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Transposon-mediated targeted and specific knockdown of maternally expressed transcripts in the ascidian *Ciona intestinalis*

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Maternal mRNAs play crucial roles during early embryogenesis of ascidians, but their functions are largely unknown. In this study, we developed a new method to specifically knockdown maternal mRNAs in *Ciona intestinalis* using transposon-mediated transgenesis. We found that *GFP* expression is epigenetically silenced in *Ciona intestinalis* oocytes and eggs, and this epigenetic silencing of *GFP* was used to develop the knockdown method. When the 5′ upstream promoter and 5′ untranslated region (UTR) of a maternal gene are used to drive *GFP* in eggs, the maternal gene is specifically knocked down together with *GFP*. The 5′ UTR of the maternal gene is the major element that determines the target gene silencing. Zygotic transcription of the target gene is unaffected, suggesting that the observed phenotypes specifically reflect the maternal function of the gene. This new method can provide breakthroughs in studying the functions of maternal mRNAs.

Eggs store a wide variety of mRNAs. These maternal transcripts, or maternal mRNAs, play crucial roles in the developmental processes of multicellular organisms. In order to understand these early developmental stages, it is necessary to determine the functions of maternal mRNAs. Ascidians, a group of chordates, are a good model to investigate the role of maternal mRNAs in developmental processes. Ascidian eggs are typical mosaic eggs[1], and the factors that determine cell fates and morphogenetic movement are prelocalized to specific parts of the egg[2]. Maternal mRNAs are potential candidates for these factors. Indeed, the maternal transcript of a gene named *macho1* determines the differentiation of muscle cells[3]. However, the functions of many ascidian maternal mRNAs remain unknown, mainly due to the limitation of techniques to investigate their functions.

In order to study maternal mRNAs, it is important to disrupt their functions. In ascidians, several approaches are currently used to disrupt maternally expressed genes. However, these approaches have disadvantages and are insufficient. For example, knockdown approaches, as represented by RNA interference (RNAi) and morpholino oligonucleotide (MO)−based knockdown, are convenient methods for disrupting maternal mRNAs of ascidians[4,5]. Generally speaking, RNAi has a disadvantage in that small RNAs can disrupt zygotic gene expression. Thus, it is difficult to determine whether the observed phenotype reflects the maternal or zygotic function of the gene if the target maternal gene has zygotic transcription. MOs are usually introduced into matured ascidian eggs to disrupt mRNA splicing or translation. Therefore, the functions of maternal genes that are already translated during oogenesis cannot be disrupted using MOs. Thus, it is important to establish a new method that efficiently and specifically disrupts ascidian maternal transcripts. Although forward genetics present one promising method, this approach requires extensive labor to isolate mutants. Screening maternal mutants takes one more generation than zygotic mutants, since it is necessary to create mutant females. Furthermore, if the mutation causes lethality during growth and development, maternal mutants cannot be obtained. This is also a disadvantage of knockout of *Ciona* genes using engineered nucleases[6,7].

We recently established a method of germline transformation for *Ciona intestinalis* using a transposon *Minos*[8]. With this method, we have created many transgenic lines that express green fluorescent protein (GFP) in a variety of tissues. By observing GFP expression in these lines, we noticed a curious phenomenon; namely, GFP expression in oocytes and eggs is epigenetically suppressed. Using this phenomenon, we established a new method to knockdown maternally expressed genes that does not affect zygotic mRNAs. Thus, we can specifically investigate

the functions of maternal mRNAs even though some genes exhibit both maternal and zygotic expression patterns. This new method will provide breakthroughs in the study of maternal mRNA function in *Ciona*.

Results

GFP expression is epigenetically suppressed in *Ciona intestinalis* oocytes and eggs. Transgenic lines that express GFP in oocytes and eggs were created using the 5′ upstream regions of maternally expressed genes or by transposon-mediated enhancer detection that entraps enhancers for maternal gene expression. GFP expression was typically observed in only a few oocytes and eggs of these maternal GFP lines (Fig. 1a). The percentage of GFP-positive or GFP-negative eggs ranged from 0 to 100% among transgenic lines, even though the lines were created with the same transposon vector. Whole-mount *in situ* hybridization (WISH) showed that *GFP* mRNA was absent in GFP-negative eggs (Fig. 1b, c), suggesting that transcriptional or post-transcriptional regulation is a likely cause of maternal *GFP* suppression. Because *Ciona* oocytes and unfertilized eggs are diploid, these cells of GFP-transgenic lines must have the *GFP* gene. Indeed, when transgenic lines expressing GFP in both a maternal and zygotic fashion showed epigenetic GFP silencing in eggs, zygotic GFP expression was observed in animals that developed from GFP-negative eggs (Supplementary Fig. 1), suggesting that GFP-negative eggs contain an intact *GFP* gene. Thus, the absence of GFP expression in oocytes and eggs was caused by epigenetic gene silencing. In addition, zygotic GFP expression was comparable in animals derived from GFP-negative

Figure 1 | **Maternal expression of GFP is epigenetically silenced in *Ciona*.** (a) A typical GFP expression pattern in the ovary. An ovary of an enhancer detection line EJ[MiTSAdTPOG]78, which entrapped an enhancer responsible for expression in oocytes. Only a few oocytes express GFP. Bar, 100 μm. (b, c) Expression of *GFP* mRNA in unfertilized eggs of a maternal GFP line, as revealed by whole-mount *in situ* hybridization (WISH). Dark blue staining suggests the presence of *GFP* mRNA. (b) An egg that had GFP fluorescence. (c) An egg that lacked GFP fluorescence.

eggs and GFP-positive eggs, suggesting that suppressed GFP expression is specific for maternal expression but not zygotic GFP expression.

Knockdown of maternal *Ci-pem* mRNA. The aptly named gene *posterior end mark* (*pem*) encodes a maternal mRNA that localizes to the posterior end of eggs[9]. *Ciona intestinalis pem* (*Ci-pem*) exhibits exclusive maternal expression throughout embryonic development[10]. Using the 5′ upstream region of *Ci-pem*, we created a transposon vector that drives GFP expression in oocytes and eggs. The 5′ upstream region of *Ci-pem* includes the 5′ untranslated region (UTR) and initiation codon of this gene. A fusion of the 5′ upstream region/5′UTR of a muscle gene *Ci-TnI* (which encodes Troponin I) with *GFP* was introduced next into the *Ci-pem* > *GFP* cassette (Fig. 2a). The *Ci-TnI* promoter drives GFP in muscle tissue but not in oocytes or eggs[11]. GFP expression from the *Ci-TnI* > *GFP* cassette was used as a marker to select transgenic animals during culture. Using this transposon vector, we created several transgenic lines expressing GFP in oocytes and eggs. Hereafter, these lines are called "*pem* > *GFP* lines". As described above, GFP expression appeared in a mosaic pattern in oocytes and eggs in *pem* > *GFP* lines (Fig. 2b).

Progeny were obtained by crossing these *pem* > *GFP* lines with wild-type animals. When sperm from *pem* > *GFP* lines were crossed with wild-types eggs, the progeny showed normal embryogenesis and larval development (Fig. 2c). In contrast, when eggs of *pem* > *GFP* lines were crossed with wild-type sperm, many progeny exhibited abnormal embryogenesis (Fig. 2d). At the larval stage, their embryonic axis could not be recognized. The body of the abnormal larvae usually separated into two parts, one of which had vacuolated notochord cells (Fig. 2d). When the tissue differentiation of these animals was examined, the major tissues of the larval body, namely the epidermis, muscle, notochord, neural tissues and endoderm, had properly differentiated (Fig. 2e–i). However, their relative position was abnormal. In normal larvae, muscle and notochord cells were placed in the tail, while most endodermal cells localized in the trunk, separate from the muscle and notochord. In abnormal larvae, cells of the muscle, notochord and endoderm were clustered in the same position. This positional pattern looks like that of the vegetal hemisphere of 110-cell stage embryos. Because endodermal cells move toward the trunk by gastrulation that starts around the 110-cell stage, the abnormality of *pem* > *GFP* embryos is likely associated with defects in gastrulation.

The ratio of normal and abnormal embryos derived from *pem* > *GFP* eggs differed among transgenic lines (Table 1) and correlated with the ratio of GFP silencing. For example, all of the eggs of *pem* > *GFP* line 2, which did not express GFP, developed abnormally, while all of the eggs of *pem* > *GFP* line 9, which expressed GFP, developed normally. In concert to the correlation between GFP silencing and abnormal development, all of the normally developed larvae showed maternal GFP expression, while abnormal larvae never exhibited maternal GFP expression even though zygotic GFP expression from the *Ci-TnI* > *GFP* cassette was observed (Fig. 3a). Therefore, the abnormal development observed in *pem* > *GFP* lines is strongly associated with epigenetic GFP silencing in eggs.

To investigate the mechanisms by which embryos derived from eggs of *pem* > *GFP* lines become abnormal, we performed quantitative RT-PCR and WISH for *Ci-pem* mRNA (Figs. 3b–g). WISH showed that *Ci-pem* mRNA was reduced to undetectable level in the GFP-negative eggs of *pem* > *GFP* lines (Figs. 3b–f). Quantitative RT-PCR showed that the level of *Ci-pem* mRNA was reduced to approximately 2.3–21% of the level in wild-type eggs (Fig. 3g). The abnormal development observed in *pem* > *GFP* lines was ameliorated by introducing *in vitro*-transcribed *Ci-pem* mRNA (Fig. 3h,i), suggesting that abnormal development was specifically caused by loss of *Ci-pem* mRNA. We investigated the quantities of four maternal mRNAs,
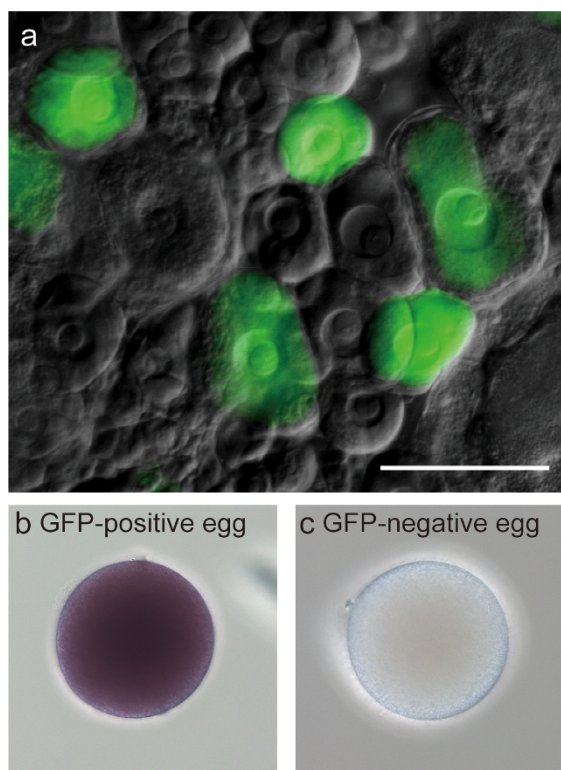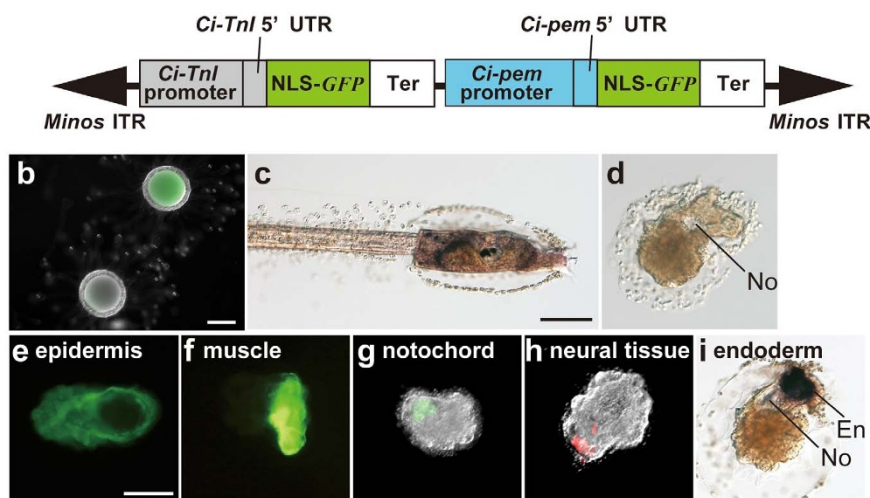
Figure 2 | Morphological defects seen in *pem > GFP* lines. (a) The transposon vector used to knockdown *Ci-pem*. Black arrowheads indicate inverted repeats (ITR) of *Minos*. UTR, untranslated region; NLS, nuclear localization signal sequence; Ter, transcription termination sequence. (b) GFP expression in unfertilized eggs of *pem > GFP* line 1. The egg in the upper right corner emitted GFP fluorescence, while the egg in the lower left corner did not. Bar, 100 μm. (c) A larva derived from sperm of *pem > GFP* line 4 and a wild-type egg. Bar, 100 μm. (d) A larva derived from an egg of *pem > GFP* line 4 and wild-type sperm. No, notochord. (e–i) Differentiation of major tissues in abnormal larvae derived from eggs of *pem > GFP* lines. (e) Epidermis (green). (f) Muscle (green). (g) Notochord (green). (h) Neural tissues (red). (i) Endoderm (En).

including *Ci-mT*[12], *Ci-Nut*[13], *Ci-Wnt5*[14], and *Ci-POPK1*[15], in *Ci-pem*–knockdown eggs derived from *pem > GFP* line 2. None of these mRNAs were present at lower levels in *Ci-pem*–knockdown than in wild-type eggs (Supplementary Fig. 2a), suggesting that the effect of the knockdown is specific to this gene. These results suggest that *Ci-pem* is silenced through a mechanism associated with epigenetic silencing of *GFP* in *Ciona* eggs.

There was a possibility that the transposon vector might have been inserted into the genomic region near *Ci-pem* gene to disrupt its expression in the *pem > GFP* lines. To examine this possibility, we identified the insertions sites of three *pem > GFP* lines in which *Ci-pem* knockdown was observed (Supplementary Table 1). All integration sites in these lines are distant from the *Ci-pem* locus (the gene model of *Ci-pem* is KH.C1.755), suggesting that transposon insertion is not the cause of knockdown of *Ci-pem*.

Silencing of maternal mRNAs depends on their promoters and 5′ UTRs. To understand mechanisms how *Ci-pem* gene was silenced in *pem > GFP* transgenic lines, the elements in the transposon vector for *pem > GFP* lines (pMiCiTnIGCipemG) were exchanged and the effects of the variant vectors on gene knockdown were examined. We paid particular attention to the 5′ UTR since this element is transcribed together with *GFP*. We deleted the 5′ UTR of *Ci-pem* from the pMiCiTnIGCipemG vector (Fig. 4a), and three transgenic lines were established with this vector. None of the three lines showed the *Ci-pem* knockdown phenotype (Fig. 4b), suggesting the important role of the 5′ UTR for knockdown of this gene. Then,

we exchanged the 5′ UTR of *Ci-pem* with that of another maternally transcribed gene encoding an Opsin-related protein, *Ci-Nut*[13], and created three transgenic lines (Nut$^{5'UTR}$ lines) with the vector (the marker cassette was exchanged with a cassette expressing *DsRed*, according to the result described below; Fig. 4c). Eggs of the Nut$^{5'UTR}$ line did not exhibit significant reduction of *Ci-pem* mRNA (Fig. 4d,e). By contrast, the level of *Ci-Nut* mRNA was reduced to approximately 27% of the level in wild-type eggs (Fig. 4d). This reduction is less efficient than the knockdown of this gene observed in the *Ci-Nut > GFP* line, as described below (Fig. 5c). From these data, we conclude that the 5′ UTR is a major element for the selection of the target gene for silencing; however, it is possible that the promoter region also participates in efficient silencing.

Next, we tested the necessity of *GFP* for *Ci-pem* silencing. The reporter gene driven by the *Ci-pem* promoter was changed from *GFP* to *Kaede* (Fig. 4f), and four transgenic lines (*pem > Kaede* lines) were created with the vector. Among them, two lines showed silencing of Kaede expression in eggs (Supplementary Fig. 3a–d). Larvae derived from the Kaede-silenced eggs showed phenotypes characteristic of *Ci-pem* knockdown (Fig. 4g), suggesting that the reporter gene does not have to be *GFP* to achieve *Ci-pem* knockdown. Finally, the necessity of the marker cassette was investigated. The *Ci-TnI > GFP* cassette was exchanged with a cassette expressing *DsRed* driven by the *Ci-musashi* Fr3 enhancer and *Ci-TPO* promoter (Fig. 4h)[16]. Neither the Fr3 enhancer nor the *Ci-TPO* promoter drove maternal expression. Among three transgenic lines created with this vector (DsRed^{marker} lines), two of them showed *Ci-pem* knockdown (Fig. 4i and Supplementary Fig. 3e–g), suggesting that the marker cassette does not affect maternal knockdown.

Knockdown of various maternal mRNAs. We tested whether maternal mRNAs other than *Ci-pem* can be knocked down using a similar experimental design. We chose two maternal genes as targets, namely *Ci-mT* and *Ci-Nut*. *Ci-mT* encodes a T-box transcription factor that shows maternal expression throughout embryonic stages[12]. To construct the knockdown vectors for these genes, the 5′ upstream region and 5′ UTR of the two genes were utilized (Fig. 5a,b). *Ci-Nut* shows zygotic expression in the neural tissues in

Table 1 | Frequency of larvae derived from eggs of *pem > GFP* lines showing *Ci-pem* knockdown phenotypes

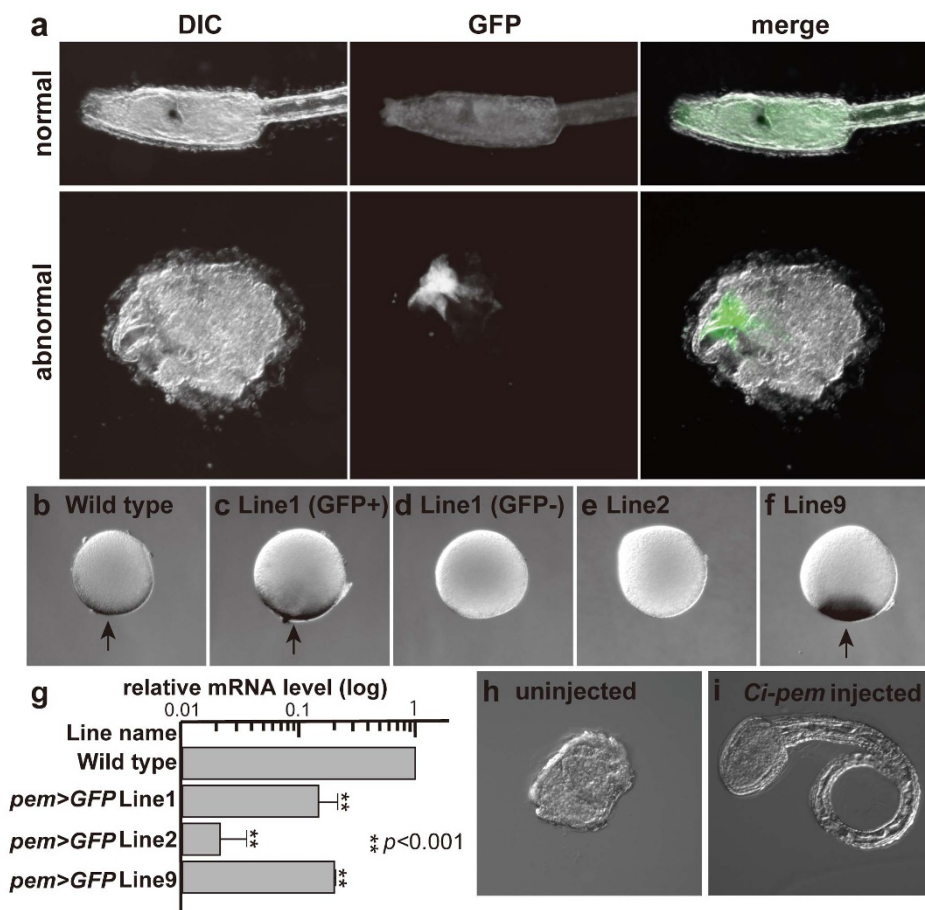| Line ID	% of normal larvae	% of abnormal larvae	no. of larvae examined
1	16	84	39
2	0	100	191
4	22	78	296
9	100	0	>100

Figure 3 | Knockdown of *Ci-pem*. (a) Abnormal larvae of *pem > GFP* lines were derived from GFP-negative eggs. One normal and one abnormal larvae derived from the same individual of a *pem > GFP* line are shown. In the normal larva, GFP fluorescence was detected throughout the body, suggesting that the fluorescence was derived from maternal GFP expression. Such maternal GFP fluorescence was not detected in the abnormal larva, although zygotic GFP expression derived from the *Ci-TnI > GFP* cassette was evident. (b–f) *Ci-pem* maternal mRNA was decreased in GFP-negative eggs of *pem > GFP* lines, as revealed by WISH. Arrows indicate the position of the signal. (b) An egg derived from a wild-type animal. (c) An egg of *pem > GFP* line 1 that had GFP fluorescence. (d) An egg of line 1 that lacked GFP fluorescence. (e) An egg of *pem > GFP* line 2. All of the eggs were GFP negative. (f) An egg of *pem > GFP* line 9. All of the eggs were GFP-positive. (g) Relative expression levels of *Ci-pem* in 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. (h) A larva derived from an egg of *pem > GFP* line 2. (i) A larva derived from an egg of *pem > GFP* line 2 that had been microinjected with *in vitro*-synthesized *Ci-pem* mRNA.

addition to maternal expression, and the 5′ upstream region of *Ci-Nut* can drive GFP in neural tissues like endogenous *Ci-Nut*[17]. For this reason, the marker cassette was omitted from the *Ci-Nut* knockdown construct (Fig. 5b). Using these vectors, three transgenic lines, namely Tg[MiCiTnIGCimTG]1, Tg[MiCiNutG]3 and Tg[MiCiNutG]4, were established (Tg[MiCiNutG]3 and Tg[MiCiNutG]4 were previously described[18]). Among them, Tg[MiCiTnIGCimTG]1 and Tg[MiCiNutG]3 showed silencing of GFP expression in eggs. The *Ci-mT* and *Ci-Nut* expression levels in GFP-negative eggs were examined by WISH and quantitative RT-PCR. There was a dramatic reduction in *Ci-mT* and *Ci-Nut* in GFP-negative eggs of the corresponding lines (Fig. 5c and Supplementary Fig. 4). *Ci-mT* mRNA in Tg[MiCiTnIGCimTG]1 eggs was reduced to 4.1% of that in wild-type eggs, and *Ci-Nut* mRNA was reduced to 1.4% in Tg[MiCiNutG]3 eggs (Fig. 5c). Embryos derived from *Ci-mT*-knocked down eggs showed an abnormality in the tail (Fig. 5d), which was distinct from the phenotypes observed in *Ci-pem* knocked down embryos. This phenotype could not be rescued by introducing *in vitro*-transcribed *Ci-mT* mRNA. *Ci-mT* is probably translated during oogenesis, and the maternally supplied *Ci-mT* protein may be necessary for morphogenesis. *Ci-Nut*−knocked down eggs showed normal embryogenesis and developed into normal larvae (Fig. 5e).

This suggests that the knockdown method itself does not affect embryogenesis. The knockdown lines of *Ci-pem*, *Ci-mT* and *Ci-Nut* showed different phenotypes, suggesting that the phenotypes obtained from this method reflect the loss of the target gene. We investigated expression of four maternal transcripts in *Ci-mT*− or *Ci-Nut*−knockdown eggs by quantitative RT-PCR (Supplementary Fig. 2b,c). 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.

Ci-Nut is both maternally and zygotically expressed[13]. Using this characteristic, we investigated whether zygotic expression of the target gene is affected by maternal gene silencing. Eggs in which maternal *Ci-Nut* mRNA was knocked down were fertilized with wild-type sperm, and expression of zygotic *Ci-Nut* was investigated using quantitative RT-PCR and WISH. Quantitative RT-PCR revealed that expression levels of *Ci-Nut* in tailbud embryos from maternal *Ci-Nut*–knockdown eggs did not differ significantly from the levels in wild-type embryos at the same stage (Fig. 5f), suggesting that zygotic expression occurred in embryos derived from maternal *Ci-Nut*−knockdown eggs. WISH of maternal *Ci-Nut*−knockdown embryos revealed that zygotic *Ci-Nut* expression begins in the neural tissues at the late gastrula stage, as in embryos derived from wild-type eggs, and that this expression continued until the tailbud stage
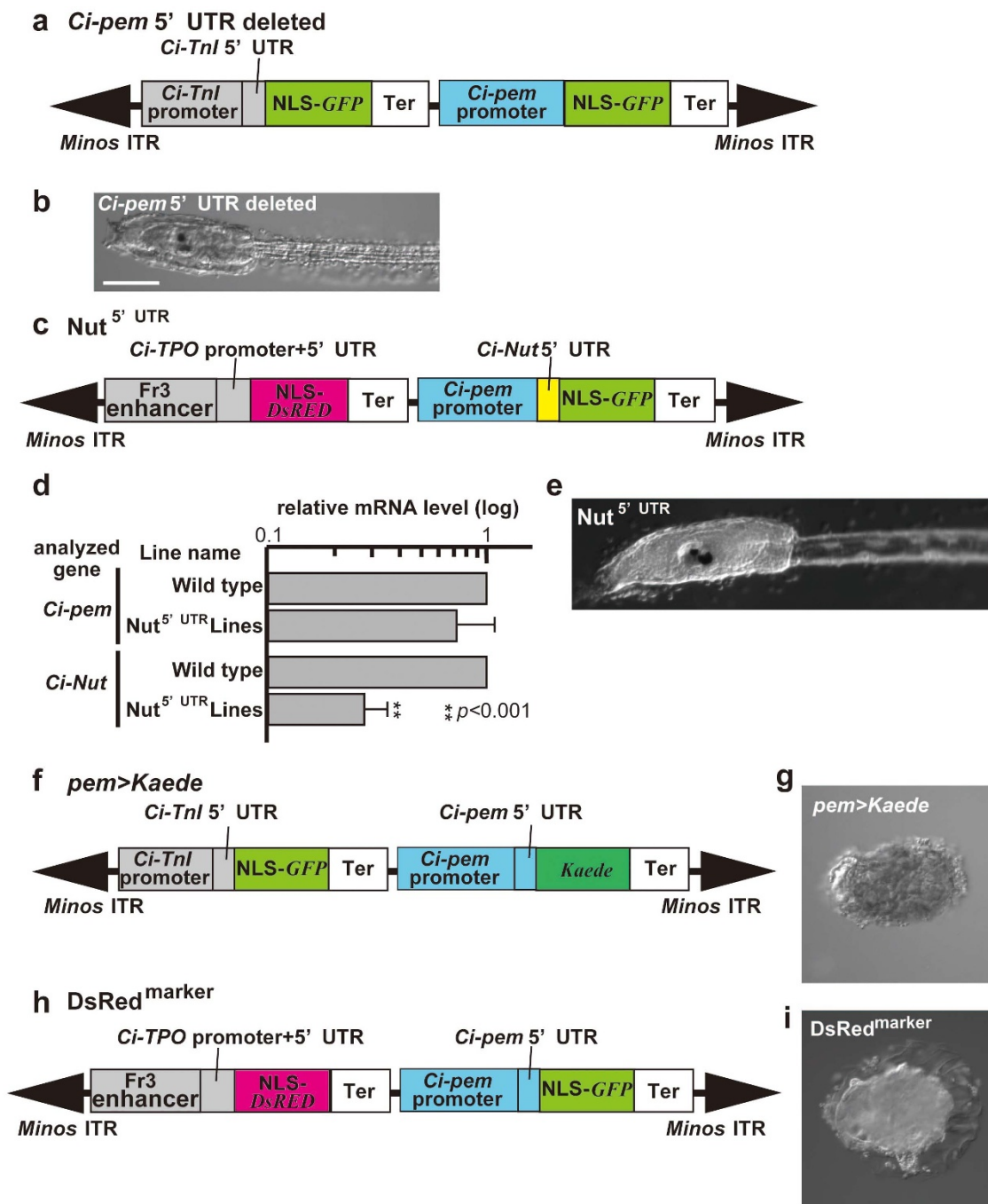
Figure 4 | The 5′ UTR is essential for maternal gene silencing of the target gene. (a) The transposon vector from which the *Ci-pem* 5′ UTR was omitted. (b) A larva derived from a transgenic line created by the vector shown in (a). Bar, 100 μm. (c) The transposon vector in which the 5′ UTR of *Ci-pem* was substituted with the 5′ UTR of *Ci-Nut*. (d) Relative expression levels of *Ci-pem* and *Ci-Nut* in eggs of Nut[5′UTR] lines, as revealed by quantitative RT-PCR. n = 3. Note that three samples were derived from different Nut[5′UTR] lines. *P* values were calculated using two-tailed Student's t test. (e) Typical morphology of larvae derived from eggs of Nut[5′UTR] lines. (f) The transposon vector in which the *Kaede* reporter was fused to the *Ci-pem* promoter and 5′ UTR. (g) A larva derived from an egg of the *pem > Kaede* line showed a typical morphology associated with *Ci-pem* knockdown. (h) The transposon vector in which the DsRed-based marker cassette was utilized. (i) A larva derived from a transgenic line created with the vector in (h) showed the typical morphology associated with *Ci-pem* knockdown.

(Fig. 5g). We conclude that the knockdown of genes investigated in this analysis is specific to maternal transcripts as was observed with GFP (Fig. 3a).

Discussion

Here we established a new method to knockdown maternal mRNAs in the ascidian *Ciona intestinalis*. We named this method MASK, after maternal mRNA-specific knockdown. MASK has four advantages over previously reported methods for maternal mRNA disruption in ascidians. First, MASK is a reverse genetic approach and we can disrupt the function of a gene of interest without laborious screenings. Second, maternal expression can specifically be knocked down using MASK and the obtained phenotypes reflect the maternal function of the targeted gene. Third, this method utilizes genetic modification; once the transgenic lines have been established, we can obtain maternal mRNA-knocked down eggs and embryos without further experimentation.
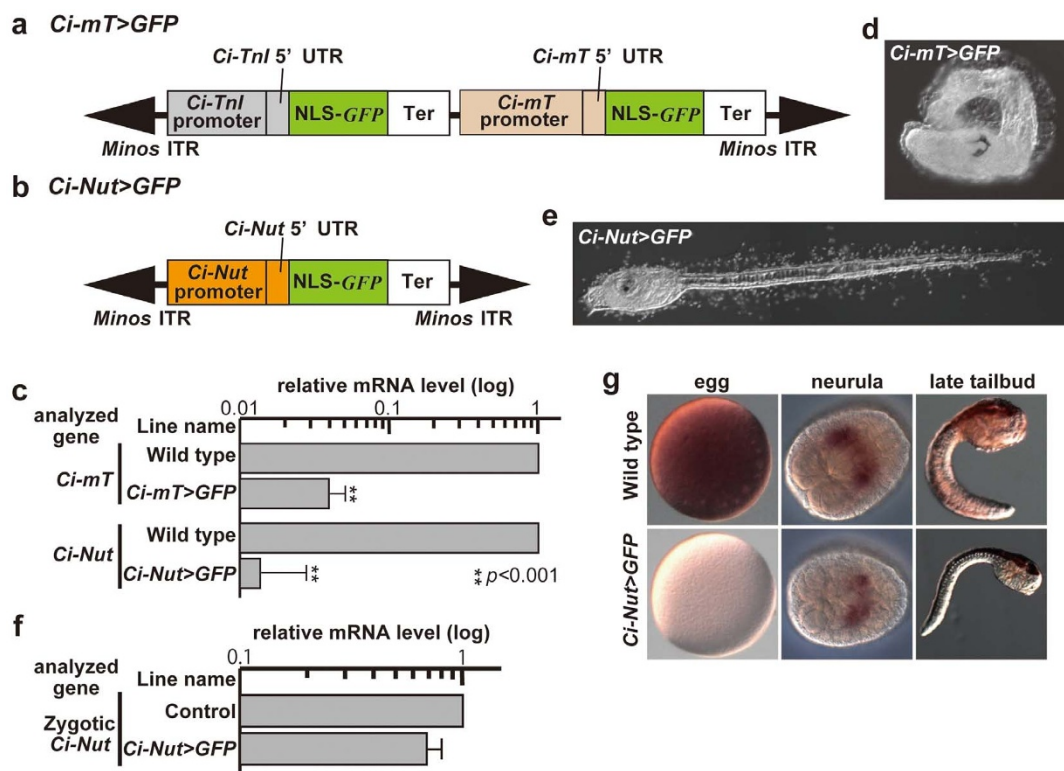
Figure 5 | Targeted knockdown of maternal transcripts. (a) The transposon vector for *Ci-mT* knockdown. (b) The transposon vector for *Ci-Nut* knockdown. (c) Relative expression levels of *Ci-mT* and *Ci-Nut* in *Ci-mT−* or *Ci-Nut−*knockdown eggs, as revealed by quantitative RT-PCR. n = 2. *P* values were calculated using the two-tailed Student's t test. (d) Morphology of *Ci-mT−*knockdown larva derived from eggs of Tg[MiCiTnIGCimTG]1. (e) Morphology of maternal *Ci-Nut−*knocked down larva derived from eggs of Tg[MiCiNutG]3. (f) Relative expression levels of *Ci-Nut* in tailbud embryos, as revealed by quantitative RT-PCR. n = 2. Control, embryos derived from wild-type eggs fertilized with sperm from Tg[MiCiNutG]3. *Ci-Nut* > GFP, embryos derived from *Ci-Nut−*knockdown eggs fertilized with wild-type sperm. *P* values were calculated using the two-tailed Student's t test. (g) Zygotic *Ci-Nut* expression was not affected by knockdown of maternal *Ci-Nut*, as revealed by WISH. Upper, *Ci-Nut* expression in a wild-type egg (left), a late gastrula-stage embryo (middle), and a late tailbud-stage embryo (right). Bottom, *Ci-Nut* expression in an egg (left), a late gastrula-stage embryo (middle), and a late tailbud-stage embryo (right) derived from maternal *Ci-Nut−*knockdown eggs. In embryos from *Ci-Nut−*knockdown eggs, the maternal *Ci-Nut* transcript was not detected, whereas zygotic expression of *Ci-Nut* was observed in the neural tissue.

Fourth, MASK is simple and easy and the only steps required to create the knockdown vector are isolation of the 5′ upstream region and 5′ UTR and their fusion with *GFP/Kaede* in *Minos*. With these advantages, MASK will be a powerful approach to study the function of maternal mRNAs in ascidian eggs.

The basis of MASK is epigenetic knockdown of *GFP* and *Kaede*, which occurs in oocytes and eggs. How do these reporter genes become targets of epigenetic silencing in eggs? A plausible explanation is that epigenetic gene modification occurs during oogenesis of *Ciona* to silence some endogenous genes, and the reporter genes may be accidental targets of the epigenetic regulation. Silencing occurs at the mRNA level, suggesting that transcriptional gene silencing or post-transcriptional degradation of mRNAs is a potential mechanism for silencing. MASK does not affect zygotically transcribed mRNAs. If silencing occurs at the post-transcriptional level, residual MASK factors could silence zygotically transcribed mRNAs. This mechanism resembles maternal *gfp*/gene silencing (MGS), which is another form of epigenetic silencing in *Ciona* that degrades both maternal and zygotic *GFP* mRNAs[18]. Therefore, we favor the idea that transcriptional silencing is the mechanism of MASK. A portion of oocytes and eggs that escaped MASK often showed strong GFP expression (see Fig. 1a). Transcriptional silencing can explain this phenomenon; once silencing is canceled in some oocytes, continuous transcription of *GFP* causes the accumulation of GFP proteins in cells, resulting in strong fluorescence. We assume that the *cis* elements and 5′ UTRs of *Ciona* genes transcribed together with

GFP and *Kaede* are regulated to make factors like microRNAs that suppress transcription of the endogenous gene[19]. This silencing may be inhibited after fertilization, probably through epigenetic remodeling in chromosomes, causing zygotic transcription not to be affected. Similarly, transcriptional silencing may accidentally be inhibited in a portion of oocytes during oogenesis, and therefore silencing was seen in a mosaic fashion. MASK may be affected by the genomic context around the transposon vector insertion sites since the degree of silencing differs across transgenic lines created with the same knockdown vector. This suggests that production of the factor that generates MASK may depend on the genomic context around the insertion site. We identified some of the insertion sites of the knockdown vectors, although we did not identify differences between MASK-positive and -negative loci. The dependency of MASK on the genomic context suggests that some maternal genes might be resistant to MASK based on their genomic location.

Knockdown of maternally expressed genes with MASK will disclose the function of maternal genes in *Ciona*. Moreover, future studies will elucidate the detailed mechanisms of MASK and reveal the endogenous function of maternal gene silencing in *Ciona intestinalis*. Based upon previous reports of epigenetic silencing in other organisms[20–22], possible approaches to uncover the mechanism of MASK include, exchanging GC composition or codon usage of *GFP/Kaede*, mutating inverted repeats of *Minos* transposons, and characterizing *trans* factors that binds to the target gene to cause MASK. In addition, other chordates should be examined in future

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studies to determine whether MASK is more broadly applicable, which would facilitate the study of maternal mRNAs in other eukaryote lineages.

Methods

Constructs. The 5′ upstream region and 5′ UTR of genes were isolated by PCR using *C. intestinalis* genomic DNA as a template. The PCR fragments were digested with *Bam*HI and subcloned into the *Bam*HI site of pSPeGFP[8] or pSPKaede[23]. The fusion cassettes were PCR amplified, and subcloned into pMiCiTnIG. For pMiFr3dTPORCipemG, the *Ci-pem-GFP* cassette was subcloned into pMiFr3dTPORDestR using gateway technology (Invitrogen).

Transgenic lines. Tg[MiCiNutG]3 and Tg[MiCiNutG]4 were previously described[18]. EJ[MiTSAdTPOG]78 is an enhancer detection line created by the method described in a previous report[24]. The other transgenic lines were created by *Minos*-mediated transgenesis as previously described[25].

Identification of insertion sites. The insertion sites of *pem > GFP* lines were characterized by thermal asymmetric interlaced (TAIL) PCR, according to previous reports[26,27]. Genomes isolated from sperm were used as templates for PCR. PCR fragments were subcloned into the 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[28] to create pRN3CipemFL. mRNA was synthesized 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[29]. The concentration of mRNA in the injection medium was adjusted to 500 ngμl^{-1}.

Tissue differentiation. Differentiation of epidermis, notochord and neural tissues in *C. intestinalis* larvae was investigated using marker transgenic lines that express *GFP* or *Kaede* reporter genes under the control of *cis* elements of the *Ci-EpiI*, *Ci-Bra* and *Ci-β2TB* genes[30,31]. The sperm of these transgenic lines was used to fertilize eggs of *pem > GFP* lines, and fluorescence was detected at the larval stage. To monitor muscle differentiation, GFP expression from the *Ci-TnI-GFP* cassette in the *Ci-pem* knockdown vector was utilized. For endoderm differentiation, histochemical staining of alkaline phosphatase was performed as previously described[32].

Gene expression. Eggs of transgenic lines were divided according to the presence and absence of GFP fluorescence prior to sampling for the following experiments. Whole-mount *in situ* hybridization (WISH) was performed as previously described[18,33]. Quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed as previously described[18] using *GAPDH* as normalizing gene. A 0.5–1.0 egg or embryo equivalent quantity of cDNA was used as a template for quantitative reverse transcription-PCR (RT-PCR). Relative quantification of mRNA was carried out using standard curves created by cloned cDNAs. The primer sequences were 5′-gtcctcgtacagtttagccatgtcg-3′ and 5′-caattcactgatcgtatagtgttgg-3′ for *Ci-pem*, 5′-gtggatgctccattccaag-3′ and 5′-gtcatacgcacgggttctg-3′ for *Ci-Par-1*, 5′-gtcgcaaacgtcatcacc-3′ and 5′-ggcctactgggtctgtttcg-3′ for *Ci-mT*, 5′-cgtggattgccattgacag-3′ and 5′-cgctctcataagccccaaac-3′ for *Ci-Nut*, and 5′-gatcgcatcataggatgctgg-3′ and 5′-tgtatccgtggttgaccttacag-3′ for *GAPDH*. *P*-values were calculated using a two-tailed Student's t test.

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Author contributions
T.I. and Y.S. designed research. T.I., K.M., A.H., M.H. and Y.S. performed experiments. T.I., K.M., A.H., M.H., N.S. and Y.S. analyzed data. Y.S. wrote the paper and T.I., K.M., M.H. and N.S. contributed in the form of discussion and critical comments.

Additional information
Supplementary information accompanies this paper at http://www.nature.com/scientificreports