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Aberrant replication timing induces defective chromosome condensation in *Drosophila* ORC2 mutants

Marie-Louise Loupart, Sue Ann Krause* and Margarete M.S. Heck

Background: The accurate duplication and packaging of the genome is an absolute prerequisite to the segregation of chromosomes in mitosis. To understand the process of cell-cycle chromosome dynamics further, we have performed the first detailed characterization of a mutation affecting mitotic chromosome condensation in a metazoan. Our combined genetic and cytological approaches in *Drosophila* complement and extend existing work employing yeast genetics and *Xenopus in vitro* extract systems to characterize higher-order chromosome structure and function.

Results: Two alleles of the *ORC2* gene were found to cause death late in larval development, with defects in cell-cycle progression (delays in S-phase entry and metaphase exit) and chromosome condensation in mitosis. During S-phase progression in wild-type cells, euchromatin replicates early and heterochromatin replicates late. Both alleles disrupted the normal pattern of chromosomal replication, with some euchromatic regions replicating even later than heterochromatin. Mitotic chromosomes were irregularly condensed, with the abnormally late replicating regions of euchromatin exhibiting the greatest problems in mitotic condensation.

Conclusions: The results not only reveal novel functions for ORC2 in chromosome architecture in metazoans, they also suggest that the correct timing of DNA replication may be essential for the assembly of chromatin that is fully competent to undergo mitotic condensation.

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Background

The origin recognition complex (ORC) is composed of six subunits, ORC1–6 [1]. ORCs have been identified in yeast, flies, frogs, mice and humans [2], and several of the homologous subunits or even whole complexes are functionally interchangeable between species, suggesting a high degree of conservation of ORC function [3–5]. The *Drosophila* ORC complex was isolated from embryonic extracts by functional homology to the *Saccharomyces cerevisiae* complex, and all six *Drosophila ORC* genes (*DmORC1–6*) have now been cloned [4,6–8]. A fully functional *Drosophila* ORC has also been reconstituted from the six recombinant proteins [4].

In *S. cerevisiae*, ORC is bound throughout the cell cycle to specific DNA elements of replication origins [9–11]. Binding of Cdc6p to the origin is the initial step in the formation of a pre-replication complex (pre-RC) [12,13]. Subsequently, the minichromosome maintenance (MCM) proteins are loaded onto the origin, S-phase cyclin-dependent kinase is activated and Cdc45p associates with the pre-RC in preparation for S-phase entry [13,14]. Assembly of the pre-RC is likely to correspond to the 'licensing' of replication origins first described in the *Xenopus* cell-free system [15–19]. Although there are a number of differences in detail, a similar mechanism for the assembly of the pre-RC appears to exist in metazoans.

Although ORC is principally involved in the initiation of DNA replication, additional roles in the establishment and maintenance of transcriptional silencing and heterochromatin have been suggested. In both *Xenopus* and *Drosophila*, ORC and HP-1 interact [7,20] and a single mutant copy of *DmORC2* will suppress position-effect variegation [7]. Replication and transcriptional silencing have also been linked by *ORC2* and *ORC5* mutants in *S. cerevisiae* [21]. As transcriptional silencing at the yeast mating-type loci appears functionally similar to heterochromatin in higher eukaryotes, these experiments provide additional evidence for the involvement of ORC in the specification of transcription-rich euchromatin versus transcription-poor heterochromatin.

As ORC is not required for the activation of origins in yeast and *Xenopus* once Cdc6 and MCM proteins have bound [22,23], ORC may only be necessary for the recruitment of these proteins to origins. ORC's role in transcriptional regulation suggests that it may also provide a 'landing pad' for other protein complexes important for chromosome dynamics [24]. Regulation of different complexes recruited

by ORC could be achieved through a conformational change in the complex, either as a result of the dissociation of one or more subunits, or hydrolysis of bound ATP [25].

Although ORC is bound to the origin throughout the cell cycle in yeast, the protein levels of at least two ORC subunits in *Drosophila* may fluctuate during the cell cycle and/or development. In the ovary, a developmentally regulated switch in the follicle cells from endoreduplication of the entire genome to specific amplification of the chorion genes causes a shift in the distribution of both ORC1 and ORC2 within the nucleus to the foci of amplifying chorion genes [26,27]. *ORC1* is an E2F-responsive gene and the protein accumulates in late G1 and S phase in the eye imaginal disc [26]. Thus, although all six subunits appear to be necessary for ORC's function in replication initiation, it is not clear whether all subunits remain associated with the origin throughout the cell cycle in metazoans.

The *Drosophila* lethal mutant *l(3)k43* [28,29] has a disc-less phenotype and is generally defective in cell proliferation during the larval stages of the *Drosophila* life cycle [30], exhibiting mitotic chromosome fragmentation and condensation defects [31]. An additional female sterile allele results in a reduction of chorion gene amplification and *DmORC2* was identified as the gene responsible for these phenotypes [32]. To learn more about the role(s) ORC2 may play in chromosome dynamics through the cell cycle, we have characterized in detail the cell-cycle progression and striking chromosomal defects of two late larval lethal alleles of *DmORC2*.

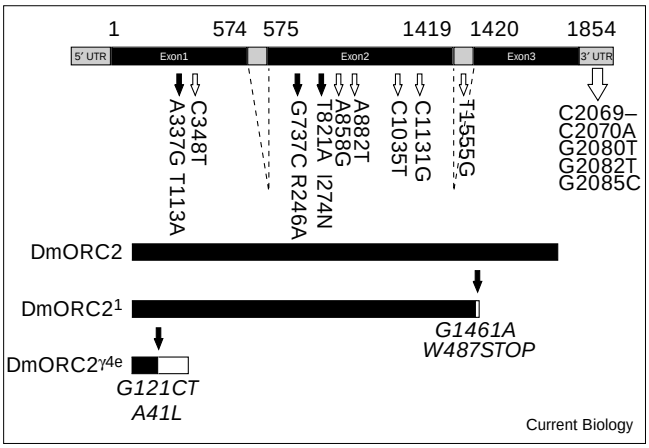
Results

DmORC2^1 is truncated by a premature stop codon

The DmORC2 protein is 618 amino acids in length [6] and the lesions for the female sterile and the *l(3)k43*$^{\gamma 43}$ alleles have been reported [32]. We sequenced the *l(3)k43*^{1} allele after amplifying the gene from homozygous mutant larval genomic DNA and identified a single base substitution, which introduces a premature stop codon at residue 487 (Figure 1). We also confirmed the frameshift near the start of the coding sequence of the *l(3)k43*$^{\gamma 4e}$ allele [32]. Therefore, the evolutionarily conserved carboxy-terminal portion of the protein was missing from both DmORC2^1 and DmORC2$^{\gamma 4e}$ (Figure 1). We also sequenced two *Drosophila ORC2* expressed sequence tags (ESTs) and the gene from a wild-type stock, finding eight additional base changes in the coding region of all sequences analyzed, compared with the published sequence [6]. Three cause amino-acid changes in the predicted protein: T113A, R246A, and I274N; the other five base changes are silent (Figure 1).

Immunoblots of third instar larval protein extracts using two independently raised antibodies to DmORC2 detected a band of the expected size (82 kDa) in wild-type and heterozygous larval extracts but not in either of the homozygous mutant larval extracts (data not shown). We failed to detect any truncated ORC2 forms in either the *l(3)k43*^{1} or *l(3)k43*$^{\gamma 4e}$ homozygotes, suggesting that these forms may be unstable or not produced.

Figure 1



DmORC2 is truncated in the *l(3)k43*^{1} and *l(3)k43*$^{\gamma 4e}$ alleles. Diagram of the *l(3)k43*^{1} mutation (indicated by the black arrow on DmORC2^1), with the wild-type and *l(3)k43*$^{\gamma 4e}$ alleles shown for comparison. The mutation of the *l(3)k43*$^{\gamma 4e}$ allele was published previously [32]. Within the *DmORC2* coding sequence of all alleles sequenced, there were eight base changes, resulting in three amino-acid substitutions (T113A, R246A and I274N; filled arrows) and five silent base changes (open arrows). There were also base changes in intron 2 and in the 3′ untranslated region (UTR; open arrows), which would not affect the protein product. The adjusted sequence of the wild-type DmORC2 protein has been deposited in GenBank under accession number AF246305.

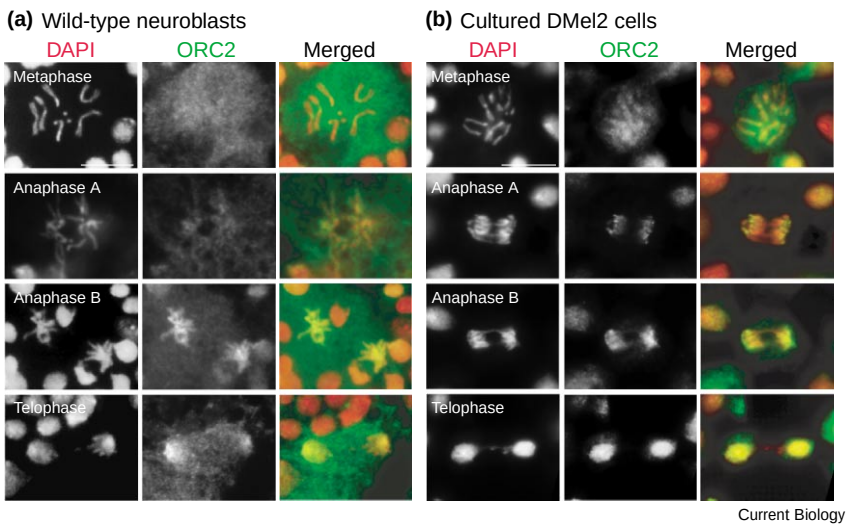
DmORC2 accumulates on chromosomes in late anaphase/telophase

During interphase, ORC2 localization was strong in the nucleus, though also present at a low cytoplasmic level (data not shown). ORC2 was not detected on chromosomes of mitotic wild-type neuroblasts from prophase through early anaphase (Figure 2a). In late anaphase, staining of the segregating chromosomes became intense along the length of the chromatids, and persisted into telophase. This pattern of staining was also observed using affinity-purified ORC2 antibody and in three *Drosophila* cell lines following either paraformaldehyde or methanol fixation (Figure 2b). ORC2 staining is remarkably similar to that of *Xenopus* ORC1, which is also absent from metaphase chromosomes but present on anaphase chromatids [33]. It appears likely that as the chromatids segregate in anaphase and reach the spindle poles, ORC is deposited on the replication origins in preparation for the next cell cycle.

Irregular chromosome condensation in *DmORC2* mutant mitotic neuroblasts

Examination of DAPI-stained larval neuroblasts from both *l(3)k43*^{1} and *l(3)k43*$^{\gamma 4e}$ alleles revealed many severe mitotic

Figure 2

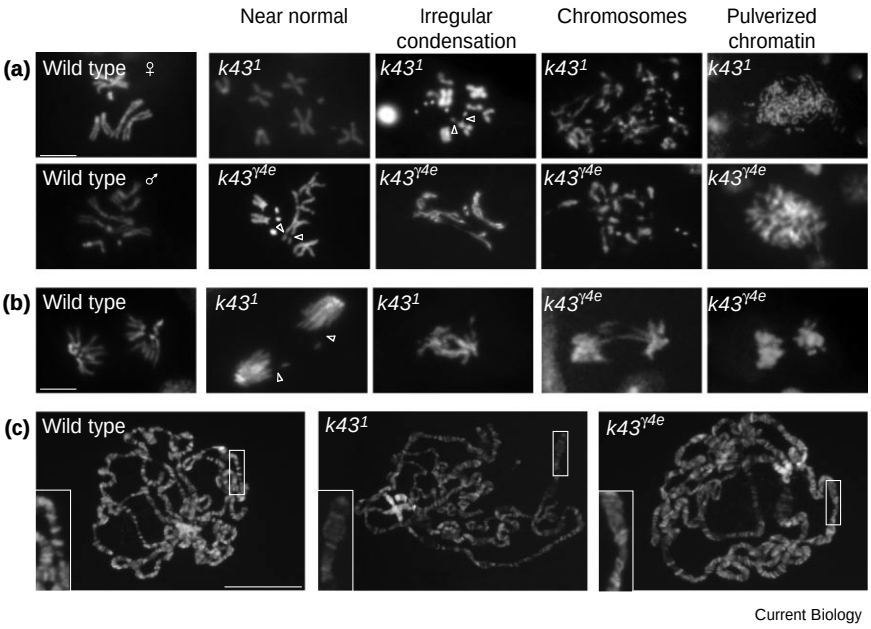


DmORC2 accumulates on chromosomes in anaphase. Immunofluorescence of **(a)** wild-type neuroblasts (paraformaldehyde fixation) and **(b)** cultured *Drosophila* Dmel2 cells (methanol fixation) in metaphase, anaphase and telophase of the cell cycle. In the merged images, DNA is red, and ORC2 is green. DAPI, 4,6-diamidino-2-phenylindole. The corresponding pre-immune serum produced faint diffuse nuclear and cytoplasmic staining, but not the accumulation on late anaphase and telophase chromosomes observed with the immune serum. The scale bars represent 5 µm.

defects (Figure 3a). More than 90% of mitotic figures were abnormal. Most frequently, mitotic chromosomes contained regions of undercondensed chromatin connected by normally condensed or even highly condensed chromatin, the same undercondensed region often visibly affected on both sister chromatids (Figure 3a, arrowheads). While the undercondensed chromatin could consist of a very fine though discernible thread, it was also clear that chromosome breaks and rearrangements had occurred, and that polyploidy was possible. In ~1% of mitotic figures, the chromatin appeared to be 'pulverized.' These figures were highly reminiscent of S-phase prematurely condensed chromosomes (PCCs) [34], suggesting these *l(3)k43* PCC-like cells may have precociously exited S phase and entered mitosis.

Although we have observed anaphase figures in *DmORC2* mutants (Figure 3b), the frequency of anaphase cells was

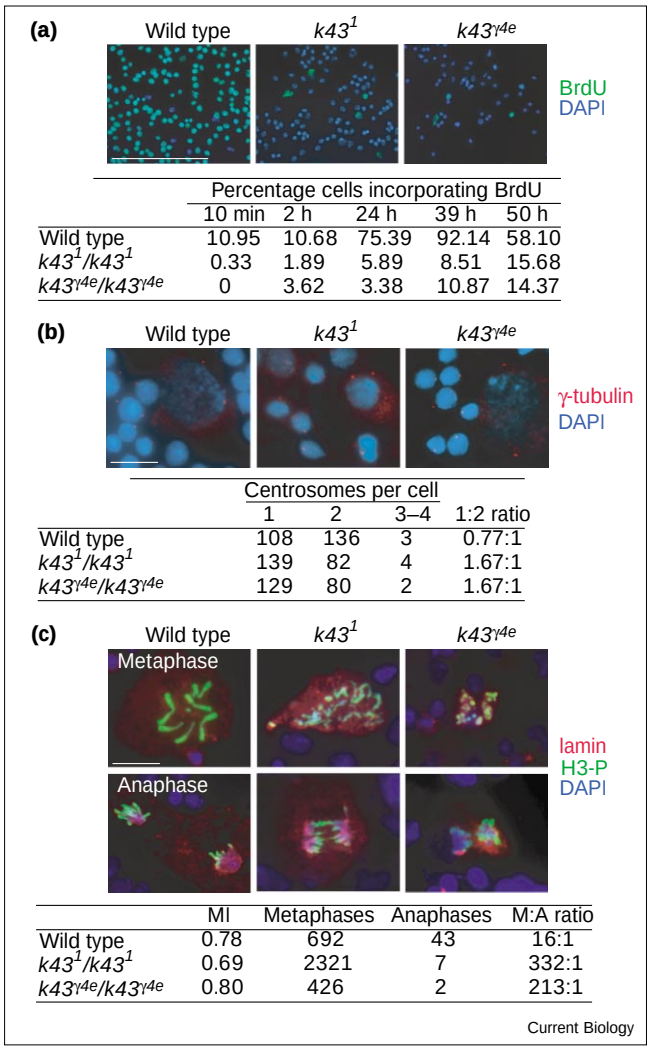
Figure 3



The mitotic chromosomes of *DmORC2* mutant neuroblasts are irregularly condensed or fragmented whereas the polytene chromosomes appear normal. **(a)** Metaphase and **(b)** anaphase figures of third instar wild-type, *l(3)k43*^{1} and *l(3)k43*$^{\gamma 4e}$ neuroblasts. The scale bars represent 5 µm. Chromosomes in which the same region on both sister chromatids was affected are indicated by open arrowheads. **(c)** Polytene chromosomes of third instar wild-type, *l(3)k43*^{1} and *l(3)k43*$^{\gamma 4e}$ salivary glands, each with a portion of the genome shown at higher magnification, demonstrating normal chromosome banding (inset). The scale bar represents 50 µm.

greatly reduced compared with the wild type. The anaphase figures we observed were all abnormal, showing either condensation defects on sister chromatids (Figure 3b, arrowheads) or chromatid bridges. Therefore, even though apparently normal bipolar spindles had formed in the *DmORC2* mutants (as judged by α- and γ-tubulin immunostaining), cells appeared unable to complete mitosis.

Mutant salivary glands were of normal size and the polytene chromosomes of both alleles were unaffected with respect to banding pattern and level of polyploidization (Figure 3c). Our data differ from a previous observation that the *l(3)k43*$^{\gamma 4e}$ polytene chromosomes are under-replicated with a poor, irregular banding pattern [20], but may be explained by the use of a modified feeding regime that increases larval health. Either ORC2 is not needed for the endoreduplication cycles necessary for the formation of salivary gland polytene chromosomes or, more likely, maternally deposited ORC2 is sufficient to support replication in the salivary glands (augmented by a lower turnover of ORC in polyploid cells than in mitotically active cells).



	Percentage cells incorporating BrdU				
	10 min	2 h	24 h	39 h	50 h
Wild type	10.95	10.68	75.39	92.14	58.10
k43^{1}/*k43*^{1}	0.33	1.89	5.89	8.51	15.68
k43$^{\gamma 4e}$/*k43*$^{\gamma 4e}$	0	3.62	3.38	10.87	14.37

	Centrosomes per cell			
	1	2	3–4	1:2 ratio
Wild type	108	136	3	0.77:1
k43^{1}/*k43*^{1}	139	82	4	1.67:1
k43$^{\gamma 4e}$/*k43*$^{\gamma 4e}$	129	80	2	1.67:1

	MI	Metaphases	Anaphases	M:A ratio
Wild type	0.78	692	43	16:1
k43^{1}/*k43*^{1}	0.69	2321	7	332:1
k43$^{\gamma 4e}$/*k43*$^{\gamma 4e}$	0.80	426	2	213:1

Perturbation of cell-cycle kinetics in *DmORC2* mutants

Feeding 5-bromo-2′-deoxyuridine (BrdU) to wild-type larvae for 10 minutes is sufficient to detect S-phase neuroblasts (data not shown). Thus, it is possible to estimate the proportion of replicating neuroblasts in brains. Neuroblast preparations from wild-type and *DmORC2* mutant larvae fed BrdU for 10 minutes to 50 hours were processed for BrdU incorporation and the percentage of cells positive for BrdU was determined (Figure 4a). Wild-type neuroblasts were actively proliferating with rapid incorporation of BrdU, in contrast to both alleles and there appeared to be, in 24 hours, a 13–22-fold decrease in the percentage of *DmORC2* mutant cells that had entered S phase as compared with the wild type; this was not alleviated with longer BrdU incorporation periods.

Determination of the ratio of cells with single centrosomes to those with conspicuously duplicated centrosomes clearly confirmed that *DmORC2* mutant cells were delayed in cell-cycle progression (Figure 4b). During mitosis, the two poles of the spindle were marked by γ-tubulin staining and, when neuroblasts entered G1 phase, only a single centrosome was apparent. Coimmunostaining for BrdU and γ-tubulin indicated that centrosomes were visibly separated in all S-phase wild-type neuroblasts (detected by 10 minutes BrdU feeding), a correlation that was also observed in *l(3)k43*^{1} and *l(3)k43*$^{\gamma 4e}$ neuroblasts. Using this assay, the ratio of G1 cells was found to be increased by a factor of ~2 in the *DmORC2* mutants, demonstrating an accumulation of G1 cells when ORC2 was defective.

Despite the delay early in the cell cycle in *DmORC2* mutant neuroblasts, the overall mitotic index was not very different from that of wild-type neuroblasts. This paradox was reconciled when the metaphase to anaphase ratio was

Figure 4

Cell-cycle progression in *DmORC2* mutant neuroblasts is abnormal. **(a)** BrdU uptake in third instar wild-type, *l(3)k43*^{1} and *l(3)k43*$^{\gamma 4e}$ neuroblasts. Each panel shows neuroblasts after 24 h feeding. BrdU, green; DNA, blue. The table shows the quantitation of BrdU uptake from 10 min to 50 h of continuous feeding. Between 614 and 2702 cells were counted for each time point and genotype. The scale bar represents 50 μm. **(b)** Immunofluorescence analysis of third instar wild-type, *l(3)k43*^{1} and *l(3)k43*$^{\gamma 4e}$ neuroblasts. DNA, blue; γ-tubulin, red. Each panel shows neuroblasts with one or two centrosomes, and the table shows the number of cells counted and the ratio of G1 (one centrosome) to S/G2/M (two centrosomes) cells in brains. The scale bar represents 5 μm. **(c)** Immunofluorescence analysis of third instar wild-type, *l(3)k43*^{1} and *l(3)k43*$^{\gamma 4e}$ neuroblasts in metaphase and anaphase of the cell cycle. Lamin, red; phosphorylated histone H3 (H3-P), green; DNA, blue. The number of cells counted for the mitotic index was: wild type, 171,967; *l(3)k43*^{1}, 113,627; *l(3)k43*$^{\gamma 4e}$, 8,985. The irregular condensation in mutant neuroblasts does not affect the mitotic-associated phosphorylation of histone H3 or the dispersal of the nuclear lamina. The table shows the number of cells counted and the ratio of metaphase to anaphase (M:A) figures in larval brains. The scale bar represents 5 μm. MI, mitotic index.

determined after staining for the mitosis-specific phosphorylated form of histone H3 [35]. In *DmORC2* mutants, the metaphase to anaphase ratio was 13–21-fold higher than in wild-type cells (Figure 4c). Thus, we believe that the delay in exiting mitosis compensates for the delay early in the cell cycle to result in an overall mitotic index similar to that in the wild type.

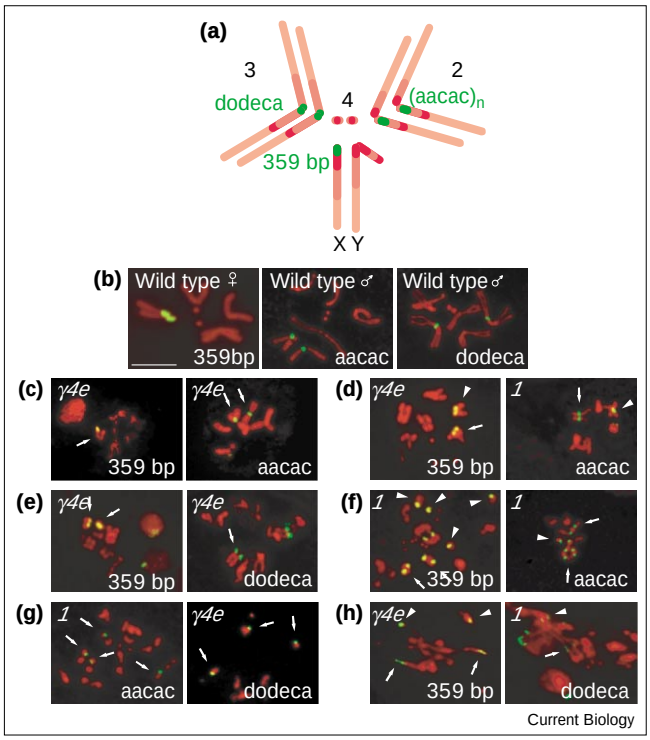
The condensation defect rarely involves heterochromatin in *DmORC2* mutant neuroblasts

We examined the condensation fate of pericentric heterochromatic sequences [36] on each of the three large *Drosophila* chromosomes by fluorescence *in situ* hybridization (FISH, Figure 5a). These sequences also served to mark each chromosome, facilitating the identification of rearranged chromosomes including chromosome fragments, in the more severely affected mutant mitotic figures. Male and female wild-type brains were used to confirm the localization of each probe on mitotic neuroblast chromosomes (Figure 5b). The three probes gave remarkably similar results with both of the *DmORC2* alleles, showing that the heterochromatic regions were properly condensed in 99% of spreads examined. Strikingly, the probes also illustrated a variety of chromosomal abnormalities in both *l(3)k43*^{1} and *l(3)k43*$^{\gamma 4e}$ figures, with more than one kind of abnormality identified within some mitotic figures. These abnormalities could be placed in the following categories and representative images are shown in Figure 5: fairly normal chromosomes, though usually with some irregularities in condensation (Figure 5c, arrows); single sister chromatid breaks (Figure 5d, arrows); double sister chromatid breaks (Figure 5e, arrows); complex rearrangements, possibly involving more than one chromosome (Figure 5f, arrows); small supernumerary chromosomes containing sequences derived from chromosome X, 2 or 3 (Figure 5g, arrows). However, in all of these chromosomes, heterochromatic sequences as identified by the site of hybridization were only very rarely undercondensed (Figure 5h, arrows).

Temporal control of DNA replication is disrupted in *DmORC2* mutant neuroblasts

The FISH experiments, along with the frequent observation of condensation defects on equivalent regions of sister chromatids, suggested that euchromatin may be more prone to defective condensation than heterochromatin. This could be explained if condensation defects were a consequence of aberrant DNA replication. For example, alterations in the replication timing of particular loci might lead to changes in protein association that result in subsequent condensation defects. To determine whether there was a correlation between replication timing and the irregular condensation observed in mitotic figures, we fed larvae BrdU for varying times and examined the pattern of BrdU incorporation in chromosomes at the following mitosis (Figure 6a).

Figure 5



FISH analysis of wild-type and *DmORC2* mutant neuroblasts with heterochromatic probes. **(a)** Karyotype of *D. melanogaster* showing the localization of each of the loci detected by the probes used for FISH. **(b)** Hybridization of the 359 bp probe to female wild-type larval neuroblasts, and the $(aacac)_n$ and dodeca repeat probes to male wild-type neuroblasts. **(c–h)** Examples of *l(3)k43*^{1} and *l(3)k43*$^{\gamma 4e}$ mitotic figures grouped by particular rearrangement (arrows), although additional rearrangements (arrowheads) occurred in many figures: (c) near-normal chromosomes could be identified in 18% of mitotic figures (n = 386), that is, metacentric chromosomes 2 or 3, telocentric X chromosomes, though usually with some irregularities in condensation (arrows); (d) 8% of events were single sister chromatid breaks identified in the tagged chromosome; (e) double sister chromatid breaks (21% of events) were also observed in the tagged chromosome (arrows); (f) 33% of events were complex rearrangements, possibly involving more than one chromosome (arrows); (g) small supernumerary chromosomes (19%) could be formed from heterochromatic sequences that resembled chromosome 4 except that the supernumeraries were tagged by one of the probes from chromosome X, 2 or 3 (arrows); and (h) the site of hybridization was extremely undercondensed in only 1% of cases (arrows). In all images, the probe is green and the DNA is red. The scale bar represents 5 μm.

Long exposures (4–24 hours) of wild-type larvae to BrdU generated substantial numbers of totally labeled mitotic figures, indicating that these cells had progressed through most of S phase and G2 within as little as 4 hours (Figure 6b). Shorter exposures of 2–4 hours resulted in partial BrdU incorporations in wild-type mitotic chromosomes. As expected, centromeric heterochromatic DNA was late replicating in all wild-type cells (Figure 6c) and the centromere of chromosome 3 appeared to replicate latest by this assay (Figure 6d). As described above, the

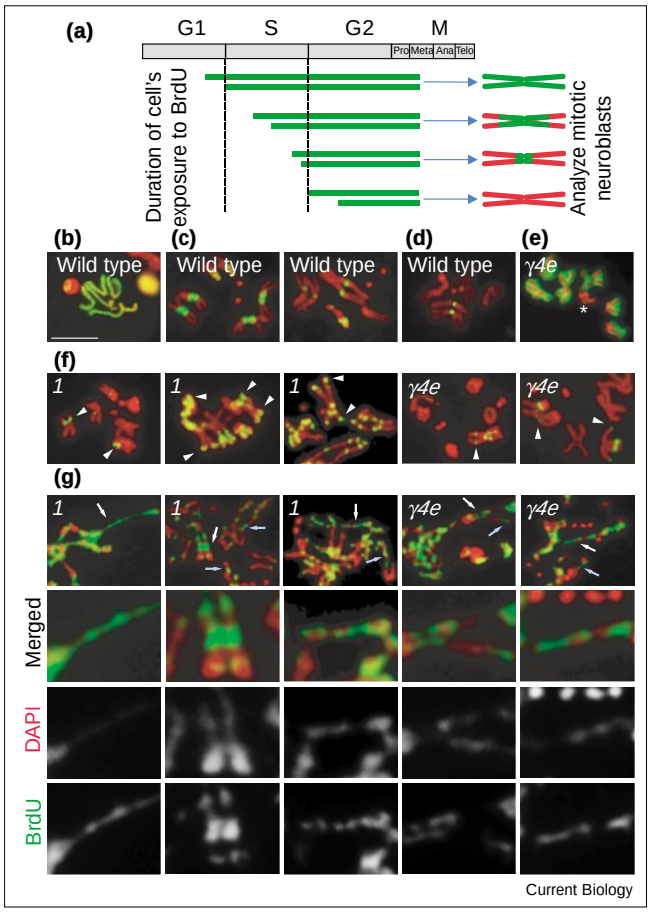


Figure 6

Analysis of replication timing with BrdU incorporation in wild-type and *DmORC2* mutant neuroblasts. **(a)** Diagram showing the expected pattern and extent of BrdU incorporation (green) in wild-type mitotic chromosomes (red) depending on when in the S phase BrdU incorporation had occurred. If neuroblasts incorporated BrdU early in S phase, all of the genome would be labeled and mitotic chromosomes would exhibit full BrdU incorporation. If incorporation commenced sometime during S phase, only a fraction of the genome, proportional to the amount of time spent in S phase, would be labeled, and mitotic chromosomes would show a partial labeling. If incorporation occurred only at the end of S phase, then only the very latest replicating parts of the genome (the pericentromeric heterochromatin) would be labeled. **(b)** Total BrdU incorporation in wild-type neuroblasts. **(c)** Partial incorporation of BrdU in wild-type neuroblasts at pericentromeric heterochromatin. **(d)** The centromere of chromosome 3 is the very last region of the genome to replicate in wild-type neuroblasts. In 93% of the observed wild-type mitotic figures (n = 55), the late-replicating regions were restricted to heterochromatin. **(e)** Near-total BrdU incorporation in a *l(3)k43*$^{\gamma 4e}$ mitotic figure, except for both homologues of chromosome 4 (asterisk). **(f)** Regions of euchromatin (arrowheads) can be later replicating than areas of pericentromeric heterochromatin. Heterochromatic regions were last to replicate in only 10% of mutant mitotic figures (n = 30) whereas 50% of mutant mitotic figures showed both heterochromatin and euchromatin could be late-replicating. Areas of euchromatin in 40% of the mutant mitotic figures had actually replicated after heterochromatic regions. **(g)** Undercondensed regions of paired and unpaired chromatids of *l(3)k43*^{1} and *l(3)k43*$^{\gamma 4e}$ mitotic figures are late replicating (white and blue arrows). Examples from each of these panels (white arrows) are also shown at higher magnification. BrdU labeling was restricted to the undercondensed regions of mitotic chromosomes in 38% of partially incorporated mutant mitotic figures (n = 60), and 55% of mutant mitotics showed both undercondensed and condensed DNA that was late replicating. BrdU incorporation was found to be condensed in 100% of the wild-type mitotic figures. All panels were counterstained with DAPI (red) and BrdU (green). The scale bar represents 5 µm.

frequency of BrdU labeling of *l(3)k43*^{1} and *l(3)k43*$^{\gamma 4e}$ neuroblasts was considerably less than in the wild type. Mitotic figures with partial BrdU incorporation were observed from 6–24 hours, but totally incorporated mitotic figures were only clearly identified with much longer BrdU feeding. A near-normal metaphase from *l(3)k43*$^{\gamma 4e}$ with every chromosome labeled except for chromosome 4 is shown in Figure 6e. This result suggests that the cell cycle is greatly elongated in *DmORC2* mutant neuroblasts, with more than 24 hours required between the initiation of DNA replication and mitosis.

Two extraordinary patterns of partial BrdU incorporation were observed in both *DmORC2* alleles. The first pattern was apparent in those mitotic figures with relatively 'normal' chromosome condensation. Partially labeled mitotic figures showed that late incorporation was not confined solely to centromeric heterochromatin, as it exclusively was in wild-type neuroblasts. Mitotic figures clearly showed BrdU-labeled euchromatin (at sites along chromosome arms) and only some BrdU-labeled centromeric/heterochromatic DNA (Figure 6f). Thus, it was not necessarily heterochromatic DNA that was latest replicating in *DmORC2* mutants. Some euchromatic regions replicated even after heterochromatic domains in *DmORC2* mutants, in stark contrast to wild-type cells. The second startling pattern of BrdU incorporation was evident in those mitotic figures exhibiting irregular chromosome condensation: regions of compact chromatin alternated with regions of BrdU incorporation (clearly evident in the merged images of Figure 6g). These mitotic figures indicated two things: the labeled thin strands were BrdU-positive and therefore replicated, and the condensation machinery was unable to properly assemble or fully function on at least some regions of the late-replicating chromatin. These patterns suggested that the temporal control of replication was altered in *DmORC2* mutant larvae and, furthermore, that defective condensation appeared to be linked with the abnormally late replication of euchromatin.

Discussion

We have analyzed in detail the mutant phenotype of two late larval lethal alleles of the *ORC2* gene in *Drosophila*, gaining insight into the roles ORC2 plays through the cell cycle in a metazoan. *A priori*, one might think that mutations in genes solely important for replication should arrest the cell cycle at some point in S phase. That the mutant phenotype for *DmORC2* included striking chromosomal defects in mitosis, indicates that ORC2, and probably

ORC, plays other roles in cell-cycle progression. Although *DmORC2* mutations had a dramatic effect on mitotic chromosome structure, a number of events associated with mitosis occurred normally. The depolymerization of the nuclear lamina occurred, as did the mitosis-specific phosphorylation of histone H3. Bipolar spindles could be observed, and the kinetochore attachment checkpoint appeared also to be functional. However, more subtle defects were identified that demonstrated a crucial role for ORC2 in determining proper replication timing.

The phenotype reported here is specific to mutations in *ORC2*, and not observed generally for defects in other replication proteins. We have also analyzed mutations in *DmRfc4*, subunit 4 of replication factor C (important for loading proliferating cell nuclear antigen (PCNA) to allow processive replication). Although we observed mitotic defects, they differ from the *DmORC2* phenotype described here (resulting in either PCC-like figures or premature sister chromatid separation), and are primarily due to defective checkpoint control (S.A.K., M-L.L., S. Vass, S. Harrison and M.M.S.H., unpublished work). The localization of Rfc4 during mitosis is also different from that of ORC2, as the protein does not appear to rebind chromatin during anaphase. Thus, the *DmORC2* phenotype is specific to this protein, and not a general consequence of inhibiting proteins essential for replication. Mitotic phenotypes may exist for other mutations in replication proteins. Proliferation defects and decreased BrdU incorporation have been reported for *Drosophila* ORC3 [8], MCM2 [37] and MCM4 [38] mutants, but the phenotype of mitotic cells has not yet been described for any of these mutations. Neuroblasts of PCNA mutants do not exhibit mitotic abnormalities (Daryl Henderson, personal communication).

Developmental control of chromosome architecture

The intense ORC2 accumulation on mitotic chromosomes in late anaphase and telophase is striking, and similar to the observed localization of ORC1 in *Xenopus* cultured cells [33]. DmORC2 is strongly localized to the centromeres of metaphase and anaphase chromosomes of early syncytial *Drosophila* embryos and physically associates with the heterochromatin-binding protein, HP-1 [7]. We have observed a low, but detectable, concentration of ORC2 on pericentric regions of anaphase chromosomes in cultured cells (Figure 2b). These differences in ORC concentration on centromeres could be attributable to the inherent biological differences of the two stages of *Drosophila* development: the rapid S/M cycling occurring in embryos versus the slower cell cycles with Gap phases of neuroblasts. If DmORC2 is retained more strongly at centromeric regions during embryogenesis, ORC may aid in the establishment of heterochromatin, which occurs concomitant with cellularization in *Drosophila* embryos. Clearly, additional information will be gleaned from the analysis of diploid and polyploid chromosomes from the *DmORC3* mutant [8].

Delay in entering S phase is accompanied by temporal disruption of replication

The reduced frequency of BrdU incorporation and increased occurrence of cells with a single centrosome both indicate an early cell-cycle delay in *DmORC2* mutant neuroblasts. If the ORC complex is unstable when ORC2 is mutated, then assembly of the pre-RC may be affected and the time taken to enter S phase prolonged. However, at least some *DmORC2* mutant neuroblasts did enter S phase, albeit with slow progression. Detailed analysis of the mitotic chromosomes following S phase in the presence of BrdU highlighted significant changes to the replication timing of at least some regions of euchromatin. Heterochromatin always replicated later than euchromatin in wild-type neuroblasts. In contrast, this temporal relationship was perturbed in the *DmORC2* mutants, such that some euchromatic regions became late replicating, even later than heterochromatin. This could be attributed to defects in RC formation and function in euchromatin.

This effect on euchromatin could be the result of a higher affinity of ORC for heterochromatin. ORC2, at least, appears to be present on pericentric heterochromatin in early anaphase, but not on euchromatic arms until later in anaphase. Perhaps the interaction of ORC with HP-1 at heterochromatin stabilizes the assembly of RCs, ensuring the appropriate replication of these regions in the next cell cycle even when ORC2 function is compromised.

Elements of chromatin structure and function specific to either heterochromatin or euchromatin exist. The RAD53 kinase in *S. cerevisiae* is involved in distinguishing between early and late replication origins and preventing late origins from firing prematurely [39] by delaying the recruitment of replication protein A (RPA) to these origins [40]. It is certainly possible in *DmORC2* mutants that RPA has not been properly recruited to the euchromatin that replicates very late. Other aspects of chromatin modeling such as histone acetylation may also be affected [41,42]. The SAS2 acetyltransferase in *S. cerevisiae*, which is important for gene silencing [43], interacts genetically with ORC2 and ORC5 [44], while human HBO1 acetyltransferase interacts biochemically with ORC1 [45]. A defective ORC subunit may result in decreased acetylation of particular chromatin regions which, in turn, may induce that region to take on more heterochromatin-like (that is, late-replicating) characteristics.

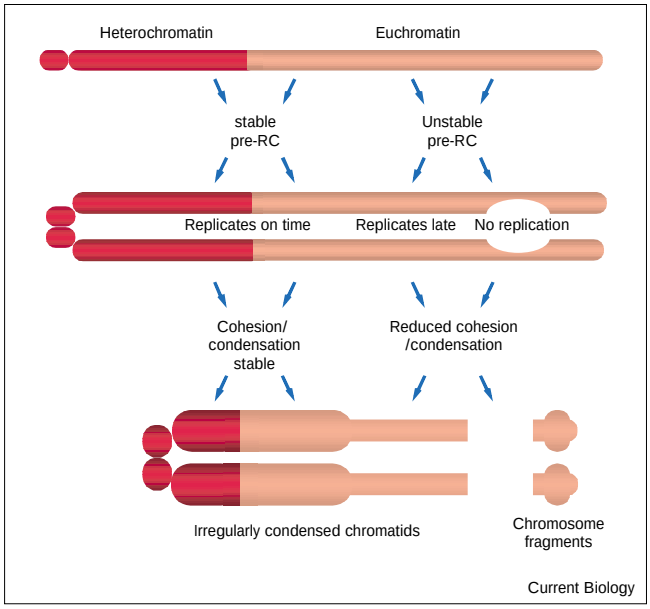
Late-replicating euchromatin is more prone to condensation defects than heterochromatin

We concluded from two lines of evidence that abnormally late-replicating euchromatin was most affected in its ability to condense properly in *DmORC2* mutant cells. First, FISH with probes for pericentric repeats on the three large chromosomes showed that heterochromatin appeared to condense normally. Therefore, it is likely that

euchromatin is the major source of inadequately condensed chromatin. Second, analysis of BrdU incorporation in the subsequent mitosis demonstrated that late-replicating euchromatin often exhibited condensation defects. DNA replication and the establishment of chromatid cohesion are intimately linked, elements of the latter process being laid down during S phase [46–50]. Perhaps aspects of the condensation machinery are also 'templated' during S phase, and if replication is altered, then subsequent assembly may be affected.

Intriguingly, the irregular condensation of mitotic chromosomes occurred frequently in the same place on both sister chromatids. The existence of distinct sister chromatids strongly suggested that these regions of the chromosome had been replicated (in addition, undercondensed and condensed regions could all be labeled with long BrdU incorporation periods). However, defects in replication-dependent chromatin assembly could be responsible for the phenotypes that we observed. CAF-1 has been shown to interact biochemically with PCNA [51], and is also associated with a specific cycle of histone acetylation/deacetylation during replication of heterochromatin [52]. Cohesion has been genetically linked with PCNA-dependent replication by *Ctf7/Eco1* mutations in *S. cerevisiae* [49,50]. Histone expression occurs during S phase, while CENP-A (a centromere-specific histone H3) must be expressed after histones, in order to be properly targeted to centromeres [53]. If CENP-A is expressed during S phase, centromere localization is abolished. Inappropriately late replication timing is observed when the locus control region (LCR) is deleted from the β-globin locus in certain thalassemias, resulting in the failure to transcriptionally activate the *cis*-linked globin genes [54]. Therefore, these examples all point to the necessity of coupling DNA replication temporally to the titration of specific chromatin components that may be important for chromosome structure and gene expression.

Figure 7



Model of the effect of *DmORC2* mutations on replication and mitotic chromosome condensation. A generic chromosome is shown, with the heterochromatin in red and euchromatin in pink. Origins of replication are required to be active in both heterochromatin and euchromatin for the chromosome to be completely replicated in S phase. We postulate that, in *DmORC2* mutants, any wild-type ORC2 binds to heterochromatic and some euchromatic origins during late anaphase, but is absent from other euchromatic origins. This affects the ability to form pre-RCs, and the conversion to RCs at the appropriate time in S phase. If RCs are formed, the region will be replicated, whereas the region will be replicated at an inappropriate time or not at all when ORC2 is mutated. As a result of the altered replication dynamics, cohesion and condensation machinery fail to assemble properly at many of the abnormal euchromatic origins and the mitotic chromosomes are not properly condensed. Where origins fail to fire, chromosome breaks occur and the resulting fragments are visible in mitosis.

ORC may have a central role in organizing higher eukaryotic chromosomes. In *S. cerevisiae*, ORC has been proposed to act as a landing pad for assembly of the pre-RC and for transcriptional control. The results presented here raise the possibility that, in metazoans, the ORC landing pad may interact with many additional protein complexes, such as those necessary for cohesion, repair, condensation, and decatenation [55]. We propose a model to account for the mitotic chromosomal defects that we have observed (Figure 7). This model takes into account the consequences of faulty ORC2 and pre-RC formation, with downstream effects on cohesion/condensation. We postulate that, because of a higher affinity of ORC for heterochromatin, as pools of wild-type (maternal) protein dwindle during development, these will selectively be targeted to heterochromatin, enabling DNA replication with appropriate timing and facilitating cohesion and condensation. Regions of euchromatin deficient in ORC would not be replicated at the right time in S phase, and therefore lose the opportunity to assemble the chromatin structures required for metaphase chromosome condensation. ORC is depicted as a central landing pad in this model, though it could just as likely serve as the structural focus for the subsequent events of cohesion and condensation, each of which is dependent on the previous event occurring correctly. We are currently examining the status of cohesin and condensin subunits to ascertain how structural defects manifest themselves in cells exhibiting abnormal mitotic chromosome condensation.

Conclusions

Our detailed analysis of two lethal alleles of the *DmORC2* gene has revealed novel roles for ORC2 in the coordination of mitotic chromosome architecture with replication timing. Both mutations caused death late in larval development, with striking defects in cell-cycle progression and

chromosome condensation in mitosis. These alleles also disrupted the normal pattern of chromosomal replication, with some euchromatic regions replicating even later than heterochromatin. Mitotic chromosomes were irregularly condensed, with the abnormally late replicating regions of euchromatin exhibiting the greatest problems in mitotic condensation. Our results also suggest that the correct timing of DNA replication may be essential for the assembly of chromatin that is fully competent to undergo mitotic condensation.

Materials and methods

Fly stocks

Canton S and *Df(3)red*^{p52} (88A4-88B4,5) were obtained from the Bloomington Stock Center; *l(3)k43*^{1} was obtained from M. Gatti (University of Rome) and *l(3)k43*$^{\gamma 4e}$ from R. Kellum (University of Kentucky). The Df and both alleles were each maintained over the TM6B balancer, *In(3LR)TM6B* (markers: *Antp*^{Hu} *e*^{1} *ry*^{CB} *Tb*^{1}) for all the experiments described. Complementation tests confirmed that *l(3)k43*^{1} and *l(3)k43*$^{\gamma 4e}$ are allelic and that both are uncovered by *Df(3R)red*^{p52}. Transheterozygotes of the two alleles or with the deficiency *Df(3R)red*^{p52} showed the same overall mitotic defects, namely a range in severity of irregularly condensed and broken chromosomes.

Immunofluorescence staining of larval neuroblasts

Third instar larvae were rinsed and dissected in 1 × EBR (130 mM NaCl, 4.7 mM KCl, 1.9 mM $CaCl_2$, 10 mM HEPES pH 6.9); the brains (without imaginal discs) were removed and hypotonically swollen for 3 min in 0.25 × EBR. The brains were then fixed for 5 min in PFA/HOAc/TX-fix (4% paraformaldehyde, TAAB Laboratories, 5% acetic acid, 0.1% triton X-100, 0.1725 × EBR), during which time they were flattened onto polylysine-treated slides under siliconized coverslips. Preparations were frozen in liquid nitrogen, and the coverslips flicked off with a razor blade. After 2 min hydration in PBS, the brains were permeabilized for 3 × 10 min in PBS with 0.05% Triton X-100 (PBTx) and blocked in 10% normal goat serum (Sigma) in PBS (PBS-NGS) or in 0.3% BSA in PBS (PBS-BSA) for 1 h at room temperature. The brains were washed for 5 min in PBTx and then incubated for 16 h at 4°C in the primary antibody (pre-immune or immune rabbit anti-ORC2 antiserum at 1:200 or affinity-purified) diluted in PBS-NGS or in PBS-BSA. Brains were then washed for 6 × 5 min in PBTx and incubated for 2 h at room temperature in the secondary antibody (Alexa 594 goat anti-rabbit at 1:500, Molecular Probes), then washed again. Additional washes were performed as necessary before the brains were stained with 75 ng/ml DAPI (Sigma). Coverslips were mounted onto the slides with Mowiol 4-88 (Calbiochem) and viewed using an Olympus Provis epifluorescence microscope equipped with a Photometrics Sensys CCD camera; images were captured using Vysis SmartCapture software and processed with Adobe Photoshop.

BrdU incorporation into larval neuroblasts

Larvae were fed for 10 min to 24 h with 50 mg/ml BrdU (Boehringer Mannheim) in *Drosophila* instant food (Sigma). The food contained food colouring to unambiguously discern which larvae had ingested the drug. To visualize the BrdU, the larval brains were dissected, hypotonically swollen, fixed and squashed onto polylysine-treated slides as described above for analysis of neuroblast mitotic figures. Preparations were then rinsed for 2 min in PBS, permeabilized for 5 min in PBTx, fixed again for 10 min with 4% PFA in PBS and washed for 5 min in PBTx. Brains were then treated for 30 min with freshly prepared 2N HCl and washed for 10 min in PBTx, blocked for 30 min in 0.3% BSA in PBS and washed for 5 min in PBTx. Brains were incubated with rat anti-BrdU antibody (1:2 dilution, Harlan Sera) for 16 h at 4°C. The slides were washed 3 × 10 min at RT in PBTx and then incubated with either FITC-conjugated goat anti-rat antibody (1:100, Harlan Sera) or Alexa 488-goat anti-rat conjugate (1:500, Molecular Probes) for 4 h at 4°C. The washes were repeated and the brains stained with 200 ng/ml DAPI during the second wash. Coverslips were mounted onto the slides with Mowiol and viewed as described above.

Supplementary material

Supplementary material including methodological detail of the cloning and sequencing of mutant *DMORC2* alleles, immunofluorescence staining of cultured *Drosophila* cells, DAPI staining of larval chromosomes and fluorescence *in situ* hybridization is available at http://current-biology.com/supmat/supmatin.htm.

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