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Genomes and Developmental Control

Transcriptome dynamics in early embryos of the ascidian, *Ciona intestinalis*

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ABSTRACT

Maternally provided mRNAs and proteins direct early development and activate the zygotic genome. Using microarrays, we examined the dynamics of transcriptomes during the early development of a basal chordate, *Ciona intestinalis*. Microarray analysis of unfertilized eggs, as well as 8-, and 16- and 32-cell embryos revealed that nearly half of the genes encoded in the genome were expressed maternally, and that approximately only one-fourth of these genes were expressed at similar levels among eggs obtained from different individuals. Genes encoding proteins involved in protein phosphorylation were enriched in this latter group. More than 90% of maternal RNAs were not reduced before the 16-cell stage when the zygotic developmental program begins. Additionally we obtained gene expression profiles of individual blastomeres from the 8- and 16-cell embryos. On the basis of these profiles, we concluded that the posterior-most localization, which has been reported for over 20 different transcripts, is the only major localization pattern of maternal transcripts. Our data also showed that maternal factors establish only nine distinct patterns of zygotic gene expression at the 16-cell stage. Therefore, one of the main developmental functions of maternally supplied information is to establish these nine distinct expression patterns in the 16-cell embryo. The dynamics of transcriptomes in early-stage embryos provides a foundation for studying how maternal information starts the zygotic program.

Introduction

The earliest developmental program in animal embryos is carried out with maternally supplied information, which subsequently triggers the zygotic program. This transition has been called the maternal-to-zygotic transition (MZT) (Tadros and Lipshitz, 2009). The MZT often coincides with the mid-blastula transition (MBT) (Newport and Kirschner, 1982; Schier, 2007); at the MBT, cell divisions become asynchronous and cell cycles become longer.

In embryos of a basal chordate, the ascidian *Ciona intestinalis*, cell division becomes asynchronous and the duration of cell cycles becomes longer after the 16-cell stage. In addition, 20 regulatory genes are specifically expressed to start zygotic developmental programs at the 16-cell stage (Bertrand et al., 2003; Hamaguchi et al., 2007; Hudson and Yasuo, 2005; Imai et al., 2004, 2006; Shi and Levine, 2008), while the earliest known zygotic gene expression (*FoxA-a* and *SoxB*1) starts at the 8-cell stage. These previous observations suggest that the MZT begins between the 8- and 16-cell stages in the ascidian embryo. In the present study, we examined the transcriptome dynamics of early *Ciona* embryos around the beginning of the MZT to understand how maternal factors begin zygotic programs. We especially focused on the following three points.

First, since no inbred strains of *C. intestinalis* are available, animals with different genetic backgrounds have been used for experiments in this species. While expression patterns of most of the genes that have been examined are highly reproducible in different animals, several genes are known to show variability in expression patterns. For example, the expression of *Otx* in a6.7 blastomeres at the 32-cell stage is seen in 6% of embryos (Tassy et al., 2006). We also empirically know that the expression of *AP2-like-2* in A5.2 at the 16-cell stage (Imai et al., 2004) is not observed in all embryos (see below). In the present study, to examine whether and how extensively such variation is seen in the maternal mRNA population, we examined the transcriptomes of unfertilized eggs obtained from 11 different adult animals.

Second, localized maternal mRNAs and the spatially controlled destabilization of maternal mRNAs are one of the key mechanisms evoking zygotic programs in a spatially regulated manner (Bashirullah et al., 2001). Because ascidian eggs have historically been thought to be typical “mosaic” eggs, extensive efforts have been made to identify localized mRNAs in early embryos, and over 20 localized maternal mRNAs encoded in the nuclear genome have been identified (Nishida and Sawada, 2001; Sardet et al., 2005;

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Sasakura et al., 2000; Satou and Satoh, 1997; Yamada et al., 2005; Yoshida et al., 1996). These mRNAs are all localized in the posterior pole of the embryo, and therefore they are called posterior end mark (pem) RNAs (or postplasmic RNAs). In an attempt to identify localized RNAs distinct from pem RNAs, a previous study compared the animal half of the 8-cell embryo with the vegetal half, and also the posterior vegetal cells of the 8-cell embryo with the remaining part of the embryos using microarrays (Yamada et al., 2005). However, it remains unclear whether there are maternal mRNAs localized in other subcellular regions or in particular cells of early *Ciona* embryos. For instance, mRNAs localized in the anterior animal cells would not have been identified in this previous microarray study (Yamada et al., 2005). In order to address this ongoing question, here, we analyzed the transcriptomes of individual blastomeres from the 8-cell and 16-cell embryos.

Third, localized and unlocalized maternal gene activity initiates zygotic gene activity. As described earlier, previous comprehensive expression profile assays of regulatory genes identified 20 zygotically activated genes at the 16-cell stage (Bertrand et al., 2003; Hamaguchi et al., 2007; Hudson and Yasuo, 2005; Imai et al., 2004, 2006; Shi and Levine, 2008) (see Fig. S5A). There were only seven variations on the expression profiles of these 20 genes, a much smaller number than the theoretically possible upper limit of 255 ($=2^{(16/2)}-1$: the ascidian embryos are believed to be bilaterally symmetrical). This observation indicates that maternal factors set up a simpler pre-pattern to restrict subsequent patterning decisions to a subset of options. However, it still remains unclear whether these seven identified patterns of gene expression represent the entirety of the early-stage zygotic expression patterns. To understand how maternal factors initiate zygotic programs, in the present study, we tried to obtain the whole repertoire of zygotic genes initially activated by maternal factors.

Materials and methods

C. intestinalis embryos and cDNA clones

All of the *C. intestinalis* adults we used in the present study were raised in the sea near the Maizuru Fisheries Research Station of Kyoto University under the National Bio-Resource Project for *Ciona*, and were maintained in aquaria. Eggs from batches c and d (Fig. 1A) were pushed out by hand from their gonoducts. For the rest of the animals, eggs and sperm were surgically obtained from gonoducts. Unfertilized eggs were collected after dechorionation. Embryos were dechorionated after insemination, and reared at 18 °C in filtered seawater. We kept a fraction of embryos at each sampling for checking the ratio that they developed into morphologically normal larvae. We used only eggs and embryos, for which at least 60% of the cohort developed into normal larvae (Table S1). cDNA clones were obtained from our EST clone collection (Satou et al., 2005). Blastomeres were isolated with a fine glass needle under a binocular microscope, and isolated blastomeres were immediately stored in RNAlater (Ambion) until RNA extraction.

Microarray

Total RNA was prepared from unfertilized eggs, embryos and isolated blastomeres with an RNeasy Mini Kit (QIAGEN). After checking the quality of the extracted RNA with a Bioanalyzer 2100 (Agilent), 50 ng of RNA was amplified and labeled with Cy3 using a Low Input Quick Amp Labeling Kit (one-color kit; Agilent Technologies). We designed two microarrays, manufactured by Agilent Technologies, which contained 39,652 and 218,674 non-overlapping probes covering 15,243 and 15,255, respectively, out of 15,274 KH gene models (version 2010) (Satou et al., 2008a) (we call them 40k- and 220k-arrays; GEO accession numbers: GPL16870 and GPL16886). We followed the manufacturer's instructions for hybridization and washing. Fluorescence intensity was scanned with a G2505C microarray scanner (Agilent Technologies) and quantified with Agilent Feature Extraction software (version 10). Some probes that were judged beyond analysis by the software were eliminated from the following analysis. The microarray data is deposited in the GEO database under the accession number GSE45575.

Raw signal values were normalized for comparison among different arrays on the basis of an assumption that the majority of genes are not differentially expressed. This assumption is commonly made, and is not likely to be violated in our experiment. For calculations, we first excluded both the 30% of probes exhibiting largest differences between the arrays, and the probes that corresponded to the top and bottom 5% with respect to signal strength. Then, the average signals of the remaining probes were calculated. Next, we adjusted all signal values so that the average signals were equal. After this normalization among arrays, we calculated the average signals for each gene; the 40k- and 220k-arrays contained an average of 2.6 and 14.3 probes per gene, respectively. The sensitivities, and thus signal strengths, differed among the probes for a single gene. Therefore, when we performed statistical tests by using multiple probes for a single gene as technical replicates, we normalized the signal values of these probes as follows. First, we summed up all of the values for each probe across arrays. Second, for every gene, the maximum value among the sums was selected, and the signal values for each probe were adjusted so that the sums of the values were equal to this maximum value. All values were log-transformed for statistical tests. Gene ontology analysis was performed with the Blast2Go program (Conesa et al., 2005). The enrichment analysis with the two-tailed Fisher's exact test is implemented in this software.

In situ hybridization and RT-qPCR

DIG-RNA probes for whole-mount in situ hybridization (WMISH) were synthesized by in vitro transcription with T7 RNA polymerase. A detailed procedure for WMISH has been previously described (Imai et al., 2004).

For reverse transcription and quantitative PCR (RT-qPCR), the same amount of total RNA from five different batches was reverse transcribed by an MMLV reverse transcriptase (Invitrogen). Quantitative PCR was performed using a StepOnePlus PCR machine with the SYBR green method (Invitrogen). The same cDNA pools were used for all reactions. Amplification of a specific product in each reaction was confirmed by determining a dissociation curve. The following primers were used: 5′-AGTTACAGCACCCAGAGAACCAA-3′and 5′-CGTCAATGAGCCGTTTTGTG-3′ for KH.C1.29; 5′-GGAA ATGGACGTTGACCAGAGA-3′ and 5′-GGCGAGAGGAGCGGACTT-3′ for KH.C7.98; 5′-AGTGACGTCGGCGAGGAA-3′ and 5′-CCAAATCGT-TAACCAAGTACTCTGTGT-3′ for KH.C3.605; 5′-TAGGCTTAGCGGAG CCACAA-3′ and 5′-CACACACCGGATCGAATACG-3′ for KH.C4.191; 5′-GGTCCTCCTGGGAATAAAGGTTT-3′ and 5′-TGGGAAGCGTATGAA GTTGCT-3′ for KH.C4.36; 5′-TGCTTACGCCTGGAATGTCA-3′ and 5′-CAAGCACTGTGCCCTGAATG-3′ for KH.C9.710; 5′-TCGGAGCCAGT-GAGCATCT-3′ and 5′-TCGTGCATTCCTCCAACCAT-3′ for KH.C11.314; 5′-AAGAAATGCAATCACATACTGCAATAG-3′ and 5′-TAGCAGACATCG GTTCTACGATTC-3′ for KH.S1081.1; 5′-CTTGTGGGCCTGGGTGTAGA-3′ and 5′-TGACATCCCGCCACACACT-3′ for KH.S1462.1; 5′-ACCA-GAGTTACGGCCATCATTAC-3′ and 5′-ACTCGCTGATGATAAACTTCCAT GT-3′ for KH.S606.2.

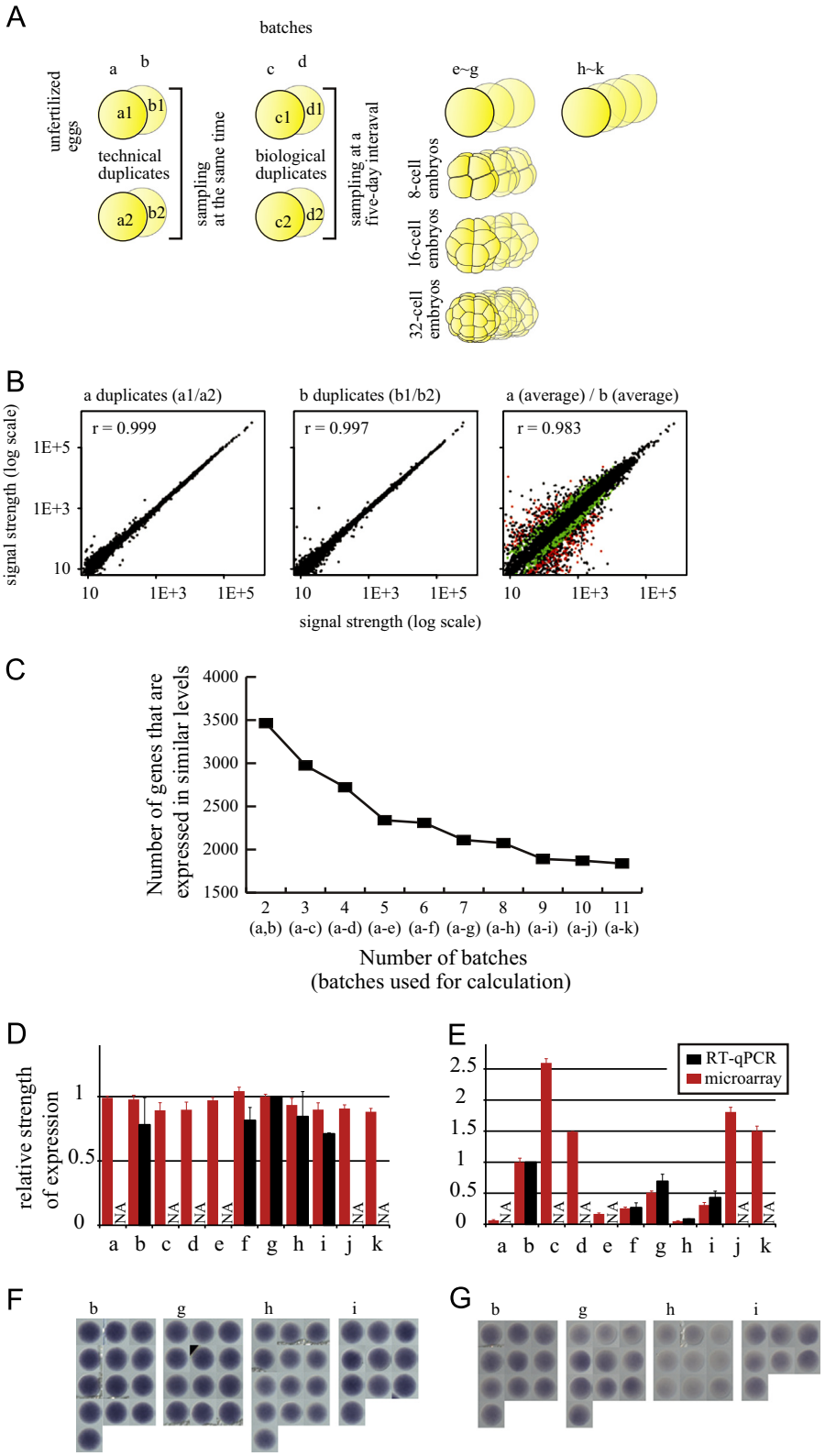

Fig. 1. A significant fraction of maternal genes are differentially expressed. (A) Diagram showing the samples used in the microarray experiments analyzing whole eggs and embryos. (B) (Left and middle graphs) Comparisons of the signal intensities observed in microarray data for technical duplicates of unfertilized eggs from batches a and b. (Right graph) A comparison between the biological duplicates (*y*-axis, batch a; *x*-axis, batch b). Red and green dots indicate genes that showed a > 4-fold significant difference and a 2 to 4-fold significant difference, respectively, between the duplicates (FDR < 5%). Both axes are on a log scale. (C) Relationship of the number of genes that are expressed at similar levels among batches (*y*-axis) and the number of batches tested (*x*-axis). (D, E) Relative strength of expression among the 11 batches of eggs for (D) an S-group gene, KH.C7.98 and (E) an HDF-group gene, KH.C1.29. Red and black bars indicate the relative expression as measured by microarrays and RT-qPCR, respectively. The relative strength was calculated against the signal from the batch with the highest expression detected by RT-qPCR (batches g and b in (D) and (E), respectively). RT-qPCR was not performed for eggs in batches a, c, d, e, j and k (indicated by 'NA'), because RNA was not available. Error bars indicate standard errors. (F, G) In situ hybridization of unfertilized eggs from batches b, g, h and i for genes shown in (D) and (E).

Results and discussion

Maternal genes are differentially expressed among different batches of embryos

First, we used microarrays to determine the gene expression profiles of unfertilized eggs from the ascidian *C. intestinalis*. Two biological duplicates, which were obtained from two different adults, were examined separately (batches, a and b; Fig. 1A). For these two biological duplicates, we also prepared technical duplicates (a1/a2 and b1/b2), in which the same RNA sources were separately labeled. The microarrays used in this experiment (40k-array) include 39,652 different probes for 15,243 genes.

As shown in Fig. 1B, microarray signals were highly reproducible between the technical duplicates (correlation coefficients, $r=0.999$ for a1 and a2, and $r=0.997$ for b1 and b2), but were not so much strongly reproducible between the biological duplicates ($r=0.983$ between the averages of a1/a2 and b1/b2, and $r=0.981$–0.985 among individual arrays; Fig. S1). A statistical test revealed that of the 7377 genes whose expression was detectable in the microarrays, 1890 were differentially expressed between the eggs from batches a and b (Welch's *t*-test with multiple testing correction by the Benjamini and Hochberg method, BH-method; False discovery rate (FDR) $<5\%$), and 589 of these differed by more than 2-fold in expression levels (226 differed by more than 4-fold). These observations raised the possibility that a substantial number of maternal genes are expressed differentially among eggs from different individuals.

To test this possibility, we collected eggs twice from the same two adult animals (c and d) at a five-day interval (c1/c2, and d1/d2). The correlation coefficients between duplicates from the same batches ($r=0.996$ for c1 and c2, and $r=0.997$ for d1 and d2) were higher than those between batches c and d ($r=0.953$–0.957; Fig. S1). Since these animals were incubated in the same tank during the interval between the two sampling time points, genetic differences, rather than environmental factors, were likely to be the primary reason for these differences in transcriptomes.

We examined seven additional batches (e–k) to determine the extent of differential expression of maternal genes (Fig. 1A). The expression of 7388 genes was observed at least once in the eleven batches (a–k). As shown in Fig. S1, all of these replicates demonstrated larger inter-batch differences ($r=0.883$–0.989) than the intra-batch differences we observed between the duplicates of a1/a2, b1/b2, c1/c2 and d1/d2 ($r=0.996$–0.999). This differential expression is unlikely to be related to the rates, in which eggs successfully develop into morphologically normal larvae, for the following three reasons. First, we used only batches from which more than 60% of the eggs successfully developed into larvae (Table S1). Second, the lowest correlation coefficient was observed between batches c and i (Fig. S1), which both displayed high developmental success rates (92–93% and 96%). Third, the correlation coefficient between batches with the highest and lowest rates of successful larval development (j and e) was not low ($r=0.973$) compared with coefficients between the other batches.

In the following analysis, we consider that genes with coefficients of variation (CVs, which are defined as the ratio of the standard deviation to the average and provide a normalized measure of dispersion) of <0.15 among the replicates were expressed at similar levels among the batches, and collectively call them the S-group. This cut-off value was empirically determined on the basis of the observation that the CVs of over 95% of genes between the technical duplicates (95.4% for batch a and 95.3% for batch b) were <0.15. The S-group contained 1838 genes with a maximum of 2.05-fold differences in expression. We referred to the remainder of the genes as the DF-group (5550 genes). Among the DF-group genes was a subset, called the HDF-group (2391 genes), that showed a difference of >4-fold in expression. Maximum signal values of individual S-, DF- and HDF-group genes among the microarrays are plotted against the CVs in Fig. S2, showing that more S-group genes are expressed in high levels, and more DF-group genes are expressed in low levels.

All of the genes in these groups are listed in Table S2. Fig. 1C shows that the number of genes that were categorized into the S-group decreased as the number of replicates increased. However, it also shows that the rate of decrease in the number of the S-group genes became smaller after the number of replicates had reached five, and the number of the S-group genes rarely decreases after the number of replicates had reached nine. Therefore, the number of the S-group genes was unlikely to decrease greatly from the current estimation, even if we examined more batches of eggs.

Experimental validation of maternal genes that are expressed stably and differentially between batches

We randomly picked up two S-group genes and eight HDF-group genes, and examined their expression levels of them in five batches (b, f, g, h and i) using an RT-qPCR assay (Fig. 1D, E and Fig. S3). Unfertilized eggs from batches b, g, h and i were further examined by in situ hybridization; we could not obtain sufficient quantities of eggs from the batch f, and therefore this batch was not examined by in situ hybridization. We examined all of the eggs under the same conditions, and the visualization reaction was stopped after the same duration for each of the genes examined. Photographs of all of the eggs were taken using the same conditions for each gene (Fig. 1F, G and Fig. S3).

The expression of an S-group gene, KH.C7.98 encoding a homolog of serine/threonine-protein kinase PAK1, was detected almost uniformly among the batches using the microarray assay, and our RT-qPCR assay of eggs from batches b, f, g, h and i confirmed this result (Fig. 1D). In situ hybridization also supported this finding; every egg from batches b, g, h and i showed a similar level of signal strength (Fig. 1F). In addition, the expression was detected almost uniformly within each of batches b, g, h and i. Similarly, we confirmed that another S-group gene, KH.S606.2 encoding a homolog of mitogen-activated protein kinase 6, was expressed almost equally in eggs from batches b, f, g, h and i using RT-qPCR, and in eggs from batches b, g, h and i using in situ hybridization (Fig. S3A). By contrast, the microarray and qPCR assays both consistently indicated that the HDF-group gene, KH.C1.29 encoding a protein without similarity to known protein, was expressed most strongly in batch b, moderately in batches f, g and i, and weakly in batch h (Fig. 1E). In agreement with this result, in situ hybridization gave stronger signals in eggs from batches b, g and i, and weaker signals in eggs from batch h (Fig. 1G). The expression was not necessarily detected uniformly within each of batches b, g, h and i. Using the same approach, we confirmed the differential expression of five additional genes (Fig. S3B–F). Thus, a significant fraction of maternal genes were indeed expressed differentially between batches.

Characterization of the S- and HDF-group genes

To understand why only a fraction of maternal genes are expressed at similar levels among batches, we examined whether the S-group genes shared specific functions using Gene Ontology (GO) annotation. A comparison of the S-group genes with the whole genome showed that 22 GO terms in a Gene Function category were significantly overrepresented among the S-group genes (FDR $<1\%$; Table 1). Among these, 18 were related to kinase/phosphatase activity (kinase activity, pyrophosphatase activity, and ATP binding). Perhaps in relation to this observation, a total of seven overrepresented Biological Process terms concerned

Table 1
GO terms in the Molecular Function Ontology that were significantly over-represented in the S-group.

GO terms	FDR
Kinase Activity	
GO:0004674 protein serine/threonine kinase activity	4.2E−03
GO:0004672 protein kinase activity	2.5E−03
GO:0016773 phosphotransferase activity, alcohol group as acceptor	9.2E−04
GO:0016301 kinase activity	4.4E−04
GO:0016772 transferase activity, transferring phosphorus-containing groups	4.4E−04
Pyrophosphatase activity	
GO:0016462 pyrophosphatase activity	4.7E−03
GO:0016817 hydrolase activity, acting on acid anhydrides	4.0E−03
GO:0016818 hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides	4.7E−03
GO:0017111 nucleoside-triphosphatase activity	2.2E−03
ATP binding	
GO:0000166 nucleotide binding	1.1E−04
GO:0032553 ribonucleotide binding	1.8E−04
GO:0035639 purine ribonucleoside triphosphate binding	5.6E−05
GO:0032555 purine ribonucleotide binding	1.8E−04
GO:0017076 purine nucleotide binding	3.1E−04
GO:0005524 ATP binding	2.6E−04
GO:0030554 adenyl nucleotide binding	5.6E−04
GO:0032559 adenyl ribonucleotide binding	5.6E−04
GO:0005488 binding	7.8E−08
Miscellaneous	
GO:0003676 nucleic acid binding	5.3E−03
GO:0003743 translation initiation factor activity	8.4E−03
GO:0005515 protein binding	3.0E−13
GO:0019899 enzyme binding	2.2E−05

Table 2
Cell-cycle related GO terms in the Biological Process Ontology that were significantly over-represented in the S-group.

GO terms	FDR
GO:0000087 M phase of mitotic cell cycle	2.0E−04
GO:0000278 mitotic cell cycle	6.2E−07
GO:0000279 M phase	4.3E−06
GO:0007049 cell cycle	6.2E−08
GO:0007067 mitosis	2.2E−03
GO:0022402 cell cycle process	2.8E−08
GO:0022403 cell cycle phase	1.2E−07

mitosis and the cell cycle (Table 2). Indeed, *Cdk*1, *Cdk*6, *Cdk*9, and *Cdk*11 were included in this gene set. This observation suggests the possibility that the expression of these genes needs to be maintained at fixed levels for their function.

Next, we compared the HDF-group genes with the whole genome, and found that only two terms among the Molecular Function category were overrepresented (FDR < 1%). These terms were related to transporter activity (GO:0005342:organic acid transmembrane transporter activity, FDR=8.7E−03; GO:0046943:carboxylic acid transmembrane transporter activity, FDR=8.7E−03). Although this result cannot be directly interpreted, the existence of these enriched GO terms suggests that the difference in expression levels of at least a portion of maternal genes is regulated but not random.

Finally we examined genes for transcription factors and signaling molecules. We previously comprehensively identified these genes in the *Ciona* genome (Hino et al., 2003; Imai et al., 2004; Satou et al., 2003a, 2003b, 2008b; Wada et al., 2003; Yagi et al., 2003; Yamada et al., 2003). First, we examined genes for transcription factors with well-known motifs, i.e. bHLH, bZIP, Ets, Fox, HMG, homeodomain, nuclear receptor and T-box. There are 259 genes encoding transcription factors with these motifs (Table S3). Of them, 115 genes were maternally expressed. Among them, 29 were the S-group genes. A statistical test did not support a hypothesis that the transcription factor genes are under- or over-represented in the S-group (a two-tailed Fisher's exact test, p-value=0.77). Similarly, 40 were the HDF-group genes, and a hypothesis that the transcription factor genes are under- or over-represented in the HDF-group was not statistically supported (a two-tailed Fisher's exact test, p-value=1.00). Second, we examined genes for signaling ligands of the Ephrin, FGF, TGFβ, Wnt, hedgehog and Delta/Notch families. There are 38 genes encoding signaling ligands of these families (Table S4). Of them, 25 genes were maternally expressed. Among them, three were grouped into the S-group. A statistical test did not support a hypothesis that the signaling ligand genes are enriched in the S-group (a two-tailed Fisher's exact test, p-value=0.62). Similarly, 11 were regarded as HDF-group genes, and a hypothesis that the signaling ligand genes are under- or over-represented in the HDF-group was not statistically supported (a two-tailed Fisher's exact test, p-value=0.040). Thus, these regulatory genes were not over- or under-represented in the S- and HDF-groups.

In summary, among approximately 16,000 genes encoded in the *Ciona* genome (Dehal et al., 2002; Satou et al., 2008a), 7388 genes were expressed at least in one batch that we tested. This ratio of maternally expressed genes is not greatly different from that observed in other animals; 40% in mice, 65% in flies and 75% in sea urchins (Lecuyer et al., 2007; Tadros and Lipshitz, 2009; Wang et al., 2004; Wei et al., 2006). However, in *Ciona*, only a quarter of these maternal mRNAs were expressed at similar levels among eggs from different animals. At least part of these S-group genes are likely to be carefully regulated (with similar levels in every batch) to ensure proper function. Expression of at least a part of the HDF-group genes is also likely to be controlled for their function. However, this does not necessarily mean that the expression levels of all of the maternal genes are regulated; difference in the expression level of certain classes of genes may not be important in early embryos.

Dynamics of maternal RNAs in early embryos

To examine the dynamics of maternal mRNAs, we examined the gene expression profiles of 8-, 16- and 32-cell embryos from batches e, f and g (Fig.1A). We first confined our analysis to the S-group genes. The correlation coefficients of the S-group genes were 0.989–0.991 in unfertilized eggs from these batches (Table S5). Similarly, the correlation coefficients of these genes at the 8-, 16, and 32-cell stages among batches e, f and g were not very different (r=0.987–0.994, 0.988–0.990, 0.990–0.992; Table S5). Most of the S-group genes likely behaved similarly among these three batches up to the 32-cell stage.

Among the 1838 S-group genes, there were only 41 genes that showed > 2-fold and statistically significant differences in expression from the unfertilized egg to the 32-cell stages (analysis of variance, ANOVA, with the three biological replicates, batches e, f and g, with multiple testing correction by the BH method; FDR < 1%). Of these, 37 genes were expressed strongly in unfertilized eggs, and decreased gradually, while the remaining four genes were strongly expressed in 32-cell embryos. In summary, as shown at the left of Fig. 2A, only 2.0% (=37/1838) of the S-group maternal RNAs decreased before the 32-cell stage (e.g., Fig. 2B), 0.2% (=4/1838) increased, and the remaining 97.8% did not change significantly before the 32-cell stage (e. g., Fig. 2C).

Because the DF-group genes were differentially expressed in unfertilized eggs, we examined these three batches individually. In batch e, 438 DF-group genes (7.8%=438/5620) showed a > 2-fold and statistically significant difference between the unfertilized egg and the 32-cell stage (ANOVA with two or three different

probes per gene in single arrays: FDR < 1%). For batches f and g, the corresponding numbers were 8.1% and 4.8%, respectively. Of the 438 DF-group genes, 180 genes in batch e (3.2%=180/5620) were more strongly expressed in unfertilized eggs (e.g., Fig. 2D); the corresponding numbers were 3.6% and 2.1% for batches f and g, respectively. On the other hand, 258 genes in batch e (4.6%=258/5620) were more strongly expressed in 32-cell embryos (e.g., Fig. 2E and F); the corresponding numbers were 4.5% and 2.7% for batches f and g, respectively. The ratio of the latter group of genes was markedly higher in the DF-group than in the S-group (black bars in Fig. 2A). It is possible that initial differences in the expression of some genes, e.g., KH.C12.648, in this latter group were partially resolved at later stages by up-regulation (Fig. 2F). However, this is obviously not the only reason for the higher ratio, because the difference in expression levels of KH.C7.805 increased at the 32-cell stage (Fig. 2E). More importantly, the expression of a majority of the group-DF genes (91.9% or more) was not markedly changed prior to the 32-cell stage (e.g., Fig. 2G).

In the former group of genes, 68 genes were expressed more strongly in unfertilized eggs of batches e, f and g in common, although no specific GO terms were enriched. On the other hand, in the latter group of genes, 106 genes were more strongly expressed in 32-cell embryos of batches e, f and g in common. Compared with the whole genome, four GO terms among the Molecular Function category were overrepresented in these 106 genes (FDR < 1%). These terms were 'GO:0008083:growth factor activity' (FDR=3.9E−05), 'GO:0008047:enzyme activator activity' (FDR=3.0E−03), 'GO:0005102:receptor binding' (FDR=5.2E−3), and 'GO:0001078:RNA polymerase II core promoter proximal region sequence-specific DNA binding transcription factor activity involved in negative regulation of transcription' (FDR=5.9E−3), although this result cannot be directly interpreted.

Gene expression profiles of individual blastomeres at the 8-cell and 16-cell stage

With the start of MZT around the 8- to 16-cell stages, developmental fates indeed begin to be specified (Fig. 3A). We manually isolated individual blastomeres from 8- and 16-cell embryos to obtain gene expression profiles with single cell resolution. Individual blastomeres of the early embryos can be easily identified by their morphology. Approximately 1000 isolated blastomeres were used for a single experiment. We did not take biological replicates, because for each experiment the blastomeres were obtained from multiple batches of embryos, and also because the manual isolation of blastomeres was time-consuming; 2 or 3 months were required to collect sufficient material to represent a single blastomere. Instead we used a microarray bearing up to 15 different probes per gene (220k-arrays), and deviations among probes within the same gene were used for statistical evaluation. In addition, as shown below, we used in situ hybridization to confirm the microarray results.

First, we established that previously known localized maternal mRNAs were successfully identified in our microarray data. An earlier microarray study (Yamada et al., 2005) showed that 17 mRNAs were asymmetrically localized in the posterior vegetal blastomere (B4.1). Of these maternal mRNAs, 12 are called type-I pem RNAs, and are strictly localized in the posterior blastomeres. The remaining five pem mRNAs are called type-II pem mRNAs, and are distributed in all blastomeres, but are enriched at the posterior pole. The localized expression of these 17 pem-mRNAs was reproduced at the 8-cell and 16-cell stages in our assay (Fig. S4; *Ci-POPK*1 and *Ci-pen*1 in Fig. 3B and D). Specifically, signals in B4.1 were stronger than those in the other three pairs of blastomeres and the difference was statistically significant (ANOVA corrected with the BH-method, FDR < 1%). One type-I pem RNA, *Ci-midnolin*, showed a slightly smaller difference than did two type-II pem RNAs, *Ci-pem*13 and *Ci-DOPZ*. Because these genes are DF-group genes, differential expression of these maternal mRNAs among batches might affect the results. It is also possible that these mRNAs might have been improperly categorized into the type-I or type-II groups in the previous study (Yamada et al., 2005).

Next, we examined the expression of zygotically activated mRNAs. Previous studies showed that 13 transcription factor genes and seven signaling ligand genes begin to be expressed zygotically at the 16-cell stage (Bertrand et al., 2003; Hamaguchi et al., 2007; Hudson and Yasuo, 2005; Imai et al., 2004, 2006; Shi and Levine, 2008) (Fig. S5A). The described expression patterns of these genes were nearly reproduced by our microarray experiment (Fig. S5B), with a few exceptions. First, the zygotic expression of genes with strong maternal expression was not well reproduced. These genes were *Orphan Fox*-1, *Orphan Fox*-2, *Hes-a*, *Zinc Finger C2H2*-2, *Orphan TGFβ* (*Gdf*1/3-*like*), *SoxF*, *GABPα* and *Smad*2/3*b* (shown in blue in Fig. S5AB). Second, the probes for *SoxF* did not give positive signals. Furthermore, the gene model set that we used did not predict the *Admp* locus correctly, and therefore no probes were designed for *Admp*. For these reasons, we could not obtain data on *SoxF* and *Admp*. Third, the expression of *AP*-2-*like*2 in A5.2 was barely detected in the microarrays. We re-examined this expression by in situ hybridization, and found that the expression of *AP*-2-*like*2 in A5.2 was not observed in all embryos, which might explain why the expression in A5.2 was barely detected in the microarrays (Fig. S5C). Similarly, a re-examination of *GABPα* yielded consistent results between the microarray and in situ hybridization data, both showing that this gene was expressed in A5.2 and B5.1 (shown in gray in Fig. S5A).

Thus, previously identified maternal and zygotic gene expression was successfully confirmed in our microarray, indicating the reliability and quality of our data. Accordingly, our microarray data will provide a foundation for understanding transcriptome dynamics with single cell resolution in early *Ciona* embryos.

Identification of localized mRNAs in the 8-cell and 16-cell embryos

We used the microarray results to examine whether there were unknown localized or asymmetrically distributed maternal mRNAs. To avoid the unpredictable effects of differential expression of maternal mRNAs among different batches of embryos, we confined our analysis to the S-group genes. There were six genes that were observed in a single pair of blastomeres of the 8-cell embryo at > 2-fold the levels detected in any of the other blastomeres (ANOVA with the BH-correction; FDR < 1%) (Fig. 3B). Of these, two were known pem genes (*Ci-POPK*1 and *Ci-pen*1), which are the only type-I pem genes in the S-group. In addition, in situ hybridization showed that KH.C9.853 mRNA, which encodes a protein with four tudor domains, was indeed localized in the posterior pole of the embryo (Fig. 3C); therefore, this gene was classified as a novel member of the pem-group. In situ hybridization failed to confirm localization of the remaining three mRNAs (KH.C2.638, KH.C2.562, and KH.C8.816).

Pem mRNAs are localized in a small region of the posterior vegetal blastomeres, not throughout the entire cytoplasm. Because of this trait, pem mRNAs are relatively easily detected by in situ hybridization. By contrast, if KH.C2.638, KH.C2.562, and KH.C8.816 mRNAs were enriched throughout the entire cytoplasm of specific cells, such localization would not be detected by in situ hybridization. Therefore, even if these latter three mRNAs are localized differently from pem mRNAs, we can conclude that there are no S-group mRNAs that are localized more clearly than the pem mRNAs in the 8-cell embryo.

There were 58 genes that passed the same criteria of preferential localization to one pair of blastomeres at the 16-cell stage (Fig. S6). Two pem mRNAs, *Ci-POPK*1 and *Ci-pen*1, showed the largest

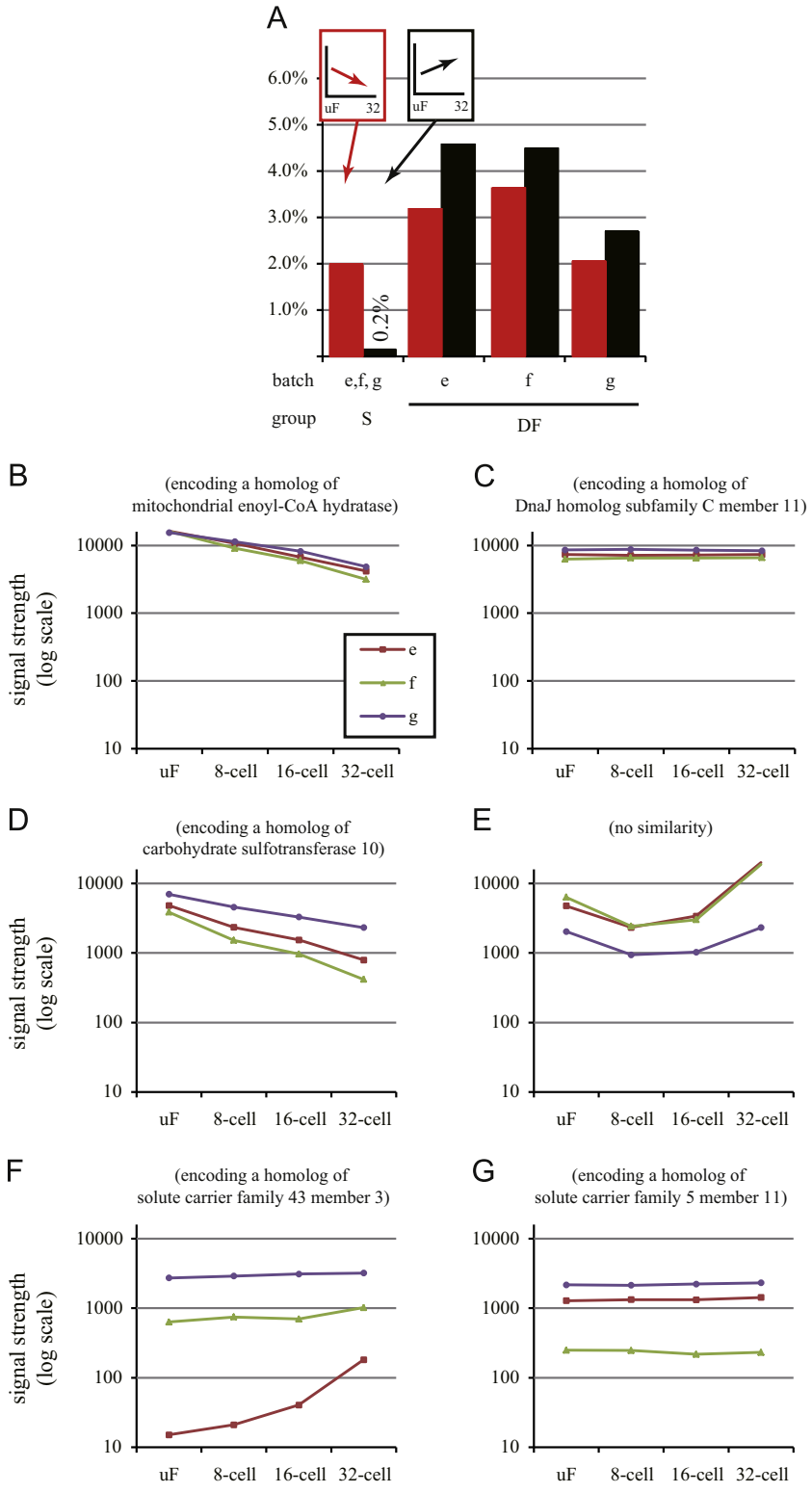

Fig. 2. Expression profiles of maternal genes in unfertilized eggs, and in 8-, 16-, and 32-cell embryos. (A) Summary of the expression profiles of the S-group and DF-group genes in early embryos. Genes with expression levels that decreased or increased more than 2-fold (FDR < 1%) between the unfertilized egg and the 32-cell stage are shown by red and black bars, respectively. Note that the rest of the genes (90%~) do not change greatly between the unfertilized egg and the 32-cell stage. (B–G) Expression profiles (B, C) of two S-group genes (KH.C2.338 and KH.C4.798) and (D–G) four DF-group genes (KH.C6.217, KH.C7.805, KH.C12.648, and KH.C1.909) from the unfertilized egg to the 32-cell stage. Red, green, and purple lines indicate the expression levels in batches e, f, and g, respectively.

differences in localized signal strength. Therefore, there are unlikely to be S-group mRNAs that are localized more clearly than the pem mRNAs at the 16-cell stage. Indeed, the expression of seven of the remaining 56 genes was previously examined at the 8-cell stage by in situ hybridization (Nishikata et al., 2001), and none of them are localized (these seven genes are indicated in red in Fig. S6).

For further confirmation, we chose genes that showed clear binomial distributions of signal values, because gradual changes

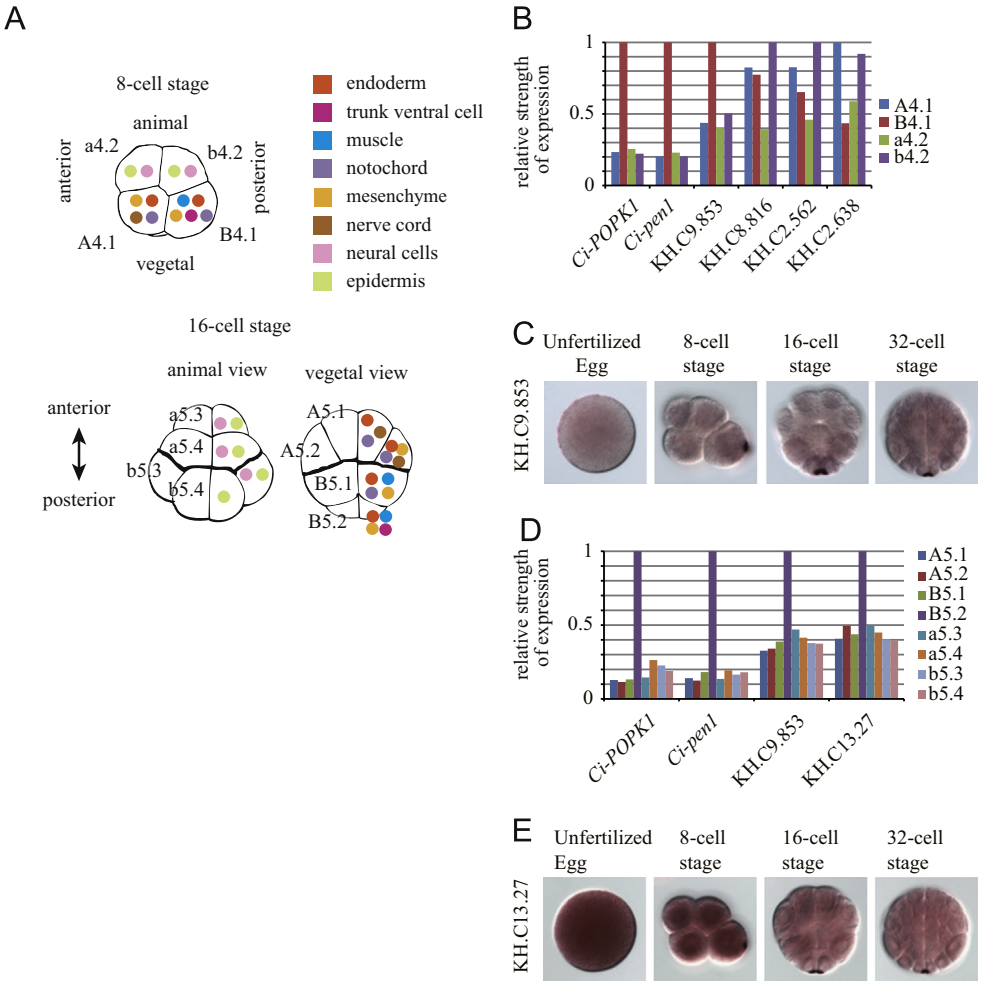

Fig. 3. Asymmetrical distribution of maternal genes in individual blastomeres at the 8- and 16-cell stages. (A) Schematic representation of the bilaterally symmetrical 8- and 16-cell embryo. Developmental fates of individual blastomeres are depicted as colored circles. The color code is shown in the upper right. Each of these blastomeres was isolated with a fine glass needle and analyzed by microarray. (B) The relative expression levels of six genes that are detected in a single blastomere of the 8-cell embryo with a signal at least 2-fold stronger than that observed in either of the remaining blastomeres (FDR < 1%). The relative strength for each gene was calculated against the signal from the blastomere that showed the strongest expression. (C) Expression of KH.C9.853 as revealed by in situ hybridization at the unfertilized egg, and the 8-, 16-, and 32-cell stages. (D) The relative expression levels of four genes that exhibited binomial distribution of signals at the 16-cell stage (see the text for details). The relative signal strength for each gene was calculated against the signal from the blastomere that showed the strongest expression (B5.2 in all cases). (E) Expression of KH.C13.27 as revealed by in situ hybridization at the unfertilized egg, and the 8-, 16-, and 32-cell stages.

were unlikely to be detectable by in situ hybridization. For this purpose, we sorted the signal values of the individual blastomeres for each of the 58 genes of interest. Then we compared the neighboring values in the sorted order, and picked up the maximum ratio of the difference between the neighboring values for each gene. The ratios of four genes exceeded two (Fig. 3D). Of these, three were pem genes (*Ci-POPK*1, *Ci-pen*1, and KH.C9.853), and the remaining one (KH.C13.27, which encodes a protein with no similarity to known proteins) was also a novel pem-group gene, as we revealed by in situ hybridization (Fig. 3E). Again, we could not find S-group mRNAs that were localized more clearly than pem mRNAs in the 16-cell embryo.

In addition to the pem mRNAs, a previous study (Yamada et al., 2005) showed that mRNAs encoded in the mitochondrial genome are asymmetrically distributed within early embryos; their distribution pattern resembles the distribution of mitochondria. The microarrays used in the present study did not include probes for mitochondrial genes.

The above observation cannot rule out the possibility that in the DF-group there were maternal mRNAs asymmetrically distributed in early embryos, and that their asymmetrical pattern was different from pem-mRNAs. However, there is no indication that such localized mRNAs would be included only in the DF-group. In addition, only pem mRNAs have been identified as localized maternal mRNAs despite extensive previous efforts (Imai et al., 2004; Nishida and Sawada, 2001; Nishikata et al., 2001; Sasakura and Makabe, 2002; Satou, 1999; Satou and Satoh, 1997; Yoshida et al., 1996). Therefore, we prefer the hypothesis that there are no mRNAs that were more clearly localized than pem mRNAs, even in the DF-group genes.

Zygotically expressed genes at the 16-cell stages

Since the zygotic expression of genes with maternal expression was poorly discriminated using the microarrays (Fig. S5), here, we considered genes that were absent or rarely expressed in the maternal transcriptome, but strongly zygotically expressed in specific cells of the 16-cell embryo. For this purpose, we first selected genes that were not maternally expressed, and genes that were expressed maternally at low levels as defined by the following criteria: <50 signal strength for all probe singals for a given gene on the 40k-microarrays when hybridized to samples from unfertilized eggs. Second, of these candidates, we picked only genes with strong expression in at least one cell of the 16-cell

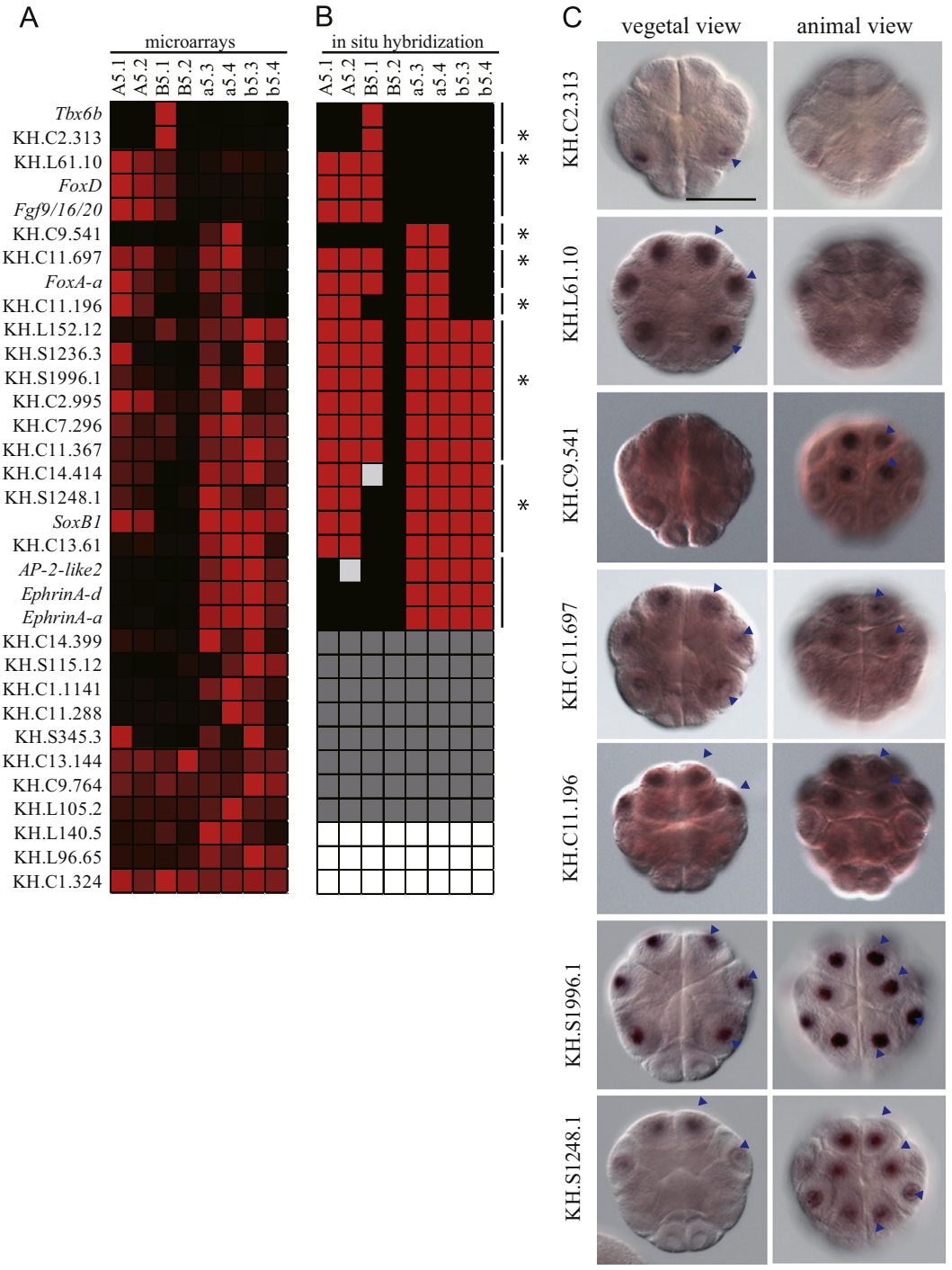

Fig. 4. Genes zygotically activated at the 16-cell stage. (A) A heat map of the microarray data showing expression of genes that were zygotically activated at the 16-cell stage. (B, C) Gene expression patterns examined by in situ hybridization. (B) A summary of the in situ hybridization results. Red boxes indicate clear zygotic expression, whose signal is detected in nuclei. Light gray boxes indicate expression that was observed in only part of the examined embryos. Dark gray boxes demark genes for which expression was not clearly revealed. White boxes specify genes for which expression was not investigated. Eight lines to the right of the heat map show groups with similar gene expression patterns. Asterisks indicate genes whose expression patterns are shown in (C). Expression patterns of the remaining genes are shown in Fig. S7, with the exception of the previously analyzed regulatory genes. (C) Photographs depicting the in situ hybridization of genes at the 16-cell stage. Expression in the right halves is indicated by arrowheads. The scale bar indicates 100 μm.

embryo (microarray signals > 50). Third, of these, we chose genes with at least 2-fold stronger expression at the 16-cell stage as compared to unfertilized eggs on the 40k-microarrays. There were 33 genes satisfying these three criteria (Fig. 4A).

As described earlier, among the 20 regulatory genes that are known to be expressed at the 16-cell stage, eight of these are maternally expressed (Hamaguchi et al., 2007; Imai et al., 2004). Probes for *Admp* were not included in our array. *Tbx6a*, *Wnt-NAe* and *Lefty* did not satisfy the above criteria, because we observed stronger expression in unfertilized eggs from at least one batch as compared to 16-cell embryos. Thus the criteria that we employed probably did not discover all genes expressed at the 16-cell stage. On the other hand, these criteria were unlikely to specifically exclude a subset of genes that may have been expressed in a different expression

pattern. Therefore, the 33 identified genes likely represent mRNAs that were zygotically expressed up to the 16-cell stage.

Next, we examined the expression patterns of 30 of these 33 genes using in situ hybridization; we could not find good cDNA clones for the remaining three genes, which we did not examine (white boxes in Fig. 4B). Furthermore, we failed to obtain clear results for eight of these targets (dark gray boxes in Fig. 4B).

In situ hybridization of the remaining 22 genes nearly recapitulated the microarray results (Fig. 4B and C; Fig. S7). We confirmed the previously identified regulatory genes that exhibit distinct expression patterns. In addition, 12 genes followed one of the gene expression patterns established by the regulatory genes. As shown in Fig. 4C, zygotic expression can be identified clearly in the ascidian embryo, because the first zygotic expression is seen in nuclei. For instance, KH.C2.313, which encodes a protein with a insulin-like domain, is expressed in B5.1, and this expression pattern is the same as the expression pattern of *Tbx6b* (Fig. 4B and C). KH.L61.10, which encodes a protein with no similarity to known proteins, is expressed in A5.1, A5.2, and B5.1, and this expression pattern is the same as the expression pattern of *FoxD* and *Fgf*9/16/20. KH.C14.414, which encodes a homolog of serine/threonine-protein kinase pim-1, is expressed in A5.1, A5.2, a5.3, a5.4, b5.3 and b5.4 in most embryos, and this expression pattern is the same as the expression pattern of *SoxB*1. However, KH.C14.414 was expressed in B5.1 of some embryos, while the microarray showed that this gene is expressed in the entire animal cells and the anterior vegetal cells (Fig. S7). As in the case of the expression of *AP-2-like2* in A5.1, the microarrays failed to detect variations in expression between individual embryos. We did not find novel genes that showed the same expression patterns as *AP-2-like2*/*EphrinA-d*/*EphrinA-a* and *Smad2*/*3b*/*GABPα*. Instead, KH.C9.541, which encodes a homolog of regulator of G-protein signaling 18, was expressed in the anterior animal cells (a5.3 and a5.4), and KH.C11.196, which encodes a homolog of acheron, was expressed in the anterior animal and vegetal cells (A5.1, A5.2, a5.3 and a5.4). These two expression patterns were not found in the previously studied regulatory genes.

It is possible that some of maternal genes, which we did not examine by in situ hybridization, are zygotically expressed in different patterns. However, the current data strongly indicate that there are no substantial differences in zygotic expression between A5.1 and A5.2 sister cells, between a5.1 and a5.2, and between b5.1 and b5.2. Only B5.1 and B5.2 sister cells are different from each other. This is probably because B5.2 cells are transcriptionally silent germ-line cells, where pem mRNAs are localized (Kumano et al., 2011; Shirae-Kurabayashi et al., 2011).

Conclusions

In the present study, we showed that the transcriptomes of unfertilized *Ciona* eggs are very different among batches. Only 25% of maternal mRNAs (1838/7388) were expressed at relatively similar levels in every batch. Expression of these maternal mRNAs may be tightly regulated in order to control their gene product function, including cell cycle regulation. The observation that genes encoding proteins related to transporter activities were enriched in the highly differentially expressed gene group indicates that a fraction of the maternal genes were regulated to be expressed differentially.

RNA localization is one of the mechanisms that are widely used to regulate cell differentiation. Our assay showed that there were no maternal mRNAs that were localized more clearly than pem mRNAs, at least among the maternal mRNAs that were expressed at similar level in eggs from different animals (S-group genes).

Our study demonstrated that there were no substantial differences in gene expression among each of the quadrants of the 16-cell embryo, except for the posterior vegetal one, where the pem-group genes localized in B5.2 distinguished B5.1 from B5.2. Previous studies have identified three maternal factors important for the establishment of the initial zygotic gene expression patterns; β-catenin and GATA-a, which specify the vegetal and animal hemispheres, respectively, and *macho*-1, which is one of the pem-group genes that determines the posterior vegetal fate (Bertrand et al., 2003; Imai et al., 2000; Nishida and Sawada, 2001). Genes encoding secreted signaling molecules including Wnt5 and Tolloid are localized in the posterior pole, and they might be involved in establishment of the expression patterns at the 16-cell stage. In addition, *FoxA-a* and *SoxB*1 are zygotically activated at the 8-cell stage, and possibly involved in establishment of the expression patterns at the 16-cell stage. On the other hand, because of the delay between gene expression and protein translation, it is unlikely that regulatory genes expressed at the 16-cell stage immediately start to regulate other genes at the 16-cell stage. The nine gene expression patterns established at the 16-cell stage cannot be fully explained by these maternal factors and two zygotic factors. In other words, to systematically understand how maternal factors initiate the zygotic programs, we need to explain how these nine gene expression patterns are established directly and indirectly by maternal factors including β-catenin, GATA-a, and macho-1.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.10.003.

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