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# Extrinsic apoptosis participates to tail regression during the metamorphosis of the chordate *Ciona*

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1 **Title**

2 Extrinsic apoptosis participates to tail regression during the metamorphosis of the chordate *Ciona*

3

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7

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27 **Abstract**

28 Apoptosis is a regulated cell death ubiquitous in animals defined by morphological features  
29 depending on caspases. Two regulation pathways are described, currently named the intrinsic and  
30 the extrinsic apoptosis. While intrinsic apoptosis is well studied and considered ancestral among  
31 metazoans, extrinsic apoptosis is poorly studied outside mammals. Here, we address extrinsic  
32 apoptosis in the urochordates *Ciona*, belonging to the sister group of vertebrates. During  
33 metamorphosis, *Ciona* larvae undergo a tail regression depending on tissue contraction, migration  
34 and apoptosis. Apoptosis begin at the tail tip and propagates towards the trunk as a polarized wave.  
35 We identified *Ci-caspase 8/10* by phylogenetic analysis as homolog to vertebrate caspases 8 and 10  
36 that are the specific initiator of extrinsic apoptosis. We detected *Ci-caspase 8/10* expression in  
37 *Ciona* larvae, especially at the tail tip. We showed that chemical inhibition of *Ci-caspase 8/10* leads  
38 to a delay of tail regression, and *Ci-caspase 8/10* loss of function induced an incomplete tail  
39 regression. The specificity between apoptotic pathways and initiator caspase suggests that extrinsic  
40 apoptosis regulates cell death during the tail regression. Our study presents rare *in vivo* work on  
41 extrinsic apoptosis outside mammals, and contribute to the discussion on its evolutionary history in  
42 animals.

43  
44 **Key words**

45 Extrinsic apoptosis, metamorphosis, urochordates, caspase.

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## 53 **Introduction**

54 Apoptosis is a regulated cell death allowing the elimination of unwanted cells during various  
55 morphogenetic process such as embryogenesis, metamorphosis or homeostasis<sup>1</sup> and able to  
56 influence its immediate cellular environment<sup>2</sup>. Apoptosis is characterised by a conserved set of  
57 morphological features (loss of adhesion, cells shrinking, chromatin condensation, membrane  
58 blebbing, fragmentation in apoptotic bodies) depending, at the molecular level, on the caspases  
59 multigene family<sup>3-5</sup>. Based on these morphological and molecular features, apoptosis has been  
60 detected in most metazoan phyla<sup>6-10</sup>. Currently, two main apoptotic regulation pathways are  
61 described, acknowledged as the intrinsic (formerly known as mitochondrial pathway) and the  
62 extrinsic apoptosis (previously named death receptors pathway) by the most recent Nomenclature  
63 Committee on Cell Death (NCCD-2018)<sup>11</sup>. Each of these apoptotic signalling pathways presents the  
64 particularity to depend on specific initiator caspases having long pro-domains CARD (Caspase  
65 Recruitment Domain) or DED (Death Effector Domain) for the intrinsic and extrinsic apoptotic,  
66 respectively. Initiator caspases operate upstream of the regulation and next activated the common  
67 downstream executioner caspases.

68 The characterisation of intrinsic apoptosis in nematode, fly and vertebrates makes it the best  
69 known among animals<sup>3,12,13</sup>. Even if recent research suggests divergences between intrinsic  
70 apoptosis<sup>14</sup>, this pathway share the specificity to be initiated by a caspase with a pro-domain CARD  
71 activated by an APAF-1 protein and is considered ancestral and conserved among<sup>11,15</sup>.

72 In mammals, extrinsic apoptosis is initiated by perturbations of the extracellular  
73 microenvironment that trigger the binding of specific ligands like CD95 to the TNFr (Tumor  
74 Necrosis Factor receptors)<sup>11,16</sup>. The activated TNFr recruits FADD (FAS-Associated Death Domain)  
75 or TRADD (Tumor necrosis factor Receptor type 1-Associated Death Domain) which bind to the  
76 pro-caspases 8 or 10, respectively. The resulting proteolytic platform called DISC (Death-Inducing  
77 Signalling Complex) allows next the fully activation of the initiator caspases.

78 In opposite to intrinsic apoptosis, extrinsic apoptosis is not present neither in fly nor in

79 nematode and most knowledge about extrinsic apoptosis come from mammals. Consequently,  
80 despite the in-depth understanding of extrinsic apoptosis in mammals, this pathway remain largely  
81 unexplored at a large evolutionary scale, excepted few punctual works made on cephalochordates,  
82 echinoderms or cnidarians<sup>17,18</sup>. Even if a previous study on *Amphioxus* suggest presence of extrinsic  
83 apoptosis in chordates, current knowledge is insufficient to understand the operation and evolution  
84 of extrinsic apoptosis among metazoans. However, in the last decades, genomic and transcriptomic  
85 analysis identified numerous of proteins potentially involved in apoptosis regulation, including  
86 DED-caspases, TNFr, TRADD or FADD like proteins suggesting the presence of extrinsic  
87 apoptosis in several animal groups, including ascidians<sup>17,19-21</sup>.

88         Ascidians, as the sister group of vertebrates, offers an ideal opportunity to analyse the  
89 evolution of molecular pathways in chordates, including vertebrates. The ascidian *Ciona* presents a  
90 bi-phasic life cycle; sessile adults release gametes in environment where fertilization occurs,  
91 followed by a rapid embryogenesis that give rise to a swimming tadpole larva. After a short  
92 swimming period, the larvae settle to a substrate and undergo a metamorphosis. The most dramatic  
93 event of this phenomenon is the tail regression, depending on multiples factors and molecular  
94 pathways including, among others, actomyosin dependant contraction of epidermal cells, GABA,  
95 ERK, or D-serine<sup>22-27</sup>. Importantly, tail regression is characterised by a polarised apoptotic wave  
96 starting at the tip of the tail and propagates toward the trunk<sup>7</sup>. However, the apoptotic pathway  
97 triggering cell death has not been investigated yet while a previous study identified several potential  
98 apoptotic actors in *Ciona* based on sequence similarity with mammals, including a caspase with  
99 DED domain<sup>28</sup>.

100         In this study, we took advantage of the easily predictable apoptotic profile during larvae  
101 metamorphosis to explore the initiation of apoptosis by focusing on the unique DED-caspase found  
102 in the *Ciona* genome, *Ci-caspase 8/10* (gene model KH.C8.550)<sup>28,29</sup>. We first clarified the  
103 evolutionary relationship between *Ciona* and vertebrates' caspases using phylogenetic analysis. We  
104 characterised expression of *Ci-caspase 8/10* in larvae, and discovered that apoptotic wave

105 propagation and tail regression is delayed in larvae exposed to a caspase 8 inhibitor. In addition,  
106 loss of function of *Ci-caspase 8/10* using Crispr/CAS9 technology significantly increased the  
107 number of larvae presenting an incomplete tail regression phenotype. Globally, we demonstrated  
108 here that Ci-caspase 8/10 participates to apoptosis regulation during *Ciona* tail regression, arguing  
109 in favour of extrinsic apoptosis implication in the process. In addition, comparative effects of  
110 various specific caspase inhibitors indicated that among initiator caspases of *Ciona*, Ci-caspase 8/10  
111 has a central role in apoptosis regulation. Ultimately, our data suggest a conservation of the  
112 extrinsic apoptosis between ascidians and vertebrates, and also at the chordates scale according to  
113 previous work conducted on *Amphioxus*.

114

## 115 **Materiel and methods**

### 116 **Animals**

117 Wild type *Ciona intestinalis* Type A from Onagawa-bay (Miyagi, Japan) and Onahama-bay  
118 (Fukushima, Japan) were cultivated by the National BioResource Project (Japan) and then  
119 maintained in seawater at the Shimoda Marine Reasearch Station (Tsukuba University Shimoda,  
120 Japan). Wild type *Ciona intestinalis* Type B collected on the field by the Biological Sample  
121 Collection Service of the Biological Station of Roscoff (Sorbonne University, Roscoff, France)  
122 were maintained in artificial seawater at the Aquatic Model Animal Facility and Engineering of the  
123 Institute of Biology Paris-Seine (Sorbonne University, Paris, France). In laboratory, *Ciona* were  
124 maintained at 18 °C in 35‰ salinity artificial seawater in a constant light condition to prevent  
125 spawning. Eggs and sperm were surgically collected from gonadal ducts, followed by cross-  
126 fertilisations in plastic Petri dishes.

127 Caspases inhibitors treatments and corresponding TUNEL labelling, *in situ* hybridization of  
128 Ci-caspase-8/10 like and TNF receptors, and real-time PCR were conducted on *Ciona* type B. Loss  
129 of function experiment and corresponding TUNEL labelling were made on *Ciona* type A. Genome  
130 used for phylogenetic analysis and primers design is the genome of *Ciona* type A<sup>29</sup>.

131

## 132 **Pharmacological treatments**

133 Swimming larvae were distributed in plastic Petri dishes post-hatching. When about 70% of  
134 the larvae had settled (eye assessment), the supernatant was replaced by filtered ASW containing  
135 specific chemicals or DMSO as control. Chemicals were stored in DMSO at -20 °C and used at 10  
136 µM of final concentration. Chemicals used were the pan-caspases inhibitor Z-VAD-fmk (Sigma-  
137 Aldrich; Merck KGaA), the caspase-2 inhibitor Z-VDVAD-fmk (Sigma-Aldrich; Merck KGaA),  
138 the caspase-8 inhibitor Z-IETD-fmk (Sigma-Aldrich; Merck KGaA), the caspase-9 inhibitor Z-  
139 LEHD-fmk (Sigma-Aldrich; Merck KGaA), and the caspase-10 inhibitor Z-AEVD-fmk (Sigma-  
140 Aldrich; Merck KGaA).

141

## 142 **TUNEL labelling**

143 TUNEL labelling were done using the *In Situ* Cell Death Detection Kit (Roche) as  
144 previously described<sup>30</sup>, or with the Click-iT® TUNEL Alexa Fluor® Imaging Assay (Invitrogen)  
145 according to manufacturer instructions.

146

## 147 **Real-time PCR**

148 RNA was extracted using the RNAqueous™-Micro Total RNA Isolation Kit (ThermoFisher  
149 Scientific), followed by a DNase treatment made with the TURBO DNA-*free*™ Kit (ThermoFisher  
150 Scientific) and finally purified a second time using the RNeasy MinElute Cleanup Kit (Qiagen). All  
151 steps were done according to the manufacturer's protocol.

152 Real-time PCR was performed using the SYBR Green Supermix (Biorad) with a Biorad's  
153 thermal cycler using the following profile: 95°C for 10 min; 40 cycles of amplification with  
154 successively 95°C for 15s, 60°C for 10s, and 72°C for 20s; one cycle for melting curve analysis with  
155 an acquisition every 0.5°C from 65°C to 95°C to verify the presence of a single product. Each assay  
156 included a no-template control for each primer pair, and five successive dilutions to determine the

157 Ct values and the reaction efficiencies. Real-time PCR reactions were done in triplicate. Gene  
158 expression level was normalized using *Ci-actin* as reference gene as previously described<sup>31-33</sup>.  
159 Primer pair used for *Ci-casp8/10* are: forward 5'-AAGACTGCTTTGTGTGCGTG-3', reverse 5'-  
160 GGCAGGCTTGGAAGAAAATAT-3'. PCR products length were between 140 and 160bp.

161

### 162 **Whole *in situ* hybridization (WISH)**

163 We performed WISH as previously described<sup>30</sup>. Concerning settled larvae used for *Ci-*  
164 *caspase 8/10* WISH, we manually removed the tail tunic with forceps as we already done<sup>30</sup>,  
165 allowing the complete elimination of the well-known strong background generated by this tissue.  
166 TNF receptors WISH were conducted on dechorionated larvae.

167

### 168 **Statistic and phylogenetic analysis**

169 Statistical differences were evaluated by Wilcoxon Mann Whitney test using the software R  
170 2.14.1. Effects were considered significant with a p-value < 0.05 and statistical significance is  
171 notated in figures by an \*.

172 Multiple alignments of protein sequences were generated using the MAFFT 7<sup>34</sup> and Gblocks  
173 0.91b<sup>35</sup> was used to removed vacancies and blur sites. Phylogenetic analyses were carried out by  
174 Maximum-Likelihood (ML) method under LG model using PhyML 3.1<sup>36</sup>, combined ML tree search  
175 with 1000 bootstrap replicates. Bayesian analyses were performed using MrBayes 3.2.6<sup>37</sup> under  
176 mixed model. One fourth of the topologies were discarded as burn-in values, while the remaining  
177 ones were used to calculate the posterior probability. The run was carried out for 500 000  
178 generations with 5 randomly started simultaneous Markov chains (1 cold chain, 14 heated chains)  
179 and sampled every 100 generations.

180

### 181 **Microscopy and images acquisition**



182 Images of figure 2 and 3 were taken with a BX61 (Olympus) and images of figure 4 an  
183 AxioImager Z1 (Carl Zeiss MicroImaging) microscopes. Images from Supplementary figure 2 were  
184 taken with a DM5000B (Leica) microscope. Images from Supplementary figure 3 were taken with a  
185 Leica SP5 confocal microscope. Images were analysed with ImageJ 1.46a-1 and AxioVision Rel.4.6  
186 softwares. Samples were mounted in Mowiol 4–88 (Calbiochem) or CitiFluor AF1 (Science  
187 Services).

188

### 189 **Microinjection and loss of functions**

190 Eggs were dechorionated and injected with a mixture composed of 250 ng/μL of crRNA, 1  
191 ng/μL of tracrRNA, 10 μg/μL of Cas9 IDT, and blue dye<sup>38</sup>. Larvae used for experiment are the T0  
192 developed from eggs successfully injected with the mix (sorted thank to the presence of the dye).  
193 DNA were extracted from about 50 larvae labelled by the dye next day after injection. The crRNA  
194 number 1 was 5'-TTTTTCTTCCAAGCCTGCCA-3' and genomic region of Ci-casp.8/10 targeted by  
195 the gRNA was amplified by PCR (forward 5'-ctgcaggaattcgatGTGTATGGTGTGATGGCTTGT-3';  
196 reverse 5'-atcgataagcttgatAACATCATCAACTGGAGCGGAA-3') using the ExTaq (Takara-bio). The  
197 crRNA number 2 was 5'- TCGCTTTCAACATCATCAAC -3' and genomic region of Ci-casp.8/10  
198 targeted by the gRNA was amplified by PCR (forward 5'-  
199 ctgcaggaattcgatTTCAGACCAGGCATGTGC-3'; reverse 5'-  
200 atcgataagcttgatAAGAAGTCAGACTCACTT-3'). PCR products were run on SDS poly-acrylamide  
201 gel electrophoresis to verify size heterogeneity induced by mutations. Then, PCR products were  
202 subcloned into the *EcoRV* site of pBluescript SKII (+) and sequenced by the conventional Sanger  
203 method to evaluate the mutation rate.

204

### 205 *In silico* docking experiment

206 Caspases amino acids sequences were converted to .pdb format using the Expasy webserver  
207 <https://swissmodel.expasy.org/interactive>. SMILES formats of inhibitor chemicals were obtained on

208 PubChem database and converted to .pdb format with the NovoPro Labs Smiles2PDB webserver  
209 <https://www.novoprolabs.com/tools/smiles2pdb>. *In silico* analysis were run with iGemdock<sup>39</sup> under  
210 Drug screening settings for 70 generations and population size of 200.

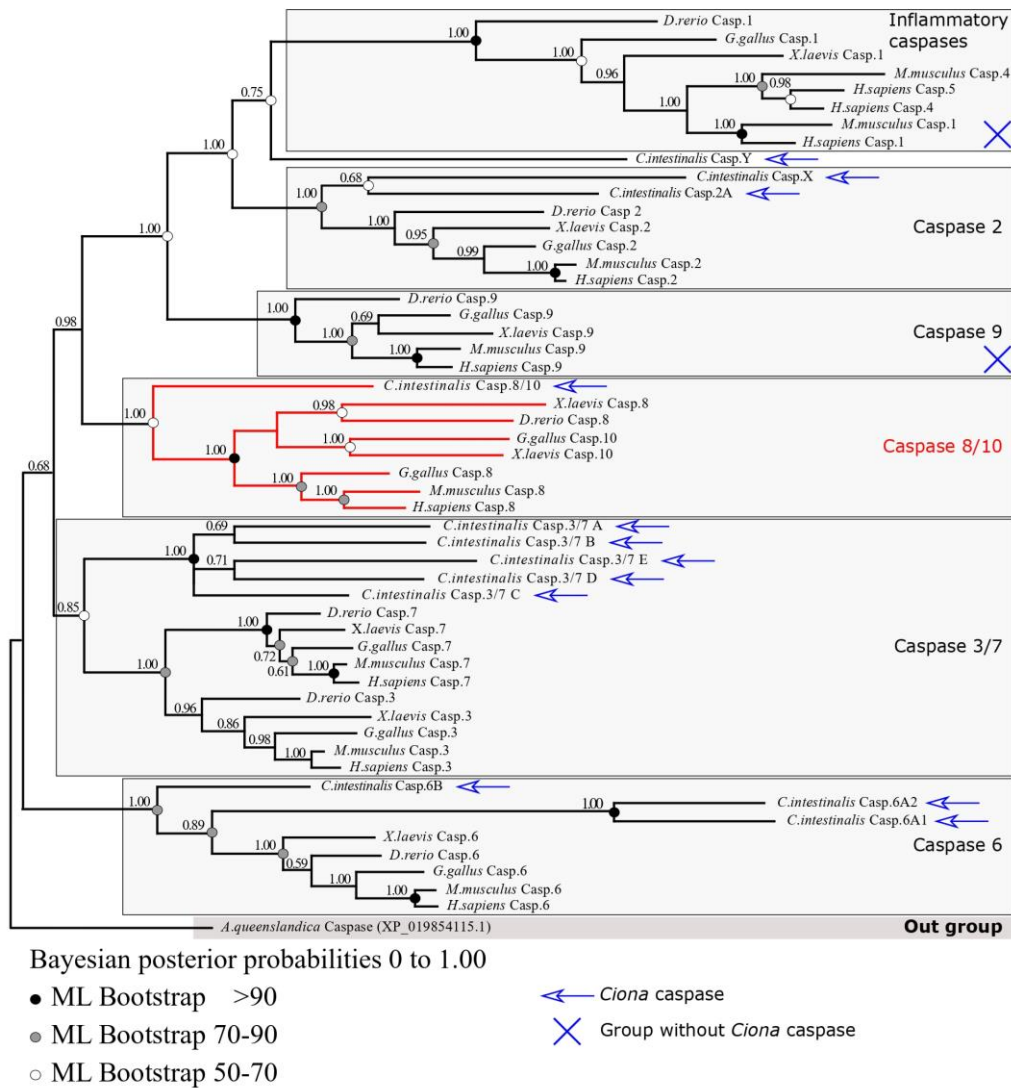
211

## 212 **Results**

### 213 ***Ciona* caspases repertory is similar to vertebrates**

214 We named *Ciona* caspases according to their vertebrate's relatives (Figure 1, Supplementary  
215 table 1, Supplementary figure 1) thanks to phylogenetic analysis. Our topology is similar between  
216 both Bayesian inference and maximum likelihood methods, and sequence relationships is consistent  
217 with the species one (Figure 1). We identified six distinct clades corresponding to the groups  
218 described in vertebrates: inflammatory caspases, caspase 8 and 10 (paralogues), caspase 2, caspase  
219 9, caspases 6 and caspases 3 and 7. All of the CARD-caspases formed a monophyletic group  
220 (posterior probability 1.00). Consistently with previous study, cCaspase 8 and 10 of vertebrates are  
221 paralogues and monophyletic (posterior probability 1.00) and form a clade with the orthologue  
222 relative Ci-caspase 8/10, the only *Ciona* caspase presenting the typical two DED pro-domains,  
223 characteristic of extrinsic apoptosis initiator caspases in mammals. Both the inflammatory caspases  
224 (posterior probability 1.00) and the caspases 9 group (posterior probability 1.00) contain vertebrate  
225 sequences only, suggesting the loss of *Ciona* caspase 9 as previously reported<sup>14,28</sup>. The caspase 2  
226 group (posterior probability 1.00) is composed of the vertebrate sequences and *Ciona* Ci-caspase 2  
227 and Ci-caspase X, this later do not have a CARD pro-domain. This suggested a caspase 2  
228 duplication in *Ciona* as already reported in tunicates<sup>14</sup>, but one of the two paralogs having a high  
229 divergent sequence making the CARD pro-domain unidentifiable (reason why by caution we named  
230 it Ci-caspase X). Executioner caspases are separated between caspases 6 (posterior probability 1.00)  
231 and caspases 3+7 (posterior probability 0.85). *Ciona* presents three probable paralogs of caspases 6  
232 (Ci-caspase 6A1, A2, and B) with a high divergence illustrated by long branch possibly explaining  
233 the decrease in nodes robustness. Caspase 3 and 7 of vertebrates are paralogues and formed a

234 monophyletic group. Five paralogues of *Ciona* are orthologous to both caspase 3 and 7. We named  
 235 them Ci-caspase 3/7 A to E. Last, Ci-caspase Y branch with the inflammatory caspases, but shows a  
 236 long branch indicating high divergence making its identification unclear hence why we chose this  
 237 denomination.  
 238



239  
 240 **Figure 1: Phylogenetic analysis of caspases of *Ciona* and vertebrates obtained by Bayesian**  
 241 **inference.** Same topology made by maximum likelihood was obtained. DED-caspases are grouped  
 242 together (red). CARD-caspases (caspases 2, 9 and inflammatory) formed a monophyletic clade.  
 243 Executioner caspases are separated between caspase 6 clade and caspase 3 plus 7 clade. Sequences  
 244 come from *Ciona* Type A genome.

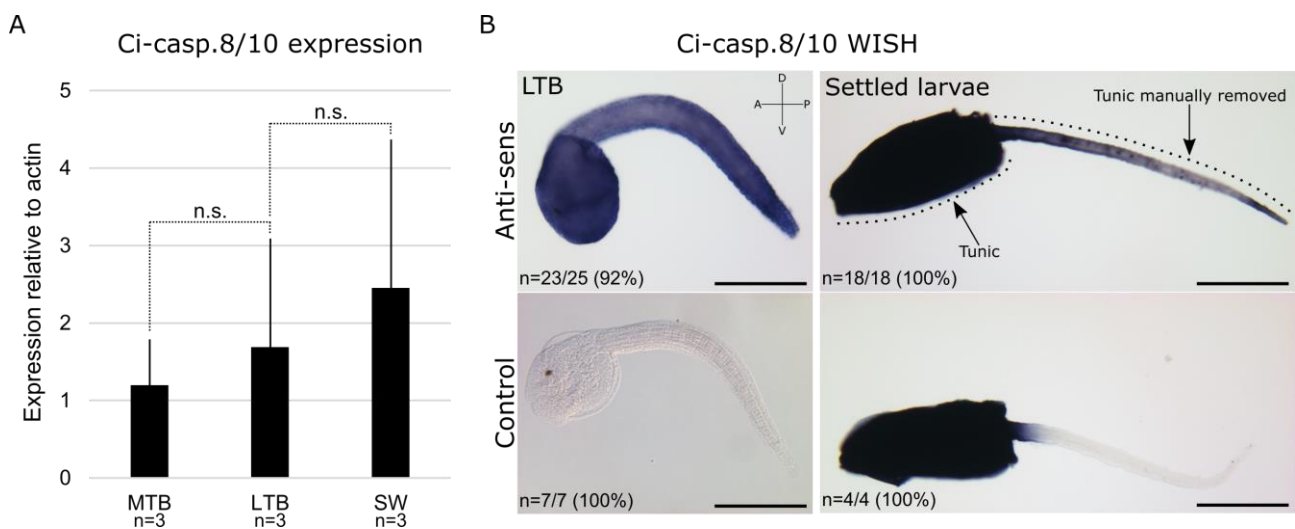
245

246 **Ci-casp-8/10 is expressed in the larvae**

247 Relative expression level of Ci-caspase 8/10 was determined by real-time PCR during  
248 embryogenesis at the mid-tail bud (MTB) and late-tail bud (LTB) stages as well as in swimming  
249 larvae (Figure 2A). Expression level increased overtime although differences were not statistically  
250 significant. We confirmed expression of Ci-caspase 8/10 by whole *in situ* hybridisation (WISH) on  
251 LTB and settled larvae (Figure 2B). Ci-caspase 8/10 expression is ubiquitous in LTB larvae with a  
252 stronger labelling at the posterior half of the tail. We manually removed the larval tunic from the tail  
253 of the settled larvae as we did previously to prevent the background generated by this tissue<sup>30</sup>.  
254 Settled larvae presented an expression pattern similar to the LTB, seems even reinforced at the tip of  
255 the tail, whereas remaining tunic makes impossible any expression pattern observation in the trunk  
256 due to the high background. Usually, extrinsic apoptosis also depends on TNF receptors and FADD  
257 which we identified in the *Ciona* genome (Supplementary Table 2). Interestingly, the three TNF  
258 receptors are expressed in the tail at the LTB stage, suggesting a possible role during tail regression  
259 (Supplementary Figure 2).

260

261



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263

264 **Figure 2: Ci-caspase 8/10 is expressed in the larvae.** **A**, real-time PCR showing expression of Ci-  
265 Casp8/10 relative to Ci-*actin* on embryonic larvae at mid tail-bud (MTB), late tail-bud (LTB), and  
266 hatching swimming larvae (SW). Error bars correspond to standard deviation. Gene expression is  
267 not statistically significant (n.s.; Wilcoxon Mann Whitney test). **B**, whole *in situ* hybridisation on  
268 dechorionated LTB larvae and settled larvae. Tunic from settled larvae has been manually delated  
269 from the tail to remove the so-known background induced by it. Strong dark blue colour of the  
270 trunk results of presence of the tunic. Scale bars: **B** = 200  $\mu$ m. Experiment were conducted on  
271 *Ciona* Type B. Orientation: A, anterior; P, posterior; D, dorsal; V, ventral.

272

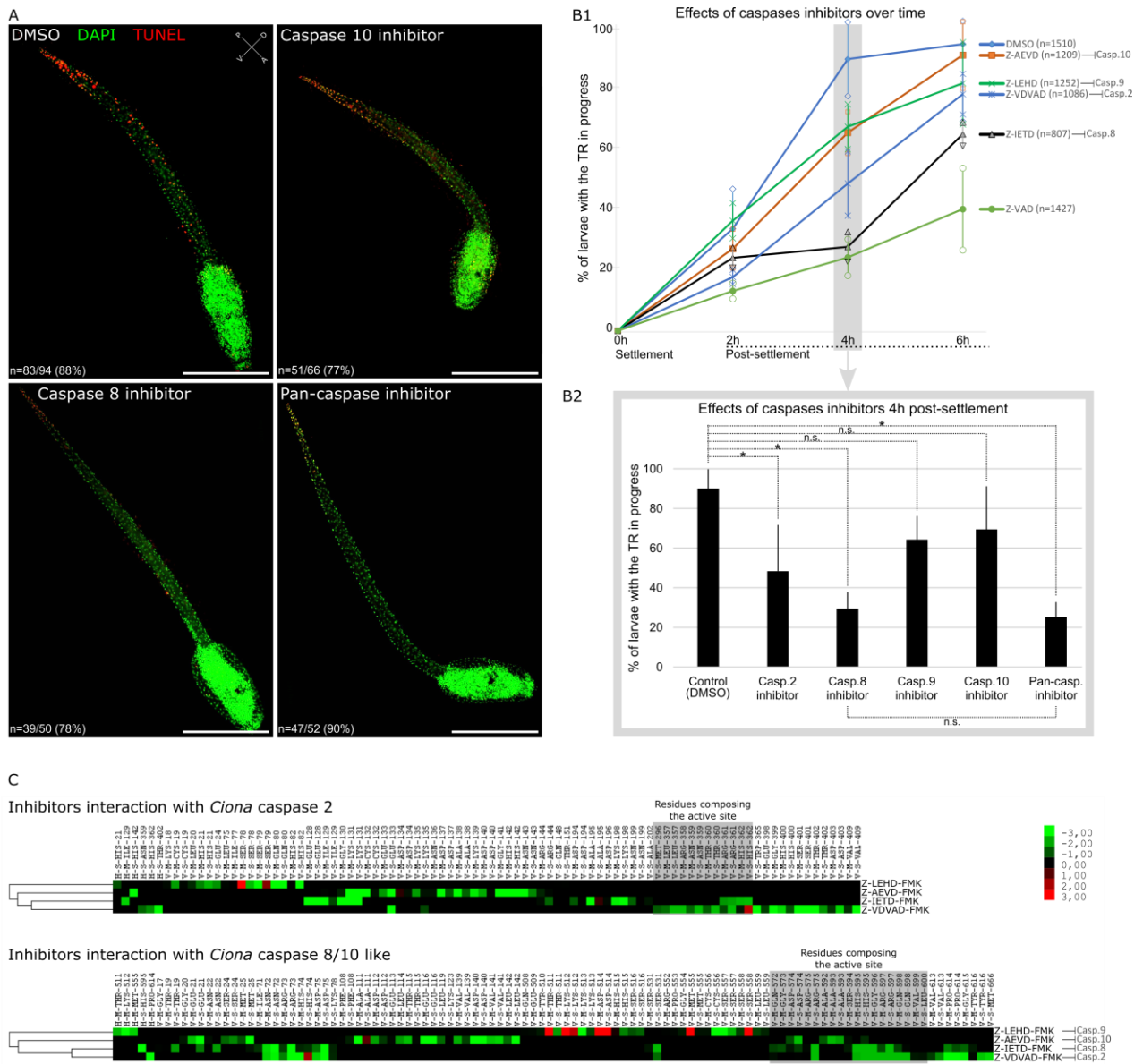
### 273 **Caspase 8 inhibition delayed the tail regression**

274 We exposed settled larvae to the caspase 8 inhibitor (Z-IETD-Fmk), the caspase 10 inhibitor  
275 (Z-AEVD-Fmk), and the pan-caspases inhibitor (Z-VAD-Fmk; as positive control) and performed  
276 TUNEL labelling when the tail regression started in the control (DMSO). Both control and caspase  
277 10 inhibitor treated larvae presented a similarly high number of TUNEL positive nuclei at the tip of  
278 the tail, while pan-caspase and caspase 8 inhibitors treated larvae presented only few apoptotic cells  
279 (Figure 3A). In order to evaluate potential differences in the apoptotic profile between control  
280 (DMSO) and caspase-8 inhibitor treated larvae, we observed shape and position of TUNEL positive  
281 nuclei we detected (Supplementary figure 3). Consistently with several previous studies<sup>7,26</sup>, we  
282 deduced that tunic, epidermal, and muscle cells undergo apoptosis in the tail. In the case of caspase  
283 8 inhibitor treatment, the same tissues were affected, indicated that the inhibitor do not modified the  
284 type of tissue undergoing apoptosis, but decrease the number of apoptotic cells. Next, we counted  
285 the number of larvae with the tail regression in progress every two hours from its beginning in the  
286 control (Figure 3B1). The caspase 10 inhibitor had no effects on the tail regression, whereas the  
287 caspase 8 inhibitor significantly reduces the number of larvae with the tail regression in progress,  
288 especially at the 4 hours' time point (Figure 3B2). Importantly, the caspase 8 inhibitor showed a  
289 similar result as the pan-caspase inhibitor already successfully used in *Ciona*, highlighting the

290 strong implication of Ci-caspase 8/10 in this process<sup>7,30</sup>. We also treated larvae with caspase 2  
291 inhibitor (Z-VDVAD-Fmk) which induce a moderate slowdown of tail regression, but still  
292 significant. This suggest that another pathway, based on a CARD-caspases, could be involved in  
293 apoptosis during tail regression. Conversely, caspase 9 inhibitor (Z-LEHD-Fmk) do not have  
294 significant effect, which can likely be explained by the absence of caspase 9 in *Ciona* genome.

295 Finally, as chemical specificity can be variable or uncertain, we tested in silico docking  
296 ability of the four specific inhibitor we used with the two *Ciona* initiator caspases, Ci-caspase 2 and  
297 Ci-caspase 8/10 like (Figure 3C). Inhibitors acts by binding the active site of caspases, creating a  
298 physical blocking preventing cleavage capacity of the protease. Our modelling indicate that caspase  
299 8 inhibitor present more binding residues affinities than caspase 10 inhibitors on active site of Ci-  
300 caspase 8/10 like, which could explain absence of caspase 10 inhibitor effect. This may be due to  
301 the fact that Ci-caspase 8/10 active site sequence is closest to the vertebrate caspase 8 than the  
302 caspase 10 (Supplementary figure 4). Ci-caspase 2 could be successfully inhibited by caspase 2  
303 inhibitor, and seems not sensitive to caspase 8 and caspase 10 inhibitor. However, Ci-caspase 8/10  
304 like could be theoretically inhibited by caspase 2 inhibitor, maybe explaining likely the effect of this  
305 drugs on tail regression. We observed that effects of treatments were transitory given that treated  
306 larvae completed the tail regression during the night post-treatment, probably due to degradation of  
307 chemicals, functional redundancy between caspases, or because the extrinsic apoptosis is likely not  
308 the only pathway implicated to trigger apoptosis during tail regression.

309



310

311 **Figure 3: Effects of caspase inhibitors on the tail regression. A**, TUNEL labelling (red)  
 312 counterstaining with DAPI (green) of larvae at the onset of tail regression. Apoptosis starts at the tip  
 313 of the tail in control (DMSO) as in presence of the caspase 10 inhibitor. Only few apoptotic cells are  
 314 detected in caspase 8 inhibitor and Z-VAD-Fmk treated larvae. **B1**, Larvae' proportion with the tail  
 315 regression in progress overtime after settlement. **B2**, Statistical differences of larvae' proportion  
 316 with the tail regression in progress at 4h post-settlement from figure B1. Scale bars: 200  $\mu$ m. **C**, *in*  
 317 *silico* analysis of theoretical docking ability between specific caspase inhibitors and *Ciona* initiator  
 318 caspases. In the profile, coloured raw indicate the preferences of the interacting residue for  
 319 chemicals, with maximum red or maximum green indicating high docking probability through-

320 black indicating low/absence of interactions. The following rows show the interactions types  
321 between the active compounds and the residues, noted H (hydrogen-bonding), E (electrostatic  
322 interaction), and V (Van der Waals interaction). Next, M and S display the main chain and the side  
323 chain of the interacting residue, respectively. Last, residue names and positions are indicating.  
324 Experiments were conducted on *Ciona* Type B. Error bars (B1, B2) correspond to standard  
325 deviation. \* Wilcoxon Mann Whitney test p-value < 0.05. Orientation: A, anterior; P, posterior; D,  
326 dorsal; V, ventral.

327

### 328 **Loss of function of Ci-caspase 8/10 generates an incomplete tail regression**

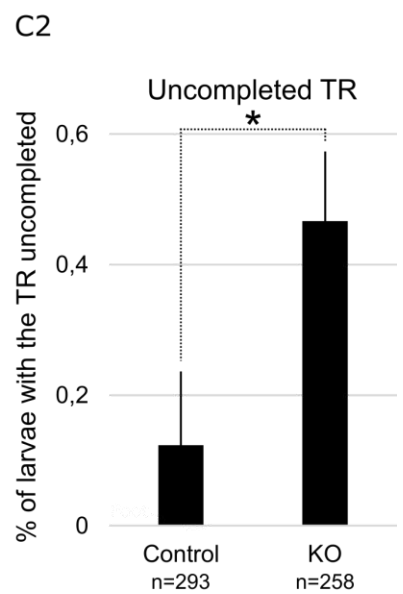
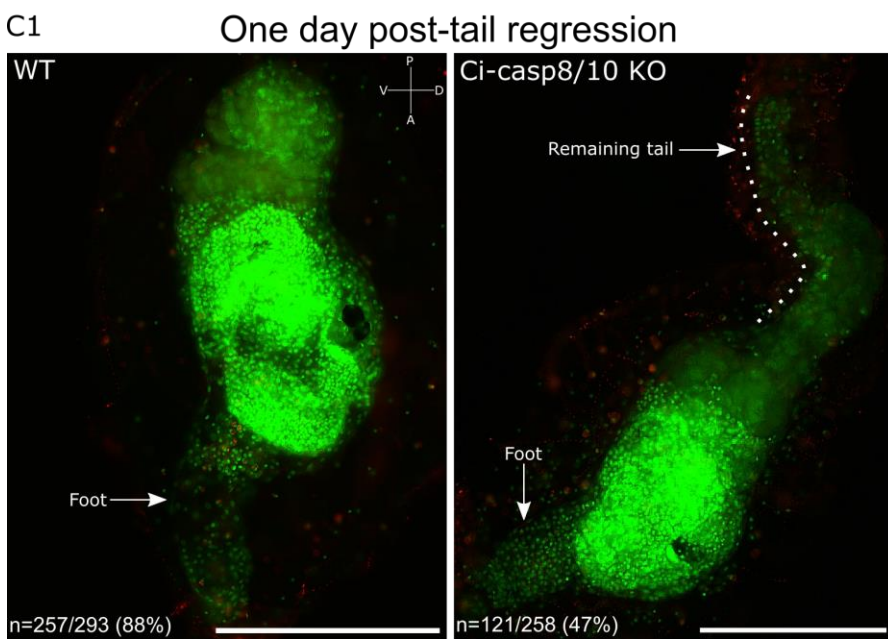
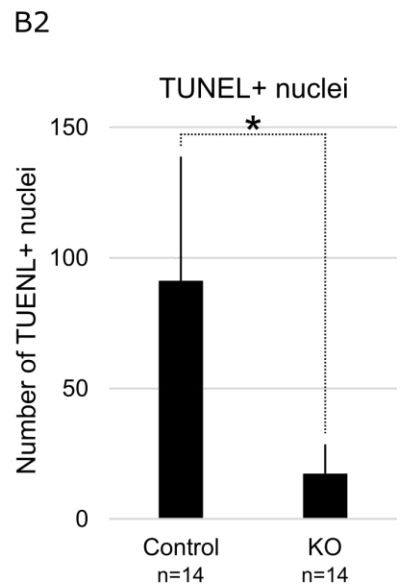
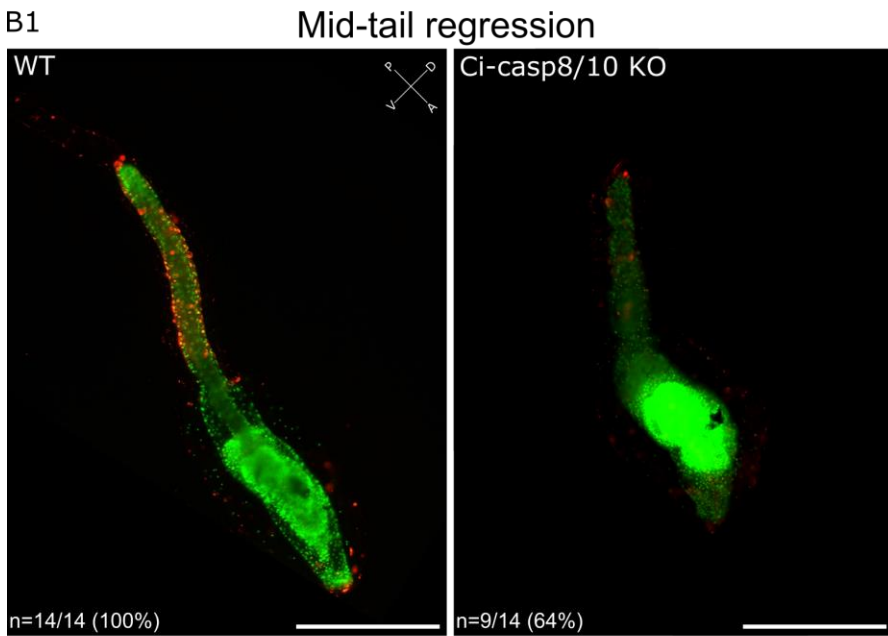
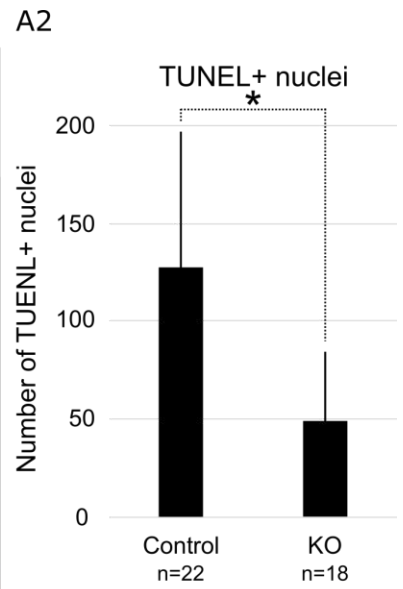
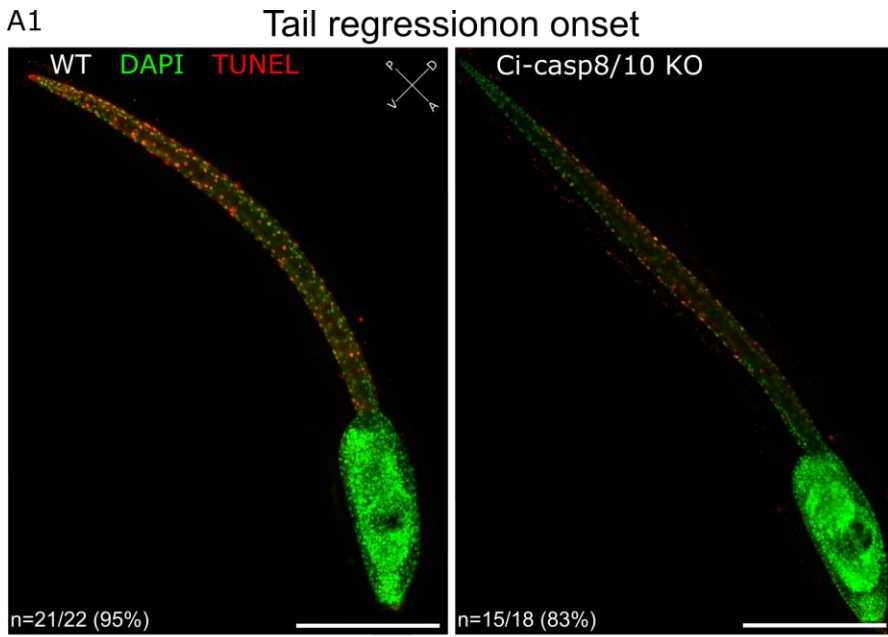
329 We injected dechorionated eggs with guide RNA against Ci-caspase 8/10. We tried two  
330 gRNA, first and second one inducing a mutation rate of 66.7% and 17%, respectively  
331 (Supplementary figure 5). Consequently, we pursue experiment with the gRNA number one. We  
332 performed a TUNEL labelling on settled larvae at the beginning of the tail regression and observed  
333 a significant decreasing of TUNEL-positive nuclei number in the Ci-caspase 8/10 KO larvae,  
334 especially at the tip of the tail, where the apoptosis is supposed to start (Figure 4A). Later, at the  
335 mid-tail regression, the significant difference of TUNEL-positive nuclei number persisted and  
336 accentuated (Figure 4B). First day post-settlement, when the tail is normally fully regressed, we  
337 observed a significant number of larvae presenting a remaining part of the tail in the Ci-caspase  
338 8/10 KO larvae (Figure 4C). Interestingly, this remaining tail stopped moving and did not display  
339 any apoptotic cells either, that could explain the incomplete regression. We conclude that Ci-caspase  
340 8/10 is an initiator triggering apoptosis during the tail regression, but this caspase should not be the  
341 only one knowing that some apoptotic cells can be still detected and that the tail can undergo a part  
342 of the regression. Importantly, the significant reduction of apoptotic cells at the tail tip is consistent  
343 with the fact that the apoptotic wave starts at the most posterior part of the tail. This default in  
344 apoptosis initiation may explain secondarily the slowdown/inhibition of the regression.

345 According to our observations, the half tail regressed is partially accumulate into the



346 epidermal pocket, the structure allowing tail tissues accumulation during tail regression<sup>23,24</sup>, which  
347 seems underdeveloped in caspase disrupted larvae. The remaining part of the tail can persist on the  
348 juvenile or disappeared, likely by progressive degradation. We did not notice any impacts on  
349 survival rate of juveniles having this phenotype, as next step of the metamorphosis went normally  
350 and the juveniles are able to feed few days after larvae settlement.

351



353 **Figure 4: Effects of Ci-caspase 8/10 loss of function on the tail regression. A1**, TUNEL labelling  
354 (red) counterstaining with DAPI (green) of larvae at the onset of tail regression (TR). Ci-caspase  
355 8/10 disrupted larvae exhibit few TUNEL+ nuclei through the tail. **A2**, the number of TUNEL+  
356 nuclei at the onset of tail regression in Ci-caspase 8/10 disrupted larvae is significantly reduced  
357 according to the control. **B1**, TUNEL labelling of larvae at the middle of tail regression. Ci-caspase  
358 8/10 disrupted larvae exhibit less TUNEL+ nuclei than the control. **B2**, the number of TUNEL+  
359 nuclei at the middle of tail regression in Ci-caspase 8/10 like disrupted larvae is significantly  
360 reduced according to the control. **C1**, while tail regression is over next day after settlement, a part of  
361 the Ci-caspase 8/10 disrupted larvae presents a remaining part of the tail (uncompleted TR) without  
362 any TUNEL+ nuclei. **C2**, Proportion of settled larvae having an uncompleted TR is significantly  
363 higher in Ci-caspase 8/10 disrupted larvae. Scale bars: 200  $\mu$ m. WT: wild-type. Experiment were  
364 conducted on *Ciona* Type A. Error bars (A2, B2, C2) correspond to standard deviation. \* Wilcoxon  
365 Mann Whitney test p-value < 0.05. Orientation: A, anterior; P, posterior; D, dorsal; V, ventral.

366

367

## 368 **Discussion**

369 We showed that Ci-caspase 8/10 is orthologous to the mammalian paralogs caspase 8 and 10.  
370 Involvement of orthologous actors is a prerequisite to consider regulation pathways as homologs.  
371 Consequently, with respect to the NCCD-2018 and according to the consensus that the caspase 8 is  
372 the initiator of extrinsic apoptosis in mammals, we hypothesised that extrinsic apoptosis regulates  
373 cell death in *Ciona*. Interestingly, *Ciona*'s genome possesses four and three members of the TNF  
374 and TNFr family, respectively<sup>28</sup>, in addition to a FADD like protein (KH.S1574.1). Next step will  
375 be to dissect their functions and characterised their interaction with Ci-caspase 8/10 to better  
376 understand the extrinsic apoptosis in *Ciona* and draw a deeper comparison with the mammals  
377 one.

378

379 **Ci-caspase 8/10 participates to the apoptotic event during the tail regression**

380 Tail regression is triggered by a set of coordinate mechanisms from larvae settlement to the  
381 apoptotic-dependant tail regression<sup>27</sup>. Activation of the ERK at the anterior adhesive papilla in  
382 followed by JNK and b-adrenergic receptor activation, mediating the signalling among the CNS  
383 toward the tip of the tail, leading to ERK phosphorylation at the most posterior extremity of the  
384 larvae<sup>26</sup>. Here, due to larvae settlement, these first steps of tail regression competency acquisition  
385 likely occur normally. Ultimately, this set of events, and especially ERK phosphorylation which  
386 transduces the death-activating signal at the metamorphosis stage, lead to the activation of  
387 executioner caspases at the tip of the tail, leading to apoptosis<sup>7,26,27</sup>. Executioner caspase activation  
388 propagate as a wave toward the trunk, given the typical polarised apoptotic profile requires for the  
389 caudal regression of ascidians tadpoles<sup>7,30,40</sup>. Consequently, the posterior initiation of apoptosis is  
390 the fundamental starting point of the apoptotic wave. Here, our data show the absence of apoptotic  
391 cells at the tip of the tail of initiator Ci-caspase 8/10 disrupted larvae, indicating a default in the  
392 apoptosis initiation. Usually, executioner caspases have to be activated by initiator caspases during  
393 the course of the apoptotic signalling pathways<sup>3,11,41</sup>. Consequently, Ci-caspase 8/10 disruption  
394 likely prevent the activation of the executioner caspases involve in tail regression, and apoptosis  
395 initiation failure likely explain the uncompleted tail regression phenotype observed, knowing that  
396 apoptosis is required for the tail regression<sup>7</sup>.

397 The reduction of TUNEL+ nuclei number and the incomplete tail regression in our Ci-  
398 caspase 8/10 disrupted larvae argue on the implication of an apoptotic pathway regulated by this  
399 initiator, potentially extrinsic apoptosis due to the presence of DED prodomain, in accordance with  
400 multiple report on caspase 8 and 10 as the specific and exclusive initiator caspase of extrinsic  
401 apoptosis in animals. However, apoptosis is not completely abolished and tail regression not fully  
402 prevented. Even if we keep in mind that the incapacity to totally blocked apoptosis in Ci-caspase  
403 8/10 like KO larvae may be due to the mutation rate of our Crispr/CAS9 and likely reflect a  
404 mosaicism effect it suggests that Ci-caspase 8/10 may not be the only initiator regulating the

405 polarised apoptotic wave. In mammals, several studies discovered simultaneous activations of  
406 intrinsic and extrinsic apoptosis, and that the initiation of one could trigger the activation of the  
407 second one due to interaction between their regulation systems<sup>42-44</sup>. Due to redundancy between  
408 intrinsic and extrinsic apoptosis we may hypothesise that the *Ciona* CARD-caspase could be  
409 responsible of an additional apoptotic signalling pathway, which works together with the extrinsic  
410 apoptosis to conduct cell death during the tail regression.

411

### 412 **Is the extrinsic apoptosis conserved?**

413 DED-caspases, FADD or TNFr have been identified in most animals phyla<sup>19,21,45-48</sup> and are  
414 usually considered homologs to their mammalian relatives. However, functional studies on extrinsic  
415 apoptosis outside of mammals remain drastically limited, and most of them were conducted on  
416 mammalian cultured cells. At the deuterostomians scale, activity of a DED-caspase has been shown  
417 concomitantly with detection of FAS and FADD in the ascidian *Botryllus schlosseri*<sup>49</sup>. The larvae of  
418 ascidians *Molgula occidentalis* presents a similar apoptotic profile as *Ciona* during the tail  
419 regression<sup>40</sup>. Characterisation of initiator caspases triggering apoptosis in this species could bring  
420 light on the conservation of extrinsic apoptosis in chordates. In the cephalochordate *Branchiostoma*  
421 *belcheri tsingtauense*, two FADD proteins and a TNF are functional in human HeLa cells and seem  
422 able to form a DISC complex<sup>18</sup>. In the echinoderm *Holothuria leucospilota*, over-expression of  
423 FADD induces apoptosis in HEK293 human cells, suggesting conservation of its function<sup>50</sup>. DED-  
424 caspase of *Molgula tectiformis* (ascidian), *Paracentrotus lividus* (sea urchin), and  
425 *Strongylocentrotus purpuratus* (sea urchin) can induce apoptosis in HeLa cells<sup>17</sup>. Taken together,  
426 our work with previous studies on *Amphioxus* and echinoderms suggest similar activation of  
427 extrinsic apoptosis base on DED-caspases at the chordate scale and among deuterostomians.

428 Finally, DED-caspases from protostomians *Leucoraja erinacea* (skate), *Tubifex tubifex*  
429 (sludge worm), *Mytilus californianus* (mussel), and *Schmidted mediterranea* (planarian) are able to  
430 triggers apoptosis in human cell lines, as well as the DED-caspase from the cnidarian *Acropora*

431 *millepora*<sup>17</sup>. Taken together, while keeping in mind that the evolutionary relationships between  
432 genes is unknown, the ability of DED-caspases to initiate apoptosis seems shared by several animals.  
433 However, the understanding of extrinsic apoptosis evolution will need further studies on animals  
434 having key phylogenetic positions.

435

### 436 **Conflicts of interest**

437 The authors declare no competing interests.

438

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456

## 457 **Authors' contributions**

458 JP, YS, and GK managed the project. GK made loss of function experiment, chemical  
459 treatments, TUNEL labelling, statistics, imaging, figures, and wrote the manuscript. GK and CR  
460 made *in situ* hybridization. All authors contributed to comment the manuscript.

461

## 462 **Data availability**

463 All data needed to evaluate the conclusions in this paper are present in the paper and/or the  
464 Supplementary Materials. Any requests can be addressed to corresponding author.

465

## 466 **Ethics declarations**

467 According to the French legislation (Décret n° 2013–118 du 1er février 2013 relatif à la protection  
468 des animaux utilisés à des fins scientifiques), experiments on *Ciona intestinalis* do not require an  
469 approval from Animal Ethics Commission because its not a “live vertebrate animals” and not a “live  
470 cephalopods”.

471

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584 characterization, inducible expression and involvement of apoptosis. *Fish Shellfish Immunol.* **89**,  
585 548–554 (2019).
- 586

587

588 **Supplementary Table 1:** List of *Ciona* Type A caspases.

Names	Domains	Accession numbers
Ci-Casp.2	CARD-P20-P10	KH2012:KH.C4.463
Ci-Casp.3/7A	P20-P10	KH2012:KH.C12.7
Ci-Casp.3/7B	P20-P10	KH2012:KH.C12.350
Ci-Casp.3/7C	P20-P10	KH2012:KH.C12.220
Ci-Casp.3/7D	P20-P10	KH2012:KH.C12.104
Ci-Casp.3/7E	P20-P10	KH2012:KH.C8.922
Ci-Casp.6A1	P20-P10	KH2012:KH.S1499
Ci-Casp.6A2	P20-P10	KH2012:KH.C2.896
Ci-Casp.6B	P20-P10	KH2012:KH.C12.449
Ci-Casp.8/10	DED-P20-P10	KH2012:KH.C8.550
Ci-Casp.X	P20-P10	KH2012:KH.C12.451
Ci-Casp.Y	P20-P10	KH2012:KH.C7.203

589

590

591 **Supplementary Table 2:** FADD and TNF receptors genes detected in the *Ciona* Type A genome

592 and potentially involved in extrinsic apoptosis.

Names	Accession numbers
TNF receptor 1	KH.C11.251
TNF receptor 2	KH.C3.453
TNF receptor 3	KH.L116.73
FADD	KH.S1574.1

593

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597 Hs\_Casp.1 MA-----  
598 Hs\_Casp.2 MAAPSA-----GSWSTFQH--KELMAADRGRRI LGVCGMH-----  
599 Ci\_Casp.2A MKKR-----  
600 Ci\_Casp.X MD---DAPS-----  
601 Hs\_Casp.3 M-----  
602 Hs\_Casp.7 MD-----CVGWP  
603 Ci\_Casp.3/7A MER-----  
604 Ci\_Casp.3/7B MNGK-----  
605 Ci\_Casp.3/7C MDPHPADFPPEPPPQPRITLRS DTV DSSVFHD--DDNLF SMDDAVEQPD-----SFDAV  
606 Ci\_Casp.3/7D MK-----  
607 Ci\_Casp.3/7E M-----  
608 Hs\_Casp.6 MSS-----  
609 Ci\_Casp.6A1 TP-----  
610 Ci\_Casp.6A2 M-----  
611 Ci\_Casp.6B -----  
612 Hs\_Casp.8 MDFS-----RNLYDIGEQLDSEDLASLKFSLDYI  
613 Hs\_Casp.10 MKSQG-----QHWYSSDKNCKVFR---EKLIIIDS NLGVQDVENLKF LCI GLV  
614 Ci\_Casp.8/10 MDSE-----GEFR---LLLHQICEGLTGENVSMMKFLCKDAI  
615 Hs\_Casp.9 MDEAD-----  
616 Ci\_Casp.Y MPIEDG-VPGLGENDQIY----AILGKFNHKEFLKFAQLEQGLGLSN-----  
617  
618  
619 Hs\_Casp.1 -----DKVLKEKRKLFIRSM-----GEGTINGL  
620 Hs\_Casp.2 P-----HHQETLKKNRVVLAKQL-----LISEL  
621 Ci\_Casp.2A -----HRNTLIKNRVKLAKCL-----HLDEV  
622 Ci\_Casp.X PTDSL NIRSTA-----SVHSL  
623 Hs\_Casp.3 -----  
624 Hs\_Casp.7 PGRKW-----HLEKNTSCGGSSGI  
625 Ci\_Casp.3/7A -----MSDETPRKPAGDV  
626 Ci\_Casp.3/7B -----ENSSAGDVLKNFHP-----NGNQGNQTAGKV  
627 Ci\_Casp.3/7C PPNSY PPNSSL--STSYSGGDILSMASPL-----PVTRITERTQTAGEV  
628 Ci\_Casp.3/7D -----  
629 Ci\_Casp.3/7E -----QVENL  
630 Hs\_Casp.6 -----ASGLRRGHP-----AGGEENM  
631 Ci\_Casp.6A1 -----  
632 Ci\_Casp.6A2 -----  
633 Ci\_Casp.6B -----  
634 Hs\_Casp.8 PQRKQEP IKDALMLFQRLQEKRMLEESNLSFLKELFRINRLDLL-ITYLNTRKEEMERE  
635 Hs\_Casp.10 PNKKLEKSSASDVFEHLLAEDLLSEEDPFFLAELLYII-RQKKL-LQHLNCTKEEVERL  
636 Ci\_Casp.8/10 PRSR--ELETALDFMEYFEAKGWIQEGDLGFLAEVLYRINRHDLKLLPGVKNRDRYEEN  
637 Hs\_Casp.9 -----RLLRRCRLRLVEEL-----QVDQL  
638 Ci\_Casp.Y -----KIIDGIESDCANKGVVEEQQFMVRKW-----KMRNGSKATVEV  
639  
640  
641 Hs\_Casp.1 LD-----ELLQT-RVLNKEEMEKVKRENA  
642 Hs\_Casp.2 LE-----HLEK-DITTEMRELIQAKVG  
643 Ci\_Casp.2A LQ-----YLVQC-GILTDVTVERITSKPT  
644 Ci\_Casp.X FS-----R-----SDVRT-----  
645 Hs\_Casp.3 -----ENTEN-----  
646 Hs\_Casp.7 CA-----SYVT-----QMADD-----  
647 Ci\_Casp.3/7A LK-----IY-----KSEVKTN--ATSESD--ENIDA-----  
648 Ci\_Casp.3/7B LM-----SYVDK-----PVFEKIDR-VITPEEFLQT-----  
649 Ci\_Casp.3/7C IR-----TYNPS-----P--EKRDH-EVSYDELEEDMKFSA  
650 Ci\_Casp.3/7D -----  
651 Ci\_Casp.3/7E CQ-----VCQ-----ATFDR-----  
652 Hs\_Casp.6 TET-----D-----AFYKR-----EMFDP-----  
653 Ci\_Casp.6A1 -----EQVYR-----  
654 Ci\_Casp.6A2 -----  
655 Ci\_Casp.6B -----SIYNSLFL-----  
656 Hs\_Casp.8 LQTPGRAQISAYRFHFCRMSWAEAN--SQCQTQSVFWRVDH-LLIRVMLYQISEEVSR  
657 Hs\_Casp.10 LP-----TRQRV-SLFRNLLYELSEGIDS  
658 Ci\_Casp.8/10 FQ-----AQQNNFTTYRVVCFMLADELTE  
659 Hs\_Casp.9 WD-----ALLSS-ELFRPHMIEDIQRAGS  
660 Ci\_Casp.Y LKS---KANRY---LGTTYITPEEASATPTQPSPIGQQTNN---PDTPFVILLRRVSS  
661  
662  
663 Hs\_Casp.1 TVMDK-TRALIDSVIP----KGAQACQICITYIC---EEDSYLAGTLGLSADQTSNGYL  
664 Hs\_Casp.2 SFSQN-VELL-----NLLPKRGPQAFDAFCE-----ALRETKQGH  
665 Ci\_Casp.2A RFSQN-VAFL-----TCLPTRGPRAYGTFL-----ALRISSQEHL  
666 Ci\_Casp.X -----QSNTAINHI  
667 Hs\_Casp.3 -----  
668 Hs\_Casp.7 -----QGCIEEQ-----GVEDSANED-  
669 Ci\_Casp.3/7A -----IRNSSDDF-  
670 Ci\_Casp.3/7B -----LTEDSIGNF-

671	Ci_Casp.3/7C	EGVSDSVKHFYGAAPVTCDDRDVDTGAGY-----DTVDAGADY-
672	Ci_Casp.3/7D	-----
673	Ci_Casp.3/7E	-----GDSFDAGFDPI
674	Hs_Casp.6	-----
675	Ci_Casp.6A1	-----FCLE-----
676	Ci_Casp.6A2	-----
677	Ci_Casp.6B	-----MSLKSAADV
678	Hs_Casp.8	SELRS-FKFLQEEISKCKLDDMNLDDIFIEM---EKR-----VILGEGKLDIL
679	Hs_Casp.10	ENLKD-MIFLLKDSL---KTEMTSLSLFLAFL---EKQ-----GKIDEDNLTCL
680	Ci_Casp.8/10	GDLKI-LKRLCAGKMSKANVRRSVDVLSWLTCM---EEE-----DLLESGDLDFI
681	Hs_Casp.9	GSRRDQARQLIID---LETRGSQALPLFIS-----CLEDTGQDML
682	Ci_Casp.Y	QLDENLRLRLRNNYAYCTGARNFRTALELFOYL---EENNL---WCGTEEEKVEIL
683		
684		
685	Hs_Casp.1	-----NMQ-----
686	Hs_Casp.2	EDML-----
687	Ci_Casp.2A	VDIL-----
688	Ci_Casp.X	-----
689	Hs_Casp.3	-----
690	Hs_Casp.7	-----
691	Ci_Casp.3/7A	-----
692	Ci_Casp.3/7B	-----
693	Ci_Casp.3/7C	-----
694	Ci_Casp.3/7D	-----
695	Ci_Casp.3/7E	SV-----
696	Hs_Casp.6	-----
697	Ci_Casp.6A1	-----
698	Ci_Casp.6A2	-----
699	Ci_Casp.6B	MSGV-----P-----
700	Hs_Casp.8	KRVC-----
701	Hs_Casp.10	EDLC-----
702	Ci_Casp.8/10	MSILQHLDNQKPYKLFQVRMNDNTVTLPKNQKSNAFQPNSHLPVASGFNSLSKQEYNH
703	Hs_Casp.9	ASFLR-----TNRQ-----
704	Ci_Casp.Y	VRKMRQLGRHDVVEI IQNFQLH-----ANQQ-----
705		
706		
707	Hs_Casp.1	-----DSQGVLSAFP-----
708	Hs_Casp.2	-----LTTLSGLQ-----
709	Ci_Casp.2A	-----EKVDISNAIP-----
710	Ci_Casp.X	-----
711	Hs_Casp.3	-----
712	Hs_Casp.7	-----
713	Ci_Casp.3/7A	-----
714	Ci_Casp.3/7B	-----
715	Ci_Casp.3/7C	-----
716	Ci_Casp.3/7D	-----
717	Ci_Casp.3/7E	-----
718	Hs_Casp.6	-----
719	Ci_Casp.6A1	-----
720	Ci_Casp.6A2	-----
721	Ci_Casp.6B	-----
722	Hs_Casp.8	-----AQINKSLLKIINDYE-----
723	Hs_Casp.10	-----KTVVPKLLRNIEKYKREKAIQIV-----
724	Ci_Casp.8/10	TSKLEPQYVSMENHRAFNGGYNQYNNYQSGQYENSHTTTPKQSLNFHNNNREKTFQNV
725	Hs_Casp.9	-----AAKLSKPTLENL-----
726	Ci_Casp.Y	-----QDVTLTHEIVRELAE-----
727		
728		
729	Hs_Casp.1	-----AP-----
730	Hs_Casp.2	-----HVLPPSLCDYDLSLPPVCECPLYK-----KLR
731	Ci_Casp.2A	-----NNKPITPTLHATITSNVHGEFVCAR-----RQP
732	Ci_Casp.X	-----NTVQSPTSTKAATSYQINLHQ-----
733	Hs_Casp.3	-----
734	Hs_Casp.7	-----
735	Ci_Casp.3/7A	-----
736	Ci_Casp.3/7B	-----
737	Ci_Casp.3/7C	-----
738	Ci_Casp.3/7D	-----
739	Ci_Casp.3/7E	-----
740	Hs_Casp.6	-----
741	Ci_Casp.6A1	-----
742	Ci_Casp.6A2	-----
743	Ci_Casp.6B	-----TP-----
744	Hs_Casp.8	-----
745	Hs_Casp.10	-----TPPVDKEAESYQGEELVS-----QTDVKTF
746	Ci_Casp.8/10	ESDTTWKQNTTSGSSTAPYSYSYPHRQNEPMTENPSFYTTGNNSTTMPSPVPIQAGSSET
747	Hs_Casp.9	-----TPVVLRLPEIRKP-----
748	Ci_Casp.Y	-----RSNSSTPFVENVDATY-----
749		
750		

751 Hs\_Casp.1 -QAVQDNP-----AMPTSSGSE-----GNVKLCSL-----  
752 Hs\_Casp.2 LSTDTVEH-----SLDNKDG-----  
753 Ci\_Casp.2A MEIGKCED-----KNDKADG-----  
754 Ci\_Casp.X -----GMDLFDG-----PTV-----  
755 Hs\_Casp.3 -----SVDSKSI-----KNLEPKI-----  
756 Hs\_Casp.7 -----SVDKPD-----RSSFVPSL-----  
757 Ci\_Casp.3/7A -----YKDDDD-----GKGLVPTV-----  
758 Ci\_Casp.3/7B -----GDTVGD-----ESHKIPew-----  
759 Ci\_Casp.3/7C -----GSDAGPD-----NSDTGPPL-----  
760 Ci\_Casp.3/7D -----  
761 Ci\_Casp.3/7E -----  
762 Hs\_Casp.6 -----  
763 Ci\_Casp.6A1 -----  
764 Ci\_Casp.6A2 -----  
765 Ci\_Casp.6B -HTNENNN-----VGQSEVDAL-----GSETPRI-----  
766 Hs\_Casp.8 -EFSKGEELCG-----VMTLSDS-----  
767 Hs\_Casp.10 LEALPQESWQN-----KHAGSNGNR-----ATNGAPSL-----  
768 Ci\_Casp.8/10 LELNRQQDYSGGQSNQFPYPTQYSGVSSNGNPIVGTNNV-----GCDGTPMVRDYPN  
769 Hs\_Casp.9 -EVLRPET-----PRPVDIGSG-----GFGDVGAL-----  
770 Ci\_Casp.Y VSVPRTES-----PVPASISSYLGNMALGDQSDPISEIDICS  
771  
772  
773 Hs\_Casp.1 -----EEAQRWQKKS-----AEIYPIMDKSSRTRLALIIICNEEF-  
774 Hs\_Casp.2 -----PVCLQVKPCTPEFYQTHFQ-----LAYRLQ--SRPRGLALVLSNVHFT  
775 Ci\_Casp.2A -----HNRLRVQNSTVAFFNETYN-----SGYKMT--TKSRGYALIVCIGRFN  
776 Ci\_Casp.X -----SDLTVRKSTHQHLMQHTDC-----YQMTMRGRPKGAALIIISVEKFFH  
777 Hs\_Casp.3 -----IHGSESMDSGISLDNS-----YKMD--YPEMGLCIIINNKNFH  
778 Hs\_Casp.7 -----FSKKNVTMRSIKTTRDRVP-----TYQYNMN--FEKLGKCIINNKNFD  
779 Ci\_Casp.3/7A -----LRQHMLEYMK--NERRGTFVVFNQENFD  
780 Ci\_Casp.3/7B -----SETTD-----FKYKMN--HKERGCFMIFNQEVYD  
781 Ci\_Casp.3/7C -----PYKQKSKVIEPEAIAVNRE-----FQYPMN--HKQGCCIIFNQKHF  
782 Ci\_Casp.3/7D -----YTIT-----QKFD  
783 Ci\_Casp.3/7E -----PIRHPDRTTVRRELKGDGDKIEPSVWDVKFDYFEYNMS--FPKRGIFLIFDQENFD  
784 Hs\_Casp.6 -----AEKYKMD--HRRRGIALIFNHERFF  
785 Ci\_Casp.6A1 -----KFYKNNRKMQEEWIDDC-----YDINY-SGKIGIAAVFLKRN  
786 Ci\_Casp.6A2 -----EISINES-----YKIDYSHEKIGLAVVFLSEMKS  
787 Ci\_Casp.6B -----YSLPDVTKMASSEVEC-----YQMD--RESRGLALIIINNENFH  
788 Hs\_Casp.8 -----PREQDSQTLT-----KVYQMK--SKPRGYCLIIINNHNFA  
789 Hs\_Casp.10 -----VSRMGQASANTLNSSTTKR-----AAVYRMN--RNHRGLCVIINNHSF-  
790 Ci\_Casp.8/10 GFETPPPPRAQPKAPVSEIKDTSTMGII-----LDKYPME-----RRGYCLIIINNENFE  
791 Hs\_Casp.9 -----ESLRGNAD-----LAYILS--MEPCGHCLIIINNWFNC  
792 Ci\_Casp.Y -FQGNVQVLESDDMSYVTHFSK-----VGVYTVRS--KEPKGHVLIILNNYEGF  
793 \* :  
794  
795 Hs\_Casp.1 -----DSIPRRTGAEVDITGMTLLQNLGYS-VDVKK-NLT  
796 Hs\_Casp.2 GE-----KELEFRSGGDVDHSTLVTLFKLLGYD-VHVLC-DQT  
797 Ci\_Casp.2A AG-----VRLPDRQGTADKKNLLAIFEQINYK-TILVE-NCN  
798 Ci\_Casp.X PE-----SDLLNREGSEKDRVRLVLLQIGFQ-CYVLI-NGT  
799 Hs\_Casp.3 KS-----TGMTSRSGTDVDAANLRETFRNLYE-VRNKN-DLT  
800 Hs\_Casp.7 KV-----TGMGVRNGTDKDAEALFKCFRSLGFD-VIVYN-DCS  
801 Ci\_Casp.3/7A RM-----RHPRKSGVDVEMVLKSAKLGFDVRLK-NQT  
802 Ci\_Casp.3/7B AKTS-----LNLGERIGSSTDARNLASAAAYLGFYIQVFN-NLT  
803 Ci\_Casp.3/7C AH-----LRMDTREGTNHDAENIKHSMEMLGFEHVRIIK-DST  
804 Ci\_Casp.3/7D KH-----LKLSSERTGSDKAAALLKTAELLGFE-TEEYL-DLE  
805 Ci\_Casp.3/7E -----YGLQQRVGSKLDRIVLEETAIKLGFT-PNVCH-DYT  
806 Hs\_Casp.6 WH-----LTLPERRGTCADRNLTRRFSDLGFE-VKCFN-DLK  
807 Ci\_Casp.6A1 DATTNEL-----ENEKIEQALQQDFDDFTLFFENMQLD-VMKFGKMK  
808 Ci\_Casp.6A2 -----DSPETKKALLEDEDFKFLFADMRFD-VKSYSGEMK  
809 Ci\_Casp.6B HA-----TRMNKRSQTDVARNLSRIFFKLGFD-VQVYK-DLS  
810 Hs\_Casp.8 KAREK-----VPKLHSIRDRNGTHLDAGALTTTFEELHFE-IKPHH-DCT  
811 Hs\_Casp.10 -----TSLKDRQGTGTHKDAEILSHVFQWLGFT-VHIHN-NVT  
812 Ci\_Casp.8/10 RQVNEDEIQRLQMTERDFPVPN-VGLKDRGTGSGNTERLENLFKEFGFI-LDVRK-DLD  
813 Hs\_Casp.9 RE-----SGLRTRTGSNIDCEKLRRRFSSPHFM-VEVKG-DLT  
814 Ci\_Casp.Y AW-----GPDHDKGAKRKGELMKQLWEGFQCK-VIVKE-NRT  
815 \* :  
816  
817 Hs\_Casp.1 ASDMTTELEAFHRP-EHKTSDSTFLVFMHSHGIR-----EGICGKHKHSEQVPDILQLN  
818 Hs\_Casp.2 AQEMQEKLQNFQAQLP-AHRVTDSCIVALLSHGVE-----GAIYGVGDK-----LLQLQ  
819 Ci\_Casp.2A KFEMERRVKQFAQME-EHRKCDSCAVAILSHGSK-----TDIYASDGR-----SIPIE  
820 Ci\_Casp.X AEQIVSTLQTFAAELE-EHYYNCSCLVAAMSHGDA-----GCFYGSQGV-----SVAID  
821 Hs\_Casp.3 REEIVELMRDVSKE--HSKRSSFVCLLSHGEE-----GIIFGTNG-----PVDLK  
822 Hs\_Casp.7 CAKMQDLLKASEED--HTNAACFACILLSHGEE-----NVIYKGDG-----VTPIK  
823 Ci\_Casp.3/7A TDEIRDHLQELSQQD--HSNCDFVCVLLSHGES-----DGVLYTKDG-----DIHLK  
824 Ci\_Casp.3/7B RSKILKWLKDVAAAS--HESYDFCGCAILTHGDK-----NNDY-----IMNIT  
825 Ci\_Casp.3/7C KVEIINWIAAVSKAD--HSNYDCFACVILTHGGD-----KDVLYARD--KMKELK  
826 Ci\_Casp.3/7D RKQIRRKLYELSEMD--HGDHDCFACAILTHGGK-----DNILYSHDD-----EMKLLK  
827 Ci\_Casp.3/7E KDDVYAELRKISQMN--HSQYDFCFAAILTHGEE-----DEMVIYAKDD-----SMKLLK  
828 Hs\_Casp.6 AEELLKIHVESTVS--HADADCFVCVFLSHGEG-----NHIYAYDA-----KIEIQ  
829 Ci\_Casp.6A1 FSEAVANLENVSKLKSSENNYSFCIFMFIGHSD-----GCIKATDGK-----LDIHE  
830 Ci\_Casp.6A2 FSTVVENLEAVLEEIQRNPLISCFICMFIGHGGP-----GFINTADGK-----LDIHE

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831 Ci_Casp.6B CISMLEVINVESTMD--HTGNDCCFVAFLSHGDD-----GCVYGTGDG-----IVQIK
832 Hs_Casp.8 VEQIYEILKIYQLMD--HSNMDCFCICILSHGDK-----GI IYGTGDGQ-----EAPIY
833 Hs_Casp.10 KVEMEMVLQKQKCNP-AHADGDCFVFCILTHGRF-----GAVYSSDEA-----LIPIR
834 Ci_Casp.8/10 QREMEKVIQIYTKKD--HSDKDCFVCMVMSHGLS-----GCVYGVVDGL-----SLSTS
835 Hs_Casp.9 AKKMVLALLELAQQD--HGALDCCVVVILSHGCQASHLQFPGAVYGTDCG-----PVSVE
836 Ci_Casp.Y AGAMPKFLNDFSRSS-FHQSCDFCAVVMIMSHGGLVDN---RDVFGVDCR-----TIAAN
837 :
838 :
839 :
840 Hs_Casp.1 AIFNMLNTKNCPSLKD KPKVII IQACRGDSPGV-VWFKDSVGVSGNL---SLPTTEEFE
841 Hs_Casp.2 EVFQLFDNANCP SLQNKPKMFFIQACRGDETRDGVQDQDGNHAGSPGCEESDAGK----
842 Ci_Casp.2A SLIKMFDNVNCPPLRNKPKIFFIQCCRGNNDQGGIDATDGPPTLNSETSDSYNSTTHNIT
843 Ci_Casp.X TVVNFNSQNCHSLQKPKKIFLQACQGDYDMGVDEVDPVQAPVGDVD--NTSTSSSN
844 Hs_Casp.3 KITNFFRGDRCSRSLTGKPKLFI IQACRGTELDGCIETDSGVDDM-----
845 Hs_Casp.7 DLTAHFRGDRCKTLEKPKLFFIQACRGTELDGCIQADSGPINDTD-----
846 Ci_Casp.3/7A EILDSFKASRCPSLAGKPKLFFIQACRGEKRSTPVEIEYKFDV-----TDSTPPEEQ
847 Ci_Casp.3/7B DFTNFTADKCP TLAGKPKLFFVQACRGHKRDTAVPFTLNVA STI-----
848 Ci_Casp.3/7C DFMQPFGRDNCPSLATKPKLFFIQACRGFKLAEPVKVRPVQCDSLNY--DTTDGGSATVD
849 Ci_Casp.3/7D DFTTFEADKCRSLASKPKLFFVQACRGDLDLRGAKVIHLKQSTGDV---LDRNPKSKE
850 Ci_Casp.3/7E TLISRVSATECPSLAGKPKLFFVQACRGEISQPAICTTACLKRQV---QSDSIPDVD
851 Hs_Casp.6 TLTGLFKGDKCHSLVGPVKFI IQACRGNQHDVPVPLD VVDNQTEK---LDTNITEVD
852 Ci_Casp.6A1 NILAKFMPTHKC--LEGKPKIFIQACRGTKKNVKS DSSLSTLCDKEI-----
853 Ci_Casp.6A2 NILSKFMPQDC--LKGKPKIFIQACRGTTRHNFDSLACSGTLTVPTV-----
854 Ci_Casp.6B KIVDQFRGDVCP SLAGKPKIFLQACRGT L HETS VQLSVDVVDGE-----DQPCVEVD
855 Hs_Casp.8 ELTSQFTGLKCP SLAGKPKVFFIQACQGDNYQKGI PVETDSEEQPYL-----EMD
856 Hs_Casp.10 EIMSHTALQCPR LAEKPKLFFIQACQGEIEQPSVSI EADALNPEQA-----
857 Ci_Casp.8/10 KISKFRPAGMCS SLTGKPKIFFVQACQGDRTMDGHSAPVDDVESDGA-----
858 Hs_Casp.9 KIVNLFNGTSCPSLGGKPKLFFIQACGGEQKDHGFEVASTSPEDES PGSN-PEPDATPFQ
859 Ci_Casp.Y DVLALFKNTHSKYLIGRPKLIFFQCCRGGSSSRGVPFVDAADAVNRL----LPPSDQTD
860 . . . * :*: : * * *
861 Hs_Casp.1 -----DDAIKKAHIEKDFIAFCSSTPDNVSW---RHPTMGSVFIGRLIEHM----
862 Hs_Casp.2 -----EKLPKMRLPTRSDMICGYACLKGTAA M---RNTKRGSWYIEALAQVF----
863 Ci_Casp.2A ---TDVDIISTASKRLPTSSDMIIGYATLQGNAA L---RNRHRGWSYIQVLVKKI----
864 Ci_Casp.X -----DHIRNKLPQKSDMLIGQATMKGFAAM---RNTKHGWSYIQAFVRVL----
865 Hs_Casp.3 -----ACHKIPVEADFLYAYSTAPGYYSW---RNSKDGSWFIQSLCAML----
866 Hs_Casp.7 -----ANPRYKIPVEADFLYAYSTVPGYYSW---RSPGRGSWVQALCSIL----
867 Ci_Casp.3/7A -----TTTVFTIPAEADFLIAHATPEEFCAW---RNRREGSRFIKALCGCL----
868 Ci_Casp.3/7B -----EKTELKLI PCQADFLIASSTPEGYSSW---RNKANGSWFIQALYL CI----
869 Ci_Casp.3/7C -----EFATIPAEADFLIAQSTVPKYYSW---RNSNGSFIQALCLTF----
870 Ci_Casp.3/7D -----SYTIPVQADFLISQATAPDYAW---RSSDKGSIFIQTLCSVF----
871 Ci_Casp.3/7E -----SEETPKIPAEADILVAYSTQPGQVSV---RNEYTGSVVFVQTMCAAL----
872 Hs_Casp.6 -----AASVYTL PAGADFLMCSVAEGYYSH---RETVNGSWYIQDLCEML----
873 Ci_Casp.6A1 -----KADGSDVLLCYA AWSGYVS YALTTNEYERGTQYLQDLHKAL----
874 Ci_Casp.6A2 -----LNPEGS DMLLCYASWQGFVSYC---VNTKKGKTCYLQELHSM L----
875 Ci_Casp.6B -----AAPVPTLP SSGSYLFCYSVAEGYYSH---RDTLYGSWFIQDFTDVM EQLV
876 Hs_Casp.8 -----LSSPQTRYIPDEADFL LGMATVNNCVSY---RNPAEGTWYIQSLCQSL----
877 Hs_Casp.10 -----PTSLQDSIPAEADFLIAGLATVPGYVSF---RHVEEGSWYIQSLCNHL----
878 Ci_Casp.8/10 -----ASHGRQVLPSESDFLIGHSTVPGYLSY---RSRTDGSWFISTLVDSL----
879 Hs_Casp.9 EGLRFFD-QLDAISSLPTPSDIFVSYSTFPGFVSW---RDPKSGSWYVETLDDIF----
880 Ci_Casp.Y -----GIDKTTLP HMSDILVAYSTLEGDMSF---RNEATGSWF INAIATVF----
881 * : : : * : : :
882 :
883 Hs_Casp.1 ---QEY-ACSC-DVEE IFRKVRFSFEQP-----DGRAQMP TERTVTLTRCFY LFP
884 Hs_Casp.2 ---SER-ACDM-HVADMLVKVNALIKDR-EGYAPGTEFHRCCKEMSEYCS-TLCRHLYLFP
885 Ci_Casp.2A ---AQH-AHEM-DLLEILTLVNSAIKSK-EGHSTDFDSQGVKEMSEFHS-TLCKLFFYP
886 Ci_Casp.X ---ARH-ACDT-DLLDMMTKVNNILKHK-EGWCPSGVYHSC TYLHILPA-KLNNHHFCCP
887 Hs_Casp.3 ---KQY-ADKL-EFMHILTRVNRKVATEFESFSFDATFHAKKQIPCIVS-MLTKELYFYH
888 Hs_Casp.7 ---EEH-GKDL-EIMQILTRVNRVARHFESQSDDPHFHEKKQIPCVVS-MLTKELYFSQ
889 Ci_Casp.3/7A ---DRYAGDDI-ELLQILTVNHHVSHGWDGEVY---KYEKSKQIPSI TT-QLTAQLFFTK
890 Ci_Casp.3/7B ---QRY--PSL-EIMQIMTRVNRMV ALHFENTNTE-SPDIYKQIPSICT-RLTGELYFHK
891 Ci_Casp.3/7C ---NLF-GEKQ-EIMQMMTKVNRMVAYDYESASKDPKMNQKQIPSI TT-QLTAELYFPK
892 Ci_Casp.3/7D ---ERY-SDEM-DIMKILTRVNRVAFNFESWTQRPDMNHMKQIPSI TS-QLTAELFIKK
893 Ci_Casp.3/7E ---KQR-GGEL-ELVQLLTVNRNIALTFETNMCS PKFDKQMP SIVS-QLTAELYFRP
894 Hs_Casp.6 ---GKY-GSSL-EFTELLTLVNRKVSQRRVDFCKDPSAIGKKQVPCFAS-MLTKKLHFFP
895 Ci_Casp.6A1 ---RMF-TKP--DKPQSISS TN-----
896 Ci_Casp.6A2 ---RKF-ADGTNHLIDILTVLNRQVSSS--PIMNSQRTDYFYQMP SFES-SLRKKLFLLV
897 Ci_Casp.6B LNKGGQ-QDKV-DFIDVLTVNRKVSARRVERSYSQQALGKKQMPCYLS-MLTKKLYLTP
898 Hs_Casp.8 ---RERCPRGD-DILTILTEVNYEVS NK-----DDKKNMGKQMPQPTF-TLRKKLVFPS
899 Hs_Casp.10 ---KKLVPRHE-DILSILTA VDDVSR RVD-----KQGTKKQMPQPAF-TLRKKLVFPV
900 Ci_Casp.8/10 ---EKY-HEKE-DLLSIMINVNQEMASK-----PYKQMPPIA-TLRKKVFFSK
901 Hs_Casp.9 ---EQW-AHSE-DLQSLLRVANAVSVK-----GIYKQMPGCFN-FLRKKLFFKT
902 Ci_Casp.Y ---SKK-ARTE-HVVDMLTEVGRGVSHRAANTPDNMATHRCCKEMSEYKS-TLRNKLYLFP
903 .
904 :
905 Hs_Casp.1 -----GH-----
906 Hs_Casp.2 -----GHPPT-----
907 Ci_Casp.2A -----GIVQTEKE-----
908 Ci_Casp.X -----
909 Hs_Casp.3 -----
910 Hs_Casp.7 -----

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911	Ci_Casp.3/7A	KSKNKICQEK-----PQGTIKENENANAQANDDFIAHEGQ-----ETEKEISR-
912	Ci_Casp.3/7B	PGSTVVESH-----ISNNDASN--AYDQSIAIETTTEERTYKRSKSTF---
913	Ci_Casp.3/7C	KREGHPASEYSVNNQVTSR-QLQHNQVTARCEYDQYSSHTVRSADNAFVETTTQFSRL
914	Ci_Casp.3/7D	PKRNTNPST-----
915	Ci_Casp.3/7E	-KDTVS-----
916	Hs_Casp.6	KSN-----
917	Ci_Casp.6A1	-----
918	Ci_Casp.6A2	DQR-----MPE-----TAPTGDRGDSGDFQS-----
919	Ci_Casp.6B	KK-----
920	Hs_Casp.8	D-----
921	Hs_Casp.10	-----
922	Ci_Casp.8/10	QNSNSSSNQN-----CNTPEGCL-----
923	Hs_Casp.9	S-----
924	Ci_Casp.Y	-----GFPKAEEE-----
925		
926		
927	Hs_Casp.1	-----
928	Hs_Casp.2	-----
929	Ci_Casp.2A	-----
930	Ci_Casp.X	-----
931	Hs_Casp.3	-----
932	Hs_Casp.7	-----
933	Ci_Casp.3/7A	-----PIPSP-----
934	Ci_Casp.3/7B	-----
935	Ci_Casp.3/7C	AMQAGDASRLLEEAYMDPRTQGAAANPPSHASVRQRDLFPRPPPLPTKQRQTRRSNDSSHR
936	Ci_Casp.3/7D	-----
937	Ci_Casp.3/7E	-----
938	Hs_Casp.6	-----
939	Ci_Casp.6A1	-----
940	Ci_Casp.6A2	-----
941	Ci_Casp.6B	-----
942	Hs_Casp.8	-----
943	Hs_Casp.10	-----PL-----
944	Ci_Casp.8/10	-----
945	Hs_Casp.9	-----
946	Ci_Casp.Y	-----
947		
948		
949	Hs_Casp.1	-----
950	Hs_Casp.2	-----
951	Ci_Casp.2A	-----
952	Ci_Casp.X	-----
953	Hs_Casp.3	-----
954	Hs_Casp.7	-----
955	Ci_Casp.3/7A	-----
956	Ci_Casp.3/7B	-----LQIRKSF
957	Ci_Casp.3/7C	RSGDYTRRPPQEVYSSPTGSPRDVTPHYVTGYDAPPRSSHETSPYGSQHNVARRRPSY
958	Ci_Casp.3/7D	-----
959	Ci_Casp.3/7E	-----
960	Hs_Casp.6	-----
961	Ci_Casp.6A1	-----
962	Ci_Casp.6A2	-----
963	Ci_Casp.6B	-----
964	Hs_Casp.8	-----
965	Hs_Casp.10	-----
966	Ci_Casp.8/10	-----
967	Hs_Casp.9	-----
968	Ci_Casp.Y	-----
969		
970		
971	Hs_Casp.1	-----
972	Hs_Casp.2	-----
973	Ci_Casp.2A	-----
974	Ci_Casp.X	-----
975	Hs_Casp.3	-----
976	Hs_Casp.7	-----
977	Ci_Casp.3/7A	-----RHK-----
978	Ci_Casp.3/7B	-----RRGKTKT-----
979	Ci_Casp.3/7C	ETSFPAQSRAIPAVTLRKQSLPVSSMYSSSADSHLLTPNRRHLHPSETVPARLSVSPNAAR
980	Ci_Casp.3/7D	-----
981	Ci_Casp.3/7E	-----
982	Hs_Casp.6	-----
983	Ci_Casp.6A1	-----
984	Ci_Casp.6A2	-----
985	Ci_Casp.6B	-----
986	Hs_Casp.8	-----
987	Hs_Casp.10	-----
988	Ci_Casp.8/10	-----
989	Hs_Casp.9	-----
990	Ci_Casp.Y	-----

```

991
992
993 Hs_Casp.1 -----
994 Hs_Casp.2 -----
995 Ci_Casp.2A -----
996 Ci_Casp.X -----FQI
997 Hs_Casp.3 -----
998 Hs_Casp.7 -----
999 Ci_Casp.3/7A -----KTETQV
1000 Ci_Casp.3/7B -----GRHSATF
1001 Ci_Casp.3/7C NSVNRRSLPLGTDPKPQAVSSWGSGINRAGLRSTKQTEV
1002 Ci_Casp.3/7D -----
1003 Ci_Casp.3/7E -----
1004 Hs_Casp.6 -----
1005 Ci_Casp.6A1 -----
1006 Ci_Casp.6A2 -----SQLNVNV
1007 Ci_Casp.6B -----
1008 Hs_Casp.8 -----
1009 Hs_Casp.10 -----DALSL
1010 Ci_Casp.8/10 -----
1011 Hs_Casp.9 -----
1012 Ci_Casp.Y -----DNSLFM
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1014 **Supplementary Figure 1: Alignment of *Ciona* Type A and human caspases.**

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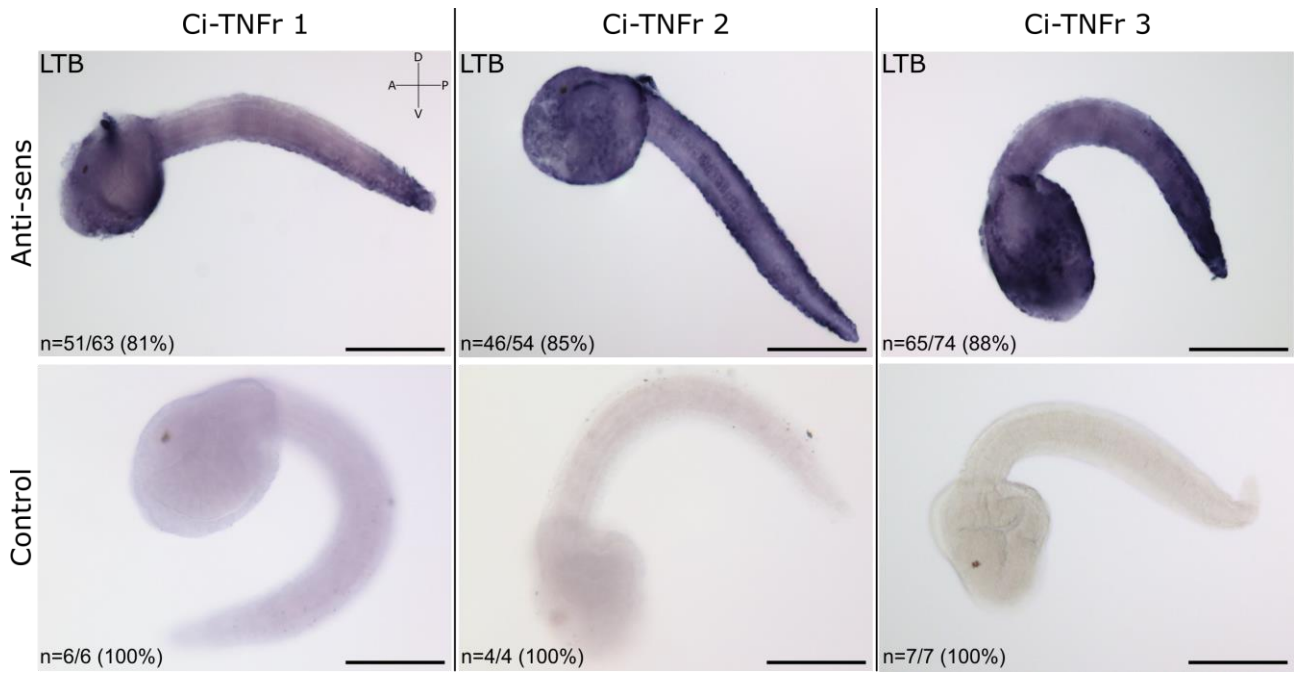
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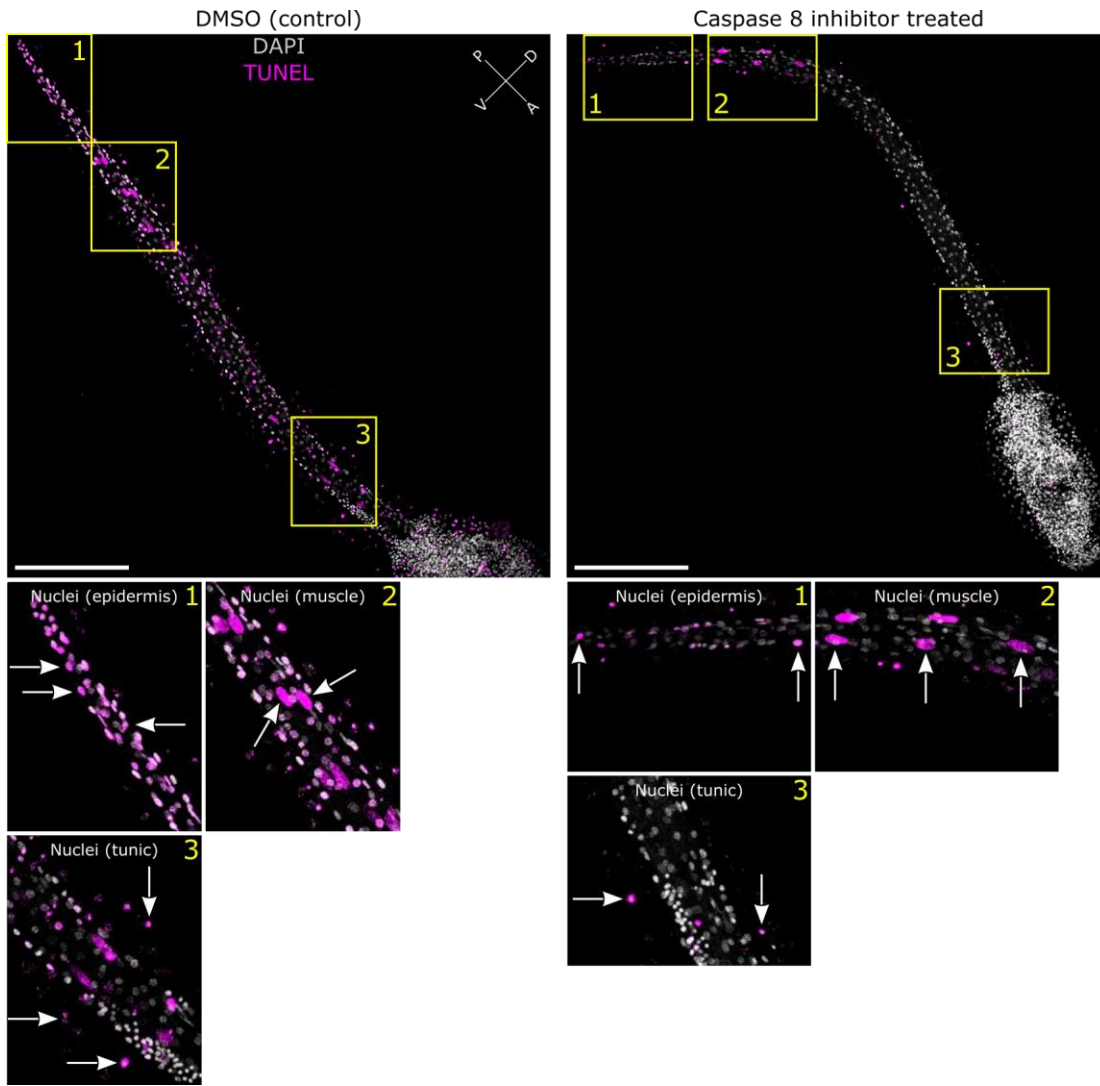
1024



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1026 **Supplementary Figure 2:** TNF receptors are expressed in the tail during development. TNFr 1 is  
 1027 mainly expressed at the tip of the tail in epidermal cells. TNFr 1 and 2 are expressed in the trunk  
 1028 and the tail with a stronger expressed at the posterior extremity. Experiment were conducted on  
 1029 *Ciona* Type B. Orientation: A, anterior; P, posterior; D, dorsal; V, ventral. Scale bars = 200  $\mu$ m.

1030



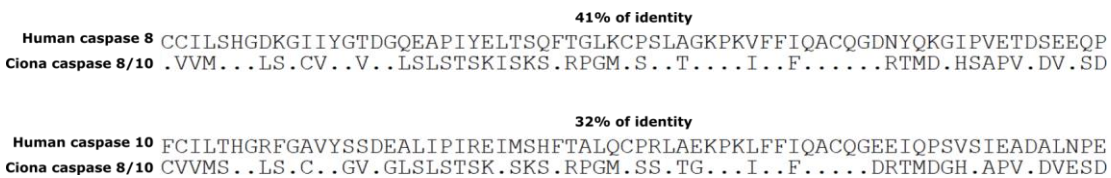
1031

1032 **Supplementary Figure 3:** Z-projection of *Ciona* type B labelled by TUNEL and imaged by  
 1033 confocal microscopy. Experiment were conducted on *Ciona* Type B. Orientation: A, anterior; P,  
 1034 posterior; D, dorsal; V, ventral. Scale bars = 200  $\mu$ m.

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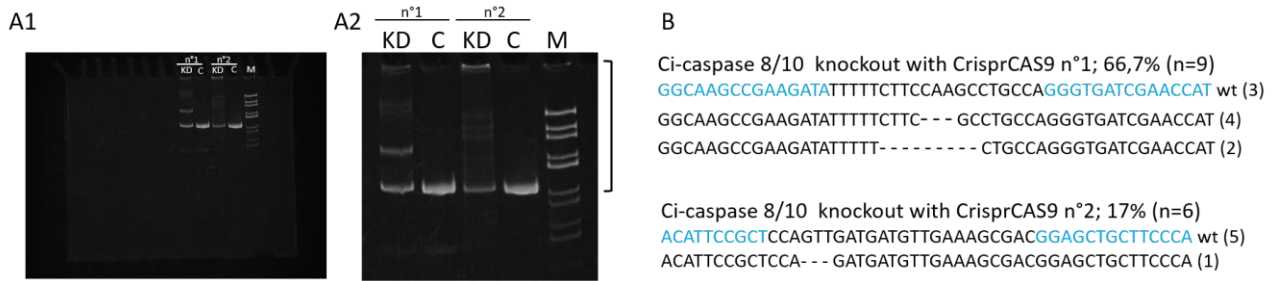
1039

1040 **Supplemental figure 4:** Alignments of active sites of human caspases 8 and 10 with Ci-caspase  
 1041 8/10. Dots represent same amino acids between *Ciona* Type A and human caspases.

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1046 **Supplemental figure 5: A**, heteroduplex mobility shift assay showing heterogeneous mutated DNA  
1047 sequence in the amplified Ci-Casp8/10 amplicons from *Ciona* type A (A1). Heterogeneous mutated  
1048 DNA is highlighted (*bracket*) in gel magnification (A2). M: marker (pBluescript digested by HaeIII);  
1049 C: Control lane without mutated Ci-Casp8/10; KD: the lane from CrisprCAS9 mix introduced  
1050 animals. **B**, sequencing of Ci-caspase 8/10 genomic sequence targeted by the gRNA showing  
1051 example of mutations. “-” indicates nucleotide deletions. wt: wild-type sequence. *Numbers in*  
1052 *parentheses* indicate the number of clones detected during sequencing.

1053