**Regulation of cnidarian-dinoflagellate mutualisms: Evidence that activation of a host TGFβ innate immune pathway promotes tolerance of the symbiont**

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**ABSTRACT**

Animals must manage interactions with beneficial as well as detrimental microbes. Immunity therefore includes strategies for both resistance to and tolerance of microbial invaders. Transforming growth factor beta (TGFβ) cytokines have many functions in animals including a tolerance-promoting (tolerogenic) role in immunity in vertebrates. TGFβ pathways are present in basal metazoans such as cnidarians but their potential role in immunity has never been explored. This study takes a two-part approach to examining an immune function for TGFβ in cnidarians. First bioinformatic analyses of the model anemone *Aiptasia pallida* were used to identify TGFβ pathway components and explore the hypothesis that an immune function for TGFβs existed prior to the evolution of vertebrates. A TGFβ ligand from *A. pallida* was identified as one that groups closely with vertebrate TGFβs that have an immune function. Second, cellular analyses of *A. pallida* were used to examine a role for a TGFβ pathway in the regulation of cnidarian-dinoflagellate mutualisms. These interactions are stable under ambient conditions but collapse under elevated temperature, a phenomenon called cnidarian bleaching. Addition of exogenous human TGFβ suppressed an immune response measured as LPS-induced nitric oxide (NO) production by the host. Addition of anti-TGFβ to block a putative TGFβ pathway resulted in immune stimulation and a failure of the symbionts to successfully colonize the host. Finally, addition of exogenous TGFβ suppressed immune stimulation in heat-stressed animals and partially abolished a bleaching response. These findings suggest that the dinoflagellate symbionts somehow promote host tolerance through activation of tolerogenic host immune pathways, a strategy employed by some intracellular protozoan parasites during their invasion of

vertebrates. Insight into the ancient, conserved nature of host-microbe interactions gained from this cnidarian-dinoflagellate model is valuable to understanding the evolution of immunity and its role in the regulation of both beneficial and detrimental associations.

Keywords: *Aiptasia,* cnidarian, coral bleaching, dinoflagellate, innate immunity, *Nematostella,* Smads, *Symbiodinium,* symbiosis, transforming growth factor beta

Abbreviations: BMP, bone morphogenetic protein; DAF-FM DA, 4-amino-5-methylamino2',7'-difluorofluoroscein diacetate; DAN, 2,3-diaminonaphtalene; Dpp, decapentaplegic; DVR, decapentaplegic Vg-related; LPS, lipopolysaccharide; NO, nitric oxide; TGFβ, transforming growth factor beta

## 1. INTRODUCTION

Transforming growth factor beta (TGFβ) cytokines are members of a large superfamily of signaling molecules, found throughout the Metazoa, that control a variety of cellular functions including developmental programming, tissue homeostasis and immunity (Massagué, 1998). TGFβ ligands group into several clades including: TGFβ *sensu stricto*, activins and bone morphogenetic proteins (BMPs), and all participate in highly conserved multi-component downstream signal transduction pathways (Fig. 1) (Heldin et al., 1997). Furthermore, there is crosstalk between different TGFβ ligands and different downstream pathways, greatly increasing the complexity of these pathways and resulting in functional versatility and cell-specific outcomes (Feng and Derynck, 2005; Herpin and Cunningham, 2007). Homologs to these pathway components have been identified in cnidarians where they have been implicated in several aspects of developmental programming but, to date, not in immunity (Hayward et al., 2002; Matus et al., 2006a; Matus et al., 2006b; Saina and Technau, 2009; Samuel et al., 2001; Technau et al., 2005).

Functional characterization of TGFβs *sensu stricto* have been described only in vertebrates where they are involved in wide-ranging and largely anti-inflammatory mechanisms (Bogdan and Nathan, 1993; Hausmann et al., 1994; Ruscetti et al., 1993). Whether other TGFβ homologs play a role in immunity and self defense in invertebrates is an open question. A phylogenetic analysis of known TGFβs and putative TGFβ homologs from a variety of organisms identified a cluster of sequences with close phylogenetic placement to TGFβ *sensu stricto* that included three sequences from invertebrates (Herpin et al., 2004). This led Herpin and colleagues to suggest that these

sequences were homologs of TGFβ *sensu stricto* and to hypothesize that a role for TGFβs in animal immunity existed prior to the divergence of these two groups. They therefore called for functional studies to explore this hypothesis. This has recently been verified in a report showing that TGFβ-like and BMP-like signals are anti-inflammatory in *Drosophila* (Clark et al., 2011). Another study identified two cDNA sequences closely related to the TGFβ *sensu stricto* group from the ctenophore (comb jelly) *Mnemiopsis* (Pang et al., 2011). Since ctenophores may be the earliest branching phylum on the animal tree of life (Dunn et al., 2008), it now seems likely that TGFβ *sensu stricto* ligands have existed since the origin of metazoans.

A key component of animal immunity is the management of host-microbe interactions both negative and beneficial (McFall-Ngai, 2008). To obtain a complete picture of the role of host immunity in host-microbe interactions, it is critical to consider both mechanisms of resistance - the ability to limit the burden of a microbial invader, and mechanisms of tolerance - the ability to limit the health impact of this burden (Schneider and Ayres, 2008). The relative contribution of these two processes could help determine the cost/benefit balance of an interaction, for example tipping a relationship over evolutionary time from a parasitic to a mutualistic one or *vice versa*. It is now well recognized that negative and beneficial interactions share many of the same host-microbe signalling pathways and cellular responses, including host innate immune responses to invading microbes (Hentschel et al., 2000; Relman, 2008; Schwarz, 2008). One tolerance-promoting (tolerogenic) mechanism employed by some parasites and pathogens of vertebrates involves modulation of the TGFβ pathway during invasion (Ndungu et al., 2005; Simmons et al., 2006; Waghabi et al., 2005). In this study, we explore the presence

of a tolerogenic host innate immune response involving TGFβ in a cnidarian-dinoflagellate mutualism.

Cnidarian-dinoflagellate associations such as those that form reef-building corals are fundamentally important mutualistic symbioses in the marine environment. These partnerships provide the trophic and structural foundation of coral reef ecosystems (Dubinsky, 1990). This intracellular association is centered around nutrient exchange and is essential for both partners to thrive in nutrient-poor tropical seas. Cnidarian hosts, such as corals and anemones, harbor photosynthetic dinoflagellate endosymbionts, from the genus *Symbiodinium*, within gastrodermal cells in vacuoles of phagosomal origin known as symbiosomes. Initial colonization most often occurs when host gastrodermal cells lining the gastric cavity phagocitize symbionts ingested through the mouth during feeding (Colley and Trench, 1985).

Although cnidarian-dinoflagellate symbioses are stable in non-stressed conditions, various environmental stressors, most notably elevated temperature caused by global warming, can cause breakdown the partnership resulting in loss of symbionts from host tissues (Douglas, 2003). This phenomenon, known as coral bleaching, results in greatly reduced host fitness and can lead to reef destruction (Hoegh-Guldberg et al., 2007; van Oppen and Lough, 2009). The cellular mechanisms leading to symbiosis dysfunction and bleaching are largely unknown, but studies to date indicate that reactive oxygen and nitrogen species, host innate immunity, and host cell apoptosis all play a role (Detournay and Weis, 2011; Weis, 2008a). Important to the present study are experiments that show elevated nitric oxide (NO) in host tissues of symbiotic anemones when they are subjected to a hyperthermic stress similar to levels that elicit a bleaching response (Detournay and

Weis, 2011; Perez and Weis, 2006). Furthermore, addition of exogenous NO to anemones causes bleaching at ambient temperature. NO is a cytotoxic signaling molecule that plays a key role in the innate immune response in a variety of organisms (Fang, 2004). These results suggest that heat stress causes a cellular response related to an innate immune response that results in the elimination of the symbiont (Weis, 2008a).

We were interested, therefore, in functional investigations of host innate immunity in cnidarian-dinoflagellate mutualisms. The goal was to provide evidence of TGFβ pathway components in a symbiotic cnidarian and evidence of their role in a tolerogenic immune mechanism that regulates these partnerships. We hypothesized that symbionts cause upregulation of a TGFβ and corresponding modulation of an immune response that results in persistence of symbionts in host tissues. Studies were performed in the model anemone *Aiptasia pallida* which harbors the same genus of dinoflagellate, *Symbiodinium* sp., as corals. Using the production of NO as a measure of immune activation, we show evidence of activation of a TGFβ pathway by the presence of symbionts, indicating that this pathway may have an immune function in cnidarians and may play a role in establishment and maintenance of a successful cnidarian-dinoflagellate symbiosis.

## 2. MATERIALS AND METHODS

### *2.1. Identification of TGFβ pathway components from Aiptasia pallida*

TGFβ pathway components were identified in *Aiptasia pallida* through tBLASTn searches of AiptasiaBase, a publicly available *A. pallida* EST database consisting of

~5,000 unique sequences (http://aiptasia.cs.vassar.edu/AiptasiaBase/index.php) (Sunagawa et al., 2009) using human TGFβ pathway sequences as queries (see Table 1 for query sequences). An additional data set of 58,018 *A. pallida* contigs (Lehnert et al., 2012) was also searched in the same manner. Resulting *A. pallida* EST sequences were used as query sequences in a reciprocal BLASTx search of GenBank. The *A. pallida* TGFβ ligand sequence has been deposited in GenBank (GenBank ID: JX113692).

The alignment provided in the recent paper by Pang et al. (2011) formed the basis of a phylogenetic analysis of TGFβ ligands. The alignment was pruned to remove extraneous sequences, including several *Mnemiopsis* sequences that were not placed in known groupings in the Pang et al. analysis. Since the primary goal was to place *A. pallida's* TGFβ ligand on the tree and determine its homology with human TGFβ ligands, ApTGFβ plus human TGFβ ligands 1-3 were added to this set of sequences. A single TGFβ sequence identified from the genome of the poriferan *Amphimedon queenslandica* (Srivastava et al., 2010) was also added to the set. A BLASTp search of the set of *Aphmimedon* predicted gene models was used to identify this sequence. The gene models were downloaded from the link provided in the *Amphimedon* genome paper (ftp://ftp.jgi36psf.org/pub/JGI_data/Amphimedon_queenslandica/assembly/). Alignment of the final sequence set was performed in MUSCLE (http://www.drive5.com/muscle/).

Similarities and identities between sequences were calculated using the "Calculate identity/similarity for two sequences" option in BioEdit v 7.0.5.3 using the BLOSUM62 similarity matrix. Prottest v 2.4 (Abascal et al., 2005) was run on the alignment to estimate the empirical model of amino acid substitution that fit the data best. The LG + I + Γ model had the overall best fit according to all selection scenarios (AIC, AICc and

BIC). Maximum likelihood (ML) analyses were performed with the MPI version of RAxML v7.2.8 (RAXML-HPC-MCI) (Stamatakis, 2006). For each alignment, we conducted two independent ML searches: one with 25 parsimony starting trees (`raxmlHPC-MPI -f d -m PROTGAMMAILGF -s input.phy -#25 -k`) and another with 25 random starting trees (`raxmlHPC-MPI -f d -m PROTGAMMAILGF -s input.phy -#25 -d -k`). For all analyses, 1000 bootstrapped trees were computed.

Bayesian analyses were performed with MrBayes3.2 (Ronquist and Huelsenbeck, 2003). MrBayes does not support the LG model of evolution, so we used the second best fit model in ProtTest for each analysis (WAG+ I + Γ) with two independent five million generation runs of five chains, with trees sampled every 500 generations using the following execution block (`prset aamodelpr=fixed(rtrev); lset rates=Invgamma; prset statefreqpr=fixed(empirical); mcmp mcmcdiagn=no nruns=1 ngen=5000000 printfreq=5000 samplefreq=500 nchains=5 savebrlens=yes; mcmc;`). We evaluated all trees in a likelihood framework by computing likelihood scores for all trees using the LG matrix in PHYML v3.0 (Guindon and Gascuel, 2003) with the following command (`phyml -i 01-Input.phy -c 4 -m LG -a e -o lr -f e -u 01-Input.tre -v e -d aa -b 0 -s NNI`). We then chose the tree with the highest likelihood from all 50 ML searches and both Bayesian trees and applied both ML and bayesian consensus support values to the most likely tree, which was arranged and visualized using FigTree v1.3.1 (Rambaut, 2007). The tree was rooted with three GDNF sequences (Xl, Mm, Dre) and Mm neurturin using FigTree1.3.1 and then annotated manually using Adobe Illustrator. Nodes with bootstrap support between 60% and 100% were labelled on the trees.

*2.2. Maintenance of anemone and dinoflagellate cultures*

One advantage to the *Aiptaisa pallida-Symbiodinium* model system is that, unlike corals, *A. pallida* can survive without symbionts in an aposymbiotic state indefinitely if kept in the dark and fed regularly (Weis et al., 2008b). This allows for the experimental infection of aposymbiotic animals with cultured symbionts.

Mass cultures of *A. pallida* were maintained in aquaria of artificial seawater (Instant Ocean; Aquarium Systems, Mentor, OH, USA) at rt on a 12h light:12h dark photoperiod with a light irradiance of 70 µmol quanta $m^{-2}$ $s^{-1}$. Aposymbiotic anemones were kept in the same conditions, but in total darkness. Approximately four weeks prior to any experiments, both symbiotic and aposymbiotic anemones were transferred into bowls containing 0.22 µm filtered artificial sea water (FASW), placed in a 25°C incubator and kept in their respective light regimes. Anemones were fed once a week with freshly hatched brine shrimp. One week before an experiment, animals were starved and transferred to 24-well plates (~5 ml per well) and kept in the same 25°C incubator.

Cultured dinoflagellates, *Symbiodinium sp.*, clade B (CCMP830) were maintained in 50 ml flask culture in sterile Guillard's f/2 enriched seawater culture medium (Sigma, St Louis, MO, USA). Dinoflagellate cultures were kept in the same light and temperature conditions as the symbiotic anemones.

*2.3 PCR verification of ApTGF*β *sensu stricto*

Aposymbiotic *Aiptasia pallida* were treated with 1µg/mL LPS from *E.coli* (L2880 Sigma, St Louis, MO, USA) in the dark for 18 hours and then frozen down in liquid nitrogen for RNA extractions. To prepare for this treatment, a 1mg/ml LPS solution in

DMSO was prepared by a 1:5 dilution of a 5mg/ml stock solution. 2µl of the 1mg/mL LPS solution was then added to a well that contained one anemone in 2mL of FASW. Total RNA extractions were performed using a combination of the Trizol® (Invitrogen, Carlsbad, CA, USA) and RNeasy Mini Kit (Qiagen, Valencia, CA, USA) protocols. Quality and quantity of the RNA was assessed using the Nanodrop 2100 (Thermo Scientific) and visualization on a 1% agarose gel. cDNA synthesis was then carried out using the SuperScript® III first- strand synthesis kit (Invitrogen, Carlsbad, CA, USA) starting with 750 ng of RNA. PCR amplification was performed with the primers AipTGFb_for ( 5'- GCT CTC GAC TCG GCA TTT T - 3') and AipTGFb_rev (5'- AGT CCT GCC TCC CTT ACG TT - 3') using the following protocol: 94°C for 3 minutes followed by 35 cycles of 95°C for 45 seconds, 50° for 45 seconds, and 72°C for 1 minute, and a final extension step at 72°C for 10 minutes. A 377 bp product was obtained and then cloned using the PGEM T-easy vector (Promega, Fitchburg, WI, USA). Plasmids obtained from the cloning process were purified using the Qiaprep® Spin Mini-prep Kit (Qiagen, Valencia, CA, USA), and checked for an insert of the appropriate size by EcoRI FastDigest® (Fermentas, Glen Burnie, MD, USA). Plasmids with the correct insert were sequenced on the ABI Prism® 3730 Genetic Analyzer using the T7 promoter primer (5' - TAA TAC GAC TCA CTA TAG GG - 3' ) at the Center for Genome Research and Biocomputing at Oregon State University.

*2.4. Experimental manipulation of anemones*

Symbiotic and aposymbiotic anemones were subjected to a variety of experimental manipulations to examine the immune status of both anemone types and the

role of TGFβ pathway components in (1) modulating an immune response and (2) the regulation of symbiosis. In some experiments, lipopolysaccharide (LPS) was used to elicit an immune response in host tissues (Detournay and Weis, 2011; Perez and Weis, 2006). Changes in the strength of an immune response were measured after alteration of components of the TGFβ pathway. Immune response was quantified by measurement of NO production. In some experiments, anemones were subjected to a high temperature stress to elicit both an immune response and a bleaching response. Changes in symbiotic state, i.e. gain or loss of symbionts in host tissues, were determined by counting symbionts in host tissues (described below).

2.4.1. Determination of the immune status of symbiotic and aposymbiotic anemones. To determine if the presence of symbionts affected the immune status of *A. pallida*, symbiotic and aposymbiotic anemones were incubated overnight in 1 µg/ml LPS (Sigma, St Louis, MO, USA) or the vehicle control dimethylsulfoxide (DMSO; Sigma, St Louis, MO, USA). Immune response was measured by quantifying NO in host tissues fluorometrically by confocal microscopy (described below). To examine the effect of the NOS inhibitor $N^G$-methyl-L-arginine (L-NMA) on NO production after LPS treatment, some anemones were incubated for 10·min in l0·mmol·l–1 L-aminoguanidine (LAG; Sigma) before processing for confocal microscopy.

2.4.2. Incubation of aposymbiotic anemones in TGFβ *sensu stricto*. To test the influence of the addition of TGFβ and possible subsequent activation of a TGFβ pathway on the immune status of aposymbiotic anemones, anemones were incubated for 4 h in FASW supplemented with water, the vehicle control DMSO (14 mM) or recombinant

human TGFβ *sensu stricto* at varying concentrations (Sigma, St Louis, MO, USA). Following this, anemones were incubated overnight in 1 µg/ml of LPS or the vehicle control DMSO (14 mM) to elicit an immune response. Immune response was measured by quantifying NO in host tissues fluorometrically by confocal microscopy.

2.4.3. Addition of anti-TGFβ to aposymbiotic anemones during onset of symbiosis. To test the effect of blocking putative TGFβ pathway activation on the onset of symbiosis, aposymbiotic anemones were incubated in FASW supplemented with (1) 10 µg/ml of rabbit IgG-purified anti-human TGFβ *sensu stricto* (anti-TGFβ; cat# T-9429, Sigma, St Louis, MO, USA), (2) 10 µg/ml of rabbit IgG control isotype or (3) a vehicle control (PBS BSA 5%) for 1 h. Subsequently, cultured dinoflagellates were added to each well at a final concentration of $2x10^5$ symbionts $ml^{-1}$. After 12 h, the wells were rinsed twice with FASW and anti-TGFβ or controls were replaced. Anemones were sampled at 24, 72 and 144 h post infection (n=3 anemones for each treatment for each time point). Infection success and NO production were assessed fluorometrically by confocal microscopy.

2.4.4. Addition of TGFβ to symbiotic animals before hyperthermic stress. To investigate the influence of the putative activation of a TGFβ pathway on host immune and bleaching responses, animals were incubated with human TGFβ *sensu stricto* prior to being subjected to a hyperthermic stress. Anemones were incubated with vehicle control or varying concentrations of TGFβ for 2 h prior to a 24 h incubation at 33ºC, a stress known to elicit bleaching (Detournay and Weis, 2011; Dunn et al., 2004; Perez and Weis, 2006) (n=3 animals per concentration). Controls were subjected to 2 h incubation in the

vehicle control followed by incubation at 25ºC for 24 h. Immune response was measured by quantifying NO in host tissues by the DAN assay (described below).

In another experiment, animals were incubated for 2 h in either vehicle control or 100 ng/ml of human TGFβ *sensu stricto* before being subjected to a 24 h hyperthermic stress at 33ºC. Loss of symbionts from host tissues, or bleaching was quantified using methods published previously (Detournay and Weis, 2011; Dunn et al., 2007; Perez and Weis, 2006). Both symbionts expelled into the media from anemones and those remaining in the host (*in hospite* symbionts) were quantified using haemocytometer counts. Percent bleaching was calculated as the number of expelled symbionts/(expelled symbionts + number of symbionts in host tissues) X 100.

*2.5. Confocal microscopy to measure NO production and infection success in host tissues*

Confocal microscopy on whole anemones was used to visualize NO and the presence of symbionts. In all cases, after experimental manipulation of anemones, FASW was replaced with 1 ml of relaxing solution (1:1, 370 mM $MgCl_2$:FASW) containing the NO dye when appropriate. Anemones were incubated for 30 min in the dark and then rinsed twice with relaxing solution. Samples were observed under a Zeiss LSM 510 Meta microscope with a 40x/0.8 water objective lens and a working distance of 0.8-3.2 mm. To measure and visualize production of NO, animals were loaded with 15 µM 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA, Molecular Probes, Eugene, OR, USA) (Detournay and Weis, 2011; Nagano and Yoshimura, 2002; Perez and Weis, 2006) with excitation and emission wavelengths of 488 and 510-530 nm respectively. *Symbiodinium* present in symbiotic anemones were visualized by detecting

chlorophyll autofluorescence with excitation and emission wavelengths of 543 and 600-700 nm respectively. Before image scanning, the focal plane of the optical section was adjusted to include the gastrodermal layer of tentacles. For each experiment, all images were obtained with the same software scanning settings, including detector gain and laser intensity. Quantification of fluorescence was achieved by first defining the gastrodermal tissue of anemone tentacles as regions of interest and measuring the mean fluorescence intensity (MFI) for that region with the LSM 5 software (Zeiss).

In experiments that measured onset of symbiosis, symbiont numbers were quantified in host tentacles using confocal imaging. Chlorophyll autofluorescence intensity for each pixel was measured and a threshold value corresponding to the background was defined by measuring the MFI at 600 nm of a gastrodermal section without symbionts (MFI≈20). Infection success was expressed as percent of pixels with an autofluorescence intensity above the threshold.

*2.6. $NO_2^-$ quantification as a measurement for NO (DAN method)*

In some experiments, NO production was measured fluorometrically in homogenized animals using 2,3-diaminonaphtalene (DAN) (Dojindo, Gaithersburg, MD, USA) with methods modified from (Guindon et al., 2005; Nussler et al., 2006) as previously used (Detournay and Weis, 2011). Since NO is a highly reactive free radical, several metabolites are produced under physiological conditions. NO reacts with oxygen to form $NO_2^-$ in aqueous solution. DAN reacts with $NO_2^-$ in acidic conditions to produce fluorescent naphthalenetriazole that is quantifiable with a plate reader.

After experimental treatment, anemones were rinsed in 100 ml of 370 mM $MgCl_2$ just before being flash frozen in liquid nitrogen and stored at -80°C. Anemones were thawed on ice and homogenized in 340 µl of deionized water at 4°C. The solution was centrifuged at 14,000 x g for 10 min at 4°C. After centrifugation, 5 µl of the supernatant was used for protein determination (Bradford assay). The remaining supernatant was filtered through a prewashed (3 times in 500 µl of deionized water) Centricon column (10 kDa cut off). A standard curve was constructed using 10,000, 5,000, 2,500, 1,250, 625, 312, and 156 nM $NaNO_2$. One hundred fifty µl of standard or sample were added to wells of a 96-well black-bottom plate (Nunc Black 96F polysorp; Fisher Scientific, Rochester, NY, USA) and 75 µl of a DAN solution at 158 µM/HCl 0.62 N was added to each well. The plate was mixed for 10 sec and incubated at 28°C in the dark for 10 min before adding 35 µl of 2 N NaOH. Finally, the DAN $NO_2^-$-dependent fluorescence at 410 nm was quantified after excitation at 365 nm in a fluorescent platereader.

*2.7. Immunoprecipitation and immunoblot analysis of Smads*

Smads are transcription factors that are activated by phosphorylation as a part of TGFβ pathways (See Fig. 1). We tested for Smad phosphorylation as evidence of TGFβ pathway activation in *A. pallida* using immunoanalyses. Sets of eight aposymbiotic or symbiotic anemones were ground in 1 ml of ice-cold homogenization buffer (50mM Tris-HCl, pH 7.4, 300mM NaCl, 5mM EDTA) with protease inhibitor cocktail (BD Biosciences, San Jose, CA, USA). In another set of experiments, sets of eight symbiotic anemones were pre-treated overnight with either 10 µg/ml IgG-purified rabbit anti-human TGFβ *sensu stricto*, 10 µg/ml rabbit IgG isotype control or FASW only and

ground as described above. Homogenates were centrifuged at 4°C for 15 min at 14,000 x g. The supernatants were pre-cleared by adding 100 µl of protein G beads according to the manufacturer's instructions (Sigma, St Louis, MO, USA). After centrifugation at 14,000 x g at 4°C for 10 min, supernatants were decanted and protein concentrations were determined using a Bradford assay. One mg of pre-cleared homogenate was immunoprecipitated overnight at 4°C with 20 µg of rabbit anti-human phosphorylated-Smad2/3 (anti-pSmad2/3, cat# SC-11769-R Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by capture of the immunocomplex with a protein G bead for 1 h at 4°C. After a pulse centrifugation, the immunocomplexes were harvested and washed 3 times with 800 µl PBS. The immunocomplexes were then resuspended in Laemmli sample buffer (Biorad, Hercules, CA ,USA) and boiled for 5 minutes.

For Western blot analysis, 20 µl of immunocomplexes were resolved on a 7 % SDS-PAGE gel. Proteins were electrophoretically transferred overnight onto nitrocellulose membrane. After blocking with 5% nonfat dry milk in TBS-Tween 20 (0.1%) for 1 h at 37 °C, membranes were incubated with anti-pSmad2/3 or an IgG isotype control, both at a concentration of 0.66 µg/ml, for 2 h at rt. The blots were washed three times in TBS-Tween 20 followed by incubation in a secondary HRP-conjugate goat anti-rabbit IgG (0.2 µg/ml, Sigma, St Louis, MO, USA) for 1 h. Bands were detected by enhanced chemiluminescence (Millipore, Temecula, CA , USA). Relative band intensities were quantified with IMAGE J software.

## 3. RESULTS

### *3.1. Major components of TGFβ pathways are present in cnidarians*

tBLASTn searches of two sequence databases identified several components of TGFβ pathways in *A. pallida* (Table 1), including ligands, receptors and Smads. Three sequences were identified as proteins belonging to the TGFβ *sensu stricto* pathway. The ApTGFβ *sensu stricto* sequence was identified with a top BLAST hit to a sequence from the chordate *Branchiostoma japonicum*. CCAS4061.g1 displayed a 41% sequence identity to the serine/threonine kinase domain of human TGFβ *sensu stricto* receptor II. CCAS2691.g1 was identified as a homolog to Smad2, which is specifically activated by TGFβ *sensu stricto* and activin proteins (Herpin et al., 2004).

ApTGFβ aligned closely with the TGFβ *sensu strico* sequence group (Fig. 2). The ApTGFβ shares nine canonical cysteines with TGFβ *sensu strictos*. Only seven of these cysteines are present in the BMPs. The cysteines are important in the tridimensional structure, or 'cysteine knot motif' of the mature ligand (Herpin et al., 2004). The TGFβ *sensu stricto* homolog from *A. pallida* shares 55% sequence identity and 76% similarity with the full pre-pro-protein domain of *B. floridae* TGFβ. Identities and similarities of ApTGFβ with Hs TGFβ1, 2 & 3 averaged 51 and 70%, respectively.

Phylogenetic analysis of pre-pro-protein sequences of TGFβ homologs from a variety of organisms is shown in Fig. 3. Overall, orthologs from vertebrates and invertebrates grouped together within clades of ligand types closely mirroring the results of Pang et al. (2011). The TGFβ-like group includes TGFβs *sensu stricto*, activins/inhibins, myostatins and lefty subgroups. BMP-like ligands branch ladder-like from the TGFβ-like group and includes nodal, BMP3, ADMP, Dpp/BMP2/4, Vg1, BMP5-8 and GDF subgroups. ApTGFβ groups with strong support with the TGFβ *sensu stricto* sequences, falling next to the sponge sequence (*A. queenslandica*) and between

chordate sequences from *B. floridae* and *C. intestinalis* and the two ctenophore (*M. leidyi)* TGFβ sequences.

*3.2. Symbiotic anemones have impaired capacity to respond to an immune elicitor*

To examine the capacity of *A. pallida* to respond to a major immune response elicitor and to determine if symbiosis influences this capacity, LPS was used to stimulate both symbiotic and aposymbiotic anemones. After 12 h, NO induction was stronger in aposymbiotic compared to symbiotic anemones (Fig. 4). This suggests that the presence of symbionts somehow impairs the capacity of the host to mount an immune response when challenged by LPS. Incubation of anemones with L-NMA resulted in a complete inhibition of NO production (data not shown), suggesting that the NO signal was coming from NOS activity in the host. These results are similar to earlier studies in *A. pallida* using another NOS inhibitor (Perez and Weis, 2006).

*3.3. Addition of exogenous TGFβ modulates LPS-induced NO production*

To explore the possibility that activation of a TGFβ pathway regulates an immune response in *A. pallida*, aposymbiotic anemones were incubated with a series of concentrations of exogenous human TGFβ *sensu stricto* before being challenged overnight by LPS. Animals incubated in exogenous ligand displayed reduced NO-specific DAF-FM DA staining compared to control animals (Fig. 5A). There was a dose-dependent decrease in immune activation, with incubation in increasing concentrations of ligand (Fig. 5B) and a significant decrease in NO staining in animals incubated in 100 ng/ml of ligand compared to control animals (Fig. 5C). This suggests that *A. pallida*

possesses a pathway that is activated by TGFβ ligands and induces an anti-inflammatory signal.

*3.4. Addition of anti-TGFβ prevents the onset of symbiosis.*

To explore the possibility that a TGFβ pathway plays a role in the onset of symbiosis, aposymbiotic animals were incubated in anti-human TGFβ *sensu stricto* before being inoculated with symbionts. Control animals, both those incubated in vehicle alone and in a rabbit IgG isotype, displayed very low NO-dependent DAF-FM DA staining (Fig. 6A & C). In contrast, anti-TGFβ-treated animals displayed higher DAF-FM DA staining than control animals at 72 and 144 h post-infection, suggesting the induction of an immune response. By 72 h post-infection, both the vehicle and rabbit isotype controls exhibited increases in symbiont numbers compared to anemones at 24 h post-infection (Fig. 6B). In contrast, anti-TGFβ-treated anemones contained almost no symbionts at 72 and 144 h post infection and were significantly less infected than control animals at these times (Fig. 6B & C). This could indicate that when a tolerogenic TFGβ pathway is blocked, the host resists successful infection by the symbionts through induction of an immune response.

*3.5. pSmad2/3 is expressed in A. pallida as a function of symbiotic state*

In vertebrates, the transcription factors Smad 2 & 3 are phosphorylated downstream of TGFβ *sensu stricto* and activin ligand binding. Functional genomic studies have identified Smads in cnidarians (Technau et al., 2005) and we show evidence of them in *A. pallida* (Table 1). We were therefore interested in examining

phosphorylated Smad2/3 in *A. pallida* both to seek evidence of a TGFβ *sensu stricto*-like pathway as well as evidence for a role in symbiosis. Immunoblots of immunoprecipates of *A. pallida* protein with anti-human pSmad2/3 revealed a strong band at 50 kD in every conditions due to the presence of the heavy chain from the rabbit anti-pSmad2/3 IgG used in the immunoprecipitation step. The immunoblot revealed also a band at approximately 60 kD present in symbiotic animals. This signal was very week in aposymbiotic animals. No 60 kD band was present in blots incubated in isotype IgG for the primary antibody (Fig. 7A). This suggests that a Smad, related to Smad 2/3, is present in *A. pallida* and more highly phosphorylated in the symbiotic than in the aposymbiotic state. This could indicate activation of a tolerogenic TGFβ pathway when symbionts are present. When anti-human TGFβ *sensu stricto* was added to anemones to block TGFβ pathway activation, expression of putative pSmad2/3, detected with immunoblots, significantly decreased compared to animals incubated in the isotype control IgG (Fig. 7B).

*3.6. Addition of TGFβ to A. pallida during heat stress reduces bleaching*

Perez and Weis (2006) demonstrated that heat-stressed anemones display a strong increase in NO and proposed that this plays a key role in loss of symbionts during hyperthermic-stress-induced bleaching. To determine if a TGFβ pathway could modulate the NO response to hyperthermic stress, heat-stressed anemones were incubated in a range of exogenous TGFβ concentrations and measured for NO activity using the DAN assay. Control anemones incubated at 25ºC with vehicle only contained 7.9 ± 4.7 µM $NO_2^-$/µg anemone protein (n=3) (data not shown). In contrast, anemones incubated at 33ºC displayed high NO activity in the absence of TGFβ, 18.7 ± 5.8 µM $NO_2^-$/µg

anemone protein (Fig. 8A). However with increasing TGFβ concentrations, NO production decreased in a dose-dependent manner to a minimum of 2.9±1.6 μM $NO_2^-$/μg anemone protein at 100 ng/ml TGFβ. Finally bleaching, or loss of symbionts from anemones, was measured in heat-stressed animals incubated with and without the addition of human TGFβ *sensu stricto* (Fig. 8B). The presence of the TGFβ ligand significantly reduced symbiont loss from host tissues compared to animals without TGFβ. If bleaching is a modified immune response to compromised symbionts (Detournay and Weis, 2011; Dunn et al., 2007; Perez and Weis, 2006), these data suggest that this is at least partially mediated by an active TGFβ pathway.

## 4. DISCUSSION

### *4.1. Genomic evidence of TGFβ pathways in A. pallida and other cnidarians*

Searches of the 5,000 unigene AiptasiaBase and an additional dataset revealed the presence of homologs to TGFβ pathway components in *A. pallida*, including a homolog to TGFβ *sensu stricto* (Table 1). These data add to other studies that have characterized TGFβ pathway components in cnidarians (Matus et al., 2006b; Saina and Technau, 2009; Samuel et al., 2001; Technau et al., 2005; Zoccola et al., 2009). The discovery of a TGFβ *sensu stricto* homolog in *A. pallida* is notable because previously, the function of this ligand had been described only in vertebrates where it is involved in immunity, mostly in anti-inflammatory roles. Although TGFβ *sensu stricto* sequences have been identified in the chordates *Ciona* and *Branchiostoma* and the hemichordate *Saccoglossus*, prior to this study, the only non-deuterostome with an identified TGFβ *sensu stricto* was the

ctenophore *Mnemiopsis leidyi* (Pang et al., 2011), a member of one of the earliest-branching metazoan phyla (Dunn et al., 2008; Hejnol et al., 2009). We also identified a TGFβ ligand from *A. queenslandica*, a member of the Porifera, another early-branching phylum. The closest homolog found in the genome of the cnidarian *Nematostella vectensis* is Nv myostatin, reported by Saina and Technau (2009). Thus, this is the first description of an immune function for a TGFβ *sensu stricto* in any non-deuterostome.

Based on their phylogenetic analyses of Related TGFβs and their close placement to vertebrate TGFβ *sensu strictos*, Herpin et al. (2004) hypothesized that an immune function for TGFβ ligands could have arisen by gene duplication prior to the protostome/deuterostome divergence. The addition of the *M. leidyi*, *A. queenslandica*, and *A. pallida* TGFβ *sensu stricto* ligands to the TGFβ tree, and the immune function of *A. pallida's* TGFβ pathway reported here suggests instead that the original *sensu stricto* ligand was present at the base of the metazoa and may have had an immune function, at least by the time cnidarians diverged. It is unclear why *sensu stricto* molecules have been retained in some basal lineages, while they appear to have diverged or have been lost in others, such as *N. vectensis*. Genome data from additional early-branching animals will help to resolve precisely how the TGFβ superfamily evolved. Additional functional studies will determine the roles that related TGFβ molecules play in these early-branching metazoans.

*4.2. Functional evidence of a role for a TGFβ pathway in immunity in A. pallida*

*A. pallida* showed a dose-dependent decrease in immune activity in response to addition of recombinant human TGFβ *sensu stricto* (Fig. 5), suggesting that a TGFβ can

act in an immunomodulatory, tolerogenic fashion in cnidarians. Such cross-taxa experiments demonstrating the ability of TGFβs in one species to functionally mimic putative homologous counterparts in another have been performed in other cellular contexts (Franchini et al., 2006; Padgett et al., 1993; Sampath et al., 1993; Zoccola et al., 2009). That these cross-taxa experiments work is not surprising given that TGFβ ligands are pleiotropic within a given animal with crosstalk occurring between different ligands and different downstream pathways (Feng and Derynck, 2005; Herpin and Cunningham, 2007). To date, no studies have investigated an immune function for TGFβs in cnidarians. Functional investigations have instead focused on the role of TGFβs in developmental programming, including muscle growth in the case of Nv myostatin (Saina and Technau, 2009). *In situ* hybridizations of myostatin in *N. vectensis* did not show expression in areas of muscle growth and morphogenesis. The authors suggest that there could be other yet-to-be-described functions for this gene in the anemone. Since a TGFβ *sensu stricto* has not been identified in the *N. vectensis* genome, it is possible that Nv myostatin may have retained multiple functions, including role(s) in innate immunity.

*4.3. The role of a TGFβ pathway in promoting host tolerance of symbionts*

Several lines of evidence point toward a tolerogenic role for a host TGFβ in maintenance of the anemone host-dinoflagellate symbiont partnership. The inability of symbionts to invade aposymbiotic anemones when a TGFβ is putatively blocked by anti-TGFβ (Fig. 6) suggests that a TGFβ's tolerogenic activity plays a role in the successful establishment of symbiosis in *A. pallida*. The higher expression of putative phosphorylated Smad2/3 in symbiotic compared to aposymbiotic anemones (Fig. 7A) and

the decrease in its expression in symbiotic anemones when a TGFβ is putatively neutralized (Fig. 7B) further suggest the presence of an active TGFβ pathway during symbiosis.

Previous studies examining the cellular mechanisms underlying cnidarian bleaching after an elevated temperature stress have demonstrated an increase in NO in the host-symbiont partnership, leading to the hypothesis that cnidarian bleaching is a modified immune response to a symbiont compromised by heat and oxidative stress (Detournay and Weis, 2011; Perez and Weis, 2006). Our findings in the current study show that this putative immune response is modulated by the addition of exogenous TGFβ *sensu stricto* from humans. Both amount of NO produced by the heat-stressed holobiont (Fig. 8A) and numbers of symbionts released from hosts (bleaching) decrease with addition TGFβ (Fig. 8B), suggesting that it can act in a tolerogenic, immunomodulatory fashion on the symbiosis.

Other work is providing evidence that TGFβ pathway components (see Fig. 1) are involved in the regulation of cnidarian-dinoflagellate symbiosis. A transcriptomic study of *Anthopleura elegantissima*, another symbiotic anemone species, showed an upregulation of CD36, a TGFβ pathway activation factor, in the symbiotic state (Rodriguez-Lanetty et al., 2006). In addition, preliminary studies by our group demonstrate a direct correlation between CD36-Thrombospondin (TSP) binding and infection success of *A. pallida* (Dicks, Detournay and Weis, unpublished data).

It is increasingly recognized that negative and beneficial interactions share many of the same host-microbe signalling pathways and cellular responses, including host innate immune responses to invading microbes (Hentschel et al., 2000; Relman, 2008;

Schwarz, 2008). The results of our study suggest that mutualistic symbionts somehow upregulate a tolerogenic TGFβ pathway in host anemones. Studies of the protozoan parasites *Trypanosoma* and *Plasmodium* show a similar promotion of vertebrate host immune modulation during invasion. *T. cruzi* activates the TGFβ pathway by causing overexpression of host TSP (Simmons et al., 2006; Waghabi et al., 2005). Studies of *Plasmodium* spp. infection suggest that binding of CD36 by parasite proteins triggers a complex tolerogenic response that includes a variety of innate immune pathways (Ndungu et al., 2005; Patel et al., 2007). The parallels between dinoflagellate and *Plasmodium* invasion and usurpation of the host immune response are particularly striking given the close phylogenetic placement of these two groups as sister taxa in the Alveolata (Adl et al., 2005). Furthermore, these parallels point to an ancient history of symbiosis (negative and cooperative) between this group and animals (Okamoto and McFadden, 2008; Schwarz, 2008).

*4.4. The apparent paradox of immunosuppression in symbiotic A. pallida*

Compared to aposymbiotic animals, symbiotic *A. pallida* displayed a greatly attenuated response to the classic microbial immune elicitor LPS (Fig. 4). This result taken in isolation indicates that symbiotic animals are immunosuppressed and could even suggest that symbionts are a burden on the host by making them more susceptible to infection. Yet decades of study on cnidarian-dinoflagellate mutualisms confirm the beneficial basis of the interaction. Furthermore, host corals that lose their symbionts after coral bleaching show greatly increased disease susceptibility compared to healthy, non-bleached counterparts (Harvell et al., 2001; Jones et al., 2004). This increased resistance

to pathogenic invasion while in the symbiotic state is a trait shared by many other mutualisms (Relman, 2008). This leads us to hypothesize that in *A. pallida*, symbionts elicit tolerogenic responses in the host without inducing a decrease in host resistance to pathogens.

Other studies provide some hints about how hosts might tolerate symbionts while mounting a full immune response to fight pathogens (Lhocine et al., 2008). TGFβ activation in dendritic cells induces an autocrine tolerogenic signal (Belladonna et al., 2008). A similar signal, restricted to the gastrodermal microenvironment harboring symbionts, could explain the apparent paradox of symbiont tolerance along with resistance to pathogenesis in cnidarians. Finally, consideration of the holobiont - that is the animal host, dinoflagellate symbionts and, in the case of corals, a complex microbial flora (Rosenberg et al., 2007) - is likely key to understanding the tolerance-resistance balance. For example, anti-microbial compounds produced by the coral holobiont contribute to resistance of pathogenic microbial invaders (Ritchie, 2006) but it is unclear which partner or multiple partners contain the biosynthetic pathways responsible for production of these compounds.

*4.5. Is host innate immunity central to symbiosis stability?*

This work suggests that the host innate immune response and its putative modulation by symbionts may be central to the stability of the symbiosis. These may be underlying processes common to the onset and breakdown of cnidarian-dinoflagellate symbiosis, two events that have to date been considered separately. The data presented here represent just a first step in describing innate immune pathways in cnidarians and

their role in symbiosis regulation. Numerous studies of other host-microbe interactions document multiple, pleiotropic inter-partner communication and regulation processes. Functional genomic studies of cnidarian-dinoflagellate symbiosis and bleaching are giving similar glimpses of the underlying complexity at play in this interaction including the presence of innate immunity genes (DeSalvo et al., 2008; Rodriguez-Lanetty et al., 2009; Rodriguez-Lanetty et al., 2006; Schwarz et al., 2008; Vidal-Dupiol et al., 2009) and in one of these studies (Rodriguez-Lanetty et al., 2006) a putative TGFβ pathway component (SR-BI/CD36 homolog) was upregulated in symbiotic anemones compared to non-symbiotic anemones, consistent with the results presented here. Insight into the ancient, conserved nature of host-microbe interactions gained from this cnidarian-dinoflagellate model is valuable to understanding the evolution of immunity and its implication in numerous health disorders in higher organisms.

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Figure 1. A simplified TGFβ signalling pathway. TGFβ is secreted in a latent form (Li et al., 2006). The mechanisms of activation are not absolutely clear, but in many cases they involve interaction with extracellular matrix proteins including thrombospondin (TSP), specifically when TSP is bound to the scavenger receptor CD36 (Li et al., 2006; Masli et al., 2006; Yang et al., 2007). Once activated, TGFβ binds to a TGFβ receptor type II, a transmembrane protein. This complex in turn binds to a type I receptor to form a heterotetramer which proceeds to activate, by phosphorylation, a family of transcription factors called Smads (ten Dijke et al., 1996). Smads migrate to the nucleus and promote or inhibit transcription of a large variety of genes (Massague and Wotton, 2000).

Figure 2. Multiple sequence alignment of mature TGFβ ligands including ApTGFβ. Asterisks mark the nine cysteines required in TGFβs *sensu stricto* required for formation of a cysteine knot motif. Taxa abbreviations are listed in Supplemental Table 1. Columns of residues are shaded by similarity group conservation (defined by GeneDoc and the BLOSUM62 matrix) where black shows 100%, dark grey shows 80% and light grey shows 60% similar residues in a column.

Figure 3. A phylogenetic tree generated using maximum likelihood analysis of pre-pro-protein sequences of TGFβ family members. Bayesian posterior probabilities greater than 60% are displayed at the nodes. RAxML bootstrap support values are shown as circles, where an open circle represents 100%, a partially filled circle represents 90% and a filled circle represents 50%. Taxa abbreviations are listed in Supplemental Table 1.

Figure 4. Effect of immune stimulation by LPS on NO production in aposymbiotic *vs*

symbiotic *A. pallida*. Anemones were exposed overnight to 1 µg/ml LPS. (A) Representative confocal images of DAF-FM DA-loaded tentacles of anemones stimulated overnight in 1µg/ml LPS. The DAF-FM DA (510-530nm) NO-dependent fluorescent signal appears in yellow and symbiont autofluorescence (600-700nm) in red. (B) DAF-FM DA fluorescence in anemones incubated in DMSO (LPS-) or 1µg/ml LPS (LPS+) (Bars represent means + SD; n=3 anemones; ANOVA P=0.039 Post Hoc Bonferroni, * different from control value by P<0.05)

Figure 5. Addition of exogenous human TGFβ *sensu stricto* reduces NO production in aposymbiotic *A. pallida*. Anemones were incubated in ligand for 2 h before being immune-stimulated overnight in 1µg/ml LPS. (A) Representative confocal images of DAF-FM DA-loaded tentacles of anemones incubated, in vehicle only or 100 ng/ml TGFβ. (B) DAF-FM DA fluorescence in anemones as a function of varying concentrations of exogenous TGFβ *sensu stricto.* (C) DAF-FM DA fluorescence in anemones incubated in vehicle only and 100 ng/ml TGFβ *sensu stricto* (Bars are means + SD, n=3, *t*-test P=0.039.)

Figure 6. Addition of anti-human TGFβ *sensu stricto* to putatively block a TGFβ pathway increases NO production and reduces infection success in *A. pallida.* Aposymbiotic anemones were incubated in: vehicle only control, rabbit isotype control (IgG) or rabbit anti-human TGFβ *sensu stricto,* for 1 h prior to being inoculated overnight with symbionts. (A) NO-dependent DAF-FM DA fluorescence expressed as mean fluorescence intensity (MFI) at 24, 72 and 144 h post infection. NO production was

significantly higher in animals incubated in anti-TGFβ *sensu stricto* compared to control animals at 72 and 144 h after inoculation. (B) Relative infection success in the same anemones at the same time points, expressed as percentage of pixels displaying fluorescence at 600-700 nm. Animals incubated in anti-TGFβ *sensu stricto* were significantly less infected than control animals at 72 and 144 h after inoculation (For A and B: points represent means + SD; n=3 anemones. *t*-tests: values of NO levels and infection success of anti-TGFβ-incubated animals different from isotype controls at a given timepoint by * $P<0.05$, ** $P\leq0.005$. (C) Confocal images of DAF-FM DA-loaded tentacles, from animals incubated in the three treatments 144 h post-infection. DAF-FM DA fluorescence is colored yellow and symbiont autofluorescence is colored red.

Figure 7. Phosphorylated-Smad 2/3 is more highly expressed in symbiotic than in aposymbiotic *A. pallida*. Anemone homogenates were immunoprecipitated with an anti-p-Smad, resolved using SDS-PAGE, transferred and probed with the same anti-pSmad antibody or a rabbit IgG isotype control. (A) Immunoblots of symbiotic and aposymbiotic samples showing bands at ~60kD (pSmad2/3) and 50kD (rabbit IgG heavy chain from the immunoprecipitation step) (each sample was a pool of 8 anemones). (B) Band intensities of anti-pSmad 2/3 immunoblots of symbiotic anemone homogenates after an overnight incubation in anti-human TGFβ *sensu stricto* or in a rabbit IgG isotype, both at a concentration of 10 µg/ml. Pre-incubation in anti-human TGFβ *sensu stricto* caused a decrease in pSmad 2/3 expression. Band intensities were quantified from immunoblots of immunoprecipitates and expressed as a percentage of intensities from control animals

incubated in FASW only (Bars represent means + SD; n=3 experiments, using 8 anemones each, *t*-test $P<0.05$).

Figure 8. Addition of exogenous human TGFβ *sensu stricto* decreases heat-stress-induced NO production and bleaching in *A. pallida*. (A) Symbiotic anemones were incubated in a range of human TGFβ *sensu stricto* concentrations for 2 h before being subjected to a heat stress at 33°C for 24 h. NO-dependent DAN fluorescence was quantified in anemone homogenates and expressed as µM of $NO_2^-$/µg of protein. DAN fluorescence decreased in a dose-dependent manner and was significantly lower when anemones were treated with 100 ng/ml TGFβ compared to control. (Bars represent means + SD; n=3 anemones; ANOVA P=0.042 Post Hoc Bonferroni, * different from control value by $P<0.05$). (B). Bleaching (% symbionts lost) was quantified in anemones incubated for 20-24 h at 33ºC after a 2 h incubation in 100 ng/ml human TGFβ *sensu stricto*. The exogenous ligand significantly decreased heat-stress-induced bleaching compared to controls. (Bars represent means + SD; n=3 anemones; *t*-test $P<0.05$).

Table 1. List of TGFβ pathway sequences identified to date in *Aiptasia pallida*.

| Group of genes/gene name (GenBank Accession of query sequence) | UniSeq in AiptasiaBase or GenBank Accession | Species of highest Blastx hit | e value of Blastx hit | Percent amino acid identity of Blastx hit | GenBank accession # of Blastx hit |
|---|---|---|---|---|---|
| TGFβ ligands | | | | | |
| TGFβ *sensu stricto* (P01137; P61811; P10600) | JX113692 | *Branchiostoma japonicum* | 1.00E-38 | 51% | AEE90023.1 |
| BMP7 (NP_001710.1) | EST CCAS2380.g1 | *Nematostella vectensis* | 6.00E-41 | 48% | XP_001639178 |
| TGFβ receptors | | | | | |
| TGF-R III (AAA67061) | EST CCAS468.g1 | *Nematostella vectensis* | 9.00E-21 | 39% | XP_001628545 |
| TGF-R II (AAA61164) | EST CCAS4061.g1 | *Ovis aries* | 7.00E-40 | 42% | AAV49298 |
| SMADs | | | | | |
| SMAD2 (NP_005892) | EST CCAS2691.g1 | *Nematostella vectensis* | 1.00E-149 | 88% | XP_001631657 |
| SMAD6 (AAB94137) | EST CCAS4346.g1 | *Nematostella vectensis* | 2.00E-55 | 41% | XP_001631691 |
| SMAD7 (NM_005904) | EST CCAS4909.g1 | *Nematostella vectensis* | 9.00E-43 | 40% | XP_001631691 |

Supplementary Table 1. Taxa used in phylogenetic analyses

| Phylum | *Species name* | Abbreviation | Gene | GenBank Accession Number |
|---|---|---|---|---|
| | | | Ml_TGFbA | AEP16383 |
| | | | Ml_TGFbB | AEP16386 |
| | | | Ml_Bmp3 | AEP16388 |
| Ctenophora | *Mnemiopsis leidyi* | Ml | Ml_Bmp58 | AEP16382 |
| Porifera | *Amphimedon queenslandica* | Aq | Aq_TGF | Aqu1.222201 |
| Cnidaria | *Acropora millepora* | Ami | Ami_dpp | AAM54049 |
| | *Aiptasia pallida* | Ap | Ap_TGFb | JX113692 |
| | *Hydra magnipapillata* | Hm | Hm_Bmp58 | XP_002165964 |
| | | | Nv_gdf5 | AAR27581 |
| | | | Nv_Bmp24 | XP_001631192 |
| | | | Nv_Bmp58 | ABC88372 |
| | | | Nv_mst | XP_001641598 |
| | *Nematostella vectensis* | Nv | Nv_act | ABF61781 |
| | *Podocoryne carnea* | Pca | Pca_Bmp58 | ABA42602 |
| Annelida | *Platynereis dumerilii* | Pdu | Pdu_dpp | CAJ38807 |
| Mollusca | *Aplysia californica* | Aca | Aca_nodl | ACM50754 |
| | *Biomphalaria glabrata* | Bgl | Bgl_nod | ACB42422 |
| | *Crassostrea gigas* | Cgi | Cgi_gdf3 | CAD67715 |
| Arthropoda | *Apis mellifera* | Ame | Ame_act | XP_001123044 |
| | *Bombyx mori* | Bmi | Bmi_dpp | NP_001138801 |
| | *Drosophila melanogaster* | Dme | Dme_act | AAL51005 |
| | | | Tca_actl | XP_970355 |
| | | | Tca_bmp10 | XP_973577 |
| | *Tribolium castaneum* | Tca | Tca_myo | XP_966819 |
| | | | Spu_lefty | NP_001123281 |
| | | | Spu_actB | NP_001121540 |
| | | | Spu_nod | NP_001091919 |
| | | | Spu_bmp3 | XP_786367.2 |
| | | | Spu_Bmp24 | ACA04460 |
| | | | Spu_uni | NP_999793 |
| | | | Spu_Bmp58 | NP_999820 |
| Echinodermata | *Strongylocentrotus purpuratus* | Spu | Spu_gdf11 | XP_789990 |
| | | | Sko_admp | NP_001158394 |
| | | | Sko_Bmp24 | NP_001158387 |
| Hemichordata | *Saccoglossus kowaleskii* | Sko | Sko_Bmp58 | NP_001158388 |
| | | | Bfl_Tgf | AEE90023 |
| | | | Bfl_nodr | AAL99367 |
| | | | Bfl_Bmp3 | XP_002596217 |
| | | | Bfl99205 | XP_002602867 |
| | | | Bfl_Bmp24 | XP_002596858 |
| Chordata | *Branchiostoma floridae* | Bfl | Bfl_Vg1 | XP_002589220 |

| | | | |
|---|---|---|---|
| | | Bfl_Mstn | XP_002599461 |
| | | Bfl244225 | XP_002606835 |
| *Ciona intestinalis* | Cin | Cin_Tgfb2 | NP_001071838 |
| *Ciona savignyi* | Cs | Cs_lefty | BAB68348 |
| | | Dre_gdnf | NP_571807 |
| | | Dre_lefty | NP_571035 |
| | | Dre_lefty2 | NP_571036 |
| | | Dre_Tgfb1 | NP_878293 |
| | | Dre_Tgfb2 | NP_919366 |
| | | Dre_Tgfb3 | NP_919367.2 |
| | | Dre_bmp3 | XP_003414176 |
| | | Dre_Bmp10 | NP_001124072 |
| | | Dre_con | XP_002662587 |
| | | Dre_gdf6 | XP_002934530 |
| | | Dre_gdf7 | XP_694563 |
| | | Dre_Dvr1 | NP_571023 |
| | | Dre_Bmp6 | NP_001013357 |
| | | Dre_inhBb | AAX68505 |
| | | Dre_actBb | NP_571143 |
| | | Dre_mstn | NP_571094 |
| *Danio rerio* | Dre | Dre_mstn2 | AAI63304 |
| *Halocynthia roretzi* | Hro | Hro_nod | BAC11909 |
| | | Hs_TGFb1 | NP_000651 |
| | | Hs_TGFb2 | NP_003229 |
| *Homo sapiens* | Hs | Hs_TGFb3 | NP_003230 |
| *Lethenteron japonicum* | Lj | Lj_Bmp24 | BAB68395 |
| | | Mm_gdnf | NP_034405 |
| | | Mm_neurt | NP_032764 |
| | | Mm_bmp3 | NP_775580 |
| | | Mm_Bmp6 | BAC30864 |
| | | Mm_bmp10 | NP_033886.2 |
| | | Mm_InhBe | NP_032408 |
| | | Mm_actBc | NP_034695 |
| | | Mm_mst | AAI05675 |
| | | Mm_gdf2 | NP_06237 |
| *Mus musculus* | Mm | Mm_gdf10 | BAC31332 |
| *Xenopus laevis* | Xl | Xl_Gdnf | NP_001090196 |
| | | Xl_lefty | AAG35771 |
| | | Xl_Tgfb2 | NP_001079195 |
| | | Xl_Xnr2 | NP_001081436 |
| | | Xl_Xnr6 | NP_001079033 |
| | | Xl_Admp1 | NP_001081792 |
| | | Xl_admp2 | NP_001090587 |
| | | Xl_Bmp2 | NP_001095136 |
| | | Xl_Bmp3 | NP_001082633 |
| | | Xl_Gdf5 | NP_001086466 |
| | | Xl_Gdf6 | AAI69872 |
| | | Xl_Vg1 | AAW30007 |
| | | Xl_der | NP_001080966 |

| | |
|---|---|
| Xl_Bmp7 | NP_001080866 |
| Xl_actD | NP_001079333 |