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EMBRYO BIOLOGY

Embryonic poly(A)-binding protein interacts with translation-related proteins and undergoes phosphorylation on the serine, threonine, and tyrosine residues in the mouse oocytes and early embryos

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Abstract

Expression of the embryonic poly(A)-binding protein (EPAB) in frog, mouse, and human oocytes and early-stage embryos is maintained at high levels until embryonic genome activation (EGA) after which a significant decrease occurs in EPAB levels. Studies on the vertebrate oocytes and early embryos revealed that EPAB plays key roles in the translational regulation, stabilization, and protection of maternal mRNAs during oocyte maturation and early embryogenesis. However, it remains elusive whether EPAB interacts with other cellular proteins and undergoes phosphorylation to perform these roles. For this purpose, we identified a group of Epab-interacting proteins and its phosphorylation status in mouse germinal vesicle (GV)- and metaphase II (MII)-stage oocytes, and in 1-cell, 2-cell, and 4-cell preimplantation embryos. In the oocytes and early preimplantation embryos, Epab-interacting proteins were found to play roles in the translation and transcription processes, intracellular signaling and transport, maintenance of structural integrity, metabolism, posttranslational modifications, and chromatin remodeling. Moreover, we discovered that Epab undergoes phosphorylation on the serine, threonine, and tyrosine residues, which are localized in the RNA recognition motifs 2, 3, and 4 or C-terminal. Conclusively, these findings suggest that Epab not only functions in the translational control of maternal mRNAs through binding to their poly(A) tails but also participates in various cellular events through interacting with certain group proteins. Most likely, Epab undergoes a dynamic phosphorylation during the oocyte maturation and the early embryo development to carry out these functions.

Keywords Epab · Oocytes · Phosphorylation · Preimplantation embryos · Proteomics · Translational control

Introduction

In mammals, maternally stored messenger ribonucleic acids (mRNAs) undergo a translational control to promote timely synthesis of the required proteins during oocyte maturation and early embryo development [1, 2]. RNA-binding proteins (RBPs) as a major component of translation complex play key roles in these processes. One of these RBPs is the embryonic poly(A)-binding protein (Epab, also known as Pabpc1l or Epabp) that specifically binds to poly(A) tails and ARE (AU-rich element; AUUUA) sites, both of which localize at the 3′ end of maternal mRNAs [3]. The Epab protein consists of four RNA recognition motifs (RRM1-4) at the N-terminal, proline-rich linker region (PrLR), and poly(A)-binding protein C-terminal (PABC) domain at the carboxyl terminal [4, 5]. RRMs are conversed among vertebrates and mediate interaction with poly(A) tails of mRNAs to facilitate their translation [3, 6]. Furthermore, RRMs participate in establishing a relationship with the translation-related factors such as eIF4G (eukaryotic translation initiation factor 4 gamma) [7] and PAIP1 (PABP-interacting protein 1) [8]. Although an exact role of PrLR has yet to be fully identified, the PABC domain with own poly(A)-binding protein-interacting motif 2 (PAM2) is involved in recruiting several translation factors to poly(A) tail [9].

The spatiotemporal *EPAB* gene expression has been comprehensively evaluated in the vertebrates from frogs to humans. In *Xenopus laevis* (*X. laevis*), Epab was at the minimal levels in the early (stage I) oocytes, increased in

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the fully grown (stage VI) oocytes and then remained constant toward the 30-h embryos after fertilization [3, 10]. Notably, *Epab* expression was also detected in the *X. laevis* somatic tissues including kidney, heart, and testis [11]. It was also characterized in mouse and human ovaries and testes [11–14]. Although the mouse somatic tissues did not express *Epab* gene, it was demonstrated in human pancreas, liver, thymus, prostate, kidney, and spleen tissues [15].

Epab was also expressed in the germinal vesicle (GV)- and metaphase II (MII)-stage mouse oocytes, and 1-cell and 2-cell embryos [12, 16]; however, no expression was reported in the 4- to 8-cell embryos and blastocysts [12]. In a recent study, Epab protein was localized in both the cytoplasm and nucleus of GV and MII oocytes, and 1-cell and 2-cell embryos in mice [17]. In humans, *EPAB* gene expression was detected throughout the oocyte maturation and from 1-cell to blastocyst stages [18]. In a more detailed study by Guzeloglu-Kayisli et al. [15], *EPAB* mRNA was noted at high levels in human GV and MII oocytes, then significantly decreased in the 8-cell and blastocyst-stage embryos [15]. Although these oocytes express only the full-length of *EPAB* transcripts, the somatic tissues such as kidney and liver are capable of generating the alternatively spliced forms, together with both variants in the ovaries, as expectedly. Since alternatively spliced *EPAB* transcripts (not including part of exons 8, 9, or 9 and 10) include a premature stop codon, only the truncated isoforms can be produced [15]. Importantly, the spliced *EPAB* transcripts were further characterized in the 8-cell and blastocyst-stage human embryos, but not in the oocytes [19]. Collectively, in addition to being *EPAB* expression in the mouse and human oocytes, its level exhibits embryonic genome activation (EGA)-dependent change in the embryos. EGA mainly occurs at the 2-cell stage in mice [20] and 4- to 8-cell embryos in humans [21]. It is noteworthy that a recent study reported that human EGA initiates at the 1-cell stage [22]. Following EGA, the poly(A)-binding protein cytoplasmic 1 (PABPC1) seems to take over these missions in the late preimplantation embryos, as observed in the somatic cells [12, 15].

As Epab plays crucial roles in the translational regulation during oocyte maturation and early embryo development, its absence or decreased levels may lead to various types of early developmental defects. Depletion of Epab levels in *X. laevis* oocyte extracts resulted in ARE-mediated or default deadenylation of maternal mRNAs, and administration of Epab to this extract rescued ARE-mediated deadenylation [3]. On the other hand, Epab depletion from 2-cell embryos in *X. laevis* by using the morpholino-mediated knockdown technology led to certain abnormalities in the anterior-posterior axis formation and movement, and eventually embryonic lethality [23]. Furthermore, knocking out *Epab* gene in mice caused female infertility due to misproduction of mature oocytes and early embryos in vitro or in vivo, whereas no infertility was encountered in the males [24]. As expected, Epab deficiency impaired translation and cytoplasmic polyadenylation of the maternal mRNAs, *c-Mos*, cyclin B1 (*Ccnb1*), and deleted in azoospermia-like (*Dazl*), during oocyte maturation. Disrupted meiotic division and chromosome mis-alignment were also noted in *Epab* null mouse oocytes upon in vitro maturation, and the other phenotypes such as decreased ovulation rates and reduced expression levels of the epidermal growth factor (EGF)-like growth factors, amphiregulin (*Areg*), epiregulin (*Ereg*) and betacellulin (*Btc*), and their downstream targets [24]. These EGF-related factors participate in the extracellular matrix synthesis and expansion of cumulus cells [25, 26].

An important finding by Friend et al. [27] in frogs was that Epab undergoes dynamic phosphorylations at the four residues (serine 460, serine 461, serine 464, and threonine 465) [27]. Although these phosphorylations are essential for cytoplasmic polyadenylation, there is no need for translational activation of stored mRNAs and subcellular localization of Epab in immature and mature oocytes. Nevertheless, phosphorylated Epab levels increase during oocyte maturation. While hyperphosphorylated Epab protein is able to interact with the complexes involving in translation and polyadenylation processes, no interaction was observed between hypophosphorylated Epab and polysomes or cap-dependent translation complexes [27].

As a result, it is evident that Epab plays pivotal roles in protecting maternally stored mRNAs from undergoing deadenylation and their translational control during oocyte maturation and early embryo development in vertebrates. Consistent with these roles, Epab further contributes to regulating several basic cellular processes, such as glycolysis, proliferation, and apoptosis, especially in granulosa and cumulus cells [28, 29]. However, it still remains elusive whether Epab interacts with some other proteins and undergoes phosphorylation to perform all these functions. In the present study, we aimed to identify Epab-interacting proteins in mouse oocytes at GV and MII stages and early preimplantation embryos at 1-cell, 2-cell, and 4-cell stages. Additionally, novel findings were also noted regarding phosphorylation status of Epab in these oocytes and embryos.

Material and methods

Animal and sample collection

The GV and MII oocytes and early embryos at 1-cell, 2-cell, and 4-cell stages were recovered from 6- to 8-week-old BALB/c female mice provided from the Akdeniz University Experimental Animals Application and Research Center and Kobay Experimental Animals Laboratory. They were kept

under a 12-h light-dark cycle without water and food restrictions. All experimental protocols were performed in accordance with relevant guidelines and regulations approved by the Akdeniz University Institutional Animal Care and Use Committee (protocol number: 1286/2021.04.011). We collected oocytes and embryos as described in our previous studies [16, 17].

For obtaining GV oocytes, female mice were superovulated with an intraperitoneal (i.p.) injection of 0.1 mL 5 IU pregnant mare serum gonadotrophin (PMSG; Sigma-Aldrich, MO, USA), diluted in sterile water. The ovaries recovered from superovulated female mice 22 h after PMSG injection were punctured with a 23-gauge needle to provide release of the GV oocytes into human tubal fluid (HFT) medium (Vitrolife, Gothenburg, Sweden). The denuded (cumulus cell-free) GV-stage oocytes around 85 µm in diameter with normal morphology (no cytoplasmic granularity, normal perivitelline space and zona pellucida thickness) and at proper maturation status were collected using a mouth-controlled pipette under a dissecting microscope (Zeiss, Oberkochen, Germany) (Fig. 1). A total of 208 GV-stage oocytes were obtained from 30 mice for liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS), and 322 GV-stage oocytes from 30 mice for matrix-assisted laser desorption/ionization-time-of flight/mass spectrometry (MALDI-TOF/MS) analysis. MII oocytes were obtained by injecting 0.1 mL 5 IU of human chorionic gonadotrophin (hCG; Sigma-Aldrich) i.p. to the female mice primed with 5 IU PMSG 48 h earlier. Then, cumulus-oocyte complexes (COCs) were recovered from the oviducts 14 h after hCG injection. Morphologically normal MII oocytes containing single polar body were isolated from COCs via treating with 1 mg/mL hyaluronidase (Sigma-Aldrich) (Fig. 1). Of note, a total of 201 MII oocytes were collected from 18 mice for LC-MS/MS, and 310 MII oocytes from 26 mice for MALDI-TOF/MS analyses.

The 1-cell, 2-cell, and 4-cell embryos were collected from female mice injected with 8 IU PMSG followed by 8 IU hCG, as described above. Immediately after hCG injection, they were mated with 12-week-old male mice (at a rate of 2 females:1 male per cage). One cell, 2-cell, and 4-cell embryos were recovered from the oviducts of vaginal plug-positive mice following 22 h, 42 h, and 56 h after hCG injection, respectively (Fig. 1). It is noteworthy that cumulus cells surrounding 1-cell embryos were removed by exposing to 1 mg/mL hyaluronidase. As a result, a total of 204 1-cell embryos from 30 mice, 211 2-cell embryos from 28 mice, and 143 4-cell embryos from 30 mice were recovered for LC-MS/MS analysis. Furthermore, we obtained 307 1-cell embryos from 23 mice, 347 2-cell embryos from 24 mice, and 305 4-cell embryos from 28 mice for MALDI-TOF/MS analysis.

Co-immunoprecipitation experiments

Co-immunoprecipitation of Epab protein before the LC-MS/MS and MALDI-TOF/MS analyses was carried out according to the manufacturer's instructions (catalog no.: ab206996, Abcam, Cambridge, UK). The isolated oocytes and early embryos were lysed in the lysis buffer (non-denaturing), including 1 × protease inhibitor cocktail (catalog no.: M221, Amresco, OH, USA). Importantly, we added phosphatase inhibitor cocktails 2 (catalog no.: P5726, Sigma-Aldrich) and 3 (catalog no.: P0044, Sigma-Aldrich) to all solutions used while preparing the samples for phosphorylation analysis. The extracts were centrifuged at 15,000 *g* at 4 °C for 10 min, and then supernatants were transferred into sterile tubes. Next, we added 10 µL Epab polyclonal antibody (produced by [17]) onto the supernatants and incubated at 4 °C overnight on a rotary mixer (VWR, PA, USA). Following incubation, 25 µL of Protein A/G Sepharose® was washed with the manufacturer-supplied buffer twice. The mixture was reincubated on the rotary mixer at 4 °C for 1 h. Subsequently, tubes were centrifuged at 2000 *g* at 4 °C for 2 min to collect sepharose beads. The beads were washed three times with 1 mL 1 × washing buffer. After removing washing solution, we eluted Epab-co-immunoprecipitated proteins from beads by treating with the solution composed of 25 µL from 50 mM ammonium bicarbonate and 1.5 µL from 100 mM DTT for LC-MS/MS analysis. For

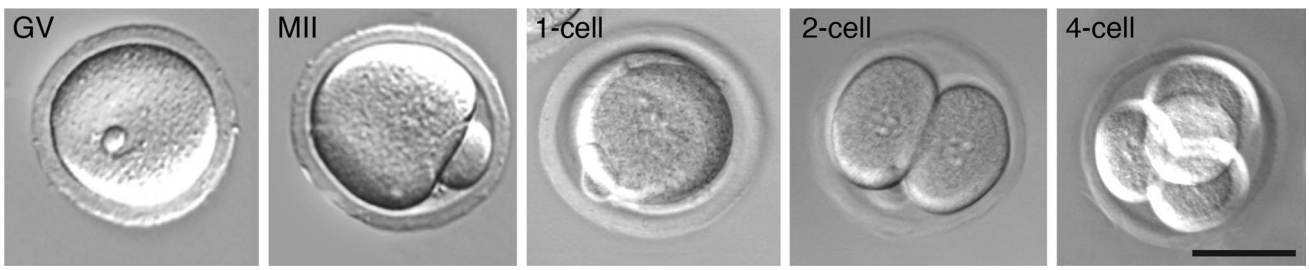


Fig. 1 The denuded oocytes (from GV- to MII-stage) and 1-cell to 4-cell embryos were collected from BALB/c mice at various time points and initially scoped under a DIC microscope for proper staging, size measurement, and cytoplasmic and nuclear maturity assessment. Scale bar: 50 µm

MALDI-TOF/MS analysis, Epab-co-immunoprecipitated proteins were eluted using TBS containing 0.5% SDS on a shaker for 2 h and then passed through a 0.2-µm column.

Identification of Epab-related proteins by LC-MS/MS

We identified Epab-interacting proteins according to the previously published studies [30, 31] by the contribution of Kocaeli University Research and Application Center (Kocaeli, Turkey). The tryptic peptides revealed following in-gel digestion were evaluated by the LC-MS/MS system (nanoACQUITY UPLC and SYNAPT high-resolution mass spectrometer with nanolockspray ion source). Columns were equilibrated with 97% mobile phase A (0.1% formic acid in LC–MS grade water (Merck, USA)), and column temperature was adjusted to 45 °C. Peptides were separated from the trap column ((Symmetry C18 5 µm, 180 µm i.d. × 20 mm) (Waters, USA)) by the gradient elution onto an analytical column (BEH C18, 1.7 µm, 75 µm i.d. × 250 mm) (Waters) at 300 nL/min flow rate with a linear gradient from 5 to 40% mobile phase B (0.1% formic acid in hypergrade acetonitrile) (Merck, USA) over 30 min.

The data-independent acquisition mode (MSE) was performed by operating the instrument at positive ion V mode, applying the MS and MS/MS functions over 1.5-s intervals with 6 V low energy and 15-40 V high-energy collusion. Glu-fibrinopeptide (internal mass calibrant) was infused at 300 nL/min flow rate, and the m/z values over 50-1600 were evaluated. Tandem mass data extraction, charge state deconvolution, and deisotoping were conducted with ProteinLynx Global Server v2.5 (Waters, USA) and searched with the IDENTITY^{E} algorithm with a fragment ion mass tolerance of 0.025 dalton (Da) and a parent ion tolerance of 0.0100 Da against the reviewed *Mus musculus* protein database from Uniprot. Carbamidomethyl-cysteine fixed modification and Acetyl N-TERM, deamidation of asparagine and glutamine, and oxidation of methionine variable modifications were adjusted. The Scaffold (version Scaffold_3.6.1, Proteome Software Inc., Portland, OR, USA) was employed to confirm MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at a greater than 95% probability as specified by the Peptide Prophet algorithm [32]. It is important to note that among the all identified proteins, only the ones that showed co-precipitation probability greater than 95% ($P < 0.05$) were accepted. Benjamini and Hochberg's procedure was applied in false discovery rate (FDR) analysis. FDR values from our analyses were applied as 0.05 for "Relaxed" and 0.01 for "Strict." Protein probabilities were assigned by the Protein Prophet algorithm [33].

As a bioinformatics analysis, the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) assessment was performed according to the protocol published previously [34]. We used UniProt accession numbers of the defined proteins in the software program (https://string-db.org/) to determine functional relationships. The search engine was adjusted to "multiple proteins by identifiers" and we set the organism as *Mus musculus*. In addition to that, the protein analysis through evolutionary relationships (PANTHER, http://PANTHERdb.org/) analysis [34] was made by using UniProt accession numbers of the defined proteins. The organism was selected as *Mus musculus*. For this purpose, we specified the ontologies as molecular and cellular functions. Please, refer to the keys for nodes and lines in Figs. 2 and 3 for the assessment of protein-protein interactions.

Phosphorylation analysis using MALDI-TOF/MS

Applied Biomics (Hayward, CA, USA) service was employed for the identification of phosphorylation sites in Epab by MALDI-TOF/MS following a standard protocol [35]. In brief, after co-immunoprecipitating Epab protein in the mouse oocytes and embryos using an Epab-specific primary antibody according to the manufacturer's instructions (catalog no.: ab206996, Abcam, Cambridge, UK), the samples including phosphorylated Epab were digested in a trypsin solution overnight at 37 °C. Supel-Tips (Sigma-Aldrich) were used for phosphopeptide enrichment. Tryptic peptides were desalted and concentrated using the Millipore C18 reverse phase Zip-Tips column (catalog no.: ZTC 18S096, Millipore, USA), eluted in 0.5 µL of matrix solution (α-cyano-4-hydroxycinnamic acid (5 mg/mL in 50% acetonitrile, 0.1% trifluoroacetic acid, 25 mmol/L ammonium bicarbonate)), and spotted on the MALDI plate (model ABI 01-192-6-AB). MALDI-TOF/MS was carried out on an AB Sciex Proteomics Analyzer (AB Sciex, CA, USA). MS spectra were acquired in the reflection positive ion mode, averaging 4000 laser shots per spectrum. A virtual digest was performed by submitting protein sequences of interest to University of California-San Francisco Protein Prospector (http://prospector.ucsf.edu/prospector/mshome.htm). The MS precursors matching the virtual digest were submitted for collision-induced dissociation (CID) fragmentation. Peptide masses and associated CID spectra were submitted to GPS Explorer workstation equipped with MASCOT search engine (Matrix Science) to search the database of Swiss-Prot. Candidates either with protein score confidence interval percentage or ion confidence interval percentage of > 95% were considered significant ($P < 0.05$). The spectra of all the peptides containing potential phosphorylation sites were manually evaluated for the loss of phosphate.

Results

In this study, we first identified the Epab-interacting proteins (Figs. 2, 3, and 4) and phosphorylated amino acids (Fig. 5) in GV- and MII-stage oocytes, and in 1-cell, 2-cell, and 4-cell embryos.

Epab-interacting proteins in the oocytes

In GV-stage oocytes, we defined a group of proteins ($n=58$) showing co-precipitation with Epab ($P<0.05$) (Fig. 2, Suppl. Table 1). However, no functional relationship was predicted between Epab and these proteins based on an assessment by the STRING program. When analyzing their molecular and cellular features, Epab-interacting ones were predominantly (≥3 per category) chromatin/chromatin-binding, or chromatin-regulatory proteins, cytoskeletal proteins, gene-specific transcriptional regulators, metabolite interconversion enzymes, protein-modifying enzymes, and protein-binding activity modulators (Fig. 4). Importantly, we further identified two translation-related (60S ribosomal protein L27a (Rpl27a) and 28S ribosomal protein S30 mitochondrial (Mrps30)) and three transcriptional regulation-related (MAF BZIP transcription factor G (Mafg), E4F transcription factor 1 (E4f1), and zinc finger protein 169 (Zf169)) proteins (Fig. 4).

In MII-stage oocytes, we defined a group of proteins ($n=96$) showing co-precipitation with Epab ($P<0.05$) (Fig. 2, Suppl. Table 2). A functional relationship between Epab and DEAD-box helicase 55 (Ddx55) was predicted by the STRING program. Additionally, there were a number of proteins showing physical interaction with Epab. They are classified (≥3 per category) as chromatin/chromatin-binding, regulatory proteins, cytoskeletal proteins, gene-specific transcriptional regulators, membrane traffic proteins, metabolite interconversion enzymes, nucleic acid metabolism proteins, protein-modifying enzymes, scaffold/adaptor proteins, and transporters (Fig. 4). Considering the basic role of Epab in translation control, the gene-specific transcriptional regulators were PR/SET domain 15 (Prdm15, a C2H2 zinc finger transcription factor), peroxisome proliferator activated receptor alpha (Ppara, zinc finger nuclear receptor), THO complex 2 (Tho2, a transcription cofactor), nascent polypeptide-associated complex subunit alpha (Naca, a basic helix-loop-helix transcription factor), SapP30-binding protein (Sap30BP, a DNA-binding transcription factor), E4f1 (a C2H2 zinc finger transcription factor), and zinc finger protein 362 (Zfp362, a C2H2 zinc finger transcription factor).

Epab-interacting proteins in the early embryos

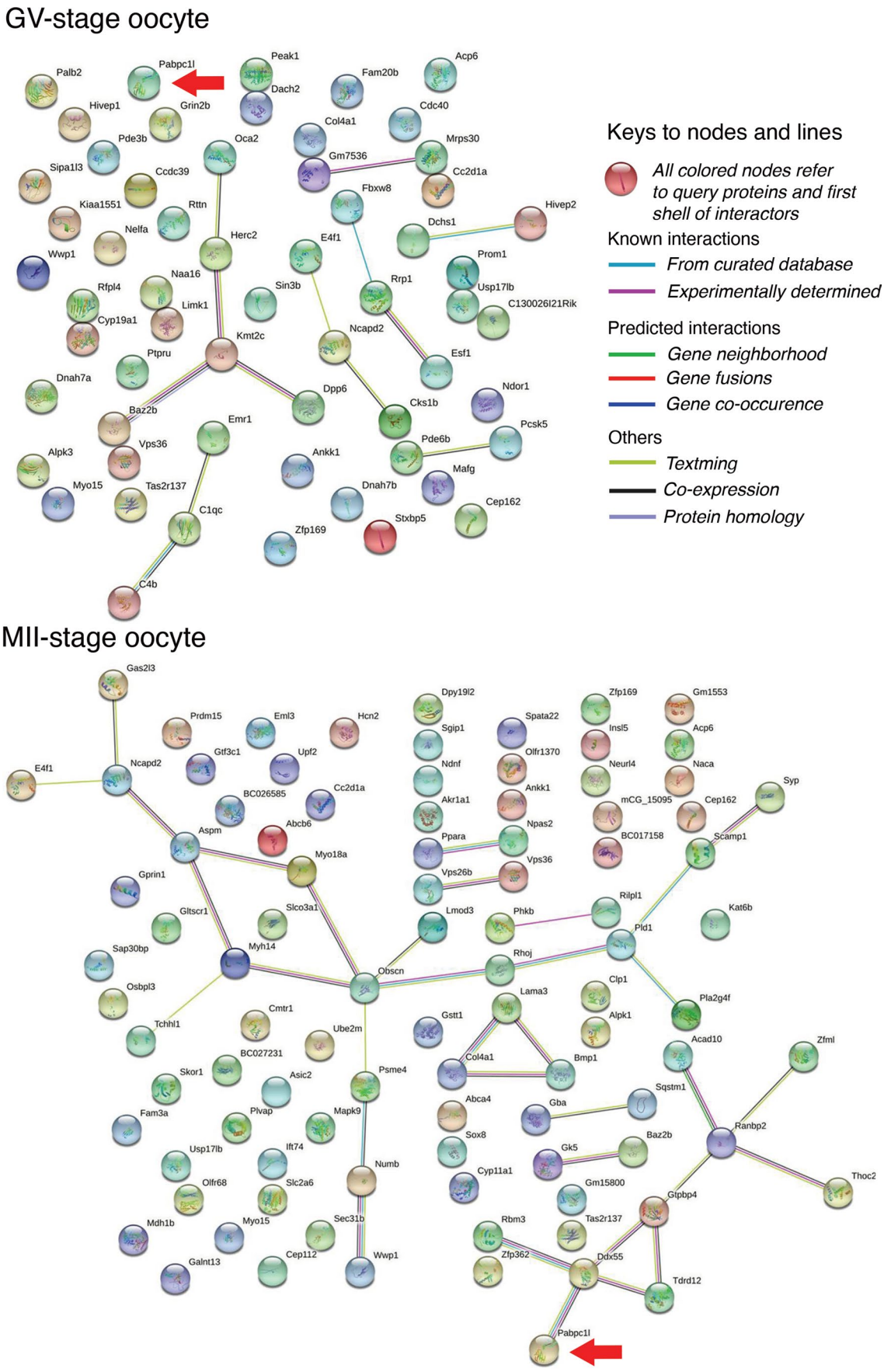
In 1-cell embryos, we defined a number of proteins ($n=61$) showing co-precipitation with Epab. No functional relationship was detected between Epab and these proteins (Fig. 3, Suppl. Table 3). However, these co-precipitated proteins (≥3 per category) serve as gene-specific transcriptional regulators, membrane traffic proteins, metabolite interconversion enzymes, protein-modifying enzymes, protein-binding activity modulators, and transporters (Fig. 4). In the transcriptional regulator category, Epab established associations with Prdm15, nucleolar transcription factor 1 (Ubtf, a DNA-binding transcription factor), Naca, and E4f1.

In 2-cell embryos, we defined a group of proteins ($n=55$) showing co-precipitation with Epab (Fig. 3, Suppl. Table 4). These proteins (≥3 per category) play roles as gene-specific transcriptional regulators, metabolite interconversion enzymes, protein-modifying enzymes, protein-binding activity modulators, transmembrane signal receptors, and transporters (Fig. 4). In more detail, the gene-specific transcriptional regulators were single-minded homolog 1 (Sim1, a basic helix-loop-helix transcription factor), short stature homeobox protein 2 (Shox2, a homeodomain transcription factor), Naca, Sap30bp, and Zxd family zinc finger c (Zxdc, a C2H2 zinc finger transcription factor).

In 4-cell embryos, we also defined a series of proteins ($n=45$) showing co-precipitation with Epab (Fig. 3, Suppl. Table 5). Among these proteins, the eukaryotic translation initiation factor 4 gamma 2 (Eif4g2, a translational activator) and zinc finger GATA-like protein 1 (Zglp1, a transcriptional regulator) showed functional interactions with Epab (Fig. 3). These interactions are compatible with the known role of Epab in translation regulation. Additionally, we detected many Epab-linked proteins (≥3 per category) that function as protein-modifying enzymes (Fig. 4). Among the gene-specific transcriptional regulators, Epab exhibited an association with Sap30bp and zinc finger protein 169 (Zfp169, a C2H2 zinc finger transcription factor).

Phosphorylation of Epab in the oocytes

We further analyzed phosphorylation status of Epab protein in GV- and MII-stage oocytes. Several phosphorylation sites were detected in the serine (S) and threonine (T) amino acids. In GV oocytes, phosphorylation was discovered in S226, S230, S342, T360, and T351 residues (Fig. 5, Suppl. Fig. 1). Importantly, while S226 and S230 phosphorylation sites localize in RRM3, the remaining ones are in RRM4 region. In MII oocytes, phosphorylation was detected in S603, T157, T347, T351, and T604 residues (Fig. 5, Suppl. Fig. 1). Although phosphorylated T157 localizes in RRM2, the T347 and T351 phosphorylations take part in RRM4 region. Moreover, we observed the S603 and T604 phosphorylations, located in the C-terminal region of Epab (Fig. 5).


GV-stage oocyte
Palb2
Pabpc1l
Peak1
Dach2
Fam20b
Acp6
Hivep1
Grin2b
Cdc40
Col4a1
Pde3b
Oca2
Mrps30
Gm7536
Sipa1l3
Ccdc39
Cc2d1a
Fbxw8
Kiaa1551
Rttn
Hivep2
Dchs1
Nelfa
E4f1
Herc2
Wwp1
Prom1
Naa16
Rrp1
Rfpl4
Sin3b
Usp17lb
Limk1
C130026I21Rik
Cyp19a1
Ncapd2
Kmt2c
Ptpru
Esf1
Dnah7a
Dpp6
Ndor1
Cks1b
Baz2b
Emr1
Pcsk5
Vps36
Pde6b
Alpk3
Ankk1
Mafg
Myo15
Tas2r137
Dnah7b
Cep162
C1qc
Stxbp5
Zfp169
C4b
Keys to nodes and lines
All colored nodes refer to query proteins and first shell of interactors
Known interactions
From curated database
Experimentally determined
Predicted interactions
Gene neighborhood
Gene fusions
Gene co-occurence
Others
Textming
Co-expression
Protein homology
MII-stage oocyte
Gas2l3
Dpy19l2
Zfp169
Gm1553
Prdm15
Eml3
Hcn2
Sgip1
Spata22
Insl5
Acp6
Gtf3c1
Upf2
Ndnf
Olfr1370
Neurl4
Naca
E4f1
Ncapd2
Cc2d1a
Akr1a1
Ankk1
Syp
BC026585
Abcb6
Ppara
Npas2
mCG_15095
Cep162
Aspm
Myo18a
Vps26b
Vps36
BC017158
Scamp1
Gprin1
Slco3a1
Lmod3
Phkb
Rilpl1
Kat6b
Gltscr1
Myh14
Obscn
Rhoj
Pld1
Sap30bp
Osbpl3
Clp1
Lama3
Pla2g4f
Tchhl1
Cmtr1
Gstt1
Alpk1
Ube2m
Acad10
Zfml
BC027231
Col4a1
Bmp1
Skor1
Asic2
Psme4
Sqstm1
Abca4
Gba
Fam3a
Plvap
Mapk9
Sox8
Baz2b
Ranbp2
Usp17lb
Ift74
Gk5
Numb
Cyp11a1
Thoc2
Olfr68
Slc2a6
Gm15800
Gtpbp4
Mdh1b
Myo15
Sec31b
Rbm3
Tas2r137
Galnt13
Cep112
Wwp1
Zfp362
Ddx55
Tdrd12
Pabpc1l

◂**Fig. 2** The Epab-interacting proteins in GV- and MII-stage oocytes. The LC-MS/MS data were evaluated using the STRING program to determine functional relationship between Epab (defined herein as Pabpc1l (poly(A)-bind protein cytoplasmic 1 like)) and the co-precipitated proteins. Epab is located at the top left (red arrow) in GV oocytes and at the bottom (red arrow) in MII oocytes. Although no functional relationship was detected in GV-stage oocytes, several proteins co-precipitated ($P < 0.05$) with Epab as depicted in colors. In MII-stage oocytes, we found a strong functional relationship between Epab and the DEAD-box helicase 55 (Ddx55). According to the keys shown in upper right, this interaction is based on the curated, experimentally determined, and co-expression database

Phosphorylation of Epab in the embryos

Upon evaluating phosphorylation sites of Epab in 1-cell, 2-cell, and 4-cell stage embryos, we detected on S, T, and tyrosine (Y) amino acids. In 1-cell embryos, phosphorylation was discovered only in S230 and T347 residues (Fig. 5, Suppl. Fig. 1). While phosphorylated S230 localizes in RRM3, RRM4 includes T347 phosphorylation.

Different from the oocytes, 1-cell and 4-cell embryos, we identified a phosphorylation site in the tyrosine residue at 194 (Y194) in 2-cell embryos (Fig. 5, Suppl. Fig. 1). Moreover, the other phosphorylation sites in S315, S322, S332, T316, and T321 residues were noted in 2-cell embryos. While phosphorylated Y194 residue is in RRM3, the others are located in RRM4 region (Fig. 5). In 4-cell embryos, phosphorylation sites were only defined in S330 and T316 residues, both of which localize in the RRM4 region (Fig. 5, Suppl. Fig. 1).

Discussion

In the present study, for the first time in the literature, we characterized the Epab-interacting proteins, which may play roles in the major cellular events from translational and transcriptional regulation to chromatin remodeling in the GV- and MII-stage mouse oocytes as well as in the early embryos at 1-cell, 2-cell, and 4-cell stages. Moreover, we revealed that Epab is phosphorylated on the serine, threonine, and tyrosine residues in these oocytes and embryos.

The well-known functions of Epab are to regulate translational activity of maternally stored mRNAs and their protection from deadenylation during oocyte maturation and early embryo development [36, 37]. In the proteomics analysis, although the STRING program did not predict any functional relationship between Epab and the defined proteins in GV oocytes, 1-cell and 2-cell embryos, several proteins exhibited functional interactions in the MII oocytes and 4-cell embryos.

In MII oocytes, we identified a relationship between Epab and Ddx55 proteins. Ddx55 exhibits a DNA- and RNA-dependent ATPase activity and is involved in ribosome biosynthesis through binding to 28S ribosomal RNA in nucleoplasm [38]. As transcriptional activity ceases in the fully grown GV oocytes before luteinizing hormone surge [39], ribosome biosynthesis gains importance for producing necessary proteins from maternal mRNAs in a time-dependent manner. Considering this, potential interaction between Epab and Ddx55 may derive from their common roles in the same ribosome complex, since Epab is known to associate with ribosomes, evidenced in the *X. laevis* oocytes [11]. Regarding this prediction, further studies are needed to uncover the mechanistic relationship and the importance of this interaction in the oocytes during maturation.

In the functional analysis of 4-cell embryos, we found a relationship between Epab and Zglp1 (also known as Glp1), which is a preserved transcriptional regulator, having two zinc finger motifs that promote DNA binding ability. A recent study by Nagaoka et al. [40] reported that Zglp1 implicates in determining oogenic fate and entry into meiosis in mice [40]. As expected, its deficiency resulted in lack of embryonic germ cells [41]. Understanding the precise functional importance of Epab interaction with Zglp1 in 4-cell embryos requires further investigation with special emphasis on the potential effects of disrupting this interaction on early embryogenesis. Another Epab-related protein Eif4g2 acts as a translation-initiating factor and participates in cap-dependent [42] and cap-independent translation in the human embryonic stem cells [43]. As Eif4g2 exhibits a high homology with Eif4g [44], which is known to bind Epab [45], Eif4g2 and Epab seem to work together in the translational regulation in 4-cell embryos.

In addition to detected functional relationship in MII oocytes and 4-cell embryos, we also defined many Epab-related proteins participating in various cellular events including translational and transcriptional regulation, transport, intracellular signaling, protein-binding and modifications, maintenance of structural integrity, metabolic processes, and chromatin remodeling in the oocytes and early embryos. These potential interactions may originate from indirect associations through being involved in the same complexes, acting in these events. On the other hand, these interactions suggest that Epab might have additional roles apart from its primary role in translation regulation. Consistent with this prediction, it was demonstrated that Epab enables to establish an association with other proteins via its PABC domain, which facilitates binding to some proteins such as deadenylase, eukaryotic release factor 3 (Erf3), Pabpc1, and Eif4b [37].

As Epab has a central role in the translational control of maternal mRNAs during oocyte maturation and early embryo development [11, 15, 27], we focused on the Epab-related proteins which particularly take part in translational and transcriptional regulation. These proteins mainly function as components of ribosomal complex, transcription

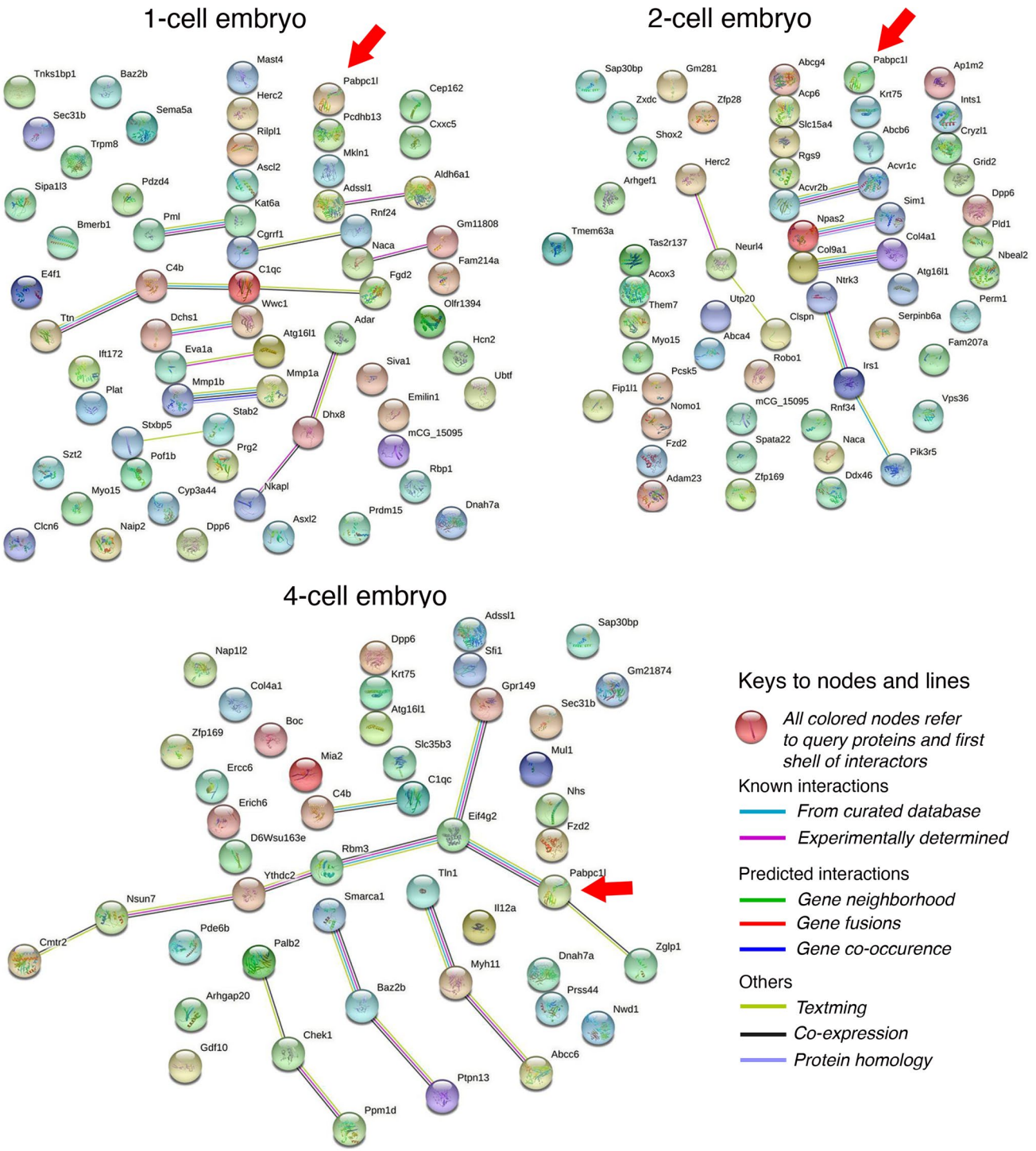


Fig. 3 The Epab-interacting proteins in 1-cell, 2-cell, and 4-cell embryos. The LC-MS/MS data were evaluated using the STRING program to determine functional relationship between Epab (defined herein as Pabpc1l, red arrow) and the co-precipitated proteins. In 1-cell and 2-cell embryos, no functional relationship was noted, while in 4-cell embryos, Epab showed functional interaction with the eukaryotic translation initiation factor 4 gamma 2 (Eif4g2, a translational activator) and the zinc finger GATA-like protein 1 (Zglp1, a transcriptional regulator). According to the keys shown in the lower right, these interactions are based on the curated, experimentally determined, and co-expression databases

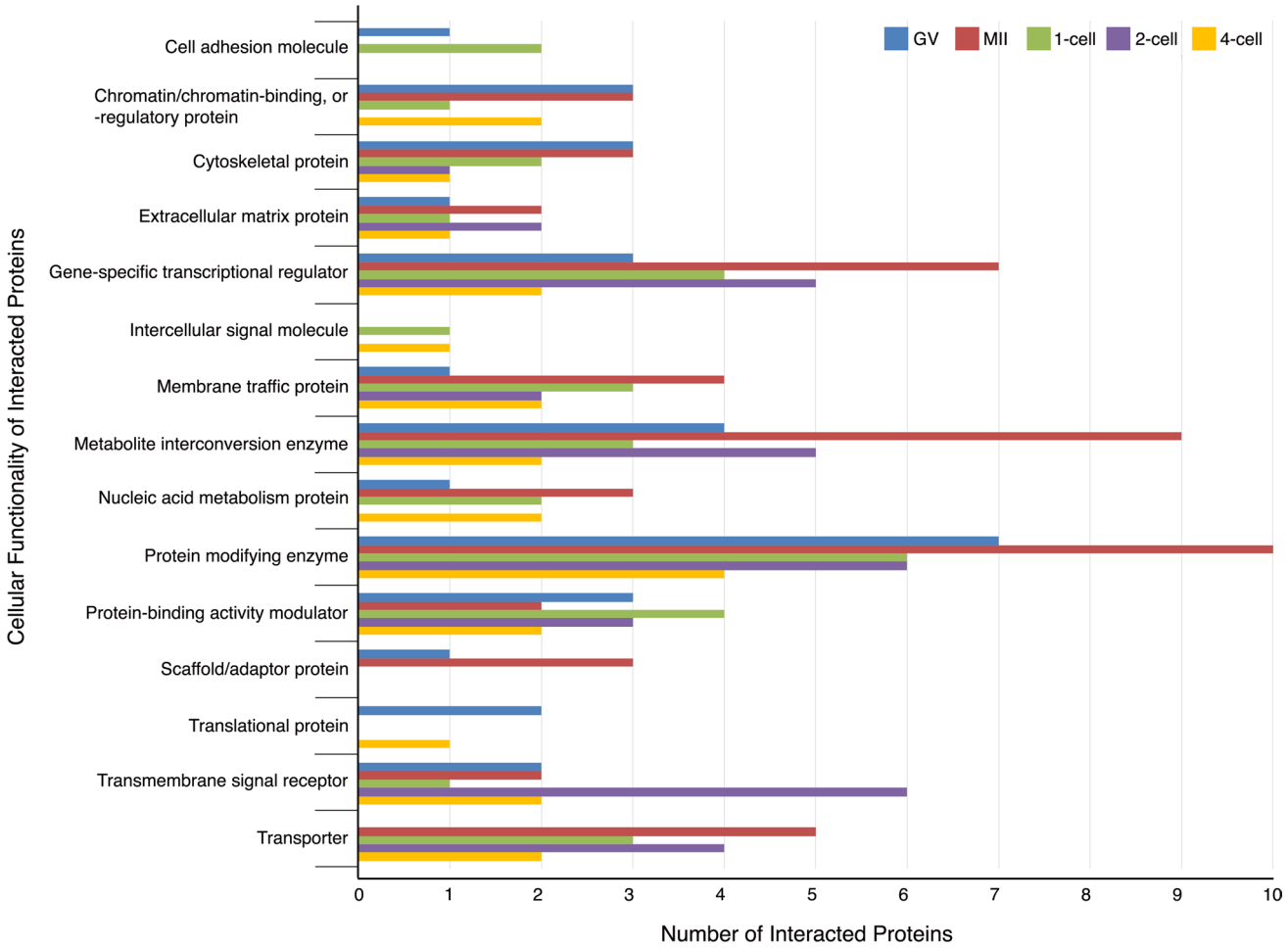


Fig. 4 Upon analyzing LC-MS/MS data extracted from mouse oocytes and embryos (stages are shown in five different colors), Epab protein interacted with a varying number of proteins (min = 0, max = 10, shown in *x*-axis) that are responsible for 15 different cellular and molecular functions (shown in *y*-axis)

factors having zinc finger domain, basic helix-loop-helix domain or homeodomain, and transcription cofactors in the oocytes or early embryos. Interaction of Epab with transcriptional factors may be required for promoting rapid translation of certain mRNAs via binding their poly(A) tails, as the nuclear poly(A)-binding protein (Pabn) does [4, 46]. In fact, we found a potential Epab-E4f1 relationship in GV oocytes, MII oocytes, and 1-cell embryos; Epab-Sap30bp30BP relationship in MII oocytes, 2-cell and 4-cell embryos; and Epab-Naca relationship in MII oocytes, 1-cell and 2-cell embryos. Based on these interactions, we suggest that a number of phenotypes emerging upon Epab deficiency such as chromatin configuration defects, altered EGF-like growth factor levels, changed EGF signaling, impaired gene expression, abnormal oocyte maturation, and embryo production in the female mice [24, 29, 47] may arise from loss of its additional roles in transcriptional regulation.

Despite identifying many Epab-related proteins in the oocytes and embryos, we were not able to describe any relationship with the common translation-related proteins such as Eif4b, Eif4e, other Eif4g isoforms, Paip1, Dazl, Cpeb, Erf3, and Cpsf, by which Epab establishes interactions during translational control [8, 37, 48, 49]. Importantly, as all these interactions were characterized in the *Xenopus* oocytes, there may be species-specific differences in the oocytes and early embryos. Another possible explanation of not observing any interaction may result from broken physical connections between Epab and those proteins during the proteomic processes. Thus, possible relationships between Epab and the translation-related proteins must be evaluated separately in these oocyte and embryonic stages.

In the present study, as another novel finding, we uncovered phosphorylation sites of Epab in the oocytes and early embryos. To the best of our knowledge, there is only one report in the literature analyzed Epab phosphorylation state in *X. laevis* oocytes [27]. In that study, it was revealed that Epab is dynamically phosphorylated in the mature and immature oocytes. For successful oocyte maturation, Epab

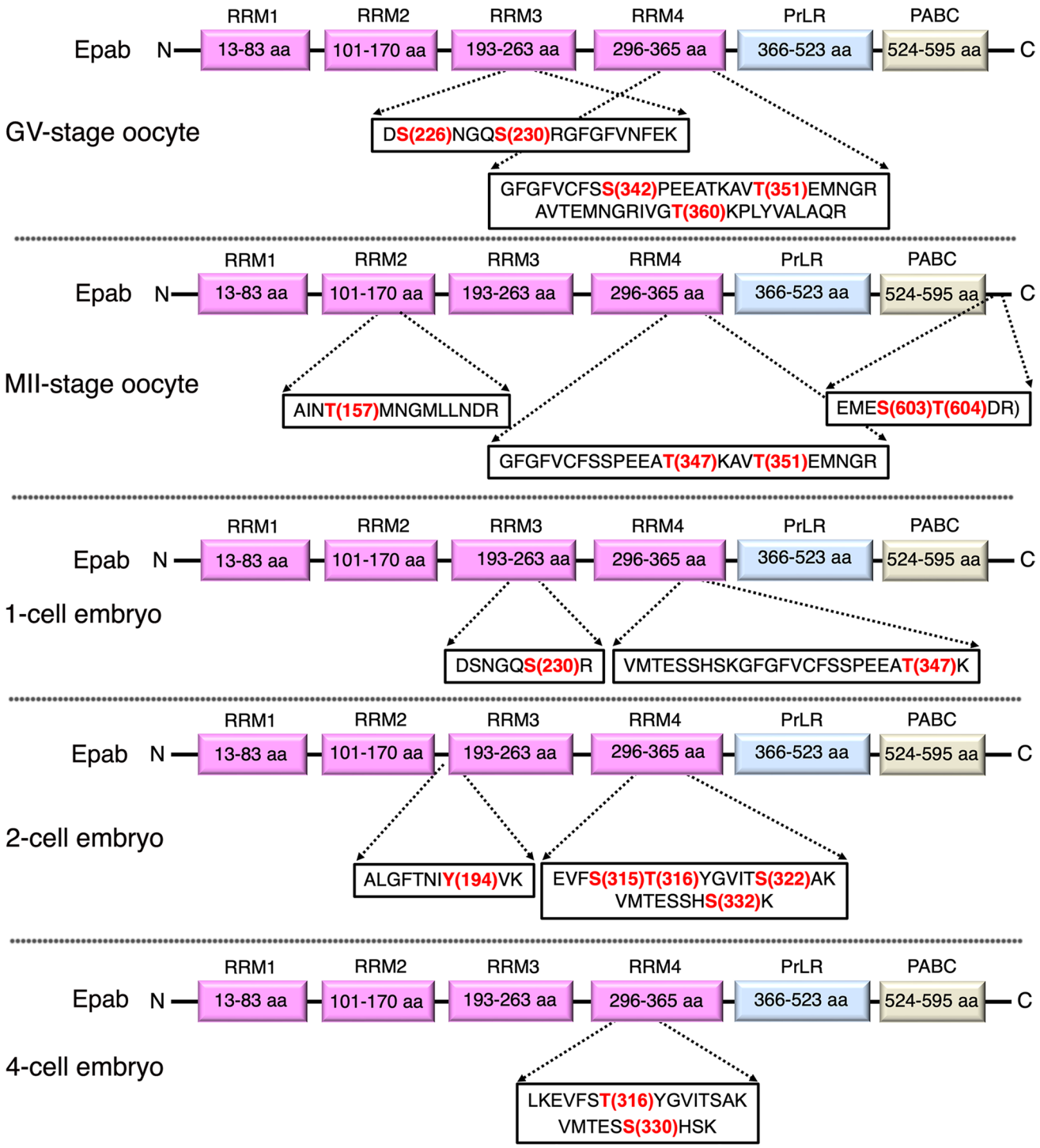


Fig. 5 Phosphorylation sites of Epab protein in GV- and MII-stage oocytes, and 1-cell, 2-cell, and 4-cell embryos. Epab underwent phosphorylation on the serine (S), threonine (T), and tyrosine (Y) residues, which are localized in the RNA recognition motifs (RRMs) 2, 3, and 4 or C-terminal region ($P < 0.05$). N, amino terminal; C, carboxyl terminal; PrLR, proline-rich linker region; PABC, poly(A)-binding protein C-terminal; aa, amino acid

undergoes phosphorylation on the serine residues at the positions of 460, 461, and 464, and from threonine residue at 465 [27]. These residues are located in the PrLR site between RRM4 and PABC domain. In the failure of establishing these phosphorylations, oocyte maturation cannot be resumed. It was also demonstrated herein that while Epab

phosphorylation is required for cytoplasmic polyadenylation, this modification is not essential for translational activation of the maternal mRNAs and subcellular localization (cytoplasm or nucleus) of Epab [27].

Similar to the previous results, we detected Epab phosphorylation in the serine and threonine residues in mouse GV and MII oocytes. However, these phosphorylation sites were located in the RRM2, RRM3, RRM4, or C-terminal regions of Epab, but not in the PrLR site. Importantly, phosphorylation sites in the serine and threonine residues exhibited differences in the GV and MII oocytes, except for the T351 phosphorylation, detected in both stages. In the 1-cell, 2-cell, and 4-cell stage embryos, additional phosphorylation sites were defined in the serine and threonine residues, localizing in the RRM3 and RRM4 regions. It is worth noting that we discovered a further phosphorylation in the tyrosine residue (Y194) of RRM3 in the 2-cell embryos. These findings suggest that Epab exhibits different phosphorylation patterns in the mammalian model organism, mouse, oocytes and early embryos.

As RRM2-4 are the key players in regulating translational activity through binding to poly(A) tails of maternal mRNAs [3, 6], Eif4g [7], and Paip1 [8], Epab phosphorylation may provide functional divergence during oocyte maturation and early embryo development. Given the reported functions of Epab in the frog oocytes upon phosphorylation [27], the potential effects of Epab phosphorylation on cytoplasmic polyadenylation, subcellular localization, translation regulation, and even its additional roles should be investigated in the mammalian oocytes and early embryos. Besides, further studies are required for describing intracellular protein kinases and phosphatases, acting on modulation of Epab phosphorylation.

All findings in the current study led us to debate on potential roles of Epab in mammalian reproductive biology and in assisted reproduction. As Epab primarily functions in translational regulation of maternally stored mRNAs during oocyte maturation and early preimplantation embryo development, its interaction status with cytoplasmic proteins and phosphorylation states may be an important determinant to predict oocyte and embryo development potential. For this purpose, Epab interaction capacity and phosphorylation status can be examined in the developmentally arrested oocytes and embryos because impairment in the transcriptional control may occur in these situations [50, 51]. For maintaining a success rate of developmental progression of oocytes and embryos, maternal mRNA clearance must also take place in a time-dependent manner [52, 53]. It would be interesting to analyze Epab levels in the oocytes and embryos, failing in timely clearing maternal mRNAs following EGA. As a result, evaluating phosphorylation state, expression level, and interaction capacity of Epab in oocytes and embryos may contribute to creating a biomarker to predict developmental competency of oocytes and embryos. Novel findings on this subject might help to increase the success rate of assisted reproductive technology (ART) applications.

As couples delay childbearing due to various reasons over the last decades, elucidating the molecular background of fertility loss accompanying to the advancing maternal age becomes more important. Although no study has examined any effect of maternal aging on Epab levels, compromised global transcriptome was noted in the aged oocytes [54] and embryos [55]. Therefore, considering the basic role of Epab in protecting mRNAs from deadenylation and thereby degradation, investigating phosphorylation state and protein interaction status of Epab in aging oocytes and embryos may contribute to determining the transcriptomic changes accompanying with biological aging.

Despite revealing important findings and future perspectives in the current study, there are some limitations and promising future studies based on these preliminary findings: (i) Identified Epab-interacting proteins should also be confirmed by further experiments (such as immunoprecipitation followed by western blotting) to increase reliability of the proteomics data. (ii) Defined phosphorylations in Epab protein should also be confirmed by gel mobility shift assay and/or specific antibody detection experiments. In the future studies, (iii) dynamic existence of these phosphorylations can be evaluated by a site-specific mutagenesis to reveal their functional importance. Furthermore, (iv) physiological significance of these phosphorylations on Epab interaction with mRNAs and other proteins merits further investigation in oocytes and embryos.

Conclusion

In this study, we firstly characterized the Epab-interacting proteins in developing mouse oocytes and early embryos, in which those proteins may play roles from transcriptional control to a wide range of cellular events such as protein modifications, metabolic processes, intracellular signaling, and chromatin remodeling. Secondly, we demonstrated that Epab undergoes a dynamic phosphorylation pattern on the serine, threonine, and tyrosine residues during these developmental stages. From that clinical point of view, we conclusively suggest that monitoring the Epab-interacting protein level and its phosphorylation status may yield clues to the molecular background of failed oocytes and embryos that exhibit developmental progression defects and implantation failures in ART.

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Data availability The datasets generated during the current study are available from the corresponding author on request.

Declarations

Ethical approval All experimental protocols were performed in accordance with relevant guidelines and regulations approved by the Akdeniz University Institutional Animal Care and Use Committee (protocol number: 1286/2021.04.011).

Informed consent Not applicable.

Conflict of interest The authors declare no competing interests.

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