Published in final edited form as:
Development. 2008 August ; 135(15): 2627–2636. doi:10.1242/dev.016329.

Role for PADI6 and the CPLs in Ribosomal Storage in Oocytes and Translation in the Early Embryo

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Abstract

The mechanisms mediating the establishment of totipotency during the egg-to-embryo transition in mammals remain poorly understood. The cytoplasmic lattices (CPLs), a structure unique to mammalian oocytes and preimplantation embryos, have long been predicted to function as a storage form for the maternal contribution of ribosomes and mRNA to the early embryo. Here, we demonstrate that oocyte- and embryo-restricted peptidylarginine deiminase 6 (PADI6) is required for incorporation of ribosomal components into the oocyte CPLs. Next, we show that the abundance and localization of ribosomal components is dramatically affected in *PADI6*$^{-/-}$ two-cell embryos and that *de novo* protein synthesis is also disregulated in these embryos. Finally, we demonstrate that embryonic genome activation (EGA) is defective in *PADI6* -/- two-cell embryos. These results suggest that, in mammals, ribosomal components are stored in the oocyte CPLs and are required for protein translation during early development.

Eggs of most lower organisms contain an abundance of stored ribosomes that facilitate protein translation in the early embryo until EGA (1). In *Xenopus* and *Drosophila*, EGA occurs after more than 12 cell divisions. In mammals, however, EGA initiates after just one cell division in the mouse and two or three cell divisions in humans, cattle, and sheep (2). Given this observation, it has remained unclear whether mammalian oocytes need to maintain a storehouse of maternal ribosomes. Nevertheless, biochemical data in the mouse indicate that 75-80% of ribosomes are not incorporated into polysomes at ovulation and do not engage in protein synthesis *in vitro* (3). Several lines of evidence suggest that these inactive ribosomes are actually embedded in the oocyte CPLs, a fibrillar matrix composed of a proteinaceous component and RNA. Previous investigators have observed that a precursor-product relationship exists between ribosomes and the CPLs during oocyte growth, with the number of ribosomes decreasing as the density of CPLs increases (4-6). Further, roughly two-thirds of the ribosomes predicted to exist, based on total rRNA levels, cannot be visualized at the ultrastructural level in fully grown oocytes (4,7). The observation that the CPL fibrils contain repeating units of electron-dense ribosome-sized particles suggested that these “missing” ribosomes are actually contained within the CPLs (6). Finally, unlike somatic cell ribosomes, which require centrifugal forces of at least 100,000 g (>1h) for pelleting, roughly 70% of oocyte rRNA partitions in the cell pellet following centrifugation at 9,000 g (5 min), suggesting that

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most egg ribosomes are associated with a large supramolecular complex (8). Subsequent analysis of the potential relationship between ribosomes and CPLs has likely been muted by the more recent suggestion that the lattices contain intermediate filaments such as keratin (9, 10). However, this interpretation does not diminish the possibility that the CPLs also contain ribosomes, given that many components of the protein synthetic machinery form associations with intermediate filaments (11,12).

We have previously characterized PADI6 and found, by immuno-electron microscopy, that this highly abundant protein primarily localizes to the CPLs in mature oocytes (13). In order to investigate PADI6 function, we inactivated the *PADI6* gene in mice and found that *PADI6*$^{-/-}$ females are infertile with the developmental arrest occurring at the two-cell stage, thus establishing that *PADI6* functions as a maternal effect gene. Further, we also found that the CPL structure cannot be visualized in *PADI6*$^{-/-}$ metaphase II-arrested oocytes, suggesting that PADI6 is required for CPL formation and/or maintenance and that the lattices are required for development beyond the two-cell stage (*In Press*).

In this study, we first utilized transmission electron microscopy (TEM) to investigate when the *PADI6*$^{-/-}$ defect becomes evident during oogenesis and also to study the relationship between PADI6, CPLs, and ribosomes in growing oocytes. Analysis of primordial follicle oocytes found no clear morphological differences between *PADI6*$^{+/+}$ oocytes (Fig. 1A) and *PADI6*$^{-/-}$ oocytes (Fig. 1B). This result is somewhat surprising given that we have previously shown that the PADI6 protein is abundantly expressed at this developmental stage (13). Similar to previous reports (7), we next found that the CPLs first become evident in *PADI6*$^{+/+}$ oocytes at approximately the 40 μm stage of growth and appear to be derived from curvilinear "intermediate structures" (IS), which have properties of both aggregated ribosomes (Rb) and mature lattices (Fig. 1C). However, no CPLs or IS were observed in ~40 μm *PADI6*$^{-/-}$ oocytes (Fig. 1D). CPLs were also evident in fully grown germinal vesicle (GV) stage *PADI6*$^{+/+}$ oocytes (Fig. 1E), while CPLs were not observed in similarly staged *PADI6*$^{-/-}$ oocytes (Fig. 1F). Cytosolic ribosome levels appeared similar between stage-matched *PADI6*$^{+/+}$ and *PADI6*$^{-/-}$ oocytes. We found that CPLs were also not observed in *PADI6*$^{-/-}$ two-cell embryos (Fig. 1H) while lattices in *PADI6*$^{+/+}$ two-cell embryos were visible (arrows, Fig. 1G). Lower magnification TEM images of each field in Fig. 1 have been included (fig. S1).

To further evaluate the relationship between PADI6 and ribosomes, we next investigated whether total ribosome levels were altered in *PADI6*$^{-/-}$ oocytes. Analysis of 18S rRNA levels by RT-PCR and 40S subunit levels by immunoblot analysis using an antibody reactive with the small ribosomal subunit protein, S6, found that loss of PADI6 does not appear to affect levels of these ribosomal components (Fig. 2A). Next, we ruptured *PADI6*$^{+/+}$ and *PADI6*$^{-/-}$ oocytes in a hypotonic 0.05 M KCl buffer containing 0.2 M sucrose and 0.5% Triton X-100 and serially centrifuged the oocyte lysates for 5 min at 650 and 9,000g. Ribosomal protein levels were then evaluated by immuno-slot-blot analysis using the S6 antibody. Results show that the majority of the S6 protein partitioned in either the 650 or 9,000g pellet in *PADI6*$^{+/+}$ oocytes. In *PADI6*$^{-/-}$ oocytes, however, most of the S6 protein partitioned in the supernatant fraction following rupture and centrifugation (Fig. 2B). This finding supports the hypothesis that, in oocytes, ribosomal components are associated with a supramolecular complex. We next performed TEM analysis of the *PADI6*$^{+/+}$ and *PADI6*$^{-/-}$ 9,000g oocyte lysate pellets to test the hypothesis that the CPLs represent this complex. Results show that the major distinction between these two samples was that an abundance of lattice-like structures were found in the *PADI6*$^{+/+}$ oocyte pellet (arrow, Fig. 2C), while these structures were not observed in the *PADI6*$^{-/-}$ oocyte pellet (Fig. 2D). Interestingly, ribosomes appear to be directly associated with some of the putative CPL structures (arrowheads). In further support of the hypothesis that ribosomes are contained within CPLs, we also found by immuno-slot-blot analysis that PADI6, which localizes to CPLs, mainly partitions in the 9,000g pellet of *PADI6*$^{+/+}$ oocyte lysates (fig.

S2). Taken together, the above findings support the hypothesis that PADI6 is required for both CPL formation and for incorporation of ribosomal components into the CPL structure.

We further tested the hypothesis that the CPLs contain ribosomal components using laser scanning confocal microscopy and immunofluorescence analysis to determine if PADI6 and S6 co-localize in *PADI6*$^{+/+}$ oocytes and also if S6 localization is altered in *PADI6*$^{-/-}$ oocytes as would be expected in the absence of CPL. Due to the high abundance and diffuse staining pattern of PADI6 and S6 in GV oocytes (fig. S3), our initial co-localization analysis was not reliable. It has previously been demonstrated that the CPLs are detergent insoluble following 1% Triton X-100 (Triton) extraction (9,10), and we found that Triton extraction before fixation allowed us to more reliably test for co-localization. Similar techniques are used for visualization of proteins associated with the cytoskeleton (14). Results show that, in Triton extracted oocytes, PADI6 and S6 appear to co-localize in regions throughout the cytoplasm and at granular foci in the oocyte cortex (Fig. 3A). Strikingly, while S6 is still retained in the Triton extracted *PADI6*$^{-/-}$ oocytes, the staining is less diffuse and more punctate than in *PADI6*$^{+/+}$ oocytes. Additionally, in these oocytes, S6 is absent from the oocyte cortex (arrow, Fig. 3A). These results further strengthen the interrelationship between PADI6, ribosomal components, and the CPLs.

Given that lattices do not form in *PADI6*$^{-/-}$ oocytes and early embryos, these cells represent a unique model system in which to study the function of stored ribosomes during oogenesis and early development. While previous investigators predicted that the stored ribosomes are involved in translation following fertilization (8), it is also possible that these ribosomes play a role in protein synthesis in oocytes. To test this hypothesis, we metabolically labeled *PADI6*$^{+/+}$ and *PADI6*$^{-/-}$ GV stage oocytes for two hours using [^{35}S]methionine and evaluated *de novo* protein synthesis by fluorography of resolved proteins. Results showed that, with few exceptions, the repertoire of nascently synthesized proteins appears similar between *PADI6*$^{+/+}$ and *PADI6*$^{-/-}$ oocytes (Fig. 3B). Note: the 72 kDA protein (arrow) missing from *PADI6*$^{-/-}$ oocytes is likely PADI6. This result, coupled with the observation that oocyte growth and fertilization are not affected by loss of PADI6, supports the hypothesis that CPL-associated ribosomal components do not play a critical role in protein translation in oocytes.

Our previous finding that *PADI6*$^{-/-}$ embryos arrest at the two-cell stage, suggested that CPL-associated ribosomal components are likely required for protein synthesis in the early embryo. To begin testing this hypothesis, we next evaluated S6 levels and localization by western blot and confocal immunofluorescence analysis in *PADI6*$^{+/+}$ and *PADI6*$^{-/-}$ two-cell embryos. Results show that both PADI6 and S6 strongly co-localize at the non-apposed cortical regions of each blastomere in *PADI6*$^{+/+}$ embryos (Fig. 3C). This localization pattern is surprising, given that this region is rich in microfilaments and contains few CPL (15), and suggests that both PADI6 and ribosomal components are released from the CPL and targeted to microfilaments at the two-cell stage. In *PADI6*$^{-/-}$ two-cell embryos, we found that total amounts of S6 were reduced (Fig. 3C and 3D) and, strikingly, S6 no longer localizes to the embryonic cortex (arrows, Fig. 3C). To investigate whether the reduced levels and localization of S6 in *PADI6*$^{-/-}$ two-cell embryos affects protein translation, we next metabolically labeled the embryos with [^{35}S]methionine for two hours and then evaluated *de novo* protein synthesis by scintillation counting and by fluorography of resolved proteins. Results show that total protein synthesis rates appear lower in *PADI6*$^{-/-}$ two-cell embryos compared to *PADI6*$^{+/+}$ embryos (fig. S4). In addition to reduced global levels of protein synthesis, fluorographic analysis of newly synthesized proteins found that expression levels of specific proteins in *PADI6*$^{-/-}$ two-cell embryos are altered when compared to *PADI6*$^{+/+}$ embryos (Fig. 3E). In particular, synthesis of one protein at ~30 kDa is dramatically upregulated in the *PADI6*$^{-/-}$ two-cell embryo. Based on previous reports, we hypothesized that this protein was likely spindlin, the product of a stored maternal transcript thought to be involved in regulating the cell cycle during

meiosis and the first mitotic cell division (16). We next tested the hypothesis that spindlin protein synthesis is disregulated in *PADI6*$^{-/-}$ two-cell embryos by confocal analysis using anti-spindlin antisera and found that spindlin levels were, in fact, increased in *PADI6*$^{-/-}$ two-cell embryos (Fig. 3F).

Taken together, these observations support the hypothesis that improper storage of CPL-associated ribosomes in *PADI6*$^{-/-}$ oocytes leads to an overall reduction in protein synthesis in two-cell embryos. Further, the altered translation efficiency in *PADI6*$^{-/-}$ two-cell embryos, suggests that the CPL complex may also play a role in organizing and regulating the translational machinery such that specific maternal mRNAs are translated at different efficiencies in *PADI6*$^{-/-}$ embryos compared to *PADI6*$^{+/+}$ embryos. Further, the observation that spindlin levels are increased in *PADI6*$^{-/-}$ embryos suggests that the observed overall reduction in total protein synthesis is not simply due to cell death.

Previous investigators have shown that protein synthesis is required for major genome activation at the two-cell stage in mice (17). Therefore, we next tested the hypothesis that the observed translational defects in *PADI6*$^{-/-}$ two-cell embryos lead to defective EGA. In mammals, nuclear translocation of maternally inherited RNA Polymerase II (RNA Pol II) is thought to be a major determinant of EGA (18). Therefore, we first investigated whether RNA Pol II levels and/or localization was specifically affected by loss of PADI6. We first probed two-cell embryos with an antibody that recognizes the N-terminus of RNA Pol II and found that levels of RNA Pol II were reduced by ~20% in the nucleus of *PADI6*$^{-/-}$ two-cell embryos when compared to *PADI6*$^{+/+}$ embryos (Fig. 4A). Further, we also found that levels of phosphorylated (Ser 2) RNA Pol II (a well characterized marker for active transcription) (19) were reduced by ~30% in the nucleus of *PADI6*$^{-/-}$ embryos (Fig. 4A). These results suggest that both RNA Pol II nuclear translocation and RNA Pol II-mediated transcription are reduced in *PADI6*$^{-/-}$ embryos. Further, our metabolic labeling data suggests that the basis for the nuclear translocation deficiency may lie in the improper translation of factors required for the transport of RNA Pol II from the cytoplasm to the nucleus. This hypothesis is supported by the observation that RNA Pol II appears to aggregate outside of the nuclear envelope in *PADI6*$^{-/-}$ embryos (arrow, Fig. 4A). Given that histone H4 acetylation is well correlated with active gene transcription (20), we next probed *PADI6*$^{+/+}$ and *PADI6*$^{-/-}$ two-cell embryos with an anti-histone H4 acetyl antibody (histone H4K5) as a further test of the effects of loss of PADI6 on embryonic transcription. Results show a small, but statistically significant, ~10% reduction in histone H4 acetylation in *PADI6*$^{-/-}$ two-cell embryo nuclei when compared to *PADI6*$^{+/+}$ embryos (Fig. 4A). We next measured nuclear incorporation of BrUTP in *PADI6*$^{+/+}$ and *PADI6*$^{-/-}$ two-cell embryos using an anti-BrUTP antibody and fluorescence microscopy (21). Fluorescence analysis showed that transcription levels were reduced by ~73.7% in *PADI6*$^{-/-}$ embryos compared to *PADI6*$^{+/+}$ embryos (Fig. 4B). Finally, we investigated the *de novo* synthesis of the transcription requiring complex (TRC), a group of Triton X-100 insoluble proteins that are the first major products of embryonic transcription and thus function as a marker for EGA (22). Results show that TRC synthesis is reduced by ~53% in *PADI6*$^{-/-}$ two-cell embryos when compared to *PADI6*$^{+/+}$ embryos (Fig. 4C), thus further supporting the hypothesis that *PADI6*$^{-/-}$ two-cell embryos fail to undergo proper embryonic genome activation.

Taken together, our results support the hypothesis that the mechanism of the *PADI6*$^{-/-}$ two-cell arrest actually initiates during oocyte growth. In *PADI6*$^{-/-}$ growing oocytes, we demonstrate that CPLs do not form, thus likely disrupting the proper storage of a subset of maternal ribosomes. While this defect does not directly affect oocyte viability, the improper storage of ribosomal components leads to a reduction in total *de novo* protein synthesis at the two-cell stage, as well as a disruption in the translation efficiency of numerous transcripts. We then predict that this alteration in protein synthesis efficiency reduces targeting of specific

reprogramming factors, such as RNA Pol II, to the nucleus, thus leading to incomplete EGA and embryonic arrest.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Roger Gosden, Rosemary Bachvarova, and Elizabeth Lacy for their technical advice and critical reading of this manuscript. We thank Dr. Barbara Knowles for the anti-spindlin antibody. This work was supported by NICHD grant RO1 38353 (SAC) and by a grant from the Tri-Institutional Stem Cell Initiative funded by the Starr Foundation.

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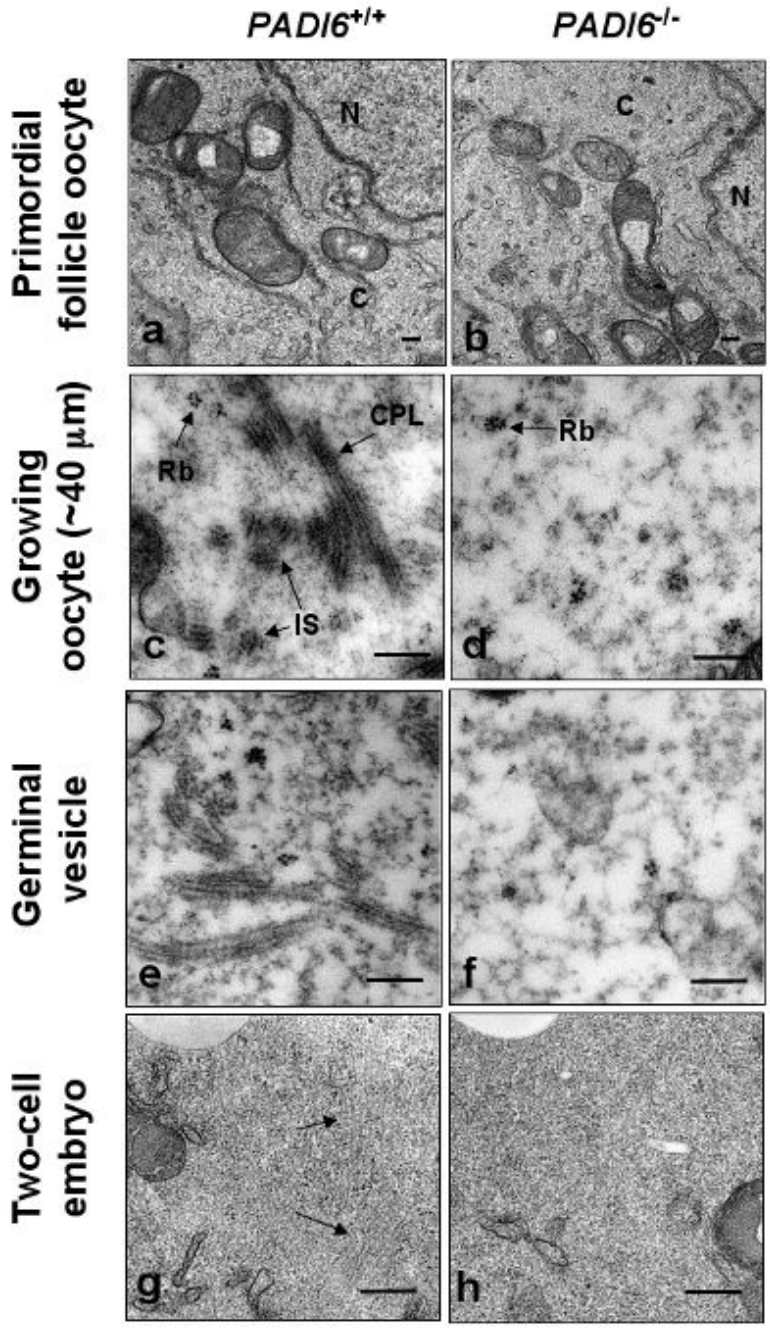


Figure 1. PADI6 is required for cytoplasmic lattice formation in oocytes and two-cell embryos

(A) *PADI6*$^{+/+}$ primordial follicle oocyte. N – nucleus. C – cytoplasm. **(B)** *PADI6*$^{-/-}$ primordial follicle oocyte. **(C)** CPLs are first observed in *PADI6*$^{+/+}$ growing oocytes. Rb – ribosome. IS – intermediate structure. CPL – cytoplasmic lattice. **(D)** CPLs do not form in *PADI6*$^{-/-}$ growing oocytes. **(E)** CPLs are also observed in *PADI6*$^{+/+}$ fully grown germinal vesicle stage oocytes. **(F)** CPLs are not observed in *PADI6*$^{-/-}$ fully grown germinal vesicle stage oocytes. **(G)** Arrows highlight CPLs in *PADI6*$^{+/+}$ two-cell embryos. **(H)** CPLs are not observed in *PADI6*$^{-/-}$ two-cell embryos. Scale bar equals 200 nm.

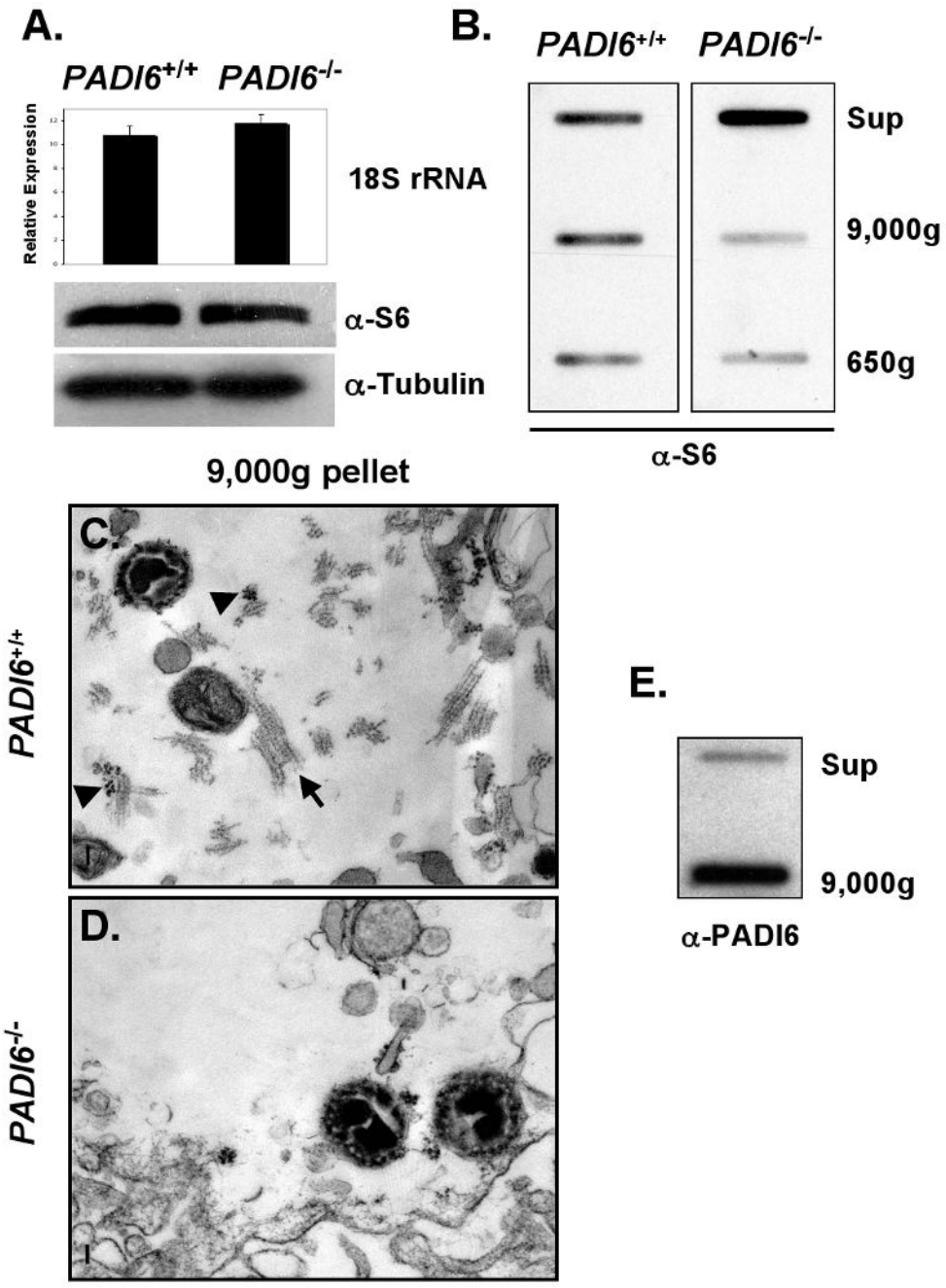


Figure 2. PADI6 is required for incorporation of ribosomal components into the oocyte cytoplasmic lattices

(A) RT-PCR analysis using 18S rRNA primers and western blot analysis using anti-S6 antibodies indicates that levels of ribosomal components are similar between *PADI6*$^{+/+}$ and *PADI6*$^{-/-}$ oocytes. **(B)** In contrast with *PADI6*$^{+/+}$ oocytes, the majority of ribosomal protein S6 partitions in the supernatant of ruptured *PADI6*$^{-/-}$ oocytes. Oocytes were ruptured in hypotonic buffer, serially centrifuged at 650 and 9,000g for 5 min, and the partitioning of ribosomal components into the supernatant (Sup) and pellet fractions was evaluated by immuno-slot-blot analysis using anti-S6 antibodies. **(C)** Transmission electron microscopic analysis indicates that while putative CPLs (arrows) are observed in the *PADI6*$^{+/+}$ 9,000g oocyte pellet, lattices

are not observed in the *PADI6*$^{-/-}$ oocyte pellet. Arrowheads highlight ribosome-like particles associated with CPLs. Scale bar equals 100 nm.

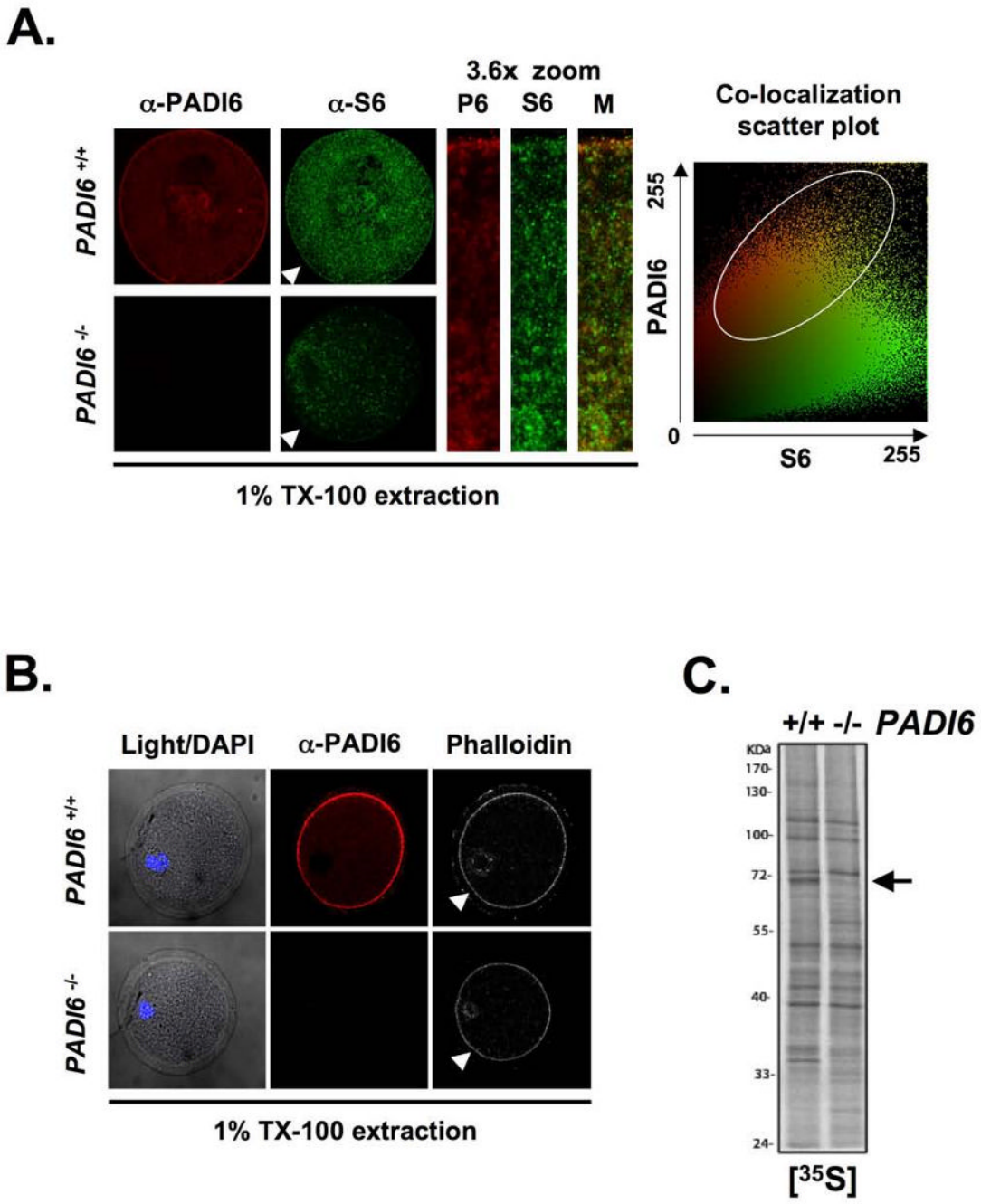
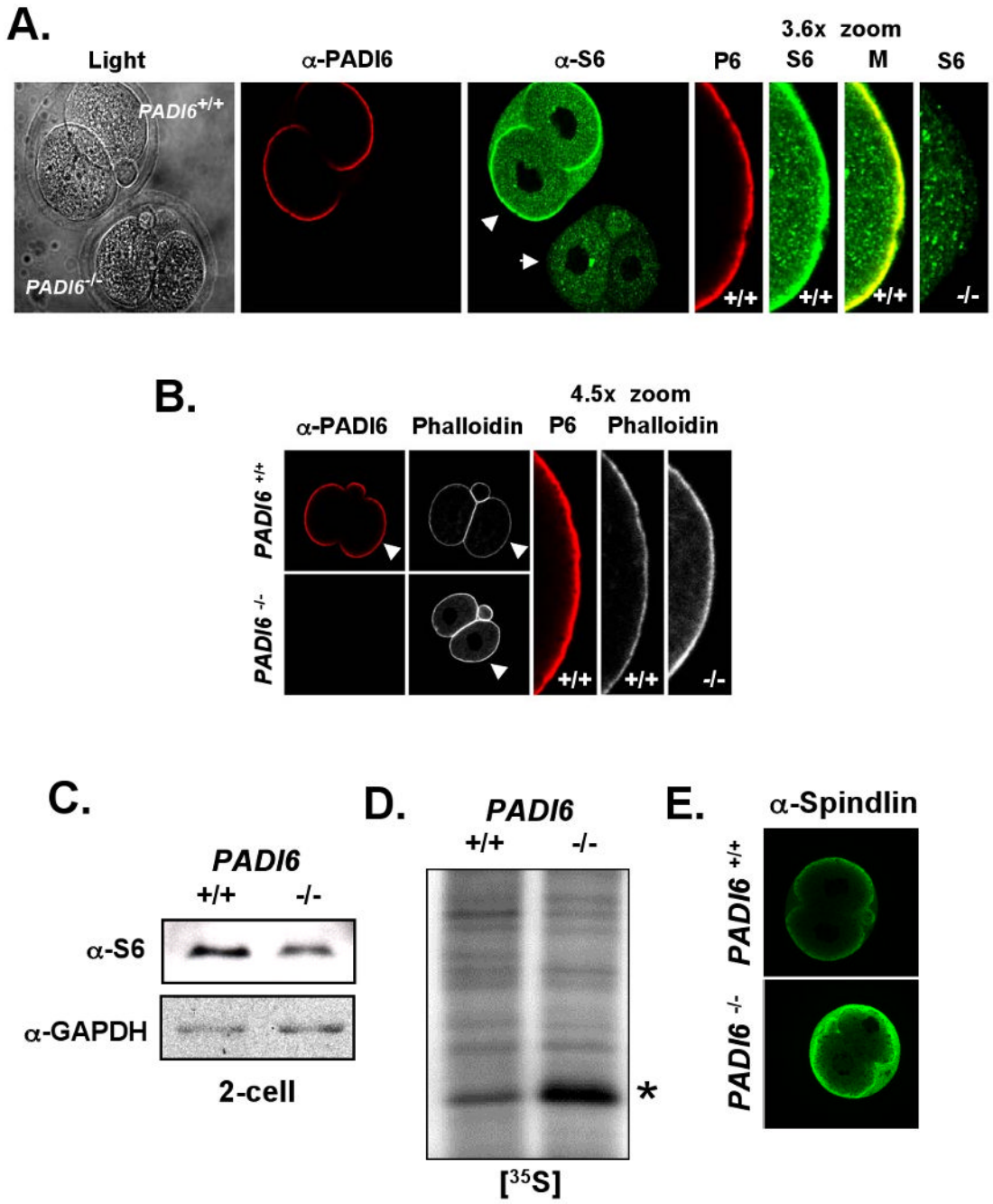


Figure 3. Loss of PADI6 alters the levels and localization of ribosomal protein S6 and affects protein synthesis in two-cell embryos

(A) PADI6 (P6) colocalizes with ribosomal protein S6 in Triton X-100 extracted GV stage *PADI6*$^{+/+}$ oocytes and S6 localization is altered in *PADI6*$^{-/-}$ oocytes. PADI6 and S6 co-localization in *PADI6*$^{+/+}$ oocytes is highlighted in merged image (M). The degree of co-localization (oval) is shown in the scatter plot. **(B)** Fluorographic analysis of [^{35}S]methionine labeled proteins from *PADI6*$^{+/+}$ and *PADI6*$^{-/-}$ GV stage oocytes. Arrow indicates a protein that, based on its molecular weight (~72 kDa) and absence from *PADI6*$^{-/-}$ oocytes, is likely PADI6. **(C)** PADI6 (P6) colocalizes with S6 at the non-apposed cortical regions of *PADI6*$^{+/+}$ two-cell embryo blastomeres while S6 is absent from the embryonic cortex in *PADI6*$^{-/-}$ two-cell

embryos. **(D)** Western blot analysis of protein extracts indicates that S6 levels are reduced in *PADI6*$^{-/-}$ two-cell embryos. **(E)** Fluorographic analysis of [^{35}S]methionine labeled proteins indicates that specific mRNAs are translated at different efficiencies in *PADI6*$^{-/-}$ two-cell embryos. Asterisk indicates putative spindlin protein. **(F)** Confocal immunofluorescence analysis confirms that spindlin expression is upregulated in *PADI6*$^{-/-}$ two-cell embryos.



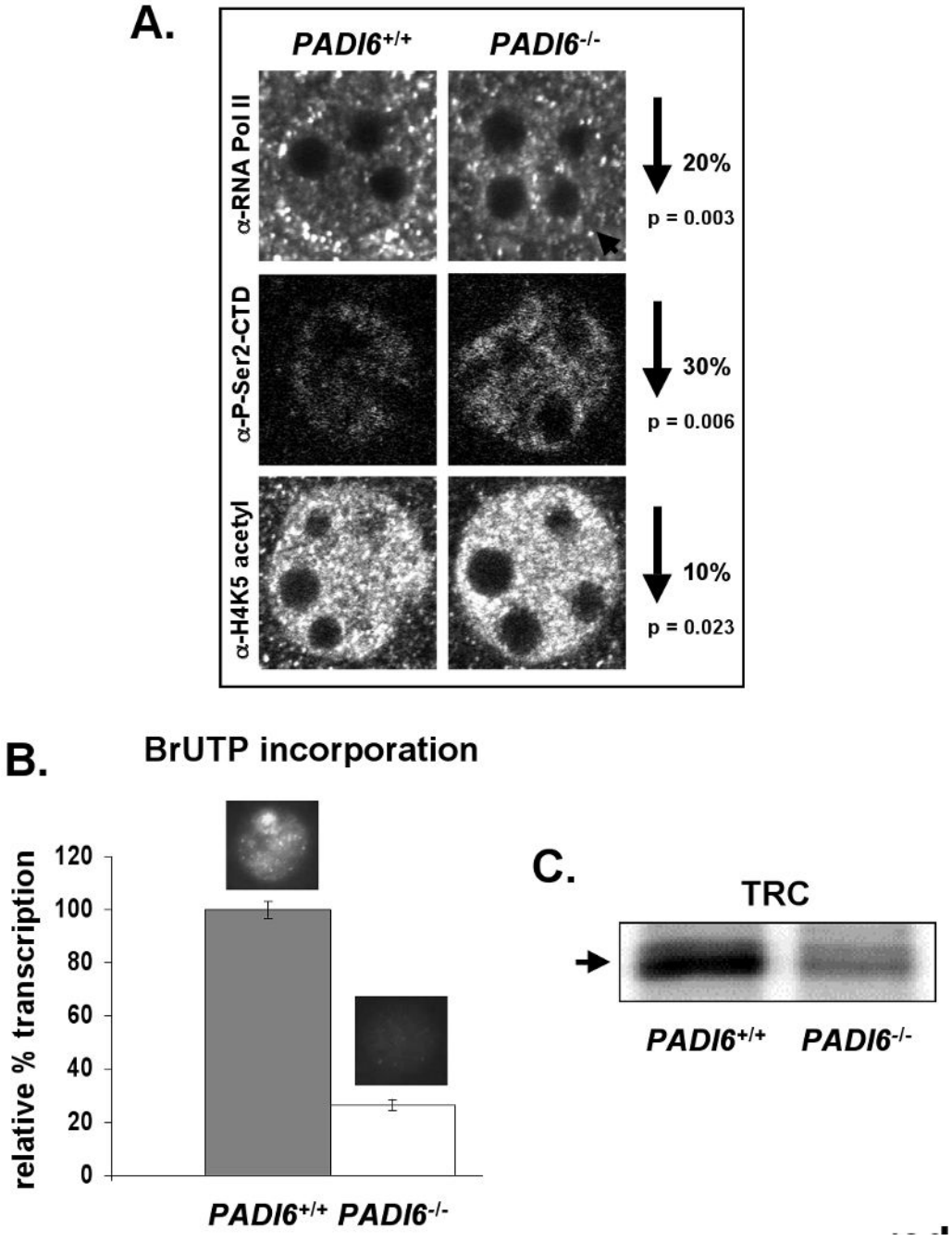


Figure 4. Embryonic genome activation is defective in *PADI6*$^{-/-}$ two-cell embryos

(A) Levels of RNA polymerase II (RNA Pol II), phosphorylated RNA Pol II (P-Ser2-CTD), and acetylated histone H4 (H4K5 acetyl) are significantly reduced in *PADI6*$^{-/-}$ two-cell embryos. Representative nuclei were chosen based on their average signal intensity matching the respective overall average intensity for *PADI6*$^{+/+}$ and *PADI6*$^{-/-}$ embryo nuclei. Arrow indicates accumulation of RNA Pol II outside of the nuclear envelope in *PADI6*$^{-/-}$ two-cell embryos **(B)** BrUTP incorporation into the nucleus of *PADI6*$^{-/-}$ two-cell embryos is significantly reduced. Representative nuclei are shown above bars in histogram. **(C)** Fluorographic analysis of [^{35}S]methionine labeled and Triton X-100 extracted two-cell

embryos indicates that synthesis of Transcription Requiring Complex (TRC) proteins is reduced in *PADI6*$^{-/-}$ embryos.