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## RNAi-Based Techniques for the Analysis of Gene Function in *Drosophila* Germline Stem Cells

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### Abstract

Elucidating the full repertoire of molecular mechanisms that promote stem cell maintenance requires sophisticated techniques for identifying and characterizing gene function in stem cells in their native environment. Ovarian germline stem cells in the fruit fly, *Drosophila melanogaster*, are an ideal model to study the complex molecular mechanisms driving stem cell function in vivo. A variety of new genetic tools make RNAi a useful complement to traditional genetic mutants for the investigation of the molecular mechanisms guiding ovarian germline stem cell function. Here, we provide a detailed guide for using targeted RNAi knockdown for the discovery of gene function in ovarian germline stem cells and their progeny.

### Keywords

Germline; Germ cell; Stem cell; *Drosophila*; Ovary; Oogenesis

### 1 Introduction

Unlike most specialized cells, which divide to produce identical daughter cells, stem cells are endowed with the property of long-term self-renewal [1]. During cell division, a stem cell retains its unspecialized fate while producing a non-identical daughter destined for differentiation. Stem cells in the developing embryo and in some adult tissues allow for the continuous production of cells that can be instructed to differentiate into a variety of specialized fates. This unique ability, combined with recent advances in cell reprogramming and gene editing, has heightened clinical interest in the properties of stem cells [2]. Safe and effective use of stem cells and their progeny in regenerative therapies will require a detailed knowledge of the molecular factors that regulate stem cell function.

Maintenance of stem cell self-renewal is influenced by a variety of factors, including the cell's chromatin state, metabolic status, and responsiveness to both the local microenvironment (the stem cell niche) and long-range physiological cues [3–6]. Identification and characterization of the molecular pathways that drive stem cell self-renewal thus require a model system wherein stem cells can be observed and manipulated within their native environment. While a variety of model systems have been employed to study stem cells, many of our fundamental models of stem cell function were developed as a result of experiments performed in vivo in the fruit fly, *Drosophila melanogaster* [7]. Like humans, *Drosophila* have a variety of tissue-resident stem cell populations that are critical

for the continued production of specialized progeny [8–12]. The ease with which *Drosophila* are reared, the broad variety of genetic tools for cell-specific gene manipulation, and the high degree of conservation between many human and *Drosophila* cell signaling pathways make fruit flies a powerful model organism for biomedical research [13]. *Drosophila* are well-suited for rapid, large-scale genetic screens [14], and the availability of transgenic libraries, stock centers, and genome and bioinformatics tools [15–18] make them an efficient and cost-effective resource to study stem cell biology within the context of the whole organism.

In particular, germline stem cells (GSCs) in the *Drosophila* ovary have provided fundamental insights into stem cell regulation. GSCs are a specialized population of stem cells that are maintained to replenish stocks of germ cells (oocytes), whose numbers are depleted by gamete production. GSCs reside in a somatic niche composed of cap cells and terminal filament cells, located at the anterior tip of each ovariole (the functional unit of the adult *Drosophila* ovary; Fig. 1a) in a specialized structure called the germarium (Fig. 1b) [12, 19]. GSCs divide asymmetrically to self-renew and produce a differentiating daughter cell (cystoblast), which undergoes four subsequent rounds of mitosis with incomplete cytokinesis (Fig. 1b, c). During this process, germ cells are specified into either oocyte or nurse cell fates, and oocytes will transition into meiosis. While all germ cells in the germarium contain endoplasmic reticulum-like organelles called fusomes (Fig. 1b, c), GSCs are readily identifiable by their anteriorly localized fusome juxtaposed to the somatic cap cells [20–23]. Additional somatic cells guide the development of the germ cells. Triangular escort cells extend long projections to move the dividing cysts laterally across the germarium [24]. Follicle cells (the daughters of a second population of stem cells, follicle stem cells) envelop each germline cyst, forming an epithelial monolayer that accompanies the developing germline cyst outside of the germarium [25–27]. The resulting follicle undergoes 14 distinct phases of development, culminating in a fertilizable oocyte [19].

Targeted RNA interference, or RNAi, is a widely used experimental technique for gene silencing that has been successfully applied to identify novel genes that control stem cell fate and proliferation in *Drosophila* [28–32]. While the creation of genetic mutant alleles can be time-consuming and labor intensive, generation of RNAi against a target of interest is relatively easy and inexpensive [32–34]. Further, while complete loss-of-function genetic mutant alleles are useful tools, they may result in severe phenotypes that are difficult to interpret or developmentally lethal, precluding analysis of gene function in adult cells. In contrast, RNAi frequently reduces, but does not abolish, the targeted mRNA, resulting in hypomorphic phenotypes. By inducing RNAi using the *UAS-GAL4* system, spatial control over gene knockdown in developing or adult fly tissues is readily accomplished (Fig. 2a). In this system, promoter or enhancer elements drive the expression of the yeast transcription factor *Gal4* [35]. Once expressed, Gal4 binds a DNA response element, the *upstream activating sequence (UAS)*, which precedes a gene fragment containing an inverted repeat with exact complementarity to a given gene of interest. Expression of the inverted repeat results in the formation of double-stranded RNA hairpins that trigger a sequence-specific RNAi silencing response in the cells in which it is expressed [36–39]. Thus, RNA hairpins are generated under the control of tissue- or cell-specific drivers, allowing for targeted knockdown of a gene of interest in specific cell population(s). RNAi experiments can be

combined with additional genetic tools to restrict knockdown to specific temporal windows (*Gal80t.s.*; Fig. 2b) or to a clonal population of cells within a wild-type environment (mosaic analysis with a repressible cell marker; MARCM) [40–42]. RNAi in *Drosophila* ovarian GSCs thus presents an elegant experimental system to discover the molecular regulations that govern stem cell fate and function in vivo.

In this protocol, we provide an experimental strategy utilizing targeted RNAi to knock down gene function in *Drosophila* ovarian GSCs. This protocol can be adapted to analyze the function of any number of genes in GSCs and their progeny and is particularly useful for gene discovery. In general, the phases of the protocol include (1) selecting or creating an appropriate *UAS-RNAi* transgenic line against a gene of interest, (2) choosing a germline-specific *Gal4* driver, (3) *Drosophila* husbandry to generate transgenic flies, (4) dissecting adult ovaries, (5) immunostaining, (6) image acquisition via confocal microscopy, and (7) image analysis. This basic protocol also provides a platform for further analysis of stem cell function, including gene expression, cell cycle progression, and known markers of GSC self-renewal and daughter cell differentiation. Taken together with other recently published methods [43–49], this protocol highlights the power of the *Drosophila* ovarian GSC as a model for the study of stem cell function in its native environment.

## 2 Materials

### 2.1 *Drosophila* Strains and Culture

1. *Drosophila* strains (*see Note 1*), including a germline-compatible *UAS-RNAi* responder strain and a germline-specific *Gal4* strain.
2. Standard fly culture medium (cornmeal/molasses/yeast/agar) in bottles and vials (*see Note 2*).
3. Yeast paste: 1–1 mixture of dry active yeast (*see Note 3*) and distilled water, mixed to the consistency of smooth peanut butter. Store covered with Parafilm at 4 °C to prevent drying.
4. Dissecting stereomicroscope with dual goose-neck illumination, 0.6–4× magnification range, and a flat black dissection base, equipped with a fly pad and air needle to deliver CO<sub>2</sub> (*Drosophila* anesthetic; *see Note 4*).

### 2.2 Ovary Dissection and Immunostaining

1. 1.5 mL microfuge tubes, pre-coated (*see Note 5*).

<sup>1</sup>Many of these lines can be obtained from the Bloomington *Drosophila* Stock Center (<http://flystocks.bio.indiana.edu>), the Vienna *Drosophila* Resource Center (<http://stockcenter.vdrc.at>), or the Kyoto *Drosophila* Stock Center (<http://www.dgrc.kit.ac.jp>).

<sup>2</sup>We recommend Nutri-Fly MF, mixed using the manufacturer's instructions and supplemented with Tegosept to prevent mold growth. Nutri-Fly MF is a molasses-based media formulation available from Genesee Scientific (<https://genesee.com>) that is easily mixed using an immersion blender and convection hot plate. Because it can be made in relatively small batches, it is a good option for small *Drosophila* labs. Fly culture medium is best when used fresh (within 3–4 weeks of pouring). We use 6 oz. square bottom polypropylene bottles and narrow polystyrene vials, both sealed with Flugs.

<sup>3</sup>We use Fleischmann's instant dry yeast, available in 1 lb. packages from Sam's club.

<sup>4</sup>*Drosophila* husbandry and anesthetic supplies can be purchased from Genesee Scientific.

<sup>5</sup>Tubes are pre-coated in a 3% bovine serum albumin (BSA) solution [diluted from a 30% solution (Sigma) in distilled water] to prevent the ovaries from sticking to the sides of the tubes. Add 250 µL of 3% BSA into microfuge tubes. Place tubes on a nutator at room temperature for 1 h to ensure complete coverage of inside of tube. Store tubes at 4 °C.

2. 15-mL polypropylene centrifuge tubes.
3. 50-mL polypropylene centrifuge tube.
4. Glass or plexiglass dissection dish.
5. Kimwipes.
6. Glass Pasteur pipettes and bulbs.
7. Two pairs of sharpened forceps (INOX, Dumont #5, Biologie point).
8. Two 27 × 1¼ gauge needles with 1 mL syringes.
9. Cold room-safe orbital nutator (also called a GyroMixer).
10. Grace's Insect Medium without additives (Lonza; *see* Note 6).
11. Phosphate-buffered saline (PBS).
12. Wash solution: 0.1% Triton-X-100 in PBS.
13. Fixative: 5.3% formaldehyde in Grace's media (*see* Note 7). Prepare fresh prior to dissection. For each sample, add 300 µL 16% formaldehyde (Ted Pella; *see* Note 8) and 600 µL Grace's media. Keep on ice.
14. Blocking Solution: 5% bovine serum albumin (BSA), 5% normal goat serum, 0.1% Triton-X-100 in PBS (*see* Note 9). Prepare using sterile technique, and store at 4 °C. Discard if cloudy.
15. Primary Antibodies: In nearly all initial experiments, we use two primary antibodies that allow for identification of germ-line and somatic cells in the ovary (*see* Fig. 3c, d). Mouse anti-Hts (1B1-s; Developmental Studies Hybridoma Bank) is diluted in blocking solution to a final concentration of 1:10 and labels both the germline-specific fusome and follicle cell plasma membranes. Mouse anti-LaminC (LC28.26-s; Developmental Studies Hybridoma Bank) is diluted in blocking solution to a final concentration of 1:100 and strongly labels the nuclear envelope of cap cells (*see* Notes 10 and 11).
16. Secondary Antibodies: Species-matched secondary antibodies tagged with a fluorophore of interest. For the detection of anti-Hts and anti-LaminC (*see* Fig.

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<sup>6</sup>We make 50 mL sterile aliquots of Grace's media and store at 4 °C until ready for dissection. A fresh batch should be used if the aliquot appears cloudy.

<sup>7</sup>Optimal fixative concentration and fixation time may vary between primary antibodies and should be experimentally determined. We have found that the concentration and incubation time given here are optimal for the primary and secondary antibodies used in this protocol.

<sup>8</sup>Formaldehyde rapidly degrades when in contact with air. For best results, we order 10 mL ampules of ultrapure 16% formaldehyde, transfer the liquid to a 15 mL conical tube, and store for use no longer than 1 week at 4 °C.

<sup>9</sup>Ideal blocking solutions help to reduce background staining and may vary with primary and secondary antibodies. It is important to choose serum for blocking solution that matches the host species of the secondary antibodies used in the immunostaining reaction.

<sup>10</sup>We have also found that some concentrated aliquots of LC28.26 will also label the nuclear envelopes of GSCs, germ cells, and, occasionally, follicle cells (faintly).

<sup>11</sup>This protocol can be adapted to stain with other molecular markers, including apoptosis assays (TUNEL) and proliferation markers (EdU) or other primary antibodies. Simply adjust the antibody concentration (if necessary) and select the appropriate secondary antibody in order to maintain specificity and avoid cross reactivity.

3c, d), we use Alexa Fluor goat anti-mouse-568 (Life Technologies; *see* Note 12), diluted to a final concentration of 1:200 in blocking solution.

17. 4',6-Diamidino-2-phenylindole (DAPI): For working concentration, prepare a 1:500 dilution of a 5.0 mg/mL DAPI stock solution (*see* Note 13) in 0.1% Triton-X-100 in PBS.
18. Mounting Media: 20 mg/mL *n*-propyl gallate in glycerol (*see* Note 14). In a 50 mL conical tube, combine 1.0 g *n*-propyl gallate (Sigma) with 5 mL PBS; vortex to mix. Add 45 mL 100% glycerol. Cover the conical tube in foil and rotate on a nutator at room temperature overnight. Media should be clear but very viscous. Store protected from light at 4 °C. A working stock can be poured into an opaque dropper bottle for ease of application.

### 2.3 Sample Preparation and Confocal Microscopy

1. Two needle holders with sharpened tungsten needles; one should have an “L”-shaped bend at the tip (*see* Note 15).
2. Glass microscope slides and coverslips (1 μm thickness, 22 × 22 mm).
3. Steel weight, measuring approximately 250 g.
4. Fingernail polish (Sally Hansen Tough as Nails, any color).
5. Laser scanning confocal microscope, inverted, equipped with 63× oil immersion lens (n.a. = 1.4) and 1.5–3× optical zoom (*see* Note 16).
6. Confocal image acquisition and analysis software (*see* Note 17).

## 3 Methods

### 3.1 *Drosophila* Strains and Culture

**3.1.1 Selecting a Gene-Specific UAS-RNAi Responder Transgene**—Although a wide variety of target-specific *UAS-RNAi* transgenes are available in *Drosophila* [32], care must be taken to ensure successful knockdown of your gene of interest in female GSCs. A variety of commonly used *Drosophila* genetic tools, including the *UAS* responder lines that promote strong expression in somatic cells, do not work effectively in the female germline [39, 40, 50, 51]. To circumvent this problem, Ni and colleagues developed two RNAi vectors

<sup>12</sup>Upon arrival, we add an equal volume of 100% glycerol to each commercial secondary antibody, mix well, and store at –20 °C in 100 μL aliquots.

<sup>13</sup>Prepare a 5 mg/mL DAPI (Life Technologies, Cat #D1306) stock solution according to manufacturer’s instructions in deionized water.

<sup>14</sup>While a variety of commercially available glycerol-based anti-fade mounting media are available, we prefer to make our own solution. We have found that it is less expensive and preserves fluorescence intensity equally (if not better) than commercially available brands. We have previously used mounting media directly supplemented with DAPI but have found that these formulations do not penetrate well into the ovary, resulting in uneven nuclear staining.

<sup>15</sup>Different members of our lab separate ovarioles (“teasing”) using different methods. Having one needle with an “L” or “shepherd’s crook” shape helps some of our lab members pull the largest follicles off the ovarioles in a left-right motion (think of a cane pulling a person off a stage in old movies).

<sup>16</sup>We recommend the Zeiss LSM700 system, particularly for small labs with routine use.

<sup>17</sup>We use Zeiss Black image acquisition software, and Zeiss Blue image analysis software. Many common analyses can also be conducted in ImageJ, which is freely available (<http://imagej.nih.gov/ij/>).

(*Valium20* and *Valium22*) specifically designed for optimal expression of RNAi hairpins in the germline [50]. *Valium20* promotes strong knockdown in both the ovarian germ-line and soma; in contrast, the *Valium22* vector, based on the germline-compatible *UASp* vector [51], promotes strong expression in the germline, but only moderate to weak expression in the soma [50, 52]. A large collection of target-specific *Valium20*- and *Valium22*-based *UAS-RNAi* transgenic lines (henceforth referred to as “*UAS-RNAi* responders”) are now available from the Transgenic RNAi Project at Harvard Medical School (TRiP; [www.flyrnai.org](http://www.flyrnai.org); see Note 18) via the Bloomington *Drosophila* Stock Center (see Note 1; Table 1). Another large collection of *UAS-RNAi* responders are available from the Vienna *Drosophila* Stock Center (VDRC). The VDRC maintains two libraries of *UAS-RNAi* responders that differ primarily in the insertion site of the *UAS-RNAi* into the *Drosophila* genome [36]. Although neither of these libraries were created in germline-optimized vectors, reliable expression of gene-specific RNAi hairpins has been achieved in the germline via co-expression of Dicer2 (*UAS-Dcr2*) [29, 30].

While an ever-increasing number of germline-compatible *UAS-RNAi* responders are being made public (see FlyBase, [www.flybase.org](http://www.flybase.org), for a current listing), these tools may not yet be available for some genes of interest. In this case, it is possible to create a *Valium20*- or *Valium22*-based *UAS-RNAi* transgene using standard molecular cloning techniques and have this transgene commercially injected into embryos for the establishment of transgenic lines (see Note 19). While creation of customized *UAS-RNAi* lines does require time to establish new transgenic lines, it is a viable and cost-effective option for genes with few existing tools. Detailed protocols, cloning vectors, and primer sequences are available from the TRiP ([www.flyrnai.org](http://www.flyrnai.org)) [50].

Three common issues arise when using targeted *UAS-RNAi* approaches. First, the strength of knockdown of a gene of interest can vary between *UAS-RNAi* transgenes. This can be assessed by measuring RNA or protein levels using a variety of techniques but is perhaps best visualized in vivo by immunofluorescence against the protein of interest if suitable reagents are available. Second, a given RNAi hairpin may target multiple genes, resulting in phenotypes not due to loss of the gene of interest (“off-target effects”). This is particularly true for the VDRC KK library [53]. To minimize the chances of off-target effects, choose more than one hairpin, targeted at different regions of the gene of interest, for RNAi analysis. The most effective control is to design hairpins with high sequence similarity to the experimental hairpin, but with several mismatched bases, thus precluding the induction of RNAi [33, 34]. Lastly, for genes that encode multiple isoforms, hairpins could target one, many, or all isoforms of the mRNA, depending on the targeted region. It is important to recognize the location of the hairpin sequence with regard to the predicted transcript, as this could impact the interpretation of results.

<sup>18</sup>Information on the specific vector used to generate a *UAS-RNAi* of interest can be found in FlyBase by clicking on the “inserted element” link, followed by the “associated sequence features” link. In general, *UAS-RNAi* lines beginning with “JF” or “HMS” were created in non-germline-compatible vectors (*Valium1* or *Valium10*), while “HMJ,” “HMC,” “GL,” or “GLC” were created in the *Valium20* or *Valium22* vectors.

<sup>19</sup>A wide variety of companies now provide transgenic injection services for *Drosophila*, including BestGene ([www.thebestgene.com](http://www.thebestgene.com)), Rainbow Transgenic Flies, Inc. ([www.rainbowgene.com](http://www.rainbowgene.com)), and Genetic Services, Inc. ([www.geneticservices.com](http://www.geneticservices.com)).



**3.1.2 Selecting a Germline-Specific Gal4 Driver Transgene**—As in the case of the *UAS-RNAi* responder, a germline-compatible *Gal4* driver must also be selected to achieve gene-specific knockdown in GSCs. As mentioned above, ubiquitous expression of a *UAS-RNAi* responder could cause developmental lethality, thus precluding analysis of adult GSC phenotypes. Further, some drivers that are considered to be “ubiquitous” (such as the *hsGal4* used in many somatic experiments) do not work well in the germline [35, 40, 51]. Thus, a germline-specific *Gal4* driver is required to restrict *UAS-RNAi* responder expression to GSCs. Unfortunately, very few germline-specific drivers exist, and even fewer drive expression robustly in GSCs; however, a few lines can be used to successfully knock down gene expression in GSCs and their daughters without directly modifying the surrounding somatic cells (Table 1).

Perhaps the most frequently used germline drivers in the *Drosophila* ovary are the *nos-Gal4::VP16* and the “maternal triple” *MTD-Gal4* [43, 51, 54, 55]. Two important caveats should be noted, however, when employing the *nos-Gal4::VP16* driver in GSC experiments. First, *nos-Gal4::VP16* expression is not limited to adult GSCs [51, 55]. This driver is expressed in the developing primordial germ cells, larval/pupal germ cell precursors, adult GSCs, and nearly all GSC progeny. It is therefore difficult to conclusively demonstrate that a given gene of interest functions solely in adult GSCs (i.e., GSC function versus GSC establishment). (Unfortunately, the *Gal80<sup>LS</sup>* system cannot help in this regard, as *Gal4::VP16* lacks the *Gal80*-binding site [56].) Second, while *nos-Gal4::VP16* is expressed in adult GSCs, it is expressed at much higher levels in differentiating daughter cells (particularly 2-, 4-, 8-, and 16-cell cysts) [51, 55]. Thus, any knockdown experiments that result in phenotypes impacting germline differentiation must be carefully interpreted. For example, *nos-Gal4::VP16*-mediated knockdown of the differentiation factor *bag of marbles* (*bam*) using targeted RNAi results in a block in germline differentiation, leading to the accumulation of single germ cells and 2-cell germ cysts at the expense of 4-, 8-, and 16-cell cysts (Fig. 3). As an isolated experiment, these results could indicate that *bam* is required for the differentiation of 2-cell cysts; however, analysis of Bam protein expression and a *bam* genetic null allele clearly demonstrates that *bam* is required for the initial differentiation of the immediate GSC daughter cell (the cystoblast) [57–60]. Similar considerations should be made when using *MTD-Gal4*, a compound driver consisting of three independent *Gal4* insertions (*nos-Gal4::VP16* plus *pCOG-Gal4::VP16* and *nos.NGT40-Gal4*) [43, 51, 54]. Intriguingly, neither *pCOG-Gal4::VP16* (also called *otu-Gal4::VP16*) nor *nos.NGT40-Gal4* are highly expressed in adult GSCs (E. Ables unpublished and Ref. [51]); however, several research groups have obtained GSC-related phenotypes when using this driver (in combination with *UAS-Dcr2*) in large-scale screens [29, 30], indicating that low levels of transgene expression are sufficient to induce phenotypes in some situations. Finally, a newly characterized *Gal4* driver, *ET-Flpx2Gal4-398A*, is also available for clonal expression of *UAS-RNAi* responders in the germ-line [41], but has not yet been widely utilized.

A variety of somatic *Gal4* drivers are also available that can be used to identify microenvironmental factors that influence GSC function (Table 1). Most of these somatic *Gal4* drivers robustly promote the expression of a wide variety of *UAS-RNAi* transgenes (except the *Valium22* series). Researchers should note, however, that many of these drivers

are expressed in overlapping populations of somatic cells and may also be expressed outside of the ovary. For example, *bab1-Gal4* is frequently used to drive *UAS-RNAi* expression in the somatic cap cells of the ovary, but it also shows strong expression in the somatic polar and stalk cells present in more developed stages of oogenesis [61, 62]. Researchers should test driver expression in multiple tissues to preclude non-autonomous effects prior to any knockdown experiment and check the expression pattern of the driver over the course of the experiment to ensure the pattern does not change due to the genetic manipulation or nutrient status of the fly.

### 3.1.3 *Drosophila* Husbandry

1. Maintain fly cultures using standard procedure (*see* Note 20).
2. Culture at least two bottles each of the *Gal4* driver line and an isotype control (typically *yw*; *see* Note 21) and one bottle of the *UAS-RNAi* responder line (*see* Note 22). Collect virgin females from the *Gal4* driver line and the isotype control.
3. Set control (*Gal4* driver with *yw* males; *UAS-RNAi* responder with *yw* female virgins) and experimental (*Gal4* driver with *UAS-RNAi* responder) crosses (Fig. 2a) in vials at a density of five pairs of flies per vial (*see* Note 23). Crosses should be set in replicates. Addition of dry yeast to the culture medium provides optimal conditions to increase egg laying. Maintain vials at 25 °C, transferring adults to new vials every 3 days. New flies should emerge within 10 days of this date (at this temperature). For experiments using a *Gal80ts* repressor (Fig. 2b), crosses should be maintained at 18 °C, and new flies will emerge within 18–20 days.
4. Allow crosses to eclose for 1–2 days. Collect the female flies you will dissect and an equal number of accompanying males (typically 12–15 pairs of flies per vial), and add them to fresh vials supplemented with wet yeast paste (*see* Note 24).
5. Transfer adults into freshly yeasted vials daily, and maintain at a constant temperature (typically 25 °C or 29 °C; *see* Note 25) until the day of dissection. For experiments using a *Gal80ts* repressor, flies should be maintained at 29 °C. The age of the flies at dissection (determined by the number of days post-

<sup>20</sup>Methods for *Drosophila* culture maintenance and husbandry have been described elsewhere.

<sup>21</sup>*y1w1* (abbreviated *yw* in this protocol) is frequently used as an inbred isotype control because of its prevalent use in generating transgenic flies. These are available from the Bloomington Stock Center (BLM #1495).

<sup>22</sup>Two control crosses are appropriate to ensure that any experimental phenotypes are the result of knockdown of the gene of interest. Since the *UAS-RNAi* should not be expressed in the absence of *Gal4*, cross *UAS-RNAi* responder males with *yw* female virgins. Likewise, the *Gal4* should not cause ovarian defects due to genomic insertion site or a background mutation. Thus, cross *Gal4* driver female virgins with *yw* males.

<sup>23</sup>Experimental crosses and their controls should always have the same diet, ambient temperature, and be age-matched.

<sup>24</sup>Oogenesis is exquisitely tuned to the female fly's diet [64]. To ensure that any phenotypes identified are the result of gene knockdown and not effects of suboptimal diet, flies must be carefully cultured. Feeding flies daily with fresh yeast paste is necessary to maintain the optimal conditions for egg laying. Despite the waste of vials, it is also critical to discard vials with old, dried yeast paste: flies will reduce egg production in the absence of fresh yeast.

<sup>25</sup>As with any *UAS/Gal4* experiment, the strength of RNAi knockdown can be temperature-dependent, even in the absence of the *Gal80* system. The *nos-Gal4::VP16* driver is particularly sensitive to temperature; weak phenotypes may be enhanced by maintaining flies at 29 °C rather than 25 °C.



eclosion) is determined by the experimenter. For the analysis of GSC self-renewal, it is appropriate to collect flies at a series of timepoints (i.e., 0 days, 4 days, 8 days, and 12 days after eclosion) to chart GSC maintenance over time. The timing of dissection post-eclosion will depend on the number of lines being screened and the exact phenotypes expected/discovered and should be determined experimentally.

### 3.2 Ovary Dissection and Immunostaining (Fig. 4)

1. Use CO<sub>2</sub> to anesthetize flies. Remove males and discard. Dissect ovaries from female flies in cold Grace's medium in a glass or plexiglass dissecting dish. To dissect ovaries (*see Note 26*), grasp the anterior end of the female's abdomen with forceps in the left hand. Using forceps in the right hand, pinch the last stripe in the female's abdomen and pull away from the body. The forceps will grab not only the cuticle but also the urogenital system (frequently including the ovaries). Repeat dissection for all of the females in that vial. Collect dissected ovaries in cold Grace's medium, and quickly break open the outer muscle sheath surrounding the ovarioles to separate individual ovarioles. Using a glass Pasteur pipet, remove BSA from a labeled, pre-coated microfuge tube and discard. Using the same pipet (which is now also coated with BSA), move the dissected ovaries in Grace's medium to the microfuge tube. Place the tube on ice.
2. Repeat dissection with additional vials of flies. Proceed with fixation (below) within an hour of dissection of the first fly.
3. Allow ovaries to collect in the bottom of the microfuge tube by gravity. Remove as much Grace's media as possible (*see Note 27*), and add 1.0 mL of Fixative. Invert the microfuge tube several times to ensure all ovaries are suspended, and rotate on a nutator for 13 min at room temperature.
4. Remove fixative to an appropriate waste container, and quickly add 1.0 mL of wash solution. Invert the tube several times, and remove the wash to the waste container. Add fresh wash solution, invert the tube to suspend the ovaries, and rotate on a nutator for 15 min.
5. Discard the solution, and wash ovaries two more times in 1.0 mL of wash solution for greater than 10 min.
6. Discard the solution, and add 1.0 mL of blocking solution. Rotate ovaries for 3 h on nutator at room temperature.
7. Discard the solution, and add 400 µL of primary antibody mixture (*see Notes 28 and 29*). Incubate ovaries overnight or over two nights at 4 °C on a nutator.

<sup>26</sup>A variety of reviews have recently published excellent descriptions of ovary dissection techniques [65–68]; we therefore refer readers there for additional photographs of ovary dissection and separating ovarioles. In particular, we have found that a YouTube video from Scott Ferguson (<https://www.youtube.com/watch?v=T94be2i5qb4>) is helpful for first-time ovary dissectors.

<sup>27</sup>At each liquid exchange throughout the rest of the protocol, allow a few minutes for ovaries to fall to the bottom of the microfuge tube by gravity before removing the solution. With the exception of the fixative and primary antibody solutions, we use a vacuum trap flask equipped with a thin pipet tip to remove liquid from the ovaries. We typically leave about 50–100 µL of solution on dissected ovaries at any given time in this protocol. This helps to avoid accidental suction of the ovaries into waste and prevents the samples from drying.

8. Remove antibody (*see* Note 30) and wash ovaries four times in 1.0 mL wash solution for at least 30 min each on a nutator.
9. Discard the last wash, and add 500  $\mu$ L of secondary antibody mixture. Incubate ovaries for 1–2 h at room temperature on a nutator. From this step forward, all samples should be protected from light to minimize loss of fluorescence.
10. Discard antibody and wash ovaries four times in 1.0 mL wash solution for at least 30 min each on a nutator.
11. Discard the last wash, and add 500  $\mu$ L of DAPI. Incubate ovaries for 15 min on a nutator.
12. Discard DAPI, and wash ovaries two times in 1.0 mL wash solution for 10 min each on a nutator.
13. Discard the last wash, removing as much liquid as possible. Add four drops of mounting media directly on top of the ovaries. Samples can be stored upright at 4 °C for up to a month.

### 3.3 Sample Preparation and Confocal Imaging

1. Prepare samples on glass slides for imaging on the day of or day before imaging (*see* Note 31).
2. Using a glass Pasteur pipet, move ovaries and mounting media onto a slide. Remove excess mounting media. Isolate (tease) individual ovarioles apart. For optimal imaging of GSCs, remove all large follicles (*see* Note 32). Spread ovarioles out across the slide, making sure there are no large clumps.
3. Once all large follicles are removed and ovarioles are sufficiently spread away from each other in the center of the slide, add 1–2 small drops of mounting media back to slide. Drop a glass coverslip onto the center of the slide, and allow capillary action to spread the mounting media and ovarioles across the coverslip. If any air remains between the coverslip and the slide, use a Pasteur pipet to slowly add a little more mounting media at the edges of the coverslip.

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<sup>28</sup>Although this protocol details immunostaining for two primary antibodies raised in the same species (and thus detected using a single secondary antibody), we routinely use multiple antibodies to localize distinct proteins or cell labels in the ovary. In theory, immunostaining with multiple antibodies of different species should work well by combining all primary antibodies together at this step. We achieve vastly decreased background staining, however, by incubating primary antibodies in series over several nights, separated by extensive wash steps (four times 30 min each). In effect, primary antibodies are layered on top of the sample. In contrast, secondary antibodies against different species are typically grouped together into a single solution.

<sup>29</sup>If two antibodies are raised in the same species, but it is necessary to image them separately, apply one antibody followed by washes, and then the appropriate secondary, as written. Then wash ovaries five times for 30–45 min each in wash solution, re-block for 1 h, and apply the second primary antibody. Following incubation, proceed with wash and second secondary (with a different fluorophore) as written.

<sup>30</sup>The mixture of anti-Hts and anti-LamC at the concentrations given can be collected, stored at 4 °C, and reused for one additional experiment.

<sup>31</sup>Samples on slides will dry out over time, decreasing fluorescence intensity. Since image acquisition is frequently the bottleneck to these experiments, we store all samples in mounting media until the day before imaging.

<sup>32</sup>Follicles can be staged based on a variety of morphological features [19]. Stage 10 follicles are easily identified under a dissecting stereomicroscope, because the size of the oocyte approximates one-half the size of the whole follicle. We remove all follicles larger than a Stage 10 for optimal imaging of GSCs.

4. Place Kimwipes and a steel weight on top of the coverslip/slide to flatten the ovarioles. After 10–15 min, remove the weight and seal the coverslip to the slide using fingernail polish. Allow slides to dry before imaging.
5. Use an inverted confocal microscope with a 63× oil immersion lens and an optical zoom (1.5–3×) to image samples. Images are collected as confocal z-stacks (1 μm optical sections). Each germarium on the slide should be imaged for analysis (typically 75–100 germaria per slide).

### 3.4 Phenotypic Analysis, Quantification, and Next Steps

RNAi-mediated knockdown of genes that function in the *Drosophila* germline may result in a variety of phenotypes. Loss of expression of genes that function in an early step of germline establishment (i.e., during development) or germline differentiation may result in a complete block to oogenesis. Knockdown of genes that maintain the GSC fate may result in a complete loss of GSCs (and, thus, all daughter germ cells), resulting in rudimentary ovaries devoid of germ cells. Loss of expression of genes that function in early cyst growth (independent of GSC function or germ-line differentiation) may result in ovaries lacking more developed follicles. Although each of these possible scenarios results in a similar “small ovary” phenotype, knockdown of some genes may result in subtle phenotypes that do not alter overall ovarian morphology. It is therefore important to perform a thorough characterization of ovariole structure and cellular composition as a starting point to more detailed molecular analyses.

Following the immunofluorescent detection of anti-Hts and anti-LaminC, early germ cells can be recognized by the presence of the Hts-positive fusome (*see* Fig. 3c, c'). GSCs are unambiguously identified via their anteriorly localized fusome adjacent to the cap cells (which express high levels of LaminC). While the fusome is typically round in shape, changes in fusome morphology (elongation and fragmentation) correlate with the different phases of the GSC cell cycle and can be used as an indicator of cell cycle progression [20, 23]. When the GSC divides to form a cystoblast, a new fusome forms, located in the posterior of the cystoblast but with a similar round shape as the GSC fusome (arrowheads, Fig. 3c). As the cystoblast continues to divide with incomplete cytokinesis to form multicellular germline cysts, the fusome branches such that a single branch endpoint is located in each cell cytoplasm (open arrowheads, Fig. 3c). Initial quantification of germ cell number at each stage of differentiation (GSCs, cystoblasts, 2-cell, 4-cell, 8-cell cysts) can thus provide a snapshot of GSC maintenance and the ability of the daughter germ cells to divide and differentiate.

Alterations in GSC or cystoblast number are the initial phenotypic indicators of a change in GSC function. Reduction or complete loss of GSCs (quantified as the average number of GSCs per germarium) is indicative of a failure to maintain the GSC population. Additional analyses can then be performed to elucidate the cause of GSC loss. For example, apoptosis assays (such as immunofluorescence for TUNEL or cleaved caspase) can indicate whether GSCs are lost due to cell death, and molecular markers for each of the differentiated stages can be assessed to determine whether GSCs are lost due to premature differentiation. Such markers may include phosphorylated Mothers Against Decapentaplegic (pMad, highly

expressed in GSCs), Bam (expressed in cystoblasts and 2- and 4-cell cysts), Ataxin-2-binding protein 1 (A2BP1, expressed in 4- and 8-cell cysts) [63], and C-3-G and Orb (expressed in oocytes). Expansion of the number of GSCs and/or cystoblast-like cells per germarium may indicate an increased rate of GSC proliferation or a failure of the immediate daughter cell to initiate differentiation. To evaluate differences in GSC proliferation, the S-phase indicator 5-ethynyl-2'-deoxyuridine (EdU) or the M-phase indicator phospho-histone H3 can be added to the analysis. These markers are also useful in examining changes in cystoblast differentiation and proliferation. Immunofluorescence analysis in targeted RNAi knockdown ovaries can be complemented by molecular analysis of the ovary, including gene expression (RT-PCR, RNA-seq, or in situ hybridization) or protein analysis (Western blotting). All phenotypes derived from targeted RNAi should be validated using other gene loss-of-function models, including the phenotypic assessment of genetic mutants by mosaic clonal analysis [45, 48].

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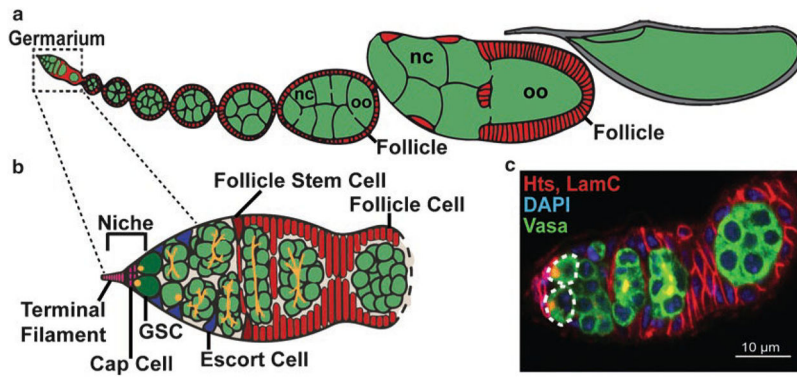
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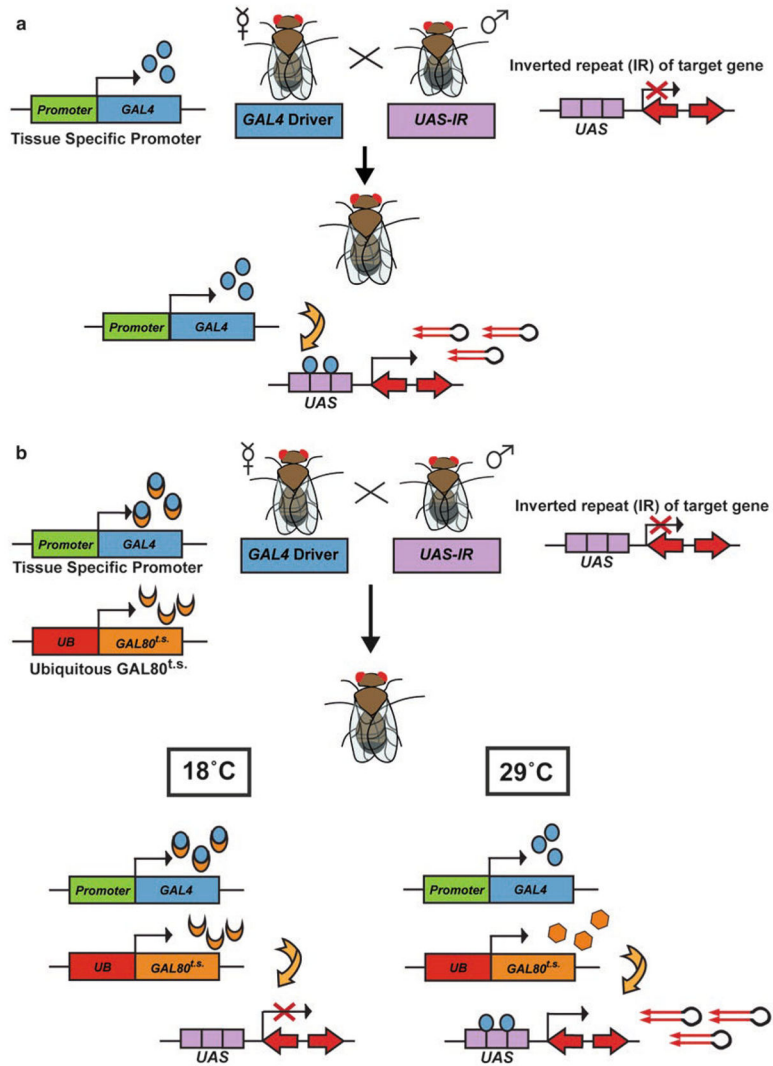
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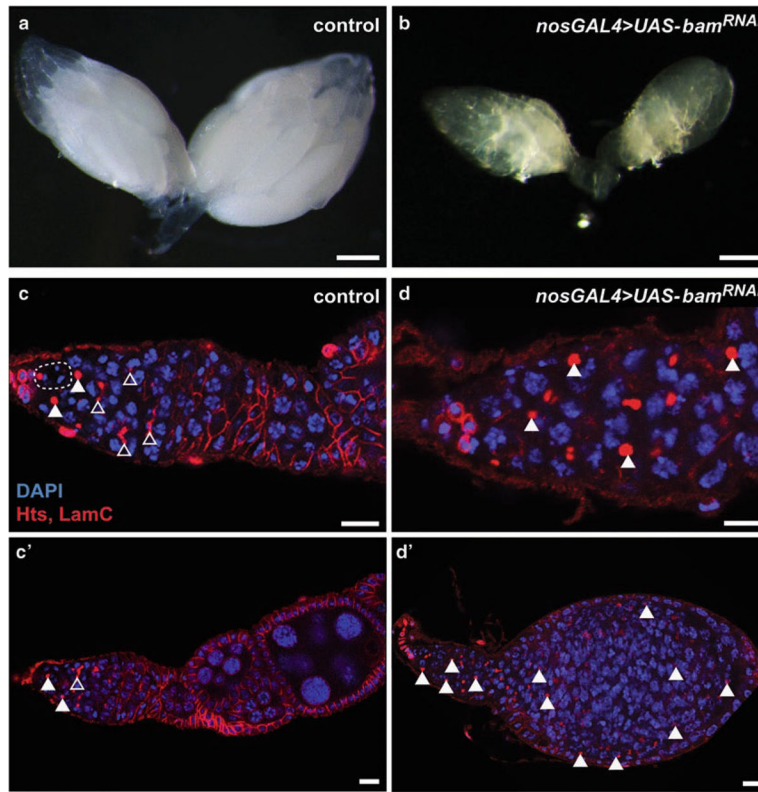
**Fig. 1.**

*Drosophila* ovarian germline stem cells fuel oogenesis. **(a, b)** The *Drosophila* ovary is composed of 15–20 ovarioles (one is represented in **a**), consisting of a germarium (**b**) and a progressive series of follicles in different stages of development. The germarium houses two stem cell populations: germline stem cells (GSC; *dark green*) and follicle stem cells (FSC; *dark red*). GSCs reside in a somatic niche composed of terminal filament cells and cap cells (*pink*). Escort cells (*blue*) help guide developing germline cysts posteriorly, where they are encapsulated by follicle cells (*red*), forming a follicle (also called an egg chamber). Germ cells (*green*) differentiate into oocytes (oo) or nurse cells (nc). **(c)** Confocal micrograph of a germarium labeled with anti-Vasa (all germ cells; *green*), anti-Hts (fusomes and follicle cell membranes; *red*), anti-LaminC (nuclear envelope of cap cells; *red*), and DAPI (all nuclei). *Dotted lines* designate GSCs. *Scale bar* = 10 μm

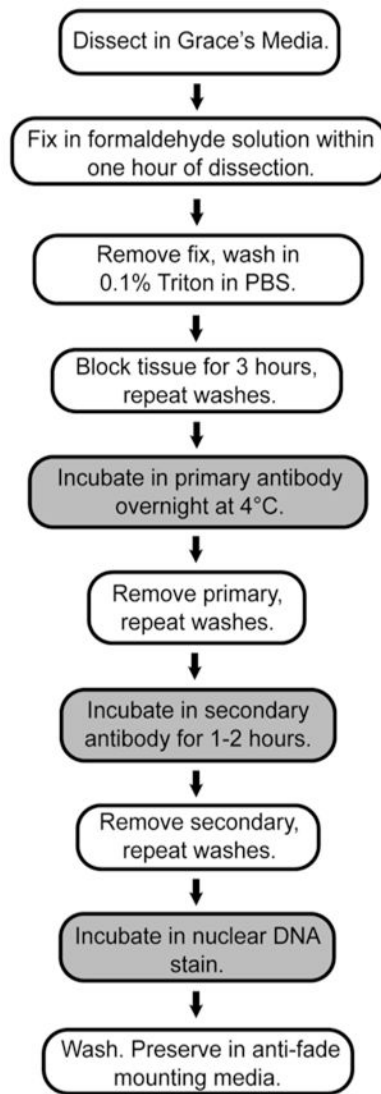


**Fig. 2.**

Targeted RNAi using the *UAS-GAL4* system. **(a)** Virgin female tissue-specific *Gal4* driver flies are crossed to male *upstream activating sequence (UAS)-RNAi* responder flies (*UAS-IR*), resulting in hairpin expression and RNAi-mediated gene knockdown in specific cells in all progeny. **(b)** For temporal control over *Gal4* expression, *Gal4* driver flies also carry a transgene in which the Gal4 inhibitor, *Gal80<sup>ts</sup>*, is ubiquitously expressed. In the resulting progeny, Gal4 expression is temperature-dependent. At 18 °C, Gal80<sup>ts</sup> binds Gal4, suppressing hairpin production. At 29 °C, the conformation of the Gal80<sup>ts</sup> protein changes, such that it can no longer bind Gal4, making Gal4 available to initiate transcription of *UAS-RNAi*



**Fig. 3.** Germline-targeted RNAi knockdown of *bam* results in small ovaries filled with undifferentiated germ cells. **(a, b)** Bright-field images of intact control **(a)** and germline-specific *bam* knockdown **(b)** ovaries 5 days after eclosion. **(c, d)** Confocal micrographs of representative ovarioles labeled with anti-Hts (fusomes and follicle cell membranes; red), anti-LaminC (nuclear envelope of cap cells; red), and DAPI (all nuclei). While *nos-Gal4* control germaria **(c)** contain GSCs (*dashed line*), cystoblasts (*filled arrowheads*), and differentiating germ cells (recognized by branched fusomes; *open arrowheads*), *bam* knockdown germaria have accumulated GSC- and cystoblast-like cells throughout the germarium, at the expense of differentiated germ cells. Further, *bam* knockdown results in a block to oogenesis, as evidenced by the lack of progressively developed follicles **(d')** typically found in control ovarioles **(c')**. Scale bar = 200  $\mu\text{m}$  **(a, b)**, 10  $\mu\text{m}$  **(c, d)**



**Fig. 4.** Experimental work-flow for ovary immunostaining. Preparation of *Drosophila* ovaries for confocal imaging is outlined, highlighting the addition of primary and secondary antibodies and nuclear counterstain



Table 1

Resources for RNAi-mediated gene knockdown in *Drosophila* ovarian GSCs and somatic support cells

Transgenes	Source	Adult ovarian expression pattern	References
<i>Gal4</i> drivers			
Germline			
<i>nos-Gal4::VP16 (III)<sup>β</sup></i>	BLM <sup>b</sup> #4937	Moderate expression in GSCs, strong expression in germline cysts	[51, 55]
<i>nos-NGT40; UAS-Dcr2</i>	BLM #25751	Very weak expression in GSCs	[29, 30]
<i>MTD-Gal4</i>	BLM #31777	Moderate expression in GSCs, very strong expression in germline cysts	[54]
<i>ET-Flpx2Gal4-168</i>	<i>Drosophila</i> community	Germline clones	[41]
Niche			
<i>bab1-Gal4</i>	BLM #6802; <i>Drosophila</i> community	Cap cells and escort cells	[61, 62]
<i>c587-Gal4</i>	<i>Drosophila</i> community	Escort cells, follicle stem cells, prefollicle cells	[69]
<i>Tj-Gal4</i>	KYO <sup>c</sup> #104055	Terminal filament & cap cells	[70]
<i>ET-Flpx2Gal4-688A</i>	<i>Drosophila</i> community	Escort cell clones	[41]
<i>I3C06-Gal4</i>	BLM #47860	Escort cells, follicle stem cells, and prefollicle cells	[71]
<i>ET-Flpx2Gal4-398A</i>	<i>Drosophila</i> community	Escort cells, follicle stem cells, and prefollicle cell clones	[41]
<i>UAS-RNAi</i> responders			
Long hairpins			
VDRC KK	VDRC <sup>d</sup>	Germline expression with <i>UAS-Dcr2</i>	[36]
VDRC GD	VDRC	Germline expression with <i>UAS-Dcr2</i>	[36]
Short hairpins			
TRiP <sup>e</sup> Valium10	BLM	Expression limited to somatic cells	[39]
TRiP Valium20	BLM	Expression in somatic and germline cells	[50]
TRiP Valium22	BLM	Strong expression in germline cells, weaker in somatic cells	[50]

<sup>a</sup>Note that a second insertion is available on the X chromosome; however, its expression is weaker than the insertion on the III<sup>b</sup>Bloomington *Drosophila* Stock Center at Indiana University, Bloomington, IN, USA<sup>c</sup>Kyoto Stock Center at the Kyoto Institute of Technology, Kyoto, Japan<sup>d</sup>Vienna *Drosophila* Resource Center, Vienna, Austria

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