

Embryonic Poly(A)-Binding Protein Is Required During Early Stages of Mouse Oocyte Development for Chromatin Organization, Transcriptional Silencing, and Meiotic Competence¹

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ABSTRACT

During oocyte maturation, fertilization, and early embryo development until zygotic genome activation (ZGA), transcription is suppressed, and gene expression is dependent upon the timely activation of stored mRNAs. Embryonic poly(A)-binding protein (EPAB) is the predominant poly(A)-binding protein in *Xenopus*, mouse, and human oocytes and early embryos and is important for regulating translational activation of maternally stored mRNAs. EPAB is critical for early development because *Epab*^{-/-} female mice do not produce mature eggs and are infertile. In this study, we further characterize morphological and molecular aspects of *Epab*^{-/-} oocytes. We demonstrated that *Epab*^{-/-} oocytes are smaller in size, contain peripheral germinal vesicles, and are loosely associated with cumulus cells. The chromatin reorganization of the surrounded nucleolus (SN) configuration and transcriptional silencing that normally occurs during oocyte growth does not occur in *Epab*^{-/-} oocytes. Interestingly, microinjection of *Epab* mRNA into *Epab*^{-/-} preantral follicle-enclosed oocytes rescues reorganization of chromatin and oocyte maturation to metaphase II. Overall, these results demonstrate an important role for EPAB during oocyte growth and the acquisition of meiotic competence.

EPAB, meiotic competence, oocyte, translational regulation

INTRODUCTION

In mammals, oocytes enter meiosis during embryonic development and become arrested at prophase of the first meiotic division [1, 2]. Following attainment of reproductive maturity, follicle-enclosed oocytes are selectively recruited to grow in response to the pituitary gonadotropin follicle-stimulating hormone (FSH). Follicular growth is characterized by proliferation of granulosa cells and formation of an antral

cavity, as well as oocyte growth and acquisition of meiotic competence. The antrum separates the granulosa cells into 2 functionally distinct compartments: the outer mural granulosa cells that line the basal lamina and the inner cumulus cells that directly surround the oocyte [3]. In response to a preovulatory surge of luteinizing hormone (LH), the oocyte resumes meiosis, and the cumulus and granulosa cells undergo terminal differentiation [4, 5]. These events are coordinated such that a cumulus-surrounded oocyte at metaphase II (MII) is ovulated at the appropriate stage to be fertilized.

Fully grown oocytes become transcriptionally silent prior to meiotic resumption. Transcription remains suppressed during oocyte maturation, fertilization, and early cleavage divisions, until zygotic genome activation (ZGA) [6, 7], which occurs at the 2-cell stage in mice and 4- to 8-cell stage in humans [8–10]. As transcriptional activity is suppressed during this critical stage of development, gene expression is regulated by the timely activation of maternally derived mRNAs synthesized in advance and stored in the oocyte during the first meiotic arrest. These mRNAs are uniquely stable, with a half-life of 8 to 12 days [11, 12], and upon the appropriate stimulus, will drive re-entry into meiosis and control the rate of mitotic cell divisions during cleavage of the early embryo [7, 13].

A main mechanism by which translation is regulated in oocytes is by cytoplasmic polyadenylation [14], whereby maternally derived mRNAs are translationally activated through elongation of their poly(A) tail in the cytoplasm. Although this process has been better delineated in other organisms, conserved sequences and proteins have been identified in mouse oocytes (reviewed in [15]). The best understood pathway involves cytoplasmic polyadenylation element-binding protein 1 (CPEB1), which promotes cytoplasmic lengthening of poly(A) tails on mRNAs that contain cytoplasmic polyadenylation element (CPE) motifs [16, 17]. CPEB1 acts with other regulatory proteins such as symplekin (SYMPK), cytoplasmic polyadenylation specificity factor (CPSF), and GLD2, an atypical poly(A) polymerase [17–19], to regulate poly(A) tail length and thus translation. The process of translational activation during oocyte maturation is complex [20] and additional pathways independent of cytoplasmic polyadenylation, such as those involving deleted in azoospermia-like (DAZL) [21–23], have also been identified. Indeed, many mRNAs contain multiple regulatory elements that facilitate intricate patterns of activation [24].

Another class of highly conserved proteins involved in translational regulation are poly(A)-binding proteins (PABPs), which bind to the poly(A) tail and bring the translational machinery together in a “closed loop” configuration [25, 26]. *Xenopus*, mouse, and human oocytes, and early embryonic cells express an embryo-specific poly(A)-binding protein (EPAB) as the predominant PABP that is then replaced by

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somatic poly(A)-binding protein cytoplasmic 1 (PABPC1) after ZGA [27–30]. In *Xenopus*, EPAB is involved in both polyadenylation-dependent and -independent pathways that temporally regulate translational activation of repressed mRNAs upon stimulation of oocyte maturation [31]. Importantly, EPAB prevents deadenylation of mRNAs [29], promotes cytoplasmic polyadenylation [32], enhances translation initiation [30], and is required for maturation of *Xenopus* oocytes [32].

In a previous study, we demonstrated that EPAB is required for female fertility in mice [33]. EPAB-deficient mice are infertile and do not generate mature eggs or embryos in vivo or in vitro. *Epab*^{-/-} oocytes fail to achieve translational activation of maternally stored mRNAs upon stimulation of oocyte maturation, including *CyclinB1* and *Dazl* mRNAs. In addition, late antral follicles in the ovaries of *Epab*^{-/-} mice exhibit impaired cumulus expansion and an 8-fold decrease in ovulation associated with a significant down-regulation of mRNAs encoding EGF-like growth factors and their downstream regulators [33].

Because protein synthesis is not required for germinal vesicle (GV) breakdown in mice [34, 35], it is possible that EPAB is required early during oogenesis for expression of mRNAs that support oocyte growth and acquisition of meiotic competence. In support of this, microinjection of *Epab* mRNA into denuded GV-stage *Epab*^{-/-} oocytes does not rescue oocyte maturation [33]. The goal of the current study was to further delineate the role of EPAB during oogenesis. Our findings collectively suggest that EPAB is a global regulator of gene expression in the oocyte and is required during oocyte development for factors that promote nuclear maturation and meiotic competence.

MATERIALS AND METHODS

Collection of Ovaries, Oocytes, and Follicle-Enclosed Oocytes

Mice were bred and maintained according to Yale University animal research requirements, and all procedures were approved by the Institutional Animal Care and Use Committee (protocol 2011-11207).

To collect GV-stage oocytes, ovaries were obtained from 12-week-old wild-type *Epab*^{+/+} C57BL/6 and *Epab*^{-/-} mice [33] 44 to 48 h after intraperitoneal injection of 5 IU of equine chorionic gonadotropin (eCG; Sigma, St. Louis, MO). Antral follicles were selectively punctured, and GV-stage oocytes were collected in α -minimum essential medium (MEM α ; Life Technologies, Grand Island, NY) supplemented with 20 mM Hepes, 75 μ g/ml penicillin G (Sigma), 50 μ g/ml streptomycin sulfate (Sigma), 0.1% polyvinyl alcohol (PVA; Sigma), and 10 μ M milrinone (Sigma) to prevent meiotic resumption. Prior to removal of the surrounding cumulus cells, oocytes were scored for the presence of cumulus cells as fully enclosed, partially enclosed, or denuded. For overnight culture, oocytes were transferred to MEM α supplemented with 25 mM NaHCO₃, 75 μ g/ml penicillin G, 50 μ g/ml streptomycin sulfate, 5% fetal bovine serum (FBS; product no. 12000-022; Life Technologies), and 10 μ M milrinone and incubated in a humidified atmosphere at 37°C with 5% CO₂ and 95% air. To examine in vitro maturation, 10 μ M milrinone was excluded from the medium, and oocytes were assessed after 18 hours of culture for GV breakdown (GVBD) and progression to MII.

Preantral follicles (~120–140 μ m in diameter) were dissected from ovaries of unprimed 23- to 29-day-old wild-type and *Epab*^{-/-} mice. Ovaries were removed from the bursa, and follicles were gently teased away from the ovary, using a forceps and a needle [36]. The culture medium for follicle collection and culture was MEM α supplemented with 25 mM NaHCO₃, 75 μ g/ml penicillin, 50 μ g/ml streptomycin sulfate, 20% FBS, 50 μ g/ml insulin, 5 μ g/ml transferrin, 5 ng/ml selenium (ITS; Sigma), and 10 ng/ml ovine FSH (National Hormone and Peptide Program, Torrance, CA).

Measurement of Oocyte Size and GV Position

Oocyte diameter was measured using transmitted images of freshly isolated, denuded oocytes obtained by confocal microscopy. Excluding the zona

pellucida, the vertical, horizontal, and diagonal diameters were measured for each oocyte by using Image J software (National Institutes of Health, Bethesda, MD) after calibration with a scale bar. The diameter of the oocyte was recorded as the average of the 3 measurements. The same images were used to determine the location of the GV. Oocytes were scored as having either central or peripheral GVs by drawing a horizontal and vertical line through the oocyte to designate the center. GVs that crossed the center were scored as central, and GVs located outside the center were scored as peripheral.

Assessment of Chromatin Configuration

GV-stage oocytes were collected from eCG-primed 12-week-old *Epab*^{+/+} and *Epab*^{-/-} mice by selectively puncturing antral follicles. Directly after isolation, oocytes were washed with phosphate-buffered saline (PBS) with 0.1% PVA and fixed with 2% formaldehyde (Sigma) in 100 mM Hepes, 50 mM EGTA, 10 mM MgSO₄, and 0.2% Triton X-100 for 1 h at 37°C. Following fixation, oocytes were incubated in 5 μ M SYTOX Orange (Life Technologies) for 10 min at room temperature, and washed 3 times in PBS/PVA before being imaged on a 510 model confocal microscope (Carl Zeiss Microscopy, Thornwood, NY). Oocytes were observed with a 63 \times 1.2-numerical aperture lens, using excitation at 543 nm and emission at 570 nm. Chromatin was classified as nonsurrounded nucleolus (NSN), surrounded nucleolus (SN), or partially surrounded nucleolus (PSN).

Assessment of Transcriptional Activity

GV-stage oocytes were isolated from 12-week-old eCG-primed *Epab*^{+/+} and *Epab*^{-/-} mice by selectively puncturing antral follicles, and transcriptional activity was determined by incorporating 5-bromouridine (BrUrd) into nascent RNA transcripts [37]. Oocytes were microinjected with 10 μ l of 5-bromouridine 5' triphosphate sodium salt (Br-UTP) (Sigma; 100 mM in 2 mM PIPES buffer [pH 7.4] containing 140 mM KCl), cultured for 45 min at 37°C and fixed in 4% formaldehyde (Sigma) in PBS/PVA for 30 min at 37°C. Following fixation, oocytes were treated with 0.5% Triton X-100 in PBS/PVA, blocked in 2% bovine serum albumin (BSA), and incubated in primary antibody (anti-BrdU; Sigma) diluted 1:300 in blocking buffer overnight at 4°C. Oocytes were then washed in blocking buffer, incubated in secondary antibody (anti-mouse-488; Life Technologies) diluted 1:200 in blocking buffer for 2 h at room temperature, washed again, and imaged on a 510 model confocal microscope using a 63 \times , 1.2 NA lens with excitation at 488 nm and emission at 530 nm. Oocytes were scored as transcriptionally active based on positive Br-UTP staining in the GV.

Preparation and Microinjection of *Epab* mRNA into Preantral Follicle-Enclosed Oocytes

pCR2.1-mEpab-HA vector was prepared as previously described [33]. To generate an empty pCR2.1-GFP vector, green fluorescent protein (GFP) was amplified from the pCMV6-GFP vector (Origene, Rockville, MD) using the primers: 5' GGGAAATTCGAAATGGA-GAGCGACGAGAGC 3' and 5' GGGAAAAGCTTCTCGAGTTAAACTCTTCTCCACCG-GC 3'. The PCR product was then cloned into the pCR2.1 vector by using a TOPO-TA cloning kit (Invitrogen). Insert direction and sequences were confirmed by sequencing with M13 forward and reverse primers. The pCR2.1-mEpab-HA and pCR2.1-GFP vectors were linearized with *Hind*III and used as a template for in vitro transcription using mMMESSAGE mMACHINE T7 kit (Ambion, Austin, TX). Following in vitro transcription, mRNAs were polyadenylated using a poly(A) tailing kit (Ambion) and stored at -80°C in nuclease-free water until microinjection.

Microinjection of oocytes and follicle-enclosed oocytes was carried out as described previously [36, 38]. Follicles were loaded into an injection chamber between 2 coverslips spaced ~100 μ m apart. Quantitative microinjection was performed using pipettes backfilled with mercury, and concentrations of injected substances were calculated based on an oocyte volume of 200 μ l [38]. mRNA was injected in a total volume of 10 μ l. Injected follicle-enclosed oocytes were plated on collagen type I culture inserts (Becton Dickinson Biosciences, San Jose, CA) and cultured in a humidified atmosphere at 37°C with 5% CO₂ and 95% air. Following 9 to 11 days of culture, oocytes were removed from the follicle and assessed either immediately or following 17 to 18 h of in vitro maturation.

Determination of Metaphase II Arrest by Spindle Immunofluorescence

Following in vitro maturation, oocytes were fixed in 2% formaldehyde (in buffer containing 100 mM Hepes, 50 mM EGTA, 10 mM MgSO₄, and 0.2%

Triton X-100) for 1 h at 37°C, washed 3 times in PBS/PVA with 0.01% Triton X-100, incubated in blocking buffer (PBS containing 0.01% Triton X-100, 0.1% PVA, and 3% BSA) for 30 min at room temperature and then in anti-tubulin primary antibody (AbD Serotec, Raleigh, NC) diluted 1:100 in blocking buffer overnight at 4°C. Oocytes were then washed 3 times in blocking buffer and incubated in secondary antibody (anti-RAT 488; Invitrogen) diluted 1:200 in blocking buffer for 1 h at room temperature. Finally, oocytes were washed 4 times with PBS/PVA, with 5 μ M SYTOX Orange in the first wash. Oocytes were examined using a 510 confocal microscope, with 40 \times 1.2 NA lens using excitation at 488 nm and emission at 530 nm (tubulin) and excitation at 543 nm and emission at 570 nm (Sytox).

Statistical Analysis

Data are representative of at least 3 independent experiments, unless otherwise specified. Values were analyzed either by Student *t*-test, 1-way ANOVA, or 2-way ANOVA, as described in each figure legend. Percentages of data were transformed via arcsin square root prior to statistical analysis [39]. All statistical analyses were performed using Prism software (Graph Pad, San Diego, CA) and significance was assessed at a *P* value of ≤ 0.05 .

RESULTS

Epab^{-/-} Oocytes Exhibit Morphological Abnormalities

Because *Epab*^{-/-} oocytes are incapable of progressing to MII, we examined morphological characteristics associated with meiotic competence such as the association with cumulus cells, oocyte size, and GV location [40–44]. Due to the increase in secondary follicle formation in *Epab*^{-/-} ovaries [33], antral follicle-enclosed oocytes were selectively punctured to include only this population of oocytes. Approximately 50% of *Epab*^{-/-} oocytes released from antral follicles were completely denuded, and only ~25% were fully enclosed by cumulus cells (Fig. 1). This is in contrast to *Epab*^{+/+}, where 14% were denuded and 62% were fully enclosed. Percentages of partially enclosed oocytes for *Epab*^{+/+} were similar to that for *Epab*^{-/-} oocytes (Fig. 1B). Oocytes were then stripped of cumulus cells and evaluated for size and GV location (Fig. 2A). *Epab*^{-/-} oocytes were slightly but significantly smaller in size than *Epab*^{+/+} oocytes (69.7 μ m vs. 71.5 μ m, respectively, *P* < 0.0001) (Fig. 2, B and C). In addition, 89% of *Epab*^{-/-} oocytes contained peripheral GVs (Fig. 2D) compared to only 11% of *Epab*^{+/+} oocytes. Overall, *Epab*^{-/-} oocytes isolated from antral follicles exhibit morphological abnormalities such as peripheral GV location and fewer connections with cumulus cells. The small decrease in size of *Epab*^{-/-} oocytes may not be meaningful in terms of oocyte development because the oocytes grew beyond 60 μ m [44].

Epab^{-/-} Oocytes Fail to Progress to the SN-Type Chromatin Configuration and Become Transcriptionally Silent

Chromatin configuration and transcriptional activity are often used as markers of meiotic and developmental competence as well as ovulation rates [45–47], all of which are abnormal in *Epab*^{-/-} mice [33]. Chromatin configuration and transcriptional activity also correlate with oocyte size and GV location [37, 48, 49]. Oocytes initially contain uncondensed chromatin (NSN) that is permissive to transcriptional activity. However, as a follicle-enclosed oocyte grows, the DNA condenses and forms a tight ring around the nucleolus (SN). This transition from NSN to SN is thought to occur around the time of antrum formation and precedes oocyte maturation [46, 49, 50]. Generally, smaller oocytes with peripheral GVs are isolated at a more immature follicular stage and thus have NSN-type chromatin. Larger oocytes with central GVs are isolated from large antral follicles and have SN-type chromatin. Additionally, the somatic cells surrounding the oocyte are

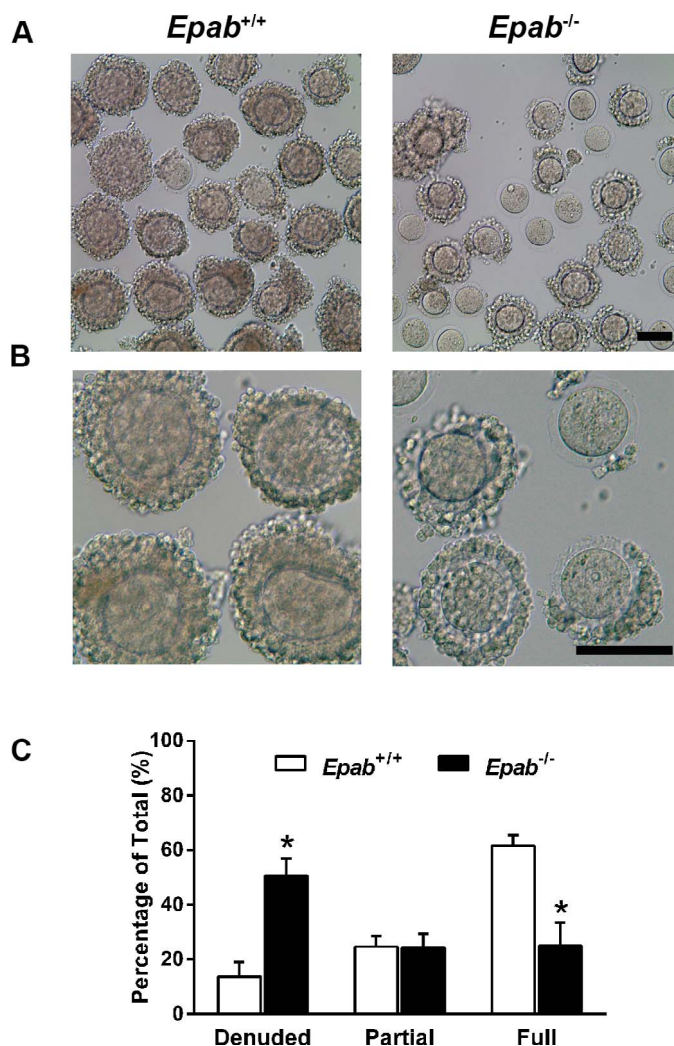


FIG. 1. *Epab*^{-/-} oocytes isolated from antral follicles have fewer connections with cumulus cells. **A**) Representative images of *Epab*^{+/+} and *Epab*^{-/-} cumulus-oocyte complexes directly after antral follicles were punctured. Bar = 100 μ m. **B**) Higher 3 \times magnification of images shown in **A**. Bar = 100 μ m. **C**) Oocytes were scored as either denuded (lacking cumulus cells), partial (partially enclosed), or full (fully enclosed). Results are means \pm SEM. *Significant difference compared to *Epab*^{+/+}, as determined by Student *t*-test, *P* ≤ 0.05 . Four *Epab*^{+/+} and 3 *Epab*^{-/-} mice from 2 litters were evaluated, and a total of at least 75 oocytes were scored for each morphological characteristic.

important for modulating chromatin reorganization and transcriptional silencing [51].

Chromatin configurations were examined in oocytes isolated from antral follicles of *Epab*^{+/+} and *Epab*^{-/-} ovaries by fixing and staining DNA with SYTOX Orange. Approximately 55% of *Epab*^{-/-} oocytes contained uncondensed NSN chromatin, and only 25% of *Epab*^{-/-} oocytes progressed to the SN configuration. In contrast, 25% of *Epab*^{+/+} oocytes contained NSN chromatin, and 75% contained SN chromatin. The number and morphology of the heterochromatin spots in *Epab*^{+/+} and *Epab*^{-/-} NSN oocytes were similar (data not shown). Interestingly, 19% of *Epab*^{-/-} oocytes exhibited chromatin that did not completely surround the nucleolus, which we termed PSN (Fig. 3, A and B). The PSN chromatin configuration was not observed in *Epab*^{+/+} oocytes.

It has previously been shown that the SN configuration begins to form when oocytes reach ~50 μ m and the percentage of SN oocytes increases significantly when antral follicles

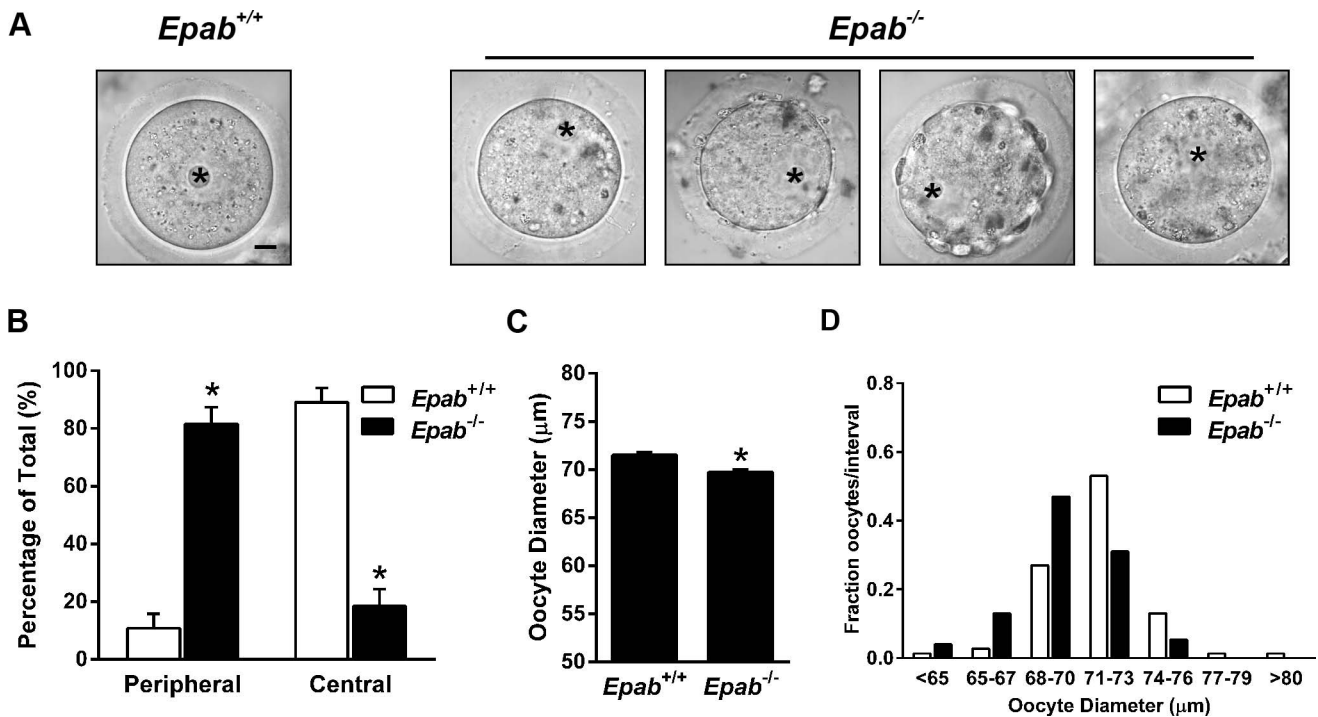


FIG. 2. Morphological characteristics of *Epab*^{-/-} oocytes. Oocytes collected from eCG-primed *Epab*^{+/+} and *Epab*^{-/-} mice were characterized based on location of the GV and oocyte size. **A**) Representative transmitted light confocal images of *Epab*^{+/+} and *Epab*^{-/-} oocytes. Bar = 10 µm. *GVs. **B**) Location of the GV was scored by confocal microscopy as either peripheral or central. Results are means ± SEM. *Significant differences compared to *Epab*^{+/+} as determined by Student *t*-test, *P* ≤ 0.01. **C**) Oocyte size was determined using Image J analysis after calibration with a scale bar. Results are means ± SEM. *Significant differences compared to *Epab*^{+/+} as determined by Student *t*-test, *P* ≤ 0.0001. **D**) Distribution of *Epab*^{+/+} and *Epab*^{-/-} oocyte sizes in 3-µm intervals. Four *Epab*^{+/+} and 3 *Epab*^{-/-} mice from 2 litters were evaluated, and a total of at least 75 oocytes were scored for each morphological characteristic.

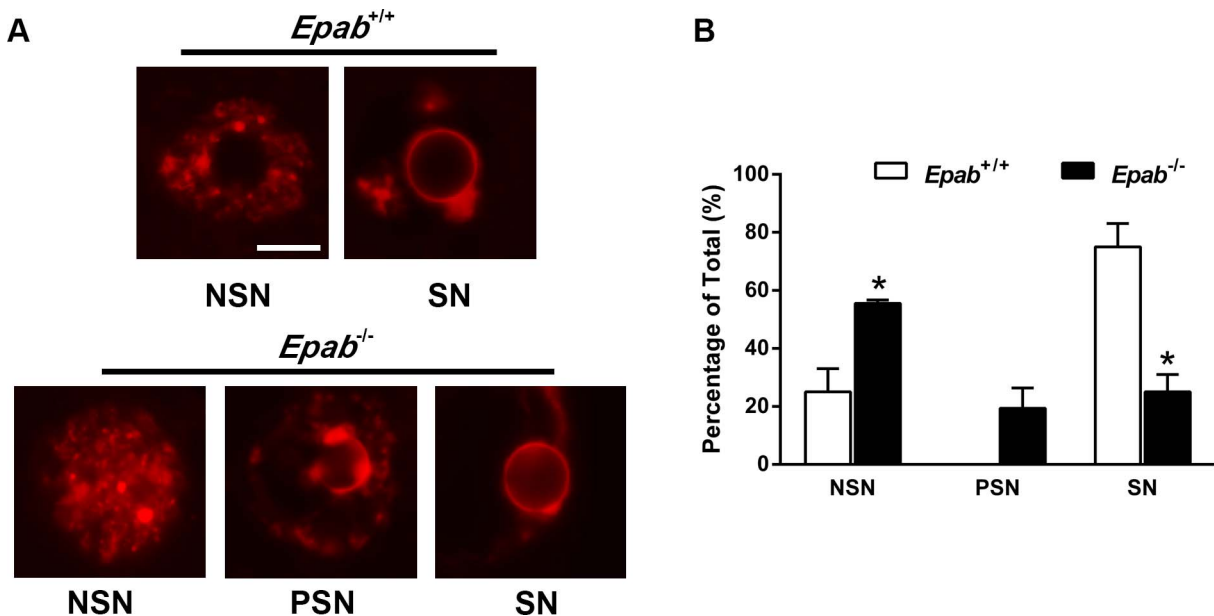


FIG. 3. *Epab*^{-/-} oocytes did not progress to the SN-type chromatin configuration. Oocytes collected from eCG-primed mice were fixed and stained with SYTOX Orange. Chromatin configuration was evaluated by confocal microscopy and classified as nonsurrounded (NSN), surrounded (SN), or partially surrounded (PSN). **A**) Representative images of the chromatin configuration in oocytes isolated from *Epab*^{+/+} and *Epab*^{-/-} mice. Bar = 10 µm. **B**) Percentages of *Epab*^{+/+} oocytes (n = 98) and *Epab*^{-/-} oocytes (n = 84) exhibiting the different chromatin configurations are shown. Results are means ± SEM. *Significant differences compared to *Epab*^{+/+} as determined by Student *t*-test, *P* ≤ 0.05. Four *Epab*^{+/+} and 3 *Epab*^{-/-} mice from 2 litters were evaluated.

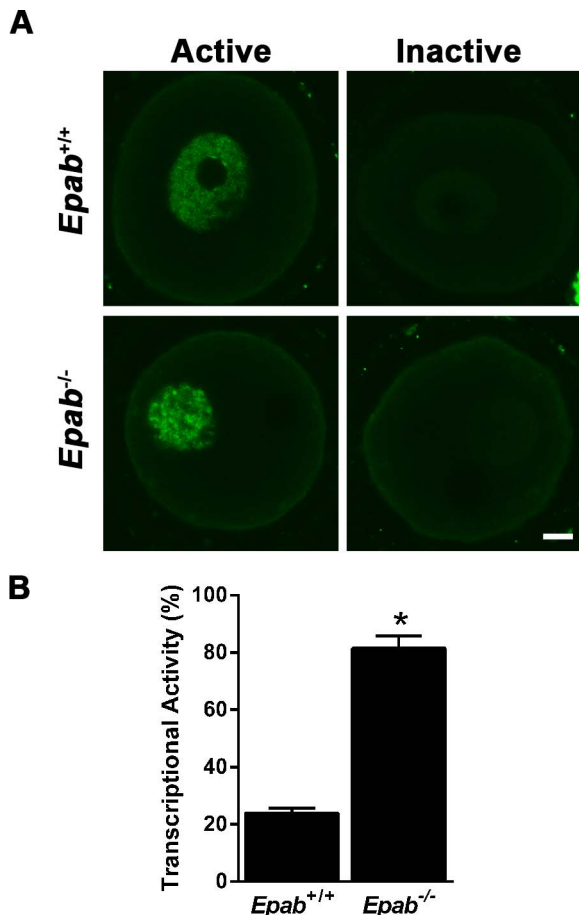


FIG. 4. *Epab*^{-/-} oocytes remain transcriptionally active. Oocytes were collected from eCG-primed *Epab*^{+/+} and *Epab*^{-/-} mice, injected with Br-UTP, and examined for incorporation into nascent mRNA transcripts by immunofluorescence and confocal microscopy. **A**) Representative images show transcriptional activity. Bar = 10 μ m. **B**) Percentages of *Epab*^{+/+} oocytes (n = 46) and *Epab*^{-/-} oocytes (n = 51) were scored as transcriptionally active. Results are means \pm SEM. *Significant differences in transcriptional status between *Epab*^{+/+} and *Epab*^{-/-} oocytes, as determined by Student *t*-test, $P \leq 0.05$. Four *Epab*^{+/+} and 4 *Epab*^{-/-} mice from 4 litters were evaluated, and a total of at least 75 oocytes were scored.

develop and oocytes reach ~ 70 μ m [49]. Although *Epab*^{-/-} oocytes were smaller in size, the average size of *Epab*^{-/-} oocytes from antral follicles was 69.7 μ m, and most oocytes were larger than 68 μ m (Fig. 2C). This finding suggests that size alone is most likely not responsible for the fewer SN oocytes in *Epab*^{-/-} ovaries. Rather, the finding that *Epab*^{-/-} oocytes maintain fewer connections with cumulus cells may be a contributing factor [51]. Overall, even in the presence of antrum formation, most *Epab*^{-/-} oocytes fail to reach the SN configuration.

Global transcriptional silencing occurs concomitantly with changes in chromatin structure. Oocytes with SN-type chromatin configuration are generally transcriptionally silent, whereas oocytes with the NSN-type chromatin configuration are generally transcriptionally active [45]. Transcriptional activity was detected by injecting oocytes with Br-UTP and examining the incorporation into nascent mRNAs by immunofluorescence and confocal microscopy (Fig. 4A). Consistent with previous reports, $\sim 24\%$ of *Epab*^{+/+} oocytes remained transcriptionally active, whereas most underwent transcriptional silencing. In contrast, $\sim 82\%$ of *Epab*^{-/-} oocytes remained

transcriptionally active and failed to undergo transcriptional silencing (Fig. 4B). Overall, the number of oocytes that exhibited NSN chromatin and transcriptional activity was significantly higher in *Epab*^{-/-} mice than in *Epab*^{+/+} mice.

Microinjection of *Epab* mRNA into *Epab*^{-/-} Follicle-Enclosed Oocytes at Preantral Stage Rescues Follicle Growth, Chromatin Reorganization, and Oocyte Maturation

Previously, we showed that microinjection of *Epab* mRNA into denuded GV-stage oocytes from *Epab*^{-/-} mice failed to rescue in vitro maturation [33], suggesting that EPAB is required at earlier steps during oogenesis. To determine the stage of oocyte development at which EPAB becomes necessary, we injected *Epab* mRNA into preantral follicle-enclosed oocytes (FEOs) isolated from *Epab*^{-/-} mice. Oocytes within follicles at the preantral stage have not completed growth and are meiotically incompetent. Therefore, after microinjection, the follicle-enclosed oocytes were cultured on a collagen membrane for 9 to 11 days in order to promote oocyte and follicle growth. Uninjected *Epab*^{-/-} follicles did not grow beyond 230 μ m during the culture period, and only 38% resumed meiosis when isolated from the follicle (Fig. 5, A and C). Maturation did not proceed normally in *Epab*^{-/-} oocytes because none of the oocytes that resumed meiosis formed morphologically normal spindles (Fig. 5, B and C). In contrast, uninjected *Epab*^{+/+} follicles and *Epab*^{-/-} follicles that were injected with *Epab* mRNA grew larger than 320 μ m (Fig. 5A). A majority of these oocytes underwent GVBD following isolation from the follicle (97% and 77%, respectively) and formed morphologically normal spindles (84% and 75%, respectively) (Fig. 5, B and C). Injection of *Gfp* mRNA into follicle-enclosed *Epab*^{-/-} oocytes served as a negative control; 29% of these oocytes underwent GVBD but did not progress normally to MII (Fig. 5, A–C).

In addition to oocyte maturation, we also examined whether microinjection of *Epab* mRNA into preantral follicle-enclosed *Epab*^{-/-} oocytes could restore the progression from NSN- to SN-type chromatin configuration. Following the culture period, oocytes were isolated from the follicles, fixed, and stained with SYTOX Orange. The chromatin configurations for *Epab*^{+/+} and *Epab*^{-/-} follicle-enclosed oocytes grown in vitro were similar to that of our previous results where the oocytes were freshly isolated from antral follicles. A majority of *Epab*^{+/+} oocytes (91%) contained SN-type chromatin, compared to only 33% of *Epab*^{-/-} oocytes. Injection of *Epab* mRNA into *Epab*^{-/-} follicle-enclosed oocytes significantly increased the percentage of oocytes that transitioned to the SN configuration (84%) (Fig. 6). We also measured the sizes of uninjected *Epab*^{+/+} and *Epab*^{-/-} oocytes and those injected with *Epab* mRNA after the culture period. The average size for uninjected *Epab*^{-/-} oocytes was ~ 67 μ m, whereas *Epab*^{-/-} oocytes injected with *Epab* mRNA grew to ~ 75 μ m, which was similar to the average size of 76 μ m for *Epab*^{+/+} oocytes (data not shown). Thus, injection of *Epab* mRNA into *Epab*^{-/-} preantral follicle-enclosed oocytes restores follicle and oocyte growth, as well as the progression from NSN to SN-type chromatin configuration.

DISCUSSION

In the present study, we demonstrated that EPAB is essential during early stages of oogenesis for acquisition of meiotic competence. *Epab*^{-/-} oocytes are smaller, with peripheral GVs, and have fewer connections with cumulus cells. *Epab*^{-/-}

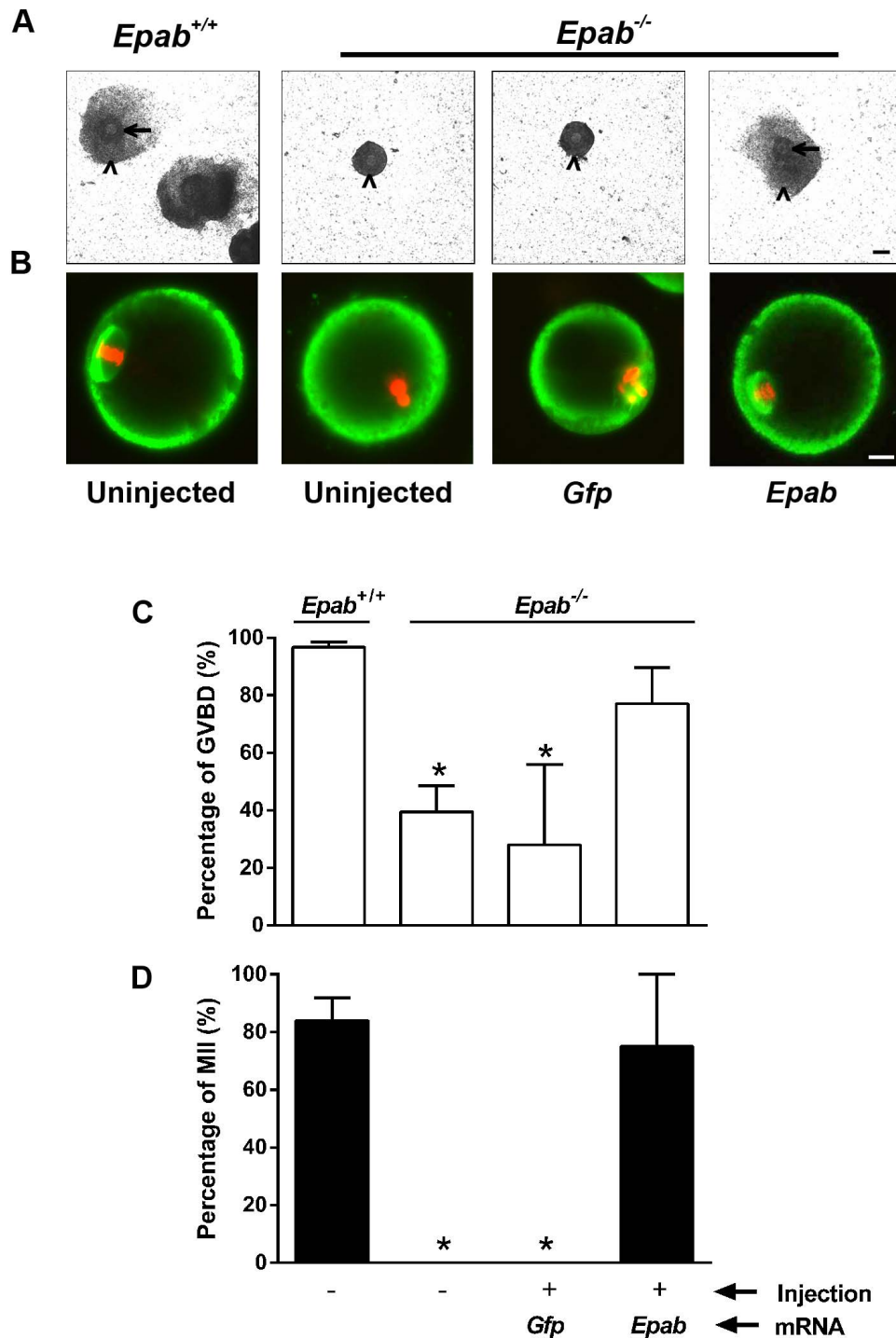


FIG. 5. Microinjection of *Epab* mRNA into *Epab*^{-/-} preantral follicle-enclosed oocytes rescued oocyte maturation. Preantral follicle-enclosed oocytes were injected with *Epab* or *Gfp* mRNA and cultured on a collagen membrane for 9 to 11 days. After the culture period, oocytes were evaluated for GVBD and MII spindle formation. **A**) Representative images of follicles are shown after the culture period. Bar = 100 μ m. Arrowhead indicates granulosa cells, and arrow indicates cumulus-enclosed oocyte complexes within the follicle. **B**) Representative images of spindle immunofluorescence shown after overnight maturation. Tubulin is shown in green, and DNA is shown in red. Bar = 10 μ m. **C** and **D**) Percentage of oocytes that underwent GVBD (**C**) and matured normally to metaphase II (**D**) is shown. Results are means \pm SEM. *Significantly lower number of *Epab*^{-/-} oocytes underwent GVBD or formed normal MII spindles than *Epab*^{+/+} oocytes ($P \leq 0.01$). Significance was determined by one-way ANOVA, Bonferroni's multiple comparisons test ($n = 70$ for *Epab*^{+/+}; $n = 27$ for uninjected *Epab*^{-/-}; $n = 10$ for *Gfp* injected *Epab*^{-/-}; and $n = 18$ for *Epab*-injected *Epab*^{-/-} FEOs).

oocytes also fail to progress to SN-type chromatin configuration and become transcriptionally silent. The requirement of EPAB during oocyte growth is further supported by the finding that chromatin configuration and oocyte maturation can be rescued when *Epab* mRNA is introduced into follicle-enclosed

Epab^{-/-} oocytes at the preantral stage. Overall, these results demonstrate that EPAB is required not only during oocyte maturation for translational activation of maternal mRNAs, but also during oocyte development for establishing factors that regulate nuclear maturation and meiotic competence.

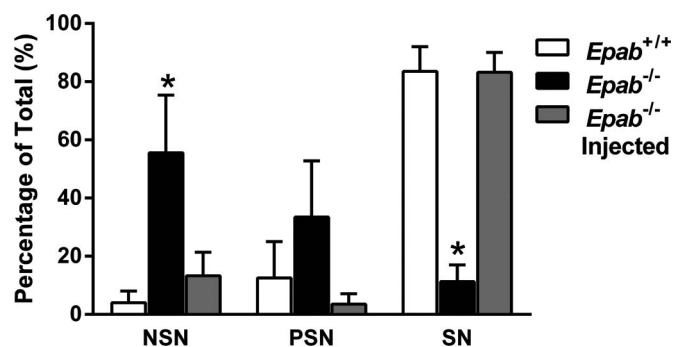


FIG. 6. Microinjection of *Epab* mRNA into *Epab*^{-/-} preantral follicle-enclosed oocytes rescued progression to SN-type chromatin. Preantral follicles were dissected from *Epab*^{+/+} and *Epab*^{-/-} ovaries, and *Epab*^{-/-} FEOs were injected with *Epab* mRNA. After 9 to 11 days of culture on a collagen membrane, oocytes were isolated from the follicles, fixed, and stained with SYTOX Orange for assessment of chromatin configuration. Graph represents the percentage of oocytes that contained NSN, PSN, or SN-type chromatin for uninjected *Epab*^{+/+} (n = 23), uninjected *Epab*^{-/-} (n = 27), and injected *Epab*^{-/-} FEOs (n = 19). Data are means \pm SEM. *Statistically significant differences compared to *Epab*^{+/+} as determined by two-way ANOVA, multiple comparisons test, $P \leq 0.05$.

The oocyte nucleus, or GV, contains a unique chromatin configuration that is subject to dynamic modifications during oogenesis. Initially, oocytes contain uncondensed NSN-type chromatin that is dispersed throughout the GV. Around the time of antrum formation, the chromatin condenses and reorganizes into the SN-type configuration that surrounds the nucleolus [46, 49, 50, 52]. Chromatin configuration is associated with transcriptional activity such that oocytes with NSN chromatin have increased levels of transcriptional activity, whereas oocytes with SN chromatin exhibit repressed transcription (reviewed in [45]). Although the NSN-to-SN transition occurs concomitantly with transcriptional silencing, these 2 processes are likely to be regulated by different mechanisms [53]. Importantly, chromatin condensation and the associated decrease in transcriptional activity are often used as markers of meiotic and developmental competence. Although both NSN and SN oocytes can mature, they do so with different rates and characteristics [50]. Studies have shown that a higher percentage of oocytes with the SN configuration undergo GVBD and progress to MII compared to oocytes with the NSN configuration. More striking is the association between chromatin configuration and developmental competence because NSN oocytes are incapable of developing past the 2-cell stage in vitro [54, 55]. The timing of transcriptional silencing also appears to be important. If the period between transcriptional repression and maturation is experimentally extended, cleavage rates and blastocyst formation are significantly reduced [51]. Despite the association of chromatin configuration and transcriptional activity with early development, these processes alone do not determine competence [54]. Rather, changes in nuclear and cytoplasmic factors that occur simultaneously are more likely to be important [56]. It remains to be determined what these factors are and whether EPAB is directly responsible for regulating their expression.

Interestingly, the surrounding cumulus granulosa cells play an active role in modulating chromatin structure and transcriptional activity in the oocyte and ultimately coordinate nuclear and cytoplasmic meiotic competence [51, 57–59]. Somatic cells also have been shown to regulate translational activation of maternal mRNAs during oocyte maturation [60]. However, the signal(s) originating from the cumulus cells that are important for these processes remain to be identified. Most

oocytes isolated from *Epab*^{-/-} ovaries are either denuded or have fewer connections with cumulus cells. It is possible that the lack of oocyte–granulosa interactions could be a contributing factor associated with meiotic incompetence and failed chromatin reorganization and transcriptional silencing. In addition, this finding suggests that the somatic cells are also affected by EPAB deficiency, and this is consistent with impaired cumulus expansion and ovulation in *Epab*^{-/-} mice. Ultimately, the somatic cell phenotype in *Epab*^{-/-} mice must derive from a problem with the oocyte because EPAB is oocyte-specific. Because bidirectional communication between oocytes and somatic cells is critical for the function of both compartments [43, 61], the lack of EPAB in the oocyte could impair the differentiation or function of somatic cells, which could then lead to further problems in the oocyte. Therefore, miscommunication between the oocyte and somatic compartment could contribute to meiotic incompetence of *Epab*^{-/-} oocytes.

The significance of EPAB in the function of the somatic compartment is currently being investigated. Perhaps the expression of oocyte-derived factors is affected in *Epab*^{-/-} mice or an independent pathway that regulates folliculogenesis is involved. Another possibility is that miscommunication between the oocyte and somatic compartment is physical, such that gap junctions, transzonal processes, or other channels do not function properly. This would disrupt not only the transfer of oocyte-derived factors, but it would also disrupt signals initiated by the somatic cells. Whether the abnormalities we observed in *Epab*^{-/-} oocytes are directly the result of EPAB deficiency in the oocyte or are an indirect secondary effect of an abnormal follicular environment is not known.

Two RNA binding proteins that are also essential for translational regulation during oocyte maturation are CPEB and DAZL. Similar to EPAB, both CPEB and DAZL are not only required for regulation of gene expression during oocyte maturation, but they are also important in the oocyte at earlier stages of gametogenesis. Deletion of CPEB results in germ cells that are arrested at the pachytene stage of prophase I. This arrest is due to loss of synaptonemal complex protein and failure of homologous chromosome pairing [62]. When CPEB is specifically reduced in prophase I oocytes, the poly(A) tail elongation of a number of key mRNAs, including *Gdf9*, *Smad5*, and *Hifoo*, is lost [63]. There is also a decreased number of oocytes and follicles, increased apoptosis, abnormal polar body formation, parthenogenetic cell division, and oocyte detachment from cumulus cells [63]. DAZL has been identified as an intrinsic factor required for germ cell entry into meiosis [64], and deletion of DAZL results in the complete loss of female germ cells before birth [65, 66]. Furthermore, specific knockdown of *Dazl* in prophase I-arrested oocytes followed by in vitro maturation results in lower percentage of oocytes that undergo GVBD and expel a polar body as well as formation of abnormal spindles. DAZL knockdown also completely prevents fertilization, as demonstrated by the absence of 2-cell embryos [21]. Overall, these studies emphasize the importance of RNA binding proteins at several steps during gametogenesis.

Deletion of other RNA binding proteins in the oocyte also produces similar phenotypes associated with chromatin organization and transcriptional activity. For example, MSY2 is an oocyte-specific RNA binding protein that regulates mRNA stability [67–71]. The absence of MSY2 perturbs oocyte growth and maturation, RNA stability, and the transcriptome. MSY2-deficient oocytes also do not undergo transcriptional silencing or progression to SN and are loosely associated with cumulus cells [72]. Another example is

ELAVL2, an AU-rich element binding protein specific to the oocyte that acts as a translational repressor. Interestingly, ELAVL2 is abundant in NSN oocytes but is ablated during progression from NSN to SN. ELAVL2 appears to be important for oocyte growth and meiotic competence because prematurely reduced *Elavl2* expression results in lower yields of fully grown and meiotically matured oocytes. Furthermore, *Elavl2* knockdown promotes translational activity in fully grown oocytes [73]. Thus, several RNA binding proteins in addition to EPAB are important for producing fully grown oocytes that have complete developmental competence, underscoring the importance of additional pathways that regulate gene expression during oocyte development.

In summary, EPAB is important in the oocyte not only for translational activation of maternal mRNAs during maturation but also for acquisition of meiotic competence during oocyte development. It is not known whether the observed phenotypes are a secondary effect of a disrupted follicular environment or a more direct role of EPAB in regulating specific mRNAs in the oocyte. Overall, based on these findings, it is reasonable to propose that EPAB interacts with different complexes to regulate translation at different times during oocyte growth and oocyte maturation. Future studies are aimed toward identifying which mRNAs EPAB binds to and is directly involved in regulating their translation, as well as other regulatory proteins that EPAB associates with in mouse oocytes. In *Xenopus* oocytes, EPAB associates with CPEB and components of the cytoplasmic poly(A) machinery [31], PUM-2 [23] and DAZL [22]. It will be interesting to determine if these interactions are conserved in mouse oocytes and whether EPAB-bound mRNAs can be identified based on CPE motifs and DAZL-binding sites. Such studies will not only provide insight into additional pathways that regulate post-transcriptional gene expression but may also uncover novel mRNAs that are essential during oocyte formation and early embryonic development.

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