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ERK- and JNK-signalling regulate gene networks that stimulate metamorphosis and apoptosis in tail tissues of ascidian tadpoles

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In ascidian tadpoles, metamorphosis is triggered by a polarized wave of apoptosis, via mechanisms that are largely unknown. We demonstrate that the MAP kinases ERK and JNK are both required for the wave of apoptosis and metamorphosis. By employing a gene-profiling-based approach, we identified the network of genes controlled by either ERK or JNK activity that stimulate the onset of apoptosis. This approach identified a gene network involved in hormonal signalling, in innate immunity, in cell-cell communication and in the extracellular matrix. Through gene silencing, we show that *Ci-sushi*, a cell-cell communication protein controlled by JNK activity, is required for the wave of apoptosis that precedes tail regression. These observations lead us to propose a model of metamorphosis whereby JNK activity in the CNS induces apoptosis in several adjacent tissues that compose the tail by inducing the expression of genes such as *Ci-sushi*.

KEY WORDS: Ascidian tadpoles, Apoptosis, *Ci-sushi*, Metamorphosis, MAPK

INTRODUCTION

During embryonic development, apoptosis plays a central role in the successful outcome of the developmental program, and has been studied using genetic and cell-biological means in many model organisms (for a review, see Meier et al., 2000). Our ability to study the full extent of processes such as apoptosis during embryonic development has been advanced by the sequencing of the genomes of model organism and by the subsequent development of DNA-chip technologies coupled with gene-silencing-based approaches that can provide a mass of testable information about the dynamics of gene regulatory networks. Programmed cell death has been found to operate in all multicellular animals studied so far, including cnidarians, nematodes, insects, ascidians, amphibians, fish, birds and mammals (Cikala et al., 1999; Ellis et al., 1991; Steller, 1995; Chambon et al., 2002; Vaux and Korsmeyer, 1999; Jacobson et al., 1997). Metamorphosis represents a dramatic example of apoptosis and occurs throughout the animal kingdom (Vaux and Korsmeyer, 1999; Jacobson et al., 1997). In basal chordates such as ascidians, metamorphosis is characterized by a period of intense cell reorganization and remodelling that is dependent on programmed cell death events (Chambon et al., 2002; Jeffery, 2002).

Although the execution of apoptosis occurs through the activation and function of a highly conserved family of cysteine proteases, termed caspases, present from hydra to man (Cikala et al., 1999; Thornberry and Lazebnik, 1998), the initiation of apoptosis also depends on the activation of extrinsic and/or intrinsic death pathways (Ashkenazi and Dixit, 1998). In addition, the apoptotic process is also regulated by many intracellular signalling pathways, including the mitogen-activating-protein kinase (MAPK) pathways (Deng et al., 2003; Lin and Dibling, 2002; Chen et al., 2005).

MAPK proteins play both pro-apoptotic and anti-apoptotic roles depending on the cellular environment (Tibbles and Woodgett, 1999; Davis, 2000; Chang and Karin, 2001). Conventional MAPK proteins consist of three family members (Johnson and Lapadat, 2002): the extracellular signal-regulated kinase (ERK) (Seger and Krebs, 1995); the c-Jun NH2-terminal kinase (JNK); and p38 (Waskiewicz and Cooper, 1995). ERK1-2 was initially reported to be a pro-survival factor (Wada and Penninger, 2004; Johnson and Lapadat, 2002), although a recent study suggests that it also has a key pro-apoptotic role in neuronal apoptosis induced by potassium (K^+) withdrawal (Subramaniam et al., 2004). JNK can exhibit either pro- or anti-apoptotic functions, depending on the cell type, the nature of the death stimulus, the duration of JNK activation and the activity of other signalling pathways (for a review, see Liu and Lin, 2005). Similar to JNK, the involvement of p38-MAPK in apoptosis is also diverse (Sarkar et al., 2002).

MAPK plays a pro-apoptotic role during ascidian metamorphosis. The activation of the *Ciona intestinalis* MAPK ERK (Ci-ERK) in tail cells precedes the wave of apoptosis, suggesting that the phosphorylated form of Ci-ERK transduces the death-activating signal in tail tissues during metamorphosis (Chambon et al., 2002). Moreover, inhibition of Ci-ERK blocks metamorphosis (Chambon et al., 2002). Finally, it was recently reported that programmed cell death in *C. intestinalis* larvae also correlates with JNK activity (Tarallo and Sordino, 2004). However, it is not yet fully known what transcriptional events are targeted by MAPK to induce apoptosis in vivo. The simplicity of the ascidian tadpole, which consists of approximately 2600 cells, the rapid rate of development and the predictable wave of apoptosis during tail regression make the ascidians an amenable system in which to study apoptosis. In addition, the recent development of comprehensive microarrays

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(75% representation of the genome), coupled with extensive in situ gene expression profiles during embryogenesis (Satou et al., 2002) (http://ghost.zool.kyoto-u.ac.jp/indexr1.html), make the ascidian a useful organism for identifying the gene-regulatory network that controls the onset of metamorphosis and also for identifying the subset of genes regulated by MAPKs that induce apoptosis. Here, using ascidian metamorphosis as a model, we set out to identify the genes downstream of either JNK- or ERK-activity that control the onset of apoptosis during development. In addition, through a gene-silencing-based approach, we demonstrate that one of the genes identified by our screen, *Ci-sushi*, links JNK activation to the wave of apoptosis that precedes tail regression. As well as identifying 110 genes regulated by either JNK or ERK, we describe how the activation of JNK in the CNS controls apoptosis in adjacent tissues (notochord, epithelia and muscle), and propose a model for the synchronous apoptosis that occurs in these tissues.

MATERIALS AND METHODS

Animal husbandry

Adult *C. intestinalis* were collected in the bay of Roscoff (Finistère, France) or were cultivated at the Maizuru Fisheries Research Station of Kyoto University; at the Field Science Center of Tohoku University; or at the International Coastal Research Center of the Ocean Research Institute, the University of Tokyo, Japan. Oocytes and sperm were obtained by dissection of gonoducts and cross-fertilization was performed in plastic Petri dishes. Embryos were cultured at 18°C in 0.2 μm filtered seawater containing 100 U/ml penicillin and 100 μg/ml streptomycin sulphate. Tadpole-type larvae hatched at approximately 18 hours of development (18 hpf). They then swam freely for more than 7 hours (25 hpf). The onset of metamorphosis started at approximately 8 hours after hatching (26 hpf), when juveniles adhered to plastic dishes, and the resorbtion of the tail began 2 hours after the attachment (28 hpf).

Indirect immunofluorescence

Larvae were fixed for 20 minutes with 3.7% formaldehyde in filtered seawater. Embryos were permeabilized with 0.2% Triton in phosphate-buffered saline (PBS) and washed once in PBS. Phosphorylated ERK was visualized by incubating larvae for 1 hour at room temperature with a monoclonal antibody raised against the active form of MAPKs ERK1 and ERK2 (dual-phosphorylated 'HTGFLT(p)EY(p)VAT' peptide) (BD Transduction Laboratories). Embryos were then washed three times in PBS 0.008% Triton and incubated for 1 hour at room temperature with FITC-conjugated donkey-anti-mouse immunoglobulin as a secondary antibody (Jackson ImmunoResearch Laboratories) diluted 1:500 in PBS. Larvae were then washed three times in PBS, once in tris-buffered saline (TBS), were rinsed in distilled water and were then mounted in Vectashield containing DAPI (Vecta Laboratory). The slides were analyzed with a Zeiss Microscope. Phosphorylated JNK and p38 were both detected with a MAP Kinase Activation Monoclonal Antibody kit (BD Transduction Laboratories). Appropriate secondary antibodies were TRITC-conjugated donkey-anti-mouse or FITC-conjugated donkey-anti-mouse immunoglobulin (Jackson Laboratories).

Treatment with JNK and MEK inhibitors

The JNK inhibitor SP600125 (Sigma-Aldrich) was dissolved to DMSO and added at a final concentration of 10 μM immediately after hatching. The MEK inhibitor U0126 (Promega) was dissolved in DMSO and added either once (6 μM) immediately after hatching, or once at this time-point (6 μM) followed by every 6 hours thereafter (6 μM at each administration). Embryos were cultured at 18°C during 48 hours. Treated and control larvae were scored for signs of metamorphosis. Results were the mean of three independent experiments.

SDS-PAGE and western immunoblotting

For activated-ERK analysis, larvae were sonicated on ice in RIPA lysis buffer [150 mM NaCl, 50 mM Tris-Cl (pH 7.6), 5 mM EDTA, 0.5% NP-40, 1 mM PMSF, 1 mM orthovanadate, 0.5% sodium deoxycholate, 0.1% SDS] supplemented with Complete Protease Inhibitor Cocktail Tablets (Roche Molecular Biochemicals). Lysates were then clarified by centrifugation. Samples were diluted in sample buffer (Laemmli, 1970) and incubated at 85°C for 5 minutes. Total proteins were separated on 12.5% SDS gels and transferred onto PVDF membranes. The blots were blocked with 5% milk powder in PBS-Tween, were incubated for 1 hour with a monoclonal anti-dual-phosphorylated ERK (BD Transduction Laboratories) that was diluted 1:500 in PBS-Tween, were washed in TBS-Tween, were incubated for 1 hour with the secondary antibody (HRP-conjugated anti-mouse IgG antibody diluted 1:10,000) and were then washed in PBS-Tween. Labelled proteins were detected using the Supersignal West Pico Chemiluminescent Substrate (Pierce). Additional analysis by western blotting with different antibodies was performed using 12.5% SDS polyacrylamide gels containing 0.13% bisacrylamide. The various primary antibodies used were MAP Kinase Activation Sampler kit and MAP Kinase Sampler kit (BD Transduction Laboratories), and, as a secondary antibody, we used horseradish peroxidase linked to rabbit antibodies directed against mouse-immunoglobulin Fc fragments (Sigma, Saint Louis, MO).

Microarray design

A *C. intestinalis* microarray (oligonucleotide-based chip version 1) was made by Agilent Technologies (ink-jet-based Sure Print technology). A total of 21,939 features on the chip consisted of 21,617 independent 60-mer oligonucleotide probe sequences. These probes represent 22,445 cDNA/EST sequences selected from over 450,000 *C. intestinalis* ESTs and 4062 cDNAs (Satou et al., 2002) (http://ghost.zool.kyoto-u.ac.jp/indexr1.html), with at least one sequence per gene. Information on the *C. intestinalis* genome (Dehal et al., 2002) indicates that 17,490 of these features represent 11,903 (75.1% of the total 15,852) *C. intestinalis* gene models, according to computational analysis. Manual checking indicated that approximately 5% of the remaining 3949 gene models are estimated to have representative features also. The additional 4127 features not corresponding to any gene models represent approximately 3500 independent cDNA clusters. As a result, the total features on the array are estimated to cover approximately 75-80% of the genes in the *C. intestinalis* genome.

RNA extraction, labelling and hybridization

Total RNA of tadpole-type larvae was extracted 7 hours after hatching (25 hpf) using TRIZOL reagent and was then treated with DNase (Promega). Labelling of amplified cRNA with either cyanine-3 CTP or cyanine-5 CTP (Perkin-Elmer/NEN Life Sciences) was performed using 250-ng aliquots of total RNA with a Low RNA Input Fluorescent Linear Amplification kit (Agilent Technologies), following the manufacturer's instruction. The quality and size distribution of targets was determined by the RNA 6000 Nano Lab-on-chip Assay (Agilent Technologies), and quantification was performed using a NanoDrop microscale spectrophotometer (NanoDrop). A set of cRNA targets from each sample was assembled into a hybridization reaction using an In Situ Hybridization kit (Agilent Tech.). Each hybridization was compared with that of the 0-hour larvae sample (18 hpf). Hybridization was performed twice with a dye swap. Hybridized microarrays were washed according to the manufacturer's protocol and then scanned using a G2565BA Micro-Array Scanner System with SureScan technology (Agilent Technologies).

Data analysis

The intensity of 21,939 gene features per array was extracted from scanned microarray images using Feature Extraction 7.1 software (Agilent Technologies), which performs background subtractions and dye normalization. The data were analyzed using Excel (Microsoft). Hierarchical clustering was performed using Cluster and Treeview (Eisen et al., 1998).

The gene features were searched against the *C. intestinalis* draft genome sequence (Dehal et al., 2002) to obtain information about corresponding genes. The features that had several hits in the draft genome sequences were excluded from the present analyses and the remaining 18,222 gene features were picked up to be included in the analyses. Gene features that coincided with a predicted gene model or cDNA sequences from the cDNA/EST database (Satou et al., 2002) and the deduced protein sequences were used

to characterize corresponding genes with the Genome browser of JGI (http://genome.jgi-psf.org/ciona4/ciona4.home.html). The cDNA and EST sequences were used in combination with the JGI gene model to obtain more-complete gene models. The GENES Human ENTRY number concerning JGI gene models was used for analysis on the Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.ad.jp/kegg) GENES Human database and KEGG PATHWAY database (Kanehisa and Goto, 2000; Kanehisa, 2002).

mRNA isolation and reverse transcriptase-PCR

For mRNA isolation, larvae were lyzed in TRIzol (Invitrogen), according to the supplier's instructions. mRNA was reverse transcribed. For each gene, a set of specific forward (For) and Reverse (Rev) oligonucleotides was designed to amplify a small part of coding region (from 200-600 bp):

C-JUN: (For: CCGTCTTGAAGGGTATGAGC, Rev: GTTCCAGTTTCCGCTTTCTG);

SRF: (For: CCACGACGATGAACATTACG, Rev: GTCGGCGTTTTATGTTCGTT);

MKP: (For: CCACTTTCCAGACCGATTTC, Rev: CCTCACAGGTCCACTCCATT);

Ci-GNRH: (For: TGTGTGTTACTTGTCGTTCTAGCC, Rev: GGATCCGTTGCAAGAGTTGT);

Ci-sushi: (For: TTGCAAGTCTTTGCACAGTTG, Rev: CCAACGGCTGTGATATGTGA);

Ci-ETS: (For: CAAAGCACACCAAGCCAGTA, Rev: GTTGGGGTAGCATGGTTCAT);

Ci-LyOx: (For: TGGGTGGGACTTGAACAC, Rev: TTCCCTCTTGCGTACTTTGG);

Ci-Vwa1a: (For: TGGTTGCAAACAAGAAGCTG, Rev: ATCCTCATTTGCATCGAACC);

Ci-Vwa1b: (For: GCACTGTCGGTTCAGTGTGT, Rev: CCGAAACTAGGTTGCGTTGT);

Ci-Vwa1c: (For: ATACTTCGACCCAGCACGTC, Rev: AAACTCCGTTACGCCTCAGA);

Ci-OATP: (For: CGGTTGGGTTGATCTTGAGT, Rev: GACGATCCCAACTTTTTCCA);

Ci-dhg: (For: GTCACCGTTTCCTCTGAAGC, Rev: GCGCCGTGTATTATGGTCTT);

Ci-Mx: (For: ACCCAGACATTGCAGGAAC, Rev: GAGCCGCTACAATTCTCAGG).

Reverse transcriptase (RT)-PCR was performed on equal amounts of input RNA and cDNA using the amplimers describe above. S26 mRNA was used as a control using the amplimer For: TCCCCTTCTTCCTCAAGCAC and Rev: GCCCAACCACCATCCTGTA. For the control S26, PCR was first performed on cDNA from untreated larvae or from 18 hpf larvae, and the number of cycles adjusted so as to produce a non-saturating signal on ethidium bromide-stained 1.2% agarose slab gels. Semi-quantitative PCR was next performed on cDNA from each time-point or from untreated and treated larvae using the number of cycles determined above. PCR products were run on 1.2% agarose slab gels.

Microinjection of morpholino oligo

Chorionated *C. intestinalis* oocytes were vigorously pipetted to remove the outer follicular cells. These partially dechorionated oocytes were then fertilized and transferred to cleaned Petri dishes (cleaned with ethanol and rinsed in distilled water) and allowed to settle by gravity. As the oocytes settled, a substantial proportion fixed to the plastic Petri dishes. Those zygotes that became fixed were injected (injection-pipette holder mounted at ~30°), with the injection needle controlled by a hydraulic micromanipulator (Narishige) coupled to a high-pressure injection device (Narishige IM300). *Ci-sushi* morpholino antisense DNA (Gene Tools) at a pipette concentration of 1 mM was mixed 10:1 with 20 mM Texas Red-Dextran 10 kDa (Molecular Probes) and injected into the zygotes (~1% injection volume) after extrusion of the second polar body and before the first cleavage. The following day (at 18 hpf), those tadpoles that were fluorescent were isolated and further analyzed through metamorphosis and TUNEL labelling. As a control, we used standard control morpholino (Gene Tools). Results were the mean of three independent experiments.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed by using digoxigenin (DIG)-labelled antisense probes as described previously (Satou, 1999). RNA probes were prepared using a DIG RNA-labelling kit (Roche). Control embryos hybridized with a sense probe did not show any signal above background levels.

RESULTS

ERK- and JNK-pathways, but not p38, are activated in swimming larvae

Following a rapid embryogenesis, which lasts 18 hours at 18°C, *C. intestinalis* larvae hatch and swim within the plankton for a variable period of a few hours. The larvae then become competent to respond to either natural or artificial settlement cues and start a series of rapid morphological changes (Arnold et al., 1997). After settlement, *C. intestinalis* metamorphose into sessile filter feeders, a process involving a massive reorganization of the body plan and, most notably, apoptosis-dependent tail regression (Chambon et al., 2002). The potential role of the CNS in initiating metamorphosis by selecting sites for settlement was previously proposed (Cloney, 1978; Cloney, 1982).

We chose to focus on MAPK signalling cascades for two reasons. First, we and others have shown that the inhibition of ERK prevents tail regression and apoptosis during ascidian metamorphosis (Chambon et al., 2002; Tarallo and Sordino, 2004). Second, it has previously been reported that MAPK pathways are well conserved in the *C. intestinalis* genome (Satou et al., 2003; Hotta et al., 2003) (see Table S1 in the supplementary material) and that some components of these pathways are strongly and/or exclusively expressed in the CNS of *C. intestinalis* during the tadpole stage (Imai et al., 2004) (see also http://ghost.zool.kyoto-u.ac.jp/ST2005.html). We examined whether activation of the three main MAPK pathways occurs during the swimming phase of *C. intestinalis* larvae. Given the perfect match between the *Ciona* and human peptide sequences in each of the two proteins (see Fig. S1 in the supplementary material), we used antibodies directed against the human dual-phosphorylated peptides to detect activated and non-phosphorylated Ci-ERK, Ci-JNK and Ci-p38 from *C. intestinalis* protein extracts and/or by indirect immunofluorescence (Fig. 1A-C and D). *C. intestinalis* larvae from hatching (18 hours post-fertilization; hpf) to the onset of metamorphosis (28 hpf) were analyzed by western blotting. The level of total protein extract of each sample was visualized by Coomassie Blue staining (Fig. 1A, upper panel). An activated form of Ci-JNK was present during the swimming phase of the larvae at 20 and 22 hpf. Ci-ERK was activated between 20 and 24 hpf, with peaks at 20 and 22 hpf, and was then activated again at 28 hpf, as we previously described (Chambon et al., 2002). No activation of Ci-p38 was detected at any time during the swimming period. We confirmed that Ci-ERK, Ci-JNK and Ci-p38 protein levels did not vary significantly during this period (Fig. 1A, lower panels).

In order to determine where in the larvae JNK and ERK activation occurred, we used the same antibodies for indirect immunofluorescence studies on larvae fixed at various times. In agreement with protein expression analysis and our activation study (Fig. 2C), Ci-JNK and Ci-ERK were found to be activated at 20 and 22 hpf. No Ci-p38 was detected in any region of the swimming larvae (see Fig. S2 in the supplementary material).

Ci-JNK was phosphorylated in the CNS of larvae (Fig. 1B,C). Activated Ci-JNK was detected in the CNS localized in the trunk of the larvae: specifically, in the posterior part of the sensory vesicle

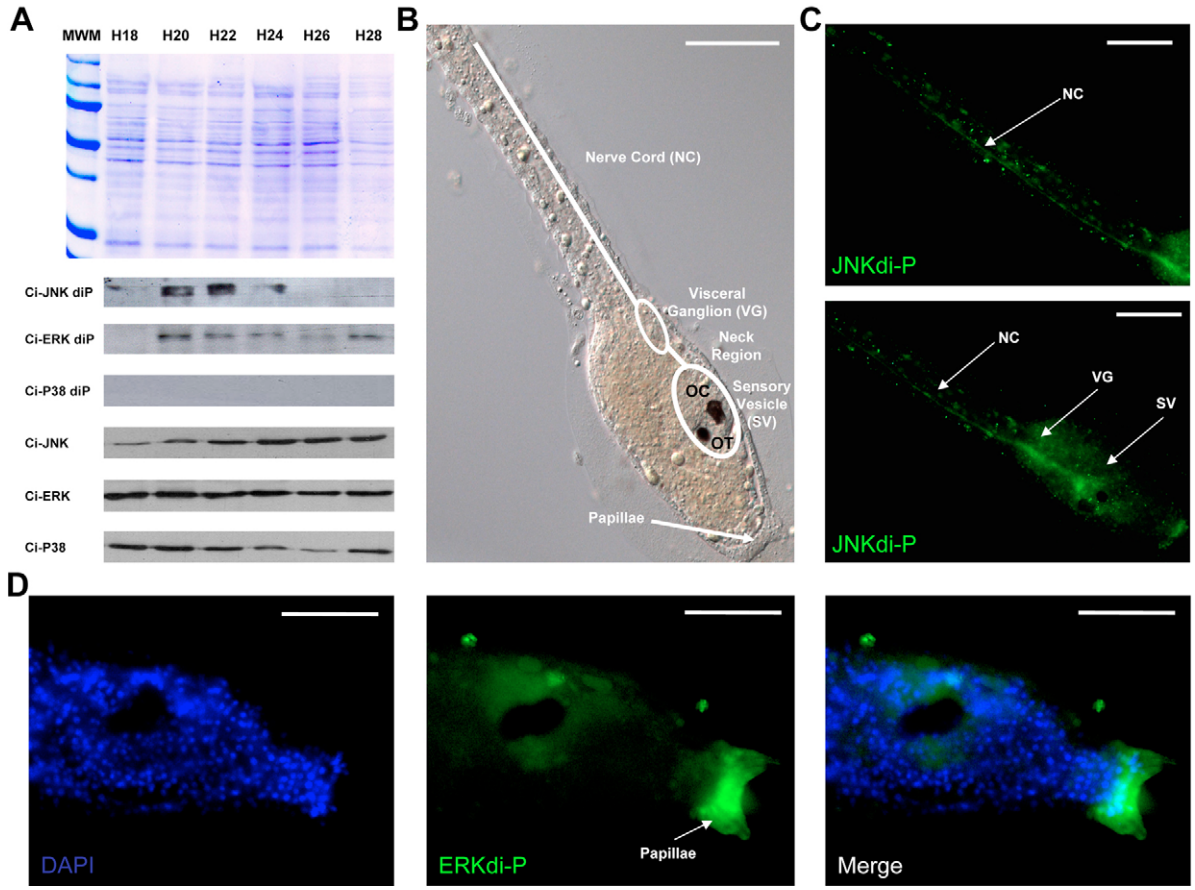

Fig. 1. MAPK activation in swimming larvae of *Ciona intestinalis*. (**A**) Comparative western blot analysis of *Ciona*-tissue homogenates with antibodies against dual-phosphorylated ERK, JNK and p38, and non-phosphorylated ERK, JNK and p38. Top: Coomassie Blue-stained gel showing the total protein. Bottom: western blotting was performed at various stages of the swimming larval phase and during metamorphosis. ERK- and JNK-proteins were expressed and activated during the acquisition of metamorphosis competence. (**B**) The CNS and papillae in swimming larvae of *C. intestinalis*. OC, ocellus; OT, otolith. (**C**) Ci-JNK activation was localized in the CNS. Ci-JNK phosphorylation (green) was detected in the sensory vesicles, the neck region, the visceral ganglion and along the nerve cord. (**D**) Ci-ERK activation was localized in the papillae of *C. intestinalis* larvae. Ci-ERK phosphorylation (green) was detected in the three posterior palps of the papillae of the tadpole. Scale bars: 120 μm.

that contains the pigmented ocellus and otolith, the visceral ganglion composed of motoneurons and between these the neck region. The caudal nerve cord also contained activated Ci-JNK.

Ci-ERK was activated in the papillae of *Ciona* larvae (Fig. 1D). *C. intestinalis* papillae are constituted of secreting cells, axial columnar cells, primary sensory neurons and undifferentiated ectodermal cells (Manni et al., 2004). Activated Ci-ERK was detected in all the papillae, from the anterior part to the extremity of each of the three palps. Some nuclei of the anterior epidermis of the trunk were also positively stained for phosphorylated Ci-ERK. As we previously described, Ci-ERK was also later activated in tail cells at 28 hpf (Chambon et al., 2002).

ERK- and JNK-inhibitor treatment blocks initiation of apoptosis and metamorphosis

We next compared the status of apoptosis in larvae that had initiated metamorphosis and that had been treated with MAPK inhibitors. The hatching larvae were treated with a MAPK inhibitor and, after 10 hours (at 28 hpf), were fixed and processed for TUNEL labelling (Fig. 2A). Major differences were observed between larvae treated with the MEK inhibitor (U0126) or JNK inhibitor (SP600125) to those larvae untreated or treated with the p38 inhibitor (SB203580). Consistent with our previous work (Chambon et al., 2002), the untreated larvae that began metamorphosis exhibited TUNEL-positive cells at the tip of the tail (Fig. 2A). No difference was observed when larvae were treated with the p38 inhibitor. By contrast, the inhibition of JNK (SP600125) and ERK (U0126) phosphorylation inhibited initiation of apoptosis at the tip of the tail (Fig. 2A).

Hatched larvae were incubated at 18°C in filtered seawater with DMSO (0.1%), U0126 (6 μM) or SP600125 (10 μM). Inhibition of the JNK cascade with SP600125 completely blocked metamorphosis (Fig. 2D) at 2 days post-fertilization. Similarly, but to a lesser extent, inhibition of MEK with U0126 blocked metamorphosis in a significant proportion of the tadpoles (Fig. 2D) (Chambon et al., 2002). Given that U0126 has a short half-life, U0126 treatment was repeated every 6 hours. In this condition, inhibition of MEK significantly decreased the number of larvae that reached the metamorphosis stage and increased the number of swimming larvae (Fig. 2D). As depicted in Fig. 2D, at 2 days after fertilization, inhibition of the JNK pathway with 10 μM SP600125 induced ~100% blockade of metamorphosis, and inhibition of the ERK pathway with 6 μM U0126 led to a significant decrease (94%) in the number of metamorphosed larvae. In the control (DMSO treated) group, 95% of larvae underwent metamorphosis.

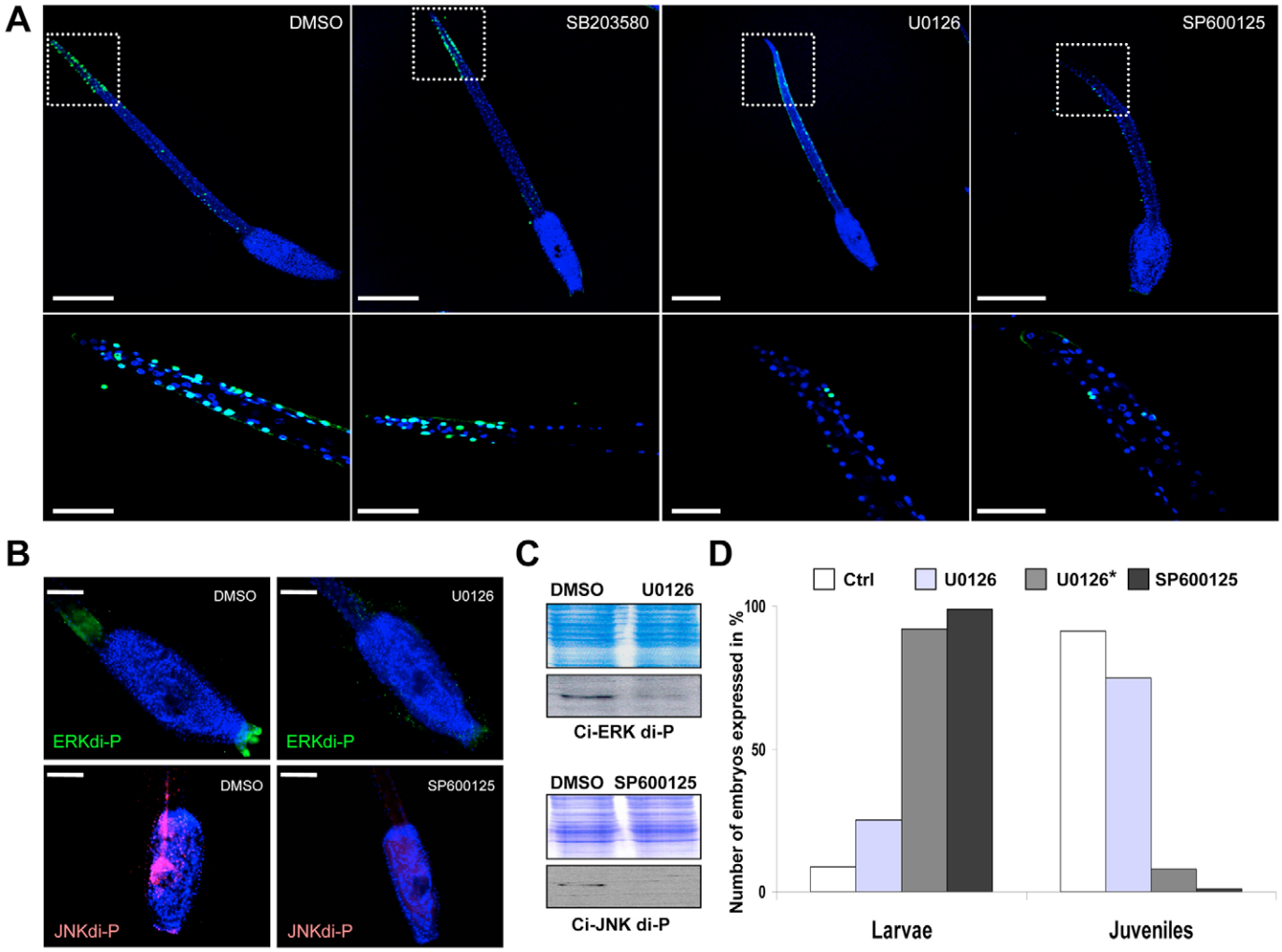

Fig. 2. Inactivation of MAPK blocks metamorphosis and associated apoptosis-dependent tail regression. (**A**) Double detection of apoptosis and nuclei in the tail of *Ciona intestinalis* during metamorphosis (at 28 hpf). Digitized images were merged to superimpose nuclei (blue) over the respective TUNEL-labelled field (TUNEL-positive nuclei appear in green). Notice that the nuclei of numerous cells of the tail extremity are TUNEL positive in the control panel (DMSO) and in p38 inhibitor (SB203580)-treated larvae. By contrast, TUNEL-positive nuclei were detected very rarely in the presence of the ERK inhibitor (U0126) or the JNK inhibitor (SP600125) in treated larvae. The white square corresponds to the region of higher magnification displayed in the lower panel. (**B**) Double detection of ERK phosphorylation (green) or JNK phosphorylation (red) and nuclei (blue) in larvae at 22 hpf. ERK phosphorylation was detected in papillae and JNK phosphorylation was detected in the CNS of larvae treated with DMSO at 22 hpf. Larvae treated with U0126 MEK inhibitor were negative for ERK activation in papillae, and the larvae treated with SP600125 JNK inhibitor were negative for JNK activation in the CNS (red). (**C**) Extracts from untreated larvae at 22 hpf and larvae treated with U0126 or SP600125 at this time were run on SDS-PAGE and western blotted with the anti-phosphorylated ERK and anti-phosphorylated JNK monoclonal antibodies. (**D**) U0126 MEK inhibitor and SP600125 JNK inhibitor blocked metamorphosis of *C. intestinalis*. From hatching, larvae were treated with 6 μm of U0126 or 10 μm of SP600125. In one condition (U0126*) the treatment was repeated every 6 hours due to the loss of activity of the MEK inhibitor U0126. Data represent the mean of three independent experiments (400 animals per experiment) expressed as a percentage of the total number of larvae. Scale bars: 225 μm in A, upper panel; 50 μm in A, lower panel; 45 μm in B.

Although Ci-ERK and Ci-JNK activation was observed in DMSO-treated larvae, the activated Ci-ERK and Ci-JNK labelling was lost at 24 hpf with SP600125 and U0126 treatment, respectively (Fig. 2B). Moreover, incubation with SP600125 or U0126 significantly decreased Ci-JNK or Ci-ERK phosphorylation when compared with the control by western blot analysis (Fig. 2C).

Modulation in gene expression of MAPK pathway components in larvae

We performed a kinetic study based on cDNA microarray experiments (Azumi et al., 2003) to identify genes that are differentially transcribed during the swimming phase. An overview of the results for each MAPK pathway is displayed in Fig. 3A. Although very few modulations in gene expression were observed immediately after hatching, the situation changed between 26 and 28 hpf, which corresponded approximately to the start of tail absorption (Fig. 3A). Interestingly, the two transcription factors *SRF* (serum response factor) and *c-jun*, which are known to be targeted by ERK and JNK, respectively, were upregulated at 26 and 28 hpf (Fig. 3B). The expression of *SRF* was maximum at these times before declining to its initial level, whereas *c-jun* expression was highly up-regulated from 26 to 50 hpf (Fig. 3B, upper and middle panel). We confirmed the microarray expression profile of *SRF* and *c-jun* by semi-quantitative reverse transcriptase (RT)-PCR (Fig. 3C).

SRF is a target of the ERK signalling pathway and provides a positive-feedback loop (Kasza et al., 2005). A similar positive feedback exists for JNK, as demonstrated in hypoxic HepG2 cells, through regulation of the *c-jun* promoter (Minet et al., 2001). Moreover, the *MKP* (*MAP kinase phosphatase*) gene, a key protein of MAPK pathways, was upregulated from 26 to 30 hpf (Fig. 3B, lower panel; Fig. 3C). MKP is a member of the dual specific phosphatase (DSP) protein family, members of which inactivate MAPKs through dephosphorylation. The increase in *MKP* gene expression could provide a negative-feedback loop on activated MAPK pathways in *C. intestinalis* larvae; as has been described for the ERK pathway in *Drosophila* and *Xenopus* (Gomez et al., 2005).

Target genes of the JNK and ERK MAPK pathways

Because inhibition of either ERK or JNK prevented the wave of apoptosis that preceded tail regression, we undertook a gene-profiling study using oligonucleotide-based microarrays (Yamada et al., 2005; Ishibashi et al., 2005) to determine the transcriptional differences between larvae treated with MAPK inhibitors or with DMSO (control). We chose tadpole larvae that were at 25 hpf for two reasons. First, because both ERK and JNK displayed a peak of activity starting at 20 hpf (Fig. 1); and second, because the major change in gene expression began between 24 and 26 hpf (Fig. 3). Among the many genes where the expression pattern is altered at 25 hpf, we anticipated identifying the subset that is involved in the initiation of the wave of apoptosis, because this is blocked by both inhibitors.

To identify genes regulated by these two MAPK pathways, we determined the expression profile of DMSO-treated larvae aged 25 hpf with identically aged larvae that had been treated with either SP600125 or U0126. Only genes that consistently displayed greater than threefold changes during SP600125 treatment or greater than twofold changes in transcript abundance during U0126 treatment were scored. This conservative approach, which has been used in a

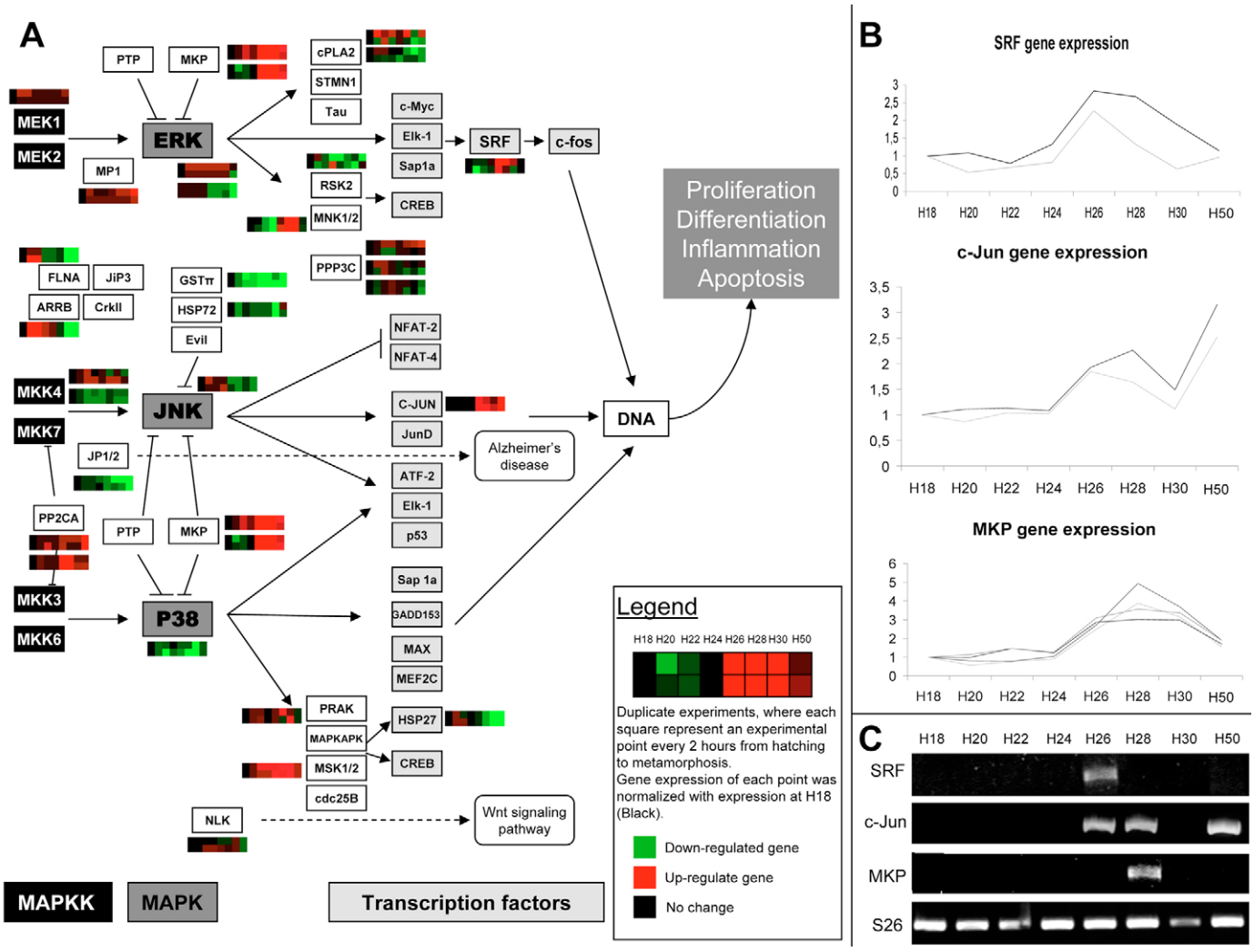

Fig. 3. Gene expression of MAPK pathway components in *Ciona intestinalis* larvae. (**A**) Microarray analysis of MAPK gene expression during *C. intestinalis* swimming larval phase. Diagram of mammalian MAPK pathways superimposed with oligonucleotide-based chip data. The microarray data are represented by a square composed of two rows (two independent experiments) and eight smaller squares corresponding to each experimental point (every 2 hours from 18 hpf to 30 hpf, the last experimental point is at 50 hpf). Downregulated genes are displayed in green and upregulated genes are displayed in red. The expression level of each gene at each experimental point was normalized with its expression level at 18 hpf (first small black square). (**B**) Expression level of the *SRF*, *c-jun* and *MKP* genes from hatching to metamorphosis. (**C**) Semi-quantitative RT-PCR for SRF, c-jun and MKP. mRNA was extracted from embryos, reverse transcribed and semi-quantitative specific PCR was performed (see Materials and methods). S26 ribosomal protein RNA represents an internal control for the level of expression (Vincent et al., 1993).

variety of microarray studies (e.g. Butler et al., 2003; Munoz-Sanjuan et al., 2002), is likely to miss a number of genes that are differentially expressed in response to MAPK-inhibitor treatment. The genes affected by the MAPK-inhibitor-treatment protocol were categorized into four major classes based on Lee et al. (Lee et al., 1999) (Tables 1, 2). Class A coded for proteins common to many cell types, class B were proteins associated with cell-cell communication, class C coded for proteins that function as transcription factors and other regulatory proteins, and class D were proteins of an unknown function (DI) or with no similarity (DII).

In total, 38 genes were upregulated and 21 genes were downregulated by inhibition of the JNK pathway (Table 1). These included homologues of genes and genes previously shown to be involved in metamorphosis of other ascidians species, as well as of *C. intestinalis* (Nakayama et al., 2001; Nakayama et al., 2002; Davidson and Swalla, 2002; Woods et al., 2004). For example, *Ci-meta5*, a gene previously shown to be involved in metamorphosis (Nakayama et al., 2001; Nakayama et al., 2002), was identified by our screen.

A total of 12 genes were upregulated and 41 genes were downregulated by U0126 MEK-inhibitor treatment (Table 2). In addition to genes previously shown to be involved in ascidian metamorphosis, treatment with MAPK inhibitors identified other genes not previously implicated in this process. We will come back to some of these genes later in the discussion.

In order to confirm the microarray data, we performed RT-PCR of the genes identified by cDNA chips between larvae treated with MAPK inhibitors or with DMSO (control), including three *Vwa1* genes (*Ci-Vwa1a*, *Ci-Vwa1b* and *Ci-Vwa1c*), *dehydrogenase* (*Ci-Dhg*), *matrix metalloprotease* (*Ci-Mx*), *gonadotropin releasing hormone* (*GNRH*; *Ci-GNRH*), *lysil oxidase* (*Ci-LyOx*), *ets* (*Ci-ets*) and *organic anion transporting polypeptide 14* (*oatp14*; *Ci-oatp*) (Fig. 4A,B).

Due to the substantial EST programme coupled with extensive in situ data (Satou et al., 2002) (http://ghost.zool.kyoto-u.ac.jp/indexr1.html), many of the genes identified by our screen had already been described. For example, genes under the control of Ci-ERK, such as *rev-erb*, are expressed exclusively in the palps (Kusakabe et al., 2002) (see also http://ghost.zool.kyoto-u.ac.jp/cgi-bin3/photoget2.cgi?CLSTR03308). *Selectin*, which is also controlled by Ci-ERK, was observed in the palps of competent and attached larvae during metamorphosis of the ascidian *Boltenia villosa* (Davidson and Swalla, 2002). Among genes that we identified to be controlled by Ci-JNK, *Vwa1* had already been detected in the tadpole brain (Satou et al., 2001) (see also http://ghost.zool.kyoto-u.ac.jp/cgi-bin3/photoget2.cgi?CLSTR01650), and *Emc* in the nervous system and the nerve corde (Imai et al., 2004) (see also http://ghost.zool.kyoto-u.ac.jp/cgi-bin3/photoget2.cgi?cicl010f24). We also conducted a series of whole-mount in situ hybridizations for several of the genes identified by our screen,

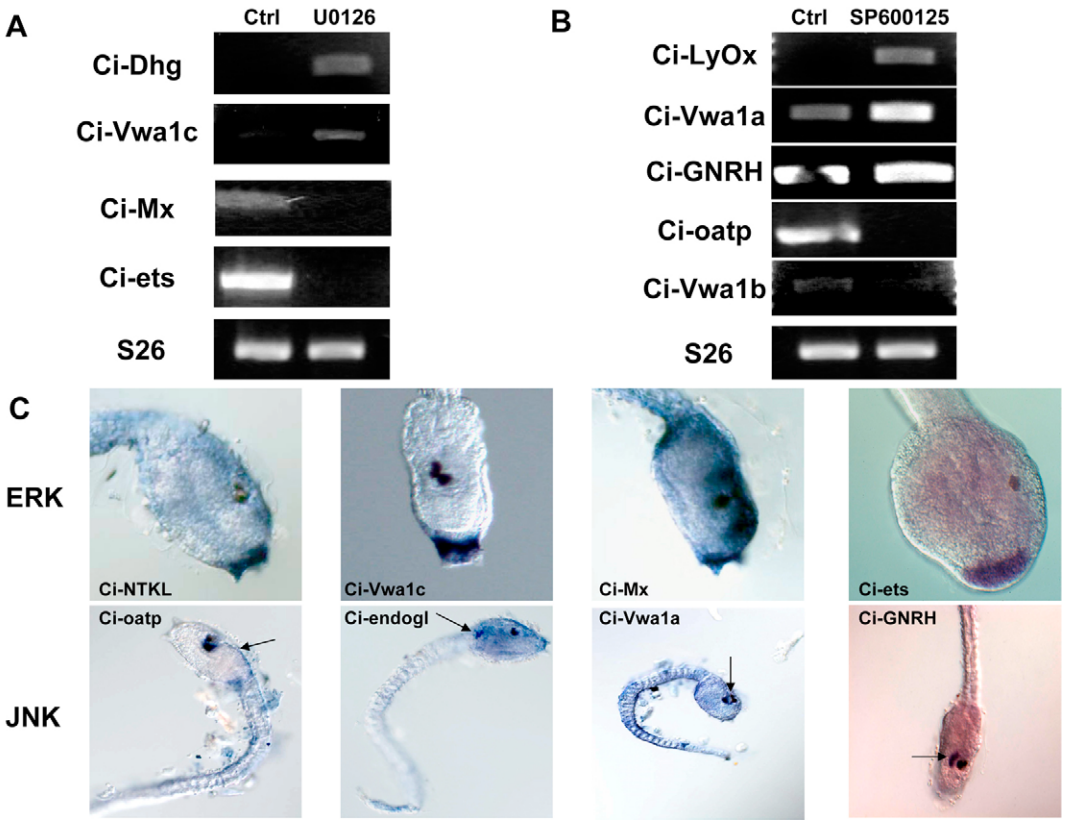

Fig. 4. Genes controlled by the MAPK pathways. (**A**) mRNA expression of ERK target genes at 25 hpf. mRNA was extracted from untreated embryos or embryos treated with 6 μM U0126, reverse transcribed and semi-quantitative specific PCR was performed (see Materials and methods). (**B**) mRNA expression of JNK target genes at 25 hpf. mRNA was extracted from untreated embryos or embryos treated with 10 μM SP600125, reverse transcribed and semi-quantitative specific PCR was performed (see Materials and methods). (**C**) Whole-mount in situ hybridization of genes controlled by ERK and JNK displaying, respectively, specific expression in papillae and in the nervous system (arrows) of *Ciona intestinalis* larvae. *Ci-Vwa1* and *Ci-GNRH* were detected in sensory vesicle (arrows); *Ci-endogl* was observed in visceral ganglion and *Ci-oatp* in the neck region (arrows).

Table 1. *Ciona intestinalis* genes, identified by microarray analysis, that have an altered level of expression at 25 hpf when treated with SP600125 JNK inhibitor

Genes upregulated by SP600125 treatment

Chip feature	KyotoGrail gene model	JGI version 1 gene model	Identity and/or GO function	Class	References*	In situ†
28253 17330	2004.159.24.1 2005.159.20.1	ci0100131547	gi|12057020|emb|CAC19873.1| putative notch receptor protein [*Branchiostoma floridae*]	B	(Davidson and Swalla, 2002)	
12297 25660	2004.377.2.1 2005.377.2.1	ci0100143565	No hit found	DII		
31511	2004.631.2.1 2005.631.2.1	ci0100132472	gi|16754895|dbj|BAB71805.1| chitin binding protein b04 [*Bos taurus*]	A		
23448- 10342	2004.53.18.1 2005.53.14.1	ci0100143017	gi|345831|pir||A44351 transcription repressor E4BP4–human]	C		
28537	2004.779.2.1 2005.779.2.1	ci0100130955	IPI:IPI00138001.1|ENSEMBL:ENSMUSP0 0000049012 [*Homo sapiens*]; ref:NP_005413.1–tectorin alpha precursor; Tectorin, alpha [*Homo sapiens*] (***Ci-tectorin***)	B		
28519 34510 20292	2004.3.4.1 2005.3.4.1	ci0100132558	gi|22208523|gb|AAM94335.1| lysyl oxidase-like protein [*Bos taurus*] (***Ci-LyOx***)	A		
34861 20491	2004.210.26.1 2004.210.26.2 2005.210.24.1 2005.210.24.2	ci0100130675	ddi:PIKB pikB, pik2; phosphatidylinositol 3-kinase 2 (pi3-kinase) (ptdins-3-kinase) (pi3k). [EC:2.7.1.137] [SP:P3K2_DICDI] [*Dictyostelium discoideum*]	B		
22493	2004.17.66.1 2005.17.44.1	ci0100131125	gi|21703186|gb|AAM76090.1| Vwa1 protein [*Boltenia villosa*] (***Ci-Vwa1a***)	B	(Davidson and Swalla, 2002)	Brain (Satou et al., 2001)
10737	2004.11.71.1 2005.11.75.1	ci0100134540	No hit found	DII		
39860	2004.232.19.1 2005.232.18.1	ci0100138016	gi|21298585|gb|EAA10730.1| ebiP4359 [*Anopheles gambiae* str. PEST], sp:O00339–MTN2_HUMAN Matrilin-2 precursor	B		
35270	2004.12.8.1 2005.12.7.1	ci0100142643	gi|21703186|gb|AAM76090.1| Vwa1 protein [*Boltenia villosa*] (***Ci-Vwa1d***)	B	(Davidson and Swalla, 2002)	Brain (Satou et al., 2001)
20083	No model found	No model found	No hit found	DII		
23861	2004.397.4.1 2005.397.4.1	No model found	No hit found	DII		
26160	2004.144.18.1 2005.144.15.1	ci0100134472	gi|21703186|gb|AAM76090.1| Vwa1 protein [*Boltenia villosa*]	B	(Davidson and Swalla, 2002)	Brain (Satou et al., 2001)
43709	2004.35.3.1 2005.35.1.1	ci0100130197	gi|1083074|pir||A39808 proteoglycan core protein, cartilage–bovine (fragments) [*Bos taurus*]	A		
5087	No model found	ci0100138545	IPI:IPI00111448.1NP_034626|ENSEMBL: DNA-binding protein inhibitor ID-2 [*Homo sapiens*] (***Ci-Emc***)	C		Nervous system and nerve cord (Imai et al., 2004)
42327	2004.70.51.1 2005.70.44.1	ci0100144324; 136070; 144926	gi|22036090|dbj|BAC06583.1| Tbx6 related protein [*Molgula tectiformis*]	A		
38046 5086 9822 33152	No model found	ci0100138545	IPI:IPI00111448.1| NP_034626|ENSEMBL: DNA-binding protein inhibitor ID-2 [*Homo sapiens*]	C		
25056	2004.158.5.1 2005.158.4.1	ci0100130544	gi|19032247|emb|CAD24309.1| putative coagulation serine protease [*Ciona intestinalis*] (***Ci-Pcsp***)	A	(Davidson and Swalla, 2002)	Entire trunk (Kusakabe et al., 2002)
18180	2004.631.2.1 2005.631.2.1	ci0100132472	gi|16754895|dbj|BAB71805.1| chitin binding protein b04 [*Bos taurus*]	A		
28215	2004.17.66.1 2005.17.44.1	ci0100131125	gi|21703186|gb|AAM76090.1| Vwa1 protein [*Boltenia villosa*] (***Ci-Vwa1e***)	B	(Davidson and Swalla, 2002)	Brain (Satou et al., 2001)
32348	2004.63.1.1 2005.63.1.1	ci0100130316	hsa:8061 FOSL1; FOS-like antigen 1 [SP:FRA1_HUMAN] [*Homo sapiens*]	C		

Table 1 continued on next page

Table 1. *Continued*

Genes upregulated by SP600125 treatment						
Chip feature	KyotoGrail gene model	JGI version 1 gene model	Identity and/or GO function	Class	References*	In situ†
27101 2749	No model found	No model found	No hit found	DII		
19514	No model found	ci0100143016	No hit found	DII		
25903	No model found	ci0100144839	sco:SCO2838 SCE20.12c; putative secreted endoglucanase [*Streptomyces coelicolor* A3(2)] (***Ci-endogl***)	A		
6841	2004.1104.3.1 2005.1104.3.1	ci0100138973	Homologue of gonadoliberin II precursor (gonadotropin releasing hormone II [GnRH-II]. Protein residues 1-21=signal peptide. Residues 22-37=mature peptide (***Ci-GNRH***)	B		
12310	2004.51.66.1 2005.51.82.1	ci0100154161	rno:83517 Fcna; ficolin A [SP:FCN1_RAT] [*Rattus norvegicus*] (***Ci-ficolin***)	B		
39245	2004.168.9.1 2005.168.9.1	ci0100130565	IPI:IPI00166146.1|TREMBL:Q8N1E9 Similar to selectin P [*Homo sapiens*] (***Ci-Sccpb***)	B	(Davidson and Swalla, 2002)	
6829 10084 34200 33941 36706	2004.605.4.1 2005.605.2.1	ci0100142092	gi|117420|sp|P10112|CRBS_CYPCA BETA CRYSTALLIN S (GAMMA CRYSTALLIN S) [*Cyprinus carpio*]	A		
15021	2004.70.51.1 2005.70.44.1	ci0100144324 ci0100136070 ci0100144926	gi|22036090|dbj|BAC06583.1| Tbx6 related protein [*Molgula tectiformis*]; gi|14331129|dbj|BAB60697.1| carboxylesterase RL1 [*Rattus norvegicus*]; gi|20072612|gb|AAH27185.1| Unknown (protein for MGC:28542) [*Mus musculus*]	C A		
17749	2004.150.3.1 2005.150.4.1	ci0100150529	dme:CG17386 CG17386 gene product [*Drosophila melanogaster*]	DI		
21814 29688	2004.41.75.1 2004.41.75.2 2005.41.74.1 2005.41.74.2	ci0100131231	gi|9055334|ref|NP_061214.1| sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6A [*Mus musculus*]	B		
20532	2004.112.43.1 2005.112.33.1	ci0100141941	IPI:IPI00008901.1|:NP_001416| Epithelial membrane protein-3 [*Homo sapiens*]	B		
23665 22383	2004.27.35.1 2005.27.39.1	ci0100134401	hsa:113746 SHIPPO1; sperm tail protein SHIPPO1 [*Homo sapiens*]	DII		
33272 16612	2004.83.15.1 2005.83.17.1	ci0100147675	hsa:50515 C4ST; chondroitin 4-sulfotransferase [*Homo sapiens*] (***Ci-Chslf***),	A		
13038	2004.267.14.1 2005.267.14.1	ci0100152461	No hit found	DII		
6443	2004.132.48.1 2005.132.48.1	ci0100131471	gi|21703186|gb|AAM76090.1| Vwa1 protein [*Boltenia villosa*] (***Ci-Vwa1f***)	B	(Davidson and Swalla, 2002)	Brain (Satou et al., 2001)
30057	2004.89.8.1 2005.89.8.1	ci0100149190	gi|86419|pir||JH0675 restrictin precursor–chicken [*Gallus gallus*]	B		

Genes downregulated by SP600125 treatment						
Chip feature	KyotoGrail gene model	JGI version 1 gene model	Identity	Class	References*	In situ†
24274	2004.147.18.1 2005.147.17.1	ci0100144083	gi|21703186|gb|AAM76090.1| Vwa1 protein [*Boltenia villosa*] (***Ci-Vwa1b***)	B	(Davidson and Swalla, 2002)	Brain (Satou et al., 2001)
37588 19322	2004.261.15.1 2005.261.14.1	ci0100149576 ci0100150380	gi|13629372 |Q9XSC6|KCRM_BOVIN Creatine kinase, M chain [*Bos taurus*]; IPI:IPI00118413.1|NP_035710| Thrombospondin 1 precursor [*Homo sapiens*]	A	(Davidson and Swalla, 2002)	

Table 1 continued on next page

Table 1. *Continued*

Genes downregulated by SP600125 treatment						
Chip feature	KyotoGrail gene model	JGI version 1 gene model	Identity	Class	References*	In situ†
8514 35944	2004.179.6.1 2005.179.6.1	ci0100143863	gi|16156758|dbj|BAB62269.1| HrPost-1 [*Halocynthia roretzi*]	B	(Davidson and Swalla, 2002)	
17552 35388	2004.221.2.1 2005.221.2.1	ci0100142296	gi|113051|sp|P22456|ACH1_XENLA Acetylcholine receptor protein, alpha-1A chain precursor [*Xenopus laevis*]	B		
34251	2004.294.9.1 2005.294.11.1	ci0100134133	No hit found	DII		
25758	2004.75.37.1 2004.75.37.2 2005.75.31.1 2005.75.31.2	ci0100131340	gi|717079|gb|AAA63789.1| cell adhesion molecule [*Canis familiaris*]	B		
16009	2004.45.52.1 2005.45.51.1	ci0100138889 ci0100139478	IPI:IPI00121222.1|TREMBL:Q9JJ96| Brain cDNA, clone MNCb-0671 [*Homo sapiens*] gi|2136497|pir||JC5092 E-selectin–pig [*Sus scrofa domestica*]	B	(Davidson and Swalla, 2002)	
19227 39927	No model found	No model found	No hit found	DII		
34732 11583	2004.140.15.1 2005.140.14.1 2005.140.14.2	ci0100145640	IPI:IPI00009826.2|SWISS- NP_001862| Carboxypeptidase B precursor [*Homo sapiens*]	A	(Nakayama et al., 2001) (Nakayama et al., 2002)	
11115	2004.79.62.1 2005.79.84.1	ci0100133108	No hit found	DII		
38111 6788	2004.51.60.1 2005.51.77.1	ci0100135686	gi|6630974|gb|AAF19631.1|AF194427_1 cysteine proteinase precursor [*Myxine glutinosa*]	B		
14883	2004.82.31.1 2005.82.27.1 2005.82.27.2 2005.82.27.3	ci0100147945	IPI:IPI00112858.1NP_073725| Polydom protein precursor [*Homo sapiens*] (***Ci-polydom***)	B		
23969 3282	2004.65.36.1 2005.65.36.1	No model found	No hit found	DII		
8515	No model found	No model found	Nuclear DNA binding protein	C		
10333 39015 20529 41121	2004.51.20.1 2005.51.23.1	ci0100154804 ci0100140937	IPI:IPI00165235.1|TREMBL:Q9BRA7| Similar to carboxypeptidase Z [*Homo sapiens*] gi|22761698|dbj|BAC11661.1| unnamed protein product [*Homo sapiens*] (***Ci-carboxypeptidase***)	A	(Nakayama et al., 2001) (Nakayama et al., 2002)	
41891	2004.195.11.1 2005.195.10.1	ci0100146733	gi|18251452|gb|AAL66021.1|AF449798_1 organic anion transporting polypeptide Oatp [*Raja erinacea*] (***Ci-OATP***)	A		
13741	No model found	No model found	Ni hit found	DII		
15499	2004.447.2.1 2005.447.2.1	ci0100135925	gi|18143343|dbj|BAB79624.1| Ci-META5 [*Ciona intestinalis*]	DI	(Nakayama et al., 2001) (Nakayama et al., 2002)	
41130	2004.6.94.1 2005.6.94.1	ci0100139289	gi|717079|gb|AAA63789.1| cell adhesion molecule [*Canis familiaris*] (***Ci-sushi***)	B		
30103	No model found	No model found	Ni hit found	DII		
21008	2004.4.32.1 2005.4.31.1	ci0100145184	IPI:IPI00153299.1|REFSEQ_NP:NP_080788|TREMBL:Q8R164| Similar to RIKEN cDNA 2010012D11 gene [*Homo sapiens*]; pir:A56716–A56716 aromatic ester hydrolase	A		

Identification of genes under the control of the JNK pathway. Column one refers to the oligo-based microarray produced in Kyoto. Column two lists gene models predicted by Grailexp with Kyoto ESTs. Column three lists gene models predicted by JGI. Column four lists the best-hit protein. Bold indicates genes observed in this study by RT-PCR and/or in situ. Gene code: classification of genes was determined according to Lee et al. (Lee et al., 1999). Class A, genes associated with functions in many cell types; Class B, genes associated with cell-cell communication; Class C, genes for transcription regulatory proteins; Class DI, sequences that match ESTs or reported proteins with unknown function; Class DII, sequences with no significant sequence similarity to known genes.

***** indicates publications where the same or similar genes were identified during metamorphosis of other ascidian species.

† indicates localization of the expression of the same or similar genes already published in *Ciona* or other ascidians species.

Table 2. *Ciona intestinalis* genes, identified by microarray analysis, that have an altered level of expression at 25 hpf when treated with U0126 MEK inhibitor

Genes upregulated by U0126 treatment

Chip feature	KyotoGrail gene model	JGI version 1 gene model	Identity and/or GO function	Class	References*	In situ†
26102 0421	2004.972.4.1 2005.972.4.1	ci0100137018	IPI:IPI00114285.1| NP_034492|ENSEMBL: Glutathione transferase omega 1	A	(Woods et al., 2004) (Davidson and Swalla, 2002)	
12481 1849	2004.573.7.1 2005.573.7.1	ci010046721	Q21945 Hypothetical 23.3 kDa protein *Caenorhabditis elegans*; KOG1695 Glutathione–S-transferase	DI	(Woods et al., 2004) (Davidson and Swalla, 2002)	
40275	2004.59.32.1 2005.59.33.1	ci0100130315	dme:CG17986 CG17986 gene product; KOG1208 dehydrogenases with different specificities (***Ci-dhg***)	A		
6534 37997	2004.158.11.1 2005.158.9.1	ci0100139721	gi|3511226|gb|AAC79993.1| glutathione S-transferase [*Anopheles gambiae*]	A	(Woods et al., 2004) (Davidson and Swalla, 2002)	
34118	2004.1136.5.1 2005.1136.5.1	ci0100152173	gi|14041150|emb|CAC38761.1| leukotriene B4 [*Geodia cydonium*]	A		
16193	No model found	ci0100153140	hsa:6819 SULT1C1; sulfotransferase family, cytosolic, 1C, member 1	A		
20200	2004.149.8.1 2005.149.9.1	ci0100146267	gi|1008046|dbj|BAA09124.1| cytochrome P-450 [*Xenopus laevis*]	A	(Woods et al., 2004)	
22885	2004.350.2.1 2005.350.2.1	ci0100140514	gi|21299591|gb|EAA11736.1| agCP5786 [*Anopheles gambiae* str. PEST]; KOG1205 Predicted dehydrogenase	A		
10258	2004.280.16.1 005.280.15.1	ci0100145025	gi|13432102|sp|P08243|ASNS_HUMAN asparagine synthetase [glutamine-hydrolyzing] (Glutamine-dependent asparagine synthetase)	A		
36290	2005.2.118.1 2004.2.117.1	ci0100144489	IPI:IPI00556036 Inter-alpha fragment; Trypsin inhibitor heavy chain 3	DII		
35270	2004.12.8.1 2005.12.7.1	ci0100142643	gi|21703186|gb|AAM76090.1| Vwa1 protein [*Boltenia villosa*] (***Ci-Vwa1c***)	B	(Davidson and Swalla, 2002)	
20083	No model found	No model found	No hit found	DII		

Genes downregulated by U0126 treatment

Chip feature	KyotoGrail gene model	JGI version 1 gene model	Identity	Class	References*	In situ†
21484 28505	2004.18.40.1 2004.18.40.2 2005.18.42.1 2005.18.42.2	ci0100145332	NM112885 protein binding/ubiquitin protein ligase/Zn-ion binding [*Arabidopsis thaliana*]	DI		
40650	2004.132.46.1 2005.132.44.1	No model found	Rat nuclear pore complex N153 (Nup153) sp:P49791	A		
10051	2004.43.38.1 2005.43.38.1	ci0100148033	O61785 (O61785) Hypothetical 29.0 kDa protein [*Caenorhabditis elegans*]	DI		
19579	2004.58.13.1 2005.58.13.1	ci0100131282	gi|19032245|emb|CAD24308.1| putative coagulation serine protease [*Ciona intestinalis*]	A	(Davidson and Swalla, 2002)	
20246 30477	2004.305.11.1 2005.305.12.1	ci0100149359	IPI:IPI00001729 Matrix metalloprotease-24-precursor (***Ci-Mx***)	E		
14883	2004.82.31.1 2005.82.27.1 2005.82.27.2 2005.82.27.3	ci0100147945	IPI:IPI00112858.1|NP_073725| Polydom protein precursor [*Homo sapiens*] (***Ci-polydom***)	B		
41165	2004.18.53.1 2005.18.55.1	ci0100146122	gi|4468715|emb|CAB38105.1| beta-microseminoprotein [*Saguinus oedipus*]	DI		
33659 25306 6228	2004.163.38.1 2005.163.33.1	ci0100139661	gi|12381859|dbj|BAB21104.2| SMaf1 [*Danio rerio*].0006355, regulation of transcription, DNA-dependent	C		
23758 2153	2004.272.9.1 2005.272.10.1	ci0100141699	No hit found	DII		
10333 39015 20529 41121	2004.51.20.1 2005.51.23.1	ci0100154804	IPI:IPI00165235.1| Similar to carboxypeptidase Z [*Homo sapiens*] (***Ci-carboxypeptidase***)	A	(Nakayama et al., 2001) (Nakayama et al., 2002)	
29978	2005.96.7.1 2004.96.7.1	ci0100141212	gi|17538868|ref|NP_502249.1|ankyrin repeats [*Caenorhabditis elegans*]	DII		

Table 2 continued on next page

Table 2. ***Continued***

Genes downregulated by U0126 treatment						
Chip feature	KyotoGrail gene model	JGI version 1 gene model	Identity	Class	References*	In situ†
35781	2004.171.6.1 2005.171.6.1	ci0100151338	gi|12644578|sp|Q9I8B0|SUR6_XENLA Surfeit locus protein 6 homologue [*Xenopus laevis*]	DI		
22776 43913	2004.342.4.1 2005.342.5.1	ci0100136887	IPI:IPI00110325.1|NP_075359|TREMBL:Q9EQ31| Lysophosphatidic acid receptor [*Homo sapiens*]	B		
34161	2004.269.19.1 2005.269.28.1	ci0100151642	No hit found	DII		
8523	2004.9.52.1 2005.9.53.1	ci0100149839	gi|7799191|emb|CAB90827.1| tomoregulin-1 [*Mus musculus*] (***Ci-tomoregulin***)	B		
43089 9784 33082	2004.780.6.1 2005.780.10.1	ci0100133150	IPI:IPI00002502.1| NP_001665| Cyclic-AMP-dependent transcription factor ATF-3 [*Homo sapiens*]	C		
41212	2005.492.2.1	ci0100150570	gi|15623822|dbj|BAB67881.1| hypothetical protein~similar to *Arabidopsis thaliana* chromosome 3, T22E16.10 [*Oryza sativa*]	DI		
18277 38399	2004.224.7.1 2005.224.5.1	ci0100152617	gi|3694666|gb|AAC62435.1| ETS-related transcription factor (binds CGGAW) [*Homo sapiens*] (***Ci-ETS***)	C		
34792	No model found	No model found	LEM3-human P selectin precursor (***Ci-Sccpa***)	B	(Davidson and Swalla, 2002)	Papilla (Davidson and Swalla, 2002)
19963	2004.546.7.1 2005.546.7.1	ci0100146623	Q9VQA9 (Q9VQA9) CG15388 protein [*Drosophila melanogaster*]	DI		
8514	2004.179.6.1 2005.179.6.1	ci0100143863	gi|16156758|dbj|BAB62269.1| HrPost-1 [*Halocynthia roretzi*]; 0006886, intracellular protein transport	B	(Davidson and Swalla, 2002)	
3279	2004.1.165.1 2005.1.179.1	ci0100148521	gi|19697349|gb|AAL92989.1| hypothetical protein [*Dictyostelium discoideum*]	DI		
10655 23352 10654 25668 42958 10653	2004.148.20.1 2005.148.22.1	ci0100143927	gi|1110451|dbj|BAA08728.1| HrHox-1 [*Halocynthia roretzi*]	C		
24373	2004.86.21.1 2005.86.21.1	ci0100139734	No hit found	DII		
34078	2004.129.22.1 2005.129.20.1	ci0100132083	gi|10441571|gb|AAG17109.1| P2.6 melatonin receptor Mel-1B [*Esox lucius*]; 0007186, G-protein coupled receptor protein signalling pathway	B		
33654	2004.145.2.1 2005.145.2.1	ci0100133750	gi|18549022|ref|XP_059205.2| similar to RIKEN cDNA 2810004N23 [*Homo sapiens*]	DI		
43630 31284	2004.88.19.1 2005.88.22.1	ci0100143297	gi|14994043|gb|AAK76396.1| Rev-Erb beta 2 [*Danio rerio*]; 0003707, steroid hormone receptor activity (***Ci-Rev-erb***)	B		Palps (Kusakabet et al., 2002)
22053	2004.27.26.1 2004.27.26.2 2005.27.28.1 2005.27.28.2	ci0100142399	IPI:IPI00128222.1|ENSEMBL:ENSMUSP00000037887 [*Homo sapiens*]	DI		
19227	2005.1063.1.1 2004.1063.1.1	ci0100130992	Gi|20890036|ref|XP8129286.1| Similar to RRP5 protein homologue [*Mus musculus*]	DII		
16998	2004.62.15.1 2005.62.15.1	ci0100148566	IPI:IPI00132628.1|ENSEMBL:ENSMUSP00000046720) [*Homo sapiens*]	DI		
39245	2004.168.9.1 2005.168.9.1	ci0100130565	IPI:IPI00166146.1|TREMBL:Q8N1E9| Similar to selectin P [*Homo sapiens*] (***Ci-Sccpb***)	B	(Davidson and Swalla, 2002)	Papilla (Davidson and Swalla, 2002)
21530	2004.14.52.1 2005.14.53.1	ci0100148511	IPI:IPI00029521.4 NTKL-Binding protein I (***Ci-NTKL***)	DI		
37229	2004.102.45.1 2005.102.59.1	ci0100134538	Q9VHK5 (Q9VHK5) CG8379 protein (LD21733p) [*Drosophila melanogaster*]	DI		

Table 2 continued on next page

Table 2. *Continued*

Genes downregulated by U0126 treatment						
Chip feature	KyotoGrail gene model	JGI version 1 gene model	Identity	Class	References*	In situ †
12589 28225	2004.22.90.1 2005.22.85.1	ci0100143826	hsa:79987 POLYDOM; probable orthologue of mouse polydom [*Homo sapiens*]	B		
2772	2004.409.5.1 2005.409.5.1	ci0100130842	mmu:73690 2410114O14Rik; RIKEN cDNA 2410114O14 gene [*Mus musculus*]; 0005576, extracellular; Defense-related protein containing SCP domain.	B	(Davidson and Swalla, 2002)	
31955	2004.1460.1.1 2005.1460.1.1	ci0100141080	hsa:10352 WARS2; tryptophanyl tRNA synthetase 2 (mitochondrial) [*Homo sapiens*]	A	(Woods et al., 2004)	
34464 6947	2004.229.19.1 2005.229.21.1	ci0100133625	IPI:IPI00117008.1|ENSEMBL:ENSMUSP00000 028486 [*Homo sapiens*]	DI		
43522	No model found	ci0100132461	IPI:IPI00016046.1|REFSEQ_NP:NP_542786Pr otein c20orf52 [*Homo sapiens*]	DI		
2255	2004.1067.1.1 2005.1067.2.1	ci0100150638	No hit found	DII		
5797	2005.31.74.1	ci0100134193	IPI:IPI00028491.3| XP_005298|1-acyl-sn-glycerol-3-phosphate acyltransferase epsilon [*Homo sapiens*]	A		
7913	2004.49.39.1 2005.49.45.1	ci0100140291	gi|11527806|dbj|BAB18672.1| fucosyltransferase [*Pongo pygmaeus*]	A		

Identification of genes under the control of the ERK pathway. See legend to Table 1.

including *Ci-GNRH*, *Ci-endoglucanase (Ci-endogl)*, *NTKL* (*Ci-NTKL*), *Ci-ets*, *Ci-Vwa1a*, *Ci-Vwa1ac*, *Ci-metalloprotease* (*Ci-Mx*) and *Ci-oatp* (Tables 1, 2). An overview of the results for each MAPK pathway is displayed in Fig. 4.

Silencing of the JNK-controlled gene *sushi* inhibits apoptosis in tail cells

We previously demonstrated that JNK activation is required for apoptosis induction in tail cells and for tail regression during metamorphosis. In order to identify candidate genes involved in the initiation of the wave of apoptosis, we next identified, by cDNA microarray analysis, genes that are controlled by the JNK pathway. To address this issue, we examined the effect of the functional suppression of JNK-controlled genes on apoptosis induction at the onset of metamorphosis. Among the many genes identified, *Ci-Sccpb* and *Ci-sushi* (Table 1) showed, respectively, expression at the tip of the tail and in tail epithelia (Fig. 5A). Out of these two genes, we choose *Ci-sushi* (Table 1) for gene silencing for two reasons. First, based on its EST count (Satou et al., 2002) and RT-PCR (Fig. 5B), *Ci-sushi* is expressed between 24 and 26 hpf only, just before the initiation of apoptosis and the onset of metamorphosis; therefore, the *Ci-sushi* knockdown avoids possible lethal phenotypes due to *Ci-sushi* having a role in earlier steps of *Ciona* development. Second, we confirmed the microarray data by RT-PCR and found that *Ci-sushi* expression is downregulated by Ci-JNK inhibitor (Fig. 5B and Table 1). By contrast, *Ci-Sccpb* expression is upregulated by SP600125 (Table 1). These data thus suggested that *Ci-sushi* would be an ideal candidate for a functional suppression experiment.

When a morpholino against *Ci-sushi* was injected into chorionated fertilized eggs, all larvae examined (18/18) were unable to initiate apoptosis in tail cells at the onset of metamorphosis (Fig. 5C). At this stage, most (22/25) of the control larva exhibited TUNEL-positive cells at the tip of the tail, and began tail regression (Fig. 5C). Moreover, as a control for synchronization and methodology, the tunic cells in both morpholino-injected and control larvae were apoptotic at this time (Chambon et al., 2002) (Fig. 5C). These data demonstrate that *Ci-sushi* is necessary to induce apoptosis in cells that compose the tail of *C. intestinalis* larvae at the onset of metamorphosis.

DISCUSSION

The MAPK signalling cascade plays a central role in mediating apoptosis during embryogenesis in invertebrates such as *Drosophila* (Kuranaga et al., 2002; Moreno et al., 2002) and *Caenorhabditis elegans* (Gumienny et al., 1999), and, in the mouse, double knockout of the two MAPK family members JNK1 (also known as MAPK8 – Mouse Genome Informatics) and JNK2 (also known as MAPK9 – Mouse Genome Informatics) leads to embryonic lethality associated with severe dysregulation in the control of cell death in the hindbrain and forebrain (Kuan et al., 1999; Sabapathy et al., 1999). There are also indications that the MAPK signalling cascade mediated via the ERK family plays a role in the onset of apoptosis during embryogenesis (Kling et al., 2002; Yao et al., 2003). Although the role of JNK (and to a lesser extent ERK) is well established in many experimental systems, it is less clear what the gene targets of JNK (or ERK) are that trigger the onset of apoptosis. We addressed this issue by initially measuring the dynamics of the gene-expression network during the onset of metamorphosis in *Ciona*. By exploiting the observation that inhibition of the MAPK signalling cascade specifically blocks the onset of apoptosis (Chambon et al., 2002) (this study), we used these pharmacological tools together with a microarray-based approach to identify the subset of genes, controlled by either Ci-ERK or Ci-JNK, that are involved in triggering the onset of apoptosis. This strategy revealed 50 potential target genes that were upregulated and 63 that were downregulated when the MAPK signalling cascades, and therefore apoptosis, were inhibited.

Apoptosis in the ascidian tadpole larvae

At the end of the period of swimming, ascidian tadpole larvae undergo metamorphosis, which usually begins with settlement through adhesive papillae and subsequent tail regression and loss of adhesive papillae (Cloney, 1978; Cloney, 1982). A larva capable of

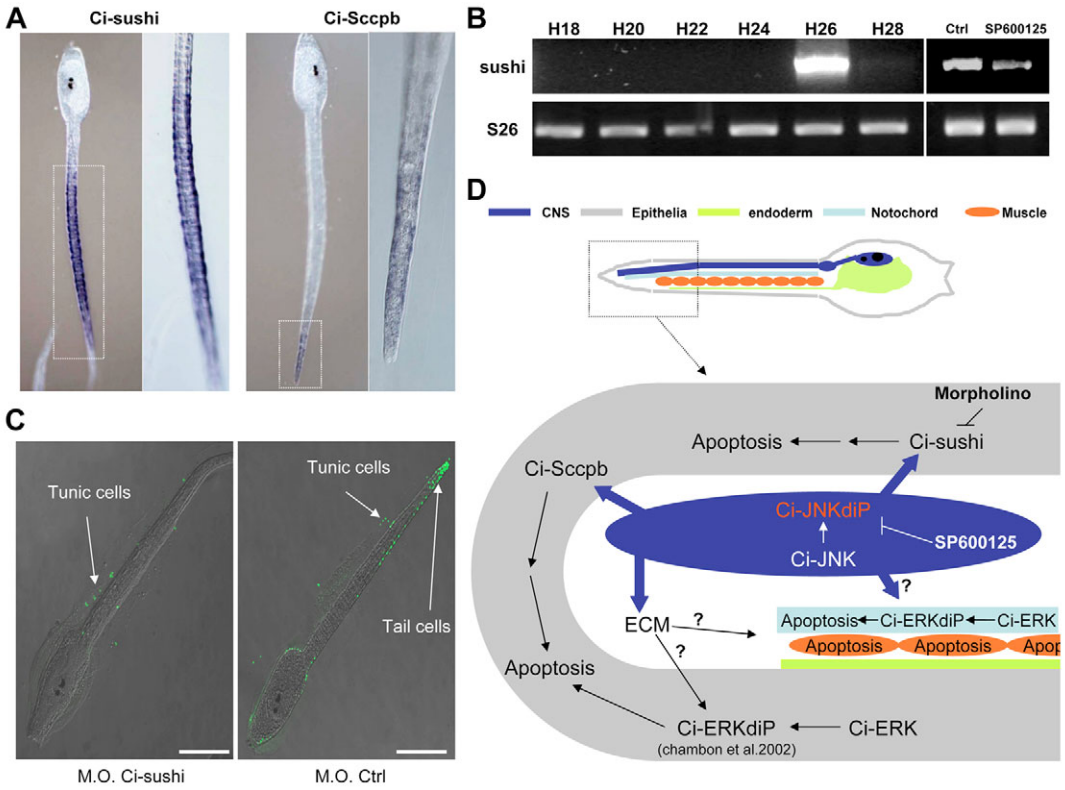

Fig. 5. Sushi antisense morpholino blocks apoptosis-dependent tail regression during metamorphosis. (**A**) Whole-mount in situ hybridization of Ci-sushi and Ci-Sccpb displaying, respectively, specific expression in tail and at the tip of the tail of *Ciona intestinalis* larvae. The white square corresponds to the region of higher magnification displayed in the right panel. (**B**) JNK activation controls *Ci-sushi* expression. *Ci-sushi* mRNA expression from hatching to metamorphosis. mRNA was extracted from embryos at various time points, reverse transcribed and semi-quantitative specific PCR was performed (see Materials and methods). *Ci-sushi* mRNA expression at 25 hpf was extracted from untreated embryos or from those treated with 10 μM SP600125, reverse transcribed and semi-quantitative specific PCR was performed (see Materials and methods). (**C**) Detection of apoptosis in the tail of *C. intestinalis* tadpoles at the onset of metamorphosis (28 hpf). Apoptotic cells were TUNEL labelled (TUNEL-positive nuclei appear green). Notice that numerous nuclei of cells of the tail extremity were TUNEL-positive in the control panel. By contrast, TUNEL-positive nuclei were detected very rarely in *Ci-sushi*-morpholino antisense-injected larva. At this time, tunic cells are TUNEL-positive in both cases (arrows), as described in our previous work (Chambon et al., 2002). (**D**) Model of the role played by the CNS in the regulation of apoptosis during metamorphosis. Ci-JNK activation in the CNS leads to *Ci-sushi* and *Ci-Sccpb* gene expression in epithelia. These genes are essential for initiating apoptosis at the onset of metamorphosis. ECM modification is also a result of Ci-JNK activation in the CNS, which could promote the induction of apoptosis through Ci-ERK activation in adjacent tissues. Scale bars: 200 μm.

undergoing these metamorphic changes successfully is termed competent, and the acquisition of metamorphic competence during the larval period has been shown to occur in response to a wide variety of external and endogenous signals (Jackson and Strathmann, 1981; Cloney, 1982; Davidson and Swalla, 2002; Jackson et al., 2002). The Ci-JNK cascade is activated at the time of competence in the tadpole CNS; more specifically, in the pharyngeal rudiment, anterior sensory vesicle, neck, internal neurons of the posterior sensory vesicle, visceral ganglion and nerve corde (Tarallo and Sordino, 2004; Chambon et al., 2002) (this study). More importantly, inhibition of the JNK pathway completely blocked metamorphosis. Similarly, Ci-ERK activation correlates with the time of metamorphic competence. Ci-ERK is activated in: tail muscle, the cytoplasm of proximal palp cells, the stomodeum, the anterior and posterior sensory vesicle, the epidermis overlying the sensory vesicle, the neck region, atrial primordia, the notochord and in the epidermis of the tail (Tarallo and Sordino, 2004; Chambon et al., 2002) (this study). Similarly, blocking activation of Ci-ERK inhibited metamorphosis in a significant percentage of the tadpole larvae (Chambon et al., 2002) (this study).

Genes modulated by JNK and/or ERK

Among the genes identified, we will discuss some that we have separated for convenience as either upregulated or downregulated by JNK or ERK activity into four categories. First, there are genes involved in innate immunity. Second, there are genes involved in hormone signalling. Third, there are genes involved in metabolism of the extracellular matrix (ECM) or coding components of the ECM. And fourth, we grouped the remaining genes into one category that we term diverse genes. Before discussing the four categories, it was also gratifying to notice that our screen identified genes that had previously been shown to be specifically expressed during ascidian metamorphosis (Woods et al., 2004; Davidson and Swalla, 2002; Nakayama et al., 2002), such as *Ci-meta5*, which is downregulated in response to JNK-inhibitor treatment. *Ci-meta5* was previously identified by differential screening of a cDNA library of swimming larvae and metamorphosing juveniles (Nakayama et al., 2002). We also identified three genes (*glutathione S-transferase*, *Cytochrome p450* and *Gluthathione-requiring prostaglandin D synthase*) that are under the control of Ci-ERK and are orthologues of or closely related to genes expressed in papillae of the ascidians

Herdmania curvata that have been implicated in metamorphosis and are downstream genes of the EGF-like Hemps pathway (Woods et al., 2004; Arnold et al., 1997). Although it is not known whether Hemps activates ERK, it is tempting to speculate that it does, because activation of the Ras/Raf/ERK pathway by EGF is well described in many species (Hornstein et al., 2003). Moreover, our identification of genes controlled by Ci-ERK in papillae, the observation that metamorphosis does not occur with MEK inhibition and data on Hemps in *H. curvata* that shows that it induces settlement and metamorphosis (Eri et al., 1999) are consistent with our observations that one of the effects of the Hemps pathway is to activate the ERK cascade in papillae cells.

Innate immunity

In our screen, 20 genes known to be involved in innate immunity were identified. It has been suggested that the activation of innate immunity genes during metamorphosis may represent the maturation of the adult immune system, and may be necessary for phagocytosis and for the re-structuring of larval tissues (Davidson and Swalla, 2002). Among the genes controlled by activation of Ci-ERK in papillae, *Ci-Vwa1c* and *Ci-polydom* could coordinate papillae resorption during metamorphosis. In the same way, *Ci-Pgly*, *Ci-ficolin* and the five genes similar to *Vwa1* (*Ci-Vwa1a*, *Ci-Vwa1b*, *Ci-Vwa1d*, *Ci-Vwa1e* and *Ci-Vwa1f*) that are controlled by phosphorylation of Ci-JNK in the CNS, could lead to phagocytosis of the visceral ganglion and sensory organs, which has been observed during metamorphosis of many ascidian species (Cloney, 1978). It is also of interest that the modulation of expression of genes such as *Ci-Sccp* could also enhance cell-cell communication before the extended period of cell reorganization and the co-ordinated massive wave of apoptosis that occurs during tail regression.

Hormone signalling

Among the genes identified that are controlled by the JNK pathway, two are interesting: *Ci-GNRH* and *Ci-oatp*, which are involved, respectively, in the reproductive and thyroid axes. The mouse *Oatp14* (also known as *Slco1c1*) was described in the transport of thyroxine across the blood-brain barrier (Tohyama et al., 2004). It is interesting to notice that the role of thyroid hormones in metamorphosis had been reported previously in ascidians (Patricolo et al., 1981; Patricolo et al., 2001), and also in amphibians (Dodd and Dodd, 1976; Nakajima et al., 2005) and lamprey (Youson and Sower, 2001). Moreover, in four ascidian species, thyroxin is present in larval mesenchyme and seems to be involved in the control of metamorphosis (D'Agati and Cammarata, 2005). The expression of *Ci-oatp* via JNK activation in the CNS may enhance thyroid signalling in larvae. Concerning GNRH, no report describes any function of this hormone in invertebrate metamorphosis. However, it is possible that GNRH may have a role in lamprey metamorphosis because, in sea lamprey, the level of GNRH increases throughout the stage of spontaneous metamorphosis (Youson and Sower, 2001; Youson et al., 2006).

Cell-cell communication

In addition to identifying genes involved in the immune system and hormonal signalling, we also identified a number of genes coding for proteins involved in the composition or processing of the ECM. For example, we identified *Ci-LyOx* (Table 1, Fig. 4B), which is responsible for the cross linking and deposition of collagen fibres, elastin fibres and *Ci-Mx*, a matrix metalloprotease (Table 2, Fig. 4A). The regulation of matrix metalloprotease and the ECM remodelling have been shown to affect apoptosis in different systems, including the apoptotic remodelling of the intestine during *Xenopus laevis* metamorphosis and post-lactation involution of the mouse mammary gland (Nakajima et al., 2005; Fata et al., 2004). Anoikis is apoptosis induced by the loss of, or inappropriate, cell adhesion. It is tempting to hypothesize that one of the inductive signals from Ci-JNK in the CNS controls apoptosis by changing ECM composition. The role of JNK in ECM degradation has already been reported in rat aortic walls (Yoshimura et al., 2005). In the tail of the tadpole, nerve corde is surrounded by matrix, which leads us to speculate that remodelling the ECM could provide a means to coordinate the response of tail cells in promoting either cell death or survival. In support of such a scenario, it was reported that, after a modification in ECM components, activation of the MAPK ERK leads to anoikis-type death (Zugasti et al., 2001). Because Ci-ERK activation precedes apoptosis in tail cells (Chambon et al., 2002), this cell death could be regulated by JNK-controlled anoikis in the tail of ascidian tadpoles.

Diverse genes

In addition, we also observed that 45.5% of the identified genes controlled by the ERK pathway had no significant identity or similarity with any sequence in the GenBank Database, or matched with hypothetical proteins from different organisms. This observation might be explained by the highly specialized adhesive organs (papillae) in which these genes are expressed. For example, there are eight types of adhesive papillae among ascidians species, which could explain these species-specific components (Cloney, 1978).

JNK activation and *Ci-sushi* expression are both required for apoptosis induction

We identified *Ci-sushi* as a target gene of the JNK pathway through the microarray-based approach. *Ci-Sushi* expression decreased following inhibition of the JNK pathway (SP600125 treatment). Moreover, under physiological conditions, *Ci-sushi* was only expressed in tail epithelia at 26 hpf, just before the onset of metamorphosis and of apoptosis at the tail extremity. *Ci-Sushi* encodes a protein containing domains known as complement control protein (CCP) modules, or short consensus repeats (SCR), which exist in a wide variety of complement and adhesion proteins. The abolition of apoptosis initiation by silencing of *Ci-sushi* with an antisense morpholino oligonucleotide demonstrates that JNK-induced activation of *Ci-sushi* expression in the tail is required to trigger the onset of apoptosis in tail tissues. This result strengthens the hypothesis that cross-talk exists between the different tail tissues and the CNS before the onset of metamorphosis.

Ascidians as a model for apoptosis

Here, we observed that activation of ERK in the papillae and JNK in the CNS are able to control, either directly or indirectly, apoptosis of different tissues composing the tail. Because the papillae are innervated in *C. intestinalis* (Manni et al., 2004), it is possible that the coordination of settlement with tail regression is controlled by the CNS. The potential role of the CNS in metamorphosis was previously raised in a review and analysis of ascidian metamorphosis in 1978 by R. A. Cloney. He proposed a preponderant role for the larval nervous system and sensory organs in selecting sites for settlement and in the onset of metamorphosis (Cloney, 1978; Cloney, 1982). Moreover, he hypothesized that the nervous system, and conduction and diffusion of one or more humoral factors are likely to be involved in metamorphosis (Satoh, 1994). Taken together with our previous results on apoptosis-dependent tail regression (Chambon et al., 2002), and with the activation of JNK in the CNS that we report here, these results raise two interesting questions: (1)

How can JNK signalling in the CNS control apoptosis of the different tissues composing the tail during its regression? and (2) What is the nature of the JNK inductive signal that leads to apoptosis?

We propose a model (Fig. 5D) whereby the CNS enhances cell-cell communication in adjacent tissues through the expression of genes, such as *Ci-sushi* or *Ci-Sccpb*, that are essential for apoptosis-dependent tail regression. In addition, the CNS may also modify ECM composition trough Ci-JNK activation, thus leading to the induction of apoptosis in adjacent tissues that are receptive (the CNS and the endoderm escape apoptosis) (Chambon et al., 2002). The model we propose places the CNS centrally in the coordination of the wave of apoptosis that precedes tail regression during *Ciona* metamorphosis

Investigation of these questions in *C. intestinalis* is particularly interesting because this organism contains the genetic rudiments of many vertebrate characteristics and the larvae represent the basic chordate body plan (Satoh, 1994; Satoh, 2003). It is interesting to notice that, during development in humans, cell death has been shown to be an important morphogenetic mechanism for the formation of the vertebral column. For example, some of the notochord exhibits cell death while the remaining cells contribute to the formation of the nucleus pulposus in the human inter-vertebral disc (Saraga-Babic et al., 1994). An excess of cell death in these structures leads to neural tube defects or tailless mutants in mouse (Alles and Sulik, 1990; Grüneberg, 1963). *Ciona* may therefore provide a pertinent model to study in vivo regulation of apoptosis in these different tissues.

By contrast, apoptosis in mammalian skeletal muscle is a rather rare event. It has been reported as a mechanism for removal of undesired myotubes (mononucleated cells) during development (Sandri and Carraro, 1999). Additionally, apoptotic death of single nuclei in otherwise normal muscle fibre has been shown with an incidence of 0.1% (Sandri et al., 1998) to 0.3% (Migheli et al., 1997) TUNEL-positive nuclei. Muscle satellite cells are believed to form a stable, self-renewing pool of stem cells in adult muscle, where they function in tissue growth and repair. A regulatory disruption of growth, differentiation and apoptosis of these cells is assumed to result in tumour formation (Koleva et al., 2005).

The future use of microarray technology coupled with a gene-silencing strategy should permit a better understanding of apoptosis regulation in the tail of *Ciona* larvae during metamorphosis and could create avenues of investigation that lead towards a better understanding of cell death regulation during development in mammals.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/6/1203/DC1

References

Alles, A. J. and Sulik, K. K. (1990). Retinoic acid-induced spina bifida, evidence for a pathological mechanism. *Development* **108**, 73-81.

Arnold, J. M., Eri, R., Degnan, B. M. and Lavin, M. F. (1997). Novel gene containing multiple epidermal growth factor-like motifs transiently expressed in the papillae of the ascidian tadpole larvae. *Dev. Dyn.* **210**, 264-273.

Ashkenazi, A. and Dixit, V. M. (1998). Death receptors: signaling and modulation. *Science* **281**, 1305-1308.

Azumi, K., Takahashi, H., Miki, Y., Fujie, M., Usami, T., Ishikawa, H., Kitayama, A., Satou, Y., Ueno, N. and Satoh, N. (2003). Construction of a cDNA microarray derived from the ascidian Ciona intestinalis. *Zool. Sci.* **20**, 1223-1229.

Butler, M. J., Jacobsen, T. L., Cain, D. M., Jarman, M. G., Hubank, M., Whittle, J. R., Phillips, R. and Simcox, A. (2003). Discovery of genes with highly restricted expression patterns in the Drosophila wing disc using DNA oligonucleotide microarrays. *Development* **130**, 659-670.

Chambon, J. P., Soule, J., Pomies, P., Fort, P., Sahuquet, A., Alexandre, D., Mangeat, P. H. and Baghdiguian, S. (2002). Tail regression in Ciona intestinalis (Prochordate) involves a Caspase-dependent apoptosis event associated with ERK activation. *Development* **129**, 3105-3114.

Chang, L. and Karin, M. (2001). Mammalian MAP kinase signalling cascades. *Nature* **410**, 37-40.

Chen, Y. F., Shin, S. J. and Lin, S. R. (2005). Ets1 was significantly activated by ERK1/2 in mutant K-ras stably transfected human adrenocortical cells. *DNA Cell Biol.* **24**, 126-132.

Cikala, M., Wilm, B., Hobmayer, E., Bottger, A. and David, C. N. (1999). Identification of caspases and apoptosis in the simple metazoan Hydra. *Curr. Biol.* **9**, 959-962.

Cloney, R. A. (1978). Ascidian metamorphosis: review and analysis. In *Settlement and Metamorphosis of Marine Invertebrate Larvae* (ed. F.-S. Chia and M. E. Rice), pp. 255-282. Amesterdam: Elsevier.

Cloney, R. A. (1982). Ascidian larvae and the events of metamorphosis. *Am. Zool.* **22**, 817-826.

D'Agati, P. and Cammarata, M. (2005). Comparative analysis of thyroxine distribution in ascidian larvae. *Cell Tissue Res.* **323**, 529-535.

Davidson, B. and Swalla, B. J. (2002). A molecular analysis of ascidian metamorphosis reveals activation of an innate immune response. *Development* **129**, 4739-4751.

Davis, R. J. (2000). Signal transduction by the JNK group of MAP kinases. *Cell* **103**, 239-252.

Dehal, P., Satou, Y., Campbell, R. K., Chapman, J., Degnan, B., De Tomaso, A., Davidson, B., Di Gregorio, A., Gelpke, M., Goodstein, D. M. et al. (2002). The draft genome of Ciona intestinalis: insights into chordate and vertebrate origins. *Science* **298**, 2157-2167.

Deng, Y., Ren, X., Yang, L., Lin, Y. and Wu, X. (2003). A JNK-dependent pathway is required for TNFalpha-induced apoptosis. *Cell* **115**, 61-70.

Dodd, M. H. I. and Dodd, J. M. (1976). The biology of metamorphosis. In *Physiology of the Amphibia* (ed. B. Lofts), pp. 467-599. New York: Academic Press.

Eisen, M. B., Spellman, P. T., Brown, P. O. and Botstein, D. (1998). Cluster analysis and display of genome-wide expression patterns. *Proc. Natl. Acad. Sci. USA* **95**, 14863-14868.

Ellis, R. E., Jacobson, D. M. and Horvitz, H. R. (1991). Genes required for the engulfment of cell corpses during programmed cell death in Caenorhabditis elegans. *Genetics* **129**, 79-94.

Eri, R., Arnold, J. M., Hinman, V. F., Green, K. M., Jones, M. K., Degnan, B. M. and Lavin, M. F. (1999). Hemps, a novel EGF-like protein, plays a central role in ascidian metamorphosis. *Development* **126**, 5809-5818.

Fata, J. E., Werb, Z. and Bissell, M. J. (2004). Regulation of mammary gland branching morphogenesis by the extracellular matrix and its remodeling enzymes. *Breast Cancer Res.* **6**, 1-11.

Gomez, A. R., Lopez-Varea, A., Molnar, C., de la Calle-Mustienes, E., Ruiz-Gomez, M., Gomez-Skarmeta, J. L. and de Celis, J. F. (2005). Conserved cross-interactions in Drosophila and Xenopus between Ras/MAPK signaling and the dual-specificity phosphatase MKP3. *Dev. Dyn.* **232**, 695-708.

Grüneberg, H. (1963). *The Pathology of Development. A Study of Inherited Skeletal Disorders in Animal*. Oxford: Blackwell.

Gumienny, T. L., Lambie, E., Hartwieg, E., Horvitz, H. R. and Hengartner, M. O. (1999). Genetic control of programmed cell death in the Caenorhabditis elegans hermaphrodite germline. *Development* **126**, 1001-1022.

Hornstein, I., Mortin, M. A. and Katzav, S. (2003). DroVav, the Drosophila melanogaster homologue of the mammalian Vav proteins, serves as a signal transducer protein in the Rac and DER pathways. *Oncogene* **22**, 6774-6784.

Hotta, K., Takahashi, H., Ueno, N. and Gojobori, T. (2003). A genome-wide survey of the genes for planar polarity signaling or convergent extension-related genes in Ciona intestinalis and phylogenetic comparisons of evolutionary conserved signaling components. *Gene* **317**, 165-185.

Imai, K. S., Hino, K., Yagi, K., Satoh, N. and Satou, Y. (2004). Gene expression profiles of transcription factors and signaling molecules in the ascidian embryo: towards a comprehensive understanding of gene networks. *Development* **131**, 4047-4058.

Ishibashi, T., Usami, T., Fujie, M., Azumi, K., Satoh, N. and Fujiwara, S. (2005). Oligonucleotide-based microarray analysis of retinoic acid target genes in the protochordate, Ciona intestinalis. *Dev. Dyn.* **233**, 1571-1578.

Jackson, D., Leys, S. P., Hinman, V. F., Woods, R., Lavin, M. F. and Degnan, B. M. (2002). Ecological regulation of development: induction of marine invertebrate metamorphosis. *Int. J. Dev. Biol.* **46**, 679-686.

Jackson, G. A. and Strathmann, R. R. (1981). Larval mortality from offshore

mixing as a link beween precompetent and competent period of development. *Am. Nat.* **118**, 16-25.
Jacobson, M. D., Weil, M. and Raff, M. C. (1997). Programmed cell death in animal development. *Cell* **88**, 347-354.
Jeffery, W. R. (2002). Programmed cell death in the ascidian embryo: modulation by FoxA5 and Manx and roles in the evolution of larval development. *Mech. Dev.* **118**, 111-124.
Johnson, G. L. and Lapadat, R. (2002). Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* **298**, 1911-1912.
Kanehisa, M. (2002). The KEGG database. *Novartis Found. Symp.* **247**, 91-101; discussion 101-103, 119-128, 244-252.
Kanehisa, M. and Goto, S. (2000). KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Res.* **28**, 27-30.
Kasza, A., O'Donnell, A., Gascoigne, K., Zeef, L. A., Hayes, A. and Sharrocks, A. D. (2005). The ETS domain transcription factor Elk-1 regulates the expression of its partner protein, SRF. *J. Biol. Chem.* **280**, 1149-1155.
Kling, D. E., Lorenzo, H. K., Trbovich, A. M., Kinane, T. B., Donahoe, P. K. and Schnitzer, J. J. (2002). MEK-1/2 inhibition reduces branching morphogenesis and causes mesenchymal cell apoptosis in fetal rat lungs. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **282**, L370-L378.
Koleva, M., Kappler, R., Vogler, M., Herwig, A., Fulda, S. and Hahn, H. (2005). Pleiotropic effects of sonic hedgehog on muscle satellite cells. *Cell Mol. Life Sci.* **62**, 1863-1870.
Kuan, C. Y., Yang, D. D., Samanta Roy, D. R., Davis, R. J., Rakic, P. and Flavell, R. A. (1999). The Jnk1 and Jnk2 protein kinases are required for regional specific apoptosis during early brain development. *Neuron* **22**, 667-676.
Kuranaga, E., Kanuka, H., Igaki, T., Sawamoto, K., Ichijo, H., Okano, H. and Miura, M. (2002). Reaper-mediated inhibition of DIAP1-induced DTRAF1 degradation results in activation of JNK in Drosophila. *Nat. Cell Biol.* **4**, 705-710.
Kusakabe, T., Yoshida, R., Kawakami, I., Kusakabe, R., Mochizuki, Y., Yamada, L., Shin-i, T., Kohara, Y., Satoh, N., Tsuda, M. et al. (2002). Gene expression profiles in tadpole larvae of Ciona intestinalis. *Dev. Biol.* **242**, 188-203.
Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.
Lee, Y. H., Huang, G. M., Cameron, R. A., Graham, G., Davidson, E. H., Hood, L. and Britten, R. J. (1999). EST analysis of gene expression in early cleavage-stage sea urchin embryos. *Development* **126**, 3857-3867.
Lin, A. and Dibling, B. (2002). The true face of JNK activation in apoptosis. *Aging Cell* **1**, 112-116.
Liu, J. and Lin, A. (2005). Role of JNK activation in apoptosis: a double-edged sword. *Cell Res.* **15**, 36-42.
Manni, L., Lane, N. J., Joly, J. S., Gasparini, F., Tiozzo, S., Caicci, F., Zaniolo, G. and Burighel, P. (2004). Neurogenic and non-neurogenic placodes in ascidians. *J. Exp. Zool. B Mol. Dev. Evol.* **302**, 483-504.
Meier, P., Finch, A. and Evan, G. (2000). Apoptosis in development. *Nature* **407**, 796-801.
Migheli, A., Mongini, T., Doriguzzi, C., Chiado-Piat, L., Piva, R., Ugo, I. and Palmucci, L. (1997). Muscle apoptosis in humans occurs in normal and denervated muscle, but not in myotonic dystrophy, dystrophinopathies or inflammatory disease. *Neurogenetics* **1**, 81-87.
Minet, E., Michel, G., Mottet, D., Piret, J. P., Barbieux, A., Raes, M. and Michiels, C. (2001). c-JUN gene induction and AP-1 activity is regulated by a JNK-dependent pathway in hypoxic HepG2 cells. *Exp. Cell Res.* **265**, 114-124.
Moreno, E., Yan, M. and Basler, K. (2002). Evolution of TNF signaling mechanisms: JNK-dependent apoptosis triggered by Eiger, the Drosophila homolog of the TNF superfamily. *Curr. Biol.* **12**, 1263-1268.
Munoz-Sanjuan, I., Bell, E., Altmann, C. R., Vonica, A. and Brivanlou, A. H. (2002). Gene profiling during neural induction in Xenopus laevis: regulation of BMP signaling by post-transcriptional mechanisms and TAB3, a novel TAK1-binding protein. *Development* **129**, 5529-5540.
Nakajima, K., Fujimoto, K. and Yaoita, Y. (2005). Programmed cell death during amphibian metamorphosis. *Semin. Cell Dev. Biol.* **16**, 271-280.
Nakayama, A., Satou, Y. and Satoh, N. (2001). Isolation and characterization of genes that are expressed during Ciona intestinalis metamorphosis. *Dev. Genes Evol.* **211**, 184-189.
Nakayama, A., Satou, Y. and Satoh, N. (2002). Further characterization of genes expressed during Ciona intestinalis metamorphosis. *Differentiation* **70**, 429-437.
Patricolo, E., Ortolani, G. and Cascio, A. (1981). The effect of l-thyroxine on the metamorphosis of Ascidia malaca. *Cell Tissue Res.* **214**, 289-301.
Patricolo, E., Cammarata, M. and D'Agati, P. (2001). Presence of thyroid hormones in ascidian larvae and their involvement in metamorphosis. *J. Exp. Zool.* **290**, 426-430.
Sabapathy, K., Jochum, W., Hochedlinger, K., Chang, L., Karin, M. and Wagner, E. F. (1999). Defective neural tube morphogenesis and altered apoptosis in the absence of both JNK1 and JNK2. *Mech. Dev.* **89**, 115-124.
Sandri, M. and Carraro, U. (1999). Apoptosis of skeletal muscles during development and disease. *Int. J. Biochem. Cell Biol.* **31**, 1373-1390.
Sandri, M., Minetti, C., Pedemonte, M. and Carraro, U. (1998). Apoptotic myonuclei in human Duchenne muscular dystrophy. *Lab. Invest.* **78**, 1005-1016.
Saraga-Babic, M., Lehtonen, E., Svajger, A. and Wartiovaara, J. (1994). Morphological and immunohistochemical characteristics of axial structures in the transitory human tail. *Ann. Anat.* **176**, 277-286.
Sarkar, D., Su, Z. Z., Lebedeva, I. V., Sauane, M., Gopalkrishnan, R. V., Valerie, K., Dent, P. and Fisher, P. B. (2002). mda-7 (IL-24) Mediates selective apoptosis in human melanoma cells by inducing the coordinated overexpression of the GADD family of genes by means of p38 MAPK. *Proc. Natl. Acad. Sci. USA* **99**, 10054-10059.
Satoh, N. (1994). *Developmental Biology of Ascidians*. New York: Cambridge University Press.
Satoh, N. (2003). The ascidian tadpole larva: comparative molecular development and genomics. *Nat. Rev. Genet.* **4**, 285-295.
Satou, Y. (1999). posterior end mark 3 (pem-3), an ascidian maternally expressed gene with localized mRNA encodes a protein with Caenorhabditis elegans MEX-3-like KH domains. *Dev. Biol.* **212**, 337-350.
Satou, Y., Imai, K. S. and Satoh, N. (2001). Early embryonic expression of a LIM-homeobox gene Cs-lhx3 is downstream of beta-catenin and responsible for the endoderm differentiation in Ciona savignyi embryos. *Development* **128**, 3559-3570.
Satou, Y., Takatori, N., Fujiwara, S., Nishikata, T., Saiga, H., Kusakabe, T., Shin-i, T., Kohara, Y. and Satoh, N. (2002). Ciona intestinalis cDNA projects: expressed sequence tag analyses and gene expression profiles during embryogenesis. *Gene* **287**, 83-96.
Satou, Y., Sasakura, Y., Yamada, L., Imai, K. S., Satoh, N. and Degnan, B. (2003). A genomewide survey of developmentally relevant genes in Ciona intestinalis. V. Genes for receptor tyrosine kinase pathway and Notch signaling pathway. *Dev. Genes Evol.* **213**, 254-263.
Seger, R. and Krebs, E. G. (1995). The MAPK signaling cascade. *FASEB J.* **9**, 726-735.
Steller, H. (1995). Mechanisms and genes of cellular suicide. *Science* **267**, 1445-1449.
Subramaniam, S., Zirrgiebel, U., von Bohlen und Halbach, O., Strelau, J., Laliberté, C., Kaplan, D. R. and Unsicker, K. (2004). ERK activation promotes neuronal degeneration predominantly through plasma membrane damage and independently of caspase-3. *J. Cell Biol.* **165**, 357-369.
Tarallo, R. and Sordino, P. (2004). Time course of programmed cell death in Ciona intestinalis in relation to mitotic activity and MAPK signaling. *Dev. Dyn.* **230**, 251-262.
Thornberry, N. A. and Lazebnik, Y. (1998). Caspases: enemies within. *Science* **281**, 1312-1316.
Tibbles, L. A. and Woodgett, J. R. (1999). The stress-activated protein kinase pathways. *Cell. Mol. Life Sci.* **55**, 1230-1254.
Tohyama, K., Kusuhara, H. and Sugiyama, Y. (2004). Involvement of multispecific organic anion transporter, Oatp14 (Slc21a14), in the transport of thyroxine across the blood-brain barrier. *Endocrinology* **145**, 4384-4391.
Vaux, D. L. and Korsmeyer, S. J. (1999). Cell death in development. *Cell* **96**, 245-254.
Vincent, S., Marty, L. and Fort, P. (1993). S26 ribosomal protein RNA: an invariant control for gene regulation experiments in eukaryotic cells and tissues. *Nucleic Acids Res.* **21**, 1498.
Wada, T. and Penninger, J. M. (2004). Mitogen-activated protein kinases in apoptosis regulation. *Oncogene* **23**, 2838-2849.
Waskiewicz, A. J. and Cooper, J. A. (1995). Mitogen and stress response pathways: MAP kinase cascades and phosphatase regulation in mammals and yeast. *Curr. Opin. Cell Biol.* **7**, 798-805.
Woods, R. G., Roper, K. E., Gauthier, M., Bebell, L. M., Sung, K., Degnan, B. M. and Lavin, M. F. (2004). Gene expression during early ascidian metamorphosis requires signalling by Hemps, an EGF-like protein. *Development* **131**, 2921-2933.
Yamada, L., Kobayashi, K., Satou, Y. and Satoh, N. (2005). Microarray analysis of localization of maternal transcripts in eggs and early embryos of the ascidian, Ciona intestinalis. *Dev. Biol.* **284**, 536-550.
Yao, Y., Li, W., Wu, J., Germann, U. A., Su, M. S., Kuida, K. and Boucher, D. M. (2003). Extracellular signal-regulated kinase 2 is necessary for mesoderm differentiation. *Proc. Natl. Acad. Sci. USA* **100**, 12759-12764.
Yoshimura, K., Aoki, H., Ikeda, Y., Fujii, K., Akiyama, N., Furutani, A., Hoshii, Y., Tanaka, N., Ricii, R., Ishihara, T. et al. (2005). Regression of abdominal aortic aneurysm by inhibition of c-jun N-terminal kinase. *Nat. Med.* **11**, 1330-1338.
Youson, J. H. and Sower, S. A. (2001). Theory on the evolutionary history of lamprey metamorphosis: role of reproductive and thyroid axes. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **129**, 337-345.
Youson, J. H., Heinig, J. A., Khanam, S. F., Sower, S. A., Kawauchi, H. and Keeley, F. W. (2006). Patterns of proopiomelanocortin and proopiocortin gene expression and of immunohistochemistry for gonadotropin-releasing hormones (lGnRH-I and III) during the life cycle of a nonparasitic lamprey: relationship to this adult life history type. *Gen. Comp. Endocrinol.* **148**, 54-71.
Zugasti, O., Rul, W., Roux, P., Peyssonnaux, C., Eychene, A., Francke, T. F., Fort, P. and Hibner, U. (2001). Raf-MEK-Erk cascade in anoikis is controlled by Rac 1 and Cdc42 via Akt. *Mol. Cell. Biol.* **21**, 6706-6717.