Cyclin E-dependent protein kinase activity regulates niche retention of *Drosophila* ovarian follicle stem cells

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Whether stem cells have unique cell cycle machineries and how they integrate with niche interactions remains largely unknown. We identified a hypomorphic *cyclin E* allele *WX* that strongly impairs the maintenance of follicle stem cells (FSCs) in the *Drosophila* ovary but does not reduce follicle cell proliferation or germline stem cell maintenance. CycE\textsuperscript{WX} protein can still bind to the cyclin-dependent kinase catalytic subunit Cdk2, but forms complexes with reduced protein kinase activity measured in vitro. By creating additional CycE variants with different degrees of kinase dysfunction and expressing these and CycE\textsuperscript{WX} at different levels, we found that higher CycE-Cdk2 kinase activity is required for FSC maintenance than to support follicle cell proliferation. Surprisingly, cycE\textsuperscript{WX} FSCs were lost from their niches rather than arresting proliferation. Furthermore, FSC function was substantially restored by expressing either excess DE-cadherin or excess E2F1/DP, the transcription factor normally activated by CycE-Cdk2 phosphorylation of retinoblastoma proteins. These results suggest that FSC maintenance through niche adhesion is regulated by inputs that normally control S phase entry, possibly as a quality control mechanism to ensure adequate stem cell proliferation. We speculate that a positive connection between central regulators of the cell cycle and niche retention may be a common feature of highly proliferative stem cells.

Cell cycle | niche adhesion | stem cell longevity

The ovary of *Drosophila melanogaster* provides an attractive model for studying stem cells because germline and somatic stem cells have well-defined locations and their behavior can be studied after directed genetic manipulations of single cell lineages (1, 2). *Drosophila* females have a pair of ovaries that are composed of 15–18 tube-like structures called ovarioles. Each ovariole produces eggs through a process called "assembly line". At the anterior tip of each ovariole is the germarium (Fig. 1A). Germline cells, known as germ cells, reside at the anterior end of the germarium in contact with two to three germ line stem cells (GSCs). A GSC divides asymmetrically to produce a new stem cell and a cystoblast, which divides four more times with incomplete cytokinesis to generate a cyst of 16 germ line cells (1). Starting in region 2b (Fig. 1A), each cyst is enveloped by a monolayer of follicle cells and is then separated from the next cyst by a short stalk as it buds from region 3 to form an egg chamber, which then progresses down the ovariole, increasing in size and maturity, and becomes an egg.

The follicle cells and stalk cells are derived from the follicle stem cells (FSCs) (Fig. 1A). Two FSC niches exist within each ovariole at the 2a/2b border region of the germarium (3). FSCs self-renew and produce “prefollicle” daughters, most of which proliferate for about eight cycles until reaching stage 6, before three cycles of endoreplication and overt differentiation (4–6). A wild-type, genetically marked FSC generally proliferates continuously to produce a marked lineage extending throughout an ovariole. FSCs have a half-life of ~2 weeks and are replaced by the daughters of another FSC in the same germarium (3, 4, 6). Early studies showed that signaling pathways including Hedgehog (Hh), Wnt, and BMP are important regulators of FSCs (6–9). FSC maintenance also depends on contacts with the niche through the adherens junction molecules DE-cadherin and through the integrin ligand laminin A and its receptor (10, 11). However, the molecular mechanisms that regulate FSC behavior and niche interactions remain largely unknown.

In a genetic screen for intrinsic factors selectively required for FSC maintenance, we obtained a hypomorphic *cyclin E* allele *WX*. Through studying this allele and other designed *Cyclin E* (CycE) variants, we found that the CycE-Cdk2 kinase activity required for FSC maintenance is higher than for proliferation of follicle cells or maintenance of GSCs. Furthermore, FSCs deficient for CycE function did not arrest proliferation or die by apoptosis, but were lost from the niche and could be partially rescued by excess DE-cadherin, suggesting that CycE-Cdk2 activity is a regulatory node that integrates FSC proliferation with niche retention.

**Results**

**CycE Allele That Selectively Impairs FSC Function.** In a screen for genes selectively required for FSC maintenance (see Materials and Methods) we isolated an allele we named WX. We made clones homozygous for WX in the ovary using the heat shock induced FRT/FLP technique (12) and counted the percentage of ovarioles containing WX clones as a measure of the survival of functional FSCs over time. From 9–18 days after heat-shock young adults, the percentage of ovarioles containing WX FSC clones was much lower than for control (wt) clones (Fig. 1B), indicating a severe FSC defect.

Up to 5 days after heat-shock, transient clones induced in prefollicle and follicle cells are still retained in ovarioles (4). Five days after heat-shock, we found many WX mutant clones and those WX clones in stage 10 egg chambers were similar in size to wild-type twin-spot clones generated simultaneously (Fig. 1D), indicating that the WX allele does not markedly impair follicle cell proliferation or survival. By measuring the frequency of germ line clones 12, 15, and 18 days after heat-shock, we also found that WX does not affect GSC maintenance (Fig. 1C). Hence, the WX allele selectively impairs FSC maintenance.

Animals homozygous for WX are embryonic lethal. Using recombination and deficiency mapping, WX was found to be at chromosome position 35D and failed to complement the lethality of *cyclin E* null alleles. Expression of CycE in WX FSCs was able to fully restore FSC maintenance (see below), confirming that FSC dysfunction is due to loss of CycE activity. CycE has previously been...

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performed RT-PCR. We found that the predicted splicing variant was the only altered product and was present at roughly the same level as mRNA from the wild-type allele.

The normal functions of CycE require high activity at some phases of the cell cycle and low activity at others, which is achieved partly through regulated protein degradation (14). Regulated degradation of mammalian CycE is achieved partly through phosphorylation-dependent binding of an SCF complex to a C-terminal region phosphorylated by CycE-Cdk2 and glycogen synthase kinase 3. This mechanism is broadly conserved among yeast, flies, and mammals (15–17), and the conserved “LTPP” destruction box is missing in the CycE<sup>Wx</sup> protein (Fig. 2A), suggesting possible defects in protein degradation. To test this possibility, we used a CycE antibody to stain ovary and wing disc tissues containing cycE<sup>Wx</sup> clones. CycE<sup>Wx</sup> levels were generally similar to wild-type CycE levels in twin-spot clones; uniform in wing discs (Fig. S2D) and stage 10A egg chambers, and mosaic in earlier egg chambers (Fig. S2B), suggesting that regulation according to the cell cycle was retained (18). Abnormally strong nuclear CycE staining of cycE<sup>Wx</sup> mutant clones was seen only in stage 10B and later egg chambers (Fig. S2C), suggesting inefficient degradation of CycE<sup>Wx</sup> after the end of endo-replication cycles (19). The nuclear localization of CycE<sup>Wx</sup> in these clones clearly shows that this protein can access the nucleus.

**CycE<sup>Wx</sup> and Designed CycE Variants Are Deficient for CycE-Cdk2 Kinase Activity.** CycE has diverse cellular functions in eukaryotes, many of which require CycE to bind and activate the cyclin-dependent kinase catalytic subunit Cdk2 (20). Notable kinase-independent functions include stimulating the reentry of murine G0 cells into the cell cycle (21, 22). Mammalian CycE contacts Cdk2 extensively, particularly through the N- and C-terminal cyclin boxes (Fig. 2A). These interactions promote the activated conformation of Cdk2 (23). While CycE<sup>Wx</sup> retains all residues analogous to those that contact Cdk2 in mammalian CycE (23), it might have an altered conformation that results in weaker stimulation of Cdk2 kinase activity.

We tested this possibility by making a cycE<sup>Wx</sup> mutant expression construct. The Drosophila cycE gene gives rise to two polypeptides (CycEI and CycEII) with different N-terminal sequences preceding the cyclin box, but each is capable of inducing cell proliferation (24). CycE<sup>Wx</sup> or wild-type CycEII tagged with Flag and HA epitopes were expressed in *Drosophila* S2 cells together with HA-tagged Cdk2. We immunoprecipitated CycE<sup>Wx</sup> with Flag beads followed by Western blot analysis for HA-Cdk2 and found that CycE<sup>Wx</sup> bound as well as wild-type CycE to Cdk2 (Fig. 2B). To test whether CycE<sup>Wx</sup> could activate Cdk2 kinase activity, we immunoprecipitated CycE<sup>Wx</sup> in the presence of HA-Cdk2 and performed in vitro kinase assays with histone H1 as a substrate. CycE<sup>Wx</sup> was able to stimulate histone H1 phosphorylation but was not as active as wild-type CycE (Fig. 2B). The ability of CycE<sup>Wx</sup> to bind Cdk2 without effectively stimulating kinase activity is shared by a human CycE variant lacking the C-terminal 45 residues (25).

To test whether reduced kinase activity can account for the cycE<sup>Wx</sup> FSC defect, we made a series of CycE variants predicted to alter CycE-Cdk2 kinase activity (Fig. 2A). CycE<sup>SA</sup> has five amino acids 313–317 in a Cdk2 binding pocket altered to alanine. The analogous human protein was shown to bind Cdk2 only in the presence of a CDK inhibitor, as part of a trimeric complex, suggesting possible defects in protein degra-
Arg-255 was changed to alanine. The analogous human R130A variant is cited as being unable to bind to Cdk2 or support Cdk2 kinase activity, even though R130 does not contact Cdk2 directly (23, 25, 26). We found that Drosophila CycER5A did not support kinase activity but did bind to Cdk2 (Fig. 2B). Although other factors can impact CycE-Cdk2 activity in vivo, our in vitro studies suggest that CycER5A and CycER5RA will be highly deficient or null for kinase activity, whereas CycER5A and CycER5X will have substantially reduced CycE-Cdk2 kinase activity in flies.

**Higher CycE-Cdk2 Kinase Activity Is Required for FSC Maintenance than for Follicle Cell Proliferation.** We used UAS-cycE transgenes for the CycE variants described above for in vivo FSC rescue assays to ask whether the cycER5X FSC defect was due to reduced CycE-Cdk2 kinase activity. We expressed the CycE proteins at two different levels and tested their ability to rescue cycER5X (null) and cycER5X FSCs. In the first test, we used tub-GAL4 (27) to express CycE proteins. These clones were marked by loss of a tub-lacZ marker. In the second test, we added a second GAL4 driver (act>y>Gal4) to enhance CycE and GFP expression levels, permitting positive clone marking by GFP (28).

Transient cycER5X mutant clones were rescued well by wild-type, WX, and 5A CycE variants, but not at all by 7A and RA variants (Fig. 3B), indicating that follicle cell proliferation requires CycE-Cdk2 kinase activity. Wild-type CycE rescued both cycER5X and cycER5X FSC function (Fig. 3A). 7A and 5A CycE variants did not rescue cycER5X FSCs at all (Fig. 3A), suggesting that those FSCs are impaired specifically because they are deficient for CycE-Cdk2 kinase activity.

High levels of CycER5X rescued cycER5X FSCs to a small degree, producing a phenotype very much like cycER5X FSCs. This suggests that the higher level of transgene expression was roughly functionally equivalent to endogenous levels of CycE expression. Remarkably, expression of CycER5X at this level, or even at a lower level, provided quite robust rescue of cycER5X FSCs (Fig. 3A). CycER5A behaved similarly to CycER5X, but provided more robust rescue of cycER5X FSCs. These observations suggest that CycER5X has a quantitative dysfunction. That dysfunction appears to be in its ability to stimulate Cdk2 kinase activity, because a CycE variant (5A) designed specifically to affect Cdk2 binding and limit kinase activity shares similar properties with CycER5X of low in vitro kinase activity, supporting follicle cell proliferation, and failing to support FSC maintenance when expressed at low levels (Fig. 3A).

CycE-Cdk2 Inhibitor and Effector Modify FSC Behavior. We manipulated the dosage of the CycE-Cdk2 inhibitor Dacapo (Dap) to see how it affected FSCs and follicle cell proliferation. We found that heterozygosity for a dap null allele partially rescued the persistence of cycER5X mutant FSCs, measured 12 days after clone induction in both adults (from 9 to 31% of oocytes) and larvae (from 1 to 15% of oocytes). We used alleles of two other genes required selectively for FSC function (CG31739 and CG8674, to be described elsewhere) in parallel experiments and.
found no rescue of FSC function by reducing the dosage of dap, implying that CycE-Cdk2 kinase activity is only limiting for FSC function in the cycE<sup>WX</sup> clones (Fig. S3A).

We increased Dap expression in FSC lineages by expressing a UAS-Dap transgene in positively marked clones. Excess Dap alone did not impair proliferation of follicle cells (Fig. 3D). FSCs that are lost are normally replaced by progeny of neighboring FSCs, so ovarian cells initially containing marked and unmarked FSCs are converted over time into ovarioles with either two or zero marked FSCs, in roughly equal proportions when all FSCs are wild-type (6). Very few ovarioles accumulated more than one FSC overexpressing Dap, indicating a modest FSC defect, but FSCs overexpressing Dap were generally maintained well over time (Fig. S3B). Excess Dap did, however, completely suppress the formation of large transient cycE<sup>WX</sup> follicle cell clones (Fig. 3C). Thus, increased Cdk inhibitor blocks follicle cell proliferation, while decreased Cdk inhibitor partially restores FSC function for cycE<sup>WX</sup> cells, further supporting the idea that cycE<sup>WX</sup> cells have CycE-Cdk2 kinase activity intermediate between that required for follicle cell proliferation and for FSC maintenance.

A major target of CycE-Cdk2 activity in promoting the G1-S phase transition is the Retinoblastoma protein (Rb), phosphorylation of which triggers the release of E2F/DP transcriptional activators and induction of many genes encoding S-phase functions, including cycE (22, 29, 30). Null alleles of E2F1 do not support normal cell proliferation (31). However, clonal expression of an RNAi transgene directed toward E2F1 (32) significantly impaired FSC maintenance (Fig. 4F) without reducing the size of 5-day transient follicle cell clones (Fig. 3E). Thus, FSCs appear to have a selectively high requirement for E2F1 activity, just as for CycE activity. Excess E2F1/DP, excess CycE, or loss of dap produced no major changes in FSC function (Figs. S3B and S4). However, overexpressing E2F1 and DP in FSC clones led to strikingly good rescue of cycE<sup>WX</sup> FSC clone maintenance (Fig. 4F), without affecting an unrelated FSC-selective mutation, CG31739 (Fig. S4). This result might reflect indirect rescue of cycE<sup>WX</sup> by inducing greater cycE<sup>WX</sup> transcription, since cycE is a target for E2F/DP in many species. We therefore repeated the test in cycE null clones, where UAS-CycE<sup>WX</sup> was expressed under the influence of drivers (tub-GAL4, act-GAL4) likely to be insensitive to E2F/DP. We again observed significant rescue of FSC maintenance by E2F1/DP overexpression (Fig. 4F), suggesting that Rb is a critical target for the FSC-selective function of CycE-Cdk2.

**CycE<sup>WX</sup> FSCs Are Rapidly Lost from Their Niches.** Given the critical role of CycE in driving S phase progression, we asked whether cycE<sup>WX</sup> FSCs were defective because they were cell cycle arrested. We used positive marking to visualize potential isolated quiescent FSCs from 9 to 18 days after clone induction. Over this time period, most wild-type FSCs continue to proliferate, so that GFP-positive cells stretch from the FSC throughout the ovariole (Fig. 4A, “1 FSC + clone”), while the proportion of ovarioles with isolated GFP-positive active FSCs (Fig. 4B, “2 FSC + clone”) slowly increases (Fig. 4E), presumably due to neighboring FSC loss and replacement. FSCs can be distinguished from their progeny by their more anterior position in the 2a/2b region and by the absence of Fas III expression (6). The characteristic shape and position of FSCs at the edge of the germarium allows them to be distinguished from more anterior or central marked escort cells, which are usually found in the company of their marked escort stem cell progenitors (3). For wild-type FSC clones, there were a few ovarioles with GFP-positive follicle cells but no FSC (Fig. 4C, “0 FSC + clone,” orange in Fig. 4E), indicating FSC loss in the last 3–5 days, and very few ovarioles with isolated marked FSCs (Fig. 4D, “1 FSC-clone,” white in Fig. 4E),
indicating long-term FSC quiescence. Surprisingly, we did not observe a significant increase in the frequency of isolated, quiescent marked FSCs (white in Fig. 4E) when CyclE activity was compromised. Instead, cyclE\textsuperscript{WX} FSCs were simply lost from ovarioles over time, transiently increasing the 0 FSC + clone category (orange in Fig. 4E) and then leading to the complete absence of marked cells (gray in Fig. 4E). We also examined negatively marked cyclE\textsuperscript{WX} FSC clones from 7 days onwards, looking carefully for isolated mutant FSCs, but again we did not see a significant proportion of such ovarioles. Thus, we did not observe arrested FSCs in response to reduced CyclE activity.

The rapid loss of cyclE\textsuperscript{WX} FSCs could be due to apoptosis, release from the FSC niche, or both. We saw no evidence of elevated apoptosis using a TUNEL assay, and we found no rescue of cyclE\textsuperscript{WX} FSCs by overexpressing the apoptosis inhibitor, DIA1p (Fig. 4F). DIA1p fully rescued the maintenance of FSCs mutant for another FSC-selective gene, CG31739, which did elicit elevated TUNEL staining (Fig. S4). While other cell death pathways might be activated in cyclE\textsuperscript{WX} FSCs, it seems more likely that those FSCs simply leave the niche at accelerated rates and then proliferate as nonstem cell daughters. We reasoned that strengthening the contact of cyclE\textsuperscript{WX} FSCs with their niches might rescue their loss. We therefore expressed excess DE-Cadherin, a molecule known to contribute to FSC-niche adhesion (11), in cyclE\textsuperscript{WX} FSCs and found that FSC maintenance was substantially rescued (Fig. 4F). By contrast, overexpressing DE-Cadherin in CG31739 FSCs had no rescue effect (Fig. S4). Importantly, ovarioles either contained rescued, proliferating ("FSC + clone") cyclE\textsuperscript{WX} FSCs overexpressing DE-cadherin or no such marked FSCs, but did not contain a significant proportion of quiescent FSCs. Thus, the primary defect due to cyclE\textsuperscript{WX} appears to be stem cell loss from the niche, not arrest followed secondarily by stem cell loss.

Discussion

An important unanswered question is whether special cell cycle machineries or connectivities exist in stem cells to accommodate their characteristic properties. The Drosophila ovary provides an attractive system to study this question because it houses more than one type of stem cell and allows measurement of the behavior of both stem cells and their progeny as a result of lineage-specific genetic manipulations. Hence, we were able to demonstrate that a hypomorphic CyclE allele strongly impaired FSC function without disrupting the proliferation and survival of either follicle cells or GSCs. These comparisons are especially informative because FSC and GSC function are measured by essentially identical methods in the very same tissue, while FSCs and their proliferating daughters likely have extremely similar gene expression profiles. We therefore confidently conclude that CyclE has an FSC-specific function.

By looking at additional cyclE alleles and other tissues, it is apparent that CyclE has other tissue-specific functions. For example, all five hypomorphic alleles we examined (WX, If36, P28, P28, and JP) produced homozygous mutant clones of reduced size in wing discs compared to twin-spot clones (Fig. S1), while different combinations of these alleles produced organismal lethality, female sterility, or roughened eyes (Table S1). Only a null cyclE allele failed to support proliferation of all cell types tested. Thus, there appear to be different thresholds of CyclE activity required to support different cellular activities. Is this because different levels of CyclE activity are required to accomplish the same goal of driving cell cycles in different cell types or because CyclE additionally accomplishes disparate cell-type-specific tasks? In wing discs, cyclE\textsuperscript{WX} mutant clones were substantially enlarged when juxtaposed with cells harboring a heterozygous \textit{Minute} mutation (Fig. S1), suggesting that the cyclE\textsuperscript{WX} proliferation defect is related to cell competition (33) and not to an intrinsic cell cycle defect. What is the nature of the primary cyclE\textsuperscript{WX} FSC defect?

The critical function for CyclE in Drosophila FSCs appears to be activation of high CyclE-Cdk2 kinase activity. The key supporting evidence is the reduced kinase activity of CyclE\textsuperscript{WX}-Cdk2 in vitro, the FSC-selective defect of other cyclE alleles designed to have reduced kinase activity, and the partial rescue of the cyclE\textsuperscript{WX} FSC defect by reducing CDK inhibitor activity. CyclE-Cdk2 kinase has many targets, but phosphorylation of Rb family proteins is known to be critical in activating E2F transcription factors and inducing a variety of genes required for progression into and through S phase. As for CyclE, reduced E2F1 function also produced a strong defect in FSC maintenance without impairing follicle cell proliferation. Furthermore, overexpression of E2F1 together with its obligate partner DP did not rescue proliferation of null cyclE cells, but did rescue the cyclE\textsuperscript{WX} FSC defect, even when CyclE\textsuperscript{WX} was expressed from a heterologous, likely E2F-insensitive, promoter. Thus, E2F1 appears to be a critical effector for the FSC-selective function of CyclE. Is that function to drive FSC cell cycles?

We did not observe quiescent cyclE\textsuperscript{WX} FSCs. We also found no evidence for apoptosis of cyclE\textsuperscript{WX} FSCs. Instead, cyclE\textsuperscript{WX} FSCs appear simply to be lost from the niche, likely acquiring the properties of nonstem cell prefollicle daughter cells. That conclusion is also supported by the partial rescue of FSC maintenance by overexpression of DE-cadherin in cyclE\textsuperscript{WX} FSCs. DE-cadherin is known to contribute to niche retention of normal FSCs (11). In these rescue experiments, we observed only proliferating FSCs, suggesting that cyclE\textsuperscript{WX} FSCs are intrinsically capable of continued proliferation and that their loss from the niche is a primary deficit and not a secondary consequence of a transient, unseen cell cycle arrest.

We therefore suggest that one or more E2F1-DP transcriptional targets are genes that promote FSC-niche adhesion and that the E2F1-DP activity required to induce such genes sufficiently for normal FSC-niche adhesion is higher than that required to induce genes necessary to support the G1-S phase transition and progress through S phase. We further suggest that the reason for establishing a link between CyclE-Cdk2 activity and adhesion genes may be to ensure that only FSCs that cycle robustly are retained.

Other types of stem cells might exhibit analogous connections where continuous proliferation is an important characteristic. However, if other aspects of stem cell function, such as error-free DNA replication in GSCs or extended longevity in quiescent mammalian hematopoietic stem cells, are a higher priority, we might expect regulatory circuitry linking cell cycle inputs positively to niche retention to be absent. There are several reports of altered function of mammalian cyclin-dependent kinases or their inhibitors selectively affecting stem cell function (34, 35), but the origin of those deficits has been hard to define. Our studies in a more favorable setting forecast the possibility that stem cell retention in the niche is the key property being controlled by cell cycle regulators. Our studies also revealed a quantitative connection between the degree of CyclE dysfunction and the rate of loss of FSCs. In other settings, this quantitative connection may allow stem cells with excessively high CyclE/Cdk2 activity, acquired through mutational changes, to be retained especially well and hence become amplified as a stable, precancerous stem cell population.

Materials and Methods

\textit{Drosophila} Stocks and Clonal Analysis. The screen for FSC-selective functions (to be described elsewhere) involved rescue of egg-laying by FSC homozygous for chromosome arm 2L after EMS mutagenesis. Alleles, including cyclE\textsuperscript{WX}, were retained if homozygous mutant clones produced severe FSC defects without markedly impairing growth and survival in other cell types. Other mutant cyclE alleles, described on FlyBase were supplied by the Bloom-
ingston Stock Center, Mary Lilly, and Terry Orr-Weaver. UAS-DE-Cadherin was from Ulrich Tepass, UAS-Dap from Bruce Edgar, and UAS-E2F1, UAS-AP, and UAS-E2F1-RNAi from Nicholas Dyson. Larvae or adult flies of the appropriate genotype were heat-shocked for 1 h at 37 °C to induce negatively or positively marked clones as in (28). Genotypes for positive-marking and expression of a transgene were of the general form, yw hs-ﬂp UAS-GFP tub-GAL4/yw; yw hs-FLP UAS-GFP tub-GAL4/yw; or yw hs-FLP UAS-GFP tub-GAL4/yw; or yw hs-FLP UAS-GFP tub-GAL4/yw; or so that transgene expression was restricted to the marked clone. Each genotype was repeated three times, scoring at least 80 ovarioles for each measurement.

RT-PCR, Plasmids, and Cloning. Heterozygous cveWv fly total mRNA was extracted using RNeasy Mini kit and Oligotex mRNA Mini kit (Qiagen) and then reverse-transcribed using OstepRT-PCR kit (Qiagen), followed by sequencing of the products. Constructs for Drosophila germline transformation and tissue culture cell transfection were made using Gateway Technology (Invitrogen). The cDNA of DmCycE was a gift from Christian Lehner (Zurich, Switzerland). Drosophila cDNA of Cdk2 was purchased from the Drosophila Genomics Resource Center (DGRC). The cDNAs of DmCycE and Cdk2 were inserted into the pENTR-D/TOPO vector using “TOPO cloning.” The destination vector used for germline transformation was pWV from the Drosophila Gateway Vector Collection. Destination vectors used for tissue culture transfection were pAAMW, pAHW, and pAFHW. Mutations were made in pENTR-D/TOPO using the QuikChange Site-Directed Mutagenesis kit (Stratagene), and products were sequenced in their entirety.

Immunoprecipitations, Western Blotting, and In Vitro Kinase Assay. S2 cells were cultured at 25 °C in Schneider’s Drosophila media plus 10% FBS plus 1% penicillin-streptomycin (Gibco-BRL). Several 100-mm plates were seeded with 1 × 10^6 cells in a volume of 10 mL media and were allowed to adhere overnight. Three to four hours before transfection, cells were given fresh media. Ten micromgs of each vector were transfected using the standard calcium phosphate protocol (Invitrogen), and after 24 h, the cells were given fresh media. Cells were allowed to express for approximately another 60 h and were then lysed at 4 °C for 1 h in 5 mL lysis buffer [50 mM Hepes, pH 7.5, 0.2 mM EDTA, 250 mM NaCl, 0.5% Nonidet P-40, protease inhibitor (Mini complete; Roche)]. The lysates were incubated with anti-mouse Flag antibody conjugated to agarose beads (Sigma). The IPs were washed three times for 10 min each with lysis buffer. Western blots were probed with antibodies against c-Myc (9E10, Santa Cruz Biotechnology) and HA (ab9110, Abcam). For in vitro kinase assays, IPs were incubated with 15 μL 20 mM Tris, pH 7.5, 5 mM MgCl2, 2.5 mM MnCl2, 1 mM DTT, 0.013 mM ATP, 2 μCi [γ-32P]ATP, and 2.5 μg histone H1 (Roche) for 30 min at 30 °C. The relative kinase activity was quantified using Adobe Photoshop and Odyssey programs.

Histology, Immunostaining, and Fluorescence Microscopy. TUNEL assays were performed using In Situ Cell Death Detection Kit, TMR red (Roche). Drosophila ovaries and wing discs were dissected and stained according to (28) using anti-Fasciclin III (mouse, 1:200; Developmental Studies Hybridoma Bank) and anti-Cyclin E (guinea pig polyclonal serum, 1:1,000; a gift from Tom Neufeld, Minneapolis, MN) and Alexa secondary antibodies (Molecular Probes) at 1:1,000. All samples were examined using a Bio-Rad MRC-600 confocal microscopy system (Bio-Rad Laboratories).

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Fig. S1. Wing disc and FSC clone defects for cycE alleles. (A and B) (A) WX and (B) PZ8 wing disc clones (no green) 3 days after induction are smaller than twin-spots (brighter green). (C) Large WX wing disc clones (no green) can be found in a Minute heterozygous background 4 days after induction. (D) Percentage of ovarioles that retain FSC clones 12 days after heat-shock induction in adults (HSA) or third instar larvae (HSL) for wild-type (wt) and named cycE alleles. Error bars are SD with \( n = 3 \).
Fig. S2. CycE^{WX} has an altered C terminus that does not greatly alter its stability. (A) CycE (top line) splice acceptor “ag” is altered to “aa” (red) in cycE^{WX} (second line). RT-PCR confirmed a single nucleotide shift in the splice acceptor, altering the reading frame to produce a shorter protein with an altered C terminus (purple) encoding the amino acids, TKCLWLRMRRMPCALGFKLPRPPRCALPKVF. (B–D) CycE protein (red) in (B) stage 6 or (C) stage 11 egg chambers, and (D) third instar wing discs containing cycE^{WX} clones (no green GFP; arrows in B).
Fig. S3. The effects of Dacapo dosage on FSCs. (A) Percentage of ovarioles that retain FSC clones 12 days after heat-shock induction in adults (HSA) or third instar larvae (HSL) for wild-type (wt) and named mutations in otherwise wild-type (+) or dap heterozygous (+/-) animals. (B) Percentage of ovarioles with FSC clones containing two (black) or one (brown) positively marked FSC plus follicle cells, marked follicle cells only (orange), a marked FSC but no marked follicle cells (white), or no marked cells of the FSC lineage (gray) 12 or 18 days after induction of wt clones expressing no transgene, UAS-Dap or UAS-CycE. Error bars in (A and B) are SD with n = 3.
Fig. S4. Rescue of CG31739 FSC function and effects of E2F1 and dap on wild-type FSCs. Percentage of ovarioles that retain FSC clones 12 days after heat-shock induction in adults (HSA) or third instar larvae (HSL) for wild-type (wt) and CG31739 alleles expressing different transgenes or in dap heterozygous (+/-) animals. Error bars are SD with n = 3.
### Table S1. Complementation among cycE alleles

<table>
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<tr>
<th>cycE allele</th>
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<th>P28</th>
<th>1F36</th>
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<th>AR95</th>
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<td>viable</td>
<td>viable fertile</td>
<td>viable fertile</td>
<td>viable fertile</td>
<td>viable fertile</td>
<td>viable fertile</td>
</tr>
<tr>
<td>PZ8</td>
<td>lethal (another lethal present)</td>
<td>viable female semi-sterile; few eggs</td>
<td>viable (75%) female semi-sterile; few eggs</td>
<td>viable (60%) female sterile; very few eggs</td>
<td>viable female sterile; many eggs</td>
<td>80% lethal female sterile; no eggs</td>
</tr>
<tr>
<td>P28</td>
<td>viable female semi-sterile; no eggs</td>
<td>viable female semi-sterile; many eggs</td>
<td>viable female sterile; many eggs</td>
<td>viable female sterile; many eggs</td>
<td>viable female sterile; many eggs</td>
<td>viable female sterile; many eggs</td>
</tr>
<tr>
<td>1F36</td>
<td>lethal</td>
<td>lethal</td>
<td>lethal</td>
<td>lethal</td>
<td>lethal</td>
<td>lethal</td>
</tr>
<tr>
<td>WX</td>
<td>lethal</td>
<td>lethal</td>
<td>lethal</td>
<td>lethal</td>
<td>lethal</td>
<td>lethal</td>
</tr>
<tr>
<td>AR95</td>
<td>lethal</td>
<td>lethal</td>
<td>lethal</td>
<td>lethal</td>
<td>lethal</td>
<td>lethal</td>
</tr>
</tbody>
</table>

Results of complementation tests using WX and AR95 alleles on FRT40A chromosomes, and the four other alleles as originally supplied and after recombination with FRT40A (both gave similar results; means presented here). Alleles are tabulated in increasing order of strength (left to right) according to FSC maintenance defects. That order is similar to the order of allelic strengths based on lethality and egg-laying of sterile females (except that PZ8 appears stronger than P28 by the latter criteria). All viable transheterozygotes also had rough eyes to different degrees.