

# Single-cell transcriptomics identifies a p21-activated kinase important for survival of the zoonotic parasite *Fasciola hepatica*

Oliver Puckelwaldt<sup>1</sup>, Svenja Gramberg<sup>1</sup>, Sagar Ajmera<sup>1</sup>, Janine Koepke<sup>2</sup>, Christos Samakovlis<sup>2</sup>, Simone Haeberlein<sup>1</sup>  
<sup>\*</sup>

<sup>1</sup> Institute of Parasitology, Justus Liebig University Giessen, Giessen, Germany

<sup>2</sup> Cardio-Pulmonary Institute (CPI), Giessen, Germany

\*Correspondence: Simone.Haeberlein@vetmed.uni-giessen.de

## 1 Abstract

2 Knowledge on the cell types and cell-specific gene expression of multicellular pathogens  
3 facilitates drug discovery and allows gaining a deeper understanding of pathogen biology. By  
4 utilizing single-cell RNA sequencing (scRNA-seq), we analyzed 19,581 cells of a globally  
5 prevalent parasitic flatworm, the liver fluke *Fasciola hepatica*, which causes a neglected  
6 tropical disease and zoonosis known as fascioliasis. We identified 15 distinct clusters,  
7 including cells of the reproductive tract and gastrodermis, and report the identification and  
8 transcriptional characterization of potential differentiation lineages of stem cells within this  
9 parasite. Furthermore, a previously unrecognized ELF5- and TRPM<sub>PZQ</sub>-expressing muscle  
10 cell type was discovered, characterized by high expression of protein kinases. Among these,  
11 the p21-activated kinase PAK4 was essential for parasite survival. These data provide novel  
12 insight into the cellular composition of a complex multicellular parasite and demonstrate how  
13 gene expression at single-cell resolution can serve as a resource for the identification of new  
14 drug targets.

## 15 Keywords

16 Single-cell transcriptomics, scRNASeq, flatworms, liver flukes, *Fasciola hepatica*, drug target,  
17 p21-activated kinase, TRPM, stem cells

## 18 Introduction

19 Infections with parasitic helminths pose a global health challenge. As many of these  
20 diseases affect humans and animals alike, they are of major importance considering “One  
21 Health” initiatives<sup>1</sup>. Fascioliasis is caused by liver flukes such as *Fasciola hepatica*, a  
22 parasitic flatworm heavily affecting livestock industry. Here, it causes a huge economic  
23 burden by reducing growth and milk yield<sup>2</sup>. Along with other food borne diseases, fascioliasis  
24 is recognized by the World Health Organization as a neglected tropical disease (NTD). It is  
25 estimated that up to 17 million people are infected worldwide<sup>3</sup> and 90 million are at risk of  
26 infection<sup>4</sup>. The increasing number of reports on parasites being resistant to the commonly  
27 used drug triclabendazole<sup>5,6</sup> and a lack of treatment alternatives or effective vaccines<sup>7</sup>  
28 motivates basic research on liver flukes aiming at the identification of novel drug targets.

29 *F. hepatica* has a complex life cycle, which includes an intermediate snail host and a  
30 mammalian as final host. The adult worms reside within the bile duct of the final host, where  
31 they shed tens of thousands of eggs per day in order to reproduce<sup>8</sup>. The eggs are released  
32 into the environment during defecation, followed by hatching of miracidia that infect snails.  
33 Within the snail, the parasite multiplies by asexual reproduction. Infected snails eventually  
34 shed cercariae, which encyst upon aquatic vegetation, where they can remain infectious for  
35 months. After oral ingestion of infectious metacercariae by the final mammalian host, newly  
36 excysted juveniles hatch from the cyst and migrate to the liver parenchyma, where the  
37 immature worms feed and grow into adults. Adult worms can persist for decades, hinting at  
38 an outstanding longevity of these worms<sup>9,10</sup>.

39 Insights into the biology of *F. hepatica* have been significantly advanced by the  
40 implementation of various omics technologies<sup>11</sup>. By using bulk RNA-seq, it was possible to  
41 identify genes being transcribed in different life stages<sup>12</sup>, to uncover the response to  
42 anthelmintics<sup>13,14</sup>, investigate interactions with the immune system<sup>15</sup>, and assess the role of  
43 the nervous system in development<sup>16</sup>. While being an important tool to investigate the  
44 dynamics of transcriptional gene expression, bulk RNA-seq naturally does not allow  
45 conclusions at single-cell resolution. Bulk analysis may also complicate the detection of rare  
46 genes or dynamics in rare cell types due to overrepresented cell types or highly expressed

47 genes. The advent of single-cell RNA sequencing (scRNA-seq) opened a new opportunity to  
48 investigate the molecular biology of multicellular organisms<sup>17</sup>. By profiling hundreds to  
49 thousands of cells in one experiment, this technology allows the identification and  
50 characterization of cell types and their characteristic genes even on a whole-organism level,  
51 if single-cell suspensions are accessible. This untargeted method promises unprecedented  
52 insights into the molecular biology especially of non-model organisms such as parasites, for  
53 which many other methodologies are still unavailable. In parasitic and free-living relatives of  
54 *F. hepatica*, specifically schistosomes and planarians, scRNA-seq technologies recently  
55 boosted the identification of novel cell-type markers<sup>18,19</sup> and the characterization of  
56 transcription factors in developmental trajectories<sup>20</sup>. By focusing on cell types with vital  
57 function for a parasite, scRNA-seq data may also facilitate the identification of new drug  
58 targets.

59 Protein kinases (PKs) have gained attention as promising drug targets in parasites such as  
60 cestodes, filaria and other trematodes<sup>21</sup>. For *F. hepatica*, we recently provided evidence of  
61 several druggable PKs<sup>22</sup>. PKs regulate most of the core biological processes, like signal  
62 transduction, cell cycle or motility<sup>23</sup>. Considerable effort was put into the development of  
63 inhibitors of human PKs for use in several diseases, leading to 52 already approved drugs<sup>24</sup>  
64 and several more in clinical trials<sup>25</sup>. Drug repurposing has been discussed as a valuable  
65 approach for the identification of new treatment options against NTDs, as there are fewer  
66 risks involved regarding efficacy and safety considerations<sup>26</sup>. The high number of available  
67 PK inhibitors and evidence for the druggability of kinases in parasitic worms make PKs  
68 attractive targets, potentially also for treating fascioliasis. A question to be answered is:  
69 which type of PKs can we consider important for pathogen survival, e.g. based on their  
70 expression in particular cell types?

71 In this study, we profiled the gene expression of adult *F. hepatica* at single-cell resolution  
72 using the 10X Chromium workflow. In order to achieve this, we established a cell  
73 dissociation protocol coupled with fluorescence activated cell sorting (FACS) to obtain a  
74 viable cell suspension. We uncovered several different cell populations, their characteristic  
75 marker genes, and identified several PKs with enriched expression in distinct cell types.  
76 Targeting one PK using a small-molecule inhibitor validated its suitability as drug target. This

77 work presents the first single-cell atlas for this family of parasites and will serve as a  
78 resource for future biomedical research as well as basic understanding of pathogens.

## 79 **Results & Discussion**

### 80 **Determination of nuclei number of *F. hepatica* adults shows cell density differences** 81 **along anterior-posterior axis**

82 Basic metrics on the number of cells in multicellular organisms are helpful in planning  
83 scRNA-seq experiments, but such metrics are unknown for liver flukes. In order to determine  
84 the total cell count and to assess the distribution of cells throughout the parasite, we  
85 quantified the nuclei within sections of adult worms. To exclude a potential bias depending  
86 on the tissue area, we utilized frontal sections (Fig 1 A) as well as transversal sections from  
87 representative areas of the worm (anterior part 1 and 2 and posterior part) (Fig 1 B). The  
88 total number of nuclei per worm was extrapolated from the nuclei counts per section.  
89 Independent of the section plane, we arrived at a total nuclei number of around 17 million  
90 within the parasite (Fig 1 C). It is to be noted that this nuclei number is only an approximation  
91 for the total cell number, as the multi-nucleated nature of the syncytial surface of the  
92 parasite, the tegument, or the fused rosette during spermatogenesis<sup>27</sup> do not allow a one-to-  
93 one translation.

94 Compared to the two anterior parts used for quantification, most of the cells were located  
95 within the large posterior part of the worm, which contains the male reproductive tissue as  
96 well as the vitellarium, creating a higher total cell count. This not only arises from the sheer  
97 size of these tissues, in addition, the male reproductive organ as well as the vitellarium also  
98 have a high cellular density (Fig 1 A). This disproportional tissue and cell distribution over  
99 the body axis bears the risk that cells of the overabundant reproductive organs might  
100 overrun some of the rarer cell types, which are hence not captured in a subsequent 10X  
101 workflow. Based on the cellular composition, we therefore decided to cut the worm into two  
102 parts for subsequent scRNA-seq experiments: a posterior part and an anterior part, which is  
103 enriched for proportionately underrepresented cell types.

105 **scRNA-seq captures cells of major tissue types of *F. hepatica***

106 We performed scRNA-seq on cells from anterior and posterior parts of several adult  
107 individuals. First, we developed a protocol to dissociate anterior and posterior parts of the  
108 worms into single cells using a combination of mechanical and enzymatic treatment (Fig 2  
109 A). Thereafter, viable cells were enriched by fluorescence-activated cell sorting (FACS)  
110 using calcein AM viability dye to select viable cells. By using the commercially available 10X  
111 Genomics Chromium platform, we analyzed a total of 10 samples, from either anterior (4) or  
112 posterior parts (4) of worms, or whole worms (2) for comparison. Using a combination of the  
113 10X cellranger workflow and the R package Seurat<sup>28</sup>, we analyzed sequencing data of a final  
114 number of 19,581 cells. Hereby we detected a median gene number per cell of 2,644 and on  
115 average 14,087 UMIs per cell after quality filtering (suppl. table S1). Based on the total of  
116 11,217 protein coding genes in the genome of *F. hepatica*, we detect on average 23% of the  
117 total genome as transcripts per cell. This is a touch higher compared to scRNaseq data  
118 obtained for adult *S. mansoni*<sup>19</sup>, where a median gene number of 1,600 was detected per  
119 cell, which is 16% of the total gene count of 9,896 protein coding genes in the genome of *S.*  
120 *mansoni*<sup>29</sup>.

121 Using Seurat, we identified 15 clusters (Fig 2 B), for which distinct marker genes could be  
122 derived (Fig 2 C, suppl. table S2). The clusters were annotated with the help of published  
123 cell marker genes that are conserved across taxa, as well as by comparison to cellular  
124 markers for the closely related blood fluke *S. mansoni*. We identified clusters resembling  
125 cells of the following tissues (number of clusters in brackets): muscle (2), gut (1), testes (3),  
126 stem cells (1), ovary (2), vitellarium (2), eggs/uterus (1), Mehlis gland (1), and two clusters  
127 for which we could not find an annotation. These cell types, as hypothesized earlier, were  
128 not equally distributed between the anterior and posterior samples (suppl. Fig S1). Because  
129 gut, testes and vitellarium represent the largest tissues in *F. hepatica* and cover most of the  
130 posterior body part, the whole-worm samples and posterior samples strongly resembled  
131 each other. Specific cell types are missing in the data set, like parenchymal or neuronal  
132 cells, which could be explained by several factors. First, rare cell types might, despite our  
133 enrichment strategy, still be overrun by overabundant cell types and not be captured during  
134 scRNA-seq. Another reason may be linked to the chosen tissue dissociation protocol, which

135 made use of enzymatic digestion followed by flow sorting, an established procedure for other  
136 flatworms<sup>18,19</sup>. One cannot fully exclude damage of more sensitive cells and/or a loss of  
137 syncytial cells, such as those of the tegument. Single-nuclei sequencing may be an elegant  
138 solution to this problem, but has so far never been applied to flatworms.

139 **Stem cells show distinct transcriptional profiles of proliferating and dormant states**

140 Being a multicellular organism, the growth and development of *F. hepatica* depends on stem  
141 cells that give rise to progenitor cells of reproductive and somatic tissues<sup>30</sup>. The remarkable  
142 output of thousands of eggs per day<sup>8</sup> necessitates a massive proliferative activity of germline  
143 stem cells and differentiation of germ cells. Understanding what drives that remarkable  
144 fecundity of the parasite involves understanding the gene expression controlling germline  
145 stem cells, i.e. spermatogonia and oogonia. First, the stem-cell cluster was identified based  
146 on the expression of known marker genes (Fig 3 A), like three *nanos* isoforms  
147 (D915\_007877, D915\_002111, D915\_002112) previously described by Robb et al 2022<sup>16</sup>.  
148 These RNA-binding proteins are known regulators of neoblast function in different  
149 organisms, including flatworms<sup>31-33</sup>. Other marker genes for this cluster were histone 2b  
150 (*h2b*) (D915\_007751) and several genes encoding MCM complex proteins. On closer  
151 inspection, we found that these marker genes showed two different types of expression  
152 patterns. While *nanos* expression was tightly restricted to the stem-cell cluster, other gene  
153 transcripts (e.g. *h2b*) were also detected in potential progenitor clusters of the vitelline,  
154 oogenesis and spermatogenesis lineages (Fig 3 D). Transcription of *h2b* was previously  
155 used to label actively proliferating cells in schistosomes and planarians<sup>32,34</sup>. To confirm that  
156 presence of *h2b* expression is a suitable marker for active cell proliferation in *Fasciola*, we  
157 labeled proliferating cells with the thymidine analogue 5-ethynyl-2'-deoxyuridine (EdU) and  
158 stained *h2b* transcripts with FISH. We found an overlap between *h2b* positive cells and EdU  
159 positive cells, with around 70% of EdU positive cells being also positive for *h2b* transcripts  
160 (Fig 3 C). The *h2b*/EdU double-positive cells were located in the periphery of the testicular  
161 lobes and ovary (Fig 3C), which validates prior description of stem cells in that location  
162 based on histological analysis<sup>27</sup>. Furthermore, *h2b* positive cells were located close to the  
163 tegument and gut tissue, confirming the presence of somatic stem cells in adult flukes, as  
164 described in previous studies for juvenile worms<sup>30</sup>. We also detected strong staining for *h2b*

165 transcripts in the center of the ovary, containing mature oocytes. This can be explained by  
166 the fact that unlike stem cells, in which histone transcription is coupled to the cell cycle, both  
167 processes are decoupled during oogenesis in preparation of embryogenesis<sup>35</sup>.

168 To obtain a clearer picture on the differentiation dynamics of stem cells and their progeny  
169 cluster, we reanalyzed all cells within the stem-cell cluster and obtained six subclusters (Fig  
170 3 E). Marker genes for several subclusters were enriched for genes involved in meiotic  
171 division (suppl. table S3). This included genes like *syCP2* coding for the synaptonemal  
172 complex protein 2, which was shown in immunolocalization experiments to be expressed in  
173 early progenitor cells of the reproductive organs of *F. hepatica*<sup>36</sup>, or the DAZ family member  
174 *boule*, which was shown in planarians and schistosomes to be important for germ-cell  
175 differentiation<sup>20,37</sup>. The six subclusters could be allocated to different cell differentiation states  
176 (Fig 3 E): prominent *nanos* and *h2b* expression with negligible expression of differentiation  
177 markers likely reflects actively proliferating stem cells (cluster 0); this gives rise to clusters  
178 that highly express both, *nanos* and differentiation markers (cluster 1), or mainly express  
179 differentiation markers (clusters 2 and 3). These clusters may cover stem-cell progeny on  
180 the way to germ-cell differentiation. While these previous clusters all express *h2b* to a certain  
181 extent, the two final, probably most differentiated clusters only express some differentiation  
182 markers (clusters 4 and 5). We thereby captured separate stages of differentiation in  
183 germline cells, which can aid in unraveling dynamics of germ-cell differentiation.

184 **Gene signatures of different cell states during germ-cell development**

185 *F. hepatica* is a hermaphroditic flatworm, harboring both female and male reproductive  
186 tissues. We captured clusters of both tissues, which included cells related to more  
187 undifferentiated as well as more mature cells with distinct marker profiles (Fig 4 A). The  
188 testes are represented by three clusters (Fig 4 A). Two of the three clusters were  
189 successfully annotated based on their expression of the orthologs of described marker  
190 genes of the male germline in schistosomes as well as markers predicted from the  
191 subclustering of the proliferating cells. Cells in the spermatocytes 1 cluster expressed *boule*  
192 (D915\_007531) and the transcription factor *one cut 1* (D915\_002483), both of which are  
193 described to promote male germ-cell differentiation<sup>20,37</sup>. Additional marker genes also hinted

194 at cell proliferation and the initiation of differentiation as main processes in this cluster, like  
195 genes coding for histones or RNA helicases. Furthermore, we additionally found a strong  
196 expression of a gene annotated as the meiosis specific with OB-fold (*meiob*) gene, which is  
197 known to play a role in meiotic recombination in humans<sup>38,39</sup>, within the spermatocyte 1  
198 cluster. Expression of *meiob* within the testes was confirmed by ISH (Fig 4 B). In contrast to  
199 this, the spermatocyte cluster 2 was enriched for genes encoding structural components like  
200 several tubulins and tektins known to be part of the sperm flagellum (Fig 4 B). One of the  
201 tektin genes was also shown to be strongly expressed in the testes of the worm by ISH (Fig  
202 4 C). Further support for the functions associated with each cluster was obtained by GO  
203 term analysis (suppl. table S4). The spermatocytes 1 cluster was enriched for GO terms like  
204 RNA binding or nucleotide binding as well as several metabolic processes, further  
205 underlining a more proliferative cell state. In contrast, the spermatocytes 2 cluster was  
206 enriched for terms involved in cytoskeletal organization, cilium assembly and axoneme  
207 assembly (suppl. table S4), reflecting a differentiated cell state<sup>27</sup>.

208 The ovary of *F. hepatica*, as the testes, is a branched organ consisting of three major cell  
209 types, the proliferating oogonia and the differentiated oocytes, which are further classified in  
210 oocytes 1 and 2, based on their differentiation state<sup>36</sup>. We identified two clusters of the  
211 ovarian cells based on their expression of the bone marrow proteoglycan (*bmpg*), a gene  
212 characteristic for oocytes found in schistosomes<sup>19</sup>. Transcripts of the *F. hepatica bmpg*  
213 localized to the more mature oogonia in the center of the ovary in ISH experiments (Fig 4 B).  
214 As outlined before, late and early oocyte clusters differed in their expression of  
215 synaptonemal complex proteins<sup>36</sup>. As for the spermatocyte 1 cluster, GO terms related to  
216 metabolic processes, DNA binding or DNA replication were enriched in the early oocyte  
217 cluster. Interestingly, the terms enriched within the late oocyte cluster were related to  
218 processes like signaling, phosphorylation, organization and nuclear receptor activity (suppl.  
219 table S4), emphasizing a complex control of signaling processes in these cells.

## 220 **Differentiation markers of vitellocytes are conserved across species**

221 The vitellarium is a unique organ of flatworms, essential to produce vitellocytes that enter  
222 ectolecithal eggs together with one oocyte. Vitellocytes (vitelline cells) are important for egg

223 shell formation and contain nutrients for the later development of the embryo. In *Fasciola*,  
224 like in schistosomes, the immature vitelline cells heavily proliferate and develop in four  
225 different stages<sup>40,41</sup>. While vitellocytes and their gene expression have seen interest in  
226 schistosome research<sup>19,42–44</sup>, no such data was available for *F. hepatica* and the different cell  
227 states have been mainly categorized by their morphology<sup>40</sup>. We discriminated two main  
228 clusters based on their differential gene expression, which we named early and late  
229 vitellocytes. Identification of orthologs for typical genes characterizing S1 to S4 vitelline cell  
230 stages in *S. mansoni*<sup>44</sup> allowed us to display the full vitelline cell lineage in *F. hepatica*. The  
231 ortholog for the nuclear receptor vitellogenin factor 1 (D915\_001975) was expressed in the  
232 early vitellocytes cluster (Fig 4 E), partially coexpressing the proliferative marker *h2b*.  
233 Expression of tyrosinase (D915\_002179) characterized an intermediate state (S2/S3)  
234 between the early and late vitellocyte clusters (Fig 4 E), which also agrees with earlier  
235 observations in schistosomes, where maturing cells express tyrosinases but expression is  
236 absent in mature S4 cells. The expression of this tyrosinase was additionally validated in the  
237 vitelline cells by ISH (Fig 4 D). Finally, the expression of egg-shell genes, such as *vb1*  
238 (D915\_010963)<sup>45</sup>, was found to be specific for mature S4 vitellocytes. As expected, GO  
239 terms enriched in the early vitellocyte cell cluster were related to proliferation and  
240 transcriptional activity, like nucleotide binding or RNA processing, while terms within the late  
241 cells covered terms like iron or vitamin binding (suppl. table S4). Overall, the existence of  
242 shared molecular vitelline-cell markers for blood and liver flukes suggest that the biological  
243 mechanisms guiding vitelline-cell function and maturation are conserved. Also major  
244 differentiation markers of vitelline cells appear conserved across these two major trematode  
245 species. This degree of conservation is anything but self-evident, given the highly differential  
246 sexual biology of both pathogens – with liver flukes being hermaphrodites and schistosomes  
247 dioecious. Insights in the reproductive biology of flatworms may allow the development of  
248 strategies to limit transmission of the parasites, consequently, the vitellarium has been  
249 discussed as a valuable target<sup>46</sup>.

250 **Genes involved in lipid metabolism characterize gastrodermal cells of liver flukes**

251 As for most trematodes, the intestine of liver flukes is bifurcated with numerous branches  
252 stretching throughout the parasite's body. The gastrodermal cells are known to express and

253 secrete a high number of digestive enzymes, primarily cathepsins<sup>47-49</sup>. Accordingly, we  
254 classified cells expressing known intestinal cathepsins of *F. hepatica*<sup>12</sup> (suppl. table S5) as  
255 gastrodermal cells (Fig 5 A). We validated this by ISH of cathepsin L1 (Fig 5 D). As to be  
256 expected, a high number of characteristic genes for this cluster associated with GO terms  
257 like proteolysis, cysteine-type peptidase activity or lipid binding (Fig 5 B). Related to lipid  
258 metabolism, a gene coding for a phospholipase B-like protein (D915\_003832) caught our  
259 attention, which showed specific transcript staining in the branches of the gut (Fig 5 B). It  
260 can be speculated that this gut-specific protein may be part of lipid metabolism in  
261 gastrodermal cells of the adult worm. As liver flukes have a highly reduced lipid  
262 metabolism<sup>50</sup>, processing of endogenous or host-derived lipids in gastrodermal cells  
263 warrants future investigation and could be interesting as antihelminthic target.

264 **Specialized muscle cells express several kinase genes**

265 The musculature in trematodes is important for several functions essential for the fluke's  
266 survival, like attachment to the bile duct to keep in place, or the feeding activity. The  
267 muscular system is composed of body musculature controlling the worm's movement, the  
268 sucker musculature for attachment, as well as the muscles lining the reproductive and  
269 digestive organs<sup>51</sup>. All muscles are of the invertebrate smooth type with the cell body  
270 connected to the muscle fiber via cytoplasmic connections<sup>46,52</sup>. The liver fluke musculature  
271 was represented by two clusters in our data, which were termed muscle and elf5+. The  
272 muscle cluster was identified by the expression of myosin and collagen as marker genes  
273 (Fig 6 B) as well as conserved muscle markers from other species<sup>53</sup>. The expression of  
274 collagen in muscle cells is in agreement with studies in planarians and schistosomes<sup>19,54</sup>,  
275 which described muscle cells as a main source of extracellular matrix. We further validated  
276 the expression of collagen and myosin by ISH. As a reference, we combined FISH with  
277 immunofluorescence, utilising an antibody against muscle fiber protein frequently used in  
278 planarian research<sup>55</sup>. Cells expressing collagen and myosin were widely distributed and  
279 located in close proximity to the stained muscle fibers in both the subtegumental muscle  
280 layer and throughout the body (suppl Fig S3). This pattern clearly fits the nature of  
281 invertebrate muscle fibers described above. Next to these structural molecules, we found  
282 expression of a gene coding for the 5-HT receptor 1 (D915\_001848), (Fig 6 A), which is

283 thought to be involved in the serotonin-dependent activation of muscle fibers in  
284 flatworms<sup>56,57</sup>, as well as other central regulators of cell signaling. Among these were protein  
285 kinase C, G-protein coupled receptors and phospholipase C, for which signaling in response  
286 to FMRFamides was previously suggested for *Fasciola* muscle fibers<sup>58</sup>. Unexpectedly,  
287 expression of the cystatin like stefin-2 (D915\_009861) was high within the muscle cluster.  
288 Previous work on *F. hepatica* showed the localization of stefins within gastrodermal cells, the  
289 tegumental area as well as the reproductive organs<sup>59</sup>. Our data suggest that the transcripts  
290 for stefin-1 (D915\_009335) were indeed present in cells of the reproductive organs as well  
291 as in the gut, while stefin-2 (D915\_009861) and stefin-3 (D915\_001085) were also present in  
292 the muscle cluster, which is the first time the expression of those protease inhibitors is  
293 described in this cell type (suppl. Fig S2).

294 An additional cluster had muscle-like features based on marker overlap with the muscle  
295 cluster, though it also expressed distinct marker genes (Fig 6 A). We termed this cluster  
296 elf5+ based on the highly characteristic expression of the ets transcription factor elf5  
297 (D915\_002050), which was shown to regulate extracellular matrix composition in  
298 planarians<sup>60</sup>. In order to locate these elf5+ cells within *F. hepatica*, we performed FISH. Cells  
299 positive for *elf5* transcripts located in close contact with muscle fibers (Fig 6 C), which further  
300 underlined their annotation as muscle cells. While the muscle cluster appeared to be more  
301 involved in metabolic processes, translation and transport, the elf5+ cluster was enriched in  
302 GO terms related to cell communication and signal transduction. Related to these terms are  
303 for example the neuropilin and tolloid protein (D915\_000810), which has been found in  
304 neurons as well as a type of smooth muscle cells in humans<sup>61</sup> and functions in the  
305 neuromuscular junction in *Drosophila melanogaster*<sup>62</sup>, or two genes encoding for ankyrin 2  
306 (D915\_000071, D915\_002954), which contain an ankyrin repeat region. The family of  
307 ankyrins is involved in the attachment of membrane proteins to the cytoskeleton, and  
308 especially type 2 ankyrins have been shown to function in muscle cells<sup>63,64</sup>.

309 To characterize this cell type in more detail, we focused on protein kinases, which are  
310 central regulators of a plethora of cellular process<sup>23</sup>. A notable number of 16 PKs was found  
311 among the marker genes (Fig 6 E and suppl. Table S2). These included two proposed  
312 protein kinase C (PKC) genes, D915\_006901 and D915\_003066, though while annotated as

313 PKC, D915\_003066 shows closer sequence similarity to protein kinase D. PKC signaling  
314 was previously discussed as mediator of body contraction in schistosomes and liver flukes  
315 based on studies with whole worms or body strips of worms treated with PKC activators or  
316 inhibitors<sup>58</sup>. Our expression data now proof PKC expression in muscle cells and thereby  
317 suggest an involvement of PKC signaling in muscle function<sup>65,66</sup>. Other highly characteristic  
318 kinases were the focal adhesion kinase (D915\_002353) and the p21-activated kinase 4  
319 (PAK4, D915\_004414). These kinases are typically involved in signaling related to  
320 extracellular matrix binding and focal adhesions<sup>67,68</sup>. Another interesting feature of the elf5+  
321 cluster is the high expression of the TRPM<sub>PZQ</sub> channel (D915\_003213) (Fig. 6A), hinting at  
322 the still unknown role of this channel in parasitic flatworms. We speculate on a role in cell  
323 adhesion, as TRPM channels have shown to localize in focal adhesions in humans<sup>69</sup>.  
324 Ligands targeting the schistosomal TRPM<sub>PZQ</sub> (praziquantel) or the *Fasciola* TRPM<sub>PZQ</sub> (BZQ)  
325 caused tegumental damage<sup>70,71</sup>, which might be related to the destabilization of the  
326 attachment of cells to the extracellular matrix, and to the basement membrane in particular.  
  
327 Taken together, scRNA-seq analysis revealed a previously unrecognized muscle cell type  
328 that may have a more specialized location and role, thereby being distinct from the main  
329 subtegumental musculature.

### 330 **An inhibitor of the p21-activated PAK4 kinase reduces fluke vitality**

331 Protein kinases (PKs) are well druggable targets, also in various helminths<sup>72</sup>. The important  
332 role of PKs in the formation and progression of cancer has led to the development of small-  
333 molecule inhibitors, which have potential for drug repurposing against parasites<sup>21</sup>. The  
334 enrichment for various kinases within the elf5+ cluster as well as the expression of the pan-  
335 flatworm target TRPM<sub>PZQ</sub> led us to the hypothesis that the cells within the elf5+ cluster might  
336 be valuable for the identification of novel drug targets with vital functions for the parasite.

337 We selected PAK4 as a candidate kinase. PAK4 represents a still unexplored kinase in  
338 trematodes, but has shown promise in the treatment of various forms of cancer<sup>73</sup>. PAK4 is a  
339 member of the p21-activated kinase (PAK) family, which is characterized by a p21-binding  
340 domain (PDB), also called Cdc42/Rac interactive binding (CRIB) motif<sup>74</sup>. In humans, this  
341 kinase family is involved in several developmental processes, functions in the immune

342 system and the nervous system<sup>75</sup>. In the fruit fly *D. melanogaster*, these proteins play roles  
343 in the development and control of the visual system<sup>76</sup>, while for *Caenorhabditis elegans*, they  
344 are important for axon guidance, gonad development and mechanotransduction<sup>77-79</sup>. To  
345 date, PAK kinases have not been addressed in parasitic helminths. First, we confirmed the  
346 presence of the essential protein domains within the FhPAK4 amino acid sequence by  
347 SMART analysis (suppl. Fig S3). FhPAK4 shared overall 44.6% sequence identity with  
348 human PAK4, while their kinase domains had 64.3% sequence identity. Most organisms  
349 have multiple PAK family members, which belong to two groups. Group 1 and group 2 differ  
350 in their structure as well as their function<sup>74,80</sup>. Within the genome of *F. hepatica*, we detected  
351 three more PAK kinases next to FhPAK4, based on the presence of the characteristic PDB  
352 domain. By comparing *F. hepatica* PAK sequences with sequences from model organisms,  
353 FhPAK4 could be identified as a group 2 family member, while the other PAKs allocated to  
354 group 1 (Fig 7 A). Based on these results, we termed D915\_001478 and D915\_004654 as  
355 FhPAK1 and FhPAK2, respectively. The last ortholog, D915\_006992, clustered within a  
356 previously described group<sup>77,81</sup> together with the more divergent PAK members *D.*  
357 *melanogaster* PAK3 and *C. elegans* MAX-2, which is why we named this kinase FhPAK3.  
358 While *fhpak1* and *fhpak2* were transcribed in several different cell types, *fhpak3* was  
359 expressed abundantly in mature oocytes and *fhpak4* expression was confined to the elf5+  
360 cluster, the gastrodermis and vitellocytes (suppl. Fig S4). FISH experiments localized *fhpak4*  
361 transcripts in gastrodermal cells, in oocytes, and in large, scattered muscle cells positioned  
362 clearly distant from the typical subtegumental body musculature (Fig 7 B) as seen for *elf5*  
363 transcripts. The presence of *fhpak4* transcripts within the nucleus of oocytes might hint at a  
364 role for FhPAK4 in early embryo development. In line with this hypothesis is the high  
365 abundance of transcripts within the eggs of *F. hepatica* that we found in available bulk RNA-  
366 seq data (Cwiklinski 2015). Maternal transcripts of *pak4* were also detected in the  
367 developing embryo of zebrafish, where PAK4 is essential for myelopoiesis<sup>82</sup>.

368 Due to the numerous roles of the human PAK4 in various cancer types, small molecule-  
369 inhibitors have been developed<sup>83,84</sup>. We evaluated the efficacy of the ATP-competitive  
370 inhibitor LCH-7749944, which was shown to have an impact on proliferation, migration and  
371 invasion of gastric cancer cells<sup>85</sup>. The binding mechanism of LCH-774994 was predicted to

372 involve interaction with residues within the hinge/p-loop regions of the human PAK4<sup>86</sup>. We  
373 found high sequence similarities between human and FhPAK4 for both regions, while the  
374 three other *Fasciola* PAK proteins diverged to a higher degree (Fig 7 G). This suggests that  
375 LCH-774994 may target specifically FhPAK4 in *F. hepatica*. To identify the potential of  
376 FhPAK4 as drug target, we tested this inhibitor against different disease-relevant parasite  
377 stages: newly excysted juveniles (NEJs) found in the host's intestine, immature worms  
378 migrating and feeding through liver tissue, and the bile duct-residing adult worms. For all  
379 three stages, we observed a significant reduction of the motility during *in vitro* culture after  
380 LCH-774994 treatment (Fig 7 C). Adult worms showed moderate reduction in motility after  
381 incubation with 25  $\mu$ M, but were severely affected with 50  $\mu$ M after 48 h, ending in 100%  
382 lethality after 72 h (Fig 7 D). For immature worms, treatment led to a strong reduction of  
383 motility with 50  $\mu$ M already after 24 hours, and all worms died after 48 h (Fig 7 E). Thus,  
384 immature worms appeared more susceptible compared to adults. NEJs responded even  
385 more sensitive, since all died within few hours of exposure to 25  $\mu$ M, displayed impaired  
386 tissue integrity and prominent lesions on their tegument (Fig 7 F). Previous research  
387 highlighted the druggability and role of PAKs in human host cells, for example when it comes  
388 to host-cell invasion or pathogen-induced manipulation of the host's cytoskeleton by viruses,  
389 bacteria and parasites<sup>87</sup>. Here, we provide insight that PAK also of pathogen origin is  
390 essential for pathogen survival and appears attractive for therapeutic approaches. Thus, the  
391 example of FhPAK4 illustrates that the elf5+ muscle cells may be of high interest with  
392 respect to drug development.

### 393 **Conclusions**

394 In this study, we present the first transcriptome for *F. hepatica* at single-cell resolution. The  
395 scRNA-seq dataset covers several cell types that are important for proliferation, as well as  
396 somatic cell types important for parasite vitality, like gastrodermal cells and cells of the  
397 musculature. The identification of molecular markers characteristic for each cell type  
398 delivered new information on cell lineages and cell-type specific functions. We described a  
399 previously unrecognized muscle cell type, characterized by expression of TRPM<sub>PZQ</sub> and a  
400 multitude of protein kinases. By prioritizing the family of p21-activated kinases within this cell  
401 type, we highlighted the usefulness of this scRNA-seq dataset in the discovery of novel

402 druggable targets. Thus, this dataset can serve as a resource for addressing basic and  
403 applied research questions, by providing valuable insights in the cellular biology of a  
404 multicellular pathogen.

405 **Limitations of the study**

406 We report the transcriptional characterization of several cell types. Due to the abundance of  
407 cells of the male reproductive tract, this dataset misses some of somatic cell types, like cells  
408 of the parenchyma or the nervous system. Alternative ways to enrich for such cell types  
409 would add to this dataset.

410

412 **Acknowledgements**

413 Financial support by the Deutsche Forschungsgemeinschaft (DFG) under grant HA 6963/2-1  
414 and by the State of Hesse, LOEWE Center "DRUID", is gratefully acknowledged. O.P.  
415 received a scholarship by the Justus Liebig University Giessen.

416 **Author contributions**

417 Conceptualization, O.P., S.H.; Methodology, O.P.; Investigation, S.G., O.P., S.A., JK.;  
418 Visualization, O.P.; Writing – Original Draft Preparation, O.P.; Writing – Review & Editing, all  
419 authors; Funding Acquisition, C.S., S.H; Supervision: SH.

420 **Competing interests**

421 The authors declare no conflict of interest. The funders had no role in study design, data  
422 collection and analysis, decision to publish, or preparation of the manuscript.

423

## Reference

1. van Helden, P.D., van Helden, L.S., and Hoal, E.G. (2013). One world, one health. *EMBO Rep.* 14, 497–501 – 501.
2. Beesley, N.J., Caminade, C., Charlier, J., Flynn, R.J., Hodgkinson, J.E., Martinez-Moreno, A., Martinez-Valladares, M., Perez, J., Rinaldi, L., and Williams, D.J.L. (2018). *Fasciola* and fasciolosis in ruminants in Europe: Identifying research needs. *Transbound. Emerg. Dis.* 65 *Suppl 1*, 199–216.
3. Mas-Coma, S., Maria Adela, V., and Maria Dolores, B. (2023). Fascioliasis. In *Digenetic Trematodes* (Springer).
4. Keiser, J., and Utzinger, J. (2009). Food-borne trematodiases. *Clin. Microbiol. Rev.* 22, 466–483.
5. Kelley, J.M., Elliott, T.P., Beddoe, T., Anderson, G., Skuce, P., and Spithill, T.W. (2016). Current threat of triclabendazole resistance in *Fasciola hepatica*. *Trends Parasitol.* 32, 458–469.
6. Fairweather, I., Brennan, G.P., Hanna, R.E.B., Robinson, M.W., and Skuce, P.J. (2020). Drug resistance in liver flukes. *Int. J. Parasitol. Drugs Drug Resist.* 12, 39–59.
7. Spithill, T.W., Toet, H., Rathinasamy, V., Zerna, G., Swan, J., Cameron, T., Smooker, P.M., Piedrafita, D.M., Dempster, R., and Beddoe, T. (2021). Vaccines for *Fasciola* : new thinking for an old problem. In *Fasciolosis* (CABI), pp. 379–422.
8. Happich, F.A., and Boray, J.C. (1969). Quantitative diagnosis of chronic fasciolosis. 2. The estimation of daily total egg production of *Fasciola hepatica* and the number of adult flukes in sheep by faecal egg counts. *Aust. Vet. J.* 45, 329–331.
9. Leuckart, R. (1882). Zur Entwicklungsgeschichte des Leberegels (*Distomum hepaticum*). *Arch. Naturg. Berlin.*
10. Lutz, A. (1893). Weiteres zur Lebensgeschichte des *Distoma hepaticum*. *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Abteilung.*
11. Cwiklinski, K., and Dalton, J.P. (2018). Advances in *Fasciola hepatica* research using “omics” technologies. *Int. J. Parasitol.* 48, 321–331.
12. Cwiklinski, K., Dalton, J.P., Dufresne, P.J., La Course, J., Williams, D.J., Hodgkinson, J., and Paterson, S. (2015). The *Fasciola hepatica* genome: gene duplication and polymorphism reveals adaptation to the host environment and the capacity for rapid evolution. *Genome Biol.* 16, 71.
13. Radio, S., Fontenla, S., Solana, V., Matos Salim, A.C., Araújo, F.M.G., Ortiz, P., Hoban, C., Miranda, E., Gayo, V., Pais, F.S.-M., et al. (2018). Pleiotropic alterations in gene expression in Latin American *Fasciola hepatica* isolates with different susceptibility to drugs. *Parasit. Vectors* 11, 56.
14. Miranda-Miranda, E., Cossio-Bayugar, R., Aguilar-Díaz, H., Narváez-Padilla, V., Sachman-Ruiz, B., and Reynaud, E. (2021). Transcriptome assembly dataset of anhelminthic response in *Fasciola hepatica*. *Data Brief* 35, 106808.
15. Haçarız, O., Akgün, M., Kavak, P., Yüksel, B., and Sağıroğlu, M.Ş. (2015). Comparative transcriptome profiling approach to glean virulence and immunomodulation-related

genes of *Fasciola hepatica*. *BMC Genomics* 16, 366.

16. Robb, E., McCammick, E.M., Wells, D., McVeigh, P., Gardiner, E., Armstrong, R., McCusker, P., Mousley, A., Clarke, N., Marks, N.J., et al. (2022). Transcriptomic analysis supports a role for the nervous system in regulating growth and development of *Fasciola hepatica* juveniles. *PLoS Negl. Trop. Dis.* 16, e0010854.
17. Pisco, A.O., Tojo, B., and McGeever, A. (2021). Single-Cell Analysis for Whole-Organism Datasets. *Annu Rev Biomed Data Sci* 4, 207–226.
18. Fincher, C.T., Wurtzel, O., de Hoog, T., Kravarik, K.M., and Reddien, P.W. (2018). Cell type transcriptome atlas for the planarian *Schmidtea mediterranea*. *Science* 360. 10.1126/science.aaq1736.
19. Wendt, G., Zhao, L., Chen, R., Liu, C., O'Donoghue, A.J., Caffrey, C.R., Reese, M.L., and Collins, J.J., 3rd (2020). A single-cell RNA-seq atlas of *Schistosoma mansoni* identifies a key regulator of blood feeding. *Science* 369, 1644–1649.
20. Li, P., Nanes Sarfati, D., Xue, Y., Yu, X., Tarashansky, A.J., Quake, S.R., and Wang, B. (2021). Single-cell analysis of *Schistosoma mansoni* identifies a conserved genetic program controlling germline stem cell fate. *Nat. Commun.* 12, 485.
21. Pereira Moreira, B., Weber, M.H.W., Haeberlein, S., Mokosch, A.S., Spengler, B., Grevelding, C.G., and Falcone, F.H. (2022). Drug repurposing and de novo drug discovery of protein kinase inhibitors as new drugs against schistosomiasis. *Molecules* 27. 10.3390/molecules27041414.
22. Morawietz, C.M., Houhou, H., Puckelwaldt, O., Hehr, L., Dreisbach, D., Mokosch, A., Roeb, E., Roderfeld, M., Spengler, B., and Haeberlein, S. (2020). Targeting kinases in *Fasciola hepatica*: Anthelmintic effects and tissue distribution of selected kinase inhibitors. *Front Vet Sci* 7, 611270.
23. Melnikova, I., and Golden, J. (2004). Targeting protein kinases. *Nat. Rev. Drug Discov.* 3, 993–994.
24. Roskoski, R., Jr (2020). Properties of FDA-approved small molecule protein kinase inhibitors: A 2020 update. *Pharmacol. Res.* 152, 104609.
25. Bournez, C., Carles, F., Peyrat, G., Aci-Sèche, S., Bourg, S., Meyer, C., and Bonnet, P. (2020). Comparative assessment of protein kinase inhibitors in public databases and in PKIDB. *Molecules* 25. 10.3390/molecules25143226.
26. Ashburn, T.T., and Thor, K.B. (2004). Drug repositioning: identifying and developing new uses for existing drugs. *Nat. Rev. Drug Discov.* 3, 673–683.
27. Stitt, A.W., and Fairweather, I. (1990). Spermatogenesis and the fine structure of the mature spermatozoon of the liver fluke, *Fasciola hepatica* (Trematoda: Digenea). *Parasitology* 101 Pt 3, 395–407.
28. Hao, Y., Hao, S., Andersen-Nissen, E., Mauck, W.M., 3rd, Zheng, S., Butler, A., Lee, M.J., Wilk, A.J., Darby, C., Zager, M., et al. (2021). Integrated analysis of multimodal single-cell data. *Cell* 184, 3573–3587.e29.
29. Buddenborg, S.K., Tracey, A., Berger, D.J., Lu, Z., Doyle, S.R., Fu, B., Yang, F., Reid, A.J., Rodgers, F.H., Rinaldi, G., et al. (2021). Assembled chromosomes of the blood fluke *Schistosoma mansoni* provide insight into the evolution of its ZW sex-

determination system. bioRxiv, 2021.08.13.456314. 10.1101/2021.08.13.456314.

30. McCusker, P., McVeigh, P., Rathinasamy, V., Toet, H., McCammick, E., O'Connor, A., Marks, N.J., Mousley, A., Brennan, G.P., Halton, D.W., et al. (2016). Stimulating neoblast-like cell proliferation in juvenile *Fasciola hepatica* supports growth and progression towards the adult phenotype in vitro. PLoS Negl. Trop. Dis. 10, e0004994.
31. De Keuckelaere, E., Hulpiau, P., Saeys, Y., Berx, G., and van Roy, F. (2018). Nanos genes and their role in development and beyond. Cell. Mol. Life Sci. 75, 1929–1946.
32. Collins, J.J., 3rd, Wang, B., Lambrus, B.G., Tharp, M.E., Iyer, H., and Newmark, P.A. (2013). Adult somatic stem cells in the human parasite *Schistosoma mansoni*. Nature 494, 476–479.
33. Wagner, D.E., Ho, J.J., and Reddien, P.W. (2012). Genetic regulators of a pluripotent adult stem cell system in planarians identified by RNAi and clonal analysis. Cell Stem Cell 10, 299–311.
34. Guo, T., Peters, A.H.F.M., and Newmark, P.A. (2006). A Bruno-like gene is required for stem cell maintenance in planarians. Dev. Cell 11, 159–169.
35. Hentschel, C.C., and Birnstiel, M.L. (1981). The organization and expression of histone gene families. Cell 25, 301–313.
36. Hanna, R.E.B., Moffett, D., Forster, F.I., Trudgett, A.G., Brennan, G.P., and Fairweather, I. (2016). *Fasciola hepatica*: a light and electron microscope study of the ovary and of the development of oocytes within eggs in the uterus provides an insight into reproductive strategy. Vet. Parasitol. 221, 93–103.
37. Iyer, H., Issigonis, M., Sharma, P.P., Extavour, C.G., and Newmark, P.A. (2016). A premeiotic function for boule in the planarian *Schmidtea mediterranea*. Proc. Natl. Acad. Sci. U. S. A. 113, E3509–E3518.
38. Luo, M., Yang, F., Leu, N.A., Landaiche, J., Handel, M.A., Benavente, R., La Salle, S., and Wang, P.J. (2013). MEIOB exhibits single-stranded DNA-binding and exonuclease activities and is essential for meiotic recombination. Nat. Commun. 4, 2788.
39. Souquet, B., Abby, E., Hervé, R., Finsterbusch, F., Tourpin, S., Le Bouffant, R., Duquenne, C., Messiaen, S., Martini, E., Bernardino-Sgherri, J., et al. (2013). MEIOB targets single-strand DNA and is necessary for meiotic recombination. PLoS Genet. 9, e1003784.
40. Threadgold, L.T. (1982). *Fasciola hepatica*: stereological analysis of vitelline cell development. Exp. Parasitol. 54, 352–365.
41. Erasmus, D.A. (1975). *Schistosoma mansoni*: development of the vitelline cell, its role in drug sequestration, and changes induced by Astiban. Exp. Parasitol. 38, 240–256.
42. Lu, Z., Quack, T., Hahnel, S., Gelmedin, V., Pouokam, E., Diener, M., Hardt, M., Michel, G., Baal, N., Hackstein, H., et al. (2015). Isolation, enrichment and primary characterisation of vitelline cells from *Schistosoma mansoni* obtained by the organ isolation method. Int. J. Parasitol. 45, 663–672.
43. Wang, J., and Collins, J.J., 3rd (2016). Identification of new markers for the *Schistosoma mansoni* vitelline lineage. Int. J. Parasitol. 46, 405–410.
44. Wang, J., Chen, R., and Collins, J.J., 3rd (2019). Systematically improved in vitro

culture conditions reveal new insights into the reproductive biology of the human parasite *Schistosoma mansoni*. PLoS Biol. 17, e3000254.

45. Rice-Ficht, A.C., Dusek, K.A., Kochevar, G.J., and Waite, J.H. (1992). Eggshell precursor proteins of *Fasciola hepatica*, I. Structure and expression of vitelline protein B. Mol. Biochem. Parasitol. 54, 129–141.
46. Robinson, M.W., Hanna, R.E.B., and Fairweather, I. (2021). Development of *Fasciola hepatica* in the mammalian host. In *Fasciolosis* (CABI), pp. 65–111.
47. Robinson, M.W., Menon, R., and Donnelly, S.M. (2009). An integrated transcriptomics and proteomics analysis of the secretome of the helminth pathogen *Fasciola hepatica*: associated with invasion and infection of the mammalian host. Molecular & Cellular.
48. Cwiklinski, K., Donnelly, S., Drysdale, O., Jewhurst, H., Smith, D., De Marco Verissimo, C., Pritsch, I.C., O'Neill, S., Dalton, J.P., and Robinson, M.W. (2019). The cathepsin-like cysteine peptidases of trematodes of the genus *Fasciola*. Adv. Parasitol. 104, 113–164.
49. Hanna, R.E. (1975). *Fasciola hepatica*: an electron microscope autoradiographic study of protein synthesis and secretion by gut cells in tissue slices. Exp. Parasitol. 38, 167–180.
50. Cwiklinski, K., Robinson, M.W., Donnelly, S., and Dalton, J.P. (2021). Complementary transcriptomic and proteomic analyses reveal the cellular and molecular processes that drive growth and development of *Fasciola hepatica* in the host liver. BMC Genomics 22, 46.
51. Mair, G.R., Maule, A.G., Shaw, C., Johnston, C.F., and Halton, D.W. (1998). Gross anatomy of the muscle systems of *Fasciola hepatica* as visualized by phalloidin-fluorescence and confocal microscopy. Parasitology 117 (Pt 1), 75–82.
52. Pax, R.A., Day, T.A., Miller, C.L., and Bennett, J.L. (1996). Neuromuscular physiology and pharmacology of parasitic flatworms. Parasitology 113 Suppl, S83–S96.
53. Diaz Soria, C.L., Lee, J., Chong, T., Coghlan, A., Tracey, A., Young, M.D., Andrews, T., Hall, C., Ng, B.L., Rawlinson, K., et al. (2020). Single-cell atlas of the first intra-mammalian developmental stage of the human parasite *Schistosoma mansoni*. Nat. Commun. 11, 6411.
54. Cote, L.E., Simental, E., and Reddien, P.W. (2019). Muscle functions as a connective tissue and source of extracellular matrix in planarians. Nat. Commun. 10, 1592.
55. Ross, K.G., Omuro, K.C., Taylor, M.R., Munday, R.K., Hubert, A., King, R.S., and Zayas, R.M. (2015). Novel monoclonal antibodies to study tissue regeneration in planarians. BMC Dev. Biol. 15, 2.
56. Holmes, S.D., and Fairweather, I. (1984). *Fasciola hepatica*: the effects of neuropharmacological agents upon in vitro motility. Exp. Parasitol. 58, 194–208.
57. Kreshchenko, N., Terenina, N., and Ermakov, A. (2021). Serotonin signalling in flatworms: an immunocytochemical localisation of 5-HT<sub>7</sub> type of serotonin receptors in *Opisthorchis felineus* and *Hymenolepis diminuta*. Biomolecules 11, 1212.
58. Graham, M.K., Fairweather, I., and McGeown, J.G. (2000). Second messengers mediating mechanical responses to the FARP GYIRFamide in the fluke *Fasciola hepatica*. Am. J. Physiol. Regul. Integr. Comp. Physiol. 279, R2089–R2094.

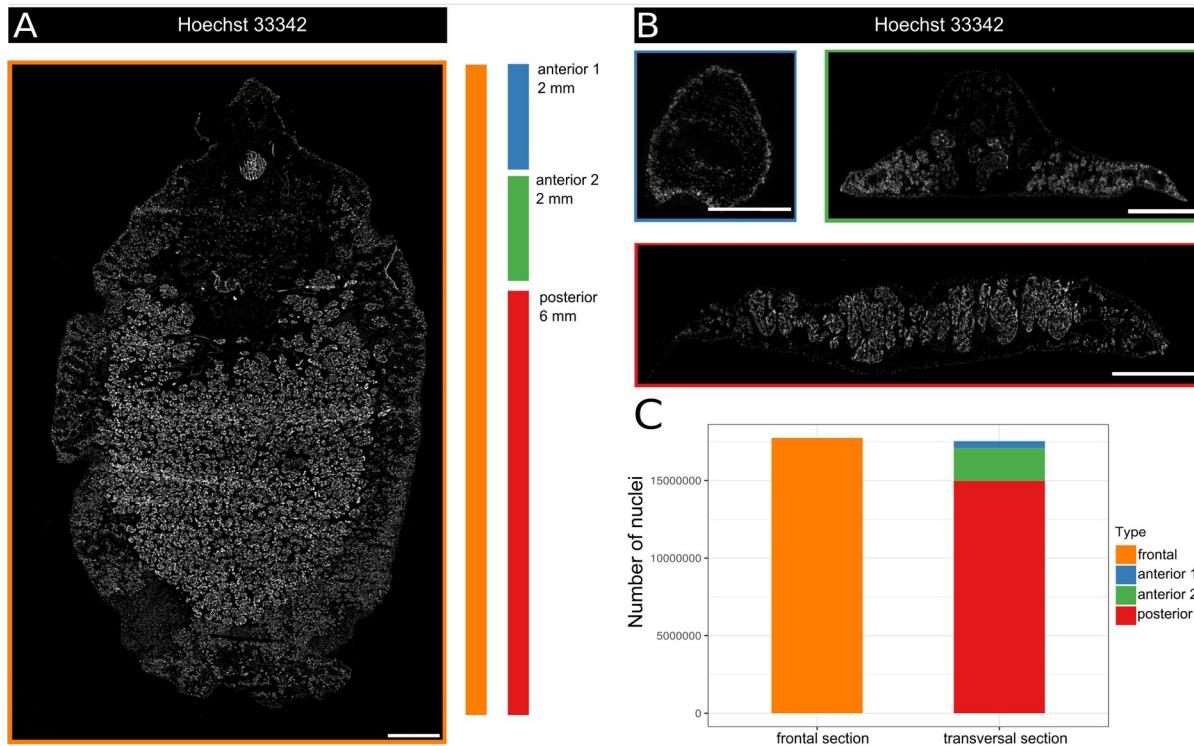
59. Cancela, M., Corvo, I., DA Silva, E., Teichmann, A., Roche, L., Díaz, A., Tort, J.F., Ferreira, H.B., and Zaha, A. (2017). Functional characterization of single-domain cystatin-like cysteine proteinase inhibitors expressed by the trematode *Fasciola hepatica*. *Parasitology* *144*, 1695–1707.
60. Dubey, V.K., Sarkar, S.R., Lakshmanan, V., Dalmeida, R., Gulyani, A., and Palakodeti, D. (2022). *S. mediterranea* ETS-1 regulates the function of cathepsin-positive cells and the epidermal lineage landscape via basement membrane remodeling. *J. Cell Sci.* *135*. 10.1242/jcs.259900.
61. Rossignol, M., Gagnon, M.L., and Klagsbrun, M. (2000). Genomic organization of human neuropilin-1 and neuropilin-2 genes: identification and distribution of splice variants and soluble isoforms. *Genomics* *70*, 211–222.
62. Kim, Y.-J., Bao, H., Bonanno, L., Zhang, B., and Serpe, M. (2012). *Drosophila Neto* is essential for clustering glutamate receptors at the neuromuscular junction. *Genes Dev.* *26*, 974–987.
63. York, N.S., Sanchez-Arias, J.C., McAdam, A.C.H., Rivera, J.E., Arbour, L.T., and Swayne, L.A. (2022). Mechanisms underlying the role of ankyrin-B in cardiac and neurological health and disease. *Front Cardiovasc Med* *9*, 964675.
64. Boskovic, S., Marín-Juez, R., Jasnic, J., Reischauer, S., El Sammak, H., Kojic, A., Faulkner, G., Radojkovic, D., Stainier, D.Y.R., and Kojic, S. (2018). Characterization of zebrafish (*Danio rerio*) muscle ankyrin repeat proteins reveals their conserved response to endurance exercise. *PLoS One* *13*, e0204312.
65. Ressurreição, M., De Saram, P., Kirk, R.S., Rollinson, D., Emery, A.M., Page, N.M., Davies, A.J., and Walker, A.J. (2014). Protein kinase C and extracellular signal-regulated kinase regulate movement, attachment, pairing and egg release in *Schistosoma mansoni*. *PLoS Negl. Trop. Dis.* *8*, e2924.
66. Blair, K.L., Bennett, J.L., and Pax, R.A. (1988). *Schistosoma mansoni*: evidence for protein kinase-C-like modulation of muscle activity. *Exp. Parasitol.* *66*, 243–252.
67. Fogh, B.S., Multhaupt, H.A.B., and Couchman, J.R. (2014). Protein kinase C, focal adhesions and the regulation of cell migration. *J. Histochem. Cytochem.* *62*, 172–184.
68. Mishra, Y.G., and Manavathi, B. (2021). Focal adhesion dynamics in cellular function and disease. *Cell. Signal.* *85*, 110046.
69. Cáceres, M., Ortiz, L., Recabarren, T., Romero, A., Colombo, A., Leiva-Salcedo, E., Varela, D., Rivas, J., Silva, I., Morales, D., et al. (2015). TRPM4 Is a novel component of the adhesome required for focal adhesion disassembly, migration and contractility. *PLoS One* *10*, e0130540.
70. Becker, B., Mehlhorn, H., Andrews, P., Thomas, H., and Eckert, J. (1980). Light and electron microscopic studies on the effect of praziquantel on *Schistosoma mansoni*, *Dicrocoelium dendriticum*, and *Fasciola hepatica* (Trematoda) in vitro. *Z. Parasitenkd.* *63*, 113–128.
71. Sprague, D.J., Park, S.-K., Gramberg, S., Bauer, L., Rohr, C.M., Chulkov, E.G., Smith, E., Scampavia, L., Spicer, T.P., Haeberlein, S., et al. (2023). Target-based discovery of a broad spectrum flukicide. *bioRxiv*. 10.1101/2023.09.22.559026.
72. Doerig, C. (2004). Protein kinases as targets for anti-parasitic chemotherapy. *Biochim.*

Biophys. Acta 1697, 155–168.

73. Yuan, Y., Zhang, H., Li, D., Li, Y., Lin, F., Wang, Y., Song, H., Liu, X., Li, F., and Zhang, J. (2022). PAK4 in cancer development: Emerging player and therapeutic opportunities. *Cancer Lett.* 545, 215813.
74. Zhao, Z.-S., and Manser, E. (2012). PAK family kinases: Physiological roles and regulation. *Cell. Logist.* 2, 59–68.
75. Liu, H., Liu, K., and Dong, Z. (2021). The role of p21-activated kinases in cancer and beyond: Where are we heading? *Front Cell Dev Biol* 9, 641381.
76. Melzig, J., Rein, K.H., Schäfer, U., Pfister, H., Jäckle, H., Heisenberg, M., and Raabe, T. (1998). A protein related to p21-activated kinase (PAK) that is involved in neurogenesis in the *Drosophila* adult central nervous system. *Curr. Biol.* 8, 1223–1226.
77. Lucanic, M., Kiley, M., Ashcroft, N., L'etoile, N., and Cheng, H.-J. (2006). The *Caenorhabditis elegans* P21-activated kinases are differentially required for UNC-6/netrin-mediated commissural motor axon guidance. *Development* 133, 4549–4559.
78. Peters, E.C., Gossett, A.J., Goldstein, B., Der, C.J., and Reiner, D.J. (2013). Redundant canonical and noncanonical *Caenorhabditis elegans* p21-activated kinase signaling governs distal tip cell migrations. *G3* 3, 181–195.
79. Zhang, H., Landmann, F., Zahreddine, H., Rodriguez, D., Koch, M., and Labouesse, M. (2011). A tension-induced mechanotransduction pathway promotes epithelial morphogenesis. *Nature* 471, 99–103.
80. Ha, B.H., Morse, E.M., Turk, B.E., and Boggon, T.J. (2015). Signaling, regulation, and specificity of the type II p21-activated kinases. *J. Biol. Chem.* 290, 12975–12983.
81. Mentzel, B., and Raabe, T. (2005). Phylogenetic and structural analysis of the *Drosophila melanogaster* p21-activated kinase DmPAK3. *Gene* 349, 25–33.
82. Law, S.H.W., and Sargent, T.D. (2013). Maternal pak4 expression is required for primitive myelopoiesis in zebrafish. *Mech. Dev.* 130, 181–194.
83. Dummler, B., Ohshiro, K., Kumar, R., and Field, J. (2009). Pak protein kinases and their role in cancer. *Cancer Metastasis Rev.* 28, 51–63.
84. Li, Y., Lu, Q., Xie, C., Yu, Y., and Zhang, A. (2022). Recent advances on development of p21-activated kinase 4 inhibitors as anti-tumor agents. *Front. Pharmacol.* 13, 956220.
85. Zhang, J., Wang, J., Guo, Q., Wang, Y., Zhou, Y., Peng, H., Cheng, M., Zhao, D., and Li, F. (2012). LCH-7749944, a novel and potent p21-activated kinase 4 inhibitor, suppresses proliferation and invasion in human gastric cancer cells. *Cancer Lett.* 317, 24–32.
86. Wu, T., Pang, Y., Guo, J., Yin, W., Zhu, M., Hao, C., Wang, K., Wang, J., Zhao, D., and Cheng, M. (2018). Discovery of 2-(4-substituted-piperidin/piperazine-1-yl)-N-(5-cyclopropyl-1H-pyrazol-3-yl)-quinazoline-2,4-diamines as PAK4 inhibitors with potent A549 cell proliferation, migration, and invasion inhibition activity. *Molecules* 23, 417.
87. John Von Freyend, S., Kwok-Schuelein, T., Netter, H.J., Haqshenas, G., Semblat, J.-P., and Doerig, C. (2017). Subverting host cell P21-activated kinase: A case of convergent evolution across pathogens. *Pathogens* 6. 10.3390/pathogens6020017.

88. McVeigh, P., McCammick, E.M., McCusker, P., Morphew, R.M., Mousley, A., Abidi, A., Saifullah, K.M., Muthusamy, R., Gopalakrishnan, R., Spithill, T.W., et al. (2014). RNAi dynamics in Juvenile *Fasciola* spp. Liver flukes reveals the persistence of gene silencing in vitro. *PLoS Negl. Trop. Dis.* 8, e3185.
89. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: An open-source platform for biological-image analysis. *Nat. Methods* 9, 676–682.
90. Le, T.H., Blair, D., Agatsuma, T., Humair, P.F., Campbell, N.J., Iwagami, M., Littlewood, D.T., Peacock, B., Johnston, D.A., Bartley, J., et al. (2000). Phylogenies inferred from mitochondrial gene orders-a cautionary tale from the parasitic flatworms. *Mol. Biol. Evol.* 17, 1123–1125.
91. Zappia, L., and Oshlack, A. (2018). Clustering trees: A visualization for evaluating clusterings at multiple resolutions. *Gigascience* 7. 10.1093/gigascience/giy083.
92. Alexa, A., and Rahnenfuhrer, J. (2010). topGO: Enrichment analysis for gene ontology. R package version.
93. Jones, P., Binns, D., Chang, H.-Y., Fraser, M., Li, W., McAnulla, C., McWilliam, H., Maslen, J., Mitchell, A., Nuka, G., et al. (2014). InterProScan 5: genome-scale protein function classification. *Bioinformatics* 30, 1236–1240.
94. Letunic, I., Khedkar, S., and Bork, P. (2021). SMART: recent updates, new developments and status in 2020. *Nucleic Acids Res.* 49, D458–D460.
95. Katoh, K., and Standley, D.M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol. Biol. Evol.* 30, 772–780.
96. Edgar, R.C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32, 1792–1797.
97. Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D.L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M.A., and Huelsenbeck, J.P. (2012). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Syst. Biol.* 61, 539–542.
98. Letunic, I., and Bork, P. (2021). Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. *Nucleic Acids Res.* 49, W293–W296.
99. Collins, J.J., 3rd, Hou, X., Romanova, E.V., Lambrus, B.G., Miller, C.M., Saberi, A., Sweedler, J.V., and Newmark, P.A. (2010). Genome-wide analyses reveal a role for peptide hormones in planarian germline development. *PLoS Biol.* 8, e1000509.
100. Cancela, M., and Maggioli, G. (2020). *Fasciola hepatica*: Methods and protocols 1st ed. M. Cancela and G. Maggioli, eds. (Springer).

## Main figures titles and legends



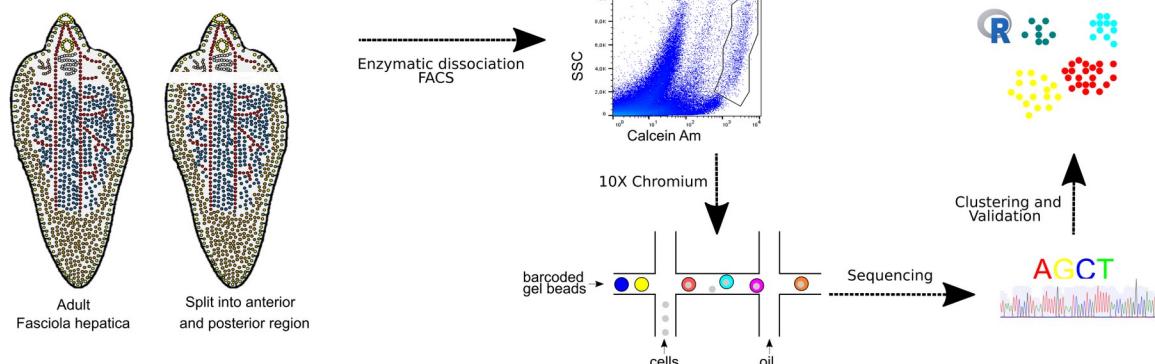
**Fig 1. *Fasciola hepatica* is structured in regions with different cellular densities. A**

Representative frontal section of an adult worm with nuclei stained using Hoechst 33342.

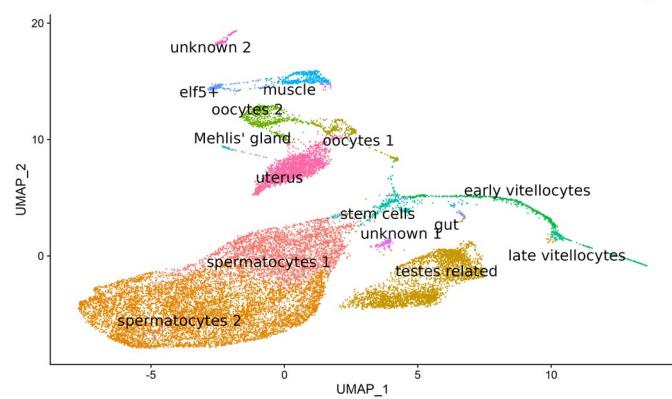
Color bars mark the worm regions selected for transversal sectioning. Scale bar: 1000  $\mu$ m. **B**

Representative images of transversal sections with nuclei stained with Hoechst 33342. Top from left to right: sections from the anterior 1 and anterior 2 regions. Bottom: section from the posterior region. Scale bar: 1000  $\mu$ m. **C** Stacked bar plot indicating the extrapolated total number of nuclei derived from either frontal or transversal sections.

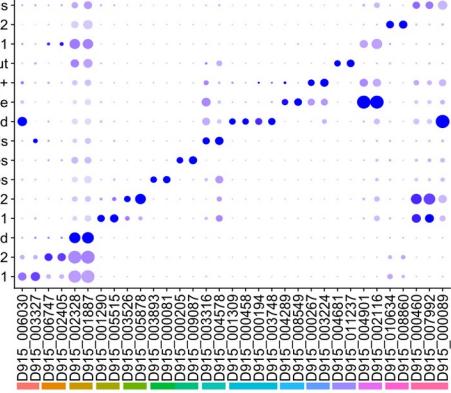
A



B

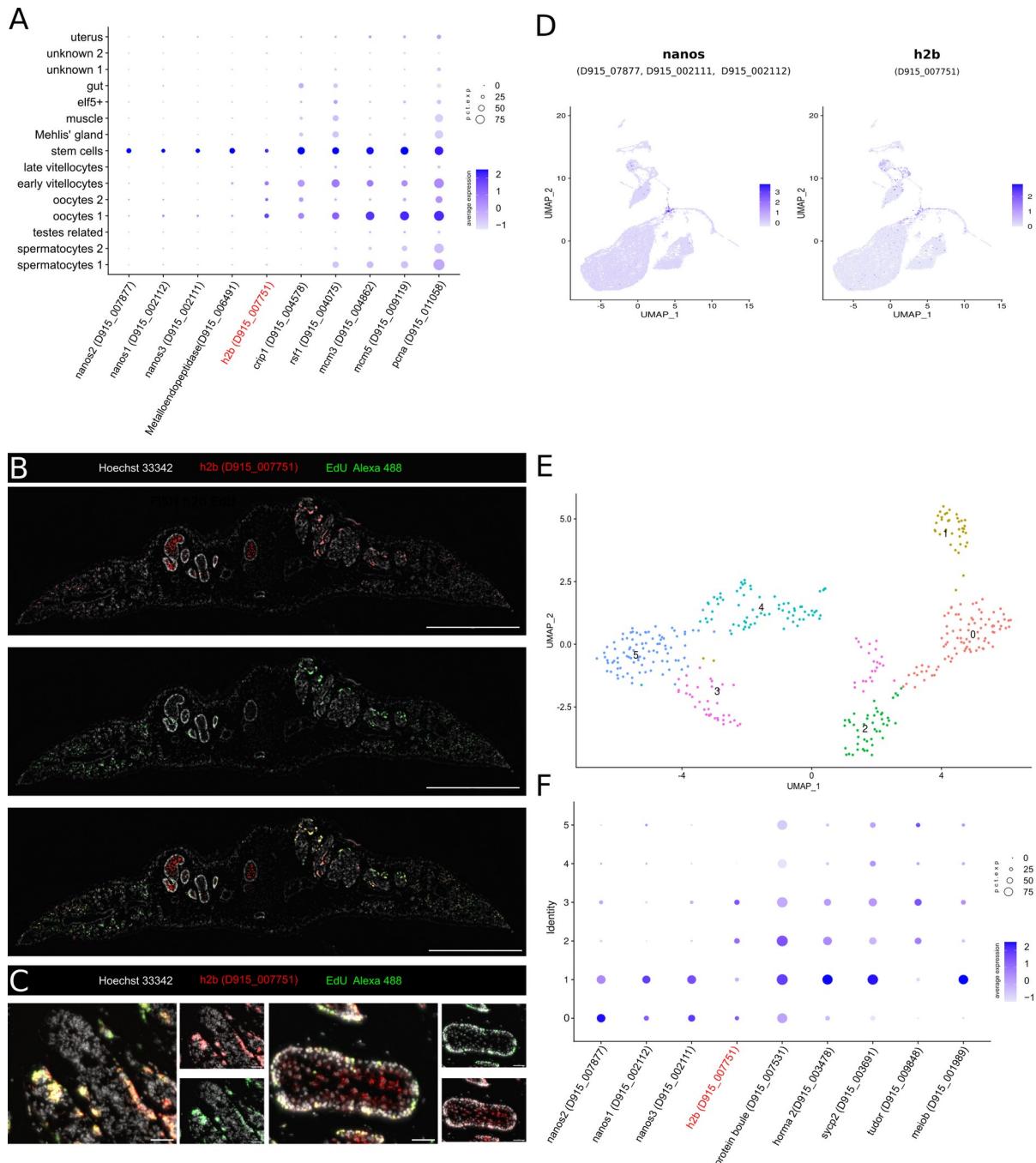


C



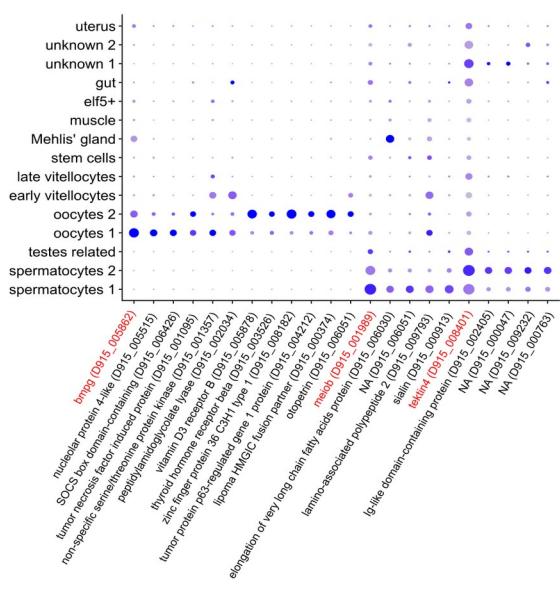
**Fig 2. scRNA-seq allowed the classification of 15 cell clusters in adult *F. hepatica*. A**

Schematic workflow outlining the major steps for data generation: adult liver flukes were first split into an anterior and posterior part before dissociating into single cells by mechanical and enzymatic processing. Next, cells were sorted based on the viability dye calcein. Cells were barcoded following the 10X Chromium protocol. Libraries were sequenced and clustering was carried out to identify transcriptionally distinct cells. **B** Uniform Manifold Approximation and Projection (UMAP) of 19,581 cells. Clusters are colored and labels added. **C** Profiles of gene expression over all clusters illustrated as dotplot. Shown is the expression of at least two marker genes for each cluster, and the cluster color is indicated below each marker pair. Level of expression is indicated by color from blue (high expression) to lavender (low expression). The percentage with which the cells of a cluster express the given gene is represented by the size of the circles.

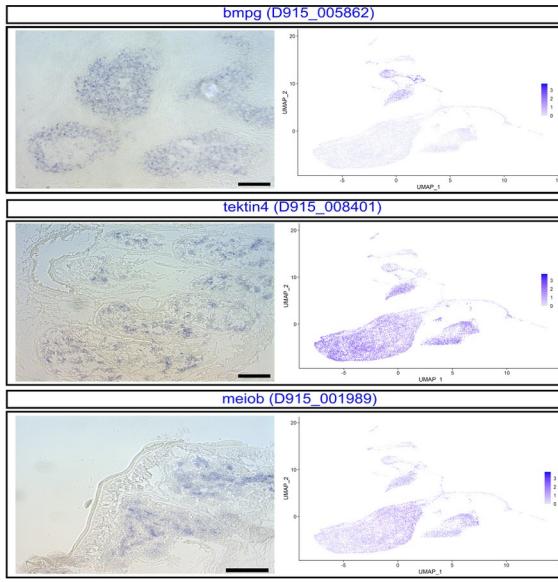


**Fig 3. Stem cells show transcriptionally distinct cell states.** **A** Dotplot showing the expression of stem-cell marker genes. ISH-validated genes are marked in red. Level of expression is indicated by color from blue (high expression) to lavender (low expression). The percentage with which cells of a cluster express the given gene is represented by the size of the circles. **B** Transversal section stained for *h2b* transcripts by FISH (red) and proliferating cells with EdU (green). Scale bar: 1000  $\mu$ m. **C** Close-up of testis (left) and ovary (right) from B. Scale bar: 50  $\mu$ m **D** UMAP plot showing the expression of *nanos* isoforms and *h2b*, **E** UMAP of subclustered cells colored by cluster. The clusters were numbered 0 to 6. **F** Dotplot showing the expression of *h2b*, *nanos* isoforms and marker genes for cell differentiation for the subclusters from E. ISH-validated genes are marked in red. Level of expression is indicated by color from blue (high expression) to lavender (low expression). The percentage with which cells of a cluster express the given gene is represented by the size of the circles.

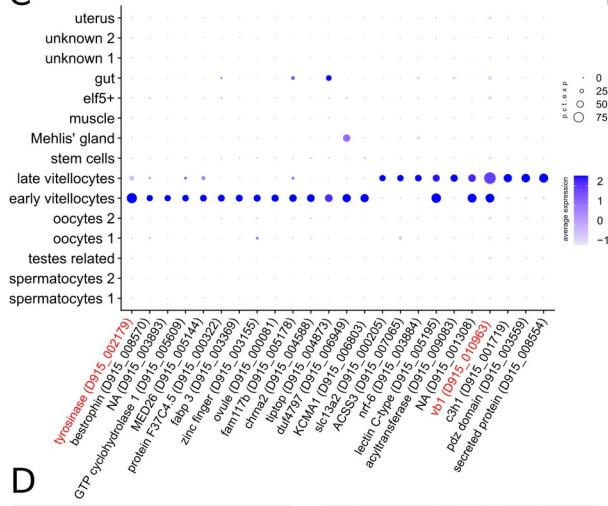
A



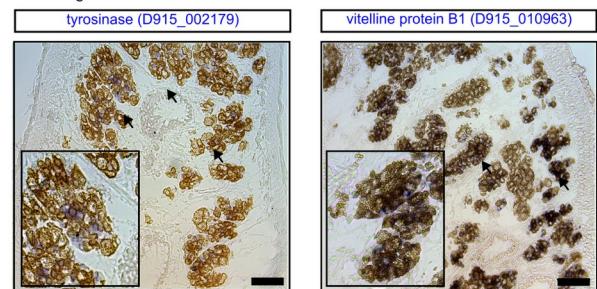
B



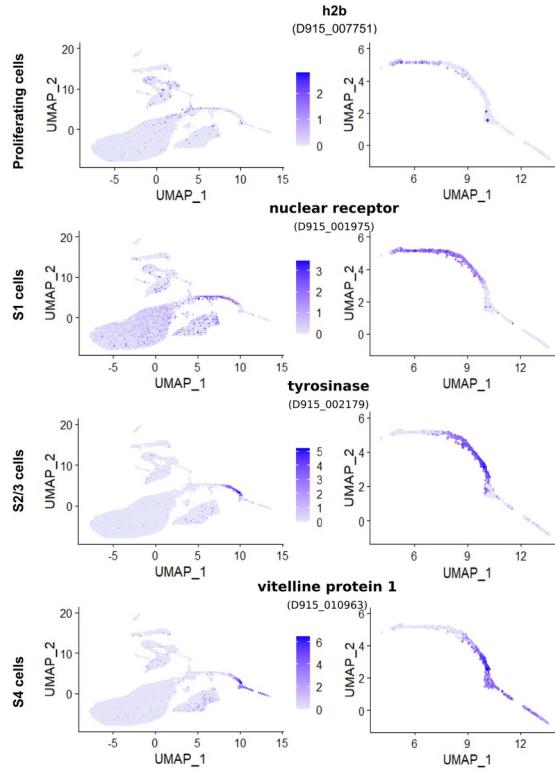
C



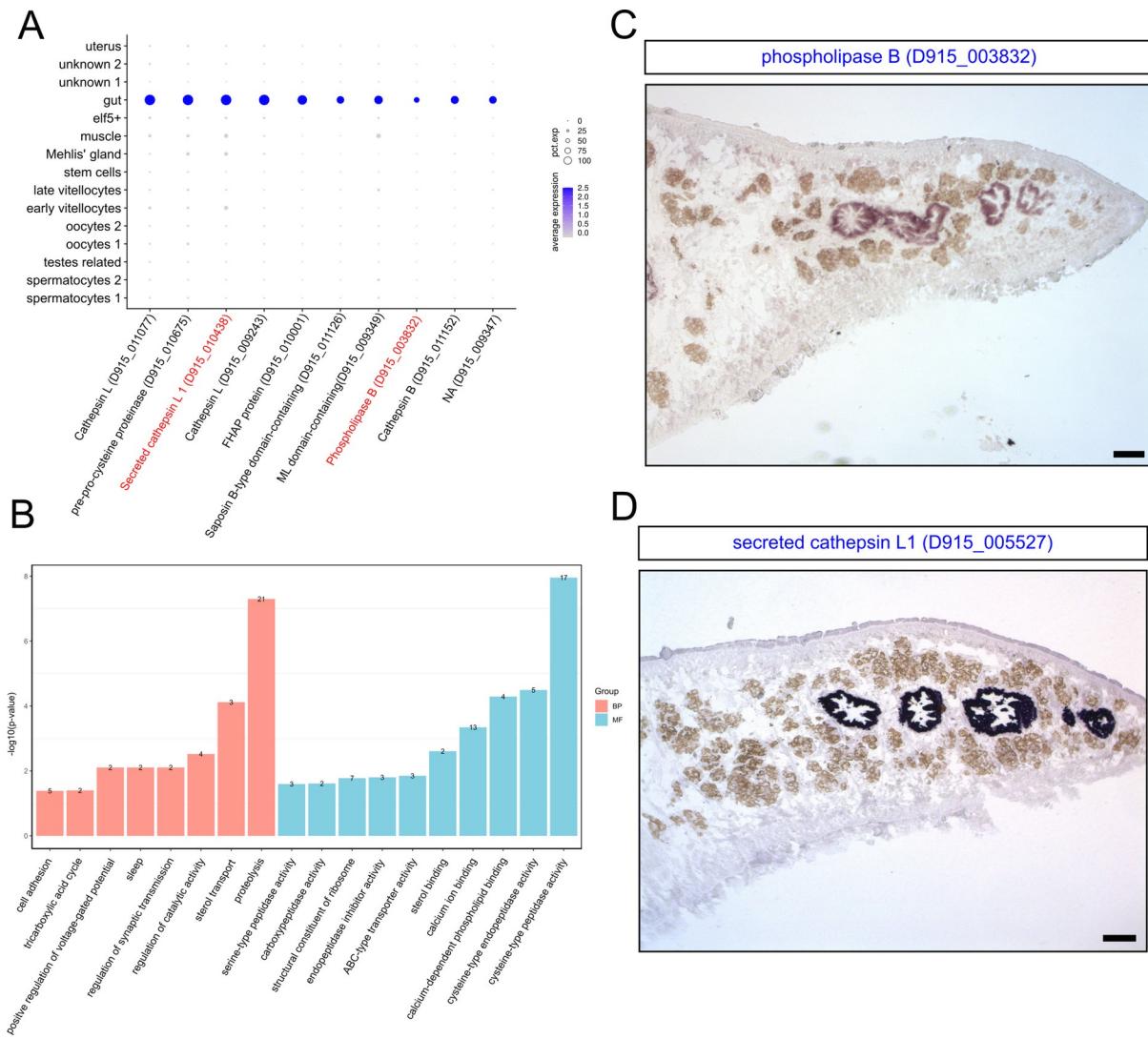
D



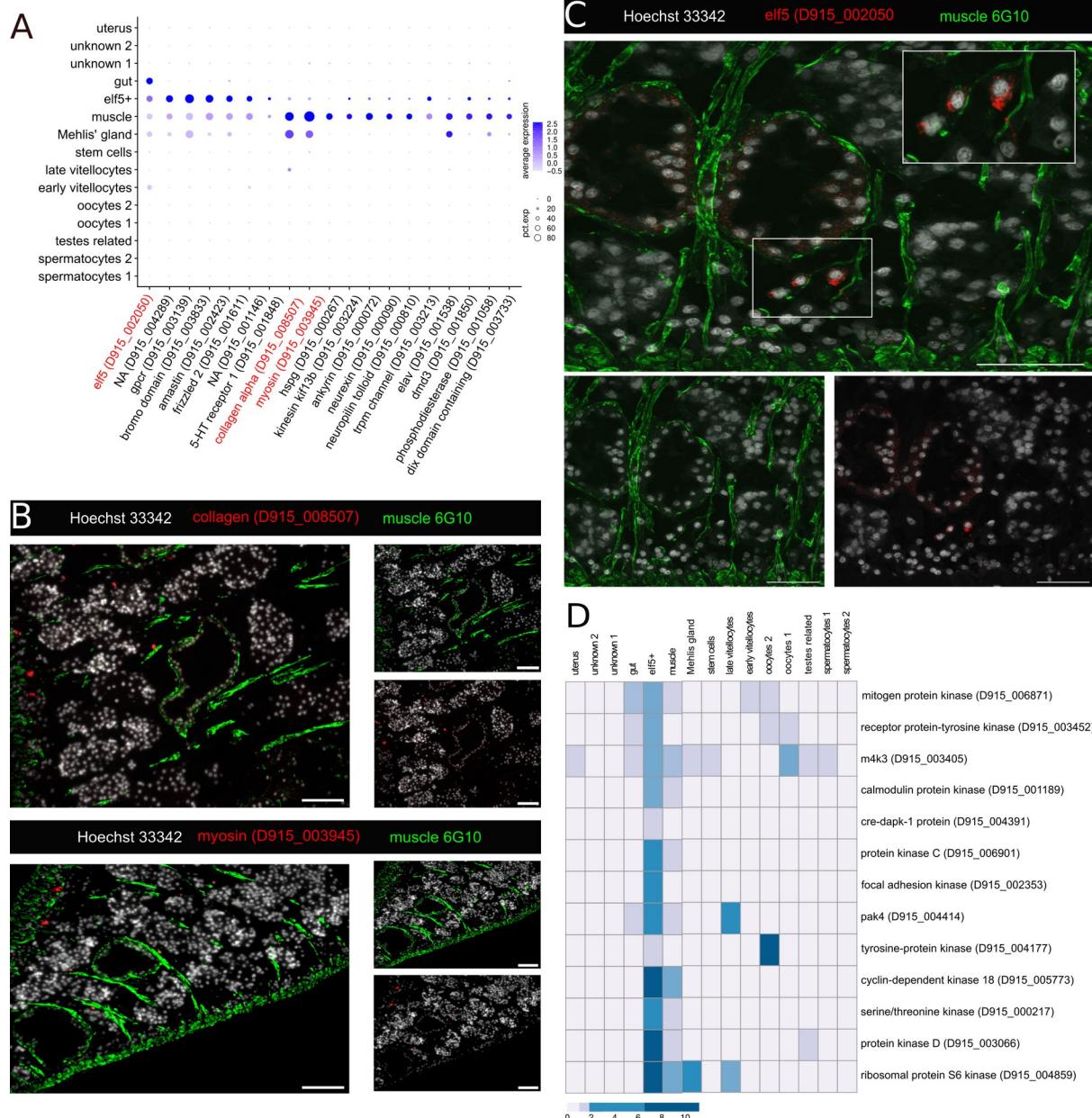
E



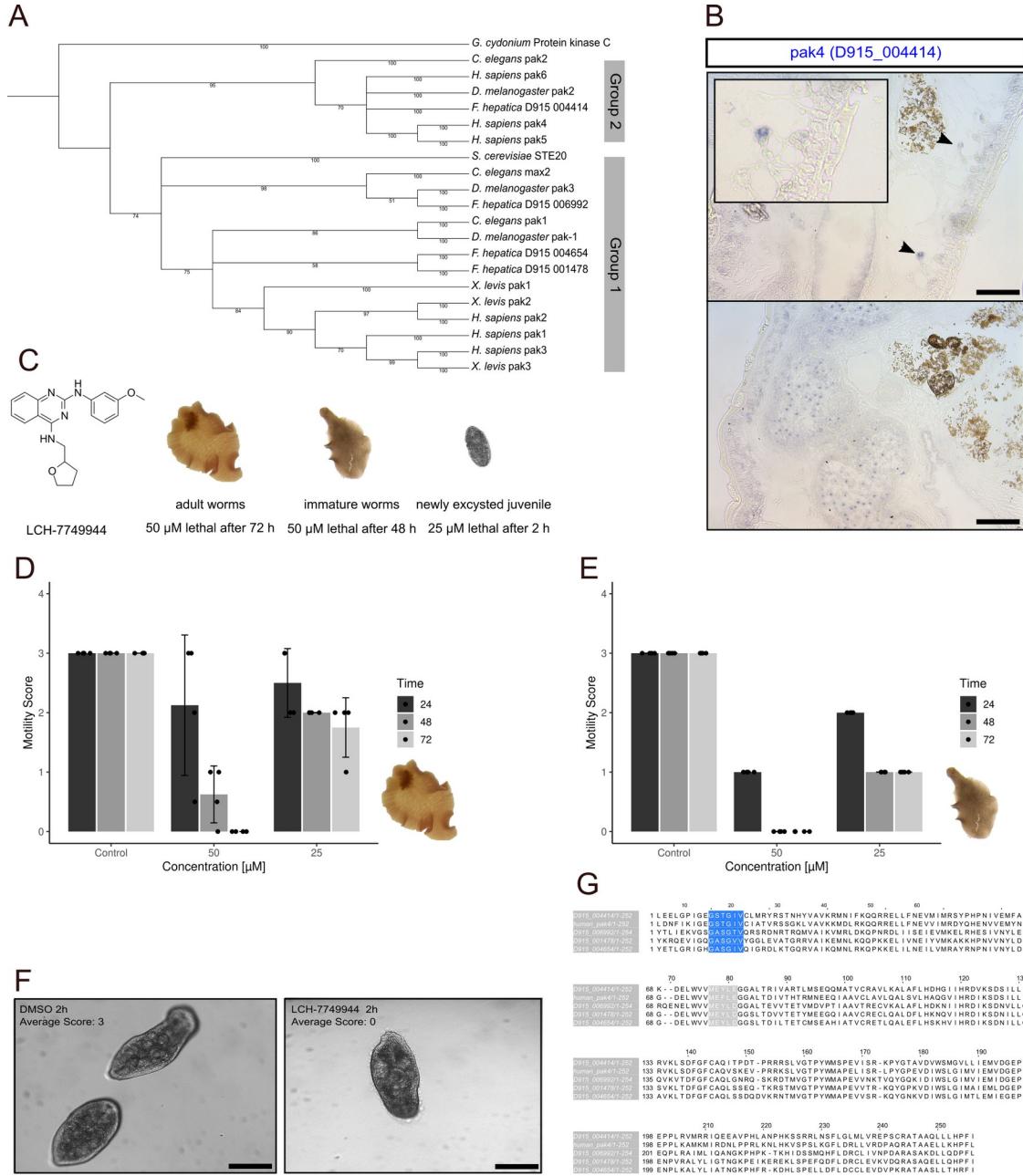
**Fig 4. Conserved marker genes in cells of reproductive tissues including a vitelline cell lineage.** **A** Dotplot showing the expression of germ cell marker genes. ISH-validated genes are marked in red. Level of expression is indicated by color from blue (high expression) to lavender (low expression). The percentage with which cells of a cluster express the given gene is represented by the size of the circles. **B** ISH stainings (top row) for transcripts of selected male and female germline markers *bmpg*, *tektin4* and *meiob* (in blue), and corresponding UMAP plots of gene expression. Scale bar: 100  $\mu$ m. **C** Dotplot showing the expression of vitelline cell marker genes. ISH-validated genes are marked in red. Level of expression is indicated by color from blue (high expression) to lavender (low expression). The percentage with which cells of a cluster express the given gene is represented by the size of the circles. **C** ISH staining for the vitelline cell markers *tyrosinase* and *VB1* transcripts (blue), arrows indicate positive staining. Scale bar: 100  $\mu$ m. **D** UMAP plots showing the expression of conserved vitelline-cell marker genes shared with vitelline-cell markers of *Schistosoma mansoni*<sup>42,43</sup>. An overview of all clusters and close-up of the stem cell, early and late vitellocytes clusters is shown.



**Fig 5. Gut cells of *F. hepatica* express genes involved in lipid metabolism. A** Dotplot showing the expression of gut cell marker genes. ISH-validated genes are marked in red. Level of expression is indicated by color from blue (high expression) to lavender (low expression). The percentage with which cells of a cluster express the given gene is represented by the sizes of the circles. **B** Gene ontology analysis of marker genes (top 75% per cluster) revealed characteristic biological processes (BP) and molecular functions (MF). The number of enriched genes is noted at the end of each bar. **C** ISH staining for *phospholipase B* transcripts and **D** for *cathepsin L1* transcripts in the gastrodermis (blue to dark blue color). Scale bar: 100  $\mu$ m.



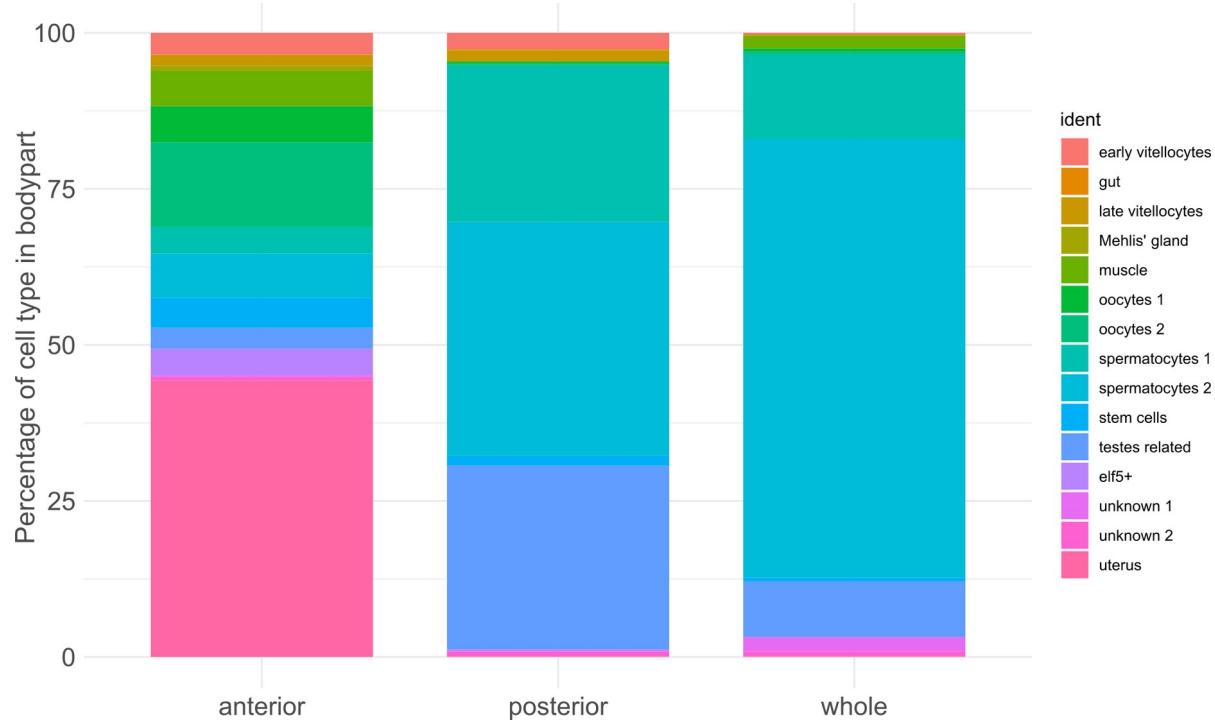
**Fig 6. Specialized muscle cells express several protein kinase genes.** **A** Dotplot showing the expression of marker genes for the muscle and elf5+ muscle cell clusters. ISH-validated genes are marked in red. Level of expression is indicated by color from blue (high expression) to lavender (low expression). The percentage with which cells of a cluster express the given gene is represented by the size of the circles. **B** Detailed view of FISH for collagen (top) and myosin (bottom) combined with immunolocalization of muscle fiber proteins. Scale bar: 100  $\mu$ m. **C** FISH for elf5 combined with immunolocalization of muscle fiber proteins. Scale bar: 100  $\mu$ m **D** Average expression of protein kinase marker genes per cluster displayed as a heatmap. Note the high and enriched expression of several protein kinases in the elf5+ muscle cluster. Expression values were centered and scaled for each row (each gene) individually.



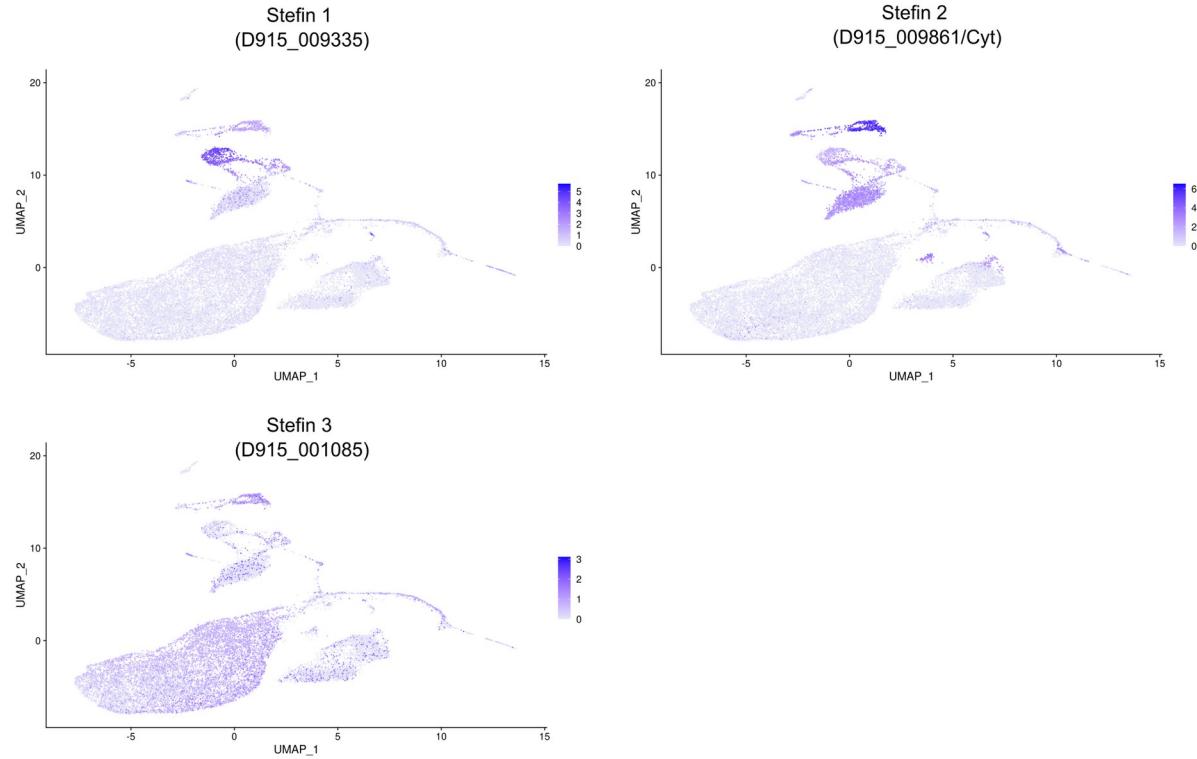
**Fig 7. An inhibitor of the p21-activated PAK4 kinase reduces parasite vitality. A**

Phylogenetic tree of PAK4 orthologs of *F. hepatica* and other species (accession numbers see suppl. table S6). p21 families are indicated, protein kinase C from *Geodia cydonium* served as outgroup. **B** ISH staining for *Fhpak4* transcripts. Scale bar: 100  $\mu$ m. **C** Chemical structure of the PAK4 inhibitor LCH-7749944 with overview of lethal effects on different liver fluke stages. **D, E** Motility scores of adult worms (D) and immature worms (E) under varying LCH-7749944 concentrations over 72 h (4 replicates per condition). Error bar shows standard deviation. **F** NEJs after 2 h treatment with 50  $\mu$ M of LCH-7749944 (right) compared to vehicle-treated NEJs (left). Representative images of 3 worms per condition are shown. Scale bar: 200  $\mu$ m. **G** Alignment of human PAK4 amino acid sequence with *F. hepatica* PAK sequences. Binding sites of LCH-7749944 are colored: P-loop in blue and hinge region in gray.

## Supplemental figure titles and legends

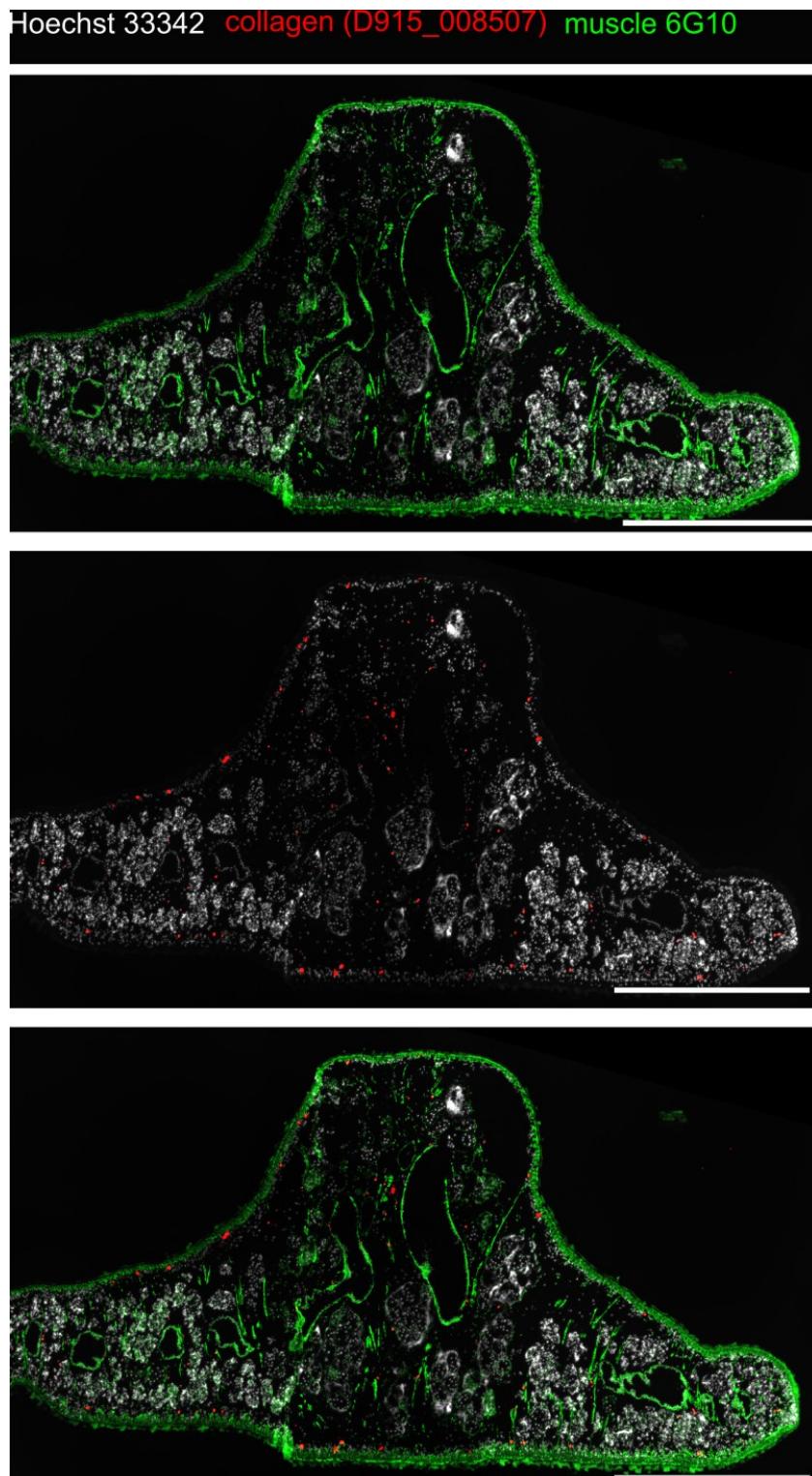


**Fig S1 Cellular composition differs between samples.** For each sample type - either anterior, posterior or whole worm sample - the number of cells within each of the 15 clusters was computed. Shown is the percentage that each cluster covers within the total cell number, per sample type.



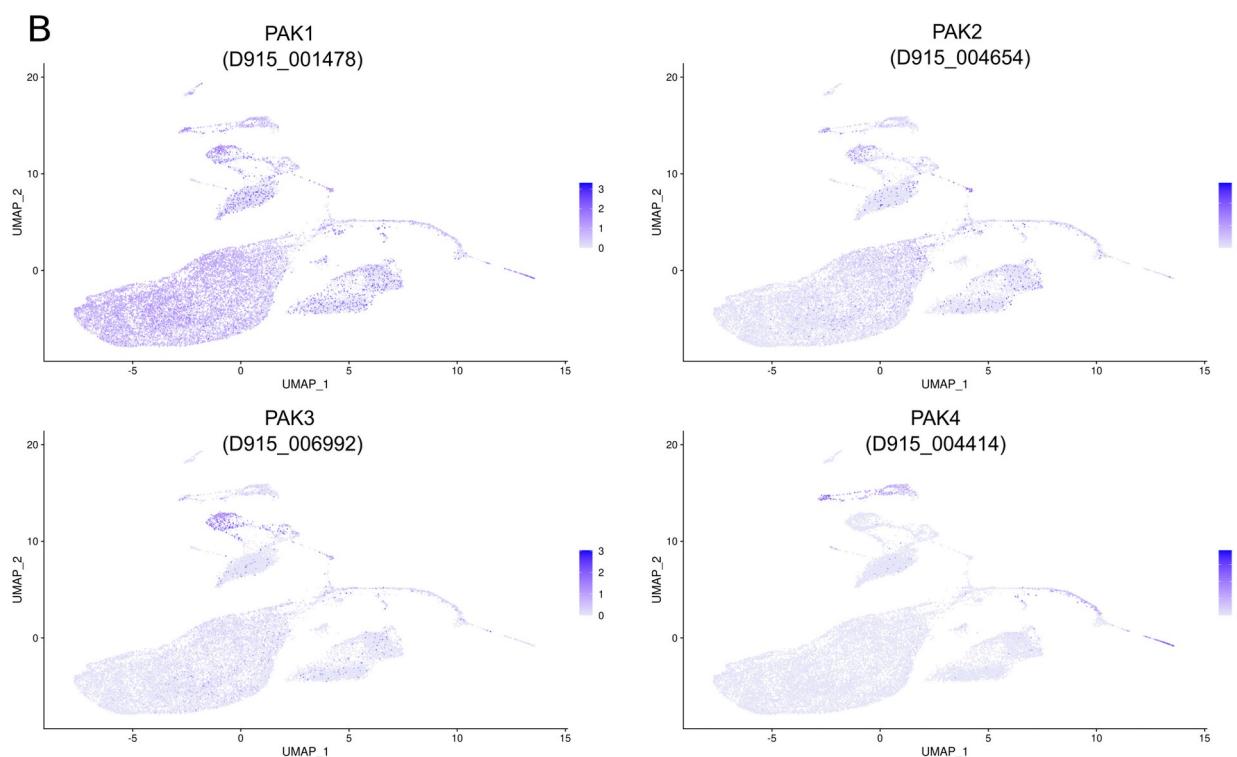
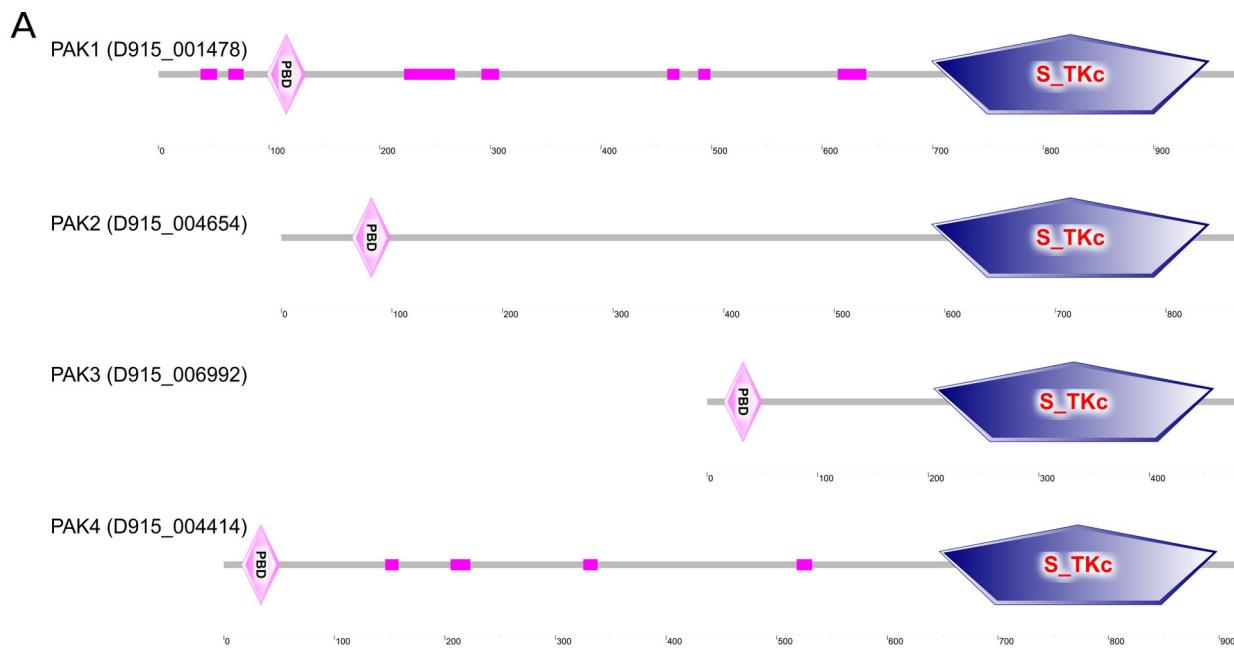
**Fig S2 Different stefins show distinct expression patterns for different cell types.**

UMAP plots colored by gene expression showing the expression of three different stefins.



**Fig S3 Muscle fibers and cells positive for collagen transcripts**

Transversal section co-stained for collagen transcripts by FISH (red) and muscle fiber proteins (green) by immunolocalization. Scale bar: 1000  $\mu\text{m.s}$



**Fig S4 Domain structure and gene expression patterns of the four different PAK kinases.** **A** SMART analysis confirmed the typical domain structure of *F. hepatica* PAK proteins, consisting of a serine/threonine kinase domain (S\_TKc) and a p21-binding domain (PBD). **B** UMAP plots colored by gene expression showing the expression of various PAK genes.

## Supplemental video and Excel table titles and legends

### **Table S1. List of samples used and related QC metrics after filtering**

### **Table S2. List of marker genes per cluster**

The FindAllMarkers() function embedded in Seurat was used to identify markers for each of the clusters by “ROC” test

### **Table S3. List of marker genes per stem cell subcluster**

The FindAllMarkers() function embedded in Seurat was used to identify positive markers for each of the clusters by “wilcox” test

### **Table S4. GO terms enriched in clusters**

### **Table S5. List of cathepsins used for definition of the gut cluster**

### **Table S6 List of sequences used for phylogenetic analysis**

### **Table S7 Primers used for cloning**

