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Ci-Pem-1 localizes to the nucleus and represses somatic gene transcription in the germline of *Ciona intestinalis* embryos

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SUMMARY

In many animal embryos, germ-cell formation depends on maternal factors located in the germ plasm. To ensure the development of germ cells, germline progenitors must be prevented from differentiating inappropriately into somatic cells. A common mechanism for this appears to be the active repression of somatic gene transcription. Species-specific germ-plasm components, such as Pgc in *Drosophila* and PIE-1 in *C. elegans,* establish germline transcriptional quiescence by inhibiting general transcriptional machineries. In the ascidian *Ciona intestinalis,* although transcriptional repression in the germline has been proposed, the factors and mechanisms involved have been unknown. We found that the protein products of *Ci-pem-1* RNA, which is an ascidian-specific component of the postplasm (the germ plasm equivalent in ascidians), localized to the nucleus of germline blastomeres, as well as to the postplasm. Morpholino oligonucleotide-mediated *Ci-pem-1* knockdown resulted in the ectopic expression of several somatic genes that are usually silent in the germline. In the *Ci-pem-1* knockdown embryos, the expression of both β-catenin- and GATAa-dependent genes was derepressed in the germline blastomeres, suggesting that Ci-Pem-1 broadly represses germline mRNA transcription. Immunoprecipitation assays showed that Ci-Pem-1 could interact with two *C. intestinalis* homologs of Groucho, which is a general co-repressor of mRNA transcription. These results suggest that *Ci-pem-1* is the *C. intestinalis* version of a germ-plasm RNA whose protein product represses the transcription of somatic genes during specification of the germ-cell fate, and that this repression may be operated through interactions between Ci-Pem-1 and Groucho co-repressors.

KEY WORDS: *Ciona intestinalis*, Germ cell, Germ plasm, Transcriptional repression, *Ci-pem-1*, PEM

INTRODUCTION

How cells adopt specific fates during development is a central topic in developmental biology. While somatic cells function in the maintenance of an animal, germ cells, the progenitors of gametes, are the only ones that transmit genetic information through sexual reproduction. In many animals, the germline and somatic lineages diverge during early embryonic development. Although somatic cell differentiation is generally initiated through transcriptional activation by specific transcription factors, germline specification often involves the active repression of mRNA transcription (Nakamura and Seydoux, 2008; Nakamura et al., 2010). For example, in *C. elegans* and *Drosophila*, the repression of mRNA transcription in the germline depends on maternal factors (i.e. PIE-1 in *C. elegans* and Pgc in *Drosophila*), which are assembled into a distinct cytoplasmic compartment called the germ plasm (Hanyu-Nakamura et al., 2008; Martinho et al., 2004; Mello et al., 1996; Nakamura et al., 1996; Seydoux et al., 1996). Pgc and PIE-1 exert their repressor function by inhibiting the phosphorylation of the C-terminal domain (CTD) of the largest subunit of RNA polymerase II (RNAPII), which is a crucial modification necessary for mRNA transcription (Batchelder et al., 1999; Hanyu-Nakamura et al., 2008; Martinho et al., 2004). Importantly, PIE-1 and Pgc are structurally unrelated, suggesting that each animal group acquired a unique molecule for germline transcriptional quiescence. Furthermore, although mouse eggs are thought not to contain germ plasm and their primordial germ cells (PGCs) are induced by signaling mechanisms (Ohinata et al., 2009; Saitou, 2009), recent studies have shown that the transcriptional repressor Blimp-1 downregulates a number of somatic genes during PGC specification (Kurimoto et al., 2008; Ohinata et al., 2005; Yabuta et al., 2006). Thus, transcriptional repression appears to have a fundamental role in germline establishment in many animal species. However, despite the importance of germline transcriptional repression, the precise mechanisms involved differ among animal groups. Therefore, the factors and mechanisms required for the process in other animal phyla remain elusive.

Ascidian embryos contain a distinct cytoplasmic compartment at the posterior pole during the cleavage stages, called the posterior-vegetal cortex/cytoplasm (PVC) or the postplasm (Nishida, 2005; Prodon et al., 2007). The postplasm contains dozens of specific maternal RNAs called postplasmic/PEM RNAs, which include *CiVH* (*Ci-vasa*), the ascidian homolog of the conserved germline factor *vasa* (Takamura et al., 2002). The postplasmic/PEM RNAs are packed into a specialized structure in the postplasm, called the centrosome-attracting body (CAB), which is connected to the centrosome through microtubules. The CAB was initially identified as an electron-dense cortical structure in detergent-treated cleavage-stage embryos (Iseto and Nishida, 1999; Hibino et al., 1998; Nishikata et al., 1999), and it is now known to be a multilayered structure, consisting of an outer layer enriched in the polarity protein complex (Patalano et al., 2006), and an inner compacted cortical endoplasmic reticulum (cER) layer, to which many postplasmic/PEM RNAs are anchored (Sardet et al., 2003; Prodon et al., 2005). Several postplasmic/PEM RNAs, including *CiVH*, are embedded within the cER mesh and form granules (Paix et al., 2009). The CAB appears to be crucial for the axis formation

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and asymmetric cell division of the posterior blastomeres during the cleavage stages (Hibino et al., 1998; Nishikata et al., 1999; Patalano et al., 2006; Prodon et al., 2010). It also plays a role in the fate determination of the posterior-vegetal blastomeres, by regulating the accumulation and translation of specific maternal RNAs (Nishida, 2005; Prodon et al., 2007), such as *macho-1*, which encodes a determinant for muscle and posterior-vegetal cells (Nishida and Sawada, 2001).

Each blastomere of the pair that inherits the postplasm in the 110-cell stage embryo, the B7.6 cells, divides once more during gastrulation to produce two morphologically distinct cells, a smaller B8.11 cell and a larger B8.12 cell. Although the CAB remnants and associated postplasmic/PEM RNAs are specifically partitioned into the B8.11 cells, a group of the postplasmic/PEM RNAs, including *CiVH*, are diffused from the postplasm during the B7.6 cell division and segregated into the B8.12 cells (Paix et al., 2009; Shirae-Kurabayashi et al., 2006; Yamada, 2006). As only the progeny of the B8.12 cells migrates into the gonad after metamorphosis, the B8.12 cells are regarded as PGCs in ascidians (Shirae-Kurabayashi et al., 2006), indicating that the postplasm-containing blastomeres at the posterior pole of ascidian embryos (hereinafter 'the germline blastomeres') retain the potential to develop into germ cells. Therefore, even though the postplasm contains multifunctional CAB and maternal RNAs involved in somatic cell differentiation, it can be considered the germ plasm equivalent in ascidians.

Transcriptional repression is proposed to occur in the ascidian embryonic germline. For example, although the postplasmic/PEM RNA that encodes a transcriptional factor for muscle cell fate determination, *macho-1*, is distributed with the postplasm during the cleavage stages, its target genes, such as *ADMP*, are never expressed in the germline blastomeres (Tomioka et al., 2002; Yagi et al., 2004). In addition, a comprehensive in situ hybridization analysis of *C. intestinalis* embryos in the ghost database (http://ghost.zool.kyoto-u.ac.jp/indexr1.html) suggested that very few zygotic genes are transcribed in germline blastomeres during the cleavage stages (Imai et al., 2004; Nishikata et al., 2001; Satou et al., 2002). Intriguingly, upon cleavage of the germline blastomere, the daughter cell that does not inherit the postplasm immediately starts the zygotic expression of genes involved in somatic development. These genes include *FoxA-a* (Di Gregorio et al., 2001; Lamy et al., 2006; Olsen and Jeffery, 1997), *SoxB1* (Miya and Nishida, 2003), *FGF9/16/20* (Imai et al., 2002; Kumano et al., 2006) and *ADMP* (Imai et al., 2004; Yagi et al., 2004). Therefore, the postplasm is likely to contain specific factors that actively repress zygotic mRNA transcription in the germline blastomeres. However, these factors have not been identified.

An ascidian-specific gene, *pem-1* (*posterior end-mark-1*), was originally identified in *C. savignyi* as a postplasmic/PEM RNA (Yoshida et al., 1996). As the *Ci-pem-1* RNA is incorporated only into the B8.11 cells upon B7.6 cell division at the beginning of the gastrulation, Ci-Pem-1 is proposed to function in germline blastomeres during the earlier, cleavage stages (Prodon et al., 2007; Shirae-Kurabayashi et al., 2006). Consistent with this hypothesis, Pem-1 plays several roles in ascidian embryogenesis during the cleavage stages. The knockdown (KD) of *pem-1* causes irregular cleavages of the posterior blastomeres because the centrosome-CAB linkage is interrupted, owing to collapse of the microtubule bundles that connect them (Negishi et al., 2007; Prodon et al., 2010). Furthermore, although β-catenin normally accumulates in the nucleus of only the vegetal somatic blastomeres, where it regulates the expression of β-catenin/TCF-dependent genes during the cleavage stages (Imai et al., 2000; Kawai et al., 2007), *pem-1* KD in *H. roretzi* embryos causes the nuclear accumulation of β-catenin in the germline blastomeres, resulting in the ectopic expression of β-catenin/TCF-dependent genes, such as *FoxA-a*, *FoxD* and *FGF9/16/20* (Kumano and Nishida, 2009). Therefore, Pem-1 regulates, directly or indirectly, the position of cleavage planes and β-catenin/TCF-dependent transcription. However, as Pem-1 is an ascidian-specific protein with no known functional domains, neither the molecular mechanism by which it exerts its function nor even whether it plays a role in germline development is known.

Here, we report that in the *C. intestinalis* germline, the phosphorylation of RNAPII CTD is downregulated compared with their somatic sisters during the cleavage stages. We also show that Ci-Pem-1 localizes to the nucleus and is crucial for the germline transcriptional repression. The knockdown of *Ci-pem-1* broadly derepressed the transcription of genes that are normally expressed only in the somatic blastomeres. Finally, we demonstrate that Ci-Pem-1 forms a complex with two *C. intestinalis* homologs of the transcriptional co-repressor Groucho. Therefore, Ci-Pem-1 is the ascidian germ plasm factor that prevents somatic gene expression in the germline, probably through interaction with a general transcriptional co-repressor.

MATERIALS AND METHODS

Animals

C. intestinalis were collected at Osaka Bay (Hyogo), Murotsu Bay (Hyogo), Maizuru Bay (Kyoto), Onagawa Bay (Miyagi), Usa bay (Kochi) and Tokyo Bay, in Japan. The eggs and sperm were isolated from adult individuals by cutting the gonads, and were kept at 18°C (eggs) or on ice (sperm) until use. The eggs were dechorionated before use as described (Shirae-Kurabayashi et al., 2006). After fertilization, the eggs were reared at 16 or 18°C in filter-sterilized seawater (FSW) containing 50 μg/ml streptomycin sulfate.

Antibody

A C-terminal fragment of the *Ci-pem-1*-coding region (corresponding to amino acids 201-405) was amplified by PCR and cloned into the pProExHTa plasmid (Gibco). Histidine-tagged Ci-Pem-1 protein was expressed in *Escherichia coli* BL21 by IPTG induction and purified with Ni-NTA agarose (Qiagen). The protein was further purified using preparative SDS-PAGE, dialyzed against PBS and used to generate rabbit polyclonal antibodies. Guinea pig antiserum against CiVH (Ci-Vasa) was generated using the recombinant protein described in Shirae-Kurabayashi et al. (Shirae-Kurabayashi et al., 2006).

Immunohistochemistry and in situ hybridization

Immunohistochemistry was carried out as described (Shirae-Kurabayashi et al., 2006) with slight modifications. In some cases, fixed embryos were permeabilized with 1% Triton X-100 in PBS for 10 minutes at room temperature before their pre-treatment in blocking solution. The primary antibodies used were rabbit anti-Ci-Pem-1 antiserum (1:3000 dilution), guinea pig anti-CiVH antiserum (1:5000 dilution), mouse anti-phosphohistone H3 6G3 (Cell Signaling; 1:3000 dilution), mouse anti-RNAPII H5 (1:1000 dilution), H14 (1:500 dilution), 8WG16 (1:500) (Covance), 4H8 (1:10,000 dilution; Millipore), rabbit anti-RNAPII CTD pSer2 (1:5000 dilution; Abcam ab5095) and anti-RNAPII CTD pSer5 (1:10,000 dilution; Abcam ab5131). The secondary antibodies were Alexa Fluor-conjugated anti-rabbit IgG, anti-guinea pig IgG, anti-mouse IgG and anti-mouse IgM (Invitrogen). Whole-mount in situ hybridization of *C. intestinalis* embryos was carried out as described (Shirae-Kurabayashi et al., 2006). Hybridized signals from digoxigenin (DIG)-labeled RNA probes were detected using an HRP-conjugated anti-DIG antibody (1:3000; Roche), followed by the TSA-PLUS Fluorescein system (Perkin Elmer). Stained embryos were mounted in ProLong Gold mounting medium

(Invitrogen), and observed under a laser confocal microscope (Leica TCS SP2 AOBS and Olympus FV1000D) or a fluorescence microscope (Leica DMRE).

Morpholino oligonucleotides

Specific morpholino oligonucleotides (MOs) were synthesized and purified by Gene Tools. The sequence of the *Ci-pem-1* MO targeted against the ATG region was 5′-atactgtgcatgtttacattcatat-3′, and of the MO against the 5′ UTR region was 5′-cttataacttttgataacgctctg-3′. As both the *Ci-pem-1* MOs produced the same phenotypes when injected into embryos (data not shown), the MO against the ATG region was used in all the experiments unless otherwise stated. The sequence of the *Ci-β-catenin* MO was 5′-ctgatatgcagtggtgagtctcaac-3′ (Rothbacher et al., 2007). The control MO was provided by Gene Tools. MOs were microinjected into unfertilized eggs, one-cell embryos (30 minutes after fertilization) or two-cell embryos, depending on the experiment. Results from at least two independent injections were combined to score the data.

Cells, transfections and immunoprecipitation

HEK293 cells were grown under 5% CO_2 in DMEM (Invitrogen) supplemented with 10% heat-inactivated FBS at 37°C. For the expression of FLAG-tagged *C. intestinalis* proteins, the *Ci-pem-1*-, *Ci-pem-1ΔC*- (a derivative in which the C-terminal coding sequence for 'WRPW' was deleted), *Ci-Gro1*- and *Ci-Gro2*-coding sequences (CDSs) were PCR amplified from their corresponding cDNAs and subcloned into the p3×FLAG-CMV-10 plasmid (Sigma). For the expression of GFP-fusion proteins, the *gfp* CDS was fused to the 5′ end of each *C. intestinalis* CDS in the vector pCMVTNT (Promega). These plasmids were transfected into HEK293 cells using Fugene 6 (Roche). Twenty-four hours after transfection, the cells were treated with 10 μM MG132 for 7 hours to prevent proteasome-mediated protein degradation. The nuclear fraction was recovered using the NE-PER regents (Pierce), and was subsequently homogenized in TNG400 [50 mM Tris-HCl (pH 8.0), 400 mM NaCl, 10% glycerol, 1% Triton X100] containing protease inhibitors (Complete EDTA-free; Roche). The nuclear lysate was spun at 10,000 ***g*** for 10 minutes. The supernatant was pre-cleared with Sepharose CL-6B (Sigma) before immunoprecipitation. Anti-FLAG M2 beads (Sigma) or anti-GFP beads (MBL) were added to the cleared lysate, and the mixture was incubated at 4°C overnight. The beads were washed extensively with TNG400 and boiled in sample buffer. Eluates were separated by 4-12% NuPAGE with the MOPS buffer system (Invitrogen) and transferred to Immobilon-P membranes (Millipore). For western analysis, anti-FLAG M2 (1:5000, Sigma), rabbit anti-GFP (1:1000, MBL), rabbit anti-Ci-Pem-1 antiserum (1:3000) or mouse anti-TLE1/2/3/4 IgG (1:1000, Cell Signaling Technology), followed by HRP-labeled anti-mouse or rabbit IgG was used. Signal detection was carried out using the ECL (GE Healthcare) or the Super Signal West Dura (Thermo Fisher) system.

RESULTS

RNAPII CTD phosphorylation is downregulated in *C. intestinalis* germline blastomeres

In *C. intestinalis* embryos, most zygotic genes are transcribed only in the somatic blastomeres, although a small number of genes appear to be expressed in the germline blastomeres during the cleavage stages (Imai et al., 2004; Nishikata et al., 2001; Satou et al., 2002). This suggests that the RNAPII-mediated transcription in germline blastomeres is significantly reduced, even though it is not completely blocked. The RNAPII-dependent transcription cycle is coupled with extensive phosphorylation and dephosphorylation events on Ser residues in the repeated heptapeptide sequences of the CTD, providing a platform for the recruitment and assembly of many factors involved in transcription and mRNA processing (Egloff and Murphy, 2008; Phatnani and Greenleaf, 2006). Phosphorylation occurs at Ser5 during transcriptional initiation and at Ser2 during transcriptional elongation. We therefore examined the status of the RNAPII CTD phosphorylation in *C. intestinalis* embryos using several independent anti-phospho-CTD antibodies. In embryos stained with a polyclonal antibody (ab5131) that specifically detects CTD phospho-Ser5 (pSer5), the nuclei of all the somatic blastomeres were positive for this epitope from the two-cell stage onwards (Fig. 1A-D; data not shown). Another anti-pSer5 monoclonal antibody (H14), which apparently had lower immunoreactivity than ab5131, labeled the nuclei from the eight-cell stage onwards (see Fig. S1A,B in the supplementary material; data not shown). Compared with the CTD Ser5 phosphorylation, the appearance of CTD phospho-Ser2 (pSer2) in the somatic blastomeres was delayed and detected only after the eight-cell (polyclonal antibody ab5095) or 16-cell (monoclonal antibody H5) stage (Fig. 1E-H; see Fig. S1C,D in the supplementary material; data not shown).

Intriguingly, with these independent anti-phospho-CTD antibodies, we observed a severe reduction in both the pSer2 and pSer5 signals in the germline blastomeres during the cleavage stages (Fig. 1A′-H′; see Fig. S1 in the supplementary material, arrows), compared with their sister somatic blastomeres (Fig. 1A′-H′; see Fig. S1 in the supplementary material, arrowheads). By superimposing the series of *z*-axis confocal images, the reduction of pSer2 and pSer5 signals in the germline was clearly detected from the eight- to the 110-cell stage embryos (Fig. 1A′-H′, arrows). Under our conditions, the downregulation of the CTD Ser2 phosphorylation was more severe than that of CTD Ser5 (Fig. 1; see Fig. S1 in the supplementary material). However, unlike in the *Drosophila* and *C. elegans* embryos, the CTD phosphorylation in the germline blastomeres did not totally vanish, and weak, often punctate, signals remained detectable in the nucleus during the cleavage stages. By using an anti-RNAPII antibody (4H8) that recognizes the CTD regardless of its phosphorylation state, we detected consistent labeling in the nucleus of germline blastomeres throughout the cleavage stages (Fig. 1A-H), indicating that the RNAPII CTD phosphorylation is specifically reduced in the germline blastomeres. These results suggest that mRNA transcription in the *C. intestinalis* germline is strongly downregulated by a specific factor during the cleavage stages, although the underlying mechanism appears to differ from that of *C. elegans* and *Drosophila*.

Ci-Pem-1 is highly concentrated in the nuclei of germline blastomeres

We have previously reported that one of the major postplasmic/PEM RNAs, *Ci-pem-1*, is never incorporated into the B8.12 PGCs (Shirae-Kurabayashi et al., 2006; Prodon et al., 2007). To analyze the distribution of the endogenous Ci-Pem-1 protein during embryogenesis, we generated an anti-Ci-Pem-1 antibody, which recognized an ~45 kDa band on immunoblot of ovarian extracts (see Fig. S2 in the supplementary material). The size of the band matched well with the predicted molecular mass of Ci-Pem-1 protein. By using this antibody for immunostaining, we detected strong signals in the postplasm of cleavage-stage embryos (Fig. 2A″-C″,E″, arrows), in patterns that were similar to those of *pem-1* RNA in *H. roretzi* and two *Ciona* species (Negishi et al., 2007; Shirae-Kurabayashi et al., 2006; Yoshida et al., 1996). We also detected the Ci-Pem-1 protein in unfertilized eggs (see Fig. S3A,B in the supplementary material), indicating that, like its mRNA, it was maternally supplied. However, contrary to the accumulation of *pem-1* mRNA in the cortex of unfertilized eggs and one-cell stage embryos (Paix et al., 2009; Prodon et al., 2005; Prodon et al., 2006), the Ci-Pem-1 protein was distributed in the cytoplasm with the enrichment to the vegetal pole (see Fig. S3B-D in the

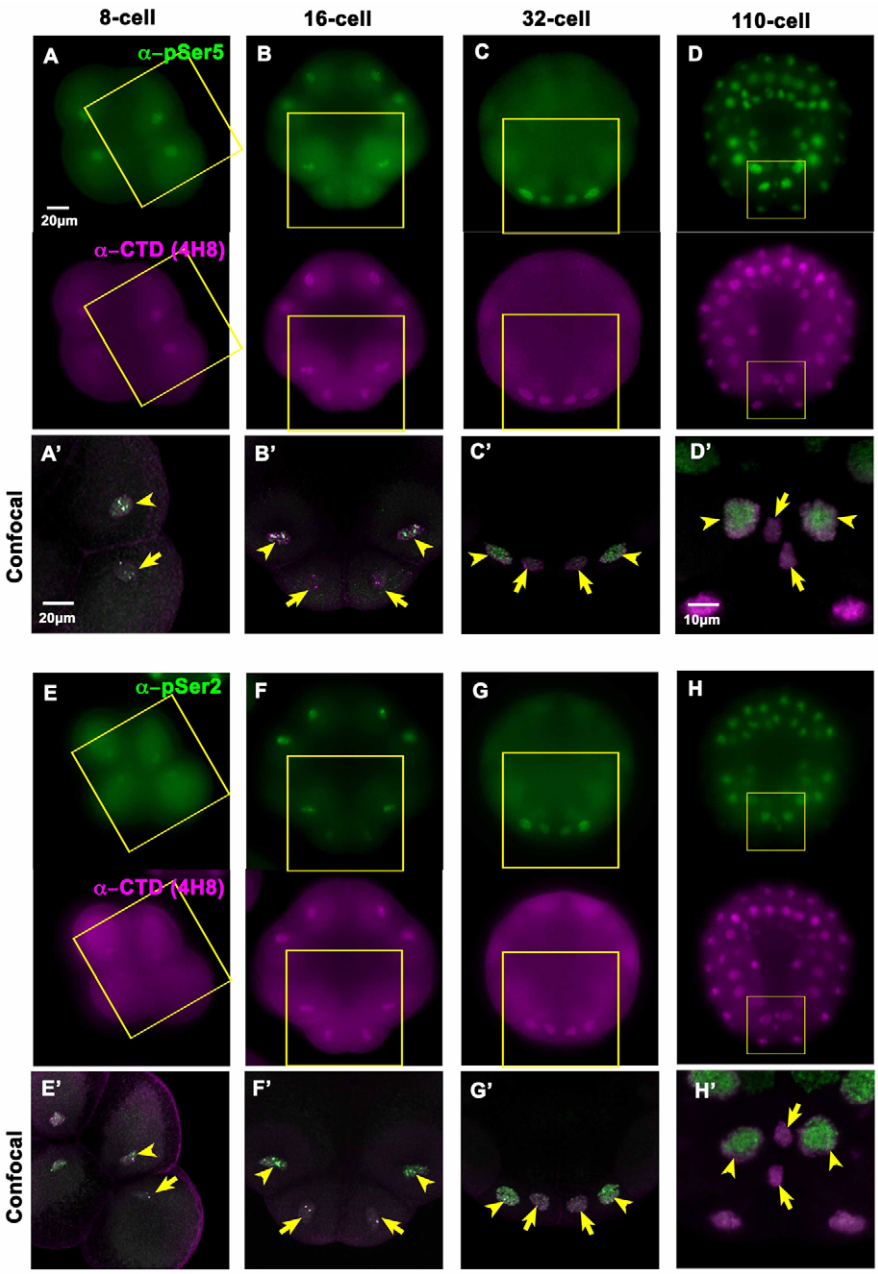

Fig. 1. Phosphorylation of the RNAP II CTD is reduced in germline blastomeres. (**A-D**) Immunostaining for pSer5 (ab5131, green) and pan-RNAPII (4H8, magenta) in eight- (A), 16- (B), 32- (C) and 110- (D) cell stage embryos. (**E-H**) Immunostaining for pSer2 (ab5095, green) and pan-RNAPII (4H8, magenta) in eight- (E), 16- (F), 32- (G) and 110- (H) cell-stage embryos. (**A′-H′**) Merged *z*-axis projection images of the boxed areas shown in the upper panels. The pSer2 and pSer5 levels in the germline blastomeres (arrows) were much lower than in their neighboring somatic sister (arrowheads).

supplementary material). In the two-cell stage embryo, it was partially concentrated in the posterior cortex, like the postplasmic/PEM RNAs, including *Ci-pem-1* (see Fig. S3F in the supplementary material).

More interestingly, we also detected anti-Ci-Pem-1 immunoreactivity in the nuclei of the posterior-most germline blastomeres during the cleavage stages (Fig. 2A″-E″, Fig. 3A′,E′,F′, arrowheads). Even in unfertilized eggs (see Fig. S3B′ in the supplementary material) and one-cell stage embryos (see Fig. S3C′ in the supplementary material), a low level of anti-Ci-Pem-1 immunoreactivity was detected in the vicinity of the female chromosomes, where F-actin was slightly accumulated. Ci-Pem-1 was consistently detected in the interphase nuclei of the posterior-most blastomeres of four-, eight-, 16-, 32- and 110-cell embryos (Fig. 2A″-E″, Fig. 3A′E′,F′). However, its nuclear accumulation was dynamically regulated during the cell-cycle progression: Ci-Pem-1 signals were undetectable around the chromosomes in germline blastomeres at prometaphase, metaphase (Fig. 3B′,C′, arrowheads), or during telophase before the nuclear envelope was reassembled (Fig. 3D′). At late telophase, when the nuclear envelope had reassembled, Ci-Pem-1 reappeared only in the postplasm-containing daughter blastomeres (Fig. 3E′,F′). The nuclear accumulation of Ci-Pem-1 in the germline blastomeres gradually increased until the 110-cell stage (Fig. 2A″-E″, arrowhead). Although the *Ci-pem-1* RNA is inherited only by the B8.11 cells upon the division of the B7.6 cell in the 110-cell-stage embryo (Paix et al., 2009; Shirae-Kurabayashi et al., 2006), the Ci-Pem-1 protein was distributed into both the B8.11 and B8.12 daughter cells (data not shown). Subsequently, Ci-Pem-1 signals in the B8.12 PGCs dropped during the neural-plate stage, and became undetectable by the tailbud stage (see Fig. S4 in the supplementary material).

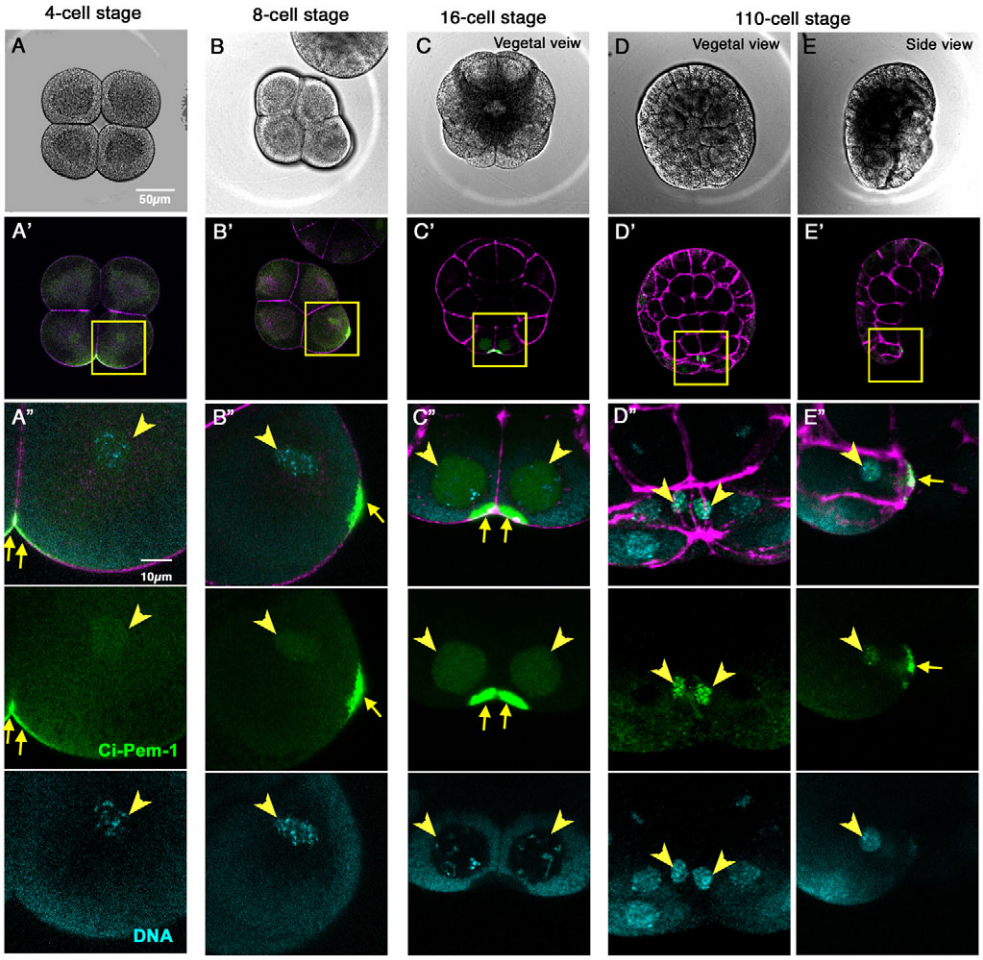

Fig. 2. Ci-Pem-1 protein signals are detected not only in the postplasm but also in the nuclei of the germline blastomeres. (**A-E**) Nomarski optics of four-, eight-, 16- and 110-cell-stage embryos. (C,D) Vegetal views. (B,E) Side views. (**A′-E′**) Embryos were stained with an anti-Ci-Pem-1 antibody (green), with phalloidin to visualize F-actin (magenta) and with DAPI to visualize DNA (cyan). (**A″-E″**) Higher magnification of the boxed areas in A′-E′. Arrows indicate Ci-Pem-1 signals in the postplasm; arrowheads indicate the Ci-Pem-1 signals in the nucleus.

To confirm that the anti-Ci-Pem-1 immunoreactivity in the postplasm and nucleus was due to labeling of the endogenous Ci-Pem-1 protein and not to crossreactivity of the antibody, we stained embryos in which *Ci-pem-1* was knocked down. We injected an MO against *Ci-pem-1* into one blastomere of two-cell stage embryos, and allowed them to develop to the 16-cell stage (Fig. 4A). We found that, although the anti-Ci-Pem-1 signal was obvious in the germline blastomere of the uninjected side, neither the postplasmic nor the nuclear anti-Ci-Pem-1 immunoreactivity was detectable in the germline blastomere on the MO-injected side (Fig. 4B, arrows and arrowheads). Furthermore, when Ci-Pem-1 was expressed in HEK293 cells, we observed strong anti-Ci-Pem-1 signals in the nucleus of the transfected cells (Fig. 4C). GFP-tagged Ci-Pem-1 also accumulated in the nucleus of HEK293 cells (data not shown). The WoLF PSORT program (Horton et al., 2007) predicted that Ci-Pem-1 contains three classical nuclear localization signals in the middle part of the protein (Fig. 4D, colored boxes). These results collectively indicate that a significant proportion of the endogenous Ci-Pem-1 protein is present in the nucleus of the germline blastomeres, and suggest that Ci-Pem-1 has nuclear functions in the germline blastomeres during the cleavage stages.

When we injected the MO against *Ci-pem-1* into unfertilized *C. intestinalis* eggs and fertilized them, their cleavage patterns were disturbed (see Fig. S5 in the supplementary material). Although gastrulation occurred, the invagination of the B7.6 cells into the vegetal site was defective (see Fig. S5G,H in the supplementary material), resulting in embryos that were spherical at the presumptive tailbud stage (data not shown). We also examined the distribution of postplasmic/PEM mRNAs in *Ci-pem-1* KD embryos. Signals for both the *Ci-pem-1* (see Fig. S6A,B in the supplementary material) and *CiVH* (see Fig. S6C,D in the supplementary material) RNAs were readily detected as a subcortical crescent in the posterior-most region of the germline blastomere pair in 16-cell stage embryos, indicating that Ci-Pem-1 is dispensable for the asymmetric inheritance of postplasm by the germline blastomeres during the cleavage stages. These results are consistent with the effects of *pem-1* KD in other ascidian species (Negishi et al., 2007; Prodon et al., 2010), and suggest that roles of Pem-1 during the cleavage stages are conserved in ascidians.

Ci-Pem-1 represses zygotic gene expression in the germline blastomeres

The nuclear accumulation of Ci-Pem-1 in the germline blastomeres led us to speculate that Ci-Pem-1 might be involved in the germline transcriptional control. We therefore examined the effects of *Ci-pem-1* KD on zygotic gene expression in cleavage stage embryos. Upon the cleavage of a parental germline blastomere, the postplasm-free daughter cell begins the zygotic transcription of many genes, but the other, postplasm-containing, daughter, does not (Di Gregorio et al., 2001; Imai et al., 2002; Imai et al., 2004; Kumano et al., 2006; Lamy et al., 2006; Miya and Nishida, 2003; Tomioka et al., 2002; Olsen and Jeffery, 1997; Yagi et al., 2004). *Ci-FoxA-a* and *Ci-SoxB1* are expressed in this manner from the eight-cell stage, the earliest stage of zygotic gene expression so far

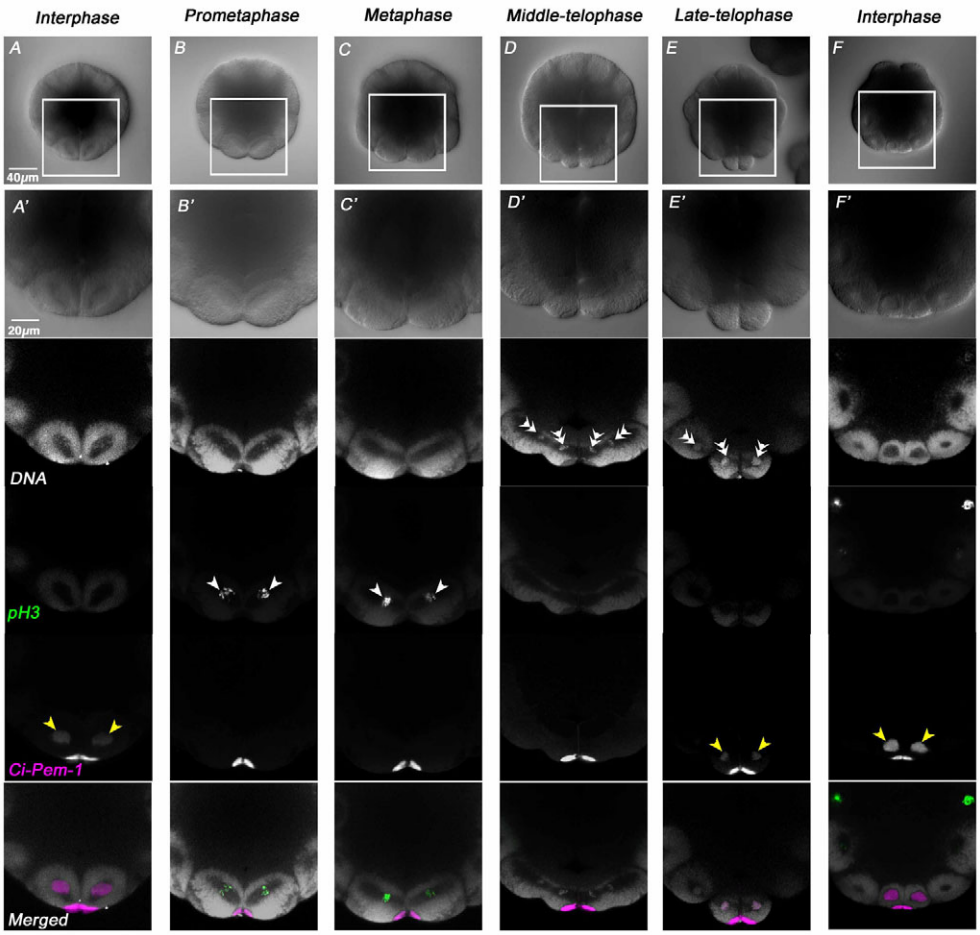

Fig. 3. The anti-Ci-Pem-1 immunoreactivity is detected only in the interphase nuclei of the germline blastomeres.
(**A-F**) Nomarski optics of 16- to 32-cell stage embryos undergoing mitosis in the germline blastomeres. (**A′-F′**) Higher-magnification of the boxed areas in A-F. The merged images show DNA (gray), phospho-histone H3 (pH3; green) and Ci-Pem-1 (magenta). Cell-cycle stages of the germline blastomeres are: (A) interphase, (B) prometaphase, (C) metaphase, (D) middle-telophase, (E) late-telophase and (F) interphase. The pH3-positive chromosomes are indicated by white arrowheads, and chromosomes in telophase are indicated by double arrowheads. The Ci-Pem-1 signals in the nucleus of germline blastomeres are indicated by yellow arrowheads.

detected (Imai et al., 2004; Lamy et al., 2006; Miya and Nishida, 2003). When *Ci-pem-1* was knocked down, we detected the ectopic transcription of *Ci-FoxA-a* and *Ci-SoxB1* in the germline blastomeres of eight- and 16-cell stage embryos (Fig. 5C,D,G,H). Interestingly, we also detected their ectopic expression in all the somatic blastomeres, indicating that Ci-Pem-1 affects the transcriptional potential of all blastomeres (see Discussion). We further tested the effects of *Ci-pem-1* KD on the expression of *Ci-FGF9/16/20* and *Ci-ADMP*, which begins at the 16-cell stage (Imai et al., 2002; Imai et al., 2004; Yagi et al., 2004). As reported previously, in normal 16-cell-stage embryos, Ci-*FGF9/16/20* was expressed in all the vegetal blastomeres, except for the germline (Fig. 5I), and Ci-*ADMP* was expressed only in the somatic sister blastomeres of the germline (Fig. 5K). In *Ci-pem-1* KD embryos, however, both genes were misexpressed in the germline (Fig. 5J,L, broken circles). Furthermore, *FGF9/16/20* was also misexpressed in the posterior-animal blastomeres in several cases (Fig. 4J′). By contrast, ectopic *Ci-ADMP* expression was detected only in the germline (Fig. 5L). Ci-Pem-1 was unlikely to regulate the timing of zygotic gene expression, as we never observed precocious mRNA transcription in the *Ci-pem-1* KD embryos (data not shown). Therefore, these data suggest that Ci-Pem-1 plays a fundamental role in repressing the somatic gene transcription in germline blastomeres.

In *H. roretzi* embryos, *Hr-pem-1* KD leads to the nuclear accumulation of β-catenin in germline blastomeres, resulting in the misexpression of its target genes, including *FoxA*, *FoxDa* and *FGF9/16/20* (Kumano and Nishida, 2009). To examine whether Ci-Pem-1 represses the germline transcription of genes besides the β-catenin/TCF pathway target genes, we used a β-galactosidase reporter, G12 (G12-bpbra::*NLS-lacZ)* (Rothbacher et al., 2007), which contains tandem arrays of GATAa-binding sites present in the *Ci-fog* promoter and recapitulates endogenous expression that is regulated by the GATAa transcription factor (Rothbacher et al., 2007) (Fig. 6A). As reported previously (Rothbacher et al., 2007), the G12 reporter was activated only in the eight blastomeres in the animal hemisphere in 16-cell-stage embryos (Fig. 6B″). The β-catenin/TCF pathway has been proposed to repress GATAa-dependent gene expression in the vegetal blastomeres, as the KD of β-catenin/TCF-pathway components results in ectopic G12 expression in them (Rothbacher et al., 2007). Although we confirmed that the *Ci-β-catenin* KD resulted in ectopic G12 expression in vegetal somatic blastomeres, careful examination of the results revealed that the GATAa activity was still repressed in the germline blastomeres (Fig. 6C; Table 1), indicating that a β-catenin/TCF-independent mechanism exists to repress the GATAa-mediated transcription in germline blastomeres.

We next examined whether the repression of the G12 reporter in the germline was dependent on Ci-Pem-1. In the *Ci-pem-1* KD embryos, the G12 reporter was ectopically activated in germline blastomeres (Fig. 6E, arrowheads in dashed circles; Table 1), indicating that Ci-Pem-1 represses the GATAa activity in germline blastomeres. Similar to the case for *FoxA-a* and *SoxB1*, the G12 reporter was misexpressed in all the vegetal blastomeres of the *Ci-pem-1* KD 16-cell-stage embryos (Fig. 6E, arrowheads; Table 1). We also examined the G12 reporter expression in the *Ci-pem-1 Ci-*

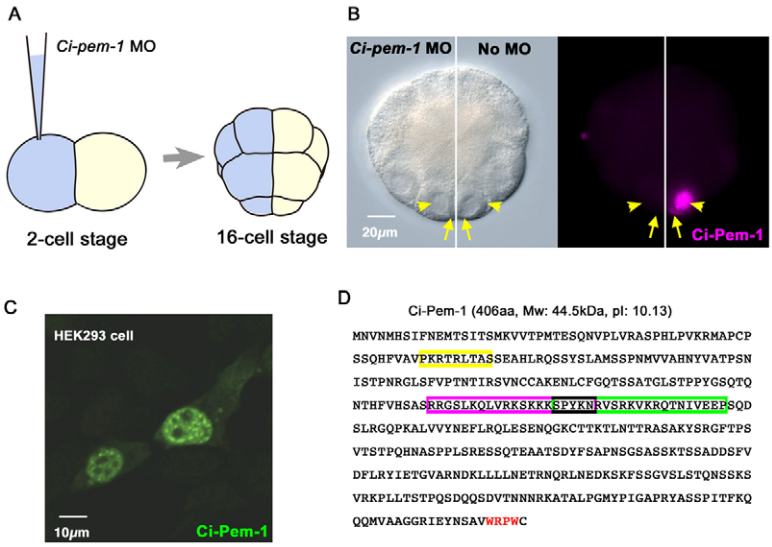

Fig. 4. Ci-Pem-1 is a nuclear protein. (**A**) Diagram of *Ci-pem-1* MO injection into one blastomere at the two-cell stage and the resulting 16-cell stage embryo. (**B**) The nuclear signal of Ci-Pem-1 was absent in one germline blastomere in the 16-cell stage embryo. Arrows and arrowheads indicate the postplasm and the nucleus of the germline blastomeres, respectively. (**C**) Nuclear distribution of Ci-Pem-1 (green) in the HEK 293 cells. Cells expressing Ci-Pem-1 under the CMV promoter were stained with the anti-Ci-Pem-1 antibody. (**D**) Amino acid sequence of Ci-Pem-1. The Ci-Pem-1 protein contains three putative nuclear localization signals (yellow, green and magenta boxes) in the middle of the sequence, and a WRPW motif in the C-terminal region (red).

β-*catenin* double-KD embryos, and found that its expression pattern was indistinguishable from that in *Ci-pem-1* single-KD embryos (Fig. 6D), suggesting that the derepression of mRNA transcription in somatic blastomeres by *Ci-pem-1* KD is attributable to its effect on the β-catenin/TCF pathway (Rothbacher et al., 2007).

Ci-Pem-1 can interact with *C. intestinalis* Groucho homologs, Ci-Gro1 and Ci-Gro2

Although Pem-1 protein is unique to ascidians, it contains a WRPW (Trp-Arg-Pro-Trp) motif in its C-terminal region (Fig. 4D, shown in red) (Negishi et al., 2007), which could act as a binding sequence for Groucho transcriptional co-repressors (Jennings et al., 2006). The WRPW sequence is perfectly conserved in Pem-1 proteins among ascidian species (Negishi et al., 2007), despite their otherwise low level of conservation (25-62% amino acid identity). Two *Groucho* homologs (*Ci-Gro1* and *Ci-Gro2*) exist in the *C. intestinalis* genome. Consistent with the expression data in *Ciona* Ghost database, RT-PCR analysis confirmed that both *Ci-Gro1* and *Ci-Gro2* were expressed maternally and deposited into eggs (data not shown).

To examine whether Ci-Pem-1 can interact with Groucho in the nucleus, we performed co-immunoprecipitation assays using HEK293 cells expressing 3×Flag-tagged Ci-Pem-1 and GFP-tagged Ci-Gro1 or Ci-Gro2. Using the nuclear lysates from the transfectants, we observed that 3×Flag-Ci-Pem-1 co-immunoprecipitated with GFP-Ci-Gro1 and GFP-Ci-Gro2, but not with the control, non-fused GFP (Fig. 7A,B). Reciprocally, both GFP-Ci-Gro1 and GFP-Ci-Gro2 were specifically co-immunoprecipitated with 3×Flag-Ci-Pem-1 from nuclear lysates (Fig. 7C,D). By contrast, only low levels of a mutant Ci-Pem-1 that lacked the WRPW motif (3×Flag-Ci-Pem-1ΔC) co-immunoprecipitated GFP-Ci-Gro1 and GFP-Ci-Gro2, even though the proteins were present at high levels in the nuclear extracts (Fig. 7). These results indicate that Ci-Pem-1 can interact with Ci-Gro proteins in the nucleus, preferentially through the WPRW sequence.

DISCUSSION

Transcriptional repression is a conserved hallmark of germ-cell specification

We showed that the germline blastomeres of *C. intestinalis* embryos experienced a severe downregulation of RNAPII CTD phosphorylation during the cleavage stages (Fig 1; see Fig. S1 in the supplementary material). A transient lack of CTD phosphorylation during germ-cell formation has been observed in *C. elegans*, *Drosophila* and *Xenopus* embryos (Seydoux and Dunn, 1997; Venkatarama et al., 2010). In *Drosophila* and *C. elegans* embryos, specific factors in the germ plasm directly inhibit the kinase responsible for CTD Ser2 phosphorylation (Batchelder et al., 1999; Hanyu-Nakamura et al., 2008; Nakamura and Seydoux, 2008; Nakamura et al., 2010). However, low levels of both pSer2 and pSer5 persist in the germline blastomeres in *C. intestinalis* embryos (Fig. 1), suggesting that it uses a different mechanism for the germline transcriptional repression. In mouse, a number of somatic genes are downregulated without a clear loss of CTD phosphorylation during the specification of PGCs (Kurimoto et al., 2008; Seki et al., 2007). Thus, although the mechanism of repression and the mode of germ-cell specification differ, the repression of somatic transcriptional programs is a fundamental hallmark of germ-cell specification (Nakamura and Seydoux, 2008; Nakamura et al., 2010).

Table 1. G12 expression in MO-injected embryos

	Animal somatic blastomeres	Vegetal somatic blastomeres	Germline blastomeres
Control MO alone (*n*=9)	100	0	0
Ci-pem-1+ control MOs (*n*=12)	100	83	100
Ci-pem-1 + *Ci*-β-*Catenin* MOs (*n*=10)	100	100	90
Ci-β-*Catenin* + control MOs (*n*=7)	100	100	0

Numbers indicate the percentage of 16-cell stage embryos that showed G12 expression in all the animal, vegetal or germline blastomeres. Concentration of each MO was 0.5 mM; in the control MO alone injection, the concentration was 1 mM.

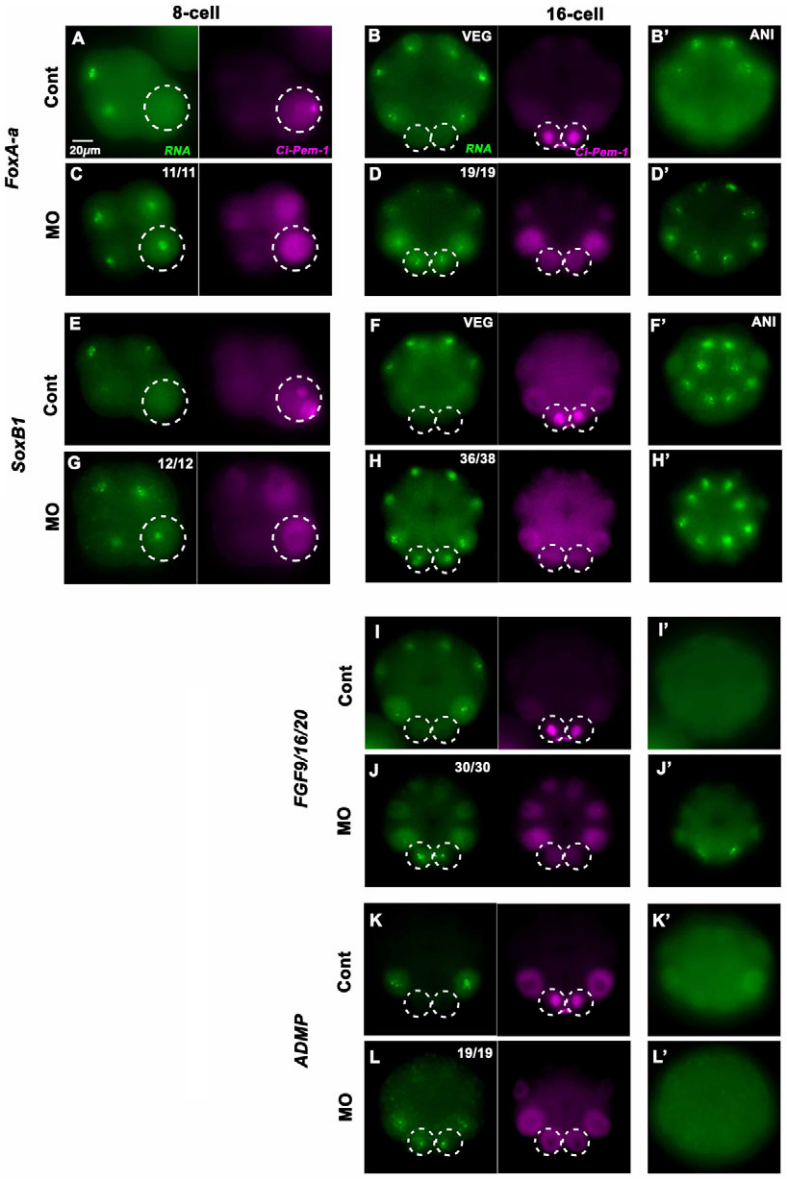

Fig. 5. Somatic genes are misexpressed in the germline blastomeres of *Ci-pem-1* KD embryos. (**A-B′,E-F′,I,I′,K,K′**) Control embryos. (**C-D′,G-H′,J,J′,L,L′**) *Ci-pem-1* MO-injected embryos. (A,C,E,G) The eight-cell stage. Side views. (B,D,F,H-L) The 16-cell stage. Vegetal views. (B′,D′,F′,H′-L′) Animal views. (A-D) *Ci-FoxA-a* RNA. (E-H) *Ci-Sox-B1* RNA. (I,J) *Ci-FGF9/16/20* RNA. (K,L) *Ci-ADMP* RNA. Broken circles indicate germline blastomeres. In control embryos (A,B,E,F,I,K), no expression of these genes was detected in the germline blastomeres. In the *Ci-pem-1* MO-injected embryos, the Ci-Pem-1 signal was undetectable and ectopic signals for the indicated mRNAs were detected in the germline nuclei. *Ci-pem-1*KD resulted in the expression of *FoxA-a* (D,D′) and *SoxB1* (H,H′) in all the blastomeres. The numbers indicated in the upper right corner of C,D,G,H,J,L show the number of embryos with ectopic expression per number of MO-injected embryos.

Nuclear localization of Ci-Pem-1 in germline blastomeres

We showed that the endogenous Ci-Pem-1 protein is highly enriched in the nucleus of the germline blastomeres during the cleavage stages. As the *Ci-pem-1* RNA is provided maternally and is detected only in the postplasm during the cleavage stages (Fujiwara et al., 2002; Nishikata et al., 2001; Shirae-Kurabayashi et al., 2006), Ci-Pem-1 protein produced in the postplasm appears to be transported into the nucleus of germline blastomeres.

It was reported that the Hr-Pem-1 signal is detected only in the postplasm (Negishi et al., 2007). Notably, under the immunostaining conditions that we normally use (Shirae-Kurabayashi et al., 2006), the nuclear Ci-Pem-1 signal was relatively faint compared with the signal in the postplasm. We found that the nuclear signal became much stronger after the protease treatment and permeabilization of embryos, processes that are used during sample preparation for in situ hybridization. These findings suggest that the nuclear Pem-1 is embedded into a complex, such that its epitopes tend to be masked. Although Hr-Pem-1 shows only 25% amino acid identity with Ci-Pem-1 (Negishi et al., 2007), the proteins share several important signature features, such as an abundance of basic amino acids and the presence of putative NLSs (data not shown). Thus, it is plausible that the nuclear localization of the Pem-1 protein is a common feature in ascidians during normal embryogenesis.

During the cleavage of the germline blastomere, the nuclear Ci-Pem-1 signals transiently disappeared from prometaphase to telophase, suggesting that nuclear Ci-Pem-1 diffuses into the cytoplasm as a result of the nuclear envelope breakdown. After the nuclear envelope was reassembled in late telophase, the Ci-Pem-1 signal in the nucleus reappeared in the germline daughter blastomere (Fig. 3). By contrast, the Ci-Pem-1 signal was hardly detected in the somatically fated sister blastomere. Thus, the nuclear localization of Ci-Pem-1 must be regulated in a cell-type-specific manner. Interestingly, when Ci-Pem-1 was expressed heterogeneously in HEK293 cells, its nuclear signal dramatically increased following the addition of a proteasome inhibitor, MG132, to the culture medium (data not shown), suggesting that Ci-Pem-1

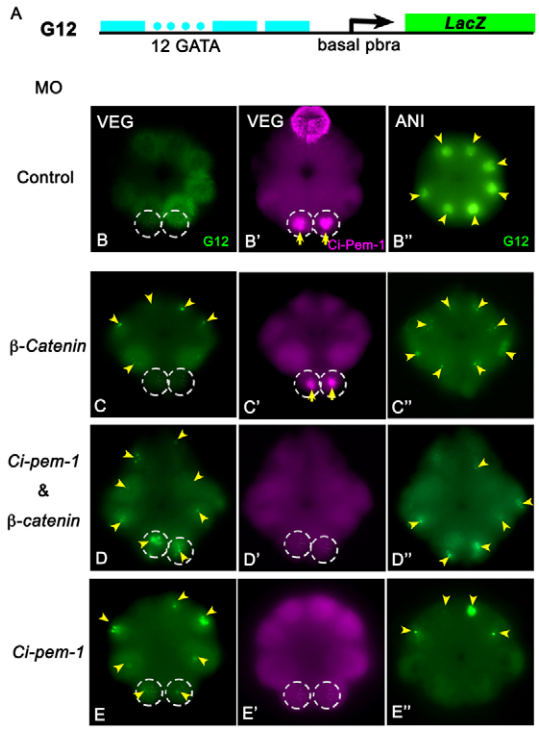

Fig. 6. GATAa-dependent transcription is blocked in germline blastomeres by Ci-Pem-1. (**A**) The G12-bpbra::*NLS-lacZ* reporter construct to monitor GATAa-mediated transcriptional activity (Rothbacher et al., 2007). (**B-E″**) The expression of β-galactosidase was visualized at the 16-cell stage by in situ hybridization. Embryos were also stained for Ci-Pem-1 (magenta) to verify the *Ci-pem-1* KD (D′,E′). (B) Control-, (C) *Ci-pem-1* MO-, (D) *Ci-pem-1* MO- and *Ci*-β-*catenin* MO- and (E) *Ci*-β-*catenin* MO-injected embryos. (B-E,B′-E′) Vegetal views; (B″-E″) animal views. Arrowheads indicate nuclei expressing the G12 reporter; broken circles indicate germline blastomeres.

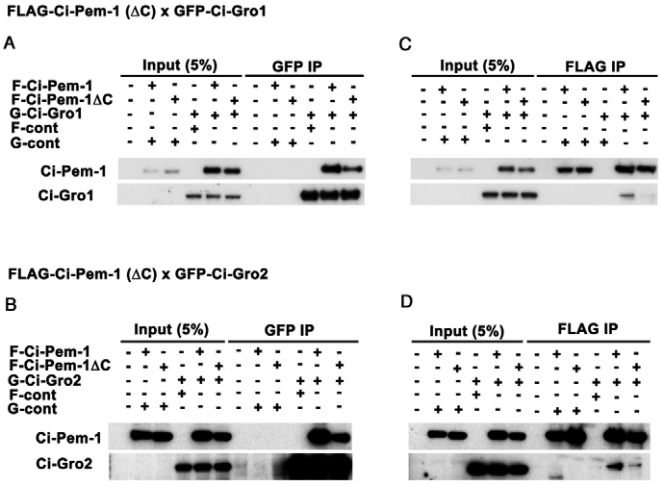

Fig. 7. Ci-Pem-1 interacts with Ci-Gro1 and Ci-Gro2 in a WRPW-motif dependent manner. (**A-D**) Nuclear lysates of HEK293 cells expressing 3×Flag-Ci-Pem-1 (F-Ci-Pem-1) and GFP-Ci-Gro1 (G-Ci-Gro1) or GFP-Ci-Gro2 (G-Ci-Gro2) were immunoprecipitated with anti-GFP (A,B) or anti-FLAG (C,D) antibodies, and the bound proteins were analyzed by western blotting. F-Ci-Pem-1 (ΔC) denotes a 3×FLAG-tagged mutant Ci-Pem-1 protein in which the WRPW sequence was deleted. Full-length Ci-Pem-1 co-immunoprecipitated with both Ci-Gro1 and Ci-Gro2, and vice versa. Ci-Pem-1 lacking the WRPW motif (Ci-Pem-1ΔC) co-immunoprecipitated Ci-Gro1 and Ci-Gro2 poorly.

is actively degraded through the proteasome pathway. Therefore, the Ci-Pem-1 that diffused into the cytoplasm during mitosis might be actively degraded to prevent its redistribution into the nucleus of the somatic daughter blastomere, whereas continuous Ci-Pem-1 translation from the postplasmic source in the germline daughter may support its accumulation in the nucleus.

Ci-Pem-1 is crucial for the transcriptional repression in germline blastomeres

We found that *Ci-pem-1* KD embryos misexpressed several genes in the germline blastomeres during the cleavage stages (Figs 5, 6). Previous studies in *H. roretzi* suggested that Hr-Pem-1 directly or indirectly prevents the nuclear localization of β-catenin and represses the transcription of its downstream genes during cleavage stages (Kumano and Nishida, 2009). However, the β-catenin/TCF pathway is not the only target interfered with by Ci-Pem-1, as *Ci-pem-1* KD also caused the derepression of GATAa-mediated transcription in the germline, independent of its effects on β-catenin (Fig. 6). Furthermore, *Ci-ADMP,* one of target genes for the muscle determinant Ci-Macho-1 (Yagi et al., 2004), was ectopically expressed in the germline by *Ci-pem-1* KD (Fig. 5K,L). Therefore, the repression of mRNA transcription by Ci-Pem-1 must be widespread, even though the germline transcription in this animal is not completely repressed (Fig. 1) (Imai et al., 2004; Nishikata et al., 2001; Satou et al., 2002). As nuclear Ci-Pem-1 signals are detectable in the germline beyond the B7.6 cell division that leads to the segregation of PGCs (Fig. 2; see Fig. S4 in the supplementary material), Ci-Pem-1 could be responsible for germline transcriptional repression throughout the cleavage stages, to ensure the germ-cell fate. Based on these results, we propose that Ci-Pem-1 is the *C. intestinalis* version of the germ plasm factor that is responsible for the repression of somatic gene expression during specification of the germline.

Interestingly, *Ci-pem-1* KD resulted in the ectopic activation of *FoxA-a* and *SoxB1* in all the blastomeres of eight- and 16-cell-stage embryos (Fig. 5A-H), and the misexpression of an exogenous G12 reporter in all the vegetal somatic blastomeres (Fig. 6). Therefore, Ci-Pem-1-mediated transcriptional repression is not restricted to the germline. As Ci-Pem-1 can be clearly detected only in germline blastomeres, and is only detected at a very low level if at all in their somatic sisters during these stages (Figs 2, 3), the broad derepression of zygotic transcription by *Ci-pem-1* KD must be a consequence of its effects during earlier stages. Given that Ci-Pem-1 is maternally supplied and accumulates around chromosomes even in unfertilized eggs (see Figs S2, S3 in the supplementary material), Ci-Pem-1 can potentially establish a transcriptionally repressed state as early as the one-cell stage. Therefore, the ectopic expression of several genes observed in the somatic blastomeres in the *Ci-pem-1* KD embryo might have been due to the failure of establishing such transcriptional potential in their ancestors during earlier stages.

Alternatively, Ci-Pem-1 may be required for the proper partitioning of transcription-factor activities that restrict somatic gene expression within specific sets of blastomeres. Notably, the G12 reporter was also misexpressed in vegetal somatic blastomeres when *Ci*-β-*catenin* was knocked down (Fig. 6), suggesting that the GATAa-mediated transcriptional repression in these cells depends on the β-catenin/TCF pathway (Rothbacher et al., 2007). As Pem-

1 is known to affect the behavior of β-catenin (Kumano and Nishida, 2009; Yoshida et al., 1996; Yoshida et al., 1998), *Ci-pem-1* KD might affect the activity of β-catenin/TCF pathway components, resulting in the misexpression of several genes in the vegetal soma. However, Ci-Pem-1 can repress transcription independent of the β-catenin/TCF pathway in the germline, as the sole knockdown of *Ci-β-catenin* did not promote the misexpression of the G12 reporter in the germline blastomeres (Fig. 6C-E).

Ci-Pem-1 may repress mRNA transcription through interactions with conserved transcriptional co-repressors

We showed that Ci-Pem-1 formed a complex with two *C. intestinalis* homologs of the co-repressor Groucho, Ci-Gro1 and Ci-Gro2 (Fig. 7). We therefore propose that Ci-Pem-1 may repress somatic gene transcription in the germline by interacting with Groucho family proteins. Although the precise mechanism by which Groucho represses transcription remains to be elucidated, it appears to target the transcriptional machinery or to involve chromatin modifications (Buscarlet and Stifani, 2007). Interestingly, the murine transcriptional repressor, Blimp-1, which is responsible for repressing the somatic gene transcription during PGC specification in early mouse embryos (Kurimoto et al., 2008; Ohinata et al., 2005), also interacts with Groucho family proteins (Kallies and Nutt, 2007; Ren et al., 1999). Groucho does not interact with DNA directly; instead, it is recruited to its regulatory regions by sequence-specific DNA-binding proteins. Therefore, determining whether Pem-1 can bind DNA and whether chromatin modifications are involved in establishing germline transcriptional quiescence in ascidians will be interesting issues for future investigation.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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