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CELLULAR & MOLECULAR BIOLOGY LETTERS
http://www.cmbl.org.pl

Received: 10 January 2012
Final form accepted: 28 May 2012
Published online: 07 June 2012

Volume 17 (2012) pp 433-445
DOI: 10.2478/s11658-012-0022-7


Short communication

# *De novo* SYNTHESIS OF PROTEIN PHOSPHATASE 1A, MAGNESIUM DEPENDENT, ALPHA ISOFORM (PPM1A) DURING OOCYTE MATURATION

DANA CHUDERLAND[1§], ZEEV DVASHI[2§], RUTH KAPLAN-KRAICER[1], DANIELLA BEN-MEIR[2], RUTH SHALGI[1]* and SARA LAVI[2]

[1]Department of Cell and Developmental Biology, Sackler Faculty of Medicine, [2]Cell Research and Immunology, The George S. Wise Faculty of Life Sciences, Tel-Aviv University, Ramat-Aviv, Tel-Aviv 69978, Israel

**Abstract:** Oocyte maturation in mammals is a multiple-stage process that generates fertilizable oocytes. Ovarian oocytes are arrested at prophase of the first meiotic division characterized by the presence of a germinal vesicle. Towards ovulation, the oocytes resume meiosis and proceed to the second metaphase in a process known as maturation; they undergo nuclear and cytoplasmic changes that are accompanied by translation and degradation of mRNA. Protein phosphatase 1A, magnesium dependent, alpha isoform (PPM1A), which belongs to the metal-dependent serine/threonine protein phosphatase family, is highly conserved during evolution. PPM1A plays a significant role in many cellular functions such as cell cycle progression, apoptosis and cellular differentiation. It works through diverse signaling pathways, including p38 MAP kinase JNK and transforming growth factor beta (TGF-β). Herein we report that PPM1A is expressed in mouse oocytes and that its mRNA level rises during oocyte maturation. Using quantitative real-time polymerase chain reaction (qPCR) and western blot analysis, we found that PPM1A mRNA is synthesized at the beginning of the maturation process and remains elevated in the mature oocytes, promoting the accumulation of PPM1A

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§ Both authors contributed equally to the work

* Author for correspondence. e-mail: shalgir@post.tau.ac.il, tel.: +972-3-6408685, fax: +972-3-6407432

Abbreviations used: GV – germinal vesicle; GVBD – GV breakdown; hCG – human chorionic gonadotropin; IVM – *in vitro* maturation; LH – luteinizing hormone; MII – second meiotic division; MAPK – mitogen-activated protein kinase; MPF – maturation promoting factor; Phos-p38 – phospho-p38-MAPK; PP1 – protein phosphatase 1; PPM1A – protein phosphates 1A, magnesium dependent, alpha isoform; RPL34 – ribosomal protein L34

protein. Since PPM1A function is mainly affected by its level, we propose that it might have an important role in oocyte maturation.

**Key words:** GV, MII, Transcription, Signal transduction, Phosphatase, P38-MAPK, Oocyte, Ovary, PPM1A, Maturation

## INTRODUCTION

Meiosis of mammalian oocytes is initiated during embryonic life and arrests around birth, at the diplotene stage (prophase of the first meiotic division), characterized by the presence of a germinal vesicle (GV). GV oocytes are enclosed in a single layer of flat granulosa cells to form primordial follicles that serve as a resting pool [1]. As females reach puberty, cohorts of primordial follicles are recruited from the resting pool to start the growth phase (folliculogenesis [2]). Following stimulation by pituitary luteinizing hormone (LH), the fully grown oocytes resume meiosis, starting with GV breakdown (GVBD), spindle formation during the first meiotic division, segregation of homologous chromosomes, extrusion of the first polar body and an arrest at metaphase of the second meiotic division (MII) until fertilization [3]. The processes of folliculogenesis and oocyte maturation involve many molecular changes that yield competent oocytes, ready to be fertilized.

Mouse oocytes undergo dramatic changes in protein synthesis during the meiotic divisions, when more than half of the oocyte mRNA is either de-adenylated or degraded. Synthesis of new transcripts is essential for progression of the oocytes through maturation, fertilization and oocyte-to-embryo transition. Proteins with up-regulated synthesis during the GV-to-MII transition are mostly those involved in DNA replication and amino acid metabolism, as well as G-protein coupled receptors and signaling molecules. The down-regulated proteins are those involved in protein metabolism and electron transport, as well as ribosomal proteins and others [4]. Since the oocyte-to-embryo transition is a transcriptional quiescent stage, the post-transcriptional and post-translational processes need to be orchestrated in a manner that will facilitate the proper execution of these multi-step biochemical processes [5].

To date, little is known regarding the network operating during oocyte maturation and activation, which is different from the one operating in blastomeres or somatic cells. It is well established that signaling-molecule-mediated phosphorylation/dephosphorylation processes play essential roles in oocyte meiotic divisions [6-8]. Maturation promoting factor (MPF) and other cyclin-dependent kinases are key molecules involved in regulating the progression of meiotic and mitotic divisions. In addition, the mitogen-activated protein kinase (MAPK) cascade was shown to regulate the progression of meiosis in oocytes by activating MPF [3]. Though much is known regarding the role of the phosphorylating protein kinases in regulating meiosis in mouse oocytes, little is known about the role of the dephosphorylating protein, phosphatases, in the process. However, there are several examples that

demonstrate their essential role: CDC25A, a dual-specificity phosphatase that functions in resumption of meiosis, formation of the MI spindle and the MI-MII transition[7]; protein phosphatase 1 (PP1) and PP2A, serine/threonine protein phosphatases that are involved in regulation of MAPK activity and in microtubule organization [9]; and PTEN, a phosphatase that regulates PI3K signaling and plays a cardinal role in oocyte growth [10].

Serine/threonine phosphatases are divided into four major families, PP1, PP2A, PP2B, and PPM1, depending on their substrate and bivalent cation dependency, as well as on their sensitivity to various inhibitors[11]. The PPM1 family members are highly conserved in evolution and are expressed by various organisms ranging from yeast to higher eukaryotes. These enzymes are monomeric phosphatases that depend on divalent ions such as $Mg^{++}$ and are insensitive to the known phosphatase inhibitors including okadaic acid [12]. At least sixteen different PPM1 genes have been identified so far in mammalian cells [13]. The latest studies suggest that PPM1A, a member of the PPM1 family, regulates key cellular functions via several signaling proteins such as p38-MAPK, JNK-MAPK, Smad 2/3, Axin and others [13, 14]. The precise regulatory mechanism of PPM1A is still unknown, though it was suggested that the protein levels of PPM1A determine its mode of action [11, 15]. Interestingly, PPM1A and its twin paralog PPM1B differ in their mRNA expression level during the early stages of mouse embryonic development; while PPM1B mRNA increases during the early developmental stages, only upon its decline does the PPM1A mRNA expression level increase [16].

In this study, we investigated the pattern of PPM1A expression during oocyte maturation at both RNA and protein level. We found that PPM1A protein is present in mouse ovaries in both granulosa cells and oocytes. We further found that PPM1A mRNA and protein are synthesized *de novo* during oocyte maturation. Elucidating the PPM1A pattern of expression may shed light on the process of oocyte maturation.

## MATERIALS AND METHODS

Animal care and all experiments were in accordance with institutional guidelines and were approved by the Institutional Animal Care and Use Committee, Sackler Faculty of Medicine, Tel-Aviv University, Permit ID number L-11-016.

### Reagents

Pregnant mare's serum gonadotropin (PMSG; Syncro-part, Sanofi, Paris, France), human chorionic gonadotropin (hCG) and M2 medium (Sigma, St. Louis, MO, USA). Primary antibodies: anti-actin (Millipore, Temecula, CA, USA), anti-PPM1A (monoclonal 9F4 produced in our laboratory against recombinant PPM1A), anti-phospho/general p38 (Sigma). Secondary antibodies: monoclonal Cy3-conjugated antibodies, monoclonal and polyclonal HRP-conjugated antibodies (Jackson Immunoresearch, PA, USA). DNA was stained with Hoechst 33342 (Sigma).

**Mouse model**
C57 BLACK (6-8 weeks old) mice, used in all experiments, were reared and housed at the animal facilities of the Sackler Faculty of Medicine at Tel Aviv University.

**Oocytes**
Germinal vesicle (GV) oocytes were isolated from ovaries of untreated mice into M2 medium [36] supplemented with 1 μM milrinone (Sigma) to prevent resumption of meiosis and maintain the oocytes at the GV stage [37]. Oocytes arrested at metaphase of the second meiotic division (MII): Female mice were injected with 7 IU of hCG, 48 h after administration of 5 IU PMSG. Ovulated, cumulus-enclosed oocytes were taken from the oviductal ampullae into M2 medium, 16-18 h after hCG administration. Cumulus cells were removed by a brief exposure to 400 IU/ml hyaluronidase (Sigma).

**Granulosa and cumulus cells**
Primary mouse granulosa cells were isolated from ovaries of 7-week-old mice, pre-treated 48 hours earlier with 5 IU PMSG. The ovaries were needle-pricked and the oocytes and granulosa cells were collected and subjected to RNA isolation. Primary mouse cumulus cells were isolated from the oviducts of 7-week-old mice, pre-treated with PMSG (5 IU) and hCG (7 IU). The cumulus-oocyte complexes were briefly exposed to 400 IU/ml hyaluronidase and the cumulus cells were collected.

**Immunofluorescence**
Zonae pellucidae (ZP) were removed from the oocytes by alpha-chymotrypsin (50 μg/ml in 1 mM HCl; Sigma). The ZP-free oocytes were fixed by 3% paraformaldehyde (Merck, Gibbstown, NJ, USA), washed in blocking solution (3% fetal bovine serum in Dulbecco's phosphate buffer (DPBS)) and permeabilized (10 min in 0.05% Nonidet P-40; Sigma). Oocytes were further incubated for 1.5 h with anti-PPM1A antibody, washed in blocking solution and incubated for an additional 1 h with Cy3-conjugated secondary antibody [38] together with Hoechst 33342. Stained oocytes were visualized and photographed by a Leica laser confocal microscope (SP5, Wetzlar, Germany).

**Immunohistochemistry**
Paraffin-embedded sections of ovaries from 7-week-old mice were deparaffinized, microwave heated while being subjected to an antigen retrieval agent (H-3300, Vector Laboratories Inc., Burlingame, CA, USA), cooled on ice to room temperature, rinsed in PBS, incubated for 1 h with PBSTg (0.2% Tween and gelatin in PBS), washed with PBS, blocked for 10 min in blocking solution (927B; Cell Marque Corporation, CA, USA) and incubated overnight with anti-PPM1A antibody. On the following day, sections were washed in PBSTg and PBS before and after applying the appropriate secondary antibodies together with nuclear marker (Hoechst). Sections were rinsed, mounted with Mowiol

(Sigma) and photographed. The photographs were de-convoluted and processed using a Leica laser confocal microscope (SP5, Wetzlar, Germany).

**Immunoblotting**

Oocytes (250) were collected into ice-cold radioimmunoprecipitation assay buffer (RIPA; 20 mM Tris•HCl pH=7.4, 137 mM NaCl, 10% glycerol, 2% NP-40 or 1% triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, pH=8) and kept at -20°C for several hours. When defrosted, an appropriate sample buffer (200 mM Tris pH=6.8, 10% glycerol, 2% SDS, 100 mM DTT and Bromo-phenol-blue) was added and the samples were subjected to SDS-PAGE, immunoblotted with the appropriate primary antibodies (anti-PPM1A, or anti-actin), followed by incubation with the corresponding horseradish peroxidase-conjugated secondary antibodies and subjected to enhanced chemiluminescence assay (ECL; Thermo Scientific, IL, USA).

**RNA isolation, reverse transcription (RT) PCR, and quantitative real-time PCR (qPCR)**

Total RNA was isolated using Trizol (Invitrogen, Grand Island, NY, USA). First-strand cDNA was created by RT (MaximaTMReverse transcriptase, Fermentas UAB, Lithuania) from the cells' total RNA. Alternatively, we synthesized cDNA from 60 oocytes using a highly sensitive kit (BioRad Reverse Transcription System, Hercules, CA, USA). We then followed the expression of mRNAs using three sets of primers: PPM1A 5'-ggc aagcaagcggaatgt aa-3' and 3'-cat atcatcggttgacgcaga a-5', HPRT1 5'-cag gccagactttgttgg at-3' and 3'-gtg cgctcatct tag gct-5', and RPL34 5'-aga aggttgggaaagcacct -3' and 3'-ata aggaaagcccgcttg at-5'. The reactions were carried out according to the manufacturer's protocol (SYBR Green qPCR Kit, Thermo Scientific) using a Corbett Research RG-6000 real-time detection system. Assays for each sample and primer set were performed in triplicate, with each reaction using 1.5 µl of diluted cDNA and 0.3 µM primer in a total reaction volume of 10 µl. Absolute quantification was determined using a standard curve for each gene in this study. Absolute levels of cDNA were calculated from a standard curve established using the cDNA standards (1:8, 1:32, 1:128, 1:512 and 1:2048), and the ratio between the gene of interest and the normalizing gene were calculated. The PCR protocol includes a denaturation program (95°C for 15 min), 40 repeats of an amplification and quantification program (95°C for 15 s, 60°C for 15 s and 72°C for 20 s) and a melting curve program (72-95°C).

**Statistics and quantification**

The fluorescence intensity of PPM1A within the oocyte as well as the relative pixel intensity of the protein of interest such as PPM1A and actin were measured with ImageJ software (NIH, Bethesda, MD) as mean pixel value/area unit. All statistics were computed using Student's t-test, two-tailed distribution and equal variance.

## RESULTS

In view of the tight regulation of PPM1A in embryogenesis, we wished to study the function of PPM1A in oocytes, prior to fertilization, and started with characterization of its expression pattern in the ovary. Histological sections of C57 Black mice ovaries were immunostained with an anti-PPM1A antibody. We found that PPM1A is highly expressed in both oocytes and granulosa cells (Fig. 1). Consequently we looked for the expression of PPM1A in isolated ovarian immature GV oocytes as well as in ovulated, MII oocytes, and detected the protein in both types of oocytes (Fig. 2A). Interestingly, quantifying the fluorescence intensity indicated an up-regulation of the PPM1A protein during the transition from GV to MII, manifested by a 4-fold higher expression of the phosphatase in MII oocytes than in GV oocytes (Fig. 2B). This change in protein expression was verified by western blot analysis (Fig. 2C, D).

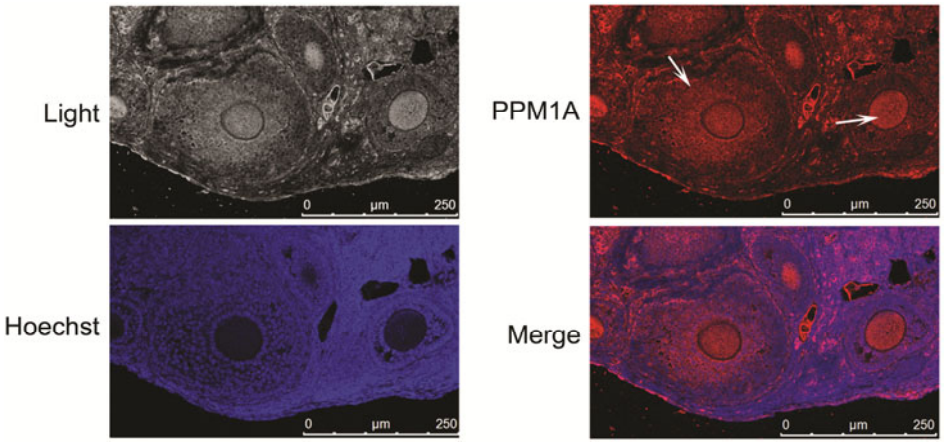


Fig. 1. Expression of PPM1A in the ovary. Histological sections of 7-week-old C57 Black mouse ovaries labeled with mouse monoclonal anti-PPM1A antibody (red) and Hoechst (blue) as a nuclear marker. The labeled sections were visualized and photographed with a Leica Laser confocal microscope. PPM1A was found within the follicles, in the oocytes and their surrounding granulosa cells (arrows).

### PPM1A expression is specifically enhanced in the oocytes

It is well established that the communications between oocytes and their surrounding granulosa cells is bidirectional, meaning that during oogenesis and maturation both cell types secrete factors that regulate each other [17]. In view of the up-regulation of PPM1A protein in mature MII oocytes, we traced the origin of its biosynthesis by qPCR analysis of oocytes collected before (GV) and after *in vivo* human chorionic gonadotropin (hCG) administration (ovulated MII). We found that PPM1A mRNA is synthesized in the oocytes during their maturation, but not in the somatic component of the follicles (Fig. 3A) since no change in its level was detected in pre-ovulatory granulosa cells or post-ovulatory cells (cumulus; Fig. 3B; [18]). Thus, while granulosa cells kept

a constant level of mRNA expression, regardless of hCG stimulation, *de novo* PPM1A biosynthesis was restricted to maturing oocytes.

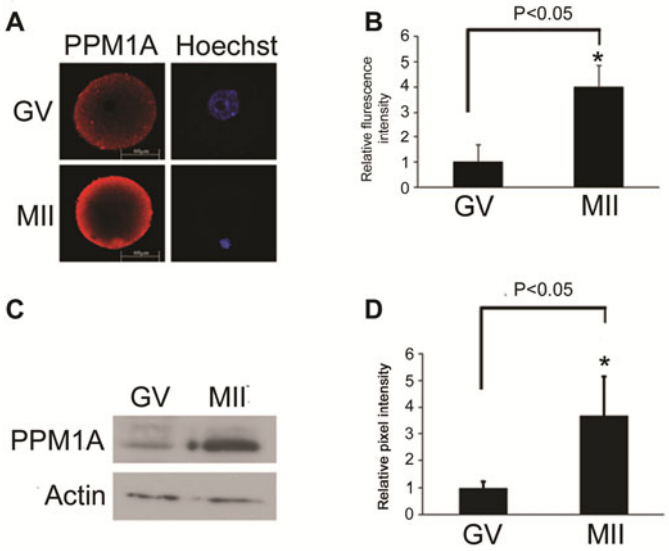


Fig. 2. PPM1A is highly expressed in mature oocytes. A – Freshly isolated, ovarian GV oocytes (top, from PMSG (5 IU) synchronized mice) and ovulated MII oocytes (bottom; from PMSG (5 IU) and hCG (7 IU) superovulated mice) labeled with anti-PPM1A antibody (red). Hoechst (blue) was used as a nuclear marker. B – Oocyte fluorescence quantified by the ImageJ analysis tools. Bars are mean ± SD, 30 oocytes per treatment. Statistical analysis includes Student's *t*-test, two-tailed distribution and equal variance. * – significantly different from GV value (arbitrarily set as 1; $P < 0.05$; *t*-test). C – Relative amounts of PPM1A in GV and MII mice oocytes. Both oocyte types were isolated from mice treated as in A. Western blot analysis of PPM1A from 250 GV or MII oocytes. The amount of total protein was calibrated with anti-actin antibody. D – Relative amounts of expressed PPM1A protein in GV and MII oocytes calibrated to actin antibody quantified by the ImageJ analysis tools. The amount in GV oocytes was arbitrarily set as 1.

Oocytes re-program their protein repertoire during maturation, including up-regulation and degradation of many mRNA species. To validate our results, we followed the ribosomal protein L34 (RPL34), known to be degraded during the process of oocyte maturation [4]. As expected, qPCR analysis confirmed reduction in the expression level of RPL34 mRNA during the transition from GV to MII (Fig. 3A), demonstrating that the enhanced PPM1A mRNA synthesis is specific to PPM1A.

In an attempt to find out at which step of the multistage process of oocyte maturation PPM1A mRNA is up-regulated, we utilized the spontaneous *in vitro* maturation (IVM) properties of GV oocytes. First we evaluated the ability of PPM1A to undergo up-regulation in oocytes during IVM [19], and found it to be similar to that of *in vivo* matured oocytes (Fig. 3A); namely, PPM1A mRNA was significantly up-regulated in *in vitro* matured oocytes compared to GV oocytes

(Fig. 4A). Next, we examined at what step of the oocyte maturation process PPM1A mRNA is elevated. We found that 2 hours after the onset of the IVM process, at the time of GVBD, an enhancement in PPM1A transcription could be detected (Fig. 4B).

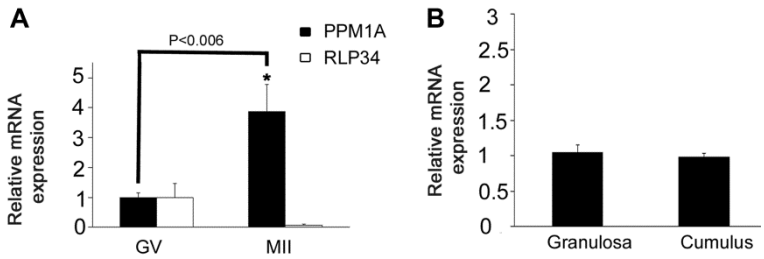


Fig. 3. PPM1A is synthesized in oocytes during maturation. GV oocytes and pre-ovulatory granulosa cells were isolated from PMSG (5 IU)-primed C57 Black mice; MII oocytes and post-ovulatory granulosa cells (cumulus) were isolated from oviductal ampullae of PMSG (5 IU) and hCG (7 IU) superovulated C57 Black mice. A – Total mRNA was extracted from equal numbers of oocytes at the GV and MII stages and subjected to qPCR analysis with specific primers for PPM1A or RPL34 and normalized with the endogenous control HPRT1. Bars are mean ± SD, representing 4 independent experiments, 60 oocytes per experiment. Statistics were computed using Student's *t*-test, two-tailed distribution, and equal variance. * – Significantly different from GV value (arbitrarily as 1; $P < 0.006$ *t*-test). B – Pre- and post-ovulatory granulosa cells were subjected to RT-qPCR analysis with specific primers for PPM1A and normalized with the endogenous control HPRT1. Pre-ovulatory granulosa cell value was arbitrarily set as 1.

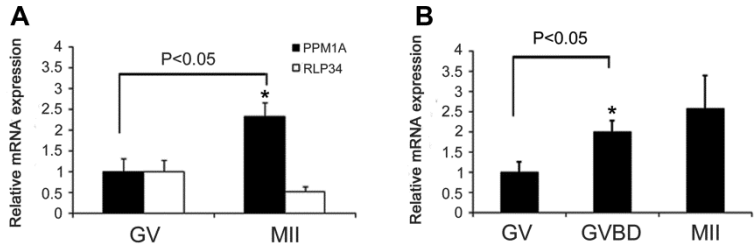


Fig. 4. PPM1A mRNA is up-regulated at the GVBD stage. GV oocytes were isolated from ovaries of PMSG (5 IU) primed C57 Black mice. Oocytes were incubated in M2 medium (37°C, 5% $CO_2$) for *in vitro* maturation. Oocytes were collected after incubation for 1.5 h (GVBD) or 16 h (MII). A – GV oocytes and *in vitro* matured MII oocytes were subjected to RT-qPCR analysis using specific primer for PPM1A or RPL34 and calibrated with the endogenous control HPRT1. Bars are mean ± SD (3 independent experiments; 60 oocytes per treatment). * – Significantly different from GV value (arbitrarily as 1; $P < 0.05$; *t*-test). B – GVBD oocytes and *in vitro* matured MII oocytes were subjected to RT-qPCR analysis with specific primers for PPM1A, calibrated with the endogenous control HPRT1. Bars are mean ± SD (3 independent experiments; 50 oocytes per treatment). * – Significantly different from GV value (arbitrarily set as 1; $P < 0.05$; *t*-test).

**De-phosphorylation of p38 is essential for the completion of oocyte maturation**

Signal transduction cascades in general, and those of the MAPK family in particular, include events of phosphorylation and de-phosphorylation. The cascade of ERK-MAPK, a member of the MAPK family, was extensively studied and was shown to be essential for the development of healthy mature rodent oocytes [20]. The less studied p38-MAPK cascade was suggested to be involved in maturation of porcine [21] and *Xenopus* [22] oocytes. In view of the fact that PPM1A is a known phosphatase of p38-MAPK [23], and of our findings showing that PPM1A is up-regulated in mouse oocytes during the transition from GV to MII, we examined the kinase activity of p38-MAPK during maturation of mouse oocytes and found that the level of phospho-p38-MAPK (Phos-p38) was higher in GV oocytes than in MII oocytes (Fig. 5). Induction of PPM1A expression together with decreased p38-MAPK phosphorylation during the process of oocyte maturation suggests a possible role of PPM1A in regulation of oocyte maturation via p38 phosphorylation.

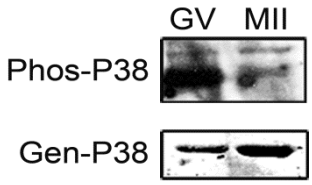


Fig. 5. The role of phosphorylated p38-MAPK in oocyte maturation. Western blot analysis of C57 Black mice oocytes: 150 freshly isolated ovarian GV oocytes (5 IU PMSG) and ovulated MII oocytes (PMSG (5 IU) and hCG (7 IU)). Blots were subjected to specific anti-phosphorylated p38-MAPK (Phos-p38) antibody. The amount of total protein was calibrated with anti-general p38-MAPK (gen-p38) antibody.

## DISCUSSION

The ovary is a highly dynamic organ, regulated by various signaling pathways to induce folliculogenesis and oocyte maturation. Several studies have recently pointed out, by gene array analysis, the presence of the PPM1 family in general and of PPM1A in particular in oocytes and pre-implantation embryos [16, 24, 25]. Still, as PPM1A activity is determined by its protein levels [11, 15], it is important to determine its expression at both RNA and protein levels.

PPM1A was found to be regulated at the level of RNA and protein expression. Several studies have demonstrated elevated PPM1A transcription during cell differentiation [26]. The stability of PPM1A protein was shown to be regulated through its association with PTEN in TGF-β treated cells [27]. In addition, we and others have demonstrated that subcellular localization may affect PPM1A activity. Stimulation of PC12 cells with nerve growth factor (NGF) induced PPM1A nuclear localization, which in turn directed them to differentiation [28]. During hepatocarcinoma progression, PPM1A expression was shifted from the nucleus to the cytoplasm [29]. PPM1A was also suggested to be regulated by

phosphorylation of casein kinase II. However, this phosphorylation was not shown to directly affect its activity [15].

In this work we showed for the first time that PPM1A is expressed in the mouse ovary in both granulosa cells and oocytes. Furthermore, we have demonstrated a significant elevation in both PPM1A mRNA and protein during oocyte maturation. Enhanced PPM1A mRNA and protein were observed at the GVBD stage. Since PPM1A protein levels were suggested to be a key factor for its activation [11, 15], we speculate that accumulation of PPM1A has an essential role in regulating post-translation modification processes during oogenesis. Moreover, the fact that PPM1B was found to be elevated during early stages of pre-implantation in embryonic development, and knockout of PPM1B led to embryonic lethality, indicates the important role of the PPM1 family in mouse embryonic development [30].

Though a growing mouse oocyte, arrested at diplotene of the first meiotic prophase, is transcriptionally and translationally active, a large number of synthesized mRNAs are not used for immediate translation but instead are stored to support oocyte maturation and early pre-implantation embryogenesis [31]. When the oocyte enters the MII phase the transcription processes are stopped. Transcription renewal resumes in the 2-4 cell embryos [24]. The stringent control of phosphorylation is important for various cellular events and for cell cycle regulation [32], but is much more significant in mouse oocytes, since they undergo a series of cell divisions and cleavages during oocyte maturation and early embryonic development, in the absence of gene transcription. We found that PPM1A is up-regulated prior to GVBD during oocyte maturation. We can therefore predict that an increase in PPM1A levels may regulate several signaling pathways within the oocyte, as in the case of adipocytes, where PPM1A expression is elevated upon differentiation [11]. For example, TGF-β signaling, which plays a crucial role in oocyte maturation and fertilization [33], may be governed by its negative regulator PPM1A. Similarly, P38-MAPK, which is known for its important role in uncoupling the communication between cumulus cells and the oocyte after LH stimulation, might be affected by this phosphatase [34, 35]. The important role of phosphatases in oocyte maturation was demonstrated by regulation of the dual specific phosphatase Cdc25, which promotes oocyte maturation by reversing the inhibitory phosphorylation that keeps the oocytes at the GV stage, and by activation of MPF to promote progression of the cell cycle [6]. The elevation of PPM1A close to GVBD may imply its role in the upcoming cell cycle events such as chromatin reorganization, spindle formation and polar body extrusion.

In summary, we have characterized the expression profile of PPM1A in mouse ovaries and shown that PPM1A mRNA and protein were significantly up-regulated at the GVBD stage during the process of oocyte maturation, indicating a putative role for PPM1A in the fine tuning of the signal transduction within the process of oocyte maturation.

**Declaration of interest.** We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

**Acknowledgments.** This work is a partial fulfillment of the requirements for the Ph.D. degree of Zeev Dvashi at the Faculty of Life Sciences, Tel-Aviv University. This work was supported by the Linda Tallen and David Paul Kane Educational and Research Foundation and the Israel Ministry of Health (MOH) to SL.

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