Development 134, 1471-1479 (2007) doi:10.1242/dev.02810

Stonewalling *Drosophila* stem cell differentiation by epigenetic controls

Jean Z. Maines[1], Joseph K. Park[1], Meredith Williams[2] and Dennis M. McKearin[1,*]

During *Drosophila* oogenesis, germline stem cell (GSC) identity is maintained largely by preventing the expression of factors that promote differentiation. This is accomplished via the activity of several genes acting either in the GSC or in its niche. The translational repressors Nanos and Pumilio act in GSCs to prevent differentiation, probably by inhibiting the translation of early differentiation factors, whereas niche signals prevent differentiation by silencing transcription of the differentiation factor Bam. We have found that the DNA-associated protein Stonewall (Stwl) is also required for GSC maintenance. *stwl* is required cell-autonomously; clones of *stwl*$^{-}$ germ cells were lost by differentiation, and ectopic Stwl caused an expansion of GSCs. *stwl* mutants acted as Suppressors of variegation, indicating that *stwl* normally acts in chromatin-dependent gene repression. In contrast to several previously described GSC maintenance factors, Stwl probably functions epigenetically to prevent GSC differentiation. Stwl-dependent transcriptional repression does not target *bam*, but rather Stwl represses the expression of many genes, including those that may be targeted by Nanos and Pumilio translational inhibition.

KEY WORDS: Stem cells, Epigenetics, *Drosophila*, Oogenesis

INTRODUCTION

Stem cells are partially defined by their ability to divide asymmetrically, producing a differentiating daughter cell and a self-renewing stem cell. This paradigm is used many times throughout development to produce and maintain mitotically active tissues, reserving stem cells as a source of cell progenitors throughout the life of an animal. Mechanisms regulating stem cell biology are relevant to studies of developmental biology as well as to regenerative medicine and to the pathogenic transformation of tissues. Loss of stem cells can disrupt normal tissue homeostasis and may cause premature aging, infertility or defects in tissue regeneration. By contrast, misregulation of stem cell self-renewal can produce a population of undifferentiated cells that may be susceptible to carcinogenic transformation.

The germline stem cells (GSCs) of the *Drosophila* ovary have emerged as a valuable model for studying stem cell behavior and its regulation. In contrast to stem cells in many other tissues, the ovarian GSCs are easily identified by location and by molecular markers. Two or three ovarian GSCs reside at the anterior of each ovariole, closely apposed to the somatic cap cells of the germarium. Mutational analysis in *Drosophila* has revealed a suite of genes that is necessary for GSC maintenance (Gilboa and Lehmann, 2004a; Spradling et al., 2001). These studies have demonstrated that a microenvironment, or niche, composed of multiple cell types influences GSC self-renewal. Factors intrinsic to the stem cell, as well as signals from the somatic cells of the niche, are important for GSC maintenance.

The emerging view of GSC maintenance in *Drosophila* indicates that GSCs are maintained largely by preventing their differentiation (Kai et al., 2005; Li and Xie, 2005; Wang and Lin, 2004). Stromal cells produce Dpp locally to prevent GSC differentiation by initiating a Smad signaling cascade in GSCs that silences transcription of the key differentiation factor *bag of marbles* (*bam*) (Chen and McKearin, 2003a; Song et al., 2004; Xie and Spradling, 1998). Adherens junctions anchor GSCs to cap cells and are essential for GSC maintenance and for Smad signaling (Song et al., 2002). Other factors required in the cap cells, such as *piwi* (Cox et al., 2000) and *fs(1)Yb* (King and Lin, 1999), are also essential GSC maintenance factors, but their mode of action remains unknown.

Gene products that are required intrinsically in stem cells for their maintenance also function to prevent germline differentiation. For example, the translational inhibitors Nanos (Nos) and Pumilio (Pum) are required in stem cells (Forbes and Lehmann, 1998; Lin and Spradling, 1997; Wang and Lin, 2004). The Nos-Pum complex is thought to repress the translation of key differentiation factors to prevent GSC differentiation, but the targets of Nos and Pum in GSCs are unknown. A recent report has also suggested that chromatin state influences GSC maintenance, because *Iswi*, a key chromatin-remodeling factor, is required in GSCs for their maintenance (Xi and Xie, 2005).

Here, we present evidence that the DNA-associated protein Stonewall (Stwl) is required in GSCs, and that it promotes their maintenance by repressing germ cell differentiation. Stwl is a nuclear factor that has protein domains that suggest an interaction with histone-modifying enzymes. We demonstrate that *stwl* mutants act as dominant suppressors of variegation, indicating a requirement for *stwl* in heterochromatin assembly or maintenance. We order the requirement for *stwl* with respect to other GSC maintenance factors and find that *stwl* is not essential for *bam* silencing, but rather that it acts in opposition to *bam* activity to prevent germ cell differentiation. We propose that Stwl represses the expression of many genes, including those targeted by Nos-Pum translational inhibition. We identify a group of genes that are specifically upregulated in the absence of *stwl* and, among those genes, find candidates for Nos-Pum translational inhibition.

MATERIALS AND METHODS

Transgenic and mutant flies

Genetic mutants identified below are also described at http://flybase.bio.indiana.edu. *stwl* mutants used included *stwl*^{Z1}, *stwl*^{95} (K. Clark, PhD thesis, University of Texas Southwestern Medical Center, 1996)

[1]Department of Molecular Biology and [2]Graduate School of Biomedical Sciences, University of Texas Southwestern Medical Center at Dallas, Dallas, TX 75390-9148, USA.

*Author for correspondence (e-mail: Dennis.McKearin@utsouthwestern.edu)

Accepted 17 January 2007

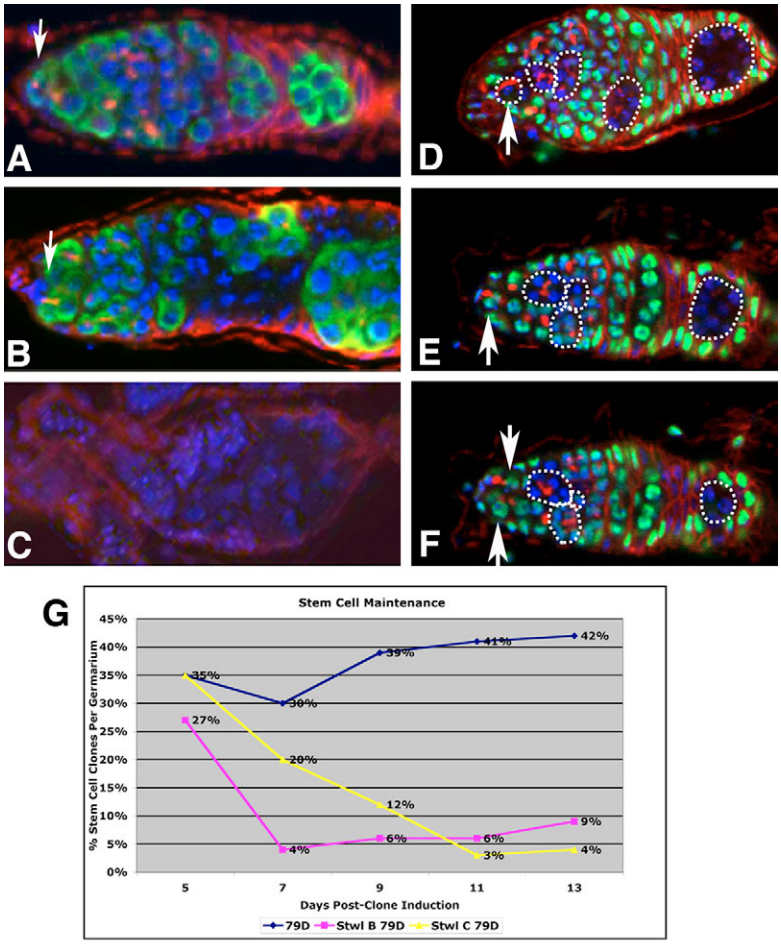

Fig. 1. Stwl is required for GSC maintenance. (**A-C**) Ovaries from heterozygous control animals (A) were dissected 10 days post-eclosion. Germ cells were labeled with the germ cell marker anti-Vasa (green), a fusome marker (anti-Hts, red) and the nuclear dye Hoechst (blue). Ovaries from $stwl^{Z1}/stwl^{\Delta 95}$ mutant animals were dissected at 4 days (B) and 10 days (C) post-eclosion. (**D-G**) GSC clones were induced by heat-shock in adult animals and identified by the absence of nuclear GFP. (D) Clones induced in control wild-type genotype. Arrow points to the GSC. (E,F) The presence of clonally-related mutant germ cell clusters at 7 days post-heat-shock (outlined) indicated that each germ cell cluster was derived from a mutant stem cell. The stem cell region of this germarium, however, contained only wild-type stem cells (arrows). (G) Graph showing the frequency of negatively-marked stem cell clones from a wild-type FRT chromosome (blue) or two *stwl*-null chromosomes (yellow, pink) over a 2-week period.

and $stwl^{EY05697}$ (Bellen et al., 2004). Other mutations included nos^{RC} (Gavis and Lehmann, 1992), Df(*nos*) Df(3R)DL-FX1 (Sonoda and Wharton, 1999) (gift from R. Wharton), pum^{ET9} (Barker et al., 1992), $bam^{\Delta 86}$ (McKearin and Ohlstein, 1995), *Su(var)*3-9^{1} (Reuter et al., 1986) and EY00986 upstream of CG3919 (*stwl* neighbor) from Bellen (Bellen et al., 2004).

Transgenic flies used included P{*bam*P-GFP} (Chen and McKearin, 2003b), P{hsp70-*stwl*} (Clark and McKearin, 1996), P{FRT 79D} and P{hsFLP} from Bloomington Stock Center, P{*nos*P-Gal4:VP16} (a gift from R. Lehmann) (Van Doren et al., 1998), DX1 P{*w*+} (a gift from S. Henikoff) (Dorer and Henikoff, 1994), P{Nos-myc} (a gift from R. Wharton) (Verrotti and Wharton, 2000). Histone-GFP fusion flies were a gift from J. Duffy (Indiana University, Bloomington, IN).

Ectoptic Stwl was produced by combining the $stwl^{EP}$ allele, which allows misexpression of $stwl^{+}$ when a source of Gal4 transactivator is introduced, with the germline-specific Nos-Gal4:VP16. Flies were transferred to wet yeast and shifted to 29°C for a minimum of 18 hours prior to ovary dissection. For each genotype, a minimum of 30 ovarioles were scored. {HS-*stwl*} produced a similar, but more variable, stem cell expansion phenotype.

For clonal analysis, flies of the genotype {hsFLP}; {histoneGFP} {FRT79D} were crossed to $stwl^{95}$ {FRT 79D} flies. To make clones of wild-type alleles, such as for Lola expression experiments, mitotic recombination was induced in P{hsFLP}/+; {histoneGFP} {FRT 79D}/{FRT 79D} females. Adult females were subjected to heat-shock (37°C) for 1 hour, three times per day for 2 days. All fly stocks were raised on standard cornmeal molasses agar at 25°C, unless noted otherwise. Stem cell maintenance was assayed by retention of mutant stem cells over time. At 5, 7, 9, 11 and 13 days post-clone-induction, ovaries were examined and scored for the number of GSC clones/ovariole. For each time-point and genotype, a minimum of 30 ovarioles were scored.

stwl bam double-mutant analysis used $stwl^{95}$ bam^{86} and $stwl^{Z1}$ bam^{86} or $stwl^{EY}$ bam^{86} chromosomes. All three *stwl* alleles acted as strong loss-of-function alleles and behaved similarly in combination with bam^{86}.

For eye pigment extraction, ten heads from male flies aged for 3 days were homogenized in 700 μl of methanol with 0.1% HCl and were then incubated overnight at 4°C. Following incubation, the extracts were cleared by centrifugation and measured for optical density at 488 nm. Each genotype was assayed in triplicate.

Immunohistochemistry

Ovaries were prepared for reaction with antibodies as described previously (Christerson and McKearin, 1994). The antibodies and dilutions used were: rabbit anti-GFP (Invitrogen) (1:10,000 dilution); mouse anti-α-Hts (1B1) (Zaccai and Lipshitz, 1996) (1:750); mouse anti-BamC (A7) (McKearin and Ohlstein, 1995) (1:10); rat anti-Pum 1637 (Macdonald, 1992) (1:500); rabbit anti-Vasa (Lasko and Ashburner, 1990) (1:1000); rabbit anti-Bru (Sugimura and Lilly, 2006) (1:3000), rabbit anti-Lola (Giniger et al., 1994) (1:100); rat anti-Stwl F6 (K. Clark, PhD thesis, University of Texas Southwestern Medical Center, 1996) (1:100); and mouse anti-Myc (9E10, DSHB) (1:1000). Alexa Fluor secondary antibodies (Molecular Probes) were used at a 1:500 dilution.

Phenotypic analysis and genetic interactions

Ovaries isolated from 3-day-old well-fed flies were incubated with anti-Hts and anti-GFP antibodies. Images were collected on a Zeiss LSM 510 Meta and projected to count the number of spherical spectrosomes/fusomes, the number of *bam*-GFP-negative cells and to identify differentiated cysts. Round spectrosome-containing cells that were negative for *bam*-GFP were scored as GSCs. GSC number was determined by scoring a minimum of 20 germaria per genotype.

Microarray analysis

Microarray analyses for *stwl bam* versus *bam* ovaries were performed at the UT Southwestern Microarray Core Facility using Affymetrix *Drosophila* Genome 2.0 chips, representing ~18,500 transcripts. Ovaries from each genotype were hand-dissected and placed immediately into a Trizol RNA isolation reagent (Invitrogen). Total RNA was used to probe microarray chips. The expression of over 18,500 transcripts represented on the Affymetrix *Drosophila* Genome 2.0 microarray was examined in ovarian tissue samples obtained from *stwl bam* and *bam* mutant females. Triplicate hybridizations were performed for each sample. Overall, ~9000 transcripts were expressed in the tissues of each genotype.

Data analysis was performed to identify differentially expressed genes between *stwl bam* versus *bam* mutant genotypes. Using GeneSpring analysis software (Silicon Genetics), data were initially screened to identify signals counted as present and showing at least a twofold-change difference between the two genotypes. The data was analyzed using a parametric *t*-test and multiple correction method (Benjamini and Hochberg False Discovery Rate; $P<0.05$). We classified the differentially expressed transcripts into functional groups based upon GO (www.geneontology.org), and functional designation in NCBI and FlyBase notation.

RESULTS

A GSC maintenance defect typically results in a progressive decline in both germ cell number and in egg chamber production. In ovaries from *stwl* mutant females, we noticed that egg chamber number declined as the females aged. Taken together with our previous studies demonstrating that *stwl* acts early in germ cell development and is expressed in stem cells and cystoblasts (K. Clark, PhD thesis, University of Texas Southwestern Medical Center, 1996), we considered the possibility that *stwl* may play a role in stem cell maintenance. Ovaries from wild-type females retained germ cells for more than 3 weeks (Fig. 1A), but *stwl* mutant females showed a dramatic and rapid loss of germ cells, with approximately 50% of ovarioles lacking germ cells by 10 days post-eclosion (Fig. 1B,C). Akiyama had also reported germ cell decline while studying another collection of *stwl* mutant alleles and proposed that these *stwl* germaria lost GSCs (Akiyama, 2002).

The microenvironment or niche that regulates GSC maintenance includes somatic cells of the cap and terminal filament, which produce survival and differentiation factors that are crucial for GSC maintenance. Stwl is expressed in both germ cells and in somatic cells (K. Clark, PhD thesis, University of Texas Southwestern Medical Center, 1996); thus, *stwl* may be required in somatic cells to produce the microenvironment necessary for GSC maintenance and/or in the stem cell to respond to that microenvironment. To differentiate between these possibilities, we used mitotic recombination to eliminate *stwl* specifically in germ cells. Under conditions that produced GFP-negative GSCs (hereafter referred to as 'clones') in approximately 35-40% of ovarioles (Fig. 1D,G), wild-type GSC clones were maintained for over 2 weeks, whereas most *stwl*$^{-}$ GSC clones were rapidly depleted (Fig. 1E-G). TUNEL labeling in both wild-type and *stwl*$^{-}$ ovaries failed to reveal any germ cell apoptosis in the more than 100 germaria that were examined (data not shown).

To distinguish further between stem cell loss by germ cell death or by differentiation, we followed cyst progression in mosaic ovaries. *stwl*$^{-}$ clones were induced by heat-shock and animals were aged for a week or more to ensure that persisting clonal germ cells were derived from a mutant stem cell. Careful analysis of ovarioles containing *stwl*$^{-}$ germline clones revealed germaria containing clonally related mutant germ cell clusters without a corresponding stem cell, indicating that the stem cell that gave rise to these germ cell clusters had been lost (Fig. 1D-F). Together with the lack of apoptotic germ cells, these studies suggested that stem cell loss in *stwl* mutants was not a consequence of cell death, but rather that *stwl*$^{-}$ stem cells were lost by differentiation.

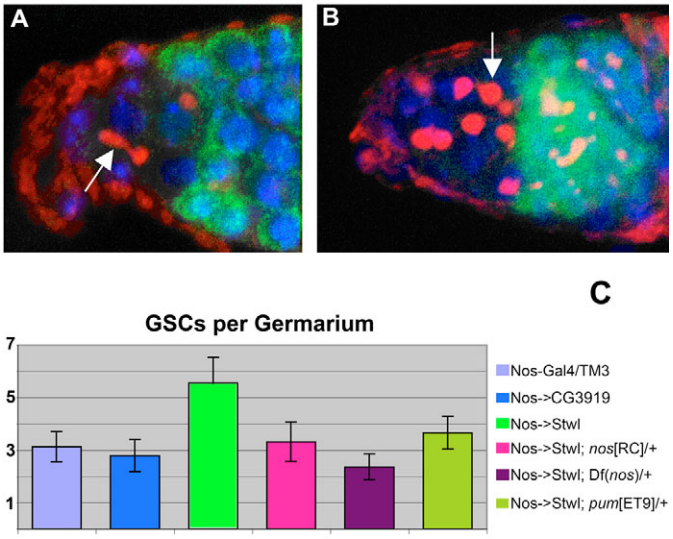

Fig. 2. Ectopic germline expression of Stwl induced an expansion of germline stem cells. (**A**) Germarium from Nos-Gal4 control animals contained a dividing stem cell that can be recognized by the elongated fusome (arrow) connecting the germline stem cell (GSC)-cystoblast pair of cells that were negative for *bam*-GFP (red, anti-Hts; blue, Hoechst; green, GFP). (**B**) Projection of three confocal sections from Nos-Gal4>*stwl*^{EY} animals. Arrow shows GSC-like cells. (**C**) Graph showing the average number of GSCs/germarium in various genotypes: Nos-Gal4 alone (light purple), Nos-Gal4 driving CG3919 (a gene neighboring *stwl*, blue), Nos-Gal4 driving *stwl* (green), Nos-Gal4 driving *stwl*/*nos*^{RC} (pink), Nos-Gal4 driving *stwl*/Df(*nos*) (dark purple) or Nos-Gal4 driving *stwl*/*pum*^{ET9} (yellow). *bam*-GFP-negative cells containing single, spherical fusomes were scored as GSCs (Xie and Spradling, 1998; Chen and McKearin, 2003a). The difference in the number of GSC-like cells in Nos-Gal4>*stwl*^{EY} (Nos->Stwl in C) ovaries was statistically significant by unpaired *t*-test ($P<0.001$) when compared individually to the number of GSC-like cells scored in the other genotypes presented in C.

In addition to its requirement for GSC maintenance, *stwl* was also essential as a cell-autonomous factor for proper oocyte maturation. Developing egg chambers derived from *stwl*$^{-}$ GSCs displayed several defects previously described in ovaries from *stwl*$^{-}$ females (Clark and McKearin, 1996), including a failure of oocyte differentiation, as evidenced by abnormal oocyte karyosome formation and by a failure to concentrate Orb within a single cell (data not shown). Likewise, pycnotic nuclei and widespread TUNEL labeling in cystocytes of stage-4 egg chambers revealed that *stwl*$^{-}$ germline clones eventually became apoptotic, as was described for homozygous *stwl* ovaries (Clark and McKearin, 1996). By contrast, egg chambers containing *stwl*$^{-}$ follicle cells but wild-type germ cells developed normally (data not shown), suggesting that, like GSC loss, *stwl* acts in germ cells rather than in soma to ensure oocyte development.

Ectopic Stonewall expression delays cystoblast differentiation

A corollary to the observation that *stwl* inactivation caused premature differentiation is that excess *stwl* expression might be sufficient to delay or block cystoblast differentiation. Although ovaries expressing ectopic Stwl in germ cells were morphologically similar to wild type,

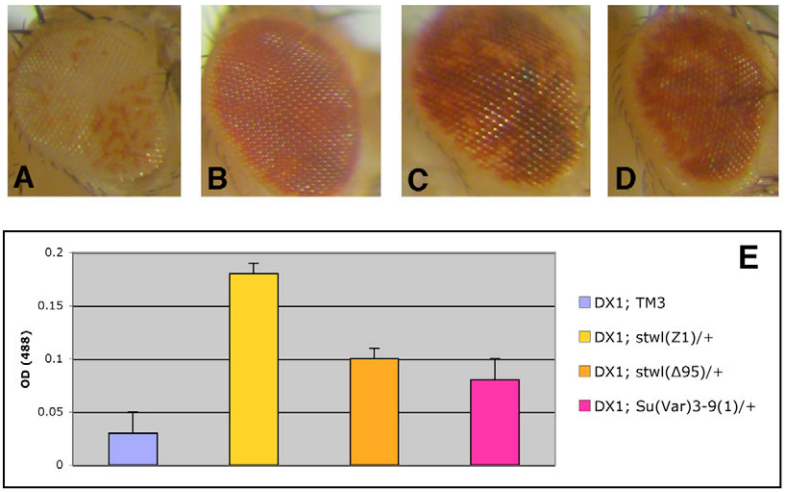

Fig. 3. *stwl* mutants are dominant suppressors of position-effect variegation. (**A**) Eye of DX1 flies showing strong variegation. (**B**,**C**) Eyes of DX1 flies also heterozygous for *stwl* alleles showing suppressed variegation. (**D**) The DX1 variegated phenotype was dominantly suppressed by a mutation in the histone methyltransferase encoded by the *Su(var)3-9* locus. (**E**) Graph of optical density of extracted pigments from flies of the indicated genotypes.

they contained extra GSC-like cells in the germarium, as measured by the presence of round fusomes (spectrosomes) and by the quiescence of a *bam* transcriptional reporter in germ cells at the anterior end of germarium (compare Fig. 2A with 2B). Ectopic Stwl resulted in two to three extra stem-cell-like cells per germarium, on average, compared with Gal4 control ovaries (Fig. 2C).

The extra GSC-like-cells phenotype induced by ectopic Stwl expression provided a valuable assay to identify genes that were required for Stwl action in the GSC differentiation pathway. We introduced single-copy mutations of several GSC maintenance genes into the Stwl overexpression background and scored GSC number. Introduction of single-copy mutations in the *dpp* pathway (e.g. *mad*, *punt*, *tkv*) did not significantly alter the *stwl* overexpression phenotype (data not shown). By contrast, mutations in *nos* and, to a lesser extent, *pum*, specifically suppressed the *stwl* overexpression phenotype, reducing the average GSC number to three per germaria (Fig. 2C). As a control, we found that the number of GSCs in Nos-Gal4/+; nos^{RC}/+ and Nos-Gal4/+; Df(*nos*)/+ ovaries was the same as in Nos-Gal4/+; TM3/+ ovaries. These findings suggested that the action of Stwl on GSC fate depended more heavily on the contribution of Nos-Pum to the GSC maintenance pathway than on niche signaling mediated by Dpp.

Stwl acts as a regulator of chromatin structure

Our data suggested that *stwl* acted autonomously in the stem cell to promote GSC maintenance or to repress cystoblast differentiation. Because the Stwl protein contains modified SANT domains, which have been implicated in chromatin regulation (Boyer et al., 2002), we considered the possibility that *stwl* may influence gene expression via the modification of chromatin structure or function. We noticed that Stwl was expressed in eye discs (data not shown), and took advantage of a well-described assay for chromatin-mediated gene repression to test for *stwl*-dependent chromatin modification (Dorer and Henikoff, 1994). Position-effect variegation (PEV) occurs when euchromatic genes are placed in, or adjacent to, heterochromatin, at which point they become subject to heterochromatic gene silencing. This silencing is often mosaic, or variegating, and can be relieved by reducing the concentration of heterochromatin-binding proteins or other associated transcriptional repressors (Karpen, 1994).

The DX1 chromosome contains a 'heterochromatinized' $white^{+}$ (w^{+}) gene array that normally produces weak w^{+} expression (Dorer and Henikoff, 1994), resulting in mostly white eyes with a few spots of red eye pigment (Fig. 3A). Mutant alleles of known chromatin-regulatory genes, such as *Su(var)*$3\text{-}9^{1}$, which encodes a histone methyltransferase required for heterochromatin structure (Rea et al., 2000), suppressed the variegated phenotype by producing more-uniform expression of w^{+} (Fig. 3D). Surprisingly, inactivating *stwl* alleles acted as strong dominant *Su(var)* mutations (Fig. 3B,C). We quantified the degree of suppression by extracting A_{488}-absorbing eye pigment from fly heads of each genotype and found that a reduction in *stwl* gene dose resulted in a two- to three-fold increase in eye pigment, a reflection of w^{+} expression level (Fig. 2E). *stwl* mutants showed a similar dominant *Su(var)* affect when tested with the variegated w^{m4} and bw^{D} alleles (data not shown).

Stwl in the hierarchy of GSC maintenance genes

One attractive candidate for a target silenced by *stwl* was the *bam* gene, because previous studies had shown that *bam* silencing was required for GSC maintenance (Ohlstein and McKearin, 1997). If

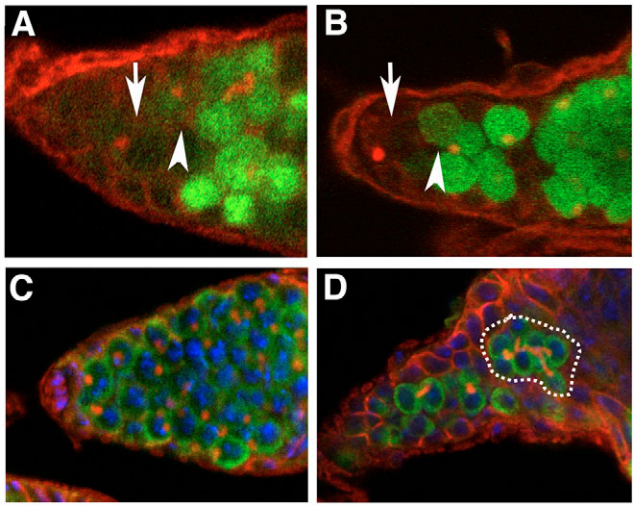

Fig. 4. *stwl* was not required for *bam* silencing and suppressed differentiation independently of *bam*. Spectrosomes/fusomes are labeled red in each panel. (**A**) *bam* transcriptional reporter (green) activity in wild-type germarium. (**B**) *bam* transcriptional reporter activity in *stwl bam* double mutants. Arrows and arrowheads indicate GSCs and cytoblasts, respectively. (**C**) Germ cells (stained green with anti-Vasa) in *bam* mutant ovaries contained spherical spectrosomes (red). (**D**) Elongated fusomes indicative of cyst formation (encircled) in *stwl bam* mutant ovaries. DNA is blue in C,D.

Table 1. Transcripts whose abundance increased in *stwl bam* double-mutant ovaries compared with *bam* ovaries

Gene	Function	*stwl bam*	*bam*	Fold difference	*P*-value
mRNA processing/translation					
CG8335	eIF-3	816	72	11.3	0.000371
CG14443	RNA helicase	155	22	7.0	0.00131
B52	mRNA splicing	1631	363	6.7	0.0165
aret	Bruno: RNA binding	1481	405	3.7	0.0155
CG10630	ds-RNA binding	4049	1199	3.4	0.00964
CG8023	eIF-4e like	953	411	2.3	0.00738
Transcription					
CG10102	Zn finger	291	11	26.5	0.000255
CG31601	Zn finger	216	17	12.7	0.0103
CG16898	Zn finger, bHLH	132	16	8.3	0.0482
east	Nucleoskeleton	356	76	4.7	0.0361
CtBP	Co-repressor	1756	460	3.8	0.0115
CG8119	SANT domain	187	56	3.3	0.00445
lola	BTB/POZ domain	350	105	3.3	0.0134
His3.3B	Histone 3.3	5137	2136	2.4	0.0357
CG12054	Repressor	158	70	2.3	0.0128
Smr	Co-repressor	772	348	2.2	0.043
Signaling					
CG31187	Diacylglycerol kinase	188	6	31.3	0.00077
Rala	Ras-related	988	128	7.7	0.00449
Pi3K21B	PI-3 kinase	330	71	4.6	0.00339
Tob	Dpp antagonist	77	19	4.1	0.00498
Src64B	Src tyrosine kinase	219	82	2.7	0.00455
Adhesion					
CG8563	Metalloprotease	266	16	16.6	0.00512
Fas2	Homophilic cell adhesion	1185	310	3.8	0.00224
CG12497	Laminin	80	37	2.2	0.0371
Structural/other					
Act88F	Actin filament	531	17	31.2	0.0116
Acp1	Cuticle protein	269	24	11.2	0.0107
up	Tropomyosin binding	3022	302	10.0	0.000511
RfaBp	Fatty acid binding	1004	350	2.9	0.0416

Bold indicates genes with the largest differential change in expression.

bam transcription were de-repressed in *stwl* mutants, GSCs would be lost because *bam* expression would cause GSC-to-cystoblast differentiation (Ohlstein and McKearin, 1997). We therefore scored *bam* expression in *stwl*$^{-}$ ovaries using both a Bam-specific antibody and a *bam* transcriptional reporter. We found, however, that *bam* transcriptional silencing was properly maintained in *stwl*$^{-}$ ovaries (Fig. 4A), indicating that Stwl is not required for *bam* silencing.

Mutations that cause GSC loss without disrupting *bam* silencing, such as those in *pum* and *pelo*, have been described previously (Chen and McKearin, 2005; Szakmary et al., 2005; Xi et al., 2005). Tests of genetic epistasis have shown that double mutants of *bam* and the GSC-loss gene (*pum bam* or *pelo; bam*) can form differentiating germ cell cysts, indicating that *bam* function is dispensable when either *pum* or *pelo* is also absent (Chen and McKearin, 2005; Szakmary et al., 2005; Xi et al., 2005). We similarly constructed *stwl*^{95} *bam*^{86} double-mutant animals to determine whether *stwl* and *bam* also constituted an antagonistic gene pair. In approximately 80% of *stwl bam* germaria, we found germ cells interconnected by branched fusomes, indicating that cystoblasts had formed and initiated cyst differentiation (Fig. 4D). On average, 30% of germ cells in these ovaries were in clusters penetrated by branched fusomes. Taken together, these findings indicated that *stwl* antagonizes *bam* action by a mechanism that is independent of *bam* transcriptional silencing.

Identifying targets of Stwl repression

To identify Stwl targets whose transcriptional silencing is required for GSC maintenance, we carried out microarray analysis of undifferentiated germ cells that lack *bam* and compared them to *stwl bam* mutants. Both of these genetic backgrounds provided a nearly homogeneous population of cell types, because *bam* mutant cells failed to differentiate into cystoblasts and *stwl bam* germ cells arrested as partially formed cysts. The homogeneity and early arrest of the mutant germ cells improved the quality of microarray data by eliminating late-stage egg chambers that produce complex and abundant populations of mRNA, which can distort microarray analysis.

We identified 501 genes that were differentially expressed twofold or more ($P<0.05$) in *stwl bam* mutants relative to *bam* mutant ovaries. Of these candidate transcripts, 235 were upregulated in *stwl bam* versus *bam* mutants. Differentially expressed transcripts designated as having functions in mRNA processing, transcription or other roles are listed in Table 1, and those with the largest differential change in expression are highlighted by bold. Transcripts that appeared as downregulated are listed in Table S1 in the supplementary material.

Because we expected Stwl to act as a transcriptional inhibitor, we concentrated our initial studies on those transcripts shown in Table 1. Among the genes upregulated in the absence of *stwl*, we expected to find those whose expression was directly influenced by *stwl*, as well as genes that are upregulated early in cyst development, because

Table 2. Upregulated mRNAs with Nanos response elements (NREs) in their 3′-UTRs

Gene	Information	*stwl bam*	*bam*	Fold difference	*P*-value
Predicted NREs: 3					
CG4068		2693	452	6.0	5.90E-05
bun	Transcription factor	744	195	3.8	0.00598
cpo	mRNA binding	441	144	3.1	0.00171
CG12054	Transcription repressor	158	69	2.3	0.0128
Smr	Transcription co-repressor	772	348	2.2	0.043
Predicted NREs: 2					
fln	Contractile fiber	153	6	25.5	0.0394
CG15056		877	65	13.5	0.00296
Rala	Ras related	988	128	7.7	0.00449
Zip3	Metal ion transport	499	116	4.3	0.0151
lola	BTB/POZ domain	350	105	3.3	0.0134
sar1	Ras family small GTPase	3869	1603	2.4	0.0191
Predicted NREs: 1					
CtBP	Transcription co-repressor	1756	460	3.8	0.0115
CanB	Ca^{2+}-dependent phosphatase	187	50	3.7	0.00381
CG3894	STAT inhibitor	161	44	3.7	0.000399
CG14440		102	31	3.3	0.0133
CG6169		357	114	3.1	0.0413
knrl	Nuclear receptor	53	17	3.1	0.0031
CG12025		358	130	2.8	0.0117
CG31323		144	56	2.6	0.0274
CG33075	Mitochondrial carrier	283	112	2.5	0.0316
CG10186	Electron transporter	266	107	2.5	0.047
Nf1	Ras GTPase activator	113	47	2.4	0.0349
pst	Olfactory learning	1514	636	2.4	0.00654
mirr	Transcription factor	199	85	2.3	0.0371
Rbp2	Translation initiation	273	118	2.3	0.0333

Bold: published mutant analysis suggests ovarian and/or germ cell function.

stwl bam mutant ovaries execute early cyst formation. Both classes were of interest, as few early germ cell differentiation factors have been identified to date.

Bruno (also known as Arrest – FlyBase), a translational repressor required for cyst formation (Parisi et al., 2001), appeared in greater abundance in *stwl bam* profiles. Bruno accumulation did not change significantly in $stwl^{+/-}$ versus $stwl^{-/-}$ germline clones (Fig. 5A,B). Bruno accumulation did increase, however, throughout *stwl bam* germaria (Fig. 5E,F) but not in *bam* mutant germaria (Fig. 5C,D), correlating with cyst differentiation. Thus, Bruno was not a direct Stwl target gene, but *bruno* mRNA was present in greater abundance in the *stwl bam* transcript profile due to cyst differentiation.

Transcripts from *lola*, a gene implicated in chromatin organization (Zhang et al., 2003), also appeared in greater abundance in *stwl bam* profiles. Likewise, Lola protein was detected in 48% of the cells in *stwl bam* ovaries, in contrast to in 12% of *bam* cells and in 18% of early germ cells (GSCs to eight-cell cysts) in $stwl^{+/-}$ ovaries. Lola is a dynamically expressed protein (Giniger et al., 1994), and its apparent increase in transcript profiles and in ovaries could be explained if a greater percentage of *stwl bam* cells was trapped at Lola-expressing stages. We therefore examined Lola expression more closely in *stwl* mutant and *stwl* mosaic backgrounds, in which cystocytes do not arrest differentiation until much later in oogenesis. Unlike Bruno, Lola protein was expressed in much greater abundance in germ cells of *stwl* homozygous mutant flies compared with $stwl^{+/-}$ (data not shown). Analysis of *stwl* mosaic germlines provided the clearest demonstration of the dependence of Lola expression on *stwl* activity. As shown in Fig. 5G,H, almost all $stwl^{-/-}$ cells in mosaic germaria expressed Lola, whereas most $stwl^{+/-}$ germ cells were Lola-negative. Lola was detected in 84% of germ cells lacking *stwl* (54/64 $stwl^{-/-}$ germ cell clones) and in only 26% of germ cells expressing $stwl^{+}$ (15/52 $stwl^{+/+}$ germ cell clones). We concluded, therefore, that induction of *lola* mRNA reflected in the transcript profiling experiments was due to upregulation of *lola* transcription in the absence of Stwl, suggesting that Stwl directly repressed *lola* transcription.

As previously noted, genetic interactions had suggested a link between Stwl and Nos-Pum action. We examined the expression of Nos and Pum in *stwl* mutant ovaries, but found no change in the levels or localization of Nos or Pum in the absence of *stwl* (see Fig. S1 in the supplementary material). Because these proteins act in different cellular compartments (nucleus versus cytoplasm), we suspected that an overlapping set of targets might account for the genetic interactions. To identify Stwl-repressed mRNAs that might also be targets of Nos-Pum translational repression, we searched the 235 genes upregulated in the absence of *stwl* for those that contained consensus Pum-binding sites (termed Nanos response element or NRE: AUUGUA) within their 3′-UTRs (Murata and Wharton, 1995; Sonoda and Wharton, 1999). We identified 25 transcripts with one or more NRE consensus sites within their 3′-UTR, including six transcripts with two NREs and five transcripts with three NREs (Table 2). Although the number of transcripts containing one NRE in their 3′-UTR sequences was not different from random occurrence, the frequency of 3′-UTRs with multiple NREs was significantly higher in the induced group of transcripts than in a group whose levels did not change in the absence of *stwl* (4.7% versus 2%). Intriguingly, several of the multiple *lola* transcripts carried two predicted NREs within their 3′-UTR, suggesting that *lola* may be a candidate for both Nos-Pum translational regulation and *stwl* transcriptional regulation.

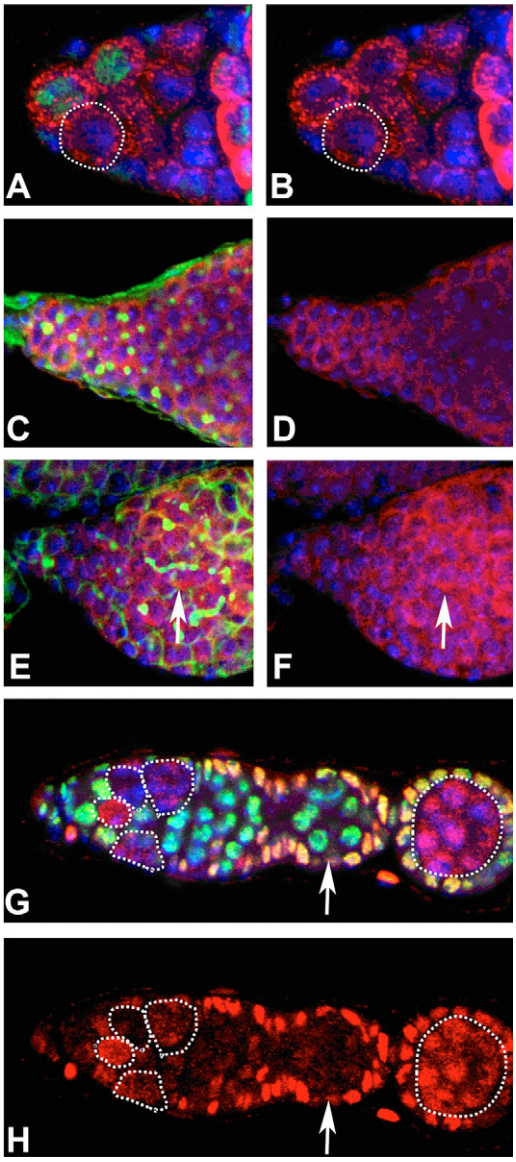

Fig. 5. Expression of Bruno and Lola in *stwl* mutant cells. (**A**,**B**) Bruno (Bru) expression (red) in *stwl* mutant GSCs (marked by the absence of GFP, green, and outlined) was similar to that of neighboring wild-type germ cells. (**C-F**) Bru expression (red) was uniform in *bam* mutant germaria (C,D) but was elevated in *stwl bam* ovaries (E,F) as differentiating germ cell clusters formed (arrows). Spectrosomes/fusomes (green) were labeled in C and E to show the degree of cyst development. (**G**,**H**). Germline clones of *stwl* mutant cells (marked by the absence of GFP, green, and outlined) contained higher levels of Lola (red) than neighboring cells.

DISCUSSION

Previous studies have shown that mechanisms that block differentiation are crucial for maintaining ovarian GSCs (Kai et al., 2005; Wang and Lin, 2004; Wong et al., 2005). Dpp signaling within the GSC niche silences Bam expression (Chen and McKearin, 2003a; Li and Xie, 2005; Song et al., 2004), and the translational repressors Nos and Pum prevent translation of putative cystoblast-promoting mRNAs (Gilboa and Lehmann, 2004b; Wang and Lin, 2004). Our finding that GSCs prominently express a marker of transcriptional quiescence is another indication that the stem cell state depends heavily on mechanisms that restrict gene expression. Similar conclusions have been made for several stem cell populations in mammals. For example, the transcriptional repressor Plzf (Zbtb16) is required for spermatogonial stem cell maintenance in the mouse (Buaas et al., 2004; Costoya et al., 2004). Plzf associates with the Polycomb family member *bmi-1* (*bmi1*) and has been implicated in chromatin-mediated repression (Barna et al., 2002). Other adult stem cells are also likely to be regulated by chromatin state, as *bmi-1* is required for the self-renewal of hematopoetic and neural stem cells in the mouse (Lessard and Sauvageau, 2003; Molofsky et al., 2003; Park et al., 2003).

Stwl carries copies of two modified SANT domains, the MADF and BESS motifs, which are found principally in flies and have been studied in several *Drosophila* proteins (Bhaskar and Courey, 2002; Cutler et al., 1998). SANT-domain proteins in humans and yeast are most frequently associated with chromatin-remodeling and histone-modifying activities (Boyer et al., 2002). In many instances, the SANT domains are essential for histone binding. Peterson (Boyer et al., 2004) has advanced the hypothesis that SANT domains bind histone tails in histone-modifying complexes, such as histone acetyl transferases (HAT) and histone de-acetylation complexes (HDACs). On the other hand, SANT domains in Myb, Adf1 and Dip3 have been shown to act as sequence-specific DNA-binding motifs (Bhaskar and Courey, 2002; Cutler et al., 1998; Gabrielsen et al., 1991). Although we cannot exclude the possibility that Stwl acts as a sequence-specific DNA-binding protein, we favor the idea that it acts as a histone-interacting protein, because the *Su(var)* phenotype has been remarkably predictive of proteins involved in histone modification and chromatin remodeling (Ebert et al., 2004). For instance, *Su(var)*3-7 is a *Drosophila* protein with a modified SANT domain. This domain interacts with a major heterochromatin protein, HP-1 [Su(var)205] (Delattre et al., 2000), and contributes to heterochromatic gene silencing (Delattre et al., 2004).

Chromatin silencing factors and GSC maintenance

The *Su(var)* activity and stem-cell-loss phenotype of *stwl* led us to propose that Stwl is a component of a chromatin-modifying complex and that chromatin state may influence GSC fate. Indeed, the chromatin remodeler *iswi* also plays an essential role in maintaining GSCs (Xi and Xie, 2005), indicating a significant function for chromatin remodeling in GSC chromosomes. Our finding that *stwl* acts as a potent *Su(var)* and is required for GSC maintenance suggests that domains of chromatin silencing are established and maintained in these stem cells to regulate differentiation.

Remarkably, loss- and gain-of-function experiments documented that Stwl was both necessary and sufficient for GSC fate (Akiyama, 2002; this paper). Our data indicates that Stwl represses the expression of differentiation-promoting genes, probably by chromatin-mediated transcriptional silencing. Strikingly, imposing Stwl-mediated silencing in germ cells caused more of them to persist as GSC-like cells. These ectopic GSCs retained properties of stem cells, including *bam* silencing. Eventually, these supernumerary GSCs differentiated, probably when they exited the crucial niche region.

Only a few genes that can expand the number of GSCs have been identified and, in each case, these genes occupy crucial positions in the genetic circuits maintaining GSCs. Constitutive activation of the Dpp signaling pathway can block germ cell differentiation and maintain all germ cells in a GSC-like state (Casanueva and Ferguson, 2004; Xie and Spradling, 1998). Prolonged somatic expression of *piwi* can expand the number of GSC-like cells, but only within the anterior-most region of the GSC niche (Cox et al., 2000; Szakmary et al.,

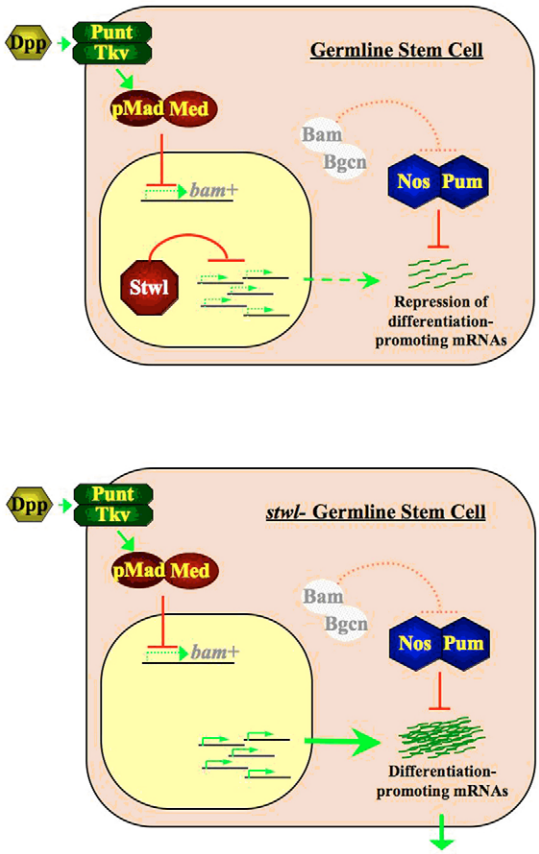

Fig. 6. A model for Stwl function in repressing the transcription of germ cell differentiation-promoting factors. In wild-type GSCs (top), transcriptional quiescence of *bam,* via the Dpp signaling cascade, allows full Nos-Pum translational repression of differentiation-promoting mRNAs. Stwl represses the transcription of these mRNAs, probably through interaction with and/or modification of histones. In *stwl*$^{-}$ GSCs (bottom), the absence of Stwl function upregulates transcription, thereby increasing the pool of differentiation-promoting mRNAs and promoting cystoblast differentiation despite full Nos-Pum activity.

2005). Ourselves and others have proposed that the region defined by the limited expansion of GSCs represents a domain of graded Dpp signaling (Casanueva and Ferguson, 2004; Chen and McKearin, 2005; Szakmary et al., 2005). Overexpression of factors that enhance Dpp signaling, such as *piwi* (Chen and McKearin, 2005; Szakmary et al., 2005), expand the niche by 'flattening' the gradient. We propose that Stwl represses GSC differentiation cell-autonomously, delaying germ cell differentiation and expanding GSC number within the limits of the niche. Our finding that an intrinsic transcriptional repressor can expand GSC number might have important implications in systems that require stem cell number to expand (for example, during growth), or in cases of cancer metastasis when cancer stem cell numbers might amplify.

In addition to *Drosophila* GSCs, epigenetic-silencing mechanisms have been implicated as maintenance factors in a few other stem cell systems (Buszczak and Spradling, 2006). For example, the Polycomb proteins are bound to a suite of promoters in embryonic stem (ES) cells and the corresponding genes become activated when ES cells begin to differentiate (Boyer et al., 2006; Lee et al., 2006). A separate study identified another PcG protein, Ezh2, as necessary for maintenance of mouse hematopoietic stem cells (Kamminga et al., 2006).

The current paradigm for the mechanisms that regulate GSCs predicts that stem cells are maintained by blocking their differentiation. GSCs are subject to repressing activities by niche signaling (Chen and McKearin, 2003a; Song et al., 2004) and translational control (Gilboa and Lehmann, 2004b; Wang and Lin, 2004). Here, we propose that Stwl preserves GSCs by imposing epigenetic transcriptional quiescence that acts downstream of *bam*-promoter silencing and depends on full *nos*$^{+}$ and *pum*$^{+}$ function for the preservation of GSC fate. We note that previous studies on transcriptional silencing in pole cells in *Drosophila* and germ cells in *C. elegans* have implicated Nos and its orthologous proteins in regulating epigenetic modifications (Schaner et al., 2003). In flies, *nos*$^{+}$ was required to delay the accumulation in pole cell genomes of lysine 4 methylation of histone H3 (H3meK4) that would signal broad transcriptional activation (Schaner et al., 2003).

The fact that Stwl action depended on the genetic dosage of *nos*$^{+}$ can be explained if Stwl silences the transcription of genes whose transcripts are also translationally inhibited by Nos-Pum. Alternatively, Stwl-dependent and Nos-dependent epigenetic mechanisms might collaborate to provide full-genome transcriptional silencing. Because transcripts bearing multiple NREs were over-represented in the class of Stwl-repressed genes, we favor the initial hypothesis, and present a model for Stwl function in Fig. 6. We propose that Stwl represses the transcription of a cohort of genes, including key differentiation factors also targeted by Nos-Pum translational repression. It is also possible that *nos* and *stwl* are both involved in regulating chromatin state in the stem cell. In pole cells, Nos activity has been shown to influence chromatin state, probably by repressing the translation of chromatin modifiers (Schaner et al., 2003). The possibility of dual mechanisms for silencing the expression of this group of genes might suggest that they can promote key steps in GSC differentiation and may require overlapping mechanisms to block their expression. Perhaps it will be possible to produce a chart of the earliest steps in differentiation from a stem cell to a differentiated daughter by noting genes that are common targets of multiple repressors.

We would like to thank Scott Cameron, Rick Jones and Bob Hammer for useful comments on the manuscript. The authors thank anonymous reviewers who offered very useful critiques to improve the manuscript. Rick Jones also provided valuable reagents and advice about gene regulation by chromatin modifications. M.W. extends her thanks to the UT Southwestern Summer Undergraduate Research Program and J.K.P. thanks the UT Southwestern Medical Scientist Training Program for advice and support. We thank members of the McKearin laboratory, past and present, as well as members of UTSW model organism laboratories for helpful discussions. We thank Mary Kuhn and Tiana Endicott for outstanding technical assistance. The UTSW Microarray Core Facility provided invaluable expertise and technical assistance. This work was supported by grants to D.M.M. from NSF (IOB-0346223) and NIH (GM45820).

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/8/1471/DC1

References

Akiyama, T. (2002). Mutations of stonewall disrupt the maintenance of female germline stem cells in Drosophila melanogaster. *Dev. Growth Differ.* **44**, 97-102.
Barker, D. D., Wang, C., Moore, J., Dickinson, L. K. and Lehmann, R. (1992). Pumilio is essential for function but not for distribution of the Drosophila abdominal determinant Nanos. *Genes Dev.* **6**, 2312-2326.
Barna, M., Merghoub, T., Costoya, J. A., Ruggero, D., Branford, M., Bergia, A., Samori, B. and Pandolfi, P. P. (2002). Plzf mediates transcriptional repression of HoxD gene expression through chromatin remodeling. *Dev. Cell* **3**, 499-510.
Bellen, H. J., Levis, R. W., Liao, G., He, Y., Carlson, J. W., Tsang, G., Evans-Holm, M., Hiesinger, P. R., Schulze, K. L., Rubin, G. M. et al. (2004). The BDGP gene disruption project: single transposon insertions associated with 40% of Drosophila genes. *Genetics* **167**, 761-781.
Bhaskar, V. and Courey, A. J. (2002). The MADF-BESS domain factor Dip3 potentiates synergistic activation by Dorsal and Twist. *Gene* **299**, 173-184.
Boyer, L. A., Langer, M. R., Crowley, K. A., Tan, S., Denu, J. M. and Peterson,

C. L. (2002). Essential role for the SANT domain in the functioning of multiple chromatin remodeling enzymes. *Mol. Cell* **10**, 935-942.
Boyer, L. A., Latek, R. R. and Peterson, C. L. (2004). The SANT domain: a unique histone-tail-binding module? *Nat. Rev. Mol. Cell Biol.* **5**, 158-163.
Boyer, L. A., Plath, K., Zeitlinger, J., Brambrink, T., Medeiros, L. A., Lee, T. I., Levine, S. S., Wernig, M., Tajonar, A., Ray, M. K. et al. (2006). Polycomb complexes repress developmental regulators in murine embryonic stem cells. *Nature* **441**, 349-353.
Buaas, F. W., Kirsh, A. L., Sharma, M., McLean, D. J., Morris, J. L., Griswold, M. D., de Rooij, D. G. and Braun, R. E. (2004). Plzf is required in adult male germ cells for stem cell self-renewal. *Nat. Genet.* **36**, 647-652.
Buszczak, M. and Spradling, A. C. (2006). Searching chromatin for stem cell identity. *Cell* **125**, 233-236.
Casanueva, M. O. and Ferguson, E. L. (2004). Germline stem cell number in the Drosophila ovary is regulated by redundant mechanisms that control Dpp signaling. *Development* **131**, 1881-1890.
Chen, D. and McKearin, D. (2003a). Dpp signaling silences bam transcription directly to establish asymmetric divisions of germline stem cells. *Curr. Biol.* **13**, 1786-1791.
Chen, D. and McKearin, D. M. (2003b). A discrete transcriptional silencer in the bam gene determines asymmetric division of the Drosophila germline stem cell. *Development* **130**, 1159-1170.
Chen, D. and McKearin, D. (2005). Gene circuitry controlling a stem cell niche. *Curr. Biol.* **15**, 179-184.
Christerson, L. B. and McKearin, D. M. (1994). orb is required for anteroposterior and dorsoventral patterning during Drosophila oogenesis. *Genes Dev.* **8**, 614-628.
Clark, K. (1996). The *Drosophila* Stonewall gene encodes a putative transcription factor that regulates oocyte differentiation and germ cell viability. PhD thesis, Department of Molecular Biology, University of Texas Southwestern Medical Center at Dallas, USA.
Clark, K. A. and McKearin, D. M. (1996). The Drosophila stonewall gene encodes a putative transcription factor essential for germ cell development. *Development* **122**, 937-950.
Costoya, J. A., Hobbs, R. M., Barna, M., Cattoretti, G., Manova, K., Sukhwani, M., Orwig, K. E., Wolgemuth, D. J. and Pandolfi, P. P. (2004). Essential role of Plzf in maintenance of spermatogonial stem cells. *Nat. Genet.* **36**, 653-659.
Cox, D. N., Chao, A. and Lin, H. (2000). piwi encodes a nucleoplasmic factor whose activity modulates the number and division rate of germline stem cells. *Development* **127**, 503-514.
Cutler, G., Perry, K. M. and Tjian, R. (1998). Adf-1 is a nonmodular transcription factor that contains a TAF-binding Myb-like motif. *Mol. Cell. Biol.* **18**, 2252-2261.
Delattre, M., Spierer, A., Tonka, C. H. and Spierer, P. (2000). The genomic silencing of position-effect variegation in Drosophila melanogaster: interaction between the heterochromatin-associated proteins Su(var)3-7 and HP1. *J. Cell Sci.* **113**, 4253-4261.
Delattre, M., Spierer, A., Jaquet, Y. and Spierer, P. (2004). Increased expression of Drosophila Su(var)3-7 triggers Su(var)3-9-dependent heterochromatin formation. *J. Cell Sci.* **117**, 6239-6247.
Dorer, D. R. and Henikoff, S. (1994). Expansions of transgene repeats cause heterochromatin formation and gene silencing in Drosophila. *Cell* **77**, 993-1002.
Ebert, A., Schotta, G., Lein, S., Kubicek, S., Krauss, V., Jenuwein, T. and Reuter, G. (2004). Su(var) genes regulate the balance between euchromatin and heterochromatin in Drosophila. *Genes Dev.* **18**, 2973-2983.
Forbes, A. and Lehmann, R. (1998). Nanos and Pumilio have critical roles in the development and function of Drosophila germline stem cells. *Development* **125**, 679-690.
Gabrielsen, O. S., Sentenac, A. and Fromageot, P. (1991). Specific DNA binding by c-Myb: evidence for a double helix-turn-helix-related motif. *Science* **253**, 1140-1143.
Gavis, E. R. and Lehmann, R. (1992). Localization of nanos RNA controls embryonic polarity. *Cell* **71**, 301-313.
Gilboa, L. and Lehmann, R. (2004a). How different is Venus from Mars? The genetics of germ-line stem cells in Drosophila females and males. *Development* **131**, 4895-4905.
Gilboa, L. and Lehmann, R. (2004b). Repression of primordial germ cell differentiation parallels germ line stem cell maintenance. *Curr. Biol.* **14**, 981-986.
Giniger, E., Tietje, K., Jan, L. Y. and Jan, Y. N. (1994). lola encodes a putative transcription factor required for axon growth and guidance in Drosophila. *Development* **120**, 1385-1398.
Kai, T., Williams, D. and Spradling, A. C. (2005). The expression profile of purified Drosophila germline stem cells. *Dev. Biol.* **283**, 486-502.
Kamminga, L. M., Bystrykh, L. V., de Boer, A., Houwer, S., Douma, J., Weersing, E., Dontje, B. and de Haan, G. (2006). The Polycomb group gene Ezh2 prevents hematopoietic stem cell exhaustion. *Blood* **107**, 2170-2179.
Karpen, G. H. (1994). Position-effect variegation and the new biology of heterochromatin. *Curr. Opin. Genet. Dev.* **4**, 281-291.
King, F. J. and Lin, H. (1999). Somatic signaling mediated by fs(1)Yb is essential for germline stem cell maintenance during Drosophila oogenesis. *Development* **126**, 1833-1844.
Lasko, P. F. and Ashburner, M. (1990). Posterior localization of vasa protein correlates with, but is not sufficient for, pole cell development. *Genes Dev.* **4**, 905-921.
Lee, T. I., Jenner, R. G., Boyer, L. A., Guenther, M. G., Levine, S. S., Kumar, R. M., Chevalier, B., Johnstone, S. E., Cole, M. F., Isono, K. et al. (2006). Control of developmental regulators by Polycomb in human embryonic stem cells. *Cell* **125**, 301-313.
Lessard, J. and Sauvageau, G. (2003). Bmi-1 determines the proliferative capacity of normal and leukaemic stem cells. *Nature* **423**, 255-260.
Li, L. and Xie, T. (2005). STEM CELL NICHE: structure and function. *Annu. Rev. Cell Dev. Biol.* **21**, 605-631.
Lin, H. and Spradling, A. C. (1997). A novel group of pumilio mutations affects the asymmetric division of germline stem cells in the Drosophila ovary. *Development* **124**, 2463-2476.
Macdonald, P. M. (1992). The Drosophila pumilio gene: an unusually long transcription unit and an unusual protein. *Development* **114**, 221-232.
McKearin, D. and Ohlstein, B. (1995). A role for the Drosophila bag-of-marbles protein in the differentiation of cystoblasts from germline stem cells. *Development* **121**, 2937-2947.
Molofsky, A. V., Pardal, R., Iwashita, T., Park, I. K., Clarke, M. F. and Morrison, S. J. (2003). Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation. *Nature* **425**, 962-967.
Murata, Y. and Wharton, R. P. (1995). Binding of pumilio to maternal hunchback mRNA is required for posterior patterning in Drosophila embryos. *Cell* **80**, 747-756.
Ohlstein, B. and McKearin, D. (1997). Ectopic expression of the Drosophila Bam protein eliminates oogenic germline stem cells. *Development* **124**, 3651-3662.
Parisi, M. J., Deng, W., Wang, Z. and Lin, H. (2001). The arrest gene is required for germline cyst formation during Drosophila oogenesis. *Genesis* **29**, 196-209.
Park, I. K., Qian, D., Kiel, M., Becker, M. W., Pihalja, M., Weissman, I. L., Morrison, S. J. and Clarke, M. F. (2003). Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. *Nature* **423**, 302-305.
Rea, S., Eisenhaber, F., O'Carroll, D., Strahl, B. D., Sun, Z. W., Schmid, M., Opravil, S., Mechtler, K., Ponting, C. P., Allis, C. D. et al. (2000). Regulation of chromatin structure by site-specific histone H3 methyltransferases. *Nature* **406**, 593-599.
Reuter, G., Dorn, R., Wustmann, G., Friede, B. and Rauh, G. (1986). Third chromosome suppressor of position-effect variegation loci in *Drosophila melanogaster*. *Mol. Gen. Genet.* **202**, 481-487.
Schaner, C. E., Deshpande, G., Schedl, P. D. and Kelly, W. G. (2003). A conserved chromatin architecture marks and maintains the restricted germ cell lineage in worms and flies. *Dev. Cell* **5**, 747-757.
Song, X., Zhu, C. H., Doan, C. and Xie, T. (2002). Germline stem cells anchored by adherens junctions in the Drosophila ovary niches. *Science* **296**, 1855-1857.
Song, X., Wong, M. D., Kawase, E., Xi, R., Ding, B. C., McCarthy, J. J. and Xie, T. (2004). Bmp signals from niche cells directly repress transcription of a differentiation-promoting gene, bag of marbles, in germline stem cells in the Drosophila ovary. *Development* **131**, 1353-1364.
Sonoda, J. and Wharton, R. P. (1999). Recruitment of Nanos to hunchback mRNA by Pumilio. *Genes Dev.* **13**, 2704-2712.
Spradling, A., Drummond-Barbosa, D. and Kai, T. (2001). Stem cells find their niche. *Nature* **414**, 98-104.
Sugimura, I. and Lilly, M. A. (2006). Bruno inhibits the expression of mitotic cyclins during the prophase I meiotic arrest of Drosophila oocytes. *Dev. Cell* **10**, 127-135.
Szakmary, A., Cox, D. N., Wang, Z. and Lin, H. (2005). Regulatory relationship among piwi, pumilio, and bag-of-marbles in Drosophila germline stem cell self-renewal and differentiation. *Curr. Biol.* **15**, 171-178.
Van Doren, M., Williamson, A. L. and Lehmann, R. (1998). Regulation of zygotic gene expression in Drosophila primordial germ cells. *Curr. Biol.* **8**, 243-246.
Verrotti, A. C. and Wharton, R. P. (2000). Nanos interacts with cup in the female germline of Drosophila. *Development* **127**, 5225-5232.
Wang, Z. and Lin, H. (2004). Nanos maintains germline stem cell self-renewal by preventing differentiation. *Science* **303**, 2016-2019.
Wong, M. D., Jin, Z. and Xie, T. (2005). Molecular mechanisms of germline stem cell regulation. *Annu. Rev. Genet.* **39**, 173-195.
Xi, R. and Xie, T. (2005). Stem cell self-renewal controlled by chromatin remodeling factors. *Science* **310**, 1487-1489.
Xi, R., Doan, C., Liu, D. and Xie, T. (2005). Pelota controls self-renewal of germline stem cells by repressing a Bam-independent differentiation pathway. *Development* **132**, 5365-5374.
Xie, T. and Spradling, A. C. (1998). decapentaplegic is essential for the maintenance and division of germline stem cells in the Drosophila ovary. *Cell* **94**, 251-260.
Zaccai, M. and Lipshitz, H. D. (1996). Role of Adducin-like (hu-li tai shao) mRNA and protein localization in regulating cytoskeletal structure and function during Drosophila Oogenesis and early embryogenesis. *Dev. Genet.* **19**, 249-257.
Zhang, W., Wang, Y., Long, J., Girton, J., Johansen, J. and Johansen, K. M. (2003). A developmentally regulated splice variant from the complex lola locus encoding multiple different zinc finger domain proteins interacts with the chromosomal kinase JIL-1. *J. Biol. Chem.* **278**, 11696-11704.