

# The Hippo Pathway Regulates Stem Cells During Homeostasis and Regeneration of the Flatworm *Macrostomum Lignano*

Turan Demircan<sup>1</sup> and Eugene Berezikov<sup>1,2</sup>

The Hippo pathway orchestrates activity of stem cells during development and tissue regeneration and is crucial for controlling organ size. However, roles of the Hippo pathway in highly regenerative organisms, such as flatworms, are unknown. Here we show that knockdown of the Hippo pathway core genes in the flatworm *Macrostomum lignano* affects tissue homeostasis and causes formation of outgrowths through hyperproliferation of stem cells (neoblasts), and leads to disruption of allometric scaling during regeneration and increased size of regenerated parts. We further show that Yap, the downstream effector of the Hippo pathway, is a potential neoblast marker gene, as it is expressed in dividing cells in *M. lignano* and is essential for neoblast self-renewal. The phenotypes we observe in *M. lignano* upon knockdown of the Hippo pathway core genes and Yap are consistent with the known functions of the pathway in other model organisms and demonstrate that the Hippo pathway is functionally conserved between flatworms and mammals. This work establishes *M. lignano* as a productive model for investigation of the Hippo pathway.

## Introduction

**T**HE HIPPO PATHWAY, A HIGHLY CONSERVED phosphorylation-based signaling pathway, regulates tissue and organ size in invertebrates and vertebrates by repressing cell proliferation and inducing apoptosis [1–3]. Although the upstream elements of the Hippo pathway still need to be explored, the main components of the pathway are well characterized. In *Drosophila melanogaster*, the core of the Hippo kinase cascade consists of two serine/threonine kinases, Hippo (Hpo) [4–8] and Warts (Wts) [9,10], and their adapter proteins Salvador (Sav) [11,12] and Mats [13]. In mammals, these components are called Mst, Lats, Sav, and Mob, respectively [14]. Once upstream signals activate Hpo, it phosphorylates and activates adapter proteins Sav and Mats [15,16] and downstream kinase Wts [8]. Active Wts phosphorylates transcriptional coactivator Yorkie (Yki), called Yes-associated protein (Yap) in mammals, and as a result inhibits its function [17]. Yap is recognized as one of the major transcriptional regulators for cell division, growth, and stem cell renewal [18]. Activated YAP binds to several transcription factors and regulates expression of genes involved in cell division and inhibition of apoptosis [12,19–22]. Defects in Hippo pathway genes have been linked to outgrowths of tissues in imaginal disc in *D. melanogaster* [23] and many cancer types in mammals [15].

Organ size control by the Hippo pathway in animals is provided through regulation of the amount of stem cells and their proliferation and differentiation potential [24], and increase or decrease in the number of the stem cells may account for cancer or premature aging, respectively [25]. Although Hippo pathway has been intensively studied in *D. melanogaster*, mice, and cell cultures, the mechanistic links between stem cell dynamics and the Hippo pathway are still not fully understood. In mammals it is difficult to perform in vivo stem cell studies due to their complex hierarchic organization and problems of accessibility to stem cells. For these reasons, in vitro examination of stem cells is a common alternative approach to unveil functions of genes/pathways in stem cells. However, complex interaction of stem cells with their niches in vivo makes in vitro studies less informative about native behavior of stem cells. In addition to technical boundaries for in vivo studies of stem cells in mammals, the restricted stem cell population and limited regeneration potential of mammalian models also complicate studies of stem cell properties and molecular mechanisms underlying their regulation.

To extend our current knowledge on stem cell biology, several nonmammalian model organisms have been used broadly [26–30], and among them flatworms are highly attractive models due to their accessible pluripotent stem cell population (called neoblasts), high regeneration capacity and

<sup>1</sup>Hubrecht Institute for Developmental Biology and Stem Cell Research and University Medical Center Utrecht, Utrecht, The Netherlands.

<sup>2</sup>European Research Institute for the Biology of Ageing, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands.

the ease of functional genetic manipulations [31–37]. Here we use the flatworm *Macrostomum lignano* (Platyhelminthes, Macrostomida) to address the role of the Hippo signaling pathway in stem cell regulation and regeneration in flatworms. *M. lignano* has several important experimental properties that make it a convenient model organism: the transparency of the animal facilitates observation of morphological changes at different conditions and treatments [38–42], whereas short generation time, ease of culturing and high number of progeny, combined with available draft genome and transcriptome data and established protocols for RNA interference (RNAi) [38,41], in situ hybridization (ISH) and bromodeoxyuridine (BrdU) labeling [38,40,41,43] provide the basis for genomics approaches in this organism. Upon amputation, *M. lignano* is able to regenerate the whole posterior part, including gonads and other organs, from a small head stump [32], and thus, it provides a powerful experimental system to study the molecular mechanisms governing regeneration and function of neoblasts [41].

In this study, we report identification of all main components of the Hippo pathway in *M. lignano*: *machpo*, *macsav*, *macwts*, *macmats*, and *macyap*. We characterized expression patterns of these genes and performed loss-of-function studies by RNAi. We showed that the Hippo pathway affects stem cell dynamics and is required for tissue homeostasis and regeneration. The obtained results contribute to a better understanding of the functions of the Hippo pathway in tissue homeostasis, stem cell dynamics, and regeneration, and establish *M. lignano* as a powerful model for detailed investigation of the Hippo pathway in the context of regeneration.

## Materials and Methods

### Animal culture

Individuals of *M. lignano* DV1 line were cultured in Petri dishes in f/2 medium, nutrient-enriched artificial sea water, and were fed *ad libitum* with diatom *Nitzschia curvilineata* [44]. During the experiments, animals were kept in incubator by following conditions: 20°C temperature, 60% humidity, and 14/10 h day/night cycle [45].

### Identification and cloning of the Hippo pathway genes of *M. lignano*

The sequences of the Hippo pathway genes in different species were retrieved from GenBank/EMBL database and used in basic local alignment search tool (BLAST) searches to identify *M. lignano* *hpo*, *sav*, *wts*, *mats*, and *yap* homologs in *M. lignano* genome and transcriptome assemblies (Simanov et al., article in preparation; www.macgenome.org). Best reciprocal hits were defined as *M. lignano* homologs of the respective genes, and their structures were verified by targeted cDNA resequencing.

Total RNA was isolated from 200 to 300 animals using Trizol Reagent (Sigma) according to manufacturer's instructions. Before RNA isolation, animals were starved for 2 days to prevent RNA contamination from diatoms. cDNA was obtained by reverse transcription reaction using oligo dT primer and moloney murine leukemia virus reverse transcriptase (Promega) according to the manufacturer's protocol. Primers used to amplify fragments are given in

Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/scd). Amplified fragments were cloned into pGEM-T vector (Promega) according to manufacturer's instructions and identity of clones verified by Sanger sequencing. ClustalW software was used to perform both amino acid and nucleotide alignments. Analyses of alignment were done by Boxshade software based on similarity shading. The full length genes were submitted to GenBank with accession numbers JX047307, JX047308, JX047309, JX047310, and JX047311 for *machpo*, *macsav*, *macwts*, *macmats*, and *macyap*, respectively. In the body of this article, the homologous genes are named as Hpo, Sav, Wts, Mats, and Yap for brevity.

### Whole-mount ISH

DNA templates used for producing digoxigenin (DIG)-labeled riboprobes were amplified by standard polymerase chain reaction using the High fidelity Taq polymerase, Pfu (Thermo Scientific). The used primer sets are given in Supplementary Table S2. DIG-labeled RNA probes (700–800 bp in length) were generated using the DIG RNA labeling KIT T7 (Roche) according to the manufacturer's protocol. Whole-mount ISH (WISH) was carried out by following an earlier described protocol [42] and using 6–8 weeks old adults. For *Yap* WISH on juveniles, animals 5–7 days after hatching were used. For *Yap* WISH on regenerating worms, young adult (6–8 weeks staged) individuals were cut below the gonads and one day after cutting they were fixed and analyzed following the same WISH protocol. For *Hpo* and *Yap* expression on irradiated animals, a batch of worm was gamma-radiated by a repetitive high dose of irradiation (210 Gy dose at day 1; 15 Gy dose at days 8 and 9) to eliminate neoblasts and gonads of *M. lignano* [46]. Color development was performed using NBT/BCIP (Roche) under visual control. After development of staining, samples were analyzed and photographed using Nomarski microscope differential interference contrast (DIC) optics and AxioCam HRC (Zeiss) digital camera.

### BrdU labeling

To label neoblasts in S-phase after RNAi treatment, the previously described protocol [43] was followed. Briefly, animals were soaked in culture medium containing 10 mM BrdU (Sigma) for 30 min. Worms were rinsed several times with f/2 medium to get rid of excess amount of BrdU, relaxed with 7.14% magnesium chloride gradually and fixed with 4% paraformaldehyde. After fixation, worms were washed several times with phosphate buffer saline containing 0.1% Triton [PBS-T(0.1%)]. Permeabilization was performed by 1 h incubation in PBS-T. For nicking epithelium layer of worms, specimens were treated with a final concentration of 0.1 mg/mL protease XIV for 20 min at 37°C. 2 M HCl was used for unwinding of DNA for 1 h at 37°C. Blocking was accomplished by bovine serum albumin (BSA) (1%)-PBS-T (0.1%) for 1 h at room temperature. Anti-BrdU antibody (Roche) was diluted in blocking solution 1/200 times and worms were incubated with BrdU antibody overnight at 4°C. Worms were washed with PBS-T(0.1%) briefly and incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse secondary antibody for 1 h at room temperature with 1/1,200 final dilution in blocking solution.

Specimens washed with PBS-T(0.1%) several times and analyzed using Leica DM6000 fluorescent microscope.

### Immunohistochemistry

Individuals, which were pretreated with *Hpo*, *Yap*, or green fluorescent protein (GFP) dsRNA for one week, were cut below the gonad and left with food to restore posterior part. dsRNA treatment was continued during the experiment. Seven days after cutting, samples were collected for immunohistochemistry (IHC) analysis. Mitotic cells labeling was performed using pH3-specific polyclonal antibody from Millipore according to the optimized protocol [47]. *M. lignano* muscle-specific and neuron-specific monoclonal antibodies (mABs) Mmu3 and MNu1 described in Ladurner et al. [44] were used for IHC according to the original protocol [44] with the following modifications: blocking by BSA(1%)-PBS-T (0.1%) was extended from 30 to 60 min, washing with PBS-T(0.1%) after primary (mAB; 1/5 dilution in BSA-T) and secondary (FITC-conjugated goat anti-mouse antibody; Dako; 1:100 in BSA-T) antibody incubation was extended from 30 to 60 min. Specimens were covered with VECTASHIELD® Mounting Medium and analyzed using Leica SPE confocal microscope.

### RNA interference

In *M. lignano*, specific knockdown by RNAi protocol based on soaking was followed as previously described [38,41]. Double-stranded RNAs aimed to target *Hpo*, *Sav*, *Warts*, *Mats*, and *Yap* were generated with an in vitro transcription system (T7 RiboMax TM Express RNAi System; Promega) by following manufacturer's protocol. Same sequences as for ISH were used for dsRNA production (700–800 bp in length), using four gene-specific primers for each gene, in which two of them included a T7 RNA polymerase promoter sequence (Supplementary Table S3). dsRNA was diluted in f/2 to a final concentration of 30 ng/μL. To exclude possible off-target effect of delivered dsRNA, control worms were treated with GFP dsRNA or just f/2 medium (mock). GFP dsRNA as a negative control was produced, diluted to 30 ng/μL and

delivered by soaking. No morphological difference was observed between mock and GFP dsRNA-treated control worms (data not shown). RNAi soaking experiments were performed with young (8–10 weeks old) adults in 24-well plates. Before RNAi, worms were treated with antibiotics (Kanamycin and Ampicillin, 50 μg/mL) for one week. Twenty-five individuals were kept in each well on algae and incubated with 300 μL dsRNA solution (30 ng/μL) in f/2 by refreshing it every day. Individuals used in imaging, BrdU labeling and IHC were chosen randomly. Images were taken by using Nikon Eclipse E600 microscope and Leica DFC 500 digital camera and edited with Adobe Photoshop CS3®.

### Regeneration experiments

Young adults (8–10 weeks old) were collected randomly and treated with *Hpo*, *Yap*, or GFP dsRNA for one week. Pretreated worms were cut just below the gonads with a fine steel surgical blade (SM62; Swann–Morton) to remove the posterior part completely. Regenerating worms were left with food, and dsRNA delivery was continued during the experiment. At days 5 and 10 after cutting, randomly chosen individuals were analyzed using Nikon Eclipse E600 microscope and Leica DFC 500 digital camera to study the regeneration capacity after RNAi treatment.

### Accession numbers

JX047307–JX047311.

## Results

### *Hippo* pathway genes are conserved in *M. lignano*

To identify the components of the Hippo pathway conserved in *M. lignano*, we used publicly available *M. lignano* draft genome and transcriptome assemblies and performed reciprocal BLAST searches against human and *Drosophila* Hippo pathway genes. We identified *M. lignano* orthologs of *Hpo*, *Sav*, *Warts*, *Mats*, and *Yap* genes, all of which share high degree of identity with their orthologs in other species

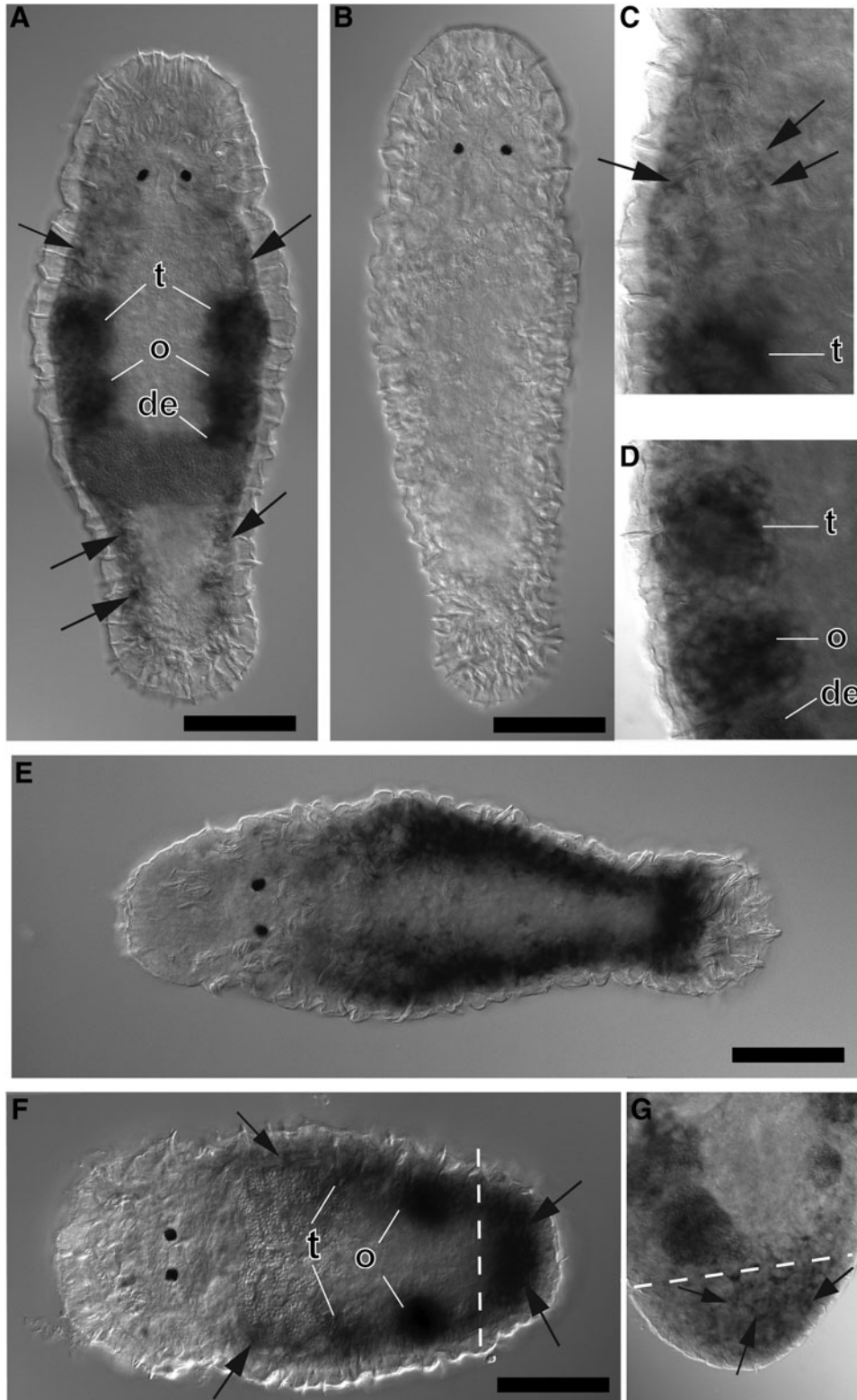


**FIG. 1.** Whole-mount in situ hybridization of the Hippo pathway core genes. (A–D) *Hpo*, *Sav*, *Wts*, and *Mats* are expressed in both proliferating and differentiated tissues. (A) *Hpo* expression is detected in testes, ovaries, developing eggs, somatic stem cells, gut, and glands. (B) *Sav* gene is mostly expressed in proliferating cells. (C) *Wts* expression is extended from proliferating cells to differentiated tissues in gut and tail. (D) *Mats* expression pattern resembles the expression of *Wts* gene, seen in testes, ovaries, developing eggs, neoblasts, gut, and glands. (E) *Hpo* expression after irradiation is detected only in differentiated tissues. t, testes; o, ovaries; de, developing eggs; me, mature eggs. Scale bars: 200 μm. The detailed description of the morphology of *Macrostomum lignano* is provided in Supplementary Fig. S10.

(Supplementary Figs. S1–S5). Since there are some differences in the Hippo pathway gene names between mammals and *Drosophila*, we named the *M. lignano* orthologs following the more frequently used names: *machpo*, *macsav*, *macwarts*, *macmats*, and *macyap*. For simplicity, in the following sections we do not use the “mac” prefix when referring to the *M. lignano* Hippo pathway genes.

### *Hippo pathway core genes are expressed in gonads, differentiated tissues, and neoblasts*

WISH revealed *Hpo* expression in both proliferating cells and differentiated tissues, such as glands (Fig. 1A). *Sav*, *Wts*, and *Mats* genes show similar expression patterns as *Hpo*. Expression of these genes is mainly enriched in gonads, with



**FIG. 2.** *Yap* is expressed in proliferating cells. (A) Whole-mount in situ hybridization of adult specimen. *Yap* expression is detected in testes, ovaries, developing eggs, and somatic stem cells (arrows) and testes. (B) No *Yap* expression detected in irradiated animals. (C) Detail of *Yap* expression in somatic stem cells (arrows) and testes. (D) Detail of *Yap* expression in testis, ovaries, and developing eggs. (E) Expression of *Yap* in 5 days posthatchling juvenile, expression in neoblasts. (F) *Yap* expression in regenerating worms, 24h after amputation. *Yap* expression is visible in testis, ovaries, neoblasts, and blastema. (G) Detail of *Yap* expression in undifferentiated cells (arrows) in blastema. Dashed lines, amputation site; t, testes; o, ovaries; de, developing eggs. Scale bars: 200 μm.

extension to neoblasts and differentiated tissues in the gut (Fig. 1B–D). To investigate *Hpo* expression in proliferating cells, we carried out WISH on animals which were exposed to gamma irradiation. In flatworms, including *M. lignano*, irradiation is routinely used to selectively deplete proliferating cells [38,48–50]. Following irradiation, *Hpo* expression disappeared in gonads but remained unchanged in differentiated tissues (Fig. 1E). This confirms that *Hpo* is expressed in both neoblasts and postmitotic cells. Expression of all Hippo pathway core genes in blastema was detectable 24 h after cutting (Supplementary Fig. S6).

### *M. lignano* *Yap* is expressed in neoblasts

WISH on adult animals revealed *Yap* expression in gonads and anterior and posterior parts of the lateral sides of the worm—the regions where neoblast are primarily located in *M. lignano* [41,43], while no clear expression in differentiated tissues was observed (Fig. 2A, C, and D). For further verification of *Yap* expression in neoblasts, its expression in juveniles, which lack developed gonads, was studied. As shown in Fig. 2E, *Yap* is expressed in the bilateral regions of the juvenile, where neoblasts are typically located, as de-

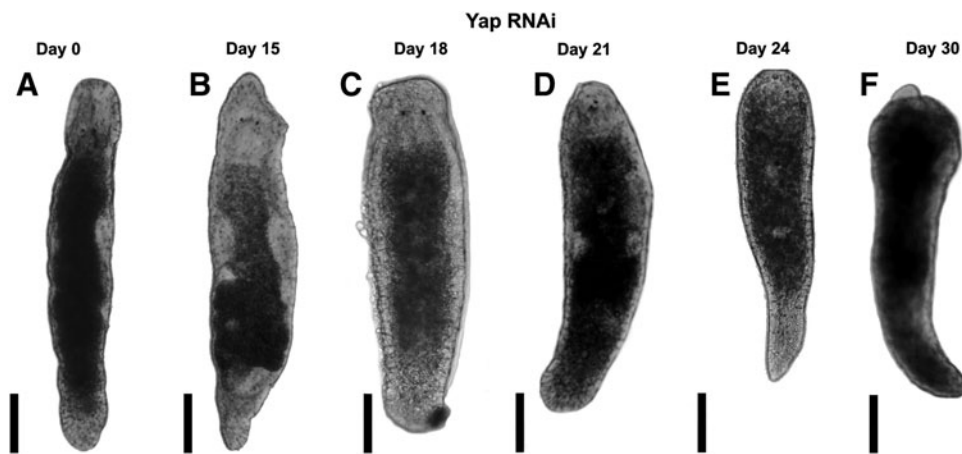
scribed previously [38]. As a next step, *Yap* expression was analyzed in regenerating animals. Upon amputation, *M. lignano* animals initially form blastema—the region at the cutting site where hyperproliferation and accumulation of undifferentiated neoblasts occur [51], and thus, gene expression in neoblasts can be tested by WISH on regenerating worms within 48 h after cutting [38]. We checked *Yap* signal one day after cutting and observed strong *Yap* expression in blastema and additionally in testes, ovaries, and neoblasts as in intact worm (Fig. 2F, G). Finally, no *Yap* expression was detected in irradiated animals (Fig. 2B); thus, confirming that it is not expressed in differentiated tissues. Based on all these data, we conclude that *Yap* expression is restricted to proliferating cells and potentially can be used as a neoblast marker.

### *Hippo* pathway core genes and *Yap* are required for *M. lignano* tissue homeostasis

To clarify the roles of the Hippo pathway in *M. lignano* during homeostasis, we used RNAi to systematically knockdown all the identified genes of the pathway. Efficiency of knockdown of *Hpo* and *Yap* genes was assessed by



**FIG. 3.** Effects of the knockdown of the Hippo pathway core genes on adult tissue homeostasis. (A, F, K) GFP(RNAi) animals do not show any morphological changes during the course of treatment. (A–E) Two weeks of treatment with dsRNA. (B–E) *Hpo*, *Sav*, *Wts*, and *Mats* RNAi worms, respectively, start forming outgrowths (arrowheads). (F–J) Four weeks of treatment with dsRNA. (G–J) Bulges are spreading in *Hpo*, *Sav*, *Wts*, and *Mats* RNAi worms, respectively. (K–O) Six weeks of treatment with dsRNA. (L–O) Severer phenotype with numerous bulges and outgrowths in *Hpo*, *Sav*, *Wts*, and *Mats* RNAi worms, respectively. Scale bars: 200  $\mu$ m. GFP, green fluorescent protein; RNAi, RNA interference.



**FIG. 4.** Effects of *Yap* gene knockdown on adult tissue homeostasis. *Yap*(RNAi) worms gradually lose rostrum integrity (A–F), leading to shrinking of the head and loss of eyes at days 21–24 (D, E). The whole head of the individual is completely lost with reduction in the body size after one month of treatment (F). Scale bars: 200  $\mu$ m.

WISH and for both *Hpo* and *Yap* genes significant decreases of mRNA levels were achieved (Supplementary Fig. S7). RNAi for *Hpo*, *Sav*, *Warts*, *Mats*, or *Yap* was performed on young adults. Morphology of treated animals could not be distinguished from the controls during the initial phase of treatment (data not shown). However, after 2 weeks of RNAi, intact worms treated with *Hpo*, *Sav*, *Warts*, or *Mats* dsRNA started to form outgrowths (Fig. 3B–E). For each of these genes outgrowths were observed in different regions of the body. Continuing RNAi for two additional weeks resulted in an increasing size of the outgrowths and the formation of epidermal bulges throughout the body (Fig. 3G–J). After 6 weeks of RNAi, more severe phenotypes in the form of additional bulges were observed for each of the treatments (Fig. 3L–O). RNAi of the Hippo pathway genes was 100% penetrant, as all treated animals showed outgrowths and bulges and died within 8 weeks of treatment. The GFP dsRNA-treated control worms had no outgrowths and showed no morphological changes within this time period (Fig. 3A, F, and K).

To characterize the function of *Yap* in *M. lignano*, intact worms were treated with *Yap* dsRNA. After 2 weeks of treatment, worms started losing the rostrum integrity (Fig. 4B). Head shrinking proceeded progressively and quickly with continuation of the RNAi (Fig. 4C–E), and individuals lost their head completely after 4 weeks of treatment (Fig. 4F), followed by lysis of the whole worms. In addition to head regression, shrinking in the body size and ventral curling (Fig. 4F) were also observed in *Yap* knockdowns.

#### *Hpo* and *Yap* genes are essential for proper regeneration

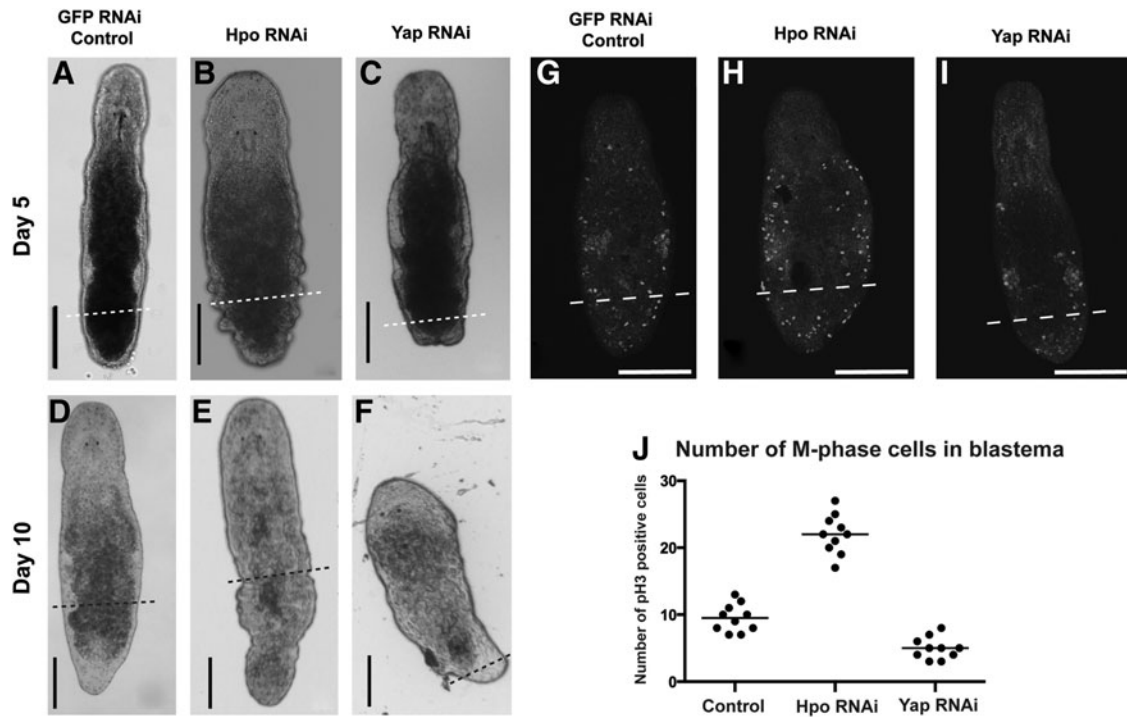
To understand how *Hpo* and *Yap* genes influence the regeneration process in *M. lignano*, worms were pretreated with *Hpo* or *Yap* dsRNA for one week and then cut below the testes. We observed that both treated and control groups can form a blastema. However, there was a clear difference in regeneration of missing tissues between controls and treated worms 5 days after cutting (Fig. 5A–C). During regeneration of the posterior part *Hpo*(RNAi) worms formed bulges around cutting site (Fig. 5B). Worms treated with *Yap* dsRNA were able to close the wound and form a blastema

but could not grow new tissues within 5 days (Fig. 5C) compared to the control group (Fig. 5A). Ten days after the cutting, control animals showed normal regeneration and restored almost whole posterior part, including missing tissues and organs (Fig. 5D). *Yap*(RNAi) worms failed to regenerate and animals started to shrink at day 10 (Fig. 5F) and died in the following 2 days. In contrast, *Hpo*(RNAi) animals regenerated the missing tissues 10 days after cutting (Fig. 5E).

To correlate regeneration capacity and accumulation of dividing cells in blastema, pH3 labeling was performed 2 days after cutting (Fig. 5G–I). The number of mitotic cells identified in blastema for *Hpo*(RNAi) animals (Fig. 5H) was higher than in control ones (Fig. 5G), and the number of pH3 labeled cells in *Yap*(RNAi) worms (Fig. 5I) was significantly lower than in control and *Hpo*(RNAi) animals, as quantified by counting labeled cells in blastemas of 10 randomly chosen individuals from each group (Fig. 5J).

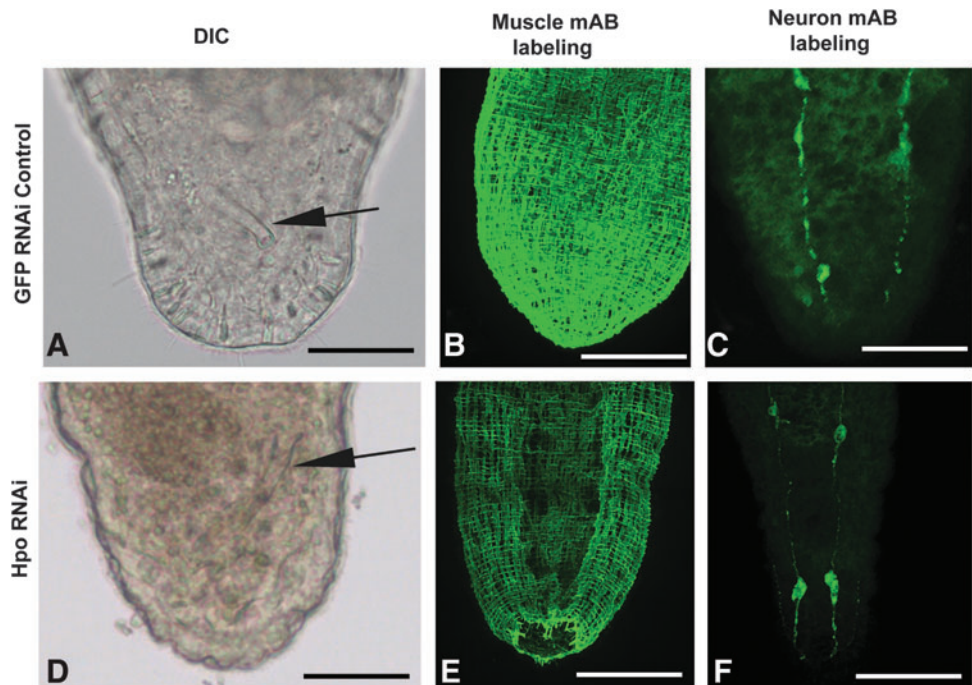
Although *Hpo*(RNAi) worms can regenerate the posterior part, we observed that regeneration occurs in a less controlled way compared to nontreated animals, as evidenced by morphological aberrations and disrupted allometric scaling. The formed posterior parts appeared to be larger than in the control group and quantification of the restored posterior part sizes revealed statistically significant differences between control and *Hpo*(RNAi) worms (Supplementary Fig. S8).

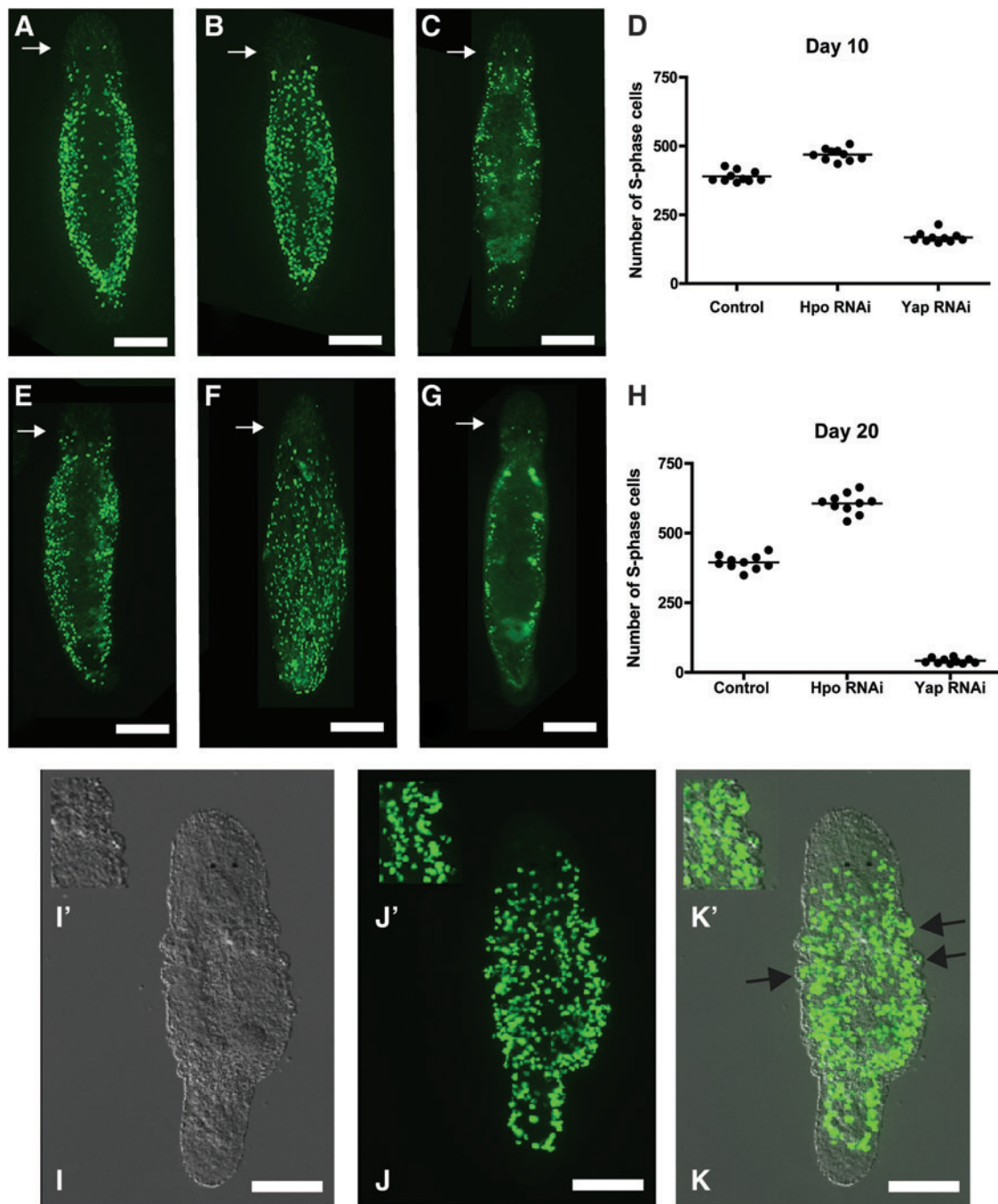
Regenerated parts of *Hpo*(RNAi) worms contained many bulges and outgrowths (Fig. 6D). However, cell differentiation was not affected in *Hpo*(RNAi) animals, as evidenced by formation of organs, such as stylet in *Hpo*(RNAi) worms (Fig. 6D) as in control worms (Fig. 6A). Further, muscle-specific mAb labeling for both control (Fig. 6B) and *Hpo*(RNAi) worms (Fig. 6E), as well as neuron-specific mAb labeling (Fig. 6C, F) showed that formation of muscle and neuronal cells during regeneration was not affected in *Hpo*(RNAi) animals. Whole-mount mAb labeling conferred the continuity in muscle fibers and ventral nerve cord between anterior and regenerated posterior part, although there were slight differences in patterning of these tissues between *Hpo*(RNAi) and control animals (Supplementary Fig. S9).



**FIG. 5.** Effects of *Hpo* and *Yap* gene knockdowns on regenerating *Macrostomum lignano*. (A, D) GFP(RNAi) worms after 5 and 10 days of treatment show normal regeneration process with formation of missing parts. (B, E) *Hpo*(RNAi) animals are able to regenerate the posterior part. (B) The regeneration process is accompanied by the appearance of outgrowths and bulges. (E) The regenerated part is larger than in control animals. (C, F) *Yap*(RNAi) worms fail to regenerate. (C) After 5 days of RNAi, the wound can be closed and a thin blastema is formed. (F) With prolonged treatment, worms are not able to regenerate tissues, shrink in size and die within 2 weeks of RNAi. (G–I) Mitotic cells were labeled by anti-pH3 mAB after 2 days after cutting. White dots show labeled cells. (G) pH3 positive cells in blastema for control worms. (H) Number of pH3 positive cells in blastema for *Hpo*(RNAi) worms. Note the increased number of labeled cells. (I) Number of pH3 positive cells in blastema for *Yap*(RNAi) worms. *Yap*(RNAi) results in decreased number of pH3 positive cells in blastema. (J) Quantitative analysis of the number of the mitotic cells in blastema for control, *Hpo*, or *Yap* RNAi worms after 2 days of cutting. Dashed lines show amputation sites. Scale bars: 200  $\mu$ m. mAB, monoclonal antibody.

**FIG. 6.** Tissue differentiation is not effected with *Hpo*(RNAi). (A–C) Control worms regenerated tail. (A) DIC image of control worm's tail, stylet indicated by an arrow. (B) Muscle-specific mAB labeling on control worms. (C) Neuron-specific mAB labeling on control worms. (D–F) *Hpo*(RNAi) regenerated tail. (D) DIC image of *Hpo*(RNAi) worm's tail. Cell differentiation is not affected, as evidenced, for example, by formation of a stylet (arrow). (E) Muscle-specific mAB labeling shows that *Hpo*(RNAi) worms can undergo tissue differentiation. (F) Neuron-specific mAB labeling is another evidence of tissue differentiation upon regeneration of *Hpo*(RNAi) worms. Scale bars: 100  $\mu$ m. DIC, differential interface contrast. Color images available online at [www.liebertpub.com/scd](http://www.liebertpub.com/scd)





**FIG. 7.** Effects of *Hpo* and *Yap* gene knockdowns on stem cell dynamics. S-phase cells of *Macrostomum lignano* were labeled by BrdU incorporation. (A–D) 10 days of dsRNA treatment. (E–H) 20 days of dsRNA treatment. (A, E) GFP(RNAi) worms show normal cell proliferation and S-phase patterning. (B, F) *Hpo*(RNAi) leads to increase in cell proliferation and changes in patterning. (F) Further increase in the number of labeled cells and a loss of lateral distribution pattern at day 20 of treatment. (C, G) *Yap*(RNAi) leads to a loss of S-labeling. (C) Significant decrease of proliferation cells at day 10 of the treatment. (G) Almost all S-phase cells are depleted at day 20 of the treatment. (D, H) Quantitative analysis of the number of S-phase cells after *Hpo* or *Yap* RNAi at days 10 and 20 of treatments, respectively. The number of BrdU labeled cells was counted for 10 individuals of control group, *Hpo*(RNAi) and *Yap*(RNAi) worms. Each dot on the graphs represents a worm, and the lines are medians of the groups. The number of S-phase cells in *Hpo*(RNAi) worms is higher than in the control worms at day 10, and further increases at day 20. *Yap*(RNAi) worms have less S-phase cells at day 10, and at day 20 worms lost the majority of the S-phase cells. (I–K) S-phase cells accumulate in bulges of *Hpo*(RNAi) animals. The distribution of BrdU labeled cells was inspected after 3 weeks of *Hpo*(RNAi). (I) DIC picture shows the morphology of the worms with several bulges. (J) Fluorescence image of BrdU labeling for same individual. Green dots represent S-phase cells. (K) Overlay of fluorescent and DIC images shows accumulation of S-phase cells in bulges (black arrows). (I'–K') Detailed representation of DIC and GFP channels and the overlay. White arrows in (A–C, E–G) show the eye level. Scale bars: 200 μm. BrdU, bromodeoxyuridine. Color images available online at [www.liebertpub.com/scd](http://www.liebertpub.com/scd)

### The Hippo pathway regulates stem cell proliferation dynamics in *M. lignano*

To elucidate the role of the Hippo pathway in neoblast regulation in *M. lignano*, whole-mount BrdU labeling was performed and the number of S-phase cells quantified. The quantification of S-phase cells in the control, *Yap*(RNAi) and *Hpo*(RNAi) animals was performed at days 10 and 20 of treatment with dsRNA. We found that at day 10 the number of S-phase cells was significantly increased in *Hpo*(RNAi) worms, and significantly decreased in *Yap*(RNAi) worms compared to the control animals (Fig. 7A–D; Supplementary Table S4, *t*-test,  $p < 0.01$ ,  $n = 10$ ). At day 20, post-RNAi, the number of S-phase cells in the *Hpo* knockdown worms was drastically higher than in the control animals, and the bilateral pattern of dividing cells was lost (Fig. 7F, H). BrdU-labeled stem cells were dispersed throughout the whole body, which is atypical for stem cell localization in *M. lignano*. At the same (day 20) time point, *Yap*(RNAi) worms lost almost all of their dividing cells (Fig. 7G, H; Supplementary Table S4). Next, we sought the cellular composition of the bulges that appear in *Hpo* knockdowns. We performed BrdU labeling of the *Hpo*(RNAi) worms at week 3 post-RNAi and observed accumulation of S-phase labeled cells in the bulges of epithelial tissues (Fig. 7I–K). This result indicates that the bulges and outgrowths are formed due to accumulation of dividing cells.

### Discussion

The Hippo signaling pathway is broadly conserved across the animal kingdom, although some of its components appear to be possibly lost in some phyla, for example, in nematodes [52]. Based on data from *Drosophila* and mammals, it has been widely accepted that the Hippo pathway is crucial for constraining cell proliferation and tumor suppression (reviewed in [2] and [15]). However, to date, there were no functional studies of the Hippo pathway in a highly allometric scaling organism, nor in an animal from Lophotrochozoan phylum, which includes a wide range of animals. Here we show that all Hippo pathway core genes and *Yap* gene are highly conserved in a Platyhelminthes representative *M. lignano*. The Hippo pathway core genes (*Hpo*, *Sav*, *Wts*, and *Mats*) are broadly expressed in both proliferating and differentiated cells. Expression in gonads and neoblasts may be due to the regulatory roles of the Hippo pathway during cell division. Since the activity of the Hippo pathway members is regulated primarily at the post-translational level [8,16,17], high expression at the mRNA level in dividing cells can be a protection mechanism of the organism to cease dividing and stimulate differentiation of mitotic cells when necessary. Elimination of neoblasts with irradiation unmasked expression of the Hippo pathway core genes in terminally differentiated tissues in *M. lignano*. These findings are consistent with the known expression of the core components of the Hippo cascade in a broad range of human tissues, including both differentiated cells and adult stem cells [53]. In mammals, the Hippo pathway acts as a tumor suppressor through its function as a negative regulator of cell proliferation (reviewed in [54]). Here we show that the knockdown of the Hippo pathway core components by RNAi in *M. lignano* leads to outgrowths due to the increased

number of proliferating cells, reminiscent of tumor formation, and results in the tissue homeostasis failure. Thus, the Hippo pathway plays the same tumor suppressor role in flatworms as in mammals. Surprisingly, in addition to increased rate of cell proliferation, bilateral patterning of proliferating cells is lost in *Hpo*(RNAi) animals: dividing cells are dispersed throughout the whole body, and this might be seen as an evidence of migration of stem cells without differentiation. The appearance of dividing cells in the middle region and epithelial tissues of the body resembles tumor cell migration in mammals. These data may account for a new function of the Hippo pathway in control of localization of stem cells in flatworms. To test this hypothesis, development of new tools for visualization of stem cell migration in *M. lignano* will be required.

Expression pattern and function of *Yap* also appear to be conserved between *M. lignano* and mammals. In mammals, *Yap* is highly expressed in embryonic stem (ES) cells and is an important player in maintaining pluripotency of both ES cells and induced pluripotent stem cells, and its inactivation is crucial for differentiation of ES cells [18]. Here we gathered several lines of evidence that support neoblast-specific expression of *Yap* in *M. lignano*. It has a clear expression in gonads and neoblasts in adults, in juveniles it is expressed only in neoblasts, its expression is abrogated by irradiation, and it is highly expressed in blastema regions. Knockdown of *Yap* by RNAi leads to a dramatic decrease in the number of S-phase cells, which is often used as a proxy to judge neoblast activity [42,43,46], and to a head regression phenotype, which is associated with stem cell loss in the flatworms and was shown in planaria before [55,56], but reported here for the first time in *M. lignano*. These data suggest that YAP is required for neoblast proliferation in *M. lignano*, and since stem cells of flatworms are thought to be pluripotent, as single neoblast is capable of making all cell lineages [57], it appears that YAP has the same pluripotency function of maintaining neoblast self-renewal in *M. lignano*. However, this should be validated by further experiments once good differentiation markers for *M. lignano* become available. Expression of *Yap* should be also studied in relation to known neoblast markers (eg, *Piwi*), which would require development of a robust protocol for double fluorescent in situ hybridization in *M. lignano*.

In contrast to the process of adult tissue homeostasis, limited regeneration capacity of mammals leaves open questions concerning the roles of the Hippo pathway during regeneration. Flatworm species, on the other hand, have enormous regeneration capacity, and for this reason are used extensively for research on regeneration [33]. Here we show that in *M. lignano* *Hpo*(RNAi) worms could form a blastema and regenerate the posterior part but with loss of proportion of the regenerates. The regenerated parts are bigger in *Hpo*(RNAi) worms than in controls but overall tissue structure is not affected, indicating that HPO is not involved in differentiation of neoblasts into various cell types but is crucial for maintaining the scale of regenerated parts. *Yap*(RNAi) worms, on the other hand, could close the wound but failed to add new tissues around the wound sites at the onset of regeneration. Subsequently, *Yap*(RNAi) worms showed shrinkage of the body due to defects in homeostasis, could not regenerate the posterior part and eventually died.

The results of the knockdown of the Hippo pathway core genes and *Yap* by RNAi are best interpreted in the context of different steps of flatworm regeneration, which is a highly coordinated and tightly regulated process [58]. During regeneration, the first step is closing the wound, followed by blastema formation through hyperproliferation of neoblasts. The next step is restoring the missing parts by differentiation and re-establishment of organs and structures in the allometric scale through cell death and tissue remodeling. Our data show that HPO or YAP activity is not necessary for wound closure, which is a process of contraction of muscles and flattening of epidermal tissues but formation of blastema and restoring of the missing part is strictly dependent on YAP activity, since *Yap*(RNAi) worms failed to make new tissues. At the same time, HPO activity is not essential for formation of blastema, cell differentiation, and addition of new tissues but it is critical for scaling and remodeling at the final steps of differentiation. Our results suggest that this function of *Hpo* is conserved between flatworms and mammals, where, for example, loss of *Hpo* leads to overgrowth of liver during regeneration [59].

Taken together, we show that the Hippo pathway is functionally conserved between flatworms and mammals, and regulates the size of regenerating parts by controlling stem cell proliferation. While significant progress in dissecting the Hippo pathway has been made during the last decade, there are still many open questions concerning upstream and downstream elements of the pathway, as well as integration of the pathway with other signaling networks involved in stem cell regulation [24]. Here we propose that many of these questions can be efficiently addressed using *M. lignano* as a model organism. Indeed, this animal has high regeneration capacity and stem cell population, that is, easily accessible for manipulation. *M. lignano* is easy to culture in the laboratory conditions, it has short generation time, and gene knockdowns are easily achieved by RNAi, whereas small size and transparency facilitate characterization of phenotypes. Genetic and genomic recourses, including genome sequence, are available, and proof-of-principle for generating transgenic animals has been obtained (Demircan and Berezikov, in preparation). Thus, *M. lignano* is well-positioned as a model for dissecting the mechanism of regeneration and the details of the various molecular pathways, such as Hippo, that are involved in regulation of stem cells.

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### Author Disclosure Statement

The authors declare no potential conflicts of interest.

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Address correspondence to:

*Dr. Eugene Berezikov  
European Research Institute for the Biology of Ageing  
University of Groningen  
University Medical Center Groningen  
Antonius Deusinglaan 1  
9713AV, Groningen  
The Netherlands*

*E-mail: e.berezikov@umcg.nl*

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