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^{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^{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^{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 an assembly line process. At the anterior tip of each ovariole is the germarium (Fig. 1*A*). Nonproliferating somatic cells, known as terminal filament and cap cells, reside at the anterior end of the germarium in contact with two to three germline stem cells (GSCs). A GSC divides asymmetrically to produce a new stem cell and a cystoblast, which divides four more times with incomplete cytokenesis to generate a cyst of 16 germline cells (1). Starting in region 2b (Fig. 1*A*), 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. 1*A*). Two FSC niches exist within each ovariole at the 2a/2b border region of the germarium (3). FSCs self-renew and produce "prefollicle cell" 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. 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-shocking young adults, the percentage of ovarioles containing *WX* FSC clones was much lower than for control (wt) clones (Fig. 1*B*), 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. 1*D*), indicating that the *WX* allele does not markedly impair follicle cell proliferation or survival. By measuring the frequency of germline clones 12, 15, and 18 days after heat-shock, we also found that *WX* does not affect GSC maintenance (Fig. 1*C*). 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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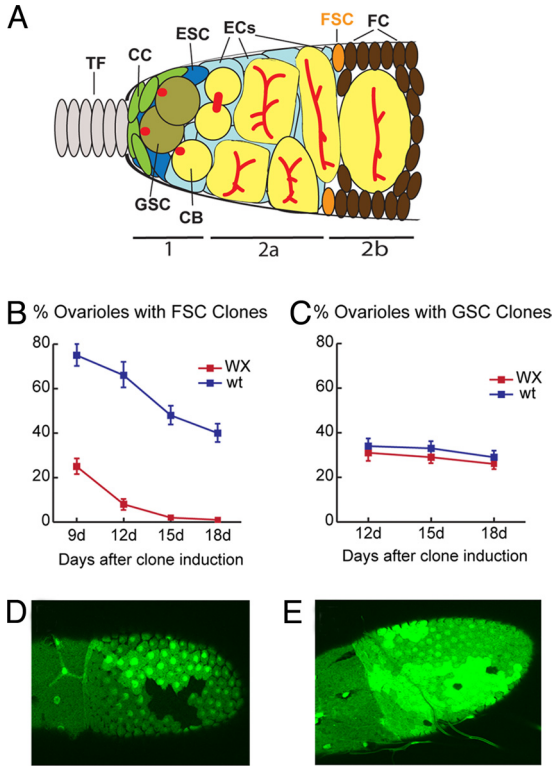

Fig. 1. A *cycE* allele that selectively impairs FSC function. (*A*) A *Drosophila* germarium harbors GSCs (olive) and escort stem cells (blue, ESCs) next to terminal filament (TF) and cap cells (green, CC). FSCs (orange) reside at the border of region 2a and 2b, anterior (left) to their FasIII staining (brown, FC) progeny, which proliferate and differentiate into follicle cells enveloping germline cysts (yellow) and stalk cells, separating egg chambers. (*B* and *C*) Percentage of ovarioles that retain wild-type (wt) or *WX* mutant (B) FSC clones and (*C*) GSC clones 9–18 days after induction in young adults. Error bars are SD with $n = 3$. (*D* and *E*) Transient follicle cell clones 5 days after induction, marked by loss of GFP (green) are as large as twin-spots (two copies of GFP; brighter green) for (*D*) *WX*, but (*E*) tiny or absent for the null *AR95 cycE* allele in stage 10 egg chambers.

shown to be essential for cell proliferation (13). Accordingly, the *AR95* and *KG00239* null alleles produced tiny or no transient follicle cell clones 5 days after heat-shock (Fig. 1*E*) and no FSC clones 12 days after heat-shock (Fig. S1*D*). $cycE^{WX}$ allows normal follicle cell proliferation, indicating that it is hypomorphic. We therefore tested other hypomorphic *cycE* alleles. *JP* and *PZ8* are P-element insertion alleles and did not impair FSC maintenance, while the chemically induced alleles *P28* and *1F36* showed significant FSC defects (Fig. S1*D*). None of these four hypomorphic *cycE* alleles reduced the size of transient follicle cell clones or GSC maintenance. From complementation tests assessing lethality and female sterility, *WX* and *1F36* appear to be the strongest alleles, *PZ8* and *P28* are of similar strength, and *JP* is the weakest of these alleles (Table S1). Thus, strong *cycE* hypomorphic mutations reduce FSC maintenance roughly according to allelic strength without affecting follicle cell proliferation or GSC maintenance.

$CycE^{WX}$ Has an Altered C Terminus. We extracted DNA from heterozygous *WX* flies and sequenced the *cycE* gene locus. An "AG" to "AA" point mutation at the acceptor site of the last intron-exon junction was identified (Fig. S2*A*). This mutation is expected to alter splicing. We predicted that the first "G" in the last exon might be used as part of a new AG splicing acceptor site, causing a frame-shift by one nucleotide and the original last 91 amino acids would be changed to 31 new amino acids (Fig. S2*A*). We extracted mRNA from heterozygous *WX* flies and 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 $CycE^{WX}$ protein (Fig. 2*A*), suggesting possible defects in protein degradation. To test this possibility, we used a CycE antibody to stain ovary and wing disc tissues containing $cycE^{WX}$ clones. $CycE^{WX}$ levels were generally similar to wild-type CycE levels in twin-spot clones; uniform in wing discs (Fig. S2*D*) and stage 10A egg chambers, and mosaic in earlier egg chambers (Fig. S2*B*), suggesting that regulation according to the cell cycle was retained (18). Abnormally strong nuclear CycE staining of $cycE^{WX}$ mutant clones was seen only in stage 10B and later egg chambers (Fig. S2*C*), suggesting inefficient degradation of $CycE^{WX}$ after the end of endo-replication cycles (19). The nuclear localization of $CycE^{WX}$ in these clones clearly shows that this protein can access the nucleus.

$CycE^{WX}$ 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. 2*A*). These interactions promote the activated conformation of Cdk2 (23). While $CycE^{WX}$ 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 $cycE^{WX}$ 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). $CycE^{WX}$ or wild-type CycEI tagged with Flag and HA epitopes were expressed in *Drosophila* S2 cells together with HA-tagged Cdk2. We immunoprecipitated $CycE^{WX}$ with Flag beads followed by Western blot analysis for HA-Cdk2 and found that $CycE^{WX}$ bound as well as wild-type CycE to Cdk2 (Fig. 2*B*). To test whether $CycE^{WX}$ could activate Cdk2 kinase activity, we immunoprecipitated $CycE^{WX}$ in the presence of HA-Cdk2 and performed in vitro kinase assays with histone H1 as a substrate. $CycE^{WX}$ was able to stimulate histone H1 phosphorylation but was not as active as wild-type CycE (Fig. 2*B*). The ability of $CycE^{WX}$ 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 $cycE^{WX}$ FSC defect, we made a series of CycE variants predicted to alter CycE-Cdk2 kinase activity (Fig. 2*A*). $CycE^{5A}$ 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, and failed to stimulate any detectable kinase activity (21). Surprisingly, *Drosophila* $CycE^{5A}$ bound well to Cdk2 and supported clearly detectable H1 kinase activity, albeit lower than for wild-type CycE (Fig. 2*B*). We therefore changed two more adjacent amino acids (311–312) to alanine in $CycE^{7A}$ and found this variant to be unable to associate with Cdk2 or support CycE-Cdk2 kinase activity (Fig. 2*B*). In a third variant ($CycE^{RA}$),

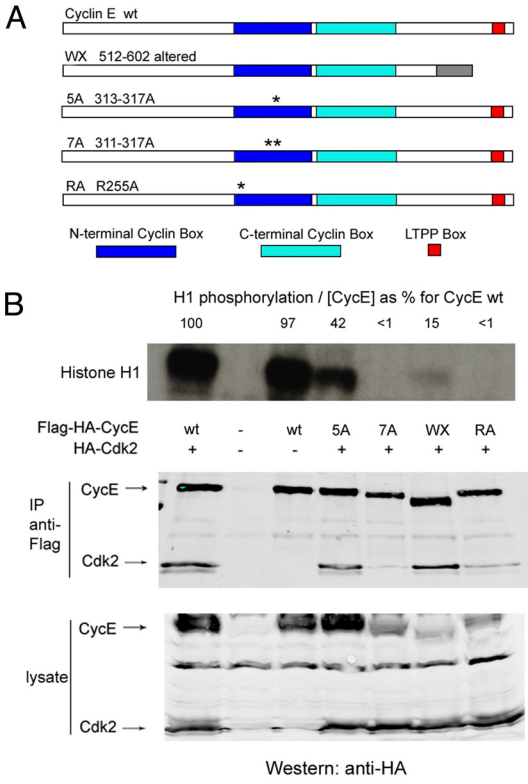

Fig. 2. Reduced CycE-Cdk2 kinase activity of CycE^{WX} and engineered CycE variants. (*A*) CycE variants with altered sequences depicted by gray box (*WX*) or asterisks. (*B*) Flag-HA-CycE variants coexpressed with HA-Cdk2 were immune-precipitated with Flag beads. One-half was incubated with histone H1 and [γ-^{32}P]ATP before SDS gel electrophoresis and autoradiography (*Top*), and the other half was probed with HA antibody after Western blot analysis (*Middle*). The amount of proteins in the cell lysates is shown in the *Bottom*. The ^{32}P signal was divided by the CycE IP signal and expressed as a percentage of that value for wild-type CycE to estimate kinase activity. In a repeat experiment, the analogous values were 100, 95, 36, <1, 21, <1, respectively.

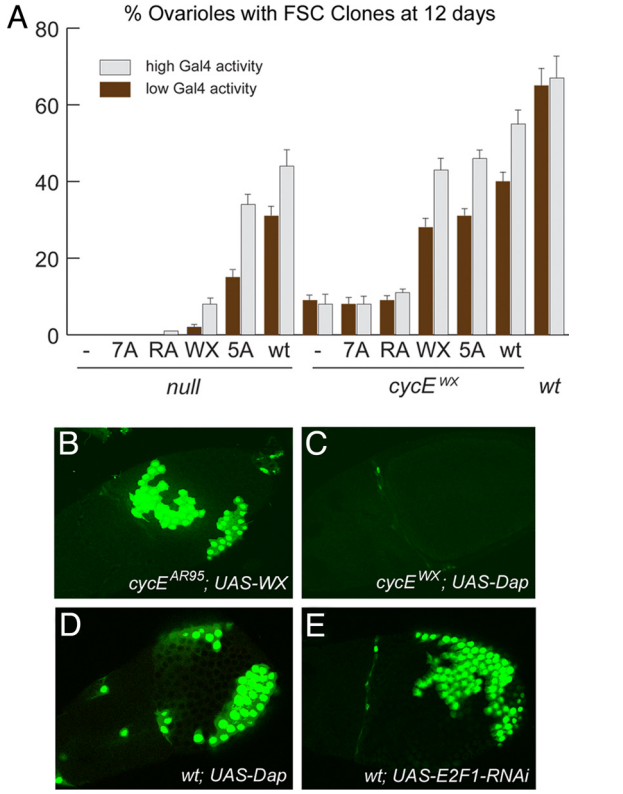

Fig. 3. FSC maintenance depends quantitatively on CycE-Cdk2 kinase activity. (*A*) Rescue of FSC maintenance measured by the percentage of ovarioles with *cycE^{AR95}* (null) or *cycE^{WX}* FSC clones when the indicated *UAS-CycE* variants are expressed in the mutant FSC lineage. *UAS-cycE* transgenes were expressed at low levels using *tub-GAL4* alone (brown) or at higher levels by also including *act>Gal4* (gray). (*B–E*) Positively marked 5-day transient follicle cell clones (green) are (*B*) restored by *UAS-CycE^{WX}* for *cycE^{AR95}*, (*C*) abolished by *UAS-Dap* for *cycE^{WX}*, and maintained in wt cells expressing (*D*) *UAS-Dap* or (*E*) *UAS-E2F1-RNAi*.

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* CycE^{RA} did not support kinase activity but did bind to Cdk2 (Fig. 2*B*). Although other factors can impact CycE-Cdk2 activity in vivo, our in vitro studies suggest that CycE^{7A} and CycE^{RA} will be highly deficient or null for kinase activity, whereas CycE^{5A} and CycE^{WX} 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 *cycE^{WX}* 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 *cycE^{AR95}* (null) and *cycE^{WX}* 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 *cycE^{AR95}* mutant clones were rescued well by wild-type, WX, and 5A CycE variants, but not at all by 7A and RA variants (Fig. 3*B*), indicating that follicle cell proliferation requires CycE-Cdk2 kinase activity. Wild-type CycE rescued both *cycE^{AR95}*and *cycE^{WX}* FSC function (Fig. 3*A*). The "kinase dead" CycE RA and 7A variants did not rescue *cycE^{WX}* FSCs at all (Fig. 3*A*), suggesting that those FSCs are impaired specifically because they are deficient for CycE-Cdk2 kinase activity.

High levels of CycE^{WX} rescued *cycE^{AR95}* FSCs to a small degree, producing a phenotype very much like *cycE^{WX}* FSCs. This suggests that the higher level of transgene expression was roughly functionally equivalent to endogenous levels of *cycE* expression. Remarkably, expression of CycE^{WX} at this level, or even at a lower level, provided quite robust rescue of *cycE^{WX}* FSCs (Fig. 3*A*). CycE^{5A} behaved similarly to CycE^{WX}, but provided more robust rescue of *cycE^{AR95}* FSCs. These observations suggest that CycE^{WX} 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 CycE^{WX} of low in vitro kinase activity, supporting follicle cell proliferation, and failing to support FSC maintenance when expressed at low levels (Fig. 3*A*).

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 *cycE^{WX}* mutant FSCs, measured 12 days after clone induction in both adults (from 9 to 31% of ovarioles) and larvae (from 1 to 15% of ovarioles). We used alleles of two other genes required selectively for FSC function (*CG31739* and *CG8674*, to be described elsewhere) in parallel experiments and

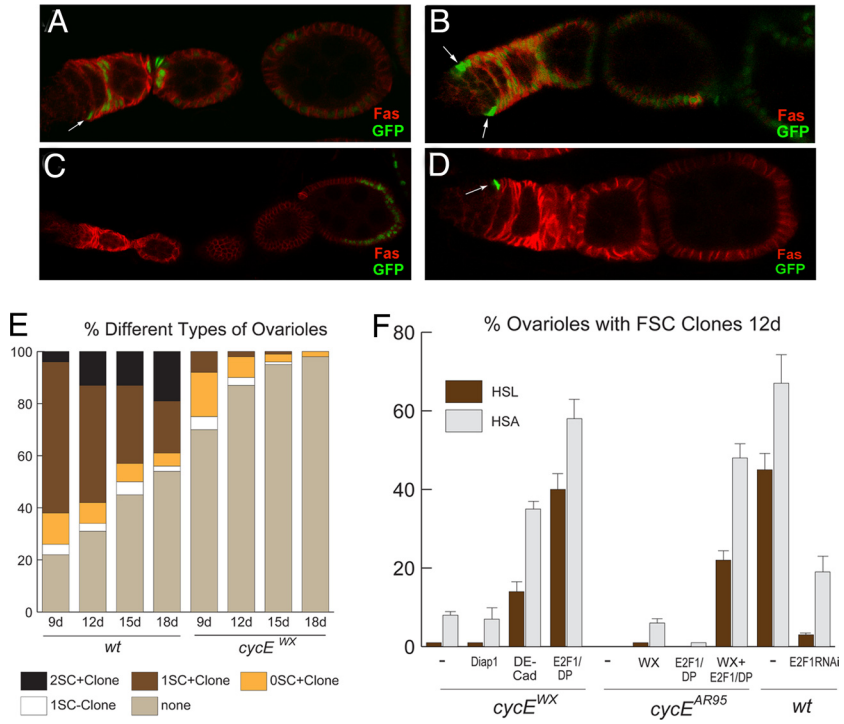

Fig. 4. *cycE*^{WX} FSCs are rapidly lost from their niches. (*A–D*) Ovarioles with positively marked FSC clones (green) and stained for Fasciclin III (red), which stains FSC progeny but not the FSC (arrows), showing clones with (*A*) one FSC and follicle cells, (*B*) two FSCs and follicle cells, (*C*) follicle cells but no FSC, (*D*) one FSC but no follicle cells. (*E*) 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) 9–18 days after induction in adults for wild-type (wt) or *cycE*^{WX}. (*F*) Rescue of larval (HSL) or adult (HSA) induced *cycE*^{WX} FSC clones by expression of indicated *UAS*-transgenes in the clones, measured 12 days after clone induction. Error bars are SD with $n = 3$.

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*^{WX} clones (Fig. S3*A*).

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. 3*D*). FSCs that are lost are normally replaced by progeny of neighboring FSCs, so ovarioles 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. S3*B*). Excess Dap did, however, completely suppress the formation of large transient *cycE*^{WX} follicle cell clones (Fig. 3*C*). Thus, increased Cdk inhibitor blocks follicle cell proliferation, while decreased Cdk inhibitor partially restores FSC function for *cycE*^{WX} cells, further supporting the idea that *cycE*^{WX} 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. 4*F*) without reducing the size of 5-day transient follicle cell clones (Fig. 3*E*). 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. S3*B* and S4). However, overexpressing E2F1 and DP in FSC clones led to strikingly good rescue of *cycE*^{WX} FSC clone maintenance (Fig. 4*F*), without affecting an unrelated FSC-selective mutation, *CG31739* (Fig. S4). This result might reflect indirect rescue of *cycE*^{WX} by inducing greater *cycE*^{WX} transcription, since *cycE* is a target for E2F/DP in many species. We therefore repeated the test in *cycE* null clones, where *UAS-CycE*^{WX} 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. 4*F*), suggesting that Rb is a critical target for the FSC-selective function of CycE-Cdk2.

***CycE*^{WX} FSCs Are Rapidly Lost from Their Niches.** Given the critical role of CycE in driving S phase progression, we asked whether *cycE*^{WX} 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. 4*A*, "1 FSC + clone"), while the proportion of ovarioles with two GFP-positive active FSCs (Fig. 4*B*, "2 FSC + clone") slowly increases (Fig. 4*E*), 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. 4*C*, "0 FSC + clone," orange in Fig. 4*E*), indicating FSC loss in the last 3–5 days, and very few ovarioles with isolated marked FSCs (Fig. 4*D*, "1 FSC-clone," white in Fig. 4*E*),

indicating long-term FSC quiescence. Surprisingly, we did not observe a significant increase in the frequency of isolated, quiescent marked FSCs (white in Fig. 4*E*) when CycE activity was compromised. Instead, $cycE^{WX}$ FSCs were simply lost from ovarioles over time, transiently increasing the 0 FSC + clone category (orange in Fig. 4*E*) and then leading to the complete absence of marked cells (gray in Fig. 4*E*). We also examined negatively marked $cycE^{WX}$ 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 CycE activity.

The rapid loss of $cycE^{WX}$ 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 $cycE^{WX}$ FSCs by overexpressing the apoptosis inhibitor, DIAP1 (Fig. 4*F*). DIAP1 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 $cycE^{WX}$ 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 $cycE^{WX}$ 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 $cycE^{WX}$ FSCs and found that FSC maintenance was substantially rescued (Fig. 4*F*). By contrast, overexpressing DE-Cadherin in *CG31739* FSCs had no rescue effect (Fig. S4). Importantly, ovarioles either contained rescued, proliferating ("FSC + clone") $cycE^{WX}$ 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 $cycE^{WX}$ 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 *cycE* 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 CycE has an FSC-specific function.

By looking at additional *cycE* alleles and other tissues, it is apparent that CycE has other tissue-specific functions. For example, all five hypomorphic alleles we examined (*WX*, *If36*, *P28*, *Pz8*, 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 *cycE* allele failed to support proliferation of all cell types tested. Thus, there appear to be different thresholds of CycE activity required to support different cellular activities. Is this because different levels of CycE activity are required to accomplish the same goal of driving cell cycles in different cell types or because CycE additionally accomplishes disparate cell-type-specific tasks? In wing discs, $cycE^{WX}$ mutant clones were substantially enlarged when juxtaposed with cells harboring a heterozygous *Minute* mutation (Fig. S1), suggesting that the $cycE^{WX}$ proliferation deficit is related to cell competition (33) and not to an intrinsic cell cycle defect. What is the nature of the primary $cycE^{WX}$ FSC defect?

The critical function for CycE in *Drosophila* FSCs appears to be activation of high CycE-Cdk2 kinase activity. The key supporting evidence is the reduced kinase activity of $CycE^{WX}$-Cdk2 in vitro, the FSC-selective defect of other *cycE* alleles designed to have reduced kinase activity, and the partial rescue of the $cycE^{WX}$ FSC defect by reducing CDK inhibitor activity. CycE-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 CycE, 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 *cycE* cells, but did rescue the $cycE^{WX}$ FSC defect, even when $CycE^{WX}$ was expressed from a heterologous, likely E2F-insensitive, promoter. Thus, E2F1 appears to be a critical effector for the FSC-selective function of CycE. Is that function to drive FSC cell cycles?

We did not observe quiescent $cycE^{WX}$ FSCs. We also found no evidence for apoptosis of $cycE^{WX}$ FSCs. Instead, $cycE^{WX}$ 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 $cycE^{WX}$ 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 $cycE^{WX}$ 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 CycE-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 CycE dysfunction and the rate of loss of FSCs. In other settings, this quantitative connection may allow stem cells with excessively high CycE/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

***Drosophila* Stocks and Clonal Analysis.** The screen for FSC-selective functions (to be described elsewhere) involved rescue of egg-laying by FSCs homozygous for chromosome arm 2L after EMS mutagenesis. Alleles, including $cycE^{WX}$, were retained if homozygous mutant clones produced severe FSC defects without markedly impairing growth and survival in other cell types. Other mutant *cycE* alleles, described on FlyBase were supplied by the Bloom-

ington Stock Center, Mary Lilly, and Terry Orr-Weaver. *UAS-DE-Cadherin* was from Ulrich Tepass, *UAS-Dap* from Bruce Edgar, and *UAS-E2F1*, *UAS-DP*, 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*; *cycE* (or +) *FRT40A/+* (or *tub-lacZ*) *tub-GAL80 FRT40A*; *UAS-X* (or +)/*act>CD2>GAL4* (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 $cycE^{WX}$ fly total mRNA was extracted using RNeasy Mini kit and Oligotex mRNA Mini kit (Qiagen) and then reverse-transcribed using Onestep RT-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 *DmcycEI* was a gift from Christian Lehner (Zurich, Switzerland). *Drosophila* cDNA of *Cdk2* was purchased from the *Drosophila* Genomics Resource Center (DGRC). The cDNAs of *DmcycEI* and *Cdk2* were inserted into the pENTR-D/TOPO vector using ''TOPO cloning.'' The destination vector used for germline transformation was pTW from the *Drosophila* Gateway Vector Collection. Destination vectors used for tissue culture transfection were pAMW, 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 Schneiders *Drosophila* media plus 10% FBS plus 1% penicillin-streptomycin (GibcoBRL). Several 100-mm plates were seeded with 1×10^7 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 micrograms 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 $MgCl_2$, 2.5 mM $MnCl_2$, 1 mM DTT, 0.013 mM ATP, 2 μCi [γ-^{32}P]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 microscope system (Bio-Rad Laboratories).

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Supporting Information

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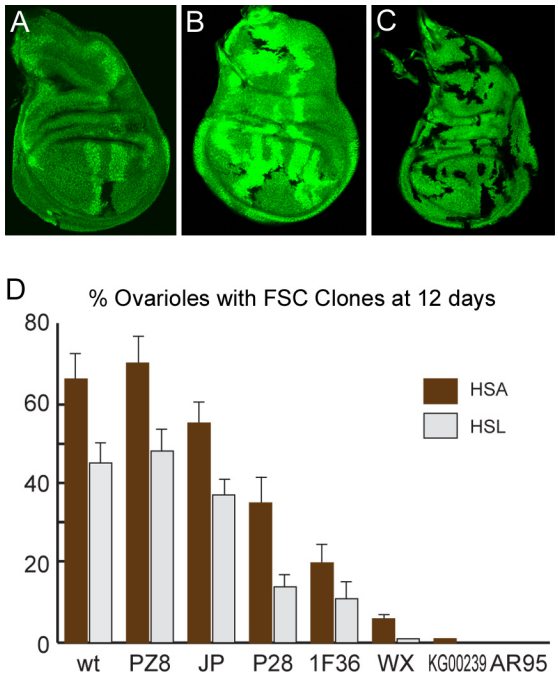

Fig. S1. Wing disc and FSC clone defects for *cycE* alleles. (*A* and *B*) (*A*) *WX* and (*B*) *PZ8* wing disc clones (no green) 3 days after induction are smaller than twin-spots (brighter green). (*C*) Large *WX* wing disc clones (no green) can be found in a *Minute* heterozygous background 4 days 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 $n = 3$.

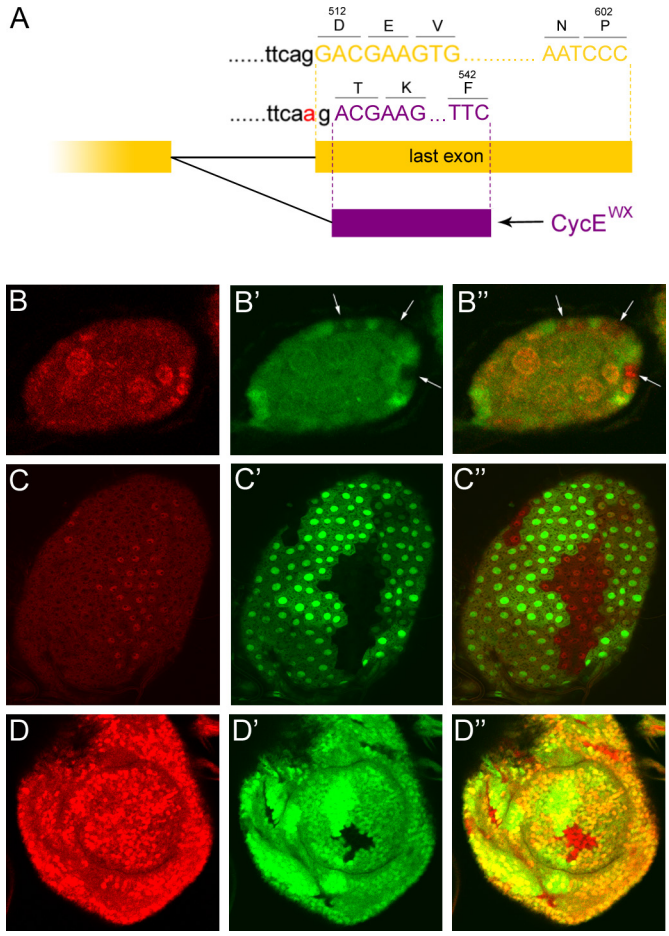

Fig. S2. CycE^{WX} has an altered C terminus that does not greatly alter its stability. (*A*) CycE (top line) splice acceptor ''ag'' is altered to ''aa'' (red) in *cycE^{WX}* (second line). RT-PCR confirmed a single nucleotide shift in the splice acceptor, altering the reading frame to produce a shorter 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*).

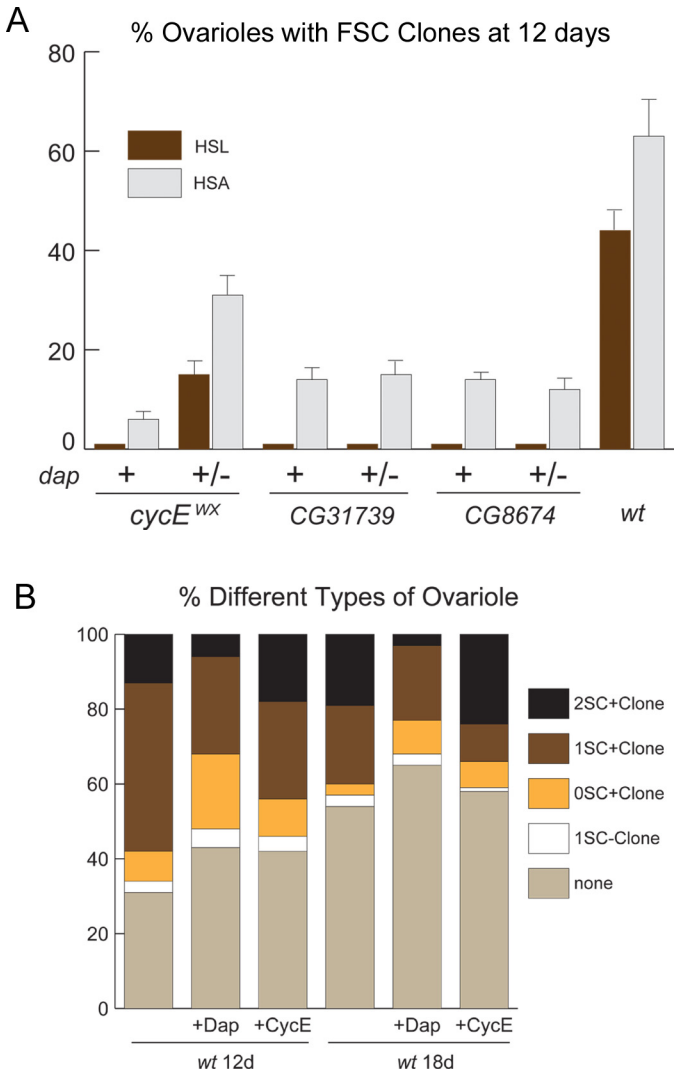

Fig. S3. The effects of Dacapo dosage on FSCs. (*A*) Percentage of ovarioles that retain FSC clones 12 days after heat-shock induction in adults (HSA) or third instar larvae (HSL) for wild-type (wt) and named mutations in otherwise wild-type (+) or *dap* heterozygous (+/−) animals. (*B*) Percentage of 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 $n = 3$.

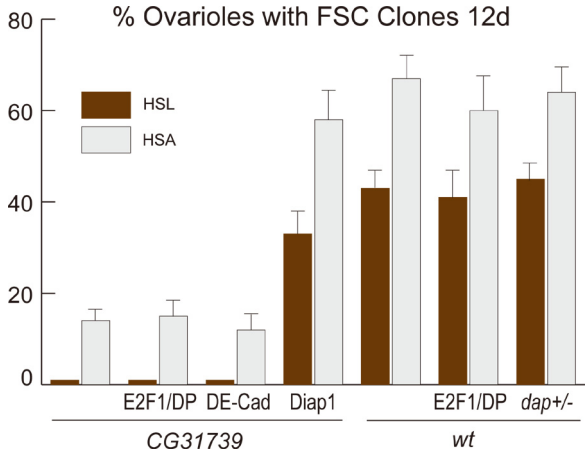

Fig. S4. Rescue of *CG31739* FSC function and effects of E2F1 and *dap* on wild-type FSCs. Percentage of ovarioles that retain FSC clones 12 days after heat-shock induction in adults (HSA) or third instar larvae (HSL) for wild-type (wt) and *CG31739* alleles expressing different transgenes or in *dap* heterozygous (+/−) animals. Error bars are SD with $n = 3$.

Table S1. Complementation among *cycE* alleles

cycE allele	JP	PZ8	P28	1F36	WX	AR95
JP	viable fertile	viable fertile	viable fertile	viable fertile	viable fertile	viable fertile
PZ8		lethal (another lethal present)	viable female semi-sterile; few eggs	viable (75%) female semi-sterile; few eggs	viable (60%) female sterile; very few eggs	80% lethal female sterile; no eggs
P28			viable female sterile; no eggs	viable female semi-sterile; many eggs	viable female sterile; many eggs	60% lethal female sterile; no eggs
1F36					lethal	lethal
WX					lethal	lethal
AR95						lethal

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.