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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.

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 of the lesions for the female sterile and the l(3)k43 allele [32]. There-fore, the evolutionarily conserved carboxy-terminal portion of 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].

The DmORC2 protein is 618 amino acids in length [6] and the lesions for the female sterile and the l(3)k43 allele have been reported [32]. We sequenced the l(3)k43 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 allele [32]. Therefore, the evolutionarily conserved carboxy-terminal portion of the protein was missing from both DmORC2 and DmORC2 frameshift (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 or l(3)k43 homozygotes, suggesting that these forms may be unstable or not produced.

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 separate 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 and l(3)k43 homozygous alleles revealed many severe mitotic
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...
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)k431 wild-type 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 polytoid cells than in mitotically active cells).

Table 1. Determination of the ratio of cells with single centrosomes

<table>
<thead>
<tr>
<th>Wild type</th>
<th>k431</th>
<th>k43γ4e</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage cells incorporating BrdU</td>
<td></td>
<td></td>
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<tr>
<td>10 min</td>
<td>72%</td>
<td>78%</td>
</tr>
<tr>
<td>24 h</td>
<td>69%</td>
<td>75%</td>
</tr>
<tr>
<td>48 h</td>
<td>54%</td>
<td>58%</td>
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<tr>
<td>50 h</td>
<td>43%</td>
<td>41%</td>
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<table>
<thead>
<tr>
<th>Wild type</th>
<th>k431</th>
<th>k43γ4e</th>
</tr>
</thead>
<tbody>
<tr>
<td>Centrosomes per cell</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:1</td>
<td>2:1</td>
<td>3:1</td>
</tr>
<tr>
<td>108</td>
<td>158</td>
<td>2321</td>
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<tr>
<td>112</td>
<td>168</td>
<td>2321</td>
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<tr>
<td>131</td>
<td>204</td>
<td>2321</td>
</tr>
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</table>

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)k431 and l(3)k43γ4e 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

(a) BrdU uptake in third instar wild-type, l(3)k431 and l(3)k43γ4e 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)k431 and l(3)k43γ4e 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)k431 and l(3)k43γ4e 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)k431, 113,627; l(3)k43γ4e, 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 3(3)k43γ and 3(3)k43γk 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).

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**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), and dodeca repeat probes to male wild-type neuroblasts. (c-h) Examples of 3(3)k43γ and 3(3)k43γk 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.

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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
frequency of BrdU labeling of l(3)k431 and l(3)k43p6 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)k43m6 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 SCC-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 αi-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 heterochromatin 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. Here 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)red22 (88A4-88B4.5) were obtained from the Bloomington Stock Center; (3)k431 was obtained from M. Gatii (University of Rome) and (3)k433 from R. Kellum (University of Kentucky). The Df and both alleles were each maintained over the TM6B balancer, In(3L)TM6B (markers: AntpF1, el2, y+ Tb1) for all the experiments described. Complementation tests confirmed that (3)k431 and (3)k433 are allelic and that both are uncovered by Df(3R)red22. Transheterozygotes of the two alleles or with the deficiency Df(3R)red22 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 CaCl2, 10 mM HEPES pH 6.9); the brains were flattened onto polylysine-treated slides under siliconized coverslips. Brains were then washed for 5 min in PBTx and then incubated for 16 h at 4°C. The washes were repeated and the brains stained with 200 ng/ml DAPI (Sigma). 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/supmat.htm.

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References


