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Transducin-Like Enhancer of Split-6 (TLE6) Is a Substrate of Protein Kinase A Activity During Mouse Oocyte Maturation[1]

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ABSTRACT

Fully grown oocytes in the ovary are arrested at prophase of meiosis I because of high levels of intraoocyte cAMP that maintain increased levels of cAMP-dependent protein kinase (PKA) activity. Following the luteinizing hormone surge at the time of ovulation, cAMP levels drop, resulting in a reduction in PKA activity that triggers meiotic resumption. Although much is known about the molecular mechanisms of how PKA activity fluctuations initiate the oocyte's reentry into meiosis, significantly less is known about the requirement for PKA activity in the oocyte after exit from the prophase I arrest. Here we show that although PKA activity decreases in the oocyte upon meiotic resumption, it increases throughout meiotic progression from the time of germinal vesicle breakdown (GVBD) until the metaphase II (MII) arrest. Blocking this meiotic maturation-associated increase in PKA activity using the pharmacological inhibitor H89 resulted in altered kinetics of GVBD, defects in chromatin and spindle dynamics, and decreased ability of oocytes to reach MII. These effects appear to be largely PKA specific because inhibitors targeting other kinases did not have the same outcomes. To determine potential proteins that may require PKA phosphorylation during meiosis, we separated oocyte protein extracts on an SDS-PAGE gel, extracted regions of the gel that had corresponding immune reactivity towards an anti-PKA substrate antibody, and performed mass spectrometry and microsequencing. Using this approach, we identified transducin-like enhancer of split-6 (TLE6)—a maternal effect gene that is part of the subcortical maternal complex—as a putative PKA substrate. TLE6 localized to the oocyte cortex throughout meiosis in a manner that is spatially and temporally consistent with the localization of critical PKA subunits. Moreover, we demonstrated that TLE6 becomes phosphorylated in a narrow window following meiotic resumption, and H89 treatment can completely block this phosphorylation when added prior to GVBD but not after. Taken together, these results highlight the importance of oocyte-intrinsic PKA in regulating meiotic progression after the prophase I arrest and offer new insights into downstream targets of its activity.

AKAP, meiosis, oocyte maturation, phosphorylation, PKA, subcortical maternal complex

INTRODUCTION

The cAMP-dependent protein kinase, protein kinase A (PKA), is a critical physiological inhibitor of meiotic resumption in mammalian oocytes. A key role for cAMP was recognized decades ago when it was observed that removal of prophase I-arrested oocytes from ovarian follicles resulted in spontaneous resumption of meiosis unless the oocytes were cultured in the presence of either cell-permeable cAMP analogs such as dibutyryl cAMP or phosphodiesterase inhibitors that prevent cAMP degradation [1–3]. Later it was demonstrated that microinjection into oocytes of a heat-stable protein inhibitor of PKA can induce resumption of meiosis even in the presence of dibutyryl cAMP, indicating that the cAMP effect is solely mediated by PKA [4]. More recent studies further clarified this signaling pathway by showing that within the ovarian follicle, oocyte meiotic arrest is maintained by the action of G protein-coupled receptor 3, which constitutively activates G_s protein and thereby causes persistent adenylate cyclase-mediated production of intraoocyte cAMP, maintaining PKA in an active state [5–7]. Granulosa cells support maintenance of meiotic arrest by generating cGMP, which enters the oocyte via gap junctions and inhibits oocyte cAMP phosphodiesterase 3A, preventing cAMP hydrolysis [8, 9]. All of these studies support the idea that appropriate levels of PKA activity are necessary and sufficient to maintain meiotic arrest, and that a decrease in PKA activity is sufficient to induce resumption of meiosis.

PKA activity maintains meiotic arrest by preventing activation of maturation-promoting factor (MPF), a protein complex comprised of cyclin B and cyclin-dependent kinase-1 (CDK1). At least two proteins that regulate MPF activity are phosphorylated by PKA in ways that result in MPF inhibition. Phosphorylation of CDC25B by PKA causes restriction of CDC25B to the oocyte cytoplasm, thereby preventing this phosphatase from dephosphorylating and activating CDK1 in the nucleus [10]. PKA-mediated phosphorylation of nuclear WEE2 protein tyrosine kinase increases its ability to phosphorylate and inactivate CDK1 [11]. These dual actions

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of PKA serve to prevent nuclear MPF-mediated phosphorylation events required for the resumption of meiosis.

PKA activity is tightly regulated by spatial localization via scaffolding proteins known as A-kinase anchor proteins (AKAPs) [12]. AKAPs bind multiple components of cAMP/PKA signaling pathways, including PKA, its downstream targets, phosphodiesterases that limit PKA activation by degrading cAMP, and protein phosphatases that dephosphorylate PKA substrates. AKAP-mediated spatial restriction serves as a mechanism to generate specific, localized, and temporally controlled actions within cells in response to elevation of cAMP levels. We have previously shown that the PKA regulatory (RI and RII) and catalytic subunits are compartmentalized in different regions of the mouse oocyte and that their localization changes during oocyte maturation, suggesting that AKAPs are important in regulating PKA actions in oocytes [13]. Oocytes contain several AKAPs that target PKA to different subcellular compartments, including AKAP1 (mitochondria), AKAP7γ (cortex and nucleus), ezrin (cortex), WAVE1 (cortex and cytoplasm) and pericentrin (centrosomes) [13–20]. AKAP1-null oocytes are defective in their ability to resume meiosis [14, 17], suggesting not only that PKA maintains meiotic arrest but that its activity at mitochondria is important for meiotic resumption. Alternatively, AKAP1 could serve to inhibit PKA activity by sequestering it in the mitochondrial compartment and away from critical substrates, so that in the absence of AKAP1, PKA inappropriately maintains its meiosis-inhibiting activities.

Although PKA is best known as an inhibitor of meiotic resumption in oocytes, less is known about its role in regulating meiotic progression following exit from the prophase I arrest. Previously we have demonstrated that PKA activity regulates meiotic spindle dynamics [21]. However, the precise role of PKA activity and the identity of specific PKA substrates phosphorylated during meiotic maturation are unknown. In this report, we use pharmacological inhibitors to determine if PKA activity is essential for meiotic progression, and we use a proteomics approach to identify PKA substrates phosphorylated during mouse oocyte maturation. We find that transducin-like enhancer of split-6 (TLE6), a highly abundant oocyte protein and component of the subcortical maternal complex (SCMC) [22], is phosphorylated by PKA at the time of germinal vesicle breakdown (GVBD).

MATERIALS AND METHODS

Animals and Collection of Oocytes and Embryos

Female CF-1 mice were obtained from Harlan Sprague-Dawley (Indianapolis, IN); B6D2F1/J males used for mating were obtained from Jackson Laboratory (Bar Harbor, ME). All procedures were performed according to protocols approved by the appropriate institutional animal care and use committees at the University of Pennsylvania and the National Institutes of Health/National Institute of Environmental Health Sciences. Chemicals were from Sigma (St. Louis, MO) unless otherwise indicated. Growing oocytes were isolated from ovarian follicles of CF-1 mouse pups as described previously [23] on the following days of age: 2 (primordial), 5 (primary), 11 (secondary), 17 (small antral), and 22 (large antral). Superovulation and collection of fully grown, germinal vesicle (GV)-intact oocytes and metaphase II (MII)-arrested eggs (MII eggs) were carried out as described previously [24] except that the collection medium was Whitten medium [25] supplemented with 15 mM HEPES and 0.01% polyvinyl alcohol. GV-intact oocytes were initially cultured in CZB medium (catalog no. MR-019-D; EMD Millipore, Bedford, MA) [26] containing 2.5 μM milrinone to inhibit resumption of meiosis [27]. To obtain cells at the GVBD, metaphase I (MI), or MII stages of meiosis, GV oocytes were washed free of milrinone and in vitro matured in CZB for 90 min, 8–9 h, or 15 h, respectively.

Kemptide Assay

PKA activity was measured in pools of 10 oocytes at various stages of maturation using the SignaTECT cAMP-Dependent Protein Kinase (PKA) Assay System (Promega, Madison, WI) according to the manufacturer's instructions. Measurements of basal PKA activity were performed in the absence of exogenous cAMP, whereas measurements of total (cAMP-stimulated) PKA activity were performed in reaction buffer containing 0.025 mM cAMP.

Inhibitor Treatments

All inhibitors were from Sigma, and stock solutions were made in dimethyl sulfoxide (DMSO) such that the final dilution of DMSO in culture medium was at least 1:100. GV-intact oocytes were washed free of milrinone and cultured in CZB medium for 0, 45 or 90 min, then cultured in CZB alone or CZB containing the following inhibitors: 25–40 μM H89, 10 or 100 μM Y-27632, 1 μM Gö 6983, or 1 μM bisindolylmaleimide X (BIMX). Of note, we used a range of H89 concentrations because we observed variability in inhibitor activity based on lot, source, and how long it had been stored. We also observed that H89 was less effective when oocytes were matured in droplets overlaid with mineral oil, likely because of partitioning of the inhibitor. Incubation times for various experiments are indicated in the text and legends. At the end of treatment, the oocytes were examined under a dissecting microscope for the presence or absence of the germinal vesicle and first polar body.

Immunofluorescence

For cytoskeleton staining, zona pellucida-intact oocytes were fixed and permeabilized in 2% formaldehyde with 2% Triton X-100, 500 mM PIPES, 25 mM $MgCl_2$, and 12 mM EGTA at 37°C for 30 min. Cells were blocked in PBS containing 3 mg/ml bovine serum albumin (BSA) and 0.01% Tween 20 (blocking buffer). The microtubule cytoskeleton was immunostained using a fluorescein isothiocyanate-conjugated mouse monoclonal anti-alpha-tubulin antibody (Sigma; 1:100 dilution in blocking buffer), and the actin cytoskeleton was detected using 2 U/ml rhodamine-phalloidin (Molecular Probes, Eugene, OR). Cells were mounted in Vectashield (Vector Laboratories, Burlingame, CA) containing 2 μg/ml propidium iodide or 1.5 μg/ml 4′,6-diamidino-2-phenylindole for chromatin visualization. For TLE6 immunocytochemistry, the zonae pellucidae were removed by a brief treatment in acid Tyrode solution, pH 1.6. Cells were fixed in 3.7% paraformaldehyde, permeabilized in PBS containing 3 mg/ml BSA and 0.1% Triton X-100, and processed as described above. The primary antibody was a rabbit polyclonal antiserum raised against recombinant TLE6 [28] diluted 1:500–1:2000 in blocking buffer. The same dilution of normal rabbit serum was used as a control. The secondary antibody was AlexaFluor 488- or 633-conjugated anti-rabbit immunoglobulin G (IgG) (1:200–500; Molecular Probes). Cells were observed on a laser-scanning confocal microscope using a 40X Plan APO objective, N.A. 0.75 (Leica DMRE; Leica Microsystems, Inc., Exton, PA). Images were acquired with Leica confocal software and processed using Photoshop (Adobe Systems, San Jose, CA).

Gel Electrophoresis and Immunoblotting

Standard gels. Total oocyte or embryo proteins were separated on either 9% (w/v) or 4–12% gradient SDS-polyacrylamide gels and transferred to either Immobilon-P (Millipore, Bedford, MA) or polyvinylidene fluoride (PVDF; Thermo Scientific, Rockford, IL) membranes. The membranes were blocked in PBS containing 0.1% Tween-20 (PBST) and 5% milk. The primary antibodies were either monoclonal rabbit anti-Phospho-PKA substrate (RRXS*/T*) (catalog no. 9624; Cell Signaling, Beverly, MA) diluted 1:1000 in PBST or rabbit polyclonal anti-TLE6 [28] diluted 1:20 000 in PBST. The secondary antibody was horseradish peroxidase-conjugated anti-rabbit IgG (Amersham, Piscataway, NJ) diluted 1:5000 in PBST. Membranes were developed using enhanced chemiluminescence reagents (Amersham) following the manufacturer's protocol.

Phospho-affinity gels. Phos-tag acrylamide (Wako Chemicals USA, Richmond, VA) [29, 30] gels were made following the manufacturer's protocol using 8% (w/v) acrylamide (acrylamide:bisacrylamide = 29:1), 30 μM Mn^{2+}-Phos-tag, and 60 μM $MnCl_2$. A standard 4.5% (w/v) acrylamide gel was used for stacking. Total protein from 50 oocytes or MII eggs per lane was electrophoretically separated, immunoblotted onto PVDF membrane, blocked using Tris-buffered saline containing 0.1% Tween-20 (TBST) containing 5% milk and 5% donkey serum (TBST blocking solution), and then probed either 2 h at room temperature or overnight at 4°C with primary anti-TLE6 antibody

diluted 1:5000 in TBST blocking solution. Secondary antibody was peroxidase-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA) diluted 1:100 000 in TBST. Membranes were developed using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's protocol.

Mass Spectrometry and Microsequencing

Total protein from 50 or 500 MII eggs was separated in adjacent lanes on a 9% (w/v) SDS-polyacrylamide gel. The lane containing protein from 50 MII eggs was cut from the gel, transferred, and immunoblotted as outlined above using a rabbit anti-Phospho-PKA substrate antibody. The regions of the gel adjacent to the strongly immunoreactive bands at ~60–66 kDa and ~110–140 kDa were excised from the gel lane containing protein from 500 MII eggs. The gel region was sent to the Proteomics Core facility at the University of Pennsylvania for analysis. Identification of proteins in the gel region was performed by matrix-assisted laser desorption/ionization (MALDI)-time of flight (TOF)/TOF mass spectrometry and microsequencing as previously described [31].

Immunoprecipitation

Immunoprecipitation was done using the Pierce Classic IP Kit (Thermo Scientific) according to the manufacturer's instructions with the following modifications. Sets of 100 ovulated MII eggs were snap frozen in 100 μl of the provided IP Lysis/Wash buffer containing 1% Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific). A combined pool of 600 MII eggs was evenly divided into two tubes and 2 μg of either PKA substrate antibody (Cell Signaling Technology, Danvers, MA) or normal rabbit IgG (Santa Cruz Biotechnology, Inc., Dallas, TX) was added. The samples were incubated overnight on a rotating platform at 4°C and then the immune complexes were captured by incubation for 1 h at 4°C on a spin column containing Protein A/G agarose resin. After three washes with IP Lysis/Wash buffer, the columns were washed once with conditioning buffer, incubated 10 min at 100°C with 25 μl of reducing sample buffer, and centrifuged to collect the eluate. The samples were separated on a 4–12% Tris-glycine gel and immunoblotted for TLE6 as described above for standard gels.

Phosphatase Treatment

GV-intact oocytes and MII eggs (50 per sample) were incubated at 30°C for 30 min in 25 μl reaction buffer containing either 400 units of Lambda Protein Phosphatase (New England BioLabs, Ipswich, MA) or 1% Phosphatase Inhibitor Cocktail 3 (Sigma). The same number of oocytes and eggs were snap frozen in T-PER Tissue Protein Extraction Reagent (Thermo Scientific) containing 1% Protease Inhibitor Cocktail (Sigma) for use as controls. The control and treated oocyte and egg lysates were boiled for 3 min in reducing sample buffer and then separated on a phospho-affinity gel and immunoblotted for TLE6 as described above.

RESULTS

PKA Activity Changes During Oocyte Maturation

Although a decrease in PKA activity occurs during meiotic resumption, less is known about its activity within the oocyte during meiotic progression. To determine how PKA activity changes over the course of oocyte maturation, we assayed its activity in extracts from cells at distinct stages of meiosis, including prophase I (GV), prometaphase I/MI (GVBD), and MII (Fig. 1). This activity assay quantifies the ability of PKA within a cell to phosphorylate Kemptide, a synthetic peptide substrate derived from pyruvate kinase [32]. Both basal levels of PKA activity and total PKA present can be determined by performing this assay in the absence or presence of exogenous cAMP, respectively (Fig. 1). As expected, we found that basal PKA activity decreased significantly between GV and GVBD stages (Fig. 1A; $P < 0.001$). However, basal PKA activity increased significantly between GVBD and MII stages (Fig. 1A; $P < 0.001$). Of note, we compared the basal levels of PKA activity in MII eggs that were either matured in vitro or ovulated. In vitro-matured MII eggs had lower PKA activity compared to those that were ovulated, albeit these differences were not statistically significant (Fig. 1A). In contrast to basal PKA activity, total PKA levels were similar among all meiotic stages (Fig. 1B). Taken together, these data suggest that basal oocyte PKA activity is precisely regulated and that its increased levels following GVBD may be important for proper progression to MII.

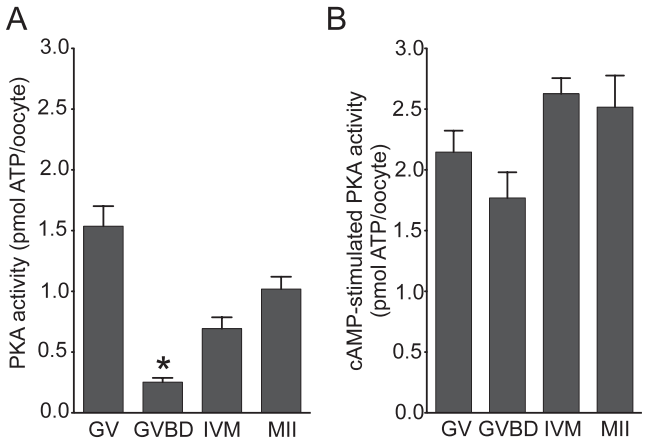

FIG. 1. Meiotic maturation-associated changes in basal and total PKA activity. Basal PKA activity (**A**) and total cAMP-stimulated PKA activity (**B**) were measured using a Kemptide assay in extracts from cells at the indicated stages of meiosis. GV, GV-intact; IVM, matured in vitro to the MII stage; MII, matured in vivo to the MII stage. The asterisk denotes that there was significantly less basal PKA activity at the GVBD stage compared to both the GV and MII stages ($P < 0.05$). Error bars indicate SEM.

PKA Activity Is Required for Meiotic Progression

To determine if the increased level of basal PKA activity observed following GVBD was required for progression to MII, we disrupted PKA activity during meiotic maturation using H89, a cell-permeable competitive inhibitor of ATP binding to the PKA catalytic subunit [33]. Specifically, we in vitro matured oocytes in the presence of between 25 and 40 μM H89 and monitored their ability to extrude the first polar body. We chose this concentration range of H89 because it was the maximum that was nontoxic but still effective (concentrations ≥50 μM resulted in oocyte death; data not shown). Culturing oocytes in the presence of 25 μM H89 resulted in a 50–60% reduction of basal PKA activity as measured using the Kemptide assay, and basal PKA activity was almost completely abolished when the inhibitor was added directly to oocyte extracts, suggesting that H89 in this system is in fact blocking PKA activity (data not shown). When oocytes were matured in the continuous presence of 25 μM H89, meiotic progression was significantly impaired. Typically, meiotic maturation takes ~12–14 h to complete in vitro. However, none of the oocytes cultured in the presence of H89 reached MII even 20 h after the onset of meiotic maturation, as evidenced by complete absence of polar body extrusion (Fig. 2A). Instead, 43% of the oocytes exposed to H89 remained GV intact and 46% underwent GVBD but did not reach MII; the remainder fragmented (Fig. 2A). In contrast, 97% of control oocytes matured in the absence of H89 reached MII in the same time frame (Fig. 2A). Inhibition of meiotic progression was also observed when H89 was added to the culture medium 90 min after meiotic resumption, a time point when most oocytes had already undergone GVBD. Under these conditions, only 5% of the oocytes reached MII compared to 100% of controls. Taken together, these results suggest that PKA activity during the first

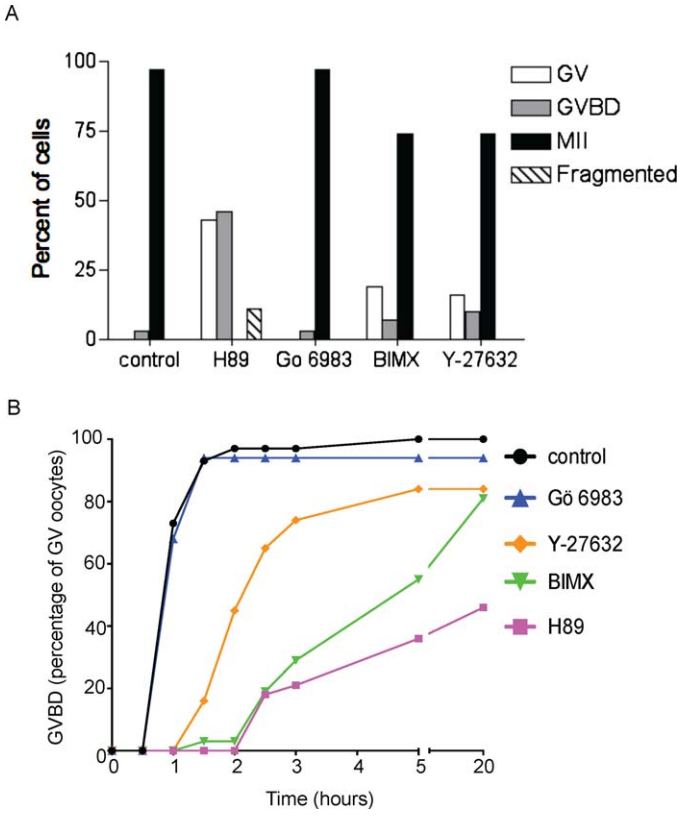

FIG. 2. The effect of H89 exposure on meiotic progression and kinetics. **A**) The effect of PKA inhibition on the ability of oocytes to progress through meiosis was monitored using the small molecular inhibitor H89. After meiotic maturation in the continuous presence of 25 μM H89, the meiotic stage of each cell was scored (GV, GV-intact). **B**) The effect of PKA inhibition on the kinetics of GVBD was also monitored over a 20-h period of meiotic maturation. As controls for both of these experiments, oocytes were either matured in medium alone (control) or in the presence of molecules with minimal inhibitory activity toward PKA (BIMX, Gö 6983, Y-27632). These experiments were repeated three times with a minimum of 10 cells evaluated in each inhibitor group.

few hours of meiotic maturation is required not only for resumption of meiosis but also for complete progression to MII.

Although H89 is a highly selective and potent inhibitor of PKA, it also potentially inhibits mitogen- and stress-activated kinase 1 (MSK1), ribosomal protein S6 kinase 1 (RPS6KB1), rho-associated coiled-coil-containing protein kinase 2 (ROCK2), and protein kinase N2 (PKN2), and can inhibit additional kinases such as protein kinase C (PKC) to some extent [34, 35]. To determine if the observed meiotic inhibition with H89 was predominantly PKA mediated, we also matured oocytes in the presence of two inhibitors with minimal inhibitory activity toward PKA: BIMX, a potent inhibitor of MSK1, RPS6KB1, and PKC, and Y-27632, a potent inhibitor of ROCK2 and PKN2 [34, 35]. About 75% of oocytes cultured continuously in either BIMX or Y-27632 progressed to MII (Fig. 2A). We also tested Gö 6983, a potent inhibitor of PKC isoforms α, β, γ, and δ, [36] and found that it had no effect on oocyte maturation.

To better define how disruption of PKA activity impacts meiotic maturation, we examined the timing of GVBD in the presence of 25 μM H89 and found that the kinetics were significantly slower compared to control oocytes (Fig. 2B). For example, after 2 h of culture, almost all control oocytes had undergone GVBD, while in the presence of H89, no oocytes had yet done so. The timing of GVBD in oocytes matured in the presence of Gö 6983 was indistinguishable from that of controls (Fig. 2B). In addition, although the kinetics of GVBD were altered when oocytes were matured in the presence of BIMX and Y-27632 compared to controls, the phenotype was not as severe as when oocytes were cultured in the presence of H89. These findings suggest that the target kinase of H89 mainly responsible for causing disruption of meiotic maturation was likely PKA.

Because PKA activity has been implicated in regulating meiotic spindle dynamics [21], we examined the effects of H89 on spindle morphology during meiotic progression. For these experiments, 40 μM H89 was added to the culture medium beginning after completion of GVBD at 90 min to allow meiosis to resume normally. After in vitro maturation for 8–9 h, the majority of control oocytes progressed through meiosis I. For example, 67% of control oocytes were in metaphase of meiosis I as indicated by the formation of a bipolar spindle (Fig. 3A) and 17% had progressed to anaphase or telophase of meiosis I. In contrast, no normal spindles were observed when oocytes were in vitro matured in the presence of H89 for 8–9 h (Fig. 3, B and C). Severely disrupted spindles were observed in 85% of oocytes matured in the presence of H89, ranging from condensed monopolar spindles with discrete chromosome pairs still discernible to condensed masses or rosettes of chromatin in which homologous pairs could not be resolved (Fig. 3, B and C). Of note, a small but similar percentage of oocytes degenerated when matured in the presence or absence of H89, indicating that there was not obvious toxicity of the inhibitor itself (15% and 13%, respectively). After in vitro maturation for 15–16 h, 96% of control oocytes had reached MII as indicated by the extrusion of the first polar body and formation of the second meiotic spindle, but no oocytes exposed to H89 did so (Fig. 3D; remaining 4% of oocytes were at MI). Instead, all cells exposed to H89 for 15–16 h remained in meiosis I and were characterized by altered chromatin and spindle structures (Fig. 3, E and F). Fifty-seven percent of H89-treated oocytes had single chromatin masses or rosette-like structures similar to those observed at 8 h, and the remaining 43% had two or more chromatin masses, with no evidence of first polar body extrusion or spindle formation (Fig. 3, E and F). Unlike H89 treatment, BIMX did not adversely impact meiotic spindle formation, as the majority of oocytes that were matured in the presence of BIMX formed both MI and MII spindles normally (Supplemental Figure S1; available online at www.biolreprod.org). Taken together, these results imply that PKA activity, rather than the activity of other H89-sensitive kinases, is required for proper kinetics, spindle dynamics, and progression of meiosis.

Identification of PKA Substrates During Meiotic Progression

To begin to elucidate the molecular mechanisms by which PKA regulates meiotic progression, we took a proteomics approach to identify putative targets of increased PKA activity following GVBD. To do this, we first performed an immunoblot analysis on protein extracts from oocytes at distinct meiotic stages using an antibody that recognizes the specific serine and threonine residues (RRXS/T) phosphorylated by PKA. Consistent with the observations that PKA is active and required throughout meiotic progression, we found that PKA substrates are also phosphorylated at GV, GVBD, MI, and MII stages (Fig. 4A). Moreover, the number and pattern of phosphorylated proteins is dynamic and changes over the course of meiosis.

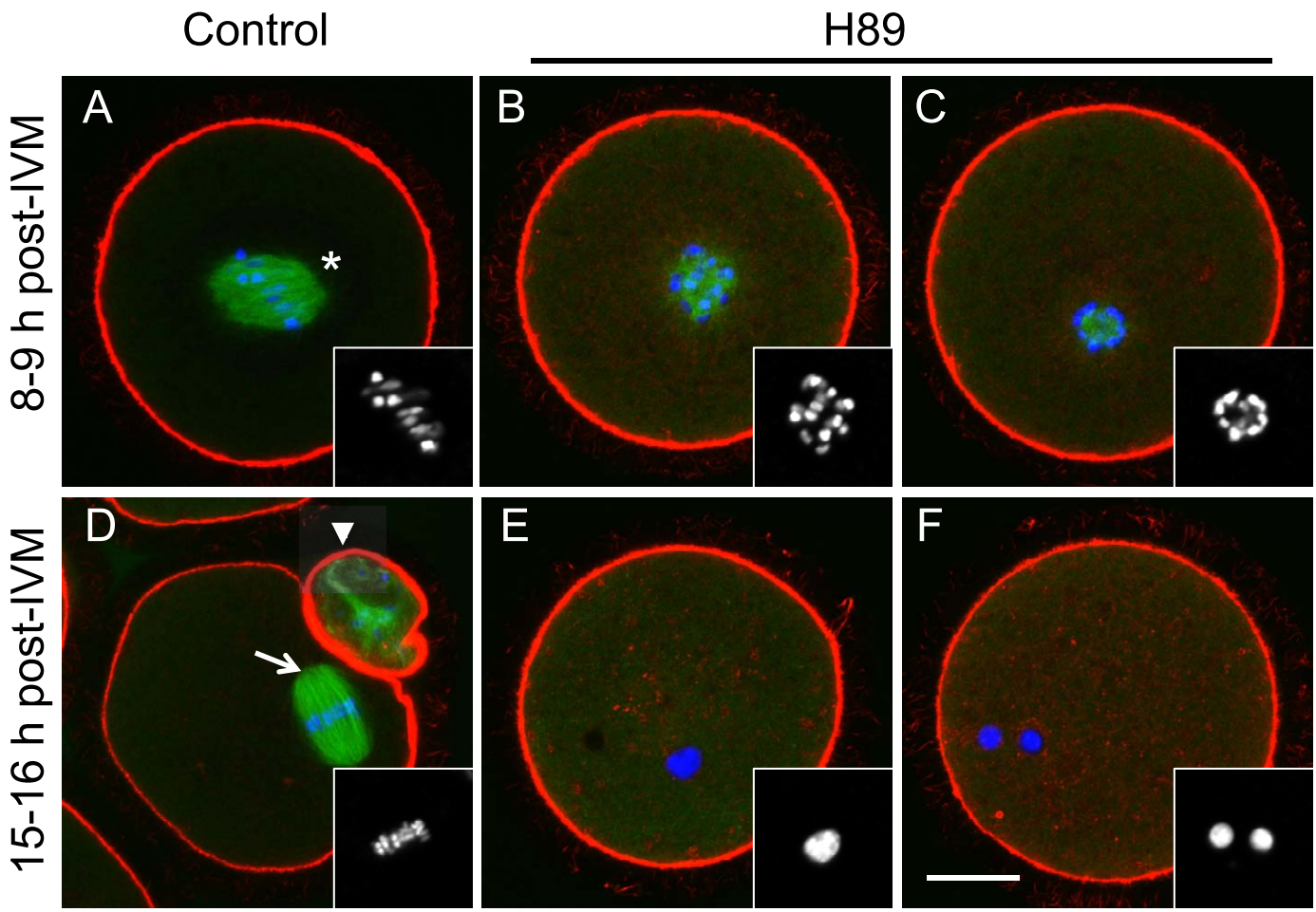

FIG. 3. The effect of H89 exposure post-GVBD on meiotic spindle morphology. Addition of 40 μM H89 to oocytes 90 min after initiation of meiotic resumption resulted in altered meiotic spindle and chromatin phenotypes following culture for a total of 8–9 h (**A**–**C**) or 15–16 h (**D**–**F**). Representative images of cells after maturation in vitro in the presence of no inhibitor (**A**, **D**) or H89 (**B**, **C**, **E**, **F**) are shown (red, F-actin; blue, DNA; green, tubulin; inset, DNA). By 8–9 h, most of the control oocytes had reached metaphase of meiosis I (asterisks, meiosis I spindle) and by 15–16 h they had reached metaphase of meiosis II (arrow, meiosis II spindle; arrowhead, polar body). In contrast, the majority of H89-treated oocytes at both time points had altered morphology including a monopolar spindle with distinct condensed homologous chromosomes (**B**), a monopolar spindle with a chromatin rosette (**C**), a single chromatin mass (**E**), or a double chromatin mass (**F**). These experiments were repeated twice, with a total of at least 21 cells evaluated for each treatment group. Bar = 20 μm.

To determine the identity of PKA substrates, we separated protein extracts from two sets of MII eggs in parallel on an SDS-PAGE gel. One set of extracts was transferred and immunoblotted with the PKA substrate antibody (Fig. 4B). The immunoreactive bands on this blot were then used as a guide to excise the two main regions of gel containing the proteins of interest from the second set of MII egg extracts (Fig. 4B). Using MALDI-TOF/TOF mass spectrometry and microsequencing of the proteins within two excised gel pieces, we identified six putative targets of PKA activity (Table 1). Of the identified targets, we were particularly interested in TLE6 because, according to information available in the BioGPS database, it is highly abundant in oocytes and fertilized eggs and is expressed in a cell type-specific manner [37] (Fig. 5A). TLE6 is also an integral part of the SCMC that plays an essential role in preimplantation embryo development [22]. We identified eight unique peptides corresponding to TLE6 from the lower gel band by mass spectrometry, and the N-terminal region had five high-probability PKA phosphorylation sites as predicted using NetPhosK software [38] (Fig. 5B). Of note, the predicted phosphorylation sites were all located in the N-terminal region of TLE6 that differs significantly from all other TLE family proteins (Fig. 5C).

To confirm that TLE6 was in fact a PKA substrate as identified using the anti-PKA substrate antibody, we took two independent approaches. First, we stripped and reprobed the meiotic maturation series immunoblot, which was initially probed with the anti-PKA substrate antibody, with a TLE6-specific antibody. The ~60 kDa immunoreactive band detected by the anti-TLE6 antibody corresponded to a similar band detected using the anti-PKA substrate antibody (Fig. 4A). Second, we performed an immunoprecipitation experiment (Fig. 6A). Using the anti-PKA substrate antibody, we were able to pull down TLE6 from MII egg extracts. This interaction was specific because nonimmune IgG did not immunoprecipitate TLE6 (Fig. 6A).

Consistent with TLE6 being a substrate of PKA activity, we observed a clear decrease in electrophoretic mobility of TLE6 over the time course of meiotic maturation, indicating a change in protein phosphorylation (Fig. 4A). To determine if this mobility shift was due to phosphorylation and not another posttranslational modification, we treated extracts from GV oocytes and MII eggs with a phosphatase (Fig. 6B). These

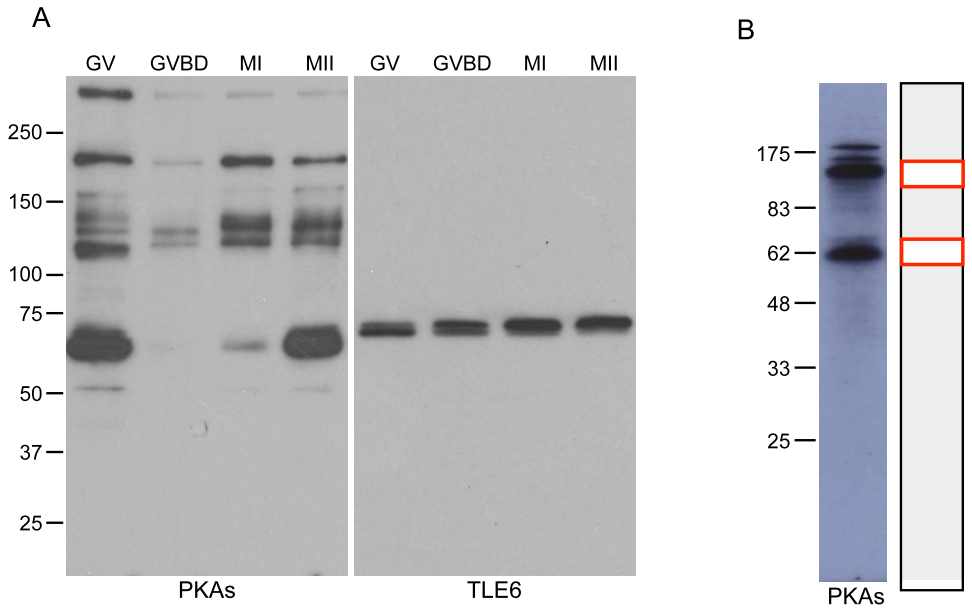

FIG. 4. Identification of PKA substrates phosphorylated during oocyte maturation. **A**) Immunoblot analysis with specific antibodies was performed to determine how PKA substrate (left) and TLE6 (right) expression change during meiotic maturation. Extracts from 100 cells at each meiotic stage were run per lane (GV, GV-intact). These experiments were repeated three times. **B**) For proteomics analysis, specific regions of an SDS-PAGE gel (schematic on right, red boxes) containing proteins from MII eggs that corresponded to PKA substrate immunoreactive bands (left) were excised and submitted for substrate identification. Numbers to left of blots indicate apparent molecular weight in kDa.

extracts were separated by phosphate-affinity PAGE (Phos-tag), in which the phosphate-affinity site is a polyacrylamide-bound dinuclear Mn^{2+} complex that enhances mobility shifts of the phosphorylated forms of proteins [29]. We found that phosphatase treatment did not impact the electrophoretic mobility of TLE6 in GV oocytes, suggesting that the protein is not phosphorylated at this stage (Fig. 6B). However, phosphatase treatment of MII egg extracts increased the electrophoretic mobility of TLE6 such that it migrated similarly to the unphosphorylated form of the protein observed in extracts from GV oocytes (Fig. 6B). Treatment of extracts with a phosphatase inhibitor alone had no effect on the electrophoretic mobility of TLE6 (Fig. 6B). Taken together, these results suggest that the meiotic maturation-associated changes in TLE6 electrophoretic mobility are attributable to phosphorylation events likely mediated by PKA given substrate specificity.

The spatial-temporal localization of PKA and TLE6 within the oocyte is also consistent with a direct or indirect interaction between these proteins. We demonstrated previously that both the catalytic and regulatory RI subunits of PKA localize to the oocyte cortex [13]. To determine if TLE6 localized to regions of the oocyte similar to where PKA was located, we performed immunocytochemistry with an anti-TLE6 antibody. We found that TLE6 was expressed throughout oogenesis—in oocytes isolated from primordial follicles all the way to those isolated from antral follicles (Fig. 7A). TLE6 was cytoplasmic in primordial and primary oocytes isolated from Postnatal Day (PND) 2 and PND5 females (Fig. 7A). In oocytes isolated on and after PND11, TLE6 localized predominantly to the cortex, although there was still some signal throughout the cytoplasm. The predominant cortical localization, therefore, was likely first present in oocytes from secondary follicles and subsequently persisted throughout all stages of meiotic maturation (Fig. 7B). The cortical staining appeared in some instances to be polarized, although this observation was not consistent. These results support that TLE6 is expressed in the correct spatiotemporal manner to be a target of PKA activity.

PKA Regulates TLE6 Phosphorylation During a Narrow Window of Meiotic Maturation

Given the likely role of PKA in regulating TLE6, we wanted to define more precisely when TLE6 is phosphorylated during meiotic maturation. Using Phos-tag gels, we found that TLE6 phosphorylation was first detected 60 min after the resumption of meiosis when oocytes were still GV intact. This phosphorylation event was evident as the appearance of a distinct doublet with an increase in intensity of a higher-molecular-weight immunoreactive band (Fig. 8A). By 90 and 180 min following meiotic resumption, when nearly all oocytes had undergone GVBD, TLE6 appeared to be almost completely

TABLE 1. Proteins identified using mass spectrometry.

Gel band	Gene	Full name (alias)	No. of peptides/ no. of matches	Mass (Da)	No. of PKA sites
Higher	*Nlrp5*	NLR family, pyrin domain containing 5 (Mater)	37/217	125 421	9
	Dnmt1	DNA methyl transferase 1	21/42	183 074	1
Lower	*Padi6*	Peptidylarginine deiminase type 6	27/463	76 729	5
	Hspa8	Heat shock protein 8	24/135	70 827	2
	Tle6	Transducin-like enhancer of split 6, homolog of Drosophila E(spl)	8/42	65 074	7
	Lcp1	Lymphocyte cytosolic protein 1 (L-plastin)	6/31	70 105	5

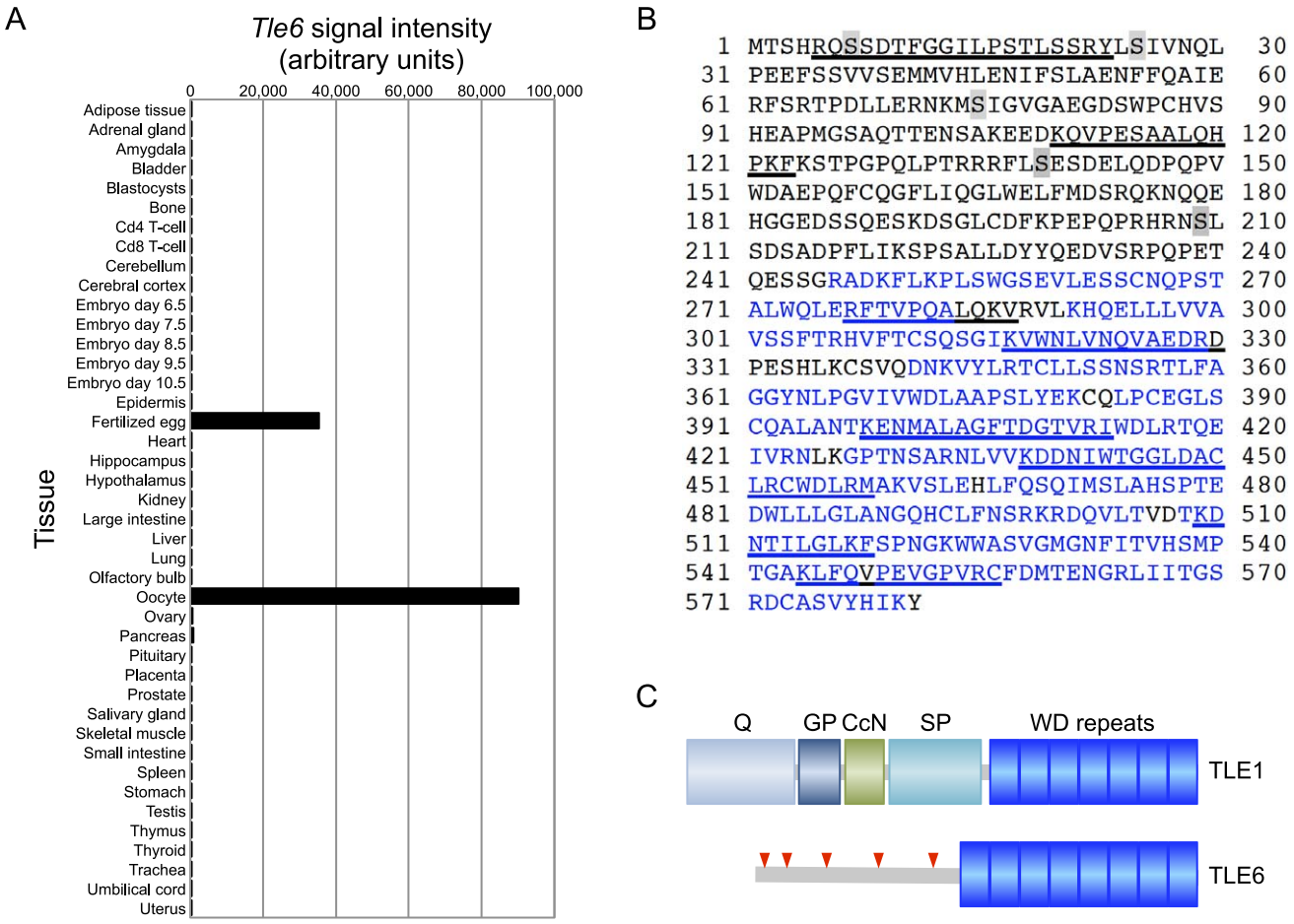

FIG. 5. Properties of TLE6. **A**) Microarray data from the BioGPS database indicates that *Tle6* has a high relative abundance in the oocyte and unfertilized egg compared to other tissues. **B**) The *Mus musculus* TLE6 protein sequence has several high-probability PKA phosphorylation sites (gray highlight) and WD repeat sequences (blue font). The peptides identified in the proteomic analysis are underlined. **C**) A schematic representation of TLE family protein domains shows that TLE6 lacks the common N-terminal domains found in the remainder of the family members. TLE1 is representative of TLE1-5.

phosphorylated (Fig. 8A). This physiological increase in meiotic maturation-associated phosphorylation of TLE6 was completely abrogated when oocytes were treated with H89 beginning 45 min after meiotic resumption was initiated by removing milrinone from the medium (Fig. 8A). In contrast, when oocytes were treated at this same time with the ROCK2 inhibitor, Y-27632, at concentrations previously demonstrated to impair GVBD and polar body emission [29], the phosphorylation status of TLE6 was unaffected (Fig. 8B). Similar treatments with either Gö 6983 or BIMX did not inhibit TLE6 phosphorylation (data not shown). To determine if PKA activity was necessary for the continued phosphorylation of TLE6 after completion of GVBD, H89 was added to the maturation medium 1.5 h after milrinone removal. The phosphorylation state of TLE6 in MI oocytes was then assessed 8 h post-meiotic resumption. In extracts from control MI oocytes, TLE6 was maximally phosphorylated at this time, as indicated by the decrease in electrophoretic mobility that occurred compared to extracts from control GV oocytes (Fig. 8C). H89 treatment, however, resulted in the presence of an additional faster-migrating TLE6-immunoreactive band that was absent in untreated MI oocytes or those treated with either Gö 6983 or BIMX (Fig. 8C). These data demonstrate that the increase in PKA activity following meiotic resumption is coincident with the timing of TLE6 phosphorylation. Moreover, PKA activity is likely not only responsible for initiating TLE6 phosphorylation at the time of GVBD but also for maintaining its complete phosphorylation throughout oocyte maturation. Of note, however, H89 treatment following GVBD did not completely return TLE6 phosphorylation levels back to those observed in GV oocytes, implying that additional kinases may be responsible for phosphorylating TLE6 during oocyte maturation.

DISCUSSION

In this study, we demonstrate that, in addition to its well-documented role in the maintenance of meiotic arrest, intra-oocyte PKA activity functions after the prophase I arrest and is essential for successful oocyte maturation. PKA activity increases during meiosis and blocking its function using the pharmacological inhibitor, H89, results in various meiotic defects including delayed GVBD kinetics, abnormal chromatin and spindle structure, and failure to progress to MII. These phenotypes are observed with only a ~50–60% reduction of PKA activity that was achieved with H89. This finding suggests that PKA activity needs only to be reduced below a critical threshold rather than to be completely abrogated to result in altered gamete quality. Consistent with this notion, we found that MII eggs that were matured in vitro tended to have less PKA activity compared to those that were ovulated, and it is well documented that in vitro matured eggs are of reduced quality [39, 40].

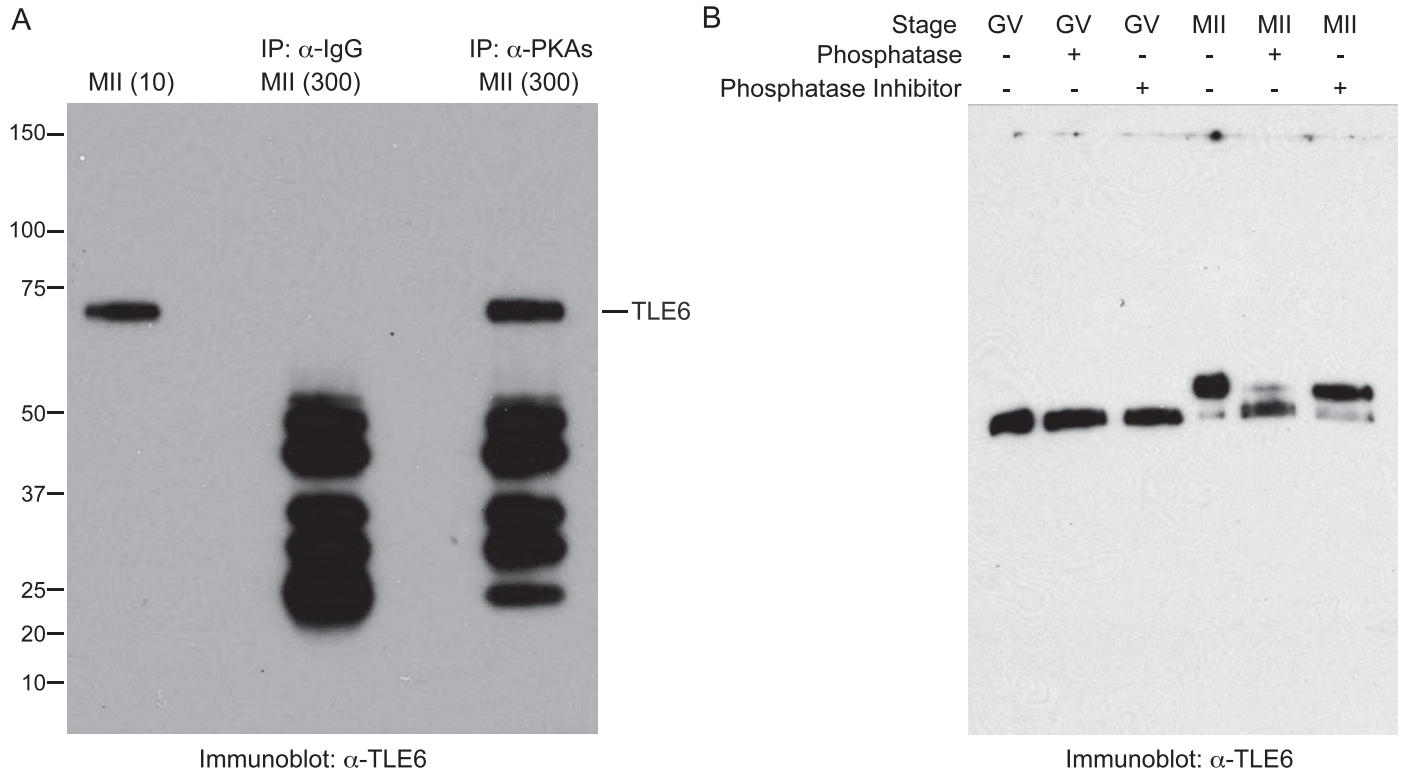

FIG. 6. TLE6 is a PKA substrate that is phosphorylated during meiotic maturation. **A**) Immunoprecipitation of extracts from 300 MII eggs with an anti-PKA substrate antibody, but not with a nonimmune IgG, pulled down TLE6 as determined by immunoblot analysis with an anti-TLE6 antibody. Immunoreactive bands at and below 50 kDa are likely heavy and light immunoglobulin chains. **B**) Protein extracts from 50 GV oocytes or MII eggs (±phosphatase or phosphatase inhibitor) were separated on Phos-tag gels and immunoblotted with an anti-TLE6 antibody. Phosphatase treatment increased the electrophoretic mobility of TLE6 in MII eggs but not in GV oocytes. Note that standard molecular weight markers cannot be used for Phos-tag gels because migration is dependent on phosphorylation status.

Although we confirmed that H89 treatment reduces PKA activity in both intact oocytes and oocyte extracts, we cannot definitively exclude the possibility that the H89 effects on meiotic maturation that we observed were due to inhibition of other kinases. Kinases that may also be affected by H89 include MSK1, RPS6KB1, and ROCK2, all of which have an IC_{50} for H89 in the low nanomolar range [35]. However, such off-target effects are highly unlikely given that alternative inhibitors BIMX, Y-27632, and Gö 6983, which target these kinases and not PKA, had minimal or no inhibitory effects on GVBD and progression to MII. In addition, one of the main phenotypes we observed with H89 treatment during meiotic progression was a defect in chromatin and spindle dynamics. These results are consistent with the observation that PKA substrates localize throughout the oocyte cytoplasm as well as to the spindle poles during meiosis I and II [21]. In the absence of PKA activity induced experimentally by RNA interference knockdown of PKA subunits, the meiosis I spindle fails to migrate completely to the oocyte cortex, resulting in cleavage plane defects and the extrusion of abnormally large polar bodies [21]. These findings suggest that phosphorylation of PKA substrates in specific locations such as the spindle and oocyte cortex, possibly directed by AKAP-mediated PKA localization, is important for the successful completion of meiosis.

To begin to elucidate the substrates of PKA activity during meiosis, we used an unbiased proteomics approach to identify oocyte proteins that migrate to the same region of an SDS-PAGE gel as those detected by an antibody that recognizes a consensus PKA phosphorylation motif. Interestingly, several of the potential top hits we identified were maternal effect genes, including NLRP5, TLE6, PADI6, and DNMT1 [22, 41–43]. In particular, NLRP5, TLE6, and PADI6 interact together as part of the SCMC, a group of proteins that localize to the oocyte cortex [22]. Maternal effect genes encode products that are made during oogenesis that when mutated cannot be rescued by the paternal allele [44]. Loss of these products compromises preimplantation embryo development without affecting oogenesis, folliculogenesis, meiotic maturation, or ovulation.

Similar to the SCMC, PKA subunits often localize to the oocyte cortex. Spatial localization of PKA in specific regions of the oocyte is required for meiotic maturation and is mediated by AKAPs [14, 17]. In the absence of cAMP, the PKA catalytic subunit is tethered to the regulatory RI and RII subunits that are localized by interactions with AKAPs. PKA RI localizes mainly to the cortical region of GV-intact oocytes, and to the MI and MII spindles during maturation, whereas PKA RII is found diffusely throughout the cytoplasm of GV-intact oocytes and then localizes to mitochondria during maturation [13, 17, 21]. Localization of the PKA catalytic subunit is largely cortical in GV-intact oocytes [13], consistent with the localization of both PKA RI and the SCMC components that we identified as possible targets of PKA phosphorylation. Thus, TLE6, NLRP5, and PADI6 are all expressed at the correct time and subcellular location to be phosphorylated by PKA in the oocyte. It is therefore possible that alterations in PKA activity may compromise the integrity and function of the SCMC, resulting in late effects in the embryo.

In this study, we performed a detailed phosphorylation analysis of TLE6 during meiotic maturation. We found that although PKA activity is high at the GV stage, TLE6 is not

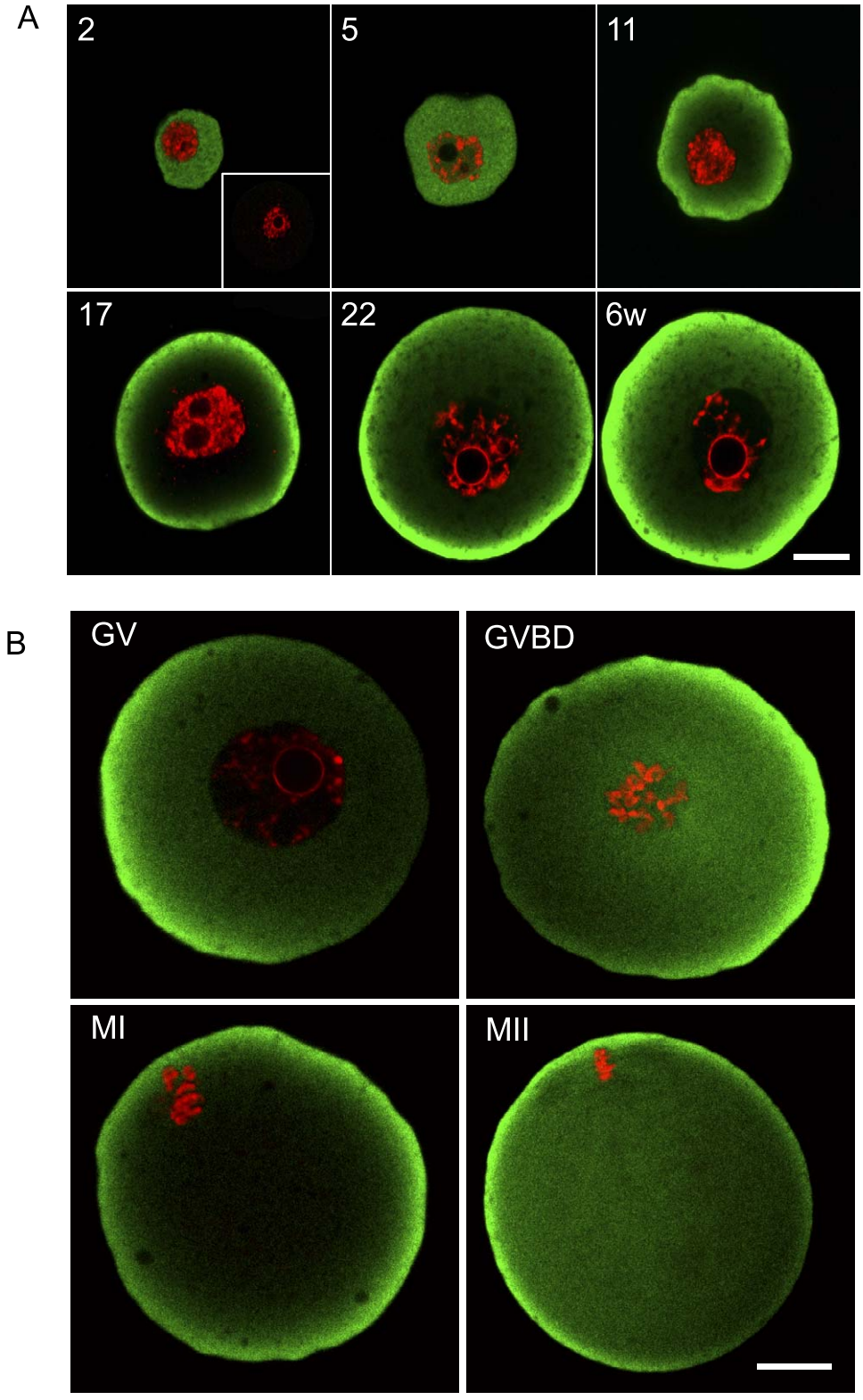

FIG. 7. TLE6 localization during oogenesis and meiotic maturation. **A**) TLE6 localization during oocyte growth. Numbers in each panel indicate the PND oocytes were collected, except in lower right panel, 6w indicates 6 wk of age. TLE6 is primarily cytoplasmic until PND11, when it becomes cortically enriched. Oocytes isolated from PND11 mice likely originate from secondary follicles. The inset shows a low-magnification view of a fully grown oocyte immunostained with nonimmune serum in place of TLE6 antiserum as a control. **B**) TLE6 is cortically enriched throughout meiotic maturation. The meiotic stage is indicated on each panel (GV, GV-intact). These experiments were repeated three times and a minimum of 35 cells were evaluated at each stage. Red, DNA; green, TLE6. Bars = 20 μm.

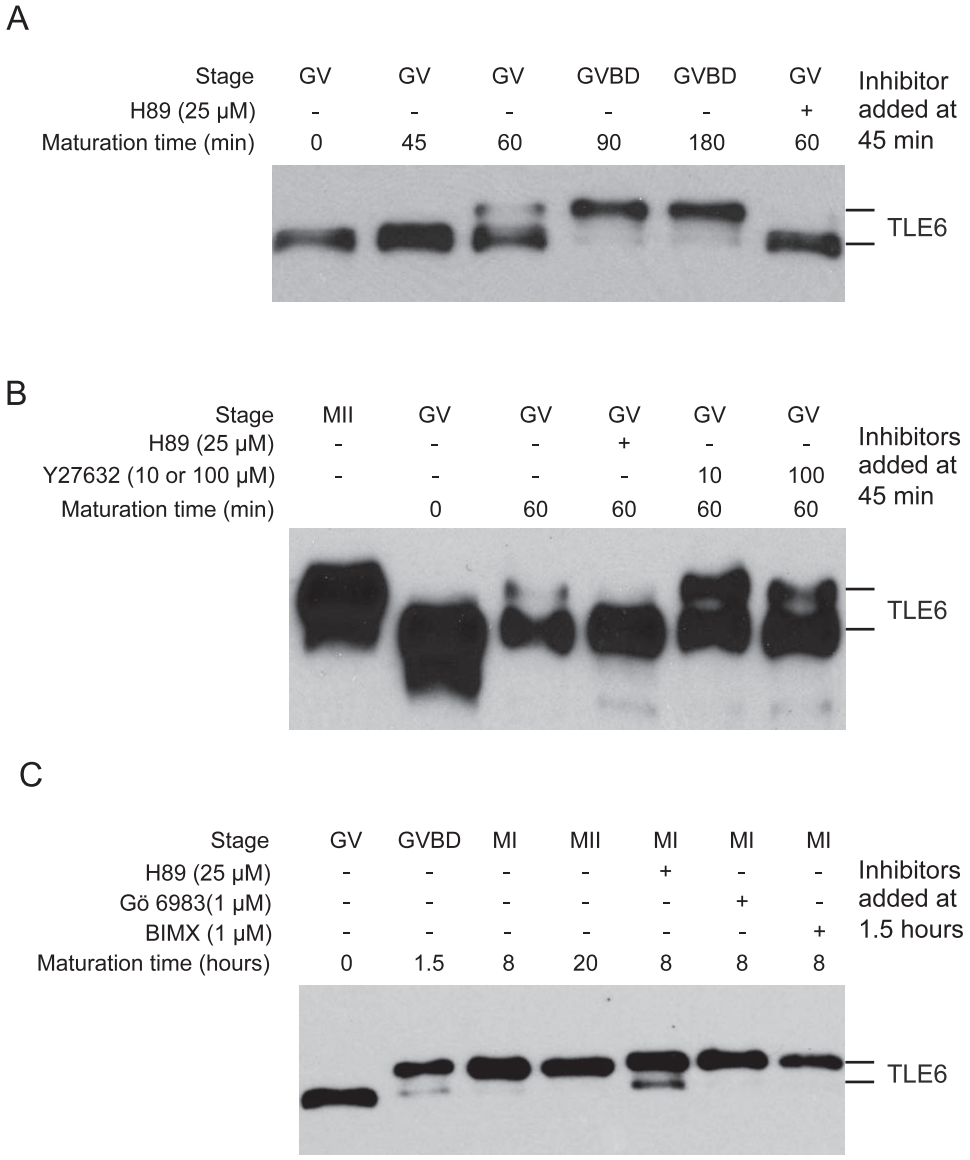

FIG. 8. The effect of H89 on TLE6 phosphorylation during meiotic maturation. Oocytes were in vitro matured for the total time indicated (maturation time), and various inhibitors (H89, BIMX, Gö 6983, Y-27632) were added at either 45 min or 1.5 h after meiotic resumption, as indicated. The final meiotic stage of cells in each treatment condition was scored upon completion of the designated culture period (stage). Following treatments, protein extracts from cells in each group were separated on Phos-tag gels and immunoblotted for TLE6. **A**) TLE6 phosphorylation was evident by 60 min post-meiotic resumption and complete by 90 min. Addition of H89 45 min post-meiotic resumption completely reversed this phosphorylation. **B**) Unlike H89, Y27632 addition to cells 45 min post-meiotic resumption had no effect on TLE6 phosphorylation. **C**) Addition of H89, but not Gö 6983 or BIMX, 1.5 h post-meiotic resumption partially reversed TLE6 phosphorylation. These experiments were repeated three times.

phosphorylated until after oocytes have undergone GVBD. At this time, PKA activity is relatively low compared to its activity in GV oocytes. This apparent discrepancy between PKA activity and TLE6 phosphorylation status in the GV oocyte could be explained by several mechanisms, including spatial regulation of PKA activity or the presence of a counteracting phosphatase that is active at the GV stage. Furthermore, not much kinase activity may be required for target substrate phosphorylation. TLE6 has several putative PKA phosphorylation sites, and future studies are planned to confirm the identity and function of these sites in regulating TLE6. Moreover, a better understanding of the precise role of TLE6 and the consequences of its phosphorylation changes during meiotic maturation is needed. Although this is speculative, the PKA-mediated phosphorylation of TLE6 could be related to the roles of two other SCMC components, FILIA and NLRP5, in spindle assembly and migration during oocyte maturation [45].

Our proteomics analysis suggests that PKA may serve as a general SCMC kinase. However, it remains to be determined whether NLRP5 and PADI6 are indeed true targets of PKA activity. NLRP5 appears to be phosphorylated during oocyte maturation, based on the appearance of new, slower migrating bands on immunoblots, but we were unable to determine the responsible kinase(s) with the methods used in this study (unpublished results). PADI6 has been shown by others to undergo maturation-associated increases in phosphorylation that mediate its interaction with 14-3-3 proteins, and although PKA has been implicated as a potential kinase for PADI6, this function has not been established [46]. Both NLRP5 and PADI6 localize to cytoplasmic lattices, which are found diffusely throughout the oocyte cytoplasm and function in

organelle positioning and redistribution during oocyte maturation [47, 48]. Furthermore, it has been suggested that cytoplasmic lattices and the SCMC may be structurally related and that proteins including NLRP5 and PADI6 shuttle between these structures [47]. Taken together with our data demonstrating that TLE6 is a PKA substrate, these findings suggest that PKA and likely other kinases phosphorylate several components of the SCMC and cytoplasmic lattices. Future studies will probe whether these phosphorylation events promote cytoplasmic reorganization and identify additional kinases that phosphorylate TLE6 and other SCMC components during oocyte maturation.

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