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Evidence for dynamic and multiple roles for huntingtin in *Ciona intestinalis.*

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Running title Dynamic role of Huntingtin

Abstract

Although mutations in the huntingtin gene (*HTT*) due to polyQ expansion cause neuropathology in humans (Huntington's disease; HD), the normal function(s) of the gene and its protein (HTT) remains obscure. With new information from recently sequenced invertebrate genomes, the study of new animal models opens the possibility of a better understanding of HTT function and its evolution. To these ends, we studied huntingtin expression pattern and dynamics in the invertebrate chordate *Ciona intestinalis. Ciona* huntingtin (*Ci-HTT*) shows a biphasic expression pattern during larval development and prior to metamorphosis. A single form of huntingtin protein product is present until the early larval stages, at which time two different mass proteins become evident in the metamorphically competent larva. An antibody against *Ci*-HTT labeled 50 cells in the trunk mesenchyme regions in prehatching and hatched larvae and probably represents the distribution of the light-form of the protein. Dual labeling with anti-*Ci*-HTT and anti aldoketoreductase confirmed the presence of *Ci*-HTT in mesenchyme cells. Suppression of *Ci-HTT* RNA by a Morpholino oligo-nucleotide reduced the number and apparent mobility of *Ci*-HTT positive cells. In *Ciona*, HTT expression has a dynamic temporal and spatial expression pattern that in ontogeny precedes metamorphosis. Although our results may reflect a derived function for the protein in pre- and post- metamorphic events in *Ciona,* we also note that as in vertebrates, there is evidence for multiple differential temporal expression, indicating that this protein probably has multiple roles in ontogeny and cell migration.

Introduction

Genes coding for the huntingtin protein have been found in all vertebrate and invertebrate genomes examined so far; Huntingtin genes (*HTT)* are present in vertebrates including mammals (Schmitt et al. 1995, Wood et al. 1996, Matsuyama et al. 2000) and fish (Baxendale et al. 1995, Karlovich et al. 1998). In invertebrates, homologues have been identified in non-vertebrate chordates such as amphioxus (Candiani et al. 2007), ascidians (Gissi et al. 2006), and in 'basal' deuterostomes such as sea urchins (Kauffman et al. 2003, Sodergren et al. 2006, Tartari et al. 2008). Homologues are also present in arthropods (Li et al. 1999), and nematodes (Wormbase sequence name F21G4.6) pointing to the universal conservation of the gene across phylogeny (see the following reviews; Sipione & Cattaneo 2001, Zuccato et al. 2010).

Despite this conservation, a detailed analysis of the protein reveals important phylogenetic differences in parts of the sequence. In vertebrates, there is an extensive poly- Q region in the n-terminus of *HTT* (expansions of which are known to cause pathology in humans (Anon 1993)). In all of the non-vertebrate animals so far examined, this region in the n-terminus is greatly reduced. In sea urchin (a basal deuterostome), HTT was detected in the genome (Kauffman et al. 2003, Sodergren et al. 2006, Idris 2007), was subsequently cloned (Tartari et al. 2008), and revealed to consist of a single Poly-Q (consisting of two repeats).   A similar state of affairs exists in the amphioxus sequence that is considered to be one of the most 'basal' living invertebrate chordates (Candiani et al. 2007).   It was therefore notable that within deuterostomes, *Ciona intestinalis* lacks two poly-Q 'repeats found in other chordates and invertebrates. Thus, it is likely that the two repeats in Amphioxus and Sea Urchin represent the 'basal' condition while *Ciona* has lost the ancestral Poly-Q repeat pattern (Idris 2007, Tartari et al. 2008). This hypothesis is partly based on the recent finding that within in the chordate phylum, tunicates (including ascidians) are likely to represent a sister group of vertebrates (Delsuc et al. 2006).

Further clues to the function of a protein may be found in its tissue specific distribution both within organisms, across development and by examining its evolutionary

trajectories. Despite its pathological effects in the nervous system, human huntingtin RNA is found in almost every organ and tissue type, although the expression level in tissues and at different developmental stages varies widely (Strong et al. 1993, Li et al. 1993, Landwehrmeyer et al. 1995, Schmitt et al. 1995). In mammals a high copy level was found in nerve ganglia, parathyroid and lymph nodes, whereas minimal expression was found in vascular and thymus tissue (NCBI UniGene UGID 909197, Hs.518450 www.ncbi.nlm.nih.gov). In invertebrates, expression data indicates that huntingtin mRNA is confined to non-neural tissues in flies and sea urchins(Kauffman et al. 2003), though it is also expressed in certain neurons in mature *Aplysia californica* (Moroz et al. 2006) and in both the neural tube and non-neural CNS cells of Amphioxus (Candiani et al. 2007).

The above considerations indicated that *Ciona* may represent at 'minimal' or derived model with which to study the function of HTT in a chordate and it seemed to us desirable to assess the function of the protein better to obtain a more complete picture of HTT evolution. To this end, we examined the expression pattern and dynamics of huntingtin in *Ciona.* We found that *HTT* has a dynamic temporal and spatial expression pattern in which one form of the protein is most evident in a population of mesenchyme – like cells before metamorphosis. Although these observations may reflect a derived state for the protein in pre- and post- metamorphic events in *Ciona,* we also note that as in vertebrates, there is evidence for multiple roles of the protein.

Materials and Methods

In situ, Immunohistochemistry and Western blotting of huntingtin

Adult Ciona intestinalis were collected from the Bay of Naples Italy and the Gullmarn Fjord Sweden in the vicinity of the Sven Lovén Centre for Marine Sciences, Kristineberg. They were maintained in tanks with running local sea water until use. Metaphase I arrested oocytes and spermatozoa were collected from different animals and fertilizated by mixing gametes in filtered seawater. Embryos were observed with a stereomicroscope and embryos of appropriate stages were identified using the morphological criteria reported by Chiba et al. (S. Chiba et al. 2004). Embryos were fixed in 4% paraformaldehyde for whole-mount In situ hybridization and immuno histochemistry; Samples were pelleted by centrifuging at 500 rpm and frozen for RNA and protein extraction. Each in-vivo experiment was performed using more than 500 embryos or larvae and repeated at least three times to confirm the results.

Total RNA isolated from the brain of adult Ciona using TRIzol reagent (Sigma, Milan, Italy) was used in preparing probes for the in situ hybridization using gene specific primers (Supplementary Table 2) from the cDNA synthesized using Random hexamers (Invitrogen, Milan, Italy). Four different probes of varying length at different regions of the huntingtin gene were used for the analysis. The probes were cloned in pCR 2.1-TOPO DUAL vector (Invitrogen, Milan, Italy) and in vitro transcription was performed for the synthesis of DIG labeled sense and anti-sense probes using DIG-RNA labelling kit (Boehringer, Mannheim, Germany). The synthesized probe was used at an appropriate concentration for the whole mount in situ hybridization on seven different stages of development, on sections such as the sections of brain complex.

Total proteins were extracted from embryos of different development stages and tissues using the BioRad Ready Prep Protein Extraction Kit (BioRad, CA, USA) for the western blot. Western blot, whole mount immunohistochemistry and Immuno histochemistry on sections were performed using the two different custom made Ciona Huntingtin protein sequence specific primary polyclonal antibodies (MCA and LCA specific for the peptide region 99-113 NH2-NSKILVELYKEINKKN-COOH and 1409-1422 NH2-

EKWKKYSRQVADV-COOH respectively from PRIMM srl, Milano, Italy). Western blots were performed using a 500 mg of total protein, Whole mount immuno histochemistry was performed on the dechorionated larva and Immuno histochemistry on tissue sections were performed on Brain complex tissue sections of 5 micron thickness using a Leica microtome.

Northern blot Assay

Total RNA of 10 μg from the mix of embryonic stages, larval stages and brain complex tissues was electrophoretically separated in a denaturing 0.8% Agarose gel containing 6% formaldehyde. The electrophoresis was performed for the different RNA samples, denatured at 90°C along with an equal volume of sample loading buffer and 0.5 μl ethidium bromide for 30 hours. The samples were run along with ssRNA ladder (NEB Biolabs, Hertfordshire, UK) under uniform power at 60 mA. Separated RNA samples in the gel were transferred by the capillary method to a nylon membrane (Hybond N+, Amersham Pharmacia, Uppsala, Sweden) overnight using 5XSSC buffer and UV cross linked. Pre-hybridization of the membrane was performed in DIG¬Easy-Hyb buffer (Roche Applied Science, Mannheim, Germany) at 55°C for 1 hour. Then, hybridization of the huntingtin gene on the membrane was performed using the cocktail mixture of antisense DIG labeled RNA probes under denaturating conditions. The hybridization was performed for 16 hours at 55°C and washed with varying stringency. The RNA-RNA hybrid on the membrane was detected using antiDIG-AP conjugate (diluted 1:2000) and BM Purple AP substrate precipitating kit (Roche Applied Science, Mannheim, Germany). The colour was developed by 20 minutes of incubation in the dark and material was washed briefly in distilled water to stop the reaction.

Real time Quantitative PCR

To estimate the huntingtin gene copy number during development and in different tissues of adult Ciona, total RNA was extracted from the seven different developmental stages and tissues of Ciona intestinalis. cDNA was synthesized from 1 μg of total RNA using random hexamers. The RT-PCR was performed using SYBR green master Mix

(Applied Biosystems, CA, USA) according to the manufacturer's protocol. Four different sets of huntingtin specific primers, specific for N, Mid 1, Mid 2 and C terminal regions of the Huntingtin gene and three sets of primers towards house- keeping genes like GAPDH and Ribosomal protein 27 and 18 as controls were used for PCR. The relative estimation of the transcript for different developmental stages and tissues were calculated from the relative estimation of Ct values of the target gene in comparison to the reference gene. Estimation of the quantification was also compared using the software called REST MCS 2006 (www.rest.gene-quantification.info). The Ct values were normalized; standard deviation and Delta Ct were calculated as per the formula of relative quantification using the control as the reference marker. Then, Delta Ct was calculated and plotted as a bar chart for the relative estimation of the level of transcript at the each stage and tissue type. The different stages and tissues on which the real time assay was performed were Gastrula, Neurula, Tailbud, Early larva, Late larva, Early juvenile, Late juvenile, 1 week adult, ganglion, neural gland, heart, muscle, gut and egg.

Gene suppression functional study

Gene suppression of huntingtin in Ciona intestinalis was performed by the microinjection of Morpholino in the dividing embryos. Eggs and sperm were obtained surgically from the gonoduct of different animals and kept in normal sea water at 18°C until dechorionation. Chemical dechorionation was performed on the eggs to remove the vitelline coat (Chorion) just before the fertilization and injection. Eggs were incubated in 1% sodium thioglycolate with 0.05% Pronase E (Protease type XIV Bacterial from Streptomycin griseous – P5147 – Sigma Aldrich, Milan, US) in filtered sea water (FSW) (pH 10.0) in agarose coated dishes for 5-6 minutes. After two washes in FSW, the eggs were fertilized. The inseminated oocytes were washed twice with FSW to remove excess sperm and to avoid polyspermy. The microinjection of the Morpholino was then performed on the inseminated eggs until the first cell division (40 minutes). The experiment was later modified with injection of Morpholino into dechorionated eggs followed by the fertilization with diluted sperm (conc. 1×106/ml) to obtain uniformity of development. The second modified method was more suitable and convenient for microinjection and development.

Microinjection was carried out as described previously by Satou et al., (Satou et al. 2001). 50 to 250 fM of Morpholino were injected along with 0.5% Rhodamine dye. The 25mer antisense oligonucleotide 5'-TGACAGACTTGACTAACTT TTCCAT-3' complementary to the huntingtin ITS (Initiation Transcription Site) region was made to order (Gene Tools, LLC, OR, USA). The control for the anti-sense morpholino oligonucleotide was synthesized as 5 mis-pair of the Morpholino oligonucleotide of the same length with the sequence 5'-TGAGAGAGTTCACTAAGTTTTCGAT-3'. The 300 nM Morpholino was diluted with sterile water to obtain the working dilution. The Morpholinos were injected using a micropipette prepared by pulling capillary tubing (Microcaps of Drummond Sci. Co., PA, USA) with an outer diameter of 1 mm, using a microelectrode puller Sutter P87 with Heat 760, pull 130, velocity 30 and Pull 130. About 5 – 10% cell volume was injected into the each of the dechorionated eggs. The microinjection was aided by stacking the dechorionated eggs against a holding pipette in a dish that was coated and dried using 10% BSA. The development of Morpholino injected embryos and their controls were monitored continuously during development. The embryos that developed into larvae and early rotation stage juveniles after microinjection were fixed in 4% PFA for immune-histochemical analysis.

Imaging

Following in situ hybridization on the whole mount and sections, material was imaged using the Axiophot inverted fluorescence microscope (Zeiss, Gottingen, Germany) using 10X, 20X and 40 X lens. For the immune- labeling, the sections and whole mount material were imaged using a 20X, 40X normal and 100X oil-immersion lens on a laser-scanning confocal microscope (Carl Zeiss micro imaging GmbH, Gottingen, Germany). The sections were viewed using the FITC/Rhodamine settings (HFT UV/488/543/633) with 30% laser power. The images were scanned at 500-530 nm for the FITC signal and the Rhodamine signal at 565-615nm. The image was calibrated against white light to normalize background fluorescence against control in every case.

Results

Huntingtin gene expression pattern during larval development and in adult tissues

Embryonic development

Huntingtin RNA was expressed in all the developmental stages of *Ciona,* from unfertilized eggs, through larval stages to adults with significant differences in the level of expression of the gene at different stages. Examination of the pattern revealed that there were different levels of expression in different cell types (Figures 1 and 2). The antisense probes against the coding region of the huntingtin gene, between exon 25 till 29 of 551 bps size (mid-region 1, probe 1) and between exon 28 till 33 of 560 bps (mid-region 2, probe 2) both showed a positive signal. Sense probes and two other antisense probes, C terminal HD and N terminal HD, did not show any staining (Figures 1 and 2). The *In situ* expression of the huntingtin gene was found from the four cell stage onwards. In 64, 110-cell and gastrula stages, expression of the gene was symmetrical in cells that give rise to; the nervous system (A8.7, A8.8, & A 8.15), mesenchyme cells (B8.5 and B7.7), TLCs (Trunk lateral Cells, A 7.6), Neural tube (A 8.5), notochord (B 8.6) and muscle (A8.61) (Figure 1). The expression of the huntingtin gene in the nervous system and mesenchyme was also confirmed in the neurula and tailbud stages where Huntingtin expression was revealed as a distinct cross-like pattern with the most significant concentrations in the mesenchyme region and less obviously in the neural tube (Figure 1). Expression of the huntingtin gene in the hatched or pre-hatched larva was found in the larval 'head' as faint spots (Figure 2) and was present in the sensory vesicle near the ocellus and otolith (with the signal absent in the sense controls). Positive signals were also seen as distinct spots in the region of the TLCs. Metamorphosed early rotation stage juveniles also showed a very faint signal (Figure 2) in the developing gut and heart.

Adult tissues

Localization of the huntingtin gene expression in adult *Ciona* nervous system was confirmed by *in situ* hybridization on sections from the *Ciona* neural complex. The huntingtin gene was expressed in the neural gland (NG), cerebral ganglion (CG) and

dorsal tubercle (DT) (Figure 3). In the ganglion (CG),   there was a positive punctuate signal around the cortex. This staining pattern confirms the presence of the gene in the cell bodies of the neurons of the cerebral ganglion and absence of the signal in the core (which consists mainly of neuropile Figure 3, E). The absence of a positive signal with the sense probe in the ganglion tissues confirmed that the pattern was due to the huntingtin gene. No conclusions could be drawn about the potential expression in the ciliated duct as there was a strong staining of duct cells in both test and controls.

Quantitative analysis of the HTT expression based on Real time PCR assay.

To better understand the changes in expression during development and in different tissues, we carried out a real-time PCR (RT-PCR) analysis. *Ciona* huntingtin expression was found in almost all of the embryonic stages and adult tissues, but the copy levels between tissues and stages were significantly different. The Ct value obtained from the target (C-terminal Huntingtin gene) and a control reference gene RPS27A (Olinski et al. 2006) were compared to give normalized expression values (Figure 4, Supplementary Table 1). The highest transcript number was found in the neural gland (NG) of the adult followed by lower levels in other tissues. In the adult, a greater number of transcripts were found in the neural gland than in the cerebral ganglion (CG). When the whole brain was considered (brain is equivalent to the CG,NG, ciliary duct and supporting tissues together), expression was lower than in the NG and CG.   Expression was also found in relatively low levels in the gut and heart and was barely detectable in mantle muscle. In the different embryonic stages of the larva, the copy number increased until the neurula stage and then decreased thereafter. The expression level of *HTT* in the egg presumably represents maternal huntingtin RNA (Figure 4).

Transcript dynamics in embryo development and adult tissues

Northern blot assays for Ciona intestinalis huntingtin transcripts at the early gastrula stage showed a single mass of approximately 12.5 Kbps. From the larval stage onwards, two dif ferent transcripts of 12.5 and 10 Kbps were detected. The brain complex of adult

Ciona intestinalis also expressed two transcripts (Figure S1).The 12 kbps transcript is equivalent to the full length huntingtin gene (8847bps) along with the noncoding 5' and 3' UTR of the gene, The transcript of around 10 kbps could represent the huntingtin gene with only 50 exons (7108bps) and the 5'UTR.

Protein expression in larval and adult stages

Next we followed the expression of the huntingtin protein at different stages using Western blotting and Immunocytochemistry (Figure 5 and Figure 6). HTT protein expression for different embryonic developmental stages with Western blots showed two different patterns using the MCA antibody (specific to the amino terminal). Embryonic development until the larval stage showed a single form of HTT of 300 KDa and two forms from the larval stage onwards of 220 and 300 KDa, which was also present in 3 week old adults (Figure 5). The LCA antibody did not detect any protein product. The 220 KDa product produced consistently stronger staining when it was present, and it thus seems likely that this fraction of the protein is that which is stained in the whole mounts (discussed in the next sections). As the heavier transcript was present in all the tissues, the fact that in tissue sections and whole mounts the pattern reflected the appearance of the lighter band reinforced this possibility. Dissection of adult tissues allowed a more detailed assessment of the tissue -specific distribution of huntingtin. In the brain, gut, ovaries and muscle, the huntingtin protein was present as a single 300 kDa band (Figure 5). The amount of protein in the muscle tissue was lower than in the brain and other tissues. It should be stressed that Western blot analysis of the developing embryos and tissues showed the huntingtin specific bands only after using 500mg total protein because of the relatively low concentration of HTT as a proportion of total protein. The expression level of the huntingtin protein for various developmental stages and tissues was estimated by normalization with ß-tubulin that showed a similar level of expression for all stages and tissues (Figure 5).

Whole mount Immuno histochemistry in larval and adult tissues

No evidence was found for expression of HTT in embryonic or other developmental stages using whole mounts, while expression of HTT was found in larval stages onwards as a punctuate signal in the 'neck' region of the larvae, mostly in the mesenchyme region and around the sensory vesicle (Figure 6). The expression of HTT was first noted around the ventral trunk region as two symmetrical clusters of cells in the hatching larva. These HTT expressing cells then apparently migrated during later-larval stages (Figure 6 B-C). As no positive expression of HTT was seen in the early embryonic stages such as 110 cell, gastrula, neurula and tailbud. It is likely that during these stages only the heavier transcript was present and that this codes for a form of the protein that the MCA antibody cannot identify in whole mounts.

The cells expressing HTT apparently migrated around the 'head' region of the animal prior to metamorphosis (Figure 6C). In the later stages of larval development, the huntingtin protein was detected around the mesenchyme regions that after metamorphosis makes some of the tissues in the adult and constitute the area around the brain complex. During the juvenile early rotation (first ascidian) stage, expression was evident as a L shaped pattern of cells between the neural ganglion and the mesenchyme region (Figure 6D). The dynamics of the HTT expressing cells was inferred by counting the number of positive cells in different planes of confocal images at each stage. As the mean number of huntingtin positive cells in the larval (51 ± 1 SEM, n = 5) and juvenile stages (55 ± 2.13 SEM, n=5) were not significantly different, we speculate in the discussion that this constancy of cell numbers represents the possibility that a fixed number of cells are mobile, rather than indicating a changing expression pattern in different cells.

To identify the HTT expressing positive cells, embryos were co-immunostained with the HTT antibody and an anti-aldoketoreductase enzyme antibody (AKR1C1). This enzyme (also called anti Oxidoreductase) has been identified as a potential marker for mesenchyme and TLC cells in a major expression study (T. Kusakabe et al. 2002). We obtained an antibody raised against the mouse version of this enzyme and tested its

capacity to stain cells in *Ciona*. We found that in the early larval stages around 50 mesenchyme cells were co-labeled by AKR1C1 and the HTT antibody (Figure 7). The pattern in the 3 week old adult shows a huntingtin signal in the brain complex region and gut (Figure 6E-F), whereas there were no signals found in the tunic and muscles.

Immunohistochemistry of HTT in adult nervous and glandular tissues

To further investigate the distribution of HTT in the adult, we carried out immuno histochemistry on semi-thin sections of *Ciona* brain complex (ganglion, neural gland complex and ciliated duct together). The results show that huntingtin protein is most abundant in the neural gland and dorsal tubercle along with the ciliated duct of the brain complex (Figure 8). HTT expression was seen a sparse pattern in the cerebral ganglion and was abundant in the neural gland, confirming the findings of the   rtPCR experiments. HTT was not seen to label neurons in the cerebral ganglion (Figure 8C and inset figure), despite the presence of RNA in the *in situ* study. The neural gland shows a strong huntingtin distribution over the glandular epithelium but not in the basal lamina of the gland. In the dorsal tubercle, the huntingtin signal was not seen in the ciliated epithelium but was present in the basal lamina. Specificity of the HTT staining in the sections was confirmed by a control assay using pre-immune serum as a primary antibody and was negative for huntingtin staining on sections, whereas the same sections showed positive staining for the β tubulin. We interpret these results above as showing that the HTT antibody stains the protein translated by the 'light' transcript

Gene suppression study

Morpholino suppression of HTT induced some notable though subtle changes in the development of larvae and in metamorphosis (Figure 9). Post-fertilization microinjection of Morpholino showed consistent results rather than injection into pre-fertilized eggs followed by fertilization. The injection of 5-mis-sense Morpholino and un-injected embryos served as dual controls and showed larva with completely normal development (such controls were carried out in parallel in each experiment).   It was also found from the morpholino suppression analysis that the expression of huntingtin protein

varied considerably in comparison with the control 5-mis-sense MO injected animals. The number of cells labeling with the HTT antibody decreased when compared to controls at the prehatching/hatching developmental stages in the MO injected developing embryos (Figure 9) and the number of cells remained consistently low in later stages. This result suggests that the MO was able to partially block production of the protein. The number of huntingtin positive cells in the morpholino injected larva was significantly less than control morpholino injected larva (significantly different at the 0.001 level in a Students t-test). Morpholino injected larvae showed 16 (±1.52 SEM, n=30) positive cells in comparison to 48 (±1.3 SEM, n = 25) cells in the controls (based on data from larvae from 3 independent fertilization batches). The other main   feature that was noted was that while there was an apparent migration of the cells in the control morpholino larvae (Figure 9B) similar to that seen in control larvae, in the morpholino injected larvae, there was very little displacement of the signal from the junction of the trunk with the tail (trunk lateral cell poutches) in morpholino antisense larvae (Figure 9 A) n=30.

Rescue of the Morpholino was not performed because of the difficulty in making the full length huntingtin construct for microinjection. The immuno histochemical analysis of the morpholino oligonucleotide suppressed larvae confirmed, in part, the partial absence of the huntingtin gene due to suppression. Complete suppression of huntingtin expression was ruled out in these experiments as there was always a minimal number huntingtin positive stained cells.

Discussion

In this study, we examined the anatomical expression pattern of *HTT* RNA as well as the translated protein during the development of *Ciona intestinalis* embryos, larvae and adults. Expression analysis by *In situ* hybridization and real-time PCR revealed that the huntingtin gene is expressed in neuronal and in some non-neuronal tissues. In humans, rodents and *Fugu,* the huntingtin gene is expressed as two differently sized transcripts in later embryonic development 13.0 and 10.0 kbps respectively (Lin et al. 1993, Schmitt et al. 1995). It must be of significance then that Huntingtin expression alters, and two proteins of different molecular weight are evident in *Ciona* prior to metamorphosis. The two different molecular weights of the protein seen in Western Blots, must be contrasted with the pattern seen in immunohistochemistry where the antibody only detected a pattern consistent with the labeling of the lighter version of the protein. We can only speculate that the lack of staining of HTT with the antibody at other stages reflects a lack of access of the antibody when fixed *in situ*. This was not the cases in the Western bots. In general, these changes stress the importance of the huntingtin protein during development (particularly between the early and late larval stages) where it is translated from the differentially expressed huntingtin form through differential polyadenylation or alternative splicing as in the case of the human huntingtin gene (Lin et al. 1993).

The differential expression and changes in the form of the protein both in development and in the stages directly prior to metamorphosis, suggests a parallel with the dual impact in vertebrates including mammals of huntingtin; first acting on early development (Lumsden et al. 2007) and later in mature individuals due to HD pathology (Anon 1993). Certainly, the expression of *Ciona* huntingtin protein and the possible migration of HTT positive mesenchyme cells indicates a role in development especially during the larval stages and immediately prior to metamorphosis.

The dynamic reorganization of the huntingtin labeled cells prior to, during, and after metamorphosis was confirmed from the expression of the protein as two different forms from the Western blots carried out on late larval stages. This is likely to be due to the presence of the two different transcripts during development due to alternative splicing of two polyadenylation sites in exon 50 of the huntingtin gene as suggested by Gissi et al

2006, who predicted 12.5 and 10 kbps transcripts. The presence of the two transcripts is unlikely to be due to degradation during apoptosis as we blocked metamorphosis by maintaining larvae on agar coated petridishes and found that the two transcripts were maintained (data not shown).

The larval huntingtin protein becomes part of the 'neurohypophysial' complex in the late larval stages followed by the Juvenile stages (Rotation of the body axes stage) as an L shaped pattern. The neurohypophysial duct, where the huntingtin protein is expressed, differentiates into the neural gland rudiment and the dorsal wall begins to proliferate as neuroblasts. These converge to form the cerebral ganglion. In the adult, huntingtin protein was found in the neural gland but not in the cortex of the ganglion. This evidence suggests that it is likely that the 'light' form of the huntingtin protein is associated strongly with the dynamic changes during metamorphosis in *Ciona* and does not have a role in the nervous system of the adult (neither that of the larvae). This could explain some of the differences seen in the literature where (by using in situ hybridization), it has been observed that HTT is apparently absent from neuronal tissues in some invertebrates (Kauffman et al. 2003). In fact, the different protein masses may play two different, as yet unknown, roles and have different spatial and temporal expression patterns.

The concentration of the huntingtin protein in early rotation stage juveniles makes an interesting comparison with the huntingtin mRNA pattern that was very low in similar stage juveniles. The Western blots showed that the protein in early rotation stage juveniles matures through post transcriptional modification (Alternative Polyadenylation splicing) or modification driven by some other factors in *HTT*, to produce the two forms of the HTT protein.

Cell counts show that the HTT positive cells in *Ciona* precede and survive the transition from larva to adult. Metamorphosis in Ciona is a dynamic event where there is cell proliferation, remodeling and cell death. Recent work has shown that the majority of the neurons in the larval nervous system of *Ciona* (apart from some interneurons in the tail) survive metamorphosis (Horie et al. 2011). A small population of cells in the neck region of the larval nervous system become a motoneurone population in the adult CNS

(Dufour et al. 2006) and TLCs in the larva are neural stem cells that also contribute to the adult CNS formation (Jeffery et al. 2008). As the HTT positive cell numbers were constant form late larva to juvenile, we may conclude that the HTT positive cells were not subject to either programmed cell death or proliferation. Though other nearby cells are clearly subjected to both processes during metamorphosis.

Interpretation of Morpholino gene suppression experiments

In vertebrates genetic manipulation has given some clues to the function of HTT. For example, in transgenic mouse experiments, huntingtin has been shown to play a role in early development, as homozygous null mice exhibit developmental defects and display an increase in apoptosis in the embryonic ectoderm (Nasir et al. 1995). Knockdown of huntingtin expression in zebrafish resulted in a variety of developmental defects such as hypochromic blood due to decreased hemoglobin (Lumsden et al. 2007). In the mouse, effects of knockdown include lethality, neurological deficits, neurodegeneration and impaired spermatogenesis suggesting that huntingtin is required in both embryonic and post embryonic stages (Nasir et al. 1995, Zeitlin et al. 1995, White et al. 1997). Further evidence for a post embryonic role comes from study of the pathology of HTT, as when protein function is impaired by repeat expansion the effects are chronic and in the later stages of life causes Huntington's chorea (Anon 1993). It has been suggested that HD pathology could be caused by a loss of function of the normal HTT protein as much as by a gain of pathological function (see; Zuccato et al. 2010). Several studies in vertebrates thus point to the neuroprotective role of normal HTT (Rigamonti et al. 2000, Rigamonti et al. 2001, Ho et al. 2001), in which removal of HTT or experimental manipulation results in an increased rate of cell death. It is intriguing that HTT partial suppression by morpholino in the present experiments results in a decreased HTT positive cell population consistent with a similar protective role of HTT. However, without parallel analysis of apoptosis in the larva it is not possible to attribute the decreased numbers to cell death *per se*.

Mesenchyme cells and the HTT –positive population

The results of this study have important implications for our understanding of the function and nature of mesenchyme and associated cells. There are apparently around 900 such cells in the tadpole larvae derived from different lines or lineages (A7.6, B8.5, and B7.7) in the 110-cell stage embryo (Tokuoka et al. 2004) These cells give rise to the adult body after metamorphosis and their migration in the early to metamorphically competent larva has been reported earlier (Satoh 1994, Swalla et al. 1994). Our HTT antibody labeled only 50 cells that comprise a relatively small fraction of the mesenchyme cell total. Of the various subsets of mesenchyme cells, HTT -positive cells resemble most closely the trunk lateral cells (TLCs). TLCs are subset of mesenchyme cells that arise from the A7.6 blastomeres and have been shown to actively migrate in the pre-metamorphic larva. They appear on or just before hatching, and have been defined as neural crest- like cells due to their behavior and positivity to the HNK-1 antigen (Jeffery et al. 2008). The numbers of TLCs and HTT positive cells are similar (although not identical; 28 in Jeffery et al. 2008, vs 50 in the present study) and in disruption experiments, the TLCs lack of migration was noted (Jeffery et al. 2008). Considering the role of neural crest cells in development and phylogeny and the importance of HD pathology, the relationship between the HNK-1 and HTT should be further investigated.

Other functions of the mesenchyme cells that should be noted include their role in the remodeling of the larval body prior to and during metamorphosis from the larva to the adult. During this process, programmed cell death is activated (Tarallo & Sordino 2004, Chambon et al. 2007, Takada et al. 2005) and cell debris and their by-products are processed and recycled. It has been noted that a population of the mesenchyme cells are autophagocytic (Mancuso & Dolcemascolo 1981) and may play a role in this restructuring process. Indeed, autophagy related genes have now been established to be present in *Ciona* (Godefroy et al. 2009) . Also during *HTT* pathology, and under experimental conditions, autophagy of HTT related debris has been observed and is now considered to be one of many important mechanisms in the neuropathology of the disease (Kegel et al. 2000, Winslow & Rubinsztein 2008). This feature and HTT-positive cells could perhaps deserve more attention in future work on *Ciona*.

Conclusions

In *Ciona*, *HTT* expression has a dynamic temporal and spatial expression pattern that precedes metamorphosis in ontogeny. Although our results may reflect a derived role for the protein in pre and post- metamorphic events we also note that as in vertebrates, there is evidence for multiple differential expression events as well as differential expression of two forms of the protein, which presumably indicates multiple roles in ontogeny. We note some parallels between HTT positive cells, the TLCs, migration, possible cell- protection, autophagy and tissue remodeling that should be further investigated.

Abbreviations

HTT, Huntington protein; *HTT,* Huntingtin gene; MO, Morpholino Oligo nucleotide.

Authors contributions

MMI, and ERB designed the initial experiments and consulted with MT. MMI carried out the experiments, and MMI and ERB analyzed the data. MMI drafted the paper and ERB finalized it. MMI, MT, and ERB edited and approved the paper.

References

Anon, 1993. A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. The Huntington's Disease Collaborative Research Group. *Cell*, 72(6), 971–983.

Baxendale, S. et al., 1995. Comparative sequence analysis of the human and pufferfish Huntington's disease genes. *Nature Genetics*, 10(1), 67–76.

Candiani, S. et al., 2007. Characterization, developmental expression and evolutionary features of the huntingtin gene in the amphioxus Branchiostoma floridae. *BMC Developmental Biology*, 7,.127.

Chambon, J.-P. et al., 2007. ERK- and JNK-signalling regulate gene networks that stimulate metamorphosis and apoptosis in tail tissues of ascidian tadpoles. *Development (Cambridge, England)*, 134(6),.1203–1219.

Chiba, S. et al., 2004. Development of Ciona intestinalis juveniles (through 2nd ascidian stage). *Zoological Science*, 21(3), 285–298.

Delsuc, F. et al., 2006. Tunicates and not cephalochordates are the closest living relatives of vertebrates. *Nature*, 439(7079), 965–968.

Dufour, H.D. et al., 2006. Precraniate origin of cranial motoneurons. *Proceedings of the National Academy of Sciences of the United States of America*, 103(23), 8727–8732.

Gissi, C. et al., 2006. Huntingtin gene evolution in Chordata and its peculiar features in the ascidian Ciona genus. *BMC Genomics*, 7, 288.

Godefroy, N. et al., 2009. Identification of autophagy genes in Ciona intestinalis: a new experimental model to study autophagy mechanism. *Autophagy*, 5(6), 805–815.

Ho, L.W. et al., 2001. Wild type Huntingtin reduces the cellular toxicity of mutant Huntingtin in mammalian cell models of Huntington's disease. *Journal of Medical Genetics*, 38(7), 450–452.

Horie, T. et al., 2011. Ependymal cells of chordate larvae are stem-like cells that form the adult nervous system. *Nature*, 469(7331), 525–528.

Hotta, K. et al., 2007. A web-based interactive developmental table for the ascidian Ciona intestinalis, including 3D real-image embryo reconstructions: I. From fertilized egg to hatching larva. *Developmental Dynamics: An Official Publication of the American Association of Anatomists*, 236(7), 1790–1805.

Idris, M.M., 2007. *Evolution, genetics and function of huntingtin gene in Ciona intestinalis.* PhD. London UK: Open University.

Jeffery, William R et al., 2008. Trunk lateral cells are neural crest-like cells in the ascidian Ciona intestinalis: insights into the ancestry and evolution of the neural crest. *Developmental Biology*, 324(1), 152–160.

Karlovich, C.A. et al., 1998. Characterization of the Huntington's disease (HD) gene homologue in the zebrafish Danio rerio. *Gene*, 217(1-2), 117–125.

Kauffman, J.S. et al., 2003. Neural expression of the Huntington's disease gene as a chordate evolutionary novelty. *Journal of Experimental Zoology. Part B, Molecular and Developmental Evolution*, 297(1), 57–64.

Kegel, K.B. et al., 2000. Huntingtin expression stimulates endosomal-lysosomal activity, endosome tubulation, and autophagy. *The Journal of Neuroscience.* 20(19), 7268–7278.

Kusakabe, T. et al., 2002. Gene expression profiles in tadpole larvae of Ciona intestinalis. *Developmental Biology*, 242(2), 188–203.

Landwehrmeyer, G.B. et al., 1995. Huntington's disease gene: regional and cellular expression in brain of normal and affected individuals. *Annals of Neurology*, 37(2), 218–230.

Li, S.H. et al., 1993. Huntington's disease gene (IT15) is widely expressed in human and rat tissues. *Neuron*, 11(5), 985–993.

Li, Z. et al., 1999. A putative Drosophila homolog of the Huntington's disease gene. *Human Molecular Genetics*, 8(9), 1807–1815.

Lin, B. et al., 1993. Differential 3' polyadenylation of the Huntington disease gene results in two mRNA species with variable tissue expression. *Human Molecular Genetics*, 2(10), 1541–1545.

Lumsden, A.L. et al., 2007. Huntingtin-deficient zebrafish exhibit defects in iron utilization and development. *Human Molecular Genetics*, 16(16), 1905–1920.

Mancuso, V. & Dolcemascolo, G., 1981. Autophagic activity in the mesenchyme cells of Ciona embryo. *Acta Embryologiae Et Morphologiae Experimentalis (Halocynthia Association)*, 2(1), 3–13.

Matsuyama, N. et al., 2000. Identification and characterization of the miniature pig Huntington's disease gene homolog: evidence for conservation and polymorphism in the CAG triplet repeat. *Genomics*, 69(1), 72–85.

Moroz, L.L. et al., 2006. Neuronal transcriptome of Aplysia: neuronal compartments and circuitry. *Cell*, 127(7), 1453–1467.

Nasir, J. et al., 1995. Targeted disruption of the Huntington's disease gene results in embryonic lethality and behavioral and morphological changes in heterozygotes. *Cell*, 81(5), 811–823.

Olinski, R.P. et al., 2006. Three insulin-relaxin-like genes in Ciona intestinalis. *Peptides*, 27(11), 2535–2546.

Rigamonti, D. et al., 2001. Huntingtin's neuroprotective activity occurs via inhibition of procaspase-9 processing. *The Journal of Biological Chemistry*, 276(18), 14545–14548.

Rigamonti, D. et al., 2000. Wild-type huntingtin protects from apoptosis upstream of caspase-3. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 20(10), 3705–3713.

Satoh, N, 1994. *Developmental biology of ascidians*, Cambridge UK: Cambridge Univesity Press.

Satou, Y, Imai, K S & Satoh, N, 2001. Action of morpholinos in Ciona embryos. *Genesis (New York, N.Y.: 2000)*, 30(3), 103–106.

Schmitt, I. et al., 1995. Expression of the Huntington disease gene in rodents: cloning the rat homologue and evidence for downregulation in non-neuronal tissues during development. *Human Molecular Genetics*, 4(7), 1173–1182.

Sipione, S. & Cattaneo, E, 2001. Modeling Huntington's disease in cells, flies, and mice. *Molecular Neurobiology*, 23(1), 21–51.

Sodergren, E. et al., 2006. The genome of the sea urchin Strongylocentrotus purpuratus. *Science.* 314(5801), 941–952.

Strong, T.V. et al., 1993. Widespread expression of the human and rat Huntington's disease gene in brain and nonneural tissues. *Nature Genetics*, 5(3), 259–265.

Swalla, B.J. et al., 1994. Heterochronic expression of an adult muscle actin gene during ascidian larval development. *Developmental Genetics*, 15(1), 51–63.

Takada, N. et al., 2005. The cell death machinery controlled by Bax and Bcl-XL is evolutionarily conserved in Ciona intestinalis. *Apoptosis* 10(6), 1211–1220.

Tarallo, R. & Sordino, P., 2004. Time course of programmed cell death in Ciona intestinalis in relation to mitotic activity and MAPK signaling. *Developmental Dynamics*, 230(2), 251–262.

Tartari, M. et al., 2008. Phylogenetic comparison of huntingtin homologues reveals the appearance of a primitive polyQ in sea urchin. *Molecular Biology and Evolution*, 25(2), 330–338.

Tokuoka, M. et al., 2004. Three distinct lineages of mesenchymal cells in Ciona intestinalis embryos demonstrated by specific gene expression. *Developmental Biology*, 274(1), 211–224.

White, J.K. et al., 1997. Huntingtin is required for neurogenesis and is not impaired by the Huntington's disease CAG expansion. *Nature Genetics*, 17(4), 404–410.

Winslow, A.R. & Rubinsztein, D. C, 2008. Autophagy in neurodegeneration and development. *Biochimica Et Biophysica Acta*, 1782(12), 723–729.

Wood, J.D. et al., 1996. Partial characterisation of murine huntingtin and apparent variations in the subcellular localisation of huntingtin in human, mouse and rat brain. *Human Molecular Genetics*, 5(4), 481–487.

Zeitlin, S. et al., 1995. Increased apoptosis and early embryonic lethality in mice nullizygous for the Huntington's disease gene homologue. *Nature Genetics*, 11(2), 155–163.

Zuccato, C, Valenza, M. & Cattaneo, E, 2010. Molecular mechanisms and potential therapeutic targets in Huntington's disease. *Physiological Reviews*, 90(3), 905–981.

Figure legends

Figure 1. Expression of huntingtin mRNA during *Ciona* development revealed by whole-mount in situ hybridization. In the first column the embryonic stage is shown diagrammatically based on images from the database http://chordate.bpni.bio.keio.ac.jp/faba/1.1/top.html (Hotta et al. 2007), with the expression signal of key regions shown in color. The second and third columns show embryos hybridized with antisense and sense probes respectively. The last column describes the detail of the expression pattern of huntingtin mRNA. (A) Four cell stage, the signal is seen in all four cells. (B) Eight cell stage, the signal is observed in all the cells. (C) 110 cell Gastrula stage, the signal was present in cells that give rise to the nervous system (A8.15, A8.8, A 8.7), the mesenchyme (B8.5 & B7.7), the muscle (A8.16), trunk lateral cells (B7.6), endoderm (A 7.2), neural tube (A8.5) and notocord (B8.6)., (D) Neurula stage, a positive signal was prominent in mesenchyme and neural tube regions. (E) Early tailbud stage.

Figure 2. Whole-mount in situ hybridization of huntingtin mRNA during *Ciona* development. *Ciona* larvae (Left panel) and juvenile (Right panel) against antisense probe and sense probes. (A) Larval 'head', the signal is seen in the sensory vesicle region, laterally to the future palps (red arrows) and around the mesenchyme region as spots. (B) Larval 'head', another view. (C) Lack of reaction to sense probe. Juvenile rotation stage *Ciona* (D), the transcript of the huntingtin seen in all the tissues. (E) Juvenile *Ciona*, dorsal view, (F) Juvenile with sense probe without any signal.

Figure 3. In-situ hybridization of huntingtin gene in adult brain sections. The sections were hybridized with the antisense huntingtin specific probes 1 and 2 (A-F). A. Neural gland and dorsal tubercle , B. dorsal tubercle ,C. neural gland along with cerebral ganglion, D. Neural gland another view, E. Cerebral ganglion, F. Neural gland another view. G-I Ganglion and neural gland, as controls on hybridization with sense probe. Red

arrow indicates the presence of background staining in the cilia of the ciliated duct in both test and controls. GG – Cerebral ganglion, NG- Neural gland, CD – Ciliated duct and DT – Dorsal tubercle

Figure 4. Real-time PCR assay of relative quantity of huntingtin expression. The graph represents the Delta Ct Ct estimation of relative quantity of the target huntingtin expression against base-line of RPS27A housekeeping genes (see supplementary Table 1 for details). The graph was plotted using Origin software against different tissues at each stage in the X-axis and the delta Ct Ct plotted on the Y axis. The bars represent the standard error of the mean for each value.

Figure 5. Western blots of larval and adult stages and tissues. A, The upper band represents 300 kDa and the lower 220 kDa. Lane thirteen contains the broad range pre -stained marker (the band represents 205 kDa). The lower panels indicate the control β-tubulin signal of approximately 45 kDa for each sample. B, Greyscale densities of 330 and 220 KDa bands from each lane normalized to the tubulin signal.

Figure 6. Whole mount immuno histochemistry for the huntingtin protein in *Ciona intestinalis* embryos. A, Mid Tailbud. B, Late tail bud stage. C, Late larva. D, Early Juvenile (First ascidian stage). E, The siphon and brain of a young adult (3 weeks old). F, Brain complex of 3 week old adult *Ciona*. The huntingtin protein signal is shown as green fluorescence in the first column and the superimposed view of the green fluorescence with bright -field is seen in the second column. The signal of the huntingtin proteins are symmetrical in the embryos and are mostly localized in the ventral trunk mesenchyme regions. The red arrow points to the siphon of the adult and the Yellow arrow indicates the adult brain ganglia.

Figure 7. Dual whole-mount immunohistochemistry for huntingtin and AKR1C1 protein. A, Larval stage, all the cells are labeled for both markers. B, Late larval stage and Juvenile stages.

Figure 8. Immunohistochemistry on *Ciona* Brain Complex sections. Column one, *Ciona* huntingtin specific signal, column two *Ciona* ß-tubulin specific signal and column three, superimposition of column one and two. Row A; the ganglion (GG) and neural gland to the right of the NG. Row B, The neural gland. Row C. The ganglion/neural gland at higher magnification, D. Negative control. GG – Ganglion; NG – Neural gland; DT- Dorsal tubercle; CD – Ciliated duct; Yellow arrow points to single neuron. Inset C3 shows a 2X view of neuron staining in the ganglion for ß-tubulin antibody.

Figure 9. Immunohistochemistry on morpholino and control injected larva used as a means of estimating the distribution of positive huntingtin protein signal in the; A, Morpholino (M+) and B, Morpholino control (M-) larvae. Top row; dorso-ventral view shows both the side of the trunk and ventral staining. The number of positive huntingtin cells is ~15 in the morpholino microinjected larval 'head', whereas in the control (bottom row) the signal was seen in ~50 cells.

Supplementary Figure 1. Northen gel blot of 1-Early embryonic stage, 2- Late larval stage, 3- Brain complex of adult Ciona and 4- ss RNA Ladder (NEB). There is a single prominent band of approximately 11 kb in lane 1 and two bands of 11 kb and 12.5 kb in lane 2. Arrows indicate the bands, numbers to the side of the gel image indicate locations of known size markers.

Supplementary Table 1. Table showing mean Ct values for *HTT* RNA and a housekeeping (RPS27a) gene along with delta Ct, std delta Ct, Delta Ct Ct and SEM for different adult Ciona tissues and developmental stages used for the expression analysis based on Real time PCR.

Supplementary Table 2. List of oligonucleotides used for probe synthesis for *in situ* expression analysis. The HTT specific oligonucleotides were designed to span Htt regions with different product sizes.

Figure 1
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Figure 1
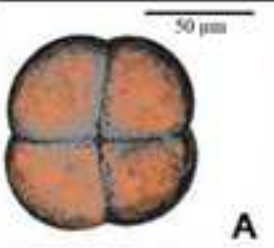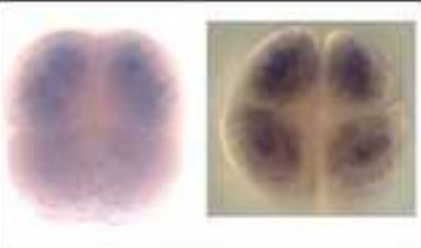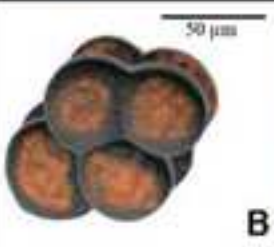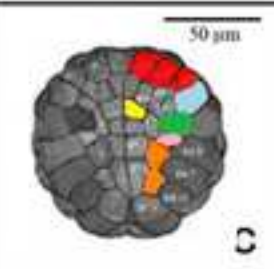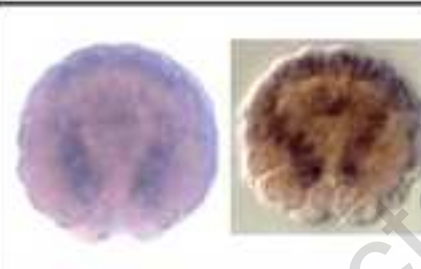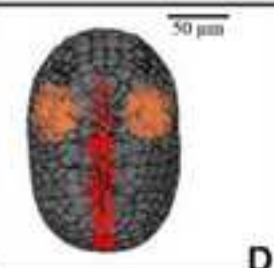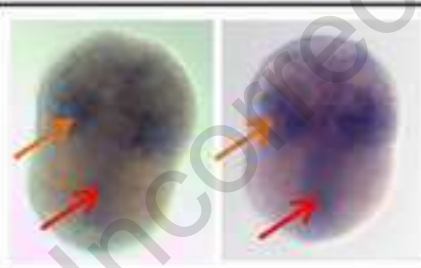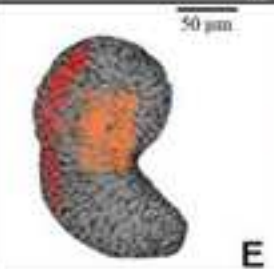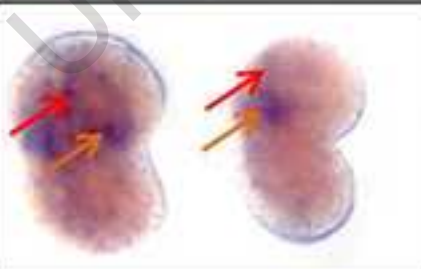
Embryo Stage	Antisense (probes 1 and 2)	sense	Stage/Staining pattern
A			4 cell- stage/all cells
B			8 cell -stage/all cells
C			Gastrula -stage/cells A8.15, A8.8, A 8.7 (NS), B8.5 & B7.7 (Mes), A8.16 (Muscle), B7.6 (TLC) A 7.2 (Endoderm), A8.5 (neural tube), B8.6 (notocord).
D			Neurula- stage/positive cells mesenchyme and neural tube
E			Early tailbud -stage /positive Cells in mesenchyme and neural tube

Figure 2

Figure 3
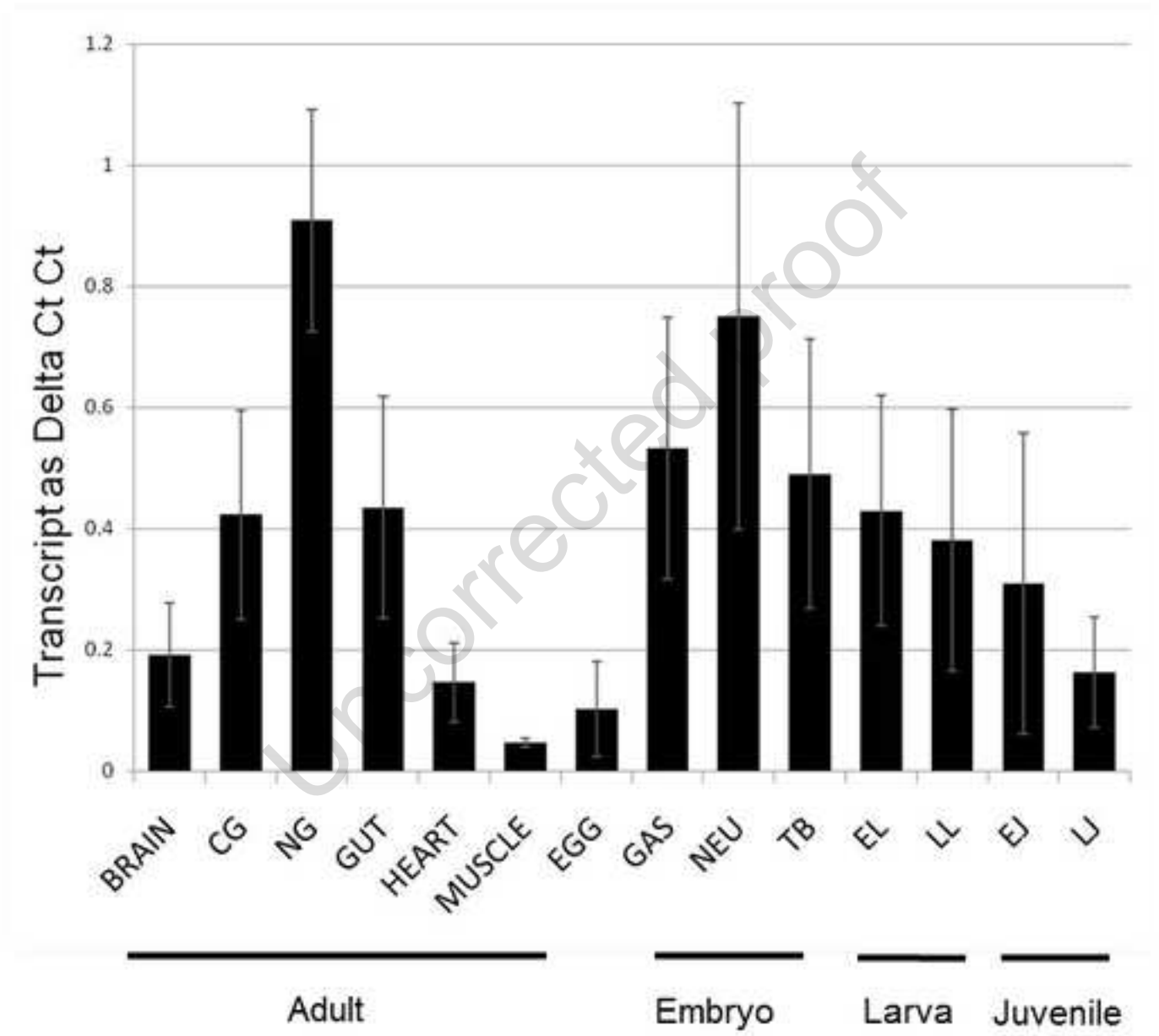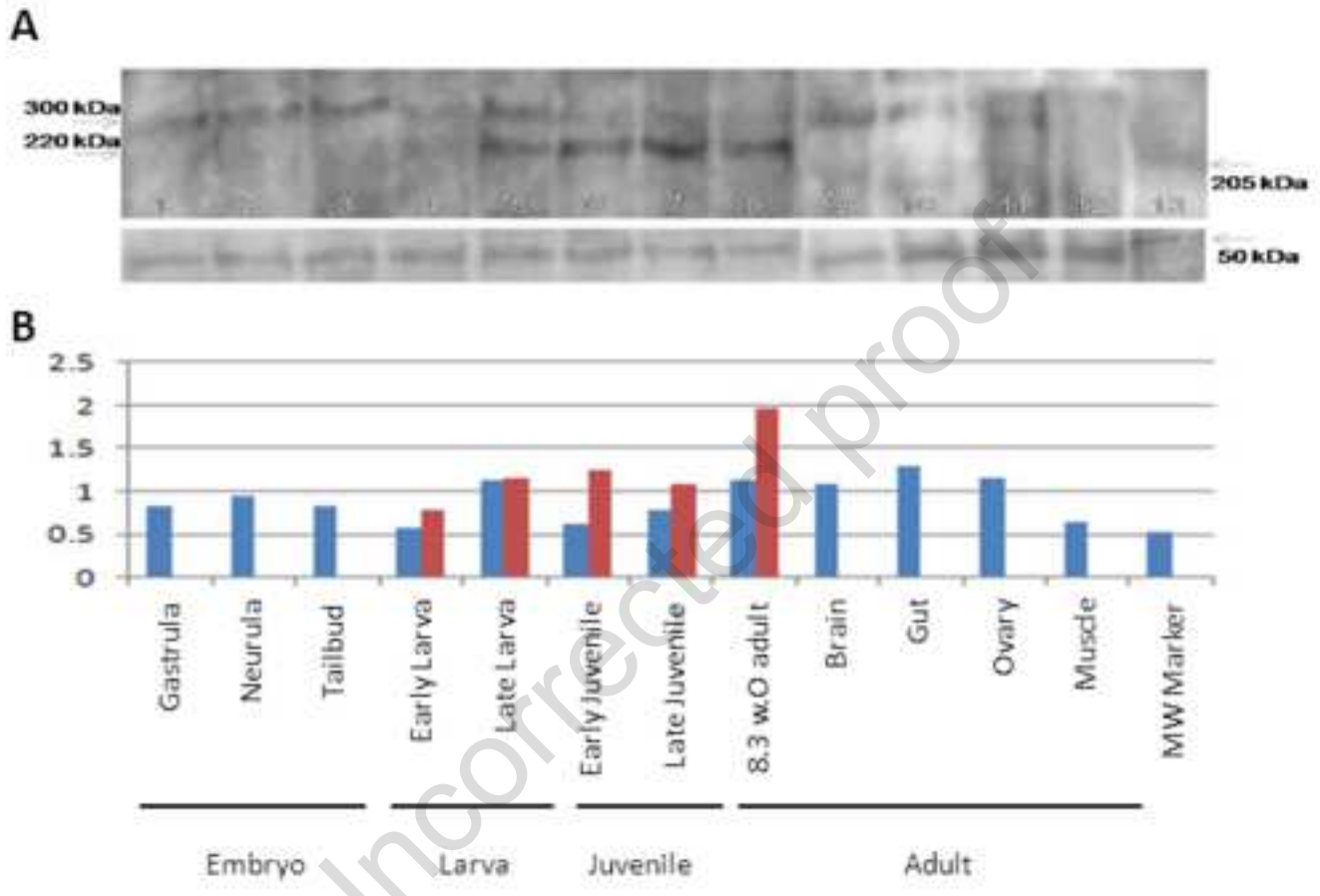Click here to download high resolution image

Figure 3

Figure 4
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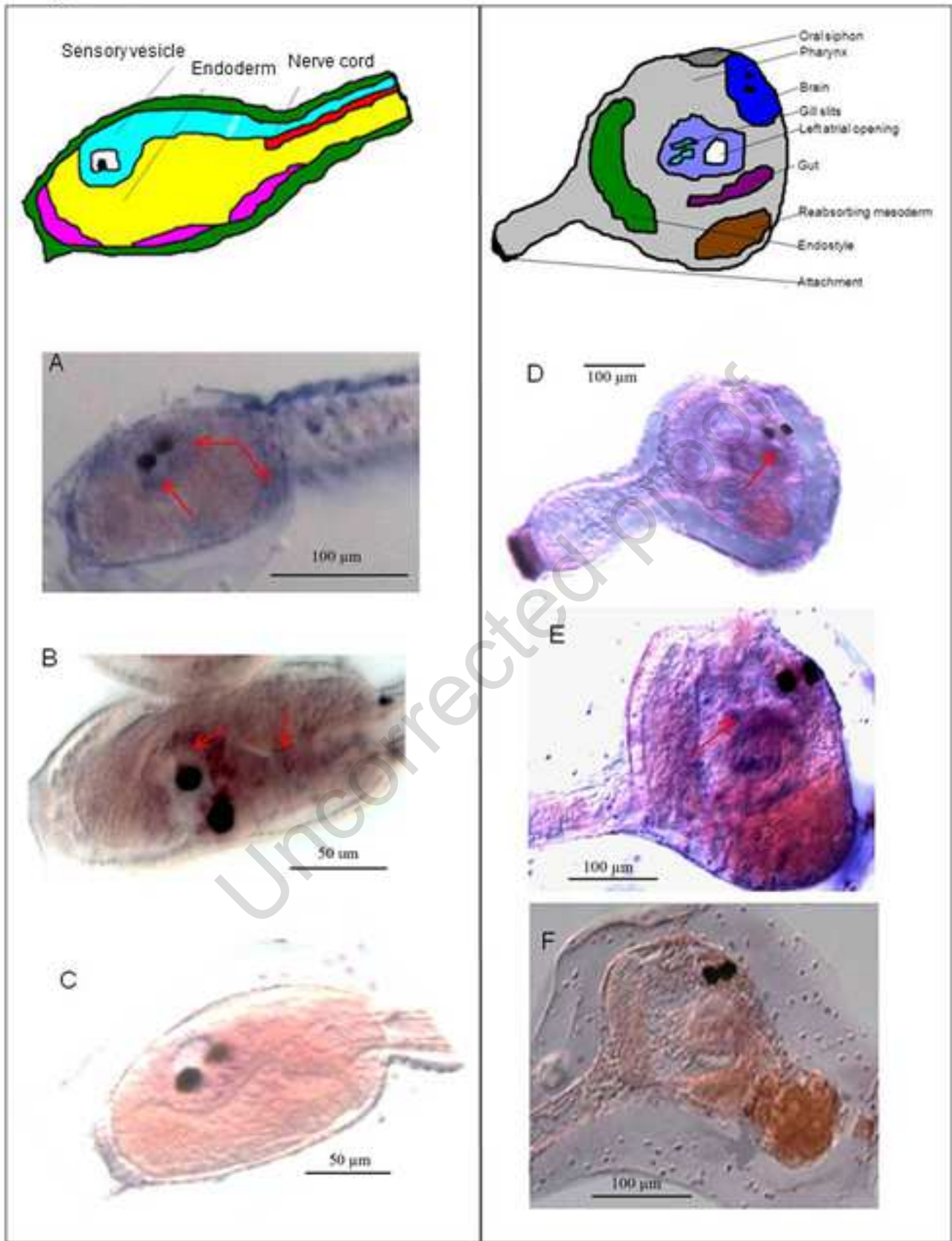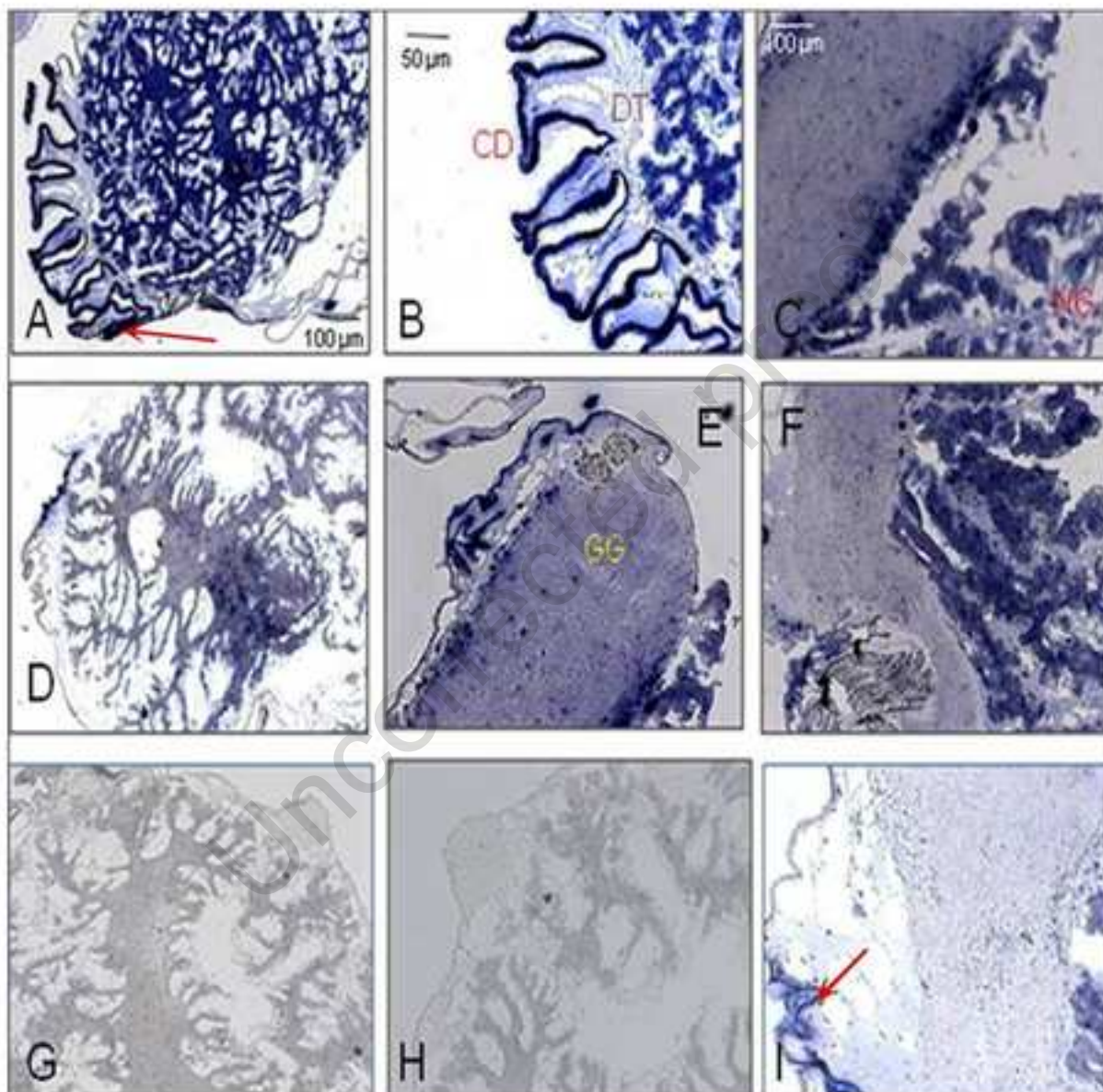
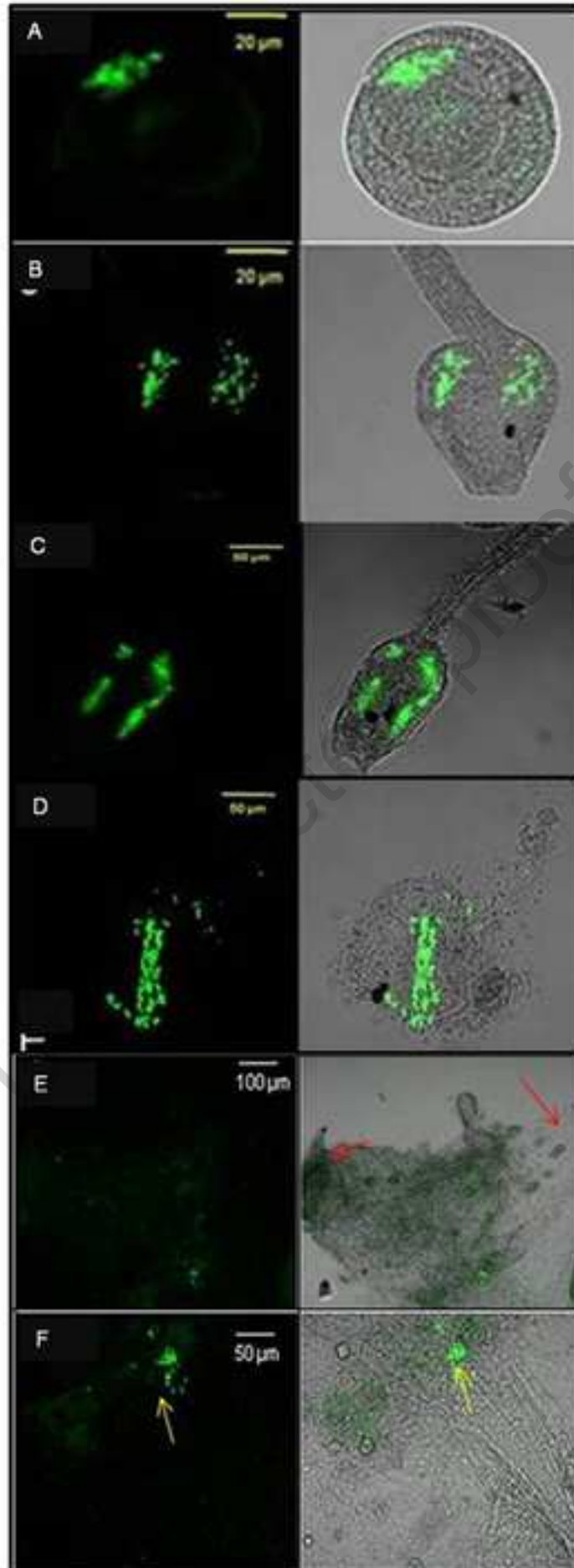Figure 4

Figure 5

Figure 6

Figure 6

Figure 7
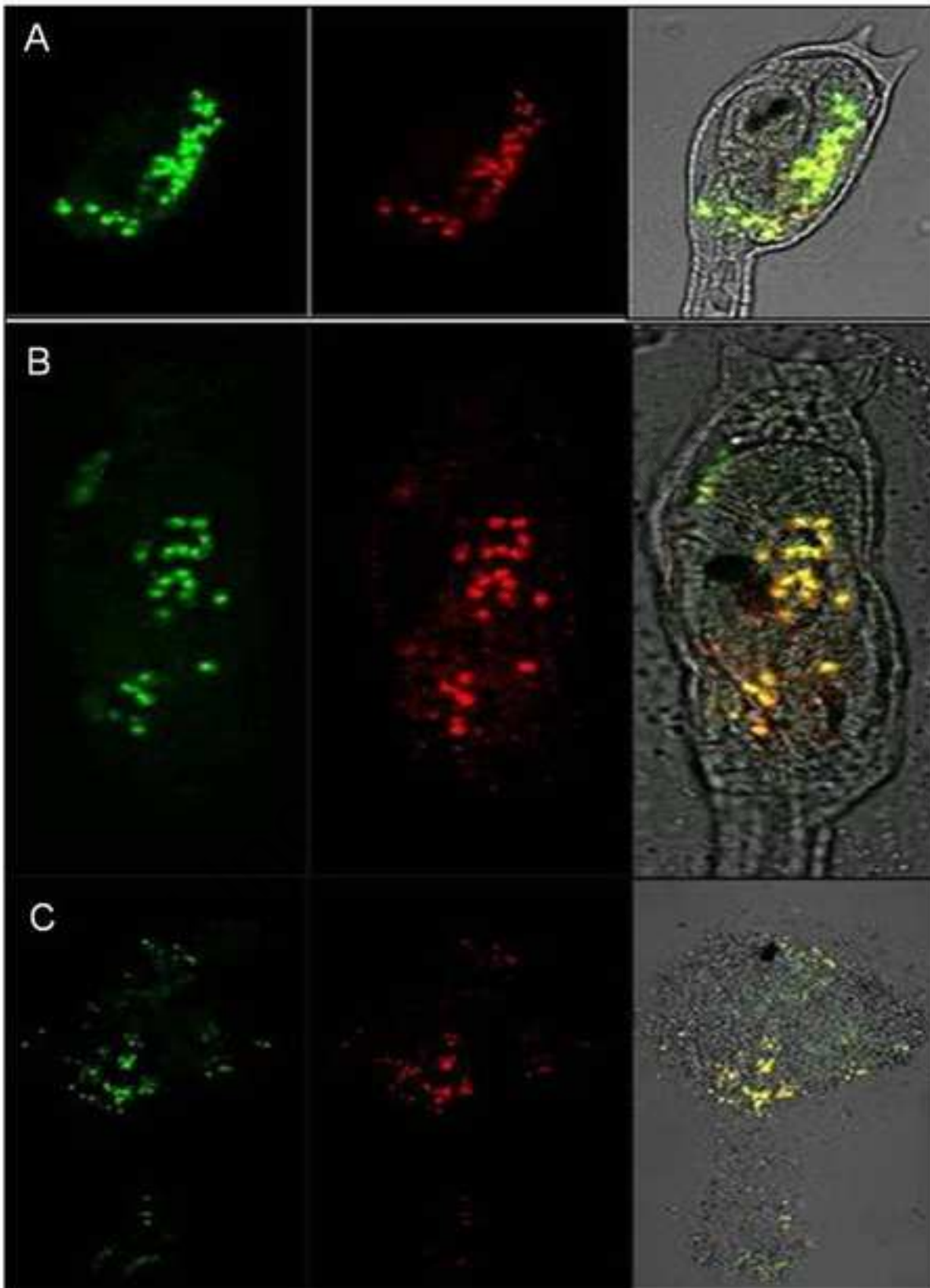Click here to download high resolution image
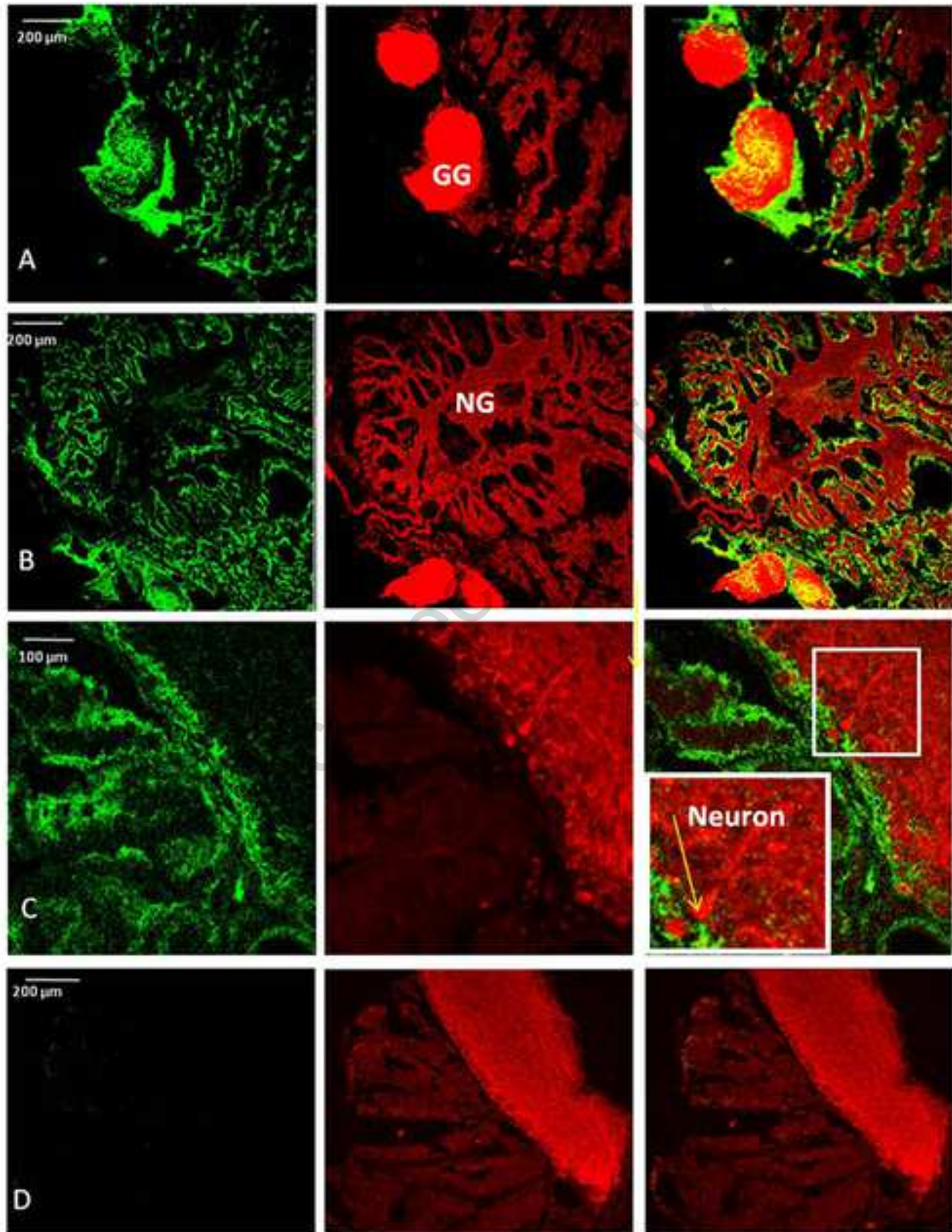
Figure 7

Figure 8

Figure 9
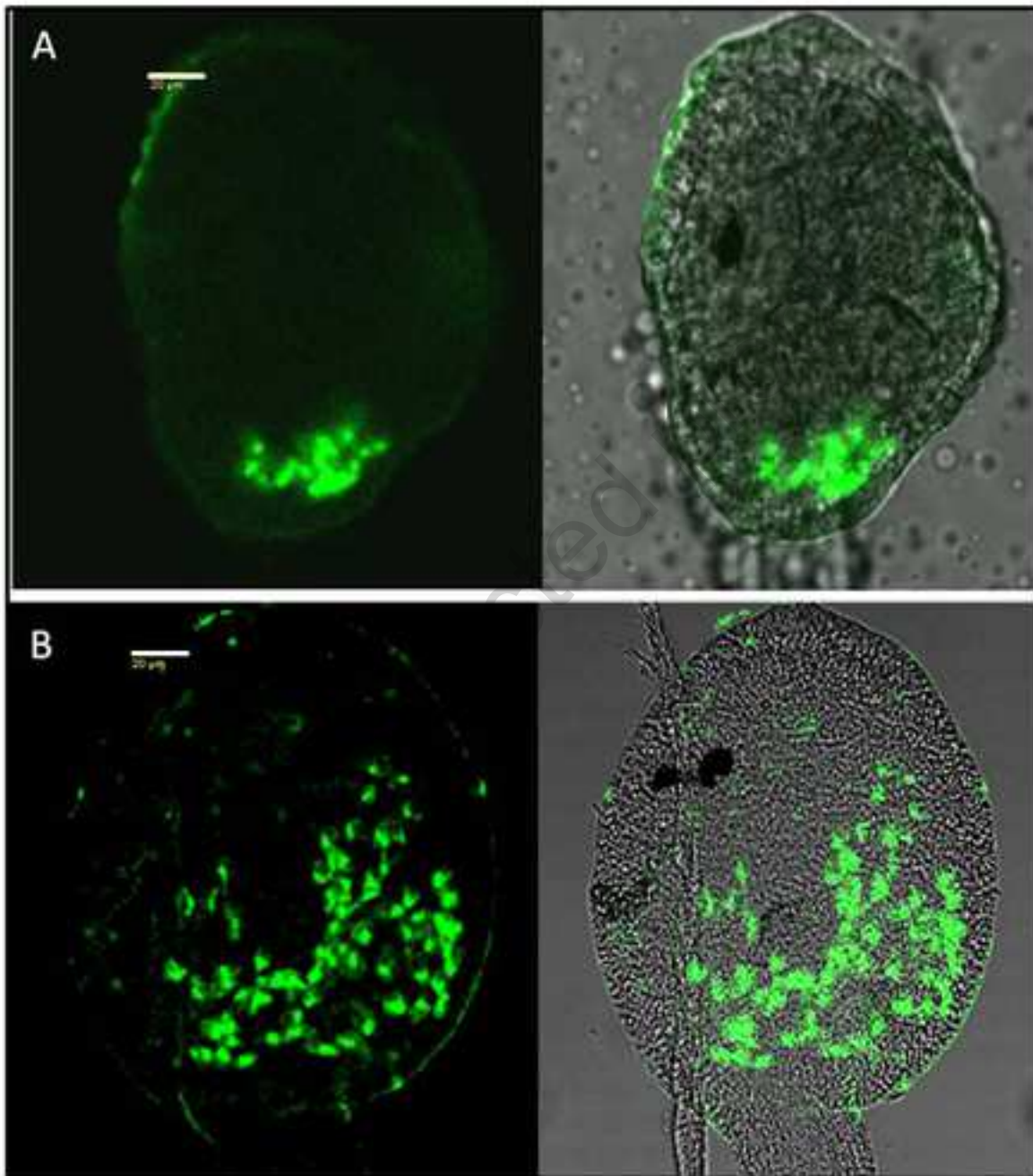
Figure S1
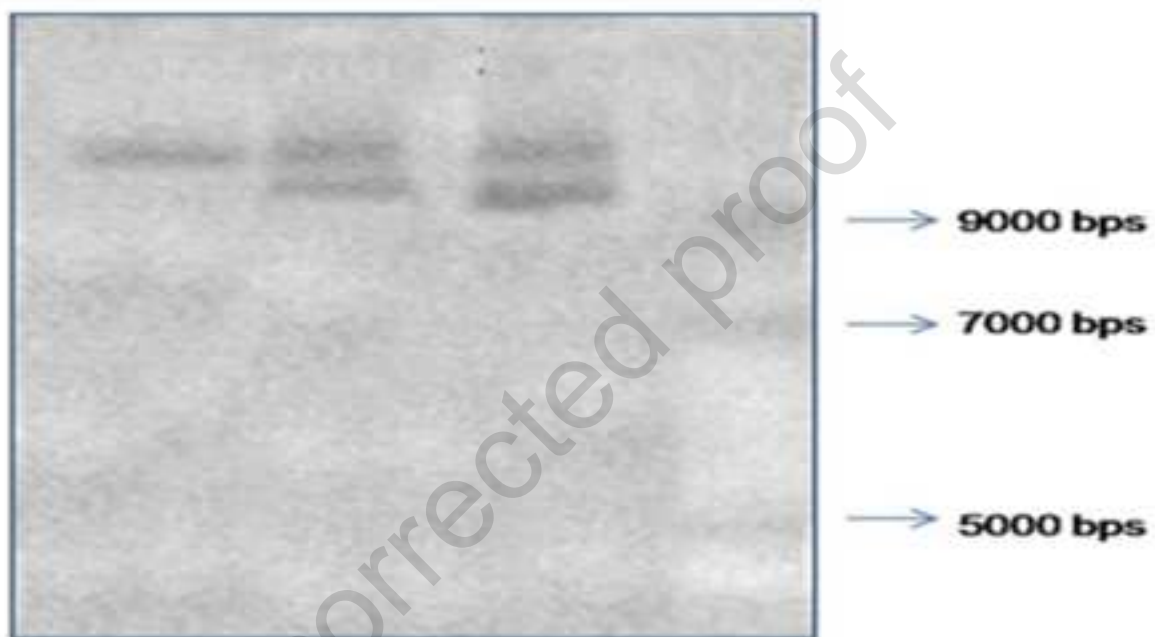
Supplementary Table 1

Tissue and Stages	Mean Ct - Ci-HTT	Mean Ct - RPS27A	Delta Ct	Std - ΔCt	ΔCt Ct	SEM
Brain	24.4	22.0	-2.4	0.6	0.2	0.1
Cerebral Ganglion	22.9	21.7	-1.2	0.6	0.4	0.2
Neural Gland	23.4	23.2	-0.1	0.3	0.9	0.2
Gut	24.2	23.0	-1.2	0.6	0.4	0.2
Heart	27.0	24.2	-2.8	0.6	0.1	0.1
Muscle	33.1	28.7	-4.4	0.2	0.0	0.0
Egg	29.2	25.9	-3.3	1.1	0.1	0.1
Gastrula	26.4	25.5	-0.9	0.6	0.5	0.2
Neurula	26.1	25.7	-0.4	0.7	0.8	0.4
Tail Bud	26.6	25.6	-1.0	0.7	0.5	0.2
Early Larva	24.2	23.0	-1.2	0.6	0.4	0.2
Late Larva	24.4	23.0	-1.4	0.8	0.4	0.2
Early Juvenile	30.0	28.3	-1.7	1.2	0.3	0.2
Late Juvenile	31.0	28.4	-2.6	0.8	0.2	0.1

Table 2
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Supplementary Table 2

Name	Type	Sequence	Size
New3F	Forward	TCAAAGAAACACAGACAGGAACACT	354
5INSR	Reverse	CAGTTACTACAGATGAAGT	
HI9	Forward	AATTCACTCTGCTCAAACTTTTACA	551
HI14	Reverse	TGTGGAATAATGTAGTAGT	
HIF1	Forward	ATTCGATTGTTTGAGATACTTGTGA	560
HIF1	Reverse	TTCTTCCATTTCTCAACATTTTCAC	
HI1	Forward	TATACAACAACTCCTCCAACCCACT	1558
HI2	Reverse	ACAGCATCATGGCATTTCTC	