RESEARCH ARTICLE

A Krüppel-like factor is required for development and regeneration of germline and yolk cells from somatic stem cells in planarians

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Abstract

Sexually reproducing animals segregate their germline from their soma. In addition to gamete-producing gonads, planarian and parasitic flatworm reproduction relies on yolk cell–generating accessory reproductive organs (vitellaria) supporting development of yolkless oocytes. Despite the importance of vitellaria for flatworm reproduction (and parasite transmission), little is known about this unique evolutionary innovation. Here, we examine reproductive system development in the planarian Schmidtea mediterranea, in which pluripotent stem cells generate both somatic and germ cell lineages. We show that a homolog of the pluripotency factor Klf4 is expressed in primordial germ cells (PGCs), presumptive germline stem cells (GSCs), and yolk cell progenitors. Knockdown of this klf4-like (klf4l) gene results in animals that fail to specify or maintain germ cells; surprisingly, they also fail to maintain yolk cells. We find that yolk cells display germ cell–like attributes and that vitellaria are structurally analogous to gonads. In addition to identifying a new proliferative cell population in planarians (yolk cell progenitors) and defining its niche, our work provides evidence supporting the hypothesis that flatworm germ cells and yolk cells share a common evolutionary origin.
Introduction

Sexually reproducing animals consist of 2 main cell types: germ cells that produce gametes (eggs and sperm) and somatic cells that make up the remainder of the body. Animal germ cells are typically specified in either of 2 ways: by determinate or inductive specification [1–4]. Determinate specification results from the segregation of specialized maternal determinants (germ plasm) at the onset of embryogenesis; those cells receiving germ plasm acquire germ cell fate. In contrast, inductive specification occurs later in embryogenesis when extrinsic signals from surrounding tissues instruct competent cells to form germ cells. Determinate specification has been studied extensively in traditional laboratory models, including *Drosophila*, *Caenorhabditis elegans*, zebrafish, and frogs [5–17]. Inductive specification, although intensively investigated in mammals, has been less well characterized mechanistically across phylogeny, even though it is the basal and most common mode of germ cell specification in the animal kingdom [1–3].

Irrespective of the mode of germ cell specification, an important commonality exists: Once formed, germ cells are set aside from the soma. The developmental decision to segregate the germ cell lineage from somatic cells is essential for species continuity; unlike the soma, which expires each generation, “immortal” germ cells pass on genetic information and serve as a perpetual link between generations. Many animals (e.g., *Drosophila*, *C. elegans*, and mice) specify their germ cells (and segregate them from their soma) only once during embryonic development [1–4]. However, some animals retain the ability to specify new germ cells throughout their lifetime. Sponges and cnidarians maintain into adulthood multipotent stem cells that fuel the continuous production of new germ cells while also giving rise to somatic cell lineages [18–24]. How do these stem cells decide between somatic and germ cell fates?

Planarian flatworms can regenerate an entire body from small tissue fragments. Intensive efforts have been devoted to understanding the mechanisms underlying this regenerative prowess. Planarian regeneration is driven by pluripotent stem cells called neoblasts that are distributed throughout the body [25–27]. Planarians can also inform our understanding of germ cell biology: The neoblasts that give rise to all somatic lineages also give rise to new germ cells [28–31]. Interestingly, neoblasts and germ cells express a shared set of conserved “germline genes,” including *piwi*, *vasa*, *pumilio*, and *tudor* [32,33], which play important roles in neoblast function [34–44]. Like mammals, planarians undergo inductive germ cell specification [28–31,45,46]. However, the mechanistic basis underlying germ cell specification from “somatic” neoblasts and the factors involved in adopting somatic versus germ cell fate remain obscure.

Here, we investigate how new germ cells are specified from neoblasts throughout postembryonic development and during regeneration in planarians. We also examine another critical aspect of the planarian reproductive system: the development of an extensive network of accessory organs known as vitellaria. Unique among animals, eggs of most flatworms are ectolecithal: Yolk is not present within oocytes themselves, but rather is made by vitellaria that produce specialized yolk cells (vitelline cells or vitellocytes). Planarians and all parasitic flatworms are characterized by ectolecithality. However, despite the importance of vitellaria in the life cycle and transmission of these parasites [47,48], little is known about the development of vitellaria or production of yolk cells.

We show that a homolog of the conserved transcription factor *Krüppel-like factor 4* (*klf4*), a critical inducer of pluripotency in mammals [49], is expressed in male and female presumptive germline stem cells (GSCs) in the planarian *Schmidtea mediterranea*, as well as in a newly discovered population of mitotically competent yolk cell progenitors. We demonstrate that *klf4-like* (*klf4l*) is required for germ cell specification and that *klf4l* knockdown leads to the loss
of both germ cell and yolk cell lineages. We provide evidence that yolk cell–producing organs in planarians consist of 2 distinct cell types: a yolk cell lineage, which is characterized by several germ cell–like attributes, and support cells, which sustain yolk cell maintenance and differentiation. Our data show that planarian vitellaria are structurally analogous to gonads and that yolk cells share several important features with both somatic neoblasts and germ cells.

Results

klf4l is expressed in planarian gonads and yolk-producing accessory organs

In the search for regulators of germ cell fate in planarians, we focused on the conserved transcription factor KLF4, a key pluripotency factor in mammals [49]. Sexual *S. mediterranea* are cross-fertilizing, simultaneous hermaphrodites. Using fluorescent RNA in situ hybridization (FISH) on sexually mature adults, we found that one of the 5 *klf* genes present in the *S. mediterranea* genome, *klf4l* (S1A and S1B Fig), is expressed at high levels within the ventrally situated ovaries, as well as in cells that are distributed along the medial posterior region of each lobe of the cephalic ganglia and appear to be arranged in a field anterior to each ovary (Fig 1A and 1B). Sparse *klf4l*+ cells are also located dorsolaterally, where the testes reside (Fig 1A and 1B). Additionally, *klf4l*+ cells are scattered ventrolaterally throughout the parenchyma (the tissue surrounding the planarian’s internal organs), in a pattern reminiscent of vitellaria, the yolk-producing organs essential for reproduction (Fig 1A and 1B). Thus, this pluripotency-associated transcription factor is expressed in areas associated with male and female reproductive tissues.

klf4l expression is restricted to a subset of nanos+ germ cells in ovaries and testes

To analyze gonadal *klf4l* expression in more detail, we performed double FISH (dFISH) to detect *klf4l* and the germline marker *nanos* [30] (Fig 1C–1E). Previous work in planarians has shown that gonadal *nanos* expression is restricted to the early spermatogonia and oogonia in the outermost layer of testes and ovaries, respectively, which have been interpreted as presumptive GSCs [29,46,50,51]. In addition to the previously described *nanos*+ germ cells found at the ovary periphery, we detected *nanos*+ cells in the same anterior ovarian fields described above (Fig 1C); a substantial proportion of *nanos*+ cells in these fields coexpresses *klf4l* (81%, *n* = 1,116 *nanos*+ cells) and all *klf4l*+ cells are *nanos*+ (100%, *n* = 908 *klf4l*+ cells) (S1 Data). *klf4l* expression is similarly restricted to a subset of *nanos*+ germ cells located at the ovary periphery, and in *nanos*+ cells clustered at the boundary between the ovary and its outlet, the tuba (the anterior-most portion of the oviduct where fertilization occurs) (Fig 1D) (90%, *n* = 1,588 *nanos*+ cells). All *klf4l*+ cells within and at the base of the ovary coexpress *nanos* (100%, *n* = 1,423 *klf4l*+ cells) (S1 Data). In the testes of sexually mature adults, *klf4l* is also expressed around the periphery, but is confined to a small subset of *nanos*+ germ cells (14%, *n* = 10,628 *nanos*+ cells) (Fig 1E). Similar to female germ cells, all *klf4l*+ male germ cells express *nanos* (100%, *n* = 1,475 *klf4l*+ cells) (S1 Data). Our observations show that in both ovaries and testes, the *nanos*+ presumptive GSCs are heterogeneous with respect to *klf4l* expression.

Since only a fraction of *nanos*+ germ cells express *klf4l*, we wondered whether *klf4l* expression represents the earliest stages of *nanos*+ germ cell development. To answer this question, we examined the developmental progression of *klf4l* and *nanos* expression, starting from the emergence of primordial germ cells (PGCs) in newly hatched planarians. Previous studies describing *nanos* expression in hatchlings failed to detect the presence of female (i.e., antero-ventral) PGCs in planarians until 1 week posthatching. Male (dorsolateral) *nanos*+ PGCs were observed in a minority of planarians during the final stages of embryonic development (stage 8...
Fig 1. klf4l is expressed in gonads and vitellaria and is restricted to a subset of nanos+ germ cells in planarian ovaries and testes.

(A) Schematics depicting the dorsal (left) and ventral (right) views of landmark structures and various reproductive organs in adult sexual S. mediterranea. (B) Maximum intensity projections of confocal sections showing FISH of klf4l (green) in ventral head region (top), ventral tail region (middle), and dorsal tail region (bottom). (C) Maximum intensity projection of confocal sections showing dFISH of klf4l (green) and germline marker nanos (magenta) in ventral head region. klf4l- and nanos-expressing cells are detected.
surrounding the tuba (tu) at the base of each ovary (ov), along the periphery of the ovaries, and in anterior ovarian fields (of) situated mediolaterally along the brain. (D) Single confocal section of a planarian ovary located posterior to the brain (br) showing klf4l (green) and nanos (magenta) dFISH. klf4l- and nanos-expressing cells are found at the ovary-tuba junction, along the periphery of the ovary, and in germ cells anterior to the ovary. Dashed line denotes ovary (white) and tuba (yellow) boundary. (E) Confocal section of klf4l (green) and nanos (magenta) dFISH showing klf4l/nanos double-positive and nanos single-positive cells along the periphery of the testis. Dashed line denotes testis boundary. (B–E) Nuclei are counterstained with DAPI (gray). Scale bars, 200 μm (B), 100 μm (C), and 50 μm (D, E). dFISH, double FISH; FISH, fluorescent RNA in situ hybridization; klf4l, klf4-like.

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embryos) and in 1-day-old hatchlings [30,51]. In contrast to these studies, by FISH, we were able to detect female nanos+ cells in 100% of 1-day-old hatchlings; however, only a fraction of these cells expresses the neoblast/germline marker piwi-1, (40%, n = 1,199 nanos+ cells), indicating that not all anteroventral nanos+ cells are germ cells (S2A Fig). While predominantly expressed in germ cells, nanos transcripts have also been detected in a population of eye cells [51]. Consistent with their ventral location and proximity to the cephalic ganglia, we postulate that nanos+/piwi-1+ cells may represent another somatic cell population, such as neurons. To determine the proportion of nanos+ PGCs that express klf4l, we performed triple FISH and found that 56% of nanos+/piwi-1+ PGCs coexpress klf4l (n = 598 nanos+/piwi-1+ cells) (S2A Fig), indicating that this heterogeneity persists throughout sexual development (72% and 75% of nanos+/piwi-1+ germ cells are klf4l+ in immature, juvenile ovaries and mature, adult ovaries, respectively) (S2B Fig).

Male germ cells are easily observed throughout testis maturation. In hatchlings, all nanos+ cells distributed dorsolaterally (where testes will develop) coexpress piwi-1 (100%, n = 517 nanos+ PGCs) (S1 Data). Essentially all nanos+ PGCs also coexpress klf4l (98%, n = 189 nanos+ cells) (Fig 2A). However, as planarians undergo sexual maturation and testes primordia continue to develop, klf4l is expressed in an increasingly smaller proportion of the nanos+ population (34%, n = 5,198 nanos+ cells in juveniles and 14%, n = 10,628 nanos+ cells in adults), and there is a marked increase in klf4l/nanos+ germ cells (Fig 2B and 2C). These data indicate that in hatchlings, newly specified PGCs express both klf4l and nanos; whereas, during sexual development a nanos single-positive germ cell population emerges and expands. These observations are consistent with a model in which klf4l+/nanos+ cells represent the most undifferentiated germ cell state (i.e. PGC and GSC), and klf4l−/nanos+ germ cells are their immediate progeny. It is important to note that essentially all klf4l+ cells coexpressed nanos and piwi-1 (S1 Data)—we did not detect a population of neoblasts (nanos−/piwi-1−) that expressed klf4l.

Thus far, we have characterized klf4l expression in the sexual strain of S. mediterranea. However, this species also exists as an obligate asexual biotype, which reproduces exclusively by fission and does not produce mature gametes or accessory reproductive organs. Although asexual planarians do not develop functional gametes, they nevertheless specify PGCs in small clusters of nanos+ gonadal primordia. These nanos+ cells do not proliferate or differentiate [29,30,51]. By comparing small (approximately 2 mm) and large (>5 mm) asexuals, we found that the number of nanos+ germ cells in female (anteroventrally located) and male (dorsolaterally located) primordia increases as animals grow (Figs 2D, 2E, S2C and S2D). We examined whether klf4l expression was restricted to these early PGCs, and by dFISH we found that klf4l is coexpressed in the majority of female nanos+ cells, in similar proportions for both small and large asexuals (91%, n = 24 nanos+ cells and 86%, n = 213 nanos+ cells, respectively) (S2C and S2D Fig). In contrast, klf4l is coexpressed in virtually all male nanos+ cells in small asexuals (98%, n = 559 nanos+ cells), whereas testis primordia in larger animals contain both klf4l+/nanos+ cells and klf4l−/nanos+ cells (76% and 24% respectively, n = 1645 nanos+ cells) (Fig 2D and 2E). Thus, in both growing asexuals and maturing sexuals, as nanos+ cells in testis primordia increase in number, klf4l expression becomes restricted to a subset of these germ cells. This
similarity suggests that in the asexual biotype germ cells can undergo the first step of development—from 
\(\text{kfl4l}^+\)/\(\text{nanos}^+\) to \(\text{kfl4l}^-\)/\(\text{nanos}^+\) cells—before reaching a block in differentiation.

**kfl4l**-expressing germ cells in ovaries and testes are mitotically active

In many animals, the production of gametes in adulthood is enabled by GSCs. Our findings raise the possibility that **kfl4l**-expressing cells are GSCs representing the top of oogonial and

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**Fig 2.** **kfl4l** expression becomes restricted to a subset of **nanos**\(^+\) male germ cells during sexual planarian maturation and asexual planarian growth. (A–C) Confocal sections showing dFISH of **kfl4l** (green) and **nanos** (magenta) in hatching testis primordia (A), juvenile testes (B), and a sexually mature adult testis (C). **kfl4l** is expressed in most **nanos**\(^+\) male PGCs in hatchlings and becomes progressively restricted to a subpopulation of **nanos**\(^+\) germ cells as planarians sexually mature. **(D, E)** Confocal sections showing dFISH of **kfl4l** (green) and **nanos** (magenta) in testis primordia in small (D) and large (E) asexual planarians. **kfl4l** is coexpressed in almost all **nanos**\(^+\) male germ cells in small asexuals and is restricted to a subset of **nanos**\(^+\) male germ cells in large asexuals. **(A–E)** Percentages reflect **nanos**\(^+\) cells that are also **kfl4l**\(^+\). Nuclei are counterstained with DAPI (gray). Scale bars, 50 μm. Underlying data can be found in S1 Data. dFISH, double FISH; **kfl4l**, **kfl4l-like**; PGC, primordial germ cell.
spermatogonial lineages. All GSCs have the ability to undergo self-renewing divisions, which give rise to differentiating daughter cells while maintaining the stem cell pool. By combining phospho-Histone H3 (pHH3) immunostaining with klf4l and nanos dFISH, we examined the mitotic profiles of cells within the germ cell hierarchy and sought to ascertain whether klf4l⁻/nanos⁻ cells are competent to divide and, therefore, fulfill a basic criterion of GSC behavior.

We found that klf4l⁻/nanos⁻ germ cells within the ovarian fields and the outer periphery of the ovaries are mitotically active (0.3%, n = 3409 klf4l⁻/nanos⁻ cells) (Fig 3A and 3B). We also detected proliferation of klf4l⁺/nanos⁻ oogonia in the ovaries, whereas nanos⁻ oogonia within the ovaries do not divide mitotically. Thus, female germ cells are specified and proliferate within the ovarian field and/or the ovary periphery, and as oogonia turn off nanos expression, they cease to divide mitotically and differentiate into oocytes.

Male germ cells actively divide throughout spermatogenesis; spermatogonia undergo 3 rounds of synchronous mitotic divisions with incomplete cytokinesis to produce 2-, 4-, and 8-cell spermatogonial cysts connected by intercellular bridges, whose cells differentiate into primary spermatocytes and divide meiotically to generate 32 spermatids that ultimately transform into mature sperm [31,52]. We detected pHH3⁺/klf4l⁻/nanos⁻ triple-positive cells in testes of both hatchlings (1%, n = 773 klf4l⁻/nanos⁻ cells) and adults (0.2%, n = 2,436 klf4l⁻/nanos⁻ cells). In mature sexuals, mitotic, single-cell spermatogonia and mitotic doublets were observed in nanos⁻ germ cells (including klf4l⁺/nanos⁻ cells) along the outermost periphery of the testis (Fig 3C and 3D). We also observed nanos⁻/pHH3⁺ singlets and doublets, which might represent mitotic nanos⁻ single-cells or 2-cell spermatogonia (Fig 3D). We never detected nanos expression in pH3⁻ 4- or 8-cell premeiotic spermatogonial cysts, or in 16- or 32-cell meiotic cysts (Fig 3D). All our observations thus far support a model in which the spermatogonial lineage consists of klf4l⁻/nanos⁻ germ cells at the top of the hierarchy, giving rise to klf4l⁺/nanos⁻ and subsequently klf4l⁻/nanos⁻ single-cell spermatogonia and that germ cells cease expressing nanos once spermatogonial cystogenic divisions have occurred.

**klf4l is required for female and male gametogenesis and is necessary for PGC specification**

Having established klf4l as an early germ cell marker, we asked whether klf4l is required for gonadal development. We induced klf4l RNA interference (RNAi) by feeding hatchlings double-stranded RNA (dsRNA) twice a week for 4 to 6 weeks—the time normally required to reach sexual maturity (Fig 4A). We examined the effects of klf4l knockdown on gonadal development by FISH to detect markers of early germ cells (nanos), oocytes (Cytoplasmic Polyadenylation Element Binding Protein 1, CPEB1), and gonadal somatic support cells (ophis, Laminin A, and dmd1) [50,53,54]. klf4l knockdown resulted in a significant reduction of early (nanos⁻) germ cells in the anterior ovarian fields and ovaries as well as a loss of mature (CPEB1⁺) oocytes (Figs 4B, 4C, and S3A). In extreme cases, ovaries were devoid of mature germ cells. Despite this dramatic loss of germ cells, klf4l RNAi ovaries were larger than their control RNAi counterparts, because of a significant expansion of (ophis⁻ or LamA⁻) somatic support cells (Figs 4B, 4C, and S3A). klf4l RNAi also led to a loss of germ cells in the testes (Fig 4D and 4E). Agametic klf4l RNAi testes consist of dmd1⁺ somatic cells and have a “collapsed” appearance due to the absence of germ cells (Fig 4D and 4E). To test for specificity of our RNAi phenotypes and to rule out the possibility of off-target effects, we independently targeted 2 nonoverlapping portions of klf4l (amino terminus versus carboxyl terminus), which induced similar levels of mRNA knockdown (S4 Fig) and indistinguishable phenotypes (S5A and S5B Fig). Taken together, these data indicate that klf4l is required for the maintenance of nanos⁺ germ cells to sustain proper gametogenesis in both ovaries and testes.
Is klfp also necessary for PGC specification? Since klfp-/+ nanos+ PGCs are specified during embryogenesis and are already present in newborn hatchlings, it is not feasible to induce RNAi by dsRNA feeding before PGC specification. However, planarians can regenerate germ cells de novo—amputated head fragments comprised solely of somatic cells can inductively specify new germ cells [28–30]. Therefore, to test the requirement of a gene during PGC specification, one can feed adult planarians dsRNA to induce RNAi, amputate heads anterior to all reproductive tissues, and examine regenerating “germ cell-free” head fragments for de novo germ cell specification (Fig 4F) [50]. Two weeks postamputation, we found that control head fragments respecified klfp-/+ nanos+ cells dorsolaterally (Figs 4G and S3B) [30]. By contrast, respecification of PGCs in klfp RNAi or nanos RNAi head fragments was significantly impaired, with several head regenerates lacking germ cells entirely (Figs 4I, 4J, and S3B). Similarly, dmd1 RNAi head fragments fail to specify new klfp-/+ nanos+ germ cells during regeneration (Figs 4H and S3B), because dmd1 (a critical somatic gonadal niche factor) is required nonautonomously for germ cell specification [50]. These data suggest that klfp and nanos are
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**Fig 4. klf4l is required for gametogenesis in adult ovaries and testes and is necessary for PGC specification.** (A) RNAi scheme during development in sexual *S. mediterranea* from newborn hatching to sexually mature adult. (B) Single confocal section of an ovary (ov) located at the posterior of the brain (br) showing dFISH of *nanos* (magenta) and *ophis* (green; somatic gonadal cells, tuba (tu), oviduct (od) in control and *klf4l* RNAi planarians. (C) Quantification of *nanos*+ germ cells, *CPEB1*+ oocytes, *ophis*+ somatic gonadal cells, and *LumA* somatic gonadal cells per ovary in control and *klf4l* RNAi animals. *N* = 3 experiments. Data are presented as mean ± SD. *klf4l* RNAi results in significantly fewer germ cells and a corresponding increase in somatic support cells compared to control RNAi ovaries, *p < 0.0001*, 2-tailed Welch t test. Underlying data can be found in S1 Data. (D) Maximum intensity projections of confocal sections showing dFISH of *dmd1* (magenta; somatic gonadal cells) and *nanos* (green) in dorsal tail region. *dmd1* and *nanos*-expressing cells are detected surrounding the DAPI-rich sperm located at the center of each testis (te) in control RNAi planarians. *dmd1*+ somatic gonadal cells are present but display a “collapsed” appearance due to the loss of germ cells in *klf4l* RNAi planarians. Dashed line denotes planarian boundary. *N* = 3 experiments, *n* = 9 to 12 planarians. (E) Confocal sections of control and *klf4l* RNAi testes. Note loss of spermatogenesis and “collapsed” appearance of *dmd1*+ somatic gonadal cells in *klf4l* RNAi testes compared to controls. (F) Amputation scheme to assay de novo respecification of germ cells. Amputation anterior to the ovaries results in a head fragment lacking any reproductive tissues (soma only). This head fragment will regenerate a new trunk and tail and will specify new germ cells. (G–J) Maximum intensity projections of confocal sections showing dFISH of *klf4l* (green) and *nanos* (magenta) in head regenerates 2 weeks postamputation. *N* = 3 to 5 experiments, *n* = 14 to 35 planarians. (G) Control RNAi head regenerates specify new *nanos*+ PGCs that coexpress *klf4l*. (H–J) *klf4l* and *nanos* RNAi head regenerates phenocopy *dmd1* knockdowns and fail to specify *klf4l*+/*nanos*+ PGCs. (B, D–E, G–J) Nuclei are counterstained with DAPI (gray). Scale bars, 100 μm (B), 200 μm (D), 50 μm (E), 200 μm for whole-planarian images, 50 μm for side panels (G–J). *klf4l*, *klf4*-like; PGC, primordial germ cell; RNAi, RNA interference.

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required cell autonomously for specification of new germ cells from neoblasts and that *klf4l*+/*nanos*+ male PGCs rely on somatic gonadal “niche” cells for their induction.

**klf4l**-expressing cells are present in vitellaria and are progenitors of the yolk cell lineage

Our results indicate that *klf4l* is an essential regulator of the establishment and maintenance of the planarian germ cell lineage. Is this crucial germline regulator also expressed in “somatic” organs: the vitellaria (Fig 1B)? In *S. mediterranea*, the vitellaria are located ventrally beneath the testes and connect to the oviducts (Fig 1A). Yolkless oocytes are fertilized in the anterior-most compartment of the oviduct (the tuba). After fertilization, zygotes are transported posteriorly through the oviducts to the genital atrium, accumulating thousands of yolk cells along the way. One or more yolk cells and numerous extraembryonic yolk cells are then enclosed within egg capsules. These capsules are laid through the gonopore, and embryonic development proceeds for 2 weeks before newborn hatchlings emerge [31,55–57]. Planarian embryos rely on vitellaria-derived yolk cells for their nutritional needs and development. However, little is known about these essential reproductive structures or how yolk cells are made.

To our surprise, ventrolateral *klf4l*-expressing cells also expressed *nanos* (97%, *n* = 1,548 *klf4l*+ cells) (Fig 5A and 5A’). Previously, *nanos* expression had only been detected in a population of eye cells and in germ cells in testes and ovaries [29,30,31]. Compared to germ cells, ventrolateral *nanos* is expressed at lower levels, but is readily detectable due to recent improvements in ISH sensitivity [58,59]. As in the gonads, ventrolateral *klf4l* expression is restricted to a subset of *nanos*+ cells (49%, *n* = 3,304 *nanos*+ cells). Are *klf4l*+/*nanos*+ cells present in the vitellaria and do they represent the progenitor of planarian yolk cells? To answer these questions and to characterize the progression of yolk cell development, we performed combinatorial dFISH analyses on mature sexual planarians to detect *klf4l* and previously reported vitellaria markers *CPEB1*, *surfactant b*, and *Monosiga brevicollis MX1 hypothetical protein* (*MX1*) [54]. We found that some *klf4l*+ cells coexpress *CPEB1* (10%, *n* = 984 *klf4l*+ cells) and *surfactant b* (9%, *n* = 822 *klf4l*+ cells), but not *MX1* (0%, *n* = 1,094 *klf4l*+ cells) (Fig 5B–5D and 5B’–5D’). These observations suggest that *klf4l* expression marks the earliest yolk cells and that *CPEB1* and *surfactant b* expression precede *MX1* expression in the yolk cell lineage. Indeed, most *CPEB1*+ cells coexpress *surfactant b* (95%, *n* = 1,752 *CPEB1*+ cells) but a much smaller fraction coexpress *MX1* (36%, *n* = 4,334 *CPEB1*+ cells) (S6A, S6B, S6A’ and S6B’ Fig). A large fraction of *surfactant b*-expressing cells are also *MX1*+ (70%, *n* = 8,057 *surfactant
Fig 5. *klf4l* cells are present in vitellaria and are the progenitors of yolk cells. (A–D) Maximum intensity projections of confocal sections showing dFISH of *klf4l* (green) with *nanos* (A), or vitellaria markers CPEB1 (B), surfactant b (C), and *MX1* (D) (magenta) in the ventral posterior region of sexually mature planarians. Dashed line denotes planarian boundary. (A'–D') Single confocal sections of dFISH corresponding to A–D. (A') dFISH of ventrally expressed *klf4l* (green) and *nanos* (magenta). Almost all *klf4l*+ cells coexpress *nanos* whereas *klf4l* is expressed in a subset of *nanos*+ cells. (B'–D') *klf4l* is expressed in a subset of CPEB1+ (B') and surfactant b+ (C') yolk cells, but not in *MX1*+ yolk cells (D'). (A'–D') Side panels are high-magnification views of outlined areas showing *klf4l* single- (white arrowheads) and double-positive cells (yellow arrowheads). Note the increase in cell size as *klf4l*+ cells differentiate into CPEB1+. surfactant
b- and ultimately MX1+ yolk cells. Underlying data can be found in S1 Data. (E) Schematic depicting genes expressed during developmental progression of yolk cell lineage. (F, G) Maximum intensity projections of confocal sections showing FISH of nanos (F) and MX1 (G) (green) in ventral tail region of control and klf4l RNAi animals. Dashed line denotes planarian boundary. N = 3 experiments, n = 8 to 15 planarians. klf4l RNAi results in loss of nanos-expressing cells and a reduction of MX1+ yolk cells in the vitellaria. (A’–D’, F–G) Nuclei are counterstained with DAPI (gray). Scale bars, 200 μm (A–D, F–G), 50 μm for overview images, 20 μm for side panels (A’–D’). klf4l, klf4-like; RNAi, RNA interference.

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Yolk cells share features with neoblasts and germ cells

Our results suggest that klf4l marks the top of both germ cell and yolk cell lineages. Yolk cells are technically somatic since they do not generate gametes, yet it has long been postulated that flatworm yolk cells may share an evolutionary origin with oocytes (the female germline) [55]. One hypothesis is that yolk cells were derived from germ cells in the course of evolution and that a split/divergence between these 2 cell types may have occurred in the common ancestor of all ectolecithal flatworms [60–62]. As we found that both yolk cells and the germline share klf4l and nanos expression, we wondered whether yolk cells share other germ cell characteristics, such as expression of piwi-1 and germininal histone H4 (gH4) (S9A Fig), 2 transcripts thought to be expressed exclusively in neoblasts and germ cells [30,57,63–66]. By dFISH, we found that the vast majority of klf4l+ cells in the vitellaria are also piwi-1+ (94%, n = 789 klf4l+ cells) and gH4+ (98%, n = 399 klf4l+ cells) (Figs 6A, 6E, S9B, and S9C). Unlike other somatic tissues, in which piwi-1 mRNA is degraded during differentiation [36], piwi-1 expression persists during yolk cell differentiation and is still detected in most CPEB1+ cells (80%, n = 2,801 CPEB1+ cells) and surfactant b+ cells (55%, n = 2,136 surfactant b+ cells), but not in MX1+ cells (0%, n = 284 MX1+ cells) (Fig 6B–6D). Similarly, gH4 is coexpressed in most surfactant b+ cells.
(64%, n = 4,410 surfactant b⁺ cells) (Figs 6F and S9D). Thus, similar to the germ cell lineages in testes and ovaries, piwi-1 and gH4 expression persist in differentiating yolk cells.

In addition to the retention of germ cell features in yolk cells, these cells are mitotically active. We detected pH3 staining in klf4l⁻/nanos⁺ as well as klf4l⁺/nanos⁻ yolk cells (Fig 6G). Taken together, these results show that even though yolk cells do not give rise to gametes (and are therefore not germ cells), they do exhibit several germ cell characteristics, including expression of the germline markers nanos, piwi-1, and gH4, and the capacity to proliferate.
Vitellaria contain distinct cell types: A yolk cell lineage and nonyolk support cells

Gonads are not composed solely of germ cells: They also contain somatic support cells (or niche cells) that govern germ cell behavior. Thus, we asked whether vitellaria contain nonyolk vitelline support cells and whether they could play a niche-like role in maintaining the klf4l1/nanos+ stem/progenitor population for sustaining the yolk cell lineage. Previously, expression of the orphan G-protein–coupled receptor ophis, a somatic gonadal cell marker, was detected in the vitellaria, but its role there was not characterized [53]. We found that in mature sexual planarians, the vitellaria are arranged in an extensively branched network containing 2 populations of ophis-expressing cells: ophis\textsuperscript{high} cells, which express ophis predominantly in the nucleus, and ophis\textsuperscript{low} cells with weak signal throughout the cell (Fig 7A). ophis\textsuperscript{+} cells are interspersed throughout the vitellaria, similar to klf4l1 cells (S10A–S10D Fig). klf4l1 cells are tightly juxtaposed with ophis\textsuperscript{high} cells; however, they never coexpress high levels of ophis (0% klf4l1 cells are ophis\textsuperscript{high}, n = 368 klf4l1 cells) (Fig 7A and 7E). On the other hand, a large fraction of klf4l1 cells are ophis\textsuperscript{low} (60% klf4l1 cells are ophis\textsuperscript{low}, n = 368 klf4l1 cells). These results led us to hypothesize that ophis\textsuperscript{low} versus ophis\textsuperscript{high} cells represent 2 distinct classes of cells in the vitellaria: ophis\textsuperscript{low} cells constitute the yolk cell lineage proper and ophis\textsuperscript{high} cells are support cells that closely associate with the yolk cells and comprise the remaining structure of the vitellaria.

If the ophis\textsuperscript{low} population represents the yolk cell lineage of which klf4l1 cells are the precursors, then we would expect klf4l1/ophis\textsuperscript{low} cells to express markers of progressive yolk cell differentiation. Consistent with this idea, almost all CPEB1\textsuperscript{+}, surfactant b\textsuperscript{+}, and MX1\textsuperscript{+} cells coexpress low levels of nuclear and cytoplasmic ophis mRNA (98%, n = 2,914 CPEB1\textsuperscript{+} cells; 100% n = 1,760 surfactant b\textsuperscript{+} cells; 96%, n = 256 MX1\textsuperscript{+} cells) (Fig 7B–7E). On the other hand, high levels of nuclear ophis were rare in CPEB1\textsuperscript{+}, surfactant b\textsuperscript{+}, and MX1\textsuperscript{+} cells (2%, n = 2,914 CPEB1\textsuperscript{+} cells; 0%, n = 1,760 surfactant b\textsuperscript{+} cells; 1%, n = 256 MX1\textsuperscript{+} cells). These results indicate that ophis\textsuperscript{low} expression emerges in a subset of klf4l1 yolk cell progenitors and subsequently persists as these cells differentiate (Fig 7H), whereas ophis\textsuperscript{high} expression defines a distinct cell type in the vitellaria.

In agreement with the model that ophis\textsuperscript{high} cells constitute a separate cell lineage, the majority of these cells do not express yolk cell markers (0%, n = 784 ophis\textsuperscript{high} cells are klf4l1; 12%, n = 519 ophis\textsuperscript{high} cells are CPEB1\textsuperscript{+}; 0%, n = 721 ophis\textsuperscript{high} cells are surfactant b\textsuperscript{+}; 1%, n = 521 ophis\textsuperscript{high} cells are MX1\textsuperscript{+}). Instead, most ophis\textsuperscript{high} cells express Laminin A (Lama) (81%, n = 440 ophis\textsuperscript{high} cells are Lama\textsuperscript{+}) (Fig 7F), a gene expressed in the vitellaria (S10E and S10F Fig) as well as in somatic gonadal cells in the testes and ovaries (S10G Fig). This result corroborates the finding that ophis\textsuperscript{high} expression marks support cells within the vitellaria. Notably, klf4l1 and Lama are never coexpressed within the vitellaria (0%, n = 540 klf4l1 cells, 0%, n = 867 Lama\textsuperscript{+} cells) (Fig 7G). Taken together, our data suggest that 2 cell lineages exist in the vitellaria: the yolk cell lineage (ophis\textsuperscript{low}), which includes klf4l1 cells, and a second population made up of ophis\textsuperscript{high}/Lama\textsuperscript{+} cells. It was previously reported that ophis transcript was expressed in the somatic gonadal cells of the ovary [53]. In addition to this expression pattern, we detect low levels of ophis expression in the oogonial lineage, similar to yolk cells (S10H Fig). The dichotomy between ophis\textsuperscript{low} versus ophis\textsuperscript{high} expression in the germline and somatic lineages of the ovary is reminiscent of what we observed in the 2 vitellarial lineages.

Gonadal niche factor ophis is required to maintain the yolk cell lineage

Previous work has shown that ophis is required for proper development of both male and female gonads in planarians [53]. To address whether ophis is a shared molecular regulator of gonads and vitellaria, we performed RNAi knockdown of ophis in hatchlings until they
Fig 7. Vitellaria contain distinct cell types: Yolk cells and nonyolk support cells. (A–D, F–G) Single confocal sections showing dFISH. Side panels are high-magnification views of outlined areas. (A) dFISH of klf4l (magenta) and vitellaria marker ophis (green). ophis\textsuperscript{low} cells do not coexpress klf4l (filled white arrowhead) but ophis\textsuperscript{high} cells do (unfilled white arrowhead). (B–D) dFISH of ophis (green) and CPEB1 (B), surfactant b (C), and MX1 (D) (magenta). ophis\textsuperscript{low} cells (unfilled white arrowheads), but not ophis\textsuperscript{high} cells (filled white arrowheads), express yolk cell lineage differentiation markers. (E) Proportion of cells in the vitellaria that coexpress low levels (left) versus high levels (right) of ophis. ophis\textsuperscript{low} cells predominantly coexpress markers of the yolk cell lineage. Conversely, most ophis\textsuperscript{high} cells coexpress LamA but do not express yolk cell markers. (F) dFISH of LamA (magenta) and ophis (green). ophis\textsuperscript{high} cells coexpress LamA (filled white arrowhead) whereas ophis\textsuperscript{low} cells do not (unfilled white arrowhead). (G) dFISH of LamA (magenta) and klf4l (green). LamA and klf4l are never
reached sexual maturity and analyzed the effects on the vitellaria by FISH (Fig 8A–8C). *ophis* knockdown resulted in a dramatic loss of the LamA\(^{+}\) cells throughout the vitellaria, but did not affect LamA expression within the gut (Fig 8A). We also observed a significant reduction of klf4l\(^{+}\) cells and complete loss of mature MX1\(^{+}\) yolk cells in *ophis* RNAi animals (Fig 8B and 8C). Although we cannot distinguish the functions of *ophis\(^{high}\) from *ophis\(^{low}\) cells in the vitellaria by available techniques in planarians, it is clear from our data that *ophis* is essential for the maintenance of support cells (*ophis\(^{high}\)/LamA\(^{+}\)) in the vitellaria and is required (perhaps through the action of support cells) for the maintenance and differentiation of klf4l\(^{+}\) yolk cell progenitors.

**Discussion**

Most animals specify PGCs and segregate them from somatic tissues only once, early in development. Within developed gonads, germ cells are generated from GSCs for the reproductive life of the organism. Planarians also specify PGCs in development but are able to continuously regenerate new germ cells from pluripotent stem cells throughout their lifetime. Whether or not *ophis* is coexpressed in the same cells. (A–D, F–G) Nuclei are counterstained with DAPI (gray). Scale bars, 50 \(\mu m\) for overview images, 20 \(\mu m\) for side panels. (H) Schematic depicting genes expressed during developmental progression of *ophis*\(^{low}\) yolk cells and associated *ophis*\(^{high}\) support cells. Underlying data can be found in S1 Data. dFISH, double FISH; klf4l, klf4-like.

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Fig 8. Germ cell niche factor *ophis* is required to sustain yolk cell production/vitellogenesis. (A–C) Maximum intensity projections of confocal sections showing FISH of LamA (A), klf4l (B), and MX1 (C) (green) in the ventral posterior region of sexually mature control versus *ophis* RNAi animals. Dashed line denotes planarian boundary. \(N = 3\) to 5 experiments, \(n = 7\) to 26 planarians. (A) *ophis* RNAi results in a dramatic loss of the LamA\(^{+}\) cells throughout the vitellaria. Note that LamA expression is only visible in the branched gut in *ophis* RNAi planarians. (B, C) *ophis* RNAi results in a reduction of klf4l\(^{+}\) yolk cell progenitors and MX1\(^{+}\) differentiated yolk cells. (A–C) Nuclei are counterstained with DAPI (gray). Scale bars, 200 \(\mu m\). (D) Model depicting similarities shared between gonads (where gametogenesis occurs) and vitellaria (where yolk cell production occurs). klf4l\(^{+/}\)nanos\(^+/\)piwi-1\(^+/\) presumptive GSCs in testes and ovaries divide and give rise to klf4l\(^{+/}\)nanos\(^+/\)piwi-1\(^+/\) progeny. These germ cells are supported by *ophis* somatic gonadal niche cells. Vitellaria are comprised of klf4l\(^{+/}\)/ nanos\(^+/\)/piwi-1\(^+/\) “germ cell-like” yolk progenitors that are mitotically competent, sustain yolk cell production, and are supported by *ophis*\(^{high}\) support cells. FISH, fluorescent RNA in situ hybridization; klf4l, klf4-like; RNAi, RNA interference.

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not planarians also maintain GSCs is less clear, especially since theoretically they could reseed gonads with new germ cells from their somatic stem cells (neoblasts) throughout adulthood. Characterizing the regulators that define planarian germ cells and function in their specification and maintenance will reveal important clues for understanding the remarkable ability of planarians to faithfully regenerate germ cells.

Elucidating early stages of the germ cell lineage

We found that klf4l expression marks the earliest/least differentiated germ cell state in planarians. Early hatchlings specify PGCs that coexpress klf4l and nanos dorsolaterally, where adult testes will ultimately reside. Thus, male PGCs likely do not undergo extensive migration to the somatic gonad. Instead, they are likely to be specified along with dmd1-expressing somatic gonadal niche cells and then differentiate in situ as the testis grows/elaborates during reproductive maturation. As hatchlings develop into juveniles, testis primordia grow in size and 2 successive populations of klf4l+ germ cells arise (nanos+ and nanos−). These populations emerge as testes develop, strongly suggesting that they represent the first steps in germ cell differentiation and that cessation of klf4l expression may be required for germ cell differentiation to proceed.

We observed that klf4l is expressed in virtually all nanos+ cells in early hatchlings and then becomes increasingly restricted to a smaller subset of nanos+ cells as planarians sexually mature. The restriction of klf4l expression to a subset of nanos+ germ cells holds true in asexual planarians as well, where the number of germ cells in both gonadal primordia increases as animals grow. Small asexual planarians express klf4l in almost all nanos+ germ cells, whereas larger asexuasl have proportionally fewer double-positive cells in their testis primordia. Our results refine the stage at which development arrests in asexuals: in growing asexuals, klf4l+/nanos+ cells can only carry out the first step of development (into klf4l−/nanos+ cells), further reinforcing the idea that the germ cell lineage progresses in this direction.

Additionally, we have shown that klf4l is required for the specification of germ cells. RNAi knockdown of klf4l in soma-only head fragments results in regenerated animals that do not respecify nanos+ germ cells, even though new testis somatic gonadal support cells (dmd1+) that form the niche are made. Our data suggest that klf4l is required cell autonomously for the de novo specification of germ cells. Taken together, these observations support a model in which klf4l expression marks the top of the germ cell hierarchy and that expression of klf4l is required for the acquisition of germ cell fate.

Although animals specify their germline in different ways (preformation versus induction), a conserved feature of newly specified PGCs is the repression of somatic differentiation transcriptional programs. Posttranscriptional regulation through the action of conserved germ-line-specific RNA regulators such as vasa, pumilio, nanos, and piwi plays an outsized role in controlling germ cell fate, survival, proliferation, and differentiation. Germ cell fate specification at the transcriptional level is less well understood. During mouse embryogenesis, PGCs are specified from pluripotent epiblast cells by BMP signals from the extraembryonic ectoderm and the visceral endoderm through the action of Smads [67–70]. Critical regulators of PGC specification have been described, including transcription factor genes Prdm1 (which encodes BLIMP1) and Prdm14 [71–78]. A key role of BLIMP1 is to induce expression of Tcfap2c (which encodes the transcription factor AP2γ) [75,79,80], and together, BLIMP1, PRDM14, and AP2γ are important for initiating PGC specification, repressing expression of somatic genes, activating expression of PGC-specific genes, and driving epigenetic reprogramming [78–82].

Recent studies on emerging models have shown that some of the molecular mechanisms regulating PGC specification may be conserved. In the cricket Gryllus bimaculatus, PGCs are
specified in response to BMP signaling via the action of Blimp-1 [83,84]. Additionally, in the cnidarian *Hydractinia symbioticocarpus*, a homolog of AP2 is an inducer of germ cell fate [24]. Although the inductive cues that control germ cell fate in *S. mediterranea* remain to be identified, here, we identify a transcription factor, Klfl4, required for germ cell specification. It is worth noting that Klfl4 is a crucial pluripotency factor in mammals. Furthermore, pluripotency genes Oct4, Sox2, and Nanog are expressed in mouse PGCs [85], reflecting the importance of maintaining pluripotency in germ cells. Therefore, future identification of Klfl4 targets in *S. mediterranea* will not only elucidate the transcriptional program required for promoting germ cell fate from pluripotent neoblasts but may also provide important clues into how germ line pluripotency is maintained.

**Are klfl4-expressing cells true stem cells?**

GSCs are characterized by the ability to undergo self-renewing divisions in which one daughter remains a stem cell and the other differentiates. Consistent with the hypothesis that *klfl4* cells are GSCs, *klfl4*-expressing cells in both testes and ovaries are mitotically active throughout postembryonic development. However, technical limitations precluded us from testing whether mitotic *klfl4* cells undergo self-renewing divisions. Alternatively, it is possible that no resident GSC population exists within the gonads themselves; instead, neoblasts residing in the gonad-adjacent parenchyma may be continually specified as new germ cells that then differentiate directly without self-renewing. Either way, dFISH experiments with *klfl4* and *nanos* have uncovered heterogeneity within the early male and female germ cell compartments. Furthermore, developmental timeline experiments have allowed us to define the early germ cell lineage with *klfl4*/*nanos* germ cells at the top of the hierarchy. Prolonged inhibition of *klfl4* via RNAi during postembryonic development and sexual maturation led to a dramatic loss of early germ cells in both testes and ovaries, resulting in agametic gonads in some animals. This result suggests that *klfl4* is required for the maintenance of GSCs (or germ cell lineal progenitors) to sustain gametogenesis. Future experiments will explore whether *klfl4*/*nanos* cells represent true GSCs and whether this newly defined lineage progresses in a unidirectional manner, or if all *nanos*-expressing cells retain GSC-like potential.

**Bidirectional soma-germ cell communication in the ovary**

Intriguingly, loss of germ cells in the ovary led to a corresponding increase in ovarian somatic gonadal cells (*ophis*+ and *LamA*+). This result reveals that soma-germline communication in the planarian ovary is bidirectional. The importance of somatic support cells for germ cell development is undisputed. However, far less is known about how germ cells signal back to their somatic microenvironment [86–88]. In planarians, somatic cell expansion in the ovary in response to germ cell loss suggests that somatic and germ cell numbers are coordinated via a feedback mechanism. What signals regulate this feedback loop? How does the planarian ovary balance somatic and germ cell numbers to achieve an equilibrium between these 2 cell types? The planarian ovary presents a unique opportunity to investigate the mechanisms involved in soma-germline coordination during development, homeostasis, and regeneration.

While both gonads contain *klfl4*/*nanos*+ putative GSCs, there is also a population of these cells anterior to each ovary. They may be germ cell progenitors that migrate posteriorly and enter the ovary, where they then give rise to *nanos*− oogonia/oocytes. Alternatively, *klfl4*/*nanos*− cells may be specified in a permissive zone along the medial posterior regions of the cephalic ganglia, but only the posterior-most germ cells located at the base of the brain (where the somatic gonad is located) are then able to associate with somatic gonadal cells and
consequently instructed to differentiate. Until we are able to specifically ablate this population, its contribution to the ovary (or lack thereof) will remain mysterious.

A shared evolutionary origin of germ cells and yolk cells?

A unique reproductive feature of flatworms is ectolecithality: a developmental novelty in which oocytes develop with little/no yolk while specialized yolk cells are produced ectopically. For embryogenesis to occur, the fertilized oocyte and numerous yolk cells must be deposited together in egg capsules. As yolk cells are the sole source of embryonic nutrients, ectolecithality has led to marked evolutionary and functional consequences on embryonic development. For example, yolkless embryos develop temporary organs (e.g., embryonic pharynx and primitive gut) that facilitate uptake of maternally provided yolk/nutrients early in embryogenesis [89].

Recent phylogenetic analyses have shed light on the origin of ectolecithality in flatworms. One group of flatworms produces oocytes and yolk cells within a single organ (the germovitel-larium); another group partitions egg- and yolk cell-production into 2 distinct organs (the ger-marium/ovary and vitellaria). This latter group is known as Euneoophora and includes planarians and parasitic flatworms. Although traditional phylogenies grouped both types of ectolecithal worms together, recent phylogenies suggest that they evolved independently [60–62]. Thus, the ectolecithal common ancestor of all euneoophorans likely evolved from more primitive endolecithal (“yolky egg”-producing) flatworms [61,62], consistent with a model in which yolk cells in ectolecithal flatworms evolved from ancestral “yolky” germ cells. These phylogenetic studies recognized that molecular similarities between germ cell and yolk cell precursors would lend further support to the shared evolutionary origin hypothesis [60–62]. Here, we provide molecular and developmental evidence suggesting that yolk cells and germ cells are homologous. Even though yolk cells do not produce gametes and, therefore, are not de facto germ cells, they share several molecular and cellular characteristics in common with germ cells (Fig 8D). Yolk cells express both klf4l and nanos: 2 markers that define male and female germ cell lineages. Similarly to testes and ovaries, klf4l expression in vitellaria is restricted to a subset of nanos+ yolk cells, suggesting that klf4l+/nanos+ cells define the lineal progenitors of yolk cells. We also find that yolk cells express piwi-1 and gH4, which until this work, were reported to be expressed exclusively in neoblasts and germ cells. piwi-1 and gH4 are highly expressed in neoblasts but downregulated in their immediate somatic progeny. In contrast to the soma, but similar to piwi-1 and gH4 expression in male and female germ cell lineages, expression of these genes is sustained in differentiating (klf4l–/nanos+) yolk cells. This sustained expression of neoblast/germ cell markers provides another molecular similarity between germ cells and yolk cells.

Surprisingly, we observed mitosis in yolk cells. Previously, the only planarian somatic cells thought to have mitotic activity were neoblasts. Although yolk cells are technically somatic, our results clearly indicate that like germ cells, a subset of yolk cells is mitotically competent. The observation that both klf4l+/nanos+ and klf4l+/nanos− yolk cells divide indicates that mitotic ability is not limited to the earliest progenitor in the yolk cell lineage. Are pH3+/klf4l+/nanos+ cells undergoing self-renewing divisions? Do piwi-1+/klf4l+/nanos− cells represent a new stem cell population in planarians? With the sole exception of planarian gonads, no other planarian organ contains a resident stem cell (or dividing cell) population. Instead, dividing neoblasts in the parenchyma are the only source of new differentiated somatic cells, which then integrate into existing tissues. The planarian vitellarium provides an intriguing case study to understand the regulation of stem cell populations in planarians.

These similarities between the female germ cell and yolk cell lineages prompted us to ask whether ovaries and vitellaria also share structural features. For example, is there a distinct
lineage of somatic support cells that acts as a niche? Gonads are typified by the presence of somatic support cells that associate intimately with germ cells and play crucial roles in their development. We discovered that in addition to the yolk cells, vitellaria contain a second population of cells (ophis<sup>high</sup>/LamA<sup>+</sup>) with long fingerlike projections that contact all stages of the yolk cell lineage. Both ophis and LamA are also expressed in the somatic gonadal cells of the ovary. ophis RNAi leads to loss of LamA<sup>+</sup> vitellaria cells, a dramatic decrease in klf4l<sup>+</sup> yolk cell progenitors, and a complete failure of vitellogenesis, suggesting that the ophis<sup>high</sup>/LamA<sup>+</sup> cells could function as a niche required to maintain the yolk cell stem/progenitor population. Because a significant number of klf4l<sup>+</sup> yolk cell progenitors coexpress low levels of ophis, we cannot yet distinguish definitively between a cell-autonomous versus nonautonomous role for ophis in yolk cell development. However, since ophis RNAi results in a dramatic loss of klf4l<sup>+</sup> cells that far outnumbers the fraction of klf4l<sup>+</sup> cells that coexpress ophis (60%), we favor the model that ophis acts nonautonomously in the maintenance of klf4l<sup>+</sup>/ophis<sup>−</sup> yolk cells.

Comparative analyses of gametogenesis and vitellogenesis in <i>S. mediterranea</i> have allowed us to investigate the biological phenomenon of ectolecithality and to better understand its origin in Platyhelminthes. Interestingly, nanos expression has been detected in early yolk cells of the parasitic flatworm <i>Schistosoma mansoni</i> [47]. Since all parasitic flatworms (trematodes, cestodes, and monogeneans) are characterized by the presence of ectolecithality, and depend on sexual reproduction to successfully propagate, the vitellaria may provide an effective antihelminthic target. Thus, the experimental accessibility of planarians provides an opportunity to dissect the mechanisms regulating vitellaria development, with the potential to help in the fight against their parasitic cousins.

**Conclusions**

This study demonstrates the functional requirement for klf4l in germ cell specification and maintenance in planarians and provides evidence that klf4l expression marks the top of the germ cell lineage. Additionally, our results suggest that klf4l is a pivotal intrinsic regulator not only of germ cells, but also of yolk cells in a somatic reproductive structure, the vitellaria. Furthermore, we identify a new population of mitotically competent yolk cell progenitors and characterize their niche. Together, these results show that planarian germ cells and somatic yolk cells exhibit a remarkable degree of similarity, supporting the hypothesis that these 2 lineages share an evolutionary origin.

**Materials and methods**

**Planarian culture**

Sexual <i>S. mediterranea</i> [63] were maintained in 0.75X Montjuïc salts [90] at 16 to 18°C. Asexual <i>S. mediterranea</i> (clonal strain CIW4) [91] were maintained in 1X Montjuïc salts at 20 to 22°C. Planarians were starved for 1 week before experimentation.

**Cloning**

Target genes were cloned by PCR amplification of cDNA generated from RNA extracted from adult sexual <i>S. mediterranea</i>. Gene-specific PCR amplicons were ligated into the pJC53.2 vector via TA-cloning as previously described [65]. Anti-sense riboprobes were generated by in vitro transcription reactions with T3 or SP6 RNA polymerases [59]. dsRNA was generated using T7 RNA polymerase [92]. Sequences used for probes and dsRNA are found in S1 Table.
In situ hybridization
FISH protocols were performed as previously described \[58,59\] with the following modifications. Asexual and sexual hatchling/sexual adult planarians were killed in 7.5% N-acetyl-L-cysteine in 1X PBS for 5/10 minutes; fixed in 4% formaldehyde in PBSTx (1X PBS + 0.1% Triton X-100) for 15/30 minutes; bleached in Bleaching Solution (1X SSC solution containing 5% deionized formamide and 1.2% hydrogen peroxide) for 2/4 hours; incubated in PBSTx containing 10 μg/ml Proteinase K and 0.1% SDS for 10/20 minutes; and refixed in 4% formaldehyde in PBSTx for 10/15 minutes. Planarians were blocked in Blocking Solution (5% heat inactivated horse serum, 5% Roche Western Blocking Buffer in TNTx [0.1 M Tris pH 7.5, 0.15 M NaCl, 0.3% Triton X-100]) for 2 hours at room temperature, and incubated in Blocking Solution containing anti-Digoxigenin-POD (1:2,000), anti-Fluorescein-POD (1:2,000), or anti-Dinitrophenyl-HRP (1:2,000) for 8 hours at 12˚C. For fluorescent development of riboprobes, TSA reactions were performed for 30 minutes.

pHH3 immunofluorescence
Immunostaining was performed after FISH development by reblocking planarians in Blocking Solution (5% heat inactivated horse serum, 5% Roche Western Blocking Buffer in TNTx) for 2 hours at room temperature, labeling mitotic cells with anti-phospho-Histone H3 (Ser10) (1:2,000) in Blocking Solution overnight at 12˚C, washing 6X in PBSTx (30 minutes each), reblocking for 2 hours at room temperature, and incubating with HRP-conjugated goat anti-mouse (1:500) in blocking solution overnight at 12˚C. Planarians were washed 6X in PBSTx (30 minutes each) and TSA was performed for 30 minutes.

Imaging
Confocal imaging was performed using a ZEISS LSM 880 with the following objectives: EC Plan-Neofluar 10x/0.3 M27, Plan-Apochromat 20x/0.8 M27, Plan-Apochromat 40x/1.3 Oil DIC M27. Image processing was performed using ZEISS ZEN 3.1 (blue edition) for linear adjustments and maximum intensity projections.

RNA interference
In vitro dsRNA synthesis was performed as previously described \[92\] by in vitro transcription from PCR amplicons flanked by T7 promoters. In vitro transcription reactions were carried out overnight at 31˚C, DNase-treated, brought up to 80 to 100 μl final volume with water, and annealed. dsRNA was added to liver (1:2–1:5) and fed to animals. dsRNA generated from the CamR and ccdB-containing insert of the pJC53.2 vector was used for all control RNAi feedings \[65\].

Quantification and statistical analysis
Z-stack images through ovaries, testes, and vitellaria were visualized on ZEISS ZEN 3.1 (blue edition) or Imaris (Oxford Instruments, UK, Bitplane AG, Switzerland) software and cell counting was performed manually. Binary decisions (positive/negative or high/low) were made for cells using single color channels at a time. Counts for all experiments are detailed in S1 Data. Statistical analyses were performed using GraphPad Prism software. Statistical tests, significance levels, number of data points, cell or planarian numbers (n), and experimental replicates (N) are provided in the text and/or figure legends.
Quantitative PCR

Total RNA was extracted using TRIzol Reagent (Invitrogen, Thermo Fisher Scientific, USA) from whole sexual animals 1 week after the final dsRNA feeding. Total RNA was DNase-treated and purified, followed by cDNA synthesis using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., USA). *klf4l* primers were designed to sequences outside of the sequence targeted by the dsRNA in order to avoid spurious amplification of dsRNA along with cDNA. For example, primers to the amino terminus of *klf4l* were used to quantify *klf4l* knockdown levels in *klf4l* RNAi (carboxyl terminus) animals in which dsRNA targeting the carboxyl terminus was used, and vice versa. All quantitative PCR (qPCR) analyses were normalized to endogenous control gene *β-tubulin*. qPCR primers are listed in S2 Table. qPCR was performed using the GoTaq qPCR Master Mix reagent system (Promega, USA) on a StepOnePlus Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, USA). Three technical replicates were run for each of 3 biological replicates. ΔΔCt calculations were performed in Microsoft Excel and 2^−ΔΔCt values (normalized to control RNAi) with 95% confidence intervals were plotted using GraphPad Prism software.

Supporting information

**S1 Fig. The Klf gene family in *S. mediterranea.* (A) The *S. mediterranea* genome contains 5 genes that encode Klf proteins [93]. Alignment of *S. mediterranea* Klf DBDs. The height of the red bars reflects percent conservation of the amino acid in the alignment. Sequence logo at bottom of the alignment depicts the consensus sequence. All Klfs are characterized by exactly 3 highly conserved C2H2 ZnF domains (each one outlined by a box) separated by intervening linker sequences and located at the carboxyl terminus. The black arrow points to a canonical aspartic acid residue (D) in ZnF2, which is important for Klf-DNA binding and is conserved in all filoza (animals and their nearest unicellular relatives) [94]. Note the additional 5 amino acids in ZnF2 in *S. mediterranea* Klf4l (SMEST031008001.1.protein). These additional 5 residues in ZnF2 are a triclad innovation and are present in Klf4l homologs in the Platyhelminthes *S. mediterranea* (Smed), *Schmidtea polychroa* (Spol), *Dugesia japonica* (Djap), *Dendrocoelum lacteum* (Dlac), *Planaria torva* (Ptor), *Polycelis nigra* (Pnig), and *Polycelis tenuis* (Pten) (all from the Tricladida order), but not in *Prostheceraeus vittatus* (Pvit) and *Stylocus ellipticus* (Sell) (Polycladida). (B) The most significant human and mouse BLASTP hits for *S. mediterranea* Klf4l are Klf4 proteins. Alignment of *Homo sapiens* (NP_004226.3), *Mus musculus* (NP_034767.2), *Xenopus tropicalis* (NP_001017280.1), and *Danio rerio* (NP_571798.1) Klf4 DBDs with *S. mediterranea* (SMEST031008001.1) Klf4l DBD. DBD, DNA-binding domain; *klf4l*, *klf4-like*; ZnF, zinc finger.

(PDF)

**S2 Fig. *klf4l* is expressed in a subset of *nanos*+ female germ cells in sexual and asexual planarians.** (A, B) Confocal section showing triple FISH of *piwi-1* (cyan), *klf4l* (green), and *nanos* (magenta) in female germ cells in hatchlings and sexually mature ovary. *klf4l* is expressed in a subset of *nanos*/^+^/ *piwi-1*+ female germ cells (compare filled (*klf4l*+) yellow arrowhead). All *klf4l*/^−^/ *nanos*+ germ cells are *piwi-1*+. A small fraction of *klf4l*/^−^/ *nanos*+ cells do not express *piwi-1* and are not germ cells (white arrowhead). (C, D) Confocal sections showing dFISH of *klf4l* (green) and *nanos* (magenta) in female germ cells (located mediolaterally along the planarian brain) in small (C) and large (D) asexual planarians. *klf4l* is expressed in a subset of *nanos*+ female germ cells. Insets show high-magnification views of heterogeneity of *klf4l* expression in *nanos*+ cells. (A–D) Percentages reflect *nanos*+ germ cells that are also *klf4l*+. Nuclei are counterstained with DAPI (gray). Scale bars, 100 μm (A, B), 50 μm for whole-brain...
images, 10 μm for insets (C, D). Underlying data can be found in S1 Data. FISH, fluorescent RNA in situ hybridization; klf4l, klf4-like.

(PDF)

S3 Fig. klf4l is required for oogenesis and restricts expansion of somatic gonadal cells in adult ovaries. (A) Single confocal section of an ovary located at the posterior of the brain (br) and anterior to the tuba/oviduct (tu/od) showing dFISH of LamA (magenta; somatic gonadal cells) and CPEB1 (green; oocytes) in control and klf4l RNAi planarians. klf4l RNAI leads to oocyte loss and a nonautonomous increase in somatic support cells. Nuclei are counterstained with DAPI (gray). Scale bars, 50 μm. (B) Quantification of newly specified PGCs in head regenerates. Data are presented as mean ± SD. N = 3 to 5 experiments, n = 14 to 35 planarians. p < 0.0001, Welch ANOVA test. Underlying data can be found in S1 Data. dFISH, double FISH; klf4l, klf4-like; RNAi, RNA interference.

(PDF)

S4 Fig. Quantification of klf4l expression in knockdown animals. qPCR analysis of klf4l mRNA expression (normalized to β-tubulin) in control and klf4l RNAI animals depicting efficient knockdown of klf4l after RNAI treatment. Top: dsRNA targeting the amino terminus of klf4l was used for RNAI-mediated knockdown of klf4l, and qPCR primers targeting the carboxyl terminus were used to quantify klf4l expression levels. Bottom: dsRNA targeting the carboxyl terminus of klf4l was used for RNAI and qPCR primers targeting the amino terminus were used to quantify klf4l expression levels. N = 3 biological replicates (3 technical replicates each). Bar graphs depict 2−ΔΔCt values (normalized to control RNAI) with 95% confidence intervals. Underlying data can be found in S1 Data. klf4l, klf4-like; qPCR, quantitative PCR; RNAI, RNA interference.

(PDF)

S5 Fig. Independent RNAI triggers targeting nonoverlapping klf4l sequences result in similar phenotypes in the gonads. (A) Single confocal section of an ovary located posterior to the brain (br) and anterior to the tuba/oviduct (tu/od) showing dFISH of LamA (magenta; somatic gonadal cells) and CPEB1 (green; oocytes) in control and klf4l RNAI planarians. RNAI resulting from dsRNA targeting near-full-length klf4l, an amino-terminal portion of klf4l, or a carboxyl-terminal portion of klf4l all lead to similar defects in oogenesis. N = 2 experiments, n = 11–16 planarians. (B) Maximum intensity projections of confocal sections showing dFISH of dmd1 (magenta; somatic gonadal cells) and nanos (green) in a dorsal tail region where testes reside. All klf4l knockdowns lead to similar defects in spermatogenesis and “collapsed” testes due to male germ cell loss. Dashed line denotes planarian boundary. N = 2 experiments, n = 10 to 14 planarians. (A, B) Nuclei are counterstained with DAPI (gray). Scale bars, 50 μm (A), 200 μm (B). dFISH, double FISH; klf4l, klf4-like; RNAI, RNA interference.

(PDF)

S6 Fig. Defining the stages of yolk cell development. (A–C) Maximum intensity projections of confocal sections showing dFISH of vitellaria markers CPEB1 (A–B), surfactant b (A, C), and MX1 (B, C) in the ventral posterior region of sexually mature planarians. Dashed line denotes planarian boundary. (A’–C’) Single confocal sections of dFISH corresponding to A–C. (A’) dFISH of ventrally expressed CPEB1 (magenta) and surfactant b (green). Almost all CPEB1+ cells coexpress surfactant b and all surfactant b+ cells are CPEB1+. (B’) dFISH of CPEB1 (magenta) and MX1 (green). A subset of CPEB1+ cells coexpress MX1 whereas all MX1+ cells are CPEB1+. (C’) dFISH of MX1 (magenta) and surfactant b (green). A subset of surfactant b+ cells coexpresses MX1 whereas virtually all MX1+ cells are surfactant b+. (A’–C’) Side panels are high-magnification views of outlined areas. (A’–C’) Nuclei are counterstained
with DAPI (gray). Scale bars, 200 μm (A–C), 50 μm for overview images, 20 μm for side panels (A’–C’). Underlying data can be found in S1 Data. dFISH, double FISH.

(PDF)

S7 Fig. Vitellaria develop postembryonically and produce differentiating yolk cells during sexual maturation. (A, B) Maximum intensity projections of confocal sections showing dFISH of klf4l (green) with nanos, or vitellaria markers CPEB1, surfactant b, or MX1 (magenta) in the ventral posterior region of hatchlings (A) or juveniles (B). Dashed line denotes planarian boundary. (A) Hatchlings do not express any of the vitellaria markers tested and are devoid of vitellaria. (B) klf4l+/nanos− yolk cell progenitors, as well as klf4l+/nanos+, CPEB1+, and surfactant b− differentiating yolk cells are detected in all juveniles. Only a fraction of juveniles express MX1+ yolk cells (B). (A, B) Nuclei are counterstained with DAPI (gray). Scale bars, 100 μm. dFISH, double FISH; klf4l, klf4-like.

(PDF)

S8 Fig. RNAi triggers targeting nonoverlapping klf4l sequences lead to similar defects in vitellogenesis. Maximum intensity projections of confocal sections showing FISH of MX1 (green; mature yolk cells) in ventral tail region of control and klf4l RNAi animals. RNAi triggered by dsRNA targeting near-full-length klf4l, an amino-terminal portion of klf4l, or a carboxyl-terminal portion of klf4l leads to similar loss of MX1+ yolk cells. Dashed line denotes planarian boundary. N = 2 experiments, n = 6 to 7 planarians. (A, B) Nuclei are counterstained with DAPI (gray). Scale bars, 200 μm. FISH, fluorescent RNA in situ hybridization; klf4l, klf4-like; RNAi, RNA interference.

(PDF)

S9 Fig. Yolk cells express neoblast/germ cell markers. (A) Single confocal sections showing dFISH of neoblast and germ cell marker gH4 (magenta) and klf4l (green). gH4 is expressed at high levels in neoblasts as well as in spermatogonia and oogonia. klf4l+ cells in the testes (top panels), ovarian field, and ovary (ov) (bottom panel) coexpress gH4 (yellow arrowheads). Note the absence of gH4 in differentiated somatic cells found in the brain (br) and tuba (tu). Nuclei are counterstained with DAPI (gray). (B, C) Maximum intensity projections of confocal sections showing dFISH of klf4l and neoblast/germline markers piwi-1 or gH4 in the vitellaria. (D) Maximum intensity projection of confocal sections showing dFISH of gH4 (magenta) and surfactant b (green). Dashed line denotes planarian boundary. Scale bars, 50 μm (A), 200 μm (B–D). dFISH, double FISH; klf4l, klf4-like.

(PDF)

S10 Fig. The vitellaria and ovary are comprised of 2 populations of ophis-expressing cells: ophis\textsuperscript{high} versus ophis\textsuperscript{low} cells. (A–F) Maximum intensity projections of confocal sections showing dFISH of vitellaria markers in the ventral posterior region of sexually mature planarians. Dashed line denotes planarian boundary. (G) Confocal section of an ovary depicting LamA expression (magenta) in somatic gonadal cells and klf4l expression (green) in early germ cells. (H) Confocal section of an ovary depicting ophis\textsuperscript{high} expression (magenta/gray) in somatic gonadal cell nuclei (filled arrowhead) and ophis\textsuperscript{low} expression in oogonia and oocytes (unfilled arrowhead). (G, H) Dashed line denotes ovary (white) and tuba (yellow) boundary. Nuclei are counterstained with DAPI (gray). Scale bars, 200 μm (A–F), 50 μm (G, H). dFISH, double FISH; klf4l, klf4-like.

(PDF)

S1 Data. Quantifications and cell counts.

(XLSX)
S1 Table. Information for transcripts mentioned in this paper.
(XLSX)

S2 Table. Quantitative PCR primers.
(XLSX)

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References


