Loss-of-Function Screen Reveals Novel Regulators Required for *Drosophila* Germline Stem Cell Self-Renewal

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**ABSTRACT** The germline stem cells (GSCs) of *Drosophila melanogaster* ovary provide an excellent model system to study the molecular mechanisms of stem cell self-renewal. To reveal novel factors required for *Drosophila* female GSC maintenance and/or division, we performed a loss-of-function screen in GSCs by using a collection of P-element-induced alleles of essential genes. Mutations in genes of various functional groups were identified to cause defects in GSC self-renewal. Here we report that a group of mutations affecting various ubiquitin-conjugating enzymes cause significant GSCs loss, including *Plenty of SH3s* (*POSH*), *Ubiquitin-conjugating enzyme 10* (*UbcD10*), and *pineapple eye* (*pie*). Ubiquitin-mediated protein degradation plays a variety of roles in the regulation of many developmental processes, including mediating stem cell division through degradation of cell cycle regulators. We demonstrated that *pie*, sharing highly conserved RING domains with human E3 ubiquitin ligase G2E3 that are critical for early embryonic development, is specifically required for GSC maintenance, possibly through regulation of bone morphogenetic protein signaling pathway. Despite the previously reported role in imaginal disc cell survival, *pie* loss-of-function induced GSC loss is not to the result of caspase-involved cell death. Further efforts are needed to elucidate the functions of ubiquitin ligases in GSC maintenance, which will ultimately contribute to a better understanding of how the ubiquitin-conjugating enzymes regulate stem cell biology in mammalian systems.

Self-renewal of female GSCs is primarily regulated by bone morphogenetic protein (BMP) signaling from the niche, mediated by the ligands decapentaplegic (*dpp*) and glassbottomed boat (*gbb*) (Xie and Spradling 1998; Song et al. 2004). Notch activity in the niche cells is shown to regulate this process (Ward et al. 2006). The BMP signaling activates cytoplasmic Mad and Medea, the *Drosophila* Smads, in GSCs, which form complex and silence the transcription of *bag-of-marble* (*bam*) gene, the key differentiation factor that is normally turned off in GSCs (Chen and McKearin 2003a; Szakmary et al. 2003; Shcherbata et al. 2007).

To reveal novel factors required for GSC self-renewal, we performed a loss-of-function screen in GSCs, which yielded a group of mutations affecting various ubiquitin-conjugating enzymes, including *Plenty of SH3s* (*POSH*), *Ubiquitin-conjugating enzyme 10* (*UbcD10*), and *pineapple eye* (*pie*). Ubiquitin-mediated posttranslational regulation...
plays a variety of roles in many developmental processes, including the maintenance of quiescence in stem cells (Onoyama and Nakayama 2008; Clague and Urbe 2010). In the following studies, we will demonstrate a nonapoptotic role of pie, which was previously reported as a survival factor (Shi et al. 2003), in GSC maintenance.

**MATERIALS AND METHODS**

**Fly stocks and culture conditions**

The following stocks and other deficiency alleles were obtained from Drosophila Genetic Resource Center, Kyoto Institute of Technology, Japan: y^{e2} w^{1118} P{[ey-FLP.N]}2; PBac[WH] pie^{65500} P{[neoFRT]}40A/CyO, y w^{1118} P{[ey-FLP.N]}2 P{[GMR-lacZ.C(38.1)]}TPN1; P{[neoFRT]}42D P{[lacW]} POSH^{135815} /CyO y+, y^{d2} w^{1118} P{[ey-FLP.N]}2 P{[GMR-lacZ.C(38.1)]}TPN1; P{[neoFRT]}42D P{[GT1]} UbcD10^{P{[GMR-lacZ.C(38.1)]}TPN1; P{[lacW]} kek^{16732} y^{d2} w^{1118} P{[ey-FLP.N]}2 P{[GMR-lacZ.C(38.1)]}TPN1; P{[lacW]} bcr^{1024611} P{[GMR-lacZ.C(38.1)]}TPN1; P{[lacW]} ab^{1024611} P{[neoFRT]}40A/CyO y+, y^{d2} w^{1118} P{[ey-FLP.N]}2 P{[GMR-lacZ.C(38.1)]}TPN1; P{[lacW]} ab^{1024611} P{[neoFRT]}40A/CyO y+, y^{d2} w^{1118} P{[ey-FLP.N]}2 P{[GMR-lacZ.C(38.1)]}TPN1; P{[lacW]} ab^{1024611} P{[neoFRT]}40A/CyO y+, y^{d2} w^{1118} P{[ey-FLP.N]}2 P{[GMR-lacZ.C(38.1)]}TPN1; P{[lacW]} ab^{1024611} P{[neoFRT]}40A/CyO y+, y^{d2} w^{1118} P{[ey-FLP.N]}2 P{[GMR-lacZ.C(38.1)]}TPN1; P{[lacW]} ab^{1024611} P{[neoFRT]}40A/CyO y+.
The following stocks are obtained from Bloomington Drosophila Stock Center at Indiana University: w; FRT40ApieE1-16/SM5-TM6, y+; hsFLP; Ubi-GFP FRT40A/Cyo w; hsFLP; FRT42BUbi-GFP/Cyo w; hsFlp; FRT42DUbi-GFP/Cyo w; w; pin/cyo; Ly/TM6B; w;; bam-GFP was a generous gift from the McKearin lab. Flies were cultured at 25°C on standard corn-meal 2 yeast 2 agar medium (protein-rich diet). For starvation assay, females of desired genotypes were fed with a protein-rich diet or in an empty vial containing a Kimwipe soaked in 10% light corn syrup (protein-poor diet) for 5 days at 25°C before analysis.

### Table 1: Deficiency screen for novel regulators in GSC self-renewal

| Function Groups | Gene Name | Allele(s) | Function | % Clonal Day 14 | GSC Loss/Day | Passed 2 Screen |
|-----------------|-----------|-----------|----------|-----------------|-------------|-----------------|
| I. Ubiquitin ligase | POSH | hsFLP; FRT42DPOSH; FRT42DGFP | E3 ubiquitin ligase | 0% | 30% | Yes |
| & Pie | hsFLP; pie; FRT40A; FRT40AGFP | E3 ubiquitin ligase (Predicted) | 0% | 25% | |
| & UbcD10 | hsFLP; FRT42DubicD10; FRT42DGFP | E2 ubiquitin ligase | 3.40% | / | Yes |
| II. EGFR signaling | Kekkon-1 | hsFLP; kek1; FRT40A; FRT40AGFP | Repressor of EGFR signaling | 11% | 9% | Yes |
| & Drk | hsFLP; FRT42Ddrk; FRT42DGFP | RTK adaptor protein | 4.80% | 7.59% | Yes |
| III. Transcription factors | Abrupt | hsFLP; ab | Transcription factor activity | 6.9% | 10.5% | Yes |
| & Cropped | hsFLP; crp; FRT40A; FRT40AGFP | Specific RNA polymerase II | 0% | 28.7% | Yes |
| IV. Housekeeping genes | CG8674 | hsFLP; k14505; FRT40A; FRT40AGFP | Proton-transporting ATP synthase complex assembly | 0% | / | Yes |
| & Df31 | hsFLP; FRT42Dk05815; FRT40A; FRT40AGFP | Chromatin organization | 6% | / | Yes |
| & snRNA:U6atac:29B | hsFLP; FRT40A; FRT40AGFP | Nuclear mRNA splicing | 0% | / | |

EGFR, epidermal growth factor receptor; GSC, germline stem cell; RTK, receptor tyrosine kinase.

**Figure 2** pie shows high similarity with human G2E3 RING domains. (A) Genomic map of pie shows locations of various mutant lines used. pie; pie; and pie; are ethyl-methanesulfonate-induced loss-of-function mutants; pie; is a P-element insertion-caused loss-of-function line. (B) Amino acid sequence alignment of pie protein N-terminal and human G2E3 RING domains. Red blocks indicate identical amino acids. The null allele pie; is caused by frame shift at Asp203 (underlined).
Immuno-fluorescence and microscopy

Ovaries of desired genotypes were dissected in phosphate-buffered saline (PBS) and immediately fixed in PBS containing 4% paraformaldehyde and then stained as described (Shcherbata et al. 2004). The following primary antibodies were used: mouse anti-adducin, mouse anti-Lamin C (Developmental Studies Hybridoma Bank, 1:20; rabbit anticleaved caspase 3 and rabbit anti-pMad (Cell Signaling Technology, 1:250 and 1:50); and rabbit anti-bgal 1:5000. Secondary fluorescence antibodies used include Alexa 488, 568, and 633 antimouse or antirabbit (1:250). Samples were mounted and analyzed on Leica SPE5 and Nikon N1 confocal laser-scanning microscopes.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

The In Situ Cell Death Detection Kit (Roche Applied Science) was used as an independent means of detecting apoptotic cells (Sgonc et al. 1994). Ovaries were dissected in cold PBS, fixed in 4% paraformaldehyde, and processed according to manufacturer’s protocol. Labeled ovaries were
counterstained with DAPI, mounted, and analyzed with fluorescent microscope.

**Generation of clones by FLP-mediated mitotic recombination**

Two- to four-day-old females or males of the following genotypes were heat shocked for 45 min in a 37°C water bath for 2 consecutive days to induce mitotic recombination. Heat shocked flies were kept at 25°C and transferred to fresh food with wet yeast paste every other day before dissection.

**Maintenance analysis**

The GSC loss per day was calculated by comparing % of geraria with clonal GSCs at two time points after the heat-shock induction: GSC loss per day = (% of geraria with clonal GSCs at day 5 − % of clonal geraria at day 11)/% of clonal geraria at day 11. This analysis was performed using a Student’s t-test.

**Loss of function screen for genes that affect Drosophila germline GSC maintenance and division**

For primary screen, third instar larvae were heat shocked for 60 min in a 37°C water bath for 2 consecutive days and dissected 14 days after the last heat shock. Ovaries were analyzed for the presence of clonal (GFP-negative) egg chambers. For secondary screen, 2-4 day old female adults were heat shocked for 45 min in 37°C water bath for 2 consecutive days and dissected at 7 and 14 days after induction.

**RESULTS**

**Genetic screen for key regulators in Drosophila female GSC maintenance**

To reveal novel factors required for Drosophila female GSC maintenance and division, we performed a loss-of-function screen using 820 P-element induced mutant lines of essential genes (Drosophila Genetic Resource Center DGRC, Kyoto). Each P-element line recombined with FRT loci was crossed to corresponding FRT, Ubi-GFP line; then, homozygous clones were generated through heat shock flippase mediated mitotic recombination in young adult females (Materials and Methods, supporting information, Table S1). The primary screen yielded 43 positive lines on the basis of the phenotype that no or very few homozygous clones were recovered in the ovarioles 14 days after heat shock induction (Figure 1C), indicating a defect in GSC maintenance or division. In contrast, the negative control lines displayed homozygous clones all along the ovarioles, suggesting that GSCs are still properly producing progenies (Figure 1B).

In the secondary screen, each candidate line was further examined by assessing the rate of mutant GSC clone loss between 7 and 14 days after clonal induction (see Materials and Methods). Ten lines of 43 candidate mutant lines from the primary screen were confirmed to result in rapid GSC loss after clone induction in the secondary screen (Figure 1, D–F; Table 1). Functions of genes affected by these 10 lines fall into various categories spanning from posttranslational modification, epidermal growth factor receptor signaling pathway, to transcription factors, chromatin regulators, and mitochondria activity. A group of genes, including Posh, UbcD10, and pie, came to our interest. Posh and UbcD10, respectively, encode for Drosophila E3 and E2 ubiquitin ligase (Figure 2B) (Tsuda et al. 2005; Yatsenko et al. 2007); whereas pie, with predicted RING/PHD domains, shares highly conserved sequence with a human E3 ubiquitin ligase G2E3 (Shi et al. 2003; Brooks et al. 2008). The authors of a recent study have demonstrated that another ubiquitin-conjugating enzyme, Effete, maintains Drosophila GSCs through regulating cyclin A degradation (Chen et al. 2009). The finding of this group of ubiquitin ligase genes to be essential for GSC maintenance suggests a potentially conserved role of ubiquitin-mediated proteolysis in stem cell self-renewal and tissue homeostasis.
caspase inhibitor protein baculovirus p35 can partially rescue the rough eye phenotype (Shi et al. 2003). To test the possibility that pie mutant phenotypes in GSC maintenance are the result of cell death, we examined the cell death marker, cleaved caspase 3 in clonal germlia. On the basis of previous evidence and our observation from TUNEL assay, germline cells in region 2 serve as a checkpoint for programmed cell death, which show an increase in apoptosis under nutrient-deprived condition (Figure 4, A and B) (Drummond-Barbosa and Spradling 2001). Cleaved caspase 3 antibody specifically recognizes apoptotic cells and detects similar pattern with TUNEL in region 2 (Figure 4C) (Baum et al. 2007; Nezis et al. 2009). To our surprise, lack of pie function in GSCs did not result in apoptosis: we never observed activated caspase3 in either mutant or wild-type GSCs (Figure 4, C and D; Table 3). To further test whether p35 overexpression could rescue pie mutant induced GSC loss, we expressed UASp-p35 in the germline under nos-Gal4 driver, in the pie clonal background and examined the pie GSC maintenance at days 5, 8, and 11 after clonal induction.

Figure 4 pie is not required for GSC survival. (A) Drosophila germarium cysts categorized by region 1–3. (B and B’). TUNEL assay recognizes cell death in region 2 cysts upon 5 days of protein-depleted diet (method). (C and D) At 8 days after heat shock induction, neither wild-type GSCs (C, dashed circle) nor pie E1-16 GSCs (D, dashed circle) are positive for cleaved caspase 3 (magenta, cell death marker), whereas in both cases, there is a basal frequency of cell death in region 2 of germaria (C’ and D’). (E) Blocking cell death by overexpressing p35 in the germline does not rescue pie mutant induced GSC loss. (F) Newly emerged w females fed on poor-diet for 5 days (blue bars) exhibit significantly more cell death in region 2, compared to rich-dieted animals of same genotype (red bars); while overexpressing p35 in well-fed females blocked the basal frequency of cell death.

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cell death in region 2, and the whole germlarium, indicated by reduced occurrence of caspase 3+ cysts (Figure 4F). However, the occurrence and loss rates of pie<sup>05500</sup>;nosGal4/UASp-p35 GSCs are respectively comparable with those without p35 expression (Figure 4F; Table 4), demonstrating that p35 expression does not rescue pie phenotype in this cell type. These data indicate that Pie loss-of-function induces GSC maintenance failure through other mechanisms than GSC cell death.

**Pie is required for GSC maintenance through regulating BMP signaling**

Self-renewal of female GSCs is primarily regulated by BMP signaling from the niche. Somatic niche cells express the ligands dpp and gbb, activating cytoplasmic Mad and Medea, the Drosophila Smads, in GSCs, which form complex and silence the transcription of the bam gene, the key differentiation factor that is normally turned off (Chen and McKearin 2003a; Song et al. 2004). Therefore, activation of this short-range BMP signal can be indicated by accumulation of BMP response gene products, phosphorylated Mad (pMad) and Daughters against dpp (Dad) primarily in the GSCs, and repressed expression of differentiation of the bam gene in GSCs (Song et al. 2004). To determine whether pie mutant induced GSC loss is attributable to altered BMP signaling in GSCs, we analyzed the pMad level in clonal germlaria 8 to 9 days after the induction, when most of the pie mutant GSCs are leaving the niche. We observed a significantly lower frequency of pMad expression in pie<sup>E1-16</sup> GSCs compared with the neighboring wild-type GSCs or FRT40A control ones (Figure 5A, arrow; C). Because of the lack of reliable antibodies against bam, we used a bam-GFP enhancer-trap allele (Chen and McKearin 2003b) to examine bam expression in clonal germlaria. Compared with the wild-type GSCs, in which bam expression is mostly turned off (Figure 5B, arrow; D), 25.9% pie mutant GSCs express bam 8 days after induction (Figure 5B, arrow head; D), matching with the decline of pMad expression. This evidence conclusively suggests that pie regulates GSC maintenance through mediating with BMP signaling.

**DISCUSSION**

Drosophila GSCs and their niche provide a perfect model system to study adult stem cell behaviors, including essential factors and signaling pathways involved in stem cell maintenance, division, and differentiation. With this system and the powerful Drosophila genetics, we performed a loss-of-function screen and discovered a group of genes encoding various ubiquitin ligases in flies that are essential for GSC self-renewal. We followed up with pie and found that pie is cell autonomously required for GSC maintenance. Despite the previous finding in imaginal disc and eye tissue that pie mutant leads to apoptosis-related cell death (Shi et al. 2003), we provided evidence that pie loss-of-function induced GSC loss is not related to programmed cell death.

Ubiquitin-mediated protein degradation plays a variety of roles in the regulation of many developmental processes, including mediating stem cell proliferation and stem cell division through degradation of cell cycle regulators (Rathinam 2011). An E2 ubiquitin-conjugating enzyme in Drosophila, Effete (Eff), has been reported to regulate GSC maintenance through targeting cyclin A for degradation (Chen et al. 2009). Evidence from mammalian system has demonstrated that Fbxw7, an F-box protein subunit of an SCF-type ubiquitin ligase complex, targets positive regulators of the cell cycle—including Cyclin E, c-Myc, Notch, and c-Jun, and maintains mouse hematopoietic stem cell quiescent to preserve their capacity for self-renewal (Onyama and Nakayama 2008). pie shares a highly similar sequence with the PHD/RING domains of human G2E3, an E3 ligase essential for mammalian early embryo development. Biochemical evidence has demonstrated that these PHD/RING domains are responsible for the catalytic function of G2E3 (Brooks et al. 2008). Therefore, we speculate a novel role of pie as an ubiquitin ligase other than a cell survival factor in Drosophila GSC maintenance, in a tissue-specific manner. It is possible that, as an ubiquitin ligase, pie might regulate targets that in one cell type involve in self-renewing division, whereas in another cell type result in survival against apoptotic cell death.

The BMP pathway is the major signaling pathway regulating Drosophila GSC self-renewal as well as proliferation (Song et al. 2004; Li and Xie 2005). Two BMP ligands, dpp and gbb, expressed in TF/cap cells, directly act on GSCs to control their self-renewal and division (Song et al. 2004). Activation of this short-range BMP signal is proved by the accumulation of BMP response gene products, pMad and Dad primarily in the GSC, and repressed expression of differentiation gene bam in GSCs (Song et al. 2004). We observed reduced pMad level in

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### Table 3 The requirement for pie in GSC maintenance is not attributable to cell death

| Genotype                        | %Caspar3+ | Clonal GSCs | Nonclonal GSCs | Anterior Clonal Cysts | Anterior Nonclonal Cysts | Posterior Clonal Cysts | Posterior Nonclonal Cysts |
|---------------------------------|-----------|-------------|----------------|-----------------------|-------------------------|------------------------|--------------------------|
| hsFLP; Ubi-GFP FRT40A; pie<sup>05500</sup> FRT40A                      | 0% (n = 34) | 0% (n = 109) | 17% (n = 64) | 1.4% (n = 218) | 0% (n = 50) | 0% (n = 156) |
| hsFLP; Ubi-GFP FRT40A/pie<sup>E1-16</sup> FRT40A                      | 0% (n = 12) | 0% (n = 68) | 18% (n = 33) | 8.1% (n = 111) | 3.8% (n = 26) | 1.2% (n = 83) |
| hsFLP; Ubi-GFP FRT40A/FRT40A                                         | 0% (n = 78) | 0% (n = 94) | 3.0% (n = 168) | 8.3% (n = 218) | 1.3% (n = 75) | 0% (n = 157) |

GFP, green fluorescent protein; GSC, germline stem cell.

### Table 4 p35 overexpression cannot rescue pie-induced GSC loss

| Genotype           | Days after Induction | Exp. I | Exp. II | Exp. III |
|--------------------|----------------------|--------|---------|----------|
| hsFLP; pie<sup>05500</sup> FRT40A/Ubi-GFP FRT40A | 5 days | 27% (n = 85) | 34% (n = 100) | 27% (n = 62) |
|                    | 8 days | 7% (n = 90)  | 8% (n = 91)  | 5% (n = 80)  |
|                    | 11 days| 3.6% (n = 55) | 6% (n = 47)  | 1% (n = 100) |
| hsFLP; pie<sup>05500</sup> FRT40A/Ubi-GFP FRT40A; nosGal4/UASp-P35 | 5 days | 25.9% (n = 116) | 21% (n = 85) | / |
|                    | 8 days | 6.6% (n = 121) | 6.3% (n = 111) | / |
|                    | 11 days| 3.9% (n = 76)  | 1.7% (n = 59)  | / |

GSC, germline stem cell.
accord with premature expression of bam in pie mutant GSCs, suggesting a role of pie in the regulation of BMP signaling to maintain the stem cell identity. In contrast to Mad, Dad functions as an antagonist of Dpp, thus forming a negative-feedback loop of BMP signaling (Tsuneizumi et al. 1997). A possible mechanism would be that pie serves as an E3 ubiquitin ligase in GSCs, which targets Dad protein for poly-ubiquitination and degradation by proteasome. Further evidence and exploration are needed to test the hypothesis.

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