

RESEARCH ARTICLE

Induction of endocycles represses apoptosis independently of differentiation and predisposes cells to genome instability

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ABSTRACT

The endocycle is a common developmental cell cycle variation wherein cells become polyploid through repeated genome duplication without mitosis. We previously showed that *Drosophila* endocycling cells repress the apoptotic cell death response to genotoxic stress. Here, we investigate whether it is differentiation or endocycle remodeling that promotes apoptotic repression. We find that when nurse and follicle cells switch into endocycles during oogenesis they repress the apoptotic response to DNA damage caused by ionizing radiation, and that this repression has been conserved in the genus *Drosophila* over 40 million years of evolution. Follicle cells defective for Notch signaling failed to switch into endocycles or differentiate and remained apoptotic competent. However, genetic ablation of mitosis by knockdown of *Cyclin A* or overexpression of *fzr/Cdh1* induced follicle cell endocycles and repressed apoptosis independently of Notch signaling and differentiation. Cells recovering from these induced endocycles regained apoptotic competence, showing that repression is reversible. Recovery from *fzr/Cdh1* overexpression also resulted in an error-prone mitosis with amplified centrosomes and high levels of chromosome loss and fragmentation. Our results reveal an unanticipated link between endocycles and the repression of apoptosis, with broader implications for how endocycles may contribute to genome instability and oncogenesis.

KEY WORDS: Endocycle, Apoptosis, Cell cycle, *Drosophila*, Oogenesis

INTRODUCTION

During each cell division cycle, chromosomes are duplicated and segregated to two daughter cells. Cell cycle checkpoints ensure the proper order and fidelity of cell cycle events and are fully engaged when problems are encountered (Vleugel et al., 2012; Weinert and Hartwell, 1993). The DNA damage checkpoint arrests the cell cycle and mobilizes repair proteins to fix the damage (Ciccio and Elledge, 2010). If the level of genomic DNA damage is severe, however, the cell can actively withdraw from the cell cycle into an inactive state known as senescence, or engage a programmed cell death (PCD), often through a pathway known as apoptosis (Bartkova et al., 2006; Fuchs and Steller, 2011). Cell division cycles and checkpoints are altered during both development and cancer (Hanahan and Weinberg, 2011). These variations on the cell cycle theme in development and disease, however, remain incompletely defined. In this study, we use *Drosophila* as a model to examine the cell cycle variation known as the endocycle, and find that it has an unanticipated relationship with the repression of apoptosis.

The endocycle is composed of alternating gap (G) and DNA synthesis (S) phases without mitosis (Calvi, 2013; Davoli and de Lange, 2011; Fox and Duronio, 2013). Cells are induced to switch from canonical mitotic cycles to variant endocycles at specific times of development in a wide variety of organisms. Although the details of this regulation can differ among organisms and cell types, the unifying theme is that mitotic functions are repressed, thereby promoting entry into endocycles. Subsequent cell growth and repeated genome duplications during alternating G/S endocycles results in large, polyploid cells. Other cells polyploidize through a variation of the endocycle known as endomitosis, wherein cells initiate mitosis but do not divide, including glial cells in *Drosophila* and megakaryocytes and liver cells in humans (Calvi, 2013; Fox and Duronio, 2013; Unhavaithaya and Orr-Weaver, 2012).

In *Drosophila melanogaster*, cells switch from mitotic cycles into endocycles during development of many tissues from the embryo, larva and adult ovary (Lee et al., 2009; Mahowald et al., 1979; Mulligan and Rasch, 1985; Smith and Orr-Weaver, 1991). Endocycle entry in these tissues is promoted by transcriptional repression of genes that are required for mitosis, for example mitotic cyclins *Cyclin A* (*CycA*) and *Cyclin B* (*CycB*), and increased expression of *fzr/Cdh1* (*rap* – FlyBase), which encodes a subunit of the anaphase-promoting complex (APC) ubiquitin ligase (Maqbool et al., 2010; Narbonne Reveau et al., 2008; Schaeffer et al., 2004; Sigrist and Lehner, 1997; Zielke et al., 2008). APC^{Cdh1} ubiquitinates *CycB* and other proteins required for mitosis, targeting them for destruction by the proteasome (Manchado et al., 2010; Pesin and Orr-Weaver, 2008; Wäsch et al., 2010). Thus, endocycle entry is enforced by repressing mitosis at both transcriptional and post-transcriptional levels. Subsequent oscillating levels of APC^{Cdh1} and Cyclin E/Cdk2 (*Cdc2c* – FlyBase) activity promote alternating G and S phases of the endocycle, respectively (Narbonne Reveau et al., 2008; Zielke et al., 2008). Endocycle regulation in *Drosophila* is similar in many respects to that in mammals, including regulation by Cyclin E/Cdk2, APC^{Cdh1}, and dampened expression of genes regulated by the E2F family of transcription factors (Calvi, 2013; Chen et al., 2012; Maqbool et al., 2010; Meserve and Duronio, 2012; Narbonne Reveau et al., 2008; Pandit et al., 2012; Sher et al., 2013; Ullah et al., 2009; Zielke et al., 2011). Although much progress has been made, the mechanisms of endocycle regulation and its integration with development remain incompletely defined.

Whereas polyploidization occurs during the endocycles of normal development, aberrant polyploidy is also common in solid tumors from a variety of human tissues (Davoli and de Lange, 2011; Fox and Duronio, 2013). Over the last 100 years there has been a growing appreciation that genome instability in these polyploid cells contributes to cancer progression (Boveri, 2008; Carter et al., 2012; Dutrillaux et al., 1991; Fujiwara et al., 2005; Gretarsdottir et al., 1998; Navin et al., 2011; Shackney et al., 1989). Evidence suggests that some cancer cells may polyploidize by switching to a variant G/S cell cycle that shares many attributes with normal

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developmental endocycles, and that these polyploid cells contribute to oncogenesis (Davoli and de Lange, 2011; Davoli and de Lange, 2012; Davoli et al., 2010; Varette and Pellman, 2012; Vitale et al., 2011; Wheatley, 2008). Examination of normal developmental endocycles, therefore, may lead to a better understanding of the mechanisms and consequences of polyploidy in cancer cells.

We have previously shown that another common attribute of endocycling cells in *Drosophila* is that they do not apoptose in response to DNA replication stress (Mehrotra et al., 2008). In mitotic cycling cells, replication stress activates the ataxia telangiectasia mutated/ataxia telangiectasia and Rad3 related (ATM/ATR) checkpoint kinases as part of an apoptotic pathway mediated by the *Drosophila* ortholog of the human p53 tumor suppressor, whereas in endocycling cells this pathway is repressed (Fuchs and Steller, 2011; Mehrotra et al., 2008). Although the repression of apoptosis is a common attribute of endocycling cells from different tissues in *Drosophila*, it has remained unclear whether it is endocycle reprogramming or the differentiation of these cells that is responsible for apoptotic repression.

In this study, we use *Drosophila* oogenesis as a model to investigate the relationship between endocycle reprogramming and apoptotic competence. Our findings reveal an unsuspected link between the endocycle program and apoptosis that is independent of cell differentiation, and show that when endocycling cells return to mitosis it has dire consequences for genome stability, with important implications for how endocycles may contribute to oncogenesis.

RESULTS

The repression of apoptosis during endocycles is conserved in the genus *Drosophila*

To examine the link between cell cycle programs and apoptosis, we used the *Drosophila* ovary as a model system because cells switch from mitotic cycles to endocycles at precise times during oogenesis (Calvi et al., 1998; Dej and Spradling, 1999; Deng et al., 2001; López-Schier and St Johnston, 2001; Mahowald et al., 1979; Mulligan and Rasch, 1985; Painter and Reindorp, 1939). A *Drosophila* ovary is composed of 16–20 ovarioles that each contain a series of egg chambers that migrate posteriorly as they mature through 14 stages (Fig. 1A,B) (King, 1970). Each egg chamber is composed of an oocyte and 15 sister germline nurse cells surrounded by an epithelial layer of somatic follicle cells (Fig. 1A) (Spradling, 1993). These cells originate from germline and somatic stem cells within the germarium. The germline nurse cells begin endocycles just before stage 1. Somatic follicle cells, however,

continue to proliferate by mitotic divisions through stage 6, and then switch into endocycles at stages 6–7 in response to Notch signaling (Fig. 1B) (Deng et al., 2001; Domanitskaya and Schüpbach, 2012; López-Schier and St Johnston, 2001; Schaeffer et al., 2004; Sun and Deng, 2005; Sun and Deng, 2007). Follicle cells cease genomic endoreplication in stage 10 and begin developmental amplification of genes required for eggshell synthesis (Fig. 1B) (Calvi, 2006; Calvi et al., 1998; Kim et al., 2011; Spradling and Mahowald, 1980).

To evaluate the relationship between cell cycle programs and the apoptotic response to DNA damage, we irradiated *D. melanogaster* adult females with 4000 rads of gamma rays, and assayed cell death by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), and mitotic divisions with anti-phosphohistone H3 (PH3) antibodies that label mitotic chromosomes (Gavrieli et al., 1992; Hendzel et al., 1997). We assayed cell death 24 hours after irradiation to detect both p53-dependent and delayed p53-independent cell death (McNamee and Brodsky, 2009; Wichmann et al., 2006; Wichmann et al., 2010). After irradiation, germline cells were often TUNEL positive during mitotic cycles in the germarium, but these cells were rarely TUNEL positive during endocycles of stage 1 and older egg chambers, and were similar in frequency to unirradiated controls (Fig. 1C; data not shown). Similarly, many mitotically proliferating follicle cells were TUNEL positive up until stage 6 (~10%), but were rarely TUNEL positive after stage 6 during both endocycle and developmental gene amplification stages (<0.1%) (Fig. 1C). In response to irradiation, mitotic cycling germline and somatic cells also labeled with antibodies against cleaved Caspase 3, and had small, pycnotic nuclei, indicating that these cells were undergoing an apoptotic cell death (data not shown) (Jänicke et al., 1998). Similar results were obtained 6 hours after ionizing radiation (IR) (supplementary material Fig. S1). These results indicate that the apoptotic response to DNA damage is repressed during endocycles of both germline and somatic cells of the ovary.

The developmental timing of the follicle cell mitotic to endocycle transition at stages 6–7 is conserved among species in the genus *Drosophila*, suggesting a possible conserved role for Notch in this cell cycle remodeling (Calvi et al., 2007). To determine if the repression of apoptosis during ovarian endocycles is also conserved, we irradiated females of eight different *Drosophila* species. In all species tested, the apoptotic response to IR was repressed when germline and somatic cells switched to the endocycle (Fig. 1D) (Table 1). This included *D. virilis*, which diverged from the *D. melanogaster* lineage ~42 million years ago (Fig. 1D) (Table 1)

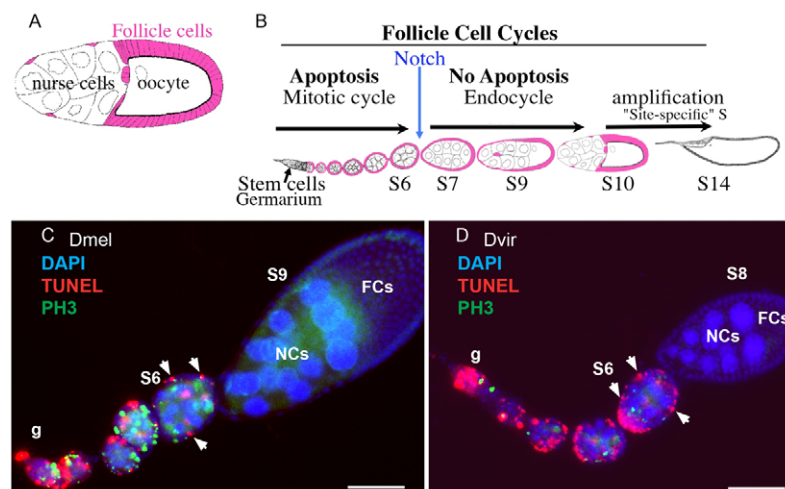


Fig. 1. The repression of apoptosis during ovarian endocycles is conserved in the genus *Drosophila*.

(A) Drawing of a longitudinal section through a stage-10 egg chamber. Somatic follicle cells (magenta) surround the germline nurse cells and oocyte. (B) An ovariole with egg chambers that mature through 14 stages (S) as they migrate posteriorly (left to right) down the ovariole (King, 1970). Germline and somatic follicle stem cells and their daughters (magenta) divide mitotically in the germarium. (C,D) TUNEL, PH3 and DAPI labeling of ovaries from *D. melanogaster* (Dmel) (C) and *D. virilis* (Dvir) (D) females 24 hours after irradiation. Arrowheads point to three of the many TUNEL-labeled follicle cells in stage 6 (S6) in both species. The germarium, follicle cells and nurse cells are indicated. See Table 1 for a complete list of species analyzed. Scale bars: 50 μ m. FC, follicle cell; g, germarium; NC, nurse cell.

Table 1. Endocycle repression of apoptosis is conserved in the genus *Drosophila*

Species analyzed	Approximate millions of years since divergence from <i>D. melanogaster</i> *
<i>D. melanogaster</i>	0
<i>D. simulans</i>	3
<i>D. yakuba</i>	10
<i>D. erecta</i>	10
<i>D. teissieri</i>	10
<i>D. santomea</i>	10
<i>D. ananassae</i>	14
<i>D. willistoni</i>	37
<i>D. virilis</i>	40

*(Bachtrog et al., 2006; Clark et al., 2007).
None of the species analyzed exhibited an apoptotic response to ionizing radiation during nurse cell or follicle cell endocycles.

(Clark et al., 2007). The apoptotic response to IR was also repressed in endocycling tissues of the larva in *D. melanogaster* and other species (supplementary material Fig. S2) (Mehrotra et al., 2008). These results indicate that the repression of apoptosis during endocycles is conserved in the genus *Drosophila*.

Notch signaling is required for the repression of apoptosis in follicle cells

Given that Notch signaling induces endocycles in follicle cells, our results suggested that the Notch pathway may coordinate endocycle entry with the repression of apoptosis. To determine if Notch is required for the repression of apoptosis, we used flippase/flippase recognition target (Flp/*FRT*) mitotic recombination to create clones of follicle cells homozygous mutant for *Presenilin* (*Psn*) in

heterozygous *Psn*⁺ females (Xu and Rubin, 1993). *Psn* is the gamma secretase that cleaves the transmembrane Notch receptor in follicle cells upon binding to its germline ligand Delta, and is required for Notch signaling (Bray, 2006; De Strooper et al., 1999; Ye et al., 1999). Clones of *Psn* mutant follicle cells were compromised for Notch signaling and failed to switch into the endocycle after stage 6, as evidenced by smaller nuclei relative to the polyploid *Psn*⁺ follicle cells in the same egg chamber, consistent with previous reports (Fig. 2A-A'') (Sun and Deng, 2007). Antibody labeling for the immature follicle cell marker Fasciclin III (Fas III; Fas3 – FlyBase) indicated that it persisted inappropriately past stage 6 in the *Psn* mutant clones, confirming that Notch-dependent follicle cell differentiation was also impaired (Fig. 2A''-A'''). Upon irradiation, these *Psn* mutant clones frequently had cleaved Caspase 3 or TUNEL labeling after stage 6, whereas control *Psn*⁺ follicle cells in the same egg chambers were only rarely positive for these apoptotic markers (Fig. 2B-D). These results suggest that the Notch pathway is genetically required for both endocycle entry and the repression of apoptosis in follicle cells after stage 6 of oogenesis.

Knockdown of *Cyclin A* or overexpression of *fzr/Cdh1* induces precocious endocycles in follicle cells

Although our results with *Psn* clones suggested that Notch is required to repress apoptosis, they did not resolve whether Notch does so directly, or rather indirectly through promotion of cell cycle reprogramming. To address this question, we sought to uncouple endocycle entry from Notch signaling. Previous studies in *Drosophila* indicated that knockdown of CycA or overexpression of *fzr* (*cdh1*) promotes polyploidization (Mihaylov et al., 2002; Sauer et al., 1995; Schaeffer et al., 2004; Sigrist and Lehner, 1997). To

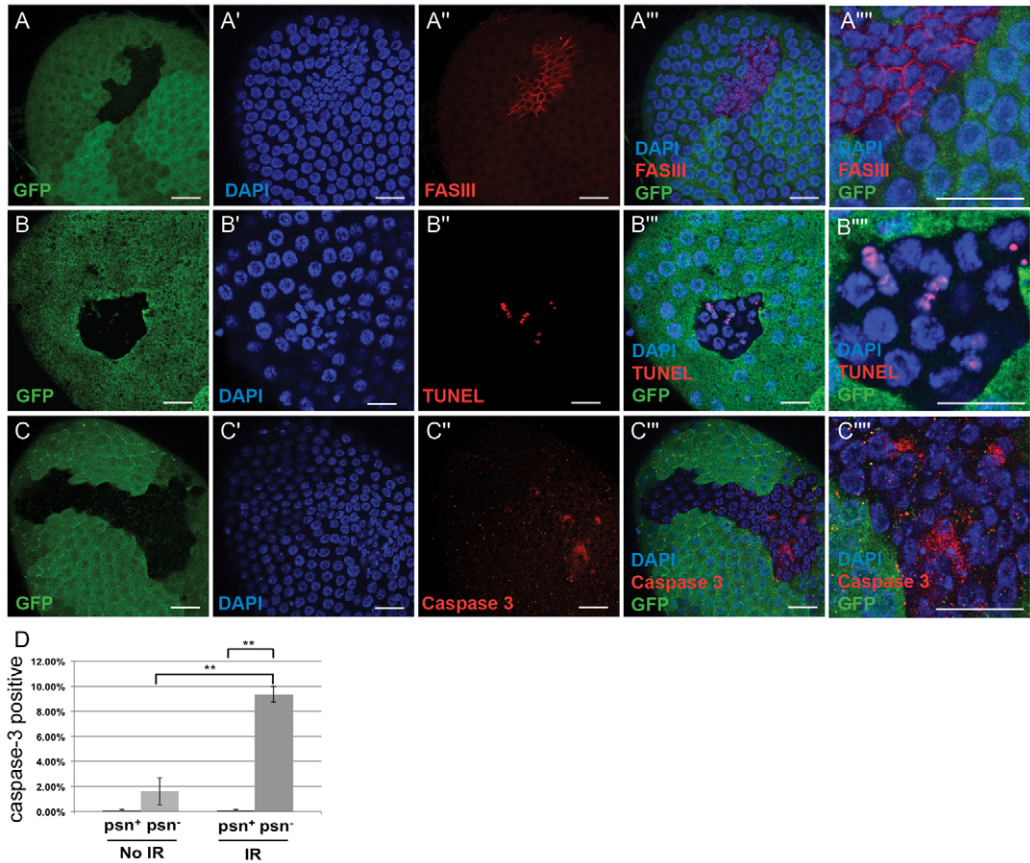


Fig. 2. Notch signaling is required to repress apoptosis in follicle cells. (A-A''') *Presenilin* (*Psn*) mutant follicle cell clone in a stage-8 egg chamber identified by the absence of green fluorescent protein (GFP; not green) (A). *Psn* mutant clones had small nuclei (A') and retained the expression of the immature follicle cell marker Fasciclin III (A''-A'''), indicating that they had failed to switch into the endocycle and differentiate. (B-C''') After irradiation, some *Psn* mutant follicle cells labeled for TUNEL (B-B''') or anti-cleaved Caspase 3 (C-C'''), whereas wild-type endocycling follicle cells in the same chamber did not. (D) Quantification of cleaved Caspase labeling in *Psn* mutant (*Psn*⁻) and control (*Psn*⁺) follicle cells in the same egg chambers with (IR) and without (No IR) irradiation (*n*=3 biological replicates of ten *Psn*^{C1} clones each, representing ~2000 total cells, ***P*=0.003 by Student's *t*-test). Scale bars: 25 μm.

evaluate if this polyploidization occurs through endocycles, we knocked down *CycA* in S2 cells by RNAi, which inhibited mitotic proliferation and resulted in cells that increased in cell and nuclear size over time (supplementary material Fig. S3A–C). Flow cytometry revealed distinct peaks of 2C, 4C, 8C, 16C and 32C DNA content (supplementary material Fig. S3D,E). These quantum doublings of the genome suggested that polyploidization after *CycA* knockdown occurred largely through periodic G/S endocycles rather than re-replication, which is a repeated and unbalanced origin firing during a single S phase (Mihaylov et al., 2002).

We then used fly strains with GAL4-inducible *UAS:CycA^{RNAi}* hairpin RNA or *UAS:fzr* transgenes and a heat-inducible *hsp70:GAL4* to induce follicle cells to precociously switch into the endocycle before stage 7 of oogenesis (Brand and Perrimon, 1993). This GAL4/UAS system is not expressed in the germline, which served as an internal control (Brand and Perrimon, 1993; Rørth, 1998). Adult females from *hsp70:GAL4; UAS:CycA^{RNAi}* and a control *y w* strain were heat treated at 37°C for 30 minutes twice a day, for a total of seven heat pulses over 4 days. Expression of *CycA^{RNAi}* resulted in *CycA* protein being undetectable by western blot (Fig. 3A). However, there are two abundant sources of ovarian *CycA* protein, somatic follicle cells before stage 7 and germline cells after stage 11 (Sugimura and Lilly, 2006; Vardy et al., 2009). Expression of *hsp70:GAL4* alone or with *UAS:CycA^{RNAi}* resulted in nonspecific cell death and degeneration of late stage egg chambers, but did not affect earlier stages of oogenesis, as we have previously described (McConnell and Calvi, 2009). This destruction of late stage egg chambers eliminated the germline contribution of *CycA*, explaining why *CycA* protein abundance was reduced in the *hsp70* females, and undetectable when *CycA* protein was also knocked down in the follicle cells in the *hsp70:GAL4; UAS:CycA^{RNAi}* females (Fig. 3A). These results indicate that expression of *CycA^{RNAi}* results in a strong knockdown of *CycA* protein in somatic follicle cells.

CycA knockdown greatly reduced follicle cell number and increased both cell and nuclear size of all follicle cells as early as stage 1 of oogenesis (Fig. 3B,C). Measurement of nuclear diameter and total 4',6-diamidino-2-phenylindole (DAPI) fluorescence in these cells before stage 7 of oogenesis indicated that they had increased DNA content relative to mitotic cycling controls, and continue to endoreplicate their genome after stage 7, achieving a final DNA content in excess of wild type (Fig. 3D). Five pulses of *fzr* overexpression in the *hsp70:GAL4; UAS:fzr* females also repressed follicle cell mitotic divisions and resulted in premature endocycles before stage 7, consistent with previous reports (supplementary material Fig. S4) (Schaeffer et al., 2004; Sigrist and Lehner, 1997). The frequency of mitotic PH3-positive follicle cells during stages 1–6 was greatly reduced in *CycA^{RNAi}* and *fzr* expressing ovaries compared with control, with none having fully condensed mitotic chromosomes (Fig. 3B,C,E; supplementary material Fig. S4A,B). Despite a repression of mitosis, periodic DNA synthesis continued in these cells, as evidenced by incorporation of the nucleotide analog bromodeoxyuridine (BrdU) (Fig. 3F,G; supplementary material Fig. S4C,D) (Calvi and Lilly, 2004). The MPM-2 antibody detects a phospho-epitope at the *Drosophila* histone locus body (HLB) whose cell cycle phosphorylation is dependent on Cyclin E/Cdk2 (Calvi et al., 1998; Davis et al., 1983; Liu et al., 2006; White et al., 2007). MPM-2 labeling was mosaic among precociously endocycling follicle cells, indicating that *CycE/Cdk2* activity oscillates during these unsynchronized induced endocycles (Fig. 3H).

To examine the kinetics of the precocious endocycle entry, we used the 'FLP On' recombination system to create clones of follicle cells expressing *CycA^{RNAi}* or *fzr* and *UAS:RFP* (Pignoni and Zipursky, 1997). In contrast to control clones that had ~21 cells positive for red fluorescent protein (RFP) 5 days after heat induced recombination, *CycA^{RNAi}* and *fzr* clones contained only one to four cells with very large nuclei (Fig. 3I–J'; supplementary material

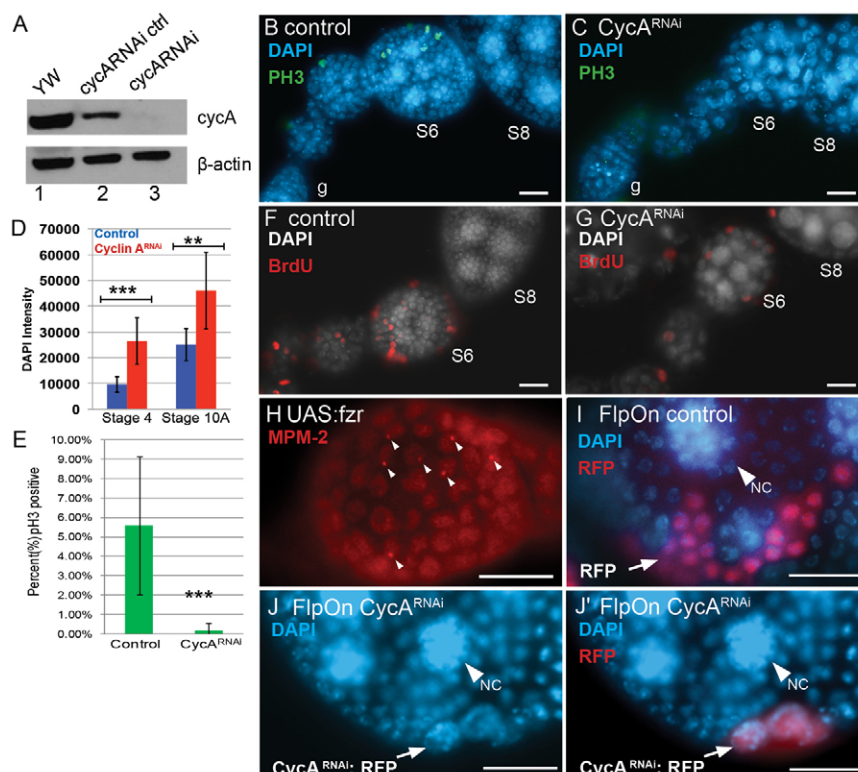


Fig. 3. Induction of precocious endocycles in follicle cells. (A) Western blot for Cyclin A (*CycA*) and β -actin loading control from ovarian extracts of *y w* control (lane 1), *hsp70:GAL4; UAS:CycA^{RNAi}* without (lane 2) or with (lane 3) seven pulses of heat induction. (B,C) Anti-PH3 and DAPI labeling in control (B) and *CycA^{RNAi}*-expressing (C) ovaries. (D) Total DAPI fluorescence in nuclei of follicle cells of stage 4 ($n=25$) and stage 10A ($n=20$). *** $P<0.0001$, ** $P=0.0002$. (E) The percentage of PH3-positive cells in control (5.58%) and *CycA^{RNAi}* (0.18%) ovaries between stages 1 and 6 (two biological replicates representing in total 80 ovaries and ~14,520 wild-type and ~5600 *CycA^{RNAi}* cells, *** $P<0.0001$ by Student's *t*-test). (F,G) DAPI and BrdU labeling in control (F) and *CycA^{RNAi}*-expressing (G) ovaries. (H) Cyclin E/Cdk2 activity oscillates in induced endocycling cells (IECs). Anti-MPM-2 labeling of a stage-4 egg chamber with follicle cells in a precocious endocycle induced by *fzr* overexpression. Histone locus bodies are indicated by arrowheads. (I) A wild-type control clone of follicle cells expressing RFP in a stage-6–7 egg chamber (arrow). (J,J') A clone of follicle cells expressing *CycA^{RNAi}* and RFP in a stage-6 egg chamber (arrow). Scale bars: 15 μ m. NC, nurse cell.

Fig. S4E,E'). These small clones of highly polyploid cells suggested that upon *CycA^{RNAi}* or *fzr* expression cells rapidly switch from mitotic proliferation to endocycles within the same cell cycle, or after only one to two mitotic divisions. Together, the results suggested that *CycA* knockdown or *fzr* overexpression induces follicle cells to precociously switch into a bona fide endocycle before stage 7 of oogenesis. We hereafter refer to these as induced endocycling cells (iECs).

iECs repress apoptosis downstream of ATM

To determine whether the precocious iECs were resistant to apoptosis, we gamma irradiated *hsp70:GAL4; UAS:CycA^{RNAi}* and control females and assayed cell death before stage 7. After irradiation, iECs had a much lower frequency of labeling for anti-cleaved Caspase 3 compared with control mitotic cycling follicle cells (0.17% versus 9.05%) (Fig. 4A-C). Induction of precocious endocycles by overexpression of *UAS:fzr* also repressed the apoptotic response to irradiation (Fig. 4D,D'). Radiation caused DNA damage in iECs, as evidenced by numerous repair foci labeled with antibodies against the phospho-epitope γ H2Av, which is phosphorylated by ATM, a proximal checkpoint kinase in the apoptosis pathway (Fig. 4E,F) (Lake et al., 2013; Madigan et al., 2002). These results indicate that a precocious switch into the endocycle represses the apoptotic response to DNA damage downstream of ATM activation and upstream of Caspase 3 cleavage.

iECs repress apoptosis independently of Notch signaling and differentiation

Normal endocycle entry is associated with a Notch-dependent differentiation program. Therefore, although *CycA* and *fzr* are formally downstream of Notch signaling in follicle cells, it remained possible that induction of a precocious endocycle may have activated a premature differentiation program that contributed to the

repression of apoptosis. To investigate this possibility, we labeled *CycA^{RNAi}* and control ovaries with antibodies against Cut, Hnt (Peb – FlyBase) and FasIII, proteins diagnostic for follicle cell developmental status. The results indicated that the normal developmental timing of these differentiation markers was not altered by *CycA^{RNAi}* or *fzr* expression (López-Schier and St Johnston, 2001; Sun and Deng, 2005; Sun and Deng, 2007; Sun et al., 2008) (Fig. 5A-F'; supplementary material Fig. S5A-B'). These results indicate that *CycA* knockdown or *fzr* overexpression induces follicle cells to enter a precocious endocycle that is uncoupled from a Notch-activated differentiation program, and that this cell cycle reprogramming represses the apoptotic response to DNA damage.

Recovery from *CycA^{RNAi}* results in polytene chromosomes and a low frequency of apoptosis

Most normal endocycling cells in development do not switch back to mitotic cell cycles. We wondered, therefore, whether the iECs could switch back into a mitotic division cycle, and, if so, whether they would regain apoptotic competence. To test this, we heat induced *hsp70:GAL4; UAS:CycA^{RNAi}* twice a day for a total of seven times over 4 days as before, and then ceased heat pulses and allowed females to recover for up to 5 days (~9 days total) (Fig. 6A). Labeling of ovaries with DAPI and anti-PH3 indicated that follicle cells do not resume mitosis upon recovery from heat-induced expression of *CycA^{RNAi}* hairpin RNA. Instead, many polyploid follicle cells developed large polytene chromosomes, a phenotype that was most prevalent during days 3 to 5 of recovery (Fig. 6B,C). Assuming normal rates of oogenesis, these follicle cells would probably have been follicle stem cell daughters in the germarium during the time of heat treatments (Nystul and Spradling, 2007; Nystul and Spradling, 2010). The follicle cell polytene chromosomes were similar in morphology to the classic polytene chromosomes from larval salivary glands, which have both

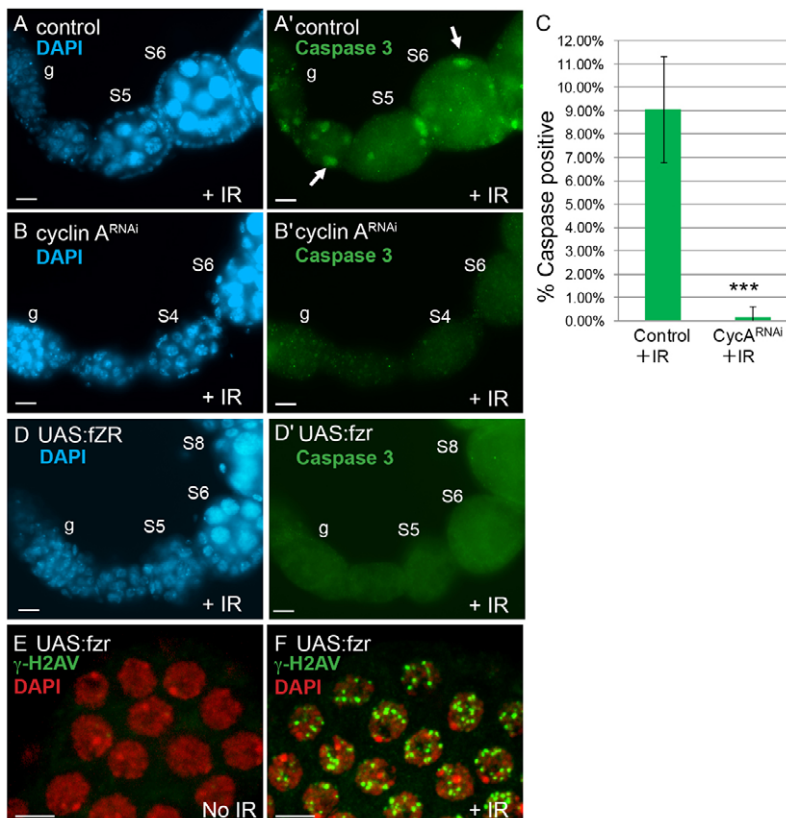


Fig. 4. iECs repress apoptosis downstream of ATM.

(A-B') DAPI (A,B) and anti-cleaved Caspase 3 labeling (A',B') of ovaries 24 hours after irradiation with 4000 rads of gamma rays from *hsp70:GAL4; UAS:CycA^{RNAi}* females without (A,A') or with (B,B') seven heat inductions. Arrows in A' indicate two cells labeled with anti-cleaved Caspase 3. (C) The average percentage of anti-cleaved Caspase 3-positive follicle cells in control (9.05%) and *CycA^{RNAi}*-expressing (0.17%) ovaries between stages 1 and 6, 24 hours after irradiation (two biological replicates representing in total 80 ovarioles and ~14,520 wild-type and ~5600 *CycA^{RNAi}* cells, *** $P < 0.0001$ by Student's *t*-test). (D,D') DAPI (D) and anti-cleaved Caspase 3 labeling (D') of ovaries from *hsp70:GAL4; UAS:fzr* females 24 hours after irradiation with 4000 rads of gamma rays. (E,F) Anti- γ H2Av labeling of repair foci within *UAS:fzr* iECs without (E) or with (F) irradiation. Scale bars: 15 μ m for A-B', D,D'; 5 μ m for E,F.

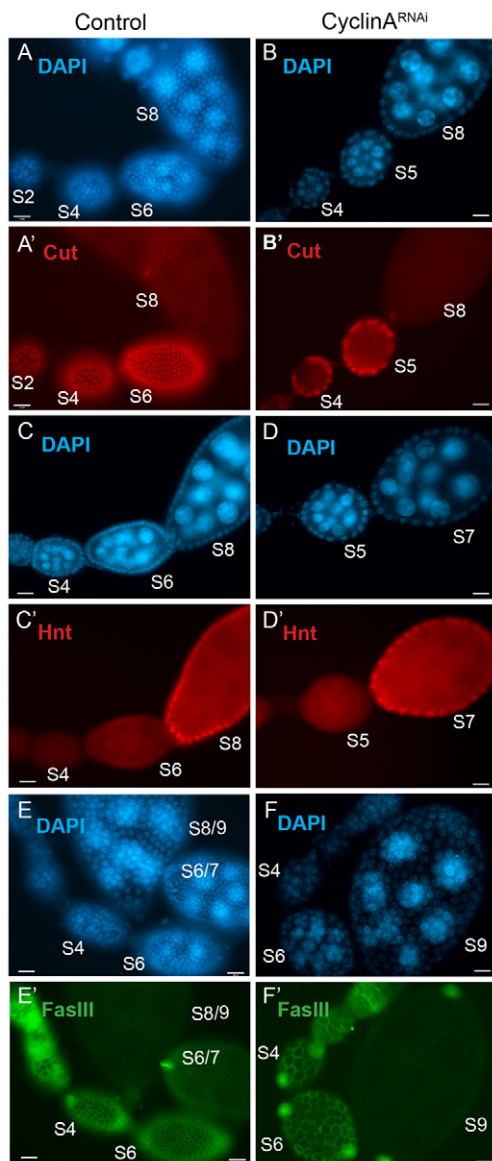


Fig. 5. iECs repress apoptosis independently of Notch signaling and differentiation. (A-F') Ovaries from *hsp70:GAL4; UAS:CycA^{RNAi}* females without (control A,A',C,C',E,E') or with (B,B',D,D',F,F') seven heat inductions were labeled with DAPI (A-F) and the indicated antibodies (A'-F'). The brightest FasIII labeling (E',F') corresponds to polar follicle cells. Scale bars: 15 μ m.

homologs and all sister chromatids synapsed (Fig. 6D). Control *hsp70:GAL4; UAS:CycA^{RNAi}* females that were given heat pulses twice a day for the entire nine-day period did not have polytene chromosomes, indicating that it was the cessation of *CycA^{RNAi}* expression and not total time from the first heat pulse that promoted polytene chromosome formation (data not shown). Nevertheless, CycA protein remained undetectable by western blotting on day 5 of recovery, suggesting why these cells were incapable of mitosis (Fig. 6E). Labeling with the nucleotide analog 5-ethynyl-2'-deoxyuridine (EdU) indicated that many of these polytene cells continued to endoreplicate their DNA (Fig. 6F). These results suggest that partial recovery from *CycA* RNAi promotes the formation of polytene chromosomes in endocycling follicle cells.

Although mitotic divisions were not observed in follicle cells recovering from *CycA^{RNAi}* expression, a few polyploid iECs

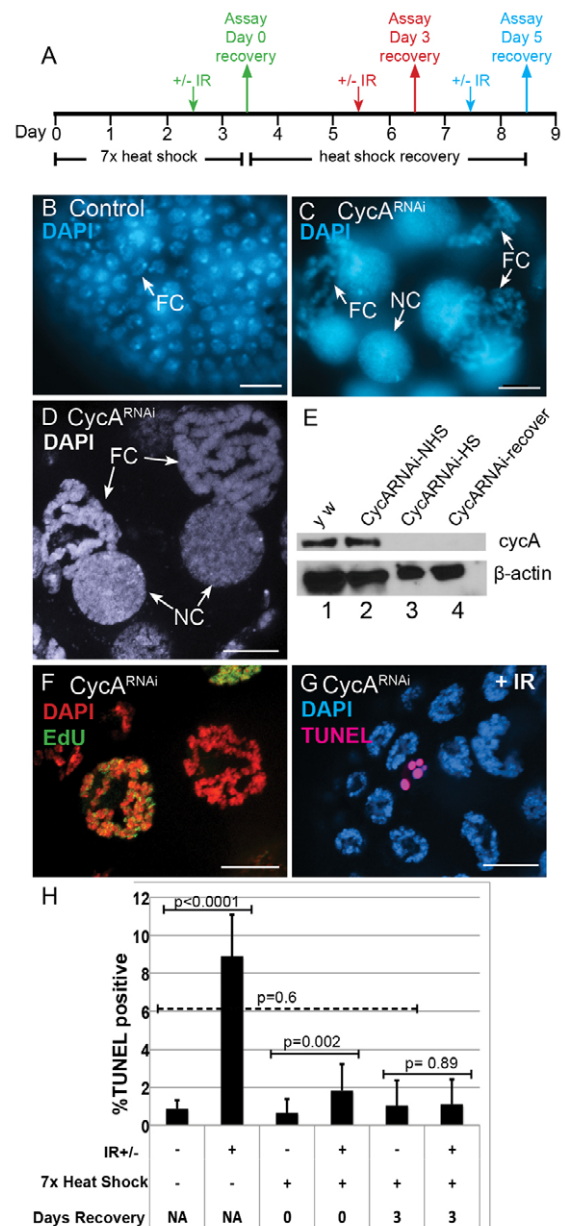


Fig. 6. Recovery from *CycA^{RNAi}* expression results in polytene chromosomes and persistent repression of apoptosis. (A) Timeline of the *CycA* RNAi recovery experiment. Similar colors represent matched irradiation and assay 24 hours later. (B,C) Wide-field image of DAPI labeled egg chambers from wild-type control (B) or *CycA^{RNAi}* females on day 5 of recovery (C). Arrows in C point to three follicle cell (FC) nuclei with polytene chromosomes and one nurse cell (NC) nucleus. (D) Confocal image of DAPI labeled polytene chromosomes in follicle cells (FC) from a *CycA^{RNAi}* recovery female. (E) Cyclin A protein is undetectable upon recovery from *CycA^{RNAi}* expression. Western blot of ovarian protein extracts for Cyclin A and β -actin (loading control) proteins from *y w* control strain (lane 1), *hsp70:GAL4; UAS:CycA^{RNAi}* without heat induction (lane 2) or after seven heat inductions with 1 day (lane 3) or 5 days (lane 4) of recovery. (F) EdU labeling of polytene follicle cells on day 3 of recovery. (G) TUNEL labeling of one follicle cell undergoing apoptosis after IR on day 5 of recovery. (H) Most follicle cells do not apoptose upon recovery from *CycA* RNAi. Quantification of TUNEL-positive cells on days 0 and 3 of recovery with or without IR. Control females were not heat shocked and did not express *CycA^{RNAi}* ($n \sim 2800$ heat shocked or 7260 control follicle cells per sample). Lines indicate P values for pairwise comparisons. The dotted line indicates the P value for the comparison of no IR treatment without heat shock to no IR treatment on day 3 of recovery from heat shock.

underwent spontaneous apoptosis as evidenced by large pycnotic nuclei and labeling with TUNEL or cleaved Caspase 3 (Fig. 6G; supplementary material Fig. S6A,A'). These apoptotic polyploid cells, however, occurred at a very low frequency (Fig. 6H). Gamma irradiation did not significantly increase the frequency of apoptotic cells (Fig. 6H; supplementary material Fig. S6B,B'). These data suggest that most cells that are recovering from *CycA*^{RNAi} expression remain in the endocycle and continue to repress apoptosis.

Recovery from *fzr* overexpression results in an error-prone polyploid mitosis, chromosome instability and apoptosis
The inability of iECs to resume mitotic divisions upon recovery from heat induction of *CycA*^{RNAi} hairpin RNA expression could be explained by persistent RNAi that kept Cyclin A protein levels low. Alternatively, it may be that iECs are fundamentally incapable of returning to a mitotic division program. To address this question, we examined the follicle cells that were induced into the endocycle by overexpression of *fzr* instead of RNAi. The *hsp70:GAL4; UAS:fzr* females were heat pulsed as before to induce all follicle cells into an endocycle, and then allowed to recover from 1 to 5 days before fixation and labeling (Fig. 7A). By day 3 of recovery from *fzr* expression, each ovariole had numerous large follicle cells with condensed PH3-labeled chromosomes. Mitotic cells were observed only before stage 7, suggesting that Notch signaling was promoting follicle cell endocycles in later chambers, as it does in wild type. Follicle cells from control females had the normal four chromosome arms visible in anaphase (Fig. 7B). By contrast, the large, mitotic follicle cells in *fzr* recovery females clearly contained many more chromosomes (Fig. 7C). Some of these polyploid cells appeared to have a normal anaphase segregation of chromosomes, except that many extra chromosomes were left at the division mid-plane

(Fig. 7C). Other cells had much more disordered mitotic figures with frequent anaphase chromosome bridges (Fig. 7D). In many cells, small chromosome fragments were observed, indicative of DNA breakage (Fig. 7D). Indeed, some cells had multilobed nuclei or micronuclei that were separate from the main chromosome mass (Fig. 7E; data not shown). To determine whether the chromosome instability and fragmentation in these cells could be augmented by centrosome amplification, we labeled with antibodies against the centrosome marker Centrosomin (Cnn), which indicated that many of the polyploid mitotic cells had several fold more than the normal two centrosomes (Fig. 7E) (Eisman et al., 2009). Labeling for some of the centrosomes was comparable in size to that of wild type, whereas other centrosome foci were larger, suggestive of centrosome clustering that has been described in polyploid cancer cells (Fig. 7E) (Ganem et al., 2009).

Labeling with TUNEL indicated that recovery from *UAS:fzr* overexpression was also associated with the reacquisition of apoptotic competence (Fig. 7F,G). Spontaneous apoptosis was observed as early as day 1 of recovery, and gamma irradiation increased the apoptotic frequency in recovering iECs (Fig. 7G; data not shown). Spontaneous and IR-induced cell death increased with subsequent days of recovery, and by day 5 cumulative spontaneous cell death resulted in large gaps in the follicle cell epithelium (Fig. 7G; data not shown). These results indicate that polyploid follicle cells recovering from *fzr* overexpression reacquire apoptotic competence, and can execute an error-prone, polyploid mitosis with centrosome amplification, chromosome loss and chromosome fragmentation.

DISCUSSION

We have found that when nurse and follicle cells switch into the endocycle they strongly repress the apoptotic response to DNA

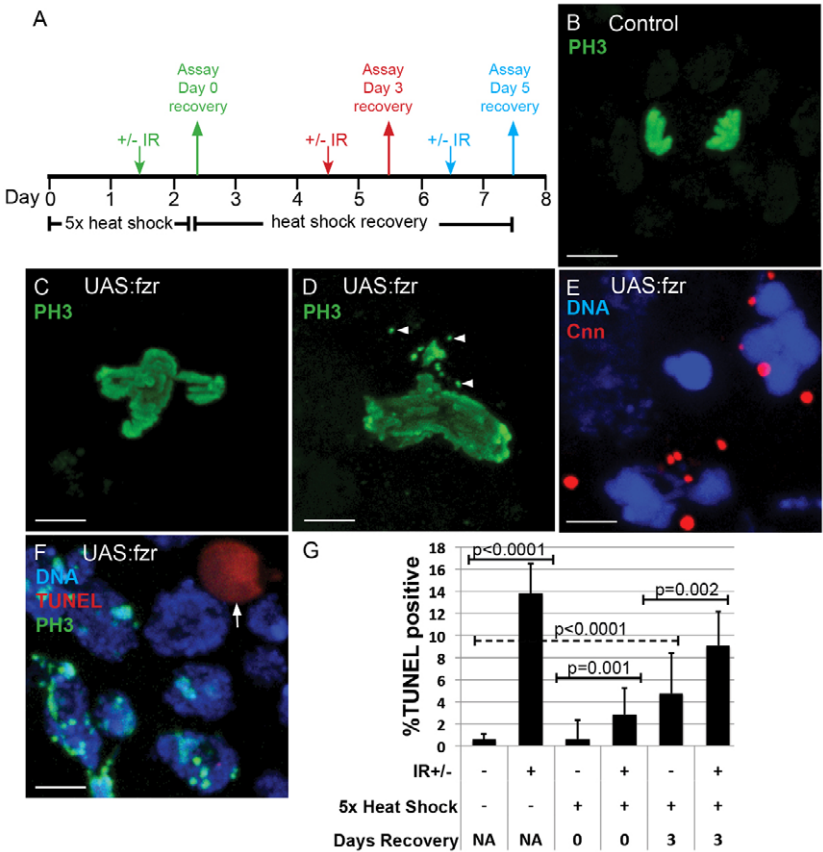


Fig. 7. Recovery from *fzr/Cdh1* overexpression results in an error-prone mitosis and apoptosis. (A) Experimental timeline for the *UAS:fzr* overexpression and recovery experiment. (B-D) Confocal image of anti-PH3 labeling of anaphase chromosomes in single follicle cells from control (B) or *hsp70:GAL4; UAS:fzr* females (C,D) on day 3 of recovery from heat shock. Arrowheads in D point to chromosome fragments. (E) Centrosome amplification and clustering in two follicle cells on day 3 of recovery labeled with DAPI and antibodies against the centrosome protein Centrosomin (Cnn). (F) TUNEL labeling (arrow) of one polyploid follicle cell undergoing spontaneous apoptosis on day 3 of recovery. (G) iECs recovering from *fzr* overexpression regain apoptotic competence. Quantification of TUNEL-positive cells on days 0 and 3 of recovery with or without IR. Control females were not heat shocked and did not express *UAS:fzr* (*n*~2800 heat shocked or 7260 control follicle cells per sample). Lines indicate *P* values for pairwise comparisons. The dotted line indicates the *P* value for the comparison of no IR treatment without heat shock to no IR treatment on day 3 of recovery from heat shock. Scale bars: 5 μ m.

damage caused by ionizing radiation. Genetic ablation of mitosis induced precocious endocycles and repressed apoptosis independently of Notch pathway activity or differentiation. This endocycle repression of apoptosis was reversible, with cells recovering from an induced endocycle regaining apoptotic competence, and entering an error-prone, polyploid mitosis associated with chromosome loss and fragmentation. These findings suggest that there is a link between the endocycle program and the apoptotic response to DNA damage, which has important implications for understanding the impact of endocycles on genome stability and cancer.

Notch represses apoptosis indirectly through promoting follicle cell endocycles

It was previously shown that Notch signaling is required to induce endocycles in follicle cells beginning in stages 6–7 of oogenesis (Deng et al., 2001; López-Schier and St Johnston, 2001). Our results with *Psn* mutant clones confirm those findings, and also show that when follicle cells fail to enter endocycles they remain competent to apoptose in response to DNA damage. The formal interpretation of this result is that the Notch pathway is genetically required for the repression of apoptosis in follicle cells after stage 6 of oogenesis. Indeed, Notch is known to modulate the apoptotic response in other developmental contexts in flies and other organisms, and defects in this regulation contribute to a variety of cancers (Carthew, 2007; Dang, 2012; Miller and Cagan, 1998). Moreover, endocycle entry is frequently associated with cell differentiation in a variety of organisms, suggesting that it may be differentiation that represses apoptosis (Caro et al., 2008; Chen et al., 2012; Fox and Duronio, 2013; Pandit et al., 2012; Ullah et al., 2008). Genetic ablation of mitosis, however, induced precocious follicle cell endocycles before stage 7, and repressed apoptosis independently of Notch signaling or differentiation (Fig. 8). These results strongly suggest, therefore, that Notch signaling inhibits apoptosis indirectly through its promotion of endocycles, but do not eliminate the possibility that the Notch and apoptotic pathways intersect in other ways in endocycling follicle cells.

iECs repress the apoptotic pathway downstream of ATM

Gamma irradiation resulted in numerous γ H2Av foci in both developmental endocycles and iECs, indicating that ATM kinases were active at damage sites in endocycling cells, yet there was no evidence of downstream Caspase cleavage (Fig. 8). This suggests that the mechanism for repression of apoptosis downstream of ATM may be similar between developmental and induced endocycles, perhaps at the level of silencing of p53 target genes (Mehrotra et al., 2008). Why do endocycling cells repress apoptosis? It is known that some heterochromatic regions of the genome do not duplicate every endocycle S phase, and that this under-replication is associated with DNA fragile sites, probably due to stalling and collapse of nested replication forks, suggesting that endocycle program must be coupled to the repression of apoptosis so that these cells can survive in the presence of constitutive DNA damage (Endow and Gall, 1975; Hammond and Laird, 1985a; Hammond and Laird, 1985b; Hong et al., 2007; Leach et al., 2000; Lilly and Spradling, 1996; Mehrotra et al., 2008; Renkawitz-Pohl and Kunz, 1975; Sher et al., 2012).

We found that iECs undergo mitotic division and regain apoptotic competence upon recovery from *fzr* overexpression (Fig. 8). This result further demonstrates that there is a relationship between cell cycle programs and apoptotic competence, and that the repression of apoptosis is dynamic and reversible. The chromosome loss and

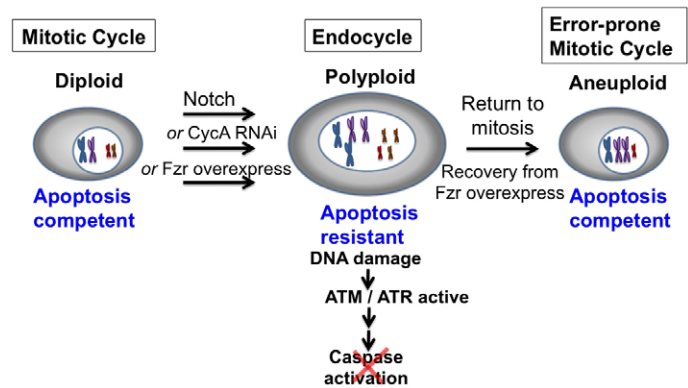


Fig. 8. Endocycles repress apoptosis and contribute to aneuploidy.

Notch signaling normally induces follicle cells to switch from mitotic cycles to endocycles and is associated with the repression of the apoptotic response to DNA damage. Genetic ablation of mitosis by knockdown of *Cyclin A* or overexpression of *fzr* results in induced endocycling cells (iECs), which repress apoptosis independently of Notch signaling or differentiation. Both developmental endocycling cells and iECs repress the apoptotic response to DNA damage downstream of ATM. Upon recovery from *fzr* overexpression, iECs regain apoptotic competence and undergo an error-prone polyploid mitosis. Although this error-prone mitosis probably results in severe aneuploidy and the death of many of these cells, some may survive.

damage incurred during this error-prone mitosis may be one trigger for the observed spontaneous apoptosis. In addition, endocycles, or exit from endocycles, may cause an endogenous cellular stress that later triggers apoptosis.

Programmed cell death in endocycling cells depends on the type of stress

It is important to point out that our analysis has focused on the apoptotic response to genotoxic damage, and that endocycling cells can undergo programmed cell death (PCD) in response to other stimuli. The endocycling nurse and follicle cells normally undergo developmental PCD during late oogenesis, but this cell death does not require the pro-apoptotic, p53 target genes or Caspase activation, instead occurring through a distinct autophagic cell death pathway that has yet to be fully defined (Foley and Cooley, 1998; Mazzalupo and Cooley, 2006). A similar type of autophagic cell death occurs during the developmental demise of endocycling larval salivary glands during metamorphosis, although in those cells the apoptotic pathway also contributes to cell destruction (Berry and Baehrecke, 2007; Jiang et al., 1997; Yin et al., 2007). Endocycling nurse and follicle cells can also undergo PCD beginning in stage 7 of oogenesis in response to metabolic and other stresses (Pritchett et al., 2009). This ‘vitellogenic checkpoint’ also has hallmarks of autophagic cell death and does not require the pro-apoptotic p53 target genes (Peterson et al., 2007). Thus, although endocycling nurse and follicle cells do not apoptose in response to genotoxic damage, they are competent to undergo PCD in response to other stresses and developmental signals.

Endocycles and apoptotic repression are coupled during development and evolution

Our analysis of different *Drosophila* species indicates that the temporal correlation between endocycle entry and the repression of apoptosis in nurse and follicle cells has been conserved over at least 40 million years of *Drosophila* evolution (Clark et al., 2007). Other evidence suggests that endocycle regulation and the repression of apoptosis are conserved beyond *Drosophila*. Endocycles in

Drosophila and mammals share many similarities, including regulation by CycE/Cdk2 and APC^{cdh1} (Lee et al., 2009; Narbonne Reveau et al., 2008; Zielke et al., 2008). Moreover, a transcriptional repression by the E2F family of transcription factors is important for endocycling cells of the *Drosophila* salivary gland, mouse trophoblast giant cells (TGCs) and mouse liver cells (Calvi, 2013; Chen et al., 2012; Maqbool et al., 2010; Pandit et al., 2012; Sher et al., 2013; Weng et al., 2003; Zielke et al., 2011). Importantly, the transition of mouse trophoblast stem cells into endocycling TGCs *in vitro* is associated with a dampened apoptotic response to DNA damage (MacAuley et al., 1998; Ullah et al., 2008). The repression of apoptosis, therefore, may be a common attribute of endocycling cells.

Intermediate levels of Cyclin A promotes polytene chromosome condensation

Cells recovering from *CycA* knockdown did not return to mitosis, but instead formed giant polytene chromosomes. Our data suggest that this phenotype is likely caused by persistence of a low level of RNAi and only partial recovery of *CycA* expression. This is similar to the behavior of nurse cells during normal oogenesis, which transiently express a very low level of mitotic cyclins in stage 4-5 and form polytene chromosomes (Dej and Spradling, 1999; Painter and Reindorp, 1939; Reed and Orr-Weaver, 1997). Mutation in the Apc2 ubiquitin ligase subunit called *morula*, or the Cyclin A translational repressor *arrest*, increases mitotic cyclin levels in these nurse cells and results in an inappropriate mitosis in stage 4-5 (Kashevsky et al., 2002; Reed and Orr-Weaver, 1997; Sugimura and Lilly, 2006). These observations suggest that high levels of mitotic cyclins promote mitosis, whereas intermediate levels induce distinct polytenes in both nurse and follicle cells.

Endocycles, apoptosis, genome instability and cancer

Unlike most endocycling cells in development, we found that cells recovering from *fzr* overexpression could return to mitosis, but this polyploid division was error prone (Fig. 8). Our results in iECs are consistent with previous evidence from *Drosophila* and mouse that a polyploid mitosis in an otherwise normal cell can have severe consequences for genome integrity (Duncan et al., 2010; Fox et al., 2010; Unhavaithaya and Orr-Weaver, 2012). Although some of the iECs subsequently apoptosed, others survived, and it remains possible that some of these cells can divide back to near euploid DNA content and continue to proliferate. Future lineage and live cell analysis will be necessary to reveal the ultimate fate and ploidy of iECs that return to mitosis.

The errors we observed in iECs that return to mitosis resembled the genome instability seen during division of tetraploid cancer cells, including amplified centrosomes, chromosome instability (CIN) and DNA damage (Crasta et al., 2012; Fujiwara et al., 2005; Ganem et al., 2009; Janssen et al., 2011; Meyerson and Pellman, 2011; Stephens et al., 2011). We also saw evidence for centrosome clustering, which in tetraploid cancer cells can result in a normal-looking bipolar spindle, but mal-attachment of kinetochores to these spindles also often results in CIN (Ganem et al., 2009). Although tetraploid cancer cells often arise because of a failure to complete mitosis, evidence suggests that some cancer cells polyploidize by switching into a G/S endocycle (Castedo et al., 2006; Davoli and de Lange, 2011; Davoli and de Lange, 2012; Fox and Duronio, 2013; Fujiwara et al., 2005). Treatment of human cells with DNA-damaging agents, or telomere erosion during crisis, can induce a subset of cells to polyploidize by a G/S cycle, and the subsequent error-prone mitosis in these cells contributes to aneuploidy and oncogenesis (Davoli and de Lange,

2012; Davoli et al., 2010; Erenpreisa et al., 2008; Puig et al., 2008; Varette and Pellman, 2012; Wheatley, 2008; Zheng et al., 2012). Thus, similar to the genetic ablation of mitosis in iECs, it may be that some cancer cells can escape a checkpoint arrest that inhibits mitosis by entering an oscillating G/S cycle. Our evidence that *Drosophila* endocycling cells repress apoptosis downstream of ATM suggests a possible mechanism by which cancer cells without p53 or Rb mutations may survive and escape therapy. Consistent with this hypothesis, recent evidence suggests that apoptosis and senescence are repressed in some tetraploid cells through epigenetic silencing of p53 target genes (Zheng et al., 2012). Although the ultimate fate for many of these aneuploid mitotic cells may be cell death, if only a few survive they have the potential to contribute to cancer progression. The endocycle, therefore, appears to be another example of a normal developmental program that can go awry and contribute to oncogenesis.

MATERIALS AND METHODS

Drosophila genetics

Unless otherwise noted, fly strains were raised at 25°C and were obtained from the Bloomington *Drosophila* Stock Center (BDSC, Bloomington, IN, USA), including *P[GAL4-Hsp70.PB]89-2-1* (Brand and Perrimon, 1993). *UAS:CycA^{RNAi}* was obtained from the Vienna *Drosophila* RNAi Center (VDRC, Vienna, Austria), whereas *UAS:fzr-III* was provided by C. Lehner (Sigrist and Lehner, 1997). For *CycA* RNAi or *fzr* expression, flies were heat treated at 37°C for 30 minutes twice per day for a total of five (*UAS:fzr*) or seven (*UAS:CycA^{RNAi}*) treatments. *FLP-On* clones were created in *hsp70:FLPase/+; Actin <CD2>GAL4 UAS:RFP/UAS:CycA^{RNAi}* (or *UAS:fzr*) females by one 45-minute interval of heat-induction at 37°C (Pignoni and Zipursky, 1997). *Presenilin (Psn)* mutant strains were provided by M. Fortini (Thomas Jefferson University, Philadelphia, PA) and *y w; hsp70:FLPase; Psn^{C1}, FRT2A/2x Ubi-GFP, FRT2A* adult females were heat shocked at 37°C for 1 hour and allowed to recover for 4 days before labeling.

Immunoblotting

Western blots were labeled with primary mouse anti-cyclin A (A12, Developmental Studies Hybridoma Bank, University of Iowa) and mouse anti-β-actin (A1978, Invitrogen), both at 1:1000, and secondary antibody anti-mouse, peroxidase labeled (KPL) at 1:5000. The SuperSignal Pico ECL kit (Thermo Scientific) was used to detect peroxidase activity.

Immunofluorescence microscopy

Ovaries were fixed and labeled as previously described (Schwed et al., 2002), using the following antibodies and concentrations: rabbit anti-phosphohistone H3 (pH3), 1:200 (Millipore); rabbit anti-GFP, 1:500 (Invitrogen); mouse anti-MPM-2, 1:1000 (Millipore); mouse anti-γH2Av (1:8000) (Lake et al., 2013); rabbit anti-cleaved Caspase3, 1:50 (Cell Signaling); mouse anti-Cut, 1:15 (Developmental Studies Hybridoma Bank, DSHB); mouse anti-Hnt, 1:15 (DSHB); and mouse anti-FASIII, 1:30 (DSHB). BrdU and EdU incorporation was for 1 hour as previously described (Calvi and Lilly, 2004). Micrographs were taken on a Leica DMRA2 and analyzed using OpenLab (Improvision) software or using a Leica SP5 confocal. Nuclear DNA content was quantified using total DAPI fluorescence intensity per nucleus with background subtraction using OpenLab.

Cyclin A RNAi in tissue culture

CycA was knocked down in S2 cells with double-stranded RNA using standard methods (Kao and Megraw, 2004). The *CycA* double-stranded RNA corresponded to the sequence of *CycA* in the *UAS:CycA^{RNAi}* fly strain from the VDRC.

Flow cytometry

DNA content in fixed S2 cells was evaluated by standard methods using propidium iodide and a FACSCalibur flow cytometer (BD Biosciences).

Nuclear DNA content in ovaries was measured as previously described (Calvi and Lilly, 2004; Calvi et al., 1998), using DAPI, and analyzed using a FACSaria II flow cytometer (BD Biosciences).

Gamma irradiation

Flies were irradiated with a total of 4000 rads from a cesium source, and 6 or 24 hours later labeled with anti-cleaved Caspase 3 (Cell Signaling), anti- γ H2Av or TUNEL (*In Situ* Cell Death Detection Kit, TMR red. Roche ver.11 #12 156 792 910).

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Competing interests

The authors declare no competing financial interests.

Author contributions

All authors performed experiments. C.H. and B.R.C. wrote the manuscript, with editorial input from B.Z. and M.D.

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Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.098871/-/DC1>

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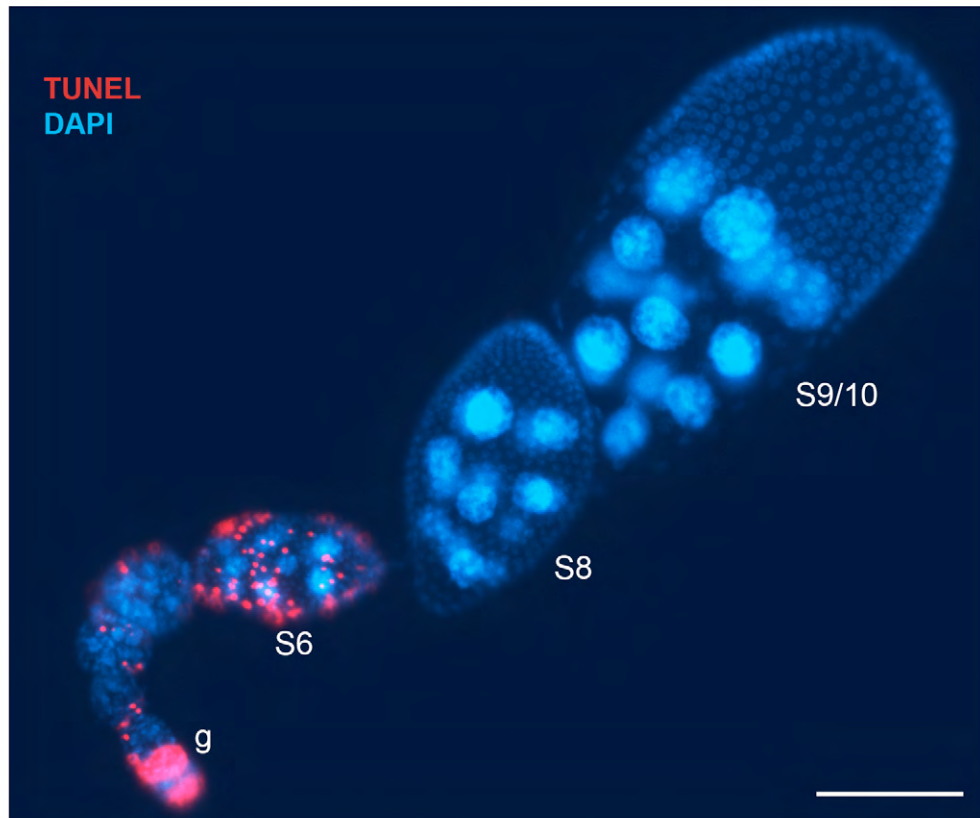


Fig. S1. TUNEL labeling of a *D. melanogaster* ovariole six hours after IR. Scale bar is 50 microns.

D. virilis

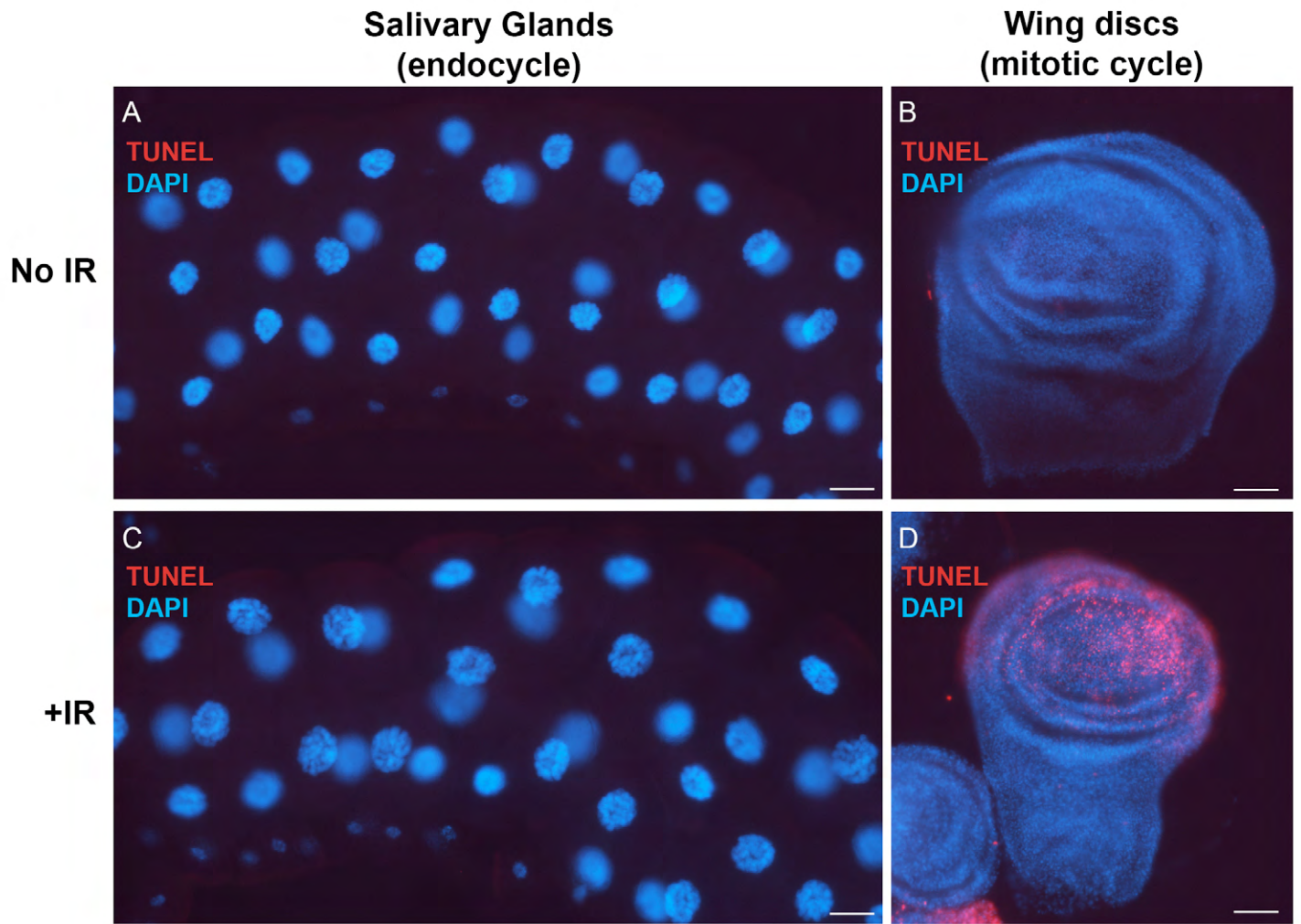


Fig. S2. The repression of apoptosis in endocycling larval tissues is conserved in the genus *Drosophila*. TUNEL labeling of endocycling salivary gland cells (A, C) and mitotic cycling wing disc cells (B, D) from *D. virilis* without (A, B) or with (C, D) ionizing radiation (IR). Scale bars are 50 microns.

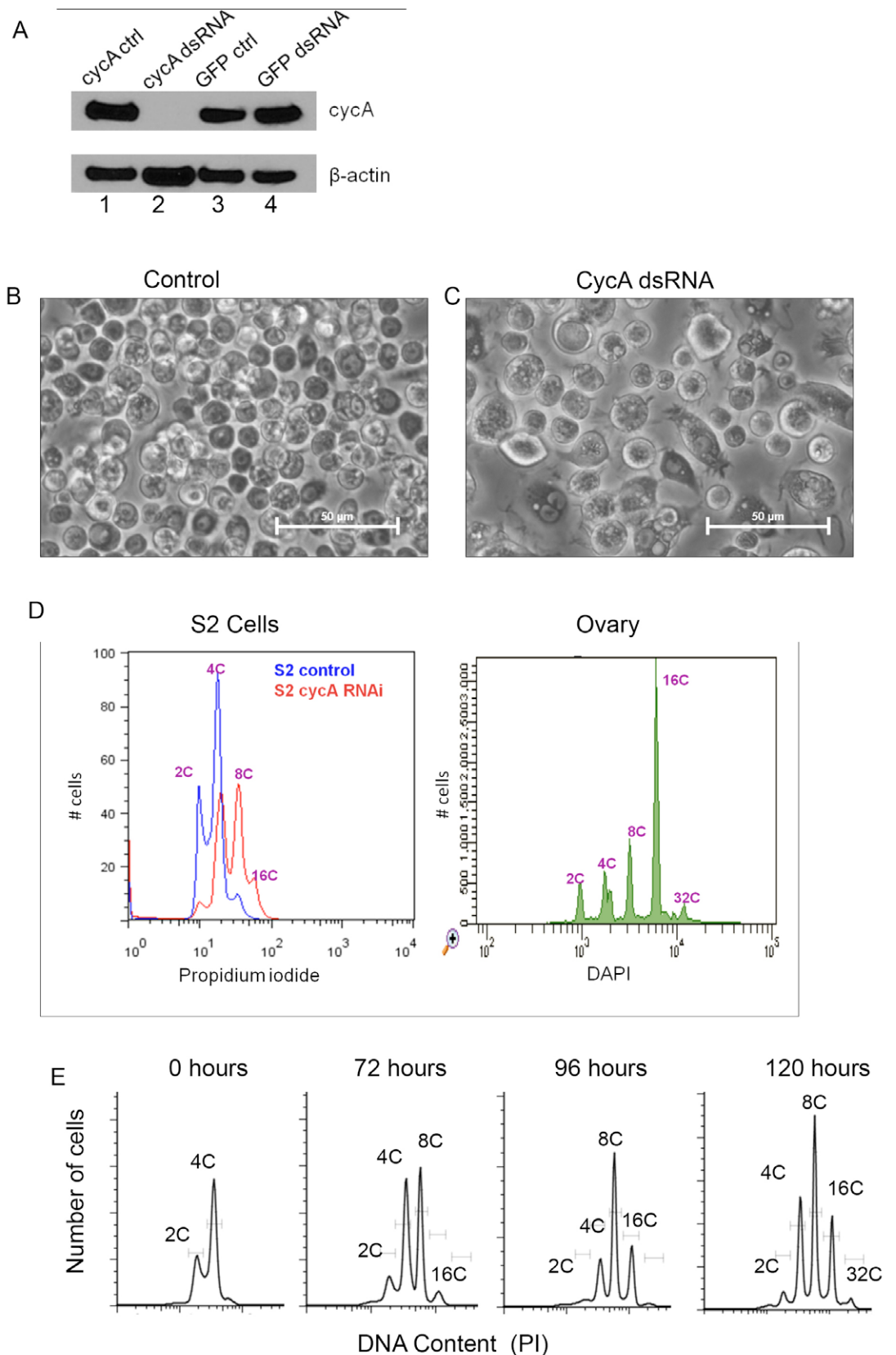


Fig. S3. Cyclin A knockdown in *Drosophila* tissue culture cells induces endocycles. (A) Cyclin A protein is not detectable by Western blot 72 hours after treatment of S2 cells with Cyclin A double-stranded RNA (dsRNA) (lane 2). Controls cells were either mock treated (lanes 1, 3) or treated with dsRNA against GFP (lane 4). β-actin was used as a loading control. (B-C) 72 hours after treatment, control cells continue mitotic proliferation (B), while cells treated with CycA dsRNA ceased proliferating, had no evidence of mitosis, and increased in cell and nuclear size (C). (D) Flow cytometry analysis of S2 cell nuclei 72 hours after treatment with CycA dsRNA (red) revealed that they were polyploid with quantum doublings of diploid genome content. Control S2 cells at 72 hours (blue). The profile of genome doublings in S2 cells resembles that of endocycling follicle cells from the ovary (right panel, green). (E) Time course of S2 cell polyploidization at different times after a single CycA dsRNA treatment.

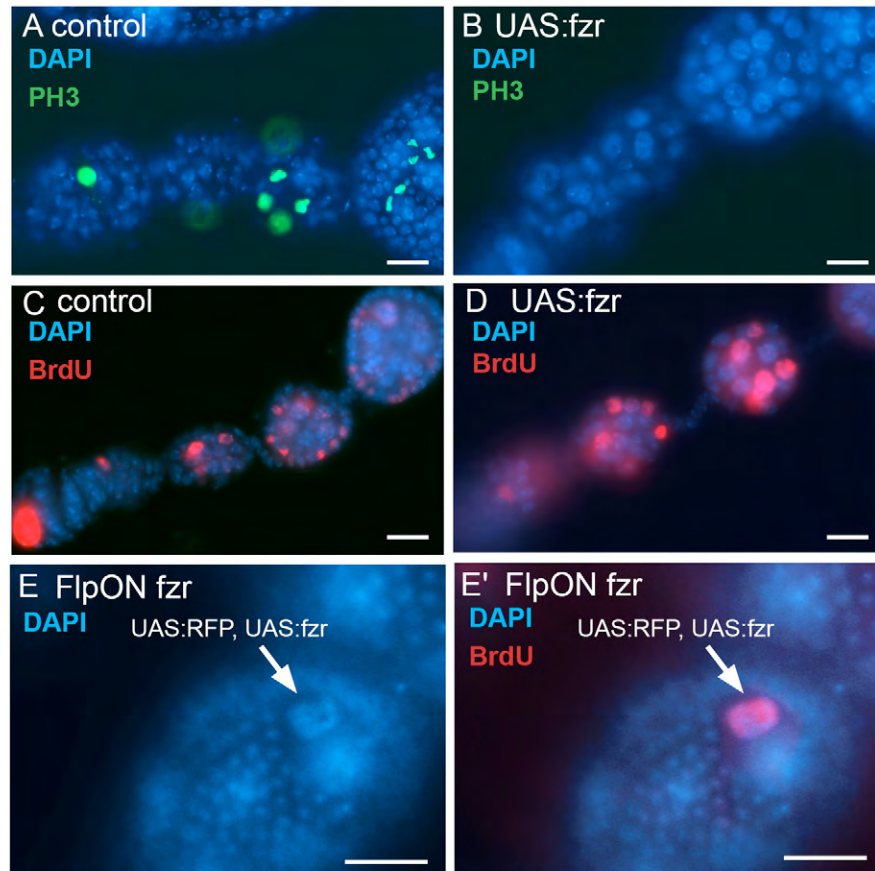


Fig. S4. Overexpression of *fzr* induces precocious endocycles in follicle cells. (A, B) Anti-PH3 and DAPI labeling in control (A) or *hsp70:GAL4 ; UAS:fzr* ovaries after five heat treatments (B). (C, D) BrdU labeling in control (C) or *hsp70:GAL4 ; UAS:fzr* ovaries after five heat treatments (D). (E, E') FLP-On follicle cell clone expressing RFP and *fzr*. Scale bars are 15 microns.

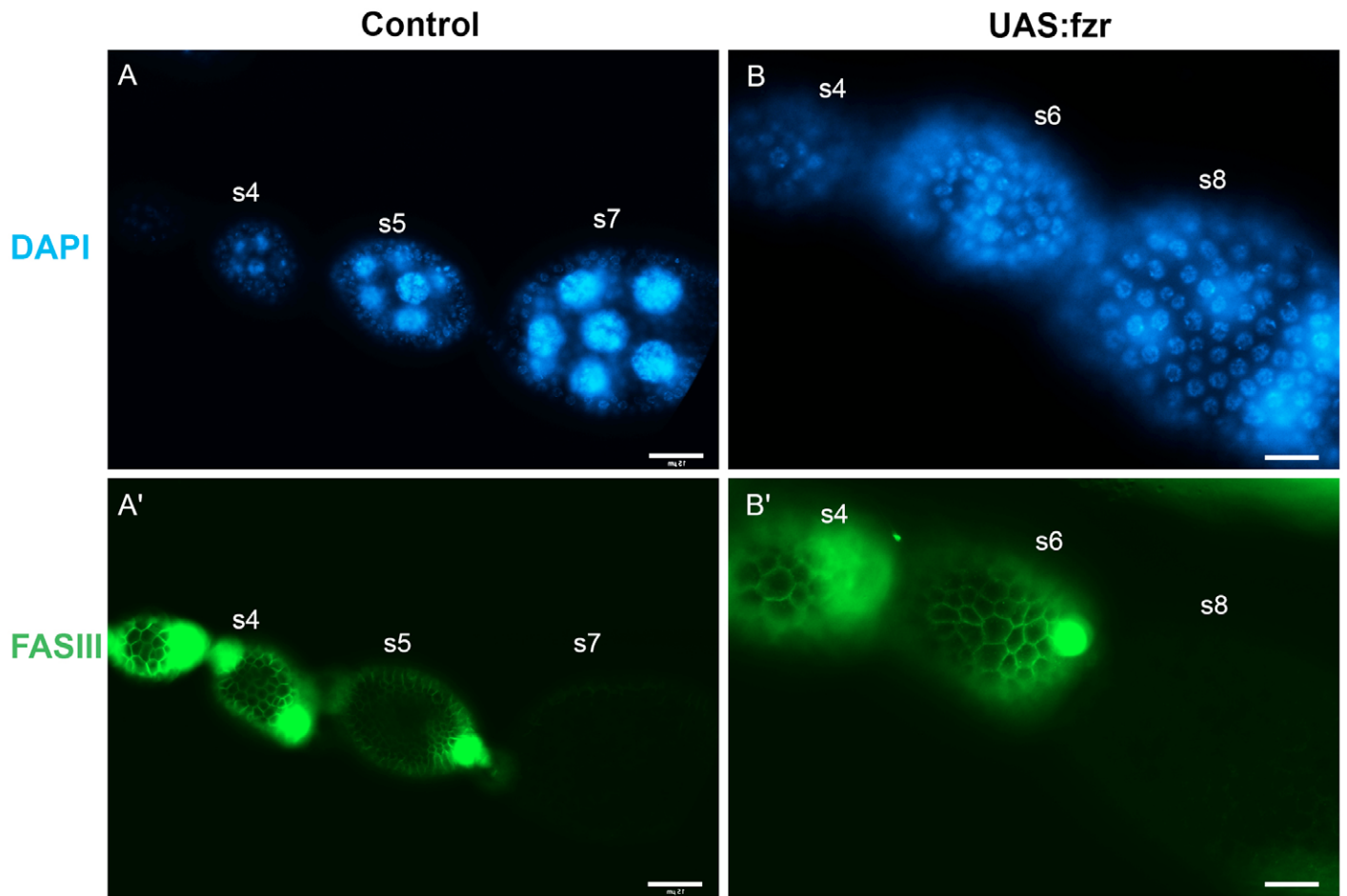


Fig. S5. *fzr* induces precocious endocycles but does not alter the developmental timing of follicle cell differentiation. DAPI (A, B) and anti-FASIII (A', B') labeling of ovarioles from control (A, A') and *hsp70:GAL4 ; UAS:fzr* after five heat treatments (B, B'). Scale bar is 15 microns.

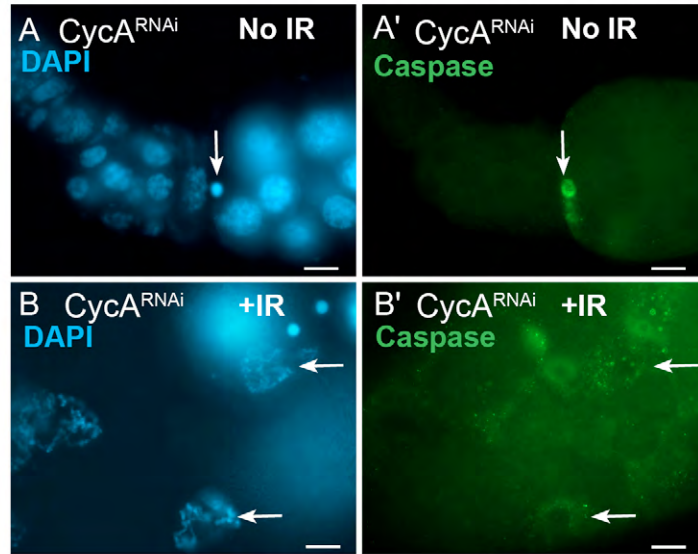


Fig. S6. Apoptosis of iECs recovering from CycA RNAi

After five days of recovery from *CycA^{RNAi}* expression ovaries were labeled with DAPI (A, B) or anti-cleaved Caspase antibodies (A', B'), either without (A, A') or with (B, B') irradiation. Arrows in A, A' point to a rare Caspase 3-positive follicle cell with a large pycnotic nucleus that is undergoing spontaneous apoptosis. Arrows in B, B' point to two polytene follicle cells that labeled faintly with anti-cleaved Caspase 3 24 hours after irradiation, but did not have other hallmarks of apoptosis. All scale bars are 15 microns.