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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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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 homeostasis¹ and able to
56 influence its immediate cellular environment². 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 family³⁻⁵. Based on these morphological and molecular features, apoptosis has been
60 detected in most metazoan phyla⁶⁻¹⁰. 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)¹¹. 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^{3,12,13}. Even if recent research suggests divergences between intrinsic
70 apoptosis¹⁴, 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^{11,15}.

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)^{11,16}. 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^{17,18}. 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^{17,19-21}.

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-serine²²⁻²⁷. Importantly, tail regression is characterised by a polarised apoptotic wave
96 starting at the tip of the tail and propagates toward the trunk⁷. 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 domain²⁸.

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)^{28,29}. 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 A²⁹.

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 described³⁰, 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 described³¹⁻³³.
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 described³⁰. Concerning settled larvae used for *Ci-*
164 *caspase 8/10* WISH, we manually removed the tail tunic with forceps as we already done³⁰,
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 7³⁴ and Gblocks
173 0.91b³⁵ was used to removed vacancies and blur sites. Phylogenetic analyses were carried out by
174 Maximum-Likelihood (ML) method under LG model using PhyML 3.1³⁶, combined ML tree search
175 with 1000 bootstrap replicates. Bayesian analyses were performed using MrBayes 3.2.6³⁷ 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 dye³⁸. 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 iGemdock³⁹ under
210 Drug screening settings for 70 generations and population size of 200.

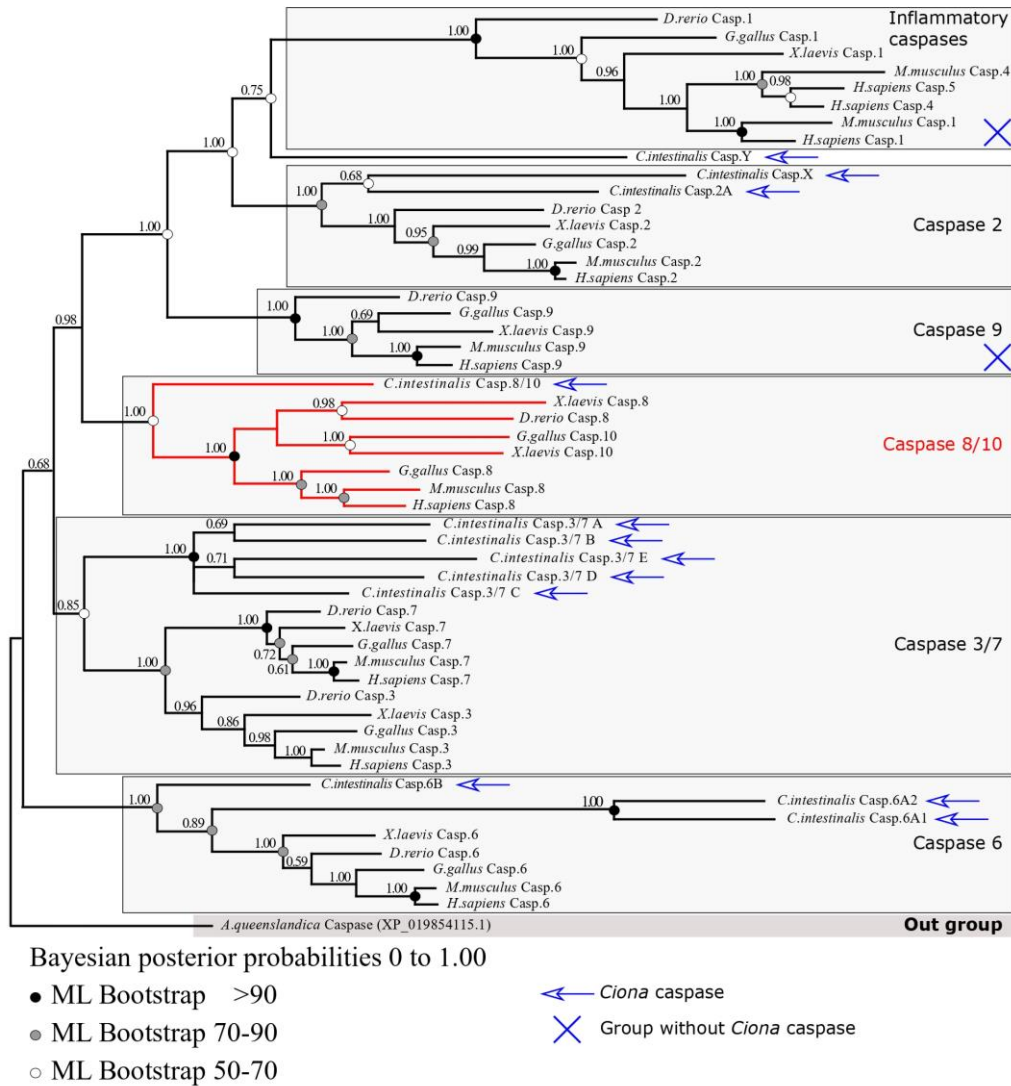
211

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^{14,28}. 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 tunicates¹⁴, 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.

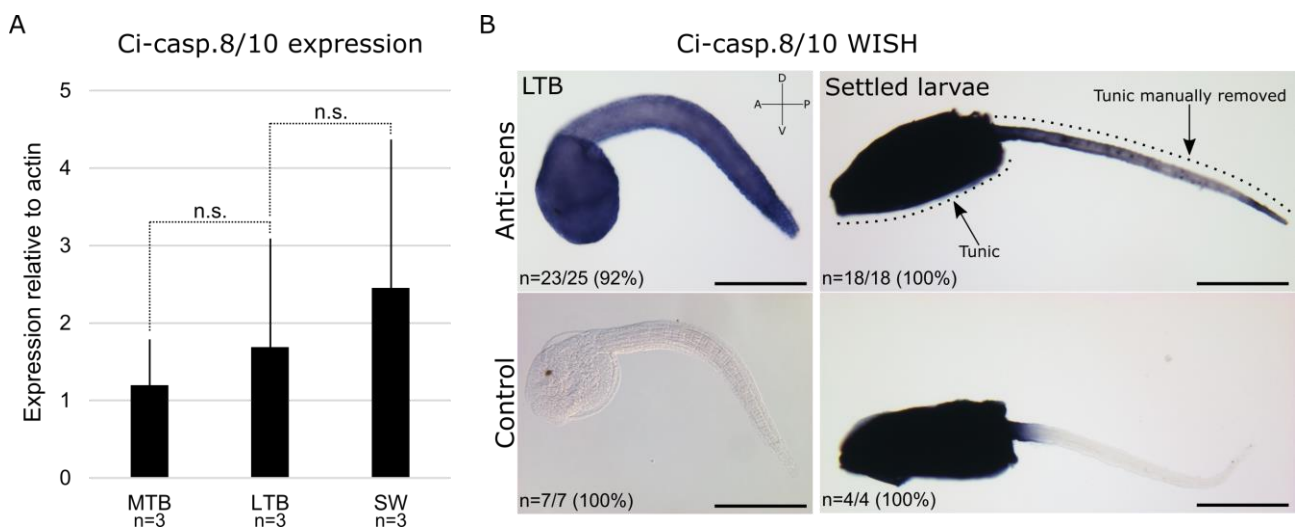
245

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 tissue³⁰.
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



262

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 μ m. Experiment were conducted on
271 *Ciona* Type B. Orientation: A, anterior; P, posterior; D, dorsal; V, ventral.

272

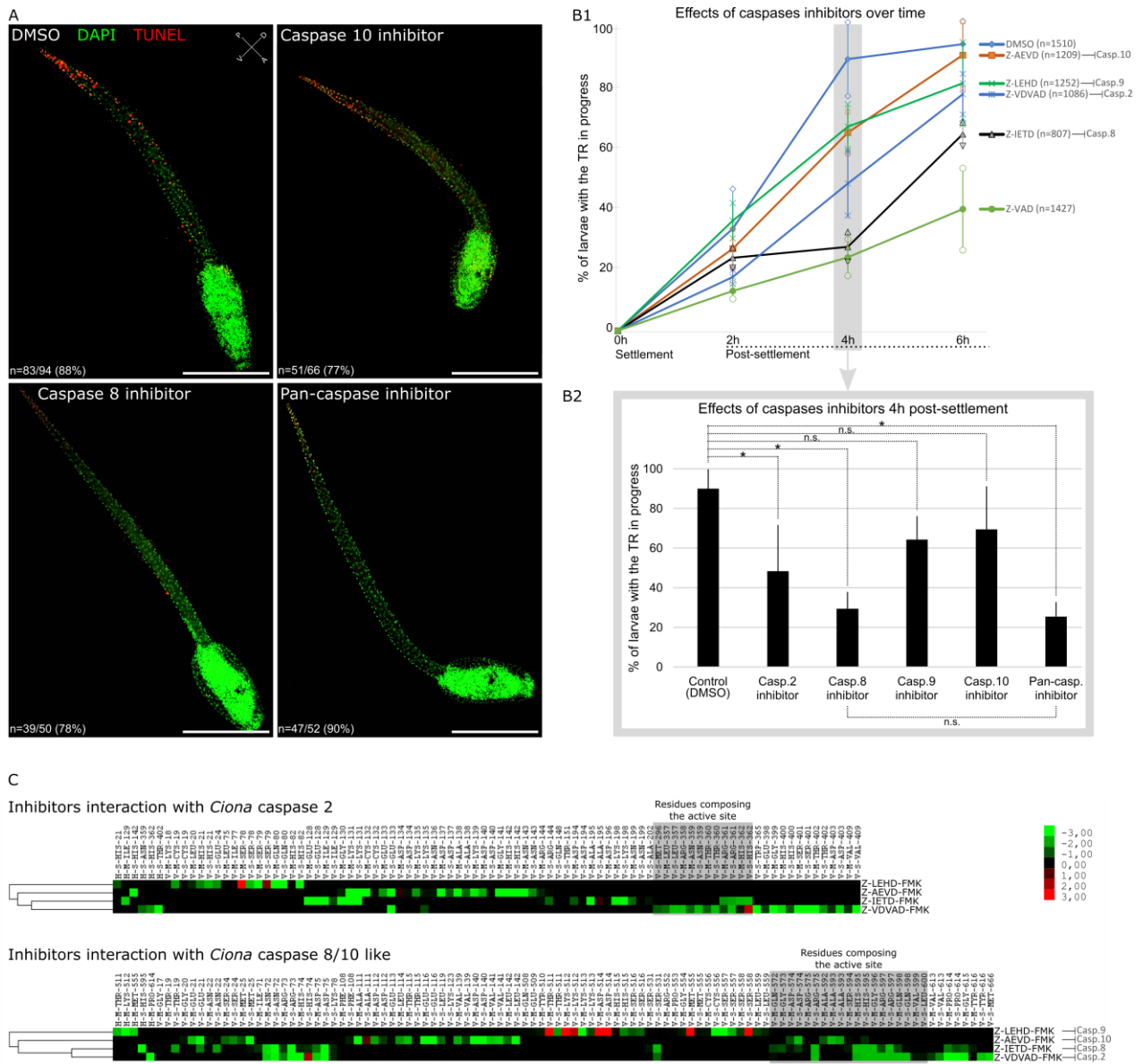
273 **Caspase 8 inhibition delayed the tail regression**

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^{7,26}, 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^{7,30}. 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 μ 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.

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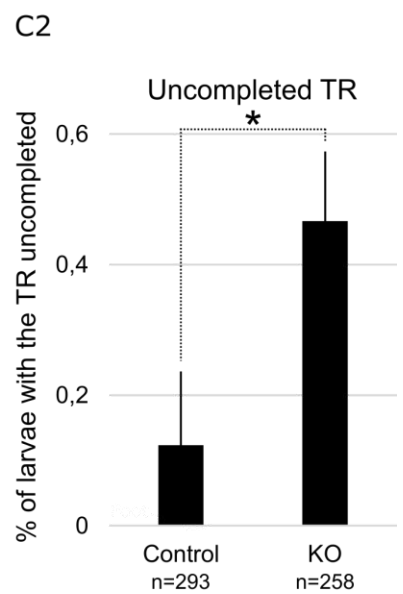
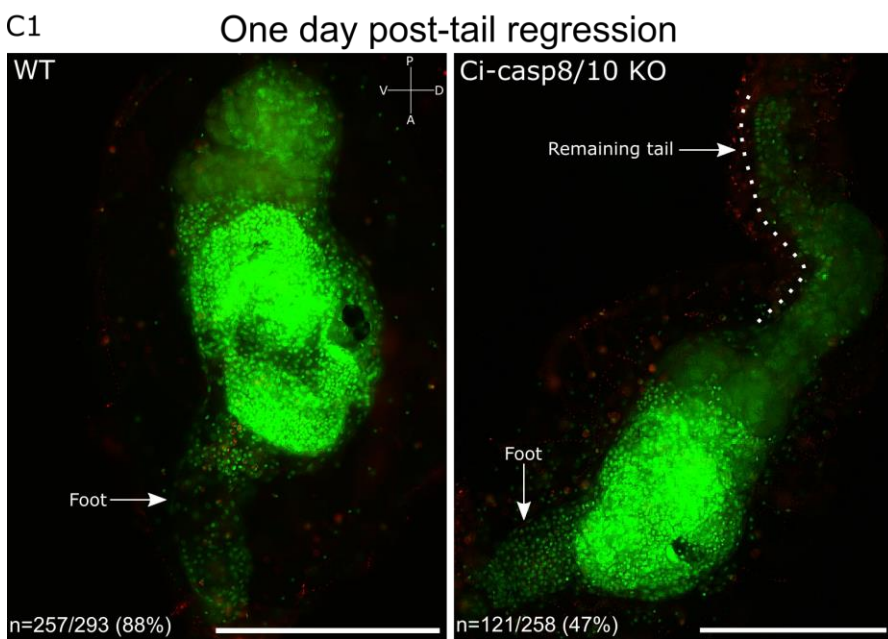
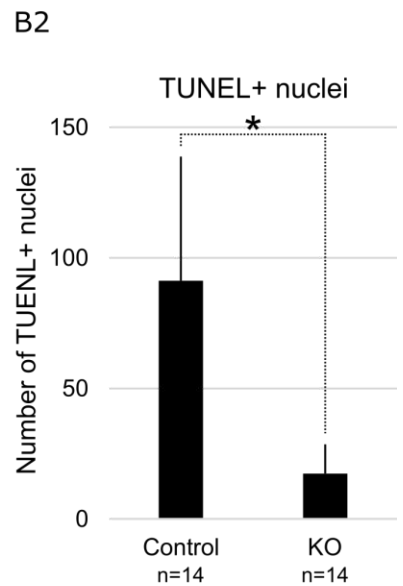
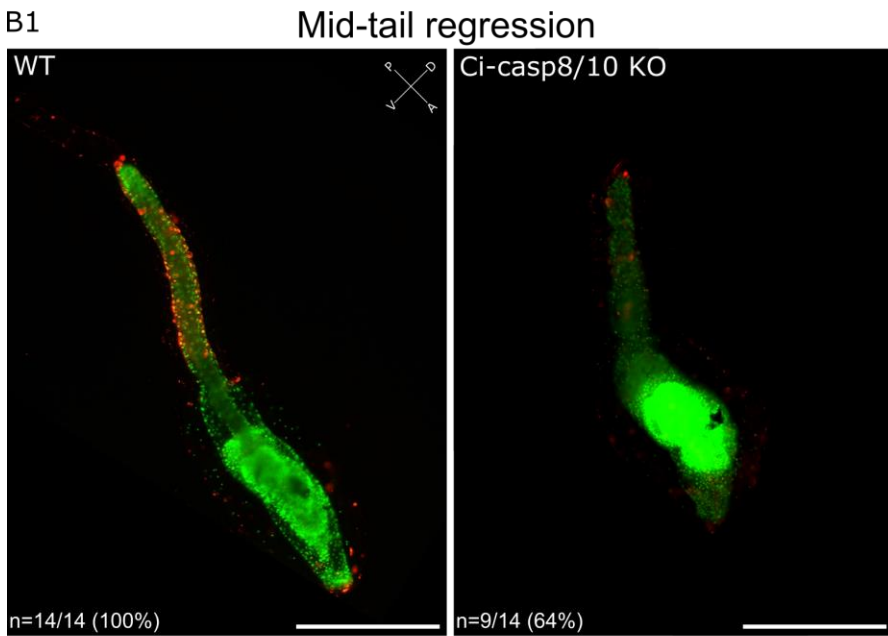
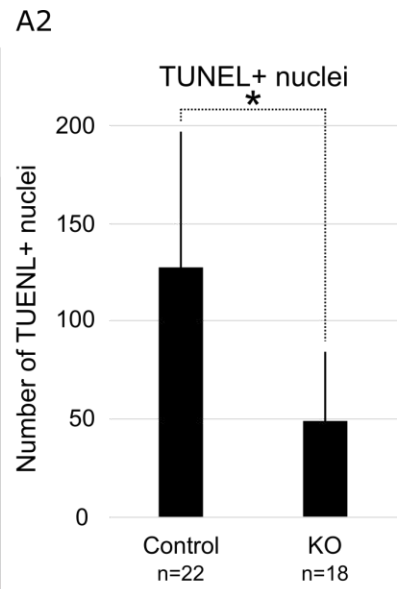
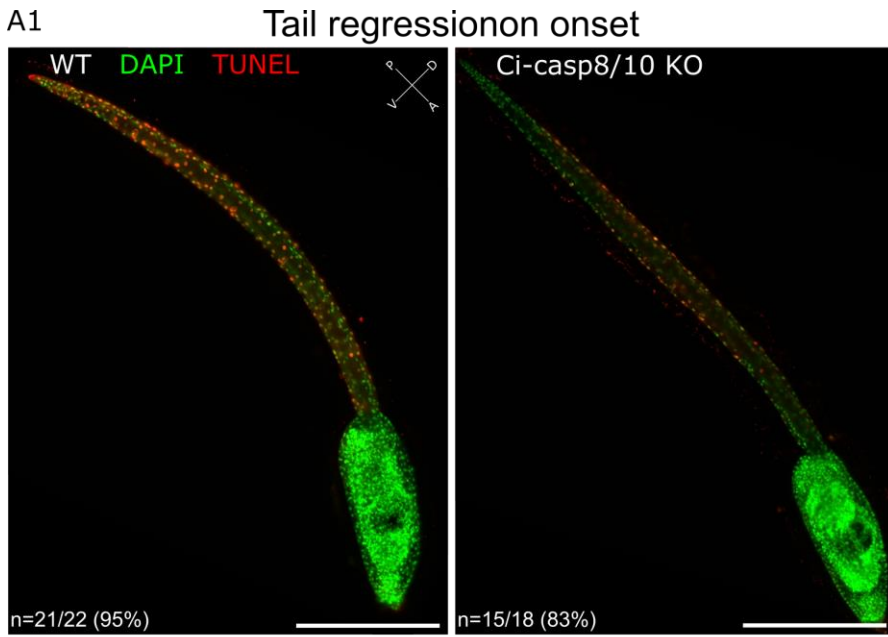
328 **Loss of function of Ci-caspase 8/10 generates an incomplete tail regression**

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^{23,24}, 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 μ 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, respectively²⁸, 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 regression²⁷. 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 larvae²⁶. 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^{7,26,27}. 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^{7,30,40}. 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^{3,11,41}. 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 regression⁷.

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 systems⁴²⁻⁴⁴. 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^{19,21,45-48} 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*⁴⁹. The larvae of
418 ascidians *Molgula occidentalis* presents a similar apoptotic profile as *Ciona* during the tail
419 regression⁴⁰. 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 complex¹⁸. In the echinoderm *Holothuria leucospilota*, over-expression of
423 FADD induces apoptosis in HEK293 human cells, suggesting conservation of its function⁵⁰. DED-
424 caspase of *Molgula tectiformis* (ascidian), *Paracentrotus lividus* (sea urchin), and
425 *Strongylocentrotus purpuratus* (sea urchin) can induce apoptosis in HeLa cells¹⁷. 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*¹⁷. 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**,
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- 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

594

595

596

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 NLGVDVENLKFLCIGLV
614 Ci_Casp.8/10 MDSE-----GEFR---LLLHQICEGLTGENVSMKFLCKDAI
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 -----MSDET PRKPAAGDV
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--ELETALDFMEYFEAKGWIQEGDLGFLAEVLYRINRH DLLKLLPGVKNRDRYEEN
637 Hs_Casp.9 -----RLLRRCRLRLVEEL-----QVDQL
638 Ci_Casp.Y -----KIIDGIESDCANKGVVEEQFQVMVRKW-----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-EVSYDELLEEDMKFSA
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---EEDSYLAGTGLSADQTS GNYL
664 Hs_Casp.2 SFSQN-VELL-----NLLPKRGPQAFDAFCE-----ALRETKQGH
665 Ci_Casp.2A RFSQN-VAFL-----TCLPTRGPRAYGTFL E-----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	QLDENLRLRLRNNYAYCTGARNFRTALELFQYL---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-----QTDVKT
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-----VMTISDS-----
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--YPEMGLCIIINKNFH
778 Hs_Casp.7 -----FSKKNVTMRSIKTTRDRVP-----TYQYNMN--FEKLGKCIINKNFD
779 Ci_Casp.3/7A -----LRQHMLEYMK--NERRGTFVVFNQENFD
780 Ci_Casp.3/7B -----SETTD-----FKYKMN--HKERGCFMIFNQEVYD
781 Ci_Casp.3/7C -----PYKQKSKVIEPEAIAVNRE-----FQYPMN--HKQRGCCIIFNQKHF
782 Ci_Casp.3/7D -----YTIT-----QKFD
783 Ci_Casp.3/7E -----PIRHPDRTTVRRELKDGDDKIEPSVWDVKFDYFEYNMS--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--RNHRGLCVIVNHNFSF
790 Ci_Casp.8/10 GFETPPPPRAQPKAPVSEIKDTSTMGII-----LDKYPME-----RRGYCLIIINNENFE
791 Hs_Casp.9 -----ESLRGNAD-----LAYILS--MEPCGHCLIIINNWNFC
792 Ci_Casp.Y -FQGNVQVLESDDMSYVTHFSK-----VGVYTVRS--KEPKGHVLIILNNYEGF
793 * :
794
795
796 Hs_Casp.1 -----DSIPRRTGAEVDITGMTLLQNLGYS-VDVKK-NLT
797 Hs_Casp.2 GE-----KELEFRSGGDVDHSTLVTLFKLLGYD-VHVLC-DQT
798 Ci_Casp.2A AG-----VRLPDRQGTADKKNLLAIFEQINYK-TILVE-NCN
799 Ci_Casp.X PE-----SDLLNREGSEKDRVRLVLLQIGFQ-CYVLI-NGT
800 Hs_Casp.3 KS-----TGMTSRSGTDVDAANLRETFRNLYE-VRNKN-DLT
801 Hs_Casp.7 KV-----TGMGVRNGTDKDAEALFKCFRSLGFD-VIVYN-DCS
802 Ci_Casp.3/7A RM-----RHPRKSGVDVEMVLKSAKLGFDVRLK-NQT
803 Ci_Casp.3/7B AKTS-----LNLGERIGSSTDARNLASAAAYLGFYIQVFN-NLT
804 Ci_Casp.3/7C AH-----LRMDTREGTNHDAENIKHSMEMLGFEHVRIIK-DST
805 Ci_Casp.3/7E KH-----LKLSSERTGSDKAAALLKTAELLGFE-TEEYL-DLE
806 Hs_Casp.6 -----YGLQQRVGSKLDRIVLEETAIKLGFT-PNVCH-DYT
807 Hs_Casp.6 WH-----LTLPERRGTCADRNLTRRFSDLGFE-VKCFN-DLK
808 Ci_Casp.6A1 DATTNEL-----ENEKIEQALQQDFDDFTELFENMQLD-VMKFGKMK
809 Ci_Casp.6A2 -----DSPETKKALLEDEDFKFLFADMRFD-VKSYSGEMK
810 Ci_Casp.6B HA-----TRMNKRSGTDVDARNLSRIFKKLGFD-VQVYK-DLS
811 Hs_Casp.8 KAREK-----VPKLHSIRDRNGTHLDAGALTTTFEELHFE-IKPHH-DCT
812 Hs_Casp.10 -----TSLKDRQGTGTHKDAEILSHVFQWLGFT-VHIIHN-NVT
813 Ci_Casp.8/10 RQVNEDEIQRRQLQMTERDFPVPN-VGLKDRGTGSGNTERLENLFKEFGFI-LDVRK-DLD
814 Hs_Casp.9 RE-----SGLRTRTGSNIDCEKLRRRFSSPHFM-VEVKG-DLT
815 Ci_Casp.Y AW-----GPDHDRKGAKRGDGELMKQLWEGFQCK-VIVKE-NRT
816 * :
817
818 Hs_Casp.1 ASDMTTELEAFahrP-EHkTSDSTFLVFMShGIR-----EGICGKKHSEQVPDILQLN
819 Hs_Casp.2 AQEMQEKLQNFaQLP-AHRVTDSCIVALLSHGVE-----GAIYGVdGK-----LLQLQ
820 Ci_Casp.2A KFEMERRVKQFAQME-EHRKCDSCAVAILSHGSK-----TDIYASDGR-----SIPIE
821 Ci_Casp.X AEQIVSTLQTFaELE-EHYyNSCSLVAAMSHGDA-----GCFYGSdGV-----SVAID
822 Hs_Casp.3 REEIVELMRdVSKED--HSKRSSFVcVLLSHGEE-----GIIFGTNG-----PVDLK
823 Hs_Casp.7 CAKMqDlLkKASEED--HTNAACFACILlSHGEE-----NVIYgKdG-----VTPiK
824 Ci_Casp.3/7A TDEIRdHLQELsYQD--HSNCDFVCVvLLSHGES-----DGVLYTKdG-----DIHLK
825 Ci_Casp.3/7B RSKiLkWLkDVAAAS--HESyDCFCGAILTHGDK-----NNDY-----IMNIT
826 Ci_Casp.3/7C KVEiLNIWAaVSKAD--HSNyDCFACVILTHGGD-----KdVLYARdD-----KMElK
827 Ci_Casp.3/7D RKQIRrKLYELSEMD--HGdHdCFACAILTHGGK-----DNIlySHdD-----EMKlK
828 Ci_Casp.3/7E KdDVyAElRkISQMN--HSyQdCFACAILTHGEE-----DEmVYAKdD-----SMKlK
829 Hs_Casp.6 AEELLKIHEVSTVS--HADADCFVCVfLShGEG-----NHiyAYDA-----KIEiQ
830 Ci_Casp.6A1 FSEAVANLENVSKLKSennYScFiCMFiGHGSd-----GCIkATdGK-----LDIHE
831 Ci_Casp.6A2 FSTVVENLEAVLEEiQRNPLIScFiCMFiGHGGP-----GFINTAdGK-----LDIHE

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831 Ci_Casp.6B CISMLEVINVESTMD--HTGNDCCFVAFLSHGDD-----GCVYGTGDG-----IVQIK
832 Hs_Casp.8 VEQIYEILKIYQLMD--HSNMDCFCICILSHGDK-----GIYGTGDGQ-----EAPIY
833 Hs_Casp.10 KVEMEMVLQKQKCNP-AHADGDCFVFCILTHGRF-----GAVYSSDEA-----LIPIR
834 Ci_Casp.8/10 QREMEKVIQIYTKKD--HSDKDCFVCMVMSHGLS-----GCVYGVVDGL-----SLSTS
835 Hs_Casp.9 AKKMVLALLELAQQD--HGALDCCVVVILSHGCQASHLQFPGAVYGTGDC-----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 SLQNKPKMFFIQACRGDETRDGVQQDGKNHAGSPGCEESDAGK----
842 Ci_Casp.2A SLIKMFDNVNCPPLRNKPKIFFIQCCRGNNDQGGIDATDGPPTLNSETSDSYNSTHNTIT
843 Ci_Casp.X TVVNFSSNQNC HSLQKPKKIFLQACQGDYDMGVDEVDPVQAPVGDVD--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 TLISRVSATECP SLAGKPKLFFVQACRKEISQPAICTTACLKRQV---QSDSIPDVD
851 Hs_Casp.6 TLTGLFKGDKCHSLVGPKKIF I IQACRGNQHDVPV I PLDVVDNQTEK---LDTNITEVD
852 Ci_Casp.6A1 NILAKFMPTHKC--LEGKPKIF I FQACRGT KKNVKS DSSLSTLCDKEI-----
853 Ci_Casp.6A2 NILSKFMPQDC--LKGKPKIF I FQCRGRTRHNFDSLACSGTLTVPTV-----
854 Ci_Casp.6B KIVDQFRGDVCP SLAGKPKIFLQACRGT L HETS VQLSVDVVDGE-----DQPCVEVD
855 Hs_Casp.8 ELTSQFTGLKCP SLAGKPKVFFIQACQGDNYQKGI PVETDSEEQPYL-----EMD
856 Hs_Casp.10 EIMSHTALQCPR LAEKPKLFFIQACQGE EIQPSVSI EADALNPEQA-----
857 Ci_Casp.8/10 KISKFRPAGMCS SLTGKPKIFFFQACQGDRTMDGHSAPVDDVESDGA-----
858 Hs_Casp.9 KIVNIFNGTSCPSLGGKPKLFFIQACGGEQKDHGFEVASTSPEDES PGSN-PEPDATPFQ
859 Ci_Casp.Y DVLALFKNTHSKYL IGRPKL I FFQCCRGGSSSRGV PFVDAADAVNRL-----LPPSDQTD
860 . . . * :*: : * * *
861 Hs_Casp.1 -----DDAIKKAHIEKDFIAFCSSTPDNVSW---RHPTMGSVFIGRLIEHM----
862 Hs_Casp.2 -----EKLPKMRLPTRSDMICGYACLKGTAA M---RNTKRGSWYIEALAQVF----
863 Ci_Casp.2A ---TDVDI I STASKRLPTSSDMI IGYATLQGNAA 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---RNSKNGSFIQALCLTF----
870 Ci_Casp.3/7D -----SYTIPVQADFLISQATAPDYAW---RSSDKGSIFIQTLCSVF----
871 Ci_Casp.3/7E -----SEETPKIPAEADILVAYSTQPGQVSV---RNEYTGSVVFVQTMCAAL----
872 Hs_Casp.6 -----AASVYTL PAGADFLM CYSVAEGYYSH---RETVNGSWYIQDLCEML----
873 Ci_Casp.6A1 -----KADGSDVLLCYA AWSGYVS YALTTNEYERGTQYLQDLHKAL----
874 Ci_Casp.6A2 -----LNPEGS DMLLCYASWQGFVSYC---VNTKKGKTCYLQELHSM L----
875 Ci_Casp.6B -----AAPVPTLP SSGSYLFCYSVAEGYYSH---RDTLYGSWFIQDFTDVMEQLV
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 I FRKVRFSFEQP-----DGRAQMP TERTVTLTRCFYLF P
884 Hs_Casp.2 ---SER-ACDM-HVADMLVKVNALIKDR-EGYAPGTEFHRCCKEMSEYCS-TLCRHLYLFP
885 Ci_Casp.2A ---AQH-AHEM-DLLEILTLVNSA I KSK-EGHSTDFDSQGVKEMSEFHS-TLCKLFFYP
886 Ci_Casp.X ---ARH-ACDT-DLLDMMTKVNNILKHK-EGWC PGSVYHSC TYLHLIPA-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 LNKGKQ-QDKV-DFIDVLT VVNRKVSARRVERSYSQQALGKQMP CYLS-MLTKKLYLTP
898 Hs_Casp.8 ---RERCPRGD-DILTILTEVNYEVS NK-----DDKKNMGKQMPQPTF-TLRKKLVFPS
899 Hs_Casp.10 ---KKLVPRHE-DILSILTA VNDVSRV D-----KQGT KQMPQPAF-TLRKKLVFPV
900 Ci_Casp.8/10 ---EKY-HEKE-DLLSIMINVNQEMASK-----PYKQMPPIA-TLRKKVFFSK
901 Hs_Casp.9 ---EQW-AHSE-DLQSLLLRVANAVSVK-----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	ETSFPAGSRAIPAVTLRKQSLPVSSMYSSSADSHLLTPNRRHLHPSETVPARLSVSPNAAR
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
1013

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1014 **Supplementary Figure 1: Alignment of *Ciona* Type A and human caspases.**

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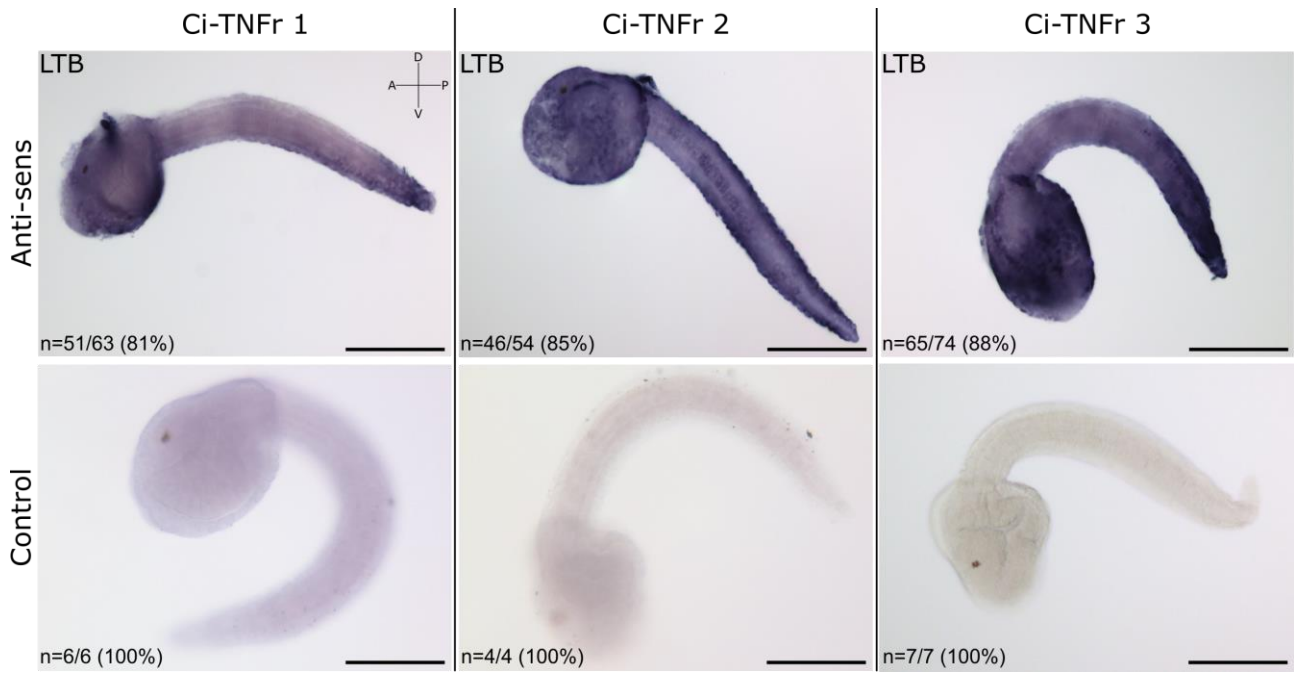
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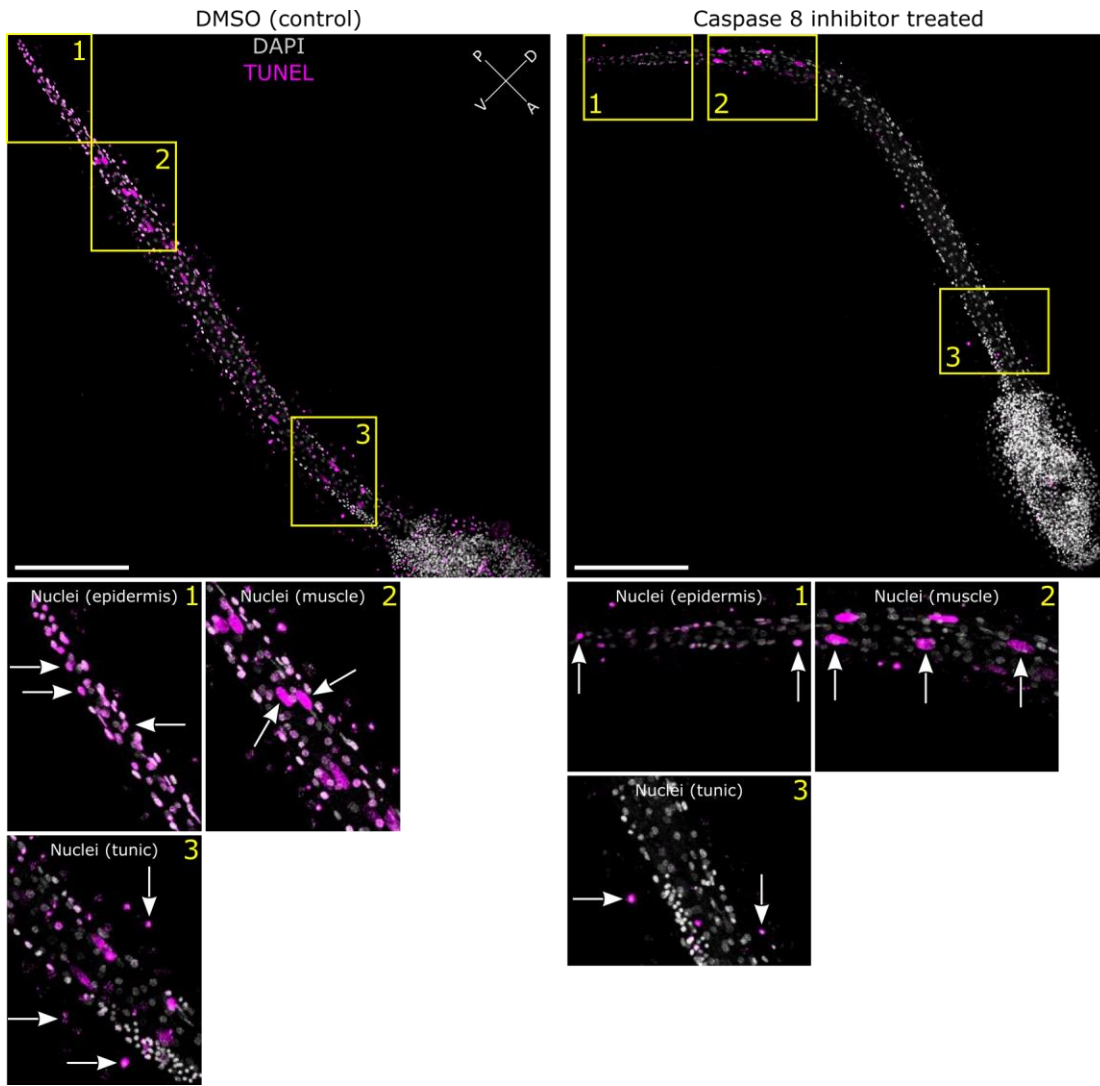
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1026 **Supplementary Figure 2:** TNF receptors are expressed in the tail during development. TNFr 1 is
 1027 mainly expressed at the tip of the tail in epidermal cells. TNFr 1 and 2 are expressed in the trunk
 1028 and the tail with a stronger expressed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

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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 μ m.

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41% of identity

Human caspase 8 CCILSHGDKGIIYGTGQEAPIYELTSQFTGLKCPKSLAGKPKVFFIQACQGDNYQKGPVETDSEEQP
Ciona caspase 8/10 .VVM...LS.CV..V..LSLSTSKISKS.RPGM.S..T...I..F.....RTMD.HSAPV.DV.SD

32% of identity

Human caspase 10 FCILTHGRFGAVYSSDEALIPREIMSHFTALQCPRLAEKPKLFFIQACQGEETQPSVSIADALNPE
Ciona caspase 8/10 CVMMS...LS.C..GV.GLSLSTSK.SKS.RPGM.SS.TG...I..F.....DRTMDGH.APV.DVESD

1038

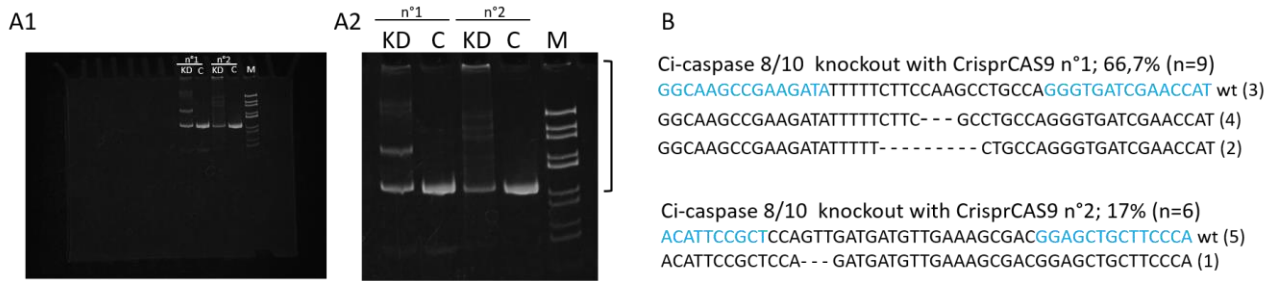
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1040 **Supplemental figure 4:** Alignments of active sites of human caspases 8 and 10 with Ci-caspase
 1041 8/10. Dots represent same amino acids between *Ciona* Type A and human caspases.

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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.

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