EP45 accumulates in growing *Xenopus laevis* oocytes and has oocyte-maturation-enhancing activity involved in oocyte quality

Gaëlle Marteil[1], Romain D'Inca[1], Aude Pascal[1], Nathalie Guitton[2], Torbjørn Midtun[3], Anders Goksøyr[3], Laurent Richard-Parpaillon[1] and Jacek Z. Kubiak[1,*]

[1]CNRS UMR 6061, Institute of Genetics and Development of Rennes, Mitosis and Meiosis Group, University of Rennes 1, IFR 140 GFAS, Faculty of Medicine, 35 043, Rennes, France
[2]Proteomics Core Facility Biogenouest, Inserm U625, Campus de Beaulieu, 35 042, Rennes, France
[3]Department of Molecular Biology, University of Bergen, Bergen, N-5020, Norway
*Author for correspondence (jacek.kubiak@univ-rennes1.fr)

Accepted 1 March 2010
Journal of Cell Science 123, 1805-1813

doi:10.1242/jcs.063305

Summary

The capacity of oocytes to fully support meiotic maturation develops gradually during oocyte growth. Growing oocytes accumulate proteins and mRNAs required for this process. However, little is known about the identity of these factors. We performed a differential proteomic screen comparing the proteomes of growing stage-IV oocytes, which do not undergo meiotic maturation in response to progesterone, with fully grown stage-VI ones, which do. In 2D gels of stage-VI oocytes, we identified a group of four protein spots as EP45 (estrogen-regulated protein 45 kDa), which belongs to the family of serine protease inhibitors and is also known as Seryp or pNiXa. Western blot analysis after mono- and bi-dimensional electrophoreses confirmed the accumulation of certain forms of this protein in oocytes between stages IV and VI. *EP45* mRNA was not detectable in oocytes or ovaries, but was expressed in the liver. A low-mobility isoform of EP45 was detected in liver and blood, whereas two (occasionally three or four) higher-mobility isoforms were found exclusively in oocytes, suggesting that liver-synthesized protein is taken up by oocytes from the blood and rapidly modified. Alone, overexpression of RNA encoding either full-length or N-terminally truncated protein had no effect on meiotic resumption in stage-IV or -VI oocytes. However, in oocytes moderately reacting to low doses of progesterone, it significantly enhanced germinal-vesicle breakdown, showing a novel and unsuspected activity of this protein. Thus, EP45 accumulates in growing oocytes through uptake from the blood and has the capacity to act as an 'oocyte-maturation enhancer' ('Omen').

Key words: EP45, pNiXa, Seryp, Oocyte-maturation-enhancing activity, Oocyte growth, Oocyte quality, M-phase induction, GVBD, Serpin, Bet-hedging

Introduction

Oocytes of vertebrates are arrested in the first meiotic prophase for weeks, months or years. During this long-lasting period, they undergo considerable growth, which occurs through the accumulation of different types of molecules (proteins, lipids, mRNAs) of maternal origin. Some of them are synthesized in ovo (through mRNA transcription and translation), others are incorporated from blood. Both oocyte transcription and protein uptake are extremely active during oocyte growth. In mesolecithal amphibian oocytes, including those of *Xenopus laevis*, nutrients are certainly the most abundant constituents of the oocyte mass accumulated during the growth period. However, regulatory molecules, which are less abundant, are certainly of key importance for the functionality of female gametes (Gosden, 2002). When fully grown amphibian oocytes enter meiotic maturation, they become transcriptionally silent and this state persists after fertilization, until the mid-blastula transition (MBT) (Maller, 1998). Thus, both oocyte maturation and early-embryo development depend entirely on the maternal genetic information. As such, to generate an oocyte suitable for fertilization and embryonic development, all regulatory molecules ensuring the normal course of oocyte maturation and early development must properly accumulate during oocyte growth. Oocyte maturation is a highly regulated process, leading to the formation of fully mature female gametes with an appropriate number of chromosomes and the full potential to undergo successful fertilization and embryonic development until the MBT. Thus, the quality of mature oocytes depends largely on the quality of stockpiles accumulated during oogenesis. These processes are of key importance for embryonic developmental and reproductive success (Marteil et al., 2009).

Oogenesis and oocyte maturation are well characterized in *X. laevis*. The ovary of sexually mature females contains oocytes in all stages of oogenesis (stages I to VI according to Dumont's classification; Dumont, 1972). Oocytes at all stages are arrested in meiotic prophase I and undergo continuous growth by accumulating yolk proteins, among which the large conserved phosphoglycolipometalloprotein vitellogenin plays a major role (Stifani et al., 1990). Interestingly, only stage-VI postvitellogenic oocytes are responsive to progesterone, the major steroid that induces meiotic resumption and leads to a mature oocyte. Progesterone stimulation induces not only the inhibition of adenylate cyclase, leading to a rapid drop in cAMP levels and a reduction in protein kinase A (PKA) activity, but also de novo synthesis of protein (such as the Mos proto-oncogene) (Tunquist and Maller, 2003). These events trigger a cascade of protein

phosphorylation events, culminating in the abrupt activation of M-phase-promoting factor (MPF), also known as the cyclin-dependent kinase 1 (CDK1)-cyclin B complex (Masui and Markert, 1971). Active MPF induces germinal-vesicle (oocyte nucleus) breakdown (GVBD), chromatin condensation and meiotic-spindle formation (Jessus et al., 1991). The first meiotic division terminates with the first polar-body extrusion followed by arrest in metaphase II (MII) under the control of the cytostatic factor (CSF) (Masui and Markert, 1971). At this step, the mature oocyte is now ready for ovulation and fertilization.

As mentioned above, smaller oocytes cannot resume meiosis upon progesterone stimulation (Reynhout et al., 1975). Indeed, progesterone is not able to release prophase I arrest in stage-IV oocytes, despite the fact that these small pigmented oocytes express functional progesterone receptors that are able to inhibit PKA (Mulner et al., 1983; Sadler and Maller, 1983). Even upon PKA inhibition, stage-IV oocytes are unable to activate MPF (Masui and Markert, 1971) and enter the first meiotic M phase (Sadler and Maller, 1983). However, injection of low doses of active MPF in stage-IV oocytes is sufficient to initiate MPF activation followed by GVBD. This experiment clearly indicates that part of the molecular machinery responsible for meiotic maturation is already present in a silent form in these oocytes (Wasserman et al., 1984; Rime et al., 1991), but that the coupling between PKA and CDK1 activation is not functional (Jessus and Ozon, 2004).

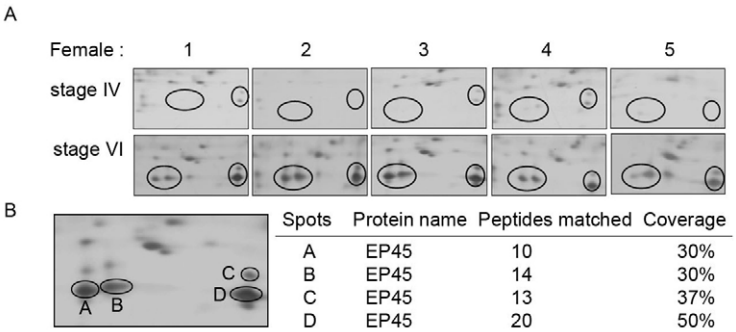
To understand the molecular mechanisms leading to the acquisition of the capacity of an oocyte to proceed to GVBD, comparison of oocyte morphological and functional changes between stages IV and VI is particularly interesting. The best-known factor essential for CDK1 activation and lacking in stage-IV oocytes is Plx1 (a polo-like kinase) (Karaiskou et al., 2004). However, Plx must be one of further regulatory proteins that accumulate following stage IV, because providing stage-IV oocytes with Plx1 does not confer the capacity to respond to progesterone. Other proteins might not necessarily be involved in the core mechanism of CDK1 activation. To gain insight into the nature of the molecules potentially involved in these processes, we performed a proteomic screen in which we compared the proteomes of stage-IV and -VI oocytes. We focused on cytoplasmic proteins that were absent or only weakly present in stage-IV oocytes and became particularly abundant in stage-VI oocytes. Such behavior suggests potential involvement in the development of the capacity of stage-VI oocytes to undergo GVBD. EP45 (estrogen-regulated protein 45 kDa), a member of the superfamily of serine protease inhibitors (serpins), was one such protein identified in our proteomic screen. This protein has a high affinity for nickel because of its histidine-rich N terminus (Beck et al., 1992; Sunderman et al., 1996). This enabled its purification and identification as a 45 kDa protein named pNiXa (for protein-Ni-Xenopus-a), because of its affinity for nickel (Beck et al., 1992). More interestingly, purified pNiXa, when injected into stage-VI *X. laevis* oocytes, was reported to induce GVBD (Haspel et al., 1993). Moreover, this effect was shown to be synergistic with Ras-induced maturation (Haspel et al., 1993). In the light of these results, the identification of this protein in our proteomic screen as a cytosolic protein that accumulates in oocytes between stages IV and VI raised an intriguing question of why the endogenous protein does not induce GVBD by itself without progesterone stimulation. Recently, EP45/pNiXa was found to also be an important component of yolk platelets and was named Seryp (for Serpin yolk protein) (Jorgensen et al., 2009). Here, we characterize in detail the accumulation of cytosolic EP45/pNiXa/Seryp in growing *X. laevis* oocytes. We show that it is not synthesized in oocytes, but rather in the liver and then delivered to oocytes from the blood. Moreover, we show that cytoplasmic expression of this protein does not induce GVBD in either stage-IV or -VI oocytes, but enhances GVBD in oocytes responding moderately to progesterone. This protein is therefore a candidate for membership of a novel class of positive regulators of oocyte maturation, which we propose to name 'oocyte-maturation enhancers' (or 'Omen') and which are involved in non-core pathways stimulating CDK1 activation, GVBD and the process of oocyte maturation.

Results

EP45 is a novel protein that accumulates in the cytoplasm of growing oocytes

Accumulation of proteins during oocyte growth participates in the acquisition of the capacity of the oocyte to undergo meiotic maturation. To identify novel proteins that accumulate in *X. laevis* oocytes before they acquire the ability to undergo meiotic maturation, we compared the proteomes of small stage-IV oocytes and fully grown stage-VI oocytes (Dumont, 1972) by 2D electrophoresis. Among proteins less abundant in stage-IV than in stage-VI oocytes, we found a family of four spots in the same region (molecular weight 45 kDa; pHi 7.8) that was always clearly more abundant in stage-VI oocytes (Fig. 1A, females 1-5). All four spots were identified as the same protein, EP45, by mass spectrometry (MS) (Fig. 1B).

To validate our screen, polyclonal antibodies raised against two different regions of EP45 (Fig. 2A) were used to detect the expression of EP45 in stage-IV and -VI oocytes by western blotting (WB) following mono-dimensional PAGE (Fig. 2B). In stage-VI oocytes obtained from most females, EP45 migrates as two bands: a more abundant slow-migrating one (band 1, corresponding to a



Spots	Protein name	Peptides matched	Coverage
A	EP45	10	30%
B	EP45	14	30%
C	EP45	13	37%
D	EP45	20	50%

Fig. 1. Differential expression of EP45 between stage-IV and stage-VI oocytes. (**A**) Cytosolic proteomes of stage-IV and -VI oocytes from five different females were analysed by 2D electrophoresis as described in the Materials and Methods. Gels were fixed overnight and then silver stained. Spots in circles correspond to different isoforms of EP45. (**B**) Identification of EP45 isoforms was performed using peptide mass fingerprints from MALDI-TOF analysis. Peptides matched: number of peptides whose sequences match the sequence of the identified protein. Coverage: percentage of the protein sequence covered by the peptides.

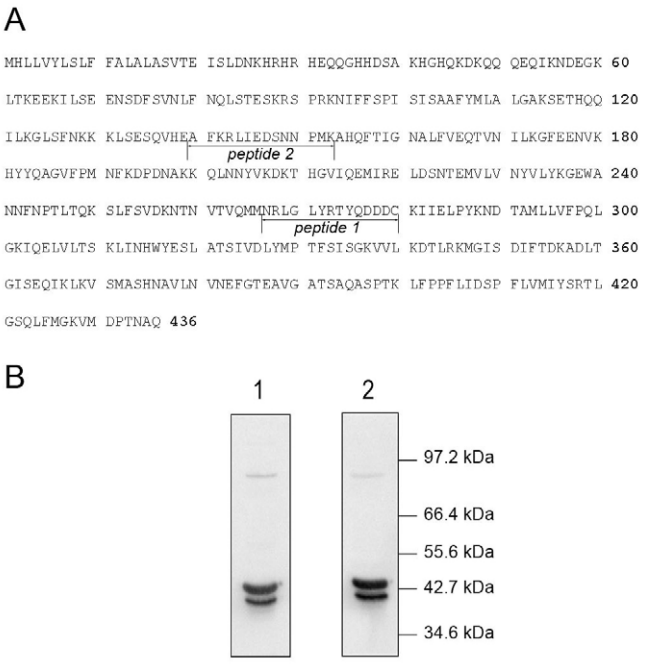


Fig. 2. Characterization of EP45 antibodies. (**A**) Two polyclonal antibodies produced in rabbits were raised against synthetic peptides (1 and 2, underlined) selected from the sequence of *X. laevis* EP45. (**B**) WB showing the specificity of antibodies (antibodies 1 and 2 raised against peptides 1 and 2, respectively) used to detect *X. laevis* EP45: both antibodies (1/2000) give the same detection pattern for stage-VI oocyte extracts (two major bands are visible at 45 and 41 kDa and one minor at 91 kDa).

protein of 45 kDa) and a less abundant fast-migrating one (band 2, corresponding to a protein of 41 kDa) (Fig. 2B). Interestingly, in oocytes obtained from some females, we found one additional fast-migrating band (band 3, corresponding to a protein of about 36 kDa; Fig. 3A, female e, stage VI), illustrating the high degree of heterogeneity of the WB profile of EP45. Apart from the two (or three) major bands, each antibody detects only a faint higher molecular weight band (around 91 kDa; Fig. 2B), which might correspond to a covalent complex between the EP45 serpin and a target protease (Lawrence et al., 1995). The comparison of stage-IV and -VI oocytes shows that both the 45 and 41 kDa bands, as well as the 36 kDa band present in some pools of oocytes, are less abundant in stage-IV than in stage-VI oocytes (as normalized to tubulin WB; Fig. 3A,B). However, the most striking accumulation is observed for the EP45 form corresponding to band 2 (41 kDa), with a threefold increase in stage-VI oocytes compared with stage IV (Fig. 3B). Thus, EP45 accumulates extensively in the cytoplasm of stage-VI oocytes and its quantity increases in stage-VI oocytes in comparison to stage IV, confirming the results of our proteomic screen. In addition, EP45 shows an important heterogeneity in respect to its migration pattern in oocytes from different females.

Because the proteomic screen was carried out by 2D electrophoresis, we compared the proteomes obtained from stage-IV and -VI oocytes by 2D PAGE followed by 2D WB with EP45 antibody (Fig. 4). 2D WB revealed the presence of multiple spots corresponding to EP45 bands 1, 2 and 3 (Fig. 4A,C) in oocytes from the two stages, suggesting complex regulation of this protein. Numerous spots were absent or less abundant in stage-IV oocytes compared with stage VI (Fig. 4A,C). These results confirm that EP45 accumulates between stages IV and VI as multiple spots, not just as the four spots previously identified by silver staining of 2D gels (Fig. 1). Fast-migrating isoforms of EP45 (bands 2 and 3) are mainly responsible for the accumulation of EP45 in stage-VI oocytes (Figs 3 and 4). The heterogeneity of EP45 was also confirmed in 2D WB (compare the patterns of EP45 in Fig. 4A and Fig. 4C).

Accumulation of EP45 in oocytes

To obtain more information about the behavior of EP45 during oogenesis, we compared the WB profiles of this protein during all stages of oogenesis and throughout oocyte maturation. EP45 is barely detectable in the smallest stage-I and stage-II oocytes, and starts to accumulate in stage-III oocytes as the slow-migrating form (Fig. 5A). The levels of EP45 increase through stages IV, V and VI. The quantification of the WBs of five different pools of oocytes from stages IV and VI shows that the intensity of the two major bands (1 and 2) increases, and that the fast-migrating form accumulates in the most dynamic manner between stages IV and VI (Fig. 3A). Generally, apart from the changes in abundance of

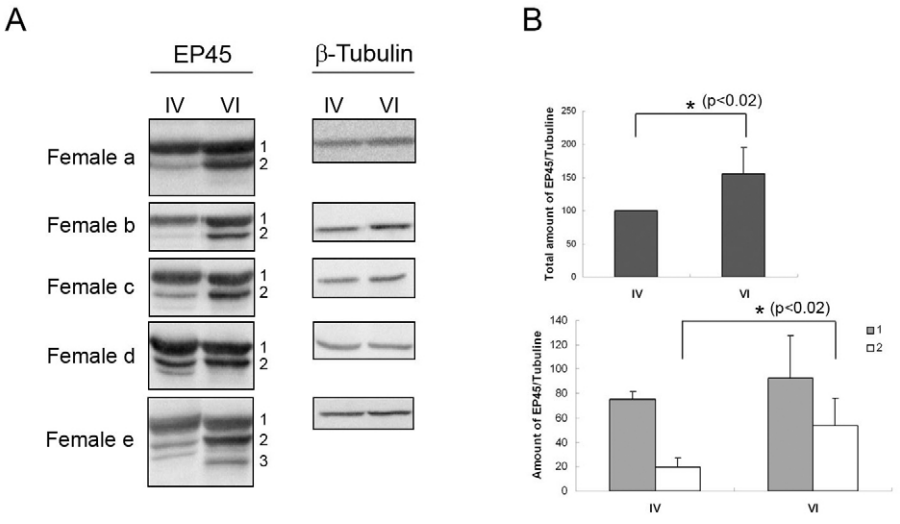


Fig. 3. Proteomic screen validation: 1D electrophoresis and WB analysis of EP45 in stage-IV and stage-VI oocytes. (**A**) EP45 is less abundant in stage-IV than in stage-VI oocytes. High heterogeneity of the EP45 protein pattern is observed among pools of oocytes. Extracts from stage-IV and -VI oocytes of five different females (females a-e, different to those used for the screen; Fig. 1) were immunoblotted with antibody 1 against EP45. (**B**) The high-mobility isoform of EP45 (band 2 of 41 kDa) is the main form of EP45 that accumulates between stage-IV and -VI oocytes. Histograms correspond to quantification of the WB shown in A normalized the to β-tubulin signal (shown in A). Quantification of WB from A was performed using Image-Quant 5.2 software.

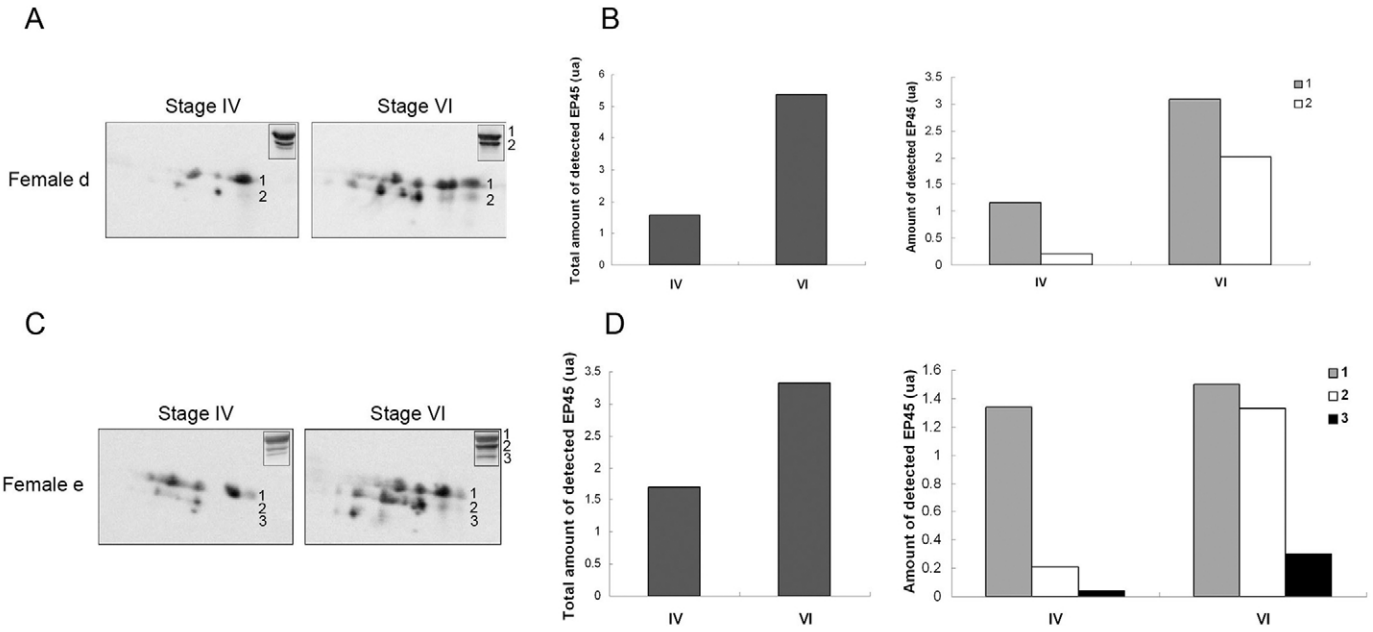


Fig. 4. Proteomic screen validation: 2D electrophoresis and WB analysis of EP45 in stage-IV and stage-VI oocytes. (**A**) Multiple spots corresponding to different EP45 isoforms accumulate between stages IV and VI. Proteomes (15 µg of total protein) of stage-IV and -VI oocytes from female d (the same female as in Fig. 3A) were separated by 2D electrophoresis and then immunoblotted with antibody 1 against EP45. (**B**) Accumulation of EP45 between stages IV and VI mostly concerns fast-migrating spots. Quantification of 2D WB from A was performed using Image-Quant 5.2 software (1: slow-migrating spots, 2: fast-migrating spots). (**C**) The heterogeneity of the EP45 protein profile, visible in 1D analysis as additional fast-migrating band(s), is also found in 2D analysis (female e, the same female as in Fig. 3A). Experimental procedures are the same as those described in A. (**D**) Quantification of 2D WB from C was performed using Image-Quant 5.2 software (1: slow-migrating spots, 2: fast-migrating spots, 3: fastest-migrating spots).

each band, no other change in the migration pattern of EP45 was found during oocyte maturation (Fig. 5A). However, some minor differences could be observed in pools of oocytes with atypical patterns of EP45 (Fig. 3A, females d and e). Similarly, there was no change in the WB pattern of EP45 during oocyte maturation (data not shown). Reverse transcriptase (RT)-PCR showed that *EP45* mRNA is highly present in liver, but remains undetectable both in oocytes, as recently reported by Jorgensen and colleagues (Jorgensen et al., 2009), and in the whole ovary (Fig. 5B). The protein was found by WB both in liver and in blood plasma, but as a slightly less mobile single band (around 50 kDa; Fig. 5C). Interestingly, this lower-mobility form of EP45 was never detected, even in small proportions, in oocytes or in embryos (Fig. 5C), suggesting that EP45 is taken up by oocytes from blood and immediately modified in oocytes to the faster-migrating forms.

EP45 overexpression enhances GVBD only in stage-VI oocytes, which are partially responsive to progesterone

Injection of pNiXa alone into stage-VI oocytes was shown to induce GVBD (Haspel et al., 1993). Paradoxically, we show in this paper that such oocytes already contain large amounts of EP45. In addition, we have not observed any change in the PAGE mobility of EP45 after oocyte maturation, indicating that there is no major change in the state of EP45 between meiotic prophase I and metaphase II oocyte stages (Fig. 5A). These data suggest that the stockpile of endogenous EP45 is not sufficient to induce GVBD in stage-VI oocytes. To gain information on the potential role of EP45 in oocytes arrested in prophase I, we injected mRNA encoding EP45 in order to overexpress this protein in stage-IV and stage-VI oocytes, and to assess the effect on oocyte maturation.

First, we injected *EP45* mRNA into stage-IV oocytes. These oocytes do not undergo GVBD following progesterone treatment, partly because of the absence of Plx (Karaiskou et al., 2004). In this series of experiments, stage-IV oocytes were injected with *EP45* mRNA and treated with 1 µM progesterone (saturating dose for stage-VI oocytes) to facilitate any changes triggered by EP45 overexpression. Such a combination could reveal a potential synergistic effect of progesterone and EP45. Despite this double treatment, as expected, no stage-IV oocyte underwent GVBD, as judged by the absence of white spot appearance (Fig. 6A). In addition, no changes in cell-cycle markers of oocyte maturation, such as cyclin B2 and ERK2 phosphorylation states, were detected by WB (Fig. 6B). We also followed the level of EP45 overexpression with anti-EP45 antibody (Fig. 6B). In all cases, the levels of overexpression were moderate (Fig. 6B). These results show unequivocally that EP45 expressed in stage-IV oocytes does not induce GVBD.

Next, we injected stage-VI oocytes using a very similar protocol (with the injection of mRNA encoding β-galactosidase as a control, without or with progesterone). The level of EP45 overexpression was detected using anti-EP45 antibodies or anti-myc antibodies in experiments in which a myc-tagged form of EP45 was expressed (Fig. 6D; Fig. 7B). In experiments using stage-VI oocytes, we reduced the concentration of progesterone to a non-saturating dose of 50 nM. Similarly to stage-IV oocytes, EP45-injected stage-VI oocytes that were not treated with progesterone did not undergo GVBD (Fig. 6C) and did not show changes in cyclin B2 or ERK2 phosphorylation states (Fig. 6D). Moreover, oocytes treated with 50 nM progesterone matured with very similar dynamics and rate, regardless of EP45 or β-galactosidase expression (Fig. 6C,D).

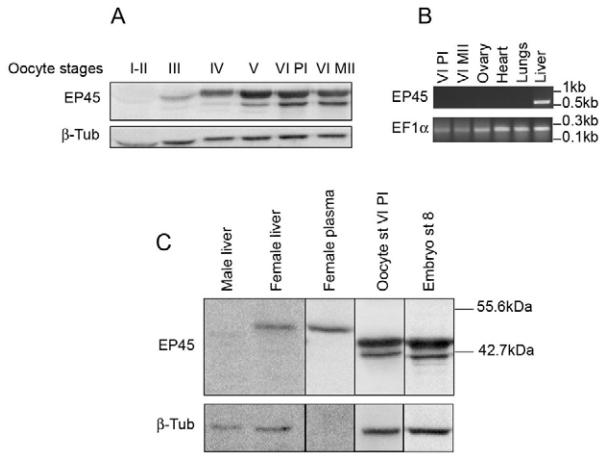
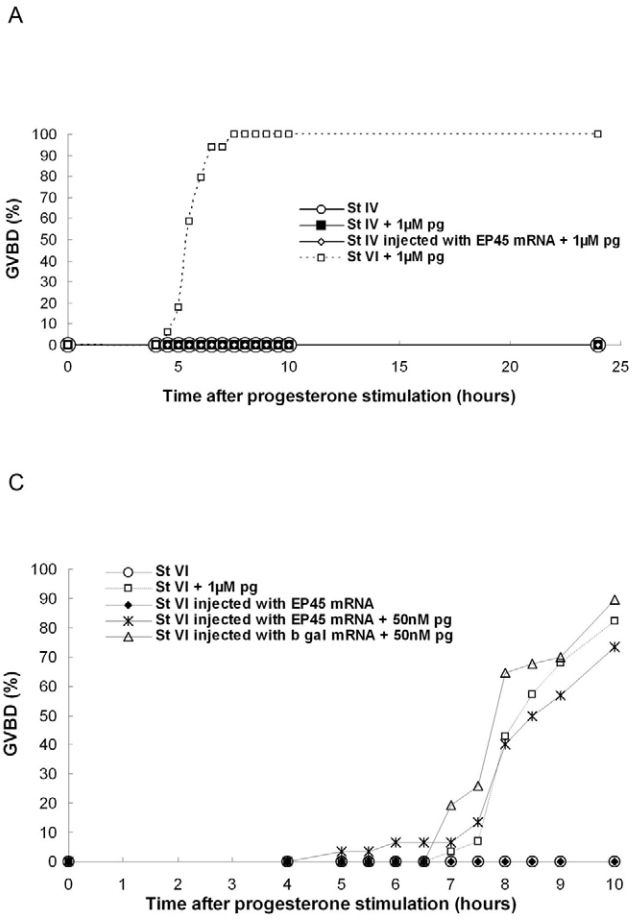


Fig. 5. **Expression profile of EP45.** (**A**) Traces of EP45 are barely detectable in stage I-II oocytes. EP45 protein accumulates clearly in stage-III oocytes as two isoforms of 45 and 41 kDa. Extracts derived from all stages of oogenesis were immunoblotted with specific antibodies for EP45 and β-tubulin (loading control). (**B**) EP45 can be synthesized only in liver among analysed tissues, because the mRNA encoding this protein is only detected in liver. RT-PCR experiments were performed with specific primers for *EP45* and *EF1α* mRNA (control). (**C**) EP45 is synthesized in liver, delivered in blood and taken up by oocytes. WBs of protein extracts derived from liver, plasma, stage-VI oocytes and stage-8 embryos were performed using specific antibodies against EP45 and β-tubulin (loading control). Note the absence of the β-tubulin signal in blood plasma.

These results show that EP45 expression does not induce GVBD and does not modify the response of stage-VI oocytes to 50 nM progesterone.

Because 50 nM progesterone elicits almost the maximum response of oocytes – such treatment usually induces GVBD in about 90% of oocytes (Fig. 6C) – we assessed the reactions of stage-VI oocytes injected with full-length *EP45* mRNA and treated with 10 nM progesterone. In non-injected oocytes, as well as oocytes injected with *β-galactosidase* mRNA, 10 nM progesterone induces a whole spectrum of oocyte reactions, depending on the batch and characteristics of each female. Some batches did not react to such treatment (no GVBD; non-responsive), some pools of oocytes underwent almost 100% GVBD (maximally responsive), and an intermediate group varied between these two extremes (mid-responsive) (Fig. 7A). Non-responsive batch oocytes (no GVBD in control) injected with *EP45* mRNA still did not undergo GVBD. Maximally responsive batch oocytes (almost 100% GVBD in control) remained at the highest level of response to 10 nM progesterone (Fig. 7A). However, the percentage of maturation was clearly increased in the mid-responsive batches of oocytes (Fig. 7A). Entry into oocyte maturation was also confirmed by cyclin B2 and ERK2 phosphorylation in these oocytes (Fig. 7B). The EP45 WB pattern (the presence of two or three bands) did not correlate with either the responsiveness of oocytes to 10 nM progesterone or their response to exogenous EP45 expression (Fig. 7B). These results show that EP45 overexpression enhances oocyte maturation without provoking GVBD by itself.



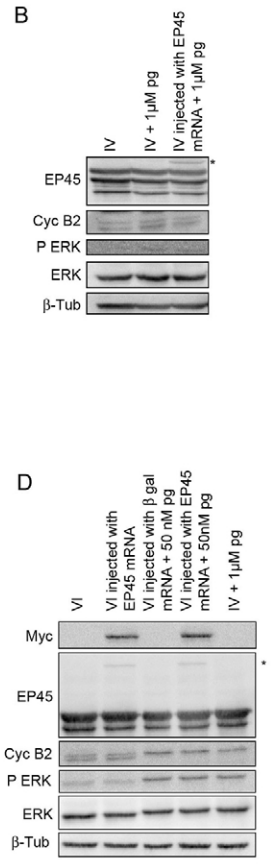


Fig. 6. EP45 expression in stage-IV and stage-VI oocytes. (**A**) EP45 expression in stage-IV oocytes does not trigger oocyte maturation. *EP45* mRNA was microinjected into stage-IV and -VI oocytes. 14 hours after microinjection, oocytes were incubated with 1 μM progesterone (pg) and the time course of oocyte maturation was followed on the basis of the appearance of a white spot. Stage-VI oocytes from the same female incubated with 1 μM progesterone were used here as a control of progesterone action. (**B**) Immunoblots of oocyte protein extracts from stage-IV oocytes either expressing EP45 or not in the presence or absence of 1 μM progesterone using specific antibodies for EP45 (EP45), cyclin B2 (Cyc B2), phosphorylated ERK2 (P ERK), ERK2 (ERK) and β-tubulin (β-Tub). Exogenous unprocessed EP45 is labeled with an asterisk. (**C**) EP45 expression does not trigger oocyte maturation (absence of the white spot) in stage-VI oocytes, and does not modify the time course and rate of GVBD in oocytes expressing EP45 and incubated with 50 nM progesterone. Experimental procedures are the same as those described in A, except that a control of *β-galactosidase* mRNA was used (β-gal). (**D**) WBs of oocyte extracts derived from stage-VI oocytes either microinjected with *EP45* or *β-gal* mRNA or left untreated in the presence or absence of either 50 nm or 1 μM progesterone. myc-tagged EP45 was detected using anti-myc and anti-EP45 antibodies. Exogenous unprocessed EP45 is labeled with an asterisk in the EP45 WB.

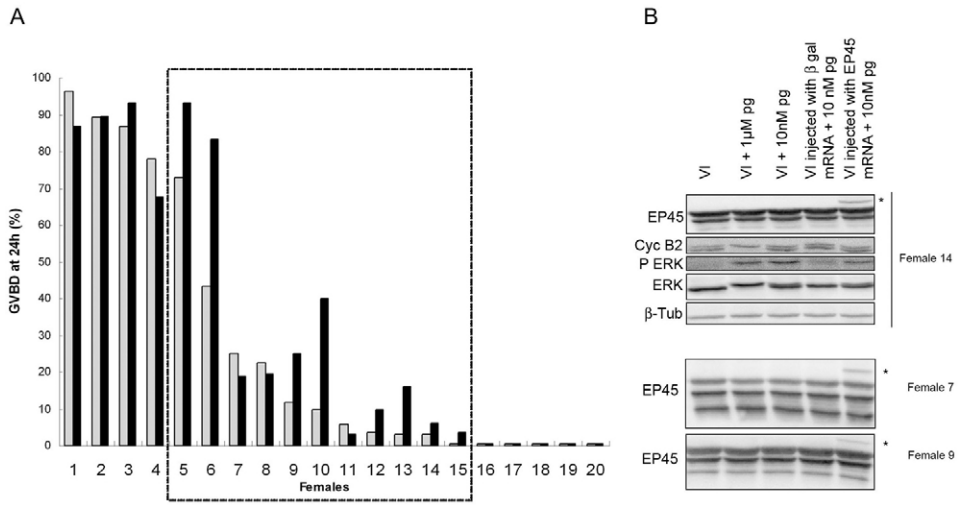


Fig. 7. Oocyte-maturation-enhancing activity (Omen) in stage-VI mid-responsive oocytes. (**A**) EP45 expression enhances oocyte maturation in stage-VI oocytes that are partially responsive to 10 nM progesterone (females 5, 6, 9, 10, 12-15 within dotted frame). Experimental procedures are the same as those described in Fig. 6A. Histograms show the percentage of maturation of oocytes microinjected with RNAs encoding β-galactosidase (grey) or EP45 (black). (**B**) WB of oocyte extracts from stage-VI oocytes either microinjected with *EP45* or *β-gal* mRNA or left untreated in the presence or absence of either 10 nM or 1 µM progesterone (additional positive control) using specific antibodies (the same as in Fig. 6B). Examples of **WB** of typical oocytes with two bands of EP45 (female 14), and two pools of oocytes possessing three bands of EP45 (females 7 and 9) are shown. Note that the two atypical oocyte pools react differently to EP45 expression: those from female 7 are not stimulated by EP45, whereas those from female 9 are. Exogenous unprocessed EP45 is labeled with an asterisk.

Discussion

We show here that EP45 accumulates during *X. laevis* oocyte growth, and that some isoforms of this protein accumulate specifically and in large amounts between stages IV and VI of oogenesis (1D gels). EP45 in oocytes is extensively modified, forming a complex pattern as revealed in 2D gels. We confirm that EP45-encoding mRNA is absent in oocytes and ovaries, but is present in liver. EP45 protein was found in both liver and blood of females (absent in liver of males), as well as in oocytes, confirming the exogenous origin of EP45 in oocytes by means of uptake from blood (Holland and Wangh, 1987; Holland et al., 1992; Beck et al., 1992; Jorgensen et al., 2009). The electrophoretic pattern of EP45 (in both 1D and 2D gels) in oocytes from different females shows an unusually high degree of heterogeneity in comparison to other proteins. Both the quantity and electrophoretic pattern of EP45 are unchanged during oocyte maturation, fertilization and early development preceding embryonic genome expression at the MBT. Finally, the expression of EP45 in stage-IV and -VI oocytes does not induce GVBD, contrary to previous results showing that protein purified from MII oocytes induces GVBD in stage VI (Haspel et al., 1993). Most significantly, we found that, in oocytes that respond moderately to low doses (10 nM) of progesterone, EP45 expression clearly stimulates GVBD, showing that EP45 has previously unexpected activity as an oocyte-maturation enhancer (Omen).

Cytoplasmic and yolk-platelet-associated EP45

EP45 was characterized independently as a liver-synthesized estrogen-regulated protein (Holland and Wangh, 1987; Holland et al., 1992) and as a nickel-binding protein in oocytes named pNiXa (Beck et al., 1992). Recently, the same protein was found to be associated with yolk platelets and was called Seryp (for Serpin yolk protein) (Jorgensen et al., 2009). Here, we identified the same protein in our proteomic screen designed to identify proteins that accumulate in oocytes during their growth. The presence of a 43 kDa serpin identical to pNiXa in the cytosolic fraction of *X. laevis* oocytes was also reported by the Hashimoto group (Hashimoto et al., 1995; Hashimoto et al., 1996; Goto et al., 2001; Yoshitome et al., 2003). Moreover, isopycnic centrifugation of gently lysed eggs shows the presence of Seryp in two different fractions: a high-density one accompanied by LV1 and LV2 vitellogenin derivatives, clearly corresponding to Seryp in yolk platelets; and a second, vitellogenin-free, one at the top of the density gradient, corresponding to Seryp in the cytoplasmic compartment. The presence of the latter fraction is interpreted by the authors as artefactual contamination from yolk platelets (Jorgensen et al., 2009). However, in a proteomic experiment with a cytoplasmic fraction of MII oocytes, unrelated to the current study, we found EP45 to be among the most abundant proteins, whereas vitellogenin is barely detectable (identified by a single peptide). This confirms by another method the presence of high levels of vitellogenin-free EP45, supporting the hypothesis that two distinct pools of EP45/Seryp are present in oocytes: one associated with yolk platelets and the other cytoplasmic. However, the translocation process of EP45 to the cytoplasm remains unknown.

EP45 isoforms

We discovered several isoforms of EP45 in the liver, plasma and oocytes, and an intriguing heterogeneity between pools of oocytes obtained from different females. It is possible that certain isoforms correspond to covalent complexes of EP45 with proteinases (e.g. the 91 kDa band; Fig. 2B). Indeed, serpins inhibit proteinases by acting as pseudosubstrates. The interaction between a serpin and a protease leads to two different pathways: the ‘inhibitory pathway’, with the formation of an inactive serpin-proteinase covalent complex, and the ‘substrate pathway’, delivering a cleaved serpin and an inactive protease. Therefore, the inhibitory pathway delivers higher molecular weight complexes of different molecular weights depending on the mass of the protease, whereas the substrate

pathway produces lower molecular weight forms of cleaved EP45. However, novel biological functions for serpins have also been demonstrated: they can act as hormone carriers (serpin A6), neurotrophic factors (serpin F1) and chaperones (serpin H1) (for a review, see van Gent et al., 2003). These various functions might also be linked to formation of the different forms of EP45 detected in oocytes.

In liver and plasma, an isoform of 50 kDa is found, whereas two or three forms are present in oocytes (45, 41 and 36 kDa). We have never found even traces of the precursor p50 protein or *EP45* mRNA in oocytes [this paper and Jorgensen et al. (Jorgensen et al., 2009)]. These results show that the three fast-migrating isoforms correspond to post-translation modification of the p50 precursor that occurs immediately after uptake from the blood. 2D analysis of oocyte EP45 shows a high number of additional spots corresponding to the two bands (1 and 2) found in 1D gels of stage-VI oocytes compared with stage IV. The stage-VI-specific spots appear on 2D gels both at lower and at higher pHi with respect to stage IV, and we cannot rule out that some modifications could also result in vertical mobility shift in 2D gels. Some of these changes might be, at least in part, attributed to differential phosphorylation. However, stage-IV and -VI oocytes remain in the same cell-cycle stage; thus, it is certainly not a cell-cycle-dependent process. EP45 also has an amino acid signal for *N*-linked glycosylation (Beck et al., 1992) and we found that it is potentially ubiquitinated during embryonic mitosis (Bazile et al., 2008) (protein number 62 in the latter reference). Identification of oocyte-specific post-translational modifications of EP45 will be of great value for a full understanding of the role of EP45 in oogenesis and oocyte maturation.

Ooocyte-maturation-enhancing function of EP45

Microinjection of EP45-encoding mRNA in stage-IV and -VI oocytes, with or without progesterone stimulation, shows that EP45 alone is not able to induce oocyte maturation. This contradicts data showing that MII-oocyte-purified pNiXa induces GVBD in stage-VI oocytes (Haspel et al., 1993). Moreover, in our hands, expression of *EP45* mRNA in stage-VI oocytes stimulated with 50 nM progesterone has no effect. Haspel and colleagues (Haspel et al., 1993) observed at least three events provoked by pNiXa microinjection into oocytes: GVBD induction in non-stimulated stage-VI oocytes; a clear decrease in GVBD rate upon 10 μg/ml (corresponding to 31 μM) progesterone treatment (in comparison to progesterone alone); and synergy in the rate of GVBD upon co-injection of pNiXa with ras-p21 (compared with ras-p21 injection alone). Apparent discrepancies between these and our results could have different explanations. Firstly, pNiXa purified from MII-arrested oocytes is post-translationally modified in a MII-oocyte-specific manner, whereas the modifications of experimentally expressed EP45 are specific for meiotic prophase. Secondly, contaminants could have been co-injected with pNiXa, inducing GVBD, because MII oocytes contain all components of the M-phase-inducing molecular machinery in a highly active state, including MPF and its substrates in fully phosphorylated and active forms. To fully elucidate the role of each isoform of EP45 in oocyte physiology, further analysis will be necessary to understand the nature of post-translational modifications of this protein following its uptake.

Most interestingly, in stage-VI oocytes stimulated with 10 nM progesterone and injected with EP45-encoding mRNA (this paper), maturation of the mid-responsive oocytes was clearly enhanced. In accordance with the absence of GVBD induction without progesterone stimulation in stage-VI oocytes, the low-responsive oocytes treated with 10 nM progesterone were not stimulated to undergo GVBD upon EP45 overexpression. For the highly responsive oocytes, which respond similarly to low and high doses of progesterone, no increase in the GVBD rate upon EP45 overexpression was observed. Thus, we show that EP45 expressed in stage-VI oocytes has a previously unexpected capacity to enhance, but not to induce, GVBD. This is in agreement with the presence of high quantities of cytoplasmic EP45 in stage-VI oocytes, which do not undergo GVBD without stimulation. Further studies will be required to elucidate how EP45 enhances oocyte maturation. As an example, kallistatin, another serpin, stimulates cell proliferation through activation of the ERK1/2 pathway (Miao et al., 2000). As ERK2 mitogen-activated protein (MAP) kinase pathway activation is crucial to GVBD, one hypothesis might be that EP45 could enhance oocyte maturation by modulating this pathway.

Intriguingly, an activity-enhancing mitotic exit was recently reported (Garnett et al., 2009). Namely, UBE2S, an anapahase-promoting complex/cyclosome (APC/C) auxiliary protein, is dispensable for physiological mitotic exit, but crucial for spindle assembly checkpoint (SAC) slippage (Garnett et al., 2009). Mitotic-exit enhancement of UBE2S therefore seems to be analogous to the Omen function of EP45 in M-phase entry (this paper). Thus, the existence of activity enhancing M-phase entry not restricted to meiosis could be envisaged. It might depend on various pathways independent of the core mechanism triggering M-phase entry, for example, upon mobilization of energy sources necessary for cell-cycle transitions. EP45 is probably not the only protein with Omen activity and numerous proteins acting in synergy might enhance M-phase entry. In oocytes, they could collectively contribute to the quality of oocytes, without individually affecting the core pathway leading to CDK1 activation (Marteil et al., 2009). We have recently found other potential Omen proteins as well as potential oocyte-maturation repressors (Omre) in additional proteomic screens (D'Inca et al., 2010).

The Omen activity of EP45 linked to the responsiveness of oocytes to progesterone could be involved in an evolutionary bet-hedging strategy (Kussel and Leibler, 2005). This strategy, well studied in bacteria (Beaumont et al., 2009), relies on the stochastic presence of molecules and processes in cells and organisms that are unnecessary in a normal environment, but become essential for survival in the face of drastic environmental changes. Various environmental factors could induce hormonal disorder, including decrease in progesterone. In such cases, oocytes with higher EP45 levels and activity (equivalent to mid-responsive oocytes expressing exogenous EP45 in our experiment) will mature in a higher proportion than those with lower EP45 levels and will deliver functional gametes supporting relative reproductive success of females. To our knowledge, EP45 is the first gene product identified in vertebrates that could play a role in such a bet-hedging strategy.

Materials and Methods

Oocyte preparation

X. laevis females were purchased from NASCO (Fort Atkinson, WI, USA). All stages of oocytes were manually isolated from the ovary after digestion in calcium-free OR2 (82.5 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 5 mM Hepes pH 7.4) containing 275 units/ml collagenase (type 1A, Sigma).

Sample preparations

Oocytes and embryos were homogenized in MPF (80 mM β-glycerophosphate, 50 mM NaF, 20 mM EGTA, 20 mM Hepes, 15 mM $MgCl_2$, 1 mM DTT, pH 7.5),

supplemented with aprotinin (10 μg/ml), leupeptin (10 μg/ml), pepstatin (10 μg/ml), AEBSF (1 mM) and Na orthovanadate (1 mM), and centrifuged (16,000 ***g***, 15 minutes, 4°C). The oocyte extract was then collected and either Laemmli buffer (Laemmli, 1970) was added for 1D WB or PBS buffer for 2D analysis. For 2D analysis, lipids were finally removed by freon extraction. A piece of liver was sonicated in EB buffer (20 mM Hepes, 100 mM NaCl, 0.05% Triton, pH 7.5), supplemented with DTT (1 mM), β-glycerophosphate (5 mM), Na orthovanadate (0.5 mM), EDTA (1 mM), AEBSF (0.5 mM), aprotinin (10 μg/ml), leupeptin (5 μg/ml), pepstatin (2 μg/ml). The homogenate was then centrifuged (13,000 rpm, 15 minutes, 4°C) and the supernatant was collected. Two additional centrifugations (13,000 rpm, 15 minutes, 4°C) enabled extract purification. After protein quantification, Laemmli buffer was added to the extract.

2D electrophoresis for MS analysis

Protein extract (40 μg for silver-stained gels and 310 μg for Coomassie-blue-stained gels) was diluted in DeStreak buffer (GE Healthcare) to a final volume of 450 μl. First-dimension isoelectric focusing was carried out on an IPGphor system (GE Healthcare) using precast immobilized pH gradient strips (pH 3 to 10 linear, GE Healthcare). After rehydration of the strips, isoelectric focusing was performed over 19 hours with a gradually increasing voltage. The strips were then allowed to equilibrate with two incubations for 15 minutes each at room temperature in a solution of 20 ml of 50 mM Tris-HCl, pH 8.2, 6 M urea, 30% glycerol, 2% SDS, containing 65 mM DTT for the first incubation and later for 15 minutes with 250 mM iodoacetamide. Strips were applied to the top of a 12.5% SDS polyacrylamide gel and run in a vertical Ettan DALT Six System (GE Healthcare). For preliminary experiments, gels were silver stained as previously described (Shevchenko et al., 1996). For MS analysis, gels were stained in a solution of 0.1% Coomassie blue and then destained in a solution of 18% ethanol and 9% acetic acid. After staining, gels were scanned and then processed for image analysis using Image Master 2D platinum 5.0 software (GE Healthcare).

MS analysis and protein identification

Spots of interest were excised from the gel using an Ettan Spot Handling Workstation (GE Healthcare). Gel plugs were washed in MilliQ water, once in 100 nM $Na_2S_2O_3$, once in 50% methanol and 50 mM ammonium bicarbonate, and once in 75% acetonitrile, and dried. In-gel digestion was performed by incubation for 60 minutes with 340 UI/ml sequencing-grade modified porcine trypsin (Promega, Charbonnières-les-Bains, France) in 20 mM NH_4HCO_3. Extraction was performed in two successive steps, by adding 50% acetonitrile and 0.1% trifluoroacetic acid. Digests were dried out and dissolved in 2 mg/ml α-cyano-4-hydroxycinnamic acid in 70% acetonitrile and 0.1% trifluoroacetic acid, before spotting onto matrix-assisted laser desorption/ionisation (MALDI) targets (384 Scout MTP 600 μm AnchorChip; Bruker Daltonik, Bremen, Germany). Mass fingerprints were acquired using a MALDI–time-of-flight (TOF)/TOF mass spectrometer (Ultraflex MALDI mass spectrometers; Bruker Daltonik) and processed with FlexAnalysis software (version 2.2; Bruker Daltonik). After internal calibration with trypsin autodigestion peptides, the monoisotopic masses of the tryptic peptides were used to query NCBInr sequence databases (version 20060609, 3,682,060 sequences) using the Mascot search algorithm (Mascot server version 2.1.04; http://www.matrixscience.com). Search conditions were as follows: initial rather-open mass window of 70 ppm for an internal calibration, one missed cleavage allowed, modification of cysteines by iodoacetamide, and methionine oxidation and N-terminal pyroglutamylation as variable modifications. To ascertain unambiguous identification, each identification was carefully checked as previously described (Com et al., 2003).

Immunoblotting

For 2D WB, samples containing 15 μg oocyte extract proteins were diluted in rehydration buffer (8 M urea, 2% CHAPS, 50 mM DTT, 0.2% Bio-Lyte 3/10 ampholyte, 0.001% Bromophenol Blue). Rehydration of the strip (3-10 Ready strip IPG strips, 7 cm, BioRad) was performed in the Protean IEF Cell System (BioRad), 12 hours, 50 V, 20°C. Immediately after rehydration, isoelectrofocusing was carried out in the same apparatus for 2 hours, 5000 V, 20°C. Then, strips were equilibrated first in equilibration buffer (6 M urea, 2% SDS, 0.375 M Tris-HCl, pH 8.8, 20% glycerol) containing 130 mM DTT and second in equilibration buffer containing 135 mM iodoacetamide. Strips were finally rinsed in migration buffer to be ready for the second dimension. For both 1D and 2D WB, oocyte extracts were subjected to 9-10% SDS-PAGE and separated proteins were transferred to nitrocellulose membranes (Hybond C, GE Healthcare). Membranes were blocked with 5% nonfat dry milk in PBS containing 0.1% Tween 20 and then probed with primary antibodies raised against EP45 (AB1 raised against peptide 1 and AB2 against peptide 2; Fig. 2A), cyclin B2 (gift from Thierry Lorca, CRBM, France), ERK2 (Santa Cruz Biotechnology), phosphorylated ERK2 (Santa Cruz Biotechnology) and tubulin (Sigma Aldrich). Antigen-antibody complexes were revealed using alkaline-phosphatase-conjugated anti-rabbit or anti-mouse secondary antibodies (diluted 1:10,000) in combination with Enhanced ChemiFluorescence reagent (GE Healthcare). Signal quantification was performed using Image-Quant 5.2 software (GE Healthcare).

RT-PCR

Total RNA was extracted from tissues using TRI Reagent according to the manufacturer's protocol (Molecular Research Center). cDNA was obtained from reverse transcription of 5 μg total mRNA. PCR was then performed using 2 μl cDNA. The following primers were used for EP45 and for EF1-α (control): EP45 up (5′ CTC TAC AAA GGA GAA TGG GCT 3′), EP45 rev (5′ AGC CTC GAG TCA TTG TGC ATT AGT TGG GTC 3′), EF1-α for (5′ GTG GAA ATT TGA GAC CAG C 3′) and EF1-α rev (5′ TTT ACA CCC AGA GTG TAG GC 3′).

Oocyte microinjections

After dissection, oocytes were cultured in Maller and Koontz buffer (83 mM NaCl, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, pH 7.8). Full-length *EP45* cDNA and *β-galactosidase* cDNA (control) were subcloned into pCS2+ vector and transcribed using the Ambion Message Machine kit. Typically, 32.2 ng RNA (EP45 or β-galactosidase) was injected into oocyte. About 14 hours post microinjection, oocytes were stimulated with 10 nM, 50 nM or 1 μM progesterone, or were left untreated. The time course of maturation was followed by calculating the percentage of GVBD, as measured by the appearance of a white spot centered on the animal pole of the oocyte.

The authors thank Catherine Jessus, Marc W. Kirschner, Maciej Dobrzynski and Daniel Fisher for critical reading of this article at different stages of writing, and for stimulating discussions and English-language corrections. Proteomics analyses were supported by the Proteomics Core Facility, Biogenouest. The authors thank Régis Lavigne and Charles Pineau for valuable assistance during the MS experiments. This work was supported by grants from ARC and LCC to J.Z.K. G.M. was a recipient of a fellowship from the French Ministère de la Recherche et de l'Enseignement supérieur.

References

Bazile, F., Gagne, J. P., Mercier, G., Lo, K. S., Pascal, A., Vasilescu, J., Figeys, D., Poirier, G. G., Kubiak, J. Z. and Chesnel, F. (2008). Differential proteomic screen to evidence proteins ubiquitinated upon mitotic exit in cell-free extract of Xenopus laevis embryos. *J. Proteome Res.* **7**, 4701-4714.

Beaumont, H. J., Gallie, J., Kost, C., Ferguson, G. C. and Rainey, P. B. (2009). Experimental evolution of bet hedging. *Nature* **462**, 90-93.

Beck, B. L., Henjum, D. C., Antonijczuk, K., Zaharia, O., Korza, G., Ozols, J., Hopfer, S. M., Barber, A. M. and Sunderman, F. W., Jr (1992). pNiXa, a Ni(2+)-binding protein in Xenopus oocytes and embryos, shows identity to Ep45, an estrogen-regulated hepatic serpin. *Res. Commun. Chem. Pathol. Pharmacol.* **77**, 3-16.

Com, E., Evrard, B., Roepstorff, P., Aubry, F. and Pineau, C. (2003). New insights into the rat spermatogonial proteome: identification of 156 additional proteins. *Mol. Cell. Proteomics* **2**, 248-261.

D'Inca, R., Marteil, G., Bazile, F., Pascal, A., Guitton, N., Lavigne, R., Richard-Parpaillon, L. and Kubiak, J. Z. (2010). Proteomic screen for potential regulators of M-phase entry and quality of meiotic resumption in *Xenopus laevis* oocytes. *J. Proteomics* (in press).

Dumont, J. N. (1972). Oogenesis in Xenopus laevis (Daudin). I. Stages of oocyte development in laboratory maintained animals. *J. Morphol.* **136**, 153-179.

Garnett, M. J., Mansfeld, J., Godwin, C., Matsusaka, T., Wu, J., Russell, P., Pines, J. and Venkitaraman, A. R. (2009). UBE2S elongates ubiquitin chains on APC/C substrates to promote mitotic exit. *Nat. Cell Biol.* **11**, 1363-1369.

Gosden, R. G. (2002). Oogenesis as a foundation for embryogenesis. *Mol. Cell. Endocrinol.* **186**, 149-153.

Goto, N., Muto, S., Sugimoto, I., Ikawa-Kitayama, K., Igarashi, K., Ito, S. and Hashimoto, E. (2001). A serpin with M(r) 43,000 is a binding protein of M(r) 25,000 protein, a substrate for protein ser/thr kinase detected in Xenopus laevis oocytes. *J. Biochem.* **129**, 229-236.

Hashimoto, E., Takeuchi, F., Tanaka, Y. and Yamamura, H. (1995). Purification and phosphorylation of a M(r) 25,000 protein, an effective phosphate acceptor for casein kinase II and protein kinase C, detected in the cytosolic fraction of Xenopus laevis oocytes. *J. Biochem.* **118**, 453-460.

Hashimoto, E., Kobayashi, N., Kubota, N., Tanaka, Y. and Yamamura, H. (1996). Phosphorylated sites of M(r) 25,000 protein, a putative protein phosphatase 2A modulator, and phosphorylation of the synthetic peptide containing these sites by protein kinase C. *J. Biochem.* **119**, 626-632.

Haspel, J., Sunderman, F. W., Jr, Hofper, S. M., Henjum, D. C., Brandt-Rauf, P. W., Weinstein, I. B., Nishimura, S., Yamaizumi, Z. and Pincus, M. R. (1993). A nickel-binding serpin, pNiXa, induces maturation of Xenopus oocytes and shows synergism with oncogenic ras-p21 protein. *Res. Commun. Chem. Pathol. Pharmacol.* **79**, 131-140.

Holland, L. J. and Wangh, L. J. (1987). Estrogen induction of a 45 kDa secreted protein coordinately with vitellogenin in Xenopus liver. *Mol. Cell. Endocrinol.* **49**, 63-73.

Holland, L. J., Suksang, C., Wall, A. A., Roberts, L. R., Moser, D. R. and Bhattacharya, A. (1992). A major estrogen-regulated protein secreted from the liver of Xenopus laevis is a member of the serpin superfamily. Nucleotide sequence of cDNA and hormonal induction of mRNA. *J. Biol. Chem.* **267**, 7053-7059.

Jessus, C. and Ozon, R. (2004). How does Xenopus oocyte acquire its competence to undergo meiotic maturation? *Biol. Cell* **96**, 187-192.

Jessus, C., Rime, H., Haccard, O., Van Lint, J., Goris, J., Merlevede, W. and Ozon, R. (1991). Tyrosine phosphorylation of p34cdc2 and p42 during meiotic maturation of Xenopus oocyte. Antagonistic action of okadaic acid and 6-DMAP. *Development* **111**, 813-820.

Jorgensen, P., Steen, J. A., Steen, H. and Kirschner, M. W. (2009). The mechanism and pattern of yolk consumption provide insight into embryonic nutrition in Xenopus. *Development* **136**, 1539-1548.

Karaiskou, A., Lepretre, A. C., Pahlavan, G., Du Pasquier, D., Ozon, R. and Jessus, C. (2004). Polo-like kinase confers MPF autoamplification competence to growing Xenopus oocytes. *Development* **131**, 1543-1552.

Kussell, E. and Leibler, S. (2005). Phenotypic diversity, population growth, and information in fluctuating environments. *Science* **309**, 2075-2078.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lawrence, D. A., Ginsburg, D., Day, D. E., Berkenpas, M. B., Verhamme, I. M., Kvassman, J. O. and Shore, J. D. (1995). Serpin-protease complexes are trapped as stable acyl-enzyme intermediates. *J. Biol. Chem.* **270**, 25309-25312.

Maller, J. L. (1998). Recurring themes in oocyte maturation. *Biol. Cell* **90**, 453-460.

Marteil, G., Richard-Parpaillon, L. and Kubiak, J. Z. (2009). Oocyte quality to support meiotic maturation and embryonic development. *Reprod. Biol.* **9**, 203-224.

Masui, Y. and Markert, C. L. (1971). Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. *J. Exp. Zool.* **177**, 129-145.

Miao, R. Q., Murakami, H., Song, Q., Chao, L. and Chao, J. (2000). Kallistatin stimulates vascular smooth muscle cell proliferation and migration in vitro and neointima formation in balloon-injured rat artery. *Circ. Res.* **86**, 418-424.

Mulner, O., Belle, R. and Ozon, R. (1983). cAMP-dependent protein kinase regulates in ovo cAMP level of the Xenopus oocyte: evidence for an intracellular feedback mechanism. *Mol. Cell. Endocrinol.* **31**, 151-160.

Reynhout, J. K., Taddei, C., Smith, L. D. and LaMarca, M. J. (1975). Response of large oocytes of Xenopus laevis to progesterone in vitro in relation to oocyte size and time after previous HCG-induced ovulation. *Dev. Biol.* **44**, 375-379.

Rime, H., Yang, J., Jessus, C. and Ozon, R. (1991). MPF is activated in growing immature Xenopus oocytes in the absence of detectable tyrosine dephosphorylation of P34cdc2. *Exp. Cell Res.* **196**, 241-245.

Sadler, S. E. and Maller, J. L. (1983). The development of competence for meiotic maturation during oogenesis in Xenopus laevis. *Dev. Biol.* **98**, 165-172.

Shevchenko, A., Wilm, M., Vorm, O. and Mann, M. (1996). Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal. Chem.* **68**, 850-858.

Stifani, S., Nimpf, J. and Schneider, W. J. (1990). Vitellogenesis in Xenopus laevis and chicken: cognate ligands and oocyte receptors. The binding site for vitellogenin is located on lipovitellin I. *J. Biol. Chem.* **265**, 882-888.

Sunderman, F. W., Jr, Varghese, A. H., Kroftova, O. S., Grbac-Ivankovic, S., Kotyza, J., Datta, A. K., Davis, M., Bal, W. and Kasprzak, K. S. (1996). Characterization of pNiXa, a serpin of Xenopus laevis oocytes and embryos, and its histidine-rich, Ni(II)-binding domain. *Mol. Reprod. Dev.* **44**, 507-524.

Tunquist, B. J. and Maller, J. L. (2003). Under arrest: cytostatic factor (CSF)-mediated metaphase arrest in vertebrate eggs. *Genes Dev.* **17**, 683-710.

van Gent, D., Sharp, P., Morgan, K. and Kalsheker, N. (2003). Serpins: structure, function and molecular evolution. *Int. J. Biochem. Cell Biol.* **35**, 1536-1547.

Wasserman, W. J., Houle, J. G. and Samuel, D. (1984). The maturation response of stage IV, V, and VI Xenopus oocytes to progesterone stimulation in vitro. *Dev. Biol.* **105**, 315-324.

Yoshitome, S., Nakamura, H., Nakajo, N., Okamoto, K., Sugimoto, I., Kohara, H., Kitayama, K., Igarashi, K., Ito, S., Sagata, N. et al. (2003). Mr 25 000 protein, a substrate for protein serine/threonine kinases, is identified as a part of Xenopus laevis vitellogenin B1. *Dev. Growth Differ.* **45**, 283-294.