Developmental Biology 433 (2018) 433–447

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Resource

Girardia dorotocephala transcriptome sequence, assembly, and validation through characterization of *piwi* homologs and stem cell progeny markers

Eugene Matthew P. Almazan[a], Sydney L. Lesko[a], Michael P. Markey[b], Labib Rouhana[a,*]

[a] Department of Biological Sciences, Wright State University, 3640 Colonel Glenn Highway, Dayton, OH 45435, United States
[b] Department of Biochemistry and Molecular Biology, Wright State University, 3640 Colonel Glenn Highway, Dayton, OH 45435, United States

ARTICLE INFO

Keywords:
Planarian
Platyhelminthes
RNA-seq
de novo transcriptome assembly
Stem cells
PIWI

ABSTRACT

Planarian flatworms are popular models for the study of regeneration and stem cell biology *in vivo*. Technical advances and increased availability of genetic information have fueled the discovery of molecules responsible for stem cell pluripotency and regeneration in flatworms. Unfortunately, most of the planarian research performed worldwide utilizes species that are not natural habitants of North America, which limits their availability to newcomer laboratories and impedes their distribution for educational activities. In order to circumvent these limitations and increase the genetic information available for comparative studies, we sequenced the transcriptome of *Girardia dorotocephala*, a planarian species pandemic and commercially available in North America. A total of 254,802,670 paired sequence reads were obtained from RNA extracted from intact individuals, regenerating fragments, as well as freshly excised auricles of a clonal line of *G. dorotocephala* (MA-C2), and used for *de novo* assembly of its transcriptome. The resulting transcriptome draft was validated through functional analysis of genetic markers of stem cells and their progeny in *G. dorotocephala*. Akin to orthologs in other planarian species, *G. dorotocephala Piwi1* (*GdPiwi1*) was found to be a robust marker of the planarian stem cell population and *GdPiwi2* an essential component for stem cell-driven regeneration. Identification of *G. dorotocephala* homologs of the early stem cell descendent marker PROG-1 revealed a family of lysine-rich proteins expressed during epithelial cell differentiation. Sequences from the MA-C2 transcriptome were found to be 98–99% identical to nucleotide sequences from *G. dorotocephala* populations with different chromosomal number, demonstrating strong conservation regardless of karyotype evolution. Altogether, this work establishes *G. dorotocephala* as a viable and accessible option for analysis of gene function in North America.

1. Introduction

Planarians are free-living flatworms (class Turbellaria) that belong to the phylum Platyhelminthes, which also includes parasitic flatworms classified as cestodes, trematodes, and monogeneans. For more than a century, scientists have been captivated by the regenerative abilities of planarian flatworms, which are able to redevelop all missing body parts and can give rise to whole organisms from small body fragments (reviewed by Brøndsted (1969) and Elliott and Sanchez Alvarado (2013)). Planarians were the subject of intensive research during the 19th century and the early 20th century, which included significant contributions by Morgan (1900). However, advancements in genetics and biochemistry amenable to the study of biological phenomena in other organisms shifted the interest of many scientists during the late 1900s, decreasing the number of laboratories with expertise in planarian research.

The development of research tools to study gene expression and function in planarians, which include genome (Nishimura et al., 2015; Robb et al., 2015, 2008) and transcriptome (Brandl et al., 2016; Ishizuka et al., 2007; Nishimura et al., 2012; Sanchez Alvarado et al., 2002; Sasidharan et al., 2013; Zayas et al., 2005) sequences, as well as protocols for whole-mount *in situ* hybridization (King and Newmark, 2013; Pearson et al., 2009; Umesono et al., 1997) and RNA-interference (RNAi) (Newmark et al., 2003; Orii et al., 2003; Rouhana et al., 2013; Sanchez Alvarado and Newmark, 1999), have revitalized the use planarians as a model for molecular studies of stem cell-driven developmental processes (reviewed by Elliott and Sanchez Alvarado (2013), Newmark and Sanchez Alvarado (2002), Reddien and Sanchez Alvarado (2004), Rink (2013), Salo et al. (2009) and Shibata et al. (2010)). Planarians have also been the subject of recent work in toxicology, as well as behavioral, ecological, and evolutionary biology research (Alvarez-Presas et al., 2008; Hagstrom et al., 2016, 2015;

* Corresponding author.
E-mail address: labib.rouhana@wright.edu (L. Rouhana).

http://dx.doi.org/10.1016/j.ydbio.2017.07.022
Received 19 March 2017; Received in revised form 5 July 2017; Accepted 27 July 2017
Available online 01 August 2017

Hicks et al., 2006; Inoue et al., 2015; Levin et al., 2016; Lombardo et al., 2011; Majdi et al., 2014; Shomrat and Levin, 2013), broadening their use as a model beyond fields related to developmental biology. In the classroom, these charismatic organisms have been an important component in K-12 science education and tools are being developed to modernize their use in college level courses (Accorsi et al., 2017; Eberhardt et al., 2015; Pagan et al., 2009; Valverde, 2015). For these reasons, planarians are poised to become more than an emerging model organism, but an integral part of modern research and education in the biological sciences.

The vast majority of tools made available by planarian researchers over the last decade, have been largely developed for laboratory lines of sexual (Zayas et al., 2005) and asexual (Sanchez Alvarado et al., 2002) strains of *Schmidtea mediterranea*, as well as an asexual line of *Dugesia japonica* (Orii et al., 1993). As useful as these species have been to the advancement of this field, they are not readily available in the Western Hemisphere and maintenance by novices is often challenging. Furthermore, the fact that *S. mediterranea* and *D. japonica* are not natural habitants of the region, impedes their broad availability to educators in North America who try to implement hands-on pedagogy using planarians (Accorsi et al., 2017).

In this study, we establish a clonal line of the commercially available North American planarian *Girardia dorotocephala*: a species that is larger and more active than other planarian species, as well as the subject of classical work in planarian regeneration (Child, 1909, 1911; Coward, 1974; Flickinger and Coward, 1962), neurobiology and behavior (Best et al., 1975, 1967; Block and McConnell, 1967; Morita and Best, 1965), reproduction (Hyman, 1925; Legner and Tsai, 1978), and toxicology (Grebe and Schaeffer, 1991; Kapu and Schaeffer, 1991; Kostelecky et al., 1989). We analyze the karyotype, regenerative capacity and neoblast distribution in this strain, as well as provide a draft of its transcriptome. We validate the utility of this transcriptome sequence draft by analyzing the expression and function of *piwi* homologs in *G. dorotocephala*, which corroborate with previous studies of *S. mediterranea* and *D. japonica* orthologs, as well as through identification of a family of planarian-specific lysine-rich proteins with homology to markers of "early" stem cell differentiation processes. Finally, we measure the usefulness of this clonal-derived transcriptome sequence draft on samples from a *G. dorotocephala* population with a vastly different karyotype, and determine that there is nearly 99% nucleotide sequence identity between orthologous transcripts from these two populations. This work validates the conserved function of planarian *piwi* homologs in stem cell-driven regeneration and provides a tool for expanding the use of *G. dorotocephala* in research and education.

2. Results

2.1. Characterization of Girardia dorotocephala clonal line MA-C2

The genus (formerly subgenus) *Girardia* has been used to catalog planarians of North American origin, with *G. dorotocephala* as one of the most commonly found members of the genus (reviewed by Artois and Tyler (2015), Kenk (1989) and Sluys et al. (2005)). Native populations of *G. dorotocephala* inhabit Mexico, Canada, and the contiguous United States, but is also found as an invasive species in Hawaii (Kawakatsu et al., 1984) and Japan (Kawakatsu et al., 2007; Fig. 1A). Within the continental United States, *G. dorotocephala* populations are found broadly throughout Southwestern and Midwestern states (Fig. 1A′; Ball, 1971; Benazzi, 1974; Hampton, 1988; Hyman, 1925; Hyman, 1929; Hyman, 1939; Hyman, 1956; Jenkins, 1967; Kawakatsu and Mitchell, 1981; Kenk, 1944; Kenk, 1989; Muttkowski, 1918; Nixon, 1981; Puccinelli and Deri, 1991; Sluys et al., 2010; Stringer, 1909; Watermolen, 2005; Woodworth, 1897). Unlike planarian species commonly used in developmental and regenerative biology research, more specifically *Schmidtea mediterranea* (native to Spain) and *Dugesia japonica* (native to Japan), comprehensive genetic information that has been functionally validated is not available for *G. dorotocephala*. Therefore, we decided to establish a clonal line of *G. dorotocephala*, characterize its stem cell distribution, sequence the transcribed component of its genome, and use this transcriptome sequence to analyze the expression and function of genes involved in planarian adult stem cell biology.

Starting from a single individual obtained through a commercial vendor (Carolina Biological Supply Company, Burlington, NC), we established a clonal line of *G. dorotocephala* (*G. dorotocephala* MA-C2). Individuals from the MA-C2 clonal line have been maintained in the laboratory as an exclusively fissiparous population for over two years. *G. dorotocephala* MA-C2 has an orange to light brown pigmentation, a pointed head, and the characteristic prominent auricles for which it was originally named "*Dugesia diabolis*" (Hyman, 1956; Fig. 1B). Individuals in this population range in size from 1 mm to over 3 cm in length, and from 0.5 to 3 mm width, depending on nutritional status and time after fissioning (Fig. 1B and B′). Previous surveys have found diploid populations of *G. dorotocephala* carrying either four (2n = 8) or eight (2n = 16) chromosomal pairs (Benazzi, 1974; Nascetti et al., 1990; Puccinelli and Deri, 1991). However, analysis of MA-C2 cells revealed a karyotype of twelve small chromosomal pairs for this strain of *G. dorotocephala* (2n = 24; Fig. 1C), suggesting that multiple chromosomal fragmentation events occurred since isolation from 2n = 8 or 2n = 16 ancestral populations.

2.2. Assessment of G. dorotocephala MA-C2 stem cell distribution and regenerative capacity

The extraordinary ability of planarians to regenerate damaged or missing body parts requires a population of adult pluripotent stem cells called neoblasts (reviewed by Newmark and Sanchez Alvarado (2002), Reddien and Sanchez Alvarado (2004), Rink (2013) and Shibata et al. (2010)). To assess the presence and distribution of neoblasts in *G. dorotocephala* MA-C2, we performed immunofluorescence analyses using Y12 antibodies (Lerner et al., 1981), which have been used to visualize cytoplasmic ribonucleoprotein granules characteristic of planarian stem cells (Kashima et al., 2016; Rossi et al., 2014; Rouhana et al., 2012, 2014), and anti-phospho-histone H3(Ser10) (PH3(S10)), which labels the fraction of neoblasts in M-phase (Newmark and Sanchez Alvarado, 2000). Indeed, cells resembling the morphology, size and distribution of neoblasts were labeled in *G. dorotocephala* MA-C2 by these antibodies (Fig. 2A-A‴). Y12 labels the cytoplasm of a population of ovoid-shaped cells of slightly less that 10 μm diameter in the mesenchyme of *G. dorotocephala* (Fig. 2A′ and A‴), a subset of which are also labeled by PH3(S10) antibodies (Fig. 2A″ and A′‴). Mitotic neoblasts were readily detected throughout the anterior-posterior (AP) axis in whole-mount samples, with the pharyngeal and head regions being the least populated (Fig. 2B and B′). A region particularly rich in neoblasts in *S. mediterranea* and *D. japonica* was also distinguishable along the midline of *G. dorotocephala* between the pharynx and tail (Fig. 2B and B″). These results are consistent with previous observations of adult stem cell distribution in using these antibodies in other planarians species, such as *S. mediterranea* (Newmark and Sanchez Alvarado, 2000) and *D. japonica* (Rouhana et al., 2010), as well as the distribution of proliferating cells labeled by bromodeoxyuridine uptake in *G. dorotocephala* (Newmark and Sanchez Alvarado, 2000). From these results, we conclude that a distribution of adult stem cells comparable to that observed in other planarian models can be visualized with PH3(S10) and Y12 antibodies in *G. dorotocephala*.

The presence of neoblasts is essential, but not sufficient, for regeneration. Some planarian species are unable to regenerate a head depending on the position of amputation along their AP axis, regardless of the availability of vast neoblast populations (Liu et al., 2013; Sikes and Newmark, 2013; Umesono et al., 2013). To confirm the

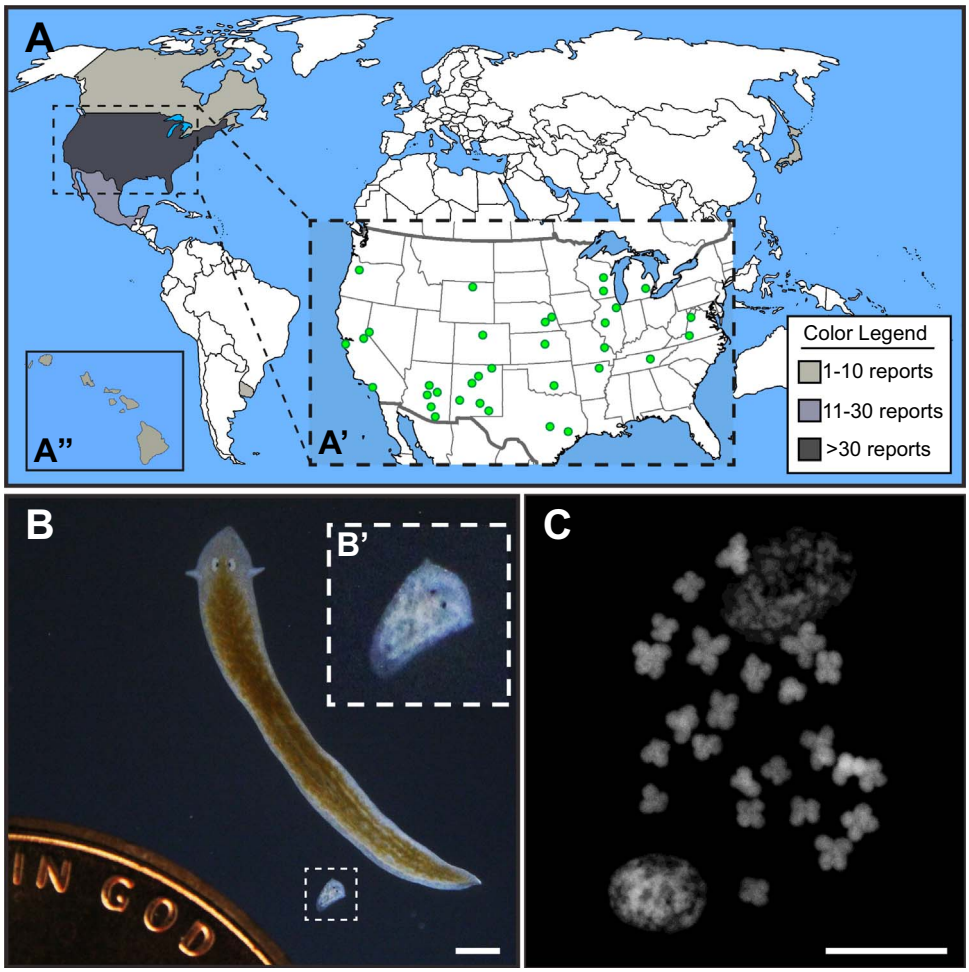

Fig. 1. The MA-C2 clonal line of the North American planarian species *Girardia dorotocephala*. (A) Reported records of *G. dorotocephala* populations in North America, Uruguay, and Japan, with specific locations indicated for the contiguous United States (green circles in (A'); references in text). (B) Appearance of large and small (B') individuals of the fissiparous *G. dorotocephala* clonal line MA-C2 imaged by dark field microscopy. A United States penny is shown for size reference. (C) Karyotypic analysis by DAPI staining and confocal microscopy reveals a total of 24 chromosomes (2n = 24) in *G. dorotocephala* MA-C2 cells. Scale bars = 1 mm (B) and 10 μm (C).

regenerative potential of *G. dorotocephala* described by Child (Child, 1909, 1911) and more recently reported by Newmark and Sanchez Alvarado (Sanchez Alvarado and Newmark, 2001) in the MA-C2 strain, amputations along the AP axis were made that resulted in head tip (Tip), head (Head), pre-pharyngeal (Pre-Ph), pharyngeal (Ph), post-pharyngeal (Post-Ph), and tail (Tail) fragments. These fragments were monitored for the ability to regenerate over the course of 7 days, which was determined by the presence of a pharynx, auricles, and a pair of photoreceptors (Fig. 3). Blastema formation was evident in all fragments two days post-amputation (DPA), with the exception of Tip fragments, which disintegrated after failing to regenerate (Fig. 3A). Regeneration was evident 7 DPA in Head, Pre-Ph, Ph, Post-Ph, and Tail fragments (Fig. 3A). To quantitatively measure the observed regeneration patterns, 23 animals were amputated in the same manner and allowed to regenerate for a period of 10 days, at which point, fragments were assessed for the presence of a pharynx, auricles and photoreceptors. All of the Pharynx fragments were able to regenerate, while Pre-Ph fragments showed a slight decrease with 95.7% of the samples scoring for successful regeneration (Fig. 3B). Post-Ph and Tail fragments, which most closely mimic the natural location of fission events in this species during asexual reproduction, both displayed successful regeneration in 73.9% of the samples (Fig. 3B), with the rest of the samples (26.1%) developing undistinguishable or abnormal heads. The Tip fragments, which are composed of an anatomic region of the head where neoblasts were not detected (Fig. 2B), were all unable to regenerate (Fig. 3B). The Head fragments, which included the photoreceptors, auricles and "neck", displayed regeneration of a pharynx in only 35% of the samples under tested conditions (Fig. 3B). These results indicate that there is some variability in regeneration potential along the AP axis in the MA-C2 strain, which was also observed by Child in his experiments with *G. dorotocephala* (Child, 1911). In our experiments, the pharyngeal and pre-pharyngeal regions displayed the most robust regenerative capacity, as compared to the anterior and posterior ends of the animal.

2.3. Generation of the G. dorotocephala MA-C2 transcriptome sequence draft by next-generation RNA sequencing and de novo assembly

Functional genomic studies based on comparative transcriptomics, *in situ* hybridization, and RNAi, have been a fundamental approach for advancement of modern research using planarian flatworms (Elliott and Sanchez Alvarado, 2013; Newmark, 2005; Reddien et al., 2005a). Thus, we sequenced the transcribed fraction of the MA-C2 genome, in order to establish *G. dorotocephala* as a viable option for research and education in the era of functional genomics. Pools of ribosomal RNA (rRNA)-depleted total RNA from groups of MA-C2 intact and regenerating specimens (one and four days post-amputation), as well as freshly dissected auricle fragments, were subjected to paired-end sequencing using an Illumina platform (Fig. 4). Over 50 million pairs of quality reads were obtained for each of these groups, which were combined and used for *de novo* assembly using Trinity (Grabherr et al.,

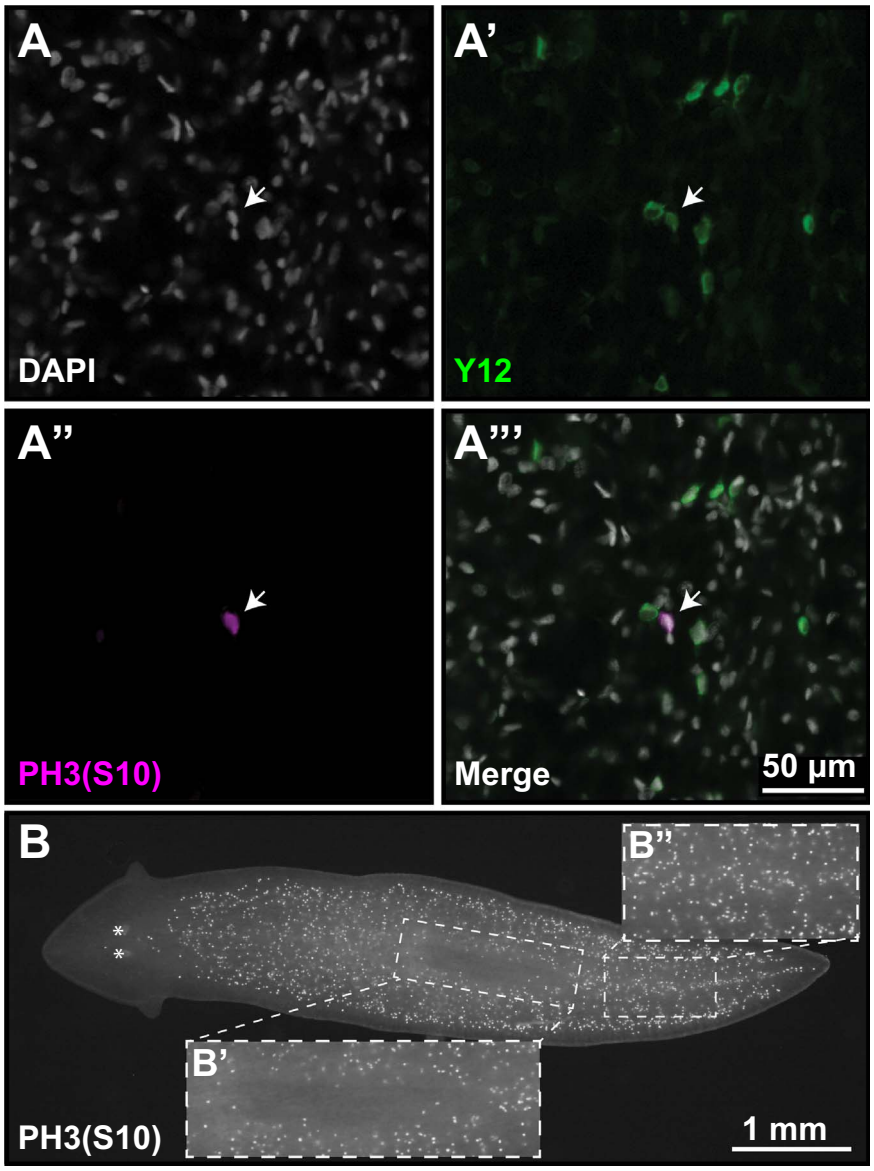

Fig. 2. Adult stem cell distribution in *G. dorotocephala*. (A-A′″) Stem cells in a field of mesenchymal cells stained with DAPI (A, A″) are visualized by immunofluorescence and confocal microscopy using the Y12 antibody (green) (A′, A‴), whereas stem cells in M-phase are detected using phospho-Histone H3 (Ser10) antibodies (PH3(S10); magenta) (A″, A‴). (B) Distribution of M-phase neoblasts throughout the anatomy of a whole-mount sample, with magnified view insets for areas of low (pharyngeal region; B′) and high (posterior midline; B″) mitotic cell density. Position of photoreceptors is denoted by asterisks. Scale bars = 50 µm (A) and 1 mm (B).

2011; Haas et al., 2013). A transcriptome draft with a total of 634,246 contigs, which includes predicted alternative splicing isoforms for 214,308 unique contig groups, was produced from this assembly (available at corescholar.libraries.wright.edu/biology/318). The completeness of this MA-C2 transcriptome draft is 91.8% according to Benchmark Universal Single-Copy Orthologs (BUSCO; Simao et al., 2015) assessments (Supplementary Fig. S1), which is comparable to scores obtained for transcriptomes asexual and sexual strains of *S. mediterranea* (94.7% and 92.4%, respectively), as well as *Dendrocoelum lacteum* (96%) (Grudniewska et al., 2016; Liu et al., 2013; Xiang et al., 2014), and an improvement over the score of 70% reported for a *S. polychroa* transcriptome (Levin et al., 2016) (Supplementary Fig. S1; Supplementary Table S1). Contig size length in the MA-C2 transcriptome draft ranges from 224 to 29,554 nucleotides (nts), with a mean of 594.4 nts and median length of 367 nts. We were able to annotate and assign Gene Ontology (GO) groups to 99,716 MA-C2 contig sequences via BLAST2GO analysis (Supplementary Fig. S2-S5; Supporting Information), which are also available as a separate FASTA file (corescholar.libraries.wright.edu/biology/337). The average size of sequences mapped to proteins in the SwissProt database for metazoan species is twice the length of that of unmapped contigs (1082.1 nts vs. 503.4 nts; Supplementary Fig. S2). The longest transcript in the assembly (TR59653|c2_g1_i1; 29554 nts) encodes for an ortholog of a human protein that is 8797 amino acids in length (Nesprin-1; NCBI Reference Sequence: NP_892006.3), which attests to the quality of our transcriptome assembly. On the other hand, the number of contigs in this draft is likely to represent an over-estimation of gene number in the *G. dorotocephala* genome, but we refrained from further selection in order to avoid exclusion of non-coding transcripts as well as small and novel protein-coding transcripts. Instead, we opted to evaluate the functionality of the *G. dorotocephala* MA-C2 transcriptome draft through functional analyses of genes involved in planarian adult stem cell biology. First, we characterized *G. dorotocephala* homologs of *piwi*, a family of PIWI-interacting RNA (piRNA) regulators whose function in planarian stem cells has been well documented in other planarian species (Nakagawa et al., 2012; Palakodeti et al., 2008; Reddien et al., 2005b; Shibata et al., 2016). We also characterized homologs of *PROG-1*, as well as *arginine:glycine*

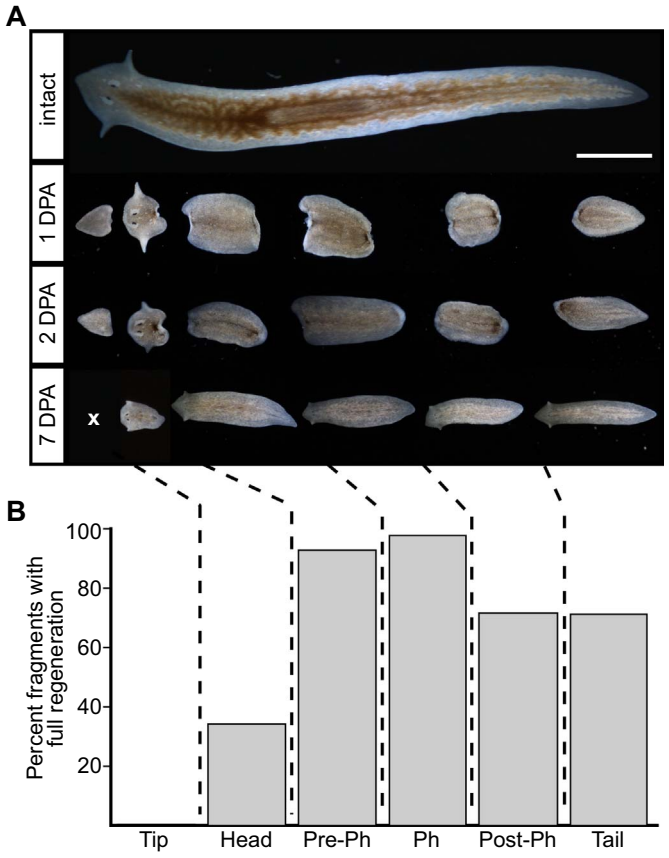

Fig. 3. *G. dorotocephala* regeneration observed in regions with detectable populations of neoblasts. (A) Time course analysis of planarian regeneration after transverse amputation into six different fragments visualized 1 day post-amputation (DPA), 2 DPA, and 7 DPA by dark field microscopy. Lethality observed in head tip region fragments is indicated ("x"). Scale Bar = 1 mm. (B) Percentage of amputation products able to regenerate as indicated by the presence of auricles, photoreceptors and pharynx observed in head tip (Tip), head region (Head), pre-pharyngeal region (Pre-Ph), pharyngeal region (Ph), post-pharyngeal region (Post-Ph), and tail region (Tail) fragments 10 DPA (n = 23 individuals).

amidinotransferase-1 (*AGAT-1*) and *AGAT-2*, which are stem cell descendant markers established in *S. mediterranea* (Eisenhoffer et al., 2008).

2.4. Validation of the MA-C2 transcriptome draft through analysis of piwi homologs in G. dorotocephala

To test the functionallity of the *G. dororocephala* MA-C2 transcriptome assembly we searched for homologs of the human PIWI-like protein HIWI (NCBI Gene ID: 9271; Protein Reference ID: NP_001177900.1) using the stand alone TBLASTN program BlastStation Local-64 (TM Software, Inc., Arcadia, CA). Five unique contigs (plus corresponding alternatively spliced isoform contigs) were identified as homologs of HIWI; all with Expect-values (E-values) lower than $1e^{-28}$ (Table 1). The three unique MA-C2 contigs with the best E-value for HIWI homology correspond to *Smedwi-3, Smedwi-2,* and *Smedwi-1* orthologs in *S. mediterranea*, and were therefore named *GdPiwi3*, *GdPiwi2*, and *GdPiwi1*, respectively (Table 1). The three least identical unique contigs identified as HIWI homologs, actually shared highest identity with Argonaute-2 (AGO-2) proteins from other planarian species (Table 1), and were concluded not to be *piwi* orthologs. The protein sequence encoded by contigs representing *GdPiwi3*, *GdPiwi2*, and *GdPiwi1*, matched orthologs in *S. mediterranea* and *D. japonica* with 80% or higher sequence identity and complete or nearly complete coverage [Smedwi-1 (808 amino acids (a.a.)), M1-end; Smedwi-2 (833 a.a.), N14-stop; Smedwi-3 (974 a.a.), Q18-stop; DjPiwiA (809 a.a.), S12-end; DjPiwiB (828 a.a.), N7-stop; DjPiwiC (966 a.a.), Q18-stop], once again demonstrating deep and accurate assembly of our transcriptome reads.

Amongst planarian *piwi* homolos, *Smedwi-1* and its orthologs (*DjpiwiA*; *Drpiwi-1*) have long been utilized as robust neoblast markers, although their expression is not essential for regeneration or homeostasis in asexual individuals (Nakagawa et al., 2012; Palakodeti et al., 2008; Reddien et al., 2005b; Shibata et al., 2016). On the other hand, *Smedwi-2* and its orthologs (*DjpiwiB*; *Drpiwi-2*) are all required for planarian regeneration and homeostasis (Nakagawa et al., 2012; Palakodeti et al., 2008; Reddien et al., 2005b; Shibata et al., 2016). We cloned *GdPiwi1* and *GdPiwi2* partial cDNAs based on sequences identified in the MA-C2 transcriptome, and tested the expression of both genes in *G. dorotocephala* by whole-mount *in situ* hybridization (Fig. 5A and B). Similar expression patterns were observed for both *GdPiwi1* (Fig. 5A) and *GdPiwi2* (Fig. 5B), which resembled the distribution of mitotic neoblasts observed using PH3(S10) antibodies (Fig. 2B), as well as a recent report of *Piwi1* expression in *Dugesia (Girardia) dorotocephala* by (Accorsi et al., 2017).

Next, we tested the conservation of *GdPiwi1* and *GdPiwi2* function during planarian regeneration and homeostasis. To do this, we fed planarians *in vitro* synthesized double-stranded RNA (dsRNA) corresponding to *GdPiwi1*, *GdPiwi2*, or firefly *luciferase* sequence (as negative control) twice per week. For regeneration analyses, heads and tails were amputated 3-days after the fourth dsRNA feeding and allowed to regenerate for a week. Initiation of blastema formation was observed 2 DPA and head regeneration 7 DPA in all the planarians fed with either control or *GdPiwi1* dsRNA (*GdPiwi1(RNAi)*) (Fig. 5C–F). On the other hand, 100% of the planarians subjected to *GdPiwi2* RNAi (*GdPiwi2(RNAi)*) failed to regenerate and eventually died, regardless of initiating blastema formation 2 DPA (Fig. 5G and H). For homeostatic integrity assays, planarians were fed twice per week for 4-weeks without further manipulation. Similarly to regeneration assays, no homeostatic defects were obseved in control or *GdPiwi1(RNAi)* groups after more than four weeks from the initial dsRNA feedings (Fig. 5I and J). On the other hand, *GdPiwi2* RNAi lead to defects in homeostasis, such as head regression, curling and death (Fig. 5K). These experiments show that *GdPiwi2* expression is required for stem cell driven regeneration and homeostaisis in *G. dorotocephala*.

Previous studies have shown that neoblasts are lost approximately a week prior to the manifistation of defects in regeneration and homeostasis caused by irradiation (Wagner et al., 2011), or knockdown of genes such as micro-RNA factor *Argonaute-2* (Rouhana et al., 2010). We analyzed whether the defects in regeneration and homeostasis observed in *GdPiwi2(RNAi)* were due to the loss of stem cells. To do this, we assessed the presence of mitotic stem cell populations in all experimental groups a week after the fourth dsRNA feeding, when homeostasis and regeneration defects where first detected in *GdPiwi2(RNAi)*. As a control for loss of stem cells, we analyzed a group of planarians subjected to x-ray irradiation, which specifically depletes mitotically active neoblasts (Bardeen and Baetjer, 1904; Curtis and Hickman, 1926). As expected, mitotic neoblasts were detected in control*(RNAi)* samples (Fig. 6A). M-phase neoblasts were also readily detectable in *GdPiwi1(RNAi)* (Fig. 6B) and *GdPiwi2(RNAi)* (Fig. 6C), but absent in irradiated samples (Fig. 6D). Quantitative measurements of biological replicates showed no significant difference in mitotic neoblast population between control*(RNAi)* and *GdPiwi1* or *GdPiwi2* knockdown (Fig. 6E), suggesting that deffects in regeneration and homeostasis observed after *GdPiwi2* RNAi were not due to complete loss of neoblasts. Altogether, these results correlate with previous studies of *piwi* homologs in *S. mediterranea* (Palakodeti et al., 2008; Reddien et al., 2005b) and *D. japonica* (Shibata et al., 2016) demonstrating a conserved role for *GdPiwi2* orthologs as essential components for homeostasis and regeneration in planarians, as well as for *GdPiwi1* orthologs as neoblast markers whose expression is not essential for somatic homeostatis and regeneration in asexual planarians.

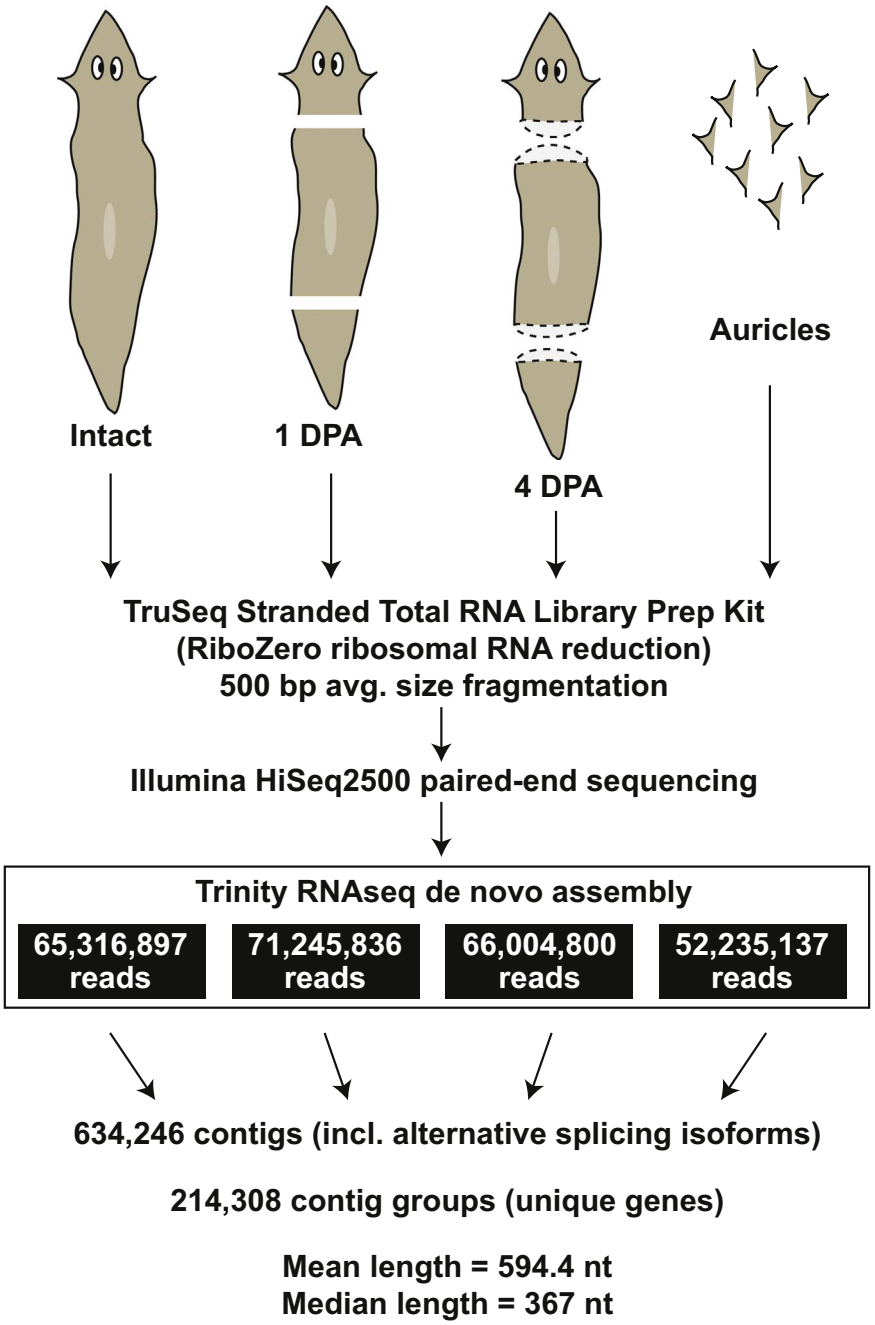

Fig. 4. *G. dorotocephala* MA-C2 transcriptome sequencing and assembly. Total RNA extracted from intact and regenerating fragments 1 and 4 days post-amputation (DPA), as well as excised auricles, was subjected to ribosomal RNA depletion, library synthesis, and paired-end sequencing, using Illumina technologies. The sum of 254,802,670 read pairs from sequenced samples was used for *de novo* assembly, yielding a total of 634,246 contigs (214,308 contig groups) in the current draft of the MA-C2 transcriptome draft.

2.5. Identification of a Lysine-rich family of proteins with homology to the stem cell differentiation marker PROG-1

To further validate the usefulness of the MA-C2 transcriptome draft we sought to identify genes expressed during stem cell differentiation in *G. dorotocephala*. The planarian-specific *PROG-1* (NB.21.11e) and *PROG-2* (NB.32.1g) genes were identified as "early" stem cell progeny markers, as compared to L-Arginine: Lysine Amidinotransferase homologs *AGAT-1, -2,* and *-3*, which are "late" stem cell progeny markers (Eisenhoffer et al., 2008). These genes are expressed in *S. mediterranea* during epithelial cell differentiation from a subset of adult stem cells (zeta-neoblasts; van Wolfswinkel et al., 2014) and during embryoic development (Davies et al., 2017). Three unique contigs with homology to PROG-1, four unique contigs with homology to PROG-2, and potential orthologs for each *AGAT* gene, were identified in the MA-C2 transcriptome draft by TBLASTN analysis (Table 2). Whole-mount *in situ* hybridization analyses revealed that two *G. dorotocephala PROG-1* homologs (*GdPROG1-L1* and *GdPROG1-L2*) are expressed in cells scattered throughout the AP axis and concentrated in the proximity of the photoreceptors (Fig. 7A and B), which resembles the distribution of *PROG-1* in "early" neoblast progey of *S. mediterranea* (Eisenhoffer et al., 2008). On the other hand, cells expressing *GdPROG1-L3* (the most divergent *PROG-1* homolog from this group) ressembled the distribution of *AGAT-1* and *AGAT-2* expression in *G. dorotocephala* (Fig. 7C–E). The abundance of cells expressing *GdPROG1-L3*, *GdAGAT-1* and *GdAGAT-2* peaked near the anterior edge, akin to "late" neoblast progeny *S. mediterranea* (Eisenhoffer et al., 2008), and differently from neuronal markers such as *CPEB-2* (Fig. 7F; Rouhana et al., 2017).

Table 1
Identification of PIWI homologs in *G. dorotocephala*. TBLASTN analysis of Human PIWI (HIWI) revealed three *piwi* paralogs in *G. dorotocephala* MA-C2 (*GdPiwi1*, *GdPiwi2* and *GdPiwi3*). Contig IDs for top matches ranked based on lowest TBLASTN E-value are listed, including alternatively spliced contig isoforms and percent sequence identity with HIWI. Paralogs with highest protein sequence identity in *S. mediterranea* and *D. japonica* (sixth and seventh columns) was confirmed by pair-wise BLASTP analyses. Three *G. dorotocephala* contigs are more identical to AGO-2 orthologs in *S. mediterranea* and *D. japonica*, and were therefore labeled as GdAgo2, GdAgo2-like1 and GdAgo2-like2. Value in parentheses represents E-value from BLASTX alignments of *G. dorotocephala* contig sequences with protein sequences corresponding to AGO-2 in *S. mediterranea* and Argonaute-2 in *D. japonica*.

MA-C2 Contig ID	Length	TBLASTN E-value (vs. HIWI)	Identity (%)	*G. dorotocephala*	*S. mediterranea*	*D. japonica*
TR184850|c2_g1_i1	3263	7.00E−144	37	GdPiwi3	Smedwi-3	DjPiwiC
TR184850|c2_g2_i1	792	2.00E−13	33			
TR3618|c1_g2_i4	10162	5.00E−59	24	GdPiwi2	Smedwi-2	DjPiwiB
TR3618|c1_g2_i1	10144	5.00E−59	24			
TR3618|c1_g2_i2	1889	1.00E−24	21			
TR8465|c0_g1_i1	2892	2.00E−55	25	GdPiwi1	Smedwi-1	DjPiwiA
TR6781|c1_g1_i3	4112	6.00E−41	24	GdAgo2	AGO-2 (0.0)	Argonaute-2 (0.0)
TR6781|c1_g1_i1	4115	6.00E−41	24			
TR8480|c0_g1_i1	3561	7.00E−31	24	GdAgo2-like1	AGO-2 (2e−118)	Argonaute-2 (7e−119)
TR8480|c0_g1_i2	2422	1.00E−30	25			
TR9006|c5_g1_i5	3002	1.00E−29	25	GdAgo2-like2	AGO-2 (2e−112)	Argonaute-2 (1e−112)
TR9006|c5_g1_i3	3151	1.00E−29	25			
TR9006|c5_g1_i2	3115	1.00E−29	25			
TR9006|c5_g1_i1	2010	1.00E−29	25			

The identification of three non-allelic sequences expressed in a pattern that ressembled stem cell progeny on course to epithelial differentiation, suggested the possiblility of functional redundance between *PROG-1* paralogs in *G. dorotocephala*. To see whether potential redundancy is also present in other planarian species, we searched for *PROG-1* paralogs in the *S. mediterranea*. A total of nine sequences homologous to SmedPROG-1 and GdPROG1-L1 were identified from BLASTP homology searches against Unigene proteins in the *S. mediterranea* genome database (smed.stowers.org; Robb et al., 2015; Supplementary Fig. S6A). All of the PROG-1 homologs identified in both *S. mediterranea* and *G. dorotocephala* contain a higher concentration of Lysine residues (average = 14.6%; range = 10.1–19.1%; Supplementary Fig. S6B), than expected from the general 8% predicted from planarian genomic studies (Nishimura et al., 2015). Interestingly, Lysine residuess were scattered in different positions across these proteins, but nearly complete or complete conservation was observed for over a handful of Cysteine and Aspartic Acid positions amongst PROG-1 homologs within and between species (Supplementary Fig. S6C). From these observations, we predict that there is a conserved family of lysine-rich proteins that function during stem cell differentiation into planarians ephilial lineages.

2.6. MA-C2 transcriptome sequences are representative of G. dorotocephala populations with different karyotypes

Although the presence of *G. dorotocephala* has been documented to occur in a wide range of locations across North America (Fig. 1A), there are several reports of karyotype variability between populations of this species (Benazzi, 1974; Nascetti et al., 1990; Puccinelli and Deri, 1991). Given that many of these populations reproduce asexually, and that little or no genetic information from them is available, it remains to be determined whether these populations are indeed likely to represent a single species. We decided to address this question by sequencing the transcriptome of individuals from a separate population *G. dorotocephala* that carry a different chromosomal number than MA-C2 (2n = 16; Fig. 8A and B). Total RNA extracted from intact and regenerating fragments (1 and 4 DPA) from this strain (from here on referred to as *G. dorotocephala* (2n = 16)) were pooled and submitted for paired-end Next Generation sequencing. A total of 61,620,720 pairs of reads were obtained and used as starting material for *de novo* transcriptome assembly (as above), which yielded 104,992 unique contigs (225,685 contigs including alternatively spliced isoforms). We searched for HIWI homologs in *G. dorotocephala* (2 n = 16) contigs by TBLASTN and were able to find a representative for each *PIWI1/A* (*Smedwi-1*, *DjPiwiA*, and *GdPiwi1*) *PIWI2/B* (*Smedwi-2*, *DjPiwiB*, and *GdPiwi2*), and *PIWI3/C* (*Smedwi-3*, *DjPiwiC*, and *GdPiwi3*) ortholog groups (Fig. 8C). In order to estimate the nucleotide sequence conservation between *G. dorotocephala* MA-C2 and *G. dorotocephala* (2n = 16) transcriptomes, we aligned transcript sequences of more than 2000 nt-length from orthologous representatives of each *G. dorotocephala* strain, as well as those of *S. mediterranea* and *D. japonica*. Surprisingly, we found that orthologous sequences obtained from *G. dorotocephala* MA-C2 and (2n = 16) were almost completely identical (98–99%; Fig. 8D), regardless of differences in chromosome number between these two strains (Figs. 1C and 8B). On the other hand, *G. dorotocephala* sequences were only 67–75% identical to orthologs in *S. mediterranea* and *D. japonica* (Fig. 8D). In fact, *S. mediterranea* and *D. japonica* orthologs were more identical to one another (78% to 79%) than they were to *G. dorotocephala* orthologs (Fig. 8D). These results suggest that *G. dorotocephala* MA-C2 and (2n = 16) are very likely to represent a single species, regardless of vast differences in their karyotypes. These results also show that the MA-C2 transcriptome shares almost perfect sequence identity with *G. dorotocephala* populations freshly collected from the wild, and therefore serves as a good reference for genetic sequences in diverse populations of *G. dorotocephala*. Finally, the closer sequence identity amongst *S. mediterranea* and *D. japonica*, than between species of the Old World and *G. dorotocephala*, supports the classification of *Dugesia* and *Girardia* as separate genera (Sluys et al., 2009), and the inclusion of *dorotocephala* as a member of the latter.

3. Discussion

In this work, we analyzed the stem cell distribution and regenerative capacity of *G. dorotocephala*: one of the most widely-distributed planarian species in North America. Additionally, we provide a transcriptome sequence draft of a clonal line of *G. dorotocephala* (MA-C2), which was obtained through Next Generation RNA sequencing and *de novo* alignment. We used the MA-C2 transcriptome assembly to identify, clone, and characterize the expression and function of *piwi* homologs in *G. dorotocephala*; validating *PIWI1/A* orthologs as robust neoblast markers and *PIWI2/B* orthologs as crucial components for planarian regeneraiton and homeostasis. Additionally, we uncovered a family of Lisyne-rich proteins expressed in early and late neoblast progeny. Finally, we show that MA-C2 transcript sequences are nearly identical to genes from *G. dorotocephala* populations with vastly different chromosomal number.

3.1. The G. dorotocephala MA-C2 transcriptome as a tool for regenerative biology research and education

Planarian flatworms have surged as model organisms for stem cell and regenerative biology research, with *S. mediterranea* and *D. japonica* being

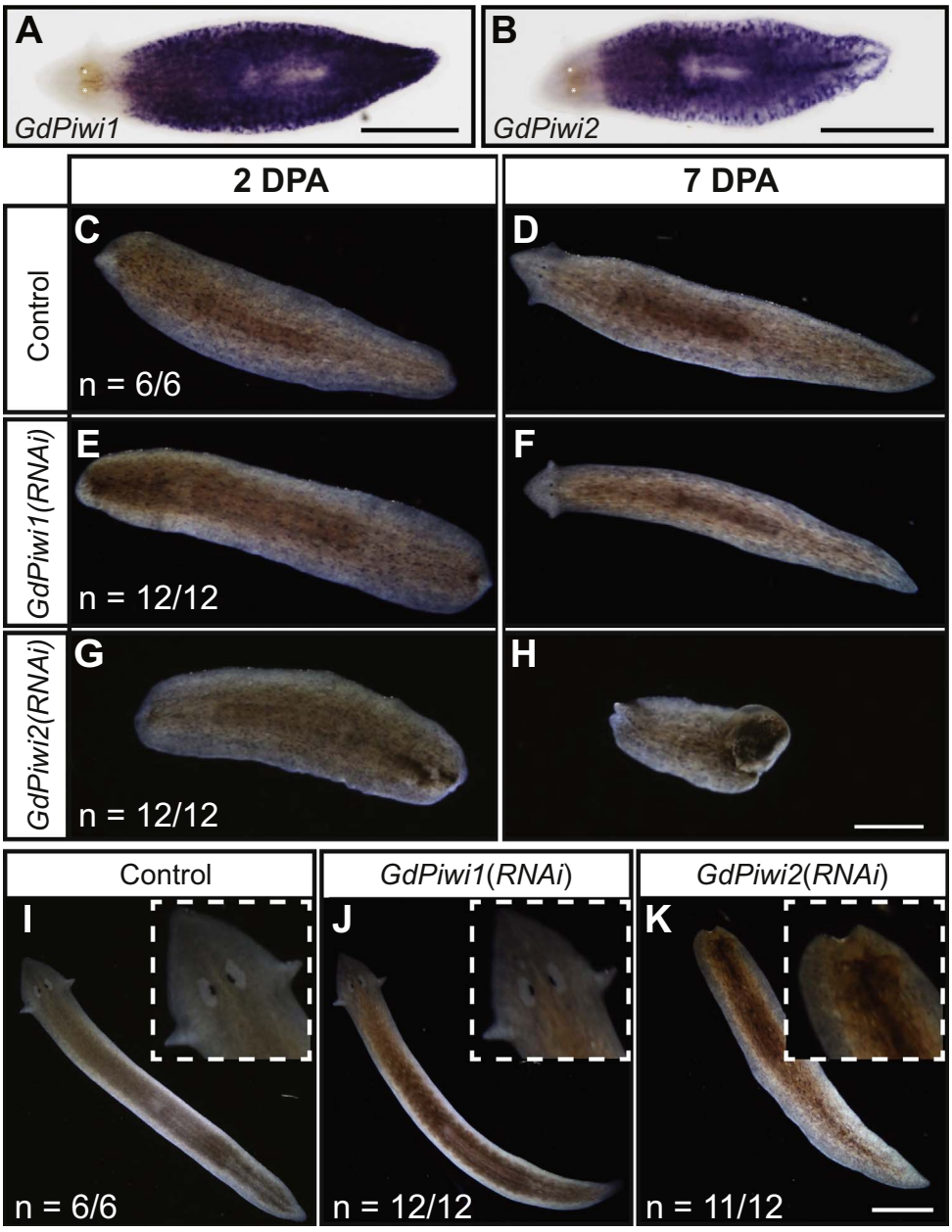

Fig. 5. *GdPiwi2* is expressed in planarian stem cells and required for regeneration and homeostasis. (A-B) Whole-mount *in situ* hybridization analysis reveals that cells expressing *GdPiwi1* (A) and *GdPiwi2* (B) reflect neoblast distribution patterns. (C-H) Control (C-D) and *GdPiwi1(RNAi)* (E-F) fragments subjected to head and tail amputation initiate blastema formation 2 days post-amputation (DPA) (C, E) and regenerate 7 DPA (D, F), whereas and *GdPiwi2(RNAi)* fragments initiate blastema formation 2 DPA (G) but are unable to regenerate and collapse 7 DPA (H). (I-K) Extended dsRNA treatments have no discernible effect in control (I) or *GdPiwi1(RNAi)* (J), but cause head regression (K) and ultimately death in *GdPiwi2(RNAi)*. Scale bars = 1 mm.

the most utilized species in the last couple of decades. The use of *G. dorotocephala* was prominent amongst North American researchers in the past, but the unavailability of accessible and functionally validated genetic sequence minimized the use of this species in modern research. Recently, some groups have begun to exploit this model for validation of genetic networks uncovered in *S. mediterranea* (Wurtzel et al., 2015), as well as in comparative development studies using chemical genetics (Emmons-Bell et al., 2015). Planarians have also played a major role in classrooms, where their charisma and amazing ability for regeneration have been used to spark student interest in science for decades (Jensen and Allen, 1983; Mairs, 1965; Scadding, 1985). However, the use of planarians in current hands-on pedagogy in North America has also been limited by the lack of genetic information, which has only been available for foreign planarian species whose distribution to classrooms across the United States is restricted. A recent publication recognizing the benefits of expanding the use of planarians in schools and universities, has provided a wonderful tool to access protocols and genetic information of four planarian species commercially available in North America (Accorsi et al., 2017). The MA-C2 transcriptome provides an additional resource validated through studies of stem cell gene expression and function, as well as through comparing sequences from different populations of *G. dorotocephala*.

3.2. Conserved expression and function of piwi homologs planarian stem cells, regeneration, and homeostasis

Orthologous members of the *PIWI1/A* family of planarian *piwi* genes in *S. mediterranea* (*Smedwi-1*) and *Dugesia* species (*DjPiwiA* and *Drpiwi-1*) have all been shown to serve as robust neoblasts markers. Here, we demonstrate that *GdPiwi1* also serves as a reliable neoblast marker in the genus *Girardia*. These results corroborate with

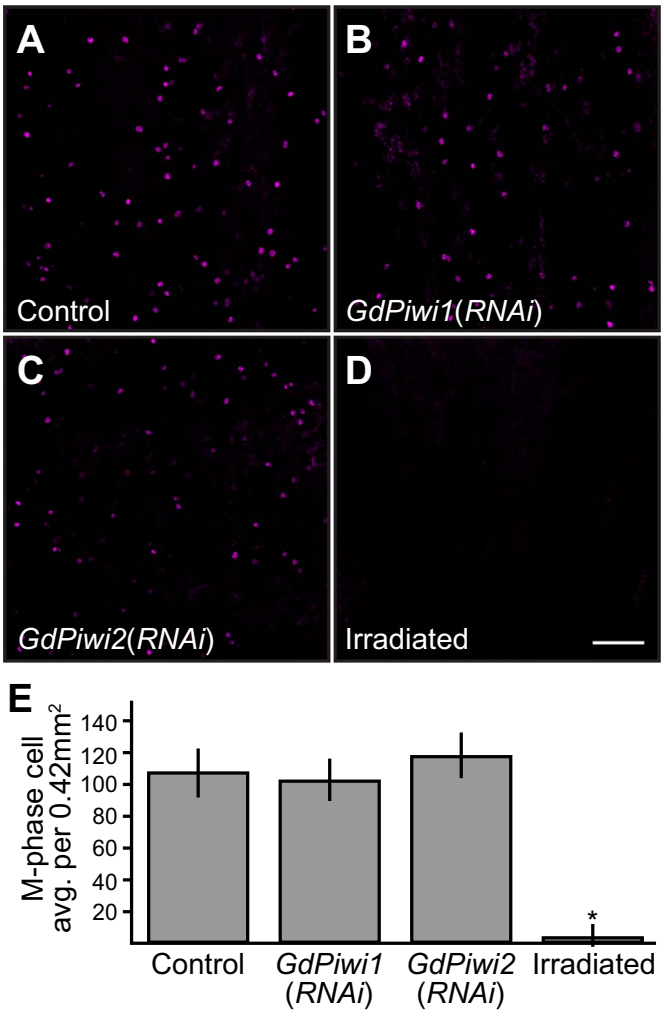

Fig. 6. Loss of neoblasts does not precede *GdPiwi2(RNAi)* homeostasis and regeneration defects. (A-D) Single confocal plane of representative phospho-Histone H3 (Ser10) (PH3(S10)) immunofluorescence analysis of mitotic neoblasts located posterior to the pharynx shows similar distributions in control (A), *GdPiwi1(RNAi)* (B), and *GdPiwi2(RNAi)* (C) three weeks into RNAi treatment. PH3(S10) analysis of planarians subjected to x-ray irradiation (D) is shown as control for loss of neoblasts. Scale bar = 100 µm. (E) Average number M-phase neoblasts in control, *GdPiwi1* and *GdPiwi2* knockdowns (3-weeks after initiation of RNAi treatment), as well as in irradiated planarians. The total number of cells labeled by PH3(S10) in a 650 µm × 650 µm region posterior to the pharynx was quantified from four confocal sections 10 µm apart and averaged from biological replicates ($n \geq 4$; error bars represent standard deviation from the mean). Only irradiated samples were significantly different in a two-tailed Student's T-test (asterisk; $p \leq 0.05$).

the recently reported use of *PIWI1/A* orthologs as neoblast markers in planarians regarded to as *Girardia* sp. and *Dugesia dorotocephala* (Accorsi et al., 2017).

PIWI1A transcripts are specifically detected in neoblasts, but their protein products are most abundant in neoblast progeny for a brief period during differentiation (Guo et al., 2006; Tasaki et al., 2011). Regardless of strong expression in neoblasts, no obvious function for *PIWI1/A* orthologs in regeneration and homeostasis has been observed in asexual planarians (Palakodeti et al., 2008; Reddien et al., 2005b; Rossi et al., 2006; Shibata et al., 2016; this study). Detailed studies of cytoplasmic ribonucleoprotein particles in neoblasts (chromatoid bodies) showed that SMEDWI-1 aids SMEDWI-3 in transcript localization to these structures (Rouhana et al., 2014), suggesting that these paralogs serve partially redundant functions. However, an essential role *Drpiwi-1* has been demonstrated in germline development of *D. ryukyuensis* (Nakagawa et al., 2012), suggesting that members of the *PIWI1/A* family may have non-redundant functions in planarian germline development.

Expression of *PIWI2/B* family members in *S. mediterranea* and *D. japonica* can be detected in neoblasts and in differentiated cells of the head region by *in situ* hybridization (Reddien et al., 2005b; Shibata et al., 2016; Solana et al., 2012). Indeed, the distribution of cells expressing *GdPiwi2* mRNA closely resembles neoblast distribution in *G. dorotocephala* (Fig. 5B), but no evident signal was detected in the head region with our current *in situ* protocol. Nevertheless, disruption of expression of *PIWI2/B* members in *S. mediterranea*, *D. japonica*, and *G. dorotocephala*, all result in the same robust phenotype: the loss of regeneration and homeostatic integrity regardless of neoblast availability (Palakodeti et al., 2008; Reddien et al., 2005b; Shibata et al., 2016; this work). These observations can be explained by the model proposed by Shibata et al. (2016), which puts forward that DjPiwiB is a nuclear protein inherited by neoblast progeny to secure continious silencing of transposable elements during and after differentiation. It remains to be seen whether the function of *PIWI2/B* members goes beyond inhibition of transposable element expression. Measuring the degree in which target specificity is conserved between *S. mediterranea*, *D. japonica*, and *G. dorotocephala* orthologs, is an important step to determine the molecular mechanisms behind *PIWI2/B* function.

3.3. Expression of PROG-1 homologs during stem cell differentiation

The identification of *prog-1* as a marker for early neoblast progeny has had considerable impact in planarian stem cell research (Fraguas et al., 2011; Pearson and Sanchez Alvarado, 2010; Scimone et al., 2010; Tu et al., 2015; van Wolfswinkel et al., 2014; Wagner et al., 2012, 2011). However, the molecular function of *prog-1*, how it contributes

Table 2
Identification of neoblast progeny and neuronal markers in *G. dorotocephala*. *G. dorotocephala* MA-C2 contig sequences with homology to "early" (PROG-1, PROG-2) and "late" (AGAT-1, −2, and −3) neoblast progeny markers in *S. mediterranea* (Eisenhoffer et al., 2008), as well as neuronal marker CPEB-2, identified through TBLASTN analyses. Name for *S. mediterranea* neoblast progeny markers as recorded in NCBI are shown in parenthesis (left column). MA-C2 contig IDs are shown for *G. dorotocephala* homologs (third column) with given name in parenthesis for genes with validated expression by whole-mount *in situ* hybridization. Distribution of expression of homologs in *S. mediterranea* (second column) and *G. dorotocephala* (last column) are listed. Abbreviations used in the table include "n.d." for "not detected" and "n.a." for "not assessed.".

S. mediterranea homolog	Expression in *S. mediterranea*	*G. dorotocephala* homolog	Contig length (nts)	TBLASTN E-value	Expression in *G. dorotocephala*
PROG-1 (NB.21.11e)	early progeny	TR46486|c0_g3_i3 (*PROG1-L1*)	557	9.00E−18	early progeny
		TR33595|c0_g9_i1 (*PROG1-L2*)	542	1.00E−10	early progeny
		TR51252|c2_g1_i4 (*PROG1-L3*)	743	1.00E−07	**late progeny**
PROG-2 (NB.32.1g)	early progeny	TR28058|c0_g3_i2	658	1.00E−09	n.d.
		TR194305|c0_g1_i3	1091	9.00E−09	n.d.
		TR206301|c0_g1_i2	784	9.00E−05	n.d.
		TR177408|c0_g1_i2	542	2.00E−04	n.d.
AGAT-1 (NB.8.8b)	late progeny	TR154437|c0_g1_i1 (*AGAT-1*)	1841	0.00E+00	late progeny
AGAT-2 (H.56.4 h)	late progeny	TR197413|c0_g1_i1 (*AGAT-2*)	1504	2.00E−34	late progeny
AGAT-3 (NB.2.5d)	late progeny	TR15918|c3_g2_i1	1711	7.00E−20	n.a.
CPEB-2	central nervous system	TR16227|c2_g2_i1 (*CPEB-2*)	2563	0.00E+00	central nervous system

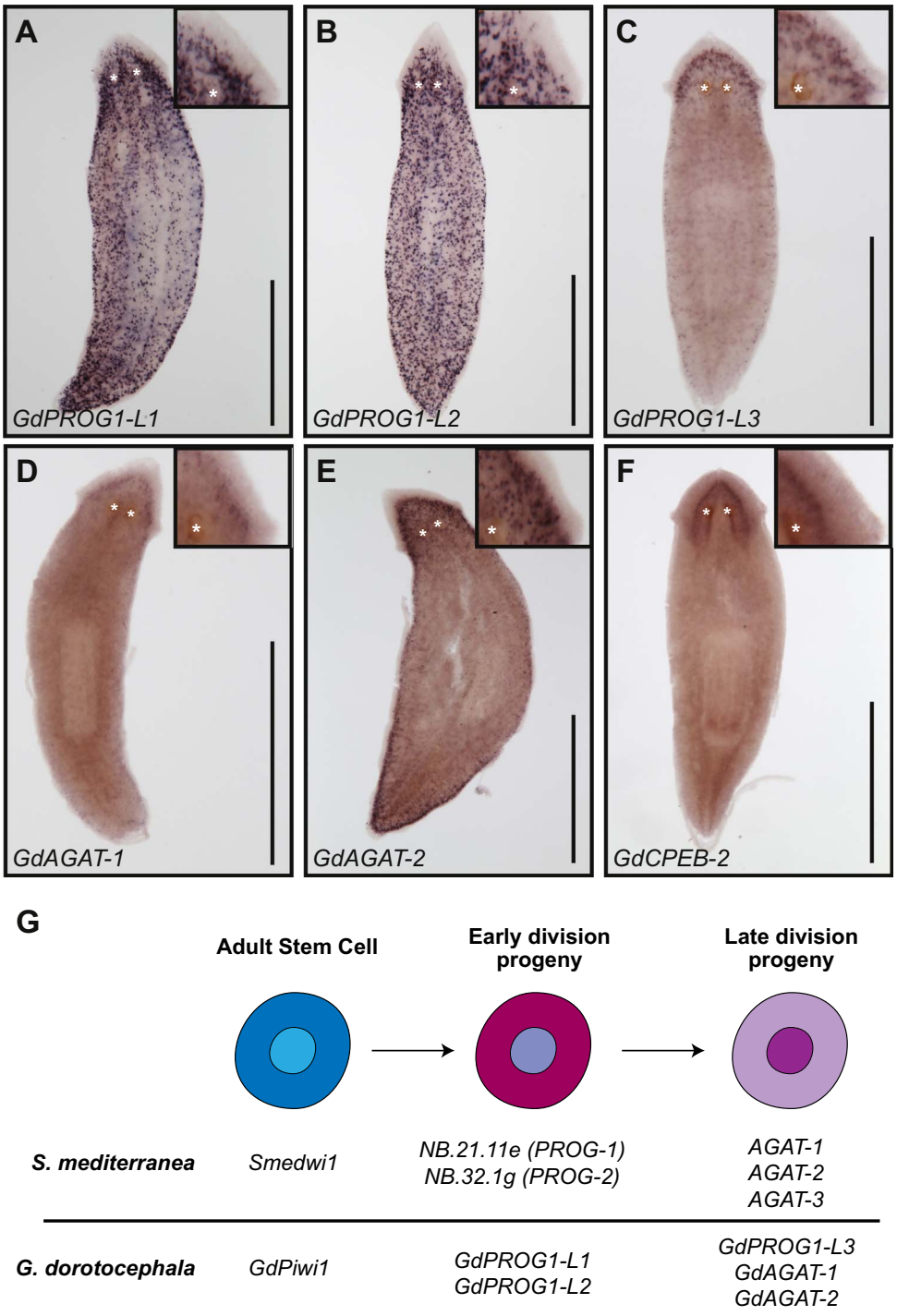

Fig. 7. *G. dorotocephala PROG-1* paralogs are expressed during early and late differentiation of epithelial lineages. Expression of (A) *GdPROG1-L1*, (B) *GdPROG1-L2*, (C) *GdPROG1-L3*, (D) *GdAGAT-1*, (E) *GdAGAT-2*, and (F) *GdCPEB-2* detected by whole-mount *in situ* hybridization in *G. dorotocephala*. (G) Graphical representation of gene activation during epithelial cell differentiation in *S. mediterranea* (top) and *G. dorotocephala* (bottom) according to Eisenhoffer et al. (2008) and this study, respectively.

to the formation of epithelial cells, or its relationship to other genes expressed during differentiation of (zeta)neoblasts (*e.g. AGAT* homologs) remain unknown. We found that at least three homologs of *prog-1* are present in the *G. dorotocephala* genome (*GdPROG1-L1*, *GdPROG1-L2*, and *GdPROG1-L3*) and demonstrate that all three are expressed ressembling the discribution of cells on course to epithelial differentiation (Fig. 7A–C). Whereas *GdPROG1-L1* and *GdPROG1-L2* expression is identical to that of *prog-1* and other "early" neoblast progeny markers (Eisenhoffer et al., 2008), expression of *GdPROG1-L3* more closely resembles the distribution of "late" neoblast progeny markers such as *AGAT-1* and *–2*. This suggests that the function of PROG1-like proteins is not restricted to an early transitional state. Computational analyses revealed the presence of a signal peptide at the N-terminus of PROG1-like proteins, suggesting that these may be secretory or membrane proteins processed in the endoplasmic reticulum. However, the absence of significant conservation with sequences in protein repositories provides little insight into the potential function of this family of proteins. In fact, we found no evidence for conservation of PROG1-like proteins even in sequenced genomes of parasitic flatworms, and only a few positions between PROG1-like paralogs in *G.*

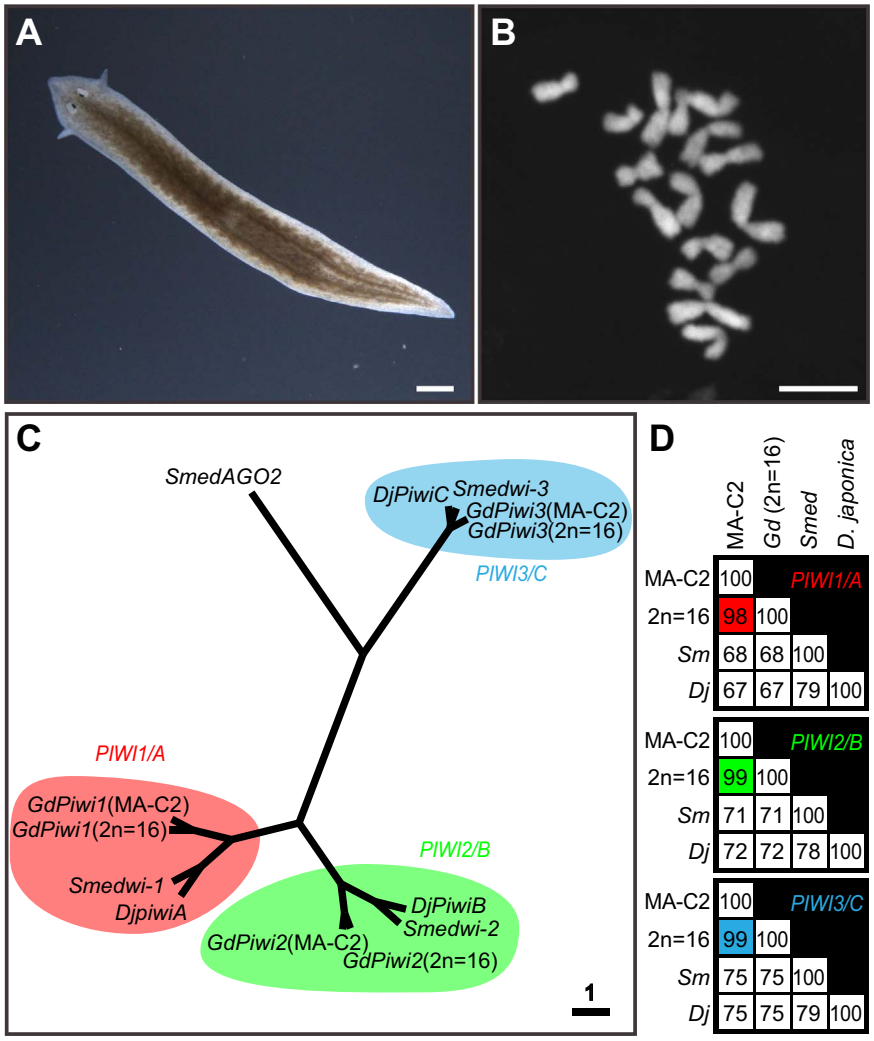

Fig. 8. Nearly identical nucleotide sequence between *piwi* transcripts from *G. dorotocephala* populations with different chromosomal number. (A) Appearance of an individual from a wild population of fissiparous *G. dorotocephala* imaged by dark field microscopy. (B) Karyotypic analysis by DAPI staining and confocal microscopy reveals a total of 8 chromosomal pairs (2n = 16) in cells of *G. dorotocephala* population shown in (A). (C) Phylogenetic tree representing maximum likelihood analysis of relationship between mRNA sequences of planarian *piwi* families. The tree was constructed using Phylogeny.fr (Dereeper et al., 2008) under "one-click" settings with *Smedwi-1* (DQ186985.1), *Smedwi-2* (DQ186986.1), *Smedwi-3* (EU586258.1), *DjpiwiA* (AB530671.1), *DjPiwiB* (AB530672.1), and *DjPiwiC* (AB530673.1) as representatives downloaded from GenBank; TR8465|c0_g1_i1, TR3618|c1_g2_i1, and TR184850|c2_g1_i1, as representatives of the *G. dorotocephala* MA-C2 transcriptome draft (MA-C2); and MX27184_C0_g1_i1, MX25326_C0_g1_i1, and MX62556_C0_g1_i2, as representatives of the *G. dorotocephala* (2n = 16) transcriptome draft. The *SmedAGO-2* (JF263459.1) sequence is used as an outgroup. Members of the *PIWI1/A* (red), *PIWI2/B* (green), and *PIWI3/C* (blue) families are highlighted. Scale bar represents one substitution per position. (D) Percent nucleotide sequence identity between entire cDNA sequence of orthologs in each planarian *piwi* family. Scale bars = 1 mm (A) and 10 μm (B).

dorotocephala and *S. mediterranea* are conserved (Supplementary Fig. S6). We attempted to gain evidence for *GdPROG1-L1*, *GdPROG-L2* and *GdPROG1-L3* function through individual and combined RNAi experiments, but failed to observe defects in homeostasis or regeneration under tested conditions. Further study will be required to uncover how this unique family of proteins contributes to the development and/or function of the dynamic planarian epidermis.

3.4. *G. dorotocephala transcriptome, karyotype, and taxonomy*

Without genetic sequence data, researchers have historically relied on external morphology, anatomy of reproductive structures, and karyotypic qualities, for classification of planarian species (Ball and Gourbault, 1978; Kenk, 1974). The taxonomical identification of fissiparous populations of planarian species is particularly challenging (Baguña et al., 1999). In the case of *G. dorotocephala*, confusion arises from strong physical similarities with different species, karyotype variability, and the use of alternative names for this species (*e.g. Girardia dorotocephala, Dugesia dorotocephala, Planaria dorotocephala, Euplanaria dorotocephala*, and *Dugesia* (*Girardia*) *dorotocephala*) (Kenk, 1989; Sluys et al., 2005). Confusion increases when commercial sources provide customers with a different species of planarian than advertised, which has occurred to researchers in the field. Possible discrepancies in species designation may be present in genetic records. For example, the top MA-C2 *cytochrome oxydase 1* BLASTN match amongst sequences in the National Center for Biotechnology Information (NCBI) repository, is a *G. dorotocephala* sequence with 99% identity (GenBank ID: KM200929; Sluys et al., 2015). However, MA-C2 ribosomal RNA sequence matched with 100% identity (and 100% coverage) to records from both *Girardia tigrina* and *Dugesia dorotocephala* (GenBank ID: AY216702 and AY216704, respectively; Burke et al., 2003), as well as 98% with a record from *Dugesia tigrina* (Genbank ID: U78718.1), and 90% with records from *Dugesia dorotocephala*, *Girardia trigrina*, and *Procotyla fluviatilis* (GenBank IDs: AY216705.1, AY216706.1 and KC869877.1), demonstrating a complex history of confusion regarding the taxonomy of this flatworm. Our comparative analysis of sequences obtained by RNA-seq showed nucleotide conservation of 98% and 99% between *piwi* transcripts from two fissiparous *G. dorotocephala* populations with

vastly different chromosomal number (2n = 24 and 2n = 16). Additionally, MA-C2 *piwi* contigs are ~99% identical to sequences reported by other laboratories for what is regarded as commercially purchased *Dugesia dorotocephala* (Accorsi et al., 2017) and *Girardia tigrina* (Wheeler et al., 2015), suggesting that all three of these studies were done on the same species. Altogether, these results suggest that the MA-C2 transcriptome is a reliable resource for genetic studies of *Girardia dorotocephala* populations, and we hope that it facilitates the use of this North American planarian species in molecular, developmental, and ecological studies.

4. Materials and methods

4.1. Planarian colony and manipulation

A clonal line of *Girardia dorotocephala* (*G. dorotocephala* MA-C2) was established from commercially purchased planarians (Carolina Biological Supply Company, Burlington, NC; Item #132970). Starting from a single individual, the population was amplified by transverse fission, as well as through regeneration of amputated fragments. Planarians were fed pureed organic calf liver once or twice per week and maintained at 21 °C in Ziploc containers filled halfway with 0.75x Montjüic salts (Cebria and Newmark, 2005). For regeneration experiments, individual planarians close to 1.5 cm in size were amputated and allowed to regenerate as isolated fragments or in groups.

4.2. Karyotype analyses

Freshly amputated planarian tail fragments were incubated in 0.75x Montjüic salts for 48 h at 21 °C. Tail fragments were then placed in a Petri dish filled with MilliQ water for 20 min at room temperature and transferred to 1.5 ml microcentrifuge tubes. Water was replaced with a solution of 60% acetic acid for a 5-min incubation at room temperature, and samples were then transferred with minimal carry over of excess fluid to a Superfrost Plus positively charged microscope slide (Fisher Thermo Scientific, Waltham, MA). A cover slip was placed on top of the samples with a folded paper towel serving as cushion and pressed with a 35-pound weight for 5 min. The slide was then placed on an aluminum block pre-cooled to −80 °C, the cover slip removed using a razor blade, and the planarian tissue stained for 5 min with a solution containing 1 µg of DAPI per ml of PBS. After 3 washes with PBS, the samples were mounted in an 80% glycerol:20% PBS solution and analyzed by confocal microscopy.

4.3. Immunofluorescence

Immunofluorescence was carried out according to (Forsthoefel et al., 2014) with minor modifications. Briefly, animals starved for 1–2 weeks were sacrificed by incubation in 2% HCl for 6 min, and were then fixed with a Methacarn solution (6:3:1 Methanol:Chloroform:Acetic acid) for 20 min at room temperature. The samples were bleached in methanol containing 6% hydrogen peroxide, rinsed in PBS containing 0.3% Triton-X (PBSTx), and incubated overnight at 4 °C in a blocking solution containing BSA and fish gelatin (Forsthoefel et al., 2011). Samples were then incubated overnight in a primary antibody solution comprised of blocking solution supplemented with Y12 monoclonal antibody (1:250 dilution; NeoMarkers; Cat. # MS-450-P0) and/or anti-phospho-histone H3(Ser10) (PH3(S10)) antibody (1:250 dilution; Life Technologies; Cat. # 44–1190 G), rinsed four times in PBSTx, incubated overnight blocking solution containing Alexa Fluor® 488 anti-mouse or Alexa Fluor® 568 anti-rabbit secondary antibodies (1:500 dilutions; Thermo Fisher Scientific, Waltham, MA), washed four more times with PBSTx and mounted in a 4:1 glycerol:PBS solution.

4.4. RNA preparation and sequencing

RNA extraction using Trizol® Reagent was performed as per the manufacturer's protocol (Invitrogen, Carlsbad, CA). Experimental groups for *G. dorotocephala* MA-C2 included intact animals, freshly dissected auricles, and regenerating fragments both 1 and 4 days post-amputation (DPA). Isolated RNA was treated with DNase, extracted by ethanol precipitation, resuspended in RNase-free water, and submitted to the Roy J. Carver Biotechnology Center (University of Illinois at Urbana-Champaign) for sequencing. Briefly, a cDNA library was generated for each group using Illumina's TruSeq Stranded RNA-seq Sample Prep Kit, yielding cDNA fragments ranging from 80 bp to 1.2 kb (500 bp average size). The libraries were then quantified by qPCR and sequenced on two lanes from each end of the fragments on an Illumina HiSeq® 2500 Sequencing System. Raw reads used for the *G. dorotocephala* MA-C2 assembly are available under NCBI BioProject ID: PRJNA317859. Raw reads used for the *G. dorotocephala* (2n = 16) assembly are available under NCBI BioProject ID: PRJNA320631.

4.5. De novo assembly of G. dorotocephala transcriptomes

Adaptor sequences were trimmed using Trimmomatic (Bolger et al., 2014). After trimming, all the forward (R1) reads for each of the four groups (intact, 1 DPA, 4 DPA, and auricles) were then combined using the *Concatenate datasets* tool in Galaxy (Afgan et al., 2016), and the reverse complement (R2) reads were likewise combined. These R1 and R2 reads were then used as the input for *de novo* assembly of RNA-seq data using Trinity version 2.3.2 (Haas et al., 2013). The draft assembly for *G. dorotocephala* MA-C2 is available online at http://corescholar.libraries.wright.edu/biology/318. The draft assembly for *G. dorotocephala* (2n = 16) is available at http://corescholar.libraries.wright.edu/biology/317.

4.6. Gene constructs

PIWI homologs and stem cell progeny markers found in the MA-C2 transcriptome draft were identified using BlastStation Local-64 (TM Software, Arcadia, CA) and corresponding fragments for *GdPiwi1* and *GdPiwi2* were amplified from *G. dorotocephala* MA-C2 cDNA. Total RNA extracted as described above was used as a template for reverse transcription using the GoScript™ System (Promega, Madison, WI). Then, the primers TR8465F [5′-GACATGGTGAAGTTCTTGACCTTG-3′] and TR8465R [5′-GTGGATTTTCTACGACAATCACCC-3′] were used to amplify *GdPiwi1*, whereas TR3618F [5′-GGAGAGACCGATATCCAAGAGTTC-3′] and TR3618R [5′-ATTCCGCATATGACAACGTTCTTC-3′] were used to amplify *GdPiwi2*. Amplicons were cloned into the pGEM-T Easy (Promega, Madison, WI) and their identity verified through Sanger sequencing (Retrogen Inc., San Diego, CA). Complementary DNA (cDNA) corresponding to *GdPROG1-L1*, *-L2*, *-L3*, *GdAGAT-1*, *GdAGAT-2*, and *GdCPEB-2* sequences, flanked by SP6 and T3 promoters, were synthesized as GeneArt Strings (ThermoFisher, Waltham, MA). GeneArt construct sequences are provided in Supplementary Table S2.

4.7. Whole-mount in situ hybridization

Digoxigenin-11-UTP (Roche, Mannheim, Germany) -labeled riboprobes were synthesized from pGEM-T Easy amplicons using SP6 RNA Polymerase *in vitro* transcription or from GeneArt construct amplicons using T3 RNA Polymerase. Probes were DNase treated, extracted by ethanol precipitation, and used to examine gene expression in whole-mount planarian samples as per (Umesono et al., 1997) with modifications as per (Pearson et al., 2009) and (King and Newmark, 2013). Briefly, planarians of 0.5–1.0 cm length were killed with 10% N-acetyl-cysteine in PBS for 12 min and fixed with formaldehyde as per (Pearson et al., 2009) for seven minutes, bleached with hydrogen

peroxide, Proteinase K-treated and post-fixed as per (King and Newmark, 2013), and subjected to riboprobe hybridization, SSC washes, anti-DIG-Alkaline Phosphatase antibody binding, washing, and developed in a 10% polyvinylalcohol-based solution containing NBT/BCIP as substrate as per (Pearson et al., 2009).

4.8. RNA-interference

Double-stranded RNA was synthesized from T7 promoter-flanked amplicons generated from pGEM-T easy constructs with the following oligo primers: 5′-GCGCGAATTAACCCTCACTAAAGTAATACGACTCACTATAGGG-3′ and 5′-CGCGCGCTAATACGACTCACTATAGGGATTTAGGTGAC-3′. Double-stranded RNA synthesized by T7 RNA Polymerase *in vitro* transcription was annealed by successive 3-min incubations at 90 °C, 75 °C, 50 °C and room temperature, and were then stored at −20 °C or used in feedings. Planarians were fed twice a week in a solution containing 1.5 μg dsRNA per 10 μl of liver paste (2:1 liver purée: water with food dye) as previously described (Rouhana et al., 2013). For regeneration assays, planarians were amputated five days after the fourth feeding, whereas planarians in homeostasis were continually fed for a total of 4 weeks or until phenotypes were observed.

4.9. Imaging/Microscopy

Images of planarians, planarian fragments, and *in situ* hybridization samples were captured by dark field microscopy using an Axio Zoom. V16 stereomicroscope from Zeiss (Oberkochen, Germany) connected to a Canon EOS Rebel T3 digital camera (Tokyo, Japan). Whole-mount immunofluorescence was also imaged using the Zeiss Axio Zoom. V16 stereomicroscope. Images of neoblasts subjected to Y12/PH3(S10) double immunofluorescence and chromosomal spreads for karyotype analysis were obtained using a C2+ confocal microscopy system running NIS Elements software (Nikon, Tokyo, Japan) under a 20X and a 60X objective, respectively.

Author Contributions

E.M.P.A. established and characterized the MA-C2 clonal line, cloned *G. dorotocephala piwi* homologs, and assisted with RNA extraction for RNAseq. S.L.L. performed karyotype analyses. M.P.M. contributed with *de novo* assembly of RNA-seq reads. L.R. performed RNA extractions, sequence analysis of *piwi* homologs and experiments involving neoblast progeny markers. E.M.P.A and L.R. designed the project and wrote the manuscript.

Supporting information

Draft assemblies for *G. dorotocephala* MA-C2 and *G. dorotocephala* (2n = 16) are available online at http://corescholar.libraries.wright.edu/biology/318 and http://corescholar.libraries.wright.edu/biology/317, respectively. The full BLAST2GO results as well as GO annotation for the MA-C2 transcriptome is available at http://corescholar.libraries.wright.edu/biology/336 and a FASTA file the MA-C2 transcriptome sequences matched by BLAST2GO annotation is available at http://corescholar.libraries.wright.edu/biology/337. Raw reads from *G. dorotocephala* MA-C2 and *G. dorotocephala* (2n = 16) RNA-seq are available under NCBI BioProject ID: PRJNA317859 and NCBI BioProject ID: PRJNA320631, respectively.

Acknowledgments

We thank Junichi Tasaki for assistance with microscopy, Scott Baird for feedback during manuscript preparation, and Dr. Alvaro Hernandez from the Roy J. Carver Biotechnology Center (Univ. of Illinois) for guidance with RNA-seq. E.M.P.A. received support from the BioSTAR [NIH Project No. 5R25GM090122-05] and STREAMS [NIH Project No. 5R25HL103168-05] programs at Wright State University. S.L.L. is a Wright State University Considine Scholar. This work was supported by a Biology Award for Research Excellence (BARE) and a Dale True Grant for student experimental research to E.M.P.A., as well as by start-up funds from Wright State University and support from the NIH [Project No. R15HD082754-01] to L.R.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2017.07.022.

References

Accorsi, A., Williams, M.M., Ross, E.J., Robb, S.M.C., Elliott, S.A., Tu, K.C., Alvarado, A.S., 2017. Hands-on classroom activities for exploring regeneration and stem cell biology with Planarians. Am. Biol. Teach. 79, 208–223.

Afgan, E., Baker, D., van den Beek, M., Blankenberg, D., Bouvier, D., Cech, M., Chilton, J., Clements, D., Coraor, N., Eberhard, C., et al., 2016. The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2016 update. Nucleic Acids Res. 44, W3–W10.

Alvarez-Presas, M., Baguna, J., Riutort, M., 2008. Molecular phylogeny of land and freshwater planarians (Tricladida, Platyhelminthes): from freshwater to land and back. Mol. Phylogenet. Evol. 47, 555–568.

Artois, T., Tyler, S., 2015. *Girardia dorotocephala* (Woodworth, 1897). In: Tyler, S., Artois, T., Schilling, S., Hooge, M., and Bush, L.F. (Eds.) (2006–2017). World list of Platyhelminthes. Accessed through: World Register of Marine Species at ⟨http://www.marinespecies.org/aphia.php?P=taxdetails&id=482171⟩.

Baguña, J., Carranza, S., Pala, M., Ribera, C., Giribet, G., Arnedo, M.A., Ribas, M., Ruitort, M., 1999. From morphology and karyology to molecules. New methods for taxonomical identification of asexual populations of freshwater planarians. A tribute to professor Mario Benazzi. Ital. J. Zool. (Modena) 66, 207–214.

Ball, I.R., 1971. Systematic and biogeographical relationships of some *Dugesia* species (Tricladida, Pauludicola) from Central and South America. Am. Mus. Novit. No. 2472, 1–25.

Ball, I.R., Gourbault, N., 1978. Phyletic status of genus *Planaria* (Platyhelminthes, Turbellaria, Tricladida). Bijdr. Dierkd. 48, 29–34.

Bardeen, Baetjer, F.H., 1904. The inhibitive action of the Roentgen rays on regeneration in planarians. J. Exp. Zool. i, 191–195.

Benazzi, M., 1974. New Karyotype in American Freshwater Planarian *Dugesia dorotocephala*. Syst. Zool. 23, 490–492.

Best, J.B., Elshtain, E., Wilson, D.D., 1967. Single monophasic square-wave electric pulse excitation of the planarian Dugesia dorotocephala. J. Comp. Physiol. Psychol. 63, 198–207.

Best, J.B., Abelein, M., Kreutzer, E., Pigon, A., 1975. Cephalic mechanism for social control of fissioning in planarians: iii. Central nervous system centers of facilitation and inhibition. J. Comp. Physiol. Psychol. 89, 923–932.

Block, R.A., McConnell, J.V., 1967. Classically conditioned discrimination in the planarian, *Dugesia dorotocephala*. Nature 215, 1465–1466.

Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30, 2114–2120.

Brandl, H., Moon, H., Vila-Farre, M., Liu, S.Y., Henry, I., Rink, J.C., 2016. PlanMine–a mineable resource of planarian biology and biodiversity. Nucleic Acids Res. 44, D764–D773.

Brøndsted, H.V., 1969. Planarian Regeneration 1st edn. Pergamon Press, Oxford, New York.

Burke, W.D., Singh, D., Eickbush, T.H., 2003. R5 retrotransposons insert into a family of infrequently transcribed 28S rRNA genes of planaria. Mol. Biol. Evol. 20, 1260–1270.

Cebria, F., Newmark, P.A., 2005. Planarian homologs of netrin and netrin receptor are required for proper regeneration of the central nervous system and the maintenance of nervous system architecture. Development 132, 3691–3703.

Child, C.M., 1909. The regulatory change of shape in Planaria dorotocephala. Biol. Bull. 16, 277–296.

Child, C.M., 1911. Studies on the dynamics of morphogenesis and inheritance in experimental reproduction the I Axial gradient in *Planaria dorotocephala* as a limiting factor in regulation. J. Exp. Zool. 10, 265–320.

Coward, S.J., 1974. Chromatoid bodies in somatic cells of the planarian: observations on their behavior during mitosis. Anat. Rec. 180, 533–545.

Curtis, W.C., Hickman, J., 1926. Effects of x-rays and radium upon regeneration in planarians. Anat. Rec. Phila. 34, 145–146.

Davies, E.L., Lei, K., Seidel, C.W., Kroesen, A.E., McKinney, S.A., Guo, L., Robb, S.M., Ross, E.J., Gotting, K., Sanchez Alvarado, A., 2017. Embryonic origin of adult stem cells required for tissue homeostasis and regeneration. Elife 6.

Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J.F., Guindon, S., Lefort, V., Lescot, M., et al., 2008. Phylogeny.fr: robust phylogenetic analysis for the non-specialist. Nucleic Acids Res. 36, W465–W469.

Eberhardt, L.S., Swanson, R.J., Dulin, G.S., 2015. Engaging students in inquiry through behavioral bioassays of aquatic macroinvertebrates. Am. Biol. Teach. 77, 681–688.

Eisenhoffer, G.T., Kang, H., Sanchez Alvarado, A., 2008. Molecular analysis of stem cells and their descendants during cell turnover and regeneration in the planarian Schmidtea mediterranea. Cell Stem Cell 3, 327–339.

Elliott, S.A., Sanchez Alvarado, A., 2013. The history and enduring contributions of planarians to the study of animal regeneration. Wiley Interdiscip. Rev. Dev. Biol. 2, 301–326.
Emmons-Bell, M., Durant, F., Hammelman, J., Bessonov, N., Volpert, V., Morokuma, J., Pinet, K., Adams, D.S., Pietak, A., Lobo, D., et al., 2015. Gap junctional blockade stochastically induces different species-specific head anatomies in genetically wild-type *Girardia dorotocephala* Flatworms. Int. J. Mol. Sci. 16, 27865–27896.
Flickinger, R.A., Coward, S.J., 1962. The induction of cephalic differentiation in regenerating *Dugesia dorotocephala* in the presence of the normal head and in unwounded tails. Dev. Biol. 5, 179–204.
Forsthoefel, D.J., Park, A.E., Newmark, P.A., 2011. Stem cell-based growth, regeneration, and remodeling of the planarian intestine. Dev. Biol. 356, 445–459.
Forsthoefel, D.J., Waters, F.A., Newmark, P.A., 2014. Generation of cell type-specific monoclonal antibodies for the planarian and optimization of sample processing for immunolabeling. BMC Dev. Biol. 14, 45.
Fraguas, S., Barberan, S., Cebria, F., 2011. EGFR signaling regulates cell proliferation, differentiation and morphogenesis during planarian regeneration and homeostasis. Dev. Biol. 354, 87–101.
Grabherr, M.G., Haas, B.J., Yassour, M., Levin, J.Z., Thompson, D.A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q., et al., 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat. Biotechnol. 29, 644–652.
Grebe, E., Schaeffer, D.J., 1991. Neurobehavioral toxicity of cadmium sulfate to the planarian *Dugesia dorotocephala*. Bull. Environ. Contam. Toxicol. 46, 727–730.
Grudniewska, M., Mouton, S., Simanov, D., Beltman, F., Grelling, M., de Mulder, K., Arindrarto, W., Weissert, P.M., van der Elst, S., Berezikov, E., 2016. Transcriptional signatures of somatic neoblasts and germline cells in *Macrostomum lignano*. Elife 5.
Guo, T., Peters, A.H., Newmark, P.A., 2006. A Bruno-like gene is required for stem cell maintenance in planarians. Dev. Cell 11, 159–169.
Haas, B.J., Papanicolaou, A., Yassour, M., Grabherr, M., Blood, P.D., Bowden, J., Couger, M.B., Eccles, D., Li, B., Lieber, M., et al., 2013. De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. Nat. Protoc. 8, 1494–1512.
Hagstrom, D., Cochet-Escartin, O., Zhang, S., Khuu, C., Collins, E.M., 2015. Freshwater Planarians as an Alternative Animal Model for Neurotoxicology. Toxicol. Sci. 147, 270–285.
Hagstrom, D., Cochet-Escartin, O., Collins, E.M., 2016. Planarian brainregeneration as a model system for developmental neurotoxicology. Regeneration 3, 65–77.
Hampton, A.M., 1988. Altitudinal range and habitat of Triclads in streams of the Lake Tahoe Basin. Am. Midl. Nat. 120, 302–312.
Hicks, C., Sorocco, D., Levin, M., 2006. Automated analysis of behavior: a computer-controlled system for drug screening and the investigation of learning. J. Neurobiol. 66, 977–990.
Hyman, L.H., 1925. The reproductive system and other characters of *Planaria dorotocephala* Woodworth trans. Am. Microsc. Soc. 44, 51–89.
Hyman, L.H., 1929. Studies on the morphology, taxonomy, and distribution of North American Triclad Turbellaria. II. On the distinction between *Planaria agilis* and *Planaria dorotocephala* with notes on the distribution of ayilis in the Western United States. Trans. Am. Microsc. Soc. 48, 406–415.
Hyman, L.H., 1939. North American Triclad Turbellaria. ix. The priority of *Dugesia Girard* 1850 over Euplanaria Hesse 1897 with notes on American species of Dugesia. Trans. Am. Microsc. Soc. 58, 264–275.
Hyman, L.H., 1956. North American triclad Turbellaria. 15. Three new species. Am. Mus. Novit. No. 1808, 1–14.
Inoue, T., Hoshino, H., Yamashita, T., Shimoyama, S., Agata, K., 2015. Planarian shows decision-making behavior in response to multiple stimuli by integrative brain function. Zool. Lett. 1, 7.
Ishizuka, H., Maezawa, T., Kawauchi, J., Nodono, H., Hirao, Y., Nishimura, O., Nakagawa, H., Sekii, K., Tasaka, K., Tarui, H., et al., 2007. The *Dugesia ryukyuensis* database as a molecular resource for studying switching of the reproductive system. Zool. Sci. 24, 31–37.
Jenkins, M.M., 1967. Aspects of Planarian Biology and Behavior. Chemistry of Learning. Springer, 116–143.
Jensen, L.A., Allen, A.L., 1983. Transparency master: planaria in the classroom. Am. Biol. Teach. 45, 170–171.
Kapu, M.M., Schaeffer, D.J., 1991. Planarians in toxicology. Responses of asexual *Dugesia dorotocephala* to selected metals. Bull. Environ. Contam. Toxicol. 47, 302–307.
Kashima, M., Kumagai, N., Agata, K., Shibata, N., 2016. Heterogeneity of chromatoid bodies in adult pluripotent stem cells of planarian *Dugesia japonica*. Dev. Growth Differ. 58, 225–237.
Kawakatsu, M., Mitchell, R.W., 1981. Freshwater planarians from the southern United States and Mexico: *Dugesia dorotocephala* and Dugesia sp. (Turbellaria, Tricladida, Paludicola.). Annot. Zool. Jpn. 54, 191–206.
Kawakatsu, M., Mitchell, R.W., Hirao, Yh, Tanaka, I., 1984. Occurrence of *Dugesia dorotocephala* (Woodworth, 1897) (Turbellaria, Tricladida, Paludicola) in Honolulu, Hawaii. Biol. Mag. Okinawa, 1–9.
Kawakatsu, M., Nishino, M., Ohtaka, A., 2007. Currently known exotic planarians from Japan. Jpn. J. Limnol. 68, 461–469.
Kenk, R., 1944. The fresh-water tri-clads of Michigan. Misc. Publ. Mus. Zool. Univ. Mich. No. 60, 44.
Kenk, R., 1974. History of the study of Turbellaria in North America. In: Riser, N.W., Morse, M.P. (Eds.), Biology of the Turbellaria. vii-xv, 1-530. McGraw-Hill, New York, 17–22.
Kenk, R., 1989. Revised list of the North American freshwater Planarians (Platyhelminthes:Tricladida:Paludicola). Smithsonian Institution Press, Washington, D.C..
King, R.S., Newmark, P.A., 2013. In situ hybridization protocol for enhanced detection of gene expression in the planarian *Schmidtea mediterranea*. BMC Dev. Biol. 13, 8.
Kostelecky, J., Elliott, B., Schaeffer, D.J., 1989. Planarians in toxicology. I. Physiology of sexual-only *Dugesia dorotocephala:* effects of diet and population density on adult weight and cocoon production. Ecotoxicol. Environ. Saf. 18, 286–295.
Legner, E.F., Tsai, S.C., 1978. Increasing fission rate of planarian mosquito predator, Dugesia-dorotocephala, through biological filtration. Entomophaga 23, 293–298.
Lerner, E.A., Lerner, M.R., Janeway, C.A., Jr., Steitz, J.A., 1981. Monoclonal antibodies to nucleic acid-containing cellular constituents: probes for molecular biology and autoimmune disease. Proc. Natl. Acad. Sci. USA 78, 2737–2741.
Levin, M., Anavy, L., Cole, A.G., Winter, E., Mostov, N., Khair, S., Senderovich, N., Kovalev, E., Silver, D.H., Feder, M., et al., 2016. The mid-developmental transition and the evolution of animal body plans. Nature 531, 637–641.
Liu, S.Y., Selck, C., Friedrich, B., Lutz, R., Vila-Farre, M., Dahl, A., Brandl, H., Lakshmanaperumal, N., Henry, I., Rink, J.C., 2013. Reactivating head regrowth in a regeneration-deficient planarian species. Nature 500, 81–84.
Lombardo, P., Giustini, M., Miccoli, F.P., Cicolani, B., 2011. Fine-scale differences in diel activity among nocturnal freshwater planarias (Platyhelminthes: tricladida). J. Circadian Rhythms 9, 2.
Mairs, J.D., 1965. A Guide to Laboratory Activities Using Live Planaria for Secondary School Advanced Biology. Drake University.
Majdi, N., Boiche, A., Traunspurger, W., Lecerf, A., 2014. Predator effects on a detritus-based food web are primarily mediated by non-trophic interactions. J. Anim. Ecol. 83, 953–962.
Morgan, T.H., 1900. Regeneration in planarians. Arch. Entwickl. Org. 10, 58–119.
Morita, M., Best, J.B., 1965. Electron microscopic studies on Planaria. II. Fine structure of the neurosecretory system in the planarian *Dugesia dorotocephala*. J. Ultrastruct. Res. 13, 396–408.
Muttkowski, R.A., 1918. The fauna of Lake Mendota. University of Wisconsin–Madison..
Nakagawa, H., Ishizu, H., Hasegawa, R., Kobayashi, K., Matsumoto, M., 2012. Drpiwi-1 is essential for germline cell formation during sexualization of the planarian *Dugesia ryukyuensis*. Dev. Biol. 361, 167–176.
Nascetti, G., Bullini, L., Benazzi, M., 1990. Genetic-divergence in North-American fresh-water planarians of the Dugesia-dorotocephala Group (Turbellaria, Tricladida, Paludicola). Z. Zool. Syst. Evol. 28, 261–268.
Newmark, P.A., 2005. Opening a new can of worms: a large-scale RNAi screen in planarians. Dev. Cell 8, 623–624.
Newmark, P.A., Sanchez Alvarado, A., 2000. Bromodeoxyuridine specifically labels the regenerative stem cells of planarians. Dev. Biol. 220, 142–153.
Newmark, P.A., Sanchez Alvarado, A., 2002. Not your father's planarian: a classic model enters the era of functional genomics. Nat. Rev. Genet. 3, 210–219.
Newmark, P.A., Reddien, P.W., Cebria, F., Sanchez Alvarado, A., 2003. Ingestion of bacterially expressed double-stranded RNA inhibits gene expression in planarians. Proc. Natl. Acad. Sci. USA 100 (Suppl 1), 11861–11865.
Nishimura, O., Hirao, Y., Tarui, H., Agata, K., 2012. Comparative transcriptome analysis between planarian *Dugesia japonica* and other platyhelminth species. BMC Genom. 13, 289.
Nishimura, O., Hosoda, K., Kawaguchi, E., Yazawa, S., Hayashi, T., Inoue, T., Umesono, Y., Agata, K., 2015. Unusually large number of mutations in asexually reproducing clonal planarian *Dugesia japonica*. PLoS One 10, e0143525.
Nixon, S.E., 1981. Freshwater triclads (Turbellaria) of Arizona. Southwest. Nat. 25, 469–477.
Orii, H., Agata, K., Watanabe, K., 1993. POU-domain genes in planarian *Dugesia japonica*: the structure and expression. Biochem. Biophys. Res. Commun. 192, 1395–1402.
Orii, H., Mochii, M., Watanabe, K., 2003. A simple "soaking method" for RNA interference in the planarian *Dugesia japonica*. Dev. Genes Evol. 213, 138–141.
Pagan, O.R., Coudron, T., Kaneria, T., 2009. The flatworm planaria as a toxicology and behavioral pharmacology animal model in undergraduate research experiences. J. Undergrad. Neurosci. Educ. 7, A48–A52.
Palakodeti, D., Smielewska, M., Lu, Y.C., Yeo, G.W., Graveley, B.R., 2008. The PIWI proteins SMEDWI-2 and SMEDWI-3 are required for stem cell function and piRNA expression in planarians. RNA 14, 1174–1186.
Pearson, B.J., Sanchez Alvarado, A., 2010. A planarian p53 homolog regulates proliferation and self-renewal in adult stem cell lineages. Development 137, 213–221.
Pearson, B.J., Eisenhoffer, G.T., Gurley, K.A., Rink, J.C., Miller, D.E., Sanchez Alvarado, A., 2009. Formaldehyde-based whole-mount in situ hybridization method for planarians. Dev. Dyn.: Off. Publ. Am. Assoc. Anat. 238, 443–450.
Puccinelli, I., Deri, P., 1991. Comparative Karyological analysis of some American planarians belonging to the genus *Dugesia* (Subgenus *Girardia*) (Platyhelminthes, Tricladida). Caryologia 44, 225–232.
Reddien, P.W., Sanchez Alvarado, A., 2004. Fundamentals of planarian regeneration. Annu. Rev. Cell Dev. Biol. 20, 725–757.
Reddien, P.W., Bermange, A.L., Murfitt, K.J., Jennings, J.R., Sanchez Alvarado, A., 2005a. Identification of genes needed for regeneration, stem cell function, and tissue homeostasis by systematic gene perturbation in planaria. Dev. Cell 8, 635–649.
Reddien, P.W., Oviedo, N.J., Jennings, J.R., Jenkin, J.C., Sanchez Alvarado, A., 2005b. SMEDWI-2 is a PIWI-like protein that regulates planarian stem cells. Science 310, 1327–1330.
Rink, J.C., 2013. Stem cell systems and regeneration in planaria. Dev. Genes Evol. 223, 67–84.
Robb, S.M., Ross, E., Sanchez Alvarado, A., 2008. SmedGD: the Schmidtea mediterranea genome database. Nucleic Acids Res. 36, D599–D606.
Robb, S.M., Gotting, K., Ross, E., Sanchez Alvarado, A., 2015. SmedGD 2.0: the Schmidtea mediterranea genome database. Genesis 53, 535–546.

Rossi, A., Ross, E.J., Jack, A., Sanchez Alvarado, A., 2014. Molecular cloning and characterization of SL3: a stem cell-specific SL RNA from the planarian Schmidtea mediterranea. Gene 533, 156–167.
Rossi, L., Salvetti, A., Lena, A., Batistoni, R., Deri, P., Pugliesi, C., Loreti, E., Gremigni, V., 2006. *DjPiwi-1*, a member of the PAZ-Piwi gene family, defines a subpopulation of planarian stem cells. Dev. Genes Evol. 216, 335–346.
Rouhana, L., Shibata, N., Nishimura, O., Agata, K., 2010. Different requirements for conserved post-transcriptional regulators in planarian regeneration and stem cell maintenance. Dev. Biol. 341, 429–443.
Rouhana, L., Vieira, A.P., Roberts-Galbraith, R.H., Newmark, P.A., 2012. PRMT5 and the role of symmetrical dimethylarginine in chromatoid bodies of planarian stem cells. Development 139, 1083–1094.
Rouhana, L., Weiss, J.A., Forsthoefel, D.J., Lee, H., King, R.S., Inoue, T., Shibata, N., Agata, K., Newmark, P.A., 2013. RNA interference by feeding in vitro-synthesized double-stranded RNA to planarians: methodology and dynamics. Dev. Dyn.: Off. Publ. Am. Assoc. Anat. 242, 718–730.
Rouhana, L., Weiss, J.A., King, R.S., Newmark, P.A., 2014. PIWI homologs mediate histone H4 mRNA localization to planarian chromatoid bodies. Development 141, 2592–2601.
Rouhana, L., Tasaki, J., Saberi, A., Newmark, P.A., 2017. Genetic dissection of the planarian reproductive system through characterization of *Schmidtea mediterranea* CPEB homologs. Dev. Biol. 426, 43–55.
Salo, E., Abril, J.F., Adell, T., Cebria, F., Eckelt, K., Fernandez-Taboada, E., Handberg-Thorsager, M., Iglesias, M., Molina, M.D., Rodriguez-Esteban, G., 2009. Planarian regeneration: achievements and future directions after 20 years of research. Int. J. Dev. Biol. 53, 1317–1327.
Sanchez Alvarado, A., Newmark, P., 2001. Regeneration in Planaria, Encyclopedia of Life Sciences. Nature Publ. Group, London, 1–7.
Sanchez Alvarado, A., Newmark, P.A., 1999. Double-stranded RNA specifically disrupts gene expression during planarian regeneration. Proc. Natl. Acad. Sci. USA 96, 5049–5054.
Sanchez Alvarado, A., Newmark, P.A., Robb, S.M., Juste, R., 2002. The *Schmidtea mediterranea* database as a molecular resource for studying *platyhelminthes*, stem cells and regeneration. Development 129, 5659–5665.
Sasidharan, V., Lu, Y.C., Bansal, D., Dasari, P., Poduval, D., Seshasayee, A., Resch, A.M., Graveley, B.R., Palakodeti, D., 2013. Identification of neoblast- and regeneration-specific miRNAs in the planarian *Schmidtea mediterranea*. RNA 19, 1394–1404.
Scadding, S.R., 1985. Classroom study of the control of mitosis in planaria. Am. Biol. Teach. 47, 428–429.
Scimone, M.L., Meisel, J., Reddien, P.W., 2010. The Mi-2-like Smed-CHD4 gene is required for stem cell differentiation in the planarian *Schmidtea mediterranea*. Development 137, 1231–1241.
Shibata, N., Rouhana, L., Agata, K., 2010. Cellular and molecular dissection of pluripotent adult somatic stem cells in planarians. Dev., Growth Differ. 52, 27–41.
Shibata, N., Kashima, M., Ishiko, T., Nishimura, O., Rouhana, L., Misaki, K., Yonemura, S., Saito, K., Siomi, H., Siomi, M.C., et al., 2016. Inheritance of a nuclear PIWI from pluripotent stem cells by somatic descendants ensures differentiation by silencing transposons in planarian. Dev. Cell 37, 226–237.
Shomrat, T., Levin, M., 2013. An automated training paradigm reveals long-term memory in planarians and its persistence through head regeneration. J. Exp. Biol. 216, 3799–3810.
Sikes, J.M., Newmark, P.A., 2013. Restoration of anterior regeneration in a planarian with limited regenerative ability. Nature 500, 77–80.
Simao, F.A., Waterhouse, R.M., Ioannidis, P., Kriventseva, E.V., Zdobnov, E.M., 2015. BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. Bioinformatics 31, 3210–3212.
Sluys, R., Kawakatsu, M., Ponce De Leon, R., 2005. Morphological stasis in an old and widespread group of species: contribution to the taxonomy and biogeography of the genus Girardia (Platyhelminthes, Tricladida, Paludicola). Stud. Neotrop. Fauna Environ. 40, 155–180.
Sluys, R., Kawakatsu, M., Riutort, M., Baguna, J., 2009. A new higher classification of planarian flatworms (Platyhelminthes, Tricladida). J. Nat. Hist. 43, 1763–1777.
Sluys, R., Kawakatsu, M., Yamamoto, K., 2010. Exotic freshwater planarians currently known from Japan. Belg. J. Zool. 140, 103–U138.
Sluys, R., Vila-Farre, M., Alvarez-Presas, M., Riutort, M., Kawakatsu, M., Tulp, A.S., 2015. The diet and distribution of *Pentacoelum kazukolinda* (Kawakatsu & Mitchell, 1984), a maricolan planarian with a freshwater ecology. Zool. Scr. 44, 72–91.
Solana, J., Kao, D., Mihaylova, Y., Jaber-Hijazi, F., Malla, S., Wilson, R., Aboobaker, A., 2012. Defining the molecular profile of planarian pluripotent stem cells using a combinatorial RNAseq, RNA interference and irradiation approach. Genome Biol. 13, R19.
Stringer, C.E., 1909. Notes on Nebraska Turbellaria with descriptions of two new species. Zool. Anz. 34.
Tasaki, J., Shibata, N., Nishimura, O., Itomi, K., Tabata, Y., Son, F., Suzuki, N., Araki, R., Abe, M., Agata, K., et al., 2011. ERK signaling controls blastema cell differentiation during planarian regeneration. Development 138, 2417–2427.
Tu, K.C., Cheng, L.C., H, T.K.V., Lange, J.J., McKinney, S.A., Seidel, C.W., Sanchez Alvarado, A., 2015. Egr-5 is a post-mitotic regulator of planarian epidermal differentiation. Elife 4, e10501.
Umesono, Y., Watanabe, K., Agata, K., 1997. *A planarian orthopedia homolog is* specifically expressed in the branch region of both the mature and regenerating brain. Dev. Growth Differ. 39, 723–727.
Umesono, Y., Tasaki, J., Nishimura, Y., Hrouda, M., Kawaguchi, E., Yazawa, S., Nishimura, O., Hosoda, K., Inoue, T., Agata, K., 2013. The molecular logic for planarian regeneration along the anterior-posterior axis. Nature 500, 73–76.
Valverde, P., 2015. Implementation of biotechnology projects of different Inquiry level to promote Career success of non-science majors. FASEB J. 29, LB73.
van Wolfswinkel, J.C., Wagner, D.E., Reddien, P.W., 2014. Single-cell analysis reveals functionally distinct classes within the planarian stem cell compartment. Cell Stem Cell 15, 326–339.
Wagner, D.E., Wang, I.E., Reddien, P.W., 2011. Clonogenic neoblasts are pluripotent adult stem cells that underlie planarian regeneration. Science 332, 811–816.
Wagner, D.E., Ho, J.J., Reddien, P.W., 2012. Genetic regulators of a pluripotent adult stem cell system in planarians identified by RNAi and clonal analysis. Cell Stem Cell 10, 299–311.
Watermolen, D.J., 2005. Aquatic and terrestrial flatworm (Platyhelminthes, Turbellaria) and ribbon worm (Nemertea) records from Wisconsin. Wis. Dep. Nat. Resour. Res. Manag. Find. 55, 8.
Wheeler, N.J., Agbedanu, P.N., Kimber, M.J., Ribeiro, P., Day, T.A., Zamanian, M., 2015. Functional analysis of *Girardia tigrina* transcriptome seeds pipeline for anthelmintic target discovery. Parasit. Vectors 8, 34.
Woodworth, W.M., 1897. Contributions to the Morphology of the Turbellaria: On Some Turbellaria from Illinois. Museum.
Wurtzel, O., Cote, L.E., Poirier, A., Satija, R., Regev, A., Reddien, P.W., 2015. A generic and cell-type-specific wound response precedes regeneration in planarians. Dev. Cell 35, 632–645.
Xiang, Y., Miller, D.E., Ross, E.J., Sanchez Alvarado, A., Hawley, R.S., 2014. Synaptonemal complex extension from clustered telomeres mediates full-length chromosome pairing in *Schmidtea mediterranea*. Proc. Natl. Acad. Sci. USA 111, E5159–E5168.
Zayas, R.M., Hernandez, A., Habermann, B., Wang, Y., Stary, J.M., Newmark, P.A., 2005. The planarian Schmidtea mediterranea as a model for epigenetic germ cell specification: analysis of ESTs from the hermaphroditic strain. Proc. Natl. Acad. Sci. USA 102, 18491–18496.