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

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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 (Petrermann 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 germ-line 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 regions including the chorion (eggshell) gene amplification, which occurs in follicle cells in the absence of global genomic DNA replication.
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.

## 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\textsuperscript{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\textsuperscript{45} and Df(3L)ED4342, which results in lack of Claspin protein (Claspin\textsuperscript{45}/Df(3L)ED4342), and those from females with a hypomorphic allele (Claspin\textsuperscript{q5}) exhibited similar defects as eggs from females carrying Claspin\textsuperscript{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\textsuperscript{K451} and mei-41\textsuperscript{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\textsuperscript{45} mutant embryos (73.8%, SD 9.6) (Fig. 1B). Of interest, the eggshell defect in mei-41\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{K451} mutant embryos is caused by a substantial reduction in chorion gene
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, Claspin45 and Claspin45/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, Claspin45, and Claspin45/Df(3L)ED4342, respectively).

**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.
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<sup>RT1</sup>, Claspin<sup>45</sup>, and mus101<sup>K451</sup> 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.
Consistent with its severely defective eggshell phenotype, the Claspin<sup>45</sup> hypomorphic allele showed substantial amplification reductions similar to those of the null allele. In the mus<sup>101<sup>K451</sup> 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 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.

Similarly, we found that the relative amplification levels of ACE1 and ACE3 were not reduced in the mei-41<sup>RT1</sup> and mei-41<sup>RT1/D3</sup> 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<sup>RT1</sup>, Claspin<sup>45</sup>, mus<sup>101<sup>K451</sup></sup>, and gpr<sup>61</sup>), 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<sup>45</sup> and mus<sup>101<sup>K451</sup></sup> mutant follicle cells, we quantified the EdU intensity. The intensity of the EdU foci in Claspin<sup>45</sup> and mus<sup>101<sup>K451</sup></sup> 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 and gpr 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 gpr are dispensable.
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 Claspin45 and mus101K451 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 Claspin45 and mus101K451 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 Claspin45, and mus101K451 mutant ovaries. In Claspin45 and mus101K451 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 Claspin45 and mus101K451 mutants, when the initiation of chorion gene amplification occurs. In the Claspin45 and mus101K451 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). These findings suggest that both Claspin and mus101 are dispensable for the endocycle, but are required for the initiation of chorion gene 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 Claspin45 mutant (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 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 Claspin45 mutant, while it was not significantly different from wild type in the mus101K451 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 replication. In addition, the efficient progression of replication forks requires Claspin, but not mus101.
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<sup>RT1</sup> or mus101<sup>K451</sup> mutant follicle cells. The Claspin staining pattern in the mei-41<sup>RT1</sup> 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<sup>K451</sup> 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<sup>K451</sup> 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<sup>K451</sup> 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<sup>45</sup> 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<sup>45</sup> 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<sup>K451</sup> 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 endocyte 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 Mre1 (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 (Whitaker et al., 2000).

We found that ORC2 localization to amplification loci was significantly reduced in the mus101K451 mutant compared with the wild type, whereas it was not significantly different in Claspin mutant (Fig. 5). Compared with the wild type (47.3-fold relative to actin), the amplification of ACE3 in Claspin and mus101K451 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 (Royozman 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 mus101K451 mutations (May et al., 2009). 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 (Whitaker et al., 2000), the reduction in ORC2 intensity found in mus101K451 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-41RT1 and mus101D1, 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 in the replication stress checkpoint and exhibit reduced fork progression. The data obtained from the experiments described (Lee et al., 2012) and mus101K451 and grpfs1 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 mus101K451 and grpfs1 mutant flies 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 (Royozman et al., 1999). Quantitative PCR was performed to amplify the amplification control elements for the first and third chromosome chorion clusters (ACE1 and ACE2) 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′-GGTACCCGTGACCGCAGCAACTTCATACT-3′ and 5′-CCACCTTTCAAATCTCCGACATAGCACT-3′ for ACE1; and 5′-CCTTCTCTTCCTGAAAGCCAAA-3′ and 5′-CTTCTGTTGCAAGATTTGGAATG-3′ for ACE3. The primers for actin have been reported previously (Royozman 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 (Pfafli, 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 (Royozman 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 antibodies were 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.

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-41RT1, and mei-41RT1 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 mus101K451 and grpfs1 mutant flies 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.

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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