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The origin recognition complex is dispensable for endoreplication in *Drosophila*

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The origin recognition complex (ORC) is an essential component of the prereplication complex (pre-RC) in mitotic cell cycles. The role of ORC as a foundation to assemble the pre-RC is conserved from yeast to human. Furthermore, in metazoans ORC plays a key role in determining the timing of replication initiation and origin usage. In this report we have produced and analyzed a *Drosophila orc1* allele to investigate the roles of ORC1 in three different modes of DNA replication during development. As expected, ORC1 is essential for mitotic replication and proliferation in brains and imaginal discs, as well as for gene amplification in ovarian follicle cells. Surprisingly, however, ORC1 is not required for endoreplication. Decreased cell number in *orc1* mutant salivary glands is consistent with the idea that undetectable levels of maternal ORC1 during embryogenesis fail to support further proliferation. Nevertheless, these cells begin endoreplicating normally and reach a final ploidy of >1000C in the absence of zygotic synthesis of ORC1. The dispensability of ORC is further supported by an examination of other ORC members, whereas Double-parked protein/Cdt1 and minichromosome maintenance proteins are apparently essential for endoreplication, implying that some aspects of initiation are shared among the three modes of DNA replication. This study provides insight into the physiologic roles of ORC during metazoan development and proposes that DNA replication initiation is governed differently in mitotic and endocycles.

Tightly controlled cell cycle progression is essential for normal proliferation, development, and differentiation in every organism. In organisms as diverse as yeast and mammals the initiation of DNA replication commits cells to progress through a complete cell cycle. Molecular mechanisms that govern the initiation of DNA replication have been the focus of efforts to understand how cell cycle progression is controlled (1, 2).

There are three modes of DNA replication, and each fulfills specific requirements at different stages of development. Mitotic replication takes place in every proliferating cell to increase cell number, whereas localized gene amplification is specialized to increase particular gene products, which permits cells to acquire advantages in areas such as proliferation or drug resistance (3, 4). The third mode of replication, endoreplication, takes place in terminally differentiated cells that often lack the ability to further proliferate. Endoreplicating cells acquire high ploidy by multiple rounds of replication without cell division, thus increasing their metabolic capacity to support growth and development. In mammals the endocycle occurs in megakaryocytes to produce a large number of platelets and in placental giant trophoblasts to support fetal development. In *Drosophila* ovarian nurse cells and most larval tissues use endoreplication to support early embryogenesis and larval growth, respectively (5, 6).

Regulation of mitotic replication and amplification has been extensively studied during the last two decades in various organisms, revealing that DNA replication is initiated by a stepwise assembly of the prereplication complex (pre-RC) (1, 2). The components of pre-RC are well conserved among species, and it has been generally assumed that all three replication modes are initiated by the same pre-RC formation. Studies of endoreplication to date have focused mainly on cyclin E–mediated controls of origin licensing and relicensing, and developmental regulation of the transition from mitotic to endoreplication (6).

Previously, we and others have shown that in metazoans, activities of the origin recognition complex 1 (ORC1), the largest of six ORC components, are regulated in a cell cycle–dependent manner (7–9). Moreover, ectopic expression of *Drosophila* ORC1 can induce ectopic replication in the cells going through gene amplification (7). These results suggest that ORC1 is one of the major determinants in the timing of pre-RC formation in metazoans.

In this report we have prepared an $orc1^{-}$ allele to examine roles of ORC during *Drosophila* development. $orc1^{-/-}$ cells are defective both in proliferation and amplification, demonstrating that ORC function is evolutionarily conserved. To our surprise, however, $orc1^{-/-}$ cells successfully go through developmentally programmed endocycles in salivary glands (SGs), endoreplicate as efficiently as WT cells, and reach a final ploidy of >1000C in the absence of zygotic ORC1 synthesis. An examination of other ORC components further supports the notion that ORC is dispensable for endoreplication. In contrast, down-regulation of Double-parked protein (Dup)/Cdt1 or minichromosome maintenance protein 5 (MCM5) results in severe endoreplication defects, demonstrating that endoreplication shares some of the downstream replication factors used in mitotic replication.

Our work suggests that an ORC-independent, unconventional initiation mechanism controls endoreplication.

Results

Establishment of the $orc1^{-}$ Null Mutant. To study the involvement of ORC1 during development, we prepared $orc1^{-}$ mutants by homologous recombination (10, 11). Two stop codons were inserted at amino acid positions 25 and 643 (Fig. 1 *A* and *B*).

Both $orc1^{-/-}$ homozygotes or transheterozygotes over *Df(2R)ST1*, a deficiency that uncovers the *orc1* gene, died soon after pupation. They also demonstrated some developmental delays in the larval stages (molting and pupation of the $orc1^{-/-}$ homozygotes occurs with a ≤1 day delay) (Fig. 1*C*, Fig. 4*H*). These phenotypes are fully rescued by a single copy of genomic $orc1^{+}$ transgene, demonstrating that they are caused solely by the disruption of the *orc1* gene. The mutants are also fully rescued by the *porc1>orc1gfp* transgene that encodes an ORC1-GFP fusion protein under the control of the native promoter. ORC1-GFP from this transgene and endogenous ORC1 show essentially the same cell cycle–dependent distribution in imaginal discs (12).

Western blot analyses revealed no detectable endogenous ORC1 in the imaginal disc samples of $orc1^{-/-}$ mutants rescued by *porc1>orc1gfp* (Fig. 1*D*, lane 2). Similar analyses using various antibodies against three different portions of ORC1 (7) also failed to detect any endogenous ORC1 either in full-length or truncated forms (data not shown). Based on these results, the $orc1^{-}$ allele produces no stable ORC1 and is a functional null.

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The authors declare no conflict of interest.

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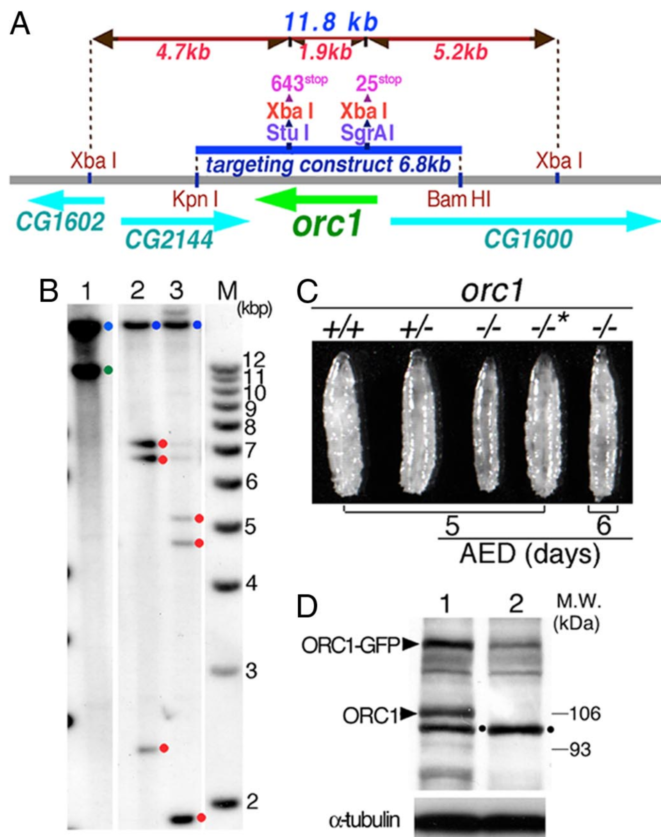

Fig. 1. Production of an *orc1* mutant. (*A*) Genomic map of the *orc1* locus on 2R. The 6.8-kb *Bam*H I-*Kpn*I genomic DNA fragment with 2 stop codons was used for homologous recombination to replace the endogenous $orc1^+$ gene. (*B*) Southern blot of *Xba*I-digested genomic DNA probed for *orc1*. The $orc1^+$ allele yields an 11.8-kb fragment from the *wt* chromosome (green dot) (lane 1). The mutated allele generates 3 fragments (red dots): (lane 2) 7.1, 6.7, and 2.4 kbp after the first homologous recombination (HR) that results in two tandem copies of the *orc1* gene; (lane 3) 5.2, 4.7, and 1.9 kbp after the second HR (11). An ≈19-kb fragment (blue dots in lanes 1–3) is from the $orc1^+$ allele of the *CyO* balancer chromosome that is required to maintain the $orc1^-$ mutant lines. (*C*) Day 5 after egg deposition (AED) larvae of WT (+/+), heterozygous *orc1* (+/−), homozygous *orc1* (−/−), and homozygous *orc1* rescued by one copy of the *porc1*>*orc1-gfp* transgene (−/−*). After an additional day of development, $orc1^{-/-}$ larvae attain nearly a normal size. (*D*) Western blot of the imaginal disc samples probed with anti-ORC1 antibody: the transgenic line carrying *porc1* > *orc1-gfp* (lane 1) and $orc1^{-/-}$ rescued by the transgene (lane 2). The ≈100-kDa nonspecific bands are marked by dots. α-tubulin is a loading control.

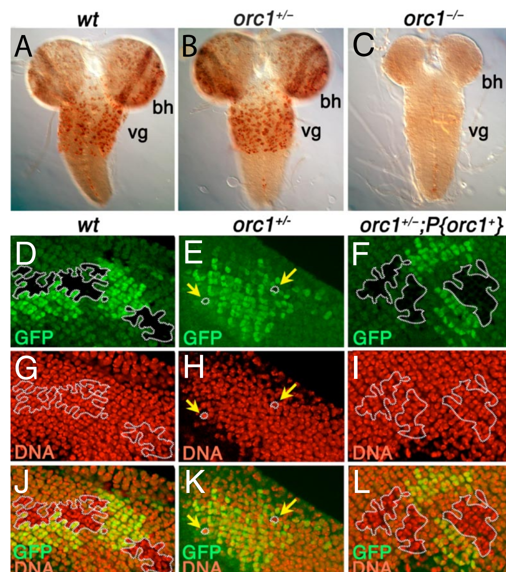

Fig. 2. ORC1 is essential for proliferation. (*A*–*C*) BrdU incorporation in late third instar (developmental stage-matched) larval brains from day-5 (*A* and *B*) and day-6 (*C*) larvae. bh, brain hemisphere; vg, ventral ganglion. Note that vg develops earlier than bh and reaches a comparable size in all 3 genotypes, whereas bh is smaller in *C*. Relatively small defects in brain development are consistent with persistent maternal ORC1 in CNS, as was previously reported (7), and could be due to cell-autonomous effects or developmental delays as shown in Fig. 4*H*. (*D*–*L*) Somatic clones in the eye-antennal imaginal discs, marked by the absence of GFP (outlined with dotted lines), generated in WT (*D* and *J*), $orc1^{+/-}$ heterozygotes with absence (*E* and *K*) and presence (*F* and *L*) of a genomic $orc1^+$ transgene. (*G*–*I*) DNA staining with TOPRO-3, (*J*–*L*) merge. Note that $orc1^{-/-}$ cells survive but do not proliferate (arrows in *E*, *H*, and *K*), and the proliferation defect is rescued by an $orc1^+$ transgene (*F*, *I*, and *L*).

ORC1 Is Essential for Proliferation. To study the role of ORC1 in the mitotic cycle, we examined the CNS and the imaginal discs in $orc1^{-/-}$ homozygotes. The hemispheres of $orc1^{-/-}$ brains are significantly smaller compared with controls (Fig. 2 *A*–*C*), indicating that brain development stops prematurely when maternal ORC1 is depleted (7). BrdU incorporation is severely reduced in the entire $orc1^{-/-}$ brain (Fig. 2*C*), demonstrating a lack of DNA synthesis. These phenotypes represent proliferation defects, as was also observed in other replication initiation factor mutants, such as *orc2*, *orc5*, and *Rfc4* (13–15). No imaginal discs were detectable in late third instar larvae, indicating that this tissue, which normally increases exponentially in cell number during larval development (16), has failed to proliferate.

To circumvent the lethality of the $orc1^{-/-}$ homozygotes and examine phenotypes of the $orc1^{-/-}$ imaginal disc cells, we used the FRT-FLP system to produce mitotic clones in early larval stages (17). In the WT control, cell number and cell size are indistinguishable between GFP(+) and GFP(-) clones (Fig. 2*J*), demonstrating that an abundance of GFP has no effects on proliferation. In an $orc1^{-/+}$ heterozygous background the *gfp* transgene is linked to the WT chromosome, thus the absence of GFP signals mark $orc1^{-/-}$ clones. Each $orc1^{+/+}$ clone is accompanied by an $orc1^{-/-}$ twin sister clone that contains a single cell (Fig. 2 *E* and *K*). We never detected apoptotic figures in $orc1^{-/-}$ clones, which have been reported in $e2f^{729^{-/-}}$ clones (18). A single copy $orc1^+$ transgene supplied in *trans* recovered proliferation of GFP(-) clones (Fig. 2 *F* and *L*), demonstrating that proliferation defect in the GFP(-) clones are caused solely by a lack of the $orc1^+$ gene. These results indicate that ORC1 is essential for proliferation, as expected, but not for cell viability.

ORC1 Is Essential for Gene Amplification. We next examined the role of ORC1 in amplification, a specialized mode of replication that takes place in the ovarian follicle cells (19). We induced mitotic recombination in early oogenesis while follicle cells were still proliferating and examined amplification in the $orc1^{-/-}$ and $orc1^{+/+}$ clones in late oogenesis. During amplification specific origins fire repeatedly while the rest of the chromosome is quiescent, resulting in limited BrdU incorporation at discrete subnuclear foci during pulse-labeling experiments (Fig. 3*B*). No BrdU incorporation was detectable in the $orc1^{-/-}$ clones (Fig. 3 *E* and *F*), indicating that amplification was abolished in these cells.

Histochemical analyses revealed a lack of characteristic subnuclear localization of ORC1 to several foci in $orc1^{-/-}$ clones (Fig. 3*G*). Furthermore, ORC2 also failed to localize to foci (Fig. 3*H*), indicating that no pre-RC was assembled at amplification sites in these cells. These results support previous biochemical studies that determined that ORC assembly requires all 6 components (20, 21).

$orc1^{-/-}$ follicle cell clones contained two to three cells, always fewer than their sister clones, consistent with the idea that ORC1

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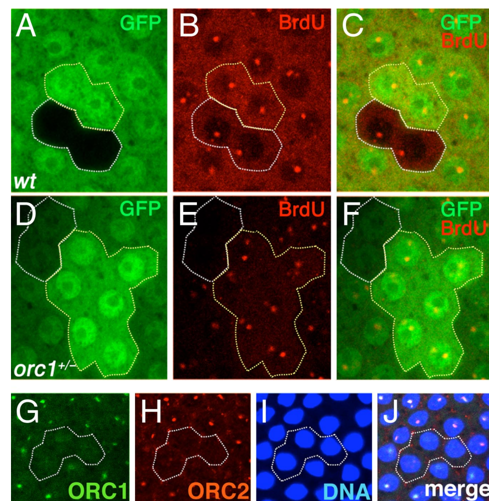

Fig. 3. ORC1 is essential for amplification. (*A–F*) Two-cell $orc1^{-/-}$ somatic clones of ovarian follicle cells (stage 11) generated in WT (*A–C*) and $orc1^{+/-}$ heterozygous (*D–F*) flies. GFP(−/−) or GFP(+/+) clones are outlined. DNA synthesis occurs only at the specific amplification loci at this stage of oogenesis. (*G–J*) Histochemical analyses of ORC1 (*G*), ORC2 (*H*), and DNA (*I*). A 3-cell somatic clone was generated in an $orc1^{+/-}$ heterozygote. Note that ORC2 also fails to localize to the amplification loci in $orc1^{-/-}$ cells.

is required for proliferation, just as it is for imaginal disc cells (Fig. 3 *D–F*). ORC1 does not completely disappear in follicle cells during mitotic cycles (7), unlike in imaginal disc cells in which ORC1 is down-regulated to undetectable levels at the end of each cycle (7, 12). Presumably, residual ORC1 carried over from the cycle in which mitotic recombination took place allowed partial proliferation of $orc1^{-/-}$ clones.

Taken together, our observations demonstrate that ORC1 is required for proliferation and amplification as expected.

Endoreplication Is Essentially Normal in $orc1^{-/-}$ SGs. SG precursor cells differentiate during embryonic development (22 for review). During larval development, extensive endoreplication drives an exponential increase in the size of the SG (23). The absence of imaginal discs in the third instar suggests that insufficient maternal stores of ORC1 exist to support mitotic replication (of these diploid cells) from early on in larval development. Thus, we expected that the third instar SGs would be undetectably small in $orc1^{-/-}$ mutants, assuming that ORC1 would also be required for the extensive DNA replication that drives these cells to a ploidy of 1000C in the WT organism (24).

To our surprise, third instar $orc1^{-/-}$SGs are large: they also incorporate BrdU in pulse-labeling experiments, showing that these cells continue to synthesize DNA late in larval development (Fig. 4 *A* and *B*). ORC1 does not seem to persist aberrantly in the mutant SGs, because it is undetectable either by Western blot (Fig. 4*C*) or immunochemical staining (data not shown). The levels of other ORC subunits are normal in $orc1^{-/-}$SGs (Fig. 4*C*), unlike in proliferating mammalian cells in which down-regulation of ORC2 resulted in reduced levels of other ORC components (25, 26). The reduced size of $orc1^{-/-}$ glands seems to be due primarily to the presence of fewer cells than there are in the corresponding WT glands (Fig. 4*D*), consistent with proliferation defects observed in the $orc1^{-/-}$ clones in imaginal discs (Fig. 2 *E* and *K*) and in the *orc2* and *orc5* mutant cells that arrest either in G_1 or M phase (13, 14). $orc1^{-/-}$ cells are the same size as WT cells (Fig. 4 *E* and *F*), further supporting the idea that endoreplication, which drives the increase in cell size, is essentially normal in the absence of ORC1 function.

We performed 2 additional experiments to determine whether the efficiency of endoreplication in $orc1^{-/-}$ cells is normal. First, we measured the DNA content of individual nuclei of the SG cells throughout larval development by staining fixed preparations with Hoechst 33342 and summing fluorescence in 3D-reconstructed nuclear images. The rate of DNA synthesis is essentially indistinguishable in WT and ORC1 mutant, even 100 h into larval development, just before the onset of pupation (Fig. 4 *G* and *H*). We observed two minor but reproducible differences between the WT and mutant SGs. First, at the end of larval development, WT cells have approximately twofold more DNA (i.e., 2000C vs. 1000C in mutant cells). Second, DNA accumulates continuously throughout development in the WT nuclei, whereas accumulation is punctuated by two pauses that correspond to the larval molts in mutant nuclei (** in Fig. 4*H*). It is possible that these phenotypes and developmental delays observed in $orc1^{-/-}$ homozygous larvae (Fig. 1*C*) reflect the lack of presumptive function of ORC outside of DNA replication. Taken together, these observations suggest that endoreplication is nearly normal in the absence of detectable ORC1, even late in larval development when the DNA content of each cell is almost 300-fold higher than in diploid imaginal disc cells.

Second, we examined the levels of DNA double-strand breaks (DSBs) in SGs. If residual (but undetectable) ORC1 were required to license origins during endoreplication, the progressive per-genome shortage of ORC1 would be expected to increase the

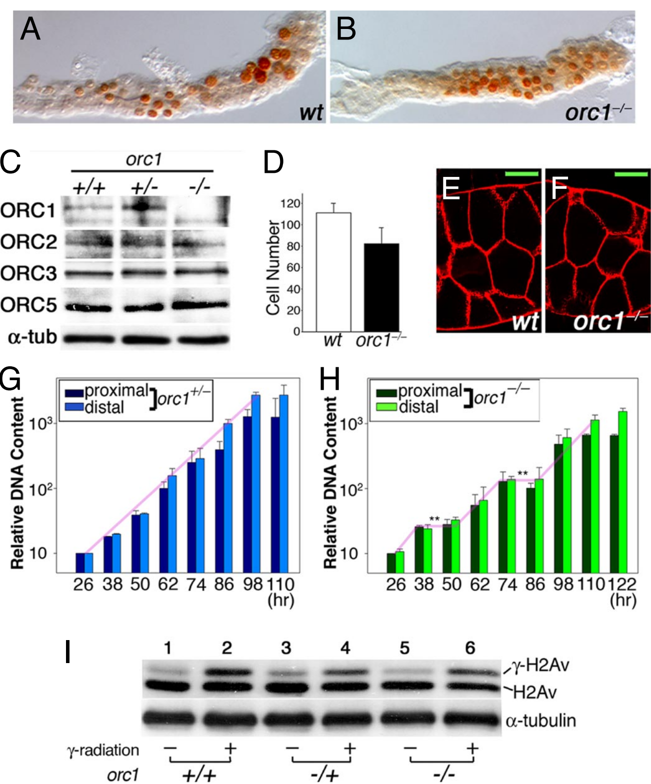

Fig. 4. ORC1 is dispensable for endoreplication. (*A* and *B*) BrdU incorporation in stage-matched SGs from day-5 WT (*A*) and day-6 $orc1^{-/-}$ homozygous (*B*) larvae. (*C*) Western blots of the SG samples from various genotypes as indicated, probed with anti-ORC1, 2, 3, or 5 (7, 21, 49). Alpha-tubulin is a loading control. (*D*) Cell numbers in SGs. *wt*: 110 ± 9.74 SD (n = 18), $orc1^{-/-}$: 83.8 ± 13.5 SD (n = 17); $P < 0.0001$. (*E* and *F*) Cell sizes in WT (*E*) and $orc1^{-/-}$ homozygous (*F*) SGs. The cell membranes are stained with Phalloidin. Scale bar, 50 μm. (*G* and *H*) DNA synthesis during larval development. DNA content (shown in a log scale) of larval SG cells from $orc1^{+/-}$ heterozygotes (*G*) and $orc1^{-/-}$ homozygotes (*H*). Note that after two synchronous endocycles, synchrony among cells gradually disappears (24). Also note two pauses (indicated by **) that correspond to larval molts. (*I*) Western blots of the endoreplicating SG samples from various genotypes as indicated probed with anti-H2Av (28), before (−) and after (+) γ-irradiation. Alpha-tubulin is a loading control.

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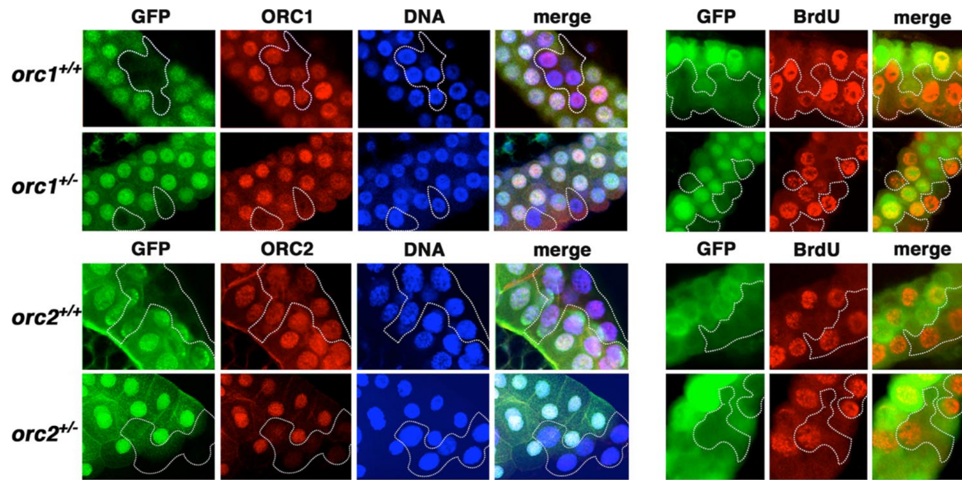

Fig. 5. ORC is dispensable for endoreplication. Somatic clones in the SGs, marked by the absence of GFP (outlined with dotted lines), generated in WT (first and third rows), *orc1*$^{+/-}$ heterozygous (second row), and *orc2*$^{+/-}$ heterozygous (fourth row) flies. Note that endocycle is not synchronized in the late larval stages, so that the BrdU incorporation levels vary from cells to cells.

distance between active origins, resulting in the collapse of stalling replication forks and the generation of DSBs (27). To test this idea, we assayed the accumulation of γ-H2Av, a phosphorylated H2A histone variant that accumulates in response to DSBs (28). As shown in Fig. 4*I* (lanes 1, 3, and 5), the level of γ-H2Av is not elevated in *orc1*$^{-/-}$ SGs. Gamma irradiation induces phosphorylation of H2Av (Fig. 4*I*, lanes 2, 4, and 6), demonstrating that H2Av remains responsive to DSBs in the absence of ORC1 function. The basal levels of γ-H2Av in SGs are higher than those in proliferating tissues (data not shown), presumably owing to physiologic DSBs that arise at borders between fully replicated euchromatin and underreplicated heterochromatin during endoreplication (29, 30). Those observations are consistent with the idea that the efficiency of endoreplication is essentially normal in the absence of zygotic ORC1 function and detectable ORC1 protein.

ORC Is Dispensable for Endoreplication. Next, we wished to determine whether other ORC subunits are also dispensable for endoreplication. To address this issue, we carried out clonal analyses in an *orc2*$^{-}$ allele, *k43*$^{\gamma 4}$ (31). Somatic clones were induced in proliferating embryonic SGs (at 3–5 h after egg deposition [AED]) and examined in the late larval stages, at which the ploidy of the SGs reach >1000C after 9 to 10 rounds of endocycle (at 98 h AED in Fig. 4*G*) (6).

An examination of *orc1*$^{-/-}$ and *orc2*$^{-/-}$ somatic clones revealed results that were essentially identical. The *orc1*$^{-/-}$ and *orc2*$^{-/-}$ homozygous clones contain fewer cells compared with their twin sisters, which parallels the reduced cell number in the homozygous SGs (Fig. 4*D* and Fig. 5). We attribute the limited proliferation in the clones to the residual maternal proteins that are still detectable before embryonic stage 13 (7).

Although proliferation was perturbed in the absence of the WT *orc1*$^{+}$ or *orc2*$^{+}$ allele, the cell size, the nuclear size, and DNA content of the twin clones were all indistinguishable between homozygous and WT clones (Fig. 5), demonstrating that homozygous clones have gone through approximately 10 rounds of endocycles without newly synthesized protein. Moreover, in the absence of any detectable ORC in homozygous clones, pulse-labeling experiments demonstrated no difference in BrdU incorporation between *orc1*$^{-/-}$ homozygous, *orc2*$^{-/-}$ homozygous, and WT cells (Fig. 5). Thus, ongoing DNA synthesis is also unperturbed, even during the late rounds of endocycles just before pupation. These results are consistent with the previous report on unaffected SG replication in *orc2*$^{-}$ mutants (31).

Taken together, our study strongly indicates that ORC is essential for proliferation but dispensable for endoreplication.

Dup/Cdt1 and MCM Are Required for Endoreplication. As a next step to understand the molecular mechanisms needed to regulate the initiation of endoreplication, we wished to determine whether endoreplication and mitotic replication share any downstream replication factors for pre-RC formation. We down-regulated the levels of various pre-RC components by RNA interference (RNAi) specifically in SGs using the *patched* (*ptc*)>*Gal4* driver (48) and assessed the effects on endoreplication.

Consistent with clonal analyses results, RNAi against either *orc2* or *orc5* revealed no effect on endoreplication in SGs (Fig. 6 *C–F*), even though the protein level produced by each gene was drastically reduced (Fig. 6*K*).

In contrast, a *dup* (fly homologue of *cdt1*)-RNAi expression caused a dramatic decrease in nuclear size, cell size, and DNA content of SG cells, whereas no detectable change was observed in fat bodies in which the Gal4 driver was inactive (Fig. 6 *G* and *H*). This result demonstrates that Dup is essential for endoreplication, consistent with the previous reports that a *dup* mutant has defects in endoreplication (32) and that overexpression of Dup induces overendoreplication in the ovarian follicle cells or the imaginal disc cells (33). It also partially rescues endoreplication defects in *dap*/p27 mutants (34).

There has been disagreement over the involvement of MCM in endoreplication in flies. MCM 4 and MCM 5 have been proposed to be dispensable for endoreplication (35, 36) on the basis of the observations that larval endoreplicating tissues are normal in *mcm4* homozygotes and that endoreplication is completed in *mcm5* SGs. In contrast, *mcm6* larval size is reduced by 50%, indicating severe endoreplication defects (37).

An RNAi experiment to down-regulate *mcm5* messages, however, resulted in significant suppression of growth in SGs (Fig. 6 *I* and *J*). Thus, it seems that at least some MCM members are engaged in endoreplication.

Together, these results show that endoreplication is initiated in an ORC-independent manner and that at least some downstream components of the replication machinery are required both for mitotic and endoreplication.

Discussion

Our analysis of an *orc1*$^{-/-}$ mutant has revealed that endoreplication takes place in the *orc1*$^{-/-}$ SG cells throughout development as

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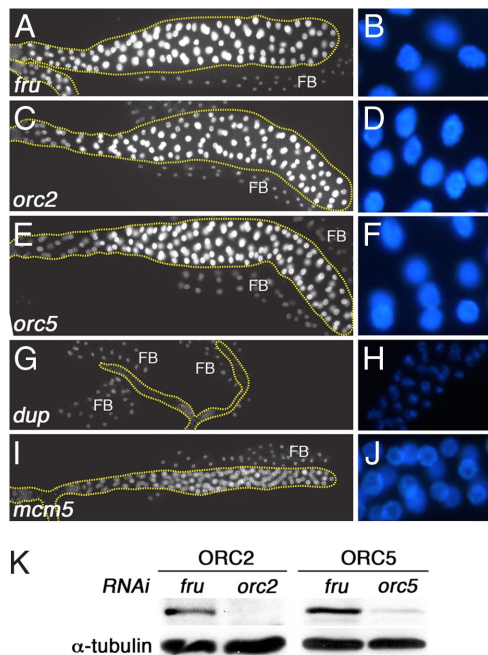

Fig. 6. Dup/Cdt1 and MCM are required for endoreplication. Each transgene endcoding RNAi against *fruitless* (*fru*) (*A* and *B*), *orc2* (*C* and *D*), *orc5* (*E* and *F*), *dup* (*G* and *H*), or *mcm5* (*I* and *J*) was expressed by a *patched (ptc)*>*gal4* driver, a SG–specific promoter (48). (*B*, *D*, *F*, *H*, and *J*) High-magnification views of the nuclei by Hoechst 33342 staining. *fru*, an adult brain–specific gene (50), was used as a negative control. FB is the fat body. (*K*) Western blot shows that ORC2 and ORC5 were down-regulated by *orc2*- or *orc5*-RNAi expression. Alpha-tubulin is a loading control.

efficiently as it does in the $orc1^{+/+}$ WT cells. DNA replication in $orc1^{-/-}$ cells occurs despite the absence of zygotic ORC1 expression, no detectable ORC1 in the SG cells either by immunostaining or Western blot analysis, and a lack of sufficient ORC1 function to support proliferation of the SG precursors before endoreplication starts.

There are two possible interpretations of these results. The first is that an undetectable amount of remaining maternal ORC1 is enough to support the entire course of endocycles during embryonic and larval development. The second is that endoreplication does not require ORC.

For the first interpretation to be the case, the undetectable residual maternal ORC1 must be able to support the 10-round endocycles to reach >1000C DNA content, even though it apparently is not enough to support proliferation in the same cells before endoreplication starts (Fig. 4*D*). Moreover, DNA synthesis during endocycles in the $orc1^{-/-}$ SG cells occurs as efficiently as in WT cells, with no indication of increased DSBs, suggesting that the number of firing origins is not reduced significantly during endoreplication, a sharp contrast to the inefficient DNA synthesis observed in many replication gene mutants (13, 14). This interpretation would also require the cells to be in a state in which ORC1 is somehow immune to Anaphase Promoting Complex/Fizzy-related (APC/Fzr)-mediated degradation (12). The surge of APC/Fzr activity at the start of the endocycle stage in mid-embryogenesis (38) and cyclic APC/Fzr activity at every round of endocycles (39) would target any remaining maternal ORC1. We cannot rule out the possibility that undetectably low levels of ORC remain associated with the chromosomal DNA throughout endocycles and are protected from the APC/Fzr by unknown mechanisms. However, this hypothetical pool of ORC would have to have special properties, capable of efficiently replicating 500 genome equivalents in the third instar SG cells but incapable of replicating the single genome in imaginal disc cells.

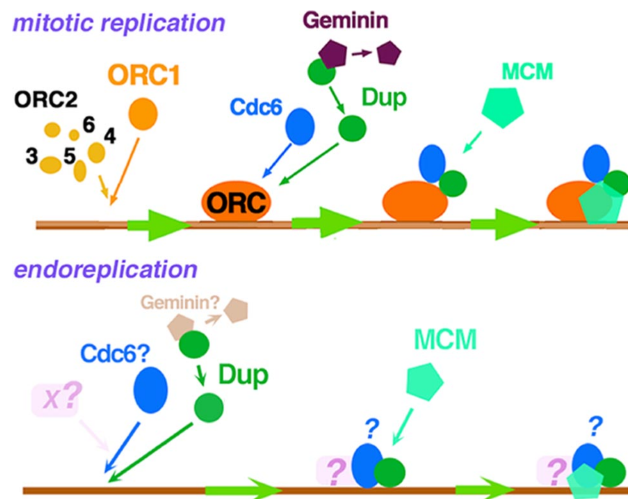

Fig. 7. Endoreplication initiation model. In mitotic replication ORC assembles on DNA late in G_1 phase when ORC1 accumulates, which recruits Cdc6, Dup/Cdt1, and then MCM complexes to form pre-RC. In endoreplication Dup/Cdt1 and possibly Cdc6 are recruited to DNA by unknown mechanisms, either directly or indirectly (mediated by protein *X*), which recruit MCM complexes to form pre-RC.

Alternatively, ORC is in fact dispensable for endoreplication. If this is the case, whether or not ORC is required for the transition from mitotic to endoreplication remains to be determined and needs further investigation. In either case, how might DNA replication be initiated at each round of endocycles in the absence of ORC? The requirement of Dup/Cdt1 and MCM for endoreplication (Fig. 6) indicates that at least some components of the replication initiation cascade are shared between the 2 modes of replication.

A simple model would be that there is an endoreplication-specific protein that brings in Dup/Cdt1 (and Cdc6 if it is required) to replication origins (Fig. 7). One possibility is that there is an ORC1 homologue that is specific to endoreplication. In *Arabidopsis* there are two ORC1 proteins (ORC1a and ORC1b); one is specifically expressed during mitotic cycle and the other during the endocycle (40). ORC1a and ORC1b are highly homologous, and both associate with ORC2–6. However, in the case of *Drosophila* the entire ORC seems to be dispensable for endoreplication (Figs. 5 and 6) (31), which argues against this model.

Another possibility is that there is a functional ORC homologue, protein X (Fig. 7). It has been shown that Cdc6 can bypass ORC for replication initiation when tethered to an artificial origin on a plasmid DNA through a Gal4 binding site (41). Many Cdt1- and/or Cdc6-associated proteins reported in various organisms (42, 43) are candidates for protein X to bring Dup (and Cdc6) to origins and initiate endoreplication.

It is also possible that ORC-independent endoreplication in flies involves an endoreplication-specific chromosomal structure. Structure-mediated replisome assembly occurs in bacteria when replication forks are frequently inactivated under normal growth conditions (44). It has been proposed that D-loops resulting from homologous recombination at DSBs mediate a DnaA-independent loading of DnaB onto arrested replication forks (45). Similar mechanisms that reactivate collapsed forks have been proposed in yeast (46) and in telomeres where protein-mediated loop structures mimic the D-loop (47). Tightly controlled replication initiation by multiple layers of mechanisms (including regulation by ORC) and various checkpoint systems would preclude such DNA structure–dependent replisome formation during normal proliferation (44).

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However, it might be used in specialized replication, such as endoreplication, during which there is a physiologic increase of DSBs and checkpoint pathways are shut down. It would be of great interest in future to find the alternative mechanisms that regulate the ORC-independent initiation of DNA replication during endocycles.

Materials and Methods

Production of *orc1* Mutant. The *Bam*H I-*Kpn*I 6.8-kb fragment was used for ''ends-in'' homologous recombination (10, 11), which introduced stop codons in all three reading frames into the *orc1* ORF at its *Sgr*A I and *Stu*I sites. All six lines that were independently isolated showed essentially identical phenotypes, thus the progeny from a cross of two lines were used for further study.

Fly Lines. Mosaic animals were obtained from crosses between P{hsFLP}1;P{FR*T*(*w^{hs}*)}G13 P{Ubi-GFP.nls}2R1 P{Ubi-GFP.nls}2R2 and P{FRT(*w^{hs}*)}G13 *bw^1 sp^1*,*$orc1^-$*/*CyO* for *orc1* or *y w* P{hsFLP}1;P{neo FRT}82B P{Ubi-GFP(S65T)nls}3R and P{neo FRT}82B *ry^{506}*, *$k43^{\gamma4}$e*/*TM2* for *orc2* (31). Somatic clones in various tissues were induced by a single heat-shock administration at 37°C as follows: 90 min in first instars (50 ± 2 h AED) for imaginal discs, 2 h in embryos (4 ± 1.5 h AED) for SG clones, and 1 h in day 3 to 4 adult females for ovaries. RNAi expression was driven by *ptc-GAL4* (48) in SGs in combination with *UAS>RNAi* lines as follows: *orc5* (7833R-1), *dup*/*Cdt1* (8171R-2), *mcm5* (4082R-2) (National Institute of Genetics, Japan), and *orc2* (47602) (Vienna *Drosophila* RNAi Center, Austria). The *fruitless*-RNAi line (50) was used as a control.

Quantification of DNA Content in SG Polytene Chromosomes. DNA was stained by Hoechst 33342 (0.5 μg/ml) (Sigma). DNA content was measured as the sum of fluorescent signal intensities in 3D images of nuclei using Huygens SVI deconvolution software (Scientific Volume Imaging, Hilversum, The Netherlands). Each 3D image was reconstituted by assembling a series of 2D images taken every 0.3 μm throughout a nucleus using a Zeiss LSM 510 inverted confocal microscope, resulting in between 40 (first instar nucleus) and 160 (late third instar) layers. Between 15 and 65 nuclei from each genotype were measured at each time point during the course of the experiments. Twenty-six-hour AED SGs included in each sample served as internal standards both for staining and signal measurement.

Immunohistochemistry and Western Blot Analyses. BrdU labeling, immunostaining, and Western blot analysis were performed as described previously (7, 12, 39).

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