

Potential role of protein tyrosine phosphatase nonreceptor type 13 in the control of oocyte meiotic maturation

Taku Nedachi and Marco Conti*

Division of Reproductive Biology, Department of Obstetrics and Gynecology, Stanford University School of Medicine, Stanford, CA 94305, USA

*Author for correspondence (e-mail: marco.conti@stanford.edu)

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Summary

Protein tyrosine phosphatase nonreceptor type 13 (PTPN13) is a tyrosine phosphatase with multiple interacting domains that has been implicated previously in the regulation of apoptosis. We provide evidence that PTPN13 plays an important role in the control of the meiotic cell cycle. A cDNA coding for PTPN13 was isolated during the screening for the substrate of protein kinase A expressed in mammalian oocytes. PTPN13 is expressed in both mouse and *Xenopus* oocytes and is a substrate for protein kinase A *in vitro* and *in vivo*. Expression of a truncated constitutively-active PTPN13 in *Xenopus* oocytes synergizes with progesterone in the induction of germinal

vesicle breakdown, the translation of Mos, the phosphorylation of Erk and the dephosphorylation of Cdc2. The phosphatase activity of PTPN13 is required for this synergism. Oocyte injection with specific small interference RNA downregulates the expression of mRNA for PTPN13 and blocks oocyte maturation induced by progesterone, a blockade that can be overcome by Cdc25 overexpression. These findings indicate that PTPN13 is involved in the regulation of the meiotic cell cycle.

Key words: *Xenopus* oocyte, Cyclic AMP, Meiotic resumption, Tyrosine phosphatase

Introduction

During the growth and maturation of ovarian follicles of most species, oocytes are arrested in a dictyate stage of meiosis. This state, which has the properties of the G2 phase of mitosis, is characterized by the presence of a morphologically distinguishable nucleus with a prominent nucleolus (germinal vesicle, GV) and is associated with partial decondensation of the chromosomes. In all species studied, a gonadotropin-dependent signal derived from somatic cells surrounding the oocyte induces reentry into the meiotic cell cycle, and completion of the first meiotic division involves chromosome condensation, dissolution of the nuclear membrane (germinal vesicle breakdown, GVBD) and spindle organization. This first meiotic division is completed with the emission of the first polar body. Unlike the mitotic cycle where DNA synthesis occurs during the S phase, oocytes immediately enter a second division without an S phase and are arrested in metaphase II. Only metaphase II stage oocytes are able to undergo fertilization by the spermatozoa, complete the second meiotic maturation and form pronuclei.

The signaling network controlling meiotic resumption includes early components proximal to plasma membrane receptors and distal components, which are centered around the regulation of the Cdc2/cyclinB complex (maturation promoting factor, MPF) (Ferrell, 1999a; Ferrell, 1999b). The distal components are well characterized (Karaiskou et al., 2001; Nebreda and Ferby, 2000; Schmitt and Nebreda, 2002b). Extensive data are available on the control of MPF activity and the most immediate partners, including Cdc25 (Gautier et al., 1991; Kumagai and Dunphy, 1991; Rime et al., 1994) and

Myt1 (Palmer et al., 1998). Activation of MAP kinase cascade (Gotoh et al., 1995; Nebreda and Hunt, 1993) and de novo synthesis of Ringo/Speedy (Ferby et al., 1999; Lenormand et al., 1999) are additional components involved in the activation of MPF and resumption of meiosis.

Whereas the early events causing resumption of meiosis in mammalian oocytes are still a matter of debate, at least two receptor-activated pathways have been shown to cause meiotic resumption in *Xenopus* oocytes. One pathway involves progesterone binding and activation of a putative membrane steroid receptor that is coupled to a decrease in adenylyl cyclase (AC) activity and cAMP (Masui, 1967; Schuetz, 1967a; Schuetz, 1967b; Zhu et al., 2003a; Zhu et al., 2003b). In a distinct pathway of less clear physiological significance, insulin-like growth factor 1 (IGF1) binds and activates a tyrosine kinase receptor that most likely signals through activation of a phosphatidylinositol 3 kinase (PI3K) pathway (Liu et al., 1995; Sadler and Maller, 1987; Sadler and Maller, 1989). In this pathway, PKB/AKT is activated and a phosphodiesterase (PDE) is one of the downstream targets of the kinase producing a decrease in cyclic AMP (cAMP) (Andersen et al., 1998; Andersen et al., 2003). It is then possible that both the progesterone and the IGF1 pathways converge on cAMP regulation.

Convincing evidence is available that cAMP plays an important role in maintaining meiotic arrest in both mammals and amphibians (Conti et al., 2002; Maller et al., 1979). Inhibition of the activity of Gs in mice and frogs induces oocyte maturation (Gallo et al., 1995; Mehlmann et al., 2002), suggesting that G α s or G $\beta\gamma$ maintain the oocyte AC in an active

state leading to a constitutive production of cAMP. In agreement with this view, it has been reported recently that AC3 and AC9 are expressed in mouse oocytes and that inactivation of the *Adcy3* gene causes a leaky meiotic arrest and possibly infertility (Horner et al., 2003).

Downstream from cAMP, protein kinase A (PKA) is probably involved in the maintenance of meiotic arrest. Early studies have demonstrated that injection of a PKA catalytic subunit completely blocks hormonal induction of oocyte maturation (Maller and Krebs, 1977). Conversely, injection of the regulatory subunit of PKA or the heat stable inhibitor of PKA (PKI) causes meiotic resumption in *Xenopus* and mouse oocytes (Bornslaeger et al., 1986; Huchon et al., 1981; Maller and Krebs, 1977). More recent data by Schmitt et al. indicate that an inactive PKA catalytic subunit also blocks progesterone-induced *Xenopus* oocyte maturation (Schmitt and Nebreda, 2002a), suggesting that PKA may prevent oocyte maturation by PKA catalytic-dependent and -independent mechanisms.

In contrast to the above findings, little is known about the mechanisms by which an active PKA maintains meiotic arrest. It has been hypothesized that PKA phosphorylates and maintains key components of the meiotic machinery in an inactive state, but evidence for direct substrates of PKA is scant. Recently, it has been proposed that *Xenopus* Cdc25 phosphorylation fulfills these criteria (Duckworth et al., 2002). PKA phosphorylates Cdc25 in a cell-free system in S287, which is thought to be an inhibitory site also involved in 14-3-3 interaction. However, other PKA substrates are probably present in the cascade controlling meiotic arrest. For example, it is unclear how the translation machinery is maintained in an inactive state during meiotic arrest. Here, we have used a small-pool strategy to identify PKA substrates that function in the signaling pathways involved in meiotic control.

Materials and methods

In vitro expression screening

A cDNA library was generated with oocytes from 22-day-old mice (1.3×10^6 recombinants; gift from Dr John J. Eppig). This pSPORT-P (Invitrogen, Carlsbad, CA) plasmid library in the *Escherichia coli* (*E. coli*) was diluted to 140 pools that included ~100 independent clones and plated on 150 mm plates. After overnight culture, a replica plate was made. The original plates were then washed with 5 ml of LB medium to collect all the transformed *E. coli* and were continuously cultured in the LB medium overnight. Plasmids were isolated from pools using a plasmid miniprep kit (Qiagen, Valencia, CA). For making an arrayed library, independent colonies were taken from the replica plates and placed in 96-well plates with glycerol stock solution. In vitro transcription and translation for each pool was performed using TNT^R-coupled reticulocyte transcription/translation kit (Promega, Madison, WI) with [³⁵S]methionine (New England Nuclear, Boston, MA). Translated protein pools were then incubated for 30 minutes at 30°C with or without 0.1 U protein kinase A catalytic subunit (Promega) in the kinase assay buffered solution (50 mM Tris, pH 7.4, 5 mM MgCl₂, 4 µg/ml aprotinin, 0.7 µg/ml pepstatin, 0.2 mM PMSF, 0.5 µg/ml leupeptin, 0.1 µg/ml cycloheximide, 0.3 µM okadaic acid, 1 mM ATP). Incubation mixtures were subjected to 8% SDS-PAGE (1:30 bis: acrylamide), gels were dried and exposed to X-ray film (Eastman Kodak, Rochester, NY).

Plasmids and mutagenesis

Isolated mouse C-terminal PTPN13 (amino acid 1917-2460) was subcloned into a pCMV-HA vector for tagging the influenza virus HA

epitope on the N terminus. HA-tagged C-PTPN13 was then inserted into the pSP64-poly (A) vector (Promega) for mRNA transcription. Mutagenesis of mouse C terminus PTPN13 (Cys2374Ser) was performed by using a Quick change site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the protocol provided by the manufacturer. The mutation was checked by sequence analysis. The pSP64-poly (A)-PDE3A construct was prepared as described previously (Andersen et al., 2003). Full-length mouse Cdc25B cDNA was amplified from mouse lung total RNA by RT-PCR using forward, 5'-GCCGGATCCGCCACGATGGAGGT-3' and reverse, 5'-TGG-CAGCAGGCTCATCATCACT-3' primers. Amplified PCR products were then inserted into pcDNA3.1/V5-His-TOPO TA cloning vector (Invitrogen), and the insert excised by *Bam*HI and subcloned into pSP64-poly (A) vector (Promega Corp.). The insert was checked by sequence analysis.

Reverse transcription and polymerase chain reaction

For semiquantitative measurements of gene expression, total RNA was extracted from *Xenopus* oocytes using TRIzol solution (Invitrogen). Total RNA was then treated with DNase I (Invitrogen) to remove genomic DNA. For reverse transcription, first-strand cDNA was synthesized from 2 µg of total RNA with random hexanucleotides as primer (SuperScript First-Strand Synthesis System for RT-PCR, Invitrogen). PCR incubation mixtures contained 50 mM KCl, 20 mM Tris/HCl (pH 8.4), 1.5 mM MgCl₂, 200 µM of deoxynucleoside triphosphate, 0.5 µM of each primer and 2.5 units of *Taq* DNA Polymerase (Invitrogen). The PCR conditions were as follows: denaturation at 94°C for 2 minutes; followed by 27 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute, and final extension at 72°C for 10 minutes. The numbers of cycles were chosen by the pilot experiments that showed the reactions were in the exponential phase. Specific primers were used to amplify cDNAs: xPTPN13 forward, 5'-AAGTCAGGACCCATCATTACA-3'; xPTPN13 reverse, 5'-CTT-GATAGCAGAAAATATATT-3'; xGAPDH forward, 5'-CTCCTCTC-GCAAAGGTCATC-3'; xGAPDH reverse, 5'-GGAAAGCCATT-CCGGTTATT-3' (Stanford PAN Facility, Stanford, CA). PCR products were analyzed by 1% agarose gel electrophoresis. RNA without RT did not yield any amplicons indicating there is no contamination of genomic DNA.

For sequence analysis of the PTPN13 catalytic subunit, primers were designed from EST clones (GenBank accession number BJ063819 and BJ070269). Specific primers were used to amplify cDNA coding tyrosine phosphatase domain of PTPN13: (forward primers) xPTPN13F1, 5'-GTCATCCAATCTCTACTAG-3'; xPTPN13F2, 5'-GAGGACACAGACTGTGATG-3'; (reverse primers) xPTPN13R1, 5'-TGCCTCTGAAGTCGCATTG-3'; xPTPN13R2, 5'-GAGTATAACTTGATAGCAG-3'. Four different combinations of the primers (F1 and R1, F1 and R2, F2 and R1, F2 and R2) were used for RT-PCR and amplified PCR products were subcloned into pcDNA3.1/V5-His-TOPO TA cloning vector (Invitrogen) followed by sequence analysis.

Immunohistochemistry of mouse ovaries

PTPN13 protein expression was visualized by immunohistochemical detection with the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA). Ovaries were fixed in Bouin's solution, embedded in paraffin and then cut into 5 µm sections. Deparaffinized sections were rehydrated and rinsed in PBS, and endogenous peroxidases were blocked by incubation in hydrogen peroxide followed by incubation in normal horse serum. The sections were then incubated overnight in an anti-mouse PTPN13 antibody (1:80; Santa Cruz Biotechnology) in a humidified chamber at 4°C. The distribution of the primary antibody was revealed with a biotinylated horse anti-goat secondary antibody, and the avidin-biotin-peroxidase complex was visualized with DAB. Sections were rinsed in PBS between each step. The specificity of the PTPN13 staining was checked by

replacing the antibody with nonimmune IgG (Santa Cruz Biotechnology).

In vitro mRNA synthesis

For in vitro mRNA synthesis, the pSPORT-P or pSP64-poly (A) constructs were transcribed using the T7 or SP6 polymerases according to the procedure supplied by the manufacturer (mMessage mMachine Kit, Ambion, Austin, TX). The transcribed mRNA was purified by phenolchloroform extraction, precipitated at -20°C with one volume isopropanol and subsequently resuspended in DEPC water. The mRNA concentration was measured by OD²⁶⁰ and agarose gel electrophoresis. The mRNA was diluted to a concentration of 1 mg/ml in DEPC water and stored at -80°C .

Injection into *Xenopus* oocytes

Ovary fragments were surgically removed from PMSG-primed *Xenopus laevis* and defolliculated oocytes were isolated after treatment with collagenase (2.5 mg/ml) in MBS buffer [10 mM HEPES (pH 7.4), 88.0 mM NaCl, 1 mM KCl, 0.82 mM MgSO₄, 2.4 mM Na₂ HCO₃] for 1-1.5 hours. Dumont Stage VI oocytes were selected for all experiments. Oocyte storage and experiments were carried out in OR2 solution [5 mM HEPES (pH 7.8), 82.5 mM NaCl, 2.5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 1 mM Na₂HPO₄] supplemented with 0.1% BSA. Oocytes were routinely tested for their progesterone responsiveness by incubation of 10 oocytes in 500 nM of progesterone in OR2 solution overnight at 16°C. Oocytes that exhibited a progesterone-stimulated GVBD of 80-100% were used. The mRNAs, siRNAs or H₂O (vehicle) were injected using a micromanipulator (Narishige USA, Long Island, NY) into defolliculated *Xenopus* oocytes. The oocyte maturation was induced by stimulating 0-500 nM of progesterone, or injecting Cdc25B mRNA (1 ng/oocyte) or PDE3A mRNA (20 ng/oocyte). Resumption of meiosis was scored by the appearance of a white spot on the animal pole of the oocyte.

Western blot analysis

Expression of HA-tagged clones and intracellular signaling molecules was analyzed after lysing injected oocytes in 10 μl lysis buffer (250 mM sucrose, 1 mM KCl, 1 mM MgCl₂, 0.2 mM PMSF) per oocyte. The oocyte extract was isolated by centrifugation in a Beckman model B centrifuge for 4 minutes at 4°C. The lipid supernatant was removed and the clarified supernatants were transferred to new microtubes. Approximately 25 μl of oocyte extract was analyzed by gel electrophoresis on 8% SDS-PAGE (1:30 bis: acrylamide). After transfer, PVDF membranes (Immobilon, Millipore, Bedford, MA) were blocked overnight at 4°C in 0.2% blocking grades BSA (BioRad Laboratories, Hercules, CA) in Tris buffered saline with 0.1% Tween 20. Immunostaining to detect HA-clone expression was performed by incubating 1 hour with a 1:1000 dilution of an anti-mouse PTPN13 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-human PTPN13 antibody (ScienceReagent, El Cajon, CA), HA.11 monoclonal antibody (COVANCE, Princeton, NJ), anti-*Xenopus* Mos antibody (Santa Cruz Biotechnology), antiphospho-p42/p44 antibody (Cell Signaling, Beverly, MA), DC3 MAP kinase monoclonal antibody (gift from Dr James J. Ferrell, Jr), antiphospho-Cdc2 antibody (Cell Signaling) or anti-Cdc2 antibody (Oncogene Research Products, San Diego, CA). Specific proteins were visualized after subsequent 1:5000 dilution of anti-mouse, -rabbit, or -goat IgG conjugated to horseradish peroxidase (Amersham-Pharmacia Biotech, Santa Cruz Biotechnology) and ECL procedure (Amersham Pharmacia Biotech).

In vitro phosphorylation of PTPN13

HEK293 cells were cultured until 50% confluency in 6 ml of DMEM (Invitrogen) + 10% FBS media using 60 mm plates. After 6 hours of serum starvation of the cells using serum-free DMEM, cells were harvested by using 50 mM Tris (pH 7.6), 150 mM NaCl, 1 mM EDTA,

0.1% NP-40, 4 $\mu\text{g/ml}$ aprotinin, 0.7 $\mu\text{g/ml}$ pepstatin, 0.2 mM PMSF, 0.5 $\mu\text{g/ml}$ leupeptin, 1 μM microcystin-LR), immunoprecipitated with 2 μg of anti-human PTPN13 antibodies (ScienceReagent) or 2 μg of nonimmune IgG (Santa Cruz Biotechnology) and protein G-sepharose (Amersham Pharmacia Biotech). Immunoprecipitated PTPN13 was duplicated then half were incubated with a kinase assayed solution (50 mM Tris, pH 7.6, 5 mM MgCl₂, 1 mM ATP, 4 $\mu\text{g/ml}$ aprotinin, 0.7 $\mu\text{g/ml}$ pepstatin, 0.2 mM PMSF, 0.5 $\mu\text{g/ml}$ leupeptin and 500 $\mu\text{Ci/ml}$ [γ -³²P]ATP (3000 Ci/mmol, Amersham Pharmacia Biotech)) with or without 0.1 unit of PKA catalytic subunit at 30°C for 0, 5, 15 or 30 minutes. The reactions were terminated by adding 4 \times Laemmli's sample buffered solution and boiling for 5 minutes. The samples then were subjected to 6% SDS-PAGE, followed by autoradiography for 12 hours. The remaining half was used for western blot analysis using anti-human PTPN13 antibodies (ScienceReagent).

Metabolic labeling of HEK293 cells

HEK293 cells were cultured until 50% confluency in 6 ml of DMEM (Invitrogen) + 10% FBS media using 60 mm plates (Corning, Corning, NY). For the metabolic labeling, the cell medium was exchanged to 3 ml of phosphate-free DMEM for 1 hour, then 500 μCi of ³²P orthophosphate (Phosphorus ³²P, Amersham Pharmacia Biotech) was added in each plate for 6 hours. Metabolically labeled cells were stimulated with 100 μM forskolin, harvested by using 1 ml of Tris-NP40 lysis buffered solution [50 mM Tris (pH 7.6), 150 mM NaCl, 1 mM EDTA, 0.1% NP-40, 4 $\mu\text{g/ml}$ aprotinin, 0.7 $\mu\text{g/ml}$ pepstatin, 0.2 mM PMSF, 0.5 $\mu\text{g/ml}$ leupeptin, 1 μM microcystin-LR), and immunoprecipitated with 2 μg of anti-human PTPN13 antibody (ScienceReagent) and protein G-sepharose (Amersham Pharmacia Biotech). The immunoprecipitated PTPN13 was duplicated and half were subjected to 6% SDS-PAGE, followed by autoradiography for 12 hours. The remaining half was used for western blot analysis by anti-human PTPN13 antibodies.

Measurement of protein tyrosine phosphatase activity

Myelin basic protein (MBP; Sigma) and constitutively active EGF receptor (Calbiochem) were incubated at 30°C for 6 hours in 40 mM imidazole-HCl (pH 7.2) containing 50 mM NaCl, 15 mM Mg(CH₃COOH)₂, 100 mM MgCl₂, 100 μM Na₃VO₄, 200 μM EDTA, 0.05% (vol/vol) Triton-X 100, 3% glycerol, and 40 μM [γ -³²P]ATP. At the end of the incubation, the sample was subjected to gel filtration on a Sephadex G-15 fine column (20 ml column volume; Amersham Pharmacia Biotech). Fractions containing the ³²P-labeled MBP were pooled and stored at 4°C before use in protein-tyrosine phosphatase (PTPase) assay. The PTPN13 immunoprecipitates from HEK293 cell extracts were prepared as described above. Immunoprecipitates were then subjected to in vitro phosphorylation with 1 unit of PKA catalytic subunit (PKA; Promega Corp.). The following controls were used: PKA + protein kinase A inhibitor peptide (PKI; Sigma), heat inactivated PKA or 1 μM sodium orthovanadate for 30 minutes, followed by washing three times with Tris-NP40 lysis buffered solution. The washed immunoprecipitates were incubated with ³²P-labeled MBP in 25 mM imidazole (pH 7.4), containing 1 mg/ml BSA and 0.1% (vol/vol) 2-mercaptoethanol at 30°C for 15 minutes. The reaction was terminated by applying the aliquots to P81 cellulose filter paper, followed by washing once at 4°C in 10% trichloroacetic acid (TCA) and then three times in 5% TCA. The paper filters were dried with methanol, and counted using a liquid scintillation counter. Tyrosine phosphatase activity was calculated by subtracting the counts from each sample from the total count (without immunoprecipitates). The experiments were repeated at least three times and representative data are reported.

siRNA preparation

Four sets of primers from EST *Xenopus* PTPN13 sequences (GenBank Accession Number BJ063819 and BJ070269)

corresponding to the mouse PTPN13 sequence (2020-2446) were used for generating small interference RNA (siRNA) targeting *Xenopus* PTPN13. Twenty-one nucleotide DNA oligos for target sequences, with an additional T7 promoter binding site, were chemically synthesized (Stanford PAN Facility). The siRNA sequences targeting PTPN13 mRNA are shown in Table 1. SiRNAs were made according to the procedure supplied by the manufacturer (Silencer siRNA construction kit, Ambion). Concentration of synthesized siRNAs was determined by measuring the OD²⁶⁰. Aliquoted siRNA (1 mg/ml) was kept at -80°C until use.

Bioinformatic tools

BLAST (Basic Local Alignment Search Tool) search was performed using NCBI BLAST 2.2.6 (<http://www.ncbi.nlm.nih.gov/BLAST/>). Scansite (<http://scansite.mit.edu/>) was used for identification of motifs within proteins likely to be phosphorylated by protein kinase A.

Results

Small pool screening strategy for PKA substrates

A small pool strategy was used to identify PKA substrates involved in the control of oocyte maturation. A complementary DNA plasmid library of 1.3×10^6 recombinants from mouse oocytes was subdivided into pools of ~100 clones. Each plasmid pool was in vitro-transcribed/translated in the presence of [³⁵S]methionine, then incubated in the absence or presence of a PKA catalytic subunit and ATP followed by fractionation on SDS-PAGE (Fig. 1A). Pools containing translated proteins that shifted in mobility after incubation with PKA were further purified in a second and third round of screening until a single plasmid was obtained. One hundred and forty independent pools of ~100 clones were screened and 10 independent clones were identified as potential PKA substrates by this in vitro expression screening (four representative positive clones are shown in Fig. 1B). Confirmed clones were then transcribed into mRNAs and injected into *Xenopus* oocytes to determine their effect on progesterone-induced oocyte maturation. In the presence of progesterone, mRNA from clone 1-4B, but not three other clones, produced a consistent acceleration of oocyte maturation as compared to the H₂O-injected controls (Fig. 1C).

Sequencing of the insert and a BLAST homology search revealed that the 1-4B clone codes for the C terminal fragment (amino acid 1917-2460) of the protein tyrosine phosphatase nonreceptor type 13 (PTPN13, also known as FAP1, PTP1L1, PTP1E, PTP-BL or PTP-BAS). The mouse full-length protein consists of 2460 amino acids with a long N terminus containing a FERM domain and five PDZ domains (Fig. 2A). Partial *Xenopus* PTPN13 sequences are available as EST sequences (GenBank Accession Numbers BJ052929, BJ063819 and BJ070269) that corresponds to amino acids 2020-2259 and 2345-2446 of the mouse PTPN13 sequence. Several primers

were designed according to these EST sequences and RT-PCR was performed using total RNA purified from *Xenopus* oocytes as a template. With this strategy, the entire amino acid sequence of the tyrosine phosphatase domain of *Xenopus* PTPN13 was identified (Fig. 2A,B). This PTPase domain is very similar to the PTPase domain of mouse or human PTPN13 (72.49% *Xenopus* versus mouse, 75.11% *Xenopus* versus human, 90.83% mouse versus human).

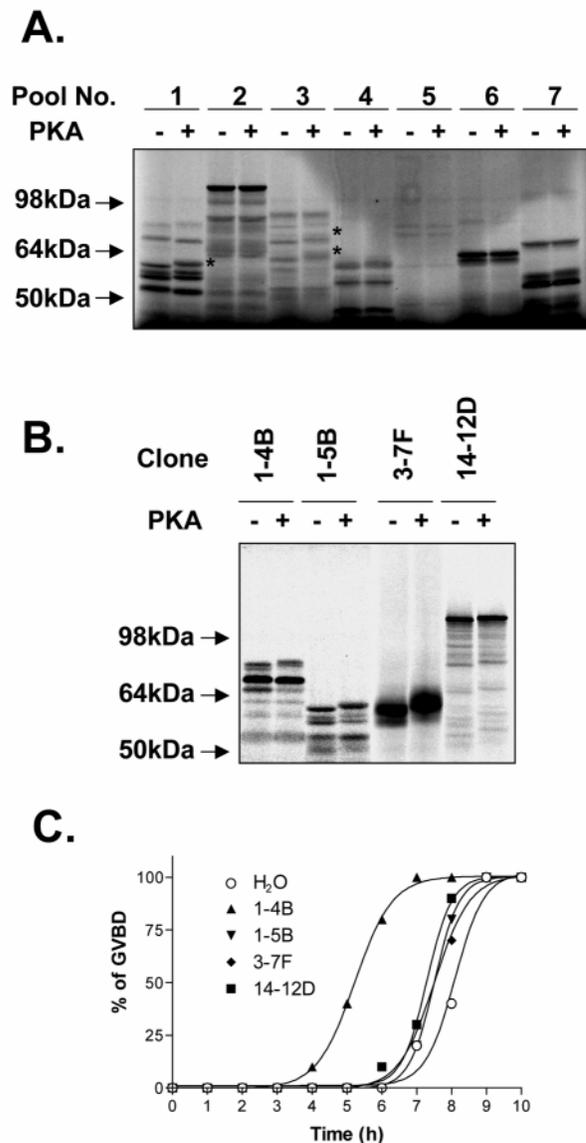


Fig. 1. Screening for potential PKA substrates from a mouse oocyte library. (A) Radiolabeled protein pools were generated and analyzed by a SDS-gel mobility shift assay. Asterisks indicate the proteins where mobility was shifted by incubation with PKA. (B) Radiolabeled proteins derived from isolated single clones (1-4B, 1-5B, 3-7F and 14-12D) were incubated with or without the PKA catalytic subunit, followed by a SDS-gel mobility shift assay. (C) mRNA from each independent clone were synthesized in vitro. Twenty nanograms of each mRNA (in 20 nl of H₂O) or 20 nl of H₂O (vehicle) were injected into 30 *Xenopus* oocytes 12 hours before 500 nM progesterone stimulation. Percentage of GVBD was measured by counting white spots on the animal pole of *Xenopus* oocytes.

Table 1. Sequence of PTPN13 siRNAs and scrambled siRNAs

| Primer | Sequence |
|-------------|-----------------------------|
| siRNA-1 | 5'-AAGTCAGGACCCATCATTAC-3' |
| siRNA-2 | 5'-AAGGACCTCTCCTAGTACAG-3' |
| siRNA-3 | 5'-AAAATTTTGCTCCCATACCAT-3' |
| siRNA-4 | 5'-AATTCCTCAGTGATTCTATG-3' |
| Scrambled 1 | 5'-AAGAAACCCGGTTAAACCCTT-3' |
| Scrambled 2 | 5'-AAGCCCTTGGCACACATATAA-3' |
| Scrambled 3 | 5'-AAGTGTGCCCCCAAATTTAAA-3' |

PTPN13 is expressed in mouse and *Xenopus* oocytes

Several strategies were followed to determine the expression of PTPN13 in mouse and *Xenopus* oocytes. For RT-PCR analysis, 5' and 3' primers that produce 248 bp PCR products were designed from the C-terminal *Xenopus* PTPN13 sequence. Using this strategy, PTPN13 mRNA was detected in stage VI *Xenopus* oocytes (Fig. 3A). Similar results were obtained with mouse GV oocyte mRNA (data not shown). Moreover, immunohistochemistry of mouse ovary sections using an anti-mouse PTPN13 antibody demonstrated immunoreactivity in the oocytes, whereas little signal was observed in granulosa cells (Fig. 3B). However, this antibody cross-reacted poorly with the *Xenopus* PTPN13 and could not be used to evaluate protein expression in the frog oocytes.

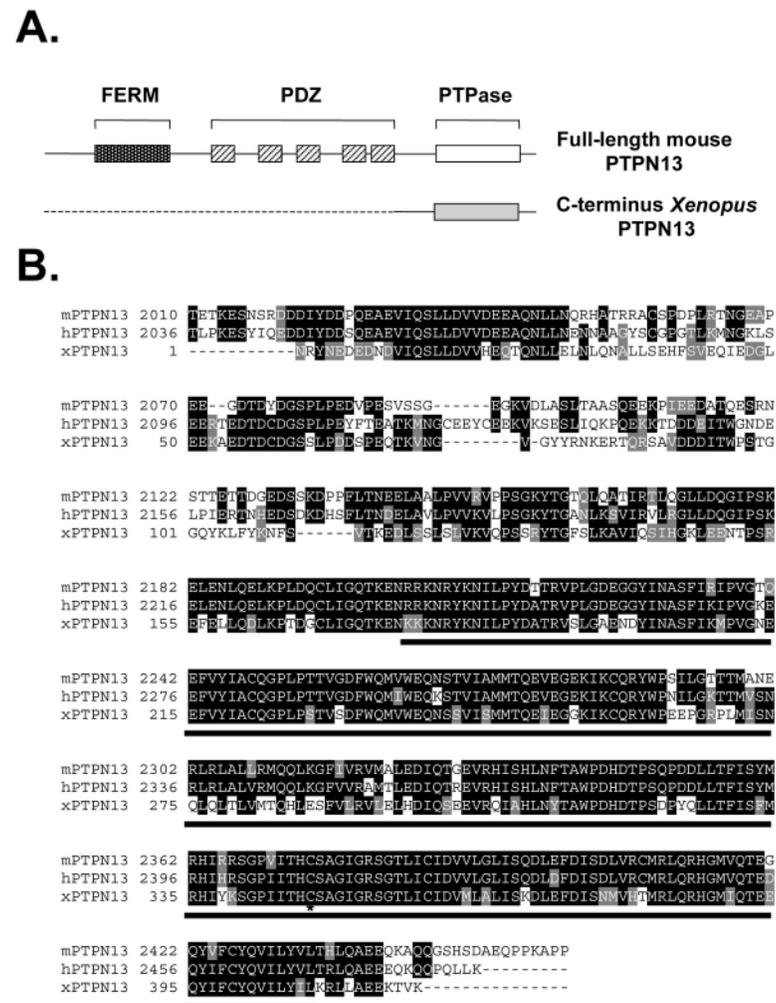


Fig. 2. Structure of PTPN13. (A) Full-length mouse PTPN13 and C-terminus *Xenopus* PTPN13. The full-length PTPN13 contains N-terminal FERM domain (also known as band 4.1 homology membrane-binding domain), five PDZ domains and a protein tyrosine phosphatase (PTPase) domain. (B) Alignment of predicted PTPN13 catalytic domain amino acid sequences of *Xenopus* (xPTPN13), and the sequences of mouse (mPTPN13) and human (hPTPN13). C terminus *Xenopus* PTPN13 is based on RT-PCR analysis, and the sequences are from databases (GenBank Accession Numbers BJ052929, BJ063819 and BJ070269). A solid line indicates the protein tyrosine phosphatase domain. An asterisk indicates the catalytically important residue in this domain that we used for mutagenesis in this report.

Endogenous PTPN13 is phosphorylated by PKA

Scanning of the mouse PTPN13 sequence for putative PKA phosphorylation sites indicated the presence of 12 potential sites located around S40, S145, T401, S493, S503, T827, S919, S929, S1073, S1233, S1641 and T2383, sites that are conserved in the human sequence. To determine whether full-length PTPN13 is phosphorylated by PKA, both cell-free and intact cell experiments were performed. Endogenous PTPN13 from HEK293 cells was immunoprecipitated using anti-PTPN13 antibodies or control IgG. Immunoprecipitated PTPN13 was subjected to *in vitro* phosphorylation using [³²P]ATP and a catalytic subunit of PKA, followed by SDS-PAGE. As shown in Fig. 4A, specific phosphorylation of the immunoprecipitated PTPN13 by PKA was evident; however, no clear shift in mobility of the band could be detected. In addition, PTPN13 was immunoprecipitated from lysates of metabolically labeled HEK293 cells incubated with or without forskolin. SDS-PAGE indicated that PTPN13 is phosphorylated under basal conditions in an intact cell. More important, forskolin treatment caused an increase in [³²P] incorporation in the protein (Fig. 4B). To determine whether the PKA-mediated phosphorylation of PTPN13 impacts its activity, PTPase activity in PTPN13 immunoprecipitates was measured after *in vitro* phosphorylation with PKA. PTPN13 activity was decreased 50% by incubation with PKA, whereas little change in PTPase activity was observed by incubation with a PKA + PKA inhibitor peptide (PKI) or heat-inactivated PKA (HI-PKA) (Fig. 4C). These results indicate that PTPN13 can be phosphorylated by PKA, and this PKA-dependent phosphorylation reduces its tyrosine phosphatase activity.

Injection of C terminus PTPN13 accelerates *Xenopus* oocyte maturation induced by progesterone

In order to define the roles of PTPN13 in *Xenopus* oocyte maturation, we constructed an HA-tagged C terminus constitutively active PTPN13 (C-PTPN13) that codes for amino acid 1917-2460 of the mouse PTPN13 sequence. Twenty nanograms of C-PTPN13 mRNA in 20 nl H₂O or 20 nl H₂O (vehicle) were injected into stage VI *Xenopus* oocytes. Twelve hours after the injection, each group of oocytes was stimulated with different concentrations of progesterone and the meiotic progression was determined by scoring GVBD. As shown in Fig. 5A-C, expression of C-PTPN13 accelerates GVBD in response to progesterone. When oocytes were stimulated with a threshold progesterone concentration (100 nM), ~50% of the oocytes injected with C-PTPN13 mRNA underwent GVBD, whereas little meiotic resumption was observed in oocytes injected with vehicle (Fig. 5A). At a higher concentration of progesterone, an acceleration of the time course of GVBD was observed (Fig. 5B,C). Finally, the effect of C-PTPN13 was concentration dependent (Fig. 5D).

These results indicated that the expression of PTPN13 potentiates the progesterone effects on *Xenopus* oocyte maturation.

C-PTPN13 expression affects the MAP kinase cascade

The expression of Mos and phosphorylation of Erk2 and Cdc2 was monitored in oocytes expressing a constitutively active PTPN13. In addition to promoting GVBD, the expression of C-PTPN13 in the presence of a threshold progesterone concentration induced the accumulation of Mos, the phosphorylation of Erk2 and the dephosphorylation of Cdc2 (Fig. 6). No effect of C-PTPN13 was observed when progesterone was omitted (data not shown). Thus, these data indicate that the expression of a constitutively active PTPN13 increases the sensitivity of the oocyte to progesterone as well as shortening the response time.

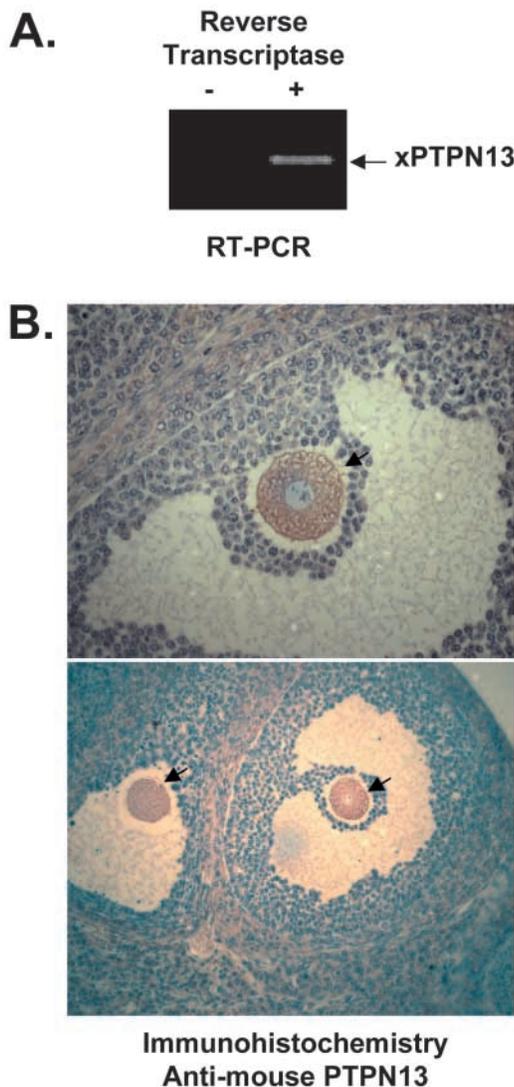


Fig. 3. PTPN13 expression in the oocytes. (A) The expression of PTPN13 mRNA in *Xenopus* oocytes was determined by RT-PCR. Minus RT was used as a negative control. (B) Immunohistochemistry of mouse ovary was performed using a polyclonal antibody against mouse PTPN13. Arrows indicate the cytoplasm of the oocytes.

Phosphatase activity of PTPN13 is necessary for synergism with progesterone

To determine whether the catalytic activity of PTPN13 is necessary for the acceleration of progesterone-induced oocyte

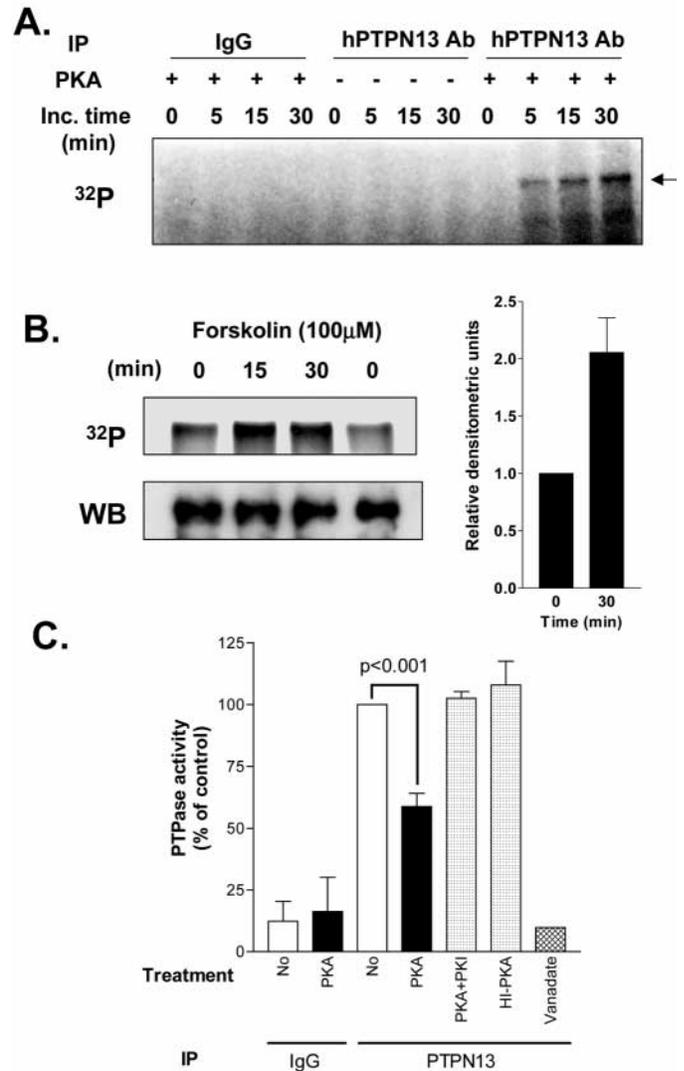


Fig. 4. Phosphorylation of PTPN13 by PKA in vitro and in vivo. (A) HEK293 cell lysate was subjected to immunoprecipitation using control IgG (lane 1-4) or anti-human PTPN13 antibodies (lane 5-12). Immunoprecipitates were then incubated with or without PKA catalytic subunits for the indicated time followed by 6% SDS-PAGE analysis. Radiolabeled PTPN13 was detected by autoradiography. (B) [³²P]-metabolic-labeled HEK293 cells were stimulated with 100 μM forskolin for the indicated time. Harvested cell lysates were then subjected to immunoprecipitation using an anti-human PTPN13 antibody or control IgG, followed by 6% SDS-PAGE analysis. Radiolabeled and total PTPN13 were detected by autoradiography (upper panel) or western blotting analysis (lower panel). Densitometric analysis was performed on four independent experiments and the average and SEM are reported in the bar graph. (C) Human PTPN13 was immunoprecipitated by anti-PTPN13 antibody or normal rabbit IgG from HEK293 cell lysates. Immunoprecipitated PTPN13 was then subjected to in vitro phosphorylation with or without PKA catalytic subunit (PKA), PKA + PKI, or heat-inactivated PKA (HI-PKA). After washing three times with lysis buffered solution, tyrosine phosphatase activity in the immunoprecipitates was measured.

maturation, Cys2374Ser point mutations were introduced in the catalytic site of mouse PTPN13. Nakai et al. have shown that the corresponding mutation of human PTPN13 resulted in an inactive PTPN13 protein (Nakai et al., 2000). When this mutant was used for injection, acceleration of progesterone-

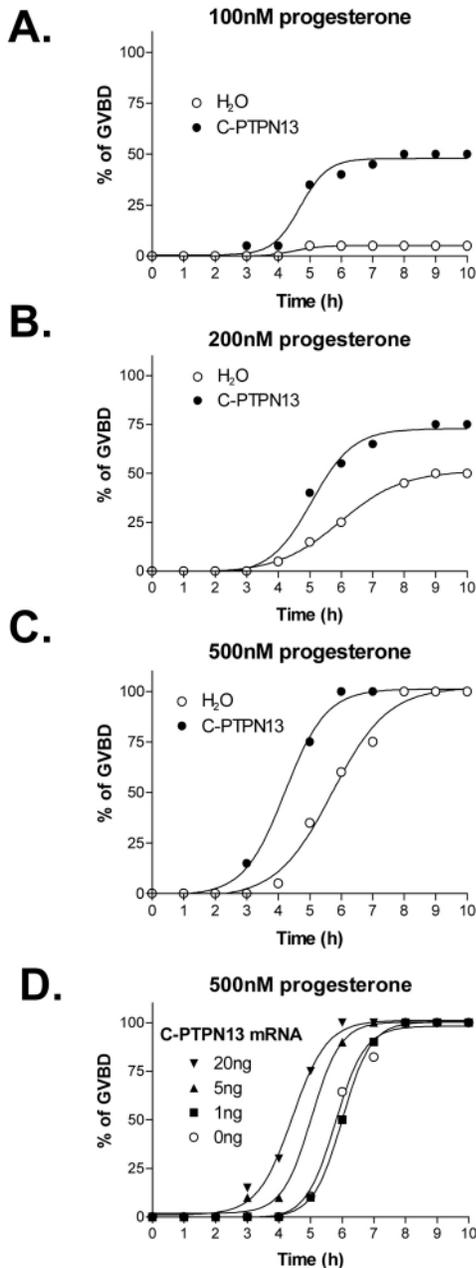


Fig. 5. Effects of C-PTPN13 injection on progesterone-inducing *Xenopus* oocyte maturation. (A-C) Twenty nanograms of HA-tagged C-PTPN13 mRNA (in 20 nl H₂O) or 20 nl of H₂O were injected into 30 *Xenopus* oocytes and 12 hours later oocytes were stimulated with the indicated concentration of progesterone. Resumption of meiosis was scored at the indicated time by the appearance of a white spot on the animal pole of the oocyte. (D) Different amounts (as indicated) of HA-tagged C-PTPN13 mRNA were injected into *Xenopus* oocytes and 12 h later oocytes were stimulated with 500 nM progesterone. Results shown are representative of the two independent experiments performed.

induced GVBD was not observed suggesting that phosphatase activity is necessary for the potentiation (Fig. 7).

Downregulation of PTPN13 causes an arrest in progesterone-induced oocyte maturation

To further clarify the role of the endogenous PTPN13 in *Xenopus* oocyte maturation, small interference RNA (siRNA) was used to downregulate the PTPN13 expression. Sequences targeted for siRNA were selected from available *Xenopus* PTPN13 sequences. Four 21 bp sequences specific for PTPN13, and with no homology to other known sequences, as well as corresponding scrambled sequences were used to prepare siRNA (Table 1). Total RNA from siRNA-injected oocytes or control oocytes were then purified followed by RT-PCR analysis using *Xenopus* PTPN13 or *Xenopus* GAPDH primers. Two out of four PTPN13 siRNAs (siRNA-1 and siRNA-2) significantly decreased the expression of the endogenous PTPN13 mRNA, albeit to a different degree, whereas GAPDH expression was not affected (Fig. 8A,C). After injecting siRNA, the *Xenopus* oocytes were stimulated by progesterone, and the number of matured oocytes was recorded. As shown in Fig. 8A,C, only those siRNAs that significantly decreased PTPN13 mRNA blocked progesterone-induced oocyte maturation. In addition, none of the scrambled siRNA decreased the expression of the PTPN13 mRNA (Fig. 8B). A complete time course and concentration dependence of the effect of the most potent siRNA and its corresponding scrambled siRNA are reported in Fig. 8C,D.

We confirmed that expression of PTPN13 is required for the activation of the signaling pathways necessary for resumption of meiosis by monitoring the effect of siRNA on MAPK and MPF. PTPN13 siRNA-1, scrambled siRNA-1 or vehicle were injected into *Xenopus* oocytes that were then incubated with or without progesterone for 8 hours. Accumulation of Mos, phosphorylation of Erk2 and dephosphorylation of Cdc2 in response to progesterone were blocked by injecting PTPN13 siRNA-1 whereas a scrambled siRNA-1 had no effect (Fig. 9).

Cdc25 is the dual-specific phosphatase that dephosphorylates and activates Cdc2. In view of the data indicating that PKA phosphorylates PTPN13, we investigated

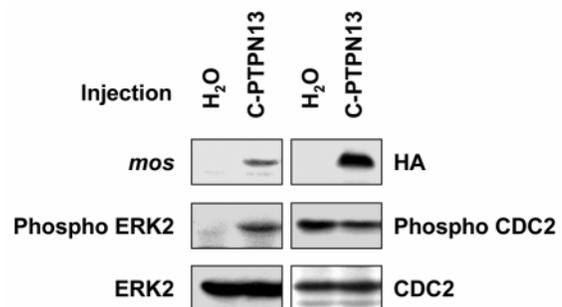


Fig. 6. Effect of C-PTPN13 injection on progesterone-dependent changes in signaling molecules in *Xenopus* oocytes. Twenty nanograms of HA-tagged C-PTPN13 mRNA (in 20 nl H₂O) or 20 nl of H₂O were injected into *Xenopus* oocytes and 12 hours later oocytes were stimulated with 100 nM progesterone for 8 hours. The oocyte lysates were subjected to 8% SDS-PAGE and the expression and phosphorylation of each protein were determined. Similar results were observed in three independent experiments.

whether this phosphatase functioned upstream of Cdc25. siRNA-1, scrambled siRNA-1 or vehicle were injected into the *Xenopus* oocytes. After 12 hours, 1 ng of mouse Cdc25B mRNA was injected into one group of oocytes, whereas another group was stimulated by 500 nM of progesterone. As shown in Fig. 10, Cdc25B injection overcame the blockade in oocyte maturation induced by PTPN13 siRNA. These data suggest that Cdc25 is downstream of PTPN13. Conversely, overexpression of PDE3A, which is sufficient to induce maturation in oocytes (Andersen et al., 2003), could not

overcome the blockade induced by downregulation of PTPN13 indicating that PTPN13 is downstream of cAMP/PKA. These data also confirm that the effect of the siRNA is specific and that the machinery involved in meiotic resumption functions properly after the siRNA blockade is bypassed.

Discussion

PTPN13 as a physiological substrate for protein kinase A in oocytes

Numerous reports have investigated the role of the cAMP signaling pathway in meiotic resumption of oocytes (Karaiskou et al., 2001; Nebreda and Ferby, 2000; Schmitt and Nebreda, 2002b); however, little information is available on the nature of the substrates of the cAMP-dependent kinase (PKA) responsible for maintaining meiotic arrest (Duckworth et al., 2002). In this report, we identify protein tyrosine phosphatase nonreceptor type 13 (PTPN13) as a PKA substrate expressed in mouse and *Xenopus* oocytes. Using constitutively active mutants or RNA interference, we provide evidence that this phosphatase is necessary for meiotic resumption, and although not sufficient to promote maturation by itself, it potentiates the progesterone effects. We propose that, in addition to the recently identified Cdc25, PTPN13 is a target for PKA phosphorylation and that it acts at a step distal to PKA in the program activated during oocyte maturation. Thus, our results suggested this phosphatase, which was previously thought to be involved in apoptosis, has an important additional role in the control of the meiotic, and perhaps mitotic, cell cycle.

Protein tyrosine phosphatase nonreceptor type 13 (PTPN13; also known as FAP1, PTPL1, PTP1E, PTP-BL or PTP-BAS) is a 250 kDa protein tyrosine phosphatase expressed in most cells (Hendriks et al., 1995). The protein is composed of a C-terminal catalytic domain and a large N terminus. The N terminus contains one FERM and five PDZ domains that are most probably involved in protein/protein interaction. This protein was identified by yeast two-hybrid screening as a molecule that interacts with the intracellular domain of Fas (APO1/CD95) (Sato et al., 1995), and several reports have explored the anti- or proapoptotic role of this protein. Tumor cell lines expressing a high level of PTPN13 protein are resistant to Fas-mediated apoptosis (Meinhold-Heerlein et al., 2001). Likewise, Jurkat cells transfected with PTPN13 cDNA become resistant to Fas-mediated apoptosis, and downregulation of PTPN13 correlates with sensitization to Fas-mediated apoptosis in interleukin 2 (IL2)-activated T cells and myelodysplastic transferred cells (MDS) (Mundle et al., 1999). However, Bompard et al. showed the expression of PTPN13 has a negative effect on the insulin-like growth factor 1 (IGF1)-induced antiapoptotic effect (Bompard et al., 2002). Therefore, further studies are required to assess the role of PTPN13 in oocyte apoptosis.

Constitutive activation of PTPN13 modulates *Xenopus* oocyte maturation

Recently, it has been shown that PTPN13 is present in the centrosomal area during interphase/metaphase and moves to the spindle mid-zone in anaphase, underscoring the possibility that PTPN13 functions during the cell cycle (Herrmann et al., 2003).

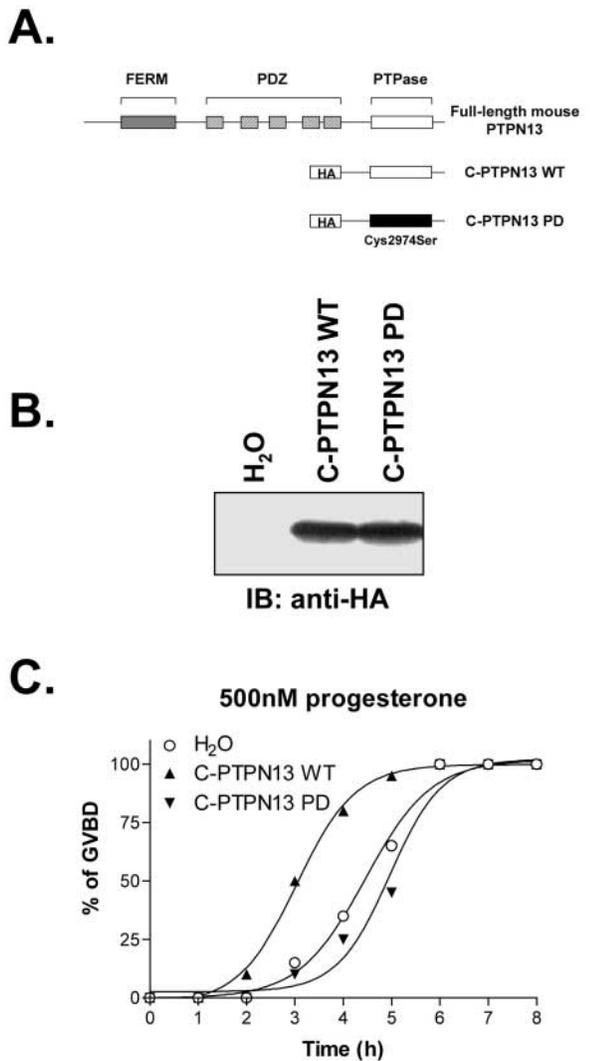


Fig. 7. Effect of phosphatase-dead C-PTPN13 injection on *Xenopus* oocyte maturation. (A) Schematic diagram of full-length mouse PTPN13 (mPTPN13), HA-tagged C-terminus mPTPN13 (C-PTPN13) or C-PTPN13 PD and HA-tagged C-terminus C2374S mutant mPTPN13 (C-PTPN13 PD), a construct where the conserved cysteine 2374 was mutated to serine. (B) mRNA coding for a HA-tagged C-PTPN13 (C-PTPN13; C-PTPN13 PD) or vehicle (H₂O) was injected into *Xenopus* oocytes. Twelve hours later, oocyte extracts were prepared and the expression of the two constructs was detected using HA antibodies. (C) Twelve hours after injection of these mRNAs, oocytes were treated with 500 nM progesterone and resumption of meiosis was scored at different times of incubation. Results shown are representative of three independent experiments.

In the functional studies we report here, we found that progesterone-induced oocyte maturation is significantly accelerated by the overexpression of C-terminal constitutively active PTPN13 (C-PTPN13). Because a construct containing only the catalytic domain was effective, we conclude that neither the PDZ domains nor the FERM domain is necessary for the meiotic promoting effects. Nevertheless, it is unclear at present whether full-length PTPN13 would affect progesterone-induced maturation because very little accumulation of PTPN13 protein was obtained even after injecting large amounts of full-length PTPN13 mRNA. Moreover, injection of the phosphatase-dead mutant indicated phosphatase activity is required for accelerating oocyte maturation induced by progesterone. These findings suggest that dephosphorylation of an unknown PTPN13 substrate transduce signal(s) is important for meiotic resumption.

It is well established that the dual-specific phosphatase

Cdc25 is involved in oocyte maturation by controlling the phosphorylation state of one of the components of the maturation promoting factor (MPF), Cdc2 (Gautier et al., 1991; Kumagai and Dunphy, 1991; Lincoln et al., 2002; Rime et al., 1994). Therefore, one possibility is that PTPN13 substitutes for Cdc25 by dephosphorylating Tyr15 on Cdc2. However, in the absence of progesterone, little dephosphorylation of Cdc2 was observed after overexpression of C-PTPN13 (data not shown). In addition, preliminary experiments incubating immunoprecipitated PTPN13 with phosphorylated Cdc2 did not show appreciable dephosphorylation (data not shown). These findings suggest that C-PTPN13 does not directly dephosphorylate Cdc2 and that other intermediates are dephosphorylated on tyrosine residues. Identification of these physiological substrates of PTPN13 should clarify the role of this phosphatase in meiotic control. At least two candidate substrates for PTPN13, ephrin B and I κ B, already have been

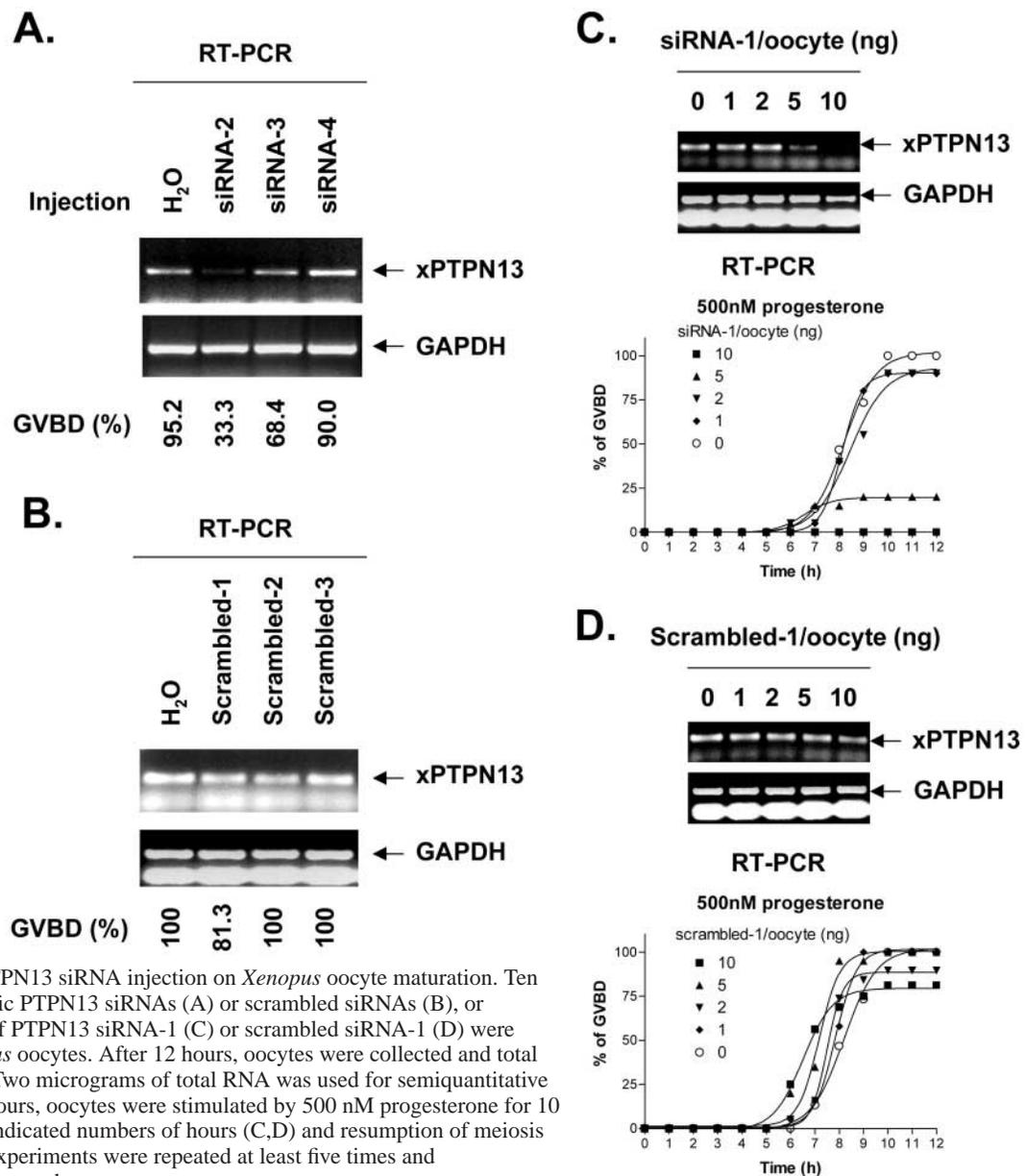


Fig. 8. Effects of PTPN13 siRNA injection on *Xenopus* oocyte maturation. Ten nanograms of specific PTPN13 siRNAs (A) or scrambled siRNAs (B), or indicated amounts of PTPN13 siRNA-1 (C) or scrambled siRNA-1 (D) were injected into *Xenopus* oocytes. After 12 hours, oocytes were collected and total RNA was isolated. Two micrograms of total RNA was used for semiquantitative RT-PCR. After 12 hours, oocytes were stimulated by 500 nM progesterone for 10 hours (A,B) or the indicated numbers of hours (C,D) and resumption of meiosis was scored. These experiments were repeated at least five times and representative results are shown.

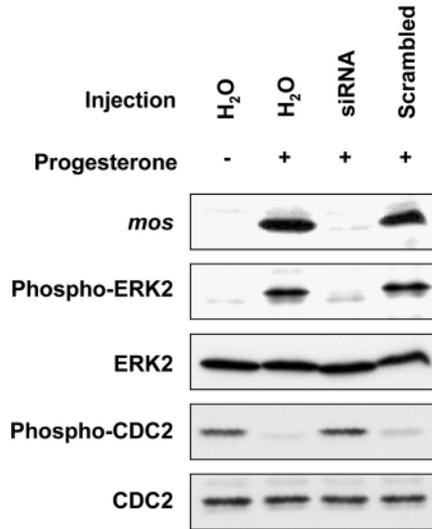


Fig. 9. Effects of PTPN13 siRNA injection on progesterone-dependent changes in signaling molecules in *Xenopus* oocytes. Ten nanograms of PTPN13 siRNA-1 or scrambled siRNA-1 were injected into *Xenopus* oocytes. After 12 hours, oocytes were stimulated with 500 nM progesterone for 8 hours. The oocyte lysates were subjected to 8% SDS-PAGE, and the expression and phosphorylation of each protein were detected by western blotting analysis.

identified (Nakai et al., 2000; Palmer et al., 2002). It is well established that I κ B is involved in NF κ B signaling, but the significance of the phosphorylation of I κ B in NF κ B regulation is unclear, nor is information available on the role of NF κ B in meiosis. Ephrin B was identified as a membrane-bound ligand for the Eph receptor, and not only the Eph receptor but also the ephrin B can transduce intracellular signals. Cowan and Henkemeyer reported that several cytoskeletal regulators were recruited by the tyrosine phosphorylation of ephrin B (Cowan and Henkemeyer, 2002); however, the expression and function of ephrin B in oocytes is unknown.

PTPN13 is required for progesterone-induced *Xenopus* oocyte meiotic maturation

RNA interference (RNAi) demonstrated that downregulation of the PTPN13 mRNA produces a meiotic blockade. Our efforts to generate antibodies specific for the *Xenopus* PTPN13 have been unsuccessful and the available antibodies against the human or mouse PTPN13 do not cross-react with the *Xenopus* protein. Therefore, we have been unable to verify that the PTPN13 protein is also downregulated by siRNA treatment. However, several findings suggest that the siRNA treatment is indeed specific. The ability to produce meiotic blockades varied among the different siRNA produced and there was a proportionality between decreased mRNA and inhibition of maturation. Furthermore, all scrambled siRNAs were ineffective in inducing maturation. That the effect of the siRNA is selective is confirmed by the observation that expression of several proteins involved in meiotic control, including Cdc2 and Erk, is not affected by the siRNA. Finally, the siRNA blockade could be overcome by the expression of Cdc25, indicating that the arrest is not due to nonspecific toxic effects and that direct activation MPF causes resumption of meiosis.

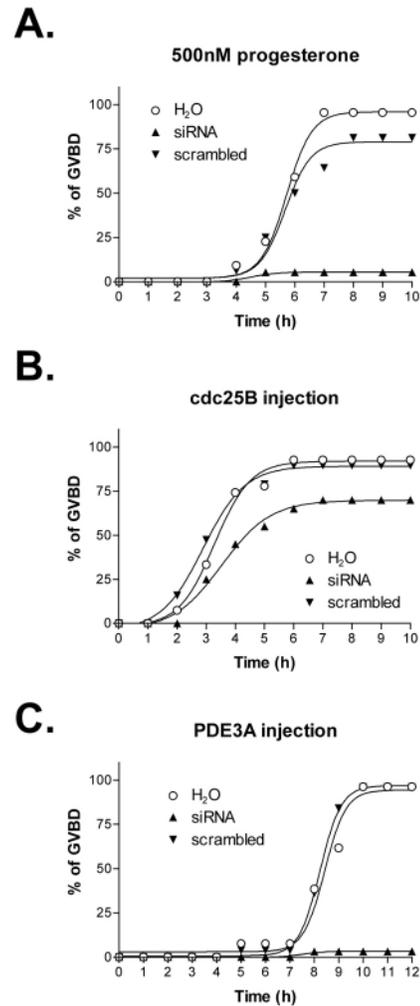


Fig. 10. Cdc25B overexpression, but not PDE3A overexpression, overcomes the meiotic block induced by PTPN13 siRNA. Ten nanograms of siRNA-1, scrambled siRNA-1 or vehicle were injected into *Xenopus* oocytes. After 12 hours, oocytes were stimulated with (A) 500 nM progesterone, or injected with (B) 1 ng of Cdc25B mRNA or (C) 20 ng of PDE3A mRNA to induce oocyte maturation, and resumption of meiosis was scored. Results shown are representative of the two experiments performed.

The whole of these data suggest that downregulation of PTPN13 interferes with the signaling pathway activated by progesterone and therefore blocks meiotic resumption.

Based on the above findings, we propose that PTPN13 plays a role in the control of the meiotic cell cycle. The observation that the mammalian PTPN13 localizes in the centrosomal area or on the spindle of dividing HeLa cells is consistent with this hypothesis (Herrmann et al., 2003). In the report involving PTPN13 in mitosis, it was proposed that PTPN13 also may function during anaphase or more likely during cytokinesis. An important difference between our data and the previous report is that in our case the phosphatase activity is necessary for the effect on meiosis, whereas the phosphatase inactive mutant was still effective in disrupting cytokinesis in HeLa cells. Without excluding a possible effect later in the cell cycle, our data suggest that PTPN13 may have an additional function earlier

during the prophase/metaphase or G2/M transition. The experiment manipulating cAMP levels by PDE overexpression and Cdc25 rescue suggest that PTPN13 acts at a step distal to cAMP and PKA, but upstream of Cdc25. Taken together with our finding that PTPN13 is inactivated by PKA phosphorylation in vitro, we propose that the inactivation of PKA that follows the progesterone-induced decrease in cAMP causes PTPN13 activation; this in turn causes dephosphorylation of substrates critical for MPF activation. This may not be the primary event, however, because PTPN13 does not function by itself.

Recently, it has been proposed that microtubules play a role in nuclear envelope breakdown (Aitchison and Rout, 2002) and that the FERM domain in PTPN13 has been shown to be necessary for microtubule interaction (Herrmann *et al.*, 2003). Thus, it is possible that PTPN13 localizes to the microtubules and controls the phosphorylation of a microtubule regulatory protein required for nuclear envelope breakdown. We cannot exclude, however, the possibility that the downregulation of PTPN13 by siRNA also disrupts macromolecular complexes crucial for the progression of the cell cycle.

In conclusion, our findings strongly suggest that PTPN13 is a phosphatase that functions downstream of cAMP signaling and plays an essential role in controlling meiotic resumption in *Xenopus* oocytes. Identification of the tyrosine substrate dephosphorylated by PTPN13 will help to uncover novel regulatory circuits involved in meiotic, and perhaps mitotic, control.

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