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Persistent competition among stem cells and their daughters in the *Drosophila* ovary germline niche

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Cell competition is a short-range cell-cell interaction leading to the proliferation of winner cells at the expense of losers, although either cell type shows normal growth in homotypic environments. *Drosophila* Myc (dMyc; Dm – FlyBase) is a potent inducer of cell competition in wing epithelia, but its role in the ovary germline stem cell niche is unknown. Here, we show that germline stem cells (GSCs) with relative lower levels of dMyc are replaced by GSCs with higher levels of dMyc. By contrast, dMyc-overexpressing GSCs outcompete wild-type stem cells without affecting total stem cell numbers. We also provide evidence for a naturally occurring cell competition border formed by high dMyc-expressing stem cells and low dMyc-expressing progeny, which may facilitate the concentration of the niche-provided self-renewal factor BMP/Dpp in metabolically active high dMyc stem cells. Genetic manipulations that impose uniform dMyc levels across the germline produce an extended Dpp signaling domain and cause uncoordinated differentiation events. We propose that dMyc-induced competition plays a dual role in regulating optimal stem cell pools and sharp differentiation boundaries, but is potentially harmful in the case of emerging *dmyc* duplications that facilitate niche occupancy by pre-cancerous stem cells. Moreover, competitive interactions among stem cells may be relevant for the successful application of stem cell therapies in humans.

KEY WORDS: Dpp, Cell competition, Stem cells, *Drosophila*

INTRODUCTION

The integrity of stem cells is vital for an organism as they constantly replenish tissues throughout adult life. As long-lived cells with self-renewing capacities, they are equipped with efficient repair systems to maintain their genome intact. The quality of the germline stem cell is especially crucial because defects in this type of stem cell will be passed on to the next generation.

The fly ovary germline is a well-characterized stem cell system that allows complex questions of stem cell behavior to be addressed. Approximately 16 ovarioles constitute an ovary. Each ovariole harbors two to three ovarian germline stem cells (GSCs) at the tip of a germarium (reviewed by Fuller and Spradling, 2007), which acts as a stem cell niche (Xie and Spradling, 2000), a special environment where adult stem cells are maintained. GSCs in this niche are anchored via E-cadherin-mediated adhesion to stromal cap cells (Song et al., 2002), which secrete the stem cell factor Dpp to the adjacent stem cells to prevent GSC differentiation (Xie and Spradling, 1998) (Fig. 1A). Phosphorylated Mad (pMad) transduces the Dpp signal in GSCs and represses the differentiation factor *bag of marbles* (*bam*) by direct binding to the *bam* promoter (Chen and McKearin, 2003).

Stem cells divide asymmetrically and give rise to daughters with different fates. The niche proximal daughter cell maintains GSC identity, cap cell anchorage and high Dpp signaling levels, whereas the distal daughter differentiates into a Bam-expressing cystoblast (CB). CBs undergo several rounds of divisions with incomplete cytokinesis to form cysts of 16 cystocytes connected by branched fusomes (Deng and Lin, 1997). Alternatively, GSCs can also divide symmetrically to substitute stem cells that are lost from the niche due to natural turnover (Fig. 1A) (Xie and Spradling, 2000). Under certain conditions, even differentiated CBs up to 8-cell cysts can revert to stem cells to repopulate an empty niche (Kai and Spradling, 2004).

Mutations in both *bam* and *bgcn* (benign gonial cell neoplasm) block the differentiation of GSCs and produce stem cell tumors in homozygous flies. It has been recently reported that *bam* and *bgcn* mutant stem cells, which express high levels of E-cadherin, are able to gradually take over a niche shared with wild-type GSCs, suggesting a competitive relationship among stem cells (Jin et al., 2008). Originally, cell competition has been discovered in fly wing imaginal discs where clones heterozygous for mutations in ribosomal genes (*Minutes*) were found to be outcompeted by wild-type cells without changing tissue morphology (Morata and Ripoll, 1975; Lambertsson, 1998). Such *Minute* and wild-type cells grew normally in a homotypic environment and only apposition of both cell types triggered apoptosis of the loser cells. The unequal fates are thought to be mediated by differential competition for the survival factor Dpp. Lower Dpp signaling levels in *Minutes* allow the expression of *brinker*, which in turn triggers JNK-dependent apoptosis (Morata and Ripoll, 1975; Moreno et al., 2002) (reviewed by Moreno, 2008). More recent studies demonstrated that dMyc-overexpressing cells act as supercompetitors that outcompete surrounding wild-type cells in the wing epithelium (Johnston et al., 1999; de la Cova et al., 2004; Moreno and Basler, 2004).

Because cells with lower levels of protein synthesis (*Minutes*) or dMyc are outcompeted, cell competition was proposed to serve as a quality control mechanism that improves organ function (Diaz and Moreno, 2005). In order to test this hypothesis, we turned to the *Drosophila* ovary germline, a stem cell-based tissue where quality

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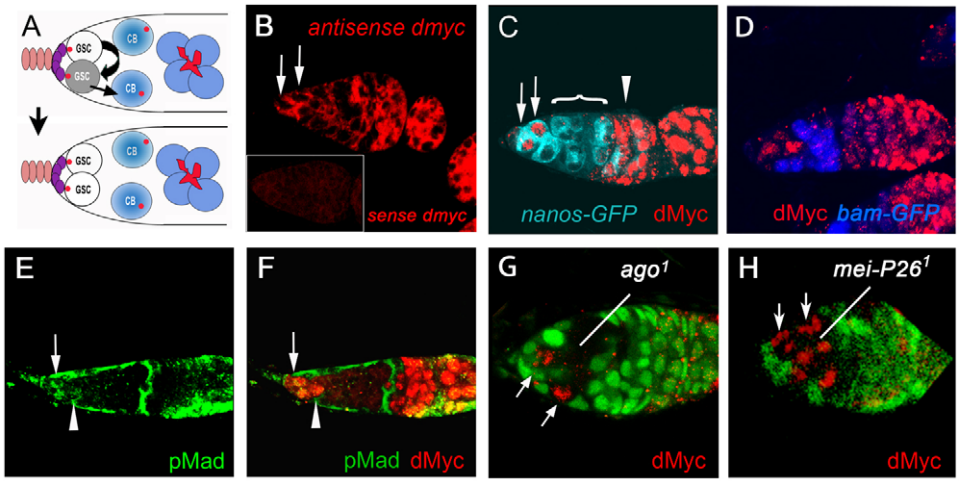

Fig. 1. GSCs express high levels of dMyc. (**A**) Ovariole tip. Anterior is oriented to the left in all panels. Cap cells and the terminal filament (TF) are shown in magenta and pink, respectively. Diagram illustrating germ cell replacement by horizontal division of a GSC. (Top) A suboptimal stem cell (GSC, gray) located next to an optimal stem cell (GSC, white) is forced to differentiate. The optimal stem cell divides symmetrically to occupy the niche. (**B**) In situ hybridization reveals the presence of *dmyc* mRNA in GSCs (arrows) and late cysts. Inset shows control in situ with the sense *dmyc* RNA probe. (**C**) Staining of ovarioles with anti-dMyc antibody (red) shows that in addition to the previously described expression in late cysts (arrowhead), there is also strong expression at the tip of the germarium where GSCs are located (arrows). However, dMyc levels are markedly lower in 1- to 2-day-old cysts up to the 16-cell cyst stage (bracket). Early cystoblasts (CBs) typically express low levels of dMyc. Note that *dmyc* mRNA and protein expression patterns are identical (compare B and C). *nanos*, a germline marker, is shown in light blue. (**D**) Patterns of dMyc (red) and Bam-GFP (blue) expression in the germarium. (**E**,**F**) Staining for pMad (E, green) and merge of pMad and dMyc (red) staining (F) of the same germarium, revealing that two pMad-positive GSCs express high levels of dMyc (arrows). Occasionally pre-cystoblasts are also positive for dMyc (arrowhead). (**G**) *ago* mutant clones, identified by the absence of GFP (green), were stained with anti-dMyc (red, arrows). Note that the differentiating progeny of *ago* mutant stem cells (arrows) show normal downregulation of dMyc in cystoblasts. (**H**) *mei-P26* mutant clones, visualized by the absence of GFP (green), were stained with anti-dMyc (red, arrows). Remarkably, *mei-P26* mutant cystoblasts fail to downregulate dMyc protein, suggesting a role of Mei-P26 in dMyc repression during the stem cell-cystoblast transition.

control is thought to be of paramount importance. In the *Drosophila* ovary germline, *dmyc*, the homolog of the human *c-myc* oncogene, is known to be required for endoreplication of the differentiating cysts (Maines et al., 2004), but its role in GSCs is not known. Here, we show that differential expression of dMyc in GSCs triggers competitive interactions. dMyc-mediated competition in the germline is non-apoptotic and does not affect total stem cell numbers. In addition, we present supporting data for a naturally ongoing level of competition between high dMyc stem cells and low dMyc differentiating daughters, which seems to favor efficient launching of the differentiation program.

MATERIALS AND METHODS

Fly stocks and generation of marked clones

nanos-gal4VP16 flies were used to mark the germline and overexpress several genes, when crossed to males containing *EPgy-bam* and *EPgy-dIAP1* (H. Bellen, Baylor College of Medicine, TX, USA), *UASp-tub-GFP* (Bloomington Stock Center) and *UASp-tkv^{ACT}* (Casanueva and Ferguson, 2004) transgenes. The *bamP-GFP* line (D. McKearin, University of Texas, TX, USA) was used to monitor *bam* expression.

Clones in the GSCs were typically generated by heat-shocking 2-day-old flies twice per day. Flies were heat-shocked at 37°C for 1 hour, then allowed to recover for a period of 6-7 hours before they were subjected to a second identical heat-shock. Flies were then kept at 25°C and transferred to fresh food every 2 days. For the induction of *tub>dmyc* mutant GSC clones, females of genotype *tub-FRT-CD2-FRT-dmyc* were generated and heat-shocked for 30 minutes at 37°C.

We used 1-week-old adults of genotype *dmyc^{P0} FRT18A* and *Dp (1;1) Co hsp70-Flp FRT18A* to study the GSC niche. As a control niche, we used *arm LacZ FRT18A; hs-Flp38*. For the induction of *dmyc^{P0}* and 4×*dmyc* (*Dpmyc*) mutant GSC clones, females of genotype *dmyc^{P0} FRT18A/arm-lacZ FRT18A; hs-Flp* and *yw Dp (1;1)Co hs-Flp FRT18A/arm-lacZ FRT18A* were generated, respectively. As a control, we generated *FRT18A/arm-lacZ FRT18A; hs-Flp*. For the induction of other mutant GSCs, females of the following genotypes were generated: *hs-Flp Ubi-GFP FRT40/Pten^{2L100} FRT40*, *hs-Flp Ubi-GFP FRT40/Pten^{2L117} FRT40*, *hs-Flp; Ubi-GFP FRT18A/mei-P26^{1}FRT18A*, *hs-Flp; Ubi-GFP FRT40/lgl^{4}FRT40* (Gateff and Schneiderman, 1974), *hs-Flp; Arm-Lacz FRT42/ptc^{s2}FRT42* (Simcox et al., 1989), *hs-Flp; Ubi-GFP FRT80/sty$^{\Delta 5}$FRT80* (Hacohen et al., 1998), *hs-Flp;Ubi-GFPFRT82/bam$^{\Delta 86}$FRT82*, *hs-Flp;Ubi-GFP FRT82/sav^{shrp6B21}FRT82* (Kango-Singh et al., 2002), and *hs-Flp; Ubi-GFP FRT82/scrib^{1}FRT82* (Bilder et al., 2000). To analyze GSC competition in *tkv^{ACT}* germaria, flies of genotypes *Dp (1;1) Co/arm-lacZ FRT18A; UASp-tkv^{ACT}/+; nanos-Gal4:VP16/+* and *dmyc^{P0}FRT18A /arm-lacZ FRT18A; UASp-tkv^{ACT}/+;nanos-Gal4:VP16/+* were generated (Van Doren et al., 1998; Johnston et al., 1999).

We used *tub>dmyc>Gal4/Cyo* adult females, and generated *dmyc^{P0} FRT18A/ dm^{4}*, *dmyc^{PG45}/dmyc^{PG45}*; *tub>dmyc>Gal4/+* and *dmyc^{PG45}/ dm^{4}; tub>dmyc>Gal4/+*. Ovaries were processed from 1-week-old flies. The *dmyc* sequence was amplified by PCR, subcloned into the pUASp vector and constructs sent to generate transgenic *UASp dMyc* flies (BestGene). They were used to overexpress dMyc in the germline using the *nanosGal4* driver by generating *dmyc^{P0}/dmyc^{PG45}; UASp Myc/+; nanosGal4 UASp tub-GFP* flies.

Calculations

The percentage of GSCs inside a marked clone was quantified after processing the corresponding samples for immunostaining with anti-β-Gal and or anti-Hts antibodies. GSCs were identified by their attachment to cap cells and by the presence of a round fusome.

The decay time of clones in the niche was calculated as follows. For homogenous niches, the number of GSCs was counted at 1-4 weeks, then data were modeled with a linear regression N=b0+b1*T (T in weeks and N is the number of GSCs. Example for *dmyc^{P0}* homogenous niche. 2.4 (1 week), 2.1 (2 weeks), 1.53 (3 weeks), 1 (4 weeks), Regression b0=2.96 (P<1e-5), b1=–0.48 (P<1e-15), degrees of freedom=50. Decay time: time to reach half

of the 1st week value=3.67 weeks. To quantify GSCs decay in mosaic niches, we modelled R1 scores with a linear or nonlinear regression for the following scenarios (y, R1; x, time in unit of days; goodness of fit scored by the norm of residuals). Example for $dmyc^{P0}$ mosaic niche. R1: 60.0% (4 days), 33.3% (1 week), 14.3% (2 weeks), 14.3% (3 weeks). Linear regression y=p1*x+p2, coefficients: p1=–0.024773, p2=0.58965, norm of residuals=0.18504. Decay time: time to reach half of the 4-day value=1.67 weeks (cubic regression 1.07 weeks). To test if differences between decays are significant, we performed *t*-tests. Complete calculations are available upon request.

To measure stem cell division rates, we used the same system as Xie and Spradling (Xie and Spradling, 1998). We determined the relative number of wild-type and mutant cysts in germaria that contained one control and one mutant stem cell. For a given genotype, these values were similar at each time point, and the average is presented in the text. Marked wild-type stem cells gave a value of 0.8. Mutant 4×*dmyc* GSCs occupy the niche very early after clone induction. In this case, the GSC division rate could only be scored at very early time points.

GSC size was measured after staining with Alexa-conjugated phalloidin (Molecular Probes) to reveal the cortical actin that delineates cellular contours. Serial confocal images of 2 μm each were obtained. Cell size was calculated as the maximum area of the whole collection of sections per individual germarium by using the appropriate Leica Confocal Software (LCS) tool.

Immunohistochemistry and apoptosis assays

We used the following antibodies: monoclonal (Promega) or polyclonal (Cappel) anti-β-Gal, anti-pMad (P. ten Dijke and G. Morata, University of Leiden, The Netherlands), polyclonal or monoclonal anti-dMyc (R. Eisenman and B. Edgar, Fred Hutchinson Cancer Research Center, WA, USA), anti-Hts (1B1; Developmental Studies Hybridoma Bank; DSHB). All images were obtained with a LEICA TCS-SP2-AOBS. For each germarium, six to ten image stacks of 2 μm were obtained through the z dimension in order to analyze the whole GSC population. For detection of apoptosis, we used anti-cleaved caspase-3 (Cell Signaling Tech), anti-Hid antibody (H. Steller, The Rockefeller University, NY, USA) or analysis of nuclear fragmentation by DAPI staining. As a positive control of apoptosis in the GSCs, we stained ovaries 7 hours after the induction of apoptosis by heat-stress (45 minutes at 38°C).

In situ hybridization

FISH was performed using standard protocols. Briefly, ovaries were fixed in 4% formaldehyde (FA) and hybridized with a digoxigenin-labeled RNA probe overnight or for 48 hours at 56°C. After washing, signal was developed by using an anti-digoxigenin antibody (Jackson ImmunoResearch) and the Tyramide Signal Amplification System (Invitrogen).

RESULTS

dMyc expression is developmentally regulated in stem cells and differentiating cystoblasts

In order to study the role of dMyc in stem cells and early differentiation events, we analyzed the endogenous expression pattern of *dmyc* RNA and protein in ovarioles. We found that *dmyc* mRNA was expressed in several cells at the germarium tip using in situ hybridization (Fig. 1B). In addition, we detected the previously described dMyc expression in differentiating cysts that is required for endoreplication (Maines et al., 2004). However, *dmyc* transcripts were tightly downregulated in the intermediate zone where cystoblast differentiation up to the 16-cell cyst stage takes place. A similar pattern could be observed by immunohistochemistry with an anti-dMyc antibody (Fig. 1C). dMyc was highly expressed in cells showing characteristic features of GSCs: they expressed *nanos*, were negative for the differentiation marker *bam* and expressed high levels of active (phosphorylated) Mad (pMad) (Fig. 1C-F), indicating that they were transducing high levels of Dpp (Kai and Spradling, 2004). dMyc was also present in cells that had already lost niche contact but that did not yet upregulate the differentiation factor Bam. Such pre-cystoblasts (pre-CBs) (Gilboa et al., 2003) showed intermediate levels of pMad (Fig. 1E,F). In differentiating cystoblasts, however, expression of dMyc protein was strongly reduced, consistent with the pattern observed using mRNA in situ hybridization against *dmyc*.

Because dMyc expression in the ovary appeared to be developmentally regulated, we sought to determine how dMyc downregulation is established. The F-box protein *archipelago* (*ago*) has been shown to regulate dMyc levels in *Drosophila* wing and eye tissues (Moberg et al., 2004), therefore we tested whether it also modulated dMyc expression in the germline. However, *ago* mutant GSCs gave rise to differentiating CBs that showed normal dMyc downregulation (Fig. 1G). More recently, the cytoplasmic protein Brat was found to regulate dMyc in the daughter cell of neuronal stem cells (Betschinger et al., 2006). *Brat* forms part of a family of tumor suppressors that includes the gene *mei-P26*, which is known to produce ovarian tumors when mutated (Page et al., 2000). Interestingly, we found that progeny derived from $mei\text{-}P26^{1}$ mutant stem cells failed to downregulate dMyc (Fig. 1H). This does not prove that Mei-P26 is indeed regulating dMyc expression because *mei-26* mutations might simply block GSC differentiation leading to an accumulation of dMyc-positive GSC-like cells. However, recent findings demonstrate that *mei-p26* mutant GSCs are able to differentiate into cystocyte marker-expressing cells, but that these *mei-p26* mutant CBs later fail to form oocytes because they proliferate indefinitely (Neumüller et al., 2008). We therefore consider it likely that Mei-p26, whose levels are low in stem cells, but high in cysts, might control the expression of dMyc.

Stem cells with elevated levels of dMyc outcompete wild-type GSCs

In order to test whether dMyc can induce competitive interactions in the niche, we manipulated dMyc levels by generating one unmarked GSC with higher dMyc levels (*Dpmyc/Dpmyc*, with four copies of *dmyc*) next to a resident marked stem cell using a heat-shock-inducible Flipase, which mediates FRT mitotic recombination (Xie and Spradling, 1998). Four days after clone induction (ACI), we verified the presence of such mosaic niches. Already one week ACI, a subset of 4×*dmyc*-containing GCSs had replaced adjacent stem cells (*Dpmyc*/+) and, after 2 to 3 weeks, 4×*dmyc* stem cells constituted the entire germline (47.2% of the total GSCs were 4×*dmyc*-containing cells at 4 days ACI, but they expanded to 95.8% of the total GSCs after 4 weeks; Fig. 2A-C, Table 1, Fig. 3A). Although the marked control stem cells (which carried one *lacZ* copy) disappeared from the niche, a constant number of GSCs was maintained, possibly by horizontal division of the 4×*dmyc* GSCs (see Fig. 1A). The elimination of resident GSCs by 4×*dmyc* stem cells did not seem to be caused by apoptosis, as we found no evidence of induction of the pro-apoptotic gene *hid* (Grether et al., 1995) (Fig. 2D, inset shows positive control for *hid* induction after heat stress). Furthermore, we did not detect activation of caspase-3, nuclear fragmentation or aberrant mitochondrial function (in vivo imaging with mitotracker; data not shown) suggesting that wild-type GSCs did not die, but were expelled from the niche and differentiated.

Why did cells with more dMyc take over the niche? Elevated dMyc levels could lead to increased division rates of GSCs leading to the loss of more slowly proliferating wild-type stem cells. Because the insulin pathway is known to promote GSC proliferation (LaFever and Drummond-Barbosa, 2005), we generated clones mutant for the negative regulator *Pten* to test whether increased division rates of *PTEN* mutant GSCs are sufficient to drive surrounding stem cells out of the niche. *Pten* mutant GSCs showed increased proliferation (1.6 versus 0.8 in the wild type, Table 1) but

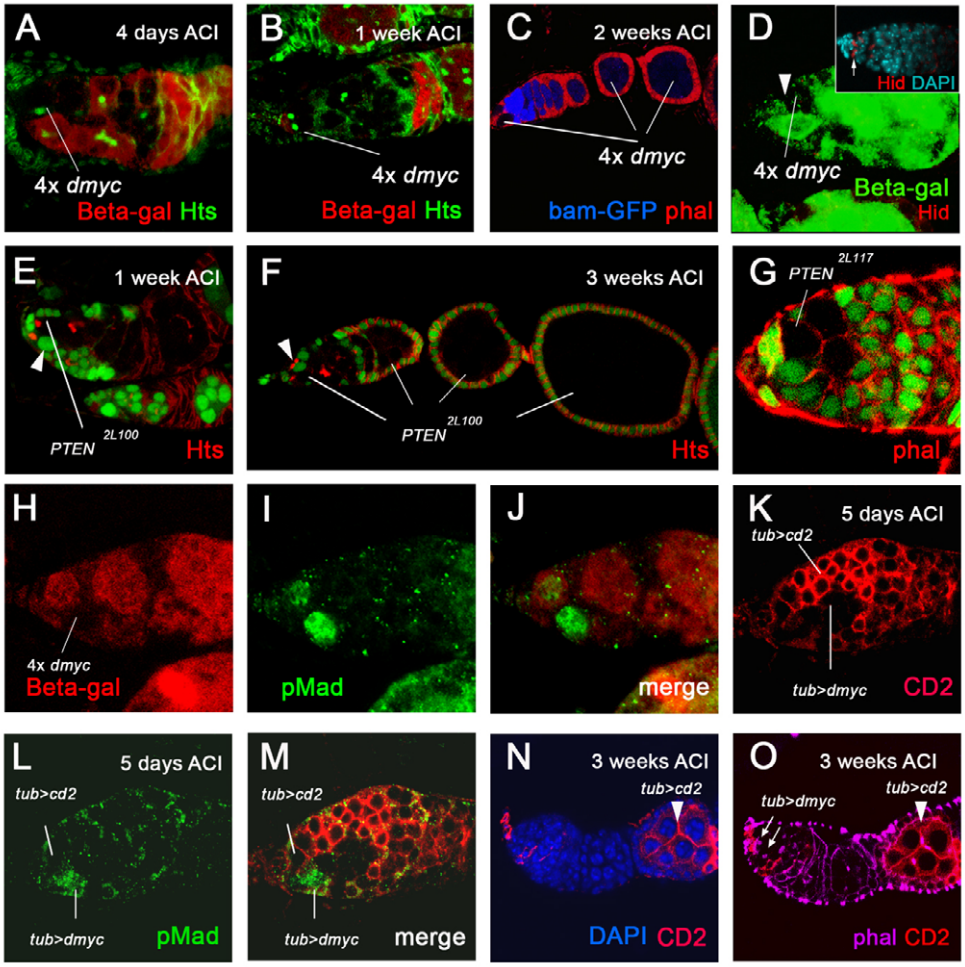

Fig. 2. dMyc-overexpressing stem cells induce competition in mosaic niches. (**A-C**) Mosaic niches containing control (*Dpmyc/+*) (βGal-positive, red) and *Dpmyc/Dpmyc* (4×*dmyc*) mutant (βGal-negative) GSCs were generated and their progeny followed over time. GSCs were identified by the presence of a round fusome (anti-Hts staining, green) and their close position to the terminal filament, or, alternatively, by the lack of Bam-GFP expression (blue, C). Four days ACI, mosaic stem cell niches can be observed (A). However, stem cell niches are taken over by mutant 4×*dmyc* GSCs 1 and 2 weeks ACI (B,C). (**D**) GSC-GSC interaction is non-apoptotic. Expression of Hid (red) is not induced in the *Dpmyc/+* (GFP-positive) out-competed stem cell next to a *Dpmyc/Dpmyc* GSC (arrowhead). Inset in D shows positive control staining (arrow) for the same antibody in wild-type GSCs from heat-stressed flies (nuclei stained with DAPI, cyan). (**E**,**F**) Mosaic niches containing $Pten^{2L100}$ mutant (GFP-negative) GSCs and control stem cells (GFP-positive), heterozygous for $Pten^{2L100}$ ($Pten^{2L100}$/+). GSC offspring was examined over time (1 and 3 weeks ACI in E and F, respectively). Anti-Hts staining, red. (**G**) Mosaic niches containing a $Pten^{2L117}$ mutant (GFP-negative) and a control ($Pten^{2L117}$/+) stem cell (GFP, green) were stained with phalloidin (red) to reveal cellular contours. This confocal plane is representative of the relative difference in GSC territory. (**H-J**) A mosaic GSC niche containing a control (*Dpmyc/+)* (βGal, red) and a 4×*dmyc* mutant GSC (black, H) were stained with anti-pMad (green, I). Merge is shown in J. 4×*dmyc* mutant GSCs show higher levels of Dpp signaling. (**K**) A mosaic niche inhabited by a dMyc-overexpressing GSC (*tub>dmyc*) and a control stem cell (*tub>cd2,* red) is shown 5 days ACI. (**L**) pMad staining (green) for the same germarium. (**M**) Merged image of K and L. The dMyc-overexpressing GSC displays higher levels of pMad, compared with the wild-type cell. (**N**,**O**) Mosaic germarium 3 weeks ACI. Control cells (*tub>cd2*, red) have been lost from the germline or appear far downstream (arrowhead) and *tub>dmyc* GSCs have occupied the niche (CD2-negative, arrows, O). Germaria were stained with DAPI (blue, N) to visualize cell nuclei and phalloidin (purple, O) to reveal cellular contours and fusomes of stem cells (arrows, O).

did not extrude neighboring GSCs and co-existed with them instead (Fig. 2E,F; Table 1). Moreover, dMyc-overexpressing GSCs showed only slightly elevated proliferation rates compared with the wild-type GSCs (1 versus 0.8 in the wild-type, Table 1). These results suggest that the competitive advantage of dMyc-overexpressing GSCs does not rely on increased proliferation. As a second possibility, we envisaged that larger cells might displace smaller ones, as *dmyc* is implicated in cell size control (Johnston et al., 1999). GSCs that express higher levels of dMyc in a homogeneous niche were slightly larger than wild type (4×*dmyc* GSCs 68±17 μm^2 versus 60±10 μm^2 in the control, *P*=not significant). Previous studies have shown that Pten negatively regulates cell size (Goberdhan et al., 1999). Therefore, we used two alleles (Oldham et al., 2002) ($Pten^{2L100}$ and $Pten^{2L117}$) to generate GSC mutant clones. Pten clearly affected GSC size (Fig. 2G), ($Pten^{2L100}$ GSCs, 83±15 ; $Pten^{2L117}$ GSCs, 86±16 μm^2; versus 54±18 μm^2 in the wild type; $P<0.001$) but did not eliminate neighboring stem cells, speaking against cell size as a factor influencing stem cell competition. In a third scenario, dMyc might stimulate the capture and/or transduction of extracellular factors like Dpp (Moreno and

Table 1. Differential behavior of mutant stem cells in the GSC niche

Genotypes in marked mosaic niches (mutant versus control GSCs)*	Percentage of mutant GSCs in a clone (number of mutant GSCs in a clone/ total number of GSCs × 100) (*n* germaria)				GSC decay time† (weeks)	GSC relative division rate‡ (*n*)
	4 days	1 week	2 weeks	3 weeks		
Wild type versus wild type	52.3 (21)	57.8 (19)	52.5 (30)	41.2 (17)	4.46	0.8 (48)
$dmyc^{P0}/dmyc^{P0}$ (versus $dmyc^{P0}$/+)	60.2 (25)	28 (34)	6.2 (10)	5.2 (25)	1.07	0.7 (66)
Dpmyc/Dpmyc (Dpmyc/+)	47.2 (11)	73.3 (15)	83.3 (18)	95.8 (24)	N/A	1 (12)
tub>dmyc (versus *tub>cd2*)	49.7 (107)	N/A	62.0 (103)	70.1 (146)	N/A	N/A
$Pten^{2L100}/Pten^{2L100}$ (versus $Pten^{2L100}$/+)	40 (40)	41.6 (62)	31 (29)	45 (31)	4.43	1.6 (48)
lgl^4/lgl^4 (versus lgl^4/+)	43 (41)	41.2 (44)	50 (64)	40.7 (27)	N/A	0.9 (7)
$scrib^1/scrib^1$ (versus $scrib^1$/+)	27.7 (19)	27 (18)	30 (8)	N/A	N/A	0.9 (6)
CyclinE, DIAP						
ago^1/ago^1 (versus ago^1/+)	30.4 (21)	37.5 (26)	28.5 (18)	23.8 (15)	N/A	0.8 (10)
$sav^{shrp6B21}/sav^{shrp6B21}$ (versus $sav^{shrp6B21}$/+)	18.3 (35)	21.4 (14)	39.3(36)	44.4 (15)	N/A	1.1 (9)
Hedgehog pathway						
ptc^{S2}/ptc^{S2} (versus ptc^{S2}/+)	16.6 (17)	13.2 (20)	14.3 (15)	13.3 (6)	N/A	0.9 (6)
EGF pathway						
sty^{D5}/sty^{D5} sty^{D5}/+	23 (57)	33.3 (21)	41.2 (64)	43.9(41)	N/A	1.1 (7)

*Mutant or control stem cells were marked (*lacz* or *gfp*) to distinguish the two populations (see Materials and methods).
†Decay time: time required to halve the initial stem cell population of a given genotype.
‡Stem cell division rates were measured according to the system used by Xie and Spradling (Xie and Spradling, 1998).
N/A, not assessed.

Basler, 2004) that are required to maintain GSC fate. Consistent with this idea, 4×*dmyc* GSCs showed higher levels of pMad than did control GSCs in a mosaic niche (6/6, 4 days ACI; Fig. 2H-J).

Because all the dMyc overexpression experiments in 4×*dmyc* GSCs have been obtained using the *dmyc* duplication [Dp(1;1)Co], which harbors about 12 additional genes (Moreno and Basler, 2004), we devised a second genetic setup to verify the effect of dMyc levels on GSC competition.

To this end, we heat-shocked *hs-flp*; *tub>cd2stoP>dmyc* transgenic flies for 30 minutes to induce a moderate amount of *tub>dmyc* clones, which could be visualized by the loss of the membrane marker CD2, next to *tub>cd2* control cells. Five days ACI, a high percentage of germaria showed mosaic niches containing both a marked control and an unmarked *tub>dmyc* stem cell (Fig. 2K-O). After 2 weeks, the loss of CD2-positive GSCs became evident, concomitant with a decrease in number of mosaic niches (49.7% of the total GSCs were *tub>dmyc*-containing cells at 4 days ACI, but they had expanded to 62% of the total GSCs after 2 weeks). Three weeks ACI, a high proportion of germaria showed complete absence of CD2-positive germ cells (70% of the total GSCs were *tub>dmyc*-containing cells at 3 weeks ACI), indicating that dMyc-overexpressing cells had occupied the majority of niches (Fig. 2N,O; see also Table 1). The analysis of pMad expression in *tub>cd2/tub>dmyc* mosaic niches reinforced the previous results that dMyc-overexpressing GSCs typically showed higher levels of pMad. Differences in pMad levels were strongest at 5 days ACI (11/16 germaria, five germaria showed identical pMad levels) (Fig. 2L,M). Importantly, *tub>dmyc* GSCs were not differentiation-defective and produced normal cysts and oocytes, as verified by phalloidin and Hts staining (fusome marker; Fig. 2O; data not shown).

Stem cells expressing low levels of dMyc are expelled from the niche and differentiate

To investigate whether wild-type stem cells have a competitive advantage over suboptimal GSCs, in this case GSCs expressing low levels of dMyc, we first examined niches containing only *dmyc* mutant stem cells in flies homozygous for the hypomorphic $dmyc^{P0}$ allele (Johnston et al., 1999). Although $dmyc^{P0}$ mutant stem cells express lower levels of dMyc, this reduction does not affect the ability of $dmyc^{P0}$ GSCs to self-renew and generate differentiated progeny, ruling out the possibility that such GSCs might be lost because they are not fully functional (Fig. 4A). We then created mosaic niches harboring unmarked $dmyc^{P0}$ mutant GSCs next to marked $dmyc^{P0}$/+ control stem cells. Four days ACI, we were able to detect such mosaic niches; however, after 1 week, half of the homozygous mutant $dmyc^{P0}$ GSCs had abandoned the niche. This elimination trend continued in later weeks (60.2% of the total GSCs were $dmyc^{P0}/dmyc^{P0}$ cells at 4 days ACI, but they had decreased to 6.2% of the total GSCs after 2 weeks) (Table 1, Fig. 3A, Fig. 4B-D). As a second measurement, we quantified the time it takes to halve the initial $dmyc^{P0}$ homozygous stem cell population (decay time) in a mosaic niche with control GSCs, and compared it with that exhibited in a homogeneous niche of only $dmyc^{P0}$ GSCs. The decay time in the latter niche was around four weeks but dropped to one week in a mosaic niche (see Materials and methods). The fact that $dmyc^{P0}$ mutant cells were still present in the germaria 2 weeks ACI, forming part of developing cysts outside the niche (Fig. 4D), suggested that suboptimal stem cells did not undergo apoptosis, but had rather moved out of the niche to differentiate. By using various methods to detect apoptotic cells, such as Caspase 3 activation, lack of Hid staining (Grether et al., 1995), absence of nuclear fragmentation and intact mitochondrial function, we were unable to detect cell death of $dmyc^{P0}$ mutant GSCs even under intense monitoring. However, we could easily detect activated Caspase 3 in wild-type GSCs after heat-shock-induced stress or irradiation (Fig. 4E, inset; data not shown). Finally, the elimination of mutant stem cells along the differentiation pathway still took place when apoptosis was blocked in the germline by overexpressing the apoptosis inhibitor dIAP-1 (Hay et al., 1995) from the early germline nanos-Gal4 driver (Rørth, 1998; Van Doren et al., 1998) (Fig. 4F). These experiments show that suboptimal cells are reliably detected and driven out of the niche, which suggests that dMyc-induced cell competition might represent a continuous survey instrument used to maintain optimal stem cell pools at any time.

The competitive advantage of differentiation-defective stem cells was shown to rely on their higher E-cadherin levels, which seem to confer physical strength with which to push out competitor stem cells (Jin et al., 2008). By contrast, we did not observe noticeable differences in E-cadherin expression between suboptimal $dmyc^{P0}$ and $dmyc^{P0}$/+ control cells (see Fig. S1 in the supplementary

material). In order to test whether the competitive advantages of stem cells depended on Dpp signaling (higher pMad levels), we overexpressed a constitutively active version of the Dpp receptor (tkv^{ACT}) in the germline and monitored mosaic niches harboring stem cells with different dMyc levels. We found that expression of tkv^{ACT} blocked not only the loss of $dmyc^{P0}$ homozygous GSCs sharing the niche with wild-type GSCs but also the expulsion of wild-type stem cells facing a 4×*dmyc* competitor (4/4 and 5/5, 3 weeks ACI, respectively; Fig. 4G,H), suggesting that tkv^{ACT} could equalize competition for Dpp in the niche. However, the expression of tkv^{ACT} on its own causes the accumulation of stem cell-like cells. Therefore, we cannot exclude that the observed rescue of the loser GSCs might be partially caused by difficulties in niche exit of such stem cells due to impaired differentiation.

dMyc-induced competition and other growth-promoting mutations

GSCs carrying *dmyc* duplication and *Pten* mutations might have been expected to behave more similarly, as both genes are growth-promoting and related to oncogenic transformation. We therefore decided to test other mutations in oncogenic pathways to determine their invasive potential in the fly ovary stem cell niche. To this purpose, we monitored again mosaic niches and studied the integration of the mutant GSCs into the stem cell niche. None of the tested mutations (*Pten*, *scribble*, *salvador*, *patched*, *ago*, *sprouty*, *lgl*) induced competitive interactions that were as potent as those observed for the *dmyc* duplication (Fig. 3B, data not shown). Although, *salvador* (*sav*) and *sprouty* (*sty*) mutant stem cells also improved niche occupancy during the observed 20 days ACI, they never exceeded a ratio of 0.5, where half of all GSCs in a clone are still wild type. *sav* is a component of the Salvador-Warts-Hippo pathway, which inhibits cell growth, whereas *sty* is a negative regulator of receptor tyrosine kinase signaling. In the absence of strong invasive behavior, we therefore classified such mutations as 'settler' mutations that successfully establish themselves in the germline, but co-exist with resident GSCs (Fig. 3B and Fig. 4I and 4K), despite higher proliferation rates in the case of *sav* and *Pten* stem cells (Table 1). Genetic lesions in *bam* represent a second category of mutations that produced a 'stem cell plague' (Fig. 4J,M), as it has been previously described. Such mutant stem cells expand by blocking the differentiation of their daughters, thereby increasing the total number of stem cell-like cells. Only stem cells with more dMyc behaved differently, occupying the niche by eliminating and replacing normal stem cells without affecting total stem cell numbers. Because of these characteristic differences, we refer to the gain-of-function mutation in *dmyc* as a 'squatter' mutation to describe that mutant stem cells take over the space formerly inhabited by resident stem cells (Fig. 2A-C, Fig. 4L).

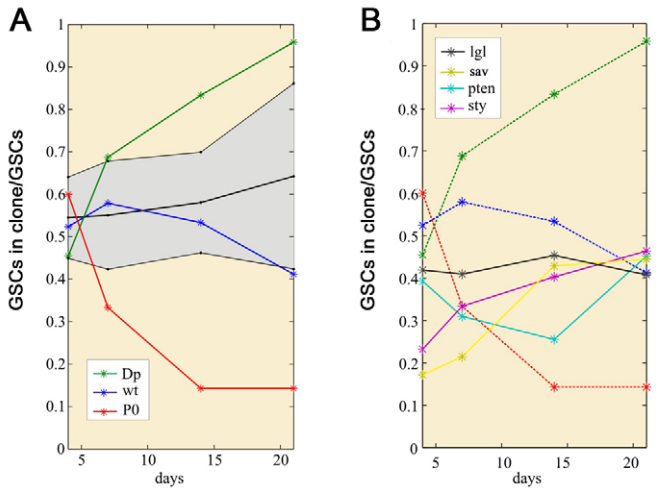

Fig. 3. Competitive interactions in mosaic stem cell niches. (**A**) Graph depicting the ratio of GSCs in a clone over total GSCs in mosaic niches as a function of time after clone induction (ACI). The behavior of the wild type (marked versus unmarked wild-type stem cells) is shown in blue, $dmyc^{P0}$ ($dmyc^{P0}$ mutant versus $dmyc^{P0}$/+ cells) is marked in red and DpMyc (*DpMyc* versus *DpMyc*/+) in green. We also plotted the null behavior (shaded area), obtained by a permutation test (mean, continuous gray line, ±2 standard deviations; random sampling of the corresponding set preserving group size; see Fig. S2 in the supplementary material). The decrease in the ratio of $dmyc^{P0}$ niches, as well as the increase in DpMyc niches, compared with the wild-type situation, illustrates that dMyc induces cell competition in mosaic stem cell pools. (**B**) Integration of mutant stem cells into the stem cell pool. The ratio of GSCs in the clone over total GSCs in mosaic niches (*mut*/*mut* versus *mut*/+ GSCs for $dmyc^{P0}$, *Pten*, *sty*, *sav* and *lgl*) as a function of time after clone induction for several oncogenic pathways [*Pten* (cyan), *sty* (purple), *sav* (yellow), *lgl* (black)] did not exhibit competition compared with the respective dMyc mosaic niches (dashed lines, color coding for *dmyc* behavior as in Fig. 4A).

A competitive interaction between high dMyc stem cells and low dMyc progeny for the stem cell factor Dpp may facilitate differentiation

Our previous results suggest that GSCs can adopt a competitive behavior in the case of emerging differences in dMyc levels. The physiological expression pattern of dMyc in the germline (Fig. 1B-F), however, may continuously promote a certain level of competition between high dMyc stem cells and the lower dMyc differentiating progeny if differences in dMyc levels inevitably trigger competition. It is intriguing to speculate that a naturally occurring dMyc border at the GSC/CB interface might ensure that most niche-secreted Dpp factor is efficiently internalized and exhausted by stem cells as a result of their increased metabolic rates promoted by high dMyc expression, thereby leaving CBs with very little remaining stem cell factor.

To examine this hypothesis, we altered the endogenous dMyc expression pattern along the germarium. First, we added ubiquitous overexpression of dMyc to the physiological levels by using a *dmyc* transgene under the *tubulin* promoter (*tub>dmyc*). Under these conditions, we observed an unusually high number of undifferentiated GSC-like cells that resided in the anterior tip of the germarium, identified by a round fusome, compared with the wild-type situation (Fig. 5A-H). To further minimize differences in dMyc between GSCs and their daughters, we next expressed the *tub>dmyc* transgene in females mutant for very strong lethal *dmyc* alleles ($dmyc^{PG45}/dmyc^{PG45}$ or $dmyc^{PG45}/dm^{4}$) (Bourbon et al., 2002; Pierce et al., 2004). We found that even more daughter cells exhibited a GSC-like morphology at positions that were very distal from the niche, until they finally entered differentiation (Fig. 5I-P).

If changes in dMyc levels induced competition for Dpp uptake in the niche, one would predict to find more Dpp-transducing cells when dMyc differences, and therefore competition is eliminated. This is expected because stem cells would lose their competitive power to turn over most diffusible Dpp. As a consequence, the available Dpp could travel farther and could now be acquired by cells more distal from the niche. Indeed, our experiments showed that pMad activation expanded substantially in the $dmyc^{PG45}/dmyc^{PG45}$; *tub>dmyc*/+ flies compared with in *tub>dmyc* flies, where differences in dMyc levels still exist (Fig. 5F,J,N; Table 2).

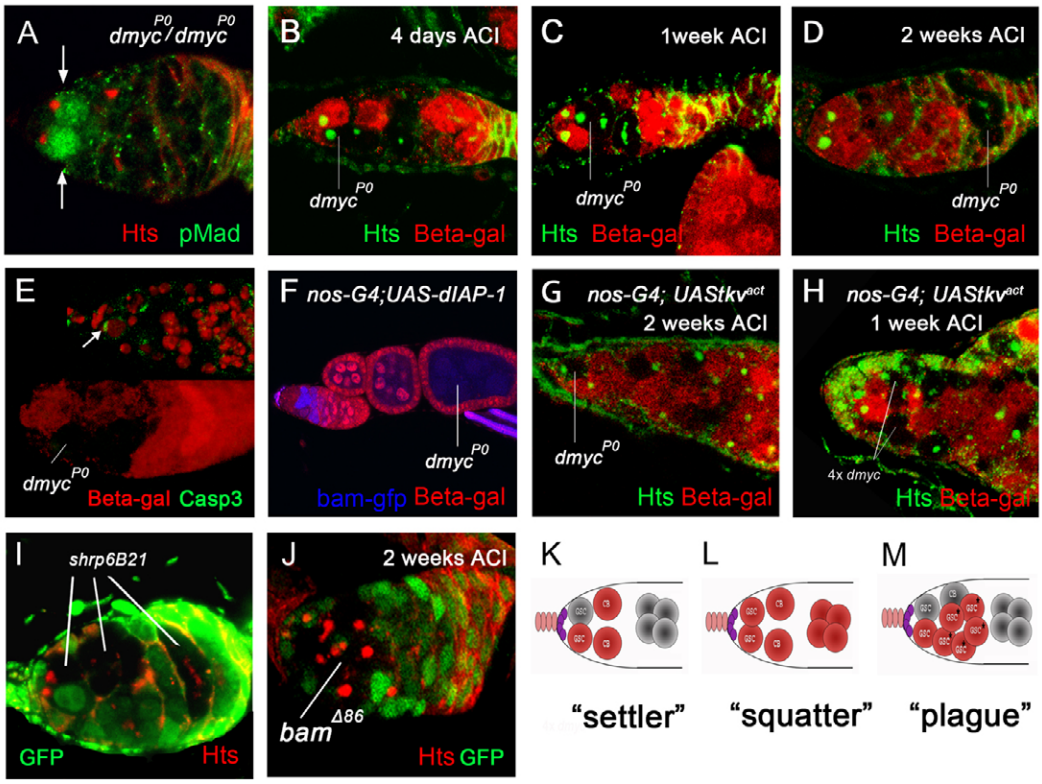

Fig. 4. Elimination of suboptimal stem cells and the accumulation of pre-cancerous mutations. (**A**) Germaria of 1-week-old flies homozygous for *dmyc*^{P0} stained with anti-Hts (red) and anti-pMad (green) to identify GSCs (arrows). Note that *dmyc*^{P0} mutant GSCs display a normal morphology. (**B-D**) Mosaic niches containing *dmyc*^{P0} mutant (βGal-negative) and control GSCs (*dmyc*^{P0}/+; βGal-positive, red) were generated and differentiating progeny was followed over time. GSCs were identified by the presence of a round fusome (anti-Hts staining, green) and their proximity to the terminal filament, or, alternatively, by the lack of Bam expression (blue, see F). Four days after clone induction (ACI), mosaic stem cell niches are observed (B). However, stem cell niches become depleted of *dmyc*^{P0} mutant GSCs over time, visualized by βGal-negative cells distal from the niche. (C,D) *dmyc*^{P0} mutant cystoblasts (C) and cysts (D) 1 week and 2 weeks ACI, respectively, derived from formerly mosaic niches. (**E**,**F**) GSC-GSC interaction is non-apoptotic. (E) Mosaic germarium, containing control [*dmyc*^{P0}/+; βGal positive (red)] and *dmyc*^{P0} mutant GSCs, was stained with anti-active Caspase 3 (green). Inset shows positive control staining (arrow) for the same antibody in wild-type GSCs from heat-stressed flies (nuclei stained with DAPI, red). (F) Competition between GSCs proceeds in the absence of a fully active apoptotic pathway, i.e. in a background of dIAP-1 overexpression. Mosaic germarium, containing control [*dmyc*^{P0}/+; βGal-positive (red)] and *dmyc*^{P0} mutant GSCs. Bam-GFP is shown in blue. (**G**,**H**) Expression of *tkv*^{ACT} in the germline prevents both loss of *dmyc*^{P0} GSCs from the niche (black) under competition with control *dmyc*^{P0}/+ GSCs (βGal, red) (G) and differentiation of *Dpmyc*/+ stem cells (βGal, red) when in competition with 4×*dmyc* mutant GSCs (black; H; note Hts staining, green). (**I**) Mosaic niche containing *sav*^{shrp6B21} mutant (black) and control *sav*^{shrp6B21}/+ (GFP-positive, green) GSCs. GSC progeny was analyzed 2 weeks ACI. Anti-Hts, red. The *sav*^{shrp6B21} mutant GSC gave rise to more offspring (see Table 2), but did not expel the control GSC. (**J**) Mosaic niche containing control (*bam*^{Δ86}/+) stem cells (GFP-positive, green) and *bam*^{Δ86} mutant (black) GSCs and their progeny 2 weeks ACI. Anti-Hts, red. *Bam*^{Δ86} mutant 'GSC-like' cells accumulate in the niche. (**K-M**) Diagrams illustrating the methods by which tumor-promoting mutations may be established within a stem cell-based adult tissue, i.e. a germarium containing wild-type stem cells (GSC, gray). GSCs are attached to the cap cells (magenta) and divide asymmetrically to produce cystoblasts (CB), which in turn will divide to form cysts. Mutations affecting tumor-promoting genes take place originally in one stem cell but are inherited by its progeny as shown in red. Mutations may establish within the tissue by the following strategies. (K) 'Settler' strategy. Mutant GSCs (red) remain in the stem cell niche and produce differentiated progeny, usually at higher rates than the wild-type GSCs (gray). The number of mutant GSCs does not change. (L) 'Squatter' strategy. Mutations expand among the stem cell population, which leads to an increasing number of mutant GSCs, without affecting total GSC numbers as wild-type cells are forced to differentiate and are being replaced by mutant stem cells. (M) 'Plague' strategy. Mutations expand by increasing total stem cell-like numbers (black asterisks), thereby producing a 'plague of stem cell-like cells'.

Thus, minimizing the difference in dMyc levels between GSCs and differentiating CBs increases the range of the extracellular Dpp signal.

The manipulation of dMyc levels also led to aberrant differentiation patterns. GSC-like cells could be found intermingled with late cyst-stage cells (Table 2, Fig. 5N), suggesting that differentiation is not properly initiated in certain cells. It is conceivable that dMyc expression in the somatic cells of the niche might influence GSC numbers. In order to eliminate this complication, we employed two controls: (1) we reduced dMyc in the whole animal (*dmyc*^{P0}/*dm*^{4} and *dmyc*^{P0}/*dm*^{PL35}); and (2), in those *dmyc* mutant backgrounds, we overexpressed dMyc in the germline only, using a *UASp-dmyc* transgene driven by the germline promoter nanos-Gal4 (Fig. 5Q-X). Restricting dMyc-overexpression to the germline in a *dmyc* mutant background also resulted in the accumulation of GSC-like cells and an extended pMad gradient, confirming the above results. The extension of the pMad-signaling domain was less pronounced in the *dmyc* mutant background alone (Fig. 5R). Although the realm of Dpp diffusion is probably increased in this situation, this might not be reflected in the pMad read-out, as Dpp uptake and therefore transduction is compromised in such *dmyc*-deficient cells.

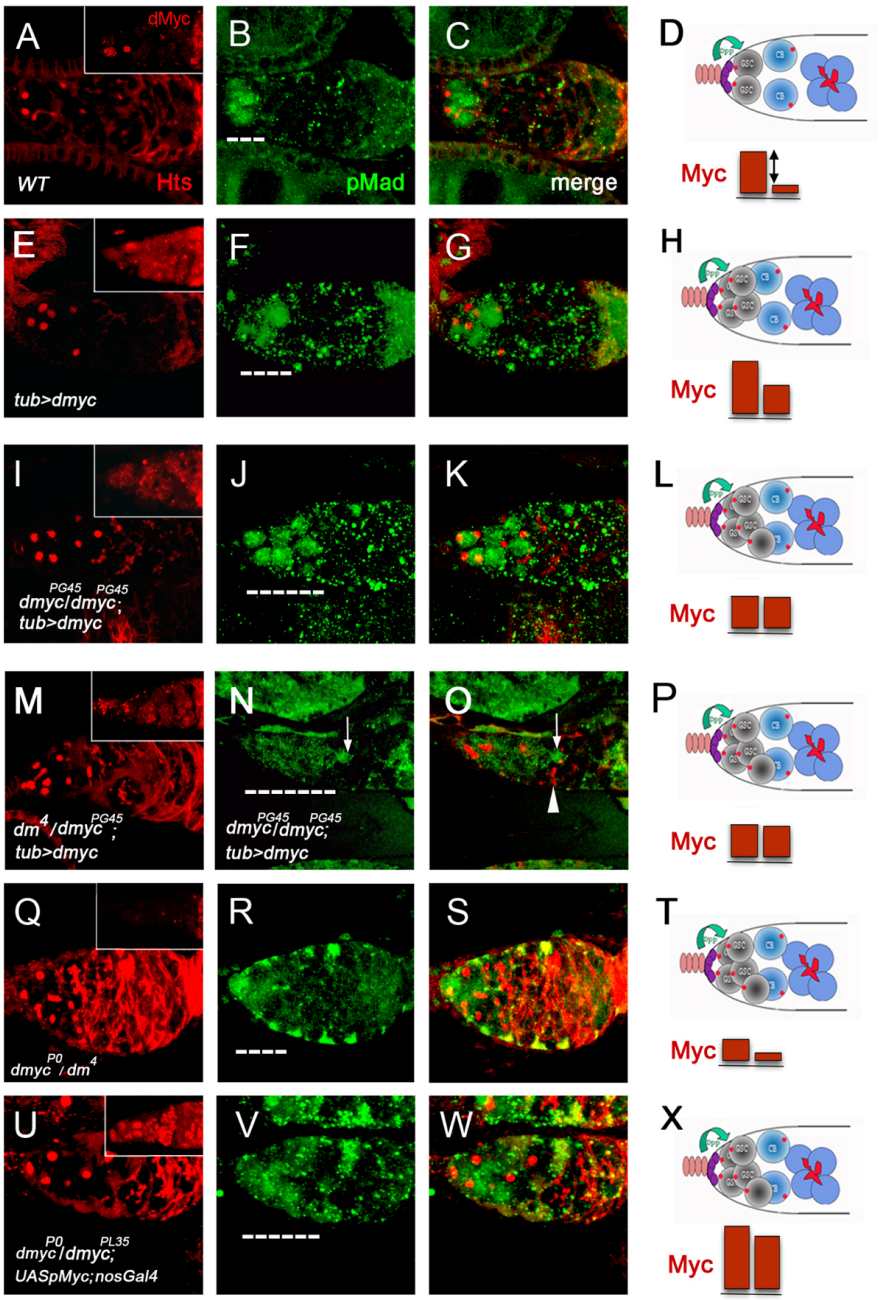

Fig. 5. Equalizing dMyc levels between stem cells and their daughters leads to ectopic Dpp signaling outside the niche. (**A-C**) Control germaria of 1-week-old flies were stained with anti-Hts, a fusome marker (A, red), and anti-pMad (B, green) to identify GSCs. (C) Merged image. Insets in A,E,I,M,Q and U show dMyc expression [anti-dMyc antibody (red)] in the germaria of the corresponding genotype. (**E-G**) Germaria from 1-week-old *tub>dmyc* flies stained with anti-Hts (E, red) and anti-pMad (F, green). The extension of pMad-positive cells is visualized by a white dotted line. (G) Merged image. (**I-K**) Germaria from 1-week-old $dmyc^{PG45}/dmyc^{PG45}$; *tub>dmyc* flies stained with anti-Hts (I, red) and anti-pMad (J, green). The merged image (K) shows an increase in the population of GSCs that transduce high levels of Dpp signaling (extended pMad activation, dotted line). (**M**) Germaria from 1-week-old $dm^{4}/dmyc^{PG45}$; *tub>dmyc* flies stained with anti-Hts (red) to identify GSCs. (**N**,**O**) Germarium from a 1-week-old $dmyc^{PG45}/dmyc^{PG45}$; *tub>dmyc* fly showing abnormal location of a GSC (arrow, round fusome) in the region of 8- to 16-cell cysts (arrowhead points to branched fusome of cysts). This mixing of very early cystoblasts (stem cell-like) and late (8- to 16-cell) cysts never occurs in wild-type germaria. pMad staining (green, N) and merge with anti-Hts staining (red) of the same germaria (O). (**Q-S**) Germaria from 1-week-old $dmyc^{P0}/dm4$ flies were stained with anti-Hts (Q, red) and anti-pMad (R, green). S shows the merged image. (**U-W**) Germaria from 1-week-old $dmyc^{P0}/dmyc^{PL35}$; *UASpMyc*; *nanosGal4* flies were stained with anti-Hts (U, red) and anti-pMad (V, green). W shows the merged image. (**D**,**H**,**L**,**P**,**T**,**X**) Schematics of germaria, illustrating the different situations that occurred when dMyc levels were manipulated. GSCs are represented in gray and differentiated progeny (cystoblasts, CB) in blue. The changes of dMyc occur at some point during the transition of GSC to pre-cystoblast and cystoblast. For simplicity, the pre-cystoblast stage is not shown. The lower diagram in each case shows the relative levels of dMyc in GSC verus daughter cells (left and right red bars, respectively). (D) Wild type; (H) *tub>dmyc*; (L) $dmyc^{PG45}/dmyc^{PG45}$; *tub>dmyc*; (P) $dm^{4}/dmyc^{PG45}$; *tub>dmyc*; (T) $dmyc^{P0}/dm^{4}$; (X) $dmyc^{P0}/dmyc^{PL35}$; *UASpMyc; nos-Gal4*. A-K,O and Q-W are overlays of several confocal planes throughout the germarium. N and O show a single confocal plane. Anterior is to the left in all pictures and diagrams.

Table 2. Competition between stem cells and their daughters

Drosophila strains	GSCs* and GSC-like cells† (average number per germarium)	Percentage of total germaria with aberrant differentiation (mixed stages)‡
Wild type	2.8±0.7 (*n*=32)	0 (*n*=21)
tub>dmyc	3.8±0.6 (*n*=25) *P*=2.1800×10^{-6}§	5 (*n*=25)
dmyc^{PG45}/dmyc^{PG45}; tub>dmyc	5.6±1.3 (*n*=26) *P*=6.3292×10^{-11}§	40 (*n*=28)
dmyc^{PG45}/dm^{4}; tub>dmyc	6.1±2 (*n*=11) *P*=5.1650×10^{-7}§	26 (*n*=11)
dmyc^{P0}/dm^{4}	4.0±1.2 (*n*=48) *P*=2.9781×10^{-6}§	–
dmyc^{P0}/dmyc^{PL35}; UASpMyc; nosGal4	4.3±1.3 (*n*=24) *P*=1.3118×10^{-5}§	–

*All undifferentiated cells with a round fusome were counted (including pre-cystoblasts).
†GSC-like cells: they do not always represent functional stem cells, but rather cells with a GSC-like morphology blocked in differentiation. Numbers are shown±s.d.
‡Percentage of germaria containing GSC-like cells intermingled with late cysts was determined by counting germaria with GSC-like cells in the 8- or 16-cyst germarium region (see Fig. 2N). GSC-like appearance was identified by anti-Hts and anti-pMad staining.
§*P*-values for Wilcoxon rank sum test: compared with wild type (all *P*-values are significant).

We conclude that equalizing dMyc levels across the germarium leads to ectopic pMad signaling, an effect explained by a model that implements competitive interactions (see Fig. 6), but which is unexpected if the presented dMyc manipulations simply interfered with stem cell differentiation.

DISCUSSION

In this study we describe competitive interactions among stem cells that arise as a consequence of differential dMyc levels in individual GSCs. We provide evidence that such dMyc-induced competition leads to the replacement of suboptimal (*dmyc* mutant) stem cells by wild-type GSCs or, in the case of emerging *dmyc* duplications, to the niche take-over by dMyc-overexpressing stem cells. In both cases, the outcompeted stem cells are forced to leave the niche, by a mechanism that is likely to involve Dpp-signaling, and differentiate. Furthermore, we propose that competitive interactions for niche-secreted Dpp between stem cells and their progeny lead to an efficient initiation of the differentiation process.

Non-apoptotic competition in GSCs induced by dMyc

In our analysis of mosaic niches, we found that stem cells mutant for the hypomorphic allele *dmyc^{P0}* (*dmyc^{P0}/dmyc^{P0}*) were gradually outcompeted by neighboring GSCs with higher levels of dMyc (*dmyc^{P0}/+*). The same effect was observed with stem cells overexpressing dMyc relative to their counterparts, resulting in the niche conquest by such dMyc-overexpressing stem cells. Comparison of mosaic niches with niches harboring a homogenous population of stem cells regarding dMyc levels showed that dMyc was able to induce competition, measurable in shorter decay times of the outcompeted cells.

Our results contrast, however, with a recent study by Ting Xie and colleagues, who also examined *dmyc* in GSC competition. They tested the lethal *dmyc* alleles *dm^{2}* and *dm^{4}*, which behave genetically as null alleles and found that such dMyc-deficient GSCs were not outcompeted by control GSCs (Jin et al., 2008). Strikingly, they also obtained different results with stem cells carrying two copies of the *nos-gal4VP16* driver to overexpress *UASdmyc* in the germline. Such GSCs did not compete with control GSCs carrying only one copy of *nos-gal4VP16*, rather they were lost slightly faster from the niche.

Part of the explanation for the discrepancies might lay in differences in experimental design and chosen *dmyc* alleles. In our hands, GSCs carrying strong or null mutations in *dmyc* (*dmyc^{PG45}* or *dm^{4}*, respectively) showed poor viability and, in the few mosaic niches found, *dmyc*-deficient stem cells were not lost from the niche. We therefore decided to study hypomorphic *dmyc^{P0}* stem cells that express dMyc, but at lower levels – an ideal case of viable, but suboptimal stem cells. GSCs devoid of any *dmyc* (*dm4* deletion) may not enter competitive interactions because some basal dMyc levels might be required to permit an intercellular comparison of relative competitiveness. Wing pouch cells of the imaginal disc have been proposed to be able to compare their Dpp signaling levels with those of cells outside of the pouch (Rogulja and Irvine, 2005). Intriguingly, this differential behavior coincides with the dMyc expression pattern (Johnston et al., 1999). We also believe that dMyc-induced cell competition acts only within a certain range of dMyc fluctuation, and will be overrun by apoptosis as a consequence of the well-characterized effect of dMyc to induce cell-autonomous apoptosis when expressed at high levels (Hueber and Evan, 1998).

Both genetic systems employed here to raise dMyc levels (*dmyc* duplication and *tub>cd2>dmyc* flip-out cassette) are compatible with GSC viability. The overexpression of dMyc using Gal4 amplification systems with two copies of *nos-gal4VP16* driver may not be so well tolerated by stem cells, compromising their competitiveness.

In contrast to studies that have focused on cell adhesion (O'Reilly et al., 2008; Jin et al., 2008) and mechanical models of GSC extrusion from the niche, we provide evidence that Dpp signaling and cell-cell communication play a role for dMyc-induced competition. We do not completely understand how cells compare the Myc levels of one another, but we present evidence that the ability to compete for 'stemness' factors, like Dpp, is important: when GSCs with more dMyc contact GSCs with less dMyc, the cells with higher levels become more sensitive to Dpp and accumulate pMad, which ensures a long-lasting stem cell fate, whereas the cells with lower relative levels of dMyc lose responsiveness and eventually differentiate. A possible connection between dMyc and the acquisition of an extracellular ligand(s) has been proposed before. This relationship seems to be indirect and is likely to involve the coordinated control of several target genes, resulting in a gain of several aspects of biometabolic functions, e.g. modifying rates of endocytosis (Moreno and Basler, 2004).

The identification of paracrine and/or juxtacrine signals that are specific to loser and/or winner cells is likely to become a fascinating line of future research, as in the case of the apoptotic elimination of 'loser' cells (Ryoo et al., 2004; Perez-Garijo et al., 2004; Huh et al., 2004). In fact, the possibility that cells can compare their relative Dpp signaling levels has been postulated to explain both apoptotic cell competition (Diaz and Moreno, 2005; Senoo-Matsuda and Johnston, 2007; Moreno, 2008) and the regulation of cell proliferation (Rogulja and Irvine, 2005), but the molecules that mediate this comparison are unknown. Further studies will also clarify how similar apoptotic and non-apoptotic cell competition really are.

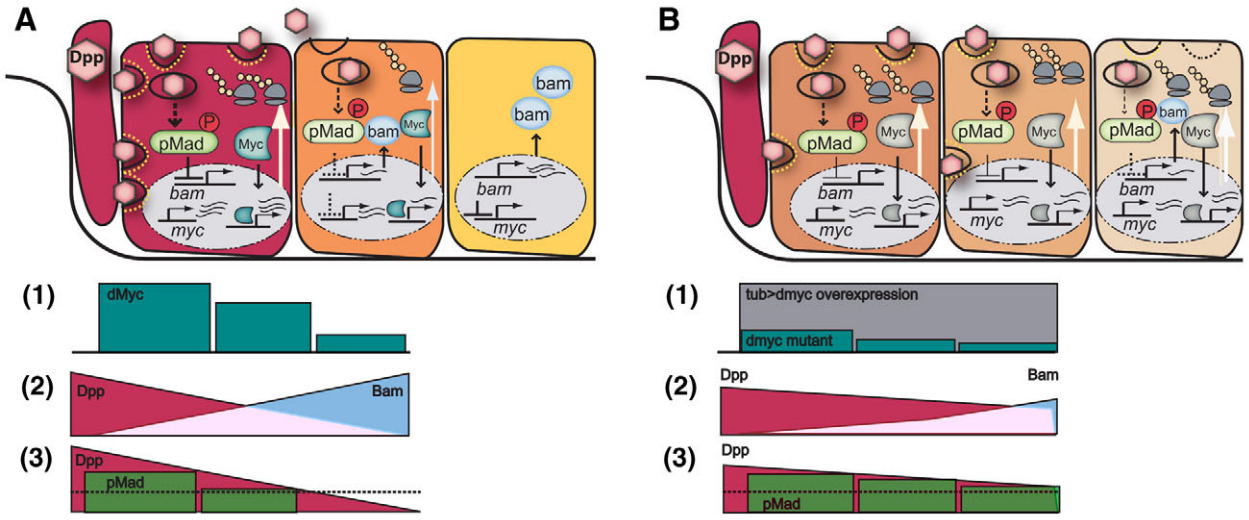

Fig. 6. Model of programmed cell competition between GSCs and their daughters. Schematic of the ovary stem cell niche. A cap cell (oval-shaped cell), which secretes diffusible Dpp molecules, is depicted in dark red, followed by a GSC, a pre-CB and a CB cell. (**A**, top) Physiological dMyc expression (dark green) is high in GSCs and activates multiple target genes, leading to high protein synthesis (depicted by ribosomes, gray) and elevated endocytosis rates (yellow dotted line). Most niche-secreted Dpp is internalized by competitive stem cells and transduced into pMad, which directly represses the transcription of *bam* by binding to its promoter. dMyc downregulation starts in pre-CBs and is complete in CBs, probably mediated by Mei-P26 or similar factors, leading to decreased endocytosis rates of Dpp and signal transduction. GSCs differentiate after asymmetric division owing to the expression of the differentiation factor Bam, turned on as a consequence of the drop in pMad levels. Bars below show (1) the physiological expression of dMyc (dark green) in GSCs, pre-CBs and CBs, (2) the overlay of the Dpp gradient and inverse Bam expression, and (3) the Dpp gradient and threshold (dotted line) required for signal transduction (pMad activation levels, green). (**B**) If physiological dMyc levels are reduced to a minimum (*dmyc* mutant background) or masked by ubiquitous overexpression of dMyc (gray), pre-CBs and CBs compete more efficiently for Dpp, leading to a more equal distribution of the available stem cell factor. Consequently they show higher pMad levels and delayed differentiation as a result of the prolonged repression of *bam*. Dpp signaling thresholds are still met by daughter cells more distal to the niche owing to the flatter trajectory of the Dpp gradient, depicted below. Bars below show (1) uniform dMyc levels achieved in a *dmyc* hypomorph (dark green, remaining endogenous dMyc) combined with dMyc overexpression using a *tub>dmyc* transgene (gray), (2) flatter Dpp gradient and delayed Bam upregulation in the absence of competition, and (3) resulting higher pMad levels in pre-CBs and CBs (green).

dMyc expression in stem cells: implications for quality control and cancer

Our results with $dmyc^{P0}$ mutant stem cells have demonstrated that suboptimal GSCs are efficiently recognized and eliminated from the stem cell pool, suggesting that dMyc-induced cell competition plays a physiological role in controlling tissue quality. Because dMyc is always expressed at high levels in GSCs (Fig. 1B-F), the quality control system seems to be especially suited to detecting subtle drops in dMyc levels in suboptimal GSCs. In a similar way, fluctuation in the repression of the differentiation factor *bam* by Dpp signaling has been suggested to regulate stem cell quality (Jin et al., 2008), which would be consistent with our model that competitive stem cells show high pMad levels and, hence, tightly repressed *bam* expression.

Although cell competition seems an excellent tool with which to select the 'fittest' stem cell when compromised GSCs are present, it bears the risk that stem cells with modest dMyc overexpression are selected as being preferable over the wild type. Niches occupied by stem cells harboring dMyc duplications will give rise to differentiated tissue containing identical genetic alterations. Such 'pre-cancerous' fields are then more likely to accumulate secondary or tertiary hits that will lead to tumor formation (Braakhuis et al., 2005). Given that stem cells are long-lived, we believe that the characteristics and consequences of dMyc-induced competition are relevant for cancerous transformation, especially for tissues with a high turnover (Merlo et al., 2006). The most notable features of niche occupancy by dMyc-overexpressing stem cells are (1) the replacement of normal GSCs, which was not observed by other proliferation-promoting mutations (*Pten*, *sty*), and (2) the absence of tissue alteration. The property to outcompete wild-type stem cells renders dMyc mutations potentially dangerous because they are capable of establishing a mutant stem cell population that can remain long enough to accumulate further cooperating mutations (Moreno, 2008; Rhiner and Moreno, 2009). This could be a reason why *myc* is the target of early mutations in cancers and *Pten* inactivation occurs only at later stages.

dMyc expression at the GSC/CB interface

So far, cell competition has been analyzed extensively using artificial means to create mosaic tissues, but few attempts have been undertaken to reveal competitive interactions in a more physiological situation. An exception is the study on the competitive interactions of cells during liver repopulation after hepatectomy (Oertel et al., 2006).

In this study, we have tested the novel idea that developmentally regulated expression of dMyc in the germline triggers competition for the stem cell factor Dpp between high dMyc-expressing stem cells and low dMyc-expressing progeny. This competition might be classified as 'low level', compared with stem cell-stem cell interactions in the niche, as the daughter cells compete with the handicap of being located more distally from the source of Dpp than are the stem cells. Nonetheless, our experiments, in which we perturbed the physiological dMyc pattern and equalized GSCs and CBs in terms of dMyc levels, strongly suggested a contribution of competition in the initial step

of differentiation. The ability of dMyc to activate a variety of genes encoding components of protein synthesis pathways (Grewal et al., 2005) indicates that it may have the capacity to stimulate protein translation in a coordinate manner. We propose a model in which high dMyc levels in stem cells stimulate high metabolic rates, including increased protein synthesis and endocytosis, through the activation of multiple target genes (Fig. 6). This enables the stem cells to compete efficiently and turn over a high amount of niche secreted Dpp, resulting in elevated pMad levels, which in turn ensure a tight repression of the differentiation gene *bam*. During the pre-CB to CB transition, dMyc levels are downregulated, probably because of the oncoming expression of Mei-P26, which lowers the efficiency of pre-CBs/CBs to take up Dpp. The consequence is a steep decline of the Dpp gradient across the niche, where remaining Dpp input in CBs is too low to activate pMad and Bam-mediated differentiation is fully initiated. In the absence of competition, achieved by imposing equivalent dMyc levels in all three cell types (see Fig. 5), available Dpp molecules distribute more uniformly over several cell diameters because cells compete on a more similar basis. As a consequence, Dpp signaling thresholds are still attained in cells distal from the niche (Fig. 6, lower part), which still present stem cell-like morphology owing to repressed *bam* transcription by pMad. We do not intend to play down the role of Bam, which is the main trigger for differentiation, but we suggest that dMyc-induced competition for Dpp reinforces the tight repression of Bam in GSCs and the efficient derepression of Bam in the differentiating daughter cells. If competition is impaired, the differentiation process is delayed and less defined, occasionally leading to the mixing of cystoblasts at different stages of differentiation.

The competitive interaction between stem cells and their daughters containing different relative levels of dMyc described here is of particular interest because the interface along which the competition takes place is created through gene regulation. Therefore, we propose the term ‘programmed cell competition’ to distinguish it from competitive interactions that arise as a result of genetic alterations. Programmed cell competition will occur along boundaries of gene expression that are epigenetically defined (i.e. by gene regulation) and does not require a mutational event, as in previously described forms of competition.

Because Myc proteins play important roles in the adult stem cells of several mammalian niches (reviewed by Murphy et al., 2005), it is possible that the interactions described here are conserved, at least in certain tissues (Muncan et al., 2006). In tissues where stem cells are not grouped together within the same niche, the process may be aided by migration of the stem cells from one niche to the other, as has been recently described for the somatic stem cell niches of the *Drosophila* ovary (Nystul and Spradling, 2007). More generally, the concept of competition among cells could be of use to describe several aspects of development and homeostasis, an idea nicely supported, for example, by the competition that occurs between the soma and the germline for lipid phosphate uptake (Renault et al., 2004). Stem cell interactions such as those described here significantly contribute to the balance between differentiation and self-renewal, and may be relevant for diverse processes such as aging, the accumulation of pre-cancerous mutations and the successful application of stem cell therapies.

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Supplementary material

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