

Effects of U0126 and fibroblast growth factor on gene expression
profile in *Ciona intestinalis* embryos as revealed by microarray
analysis

Eriko Sakabe,¹ Nobuhiko Tanaka,² Naoki Shimosono,¹ Takashi Gojobori,²
Shigeki Fujiwara^{1,*}

¹Department of Materials Science, Kochi University, 2-5-1 Akebono-cho, Kochi-shi, Kochi
780-8520 and ²Center for Information Biology and DDBJ, National Institute of Genetics,
1111 Yata, Mishima, Shizuoka 411-8540, Japan

Running title: Effects of U0126 on gene expression in *Ciona*

* Author to whom all correspondence should be addressed.

Shigeki Fujiwara

Department of Materials Science, Kochi University, 2-5-1 Akebono-cho, Kochi-shi, Kochi
780-8520, Japan

Phone: +81-88-844-8317

Fax: +81-88-844-8356

E-mail: tatataa@kochi-u.ac.jp

Abstract

Fibroblast growth factor (FGF) induces the notochord and mesenchyme in ascidian embryos, via extracellular signal-regulated kinase (ERK) that belongs to the mitogen-activated protein kinase (MAPK) family. A cDNA microarray analysis was carried out to identify genes affected by an inhibitor of MAPK/ERK kinase (MEK), U0126, in embryos of the ascidian *Ciona intestinalis*. Data obtained from the microarray and *in situ* hybridization suggest that the majority of genes are downregulated by U0126 treatment. Genes that were downregulated in U0126-treated embryos included *Ci-Bra* and *Ci-Twist-like1* that are master regulatory genes of notochord and mesenchyme differentiation, respectively. The plasminogen mRNA was downregulated by U0126 in presumptive endoderm cells. This suggests that a MEK-mediated extracellular signal is necessary for gene expression in tissues whose specification does not depend on cell-to-cell interaction. Among 85 cDNA clusters that were not affected by U0126, 30 showed mitochondria-like mRNA localization in the nerve cord/muscle lineage blastomeres in the equatorial region. The expression level and asymmetric distribution of these mRNAs were independent of MEK signaling.

Key words: ascidian, MAPK cascade, fibroblast growth factor, microarray, induction

Introduction

Localization of maternal cytoplasmic components and cell-to-cell communications are important for embryonic cells to determine their developmental fate (Davidson 1986; Nishida 2005). In ascidian embryos, blastomeres around the vegetal pole are specified independent of cell-to-cell interaction (Whittaker 1990; Nishida 1993). Nuclear localization of β -catenin is an important step for endoderm differentiation in the *Ciona savignyi* embryo (Imai *et al.* 2000). β -catenin also confers on the vegetal blastomeres the ability to induce the notochord and mesenchyme in the equatorial region (Imai *et al.* 2000). Fibroblast growth factor (FGF) is responsible for the induction both in *Halocynthia* and *Ciona* embryos (Nakatani *et al.* 1996; Kim *et al.* 2000; Imai *et al.* 2002). FGF receptor (FGFR) activates extracellular signal-regulated kinase (ERK), which belongs to the mitogen-activated protein kinase (MAPK) family, through a sequential activation of protein kinases (MAPK cascade; for a review see Widmann *et al.* 1999). MAPK/ERK kinase (MEK) is a component of the MAPK cascade that directly phosphorylates and activates ERK (Widmann *et al.* 1999). Several lines of evidence suggest that the MAPK cascade is involved in the induction of the notochord, mesenchyme and central nervous system in ascidian embryos (Nakatani & Nishida 1997; Kim & Nishida 2001; Bertrand *et al.* 2003; Hudson 2003; Nishida 2003).

Mechanism of the notochord/mesenchyme induction has been well analyzed at the

cellular level using *Halocynthia roretzi*. The induction occurs at the 32-cell stage (Nakatani & Nishida 1999). Blastomeres that received the inductive signal undergo asymmetric cell division in normal embryos. For example, the A6.2 blastomere divides into presumptive notochord (A7.3) and nerve cord (A7.4) blastomeres. If A6.2 is isolated at the 32-cell stage, both of its daughter cells express nerve cord-specific genes (Minokawa *et al.* 2001) and no notochord cell is produced (Nakatani *et al.* 1996). FGF can induce expression of notochord-specific differentiation markers in both daughter cells derived from isolated A6.2 (Nakatani *et al.* 1996; Minokawa *et al.* 2001). Treatment of isolated A6.2 blastomeres with a MEK inhibitor, U0126, causes nerve cord differentiation in both daughter cells (Minokawa *et al.* 2001). Similarly, asymmetric division of B6.4 produces a mesenchyme-lineage (B7.7) and muscle-lineage (B7.8) daughter cells. The muscle differentiation pathway is suppressed in B7.7, in response to the inductive signal (Kim & Nishida 1999). These observations suggest that the FGF/MAPK cascade suppresses genes involved in nerve cord/muscle differentiation as well as it activates those involved in notochord/mesenchyme differentiation. Many transcription factors and signaling molecules are identified as downstream targets of β -catenin and FGF using *Ciona intestinalis* embryos (Imai *et al.* 2004). They are involved in endoderm differentiation and notochord/mesenchyme induction. In contrast, genes that are suppressed upon the induction are expected to be involved in nerve cord/muscle differentiation. However, we have little information about the suppression of gene

expression dependent on the induction. In addition, function of other receptor tyrosine kinases during ascidian embryogenesis is not understood.

Mitochondria in the ascidian embryos are abundant in A6.2, A6.4, B6.2 and B6.4 blastomeres at the 32-cell stage (Zalokar & Sardet 1984). They are asymmetrically segregated into one of their daughter cells (A7.4, A7.8, B7.4 and B7.8) during the next cleavage (Zalokar & Sardet 1984). These blastomeres give rise to the nerve cord and muscle (Nishida 1987). The other daughter cells (A7.3, A7.7, B7.3 and B7.7) differentiate into the notochord and mesenchyme depending on the inductive signal from vegetal pole cells (Nishida 1987; Nakatani & Nishida. 1994; Kim & Nishida 1999). Many different types of mRNA show a mitochondria-like localization (Makabe *et al.*, 2001; Nishikata *et al.* 2001; Fujiwara *et al.* 2002; Yamada *et al.* 2005). Since most of them encode proteins whose function seems not related to mitochondria, some of them are expected to be involved in nerve cord/muscle specification. However, mechanism of asymmetric distribution and function of these mRNAs have not yet been revealed.

In the present study, we conducted a cDNA microarray analysis using U0126-treated embryos of the ascidian, *Ciona intestinalis*. The majority of genes seemed to be suppressed by U0126. Spatial expression of selected cDNA clones was examined by *in situ* hybridization. Many of genes whose expression was not affected by U0126 showed the mitochondria-like localization. Asymmetric distribution of their mRNA correctly occurred

in U0126-treated embryos. Our results suggest that asymmetric cell division that occurs after notochord/mesenchyme induction is a cell autonomous process.

Materials and Methods

Biological materials

C. intestinalis juvenile adults were kindly provided by Nori Satoh and Kazuko Hirayama at Kyoto University. They were cultured near the Usa Marine Biological Institute of Kochi University. Mature adults were collected, and gametes were surgically obtained from the gonoducts. Eggs were inseminated with non-self sperm. Embryos were treated with 2 μ M U0126 (Promega, Madison, WI, USA) from the 24-cell stage. As a reference, embryos were treated with 0.1% dimethylsulfoxide (DMSO), since 2 mM U0126 stock was dissolved in DMSO. For FGF treatment, fertilized eggs were dechorionated with 0.05% actinase E (Kaken Pharmaceutical, Tokyo, Japan) and 1% sodium thioglycolate. Embryos were treated with 2 ng/ml recombinant human FGF (Roche, Mannheim, Germany) from the 24-cell stage. As a reference, embryos were treated with 0.1% bovine serum albumin (BSA), since FGF treatment was carried out in a seawater containing 0.1% BSA.

Preparation of fluor-tagged target cDNA

Total RNA was extracted from drug-treated embryos as described by Fujiwara *et al.* (1993). Two micrograms of RNA was incubated with 3 μ g hexanucleotide mix (Roche) at 70°C for 10 min. Samples were then mixed with 2 mM dTTP, 2 mM dCTP, 5 mM dATP, 5 mM dGTP, 1

mM Cy3 (or Cy5) dCTP, 1 mM Cy3 (or Cy5) dUTP (Amersham, Piscataway, NJ, USA), 10 mM dithiothreitol, 400 units of SuperScript II reverse transcriptase (Gibco BRL, Gaithersburg, MD, USA) and the First Strand Buffer (Gibco BRL). The reaction solution was incubated at 25°C for 10 min and 42°C for 1 h. Two hundred units of SuperScript II was added to the reaction solution. The solution was further incubated at 42°C for 1 h. The reaction was stopped by adding 50 mM ethylenediaminetetraacetic acid (EDTA). Template RNA was hydrolyzed by incubation with 0.2 N NaOH at 65°C for 1 h. After neutralization with Tris-HCl (pH 8.0), the cDNA was washed with TE [10 mM Tris-HCl (pH 7.5), 1 mM EDTA] and concentrated by using Microcon-YM30 (Millipore, Tokyo, Japan).

Microarray analysis

Fluor-linked cDNA was dissolved in 4x SSC solution [600 mM NaCl, 60 mM sodium citrate (pH 7.0)], and denatured at 95°C for 2 min. After adding 0.2% sodium dodecylsulfate, the target cDNA was placed on the cDNA chip prepared by Ishibashi *et al.* (2003).

Hybridization was performed as described in Ishibashi *et al.* (2003). Fluorescent signal was detected by FLA-8000 (Fuji Film, Tokyo, Japan) and analyzed with Array Gauge ver. 2.0 (Fuji Film, Tokyo, Japan). The fluorescent intensity of each dye was separately scanned.

We calculated the ratio of fluorescent intensities obtained from U0126-treated and reference probes (U:D), where U and D represent the fluorescent intensity resulted from an

U0126-treated probe and DMSO-treated probe, respectively. We conducted three independent experiments. The geometrical average of the U:D ratio across three experiments was calculated for each spot. Similarly, the ratio of fluorescent intensities obtained from FGF-treated and reference probes (F:B) was calculated, where F and B represent the fluorescent intensity resulted from FGF-treated probe and BSA-treated probe, respectively. The geometrical average of the F:B for four independent experiments was calculated on each spot.

Whole-mount in situ hybridization

The templates for synthesizing RNA probes were prepared from cDNA clones (Fujiwara *et al.* 2002; Kusakabe *et al.* 2002; Satou *et al.* 2001; 2002) by PCR using primers SK (5'-TCTAGAACTAGTGGATC-3') and T7 (5'-AATACGACTCACTATAG-3'). RNA probes were labeled with digoxigenin (DIG) using T7 RNA polymerase, according to the protocol supplied by Roche. *In situ* hybridization was carried out essentially as described by Nagatomo *et al.* (2003), except that the temperature for hybridization was 54°C and that for washing was 60°C.

Results

Microarray analysis of gene expression in embryos treated with a MEK inhibitor

We used a microarray, prepared by Ishibashi *et al.* (2003), which contained 9287 *C. intestinalis* cDNA clusters. Embryos were continuously treated with 2 μ M of a MEK inhibitor U0126 from the 24-cell stage, and were collected for preparation of Cy3- or Cy5-conjugated cDNA at the 110-cell stage. The microarray was hybridized with a mixture of fluor-linked target cDNA prepared from U0126-treated embryos and DMSO-treated (reference) embryos. Three independent hybridization experiments were carried out. In each experiment, the U:D value of each spot was normalized so that the average U:D of 9287 spots was 1.00 (data set 1). The geometrical average was calculated for each spot based on three experiments. Distribution of the average U:D is shown in Fig. 1A. The U:D of 371 spots was greater than 2.00 (Table 1, Fig. 1A) and that of 39 spots was less than 0.5 (Fig. 1A). Forty nine of the top 50 cDNA clusters in the data set 1 showed the U:D greater than 1.0 in all three experiments. Downregulation was less reproducible. Twenty eight of the last 50 clusters in the data set 1 showed the U:D less than 1.0 in all three experiments.

Expression of 85 clusters that showed high U:D values was examined by whole-mount *in situ* hybridization. However, no obvious upregulation was observed, as described below. Therefore, we attempted another normalization method. We selected 20 clusters (00084r1,

00538r1, 00599r1, 00961r1, 01200r1, 02767r1, 02780r1, 03280r1, 03640r1, 03926r1, 04873r1, 07226r1, 12330r1, 12876r1, 13312r1, 14460r1, 14497r1, 14774r1, 14966r1, and 14987r1). *In situ* hybridization revealed that the amount and localization of mRNAs for these genes were not affected by U0126 (see below). The U:D value of each spot was then adjusted so that the average U:D of the 20 spots became 1.00. In this case (data set 2), the maximum U:D was 1.57 (Table 1). The U:D of 26 spots was greater than 1.00 (Table 1, Fig. 1A), and that of 109 spots was less than 0.10 (Fig. 1A). Thirty one among the top 50 cDNA clusters in the data set 2 also ranked in the top 50 in the data set 1 (Table 1). In contrast, only 7 among the last 50 clusters in the data set 2 were found in the last 50 of the data set 1 (data not shown).

Microarray analysis of FGF target genes

We also conducted a microarray analysis of FGF target genes. Embryos were treated with 2 ng/ml recombinant human FGF from the 24-cell stage. The cDNA chip was hybridized with a mixture of fluor-linked probes, prepared from FGF-treated and BSA-treated (control) 110-cell embryos. Hybridization was carried out four times. In each experiment, the F:B value of each spot was normalized so that the average F:B was 1.00. The geometrical average was calculated for each spot based on four hybridization experiments. Distribution of the average F:B is shown in Fig. 1B. The range of up- and down-regulation was narrower

than that obtained from U0126-treated samples (Fig. 1B). The F:B of 9148 spots (98.5%) lay between 2.0 and 0.5 (Fig. 1B).

Genes that showed high F:B value includes those involved in signal transduction pathways, such as 07270r1 (similar to a Ras-related protein Rab-14; F:B 2.59) and 02731r1 (*Ci-Stbm/Vang*; F:B 2.42). Genes encoding motor proteins, such as 07716r1 (similar to myosin-18A; F:B 2.39), 05057r1 (similar to myosin regulatory light chain, MRLC5; F:B 2.31) and 08887r1 (kinesin-like protein KIF12; F:B 2.22), also showed high F:B. The F:B of both *Ci-Bra* and *Ci-Twist-like1* was 0.89 (data not shown).

Spatial expression of genes that showed low U:D values

Spatial expression of 95 cDNA clusters, which showed low U:D values, was also examined by whole-mount *in situ* hybridization (Fig. 2). In this section, U:D values are mentioned based on the data set 2. Most of them were ubiquitously expressed or not detected (data not shown). The U:D ratio of 11351r1 (*Ci-Bra*) and 15255r1 (*Ci-Twist-like1*) was 0.11 and 0.20, respectively. The notochord-specific expression of 11351r1 and mesenchyme-specific expression of 15255r1 were completely suppressed in U0126-treated embryos (Fig. 2A, B). Specific expression of 00144r1 (plasminogen precursor; U:D 0.19) in the endodermal lineage cells (A7.1, A7.2 and A7.5) was downregulated in U0126-treated 110-cell embryos (Fig. 2C). Expression of the other genes were not affected by U0126 or not detectable (data not shown).

Spatial expression of genes that showed high U:D values

Whole-mount *in situ* hybridization revealed that 30 among the 85 cDNA clusters that showed high U:D values exhibited a characteristic localization pattern (Table 1, Figs 3, 4). In the vegetal hemisphere, mRNA for these genes was observed in A6.2, A6.4, B6.2, B6.3, and B6.4 blastomeres of the 24~32-cell embryo (Figs 3, 4). The mRNA was observed in one of their daughter cells (A7.4, A7.8, B7.4, B7.5 and B7.8) after the next cleavage. The amount of mRNA was small in the other daughter cells (A7.3, A7.7, B7.3, B7.6, and B7.7). The mRNA was preferentially distributed mainly into nerve cord/muscle-lineage cells (Fig. 3C, D). All the blastomeres in the animal hemisphere also contained a significant amount of mRNA (Fig. 3B). This distribution pattern is similar to that of mitochondria (Zalokar & Sardet 1984; see Fig. 5). The 30 cDNA clusters included above-mentioned 20 clusters and 01241r1, 02653r1, 02772r1, 05327r1, 06428r1, 07517r1, 11140r1, 11329r1, 11372r1, and 14463r1 (Table 1). Five of the 30 clusters were located on the mitochondrial genome (accession number AJ517314). Among them 00599r1, 01241r1, 14460r1, and 14497r1 encoded 16S rRNA. The nucleotide sequence of 14774r1 corresponded to a tRNA gene cluster on the mitochondrial genome. Except for them and those appeared in Table 1, genes that showed mitochondria-like mRNA localization included basic transcription factor 3 (00961r1), *C. intestinalis* endostyle-specific secretory protein (02653r1), *C. intestinalis* endostyle-specific

protein 10 (02772r1, similar to thyroid hormone receptor interactor protein), γ -aminobutyric acid receptor subunit α 4 (05327r1), ADP-ribosylation factor-like 10C (11329r1), and a WD repeat-containing protein (14463r1). Note that high U:D means the values greater than 4.00 in the data set 1, but approximately 1.00 in the data set 2 (Table 1, Fig. 1A). None of them was upregulated or ectopically activated in U0126-treated embryos (Fig. 3). The A-line muscle precursor cells moves posteriorly and form a single cell cluster, on each side, with the B-line muscle precursor cells (Fig. 3D). U0126 inhibited this movement, but did not alter the uneven distribution and localization of the mRNA (Fig. 3D). Distribution of mitochondria was visualized by vital fluorescent staining using 1 mg/ml of 3,3'-diethyloxycarbocyanine (Fig. 5). U0126 did not affect asymmetric distribution and fluorescent intensity of mitochondria (Fig. 5). Remaining 55 cDNA clusters were either expressed ubiquitously or not detected (data not shown). None of them seemed upregulated by U0126 treatment (data not shown). Expression of 6 cDNA clusters (00480r1, 01158r1, 02286r1, 02795r1, 03280r1, and 14987r1) was examined by reverse transcription-PCR (RT-PCR) using specific primers. The average U:D of these 6 clusters was 6.15 in the data set 1, while that was 1.07 in the data set 2 (Table 1). U0126 did not upregulate any of them (data not shown).

Discussion

U0126 suppresses the majority of genes

In the present study, two methods were applied to normalize the microarray data obtained from U0126-treated embryos. In the data set 1, the average U:D of 9287 spots was adjusted to be 1.00. This normalization strategy was based on the expectation that most genes were not affected by U0126 treatment. However, genes that showed high U:D values were not upregulated when examined by *in situ* hybridization and RT-PCR. Many of them exist in the fertilized egg as maternal mRNAs, as judged by the frequency of expressed sequence tags in cDNA libraries constructed from embryos at various different developmental stages (Nishikata *et al.* 2001; Satou *et al.* 2002). We therefore thought that U0126 suppressed most zygotic genes, either specifically or non-specifically. In the data set 2, 20 cDNA clusters were selected as the standard for normalization. The expression level and mitochondria-like localization of these 20 clusters were not affected by U0126 treatment, as revealed by *in situ* hybridization. Although *in situ* hybridization-based quantitation is not accurate, this normalization method produced a more reasonable data set. For example, *Brachyury* and *Twist-like1* are downstream genes of FGF signaling in the *Ciona* embryo (Imai *et al.* 2002; 2003; Tokuoka *et al.* 2004). Expression of these genes were suppressed by microinjection of *Fgf9/16/20*-specific morpholino oligos (Imai *et al.* 2002; 2003). The U:D values of 11351r1

(*Ci-Bra*) and 15255r1 (*Ci-Twist-like1*) were 0.11 and 0.20, respectively, in the data set 2, while they were 0.69 and 0.97 in the data set 1. Similarly, the U:D value of 00144r1 (plasminogen precursor) was greater than 1.00 in the data set 1, although *in situ* hybridization revealed that its expression was completely suppressed by U0126.

Mitochondria-like localization of mRNA is MEK-independent

Among 85 cDNA clusters that showed high U:D values, 30 (35%) exhibited mitochondria-like mRNA localization. This type of localized mRNA are mostly provided maternally (Makabe *et al.*, 2001; Nishikata *et al.* 2001; Fujiwara *et al.* 2002; Yamada *et al.* 2005). It seems reasonable that maternally transcribed genes are not affected by U0126 after fertilization. However, a large scale *in situ* hybridization analysis revealed that only 12 (1.2%) of 1003 maternal genes showed mitochondria-like localization (Nishikata *et al.* 2001). The proportion of mitochondria-like genes was much higher in the U0126-untreated genes. FGF/MEK signaling is required for suppression of nerve cord/muscle differentiation program in A7.4, A7.8, B7.4 and B7.8 (Kim & Nishida 1999; Minokawa *et al.* 2001). U0126 causes nerve cord differentiation in both daughter cells derived from isolated A6.2 (Minokawa *et al.* 2001). These observations suggest that asymmetric division also depends on the induction. However, in the present study, neither upregulation nor mis-localization was observed. Mitochondria themselves are also normally localized in U0126-treated embryos. These

results suggest that asymmetric division is a cell-autonomous process in the *C. intestinalis* embryo.

Responsiveness of non-recipient cells is suppressed in normal embryos

The distribution of F:B was narrower than that of U:D. One possible explanation is that U0126 blocked the MEK activity stimulated by various other signaling molecules. Although the *C. intestinalis* genome contains many receptor tyrosine kinases, such as epidermal growth factor receptor, insulin receptor and Eph (Satou *et al.* 2003), their involvement in embryogenesis is poorly understood. Future investigation of these signaling pathways is important for comprehensive understanding of ascidian embryogenesis. Another explanation is that the FGF signal transduction is suppressed in non-recipient cells.

Although both daughter cells of isolated A6.2 can respond to FGF and differentiate into the notochord (Minokawa *et al.* 2001), only one daughter cell (A7.3) differentiates into the notochord in normal embryos. FGF treatment did not induce notochord differentiation in blastomeres isolated from the animal hemisphere (a4.2 and b4.2) (Nakatani *et al.* 1996).

Upregulation of *Ci-Bra* was not observed in our microarray analysis. FGF did not induce ectopic *Ci-Bra* expression in intact embryos (E. Sakabe, unpublished observation). The FGF signal transduction is therefore thought to be suppressed in non-recipient cells. Among candidate FGF target genes, *Ci-Stbm/Vang* encodes a component of the planar cell polarity

(PCP) pathway that is thought to be involved in the gastrulation movement (Keller 2002; Hotta *et al.* 2003).

Expression of some genes in autonomously specified cell types is MEK-dependent

In ascidian embryos, most of the endoderm and tail muscle cells are specified autonomously by localized maternal determinants (Nishida 1992; 1993). In the present study, U0126 suppressed expression of 00144r1 (plasminogen precursor) in the endodermal lineage cells (A7.1, A7.2 and A7.5). Although the FGF/MEK signal induces differentiation of a posterior endoderm cells derived from B4.1 (Kondoh *et al.* 2003), A-line endoderm cells are thought to differentiate autonomously. U0126 caused ectopic activation of the *Ci-ZicL* enhancer in the presumptive epidermal lineage blastomeres (Anno *et al.* submitted). These observations suggest that extracellular signal is necessary for gene expression even in those cells whose specification does not require cell-to-cell communication. However, the present results should be carefully analyzed further, because U0126 is not an endogenous factor.

Acknowledgements

We thank Nori Satoh and Kazuko Hirayama for providing us with farm-raised animals. This work was supported by MEXT Japan and Japan Society for Promotion of Science. ES was supported by The Japan Science Society.

References

- Bertrand, V., Hudson, C., Caillol, D., Popovici, C. & Lemaire, P. 2003. Neural tissue in ascidian embryos is induced by FGF9/16/20, acting via a combination of maternal GATA and Ets transcription factors. *Cell* **115**, 615-627.
- Davidson, E. H. 1986. *Gene activity in early development*. Academic Press, Orlando.
- Fujiwara, S., Kawahara, H., Makabe, K. W. & Satoh, N. 1993. A complementary DNA for an ascidian embryonic nuclear antigen Hgv2 encodes a protein closely related to the amphibian histone-binding protein N1. *J. Biochem.* **113**, 189-195.
- Fujiwara, S., Maeda, Y., Shin-i, T., Kohara, Y., Takatori, N., Satou, Y. & Satoh, N. 2002. Gene expression profiles in *Ciona intestinalis* cleavage-stage embryos. *Mech. Dev.* **112**, 115-127.
- Hotta, K., Takahashi, H., Asakura, T., Saitoh, B., Takatori, N., Satou, Y. & Satoh, N. 2000. Characterization of *Brachyury*-downstream notochord genes in the *Ciona intestinalis* embryo. *Dev. Biol.* **224**, 69-80.
- Hotta, K., Takahashi, H., Ueno, N. & 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.

- Hudson, C., Darras, S., Caillol, D., Yasuo, H. & Lemaire, P. 2003. A conserved role for the MEK signalling pathway in neural tissue specification and posteriorization in the invertebrate chordate, the ascidian *Ciona intestinalis*. *Development* **130**, 147-159.
- Imai, K. S., Hino, K., Yagi, K., Satoh, N. & 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.
- Imai, K. S., Satoh, N. & Satou, Y. 2002. Early embryonic expression of *FGF4/6/9* gene and its role in the induction of mesenchyme and notochord in *Ciona savignyi* embryos. *Development* **129**, 1729-1738.
- Imai, K. S., Satoh, N. & Satou, Y. 2003. A twist-like bHLH gene is a downstream factor of an endogenous FGF and determines mesenchymal fate in the ascidian embryos. *Development* **130**, 4461-4472.
- Imai, K. S., Takada, N., Satoh, N. & Satou, Y. 2000. β -catenin mediates the specification of endoderm cells in ascidian embryos. *Development* **127**, 3009-3020.
- Ishibashi, T., Nakazawa, M., Ono, H., Satoh, N., Gojobori, T. & Fujiwara, S. 2003. Microarray analysis of embryonic retinoic acid target genes in the ascidian *Ciona intestinalis*. *Dev. Growth Differ.* **45**, 249-259.
- Keller, R. 2002. Shaping the vertebrate body plan by polarized embryonic cell movements. *Science* **298**, 1950-1954.

- Kim, G. J. & Nishida, H. 1999. Suppression of muscle fate by cellular interaction is required for mesenchyme formation during ascidian embryogenesis. *Dev. Biol.* **214**, 9-22.
- Kim, G. J. & Nishida, H. 2001. Role of FGF and MEK signaling pathway in the ascidian embryo. *Dev. Growth Differ.* **43**, 521-533.
- Kim, G. J., Yamada, A. & Nishida, H. 2000. An FGF signal from endoderm and localized factors in the posterior-vegetal egg cytoplasm pattern the mesodermal tissues in the ascidian. *Development* **127**, 2853-2862.
- Kondoh, K., Kobayashi, K. & Nishida, H. 2003. Suppression of macho-1-directed muscle fate by FGF and BMP is required for formation of posterior endoderm in ascidian embryos. *Development* **130**, 3205-3216.
- Kusakabe, T., Yoshida, R., Kawakami, I., Kusakabe, R., Mochizuki, Y., Yamada, L., Shin-i, T., Kohara, Y., Satoh, N., Tsuda, M., Satou, Y. 2002. Gene expression profiles in tadpole larvae of *Ciona intestinalis*. *Dev. Biol.* **242**, 188-203.
- Makabe, K. W., Kawashima, T., Kawashima, S., Minokawa, T., Adachi, A., Kawamura, H., Ishikawa, H., Yasuda, R., Yamamoto, H., Kondoh, K., Arioka, S., Sasakura, Y., Kobayashi, A., Yagi, K., Shojima, K., Kondoh, Y., Kido, S., Tsujinami, M., Nishimura, N., Takahashi, M., Nakamura, T., Kanehisa, M., Ogasawara, M., Nishikata, T. & Nishida, H. 2001. Large-scale cDNA analysis of the maternal genetic information in the egg of *Halocynthia roretzi* for a gene expression catalog of ascidian development.

Development **128**, 2555–2567.

Minokawa, T., Yagi, K., Makabe, K. W., Nishida, H. 2001. Binary specification of nerve cord and notochord cell fates in ascidian embryos. *Development* **128**, 2007-2017.

Nagatomo, K., Ishibashi, T., Satou, Y., Satoh, N. & Fujiwara, S. 2003. Retinoic acid affects gene expression and morphogenesis without upregulating the retinoic acid receptor in the ascidian *Ciona intestinalis*. *Mech. Dev.* **120**, 363-372.

Nakatani, Y. & Nishida, H. 1994. Induction of notochord during ascidian embryogenesis. *Dev. Biol.* **166**, 289-299.

Nakatani, Y. & Nishida, H. 1997. Ras is an essential component for notochord formation during ascidian embryogenesis. *Mech. Dev.* **68**, 81-89.

Nakatani, Y. & Nishida, H. 1999. Duration of competence and inducing capacity of blastomeres in notochord induction during ascidian embryogenesis. *Dev. Growth Differ.* **41**, 449-453.

Nakatani, Y., Yasuo, H., Satoh, N. & Nishida, H. 1996. Basic fibroblast growth factor induces notochord formation and the expression of *As-T*, a *Brachyury* homolog, during ascidian embryogenesis. *Development* **122**, 2023-2031.

Nishida, H. 1987. Cell lineage analysis in ascidian embryos by intracellular injection of a tracer enzyme. III. Up to the tissue restricted stage. *Dev. Biol.* **121**, 526-541.

Nishida, H. 1992. Regionality of egg cytoplasm that promotes muscle differentiation in

- embryo of the ascidian, *Halocynthia roretzi*. *Development* **116**, 521-529.
- Nishida, H. 1993. Localized regions of egg cytoplasm that promote expression of endoderm-specific alkaline phosphatase in embryos of the ascidian *Halocynthia roretzi*. *Development* **118**, 1-7.
- Nishida, H. 2003. Spatio-temporal pattern of the activation of MAP kinase in embryos of the ascidian, *Halocynthia roretzi*. *Dev. Growth Differ.* **45**, 27-37.
- Nishida, H. 2005. Specification of embryonic axis and mosaic development in ascidians. *Dev. Dyn.* **233**, 1177-1193.
- Nishikata, T., Yamada, L., Mochizuki, Y., Satou, Y., Shin-i, T., Kohara, Y. & Satoh, N. 2001. Profiles of maternally expressed genes in fertilized eggs of *Ciona intestinalis*. *Dev. Biol.* **238**, 315-331.
- Satou, Y., Sasakura, Y., Yamada, L., Imai, K. S., Satoh, N. & 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.
- Satou, Y., Takatori, N., Fujiwara, S., Nishikata, T., Saiga, H., Kusakabe, T., Shin-i, T., Kohara, Y. & Satoh, N. 2002. *Ciona intestinalis* cDNA projects: expressed sequence tag analyses and gene expression profiles during embryogenesis. *Gene* **287**, 83-96.
- Satou, Y., Takatori, N., Yamada, L., Mochizuki, Y., Hamaguchi, M., Ishikawa, H., Chiba, S., Imai, K., Kano, S., Murakami, S. D., Nakayama, A., Nishino, A., Sasakura, Y., Satoh, G.,

- Shimotori, T., Shin-i, T., Shoguchi, E., Suzuki, M. M., Takada, N., Utsumi, N., Yoshida, N., Saiga, H., Kohara, Y. & Satoh, N. 2001. Gene expression profiles in *Ciona intestinalis* tailbud embryos. *Development* **128**, 2893-2904.
- Tokuoka, M., Imai, K. S., Satou, Y. & Satoh, N. 2004. Three distinct lineages of mesenchymal cells in *Ciona intestinalis* embryos demonstrated by specific gene expression. *Dev. Biol.* **274**, 211-224.
- Whittaker, J. R. 1990. Determination of alkaline phosphatase expression in endodermal cell lineages of an ascidian embryo. *Biol. Bull.* **178**, 222-230.
- Widmann, C., Gibson, S., Jarpe, M. B. & Johnson, G. L. 1999. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiol. Rev.* **79**, 143-180.
- Yamada, L., Kobayashi, K., Satou, Y. & 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.
- Zalokar, M. & Sardet, C. 1994. Tracing of cell lineage in embryonic development of *Phallusia mammillata* (Ascidian) by vital staining of mitochondria. *Dev. Biol.* **102**, 195-205.

Figure Legends

Fig. 1. The distribution of the U:D and F:B of 9287 spots. The horizontal axis indicates the U:D or F:B, as 1.2^n . The vertical axis indicates the number of spots in a logarithmic scale. (A) The data set 1 was calculated so that the average U:D of 9287 spots was 1.00. The data set 2 was calculated so that the average U:D of 20 U0126-unaffected genes was 1.00. (B) The distribution of the average F:B was compared with the average U:D (data set 1).

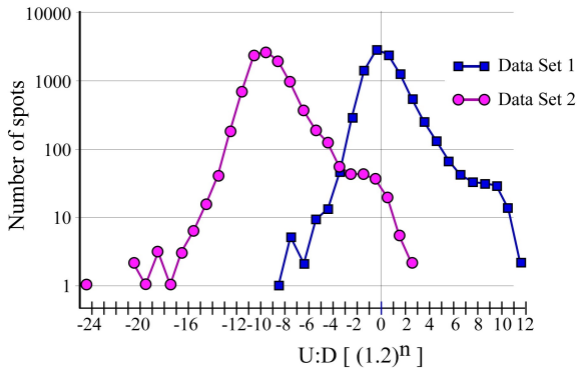
Fig. 2. Expression of selected genes that were downregulated by U0126. The upper panels show DMSO-treated (control) embryos and the lower panels show U0126-treated embryos. Gastrulae are oriented to show the vegetal hemisphere (dorsal side). (A) Expression of *Ci-Bra* (11351r1) at the late gastrula stage (left) and initial tailbud stage (right). The upper right panel is a lateral view, the anterior is to the left. The lower right panel is a dorsal view, the anterior is to the left. (B) Expression of *Ci-Twist-like1* (15255r1) in the pair of B7.7 blastomeres at the early gastrula stage. (C) Expression of *Ci-plasminogen* (00144r1) in A7.1, A7.2 and A7.5 blastomeres at the early gastrula stage.

Fig. 3. Mitochondria-like distribution of mRNA. (A-D) DMSO-treated embryos. (E-H) U0126-treated embryos. (A, B, E, F) The 32-cell embryos. (C, G) The 110-cell embryos. (D, H) Late gastrula. (A, C, D, E, F, H) The vegetal (dorsal) view. (B, F) The animal view. Blastomeres in which the mRNA was predominantly distributed were indicated by their names (A, B, C) or by asterisks (E, F, G). Since the cleavage pattern of ascidian embryos is bilaterally symmetrical, the blastomeres were indicated only on the right side. Arrows in (H) indicate the A-line muscle precursor cells, whose movement was inhibited by U0126.

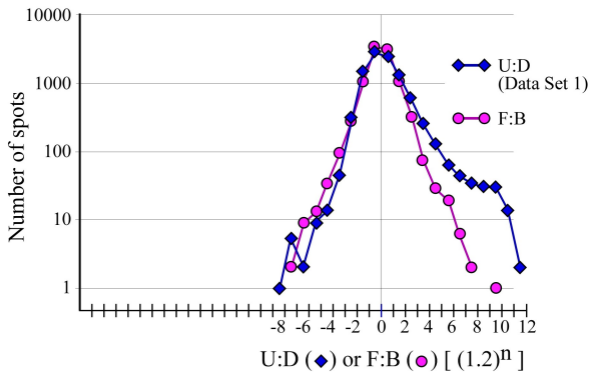
Fig. 4. Mitochondria-like mRNA localization of selected cDNA clusters in the 32-cell embryos. The cDNA cluster ID (Satou et al., 2002) is indicated above each panel. All embryos are at the 32-cell stage, and oriented to display the vegetal hemisphere.

Fig. 5. Distribution of mitochondria in U0126-treated and control embryos as revealed by a vital fluorescent staining. (A, B) DMSO-treated (control) embryos. (C, D) U0126-treated embryos. (A, C) The 32-cell embryos. (B, D) The 110-cell embryos. Blastomeres in which mitochondria are preferentially distributed are indicated by their names (A, B) or by asterisks (C, D). All the embryos were oriented to display the vegetal hemisphere.

A

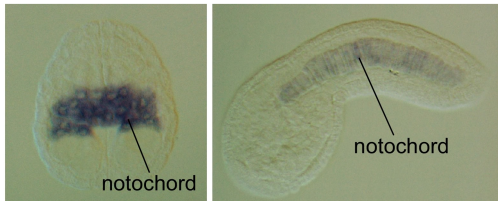


B



DMSO

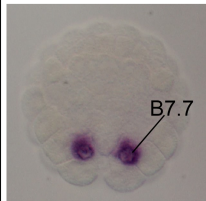
A

Ci-Bra
(11351r1)

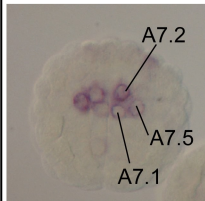
U0126

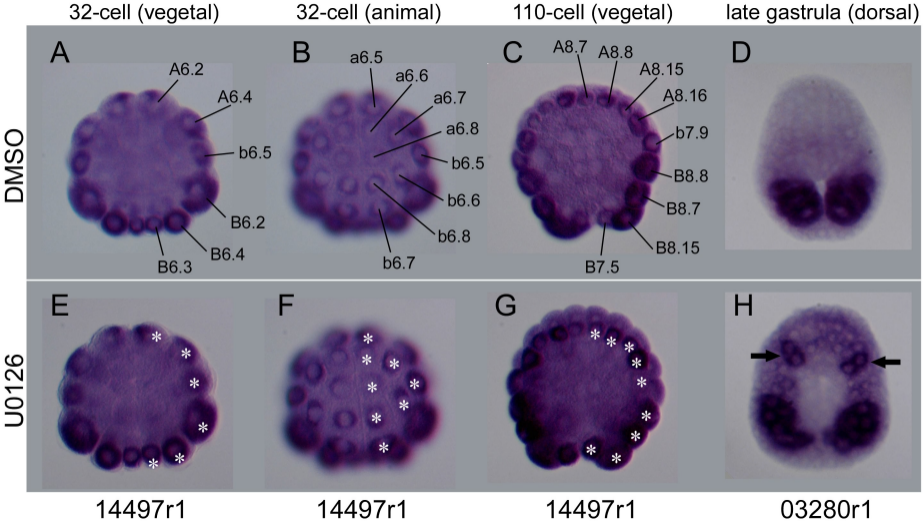


B

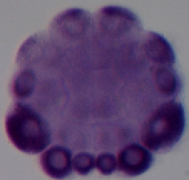
Ci-Twist-like1
(15255r1)

C

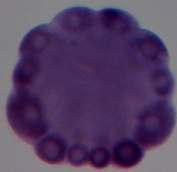
Ci-plasminogen
(00144r1)



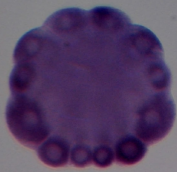
13312r1



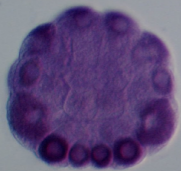
00599r1



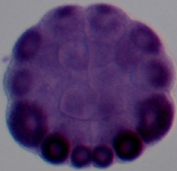
00538r1



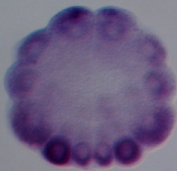
01200r1



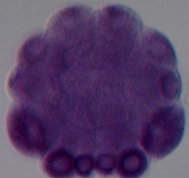
14987r1



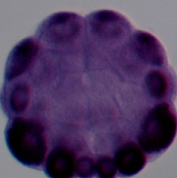
04873r1



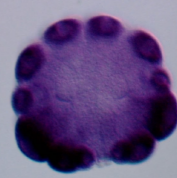
14966r1



14460r1



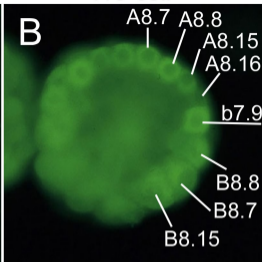
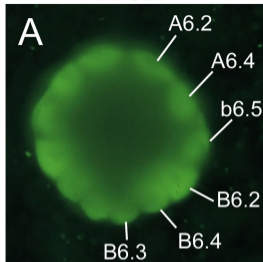
12330r1



32-cell

110-cell

DMSO



U0126

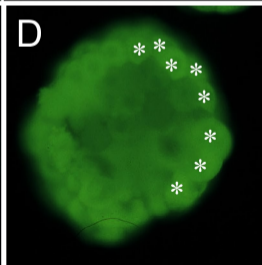
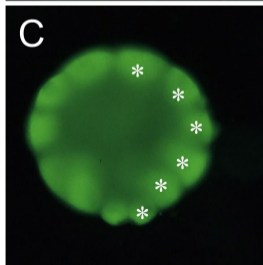


Table 1. Description of the top 50 genes that showed high U:D values in the data set 2

Cluster ID [†]	EST sequence similarities (BLASTX results)	In situ [‡]	U:D (2) [§]	U:D (1) [§]
13409r1	Chromosome-associated kinesin KIF4A		1.57	2.17
02077r1	Intelectin 2		1.47	0.94
14497r1	Mitochondrial 16S rRNA	M	1.42	7.94
13312r1	Hypothetical protein (<i>Xenopus tropicalis</i> MGC69312)	M	1.31	7.61
00599r1	Mitochondrial 16S rRNA	M	1.27	7.36
01158r1	Ci-FoxH-a		1.22	6.88
00538r1	Heterogeneous nuclear ribonucleoprotein R	M	1.21	6.78
02617r1	Cornifin		1.18	5.86
01200r1	No significant similarity to known proteins	M	1.17	6.73
14987r1	No significant similarity to known proteins	M	1.17	6.75
04873r1	Ribulose-1,5-bisphosphate carboxylase small subunit	M	1.17	6.61
14966r1	Solute carrier family 22-like	M	1.14	6.49
00480r1	Fibrillarlin		1.13	6.56
01300r1	Hypothetical protein (<i>Caenorhabditis briggsae</i> CBG00117)		1.11	4.05
07785r1	Ci-Wnt5a		1.11	6.29
14460r1	Mitochondrial 16S rRNA	M	1.10	6.72
12876r1	Transcriptional cofactor subunit 9 required for Sp1 transactivatio	M	1.09	6.16
12330r1	Radical S-adenosyl methionine and flavodoxin domains 1	M	1.08	6.24
13091r1	No significant similarity to known proteins		1.08	6.11
03640r1	No significant similarity to known proteins	M	1.08	6.23
13104r1	No significant similarity to known proteins		1.04	1.65
<i>citb006m15</i>	No significant similarity to known proteins		1.04	6.20
06691r1	Plectin		1.03	0.93
03280r1	Splicing factor 3B subunit 1	M	1.02	5.90
02767r1	Developmentally regulated G protein-coupled receptor (DREG)	M	1.01	5.89
04888r1	No significant similarity to known proteins		1.01	5.69
01835r1	Transmembrane 9 superfamily protein member 2		0.99	2.07
02780r1	Solute carrier family 35 member B3	M	0.98	5.62
02795r1	No significant similarity to known proteins		0.98	5.61
00877r1	Ci-MHC2 (myosin heavy chain)		0.97	5.70
07226r1	Eukaryotic translation initiation factor 3 subunit 10	M	0.97	5.97
<i>cicl006n06</i>	No significant similarity to known proteins		0.96	5.30
02588r1	No significant similarity to known proteins		0.95	5.47
03212r1	Hypothetical protein (<i>Homo sapiens</i> FLJ12672)		0.95	5.75
<i>citb002m10</i>	No significant similarity to known proteins		0.94	5.74
00551r1	No significant similarity to known proteins		0.94	5.53
08118r1	No significant similarity to known proteins		0.94	4.79
00410r1	Mitochondrial ribosomal protein S5		0.94	5.56
11372r1	No significant similarity to known proteins	M	0.93	5.43
02286r1	No significant similarity to known proteins		0.92	5.18
15237r1	Hypothetical protein (<i>Homo sapiens</i> DKFZp762N1412)		0.92	5.28
00084r1	Protein kinase C-like N2 (PRKCL2)	M	0.91	5.27
00325r1	No significant similarity to known proteins		0.91	5.33
15201r1	No significant similarity to known proteins		0.91	5.17
00469r1	No significant similarity to known proteins		0.91	5.22
02503r1	Ectonucleotide pyrophosphatase/phosphodiesterase 3 (E-NPP3)		0.90	5.31
11002r1	No significant similarity to known proteins		0.89	4.88
04506r1	No significant similarity to known proteins		0.87	5.22
14774r1	Mitochondrial tRNA gene cluster	M	0.86	5.18
14249r1	Non-ATPase proteasome 26S subunit 2		0.86	5.04

[†] Cluster ID is according to the *Ciona intestinalis* EST project web site (<http://ghost.zool.kyoto-u.ac.jp/indexr1.html>). When cluster ID is not available, clone ID (italicized) were indicated. [‡] M, mitochondria-like localization of mRNA. [§] U:D (1) and U:D (2) are calculated from data set 1 and 2, respectively.