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APC/C$^{Fzr/Cdh1}$ promotes cell cycle progression during the *Drosophila* endocycle

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The endocycle is a commonly observed variant cell cycle in which cells undergo repeated rounds of DNA replication with no intervening mitosis. How the cell cycle machinery is modified to transform a mitotic cycle into endocycle has long been a matter of interest. In both plants and animals, the transition from the mitotic cycle to the endocycle requires Fzr/Cdh1, a positive regulator of the Anaphase-Promoting Complex/Cyclosome (APC/C). However, because many of its targets are transcriptionally downregulated upon entry into the endocycle, it remains unclear whether the APC/C functions beyond the mitotic/endocycle boundary. Here, we report that APC/C$^{Fzr/Cdh1}$ activity is required to promote the G/S oscillation of the *Drosophila* endocycle. We demonstrate that compromising APC/C activity, after cells have entered the endocycle, inhibits DNA replication and results in the accumulation of multiple APC/C targets, including the mitotic cyclins and Geminin. Notably, our data suggest that the activity of APC/C$^{Fzr/Cdh1}$ during the endocycle is not continuous but is cyclic, as demonstrated by the APC/C-dependent oscillation of the pre-replication complex component Orc1. Taken together, our data suggest a model in which the cyclic activity of APC/C$^{Fzr/Cdh1}$ during the *Drosophila* endocycle is driven by the periodic inhibition of Fzr/Cdh1 by Cyclin E/Cdk2. We propose that, as is observed in mitotic cycles, during endocycles, APC/C$^{Fzr/Cdh1}$ functions to reduce the levels of the mitotic cyclins and Geminin in order to facilitate the relicensing of DNA replication origins and cell cycle progression.

KEY WORDS: APC/C, Cdh1, Cyclin E, *Drosophila*, endoreplication, endocycle, Fzr/Cdh1, Geminin

INTRODUCTION

During the endocycle, also called the endoreplicative cycles, cells undergo repeated rounds of DNA replication without undergoing a cellular division. Both plants and animals use this common variant cell cycle to increase ploidy and cell size (Edgar and Orr-Weaver, 2001; Traas et al., 1998; Zybina and Zybina, 1996). The decoupling of S phase from mitosis makes the endocycle a useful model for defining the minimum cell cycle requirements to achieve a G/S oscillation and the associated once-per-cell-cycle replication of the genome.

Current data strongly suggest that the *Drosophila* endocycle is driven by the oscillations of Cyclin E/Cdk2 (Cdc2c – FlyBase) activity (reviewed by Edgar and Orr-Weaver, 2001; Lilly and Duronio, 2005). In *Drosophila*, Cyclin E/Cdk2 activity is required for DNA replication during both endocycles and mitotic cycles (Knoblich et al., 1994). While Cyclin E levels oscillate during endocycles, the continuous overexpression of Cyclin E results in a block of endoreplication (Follette et al., 1998; Weiss et al., 1998). These data are consistent with the model that endocycles require a Gap phase, when overall Cyclin E/Cdk2 activity is low, in order to relicense DNA replication origins for the subsequent S phase. Thus, the well-documented inhibitory effects of Cyclin/Cdks on pre-replication complex formation observed during the mitotic cycle (reviewed by Bell and Dutta, 2002) appear to be conserved during endocycles. An important factor controlling the periodicity of Cyclin E/Cdk2 activity is the regulated accumulation and destruction of Cyclin E protein, as well as the oscillations of the Cdk inhibitor Dacapo (Dap) (Doronkin et al., 2003; Hong et al., 2007; Moberg et al., 2001). A theoretical model, outlining the possible feedback relationships that might control the periodicity of Cyclin E/Cdk2 activity during *Drosophila* endocycles has been proposed (Edgar and Orr-Weaver, 2001). However, the precise mechanism driving endocycle progression and the accompanying oscillation of Cyclin E remains unclear.

The mitotic kinase Cdk1 (Cdc2 – FlyBase) drives entry into mitosis. In *Drosophila*, entry into the endocycle is contingent on the downregulation of Cdk1 activity (Edgar and Orr-Weaver, 2001; Lilly and Duronio, 2005). Mutations in either Cdk1 or the positive regulatory subunit Cyclin A, force cells undergoing mitotic cycles to enter the endocycle (Hayashi, 1996; Sauer et al., 1995; Weigmann et al., 1997). The mitotic cyclins Cyclin A, Cyclin B and Cyclin B3 are degraded by the highly conserved Anaphase Promoting Complex/Cyclosome (APC/C), an E3 ubiquitin ligase that targets proteins for destruction by the 26S proteasome (Sigrist et al., 1995; Dawson et al., 1995; Schaeffer et al., 2004; Sigrist and Lehner, 1997). Fizzy-related (Fzr; Rap – FlyBase) is a Cdh1-like positive regulatory subunit of the APC/C that promotes the degradation of the mitotic cyclins in G1 (Jacobs et al., 2002; Sigrist and Lehner, 1997; Schaeffer et al., 2004). In *fzr* loss-of-function mutants, cells fail to enter the endocycle (Sigrist and Lehner, 1997; Schaeffer et al., 2004). Additionally, when overexpressed, *fzr* downregulates the levels of Cyclin A, Cyclin B and Cyclin B3, and inhibits entry into mitosis (Schaeffer et al., 2004; Sigrist and Lehner, 1997). Consistent with a role in promoting entry into the endocycle, *fzr* is transcriptionally upregulated in the salivary gland, as well as the follicle cells of the ovary, at the time of the mitotic/endocycle switch

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(Shcherbata et al., 2004; Sigrist and Lehner, 1997). Taken together, these data indicate that an important step towards entering an endocycle is the APC/C$^{Fzr/Cdh1}$-dependent destruction of the mitotic cyclins.

However, although the requirement for the APC/C to enter the endocycle is well established, its role beyond the mitotic/endocycle transition remains unclear (Bentley et al., 2002; Edgar and Orr-Weaver, 2001; Kashevsky et al., 2002). Because of the requirement for APC/C activity to switch from the mitotic cycle to the endocycle, it has been difficult to assess whether the APC/C contributes to the cell cycle oscillator that drives endoreplication. Current models on the function of the APC/C during the endocycle are based on the analysis of hypomorphic alleles of APC/C subunits that do not allow the separation of these two potentially temporally independent functions (Bentley et al., 2002; Kashevsky et al., 2002; Schaeffer et al., 2004; Shcherbata et al., 2004). One of the few exceptions where the APC/C is known to function during the endocycle is in the polyploid nurse cells of the ovary (Kashevsky et al., 2002; Reed and Orr-Weaver, 1997). In endocycling nurse cells, a transient increase in mitotic activity in stage 5 egg chambers initiates a developmental alteration in chromatin structure and nuclear organization (Dej and Spradling, 1999; Kashevsky et al., 2002; Reed and Orr-Weaver, 1997). In mutants that compromise APC/C activity, this transient increase becomes permanent and the nurse cells arrest in a metaphase-like state with high levels of mitotic cyclins (Reed and Orr-Weaver, 1997). These data support a model in which the APC/C restrains nurse cells from fully entering the mitotic cycle after the brief burst of mitotic activity in the fifth endocycle (Kashevsky et al., 2002; Reed and Orr-Weaver, 1997). However, because the effect of compromising the APC/C in nurse cells is restricted to a specific developmental stage and cell type, it has been difficult to assess whether these results reflect a general requirement for the APC/C in endoreplication. Thus, the precise importance of the APC/C in promoting cell cycle progression during the endocycle remains to be determined.

Here, we demonstrate that APC/C$^{Fzr/Cdh1}$ activity is required to promote the G/S oscillation of the *Drosophila* endocycle. We show, using several strategies, that compromising APC/C$^{Fzr/Cdh1}$ activity after cells enter the endocycle results in the accumulation APC/C targets and a block to endoreplication. Moreover, our data support a model in which oscillations of APC/C^{Fzr} activity during the endocycle are driven by the periodic expression of Cyclin E.

MATERIALS AND METHODS

Fly stocks

hs-FLP^{1} w^{1118}; Adv^{1}/CyO, *hs-FLP^{1} y^{1} w^{1118}; Dr^{Mio}/TM3 ry* Sb^{1}*, *yw; Act>y+>Gal4 UAS-GFP/CyO*, *y^{1}w*; UAS-nls-GFP*, *y^{1}w*; act>y+>Gal4/TM6 Tb* (Pignoni and Zipursky, 1997), *morula^{1}/SM6a* and *morula^{2}/SM6a* were obtained from the Bloomington Stock Center. The *Act>Cd2.f^{+}>Gal4, hs-flp;; UAS-lacZ/TM6 Tb* (Hazelett et al., 1998) and *hs-flp;; UAS-GFP, Act>Cd2>Gal4/TM3 Sb* (Shcherbata et al., 2004) were gifts from Jessica Treisman and Hannele Ruohola-Baker, respectively. Christian Lehner kindly provided the following overexpression lines: *UAS-CycE III*, *UAS-Fzr II* and *UAS-Fzy II* (Sigrist and Lehner, 1997). *hs-Rca1* lines (Dong et al., 1997) were provided by Barbara Thomas. The *UAS-Geminin* line used was *UAS-Geminin III* (Quinn et al., 2001).

Generation of transgenic flies

The *RNAi* constructs against *geminin* and the individual APC/C subunits *Apc6*, *Apc8* and *Apc10* were cloned in the pWIZ vector, (Lee and Carthew, 2003). The *pWIZ* vector is under the control of a GAL4-sensitive *UAS enhancer*, thus allowing the conditional or tissue specific silencing of target genes. The constructions of the *Apc6^{RNAi}* and *Apc8^{RNAi}* lines have been described previously (Pal et al., 2007). To generate the *UAS-Apc10^{RNAi}* construct, a 443 bp region was amplified by PCR using sequences corresponding to exon 2 of the *Apc10* transcript. The PCR product was cloned in opposite orientations on both sides of the white intron present in the vector (Lee and Carthew, 2003). To generate the UAS-*geminin*^{RNAi} construct, a region of 500 bp of geminin cDNA was amplified by PCR and then two copies of this sequence were cloned in opposite orientations into the *pWIZ* vector. Two transgenic lines were generated, M4-*geminin*^{RNAi} and M6-*geminin*^{RNAi}, of which M4 was shown to result in a more complete knockdown of Geminin protein (data not shown). In this study, we used the M4-*geminin*^{RNAi} line. To generate the *UASp-Rca1* construct, the 1.3 kb *Bst*BI-*Ase*I cDNA fragment containing the entire *rca1*-coding region and poly(A) signal provided by the α-tubulin 3′-untranslated region (UTR) were inserted into the pUASP(–) vector for P-element transformation. pUASP(–) is a derivative of pUASP vector (Rorth, 1998) and lacks 1.3 kb *K10* 3′-UTR. Construction of the transgene of *Orc1-GFP* fusion driven by 2.4 kb *orc1* promoter has been described previously (Araki et al., 2003). The stable Orc1 derivative transgene was prepared by the alanine substitution of L295 and N299 in the O-box that mediates Fzr-specific APC/C degradation (Araki et al., 2005).

Generation of flip-out clones

Somatic overexpression was achieved by generating Flip-out/Gal4 clones (Pignoni and Zipursky, 1997) in *hs-flp/+; Act>CD2>Gal4, UAS-GFP/+; UAS-x/+* larvae or females (x denotes *Apc6^{RNAi}*, *Apc8^{RNAi}*, *Apc10^{RNAi}*, *Geminin^{RNAi}*, *Geminin*, *CycE*, *fzr* or *fzy*. Clones were induced by heat-shocking larvae or females at 37°C for 15 minutes. Subsequently, early L3 stage larvae were dissected 3 days later and adult females were dissected 1 day later. Using this strategy, we were able to induce clones in cells after they had entered the endocycle. We verified that spontaneous clones are rare (3.1% of non heat-shocked ovarioles have one follicular clone, *n*=221 ovarioles). Clones expressing Gal4 were induced by 'flipping out' an interruption cassette Act>CD2>Gal4 transgene in a genetic background that contained the UAS-x constructs, as well as a UAS-GFP or UAS-*lacZ* transgene. Thus, the co-expressing GFP or β-galactosidase marks cells that express the UAS-x constructs. For the hs-Rca1 experiment in the nurse cells, females were heat-shocked at 37°C for 1 hour and dissected 1 day later.

Immunocytochemistry

Immunocytochemistry of adult ovary staining was performed as described previously (McKearin and Ohlstein, 1995). The following antibodies were used in this study: mouse monoclonal α-GFP 1:200 (Roche), rabbit polyclonal α-GFP 1:500 (Molecular Probes), mouse monoclonal α-β-Galactosidase 1:500 (Promega), mouse monoclonal α-Cyclin A A12 1:100 (Sigrist et al., 1995), mouse monoclonal α-Cyclin B F2F4 1:50 (Lehner and O'Farrell, 1990), rat polyclonal α-Geminin 1:1000 (Quinn et al., 2001) and purified rat polyclonal α-Orc1 1:30 (Asano and Wharton, 1999). Rabbit polyclonal α-Cyclin A antibody 1:50 (Lehner and O'Farrell, 1989) was a gift from Christian Lehner. α-Geminin staining was performed using two independent antibodies (Calvi et al., 1998; Quinn et al., 2001). Both α-Geminin antibodies gave similar staining patterns in both the embryo and the ovary (data not shown). Fluorescence-conjugated secondary antibodies were purchased from Molecular Probes and were used at a 1:1000 dilution. All samples were mounted in cytifluor (Kent). Samples were examined with an Olympus Fluoview FV1000 microscope and composite figures were prepared using Adobe Photoshop 7.0.

Semi-quantitative RT-PCR

The semi-quantitative RT-PCR was carried out according to Le and Richardson, 2004 (Le and Richardson, 2004). Salivary glands from *hs-flp/+; Act>CD2>Gal4, UAS-GFP/+; UAS-CycE/+* and *hs-flp/+; Act>CD2>Gal4, UAS-GFP/UAS-Apc10^{RNAi}; UAS-Apc8^{RNAi}/+* larvae were heat-shocked at 37°C for 1 hour twice to ensure that a large number of cells overexpress the *CycE* or *Apc^{RNAi}* constructs. Total RNA was extracted from 4- to 8-hour embryos and third instar larval salivary glands using Trizol reagent (Invitrogen) according to the manufacturer's protocol. Reverse transcription-polymerase chain reaction (RT-PCR) was performed using the SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol to produce the cDNA (first strand). Each cDNA was primed by random hexamer (Gibco). The PCR was performed according to

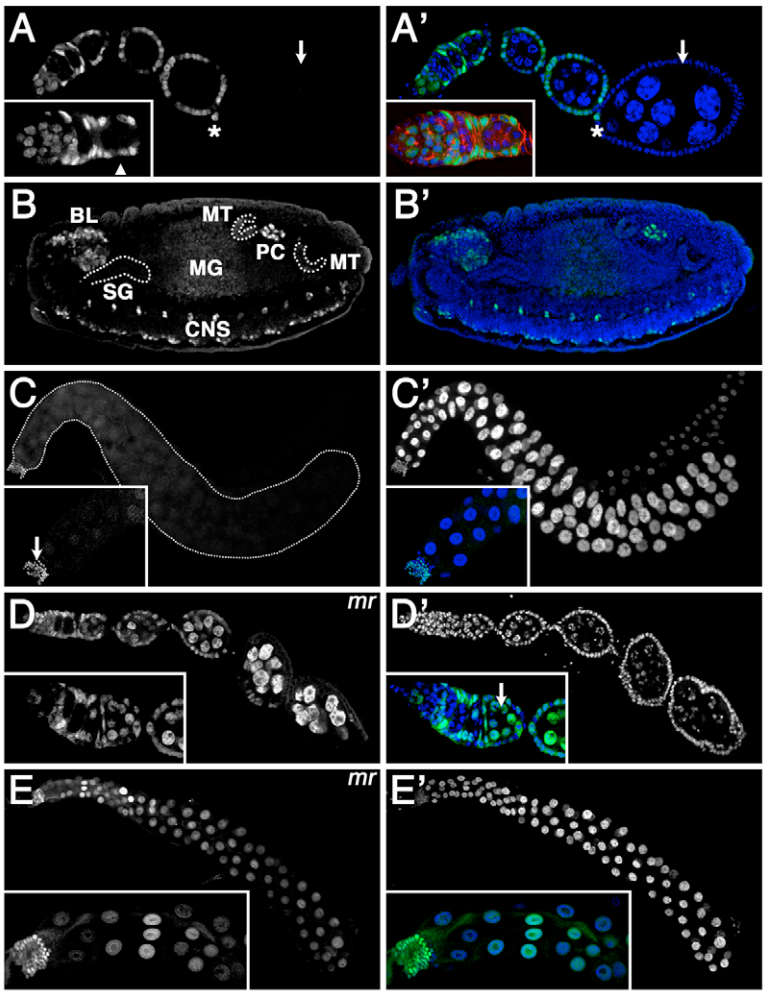


Fig. 1. Geminin levels are low during endocycles. Wild-type (A,A′) ovariole, (B,B′) stage 13 *Drosophila* embryo and (C,C′) larval salivary glands stained with (A,B,C, white; A′,B′,C′ inset, green) α-Geminin and (A′, inset, red) α-α-Spectrin antibodies, and (A′,B′,C′ inset, blue; C′, white) DAPI. (**A**,**A′**) Geminin is expressed in mitotic follicle cells of the ovary (stage 1-6), but is absent from endocycling cells after stage 6 (arrow), with the exception of the polar cells (asterisk). Geminin levels are downregulated when germline nurse cells enter into the endocycle in region 3 of the germarium (localized by the α-Spectrin antibody staining; A′, inset). (**B**,**B′**) During embryogenesis, Geminin expression is shown in mitotic cells, but is absent from endocycling cells. (**C**,**C′**) Geminin staining is also absent in the larval salivary gland, except for the imaginal ring of the salivary gland (insert, arrow), which contains diploid adult precursor cells. mr^1/mr^2 mutant (**D**,**D′**) ovariole and (**E**,**E′**) larval salivary gland, labeled with (D,E, white; D′,E′, insets, green) α-Geminin antibody and (D′,E′, white; D′,E′, insets, blue) DAPI. Geminin is ectopically expressed in *mr* mutant nurse cells and in salivary glands cells.

standard methods by using a PTC-200 Peltier thermal cycler. RT-PCR amplicons were in the linear phase of amplification. Expression of the *ribosomal protein 49* (*rp49*; *RpL32* – FlyBase) gene was used for normalization. The bands were quantified using Quantity One software (BioRad).

Primers used for RT-PCR and cloning

The primers used in this study were: cycA F, 5′-TGCGAGCGTAGCCAAACAAG-3′; cycA R, 5′-TCTTTCTCTTAGCGTCGTTG-3′; geminin F, 5′-CAAGCAAGTAGCACACGCTC-3′; geminin R, 5′-TCAGCATTGCCAAGCGGAAC-3′; orc1 F, 5′-CAGACTGCCTGAACTACTCC-3′; orc1 R, 5′-TCTTTGGCGAATAGTCCTCG-3′; rp49 F, 5′-TACAGGCCCAAGATCGTGAA-3′; rp49 R, 5′-ACGTTGTGCACCAGGAACTT-3′; Apc10, 5′-ACTCTAGAGTGTAGAGCGTTTGCGAGAC-3′; Apc10, 5′- ACTCTAGACTAACGAATGGTGGCGAACT-3′.

RESULTS

Geminin is downregulated at the mitotic/endocycle transition

In mammals, entry into the endocycle is temporally coincident with a dramatic decrease in the levels of the APC/C target Geminin (Bermejo et al., 2002; Gonzalez et al., 2006). In *Drosophila*, Geminin was reported to be present in some endocycling cell types (Quinn et al., 2001). However, upon re-examination we found that, as is observed in mammals, Geminin levels are dramatically downregulated upon entry into the endocycle. In the follicle cells of the ovary, Geminin levels are reduced, coincident with entry into the first endocycle in stage 6 of oogenesis (Fig. 1A,A′, arrow). Additionally, Geminin levels are low as the nurse cells enter the endocycle in region 3 of the germarium (Fig. 1A,A′, inset, arrowhead). Consistent with what is observed in the ovary, during embryogenesis Geminin levels are dramatically downregulated as cells enter the endocycle in multiple cell types. Geminin is present at high levels early in embryogenesis during the syncytial divisions and cellularization, irrespective of cell cycle stage (Quinn et al., 2001) (see Fig. S1A,A′ in the supplementary material). Thereafter, Geminin is expressed in mitotically dividing cells, including the central nervous system cells (CNS) and brain lobe cells (BL) (Fig. 1B,B′) (Quinn et al., 2001). By contrast, Geminin levels are low in the population of cells that have entered the endocycle, including the embryonic salivary gland (SG), the midgut (MG), the hindgut (HG) and the Malpighian tubules (MT) (Fig. 1B,B′). Geminin remains low throughout the process of endoreplication, with low levels of the protein observed in the endocycling cells of the larval salivary glands (Fig. 1C,C′) and Malpighian tubules (see Fig. S1B,B′ in the supplementary material). Thus, as is observed in mammalian trophoblasts, in *Drosophila* the levels of Geminin are downregulated at the mitotic/endocycle transition.

The downregulation of Geminin observed upon entry into the endocycle mirrors the behavior of the APC/C targets Cyclin A and Cyclin B (Lehner and O'Farrell, 1989; Lilly and Spradling, 1996; Reed and Orr-Weaver, 1997; Sauer et al., 1995). *morula* (*mr*) encodes the APC/C subunit APC2 (Kashevsky et al., 2002). In *mr*/*Apc2* hypomorphic female-sterile mutants, nurse cells proceed through the first four rounds of endocycle in a manner

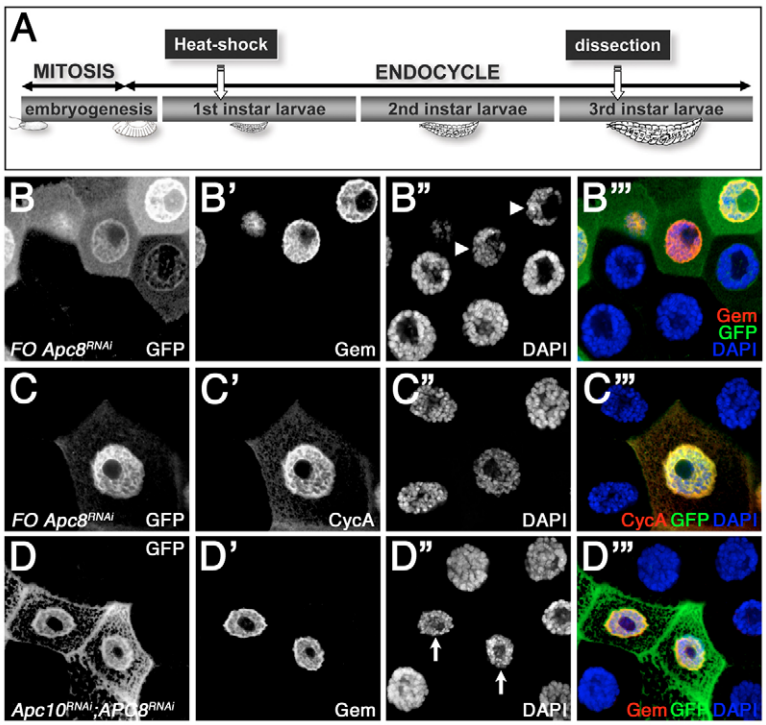


Fig. 2. APC/C$^{Fzr/Cdh1}$ functions during endocycles. (**A**) Schematic representation of Flipout/Gal4 clones induction in the *Drosophila* salivary gland. Clones are induced during the first larval instar, after the cells have entered the endocycle (Smith and Orr-Weaver, 1991). The Flipout/Gal4 technique was used to clonally express *UAS-Apc*^{RNAi} constructs with *GFP*. (**B-D‴**) salivary gland containing (B-B‴ and C-C‴) *Apc8*^{RNAi} or (D-D‴) *Apc8*^{RNAi}*; Apc10*^{RNAi} Flipout/Gal4 clones stained with (B,C,D, white; B‴,C‴,D‴, green) α-GFP, (B′,D′, white; B‴, D‴, red) α-Geminin and (C′, white; C‴, red) α-Cyclin A antibodies and (B″,C″,D″, white; B‴ ,C‴,D‴, blue) DAPI. (B′,D′) Geminin and (C′) Cyclin A are ectopically expressed in cells that express the *Apc8*^{RNAi} or the *Apc8*^{RNAi}*; Apc10*^{RNAi} constructs. Expression of *Apc8*^{RNAi}, as well as the co-expression of *Apc8*^{RNAi} and *Apc10*^{RNAi}, results in a decrease in the nuclear size (B″, arrowheads; D″, arrows).

indistinguishable from wild type, but in stage 5 of oogenesis, the nurse cells condense and arrest in a metaphase-like state (Reed and Orr-Weaver, 1997). This arrest is accompanied by the accumulation of the mitotic cyclins Cyclin A and Cyclin B (Kashevsky et al., 2002; Reed and Orr-Weaver, 1997; Sugimura and Lilly, 2006). We find that Geminin is also ectopically expressed in the polyploid nurse cells in *mr*^{1}/*mr*^{2} mutants (Fig. 1D,D′). Intriguingly, the increase in Geminin levels in *mr*/*Apc2* mutants is observed much earlier in oogenesis, in region 3 of the germarium, than the rise in the levels of the mitotic cyclins, which is not observed until stage 5 (Reed and Orr-Weaver, 1997) (Fig. 1D,D′, inserts, arrow). Additionally, Geminin levels are increased in the endocycling cells of the larval salivary gland in *mr*/*Apc2* mutant larvae (Fig. 1E,E′). Thus, as is observed with the mitotic cyclins, the APC/C functions to keep Geminin levels low during *Drosophila* endocycles.

APC/C$^{Fzr/Cdh1}$ functions beyond the mitotic/endocycle transition

The increase in the levels of the APC/C target Cyclin B in the polyploid nurse cells of *mr*/*Apc2* female-sterile mutants, first suggested that the APC/C functions during *Drosophila* endocycles (Kashevsky et al., 2002; Reed and Orr-Weaver, 1997). However, it was unclear whether this requirement was cell type specific, only being necessary to progress through the unusual pseudomitotic-like fifth endocycle that occurs in nurse cells (Kashevsky et al., 2002) or whether APC/C activity was generally required for endocycle progression. To examine this issue, we used an inducible RNAi system to knockdown the levels of several APC/C subunits after cells had successfully entered the endocycle. This strategy, which uses the inducible hs-FLP/Gal4 (Flipout/Gal4) system (Pignoni and Zipursky, 1997), allows us to examine the role of the APC/C during endocycles, independent of the requirement for APC/C activity to transit the mitotic/endocycle boundary. To examine the role of the APC/C during endocycles in the salivary gland, RNAi constructs are induced during the L1 larval stage, after cells have initiated endocycles (Smith et al., 1993) (Fig. 2A). In our studies we used RNAi constructs against Apc6/Cdc16 and Apc8 (Pal et al., 2007) and Apc10. Apc6 and Apc8 are tetratrico peptide repeat (TPR) subunits, which, in humans, are part of the core APC/C complex (Vodermaier et al., 2003). In budding yeast, Apc10/Doc1 is required for the processivity of APC/C-mediated ubiquitination reactions (Carroll and Morgan, 2002) and for substrate binding (Passmore et al., 2003).

When expressed in the salivary gland after cells have entered the endocycle, all three *Apc*^{RNAi} constructs result in the accumulation of the APC/C targets Geminin, Cyclin A and Cyclin B (Fig. 2B-B‴, 2C-C‴; see Fig. S2 and Table S1 in the supplementary material). Additionally, although no abrogation of the endocycle was associated with the expression of *Apc6*^{RNAi} or *Apc10*^{RNAi} (Fig. S2 in the supplementary material), we observe a notable decrease in the size of salivary gland nuclei, as measured by DAPI staining, in a proportion of cells (20.6%, *n*=218) that express the *Apc8*^{RNAi} construct (Fig. 2B″, arrowheads). Moreover, the co-expression of the two *Apc*^{RNAi} constructs, *Apc8*^{RNAi} and *Apc10*^{RNAi}, results in a dramatic decrease in nuclear size in a large fraction (42.9%, *n*=291) of salivary gland nuclei from early third instar larvae (Fig. 2D-D″, arrows). Consistent with the reduced ploidy values observed with the expression of *Apc8*^{RNAi}, we find that the APC/C targets Cyclin A and Geminin accumulate to higher levels after the expression of *Apc8*^{RNAi} relative to what is observed after the expression of *Apc6*^{RNAi} or *Apc10*^{RNAi} (see Table S1 in the supplementary material). Thus, the minimal effect on DNA content observed after *Apc6*^{RNAi} and *Apc10*^{RNAi} expression correlates with reduced APC/C target accumulation. There are several possible explanations for why *Apc8*^{RNAi} shows a more robust response. First, this may reflect the reduced on target efficiency of the *Apc6*^{RNAi} and *Apc10*^{RNAi} constructs relative to the *Apc8*^{RNAi}. Alternatively, the Apc6 and Apc10 proteins may have increased stability relative to the Apc8 protein when assembled in the large multiprotein APC/C complex (Davis et al., 2002; Shakes et al., 2003). These data support the model that the APC/C functions beyond the mitotic/endocycle transition to promote endoreplication.

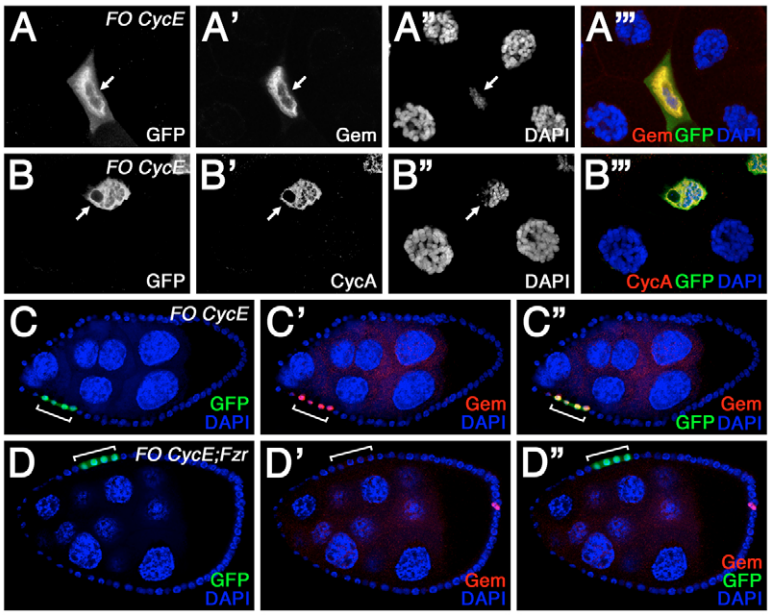


Fig. 3. Cyclin E overexpression during the endocycle results in the unscheduled accumulation of APC/C targets. The Flipout/Gal4 technique was used to clonally overexpress Cyclin E and Fizzy-related with GFP. (**A-B‴**) *Drosophila* salivary glands and (**C-D″**) egg chambers containing (A-C″) *UAS-Cyclin E* and (D-D″) *UAS-Cyclin E; UAS-Fzr* Flipout clones labeled with (A,B, white; A‴,B‴,C,C″,D,D″, green) α-GFP, (A′, white; A‴,C′,C″,D′,D″, red) α-Geminin and (B′, white; B‴, red) α-Cyclin A antibodies and (A″,B″, white; A‴,B‴,C-C″,D-D″, blue) DAPI. (A′,C′) Geminin and (B′) Cyclin A are ectopically expressed in cells that overexpress Cyclin E, both in the salivary gland (arrows) and in follicle cells of the ovary (brackets). (D-D″) In follicle cells, the co-expression of Fzr/Cdh1 with Cyclin E inhibits the expression of Geminin (brackets).

In somatic tissues, the APC/C has two potential activators: Fizzy/Cdc20 (Fzy/Cdc20) and Fizzy-related/Cdh1 (Fzr/Cdh1) (Dawson et al., 1995; Sigrist et al., 1995; Sigrist and Lehner, 1997). In *Drosophila*, Fzr/Cdh1 but not Fzy/Cdc20, is required for cells to enter the endocycle (Sigrist and Lehner, 1997). Previous works indicate that APC/C$^{Fzr/Cdh1}$ activity is downregulated by Cyclin E/Cdk2 (Sigrist and Lehner, 1997). We find that in both the salivary gland and follicle cells of the ovary, the overexpression of Cyclin E after cells have entered the endocycle results in the accumulation of the APC/C targets Geminin (Fig. 3A-A‴,C-C″), Cyclin A (Fig. 3B-B″) and Cyclin B (data not shown). Consistent with the model that the accumulation of these APC/C targets is due to the Cyclin E-dependent inhibition of Fzr/Cdh1, the co-expression of Fzr/Cdh1 with Cyclin E prevents the accumulation of these APC/C targets during follicle cell endocycles (Fig. 3D-D″). By contrast, the co-expression of Fzy/Cdc20 with Cyclin E has no effect on preventing the Cyclin E-induced accumulation of the APC/C targets Cyclin A and Geminin in follicle cells (data not shown). In the larval salivary gland, the co-expression of Fzr/Cdh1 with Cyclin E did not inhibit the expression of APC/C targets (data not shown). This may reflect the different time frame over which endoreplication occurs in the follicle cells versus salivary glands. Although most follicle cells undergo only three endocycles to attain ploidy values of 16C, many of the cells in the salivary gland undergo at least 10 endocycles to achieve ploidy values of greater than 2000C (Hammond and Laird, 1985a; Hammond and Laird, 1985b; Lilly and Spradling, 1996). Cyclin E/Cdk2 acts catalytically to inhibit Fzr/Cdh1, whereas the ability of Fzr/Cdh1 to activate the APC/C is achieved stoichiometrically (Passmore and Barford, 2005). Therefore, we reasoned that the ability of Fzr to overcome inhibition by Cyclin E/Cdk2 activity might diminish as Cyclin E levels increase over time. Thus, the accumulation of APC/C targets in *UAS-Cyclin E; UAS-Fzr* salivary glands may reflect the inability of the exogenously supplied Fzr/Cdh1 to overcome the inhibitor effects of Cyclin E in this time frame.

To examine more directly the effects of inhibiting Fzr/Cdh1 during endocycles, we expressed the Fzr/Cdh1 inhibitor Regulator of Cyclin A 1 (Rca1) (Dong et al., 1997; Grosskortenhaus and Sprenger, 2002). In *rca1* mutants, mitotic cells are rushed into an endocycle, presumably because of unrestrained APC/C$^{Fzr/Cdh1}$ activity, which prevents the accumulation of the mitotic cyclins and progression into mitosis (Dong et al., 1997; Grosskortenhaus and Sprenger, 2002). In both the salivary gland and the follicle cells the expression of *Rca1* after cells have entered the endocycle, using the FLPout/Gal4 technique, results in the accumulation of APC/C targets (Fig. 4A-A″,B-B″,C-C″). Additionally, using a transgenic line that contains *Rca1* under the control of the inducible heat-shock promoter, we find that the induction of *Rca1* expression results in the accumulation of APC/C targets in the endocycling nurse cells (Fig. 4D-D″). The observation that expressing an inhibitor of the APC/C$^{Fzr/Cdh1}$ triggers the accumulation of APC/C targets in multiple polyploid cell types further supports the model that APC/C$^{Fzr/Cdh1}$ functions beyond the mitotic/endocycle transition.

Geminin is not the only essential downstream target of the APC/C during endocycles

In *Drosophila*, the overexpression of Cyclin E results in a block to S phase during endocycles but not mitotic cycles (Follette et al., 1998; Weiss et al., 1998). As described above, we find that the overexpression of Cyclin E in the endocycling cells of the salivary gland, results in the accumulation of APC/C targets, including the Cdt1/Dup inhibitor Geminin (Fig. 3A-A‴,C-C″). Because of its ability to inhibit the activity of the licensing factor Cdt1/Dup, the overexpression of Geminin blocks DNA replication (Fig. 5A-A‴) (McGarry and Kirschner, 1998; Quinn et al., 2001). Therefore, we reasoned that the block to endoreplication observed upon the overexpression of Cyclin E might be due to the unscheduled accumulation of Geminin. In order to test this hypothesis, we co-expressed a *UAS-Geminin^{RNAi}* construct with *UAS-Cyclin E* using the Flipout/Gal4 system (*hs-flp/+; UAS-Geminin^{RNAi}/+; Act>CD2>Gal4, UAS-GFP/UAS-CycE* larvae). Whereas the overexpression of Cyclin E results in accumulation of Geminin in the cells of the salivary gland, the co-expression of *Geminin^{RNAi}* with Cyclin E reduced Geminin below the level of detection (Fig. 5B′). However, preventing the accumulation of Geminin did not rescue the block to DNA replication associated with the constitutive expression of Cyclin E [compare the wild-type nucleus (arrowhead) to the *UAS-Geminin^{RNAi}; UAS-CycE* nucleus (arrow) in Fig. 5B″]. This may reflect the ability of high Cyclin E/Cdk2 activity to inhibit

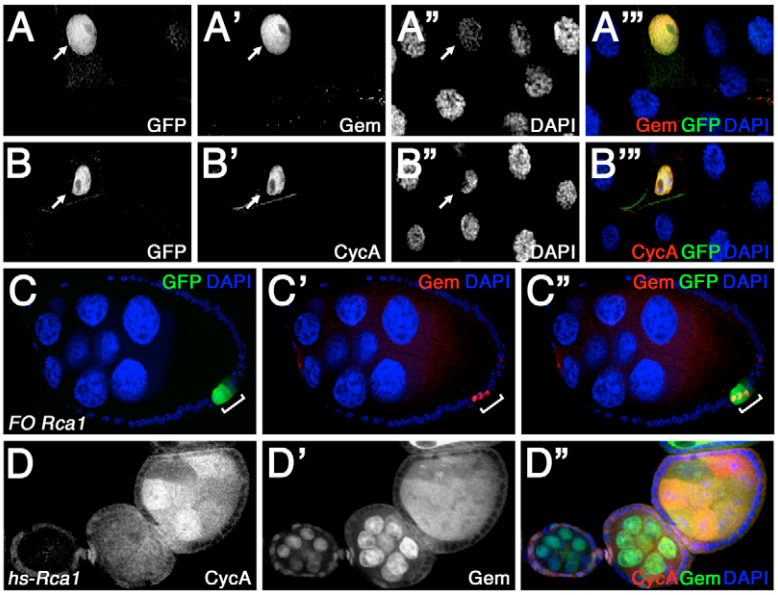


Fig. 4. Expression of the Fzr/Cdh1 inhibitor Rca1 during the endocycle. Rca1 was ectopically expressed using either (A-C) the Flipout/Gal4 technique or (D) a *hs-Rca1* construct. (**A-B‴**) *Drosophila* salivary glands and (**C-C″**) egg chamber containing *UAS-Rca1* Flipout clones stained with (A,B, white; A‴,B‴,C,C‴, green) α-GFP, (A′, white; A‴,C′,C″, red) α-Geminin and (B′, white; B‴, red) α-Cyclin A antibodies and (A″,B″, white; A‴, B‴,C,C′,C″, blue) DAPI. (**D-D″**) Egg chambers expressing a *hs-Rca1* construct labeled with (D, white; D″, red) α-Cyclin A and (D′, white; D″, green) α-Geminin antibodies and (D″, blue) DAPI. Induction of Rca1 expression leads to the accumulation of APC/C targets, in the (A,B) salivary gland (arrows), in the (C) follicle cells (brackets) and in (D) the nurse cells.

DNA replication origins directly during the endocycle and/or the presence of additional important APC/C targets that must be destroyed in order to facilitate endocycle progression.

We wanted to examine further the idea that the APC/C has additional critical targets, apart from Geminin, during endocycles. To explore this possibility, we co-expressed $Geminin^{RNAi}$ and the Apc^{RNAi} constructs $Apc8^{RNAi}$ and $Apc10^{RNAi}$ (*Act>CD2,f⁺>Gal4, hs-flp/+: UAS-Apc10*^{RNAi}*/UAS-Geminin*^{RNAi}*; UAS-lacZ/UAS-Apc8*^{RNAi}) in the larval salivary gland. If Geminin were the only essential downstream target of the APC/C during endocycles, we would predict that preventing the accumulation of Geminin should rescue the Apc^{RNAi} endoreplication defects. However, although the $Geminin^{RNAi}$ expression reduced the Geminin protein below the level of detection in the $Apc8^{RNAi}$; $Apc10^{RNAi}$ background (Fig. 5C′), the depletion did not rescue the associated endoreplication defects (Fig. 5C-C‴). We have previously observed that overexpressing $Apc6^{RNAi}$ or $Apc10^{RNAi}$ in the salivary gland often leads to low levels of Geminin expression that are not associated with a corresponding decrease in DNA content. Thus, we believe that the inability of $Geminin^{RNAi}$ to rescue the endoreplication defects associated with compromising the APC/C is not due to the residual expression of the Geminin protein. Taken together, our data support the model that Geminin is not the only essential target of APC/C during endocycles. As outlined in the discussion in detail, other potential important targets of the APC/C during endoreplication are Cyclin A and String/Cdc25 (Weiss et al., 1998).

APC/C$^{Fzr/Cdh1}$ drives the oscillation of Orc1 during the endocycle

Origin Recognition Complex 1 (Orc1) is a highly conserved component of the pre-replication complex (pre-RC) (Dutta and Bell, 1997). The levels of the Orc1 protein oscillate during the nurse cell and follicle cell endocycles (Asano and Wharton, 1999). Consistent with these earlier observations, we find that Orc1 levels oscillate during the endocycle in larval salivary glands (Fig. 6F). Intriguingly, the constitutive transcription of an Orc1 transgene from a heterologous promoter does not notable diminish the kinetics of Orc1 protein oscillations during endocycles (Araki et al., 2005). Moreover, Orc1 oscillations during the endocycle do not require the native Orc1 3′ or 5′ UTR, and therefore are unlikely to be controlled at the level of translation (Araki et al., 2005). In *Drosophila*, Orc1 is a target of APC/C$^{Fzr/Cdh1}$ (Araki et al., 2003; Araki et al., 2005). We find that compromising APC/C activity in salivary gland cells using either the Apc^{RNAi} strategy as outlined in Fig. 2A (Fig. 6A-A″) or by

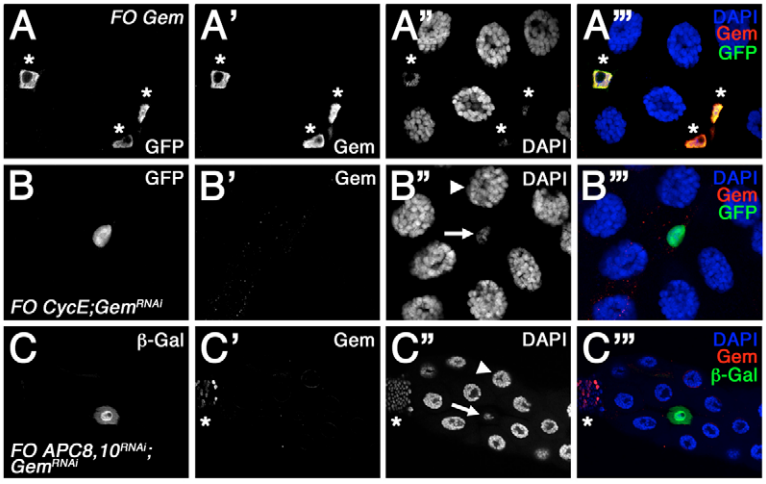
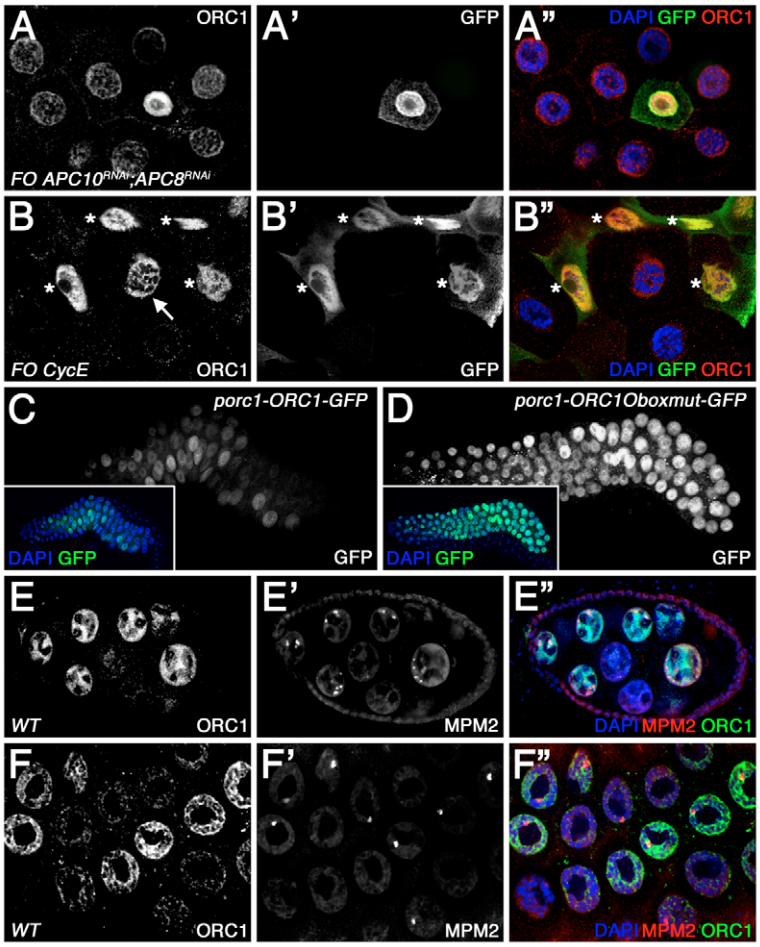


Fig. 5. Co-expressing a $Geminin^{RNAi}$ construct does not rescue the *Cyclin E* or Apc^{RNAi} overexpression phenotypes. The Flipout/Gal4 technique was used to clonally overexpress Geminin, Cyclin E Apc10^{RNAi}; Apc8^{RNAi} and Geminin^{RNAi} with GFP. *Drosophila* salivary glands containing (**A-A‴**) *UAS-Geminin*, (**B-B‴**) *UAS-Cyclin E*; *UAS-Geminin*^{RNAi} and (**C-C‴**) *UAS-Apc10*^{RNAi}*/UAS-Geminin*^{RNAi}*; UAS-Apc8*^{RNAi} Flip-out clones labeled with (A,B, white; A‴,B‴, green) α-GFP, (C, white; C‴ green) α-β-Gal and (A′,B′,C′, white; A‴,B‴,C‴, red) α-Geminin antibodies, and (A″,B″,C″, white; A‴, B‴,C‴ blue) DAPI. (A-A‴) The overexpression of Geminin, in cells marked by an asterisk, blocks DNA replication. (B-B‴,C-C‴) Co-expression of Geminin^{RNAi} does not rescue the block of DNA replication associated with overexpression of Cyclin E or Apc10^{RNAi}; Apc8^{RNAi} [compare the DNA content in flip-out clones (arrows) with the DNA content in wild-type cells (arrowheads)]. The mitotic cells of the imaginal ring cyclically express Geminin (asterisk).



Fig, 6. Orc1 is a target of the APC/C$^{Fzr/Cdh1}$ during the endocycle. *Drosophila* salivary glands containing (**A-A″**) *UAS-Apc10^{RNAi}; UAS-Apc8^{RNAi}* and (**B-B″**) *UAS-Cyclin E* Flip-out clones labeled with (A,B, white; A″,B″, red) α-Orc1 and (A′,B′, white; A″,B″, green) α-GFP antibodies, and (A″,B″, blue) DAPI. Orc1 is ectopically expressed in cells that express the *UAS-Apc10^{RNAi}; UAS-Apc8^{RNAi}* or *UAS-Cyclin E* (marked by an asterisk) constructs in the salivary gland. The arrow indicates a wild-type cell that expresses high levels of Orc1 protein. (**C**,**D**) *porc1-Orc1-GFP* (C) and *porc1-Orc1Oboxmut-GFP* (D) salivary glands labeled with (white and green) α-GFP antibody and (blue) DAPI. Higher levels of Orc1 are observed when the O box is mutated. The pictures shown in C and D were taken using the same settings. Wild-type (**E-E″**) egg chambers and (**F-F″**) salivary gland stained with (E,F, white; E″,F″,green) α-Orc1 and (E′,F′, white; E″,F″, red) α-MPM2 antibodies, and (E″,F″, blue) DAPI. There is a correlation between the MPM2 spheres and the Orc1 staining.

overexpressing Cyclin E (Fig. 6B-B″) results in the increased accumulation of Orc1 protein. Indeed, 65% of the cells that express the *Apc10^{RNAi}*; *Apc8^{RNAi}* constructs have high levels of Orc1 (n=63, Fig. 6A), compared with 14% of wild-type nuclei (n=261, Fig. 6B, arrow).

One possible model to explain the oscillation of Orc1 protein during the endocycle is that APC/C$^{Fzr/Cdh1}$ activity is periodically inhibited by high levels of Cyclin E/Cdk2. In order to determine whether the accumulation of Orc1 is coincident with high Cyclin E/Cdk2 activity, we co-stained wild-type ovaries and salivary glands with α-Orc1 and α-MPM2 antibodies. Although traditionally used to follow mitotic phosphoepitopes, the α-MPM2 antibody has proven a useful marker for monitoring Cyclin E/Cdk2 activity in *Drosophila*, with the presence of one or more α-MPM2-positive subnuclear spheres, which have recently been shown to be histone bodies (White et al., 2007), correlating with high levels of Cyclin E/Cdk2 activity (Calvi et al., 1998; Royzman et al., 1999). We find that high levels of Orc1 strongly correlate with high levels of α-MPM2 histone body staining in the endocycling nurse cells (Fig. 6E-E″ and Table 1) and follicle cells (see Fig. S3B-B″ in the supplementary material and Table 1) of the ovary, as well as in the larval salivary gland (Fig. 6F-F″ and Table 1). These data support the model that Cyclin E/Cdk2 activity, and the subsequent inhibition of the APC/C$^{Fzr/Cdh1}$, may drive the periodic accumulation of Orc1 during wild-type endocycles.

In order to confirm that Orc1 is a direct target of the APC/C$^{Fzr/Cdh1}$ during the endocycle, we compared the distribution of an *Orc1-GFP* construct under the control of the native *Orc1* promoter (*pOrc1-Orc1-GFP*), to the distribution of a similarly designed *Orc1-GFP* transgene that carried a mutated O-box (Araki et al., 2005). The O-box is a novel APC/C targeting motif that is necessary and sufficient for directing the Fzr/Cdh1-dependent degradation of Orc1 (Araki et al., 2005). In endocycling salivary glands, nurse cells and follicle cells, *porc1-Orc1-GFP* levels oscillate in a manner similar to that observed with native Orc1 protein (Fig. 6C, compared with 6F). By contrast, *porc1-Orc1Oboxmut-GFP* protein levels are increased in

Table 1. MPM2 staining correlates with the levels of *porc1-Orc1-GFP* but not with the levels of *porc1-Orc1Oboxmut-GFP*

	Tissue	% MPM2$^+$/High Orc1	% MPM2$^+$/Medium Orc1	% MPM2$^+$/No Orc1	% MPM2$^+$	% Orc1	*n*
porc1-Orc1-GFP	NC	88.4	10.9	0	26.7	40.6	165
	FC	87.6	26.0	1.75	36.8	54.9	253
	SG	94.0	40.8	4.6	36.7	55.8	147
porc1-Orc1Oboxmut-GFP	NC	27.6	–	–	27.6	100	348
	FC	35.0	32.9	–	34.2	100	222
	NC	48.5	44.3	–	46.2	100	225

NC, nurse cells; FC, follicle cells; SG, salivary gland cells.

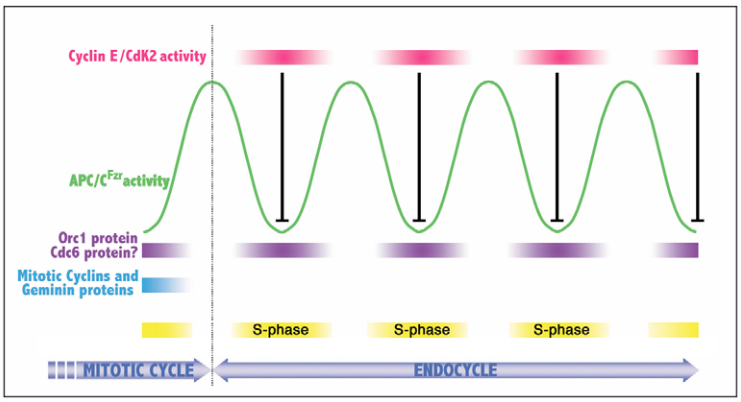


Fig. 7. A model for the regulation of the APC/C$^{Fzr/Cdh1}$ activity during endocycles in *Drosophila*. Our data indicate that APC/C$^{Fzr/Cdh1}$ activity is required for endocycle progression. During the endocycle, APC/C$^{Fzr/Cdh1}$ targets Geminin, Cyclin A, Cyclin B and Orc1, and possible additional proteins for proteolysis. We propose that APC/C$^{Fzr/Cdh1}$ activity is downregulated during the endocycle by the oscillating activity of the Cyclin E/Cdk2 complex. Oscillation of APC/C$^{Fzr/Cdh1}$ activity allows for the periodic accumulation of Orc1, a component of the pre-RC. See text for details. During the endocycle, staining for the mitotic cyclins and Geminin appears extremely low and consequently the proteins are not shown in this model. However, it is still possible that these proteins are present at low levels in some endocycling cell types.

salivary glands (Fig. 6D), nurse cells (Fig. S3A) and follicle cells (see Fig. S3C in the supplementary material). Specifically, in the nurse cells the levels of *porc1-Orc1Oboxmut-GFP* protein are uniformly high (see Fig. S3A in the supplementary material), while in the follicle cells and salivary glands there is some cell-to-cell variation in *porc1-Orc1Oboxmut-GFP* levels (see Fig. S3C in the supplementary material; Fig. 6D). Does this cell-to-cell variation indicate that cell cycle-dependent oscillations of Orc1 can occur independently of O-box-mediated destruction by APC/C^{Fzr}? In order to answer this question, we double-labeled ovaries and salivary glands with α-GFP and α-MPM2 antibodies. In the follicle cells, nurse cells and salivary gland cells, we find that *porc1-Orc1Oboxmut-GFP* levels do not correlate with α-MPM2 histone body staining (Table 1), indicating that cell cycle regulation of Orc1 levels is O-box dependent. Our data do not eliminate the possibility that there are additional inputs that influence the stability of the Orc1 protein during endocycles. However, taken together, our data strongly suggest that the oscillation of Orc1 during the endocycle is at least partially driven by the O-box-mediated destruction of the Orc1 protein by APC/C$^{Fzr/Cdh1}$.

It remains unclear why the levels of some APC/C targets, such as Cyclin A, Cyclin B and Geminin, stay below the level of detection during endocycles, whereas the levels of the Orc1 protein oscillate. We reasoned that one possible explanation for the different modes of behavior might be found at the level of transcription. Previous reports suggest that transcript levels of the mitotic cyclins are reduced upon entry into the endocycle (Lehner and O'Farrell, 1989; Lehner and O'Farrell, 1990; Whitfield et al., 1989). We used semi-quantitative RT-PCR to compared the transcript levels for *Cyclin A*, *geminin* and *Orc1*, in 4- to 8-hour-old embryos (primarily cells in the mitotic cycle) with the levels of these transcripts in salivary glands from early third instar larvae (primarily cells in the endocycle). Consistent with previous reports, we find that the levels of *Cyclin A* transcript are reduced almost sevenfold in endocycling versus mitotic cells, relative to the levels of the *ribosomal protein 49* (*rp49*), while the levels of *geminin* transcript are reduced approximately threefold (see Fig. S4 in the supplementary material). By contrast, the levels of the *Orc1* transcript are only slightly downregulated. Intriguingly, when a large number of heat shocks are performed to increase the number of cells that express either the *Apc^{RNAi}* constructs or *CycE* in endocycling cells of the larval salivary gland, we observe a modest increase in the transcript levels of *Cyclin A* and *geminin* (see Fig. S5 in the supplementary material). Thus, these data suggest that, perhaps as is observed in mammals, the regulation of APC/C activity may drive a positive-feedback circuit that controls both protein stability and mRNA expression (Verschuren et al., 2007). However, as discussed in detail below, differences in transcript levels is just one of several possible explanations for the differential expression of APC/C targets during endocycles.

DISCUSSION

The endocycle provides a useful model for determining the minimum cell cycle inputs required to achieve a G/S oscillation and the once-per-cell-cycle replication of the genome. Here, we demonstrate that APC/C activity is required for endocycle progression. During the endocycle, mitotic activities are repressed (reviewed by Edgar and Orr-Weaver, 2001; Lilly and Duronio, 2005). This is accomplished, at least in part, by preventing the accumulation of the mitotic activators Cyclin A, Cyclin B and Cdc25, which function to activate the mitotic kinase Cdk1. During the mitotic cycle, the mitotic cyclins are periodically targeted for regulated proteolysis by the E3-Ubiquintin ligase the APC/C (Dawson et al., 1995; Sigrist et al., 1995; Sigrist and Lehner, 1997). Yet the transcriptional downregulation of several APC/C targets at the mitotic/endocycle boundary, including the mitotic cyclins and String/Cdc25, suggested that the proteolytic activity of the APC/C might not be necessary during endocycles (Lehner and O'Farrell, 1989; Lehner and O'Farrell, 1990; Whitfield et al., 1989). However, we find that compromising APC/C activity, after cells have entered the endocycle, results in the accumulation of Geminin and the mitotic cyclins, and in a block of DNA replication. Thus, the transcriptional downregulation of APC/C targets observed at the mitotic/endocycle transition is either downstream of APC/C activity and/or not sufficient to maintain low levels of these proteins. Taken together, our data suggest a model in which APC/C promotes the G/S oscillation of the endocycle by preventing the unscheduled accumulation of Geminin and the mitotic cyclins.

We find that during endocycles, APC/C activity prevents the inappropriate accumulation of Geminin, an inhibitor of the DNA replication-licensing factor Cdt1/Dup. When directly expressed in endocycling cells, Geminin efficiently inhibits DNA replication (Fig. 5) (Quinn et al., 2001). These results strongly suggest that an essential function of the APC/C during the endocycle is to prevent the unregulated accumulation of Geminin. A similar role has been proposed for the APC/C during endoreplicative cycles of mouse trophoblasts (Gonzalez et al., 2006). However, our data indicate that Geminin is not the only essential target of the APC/C during endocycles. A candidate for a second important target of the APC/C during endocycles is Cyclin A. Previous studies have shown that the overexpression of Cyclin A in the salivary gland, between the first and second endocycle, results in variable inhibitory effects on endoreplication (Weiss et al., 1998). Although the majority of salivary gland cells that overexpress Cyclin A appear to be

unaffected, a small percentage show a marked decrease in ploidy values. The reason for this variability is not clear (Weiss et al., 1998). However, if the inhibitory influence of Cyclin A is mediated through binding and activation of Cdk1, this effect may be greatly amplified in the presence of high levels of String/Cdc25, which removes an inhibitory phosphate from Cdk1 (Kumagai and Dunphy, 1991; Strausfeld et al., 1991). Recent studies indicate that String/Cdc25, which contains both a consensus Ken box and D-box, is a target of the APC/C (Donzelli et al., 2002; Tanaka-Matakatsu et al., 2007) (Barbara Thomas, personal communication). Therefore, an essential function of the APC/C during endocycles may involve restricting the activity of the mitotic kinase Cdk1, by preventing the accumulation of both Cyclin A and String/Cdc25. Finally, we note that the APC/C may have additional essential targets during the endocycle, which have yet to be identified.

The periodic accumulation of the Orc1 protein during endocycles strongly suggests that the activity of the APC/C$^{Fzr/Cdh1}$ may not be continuous but cyclical. Previous work indicates that in *Drosophila* Cyclin E/Cdk2 inhibits the activity of APC/C$^{Fzr/Cdh1}$ (Reber et al., 2006; Sigrist and Lehner, 1997). These data are consistent with the observation that phosphorylation of Fzr/Cdh1 by Cdks inhibits the ability of Fzr/Cdh1 to bind and activate the APC/C in yeast, *Xenopus* and mammals (Kramer et al., 2000; Zachariae et al., 1998). During the endocycle, the levels of Cyclin E oscillate (Follette et al., 1998; Weiss et al., 1998). Taken together, these observations suggest a model in which APC/C$^{Fzr/Cdh1}$ is regulated by the periodicity of Cyclin E/Cdk2 activity, with high levels of Cyclin E resulting in the inhibition of APC/C$^{Fzr/Cdh1}$ activity and low levels of Cyclin E permitting full APC/C$^{Fzr/Cdh1}$ activity (Fig. 7). Our data support this hypothesis. First, we find that the periodicity of Orc1 levels during the endocycle requires a functional O-box, consistent with the cyclic destruction of Orc1 by APC/C$^{Fzr/Cdh1}$ (Araki et al., 2003; Araki et al., 2005). Second, the levels of Orc1 are sensitive to Cyclin E. Specifically, overexpressing Cyclin E after cells have entered the endocycle results in the accumulation of APC/C$^{Fzr/Cdh1}$ targets, including Orc1, Cyclin A, Cyclin B and Geminin. Thus, the regulatory relationship observed between Cyclin E/Cdk2 and Fzr/Cdh1 that has been reported during mitotic cycles is conserved during endocycles (Vidwans et al., 2002). Finally, in endocycling cells the accumulation of Orc1 occurs during periods of high Cyclin E/Cdk2 activity, when APC/C$^{Fzr/Cdh1}$ dependent proteolysis would be predicted to be low. These data support the idea that the oscillations of Cyclin E/Cdk2 activity drive the periodicity of APC/C$^{Fzr/Cdh1}$ activity during the endocycle.

Although we note that we have not formally demonstrated a requirement for the oscillation of APC/C$^{Fzr/Cdh1}$ activity during the *Drosophila* endocycle, it is interesting to speculate on how the cyclic, rather than the continuous, activity of the APC/C might serve to facilitate endocycle progression. Our data indicate that a period of high APC/C$^{Fzr/Cdh1}$ activity is required during the G phase of the endocycle in order to degrade the mitotic cyclins and Geminin, which can function to inhibit the formation of pre-RCs. However, a period of low APC/C activity may also be important. The continuous activation of APC/C^{Cdh1} significantly slows DNA replication in mouse tissue culture cells (Sorensen et al., 2000). This inhibition may reflect the inability of a cell to accumulate adequate levels of proteins required for DNA replication, such as the APC/C^{Cdh1} target and pre-replication complex component CDC6, in the presence of a constitutively active APC/C^{Cdh1}. In *Drosophila*, continuous APC/C$^{Fzr/Cdh1}$ activity might prevent the accumulation of two pre-RC components, CDC6 and Orc1. Intriguingly, APC/C activity also appears to oscillate during mammalian endocycles. In endocycling mouse trophoblasts, the levels of Cyclin A oscillate, consistent with the regulated destruction of the Cyclin A protein by the APC/C (MacAuley et al., 1998). Additionally, the inhibition of APC/C activity in endocycling trophoblasts results in the accumulation of the APC/C targets Cyclin A and Geminin (Gonzalez et al., 2006). Taken together, these observations support a model in which the oscillation of APC/C$^{Fzr/Cdh1}$ activity, which is driven by the regulatory influences of Cdks, promotes efficient cell cycle progression during the endocycle.

Our data raises important questions. Why do levels of some APC/C$^{Fzr/Cdh1}$ targets, such as Cyclin A, Cyclin B and Geminin, remain below the level of detection while the levels of Orc1 protein oscillate? What might account for these different modes of regulation? Currently, there is no definitive explanation. However, we envisage at least three possibilities, which are not mutually exclusive, that may contribute to this differential behavior. First, we find that relative to the *Cyclin A* and *geminin*, the levels of *Orc1* transcript are only minimally downregulated upon entry into the endocycle (see Fig. S4 in the supplementary material). Transcriptional downregulation, or changes in transcript stability, may help contribute to the low levels of Geminin and Cyclin A proteins observed during the endocycle. Second, the translational efficiency of a subset of transcripts may be reduced upon entry into the endocycle. Finally, it is possible that the Orc1 protein is not as efficiently targeted by the APC/C$^{Fzr/Cdh1}$ as the mitotic cyclins or Geminin. Indeed the cis-acting sequences that target these proteins for destruction show considerable variability. Orc1 is targeted for APC/C$^{Fzr/Cdh1}$ destruction via a novel motif called the O-box (Araki et al., 2005). By contrast, Cyclin B and Geminin are targeted by a similar but unique sequence called the destruction-box (D-box), while *Drosophila* Cyclin A is targeted for destruction by a large complex N-terminal degradation sequence (Jacobs et al., 2001; McGarry and Kirschner, 1998). There is precedence for post-translational regulation of APC/C$^{Fzr/Cdh1}$ targets, resulting in differential expression. In mammalian cells the pre-RC component CDC6, which is structurally related to Orc1, is protected from APC/C$^{Fzr/Cdh1}$ degradation by phosphorylation by Cyclin E/Cdk2 (Laronne et al., 2003; Mailand and Diffley, 2005). One or all of these potential mechanisms may contribute to the differential expression of various APC/C$^{Fzr/Cdh1}$ targets during the endocycle.

Recent evidence from mice indicates that the depletion of the APC/C inhibitor Emi1/Rca1, results in both a strong decrease in E2F target mRNAs, such as *geminin* and *Cyclin A*, as well as APC/C activation (Verschuren et al., 2007). The authors suggest that the regulation of APC/C activity, by the inhibitor Emi1/Rca1, drives a positive feedback circuit that controls both protein stability and mRNA expression. Thus, the observed decrease in the levels of at least some APC/C targets that occurs upon depletion of Emi1/Rca1, including Geminin and Cyclin A, are controlled at the levels of transcription and protein stability (Verschuren et al., 2007). Developmentally programmed endocycles may provide a natural example where cell cycle progression occurs in the context of increased APC/C$^{Fzr/Cdh1}$ activity. Thus, a similar positive-feedback circuit may be operating during *Drosophila* endocycles to downregulate the transcription of E2F target genes. Determining the precise regulatory relationships between the upregulation of APC/C$^{Fzr/Cdh1}$ activity and the transcriptional downregulation of genes such as *Cyclin A* and *geminin*, during the *Drosophila* endocycle represents an exciting area for future research.

The requirement for APC/C activity to promote endocycle progression may help answer several longstanding questions concerning the regulation of the *Drosophila* endocycle. For

example, why does the continuous expression of Cyclin E inhibit cell cycle progression during the endocycle but not the mitotic cycle (Edgar and Orr-Weaver, 2001; Follette et al., 1998; Weiss et al., 1998)? Several models have been proposed to explain this difference. First, the breakdown of the nuclear envelope that occurs during the mitotic cycle, but not the endocycle, may allow for a transient decrease in local Cyclin E/Cdk2 activity, thus allowing for the relicensing of DNA replication origins (Edgar and Orr-Weaver, 2001). Alternatively, there may be differences in the machinery required to produce a functional pre-RC in mitotic versus endocycling cells (Feger et al., 1995; Lake et al., 2007). Our results suggest an alternative model for why endocycles are unusually sensitive to continuous Cyclin E expression. This model is based on our demonstration that endocycle progression requires APC/C activity. Both Fzy/Cdc20 and Fzr/Cdh1 function as activators of the APC/C (Dawson et al., 1995; Sigrist et al., 1995; Sigrist and Lehner, 1997). However, the regulation of these APC/C activators is very distinct (Thornton and Toczyski, 2006). During the mitotic cycle, the binding of Fzy/Cdc20 to the APC/C is dependent on the phosphorylation of several APC/C subunits by the mitotic kinase Cdk1 (Rudner and Murray, 2000; Shteinberg and Hershko, 1999; Shteinberg et al., 1999; Yamada et al., 1997). By contrast, a Cdk-dependent inhibitory phosphorylation on Fzr/Cdh1 relegates $APC/C^{Fzr/Cdh1}$ activity to late M phase and G1. Because of its requirement for Cdk1 activity, $APC/C^{Fzy/Cdc20}$ is unlikely to be active during most endocycles. Indeed, *Drosophila* endocycles proceed normally in *fzy* mutants (Sigrist et al., 1995). Thus, the only available activator of the APC/C during the endocycle is Fzr/Cdh1. As previously discussed, Fzr/Cdh1 is inhibited by Cyclin E/Cdk2 activity (Sigrist and Lehner, 1997). Therefore, we propose that during the endocycle, continuous Cyclin E/Cdk2 activity results in the permanent inhibition of the only available activator of the APC/C, Fzr/Cdh1. This leads to the accumulation of Geminin, Cyclin A and other potential targets, which act to block cell cycle progression. Thus, the ability of continuous Cyclin E to inhibit DNA replication during the endocycle may reflect differences in the available activators of the APC/C present in mitotic versus endocycling cells.

In conclusion, our demonstration that the $APC/C^{Fzr/Cdh1}$ has a crucial function during endocycles will allow new models on the minimum cell cycle inputs necessary to construct a G/S oscillator to be formulated and tested.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/8/1451/DC1

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