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Orc mutants arrest in metaphase with abnormally condensed chromosomes

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SUMMARY

The origin recognition complex (ORC) is a six subunit complex required for eukaryotic DNA replication initiation and for silencing of the heterochromatic mating type loci in *Saccharomyces cerevisiae*. Our discovery of the *Drosophila* ORC complex concentrated in the centric heterochromatin of mitotic cells in the early embryo and its interactions with heterochromatin protein 1 (HP-1) lead us to speculate that ORC may play some general role in chromosomal folding. To explore the role of ORC in chromosomal condensation, we have identified a mutant of subunit 5 of the *Drosophila melanogaster* origin recognition complex (*Orc5*) and have characterized the phenotypes of both the *Orc5* and the previously identified *Orc2* mutant, *k43*. Both *Orc* mutants died at late larval stages and surprisingly, despite a reduced number of S-phase cells, an increased fraction of cells were also detected in mitosis. For this latter population of cells, *Orc* mutants arrest in a defective metaphase with shorter and thicker chromosomes that fail to align at the metaphase plate within a poorly assembled mitotic spindle. In addition, sister chromatid cohesion was frequently lost. PCNA and MCM4 mutants had similar phenotypes to *Orc* mutants. We propose that DNA replication defects trigger the mitotic arrest, due to the fact that frequent fragmentation was observed. Thus, cells have a mitotic checkpoint that senses chromosome integrity. These studies also suggest that the density of functional replication origins and completion of S phase are requirements for proper chromosomal condensation.

Key words: *Drosophila*, DNA replication, Chromosome condensation, ORC

INTRODUCTION

The condensation of interphase chromatin to form the discrete packages of DNA for segregation during mitosis is one of the more dramatic events in the cell cycle. The metaphase chromosome in metazoans is thought to be formed by a series of DNA coiling events that transform the basic 11 nanometer nucleosome fiber into ever more complex and compacted loops, giving rise to a series of fibers approx. 30 nanometers, 300 nanometers and 700 nanometers in diameter (Gasser, 1995; Paulson and Laemmli, 1977; Widom and Klug, 1985). Condensed chromosomes are often differentiated into discrete zones, visualized by characteristic banding patterns in response to a particular dye or stain. The ability of certain regions to stay condensed throughout the cell cycle (forming the interphase heterochromatin), while other regions go through rounds of condensation and decondensation, demands both spatial and temporal regulation of condensation. Many lines of evidence imply that condensation must be determined at least in part by features of the DNA itself (see, for example, Benos et al., 2000) and the enzymatic activities of topoisomerases and classes of special folding proteins (Hirano, 1999; Koshland and Strunnikov, 1996). The key outstanding issues are how many levels of regulation exist, and to what extent the differentiated structure of the condensed chromosomes depend upon specialized sequence-dependent DNA:protein interactions.

The phenotypes of mutant SMC genes (structural maintenance of chromosomes) (Lieb et al., 1998; Strunnikov et al., 1993) and the biochemical studies of the 13S condensin complex from *Xenopus* (Hirano et al., 1997; Kimura et al., 1998) have provided deep insights into the mechanisms of chromosomal condensation. 13S condensin is required for assembly of what appear to be (at low resolution) mitotic-like chromosomes in a *Xenopus* cell-free extract. Significantly, purified condensin when combined with a type I topoisomerase can reconfigure circular DNA into an ordered structure with a positive global writhe in an ATP-dependent manner (Kimura et al., 1999). Given the high abundance and biochemical activities of this complex, Kimura et al. suggest that positive solenoidal supercoiling of a nucleosomal fiber (or a higher order fiber) by the condensin complex is a key mitosis-specific strategy for chromosomal folding (Kimura et al., 1999). Condensin is clearly necessary for chromosomal folding, but it may not be sufficient.

S-phase progression and the completion of DNA replication, with concomitant production of sister chromatid fibers, is not necessary for a certain level of condensation. In G1 cells fused to metaphase cells, the membrane of the G1 nucleus breaks down and its chromosomes undergo premature chromosomal condensation (PCC) without passage through S phase (Rao and Hanks, 1980). Moreover, decondensed *Xenopus* sperm chromosomes undergo condensation in a cell-free extract when provided the 13S condensin complex, ATP and topoisomerases without detectable DNA replication (Hirano, 1999).

Normal cell cycle progression through S phase may have profound consequences upon the ultrastructure of the mitotic chromosome. For example, when G2 mammalian cells are fused to G1 cells, both nuclei enter mitosis in synchrony. However, the DNA of the G1 cell passes through S phase somewhat more rapidly and results in less condensed mitotic chromosomes (Rao and Johnson, 1970). Most importantly, PCC detected by fusion of a G1 nucleus with a mitotic nucleus show poorly condensed extended chromatin fibers much longer than normal chromatids (Gollin et al., 1984; Johnson and Rao, 1970). These experiments show that the activities present in the mitotic cells are able to condense interphase chromatin but that the morphology of those condensed chromosomes is incomplete. In any case, it is clear that completion of DNA replication is linked to condensation. The 13S condensin complex itself is biochemically inactive, unless phosphorylated by Cdc2-cyclinB. As Cdc2-cyclinB levels are controlled by S phase checkpoints, both appropriate compartmentalization and specific activities of the condensin complex are probably linked to the completion of DNA replication (Kimura et al., 1998; Sutani et al., 1999).

We have been intrigued by the possibility that the positions and density of DNA replication origins on the DNA fiber may influence chromosome folding. Such a link has been suggested by indirect evidence such as a correlation between apparent chromosome fiber loop lengths and mean distances between replication origins (Marsden and Laemmli, 1979; Paulson and Laemmli, 1977). In eukaryotes, the origin recognition complex (ORC) serves as the platform upon which replication initiation complexes assemble (Lee and Bell, 2000) and thus the distribution of ORC along the DNA determines, in part, the sites at which replication may start. The ORC protein in yeast also plays a discrete role in forming heterochromatin at the HMR locus through recruitment of Sir1 (Fox and Rine, 1996). Our discovery of the *Drosophila* ORC complex concentrated in the centric heterochromatin of mitotic cells in the early embryo and its interactions with heterochromatin protein 1 (HP-1) lead us to speculate that ORC may play some general role in chromosomal folding (Pak et al., 1997).

Drosophila is a well-suited organism with which to explore the role of ORC in chromosomal condensation. There are a small number of chromosomes whose morphology is well studied (Rubin and Lewis, 2000), and the six subunit ORC complex has been described (Austin et al., 1999; Chesnokov et al., 1999; Gossen et al., 1995). More importantly, *Orc* mutants can be identified and analyzed for defects in DNA replication and chromosomal condensation. Homozygous *Orc* mutants survive until the late-larval stage (Gatti and Baker, 1989; Landis et al., 1997; Pinto et al., 1999), owing to the large maternally deposited stores of ORC present in the fertilized egg (Gossen et al., 1995). This late larval lethality has allowed us to examine the structure of the mitotic chromosomes as ORC becomes depleted and the density of active replication origins goes down. We have identified a mutant in *Orc5* and have phenotypically characterized this mutant and a previously identified *Orc2* mutant, *k43*. We find that *Orc* mutants have severely reduced levels of BrdU incorporation and also have a significantly increased fraction of cells in mitosis. In this respect, *Orc* mutants have similar characteristics to the previously described *double-parked*/*cdt1* mutant, another protein now known to be crucial for the assembly of the pre-initiation complex (Whittaker et al., 2000). *Orc* mutants arrest in a defective metaphase with aberrantly condensed chromosomes that fail to align at the metaphase plate and a poorly assembled mitotic spindle. We suggest that DNA replication may be a requirement for proper chromosomal condensation because MCM4 (*dpa* – FlyBase) and PCNA (*mus209* – FlyBase) mutants have similar defects as observed in *Orc* mutants. In addition, we propose that DNA replication defects trigger the mitotic arrest, due to the fact that frequent fragmentation is observed in ORC, MCM4 and PCNA mutants. We discuss mechanisms by which an improper or incomplete S phase may influence the degree of chromosomal folding and mitotic checkpoints.

MATERIALS AND METHODS

Fly strains

The deletion strains *In(2L)b79h1A/SM5, al*^{2} *Cy lt*^{v} *sp*^{2}, *Df(2L)b84a9, pr pk cn sp/In(2LR)O, Cy dp*^{lvI} *b*^{81f1} *pr cn*^{2} *Stan[-], In(2LR)b81a2/In (2LR) SM5, al*^{2} *Cy lt*^{v} *sp*^{2} and *Df(2L)TE35B-GV203/CyO; Dp(2;3)17/(+),* and the EMS-induced mutant strains *l(2)34Df*^{BG1}*/CyO* and *b l(2)34Df*^{L480} *el rd*^{s} *pr cn/CyO* were provided by J. Roote (University of Cambridge, UK). The *Orc2* mutant strains *k43*^{1}*/TM6β* and *k43*^{γ4}*/TM6β* and *Orc3* mutant strains *lat*^{le344}*/CyO* and *lat*$^{vr6.35}$*/CyO* have been previously described (Landis et al., 1997) and were provided by J. Tower (University of Southern California) and S. Pinto (Cold Spring Harbor Laboratory) respectively. The *Orc2* deletion strain *Df(3R)293γ7TM6β,* the MCM4 mutant strain *dpa*^{1}*/CyO* and the PCNA mutant strain *mus209*^{2448}*/CyO* were provided by the Bloomington Stock Center.

Deletion mapping

Genomic DNA was prepared from the deletion strains (see above) and hydrolyzed with the restriction enzymes *Bgl*II and *Sac*II. The products were separated by 0.8% agarose gel electrophoresis in 1× Tris-Acetate (TAE). The DNA was then transferred to Zeta-Probe membrane (BioRad) and probed with a ^{32}P-labeled ORC5 and ORC2 (loading control) cDNA fragments. The data was then quantitated on a Fuji phosphoimager.

Rescue of the *Orc5* mutant

A Bcl fragment derived from the P1 clone DS00941 and which contained the gene for Orc5, was cloned into the *Bam*HI site of the vector pW8 (Klemenz et al., 1987). This cloned DNA fragment was then hydrolyzed with *Swa*I and *Stu*I and re-ligated to generate an Orc5-delete fragment. Both constructs were then microinjected into *w*^{1118} to generate transgenic lines containing the Orc5 or Orc5-delete transgene. The following rescue cross was then performed: *w*^{1118}*; l(2)34Df*^{BG1}*/SM6 Roi eve lacZ (SEL)*; P[DmOrc5^{+}] × *w*^{1118}*; l(2)34Df*^{L480}*/SEL*. The *Orc5* mutant heteroallelic combination *l(2)34Df*^{BG1}*/l(2)34Df*^{L480} was then scored for viability by screening for the absence of the dominant marker *Roi*. The expected number of *Orc5* mutants is 25% and the observed numbers are shown in Table 1.

Sequencing of an *Orc5* mutant allele

A *l(2)34Df*^{BG1}*/In(2LR) Gla Bc* strain containing the dominant larval marker Black cells (Bc) was constructed. *l(2)34Df*^{BG1} homozygous larvae were then selected by screening for the absence of the Bc marker. Genomic DNA was prepared from these larvae. The gene for Orc5 was then PCR-amplified from the genomic DNA preparation and directly sequenced.

BrdU labeling of third instar larval brains

Larval brains were dissected from third instar larvae and soaked in

0.7% NaCl and BrdU (Sigma) for 1.5 hours at 25°C. The BrdU incorporation was detected using a monoclonal BrdU antibody (Becton Dickinson) essentially as described (Calvi et al., 1998). Briefly, larval brains were fixed in 5% formaldehyde/PBS, washed several times in PBS + 0.1% Triton X-100 (PBT) and incubated in 1:10 BrdU antibody/PBT overnight at 4°C. The larval brains were then washed several times in PBT, incubated in 1:100 Alexa 488 goat anti-mouse IgG (Molecular Probes) for 2 hours at room temperature, washed several times in PBT, rinsed in PBS and mounted in 70% glycerol/2%n-propyl gallate/PBS.

Third instar larval brain squashes and BrdU-labeled chromosome spreads

Larval brains from third instar larvae were dissected and squashed as described (Ashburner, 1989). The mitotic chromosomes were visualized by DAPI staining. To detect BrdU incorporation in mitotic chromosomes, early first instar larvae were first fed 0.1 mg/ml BrdU (Truman and Bate, 1988). About 2 to 3 days later, at third-instar stage, brains were dissected, fixed and squashed essentially as described (Ashburner, 1989) with some exceptions (Williams and Goldberg, 1994). Briefly, larval brains were fixed for 30 seconds in 45% acetic acid, 3 minutes in 60% acetic acid, squashed and subsequently soaked in methanol for 10 minutes. Chromosome spreads were then stained with BrdU and phospho-H3 (Upstate Biotechnology) antibodies essentially as described elsewhere (Calvi et al., 1998) and above. To quantitate mitotic cells, chromosome spreads were generated as described above from non-BrdU fed larvae and stained with phospho-H3 antibody only.

Tubulin staining

Larval brains were dissected from third-instar larvae, fixed and stained with phospho-H3 and β-tubulin (Asai et al., 1982) antibodies as described in (Feiguin et al., 1998).

Image collection

All images were taken with a Zeiss 510 confocal microscope except that of the DAPI-labeled chromosome spreads which were taken on a Zeiss axiophot microscope equipped with a Leica LEI-750TD CCD camera (Optronics).

RESULTS

Identification of *Orc5* mutants

Although the ORC complex is stable and acts as replication initiator protein, any given ORC subunit may have additional functions, only a subset of which are relevant to the entire complex. For example, the Orc3 subunit has additional functions at the neuromuscular junction (Pinto et al., 1999). To uncover both shared and specific roles for each ORC subunit, we thus wanted to compare phenotypes of mutant alleles of different *Orc* genes. We started our *Orc* mutant search by focusing on *Orc5*, a gene located within the well-characterized Adh region of *Drosophila* chromosome 2 in which many recessive lethal mutations have been precisely mapped.

The strategy we used to obtain a *Orc5* mutant is illustrated in Fig. 1, and began with an alignment of the genetic and physical maps of a portion of the *Drosophila* genome. The *Orc5* gene was first located near *Sos*, as both genes were found in the P1 clone DS00941 (Ashburner et al., 1999). A series of four partially overlapping deletion strains in the *Sos* region were used to determine candidate *Orc5* complementation groups (see Fig. 1C). Genomic DNA was prepared from the four heterozygous deletion strains and analyzed by DNA-blot hybridization for the amount of the *Orc5* gene present in each strain. *Df(2L)b84a9* shown in blue was the only deletion that lowered the ratio of *Orc5:Orc2* to close to 0.5 (see Fig. 1D,E). This placed *Orc5* between the two red lines on the genetic map (see Fig. 1). Therefore, l(2)34Dd and l(2)34Df were candidate *Orc5* genes.

To identify which of the two complementation groups encoded Orc5, *Orc5* transgenes were tested for their ability to rescue the lethality associated with these two mutations. Two transgenes were used: (1) a genomic fragment containing the *Orc5* gene and (2) the same fragment from which the *Orc5*-specific *Swa*I/*Stu*I fragment had been removed (see Fig. 1F). The full-length *Orc5* transgene complemented l(2)34Df lethality, whereas the *Orc5* delete transgene did not, thereby demonstrating that this complementation group is *Orc5* (see Table 1). l(2)34Dd encodes a gene just upstream of *Orc5* as $l(2)34Dd^{CH60}$ homozygotes were complemented by both the *Orc5* and *Orc5* delete transgenes (data not shown). A second open reading frame was found upstream of *Orc5* in the rescue fragments and encodes *dSop2*, a member of the Arp2/3 complex (A. Hudson and L. Cooley, personal communication).

The *Orc5* gene was PCR-amplified from genomic DNA of homozygous $l(2)34Df^{BG1}$ larvae (see Materials and Methods) and directly sequenced. A mutation at the *Orc5* exon/intron boundary of the only 5′ splice site changed the universally conserved/GT (Mount, 1982; Mount et al., 1992) to/AT (see Fig. 1F). In other well-studied cases, a G to A 5′ splice site mutation results in a pre-mRNA species in which lariat formation but no cleavage of the 3′ end of the intron occurs (Moore et al., 1993). No abnormally sized Orc5 protein was detected by western blot analysis of heterozygous $l(2)34Df^{BG1}$ crude lysates (data not shown), implying that no aberrant splicing occurred that led to a truncated or altered form of the Orc5 protein. The homozygous *Orc5* mutant $l(2)34Df^{BG1}$ dies primarily at the 3rd instar stage of larval development.

A previously characterized chorion gene amplification mutant, $k43^{fs293}$, was found to contain a mutation in the *Orc2* gene (Landis et al., 1997). $k43^{1}$ is a recessive lethal allele of *Orc2* that, like *Orc5*, also dies at the 3rd instar larval-pupal boundary. Both *Orc* mutants also lack imaginal discs at the larval stage. Lethality at the larval/pupal boundary and poorly developed imaginal discs are diagnostic of many mutant genes that are important for cell proliferation (Gatti and Baker, 1989). As the only population of cells that still divide at this stage are in the imaginal discs and larval brain, these tissues provide the

Table 1. l(2)34Df is rescued to viability with a genomic *Orc5* transgene

Line	Chromosome	Rescued (%)
24	3	17.6 (40/227)
33	X	24.0 (18/75)
312	X	13.7 (14/102)
12Δ	X	0 (0/107)

Four transgenic lines carrying independent P-element insertions that contain either the wild-type *Orc5* gene (24,33,312) or a construct in which most of the *Orc5* gene has been deleted (12Δ) were used in the rescue cross. All the P-element insertions mapped to either the X or third chromosome, as indicated in the table. The *Orc5* gene is on chromosome 2. The percentage of rescued flies observed is reported here, with the number of rescued/total progeny in parentheses.

most sensitive assays for the effect of such mutants on cell proliferation. Therefore, the phenotypic characterization of both the *Orc5* and *Orc2* mutants concentrated on the larval brain.

Orc mutants are DNA replication-defective and arrest in mitosis

Both biochemical and genetic evidence indicate that ORC is crucial for DNA replication in vivo (reviewed by Spradling, 1999). To determine whether *Orc2* and *Orc5* mutants are also DNA synthesis defective, 5-bromo-2-deoxyuridine (BrdU) incorporation levels in larval neuroblasts were determined and compared for wild type, heterozygous and homozygous *Orc2* and *Orc5* mutants. Only a few BrdU foci were observed in either homozygous *Orc* mutant larval brain (Fig. 2B,D, see arrows) compared with the large number of foci observed in either heterozygote (Fig. 2A,C). Therefore, both *Orc2* and *Orc5* mutants had severely reduced levels of DNA synthesis.

The reduced levels of DNA replication in *Orc* mutants suggest that the majority of cells arrest outside S phase, most likely in G1. To look more closely at cell cycle progression in *Orc* mutants, larval brain squashes were prepared in the absence of hypotonic treatment and scored for the presence of mitotic cells by phospho-H3 antibody staining (see Table 2). The large majority of cells were not stained with phospho-H3 and are likely to be in G1, due to the fact that both *Orc* mutants are DNA replication defective (see Fig. 2). However, *Orc* mutants have an unexpected four- to fivefold increase in mitotic cells compared with wild type, suggesting that they have a second arrest point in mitosis. To define this mitotic arrest further, DAPI-stained chromosome spreads were then scored for the presence of chromosomes in each stage of mitosis (see Table 3). We observed a few prophase nuclei in the mutant squashes (see Table 3), which are characterized by nuclear membrane breakdown and long extended but visible chromosomes in the early stages of condensation. However, no anaphase or telophase figures were found. Instead, a large number of metaphase-like figures were observed in which a highly condensed chromosome mass was present, with a semblance of congression but where alignment was incomplete or disorganized (see Table 3). In addition, there were metaphase spreads in which the individual chromatids were distinguishable but appeared aberrantly condensed. Levels of defective metaphase figures were six times higher than the levels of metaphases of wild type (87% versus 14%). Therefore, we conclude that *Orc* mutants have a defect in establishing a true metaphase and progressing past this point.

To investigate this apparent mitotic arrest further, *Orc* mutant and wild-type larval brains were stained with phospho-H3 and tubulin antibodies to visualize the mitotic chromosomes and spindle respectively. Representative confocal images are shown in Fig. 3. The spindle alone is shown at the same magnification in insets. The *Orc* mutant mitotic spindles never assembled properly (Fig. 3). Instead, the majority of mitotic spindles observed were anastral with an amorphous microtubule organizing center and unfocused spindle poles when compared with wild type. The defects observed in *Orc* mutants were classified based on the severity of congression problems. In Class I, most chromosomes appear to congress at the metaphase plate as a highly condensed chromosome mass (Fig. 3, see arrows). The mitotic spindle is unfocused and spindle fibers are wound about the chromatid mass. In some cases, uneven spindle poles are observed (Fig. 3, see *Orc2*, Class I inset). In Class II, chromosomes do not congress at the metaphase plate. Instead, chromatids can be observed throughout the spindle and at the spindle poles (Fig. 3, arrows). In addition, the spindle appears to be diffuse but more focused

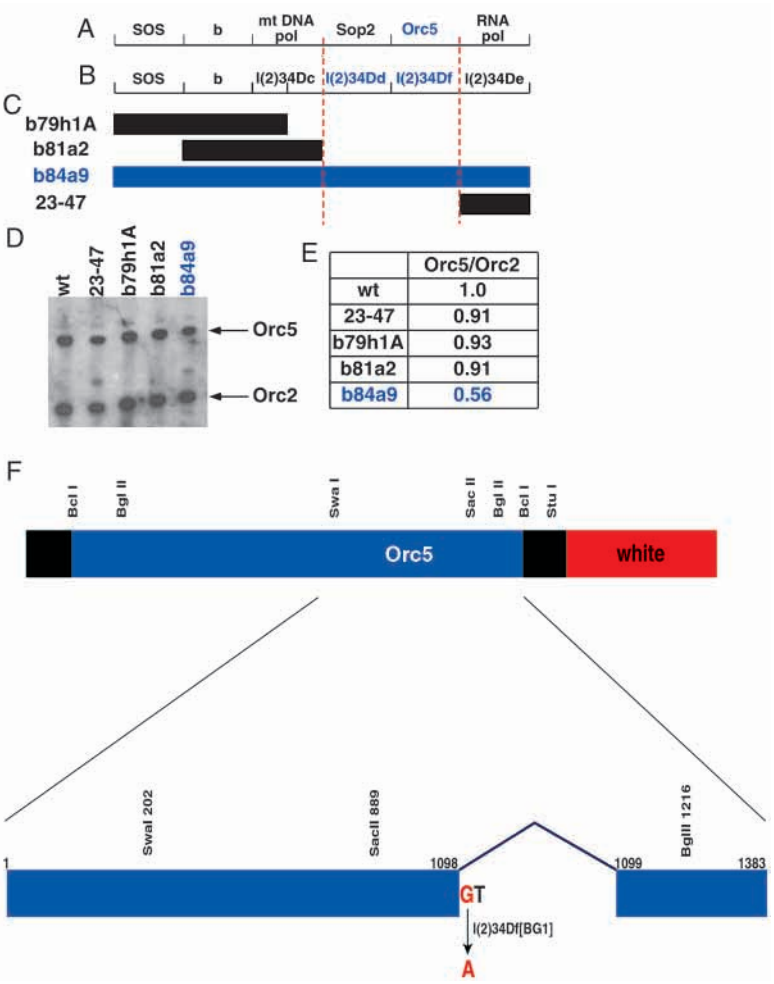

Fig. 1. Isolation of a *Orc* mutant. (A) A partial physical map of the P1 clone DS00941 and (B) the genetic map of the SOS region were aligned to help identify a *Orc5* mutant. (C) A series of four overlapping deletions used to determine candidate *Orc5* complementation groups is also shown. The deleted regions are designated by rectangles. (D) Quantitative DNA blot analysis of the deletion strains identifies *l(2)34Dd* and *l(2)34Df* as candidate *Orc5* mutants. (E) DNA blot data normalized to wild-type (wt). (F) *l(2)34Df* is *Orc5*. The *l(2)34Df* mutant is rescued by a genomic *Orc5*-containing transgene (top panel and Table 1) and the restriction sites define the genomic DNA in the rescue transgene (blue) between the poly-linker sites (black boxes). The *white* gene is in red. *l(2)34Df*^{BG1} contains a *Orc5* mutation at the exon/intron boundary (bottom panel). The cDNA structure of *Orc5* is depicted below the genomic DNA. 1 refers to the A of the initiation codon and 1383 marks the termination codon in the cDNA.

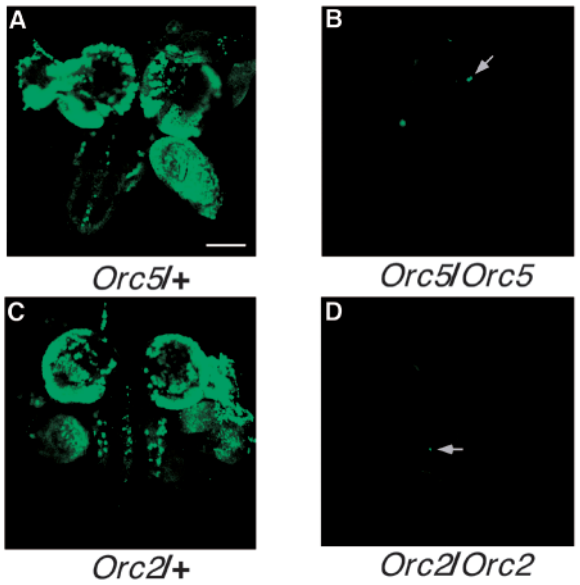

Fig. 2. BrdU incorporation levels were lower in *Orc* mutants. BrdU incorporation levels were determined in third instar larval brains of the following genotypes: (A) *l(2)34Df*^{BG1}/In(2LR) *Gla Bc*; (B) *l(2)34Df*^{BG1}/*l(2)34Df*^{BG1}; (C) *k43*^{1}/TM6β; (D) *k43*^{1}/*k43*^{1}. (A,C) Heterozygotes. (B,D**)** Homozygous *Orc5* or *Orc2* mutants, respectively. Scale bar: 100 μm.

Table 2. DNA replication mutants arrest in mitosis

	% Mitotic cells	Fold increase
Wild type	1.6±0.3 (16/966, 14/749, 111/8395)	–
Orc2	6.2±0.1 (49/799, 90/1430)	3.9
Orc5	8.1±2.2 (10/154, 18/210, 40/340)	5.5
MCM4	8.0±0.0 (85/1058, 244/3044)	5.0
PCNA	5.2±0.1 (10/193, 22/418, 43/847)	3.3

The mitotic frequency in wild-type and DNA replication mutants was determined by averaging the percentage of mitotic cells observed in two to three larval brain squashes. The number of mitotic cells/the total number of cells is given in parentheses.

Table 3. DNA replication mutants arrest in metaphase

	Wild type (%)	*Orc5* (%)	*Orc2* (%)	MCM4 (%)	*PCNA* (%)
Prophase	54 (38/70)	13 (6/45)	13 (14/105)	0	0
Metaphase*	14 (10/70)	87 (39/45)	87 (91/105)	100 (56/56)	91 (52/57)
Anaphase	23 (16/70)	0	0	0	9 (5/57)
Telophase	9 (6/70)	0	0	0	0

*In the DNA replication mutants *Orc5*, *Orc2*, MCM4 and *PCNA*, metaphase-like cells (see text) are included and represent 43% (39/91), 79.5% (31/39), 61% (34/56) and 67% (35/52) of the metaphase figures reported here.

than Class I (Fig. 3; see *Orc5*, Class II inset). In some cases, the spindles are monoastral (Fig. 3; see left arrow, *Orc2*, Class II and inset). Similar spindle defects have been previously observed in *Drosophila* mutants such as *abnormal spindle* (*asp*) and *γtub23C*, which is required for microtubule-organizing activity of the centrosome (do Carmo Avides and Glover, 1999; Sunkel et al., 1995), suggesting that centrosome function may be impaired.

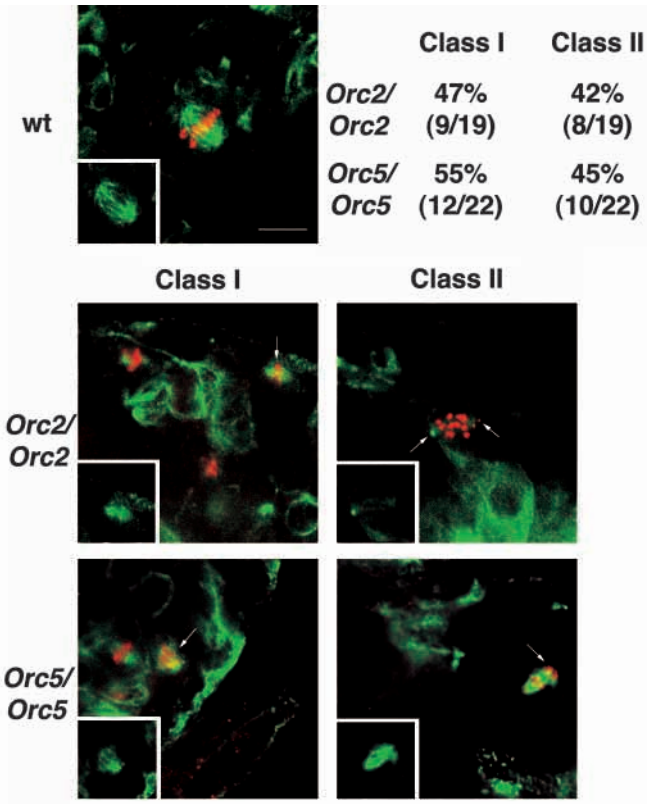

Fig. 3. *Orc* mutants arrested in a metaphase-like state with chromosome congression and spindle defects. The mitotic chromosomes (red, phospho-H3) and spindle (green, tubulin) were visualized by confocal microscopy in whole-mount third instar larval brain of the following genotypes: wild type (wt), *k43*^{1}/*k43*^{1} (*Orc2*) and *l(2)34Df*^{BG1}/*l(2)34Df*^{BG1} (*Orc5*). The mitotic spindle for each genotype (arrow) is shown in an inset without the chromosome stain. The spindle defects observed were divided into two classes (Class I and Class II) and quantitated in a table (top right). Scale bar: 10 μm.

Orc mutant mitotic chromosomes are abnormally condensed and exhibit discontinuous BrdU incorporation

Chromosome spreads were prepared from third-instar larval brains with hypotonic treatment to better define the degree of condensation present in the *Orc* mutants. Representative data from wild type and the mutants *Orc2* and *Orc5* are shown in Fig. 4. Both these mutants clearly show abnormally condensed mitotic chromosomes relative to the wild-type morphology. In general, the mitotic chromosomes appeared shorter and thicker, the centromeres were not distinct and sister chromatid cohesion was frequently lost. These chromosome condensation defects were organized by increasing severity into three classes. In Class I, *Orc* mutant chromosomes have mild defects (such as undercondensed chromosome arms and, in some cases, elongated centromeres; see Fig. 4, arrowhead). In Class II, *Orc* mutant centromeres cannot be distinguished, and chromosome arms appear far thicker and shorter than in Class I. In addition, sister chromatid cohesion is frequently lost and the chromosomes have an extremely aberrant morphology. In Class III, *Orc* centromeres are underwound (see Fig. 4, arrowhead in insets) and chromosome arms are decondensed and noticeably shorter than wild type. The ‘dumb-bell’

morphology observed in Class III has been previously observed in mutants of proliferation disrupter, a centromeric binding protein (Török et al., 1997). In such spreads, sister chromatid cohesion was almost always lost. While chromosome fragments could usually be detected in *Orc* mutant chromosome squashes, the total number of such dumb-bell structures was as expected for loss of cohesion of sister chromatids that were aberrantly condensed. It is also interesting to note that the *Orc2* chromosome defects become more severe with age, at which point ORC levels might be expected to be lower in neuroblasts (see $Orc2^{w}$, Fig. 4, table). Before the $Orc2^{w}$ third-instar larvae start wandering (denoted $Orc2^{w}$ in Fig. 4, table), 32% of the chromosome defects observed are Class I and 5% are Class III. In contrast, only 3% of third instar larval chromosomes are Class I and 21% are Class III when the larvae begin to wander.

Extensive chromosome fragmentation in both *Orc* mutant chromosome spreads (for example, see arrow in Fig. 4, Class I *Orc2*) was observed as first noted in the *Orc2* mutant (Gatti and Baker, 1989). Such chromosome breaks could readily arise from discontinuous or incomplete replication, leaving gaps in the DNA, which subsequently break either in vivo or during experimental preparation. Indeed, our attempts to perform fluorescent in situ hybridization (FISH) to determine the degree of condensation defects in *Orc* mutants were unsuccessful, as these mutant chromosomes fell apart during the experiment. To assess more directly DNA replication patterns in these abnormally folded structures, *Orc* mutant larvae were grown in the presence of BrdU and BrdU incorporation in mitotic chromosomes was determined by immunochemical methods (Fig. 5). Chromosomes from either the *Orc5* or *Orc2* mutants show a faint BrdU outline with one or more intense spots of BrdU incorporation. Even regions of the chromosomes in which the DNA replication machinery has proceeded, due to the presence of clearly distinct sister chromatids, exhibited a discontinuous punctate pattern of BrdU incorporation (see Fig. 5, *Orc2* arrows). It is important to note that the neuroblast cell cycle is normally much faster than the 2-3 day labeling period used in our experiments and yet we never saw large regions of BrdU staining in the mutant chromosomes. Moreover, we saw no incorporation at all when dissected larval brain tissue was given a 1 hour pulse of BrdU (data not shown). Therefore, we conclude that there is a slow, extended S phase in *Orc* mutants in which BrdU levels are depleted before extensive incorporation can occur.

The data presented above were obtained by phenotypically characterizing *Drosophila* strains homozygous for a single mutant allele of *Orc2* or *Orc5*. A phenotypic characterization of other allelic combinations of *Orc5*, *Orc2* and *Orc3* establishes that the defects observed are a result of a loss of ORC function and are not due to a particular special or unusual mutant allele. For example, similar replication defects, abnormally condensed chromosomes, and a metaphase-like arrest were observed in the homozygous *Orc5* mutant $l(2)34Df^{BG1}$, $l(2)34Df^{BG1}$over the deficiency DF(2L)b84a9 (see Fig. 1), as well as in a second allele of $l(2)34Df^{L480}$ over DF(2L)b84a9 (data not shown). Moreover, the heteroallelic combination $l(2)34Df^{BG}/l(2)34Df^{L480}$, which is rescued by a *Orc5* transgene shows the same phenotypes as the homozygous $l(2)34Df^{BG}$ mutant. This demonstrates that the phenotypes observed here are not due to secondary lethals present on the mutant chromosome. Furthermore, the absence of an aberrantly sized Orc5 protein in crude lysates and the nature of the mutation in the $l(2)34Df^{BG1}$ allele (see Fig. 1F) together with these results, argues that the phenotypes we report are due to an *Orc5*-null or a strong hypomorph. Similarly, the replication defects, chromosome condensation abnormalities, and the metaphase-like arrest were observed for multiple *Orc2* allelic combinations: $k43^{1}$ homozygotes, $k43^{1}$ over the deficiency $k43^{293\gamma7}$ and $k43^{1}$ over a null allele of *Orc2*, $k43^{\gamma4}$ (data not shown). For unknown reasons, we were not able to recover $k43^{\gamma4}$ homozygotes. Finally, the phenotypes observed in *Orc5* and *Orc2* mutants were also present in two mutant alleles of the *Orc3* mutant *latheo* (lat^{let344} homozygotes and the heteroallelic combination $lat^{let344}]/lat^{vr6.35}$). The data obtained for *Orc5* mutant $l(2)34Df^{BG1}$ most directly argue that these phenotypes arise from a null mutation. However, it is likely that ORC levels do not disappear within one cell cycle because of the presence of large maternal stores and that, even with a null mutation, loss of replication activity might gradually diminish.

MCM4 and PCNA mutants exhibit phenotypes similar to *Orc* mutants

We wondered whether *Orc* mutations uniquely affected the chromosome condensation process or if such mitotic phenotypes arise when the DNA replication process is

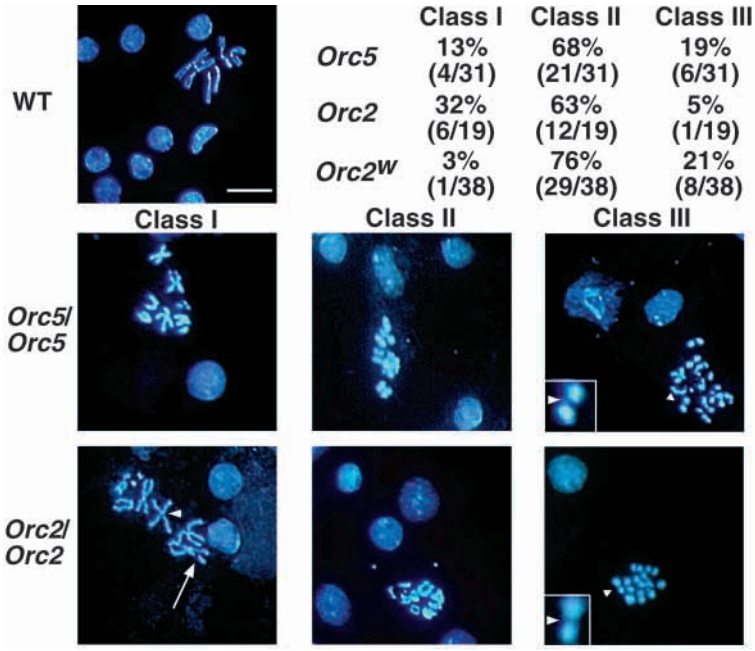

Fig. 4. *Orc* mutants had abnormally condensed chromosomes. CCD images of DAPI-stained mitotic chromosome spreads are shown. For each genotype, a representative Class I, Class II and Class III condensation defect (see text) is shown. Class III defects are also shown in insets. The defects observed were quantitated in a table (top right). In the table, *Orc2* is divided into non-wandering (*Orc2*) and wandering ($Orc2^{w}$) 3rd instar larvae. The arrows show characteristic broken chromosomes, while the arrowheads show examples of decondensed centromeres and/or arms. Scale bar: 10 μm.

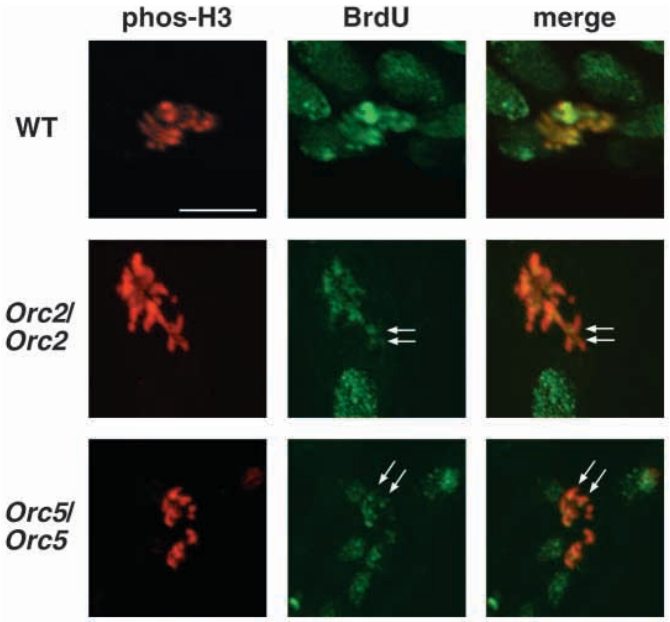

Fig. 5. BrdU incorporation in *Orc* mutant chromosome spreads was discontinuous. For each genotype, representative confocal images are presented in sets of three panels: phospho-H3 (red), BrdU (green) and a merged image (co-localization in yellow). The arrow in the *Orc2* and *Orc5* panels highlight patches of BrdU incorporation in the sister chromatids of one chromosome. Scale bar: 10 μm.

defective. We therefore characterized the phenotype of two other DNA replication mutants: *disc proliferation abnormal* (*dpa*), a MCM4 mutant and *Mus209*, a PCNA mutant. We first confirmed that both mutants were defective for DNA replication by measuring BrdU incorporation levels in larval neuroblasts. Representative confocal images are shown in Fig. 6A-D. Only a few BrdU foci were observed in the homozygous MCM4 mutant larval brain compared with the large number of foci observed in the heterozygote (Fig. 6A,C) consistent with previous studies (Feger et al., 1995). A larger number of BrdU foci were observed in the PCNA mutant when compared with the MCM4 mutant, but not nearly to the levels present in the PCNA heterozygote (Fig. 6B,D). Therefore, both MCM4 and PCNA mutants are DNA replication defective.

We then characterized the MCM4 and PCNA mutants for mitotic defects. Chromosome squashes were prepared in the absence of hypotonic treatment and scored for the presence of mitotic cells by phospho-H3 antibody staining (see Table 2). Both DNA replication mutants have a three- to fivefold increase in mitotic cells compared with wild type, suggesting that (like *Orc* mutants), they have a second arrest point in mitosis (see Table 2). Furthermore, both DNA replication mutants appear to arrest in an abnormal metaphase-like state because MCM4 and PCNA mutants have a metaphase-like frequency that is six to seven times greater than that of wild type (100% and 91% versus 14%, respectively) virtually identical to the increase observed in *Orc* mutants (see Table 3). In addition, a large number of metaphase-like figures were

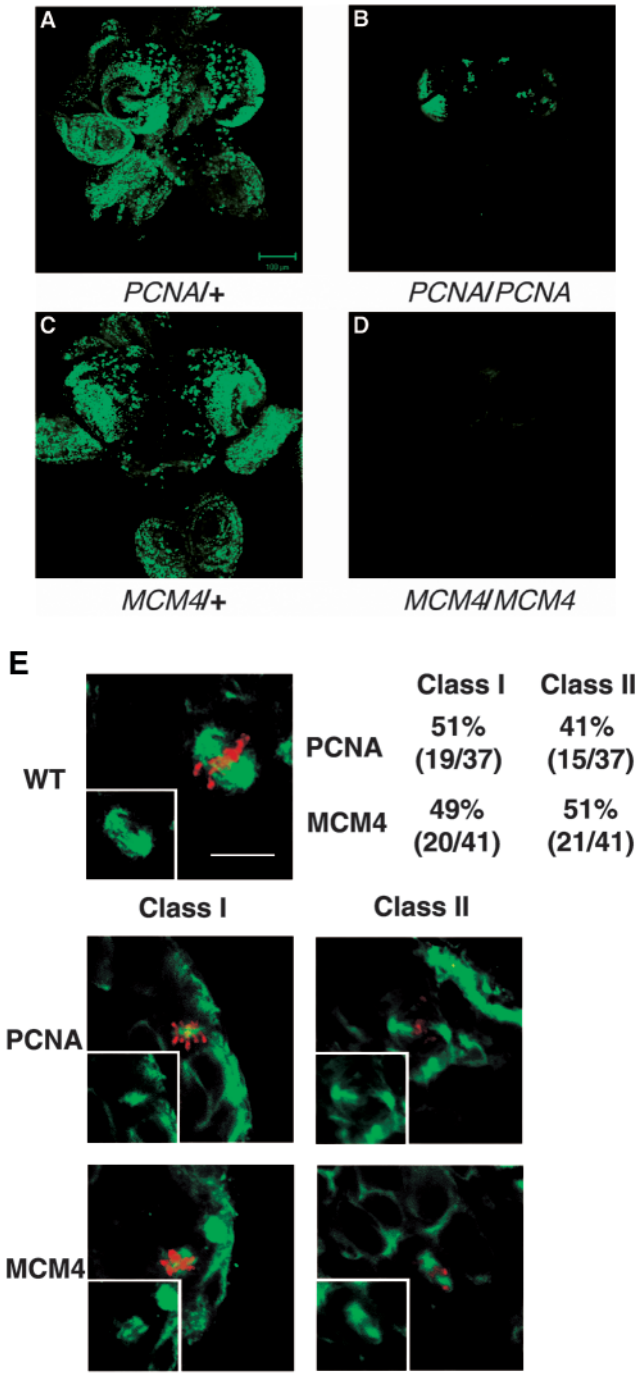

Fig. 6. The DNA replication mutants MCM4 and PCNA arrested in a metaphase-like state. (A-D) MCM4 and PCNA mutants incorporate lower levels of BrdU. BrdU incorporation levels were determined in third instar larval brains of the following genotype: (A) $mus209^{2448}$/In(2LR) *Gla Bc*; (B) $mus209^{2448}$/$mus209^{2448}$; (C) dpa^{1}/In(2LR) *Gla Bc*; and (D) dpa^{1}/dpa^{1}. (A,C) Heterozygotes. (B,D) Homozygous PCNA or MCM4, mutants respectively. (E) MCM4 and PCNA mutants have chromosome congression and spindle defects. The mitotic chromosomes (red, phospho-H3) and spindle (green, tubulin) were visualized by confocal microscopy in whole-mount third instar larval brain of the following genotypes: wild-type (wt), $mus209^{2448}$/$mus209^{2448}$ (PCNA) and dpa^{1}/dpa^{1}, (MCM4). The mitotic spindle for each genotype is shown in an inset enlarged without the chromosome stain. The spindle defects observed were divided into two classes (Class I and Class II) and quantitated in a table (top right). Scale bars: 100 μm in A-D; 10 μm in E.

observed in which a highly condensed chromosome mass was present (see Table 3), suggesting that (like *Orc* mutants) MCM4 and PCNA mutant chromosomes are unable to align appropriately at the metaphase plate. No anaphase figures were observed in MCM4 mutants. Anaphase figures were observed in PCNA mutant spreads but were very rare; the metaphase:anaphase ratio is 14 times lower in PCNA mutants than in wild type (10.5 versus 0.75; see Table 3).

We then analyzed the mitotic chromosomes and spindle in MCM4 and PCNA mutants for defects by staining with phospho-H3 and tubulin antibodies, respectively. Representative confocal images are shown in Fig. 6E. Similar to *Orc* mutants, MCM4 and PCNA mutant have abnormal mitotic spindles that are either anastral or have poorly focused poles (Fig. 6E). In addition, both DNA replication mutants had both Class I and Class II mitotic figures in which the chromosomes were either aggregated at the metaphase plate (MCM, Class I) or scattered along the metaphase-like spindle (MCM4, Class II (arrows) or PCNA, ClassII). In some cases, the individual chromosomes can be resolved in the chromosome aggregate (PCNA, Class I). In addition, reminiscent of *Orc* mutants, the spindle in Class II appears to be diffuse but more focused than Class I (see MCM4, Class II inset). The spindle poles are often clumpy and the spindle fibers are not present (see PCNA, Class II inset).

Chromosome spreads were prepared from third-instar larval brains with hypotonic treatment to better define the chromosome morphology of these DNA replication-defective mutants. Representative chromosome spreads from wild-type, MCM4 and PCNA mutants are shown in Fig. 7. Both MCM4 and PCNA mutants clearly show abnormally condensed mitotic chromosomes, similar to *Orc* mutants. The large majority of chromosome spreads (as with *Orc* mutants) are Class II, in which the centromeres cannot be distinguished, the chromosome arms appear decondensed and sister chromatid cohesion is frequently lost. A few Class III spreads containing 'dumb-bell' chromosomes in which the centromeres are underwound (see Fig. 7, arrowhead in insets) and the arms are more decondensed than Class II were also observed**.** As with the *Orc* mutants, mild condensation defects such as undercondensed chromosome arms were present in MCM4 mutants but were rare (Class I, Fig. 7). In PCNA mutants, Class I chromosome spreads were more prevalent in which the chromosomes morphology was close to wild type but their arrangement was clearly abnormal. Metaphase spreads were often quite crowded and chromatids appeared fused (see Class I, PCNA). This observation is consistent with the chromosome congression defects found in these mutants.

DISCUSSION

The analysis of *Drosophila* larvae with severely reduced ORC function has confirmed at a cellular level a role for ORC in cellular proliferation. At the simplest level, the vastly reduced BrdU labeling in the proliferative neural tissue of larvae homozygous for *Orc2* or *Orc5* mutations confirms a crucial role of ORC in cellular proliferation. At a higher magnification, the patchy and reduced levels of BrdU staining of chromosomes in *Orc* mutants was most consistent with a slowed S phase, with perhaps fewer origins used, and triggers a mitotic arrest. We suspect that this arrest is triggered by DNA damage sustained during a defective S phase. In addition, the presence of abnormally condensed chromosomes in *Orc* mutants, and in MCM4 and PCNA mutants has offered unexpected insight into a link between DNA replication and chromosome morphology. We emphasize that the severity of the chromosomal ORC phenotypes changes with developmental time, and we postulate that this directly reflects the extent to which maternal pools have been depleted. In comparing the phenotypes of genes that encode different replication proteins we were thus careful to compare the cells from the same tissue (larval neuroblasts) with equivalent defects in BrdU incorporation. This, for example, precluded observations with a recessive lethal primase mutant (*DNAprim*^{j10b2}), as we detected no deficiency in replication levels in the larval neuroblast (data not shown).

Orc mutants arrest in metaphase

One of the most striking observations of this study is that *Orc* and other mutants directly involved in DNA replication appear to be arrested at two stages of the cell cycle, in G1 and mitosis. The large majority of cells appear to be unable to enter S phase, as the severely reduced levels of BrdU incorporation observed (Figs 2, 6) probably reflect a G1 arrest. However, some mutant

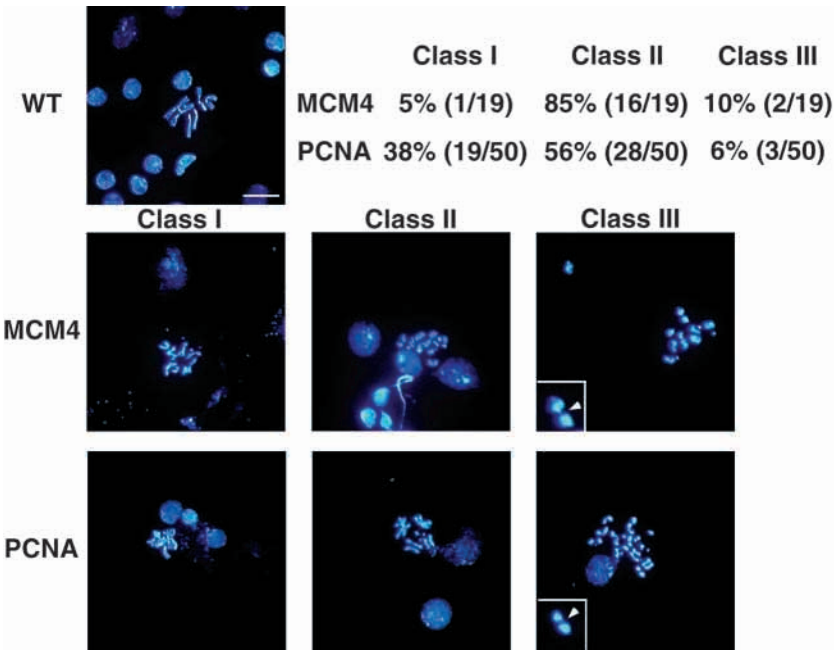

Fig. 7. DNA replication mutants MCM4 and PCNA had abnormally condensed chromosomes. CCD images of DAPI-stained mitotic chromosome spreads are shown. For each genotype, a representative Class I, Class II and Class III condensation defect (see text) is shown. Class III defects are also shown in insets. The defects observed were quantitated in a table (top right). The arrowheads indicate examples of decondensed centromeres. Scale bar: 10 μm.

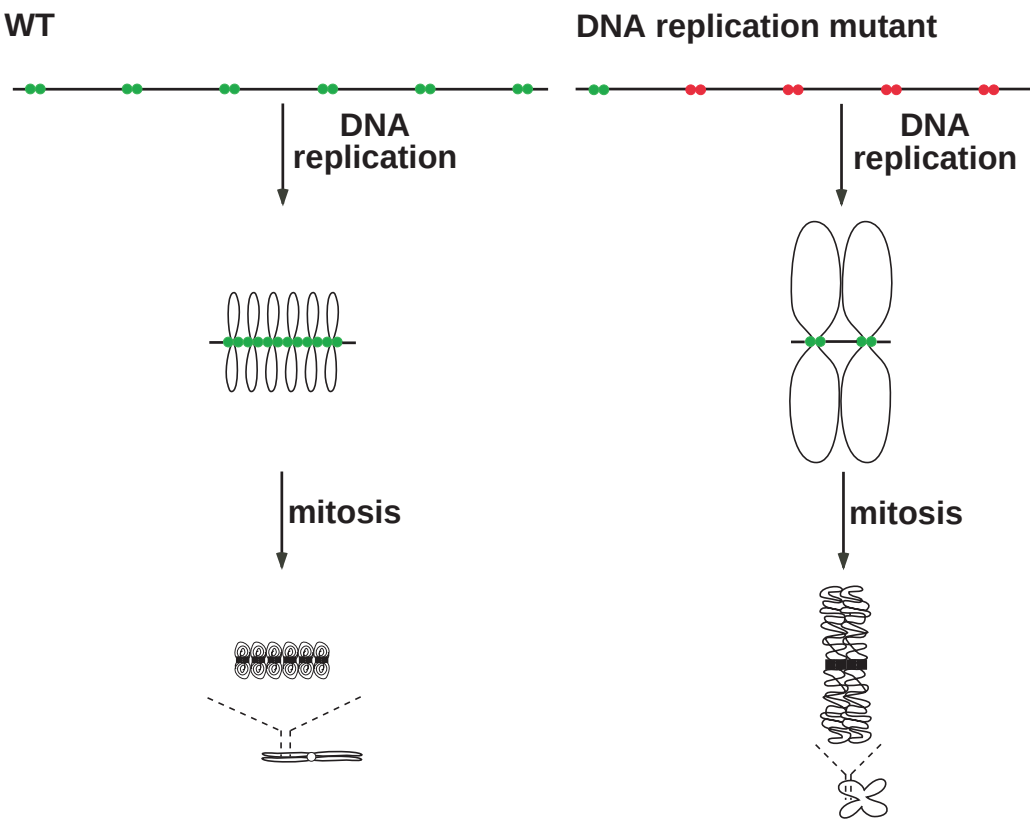

Fig. 8. Model: DNA replication is required for proper chromosomal condensation. In wild type, the density of functional DNA replication centers (green circles) in the genome is normal. During DNA replication, DNA is looped through the DNA replication machinery and serves as a first degree of chromosomal condensation. These loops are then tightly coiled in mitosis. In an *Orc* mutant, the density of functional replication origins in the genome is much lower. The majority of replication origins are inactive (red circles), presumably owing to lower levels of functional replication factors. During DNA replication, more DNA is pulled though fewer DNA polymerase complexes resulting in much larger loops. The reduced number and large size of the DNA loops then results in chromosomes that are much more disorganized and less condensed in mitosis.

cells appear to accumulate in a state with many of the hallmarks of metaphase. Specifically, they arrested at a stage that lacked a nuclear membrane, with many characteristics of a spindle. Moreover, there was no evidence of anaphase bridges, characteristic of cells at more advanced stages of mitosis. Furthermore, in most cells, sister chromatids were evident, suggesting that cells had passed through S phase. However, the mitotic state observed in these mutants was defective because of the presence of abnormally condensed chromosomes, absence of a complete bipolar spindle and congression failure.

This defective metaphase arrest with abnormally condensed chromosomes was observed in all DNA replication mutants tested. Irrespective of the condensation mechanisms that are defective or inoperative in DNA replication mutants, broken chromosomes are probably the triggers that signal the mitotic arrest. Much of the data from genetic analysis of the SMC protein complex suggests that incomplete condensation per se does not trigger mitotic arrest. SMC mutants proceed through the metaphase-anaphase transition normally with decondensed chromatin, but have chromosome segregation defects (Lieb et al., 1998; Saka et al., 1994; Strunnikov et al., 1993). In *Drosophila*, both *barren* (encoding a non-SMC protein likely associated with condensin) and *gluon* (an SMC4 homolog) mutants show chromosome defects with anaphase bridges (Bhat et al., 1996; Ouspenski et al., 2000). Instead, insufficient levels of DNA replication proteins may increase the levels of broken chromosomes and/or result in incomplete DNA replication, which would lead to a cell cycle arrest in late S phase. However, a number of such cells might break through such a checkpoint.

Cells transiently arrested in S/G2 because of incomplete replication or damaged DNA may then be halted in mitosis by checkpoints sensitive to chromosomal integrity. Treatment of wild-type embryos with the DNA synthesis inhibitor aphidicolin or irradiating embryos during mitosis results in a similar phenotype to that of DNA replication mutants: a metaphase arrest characterized by a poorly defined anastral mitotic spindle and chromosomes that do not align appropriately at the metaphase plate. This mitotic arrest is thought to operate with centrosome inactivation as mitotic spindles are anastral and the centrosomes are deficient of certain γ-tubulin components (Sibon et al., 2000). Indeed it is interesting that the γ-tubulin mutant *γtub23C* shows defective mitotic figures strikingly similar to those images reported here for *Orc* mutants (Sunkel et al., 1995). In addition, γ-tubulin staining appears abnormal in all the DNA replication mutants tested here: γ-tubulin staining often co-localizes with the chromosomes and is frequently fragmented into several pieces (data not shown). Thus, centrosome inactivation may be part of an additional pathway serving to prevent damaged or incompletely replicated DNA from finishing mitosis.

If *Drosophila* cells have two mechanisms for monitoring damaged or incompletely replicated DNA caused by insufficient ORC levels or any protein involved in DNA replication, one would expect defects in the S phase checkpoint to increase the frequency of cells arrested in mitosis. *grapes* (*grp*) encodes for the *Drosophila* homolog of Chk1, a protein kinase required for a checkpoint-mediated cell cycle arrest triggered by DNA damage or incomplete DNA replication. We thus compared *Orc2 grp* homozygous double mutants with *Orc2* homozygous larvae. No change in the frequency of mitotic cells was observed in such DAPI-stained mitotic spreads (data not shown). This result may however reflect the

multiple pathways that operate in S phase to monitor DNA damage in *Drosophila* (Brodsky et al., 2000). In *S. cerevisiae*, this multi-tiered monitoring of DNA damage, including a back-up checkpoint in mitosis may not be as effective. Dillin and Rine first reported that *Orc* mutants arrest in late stages of the cell cycle and postulated that ORC might have an M phase function (Dillin and Rine, 1998). However, recent work establishes that an *orc2 rad9* double mutant (rad9 is a protein kinase required for the DNA damage checkpoint) does not arrest at such a discrete point (P. Garber and J. Rine, personal communication).

ORC, replication and chromosome condensation

The most unanticipated aspect of this work is the impact of DNA replication mutations on the overall ultrastructure of mitotic chromosomes. The large majority of mitotic chromosomes were abnormally condensed and lacked distinct centromeres. The shorter and thicker appearance of these mitotic chromosomes suggests that an element of lateral condensation was lost.

The incomplete and abnormal chromosomal morphologies described here are interesting in light of present knowledge about mechanisms of chromosome condensation. The data add to the evidence that condensation is a multi-tiered process and not an all-or-none mechanism. Incomplete condensation in a DNA replication mutant strain may be an indirect consequence of S phase defects. For example, *Orc* deficiencies that lead to incomplete replication or damaged DNA may affect the levels of cyclin-dependent kinases, leading to reduced condensation. We emphasize that the mutant cells examined have undergone nuclear membrane breakdown, have phosphorylated histone H3, and appear well into mitosis. Thus Cdc2-cyclinB levels must be activated, but perhaps not as high as needed for normal progression. Of course, other proteins involved in condensation may be sensitive to S-phase progression. Replication timing may also play an important role in organizing chromosomal condensation and a limited level of factors might interfere with the normal coordination of folding domains. While this paper was in preparation, a study of *Orc2* mutants appeared in which the mitotic condensation defects were also documented. In that report, a mechanistic emphasis was placed upon the apparently abnormal timing of replication events and location of condensation defects, though no direct link between condensation and replication timing was shown (Loupart et al., 2000).

It is just as likely that replication plays a direct role in sculpting the morphology of the mitotic chromosome. For example, the density of active *Orc* loci on the chromatin fiber could influence the number of replication factories that would loop together such origins. Alternatively, bi-directional growing forks may be held together at a point (Lemon and Grossman, 1998) and replicated DNA would be condensed as it is spooled through the machinery.

The notion that each pair of replication forks that start at an origin remains attached was proposed by Sundin and Varshavsky as a mechanism to help avoid entanglement of independently rotating forks (Sundin and Varshavsky, 1980). Hearst et al. have further explored this idea and first pointed out that such attachment would automatically lead to a length contraction of the chromosome (Hearst et al., 1998). One of the predictions of this model is in striking concordance with our observations. In DNA replication mutants, there is a lower density of replication centers and chromosomes are abnormally shorter and wider as expected. Clearly, the number of such centers would affect the total level of chromosome condensation (Fig. 8). Fewer replication centers predict much larger loops and a much reduced lateral chromosomal condensation. While our observations to date do not distinguish between the many models discussed here, we believe that the ideas embodied in Fig. 8 fit well with our findings. Another crucial prediction of this model is that a certain level of condensation occurs during S phase and with a more detailed knowledge of the distribution of origin sites along the chromosomal DNA fiber, testing this point will become possible.

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