

Structural and functional characterizations of an Activin type II receptor orthologue from the pacific oyster *Crassostrea gigas*

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Abstract:

Members of the Transforming Growth factor beta (TGF- β) superfamily of cell signalling polypeptides are known to play important roles in cell proliferation and differentiation during development and in various physiological processes of most animal clades. Recent findings in the mollusc *Crassostrea gigas* demonstrate the occurrence of a diversity of TGF- β signalling components including various ligands, three type I receptors but only a single type II receptor. This report describes the characterization of *Cg*-ActRII, a new type II receptor displaying homology with vertebrate and *Drosophila* Activin type II receptors. The use of zebrafish embryo as a reporter organism revealed that, in a way similar to its zebrafish counterpart, overexpression of *Cg*-ActRII or its dominant negative acting truncated form resulted in a dose dependent range of dorsoventral defects coupled with anterior disorders. Expression pattern of *Cg*-ActRII transcripts examined by real time PCR and *in situ* PCR in *C. gigas* showed high levels of *Cg*-ActRII transcripts in early embryonic stages and in the developing larval central nervous system. Except for a high expression in the visceral ganglia, most oyster adult tissues displayed rather low levels of transcripts. Altogether, the data suggest a high degree of conservation at both the structural and functional levels during evolution for this class of receptors.

Keywords: Mollusc; Lophotrochozoa; Development; Nervous tissues; Activin receptor

Abbreviations: UMR M100, Unité Mixte de Recherches M100; PE2M, Physiologie et Ecophysiologie des Mollusques Marins; UCBN, Université de Caen Basse-Normandie.

1. Introduction

Molecules of the Transforming growth factor β (TGF- β) superfamily regulate various cellular and physiological processes such as cell proliferation, differentiation or early embryonic development (Driever, 1995; Ashcroft, 1999; Herpin et al., 2004; Clarke and Liu, 2008). Members of this superfamily transmit signal through heterodimeric complexes of type I and

type II serine threonine/kinase receptors (Ten Dijke et al., 1996). Binding of dimeric ligands to two type II receptors leads to the formation of a heterotetramer in which two type II receptors transphosphorylate the juxtamembrane GS (Glycine / Serine tandem repeat) domain of two type I receptors. Activated type I receptors in turn phosphorylate specific receptor substrates (SMADs) (Derynck, 1994), organized in multisubunit complexes that move into the nucleus to regulate transcription of target genes (Massague and Wotton, 2000). Based on the class of ligand they bind or transduce, both type II and type I receptors are divided into three main categories: BMP (Bone morphogenetic protein), Activin and TGF- β receptors.

Due to the wealth of genetic data available, the components of the TGF- β superfamily are well known in the deuterostome and the ecdysozoan clades. In contrast, information is less consistent in lophotrochozoans, the third clade of bilaterian animals (Adoutte et al., 2000). However in this later clade, an extensive study in the oyster *Crassostrea gigas* has led to the characterization of most categories of ligands, BMP-like ligands (Lelong et al., 2000; Herpin et al., 2004; Fleury et al., 2008), TGF- β -related ligands (Lelong et al., 2007) and Activins (unpublished results) involved in physiological processes similar to those of their vertebrate and ecdysozoan counterparts. To signal such a diversity of ligands, in Lophotrochozoans three type I receptors of each category have been identified (Herpin et al., 2002; Herpin et al., 2005a; Herpin et al., 2005b) but only a single BMP type II receptor. This later receptor however displays a quite exotic structure with two extracellular domains having the ability to interfere independently with BMP and activin ligand induced pathways respectively (Herpin et al., 2005a). Thus the possibility was suggested that this unique pleiotropic and promiscuous type II receptor constitutes a shared interface for both BMP and activin pathways. Nevertheless, in line with the situation met in deuterostomes and ecdysozoans, additional type II receptors might be required for fine-tuning the biological responses of the target cells to an already significant repertoire of ligands characterized in oyster.

In this paper we have identified of a new Activin type II receptor from the oyster *Crassostrea gigas* and have investigated its expression pattern in adult tissues and in developmental stages. To overcome technical limitations relative to our model for assessing its functionality, we have examined the ability of this receptor to interfere with the zebrafish TGF- β pathway by injecting oyster wild type or truncated receptors in zebrafish embryos.

2. Materials and methods

2.1. Animals

Adult oysters *Crassostrea gigas* were purchased from a local oyster farm (Normandie, France). For analysis of gene expression in adult tissues, mantle, mantle edge, gills, digestive gland, quick muscle of the Posterior Adductor Muscle (PAM), catch muscle of the PAM, atrium (heart), ventricle (heart), haemocytes, labial palps, visceral ganglia were dissected from these oysters and pools of 6 oysters (4 pools for each tissue) were prepared for total RNA extraction. For gonad, reproductive stage and sex were histologically determined: stage 0 (sexual resting stage), male and female stage I (gonial multiplication stage), male and female stage II (tubule development and maturation stage) and male and female stage III (sexual maturity) (Rodet et al., 2005). Embryos and larval stages were produced at the IFREMER shellfish laboratory of Argenton (France) following the procedure described in (Fabioux et al., 2004).

2.2. Cloning of an Activin type II receptor cDNA from C. gigas

A full-length sequence of Cg-Act RII was obtained by extending a single EST of 1319 bp (AM855334) of *Crassostrea gigas* obtained in the frame of a “Marine Genomics Europe” program (Tanguy et al., 2008). This extension was realized by 5'- and 3'-RACE using

Marathon cDNA amplification kit (Clontech). Doubled stranded cDNA from oyster mantle edge was ligated to adaptors and 25 ng of this template was used to PCR amplify 5'- and 3'- RACE fragments using adaptor specific primers and gene specific primers deduced from the initial 826 bp EST. Recovered fragments were gel-extracted (QIAquick[®] Gel Extraction Kit, Qiagen), sub-cloned with a TOPO-TA cloning[®] kit (Invitrogen) and then sequenced. To check the actual contiguity of 3' and 5' RACE fragments and to generate a single fragment, PCR was then performed with most 5' and 3' specific primers using the proofreading DNA polymerase *Pfu* (Promega) and the recovered fragment was sequenced again.

2.3. Phylogenetic analysis

For phylogenetic analyses, TGF- β superfamily receptor protein sequences were chosen to represent a range of type I and type II superfamily members from each main clades of bilaterian animals. The sequences were aligned using Clustal W (Thompson et al., 1994) and by manual inspection. From these alignments, distance based phylogenetic trees were constructed using the neighbour joining method of the Phylip package with one thousand bootstrap trials for each node.

2.4. Real time RT-PCR

Real time RT-PCR analysis was performed using the iCycler iQ[©] apparatus (Bio-Rad). Total RNA was isolated from embryos, larvae and adult tissues using Tri-Reagent (Sigma-Aldrich) according to the manufacturer's instructions. Recovered RNA was then further purified on Nucleospin RNAII columns (Macherey-Nagel). After treatment during 20 min. at 37°C with 1U of DNase I (Sigma) to prevent genomic DNA contamination, 1 μ g of total RNA was reverse transcribed using 1 μ g of random hexanucleotidic primers (Promega), 0.5 mM dNTPs and 200U MMuLV Reverse Transcriptase (Promega) at 37°C for 1 hour in the appropriate

buffer. The reaction was stopped by incubation at 70°C for 10 min. iQTM SYBR Green supermix PCR kit (Biorad) was used for real time monitoring of amplification (5 ng of cDNA template, 40 cycles: 95°C/15s, 60°C/15s) with the following primers: Qs-Cg ActRII 5'-CACTGGAGGACATGCAAGAA-3' and Qa-CgActRII 5'-GGCTGCAACCTGCTCTAAAC-3' as sense and antisense primers respectively. Accurate amplification of the target amplicon was checked by performing a melting curve. Using Qs-Cg-EF (5'-5'-ACCACCCTGGTGAGATCAAG-3') and Qa-CgEF (5'-ACGACGATCGCATTTCTCTT-3') primers, a parallel amplification of oyster Elongation Factor α (EF α) transcript (BAD15289) was carried out to normalize the expression data of Cg-ActRII transcript. The relative level of Cg-ActRII expression was calculated for one copy of the reference elongation factor α gene by using the following formula: $N=2^{(Ct_{EF\alpha} - Ct_{Cg-ActRII})}$.

2.5. Zebrafish maintenance and preparation of eggs

TAB zebrafish were raised and maintained on a light/dark cycle essentially as described by Westerfield (Westerfield, 1989). Synchronised eggs were obtained by mixing previously separated female and male fish at a ratio of 2:1. Eggs were then collected as described by Culp et al. (1991). Self-digested Pronase E (Sigma) at 0.5 mg/mL was used to remove the egg chorion. The reaction was stopped by removing the enzyme and then transferring the eggs into Holtfreter's solution (85 mM NaCl, 1 mM KCl, 1 mM CaCl₂ and 3.5 mM NaHCO₃) and rinsing.

2.6. Microinjections

Initially, a full length cDNA encoding Cg-ActRII was subcloned into the plasmid pRN3 (Lemaire et al., 1995). Dominant-negative controls of Cg-ActRII were obtained by inserting a stop codon downstream of the sequence encoding the transmembrane domain. Capped

synthetic mRNA encoding both full length and truncated proteins were transcribed *in vitro* with T3 RNA polymerase and the Ambion RNA capping kit using KpnI linearized plasmids as templates. Capped mRNAs were dissolved in RNase-free 0.2 M KCl containing 0.5% phenol red for injection. The mRNA solution was injected into the cytoplasm of single-cell embryos. After injection, embryos were transferred into 24-well tissue culture plates containing Holtfreter's solution (5-7 embryos per well) and incubated at 28°C for 24-30 hours then examined by microscopy for phenotype analysis. Unfertilised eggs were removed after 4 hours. Alternatively, embryos were fixed for *in situ* hybridization at 80% epiboly. Uninjected and control embryos injected with 0.2 M KCl containing 0.5% phenol red were analyzed under the same conditions. For all *in situ* hybridizations and phenotype analyses around 50-80 embryos were analyzed for each concentration of mRNA injected.

2.7. Whole-mount *in situ* hybridizations

Anti-sense probes were transcribed *in vitro* and labelled using digoxigenin-labelled UTP (Roche). The anteroaxial goosecoid (*gsc*) mesodermal marker (Thisse et al., 1994), and the ventrolateral *tbx6* mesodermal marker (Hug et al., 1997) were used as probes. Whole-mount staining of embryos was performed as described by Rissi et al. (Rissi et al., 1995).

2.8. Whole mount *in situ* RT-PCR on oyster embryos and larvae

Embryos and larvae were fixed in MEMPFA-T buffer (0.1 MOPS pH 7.4; 2mM EGTA; 1mM MgSO₄; 4% Paraformaldehyde; 0.1% Tween 20) at room temperature (RT) at least 1hour and dehydrated progressively with series of Methanol/MEMPFA mixes (25/75 : 50/50; 75/25 and 100/0) 5 min at RT and finally stored at -20°C in methanol. Whole mount *in situ* RT-PCR was adapted from the protocol described by Pesquet et al. (2004) with minor modifications. Samples were washed 3 x 10 min at 4°C in 63% (v/v) ethanol / 2% (v/v) acetic acid and

rinsed in TBS-T under agitation 2 x 10min at 4°C. Embryos and larvae were treated with proteinase K (50ng/mL in TBS-T) at 37°C during 20 min and washed in ultra-pure water 2x 10 min at RT. The genomic DNA was digested with 10U of *TaqI* restriction enzyme in appropriate buffer at 65°C during 3h. Then samples were rinsed 2x 10 min at RT in ultra pure water. Samples were then incubated in presence of 12µL of water, 1 µL of Qa-CgActrII unlabelled gene-specific reverse primer 10 µM (described above), heated at 70°C ; 10 min and placed in ice for 10 min. Reverse transcription (RT) was carried out by adding 6 µL of RT mix (4µL of MMuLV-RT 5x buffer; 2 µL of 10mM of dNTPs solution) and 1 µL of MMuLV-RT (200U) to the samples and by incubation at 37°C during 1 hour. RT was stopped by a 10 min incubation at 70°C. RNA hydrolysis was performed by replacing the RT buffer with 0.4M of NaOH at 37°C; 1 hour and washing in 100mM Tris-HCl and 150mM NaCl pH 7.5; 3 x 15 min at RT and finally in ultra pure water 2 x 15 min at RT. Amplification was carried out in 8 µL of PCR mix (2 µL of 10x PCR buffer; 2 µL 25mM of MgCl₂ and 4 µL of 200 µM of dNTPs); 1 µL of 10 µM of gene specific reverse primer Qa-CgActRII and 1 µL of 10 µM of gene-specific fluorescent-labelled forward primer (5' Cy5 – TCACTGACCAGATGCCAGTC-3'). Volume was adjusted to 19 µL with ultra-pure water and heated at 95°C during 2 min. One unit of *Taq* DNA polymerase (Promega) was added and reaction was performed by 40 cycles of 95°C/30sec; 53°C/30sec; 72°C/40sec and a final cycle at 72°C/10min. Samples were rinsed twice in ultra pure water and mounted on superfrost slides in 80% glycerol and observed in confocal microscopy

2.9. Statistical analyses

A “Chi square” test was used to check the efficacy of *Cg-ActRII* or *DN-Cg-ActRII* mRNA injections in zebrafish embryos and to compare the distribution of the recovered phenotypes at each dose. Multiple comparisons of the relative level of mRNA between tissues,

reproductive stages and developmental stages were performed using one-way ANOVA followed by a Bonferroni's test

3. Results and discussion

3.1. Cloning of a type II Activin receptor from the lophotrochozoan *C. gigas*

A full length 1983 base pair cDNA clone with an open reading frame encoding an inferred 521 amino acid long protein was isolated from *C. gigas* mantle edge (accession number FM253117). The inferred protein sequence demonstrated most resemblance to Activin type II receptors and was thus named *Cg-ActRII* (Fig.1). The intracellular region was well conserved and showed about 56.3% identity (79% similarity) with human Activin type IIB receptor (Ishikawa et al., 1998), 55.6% (75% Similarity) with zebrafish Activin type IIB receptor (Garg et al., 1999) and 50.6% identity (72% similarity) with its *Drosophila* orthologue Punt (Ruberte et al., 1995). In contrast, the extracellular domain displayed a moderate amino acid sequence identity of around 30% with *Drosophila* and vertebrate type II Activin receptors but contained 8 predictably spaced cysteine residues, four of which forming a characteristic CCCX₄C knot near the transmembrane region (Wrana et al., 1994). Despite sequence variability, the conserved spacing of all cysteines probably helps in maintaining a conserved tridimensional structure required for ligand binding. As demonstrated for vertebrate receptors, fine specificity of ligand binding of *Cg-ActRII* is probably determined by discreet residues in the extracellular domain but also likely depends of the category of type I receptor with which it interacts (Yamashita et al., 1995). Since *Drosophila* Punt, with only 20% sequence identity with its mammalian orthologue, binds human Activin with high affinity and specificity (Childs et al., 1993) we might expect *Cg-ActRII* to bind the oyster orthologue of Activin.

However, the ability of *Cg-ActRII* to bind different oyster TGF- β superfamily ligands especially in a *in vivo* context cannot be excluded.

3.2. *Cg-ActRII: the missing type II receptor in C. gigas*

A phylogenetic tree was generated using the complete inferred protein sequence of *Cg-ActRII* together with members of type I and type II TGF- β superfamily receptors from both protostomes and deuterostomes (Fig. 2). The resulting phylogenetic tree revealed two main families: the type I receptor and the type II receptor clusters. Each of these two main clusters was separated into three groups consisting of the canonical trilogy: BMP, Activin and TGF- β subfamilies of receptors. Supported by a high bootstrap value (80), *Cg-ActRII* indeed clustered within the Activin type II receptor group including human ActRIIB, zebrafish ActRIIB and *Drosophila Punt*. Interestingly, except for the TGF- β specific type II (T β RII) receptor which could have been acquired during the gene outbreak that occurred with the emergence of chordates (Herpin et al., 2004), the lophotrochozoan *Crassostrea gigas* harbours the typical assortment of TGF-beta superfamily receptors with the three family type I receptors and the BMP and Activin type II receptors shared by each bilaterian animal clade.

3.3. *Cg-ActRII is functionally active in zebrafish embryos and likely transduces a dorsalizing signal*

To determine if *Cg-ActRII* was capable of functioning in a way similar to its vertebrate orthologues, capped mRNAs encoding *Cg-ActRII* were injected into newly fertilized zebrafish embryos. Specifically, we wished to determine if expression of this molecule was able to perturb the Activin ligand-receptor pathway during embryogenesis. Expression of the full length *Cg-ActRII* in this reporter system resulted in a dose dependent range of dorsoventral defects of embryos (Fig. 3A & B) including uncoordinated embryos for the

highest doses (Fig 3A: D3 phenotype) and a range of dorsalized embryos caused by medium and low doses of *Cg-ActRII* encoding mRNA (Fig 3A: D1-D2 phenotypes). Some embryos showed defects in somite formation (Fig. 3C). Expression of a truncated version of *Cg-ActRII*, (DN-*Cg-ActRII*), in agreement with its credible dominant negative activity likely due to its capacity to subtract endogenous ligands, resulted in a concentration dependent increase in the severity of ventralized phenotypes (Fig. 3A & B). In addition, a noticeable fraction of the DN-*Cg-ActRII* injected embryos (10% for 25ng/ μ L) displayed fused eyes phenotype (Fig.3C 3-4) coupled with anterior defects. Though these later phenotypes are reminiscent of disruption of Nodal (Aoki et al., 2002; Mathieu et al., 2004) or Activin (Thomsen et al., 1990) signalling, most phenotypes were very similar to those obtained following overexpression of zebrafish activin type IIB receptor (*Dr-ActRIIB*) (Nagaso et al., 1999) supporting the idea that *Cg-ActRII* is actually functional in zebrafish in a way similar to its fish orthologue and would mediate the same endogenous ligands as *Dr-ActRIIB*. To correlate defective gastrulation with these phenotypes, whole mount *in situ* hybridization of embryos at 80% epiboly was achieved using two mesodermal markers, the anteroaxial /dorsal *gsc* mesodermal marker (Thisse et al., 1994) and the *tbx6* ventrolateral marker (Hug et al., 1997) (Fig. 3D). Compared to uninjected control embryos, expression of *Cg-ActRII* resulted in a consistent expansion in *gsc* expression at the gastrula stage (Fig. 3D). Additionally dorsal expression of *tbx6* was also strongly enhanced and appeared in some cases to cover most of the embryo (Fig. 3D). Accordingly, DN-*Cg-ActRII* injected embryos exhibited a reduced *Tbx6* expression in their dorsal part whereas *Gsc* was slightly reduced when compared to wild type embryos. Induction of *gsc* marker was rational with the dorsalized phenotypes recovered and was congruent with previous results obtained either with *Dr-ActRIIB* or other ectopically expressed oyster TGF- β superfamily components such as the *Cg-TGF- β sRII*, an exotic BMP/activin type II receptor harbouring two extracellular binding domains (Herpin et al.,

2005a). With respects to *Tbx6* expression, *Cg-ActRII* behave to some extent as most oyster ventralizing BMP-like signalling components, the oyster BMP type I receptor (*Cg-BMPRI*) (Herpin et al., 2005a) and a Tolloid homologue (Herpin et al., 2007). Hence, increase of the ventrolateral marker *Tbx6* expression was surprising in regard to the phenotypes obtained and reminds the induced expression of another ventrolateral marker *eve1* upon injection of *Dr-ActRIIB* (Nagaso et al., 1999). Although firm conclusion would require further investigations looking how specific Smad signalling pathways are modulated, it is already possible to say that *Cg-ActRII* like its zebrafish orthologue (*Dr-ActRIIB*) is likely to mediate both Activin and BMP pathways during zebrafish embryogenesis. Dual signalling may rely on the selective use of distinct type I receptors as observed for the *Drosophila* Activin type II receptor “Punt” which mediates DPP (*Decapentaplegic*) signalling in combination with the BMP type I receptor *Tkv* (Thick veins) or the Activin type I receptor *Saxophone* (Letsou et al., 1995; Ruberte et al., 1995) but achieves Activin-specific signalling with the TGF- β type I receptor *Baboon* (Brummel et al., 1999). Because oyster *Cg-T β RI* induces a similar pattern as *Cg-ActRII* on the expression of the mesodermal markers *gsc* and *tbx6* in zebrafish (Herpin et al., 2005b), the possibility that *Cg-T β RI* is one of the receptor partners of *Cg-ActRII* can be reasonably anticipated.

3.4. Cg-ActRII is highly expressed in nervous tissues during development and in adults

To gain insight into possible physiological roles of *Cg-ActRII*, determination of tissue distribution and temporal pattern of expression during development was performed by real time RT-PCR (Fig. 4). *Cg-ActRII* transcript expression was high in unfertilized oocytes (0.015 copy/copy of *EF α*), significantly dropped during the first embryonic stages (down to 0.001 copy / copy of *EF α*) and subsequently raised at the blastula and gastrula stages (up to a mean of 0.03 copy / copy of *EF α*), probably as a result of general transcription recovery. Both

maternal and early embryonic expression might be required for signalling gradients of both BMP and Activin-related ligands known to pattern the body axes in both vertebrates and *Drosophila* (Frisch and Wright, 1998; Nagaso et al., 1999). In comparison to early embryonic stages, most larval stages of development displayed a rather moderate expression level (around 0.002copy / copy of El α). Whole mount *in situ* hybridization showed a rather uniform distribution of *Cg-ActRII* transcripts in the various embryonic stages tested. For larval stages, especially in the trochophore larvae, three spots of high expression corresponding to the larval nervous ganglia clearly emerged from a faint and uniform signal covering the entire larvae (Fig. 5). Among adult tissues, *Cg-ActRII* showed a ubiquitous expression though restrained in intensity except for visceral ganglia whose significant *Cg-ActRII* expression was above five times the basal level found in other tissues. The spatial and temporal broad and basal level of *Cg-ActRII* transcript expression suggests this receptor likely plays multiple roles during development. However, specific focal expression of *Cg-ActRII* in oyster larval and adult nervous system is evocative of the situation found in *Drosophila* where an Activin signalling pathway involving Activin-like ligands, the receptors Punt and Baboon, controls axon guidance during embryogenesis (Parker et al., 2006), morphogenesis of adult specific neurons (Zheng et al., 2006) and regulates neuroblast numbers and proliferation rates in the developing larval brain (Zhu et al., 2008). In other respects, the discrete levels of *Cg-ActRII* expression in male and female gonads, whatever the sex and the reproductive stage, suggest a possible involvement of an Activin signalling pathway for regulating some aspects of gonad development and reproductive processes in oyster as described in mammals (de Kretser et al., 2002). Another possibility is that *Cg-ActRII*, in partnership with an oyster type I receptor, mediates og-TGF- β -like, an oyster gonad-specific transforming growth factor- β superfamily member presumed to play a regulatory role in the differentiation and maturation of germ cells (Fleury et al., 2008).

4. Conclusions

1. We report the characterization of *Cg-ActRII*, the first activin type II receptor from a Lophotrochozoan.
2. This receptor is actually functional in zebrafish embryos and appears to mediate the same endogenous ligands as its zebrafish orthologue.
3. The pattern of expression of *Cg-ActRII* transcripts reveals that this receptor, like its vertebrate and *Drosophila* counterparts, probably plays a key role in cell fate determination during early development and in larvae and adult nervous system.
4. The data strongly suggest a high degree of conservation at both the structural and functional levels during evolution for this class of receptors

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Figure legends

Figure 1: Multiple sequence alignment of *Cg*-ActRII with members of the Activin receptor type II family using CLUSTAL W. The predicted amino acid sequence of *Cg*-ActRII is aligned with the amino acid sequence of two type II Activin Receptors from *Homo sapiens* (Hs ActRIIB), *Danio rerio* (Dr-ActRIIB) and *Drosophila melanogaster* (Dm-Punt). Conserved residues (identity) between the *Cg*-ActRII receptor and the three other receptors are shaded in dark grey, light grey shading indicates identity with at least one of the three receptors. Amino acids of the predicted signal peptide are shown in bold letters. Transmembrane domain is underlined. Dashes indicate gaps in the amino acid sequence when compared with other sequences. The black box delimits the potential ATP binding region. Black arrowheads indicate the conserved cysteine residues in the extracellular domain. The dotted line delimits the Serine-Threonine kinase domain. (★) indicates potential N glycosylation sites .

Cg-ActRII: *C. gigas* (**FM253117**), *Hs*-ActRIIB: *H. Sapiens* (**BAA2418**), *Dr*-ActRIIB: *D. rerio* (**NP_571285**), *Dm*-Punt: *D. melanogaster* (**AAC41566**).

Figure 2: Phylogenetic relationship of TGF- β superfamily type I and type II receptors. This tree was generated by using the alignment in CLUSTAL W. From this alignment a distance based phylogenetic tree was constructed using the neighbor joining method of the Phylip package with a validation by bootstrapping method (one thousand bootstrap trials were run)

Hs ActRIIB: *H. sapiens* (**BAA24180**), *Dr* ActRIIB: *D. rerio* (**NP_571285**), *Cg*-ActRII: *C. gigas* (**FM253117**), *Dm* Punt: *D. melanogaster* (**AAAC41566**), *Cg*-TGF β sfR2: *C. gigas* (**CAD20574**), *Dm* Wishful thinking *D. melanogaster* (**AAF60175**), *Hs* BMPR-II: *H. sapiens* (**NP_001195**), *Hs* T β R-II *H. sapiens* (**P37173**), *Cg*-ALR1: *C. gigas* (**AJ309316**), *Hs* ActRI *Homo sapiens* (**NP_001096**), *Dm* Saxophone *D. melanogaster* (**I45712**), *Cg*-BMPR1 *C. gigas* (**CAE11917**), *Hs* BMP-RIA: *H. sapiens* (**NP_004320**), *Dm* Thick veins: *D. melanogaster* (**XP_079689**), *Cg*-T β R1: *C. gigas* (**AJ544074**), *Hs* T β RI *H. sapiens* (**P36897**), *Dm* Baboon (*Atr-I*): *D. melanogaster* (**A55921**), *Ce* Sma6 *C. elegans* (**AAD12261**).

Figure 3: (A) The range of zebrafish phenotypes observed after overexpression of oyster Activin type II receptor (*Cg*-ActRII). Examples showing the dorsalized phenotypes obtained after overexpression of *Cg*-ActRII. These phenotypes ranged from the least (D1) to the most severe (D3). Examples showing the ventralized phenotypes obtained after overexpression of truncated DN-*Cg*-ActRII (dominant negative) lacking the intracellular domain. These phenotypes ranged from the least (V1) to the most severe (V3). (B) Dose-dependent phenotype distribution obtained by overexpression of *Cg*-ActRII and truncated *Cg*-ActRII (DN-*Cg*-ActRII). Results are expressed as percent of embryos showing a given phenotype. The number of embryos injected for each concentration of mRNA is indicated in brackets. Paired comparisons of phenotype distributions obtained for each dose of *Cg*-ActRII mRNA (a, b, c) or DN-*Cg*-ActRII mRNA (d, e, f) were statistically tracked using χ^2 test: Significant differences were observed for a, b, c ($P < 10^{-4}$), d ($P < 0.005$), e ($P < 10^{-4}$) and f ($P < 10^{-3}$) (C)

Characteristic phenotype of somite development alteration (C1) resulting from overexpression of *Cg-ActRII* compared to wild type phenotype (C2) and of fused eyes phenotypes obtained after overexpression of truncated *Cg-ActRII* (DN-*Cg-ActRII*) (25ng/μL) (C3-4). (D) Whole mount *in situ* hybridization of zebrafish embryos using the dorsal mesoderm marker Goosecoid (*Gsc*) (D1-3-5) and the ventro-lateral mesoderm marker *Tbx6* (D2-4-6). Expression pattern of *gsc* and *tbx6* genes upon injection of *Cg-ActRII* and DN-*Cg-ActRII* (75ng/μL) at 80% epiboly.

Figure 4: Distribution of mRNAs encoding *Cg-ActRII* during developmental stages, in adult tissues and gametogenesis of *Crassostrea gigas* measured by real-time quantitative RT-PCR. Each value is the mean +SE of four pools of 6 animals (adult tissues), or the mean of four pools of embryos or larva from the same spawn (developmental stages), or the mean of four independent animals (gametogenesis). Expression levels are expressed per copy of Elongation Factor α (EF α). Significantly different means ($p < 0.01$) are indicated by letters.

M: Mantle, ME: Mantle edge, G: Gills, DG: Digestive Gland, QM: Quick muscle of the Posterior Adductor Muscle (PAM) CM: Catch Muscle of PAM, A: Atrium (heart) V: Ventricle (heart), H: Haemocytes, LP: Labial Palps, VG: Visceral Ganglia. St0: Stage 0, sexual resting stage, M: Male, F: Female, Stage I: gonial multiplication stage, II: tubule development and maturation stage, IIIA: sexual maturity stage.

Figure 5: *In situ* distribution of *Cg-ActRII* transcripts in oocytes, blastula and trochophore larvae of *Crassostrea gigas*. *In situ* RT-PCR was performed with Cy5-labelled forward primer in presence (positive) or absence of *Taq* DNA polymerase. Controls were also made by missing either *TaqI* restriction enzyme, RT or by RNA alkali digestion prior to RT-PCR (data not shown). Arrows indicate the nervous ganglia.

Cg-ActRII **-MFWINIFTSVFFILQHSTGSWSAEAKAAQ--RRGVVLYP-NCT-ENGGQS-CQRYEYCEPGSHYCFTANSQNNASA**
 Dn-ActRIIB **--MFASLLTLALLLATFAADPSHG**EVET----REGLYYNV-NWEVEKTNRSQVRCCEGEKDKRSHCYASWRNNSGS-
 Hs-ActRIIB **--MTAPWVALALLWGSICAGSGRGEA**ET----RECIYYNA-NWELERTNQSGLERCEGEQDKRLHCYASWANSSTG-
 Dm-Punt **MSKYDLLYLTAQLTLVCCLIIGHG**SILPGSHGIEGEHFDEKMCNTTQQCETRIEHCKMEADKFPSCYVLSVNETTG

NGIELKHQGCWTONQGCEN-DSCIQSNLQTSVSECCCHLDKCNINISMVSIQFSAVTEKGTAKVQSDGKAKVMEILMYSLVPLIVLVILIV
 --TQLVKKGCWLDLDFNYDRQECVATEENPQ-VFFCCCEGNFCNERFTHLPDISGPVIS--PPVSP-----SLLNVLVYSLPLSMLSMAVL
 --IELVKKGCWLDLDFNYDRQECVATEENPQ-VYFCCCEGNFCNERFTHLPEAGGPEVTYEPPTAP-----TLLTVLAYSLPLIGGLSLIVL
 -ILRIKMKGCFIDMHECNQTECVTSAEPRQGNIECCCKGSRCSNKNQYIKSTTEATTQVPKEKTQD-----GSNLIYIYIGTSVFSVLMVIVG

IVEVMWRCYYNNMYTSHLQLEACDPEMGTQEFNQPHRPIQLLELRAHGRFGEVWVANMLDAVAVKIMPFKEKASWLAEQEIYNLEHMKHDNI
 LAFWMYR---HRKPPYGHVDVNE-DGSPSPSPVLGKPLQLLELVKARGRFQVWHAQMINNEYVAVKIFPIQDKLSWQNEREMFSTFGMKHDNL
 LAFWMYR---HRKPPYGHVDIHE-DGPPPPSPVLGKPLQLLEIKARGRFQVWHAQMLMDFVAVKIFPLQDKQSWQSEREIFSTFGMKHENL
 MGLLLYR---RRKQAHFNEIPTHEAETITNSSPLLSNRPPIQLLEQKASGRFGDVMCAKLNNQDVAVKIFRMQEKESWTTEHDIYKLERMRHPNI

LLEVGGEK---HEENLWLI TEFHEKGSLECEILKGNVSVTWSQLCKIAESMVRGLAYLHDDVSLTRCQS-KPAVAHRDFKSKNVLLKEDLTACIA
 LRFIAAEKRGSNLEMEFWLIT EFHGRSITDYLGNAVSWADLCVIAESMACGLAYLHEDVPRSKGEGPKPAIAHRDFKSKNVMLKMDLTAVIG
 LQFIAAEKRGSNLELWLI TAFHDKGSLTDYLGKNIITWNELCHVAETMSRGLSYLHEDVPWCRGEGHKPSIAHRDFKSKNVLLKSDLTAVLA
 LEEFLGVKHMDD--KPEYWLITSTYQHNGLCDYLGKSHITISWPELCRIAESMANGLAHLHEEIPASKTDGLKPSIAHRDFKSKNVLLKSDLTACIA

DFGLALKEEPGKSPGETHGLVGTTRRYMAPEILEGATSERKDAFLRIDMYACGLVLWELLSRNSCSDQPADEYQLPFEEEAQSHPTLEDMQELVV
 DFGLAVRFEPEGKPPGDTHGQVGTTRRYMAPEVLEGAINFQRDSFLRIDMYAMGLVLWELVSRCKAADGPFVDEYMLPFEEEI QHPSLEDLQDAVV
 DFGLAVRFEPEGKPPGDTHGQVGTTRRYMAPEVLEGAINFQRDAFLRIDMYAMGLVLWELVSRCKAADGPFVDEYMLPFEEEI QHPSLELEQEVVV
 DFGLAMIFQPGKPCGDTHGQVGTTRRYMAPEVLEGAINFNRDAFLRIDVYACGLVLWEMVSRCDFAG-PVGFQPLPFAELGLRPSLDEVQESVV

MKKLRPTIKNHWLQHPGLEQVAATIEGCWDQDAEARVSAICVQERLSQLSRTLNVSTSSHNLISIPNQNIYHISVSN-
 HKKLRPAFKDCWLNKHSGLCQMCETMEECWDHDAEARLSAGCVQERISQIRR-VSSSTSDCLFSMVTSLTNVDLPPKESSI
 HKKMRPTIKDHWLKHPLGALQCVTIEDCWDHDAEARLSAGCVERVSLIRRSVNGTSDCLVSLVTSVTNVDLPPKESSI
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Fig1

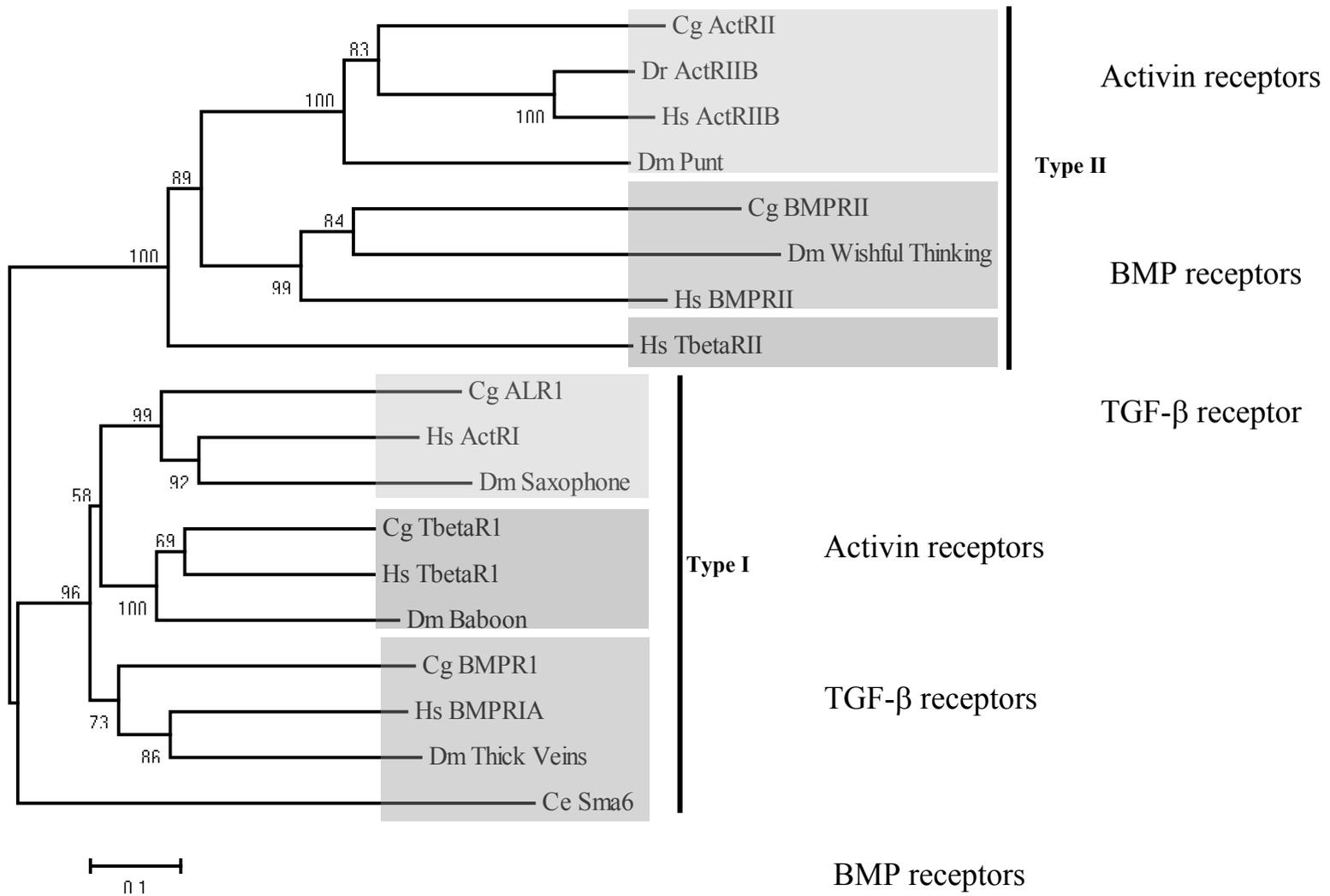


Fig. 2

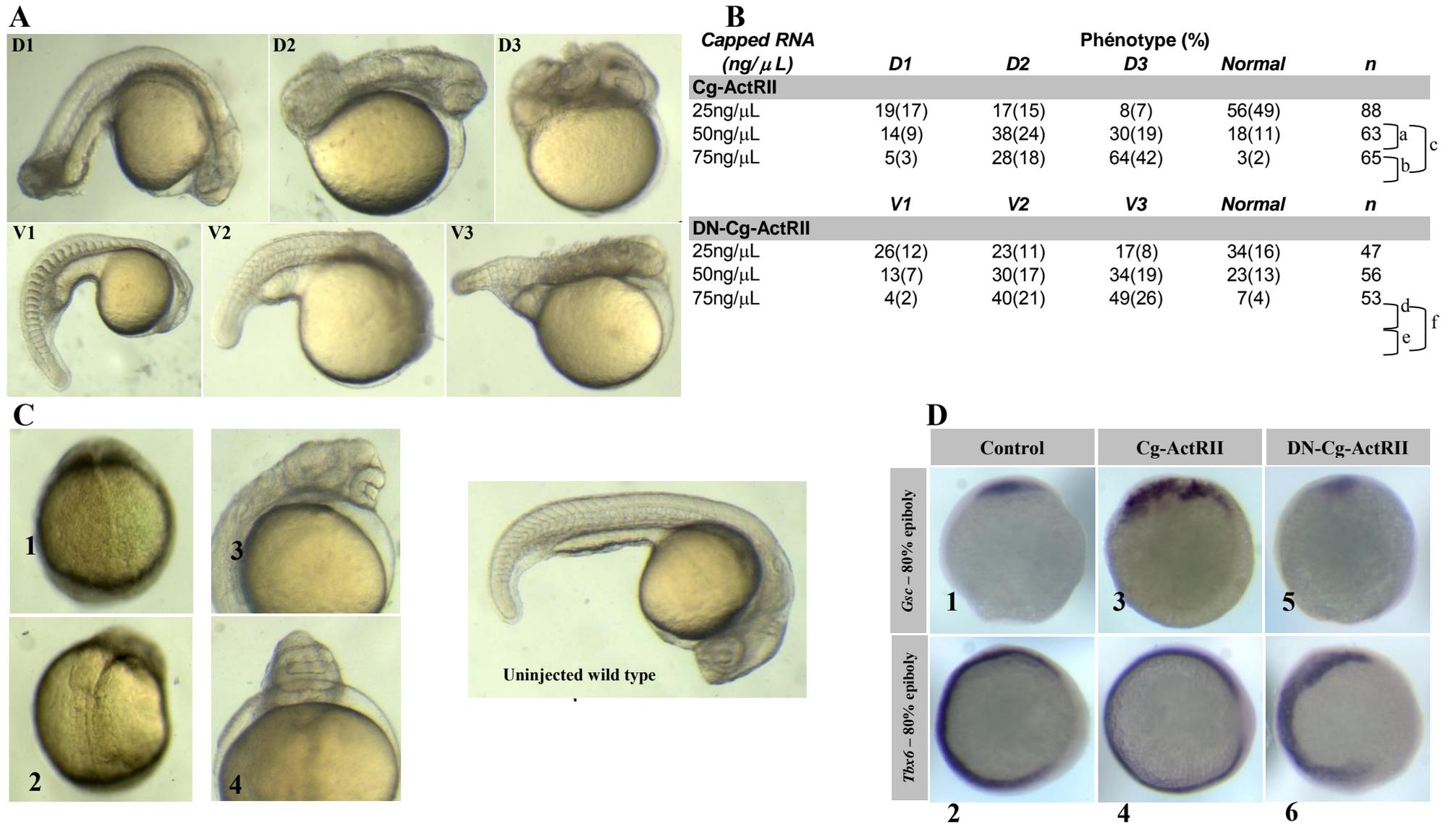


Fig. 3

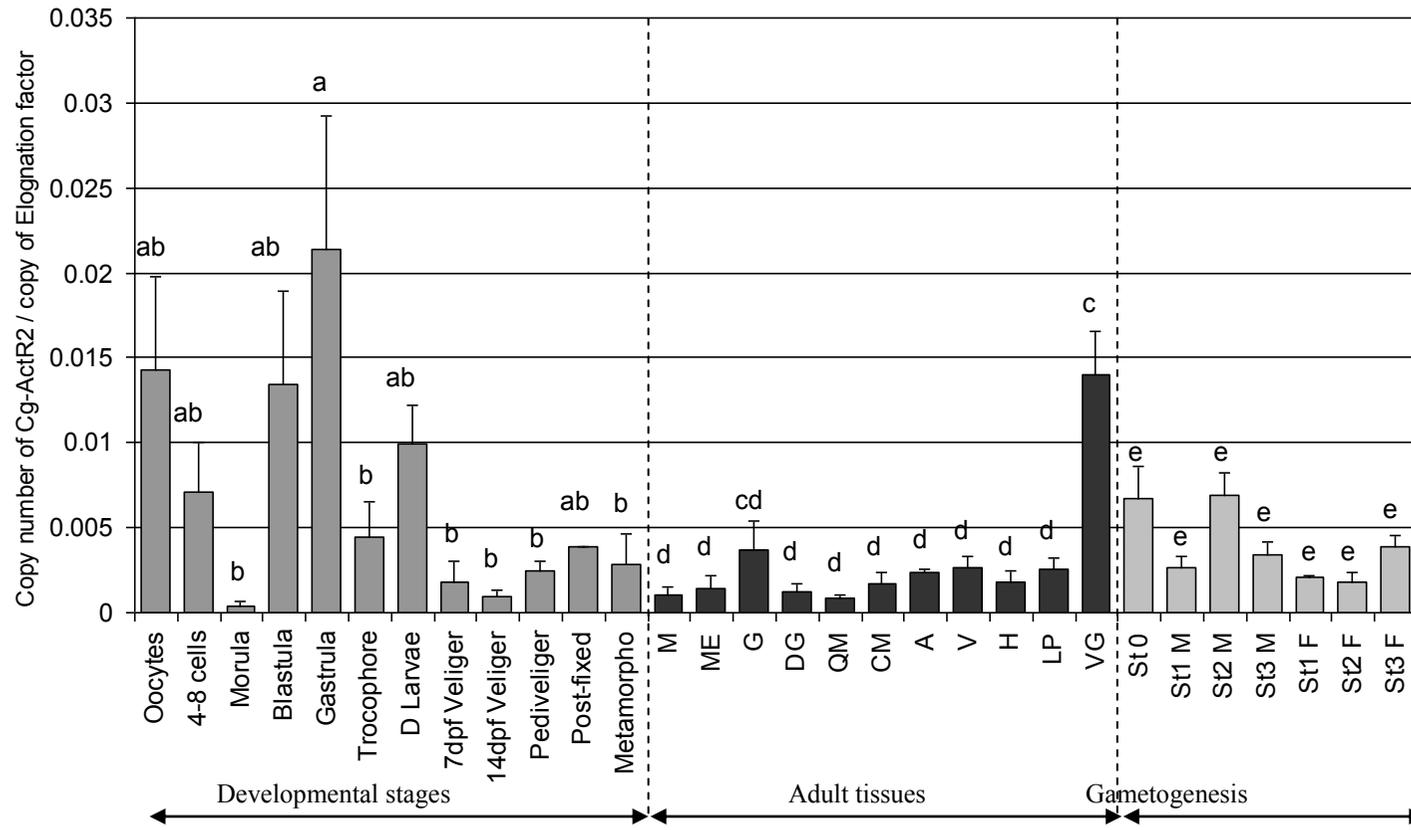


Fig. 4

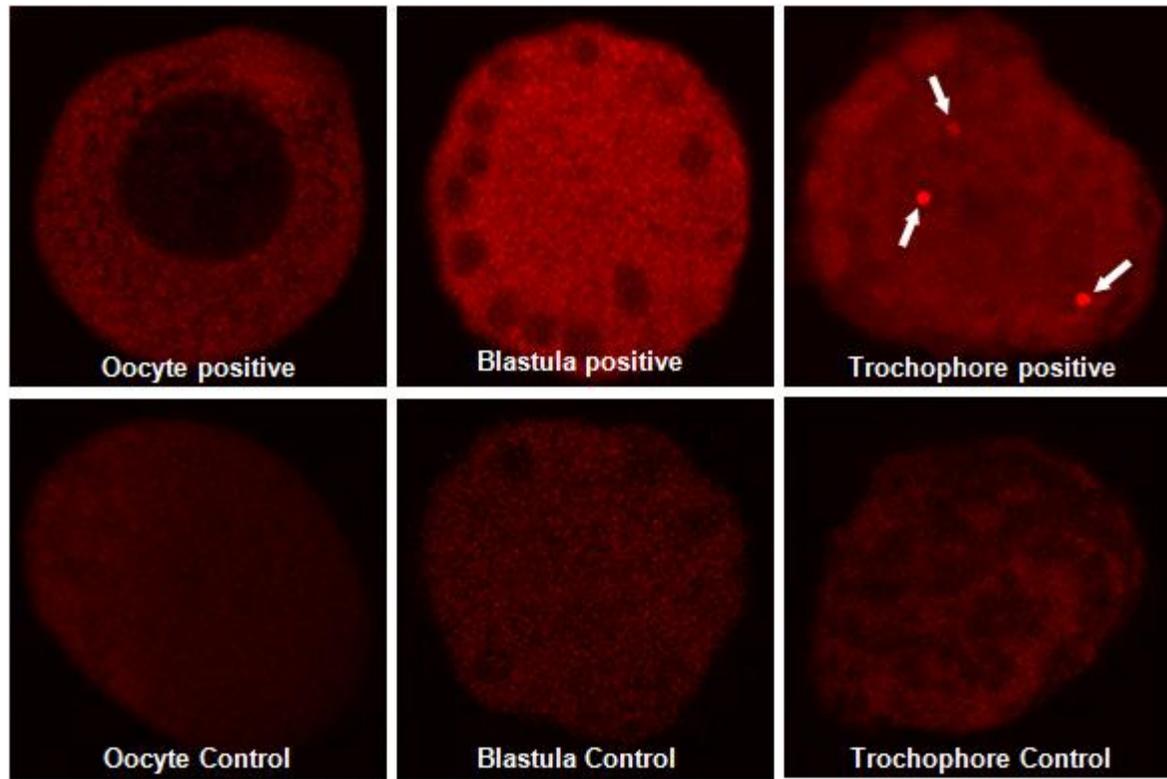


Fig. 5