Nitric Oxide Affects ERK Signaling through Down-Regulation of MAP Kinase Phosphatase Levels during Larval Development of the Ascidian *Ciona intestinalis*

Immacolata Castellano*, Elena Ercolesi*, Anna Palumbo*

Laboratory of Cellular and Developmental Biology, Stazione Zoologica Anton Dohrn, Villa Comunale, Naples, Italy

**Abstract**

In the ascidian *Ciona intestinalis* larval development and metamorphosis require a complex interplay of events, including nitric oxide (NO) production, MAP kinases (ERK, JNK) and caspase-3 activation. We have previously shown that NO levels affect the rate of metamorphosis, regulate caspase activity and promote an oxidative stress pathway, resulting in protein nitration. Here, we report that NO down-regulates MAP kinase phosphatases (mkps) expression affecting positively ERK signaling. By pharmacological approach, we observed that the reduction of endogenous NO levels caused a decrease of ERK phosphorylation, whereas increasing levels of NO induced ERK activation. We have also identified the ERK gene network affected by NO, including *mpk1*, *mpk3* and some key developmental genes by quantitative gene expression analysis. We demonstrate that NO induces an ERK-independent down-regulation of *mpk1* and *mpk3*, responsible for maintaining the ERK phosphorylation levels necessary for transcription of key metamorphic genes, such as the hormone receptor rev-erb and the van willebrand protein vwa1c. These results add new insights into the role played by NO during larval development and metamorphosis in *Ciona*, highlighting the cross-talk between different signaling pathways.

**Introduction**

Metamorphosis, the spectacular post-embryonic transformation of a larva into a juvenile, is a widespread process occurring in many metazoans from amphibians, fishes to urochordates, insects and other invertebrates, allowing the transition of the animal from vegetative to reproductive life stage. In marine organisms, this transition requires that the swimming larvae acquire the competence to receive specific environmental cues, inducing the settlement and the initiation of metamorphosis. The binding of environmental cues to cell surface receptors on the larval sensory organs transmits signals via the nervous system to activate molecular pathways that drive the subsequent morphogenetic events, implying an extensive transformation of the body plan with disappearance of larval structures and appearance and or remodeling of some organs in the adult [1]. An important signaling molecule involved in this process is nitric oxide (NO), which affects key decisions of life and death, by shutting on or off apoptotic pathways and regulating the timing of life cycle transitions [2].

The ascidian *Ciona intestinalis* provides an excellent experimental system to investigate molecular signaling pathways involved in metamorphosis [3–5]. In particular, the simplicity of the ascidian tadpole, the rapid rate of development, the availability of genome sequence [6] and the extensive *in situ* gene expression profiles during embryogenesis [7] make the ascidian a suitable organism to characterize the gene regulatory network that controls the onset of metamorphosis. Many studies have described in detail the morphological changes occurring during *Ciona* metamorphosis. After hatching, approximately 18 hours post fertilization (hpf), larvae swim for few hours, during which they acquire competence to respond to environmental cues. Then, larvae stick to a suitable substrate by adhesive papillae and metamorphosis begins (approximately 28 hpf) through a profound reconstruction of the body plan and a remarkable regression of the tail [3,4]. Adhesive papillae represent specialized organs for metamorphosis as this process is hampered in papillae-cut larvae and in mutants in which the functionality of papillae is compromised [8]. At the molecular level, several processes are involved in *Ciona* metamorphosis: 1- the production of NO [9]; 2- the activation of two members of MAP kinase proteins, the extracellular-signal-regulated kinase, ERK, and the e-Jun NH(2)-terminal kinase, JNK [10,11]; 3- the activation of the apoptosis-related peptidase caspase-3 [10]. All these events interplay together leading to metamorphosis through a massive apoptosis, starting at the extremity of the tail and propagating all along the body to all tissues [5,9,10,12].

In *C. intestinalis*, NO is produced by a NO synthase (NOS), showing the structural features of a neuronal NOS [13]. The
ERK activity, and some developmental genes whose expression is controlled by these kinases has been identified [11]. Moreover, the gene network associated with oxidative stress during Ciona development [14]. ERK and JNK play both pro-survival and pro-apoptotic roles depending on the cell type and cellular environment. In Ciona, activation of ERK and JNK is necessary for apoptosis and metamorphosis as both processes are completely blocked when these kinases are inhibited [10,11]. Moreover, the gene network controlled by these kinases has been identified [11]. In particular, ERK is activated just a few hours after hatching (first peak at 20–22 hpf) in the papillae of swimming larvae and later at metamorphosis in tail cells (second peak at 28 hpf) before the wave of apoptosis occurs, suggesting that the phosphorylated form of ERK transduces the apoptotic signal to tail tissues during metamorphosis [10,11].

Moreover, ERK and P-ERK are found to be nitrated during larval development, suggesting that NO and reactive nitrogen species levels can affect metamorphosis. Indeed, a decrease of NO or reactive nitrogen species levels by NOS inhibition or by NO scavengers markedly reduces the rate of metamorphosis, whereas NO donors or peroxynitrite cause an opposite effect [14].

The caspase-3 protein is a member of the cysteine-aspartic acid protease (caspase) family, whose sequential activation plays a key role in the execution-phase of cell apoptosis. In Ciona, the apoptotic wave, responsible for tail regression is dependent on caspase-3 activation and this event is controlled by NO levels [9,10].

In proceeding with our studies of NO signaling during C. intestinalis metamorphosis, we have focused our attention on ERK, recently recognized as a NO target [14] and whose localization during metamorphosis, first in papillae and later in the tail, overlaps the NO signal from the anterior part of the larva to the tail [9], suggesting a cross talk between the two signals. By pharmacological approaches, we now report that modulation of endogenous NO levels in the ascidian larva affects ERK phosphorylation. We have also identified the gene network associated with oxidative stress affected by NO, including ERK dual specific MAP kinase phosphatases (mkps), which provide a negative regulation on ERK activity, and some developmental genes whose expression is influenced by ERK phosphorylation.

Materials and Methods

Animal handling and incubation experiments

Adult C. intestinalis were collected at Fusaro Lake in the district of Naples (40°49’ 10.6” north latitude, 14°03’ 32” east longitude). No specific permissions were required for this location and use of animals for scientific purposes. Animals were transported to the service Marine Resources for Research and use of animals for scientific purposes. Animals were handled in strict accordance with European (Directive 2010/63) and Italian (Decreto Legislativo n. 116/1992) legislation for the care and use of animals for scientific purposes. Animals were transported to the service Marine Resources for Research and maintained at 18°C in tanks with circulating sea water and under constant light to allow gametes accumulation. Animal handling and fertilization were carried out as previously described [9,14]. In brief, eggs from a single animal were fertilized with a mixture of sperms obtained from different individuals. Embryos were cultured at 18°C in 0.2 μm filtered sea water. Just hatched larvae were obtained at about 18–20 hpf at 18°C. Development was followed on live specimen with an Olympus stereomicroscope.

Samples at appropriate stages were identified using the morphological criteria previously reported by Chiba [15] and were selected on the basis of at least 95% homogeneity. Hatched larvae (about 100 larvae/ml) were treated in tissue culture dishes in 50 ml of sea water at 18°C with the following drugs, at the final concentrations indicated in the text. These include the NOS inhibitor (1-2-trithiosemicarbazone) imidazole (TRIM) (Sigma), the slow releasing NO donor (Z-1-[N-(3-Aminopropyl)-N-(4-(3-amino(propyl)ammonio) butyl)-amino]-diazen-1-ium-1,2-diolate) (spermine NONOate, sperNO) (Alexis), spermine (Sigma), the NO scavenger (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide) (c-PTIO) (Alexis), which reacts stochiometrically with NO [16], the MEK inhibitor U0126 (Calbiochem) and the dual specificity protein phosphatase 1/6 Inhibitor (dusp 1/6 I) (Calbiochem). Stock solutions of 1 M TRIM (in DMSO), 0.1 M sperNO (in 0.01 M NaOH), 0.1 M spermine (in sea water), 0.1 M c-PTIO (in sea water), 0.025 M U0126 (in DMSO) and 0.032 M dusp 1/6 I (in DMSO) were prepared and diluted to the final experimental concentration. The experiments were performed at least in triplicate (see figure legends for details). In the case of c-PTIO, the number of live late larvae/larvae during tail regression and juveniles were counted after 24 h of treatment and expressed as 100% of total individuals, as previously reported [9,14]. Treated larvae were allowed to develop to the desired stage and then directly observed or collected by low speed centrifugation after washing in PBS buffer. The pellets were stored at −80°C until use and subsequently used for protein extraction, NO detection and RNA preparation.

Protein extraction

Larval pellets (50–100 mg) were homogenized in two volumes of RIPA lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.6, 5 mM EDTA, 0.5% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors (1 mM PMSF and Complete Protease Inhibitor Cocktail Tablets, Roche) and phosphatase inhibitors (PhosSTOP Cocktail Tablets, Roche). After centrifugation, protein concentration was determined using a Bio-Rad Protein Assay Reagent (Bio-Rad) with bovine serum albumin as a standard.

Fluorimetric determination of NO concentration

Larval pellets (50–60 mg) were homogenized in PBS and sonicated. The samples were centrifuged at 13,500 g for 5 min at 4°C and the supernatants were collected for NO analysis using 2,3-diaminonaphthalene (DAN) to form the fluorescent product 1-(H)-naphthotriazole. Briefly, samples (80 μl) were incubated at 26°C with nitrate reductase 0.06 U/ml, FAD 2.5 μM and NADPH 100 μM final for 1 hour. Then, 10 μl of DAN (0.05 mg/ml in 0.62 M HCl) was added and the samples were incubated for 20 min in the dark. After stabilization with 1N sodium hydroxide, formation of 1-(H)-naphthotriazole was measured using a spectrophotofluorimeter (Shimadzu RF-5301 PC) with excitation and emission at 365 and 425 nm, respectively. Calibration curves were performed daily with sodium nitrite (1–10 μM) in PBS. The experiments were carried out on three biological replicates.

Western blots analysis

Larva lysates were prepared as previously described [14] and examined by 10% SDS-PAGE. The gel transferred to nitrocellu-
locase membrane was analyzed with antibodies against p44/42 MAP Kinase (ERK 1/2) (ERK) (1:1000) (Cell Signaling), Phospho-p44/42 MAPK (ERK1/2) (P-ERK) (1:500) (Cell Signaling). After washing in PBS with 0.1% tween, labeled proteins were detected by ECL PLUS (ERK) and Supersignal West Pico Chemiluminescent Substrate (Pierce) (P-ERK). Densitometric analyses were carried out on scanned western blot images, using ImageJ programme.

Bioinformatic analysis

*Ciona* transcripts were retrieved from Aniseed (http://www.aniseed.cnrs.fr/). The identification of mkp1 and mkp3 was performed analyzing all the transcripts coding for dual specific phosphatases existing in *Ciona* transcrпитe by blast programme to find homology from other sources. Multiple sequence alignments of *C. intestinalis* mkp1 and mkp3 proteins with homologous counterparts were carried out by Clustal W.

RNA extraction and cDNA synthesis

Total RNA was extracted at different developmental stages using TRIzol (Invitrogen) according to the manufacturer’s instructions. Briefly, extraction with chloroform/isoamyl alcohol (24:1) was performed following RNA precipitation by addition of glycogen and isopropyl alcohol. Contaminating DNA was degraded by treating each sample with DNase (Roche) and finally removing the enzyme with RNeasy MinElute Cleanup Kit (Qiagen). The amount of total RNA was estimated by the absorbance at 260 nm and the purity by 260/280 and 260/230 nm ratios, by Nanodrop (ND-1000 UV-Vis Spectrophotometer; NanoDrop Technologies). The integrity of RNA was checked in agarose gel electrophoresis by visualizing intact rRNA subunits (28S and 18S). For each sample, 1 μg of total RNA was retrotranscribed with iScript cDNA Synthesis kit (Bio-Rad), following the manufacturer’s instructions. cDNA was diluted 1:10 with H2O prior to use in Real Time qPCR experiments.

Real Time qPCR

Data from each cDNA sample were normalized using ribosomal protein R27a as reference gene, whose levels remained relatively constant in all the developmental stages examined. The gene sequences were retrieved from Aniseed (http://www.aniseed.cnrs.fr/). For each gene, specific primers were designed on the basis of nucleotide sequence with the help of Primer 3 (see Table 1), except for *dhg* and *mkp1* for which we used the primers reported in literature [11]. The amplified fragments using Taq High Fidelity PCR System (Roche) were purified from agarose gel using QIAquick Gel extraction kit (Qiagen) and specificity of PCR products was checked by DNA sequencing (Molecular Biology Service, SZN, Naples). Specificity of amplification reactions was verified by melting curve analysis. The efficiency of each primer pair was calculated according to standard methods curves using the equation \( E = 10^{-1}\text{slope} \). Five serial dilutions were set up to determine Ct values and reaction efficiencies for all primer pairs.

Standard curves were generated for each oligonucleotide pair using the Ct values versus the logarithm of each dilution factor. PCR efficiencies were calculated for control and target genes and were found to be 2 in most of them, only for *vava15a* was 1.985 and for *mkp3* 1.926. Diluted cDNA was used as a template in a reaction containing a final concentration of 0.3 μM for each primer and 1 X FastStart SYBR Green master mix (total volume of 10 μl). PCR amplifications were performed in a ViiaATM 7 Real Time PCR System (Applied Biosystems) thermal cycler using the following thermal profile: 95°C for 10 min, one cycle for cDNA denaturation; 95°C for 15 sec and 60°C for 1 min, 40 cycles for amplification; 72°C for 5 min, one cycle for final elongation; one cycle for melting curve analysis (from 60°C to 95°C) to verify the presence of a single product. Each assay included a no-template control for each primer pair. To capture intra-assay variability all Real Time qPCR reactions were carried out in triplicate. Fluorescence was measured using ViiaATM 7 Software (Applied Biosystems). The expression of each gene was analyzed and internally normalized against R27a using REST software (Relative Expression Software Tool) based on Pfaff method [17,18]. Relative expression ratios equal or greater than ±2 were considered significant. Experiments were repeated at least three times.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA). The results of morphological and biochemical experiments were reported as means ± SEM and analyzed by unpaired t-test for comparison between the groups. \( P \leq 0.05 \) was considered statistically significant. For Real Time qPCR analysis, results were reported as means ± SD and significance was tested using the “Pair Wise Fixed Reallocation Randomisation Test”, developed by REST software [17,18]. The number of experiments was reported in the figure legends.

Results

**NO affects ERK phosphorylation**

To characterize the involvement of NO during metamorphosis, hatched larvae were treated with different compounds to modulate endogenous NO levels. In details, to reduce endogenous NO levels, two approaches were used. Larvae were treated with TRIM, the specific NOS inhibitor which interferes with the binding of both L-arginine and the cofactor BH4, and with the scavenger c-PTIO, which directly reacts with NO [16]. To increase NO levels, larvae were treated with the slow-releasing NO donor sperNO. To confirm the effectiveness of these treatments, the endogenous NO levels were measured by monitoring nitrite formation. After 6 h of TRIM (250 μM) treatment, corresponding to 24 hpf, the basal concentration of NO significantly decreased from 3.14±0.30 to 1.14±0.13 nmol nitrite/mg protein (\( P = 0.0005 \)). The NO scavenger c-PTIO (300 μM) exhibited the same effect as TRIM, resulting in a decrease of NO levels from 2.36±0.29 to 1.2±0.19 nmol nitrite/mg protein (\( P = 0.0043 \)), at 22 hpf, after 4 h of treatment. In the presence of sperNO (250 μM) after 6 h, corresponding to 24 hpf, the level of NO increased from 4.26±0.44 to 6.15±0.31 nmol nitrite/mg protein (\( P = 0.0037 \)).

The modulation of endogenous NO levels affected the rate of metamorphosis. Indeed, hatched larvae treated with c-PTIO showed a slow-down of metamorphosis, as evidenced by the increase of the percentage of late larvae or larvae during tail regression to 69% with respect to 32% in controls and a concomitant decrease of juveniles to 35% with respect to 65% in controls, observed after 24 h treatment (Figure 1A). Higher levels of c-PTIO proved to be toxic. Also TRIM and sperNO have been recently shown to affect the rate of metamorphosis. In particular, the NOS inhibitor caused a slow-down and the NO donor an acceleration of the process [14].

Larvae treated with the different compounds c-PTIO, TRIM and sperNO were withdrawn at different times after treatment and examined for ERK activation. The NO scavenger c-PTIO induced the decrease of ERK phosphorylation at 22 and 26 hpf, corresponding to 4 and 8 h of treatment, whereas a substantial
amount of P-ERK was produced at 28 hpf (Fig. 1B). Also TRIM reduced ERK phosphorylation (Figure 2A). The densitometric analysis of the P-ERK immunopositive bands respect to the ERK bands revealed a significant reduction of ERK phosphorylation at 24, 26 and 28 hpf, whereas at shorter times of 20 and 22 hpf, corresponding to 2 h and 4 h of treatment, the differences between control and treated larvae were not statistically significant (Figure 2B). Treatment of hatched larvae with the slow NO donor sperNO resulted in a significant increase of P-ERK at 26 hpf, corresponding to 8 h of treatment (Figure 3A), as confirmed by densitometric analysis of the P-ERK immunopositive bands respect to constitutive ERK (Figure 3B).

**NO regulated gene expression**

To investigate if NO affected also gene transcription related to ERK activation, we selected a series of ERK target genes mostly expressed in territories specialized for metamorphosis, such as palps and/or papillae, where NOS expression, NO production and ERK activation occur [9,11]. These genes include: dehydrogenase (*dhg*), ets-related transcription factor (*ets*), matrix metalloprotease-24-precursor (*mx*), nuclear hormone receptor (*rev-erb*), and the von Willebrand factor (*vwa1c*). We also selected serum response factor (*srf*) and NOS, whose expression in other systems has been shown to be regulated by ERK activation [19,20] (table 1). Finally, we considered dual specific MAP kinase phosphatases (*mkp*) which regulate the cellular levels of activated ERK through dephosphorylation of specific threonine and tyrosine residues [21]. By *in silico* analysis of the *Ciona* transcriptome (http://www.aniseed.cnrs.fr/), we identified two transcripts (ci0100138796, ci0100140262) belonging to type II of the dual specific phosphatase family, containing both the dual specific phosphatase domain and the N-terminal MAPK binding domain. Multiple sequence alignments of these proteins with homologous counterparts highlighted that they exhibit a significant homology with mkp1 (Figure 4) and mkp3 enzymes (Figure S1), respectively.

The relative expression of all the above mentioned genes was followed by Real Time qPCR experiments, after treating hatching larvae (18 hpf) with the NOS inhibitor TRIM to reduce endogenous NO levels (Figure 5A). Larvae were collected at 22,
24 and 26 hpf corresponding to 4, 6 and 8 h of treatment. The expression of dhg and vwa1c increased after 6–8 h and 8 h of treatment, respectively, whereas rev-erb was down-regulated at 6 h. The expression of mkp1 and mkp3 increased with respect to the control sample. In detail, the increase of expression of mkp1 already started at 24 hpf after 6 h of treatment, while the beginning of the increase of mkp3 expression occurred at 26 hpf after 8 h of treatment. The expression of the other genes, ebs, mx and srf was unaffected. To confirm that NO is responsible for the

24 and 26 hpf corresponding to 4, 6 and 8 h of treatment. The expression of dhg and vwa1c increased after 6–8 h and 8 h of treatment, respectively, whereas rev-erb was down-regulated at 6 h. The expression of mkp1 and mkp3 increased with respect to the control sample. In detail, the increase of expression of mkp1 already started at 24 hpf after 6 h of treatment, while the beginning of the increase of mkp3 expression occurred at 26 hpf after 8 h of treatment. The expression of the other genes, ebs, mx and srf was unaffected. To confirm that NO is responsible for the

Figure 1. Decrease of endogenous NO levels with the NO scavenger c-PTIO results in a slow-down of metamorphosis and a reduction of ERK phosphorylation. (A) Hatched larvae were treated with c-PTIO (300 μM) and after 24 h the number of late larvae, larvae during tail regression and juveniles were counted and reported as percent of the total. Results are representative of 10 independent experiments. Data, expressed as means ± SEM, are assessed by unpaired t-test. Asterisk represents the significance respect to the control ***P<0.001 (white bar < 0.0001, grey bar = 0.0002). (B) Hatched larvae treated with c-PTIO were examined at different times for ERK activation. Representative experiment showing the western blot analyzed with anti-P-ERK and anti-ERK antibodies. doi:10.1371/journal.pone.0102907.g001

Figure 2. Decrease of ERK phosphorylation by NOS inhibitor TRIM. Hatched larvae were treated with TRIM (250 μM) and samples at different times were examined for ERK activation. (A) Representative experiment showing the western blot analyzed with anti-P-ERK and anti-ERK antibodies. (B) Histogram showing densitometric analysis of immunopositive P-ERK respect to ERK bands. White bars, without TRIM; grey bars, with TRIM. Results are representative of 3 independent experiments. Data, expressed as means ± SEM, are assessed by unpaired t-test. Asterisk represents the significance respect to the control **P<0.01 (0.005).

doi:10.1371/journal.pone.0102907.g002

Figure 3. Increase of ERK phosphorylation by NO donor sperNO. Hatched larvae were treated with sperNO (250 μM) and samples at different times were examined for ERK activation. (A) Representative experiment showing the western blot analyzed with anti-P-ERK and anti-ERK antibodies. (B) Histogram showing densitometric analysis of immunopositive P-ERK respect to ERK bands. White bars, without sperNO; grey bars, with sperNO. Results are representative of 3 independent experiments. Data, expressed as means ± SEM, are assessed by unpaired t-test. Asterisk represents the significance respect to the control **P<0.01 (0.005).

doi:10.1371/journal.pone.0102907.g003
changes in gene expression of dhg, mkp1, mkp3, rev-erb and vwa1c, hatched larvae were treated with the slow NO donor sperNO. Under these conditions, also the expression of NOS was investigated to verify a possible feedback by NO, as recently reported in another ascidian [22]. Samples, collected at 22, 24, 26 hpf after 4, 6 and 8 h of treatment, were analyzed for quantitative gene expression (Figure 5B).

Mkp1, mkp3, dhg, rev-erb and vwa1c were down-regulated in the presence of sperNO, with respect to the control. In detail, the down-regulation of mkp1 and rev-erb reached 4 fold and 3 fold, respectively, at 24 hpf after 6 h of treatment. Dhg, mkp3 and vwa1c were down-regulated between 2 fold and more than 3 fold, at 26 hpf after 8 h of treatment. The expression of NOS was not affected by increasing NO levels.

The expression of the NO-regulated genes was followed during larval development at different times from hatching (18 hpf) to the onset of metamorphosis (28–30 hpf) (Figure 6). The expression of ERK target genes dhg and vwa1c was maximum at 22–24 hpf, whereas rev-erb was up-regulated between 20 and 24 hpf. The expression of NOS was approximately constant until 26 hpf, while it was down-regulated at 28 and 30 hpf. The gene expression pattern of mkp1 exhibited two peaks, the first one at 22–24 hpf and the second one at 28–30 hpf. On the contrary, the expression profile of mkp3 remained high from 20 to 28 hpf.

NO regulates ERK pathway through mkps expression

To confirm that mkp1 and mkp3 specifically dephosphorylate ERK during C. intestinalis larval development, we treated larvae at hatching with the dual specificity protein phosphatase 1/6 inhibitor (dusp 1/6 I), a cell-permeable compound that inhibits phosphatase activity of mkp1 and mkp3 in HeLa cells. As you can see from Figure 7A,B, ERK phosphorylation increased in the presence of the mkps inhibitor. Moreover, larvae at hatching were first treated with the NOS inhibitor TRIM, which up-regulates the expression of mkps and causes the reduction of P-ERK, and then with dusp 1/6 I, to inhibit the activity of the new produced mkps. Under these conditions, the levels of P-ERK are significantly increased with respect to the control and TRIM alone (Figure 7A,B). This suggested that the NO-mediated up-regulation of mkps led to the production of active phosphatases, thus affecting ERK phosphorylation. Finally, to understand if P-ERK, on its turn, can modulate mkps transcription, their expression was...
followed after ERK inhibition by the MEK inhibitor U0126. Larvae at hatching were treated with the inhibitor resulting in a complete block of metamorphosis after 24 h treatment, in agreement with Chambon et al., 2007 [11]. The expression of *mkp1* and *mkp3* was not significantly affected by the treatment (Figure 7C). The same result was obtained for *NOS*, *srf* and *rev-erb*. On the other hand, the expression of the previously identified ERK target genes was regulated as expected [11]. Indeed, *dhg* and *vwa1c* were up-regulated and *ets* and *mx* were down-regulated, as reported by Chambon et al., 2007 [11].

**Figure 5. Gene regulation in response to NO levels during larval development.** Histogram shows the differences in expression levels of analyzed genes, followed by Real Time qPCR. Hatched larvae (18 hpf) incubated with 250 μM TRIM (A) or 250 μM sperNO (B) were collected at 22 hpf (blue), 24 hpf (yellow) and 26 hpf (green). Data are reported as a fold difference compared to the control, larvae in sea water without TRIM or sperNO (means ± SD). Values equal or greater than ±2 were considered significant. The experiments were repeated at least 3 times.
doi:10.1371/journal.pone.0102907.g005

**Figure 6. Gene expression profiles during larval development.** Histogram shows the values of gene expression at different times of larval development respect to those obtained for hatching larvae (18 hpf), followed by Real Time qPCR. Data are reported as a fold difference compared to the control (means ± SD). Values equal or greater than ±2 were considered significant. The experiments were repeated at least 3 times.
doi:10.1371/journal.pone.0102907.g006
scavenger is depleted during the experimental time and cannot react anymore with NO newly produced by NOS [16].

During Ciona larval development, the first peak of ERK activation in the papillae (20–22 hpf) is responsible to activate a series of downstream genes, likely involved in the acquisition of competence and the attachment of larvae to the substrate. Indeed, we have demonstrated that changes in NO levels also affect the expression of ERK-target genes, including the metabolic enzyme dehydrogenase, dhg, and the developmental genes, vwa1c and rev-erb, whereas other genes such as ets, mx, srf and NOS are unaffected. Vwa domains are present in extracellular matrix molecules, adhesion proteins and cell surface receptors [29] and are also found in complement factors [29]. In the ascidian Boltenia villosa, vwa1 is expressed during metamorphosis at competence acquisition [30]. In Ciona, vwa1c is down-regulated by ERK activation and is expressed in papillae [11]. Our finding that the maximum expression of vwa1c is at 20–24 hpf, when the formation of palps and papillae occurs and the larva acquires the ability to sense external stimuli, suggests the involvement of this factor in the structural reorganization of papillae and possibly in the maturation of adult immune system and in re-structuring of larval tissues during metamorphosis [30,31]. Regarding rev-erb, this is a nuclear receptor involved in circadian rhythm in vertebrates. In Ciona, circadian rhythms have been characterized at physiological and molecular levels, although no data are available on the processes regulated by circadian clock [32]. In Ciona, rev-erb is localized in the palps [33]. Our finding that the maximum expression of this receptor occurs at 20–24 hpf is relevant considering that at this time larvae become light sensitive [34,35]. The regulation of rev-erb by NO has also been reported in other systems [36]. However, the down-regulation of rev-erb both in the presence of NOS inhibitor and NO donor suggests that, besides ERK activation, NO can mediate other ERK-independent pathways leading to rev-erb expression regulation. The fact that the previously characterized ERK target genes, ets and mx, are not affected by our pharmacological approach could be ascribed to the lower abundances of their transcripts, which fail to give detectable differences.

Regarding the molecular mechanisms that modulate ERK activation, a key role is played by the interaction between kinases and phosphatasas [37]. Different types of mkps have been reported, whose substrate specificity towards MAP kinases is dependent on the system and cell type [21,38,39]. By in silico analysis of Ciona genome, two transcripts, encoding for proteins containing all the characteristic domains responsible for ERK interaction and dephosphorylation, have been characterized. These two transcripts show significant similarity with mkp1 and mkp3 from different sources. Our results on mkps inhibition confirm that mkp1 and mkp3 are indeed specific phosphatases of ERK during Ciona metamorphosis. Moreover, the correlation between the temporal expression of mkp1 and the profile of ERK activation suggests that mkp1 is the phosphatase responsible for ERK-dependent gene transcription. Indeed, mkp1 family members are usually localized in the nucleus [40], where ERK translocates for downstream gene transcription. On the contrary, mkp3 is usually localized in the cytoplasm [40], where it could dephosphorylate ERK, thus affecting its cytoplasmic substrates.

An interesting outcome of this paper is that NO regulates mkps expression affecting finally ERK pathway. Indeed, the reduction of endogenous NO levels contributes to the up-regulation of mkp1 and mkp3 expression and to the production of active mkps with consequent ERK dephosphorylation. On the other hand, the increase of NO levels causes the down-regulation of these transcripts with consequent ERK activation. The decrease of
mkps expression by NO has also been observed in endothelial cells and it has been ascribed to destabilization of mkp-3 mRNA [41]. Our finding that ERK inhibition by MEK inhibitor does not affect mkps mRNA levels suggests that regulation of mkp expression by NO occurs through an ERK-independent pathway. Indeed, in different cell types, mkps induction can be either dependent or independent on ERK activation [42–44].

The results reported in this paper contribute to expand existing data on the role of NO on metamorphosis in a variety of taxa, including chordates, echinoderms, mollusks and annelids. In the data on the role of NO on metamorphosis in a variety of taxa, NO occurs through an ERK-independent pathway. Indeed, in different cell types, mkps induction can be either dependent or independent on ERK activation [42–44]. However, the picture emerging from literature is complex and in some cases the effect on metamorphosis seems to be dependent on the compound used, likely due to the different modulation of endogenous NO levels [9,14,45]. Therefore, a fine regulation of NO levels could differently affect the same biological process. In this study, we have effectively measured the NO levels following treatments with a NOS inhibitor, a NO scavenger and a NO donor and we have demonstrated that NO positively influences the rate of larval development by affecting ERK signaling. The constant expression of NOS during larval development until 26 hpf suggests that the modulation of endogenous NO levels could rely at these stages on the regulation of the enzyme activity by intracellular calcium concentration [46]. On the contrary, the marked decrease of NOS expression at 28–30 hpf, when larvae are attached to the substrate and metamorphosis starts, suggests that at stage NO production could be regulated at the transcriptional level. The reduction of NOS expression when metamorphosis progresses, has also been reported in other organisms [22,47–48]. Overall our findings suggest that during larval development NO induces ERK signaling, necessary for acquiring of competence, larval settlement and the initiation of the apoptotic wave. Immediately after, NO is likely no more necessary and therefore NOS expression decreases.

Conclusions

This study provides new insights into the role played by NO in regulating key molecular mechanisms responsible for life cycle transitions. We demonstrate that during Ciona larval development, changes in endogenous NO levels lead to an ERK-independent mkps regulation. These phosphatases contribute to maintain ERK phosphorylation levels and consequent transcription of downstream genes involved in key metamorphic events, such as competence acquisition and re-construction of larval tissues.

Supporting Information

Figure S1 Multiple sequence alignment of C. intestinals mkp3 with homologous counterparts. Ciona sequence c40100140262 was aligned using ClustalW with the following sequences retrieved from Uniprot database: zebrafish (Danio rerio) Q7T2L8, Drosophila melanogaster Q9V2V5, chicken (Gallus gallus) Q7T2L9, Mouse Q9DBB1. The MKB or N-terminal domain is underlined in blue (22–149 aa in D. melanogaster), the DSP or C-terminal domain is underlined in red (215–357 aa in D. melanogaster). The catalytic motif I/VHXXGXXR in DSP domain is highlighted in yellow, and the motif -PPFRRPXXG- in the MKB domain is highlighted in green. Ψ is a hydrophobic residue and X is any residue. In red are the highly conserved positions including the catalytic cysteine (C 302 in D. melanogaster). Finally the poly-lys 201–209 present in D. melanogaster but not conserved in ciona and homologous counterparts is highlighted in magenta. (TIF)

Acknowledgments

We thank Marco Borra for advice with real time qPCR experiments, the Molecular Biology Service for gene sequencing and Alberto Macina and the service Marine Resources for Research for assistance with living organisms.

Author Contributions

Conceived and designed the experiments: IC EE AP. Performed the experiments: IC EE AP. Performed the experiments: IC EE AP. Contributed reagents/materials/analysis tools: AP. Contributed to the writing of the manuscript: IC AP.

References

1. Hadfield MG (2000) Why and how marine invertebrate larvae metamorphose so fast. Sem Cell Dev Biol 11: 437–441.
2. Bisroop CD, Brandlof BP (2003) On nitric oxide signaling, metamorphosis, and the evolution of biphasic life cycles. Evol Dev 5: 542–550.
3. Cloney RA (1982) Ascidian larvae and the events of metamorphosis. Am Zool 22: 817–826.
4. Satoh N (1994) Metamorphosis and development of adult organs. In: Bard JBL editor. Developmental Biology of Ascidians. New York: Cambridge University Press. pp. 132–138.
5. Baghihiuan S, Martinand-Mari C, Mangeat P (2007) Using Ciona to study developmental programmed cell death. Semin Cancer Biol 17: 147–153.
6. Dehal P, Satou Y, Campbell R K, Chapman J, Degnan B, et al. (2002) The draft genome of Ciona intestinalis: insights into chordate and vertebrate origins. Science 298: 2157–2167.
7. Satoh Y, Takatori N, Fujisawa S, Niibata T, Saida H, et al. (2002) Ciona intestinalis cDNA projects: expressed sequence tag analyses and gene expression profiles during embryogenesis. Gene 287: 85–96.
8. Nakayama-Ishimura A, Chambon JP, Horie T, Satoh N, Sasakura Y (2009) Delineating metamorphic pathways in the ascidian Ciona intestinalis. Dev Biol 336: 357–367.
9. Comes S, Locascio A, Silvestre F, d’Ischia M, Russo GL, et al. (2007) Regulatory roles of nitric oxide during larval development and metamorphosis in Ciona intestinalis. Dev Biol 306: 772–784.

PLOS ONE | www.plosone.org 9 July 2014 | Volume 9 | Issue 7 | e102907
34. Nakagawa M, Miyamoto T, Ohkuma M, Tsuda M (1999) Action spectrum for the photophobic response of Ciona intestinalis (Ascidacea, Urochordata) larvae implicates retinal protein. Photochem Photobiol 70: 359–362.

35. Tsuda M, Kawakami I, Shiraishi S (2003) Sensitization and habituation of the swimming behavior in ascidian larvae to light. Zoolog Sci 20: 13–22.

36. Pardee KJ, Xu X, Reinking J, Schuetz A, Dong A, et al. (2009) The structural basis of gas-responsive transcription by the human nuclear hormone receptor REV-ERB beta. PLoS Biol 7: e48.

37. Murphy LO, Blenc J (2000) MAPK signal specificity: the right place at the right time. Trends Biochem Sci 31: 268–275.

38. Horsch K, de Wet H, Schuermanns MM, Allie-Reid F, Cato AC, et al. (2007) Mitogen-activated protein kinase phosphatase 1/dual specificity phosphatase 1 mediates glauococrysed inhibition of osteoblast proliferation. Mol Endocrinol 21: 2929–2940.

39. Comalada M, Lloberas J, Celada A (2012) MKP-1: a critical phosphatase in the biology of macrophages controlling the switch between proliferation and activation. Eur J Immunol 42: 1938–1948.

40. Camps M, Nichols A, Arkinall S (2000) Dual specificity phosphatases: a gene family for control of MAP kinase function. FASEB J 14: 6–16.

41. Rossig L, Haendeler J, Hermann B, Malchow P, Urbich C, et al. (2000) Nitric oxide down-regulates MKP-3 mRNA levels: involvement in endothelial cell protection from apoptosis. J Biol Chem 275: 25205–25207.

42. Bokemeyer D, Sorokin A, Yan M, Ahn NG, Templeton DJ, et al. (1996) Induction of mitogen-activated protein kinase phosphatase 1 by the stress-activated protein kinase signaling pathway but not by extracellular signal-regulated kinase in fibroblasts. J Biol Chem 271: 639–642.

43. Brandelio JM, Brunet A, Pouyssegur J, McKenzie FR (1997) The dual specificity mitogen-activated protein kinase phosphatase-1 and-2 are induced by the p42/p44MAPK cascade. J Biol Chem 272: 13661–13672.

44. Cook SJ, Beltman J, Cadwallader KA, McMahon M, McCormick F (1997) Regulation of mitogen-activated protein kinase phosphatase-1 expression by extracellular signal-related kinase dependent and Ca2+-dependent signal pathways in Rat-1 cells. J Biol Chem 272: 13309–13319.

45. Bishop CD, Pires A, Norby SW, Boudko D, Moroz LL, et al. (2008) Analysis of NO Affects ERK Signaling through mkp family for control of MAP kinase function. FASEB J 14: 6–16.

46. Bishop CD, Bates WR, Brandhorst BP (2001) Regulation of metamorphosis in the polychaete annelid Capitella teleta reveals activation of an innate immune response. Development 129: 4739–4751.

47. Griffith OW, Stuehr DJ (1995) Nitric oxide synthases: properties and catalytic mechanism. Annu Rev Physiol 57: 707–736.

48. Hens MD, Fowler KA, Leise EM (2006) Induction of metamorphosis decreases nitric oxide synthase gene expression in larvae of the marine moulus Bopyrida minuta. Biol Bull 211: 208–211.

49. Tanis N, Coentte T, Viard F (2009) Inhibitory function of nitric oxide on the onset of metamorphosis in competent larvae of Ceropidula fornicata: A transcriptional perspective. Mar Genomics 2: 161–167.

50. Pfeffer MW, Horgan GW, Dempflle L (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 30: 36–41.

51. Zhang HM, Li L, Papadopoulos N, Hodgson G, Evans E, et al. (2006) Mitogen-induced recruitment of ERK and MSK to SRE promoter complexes by ternary complex factor Elk-1. Nucleic Acids Res 36: 2394–2407.

52. Yang D, Xie P, Liu Z (2012) Ischemia-reperfusion-induced MKP-3 impairs endothelial NO formation via inactivation of ERK1/2 pathway. PLoS One 7: e40767.

53. Farooq A, Chaturvedi G, Mujtaba S, Plotnikova O, Zeng L, et al. (2001) Solution structure of ERK2 binding domain of MAPK phosphatase MKP-3: structural insights into MKP-3 activation by ERK2. Mol Cell 7: 357–399.

54. Ueda N, Degnan SM (2013) Nitric oxide acts as a positive regulator to induce metamorphosis in the ascidian Herdmania momus. PLoS One 8: e72797.

55. Bishop CD, Bates WR, Brandhorst BP (2001) Regulation of metamorphosis in ascidians involves NO/cGMP signaling and HSP90. J Exp Zool 289: 374–384.

56. Bishop CD, Brandhorst BP (2001) NO/cGMP signaling and HSP90 activity represses metamorphosis in the sea urchin Lytechinus pictus. Biol Bull 201: 394–404.

57. Leise EM, Thavaradhana K, Durham NR, Turner BE (2001) Serotonin and nitric oxide regulate metamorphosis in the marine snail Bythopsis obsoleta. Am Zool 41: 258–267.

58. Pechenik JA, Cochrane DE, Li W, West ET, Pires A, et al. (2007) Nitric oxide inhibits metamorphosis in larvae of Ceropidula fornicata, the slippershell snail. Biol Bull 213: 160–171.

59. Biggers WJ, Pires A, Pechenik JA, Johna E, Patel P, et al. (2011) Inhibitors of nitric oxide synthase induce larval settlement and metamorphosis of the polychaete annelid Capitella teleta. Inv Rep Dev doi:10.1080/07924259.2011.588006.

60. Whittaker CA, Hynes RO (2002) Distribution and evolution of von Willebrand factor A domains: widely dispersed domains with roles in cell adhesion and elsewhere. Mol Biol Cell 13: 3369–3387.

61. Tackdell D (1999) Evolution of von Willebrand factor A (VWA) domains. Biochim Soc Trans 27: 835–840.

62. Davidson B, Szwaja B (2002) A molecular analysis of ascidian metamorphosis consumes. J Biochem 147: 175–185.