenetic interval and found that *ddbt^{Z4344}/Deficiency* flies were male sterile (Table 1) but showed no other detectable abnormality. Of the 61B-C genes, *CG34264* was a prime candidate because publicly available expression profiles reported testis-enriched expression (dos Santos *et al.* 2015). We confirmed gene identity by sequencing *ddbt^{Z4344}* and identifying

a G/C to A/T transition in the *CG34264* open reading frame that changes the Gln48 codon to a premature stop. In addition, a transgene containing *CG34264* in a 3.8-kb genomic fragment was sufficient to fully rescue of *ddbt*^{Z4344} male sterility (Figure 1A and Table 1).

The predicted Ddbt protein, supported by cDNAs, has 117 amino acids, of which 31% are positively charged and mostly clustered in the C-terminal half. Predicted secondary structure consists of four α -helices spanning 60% of the protein (Figure 1A). The structural features and testis expression are characteristic of SNBPs (Eirin-Lopez and Ausio 2009). This protein class includes Mst35Ba and Mst35Bb, which are the products of duplicate genes, and Mst77F (Rathke *et al.* 2014). Consistent with the proposed role of SNBPs, males mutant for Mst35Ba and Mst35Bb or Mst77F produce spermatids or sperm with abnormal nuclear shapes (Rathke *et al.* 2010; Tirmarche *et al.* 2014). However, we did not detect abnormalities in sperm ($n = 3050$) of *ddbt*^{Z4344}/Df males, as assayed by staining with the DNA dye DAPI.

To study Ddbt conservation across species, we searched for orthologs and identified single copy orthologous loci in other Dipterans within the Schizophora (Table S1) but no similar sequences in non-Schizophoran insects. Species containing Ddbt sequences span an estimated divergence time of 65 MY (Wiegmann *et al.* 2011). Comparisons among predicted proteins revealed the following features. First, they are enriched in arginine, which comprises 14–30% of the residues. Second, three motifs of 5–11 amino acids, each located in a predicted α -helix, are conserved in sequence and order. Third, spacing between motifs is highly conserved, suggesting that a central core of 51–52 amino acids is critical for function (Figure 1B and Figure 2). There is more variability in the length of N and C tails. The Ddbt motif, PYLNFLRFLKR, is the most highly conserved and is particularly interesting since similar sequences were found in Mst35Ba, Mst35Bb, and Mst77F (Figure 1, C and D). The presence of the motif among these proteins supports classification of Ddbt as an SNBP and suggests an important role for SNBP function.

In a comparison of human and rodent orthologs, Swanson and Vacquier (2002) discovered that SNBPs are among the most rapidly evolving proteins. Their finding motivated us to ask whether Ddbt shares this property with mammalian SNBPs. To estimate rates of nonsynonymous-to-synonymous nucleotide substitutions within the *melanogaster* subgroup, we analyzed sequences from five species using PAML4 (Yang 2007). The results yielded a d_N/d_S (ratio of nonsynonymous-to-synonymous substitutions) of 0.60 averaged over all sites (Table 2), a value that meets the cutoff criteria of a $d_N/d_S > 0.5$, as indicating a high likelihood of being a target of positive selection (Swanson *et al.* 2004). To determine the fraction of sites under positive selection, we estimated d_N/d_S variation between sites and compared the results of models that assume neutral evolution or positive selection. The comparisons yielded statistically significant signatures of positive selection ($P < 0.05$), indicating 15.8% of amino acid sites analyzed with a d_N/d_S ratio of 3.9. These amino acids reside

outside of the central core (Figure 2B), supporting our conclusion from analysis of 28 Dipteran species that Ddbt has a constrained central core. Moreover, the data suggest that sequence variation in N and C tails is important and has been driven by advantageous consequences rather than relaxed selection.

Ddbt localizes to telomeres in postmeiotic stages of spermatogenesis

To determine Ddbt's subcellular localization, we constructed *enhanced green fluorescent protein-ddbt* and *mcherry-ddbt* transgenes driven by the endogenous *ddbt* promoter and inserted the transgenes into the *attP40* genomic site. Expression of *egfp-ddbt* and *mcherry-ddbt* rescued *ddbt*^{Z4344} male sterility to 90 and 100%, respectively, of control levels (Table 1), allowing their use to assay Ddbt localization.

We detected EGFP-Ddbt and mCherry-Ddbt only in postmeiotic spermatogenic stages. Progressively later stages of spermatid development can be distinguished by nuclear morphology as round, elongating, canoe, and fully condensed nuclei (Lindsley and Tokuyasu 1980). EGFP-Ddbt was first detected at early canoe stage. Each nucleus had two to four distinct foci, with one or two typically larger and brighter than the other foci (Figure 3A). Ddbt appearance preceded that of Mst35Bb, which appeared broadly distributed in late canoe-stage nuclei. Like Mst35Bb, EGFP-Ddbt persisted through spermiogenesis and foci were reliably detected in mature sperm. However, we were unable to detect EGFP-Ddbt in decondensing sperm chromatin shortly after sperm enter the egg ($n = 15$) or in the male pronucleus ($n = 31$) by EGFP fluorescence or immunostaining, perhaps due to rapid removal of Ddbt by factors in the egg cytoplasm.

Ddbt foci in spermatids and mature sperm resembled those reported for telomere capping proteins (Dubruille *et al.* 2010; Gao *et al.* 2011). To determine if Ddbt was telomeric, we coexpressed mCherry-Ddbt and GFP-K81 and observed identical patterns in canoe-stage nuclei (Figure 3B). Because large K81 foci represent aggregated telomeres (Wesolowska *et al.* 2013), we selected the smallest focus in each spermatid nucleus for a precise comparison of signal location (Figure 3, B and C). Analysis of the smallest foci of mCherry-Ddbt and GFP-K81 from spermatid nuclei yielded a Pearson correlation coefficient of 0.92 ± 0.04 ($n = 100$ nuclei). These values were comparable to those obtained from comparing signals in spermatids expressing mCherry-Ddbt and EGFP-Ddbt (Pearson correlation coefficient = 0.89 ± 0.04 , $n = 100$). We conclude that Ddbt is telomeric but cannot distinguish whether it resides in or in close proximity (within the 200-nm limit resolution of microscopy) to the TCC.

Ddbt maintains the TCC through spermiogenesis

To study the relationship of Ddbt to the TCC, we asked if distribution and dynamics of TCC components were disrupted in *ddbt*^{Z4344} spermatids. We assayed K81, which is present on meiotic telomeres and retained postmeiotically (Gao *et al.* 2011; Dubruille and Loppin 2015). We observed the expected

Table 2 d_N/d_S analysis of *ddbt* for positive selection

Models compared	$-2\Delta\ln L$	d_N/d_S estimate	% sites ^a with estimated d_N/d_S	Positively selected sites ^b
M1 (neutral) vs. M2 (selection)	8.32 $P < 0.05$ (d.f. = 2)	4.01	14.9	<i>13M, 78Q, 90A, 110K, 114R</i>
M7 (neutral) vs. M8 (selection)	8.88 $P < 0.05$ (d.f. = 2)	3.90	15.8	13M, 19N, 78Q, 90A, 110K, 113R, 114R
M8a (neutral with $d_N/d_S = 1$) vs. M8 (selection)	8.31 $P < 0.05$ (d.f. = 1)	3.90	15.8	

This analysis used *D. melanogaster*, *D. simulans*, *D. sechellia*, *D. erecta*, and *D. yakuba* sequences (Table S1).

^a Percentage of 109 codons aligned after removal of eight gaps from species comparisons.

^b Predicted with $P > 90\%$ (bold), $P > 80\%$ (italics), or $P > 70\%$ (underlined).

pattern of GFP-K81 foci in control spermatids. GFP-K81 signals were also present in *ddbt*^{Z4344} spermatids at elongating and early canoe stages, but signals were no longer detectable at late canoe stage (Figure 4A). We also tracked HP1a (Figure 4B) and HOAP (Figure 4C) by immunostaining and obtained results consistent with those for GFP-K81 in control and *ddbt*^{Z4344} spermatids. Therefore, Ddbt is required between early and late canoe stages for telomeric retention of at least three TCC components in spermatids. Hereafter, we describe *ddbt*^{Z4344} sperm telomeres as uncapped.

Since Ddbt was first detected at early canoe stage, we asked if its localization depended on K81. We used EGFP-Ddbt to monitor the protein in spermatids of males with a *k81*² null mutation (Yasuda *et al.* 1995). In the absence of K81, EGFP-Ddbt was not detected (Figure 4D). Thus, Ddbt is recruited to spermatid telomeres in a K81-dependent manner.

As previously described, Ddbt, Mst35B, and Mst77F share the NFLR motif (Figure 1C). To test whether Ddbt's motif is important for localization, we replaced its PYLNFLRFLKR with the Mst35Ba and Mst77F motifs in the context of the *egfp-ddbt* transgene. The replacements altered 4/11 and 5/11 amino acids, respectively, but preserved the invariant R (Figure 1C). Both changes led to male sterility (Table 1). Spermatids lacked EGFP signals as monitored by GFP fluorescence or immunostaining, suggesting that PYLNFLRFLKR may be important for Ddbt stability. We also created a transgene with the invariant R at amino acid position 7 replaced with aspartic acid (Ddbt^{R31D}). This change also resulted in male sterility (Table 1). We tested whether EGFP-Ddbt^{R31D} was expressed and localized to telomeres by monitoring EGFP fluorescence and HOAP immunostaining. In early canoe-stage *egfp-ddbt*^{R31D} spermatids, we detected 0–4 foci, which were weaker in intensity compared to the 2–4 foci observed in control spermatids. Of the 273 foci observed in 100 early canoe-stage *egfp-ddbt*^{R31D} spermatids, 50% were EGFP and HOAP positive, 43% were only HOAP positive, and 7% were only EGFP positive (Figure 4E). EGFP signals were not observed in late canoe-stage spermatids ($n = 500$) or mature sperm ($n = 700$). The detection of EGFP-Ddbt^{R31D} in 57% of the foci indicated that the mutant protein was imported into nuclei and recruited to telomeres but its recruitment and ability to maintain TCCs were compromised.

Loss of Ddbt induces a paternal effect on the earliest embryonic cell cycles

Since *ddbt* males produce sperm lacking TCCs, we characterized *ddbt*'s paternal effect to compare it to *k81*'s paternal effect. Previous studies showed that embryos of *k81* fathers arrest either in midcleavage or late in embryogenesis (Fuyama 1984; Yasuda *et al.* 1995). Early lethality was proposed to result from efficient paternal telomere fusions in cycle 1 leading to anaphase bridges and chromosome breaks that accumulate and cause arrest (Dubruille *et al.* 2010; Gao *et al.* 2011). Late lethality was attributed to failure of paternal chromosomes to participate in the first division, with maternal chromosomes proceeding through nuclear divisions and yielding gynogenetic haploids that develop to cuticle formation (Fuyama 1984).

To compare paternal vs. maternal chromosome behavior in *ddbt*'s embryos, we crossed *ddbt*⁺ control and *ddbt*^{Z4344} males to females that were homozygous for *T(2;3)lt^{x13}*, a cytologically visible reciprocal translocation (Wakimoto and Hearn 1990). We aged their embryos for 45–90 min AED and examined their karyotypes. In the control group, we observed the expected developmental stages from midcleavage to syncytial blastoderm and the expected karyotype, with 36% of the embryos in prometaphase or metaphase ($n = 60$). In contrast, *ddbt*'s embryos had variably sized clumps of chromatin, with an average number of 7 and up to 16 per embryo. In addition, 80% of *ddbt*'s embryos were in prometaphase or metaphase ($n = 60$). Abnormal mitotic figures were frequent and included anaphase configurations in which maternal chromosomes segregated to the poles but entangled paternal chromosomes lagged (Figure 5, A and A'). We observed only two clear cases of karyotypes with circular chromosomes indicative of intrachromosomal telomere fusions (Figure 5A'', $n = 327$ clumps with prometaphase or metaphase chromosomes analyzed from 45 embryos). Chromosome fragmentation was frequent and involved all chromosomes of the paternal and maternal sets (Figure 5A''').

To assess when *ddbt*-induced defects arose, we examined offspring of wild-type mothers and *ddbt*^{Z4344} or *ddbt*⁺ fathers. We collected newly fertilized eggs over a 15-min interval, aged them for set times before fixation, and assayed developmental stage, morphology, and cell cycle phase. We collected nine sets to capture events from 0 to 135 min AED. The first

Figure 3 Ddbt localizes to telomeric foci. (A) Stages of spermiogenesis were identified by nuclear shape (DNA, DAPI staining) and presence of DsRed-Mst35Bb. EGFP-Ddbt foci were detected at early canoe and later stages. (B) Canoe-stage nucleus with mCherry-Ddbt immunostaining (magenta) and GFP-K81 fluorescence (green). In the merged image, a line is drawn over the focus analyzed in C. (C) Signal intensities were collected along the line, normalized to the brightest intensity value, and plotted as relative values (AU, arbitrary units). Graph shows locations of peak intensity values and changes in pixel intensities. Bar, 5 μ m except 1 μ m in B.

three sets (0–15, 15–30, and 30–45 min AED) constitute the early time series and the next six constitute the late time series.

In *Drosophila*, sperm enter the egg at its anterior end. Within a few minutes, the sperm nucleus decondenses, forms the male pronucleus, moves to a central position in the egg pronuclear migration (PM) and replicates. Apposition of male and female pronuclei pronuclear apposition (PA) occurs at a characteristic internal position in the egg, about one-third the egg's length. Paternal and maternal chromosomes condense and align as separate groups on first spindle, which is called the gonameric spindle. Parental genomes remain as separate groups until mixing at anaphase. Cycle 1 is completed within 17 min of sperm entry (Foe *et al.* 1993). Cycles 2–7, each lasting $\sim$ 8–9 min, follow in rapid succession internally within the embryo. Cycles through 13 are characterized by synchronous nuclear behavior. Loss of synchrony and cellularization of blastoderm nuclei by membrane invaginations occurs in cycle 14.

In our early time series, observed stages were mostly PM/PA, cycle 1, cycle 2, and cycle 3 (Figure 5B). In the first 0- to 15-min set, stage distributions of control and *ddbt*'s embryos were indistinguishable. Over 50% in both sets were at the PM or PA stage. Pronuclear morphology and placement in the egg appeared normal, indicating that Ddbt is not required precycle 1.

Stage distributions differed markedly between the groups at 15–30 min AED ($P < 0.0001$) and 30–45 min AED ($P < 0.001$), with *ddbt* offspring showing developmental delay in cycle 1. Since our previous analysis of older embryos indicated a high proportion of *ddbt*'s embryos in prometaphase or

metaphase, we asked whether the cycle 1 delay could be in the onset of anaphase. We compared the proportion of embryos in phases before anaphase to the proportion in anaphase or later phases for control and experimental groups (Table 3). In the 15- to 30-min-AED set, a clear difference was evident, with 37.7% of control embryos at phases prior to cycle 1 anaphase compared to 51.5% of *ddbt*'s embryos ($P < 0.0001$). Developmental delay was also observed as the increased number of *ddbt*'s embryos in cycle 2 and cycle 3 in both 15- to 30- and 30- to 45-min-AED sets ($P < 0.05$ in all cases). Thus, defective paternal chromosomes result in a delay in the onset of anaphase as early as cycle 1 and with a continuing impact in the next two cycles.

Cytological observations provided morphological evidence of defects in the earliest cell cycles. Control embryos appeared normal at cycles 1, 2, and 3 ($n = 604$, Figure 5, C and D) but *ddbt*'s embryos showed defects in all three cycles ($n = 584$). We observed an occasional difference in the degree of condensation of parental chromosome sets as early as cycle 1 prometaphase (5.5%, $n = 128$) and metaphase (7.9%, $n = 114$, Figure 5E). Spindle morphology appeared normal, as assayed by α -tubulin and centrosomin (CNN) immunostaining, and maternal and paternal chromosome sets initiated anaphase. Abnormalities were striking and consistent at anaphase (Figure 5, F–H) and telophase (Figure 5I). By late anaphase, one set clearly proceeded to segregate to the spindle poles while the other lagged in the midzone. This defect was observed in all embryos in anaphase and telophase of cycle 1 ($n = 54$), cycle 2 ($n = 44$), and cycle 3 ($n = 22$), indicating consistent delay or failure in paternal chromosome

Figure 4 Ddbt requires K81 for its telomeric recruitment and maintains TCCs through spermiogenesis. TCC components in control and *ddbt*^{Z4344} spermatids: (A) GFP-K81 as assayed by GFP fluorescence, and (B) HP1 and (C) HOAP, as assayed by immunostaining. (D) EGFP-Ddbt in control and *k81*² spermatids. (E) Spermatids expressing EGFP-Ddbt^{R31D} assayed for EGFP fluorescence (green) and HOAP (magenta). The merged image shows foci containing both proteins (white), predominantly EGFP-Ddbt^{R31D} (arrowheads) or predominantly HOAP (arrow). Bar, 5 μ m.

segregation. We also noted configurations in which distal regions of chromosome arms in the lagging set remained at the midzone (Figure 5, A' and J). These appeared to be associations between extended telomeric regions of chromatids, as indicated by intense DAPI staining. We refer to these as telomere associations (TAs) rather than telomere end-to-end fusions, which are best documented as a chromatin bridge extending between segregating chromosomes. By the end of cycle 1 and cycle 2, embryos did show a thin chromatin bridge extending between well-separated daughter nuclei (Figure 5, K and L) ($n = 20$), providing evidence that at least some TAs can lead to telomere fusions. We conclude from the early time series that uncapped telomeres

cause delayed anaphase onset, frequent TAs, delay or failure in paternal chromosome segregation, and chromatin bridging in the earliest cell cycles.

The majority of *ddbt* offspring show early arrest and chromosomal catastrophes

To understand how *ddbt*-induced early defects lead to embryonic lethality, we examined the late time series, which consisted of embryos aged from 45 to 135 min AED. By this time, 83% of control embryos had developed beyond cycle 6 (Figure 6A). In contrast, *ddbt*'s embryos showed a range of phenotypes that warranted a first-level classification into two categories based on developmental progression. The majority

Figure 5 Uncapped paternal telomeres disrupt chromosome behavior and delay progression of the earliest embryonic cycles. Confocal images of chromosomes in embryos of *T(2;3)It¹³* mothers and *ddb1* fathers. (A) Maternal chromosomes, including *T(2;3)* (line), are advanced in anaphase segregation while entangled paternal chromosomes (bracket) lag. Karyotypes also include (A') telomere associations (TAs, arrow), (A'') ring chromosomes (arrows), and (A''') extensive fragmentation. (B) Comparison of stage distributions of control and *ddb1*'s embryos in the early time series. Embryo age is reported as minutes after egg deposition (AED). Embryo stage is classified as pronuclear migration/pronuclear apposition (PM/PA), or nuclear cycle. *P*-values, χ^2 test. (C and D) Normal gonameric spindles in control embryos. (E–L) Abnormalities (arrows or brackets) in *ddb1*'s embryos were: (E) hyper-condensed paternal metaphase chromosomes that were spatially distinct from maternal chromosomes; (F–J) lagging anaphase chromosomes with TAs; and (K and L) chromatin bridge. DNA, cyan; tubulin, yellow; centrosomin, magenta. *z* = focal plane. Bars, 10 μ m.

had only a few chromatin clumps indicative of early arrest and a minority had progressed further but showed haploid karyotypes (Table 4).

As noted previously, *ddb1*'s embryos in cycles 1–3 showed 100% penetrance of the TA phenotype. Analysis of the late time series confirmed that nearly all of *ddb1*'s embryos completed cycle 1 (98.7%) and cycle 2 (90.6%) (Figure 6A). Thus, TAs were apparently resolved to permit completion of these cycles. Surprisingly, only 22.8% completed cycle 3 and none finished cycle 6. Across the entire late time series, embryos produced an average of six nuclei (Table 4). Thus,

there is a fundamental difference in response to uncapped telomeres in the first two cycles compared to later cycles.

Although *ddb1*'s embryos arrest in or shortly after cycle 3, they undergo changes that are informative for identifying which nuclear or cytoplasmic processes are influenced by uncapped telomeres (Table 4). For instance, chromatin clumps became more dispersed in embryos over time. By 120 min AED, nearly 50% of the embryos show a dispersed distribution of clumps along the anterior–posterior axis. This indicates that the cytoskeleton-dependent axial expansion of nuclei, which takes place in normal cycle 4–6 embryos at

Table 3 The *ddbt*-induced developmental delay relative to anaphase

Paternal genotype no. of embryos	Minutes AED					
	0–15		15–30		30–45	
	Control 385	<i>ddbt</i> 341	Control 459	<i>ddbt</i> 408	Control 191	<i>ddbt</i> 136
Percentage of embryos						
Earlier than cycle 1A	72.5	73.3	37.7	51.5	0	0
Cycle 1A or later	27.5	26.7	62.3	48.3	100	100
	ns		$P = 5.26\text{e-}5$			
Earlier than cycle 2A	85.2	87.1	70.6	86.8	6.8	16.9
Cycle 2A or later	14.8	12.9	29.4	13.2	93.2	83.1
	ns		$P = 6.24\text{e-}9$		$P = 0.0065$	
Earlier than cycle 3A	90.7	90.9	81.5	92.2	58.1	69.1
Cycle 3A or later	9.3	9.1	18.5	7.8	41.9	30.9
	ns		$P = 3.77\text{e-}6$		$P = 0.049$	

P-value, Fisher's exact test. ns, not significant; AED, after egg deposition.

45–60 min AED (Baker *et al.* 1993), can occur in *ddbt*'s embryos, albeit with delayed timing.

We observed a suite of abnormalities, including loss of nuclear synchrony, hyper-condensation, variations in ploidy, and chromosome fragmentation. Defects generally increased in frequency with age but there were differences in when they appeared. Although embryo age varied as much as 15 min within a time set due to the egg collection interval, we observed abrupt increases in occurrence of some defects in a narrow 15- to 30-min interval. For example, only 6% of *ddbt*'s embryos in cycle 3 showed asynchronous nuclear behavior by 45 min AED. By 60 min AED, percentage increased to nearly 50% and by 90 min AED, 93.1% of embryos exhibited asynchrony. Centrosomal defects and disorganized spindles were common, reflecting disruption in coordination of chromosomal and centrosomal cycles (Figure 6, B and C). Asynchrony decreased significantly by 105 min AED due to accumulation of prometaphase or metaphase configurations. Nonuniform chromatin condensation or hyper-condensation were observed within nuclei (Figure 6D), with the most significant increase in frequency occurring 75–90 min AED. Marked rise in aneuploidy in this same interval was consistent with continued chromatin loss from spindles. While average number of nuclei per embryo did not change, multiple rounds of DNA replication continued so that nearly all embryos had at least one hyperploid chromatin clump by 90–105 min AED. Strikingly, in the 75- to 105-min-AED interval, there was a sharp increase in the frequency of extensive chromosome fragmentation, involving both parental chromosome sets.

In summary, analysis of the late series identified critical transition points that dictated how nuclei dealt with uncapped telomeres and ultimately led to defects commonly referred to as mitotic catastrophes. An important transition occurred in cycle 3 that prevented most nuclei from continuing through division. Variation in nuclear behavior may be due to differences in the number of uncapped chromosomes inherited by daughter nuclei but embryos in this category responded by developmental arrest no later than cycle 6. Maternal and

paternal chromosomes continued to show dynamic changes in condensation and replication until they arrested in a prometaphase- or metaphase-like state, then fragmented due to an unknown mechanism.

Uncapped telomeres in the earliest embryonic stages do not show detectable levels of γ -H2Av or BubR1 markers typical of DNA breaks

Because uncapped telomeres are recognized as double-strand DNA breaks (DSBs) in somatic cells (Rong 2008), we asked whether uncapped telomeres are similarly recognized as DSBs in the earliest embryonic cycles. We monitored the phosphorylation status of the DSB marker H2Av (γ -H2Av) by immunostaining (Lake *et al.* 2013). We did not detect γ -H2Av in *ddbt*'s embryos that were in cycles 1–3 ($n = 64$). However, γ -H2Av was reproducibly detected in late stage embryos ($n = 10$) that showed chromosome fragmentation (Figure S1A). As a comparison, we collected embryos from fathers that had been treated with 15,000 rads of γ -rays. This dose of irradiation results in early cell cycle arrest in offspring of treated males (Scriba 1964). We did not observe γ -H2Av foci in these embryos in cycles 1–3 ($n = 28$). However, γ -H2Av foci were detected in embryos aged 45–90 min AED when they exhibited extensive chromosome fragmentation (Figure S1A).

BubR1, a component of the spindle assembly checkpoint at the kinetochore, has also been shown to localize to HOAP-depleted telomeres and sites of DNA breaks in larval neuroblasts (Musaro *et al.* 2008; Royou *et al.* 2010). We asked whether BubR1 localized to uncapped telomeres in *ddbt*'s embryos. We assayed for GFP fluorescence in embryos of GFP-BubR1 mothers and *ddbt* fathers. We did not detect GFP-BubR1 telomeric signals in cycle 1–3 embryos ($n = 26$) (Figure S1B). As expected, GFP-BubR1 was detected at kinetochores in prometaphase chromosomes in embryos in cycles 1–3 ($n = 10$).

Although we did not detect γ -H2Av or BubR1 at uncapped telomeres in *ddbt*'s embryos in cycles 1–3, we note that γ -H2Av was not detected in early embryos with heavily irradiated paternal chromosomes that likely carry many DSBs. In

Figure 6 Uncapped paternal telomeres cause lethality due to cell cycle arrest or production of haploids or haploid mosaics. (A) Comparison of cycle completion of control and *ddbt*'s embryos from analysis of the late time series. * $P < 0.05$, ** $P < 0.001$, χ^2 test. (B) Region from a control cycle 6 embryo showing synchronous metaphases. (C) Region from an embryo of a *ddbt* father showing asynchronous phases, excessive or free centrosomes, and chromatin hyperploidy. (D and E) Two representative embryos of *ddbt* fathers. (D) Embryo arrested at cycle 3 with a mix of prometaphase chromosomes and hyper-condensed chromatin. (E) Haploid mosaic that progressed past cycle 7 with (E') hyperploidy and aneuploidy, and (E'') mitoses with different chromosome content, including haploid anaphases (arrows). Bars, 10 μm except 100 μm in D.

both cases, $\gamma\text{-H2Av}$ was detected at broken ends in later stages. More sensitive assays may be needed to detect these proteins in the early embryo. Alternatively, uncapped telomeres and DNA breaks may not be processed in the early embryo as they are in neuroblasts and so these proteins are not localized to these chromosome ends.

Selective elimination of paternal chromosomes creates lethal haploid and haploid mosaics

As predicted from *k81* studies, a minority of *ddbt*'s embryos progressed beyond the early cycles and showed haploid karyotypes. Careful inspection of these embryos yielded two unexpected findings. First, their frequency increased over time (Table 4). Second, only a subset uniformly exhibited haploid

karyotypes or interphase nuclei typical of true haploids that result from cycle 1 elimination of the paternal genome. The remaining embryos exhibited a mixture of haploid, hyper-, and hypohaploid karyotypes (Figure 6, E–E''). This larger class of haploid mosaics and their increased frequency over time can be accounted for by loss of paternal chromosomes in cycle 2 or later. We observed variability in chromosome content of nearby spindles (Figure 6E''), indicating that loss events continued within a lineage and involved a subset or all paternal chromosomes. Haploid and haploid mosaics comprised 7% of all embryos at 45–60 min AED but up to 40.6% by 120 min AED. We observed mosaics that had progressed to syncytial blastoderm stage in the late series. However, most died before late embryogenesis. This conclusion is based on

Table 4 Time course of *ddbt* paternally induced embryonic abnormalities

	Minutes AED					
	45–60	60–75	75–90	90–105	105–120	120–135
No. of embryos	57	75	76	51	64	66
Early arrest category (%)	93.0	96.0	76.3	66.7	59.4	83.3
Median no. of clumps (min, max)	6 (3, 21)	6 (1, 32)	6 (2, 13)	6 (1, 14)	6 (1, 29)	5 (2, 11)
With axial expansion of nuclei (%)	7.6	11.1	6.9	20.6	51.1	47.3
With asynchronous nuclei (%)	45.3	72.2	93.1*	58.8*	53.3	10.9*
			OR 5.2	OR 0.11		OR 0.11
			CL 1.7–16.2	CL 0.03–0.4		CL 0.04–0.3
With hyper-condensed chromatin (%)	5.7	13.9	70.7*	88.2	77.8	92.7
			OR 15.0			
			CL 6.2–35.9			
With aneuploidy (%)	30.1	38.9	74.1*	64.7	51.1	30.9
			OR 4.5			
			CL 2.2–9.6			
With hyperploidy (%)	26.4	51.4	60.3	91.2*	84.4	100
				OR 6.8		
				CL 1.9–24.8		
With fragmented chromosomes (%)	17.0	12.5	43.1*	85.3*	80.0	100
			OR 5.3	OR 7.7		
			CL 2.2–12.7	CL 2.6–22.6		
Haploid/haploid mosaic category (%)	7.0	4.00	23.7	33.3	40.6	16.7

OR, odds ratio; CL, 95% confidence limits. * $P < 0.05$, Tukey–Kramer method (significantly different from preceding time).

an assay of *ddbt*'s embryos at 24 hr AED. We found that only 7.2% ($n = 681$) survived long enough to produce the defective denticle belt pattern and mouthparts characteristic of haploid embryos (Fuyama 1984).

Our discovery of *ddbt*-induced haploid mosaics showed that paternally inherited chromosomes were subject to continued elimination, even after several rounds of DNA replication. Similarly, we observed that up to 20% of the embryos of *k81* fathers are haploid or haploid mosaics at 90 min AED (Table S2 and Figure S2). Consistent with our results with *ddbt*'s embryos, only a few of *k81*'s embryos survive to late embryogenesis (Yasuda *et al.* 1995; Langley *et al.* 2011). In these haploid and haploid mosaic embryos, a sufficient number of paternal chromosomes must have been eliminated from most spindles to avoid the cycle 3 arrest and allow haploid or aneuploid nuclei to populate the embryo.

Reduced level of maternal checkpoint kinase 2 relieves *ddbt*-induced cycle 3 arrest

Our studies showed that embryos produced by wild-type mothers and *ddbt* fathers delayed initiation of anaphase at cycle 1 and the majority arrested in or shortly after cycle 3. We asked whether either phenotype was regulated by Chk1 or Chk2 kinases, which are best characterized as regulator of checkpoints at later stages of embryogenesis (Fogarty *et al.* 1997; Takada *et al.* 2003). We analyzed embryos produced by *ddbt* fathers and mothers that were homozygous or heterozygous for *grp^{fs1}* or *mnk^{P6}*, and therefore produce eggs with reduced levels of Chk1 or Chk2, respectively. In contrast to females from our wild-type strain, *grp* and *mnk* mutant females produced fewer embryos, and even short 15-min egg collections yielded embryos at a wide variety of developmental stages. Because of these features, we were unable to

quantitatively assess the effect of maternal genotype on timing of cycle 1 anaphase initiation. However, we could examine effects on the *ddbt*-induced cycle 3 arrest. Stage distribution of embryos ($n = 57$) produced by *ddbt* fathers and *grp/+* mothers were similar to that of embryos ($n = 49$) of *grp/grp* mothers with >60% of the embryos in cycle 1–3 at 30–45 min AED (data not shown). This comparison indicates that reduced levels of Chk1 did not have a striking effect on the cycle 3 arrest phenotype.

In contrast, the Chk2 mutation, *mnk^{P6}*, profoundly affected developmental progression of *ddbt*'s embryos. The effect was observed in offspring of mothers with reduced levels of Chk2, consistent with its known haploinsufficiency (Iampietro *et al.* 2014). As expected, the majority of embryos produced by control mothers fail to progress past cycle 3 by 45 min AED. However, the majority of embryos produced by *mnk/+* or *mnk/mnk* mothers and *ddbt* fathers progressed beyond cycle 3 by 45 min AED (Figure 7A). Moreover, embryos in the later cycles frequently showed lagging chromosomes, anaphase bridges, and nuclei with variable chromosome content (Figure 7B). These features showed that nuclear cycles were allowed to continue even in the presence of damaged chromosomes. We conclude that the *ddbt*-induced cycle 3 arrest is Chk2 dependent.

Discussion

Sperm nuclear basic proteins comprise an ancient and widespread protein class in animals. Their origin has long been the topic of speculation but current data suggest derivation from histone H1 (Eirin-Lopez and Ausio 2009). Molecular and temporal profiles support the notion that SNBPs function in spermatid nuclear condensation. However, only a few SNBPs

have been knocked out to critically assess function (Rathke *et al.* 2014). Our study reveals a different role for one SNBP. We show that Ddbt is a telomere-enriched SNBP. It achieves its localization after meiotic replacement of the TCC HipHop with its sperm-specific version K81, but before spermatids incorporate Mst35Ba, Mst35Bb, and Mst77F. We propose from its temporal profile and mutant phenotypes that Ddbt is unlikely to be a capping protein. Instead, it requires TCCs for telomeric recruitment but functions to guard against TCC loss in spermatids during replacement of chromosomal proteins with other SNBPs.

Spermatid remodeling is typically envisioned as bulk histone removal and replacement by SNBPs, but there is evidence for regional retention of proteins at specific loci. In human sperm, sites of retained histones are enriched in developmentally important genes (Hammoud *et al.* 2009). There is long-standing evidence for retention of a centromeric protein in bovine sperm (Palmer *et al.* 1990). We expand the list of retained proteins to include TCCs in *Drosophila* sperm with retention achieved through action of a regionally restricted SNBP. To our knowledge, Ddbt is the first reported example of an SNBP that retains chromosomal proteins through spermiogenesis and ensures normal behavior of paternal chromosomes during embryogenesis. We predict that other SNBPs may be similarly specialized and when disrupted, could cause paternal effect defects.

The view of Ddbt as a specialized SNBP raises questions about its origin and conservation. Ddbt's function has only been assessed in *D. melanogaster*, but conservation of its core region suggests conserved function. If so, then the lineage that gave rise to Schizophoran species recruited Ddbt to meet the challenge of TCC maintenance during spermiogenesis >65 MYA.

Previous studies reported that mammalian SNBPs (Swanson and Vacquier 2002) and *Drosophila* TCC-restricted proteins (Raffa *et al.* 2011) are rapidly evolving. Ddbt is a telomere-enriched SNBP with rapidly evolving N and C tails. Its overall d_N/d_S places it in the 99.9 percentile when compared to 8510 single copy orthologs in the *melanogaster* subgroup whose d_N/d_S ratios were similarly calculated (*Drosophila* 12 Genomes Consortium *et al.* 2007). Rapid evolution of reproductive proteins has been attributed to a variety of selective forces, including sperm competition and fertilization competency (Swanson and Vacquier 2002). Rapid evolution of TCC restricted proteins has been attributed to relaxed constraints on telomere sequences in the Dipteran lineage, after the loss of telomerase (estimated 260 MYA) and with adoption of retrotransposon-based telomere elongation mechanisms (estimated 65–230 MYA) (Mason *et al.* 2015). We propose that Ddbt may be coevolving with TCCs to enable its telomere recruitment. It may also be coevolving with interacting egg proteins that remove it from sperm chromatin or use it to assemble new TCCs.

Ddbt may prevent TCC loss by directly interacting with the capping complex or by creating a protective chromatin environment. Whatever the mechanism, we expect consequences

of its loss to be as severe or more severe than K81 loss. Our results confirm those from *k81* studies in demonstrating that loss of K81, HP1a, and HOAP is not problematic for spermiogenesis. Although we cannot eliminate the possibility that Ddbt has additional nontelomeric functions, we did not observe defects in sperm nuclear condensation, production of fertilization competent sperm, or male pronuclear behavior. Instead, *ddb1*^{Z4344} offspring show striking defects. High penetrance of the paternal effect defects is likely due to complete TCC loss during spermiogenesis and complete failure to assemble TCCs on newly replicated paternal chromosomes, an expected outcome because *Drosophila* telomere capping is epigenetically determined rather than sequence dependent (Rong 2008).

Persistence of uncapped telomeres in *ddb1*'s embryos allowed us to investigate how early embryos respond to uncapped telomeres. Quantitative analysis of a carefully timed series identified responses that were not previously detected in *k81* studies but account for the unusual paternal effect shared by *ddb1* and *k81* mutants. As summarized in Figure 8, we account for observed frequencies of early arrest and haploid embryo categories. Importantly, we show that uncapped telomeres are detected in the earliest cycles and elicit different responses in cycles 1 and 2 compared to later cycles. We provide evidence that uncapped telomeres delay anaphase onset as early as cycle 1. We speculate that this delay may be due to the existence of a telomere checkpoint in the earliest cell cycles. Once anaphase initiates, the entire set of *ddb1* paternal chromosomes while present on the gonosomal spindle only infrequently fails to participate in the first division, resulting in true gynogenetic haploids. Most frequently, both maternal and paternal sets initiate anaphase, but uncapped paternal chromosomes are relatively delayed in segregation. Delay in anaphase segregation may be due to prevalent and persistent TAs. These TAs or telomere fusions may mechanically hinder chromosome movement to the poles yet *ddb1*'s embryos complete cycles 1 and 2 and enter cycle 3. In or shortly after cycle 3, uncapped telomeres are perceived as a Chk2-dependent signal to stop division. Signal strength may depend on the number of uncapped telomeres and newly generated breaks. Most cycle 3 nuclei halt division in or shortly after cycle 3. However, nuclei that lose a sufficient number of uncapped chromosomes undergo one or more additional divisions, generating embryos that may either proceed as far as cycle 6 before arresting or continue longer as haploid mosaics. Precedence for a threshold mechanism of signal strength is provided by the elegant studies of Kaul *et al.* (2012). They showed that normal human cells in culture spontaneously produce dysfunctional nonfusogenic telomeres and the accumulation of five such telomeres is sufficient to induce cell cycle arrest.

It is unclear whether embryos in cycles 1–3 are proficient at perceiving uncapped telomeres as either dysfunctional telomeres *per se* or more generically as DSBs. Detection as dysfunctional and irreparable telomeres and the response to delay anaphase onset are consistent with findings of Musaro

Figure 7 Reduced levels of maternal *chk2* permit *ddbt*'s embryos to progress past cycle 3. (A) Comparison of stage distributions of embryos produced by *ddbt* fathers and control (*w¹¹¹⁸*) mothers, to those of *mnk^{P6}* heterozygous or homozygous mothers. (B) Cycle 7 embryo of a *ddbt* father and *mnk^{P6}* homozygous mother showing asynchronous phases with prevalent chromatin bridges visible at telophase. Bars, 100 μ m in B and 10 μ m in B'.

et al. (2008) who showed that HOAP-depleted telomeres reduce the frequency of anaphases in larval neuroblasts. Detection of uncapped telomeres as DSBs is supported by the largely similar phenotypes of *ddbt*'s offspring and offspring of males treated with high doses of X rays. Scriba (1964) X-ray irradiated males with 15,000 rads. The dose was high enough to ensure that offspring were invariably lethal but the extent of damage among sperm could not be controlled. Nonetheless, Scriba noted that embryos showed asynchronous nuclei and enrichment at prometaphase and metaphase. He concluded that paternal DNA breaks delay mitotic progression but he could not pinpoint when delay occurred. He noted early embryonic arrest before cycle 6, which is similar to that observed in *ddbt*'s and *k81*'s offspring (Yasuda *et al.* 1995). As pointed out by Counce (1973), midcleavage crisis can result from a variety of experimental or genetic interventions that damage nuclei or cytoplasm in the early embryo.

In yeast and in human and *Drosophila* somatic cells, uncapped telomeres induce a DNA damage response. In *Drosophila*, mutants that lose TCC components in neuroblasts typically show high frequencies of telomere fusions (Raffa *et al.* 2011). Early embryos may show a weaker response to TCC loss. Our observation of frequent TAs is similar to the rather unusual effect of UbcD1 loss in larval neuroblasts (Cenci *et al.* 1997). We suggest that uncapped telomeres induced by *ddbt* may not be efficient substrates

for DNA repair and ligation. Alternatively, the maternal machinery required to recognize and join uncapped telomeres may not be fully functional in the earliest embryonic cycles. Either explanation is consistent with our inability to detect γ -H2Av and BubR1 at the telomeres of *ddbt*'s embryos. Notably, in their description of maternal effect mutants affecting telomere capping in embryos, Gao *et al.* (2009) reported that only a few telomere fusions occurred in embryos prior to cycle 7 but their frequency increased at later stages.

Our hypothesis of a checkpoint sensitive to telomere integrity in the first embryonic cycle may be considered surprising as it is often stated that early embryos lack cell cycle checkpoints. In their seminal paper on checkpoints, Hartwell and Weinert (1989) suggested that compared to yeast and somatic cells, embryos may lack some cell cycle checkpoints and respond differently to incomplete DNA replication and DNA breaks. They reasoned that by allowing cycle progression, embryos might sacrifice mitotic fidelity during rapid, synchronous divisions, then compensate later by eliminating damaged nuclei. Their ideas were based in part on the behavior of checkpoints and responses in *Drosophila* embryos at the syncytial blastoderm stages (O'Farrell *et al.* 2004). Our work adds to the few existing studies that have considered checkpoints and responses in *Drosophila*'s earliest cell cycles. For example, Brunk *et al.* (2007) showed that early cell cycle progression was delayed in embryos of *microcephalin* mutant

Figure 8 The *ddb1*-induced paternal effects on early embryogenesis. This sequence begins with the apposition of female and male pronuclei (red and blue PN). Parental genomes enter cycle 1 and align as separate groups on the first spindle. Normally, telomeres of maternal and paternal chromosomes are capped (top sequence). Progression through cell cycles occurs in a timely fashion, with both sets of chromosomes synchronously entering each cycle, beginning anaphase, and completing each cycle (boxed numbers 1–3, respectively), ultimately yielding viable diploids. Embryos of *ddb1* fathers have uncapped paternal telomeres. Their defects may suggest the existence of cycle 1–3 checkpoints that monitor uncapped telomeres or new breaks gener-

ated after telomere fusions (middle and bottom sequences). When paternal chromosomes are uncapped, embryos delay onset of cycle 1 anaphase. In <10% of the embryos, the entire paternal genome fails to segregate in cycle 1 anaphase (middle sequence) but the maternal genome proceeds, resulting in gynogenetic haploids. In the majority of *ddb1*'s embryos (bottom sequences), paternal chromosomes enter anaphase and exhibit frequent telomere associations. Chromatin bridges are observed in telophase. These embryos complete cycles 1 and 2 and most will arrest in or shortly after cycle 3 in a Chk-2 dependent manner. However, in some embryos, one or more nuclei may lose paternal chromosomes in cycle 2 or later. Loss of a sufficient number of paternal chromosomes allows these embryos to bypass cycle 3 arrest and develop as haploid mosaics. Although uncapped paternal chromosomes may be eliminated at different times, the result is always embryonic lethality.

mothers. These embryos exhibited uncoordinated nuclear and centrosomal cycles as early as cycle 1. Similar to the *ddb1*-induced cycle 3 defect, *microcephalin*-induced mitotic defects were partially relieved by reduced levels of the Chk2 checkpoint kinase (Rickmyre *et al.* 2007).

As noted by Rickmyre *et al.* (2007), the regulation of *Drosophila*'s earliest cycles can be challenging to study. These cycles occur before or just after egg deposition and deep within the egg, so they are refractory to live imaging and treatments with inhibitors that have been instrumental in documenting checkpoints in later stages. Maternal effect mutations have been especially useful for analyzing checkpoints in syncytial blastoderm embryos (Farrell and O'Farrell 2014). However, few maternal effect mutations affect cell cycle regulation only in embryos. They must allow egg production and so may produce a residual amount of protein product. Hence, their impact on early cycles may be minimal and difficult to detect. Our studies of *ddb1* had the advantage of analyzing embryos with completely normal maternal contributions and severely impaired and irreparable paternal defects. We contend that continued studies of *ddb1* and other strict paternal effect mutations will provide additional insights into cell cycle checkpoints and responses in early embryos.

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The Deadbeat Paternal Effect of Uncapped Sperm Telomeres on Cell Cycle Progression and Chromosome Behavior in *Drosophila melanogaster*

Takuo Yamaki, Glenn K. Yasuda, and Barbara T. Wakimoto

Figure S1. Phosphorylated H2Av and GFP-BubR1 in Early Embryos.

(A) Representative images showing detection of γ H2Av foci (magenta) on extensively fragmented chromosomes of an embryo fathered by a heavily irradiated male (top) and an embryo fathered by a *ddb1* male (bottom). Both embryos were arrested in cycle 3 and were assayed between 45 and 90 min AED. (B) In these cycle 1 embryos produced by *ddb1* fathers and mothers expressing GFP-BubR1, GFP-BubR1 signals were detected at the kinetochore during prometaphase (top) but no signals were detected at either kinetochores or telomeres at anaphase (bottom). Scale bar, 10 μ m.

Figure S2. *k81*-induced Paternal Chromosome Loss Occurs After Earliest Embryonic Cycles and Creates Haploid Mosaics. Representative haploid mosaics showing (A) chromatin bridges at cycle 7 and (B) large patches of different nuclear sizes and chromosome lagging and bridging in individual nuclei at syncytial blastoderm. Scale bars, 100 μ m in A and B, 10 μ m in A' and B'.

TABLE S1 Ddbt PROTEINS IN THE SCHIZOPHORA

Species	NCBI Gene ID or genomic location	amino acids (n)	% Arginine
<i>Drosophila melanogaster</i>	5740226	117	24
<i>Drosophila simulans</i>	6734413	109	24
<i>Drosophila sechellia</i>	6610171	110 ^a	24
<i>Drosophila erecta</i>	6545471	117	23
<i>Drosophila yakuba</i>	6532222	117	26
<i>Drosophila biarmipes</i>	scaffold7180000300910:19879..19473	117 ^b	22
<i>Drosophila suzukii</i>	scaffold3:11900223..11900629	117 ^b	21
<i>Drosophila takahashii</i>	Contig5689_22333..22740	117 ^b	23
<i>Drosophila eugracilis</i>	scaffold7180000409184:21609..21210	113 ^b	21
<i>Drosophila ficusphila</i>	scaffold7180000454105:135288..134885	117 ^b	24
<i>Drosophila elegans</i>	scaffold7180000491249:101628..101233	113 ^b	20
<i>Drosophila rhopaloa</i>	scaffold7180000777885:14618..14214	116 ^b	24
<i>Drosophila kikkawai</i>	scaffold7180000302510:882334..882743	117 ^b	23
<i>Drosophila ananassae</i>	6507379	150	20
<i>Drosophila bipectinata</i>	scaffold7180000396390:32060..131545	152 ^b	18
<i>Drosophila pseudoobscura</i>	6899976	117	29
<i>Drosophila persimilis</i>	6601101	117 ^a	30
<i>Drosophila miranda</i>	chrom XR:21661070..21660663	117 ^b	30
<i>Drosophila virilis</i>	6631187	113	23
<i>Drosophila mojavensis</i>	6572658	108	14
<i>Drosophila grimshawi</i>	scaffold15110:65546..65956	118 ^b	16
<i>Drosophila busckii</i>	scaffold4827:10905..10483	122 ^b	21
<i>Scaptodrosophila lebanonensis</i>	scaffold23557:7171..7550	107 ^b	23

<i>Phortica variegata</i>	scaffold12527:3144..3077	125 ^b	19
<i>Musca domestica</i>	105261511	178	16
<i>Bactrocera cucurbitae</i>	105212239	181	18
<i>Bactrocera dorsalis</i>	105226460	181	20
<i>Ceratitis capitata</i>	101459550	182	20

^a Modified from FlyBase reported annotation in this study

^b Annotated in this study

TABLE S2 TIME COURSE OF *k81*- PATERNALLY INDUCED EMBRYONIC ABNORMALITIES

	Minutes AED					
	45-60	60-75	75-90	90-105	105-120	120-135
Number of embryos	82	104	100	75	93	99
Early Arrest Category (%)	84.1	86.5	80.0	82.7	81.7	85.0
median number of clumps (min, max)	6 (2,11)	6 (2,12)	6 (1,13)	6 (1,10)	6 (1,10)	6 (1,12)
Haploid /Haploid Mosaic Category (%)	15.8	13.46	20.0	17.3	18.2	15.0