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Maternal PCBP1 determines the normal timing of pronucleus formation in mouse eggs

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Abstract In mammals, pronucleus formation, a landmark event for egg activation and fertilization, is critical for embryonic development. However, the mechanisms underlying pronucleus formation remain unclear. Increasing evidence has shown that the transition from a mature egg to a developing embryo and the early steps of development are driven by the control of maternal cytoplasmic factors. Herein, a two-dimensional-electrophoresis-based proteomic approach was used in metaphase II and parthenogenetically activated mouse eggs to search for maternal proteins involved in egg activation, one of which was poly(rC)-binding protein 1 (PCBP1). Phosphoprotein staining indicated that PCBP1 displayed dephosphorylation in parthenogenetically activated egg, which possibly boosts its ability to bind to mRNAs. We identified 75 mRNAs expressed in mouse eggs that contained the characteristic PCBP1-binding CU-rich sequence in the 3′-UTR. Among them, we focused on *H2a.x* mRNA, as it was closely related to pronucleus formation in *Xenopus* oocytes. Further studies suggested that PCBP1 could bind to *H2a.x* mRNA and enhance its stability, thus promoting mouse pronucleus formation during parthenogenetic activation of murine eggs, while the inhibition of PCBP1 evidently retarded pronucleus formation. In summary, these data propose that PCBP1 may serve as a novel maternal factor that is required for determining the normal timing of pronucleus formation.

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Introduction

At the end of oogenesis, mature oocytes are held in a developmentally quiescent state until an appropriate trigger initiates development. The process whereby the oocyte undergoes the final transition from this arrested state to a new cellular state that can support embryogenesis is called egg activation [1, 2]. Egg activation involves a stepwise series of events, including cortical granule exocytosis, release of oocyte meiotic arrest, and formation of haploid female pronucleus that is capable of combining with the haploid male pronucleus. Maternal mRNAs and proteins underwent dynamic changes during egg activation, including recruitment and regulated degradation of maternal mRNAs, translation, and post-translational modifications. These changes during egg activation provide necessary support to the zygote's growth and development [1–4].

Pronucleus formation, a landmark event for egg activation and fertilization, is critical for embryonic

development. The female pronucleus is assembled upon egg activation from a condensed organization of chromatin and relies on contributions from cytoplasmic components. Whilst male pronucleus development generally requires egg activation in most mammalian species, once the second polar body extrusion and female pronucleus construction begin, the sperm nuclei undergo rapid morphological and biochemical transformations. These changes include disassembly of the nuclear lamina, decondensation of chromatin, replacement of protamines by maternal histones, reassembly of a nuclear envelope, nuclear swelling, and formation of a male pronucleus [5–8]. Fusion of female and male pronuclei allows their DNA to combine, resulting in a diploid zygote and the completion of fertilization.

Increasing evidence has shown that the transition from a mature egg to a developing embryo and the early steps of development are driven by the control of maternal cytoplasmic factors (mRNAs and proteins), that are produced during oogenesis and stored in the mature egg. Upon egg activation and fertilization, these maternal factors initiate development cascades that carry out the subsequent embryonic development program [2, 9]. Furthermore, a wealth of evidence has demonstrated the existence of maternal factors that affect various nuclear functions including chromatin assembly and the structure and establishment of components of the nuclear structure, such as nucleoplasmin, histones, and nuclear lamins [10].

Despite the crucial role of maternal factors in egg activation, only a few factors have been identified. Poly(rC)-binding protein 1 (PCBP1), also referred to as hnRNP E1 or αCP1, is a maternal protein that was identified in the protein profile of mouse metaphase II (MII) eggs [11, 12]. PCBP1 contains highly conserved triple repeats of the KH domain and belongs to the superfamily of nucleic acid-binding proteins, which are involved in a variety of mRNA processing steps, including transcription [13], nucleocytoplasmic mRNA shuttling [14], mRNA stability [15–19], mRNA translation [20–22], and protein–protein interactions [23, 24]. In our previous work [25], we showed that PCBP1 plays a key role in maintaining the transcriptionally silent state in the fully grown oocyte, which is a prerequisite for preovulatory oocytes to eventually achieve meiotic maturation, fertilization, and early embryonic development.

In this study, we examined differentially expressed proteins in normal MII eggs compared with parthenogenetically activated mouse eggs through two-dimensional gel electrophoresis (2-DE). PCBP1 was identified as a differentially expressed protein and found to be required for determining the normal timing of pronucleus formation during egg activation.

Materials and methods

Reagents and animals

All reagents were from Sigma Chemicals (St Louis, MO, USA), unless stated otherwise. ICR white mice were maintained under a controlled environment of 20–22 °C, 12/12-h light/dark cycle, and 50–70 % humidity, with food and water provided ad libitum. Animal care was conducted in accordance with the Animal Research Committee guidelines of Nanjing Medical University.

Egg collection and culture

Mature cumulus-free eggs were collected from superovulated 6- to 8-week-old ICR female mice as previously described [11]. Parthenogenetically activated eggs were obtained by culturing cumulus-free eggs in Ca^{2+}-free CZB medium supplemented with 5.56 mM D-glucose containing both 10 mM $SrCl_2$ and 5 μg/mL cytochalasin B for 1 h at 37 °C in a humidified atmosphere of 5 % CO_2. The zona pellucida was then removed by treating the eggs for 3 min in acid PBS (Obiogene, Carlsbad, CA, USA) followed by mechanical shearing. Both denuded MII eggs and parthenogenetically activated eggs were extracted in lysis buffer as previously described [11] and used for proteomic studies.

Two-dimensional gel electrophoresis (2-DE) and protein identification

Lysates from MII eggs and parthenogenetically activated eggs were used for 2-DE to compare the protein expression profiles [11]. A spot was regarded as differentially expressed between groups if the difference in spot intensity was greater than twofold. The differential spots were excised and identified as previously described [26].

Phosphoprotein separation and detection

For phosphoprotein detection, MII egg protein extracts (30 μg) were separated by 2-DE and stained with Pro-Q Diamond phosphoprotein gel stain (Molecular Probes, Eugene, OR, USA) [11].

Bioinformatics analysis

PCBP1 binds to a pyrimidine-rich cluster conforming to the consensus sequence (C/U)CCAN$_x$CCC(U/A)Py$_x$UC(C/U)CC [15, 16, 18, 19, 27] in the 3′-UTR of mRNAs. We searched for this consensus sequence in the 3′-UTR of the mRNAs expressed in mouse oocytes in the UTResource

database (http://www.ba.itb.cnr.it/UTR/) [28]. An mRNA was determined to be expressed in oocytes if there was corresponding expressed sequence tags (ESTs) in the library of the Eppig Hampl Solter fully grown mouse oocyte (Library ID: dbEST:18552) [29].

RNA immunoprecipitation (RIP) assay

RNA immunoprecipitation was performed using the EZ-Magna RIP kit (Millipore, Billerica, MA, USA) following the manufacturer's protocol. Ovaries from 10 wild-type ICR mice were fully ground in liquid nitrogen and then lysed in complete RIP lysis buffer. The ovary protein extracts were then incubated with RIP buffer containing magnetic beads conjugated with a goat anti-PCBP1 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or negative control normal goat IgG (Santa Cruz Biotechnology Inc.). Samples were incubated with Protein K with shaking to digest the protein and then immunoprecipitated RNA was isolated. Purified RNA was subjected to qRT-PCR analysis to evaluate the level of *H2a.x* mRNA, and the primer sequences targeting the binding region were as follows: *H2a.x*: (sense) 5′-CCGGCCTGTGGACAAGAGT-3′ and (antisense) 5′-GGTGTTTGGGTTGTAGTTGAGT-3′. AR (androgen receptor), a well-known target that binds with PCBP1 [30], was amplified as a positive control, and *Smarca4*, which lacks the PCBP1-binding motif in its sequence, was also analyzed in the immunoprecipitated RNA. The primer sequences were as follows: AR (sense) 5′-CCCACCTTGTTCCCTTTCCA-3′ and (antisense) 5′-AGCCACAATACGCAGCAGAT-3′; Smarca4 (sense) 5′-CAACCACCCCTACATGTTCCA-3′ and (antisense) 5′-TTCGTTGCACGGAGTTTGG-3′.

RNA electrophoretic mobility shift (RNA-EMSA) assay

Ovary extract proteins (50 μg) were incubated with 50 pM biotin-labeled *H2a.x* RNA probes in RNA-EMSA buffer (50 mM KCl, 5 % (vol/vol) glycerol, 0.1 % (vol/vol) NP-40, 1 mM MgCl$_2$, 1 mM dithiothreitol, 10 mM Tris–HCl, 0.05 mg/mL heparin, 10 units of RNase inhibitor and 0.5 mg/mL yeast tRNA) for 20 min at 20 °C. Samples were subjected to electrophoresis on a 5 % polyacrylamide 0.5× TBE gels, and transferred to positive-charge nylon membranes. The signals of RNA-EMSA reaction were detected by LightShift chemiluminescent kit (Pierce Chemical, Rockford, IL, USA). For competition assays, excess unlabeled RNA probes (1.25 mM) or antibodies (1 μg) were added directly to binding reactions. All the synthesized mRNAs used in this experiment were obtained from Invitrogen [27], and the oligonucleotide sequences were as follows: wild-type H2a.x probe (WT) 5′-GCCAUCCAUCCCCUCUUCCCCAGC-3′, mutated H2a.x probe (Mut) 5′-GCCAUCCAUCCCCUCUUGCCAGC-3′ and irrelevant sequence (IS) 5′-AAGGGUGGAGCCAAAAGGGUCAUC-3′.

Western blot analysis

The proteins extracted from mouse ovaries were separated by SDS-PAGE and then electrically transferred to a nitrocellulose membrane (GE Healthcare, San Francisco, CA, USA) as described previously [26]. Following transfer, the membranes were blocked in Tris-buffered saline (TBS) containing 5 % skimmed milk for 1 h at room temperature and then incubated for 2 h at 37 °C with a goat anti-PCBP1 antibody and a polyclonal rabbit anti-H2A.X antibody (Cell signaling technology Inc., Danvers, MA, USA) diluted 1:100 and 1:500, respectively, in TBST (TBS containing 0.1 % Tween-20) with 5 % skimmed milk. After washing three times in TBST for 10 min each, the membrane was incubated for 1 h at 37 °C with horseradish peroxidase-conjugated rabbit anti-goat or goat anti-rabbit IgGs (Santa Cruz Biotechnology Inc.) diluted 1:1000 in TBST. The membrane was then washed three times in TBST for 10 min each, and processed using an enhanced chemiluminescence detection system (Alpha Innotech, San Leandro, CA, USA).

Microinjection of anti-H2A.X antibody or anti-PCBP1 antibody into MII eggs

Rabbit anti-H2A.X antibody or goat anti-PCBP1 antibody (0.2 mg/mL in PBS, pH 7.4), whose specificities were verified by western blots (Supplemental Fig. 1), was microinjected into the cytoplasm of MII eggs as described by Dai et al. [31]. The experiment was repeated six times. A Nikon Diaphot ECLIPSE TE 300 inverting microscope (Nikon UK Ltd, Kingston upon Thames, Surrey, UK) equipped with Narishige MM0-202N hydraulic three-dimensional micromanipulators (Narishige Inc., Sea Cliff, NY, USA) was used for these experiments. All microinjections were completed in 40 min. A microinjection volume of about 5–7 pl per egg was used in all experiments. The same amount of rabbit or goat IgG diluted in PBS was microinjected as a control. After microinjection, MII eggs were washed thoroughly and cultured in CZB medium for 3 h at 37 °C in 5 % CO$_2$ and then parthenogenetically activated in CZB medium supplemented with 10 mM SrCl$_2$ and 5 μg/mL cytochalasin B for 1 h, after which they were washed thoroughly and cultured in CZB medium for further observation.

siRNA microinjection into mouse eggs

Two pairs of siRNA duplexes, which have been described previously with proven inhibition efficiency [25], were microinjected into MII eggs. Scrambled siRNA nucleotides were used as a negative control. MII eggs from three groups (*Pcbp1* siRNA injected, negative control siRNA injected, and non-injected control) were cultured in CZB medium for 3 h after microinjection. Some of the injected samples were collected at this point to further confirm the knockdown efficiency of siRNA by real-time RT-PCR. The GAPDH gene was used as an endogenous control. The primer sequences and the expected sizes of PCR products were as follows: *Pcbp1* (sense) 5′-CCAGCTCGCCAGT CATCT-3′ and (antisense) 5′-TGCCCAATAGCCT TTCACC-3′ (253 bp); *Gapdh* (sense) 5′-TCCCGTAGACA AAATGGT-3′ and (antisense) 5′-TCCTGGAAGATG GTGATG-3′ (242 bp). RNAi-treated eggs were activated in Ca^{2+}-free CZB medium supplemented with 5.56 mM D-glucose containing both 10 mM $SrCl_2$ and 5 μg/mL cytochalasin B for 1 h at 37 °C under 5 % CO_2. Eggs were washed and cultured in CZB medium after parthenogenetic activation, and the number of pronuclei in each of the three groups was counted using live cell imaging and analysis.

Construction of *Pcbp1* plasmid and in vitro transcription of RNA

The plasmid-containing full-length mouse *Pcbp1* was purchased from OriGene (MD, USA), and the *Pcbp1* coding sequence was amplified and cloned at FseI and AscI of pCS2$^+$ vector. The pCS2$^+$ vector, which has a myc tag, allows in vitro transcription of polyadenylated mRNA from the SP6 promoter.

In vitro synthesis of capped RNAs was performed using linearized plasmids with the SP6 Message Machine kit (Ambion, Austin, TX, USA) and the mRNAs were purified using RNeasy cleanup kit (Qiagen, Valencia, CA, USA). Synthesized RNA was portioned into aliquots and stored at −80 °C.

Assessment of mRNA levels after PCBP1 antibody or *Pcbp1* mRNA microinjection

Goat anti-PCBP1 antibody or normal Goat IgG (0.5 mg/mL in PBS, pH 7.4) was microinjected into the cytoplasm of MII eggs as previously described. After microinjection, MII eggs were washed thoroughly and cultured in CZB medium for 3 h at 37 °C in 5 % CO_2 and then parthenogenetically activated in CZB medium supplemented with

10 mM $SrCl_2$ and 5 μg/mL cytochalasin B for 1 h, after which they were washed thoroughly and cultured in CZB medium for 3 h. Total RNA was extracted from the two groups using an Absolutely RNA Nanoprep Kit (Qiagen) according to the manufacturer's protocol. Real-time RT-PCR analysis was carried out using an Invitrogen Real-time PCR Kit and GAPDH or 18 s rRNA was used as an endogenous control. Real-time quantitative RT-PCR data were calculated using the $2^{-\Delta\Delta C_t}$ method.

To evaluate the effect of PCBP1 overexpression, in vitro transcribed myc-*Pcbp1* mRNA (10 ng/μL) was injected into the cytoplasm of MII eggs using the same procedure as the antibody microinjection. The same amount of Rnase-free PBS was injected as control. Overexpression of exogenous myc-PCBP1 protein was confirmed by immunofluorescence of anti-myc Tag antibody (Abcam, MA, USA). The mRNA levels of the genes detected after antibody microinjection were compared between the myc-*Pcbp1* mRNA microinjection group and the control group.

Live cell imaging and analysis

Antibody/IgG/siRNA microinjected eggs that had been parthenogenetically activated using $SrCl_2$ for 1 h were moved to gridded coverglass dishes (MatTek, CatNo. P35G-1.5-7-C-grid) before imaging. Images were acquired automatically at multiple locations on the coverslip using a Nikon TE2000E inverting microscope fitted with an X20 Nikon Plan Fluor objective, a linearly encoded stage (Proscan, Prior) and a Hamamatsu Orca-ER CCD camera. The microscope was controlled using Simple PCI (Compix). The microscope was housed in a custom-designed 37 °C chamber with a secondary internal chamber that delivered humidified 5 % CO_2. Differential interference contrast images were obtained every 5 min for a period of 5 h.

Statistical analysis

Each experiment was repeated at least three times. Independent samples Student's *t* tests were performed to test the differences between antibody and IgG-injected groups, as well as those between siRNA or exogenous mRNA and control groups. Before independent samples Student's *t* tests were conducted, the ratios were arcsine-root transformed to assure approximation of normality. The data were given as mean ± STDEV. Numbers of embryos examined are indicated (*n*). $P < 0.05$ was considered statistically significant.

Results

Comparison between normal MII eggs and parthenogenetically activated eggs

To systematically investigate proteins that might be involved in egg activation, we compared the total protein expression profiles of mature mouse MII eggs and parthenogenetically activated mouse eggs without zona pellucida. We performed 2-DE gel electrophoresis of the two groups over a pH range of 3–10. The volume of the stained spots was used as a measure of protein abundance. A spot was regarded differentially expressed between groups if the spot intensity showed a greater than twofold difference, and these differentially expressed spots were selected for mass spectrum (MS) analysis.

Among the identified proteins, PCBP1 exhibited differential expression patterns in MII eggs and parthenogenetically activated eggs. MS identification showed four spots to represent the PCBP1 protein. All spots had the same molecular weight but different isoelectric points (pIs). Among the four spots, spots 1 and 2 (pI values 5.57 and 5.78, respectively) were downregulated, whereas spots 3 and 4 (pI values 6.02 and 6.31) were upregulated after parthenogenetic activation (Fig. 1a, b).
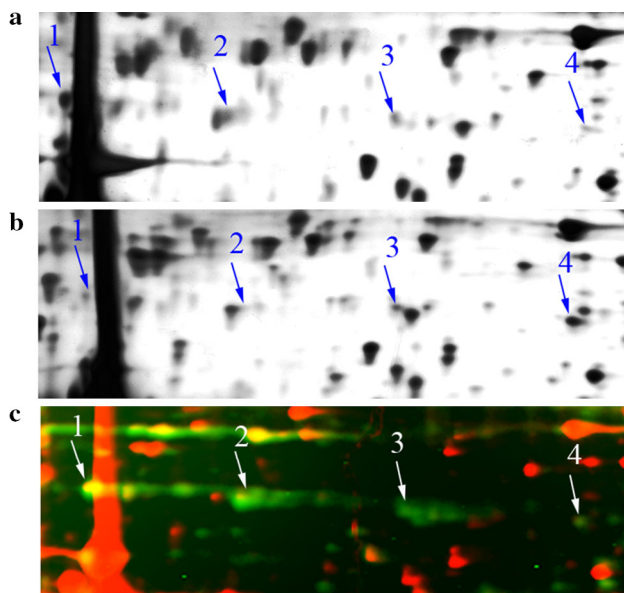
Fig. 1 Comparison of normal MII eggs and parthenogenetically activated eggs. Four spots (*arrows 1–4*) were identified as PCBP1 in gels from both normal MII mouse eggs (**a**) and parthenogenetically activated mouse eggs (**b**) by MALDI-TOF. The intensity of *spots 1* and *2* in the activated gel was downregulated by more than twofold; in contrast, *spots 3* and *4* were upregulated by more than twofold. **c** Cropped phosphoprotein gel image of normal MII mouse eggs. Four spots (*arrows 1–4*) correspond to PCBP1. The phosphorylated forms of proteins stained by Pro-Q diamond dye showed *green* fluorescence; *red* fluorescence represents silver staining proteins

The matched-peptides, sequence coverage rates and Mascot Scores of four spots from MS analysis are shown in Supplemental Table 1. These results suggest that during egg activation, PCBP1 protein undergoes post-translational modification, perhaps phosphorylation.

Phosphorylation analysis of PCBP1 protein in MII eggs

We stained a 2-DE gel of normal MII egg lysates with a fluorescently labeled Pro-Q Diamond dye that recognizes phosphorylated proteins. The four spots of PCBP1 were all labeled with fluorescence (Fig. 1c). This result suggested that PCBP1 was phosphorylated to varying degrees in normal MII eggs.

## PCBP1 might be involved in mRNA stability in mouse eggs

In eukaryotic cells, PCBP1 mostly plays an important role in mRNA stability and/or translation by interacting with a pyrimidine-rich sequence cluster conforming to the consensus sequence $(C/U)CCAN_xCCC(U/A)Py_xUC(C/U)CC$ in the 3′-UTR of mRNAs [15, 16, 18, 19, 27]. Thus, we searched for the consensus sequence in the 3′-UTR of the mRNAs expressed in the mouse oocytes as described in the Methods section. We found that 75 sequences in the 3′-UTR of mRNAs (Supplemental Table 2) expressed in mouse oocytes matched the consensus sequence, suggesting these mRNAs could be potential binding targets of PCBP1 protein. To determine whether PCBP1 was involved in regulating the stability of these mRNAs, we evaluated the levels of 11 randomly selected mRNAs by real-time quantitative RT-PCR during egg activation when PCBP1 was blocked or overexpressed. We also randomly evaluated levels of four presumably unrelated mRNAs.

Under conditions in which the PCBP1 protein was blocked during egg activation, the expression levels of the 11 putative PCBP1-binding mRNAs were all significantly decreased (Fig. 2a). We also overexpressed PCBP1 through exogenous myc-*pcbp1* mRNA microinjection and confirmed efficient overexpression by immunofluorescence with anti-myc antibody (Supplemental Fig. 2). Almost all of the putative PCBP1-binding mRNAs were elevated to varying degrees after PCBP1 overexpression, though several showed no statistical significance (Fig. 2b). Notably, the expression levels of the four mRNAs lacking the PCBP1-binding sequence showed no changes regardless of PCBP1 blocking or overexpression (Fig. 2).

H2a.x mRNA is a target of PCBP1

H2a.x was one of the mRNAs containing the PCBP1-binding sequence in its 3′-UTR that showed statistically
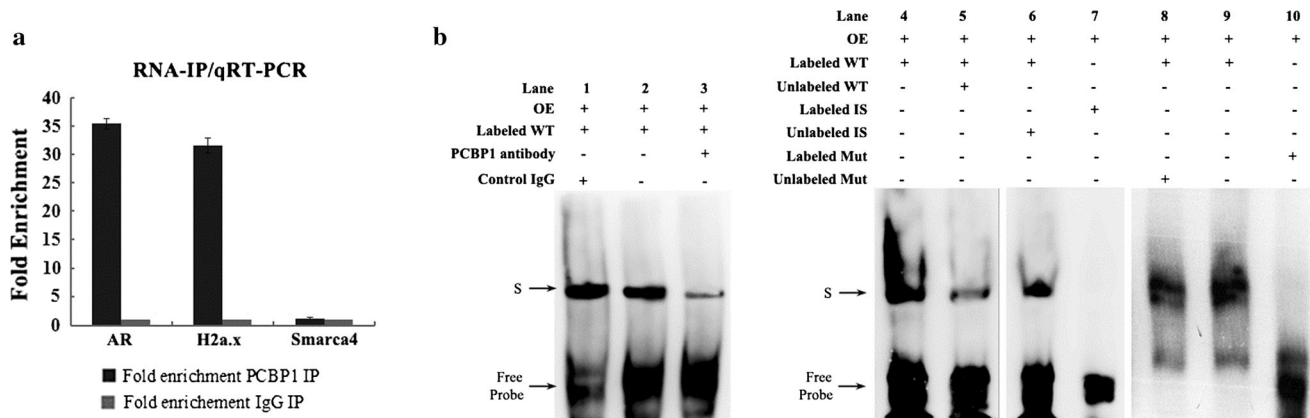
Fig. 2 Involvement of PCBP1 in mRNA stability in mouse eggs. **a** Blockade of PCBP1 by antibody microinjection led to a significant reduction in the levels of 11 putative PCBP1-binding mRNAs (including *Dia1, Foxk1, Th, Wdtc1, H2a.x, Ston2, Traf3a, Afa*p, *Znf2, Cdc42 bp* and *Rnf40*) during egg parthenogenetic activation. In comparison, the expression levels of putative non-PCBP1-binding mRNAs (including *Dppa3, Rnf114, Bmp15,* and *Smarca4*) remained unchanged significantly. **b** Overexpression of PCBP1 through exogenous *pcbp1* mRNA microinjection caused elevation of expression levels of the 11 putative PCBP1-binding mRNAs, although the changes of *Ston2, Traf3a, Cdc42 bp,* and *Rnf40* had no statistical significance. The expression levels of the four other putative non-PCBP1-binding mRNAs were also unchanged. The *y*-axis represents the relative quantification of target genes normalized to GAPDH or 18sRNA as calculated by $2^{-\Delta\Delta C_t}$ method in the real-time PCR assays. *$P < 0.05$, **$P < 0.01$
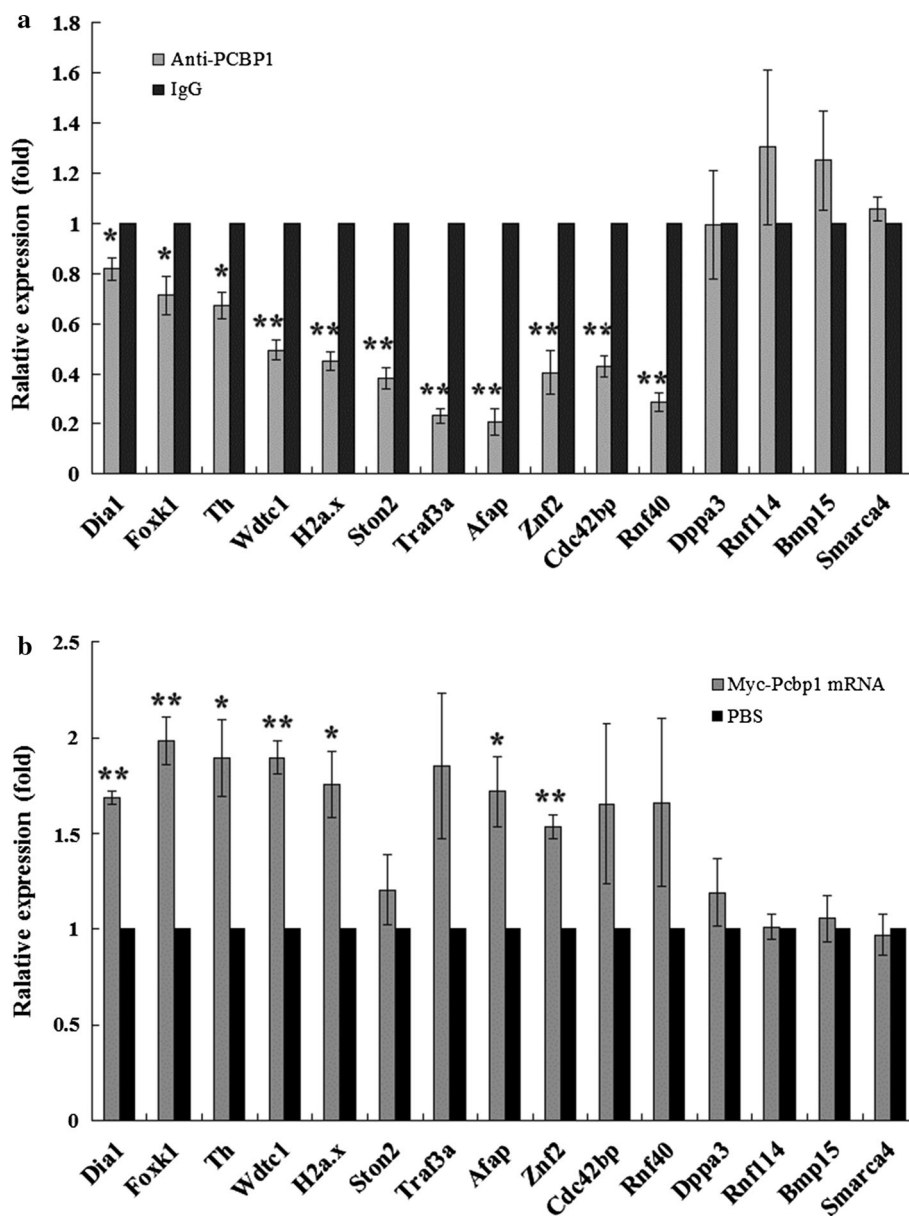
significant differences in expression upon PCBP1 blocking or overexpression. To confirm that *H2a.x* mRNA was a target of PCBP1, RIP assays were performed on ovarian tissue extracts with an antibody against PCBP1, and RNA levels in immunoprecipitates were determined by qRT-PCR. The results showed that AR, as a well-known binding target of PCBP1, was preferentially enriched (about 35-fold) in PCBP1-containing mRNPs relative to control IgG. Notably, *H2a.x* mRNA was also detected at a level of about 32-fold greater than that of control IgG. The level of *Samarca4,* one of the mRNAs lacking the PCBP1-binding motif examined above, was comparable between the anti-

PCBP1 antibody group and the control IgG group (Fig. 3a). These results indicate that PCBP1 protein could bind to *H2a.x* mRNA.

RNA-EMSA was further performed using ovarian protein extracts and biotin-labeled oligonucleotide probes encompassing the PCBP1-binding motif in the 3′-UTR of *H2a.x* mRNA. The results confirmed the presence of the *H2a.x* mRNA–protein complex, while the mutant *H2a.x* probe or irrelevant probe failed to form the RNA–protein complex. Addition of an anti-PCBP1 antibody reduced the complex formation, but IgG had no effect. The complexes were effectively competed by the unlabeled *H2a.x* probe but were not

a

RNA-IP/qRT-PCR

Fold Enrichment (0–40)

Bars: AR, H2a.x, Smarca4

■ Fold enrichment PCBP1 IP
■ Fold enrichement IgG IP

b

Lane	1	2	3
OE	+	+	+
Labeled WT	+	+	+
PCBP1 antibody	-	-	+
Control IgG	+	-	-

S → Free Probe →

Lane	4	5	6	7	8	9	10
OE	+	+	+	+	+	+	+
Labeled WT	+	+	+	-	+	+	-
Unlabeled WT	-	+	-	-	-	-	-
Labeled IS	-	-	-	+	-	-	-
Unlabeled IS	-	-	+	-	-	-	-
Labeled Mut	-	-	-	-	-	-	+
Unlabeled Mut	-	-	-	-	+	-	-

S → Free Probe →

Fig. 3 *H2a.x* mRNA is a binding target for PCBP1. **a** RNA immunoprecipitation (RIP) experiment using anti-PCBP1 or control IgG antibodies. RNA levels in immunoprecipitates were determined by qRT-PCR. AR and *H2a.x* mRNAs were enriched by 35- and 32-fold, respectively, in PCBP1-containing mRNPs relative to control IgG. But the level of *Samarca4* was comparable between the anti-PCBP1 antibody group and the control IgG group. **b** RNA-EMSA assays were performed to verify the interaction of PCBP1 and *H2a.x* mRNA. Components for every reaction are indicated above each *lane*. Protein extracts for mouse ovaries were bound to a biotin-labeled oligonucleotide probe encompassing the PCBP1-binding site in the *H2a.x* mRNA (*lane 2, 4,* and *9*). Addition of anti-PCBP1 antibody reduced the assembly (*lane 3*) but IgG had no effect (*lane 1*). Irrelevant probe (*lane 7*) or mutant *H2a.x* probe (*lane 10*) failed to form the RNA–protein complex. The prominent band was competed with the unlabeled *H2a.x* probe (*lane 5*) but not the irrelevant probe (*lane 6*) or mutated *H2a.x* probe (*lane 8*). *Arrowheads* specific RNA–protein complexes (S) and free probes. OE, ovary protein extracts; WT, *H2a.x* probe encompassing the PCBP1-binding site; Mut, mutant *H2a.x* probe; IS, irrelevant sequence

competed with an irrelevant probe or mutated *H2a.x* probe (Fig. 3b), further confirming the specificity of this binding.

Microinjection of MII eggs with H2A.X antibody retards pronucleus formation

The H2A.X protein is required for pronucleus formation in *Xenopus* oocytes. To examine whether H2A.X also plays a similar role in mice, mouse MII eggs were microinjected with an H2A.X antibody. The percentage of pronuclei formed in the H2A.X antibody-injected group was markedly lower than that of the IgG-injected group and the non-injected group at different time points from 1 to 5 h after parthenogenetic activation ($P < 0.05$). In contrast, IgG injection did not affect the rate of pronucleus formation (Fig. 4).

Inhibition of maternal PCBP1 protein by antibody microinjection retards pronucleus formation

To clarify the role of PCBP1 in pronucleus formation, we observed this process dynamically after microinjection of PCBP1 antibody. The percentage of pronuclei formed in the PCBP1 antibody-injected group was markedly lower than that in the IgG-injected group and the non-injected group at different time points from 2 to 5 h after parthenogenetic activation ($P < 0.05$). In contrast, IgG injection did not affect the rate of pronucleus formation (Fig. 5; Supplemental movie 1–3).

Depletion of maternal *Pcbp1* mRNA by stealth siRNA microinjection leads to pronucleus formation delay

Two *Pcbp1*-specific siRNAs, whose knockdown efficiencies were verified by real-time PCR (Fig. 6a), were microinjected into mouse MII eggs. The rate of pronucleus formation in the *Pcbp1* siRNA-injected group was notably lower than that of the negative control siRNA injected or non-injected group from 2 to 4 h after parthenogenetic activation, but the pronucleus formation rate recovered to a similar extent at 5 h (Fig. 6b). These results also indicated a delay in pronucleus formation after microinjection of MII eggs with stealth siRNAs targeting *Pcbp1*.

Discussion

Pronucleus formation is a landmark event for egg activation and fertilization. However, little is known about the maternal factors involved in pronucleus formation. In this study, we compared the protein profiles of parthenogenetically activated eggs to normal MII mouse eggs with the aim of searching for maternal factors facilitating egg activation. This approach makes no assumptions about the involvement of known or unknown molecules, allowing the process to be independent of any presupposed hypotheses. Our results showed that PCBP1 was one of the most significantly altered maternal proteins.

Fig. 4 Blockade of H2A.X by H2A.X antibody microinjection leads to delay in pronucleus formation. **a** A significant time-dependent inhibitory effect on pronucleus formation was observed in the H2A.X antibody-injected group compared with the non-injected group and the IgG-injected group. Data are presented as mean values ± SD; *$P < 0.05$, **$P < 0.01$ versus control. **b** The H2A.X antibody-injected group displayed an obvious delay in pronucleus formation (pronuclei indicated by *white arrows*). Time stamp is h:min after parthenogenetic activation
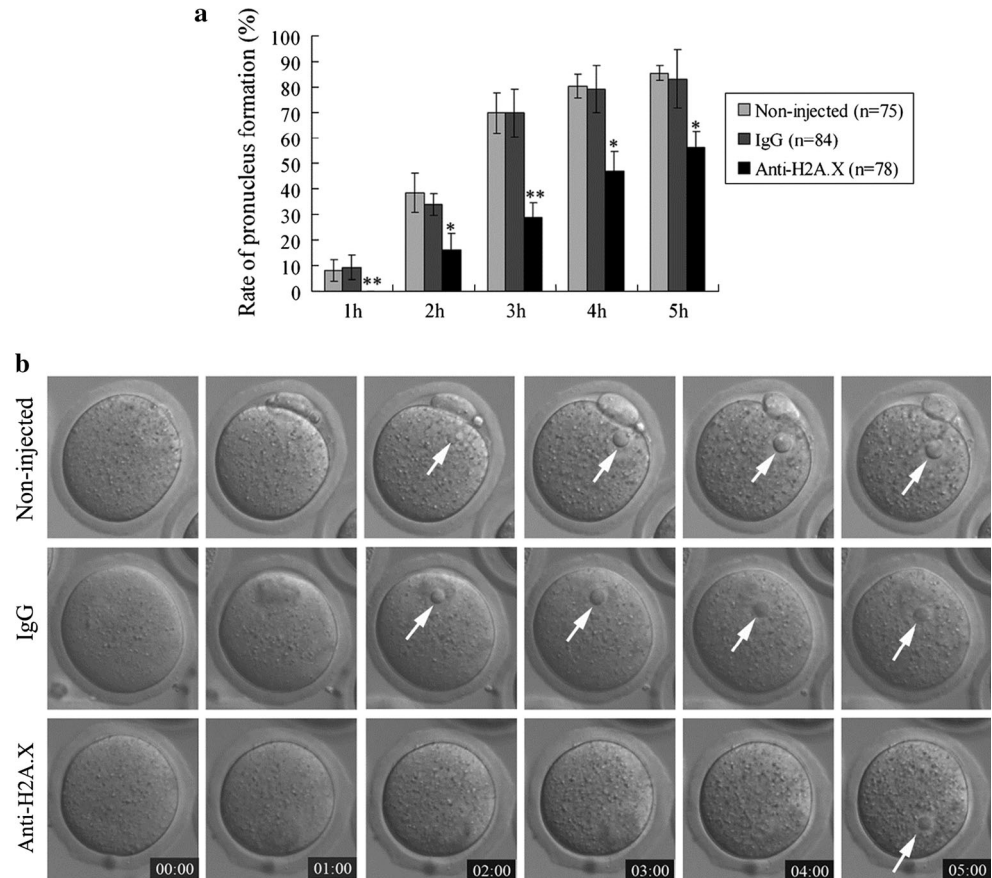
Analysis using 2-DE identified four spots in a line as PCBP1 in gels from both normal and parthenogenetically activated eggs, showing that these proteins had the same molecular weight but different pIs and suggesting that there might be some post-translational modification of PCBP1. Bioinformatics techniques have demonstrated that PCBP1 has many conserved phosphorylation sites. Thus, we anticipated that PCBP1 may be phosphorylated in mouse eggs. To confirm this hypothesis, we carried out phosphoprotein staining of 2-DE gels from normal MII eggs with a fluorescent-labeled Pro-Q Diamond dye and visualized the putative phosphorylated proteins [32]. The image verified that all four spots representing PCBP1 in the 2-DE gel from normal MII eggs displayed phosphorylation. Given that the two spots in the partial acid position were downregulated and the two spots in the partial alkaline position were upregulated in the gel from activated eggs, it is not difficult to conclude that PCBP1 might undergo dephosphorylation after parthenogenetic activation of mouse eggs, and the occurrence of such evident post-translational modification of PCBP1 also suggests that it may play an important role in egg activation. Furthermore, previous studies have shown that phosphorylation of PCBP1 regulated its binding affinity to target consensus

sequences. PAK1 kinase-mediated phosphorylation of cytoplasmic PCBP1 was shown to reduce its RNA-binding activity [33], while another study showed that dephosphorylation of PCBP1 could boost its ability to bind to mRNAs [34].

Bioinformatics analysis identified 75 mRNAs expressed in mouse oocyte that had the consensus PCBP1-binding CU-rich motif in their 3′-UTR, which implied they may be potential binding targets of PCBP1. Eleven mRNAs were randomly chosen for further investigation, and we found that their mRNA levels were significantly decreased when PCBP1 was blocked during egg activation; on the contrary, their levels were elevated to varying degrees when PCBP1 was overexpressed. However, the expressional levels of the putative non-PCBP1-binding mRNAs remained unchanged significantly even after PCBP1 was blocked or was overexpressed. The results suggested that PCBP1 protein might specifically bind the target mRNAs and be involved in regulating the stability of these mRNAs during egg activation. Among these 11 putative PCBP1-binding mRNAs, we focused on *H2a.x*.

H2A.X is a variant of H2A that is highly conserved from *Saccharomyces cerevisiae* to humans and has a major role in chromatin metabolism [35]. H2A.X plays an important

Fig. 5 Blockade of PCBP1 by PCBP1 antibody microinjection leads to delay in pronucleus formation. **a** A significant time-dependent inhibitory effect on pronucleus formation was observed in the PCBP1 antibody-injected group compared with the non-injected group and the IgG-injected group. Data are presented as mean values ± SD; *P < 0.05, **P < 0.01 versus control. **b** Dynamic observation of pronucleus formation in the non-injected group, IgG-injected group and PCBP1 antibody-injected group by live cell imaging (pronuclei indicated by white arrows). PCBP1 antibody-injected eggs displayed obvious delay in pronucleus formation. Time stamp is h:min after parthenogenetic activation. Selected frames from Supplementary Movies 1, 2 and 3
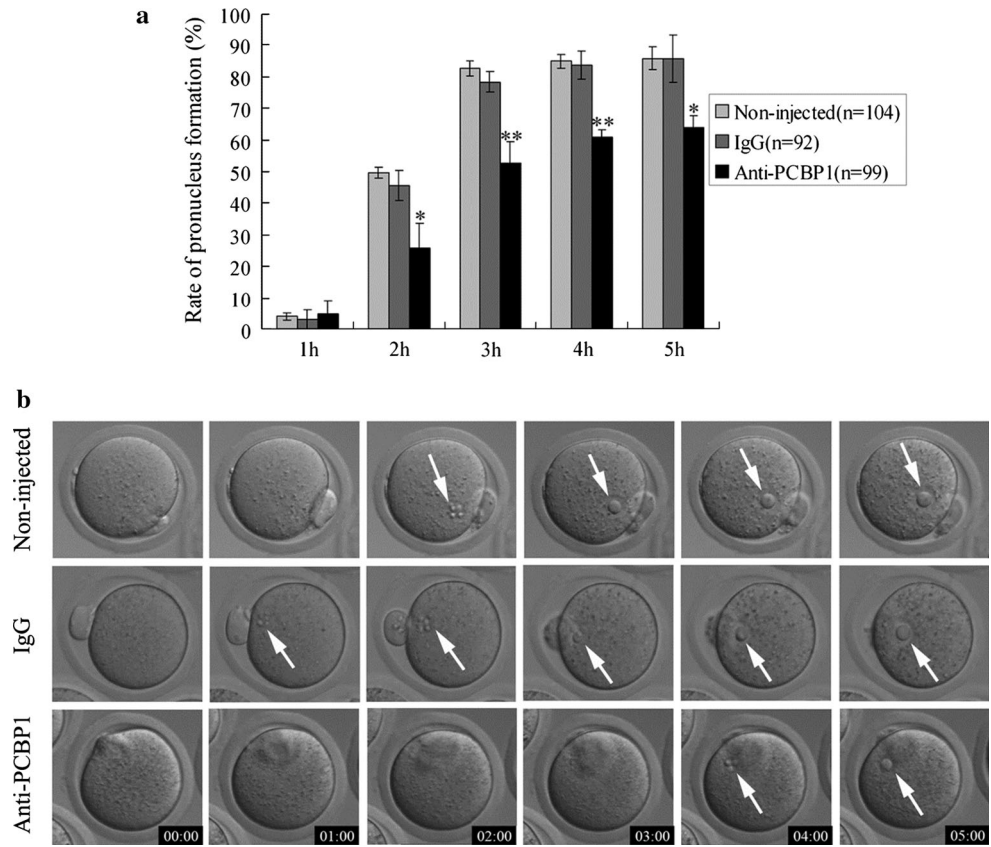
Fig. 6 Knockdown of *Pcbp1* by *Pcbp1*-siRNAs microinjection retarded pronucleus formation. **a** The knockdown efficiency of *Pcbp1*-specific siRNAs was confirmed by real-time PCR. The samples were collected from MII oocytes cultured in CZB medium for 3 h after siRNA microinjection. **b** The rate of pronucleus formation of *Pcbp1* siRNA (*upper panel*, *Pcbp1*-siRNA-1#; *lower panel*, *Pcbp1*-siRNA-2#) microinjected eggs was evidently lower than that of the two other groups at 2–4 h after injection and parthenogenetic activation. Data are presented as mean values ± SD; *P < 0.05, **P < 0.01 versus control
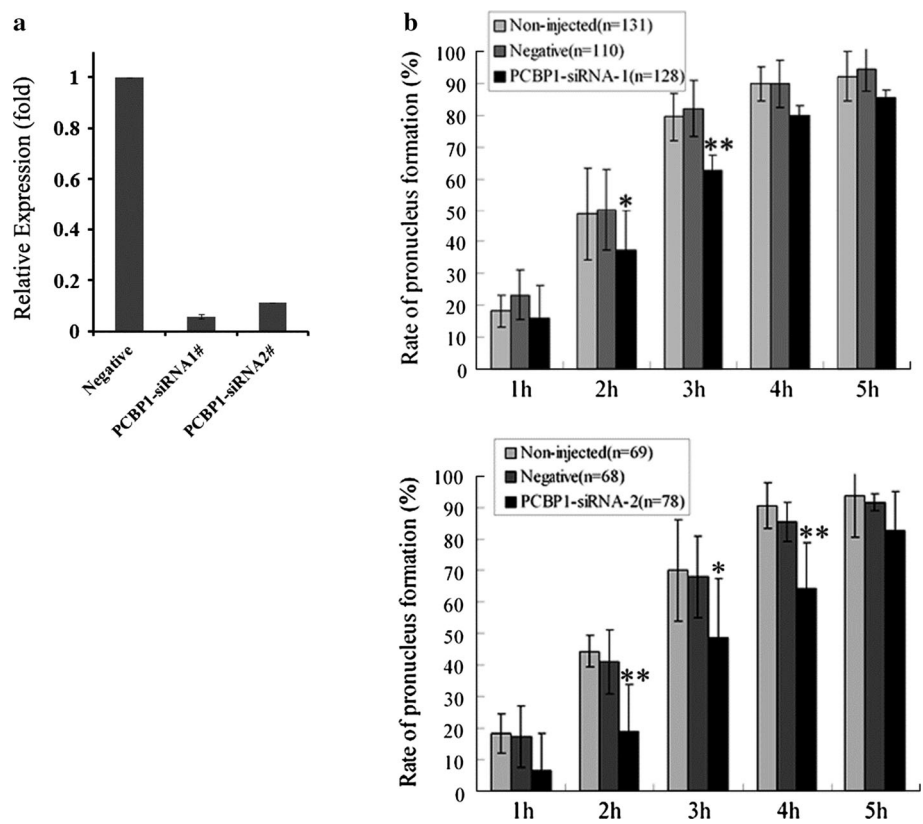
role in nucleosome assembly [36], chromatin organization [37], and DNA repair [38], in addition, H2A.X has been reported to be involved in chromatin decondensation and pronucleus formation in *Xenopus laevis* [36, 39]. RIP was performed to investigate whether PCBP1 could bind with *H2a.x* mRNA. This technique has been demonstrated to be an effective approach to identify putative targets of mRNA-binding proteins using antibodies against RNA-binding proteins [40]. As expected, the results showed that *H2a.x* mRNA was mainly enriched in the mRNP pellets immunoprecipitated by the anti-PCBP1 antibody compared with IgG, indicating it was indeed a binding target of PCBP1. In addition, RNA-EMSA further verified that PCBP1 and *H2a.x* mRNA could form an RNA–protein complex, while irrelevant or mutant *H2a.x* mRNAs failed to form the RNA–protein complex. Assembly of *H2a.x* into mRNPs was reduced by the anti-PCBP1 antibody or competed with the unlabeled self-probe but not with the mutated probe or the irrelevant probe. Thus, we concluded from the above results that PCBP1 could undoubtedly and specifically bind to *H2a.x* mRNA. Because of the limitations of materials, while the interaction of PCBP1 protein with *H2a.x* mRNA was verified in the ovary, direct evidence that PCBP1 actually binds to *H2a.x* in oocytes remains lacking. However, the obvious change in the expression level of *H2a.x* mRNA when PCBP1 was blocked or overexpressed during egg activation implied that PCBP1 could bind to maternal *H2a.x* mRNA in activated eggs and enhance its stability. H2A.X incorporation into chromatin was observed during the pronucleus formation in *Xenopus laevis* [39], however, in mammals, the direct role of H2A.X in pronucleus formation has not been proven, despite the deposition of H2A.X in the parent nuclei in mouse zygotes [41, 42]. Thus, we performed H2A.X antibody microinjection in mouse eggs, and the significantly retarded pronucleus formation in parthenogenetically activated mouse eggs after H2A.X antibody microinjection suggested that H2A.X is also involved in pronucleus formation in mice. We speculated that PCBP1 protein might contribute to pronucleus formation through its regulation on the stability of *H2a.x* mRNA.

Pronucleus formation delay was expectedly observed after inhibition of PCBP1 at the protein level or mRNA level by antibody or specific siRNAs microinjection into mouse eggs followed by parthenogenetic activation. These results indicated that PCBP1 determines the normal timing of pronucleus formation. Studies in different species (including mice, cattle and humans) have shown a positive correlation between the timing of the first cell cycle of embryo and subsequent developmental potential. Early onset of first cleavage is associated with increased blastocyst formation, higher implantation and pregnancy rates [43–45]. The appearance of pronuclei marks entry into G1

phase of the 1-cell stage of development [45], and delay in pronucleus formation was always observed in aged animals and considered to be one of the main factors causing the preimplantation wastage of embryos [46]. These observations suggest that the timing of pronuclear formation may be an important determinant of embryo development.

In summary, here we have demonstrated, using proteomic techniques, that PCBP1 is one of the most significantly altered maternal proteins during egg activation. Our results suggest that maternal PCBP1 displays dephosphorylation in parthenogenetically activated eggs, boosting its ability to bind to *H2a.x* mRNA and enhance its stability, thus determining the normal timing of pronucleus formation in mouse eggs. These findings improve our understanding of the molecular mechanisms regulating pronucleus formation and may aid in the search for the causes of failure of early embryonic development and in nuclear transplant embryonic development. In the previous work, we found PCBP1 also played an important role in the regulation of global transcriptional silencing in fully grown oocytes. Together with this work, we speculate that PCBP1 is a multifunctional protein in mouse oocytes, and might perform diverse roles according to different post-translational modifications, cellular localization, and physiological events. Further studies are required to fully investigate the roles and mechanisms of PCBP1.

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