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Embryonic Poly(A) Binding Protein (EPAB) is required during early stages of mouse oocyte development for chromatin organization, transcriptional silencing, and meiotic competence[1]

Running title: EPAB is required for meiotic competence
Summary sentence: EPAB is an oocyte-specific translational regulator that is important during oocyte growth for the acquisition of meiotic competence.
Keywords: EPAB, translational regulation, oocyte, meiotic competence

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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 since *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 demonstrate that *Epab*$^{-/-}$ oocytes are smaller in size, contain peripheral GVs, and are loosely associated with cumulus cells. Chromatin reorganization to 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.

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 two 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 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 that are synthesized in advance and stored in the oocyte during the first meiotic arrest. These mRNAs are uniquely stable with a half-life of 8-12 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 CPEB1 (cytoplasmic polyadenylation element-binding protein 1), which promotes the cytoplasmic lengthening of poly(A) tails on mRNAs that contain CPE (cytoplasmic polyadenylation element) motifs [16, 17]. CPEB1 acts with other regulatory proteins such as SYMPK (symplekin), CPSF (cytoplasmic polyadenylation specificity factor) 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 DAZL (deleted in azoospermia-like) [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 the 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 *Cyclin B1* 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 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, germinal vesicle (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 the Yale University animal research requirements, and all procedures were approved by the Institutional Animal Care and Use Committee (protocol number 2011-11207).

To collect GV stage oocytes, ovaries were obtained from 12 week old wild-type *Epab*$^{+/+}$ C57BL/6 and *Epab*$^{-/-}$ mice [33] 44-48 h after intra-peritoneal injection of 5 IU equine chorionic gonadotropin (eCG; Sigma, St. Louis, MO). Antral follicles were selectively punctured and GV stage oocytes were collected in MEMα (Life Technologies, Grand Island, NY) supplemented with 20 mM Hepes, 75 μg/ml penicillin G (Sigma), 50 μg/ml streptomycin sulfate (Sigma), 0.1% Polyvinyl Alcohol (PVA; Sigma), and 10 μM 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 MEMα supplemented with 25 mM $NaHCO_3$, 75 μg/ml penicillin G, 50 μg/ml streptomycin sulfate, 5% fetal bovine serum (FBS; #12000-022, Life Technologies) and 10 μM milrinone and incubated in a humidified atmosphere at 37°C with 5% CO_2 and 95% air. To examine *in vitro* maturation, 10 μM milrinone was excluded from the media and oocytes were assessed after 18 hours of culture for germinal vesicle breakdown (GVBD) and progression to metaphase II (MII).

Preantral follicles (~120-140 μm in diameter) were dissected from the ovaries of unprimed 23- to 29-day old wild-type and *Epab*$^{-/-}$ mice. The ovaries were removed from the bursa and follicles were gently teased away from the ovary using a forceps and a needle [36]. The culture media for follicle collection and culture was MEMα medium supplemented with 25 mM $NaHCO_3$, 75 μg/ml penicillin, 50 μg/ml streptomycin sulfate, 20% fetal bovine serum, 50 μg/ml insulin, 5 μg/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 a confocal microscope. Excluding the zona pellucida, the vertical, horizontal, and diagonal diameters were measured for each oocyte 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 three 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 that were located outside of 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% polyvinyl alcohol (PVA; Sigma) and fixed with 2% formaldehyde (Sigma) in 100 mM Hepes, 50 mM EGTA, 10 mM MgSO4, and 0.2% TritonX-100 for 1 hr at 37◦C. Following fixation, oocytes were incubated in 5 μM SYTOX orange (Life Technologies) for 10 min at room temperature, and washed three times in PBS/PVA before imaging on a Zeiss 510 confocal microscope (Carl Zeiss Microscopy, Thornwood, NY). Oocytes were observed with a 63x, 1.2 NA lens using excitation at 543 nm and emission at 570 nm. Chromatin was classified as non-surrounded nucleolus (NSN), surrounded nucleolus (SN) classification, 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 bromouridine (BrU) into nascent RNA transcripts [37]. Oocytes were microinjected with 10 pl of Br-UTP (Sigma; 100 mM in 2 mM PIPES buffer [pH 7.4] containing 140 mM KCl), cultured for 45 minutes at 37ºC, and fixed in 4% formaldehyde (Sigma) in PBS/PVA for 30 min at 37ºC. Following fixation, the oocytes were treated with 0.5% Triton X-100 in PBS/PVA, blocked in 2% BSA, and incubated in primary antibody (anti-BrdU; Sigma) diluted 1:300 in blocking buffer overnight at 4ºC. Oocytes were then washed in blocking buffer, incubated in secondary antibody (anti-mouse-488, Life technologies) diluted 1:200 in blocking buffer for 2 hrs at room temperature, washed again, and imaged on a Zeiss 510 confocal microscope using a 63x 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, GFP was amplified from the pCMV6-GFP vector (Origene, Rockville, MD) using the following primers: 5' GGGAAATTCGAAATGGA-GAGCGACGAGAGC 3'and 5' GGGAAAAAGCTTCTCGAGTTAAACTCTTTCTTCACCG-GC 3'. The PCR product was then cloned into the pCR2.1 vector 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 HindIII and used as a template for in vitro transcription using mMESSAGE mMACHINE T7 Kit (Ambion). 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 two coverslips spaced ∼100 μm apart. Quantitative microinjection was performed using pipettes backfilled with mercury and concentrations of injected substances were calculated based on an oocyte volume of 200 pl [38]. mRNA was injected in a total volume of 10 pl. Injected follicle-enclosed oocytes were plated on Collagen type 1 culture inserts (BDbiosciences, San Jose, CA) and cultured in a humidified atmosphere at 37°C with 5% CO2 and 95% air. Following 9-11 days of

culture, oocytes were removed from the follicle and either assessed immediately or following 17-18 hr 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% TritonX-100) for 1 hour at 37ºC, washed three times in PBS/PVA with 0.01% TX-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 4ºC. Oocytes were then washed three times in blocking buffer and incubated in secondary antibody (anti-RAT 488, Invitrogen) diluted 1:200 in blocking buffer for 1 hr at room temperature. Finally, oocytes were washed four times with PBS/PVA, with 5 μM SYTOX Orange in the first wash. Oocytes were observed on a Zeiss 510 confocal microscope, 40x 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 three independent experiments, unless otherwise specified. Values were analyzed either by Student's *t*-test, One-way ANOVA, or Two-way ANOVA, as described in each figure legend. Percentage data were transformed via arcsin square root prior to statistical analysis [39]. All statistical analyses were performed using Graph Pad Prism software and significance was assessed at $p \leq 0.05$.

RESULTS

Epab$^{-/-}$ *oocytes exhibit morphological abnormalities*

Since *Epab*$^{-/-}$ oocytes are incapable of progressing to metaphase II, 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 (Figure 1). This is in contrast to *Epab*$^{+/+}$ where 14% were denuded and 62% were fully enclosed. The percentage of partially enclosed oocytes was similar for *Epab*$^{+/+}$ and *Epab*$^{-/-}$ oocytes (Figure 1B). The oocytes were then stripped of cumulus cells and evaluated for size and GV location (Figure 2A). *Epab*$^{-/-}$ oocytes were slightly but significantly smaller in size compared to *Epab*$^{+/+}$ oocytes (69.7 μm vs 71.5 μm, $p<0.0001$) (Figure 2B-C). In addition, 89% of *Epab*$^{-/-}$ oocytes contained peripheral GVs (Figure 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 since the oocytes grew beyond 60 μm [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 (Non-surrounded nucleolus; 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 (Surrounded 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 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. A majority of *Epab*$^{-/-}$ oocytes, ~ 55%, 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 partially surrounded nucleolus (PSN) (Fig 3 A&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 μm and the percentage of SN-oocytes increases significantly when antral follicles develop and the oocytes reach ~70 μm [49]. Although *Epab*$^{-/-}$ oocytes were smaller in size, the average size of *Epab*$^{-/-}$ oocytes from antral follicles was 69.7 μm and the majority of oocytes were greater than 68 μm (Figure 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, a majority of *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 (Figure 4A). Consistent with previous reports, ~ 24% of *Epab*$^{+/+}$ oocytes remained transcriptionally active while a majority underwent transcriptional silencing. In contrast, ~82% of *Epab*$^{-/-}$ oocytes remained transcriptionally active and failed to undergo transcriptional silencing (Figure 4B). Overall, the number of oocytes that exhibit NSN chromatin and transcriptional activity is significantly higher in *Epab*$^{-/-}$ mice compared to *Epab*$^{+/+}$ mice.

Microinjection of Epab *mRNA into* Epab$^{-/-}$ *follicle-enclosed oocytes at the 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 when 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 9-11 days in order to promote oocyte and follicle growth. Uninjected *Epab*$^{-/-}$ follicles did not grow beyond 230 μm during the culture period and only 38% resumed meiosis when isolated from the follicle (Figure 5 A & C). Maturation did not proceed normally in *Epab*$^{-/-}$ oocytes since none of the oocytes that resumed meiosis formed morphologically normal spindles (Figure 5 B & C). In contrast, uninjected *Epab*$^{+/+}$ follicles and *Epab*$^{-/-}$ follicles that were injected with *Epab* mRNA grew to larger than 320 μm (Figure 5A). A majority of these oocytes underwent GVBD following isolation from the follicle (97% and 77%) and formed morphologically normal spindles (84% and 75%) (Figure 5 B & 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 (Figure 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, the 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%) (Figure 6). We also measured the sizes of *Epab*$^{+/+}$ and *Epab*$^{-/-}$ oocytes uninjected or injected with *Epab* mRNA after the culture period. The average size for uninjected *Epab*$^{-/-}$ oocytes was ~67 μm whereas *Epab*$^{-/-}$ oocytes injected with *Epab* mRNA grew to ~ 75 μm, which was similar to the average size of 76 μm 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 demonstrate 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*$^{-/-}$ 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 not only required during oocyte maturation for translational activation of maternal mRNAs, but also during oocyte development for establishing factors that regulate nuclear maturation and meiotic competence.

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 while oocytes with SN chromatin exhibit repressed transcription (reviewed in [45]. Although the NSN/SN transition

occurs concomitantly with transcriptional silencing, these two 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 metaphase II compared to oocytes with the NSN configuration. More striking is the association between chromatin configuration and developmental competence since 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 if 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]. The somatic cells have also 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. A majority of 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 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 since EPAB is oocyte-specific. Since bidirectional communication between oocytes and somatic cells are 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.

How EPAB is important for 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 not only disrupt the transfer of oocyte-derived factors, but it would disrupt signals initiated by the somatic cells as well. Whether the abnormalities we observed in *Epab*$^{-/-}$ oocytes is directly the result of EPAB deficiency in the oocyte, or 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 the 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.

The 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 the progression from NSN to SN. ELAVL2 appears to be important for oocyte growth and meiotic competence since 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 during oocyte development for acquisition of meiotic competence. 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 towards 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 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 an early embryonic development.

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FIGURE LEGENDS

Figure 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 puncturing antral follicles. Bar = 100 μm. **B**) Higher magnification of images shown in **A**. Bar = 100 μm. **C**) Oocytes were scored as either denuded (lack of cumulus cells), partial (partially-enclosed), or full (fully-enclosed). Results are presented as the mean ± SEM. (*) indicates significant difference compared to *Epab*$^{+/+}$ as determined by Student's *t*-test, $p \leq 0.05$. Four *Epab*$^{+/+}$ and 3 *Epab*$^{-/-}$ mice from two litters were evaluated and at least 75 oocytes total were scored for each morphological characteristic.

Figure 2. Morphological characteristics of *Epab*$^{-/-}$ oocytes. Oocytes collected from eCG-primed *Epab*$^{+/+}$ and *Epab*$^{-/-}$ mice were characterized based on the location of the GV and oocyte size. **A**) Representative transmitted light confocal images of *Epab*$^{+/+}$ and *Epab*$^{-/-}$ oocytes. Bar = 10 μm. GV's are designated by an asterisk (*). **B**) The location of the GV was scored by confocal microscopy as either peripheral or central. Results are presented as the mean ± SEM. (*) indicates significant difference compared to *Epab*$^{+/+}$ as determined by Student's *t*-test, $p \leq 0.01$. **C**) Oocyte size was determined using Image J analysis after calibration with a scale bar. Results are presented as the mean ± SEM. (*) indicates significant difference compared to *Epab*$^{+/+}$ as determined by Student's *t*-test, $p \leq 0.0001$. **D**) Distribution of *Epab*$^{+/+}$ and *Epab*$^{-/-}$ oocyte sizes in 3 μm intervals. Four *Epab*$^{+/+}$ and 3 *Epab*$^{-/-}$ mice from two litters were evaluated and at least 75 oocytes total were scored for each morphological characteristic.

Figure 3. *Epab*$^{-/-}$ oocytes do 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 either non-surrounded (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**) Percentage of *Epab*$^{+/+}$ oocytes (n=98) and *Epab*$^{-/-}$ oocytes (n=84) exhibiting the different chromatin configurations. Results are presented as the mean ± SEM. (*) indicates significant difference compared to *Epab*$^{+/+}$ as determined by Student's *t*-test, $p \leq 0.05$. Four *Epab*$^{+/+}$ and 3 *Epab*$^{-/-}$ mice from two litters were evaluated.

Figure 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 showing detection of transcriptional activity. Bar = 10 μm. **B**) Percentage of *Epab*$^{+/+}$ oocytes (n=46) and *Epab*$^{-/-}$ oocytes (n=51) scored as transcriptionally

active. Results are presented as the mean ± SEM. (*) indicates significant difference in transcriptional status between *Epab*$^{+/+}$ and *Epab*$^{-/-}$ oocytes. Significance was determined by Student's *t*-test, $p \leq 0.05$. Four *Epab*$^{+/+}$ and 4 *Epab*$^{-/-}$ mice from 4 litters, were evaluated and at least 75 oocytes total were scored.

Figure 5. Microinjection of *Epab* mRNA into *Epab*$^{-/-}$ preantral follicle-enclosed oocytes rescues oocyte maturation. Preantral follicle-enclosed oocytes were injected with *Epab* or *Gfp* mRNA and cultured on a collagen membrane for 9-11 days. After the culture period, the oocytes were evaluated for GVBD and MII spindle formation. **A**) Representative images of follicles after the culture period. Bar = 100 μm. Arrowhead indicates granulosa cells and arrow indicates cumulus-enclosed oocyte complexes within the follicle. **B**) Representative images of spindle immunofluorescence after overnight maturation. Tubulin is shown in green and DNA is shown in red. Bar =10 μm. **C-D**) Percentage of oocytes that underwent GVBD (**C**) and matured normally to metaphase-II (**D**). Results are presented as the mean ± SEM. (*) indicates a significantly lower number of *Epab*$^{-/-}$ oocytes that underwent GVBD or formed normal MII spindles compared to *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*$^{-/-}$ FEO's).

Figure 6. Microinjection of *Epab* mRNA into *Epab*$^{-/-}$ preantral follicle-enclosed oocytes rescues progression to SN-type chromatin. Preantral follicles were dissected from *Epab*$^{+/+}$ and *Epab*$^{-/-}$ ovaries, and *Epab*$^{-/-}$ FEO's were injected with *Epab* mRNA. Following 9-11 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*$^{-/-}$ FEO's (n=19). Data are presented as mean ± SEM. (*) indicates statistical significance compared to *Epab*$^{+/+}$ as determined by Two-way ANOVA, multiple comparisons test, $p \leq 0.05$.

Figure 1

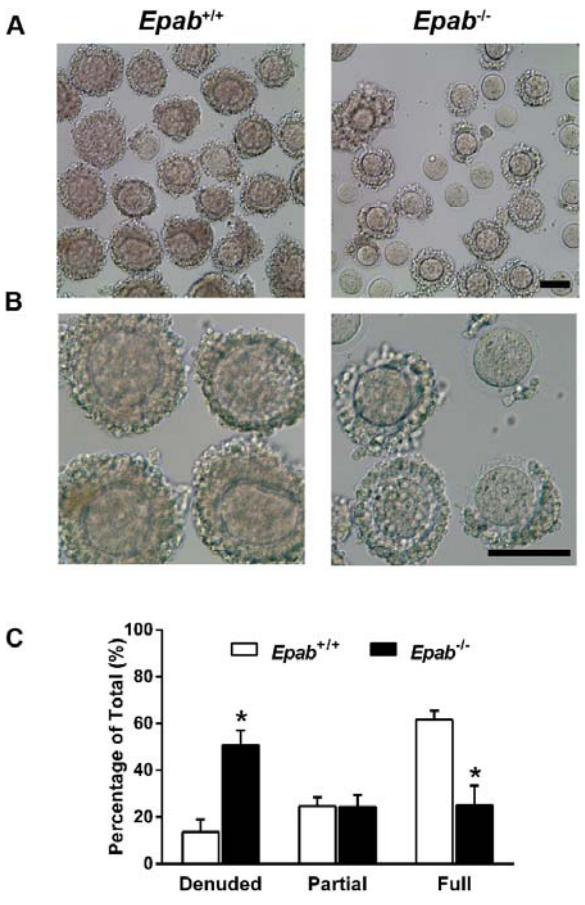

A
Epab+/+
Epab-/-
B
C
Epab+/+
Epab-/-
Percentage of Total (%)
100
80
60
40
20
0
*
*
Denuded
Partial
Full

Figure 2

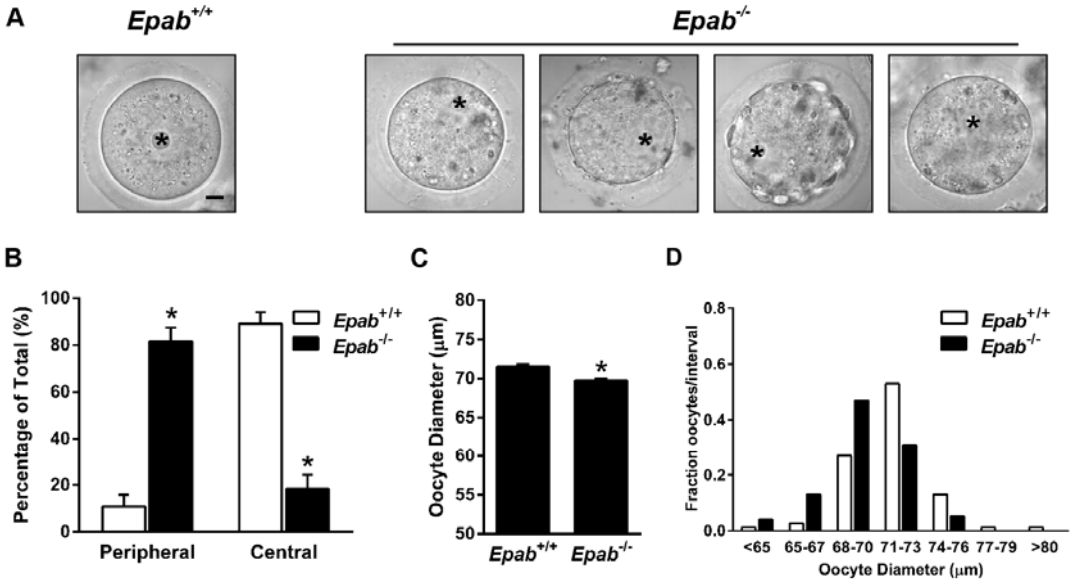

A
Epab+/+
Epab-/-
B
Percentage of Total (%)
Epab+/+
Epab-/-
Peripheral
Central
C
Oocyte Diameter (μm)
Epab+/+
Epab-/-
D
Fraction oocytes/interval
Epab+/+
Epab-/-
<65
65-67
68-70
71-73
74-76
77-79
>80
Oocyte Diameter (μm)

Figure 3

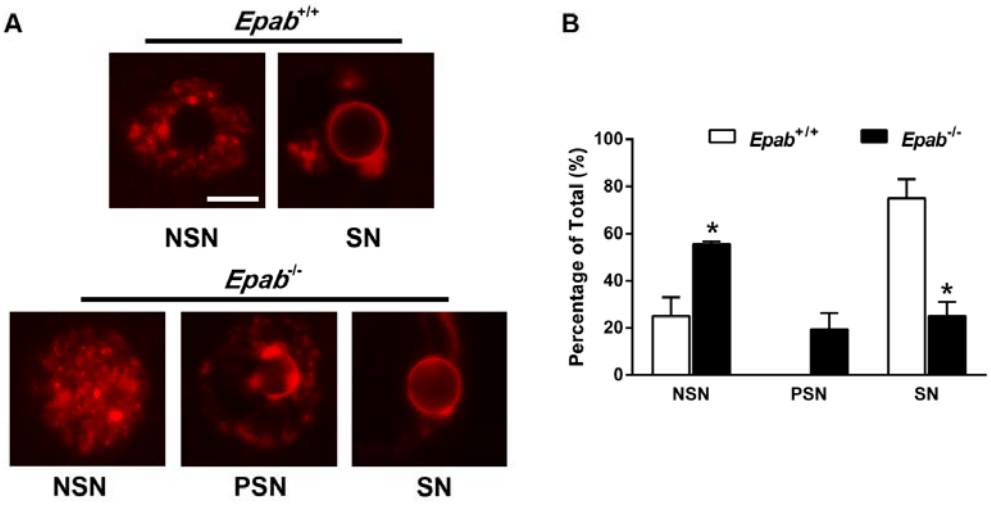

A
Epab+/+
NSN
SN
Epab-/-
NSN
PSN
SN
B
Epab+/+
Epab-/-
Percentage of Total (%)
100
80
60
40
20
0
*
*
NSN
PSN
SN

Figure 4

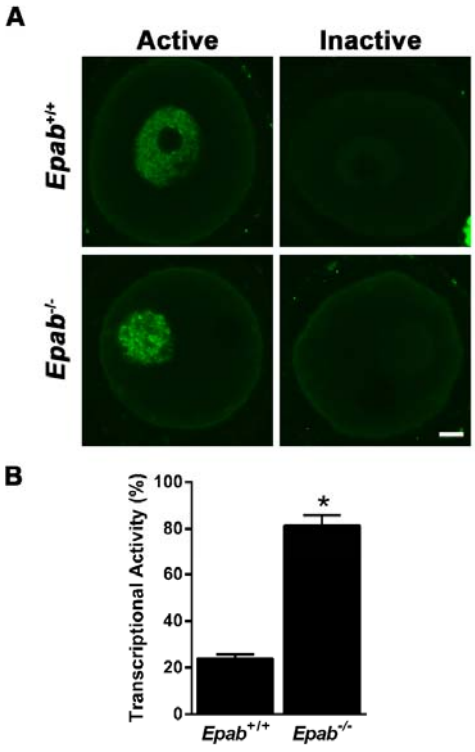

A
Active
Inactive
Epab+/+
Epab-/-
B
Transcriptional Activity (%)
100
80
60
40
20
0
*
Epab+/+
Epab-/-

Figure 5

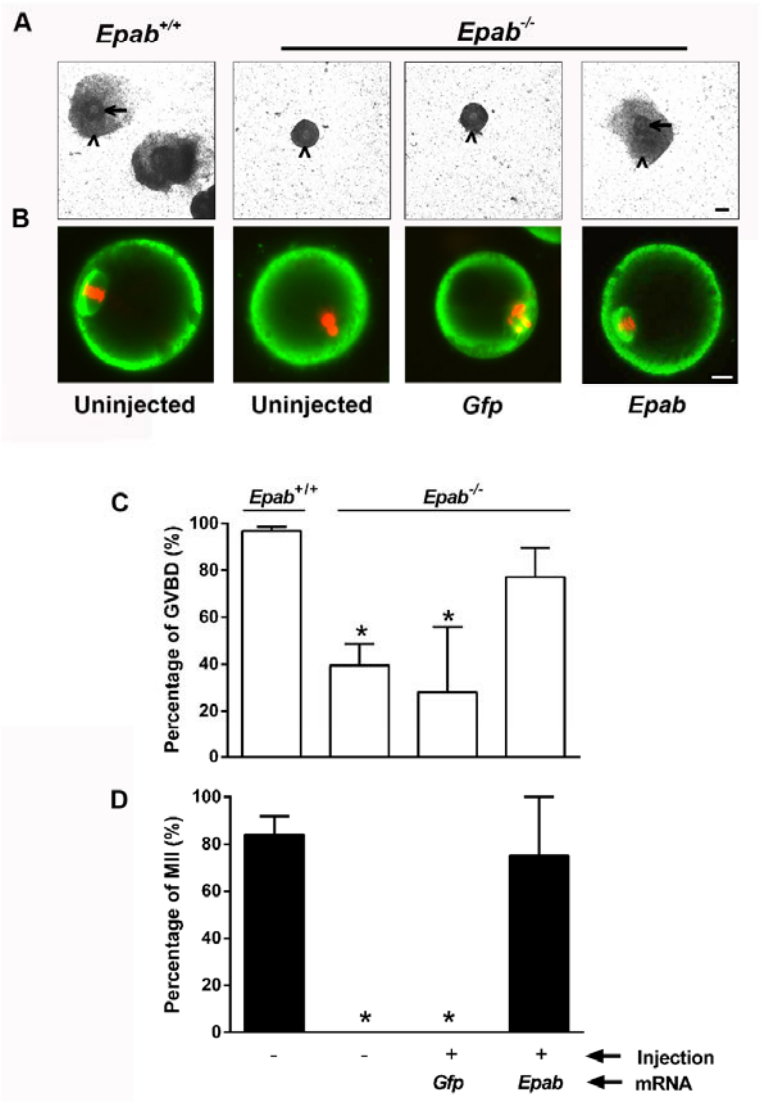
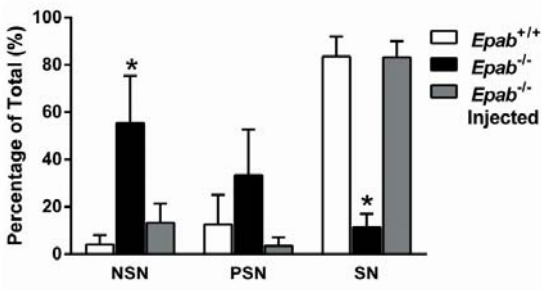

A
Epab+/+
Epab-/-
B
Uninjected
Uninjected
Gfp
Epab
C
Epab+/+
Epab-/-
Percentage of GVBD (%)
100
80
60
40
20
0
*
*
D
Percentage of MII (%)
100
80
60
40
20
0
*
*
-
-
+
Gfp
+
Epab
Injection
mRNA

Figure 6


Percentage of Total (%)
100
80
60
40
20
0
*
*
NSN
PSN
SN
Epab+/+
Epab-/-
Epab-/-
Injected