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Regeneration of the germline in the annelid *Capitella teleta*

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ABSTRACT

The germline is essential for sexual reproduction and survival of the species. In many metazoans, the developmental potential to generate a distinct germline is segregated from somatic cell lineages early in embryogenesis, suggesting that the unique features of the germline must be established from its onset. Previous studies suggest that germ cells cannot regenerate once removed from the embryo, but few animals have been experimentally tested. We investigated the ability of the germline to regenerate in a lophotrochozoan, the segmented worm *Capitella teleta*, which has a stereotyped cell lineage program by deleting the germline precursor (cell 3D) in early stage embryos using an infrared laser. Larvae and juveniles resulting from germline deletions were examined for presence of multipotent progenitor cells (MPCs), stem cells that form the germ cells and somatic stem cells. In contrast to control deletions of a non-germline macromere, most larvae resulting from deletion of cell 3D lacked MPCs as assayed by expression of germline markers *CapI-vasa*, *CapI-nanos* and *Ct-piwi1*, but showed persistent expression of these markers in the somatic posterior growth zone. However, approximately 13% of experimental larvae had MPCs, indicative of some germline regeneration. In contrast, by two weeks post-metamorphosis, all juveniles resulting from deletion of cell 3D had MPCs, as detected by *CapI-vasa* expression. Furthermore, when raised to adulthood, most animals developed reproductive structures and were fertile. In another set of deletions, both the D quadrant mesodermal and germline progenitors were removed. These juveniles also regenerated MPCs. Surprisingly, this deletion caused substantial ectopic expression of *CapI-vasa* and *CapI-nanos* in other larval tissues. Our results indicate that *C. teleta* can regenerate the germline following removal of the germline progenitors in the early embryo. The dramatic difference in ability to regenerate the germline between the larval and adult stages suggests that there are two distinct compensation events at two phases of the life cycle: a regulative event in the early stage larva and a stem cell transition event after metamorphosis, when the animals are capable of substantial body regeneration.

1. Introduction

The germline is necessary for sexual reproduction, which is imperative for the survival and evolution of species. In many well-studied bilaterian organisms, the germline separates completely from the somatic cells early in embryonic development. This can occur either by sequestration of proteins and mRNA in the cytoplasm of the zygote, or by induction via a cell-signaling event from other cells in the embryo. These two distinct mechanisms are known as preformation and epigenesis, respectively (Nieuwkoop and Sutasurya, 1979, 1981; Extavour and Akam, 2003). The segregated cells that will later form the sperm and egg are known as primordial germ cells (PGCs). It has been proposed that in many animals, germline and somatic lineages must separate early in embryonic development to avoid evolutionarily detrimental competition between different cell lineages within the organism (Buss, 1987). In addition, a number of studies have shown that the germline undergoes transcriptional and translational silencing to minimize the possibility of passing on somatic mutations to the germline, and these characteristics are not shared with somatic lineages (Blackler, 1970; Drake et al., 1998; Milholland et al., 2017; Seydoux and Braun, 2006; Strome and Updike, 2015; reviewed in Weisblat, 2006).

Removal of germ cells by excision, irradiation, or deletion results in sterile adults in many organisms, including in *Mus musculus*, *Xenopus laevis*, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Ambystoma mexicanum*, and *Gallus gallus* (Barnes et al., 2006; Blackler, 1965; Buehr and Blackler, 1970; Dubois, 1962; Dulbecco, 1946; Everett, 1943; Fargeix, 1975; Nieuwkoop, 1951; Reynaud, 1976; Sulston and Schierenberg, 1983; Züst and Dixon, 1975). These animals develop reproductive structures with no gametes. The results of such experimental manipulations support the idea of complete separation between the germline and soma early in the developmental program.

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Once segregated, the germline cannot reform from other cell types, demonstrating a single embryonic origin of the germline. In contrast, more recent experiments in the ascidian *Ciona intestinalis* have uncovered an example of germline regeneration. In *C. intestinalis*, the germline precursors are located in the tail during the larval tadpole stage, and when the larval tail is removed, the resulting juveniles lack germ cells. However, after 15 days, a few germ cells appear, and later, the adults produce sperm (Takamura et al., 2002). It is unknown from where these cells arise and if this example is a rare occurrence or whether additional sampling will reveal more cases of animals that can regenerate their germline. It is worth noting that experimental manipulations of the germline have only been performed on a small fraction of animal clades.

The superphylum Lophotrochozoa contains 14 or so highly diverse animal phyla and relative to other clades, little is known about the development of the germline, and whether its members have an ability to regenerate their germline (reviewed in Extavour and Akam, 2003). Many members of this superphylum have a shared developmental program called spiral development. Embryos that undergo spiral cleavage have a stereotypic cleavage pattern such that each cell within the embryo can be identified based on spatial relationships, cell size, and time of division (Wilson, 1892; Henry and Martindale, 1999, 1998). The predictable cleavage pattern of spiralian embryos has enabled researchers to perform single cell blastomere fate map and deletion studies on several species (Ackermann et al., 2005; Boyer et al., 1996; Damen and Dictus, 1994; Hejnol et al., 2007; Henry and Martindale, 1998; Maslakova et al., 2004; Meyer et al., 2010; Render and Render, 1997; Weisblat and Shankland, 1985). From these studies, it has been shown that several aspects of the fate map are conserved across species and even across phyla. One notable example is the apparent conservation of the embryonic origin of the germline. In all animals examined, the precursor of the germline is localized to a single cell in the 64-cell stage embryo, the cell 4d (reviewed in Lambert, 2008).

One lophotrochozoan, the annelid *C. teleta,* has several advantages for studies of the germline. The embryonic origin of the germline in *C. teleta* appears to be conserved with that of other spiralians. Cell lineage and gene expression studies using the genes *piwi*, *nanos* and *vasa* are consistent with the idea that descendants of cell 4d coincide with germline precursor stem cells (Dill and Seaver, 2008; Giani et al., 2011; Meyer et al., 2010). However, unlike in other spiralians, the germline and the mesoderm do not arise from the same precursor cell, allowing for experimental manipulation of the germline without also disrupting mesoderm formation (Meyer et al., 2010). In addition, *C. teleta* reproduces sexually with separate male and female sexes that can be successfully mated in the laboratory. Adult reproductive structures are morphologically visible and have previously been characterized in detail (Eckelbarger et al., 1984; Eckelbarger and Grassle, 1987a, 1987b). Furthermore, similar to many other annelids, *C. teleta* can regenerate (Bely, 2006; Bely et al., 2014). For example, following transverse amputation, *C. teleta* can regenerate its nervous system, musculature, and digestive tract (de Jong and Seaver, 2016). Both somatic (ovaries) and germline (oocytes) components of the reproductive tissues can also regenerate following amputation posterior of the 6th thoracic segment (Giani et al., 2011; Hill and Savage, 2009). The ability of *C. teleta* to regenerate multiple tissue types led us to hypothesize that this animal may have a unique stem cell regulatory program that allows transition of somatic stem cells to germ cells, and to potentially regenerate the germline.

Historically, morphological similarities have been observed between germ cells and stem cells in annelids (Faulkner, 1932; Potswald, 1972, 1969). Notably, both cell types have a large nuclear to cytoplasmic ratio and a characteristic morphology of undifferentiated cells. More recent molecular studies, including studies in *C. teleta,* show that the markers *vasa*, *nanos*, and *piwi* are expressed in both the germline and somatic stem cell populations in many species, emphasizing the similarities between the germline and stem cells (Dill and Seaver, 2008; Fischer and Arendt, 2013; Giani et al., 2011; Lyons et al., 2012; Mochizuki et al., 2001; Özpolat et al., 2016; Raz, 2002; Rebscher, 2014; Rebscher et al., 2012; Shibata et al., 1999; Solana, 2013). Such genes are proposed to have a role in maintaining an undifferentiated state (Mochizuki et al., 2001). These cells have been referred to as 'germline cell stem cells', 'germinal cells', 'pre-primordial germ cells (pre-PGCs)', 'presumptive primordial germ cells (PGCs)' or 'primordial stem cells (PriSCs)' and 'molecular progenitor cells' depending upon the study. In previous studies, we referred to a cluster of cells with these characteristics in *C. teleta* cells as presumptive primordial germ cells (Giani et al., 2011), and we now refer to them as the multipotent progenitor cell (MPC) cluster to better represent additional roles that the cells in this cluster appear to have during regeneration (de Jong and Seaver, 2017). The MPC cluster in *C. teleta* larvae, juveniles, and adults can be visualized with the germline/stem cell markers *CapI-vasa*, *Ct-piwi1, Ct-piwi2,* and *CapI-nanos* (Dill and Seaver, 2008; Giani et al., 2011). These cells are either pluripotent stem cells capable of forming both germline and somatic cells, or a mixed population of cells with distinct subsets destined to become either germline or somatic stem cells. We favor the latter possibility, due to recent molecular evidence suggesting heterogeneity of cells within the cluster. Specifically, only a small subset of cells in the MPC cluster expresses the marker *Ct-myc* (de Jong and Seaver, 2017).

To determine whether *C. teleta* can regenerate its germline, we performed single cell laser deletion experiments to remove the germline precursor cell and examine the resulting effects in larvae, juveniles and adults. We assessed MPC presence using molecular markers in larvae and juveniles. Adults resulting from embryonic deletion of the germline precursors were analyzed for the presence of reproductive structures, ability to mate, and viability of their offspring. In addition, we also investigated the cellular origin of the lineage capable of replacing the germline. To our knowledge, this work provides one of only a few examples of germline regeneration in bilaterian animals.

2. Materials and methods

2.1. Animal care

Embryos were acquired by separating gravid males from females for three to six days, and then combining them together in a mating dish for 11–14 h. Dishes were inspected for the presence of brood tubes made by the females (Seaver et al., 2005), and embryos were dissected from the brood tubes and placed in a dish of 0.2 μm-filtered seawater (FSW). All embryos and larval stages were raised in FSW with 60 μg/mL penicillin (Sigma-Aldrich) and 50 μg/mL streptomycin (Sigma-Aldrich) at 19 °C, which was exchanged each day until larval day nine. Animals were staged according to a published staging chart (Seaver et al., 2005). All juvenile and adult animals were maintained in organically enriched mud.

2.2. Cell deletions

Single blastomere deletions were performed using the XYClone system infrared laser (Hamilton Thorne) with the 20× objective fitted to a Zeiss Axioplan compound microscope as described in Yamaguchi et al. (2016). Embryos were placed on a Rainex-coated slide in a drop of FSW and oriented with the vegetal side of the embryo facing up. A chamber to cover the embryos was made by attaching two cover slip slivers on each end of a coverslip with melted dental wax following Lyons et al. (2012). For all blastomere deletions, the laser power was set to 100%, and the pulse length was adjusted based on the sensitivity of the brood, the size of the cell being targeted, and the stage of the cell cycle. The pulse range for cell 3D and 3B was one pulse between 150 and 250 μs, followed by a second pulse of 550–750 μs. Cell 2D and cell 2C were deleted using two pulses between 350 and 450 μs each. The

laser was targeted such that a single pulse damaged both the outer chorion of the embryo and the cell membrane, allowing the contents of the targeted cell to leave the embryo. Immediately following delivery of laser pulses, targeted blastomeres were visually monitored to ensure that the cytoplasm was leaving the cell. After completion of all deletions, the embryos were further sorted to visually confirm that the targeted cell was completely missing, with no damage to surrounding cells; all other embryos were discarded. Embryos were subsequently monitored into the next cell division to ensure that the surrounding cells were dividing normally. At least 20 control embryos for each brood were raised at the same temperature to monitor overall health of the brood. Experimental animals were scored only if at least 90% of the controls had elongated bodies and appeared morphologically normal.

2.3. Fixation and whole mount in situ hybridization

Animals were fixed as either stage nine larvae (approximately nine days after fertilization), one week (seven days post-metamorphosis) or two week-old (14 days post-metamorphosis) juveniles. Prior to fixation, larvae were placed in a 1:1 solution of FSW and 0.37 M magnesium chloride ($MgCl_2$) for ten minutes to relax their muscles, and then fixed in 3.7% paraformaldehyde (PFA) in FSW overnight at 4 °C. Juveniles were removed from the mud and placed in dishes containing 0.5% corn meal agar in FSW plus 60 μg/mL penicillin and 50 μg/mL streptomycin to remove debris from the body and allow for clearing of gut contents. Juveniles were then placed in dishes containing 0.5% cornmeal agar in a 1:1 solution of FSW plus 0.37 M $MgCl_2$ for 30 min, followed by fixation in 3.7% PFA in FSW overnight at 4 °C.

Following fixation, larvae and juveniles were washed in phosphate-buffered saline (PBS) and then dehydrated through a series of methanol washes into 100% methanol, and stored at −20 °C for at least 24 h prior to *in situ* hybridization experiments. The protocol for whole-mount *in situ* hybridization in *C. teleta* is published in Seaver and Kaneshige (2006). Digoxigenin-labeled riboprobes for *CapI-vasa* and *Ct-piwi1* were generated with the SP6 MEGAscript kit (Ambion, Inc., Austin, TX, USA), and the digoxigenin-labeled riboprobe for C*apI-nanos* was generated with the T7 MEGAscript kit (Ambion, Inc., Austin, TX, USA). Prior to the formal species description, *C. teleta* was known as *Capitella* sp. I (Blake et al., 2009). Genes named prior to the species description were named with a prefix 'CapI', and these include *CapI-vasa* and *CapI-nanos*. The *CapI-vasa* and *CapI-nanos* probe sequences and lengths are published in Dill and Seaver (2008). The *Ct-piwi1* probe sequence and length is published in Giani et al. (2011). All probes were diluted to a final concentration of between 0.5 and 1 ng/μl. Animals were hybridized with the riboprobe at 65 °C for 48–72 h, and later detected by exposure to nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyphosphate (USBiological, Salem, MA, USA) for two to six hours. The development reaction was terminated by repeated exchanges of PBS + 0.1% Tween-20 (PTw) for at least 12 h, and then were cleared by equilibration in 80% glycerol in 1× PBS with 0.125 μg/μl Hoechst 33342 (Life Technologies H3570) for at least 12 h. Animals in glycerol were then placed on glass slides with coverslips for microscopic analysis.

2.4. Scoring and analysis

Following deletion of the -3D, -3B, -2D and -2C cells, *CapI-vasa* expression was used to score resulting larvae for presence or absence of MPCs. In stage 9 larvae, MPCs are typically located near the foregut/midgut boundary along the ventral midline, but were scored as positive for MPCs in any location of the trunk of the body. Larvae were only scored if they had normally elongated bodies and if *CapI-vasa*, *CapI-nanos,* or *Ct-piwi1* expression was detected in the posterior growth zone. Juveniles were scored for the presence or absence of MPCs in the thoracic segments as well as for the number of clusters and number of countable cells. Cells were counted using either the 20× or 40× objective of a Zeiss Axioplan compound microscope in juveniles viewed from the ventral side.

Adults were scored live between 8 and 12 weeks post-metamorphosis for presence or absence of reproductive structures, and for whether the animals were male, female, or hermaphrodite. Males were then mated to females, and hermaphrodites mated with males in individual dishes of FSW and mud. One male was mated to either multiple or single females, depending on availability of females in the experimental replicate. In cases where not enough females or males of the experimental group were available, control animals were used for mating. Fertility was determined by presence or absence of brood tubes containing embryos or larvae. Larval viability was determined by ability of the larvae to swim when released manually from the brood tube.

2.5. Imaging and microscopy

Larvae and juveniles were imaged using a SPOT FLEX digital camera (Diagnostic Instruments, Inc., SterlingHeights, MI) attached to an Axioskop 2 mot-plus compound microscope (Zeiss, Gottingen, Germany). SPOT imaging software (version 5.2) was used to capture images. Adults were imaged with a Ximea camera attached to a Zeiss Stemi2000 dissecting scope using the Ximea CamTool Software (QT version 5.6.1). All images were cropped and adjusted using Adobe Photoshop CS6 (version 13.0). All figures were generated in Adobe Illustrator CS6 (version 13.0).

2.6. Statistical analyses

A one-way analysis of variance (ANOVA) was used to determine statistical differences in percent germline regeneration between different life stages. This was followed by Tukey's post- HOC analysis. A specimen was considered to have compensated for loss of germline if it developed germ cells as indicated by *CapI-vasa, CapI-nanos,* and *Ct-piwi1*-positive cells in the correct location in larvae, and *CapI-vasa*-positive cells in the correct location in juveniles. In adults, the animals were considered to have regenerated their germline if they developed sex characteristics of males and/or females, and if they successfully mated. If the groups analyzed had a p value < 0.05 when compared to other groups, they were considered to be statistically different.

3. Results

3.1. C. teleta life history and adult anatomy

During its life cycle, *C. teleta* undergoes indirect development. Following embryogenesis, larvae begin to swim at day five and actively swim in the water column by day six (Bhup and Marsden, 1982; Eisig, 1899; Reish, 1974; Werbrock et al., 2001). Metamorphosis from swimming larvae to burrowing juveniles occurs between nine and ten days post-fertilization, and adults develop reproductive structures around eight weeks post-metamorphosis (Fig. 1A). Adults reproduce sexually, and there are separate male, female and hermaphrodite sexes. Males, females and hermaphrodites can be differentiated from one another by structures that are easily visible in live specimens. The females have visible, paired ovaries (ov) on their ventral side in the anterior 10–12 abdominal segments (Fig. 1B). The males have genital spines (gs) on the dorsal side of the body in segments eight and nine as well as laterally positioned genital ducts (gd) between thoracic segments seven and eight (Fig. 1C) (Blake et al., 2009). Each male and female can reproduce multiple times. Hermaphrodites develop when females are sparse in the population and nutrient levels are high (Holbrook and Grassle, 1984). Hermaphrodites are genetically identical to males (Petraitis, 1985), and function as females. Eggs are laid in a tube, called the brood tube, which is composed of sediment and secretions from the female (Fig. 1D). The fertilized embryos develop

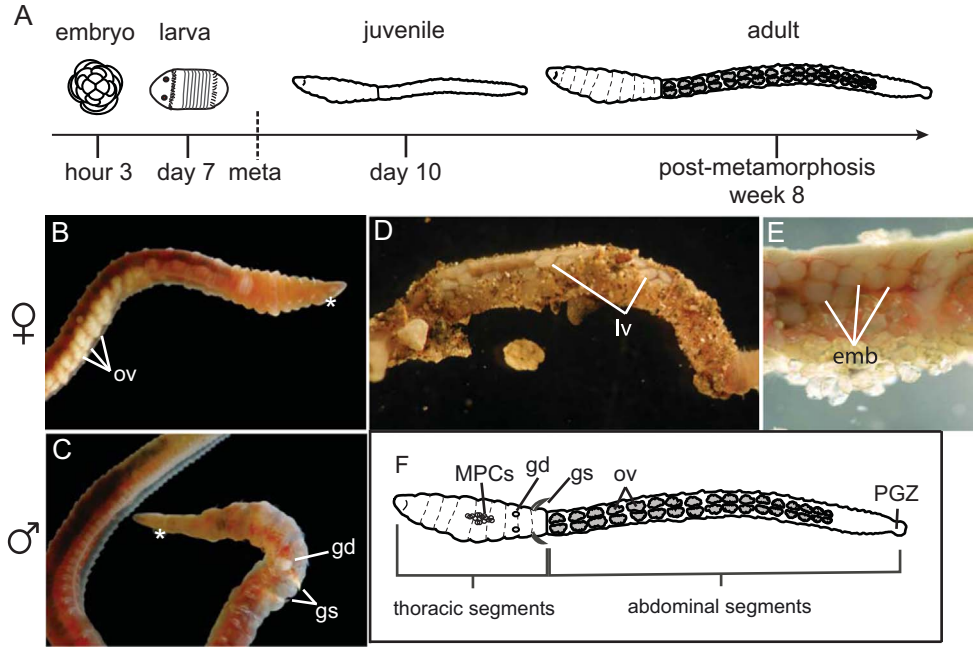

Fig. 1. Life cycle and reproductive anatomy of *Capitella teleta*. Timeline of life stages of *C. teleta* (adapted from Seaver and Kaneshige, 2006). B. Anterior end of adult female showing paired ovaries in abdominal segments. C. Adult male showing genital spines in segments eight and nine, and genital ducts positioned at the boundary of segments seven and eight. D. Brood tube containing larvae and adult female. E. Early stage embryos are visible within the brood tube in a high magnification view. F. Schematic of *C. teleta* adult showing position of reproductive structures. Asterisks in B and C indicate position of the mouth. Emb, embryo; gd, genital duct; gs, genital spines; lv, larvae; ov, ovaries; MPC, multipotent progenitor cells; pgz, posterior growth zone; meta, metamorphosis.

into larvae and remain in the brood tubes until the larvae are competent to undergo metamorphosis (Fig. 1E) (Méndez et al., 2000).

3.2. Fate map and the germline lineage of C. teleta

Early stage embryos of *C. teleta* cleave in a stereotypic fashion, and undergo spiral cleavage. Each cell can be identified based on a combination of spatial relationships, cell size, and time of birth. Similar to other spiralians, at the four-cell stage, the four cells in the embryo have a relationship to the future quadrants of the body, and are called A, B, C, and D (Fig. 2A). D is the largest cell and the B cell is on the opposite side of the embryo. The two cells contact each other at the vegetal pole, also known as the vegetal cross furrow. The micromeres are cells born from the macromere cells. Micromeres are indicated by a lowercase letter while the macromeres are given an uppercase letter. Each round of divisions is given a number, beginning with 1, and the micromeres and macromeres are identified with corresponding ascending numbers after each division. The macromere cleavages begin with a

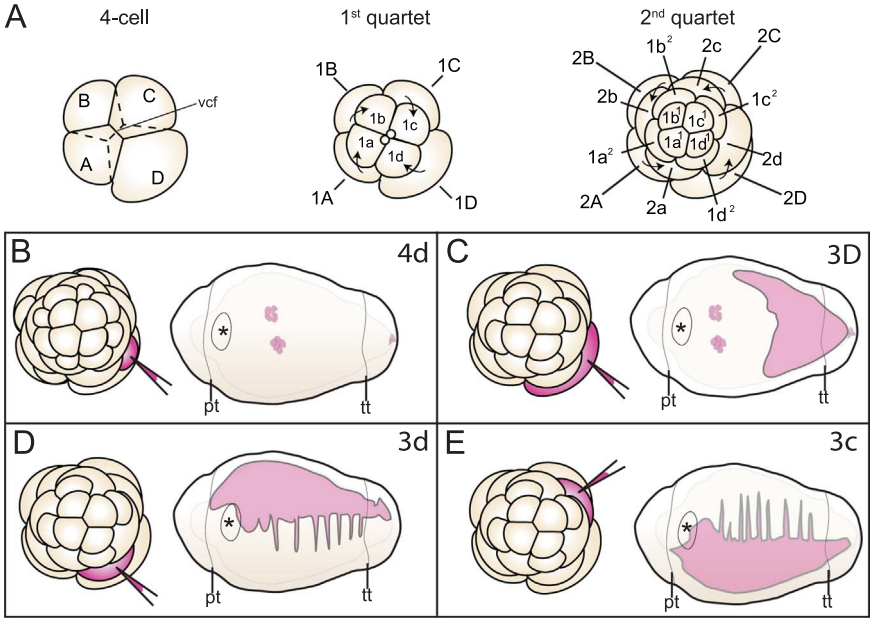

Fig. 2. *C. teleta* cleavage program and fate map of the germline and mesoderm. A. Schematic representation of spiral cleavage in *C. teleta*. B–E. Larval fates of cells. Pink shading in embryo schematics on the left shows the cell that was filled with lineage tracer, and the resulting larvae are shown in the right of each panel. Pink shading in the larvae shows the descendants of the injected precursor cell (original data from Meyer et al., 2010). B. Cell 4d makes the primordial germ cells and the anus. C. Cell 3D is the parent cell of cell 4d, and makes the primordial germ cells, anus, and the hindgut endoderm. D. Cell 3d generates the left mesodermal band. E. Cell 3c makes the right mesodermal band. The number and letter in the top right of each panel is the name of the filled cell. Asterisks indicate position of the mouth. pt, prototroch; tt, telotroch; vcf, vegetal cross furrow.

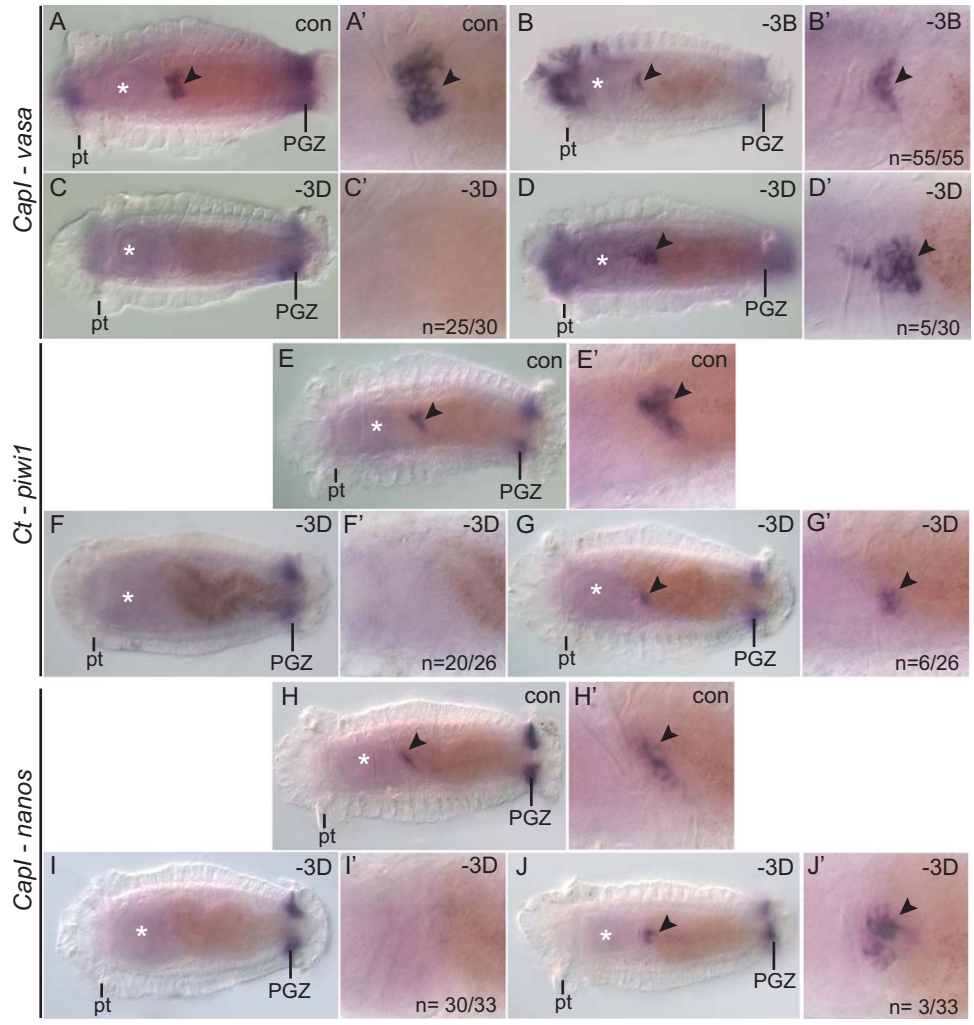

Fig. 3. Limited regeneration of the multipotent progenitor cells in larvae following deletion of the germline precursor cell, 3D. All images are ventral views, with anterior to the left. The top right indicates the identity of the deleted cell or undeleted control (con). Numbers in the bottom right of panels indicate the number of cases for the results shown over total number of cases. Larvae in panels A- D′ show *CapI-vasa* expression, larvae in panels E-G′ show *Ct-piwi1* expression, and larvae in panels H-J′ show *CapI-nanos* expression. Panels labeled with an apostrophe (for example A′ and B′) are a high magnification view of the region containing the MPC cluster from the corresponding larva to the left with the same letter (for example A and B). White asterisks indicate the location of the mouth and black arrowheads indicate the MPCs. pt, prototroch; PGZ, posterior growth zone.

clockwise division that results in the birth of the 1st quartet micromeres (1a, 1b, 1c, and 1d), and the macromeres alternate between clockwise and counter-clockwise orientations of the mitotic spindle at each division (Fig. 2A) (Eisig, 1899).

In addition to a conserved pattern of early cleavages, embryos that undergo spiralian development show conservation of fates among homologous cells. For fate mapping studies, an individual cell is labeled with an intracellular lineage tracer in the early stage embryo, and the descendants of that cell are followed to differentiated cell types, typically in larvae. A fate map of *C. teleta* has been published (Meyer et al., 2010). From this fate map, it is known that the descendant cells of cell 4d generate the multipotent progenitor cells (MPCs) as well as the anus (Fig. 2B). The embryonic origin of the germline from cell 4d is highly conserved among spiralians (reviewed in Rebscher, 2014). In *C. teleta*, the parent cell of 4d, 3D, generates the tissues from 4d as well as a portion of the midgut (Fig. 2C). The majority of mesodermal tissue in the larva is derived from the mesodermal bands, which arise from cells 3d (Fig. 2D) and 3c (Fig. 2E). Removal of blastomeres in the early stage embryo in spiralians typically results in predictable loss of structures in larvae, and this is generally true in *C. teleta* (Amiel et al., 2013).

As animals mature, there is a gradual increase in the number of cells in the MPC cluster over time in *C. teleta*. The MPCs first appear in mid-larval stages as two bilateral clusters, and by late larval stages are present as a single cluster along the ventral midline at the boundary between the foregut and midgut (Giani et al., 2011), and contain 8–20 germ cells. In one-week-old juveniles there are 20–55 germ cells in the MPC cluster, in two-week-old juveniles there are 30–55 germ cells, and in adults there are approximately 75 germ cells in the MPC cluster (Giani et al., 2011).

3.3. Limited regeneration of the germline in *C. teleta* larvae after blastomere deletion

To test for regeneration of the germline in *C. teleta* larvae, we deleted the precursor of the germline in cleavage stage embryos and analyzed larvae resulting from these embryos for presence of germline cells. Laser deletions have been demonstrated to be a very precise method for deleting single cells in *C. teleta* (Amiel et al., 2013; Pernet et al., 2012; Yamaguchi et al., 2016). In *C. teleta*, the germline precursor cell 4d is extremely small, and therefore, to improve accuracy and minimize damage to adjacent cells in the embryo, we deleted the larger, parent cell of 4d, cell 3D. Deletion of macromere 3D removes the germline as well as the anus and a portion of the midgut in larvae (Fig. 2C). In previous studies, larvae resulting from deletions of large macromeres survive and can later feed as juveniles, indicating that a functional gut forms (Pernet et al., 2012). Following 3D deletion,

embryos were raised to larval day nine, approximately nine days after fertilization. These larvae were analyzed for presence or absence of MPCs using previously characterized markers of the germline: *CapI-vasa, CapI-nanos* and *Ct-piwi1* (Dill and Seaver, 2008; Giani et al., 2011). In *C. teleta*, all three of these genes are expressed in the reproductive structures of adults (gametes and gonads), and in all stages examined, in the MPCs and the stem cells of the posterior growth zone. The posterior growth zone expression of these markers serves as an internal positive control for detection of endogenous transcripts.

At day nine of larval development, the expression of *CapI-vasa* in unmanipulated controls is restricted to the MPCs, posterior growth zone, and in some cases in the developing brain (Fig. 3A). At this stage, there is a single cluster of MPCs located at the boundary between the foregut and midgut along the ventral midline (Fig. 3A′). A control deletion of macromere 3B was also performed. This cell is of a similar size to cell 3D and contributes to the gut, but not to the germline. Deletion of this cell resulted in larvae with *CapI-vasa* expression similar to that of the unmanipulated controls, with added expression in the head ectoderm. This deletion (-3B) indicates that removal of a large part of the gut alone does not interfere with germ cell formation (Fig. 3B, B′). When the germline precursor, cell 3D, was deleted and *CapI-vasa* expression was examined in day nine larvae, the majority of cases had no germ cells (n = 25/30) (Fig. 3C, C′). However, in 16% of cases, MPCs were detected in the correct location at the ventral midline (n = 5/30) (Fig. 3D, D′). We did not observe any cases of MPCs in ectopic locations through analysis of *CapI-vasa* expression.

We also examined two other markers of the germline in larvae resulting from embryos in which 3D was deleted. *Ct-piwi1* mRNA expression in day nine larvae is similar to that of *CapI-vasa*. Expression of *Ct-piwi1* in unmanipulated controls is in the MPCs and the PGZ (Fig. 3E, E′). When cell -3D was deleted and the resulting embryos were raised to day nine larvae, the majority of cases lacked MPCs (n = 20/26)(Fig. 3F, F′). Similar to larvae resulting from -3D deletions and examined for *CapI-vasa* expression, a small number of the -3D deletions had an MPC cluster as detected by *Ct-piwi1* expression (n = 6/26)(Fig. 3G, G′). Two of these larvae had *Ct-piwi1*-labeled cell clusters with morphology similar to MPCs in ectopic locations in the mesodermal layer in the head and trunk (data not shown). *CapI-nanos* expression is also present in the PGZ and in the MPCs in day 9 control larvae (Fig. 3H, H′). When cell -3D was deleted and *CapI-nanos* expression analyzed, there were no detectable germ cells in the majority of the resulting day nine larvae (n = 30/33) (Fig. 3I, I′), although approximately 10% had detectable MPCs (n = 3/33) (Fig. 3J, J′). Therefore, the combined results of analyzing three distinct germline markers (*CapI-vasa, Ct-piwi1* and *CapI-nanos*) all show consistent expression patterns; MPC clusters were not detectable in most -3D larvae, but are present in a small proportion of cases (13%). This proportion is similar for the three different markers used. The number of germ cells in the MPC clusters appeared to be similar between the -3D larvae and the number of germ cells in MPC clusters in unmanipulated larvae. It is notable that in only two cases did we observe MPCs in an ectopic location (n = 2/89, both cases for *Ct-piwi1* expression). These results show that a small percentage of larvae can compensate for loss of the progenitor of the germline.

3.4. Germline regeneration in juveniles

We used the *CapI-vasa* probe to assess the presence or absence of the germ cell cluster in two-week-old juveniles rising from embryos in which cell 3D was deleted. Two weeks after metamorphosis, *CapI-vasa* is expressed in the MPCs of juveniles, which are typically located between thoracic segments four and five in the coelomic cavity along the ventral midline (Fig. 4A, A′). *CapI-vasa* is also expressed in immature oocytes in the developing ovaries (Fig. 4E), in the PGZ (not shown), and in cells in the coelomic cavity in segments posterior to segment six (Fig. 4A) (de Jong and Seaver, 2017). Juveniles were analyzed for presence or absence of a MPC cluster, the number of MPCs, total cluster number, and location of MPCs in each animal.

In unmanipulated control juveniles, the majority of animals have one *CapI-vasa*-positive cluster at the ventral midline between segments four and five (n = 37/42), and the cell number within the cluster ranges from 30 to 55 cells at this stage (Fig. 4A, A′). The remaining control specimens have two MPC clusters (n = 5/42), closely spaced, in an hourglass shape, with 30–55 countable cells total (data not shown). In juveniles raised from embryos in which cell -3D was deleted (germline removed), 13/32 animals had clusters similar to the controls, with 30–55 cells in one cluster located at the ventral midline (Fig. 4B, B′). In 11/32 cases, the juveniles resulting from the germline deletion had multiple MPC clusters located in the region of the ventral midline, between segments three and seven (Fig. 4C, C′). In 8/32 cases, the animals resulting from the 3D deletion had one small cluster, defined as containing fewer than 25 cells (Fig. 4D, D′). In addition, in several of the animals resulting from 3D deletion, *CapI-vasa* was also expressed in immature oocytes in the developing ovaries (Fig. 4F). Notably, following germline removal, all of the juveniles had *CapI-vasa*-positive clusters at the ventral midline near segment 5 (n = 32/32), demonstrating a dramatic ability to replace the germline in juveniles.

3.5. Regeneration of the germline in adults

Animals that had their germline removed (-3D) during embryogenesis were examined for their ability to reproduce as adults. Adult animals were scored for male and female-specific reproductive structures, and were also mated to determine if viable offspring were produced to demonstrate fertility. Approximately 41% of the -3D deletions resulted in worms with visible genital spines in segments eight and nine, and genital ducts between thoracic segments seven and eight, the same reproductive structures present in morphologically normal control males (n = 30/73) (Fig. 5A–C). Approximately 50% of the -3D deletions resulted in worms with ovaries in the anterior abdominal segments, typical of adult females (n = 37/73) (Fig. 5D and E). The remaining adults were comprised of one hermaphrodite and five immature adults with genital ducts only. Two of the immature animals did not develop reproductive structures by thirteen weeks, one died before thirteen weeks, and the other two were not followed further (data not shown). In order to demonstrate fertility, single males were mated with up to three females, and females assessed for presence of a brood tube with embryos or larvae. If any of the females produced offspring, the male was scored as fertile. The females were counted as fertile if they produced a brood tube containing larvae, or embryos that later developed into swimming larvae (Fig. 5F). The hermaphrodite was mated with one male. Broods produced by females resulting from 3D deletions produced average brood sizes relative to unmanipulated control broods. Additionally, embryos from these broods and from broods resulting from a mating with adult males resulting from 3D deletions developed into actively swimming larvae that appeared morphologically normal. These results demonstrate that both male and females resulting from 3D deletions produced functional gametes. All of the animals with complete reproductive structures (males, females, and hermaphrodite) were fertile (n = 68/68). These data show that the germline of *C. teleta* can fully regenerate after removal of the germline precursor in the early embryo.

3.6. Regeneration of the germline varies during different life history stages

Through detection of germline markers in larvae and juveniles and by fertility tests in adults, we demonstrated that the fraction of individuals with a regenerated germline vary at different stages of the life cycle. We therefore determined whether there is a statistically significant difference in percent germline regeneration at different life

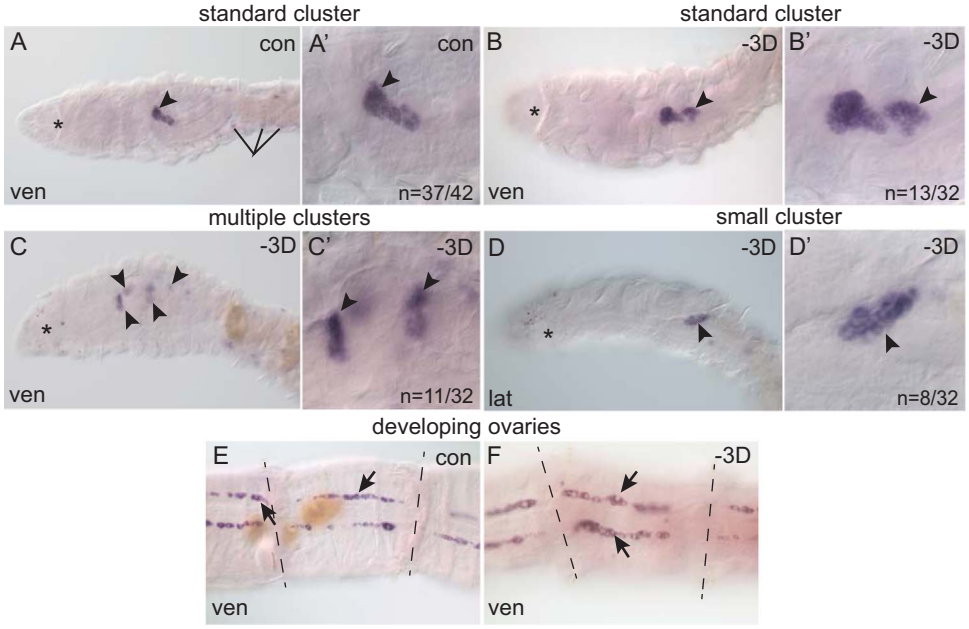

Fig. 4. Regeneration of the multipotent progenitor cell cluster in two week old juveniles following removal of the germline precursor, 3D. All images are two week post-metamorphosis juveniles with anterior to the left. Text at the top right of panels indicates either controls (con), or identity of the deleted cell. Numbers in the bottom right of panels indicate the number of cases for the results shown over the total number of cases. Black asterisks indicate the location of the mouth. Panels labeled with an apostrophe (for example A′ and B′) are a high magnification view of the region containing the MPC cluster from the same larva to the left with the same letter (for example A and B). A. Thoracic segments in an unmanipulated control show expression of *CapI-vasa* in the MPC cluster. B. Juvenile developed from -3D embryo that has a MPC cluster similar to that of the control. C. Juvenile developed from -3D embryo with multiple MPC clusters. D. Juvenile developed from -3D embryo with a small MPC cluster. E. Abdominal segments of a female or hermaphrodite control juvenile showing *CapI- vasa* expression in immature oocytes in paired ovaries (arrows). F′ Abdominal segments of a female or hermaphrodite juvenile that developed from an embryo following -3D deletion. The dotted lines mark the boundary between segments. Black arrowheads indicate the MPCs, and black arrows show immature oocytes that express *CapI- vasa*. Lines in A indicate cells expressing *CapI-vasa* in the coelomic cavity. Con, unmanipulated brood control; ven, ventral view; lat, lateral view with ventral down.

history stages. The percentage of animals with a regenerated germline is statistically different between larval and juvenile stages ($p < 0.01$), and larval and adult stages ($p < 0.01$) (Fig. 6). In contrast, there is no statistical difference between the proportion of animals that showed regeneration of the germline between juveniles and adults ($p = 0.44$). The striking difference in the presence of *CapI-vasa*-positive clusters between larvae and two week-old juveniles indicates that there may be multiple germline regeneration events: a compensation event in the early embryo that results in a small fraction of the larvae having MPCs, and a second more substantial regeneration event that occurs between larval and juvenile stages and results in the majority of juveniles and adults with germline cells.

3.7. Novel trunk expression of CapI-vasa and CapI-nanos following deletion of cell 2D

Our observation that a small proportion of larvae resulting from -3D deletion have MPCs suggests that another cell in the embryo, which would usually generate only somatic cells, can generate germline cells. We hypothesized that the cellular origin of the regenerated

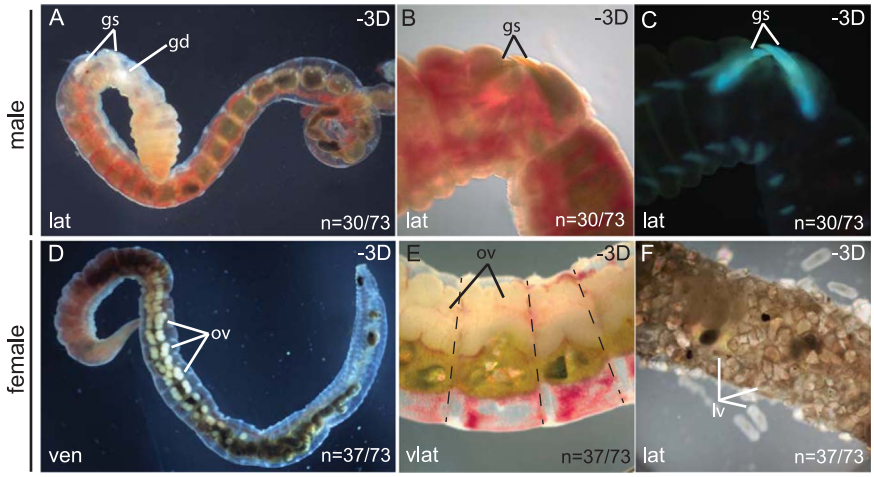

Fig. 5. Assessment of reproductive structures and fertility in adults following deletion of the germline precursor (cell 3D). All images are adults between eight and 14 weeks post-metamorphosis, and result from embryonic deletion of the germline precursor (-3D). Number of cases for each result over the total number of cases is shown in bottom right corner of each panel. Panels A–C are images of males. Panels D–F are images of females. A. Reproductive male shown with the genital ducts between segments seven and eight and the genital spines in segments eight and nine as indicated. B, C. Close up views of the genital spines. B and C are from the same individual. B. DIC image of the genital spines at high magnification. C. Genital spines are auto fluorescent. D. Reproductive female with pairs of ovaries in the abdominal segments. E. High magnification of ovaries. Dotted lines mark the boundaries between adjacent segments. F. Brood tube containing larvae and an adult female. Some larvae have emerged from the brood tube. gd, genital ducts; gs, genital spines; lat, lateral; lv; larvae; ov, ovary; ven, ventral; vlat, ventral lateral.

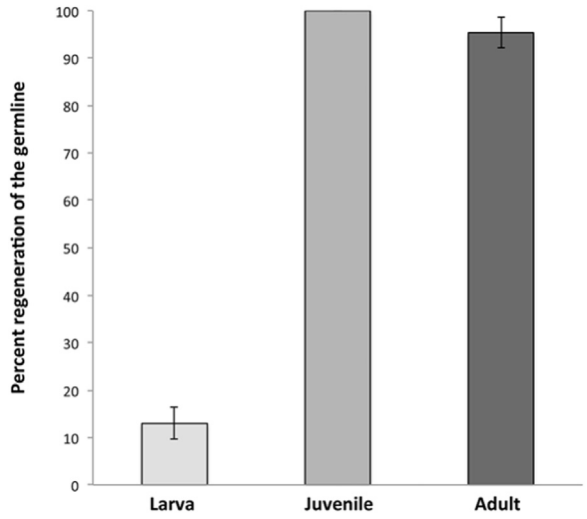

Fig. 6. Germline regeneration in *C. teleta*. Columns show the average percent regeneration for each life stage (larvae, juvenile or adult). Error bars represent the standard error of the mean.

germline is a mesodermal precursor since in most bilaterians, the embryonic origin of the germline is from the mesoderm (Extavour, 2007). In *C. teleta*, cells 3d and 3c form the majority of the mesoderm (Meyer et al., 2010) (Fig. 2D and E). Cell 2D gives rise to the left mesodermal band, the anus, as well as the germline (Meyer et al., 2010). Therefore, half of the trunk mesoderm (3d) and the germline (4d) can be deleted at once by deleting their shared parent cell, 2D. If 3d generates the regenerated mesoderm, we would expect to loose the ability to regenerate the germ line following 2D deletions, and would instead observe larvae, juveniles or adults that lack germline or gametes. After deletion of cell 2D, embryos were raised to larval day nine and expression of both *CapI-vasa* and *CapI-nanos* were analyzed.

At this stage in unmanipulated controls, *CapI-vasa* is expressed in the MPCs and in the PGZ (Fig. 7A). At day nine, the ganglia of the ventral nerve cord are visible with a nuclear stain (Fig. 7A′, bracket). When cell 2D is deleted in embryos, the larval expression of *CapI-vasa* changes dramatically relative to expression in unmanipulated controls. Expression of *CapI-vasa* in the MPCs is not present in any cases, and expression in the PGZ is faint. Surprisingly, the predominant expression of *CapI-vasa* is in a band of cells in the mesoderm on the left side that typically extends throughout the length of the trunk (n = 23/25) (Fig. 7B and C). This band of *CapI-vasa*-positive cells is on the same side of the body that normally generates mesoderm from the deleted cell. The medial edge of this band of cells abuts the lateral edge of the ventral nerve cord (Fig. 7B′, C′). Expression in these cells is perinuclear, and the cells are round with a large nuclear to cytoplasmic ratio, and appear to be undifferentiated. In the remaining two cases, there is mesodermal *CapI-vasa* expression on the left side of the trunk as in the majority of cases, but the cells are arranged in two distinct domains with a gap in the mid-trunk, and more cells expressing *CapI-vasa* in the anterior trunk mesoderm (data not shown). To determine if this change in *CapI-vasa* expression is a response to the loss of a mesodermal band, we deleted the parent cell of the right mesodermal band, cell 2C. This deletion resulted in larvae with expression similar to brood controls, with *CapI-vasa* in the MPCs, and weak expression in the PGZ (n = 69/71)(Fig. 7D, D′). The remaining two larvae resulting from 2C deletions have MPCs that are centered at the ventral midline as in the controls. However, they also have clusters of *CapI-vasa* positive cells dispersed throughout the anterior end of the larvae in the ectoderm (data not shown). This experiment indicates that the loss of a mesodermal band alone does not cause the dramatic change in *CapI-vasa* expression; instead, it is the deletion of the precursors of the germline plus one mesodermal band that leads to a novel, broad band of *CapI-vasa* expression in the trunk mesoderm.

To further investigate this unexpected result, we deleted cell 2D and analyzed the resulting larvae for expression of *CapI-nanos*. In unmanipulated day nine larvae, *CapI-nanos* is expressed in the MPCs and PGZ (Fig. 7G, G′). *CapI-nanos* expression in larvae resulting from deletion of 2D also changes dramatically, and similar to the *CapI-vasa* pattern, *CapI-nanos* is expressed in the left mesoderm in the trunk and is positioned adjacent and lateral to the ventral nerve cord, but lacks obvious MPC clusters (Fig. 7H′, I′, J′). However, unlike the expression of *CapI-vasa*, *CapI-nanos* mesodermal expression only extends midway along the length of the trunk, in the posterior trunk (n = 28/32) (Fig. 7H and I). In addition, *CapI-nanos*-positive cells can be seen distributed in the ventral ectoderm of several anterior segments. These cells are arranged in a segmentally repeated arrangement at the boundaries between segments in the ectoderm (Fig. 7H). Of the remaining three -2D larvae that do not show this pattern, two have *CapI-nanos* expression in a band of mesoderm that extends throughout the trunk, similar to the observed *CapI-vasa* expression pattern following the equivalent deletion. One larva shows expression only in the PGZ (data not shown). The differences in expression between *CapI-nanos* and *CapI-vasa* following -2D deletion suggests that *CapI-nanos*-expressing cells represent either a subset of the *CapI-vasa* expressing population, or that there are distinct subpopulations of cells that express either *CapI-vasa* or *CapI-nanos*. In summary, MPC clusters were not visible in -2D larvae by analysis of *CapI-nanos* and *CapI-vasa* expression; instead, we observed expression in a broad band of cells in the mesoderm.

3.8. CapI-vasa expression in juveniles following 2D deletion

We investigated whether the dramatic change in expression of *CapI-vasa* and *CapI-nanos* in larvae following deletion of -2D persisted into one week and two week post-metamorphic juvenile stages. We also investigated whether juveniles could regenerate their MPCs following -2D deletion, like those resulting from 3D deletion, or if they were unable to regenerate the germline due to loss of the mesodermal cell 3d.

At one week post-metamorphosis, juveniles typically contain an MPC cluster of between 20 and 55 countable cells (n = 18/24) (Fig. 8A, A′). The remaining controls contain two closely spaced clusters with 20–55 countable cells (n = 6/24). Approximately half of the juveniles resulting from 2D deletions had no MPCs present (n = 8/17) (Fig. 8B, B′). A proportion of cases had a small cluster of MPCs, defined as having ten or fewer countable cells (n = 5/17) (Fig. 8C, C'). These cells were in one cluster with the typical MPC morphology, and were located in the correct position, between segments four and six (Fig. 8C). Only 3/17 juveniles following deletion of 2D had a cluster similar in size to that of the controls (Fig. 8D, D′). The remaining juvenile had five small clusters distributed across thoracic segments three through seven (n = 1/17; data not shown). None of these juveniles had an expanded *CapI-vasa*-positive expression domain in the mesoderm comparable to that observed in the -2D larvae (Fig. 7).

At two weeks post-metamorphosis, juveniles typically possess one cluster of MPCs, containing between 30 and 55 countable cells (n = 51/58; Fig. 8E, E′). A small percentage of controls have the same number of MPCs but are organized into two clusters, closely spaced (n = 7/58; data not shown). A small cluster contains fewer than 25 countable MPCs, and multiple clusters have two or more clusters of MPCs. Unlike in the one week post-metamorphosis juveniles resulting from deletion of 2D, the majority of two-week-old juveniles following 2D deletion had multiple MPC clusters distributed across a number of thoracic segments (n = 14/22) (Fig. 8F, F′). Others had a small cluster of MPCs (n = 6/22) (Fig. 8G, G′). One two week juvenile had a single standard cluster similar to controls, while another juvenile had no detectable MPCs (data not shown). These combined results indicate that there is regeneration of the germline after metamorphosis, with the majority of

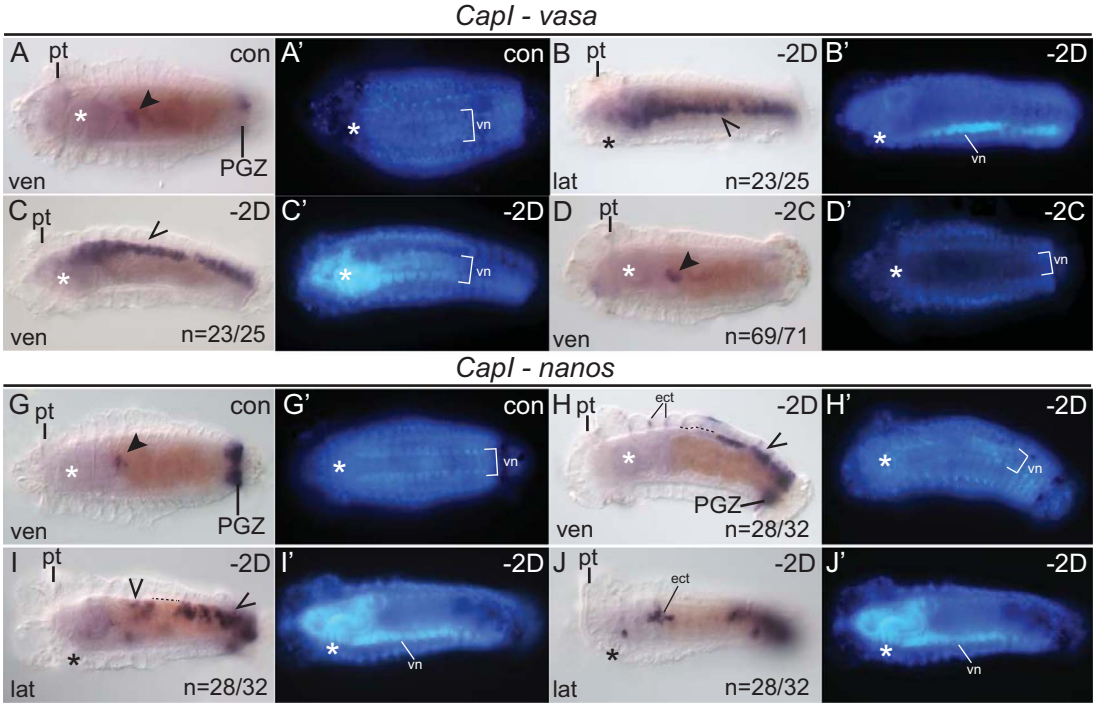

Fig. 7. Expansion of *CapI-vasa* and *CapI-nanos* trunk expression in larvae following deletion of the mesoderm and germ cell precursor, cell 2D. All images are of stage nine larvae, approximately nine days post-fertilization, with anterior to the left. Text in the top right of panels indicates the identity of the deleted cell or unmanipulated control (con). Numbers in the bottom right of panels indicates the number of cases for each results shown over the total number of cases for that deletion. Asterisks indicate the location of the mouth, closed black arrowheads indicate the MPCs, open black arrowheads indicate expression in mesodermal cells and square brackets mark the lateral edges of the ganglia in the ventral nerve cord. Panels labeled with an apostrophe (for example A′ and B′) are Hoechst nuclear labeling of the corresponding larva to the left with the same letter (for example A and B). A–D'. *CapI-vasa* expression. A, A'. Brood control. B- C'. Larvae following -2D deletion showing a band of *CapI-vasa*-expressing cells in the mesoderm to the left of the ventral midline. D, D'. Larva following -2C deletion with expression similar to the brood controls. G–J'. *CapI-nanos* expression. G, G'. Brood control. Expression is similar to that of *CapI-vasa,* but also present in the posterior growth zone at this stage. H–J'. Larvae resulting from -2D deletion. Mesodermal expression of *CapI-nanos* is to the left of the ventral midline. Expression in ectodermal cells is in a segmental pattern in anterior segments in H. The border between the mesoderm and ectoderm is labeled with a black dotted line. ect, ectodermal expression; lat, lateral; pgz, posterior growth zone; pt, prototroch; ven, ventral; vn, ventral nerve cord.

regeneration events occurring between one and two weeks post-metamorphosis. These data also show that the expansion of *CapI-vasa* expression following 2D deletion disappears following metamorphosis. Furthermore, the multiple clusters spread throughout the thoracic segments in 2-week juveniles are likely unrelated to the broad trunk expression pattern seen in larvae following 2D deletion, since expression in one week juveniles is very restricted.

3.9. Adults resulting from 2D deletion are fertile

Following 2D deletions, only 6% of animals survived from metamorphosis to adulthood (n = 12/204) compared to survival in 65% of cases in which -3D was deleted (n = 73/112), and 83% survival of unmanipulated brood controls (n = 50/60 scored for survival) (Fig. 9A). This steep decrease in survival is likely due to substantial loss of gut and mesodermal tissue, which could interfere with feeding and locomotion. Of the animals that did survive, five were male and contained genital ducts and dorsal genital spines (Fig. 9B). Five other individuals were female and contained visible ovaries (not shown). The two remaining individuals were immature with genital ducts only, and when allowed to develop longer, these immature animals later died (Fig. 9D). The males and females were mated, and all were fertile (Fig. 9 C) (n = 10/10). Therefore, removal of the precursor of the left mesodermal band and the germline does not prevent germline regeneration, meaning that the mechanism is not dependent on the presence of descendants of cell 2D in the early embryo.

4. Discussion

4.1. Regeneration of the germline

Our data provide experimental evidence for regeneration of the germline in *C. teleta*, and provide an exception to the concept of a single embryonic origin for the germline. By using three different markers, *CapI-vasa*, *CapI-nanos*, and *Ct-piwi1*, we have confidence that the loss and gain of MPCs was likely a cellular response, and not just a change in expression of a single gene. The percentage of compensation was consistent across all three genes. Our study is unique in that the germline precursor was removed in the early stage embryo, whereas in most experimental studies, the germline is removed at later developmental stages or in larval or adult stages. Following deletion of the germline precursor in the early stage embryo, we show that by 2 weeks post-metamorphosis, all animals had developed MPCs. Furthermore, most adults resulting from germline precursor removal developed reproductive structures and produced offspring. The remaining animals appeared to be immature adults, with structures resembling genital ducts by 13 weeks post-metamorphosis (n = 5/73), at which time animals are usually fully reproductive. We believe this is likely a result of tissue loss in the gut from the -3D deletion, which could hinder nutrient uptake, and thus growth when compared to controls. In contrast to our data, studies in many species support the dogma that the germline is not capable of regeneration following removal, leading to the hypothesis of a single embryonic origin of the germline. The formation of this hypothesis has likely been biased by the animals sampled, which only represent a fraction of animal diversity. Within the large and diverse clade of lophotrochozo-

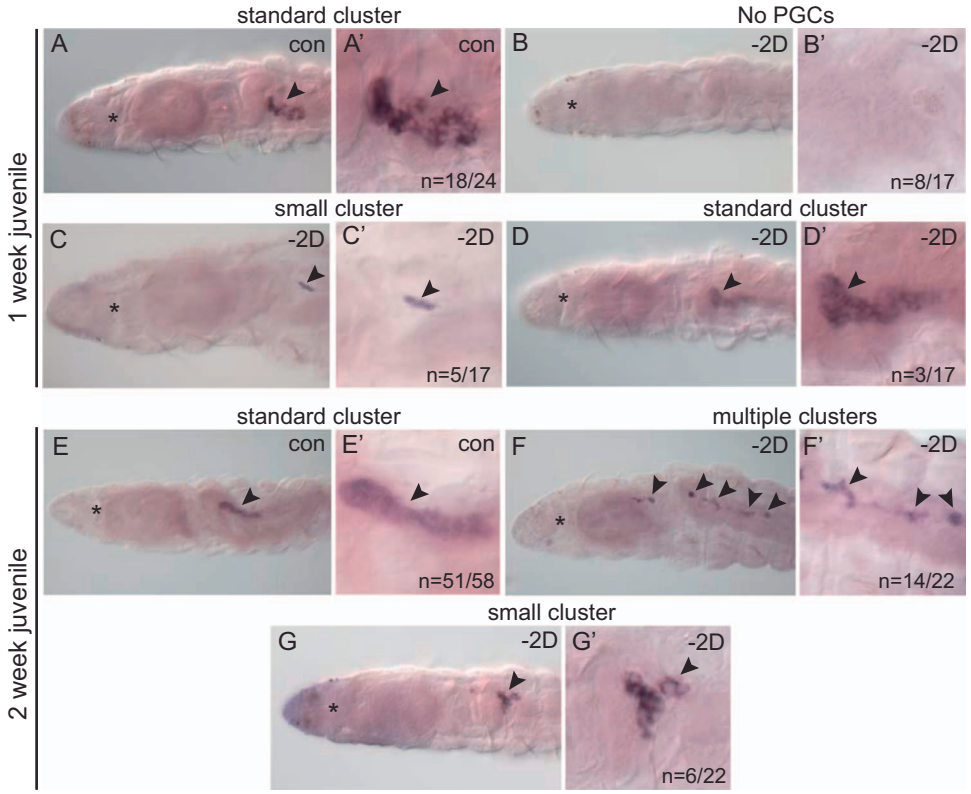

Fig. 8. Germ cell regeneration after -2D deletion occurs post-metamorphosis. All images are ventral views with anterior to the left showing expression of *CapI-vasa*. Text at the top right of panels indicates identity of deleted cell or unmanipulated control (con). Numbers in the bottom right of panels indicate the number of cases for results shown over the total number of cases. Asterisks indicate the location of the mouth, black arrowheads indicate the MPCs. Panels labeled with an apostrophe (for example A′ and B′) are a high magnification view of the region containing the MPC cluster from the corresponding larvae to the left with the same letter (for example A and B). The top two rows are juveniles one week post-metamorphosis and the bottom two rows are juveniles two weeks post-metamorphosis. A, A', E, E'. Unmanipulated control with one MPC cluster. B, B'. Juvenile lacking MPCs following -2D deletion. C, C'. Juvenile with a small cluster of MPCs. D, D'. Juvenile following -2D deletion with a standard sized cluster. F, F'. Two week old juvenile following -2D deletion that has multiple MPC clusters extending between thoracic segments 3 and 7. G, G'. Juvenile with a small MPC cluster following -2D deletion.

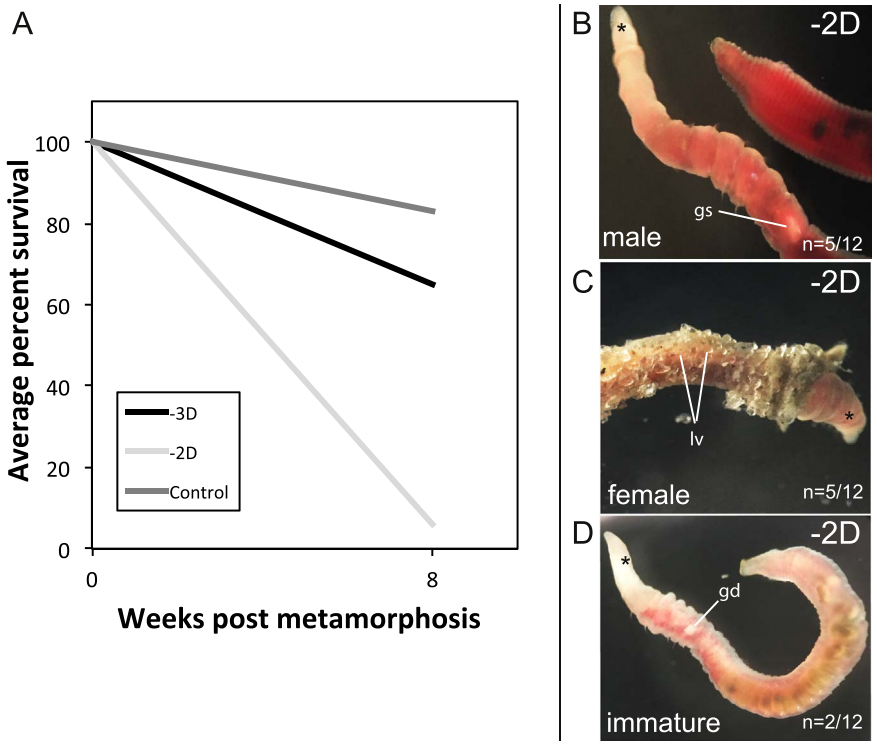

Fig. 9. Deletion of the mesodermal and germline precursor (cell 2D) results in fertile adults. A. Graph of the percent survival at 8 weeks for individuals raised to adulthood following either deletion of -2D (light gray line) or -3D (black line) in early embryos. Dark gray line indicates unmanipulated controls. B–D show the three categories of adults resulting from 2D deletion: B. Male. C. Female D. immature animal. Black asterisks indicate the approximate location of the mouth. gd, genital ducts; gs, genital spines; lv, larva.

ans, documentation of germline regeneration is limited to several species of flatworms (Sato et al., 2006; Pfister et al., 2008; Wang et al., 2007). Therefore, to our knowledge, these results add evidence germline regeneration from a second clade of lophotrochozoans, and suggest that more examples of germline regeneration in the Lophotrochozoa might be uncovered with additional sampling.

In other clades such as in amphibians, thorough experimental manipulations of the germline have been conducted. In axolotls, the lateral plate mesoderm, the source of the primordial germ cells (PGC), was surgically removed prior to PGC migration. The larvae that subsequently developed after lateral plate mesoderm removal had genital ridges and somatic reproductive tissues, but lacked germ cells (Nieuwkoop, 1951). Similarly in *X. laevis*, the region of the endoderm that forms the germ cells was removed and replaced with an anterior region of the endoderm. The resulting tadpoles developed normal gonadal anlagen, but the gonads lacked germ cells and the adults were infertile (Blackler, 1965). The ability of the germline to regenerate was also investigated by removing the vegetal pole germ plasm in early stage amphibian embryos, via both UV irradiation (Nieuwkoop and Suminski, 1959) and by pricking (Züst and Dixon, 1975); both manipulations resulted in sterile animals.

Experimental manipulations in birds and mammals also support the idea that the germline has a single origin. In birds, the founder germ cell population, the anterior germinal crescent at somite stages, was either surgically removed (Dulbecco, 1946), removed by irradiation (Dubois, 1962), or cauterized (Fargeix, 1975). All three manipulations resulted in infertile adults. A series of experiments was also performed in the chick embryo, where the germinal crescent was irradiated, and then suspensions of PGCs from a turkey were intravenously injected. All germ cells that ended up in the host chicken gonad anlagen were from the donor turkey's germ cells (Reynaud, 1976, 1970; Reynaud et al., 1969). There are fewer experimental examples from mammals, and the most convincing evidence for the single germline hypothesis is from an experiment conducted in *M. musculus*. The gonadal anlagen in embryos was irradiated and the resulting adult mice had differentiated reproductive structures, but lacked germ cells (Everett, 1943).

Results from several invertebrates also support the concept of a single embryonic origin of the germline. In *C. elegans*, the ablation of cell P4, which forms the germ cell primordia, results in an infertile adult that lacks germ cells, but has structured gonads (Sulston and Schierenberg, 1983). In *D. melanogaster*, evidence is based on a fly line with mutant *germ cell- less* (*gcl*) and *tudor* genes, both of which are necessary for germ cell specification. The mutant adult flies develop reproductive structures, but no germ cells (Barnes et al., 2006). However, the mutations in these genes crucial for germline development would have likely prevented the regeneration of the germline by another cell lineage, which might also require these genes. In sea urchin embryos, deletion of the germline precursors, the small vegetal micromeres, at the 4th embryonic cleavage, resulted in fertile animals (Ransick et al., 1996). However, when the same cells were deleted one division later, the animals produced reproductive structures, but lacked gametes (Yajima and Wessel, 2011). This suggests that the germline is not specified until after the fourth division and it cannot regenerate following germline removal.

A few studies, in addition to ours, are beginning to complicate the idea of a single embryonic origin of the germline. In the ascidian *C. intestinalis*, the larval tail, which contains the germ cells, was removed, and the resulting juveniles had no germ cells (Takamura et al., 2002). However, after approximately 15 days, using an antibody against the *C. intestinalis vasa* homologue, a few germ cells were detected, and the adults that developed produced sperm. Interestingly, recent molecular evidence suggests that these regenerated PGCs come from multiple somatic origins (Yoshida et al., 2017). In the colonial ascidian *Botryllus primigenus*, the germline is segregated from the soma in the early embryo. When all of the buds and zooids are removed, an entire animal can bud from the tunic vessels via a process called vascularization, which eventually leads to regeneration of a whole colony (Milkman, 1967; Oka and Watanabe, 1959, 1957; Sabbadin et al., 1975). Immediately after vascularization, the colony has no *vasa*-positive germ cells, but after 2 weeks of colonial regeneration, *vasa*-positive cells re-appear *de novo* (Sunanaga et al., 2006). Flatworms also provide evidence of multiple origins for the germline. In the flatworm *Macrostomum lignano*, the germline is segregated early, but during regeneration, the gonads and germ cells are formed from the somatic stem cells, demonstrating that *M. lignano* also has two mechanisms for germline formation (Pfister et al., 2008). It has long been known that the germline can regenerate from somatic tissue in flatworms (Morgan, 1902). In the planarian *Schmidtea mediterranea*, the germline can regenerate from a piece of adult tissue that lacks reproductive structures, and *nanos* is required for such germline regeneration (Wang et al., 2007). In another planarian, *Dugesia japonica*, somatic cells begin expressing germline markers *de novo* following amputation (Sato et al., 2006). There is very limited data for annelids, but *Pristina leidyi* provides some evidence for the complexity of germline origin. In this asexually reproducing animal, *vasa*-positive cells appear after the animals are induced to become sexual, and arise post-embryonically from a stem cell population (Özpolat and Bely, 2015). In summary, these studies provide a number of examples that support the idea of both an embryonic origin and a somatic stem cell origin for the germline, which allows for regeneration of the germline in adults.

Animals that use preformation (early cytoplasmic segregation) to segregate their germline have been proposed to lack the ability to regenerate germ cells when the germline precursors or primordial germ cells are removed (Saffman and Lasko, 1999). However, data in *C. intestinalis* seems to contradict this idea (Extavour, 2007). *C. intestinalis* may use both preformation and epigenetic (specification by cell-signaling in the embryo) mechanisms to specify its germline, or even specify its germline post-embryonically, and may be capable of regenerating the germline following removal (Extavour, 2007; Juliano et al., 2010). Similarly, *C. teleta* may also regenerate its germline via multiple mechanisms of specification as well as at different stages of its life cycle. Based on the observation that metazoan outgroups of bilaterians do not have a clear distinction between germline and somatic cells, it has been suggested that all stem cells in the last common ancestor of bilaterians had germline potential (Buss, 1987; Extavour, 2007; Michod, 1996; Michod et al., 2003; West-Eberhard, 2005). However, evidence from cnidarians suggests that there is a population of cells protected from mutation that more frequently gives rise to the gametes, suggesting that some form of germline segregation is a eumetazoan trait (Barfield et al., 2016; Littlefield and Bode, 1986). Whatever the case, it is likely that many bilaterian species have somatic populations that can contribute to the germline under certain circumstances, and this might be more prevalent than previously thought.

4.2. Timing of regeneration of the germline in C. teleta

C. teleta undergoes indirect development, meaning it has embryonic, larval, juvenile, and adult stages. We analyzed germline regeneration across life history stages. When the percentage of germline regeneration is compared among larvae, juveniles, and adults, regeneration ability is statistically different between larvae and adults and larvae and juveniles. Only a small proportion of animals (approximately 13%) have regenerated their MPCs by day 9 of larval development. At two weeks post-metamorphosis, all juveniles have regenerated their MPCs. We know that germline regeneration occurs post-metamorphosis, because in one week juveniles that result from deletion of cell 2D, some animals have not yet regenerated their MPCs, whereas at 2 weeks post-metamorphosis, almost all animals have regenerated their MPCs. Therefore, regeneration of MPCs occurs after tissues have been specified, and although our study does not directly address the

mechanism involved, it is likely a stem cell to germ cell transition.

It is interesting to note that the timing of the second germline regeneration event coincides with the approximate time period that the animal becomes capable of posterior regeneration (Giani et al., 2011; de Jong and Seaver, 2016). Future studies are needed to determine whether or not the ability to regenerate somatic tissues is a requirement for germline regeneration. It has been proposed that stem cells with dual germline and somatic potential are linked to the highly regenerative capabilities of some metazoans (Rebscher, 2014). Thus far, animals that can regenerate their germline (*C. intestinalis*, *M. lignano*, *D. japonica*) also have substantial somatic regenerative ability. In the future, it would be intriguing to investigate mollusks for their ability to regenerate a lost germline since they do not have substantial somatic regenerative abilities, but are closely related to annelids.

4.3. Evidence for regulation in spiralian embryos

Spiralians, particularly annelids and mollusks, have long been associated with mosaic development, meaning that each cell in the embryo is fated to form a specific structure, and if the cell is lost, the structure will also be lost. In the annelids *Lanice, Chaetopterus, Sabellaria*, *Tubifex* and *Nereis*, when blastomeres are separated during early cleavage stages and allowed to develop in isolation, each cell continues to divide as if it had not been isolated from the embryo, and each cell only forms structures consistent with its original fate (Costello, 1945; Hatt, 1932; Penners, 1926; Tyler, 1930; Wilson, 1904). In addition, when individual blastomeres are removed from the embryo in the mollusk *Ilyanassa*, the resulting larvae largely have expected loss of structures (Clement, 1967, 1962). In a previous study in *C. teleta*, 13 different blastomeres were deleted in early stage embryos, with each resulting in loss of the expected structures in larvae (Amiel et al., 2013).

However, other studies demonstrate that spiralian embryos have some capacity to replace missing structures, and are more regulative than once assumed. For example, when the eye precursors, blastomeres A and C are deleted in *I. obsoleta*, 10% of resulting larvae have eyes (McCain and Cather, 1989). Similarly, in both the gastropod *Lymnaea stagnalis* and the flatworm *Hoploplana inquiline,* when the eye precursors 1a and 1c are deleted at the 8-cell stage, both species compensate for this loss and form eyes (Arnolds et al., 1983; Boyer, 1987). In addition, in a similar experiment in *C. teleta*, regulation was seen when the blastomeres that generate the larval eyes (1a and 1c) were deleted at the 8 cell stage (Yamaguchi et al., 2016). Our experiment adds another example of the regulative ability of *C. teleta* embryos.

4.4. Embryonic origin of regenerated germline

We hypothesize that the cellular origin of the regenerated germline is from a mesodermal precursor, either 3c or 3d. Because cell 2D is the precursor of both the germline lineage (cell 4d) and the left mesodermal band (cell 3d), (Meyer et al., 2010), we deleted cell 2D in an attempt to determine if the cellular origin of the observed regenerated germline was from a descendant of one of the two mesodermal bands. Following deletion of 2D, no MPCs were visible in larvae using *CapI-vasa* and *CapI-nanos* as germline markers. This result supports the idea that the regenerating germline in larvae originates from the 3d lineage. In contrast, juveniles raised from embryos in which the macromere 2D was deleted have MPCs in 9/17 of cases and 21/22 of cases in one- and two-week old juveniles, respectively. These results indicate that the regenerating germline in juveniles originates from a source other than the 3d micromere, and that the regeneration event occurs after metamorphosis. The most likely source of cells is from the left mesodermal band precursor, 3c. If deleting both the 3c mesodermal band cell precursor and the germline precursor (3D) show results similar to the results from the 2D deletions, this would indicate that either of the mesodermal band precursors (3c or 3d) can serve as cellular sources of germline precursors. From the results of our deletion experiments, we cannot unambiguously determine the embryonic origin of the regenerating germline in larvae or adults. A more direct demonstration and important future direction would utilize long-term lineage tracers (currently in development) in combination with blastomere deletions.

4.5. Repression of a stem cell response by the germline

Following deletion of 2D (germline and one mesodermal band), no MPCs were visible using *CapI-vasa* and *CapI-nanos* as markers. However, we were surprised to observe novel expression domains of *CapI-vasa* and *CapI-nanos* in the ectoderm and mesoderm. On the side of the body in which the 2D precursor lineage was removed, there was a large band of cells in the mesodermal tissue extending through most of the trunk. The cells in this band express *CapI-vasa* and *CapI-nanos*, are large, round, and have a large nuclear to cytoplasmic ratio, consistent with their identity as stem cells. This is a novel expression pattern, and was never observed in unmanipulated animals or in any deletions other than those of 2D. Furthermore, the shape of the domain does not resemble the mesodermal band at any developmental stage, as previously described through fate mapping experiments (see Fig. 2D and E) (Meyer et al., 2010). The location and character of these *CapI-vasa* and *CapI-nanos*-expressing cells suggests that they might form part of a cellular, regenerative response to the lost tissue, and only arise when the combination of the germline and one mesodermal band are removed. In contrast, when the precursor to the right mesodermal band is deleted (cell 2C), the resulting *CapI-vasa* expression is identical to expression in control larvae, and is expressed in the MPCs and faintly in the PGZ. From these results and results of previous embryonic deletions (Amiel et al., 2013), we propose that the germ cell lineage normally has an inhibitory role. When both the mesoderm and germ cell precursors are removed, this inhibition is released, and novel mesoderm cells appear, which compensate for both somatic mesodermal precursors as well as germ line precursors as indicated by the broad *CapI-vasa* and *CapI-nanos* expression.

4.6. Working model of germline regeneration in C. teleta and final remarks

Our findings led us to propose the following model of germline regeneration in *C. teleta*. When the precursor of the germline is removed in the early embryo, another blastomere is induced to generate the germline. This process begins in larval stages, and by early juvenile stages, it is clear that somatic lineages give rise to germline precursors by one and two weeks after metamorphosis. The origin of the cells that become the new MPCs is currently unknown, but a parsimonious view would lead us to hypothesize that these germ cells arise from multiprogenitor stem cells arising from the 3c mesodermal precursor.

Almost all adults resulting from embryonic germline deletion are fertile, demonstrating a robust ability of *C. teleta* to replace its germline from somatic stem cell precursors. *C. teleta* juveniles and adults can regenerate their full posterior ends if lost, including reproductive structures. In addition, *C. teleta* can replace its eyes when the precursor blastomeres of the eyes are removed (Yamaguchi et al., 2016). This ability to regenerate the germline is another example of the developmental resiliency of *C. teleta*, allowing animals to continue to reproduce even if the cells fated to become the germline in the embryo are lost.

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