Spatio-temporal expression patterns of aurora kinases a, B, and C and cytoplasmic polyadenylation-element-binding protein in bovine oocytes during meiotic maturation.

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Key words: Aurora kinases, bovine, oocyte, meiosis

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ABSTRACT

Maturation of bovine immature oocyte requires cytoplasmic polyadenylation and synthesis of a number of proteins involved in meiotic progression and metaphase-II arrest. Aurora serine-threonine kinases - localized to centrosomes, chromosomes and midbody - regulate chromosome segregation and cytokinesis in somatic cells. In frog and mice oocytes, Aurora A regulates polyadenylation-dependent translation of several mRNAs like MOS and CCNB1, presumably by phosphorylating CPEB, and Aurora B phosphorylates histone H3 during meiosis. Here, we analyzed the expression of three Aurora kinases AURKA, AURKB and AURKC in bovine oocytes during meiosis and early embryogenesis by RT-qPCR and immunodetection. Aurora A was the most abundant in oocytes, as compared with AURKB and AURKC mRNA and protein levels. AURKA protein progressively accumulated in the oocyte cytoplasm during antral follicle growth and in vitro maturation. AURKB associated with metaphase chromosomes. AURKB, AURKC and Thr-phosphorylated AURKA were detected at a contractile ring/midbody during the first polar body extrusion. CPEB, localized in oocyte cytoplasm, was hyper-phosphorylated during prophase/metaphase-I transition. Most of CPEB was degraded in metaphase-II oocytes and remnants remained localized at a contractile ring. Roscovitine, U0126 and metformin inhibited meiotic divisions; they all induced a decrease of CCNB1 and phospho-MAPK3/1 levels and prevented CPEB degradation. However, only metformin depleted AURKA. The Aurora kinases inhibitor VX680 at 100 nM did not inhibit meiosis but led to multi-nuclear oocytes due to the polar body extrusion failure. Taken together, in bovine oocytes, Aurora kinases participate in regulating the segregation of the chromosomes, metaphase-II maintenance and the polar body formation during meiosis.

INRODUCTION

In mammal ovaries, oocytes are arrested in prophase of the first meiotic division and become transcriptionally silent when they reach the full size and enter in pre-ovulatory meiotic maturation process. Nuclear meiotic maturation of oocytes includes the condensation of chromosomes, germinal vesicle breakdown (GVBD), progression through metaphase-I (MI), and arrest at metaphase-II (MII). In cows, full-grown oocytes extracted from more than 2-mm follicles are capable to resume meiosis in vitro and then to be fertilized and to develop up to blastocyst in vitro [1]. Oocyte maturation process involves the coordinated action of several kinases. Mitogen activated protein kinases (MAPKs) and maturation promoting factor (MPF) - a complex of cyclin B1 (CCNB1) and cell cycle controller p34 kinase CDC2 (named also CDK1, cyclin-dependent kinase 1), constituted the main signalling pathways of oocyte maturation. Two forms of serine-threonine MAPKs, MAPK1 and MAPK3 (also known as p42MAPK/ERK2 and p44MAPK/ERK1 respectively) are present in mammalian oocytes, and they are at least partially activated by the upstream kinase named MOS. In bovine oocytes, MPF and MAPK3/1 activation precedes GVBD during in vitro maturation (IVM) [2]. A significant increase in overall protein synthesis occurs in bovine oocytes in accordance with GVBD at 6-10 hours after beginning of IVM and then it declines and reaches the basal level at the MII stage [3]. This process is accompanied by the polyadenylation of mRNA until the MII [4]. A number of proteins are synthesized de novo in bovine oocytes during IVM [5], including CCNB1 [6], and MOS [7]. In Xenopus and mice oocytes, cytoplasmic polyadenylation and sequential translation of such mRNA are mediated by CPEB, a protein which binds to cytoplasm polyadenylation element (CPE) - an U-rich cis-element present in 3'-untranslated region of a number of mRNAs, such as MOS or CCNB1 [8,9]. In Xenopus, activation of CPEB is triggered by the serine-threonine kinase AURKA [10],[11] which is the homologue of yeast IpL1 and Drosophila Aurora, regulating the mitotic progression in eukaryotic cells [12]. However, it is not clear whether CPEB activation by AURKA triggers the further cascade of MAPKs phosphorylations leading to MPF activation and nuclear maturation [13], or whether AURKA itself needs the active MPF to be phosphorylated and activated, while these events are independent of the MOS/MAPK pathway [14]. In mice oocytes cytoplasm, AURKA is colocalized with CPEB, CPSF (cleavage and polyadenylation specificity factor), PAP (polyA polymerase) and maskin - factors known to control polyadenylation and translation, and was reported to mediate CPEB phosphorylation in MI [15]. Phosphorylated CPEB activation stimulates both polyadenylation and translation of several mRNAs essential for oocyte maturation, including MOS, CCNB1 and SCP1/SCP3 (synaptonemal complex proteins 1 and

3) in mice [16]. AURKA might also be involved in microtubule assembly and nuclear activity in mice oocytes [17].

In higher mammals including domestic animals and human, only little is known regarding the involvement of Aurora kinases in meiosis. In contrast, in somatic cells, Aurora kinases were identified as important regulators of mitotic divisions. Three Aurora kinases - AURKA, AURKB and AURKC exist in mammals. They share a very similar kinase catalytic domain and their expression is cell cycle-regulated with a maximal protein level at G2/M. The intracellular localizations of Aurora kinases are different at least between AURKA and AURKB/AURKC [18]. AURKA localizes to centrosomes and spindle poles [19], [20]. AURKA is required for centrosome maturation and separation, bipolar spindle assembly, chromosomal alignment on the metaphase plate and for cytokinesis (review [21]). AURKB is a chromosome passenger protein essential for chromosome condensation and cytokinesis completion. It localizes to chromosome kinetochores during prophase and metaphase and to the midbody during the anaphase and telophase [22, 23]. AURKC like AURKB is a chromosome passenger protein [24], it associates with AURKB and Survivin [25], [19]. In cancer cells, Aurora proteins accumulate in cytoplasm [26, 27]. Aurora kinases activity is regulated by phosphorylation/dephosphorylation of several residues [28], [29]. Glycogen synthase kinase-3 (GSK-3) remains the only kinase identified as an upstream activator of Aurora [30]. Aurora kinases could also be activated by autophosphorylation when associated with some of its substrates, like a motor binding protein TPX2 (microtubule-associated protein homolog) for AURKA [31] or INCENP (inner centromere protein) for AURKB [29]. In turn, Aurora kinases phosphorylate a large number of proteins, including kinesin-like motor proteins, spindle apparatus proteins, kinetochore proteins, histones and tumor suppressor proteins such as p53 (for review [18]).

We previously found that in bovine oocytes, *AURKA* mRNA was abundantly highly expressed [32]. We also previously showed AURKA protein level increased during IVM in bovine oocyte and this increase was not affected by inhibition of MPF activity [33]. In porcine oocytes, Thr-phosphorylated AURKB level correlated with histone H3 phosphorylation on Ser10 [34]. In human oocyte, AURKC mRNA was reported to be over-expressed as compared to cumulus cells [35].

The aim of this study was to establish for the first time the spatio-temporal expression patterns of three Aurora kinases in bovine oocytes during meiotic maturation in order to clarify their

roles in meiosis. First, we analyzed the relative expression of *AURKA*, *AURKB* and *AURKC* mRNA by real time RT-PCR and the localization of the corresponding proteins by immunocytochemistry in bovine oocytes and preimplantation embryos. Second, mRNA and protein expression patterns of several main actors of meiotic progression such as CCNB1, CDC2, MOS and CPEB were studied in parallel with AURKA Thr-phosphorylation throughout IVM. Third, the effects of meiotic inhibitors Roscovitine, U0126 and Metformin on the levels of AURKA, phospho-MAPK3/1, CCNB1, CDC2 and CPEB were observed. Finally, the effects of VX680, the specific inhibitor of Aurora kinase activity, on meiosis progression were analyzed.

MATERIAL AND METHODS

Materials

TCM199 culture medium, Gentamicin, Epidermial Growth Factor (EGF), fetal calf serum, MEK inhibitor U0126 and Metformin were from Sigma (Saint Quentin Fallavier, France). Cyclin-dependent kinase inhibitor Roscovitine was provided by Dr L. Meijer (Centre National de la Recherche Scientifique, Station Biologique de Roscoff, France). VX680 inhibitor of Aurora kinase activity was from CAVA Technology (San Diego, USA).

Oocytes, embryos and other tissues collections.

Cattle ovaries were collected at slaughterhouse. Cumulus-oocyte complexes (COC) were obtained by aspiration of 3-6 mm antral follicles. In average, 14 $\pm$ 4 good quality COC could be obtained from one slaughtered cow. COC with more than 3 layers of compact cumulus cells around the oocyte were selected and washed several times in TCM199/Hepes medium supplemented with 50 mg/l of Gentamycin. Groups of 30 to 60 COC were subjected to in vitro maturation (IVM) in 500 µl of TCM199 medium supplemented with EGF (10 ng/ml) and 10% of fetal calf serum during 24 hours at 39°C in a humidified atmosphere of 5% CO_2 in air. In experiments with meiotic inhibitors, the substances were added in culture medium just before IVM. U0126 and Roscovitine were used at final concentration of 100µM and 50µM, respectively. Metformin was added at 10 mM, and it was reported that this concentration did not affect the cell viability [36]. We used VX680 at concentration of 100 nM, which is close to a half-maximal inhibitory concentration (IC_{50} 15-113 nM) for the proliferation of a variety of human cell types [37], and also at 1 µM – the concentration that was used in studies on mouse and human cell lines (1 and 10 µM) [38].

Routinely, the oocytes were denuded from COC by mechanical separation of cumulus cells either just after collection from ovaries (immature oocytes, IO, at germinal vesicle stage, GV) or after 3, 6, 10, 14 and 22-24 hours of IVM culture. Oocytes were rinsed in PBS and then frozen in liquid nitrogen and stored until RNA or protein extraction. Meiotic status of oocytes at different time of maturation was verified by lamin A/C immunofluorescence and chromatin labelling with Hoechst33342 (Sigma, 1µg/ml), followed by microscopic observation.
For in vitro preimplantation embryos production, COC were subjected to IVM for 22 hours, and subsequently to in vitro fertilization and embryo development as described earlier [32]. Groups of 10 or 20 embryos at 1-Cell, 2-Cell, 4-Cell, 5 to 8-Cell, morula and blastocyst stages were collected and either fixed for immunofluorescence analysis, or frozen in liquid nitrogen.
Biopsies (0.5 g) from ovary and testis were collected at INRA local slaughterhouse from two adult cows and two bulls. Calf skin fibroblast primary culture cells and cumulus cells were used for protein extraction. All samples were frozen in liquid nitrogen and kept at -80°C before experiments.

RNA and cDNA preparation and analysis.

Total RNA preparation

Total RNA was extracted from bovine oocytes, embryos and biopsies of adult ovary and testis by using TriZol reagent following the manufacturer's instructions (Invitrogen, Cergy Pontoise, France). To avoid the contamination with genomic DNA, total RNA preparations were treated by RQ1 DNAse (Promega) as described in manufacturer's protocol.

AURKA, AURKB and AURKC cDNA sequences analysis.

Full-length Aurora A (gene *AURKA*) cDNA was obtained by using SMART RACE cDNA Amplification Kit (Ozyme), 5' and 3' cDNA fragments were cloned into pCRII-dual promoter vector using the TA cloning kit (Invitrogen) and sequenced by Macrogen Company (Seoul, South Korea). Deduced cDNA and protein sequences were deposed in Genbank (accession number DQ334808). Sequences for bovine Aurora B (*AURKB*) and Aurora C (*AURKC*) were found in GenBank (accession numbers are NM_183084 and XM_870932, respectively). The sequences were analyzed using the software package proposed by Infobiogen [39]. Alignments were performed using BLAST [40] and Multalin [41]. Deduced protein sequences were analyzed through Interpro website [42] and Simple Modular Architecture Research Tool (SMART) [43].

190 *RT-PCR*

Reverse transcription (RT) was performed on RNA amounts equivalent to 5 or 10 oocytes or
192 embryos, or 1 µg of total RNA from tissue biopsies. Complementary DNA was extended from oligo $(dT)_{15}$ primers during 1 hour at 37°C by mouse Moloney leukaemia virus reverse
194 transcriptase (Invitrogen) as described in user manual. For RT-PCR analysis we used as a template the cDNA equivalent to 5% of one oocyte/embryo reversed RNA (1% of total RT
196 reaction). For ovary and testis, 1/20 of the reverse transcription products were used (50 ng of reversed RNA equivalent). PCR were performed using reagents from Interchim (Montluçon,
198 France). In negative control reaction, RNA amount equivalent to 1 oocyte or 125 ng of tissue RNA were directly subjected to PCR with specific primers. As positive control of cDNA
200 quality, the β-actin specific PCR was performed for all the samples. Primers sequences are listed in Table 1. To verify the specificity of amplified fragments, PCR products were cloned
202 and sequenced.

Real-time PCR

204 For real time RT-PCR analysis, cDNA was extended from RNA from 10 oocytes by MMLV reverse transcriptase (Invitrogen) in the presence of 20 ng of oligo $(dT)_{15}$ and 2 ng of 18S-
206 antisense-RT primer per reaction. Real-time PCR was performed on MyiQ apparatus (Bio-Rad Laboratories, Marnes La Coquette, France) using the cDNA quantity equivalent to 5% of one
208 oocyte/embryo per reaction. Reactions were performed in triplicate by using a "Real-time PCR kit" provided with a SYBR Green fluorophore (Bio-Rad) according to the manufacturer
210 instructions and by using the robotic distributor (Eppendorf). One pg of luciferase mRNA was added to each group of 10 oocytes or embryos before RNA extraction and was used as an
212 external control of RNA extraction and cDNA quality. Four independent pools of RNA were analyzed for each stage of oocyte maturation and embryo development. For each gene
214 examined, a standard curve, consisting of corresponding plasmid DNA fragments purified from with QIAquick PCR Purification kit (Qiagen) and diluted from 1 pg to 0.1 fg, was included in
216 each run. Correlation coefficients and PCR efficiencies were not less than 0.998 and 85 %, respectively. The median values of reaction triplicates were considered. Different approaches
218 were applied for relative quantification of target polyA mRNA. 1) The relative *AURKA*, *AURKB* and *AURKC* mRNA abundance in oocyte, blastocyst and testis was compared in
220 relation to the quantities of correspondent amplified products in fg obtained from the same cDNA sample. 2) The relative abundance of target mRNA in oocytes at different stages of

maturation was calculated relatively to those of 18S rRNA, internal reference gene, since its level did not change during IVM in bovine oocytes [44]. 3) The relative abundance of *AURKA* mRNA in embryos at different stages of development was calculated relatively to the external reference, luciferase polyA RNA which was added prior RNA extraction.

In all experiments, the mean value obtained for T0 immature oocytes was considered as 1. One-way ANOVA test were performed for statistical analysis of data. Difference was considered significant at $P<0.05$.

Protein analysis

Antibodies

Monoclonal mouse antibody against recombinant full-length human Aurora A kinase was produced and characterized earlier [45]. Human Aurora B (ARK-2, H-75), CPEB (H-300) and MAPK42 (MAPK1 or ERK2, C14) rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal to phospho-Thr288 Aurora A antibody was from Abcam (Cambridge, UK). Phospho-p44/42 MAPK rabbit polyclonal antibody (MAPK3/1 phosphorylated at Thr202/Tyr204) was purchased from Cell Signalling (Danvers, MA). Alpha-tubulin (TUBA) monoclonal antibody (clone DM1) and Texas Red-conjugated goat anti-mouse antibody were from Sigma (Saint Quentin Fallavier). Antibodies against human Aurora C and phosho-Ser^{10} histone H3 were kindly provided by Dr. Y. Arlot (UMR6061 CNRS, France). Cyclin B1 monoclonal antibody (clone Ab-3), horseradish peroxidase (HRP) conjugated rabbit anti-mouse, goat anti-rabbit and goat anti-mouse IgG antibodies were purchased from Lab Vision (Fremont, CA). Lamin A/C monoclonal antibody was from Ozyme, (Saint Quentin Yvelines, France); Alexa Fluor®488 goat anti-rabbit IgG and Alexa Fluor®594 goat anti-mouse IgG were from Molecular probes (USA).

Western immunoblotting

Groups of definite number of bovine oocytes or embryos were frozen in 20 µl of Tris-saline-EGTA composition buffer pH 7.5 supplemented with 2 mM sodium ortovanadate and 1 µl/ml of protease inhibitor cocktail (Sigma) and than thawed-frozen three times by rapid incubation in liquid nitrogen followed by warm water bath at 30°C. In some experiences, dephosphorylation of proteins on serine, threonine and tyrosine residues was performed by using the Lambda Protein Phosphatase (λ-PPase, Sigma). Oocyte were collected in 20 µl of 1x λ-PPase reaction buffer (50 mM, Tris-HCl, 0.1mM Na-EGTA, 5mM DTT, 0.01 Brij , 2mM

$MnCl_2$), thawed-frozen three times and then incubated with 400 units of λ-PPase 1 hour at 30°C. Before loading, concentrated reducing Laemmli buffer containing at final concentration 80 mM dithiothreitol was added to all protein extracts and samples were boiled during 8 min. Proteins extracts were resolved on 10-12% SDS-PAGE gels and transferred to nitrocellulose membranes. Blots were blocked with 5% of dry milk in Tris-buffered saline / 0.1% Tween 20 for one hour at room temperature and probed with the various antibodies overnight at 4°C. Dilutions were 1/1000 for AURKA, phospho-MAPK3/1, total MAPK3/1 and TUBA antibodies, 1/200 for CDC2, CCNB1 and AURKB, 1/2000 for CPEB. After extensive washings in 1xTBS/Tween 0.1%, immunoreactivity was detected using the appropriate HRP-conjugated secondary antibodies (diluted 1:5000, incubation 1h at room temperature) and revealed by enhanced chemiluminescence ECL Plus kit according to the manufacturer's instructions (Amersham Biosciences, Orsay, France). Densitometry was performed by scanning the original radiographs and then analysing the bands with Scion Image software Beta 4.0.2 (Fuji PhotoFilm, USA). At least three blots were analyzed for each experimental condition.

Immunohistochemistry

Ovarian biopsies were fixed for 12 hours in the solution containing 50% of saturated picric acid, 3.7% formaldehyde and 5% of acetic acid. After serial dehydration steps, the samples were embedded in paraffin and serially sectioned at a thickness of 7 µm. Sections were deparaffined, re-hydrated, and microwaved for 5 min in antigen unmasking solution (Vector Laboratories, Inc., AbCys, Paris, France) and then left to cool to room temperature. After washing in a PBS bath for 5 min, sections were immersed in peroxidase blocking reagent for 10 min at room temperature to quench endogenous peroxidase activity (Dako Cytomation; Dako, Ely, UK). After three washes in a PBS bath for 5 min, sections were blocking with 5% goat serum in PBS for 20 min, followed by incubation overnight at 4°C with PBS/0.1% BSA containing AURKA antibody (dilution 1/100). After washing three times for 10 min, sections were incubated for 30 min at room temperature with a biotinylated goat anti-mouse antibody following by serial washing and 10 min staining in streptavidin peroxidase solution at room temperature as described in kit manual (both Lab Vision Corporation). Immunoreactivity was revealed by incubation at room temperature with 3, 3'-diaminobenzidine (Lab Vision Corporation). The slides were counterstained with hematoxylin, then dehydrated and mounted in Depex (Sigma). Negative controls were performed by replacing primary antibodies by

mouse IgG (Sigma) diluted in PBS/0.1% BSA at a final concentration of 2 µg/ml. Slides were observed using the Axioplan Zeisse transmission microscope.

Immunofluorescence of bovine oocyte and embryos.

For fluorescent analysis, oocytes and blastocysts were fixed for 10 min in the solution containing 50% of saturated picric acid, 3.7% of methanol stabilized-formaldehyde (Interchim) and 5% of acetic acid, and then washed 4 times 15 min in PBS supplemented with 0.2% BSA and once 30 min in PBS/0.2% BSA/0.1% Triton. For AURKA detection during early embryogenesis, oocytes and embryos at 1-Cell, 2-Cell, 4-Cell, 5/8-Cell, morula and blastocyst stages were fixed in cold methanol at -20°C for two hours, rehydrated through serial ethanol baths, and washed three times in PBS with 0.2% BSA and 0.01% Tween20. Blocking was performed in PBS/0.5% BSA supplemented with 10% of goat inactivated serum during 2 hours. Overnight incubation with primary antibodies (diluted 1/100 for AURKA, AURKB and AURKC detection, 1/200 - 1/500 for phospho-Thr AURKA, 1/200 for phospho-Ser^{10} histone H3 and Lamin A/C) was performed at 4°C with constant shaking. At least four 30 min washes in PBS/0.2% BSA were done, and oocytes and embryos were incubated with corresponding secondary fluorochrome-conjugated antibody diluted at 1/200 for 1-2 hr at room temperature. Five 20 min washes were then performed. Oocytes and embryos were put on slides and mounted with Mowiol supplemented with DABCO anti-fading and 1µg/µl of Hoechst 33258 or DAPI (Sigma). Immunofluorescence was observed using Axioplan Zeiss fluorescent microscope supplied with appropriate filters.

ETHICS

All procedures were approved by the Agricultural and Scientific Research Government Committees in accordance with the guidelines for Care and Use of Agricultural Animals in Agricultural Research and Teaching (approval A37801).

RESULTS

Three Aurora kinases are expressed in bovine oocyte.

By RT-PCR using specific primers for *Bos taurus* Aurora kinase *AURKA*, *AURKB* and *AURKC* genes, we detected the cDNA fragments of expected sizes 234, 453 and 156 base pairs, respectively, in ovary, testis and in full grown immature oocytes isolated from 3-6 mm antral follicles (Figure 1A). *AURKA* was over-expressed in oocyte as compared with *AURKB* and *AURKC* relatively to β-actin amplification. To confirm this result, we quantified Aurora

kinases transcripts by real-time PCR. *AURKA* mRNA in oocytes was about twenty times more abundant than *AURKB* and *AURKC*, while in testis its level was only about two-fold higher (Figure 1B). *AURKA* and *AURKC* mRNA levels decreased dramatically in blastocyst, while *AURKB* was at similar level both in oocytes and blastocysts (Figure 1B).

Analysis of amino acid sequences showed that bovine AURKA, AURKB and AURKC proteins shared the very similar C-terminal, containing serine-threonine kinase catalytic domain and RXXL destruction-box (D-box), but they differed in non-catalytic N-terminal domain (Supplementary data, figure 1A). Amino acid sequences of bovine Aurora kinases were highly homologous to those of human and mice as shown for AURKA (Supplementary data figure 1B). Predicted molecular weights of bovine Aurora proteins were 46 kDa, 40 kDa and 35 kDa for AURKA, AURKB and AURKC, respectively. In order to analyse the protein expression of AURKA, AURKB and AURKC in bovine oocytes and taking in account the extreme similarity in bovine and human Aurora kinases amino acid sequences, we used the antibodies to human Aurora proteins. Monoclonal antibody to human AURKA revealed a single protein at approximately 46 kDa in an extract prepared from 10 immature or mature oocytes (IO and MO) and in testis, but neither in cumulus (CC) and nor in fibroblasts cells (Fb) (Figure 1C). Human AURKB antibodies detected a protein of about 41 kDa in a protein extract prepared from fibroblast cells or from 50 mature oocytes but not in immature oocytes. In the same conditions, the antibody to human AURKC revealed the 35 kDa protein only in testis. Thus, three Aurora kinases were expressed in oocyte and AURKA was the most abundant form in immature oocytes.

Localization of AURKA, AURKB and AURKC in bovine oocytes during meiosis and in expanded blastocysts.

AURKA, Thr-phosphorylated AURKA, AURKB and AURKC were visualized by indirect immunofluorescent detection in bovine immature oocytes at the GV stage, in MI and MII oocytes and in embryos at the stage of blastocyst (Figure 2). In GV oocytes, AURKA was uniformly present in the whole cytoplasm (Figure 2 **a**). A subpopulation of AURKA phosphorylated on threonine residue was also detected, localized to numerous patches (figure 2 **b**). No significant staining was observed when rabbit or/and mouse non-specific IgG were used instead of primary antibodies (figure 2 **d**). In MI and MII oocytes, AURKA (figure 2 **f, k**) and phospho-Thr -AURKA (figure 2 **g, l**) were dispersed through the cytoplasm. Sometimes AURKA was concentrated around the metaphase-II plate (figure 2 **k,** *MII* insert), phospho-Thr-AURKA immunoreactivity was not detected at that location (figure 2 **l**, *MII* insert). Phospho-

Thr-AURKA was concentrated at a contractile ring in the area that separates the polar body (*PB*) in oocytes at the telophase-1 and MII stages (figure 2 **m;** figure 5E **c**). AURKA was not detected in the spindle poles in MI-MII oocytes (figure 2 **f**, **g, k, l, m**). However, in the expanded blastocyst, AURKA was preferentially concentrated in the spindle poles in metaphase, anaphase and in the newly forming poles in prophase cells (figure 2 **p**, insert *M, An, P, respectively*). Thr-phosphorylated AURKA was clearly detected in the spindle poles during metaphase in the blastocyst (*M,* asterisk-labeled insert in figure 2 **p**).

AURKB was practically undetectable in the cytoplasm of immature oocytes (figure 2 **c**). In MI and MII oocytes, AURKB was detected at the chromosomes (figure 2 **h, n**) like in mitotic blastocyst cells (figure 2 **r,** inserts *M*). AURKB was also detectable in the cytoplasm of MII and to a lesser extent in MI oocytes. However, AURKB was neither detected in a germinal vesicle nor in a polar body chromatin (figure 2 **c**, **n**). In contrast, AURKB was clearly associated with the contractile ring separating the polar body in MII oocyte (figure 2 **n,** insert *PB*) and with contractile ring/midzone between two blastomers during telophase/cytokinesis (figures 2 **r,** insert *MZ*).

AURKC also showed a cytoplasmic localization throughout meiosis (figure 2 **e, i, o**). In MII oocytes, the protein was concentrated between the polar body and what might be the contractile ring (figure 2 **o,** insert *PB*). No significant AURKC specific labeling was detected in blastocyst cells.

Taken together, three known Aurora kinase genes were expressed in bovine oocyte during progression of meiosis. AURKA protein showed the most intriguing expression pattern because it is quite different from the somatic cells. In contrast, AURKB in oocyte was associated to the metaphase chromosomes and to the midbody, as it was expected accordingly to the established pattern in mitotic cells. Faint level of AURKC did not allow continuing their study at protein level. Thus, we concentrated on the analysis of AURKA.

AURKA expression pattern in ovary during folliculogenesis, in fertilized oocytes and in early embryos.

We analyzed localization of AURKA in the oocytes throughout folliculogenesis on the paraffin-embedded sections of bovine ovaries (figure 3A). All main steps of folliculogenesis were found in the sections of the adult cow ovary: i) the primary follicles in which an oocyte is surrounded by a single layer of cells; ii) the secondary and tertiary follicles with more than one granulosa layers; iii) the small antral (0.3-2 mm), antral (2-6 mm) and the pre-ovulation follicles more than 6 mm in diameter, in which the oocytes were surrounded by cumulus cells

and formed COC. AURKA was poorly detectable in the primary follicles (figure 3A **a**), in the secondary follicles (figure 3A **b**) and in the tertiary pre-antral follicles (figure 3A **c**). In small antral follicles of about 0.3-0.6 mm in diameter, AURKA immunostaining clearly appeared in the cytoplasm of oocytes, which achieved more than 80% of the size of a full-grown oocyte (figure 3A **d**). AURKA was also detected in some cumulus, granulosa and theca cells in the actively growing follicles (figure 3A **d, f**) and to a lesser extent in the large follicles (figure 3A **g**). No staining was detected without the first antibody (figure 3A **e**). Along with the follicular antrum growth, AURKA staining increased in the oocyte, being excluded from the nucleus (figure 3A **f, g**). In all analyzed oocytes originated from antral follicles, AURKA was detected exclusively in the cytoplasm.

By western blot, we showed that AURKA protein level increased during IVM, then this level remained stable up to the morula stage, and in the expanded blastocyst AURKA level was significantly declined (figure 3B, black bars). In parallel, we quantified *AURKA* polyadenylated mRNA in oocytes and embryos (figure 3B, grey bars). The level of polyA mRNA encoding AURKA was relatively stable in immature, mature and fertilized oocytes, then it slightly and progressively decreased during three first cleavages (2C, 4C, 5-8C) and it fell tremendously in embryos at the morula and blastocyst stages.

Immunofluorescence showed that AURKA was equally abundant in the cytoplasm of the unfertilized and fertilized oocytes and in 2C and 4C embryos (figure 3C) and was not concentrated significantly in the spindle poles. We observed the concentration of AURKA in the spindle poles only from the third embryo cleavage (5/8-Cell). At this stage and onward, AURKA appeared concentrated to the spindle poles and to the nearest spindle microtubules in all mitotic blastomers going through pro-metaphase, metaphase and anaphase (8-Cell and morula on figure 3C and blastocyst on figure 2 **p**).

Thus, AURKA accumulated at a very high level in the oocyte cytoplasm during the final follicular growth and maturation. AURKA was Thr-phosphorylated in oocytes. AURKA protein was not degraded up to the embryo genome activation. AURKA was definitely relocated to the spindle poles only from 8-Cell stage, i.e. from the moment of the maternal-embryo transition. In order to determine the role of AURKA in oocyte, we followed the expression of this kinase simultaneously with the several main actors implicated in the meiotic progression in vitro.

Expression of AURKA, CPEB, MOS, CCNB1 and CDC2 in bovine oocyte during IVM.

Once retired from the antral follicles and put into IVM culture medium, bovine oocytes passed through the sequential stages of nuclear maturation, and then they finally attained the MII and were arrested at that stage. In our conditions, the recovered oocytes were in the meiotic prophase and kept the intact GV for at least 6 hours, as was confirmed by lamin A/C immunostaining (not shown). First GVBD occurred at around 6 hours of IVM and the MI stage was achieved at 10 hours of IVM. At 22 hours, most of the oocytes were at the MII (figure 4A). We quantified polyA mRNA levels of *AURKA, CCNB1, MOS, CDC2* and *CPEB* in oocytes at 3, 6, 10 and 22 hours of IVM by using real-time PCR performed on oligo(dT)-extended cDNA (figure 4B). In this experiment, in order to prevent cytoplasmic polyadenylation of oocyte mRNA before IVM, the reference immature oocytes (0h, white bars) were retrieved from the ovaries transported at 4°C and were manipulated at 4°C up to RNA extraction. Total RNA level did not significantly changed during IVM as it was evidenced by 18S rRNA quantification. Levels of polyA *AURKA, CCNB1, CDC2* and *CMOS* mRNA increased during the first 3 hours of IVM and from this time onward they were relatively stable up to 10h of IVM. After 22h of IVM, AURKA polyA mRNA level did not changed as compared to 0h, while those of *CDC2* was significantly diminished. *CCNB1, CPEB* and *CMOS* mRNA levels were slightly decreased in MII oocytes (figure 4B).

We quantified AURKA, CCNB1, CPEB and CDC2 protein's levels in the oocytes at the same time of IVM (figure 4C). AURKA and CCNB1 increased significantly during maturation. Two closely migrated shifted AURKA bands were detected in the oocytes throughout IVM (figure 4D) that might be a consequence of AURKA phosphorylation. For this experiment, the special sampling and SDS-PAGE procedures were used as described [46]. In contrast to AURKA, CCNB1 was not detectable before IVM and its appearance coincided with the phosphorylation of MAPK3/1 at 3h of IVM (figure 4C). CDC2 protein was maintained at the relatively constant level throughout IVM (figure 4C). CPEB was stable in immature oocytes up to GVBD (6h of IMV), then it declined at 10h and was mostly degraded at 22h of IVM. The lower migrating CPEB-immunoreactive shifted band was detected at 6 and 10 hours of IVM (figure 5A). We performed the λ-PPase treatment of the oocytes at 10h of IVM and demonstrated that this lower migrating band corresponded to the hyper-phosphorylated form of CPEB (figure 5B). The double immunofluorescence demonstrated that both AURKA and CPEB localized to the oocyte cytoplasm (figure 5C). CPEB was fewer detected in the cytoplasm of the mature oocyte (MII) as compared with the immature oocyte (GV). However, in MII oocytes CPEB was clearly detected at the region of the polar body separation, which might be a contractile ring / midbody (figure 5C, insert *PB*).

Effect of Roscovitine, U0126 and Metformin on oocyte maturation.

Addition of 50 µM of Roscovitine, a specific inhibitor of CDC2/MPF activity, arrested the oocytes at the GV stage after 22h of IVM, but neither significantly influenced the accumulation of AURKA (figure 5D) nor AURKA phosphorylation as detected by immunofluorescence (figure 5E, d) and by gel shift (Supplementary data, figure 2). The phosphorylation of MAP kinases was inhibited by the addition of specific MAPK kinase inhibitor U0126 (100µM). The U0126-treated oocytes overcame the GVBD but did not progress to MII after 22h IVM (figure 5D). Nevertheless, the accumulation of AURKA was neither significantly affected, nor did we observe any loss of phospho-Thr- AURKA labelling in U0126 oocytes (figure 5E, f). In contrast the addition of 10 mM of metformin, an insulin-sensitizing agent and an activator of AMPK (Adenosine Monophosphate-Activated kinase), arrested the oocytes before GVBD and decreased significantly AURKA synthesis and the level of phospho-AURKA immunofluorescence (figure 5D and 5E e). The level of phospho-MAPK3/1 was significantly reduced in the presence of Roscovitine, U0126 or metformin. In contrast, CPEB level was not diminished after 22h of IVM in the oocytes treated with Roscovotine, U0126 or metformin, although in the control IVM experiments, without inhibitors or with DMSO, CPEB was largely degraded in mature oocytes (figure 5D).

Thus, in bovine oocytes, the inhibition of MPF or MAPK activation during IVM stopped meiosis before the first division. These treatments had no effect on AURKA accumulation and activation, but AURKA depletion by the metformin coincided with an arrest at GV. Interestingly, all tested inhibitors decreased the level of phospho-MAPK3/1 at 22h of IVM, and CPEB was neither hyper-phosphorylated nor degraded.

Effect of Aurora kinases inhibitor VX680 on oocyte maturation.

We performed IVM either in the presence of VX680, a small molecule inhibitor for Aurora kinase activity, or with DMSO as a control. VX680 is a potent inhibitor for AURKA, AURKB and AURKC with apparent inhibition constant values of 0.6, 18 and 4.6 nM, respectively [37]. After IVM, oocytes were denuded from the cumulus cells and nuclear status of oocytes was monitored by the chromatin Hoechst staining. When 1 µM concentration of VX680 was used, 72.4% of oocytes were arrested before the MI stage whereas 85% of control oocytes were already in MII. In VX680-treated oocytes, no phospho- Ser^{10} Histone H3 staining was detected in a compact clump of condensed chromosomes, whereas in control oocytes the phospho-Ser^{10} histone H3 was associated to the metaphase chromosomes and to the polar body chromatin (figure 6A). Practically all the VX680-treated oocytes passed through the GVBD, while no

lamin A/C staining was detected around the chromatin (not shown). In the presence of lower concentration of VX680 (100 nM), all the oocytes continued meiosis from MI onward and phospho- Ser^{10} Histone H3 was detected associated with the chromatin (figure 6A). However, many oocytes demonstrated the abnormal meiosis and showed the over-numerated chromatin structures after $VX680_{100nM}$ treatment (Table 2, figure 6B). After 14h of IVM, the control oocytes were mostly at the MI (figure 6B a), while in VX680-treated oocytes, several chromatin groups were detected into the cytoplasm (figure 6B b, c, d). Abnormally large, either non-extruded (figure 6B c) or over-numerated (figure 6B b) polar bodies were observed in $VX680_{100nM}$ -treated oocytes. The chromosomes were sometimes completely disorganized and dispersed in the cytoplasm (figure 6A d). After 24h of IVM, the most of the control oocytes were at the MII stage (figure 6B e). In contrast, the multiple chromatin structures, similar to the activated pronucleus or to the non-extruded doubling polar bodies, were detected in $VX680_{100nM}$ -treated oocytes (figure 6B f, g, h). Quantification of several polyA transcripts revealed after 3h of IVM the decrease of only *MOS* mRNA level in VX680-treated oocytes as compared to the control oocytes (figure 6C). By Western blot no differences were detected in AURKA, CDC2 or phospho-MAPK3/MAP1 protein levels between $VX680_{100nM}$ -treated and control groups (figure 6D). In contrast, yet CPEB was detectable in the control oocytes after 14h of IVM, in $VX680_{100nM}$ -treated oocytes CPEB was already degraded.

DISCUSSION

Aurora kinases in bovine oocyte: AURKB and AURKC were localized as in mitotic cells while AURKA had a different expression pattern.

We have shown experimentally that three Aurora kinases are expressed in bovine oocytes, and that AURKA is more prevalent in immature oocytes in comparison to the other Aurora kinases. Immunofluorescence and immunoblot analysis indicated that in maturing oocytes Aurora kinases were present in the cytoplasm and AURKA and AURKB levels increased during IVM. In human oocytes also, the level of AURKC mRNA was higher in MI and MII compared to immature oocytes [35]. Moreover, we found that AURKB, AURKC and the active Thr-phosphorylated AURKA were concentrated in the contractile ring/midbody zone, located at the furrow separating the first polar body. The contractile ring is an actomyosin-based structure essential for the completion of cytokinesis that is formed on the central spindle in early anaphase with the bundling of overlapping microtubules. These bundles become compacted and mature into the midbody (for review [47]). AURKB is a component of the central spindle assembly complex and it phosphorylates another component, Kinesin-6 protein MKLP1, in vitro and in vivo [48]. Recent studies reported the colocalization of AURKC with AURKB/Survivin complex in HeLa cells during mitosis [25]. Thus, the detection of bovine AURKB and AURKC in the midzone between the ooplasm and the first polar body is consistent with their expression pattern in the mitotic cells. More surprisingly, phospho-AURKA was also found in this zone, and this indicates that AURKA might have downstream substrates on at this site. Thus, in bovine oocytes, Aurora kinases might participate in the regulation of the first polar body extrusion.

AURKB in bovine oocyte was detected to be bound to the metaphase chromosomes in meiosis I and II, but neither to the chromatin in GV nor in polar body. This hypothesised the role of AURKB in the correct alignment and segregation of the chromosomes during meiotic divisions similarly to mitotic cells. These results were in concordance with the data showing that in porcine oocytes AURKB was not involved in chromosome condensation during the first meiosis while the chromosomes were condensed even upon the inhibition of AURKB and Histone H3 phosphorylation [34]. In contrast, AURKA followed the typical expression pattern of maternal mRNA, i.e. it was accumulated during oogenesis and its level was diminished after the maternal-embryo transition. AURKA protein was detected in oocytes from small antral follicles simultaneously with the appearance of the antrum and with basal follicular growth. The signals for antrum formation are not well understood, but in vitro studies performed with rodent follicles showed that FSH, LH, KL (kit ligand) and EGF are possible candidate

542 regulators (for review [49]). These signals may be also involved in the regulation of AURKA expression in the bovine oocyte. In cows, the antral follicles up to 5 mm in size are not yet
544 dependent on gonadotropins. Progesterone and androgens are likely the main steroid hormones produced by theca cells, since granulosa cells lack the expression of P450 aromatase,
546 converting steroids to estrogens [49]. In frog oocytes, progesterone induces AURKA accumulation and activation [11, 46]. It is not known whether progesterone or other factors
548 induced the initial translation of AURKA in bovine oocytes. However, our data suggests that accumulation of AURKA in oocyte cytoplasm starts a long time before the ovulation and the
550 activated form of this kinase was detected in GV oocytes retrieved from follicles that exceeded 3 mm. In Xenopus oocytes, it was shown that the upper band from two closely migrating bands
552 revealed by the AURKA antibody corresponds to Thr-phosphorylated AURKA [30], [50]. Phosho-AURKA in Xenopus oocytes was detected at GVBD [50,51], while in bovine oocytes
554 the shifted AURKA bands were already present in immature GV oocytes and also later during IVM. Immunofluorescence detection of Thr-phosphorylated AURKA confirmed that active
556 Aurora A kinase was present before the GVBD in bovine oocytes.

Thr-phosphorylated AURKA showed an intriguing cytoplasmic localization during meiosis in
558 bovine oocyte. Phosphorylated AURKA was visible on the surface and dispersed in the ooplasm of immature oocyte but was absent from the spindle zone in oocytes at MII, whereas,
560 the pan-AURKA eventually concentrated around the MII aligned chromosomes. Perhaps inactive AURKA participates to the prevention of premature mitotic divisions of unfertilized
562 oocytes and thus maintains MII arrest. The absence of the active maternal centrosomes in the spindle poles in bovine and human oocytes could also explain why AURKA was not
564 concentrated on the spindle poles [52]. In contrast, AURKA was located to the spindle in mitotic blastocyst cells and was Thr-phosphorylated at the spindle poles. The concentration of
566 AURKA in the spindle poles was clearly observed in all mitotic cells in bovine embryos from 8-Cell stage, i.e. at the stages when the novel embryo genome was already active [53].
568 Whereas AURKA mRNA disappeared progressively in the early embryo, and the AURKA protein level was stable up to the morula stage and was no longer significantly detectable in the
570 blastocyst. The same was observed in *Xenopus* embryos, where the protein level was stable during several cleavages-while AURKA activity oscillated. In contrast, in somatic cells,
572 AURKA protein level peaked at G2/M stage and was degraded during the cells exit from mitosis. Indeed, destruction of AURKA and several other mitotic regulators, such as cyclins,
574 requires an activator of the ubiquitin ligase APC/C (anaphase-promoting complex/cyclosome) called Cdh1 (for review [54]). However, in *Xenopus* eggs and early embryos, Cdh1 is missing,

and consequently AURKA is not degraded during the initial cleavages [55]. APC/C recognition sequences, the destruction-boxes, the D-box and A-box are all conserved in *Xenopus* and human Aurora genes [56], [55]. A putative A-box required for the Cdh1-dependent destruction was found within bovine AURKA amino acid sequence, in a similar to way to the human, mice and frog genes homologues (Supplementary data, figure 1B). In mice, Cdh1 is already present in MII oocytes and some APC^{Cdh1} substrates begin to degrade from the second polar body extrusion [57]. Taking in account that in mice, embryos are transcriptionally active at the late one-cell stage, bovine embryos at the eight-cell stage, and frog after 12 cleavages at midblastula, we could hypothesize that in bovine oocytes, maternal AURKA was required to be produced and to be activated in order to accomplish its role up to maternal-embryo transition, after which it might be destroyed via the APC^{Cdh1} mechanism. Therefore, the high concentration of active Aurora A in cytoplasm of fully grown immature bovine oocytes is probably required for the distinct functions related to the progression of meiosis similarly to those in frog and mice (review [18]). Thus, AURKA expression was followed throughout IVM in parallel with the main actors of meiotic progression.

AURKA synthesis and phosphorylation was independent from MAPK and MPF activation but was not required for CPEB hyper-phosphorylation during IVM.

An increase in the MOS, *CCNB1* and *CDC2* polyadenylated mRNA levels was observed during the first 3 hours of IVM and the subsequent appearance of CCNB1 protein. Our results were in agreement with the reported RACE-PAT analysis, which showed that the *CCNB1* mRNA polyA tail was progressively elongated during the first 10h of IVM and CCNB1 was synthesized from the transcripts with a long polyA [58]. MOS protein was produced in bovine oocytes from around the onset of meiotic resumption, and became phosphorylated at GVBD [59]. In pig oocytes, although MOS transcripts were polyadenylated, their translation was repressed before GVBD and then increased steadily from MI to MII [60]. According to the data, AURKA protein was already present in the active phosphorylated form and was localized together with CPEB in the cytoplasm of immature oocytes, i.e. before the massive translation of CCNB1 and MOS. AURKA level increased up to MI, and CPEB became hyper-phosphorylated at that time. *MOS, CCNB1, CDC2* and *AURKA* mRNAs contain the CPE in their 3'-UTRs. Thus, AURKA might be potentially implicated in the regulation of polyadenylation-dependent translation of these transcripts during oocyte maturation via its participation in the activation of CPEB as in *Xenopus* and mouse [11,13,15,30,61]. CPEB is a highly conserved RNA binding protein [62] which is a general regulator of meiosis. In CPEB

knock-out mice oogenesis and spermatogenesis were disrupted at pachytene [63]. In *Xenopus laevis*, phosphorylation of CPEB by Aurora A was reported to trigger the interaction of CPEB and CPSF, which in turn binds to the AAUAAA sequence and recruits poly(A) polymerase to the end of mRNA and therefore regulate the polyadenylation-dependent translation of CPE-containing transcripts (for review see [8]). It was also reported, that in human and rat cell lines the over-expression of recombinant AURKA induced the phosphorylation of CPEB and promoted the polyadenylation of CCNB1 and CDK1/CDC2 mRNAs [64]. In mice oocyte, AURKA was reported to phosphorylate CPEB first at leptotene stage of prophase and then at MI, initially triggering the translation of SCP1/SCP3 and then MOS/CCNB1, respectively [61]. We found that AURKA was active in prophase oocytes and was thus potentially capable of phosphorylating CPEB at a key serine residue in order to activate its functions during this period. Moreover, the gel shift of the CPEB band due to high phoshorylation at several residues was observed during the GVBD-MI transition (6-10h of IVM), prior to the partial degradation of CPEB during MI/MII transition and the relocation of remnants to the contractile ring/midzone. The fact that CPEB colocalizes with phospho-AURKA at the contractile ring/midzone might indicate the local translation/activation of MOS, CCNB1/CDC2 or other substrates. In synchronized HeLa cells, AURKA was shown to participate to the recruitment of the CCNB1/CDC2 complex to centrosomes, where MPF becomes activated and commits cells to mitosis [65]. In *Xenopus*, once the polyadenylation took place during oocyte maturation, most of CPEB was destroyed and all that remained stable was confined to the animal pole blastomeres in embryos where it was strongly associated with the spindle and centrosomes and was involved in the localization of CCNB1 mRNA to the mitotic apparatus [66]. Intriguingly, all tested meiotic inhibitors blocked CPEB hyper-phosphorylation and degradation, although the levels of AURKA and Thr-phosphorylation were not significantly affected, as observed during Roscovitine or U0126 treatment. The arrest prior to MI and the decrease of MAPK3/1 phosphorylation were the most common effects of Roscovitine, U0126 and metformin on oocytes after 22h of IVM. Therefore, active MAPK might be involved in CPEB hyper-phosphorylation and its consequent degradation. In fact, a recent study reported that MAPK activation is required for the phosphorylation of CPEB during meiosis in Xenopus oocytes and a lower level of MAPK activation was detected prior to MOS synthesis [50].

A significant decrease in AURKA level was observed in metformin-treated oocytes with subsequent meiotic arrest at GV stage and this was accompanied by the complete depletion of CCNB1 synthesis and a significant decrease in MAPK phosphorylation. Metformin inhibits protein synthesis through the eEF2 kinase/eEF2 axis and/or the p70S6 kinase pathway [67]. In

porcine oocytes, metformin also provoked meiotic arrest [68]. Interestingly, metformin was recently reported to be an AMPK-dependent growth inhibitor for breast cancer cells [69], where AURKA is also known to be over-expressed [26,70]. AMPK is present in bovine oocytes and cumulus cells [36], and it would be interesting to know whether there is functional relation between AMPK and AURKA. Although non-specific, the depletion of AURKA by metformin in bovine oocytes resulted in the arrest of meiotic progression. In *Xenopus* oocytes, the ablation of AURKA protein by the microinjection of a specific antibody also blocked meiosis, but the oocytes were arrested at MI [71].

In contrast to CCNB1 and MOS, CDC2 was already present in immature bovine oocytes, and no variation in protein quantity was observed during maturation, consistent with previous reports [6, 72]. This suggests that CDC2 mRNA should be polyadenylated and translated during oogenesis earlier that CCNB1 and MOS. In human cells, containing DNA damage and arrested at G2/M, the over-expression of AURKA triggered an override of the G2/M arrest through a CDK1/CDC2 reactivation, suggesting the existence of a retroactive control loop between AURKA and CDK1 [73]. In frog oocytes, it was also shown that active CDC2 was necessary for AURKA activation [14,46,74]. Our results showed that AURKA was phosphorylated before the activation of MPF and MAPK3/1, and before GVBD. Moreover, the inhibitors for CDC2/MPF or MAPK3/1 activity (Roscovitine or U0126 respectively) did not change the AURKA and phospho-Thr-AURKA expression pattern during IVM. These data confirmed that AURKA accumulation and phosphorylation was independent of MPF or MAPK activation in bovine oocytes as this has been shown in other models [30].

Effect of the Aurora kinase activity inhibitor VX680 on the meiotic progression of oocytes.

VX680 blocks the kinase activity of all three Aurora kinases, although it shows the greatest selectivity for AURKA [37]. In human cells, VX680 inhibits cell proliferation, induces DNA endoreduplication, tetraploidization and leads to apoptosis [37,75]. In our experiments, oocytes treated with 1 µM VX680 resumed GVBD but did not proceed to MI. Since the histone H3 was not phosphorylated in $VX680_{1\mu M}$ -treated oocytes, AURKB was probably inhibited at that concentration together with AURKA and AURKC. Similarly, in porcine oocytes, Aurora kinase inhibitor ZM447439, more specific for AURKB, prevented both the activation of AURKB and phosphorylation of histone H3 on Ser^{10}. ZM447439-treated oocytes were arrested just after GVBD in the late diakinesis stage [34]. Although AURKB is not required for chromosome condensation [34], it is required for maintenance of chromatin condensation in

Xenopus [76] and surf clam oocytes [77], and thus AURKB activity might be critical for the correct MI/MII transition.

At a concentration of 100 nM, VX680 neither significantly affected the Ser^{10}-phosphorylation of histone H3 nor blocked progression through meiosis. Therefore, AURKB might not be completely inactivated. However, the abnormal meiotic events, including chromosomes misalignment, chromatin decondensation and formation of pronucleus-like structures were observed. Oocytes treated with 100 nM of VX680 seemed to "accelerate" meiosis since polar bodies (often non-extruded) were already detected, whereas most of the control oocytes were only at MI after 14h of IVM. In fact, pronucleus-like chromatin structures were detected within the cytoplasm of $VX680_{100nM}$ -treated oocytes whereas the control oocytes were at MII. The same observation has been made in Xenopus oocytes in which AURKA activity was inhibited [78]. While in VX680-treated oocytes MOS polyA mRNA was diminished, it could be presumed that due to the lower level of MOS protein, the oocytes might enter the parthenogenesis. Indeed, oocytes derived from MOS-deficient female mice (MOS-/-) fail to arrest at MII and undergo parthenogenetic activation [79]. Similarly, bovine and porcine oocytes, in which the endogenous MOS mRNA was depleted with double-strand or antisense RNA microinjections, were partenogenetically activated [80-82]. VX680 also disturbed cytokinesis, and this resulted in oocyte "polyploidy". Intriguingly, CPEB was more rapidly degraded in VX680-treated oocytes, but the factors which are responsible for it degradation are not known. CPEB was probably already activated before the oocytes were recovered from the follicles, thus the polyadenylation of CCNB1 might be initiated before IVM as reported in bovine species [58], and therefore the maturation progressed and GVBD occurred. However, MOS protein must be synthesised after GVBD in order to maintain the metaphase-II arrest as a component of the cytostatic factor [83,84]. It could be supposed that while kinase activity of AURKA was at least partially inhibited by VX680, MOS might not be synthesized at a high enough level at this time, and therefore oocytes exited from MII and began DNA replication.

In conclusion, three Aurora kinases are expressed during meiosis in bovine oocytes. AURKA, AURKB and AURKC show distinct expression patterns during IVM but localize together to the midbody during the first polar body extrusion. Meiotic divisions and polar body extrusion were affected by the Aurora kinase activity inhibitor. Therefore, Aurora kinases might play a role in chromosome segregation as in mitotic cells, but also an oocyte-specific role in metaphase-II maintenance. Besides which, AURKA is highly expressed and phosphorylated in immature oocyte cytoplasm where CPEB also localizes. Nevertheless, the presence of active AURKA was not sufficient to produce the CPEB hyper-phosphorylation

observed at MI. Altogether, these observations might indicate the putative involvement of AURKA in regulating polyadenylation-dependant translation in bovine oocytes, although this role will have to be demonstrated by further studies.

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FIGURE LEGENDS

Figure 1. Aurora kinases mRNA in bovine oocyte and embryo.

A. Detection of Aurora kinases genes expression in bovine immature oocytes (Oo), ovary (Ov) and testis (Te) by RT-PCR. Total DNAse-treated RNA from testis and ovary (1 µg each) and from ten isolated full-grown immature GV oocytes was reverse transcribed. 1% of cDNA was used to detect either *AURKA*, *AURKB* and *AURKC* or β-actin (*ACTB*) expression by performing 36 or 32 PCR cycles, respectively. RT-omitted RNA from testis and ovary (125 ng) was used as negative control (-).

B. *AURKA, AURKB* and *AURKC* polyA mRNA levels measured by RT-qPCR in immature oocytes, embryos at the blastocyst stage and in testis. RT were performed on RNA from 10 oocyte/embryo using oligo-dT(15) and real time PCR was run in duplicates using 0.5 % of cDNA per reaction. Serial dilution of plasmid DNA containing *AURKA, AURKB* or *AURKC* partial cDNA were used for quantification standard. Mean quantities +/- SEM of four different cDNA samples are presented in fg per ovocyte/embryo and in fg per microgram of testicular RNA. Different small letters indicate significant difference in oocytes and embryos, and different capital letters indicate significant difference in testis ($p<0.05$).

C. Bovine AURKA, AURKB and AURKC proteins detection by Western blot.

Total proteins were extracted from immature oocytes (IO), mature oocytes 22h after IVM (MO), cumulus cells (CC), bovine fibroblasts (Fb), ovary (Ov) and adult testis (Te) biopsies and then subjected to SDS-PAGE and immunoblot. Monoclonal antibodies to human Aurora A detected 46 kDa AURKA protein in bovine oocytes (10 oocytes were loaded) and testis, and ARK-2 rabbit polyclonal antibody recognized 40 kDa AURKB protein in fibroblasts, testis and mature oocytes (50 oocytes were loaded). AURKC was detected in testis by using the antibody to human Aurora C. Control detection of α-tubulin (TUBA) was performed.

Figure 2. AURKA, AURKB and AURKC proteins localization in bovine oocytes during meiosis and in blastocyst.

AURKA, Thr-phosphorylated AURKA, AURKB and AURKC were detected by immunofluorescense in bovine GV immature oocytes (**a, b, c, e,** respectively); in metaphase-I (MI) oocytes (**f, g, h, i,** respectively); in methaphase-II (MII) oocytes (**k, l, n, o,** respectively). AURKA, phospho-AURKA and AURKB were also detected in embryos at blastula stage (**p, r,** respectively). Red (AURKA) or green (phospho-Thr AURKA, AURKB and AURKC) fluorescent images were merged with Hoechst blue chromatin staining. For the control, specific antibodies were replaced by mouse and rabbit IgG (**d**). Double-immunofluorescence was performed for simultaneous pan-AURKA and phosho-Thr-AURKA detections. In oocytes and blastocysts, the metaphase plates (*M, MI, MII*) are encompassed by white arrows and magnified in insert images. Contractile ring between the oocyte and the polar body (*PB*) in telophase-I / MII oocytes (**m, n, o**) and the midzone (*MZ*) between two blastomers during cytokinesis (**r**) are encompassed by white arrowheads and are magnified in inserts. Prophase (*P*), metaphase *(M)* and anaphase (*An*) cells in blastocyst are magnified in inserts images. Insert *M* labeled by white asterisk in (**p**) showed the phospho-Thr AURKA labeling at the

poles of the metaphase spindle (picture was selected from another blastocyst). Inserts for the pictures of the metaphase chromosomes (*M, MI, MII*) and the polar body (*PB*) in (**k**)**,** (**l**)**,** (**m**), (**n**)**,** (**o**)**,** (**r**) showed either Aurora immunostaining alone or Aurora / chromatin merged images. Bars: 0.5 mm.

Figure 3. AURKA in bovine ovary during folliculogenesis, in fertilized oocyte and in early embryo.

A. Analysis of AURKA protein expression during folliculogenesis was performed by DAB-immunoperoxidase staining on paraffin-embedded ovary sections. Images showed primary follicles (**a**), secondary follicle (**b**), tertiary pre-antral follicle (**c**), small antral follicle of about 0.6 mm in diameter (**d**), large antral follicles exceeded 2.1 mm and 3.4 mm (**f** and **g**). Control immunostaining was performed by using mouse IgG instead of primary antibodies (**e,** adjacent section to **f**). Immuno-specific staining is brown. Cell nuclei were stained with hematoxylin. Cumulus-oocyte complex (COC), oocyte (Oo), cumulus cells (CC), antrum (At), granulosa (Gr) and theca cells (Th) are designated. Grey rectangles encompassed regions which are showed magnified on the right. Bars: **a, b, c, e** – 50 µm; **d, f, g,** – 500µm.

B. Analysis of *AURKA* mRNA expression during the early embryo development was performed by relative mRNA quantification by real time RT-PCR (grey bars) and AURKA protein level was analyzed by immunoblot (black bars). Representative immunoblot is shown. IO – immature germinal vesicle oocyte, MO - mature oocyte at MII, Zyg – fertilized oocyte or zygote, 2C - 2 cell embryos, 4C - 4 cell embryos, 5-8C – 5-8 cell embryos, Mor – morulae (>16 cells), Bl – expanded blastula. Immunoblots were quantified and results are presented as AURKA / TUBA ratio. Mean of four (mRNA) or three (protein) independent quantifications ± SEM are presented. Different small letters indicate significant difference in mRNA expression and different capital letters indicate significant difference in protein levels ($p<0.05$).

C. Detection of AURKA in bovine fertilized oocyte and in early embryos by immunoluorescence. In white-framed inserts nuclear blue Hoechst staining showed the number of blastomers. Control immunofluorescence was performed with only secondary antibody**.** Regions of particular AURKA concentration are encompassed by the white arrows and the magnified pictures are shown below. Spindle poles in pro-metaphase (*Pm*), metaphase (*M*) and anaphase (*An*) cells in 8C embryo and morula (lower images) are indicated by the short arrows. Bars (A-I): 50 µm.

Figure 4. Analysis of AURKA, CPEB, MOS, CCNB1 and CDC2 expression in bovine oocyte during in vitro maturation.

A. Timing of nuclear maturation in bovine oocytes determined by lamin A/C and Hoechst chromatin analysis of oocytes at different time of IVM culture of COC in our laboratory conditions (IVM medium was TCM199 supplemented with 10% of fetal calf serum and 10 ng/ml of EGF). GV, germinal vesicle; GVBD, germinal vesicle breakdown, MI, metaphase-I, MII, metaphase-II.

B. Real time PCR quantification of relative *AURKA, CPEB, CCNB1, CDC2* and *MOS* polyA mRNA levels in bovine oocytes before or after 3h, 6h, 10h and 22h of IVM. Reference immature oocytes (0h, white bars) were retrieved from ovaries transported on ice and manipulated in cold medium up to lysis in TriZol reagent in order to prevent the RNA polyadenylation. For each time point, RT was performed using oligo-dT and 18S antisense RT primer on four independent RNA samples from 10 oocytes each; real time PCR was run in triplicates using 0.5 % of cDNA per reaction and median values were considered. Each value was normalized by corresponding 18S rRNA abundance (boxed graphic) and represented as histograms; level in immature oocytes was considered as one. Mean +/- SEM of four samples is presented. Different letters designated significant difference at $p<0.05$.

C. Immunoblot detection of AURKA, CPEB, CCNB1, CDC2 and phospho-MAPK3/MAP1 in oocytes just before IVM (0h) or after 3h, 6h, 10h and 22h of IVM COC culture. Denuded oocytes (25-50 oocytes per lane) were subjected to western-blot by using consequently AURKA, CCNB1, CDC2, CPEB, phospho-MAPK3/MAPK1 and alpha-tubulin (TUBA) antibodies on the same membrane. Representative blots from four independent experiments are shown. Blots were quantified and protein / TUB ratio is represented as histograms. The results are presented as means ± SEM; the values for the immature oocytes are considered as one. Different letters designated significant difference ($p<0.05$).

D. Immunoblot detection of the AURKA at 1h, 3h, 6h, 10h and 22h of IVM by using the special extraction procedure and Anderson SDS-PAGE [46]. 30 oocytes per line were loaded. The upper band might represent the AURKA phosphorylated protein.

Figure 5. Detection of CPEB and AURKA in bovine oocytes at different maturation stages and in the presence of meiotic inhibitors during IVM.

A. Immunoblot detection of co-expression of CPEB and AURKA in oocytes before (0h) or after 6h, 10h and 22h of IVM. Denuded oocytes (30 oocytes per lane) were immunoblotted by

using consequently CPEB, AURKA and TUBA antibodies on the same membrane. Note the double band revealed by the CPEB antibody at 6h and 10h of IVM.

B. Detection of phosphorylated forms of CPEB and MAPK1 by a gel-shift. 50 oocytes at 10 hours of IVM were lysed and either incubated (+) or not (-) with 400 units of λ-phosphatase. Anderson SDS-PAGE and successive immunoblotting with CPEB, total MAPK1 and phospho-MAPK1 antibodies were performed. 50 non-treated immature oocytes before IVM (0h) were also loaded. TUBA was used as a loading control.

C. Double-immunofluorescence detection of CPEB (a,d) and AURKA (b) in immature (GV) and mature (MII) oocytes. Images of AURKB (b) and CPEB (a) staining are presented separately or merged with DNA labelling (c, d). Merged CPEB / DNA detections in MII oocyte (d); the region of the contractile ring near the polar body (PB) is encompassed by the arrowhead and magnified in the insert. Bars: 50 µM.

D. Effect of Roscovitine, U0126 and Metformin on AURKA, CCNB1, CPEB, CDC2 and phospho-MAPK3/MAPK1 protein levels in oocytes after 22h of IVM.

Immunoblot detections of proteins were performed in immature oocytes (IO) and in oocytes after 22h of IVM. IVM was performed either in usual medium (control), or supplemented with 5% of DMSO, of with 50µM of MPF inhibitor Roscovitine (Rosco), or with 100µM of phospho-MAPK inhibitor U0126, or with 10mM of Metformin (MetF). Denuded oocytes (25 per lane) were subjected to immunoblot by using consequently AURKA, CCNB1, CDC2, CPEB, phospho-MAPK3/MAPK1 and TUBA antibodies on the same membranes. Representative blots from three independent experiments are shown. Presence or absence of GVBD / MII (nuclear maturation) is marked by (+) or by (-) respectively.

E. Immunofluorescence analysis of AURKA Thr-phosphorylation in control oocytes (**a**, GV oocyte before IVM; **b**, MI oocyte after 10h of IVM; **c**, MII oocytes after 22h of IVM) and in oocytes after 22h of IVM in the presence of 50µM Roscovitine (**d,** oocytes arrested at GV), 10mM Metformin (**e,** oocytes arrested at GV) or 100µM U0126 (**f,** oocytes arrested at pro-MI). In control immunostaining (**g**), primary antibodies were replaced by mice and rabbit non-specific IgG.

Figure 6. Effect of Aurora kinase inhibitor VX680 on oocyte maturation.

A. Immunofluorescence detection of phospho-Ser^{10} histone H3 and chromatin staining in the oocytes after 24 hours of IVM in the presence or not of Aurora kinase inhibitor VX680. Oocytes were fixed and stained with phospho-Ser^{10} histone H3. Left panel - control MII

oocyte; middle panel – oocyte after IVM in the presence of 100 nM VX680, the polar body was not extruded; right panel – oocyte after IVM in the presence of 1μM VX680, oocyte was arrested in pro-MI.

B. In vitro maturation of bovine COC in the presence or not of 100nM of Aurora kinase inhibitor VX680 was performed. IVM was stopped either 14h or 24h after the beginning, oocytes were denuded from cumulus cells and nuclear status of oocytes was monitored by staining with Hoechst. At 14h of IVM control oocytes were mostly at MI (**a**), while in VX680-treated oocytes several chromatin groups was detected (**b, c, d**). At 24h control oocytes were mostly at MII (**e**), and in VX680-treated oocytes chromatin structures similar to activated pronucleus were detected (**f, g, h**). The nuclei of the eventual cumulus cells are noted (cc).

C. RT-qPCR quantification of *AURKA*, *MOS*, *CDC2*, and *CPEB* mRNA after 3 hours of IVM in control oocytes (black bars) or in oocytes treated with 100 nM of VX680 (grey bars). For each time point, RT was performed on four independent RNA samples from 10 oocytes each. Relative mRNA values are presented as mean +/- SEM. Star designates the significant difference at $p<0.05$.

D. Immature oocytes (IO) and oocytes after 14h and 24h of IVM in the presence or not of 100nM VX680 were analyzed by western-blot (40 oocytes per line) for AURKA, CDC2, CPEB, phospho-MAPK3/MAP1 and α-tubulin (TUBA) proteins**.**

Figure 1 supplementary data. Aurora kinases sequences analysis.

A. Alignment of bovine Aurora kinases AURKA, AURKB and AURKC amino-acid sequences. Underline delimits serine-threonine kinase catalytic domains. Catalytic sites are black-boxed. **B.** Comparison of bovine, human and mouse AURKA amino acid sequences (GenBank accession numbers: bovine ABC61056, human O14965, mouse P97477). Identical amino acids are shaded. Putative APC/C recognition sequences A-box and D-boxes are outlined.

Figure 2 supplementary data.

A. Immunoblot detection of the AURKA and phospho-MAPK1 during IVM in the presence or absence of 50 μM Roscovitine by using the special extraction procedure [46]. Two shifted bands of AURKA were detected; the upper band might represent the phosphorylated form.

Tables

Table 1. Oligonucleotide primer sequences

Gene	Primer	Sequence (5'-3')	Accession number	Product size (bp)
AURKA	sense antisense	TCGGGAGGACTTGGTTTCTT TGTGCTTGTGAAGGAACACG	DQ334808	234
AURKB	sense antisense	GACATCAAGCCTGAGAATCTGC AAGGACAGGGACATTTCAGGG	NM_183084	453
AURKC	sense antisense	TCCTCATAGTAGGTTGCCGCGT TTCCTCGATGGCTCATGACCA	XM_870932	156
ACTB	sense antisense	GCGTGACATCAAGGAGAAGC TGGAAGGTGGACAGGGAGGC	NM_001033618	432
CDC2	sense antisense	ATGGCTTGGATCTGCTCTCG CATTAAAGTACGGATGATTCAGTGC	NM_174016	89
CCNB1	sense antisense	TGGGTCGGCCTCTACCTTTGCACTTC CGATGTGGCATACTTGTTCTTGATAGTCA	BT020128	332
CMOS	sense antisense	ATGGAGTTCGGGGGCAATAT TAACAGGCTCTCCTTTGAGGA	CX951994	328
CPEB	sense antisense	CACACACTCGGTACTGAGCAT ACACAGAAGCTCCTGGCCAT	XM_864691	410
luc	sense antisense	TCATTCTTCGCCAAAAGCACTCTG AGCCCATATCCTTGTCGTATCCC	X84847	149
18S	sense antisense antisense RT	CGGACCAGAGCGAAAGCATTTG GAATAACG CGCCGCATCG TCGCTCCACCAAC	DQ222453	132

Table 2. Nuclear status of the oocytes after IVM in the presence or not of 100 nM of VX680.

	pro-MI et MI oocytes	oocytes in AnaI-TeloI-Meta II	Multi-nuclear oocytes
14h IVM control	83.3%	16.7%	0
14h IVM + VX680	41.7%	41.7%	16.6%
24h IVM control	21.7%	78.3%	0
24h IVM + VX680	25%	40%	35%

Table 2. Immature bovine COC were cultured either in usual IVM medium or supplemented with 100nM of Aurora kinase inhibitor VX680 for 14h or 24h. Part of denuded oocytes was used for morphological observation by Hoechst staining of chromatin (Fig. 6B). Nuclear status of VX680-treated oocytes was compared within each experiment with control IVM oocytes. Twenty four and forty three oocytes were stained and analyzed after 14h and 24h IVM, respectively.

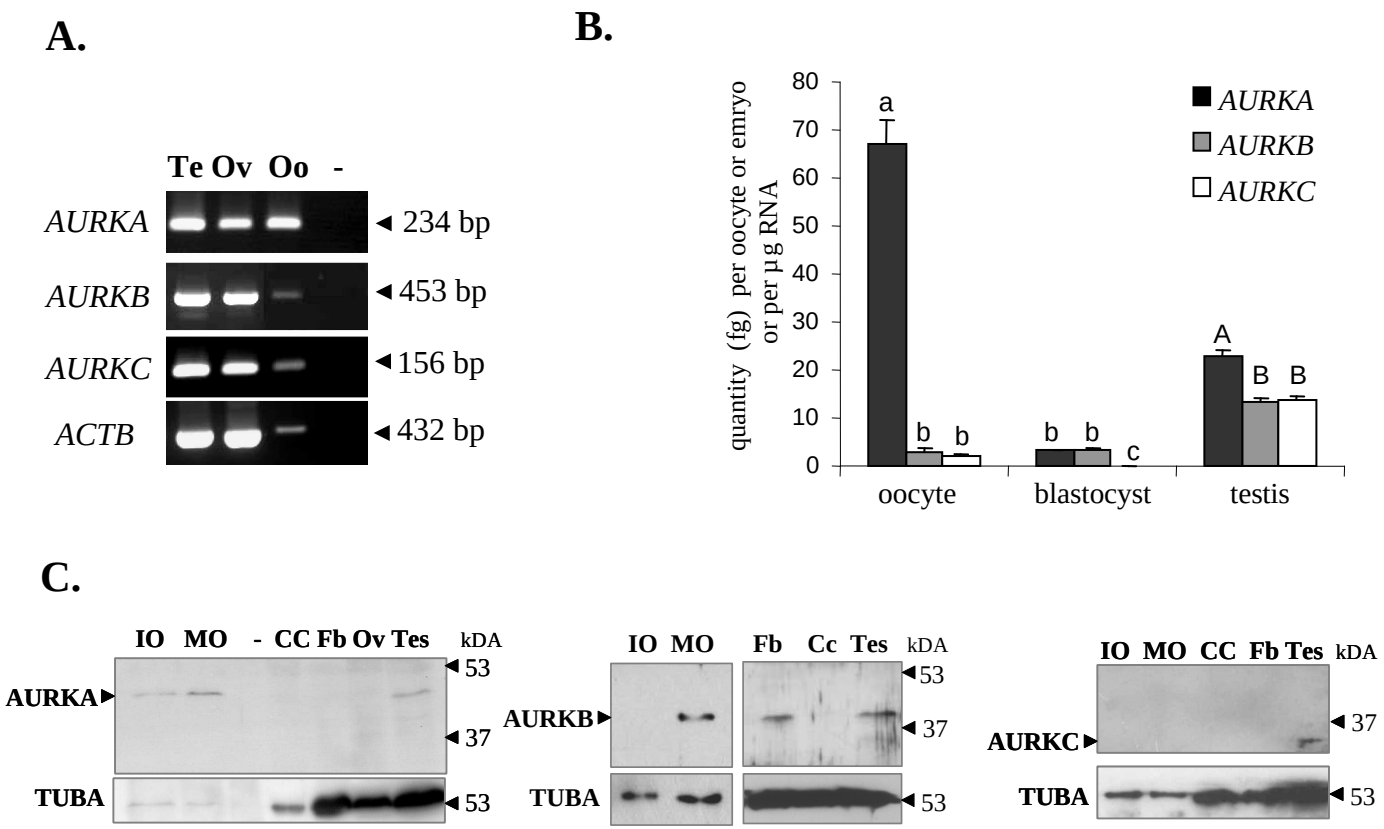
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Figure 1

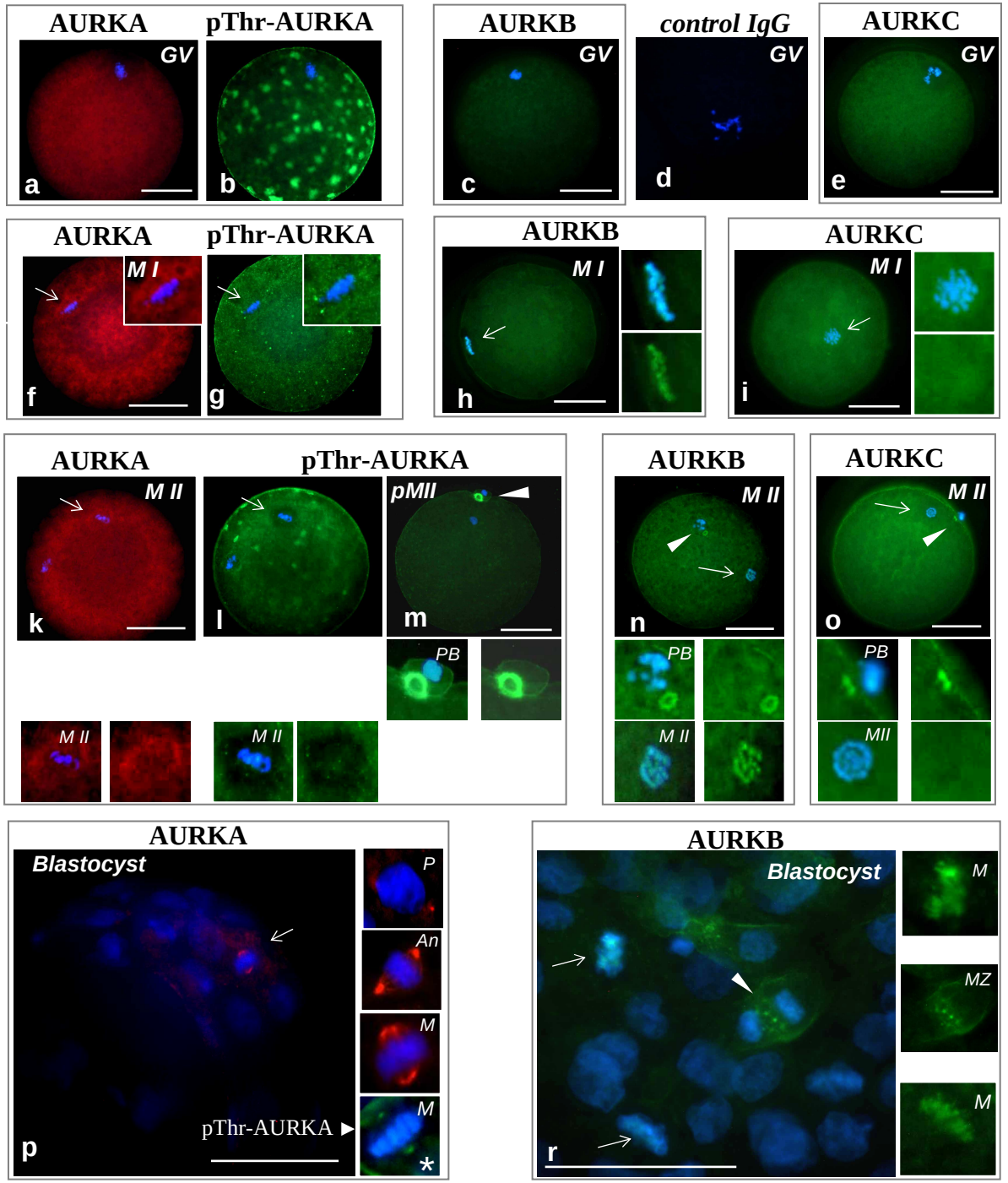
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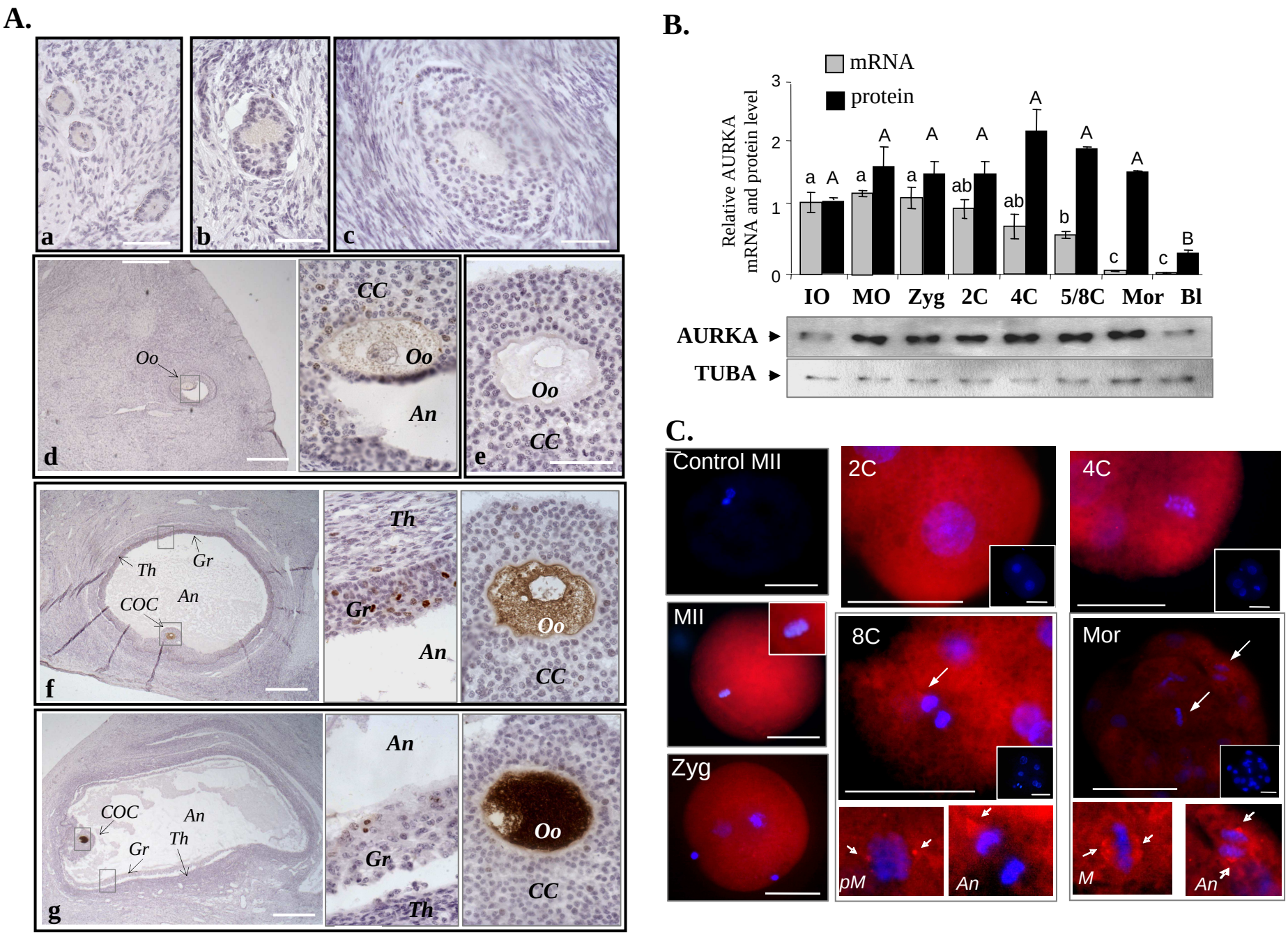
Figure 2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mRNA
protein
Relative AURKA mRNA and protein level
IO
MO
Zyg
2C
4C
5/8C
Mor
Bl
AURKA
TUBA
C.
Control MII
2C
4C
MII
8C
Mor
Zyg
pM
An
M
An

Figure 3

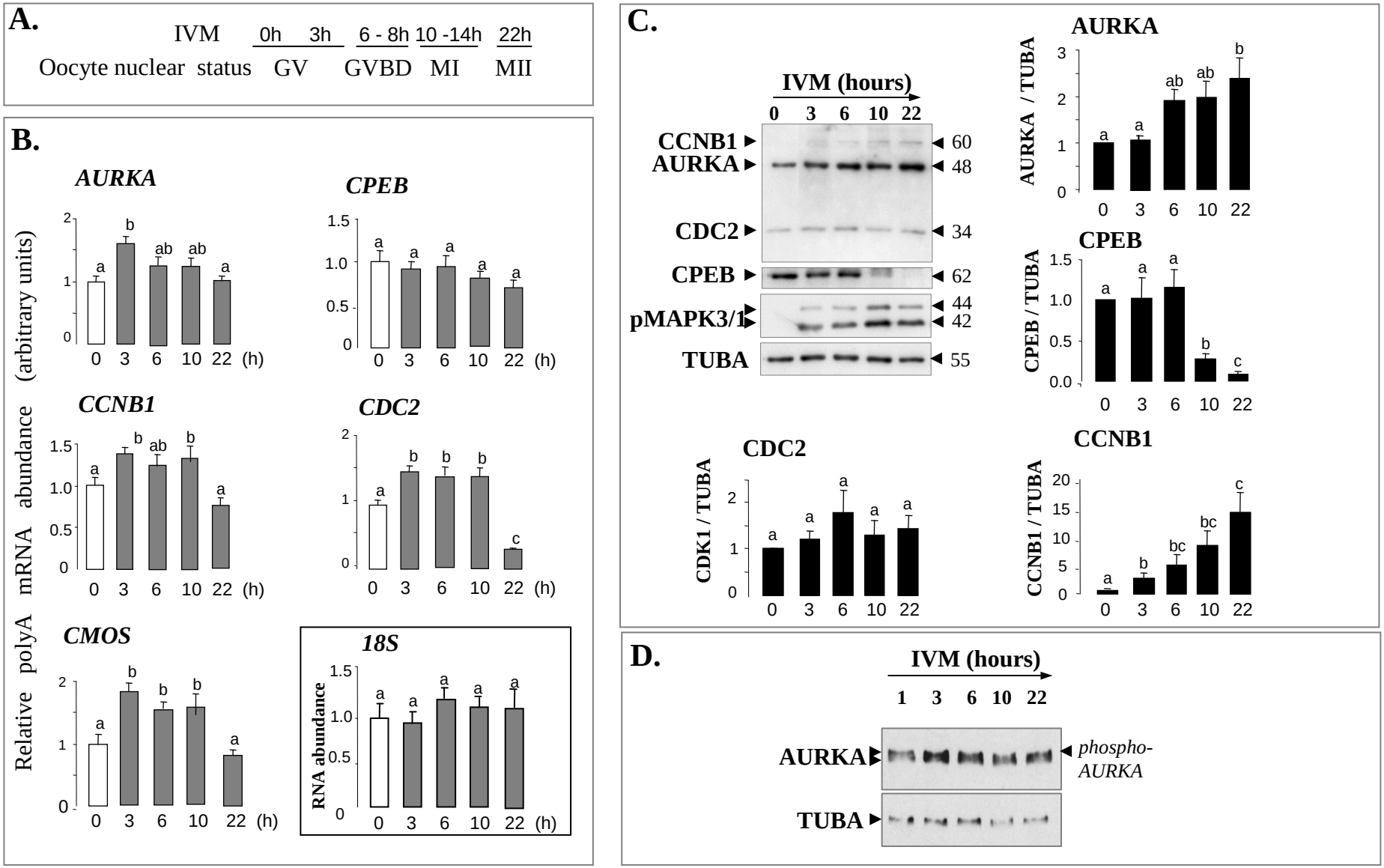

A.
IVM 0h 3h 6 - 8h 10 -14h 22h
Oocyte nuclear status GV GVBD MI MII
B.
AURKA
CPEB
CCNB1
CDC2
CMOS
18S
Relative polyA mRNA abundance (arbitrary units)
RNA abundance
0 3 6 10 22 (h)
C.
IVM (hours)
0 3 6 10 22
CCNB1
AURKA
CDC2
CPEB
pMAPK3/1
TUBA
60
48
34
62
44
42
55
AURKA / TUBA
CPEB / TUBA
CDK1 / TUBA
CCNB1 / TUBA
D.
IVM (hours)
1 3 6 10 22
AURKA
TUBA
phospho-AURKA

Figure 4

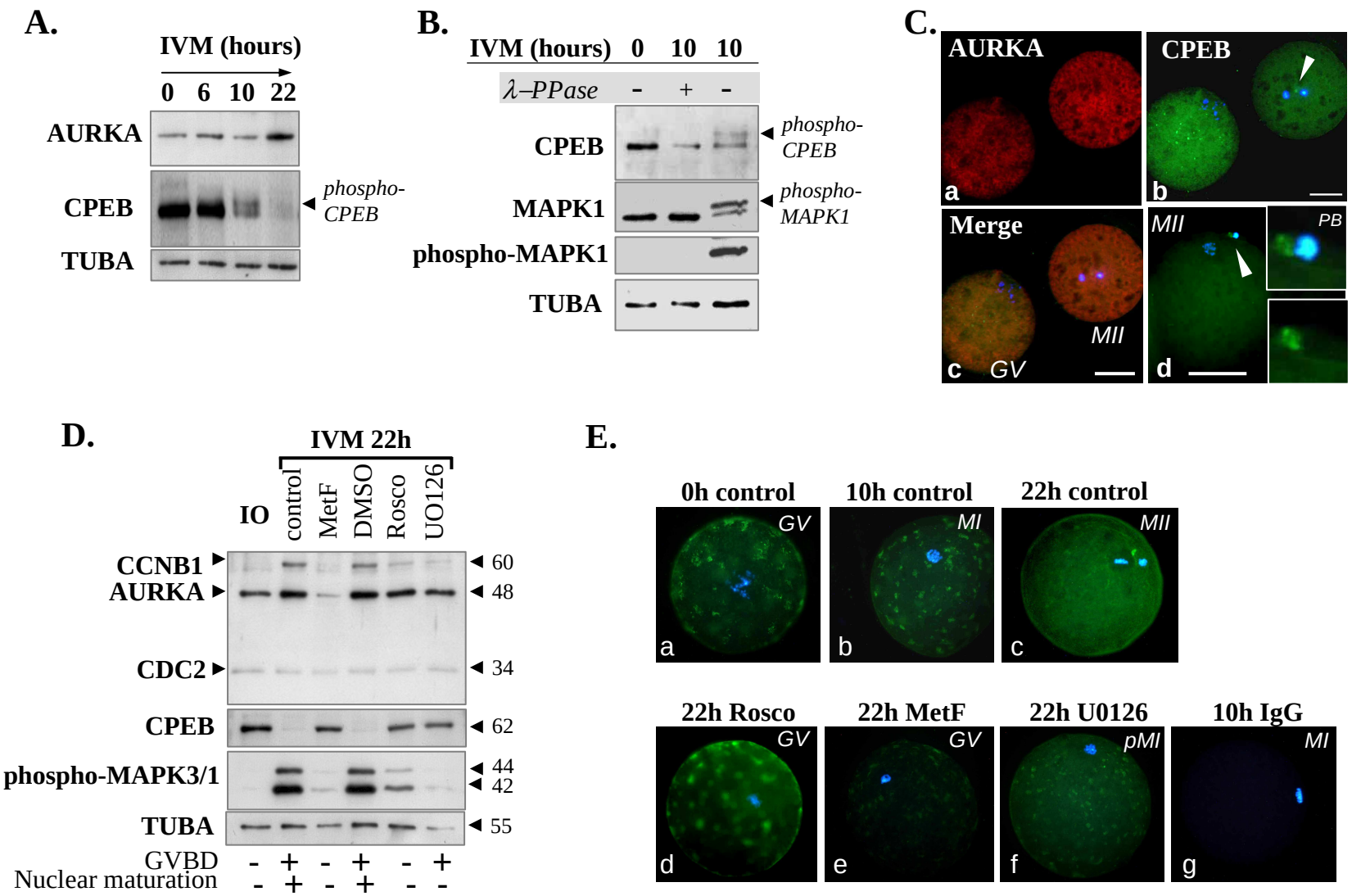

A.
IVM (hours)
0 6 10 22
AURKA
CPEB
TUBA
phospho-CPEB
B.
IVM (hours) 0 10 10
λ–PPase - + -
CPEB
MAPK1
phospho-MAPK1
TUBA
phospho-CPEB
phospho-MAPK1
C.
AURKA
CPEB
Merge
MII
PB
GV
a
b
c
d
D.
IVM 22h
IO
control
MetF
DMSO
Rosco
UO126
CCNB1
AURKA
CDC2
CPEB
phospho-MAPK3/1
TUBA
60
48
34
62
44
42
55
GVBD - + - + - +
Nuclear maturation - + - + - -
E.
0h control
10h control
22h control
GV
MI
MII
a
b
c
22h Rosco
22h MetF
22h U0126
10h IgG
GV
GV
pMI
MI
d
e
f
g

Figure 5

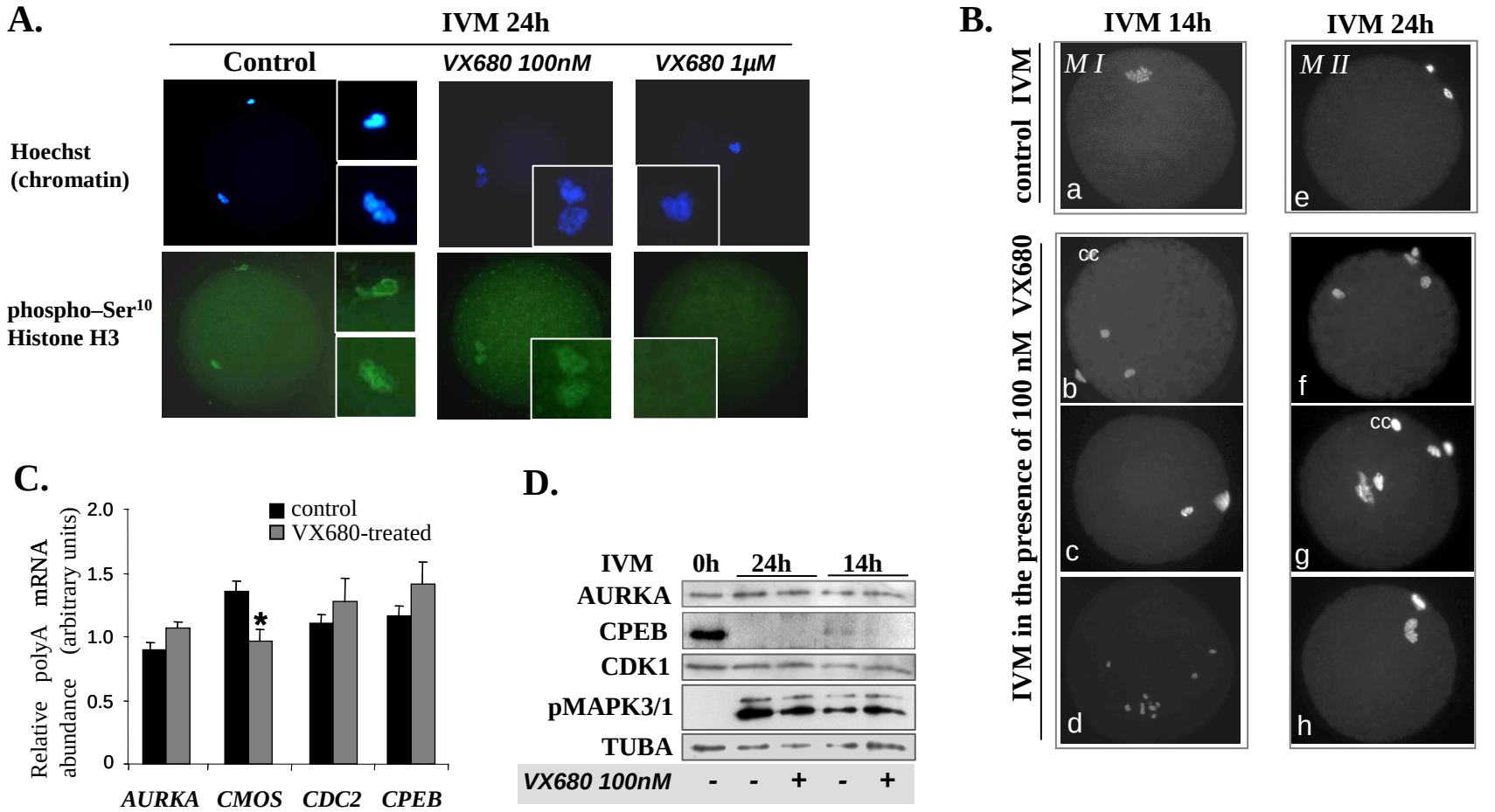
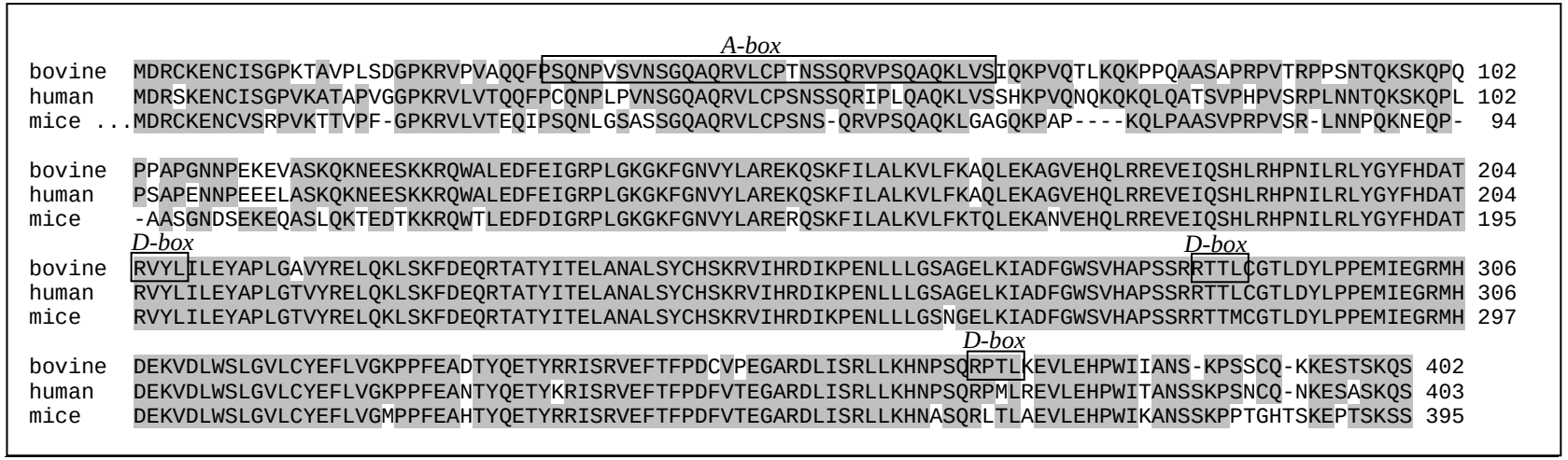

A.
IVM 24h
Control
VX680 100nM
VX680 1µM
Hoechst (chromatin)
phospho–Ser10 Histone H3
B.
IVM 14h
IVM 24h
control IVM
IVM in the presence of 100 nM VX680
M I
M II
a
b
c
d
e
f
g
h
cc
C.
control
VX680-treated
Relative polyA mRNA abundance (arbitrary units)
2.0
1.5
1.0
0.5
0
*
AURKA
CMOS
CDC2
CPEB
D.
IVM 0h 24h 14h
AURKA
CPEB
CDK1
pMAPK3/1
TUBA
VX680 100nM - - + - +

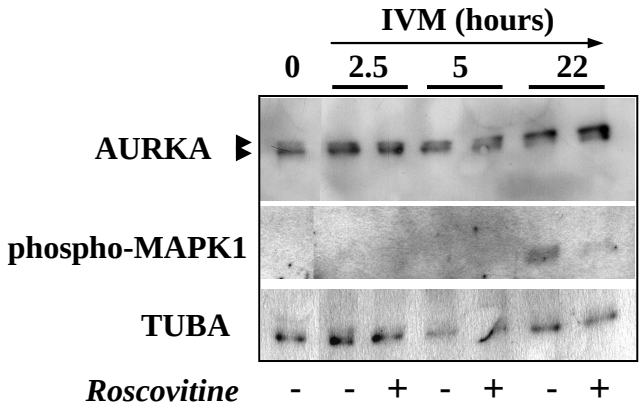
Figure 6

A.

B.



Supplementary data Fig. 1



Supplementary data Fig. 2