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Paxillin and embryonic PolyAdenylation Binding Protein (ePABP) engage to regulate androgen-dependent *Xenopus laevis* oocyte maturation - a model of kinase-dependent regulation of protein expression

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Abstract

Steroid-triggered *Xenopus laevis* oocyte maturation is an elegant physiologic model of nongenomic steroid signaling, as it proceeds completely independent of transcription. We previously demonstrated that androgens are the main physiologic stimulator of oocyte maturation in *Xenopus* oocytes, and that the adaptor protein paxillin plays a crucial role in mediating this process through a positive feedback loop in which paxillin first enhances Mos protein translation, ensued by Erk2 activation and Erk-dependent phosphorylation of paxillin on serine residues. Phosphoserine-paxillin then further augments Mos protein translation and downstream Erk2 activation, resulting in meiotic progression. We hypothesized that paxillin enhances Mos translation by interacting with embryonic PolyAdenylation Binding Protein (ePABP) on polyadenylated *Mos* mRNA. Knockdown of ePABP phenocopied paxillin knockdown, with reduced Mos protein expression, Erk2 and Cdk1 activation, as well as oocyte maturation. In both *Xenopus* oocytes and mammalian cells (HEK-293), paxillin and ePABP constitutively interacted. Testosterone (*Xenopus*) or EGF (HEK-293) augmented ePABP-paxillin binding, as well as ePABP binding to *Mos* mRNA (*Xenopus*), in an Erk-dependent fashion. Thus, ePABP and paxillin work together in an Erk-dependent fashion to enhance Mos protein translation and promote oocyte maturation.

Keywords

ePABP; paxillin; androgen; Mos; Erk; oocyte; maturation

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Introduction

Over the past several decades, research has revealed that steroids regulate both nuclear as well as extranuclear signals through the same classical steroid hormone receptors located either inside or outside of the nucleus, respectively. Extranuclear, or nongenomic, steroid responses involve modulation of G protein-coupled as well as kinase signaling at or near the plasma membrane, and appear to be just as important as the genomic actions of their nuclear counterparts (reviewed in (Hammes and Davis, 2015)). The dissection of whether a specific biologic outcome is controlled by nongenomic as opposed to genomic steroid signaling can be difficult, especially since both pathways are intricately linked (Hammes and Davis, 2015). Interestingly, *Xenopus laevis* oocyte maturation (meiotic progression) is triggered by many steroids *in-vitro* but primarily by androgens *in-vivo*, and is entirely controlled on a post-transcriptional level (Lutz et al., 2001; Lutz et al., 2003; White et al., 2005). *Xenopus laevis* oocyte maturation therefore serves as an elegant physiologic model to exclusively study nongenomic androgen receptor signaling.

Normal oocyte maturation relies on the precise regulation and timing of a multitude of intracellular signaling events (Whitaker, 1996). After duplication of the DNA early in embryogenesis, *Xenopus laevis* oocytes are held in the first meiotic arrest (prophase I) by constitutive G $\beta\gamma$ - and G α_s -mediated stimulation of cAMP production (Gallo et al., 1995; Guzman et al., 2005; Lutz et al., 2000; Sheng et al., 2005), which is at least in part mediated through G protein-coupled receptor-3 (GPR3) signaling (Deng et al., 2008; Mehlmann et al., 2004; Rios-Cardona et al., 2008). The powerful surge of gonadotropin release and subsequent sex steroid production then suppresses G protein signaling and reduces intracellular cAMP levels, leading to increased polyadenylation of *Mos* mRNA and translation of Mos protein (the germ cell equivalent of Raf). Mos then promotes activation of MEK and then Erk2, which feeds back in a positive fashion to promote more Mos protein production and Erk signaling, ultimately leading to activation of cyclin-dependent kinase 1 (Cdk1) and meiotic resumption. Meiosis is halted again at the second meiotic arrest (metaphase II) until fertilization.

We have previously shown that the versatile adaptor protein paxillin is a critical regulator of sex-steroid triggered meiotic progression in *Xenopus laevis* oocytes by functioning as a potent enhancer of the aforementioned positive feedback loop. We have shown that paxillin first acts downstream of androgen-triggered *Mos* mRNA polyadenylation to promote Mos protein expression and subsequent Erk2 activation (Rasar et al., 2006). Erk2 then regulates phosphorylation of *Xenopus* paxillin at serine residues 107 and 111, after which phosphoserine-paxillin further enhances Mos protein production and Erk2 activation, ultimately resulting in meiotic progression. In fact, replacement of endogenous paxillin with a mutated paxillin protein containing alanine mutations at serines 107 and 111 no longer supported oocyte maturation, confirming the importance of these residues (Rasar et al., 2006).

Interestingly, the ability of paxillin to modulate extranuclear androgen steroid signaling through Erk is not restricted to frog oocytes. In human prostate cancer cells, paxillin

regulates Erk signaling that is initiated at the plasma membrane through activation of the EGF receptor either directly by EGF or indirectly through androgen-triggered release of EGF receptor ligands. Specifically, as seen in frog oocytes, paxillin is required for Raf-mediated activation of Erk1/2 in prostate cancer cells, after which Erk1/2 mediates phosphorylation of human paxillin on serine residues. Phosphoserine-paxillin then travels to the nucleus and acts as a co-activator of both androgen- and growth factor-mediated gene transcription, ultimately promoting cell proliferation (Sen et al., 2010; Sen et al., 2012).

Additionally, in primary mouse granulosa cells, we have shown that paxillin mediates androgen-dependent FSH receptor protein expression exclusively on a post-transcriptional, translational level (Sen et al., 2014), reminiscent of its effects on Mos protein expression in *Xenopus laevis* oocyte maturation (Rasar et al., 2006).

How might paxillin regulate protein expression in *Xenopus laevis* oocytes and mammalian granulosa cells? Interestingly, paxillin can interact with the RNA-binding protein PABP1 (PolyAdenylation Binding Protein 1) in NIH3T3 fibroblasts (Woods et al., 2002). Abolishing this interaction resulted in nuclear trapping of PABP1, as well as impaired cell spreading and migration (Woods et al., 2005). It was suggested by the authors that paxillin may act as a carrier of RNA-binding proteins such as PABP1, and thus by inference as a carrier of mRNAs, possibly targeting mRNA for translation (Woods et al., 2002). Details regarding which signals regulate these interactions and how they might regulate biologically important processes are still not known.

We proposed that paxillin might interact with RNA-binding proteins such as the PABP family in *Xenopus laevis* oocytes, thus enhancing translation of polyadenylated *Mos* mRNA to promote Mos protein expression and subsequent oocyte maturation. We further proposed that Erk-dependent phosphorylation of paxillin might be an important regulator of its interaction with PABP. In fact, knockdown of embryonic PABP (ePABP) in *Xenopus laevis* oocytes phenocopied the effects of paxillin knockdown, with normal steroid-triggered *Mos* mRNA polyadenylation but reduced Mos protein expression, Erk2 and Cdk1 activation, as well as germinal vesicle breakdown. Furthermore, paxillin-ePABP interactions were dependent on Erk-mediated phosphorylation of serines 107 and 111 on paxillin, suggesting that Erk signaling is a critical regulator of ePABP-paxillin-mediated translation.

Materials and methods

Antibodies

Anti-Mos (sc-86) was obtained from Santa Cruz Biotechnology, mouse anti-HA (#901501) from BioLegend, mouse anti-FLAG (F3165) from Sigma-Aldrich, rabbit anti-PABP1 (#4992), rabbit anti-phospho-p44/42 MAPK (#9101) and anti-total p44/42 MAPK (#9102) from Cell Signaling Technologies. For initial experiments determining ePABP and PABP1 expression, a polyclonal antibody directed against the C-terminal tail of ePABP was kindly provided by Joan Steitz (Voeltz et al., 2001). Rabbit polyclonal antibodies directed to *Xenopus* ePABP amino acids 449-462, were used for all other experiments (generated by Biosynthesis Inc., Lewisville, TX). The phospho-Cdk1 antibody was from Cell Signaling Technologies (#9111) and the total-Cdk1 antibody from Thermofisher Scientific (#33-1800).

Plasmid construction

cDNAs encoding *Xenopus* paxillin, *Xenopus* Mos, and the 3'UTR of *Mos* mRNA were cloned into pcDNA3.1(+) (Invitrogen) for eukaryotic expression and pGEM-HE for *Xenopus laevis* oocyte expression (Rasar et al., 2006). The primers used to clone *Xenopus* paxillin and *Xenopus* Mos included a sequence to incorporate an amino-terminal HA tag. Serine mutated versions of paxillin were cloned using site-directed mutagenesis to convert serine residues to alanine.

cDNAs encoding *Xenopus* ePABP (Mammalian Gene Collection ID: BC080020) and PABP1 (Mammalian Gene Collection ID: BC052100) were cloned into pGEM-HE for *Xenopus laevis* oocyte expression or pcDNA3.1(+) for eukaryotic expression. The primers included a sequence to incorporate an amino-terminal FLAG tag.

Oocyte preparation

Oocytes were harvested from female *Xenopus laevis* (NASCO) and treated as previously described (Lutz et al., 2000). Briefly, follicular cells were removed by incubation of the ovaries in 0.8 mg/ml collagenase A (Roche) in modified Barth's solution (MBSH) without Ca^{2+} for 3 hours. Oocytes were then washed and incubated overnight at 16°C in MBSH containing 1mg/ml Ficoll, 1mg/ml bovine serum albumin, 100U/ml penicillin and 0.1mg/ml streptomycin. Testosterone-induced maturation assays (10–1000nM) were performed on stage V/VI oocytes from each preparation to determine sensitivity to the steroid. Maturation was scored as germinal vesicle breakdown, which was visualized as a white spot on the animal pole of the oocyte. Twenty oocytes were used for each data point in all experiments.

Cell culture/transfection

HEK-293 cells (CRL1573, ATCC) were cultured in 1x high glucose Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific), supplemented with 10% FBS (Seradigm), 100 units/ml penicillin and streptomycin (Thermo Fisher Scientific) at 37°C, 5% CO_2 . For transient transfections, cDNA of *Xenopus laevis* paxillin and ePABP were premixed at a 1:3 ratio (total cDNA 2µg per well of 6-well plate) with Xtreme Gene 9 (Roche) and medium (DMEM) according to manufacturer's instructions, added to 50% confluent cells and supplemented with regular medium without antibiotics as above. Experiments were carried out 72 hours after transfection following an overnight incubation in serum-free DMEM.

RNA synthesis and injections

The pGEM HE constructs were linearized with NheI for PABP1 and paxillin, PstI for ePABP, and XbaI for *Mos* 3'UTR reporter. Capped cRNA was transcribed *in vitro* with the mMessage machine T7 transcription kit according to manufacturer's protocol (Ambion). RNA was suspended in injection buffer (10mM HEPES, pH 7.4) and stage V/VI oocytes were injected with 10–20ng cRNA using a Drummond automatic injector. The sequences of the sense and antisense ePABP oligonucleotides incorporated the start codon and were C*A*C*TGGCAAACATGAATGCAAC*C*G*G and C*C*G*GTTGCATTCATGTTTGCCA*G*T*G, respectively, with phosphoro-thioated bonds indicated by asterisks. Oocytes were injected with 25ng oligonucleotides in each

experiment. For the rescue studies, FLAG-tagged ePABP was re-cloned so that nucleotides at wobble positions in the first 6 codons were changed, thus preserving the amino acid sequence but preventing antisense oligonucleotides from binding. In each rescue experiment, oocytes were co-injected with 25ng oligonucleotide and 20ng rescue cRNA. Following injections, oocytes were incubated 36–48 hours prior to starting experiments.

Steroid maturation assay

Maturation assays were conducted by incubating 20–50 oocytes per condition with the indicated concentration of testosterone (Steraloids) or ethanol in MBSH containing 1mg/ml Ficoll, 1mg/ml bovine serum albumin, 100U/ml penicillin and 0.1mg/ml streptomycin for 16 hours, at which time oocytes were scored for germinal vesicle breakdown (GVBD; visualization of a white spot on the animal pole). Dilutions were performed such that ethanol concentration was held at 0.1%.

Western blots

Xenopus laevis oocytes—Oocytes were incubated with steroid or vehicle, permeabilized in 10 μ l/oocyte RIPA lysis buffer (Santa Cruz Biotechnology), supplemented with HALT phosphatase and protease inhibitor (Thermo Scientific), and spun twice at 14,000 $\times$ g for 10 minutes to remove yolk and other debris. The cleared supernatants were then mixed 1:1 with 2x SDS buffer plus 5% 2- β -mercaptoethanol (Sigma-Aldrich). The equivalent of 0.25–1 oocytes was loaded in each lane for SDS-PAGE, transferred to PVDF membranes (BIORAD), blocked in 5% milk-TBST for 2 hours, then incubated with primary antibody overnight at 4°C (1:5000 for anti-FLAG and anti-HA, 1:1000 for anti-Mos, 1:2000 for anti-phospho/anti-total-p42 Erk 2, 1:8000 for anti-ePABP). For Cdk1 blots, antibodies were used at 1:1000 dilution in 5% BSA-TBST. Membranes were subsequently incubated with the respective HRP-conjugated secondary antibody for 1 hour, and signal detected with Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare).

HEK-293 cells—Following overnight incubation in serum-free medium, cells were incubated with 20ng/ml EGF (in DMEM, no serum) as indicated. Medium was then aspirated and cells were permeabilized in 200 μ l/well RIPA buffer, supplemented with HALT protease and phosphatase inhibitor as above. 5–10 μ l of the cleared cell lysates were then subjected to Western blotting as described above for *Xenopus laevis* oocytes.

Mos poly-adenylation assay

Polyadenylation of *Mos* cRNA was determined as described (Howard et al., 1999; Rasar et al., 2006). Briefly, 36 hours after oligonucleotide injection, each oocyte was injected with 10 ng of cRNA encoding the terminal 280 nucleotides of the 3'UTR of *Mos* mRNA and oocytes were allowed to recover for 2 hours. Oocytes were then stimulated with testosterone and permeabilized every 4 hours. RNA was extracted using 1ml Trizol (Invitrogen), and 20 μ g of total RNA was run on a 2.2% agarose formaldehyde gel in 1x MOPS buffer for ~10 hours at 60V. RNA was transferred to a Nytran SuPerCharge nylon membrane (Schleicher & Schuell) by downward transfer in 20xSSC (Schleicher & Schuell) and UV crosslinked. Membranes were pre-hybridized in ExpressHyb (Clontech) at 68°C for 30 minutes, then probed with [³²P]-dCTP labeled *Mos* 3'UTR DNA (Rediprime II, Amersham Biosciences)

for 1 hour, washed twice in 2x SSC, 0.05% SDS, then twice in 0.1x SSC, 0.1% SDS and subsequently subjected to autoradiography.

Protein/protein immunoprecipitation

Xenopus laevis oocytes—Twenty five oocytes/condition were injected with 10ng of HA-tagged paxillin cRNA and treated as indicated 36–48h after injection. Oocytes were then permeabilized in 250µl 1x RIPA (Santa Cruz Biotechnology), supplemented with HALT protease plus phosphatase inhibitor (Thermo Scientific). The cleared supernatants were first incubated with 1:50 ePABP pre-immune serum (rabbit serum obtained prior to immunization to ePABP, Biosynthesis Inc., Lewisville TX) as well as 25 µl of pre-washed protein G PLUS agarose beads (Santa Cruz Biotechnology) for a total of 1h. This was followed by an overnight incubation with 1:50 anti-ePABP plus 25 µl of protein G PLUS agarose beads at 4°C. Beads were then washed 5x with 200µl 1x RIPA and re-suspended in 2x SDS buffer with 2-β-mercaptoethanol.

HEK-293 cells—One well of 50% confluent HEK-293 cells/condition was transfected with *Xenopus* paxillin (HA-tagged) and ePABP (FLAG-tagged) as described above. Experiments were carried out as indicated 72h after transfection following overnight incubation in serum-free DMEM. One well of a 6 well-plate was subsequently permeabilized in 200µl RIPA and subjected to ePABP immunoprecipitation as described above for *Xenopus laevis* oocytes.

Protein/RNA immunoprecipitation

Fifty oocytes/condition (in triplicate) were injected with 20ng cRNA for FLAG-tagged ePABP and treated as indicated 36–48 hours after injection. Oocytes were then lysed in 250µl 1x NET (50 mM Tris-pH 7.6, 150 mM NaCl, 1mM EDTA, 0.1% NP40, HALT protease plus phosphatase inhibitor [Thermo Scientific], as well as SUPERase-In RNase inhibitor [100U/ml, Ambion]). Lysates were centrifuged at 14,000g for 10 minutes twice and then pre-cleared with 1:50 mouse IgG and 25µl pre-washed protein G PLUS agarose beads for a total of 1 hour. Pre-cleared lysates were then incubated with 1:50 anti-FLAG antibody and 25µl beads for a total of 4 hours at 4°C. Beads were washed 5x with 200µl 1x NET, then re-suspended in 2x SDS buffer (without 2-β-mercaptoethanol if RNA precipitation was to be performed).

Trizol extraction was used to purify RNA from the beads and is briefly described. First, approximately 10% of the sample was saved for Western blot (2-β-mercaptoethanol was subsequently added). To the rest of the sample, 500µl Trizol (Invitrogen) and 200µl chloroform (MP Biomedicals) were added and RNA was extracted according to manufacturer's instructions. Before isopropanol (Sigma-Aldrich) precipitation, 30µg glycogen (Thermo Scientific) was added to the sample to aid RNA recovery. RNA was re-suspended in 25µl DEPC-treated water (Sigma-Aldrich).

Quantitative PCR

Quantitative PCR was performed using the TaqMan® RNA-to-Ct™ 1-Step Kit (Applied Biosystems). Primers were designed and ordered through the Applied Biosystems Custom TaqMan Gene Expression Assay Service. Sequences of primers for ePABP, Mos and

GAPDH were as follows: ePABP - forward 5'-GATGCCTGGTCCTCTCCTT-3', and reverse 5'-CGGCATGGCAGACAAGAAATAATTT-3', Mos - forward 5'-ACCAGCGCATATACGGGC-3' and reverse 5'-GGAGTGGAAAGCCTGTGATGCTGCCCTGGGCATG-3', GAPDH - forward 5'-CTGCCACCCAGAAGACTGT-3', and reverse 5'-GTTGAGGCGGGAATAATGTTCTGA-3'. Reactions were assembled according to manufacturer's instructions. Briefly, 8.5µl of RNA was used for each 20µl reaction using the following cycling parameters: 50°C 2 min, 95°C 10 min followed by 40 cycles of 95°C 15 sec and 60°C 60 sec using the StepOnePlus™ Real-Time PCR System (Applied Biosystems).

Statistical Analysis

Data analysis was carried out using Microsoft Excel for Mac (version 15.24). Statistics were performed using unpaired student's t-tests (GraphPad Prism, version 7). A p-value of <0.05 was considered statistically significant.

Results

ePABP but not PABP1 is expressed in *Xenopus laevis* stage V/VI oocytes

PABPs are regulators of RNA stability and degradation, and also play important roles in initiation of translation (reviewed in (Bag and Bhattacharjee, 2010)). In *Xenopus laevis* oocytes and embryos, both embryonic PABP (ePABP) and PABP1 are expressed, though at different times. Whereas ePABP is expressed during oogenesis and very early embryogenesis, PABP1 dominates thereafter (Cosson et al., 2002; Voeltz et al., 2001). We first sought to confirm ePABP expression in late stage (V and VI) *Xenopus laevis* oocytes. Injected cRNA encoding either ePABP or PABP1 served as positive controls and were compared to mock injected oocytes. As reported previously, we detected endogenous expression of ePABP but not PABP1 in stage V/VI *Xenopus* oocyte lysates (Fig. 1A).

ePABP knockdown phenocopies paxillin knockdown in *Xenopus laevis* oocytes

Since we proposed that ePABP functions with paxillin to regulate oocyte maturation, we first examined the physiologic importance of ePABP by determining whether, as seen with paxillin, reduction of ePABP expression would attenuate testosterone-induced oocyte maturation. Endogenous ePABP expression was reduced by injection of phosphoro-thioated antisense oligonucleotides directed against *ePABP* mRNA; similarly, modified sense oligonucleotides were used as control conditions. Successful knockdown of ePABP was confirmed by quantitative PCR (Fig. 1B, >80% reduction in *ePABP* mRNA expression, $p<0.001$) and Western blot (Fig. 1C, approximately 50% reduction in ePABP protein expression). Testosterone-induced oocyte maturation was evaluated starting 36–48h post-injection. Notably, injection of antisense (AS) ePABP significantly reduced the ability of testosterone to promote oocyte maturation (Fig. 1D). Co-injection of FLAG-tagged cRNA encoding wild-type (wt) FLAG-tagged ePABP together with the antisense oligonucleotides reversed the suppressive effects of ePABP knockdown on oocyte maturation, confirming the specificity of the knockdown (Fig. 1D). Since each batch of oocytes had different EC₅₀s and maximal testosterone responses, we could not average together the dose response curves

from different sets of oocytes, although, as seen in Fig. 1D, supplementary Fig. 1, and supplementary Fig. 3A, similar reductions in oocyte maturation response curves were seen across all experiments. Importantly, by averaging four experiments in which the same saturating concentrations of testosterone were used (100 nM), we saw a statistically significant reduction in oocyte maturation with ePABP knockdown (average reduction by 51%, n=4 experiments, $p<0.05$, Fig. 1E).

Since ePABP is an RNA binding protein, we considered that its absence might affect overall RNA stability and thereby interfere with protein translation and subsequent oocyte maturation. However, we find that in the absence of ePABP the overall RNA yields after RNA extractions, and in particular quantities of *Mos* and *GAPDH* mRNAs were not altered (data not shown) suggesting that overall RNA stability is not dependent on the presence of ePABP.

After confirming the importance of ePABP in the process of steroid-triggered oocyte maturation, we next examined where in the maturation-promoting pathway ePABP was functioning. We had previously shown that paxillin first regulates oocyte maturation downstream of *Mos* mRNA polyadenylation but upstream *Mos* protein expression (Rasar et al., 2006). Therefore, we determined whether ePABP regulated oocyte maturation in a similar fashion. Indeed, as seen with paxillin knockdown, reduction of ePABP expression by oligonucleotides directed against *ePABP* mRNA did not significantly alter testosterone-induced polyadenylation of injected *Mos* cRNA fragments (Fig. 2A). In contrast, as seen with paxillin knockdown, ePABP knockdown attenuated testosterone-dependent downstream Erk2 phosphorylation as well as oocyte maturation (Fig. 2B, C). The negative effects ePABP knockdown on Erk2 phosphorylation, as well as *Mos* protein expression were confirmed in a dose response and ePABP rescue experiment with similar results (Fig. 3). Overall, the reductions in *Mos* protein expression and Erk2 activation by ePABP knockdown were less dramatic compared to paxillin knockdown (Rasar et al., 2006). For instance, in Fig. 3, the reduction of Erk2 phosphorylation was primarily observed with submaximal testosterone concentrations (Fig. 3, 30 nM testosterone). As seen in the maturation assay, co-injection of FLAG-tagged cRNA encoding wild-type (wt) FLAG-tagged ePABP together with the antisense oligonucleotides reversed the suppressive effects of ePABP knockdown on *Mos* expression and Erk phosphorylation (Fig. 3), confirming the specificity of the effects of ePABP knockdown. As mentioned, due to batch-to-batch variability in testosterone responses (both dose responses and timed responses), we could not average together independent experiments; however, each study was performed multiple times with very similar results. In addition to figures 2 and 3, experiments in which we detected *Mos* and/or phosphorylated Erk are shown in supplementary figures 2 and 3.

Finally, we examined the effects of ePABP knockdown on Cdk1 activation (determined by detection of Cdk1 dephosphorylation). As seen with oocyte maturation and Erk2 activation in the same experiment, testosterone-induced dephosphorylation of Cdk1 was reduced upon knockdown of ePABP expression (supplementary Fig. 3D).

Note that, contrary to *Mos* protein, expression of Erk2 and Cdk1 proteins was not affected by ePABP knockdown; their overall protein levels remained the same throughout all

conditions tested (Fig. 2, 3, supplementary Fig. 2B, 3C, 3D), just as we observed previously with paxillin knockdown (Rasar et al., 2006). Thus, ePABP knockdown does not seem to be affecting overall protein expression and perhaps is more specific to maturation-dependent proteins that are regulated by CPEP, maskin, and other molecules (Lin et al., 2010).

In *Xenopus* oocytes, testosterone enhances ePABP binding to *Mos* mRNA

Having shown that ePABP functions downstream of *Mos* mRNA polyadenylation but upstream of *Mos* protein expression, we next examined how ePABP might be functioning within this pathway. PABPs preferably bind to and stabilize poly-adenylated mRNAs (Kim and Richter, 2007), though not exclusively. They also protect nascent mRNA from deadenylation via binding to AU-rich elements, as shown for ePABP (Voeltz et al., 2001). We therefore predicted that ePABP would constitutively bind to *Mos* mRNA and that this interaction would be enhanced upon treatment with testosterone and subsequent lengthening of the polyadenylated tails. To test this hypothesis, we immunoprecipitated endogenous ePABP with the polyclonal antiserum against ePABP (pre-immune serum served as a control) and quantified *Mos* mRNA by quantitative PCR. We noted a 5–10fold enrichment of *Mos* mRNA in extracts precipitated with anti-ePABP versus control antibodies (not shown), confirming the presence of a constitutive interaction between ePABP and *Mos* mRNA. To amplify this signal, we overexpressed FLAG-tagged ePABP through injection of cRNA encoding this protein into oocytes, followed by a six-hour stimulation with 1000nM testosterone in 0.1% ethanol or 0.1% ethanol alone and precipitation of FLAG-ePABP with an anti-FLAG antibody (Fig. 4A). RNA was then extracted from precipitates, and *Mos* mRNA quantified by quantitative PCR. Using this over-expression system, a 60-fold enrichment of *Mos* mRNA was noted with anti-FLAG antibody precipitation compared to control mouse IgG precipitation (data not shown) in vehicle-treated oocytes. Importantly, testosterone treatment significantly increased the interaction between FLAG-ePABP and *Mos* mRNA (Fig. 4B). Together, these findings imply that ePABP forms a constitutive complex with *Mos* mRNA in immature oocytes. Stimulation of polyadenylation by testosterone (see Fig. 2) then further enhances this interaction.

ePABP and paxillin interact in *Xenopus* oocytes and this interaction is enhanced by testosterone

Notably, the enhanced binding of ePABP to *Mos* mRNA upon addition of testosterone was relatively modest, which suggested that this process was likely not the primary regulator of *Mos* protein expression. The striking similarities of the phenotypes observed with either paxillin (Rasar et al., 2006) or ePABP knockdown (shown here), as well as previous reports of paxillin and PABP1 interactions in somatic cells (Salaun et al., 2010; Woods et al., 2002; Woods et al., 2005), strongly suggested that paxillin and ePABP might form a dynamic testosterone-induced complex that is more significant than ePABP binding to *Mos* mRNA. Unfortunately, commercial antibodies against *Xenopus laevis* paxillin protein are not available, and a polyclonal antibody that we raised against *Xenopus* paxillin was not sensitive enough to detect endogenous paxillin by Western blot (Rasar et al., 2006) precluding the study of interactions between endogenous paxillin and ePABP. Thus, we chose to inject cRNA encoding HA-tagged *Xenopus* paxillin into *Xenopus laevis* oocytes. We then precipitated endogenous ePABP with our polyclonal rabbit antibody and performed

Western blots of the precipitates using an anti-HA antibody so that we could detect co-precipitated HA-paxillin, as well as ePABP with our anti-ePABP antibody to confirm that it had been appropriately and equally precipitated. Indeed, ePABP was expressed in oocyte lysates (Fig. 5A, bottom panel, left side) and was precipitated by the anti-ePABP antibody (Fig. 5A, bottom panel, right side). HA-paxillin was also over-expressed in lysates (Fig. 5A, top panel, left side), and, as expected (Rasar et al., 2006), migrated more slowly in oocytes treated with testosterone (due to increased Erk-dependent serine phosphorylation). Importantly, we found that a small amount of HA-paxillin co-precipitated with endogenous ePABP in the absence of testosterone. However, this interaction was markedly increased in oocytes treated with testosterone (Fig. 5A, top panel right side). In fact, in averaging four experiments (see additional studies in supplemental figure 4), testosterone induced an increase in paxillin-ePABP interaction by 4 ± 1.6 fold ($p=0.004$).

Testosterone-induced enhanced paxillin-ePABP interaction is dependent on Erk activation and phosphorylation of paxillin

How is testosterone promoting ePABP-paxillin interactions? We previously demonstrated that Erk2-dependent phosphorylation of serines 107 and 111 of paxillin is absolutely crucial for its mediation of steroid-dependent Mos protein expression and subsequent oocyte maturation (Rasar et al., 2006). We thus hypothesized that the enhanced interaction of ePABP and paxillin might be regulated by Erk2-dependent phosphorylation of paxillin at the above residues. Accordingly, we found that MEK-inhibition by U0126 in *Xenopus laevis* oocytes abrogated testosterone-induced Erk2 phosphorylation (Fig. 5B), and also significantly reduced testosterone-induced ePABP-paxillin interactions (Fig. 5C, upper panel, right side). Notably, over-expression of phosphorylation-deficient HA-tagged paxillin, where serine residues 107 and 111 were replaced by alanines (SA Pax), constitutively interacted with ePABP (seemingly more robustly than wild-type paxillin in this experiment, possibly due to greater overexpression of the mutated protein as well as differences in exposure time between the two autoradiograms). However, most importantly, the dynamic increase in ePABP-paxillin interaction upon testosterone stimulation was no longer observed, nor did the MEK inhibitor U0126 have any effect on ePABP-paxillin interactions (Fig. 5C, lower panel, right side).

Finally, to confirm that the Erk-mediated interaction between *Xenopus laevis* ePABP and paxillin is not specific to testosterone signaling or due to alternative pathways in maturing oocytes, we studied the protein-protein interaction in a heterologous system that would allow both confirmation of Erk-dependence using a different activator, as well as better quantification of this interaction. To do so, we expressed FLAG-tagged *Xenopus laevis* ePABP and HA-tagged *Xenopus laevis* paxillin in HEK-293 cells and stimulated with 20ng/ml EGF for 60 minutes. Overall, expression of HA-tagged paxillin and FLAG-tagged ePABP in cell lysates were nearly identical in all samples (Fig. 6A, left side). ePABP was directly precipitated from lysates using the aforementioned rabbit anti-ePABP antibody and equal precipitation of ePABP was confirmed by Western blot of precipitates with anti-FLAG antibody (Fig. 6A, lower panel, right side). Notably, as seen with testosterone treatment in oocytes, EGF treatment of transfected HEK-293 cells markedly enhanced the co-precipitation of HA-tagged paxillin (Fig. 6A, upper panel, right side). Averaging four

experiments, EGF increased paxillin-ePABP interactions by 3.7 ± 2 fold ($p=0.003$). In addition, MEK inhibition by U0126 attenuated the ePABP-paxillin interaction (Fig. 6A, upper panel, far right). This reduction by U0126 was significant at $51 \pm 8.4\%$ ($n=4$, $p=0.002$). The three additional experiments are shown in supplemental figure 5. Furthermore, as seen in androgen-treated *Xenopus* oocytes, mutated HA-tagged paxillin whereby serines 107 and 111 were converted to alanines, constitutively bound to ePABP; however, protein-protein interactions were not increased by EGF stimulation, nor were these interactions suppressed by U0126 (Fig. 6B). This experiment was performed two additional times with similar results, as shown in supplementary figure 5.

Discussion

ePABP is one of several polyA-binding proteins (PABPs), found in eukaryotes (reviewed in (Smith et al., 2014)). PABPs are located in both nucleus and cytoplasm and are well-known regulators of mRNA stability as well as translation. They also play a role in the control of miRNA mediated translation repression and nonsense mediated RNA decay. Their presence is particularly important under conditions that primarily rely on post-transcriptional control of protein expression, such as neuronal plasticity and germ cell maturation (Smith et al., 2014).

ePABP is overall 72% identical to the more ubiquitously expressed PABP1, in both frogs (*Xenopus laevis*) and humans (Voeltz et al., 2001). In *Xenopus laevis*, ePABP is the predominant polyA binding protein during early development and in oocytes (Voeltz et al., 2001), which we confirmed here (Fig. 1A).

What roles does ePABP play in oocytes? Firstly, ePABP appears to mitigate deadenylation of RNAs. Immunodepletion of ePABP in *Xenopus laevis* oocyte extracts promoted, and addition of recombinant ePABP prevented, mRNA deadenylation (Voeltz et al., 2001). Secondly, some evidence suggests that ePABP directly mediates steroid-triggered mRNA polyadenylation. By capturing ePABP with PABP-interacting protein 2 (PAIP2), polyadenylation of mRNAs and subsequent protein translation were inhibited in *Xenopus laevis* oocyte extracts (Kim and Richter, 2007). In this same study, ePABP was confirmed to mitigate poly(A) tail destruction and therefore stabilize polyadenylated mRNAs prior to translation. *In vivo* experiments in *Xenopus laevis* oocytes confirmed a delay in mRNA polyadenylation and oocyte maturation using a similar approach of ePABP capture by injecting *PAIP2* cRNA (Friend et al., 2012). Interestingly, a similar phenotype of reduced *Xenopus laevis* oocyte maturation but also reduced Mos protein expression was noted after overexpressing a mutant eukaryotic translation Initiation Factor 4G (eIF4G) that cannot bind endogenous PABP (Wakiyama et al., 2000). These above results, plus the fact that PABP and its interaction with eIF4G are prerequisites for normal ribosome initiation complex formation and binding of eIF4E to the mRNA cap structure, solidify a crucial role of PABPs for translation in general (Kahvejian et al., 2005). Lastly, the crucial role of ePABP in female fertility can be extended to the mammalian system: female ePABP knockout mice are infertile due to impaired follicle development, oocyte maturation but also impaired granulosa cell Erk signaling in response to LH and EGF (Guzeloglu-Kayisli et al., 2012; Yang et al., 2016). As ePABP is expressed in oocytes but not in granulosa cells, abnormal

communication between oocytes and granulosa cells during follicle development was suggested (Yang et al., 2016). Injection of ePABP mRNA into preantral but not late GV-stage oocytes restored normal oocyte maturation (Guzeloglu-Kayisli et al., 2012; Lowther and Mehlmann, 2015).

In this study, we further explored the mechanism of how ePABP regulates normal oocyte maturation by use of *Xenopus laevis* oocytes. In contrast to previous work (Friend et al., 2012), we used a more specific approach of downregulating ePABP by injecting antisense oligonucleotides directed against *ePABP* mRNA. Contrary to the above study (Friend et al., 2012), we did not detect significant changes in *Mos* cRNA polyadenylation in ePABP-depleted oocytes. The discrepancy may be explained by differences in experimental timing. The reduction in mRNA polyadenylation as seen by Friend and coauthors (Friend et al., 2012) was noted after only two hours of progesterone stimulation but was much less noticeable after 4 hours. We studied polyadenylation at 4 and 16h incubation with testosterone; thus, we may not have detected the early delay in polyadenylation. In addition, disruption of the complete poly(A) RNP structure by PAIP2 may result in effects on polyadenylation independent of ePABP. In summary, while we cannot rule out an early delay, overall polyadenylation of mRNAs appears relatively unaffected by ePABP knockdown.

Instead, as seen with paxillin knockdown, we found that knockdown of ePABP expression had a significant effect on steroid-induced *Mos* protein expression (Fig. 3, supplementary Figs. 2 and 3), which is consistent with other studies examining PABP family members that implicate PABPs as translational regulators (Kahvejian et al., 2005; Wakiyama et al., 2000). With reduced *Mos* protein expression, we saw the expected attenuation of Erk2 activation and reduction in oocyte maturation. Both *Mos* expression and Erk2 activation were rescued by co-injection of wildtype ePABP, suggesting causality (Fig. 3, supplementary Fig. 2). Of note, we found that Cdk1 activation was similarly reduced upon reduction of ePABP expression. At face value, this suggests that the canonical *Mos*/Erk2/Cdk1 pathway is disrupted in the absence of ePABP (Palmer et al., 1998; Xiong and Ferrell, 2003). With less activated Cdk1, which is known to be important for maintaining *Mos* protein stability, one would see further disruption of this canonical signaling pathway, ultimately reducing oocyte maturation. However, we cannot rule out that ePABP may be directly regulating Cdk1 activation through alternative *Mos*/Erk2-independent pathways (Fisher et al., 1999; Frank-Vaillant et al., 1999; Haccard and Jessus, 2006).

How does ePABP affect *Mos* protein expression? We found that, following testosterone stimulation, the amount of ePABP bound to *Mos* mRNA increased, potentially due to ePABP binding to newly synthesized polyA tails. That said, significant ePABP was associated with *Mos* mRNA in immature oocytes, and the increase in ePABP bound to *Mos* mRNA, though statistically significant, was not dramatic (Fig. 4B). We therefore speculate that even with the smaller basal poly(A) tail, *Mos* mRNA is still able to bind to ePABP. Alternatively, as shown previously (Voeltz et al., 2001), ePABP may bind to AU-rich elements on *Mos* mRNA, thus providing RNA stability even in the absence of active polyadenylation and/or sex steroid stimulation. Overall, we postulate that ePABP binding to *Mos* mRNA after steroid stimulation is one, but not the major, step regulating ePABP's actions in oocytes.

Instead, we propose that ePABP binding to paxillin, and in particular phosphoserine-paxillin, is the critical point in ePABP's effects on translation. Strikingly, the observed effects of ePABP knockdown on steroid-triggered *Xenopus laevis* oocytes mirror those seen with paxillin knockdown (Rasar et al., 2006). This includes unaltered *Mos* RNA polyadenylation yet reduced *Mos* protein translation, Erk2 phosphorylation, and oocyte maturation. These observations suggest that paxillin and ePABP might work together to regulate steroid-induced oocyte maturation downstream of *Mos* mRNA polyadenylation. That said, in Fig. 2, we did not examine *Mos* protein expression at the exact same time that we saw no change in polyadenylation. However, we saw significant decreases in androgen-induced Erk2 activation and maturation (Fig. 2B and C) when *Mos* mRNA polyadenylation was unchanged; and, in our studies, Erk activation and maturation always mirror *Mos* protein expression with ePABP knockdown (Fig. 3 and supplementary Fig. 3). Thus, it seems likely that ePABP is primarily, but perhaps not exclusively, functioning with paxillin between *Mos* mRNA polyadenylation and *Mos* protein expression. The subcellular localizations of paxillin and ePABP support a potential interaction. Friend et al. (Friend et al., 2012) reported the highest ePABP expression in submembranous oocyte sections, within the animal pole along with ribosomes and CPEP, which is strikingly similar to the previously reported subcellular paxillin location (Rasar et al., 2006).

Indeed, although we detected basal ePABP-paxillin complexes in immature oocytes, the interaction between the two proteins was significantly increased by stimulation of *Xenopus laevis* oocytes, with testosterone (Fig. 5A, supplementary Fig. 4). Furthermore, we discovered that MEK/Erk signaling plays a critical role in ePABP-paxillin interactions, as MEK inhibition essentially abrogated testosterone-induced ePABP-paxillin interactions (Fig. 5C, upper panel). Finally, elimination of previously determined Erk phosphorylation targets (S107, S111) on *Xenopus laevis* paxillin similarly abrogated testosterone-induced ePABP-paxillin interactions (Fig. 5C, lower panel). We had previously shown that Erk-dependent phosphorylation of these serine residues was important for paxillin's actions during oocyte maturation (Rasar et al., 2006), and these results suggest that, at least in part, serine phosphorylation of paxillin is required for ePABP interactions, which in turn may be critical for *Mos* protein translation.

Based on previous studies and the findings presented here, we propose the following model: In immature *Xenopus laevis* oocytes, a significant amount of ePABP protein is bound to *Mos* mRNA, and a small amount is bound to paxillin. Androgen stimulation of extranuclear androgen receptors triggers a decrease in cAMP, leading to increased polyadenylation of mRNAs, thus increasing ePABP binding to *Mos* mRNA. Within this increased binding, ePABP, either alone or with the help of low levels of paxillin within the complex, can then assist in initiating low levels of *Mos* protein expression. *Mos* promotes Erk2 and Cdk1 signaling, and Erk2-dependent phosphorylation of paxillin which then significantly potentiates ePABP and phosphoserine-paxillin interactions, leading to a powerful surge in *Mos* protein expression (also aided by Cdk1's ability to stabilize *Mos* protein) and persistent Erk2 activation, which ultimately culminates in promoting meiotic progression (Fig. 7). It is most likely that phosphoserine-paxillin is interacting with ePABP on the polyadenylated mRNA; however, we currently do not have data to support this hypothesis. In fact, attempts were made to perform RNA precipitation assays using overexpressed HA-tagged paxillin,

but consistent results were not obtained. Of course, additional pathways are likely also feeding those shown in figure 7, as oocyte maturation is replete with multiple back doors and positive feedback loops.

Notably, we were able to confirm the importance of Erk-mediated phosphorylation of paxillin in regulating ePABP-paxillin interactions in a heterologous system: EGF-stimulated HEK-293 cells. EGF promoted ePABP-paxillin interactions that were blocked by MEK inhibition or by the loss of the serine targets on paxillin (Fig. 6, supplementary Fig. 5). Not only do these results verify those observed in *Xenopus laevis* oocytes, but they suggest that PABP-phosphoserine-paxillin interactions may also be important in somatic cells. In fact, as mentioned in the introduction, paxillin and PABP1, the predominantly expressed PABP in most somatic cells, have been shown to interact in mouse fibroblasts, and disrupting the interaction by mutating putative Paxillin-Binding Subdomains on PABP1 (PBS1 and PBS2) interfered with cell spreading and migration (Woods et al., 2005). Though, in our hands, expressing ePABPs with mutations in either or both PBS1 and PBS2 sites in *Xenopus laevis* oocytes together with ePABP antisense oligonucleotides rescued the ePABP knockdown phenotype just like rescue with wildtype ePABP (data not shown). Thus, we did not further pursue studying the roles of PBS1 and PBS2 in ePABP-paxillin interactions.

Several studies demonstrate that regulation of phosphorylation of PABP and/or other translation initiation factors allows translation to occur (reviewed in (Bag and Bhattacharjee, 2010)). For instance, the stimulation of protein translation by serum was accompanied by increased phosphorylation of both eIF4E and eIF4G, but also increased interactions of PABP, eIF4G and eIF4E in *Xenopus laevis* kidney cells; conversely these interactions were reduced in the presence of specific kinase inhibitors (Fraser et al., 1999a; Fraser et al., 1999b), emphasizing an important role of translation regulation by phosphorylation of components of the ribosomal unit.

In *Xenopus laevis* oocytes, both translation of Mos protein and also Erk2 phosphorylation have long been known to be crucial (but perhaps not necessary) regulators normal oocyte maturation by creating a powerful positive feedback loop after only transient stimulation with sex steroids (Xiong and Ferrell, 2003). We propose that the combined actions of both ePABP and phosphoserine-paxillin contribute to this positive feedback loop by stabilizing and directing *Mos* mRNA to the ribosome, as well as stimulating and maintaining its translation, thus resulting in persistent Erk2 activation and ultimately, in progression of meiosis.

Turning to more biological processes in mammals, paxillin knockdown in human prostate cancer cells reduced not only steroid and growth factor-dependent Erk activation but also cell proliferation and migration (Sen et al., 2010). Overexpression of a constitutively active Raf (equivalent to Mos in *Xenopus laevis* oocytes) rescued Erk activation in paxillin depleted cancer cells (Sen et al., 2010) suggesting that, just like in *Xenopus* oocytes, paxillin functions upstream of Raf/Mos. Similarly, we have shown that testosterone promoted Follicle Stimulating Hormone (FSH) receptor protein expression in primary mouse granulosa cells in an Erk-dependent, post-transcriptional fashion (Sen et al., 2014), which in turn assisted in follicle development and subsequent ovulation. In fact, testosterone

promoted FSH receptor expression by enhancing Erk-dependent translation, much like we and others see in *Xenopus laevis* oocytes. We find that PABP1 is highly expressed in granulosa cells (data now shown). Thus, although further studies need to be performed, it is tempting to speculate that a similar dynamic interaction of paxillin and PABP1 in granulosa cells could be mediating FSH receptor translation, and perhaps other important protein translation as well.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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- Knockdown of ePABP phenocopies paxillin knockdown in *Xenopus laevis* oocytes.
- ePABP is necessary for testosterone-induced oocyte maturation.
- ePABP knockdown reduces testosterone-induced Mos expression, Erk2 and Cdk1 activation.
- ePABP and paxillin dynamically interact in response to testosterone (oocytes) or EGF (HEK293).
- Dynamic ePABP and paxillin interaction depends on Erk-dependent paxillin phosphorylation.

Figure 1. Knockdown of ePABP expression in *Xenopus* oocytes reduced androgen-triggered maturation

A) ePABP but not PABP1 is expressed in stage V/VI oocytes. *Xenopus* oocytes were injected with cRNA encoding ePABP, PABP1 or HEPES (mock) and subjected to Western blotting for ePABP or PABP1 after 36–48h. **B, C)** Injection of ePABP antisense oligonucleotides reduced *ePABP* mRNA (**B**) and protein (**C**) expression. **B)** *Xenopus* oocytes were injected with sense (S) or antisense (AS) oligonucleotides against ePABP, subjected to RNA extraction and quantitative PCR 36–48h later. Graphs represent means plus SEM from four independent experiments, #p < 0.001. **C)** *Xenopus* oocytes injected as above were subjected to Western blotting with rabbit anti-ePABP antibody. A representative Western blot is shown, similar results were obtained in four independent experiments. **D, E)** ePABP knockdown reduces testosterone-induced oocyte maturation. **D)** Oocyte maturation (% germinal vesicle breakdown) is shown for *Xenopus* oocytes injected with either sense (S) or antisense (AS) oligonucleotides against ePABP plus/minus cRNA encoding wildtype (wt) ePABP and subjected to 16h treatment with 0.1% ethanol or increasing concentrations of testosterone as shown. Similar results were obtained in five independent dose response

experiments, see also supplementary figure 1 and 3A. **E)** Graphs depict averages (+SEM) of oocyte maturation (% germinal vesicle breakdown) from four independent experiments at 100 nM testosterone (* $p < 0.05$).

Figure 2. ePABP knockdown does not affect *Mos* mRNA poly-adenylation upon testosterone stimulation

A) Following oligonucleotide injection with sense (S) or antisense (AS) ePABP, *Xenopus* oocytes were injected with the 3' UTR of *Mos* cRNA and subjected to Northern blotting using a *Mos* 3' UTR [P^{32}] probe following 0, 4, and 16h of incubation with 300nM testosterone. Polyadenylation is noted as decreased mobility. **B, C)** Oocytes from the experiment above were also subjected to Western blotting for phosphorylated Erk2 (**B**, pErk2, upper panel) and total Erk2 (**B**, tErk2, lower panel) as well as percent oocyte maturation (**C**) after 16h treatments with 0 or 300 nM testosterone.

Figure 3. Knockdown of ePABP reduces Mos protein expression and Erk2 activation

Xenopus oocytes were injected with ePABP sense (S) and antisense (AS) oligonucleotides plus/minus cRNA of wildtype (wt) ePABP. 36–48 hours later, oocytes were treated with 0.1% ethanol or increasing concentrations of testosterone for 16h and subjected to Western blotting for Mos (upper panel), phosphorylated Erk2 (pErk2, mid panel) and total Erk2 (tErk2, lower panel). Similar results were obtained in four independent experiments. See also supplementary figure 2 (and figure 2B).

Figure 4. Testosterone leads to a small but significant increase of ePABP binding to Mos mRNA *Xenopus* oocytes were injected with FLAG-tagged ePABP. 36–48h later they were treated with 0.1% ethanol (0nM) or 1000nM testosterone for 6h, followed by cell lysis, precipitation of FLAG-ePABP and subsequent Western blotting for FLAG-ePABP (anti-FLAG, **A**). RNA extraction was also performed with the precipitates, and quantitative PCR performed for *Mos* mRNA, depicted in graphs as mean relative to controls (0nM testosterone) plus SEM (**B**). * $p < 0.05$ (n=4)

Figure 5. Paxillin and ePABP dynamically interact in *Xenopus* oocytes

A) Paxillin and ePABP interactions are increased by testosterone stimulation. *Xenopus* oocytes were injected with HA-tagged wildtype paxillin. 36–48h later they were treated for 8h with 0.1% ethanol or 1000nM testosterone. Lysates were then subjected to immunoprecipitation of endogenous ePABP with anti-ePABP rabbit serum, followed by Western blotting for HA-paxillin (anti-HA, upper panel) and ePABP (anti-ePABP, lower panel). Shown are representative blots for HA-paxillin obtained from lysates (Input - left lanes), precipitates with pre-immune serum (Pre - center lanes) or post-immune serum (IP - right lanes) against ePABP. Upper bands in each panel represent proteins of interest (paxillin or ePABP), lower bands reflect IgG heavy chains. Averaging four independent experiments (see also supplementary figure 4), testosterone caused a 4 ± 1.6 fold increase in co-precipitated HA-paxillin relative to input when compared to control conditions ($p=0.004$).

B) The MEK inhibitor U0126 inhibits Erk2 activation. Lysates from below experiments (C) were subjected to Western blotting for phosphorylated and total Erk2 (p- and t-Erk2).

C) The presence of U0126 abrogates testosterone-induced increased paxillin-ePABP interaction for wildtype but not phosphorylation-deficient paxillin. *Xenopus* oocytes were injected as above (A) with HA-tagged wildtype (WT Pax) or mutant paxillin (S107A, S111A = SA

Pax), then treated for 8h with 0.1% ethanol, 1000nM testosterone as well as 1000nM testosterone plus 50μM U0126. Lysates were processed as in A) and subjected to Western blotting for ePABP (anti-ePABP) and paxillin (anti-HA). Shown are representative blots for HA-paxillin (WT versus SA Pax) obtained from lysates (Input - left lanes), precipitates with pre-immune (Pre - center lanes) or post-immune serum (IP - right lanes) against endogenous ePABP. Upper bands represent protein of interest (paxillin), lower bands reflect IgG heavy chains. Similar results were obtained in three independent experiments.

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Figure 6. Paxillin and ePABP dynamically interact in HEK-293 cells

A) HEK-293 cells were transfected with *Xenopus* FLAG-tagged ePABP as well as *Xenopus* HA-tagged wildtype paxillin. After an overnight incubation in serum-free DMEM, transfected cells were subjected to a 1 h stimulation with 20ng/ml EGF plus/minus 50μM U0126. Lysates were then subjected to ePABP precipitation with anti-ePABP post-immune serum, followed by Western blotting for HA-paxillin (anti-HA, upper panel) and FLAG-ePABP (anti-FLAG, lower panel). Shown are representative blots for HA-paxillin and FLAG-ePABP obtained from lysates (Input - left lanes), precipitates with pre-immune (Pre - center lanes) or post-immune serum (IP - right lanes) against ePABP. Averaging four independent experiments, EGF resulted in a 3.7 ± 2 fold increase in HA-paxillin precipitation relative to input when compared to control conditions ($p=0.003$). A $51 \pm 8.4\%$ reduction of paxillin co-precipitation relative to input was noted in the presence of U0126 compared to EGF alone ($p=0.002$). See also supplementary figure 5. **B)** EGF-mediated increase in paxillin-ePABP interaction is not observed for phosphorylation-deficient paxillin. HEK-293 cells were transfected with *Xenopus* FLAG-tagged ePABP as well as HA-tagged mutant paxillin (S107A, S111A = SA Pax) and treated as above. See also supplementary figure 5.

Figure 7. Proposed model

In immature *Xenopus laevis* oocytes, a significant amount of ePABP protein is bound to *Mos* mRNA, and a small amount is bound to paxillin. Androgen stimulation of extranuclear androgen receptors triggers a decrease in cAMP, leading to increased polyadenylation of mRNAs, thus increasing ePABP binding to *Mos* mRNA. ePABP, either alone or with the help of low levels of paxillin within the complex, can then assist in initiating low levels of Mos protein expression. Mos promotes Erk2 signaling and Erk2-dependent phosphorylation of paxillin, which then significantly potentiates ePABP and phosphoserine-paxillin interactions, leading to a positive feedback loop that enhances Mos protein expression, Erk2 and Cdk1 activation, ultimately culminating in oocyte maturation. Mos is further stabilized by active Cdk1.