scientific reports

Check for updates

OPEN

Mining differentially expressed genes during paratomy in the transcriptome of the flatworm *Stenostomum leucops*

Marcos Trindade da Rosa[1] & Elgion Lucio Silva Loreto[2]✉

Paratomy is an asexual reproductive process that occurs in annelids, flatworms and other groups. During this process, anterior structures are formed in the middle of the body, giving rise to segmented organisms, each segment being called a zooid. Once formed, the zooids detach and form new organisms. Using RNAseq of worms prior to zooid formation and with two zooids, we search for genes that are differentially expressed and may be related to the control of this process in the flatworm *Stenostomum leucops*. Several signaling pathways showed differential expression, including MAPK/ERK, PI3K-Akt, Wnt, TGFb, mTOR, FoxO and others. Forty-five genes were found to be particularly significant because they are differentially expressed and play an important role in the development of other flatworms. These include *ERK*, *MKP*, *JNK*, *PI3K*, *PTEN*, *β -catenin*, *FoxO*, *Sufu*, *GH* and others. The results suggest some similarities in gene regulation between paratomy and regeneration observed in other flatworms after fission or amputation. In worms without zooids, pathways required for cell proliferation, differentiation of cells into multiple cell lineages and determination of the body axis are activated. In worms with 2 zooids, genes involved in cell growth and apoptosis are activated. Activation of genes involved in neoblast proliferation and maintenance appears to occur at both stages.

Keywords Asexual reproduction, Planaria, Catenulida, Paratomy.

Stenostomum leucops is a small freshwater flatworm with a length of 0.5 to 2 mm. It belongs to the Catenulida, a basal group within the Platyhelminthes[1–3] (Fig. 1A). These species reproduce mainly or exclusively asexually. Although development from eggs was reported years ago[4], more recent observations have not confirmed this. We have kept this species in the laboratory for over a decade and have observed no evidence of sexual reproduction. Remarkably, the Catenulida exhibit paratomy — a unique asexual reproductive process in which anterior structures form in the middle of the body, giving rise to segmented organisms, each segment called a "zooids". Once fully formed, the zooids detach and form new organisms. In *S. leucops*, this process takes about 42 h at 28 °C, and in special cases can result in the formation of up to five zooids in a chain, although it generally produces only two zooids[5] (Fig. 1B; Suppl. Video_Paratomy). Paratomy occurs in various taxa, including annelids, platyhelminths, coelenterates and echinoderm larvae[6–9]. Although it occurs in various groups, the genetic basis of paratomy remains poorly understood.To our knowledge, only one study has implicated *ParaHox* genes in the paratomy of asexual annelids[10].

Free-living flatworms such as planarians have a remarkable regenerative capacity, which is due to the abundance of adult pluripotent stem cells, the so-called neoblasts. These organisms can regenerate an entire animal from a small fragment of tissue, making planarians excellent models for regeneration and stem cell research[11]. The genomes of planarians such as *Schmidtea mediterranea*[12], *Macrostomum lignano*[13] and *Dugenia japonica*[14] have been sequenced and the gene pathways involved in regeneration have been identified. In addition, there are strains of these species that reproduce asexually by fission, a process that bears some resemblance to paratomy, where worms divide by contraction and then each part regenerates the complete body. The main difference is that in paratomy, the differentiation of body structures occurs before the segmentation of the body.

Many studies have used RNAi, dsRNA interference, in situ RNA hybridization, whole-mount immunostaining and drug inhibition to characterize genes involved in planarian regeneration[15–18]. Some studies have also

[1]Programa de Pós-Graduação em Biodiversidade Animal, Universidade Federal de Santa Maria, Santa Maria, Rio Grande do Sul, Brazil. [2]Departamento de Bioquímica e Biologia Molecular, Universidade Federal de Santa Maria, Av. Roraima 1000, Santa Maria CEP 97105-900, RS, Brazil. ✉email: elgion.loreto@ufsm.br

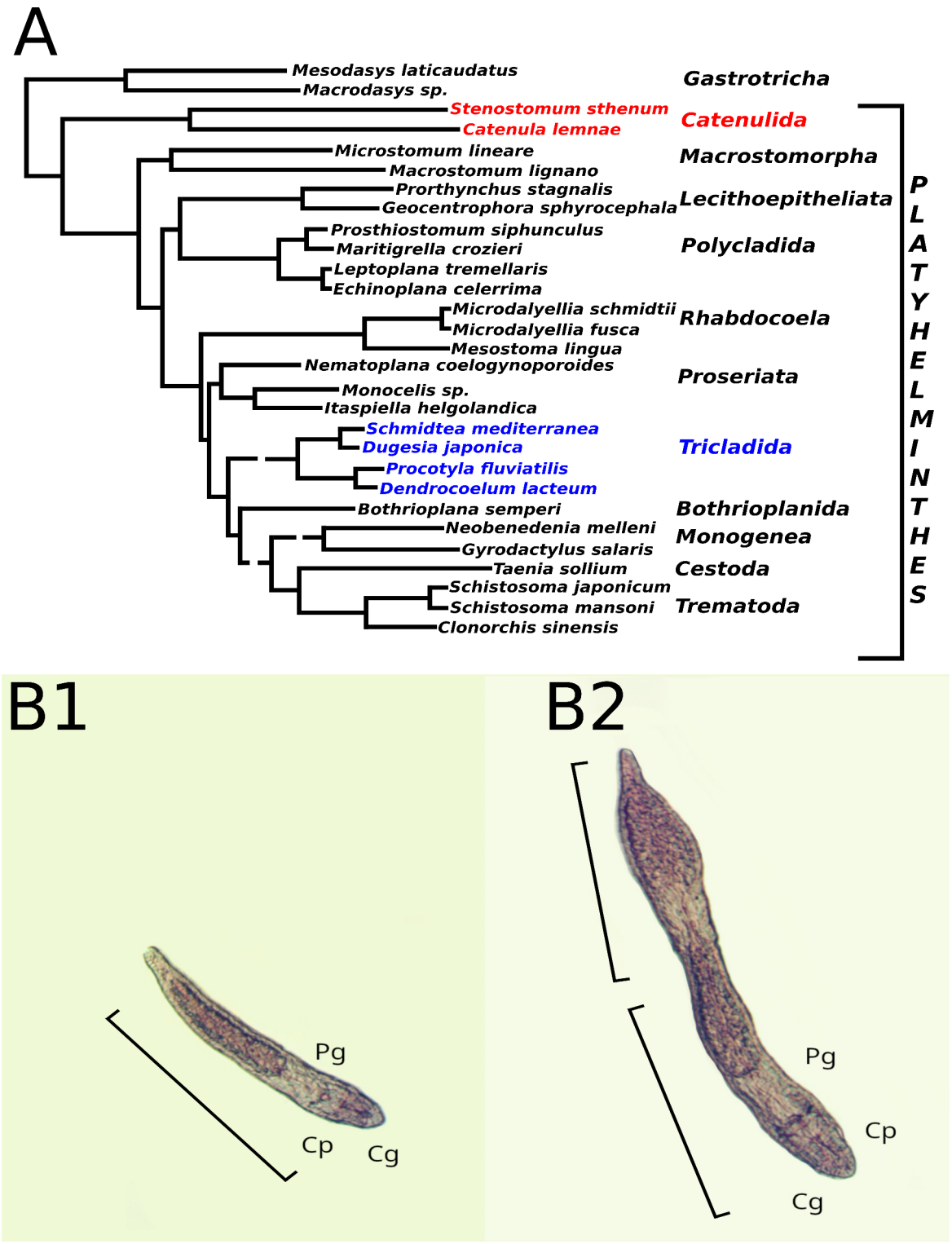

Fig. 1. **(A)** Phylogeny of Platyhelminthes highlighted the Catenulida and the planaria clade (Tricladida), based on Egger et al.[2]. The paratomy process in*S. leucops*shows worms without zooid (**B1**) and with two zooids (**B2**). The formation of anterior body structures in the midsection can be seen in the worm with two zooids. *cp* ciliated pits, *cg* cerebral ganglia, *pg* pharyngeal gland.

searched for genes involved in the fission process in planarians[19,20]. *Stenostomum leucops* with its significant regenerative ability has been used in regeneration research for over a century and Rosa & Loreto[21] have conducted a comprehensive analysis of this process. Recently, Gąsiorowski et al.[22] have shown in a detailed study that the gene *pax4/6* is involved in the formation of sensory pits during the regeneration of *Stenostomum*. Thus, today

we have an understanding of the pathways involved in planarian regeneration and genes involved in planarian fission, that can serve as a basis for investigating genes related to paratomy in Catenulida.

In this study, we have assembled and annotated a high-resolution comparative transcriptome of *S. leucops* worms previous to the zooid formation and worms with two zooids. The analysis of the differentially expressed genes at these stages aimed to identify genes potentially involved in the paratomy process. Genes associated with planarian regeneration as previously reported in the literature, were also examined to determine their presence in the *S. leucops* transcriptome and whether they exhibit differential expression between worms without zooids and those with zooids.

Materials and methods

Animals, nucleic acid isolation and sequencing

A clonally originated *S. leucops* strain (SL0-sm02) was collected in 2009 from a pond in Santa Maria, Brazil (53°17′W; 29°28′S) and maintained in the laboratory at 28 °C. Worms were kept in reconstituted water and fed with milk powder as described by Rosa et al.[5]. The use of wild animals for research purposes is regulated by local legislation and the corresponding registry was assigned the number: SisGen-Brazil -AFE70C2.

For transcriptome analysis, total RNA was isolated using Trizol and chloroform (Invitrogen®) according to the manufacturer's guidelines. The worms were caught individually under an inverted microscope with a micropipette and divided into groups without or with two zooids. For each group, two replicates of 300 worms were used for RNA extraction. RNA quality was checked with an absorbance of 1.90 at 260 nm/280 nm using a Nanodrop ND-1000 spectrophotometer (LabTech, USA) and RNA integrity was assessed using a 2100 Bioanalyzer (Agilent Technologies, USA). For library preparation, mRNA was enriched using the Dynabeads® mRNA purification kit (Invitrogen) and library preparation was performed using the Total Ion RNA-Seq v2 kit (Thermo Fisher Scientific). Sequencing was performed with the IonTorrent S5 sequencer (Thermo Fisher Scientific) using the Ion 540 TM Kit-OT2 and the Ion 540 TM Chip. The transcriptome sequences were deposited in GenBank (BioProject PRJNA1037460; accessions SAMN42159283; SAMN42159284).

Assembly and analysis of the transcriptome

The quality of reads was assessed using Fasqc software[23], and trimming was performed using Trim Galore[24] to remove adapters as well as reads and sequences with quality lesser than 20. Transcriptome assembly was performed using Trinity software[25]. The completeness of the assembly was analysed using Busco software[26] with the metazoa_odb10 database, which contains 954 basic proteins. The prediction of the coding region was performed with the Augustus software[27]. To train the software, genomes of *Macrostomum lignano* (GCA_002269645.1/GCA_001188465.1), *Caenorhabditis elegans* (NC_003279.8/NC_003280.10/NC_003281.10/NC_003282.8/NC_003283.11/NC_003284.9/ NC_001328.1) and *Schistosoma mansoni* (NC_031495.1/NC_031496.1/NC_031497.1/NC_031498.1/NC_031499.1/NC_031500.1/ NC_031501.1/NC_031502.1/NC_002545.1) were used due to their high coverage, different annotation methods, gene validation and phylogenetic proximity to *S. leucops*. The transcriptome was also annotated with the KEGG database 2023 using the Galaxy platform[28].

Once the transcriptome was assembled and annotated, the redundancy was removed (the pipeline used is shown in Fig. 2). The software Kallisto[29] was used to quantify the abundance of the transcripts. Kallisto's output was fragmented in files containing: all transcripts present in the different stages, transcriptions expressed exclusively in each stage, and a complete set of transcripts detected in our analyzes. The files containing the transcripts expressed at each stage were submitted to DESeq 2 software to identify differentially expressed genes (DEGs) by comparing expression in worms without zooids to the two zooids. A threshold of Padj = 0.05 and 2 for log 2 Fold Change was used to determine DEGs. In addition, an analysis was performed with Padj = 0.05 and

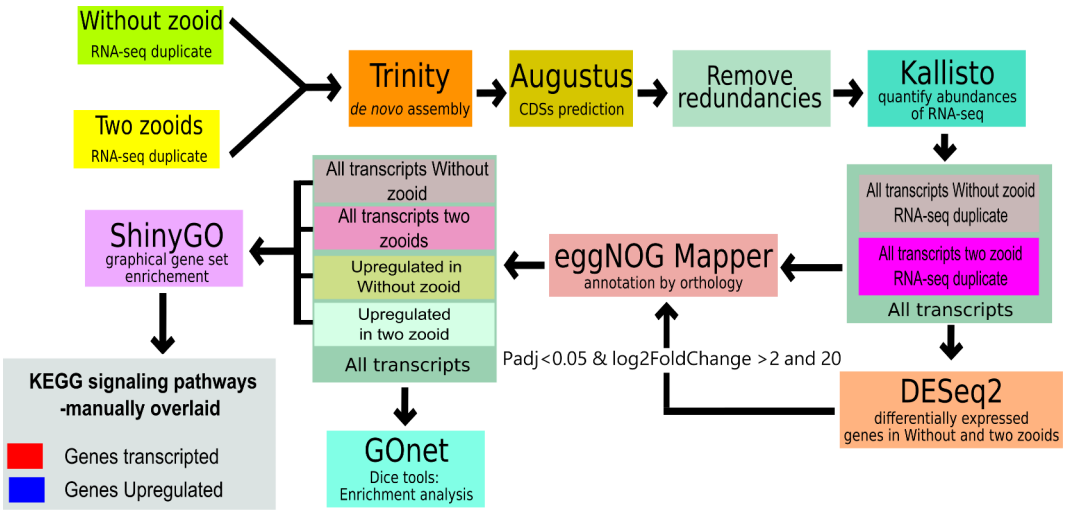

Fig. 2. Pipeline of the transcriptome analysis. The drawings show the most important steps of the analyses and the software used.

20 for log 2. This threshold was chosen to include only genes with a high degree of differential expression in the KEEG pathways. The transcript files were analyzed with EggNOG Mapper 2.1.8[30] for orthology annotation and then enriched with ShinyGO 0.80[31] for KEGG pathways[32]. In the pathways, genes that were exclusively expressed at a certain stage or were highly differentially expressed (log 2 fold change > 20) have been highlighted. The volcano plot was created with the software ggplot2[33].

The genes *Piwi*, *Ago2*, *POU2F2*, *PAF1*, *SOX*, which have been described as being involved in the maintenance of the pluripotent cell state in planaria[34–38], and the genes *C4M1*, *FBL* and *H2A*, which are neoblast markers in *Stenostomum*[39], were searched directly in the DEG genes by using the sequences of these target genes via the HMMER web server[40].

The scripts and raw data used were deposited in: https://figshare.com/articles/dataset/_b_Mining_genes_involved_in_paratomy_in_the_transcriptome_of_the_flatworm_b_b_i_Stenostomum_leucops_i_b_/27054595.

Results

The transcriptional pattern of worm with and without zooids

- The RNA-Seq data from worms without and with two zooids yielded 80,831,095 reads ranging in size from 40 to 350 bp. The assemblage with the Trinity software yielded 279,881 contigs with n50 = 849. Busco analysis showed a satisfactory completeness of genes: 67.9% complete, 20.4% fragmented and 11.7% missing (Supplementary Table 1; Supplementary Fig. 1). Principal component analysis (PCA) showed that the samples were homogeneous, and gene plot distribution analysis showed the expected scatter (Supplementary Fig. 2). The Augustus software predicted 94,161 putative coding DNA sequences (CDSs). However, after annotation with Blastx and functional annotation with eggNOG Mapper, the total number of annotated transcripts was 65,324 (Supplementary spreadsheet; Sheet_1). Analysis of differential gene expression in worms without and with 2 zooids showed that at Padj 0.05 and Log 2 Fold Change > 2, 12,045 genes of worms without zooids were upregulated (Supplementary spreadsheet- sheet_2) and 7,440 genes were upregulated in worms with two zooids (Supplementary spreadsheet, sheet_3) and 45,839 transcripts showed no differential expression (Figs. 3A and B and 4A). When the cutoof Padj used was 0.05 and Log 2 Fold Change > 20, 3,710 genes were upregulated in worms without zooids (Supplementary spreadsheet, sheet_4) and 1,299 genes were upregulated in worms with two zooids, and 60,315 showed no differential expression (Supplementary spreadsheet, sheet_5) (Figs. 3C and D and 4B).

Developmental pathways

To compare the gene expression patterns in the pathways associated with development, the transcribed genes and the upregulated genes in the KEGG pathways[32] were determined. As shown in Fig. 5 for the MAPK pathway, the upregulated genes are found in the stage without zooids. Some genes such as *Rap 1*, *ERK* and *JIP3* are only expressed at the stage without zooids, while *MKP* and *MEK5* are only expressed at the stage with 2 zooids. Importantly, the *JNK* and *p38* genes are upregulated in the without zooid stage (Table 1).

In the PI3K-Akt signaling pathway (Suppl. Material 2), the *PI3K* gene is upregulated at the two zooid stage (Table 1), *PTEM*, *Rac1* are only transcribed 1-zooid stage. On the other hand, *PDK1* and *FAK* is only transcribed in worms with two zooids. In the FoxO signaling pathway, the largest number of transcribed and upregulated genes in without zooid worms is remarkable (Fig. 6). It is also noteworthy that the *FoxO* gene and *Homer* are only active in without zooid worms. However, *USP 7* is only transcribed in worms with 2 zooids.

Analysis of several other signaling pathways involved in development revealed further differences in gene expression (Suppl. material 2). The most notable are listed in Table 1. Among the Wnt signaling pathways, for example, the *ß-catenin* gene is upregulated in without zooid. *Znrf3* is only transcribed in worms without zooid, while *PONTIN52* is upregulated in the two-zooid stage. In the mTOR signaling pathway, the *mTOR* gene is upregulated in the 2-zooid stage and *GATOR1*, *SLC7A5* are only transcribed in without zooid worms. In the Hedgehog signaling pathway, it is remarkable that in the without worms almost the entire signaling pathway of genes is UP-regulated. The *Sufu* gene is upregulated in the without zooid stage and no longer present in 2-zooids worms. In the Growth hormone synthesis, secretion and action pathway call attention that the *GH* gene (growth hormone) and *FAK* gene are only active in the 2-zooid stage. In the pluripontency pathway the *Dusp9* gene is only detected in the 2 zooids stage. In the Sphingolipid signaling pathway, there are more upregulated genes in the without zooid stage. In the TGFB (transforming growth factor beta) signaling pathway, it is noteworthy that there are many more UP-regulated genes in the without zooid stage than in the 2-zooid stage. The *p15* gene, which is associated with G1 arrest, is expressed in without zooid worms and not in 2 zooids. The SIN3A gene is expressed in 2 zooids and not in without zooid stage. In the apoptosis pathway, the most important finding is that the *CASP3* and *CASP7* genes are upregulated in 2 zooids and ENDO G is only active at this stage. In the VEGF signaling pathway *FAK* and *COX2* genes are only active in 2 zooids.

Other important genes involved in pluripotency that were differentially expressed were one of the *Piwi* and *Argonaute* genes, which were upregulated at the 2-zooid stage, while *POU2F2*, *SOX* and *PAF1* were upregulated at the without zooid stage (Table 1). The neoblast markers genes *C4M1* and *FBL*, were only present in the two zooid stages and were UP-regulated. The *H2A* gene is transcribed in both stages, but UP-regulated in the stage without zooids.

Discussion

In *Stenostomum leucops*, the paratomy is an asexual reproductive process that is accompanied by a considerable change in gene expression. Of the total annotated genes, about 13% are expressed only in the stage without zooids, while about 10% occur exclusively in the stage with two zooids. In addition, several developmental pathways are differentially enriched in these stages. Paratomy has some aspects in common with the fission and regeneration

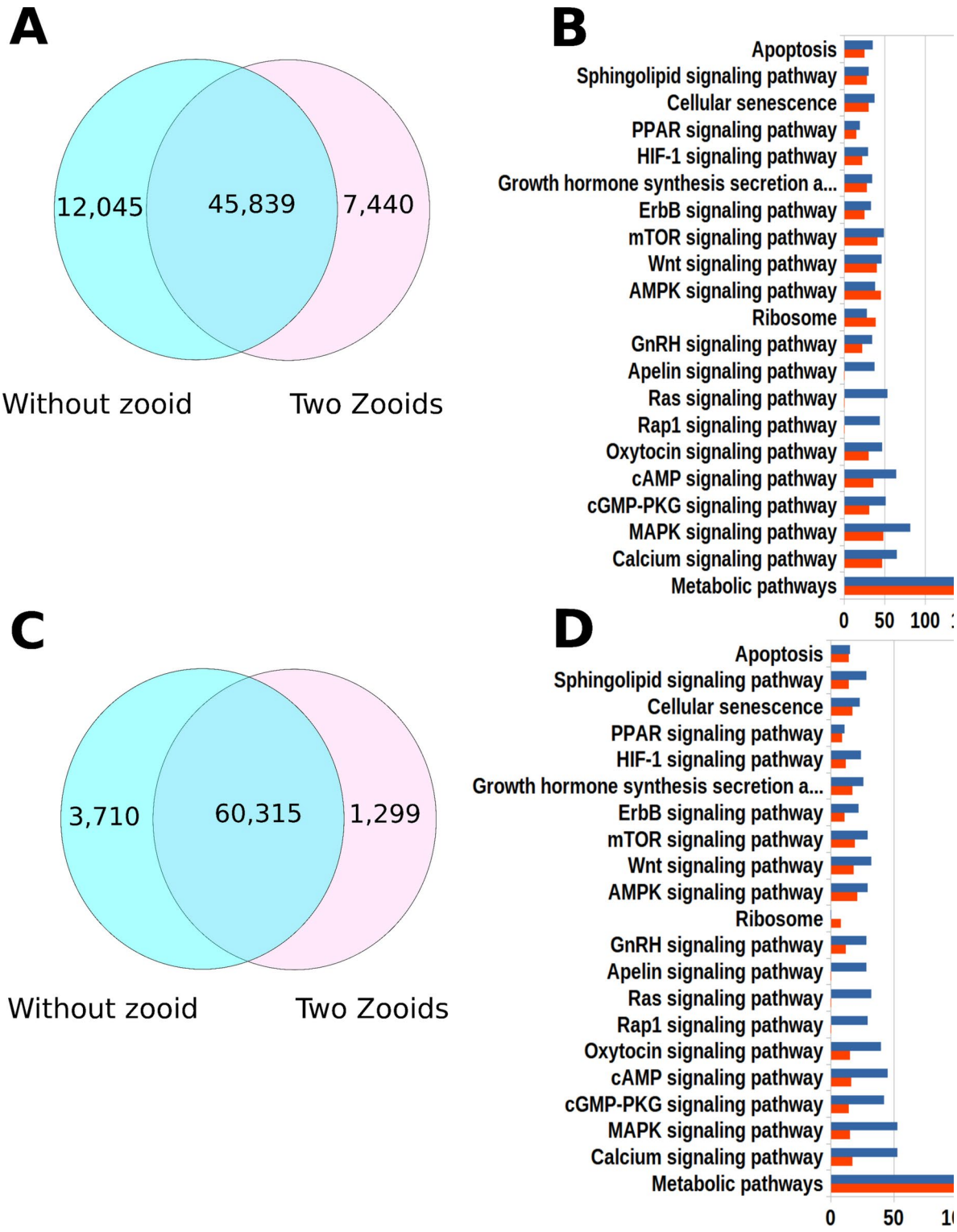

Fig. 3. Overview of the annotated genes in the transcriptome of*S. leucops*. (**A**) Venn diagram showing the number of transcripts present in worms without zooids (blue) or with 2 zooids (pink). The numbers indicate the annotated genes that are upregulated at each stage and the transcripts that are shared by worms at both stages but are not upregulated. (**B**) Classification of KEGG pathways[32] for genes that are upregulated in worms without zooids or 2 zooids. In A and B, a cutoff of Padj 0.05 and Log 2 Fold Change > 2 was used. (**C**) Venn diagram showing the number of transcripts present in worms without zooids (blue) or with 2 zooids (pink). The numbers indicate the annotated genes that are upregulated at each stage and the transcripts that are shared by worms at both stages but are not upregulated. (**D**) Classification of KEGG pathways for genest hat are upregulated in worms without zooids or 2 zooids. In (**A,B**), a cutoff of Padj 0.05 and Log 2 Fold Change > 20 was used.

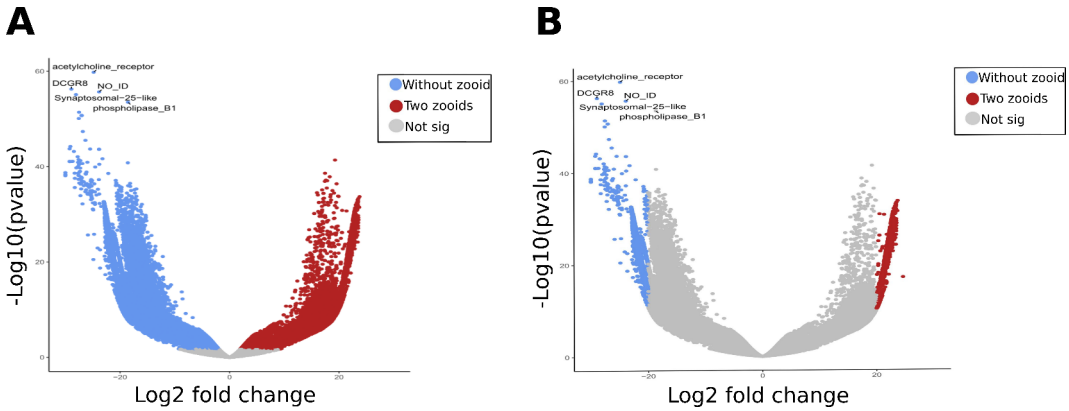

Fig. 4. Differential gene expression in worms without or with 2 zooids. (**A**) Distribution of differentially expressed genes, shown as a volcano plot in worms without and with 2 zooids. The blue dots represent the upregulated genes in worms without zooids and the red dots represent the upregulated genes in worms with 2 zooids. The gray dots represent the genes that did not reach the parameters for the plot (Padj < 0.05 and Log2 FC > 2). (**B**) Distribution of differentially expressed genes, shown as a volcano plot in worms without and with 2 zooids. The blue dots represent the upregulated genes in worms without zooids and the red dots represent the upregulated genes in worms with 2 zooids. The gray dots represent the genes that did not reach the parameters for the plot (Padj < 0.05 and Log2 FC > 20).

processes that occurs in various planarian species. However, in paratomy, the growth and differentiation of body structures precede the fission process. Theoretically, an increase in the number of neoblast cells and the first steps of differentiation are expected in the paratomy during the without zooid stage, as they occur in planaria during blastema formation after fission or amputation. In the two-zooid stage, the growth and final differentiation of these cells should proceed in parallel, as is the case in planaria during the final steps of regeneration. Indeed, the mitogen-activated protein kinase (MAPK) pathway is remarkable in that only genes are upregulated at without zooid stage. This signalling pathway is highly conserved and involved in various cellular processes such as cell division, proliferation, differentiation and migration[41]. Caution is required with this comparison, because in the regeneration of planarians many genes are activated in response to a wound[42,43]. In paratomy, on the other hand, the development of structures occurs without a wound or prior cleavage.

MAPK/ERK signalling has been shown to be required for the first steps of the regeneration process in planarian. Using RNAi and drug inhibition, Tasaki et al.[44] showed that ERK in *Dugesia japonica* is required for the differentiation of blastema cells into multiple cell lineages. It was also found that *MKP* has an inhibitory effect on *ERK* and is also involved in the differentiation process. In *S. leucops*, the *ERK* gene was observed to be expressed only at the without zooid stage, whereas *MPK* is expressed at the 2-zooid stage. These results suggest that regulation of the first steps of paratomy may correspond to blastema formation during regeneration and the second stage to the final steps of regeneration. Jaenen et al.[45] showed that in *Schmidtea mediterranea* the activation of *ERK* is mediated by H_2O_2 or reactive oxygen species. They also showed the involvement of *EGFR*. It is noteworthy that *EGRF* is only transcribed at the without zooid stage in our analyses.

In planarians, studies using RNAi and inhibitor-treated animals have shown that the *Akt* and *PI3K* genes play a role in regeneration, apoptosis and tissue maintenance, similar to other organisms[46]. In our analyses, we found a significant difference in the expression patterns of this pathway, as *PI3K* is upregulated in 2-zooids and the expression of *PTEN*, an inhibitor of *PI3K*, is restricted to without zooid worms. The *RAC1* gene, known to be involved in flatworm regeneration and to be overexpressed in blastema[47], is also only expressed only in without zooid. The *PDK1* gene, has not yet been studied in planarians, but in mice it is essential for embryonic development as it regulates cell growth[48]. This gene is active in *Stenostomum* at the 2-zooid stage, where more effective cell growth is expected. Another gene that is only expressed in the 2-zooid stage is focal adhesion kinase (*FAK*), whose function in flatworms has not yet been characterized. In humans, however, it is involved in various types of cancer and is associated with cell migration, survival, proliferation and adhesion[49]. Also, the *GH* (growth hormone) gene is only detected active in the 2-zooid stage. These genes are good candidates for functional studies once they appear to be important in the 2-zooid phase of *Stenostomum* paratomy. It should be recalled that the transcriptome approach used here assumes total RNA expression and focuses only on genes with high differential expression between paratomy stages. High expression alone is not a direct indicator of function, as many gene products must be phosphorylated or interact with other proteins to be functional. Also, genes that are only expressed in a subset of cells or a tissue can also be very important during development, but cannot be recognized in a transcriptome created from the entire body.

Consistent with the hypothesis that activation of genes involved in cell differentiation occurs at the without zooid stage, several genes associated with morphogenesis in functional studies in flatworms were found to be expressed or overexpressed only at this stage. For example, *β-catenin*, which is required for anteroposterior determination[50]. *Smad 3* and *Smad 4*, which play an important role in the dorsoventral axis and cell differentiation[5152]. *FoxO* is involved in eye and brain regeneration[53]. The Notch signalling pathway with the most upregulated genes in without zooid plays a key role in normal morphological development[54].

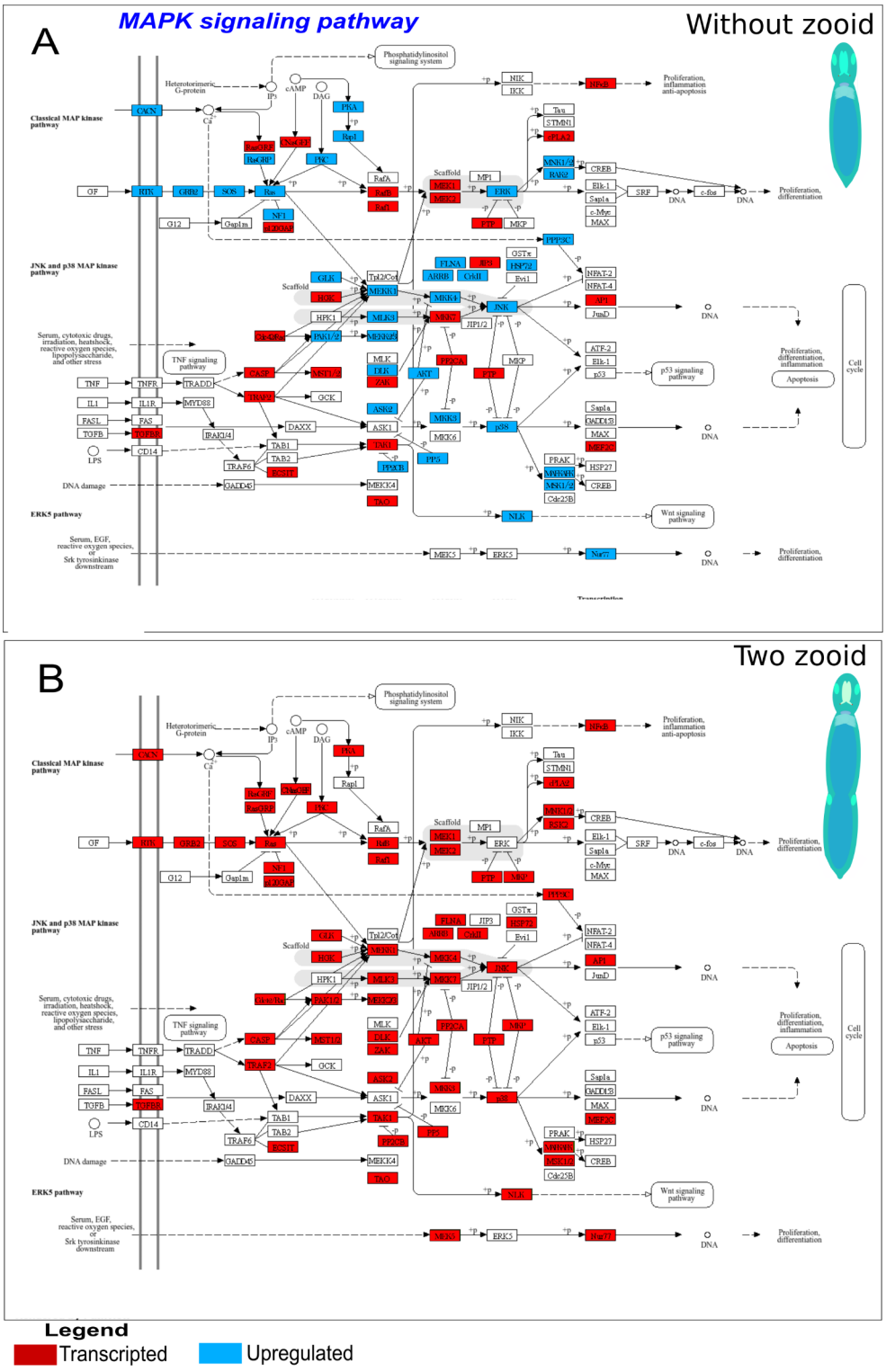

Fig. 5. KEGG graphical of MAPK signaling pathway[32]. Comparison of gene expression pattern observed in worms without zooid (**A**) and with 2-zooids stages. The KEGG signaling pathways are used with permission of the authors.

On the other hand, in agreement with the hypothesis that in the 2-zooid phase the final steps of cell differentiation and growth take place, the enhancement of the expression of the *mTOR* gene has been observed, which in flatworms shows that RNAi restricts cell proliferation and growth[5556]. Although the *PDK* and *GH* genes have not been characterized in flatworms, they have been shown to be involved in cell growth in other

uPathway	Gene	Without zooid	Two zooids	Main functions described for flatworms	Bibliography
MAPK/ERK	ERK	UP	-	Required for the differentiation of blastema cells into multiple cell lineages.	Tasaki et al.[43]
	Rap1	UP	-	Regulator for signal transduction and associated with cell adhesion and cell.	Yuan et al.[58]
	Jip3	+	-		
	MKP	-	+	Inhibitory effect on ERK/ expressed in blastema.	Tasaki et al.[43]
	MEK5	-	+	Regulation of cell proliferation and apoptosis.	Wang et al.[59]
	p38	UP	+	Module homeostasis and regeneration during infection.	Arnold et al.[60]
	JNK	UP	+	Inhibition blocks posterior regeneration.	Tejada-Romero et al.[16]
PI3K-AKT	PI3K	+	UP	Regeneration, apoptosis and tissue maintenance; PI3K signaling is required for blastema regrowth and cilia maintenance during planarian regeneration and homeostasis. Interestingly, the mitotic and apoptotic responses to amputation are substantially abated in PI3K inhibitor-treated regenerating animals.	Peiris et al.[17] Zheng et al.[45]
	PTEN	UP	-	RNAi disrupts regeneration; Inhibitor of PI3K.	Oviedo et al.[61]
	Rac1	UP	-	Involved in formation and is overexpressed in the blastema.	Xu et al.[46]
	PDK	-	UP		
Wnt	β catenin	UP	+	b-catenin is crucial for the establishment and the maintenance of the overall polarity and especially for the character 'posterior' in planarians. Posterior-facing blastemas regenerate a head instead of a tail in Smed-βcatenin-1(RNAi) animals. βcatenin-1 is required for anteroposterior blastema polarity in planarian regeneration.	Meinhardt[49] Petersen & Reddien[62]
	Pontin52	-	UP		
	Znrf 3	+	-		
TGFβ	Smad 3	+	-	Involved in specification and maintenance of the dorsoventral axis.	Molina et al.[50]
	Smad 4	UP	+	Plays an important role in tail regeneration via promoting cell differentiation.	Chen & Xu[51]
	SIM3A	-	+		
	p15	UP	-		
FoxO	FoxO	UP	-	Pro-apoptotic and is involved in eye and brain regeneration.	Pascual-Carreras et al.[52]
	Homer	UP	-		
	Usp7	-	UP	Down regulated in flatworm challenged for infection and regeneration.	Chiang et al.[63]
Notch	Notch	+	+	DAPT (inhibitor of Notch) treatment causes obvious morphological deformities during regenera- tion, such as multi-eye, asymmetric growth, and black plaques. Previous studies have shown that RNAi of Smed-notch leads to significant regen- eration abnormalities (such as cyclopia of the tail fragments).	Dong et al.[53]
	Serrate	+	-		
mTOR	mTOR	+	UP	Inhibition of mTOR with RNA interference severely restricts cell proliferation. Increases stem cell telomere length.	Peiris et al.[54] Iglesias et al.[55]
	Slc7A5	+	-		
	Gator 1	+	-		
Hedgehog	Sufu	UP	-	Negatively regulator of Hedgehog signalling; crucial in embryonic development.	Yazawa et al.[64]
Growth hormone (GH)	GH	-	+		
	FAK	-	+		
Pluripotency	Dusp9		+		
Hippo	Lg1	UP	-		
Sphingolipid	ABCC1	UP	-	Upregulated in flatworm challenged for infection and regeneration.	Chiang et al.[63]
Apoptose	Caspase 3	+	UP	Apoptosis	Yuan et al.[58]
	Caspase 7	+	UP	Apoptosis	Yuan et al.[58]
	ENDOG	-	+		
VEGF	CALN	UP	+		
	COX2	-	+		
Other	Piwi	+	UP	Maintain their pluripotent state.	Kashima et al.[33]
	AGO2	+	UP	Regulation and proliferation totipotence.	Li et al.[34]
	POU2F2	UP	-	Potentially partner with different Sox proteins.	Wood et al.[35]
	SOX	UP	+	Expression is associated with pluripotency and stem cells, neuronal differentiation.	Roberts-Galbraith et al.[36]
	PAF1	UP	+	PAF1 complex required to maintain the chromatin structure of key pluripotency genes.	Onal et al.[37]
	C4M1	-	UP	Cluster 4 marker (C4M1) is a neoblast marker gene, probably Catenulida - specific gene.	Gąsiorowski et al.[38]
	FBL	-	UP	The gene fibrillarin is a neoblast marker.	Gąsiorowski et al.[38]
	H2A	Up	+	Histone H2A is a a neoblast marker.	Gąsiorowski et al.[38]

Table 1. Genes that stand out due to a different expression pattern between the without and two zooid stages. *Legend*: ***UP*** *= upregulated;+= transcripted;-= transcription no detected.*

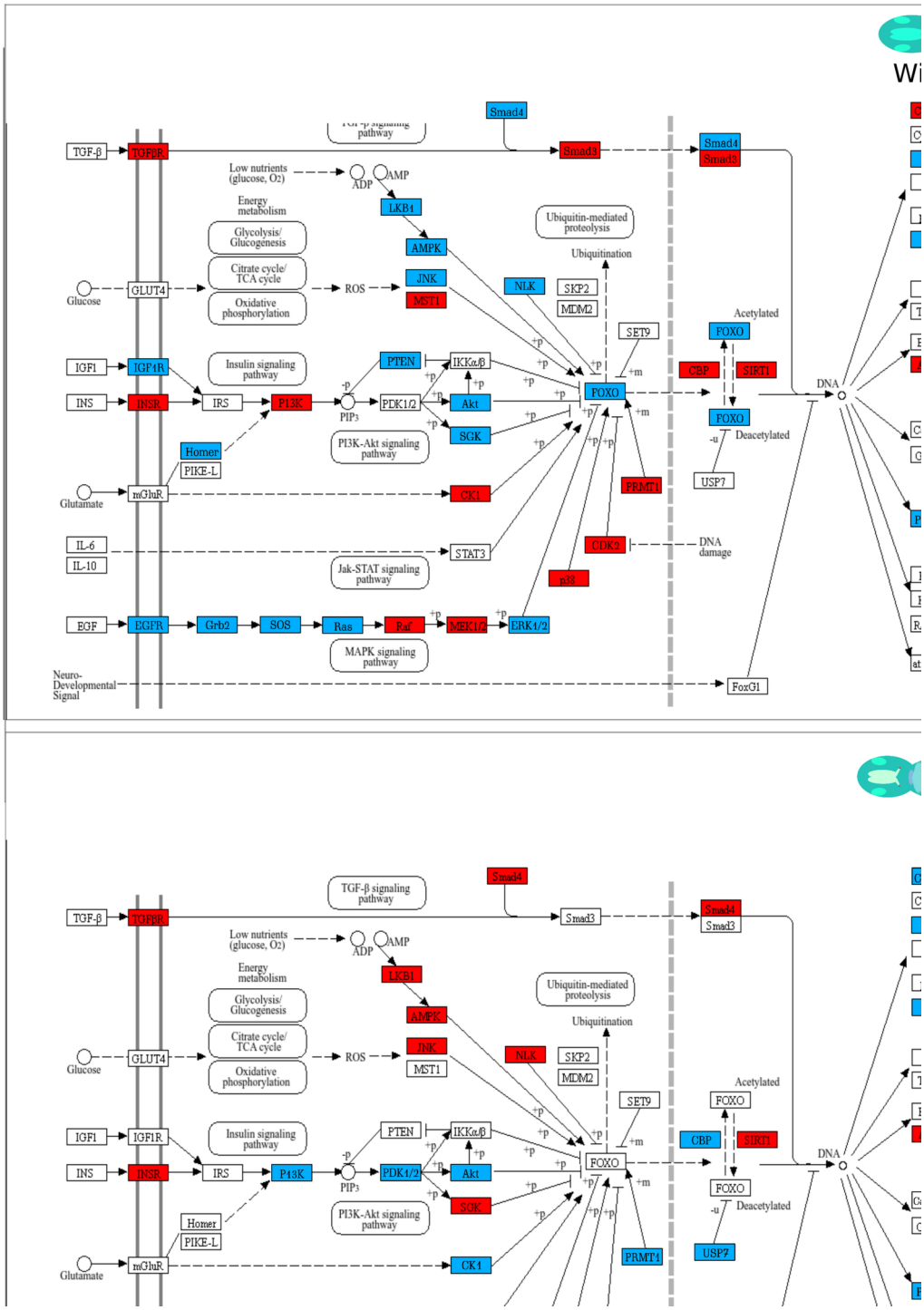

Fig. 6. KEGG graphical of FoxO signaling pathway[32]. Comparison of gene expression pattern observed in worms without zooid (**A**) and with 2-zooids stages. The KEGG signaling pathways are used with permission of the authors.

organisms[48,57]. Apoptosis is normally involved in tissue remodeling and differentiation[58] and the genes involved in this process were more active at the 2-zooid stage.

However, the data obtained in this study do not support our original hypothesis that the expansion of neoblast number occurs in the 1-zooid phase. Genes involved in neoblast maintenance in flatworms, such as *Piwi* and *Argonauta*[59,60], are upregulated at the 2-zooid stage, whereas POU2F2, SOX and PAF[61–63] are upregulated at

the without zooid stage. It is possible that neoblast proliferation occurs at both stages, starting at the 2-zooid stage, following complete differentiation and continuing to the initial period of the without zooid stage. Recently, Gąsiorowski et al.[39] have shown that the genes *Piwi* and *Argonaute* are not expressed in the neoblasts of *Stenostomum brevipharyngium*, suggesting that these genes may not be so essential for cell pluripotence in this worm. On the other hand, the authors have shown that *C4M1*, *FBL* and *H2A* are markers for neoblasts in *S. brevipharyngium*. The results in our study show that *C4M1* and *FBL* are only expressed in the second zooid stage, suggesting that neoblasts predominate in this stage.

Overall, our results give us a general picture of the genes involved in development that are activated during the paratomy process, suggesting significant similarities between this form of asexual reproduction and the regeneration observed in other flatworms after fission or amputation, since many genes share common activation pattern across both processes. In the first stage, the without zooid stage, signaling pathways required for cell proliferation, differentiation of cells into various cell lineages and body axis determination were activated. In the 2-zooid stage, the activation of genes involved in cell growth and apoptosis were observed. Activation of genes involved in neoblast proliferation and maintenance appears to occur in both stages.

The limitations of this study

The main limitations of this study are: (i) we analyzed the transcriptome obtained from the whole body of several animals. While this gives us a general picture of the genes expressed at each stage analyzed, it hides what is happening at the level of individual cells or tissue. Differences in expression that occur in different parts of the worms may be obscured when analyzing the transcriptome as a whole; (ii) we used the KEGG pathways. While the pathways are generally well conserved, some differences may occur in phylogenetically basal organisms such as Catenulida. However, we tested several other approaches and concluded that the KEGG pathways provide the most comprehensive information for our data.

Data availability

The transcriptome sequences have been deposited in GenBank (BioProject PRJNA1037460; accession SAMN42159283; SAMN42159284. The annotated orthologous genes are available in the supplementary spreadsheet. Additional data can be requested from the authors.

Received: 10 July 2024; Accepted: 22 November 2024
Published online: 26 November 2024

References

1. Telford, M. J., Lockyer, A. E., Cartwright-Finch, C. & Littlewood, D. T. J. Combined large and small subunit ribosomal RNA phylogenies support a basal position of the acoelomorph flatworms. *Proc. R Soc. Lond. B*. **270**, 1077–1083 (2003).
2. Egger, B. et al. A transcriptomic-phylogenomic analysis of the evolutionary relationships of flatworms. *Curr Biol*. **18** 25(10), 1347-53 https://doi.org/10.1016/j.cub.2015.03.034 (2015).
3. Janssen, T. et al. The first multi-gene phylogeny of the macrostomorpha sheds light on the evolution of sexual and asexual reproduction in basal Platyhelminthes. *Mol. Phylogenet Evol.* **92**, 82–107. https://doi.org/10.1016/j.ympev.2015.06.004 (2015).
4. Borkott, H. Geschlechtliche Organisation, Fortpflanzungsverhalten Und Ursachen Der Sexuellen Vermehrung Yon *Stenostomum Sthenum*. *Z. Morph Tiere*. **67**, 183–262 (1970).
5. Rosa, M. T., Pereira, C. M., Ragagnin, G. T. & Loreto, E. L. S. *Stenostomum leucops* Duges, platyhelminthesinthes, Catenulada): A putative species complex with phenotypic plasticity. *Pap Avulsos Zool.* **55**, 375–383 (2015).
6. Åkesson, B. et al. Fission in Convolutriloba longifissura: Asexual reproduction in acoelous turbellarians revisited. *Acta Zool. (Stockh)*. **82**, 231–239 (2001).
7. Kharin, A. V., Zagainova, I. V. & Kostyuchenko, R. P. Formation of the paratomic fission zone in freshwater oligochaetes. *Russ J. Dev. Biol.* **37**, 354–365. https://doi.org/10.1134/S1062360406060038 (2006).
8. Jaeckle, W. B. Multiple modes of asexual reproduction by tropical and subtropical sea star larvae: An unusual adaptation for genet dispersal and survival. *Biol. Bull.* **186** (1), 62–71. https://doi.org/10.2307/1542036 (1994).
9. Raikova, E. V. & Life cycle, cytology, and morphology of polypodium hydriforme, a coelenterate parasite of the eggs of Acipenseriform fishes. *J. Parasitol.* **80** (1), 1–22. https://doi.org/10.2307/3283338 (1994).
10. Kostyuchenko, R. P. & Amosov, A. V. Spatial colinear but broken temporal expression of duplicated ParaHoxGenes in asexually reproducing annelids, Nais communis and Pristina longiseta. *Genes (Basel)*. **7**, 1501. https://doi.org/10.3390/genes14071501 (2023).
11. Elliott, S. A. & Sánchez Alvarado, A. The history and enduring contributions of planarians to the study of animal regeneration. *WIREs Dev. Biol.* **2**, 301–326. https://doi.org/10.1002/wdev.82 (2013).
12. Grohme, M. A. et al. The genome of Schmidtea mediterranea and the evolution of core cellular mechanisms. *Nature* **7690**, 56–61. https://doi.org/10.1038/nature25473 (2018).
13. Wasik, K. et al. Genome and transcriptome of the regeneration-competent flatworm, Macrostomum lignano. *Proc. Natl. Acad. Sci. U S A*. **112** (40), 12462–12467. https://doi.org/10.1073/pnas (2015).
14. Tian, Q. et al. Whole-genome sequence of the planarian Dugesia japonica combining Illumina and PacBio data. *Genomics* **114** (2), 110293. https://doi.org/10.1016/j.ygeno.2022.110293 (2022).
15. Almuedo-Castillo, M., Sureda-Gómez, M. & Adell, T. Wnt signaling in planarians: New answers to old questions. *Int. J. Dev. Biol.* **56**, 53–65 https://doi.org/10.1387/ijdb.113451ma (2012).
16. Tejada-Romero, B., Carter, J. M., Mihaylova, Y., Neumann, B. & Aboobaker, A. A. JNK signalling is necessary for a wnt- and stem cell-dependent regeneration programme. *Development* **15** (14), 2413–2424. https://doi.org/10.1242/dev.115139 (2015).
17. Peiris, T. H., Ramirez, D., Barghouth, P. G. & Oviedo, N. J. The akt signaling pathway is required for tissue maintenance and regeneration in planarians. *BMC Dev. Biol.* **16**, 7. https://doi.org/10.1186/s12861-016-0107-z (2016).
18. Doddihal, V. et al. A PAK family kinase and the Hippo/Yorkie pathway modulate WNT signaling to functionally integrate body axes during regeneration. *Proc. Natl. Acad. Sci. U S A*. **121** (20), e2321919121. https://doi.org/10.1073/pnas.2321919121 (2024).
19. Chong, T., Stary, J. M., Wang, Y. & Newmark, P. A. Molecular markers to characterize the hermaphroditic reproductive system of the planarian Schmidtea mediterranea. *BMC Dev. Biol.* **10**, 11:69. https://doi.org/10.1186/1471-213X-11-69 (2011).
20. Arnold, C. P., Benham-Pyle, B. W., Lange, J. J., Wood, C. J. & Sánchez, A. A. Wnt and TGFβ coordinate growth and patterning to regulate size-dependent behaviour. *Nature* **572** (7771), 655–659. https://doi.org/10.1038/s41586-019-1478-7 (2019).

21. Rosa, M. T. & Loreto, E. L. S. Revisiting the regeneration of *Stenostomum leucops* (Catenulida, Platyhelminthes). *Invertebrate Reprod. Dev.* **66** (1), 1–7. https://doi.org/10.1080/07924259.2021.1994475 (2022).
22. Gąsiorowski, L. et al. Convergent evolution of the sensory pits in and within flatworms. *BMC Biol.* **21** (1), 266. https://doi.org/10.1186/s12915-023-01768-y (2023).
23. Andrews, S. & FastQC a quality control tool for high throughput sequence data [online]. http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ (2010).
24. Krueger, F. Trim Galore. In *GitHub Repository. GitHub*. (2021). https://github.com/FelixKrueger/TrimGalore.com/fenderglass/Flye. https://zenodo.org/records/7598955.
25. Grabherr, M. G. et al. Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat. Biotechnol.* **29** (7), 644–652. https://doi.org/10.1038/nbt.1883 (2011).
26. Simão, F. A., Waterhouse, R. M., Ioannidis, P., Kriventseva, E. V. & Zdobnov, E. M. BUSCO: Assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics*. **31**(19), 3210-2. https://doi.org/10.1093/bioinformatics/btv351 (2015).
27. Stanke, M., Steinkamp, R., Waack, S. & Morgenstern, B. AUGUSTUS: A web server for gene finding in eukaryotes. *Nucleic Acids Res.* 1; **32**(Web Server issue), W309-12. https://doi.org/10.1093/nar/gkh379 (2004).
28. The Galaxy Community. The Galaxy platform for accessible, reproducible, and collaborative data analyses: 2024 update. *Nucleic Acids Res.* gkae410. https://doi.org/10.1093/nar/gkae410 (2024).
29. Bray, N. L., Pimentel, H., Melsted, P. & Pachter, L. Near-optimal probabilistic RNA-seq quantification. *Nat. Biotechnol.* **34** (5), 525–527. https://doi.org/10.1038/nbt.3519 (2016).
30. Cantalapiedra, C. P., Hernández-Plaza, A., Letunic, I., Bork, P. & Huerta-Cepas, J. eggNOG-mapper v2: Functional annotation, orthology assignments, and domain prediction at the metagenomic scale. *Mol. Biol. Evol.* **38** (12), 5825–5829. https://doi.org/10.1093/molbev/msab293 (2021).
31. Ge, S. X., Jung, D. & Yao, R. ShinyGO: A graphical gene-set enrichment tool for animals and plants. *Bioinformatics* **36** (8), 2628–2629. https://doi.org/10.1093/bioinformatics/btz931 (2020).
32. Kanehisa, M. & Goto, S. K. E. G. G. Kyoto encyclopedia of genes and genomes. *Nucleic Acids Res.* **28**, 27–30. https://doi.org/10.1093/nar/28.1.27 (2000).
33. Tang, Y., Horikoshi, M. & Li, W. Ggfortify: Unified interface to visualize statistical results of popular R packages. *R J.* **8** (2), 478–489 (2016).
34. Kashima, M., Agata, K. & Shibata, N. What is the role of PIWI family proteins in adult pluripotent stem cells? Insights from asexually reproducing animals, planarians. *Dev. Growth Differ.* **62**, 407–422. https://doi.org/10.1111/dgd.12688 (2020).
35. Li, Y. et al. Argonaute-2 regulates the proliferation of adult stem cells in planarian. *Cell. Res.* **21** (12), 1750–1754. https://doi.org/10.1038/cr.2011.151 (2011).
36. Wood, S. et al. Characterization of Schistosome Sox genes and identification of a flatworm class of Sox regulators. *Pathogens* **12** (5), 690. https://doi.org/10.3390/pathogens12050690 (2023).
37. Roberts-Galbraith, R., Brubacher, J. & Newmark, P. A functional genomics screen in planarians reveals regulators of whole-brain regeneration. *eLife* **5**, e17002 . https://doi.org/10.7554/eLife.17002 (2016).
38. Onal, P. et al. Gene expression of pluripotency determinants is conserved between mammalian and planarian stem cells. *EMBO J.* **31** (12), 2755–2769. https://doi.org/10.1038/emboj.2012.110 (2012).
39. Gąsiorowski, L. et al. Regeneration in the absence of canonical neoblasts in an early branching flatworm. *bioRxiv* [Preprint]. https://doi.org/10.1101/2024.05.24.595708. (2024).
40. Finn, R. D., Clements, J. & Eddy, S. R. HMMER web server: Interactive sequence similarity searching. *Nucleic Acids Res.* **V. 39**, W29–W37. https://doi.org/10.1093/nar/gkr367 (2011).
41. Chang, L. & Karin, M. Mammalian MAP kinase signalling cascades. *Nature* **410** (6824), 37–40. https://doi.org/10.1038/35065000 (2001).
42. Wenemoser, D., Lapan, S. W., Wilkinson, A. W., Bell, G. W. & Reddien, P. W. A molecular wound response program associated with regeneration initiation in planarians. *Genes Dev.* 1; **26**(9), 988–1002 https://doi.org/10.1101/gad.187377.112 (2012).
43. Sandmann, T., Vogg, M. C., Owlarn, S., Boutros, M. & Bartscherer, K. The head-regeneration transcriptome of the planarian Schmidtea mediterranea. *Genome Biol.* 16; **12**(8), R76 https://doi.org/10.1186/gb-2011-12-8-r76 (2011).
44. Tasaki, J. et al. ERK signaling controls blastema cell differentiation during planarian regeneration. *Development* **138** (12), 2417–2427. https://doi.org/10.1242/dev.060764 (2011).
45. Jaenen, V. et al. Reactive oxygen species rescue regeneration after silencing the MAPK-ERK signaling pathway in Schmidtea mediterranea. *Sci. Rep.* **11** (1), 881. https://doi.org/10.1038/s41598-020-79588-1 (2021).
46. Zheng, H. et al. PI3K plays an essential role in planarian regeneration and tissue maintenance. *Front. Cell. Dev. Biol.* **9**, 649656. https://doi.org/10.3389/fcell.2021.649656 (2021).
47. Xu, Z., Han, Y., Li, X., Yang, R. & Song, L. Molecular cloning and characterization of DjRac1, a novel small G protein gene from planarian Dugesia japonica. *Biochem. Biophys. Res. Commun.* **526** (4), 865–870. https://doi.org/10.1016/j.bbrc.2020.03.171 (2020).
48. Lawlor, M. A. et al. Essential role of PDK1 in regulating cell size and development in mice. *EMBO J.* **21** (14), 3728–3738. https://doi.org/10.1093/emboj/cdf387 (2002).
49. Zhou, J., Yi, Q. & Tang, L. The roles of nuclear focal adhesion kinase (FAK) on cancer: A focused review. *J. Exp. Clin. Cancer Res.* **38**, 250. https://doi.org/10.1186/s13046-019-1265-1 (2019).
50. Meinhardt, H. Beta-catenin and axis formation in planarians. *Bioessays* **31** (1), 5–9. https://doi.org/10.1002/bies.080193 (2009).
51. Molina, M. D., Saló, E. & Cebrià, F. The BMP pathway is essential for re-specification and maintenance of the dorsoventral axis in regenerating and intact planarians. *Dev. Biol.* **311** (1), 79–94. https://doi.org/10.1016/j.ydbio.2007.08.019 (2007).
52. Chen, X. & Xu, C. Proteomic analysis reveals the contribution of TGFβ/Smad4 signaling pathway to cell differentiation during Planarian tail regeneration. *Appl. Biochem. Biotechnol.* **182** (2), 529–545. 10.1007/s (2017).
53. Pascual-Carreras, E. et al. Analysis of Fox genes in Schmidtea mediterranea reveals new families and a conserved role of smed-foxo in controlling cell death. *Sci. Rep.* https://doi.org/10.1038/s41598-020-80627-0 (2021).
54. Dong, Z. et al. Gamma-secretase inhibitor (DAPT), a potential therapeutic target drug, caused neurotoxicity in planarian regeneration by inhibiting notch signaling pathway. *Sci. Total Environ.* **781**, 146735. https://doi.org/10.1016/j.scitotenv.2021.146735 (2021).
55. Peiris, T. H. et al. TOR signaling regulates planarian stem cells and controls localized and organismal growth. *J. Cell. Sci.* **125** (Pt 7), 1657–1665. https://doi.org/10.1242/jcs.104711 (2012).
56. Iglesias, M. et al. Downregulation of mTOR signaling increases stem cell population telomere length during starvation of immortal planarians. *Stem Cell. Rep.* **13**, 405–418. https://doi.org/10.1016/j.stemcr.2019.06.005 (2019).
57. Guo, B., Zhang, S., Wang, S. & Liang, Y. Expression, mitogenic activity and regulation by growth hormone of growth hormone/insulin-like growth factor in Branchiostoma belcheri. *Cell. Tissue Res.* **338** (1), 67–77. https://doi.org/10.1007/s00441-009-0824-8 (2009).
58. Lorda-Diez, C. I. et al. Apoptosis during embryonic tissue remodeling is accompanied by cell senescence. *Aging (Albany NY).* **7** (11), 974–985. https://doi.org/10.18632/aging.100844 (2015).
59. Yuan, J. et al. Expression profiling of planarians shed light on a dual role of programmed cell death during the regeneration. *J. Cell. Biochem.* **119** (7), 5875–5884. https://doi.org/10.1002/jcb.26779 (2018).

60. Wang, Q. et al. Djmek is involved in planarian regeneration by regulation of cell proliferation and apoptosis. *Biochem. Biophys. Res. Commun.* **532** (3), 355–361. https://doi.org/10.1016/j.bbrc.2020.08.027 (2020).
61. Arnold, C. P. et al. Pathogenic shifts in endogenous microbiota impede tissue regeneration via distinct activation of TAK1/MKK/p38. *Elife* **5**, e16793. https://doi.org/10.7554/eLife.16793 (2016).
62. Oviedo, N. J., Pearson, B. J., Levin, M. & Sánchez-Alvarado, A. Planarian PTEN homologs regulate stem cells and regeneration through TOR signaling. *Dis. Model. Mech.* **1** (2–3), 131–143. https://doi.org/10.1242/dmm.000117 (2008). discussion 141.
63. Petersen, C. P. & Reddien, P. W. Smed-betacatenin-1 is required for anteroposterior blastema polarity in planarian regeneration. *Science* **319**(5861), 327–330. https://doi.org/10.1126/science.1149943 (2008).
64. Chiang, N. et al. Cysteinyl-specialized proresolving mediators link resolution of infectious inflammation and tissue regeneration via TRAF3 activation. *Proc. Natl. Acad. Sci. U S A.* **118** (10), e2013374118. https://doi.org/10.1073/pnas.2013374118 (2021).
65. Yazawa, S., Umesono, Y., Hayashi, T., Tarui, H. & Agata, K. Planarian Hedgehog/patched establishes anterior-posterior polarity by regulating wnt signaling. *Proc. Natl. Acad. Sci. U S A.* **106** (52), 22329–22334. https://doi.org/10.1073/pnas.0907464106 (2009).

Acknowledgements

We thank Oluwabukola Farodoye for reviewing the manuscript in English.

Author contributions

M.T.R and E.L.S.L designed the project, analyzed the data, prepared the original artwork and wrote the manuscript. All authors have made intellectual contributions to the research project and approved the final manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-024-80881-6.

Correspondence and requests for materials should be addressed to E.L.S.L.