

## Stem cells are differentially regulated during development, regeneration and homeostasis in flatworms

Katrien De Mulder<sup>a,b</sup>, Daniela Pfister<sup>a</sup>, Georg Kualess<sup>a</sup>, Bernhard Egger<sup>a</sup>, Willi Salvenmoser<sup>a</sup>, Maxime Willems<sup>b</sup>, Jessica Steger<sup>c</sup>, Katja Fauster<sup>c</sup>, Ronald Micura<sup>c</sup>, Gaetan Borgonie<sup>b</sup>, Peter Ladurner<sup>a,\*</sup>

<sup>a</sup> University of Innsbruck, Institute of Zoology and CMBl, Technikerstrasse 25, A-6020 Innsbruck, Austria

<sup>b</sup> University of Ghent, Department of Biology, Ledeganckstraat 35, B-9000 Ghent, Belgium

<sup>c</sup> University of Innsbruck, Institute of Organic Chemistry, Innrain 52a, A-6020 Innsbruck, Austria

### ARTICLE INFO

#### Article history:

Received for publication 22 February 2009

Revised 15 July 2009

Accepted 15 July 2009

Available online 23 July 2009

#### Keywords:

Evolution

Neoblasts

Planaria

Piwi

Germline

Stem cell

### ABSTRACT

The flatworm stem cell system is exceptional within the animal kingdom, as totipotent stem cells (neoblasts) are the only dividing cells within the organism. In contrast to most organisms, *piwi*-like gene expression in flatworms is extended from germ cells to somatic stem cells. We describe the isolation and characterization of the *piwi* homologue *macpiwi* in the flatworm *Macrostomum lignano*. We use *in situ* hybridization, antibody staining and RNA interference to study *macpiwi* expression and function in adults, during postembryonic development, regeneration and upon starvation. We found novelties regarding *piwi* function and observed differences to current *piwi* functions in flatworms. First, *macpiwi* was essential for the maintenance of somatic stem cells in adult animals. A knock-down of *macpiwi* led to a complete elimination of stem cells and death of the animals. Second, the regulation of stem cells was different in adults and regenerates compared to postembryonic development. Third, sexual reproduction of *M. lignano* allowed to follow germline formation during postembryonic development, regeneration, and starvation. Fourth, *piwi* expression in hatchlings further supports an embryonic formation of the germline in *M. lignano*. Our findings address new questions in flatworm stem cell research and provide a basis for comparison with higher organisms.

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### Introduction

The unique ability of stem cells to self-renew and to produce large numbers of differentiated progeny is crucial for development and tissue homeostasis (Lin and Schagat, 1997; Lin and Spradling, 1997; Morrison et al., 1997; Morrison and Spradling, 2008). In the last decennia, homologous genes have been characterized which are involved in stem cell maintenance, a process which seems to be much more evolutionary conserved than previously thought (Benfey, 1999; Cerutti and Casas-Mollano, 2006; Hutvagner and Simard, 2008; Loh et al., 2008; Pan and Thomson, 2007). Members of the Argonaute (Ago) family represent a class of proteins known to be required for stem cell self-renewal in a diversity of organisms. Ago-like genes play a crucial role during development, stem cell maintenance and differentiation, as well as for endogenous RNA interference and mobilization control of retrotransposons (Cox et al., 1998). Piwi/Ago family members were recently identified in a broad range of organisms, including ecdysozoans (Cox et al., 1998; Deng and Lin, 2001; Wang and Reinke, 2008), lophotrochozoans (Pfister et al., 2007; Rebscher et al., 2007; Reddien et al., 2005; Rossi et al., 2006; Sugio et al., 2008), deuterostomes (Kuramochi-Miyagawa et al., 2001; Sharma et al.,

2001; Song and Wessel, 2007; Tan et al., 2002), cnidarians (Bosch, 2004; Denker et al., 2008; Seipel et al., 2004), fungi (Carmell et al., 2002; Catalanotto et al., 2000), ciliates (Fetzer et al., 2002; Mochizuki et al., 2002) and plants (Carmell et al., 2002; Cox et al., 1998; Moussian et al., 2003).

Members of the Piwi/Argonaute family are further classified into two subfamilies (Hutvagner and Simard, 2008). AGO-proteins are evolutionary conserved within the eukaryotes and ubiquitously expressed in many different tissues. Ago proteins act in silencing processes through a miRNA/siRNA mediated pathway to recognize their target genes (Zhou et al., 2007). In contrast, PIWI-proteins, originally named after *Drosophila piwi* (P-element induced wimpy testis), seem to be animal specific and mostly implicated in germline specific events such as germ cell development and maintenance (Cox et al., 1998, 2000; Houwing et al., 2007; Kalmykova et al., 2005). The detailed characterization of *piwi*-like genes has further attracted attention when a new type of small non-coding RNAs, so called piRNAs (*piwi* interacting RNAs), was discovered (Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006; Watanabe et al., 2006). Recent publications describe in detail how *piwi*-like genes perform their function using a wide range of regulation machineries, such as chromatin remodelling and genome structure modification by histone and DNA methylation (Brennecke et al., 2008; Pal-Bhadra et al., 2004; Postberg et al., 2008), mRNA stability (degradation, cleavage and

\* Corresponding author. Fax: +43 512 507 2930.

E-mail address: [peter.ladurner@uibk.ac.at](mailto:peter.ladurner@uibk.ac.at) (P. Ladurner).

cleavage-independent mRNA decay) (Doi et al., 2003; Klattenhoff and Theurkauf, 2008; Ma et al., 2005; Parker et al., 2006) and translational repression (Pal-Bhadra et al., 2002; Tijsterman et al., 2002; Vaucheret et al., 2001; Hutvagner and Simard, 2008).

In most organisms studied so far, *piwi*-like genes are mainly expressed in germline stem cells. Interestingly, in triclad flatworms, *piwi* expression was found to be extended in somatic stem cells (Palakodeti et al., 2008; Reddien et al., 2005; Rossi et al., 2006). In *Schmidtea mediterranea*, three *piwi*-like genes were identified, that are expressed within the somatic stem cell population. RNAi of *smedwi2* resulted in a failure of tissue homeostasis and complete loss of regeneration capacity, suggesting a crucial role of *piwi*-like genes in somatic stem cells. Even though stem cells were present and capable to proliferate in response to wounding (Reddien et al., 2005), prolonged treatment resulted in loss of tissue homeostasis due to defects in stem cell differentiation. In the triclad flatworm *Dugesia japonica*, the *piwi*-like gene *Djpiwi-1* was expressed in a subpopulation of neoblasts (Rossi et al., 2006).

We are focussing on the stem cell system of the flatworm *Macrostomum lignano* (Platyhelminthes, Macrostomida) (Ladurner et al., 2005a, 2008). Recent work on this species demonstrates several advantages and benefits to study stem cell biology and regeneration, complementary to the well studied triclads (“planarians”). First, the vast morphological knowledge and transparency of *M. lignano* (Ladurner et al., 2005a, 2008) allows to analyze in detail the effects of starvation, gene specific RNA interference, hydroxyurea treatment, regeneration and irradiation on a morphological level (Nimeth et al., 2002, 2004, 2007; Pfister et al., 2007, 2008). Second, cell proliferation can be easily studied using a simple BrdU exposure by soaking to follow stem cell dynamics during different biological processes (Ladurner et al., 2000; Nimeth et al., 2002, 2004, 2007; Pfister et al., 2007, 2008). Next, the ease of culturing and the unlimited access to single eggs the whole year round allow the analysis of the embryonic development (Morris et al., 2006), a biological question which is not easy to analyze in triclad flatworms (Cardona et al., 2006). Furthermore, *M. lignano* is able to regenerate the whole posterior part including the gonads from a small head stump (Egger et al., 2006) and therefore provides a suitable system to elucidate the molecular mechanisms governing regeneration, stem cell- and germ cell function (Pfister et al., 2008). In addition, *M. lignano* is a solely sexual reproducing obligatory cross fertilizing hermaphrodite (Schärer and Ladurner, 2003), which allows to study the development of the gonads in detail. Finally, the availability of ESTs (<http://flatworm.uibk.ac.at/macest/>; Morris et al., 2006, Ladurner, Pfister unpublished), current efforts in genome sequencing (Berezikov, Ladurner, Schärer, unpublished), the availability of monoclonal antibodies (Ladurner et al., 2005b) and *in situ* hybridization and RNA interference protocols (Pfister et al., 2007, 2008), allow to address various developmental and evolutionary questions with this organism.

In this study, we report on the isolation and characterization of the *piwi* homologue *macpiwi* in the flatworm *M. lignano* (Platyhelminthes, Macrostomida). We used *in situ* hybridization, antibody staining and RNA interference to study *macpiwi* expression dynamics and function in adults, during postembryonic development, regeneration and starvation. We show that *macpiwi* is expressed in both testes and ovaries, as well as in a subpopulation of somatic neoblasts, demonstrating the heterogeneity of the stem cell system. We found that *macpiwi* is essential in adults for tissue homeostasis. In contrast to planarians, *M. lignano* stem cells fail to activate proliferation upon wounding under *macpiwi* treated conditions. Interestingly, stem cells seem to be differentially regulated during postembryonic development and regeneration as hatchlings were able to grow and maintain cell proliferation under the same conditions although became sensitive when adulthood was reached. Finally, our data further substantiate the hypothesis of embryonic formation of germ cells in *M. lignano*. The obtained results

contribute to a better understanding of the specific function of *piwi*-like genes in flatworms and other organisms with complex stem cell systems.

## Material and methods

### Animal culture

*M. lignano* (Platyhelminthes, Macrostomida) was kept in petri dishes in nutrient-enriched f/2 artificial sea water and fed ad libitum with diatoms (*Nitzschia curvilineata*) (Ladurner et al., 2005a). Climate chamber conditions were set at 20 °C and 60% humidity, following a 14/10 h day/night cycle (Rieger et al., 1988).

### Cloning of *macpiwi*

A partial sequence of *macpiwi* was obtained from an earlier EST project (Angu 7606) (Morris et al., 2006). Amplification of the 3' end was performed using a SMART 3' RACE cDNA amplification kit (BD Bioscience) with the following successive primers; 5'-GCCGCCA-GAGGTTTGCTTTATGAC-3', 5'-TCTCAAGGGCAACGGCATGTTTC-3', and 5'-AGCCGGTGAAGTCAAGAAGTGG-3'. The obtained fragment was cloned into a pGEM-T vector (Promega) and sequenced by GATC (Germany). The full length gene was submitted to GenBank (accession number *macpiwi*: AM942740).

### *Macpiwi* characterization and phylogenetic analysis

The sequences of *piwi* homologues, used for the phylogenetic tree construction, were obtained from the EMBL/GenBank. Potential subcellular localization was estimated using the program LOctree. Both amino acid and nucleic acid alignments were performed using the software ClustalW2. Alignments were analyzed and manually corrected using the program “GeneDoc”. Gene identity and similarity was calculated using the free online program “sequence manipulation suite”. Phylogenetic tree calculations were performed using MrBayes. To root the tree, the distantly related QDE (*Neurospora crassa*) was specified as outgroup. Four chains were run for 1 million generations, discarding the first 25,000 generations (burnin). The program FigTree was used to construct graphical trees. Phylogenetic trees were edited using the program Adobe Illustrator CS3.0.

### Whole mount *in situ* hybridization

Whole mount *in situ* hybridization (ISH) was carried out as described earlier (Pfister et al., 2007). Riboprobes were generated using the DIG RNA labelling KIT SP6/T7 (Roche), following the manufacturer's protocol. Template DNA for producing DIG-labelled probe (864 bp) was made by standard PCR using the primer couple 5'-TGCTCAAGCTGGTGTGCTGTC-3' and 5'-GTCTTGTGTGTGCCCGTGTGAG-3'. ISH conditions for *macvasa* were performed as described earlier (Pfister et al., 2008). During hybridization, riboprobes were used at working concentrations of 0.05 ng/μl. Pictures were made using a Leica DM5000 microscope and a Pixera Penguin 600CL digital camera.

### Immunocytochemistry

Antibody stainings were performed as previously described (Ladurner et al., 2005b) with the following modifications: animals were fixed for 30 min with 4% PFA at room temperature (RT). Multiple PBS-T (0.1%) washes (3×5 min, 1 h at RT) were followed by 30 min blocking in PBS-BSA-T (1%) at RT. Primary antibodies were incubated overnight in PBS-BSA-T at 4 °C (1/100 for *Macpiwi*; 1/200 for *Macvasa*). After washing with PBS-T (0.1%) (3×5 min), specimens were incubated in secondary antibody (1/200 FITC-swine-anti-rabbit, 1 h RT, DAKO) and washed again for 3×5 min in PBS-T. Specimens

were mounted with Vectashield (VECTAR) and analyzed using a Leica DM5000 microscope. Details were taken with a Zeiss LSM 510.

The primary polyclonal Macpiwi antibody was produced by GenScript (GenScript Corp, NJ, USA). The peptide (MFRKQGSQRLNPAP (C) (aa 784–797)) used for immunization was obtained from a non-conserved region within the sequence. The peptide was used for Macpiwi immunization; the generation of Macvasa antibody is described earlier (Pfister et al., 2008).

#### Double labelling of S-phase cells (BrdU) and macpiwi expressing cells

Preceding fixation, animals were pulsed for 30 min with 5 mM BrdU to label neoblasts in S-phase (Ladurner et al., 2000). *In situ* hybridization was performed as described above, except for colour development, which was carried out with Fast Red (Sigma, F4648). Following ISH signal development, specimens were rinsed twice in ddH<sub>2</sub>O and further processed through the BrdU staining protocol (Ladurner et al., 2000), except for Protease XIV treatment, which was performed at a final concentration of 0.1 mg/ml for 20 min at 37 °C.

#### Western blotting

Animals were starved for 1 day preceding protein isolation. Total protein of 650 animals was extracted in 100 µl 2× Slab/100 µl PBS and loaded onto a 12% acrylamide gel (90 min, 150 V). Protein was subsequently blotted on polyvinylidene fluoride membranes (90 min, 25 V) (Immobilon-P; Millipore) and blocked for 2 h with PBS (pH 7.4) containing 0.3% Tween-20 and 5% skimmed milk powder. Blots were incubated overnight at 4 °C in primary antibody with a final concentration of 7.4 µg/ml anti-Macpiwi antibody. After washing the blots for 3 × 10 min in PBS-Tween (0.3%), membranes were incubated in alkaline phosphatase-conjugated anti-rabbit immunoglobulin (1/10,000 Sigma, 2 h at RT). Finally, after several washing steps (8 × 10 min), immunocomplexes were detected using NBT/BCIP colour development (LifeTechnology).

#### Postembryonic development, regeneration, and starvation

During the whole postembryonic development (16 days for *M. lignano*), 50 staged juveniles were fixed each day and stored in methanol (−20 °C) until further processed for ISH and immunohistochemistry. To obtain regenerating animals, 500 specimens were cut at the female gonopore, i.e. at about two thirds of the length of the animal, and left to regenerate. In order to follow germline formation during regeneration, a second batch of worms was cut just behind the level of the pharynx. Every day, 40 animals were fixed at preset timepoints and stored in methanol (−20 °C). During starvation, worms were kept in petri dishes in culture media (f/2) without food. Medium was changed once a week.

#### RNA interference

*macpiwi* RNA interference was performed by soaking, as described earlier (Pfister et al., 2008). dsRNA probe was generated by an *in vitro* transcription system (T7 Ribomax™ Express RNAi System, Promega), and overlaps in sequence completely with the ISH probe (bp155–bp1018) (Fig. S1). As a negative control for RNA interference, a 1002 bp *Luciferase* fragment was used. dsRNA was diluted in f/2 culture medium to a final concentration of 3 ng/µl. Supernatant was changed every 12 h. Throughout the whole experiment, animals were fed ad libitum. Specimens were examined for BrdU incorporation, *piwi* mRNA and protein expression as well as the influence of *macpiwi* RNAi on the expression of the stem cell and germline specific *macvasa* after 7 days and 21 days continuous RNAi treatment. Survival, reproducibility and regeneration capacity were followed during the whole

experiment ( $d = 31$ ). Due to the delayed phenotype in hatchlings, juveniles were treated for six weeks.

In addition we have performed RNAi experiments by soaking using two *macpiwi* specific siRNAs (for sequences see Fig. S1) in concentrations ranging from 0.1 to 40 pmol/µl (siRNA 1) and from 0.1 to 200 pmol/µl for siRNA 2 (final end concentration). The siRNAs were designed using three freely available software packages for siRNA identification from the companies *Dharmacon*, *Invitrogen*, *Ambion* and the *Whitehead Institute for Biomedical Research*, based on the input sequence of the ISH probe (bp155–bp1018). Both siRNA duplexes chosen were ranked with high scores by the individual programs: siRNA 1: 5′-r(CGG AGA AGU UGC AAU UAG A)dTdT-3′ (sense) and 5′-r(U CUA AUU GCA ACU UCU CCG)dTdT-3′ (antisense); siRNA 2: 5′-r(GUG CAG GUG AUG ACG AAU U)dTdT-3′ (sense) and 5′-r(A AUU CGU CAU CAC CUG CAC)dTdT-3′ (antisense). The siRNA strands were chemically synthesized, HPLC-purified, and analyzed by LC-ESI mass spectrometry in-house (Höbartner et al., 2004; Micura, 2002).

#### Semithin sectioning and electron microscopy

These methods were performed as described earlier (Bode et al., 2006).

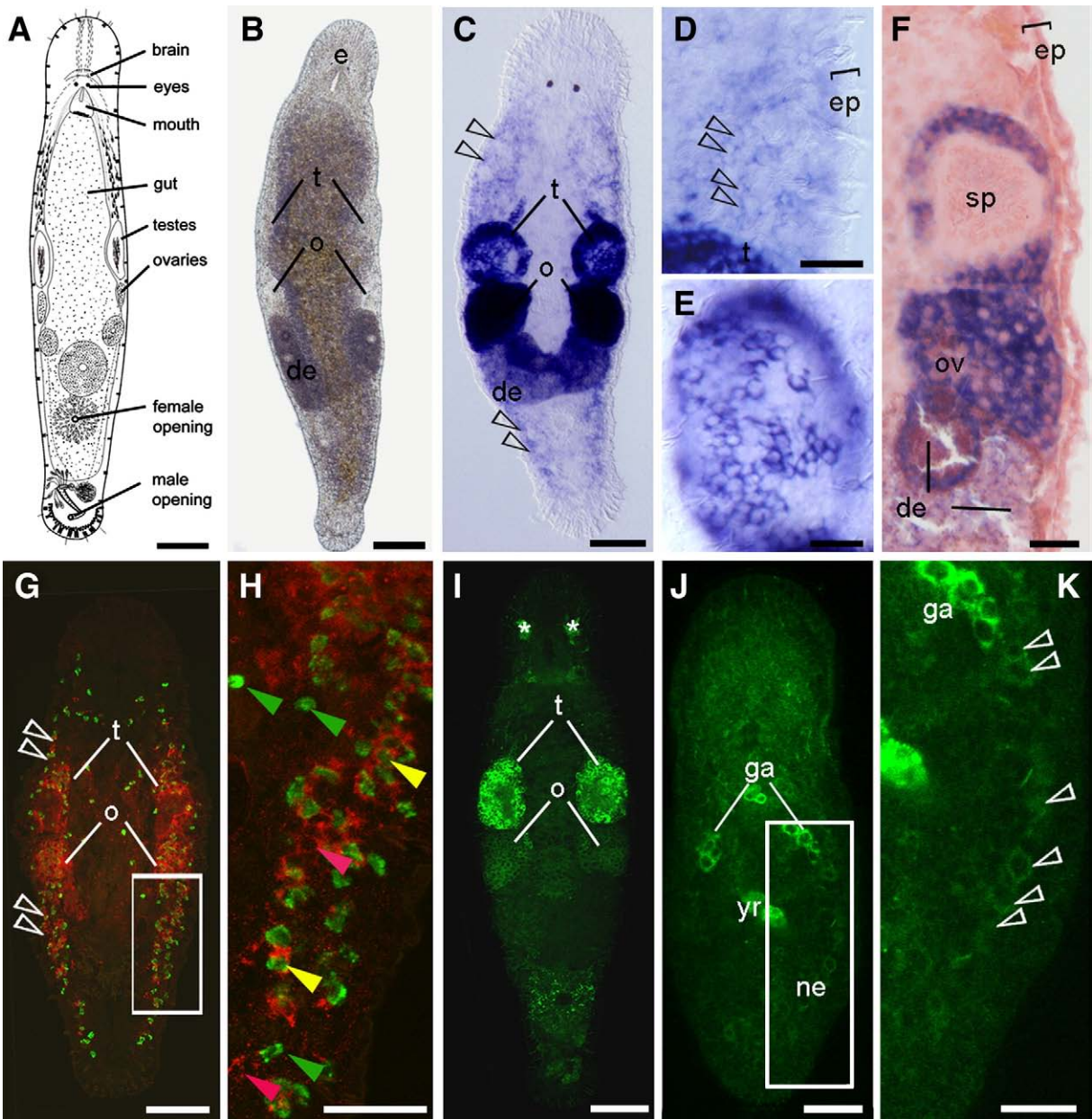
## Results

#### Cloning and characterization of the *piwi* homologue *macpiwi*

From the *M. lignano* EST database (<http://flatworm.uibk.ac.at/macest/>), clone ANGU7606 was identified showing similarity with the 5′ end of the *piwi*-like gene *seawi*. The 3′ end of *macpiwi* was obtained by RACE PCR and revealed the full length *macpiwi* cDNA of 3820 bp, coding for an 899 amino acid protein (100.01 kDa) (Fig. S1). *Macpiwi* comprises both PAZ (aa 303–426) and PIWI (aa 594–882) domain (Fig. S1), which are characteristic for members of the PIWI/Ago protein family (Cerutti et al., 2000). Phylogenetic analysis clustered *Macpiwi* clearly within the PIWI subfamily (Fig. S2). *Macpiwi* showed highest similarity with three cnidarian *piwi*-like proteins *Cniwi* (*Podocoryne carnea*, 49.5% versus 56.3%) *Chepiwi* (*Clitia hemisphaerica*, 49.1% versus 57.1%) and *Nvpiwi* (*Nematostella vectensis*, 48.2% versus 55.4%) on overall and *piwi* domain sequence, respectively. *Macpiwi* contains all seven known conserved amino acids, supposed to form the nucleic acid binding interface within the PAZ domain (Fig. S1). In addition *Macpiwi* held all 11 conserved residues which form the anchoring site for the 5′ RNA guide strand and all three amino acids (Asp–Asp–His), which outline the “cleavage” active site of the *piwi* domain (Fig. S1) (Liu et al., 2006; Tahbaz et al., 2004; Tolia and Joshua-Tor, 2007). Therefore *Macpiwi* contained all characteristics needed for participating in piRNA synthesis. LOCTree analysis predicted *Macpiwi* to be cytoplasmically localized, which was later confirmed by immunohistochemical detection of *Macpiwi* protein (see below). Surprisingly, similarity between *Macpiwi* and most other flatworm *piwi*-like proteins was low, ranging from *Smedwi-1* (34.1% versus 42.3%), *Smedwi-2* (34.6% versus 42%), *Djpiwi* (35.2% versus 42.8%) but higher with *Smedwi-3* (41.2% versus 50.6%) on overall versus *piwi* domain sequence similarities.

#### *Macpiwi* is expressed in a subpopulation of somatic stem cells and germ cells

The expression of *macpiwi* in adult animals (Figs. 1A and B) was examined using whole mount *in situ* hybridization (ISH) and immunocytochemistry. *Macpiwi* was expressed in testes, ovaries, developing eggs as well as in a subpopulation of somatic stem cells (Figs. 1C–H). Specificity of the probes was confirmed by processing animals with a *macpiwi* sense probe (Fig. S3A).



**Fig. 1.** *Macpiwi* is expressed in gonads and in a neoblast subpopulation. (A) Schematic drawing of an adult specimen; (B) DIC (Differential interference contrast) picture of an adult animal; (C) whole mount ISH of adult specimen; *macpiwi* expression is visible in testes (t), ovaries (o), developing eggs (de) and somatic stem cells (open arrowheads); (D) detail of *macpiwi* expression in somatic stem cells; note the lack of *macpiwi* expression in the epidermal layer (ep); testes (t); (E) detail of *macpiwi* expression in spermatogonia and spermatocytes in the dorsal margin of the testis; (F) semithin section through the gonads (1 μm thickness); note the complete absence of *macpiwi* expression in the lumen of the testis where mature sperm (sp) is accumulated and the homogenous expression in ovaries. (G) Confocal projection (1.02 μm) of whole mount *macpiwi* ISH double labelled with S-phase cells (BrdU); testes (t), ovaries (o); (H) confocal projection (1.02 μm) of BrdU/*macpiwi* double labelling; BrdU-only labelled cells (green arrowheads), *macpiwi* expressing cells (red arrowheads), BrdU/*macpiwi* double labelled cells (yellow arrowheads); (I) confocal projection of Macpiwi protein; Macpiwi is localized in testes (t) and ovaries (o); (J) Hatchling, stained with Macpiwi polyclonal antibody. Macpiwi is expressed in cells within the gonad anlage (ga) and in a subpopulation of somatic neoblasts (ne). Yolk remnant (yr). (K) Detail of (J) of Macpiwi in somatic stem cells (marked by open arrow heads) along the lateral side. In all pictures, anterior is to the top. Unspecific staining of the antibody in the head region is marked by asterisks. Scale bars 100 μm in (A–C, F, G, I, J); 40 μm (D, E, H, J); and 20 μm in panel K.

We found that *macpiwi* mRNA was accumulated in the outer cells of the testes, namely in spermatogonia and spermatocytes. *Macpiwi* was absent in spermatids and in the testes lumen, where mature sperm is located (Figs. 1E and F), suggesting that *macpiwi* had no function in differentiating and mature sperm. In ovaries, *macpiwi* was homogeneously expressed in both oogonia and oocytes (Figs. 1C and F). In developing eggs, *macpiwi* mRNA was located at the periphery of the egg nucleus and in mature eggs it was distributed more equally throughout the cytoplasm (Fig. 1F). However, *macpiwi* expression was not restricted to germline cells. Additionally, *macpiwi* was expressed in a subpopulation of meso-

dermally located cells, which resembled the distribution of somatic stem cells (Figs. 1C and D). Double labelling of *macpiwi* with BrdU revealed *macpiwi*/BrdU double labelled stem cells, *macpiwi*-only labelled cells as well as BrdU-only labelled cells (Figs. 1G and H). This result suggests that *macpiwi* was present in only a subpopulation of somatic stem cells.

To localize Macpiwi protein, we have generated a specific polyclonal antibody (Figs. 1I–K). Macpiwi protein was present in the cytoplasm of gonadal cells, both in testes and ovaries. Protein levels appeared to be higher in testes compared to ovaries (Fig. 1I). Macpiwi protein was uniformly present in all cells within the ovaries. In

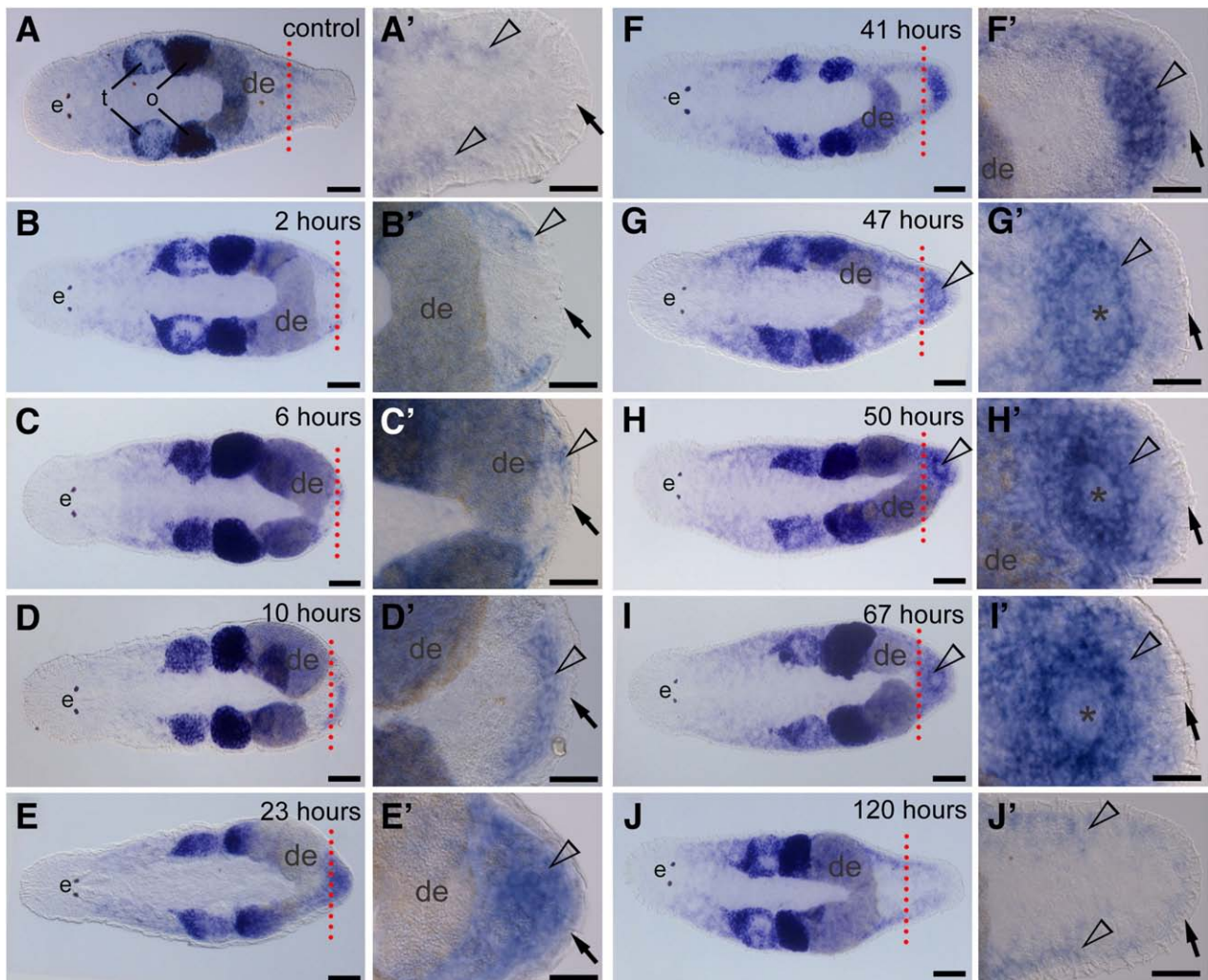
developing eggs, Macpiwi appeared as a crescent on one side of the egg nucleus. In mature eggs, Macpiwi was very weakly present within the egg cytoplasm. Cross reactivity of the antiserum was observed in head structures (Fig. 1I). This staining remained after continuous RNAi treatment (Fig. 5F), which suggested unspecific labelling. Western blot analysis showed a clear band at the expected size of Macpiwi at 100 kDa, and a weaker second band at 80 kDa, which could explain the cross reactivity (Fig. S3B). Notably, Macpiwi protein in somatic stem cells was difficult to detect in adults (Fig. 1I), although in juveniles, Macpiwi protein was weakly visible in two bands at the lateral site of the animal (Figs. 1J and K).

#### *Macpiwi is upregulated during early stages of tail regeneration*

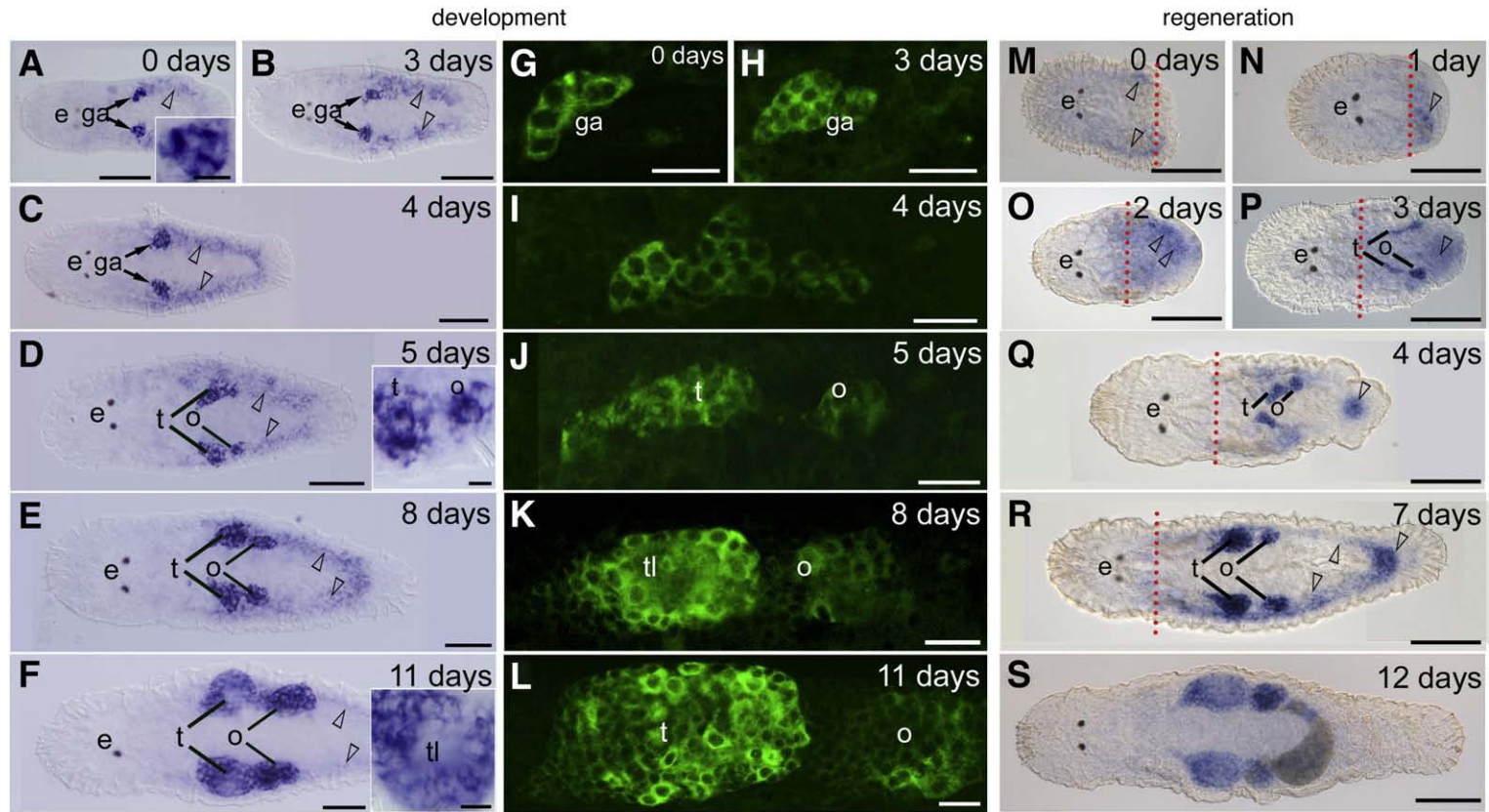
Stem cells are the sole source for regeneration in flatworms and *piwi*-like genes are known for their function in stem cell regulation (Palakodeti et al., 2008; Reddien et al., 2005; Rossi et al., 2006). The regeneration capacity and cellular facets of regeneration of *M. lignano* have been described earlier (Egger et al., 2006; Nimeth et al., 2007). Briefly, after amputation, contractions of the circular

muscle fibres close the wound. Flattening of epidermal cells covers the wound area within the first 2 h postamputation. Local proliferation of neoblasts leads to the formation of a blastema – a mass of undifferentiated cells below the epidermis. Blastemal cells differentiate into all cell types and restore all lost body parts.

Here we followed the expression of *macpiwi* at various stages of regeneration. Testes and ovaries are located in the midbody region and showed a strong expression of *macpiwi*. In order to follow changes of *macpiwi* expression in somatic stem cells, we amputated the tail plate from one batch of animals (Fig. 2A). The amputation did not lead to a marked increase of *macpiwi* expression in the anterior body region (Figs. 2A–I). Instead, changes in *macpiwi* expression remained locally at the amputation site and were observed only within the blastema throughout the regeneration period (Figs. 2A'–I'). Up to 2 h after initial cutting, *macpiwi* could not be detected at the regeneration site (Figs. 2B and B'). From 6–10 h postamputation, an upregulation of *macpiwi* expression was observed in the developing regeneration blastema (Figs. 2C–D'). From 23–41 h of regeneration, an upregulation of *macpiwi* was detected in the mass of blastemal neoblasts (Figs. 2E–F'). From 47–



**Fig. 2.** *Macpiwi* expression dynamics during regeneration. In controls (A, A'), *macpiwi* was expressed in neoblasts located along two lateral stripes in the tail region (open arrowheads in panel A') but not along the midline or the tip of the tail (arrow in panel A'). Up to 6 h after amputation *macpiwi* expression became stronger in neoblasts close to the wound site (open arrowheads in panels B' and C') and was absent at the cutting site (arrows in panels B' and C'). In the following panels D–J' open arrowheads indicate *macpiwi* expression in neoblasts of the regeneration blastema and arrows point to the epidermis of the tail tip. From 10 to 41 h of regeneration a compact mass of undifferentiated *macpiwi* expressing cells form a blastema (D–F'). Between 47–67 h postamputation the blastema increased in size and within the centre of the blastema reduced *macpiwi* expressing cells were detected (asterisks in panels G', H', I'). After 120 h, the tail plate was completely regenerated and *macpiwi* expression resembled the pattern of controls (J). In all figures, anterior is to the left. Testes (t); ovaries (o); developing eggs (de); red line indicates original level of amputation. Scale bars 100  $\mu$ m. Details, scale bars 35  $\mu$ m.



**Fig. 3.** Comparison of germline formation during postembryonic development (A–L) and regeneration (M–S). In freshly hatched worms (A, G), *macpiwi* mRNA and protein were present in a subpopulation of somatic stem cells (open arrowheads) and the “gonad anlage” (arrows); the inset in panel A displays the left gonad anlage. From three to four days of postembryonic development these gonad anlage clusters increased in size (B, C) and elongated (H, I). Ovaries appeared at day 5 (D, J); inset in panel D displays the left testis–ovary transition region. From eight days on, both testes and ovaries were clearly distinguishable (E, K). At eleven days posthatching, *macpiwi* expression was comparable to adults, with strong staining in gonads and a basic expression level in somatic neoblasts (F, L); the inset in panel F enlarges the cells surrounding the right testis lumen (tl). Note the absence of *macpiwi* expression in the mature testes lumen (F, K, L and insets). Germline formation during regeneration (M–S). In freshly cut specimens *macpiwi* is solely expressed in somatic stem cells along the lateral sides (M, open arrowheads). After one day, *macpiwi* upregulation could be observed within the formed blastema (arrowheads) (N). After two days, blastemal tissue expands in size and *macpiwi* expression is condensed posteriorly (O). Three days postamputation, gonads start to be reformed, while somatic *piwi* expression remained upregulated at the posterior end (P). After four days, animals gradually grow in size and testes and ovaries can be distinguished (Q). Note the accumulation of *piwi* positive cells at the posterior end, in the future region of the copulatory organ (open arrowhead). After seven days testes and ovaries were clearly separated (R). Twelve days postamputation, *piwi* expression in regenerates resembled adults (S). In all pictures, anterior is to the left. Unspecific staining of the antibody in the head region is marked by asterisks. eyes (e); testis (t); ovary (o); gonad anlage (ga). Scale bars 100  $\mu$ m in overviews, 20  $\mu$ m in details.

67 h of regeneration a ring-shaped distribution of *macpiwi* expressing cells became apparent (Figs. 2F–H'). This ring-shaped pattern resulted from a decrease of *macpiwi* expression in the centre of the regeneration blastema, which corresponds to the lack of cell proliferation/*macpiwi* positive cells within the inner part of the copulatory organ primordium (Egger, unpublished). After five days of regeneration, the tail plate was fully regenerated and *macpiwi* expression level was comparable to a control tail plate (Figs. 2A' and J').

*Macpiwi* is a useful marker to follow germline formation during postembryonic development and regeneration

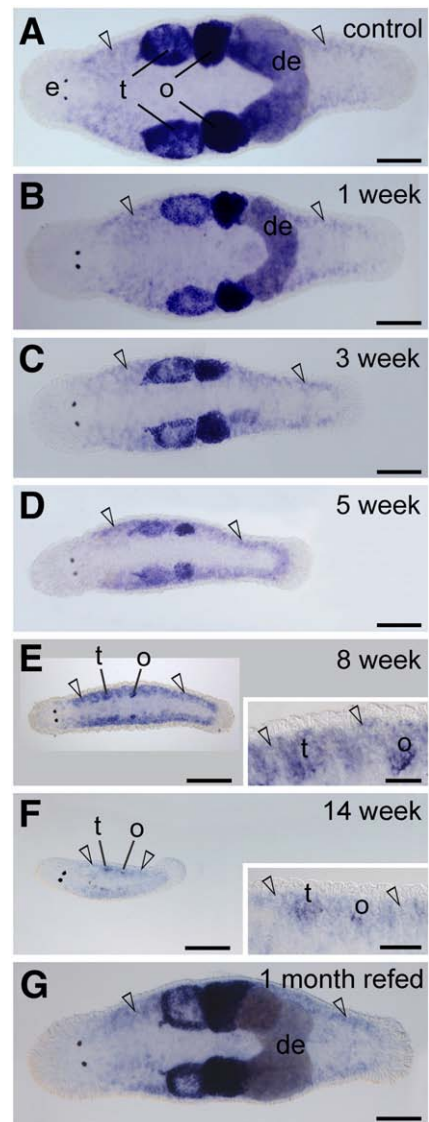
Next, we examined the expression dynamics of *macpiwi* from a 1 h old hatchling throughout various stages of postembryonic development using whole mount *in situ* hybridization and immunocytochemistry (Figs. 3A–L). Throughout postembryonic development, *macpiwi* was expressed in mesodermally located somatic stem cells in two bands along the lateral sides of the animals but lacking anterior to the eyes and within the epidermis (Figs. 3A–F). In addition, *macpiwi* mRNA (Figs. 3A–F) and protein (Figs. 3G–L) were present in developing gonads. Interestingly, in 1 h old *Macrostomum* hatchlings, *macpiwi* mRNA and protein were already strongly present in two mediolateral clusters comprising 4–6 cells each (Figs. 3A and G). These cells constitute the gonad anlage – a group of cells that gives rise to the gonads. Those clusters increased in size during the first four days of development (Figs. 3A–C, G–I), separated at day five posthatching into two smaller clusters (Figs. 3D and J) and developed into male and female gonads (Figs. 3E–L). Testes and ovaries became clearly distinguishable from days five to eight (Figs. 3D–E, J–K). Depending on the individual variation of animal development, at this stage in some animals all gonadal cells still expressed *macpiwi* (Fig. 3E). At the same time point other animals already showed a lack of *macpiwi* mRNA (data not shown) or protein (Fig. 3K) in the centre of the testes due to spermatid and sperm maturation. At day 11 posthatching, however, this lack of *macpiwi* mRNA and protein in the testes lumen was evident in all animals (Figs. 3F and L). *Macpiwi* expression level appeared to decrease in somatic stem cells in animals older than eight days (compare Fig. 3E with F).

*M. lignano* reproduces solely sexually. We could therefore follow germline reformation after the removal of the gonads by amputation. In order to trace germline formation during regeneration, one batch of animals was cut at the level of the pharynx (Figs. 3M–S). During the first few hours, wound closure takes place and *piwi* expression remained unchanged (Fig. 3M). From one to two days postamputation, *piwi* positive cells accumulated within the blastema (Figs. 3N–O). After three to four days small clusters of *macpiwi* positive cells were present which later gave rise to the gonads (Figs. 3P–Q). From seven days on, both testes and ovaries became clearly distinguishable (Fig. 3R). Subsequently, individuals gradually grew in size during the next few days and reached standard morphological proportions after 12 days (Fig. 3S).

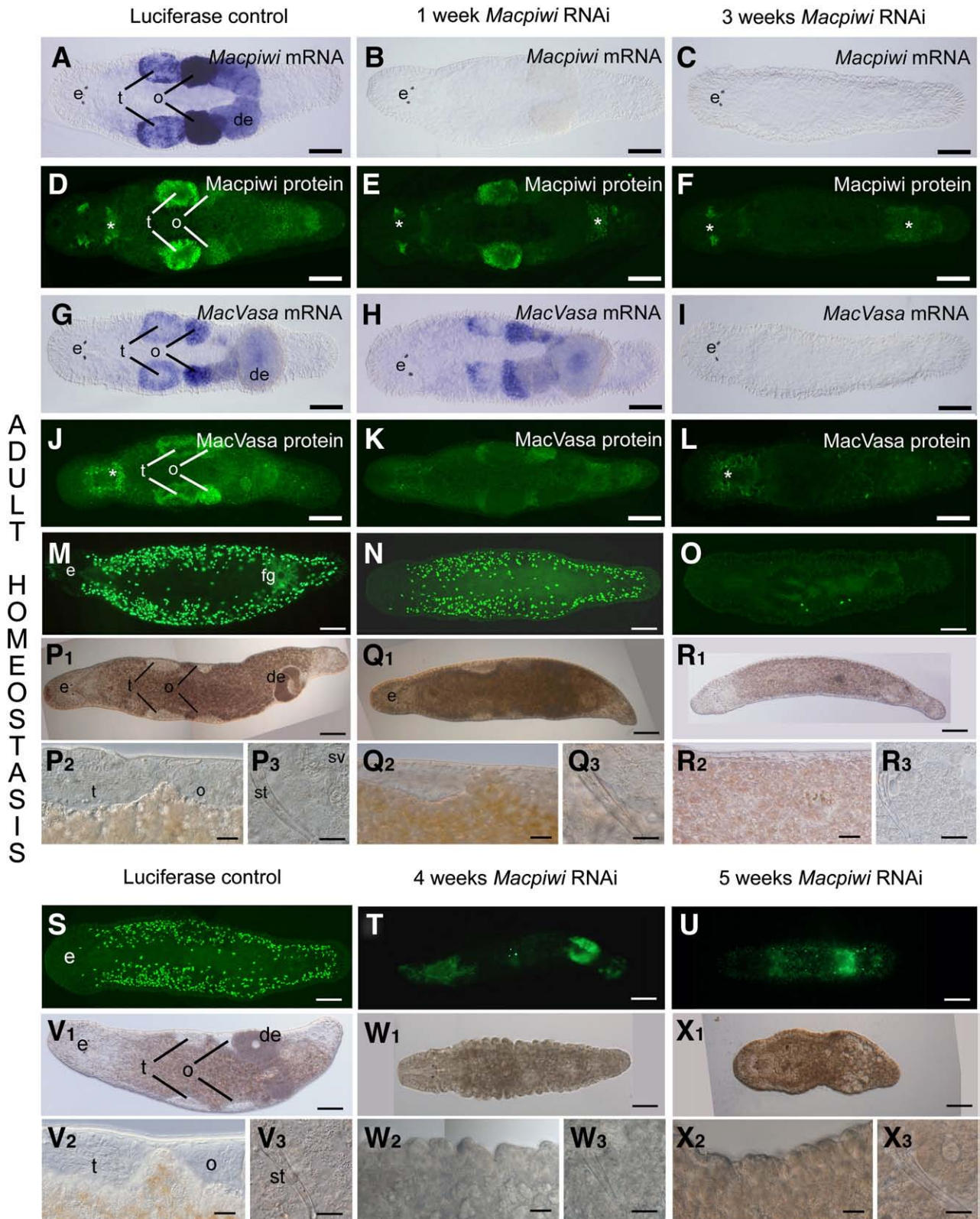
Small germline progenitors remain present after prolonged starvation

Flatworms are famous for their capacity to cope with prolonged starvation (Berninger, 1911; Gonzalez-Estevez et al., 2007; Pellettieri and Sanchez, 2007; Schultz, 1904). Animals degrow and reduce their cell number by decomposing of tissues and organs. *M. lignano* also possesses a striking ability to deal with food deprivation and reacts with a decline in the number of mitoses (Nimeth et al., 2004), the morphological disintegration of gonads and a drastic reduction of body size and *macvasa* signal (Nimeth et al., 2004; Pfister et al., 2008). This plasticity raised the question how stem cells and germ cells are regulated. We therefore sought to analyze the effects of

starvation on *macpiwi* expression. From one to five weeks of food deprivation, the size of the animals was reduced to two thirds of the initial length but *macpiwi* expression remained present in somatic stem cells and gonads (Figs. 4A–D). After eight weeks of starvation, *macpiwi* expression was prominent in somatic stem cells and weaker in two pairs of residual clusters of the gonads (Fig. 4E). At 14 weeks of starvation animals were morphologically similar to hatchlings with the exception that the larger eyes of adult worms were maintained (Fig. 4F). Remarkably, although gonads were not visible on morphological level after prolonged starvation, four gonadal clusters were still discernible by *macpiwi* expression (Fig. 4F and inset). After one month refeeding, animals had regrown completely their gonads from these remnant clusters and completely recovered the adult morphology and *macpiwi* expression pattern (Fig. 4G).



**Fig. 4.** Influence of starvation on *macpiwi* expression. Up to five weeks of starvation (A–D) *macpiwi* was expressed in testes (t), ovaries (o), developing eggs (de) and somatic stem cells (open arrowheads). After eight weeks of food deprivation (E) animals showed significant degrowth and a drastically reduced size of testes (t) and ovaries (o). *Macpiwi* expression remained high in somatic stem cells. After 14 weeks of starvation (F) animals considerably shrunk and gonad structures were morphologically completely absorbed. Small clusters of testes (t) and ovaries (o) remained detectable (E, F and insets). After refeeding (G) animals completely recovered *macpiwi* expression. In all figures, anterior is to the left. Eyes (e). Developing eggs (de). Scale bars 100 µm in panels A–G, 20 µm in insets.



**Fig. 5.** Influence of *macpiwi* RNAi on tissue homeostasis in adults. *Luciferase* dsRNA treated control animals showed normal *macpiwi* mRNA expression (A), protein pattern (B), *macvasa* expression (G), *Macvasa* protein localization (J), regular cell proliferation (M), and normal morphology (P<sub>1-3</sub>). After one week of *macpiwi* dsRNA treatment, *macpiwi* mRNA was completely abrogated (B), *Macpiwi* protein was strongly decreased (E). *Macvasa* expression (H) and protein (K) were only slightly reduced and cell proliferation was still comparable to controls at that time (N). Morphology of treated animals could not be distinguished from controls (Q<sub>1-3</sub>). After 21 days of *macpiwi* RNAi *macpiwi* and *macvasa* mRNA as well as both proteins were below detection level (C, F, I, L) and only single S-phase cells remained (O). Animals became thinner (R<sub>1</sub>) and gonads were completely disintegrated (R<sub>2</sub>). Lower panel: effect of prolonged *macpiwi* RNAi on morphology and cell proliferation (S–X). Control animal showing standard proliferation (S) and morphology (V<sub>1-3</sub>). Four weeks treated adults showing only single BrdU incorporating cells (T). Note the notched epidermis (W<sub>1</sub>) and the complete absence of gonads (W<sub>2</sub>). Stylet remained present (W<sub>3</sub>). After 5 weeks, BrdU incorporation is completely absent (U) and animals gradually disintegrated (X<sub>1-2</sub>). Stylet remained present (X<sub>3</sub>). Testes (t); ovaries (o); developing eggs (de); eyes (e); stylet (st). Unspecific staining of the antibody in the head region is marked by asterisks. In all figures, anterior is to the left. Scale bars in overviews 100 μm; scale bars in details 25 μm.

### *Macpiwi* is crucial for homeostasis in *M. lignano*

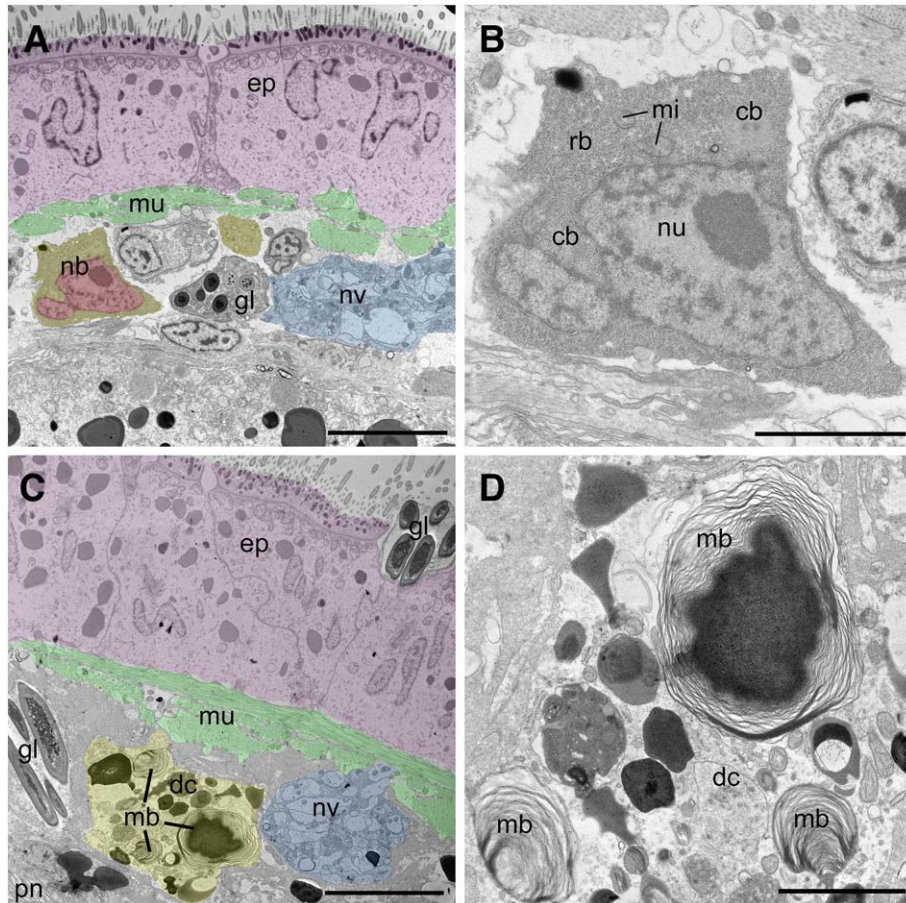
In contrast to most organisms, where *piwi*-like genes seem to have a germline specific function, it was shown that in flatworms *piwi* plays an additional role in somatic stem cells (Palakodeti et al., 2008; Reddien et al., 2005; Rossi et al., 2006; Guo et al., 2006). In order to examine the function of *macpiwi*, we applied RNA interference by soaking in adults using an 864 bp dsRNA probe (Fig. S1), during regeneration as well as during postembryonic development. We examined the effect using whole mount *in situ* hybridization, antibody staining, BrdU labelling, morphology, electron microscopy, reproduction and survival at subsequent stages of dsRNA application. In addition, we used *macvasa* as a marker to follow changes in germline and somatic stem cells (Figs. 5–8).

*Luciferase* dsRNA treated control animals substantiated that no noticeable mock effects could be observed on *macpiwi* mRNA (Fig. 5A) and Macpiwi protein (Fig. 5D), as well as the expression of the stem cell and germline specific *macvasa* (Fig. 5G), Macvasa protein (Fig. 5J) and proliferation (Fig. 5M). Furthermore, animal morphology was normal (Fig. 5P<sub>1</sub>) with distinct testes and ovaries (Fig. 5P<sub>2</sub>), developing eggs and stylet (Figs. 5P<sub>3</sub>). Continuous *macpiwi* RNAi treatment led to a complete elimination of *macpiwi* expression already after seven days (Fig. 5B) while Macpiwi protein remnants could still be detected in testes and ovaries (Fig. 5E). At this time point *macvasa* mRNA (Fig. 5H) and Macvasa protein (Fig.

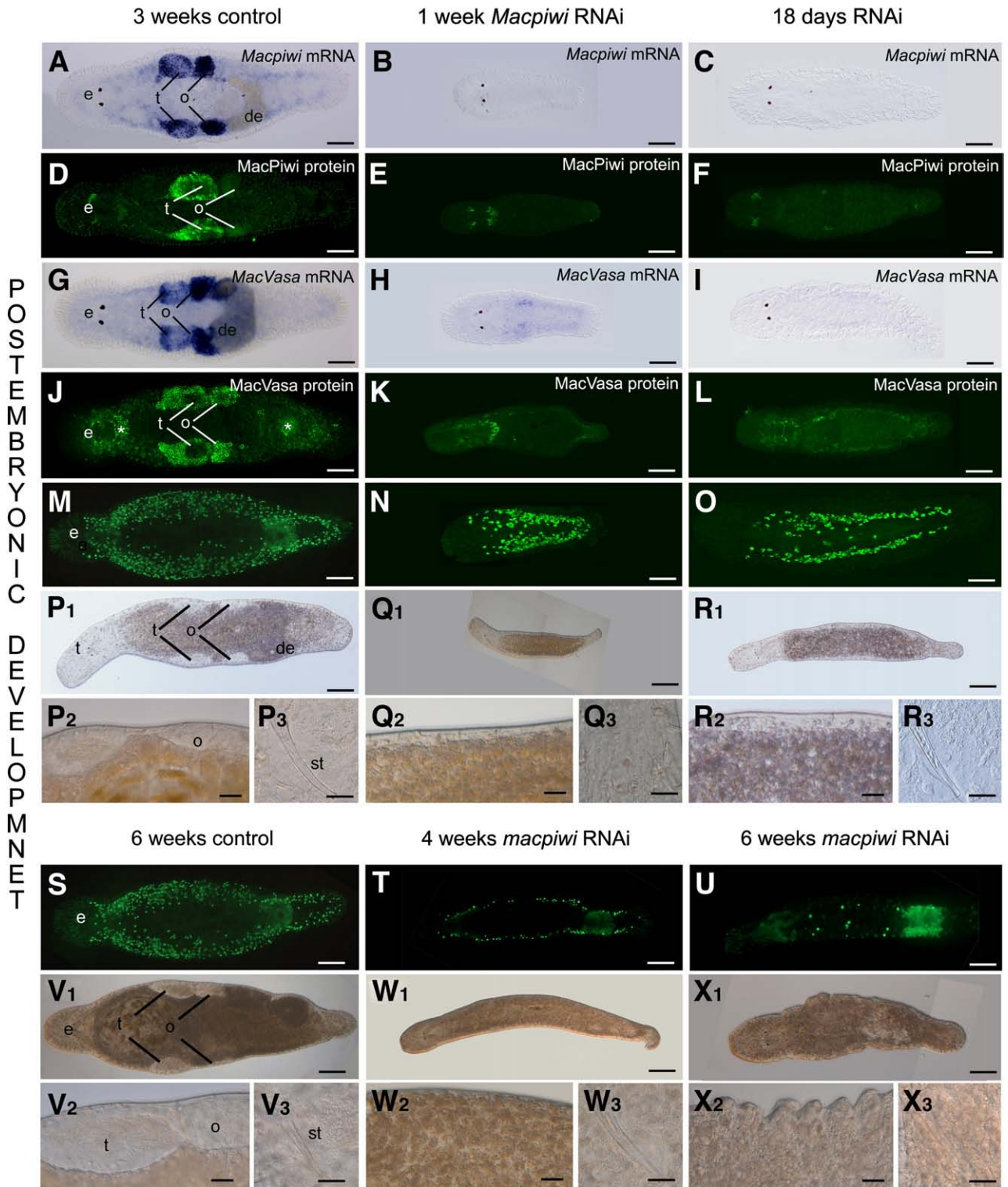
5K) were only slightly reduced and cell proliferation was not yet affected (Fig. 5N). Likewise, no drastic effect on animal morphology could be observed (Fig. 5Q<sub>1–3</sub>). After three weeks of continuous *macpiwi* dsRNA treatment however, *macpiwi* and *macvasa* mRNA (Figs. 5C, I) as well as protein (Figs. 5F and L) were completely abrogated. In contrast to the observation published so far on flatworms, *macpiwi* RNAi not only led to a disruption in the differentiation process of stem cell progenitors, but instead resulted in a drastic disruption of cell proliferation, as shown by the absence of BrdU incorporation (Fig. 5O). The absence of a functional stem cell population became also apparent in the morphology of the animals (Figs. 5R<sub>1–3</sub>). Testes and ovaries entirely disintegrated and no developing eggs were present (Figs. 5R<sub>2</sub>). Prolonged treatment for one month resulted in an even more severe effect (Figs. 5S–X). Animals gradually shrunk in size (Figs. 5W–X), the epidermis became notched (Figs. 5W<sub>1–2</sub>–X<sub>1–2</sub>), huge vacuoles were visible within the tissue (Fig. 5X<sub>1</sub>), but the male copulatory organs remained unaffected (Figs. 5P<sub>3</sub>–X<sub>3</sub>).

### Loss of tissue homeostasis is due to the lack of stem cells

We further analyzed the tissue morphology of RNAi treated animals by electron microscopy (Fig. 6). *Luciferase* dsRNA treated control animals showed normal tissue organization and morphology of epidermal cells, circular and longitudinal muscle cells, the main lateral nerve cord, and gland cells exhibited conventional



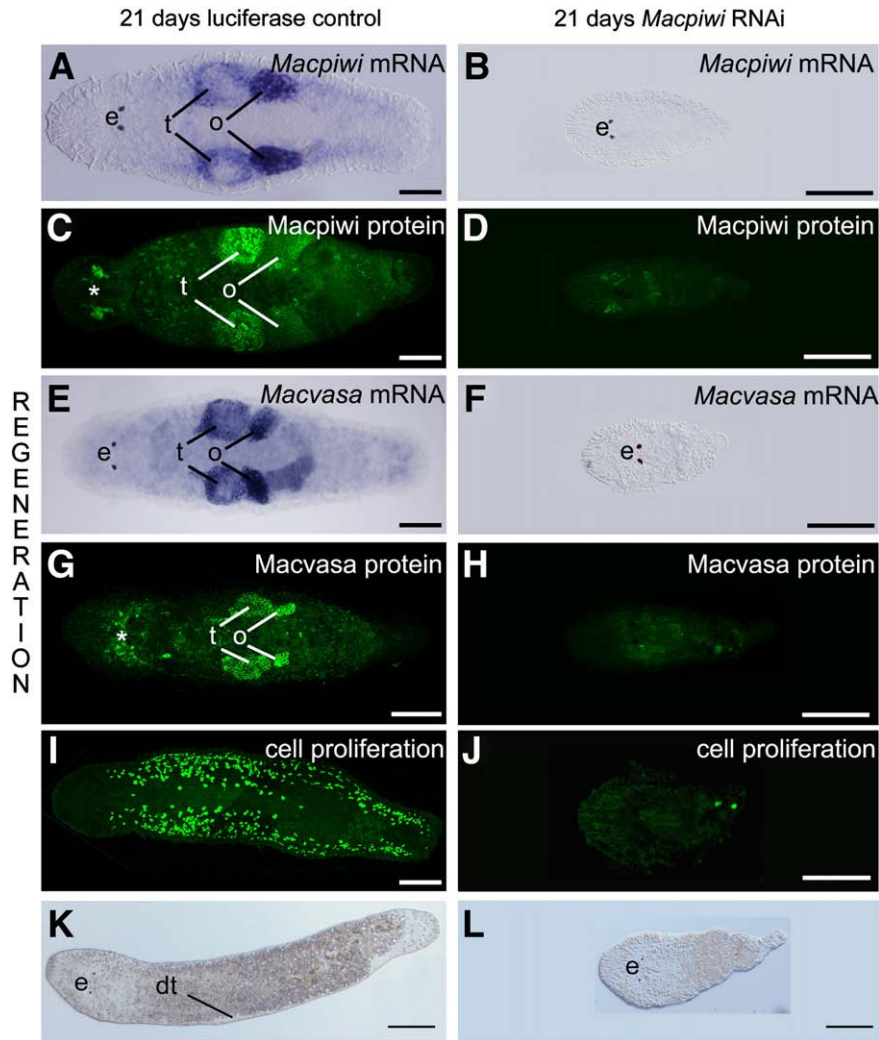
**Fig. 6.** Effect of *macpiwi* RNAi on neoblast morphology. 21 days of *luciferase* RNAi (A, B) showed normal ultrastructure (A) of epidermal cells (ep, pink), muscle cells (mu, green), the main lateral nerve cord (nv, blue), rhabdite gland cells (gl) and neoblasts (cytoplasm in yellow, nucleus in red). Of one neoblast only the cytoplasm is visible. Note the close association of neoblasts to the nerve cord. (B) Magnification of neoblast of (C) with conventional nuclear and cytoplasmic morphology; chromatoid body (cb); mitochondria (mi); nucleus (nu); free ribosomes (rb). (C, D) 21 days of *macpiwi* RNAi did not affect morphology of differentiated cells; colour code and nomenclature as in (A) but resulted in a lack of neoblasts and the presence of dying cells (dc); membrane bodies (mb); protonephridium (pn) (D) magnification of dying cell of (A). Note the membrane bodies (mb) within the dying cell (dc). Scale bars (A, C) 5  $\mu$ m; (B, D) 2  $\mu$ m.



**Fig. 7.** Effect of *macpiwi* RNAi on postembryonic development. Control RNAi with *luciferase* dsRNA did not show any effect on *macpiwi* or *macvasa* mRNA expression (A, G), protein localization (D, J) or cell proliferation (M). After seven days of *macpiwi* RNA interference, both *macpiwi* and *macvasa* mRNA and protein were dramatically reduced (B, E, H, K) and completely abrogated after 21 days of treatment (C, F, I, L). Cell proliferation was present until 18 days of *macpiwi* dsRNA treatment (N, O). During the first three weeks, treated juveniles retained normal morphology (Q, R<sub>1-3</sub>), except for their smaller body size (Q<sub>1</sub>, R<sub>1</sub>) and absence of gonads (Q<sub>2</sub>, R<sub>2</sub>). Six weeks controls showed normal cell proliferation (S) and morphology (V<sub>1-3</sub>). Once juveniles reached adulthood, *piwi* RNAi led to an effect on stem cell maintenance (lower panel). After 4 weeks, cell proliferation had significantly decreased (T) and was almost completely absent after 6 weeks (U). Note the lasting absence of gonads (W<sub>1</sub>, X<sub>1</sub>). Although animals gradually disintegrated, stylet remained present until specimens had died (X<sub>3</sub>). Eyes (e); testes (t); ovaries (o); developing eggs (de). In all figures, anterior is to the left. Unspecific stainings are marked by asterisks. Scale bars in overviews 100  $\mu$ m; scale bars in details 25  $\mu$ m.

subcellular composition (Fig. 6A). Neoblasts with their characteristic morphology (Bode et al., 2006; Ladurner et al., 2008; Rieger et al., 1999) were present close to the main lateral nerve cords (Figs.

6A and B). Up to 21 days of *macpiwi* dsRNA treatment all animals survived. During the fourth week of RNAi, mortality drastically increased and animals died within one month. Similarly, when the



**Fig. 8.** Effect of *macpiwi* RNAi on regenerating *M. lignano*. 21 days of *luciferase* RNAi showed normal *macpiwi* or *macvasa* mRNA expression (A, E), protein localization (C, G), and cell proliferation (I). After 21 days of *macpiwi* dsRNA treatment, *macpiwi* and *macvasa* mRNAs as well as proteins were completely abrogated (B, D, F, H) and animals were extremely small in size (L). Worms were not able to regenerate tissues and lacked cell proliferation almost completely (J). All animals died with prolonged treatment. In all figures, anterior is to the left. Eyes (e); testes (t); ovaries (o); developing testes (dt). Scale bars 100  $\mu$ m.

stem cell system was eliminated by radiation, we observed an initial period of survival, which was followed by a rapid decline in the number of surviving animals within the next week (De Mulder, unpublished). After 21 days, the ultrastructure of differentiated cell types was still normal with the exception of neoblasts (Fig. 6C). No stem cells were found in all ultra-thin sections of different body regions (>30  $\mu$ m analyzed). This lack was most striking in the region close to the main nerve cords where neoblasts are usually located in control animals. Instead, remnants of dying cells were located in this region (Fig. 6C) which contained peculiar membrane bodies (Fig. 6D). The lack of *macpiwi* expression and subsequent absence of neoblasts led to the failure in tissue homeostasis, resulting in tissue degradation. As such, death of the animals within one month was due to the loss of proliferating stem cells and not due to the failure in differentiation, which was shown to be the case in triclad flatworms (Reddien et al., 2005).

#### Stem cells are differentially regulated during development

We further investigated the role of *macpiwi* during postembryonic development in *M. lignano* (Fig. 7). *Luciferase* dsRNA treated control hatchlings developed normally within the 18 days of the observation period. They showed normal levels of *macpiwi* and

*macvasa* mRNA expression (Figs. 7A and G) and protein localization (Figs. 7D and J). In contrast, at seven and 18 days of *macpiwi* dsRNA treatment, *macpiwi* mRNA (Figs. 7B and C) and Macpiwi protein (Figs. 7E and F) were entirely abolished. *macvasa* mRNA was hardly visible at 7 days of *macpiwi* RNAi (Fig. 7H) and could not be detected after 18 days, respectively (Fig. 7I). Macvasa protein was absent already after seven and 18 days of *macpiwi* dsRNA treatment (Figs. 7K and L). *macpiwi* RNAi treated hatchlings retained cell proliferation activity in somatic neoblasts up to three weeks as identified by BrdU labelling (Figs. 7M–O). Juveniles formed all organs *de novo* within three weeks (Figs. 7P<sub>1</sub>–R<sub>3</sub>). However, we found that *macpiwi* dsRNA treated worms were smaller in size and no gonads were developed (Figs. 7R<sub>1–3</sub>).

Since these observations suggested that stem cells might be differentially regulated in hatchlings compared to adults, we next asked if these *macpiwi* RNAi treated juveniles remained insensitive to *macpiwi* downregulation once they reached adulthood. Notably, this was not the case. Up to three weeks posthatching, these animals showed many proliferating cells, comparable to controls (Fig. 7O). When animals reached adulthood, cell proliferation became drastically downregulated (Figs. 7S–U). Morphologically, animals remained small (Figs. 7V<sub>1</sub>–X<sub>1</sub>) the epidermal layer became thinner (Figs. 7V<sub>2</sub>–X<sub>2</sub>) but the stylet remained (V<sub>3</sub>–X<sub>3</sub>). This absence of stem cell

homeostasis led to a small morphological phenotype (Fig. 7W<sub>1–3</sub>) with a disintegrated epidermis (Figs. 7W<sub>1–2</sub>). Finally, treated juveniles died after six–seven weeks of treatment.

#### Loss of regeneration capacity in *macpiwi* RNAi treated animals

In strong contrasts to hatchlings, which were able to form and build functional organs during development despite *macpiwi* RNAi treatment, RNAi of *macpiwi* resulted in severe defects during regeneration (Fig. 8). Initially, animals were presoaked one week in dsRNA and cut at the post-pharyngeal level. To accelerate *Macpiwi* protein depletion, amputation was repeated after one week of regeneration and animals were left for another week to regenerate. *Luciferase* dsRNA treated control animals regenerated normally after two amputations, restored *macpiwi* and *macvasa* mRNA expression (Figs. 8A and E), *Macpiwi* and *Macvasa* proteins (Figs. 8C and G), and exhibited a normal cell proliferation pattern (Fig. 8I). These control animals underwent a normal regeneration process (Egger et al., 2006), although animals had not yet rebuilt morphologically visible gonads at day 21 of *luciferase* treatment, i.e. one week after the last amputation (see *Material and methods*) (Fig. 8K).

In contrast, after 21 days of *macpiwi* dsRNA treatment, *macpiwi* and *macvasa* mRNAs as well as protein expression could not be detected in regenerates (Figs. 8B, D, F and H). Although wound closure occurred normally, subsequent cell proliferation was completely absent (Fig. 8J) and animals were unable to form a regeneration blastema. Drastic effects in the morphology reflected the lack of cell proliferation and the missing *macpiwi* function. After amputation the regenerates remained tiny and were not capable to grow or to re-establish any tissues and organs (Fig. 8L). Regenerates died within one month of *macpiwi* RNAi.

## Discussion

#### *Macpiwi* is expressed in a subpopulation of somatic stem cells and germ cells

We have identified *macpiwi* expression in a subpopulation of somatic stem cells, and in male and female germ cells. Within the animal kingdom, the presence of *piwi*-like genes in somatic stem cells is not common and has been described within the Bilateria so far only in triclad flatworms (so called planarians), as well as in more basal cnidarians and sponges (Denker et al., 2008; Funayama, 2008; Reddien et al., 2005; Rossi et al., 2006; Seipel et al., 2004). For triclads, several *piwi*-like genes were found (Palakodeti et al., 2008; Reddien et al., 2005; Rossi et al., 2006). In *S. mediterranea* *piwi*-like genes were expressed in the entire neoblast population (Eisenhoffer et al., 2008; Guo et al., 2006; Palakodeti et al., 2008; Reddien et al., 2005; Wang et al., 2007). *In situ* hybridization in *M. lignano* shows a distribution of *macpiwi* staining in somatic stem cells, correlated with the distribution of neoblasts which was previously identified by BrdU labelling, electron microscopy and by *macvasa* distribution (Bode et al., 2006; Ladurner et al., 2000; Pfister et al., 2008). By *macpiwi*/BrdU double labelling, we found qualitative evidence for double labelled cells, *macpiwi*-only labelled cells as well as BrdU-only labelled cells. These data suggest that, in contrast to *S. mediterranea* where *piwi*-like genes are expressed in the entire stem cell population (Eisenhoffer et al., 2008), *macpiwi* is present only in a subset of neoblasts in *M. lignano*. A similar observation was described in *D. japonica*, in which *Djpiwi-1* was expressed only in a subpopulation of somatic stem cells along the midline, suggesting that the somatic stem cell populations might be heterogeneous (Rossi et al., 2006; Salvetti et al., 2009). Therefore, *M. lignano* might be a suitable organism to further investigate the underlying function of *piwi* gene regulation of stem cell subpopulations.

#### *Macpiwi* expression dynamics during postembryonic development, regeneration and starvation

The distinction between soma and germline is an important process in the development of animals with sexual reproduction (Torrás et al., 2004). However, in some phyla such as flatworms, Cnidarians and Porifera, the distinction between soma and germline is not clearly made, even throughout adulthood. It was generally accepted that the germline in flatworms is being formed during postembryonic development from neoblasts (Gremigni and Domenici, 1974; Sanchez, 2003; Sanchez and Kang, 2005; Wang et al., 2007). However, recently, two independent publications showed indications for the embryonic formation of the germline on a molecular level, using the germline markers *nanos* and *vasa* (Handberg-Thorsager and Salo, 2007; Pfister et al., 2008). After the removal of the entire gonads by amputation, *M. lignano* can reconstitute functional gonads during regeneration (Egger et al., 2006; Pfister et al., 2008). Gonads have been restored 59 times in a single animal after successive repeated amputations (Egger et al., 2006; Ladurner et al., 2008). Since *M. lignano* is a solely sexual reproducing obligatory cross fertilizing hermaphrodite (Schärer and Ladurner, 2003), *M. lignano* might be a suitable flatworm model organism to follow and compare germline formation during development and regeneration. The data shown here support an embryonic formation of germ cells in *M. lignano*, since already in freshly hatched worms, *macpiwi* signal was clearly visible in two clusters from which gonads developed. This observation challenges the concept that the germline is formed postembryonically in flatworms and adds evidence that this might not be representative for e.g. basal flatworm taxa.

During regeneration we observed a temporal and spatial restricted upregulation of *macpiwi* in somatic stem cells. In the blastema, *macpiwi* was expressed after 6 h of regeneration and ceased in the tail region after regeneration was accomplished. Likewise, in regenerating planaria large numbers of anti-SMEDWI-1 cells were present in the blastema region up to 15 days after amputation (Guo et al., 2006). In contrast to planarians, *M. lignano* could not form a blastema when animals were treated with *piwi* dsRNA.

Flatworms are well known for the enormous plasticity of their stem cell system upon starvation (Nimeth et al., 2004; Oviedo et al., 2003; Pellettieri and Sanchez, 2007; Pfister et al., 2008). The effect of prolonged food deprivation of *M. lignano* was already studied earlier in detail and resulted in a gradual decrease in body size, morphological degeneration of gonads as well as a significant reduction in proliferation and *macvasa* expression levels (Nimeth et al., 2004; Pfister et al., 2008). However, using *macpiwi*, we showed for the first time that some progenitors remained within the previous gonad region. The presence of these *macpiwi* positive cells within the presumptive gonad region shows resemblance with the existence of germline progenitors in asexual planarians. These cells were not visible on morphological level, but could be detected using the germline marker *nanos* (Handberg-Thorsager and Salo, 2007; Sato et al., 2006; Wang et al., 2007). Our data show that in *M. lignano*, although the somatic part of the gonads mainly disappears during prolonged starvation, small *macpiwi* positive germinal clusters remain. These observations suggest that germline reformation during regrowth after a prolonged starvation might not be based on somatic neoblasts as supposed earlier, but instead proliferation and repopulation of these remained germline clusters reconstituted gonads in *M. lignano*. Refeeding of *M. lignano* resulted in fully sexual mature adults within one month, able to produce viable offspring.

#### A new *piwi* function in somatic stem cells

In vertebrates, *piwi* homologues function in germ cells and are essential for spermatogenesis in mouse (Carmell et al., 2007; Deng and Lin, 2002; Grivna et al., 2006; Unhavaithaya et al., 2008), regulate

male and female gonad development in zebrafish (Houwing et al., 2007; Houwing et al., 2008; Tan et al., 2002) or are expressed in female gonads of *Xenopus* (Wilczynska et al., 2009). In *Drosophila*, Piwi has a role in germline cells and somatic cells of the ovary (Cox et al., 1998; Gunawardane et al., 2007; Megosh et al., 2006; Saito et al., 2006). It has been demonstrated that Piwi and its interacting piRNAs function e.g. in the epigenetic regulation of germline stem cells (Yin and Lin, 2007), in the regulation of the levels of Oskar and Vasa (Megosh et al., 2006), or are regulators of transposon activity (Brennecke et al., 2007; Vagin et al., 2006). The *C. elegans* Piwi is involved in germline development and silencing of transposons in the germline (Das et al., 2008; Wang and Reinke, 2008).

In *M. lignano*, we identified a new role of piwi-like genes in somatic stem cells of flatworms during homeostasis and regeneration. In earlier reports on planarians it was shown that neoblasts remained present in piwi treated animals. These cells were sensitive to radiation and were capable to proliferate in response to wounding (Reddien et al., 2005). However, despite the fact that a regeneration blastema was formed, lost body parts could not be rebuilt. The authors explained these observations by the fact that stem cells obtained defects in differentiation. In strong contrast to planarians, in *M. lignano* no somatic stem cells could be observed in macpiwi treated adults, and no activation of cell proliferation was detected upon wounding in regenerates. We confirmed this statement using TEM, BrdU incorporation and the absence of an additional stem cell marker macvasa. Macpiwi downregulation resulted in a block in stem cell proliferation in *M. lignano*. Therefore, we propose that failure in tissue homeostasis in *M. lignano* is most likely due to the loss of stem cells. We conclude that macpiwi has a critical role in the maintenance of the somatic stem cell population in *M. lignano*.

#### Stem cells are regulated differently during development compared to regeneration and homeostasis

We observed a significant difference in effect of macpiwi RNAi in regenerates and juveniles. It appears that a fundamental difference exists in the regulation of stem cells during regeneration and development in *M. lignano*. In contrast to macpiwi RNAi treated regenerates, which did not show any sign of proliferation activation nor organ reformation upon wounding, hatchlings were able to grow in size and even develop adult organs such as stylet and adhesive glands under macpiwi RNAi conditions. The germline on the other hand did not develop and juveniles remained sterile. Notably, when these juveniles reached adulthood animals died. These observations suggest that stem cells are differentially regulated during postembryonic development versus regeneration and homeostasis.

It is remarkable that although both regenerates and hatchlings have to form organs, hatchlings can do so under macpiwi RNAi conditions while regenerates cannot. This is the first time that such a discrepancy is demonstrated between the processes of regeneration and postembryonic development. One could speculate that macpiwi functions in neoblasts subpopulations which are not essential during postembryonic development. Alternatively, it might be possible that macpiwi is not the only piwi-like gene of *M. lignano*. Genome-sequencing will give insight on the piwi gene family in *M. lignano*. Further investigation will unravel the underlying difference in the molecular regulation of stem cells in adults, regenerates and juveniles.

#### Evolutionary implication of piwi in somatic stem cells

Within the tree of life piwi expression is largely restricted to germ cells. Notably, within sponges, cnidarians and rhabditophoran flatworms, piwi-like genes are also expressed in somatic stem cells. In representatives of these phyla, somatic stem cells can give rise to germ cells (Bosch, 2004; Denker et al., 2008; Egger et al., 2006; Funayama, 2008; Pfister et al., 2008; Seipel et al., 2004). It seems possible that

piwi-like genes are involved in the maintenance of the totipotency of the respective undifferentiated cell types. A substantial amount of evidence has been accumulated within the last years, which demonstrates that piwi is mainly involved in the maintenance of transposon silencing in the germline to preserve genome integrity (Brennecke et al., 2007; Gunawardane et al., 2007; Houwing et al., 2007). This Piwi function may be also required in undifferentiated somatic cells with totipotent differentiation capacity. Basal metazoans such as sponges or cnidarians might have evolved the Piwi/piRNA network to protect their totipotent stem cells against genome damage. This protective function of Piwi appears to be conserved in neoblasts of Platyhelminthes (Palakodeti et al., 2008; Reddien et al., 2005; Rossi et al., 2006). In the oligochaete annelid *Enchytraeus japonensis* and the compound ascidian *Botryllus primigenus* germ cells can be developed from somatic stem cells (Mukai and Watanabe, 1976; Tadokoro et al., 2006, reviewed in Agata et al., 2006). Therefore we postulate that in species with the potential to restore the germline from somatic stem cells, piwi-like genes might play a role in the molecular regulation of these cells. As more genomes are being sequenced, and representatives of many phyla are taken under study, comparison of piwi-like gene function across the animal kingdom will shed light on how piwi function is maintained and further evolved during evolution.

#### Acknowledgments

The authors want to thank Thomas Ostermann for *Macrostomum* EST-database maintenance. Furthermore, we would like to thank Helene Heiss and Hannes Mair for initial help with electron microscopy. This work was supported by a predoctoral FWO grant to K.D.M (Belgium) and a FWF grant 18099 to P.L (Austria).

#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.07.019.

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