A Novel Technique to Specifically Knockdown Maternal Gene Expression in *Ciona intestinalis*

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Abstract

Multicellular animals develop from single cell embryos. During development, a group of transcripts and proteins that are stored in eggs called maternal factors play crucial roles in constructing organized multicellular body. Therefore it is necessary to investigate the functions of maternal factors for understanding molecular mechanisms underlying development of multicellular organisms. In this study, I developed a novel technique to specifically knockdown maternal mRNAs in the chordate ascidian *Ciona intestinalis* using transposon-mediated transgenesis. I found a phenomenon in transgenic animals of *Ciona* that GFP expression is epigenetically silenced in oocytes and eggs. This epigenetic silencing of GFP was used for developing a new method to knockdown maternal gene expression. When 5' upstream promoter region and 5' untranslated region (UTR) of target maternal gene are used to drive *GFP* in eggs, the target maternal gene was knocked down together with *GFP*. In this method, 5' UTR is the essential element for deciding gene silencing target. Unlike maternal expression, there was no effect to zygotic expression of target gene, in the maternal gene-knocked down embryos. This feature indicates that phenotypes which are seen in the knockdown animals reflect maternal function of target genes. The novel technique will give us breakthroughs in studying functions of the maternal mRNAs in *Ciona*. In oocytes in which a maternal gene *Ci-pem* was knocked down, multiple copies of antisense small RNAs were transcribed from the 5' UTR of this gene compare to oocytes of control GFP transgenic line that did not show knockdown of *Ci-pem*. These small RNAs may be crucial to knockdown of *Ci-pem*.

Introduction

Multicellular animals produce eggs as reproductive cells. In animal eggs, a variety of mRNAs is stored and plays crucial roles in the developmental process. It is necessary to determine functions of these stored mRNAs in order to understand mechanism of animal development. Ascidians, a group of chordates (Satoh, 1994), are good model for studying roles of maternal mRNAs during development. Ascidian eggs are typical mosaic eggs (Conklin, 1905), and the factors that determine cell fates and morphogenetic movement show localization at specific regions of eggs (Nishida, 2005). Maternal mRNAs are promising candidates for these factors. For example, the maternal transcript of a gene named *macho-1* works as a fate determinant for muscle cells (Nishida and Sawada, 2001). Yet, the functions of many maternal mRNAs are still unclear, because of limitation of techniques to investigate their functions in ascidians.

For investigating functions of maternal mRNAs, I selected the ascidian *Ciona intestinalis* as the material. Genome sequence of *C. intestinalis* was already revealed (Dehal et al., 2002), and for that reason *Ciona* is an excellent organism to carry out functional analysis of genes. The generation time of *Ciona* is about 2-3 months which is relatively short comparable to that of zebrafish. This feature enables us to conduct genetic approached in this ascidian. Indeed, methods to create transgenic lines have been developed with transposons *Minos* and *Sleeping Beauty*, and various transgenic lines that express green fluorescent protein (GFP) in tissue-specific manner have been created (Sasakura et al., 2003, 2008; Hozumi et el., 2013). Therefore *Ciona* is a good model to study the functions of genes. Also, in *Ciona* eggs, there are about

40 maternal mRNAs which are localized in a part of eggs (Prodon et al., 2007). The functions of many of these genes are still unclear and need to be investigated.

To study functions of maternal mRNAs, it is important to inhibit their functions. In ascidians, several methods are conventionally used to disrupt maternally expressed genes. However, there are some disadvantages in these approaches, so that it is insufficient for functional study of maternal mRNAs in *Ciona*. For example, knockdown approaches like RNA interference (RNAi) and morpholino oligonucleotide (MO), are convenient ways for disrupting maternal mRNAs in ascidians (Nishiyama and Fujiwara, 2008; Satou et al., 2001). RNAi has a disadvantage that small RNAs can disrupt zygotic gene expression as well as maternal expression. Therefore, it is very difficult to know maternal function of genes by observing phenotypes, if target genes show both maternal and zygotic expression. MOs are usually injected into mature ascidian eggs to disrupt mRNA splicing or translation. Therefore, this method cannot inhibit the gene function if target genes are already translated during oogenesis. From these reasons, it is important to establish a new method that is efficiently and specifically inhibit ascidian maternal transcripts. One of the candidates is forward genetics, but it requires extensive labor to isolate mutant lines. Since eggs of mutant animals are needed in order to screen maternal gene mutants, therefore it takes one more generation than zygotic mutants. Also, if a target maternal gene has a critical role in early development, maternal mutants cannot be obtained because mutant females will not survive. This is also a disadvantage of knockout of *Ciona* genes using engineered nucleases (Kawai et al., 2012; Treen et al.,

2014).

As mentioned above, method of germline transformation using transposon *Minos* was established in *Ciona intestinalis* (Sasakura et al., 2003). By observing GFP expression in these lines, I noticed a curious phenomenon that GFP expression in oocytes and eggs is epigenetically suppressed. Using this phenomenon, I established a new technique to specifically knockdown maternal gene expression that does not affect zygotic expression. Thus, I can specifically investigate the functions of maternal mRNAs even though target genes own both maternal and zygotic transcription. This new method will be a breakthrough to study functions of maternal mRNAs in *Ciona.*

Materials and Methods

Animals

Wild type *Ciona intestinalis* was received from Maizuru Fisheries Research Station of Kyoto University, Usa Marine Biological Institute of Kochi University, Misaki Marine Biological Station of University of Tokyo, Integrated Marine Field Station in Onagawa of Tohoku University, and Marine Biological Laboratory of Hiroshima University. Transgenic animals were cultured by an inland system (Joly et al., 2007) at Shimoda Marine Research Center of University of Tsukuba. Sperm and eggs were collected either by spontaneous spawning or cutting the egg ducts and the sperm ducts.

Constructs

The 5' upstream region and 5' untranslated region (UTR) of genes were isolated from *C. intestinalis* genomic DNA as a template by PCR. The PCR fragments were digested with *BamHI* site of pSPeGFP (Sasakura et al., 2003). The fusion cassettes were PCR amplified, and subcloned into pMiCiTnIG (Sasakura et al., 2008). .To create pMiFr3dTPORCipemG, first, Fr3dTPOR cassette of pSPFr3dTPOR (Sasakura et al., 2008) was subcloned into pMiLRneo (Klinakis et al., 2000) to create pMiFr3dTPOR. Next, gateway cassette (invitrogen) was subcloned into pMiFr3dTPOR to create pMiFr3dTPORDestR. At last, the *Ci-pem-GFP* cassette was subcloned into pMiFr3dTPORDestR using gateway technology (invitrogen).

A part of the data is concealed, because it includes unpublished data.

Transgenic lines

Tg[MiCiNutG]3 and Tg[MiCiNutG]4 were described in previous report (Sasakura et al., 2010). EJ[MiTSAdTPOG]78 is an enhancer trap line created by method introduced previously (Sasakura et al., 2008). The other transgenic lines were created by *Minos*-mediated or *Sleeping Beauty*-mediated transgenesis as previously described (Matsuoka et al., 2005).

Identification of insertion sites

The insertion sites of *pem>GFP* lines were characterized by thermal asymmetric interlace (TAIL) PCR, according to previous reports (Liu et al., 1995; Hozumi et al. 2010). Genomic DNAs isolated from sperm of transgenic lines were used as templates for PCR. PCR fragments were subcloned into pGEM-T Easy vector (Promega), and their sequences were determined. The presence of characterized insertion sites was confirmed by genomic PCR with specific primers designed near the insertion sites.

Microinjection

Ci-pem cDNA was amplified by PCR and subcloned into pBS-RN3 (Lemaire et al., 1995) to create pRN3CipemFL. *Ci-pem* mRNA was synthesized from pRNCipemFL using the Megascript T3 kit (Ambion), the poly(A) tailing kit (Ambion), and Cap structure analog (New England Biolabs). Microinjection of mRNA was performed according to a previous report (Hikosaka et al., 1992). The concentration of mRNA in the injection medium was adjusted to 500 ng/μl.

Tissue Differentiation

Differentiation of epidermis, notochord and neural tissues in *C. intestinalis* larvae were investigated using marker transgenic lines that express *GFP* or *Kaede* reporter gene under control of cis elements of the *Ci-EpiI*, *Ci-Bra*, and *Ci-β2TB* genes (Joly et al., 2007; Horie et al., 2011). The sperm of these transgenic lines was used to fertilize eggs of *pem>GFP* lines and fluorescence was detected as the larval stage. To monitor muscle differentiation, GFP expression from *Ci-TnI-GFP* cassette in *Ci-pem* knockdown vector was utilized. For endodermal differentiation, histochemical staining of alkaline phosphatase was performed as previously described (Whittaker and Meedel, 1989).

Gene expression

Eggs of transgenic lines were collected either by spontaneous spawning or cutting the egg duct. Prior to sampling, I divided egg samples by the presence and absence of GFP fluorescence for the following experiments. For detecting the presence of *EGFP, Ci-pem*, *Ci-mT*, and *Ci-Nut* genes, whole-mount in situ hybridization (WISH) was performed as previously described (Yasuo and Satoh, 1994; Sasakura et al., 2010). For quantitative analysis of maternal and zygotic mRNAs of maternal genes, quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed as previously described (Sasakura et al., 2010) using *GAPDH* as normalizing gene. A 0.5-1.0 unfertilized egg or embryos equivalent quantity to cDNA was used as a template for quantitative RT-PCR. Relative quantification of mRNA was carried out using standard curves created by cloned cDNAs. The primer

sequenced used for quantifying expression levels of genes are 5'-gtcctcgtacagtttagccatgtcg-3' and 5'-caattcactgatcgtatagtgttgg-3' for *Ci-pem*, 5'-gtcgcaaacgtcatcacc-3' and 5'-ggcctactgggtctgtttcg-3' for *Ci-mT*, 5'-cgtggattgccattgacag-3' and 5'-cgctctcataagccccaaac-3' for *Ci-Nut*, 5'-gttgccggaaatatggaatcg-3' and 5'-cgatcgaccaccaaattgaac-3' for *Ci-wnt5*, 5'-ccgctgttgatttctgtcatc-3' and 5'-cgctactctgatgttgttcttc-3' for *Ci-POPK1* and 5'-gatcgcatcataggatgctgg-3' and 5'-tgtatccgtggttgaccttacag-3' for *GAPDH*. *P* values were calculated using a two -tailed Student's t test.

Small RNA sequencing

This method is consisted by unpublished data, and concealed until it is published.

Results

Epigenetic GFP suppression in *Ciona intestinalis* oocytes and eggs

Transgenic lines which express GFP in oocytes and eggs are created by either using 5' upstream region of maternally expressed genes, or trapping enhancer of maternal gene expression by transposon-mediated enhancer trapping method (Sasakura et al., 2003). In these transgenic animals, GFP expression was seen in oocytes and eggs. However, usually only a few of them carried GFP expression, and most of them did not show GFP expression (Fig. 1a). The percentage of GFP-negative eggs varied from 0% to 100% among transgenic lines, even though the lines harbored the same transposon vector, suggesting that the genomic location of the transposon vector affect on the silencing of GFP. I performed whole-mount *in situ* hybridization (WISH) to observe *GFP* mRNA expression in these GFP-positive and GFP-negative eggs. As a result, *GFP* mRNA was detected in GFP-positive eggs. However, *GFP* mRNA was not detected in GFP-negative eggs (Figs. 1b, c). Oocytes and unfertilized eggs of *Ciona* are kept in the state of diploid, in order that all of these cells contain *GFP* gene in these transgenic lines. These results suggest that maternal GFP expression in transgenic line oocytes and eggs are suppressed in the transcriptional or post-transcriptional level in the maternal GFP transgenic lines. Indeed, in transgenic lines in which GFP was expressed in both maternal and zygotic fashions, zygotic GFP was expressed in the animals developed from GFP-negative eggs (Figs. 2a, b). This suggests that *GFP* genes introduced into these transgenic lines are intact in their oocytes and have ability to express GFP fluorescence. Therefore, GFP suppression in GFP-negative oocytes and eggs is not caused by breaking or loss of *GFP* gene,

but by epigenetic gene silencing. In addition, presence of zygotic GFP expression in animals derived from GFP-negative eggs indicates that suppression specifically occurred in maternal expression of GFP but not in zygotic GFP expression.

Maternal *Ci-pem* expression is knocked down

An ascidian gene named *posterior end mark (pem)* encodes a maternal mRNA that localizes in the posterior end of fertilized eggs and early embryos (Yoshida et al., 1996). *Ciona intestinalis pem (Ci-pem)* shows exclusive maternal expression throughout embryonic development (Yoshida et al., 1997). I used this *Ci-pem* 5' upstream region and created a transposon vector which induces GFP expression in oocytes and eggs. The *Ci-pem* 5' upstream region includes 5' untranslated region (UTR) and initiation codon of this gene. Because *Ci-pem* gene is not expressed zygotically, I used a fusion of the 5' upstream and 5' UTR of the muscle gene *Ci-TnI* (which encodes Troponin I; MacLean et al., 1997) with GFP next to *Ci-pem>GFP* cassette (pMiCiTnIGCipemG; Fig. 3a) in order to identify transgenic animals during culturing. This *Ci-TnI* promoter drives *GFP* in muscle tissues, but not in oocytes and eggs (Yoshida et al., 1997). Using this vector, I made several transgenic lines which express GFP fluorescence in oocytes and eggs. Here, I named these lines *pem>GFP* lines. As I described previously, these *pem>GFP* lines oocytes and eggs showed mosaic GFP expression (Fig. 3b).

I obtained progeny of *pem>GFP* lines by crossing them with wild-type animals. When sperm from *pem>GFP* lines were crossed with wild-type eggs, larvae showed normal embryogenesis and developed into tadpole larvae (Fig.

3c). On the other hand, when wild-type sperm was crossed with *pem>GFP* line eggs, many progeny embryos showed abnormal embryogenesis (Fig. 3d). Their embryonic axis could not be recognized at the larval stage. These abnormal larvae are usually recognized into two body parts, one of which had vacuolated notochord cells (Fig. 3d). Next, I examined tissue differentiation of these animals. The major tissues of the larval body, epidermis, muscle, notochord, neural tissues and endoderm were differentiated properly (Figs. 4a-e). However, positioning of these tissues was abnormal. In normal larvae, muscle and notochord cells located in the tail, and most of endodermal cells localized in the trunk. Therefore, most of the endodermal cells locate separately from muscle and notochord cells. Unlike normal larvae, the muscle, notochord and endodermal cells located in the same region in abnormal larvae. This tissue orientation in abnormal larvae looks similar to vegetal hemisphere of 110 cell stage embryo (Kim at al., 2007). Movement of endodermal cells toward truck is caused by gastrulation that starts around the 110 cell stage, which suggests the abnormality occur in the *pem>GFP* line is likely to be caused by defects in gastrulation.

The percentage of normal and abnormal larvae derived from *pem>GFP* eggs differed among transgenic lines, and it was correlated with ratio of GFP silencing (Table 1). For example, none of *pem>GFP* line 2 eggs expressed GFP, and all of them developed into abnormal larvae. In contrast, eggs of *pem>GFP* line 9 showed GFP expression as 100% and developed into normal larvae. The correlation between GFP silencing and abnormal development was further supported by the results that all of the maternal GFP-positive eggs developed into normal larvae, while all of the GFP-negative eggs developed into

abnormal larvae (Fig. 5). These abnormal larvae had zygotic GFP expression derived from *TnI>GFP* cassette, even though maternal GFP expression was silenced (Fig. 5). Therefore abnormal development caused in *pem>GFP* line eggs strongly suggests to have a relationship with epigenetic maternal GFP silencing.

To elucidate the cause of abnormal development in *pem>GFP* line eggs, I observed *Ci-pem* mRNA expression by whole-mount *in situ* hybridization (WISH) and quantitative RT-PCR (Figs. 6a-f). WISH showed reduction of *Ci-pem* mRNA to undetectable level in GFP-negative eggs of *pem>GFP* lines (Figs. 6c, d). Quantitative RT-PCR showed that *Ci-pem* mRNA expression level dropped to approximately 2.3-21% of the level in wild-type eggs (Fig. 6f). Because *Ci-pem* is essential for early embryogenesis of ascidian (Negishi et al., 2007; Kumano and Nishida, 2009; Shirae-Kurabayashi et al., 2011), these results suggest that the abnormal development in *pem>GFP* lines is caused by the loss of *Ci-pem* mRNA. Indeed, the abnormal development occurred in *pem-GFP* lines could be recovered to tadpole larval phenotype by introducing *in vitro*-transcribed *Ci-pem* mRNA (Figs. 7a, b). This suggests those abnormal embryogenesis observed in eggs of *pem-GFP* lines are the result from specific reduction of *Ci-pem* mRNA.

To clarify the specificity of *Ci-pem* mRNA reduction in *pem>GFP* lines, I examined expression levels of four maternal mRNAs, including *Ci-mT* (Takatori et al., 2004), *Ci-Nut* (Etani and Nishikata, 2002), *Ci-wnt5* (Joly et al., 2007), and *Ci-POPK1* (Yamada et al., 2005), in *pem>GFP* line eggs. For this examination, I chose *pem>GFP* line 2 which produces 100% GFP-negative eggs. None of these four mRNAs were exhibited at lower levels in *Ci-pem*

knockdown eggs than in wild-type eggs (Fig. 8). These results suggest that *Ci-pem* mRNA is specifically knocked down in *pem>GFP* lines associated with epigenetic silencing of GFP in *Ciona* eggs.

There was a possibility that insertion sites of transposon vectors in *Ciona* genome might be close to the genomic region encoding *Ci-pem* gene, which caused disruption of *Ci-pem* expression in these *pem>GFP* lines. To examine this possibility, I identified insertion sites of three *pem>GFP* lines that exhibited *Ci-pem* knockdown (Table 2). Insertion sites of all three *pem>GFP* lines were distant from the *Ci-pem* locus (the gene model for *Ci-pem* gene is KH.C1.755), suggesting that transposon insertion site is not the cause of knockdown of *Ci-pem* expression.

Silencing of maternal mRNAs depend on their promoters and 5' UTRs

To understand factors to knockdown maternal *Ci-pem* mRNA in *pem>GFP* lines, I exchanged four regions in the vector (pMiCiTnIGCipemG) namely the marker cassette, 5' UTR, GFP reporter and inverted repeats of *Minos* transposon. Because 5' UTR is the element which is transcribed with *GFP*, I paid special attention to the element. For this reason, I eliminated *Ci-pem* 5' UTR from pMiCiTnIGCipemG vector (Fig. 9a), and three transgenic lines were created with the vector. None of these lines showed *Ci-pem* knockdown phenotype (Fig. 9b), indicating that 5' UTR has an important role in knocking down *Ci-pem*. To further investigate the roles of 5' UTR in maternal mRNA knockdown, I exchanged 5' UTR of *Ci-pem* for that of another maternally transcribed gene *Ci-Nut* (Etani and Nishikata, 2002) that encodes an Opsin-related protein (Fig. 9c). I created three transgenic lines (*Nut*$^{5'UTR}$

lines) using the transposon vector (the marker cassette was switched with that expressing *DsRed*, according to the result described in Figs. 10a, b). Larvae derived from $Nut^{5'UTR}$ line eggs showed normal morphology (Fig. 9d), Quantitative RT-PCR indicated no significant reduction of *Ci-pem* mRNA in the eggs of $Nut^{5'UTR}$ lines. On the other hand, maternal *Ci-Nut* mRNA of $Nut^{5'UTR}$ lines was reduced to approximately 27% of the level in wild-type eggs (Fig. 9e). This reduction is not efficient as the knockdown observed in the *Ci-Nut>GFP* lines described in later (Fig. 14c). These results suggest that 5' UTR is the important element which selects the target gene to be silenced. However, both promoter region and 5' UTR combination might be necessary to efficiently knockdown target genes.

Next, I investigated the necessity of marker cassettes. I switched *TnI>GFP* cassette in pMiCiTnIGCipemG vector to a cassette expressing *DsRed* driven by *Ci-musashi* Fr3 enhancer and *Ci-TPO* promoter (pMiFr3dTPORCipemG; Fig. 10a; Awazu et al., 2004). Neither of the Fr3 enhancer nor the *Ci-TPO* promoter drives maternal expression. Four transgenic lines were established, and three of them showed *Ci-pem* knockdown phenotype (Fig. 10b), suggesting that a specific organization of the marker cassette is not important for maternal gene silencing.

Some data is concealed, because it is consisted by unpublished data.

Knockdown of various maternal mRNAs

To test whether the knockdown method of *Ci-pem* can be applied to knockdown maternal mRNAs other than *Ci-pem*, I created knockdown constructs of two maternal genes. I chose *Ci-mT* and *Ci-Nut* as knockdown

targets. *Ci-mT* encodes a T-box transcription factor that shows maternal expression throughout embryonic stages (Takatori et al., 2004). To construct the knockdown vectors of these genes, their 5' upstream region and 5' UTR were isolated from *Ciona* genome (Figs. 13a, 14a). In addition to maternal expression, *Ci-Nut* shows zygotic expression in neural tissue, and the 5' upstream region of *Ci-Nut* can drive GFP in neural tissues like endogenous *Ci-Nut* (Shimai et al., 2010). Therefore the knockdown vector of *Ci-Nut* does not require a marker cassette to distinguish transgenic animals (Fig. 14a). Using these vectors, three transgenic lines were created, namely Tg[MiCiTnIGCimTG]1, Tg[MiCiNutG]3 and Tg[MiCiNutG]4 (Tg[MiCiNut]3 and Tg[MiCiNut]4 have already been described previously; Sasakura et al., 2010). Among them, Tg[MiCiTnIGCimTG]1 and Tg[MiCiNut]3 showed GFP silencing in their oocytes and eggs. *Ci-mT* and *Ci-Nut* expression in eggs were investigated by WISH and quantitative RT-PCR. There was great reduction of *Ci-mT* and *Ci-Nut* expression in GFP-negative eggs of corresponding transgenic lines (Figs. 13b, c, 14b, c). *Ci-mT* mRNA in Tg[MiCiTnIGCimTG]1 eggs were reduced to 4.1% of wild-type eggs, and *Ci-Nut* mRNA in Tg[MiCiNutG]3 eggs were reduced to 1.4% (Figs. 13c, 14c). When *Ci-mT* knockdown eggs were fertilized with wild-type sperm, larvae showed abnormal development in the tail, which is distinct from *Ci-pem* knockdown phenotype (Fig. 13d). Therefore, *Ci-mT* probably has a function in the morphogenesis of tail region. On the other hand, embryos derived from *Ci-Nut* knockdown eggs exhibited normal embryogenesis and became normal larvae (Fig. 14d). Therefore, maternal *Ci-Nut* probably does not have crucial role in the morphogenesis of larvae. This result suggests that the knockdown method

itself does not affect to embryogenesis. The knockdown line of *Ci-pem*, *Ci-mT* and *Ci-Nut* showed different phenotypes, suggesting that the phenotypes acquired from this method reflect the reduction of target gene. I examined expression of four maternal transcripts in *Ci-mT* or *Ci-Nut* knockdown eggs by quantitative RT-PCR (Figs. 15a, b). The knockdown effect was generally specific to the target mRNAs, although *Ci-pem* mRNA exhibited very weak reduction in both cases as supported by the statistical analysis.

Zygotic expressions of target genes were not affected by the maternal gene knockdown

Ci-Nut gene is expressed in both maternal and zygotic fashions. Using this characteristic, I examined whether maternal gene silencing has effect on zygotic expression of target genes. Maternal *Ci-Nut* knockdown eggs were collected and fertilized with wild-type sperm to investigate zygotic *Ci-Nut* expression by quantitative RT-PCR and WISH methods. Quantitative RT-PCR uncovered that expression level of zygotic *Ci-Nut* mRNA in larvae derived from *Ci-Nut* knockdown eggs did not differ significantly with the level of wild-type larvae (Fig. 16a). This result suggests that zygotic *Ci-Nut* is expressed normally even though maternal *Ci-Nut* expression is inhibited. WISH of maternal *Ci-Nut* knockdown embryos revealed that zygotic expression of *Ci-Nut* which begins its expression in the neural tissues at the late gastrula stage (Etani and Nishikata, 2002), in *Ci-Nut* knockdown embryos presented same expression pattern with embryos derived from wild-type eggs (Fig. 16b). Therefore I concluded that the knockdown of genes investigated in this analysis is specific to maternal transcripts as was

observed with GFP.

Antisense small RNAs derived from *Ci-pem* 5' UTR are produced in oocytes of *pem>GFP* line

This data is concealed, because it is consisted by unpublished data.

Discussion

I established a new method to knockdown maternal mRNAs in the ascidian *Ciona intestinalis*. I named this method MASK, standing after maternal mRNA-specific knockdown. MASK method has four advantages compared to previously reported methods for maternal mRNA inhibition in ascidians. First, MASK is a reverse genetic approach, so that I can inhibit gene function of interest without laborious screenings. Second, this MASK method can disrupt maternal gene expression without affecting on zygotic expression, and therefore we can observe phenotypes specifically reflecting the maternal functions of the targeted genes. Third, this method utilizes genetic modification; once transgenic lines are established I can obtain RNA knocked down eggs without further experimentation. Fourth, MASK is simple and easy and the only steps required is to create the knockdown vectors, including isolation of 5' upstream region and 5' UTR and their fusion with a fluorescence protein gene in transposon. With these advantages, MASK will be the powerful method to study the function of maternal mRNAs in ascidian eggs. Four reporter genes, namely *EGFP*, *mKO2*, *Kaede* and *wild-type GFP* can be used for MASK. I was able to test two *pem>mAG* lines and both of the lines did not show knockdown of *Ci-pem*. Therefore, currently I needed to conclude that *mAG* should be avoided for MASK. However, the number of examined *pem>mAG* lines are not sufficient, and I should further examine knockdown of *Ci-pem* with more *pem>mAG* lines in order to determine whether mAG can or cannot be used to cause MASK.

The basis of MASK is epigenetic knockdown of fluorescent protein genes, which occurs in oocytes and eggs.

Sentences that include unpublished data are concealed.

The mechanisms how these reporter genes become the target of gene silencing is an important question to understand the mechanisms of MASK and epigenetic gene regulation in *Ciona* oocytes. There is one plausible mechanism that could be the cause of silencing of reporter genes. This possible answer is a defense mechanism to the foreign genes. Since these reporter genes do not exist in *Ciona* genome, reporter genes are recognized as exogenous genes and suppressed in oocytes and eggs. Germ cells are the only cell populations that are inherited to the next generation. This importance may be an explanation why reporter gene expression is suppressed exclusively in oocytes and eggs. Sperm is another gem cells but a phenomenon like MASK had not been observed. Because transcription of genes in sperm and its progenitors is very limited to a small portion of genes compared to oocytes, such epigenetic gene silencing may not be required in the male germ cell lineage. In *Caenorhabditis elegans*, a mechanism that distinguishes between endogenous genes and exogenously introduced genes has been found in eggs (Seth et al., 2013). This phenomenon is very similar to the epigenetic gene silencing in *Ciona* in this study. In MASK, 5' UTR of target genes are fused with reporter genes, and therefore these target genes are recognized as non-self genes. One issue that could not explain with the above explanation is that antisense small RNAs were not produced from 5' UTR of *Ci-TnI* or *Ci-prm* even though they are also fused to *GFP* in this study. There might be another mechanism that could distinguish between maternally expressed and zygotically expressed genes. To elucidate this hypothesis, I will continue the studies on the molecular mechanism of MASK.

MASK method does not affect on zygotic expression of target genes. If post-transcriptional silencing (like such as degradation of target RNAs) is the mechanism of MASK, residual MASK factors (such as small RNAs) could silence zygotically transcribed mRNAs. The mechanism is similar to the maternal *gfp*/gene silencing (MGS), which is another form of epigenetic silencing in *Ciona* that degrades both maternal and zygotic *GFP* mRNAs (Sasakura et al., 2010). Therefore, I am assuming transcriptional silencing is the mechanism of MASK. Oocytes and eggs escaped MASK often showed strong GFP expression (Fig. 1a). This phenomenon can be explained if the mechanism of MASK works through transcriptional silencing. Once silencing is canceled in some oocytes, transcription of *GFP* happens continuously and accumulates GFP proteins in cells, which is the cause of strong GFP expression. If post-transcriptional silencing is assumed, it would be difficult to explain strong GFP expression in MASK-escaped oocytes, because MASK factor may continuously degrade *GFP* mRNA.

The finding that MASK does not affect on zygotic expression of target genes also suggests that the silencing ability by MASK is declined after fertilization. This phenomenon can be easily explained if transcriptional silencing is the mechanism of MASK. Probably epigenetic remodeling in chromatin occurs after fertilization, and during the remodeling transcriptional silencing by MASK factors may be canceled. It is also possible that the quantity of MASK factors decreases after fertilization. In this study I did not examine whether the quantity of antisense small RNAs decreases after fertilization, and this point should be addressed in future studies. Likewise, production of antisense small RNAs corresponding to the 5' UTR of target

genes in somatic cells should be examined.

The degree of silencing is different among the transgenic lines which are created by the same knockdown vector. The main difference of these lines is the position of the insertion sites of transposon vectors. Therefore, MASK may be affected by the genomic context of the insertion sites of transposon vector. This suggests that expression of factors to induce MASK, probably the antisense small RNAs, may be dependent on the genomic contexts around the insertion site. Although I could not identify the obvious differences between MASK-positive and -negative loci, accumulation of this kind of data will reveal the genomic condition necessary to operate MASK.

Knockdown of maternally expressed genes with MASK will be a very useful method to uncover the function of maternal genes in *Ciona*. Moreover I will continue studying the molecular mechanisms of MASK and reveal the functions of how antisense small RNAs of target genes are produced. The mechanism how this MASK occurs exclusively in oocytes and eggs needs to be investigated. I also need to examine other chordates to determine whether MASK is more broadly applicable which would promote the study of maternal mRNAs in other eukaryote lineage.

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Tables

Table 1. Frequency of larvae derived from eggs of *pem>GFP* lines with *Ci-pem* knockdown phenotype.

Line ID	% of normal larvae	% of abnormal larvae	no. of larvae examined
1	16	84	39
2	0	100	191
4	22	78	296
9	100	0	>100

Table 2. Insertion sites of the transposon vector in *pem>GFP* lines.

Line ID	Sequence near the insertion site[a]	Scaffold number in the KH2013 version of rhe genome browser[b]	Nearest gene model
1	taCCCCAACCCGCCACAACGCCCGTC TGCAACTTTGTGAACGATTTATTTTA CTCGGTTC	KhL112	KH.L 122.16
2	taTTACCTAGTGGTATTTTTGCAACG ATTCGTAAGCAATGAGATATATATAT TATAAACT	no hit[c]	no gene model
4	taTACTTAGCAACAACACCATTGTTAC GTCACACAACTTCATTGTTTCCTTCC TCTCTTG	KhL155	KH.L 155.5

[a]The targetd TA dinucleotides are shown in lower case

[b]The genome browser is available at http://ghost.zool.kyoto-u.ac.jp/cgi-bin/gb2/gbrowse/kh/

[c]The sequence shows high similarity to the whole genome shotgun reads of the *Ciona intestinalis* genome

Table 3. Frequency of *Ci-pem* knockdown with different transposons.

Table 4. Frequency of *Ci-pem* knockdown with different reporter genes.

Table 5. Antisense small RNAs specifically produced in *pem>GFP* line 2.

These tables are consisted by unpublished data, and they are concealed until data get published.

Figures

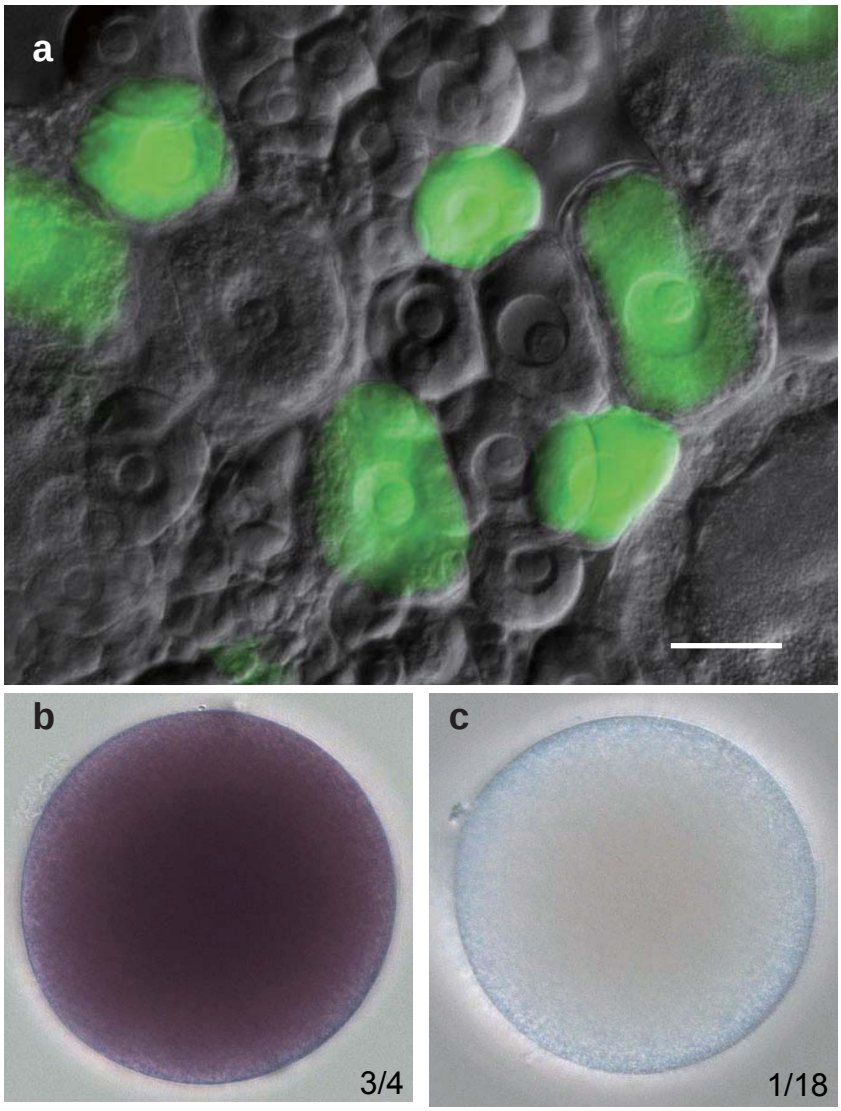

Figure 1. Epigenetically silenced maternal GFP expression in *Ciona*.
(a) A typical GFP expression pattern in the ovary of transgenic line which expresses GFP expression in oocytes. An ovary of an enhancer detection line EJ[MiTSAdTPOG]78, which entrapped an enhancer responsible for expression in oocytes. Only few oocytes were expressing GFP. Green, GFP expressing oocytes. Bar, 50 μm. (b,c) Expression of GFP mRNA in unfertilized eggs of a EJ[MiTSAdTPOG]78 eggs, as revealed by whole-mount *in situ* hybridization (WISH). Dark purple staining suggets the presence of *GFP* mRNA. (b) A GFP fluorescent positive egg. Dark purple stain was observed that represented existance of *GFP* mRNA. (c) A egg lacking GFP fluorescence was did not have dark purple stain. This result revealed *GFP* mRNA was absent in the eggs without GFP fluorescence.

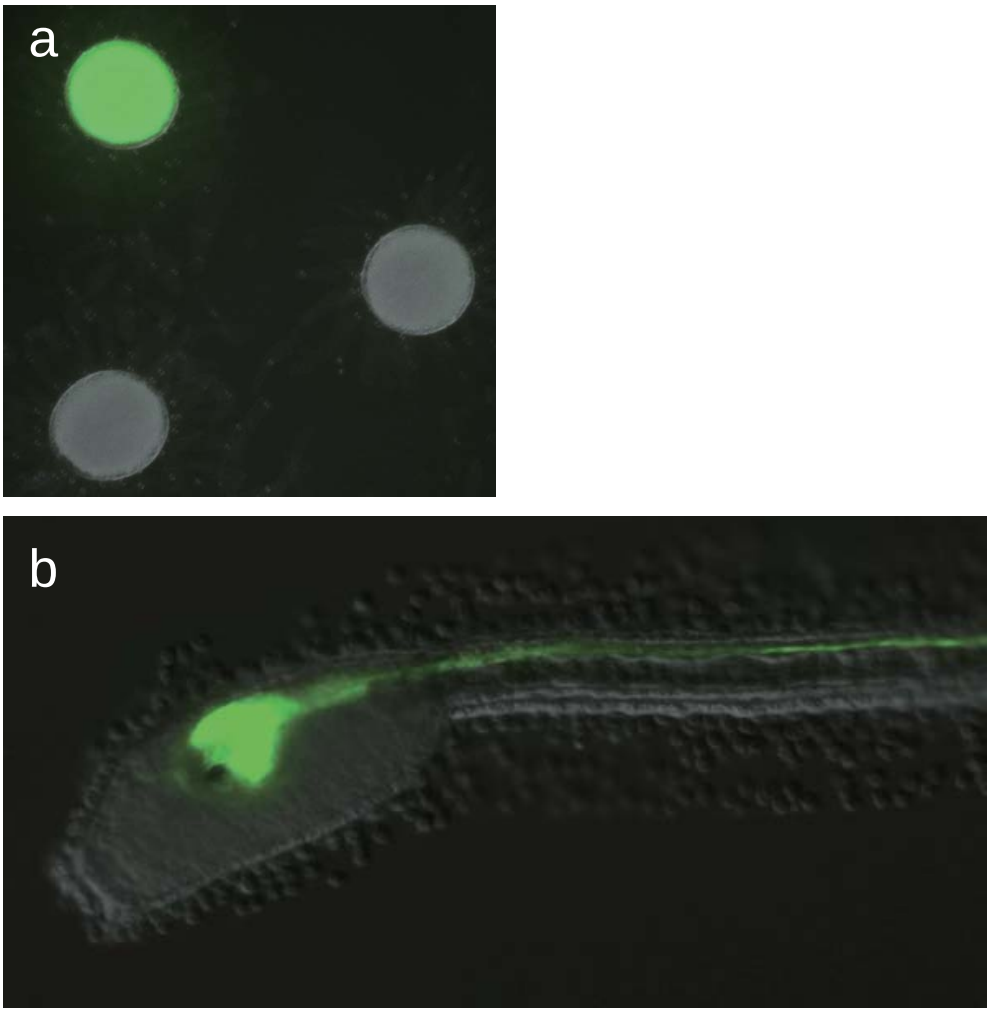

Figure 2. Larvae derived from GFP-negative eggs express zygotic GFP fluorescence.
(a) GFP expression in unfertilized eggs of Tg[MiCiNut]3, a transgenic line which expresses GFP fluorescence in oocytes. Only upper left egg showed GFP fluorescence. Other two eggs did not show GFP fluorescence.
(b) GFP-negative egg of Tg[MiCiNut]3 was fertilized by wild-type sperm and bred until larva stage. Even though maternal GFP expression was suppressed, zygotic GFP expression was presence.

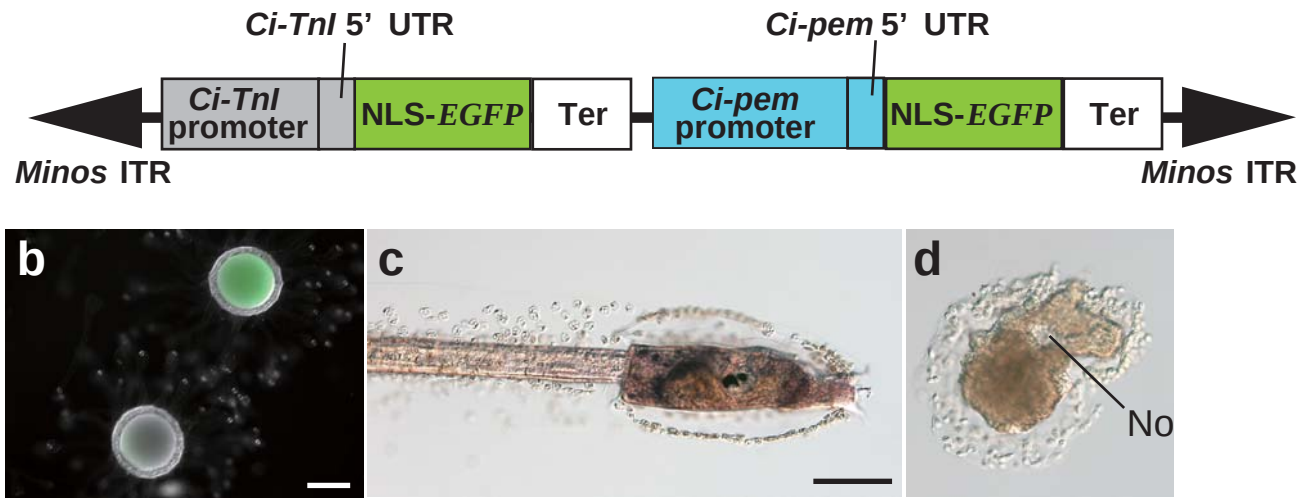

Figure 3. GFP-negative eggs of *pem>GFP* lines show abnormal development.

(a) The transposon vector used to drive *GFP* in oocytes and eggs (pMiCiTnIGCipemG). 5' upsteam region and 5' UTR of maternal gene *Ci-pem* was used to drive *GFP*. Black arrowheads indicate inverted repeats (ITR) of *Minos*. Transgenic lines created by this transposon vector was named *pem>GFP* line. UTR, untranslated region; NLS, nuclear localization signal sequence; Ter, transcription termination sequence. (b) GFP expression in unfertilized eggs of *pem>GFP* line 1. The upper egg showed GFP fluorescence but lower egg did not. Bar, 100μm. (c) A wild-type egg fertilized by *pem>GFP* line 4 sperm developed into normal larva. Bar, 100μm. (d) *pem>GFP* line 4 egg fertilized by wild-type sperm raised until larva stage showed abnormal development. No, notochord.

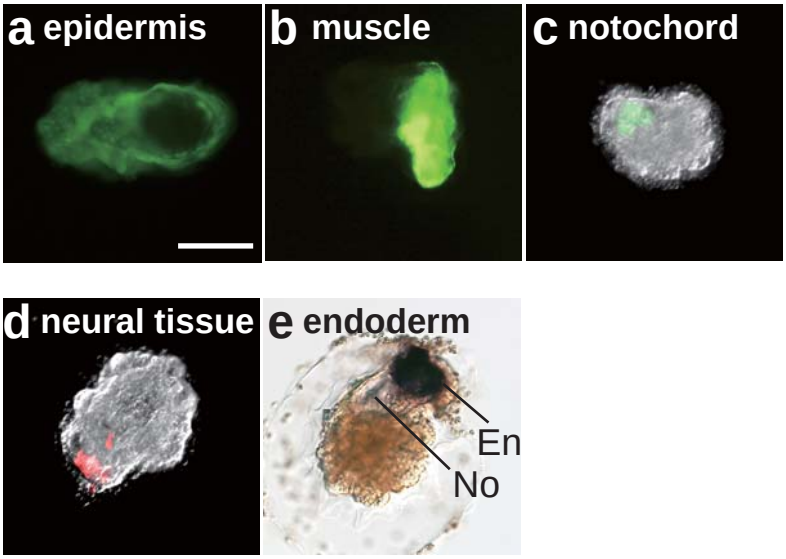

Figure 4. Tissue differentiation in *pem>GFP* lines.
(a-e) Differentiation of major tissues in abnormal larvae derived from *pem>GFP* lines. (a) Green fluorescence showed Epidermis (Ep). Bar, 100μm. (b) Green fluorescence showed Muscle (Mu). (c) Green fluorescence showed Notochord (No). (d) Red fluorescence showed Neural tissues (Ne). (e) Endoderm (En) was stained by the histochemical staining of alkaline phosphatase. Vesicular region was notochord (No). All of these tissues were differentiated properly, but their positions were abnormal.

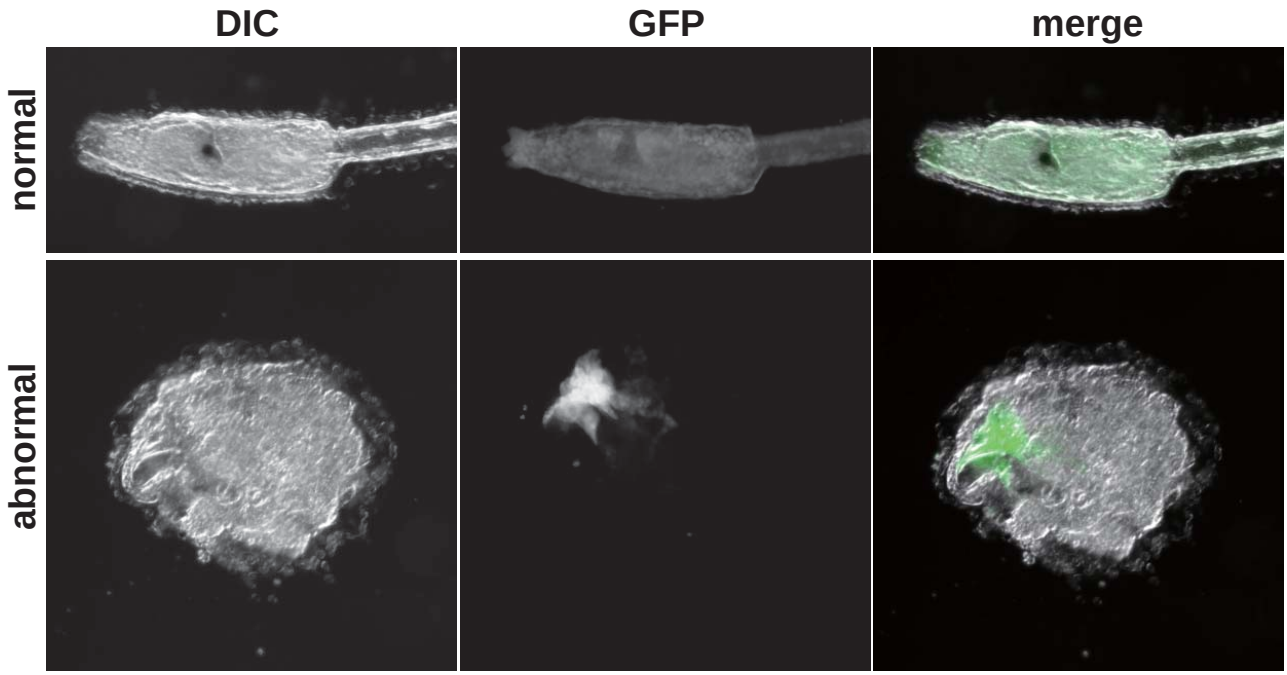

Figure 5. GFP expression of the larvae derived from eggs of *pem>GFP* lines.

A larvae derived from *pem>GFP* lines. Upper, a normal larva derived from maternal GFP-positive egg. Bottom, an abnormal larva derived from a GFP-negative egg. Left, photographs taken with differential image contrast (DIC) filter. Middle, GFP fluorescence of larva. Right, DIC and GFP expression was merged. In the normal larva, GFP expression was observed throughout the body, suggesting the fluorescence was derived from maternal GFP expression. GFP expression was also observed in the abnormal larva derived from GFP-negative egg, but not throughout the body. This result indicates that zygotic GFP is expressed even though maternal expression is suppressed.

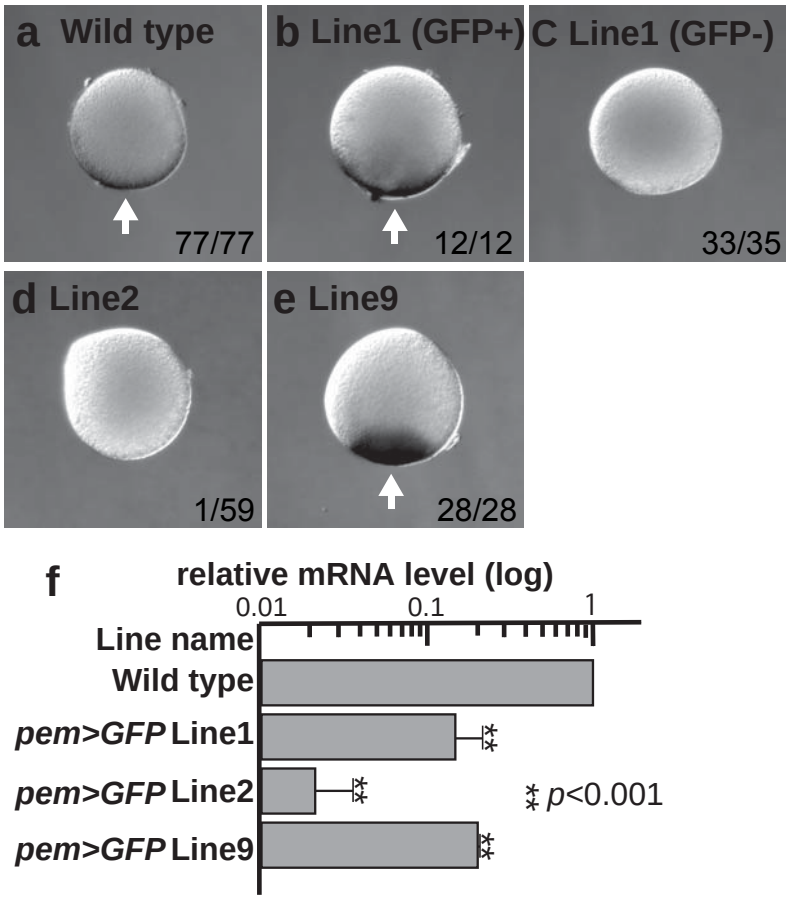

Figure 6. Knockdown of *Ci-pem* in *pem>GFP* lines.
(a-e) *Ci-pem* maternal mRNA was decreased in GFP-negative eggs of *pem>GFP* lines, as revealed by WISH. Arrows indicatethe signal of *Ci-pem* mRNA. (a) A wild-type egg with maternal *Ci-pem* expression. (b) A GFP-positive egg of *pem>GFP* line 1 showed *Ci-pem* expression. (c) A GFP-negative egg of *pem>GFP* line1 did not show *Ci-pem* expressoin. (d) A *pem>GFP* line 2 egg. All eggs from *pem>GFP* line 2 were GFP-negative. Therefore, *Ci-pem* expression was also suppressed in all of the *pem>GFP* line 2 eggs. (e) A *pem>GFP* line 9 egg. All eggs from *pem>GFP* line 9 were GFP-positive. Therefore, *Ci-pem* was expressed in all *pem>GFP* line 9 eggs. (f) Relative expression levels of *Ci-pem* in the eggs of *pem>GFP* lines, as revealed by quantitative RT-PCR. n=2 for every line. *P* values were calculated using the two-tailed Student's t test.

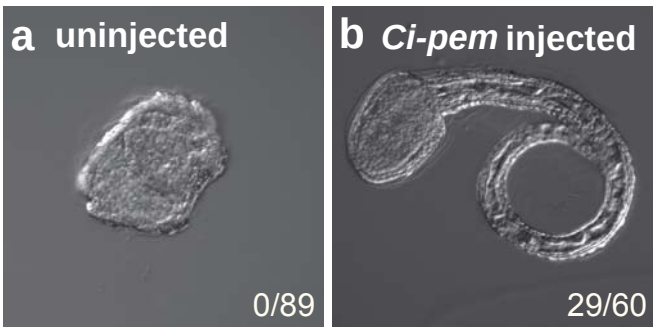

Figure 7. *Ci-pem* knockdown phenotype could be rescued by introducing *Ci-pem* mRNA.

(a) A larva derived from an egg of *pem>GFP* line 2. A larva showed abnormal morphology. (b) A larva derived from an egg of *pem>GFP* line 2 in which *in vitro*-synthesized *Ci-pem* mRNA was injected. The larva was developed into a tadpole larva.

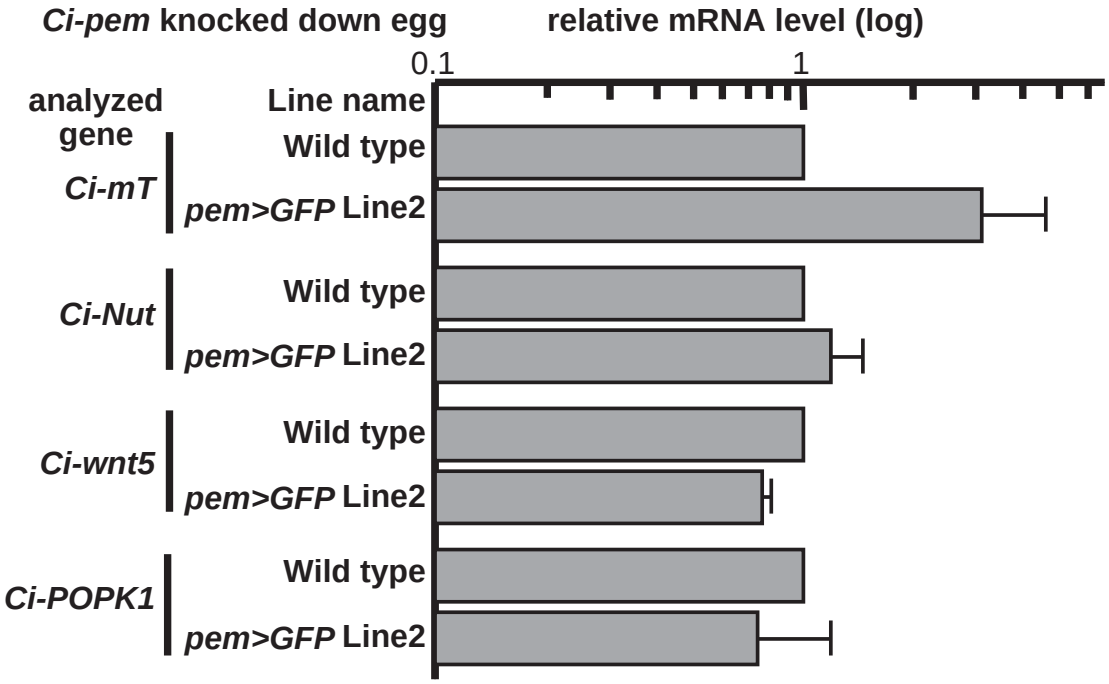

Figure 8. Target specificity of maternal mRNA knockdown in *pem>GFP* line 2.

Relative expression levels of four maternal genes including *Ci-mT*, *Ci-Nut*, *Ci-wnt5*, and *Ci-POPK1* in the eggs of *pem>GFP* line 2, as revealed by quantitative RT-PCR. n=2. *P* value is calculated using the two-tailed Student's t test. None of these four mRNAs showed signficant decrease in *Ci-pem* knockdown eggs compared with wild-type eggs.

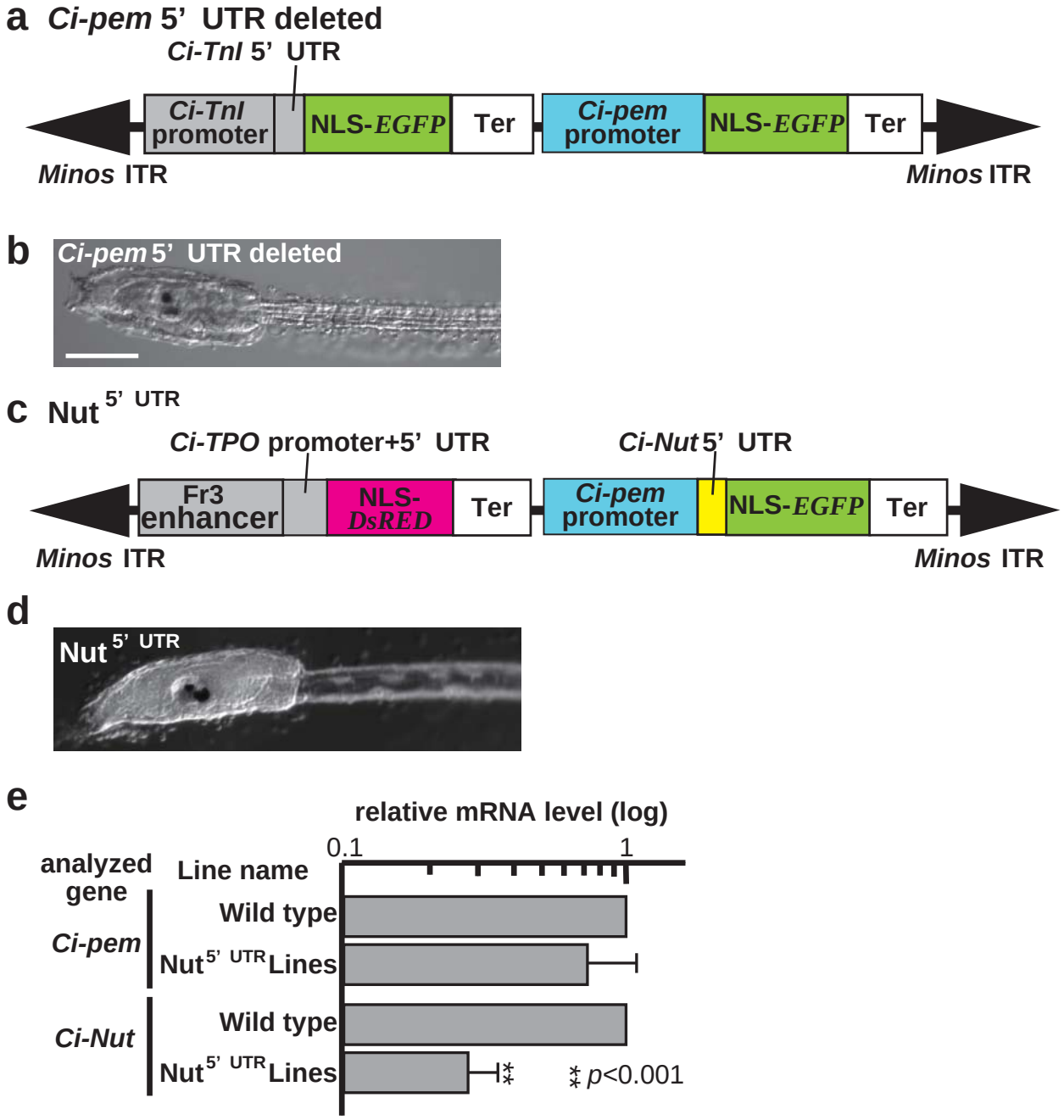

Figure 9. 5' UTR is essential for maternal gene silencing of target gene.

(a) The transposon vector with the 5' UTR of *Ci-pem* was omitted from pMiCiTnIGCipemG vector. (b) A larva derived from a egg of transgenic line created by the vector shown in (a) fertilized by wild-type sperm. A larva showed normal morphology, suggesting that 5' UTR of *Ci-pem* is the necessary region for *Ci-pem* knockdown. Bar, 100μm. (c) The transposon vector with the 5' UTR exchanged from *Ci-pem* to another maternal gene *Ci-Nut*. Transgenic line made by this vector was named Nut$^{5'UTR}$ line. (d) Relative expression levels of *Ci-pem* and *Ci-Nut* in eggs of Nut$^{5'UTR}$ lines, as revealed by quantitative RT-PCR. *Ci-Nut* expession was suppressed, but not *Ci-pem*, suggesting that the genes used for 5' UTR become the target for maternal gene knockdown. n=3. These three samples are derived from different Nut$^{5'UTR}$ lines. *P* values were calculated using the two-tailed Student's t test. (e) Typical morphology of larva derived from Nut$^{5'UTR}$ line eggs. The larval morphology was normal, suggesting that maternal *Ci-Nut* does not have critical role in *Ciona* larval development.

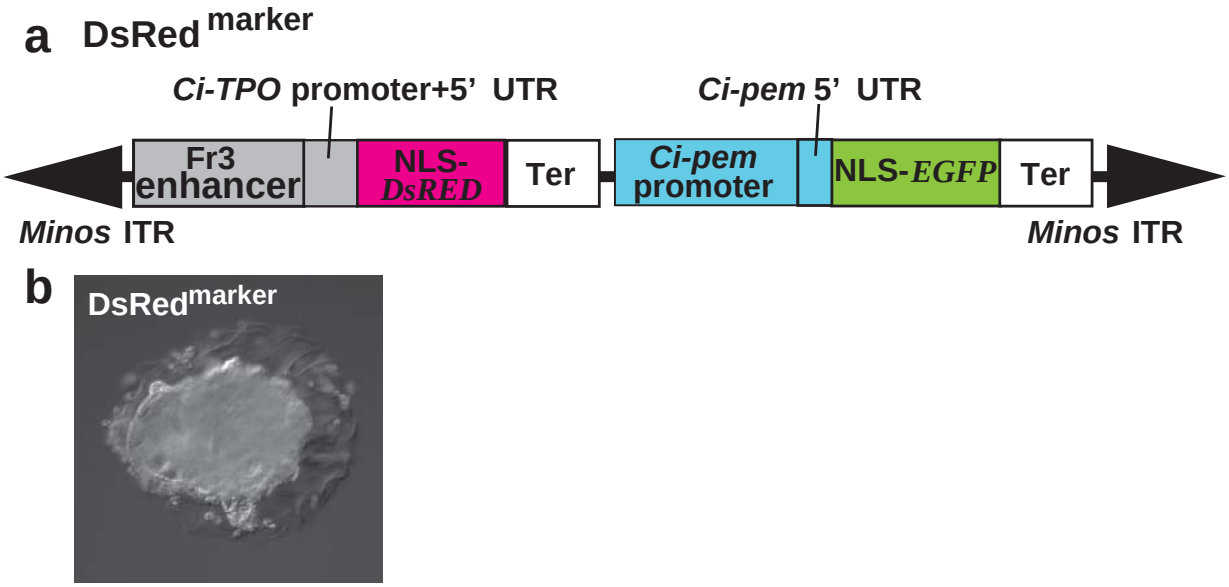

Figure 10. Marker cassette does not affect maternal gene silencing of target genes.

(a) The transposon vector with the *DsRed*-based marker cassette. (b) A larva derived from a transgenic line created by the vector in (a) showed *Ci-pem* knockdown phenotype, suggesting that the marker region does not affect on *Ci-pem* knockdown.

Figure 11. Kind of transposon do not affect maternal gene silencing of target genes.

Figure 12. Reporter genes other than *mAG* can be used for maternal mRNA knockdown.

These figures contain unpublished data, and they are concealed until data get published.

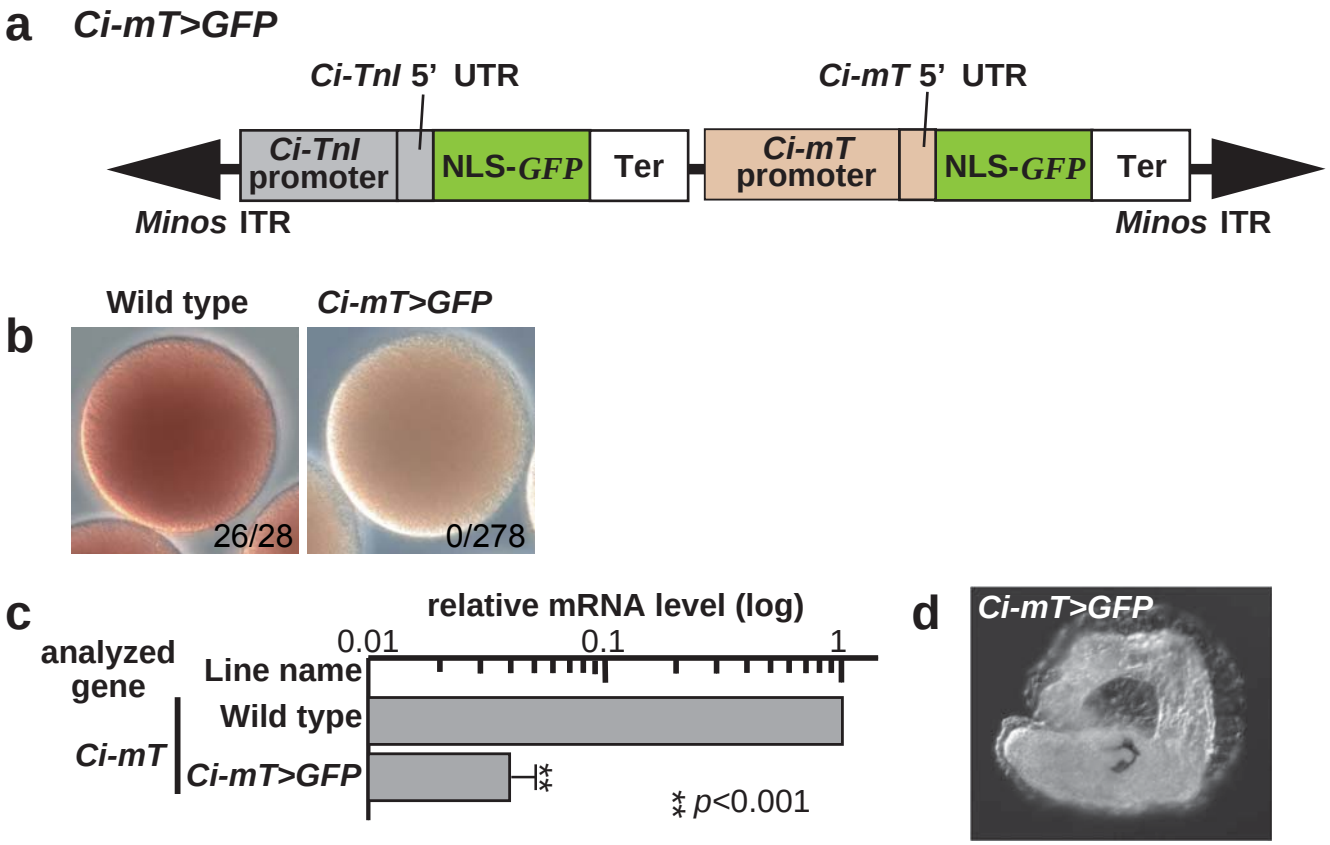

Figure 13. Targeted knockdown of *Ci-mT*.
(a) The transposon vector for *Ci-mT* knockdown, named Tg[MiCiTnIGCimtG]1 (*Ci-mT>GFP* line). 5' upstream region and 5' UTR of *Ci-mT* gene was utilized. (b) Knockdown of maternal *Ci-mT* mRNA as revealed by WISH. (Left) A wild-type egg. (Right) A *Ci-mT* knockdown egg. Signal of *Ci-mT* could not be observed. (c) Relative expression level of *Ci-mT* in eggs from *Ci-mT>GFP* line, as revealed by quantitative RT-PCR. n=2. *P* values were calculated using the two-tailed Student's t test. (d) Morphology of *Ci-mT* knockdown larva. A larva was derived from *Ci-mT>GFP* line egg fertilized by wild-type sperm showed abnormal tail development.

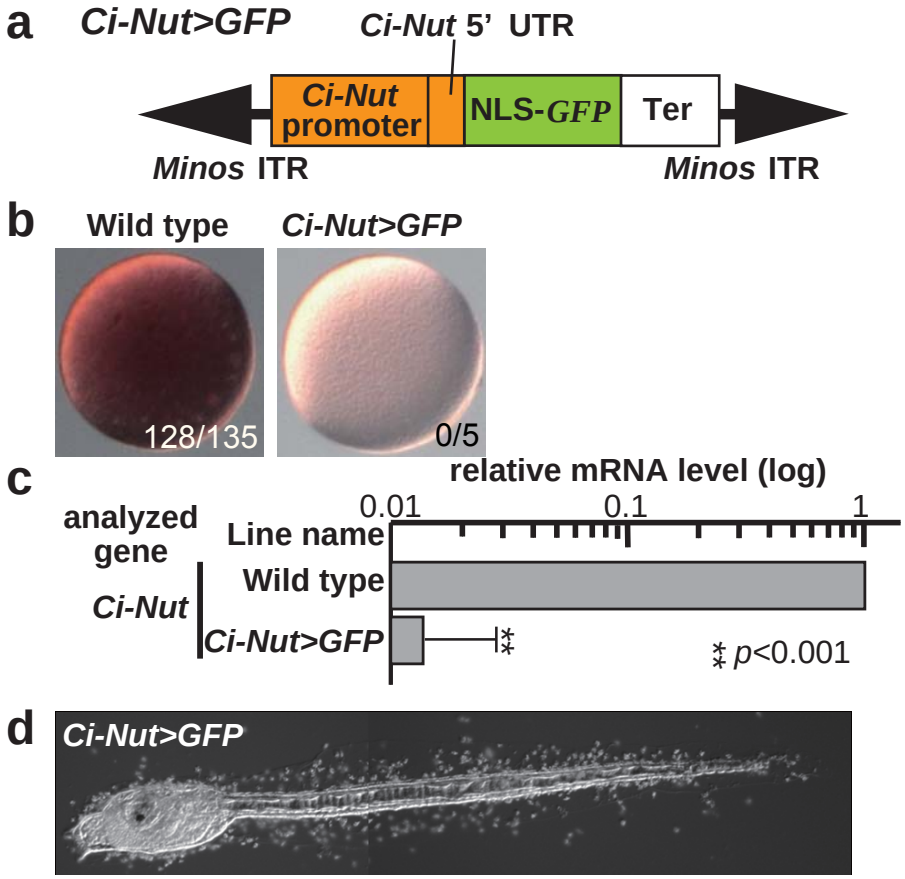

Figure 14. Targeted knockdown of maternal *Ci-Nut*.
(a) The transposon vector for *Ci-Nut* knockdown, named Tg[MiCiNutG]3, (*Ci-Nut>GFP* line). 5' upstream region and 5' UTR of *Ci-Nut* was utilized. Because *Ci-Nut* gene is expressed in both maternal and zygotic fashions, the marker cassette was omitted from the vector. (b) Knockdown of maternal *Ci-Nut* mRNA as revealed by WISH. (Left) A wild-type egg. (Right) A *Ci-Nut* knockdown egg. *Ci-Nut* mRNA could not be detected. (c) Relative expression level *Ci-Nut* in GFP-negative eggs from *Ci-Nut>GFP* line, as revealed by quantitative RT-PCR. n=2. *P* values were calculated using the two-tailed Student's t test. (d) A larva from the transgenic line created by the vector in (a), showing typical normal larval morphology.

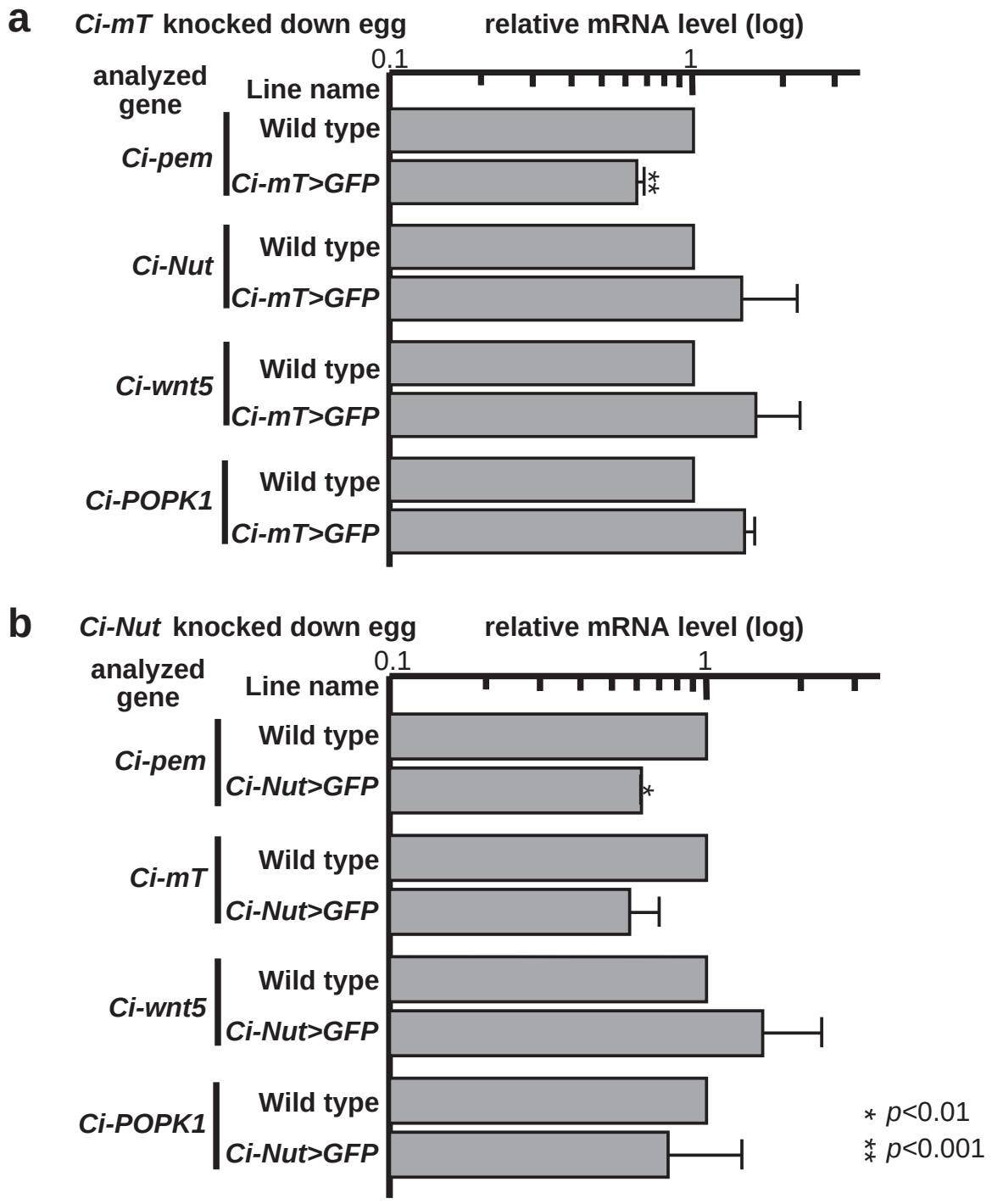

Figure 15. Target specificity of maternal mRNA knockdown.
(a) Relative expression levels of *Ci-pem*, *Ci-Nut*, *Ci-wnt5* and *Ci-POPK1* in the eggs of Tg[MiCiTnIGCimTG]1 (*Ci-mT>GFP*), as revealed by quantitative RT-PCR. n=2. *P* values were calculated using the two-tailed Student's t test. (b) Relative expression levels of *Ci-pem*, *Ci-mT*, *Ci-wnt5* and *Ci-POPK1* in the eggs of Tg[MiCiNutG]3 (*Ci-Nut>GFP*), as revealed by quantitative RT-PCR. n=2. *P* values were calculated by using the two-tailed Student's t test. In both lines, *Ci-pem* mRNA expression was reduced very weakly, but the other three maternal genes did not show significant reduction.

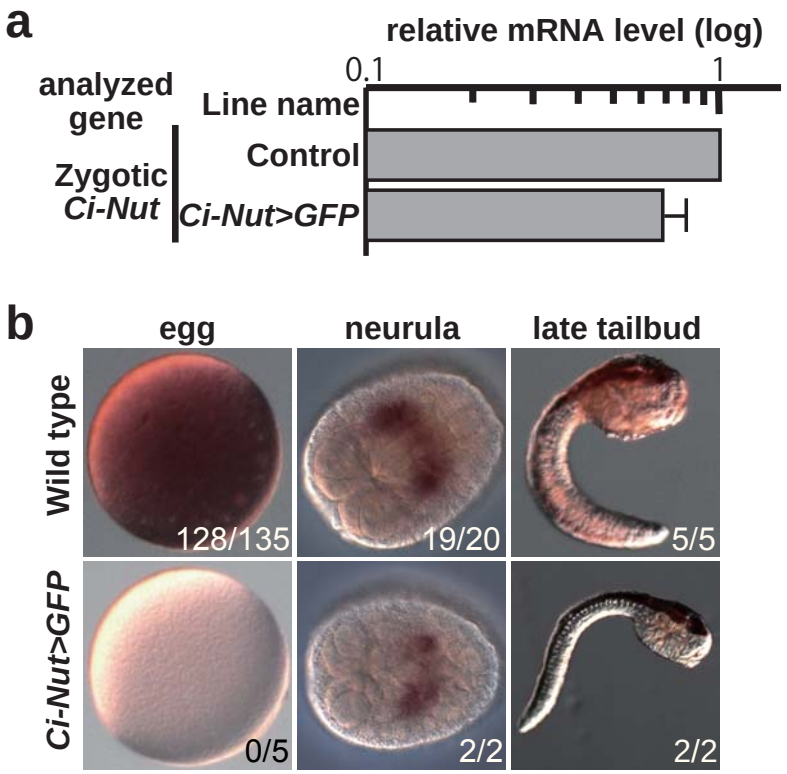

Figure 16. Zygotic *Ci-Nut* expression was not affected by maternal *Ci-Nut* knockdown.

(a) Relative expression level of zygotic *Ci-Nut* in larva, revealed by quantitative RT-PCR. n=2. Embryos derived from wild-type eggs fertilized by Tg[MiCiNutG]3 sperm were used as a control. *Ci-Nut>GFP* represent embryos derived from maternal *Ci-Nut* knockdown eggs crossed with wild-type sperm. zygotic *Ci-Nut* was expressed in the larvae derived from maternal *Ci-Nut* knockdown eggs, suggesting zygotic expression of *Ci-Nut* is not affected by maternal gene knockdown. *P* values were calculated using the two-tailed Student'st test. (b) Zygotic *Ci-Nut* expression was not affected by knockdown of maternal *Ci-Nut*, as revealed by WISH. Upper, *Ci-Nut* expression in wild-type sample. Bottom, *Ci-Nut* expression of the *Ci-Nut>GFP* line. Left, an unfertilized egg. Middle, a late gastrula-stage embryo. Right, a late tailbud-stage embryo. In embryos from *Ci-Nut* knockdown eggs, the maternal *Ci-Nut* mRNA could not be detected, whereas zygotic expression of *Ci-Nut* was detected in neural tissues.

Figure 17. Mosaic GFP expression in Tg[MiCiTnIGCiPrmG]2.
Figure 18. Antisense small RNAs produced from *Ci-pem* 5' UTR.

These figures contain unpublished data, and they are concealed until data get published.