Mouse zygote-specific proteasome assembly chaperone important for maternal-to-zygotic transition

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Summary

During the maternal-to-zygotic transition (MZT), maternal proteins in oocytes are degraded by the ubiquitin–proteasome system (UPS), and new proteins are synthesized from the zygotic genome. However, the specific mechanisms underlying the UPS at the MZT are not well understood. We identified a molecule named zygote-specific proteasome assembly chaperone (ZPAC) that is specifically expressed in mouse gonads, and expression of ZPAC was transiently increased at the mouse MZT. ZPAC formed a complex with Ump1 and associated with precursor forms of 20S proteasomes. Transcription of *ZPAC* genes was also under the control of an autoregulatory feedback mechanism for the compensation of reduced proteasome activity similar to *Ump1* and 20S proteasome subunit gene expression. Knockdown of ZPAC in early embryos caused a significant reduction of proteasome activity and decrease in Ump1 and mature proteasomes, leading to accumulation of proteins that need to be degraded at the MZT and early developmental arrest. Therefore, a unique proteasome assembly pathway mediated by ZPAC is important for progression of the mouse MZT.

Introduction

After fertilization, erasure of the oogenic program and reprogramming by establishing the embryonic program into totipotent zygotes are coordinately regulated (Pellettieri et al., 2003; DeRenzo and Seydoux, 2004; Stitzel and Seydoux, 2007). This process is called maternal-to-zygotic transition (MZT) and is accompanied by degradation of maternal mRNAs and proteins and transcription of zygotic genes (Keshet et al., 1988; Evsikov and Marín de Evsikova, 2009; Shin et al., 2010). Oocyte-derived mRNAs are degraded shortly after fertilization, and ∼90% of RNAs stored in the oocyte are degraded by the 2-cell stage, which is an essential process for embryogenesis (Stitzel and Seydoux, 2007). Degradation of maternal proteins is also suggested to be an essential component of the MZT (Mendez et al., 2002; DeRenzo and Seydoux, 2004; Huo et al., 2004).

Two major pathways for bulk degradation of intracellular proteins exist in eukaryotic cells, one of which is the autophagy-mediated lysosomal degradation. Recently, the importance of autophagy for preimplantation development has been highlighted in studies using mice (Tsukamoto et al., 2008). They reported that oocyte-specific *Atg5*-knockout mice exhibited early embryonic arrest at the 4-cell or 8-cell stage, indicating that autophagy is important for the overt morphological changes in these stages.

Another proteolytic pathway is the ubiquitin–proteasome mediated degradation. Unlike autophagy, protein degradation by UPS occurs in a selective manner, which is owed to ubiquitin ligases that specifically recognize substrate proteins and attach polyubiquitin chains to them as a degradation signal for the proteasome (Coux et al., 1996; Baumeister et al., 1998). The UPS is essential for maintenance of cellular homeostasis in eukaryotic cells (Varshavsky, 2005; Ciechanover, 2006; Tai and Schuman, 2008).

The proteasome is a highly conserved protein degradation machine made up of two complexes: the catalytic 20S proteasome (also called core particle) and the 19S regulatory particle (RP), both of which are composed of a set of multiple distinct subunits (Coux et al., 1996; Schmidt et al., 2005). The 20S proteasome is composed of 28 subunits arranged in a cylindrical particle as four heteroheptameric rings, $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$ (Baumeister et al., 1998; Maupin-Furlow et al., 2006), the correct assembly of which requires a set of dedicated chaperones named proteasome assembly chaperone (PAC) 1–4 (Psmg1–4) and ubiquitin-mediated proteolysis 1 (Ump1, also called POMP or Proteassemblin) (Ramos et al., 1998; Ellis, 2006; Fricke et al., 2007; Kusmierczyk and Hochstrasser, 2008; Ramos and Dohmen, 2008; Rosenzweig and Glickman, 2008; Murata et al., 2009).

Involvement of the UPS in the degradation of stored maternal proteins after fertilization has already been reported (Evsikov et al., 2004; Solter et al., 2004), and in general, degradation by the UPS is carefully regulated. However, the mechanisms underlying the structure and functions of the UPS at the maternal-to-zygotic transition are not well understood.

In this study, we identified a molecule that we named zygote-specific proteasome assembly chaperone (ZPAC), which is specifically expressed in the mouse gonads and zygote. In the early mouse embryo, expression of ZPAC is transiently augmented at the MZT and plays an important role in the removal of maternal proteins by enhancing the biogenesis of the 20S proteasome.

Results

Identification of ZPAC as an Ump1 interacting protein

To understand the mechanism governing entry of oocytes to totipotent zygotes during the MZT of early mouse embryos, we identified genes whose expression was specifically changed at the MZT using an mRNA differential display analysis comparing embryos at the late 1-cell stage with oocytes at the MII stage (supplementary material Fig. S1A). Of the genes identified, one was a gene that we named *ZPAC* (supplementary material Fig. S1B). Mouse *ZPAC* localized in chromosome 14 A3, which has 7 exons (supplementary material Fig. S1C,D). DNA sequences of *ZPAC* showed an open reading frame of 1056 base pairs encoding a 351 amino acid protein with a predicted molecular mass of 41.5 kDa (supplementary material Fig. S1E,F). The official symbol of the *ZPAC* gene is E330034G19Rik (GeneBank AAI39084), which was not functionally characterized but was predicted to be preferentially expressed in mouse oocytes and early embryos according to its EST profile described in the UniGene database.

To elucidate the function of ZPAC, we performed a yeast two-hybrid screen of a mouse ovary cDNA library using ZPAC as a bait and identified Ump1 as a protein interacting with ZPAC (supplementary material Fig. S2). Ump1 is known as an assembly chaperone that facilitates the formation of 20S proteasomes and is especially required for the initiation of β-ring formation. Ump1 is also degraded upon generation of the 20S proteasome (Hirano et al., 2006; Hoefer et al., 2006; Fricke et al., 2007; Murata et al., 2009). ZPAC protein interacted with Ump1 protein via its N-terminal region (Fig. 1A).

Next, we examined the expression of ZPAC in various tissues in mice. While *Ump1* mRNA was ubiquitously expressed, *ZPAC* mRNA was specifically expressed in the testis and ovary (Fig. 1B), consistent with the EST profile. We raised an antibody against recombinant ZPAC protein derived from its cDNA sequence and ascertained that ZPAC protein was expressed in these specific tissues (Fig. 1C). Ump1 mRNA and protein was also ubiquitously and constitutively expressed in all examined mouse tissues (Fig. 1B,C), but the expression levels of Ump1 protein in testes and ovaries were higher than those in other tissues except liver and heart (Fig. 1C; supplementary material Fig. S6). This suggests that specific expression of ZPAC has some correlation with Ump1 protein levels at least in testes and ovaries (Fig. 1C).

ZPAC mRNA and ZPAC protein were detected in spermatogonia of mouse testes and fully-grown oocytes of mouse ovaries (Fig. 1D). This observation was confirmed in transgenic mice carrying integrated mouse *ZPAC* promoter-driven enhanced green fluorescent protein (EGFP) cDNA (supplementary material Fig. S3). Relatively intense

signals for Ump1 proteins were also detected in spermatogonia and oocytes using immunohistochemical staining, similar to ZPAC protein (Fig. 1E), showing that both ZPAC and Ump1 are highly expressed in the germ cells.

Since ZPAC is specifically expressed in mouse gonads and Ump1 is ubiquitously expressed in mouse tissues, we examined whether ZPAC and Ump1 interact with each other in mouse gonads. Proteins extracted from testes and ovaries were subjected to immunoprecipitation using anti-ZPAC antibody. This showed that ZPAC and Ump1 formed a complex in these cells (Fig. 1F). The association of ZPAC with the proteasome assembly chaperone Ump1 led us to examine the possibility of whether a mouse gonad-specific protein ZPAC collaborates with Ump1 in the ubiquitin–proteasome system.

Unique expression of ZPAC during the MZT

Since ZPAC was expressed specifically in germ cells including oocytes, we examined gene expression profiles and subcellular localization of ZPAC as well as Ump1 during early mouse embryogenesis. In early mouse embryos, the *ZPAC* gene showed a unique expression profile, in which the level of ZPAC mRNA and protein transiently increased at the early 2-cell stage and then drastically decreased by the late 2-cell and 8-cell stages (Fig. 2A), indicating that the identification of ZPAC in the differential display screen does not fully reflect the true behavior of this transcript. The amount of *Ump1* mRNA was highest in oocytes and began to decrease as early as the 1-cell stage. However, the abundance of Ump1 protein was maintained from the 1-cell stage to the 4-cell stage, even after its mRNA level was markedly decreased, and it appears that the levels of ZPAC and Ump1 proteins were coordinately regulated (Fig. 2A). We calculated the ratio of ZPAC/Ump1 proteins at the early embryonic developmental stage (supplementary material Fig. S6). This analysis showed a nearly constant ratio of ZPAC/Ump1 protein during early zygote development, suggesting that ZPAC may form a stoichiometric complex with Ump1.

Consistent with the immunoblot analysis, intense signals of both ZPAC and Ump1 proteins were diffusely detected in the cytoplasm and nucleus at the 1-cell and 2-cell stages (supplementary material Fig. S4). However, as development proceeded, the ZPAC and Ump1 signals decreased at the 4-cell stage and were barely detectable at the 8-cell stage (supplementary material Fig. S4). In oocytes and 1-cell to 2-cell embryos, ZPAC and Ump1 proteins were partially co-localized in nuclear dot-like structures (Fig. 2B, left). Also, ZPAC co-localized with α3, a 20S proteasome subunit, in nuclear dot-like structures (Fig. 2B, right), which are quite similar to the nuclear dot-like structures in which ZPAC and Ump1 co-localized, but outside the nucleoli (Fig. 2B, left), suggesting that they work co-operatively in the cells.

Interestingly, polyubiquitinated proteins were mostly accumulated from oocytes to early 2-cell embryos and then rapidly decreased in 4-cell embryos (Fig. 2C), in which polyubiquitinated proteins disappeared most acutely between 24 and 36 hpi when proteasomal chymotrypsin-like activity from crude lysate of oocytes or early embryos was significantly upregulated (Fig. 2D). Accumulation of polyubiquitinated proteins was also observed in 2-cell embryos after treatment of proteasome inhibitor MG132 from 24 to 36 hpi (Fig. 2E). Consistently, transient augmentation of ZPAC expression coincides with a transient increase in the proteasome activity at
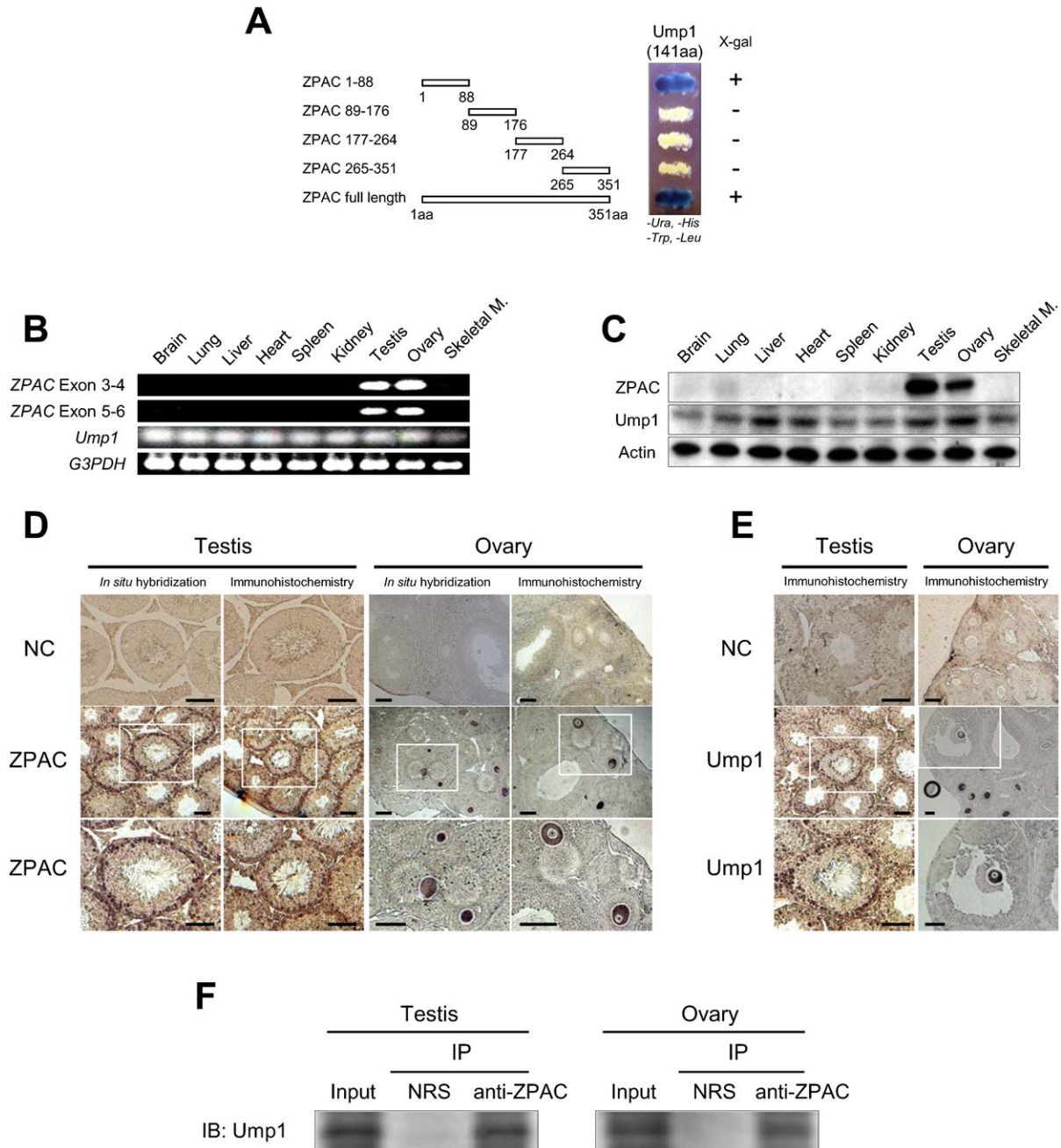
Fig. 1. Identification of ZPAC as an Ump1-interacting protein. (**A**) Characterization of the interacting region of ZPAC as an Ump1-interacting protein by a yeast two-hybrid assay. Ump1 interacting region is shown as a gray color box. (**B**) RT-PCR analysis of *ZPAC* and *Ump1* mRNAs was carried out in various mouse tissues. In *ZPAC*, two different primer sets were used for RT-PCR. *G3PDH* was used as a positive control. (**C**) Immunoblot analysis for ZPAC and Ump1 were performed in the various mouse tissues. Actin was used as a loading control. (**D,E**) *In situ* hybridization (ISH) of *ZPAC* mRNA (D), and immunohistochemical (IHC) analysis of ZPAC protein (D) and Ump1 protein (E) in the mouse testis and ovary. Positive signals (brown color) for both ZPAC and Ump1 are detected in spermatogonia of mouse testes and fully-grown oocytes of mouse ovaries. Upper panels: negative control (NC, *ZPAC* sense RNA probe for ISH and normal rabbit serum for IHC). Middle panels: ISH and IHC for ZPAC or Ump1. Lower panels: magnified images of the rectangle-enclosed areas in the middle panels. Scale bars represent 100 μm. (**F**) Immunoprecipitation by anti-ZPAC antibody followed by immunoblotting using anti-Ump1 antibody in the mouse testes and ovaries. Normal rabbit serum (NRS) was used as a negative control.

the 2-cell stage and a decrease in polyubiquitinated proteins after the late 2-cell stage.

Taken together, these results raise the possibility that ZPAC cooperates with Ump1 in the ubiquitin–proteasome mediated protein degradation during the MZT of early mouse embryos, although upregulation of chymotrypsin-like activity does not directly indicate an increase in the level of the 20S proteasome.

ZPAC is important for the development of fertilized zygotes

To uncover the role of ZPAC during early embryo development, ZPAC-knockdown zygotes were generated by pronuclei injection of ZPAC antisense DNA with internal ribosomal entry sites (IRES)-EGFP as a marker for successful expression (supplementary material Fig. S5). A nearly complete loss of ZPAC proteins and mRNA in the ZPAC antisense DNA injected embryos showing
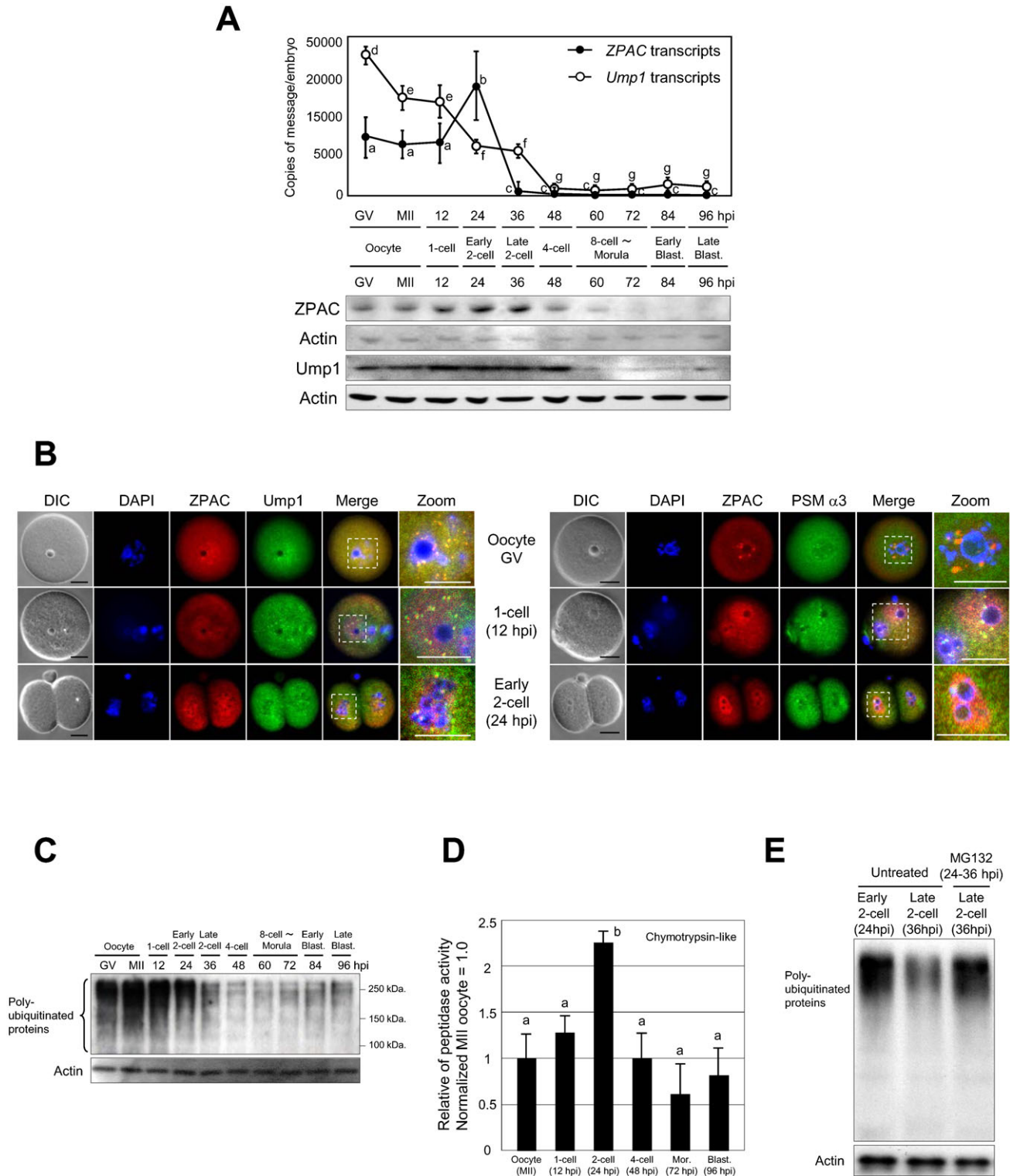
Fig. 2. Unique expression of ZPAC in early mouse embryos during the MZT. (**A**) Quantitative RT-PCR and immunoblot analyses for mRNA and protein expressions of ZPAC and Ump1 in mouse oocytes and early mouse embryos. Different letters indicate statistical significance ($P<0.05$). Actin was used as a loading control in immunoblot analyses. (**B**) Subcellular co-localization of ZPAC and Ump1 (left), and ZPAC and 20S proteasome subunit α3 (PSM α3) (right) in oocytes at the germinal vesicle (GV) stage, 1-cell embryo at 12 hpi, and early 2-cell embryo at 24 hpi. The zoom panels are magnified of merged images of the dotted rectangle-enclosed areas in the ZPAC, Ump1, PSM α3 panels. Scale bars represent 25 μm. (**C**) Immunoblot analysis for polyubiquitinated proteins in early mouse embryos. Actin was used as a loading control. (**D**) Proteasomal chymotrypsin-like activity in early mouse embryos was measured using Suc-LLVY-MCA as a substrate. Crude lysate from 300 fresh mouse oocytes or embryos were used in each stage. Three independent experiments were performed. Different letters indicate statistical significance ($P<0.05$). (**E**) Lysates prepared from untreated 2-cell embryos at 24 hpi and 36 hpi, and 2-cell embryos treated with MG132 from 24 to 36 hpi were performed immunoblotting using anti-Ub antibody. Actin was used loading control.

EGFP expression was detected by immunoblot analysis and quantitative RT-PCR analysis, respectively, (Fig. 3A, left and Fig. 6A). The majority of the EGFP-positive embryos were arrested at the 1-cell to 2-cell stages and only 33% of them developed to the 4-cell stage, whereas 73% of control embryos developed normally to the 4-cell stage (Fig. 3A, right; supplementary material Table S2). Knockdown of Ump1 is known to severely impair biogenesis of the 20S proteasome and cause cell death in mammalian cells (Heink et al., 2005; Hirano et al., 2005). In accordance with this, most of the embryos treated with Ump1 antisense DNA failed to develop beyond the 1-cell stage; only 9% and 2% of the knockdown embryos developed to the 2-cell and 4-cell stages, respectively (Fig. 3B; supplementary material Table S3). However, we observed no cell death until at least 60 hpi in the arrested 1-cell embryos by ZPAC- or Ump1-knockdown, in agreement with our previous observation that no apoptosis is seen in arrested 1-cell embryos by MG132 treatment until the same time (Shin et al., 2010).

Next, we examined whether the developmental defect observed in ZPAC-knockdown embryos was associated with proteasome activity in cells. The proteasomal chymotrypsin-like activity from crude lysate of ZPAC-knockdown arrested 1-cell embryos was significantly decreased by 77% or 83% compared with that of 1-cell or 2-cell embryos, respectively, although downregulation of its activity in ZPAC-knockdown embryos was also slightly less than Ump1-knockdown and MG132-treated embryos (Fig. 3C). Meanwhile, arrested 1-cell embryos at 24 hpi by treatment with the DNA replication inhibitor Aphidicolin, which were also arrested at the 1-cell stage as previously reported (Poueymirou and Schultz, 1987), showed similar activity to that of 2-cell embryos at 24 hpi. These results indicated that ZPAC as well as Ump1 are important for regulation of proteasome activity in early mouse embryos.

To assure inefficient protein degradation by downregulation of the proteasome activity, ZPAC-knockdown embryos were subjected to immunoblot analysis for ubiquitin along with Ump1-knockdown embryos, embryos treated with MG132 or the DNA replication inhibitor Aphidicolin. As expected, a significant accumulation of polyubiquitinated proteins was observed in MG132-treated and Ump1-knockdown embryos, but not in Aphidicolin-treated embryos (Fig. 3D), while ZPAC-knockdown embryos as well as MG132-treated embryos also accumulated polyubiquitinated proteins (Fig. 3D). Importantly, we confirmed that the accumulation of polyubiquitinated proteins in ZPAC- and Ump1-knockdown embryos was not a secondary effect of the developmental arrest.

Furthermore, to investigate the function of ZPAC in protein degradation in MII oocytes and fertilized zygotes, at which stages antisense DNA vector cannot be expressed because transcription does not occur until G2 phase at mouse 1-cell stage (Matsumoto et al., 1999), ZPAC or Ump1 antisense RNA was injected into the cytoplasm of MII oocytes and fertilized zygotes with second polar body extrusion (Fig. 4A, left and Fig. 4B, left). Accumulation of polyubiquitinated proteins was observed in ZPAC or Ump1 antisense RNA knockdown MII oocytes and fertilized zygotes (Fig. 4A, right and Fig. 4B, right). Interestingly, antisense RNA knockdown of ZPAC resulted in a nearly complete loss of Ump1 in MII oocytes and fertilized zygotes, and vice versa (Fig. 4A, right and Fig. 4B, right).

Thus, these results indicate that ZPAC does indeed play an important role in the development of fertilized zygotes and suggest that ZPAC is involved in proteasome-mediated protein degradation in unfertilized oocytes and early embryos.

ZPAC–Ump1 complex specifically associates with assembly intermediates of the 20S proteasome in early mouse embryos

To clarify the mechanism in which ZPAC is involved in protein degradation, we examined the association of the ZPAC–Ump1 complex with the proteasome in early mouse embryos.

The assembly of the mammalian 20S proteasome starts from α-ring formation assisted by PAC1 (Psmg1)–PAC2 (Psmg2) and PAC3 (Psmg3)–PAC4 (Psmg4) complexes, followed by recruitment of $\beta1$–$\beta6$, some of which are in immature forms with propeptides, on the α ring with the assistance of Ump1, resulting in half-proteasomes, which then dimerize upon incorporation of $\beta7$ to form 20S proteasomes, accompanied by cleavage of β-subunit propeptides and degradation of Ump1 (Hirano et al., 2005; Hirano et al., 2008).

Since 2-cell embryos at 24 hpi were found to have high expressions of ZPAC and Ump1 proteins (Fig. 2A) and showed a significantly higher proteasomal chymotrypsin-like activity in early mouse embryos (Fig. 2D), we used the extracts from 2-cell embryos to perform immunoprecipitation with anti-ZPAC or anti-Ump1 antibodies and subjected them to immunoblot analysis. Anti-ZPAC antibody precipitated Ump1, α-subunits and unprocessed precursor β-subunits of the 20S proteasome, but neither mature β subunits nor Rpt6, a subunit of the19S regulatory particle, were co-precipitated with ZPAC. ZPAC was also co-precipitated with PAC1 (Psmg1) and PAC3 (Psmg3), which are known to be specifically associated with assembly intermediates of the 20S proteasome (Murata et al., 2009) (Fig. 5A). Anti-Ump1 immunoprecipitation reproduced essentially the same results, as expected from previous studies (Hirano et al., 2005) (Fig. 5A). As shown in localization of ZPAC and Ump1 in Fig. 2B and supernatant fraction in immunoprecipitation analysis of Fig. 5A, free ZPAC and Ump1 protein that are not associated with each other seem to exist in the oocytes and early embryos, but the significance of these free ZPAC proteins remains to be elucidated.

It has been reported that treatment with proteasome inhibitor elevated levels of β subunit precursor forms of 20S proteasome leading to increased de novo proteasome biogenesis (Meiners et al., 2003). To ascertain the differences in the amount of precursor or mature β subunits of mouse embryos and mammalian somatic cell lines, immunoblot analysis was performed using anti-$\beta1$, $\beta2$, $\beta5$, and $\alpha3$ antibodies of 20S proteasome in the same amounts of lysates from mouse embryonic fibroblast (MEF) cells, human embryonic kidney (HEK) 293T cells, and 2-cell mouse embryos. As shown in Fig. 5B, compared with MEF and HEK293T cells, extraordinarily abundant precursor forms of examined three β-subunits was observed in 2-cell embryos.

Overall, these data demonstrate that the ZPAC–Ump1 complex associates specifically with precursor forms of 20S proteasomes and strongly suggest that ZPAC plays a role in the assembly of 20S proteasomes in early mouse embryos.

ZPAC facilitates assembly of 20S proteasomes by stabilizing Ump1 protein level in early mouse embryos

The amount of proteasomes in mammals is primarily regulated at the transcriptional level under the control of an autoregulatory feedback mechanism that allows for the compensation of reduced proteasome activity (Meiners et al., 2003). Therefore, we firstly
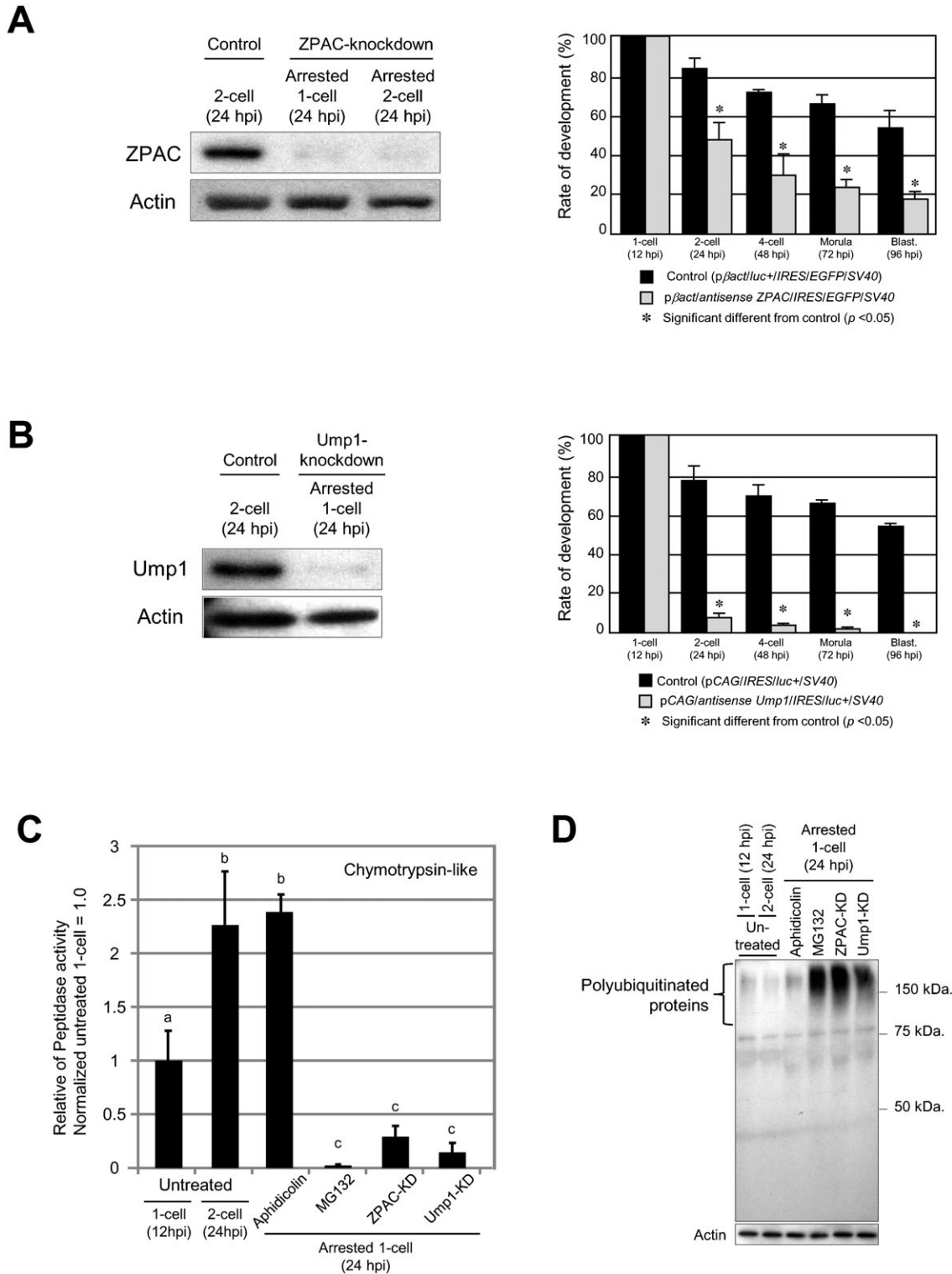
Fig. 3. ZPAC-knockdown embryos displays abnormal development and aberration of proteasome-mediated protein degradation. (**A**) Knockdown of ZPAC by a ZPAC antisense expression vector (pβ-*actin promoter/antisense ZPAC/IRES/EGFP/SV40*) was confirmed by immunoblot analysis (left). Effect of ZPAC-knockdown on the development of early mouse embryos (right). (**B**) Knockdown of Ump1 by an Ump1 antisense expression vector (p*CAG promoter/antisense Ump1/IRES/luc+/SV40*) was confirmed by immunoblot analysis (left). Effect of *Ump1*-knockdown on the development of early mouse embryos (right). (**C**) Proteasomal chymotrypsin-like activity in crude lysate from 300 fresh untreated 1-cell, untreated 2-cell, and arrested 1-cell embryos by Aphidicolin-treatment, MG132-treatment, ZPAC-, and Ump1-knockdown (KD), were measured using Suc-LLVY-MCA as a substrate. The experiment was repeated three times in duplicate. Different letters indicate statistical significance ($P<0.05$). (**D**) Effect of knockdown of ZPAC and Ump1 on the accumulation of polyubiquitinated proteins in 1-cell embryos at 12 hpi and 2-cell embryos at 24 hpi. Total protein extracts from untreated 1-cell, untreated 2-cell, Aphidicolin-treated, MG132-treated, ZPAC- and Ump1-knockdown embryos were immunoblotted with anti-Ub antibody. Actin was used as a loading control.
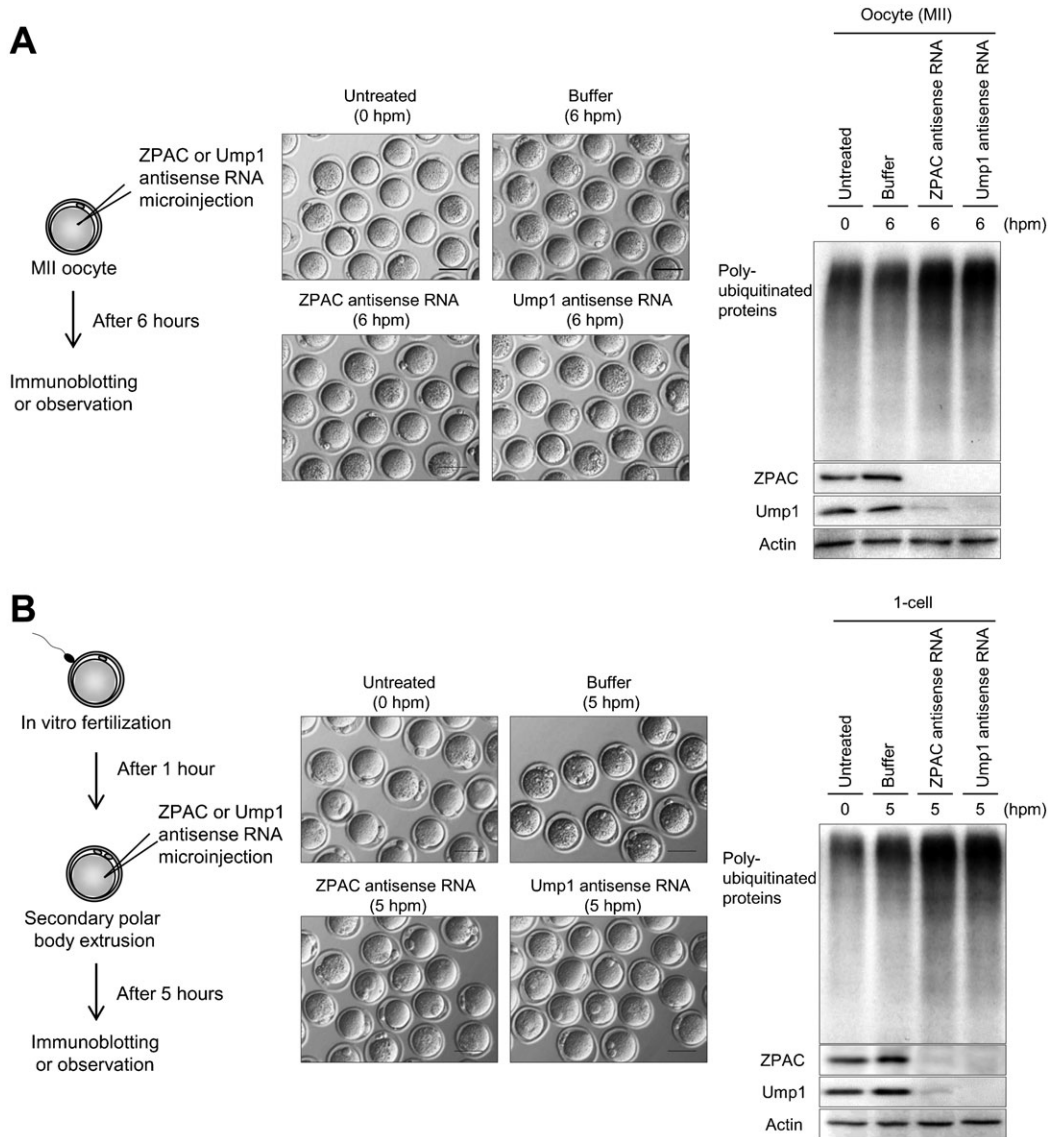
Fig. 4. Microinjection of antisense RNA of *ZPAC* and *Ump1* into the cytoplasm of mouse MII or fertilized oocytes. (A,B, left) Scheme of the experimental procedure. Dilution buffer was used a negative control. (A,B, right) ZPAC- or Ump1-knockdown embryos were selected in 6 (A) or 5 (B) hours post microinjection (hpm), followed by subjected to immunoblotting analysis using anti-Ub, anti-ZPAC, and anti-Ump1 antibodies. Actin was used a loading control in immunoblot analysis. Scale bars of middle figures represent 100 µm.

performed mRNA expression analysis of *ZPAC, Ump1*, five 20S subunits (α3/*PSMA4*, α4/*PSMA7*, β1/*PSMB6*, β2/*PSMB7* and β5/*PSMB5*), and one 19S subunit (Rpt6/*PSMC5*) genes in ZPAC-, Ump1-knockdown and MG132 treated embryos by quantitative RT-PCR with normalization of *G3PDH* mRNA levels. Treatment of embryos with MG132 resulted in a 1.3~1.6-fold or 2.6~3-fold induction of *Ump1*, α3/*PSMA4*, α4/*PSMA7*, β1/*PSMB6*, β2/*PSMB7*, β5/*PSMB5*, and Rpt6/*PSMC5* genes compared with 1-cell at 12 hpi or 2-cell at 24 hpi, respectively, whereas there was a 2.5-fold or 1.4-fold increase in mRNA of *ZPAC* gene in MG132-treated arrested 1-cell embryos compared with 1-cell at 12 hpi or 2-cell at 24 hpi, respectively (Fig. 6A). These results indicate that RNA expression of ZPAC, similar to Ump1 and the examined six components of the standard proteasome, is regulated under the control of a positive autoregulatory

feedback system of proteasome activity. Furthermore, consistent with the proteasome inhibitory effects as shown in Fig. 2E, transcriptional upregulation level of ZPAC, Ump1, and the examined six proteasomal subunits genes for the compensation of reduced proteasome activity was lower in ZPAC- or Ump1-knockdown embryos than in MG132-treated embryos (Fig. 3C and Fig. 6A). Importantly, we confirmed that mRNA expression levels of *Ump1* or *ZPAC* in ZPAC- or Ump1-knockdown embryos at 24 hpi, respectively, were upregulated as the level of each in the 1-cell embryos at 12 hpi and transcripts levels of the examined six proteasomal subunits genes in ZPAC- or Ump1-knockdown embryos at 24 hpi are also upregulated up to the level of each in the 1-cell embryos at 12 hpi (Fig. 6A). These are consistent with the previous report that silencing of different proteasomal genes using siRNA in *Drosophila* S2 cells
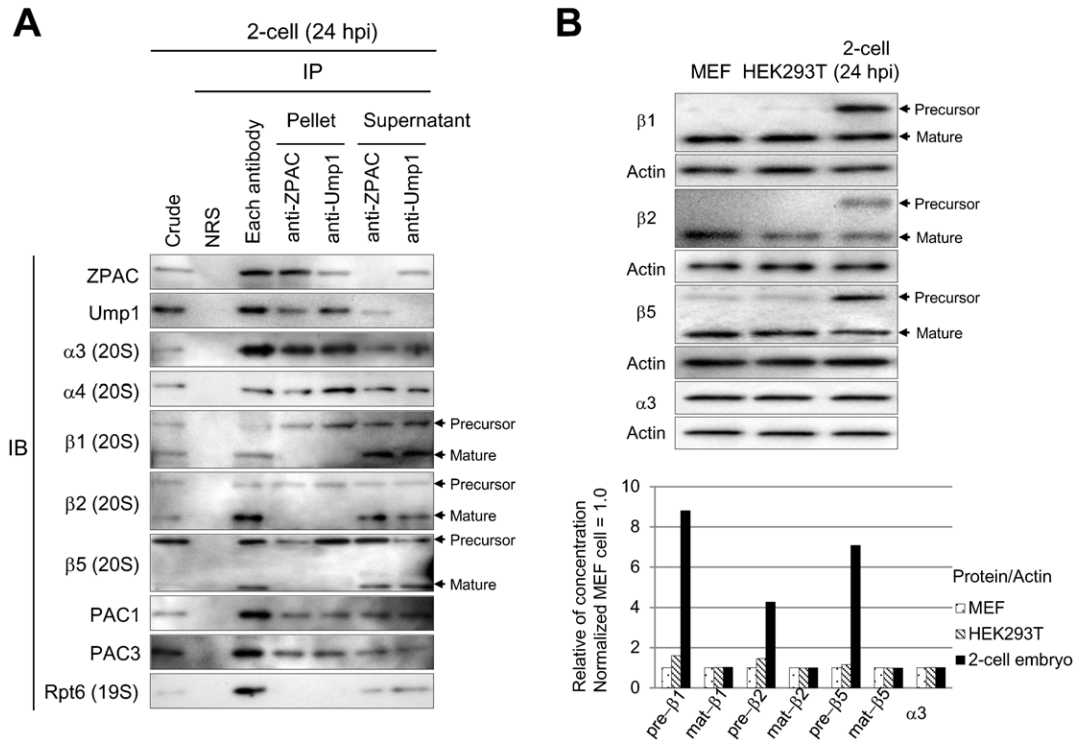
Fig. 5. ZPAC–Ump1 complex associates with precursor 20S proteasome and comparison of β subunits precursor forms of 20S proteasome between mammalian cells and 2-cell mouse embryos. (A) Lysates prepared from 2-cell embryos were immunoprecipitated with anti-ZPAC or anti-Ump1 antibody and immunoblotted with the indicated antibodies. Normal rabbit serum (NRS) was used as a negative control. (B) Immunoblot analysis of indicated proteins. Equal volume (1.5 µg) of lysates was prepared from mouse embryonic fibroblast (MEF) cell, human embryonic kidney (HEK) 293T cell, and 2-cell embryos (top). Densitometric quantification analysis of the immunoblot bands of indicated proteins (bottom). Actin was used as a loading control. pre, precursor; mat, mature.

results in the reduction of mRNA level of targeted proteasomal subunits and acceleration of mRNA levels of several non-targeted proteasomal subunits (Wójcik and DeMartino, 2002).

Next, to examine whether ZPAC is indeed involved in 20S biogenesis, we subjected the cell extracts from ZPAC-knockdown embryos as well as Ump1-knockdown embryos to immunoblot analysis. Interestingly, knockdown of ZPAC significantly reduced Ump1 proteins and likewise knockdown of Ump1 caused an almost complete loss of ZPAC proteins (Fig. 6B) in spite of almost the same level of each mRNA expression as 1-cell embryos (Fig. 6A). These are consistent with the observation that reduction of Ump1 or ZPAC in oocytes in MII stage and fertilized oocytes injected with *ZPAC* or *Ump1* antisense RNA, respectively (Fig. 4). These data suggest that the amount of Ump1 protein is greatly increased by association with ZPAC and vice versa in oocytes and early embryos, even though a certain amount of the Ump1 proteins remains despite a complete absence of ZPAC (Fig. 6B). This may also explain why a milder effect on viability of embryos is observed during ZPAC knockdown as opposed to Ump1 knockdown (Fig. 3A,B). In addition, Ump1-knockdown caused a significant reduction in 20S subunits α3, α4, β1, β2 and β5 while the amount of Rpt6 (19S subunit) was comparable to control embryos (Fig. 6B), consistent with its well-established function as a 20S assembly chaperone (Hirano et al., 2005; Hirano et al., 2006). Likewise, ZPAC-knockdown embryos also showed reduced amounts of α3, α4, β1, β2, β5 and a normal amount of Rpt6, quite similar to the observations in Ump1-knockdown embryos, although the decrease was less severe than in Ump1 knockdown (Fig. 6B).

As the reduction of the proteasome activity results in *de novo* formation of the proteasome, the newly formed proteasome enhances proteasomal degradation for a short period in a compensatory response (Meiners et al., 2003). To investigate the mechanism that underlies the observed reduction of 20S subunits in ZPAC- and Ump1-knockdown embryos, we performed immunoblot analyses in the cells extracted from embryos in the presence of MG132. As depicted in Fig. 6C, accumulation of ZPAC, Ump1, α3, β1, and Rpt6 were observed in ZPAC- and Ump1-knockdown embryos in the presence of MG132 as well as in only MG132-treated embryos. These results indicate that the reduced amount of Ump1 or ZPAC as α3, β1, and Rpt6 proteins in arrested 1-cell embryos by ZPAC or Ump1 knockdown (Fig. 6B), respectively, is not the result of secondary effects of cell arrest that lead to reduction of protein translation but the result of induced *de novo* proteasome formation of matured proteasomes in response to the proteasome inhibition by ZPAC or Ump1 knockdown. Interestingly, ZPAC or Ump1 proteins were accumulated in ZPAC- or Ump1-knockdown embryos treated with MG132, respectively, possibly resulting from gradual accumulation of ZPAC and Ump1 proteins, whose expression are also enhanced by the proteasome inhibition.

Since Ump1 is a protein with a short half-life that is degraded by newly assembled 20S proteasomes in human cell lines (Hirano et al., 2005), we tested whether ZPAC is also a short-lived protein like Ump1 in early mouse embryos. Embryos at 7 hpi were treated with or without Cycloheximide (CHX) in the presence or absence of MG132, and then subjected to immunoblot analysis using anti-ZPAC and anti-Ump1 antibodies. In embryos without
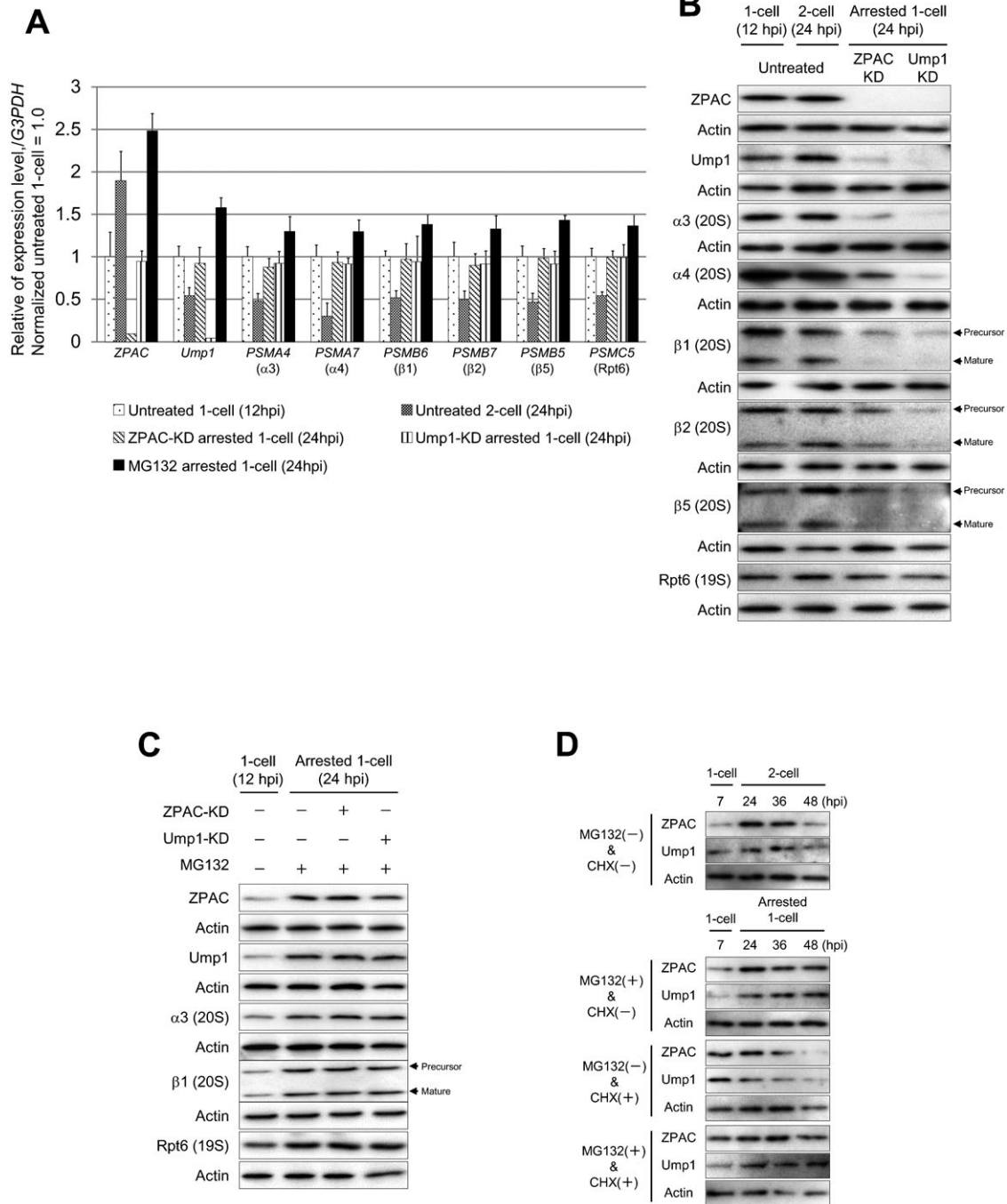
Fig. 6. ZPAC–Ump1 complex is important for proteasome biogenesis. (A) Quantitative RT-PCR analysis for mRNA of *ZPAC*, *Ump1*, and proteasome subunit genes. mRNA prepared from untreated 1-cell, untreated 2-cell, arrested 1-cell embryos by ZPAC-knockdown (KD), Ump1-knockdown, and MG132-treatment. The relative ratios were obtained by dividing the expression level of the indicated genes by the expression level of *G3PDH* gene. The mean of three independent experiments was used for the statistical analyses. **(B)** Extracts from untreated 1-cell, untreated 2-cell, ZPAC-knockdown and Ump1-knockdown embryos were subjected to immunoblot analysis with the indicated antibodies. **(C)** Immunoblot analysis of indicated proteins in lysates prepared from ZPAC- or Ump1-knockdown embryos in the presence of MG132. **(D)** One-cell embryos at 7 hpi were treated with or without cycloheximide (CHX) in the presence or absence of MG132 and lysed at the indicated time. The lysates were subjected to immunoblot analysis with anti-ZPAC and anti-Ump1 antibodies. Actin was used as a loading control (B–D).

CHX in the presence of MG132, the amount of Ump1 and ZPAC proteins were increased compared to embryos with CHX in the presence of MG132 (Fig. 6D; supplementary material Fig. S6), indicating that *de novo* translation of ZPAC and Ump1 proteins occurs in arrested 1-cell embryos at least at 24 hpi as a result of the proteasome inhibition. Ump1 proteins disappeared before 48 hpi and were stabilized by MG132 treatment (Fig. 6D), indicating that the fate of Ump1 proteins in early embryos is similar to that in human cell lines (Hirano et al., 2005; Hirano et al., 2006). Correspondingly, ZPAC also disappeared at 48 hpi

and was also a short-lived protein that is likely degraded in the proteasome, as suggested by being stabilized by MG132 (Fig. 6D).

Taken together, our data demonstrate that ZPAC is specifically associated with precursor forms of the 20S proteasome and is important for assembly of the 20S proteasome in early mouse embryos, probably by stabilizing Ump1 protein.

Discussion

The maternal-to-zygotic transition (MZT) is the first major developmental transition that occurs following fertilization (Schultz, 2002; Schier, 2007). The transition includes the degradation of many maternal mRNAs and proteins, and the beginning of zygotic gene expression resulting in a dramatic reprogramming of gene expression that is responsible for the normal development of early embryos. Our data provide evidence that the cell-type specific ubiquitin–proteasome system plays an important role in the degradation of maternal proteins during the mouse MZT. Notably, we identify ZPAC as a novel assembly chaperone for 20S proteasome at the mouse MZT, which is not found in somatic cells but is specifically expressed in germ cells and zygotes, and provide evidence that supports the role of ZPAC in specific mechanisms underlying the ubiquitin–proteasome system at the mouse MZT.

Our present findings lead us to propose a model for cell-type specific assembly of 20S proteasomes in early mouse embryos (supplementary material Fig. S7). Somatic cell-type assembly of the 20S proteasome is generally assisted by dedicated chaperones, like the PAC1 (Psmg1)–PAC2 (Psmg2) complex, the PAC3 (Psmg3)–PAC4 (Psmg4) complex, and Ump1, where Ump1 plays an important role in the assembly of immature β-subunits on β-rings (Ramos and Dohmen, 2008; Murata et al., 2009). In early mouse embryos, another assembly chaperone ZPAC is specifically expressed. ZPAC interacts with Ump1 and increases the stability of Ump1. The ZPAC–Ump1 complex promotes assembly of immature β-subunits, which are already produced and present in an excess amount in early mouse embryos, and eventually lead to the formation of half-proteasome precursor complexes. Then, the ZPAC–Ump1 complex is degraded upon generation of mature 20S proteasome. In the present model, we emphasize that correct assembly of 20S proteasomes in early mouse embryos is achieved by the general proteasome assembly factors in cooperation with another cell-type specific assembly factor.

Why is ZPAC exclusively expressed in oocytes and early embryos but not in other tissue cells if it is potentially advantageous for 20S proteasome assembly? There appear to be striking differences in strategies for 20S proteasome biogenesis between oocytes/early embryos and other tissue cells. In most tissues, cells other than oocytes and early embryos and also in rapidly proliferating cells such as cancer cell lines, precursor forms of β-subunits are hardly or only faintly detectable (Meiners et al., 2003). Indeed, we observed that early embryos have extraordinarily abundant precursor forms of β-subunits compared to the mature forms in contrast to MEF primary cell line and tumor cell line HEK293T cells (Fig. 5B). Consistent with this observation, immunoprecipitation with anti-ZPAC or anti-Ump1 antibodies did not deplete precursor β-subunits (Fig. 5A, "supernatant" lane). The propeptides of precursor β-subunits have roles to facilitate its own folding or molecular assembly, acting as intramolecular chaperones (Murata

et al., 2009). Availability of Ump1 could be the rate-limiting step in the biogenesis of 20S proteasomes in the presence of excess precursor β-subunits. Thus, increasing the stability of Ump1 protein mediated by ZPAC, possibly by stabilizing them before or during the assembly process of the 20S proteasome, might be the most effective way to increase the amount of assembled 20S proteasomes in early mouse embryos. The higher complexity of eukaryotic 20S proteasomes requires additional factors to ensure their efficient and correct assembly compared with prokaryotic 20S proteasomes (Ramos and Dohmen, 2008; Murata et al., 2009). So, proteasome chaperones are suggested to be involved in a "quality control" mechanism during the assembly of the more complex eukaryotic 20S proteasome (Le Tallec et al., 2007; Li et al., 2007; Kusmierczyk and Hochstrasser, 2008). Therefore, our findings in this study add an additional layer of regulation of 20S proteasome biogenesis. With regard to its homologs, we are unable to find them in other species with BLAST homology searches using either the full length ZPAC or the N-terminal Ump1-interacting sequences as queries. Although we do not know whether early embryos in other species also use a similar mechanism to degrade maternal proteins through the ubiquitin–proteasome system, it is possible that a functional homolog of ZPAC exists, which does not have a significant similarity in its primary amino acid sequence but may structurally resemble ZPAC. This principle is seen in the relationship between mammalian PAC1 (Psmg1) and yeast Pba1 or mammalian PAC3 (Psmg3) and yeast Pba3, both of which play a similar role in 20S assembly and are structurally very close to each other while having very low sequence homologies (Murata et al., 2009).

Besides oocytes and early zygotes, we also observed ZPAC in male germ-line cells in this study (Fig. 1D) and confirmed the co-immunoprecipitation of Ump1 with ZPAC in the testis (Fig. 1F). Spermatogenesis is known to be a complex process that originates in a small population of stem cells (Kanatsu-Shinohara et al., 2003). As the UPS is involved in the regulation of fundamental processes in mammalian stem and progenitor cells of embryonic, neural, hematopoietic, and mesenchymal origin (Naujokat and Sarić, 2007), we speculate that specific formation of proteasomes with ZPAC would be necessary for generation of male gametes.

In the course of our analysis, we noticed that the expression of *ZPAC* gene is profoundly upregulated under the control of a positive autoregulatory feedback system for sensing cellular proteasome activity, compared with general proteasome assembly factors involving Ump1 (Fig. 6A). In addition, whereas mRNA levels of *Ump1* and the other examined five 20S proteasome subunits (α3/PSMA4, α4/PSMA7, β1/PSMB6, β2/PSMB7, and β5/PSMB5) and one 19S subunit (Rpt6/*PSCM5*) were decreased from 1-cell to 2-cell embryos, only *ZPAC* gene expression was transiently elevated in this period (Fig. 6A). Mammalian cells respond to various stimuli by controlling expression of proteasome genes, which allows the cell to cope with changing demands for protein degradation (Meiners et al., 2003). Indeed, exact expression of RIKEN cDNA E330034G19 gene (Gene Symbol, E330034G19Rik; we named *ZPAC* in this study) has been described by gene array analysis of lung from mechanically ventilated knockout mice for *Nrf2* gene, which is a transcription factor that regulates the induction of several antioxidant enzymes (Papaiahgari et al., 2007), suggesting that ectopic expression of *ZPAC* gene in the lung could be induced by oxidative stress. Therefore, these results indicate that a unique

expression profile of *ZPAC* gene is directed by transcription activation in a cellular response to the increased demands for proteasomal degradation of proteins.

The mammalian fully-grown or ovulated oocyte is the largest single cell in which large amounts of maternal mRNAs and proteins are stored. Degradation of enormous amounts of maternal mRNA and proteins after fertilization contributes to dynamic changes from the oogenic program to the embryonic program (Evsikov et al., 2004; Solter et al., 2004; Pelczar et al., 2007; Li et al., 2010). A lack of this degradation and regulation would be harmful to embryonic development (Stitzel and Seydoux, 2007). What is more, damaged or misfolded proteins produced by oxidative stress during ovulation and mistakes in translation of stored maternal mRNA also need to undergo quality control by the UPS (Agarwal et al., 2005; Evsikov et al., 2006). Based on these facts, we demonstrated that the dynamic function of the 20S proteasome at the oocyte and early embryo has been demonstrated. In this context, the unique cell-type specific 20S proteasome assembly catalyzed by the ZPAC–Ump1 complex plays a pivotal role in the dynamic function of the UPS in early mouse embryos. In other words, as the demand for an increased capacity of protein degradation by the UPS might occur during MZT, the upregulation of ZPAC gene expression and the 20S proteasome biogenesis could be regarded as an adaptive response to such demand. Indeed, most of the 20S proteasome assembly is likely to be associated with the ZPAC–Ump1 complex in early mouse embryos, as suggested from our data concerning the proteasomal activity (Fig. 3C). At present, it remains to be seen whether there are any functional or compositional differences between 20S proteasome assembly by the ZPAC–Ump1 complex and 20S proteasomes assembled by only Ump1. In particular, further study is needed to investigate the capacity of each proteasome to recognize and degrade substrates.

In mammalian embryogenesis, zygotic gene activation occurring after fertilization is one of the critical events that govern the MZT for embryonic development (Li et al., 2010). The onset of zygotic gene activation is initially directed by stored maternal RNAs and proteins, and most maternal transcripts are replaced by new products of zygotic transcription. Also, the correct regulation of the onset of zygotic gene activation is an important factor for remodelling of an oocyte into a totipotent zygote. More recently, we have demonstrated that transient proteasome inhibition from 1 to 9 hpi allows fertilized oocytes to delay the onset of zygotic gene activation, indicating that proteasomal degradation of maternal proteins is implicated in the establishment of the embryonic program during the MZT (Shin et al., 2010). These findings would explain the effect of maternal protein degradation on maternal RNA decay and zygotic gene activation during the MZT.

In this study, we also observed an increase of the 20S proteasome by transient increase of ZPAC expression cooperating with Ump1 at the MZT (Fig. 2). This is likely to be in conflict with activating the degradation of Ump1 by an increased assembly of 20S proteasomes. However, the protein levels of Ump1 are also affected by its transcriptional levels. Indeed, in the early zygotes, a much higher level of Ump1 transcripts (nearly 100-fold higher) in the oocytes and 1-cell embryos than in embryos at later stages was observed (Fig. 2A). Thus, while the 20S assembly is increased by the effect of ZPAC, which stabilizes Ump1 protein, the rate of Ump1 supply exceeds

the rate of Ump1 degradation by 20S biogenesis. As a result, Ump1 protein is accumulated in early mouse embryos.

Understanding of the function of cell-type specific 20S proteasomes assembled by the ZPAC–Ump1 complex in the degradation of maternal RNAs and proteins, and zygotic gene activation during the mouse MZT helps elucidate molecular mechanisms governing the remodelling of the oocyte into the totipotent zygote and may also have implications for regulation of pluripotency.

Materials and Methods

Fluoro differential display (FDD)
Differential display was performed by the Hieroglyph mRNA profiling system (TMR-fluorescent anchored primer adaptor kit, Genomyx, Beckman Coulter) according to the manufaturer's instructions. In brief, DNase-treated mRNA prepared from 15,000 MII oocytes or 1-cell embryos at 15 hpi were used for a reverse-transcription with 9 anchored primers (dT12NN(-T) AP). The resulting cDNA mixture was amplified by PCR using one of the 20 TMR-labeled arbitrary anchored primers (M13r-ARP). PCR products were electrophoresed for 5–5.5 hours with 3,000 V on 5.6% denaturing gel (Genomyx HR-1000, Genomyx). After electrophoresis, gel on glass plates and scanned for collection of the gel images. Bands including target fragments of cDNA were excised from dried gel and eluted. Then, re-amplified FDD PCR products were cloned by TA cloning and subjected to sequencing.

Animals, collection of oocytes, *in vitro* fertilization and embryo culture
All mice were purchased from Kiwa Experimental Animals (Wakayama, Japan) and maintained in light-controlled and air-conditioned rooms. All animal procedures conformed to the Guidelines of Kinki University for the Care and Use of Laboratory Animals. Collection of oocytes and fertilized embryos was essentially performed as described previously (Ho et al., 1995; Matsuoka et al., 2008).

Yeast two-hybrid screening
Yeast two-hybrid screening was performed according to the previously described protocol (Matsuoka et al., 2008). The mouse full-length ZPAC cDNA fragment was amplified using PCR and subcloned into the p*Gilda* vector (Takara Bio). A mouse ovarian cDNA library in the vector p*B42AD* was screened. The EGY48 yeast strain used for the screening assay contained both Leu2 and lacZ reporter genes under the control of a LexA-responsive upstream activation sites. For the assay, bait and library plasmids were used to simultaneously transform yeast using the lithium acetate procedure. Double transformant cells grown on Ura−, His−, Trp− and Leu− plates were incubated for five days at 30℃. Positive colonies were picked up and assayed for the LacZ phenotype. Putative positives were detected and then further tested by assaying the colonies for β-galactosidase activity. Following confirmation of the specificity of the interaction, ZPAC-binding partners were identified by sequence analysis. The transformation of only p*Gilda* vector was used as a negative control. To identify the interaction domain of ZPAC with Ump1, partial *ZPAC* cDNA fragments (aa (amino acids) of 1–88, aa 89–176, aa 174–264, aa 265–351 and aa 1–351) were PCR-amplified and subcloned into the p*Gilda LexA* vector and the full-length ORF sequence of mouse Ump1 was PCR-amplified and subcloned into the p*B42AD* vector.

RT-PCR and quantitative RT-PCR analyses
RT-PCR and quantitative RT-PCR analyses were performed as described (Amano et al., 2009). Total RNA was isolated from oocytes and embryos by using the RNAqueous micro kit (Ambion), and from adult tissues by using the TRIzol reagent (Invitrogen). cDNA was synthesized from 1 µg of total RNA by using Superscript III RT (Invitrogen). Prepared cDNA samples were amplified and analyzed by RT-PCR and quantitative RT-PCR. RT-PCR and quantitative RT-PCR analyses were performed using the represented primer sets in supplementary material Table S1. The primers for G3PDH were described previously (Matsuoka et al., 2008). Amplifications were run in a 7300 ABI Prism Sequence Detector (Applied Biosystems).

Proteasomal activation assay
Peptidase activity was measured by using a fluorescent peptide substrate, succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (Suc-LLVY-MCA), as described (Hirano et al., 2006). 300 fresh oocytes or embryos were used in each stage.

Generation of anti-ZPAC antiserum
The design of peptide synthesis was based on the relative hydrophilicity and flexibility of regions analyzed by a computer program (GENETYX-Mac Ver. 12.0.3,

GENETYX). A synthetic peptide (LKQENRRIWGR at residues 124–134) was purified that was designed from the deduced amino acid sequence of the ZPAC protein that spans exon 3. The region used as the synthetic peptide has high hydrophilicity and no putative site of modification. Anti-ZPAC antiserum was obtained by injection of the peptide–KLH (keyhole limpet hemocyanin) complex followed by booster injections at one-week intervals, six times in total, into New Zealand White rabbits (Kitayama Labs). ELISA was used to compare the serum titer from rabbits before and after immunization with the ZPAC peptide. Finally, anti-ZPAC antiserum was fractionated with 40% ammonium sulfate and used throughout this study.

Immunohistochemical staining
The procedures for immunohistochemical staining were essentially the same as those reported previously (Mizuno et al., 2006). In brief, the sample slides were incubated with ZPAC (1:5,000) or Ump1 (Biomol, 1:1,000) in Block Ace (Dainippon Pharm). After incubation, the slides were reacted with biotinylated donkey anti-rabbit secondary antibody (Funakoshi, 1:10,000) for 1 hour at room temperature and incubated with streptavidin for 1 hour at room temperature. Signals were visualized using alkaline phosphatase (Promega).

Immunocytochemical staining
Immunocytochemical staining was performed as described (Tokoro et al., 2010). In brief, oocytes and embryos were fixed in 4% PFA (Nacalai Tesque) in phosphate-buffered saline (PBS) for 30 minutes at room temperature, and the permeated samples were then incubated in PBS containing 0.1–0.2% Triton X-100 (Nacalai Tesque) overnight at 4℃. The samples were then incubated with ZPAC (1:10,000) and/or Ump1 (Santa Cruz Biotechnology, 1:500) in PBS containing 30 mg/ml BSA overnight at 4℃. After incubation, the samples were reacted with Alexa Flour 594-labeled goat anti-rabbit IgG and/or Alexa Flour 488-labeled rabbit anti-goat IgG secondary antibodies (Invitrogen, 1:1,000) for 1 hour at room temperature. To prevent cross-reaction between secondary antibodies, we confirmed that oocytes and embryos were treated with secondary antibodies separately, and then mounted on glass slides in a Vectashield mounting medium (Vector Laboratories) containing 2–5 μg/ml DAPI (Invitrogen). The fluorescence images of oocytes and embryos were obtained using a fluorescence microscope (BIOREVO BZ-9000; Keyence).

Immunoblot analysis
The procedures for immunoblot analysis were essentially those reported previously (Mizuno et al., 2006; Matsuoka et al., 2008). In brief, we used the following antibodies: ZPAC (1:15,000), Ump1 (Biomol, 1:1,000), Ub (Santa Cruz Biotechnology, 1:300), α3 (1:1,000), α4 (1:1,000), β1 (1:1,000), β2 (1:1,000), β5 (1:1,000), PAC1 (Psmg1) (1:1,000), PAC3 (Psmg3) (1:1,000), Rpt6 (1:1,000), and Actin (Santa Cruz Biotechnology 1:7,500). Rabbit polyclonal anti-mouse α3, α4, β5, and PAC1 (Psmg1) antisera were raised against 6×His-tagged recombinant proteins encompassing residues 232–261 of mouse α3, 205–248 of mouse α4, 203–267 of mouse proteins encompassing residues. The monoclonal antibody against Rpt6 was described previously (Tanahashi et al., 2000). Polyclonal antibodies against β1, β2 and PAC3 (Psmg3) were described previously (Hirano et al., 2006; Murata et al., 2007).

In situ hybridization and immunohistochemistry
In situ hybridization was performed as described previously with some modifications (Matsumoto et al., 1999). *ZPAC* sense and antisense RNA probe were synthesized from *ZPAC* cDNA (spanning bases 506–1032 of the mouse *ZPAC* cDNA sequence) cloned into p*GEM-T-Easy* vector (Promega) with digoxigenin-labeled UTP according to the manufacturer's protocol (Boehringer Mannheim). After hybridization, the hybrids were reacted with western blue stabilized substrate for alkaline phosphatase (Promega).

Production of transgenic (Tg) mice expressing EGFP under the control of ZPAC promoter
Transgenic mice with EGFP gene regulated by ZPAC promoter (−4482/−1; 4482 bp upstream from the start codon) were produced by standard procedures. In brief, the purified DNA fragment (ZPAC promoter/EGFP/SV40 terminator) was microinjected into the male pronuclei of zygotes collected from C57BL/6 mice (C57BL/6J, Charles River Laboratory). At 24 hours after DNA injection, morphologically normal zygotes that developed to the 2-cell stage were transferred into the oviducts of Day 1 pseudopregnant female mice (MCH:ICR, CLEA Japan Inc.). A vaginal plug was recognized on this day in the mice used for this procedure (Day 1) (C57BL/6J). Four sublines of the heterozygous transgenic mice were crossed with C57BL/6J mice for two generations before use in this study. For analysis of EGFP expression in the transgenic tissues, testes and ovaries were fixed in 4% paraformaldehyde overnight, embedded and sectioned.

Treatment of inhibitor
MG132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal) was purchased from Sigma–Aldrich. To inhibit the activity of proteasomes in early embryos, embryos were cultured in KSOM medium containing 5 μM MG132. For control, the same protocol was used without MG132. For inhibition of protein synthesis, zygotes were treated with 1 μg/ml cycloheximide (CHX) (Sigma–Aldrich). Aphidicolin (A0781; Sigma Chemical) was used to inhibit DNA replication at the 1-cell stage at a concentration of 1.0 μg/ml. 1-cell embryos were incubated with each of the chemicals from 7 to 24 hours after *in vitro* fertilization.

Microinjection of antisense expression vectors
The procedure for microinjection was essentially as described previously (Matsuoka et al., 2008; Tsunemoto et al., 2008). To investigate the effects of knockdown of ZPAC or Ump1 on the development of early embryos, *ZPAC* antisense expression vector (p*β-actin promoter/antisense ZPAC/IRES/EGFP/SV40*) with bicistronic expression of both *ZPAC* antisense RNA and EGFP or Ump1 antisense expression vector (p*CAG promoter/antisense Ump1/IRES/luc+/SV40*) with bicistronic expression of both *Ump1* antisense RNA and humanized firefly codon-optimized luciferase (luc+) gene was injected into the pronucleus of zygotes at 7 to 9 hpi. The injected zygotes showing EGFP or luciferase activity at 15 hours after microinjection were selected and then cultured to examine the effect of antisense DNA expression on subsequent embryonic development to blastocyst stage. In these experiments, p*β-actin promoter/luc+/IRES/EGFP/SV40* or *pCAG promoter/IRES/luc+/SV40* were used as a control expression vector.

In vitro RNA synthesis and microinjection of antisense RNA
The *ZPAC* and *Ump1* RNA amplification was performed using Ampliscribe T7 Transcription Kit (Epicentre Technologies) from p*GEM-T-EASY/antisense ZPAC* and p*GEM-T-EASY/antisensen Ump1* vectors. For efficient translation of the proteins in embryos or oocytes, the 5′ end of each RNA was capped using RNA Cap Analog kit (Epicentre Technologies), according to the manufacturer's protocol. To investigate the ZPAC or Ump1 function in unfertilized or just fertilized eggs until 6 hpi, *ZPAC* or *Ump1 RNA* was injected into the cytoplasm of mouse MII oocytes or fertilized oocytes at 1 hpi, which were confirmed extrusion of second polar body. Dilution buffer was used as a negative control.

Co-immunoprecipitation
Co-immunoprecipitation was performed according to previous reports (Hirano et al., 2006). For co-immunoprecipitation, we used the ZPAC and Ump1 (Biomol) antibodies.

Densitometric quantification analysis
Densitometric quantification analysis of the immunoblot bands was performed using a Molecular Imager FX with Quantity One software (Bio Rad).

Competing Interests
The authors have no competing interests to declare.

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