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Initiation of *Drosophila* Chorion Gene Amplification Requires *Claspin* and *mus101*, Whereas *Claspin*, But Not *mus101*, Plays a Major Role During Elongation

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Background: Claspin and TopBP1 are checkpoint mediators that are required for the phosphorylation of Chk1 by ATR to maintain genomic stability. Here, we investigated the functions of *Drosophila Claspin* and *mus101* (TopBP1 ortholog) during chorion (eggshell component) gene amplification, which occurs in follicle cells in the absence of global genomic DNA replication. **Results:** Unlike *Drosophila mei-41* (ATR ortholog) mutant embryos, *Claspin* and *mus101* mutant embryos showed severe eggshell defects resulting from defects in chorion gene amplification. EdU (5-ethynyl-2′-deoxyuridine) incorporation assay during initiation and elongation stages revealed that *Claspin* and *mus101* were required for initiation, while only *Claspin* had a major role in the efficient progression of the replication forks. Claspin proteins were enriched in the amplification foci both in the initiation and elongation stage-follicle cell nuclei in a *mei-41*-independent manner. The focal localization of ORC2, a component of the origin recognition complex, was not significantly affected in the *Claspin* mutant, whereas it was reduced in the *mus101* mutant. **Conclusions:** *Drosophila Claspin* plays a major role in the initiation and elongation stages of chorion gene amplification by localizing to the amplification foci in a *mei-41*-independent manner. *Drosophila mus101* is also involved in chorion gene amplification, mostly functioning in initiation, rather than elongation. *Developmental Dynamics 246:466–474, 2017.* © 2017 The Authors Developmental Dynamics published by Wiley Periodicals, Inc. on behalf of American Association of Anatomists

Key words: DNA replication; checkpoint mediator; mei-41 (ATR); ORC2

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Introduction

To maintain genomic stability, the ATR and Chk1 checkpoint kinases play major roles in the DNA damage checkpoint response, which is induced by various types of DNA damage, including DNA replication stress. DNA replication stress activates these checkpoint genes, leading to inhibition of mitotic entry and stabilization of the replication fork to prevent fork collapse. Claspin and TopBP1 are checkpoint mediators that enhance ATR activity (Liu et al., 2006). In addition to their checkpoint functions, Chk1, Claspin, and TopBP1 are involved in normal DNA replication (Petermann et al., 2008). The importance of the ATR (Brown and Baltimore, 2000), Chk1 (Liu et al., 2000; Takai et al., 2000), Claspin (Yang et al., 2016), and TopBP1 (Jeon et al., 2011) genes during normal cell cycle progression is underscored by the embryonic lethality that results from mutations in these genes in mice. *Drosophila* contains the *mei-41, Claspin, mus101*, and *grp* genes, which are orthologs of ATR, Claspin, TopBP1, and Chk1, respectively. Studies of *Drosophila Claspin* mutants have demonstrated the involvement of *Claspin* in a replication stress-induced checkpoint during the midblastula transition (Lee et al., 2012), after hydroxyurea feeding (Lee et al., 2012), and in response to defective tRNA processing (Molla-Herman et al., 2015). Although the functions of *Claspin* during the checkpoint response have been extensively studied, its role during normal development is not well understood.

In the *Drosophila* ovary, somatic follicle cells encircle 16 germline cells, including the oocyte, and various cell cycle events occur in these follicle cells depending on their developmental stages. In addition to mitotic division, atypical cell cycle events, such as endoreplication and specific gene amplification in the absence of genomic replication, occur in somatic follicle cells during *Drosophila* oogenesis. During early development up to stage 6, follicle cells increase in number by undergoing mitotic divisions. Between stages 7 and 9, these cells endocycle by alternating between the S and gap phases. At stage 10, they cease genomic replication, and re-replication occurs from specific

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replication origins to amplify up to 60 copies of the chorion gene. The initiation and elongation stages of chorion gene replication occur during separate developmental stages of follicle cells; initiation occurs during stages 10B and 11, whereas only elongation from existing replication forks takes place during stages 12 and 13 (Claycomb et al., 2002).

Chorion is a major component of the eggshell and defects in chorion gene amplification result in a thin eggshell phenotype. Re-replication of the chorion gene induces DNA double-strand breaks, replication stress, and fork collapse, which is inhibited by *mei-41, mus101*, and *grp* to achieve efficient fork progression (Alexander et al., 2015). The *mus101* mutant embryo shows a thin eggshell phenotype due to defects in chorion gene amplification (Orr et al., 1984), while the *grp* mutant has a normal chorion gene copy number in amplification-stage follicle cells (Alexander et al., 2015). However, the role of *Claspin* in chorion gene amplification is unknown. Here, we investigated the functions of *Drosophila Claspin* during chorion gene amplification and compared them with the functions of *mei-41* and *mus101*.

We found that *Drosophila Claspin* and *mus101* mutant embryos show thin eggshell phenotypes due to reductions in chorion gene amplification, while *mei-41* mutant embryos do not show obvious defects in chorion gene amplification. The chorion gene amplification detected by thymidine analog incorporation was greatly affected by *Claspin* mutations in both initiation- and elongation-stage follicle cells. Although initiation was significantly reduced in the *mus101* mutant, the progression of replication forks in the elongation stage was not severely affected. The Claspin protein was enriched in chorion gene amplification foci during the initiation and elongation stages of chorion re-replication in a *mei-41*-independent manner. These results suggest that *Drosophila Claspin* and *mus101* have a *mei-41*-independent function in the initiation of chorion gene amplification and *Claspin*, but not *mus101*, is important for the efficient progression of replication forks.

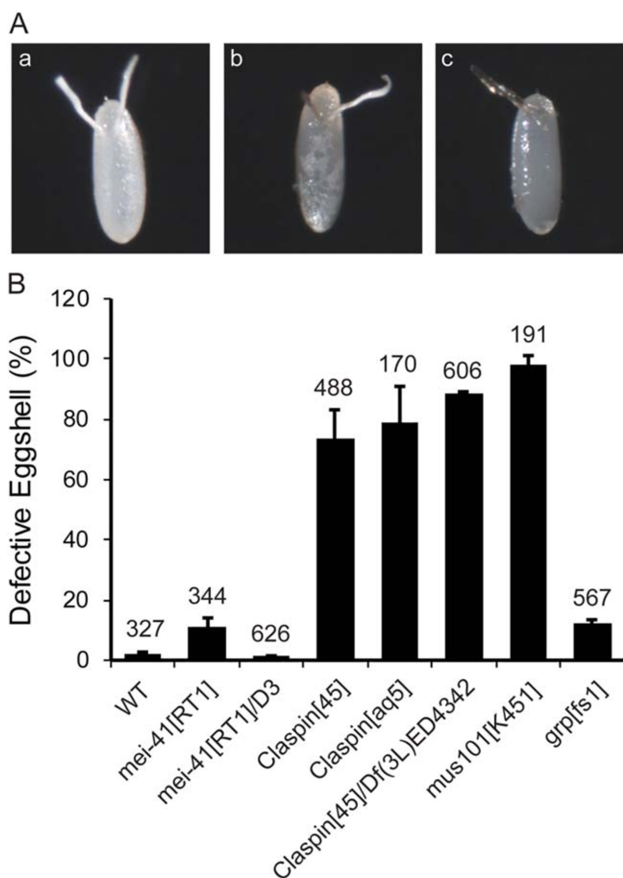

Fig. 1. *Claspin* and *mus101* mutant embryos show eggshell defects. Eggs from 4-day-old females were collected over a period of 2 hr and their eggshell phenotypes were observed. **A**: Morphology of embryos derived from *Claspin*^{45} mutant females. Representative images of embryos laid by *Claspin*^{45} homozygote females with normal (a), irregular (b), and transparent (c) egg shells are shown. **B**: The eggshell defects in embryos derived from checkpoint mutant females *(mei-41, Claspin, mus101, grp)*. Df(3L)ED4342 is the deficiency that lacks the *Claspin* gene. The graph shows the mean percentage of eggs with defective eggshells from females with the indicated genotypes from at least two independent experiments. The total embryo count for each genotype is indicated above each bar.

Results

Drosophila Claspin and *mus101* Are Required for Eggshell Production

Over 98% of eggs produced by wild type females have normal eggshells that appear opaque. We observed that the *Claspin* mutant females laid eggs with defective eggshells. Of the eggs derived from the females homozygous for the *Claspin* null allele (*Claspin*^{45}), 73.8% (SD 9.6) were defective, exhibiting either irregular (Fig. 1Ab) or transparent (Fig. 1Ac) morphologies. The remaining eggs appeared normal (Fig. 1Aa). Eggs from females carrying the heteroallelic combination of *Claspin*^{45} and *Df(3L)ED4342*, which results in lack of Claspin protein (*Claspin*^{45}*/Df(3L)ED4342*), and those from females with a hypomorphic allele (*Claspin*^{aq5}) exhibited similar defects as eggs from females carrying *Claspin*^{45} (Fig. 1B).

Because Claspin and TopBP1 function as mediators of the ATR kinase to facilitate the phosphorylation of Chk1, we tested whether *mei-41* (*Drosophila* ATR) and *grp* (*Drosophila* Chk1) were also involved in this process using mutant alleles that result in severe defects in the G2/M DNA damage checkpoint (Laurencon et al., 2003; Brodsky et al., 2004). The percentages of *grp*^{fs1} and *mei-41*^{RT1} embryos with defective eggshells (12.1%, SD 1.5; and 10.8%, SD 3.5, respectively) were slightly higher than that of the wild type embryos (1.8%, SD 0.7) but were significantly lower than that of the *Claspin*^{45} mutant embryos (73.8%, SD 9.6) (Fig. 1B). Of interest, the eggshell defect in *mei-41*$^{RT1/D3}$ was not significantly different from the wild type, suggesting that the eggshell phenotype may be allele-specific or due to an accumulation of genetic changes.

We also tested a *mus101*^{K451} mutant, which is a separation-of-function mutant that is defective in chorion gene amplification, but is normal in G2/M DNA damage checkpoint function (Orr et al., 1984; Kondo and Perrimon, 2011). We found that 98.3% (SD 2.9) of eggs from *mus101*^{K451} have severe eggshell defects as previously described (Orr et al., 1984). These results showed that together with *mus101, Claspin* is required for eggshell production, while *mei-41* and *grp* play minor roles, if any.

Drosophila Claspin and *mus101* Are Required for the Initiation of Chorion Gene Amplification

Because the eggshell defect in *mus101*^{K451} mutant embryos is caused by a substantial reduction in chorion gene

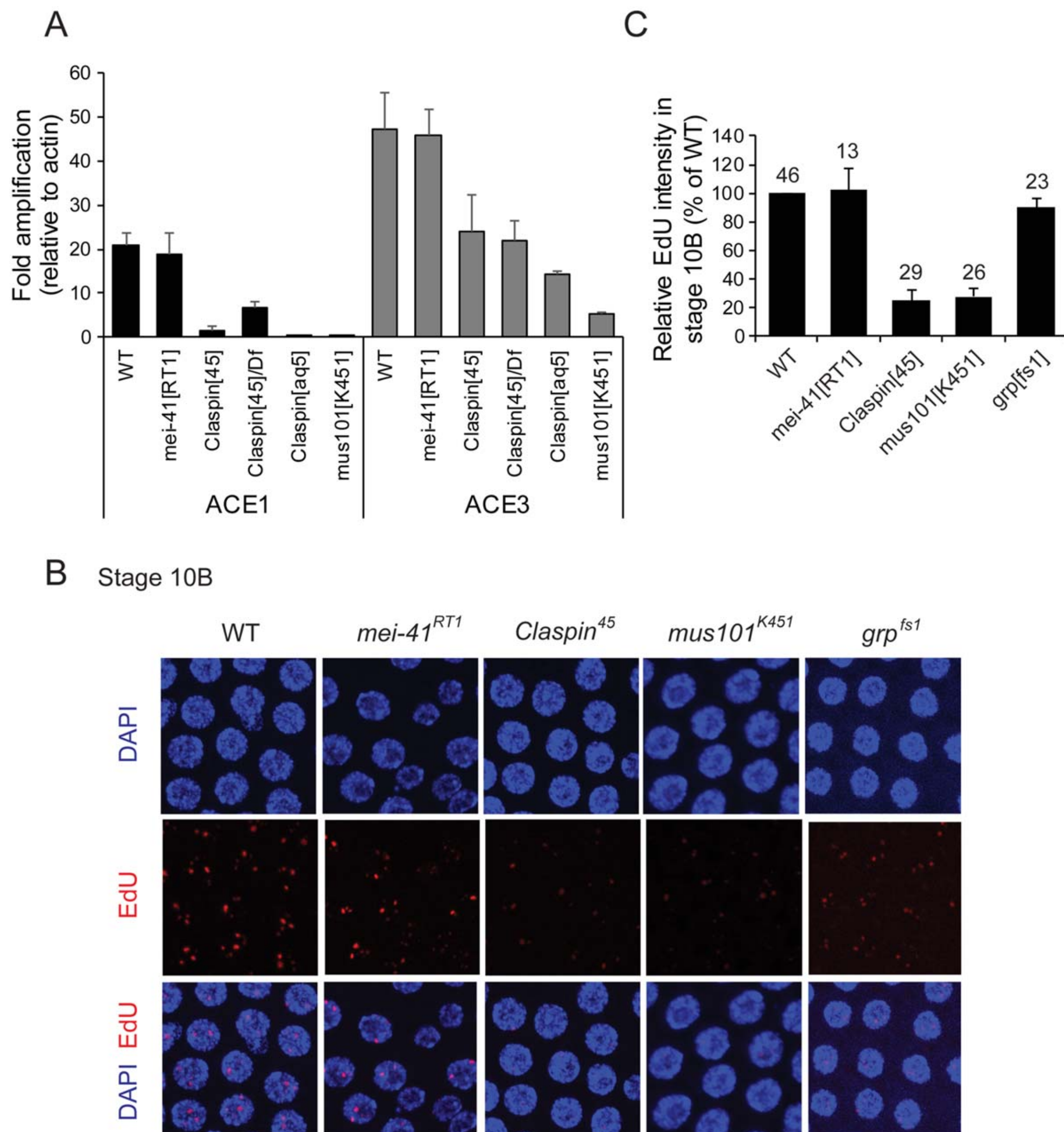

Fig. 2. *Claspin* and *mus101* mutants show defects in the initiation of chorion gene amplification. **A**: Reductions in chorion gene copy numbers in *Claspin* and *mus101* mutants. Fold amplification of loci containing *ACE3* and *ACE1* relative to actin was determined by real-time PCR using genomic DNA from stage 13 egg chambers from ovaries of the indicated genotype. Df(3L)ED4342 indicated as Df is the deficiency that lacks the *Claspin* gene. The mean and standard deviation were obtained from at least two independent experiments performed in triplicate. **B,C**: Reduction in the intensities of chorion gene amplification foci in *Claspin* and *mus101* mutant follicle cells. Chorion gene amplification foci were detected by Click-iT EdU assay in the wild type and mutant females. **B**: Representative images of stage 10B follicle cells are shown. **C**: The relative intensities of EdU foci in stage 10B follicle cells of checkpoint mutant females were compared with that of the wild type. The graph shows the mean intensity of EdU foci from at least two independent experiments. The average intensity of the EdU foci from 10 nuclei was analyzed for each egg chamber. The total number of egg chambers analyzed for each genotype is indicated above each bar.

amplification in the somatic follicle cells that overlie the oocyte (Orr et al., 1984), we tested whether this process was also defective in *Claspin* mutant embryos. To determine the relative amplification of the chorion locus, genomic DNA was isolated from stage 13 egg chambers containing follicle cells that had completed amplification of the chorion locus. Quantitative polymerase chain reaction (PCR) was performed to amplify *ACE1 and ACE3* (amplification control elements for the first and third chromosome chorion clusters). *ACE3*, located 1.5 kb away from the origin, ori-β, and *ACE1* are *cis*-acting elements that are specifically bound by the origin recognition complex (ORC) and are important for chorion gene amplification (Austin et al., 1999; Zhang and Tower, 2004).

The relative amplification level of *ACE1* was higher than that of actin by 20.8-fold in wild type stage 13 egg chambers. It was reduced in the null *Claspin* allele mutants, *Claspin*^{45} and *Claspin*^{45}/*Df(3L)ED4342* (1.4- and 6.6-fold relative to actin, respectively) (Fig. 2A). In case of *ACE3*, it was similarly reduced in the *Claspin* mutants (47.3-, 24.0-, 21.8-fold relative to actin for wild type, *Claspin*^{45}, and *Claspin*^{45}/*Df(3L)ED4342*, respectively).

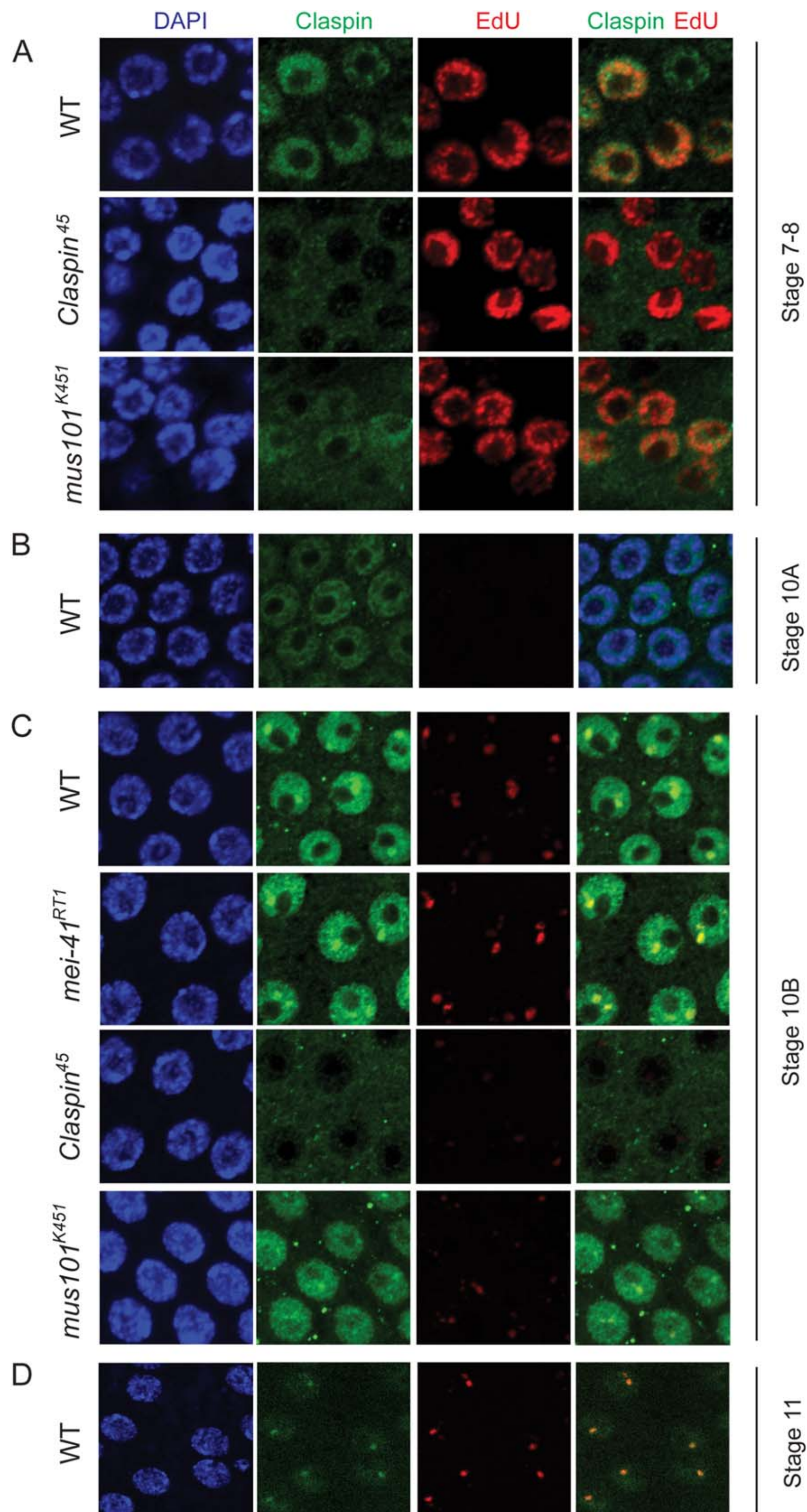

Fig. 3. Claspin protein localization in wild type, *mei-41*, and *mus101* mutant follicle cells. Click-it EdU (red) and anti-Claspin (green) antibody staining was performed for wild type, *mei-41*^{RT1}, *Claspin*^{45}, and *mus101*^{K451} mutant ovaries. **A–D**: Representative images of follicle cells during endoreplication (A), 10A (B), initiation (C, stage 10B), and elongation (D, stage 11) stages of chorion gene amplification are shown. Images (A, B, and D) are single confocal sections.

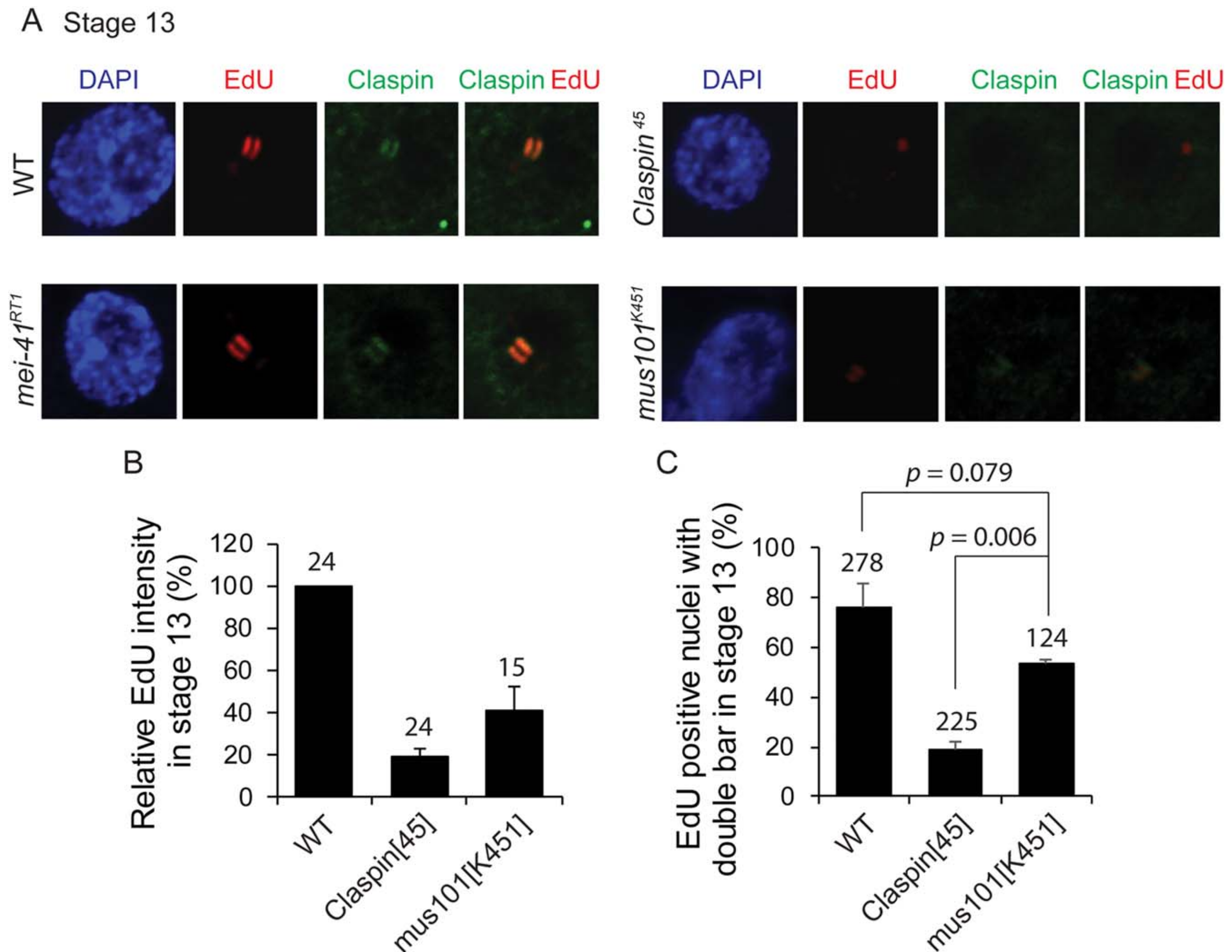

Fig. 4. Claspin localization and EdU incorporation at the elongation stage of chorion gene amplification in wild type and checkpoint mutants. Click-it EdU (red) and anti-Claspin (green) antibody staining was performed for wild type, *mei-41*^{RT1}, *Claspin*^{45}, and *mus101*^{K451} mutant ovaries. **A**: Representative images of single confocal sections of stage 13 follicle cell nuclei, in which the elongation of preformed replication forks occurs, are shown. **B**: The relative intensities of EdU foci in stage 13 follicle cells of checkpoint mutant females were compared with that of wild type cells. The graph shows the mean intensity of EdU foci from two independent experiments. The average intensity of the EdU foci from nuclei with detectable EdU incorporation was analyzed for each egg chamber. The total number of egg chambers analyzed for each genotype is indicated above each bar. **C**: The percentage of EdU-positive nuclei containing the EdU double bar structure was determined from two independent experiments. The total number of nuclei analyzed for each genotype is indicated above each bar.

Consistent with its severely defective eggshell phenotype, the *Claspin*^{aq5} hypomorphic allele showed substantial amplification reductions similar to those of the null allele. In the *mus101*^{K451} mutant, the relative amplification levels of *ACE1* (0.02-fold relative to actin) and *ACE3* (5.1-fold relative to actin) were also lower than those of the wild type (Fig. 2A). The copy number of third chromosome chorion origin in *grp*^{fs1} is not significantly different from that of the wild type according to a previous report (Alexander et al., 2015). Similarly, we found that the relative amplification levels of *ACE1* and *ACE3* were not reduced in the *mei-41*^{RT1} and *mei-41*$^{RT1/D3}$ mutants when compared with the wild type (Fig. 2A, data not shown).

To directly examine the roles of checkpoint genes in chorion amplification, we performed Click-iT EdU (5-ethynyl-2′-deoxyuridine) assays to visualize DNA replication in amplification-stage follicle cells. Six genomic loci have been found to be amplified (Kim et al., 2011), and an average of 4.2 EdU foci can be detected in wild type follicle cells at stage 10B when initiation occurs (Fig. 2B). The intensity of the foci is correlated with fold amplification, appearing as one bright, one intermediate, and two to four relatively faint dots (Fig. 2B). In the checkpoint mutants (*mei-41*^{RT1}, *Claspin*^{45}, *mus101*^{K451}, and *grp*^{fs1}), the average number of EdU foci was between 3.3 and 4.9 (data not shown). Because the intensity of the EdU foci appeared to be severely reduced in *Claspin*^{45} and *mus101*^{K451} mutant follicle cells, we quantified the EdU intensity. The intensity of the EdU foci in *Claspin*^{45} and *mus101*^{K451} mutant nuclei decreased to 25% and 28%, respectively, relative to the levels seen in the wild type (Fig. 2C). On the other hand, EdU intensity was not significantly different from that of the wild type in the *mei-41*^{RT1} and *grp*^{fs1} mutants (Fig. 2C). These results further confirmed that *Claspin* and *mus101* have major roles in the initiation step of chorion gene amplification, while *mei-41* and *grp* are dispensable.

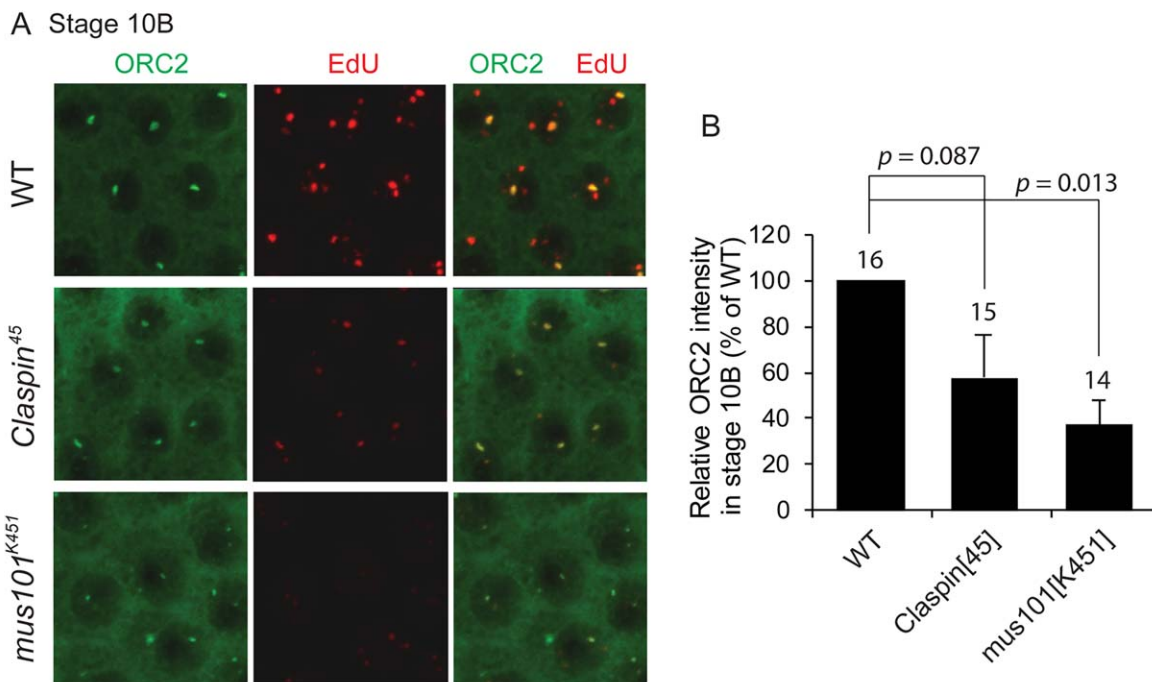

Fig. 5. ORC2 localization at chorion gene foci in *Claspin* and *mus101* mutants. Click-it EdU (red) and anti-OCR2 (green) antibody staining was performed for wild type and *Claspin*^{45}, and *mus101*^{K451} mutant ovaries. **A**: Representative images of stage 10B follicle cells are shown. **B**: The relative intensities of ORC2 foci in stage 10B follicle cells of checkpoint mutant females were compared with that of wild type cells. The graph shows the mean intensity of ORC2 foci from two independent experiments. The average intensity of the ORC2 foci from at least five nuclei was analyzed for each egg chamber. The total number of egg chambers analyzed for each genotype is indicated above each bar.

Effect of *Claspin* and *mus101* Mutations on Various Types of DNA Replication During Follicle Cell Development

To test the role of *Claspin* and *mus101* in DNA replication during follicle cell development, we compared EdU incorporation in *Claspin* and *mus101* mutant ovaries with that in wild type ovaries. In *Claspin*^{45} and *mus101*^{K451} mutant follicle cells, EdU staining in endocycling follicle cells at stages 7 and 8 was similar to that in wild type ovaries (Fig. 3A). As shown above, the intensity of EdU foci was severely affected in stage 10B follicle cells of *Claspin*^{45} and *mus101*^{K451} mutants, when the initiation of chorion gene amplification occurs (Figs. 2B,C, 3C).

In stage 13 follicle cells, when chorion gene amplification occurs only by elongation, a double bar structure of EdU staining can be detected in most of the wild type and *mei-41*^{RT1} mutant cells (Fig. 4A). In the *Claspin*^{45} and *mus101*^{K451} mutants, EdU staining cannot be detected in some follicle cells. When EdU incorporation was detected in the *Claspin*^{45} and *mus101*^{K451} mutants, the signal intensity was reduced to 19.7% and 41.3% of wild type levels, respectively (Fig. 4B). Moreover, the EdU signal in the mutants appeared as a dot or a double bar (Fig. 4A). The length of the double bar representing the number of origin firing events (Claycomb et al., 2002) was shorter in the mutants than in the wild type, confirming the defects in initiation. The dot shape of the EdU suggests that the progression of the replication forks is severely affected and EdU is not resolved into a double bar.

To quantify the defects in the elongation steps of chorion gene amplification in the *Claspin* and *mus101* mutants, the percentage of EdU-positive nuclei containing the EdU double bar structure was determined. In the stage 13 wild type follicle cells, 76% of EdU-positive nuclei exhibited a double bar structure. On the other hand, the percentage of EdU-positive nuclei with double bar structure was severely reduced to 18.8% in the *Claspin*^{45} mutant, while it was not significantly different from wild type in the *mus101*^{K451} mutant (53.8%, $p = 0.079$) (Fig. 4C). These results suggest that both *Claspin* and *mus101* are dispensable for the endocycle, but are required for the initiation of chorion gene re-replication. In addition, the efficient progression of replication forks requires *Claspin*, but not *mus101*.

Localization of *Drosophila* Claspin Protein in Follicle Cells

Claspin, a checkpoint mediator, also has a role in DNA replication and is a component of a protein complex that travels with the replisome at the replication fork (Aze et al., 2013). To test whether *Drosophila* Claspin is directly involved in amplification of the chorion locus, we performed immunofluorescence analysis of Claspin protein along with EdU incorporation assays. In the wild type ovary, a diffuse nuclear Claspin staining pattern was observed in EdU-positive S-phase follicle cells during the endocycle, from stages 7 to 9, and this pattern was not detected in the *Claspin*^{45} mutant (Fig. 3A). Claspin was detected in most EdU-negative gap-phase cells, although some exhibited weaker staining. These findings suggest that Claspin is expressed in both the S and gap phases during the endocycle. The diffuse nuclear staining pattern of Claspin persisted in stage 10A and 10B, although staining was brighter in stage 10B when compared with 10A (Fig. 3B,C).

In addition, stage 10B nuclei contained one or two bright foci of Claspin protein staining (Fig. 3C). These Claspin foci

overlapped with two bright EdU foci that corresponded to the amplified chorion gene clusters of the X and third chromosomes. We found that the localization of Claspin to the amplification foci persisted in stages 11 and 12, while the diffuse nuclear staining of Claspin was greatly diminished (Fig. 3D, data not shown). In stage 12 and 13 follicle cells, the nuclear EdU signal was detected as a double bar structure as replication forks bidirectionally move outward in the absence of initiation (Fig. 4A) (Claycomb et al., 2002). Claspin was also resolved into a double bar structure that overlapped with EdU staining (Fig. 4A), visually confirming the previous reports that Claspin moves along with the replication forks.

We tested if the localization of Claspin was affected in the *mei-41*^{RT1} or *mus101*^{K451} mutant follicle cells. The Claspin staining pattern in the *mei-41*^{RT1} mutant was not different from that of the wild type during the initiation and elongation steps of chorion gene amplification (Figs. 3C, 4A), suggesting that Claspin localizes to the amplification foci in a *mei-41*-independent manner. In *mus101*^{K451} mutant follicle cells, diffuse nuclear staining of Claspin was detected during endocycling (Fig. 3A) and in stage 10B follicle cells (Fig. 3C). Although the focal localization of Claspin was detected at stage 10B, the intensity was slightly reduced in the *mus101*^{K451} mutant (Fig. 3C), probably due to a reduction in the copy number of the origin (Fig. 2A). At stage 13, Claspin could be detected in the *mus101*^{K451} mutant follicle cells when EdU was visible (Fig. 4A). These results suggest that Claspin can be localized to amplification foci in a *mei-41*- and *mus101*-independent manner during initiation and elongation.

ORC2 Localization to Amplification Foci in *Drosophila Claspin* and *mus101* Mutant Follicle Cells

To determine whether prereplication complex (pre-RC) formation at amplification foci was affected, we examined ORC2 localization in the *Claspin* and *mus101* mutants. Because six ORC proteins form a stable complex in *Drosophila*, detection of ORC2 foci is likely to reflect the presence of the ORC complex, which is required for pre-RC formation (Tower, 2004). In stage 10B wild type follicle cells, ORC2 was detected as a single focus and was colocalized with the brightest EdU foci on third chromosome chorion loci, as previously reported (Royzman et al., 1999) (Fig. 5A).

In some *Claspin*^{45} mutant follicle cells, ORC2 foci appeared fainter than those in the wild type cells. When we quantified the relative intensities of the ORC2 foci, the average intensity was lower in the *Claspin*^{45} mutant than in the wild type, but this difference was not statistically significant (58.0% of the intensity in the wild type, $p = 0.087$, Fig. 5B). On the other hand, the intensity of ORC2 foci was significantly reduced in the *mus101*^{K451} mutant follicle cells (37.4% of the intensity in the wild type, $p = 0.013$, Fig. 5B). These results suggest that ORC2 localization to the amplification foci is affected by the *mus101* mutation.

Discussion

To understand the biological functions of *Drosophila Claspin*, we investigated the basis of the thin eggshell phenotype of *Claspin* mutants and compared it with that of *mus101* and *mei-41* mutants. We found that *Drosophila Claspin* and *mus101* are required for the initiation of chorion gene amplification. *Claspin*, but not *mus101*, plays a major role in the efficient progression of replication forks. The role of Claspin during amplification was supported by its localization to amplification foci during initiation and elongation. These characteristics were distinct from those of *mei-41*, suggesting that *Drosophila Claspin* and *mus101* have a unique and *mei-41*-independent role in DNA replication during chorion gene amplification.

During oogenesis, the mode of DNA replication in somatic follicle cells that encircle germline cells changes from mitotic replication to endoreplication, followed by chorion gene amplification in the absence of genomic DNA replication. Studies of various mutants that show defects in chorion gene amplification have revealed three different phenotypes. In addition to a lack of amplification (reviewed in Claycomb and Orr-Weaver, 2005), some mutants exhibit chorion gene overamplification (Royzman et al., 1999; Bosco et al., 2001), and other mutant follicle cells fail to exit the endocycle during the amplification stage and instead perform inappropriate genomic DNA replication throughout the follicle cell genome (Cayirlioglu et al., 2003; Aggarwal and Calvi, 2004; Beall et al., 2004; Ge et al., 2015). These results suggest that distinct signaling pathways exist for the positive and negative regulation of chorion gene amplification and for the repression of genomic DNA replication. In *Claspin* and *mus101* mutant stage 10B follicle cells, neither ectopic genomic replication nor overamplification of the chorion gene was observed. This suggests that *Claspin* and *mus101* are required for chorion gene amplification and that they are not involved in suppressing genomic DNA replication or in negatively regulating chorion gene amplification.

The functions of Claspin and TopBP1 in DNA replication are conserved from yeast to mammalian cells and both proteins are important for the initiation of DNA replication. We found that *Drosophila Claspin* and *mus101* are required for the initiation of chorion gene amplification based on the following observations. First, the intensity of EdU incorporation in follicle cells at the initiation stage (Fig. 2C) and the relative fold amplification of *ACE3*, which is located 1.5 kb away from the origin, were severely reduced in both mutants (Fig. 2A). Second, when the EdU double bar was detected in the stage 13 follicle cells of *Claspin* and *mus101* mutants, the length of the bar representing the number of origin firings (Claycomb et al., 2002) was significantly shorter than that of the wild type (Fig. 4A, data not shown). Lastly, the Claspin protein exhibited a focal localization overlapping with the largest EdU foci known to contain the ORC complex during the initiation stage (Fig. 3C).

In addition to initiation, Claspin affects the replication fork progression rate in mammalian cells (Petermann et al., 2008) and Mrc1 (yeast Claspin) found in the replisome is essential for rapid replisome progression *in vitro* (Yeeles et al., 2017). On the other hand, Dpb11 (yeast TopBP1) is not considered part of the replisome (Tanaka and Araki, 2013) and *Xenopus* TopBP1 does not seem to be required for the elongation steps of DNA replication (Hashimoto and Takisawa, 2003). Consistent with these previous reports, we found that EdU foci were not efficiently resolved into a double bar structure in the *Claspin* mutant follicle cells at the elongation-only stage, whereas a significantly higher percentage of *mus101* mutant follicle cells exhibited double bar structure formation (Fig. 4C). Moreover, Claspin staining appeared as a double bar and colocalized with EdU during the elongation stage in follicle cells (Fig. 4A), visually confirming that Claspin moves along with the replication forks. These results show that *Drosophila Claspin* and *mus101* have conserved functions during chorion gene amplification.

Drosophila chorion gene amplification begins with the binding of the ORC complex to replication origins using most of the

general DNA replication machinery. Many genes have been reported to affect chorion gene amplification and mutations in most of these genes also result in a loss of ORC foci formation (reviewed in Tower, 2004; Claycomb and Orr-Weaver, 2005). The exceptions are *Myb* and *dup* mutants; normal ORC2 foci have been detected, despite the absence of bromodeoxyuridine foci in the *Myb* mutant clones (Beall et al., 2002) and ORC2 foci are smaller in *dup* mutant follicle cells (Whittaker et al., 2000).

We found that ORC2 localization to amplification loci was significantly reduced in the $mus101^{K451}$ mutant compared with the wild type, whereas it was not significantly different in $Claspin^{45}$ mutant (Fig. 5). Compared with the wild type (47.3-fold relative to actin), the amplification of *ACE3* in $Claspin^{45}$ and $mus101^{K451}$ mutants was reduced to 24.0 and 5.2-fold relative to actin, respectively (Fig. 2A). Because *ACE3* is the region recognized by ORC2 and where the major ORC2 foci are localized at stage 10B (Royzman et al., 1999), a significant reduction in ORC2 intensity in the *mus101* mutant is likely to result from the reduced copy number of the origin.

Additionally, the Dup (*Drosophila* Cdt1) protein, which usually forms foci at chorion loci, is stabilized and delocalized by various defects in DNA replication, including $mus101^{K451}$ mutations (May et al., 2005). It is not clear if Dup localization is similarly affected in *Claspin* mutants. Because the size of ORC2 foci is smaller in *dup* mutant follicle cells than in wild type cells (Whittaker et al., 2000), the reduction in ORC2 intensity found in $mus101^{K451}$ mutants may result from the delocalization of Dup. Further analyses will be required to elucidate the detailed molecular events in the initiation steps of chorion gene amplification.

A previous study of *Drosophila* $mei\text{-}41^{RT1}$ and $mus101^{D1}$, a separation-of-function allele that shows defects in the G2/M DNA damage checkpoint, but normal DNA replication (Kondo and Perrimon, 2011), showed that cells lacking these genes are defective in the replication stress checkpoint and exhibit reduced fork progression by 25–30%, rather than the complete lack of replication (Alexander et al., 2015). We found that $mus101^{K451}$, another separation-of-function allele with the opposite phenotypes, shows defects mostly in the initiation step of chorion gene amplification. *Claspin* is directly involved in the initiation and elongation steps of chorion gene amplification, although mitotic replication and endoreplication seem to occur normally in both mutants. Because several hypomorphic mutants of pre-RC components also show phenotypic abnormalities only in chorion amplification, amplification may be more sensitive to the activity of the basal DNA replication machinery than to mitotic replication (Tower, 2004).

A recent study reporting the first example of gene amplification in normal mammalian development has identified genes that are selectively amplified in trophoblast giant cells (Hannibal and Baker, 2016). An investigation into whether the Claspin and TopBP1 play similar functions in mammals will provide useful insights. *Drosophila* chorion gene amplification will serve as a valuable model for elucidating the mechanism of action of *Claspin* and *mus101* during DNA replication, specific gene amplification, and the replication stress checkpoint.

Experimental Procedures

Drosophila Strains

All *Drosophila* strains were maintained at 25 degC. Canton S flies were used as wild type controls. *Df(3L)ED4342* (a deficiency line lacking *Claspin*), $mei\text{-}41^{D3}$, and $mei\text{-}41^{RT1}$ mutant flies were obtained from the Kyoto Stock Center and Bloomington Stock Center. The *Claspin* mutant alleles have been previously described (Lee et al., 2012) and $mus101^{K451}$ and grp^{fs1} were obtained from Dr. David Glover (University of Cambridge) and Dr. William Theurkauf (University of Massachusetts Medical School), respectively. Four- or 5-day-old adult females that were conditioned on wet yeast were used for the analysis.

Preparation of Genomic DNA and Quantitation of Amplification at Chorion Gene Loci

Ovaries were dissected and genomic DNA from stage 13 egg chambers was isolated as previously described (Royzman et al., 1999). Quantitative PCR was performed to amplify the amplification control elements for the first and third chromosome chorion clusters (*ACE1* and *ACE3*) within chorion gene loci using QuantiFast SYBR Green Mix (QIAGEN, Hilden, Germany) and a LightCycler 2.0 (Roche Life Science, Basel, Switzerland). The primers used for PCR were as follows: 5′-GGTACCCTGAGCCTGGCCAACATC-3′ and 5′-CCACTTTCCAAACTGCCGCACTAAGC-3′ for *ACE3*; and 5′-CCTTTTCTTTTGAAACGCCAAA-3′ and 5′-CTCTAGTTGCAAA GAGATTTGAAGATG-3′ for *ACE1*. The primers for actin have been reported previously (Royzman et al., 1999). Relative amplification of the chorion loci was calculated using a mathematical method based on the real-time PCR efficiencies, with actin used as a reference gene (Pfaffl, 2001).

Immunofluorescence Staining

To detect cells undergoing DNA replication, ovaries were dissected in Grace's medium at room temperature and immediately labeled using a Click-iT EdU Alexa Fluor 594 Imaging Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, ovaries were labeled with EdU (10 μM) for 1 hr and then fixed with 4% formaldehyde in phosphate-buffered saline (PBS) for 20 min. Next, the ovaries were washed twice with 3% bovine serum albumin (BSA) in PBS, permeabilized for 20 min with PBST (PBS containing 0.1% Triton X-100 and 0.05% Tween-20) and incubated with an EdU reaction cocktail for 30 min at room temperature. Then, the ovaries were washed 4 times with 3% BSA in PBS, stained with 5 μg/ml Hoechst 33342 for 30 min and mounted in Vectashield (Vector Laboratories, Burlingame, CA).

To double-label Claspin (or ORC2) and EdU, the Click-iT reaction was performed as described above. Ovaries were washed and incubated with a primary antibody, anti-Claspin (Lee et al., 2012), or anti-ORC2 (Royzman et al., 1999) (diluted 1:200), at 4 degC overnight or for 2 days, respectively. Next, the ovaries were washed in PBST and incubated with goat anti-rabbit Alexa Fluor® 488-conjugated IgG (Invitrogen) for 2 hr. The rabbit polyclonal anti-ORC2 antibody was kindly provided by Dr. Orr-Weaver (Whitehead Institute, Massachusetts Institute of Technology). The ovaries were visualized using a confocal laser scanning microscope (LSM 700, Carl Zeiss, Oberkochen, Germany). All quantitative analyses were performed with maximum intensity projections of multiple z-stack images and these images are shown unless stated otherwise.

Statistical Analysis

The experiments were performed two or more times. The quantitative data are expressed as the mean ± SD. The significance of

differences between two experimental samples was assessed using two-sided, unpaired Student's *t*-test. Differences were considered statistically significant at a $p < 0.05$.

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