PAXILLIN IS A NOVEL REGULATOR OF *XENOPUS* OOCYTE MATURATION

APPROVED BY SUPERVISORY COMMITTEE

Stephen Hammes, M.D., Ph.D.

Richard Anderson, Ph.D.

David Mangelsdorf, Ph.D

Philip Shaul, M.D.

Dedicated to my parents

For instilling in me the desire to learn,

And to my husband

For his constant support, encouragement, and optimism

Which has inspired me throughout the last several years.

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MELISSA RASAR YOUNG

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Melissa Rasar Young, Ph.D.

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Supervising Professor: Stephen R Hammes, M.D., Ph.D.

Oocyte maturation is triggered by steroids in a transcription-independent fashion that involves an unusual positive feedback loop whereby MOS (a germ cell specific Raf) activates MEK1, which in turn activates ERK2. ERK2 then acts back on MOS to enhance its expression resulting in amplification of the kinase signaling cascade. To date, little is known regarding other factors that regulate this powerful feedback kinase cascade. Here we present the scaffolding molecule, Paxillin, as a newly recognized essential regulator of meiosis in *Xenopus leavis* oocytes. Reduction of Paxillin expression using RNA interference and antisense oligonucleotides completely abrogates steroid-triggered meiotic resumption. Detailed signaling studies reveal that Paxillin is

acting early in the kinase cascade, as it is required for accumulation of MOS protein and complete activation of downstream kinase signaling in response to steroids. Surprisingly, full Paxillin activity also requires serine phosphorylation by a kinase downstream of MOS and MEK1, possibly ERK2. Together, these data suggest that Paxillin is an important regulator of the positive feedback effects of MEK/ERK signaling on MOS protein expression. The ability of Paxillin to function as a MAPK scaffold was analyzed, revealing Paxillin can interact with MOS in mammalian cells. Furthermore, the ability of Paxillin to regulate activity of proteins important for translation, specifically polyadenylation binding proteins, is briefly explored. In all, these experiments reveal a novel and critical function for Paxillin in meiosis, and support the notion that Paxillin may be general modulator of MAPK signaling and/or mRNA translation by polyadenylation binding proteins.

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LIST OF ABREVIATIONS

AC – adenylyl cylcase

AKAP – A-kinase anchoring protein

AR – androgen receptor

cAMP – cyclic adenosine monophosphate

cdc2 – also referred to as CDK1 or p34cdc2

CDK – cyclin dependent kinase

CPE – cytoplasmic polyadenylation element

CPEB – cytoplasmic polyadenylation element binding protein

CPSF – cleavage and polyadenylation specificity factor

dsRNA – double stranded RNA

Eg2 – also known as Aurora Kinase A

eIF4E – translation initiation elongation factor 4E

eIF4G – translation initiation elongation factor 4G

ePABP – embryonic PABP

ERK2 – p42 MAPK

GST – Glutathione S-Transferase

GVBD – germinal vesicle breakdown

HA – hemagglutinin eptitope tag

hCG – human chorionic gonadotropin

IGF-1 – Insulin-like growth factor 1

IP - immunoprecipitation

MAPK – mitogen activated protein kinase or ERK

MEK1 – MAP kinase kinase

MPF – maturation promoting factor, complex of CDK1 and cyclin B1

mPR – membrane progesterone receptor

mRNA – messenger RNA

Myt1 – p34cdc2 inhibitory kinase

PABP – polyadenylation binding protein

PABP1 – polyadenylation binding protein 1

PAP – polyadenylation polymerase

PBS – Paxillin binding sequence

PDE – phosphodiesterase

PI3-K – Phosphatidylinositol 3-kinase

PKA – protein kinase A

PKB/Akt – protein kinase B

Poly(A) – polyadenylated

PR – progesterone receptor

PRE – polyadenylation response element

RRM – RNA binding domain

Rsk – p90 RSK

UTR – untranslated region

Chapter One

General Introduction

Oocyte maturation

Female fertility relies on precise regulation and initiation of the meiotic cycle in oocytes, a process otherwise known as oocyte maturation. Simply, oocyte maturation is the process by which an oocyte becomes a fertilizable egg. Meiosis is unique to gametes and entails the segregation of chromosomes along the mitotic spindle to produce haploid gametes from diploid gamete precursors (reviewed in Whitaker, 1996). This is accomplished first by the replication and recombination of DNA in a precursor oocyte, followed by two rounds of segregation of the chromosomes and cell division without an intervening S phase. In most species, cell cycle pauses twice during meiosis. The first meiotic arrest occurs at prophase of meiosis I, resulting in the accumulation of immature oocytes within the ovary, and the second arrest occurs at metaphase of meiosis II producing the mature oocytes, or fertilizable eggs.

In many higher vertebrate species, the general consensus is that females are born with their full complement of oocytes and that these oocytes reach the first meiotic arrest very early in life, during *in utero* development of the female. At this point, the DNA has replicated, the chromosomes are condensed, and meiotic recombination has occurred. Only at time of sexual maturity, either weeks, months, or years after birth (depending on the species), will these oocytes re-enter the meiotic cycle. The first meiotic arrest is broken due to release of gonadotropins from the pituitary that trigger a myriad of signals

within the ovary just prior to ovulation. Upon receiving these signals, the immature oocytes are induced to resume meiosis indicated by germinal vesicle breakdown and completion of the first meiotic division and extrusion of the first polar body. However, these oocytes arrest a second time at metaphase of meiosis II. These oocytes are referred to as mature oocytes and require fertilization as the signal to complete meiosis. After fertilization, the second polar body is extruded, genetic material of the sperm and egg fuse, and the resulting embryo is capable of uninterrupted mitotic division (Albertini and Carabatsos, 1998; Maller, 1985; Maller and Krebs, 1980; Masui and Clarke, 1979; Shibuya and Masui, 1989). Clearly, understanding the signals that dictate both meiotic arrest and re-entry into the cell cycle are critical to understanding how normal reproduction takes place, allowing us to better understand causes of female infertility.

Xenopus laevis **as a model for meiotic maturation.**

Oocyte maturation occurs as the result of activation of numerous signal transduction pathways that all converge to activate a complex referred to as maturation-promoting factor (MPF). Activity of MPF has been shown to catalyze the re-entry into cell cycle in most species, although the way in which MPF is activated may vary from species to species. Previous research has indicated that MPF is made up of the cell cycle proteins cyclin-dependent kinase (CDK1) and cyclin B (Nurse, 1990; Pines and Hunter, 1990). *Xenopus laevis* oocytes have long-served as a model for studying the molecular signals that regulate meiosis, and many of the pathways elucidated in *Xenopus* oocytes now appear to be conserved in mammalian oocyte maturation as well (Gill et al., 2004; Jamnongjit and Hammes, 2005; Schmitt and Nebreda, 2002). Additionally, steroids have

been shown to trigger maturation in oocytes via classical steroid receptors in a transcription-independent, or nongenomic, manner (reviewed in Hammes, 2004). Thus, *Xenopus* oocytes not only serve as a superb model for studying the general principles of meiosis, but also as one of the few well-accepted, biologically relevant examples of nongenomic steroid-mediated signaling.

Overview of the Oocyte Maturation Signaling Cascade

Currently the general consensus is that steroids may trigger maturation in a "release of inhibition" fashion whereby oocytes are held in meiotic arrest by constitutive G protein signals that stimulate adenylyl cyclase to elevate intracellular cAMP (Gallo et al., 1995; Lutz et al., 2000; Sheng et al., 2001). In *Xenopus*, studies suggest that during ovulation, gonadotropins stimulate ovarian androgen production, leading to activation of ARs, attenuation of the constitutive G protein signaling (and likely activation of phosphodiesterases), and a drop in intracellular cAMP. Once cAMP is reduced, downstream kinases are activated, leading to germinal vesicle breakdown (GVBD) (Ferrell, 1999b; Maller and Krebs, 1980). The rapid decrease in cAMP levels is followed by a "lag period" during which cytoplasmic polyadenylation is triggered, resulting in protein synthesis of the MOS protooncogene followed by activation of the MAPK cascade, including MEK1 and p42 MAPK. Finally, after several hours, the activation of the MAPK cascade results in the activation of the cell cycle regulators, including cdc25 phosphatase and CDK1, which trigger the resumption of meiosis and germinal vesicle breakdown (Fig. 1-1).

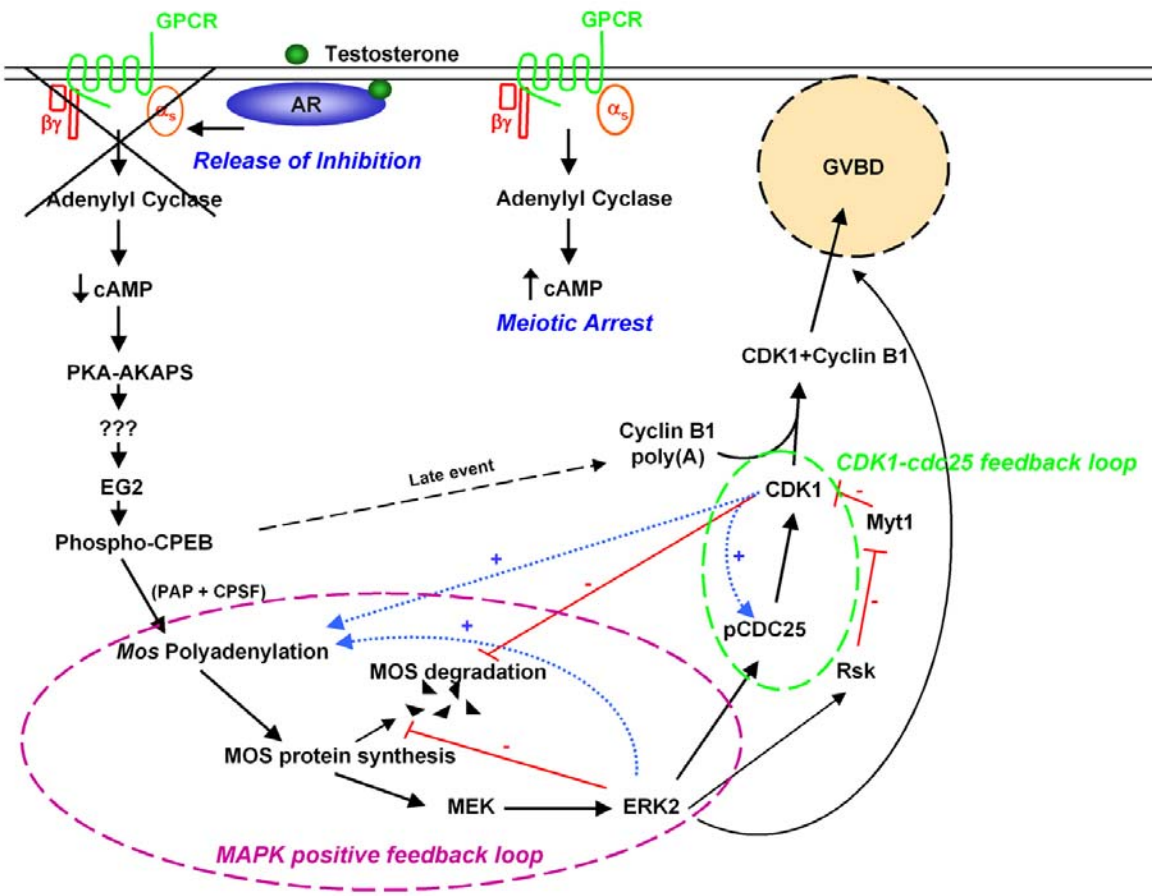


Figure 1-1: Overview of the oocyte maturation pathways. The mechanisms that maintain meiotic arrest (center), and the signals thought to be activated upon release of these signals (upper left) are listed. The signaling pathway downstream of cAMP level decline is indicated, although the steps between PKA modulation and EG2 activation are still unknown. The positive feedback kinase loop is indicated by the dashed purple oval, the CDK1-cdc25 feedback loop in green. Furthermore, specific positive feedback signals are indicated in blue dashed arrows, and inhibitory signals are indicated by red lines. A more detailed description of these steps is discussed below.

In order to understand oocyte maturation more fully, three key components must be examined in detail: 1) the factors that hold oocytes in meiotic arrest, 2) the signals that trigger meiosis to resume, and 3) the signaling pathways that lead to the eventual activation of MPF.

Meiotic arrest in the oocyte.

Meiotic arrest appears to be an active process in the oocyte. A multitude of evidence suggests inhibitory signals are responsible for holding oocytes in meiotic arrest. In lower vertebrates such as frogs and fish, oocytes can be removed from the ovary and surrounding follicular cells, yet remain in meiotic arrest indefinitely until triggered by exogenous addition of steroids or other triggers of maturation (Maller and Krebs, 1980; Smith and Ecker, 1971). In contrast, immature mammalian oocytes when removed from the ovary and their surrounding follicular cells will spontaneously mature (Edwards, 1965a, b). Together, this suggests the origin of the inhibitory signals is different across species, such that lower vertebrate oocytes have an endogenous inhibitory signal and mammalian oocytes depend on extracellular sources to maintain meiotic arrest.

Although the physical origin of the inhibitory signal may be different across species, cAMP has been identified as an essential inhibitory signal common to these species (Conti et al., 2002). A meiosis-related decrease in cAMP levels has long been observed in animals ranging from fish to frog to mouse (Conti et al., 2002; Morrill et al., 1977; Sadler and Maller, 1981, 1985). An inhibitory role for cAMP has been confirmed repeatedly based on experiments where cAMP levels were artificially elevated, by either

activating adenylyl cyclase, inhibition of cAMP phosphodiesterases, or treatment with cAMP analogs, resulting in an inhibition of maturation in both frog and mouse oocytes.

In general, regulation of intracellular cAMP levels is mediated by two families of enzymes, either adenylyl cyclases (ACs) which produce cAMP or phosphodiesterases (PDEs) that metabolize cAMP. Most of the adenylyl cyclases that have been characterized are regulated by G proteins that either promote (Gαs) or inhibit (Gαi) their activity (Freissmuth et al., 1989). On the other hand, regulation of PDEs is less well characterized but may involve feedback inhibition of PKA signaling and/or alterations in the regulation of mRNA and protein expression (Mehats et al., 2002). cAMP levels appear to be elevated in *Xenopus* oocytes, at least in part, due to constitutive Gβγ and Gαs signaling, both of which can activate adenylyl cyclase (Gallo et al., 1995; Lutz et al., 2000; Sheng et al., 2001). This also appears to be physiologically significant since inhibition of Gβγ and/or Gαs signaling results in induction of spontaneous maturation, presumably due to a reduction in intracellular cAMP (Gallo et al., 1995; Lutz et al., 2001; Sheng et al., 2001). In mice, Gαs has also been proposed as a regulator of meiotic arrest. Recently, a Gαs coupled receptor, GPR3, has been identified as having a role in maintaining meiotic arrest since oocytes from mice lacking GPR3 undergo spontaneous maturation within the ovary (Mehlmann et al., 2004).

Why does cAMP inhibit maturation? Although still not understood, it has been proposed that cAMP regulates kinase signaling cascades, such as the protein kinase A (PKA) pathway, that are critical for meiotic arrest (Conti et al., 2002; Morrill et al., 1977; Sadler and Maller, 1981). Finally, although elevation of cAMP clearly inhibits

maturation, it is important to note that it is not clear if decreased intracellular cAMP concentration is either necessary or sufficient to allow for meiotic progression (Eppig and Downs, 1988; Faure et al., 1998; Gelerstein et al., 1988).

Signals that Trigger Maturation

The study of steroid-induced *Xenopus* leavis oocyte maturation is one of the best-studied animal models of maturation available. Nearly 60 years ago, ovulation in amphibians was shown to be triggered when female frogs were treated with pituitary extracts (Rugh, 1935). Within a few years, it was hypothesized that the pituitary releases a hormone which causes release of a factor within the ovary that is responsible for inducing maturation and ovulation (Heilbrunn, 1939; Ryan and Grant, 1940; Wright, 1945). By the 1960's, it was presumed this factor was the hormone, progesterone (Smith et al., 1968). However, over the last few decades, many steroids including progestins, glucocorticoids, and androgens have been shown to induce oocyte maturation in amphibian oocytes *in vitro* (Le Goascogne et al., 1985; Lutz et al., 2001; Smith and Ecker, 1971).

Although many steroids can promote *Xenopus* oocyte maturation *in vitro*, more extensive studies have shown that androgens likely serve as the physiologic trigger *in vivo* (Lutz et al., 2001; Lutz et al., 2003). In these studies, frogs were injected with human chorionic gonadotropin (hCG) to induce oocyte maturation and ovulation, and the steroid levels in both the serum and ovarian tissue were assessed. Surprisingly, the levels of the androgens, androstenedione and testosterone, were dramatically elevated and progesterone levels were nearly undetectable. To confirm the necessity of androgens for

maturation and ovulation, the production of androgens was inhibited by administration of a CYP17 inhibitor. This treatment prevented reduced hCG-triggered oocyte maturation and delayed ovulation of the oocytes (White et al., 2005). *In vitro*, androgens are also demonstrated to be equally, if not more, potent promoters of maturation when compared to progesterone.

Further studies indicated that in the frog, the oocyte itself exclusively expresses the enzyme, CYP17, which is responsible for converting pregnenolone to dehydroepiandrosterone (DHEA) and progesterone to androstenedione, whereas all other steroidogenic enzymes were found in the surrounding follicular cells (Yang et al., 2003). These data indicate that over the last few decades, whenever studies have used progesterone as the agonist for steroid-mediated maturation in frog oocytes, most likely the oocytes were exposed to a combination of progesterone and androgens due to the rapid steroid metabolism of progesterone to androgens by the oocyte.

Interestingly, no matter which steroid hormone is used, steroid-induced maturation occurs independent of transcription as demonstrated by the failure of the transcriptional inhibitor actinomycin D to prevent maturation (Lutz et al., 2003; Maller and Krebs, 1980; Smith and Tenney, 1980; Smith and Ecker, 1971; Smith et al., 1968). In the past, this fact may have prompted many researchers to rule out the possibility that steroids were working through the classical steroid receptors. However, the relatively recent discovery of nongenomic steroid-induced signals of the classical nuclear/cytoplasmic steroid receptors, as indicated by estrogen-induced activation of MAPK and endothelial nitric oxide synthase in breast and endothelial cells (Razandi et al., 2002; Shaul, 2002; Simoncini et al., 2002) and estrogen and androgen-mediated anti-

apoptotic signals in bone cells (Kousteni et al., 2001; Kousteni et al., 2002) have reinforced the possibility that steroids could promote oocyte maturation through classical receptors. As a result, a more careful examination of the role classical steroid receptors may play in steroid-mediated oocyte maturation was begun.

Over the last decade, much research has been devoted to identifying the receptor(s) responsible for mediating oocyte maturation. Classical *Xenopus* androgen receptor (AR) and progesterone receptor (PR) have been cloned from oocytes and overexpression and antisense experiments evaluating the result of receptor level modulation have been conducted (Lutz et al., 2001; Maller, 2001). Biochemical studies of the classical *Xenopus* AR, indicated the androgen receptor mediates androgen-induced maturation in the frog. Specific antagonists of the AR and elimination of endogenous AR by RNA interference specifically reduced androstenedione-mediated maturation *in vitro* (Lutz et al., 2001; Lutz et al., 2003). Studies in which the levels of PR were altered have also been conducted, but these studies demonstrated only small changes in the ability of progesterone to induce maturation. Such moderate changes could be explained by the fact that AR may have mediated the progesterone signals due to the fact that progesterone can be metabolized to androgens by the oocyte, and progesterone is capable of binding the *Xenopus* AR in oocytes (Evaul et al., 2007; Yang et al., 2003).

Although the androgen receptor is likely the more biologically relevant receptor involved in steroid mediated maturation in *Xenopus*, nonclassical receptors have also been studied. One of these includes a member of potential membrane-associated steroid receptors, membrane progesterone receptor (mPR). mPR has homology to the G protein-coupled receptor family and has been shown to be a molecule that likely mediates

maturation in fish and can enhance progesterone mediated-maturation in *Xenopus* oocytes, but a biologically relevant role is still unclear (Josefsberg Ben-Yehoshua et al., 2007; Zhu et al., 2003a; Zhu et al., 2003b).

Steroid hormones are not the only compounds that can trigger maturation. Factors like insulin and insulin-like growth factor (IGF-1) have been identified as *in vitro* promoters of maturations via the IGF-1 receptor (Grigorescu et al., 1994; Zhu et al., 1998). The biological significance of IGF-1 receptor activation is unclear. IGF-1 receptor can activate some of the same pathways as steroid-mediated maturation, such as Aurora-A and MAPK (Baert et al., 2003; Sarkissian et al., 2004). It has been proposed that simply the overexpression of downstream signaling molecules is enough to trigger meiotic maturation without necessarily playing a role in steroid-mediated maturation (reviewed in Schmitt and Nebreda, 2002). Therefore, it's possible that strong enough activation of the IGF-1 receptor, or any other signaling pathway that shares downstream signals activated during steroid-induced maturation, may result in maturation but it does not prove that it is physiologically relevant trigger.

Release of Inhibition Model

The current model for *Xenopus* oocyte maturation is that promoters of maturation, such as steroids, overcome inhibitory signals, causing a decline in intracellular cAMP which triggers activation of the maturation signaling cascade (Fig 1-1) (Gill et al., 2004; Lutz et al., 2000). To date, it is unclear how steroid receptors can mediate such rapid changes in signaling. Interestingly, it has been shown that a scaffolding molecule, the modulator of nongenomic actions of estrogen receptor

(MNAR), binds both the AR and Gβ and its expression appears to be critical for maintaining basal levels of Gβγ signaling (Haas et al., 2005). It was further proposed that ligand binding may induce a change within the complex resulting in rapid attenuation of cAMP levels by altering βγ-signaling. This hypothesis has been supported by recent work using a βγ-sensitive potassium channel as a real-time reporter for rapid changes in βγ-signaling that showed androgens can significantly decrease βγ-signals in as little as 10 minutes (Evaul et al., 2007). This effect was confirmed to occur through the AR since the competitive antagonist, flutamide, or reduction of AR levels by RNA interference blocked the androgen-induced changes in βγ-signaling. Because βγ is known to activate AC activity, this experiment indicates one way in which steroids could affect cAMP levels rapidly. This is likely only one of many ways cAMP levels decline, and in all likelihood, other signals within the oocyte are altered upon steroid treatment. Candidates include altered Gαs and phosphodiesterase activity, both of which could alter cAMP levels (Masciarelli et al., 2004; Sadler and Maller, 1987).

A large amount of attention has been given to how steroids might decrease cAMP levels, but it is important to recognize that this is not required in all species. In some animals, such as sheep, rabbit, and pig, cAMP may not change or may actually increase at the onset of maturation (Crosby et al., 1985; Mattioli et al., 1994; Yoshimura et al., 1992). In an attempt to reconcile this difference to mouse and frog maturation, some argue that global cAMP changes may not be required, but instead local changes in cAMP concentration at key signaling sites may be important. In theory, this could be regulated by proteins that target cAMP signaling, such as A-kinase anchoring proteins

(AKAPs) (Dekel, 2005). This is supported, in part, by the observation that AKAP1 knockout mice demonstrate reduced fertility due to a defect in meiotic maturation, likely resulting from mislocalization of PKA activity (Newhall et al., 2006). Nonetheless, there are probably many signaling changes that occur to release meiotic arrest, some more important in some species than in others, and future research will undoubtedly focus on what these signals might be.

Components of the Nongenomic Signaling Pathway

Early signal transduction

Although cAMP levels decline rapidly after triggering maturation, there is a significant delay, on the order of hours, before maturation is complete suggesting a significant number of signals are activated downstream of cAMP changes. First, early immediate signals must be considered. Some have proposed that a decrease in cAMP levels may lead to reduced activity of a cAMP-dependent protein kinase, protein kinase A (PKA). Previous work has suggested that a decrease in PKA activity may be necessary for steroid-induced maturation since injection of active catalytic subunits of PKA blocks steroid-induced maturation and PKA inhibitors can induce spontaneous maturation (Maller and Krebs, 1977). Other studies have suggested that PKA may function as a negative regulator of several steps of the maturation pathway, yet this effect does not require catalytic activity of PKA (Matten et al., 1994; Schmitt and Nebreda, 2002). Finally, molecules that tether PKA regulatory subunits to specific locations within the oocyte, such as AKAPs, appear to be critical for maintenance of meiotic arrest (Brown et

al., 2002; Kovo et al., 2006; Kovo et al., 2002; Newhall et al., 2006). AKAPs are temporally and spatially regulated during meiosis and may be important for maturation since they could theoretically target cAMP responsive signals within the oocyte.

Other common kinases have been implicated in mediating early events of the maturation signaling pathway but the requirement for them is not clear (reviewed in Ferrell, 1999b). Phosphatidylinositol 3-kinase (PI3K) has been proposed to be a mediator of maturation. Since PI3-K activity has been shown to increase during steroid-induced maturation and activated forms of PI3-K can induce spontaneous maturation (Deuter-Reinhard et al., 1997; Muslin et al., 1993). Apparently contradictory data suggest it may not be required for maturation since the PI3-K inhibitors, such as wortmannin or LY294002, have no effect on steroid-mediated maturation, although inhibitors can prevent insulin-induced maturation (Carnero and Lacal, 1998; Deuter-Reinhard et al., 1997; Liu et al., 1995). Protein kinase B/Akt has also been proposed to play a role in oocyte maturation, perhaps by regulating type 3 cAMP phosphodiesterase (Andersen et al., 1998). However, a role for PI3-K and PKB/Akt during oocyte maturation is cast into doubt since activation of p70 S6K, a downstream effecter common to both pathways, is not required for either steroid or insulin-induced maturation (Morley and Pain, 1995). Overall, the data suggest that these pathways may be incidentally active during steroid-mediated oocyte maturation, and may be capable of inducing maturation when their activities are increased, but a physiologic role is uncertain.

Cellular Activation via message specific polyadenylation

Gene expression and cell fate during early animal development are regulated largely at the translational level. Oocytes contain a large store of dormant maternal mRNAs that are activated in stage and sequence specific manners during both meiotic maturation and early embryonic development. The mechanisms responsible for this are generally considered substitutes for regulated transcriptional activity typically observed in somatic cells. One of the most common and evolutionarily conserved mechanisms by which these mRNAs is regulated is cytoplasmic polyadenylation, a process that is required for oocyte maturation and has been extensively studied in both the *Xenopus* and mouse oocyte models (Barkoff et al., 1998)(reviewed in Richter, 1999).

Although mRNAs are polyadenylated with approximately 250 bases in the nucleus (Wahle, 1995), a small set of mRNAs are deadenylated to varying degrees upon reaching the cytoplasm (Huarte et al., 1992; Paynton and Bachvarova, 1994), often to less than 20 nucleotides (Paris and Philippe, 1990; Sheets et al., 1994; Stebbins-Boaz and Richter, 1994; Vassalli et al., 1989) but sometimes to 50-90 nucleotides (McGrew et al., 1989; Sheets et al., 1994). In this way, these mRNAs are maintained in a translationally dormant state until the proper time. In response to various stimuli, such as the reentry into meiosis or fertilization, a set of cytoplasmic proteins are activated, leading to the polyadenylation of subset of transcripts in a very specific order. Interestingly, some messages are translationally regulated by either the presence of a long poly(A) tail (Paris and Philippe, 1990; Vassalli et al., 1989), or by the act of polyadenylation itself (McGrew et al., 1989; Simon et al., 1992). Extensive studies have looked at the proteins and

mRNA sequences that may determine this specificity at the level of both polyadenylation and translation.

In the case of oocyte maturation, the target mRNAs have been shown to contain two cis-acting motifs, a hexanucleotide sequence, AAUAAA, seen in most transcripts, and a cytoplasmic polyadenylation element (CPE), a U-rich sequence with the consensus sequence UUUUUAU, usually located upstream of the hexanucleotide sequence anywhere from immediately adjacent to 50 nucleotides upstream of the hexanucleotide sequence (Fig 1-2)(Fox et al., 1989; Gebauer et al., 1994). Interestingly, the spacing between the hexanucleotide and the CPE sequences can determine the timing of translational activation. For instance, polyadenylation of *Mos* mRNA occurs early, whereas polyadenylation of cyclin B1 mRNA occurs late (Charlesworth et al., 2004). Furthermore, the number of urdines within the U-rich CPE also may play a part in determining if an mRNA is polyadenylated during maturation, fewer uridines, or during fertilization and early embryonic development (Paris and Philippe, 1990; Simon et al., 1992; Stebbins-Boaz and Richter, 1994).

CPEB-dependent polyadenylation

Regulated polyadenylation of maternal mRNAs in the cytoplasm require several proteins, including polyadenylation polymerase (PAP), cleavage and polyadenylation specificity factor (CPSF) (Bienroth et al., 1993; Bilger et al., 1994), and cytoplasmic polyadenylation binding protein (CPEB). Activated CPEB is believed to be responsible for the recruitment of PAP and CPSF to mRNAs in response to steroid-induced signals. CPEB was originally identified due to its ability to bind the CPE of cyclin B4 mRNA

(Hake and Richter, 1994; Paris and Pouyssegur, 1991). Further, CPEB was found to be required for cytoplasmic polyadenylation of mRNAs in response to oocyte maturation signals, especially mRNAs encoding proteins critical to maturation such as MOS, cyclins, and CDK2 (Stebbins-Boaz et al., 1996).

CPEB binds CPE-containing RNAs even when those transcripts are translationally inactive and studies suggests that it is the phosphorylation and activation of CPEB by an upstream kinase Aurora A (Eg2), that allows for CPEB to recruit the machinery for polyadenylation, 5' cap ribose methylation, and subsequently translation (Andresson and Ruderman, 1998; Mendez et al., 2000; Sarkissian et al., 2004)(reviewed in Piccioni et al., 2005). More recent evidence suggests, however, that Aurora kinase activation of CPEB may not be the only mechanism of CPEB activation. In a recent study, treatment of oocytes with the Aurora kinase inhibitor, ZM447439, does not prevent phosphorylation of CPEB (Keady et al., 2007). Instead, an early, transient increase in MAPK activation was shown to lead to partial phosphorylation of CPEB which may be permissive for an as-yet-unidentified kinase to completely phosphorylate and activate CPEB.

CPEB-independent polyadenylation

After the discovery of CPEs and CPEB mediated polyadenylation, another regulatory 3'UTR sequence motif was discovered in *Mos* mRNA, polyadenylation response element (PRE) (Charlesworth et al., 2002). In this study, oocytes were injected with a dominant-negative form of CPEB which only delayed, but did not inhibit *Mos* polyadenylation nor GVBD. Sequence analysis revealed the PRE, a 24 nucleotide

sequence that partially overlapped the CPE in *Mos* mRNA, conferred the ability for polyadenylation of transcripts in the absence of CPEB while early transient MAPK activation seemed to be enough to promote PRE-mediated polyadenylation. Furthermore, it was demonstrated that mRNAs that contain a putative PRE, such as *Mos,* Aurora-A/Eg2, FGFR1, and G10, are generally polyadenylated "early" during maturation whereas mRNAs that contain only a CPE, such as the cyclins and wee1, are generally polyadenylated "late" (Charlesworth et al., 2004). Recently, a protein called Musashi, has been identified as a regulator of PRE mediated polyadenylation, much like CPEB regulates polyadenylation of CPEs (Charlesworth et al., 2006).

It should be noted that although an mRNA that has been modified to contain only a PRE can undergo polyadenylation, the polyadenylation is less than that seen in wild-type mRNA. Therefore, it's proposed that although early CPEB-independent polyadenylation of mRNAs is capable of promoting some translation, maturation likely requires subsequent activation of CPEB to promote CPEB-mediated polyadenylation and translation which is a more robust activator of translation, guaranteeing that maturation will occur completely. Furthermore, the presence and orientation of both CPEs and PREs may, in part, regulate the temporal order in which each transcript is polyadenylated during maturation (Charlesworth et al., 2004; Charlesworth et al., 2006).

Translational regulation—Polyadenylation binding proteins

Several proteins have been implicated in mediating translation of mRNAs in Xenopus. Upon activation of polyadenylation, it is proposed that CPEB recruits factors that lead to polyadenylation and 5' cap ribose methylation of the transcript (Kuge et al.,

1998). For those transcripts that undergo cytoplasmic polyadenylation in response to steroids, it has been demonstrated that CPEB binds additional factors, Maskin and the cap-binding factor eIF4E (Stebbins-Boaz et al., 1999). However, this alone is not sufficient for translation of the transcript. In fact, Maskin prevents the association of eIF4G to eIF4E, which is required for recruitment of the complex to the 40S ribosomal subunit-containing complex (Gingras et al., 1999) (Mendez and Richter, 2001). Therefore, other proteins are required to overcome this inhibitory complex.

Recently it has been shown that another protein, polyadenylation binding protein (PABP), is responsible for dissociating the Maskin-eIF4E association so that eIF4G can bind to eIF4E and stimulate translation (Cao and Richter, 2002). Furthermore, an interaction between PABP and eIF4G has been shown to be critical for translational control of maternal mRNAs during Xenopus development (Wakiyama et al., 2000; Wilkie et al., 2005). In those studies, translational regulation of *Mos* mRNA was specifically examined, and disruption of the PABP/eIF4G association resulted in a loss of MOS protein expression and prevented steroid-induced maturation. As a result, a working model is proposed. In this model, it is believed that several molecules of PABP bind the newly synthesized poly(A) tails of CPE-containing mRNAs via one of its many RNA binding domains, and this binding leads to dissociation of the Maskin-eIF4E complex, recruitment of eIF4G due to its association with PABP, and subsequently binding of eIF4G to eIF4E, promoting the translation of polyadenylated transcripts at the ribosomes (Fig 1-2)(adapted from Cao and Richter, 2002).

PABP activity is likely not restricted to regulating transcription of CPEB-dependent polyadenylated transcripts, and probably regulates translation of several

polyadenylated transcripts during maturation through a similar mechanism. Interestingly, PABP has been identified as an important factor mediating deadenylation of mRNAs during oocyte maturation such that overexpression prevents translational inactivation of some mRNAs (Voeltz et al., 2001; Wormington et al., 1996). Therefore, PABP may play a role in the timing of protein expression during maturation and early development due to its ability to promote both translational activation and inactivation. Finally, it is unclear if the activity of PAPB is affected by another protein, or set of proteins, but some evidence suggests that at least its function in translation may be regulated, in part, by the activity of scaffolding proteins (Woods et al., 2005).

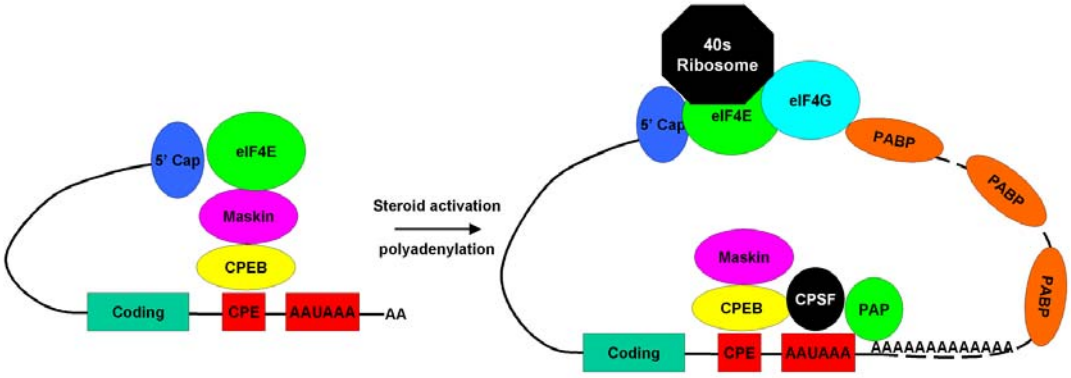


Figure 1-2: PABP regulation of translation. Adapted from (Cao and Richter, 2002). Maskin prevents association of eIF4E with eIF4G due to its association with cytoplasmic polyadenylation element binding protein (CPEB). Upon activation with steroid, CPEB is activated and it recruits polyadenylation polymerase (PAP), cleavage and polyadenylation specificity factor (CPSF), leading to polyadenylation of the transcript. Presence of a poly(A) tail then allows for PABP to be recruited to the transcript, opening up the mRNA-protein complex and bringing in its known binding partner eIF4G. After accumulation of enough PAPB molecules, Maskin association with eIF4E is disrupted, so that eIF4G can bind eIF4E and the 5'CAP. This association, in addition to other factors, allows for binding of the mRNA-protein complex to the 40s ribosome, activation translation of the transcript.

Protein Synthesis Requirement

As previously discussed, cytoplasmic polyadenylation has been extensively studied in oocyte maturation, and the polyadenylation of these messages leads to the translation of a subset of proteins. It has clearly been shown that protein synthesis is required for oocytes to undergo GVBD, indicating that translation of these proteins is necessary for initiation and completion of meiotic maturation. The requirement for protein synthesis is not unique to *Xenopus* oocytes as indicated by the inability of cow, goat, pig and sheep oocytes to undergo GVBD in the presence of protein synthesis inhibitors (reviewed in Schmitt and Nebreda, 2002). Studies suggest that there is no requirement for protein synthesis in mouse oocyte GVBD, but protein synthesis is required for MAPK activation, completion of meiosis after GVBD, and maintenance of the metaphase II arrest (Gavin et al., 1994; Verlhac et al., 1993; Verlhac et al., 1996).

Although only a few proteins are synthesized *de novo*, MOS and cyclin B1 among the most studied, there is a substantial increase in kinase activity in the oocytes as a result of the steroid-induced protein synthesis (Guerrier et al., 1977; Maller et al., 1977). A few kinases, such as Eg2 and p70 S6K, have been described as becoming active shortly after steroid induction. These have already been previously described. A few hours after steroid stimulation, an increase in activity of the members of the MAPK cascade, including MOS, MEK1, and MAPK, are observed. Finally, immediately prior to GVBD, MAPK activation leads to the activation of cell cycle regulators, such as CDK1 and cdc25. Interestingly, activation of the MAPK and cell cycle proteins appear to be dependent on each other due to an extensive positive feedback network that will be highlighted below.

The Protein Kinases

MOS

MOS is one of the earliest detected proteins that must be synthesized during maturation. MOS is a cytoplasmic homolog of the moloney murine sarcoma viral oncogene that appears to be exclusive to vertebrates and expression is largely restricted to germ cells (reviewed in Ferrell, 1999b). Functionally, MOS has a function similar to Raf-1 in that it can activate MEK1 (Resing et al., 1995). Therefore, MOS is a MAPKKK that serves as the trigger for activating the MAPK cascade in the oocytes whereby MOS activates MEK1, which in turn activates MAPK. Studies in mouse suggest that MOS may also be able to promote MAPK activity indirectly, by inhibition of a MAPK phosphatase (Verlhac et al., 2000).

In *Xenopus*, MOS is important for regulating meiosis at several stages. The accumulation of MOS protein is required for initiating meiotic re-entry of prophase I arrested oocytes. If MOS protein accumulation is inhibited by microinjection of MOS specific antisense oligonucleotides, steroids are not able to induce sustained MAPK activation nor GVBD (Keady et al., 2007). MOS has been shown to be critical for suppression of DNA replication during the meiosis I to meiosis II transition and for holding oocytes in the second meiotic arrest at metaphase II in preparation for fertilization (Dupre et al., 2002). MOS has also been described as conferring cytostatic factor (CSF) activity such that if it is injected into cleaving blastomeres, it can cause metaphase arrest in mitosis (Masui and Markert, 1971; Sagata et al., 1989). Interestingly,

upon fertilization, MOS levels decline presumably so that CSF activity is eliminated and cell cycle can occur normally.

The role for MOS in mammalian oocyte maturation differs from that of *Xenopus* oocyte maturation. MOS knockout mouse studies have indicated that although MOS does not appear to be required for GVBD in mouse oocytes, it is important for MAPK activation, suppression of DNA replication during interphase, and maintenance of meiotic arrest in mouse oocytes at metaphase II (Araki et al., 1996)(reviewed in Sagata, 1997). These data correlate with protein synthesis inhibition studies conducted in mouse oocyte maturation studies where protein synthesis was not required for GVBD, but was required for holding the second meiotic arrest (Downs, 1990; Hashimoto and Kishimoto, 1988; Kanmera et al., 1995). In these studies, MOS protein would not be made in the mouse oocytes, therefore the results phenocopy that of the MOS knockout studies.

Lastly, MOS can induce maturation without any upstream activation. Oocytes that are injected with purified MOS protein exhibit activation of CDK1 with subsequent GVBD (Yew et al., 1992). Therefore, careful regulation of MOS protein levels is critical to appropriate timing of oocyte maturation within the ovary. This appears to be guaranteed by regulation of it translation and degradation. *Mos* mRNA is abundantly present in immature oocytes, but protein is only detectible when cytoplasmic polyadenylation has been triggered by maturation promoting signals. Furthermore, it's been proposed that the immature oocyte uses proteasomal degradation to destroy any small amounts of MOS that may be translated inadvertently in the absence of maturation signals, preventing accidental activation of the maturation signaling pathway at the wrong time (Ishida et al., 1993).

MEK1

MEKs are dual-specificity threonine/tyrosine kinases that can activate either the p42/ERK2 or p44/ERK1 MAP kinases by phosphorylating key threonine and tyrosine residues within the activation loops. In *Xenopus* oocytes, MEK1 has been shown to be the kinase that is activated by MOS, and responsible for subsequent phosphorylation and activation of p42/ERK2 MAPK (Kosako et al., 1994; Resing et al., 1995). MEK1 is required for steroid-induced oocyte maturation as indicated by inhibition of maturation in response to injection of antibodies against MEK1 or treatment of oocytes with the MEK inhibitor PD98059 (Cross and Smythe, 1998; Kosako et al., 1994). Furthermore, MEK1 activation is sufficient to induce maturation since injection of constitutively active forms of MEK1 causes spontaneous maturation (Gotoh et al., 1995; Huang et al., 1995).

MAPK

MAPK (mitogen activated protein kinase) is a serine/threonine kinase that is most often recognized as a mediator of the mitogenic response to various extracellular signals. Thus, MAPK has also earned the name, extracellular signal regulated protein kinase (ERK) (Thomas, 1992). Although *Xenopus* somatic cells express two forms of MAPK, p42/ERK2 or p44/ERK1, oocytes and early embryos only express p42/ERK2 MAPK and essentially all of it is activated during steroid-mediated maturation (Ferrell et al., 1991; Gotoh et al., 1991; Jessus et al., 1991; Posada et al., 1991). If MAPK activation is prevented by expression of a MAPK phosphatase, oocytes can no longer undergo GVBD in response to steroid suggesting that MAPK activation is critically required for steroid-induced maturation (Gotoh et al., 1995).

Many studies show that MAPK is activated in a pattern that parallels the timing of MOS expression which does not occur until hours after steroid stimulation (Ferrell et al., 1991). Furthermore, injection of oocytes with activated MAPK is capable of inducing spontaneous maturation, just as is demonstrated with injection of MOS or active MEK1 (Haccard et al., 1995). Based on these and other results, most agree that MOS is responsible for activating MEK1, which subsequently activates MAPK. As alluded to earlier, some studies suggest that MAPK is actually activated in a biphasic pattern (Fisher et al., 1999; Fisher et al., 2000). In these studies, maturing oocytes were shown to exhibit an early, transient activation of MAPK that appears between 15-30 minutes after steroid addition. This activation is not sustained, however, and is subsequently followed by a dramatic increase in MAPK activity typically seen several hours after steroid induction. The first phase of MAPK activation does not require MOS expression since injection with mos antisense oligos did not prevent this activation, but inhibition of MOS expression did prevent the second wave of MAPK activation and GVBD did not occur (Keady et al., 2007). Furthermore, this work suggested that early MAPK activation may play a role in early CPEB phosphorylation and/or polyadenylation of *Mos* mRNA in a CPEB-independent fashion as described previously, but its requirement for maturation is unclear. In all, these data indicate that although MAPK may be activated early, the second phase of MAPK activation is required for maturation to occur completely and depends on the expression of MOS protein. The early transient MAPK activation may play a role in activation of early signals that lead to expression of MOS, its more potent promoter.

MPF activation

In general, reentry into either meiotic or mitotic M phase requires the activation of complexes composed of cdc2 and cyclins. cdc2 is a kinase that is responsible for initiating the G2/M transition, but it is not active unless associated with the appropriate cyclin protein. Therefore, cdc2 is also referred to as cyclin-dependent kinase 1 (CDK1) (Doree and Hunt, 2002). In *Xenopus*, the relevant complex is that of CDK1 and cyclin B1, often called MPF, but is held in an inactive state presumably by two mechanisms: inactivating phosphorylations of CDK1 and the low basal expression of cyclin B1 (reviewed in Ferrell, 1999b).

CDK1 activation is required and appears to be sufficient for GVBD (reviewed in Doree and Hunt, 2002). In immature oocytes, Myt1 is a p34cdc2 inhibitory kinase responsible for keeping CDK1 in a phosphorylated, thus inactive, state (Mueller et al., 1995). Just prior to GVBD, a protein phosphatase, cdc25, is activated by phosphorylation and cdc25 is then capable of dephosphorylating CDK1 at key residues (Izumi et al., 1992; Karaiskou et al., 1998), while Myt1 is inactivated by phosphorylation (Palmer et al., 1998). As mentioned, dephosphorylation of CDK1 alone is not sufficient for its activation—it also must complex with its cofactor, cyclin B1. Immature oocytes contain only low levels of cyclin B1 protein, therefore expression depends on translational regulation by late-CPEB dependent cytoplasmic polyadenylation (Fig 1-1)(Nakahata et al., 2003; Stebbins-Boaz et al., 1996). After enough cyclin B1 protein has been synthesized, complete activation of MPF can occur.

How does MAPK lead to MPF activation? MAPK activation has been shown to reduce inactivation of CDK1 presumably by inactivating Myt1 (Abrieu et al., 1997).

Furthermore, p90 (Rsk), a known target of MAPKs, was shown to inhibit Myt1 activity (Palmer et al., 1998). More recent evidence suggests a more direct link between MAPK and CDK1 activation whereby MAPK activates cdc25 phosphatase (Wang et al., 2007). The MAPK cascade may also affect cyclin B1 levels. deMoor and Richter show that the 3'UTR contains a specific orientation of the CPE and hexanucleotide sequences which results in a MOS "dependence" for polyadenylation of cyclin B1 mRNA (de Moor and Richter, 1997). Finally, MAPK can phosphorylate the nuclear export sequence (NES) of cyclin B1, helping promote its translocation, and as a result relocalization of its binding partner CDK1, to the nucleus where CDK1 functions (Huo et al., 2005). Therefore, the MAPK cascade may be capable of enhancing CDK1 activation by promoting dephosphorylation of CDK1, polyadenylation of cyclin B1, and relocalization of active MPF to the nucleus (Fig 1-1)(Heald et al., 1993; Izumi and Maller, 1991).

The positive-feedback kinase loops

The MAPK feedback loop

By definition of a MAPK cascade, it is logical to imagine a linear sequence such that MOS activates MEK1, which in turn activates MAPK, leading to the dephosphorylation and activation of CDK1 resulting in GVBD. However, the oocyte is an exquisite example of a cellular switch that is created in large part due to a potent positive feedback loop involving the kinase cascade which guarantees that an oocyte exhibits an all-or-none response (reentry into meiosis) upon exposure to maturation signals.

Generally, MOS is considered the trigger of the MAPK cascade, but studies have shown that microinjection of activated forms of either MEK1 or MAPK leads to the accumulation of MOS protein as long as protein synthesis is permitted (Gotoh et al., 1995; Matten et al., 1996; Roy et al., 1996). This MAPK regulation of MOS can be explained, in part, by the ability of MAPK to enhance MOS protein accumulation by two mechanisms: enhancing *Mos* mRNA polyadenylation and reducing MOS protein degradation. Cytoplasmic polyadenylation of *Mos* mRNA was shown to be specifically enhanced by MAPK activation, thus stimulating MOS translation (Charlesworth et al., 2002; Howard et al., 1999). MAPK can also stabilize MOS protein levels by phosphorylation of the serine 3 residue of MOS which prevents proteasomal degradation of the protein (Matten et al., 1996; Nishizawa et al., 1992; Sheng et al., 2002). *In vivo*, it is believed that small amounts of MOS accumulation mid-way through maturation result in the activation of small amounts of MAPK. However, this small amount of MAPK activation can feedback and enhance accumulation of the upstream kinase, MOS, resulting in stronger activation of the MAPK cascade. As a result of this potent positive feedback loop, nearly all of the molecules of MEK1 and MAPK in the oocyte are activated just prior to GVBD (Fig 1-1)(Ferrell, 1999a).

CDK1 feedback and integration with the MAPK cascade

CDK1 is also suspected to play a part in a positive feedback kinase system. In order to be activated, CDK1 must be dephosphorylated by the protein phosphatase cdc25 in a two-step process (Borgne and Meijer, 1996). The activation of cdc25 is achieved by phosphorylation by several kinases, including active MAPK (Wang et al., 2007), polo-

like kinase (PLK) and notably MPF (active CDK1/cyclin B) (Abrieu et al., 1998; Karaiskou et al., 1999). Therefore, a threshold amount of active CDK1/cyclin B1 is capable of enhancing its own activity by further activating cdc25 (Fig 1-1). Interestingly, unlike the MAPK cascade feedback loop, the CDK1-cdc25 positive feedback loop will turn itself off. After MPF has been active for some period of time, it begins to cause the degradation of cyclins, inactivating CDK1, and turning off the positive feedback loop (Ferrell, 1999a).

Finally, it is important to highlight the integration of both the MAPK and CDK1 positive feedback loops (Fig 1-1). As previously discussed, it is clear that MAPK enhances MPF activation by promoting dephosphorylation of CDK1 and nuclear localization of MPF while MOS may play a role in promoting cyclin B1 synthesis. On the other hand, CDK1 has been proposed to reciprocally feed into the MAPK feedback loop since there is evidence CDK1 can enhance MOS protein stability by phosphorylating the serine 3 residue (Castro et al., 2001; Nebreda et al., 1995). Therefore, the ability of both the MAPK and CDK1 feedback loops to partially control each other seems to ensure profound amplification of early signals such that maturation will always occur completely when an oocyte receives the correct maturation signal.

Conclusions

For decades, the study of oocyte maturation has provided valuable insight into molecular pathways that regulate cell cycle activation and cytoplasmic translational activation of mRNAs. Unfortunately, progress has been slow in understanding how early cellular changes are transmitted to late signaling events such as polyadenylation and kinase activation, let alone what factors may be required for mediation of the positive feedback loops observed during maturation. Therefore, ongoing research will likely be focused on understanding what other proteins may be required for maturation, and how they may function to integrate the large array of signals that are activated during maturation.

Chapter Two

Paxillin is Required for Oocyte Maturation

Introduction

Female fertility relies on precise regulation of oocyte meiosis. In Xenopus oocytes, maturation appears to be triggered by nongenomic actions of androgens, whereby steroids activate maturation in a "release of inhibition" fashion, causing a decline in cAMP levels, leading to activation of a series of downstream kinases and subsequently germinal vesicle breakdown (GVBD) (reviewed in Chapter 1) (Fig. 1-1). Of the kinase cascades that are activated during maturation, the MAPK cascade is one of the most studied.

At the top of the MAPK cascade is MOS, an oocyte-specific mitogen-activated protein kinase (MEK) kinase similar to Raf (Fig. 1-1 and 2-7). The expression of MOS is tightly regulated by steroid-triggered cytoplasmic polyadenylation, such that increased polyadenylation of *Mos* mRNA in response to steroids leads to increased MOS protein expression. MOS then activates MEK1 (Resing et al., 1995), which in turn activates extracellular signaling regulated kinase 2 (ERK2, or p42), and finally cyclin-dependent kinase 1 (CDK1), a component of maturation promoting factor (MPF) (reviewed in Chapter 1). A fascinating feature of this steroid-activated kinase cascade is that it constitutes a powerful, all-or-none positive feedback loop that amplifies small, reversible early signals into large, irreversible late effects (Ferrell and Machleder, 1998)(highlighted in Fig.1-1 and 2-7). Activated ERK2 then further increases MOS protein expression and activity, thus markedly enhancing the signaling cascade (Castro et al., 2001; Howard et

al., 1999; Nebreda et al., 1995). While this unusual positive feedback loop has been documented for years (Gotoh et al., 1995; Maller and Krebs, 1980; Matten et al., 1996; Roy et al., 1996), little progress has been made in identifying factors that regulate its activity.

The purpose of these studies was to identify new proteins that may be required to regulate meiotic maturation downstream of steroid activation in the oocyte. Specifically we sought to identify proteins that may regulate activation of the MAPK cascade. Using candidate approach, we focused on the adapter protein, Paxillin. Paxillin is a member of the group 3 subfamily of LIM domain proteins, that has largely been studied as a modulator of many physiological pathways, including matrix organization, cytoskeletal regulation, and focal adhesion turnover in response to integrin- and growth factor-mediated signals(Brown and Turner, 2004). Notably, Paxillin is an ideal candidate for a regulator of intracellular signaling, as it contains several protein binding domains, including many N-terminal leucine-rich LD motifs and C-terminal double zinc finger LIM domains, that are associated with Paxillin's ability to rapidly create highly dynamic scaffolds upon cellular activation (Brown et al., 1996; Schaller, 2001; Tumbarello et al., 2002). Interestingly, Paxillin and Hic-5, another group 3 LIM domain family member, have been identified as co-activators of the androgen receptor (Fujimoto et al., 1999; Kasai et al., 2003). Furthermore, in growth factor-stimulated mammalian somatic cells, Paxillin interacts with and regulates the activity of several components of the MAPK signaling cascade, including Raf, MEK, and ERK (Ishibe et al., 2003; Liu et al., 2002) and appears to be critical for normal MAPK activation in response to some extracellular stimuli (Hagel et al., 2002).

The aim of these studies was to determine if Paxillin plays a role in steroid-induced maturation, either by enhancing nongenomic AR signaling early in maturation or regulating late signals such as the MAPK cascade. We detected Paxillin expression in *Xenopus* oocytes, and, through knockdown experiments, showed that Paxillin is required for oocyte maturation. Rather than playing a role early in maturation, however, we found that Paxillin appears to be critical for the accumulation of MOS protein, perhaps by regulating the translation of *Mos* mRNA.

Materials and Methods

Antibodies—Anti-Mos (sc-86) was obtained from Santa Cruz Biotechnology, anti-HA (PRB-101) from Covance, anti-phospho-p44/42 MAPK (#9101) and anti-total p44/42 MAPK (#9102) from Cell Signaling Technologies. Polyclonal antibodies directed to XePaxillin amino acids 468-484 were generated by Biosynthesis Inc. (Lewisville, TX).

Plasmid construction—cDNAs encoding XePaxillin, XeMOS, and the 3'UTR of *Mos* mRNA were cloned into pcDNA3.1(+) (Invitrogen) for eukaryotic expression and pGEM HE (from L. Jan, University of California, San Francisco, CA) for *Xenopus* oocyte expression. Primers used to clone XePaxillin and XeMOS included sequence to incorporate an amino-terminal HA tag.

Oocyte Preparation—Oocytes were harvested from female *Xenopus laevis* (NASCO) and treated as described (Lutz et al., 2000). Briefly, follicular cells were removed by incubation of ovaries in 0.8 mg/ml collagenase A (Roche) in modified Barth's solution (MBSH) without Ca^{2+} for 3-4 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 were performed on stage V/VI oocytes from each preparation to determine sensitivity to 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.

RNA synthesis and injections—The pGEM HE constructs were linearized with NheI, except for the *Mos* 3'UTR reporter, which was linearized with XbaI. Capped cRNA was transcribed *in vitro* with T7 RNA polymerase according to manufacturers protocol (Promega). RNA was suspended in injection buffer (10mM HEPES pH 7.4) and Stage V/VI oocytes were injected with 20ng cRNA using a Drummond automatic injector. For double stranded RNA (dsRNA) injections, equal concentrations of forward and reverse XePaxillin cRNA were mixed, heated to 90°C, and allowed to slowly cool to 30°C. Oocytes were then injected with 40ng dsRNA. The sequences of the sense and antisense Paxillin oligonucleotides were C*T*A*GAGGCGACATGGATGATCT*G*G*A and T*C*C*AGATCATCCATGTCGCCTC*T*A*G, respectively, with phosphorothioated bonds indicated by asterisks.

Oocytes were injected with 25ng oligonucleotides in each experiment. For the rescue studies, XePaxillin was recloned by PCR-directed mutagenesis so that nucleotides at wobble positions in the first 6 codons were changed, preserving the amino acid sequence but preventing antisense oligonucleotides from binding. In each rescue experiment, oocytes were co-injected with 25 ng oligonucleotide and 20 ng rescue cRNA.

After all injections, oocytes were incubated at least 36-48 hours before beginning any assay.

Steroid Maturation Assays—Maturation assays were conducted by incubating 20 oocytes per condition with the indicated concentration of testosterone (Steraloids) or ethanol in MBSH for 12-16 hours, at which time oocytes were scored for GVBD (visualization of a white spot on the animal pole). Dilutions were performed such that ethanol concentration was held at 0.1%. In experiments with the proteasome inhibitor, MG132 (Calbiochem), oocytes were pretreated with 50μM MG132 or 0.1% DMSO for 2 hours. Oocytes were then incubated with the indicated concentration of testosterone in addition to either MG132 or DMSO.

Western blots—Oocytes were incubated with steroid or vehicle, permeabilized in 20 μl/oocyte oocyte lysis buffer (1% Triton X-100 50mM Tris-HCl pH 7.6, 150 mM NaCl, 2mM EDTA, 2 mM NaF, 0.5 mM NaVanadate, 100 μg/mL PMSF), and microfuged at 14,000 x g for 10 minutes to remove yolk and other debris. The cleared supernatants were then mixed 1:1 with 2x SDS buffer. The equivalent of 0.5 oocytes was loaded in each lane for SDS-PAGE, transferred to Immobilon-P membranes (Millipore), blocked in 5% TBST-milk for 1 hour, then incubated with primary antibody overnight at 4°C (1:5000 for anti-HA, 1:5000 for pre-immune and immune sera, 1:1000 for α-MOS, 1:2000 for α-phospho-p42 ERK2). Membranes were then incubated with HRP-conjugated secondary antibody for 1 hour, and signal detected by ECL (Amersham Pharmacia). Total ERK blots were generated by stripping phospho-ERK blots, then incubating with 1:1000 α-total p42 ERK2 antibody.

Mos polyadenylation assays and Northern Blots—Polyadenylation of *Mos* RNA was determined as described (Howard et al., 1999). Briefly, 36 hours after dsPax or AS injections, 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 20μg of total RNA was run on a 2.2% agarose formaldehyde gel in 1xMOPS buffer for ~10 hours at 60V. RNA was transferred to a Nytran SuPerCharge nylon membrane (Schliecher & Schuell) by downward transfer in 20xSSC (Schliecher & Schuell) and UV crosslinked. Membranes were pre-hybridized in ExpressHyb (Clonetech) at 68°C for 30 minutes, then probed with [^{32}P]-dCTP labeled mos 3'UTR DNA (Rediprime II, Amersham Biosciences) for 1 hour, washed 2x in 2xSSC, 0.05% SDS, 2x in 0.1x SSC, 0.1% SDS, then subjected to autoradiography.

To confirm knockdown of Paxillin mRNA by Northern analysis, 20μg of total RNA from sense and antisense injected oocytes were treated as in the polyadenylation assay with the following changes. Samples were run on a 1% agarose formaldehyde gel for 3 hours at 90V. The membrane was probed with [^{32}P]-dCTP labeled Paxillin DNA encoding nucleotides 585-858. The membrane was stripped by adding to boiling 0.1%SDS, allowed to cool for 15 minutes, then repeated. The membrane was pre-hybridized again, then probed with a [^{32}P]-dCTP labeled *Xenopus* GAPDH DNA probe encoding nucleotides 171-471.

Immunohistochemistry—Oocytes were injected with either vehicle, 40 ng dsXePax RNA, or 25 ng of the indicated oligonucleotides. After 48 hours, the oocytes

were fixed in paraffin, sectioned, and mounted on slides (Molecular Pathology Core Facility, UTSW). After blocking with horse serum, slides were incubated overnight with a 1:500 dilution of rabbit serum containing an anti-XePaxillin antibody directed against residues 468-484 or its corresponding pre-immune serum from the same rabbit. XePaxillin was then detected using the Vectastain ABC kit per manufacturers protocol and viewed and photographed using a Nikon microscope and digital camera.

Results

Paxillin is expressed in Xenopus laevis oocytes

A cDNA encoding a 539 amino acid isoform of Xenopus laevis Paxillin (XePaxillin) protein (BAA96456) was cloned from oocyte mRNA using reverse-transcription polymerase chain reaction (Ogawa et al., 2001). XePaxillin shares approximately 80% overall identity with human Paxillin, with 100% identity in two SH2 binding sites, five LD motifs, and four LIM domains (Fig. 2-1). Northern blot analysis confirmed the presence of Paxillin mRNA, with two isoforms detected (Fig. 2-2C), and Paxillin mRNA expression was unaffected by steroid treatment (data not shown). Immunohistochemistry using antibodies against XePaxillin revealed specific staining throughout the oocyte, including both nuclear and cytoplasmic regions (Fig. 2-2A). Interestingly, the nuclear staining of XePaxillin was the strongest, consistent with subcellular localization in mammalian somatic cells. Notably, the signal using the anti-XePaxillin serum was considerably stronger than that seen using the corresponding pre-immune serum (Fig. 2-2A), illustrating the specificity of the immunohistochemistry. Finally, the anti-Paxillin serum (but not the corresponding pre-immune serum)

recognized the over-expressed carboxyl-terminal half of XePaxillin, by Western blot (Fig. 2-2B), further confirming the specificity of the antibody. Unfortunately, although it specifically detected endogenous Paxillin by immunohistochemistry, our antibody was not sensitive enough to detect endogenous Paxillin protein by Western blot.

Paxillin is required for steroid-triggered maturation

To determine whether Paxillin was required for steroid-induced oocyte maturation, over-expression and depletion studies were performed. Overexpression of Paxillin by cRNA injection had no effect on testosterone-induced maturation as compared to mock-injected control oocytes. In contrast, knockdown of endogenous Paxillin expression by injection of full-length double-stranded XePaxillin RNA (dsRNA) dramatically inhibited oocyte maturation in response to testosterone (Fig. 2-3A). Significant reduction in Paxillin expression was confirmed by immunohistochemistry (Fig. 2-2A, left). Injection of non-specific dsRNAs did not affect maturation (data not shown), confirming the specificity of the XePaxillin dsRNA injections. Of note, RNA interference in Xenopus and mouse oocytes has been used by us and others with excellent success, and with an absence of nonspecific effects (Anantharam et al., 2003; Gordon et al., 2006; Haas et al., 2005; Lutz et al., 2003; Mehlmann, 2005; Stein et al., 2005).

The inhibitory effect of reducing Paxillin expression by dsRNA injection was confirmed by knocking down Paxillin expression using antisense phosphorothioated oligonucleotides directed against the start codon of the XePaxillin mRNA. This technique has been used extensively to reduce mRNA and protein expression in Xenopus leavis oocytes (Tao et al., 2005; Torpey et al., 1992; Vernos et al., 1995). Injection of the

XePaxillin antisense-, but not sense-oligonucleotides, markedly reduced XePaxillin mRNA expression by Northern blot, but did not affect mRNA expression of GADPH mRNA (Fig. 2-2C). As with the dsRNA injections, injection of the XePaxillin antisense oligonucleotides reduced endogenous Paxillin expression by immunohistochemistry (Fig. 2-2A, right) and inhibited steroid-induced oocyte maturation when compared to oocytes injected with the respective sense oligonucleotides (Fig. 2-3B). Importantly, the inhibitory effect of the antisense oligonucleotide injections on maturation could be rescued by over-expression of XePaxillin from injected cRNA that contained alternative DNA sequences at the region recognized by the antisense oligonucleotides (Fig. 2-3B). Confirmation of XePaxillin expression was confirmed by Western blot (inset to Fig. 2-3B). The ability of over-expressed XePaxillin to rescue antisense-injected oocytes confirmed that inhibition of maturation was a specific effect due to reduced expression of Paxillin protein in oocytes.

Paxillin functions late in the maturation signaling pathway

As mentioned, androgen-induced maturation occurs through a nongenomic AR-mediated mechanism that leads to increased *Mos* mRNA polyadenylation and subsequent MOS protein expression. MOS then activates MEK1, which activates ERK2. ERK2 then further stimulates MOS protein expression and activity, resulting in a potent positive feedback loop that ultimately activates maturation promoting factor (MPF), a complex of CDK1 and cyclin B, to trigger oocyte maturation (Ferrell, 1999b) (Fig. 2-7). To determine where in this pathway Paxillin functions, we first tested whether Paxillin was important for steroid-triggered MOS protein expression and downstream ERK2

phosphorylation. MOS accumulation and ERK2 phosphorylation were not affected when XePaxillin was over-expressed in oocytes (Fig. 2-4A). However, in oocytes injected with XePaxillin dsRNA, and therefore with decreased endogenous Paxillin, steroid-induced MOS protein expression and ERK2 activation were significantly reduced (Fig. 2-4A). These inhibitory effects were also seen in oocytes depleted of Paxillin by antisense, but not sense, oligonucleotide injection (Fig. 2-4B), confirming the specificity of the dsRNA results. Furthermore, insulin-induced ERK2 phosphorylation, which is mediated in a MOS-independent fashion, was unaffected by the loss of Paxillin by dsRNA injection, suggesting that Paxillin plays a specific role in steroid-induced activation of ERK2 by MOS (Fig. 2-4A, last two lanes).

Since Paxillin was necessary for normal MOS protein expression, we next determined whether Paxillin regulated the first step of MOS activation, *Mos* mRNA polyadenylation. The endogenous *Mos* mRNA is over 3000 base pairs long, making detection of changes in polyadenylation difficult. To overcome this problem, a reporter assay was used whereby oocytes were injected with a small cRNA that contains approximately 280 nucleotides of the 3' untranslated region of *Mos* mRNA (Howard et al., 1999). This region includes the required cis-regulatory elements that confer polyadenylation of *Mos* mRNA in response to steroid stimulation. Therefore, in oocytes, the injected small cRNA will be polyadenylated in response to steroid, and increases in its size can be detected by Northern blot. Over-expression of Paxillin had no effect on polyadenylation when compared to control oocytes, as seen by similar time-dependent shifts in mobility (Fig. 2-4C). Surprisingly, loss of Paxillin, either by dsRNA or antisense oligonucleotide injection, also had no effect on *Mos* mRNA polyadenylation

relative to mock- or sense-injected oocytes (Fig. 2-4C-D). These results suggest that Paxillin must be functioning downstream of *Mos* mRNA polyadenylation, but upstream of MOS protein expression.

Paxillin may be important for accumulation of MOS protein

To confirm that Paxillin functions upstream of MOS, we attempted to rescue ERK2 phosphorylation and oocyte maturation in Paxillin-depleted oocytes by over-expression of MOS protein. Oocytes injected with *Mos* cRNA containing a constitutive polyadenylation signal, in place of the endogenous 3'UTR, expressed MOS protein independent of steroid, leading to spontaneous activation of ERK2 and oocyte maturation (Fig. 2-5A and B, sense). As expected, *Mos* cRNA injection rescued the inhibitory effects in oocytes injected with Paxillin antisense oligonucleotides (Fig. 2-5A and B, antisense), resulting in equivalent expression of MOS, activation of ERK2, and rates of spontaneous maturation, as compared to sense-injected oocytes.

In theory, MOS protein levels could be elevated by either enhanced translation or decreased degradation. Clearly, MOS protein accumulation occurs in large part due to cytoplasmic polyadenylation of the *Mos* mRNA (Stebbins-Boaz et al., 1996), yet polyadenylation was unaffected in Paxillin deficient oocytes. On the other hand, it has been proposed that MOS protein is degraded in a proteasome-dependent manner that is inhibited by maturation-dependent phosphorylation of a key serine residue in the amino terminus of the protein (Castro et al., 2001; Nishizawa et al., 1993; Sheng et al., 2002). In an attempt to determine whether Paxillin regulates protein levels by allowing translation or decreasing degradation of MOS, we examined the effects of treatment with

a proteasome inhibitor, MG132. Inhibition with a proteasome inhibitor should rescue MOS protein levels, and perhaps maturation, in Paxillin-deficient oocytes if Paxillin is required for MOS protein stability. As expected, loss of Paxillin by dsRNA inhibited both testosterone-mediated maturation and MOS protein accumulation as compared to mock injected oocytes, but treatment with MG132 did not enhance MOS protein accumulation as was observed in MG132-treated control oocytes (Fig. 2-6B). Interestingly, MG132 did enhance maturation in response to testosterone in both sets of oocytes, although only partially in dsRNA injected oocytes (Fig. 2-6A). However, this was associated with only a modest increase of MOS and MAPK activity in control oocytes only, indicating that the effect of MG132 on maturation in Paxillin-depleted oocytes occurs in part through an alternate MOS-independent pathway. Although preliminary, these results suggest that Paxillin is not required for preventing MOS protein degradation, but instead may be important for enhancing MOS translation and subsequent MAPK activation (Fig. 2-7).

Discussion

Although oocyte maturation has been studied for decades, there is still much debate as to what signaling pathways are required, never mind how they might feed into one another. Many believe that some of the controversy will be put to rest once more novel regulatory proteins are identified. Paxillin proved to be an intriguing candidate for regulating oocyte maturation due to its extensively described ability to translate multiple extracellular signals into specific downstream intracellular signaling pathways (Brown and Turner, 2004). Our results demonstrate that Paxillin is a novel and essential regulator

of steroid-triggered oocyte maturation. Signaling studies suggest that Paxillin functions upstream of MOS protein expression, as it is important for the accumulation of MOS and activation of the downstream kinases MEK and ERK2, yet plays no role in polyadenylation of the *Mos* mRNA (Fig. 2-7). Therefore, Paxillin plays a crucial role in the MAPK signaling cascade responsible for the all-or-none switch that regulates steroid-induced oocyte maturation.

If Paxillin is enhancing MOS protein expression, then how is it doing so? One possibility is that Paxillin is inhibiting MOS protein degradation. Some studies have suggested that, without ERK signaling, MOS is generally unstable and rapidly degraded, possibly in an ubiquitin-mediated proteasome-dependent manner (Ishida et al., 1993; Nishizawa et al., 1993; Sheng et al., 2002). Upon activation with steroid, MOS degradation might be inhibited by phosphorylation of the serine 3 residue in an ERK2-dependent fashion (Matten et al., 1996; Nishizawa et al., 1992). However, in our hands, the proteasome inhibitor MG132 had no effect on MOS expression in oocytes with reduced Paxillin expression, suggesting that Paxillin was unlikely to be significantly affecting proteasome-mediated MOS degradation.

Instead, Paxillin may be enhancing MOS translation. Support for this hypothesis is found in other studies, where ERK2 seems to enhance MOS translation (Howard et al., 1999), and Paxillin appears to enhance translation of polyadenylated mRNAs at focal adhesions (Woods et al., 2005). Based on these studies, Paxillin is important for the activation of ERK2 due to its effects on MOS protein levels. It is possible that Paxillin may be required to permit translation of specific polyadenylated mRNAs, such as *Mos*. However, one must consider whether Paxillin plays a role in the positive feedback kinase

loop that is present in the oocyte (Ferrell, 1999b). ERK2 is capable of increasing MOS polyadenylation and translation, but it is unclear if other proteins are required for this activity of ERK. If Paxillin were required for ERK to enhance MOS expression, one might see results similar to the ones described here, whereby initial translation of small amounts of MOS produces only partial activation of ERK, but without Paxillin, ERK could not promote more MOS production resulting in incomplete activation of the MAPK cascade due to loss of the positive feedback loop.

Subsequent studies will be aimed at determining how Paxillin may regulate MOS protein expression. Ultimately, understanding how Paxillin regulates MOS protein expression may elucidate a novel way in which translation is regulated in the oocyte, whether it be a MAPK/positive feedback loop specific effect or a more general mechanism of translation in the oocyte.

Figure 2-1: Schematic of Paxillin functional domains. XePaxillin has 80% homology to mammalian isoforms of Paxillin. The amino terminal half has 5 LD motifs and the carboxyl terminal half has 4 LIM domains. Between the 1st and 2nd LD motif are 3 conserved phosphorylation sites. The first two regions, 31-YSFP-34 and 99-YSFP-102, are SH2 binding sites at which tyrosine phosphorylation has been reported. The 3rd region, 107-SAEPSP-112, contains two serine residues that are conserved Paxillin phosphorylation sites.

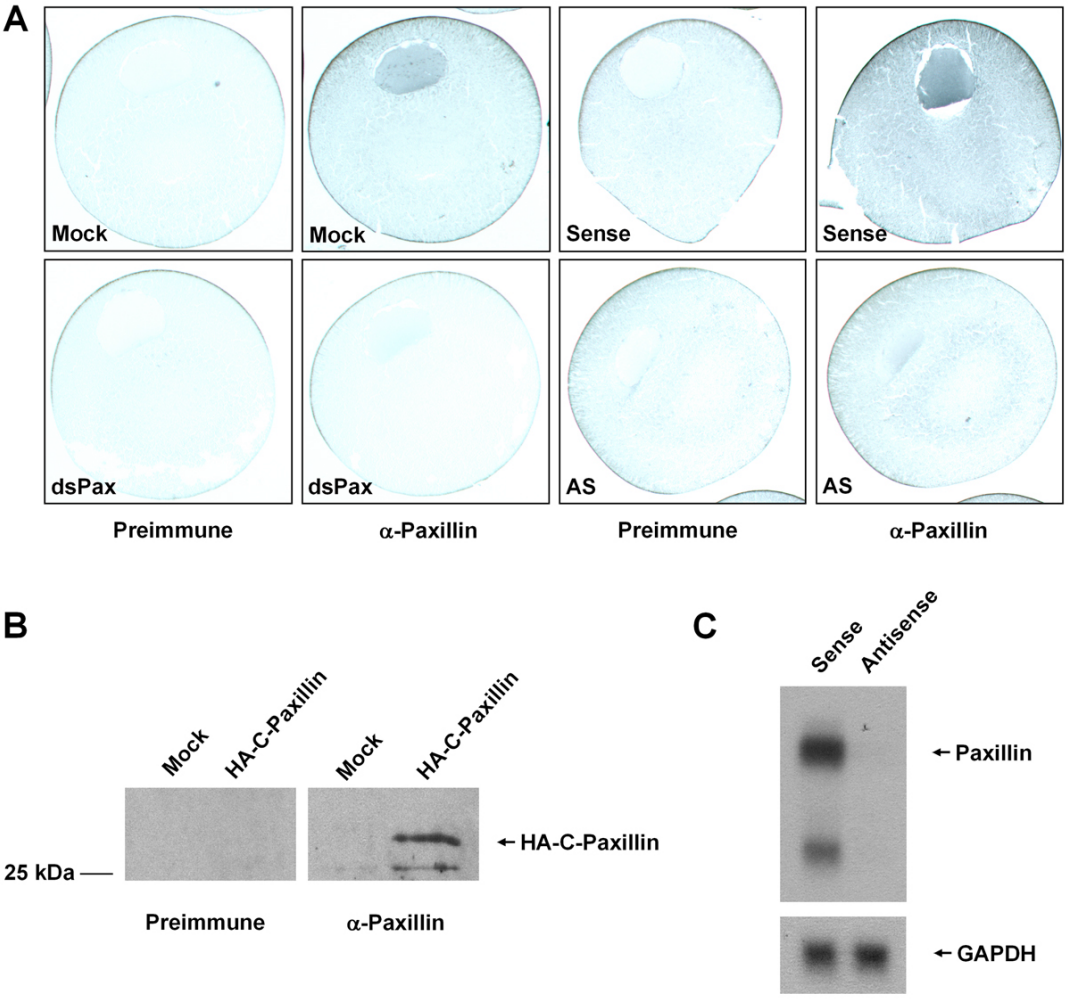


Figure 2-2: Paxillin is expressed in *Xenopus* oocytes and can be depleted. (A) Immunohistochemistry was performed on paraffin embedded sections of mock, XePaxillin dsRNA, XePaxillin sense oligonucleotide, or XePaxillin anti-sense oligonucleotide injected oocytes. Sections were incubated with equal concentrations of rabbit anti-Paxillin antisera or the corresponding preimmune serum. These are representative photos of multiple slides. (B) The anti-Paxillin, but not the corresponding pre-immune, serum recognizes the carboxyl-terminal half of XePaxillin when over-expressed in oocytes. Oocytes were injected with cRNAs encoding the indicated proteins and Western blots performed after 48 hours. (C) XePaxillin mRNA is present in sense-injected oocytes, but disappears in oocytes injected with XePaxillin anti-sense oligonucleotides, as detected by Northern blot (upper panel). Northern blots correspond to the mRNA prepared for Fig. 2-4D. GADPH mRNA levels were unchanged (lower panel).

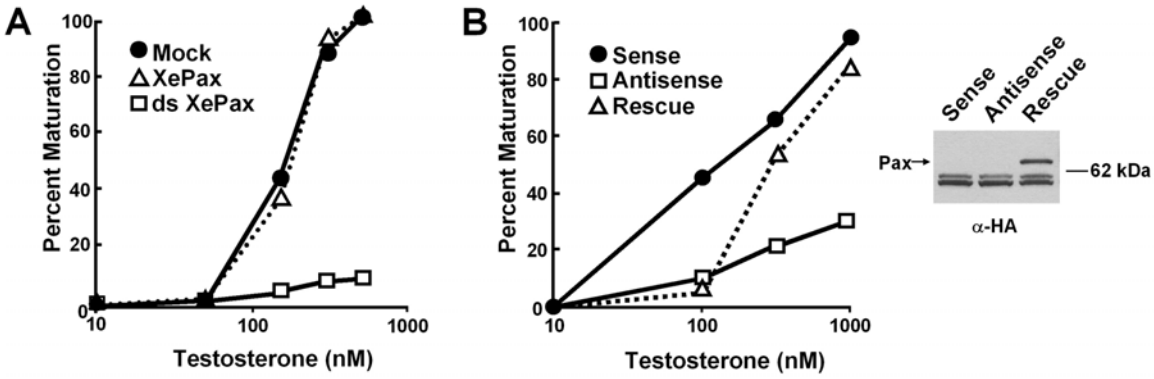


Figure 2-3: Paxillin is required for steroid-induced oocyte maturation. (A) Oocytes were injected with HA-tagged XePaxillin cRNA (XePax) to increase XePaxillin expression, double-stranded XePaxillin cRNA (ds XePax) to decrease XePaxillin expression, or 10mM Hepes (mock). After 48 hours, oocytes were incubated with the indicated concentrations of testosterone and maturation (GVBD) was scored after 16 hours. (B) Oocytes were injected with either sense or antisense oligonucleotides without and with HA-XePaxillin cRNA and then treated as in (A). Rescued oocytes contained HA-XePaxillin as measured by Western blot using an anti-HA antibody (inset). Results of dsRNA and antisense knockdown studies are representative of at least 5 experiments each.

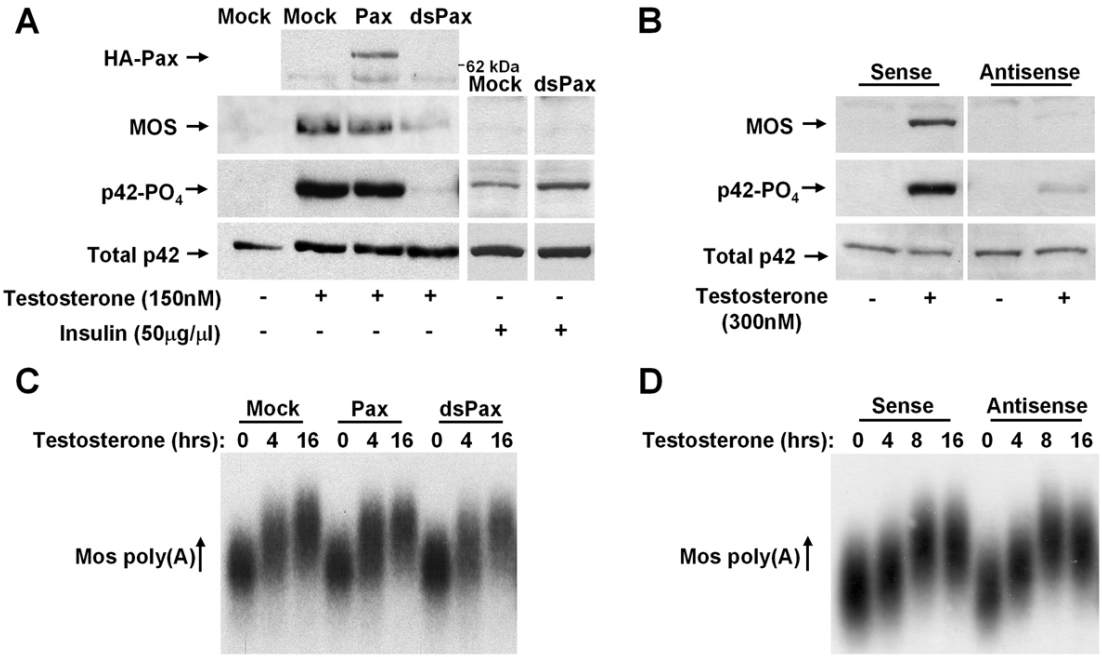
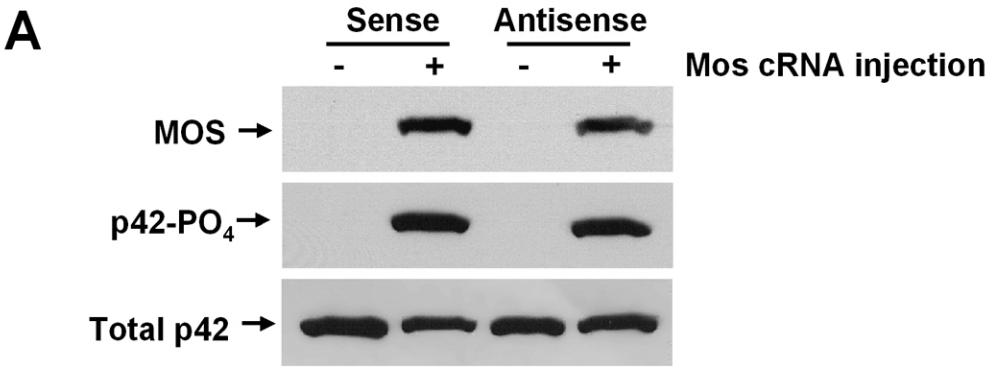


Figure 2-4: Knockdown of Paxillin blocks accumulation of MOS and activation of p42 ERK. (A) Oocytes were injected with HA-tagged XePaxillin (Pax), XePaxillin dsRNA (dsPax), or Hepes (mock). After 48 hours, oocytes were incubated with 150nM testosterone, 50 μg/ml insulin, or 0.1% ethanol. Oocytes were permeabilized at 4 and 8 hours and immunoblotted for HA-Pax, MOS (after eight hours), or phospho-p42 ERK (p42-PO_4) (after 4 hours). Phospho-p42 ERK blots were stripped and re-probed for total p42 ERK. (B) Oocytes injected with either sense or antisense Paxillin oligonucleotides were stimulated with 300nM testosterone and lysates probed as in panel A. (C-D) Oocytes were injected with the indicated reagents as described above. Oocytes were then injected with 10 ng *Mos* 3'UTR polyadenylation reporter RNA after forty hours, and stimulated with 300 nM testosterone or ethanol. RNA was extracted at the indicated times and equal amounts of RNA loaded per lane. Polyadenylation of the 3'UTR reporter was analyzed by northern blot using radiolabeled *Mos* 3'UTR DNA as the probe. Slower migration of RNA represents increased polyadenylation. All data represent one of at least three experiments with essentially identical results. Twenty oocytes per point were used for each data set, and the experiments for (A) and (C) were performed on the same set of injected oocytes at the same time with the same reagents



B

Spontaneous Maturation

	Mock	MOS
Sense	0%	66%
AS	0%	66%

Figure 2-5: MOS rescues the inhibition of maturation from decreased Paxillin expression. (A) Oocytes were injected with 5 ng *Mos* cRNA or 10mM Hepes (mock) forty hours after injection of oocytes with sense or antisense Paxillin oligonucleotides (same set of oocytes as Fig 2-4 B and D). Oocytes were permeabilized after 18 hours and lysates probed for MOS and phospho-p42 ERK (A). The lower panel represents a stripped phospho-p42 ERK blot probed for total p42 ERK. The percentage of spontaneous maturation induced by injection of *Mos* cRNA (MOS) after 18 hours was also measured (B).

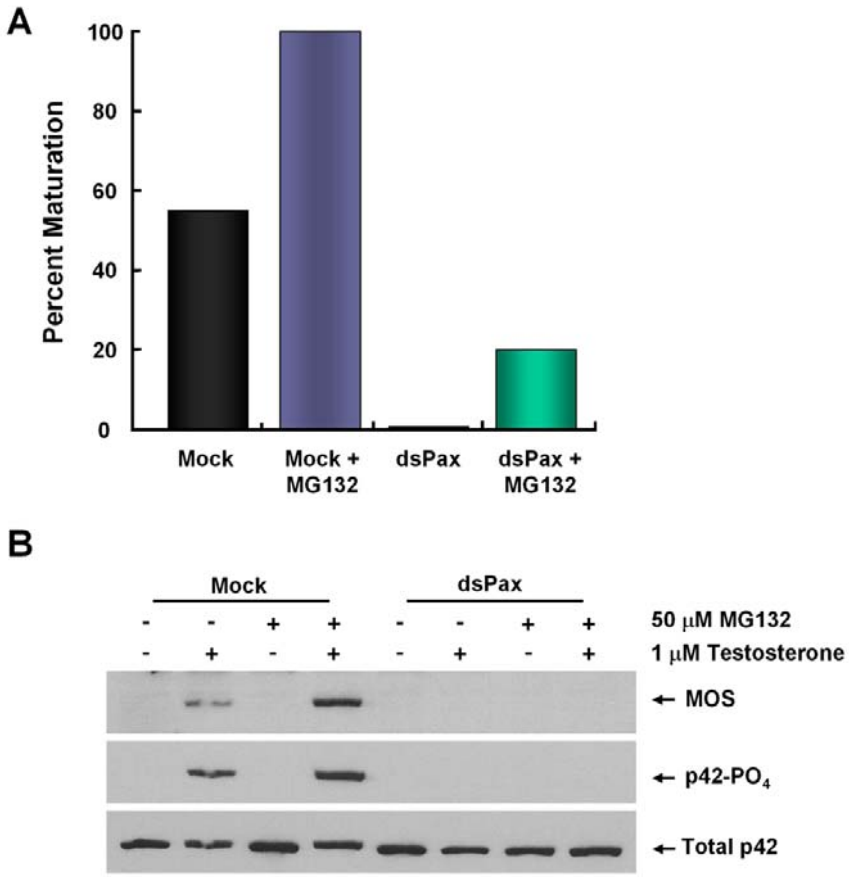


Figure 2-6: Paxillin does not affect MOS protein stability. Oocytes were injected with either 10mM Hepes (mock) or Paxillin dsRNA (dsPax). 40 hours post-injection, oocytes were pretreated for 2 hours with 50μM MG132 or 0.1% DMSO vehicle control. Oocytes were then treated with 1μM testosterone or 0.1% ethanol in the presence of 50μM MG132 or 0.1% DMSO overnight. Maturation was scored (A) and oocytes were lysed for analysis of MOS, phospho-ERK2 (p42-PO_4), and total ERK2 (total p42) (B).

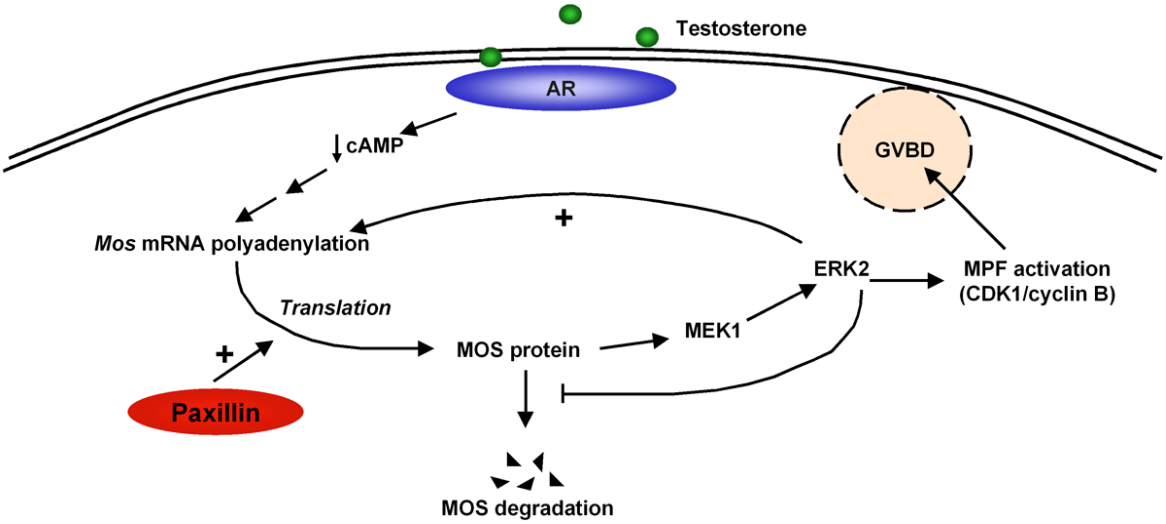


Figure 2-7: Proposed mechanism for Paxillin's function in maturation. These data suggest that Paxillin is important for regulating the expression of MOS protein, perhaps by regulating translation of the polyadenylated transcript.

Chapter Three

Regulation of oocyte maturation by Paxillin phosphorylation

Introduction

In the previous chapter, a novel role for Paxillin in oocyte maturation was identified. Results suggested that Paxillin is critical for accumulation of MOS protein, but the mechanism by which this occurs is uncertain. Due to this effect on MOS protein levels, Paxillin subsequently functions as a regulator of MEK and ERK activation in response to steroids, placing it in the positive feedback kinase loop. Understanding how Paxillin is regulated in the oocyte may elucidate how Paxillin modulates MAPK signaling in the oocyte.

Paxillin is a protein that has no known enzymatic activity, therefore it is largely believed that Paxillin function is regulated by phosphorylation. Dozens of phosphorylation sites have been identified, and phosphorylation appears to regulate both localization and protein binding which results in modulation of the signaling molecules that form complexes on the Paxillin backbone (Brown et al., 1998; Webb et al., 2005). Specificity of these interactions appears to be dictated by upstream kinases that are activated by extracellular signals. The kinase or kinases that phosphorylate Paxillin target specific sites that determine which proteins will be recruited to Paxillin, thus influencing what scaffold Paxillin will create. Interestingly, Paxillin has been shown to be phosphorylated at key tyrosine residues within the SH2 binding domains which result in creation of a MAPK scaffold including Raf, MEK, and ERK. Moreover, serine

phosphorylation of Paxillin has been proposed to be mediated by the Raf-MEK-ERK pathway.

Since Paxillin has been implicated as a regulator of MAPK signaling in somatic cells, we wished to determine whether it might be mediating the steroid-triggered positive kinase feedback loop in oocytes. Signaling studies revealed that although Paxillin functions downstream of *Mos* mRNA polyadenylation and upstream of MOS protein it is also phosphorylated downstream of MOS in a MEK-dependent manner. This phosphorylation is demonstrated to be necessary for full Paxillin function. Furthermore, a role for Paxillin as a MAPK scaffold is explored. In sum, these data indicate Paxillin is a critical regulator of meiosis, and plays an important role in the positive effects of MEK/ERK signaling on MOS protein expression and activity.

Materials and Methods

Antibodies—Anti-Mos (sc-86) was obtained from Santa Cruz Biotechnology, anti-HA (PRB-101) from Covance, anti-phospho-p44/42 MAPK (#9101) and anti-total p44/42 MAPK (#9102) from Cell Signaling Technologies. Polyclonal antibodies directed to XePaxillin amino acids 468-484 were generated by Biosynthesis Inc. (Lewisville, TX).

Plasmid construction—cDNAs encoding XePaxillin, XeMOS, and the 3'UTR of *Mos* mRNA were cloned into pcDNA3.1(+) (Invitrogen) for eukaryotic expression and pGEM HE (from L. Jan, University of California, San Francisco, CA) for *Xenopus* oocyte expression. Primers used to clone XePaxillin and XeMOS included sequence to incorporate an amino-terminal HA tag. Serine and tyrosine mutated versions of Paxillin

were cloned using site-directed mutagenesis to convert serine residues to alanine or tyrosine residues to phenylalanine. XePaxillin and corresponding mutated forms of Paxillin were cloned into pGEX4T-1(AmershamPharmacia) for purification of GST tagged protein. These were subsequently cloned into pcDNA3.1(+) for mammalian expression of the GST-tagged versions of Paxillin. A constitutively active form of B-Raf was a kind gift from William Walker (Univ. of Pittsburgh) which was cloned as described (Garcia et al., 2001) .

Steroid Maturation Assays—Maturation assays were conducted by incubating 20 oocytes per condition with the indicated concentration of testosterone (Steraloids) or ethanol in MBSH for 12-16 hours, at which time oocytes were scored for GVBD (visualization of a white spot on the animal pole). Dilutions were performed such that ethanol concentration was held at 0.1%. In experiments with the MEK inhibitor PD98059 (Calbiochem), oocytes were pretreated with 50μM PD98059 or 0.1% DMSO for one hour. Oocytes were then incubated with the indicated concentration of testosterone and either PD98059 or DMSO.

Western blots—Oocytes were incubated with steroid or vehicle, permeabilized in 20 μl/oocyte oocyte lysis buffer (1% Triton X-100 50mM Tris-HCl pH 7.6, 150 mM NaCl, 2mM EDTA, 2 mM NaF, 0.5 mM NaVanadate, 100 μg/mL PMSF), and microfuged at 14,000 x g for 10 minutes to remove yolk and other debris. The cleared supernatants were then mixed 1:1 with 2x SDS buffer. The equivalent of 0.5 oocytes was loaded in each lane for SDS-PAGE, transferred to Immobilon-P membranes (Millipore), blocked in 5% TBST-milk for 1 hour, then incubated with primary antibody overnight at 4°C (1:5000 for anti-HA, 1:1000 for α-MOS, 1:2000 for α-phospho-p42

ERK2). Membranes were then incubated with HRP-conjugated secondary antibody for 1 hour, and signal detected by ECL (Amersham Pharmacia). Total ERK blots were generated by stripping phospho-ERK blots, then incubating with 1:1000 α-total p42 ERK2 antibody. For phosphatase assays, oocytes were permeabilized in oocyte lysis buffer without NaVanadate, then 30μl was treated with 10 units calf intestinal alkaline phosphatase (New England Biolabs) for 1 hr at 37°C. Samples were then mixed with an equal volume of 2xSDS buffer and immunoblotted.

Cell culture and transfection—COS-7 cells (ATCC) were maintained at 37°C in DMEM (Fisher Scientific) containing 10% fetal bovine serum, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin (Invitrogen). Transfections were performed in six-well plates using Lipofectamine reagent (Invitrogen). Each well was transfected with 1μg total DNA as indicated. After overnight incubation in 10% serum, cells were serum starved for 24 hours. Cells were then treated with either 50μM PD98059, 10μM U0126 or 0.1% DMSO in serum-free DMEM for 2 hours. Cells were washed 2x with ice-cold PBS (pH 7.4), and permeabilized in 300μl oocyte lysis buffer. Wells were scraped, cell debris removed by centrifugation and, and the cleared lysates mixed 1:1 with 2x SDS buffer and immunoblotted as described.

GST proteins and in vitro kinase assays—XePaxillin was cloned into pGEX4T-1 (Amersham Pharmacia) and transformed into BL21. Transformed bacteria were then induced with 100μM IPTG for 2 hours and permeabilized in PBS including 10mM DTT, 1 μg/ml leupeptin, 1μg/ml aprotinin, 1μg/ml pepstatin A, and 100 μg/ml PMSF using an

Emmulsiflex C5. The cleared lysate was incubated with glutathione sepharose beads and protein eluted with 10 mM Glutathione in 50mM Tris pH 8.0, 1mM EDTA.

One microgram of GST protein was incubated in MAPK buffer with either 100 ng inactive ERK (Cell Signaling Technologies) or active ERK (New England Biolabs, NEB) per the NEB protocol. 10μCu γ-ATP was added to each reaction, and the kinase reaction incubated at 30°C for 1 hour followed by mixing with an equal volume of 2xSDS buffer. Samples were run on 10% gels by SDS-PAGE, dried, then analyzed by autoradiography. To confirm equal loading, reactions were run in duplicate without radioactive ATP, and samples were analyzed by western blot using 1:1000 anti-Paxillin antibody (Santa Cruz Biotechnologies).

GST-pulldowns—COS7 cells were transfected as described above with the indicated amount of DNA. After overnight incubation in 10% serum, cells were serum starved for 24 hours. Cells were then treated with either 50μM PD98059 or 0.1% DMSO in serum-free DMEM for 2 hours. Cells were washed 2x with ice-cold PBS (pH 7.4), then lysed in 400μl RIPA buffer (10mM sodium phosphate, 150mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1mM EDTA, 2 mM NaF, 0.5 mM NaVanadate) plus complete mini protease inhibitors (Roche). After removal of cell debris by centrifugation, samples were incubated with 25μl 50% slurry of glutathione-agarose beads (Sigma) for 2 hours at 4°C. Beads were washed 3x with 500μl RIPA and resuspended in 30μl 2xSDS buffer. Samples were immunoblotted for α-GST (1:5000 in 5%milk-TBST, Sigma) or α-MOS, α-ERK as described above.

Results

Paxillin is phosphorylated during maturation

Since mammalian Paxillin serves as a MAPK scaffold in somatic cells, and phosphorylation of Paxillin is important for this function (Ishibe et al., 2004; Ishibe et al., 2003), we determined whether Paxillin was phosphorylated during steroid-induced oocyte maturation. Oocytes were injected with HA-tagged XePaxillin cRNA, stimulated with testosterone, and solubilized every two hours for detection of HA-tagged Paxillin by Western blot. After 8 hours of steroid stimulation, a slower migrating form of Paxillin was observed that became even more abundant at 12 hours (Fig. 3-1A). This higher migrating band disappeared with alkaline phosphatase treatment, suggesting that the change in Paxillin mobility was due to phosphorylation (Fig. 3-1A). Interestingly, the time at which Paxillin phosphorylation was detected coincided with the timing of significant MOS protein accumulation and the onset of GVBD (Fig. 3-1B), providing further evidence that Paxillin function and MOS expression are linked.

Several reports have shown that phosphorylation of mammalian Paxillin occurs primarily on the amino-terminal half of the protein (Turner, 2000; Webb et al., 2005). Accordingly, the amino-terminal, but not carboxyl-terminal, half of XePaxillin was phosphorylated upon stimulation with testosterone (Fig. 3-1C).

Paxillin is phosphorylated at serine residues 107 and 111

Having demonstrated that Paxillin is phosphorylated during oocyte maturation, we next determined which residues were phosphorylated. In mammalian somatic cells,

phosphorylation of two conserved tyrosine residues appear to be important for Paxillin's actions in focal adhesion signaling and cell migration (amino acids 31 and 99 in XePaxillin, Fig. 2-1) (Ishibe et al., 2004; Ishibe et al., 2003). In addition, activation of the Raf-MEK-ERK cascade leads to phosphorylation of two serine residues of mammalian Paxillin (residues 107 and 111 in XePaxillin, Fig. 2-1) (Cai et al., 2006; Woodrow et al., 2003).

To determine the importance of these residues in regulating *Xenopus* oocyte maturation, point mutations were introduced in which the conserved tyrosine and serine residues were changed to phenylalanine and alanine, respectively (Fig. 2-1). Paxillin protein products containing an S107A mutation resulted in moderately reduced phosphorylation (Fig. 3-2A), and an S111A mutation resulted in significant loss of phosphorylation. Elimination of both serine residues (S107/111A) resulted in nearly complete loss of any slower migrating species in response to testosterone (Fig. 3-2A). On the other hand, despite mutation of the Y31F, Y99F, or Y31/99F, Paxillin was still phosphorylated in response to testosterone (Fig 3-2B).

Serine phosphorylation of Paxillin is required for its function in Xenopus oocytes

Phosphorylation of Paxillin appears to be dependent on serine residues 107/111. Therefore, we next determined if phosphorylation at these serine residues is important for XePaxillin function. Oocytes were injected with the Paxillin antisense oligonucleotides to reduce endogenous Paxillin expression, and rescue studies were performed by re-injecting with cRNAs encoding wild-type or S107/111A XePaxillin. Over-expressed S107/111A XePaxillin in oocytes with reduced endogenous XePaxillin could not rescue

maturation when compared to WT-Paxillin (Fig. 3-3A). Furthermore, S107/111A Paxillin was unable to rescue either MOS expression or activation of ERK2 as compared to WT-Paxillin rescue (Fig. 3-3B). Equal expression of the wild-type and S107/111A XePaxillin proteins, as well as confirmation that S107/111A was not being phosphorylated, was verified by Western blot (Fig. 3-3C). These data suggest that Paxillin's ability to promote maturation depends on serines 107/111 being phosphorylated.

Paxillin phosphorylation is MEK-dependent

Previous studies suggested that Raf-mediated phosphorylation of Paxillin in epithelial cells may require ERK activation (Ishibe et al., 2004; Terfera et al., 2002; Woodrow et al., 2003). Since MOS is a germ cell-specific homologue of Raf, we tested whether downstream MEK activity was required for testosterone-induced phosphorylation of Paxillin. Oocytes treated with the MEK inhibitor PD98059 showed significantly reduced testosterone-induced maturation (data not shown), as well as almost complete loss of MOS protein expression and markedly reduced ERK2 activation (Fig. 3-4A). Furthermore, PD98059 almost completely abrogated Paxillin phosphorylation. Similarly, over-expression of MOS protein in oocytes was sufficient to activate ERK2 and promote phosphorylation of Paxillin (Fig. 3-4B), and PD98059 significantly delayed and reduced these effects. Importantly, in oocytes injected with exogenous Mos cRNA, MOS protein accumulated more slowly in the presence of the MEK inhibitor, likely due to inhibition of the ERK2-mediated positive feedback loop that enhances MOS protein expression and activity. However, because MOS is such a potent activator of MEK1,

PD98059 could only delay, but not abolish, ERK2 activity, likely explaining why Paxillin phosphorylation was reduced, but not completely eliminated, in Mos cRNA-injected oocytes.

To test whether MOS could also promote MEK-dependent phosphorylation of Paxillin in somatic cells, COS cells were transfected with either HA-tagged MOS, HA-tagged Paxillin, or both. After overnight serum starvation, cells were treated for two hours with or without the MEK inhibitor PD98059. As in *Xenopus* oocytes, MOS expression led to phosphorylation of Paxillin, but here we observed only a modest increase in constitutive ERK1/2 activation (Fig. 3-5A). Nonetheless, as in oocytes, MOS-induced phosphorylation of Paxillin required MEK, as PD98059 blocked activation of ERK2 and eliminated the mobility shift of Paxillin. Since two hours with PD98059 was sufficient to eliminate virtually all detectable MOS-mediated phosphorylation of Paxillin in COS cells, persistent MOS-induced MEK activity must be needed to keep Paxillin in its phosphorylated form.

MOS is an oocyte specific form of Raf, therefore we wanted to determine if MOS-induced phosphorylation of Paxillin was specific to MOS in COS cells or if it could also be reproduced by overexpression of Raf. Therefore, cells were transfected and treated as before, replacing a constitutively active form of B-Raf for MOS (Garcia et al., 2001). Activation of ERK was significantly elevated and required inhibition of MEK using a different MEK inhibitor, U0126, to reduce ERK activation, although in this case it could not completely inhibit ERK activation (Fig. 3-5B and data not shown). As seen with MOS, active B-Raf could induce phosphorylation of XePaxillin and this was also

dependent on MEK activity, suggesting the mechanism by which MOS induces Paxillin phosphorylation is conserved among Raf family members.

ERK2 phosphorylates Paxillin in vitro

The ability of a MEK inhibitor to block MOS-mediated phosphorylation of Paxillin suggests that the serine kinase modifying Paxillin is located downstream of MEK in the MAPK cascade. Since Paxillin contains the minimal ERK2 consensus phosphorylation sequence, Ser/Thr-Pro, at residues S111-P112 (Fig. 2-1), and some studies have shown that ERK2 can phosphorylate GST-Paxillin fusion proteins *in vitro* (Ishibe et al., 2004; Liu et al., 2002; Ogawa et al., 2003)**,** we performed *in vitro* kinase assays to determine whether ERK2 could phosphorylate *Xenopus* Paxillin. GST-fusion proteins of the wild-type and S107/111A XePaxillin proteins were expressed in bacteria and purified using glutathione-sepharose beads. Activated, but not inactive, ERK2 phosphorylated GST-Paxillin significantly better than GST-S107/111A Paxillin (Fig. 3-6A). Multiple bands were observed due to phosphorylation of degradation products that contain an intact amino terminus. This difference in phosphorylation was not due to variability in substrate amounts, since Western analysis showed that the quantity of S107/111A XePaxillin was at least equal to that of WT-Paxillin (Fig. 3-6B). Thus, ERK2 is capable of phosphorylating either one or both of the serine residues 107 and 111 *in vitro*, and may be doing so *in vivo* as well.

Paxillin may serve as a MAPK scaffold

Since many pathways are associated with the activation of MAPK and other components of the MAPK cascade, many agree some sort of specificity must exist so that MAPK specifically activates a single pathway, rather than all MAPK responsive pathways within the cell. The same type of specificity probably applies to how MAPK becomes activated in the first place. One of the mechanisms by which this specificity may be attained is through MAPK scaffolds. An emerging view is that signal transduction pathways are organized in networks, and various protein scaffolds function as "relay stations" that are responsible for integrating many signals into specific multiprotein complexes. Recently, the discovery of MAPK scaffolds, such as kinase suppressor of Ras-1 (KSR), connector enhancer of KSR (CNK), Paxillin, and possibly Raf-1 have changed how MAPK activation is viewed (reviewed in Kolch, 2005). In each of these systems, MAPK activation appears to be regulated by scaffolds capable of binding various components of the pathway, theoretically enhancing the efficiency of MAPK activity by brining the components within close proximity to each other, and most likely targeting the complexes to specific downstream effectors leading to activation of discrete signals within the cell.

We wanted to determine whether XePaxillin was capable of binding the MAPK cascade components, MOS, MEK, and ERK. COS-7 cells were co-transfected with either GST, GST-Paxillin, or GST-S107/111A Paxillin and after 48 hours, treated with either 50μM PD98059 or DMSO control. After treatment, cells were lysed and the GST-tagged proteins were precipitated, along with their binding partners, using glutathione-agarose beads. GST-Paxillin specifically precipitated MOS protein, whereas GST alone

or the serine phosphorylation deficient Paxillin, GST-S107/111A, did not (Fig 3-7A). Interestingly, inhibition of MAPK activity using the MEK inhibitor appeared to enhance association of MOS with GST-Paxillin. The lower panel indicates variable association was not due to difference in protein levels in the lysates (Fig 3-7B). This may imply that the affinity for MOS and Paxillin varies depending on the kinase activity in the cell. This would be consistent with variable affinity of ERK for Paxillin in the context of MEK inhibition which was shown to increase the association of inactive ERK and Paxillin in fibroblasts (Ishibe et al., 2003). Although MOS co-precipitated with GST-Paxillin, neither MEK nor ERK were identified in the GST pulldowns (data not shown). This could be due to the difficulty associated with precipitating endogenous MEK and ERK (Melanie Cobb, personal communication), or perhaps MOS and Paxillin create a scaffold independent of MEK and ERK. GST-pulldowns or co-immunoprecipitations of Paxillin and the MAPK cascade components were attempted in oocytes, but results were difficult to interpret (data not shown).

Discussion

The use of multidomain scaffold proteins is a key mechanism by which cells translate multiple extracellular stimuli into a complex array of intracellular signaling events. Paxillin serves as an excellent example of how these scaffolding proteins function. Due to the high number of protein binding motifs interspersed throughout the protein, Paxillin can recruit a large number of signaling intermediates and effecter proteins into a single complex to allow for efficient and timely activation of multiple

pathways. In addition, phosphorylation of Paxillin regulates which molecules are recruited and the subcellular compartment in which the complex localizes (Brown and Turner, 2004). Thus, Paxillin integrates multiple upstream signals into downstream signals that regulate a myriad of processes, including cytoskeletal regulation, matrix organization, cell motility, and gene expression.

One of the most interesting regulatory functions attributed to Paxillin is its role in regulating MAPK signaling. Under many conditions, Paxillin is critical for efficient activation of the MAPK cascade. For example, Paxillin-deficient fibroblasts show reduced fibronectin-induced ERK activation (Hagel et al., 2002). In addition, Paxillin serves as a regulated MAPK scaffold that binds all three components of the MAPK cascade, Raf, MEK1, and ERK, upon growth factor stimulation of epithelial cells (Ishibe et al., 2004; Ishibe et al., 2003).

We show here that Paxillin also plays a crucial role in the MAPK signaling responsible for the all-or-none switch that regulates steroid-induced oocyte maturation, or meiotic progression. Our results demonstrate that Paxillin is a novel and essential regulator of steroid-triggered oocyte maturation. Signaling studies suggest that Paxillin functions upstream of MOS protein expression, as it is important for the accumulation of MOS and activation of the downstream kinases MEK and ERK2 (Chapter 2). In contrast, Paxillin also seems to be regulated downstream of MOS activation, as MEK-dependent phosphorylation of Paxillin on two serine residues appears to be critical for Paxillin to maintain elevated MOS expression and promote maturation (Fig. 3-3). How is it possible for Paxillin to be functioning both upstream and downstream of MOS? We propose a model whereby Paxillin is a critical positive regulator of the MOS/MEK/ERK2 feedback

loop (Fig. 3-8). Upon steroid stimulation, *Mos* mRNA is stabilized by polyadenylation, leading to low level translation of MOS protein. MOS then activates the MAPK cascade, resulting in phosphorylation of Paxillin by a kinase downstream of MEK. Paxillin phosphorylation then enhances its ability to mediate MOS expression, allowing for accumulation of MOS at levels required to fully activate the MAP kinases and trigger resumption of meiosis by MPF.

What kinase is responsible for serine phosphorylation of Paxillin? One possibility is that MOS directly phosphorylates XePaxillin. However, the specific MEK inhibitor PD98059 blocked Paxillin phosphorylation both in oocytes and COS cells, suggesting that the Paxillin kinase is located downstream of MOS. Further, PD98059 does not block Raf activity, and thus presumably would not inhibit the homolog MOS (Alessi et al., 1995).

Since the Paxillin kinase acts downstream of MEK, then one candidate is ERK2. Accordingly, we demonstrated that ERK2 is capable of phosphorylating *Xenopus* Paxillin *in vitro* (Fig. 3-6), which is consistent with other studies looking at *in vitro* phosphorylation of mammalian Paxillin isoforms by ERK2 (Cai et al., 2006; Ishibe et al., 2004; Liu et al., 2002). Although we have no direct evidence that ERK2 phosphorylates XePaxillin in steroid-triggered oocytes, the observations that *in vitro* ERK2-mediated phosphorylation requires serine residues 107 and 111, which are also essential for normal Paxillin function (Fig. 3-3), indicate that ERK2 may indeed be at least one *in vivo* kinase of Paxillin. Furthermore, other studies suggest that ERK2 phosphorylates these or equivalent serine residues *in vivo*. For example, ERK phosphorylates *Xenopus* Paxillin on serines in the frog kidney epithelial cell line A6 (Ogawa et al., 2003). In addition,

ERK2 promotes phosphorylation of murine Paxillin on serine residues 126 and 130, equivalent to residues 107 and 111 in XePaxillin (Woodrow et al., 2003). Also, a dual-kinase mechanism for serine phosphorylation has been proposed whereby ERK phosphorylates the mammalian equivalent of serine 111 which is permissive for a second kinase to phosphorylate the equivalent of serine 107 (Cai et al., 2006). At this time, it is unclear if this mechanism of serine phosphorylation is utilized in Xenopus oocytes.

Finally, similarities between our data and those examining Paxillin effects on MAPK signaling in somatic cells suggest that XePaxillin might be a scaffold for MOS/MEK/ERK signaling in oocytes. When overexpressed, we demonstrate that XePaxillin and XeMOS can interact in mammalian cells (Fig 3-7). If such an interaction exists in oocytes, this might suggest that scaffolding of the MAPK components on Paxillin enhances activation of the MAPK cascade, thus enhancing activation of ERK which would lead to increased stability and production of MOS, promoting the positive feedback loop observed in oocytes. Furthermore, the phosphorylation status of Paxillin may regulate the chances that such a MAPK scaffold will form. To test the scaffold hypothesis *in vivo*, we have attempted to demonstrate binding of XePaxillin to either MOS, MEK, or ERK by co-immunoprecipitation in oocyte extracts; however, we have been unable to detect consistent interactions between these molecules (data not shown). These negative data could be due to a variety of reasons, including difficulties with co-immunoprecipitation in oocyte extracts due to high levels of lipid and yolk contaminants, or weak/transient interactions that are disrupted upon cell lysis. More detailed precipitation studies need to be performed to determine if these components of the MAPK cascade interact *in vivo*.

In sum, we have described a novel biological role for Paxillin in the process of meiotic maturation. Further, we have shown that Paxillin functions as a critical regulator of the all-or-none response observed during oocyte maturation due to positive effects on MAPK signaling. Since this MAPK effect shares many similarities to Paxillin effects in somatic cells, elucidating the role Paxillin plays in the maturation pathway will not only be helpful in understanding meiosis, but will likely reveal a more global role for Paxillin mediated MAPK signaling in both somatic and germ cell biology.

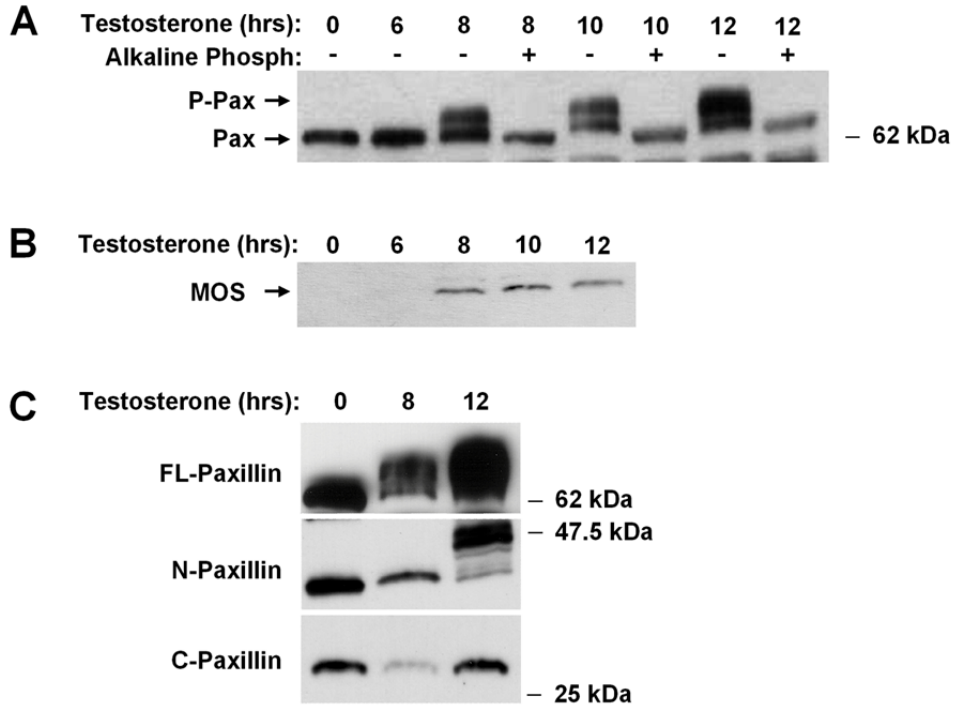


Figure 3-1: Paxillin is phosphorylated late during maturation in a MEK-dependent fashion. (A) Oocytes were stimulated with 1μM testosterone 40 hours after injection of oocytes with HA-Paxillin cRNA. Oocytes were permeabilized at the indicated times and 30μl of the sample was treated with calf intestinal alkaline phosphatase. Equal amounts of lysate were analyzed by SDS-PAGE followed by Western blot with and anti-HA antibody. The higher-mobility bands represent phosphorylated Paxillin. (B) Lysates from panel A were probed with MOS antibody. (C) Oocytes were injected with either HA-tagged cRNA encoding full-length Paxillin (FL), the amino-terminal half of Paxillin (N-Paxillin, residues 1-304) or the carboxyl-terminal half of Paxillin (C-Paxillin, residues 291-539). Forty hours post-injection, oocytes were stimulated with 1μM testosterone, permeabilized at the indicated times, and immunoblotted with HA antibody.

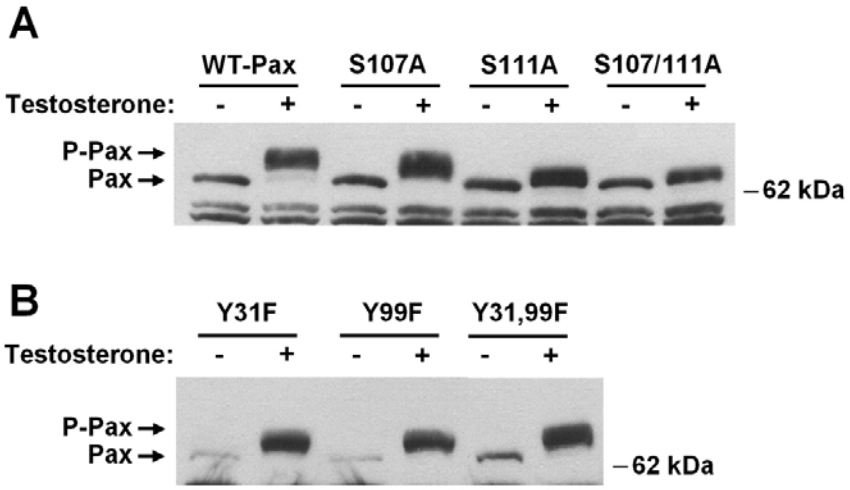


Figure 3-2: Paxillin phosphorylation depends on serine residues 107 and 111. (A) Oocytes were injected with HA-tagged Paxillin cRNA that encoded either wild type Paxillin (WT-Pax), singly mutated Paxillin (S107A or S111A), or doubly-mutated Paxillin (S107/111A). Forty hours post injection, oocytes were stimulated +/- 1μM testosterone for 16 hours and lysates probed with HA antibody. (B) Oocytes were injected with HA-tagged Paxillin cRNA encoding tyrosine to phenylalanine substitutions at residues 31 (Y31F), 99 (Y99F), or both (Y31,99F) and treated as in panel A.

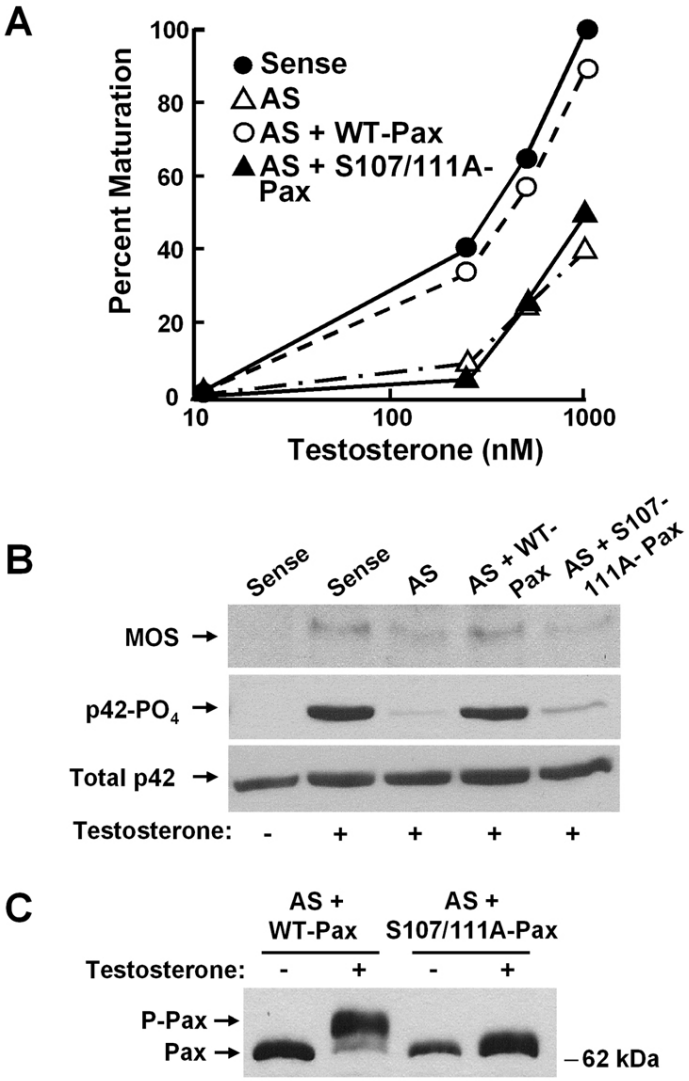


Figure 3-3: Paxillin function depends on serine residues 107 and 111. (A) Oocytes depleted of Paxillin by antisense injection (AS) were co-injected with either wild-type Paxillin (WT-Pax) or the double serine to alanine mutant Paxillin (S107/111A-Pax). Oocytes were then incubated with increasing doses of testosterone. Twenty oocytes were used for each data point. (B) Lysates from the oocytes treated with 250nM testosterone in (A) were analyzed for MOS expression and p42 ERK activation by western blot. Phospho-p42 ERK blots were stripped and re-probed for total p42 ERK. (C) Equivalent expression of wild type and S107/111A Paxillin in the oocytes from (A) was verified by HA immunoblot. Each study was performed at least three times with essentially identical results.

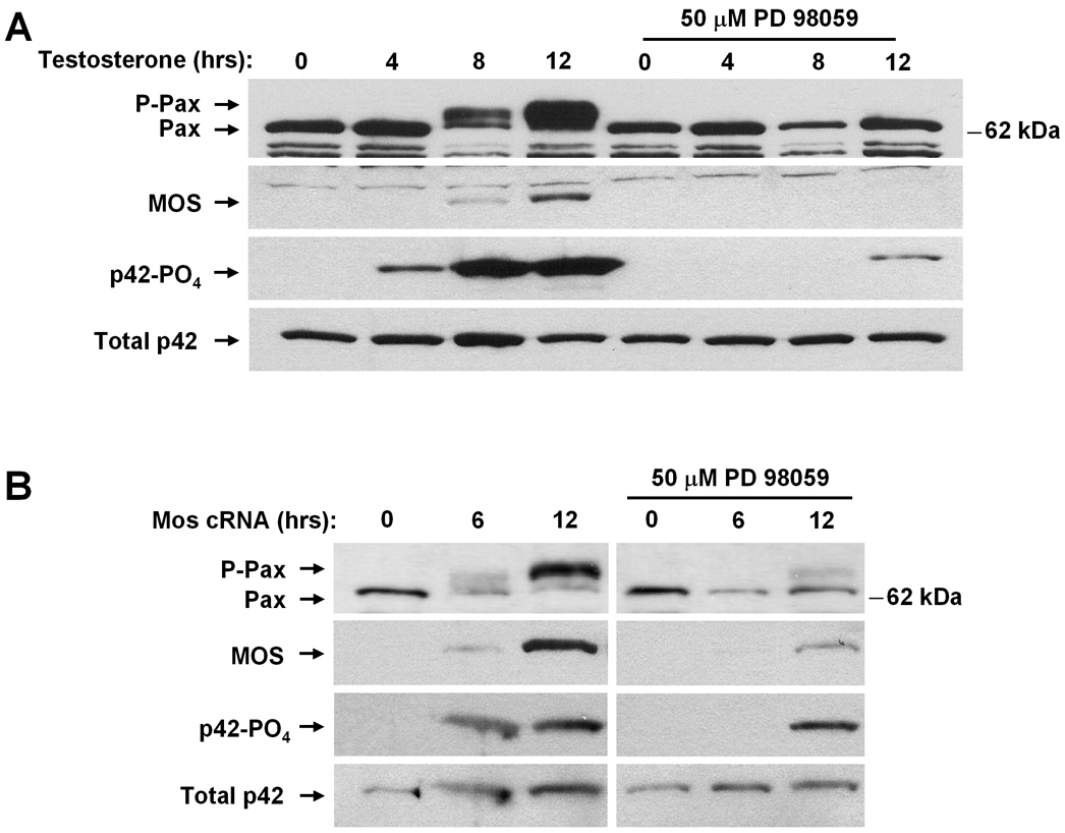


Figure 3-4: Paxillin phosphorylation in oocytes is MEK-dependent. 40 hours post-injection, HA-Paxillin expressing oocytes were pretreated with either DMSO or 50μM PD98059 for 2 hours before stimulation with 1μM testosterone (A) or injection with *Mos* cRNA (B). Either DMSO or PD98059 was maintained in the media throughout the experiment. Oocytes were permeabilized at the indicated times and lysates probed for HA, MOS, and phospho-p42 ERK2. Phospho-ERK blots were stripped and re-probed for total ERK. Each experiment was reproduced at least three times with essentially identical results.

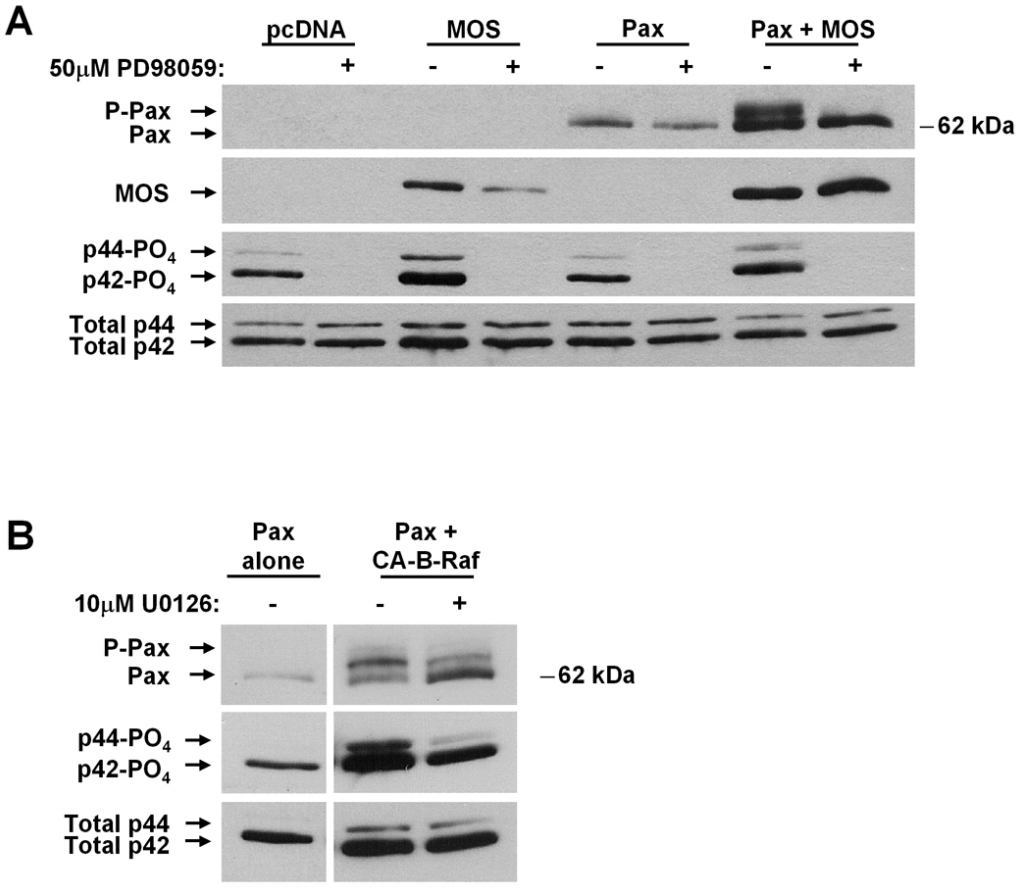


Figure 3-5: Paxillin phosphorylation by MOS and Raf in mammalian cells is also MEK-dependent. (A) COS-7 cells were transfected with a total of 1μg DNA that consisted of pcDNA3.1+, cDNAs encoding either HA-Paxillin (0.5μg) or HA-MOS (0.5μg), or both HA-Paxillin and HA-MOS (0.5 μg each). After 18 hours, cells were serum starved for 24 hours, then treated with either 50 μM PD98059 or DMSO vehicle for 2 hours. Cells were permeabilized and equal amounts of lysate probed for HA and phospho-ERKs. Phospho-ERK blots were stripped and re-probed for total ERK. (B) COS-7 cells were transfected with either HA-Paxillin (0.5μg) or both HA-Paxillin and constitutively active B-Raf (0.5ug each). Cells were treated as in panel A, except 10μM U0126 was used instead of PD98059 due to better inhibition with this compound. Each experiment was reproduced at least three times with essentially identical results.

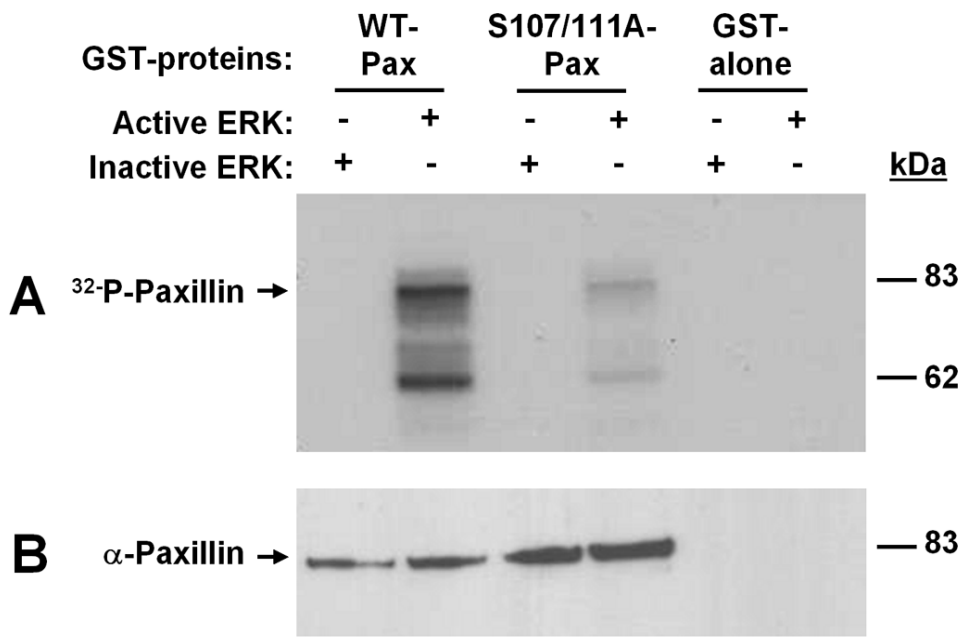


Figure 3-6: p42 ERK phosphorylates Paxillin *in vitro*. (A) GST-fusion proteins of full-length wild type Paxillin (WT-Pax), S107/111A Paxillin (S107/111A-Pax), or GST alone were incubated with either inactive or active ERK2 in the presence of 10μCi ^{32}P-ATP. (B) Western blots show equal, if not greater, loading of S107/111A Paxillin compared to WT-Paxillin.

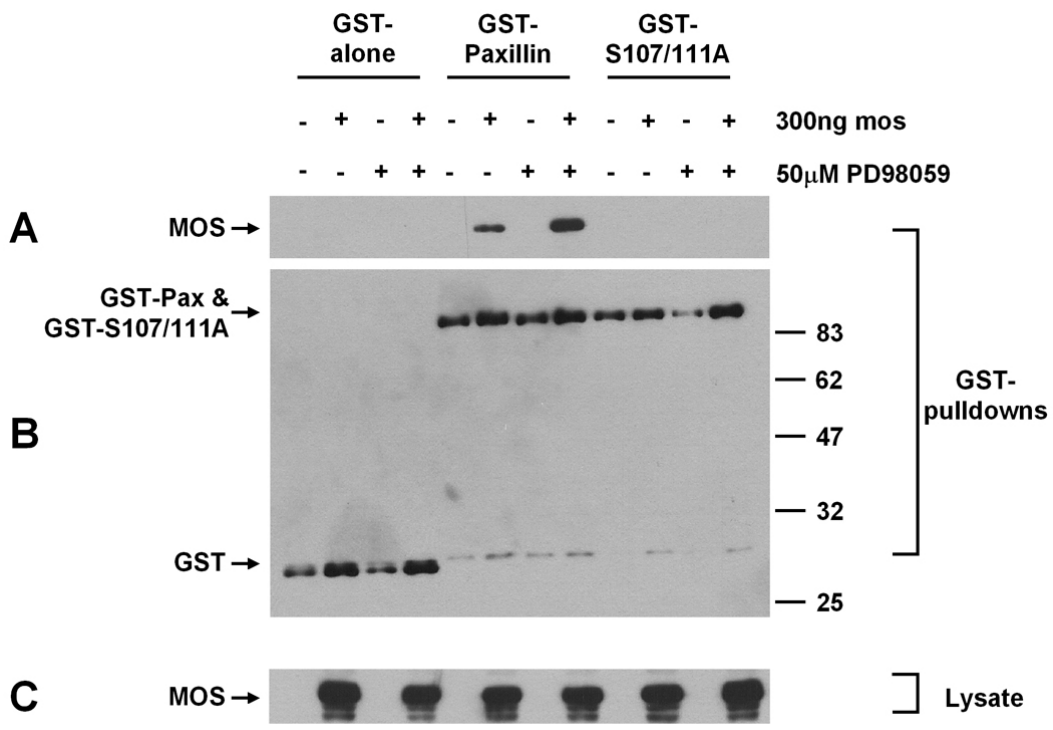


Figure 3-7: Paxillin can interact with MOS in mammalian cells. COS-7 cells were transfected with a total of 1.0μg of DNA that consisted of either pcDNA3.1+, cDNAs encoding GST alone, GST-Paxillin, or GST-S107/111A Paxillin (0.7μg), +/- 0.3μg MOS in pcDNA3.1+. After 18 hours, cells were serum starved for 24 hours. Following treatment with either 50μM PD98059 or 0.1% DMSO vehicle control, cells were lysed and GST-tagged proteins were precipitated with glutathione agarose beads. Samples of beads (A and B) or lysate (C) were immunoblotted with antibodies against MOS (A and C) or GST (B).

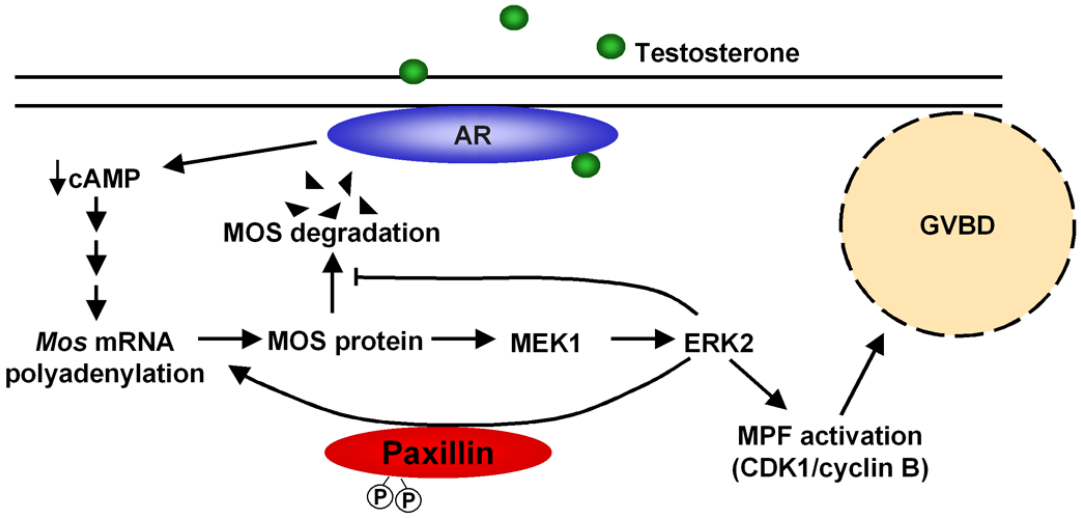


Figure 3-8: Proposed model describing Paxillin function during oocyte maturation. Testosterone stimulation of oocytes via the classical androgen receptor (AR) triggers a decrease in intracellular cAMP. This drop in cAMP leads to increased polyadenylation of *Mos* mRNA, resulting in a small increase in MOS protein expression. MOS then activates MEK1, which in turn activates ERK2. Activated ERK2, and possibly other intracellular kinases, then phosphorylate Paxillin, which acts to further enhance MOS protein expression by either increasing MOS protein translation, decreasing MOS degradation, or both. This powerful positive feedback loop eventually leads to activation of the maturation promoting factor (MPF, or CDK1 and cyclin B), resulting in meiotic resumption.

Chapter Four

Analysis of PABP during oocyte maturation

Introduction

The scaffolding protein, Paxillin, has been described as a novel regulator of Xenopus oocyte maturation (Chapters 2 and 3). Overall, it appears that Paxillin is critical for mediating the positive feedback kinase loop by regulating MOS protein accumulation. In the absence of Paxillin function, MOS protein accumulation is prevented presumably due to an inhibition of the translation, rather than enhanced degradation, of MOS protein. As described previously, even in the absence of Paxillin, there is no accumulation of MOS protein despite normal polyadenylation of *Mos* mRNA. Therefore, the defect is not due to an effect on proteins responsible for polyadenylation, but rather may involve proteins important for translation of polyadenylated mRNAs in oocytes.

As discussed in Chapter 1, one class of proteins that have been identified as critical for translation of polyadenylated mRNAs are the polyadenylation binding proteins (PABPs). Two forms of Xenopus PABP exist, PABP1 and embryonic PABP (ePABP). PABP1 is recognized as the isoform that is expressed in most somatic cells after the blastula stage, but it is absent in all stages of Xenopus oocytes except stage I oocytes, the least developed of the oocytes within the ovary. This absence of protein expression in the oocytes occurs despite the fact mRNA for PABP1 is abundantly present throughout oogenesis (Zelus et al., 1989) (Schroeder and Yost, 1996; Stambuk and Moon, 1992). On the other hand, ePABP, is highly expressed in all stages of oocytes and

testes, but disappears from eggs by 72 hours post fertilization, being replaced by PABP1 expression (Cosson et al., 2002; Voeltz et al., 2001).

Functionally, both ePABP and PABP1 appear to activate translation in either oocytes or embryos, respectively (Gray et al., 2000; Wilkie et al., 2005). Furthermore, an interaction between PABP and eIF4G has been shown to be critical for translational control of maternal mRNAs during Xenopus development (Fig 1-1)(Wakiyama et al., 2000). Interestingly, eIF4G is not the only known binding partner of PABPs—Paxillin has been shown to associate with PABP1 in mammalian fibroblasts via paxillin binding sequences (PBSs) that are present in the 1st and 4th RNA binding domains (Fig. 4-1) (Woods et al., 2005). This interaction was required for the proper localization of PABP1 within the cell and is postulated to be important for localization and translational activation of mRNAs within specific compartments in the cell. Finally, the phosphorylation status of Paxillin appeared to correlate with association of it and PABP1, such that only hypophosphorylated Paxillin was found to co-immunoprecipitate with PABP1. This could suggest that phosphorylation of Paxillin leads to its dissociation from the PABP-mRNA complex, allowing the PABP-mRNA complex to associate with eIF4G, resulting in translation of the mRNA cargo.

In this preliminary study, we have begun to investigate the role PABPs may play in regulating maturation, specifically that of MOS translation, and how Paxillin may influence its activity. We first demonstrate that indeed, embryonic PABP (ePABP) is the isoform expressed in oocytes. Initial knockdown experiments suggest that loss of ePABP phenocopies Paxillin knockdown in oocytes, such that polyadenylation of *Mos* mRNA is not affected, although MOS protein accumulation, MAPK activation, and maturation are

reduced. Immunoprecipitation of ePABP also indicates that ePABP interacts with *Mos* mRNA and possibly Paxillin, although the necessity for this still needs to be determined.

Materials and Methods

Antibodies—Anti-Mos (sc-86) was obtained from Santa Cruz Biotechnology, anti-FLAG (M2, F1804) from Sigma, and anti-PABP1 (#4992), anti-phospho-p44/42 MAPK (#9101) and anti-total p44/42 MAPK (#9102) from Cell Signaling Technologies. The antibody for ePABP was provided by Joan Steitz (Yale University) (Voeltz et al., 2001) and the CPEB antibody was provided by Laura Hake (Boston College).

Plasmid construction—cDNAs encoding *Xenopus* Paxillin, ePABP (BC080020), PABP1 (BC052100) and the 3'UTR of *Mos* mRNA were cloned into pGEM HE in both the forward and reverse direction (from L. Jan, University of California, San Francisco, CA) for *Xenopus* oocyte expression. Primers included sequence to incorporate an amino-terminal FLAG tag. Paxillin binding mutations as indicated in Fig. 4-1B were cloned using site-directed PCR mutagenesis to convert the appropriate amino acids.

Oocyte Preparation—Oocytes were harvested from female *Xenopus laevis* (NASCO) and treated as described (Lutz et al., 2000). Briefly, follicular cells were removed by incubation of ovaries in 0.8 mg/ml collagenase A (Roche) in modified Barth's solution (MBSH) without Ca^{2+} for 3-4 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 were performed on stage V/VI oocytes from each preparation to determine sensitivity to 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.

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 manufacturers protocol (Ambion). RNA was suspended in injection buffer (10mM HEPES pH 7.4) and Stage V/VI oocytes were injected with 20ng cRNA using a Drummond automatic injector. In the case of double-stranded RNA injections, equal concentrations of forward and reverse cRNA of both ePABP and PABP1 were heated to 90°C, then slowly cooled to 30°C before injection. Approximately 40ng of double-stranded RNA was injected into each oocyte. 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 phosphorothioated bonds indicated by asterisks.

Oocytes were injected with 25ng oligonucleotides in each experiment. For the rescue studies, ePABP was recloned so that nucleotides at wobble positions in the first 6 codons were changed, preserving the amino acid sequence but preventing antisense oligonucleotides from binding. In each rescue experiment, oocytes were co-injected with 25 ng oligonucleotide and 20 ng rescue cRNA. After all injections, oocytes were incubated at least 36-48 hours before beginning any assay.

Steroid Maturation Assays—Maturation assays were conducted by incubating 20 oocytes per condition with the indicated concentration of testosterone (Steraloids) or

ethanol in MBSH for 12-16 hours, at which time oocytes were scored for GVBD (visualization of a white spot on the animal pole). For timecourse experiments, GVBD was scored at the indicated hours. Dilutions were performed such that ethanol concentration was held at 0.1%.

Western blots—Oocytes were incubated with steroid or vehicle, permeabilized in 20 μl/oocyte oocyte lysis buffer (1% Triton X-100 50mM Tris-HCl pH 7.6, 150 mM NaCl, 2mM EDTA, 2 mM NaF, 0.5 mM NaVanadate, 100 μg/mL PMSF), and microfuged at 14,000 x g for 10 minutes to remove yolk and other debris. The cleared supernatants were then mixed 1:1 with 2x SDS buffer. The equivalent of 0.5 oocytes was loaded in each lane for SDS-PAGE, transferred to Immobilon-P membranes (Millipore), blocked in 5% TBST-milk for 1 hour, then incubated with primary antibody overnight at 4°C (1:5000 for FLAG, 1:1000 for MOS, 1:2000 for phospho-p42 ERK2, 1:10,000 ePABP (5%-NP40-milk). Membranes were then incubated with HRP-conjugated secondary antibody for 1 hour, and signal detected by ECL (Amersham Pharmacia). Total ERK blots were generated by stripping phospho-ERK blots, then incubating with 1:1000 total p42 ERK2 antibody.

Mos polyadenylation assays—Polyadenylation of *Mos* RNA was determined as described (Howard et al., 1999). Briefly, 36 hours after double-stranded RNA 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 20μg of total RNA was run on a 2.2% agarose formaldehyde gel in 1xMOPS buffer for ~10 hours at 60V. RNA was transferred

to a Nytran SuPerCharge nylon membrane (Schliecher & Schuell) by downward transfer in 20xSSC (Schliecher & Schuell) and UV crosslinked. Membranes were pre-hybridized in ExpressHyb (Clonetech) at 68°C for 30 minutes, then probed with [^{32}P]-dCTP labeled mos 3'UTR DNA (Rediprime II, Amerhsam Biosciences) for 1 hour, washed 2x in 2xSSC, 0.05% SDS, 2x in 0.1x SSC, 0.1% SDS, then subjected to autoradiography.

Immunoprecipitations of protein/RNA complexes—Unless otherwise indicated, 100 oocytes were injected and/or treated as indicated, then lysed in 500µl 1x NET (50 mM Tris-pH 7.6, 150 mM NaCl, 1mM EDTA, 0.1% NP40, 2 mM NaF, 0.5 mM NaVanadate) plus Roche mini-complete protease inhibitors. Lysates were centrifuged at 14,000g for 10 minutes. Lysates were precleared with 0.5 µg normal serum (rabbit for subsequent emPABP IPs or mouse for FLAG IPs) and 25µl protein A sepharose beads (Amersham Pharmacia, for emPABP IPs) or protein G-PLUS (Santa Cruz Biotechnology, for FLAG IPs) for a total of 1 hour. Precleared lysates were incubated with ~0.5µg of IP antibody and 25µl corresponding beads for 4 hours at 4°C. Beads were washed 4x with 500µl 1x NET, then resuspended in 2xSDS buffer (without 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 1/3 of the sample was saved for western blot (β-mercaptoethanol was subsequently added). To the rest of the sample, 500µl Trizol (Invitrogen) was added and RNA was extracted according to manufacturer's instructions. Before isopropanol precipitation, 30µg glycogen was added to the sample to aid RNA recovery. RNA was resuspended in 25µl DEPC-water.

Reverse transcription-PCR and Real-time quantitative RT-PCR—Semi-quantitative RT-PCR was performed using Invitrogen's Superscript III One-Step RT-PCR with Platinum Taq kit. Primers used were mos forward 5'-ACCAGCGCATATACGGGC-3', mos reverse 5'-GGAGTGGAAGCCTGTGATGCTGCCCTGGGCATG-3', GAPDH forward 5'-CGTTAAGGCTGAGAATGGCAAG-3', and GAPDH reverse 5'-AGGAGCCAGACAGTTTGTAGTG-3'. 1µl of RNA was used in each 25µl reaction with cycling parameters as 50°C-25 min, 94°C-2 min, followed by 30 cycles of 94°C-45 sec and 58°C-45 sec. Reactions were observed by ethidium bromide staining in 1% agarose gels.

Quantitative real-time RT-PCR was performed using Invitrogen's Superscript III quantitative One-Step RT-PCR with Platinum Taq kit. Primer and probes were designed and ordered through the Applied Biosystems Custom TaqMan Gene Expression Assay Service. Sequences of primers and probes for mos, GAPDH, and emPABP were as follows: mos- forward 5'- GACGGACTGCGCTTCCT3', reverse 5'-GGCGAGCAGCACATTGG-3', and probe 5' CCGGCTTCAGATCCAG-3'; GAPDH-forward 5'-CTGCCACCCAGAAGACTGT-3', reverse 5'-GTTGAGGCGGGAATAATGTTCTGA-3' and probe 5'-TCTCCACAGCTTCCC-3'; ePABP- forward 5'- GATGCCTGGTCCTCTCCTT-3', reverse 5'-CGGCATGGCAGACAAGAAATAATTT-3', and probe 5'- CCTTCCAGCAACCTGC-3'. Reactions included the supplied ROX reference dye and were assembled according to manufacturer's protocol. Briefly, 2.5µl of RNA was used for each 25µl reaction using

cycling parameters 50°C-15 min, 95°C-2 min followed by 45 cycles of 95°C-15 sec and 60°C-30 sec using the Applied Biosystems 7300 Real-Time PCR system.

Results

ePABP is expressed in Xenopus laevis oocytes and may interact with Paxillin

Two isoforms of PABP have been described in Xenopus. Studies conducted before 2001 described oocytes as containing PABP1, but subsequent work has revealed oocytes instead express another, very similar isoform, ePABP. The confusion was likely due to the fact that PABP1 antibodies used in initial studies cross-reacted with ePABP due to the high degree of similarity. Both proteins contain approximately 630 amino acids (629 for ePAPB, 633 for PABP1) and are composed of four N-terminal RNA binding motifs (RRMs) followed by the carboxy terminal third of the protein that contains an unstructured region and PABC, an alpha-helical peptide binding domain (Fig. 4-1A) (Kozlov et al., 2001). Overall, ePABP is 72% identical to PABP1, where the amino-termini share 82% identity and the carboxy-termini are 56% identical. Both share extensive identity to PABPs of other species (Voeltz et al., 2001).

In order to verify which isoform is expressed in the oocyte, we tested oocytes for the presence of mRNA and protein of each. First, cDNAs encoding the Xenopus PABP1 (BC052100) and ePABP (BC080020) were cloned from oocyte RNA by RT-PCR indicating mRNA for each is present in the oocyte. Subsequently, we confirmed which were expressed by western blot. The ePABP antibody recognized a band in oocyte

lysate, whereas PABP1 recognized none (Fig 4-2A, mock). To confirm the immunoreactive band was indeed ePABP, lysates from oocytes expressing FLAG-tagged ePABP and PABP1 were used as positive controls. An increase in immunoreactivity of a band the same size as that seen in mock was observed in FLAG-ePABP lysates (Fig 4-A, α-ePABP). On the other hand, the absence of PABP1 was confirmed since the PABP1 antibody was capable of identifying FLAG-PABP1, but nothing in mock injected oocytes (Fig 4-A, α-PABP1). Of note, the specificity of the ePABP antibody for ePABP over PABP1 was confirmed since it does not cross react with a band at the correct size in oocytes overexpressing FLAG-PABP1 (data not shown).

Studies of mammalian PABP1 have indicated Paxillin can interact with PABP1 via sequences termed Paxillin binding sites (PBSs) in the amino terminus of the protein (Fig. 4-1A) (Woods et al., 2005). ePABP shares these same PBSs, therefore, we wanted to determine if ePABP could interact with Paxillin in oocytes. Oocytes were injected with FLAG-tagged Paxillin cRNA and 40 hours post injection, were treated with steroid for 6 hours, followed by immunoprecipitation of ePABP from the lysates. As shown, a small amount of FLAG-Paxillin co-immunoprecipitated with ePABP, whereas none was detected in normal rabbit IgG control precipitates (Fig 4-2B). No steroid dependent changes in binding were detected, although under these conditions, the oocytes were not exposed to steroid long enough to cause Paxillin phosphorylation and no maturation was observed at the time of lysis. Interaction of endogenous Paxillin with ePABP in oocytes was attempted, but results could not be interpreted due to significant cross-reactivity of the immunoprecipitating antibody heavy chains by the Paxillin antibody (data not shown). Further studies need to be conducted to confirm this interaction and determine if

changes in the phosphorylation status of Paxillin alters the affinity of any such interaction as was proposed in Woods *et al*.

Knockdown of ePABP mRNA phenocopies loss of Paxillin

If Paxillin is important for ePABP function, then elimination of ePABP may mimic the effects of Paxillin knockdown. To test this, we injected oocytes with antisense phosphorothioated oligonucleotides directed against the start codon of ePABP in an attempt to reduce protein expression. Oocytes injected with antisense demonstrated markedly reduced levels of ePABP mRNA, a 97% reduction compared to sense injected oocytes (Fig 4-3B). Antisense injected oocytes also demonstrated reduced maturation in response to steroid, coupled with loss of MOS protein accumulation and absence of activated ERK (Fig 4-3 A and C). In separate experiments in which oocytes were injected with double-stranded RNA to reduce ePABP levels, polyadenylation of *Mos* mRNA was found to occur normally, but these have not been repeated with antisense injected oocytes (Fig. 4-3 D). Overall, the preservation of *Mos* polyadenylation but inhibition of MOS and active ERK phenocopy the effects of Paxillin knockdown. Unfortunately we could not detect a decrease in protein levels as assessed by western blot (data not shown). It is not believed that the antisense oligonucleotides themselves are causing non-specific inhibition of maturation because re-injection with wild-type cRNA for ePABP rescues the loss of MOS protein accumulation, ERK activation, and maturation (Fig 4-3 A and C).

ePABP binding of Mos mRNA

Based on its described function, ePABP should bind polyadenylated mRNAs during maturation. We sought to test if *Mos* mRNA is recruited to ePABP during maturation and if this is enhanced as maturation progresses. Making use of the fact that RNA-protein complexes can be immunoprecipitated with specific antibodies, and that RNA can be purified from these complexes (Martinez et al., 2005), we immunoprecipitated ePABP from oocytes and quantified the amount of *Mos* mRNA that was associated with these complexes. As controls, some oocytes were immunoprecipitated with rabbit IgG as a negative control and CPEB antibody as a positive control since CPEB has been shown to bind *Mos* mRNA using a similar method (Martinez et al., 2005). ePABP was immunoprecipitated from oocytes exposed to testosterone for 0, 2, 4, and 6 hours to see if *Mos* mRNA recruitment increases over time. By 6 hours, 50% of the oocytes had undergone GVBD. Using real-time PCR, we quantified the amount of RNA in each complex using total oocyte RNA as a quantification standard. Fold change in *Mos* mRNA binding was determined by normalizing each sample to the amount of *Mos* mRNA that was obtained in the ePABP immunoprecipitation not exposed testosterone (no T) (Fig 4-4 A). The longer oocytes were treated with steroid, the more *Mos* mRNA was found in each immunoprecipitation, up to 10-fold more after 6 hours, despite the fact approximately equivalent amounts of ePABP were immunoprecipitated in each sample (Fig 4-4 A and B). This timecourse experiment was performed only once, so these results have not been confirmed, but they do correspond with previous observations from semi-quantitative RT-PCR analysis in which RNA was only found in immunoprecipitates that had been treated with steroid,

presumably because more mRNA is polyadenylated as maturation proceeds (data not shown). Future studies will attempt to determine if the binding of mRNAs by ePABP is altered in the background of Paxillin knockdown.

Enhancement of maturation by PABPs

Some have proposed that the level of PABPs in the oocyte may be a limiting factor in maturation, therefore we assessed the effect of increased PABP expression on the kinetics of maturation. To test this, we overexpressed either FLAG-tagged PABP1 (normally not expressed in oocytes) or FLAG-ePABP. In these studies, we observed that PABP1 increased the rate of maturation whereas ePABP overexpression only slightly increased the rate of maturation, although protein expression was approximately equal (Fig 4-5A). Additionally, both appear to enhance sensitivity to steroid, such that overexpression of the PABPs leads to a greater percentage of maturation than control oocytes at EC50 concentrations of steroid (data not shown).

Since overexpression of PABPs appears to partially enhance maturation, we wanted to evaluate how *Mos* mRNA binding was affected with overexpressed proteins. In order to determine how well *Mos* mRNA binds overexpressed PABP, we compared the amount of RNA present in various precipitates from oocytes expressing FLAG-PABP1 by real-time PCR using the total oocyte RNA as a standard for quantification. Therefore, in these experiments, the y-axis represents the amount of total oocyte RNA that contains the quantity of *Mos* mRNA recovered from each immunoprecipitation. A significant amount of *Mos* mRNA was detected in FLAG immunoprecipitations (FLAG-PABP1 IP) compared to the positive control, CPEB IP (Fig 4-5 B), and this amount of *Mos* mRNA is

approximately equivalent to the amount of *Mos* mRNA recovered from endogenous ePABP immunoprecipitations of oocytes treated with steroid (data not shown).

We also tested if *Mos* mRNA binding would be greatly increased if ePABP was overexpressed. Oocytes that overexpressed FLAG-Paxillin, FLAG-ePABP, or nothing (mock) were treated with steroid for 8 hours, at which point approximately 50 % of the oocytes were mature, and lysates were subjected to FLAG antibody immunoprecipitation. In this experiment, we used FLAG-Paxillin as a control for non-specific RNA binding to proteins since significant quantities of protein can be immunoprecipitated, but little *Mos* mRNA would be expected to bind. Similar amounts of FLAG-Paxillin and FLAG-ePABP were recovered in oocytes not subjected to steroid treatment as indicated by western blot of the immunoprecipitation reactions, although greater amounts of phosphorylated Paxillin were recovered with steroid treatment (Fig 4-5 C, lower panel). Quantification of the RNA from these immunoprecipitations shows insignificant amounts of *Mos* mRNA in control immunoprecipitations (Fig 4-5 C, mock and FLAG-Paxillin) and a dramatic increase of *Mos* mRNA in the ePABP precipitations (Fig 4-5 C, FLAG-ePABP)—nearly 10 times more *Mos* mRNA than was previously observed with precipitation of endogenous ePABP or overexpressed PABP1 (Fig. 4-5 B and C and data not shown). However, only a small, undetermined fraction of total protein was immunoprecipitated in any of these studies (data not shown), so it is difficult to draw any direct quantitative relationships between experiments. Interestingly, no steroid-dependent increase in *Mos* mRNA association was observed when ePABP was overexpressed. This could indicate that saturation of an oocyte with ePABP may lead to

constitutive binding of most *Mos* mRNA in the oocyte, even those transcripts with short poly(A) tails seen in immature oocytes.

Mutation of Paxillin binding sites in PABP1 has an undetermined effect on oocyte maturation

Since we first became interested in studying PABPs due to the proposed ability to bind Paxillin, we wished to determine if binding to Paxillin was required for the activity of PABPs. At the time of this study, we were focused on PABP1 effects, rather than ePABP, since PABP1 repeatedly demonstrated a more reproducible ability to enhance maturation, whereas ePABP overexpression produced variable effects. Therefore, we examined overexpression of Paxillin binding site (PBS) mutations of PABP1 since we believed any loss in activity would be more easily observed. Oocytes were injected with FLAG-tagged wild type PABP1 (WT), or versions where the first PBS (PBS1), second PBS (PBS2), or both PBSs (PBS1/2) were altered (refer to Fig 4-1 B for locations and amino acid changes). Oocytes were then treated with steroid and maturation was monitored over time. At 8 hours, wild-type, PBS1, and PBS2 versions of PABP1 enhanced maturation compared to mock or PBS1/2 expressing oocytes (Fig 4-6 A). This difference was not due to reduced expression of PBS1/2 as expression was verified by western blot (Fig 4-6A). By 12 hours, all oocytes, including mock injected oocytes, had reached 100% maturation with the exception of oocytes expressing PBS1/2, which achieved only 50% maturation (data not shown). These studies have been repeated, and each time PABP1 wild type and versions with only one intact PBS demonstrate enhanced maturation, but the degree to which these enhance maturation

seems to depend on the level of expression—the greater the level of expression by western blot, the greater the enhancing effect. Interestingly, the PBS1/2 mutated version of PABP1 has a variable effect ranging from no effect to partial inhibition of maturation as is observed here (data not shown and Fig 4-6 A), suggesting an interaction with Paxillin may in fact be important during maturation.

To determine if the Paxillin binding sites were important for PABP1 association with *Mos* mRNA, RNA-protein immunoprecipitation studies were performed in oocytes expressing FLAG-tagged versions of each. Immunoprecipitations were performed as before, using FLAG as the precipitating antibody and mock-injected oocytes as a negative control. Semi-quantitative RT-PCR was used to identify the presence of *Mos* mRNA. Wild-type and PBS mutant versions of PABP1 each were capable of associating with *Mos* mRNA, although the association appeared to be partially steroid-dependent for the PBS mutants (Fig 4-6 B lower panels). The lack of steroid-dependent association of mRNA with wild-type PABP1 may be due to the more dramatic expression of PABP1 compared to that of the PBSs as observed by western blot of the initial lysates (Fig 4-6 B upper panel), which might cause an apparent saturation of RNA binding as was observed with overexpressed ePABP (Fig 4-5C). It is possible that if expression levels are titered down by injection of less cRNA, we may see recovery of steroid-dependent RNA association.

Discussion

This preliminary study indicates that ePABP may indeed be important for Xenopus oocyte maturation due, in part, to its ability to regulate translation of *Mos* mRNA. Similar to the results observed in Paxillin-depleted oocytes, knockdown of ePABP mRNA resulted in inhibition of the accumulation of MOS protein, MAPK activation, and maturation in response to steroid, and likely does so despite the fact *Mos* mRNA is polyadenylated normally (Fig 4-3). These results are supported by dominant negative studies in which overexpression of inactive PABPs produced similar results (Wakiyama et al., 2000). Unfortunately, in none of our studies were we able to prove that protein levels of ePABP were affected by double-stranded RNA or antisense oligonucleotide strategies, therefore future studies will be directed toward developing an approach to achieve greater reduction in protein expression to verify the observed results.

PABPs are thought to enhance translation of mRNAs due to their recruitment to transcripts as they become polyadenylated. As a result, eIF4G is carried to the mRNA due to its association with PABPs, and the localization of eIF4G to the mRNA allows it to bind eIF4E and form the initiation complex at the 40S ribosome (Fig 1-2) (Cao and Richter, 2002; Gray et al., 2000). Here, we show that PABPs bind *Mos* mRNA and postulate that it is required for its translation. However, we do not believe that PABPs specifically or preferentially bind only *Mos* mRNA during maturation. In fact, we observed a time-dependent increase in association of GAPDH mRNA with ePABP as maturation progressed, similar to that observed with *Mos* mRNA (data not shown). This indicates that PABP activity is likely generally increased during maturation, therefore

PABPs are probably important for translating a large array of messages in the oocyte. This may also suggest a more general mechanism for PABP function in somatic cells.

The mechanism by which PABPs are regulated is unclear. In some studies, PABPs have been proposed to be to regulated by phosphorylation (Gallie et al., 1997), cleavage (Bushell et al., 2001; Kuyumcu-Martinez et al., 2002), or methylation (Lee and Bedford, 2002), but none of these appear to occur in Xenopus oocytes (Wilkie et al., 2005), and the functional consequences of these modifications is not understood. Therefore, it is postulated that activity of the PAPBs may be regulated, in part, by other proteins in the cell.

An intriguing hypothesis is that Paxillin may be one of the protein factors capable of regulating PABP function. Multimerization of PABPs along polyadenylated mRNAs is thought to be an important process that enhances the activity of PABPs (Kuhn and Pieler, 1996; Melo et al., 2003) and perhaps increased multimerization could explain why overexpression of PABPs enhances steroid-induced maturation (Fig 4-5A and 4-6A). Paxillin has clearly been shown to interact with PAPB1 (Woods et al., 2005), and possibly with ePABP (Fig 4-2). Also, its scaffolding activity and tandem repeats of LD motifs and LIM domains makes Paxillin a prime candidate for coordinating the association of multiple proteins.

Here, we propose a potential, albeit very preliminary, model whereby Paxillin is important for PABP function due to an association with PABP-mRNA complexes (Fig 4-7). Perhaps hypophosphorylated Paxillin is important for stabilizing a PABP-mRNA interaction, multimerization of PABPs on mRNA, or appropriate localization of the complex within the oocyte. Others have shown phosphorylated Paxillin does not interact

with PABP, and PABP does not appear to interact with Paxillin and eIF4G at the same time (Woods et al., 2005). Therefore, once Paxillin localizes PABP to the appropriate compartment in the cell, phosphorylation of Paxillin may serve as a mechanism for release of PABP-mRNA, transferring it over to eIF4G leading to subsequent association with the ribosome. In the case of *Mos* mRNA, future studies may indicate that MOS-mediated phosphorylation of Paxillin is responsible for releasing PABP-mRNAs, thereby enhancing translation of the *Mos* mRNA itself. Such an ideal scenario could also explain how Paxillin may play a role in the MAPK positive feedback loop.

Although study of PABP1 may be informative, future work in the lab will focus on paxillin binding mutations of the endogenously expressed ePABP and how these motifs may be important for the function of ePABP in mRNA binding, MOS translation, and maturation. Furthermore, we hope to determine whether Paxillin is required for PABP function by performing the previous experiments in oocytes depleted of Paxillin. Using these strategies, we will test the proposed model above to determine whether Paxillin is required for PABP's enhancement of maturation, multimerizing, or association with polyadenylated mRNAs.

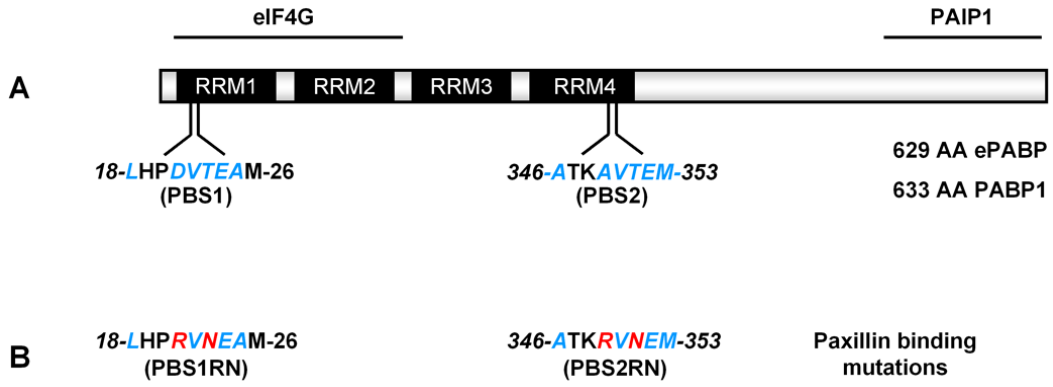


Figure 4-1: Schematic of PABP isoforms. (A) PABP1 and ePABP both consist of four RRM (RNA binding domains), followed by a C-terminal tail. Known binding domains for eIF4G and polyadenylate binding protein interacting protein 1 (PAIP1) are indicated by bars. Residues that make up the consensus Paxillin binding sites (PBS) are indicated in blue and the location in both *Xenopus* PABP isoforms is indicated by the flanking numbers. (B) Point mutations, in red, eliminate Paxillin binding activity (PBS1RN or PBS2RN). Adapted from Woods *et al*. 2005.

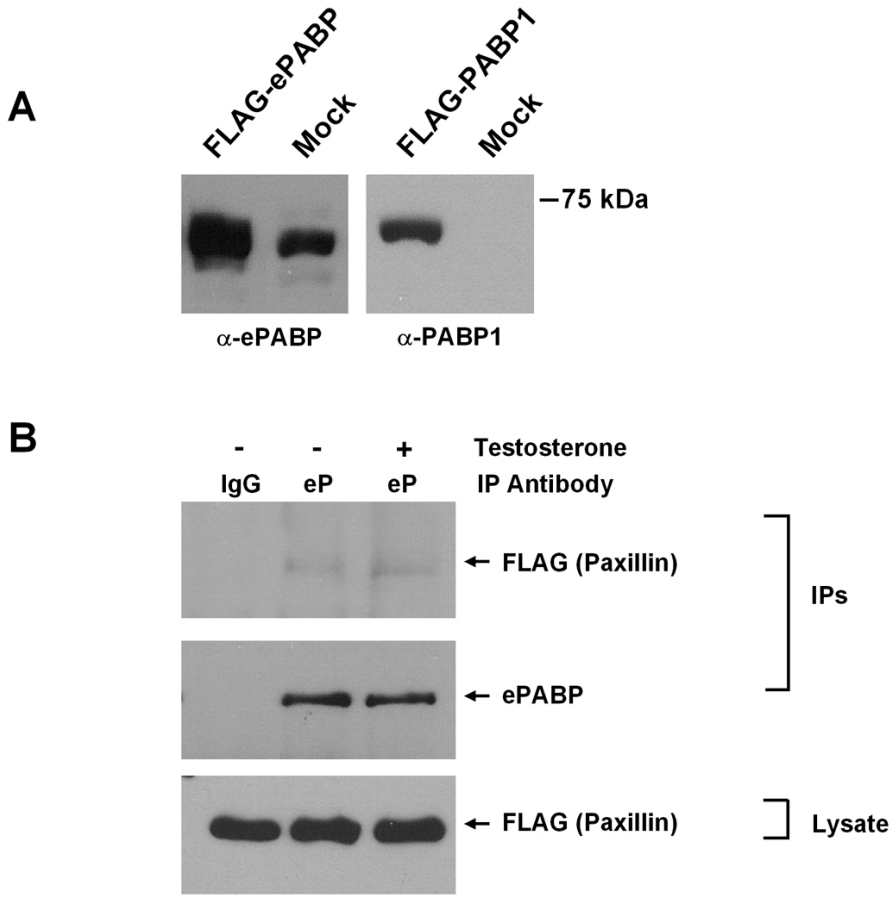


Figure 4-2: Embryonic PAPB is expressed in *Xenopus* oocytes and may bind Paxillin *in vivo*. (A) Oocytes were injected with either Flag-tagged ePABP cRNA, PABP1 cRNA or vehicle alone (mock). After 40 hours, oocytes were lysed and immunoblotted for the presence of ePABP (α-ePABP) or PABP1 (α-PABP1). (B) Oocytes were injected with FLAG-tagged Paxillin and after 40 hours, 50 oocytes were treated +/- 1000nM testosterone for 6 hours, then endogenous ePABP was immunoprecipitated (eP). As a control, a set of oocytes was immunoprecipitated with control rabbit IgG (IgG). Beads were immunoblotted for the presence of Paxillin (FLAG) and ePABP (ePABP). Initial lysates were also immunoblotted to guarantee equivalent expression of FLAG-Paxillin.

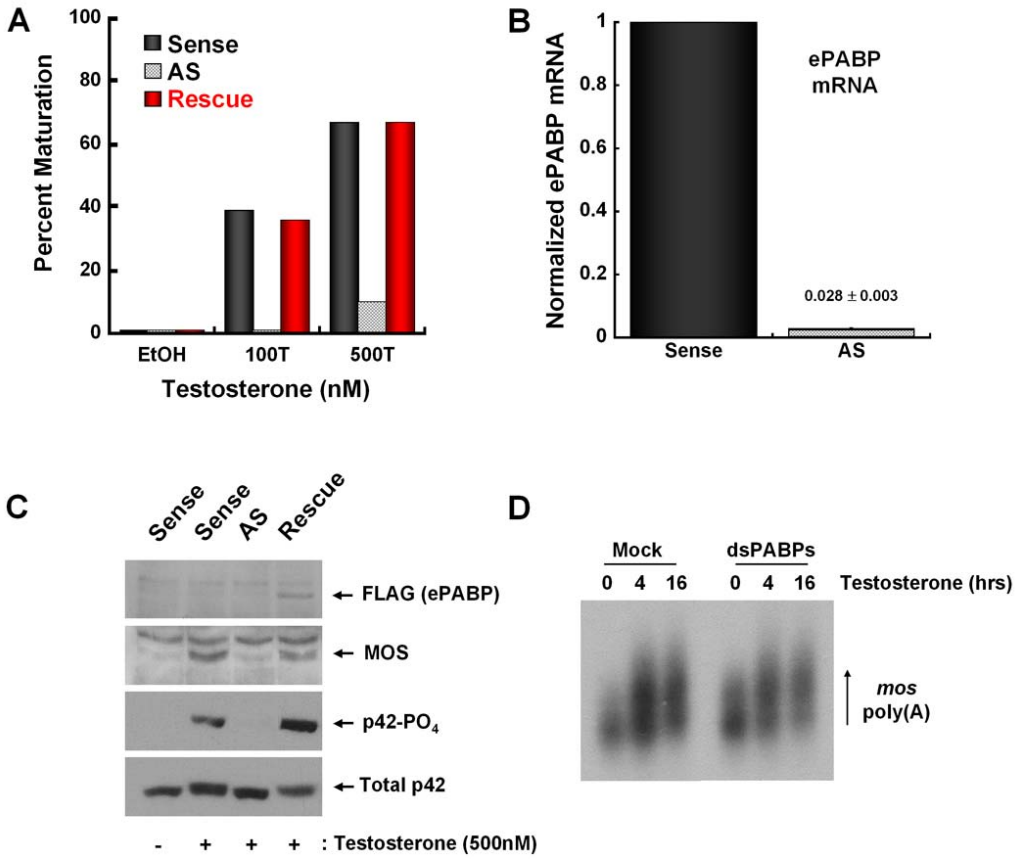


Figure 4-3: Knockdown of ePABP mRNA inhibits oocyte maturation. (A) Oocytes were injected with either 25 ng sense oligonucleotide, 25 ng antisense (AS) oligonucleotide, or 25 ng AS oligonucleotide in addition to 20 ng Flag-ePABP cRNA (Rescue). After 40 hours, oocytes were treated with increasing concentrations of steroid as indicated and GVBD was scored at 16 hours. (B) RNA was extracted from sense and AS injected oocytes. Real-time PCR was performed and levels of ePABP mRNA was normalized to an internal GAPDH control. The relative level of ePABP mRNA in both sets of oocytes was compared by setting the amount of ePABP mRNA in sense injected oocytes as 1. In comparison, the level of ePABP mRNA in AS injected oocytes was 0.02 +/- .003, only 2% of sense. (C) Lysates from oocytes in panel (A) were immunoblotted for the presence of rescue expression of FLAG-ePABP (FLAG), MOS, active ERK2 (p42-PO_4), and total ERK2 (total p42). (D) Oocytes that were injected with a combination of double-stranded PABP1 and ePABP cRNA. 36 hours post-injection, oocyte were injected with a *Mos* 3'UTR reporter, treated with 300 nM testosterone for the indicated times, and from the oocytes was subjected to Northern blot using a *Mos* 3'UTR [^{32}P] probe. Polyadenylation is indicated be decreased mobility.

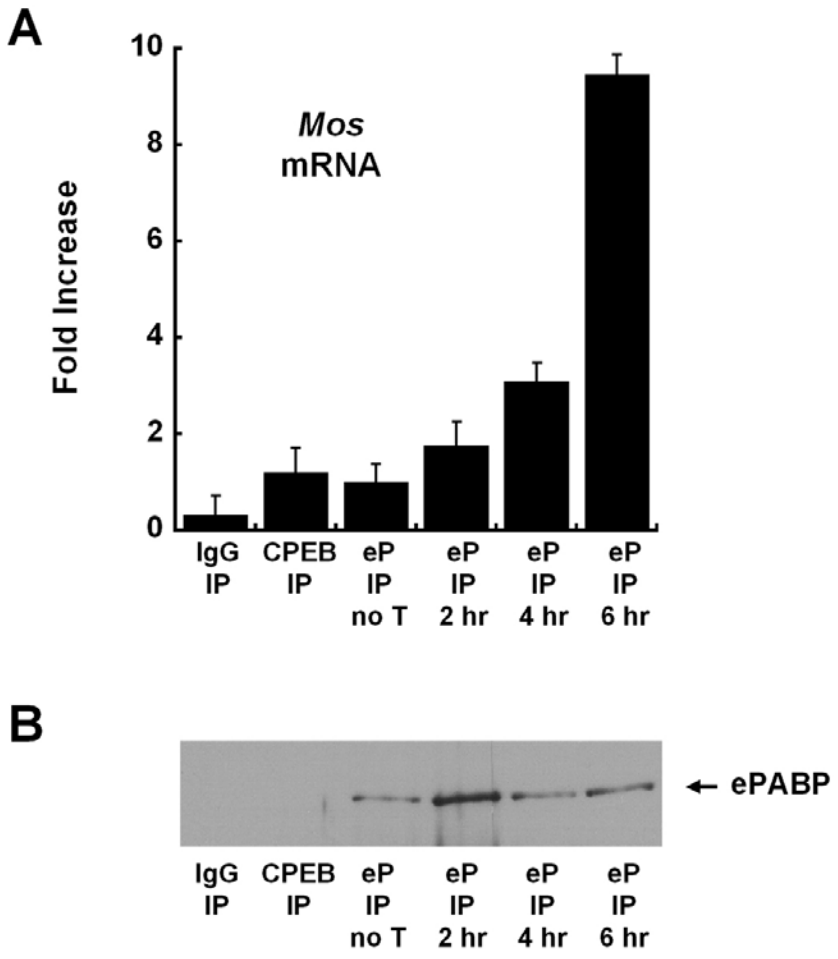


Figure 4-4: Endogenous ePABP binds *Mos* mRNA in a steroid-dependent manner. Oocytes were treated with either no steroid (IgG, CPEB, eP no T) or 300nM testosterone for the indicated period of time. Oocytes were then lysed and subjected to immunoprecipitation by the indicated antibody. (A) RNA was extracted from these immunoprecipitations and Real-Time PCR was performed to determine the amount of *Mos* mRNA associated with the precipitates. Absolute values of *Mos* mRNA were normalized to mRNA binding in ePABP precipitates at time zero (no T) to calculate fold-increase in binding. (B) Efficiency of ePABP protein pull-down was verified by western blot of the precipitates used for RNA extraction using the α-ePABP antibody. Approximately 10% of the immunoprecipitations was loaded in each well.

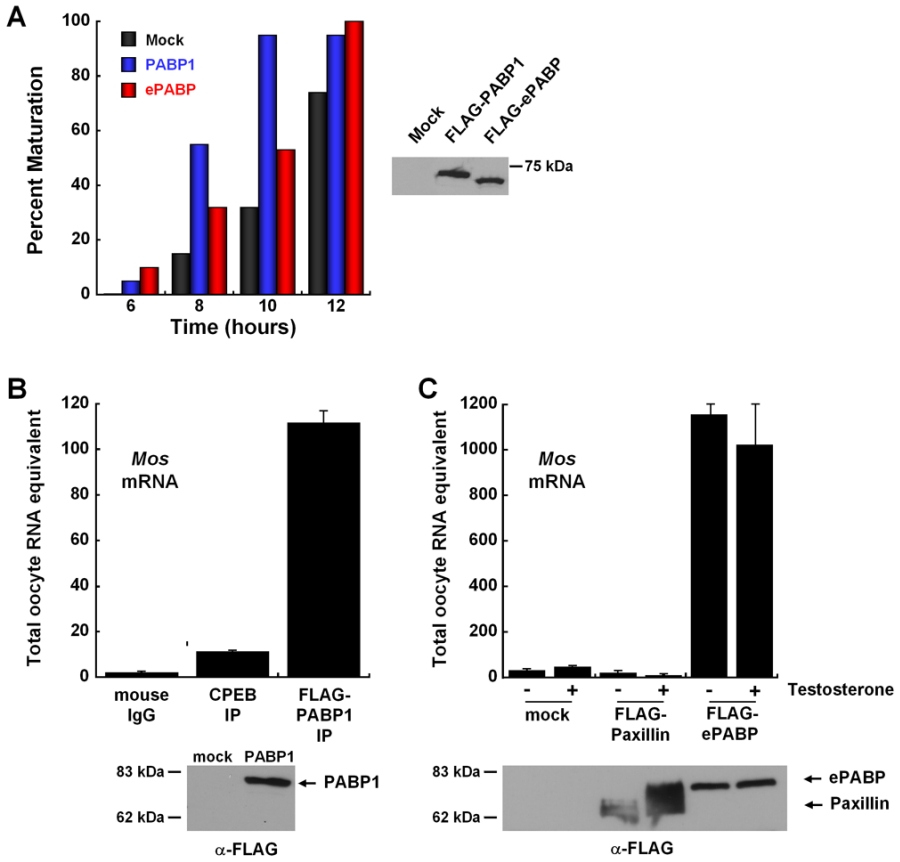


Figure 4-5: Overexpression of PABP1 and ePABP enhance maturation to varying degrees and bind *Mos* mRNA. (A) Oocytes were injected with cRNA encoding FLAG-tagged PABP1 or FLAG-tagged ePABP. After 40 hours, oocytes were treated with 100 nM testosterone and maturation was scored over time. Expression was verified by immunoblotting for the Flag tag (inset) (B) Forty mock injected or FLAG-PABP1 expressing oocytes were immunoprecipitated with either control antibody (FLAG-PABP1 oocytes-mouse IgG), CPEB antibody (mock oocytes), or FLAG (FLAG-PABP1 oocytes-FLAG-PABP1 IP). *Mos* mRNA was extracted and quantitated by real-time PCR (above) and expression of FLAG-PABP1 in the initial lysates was verified by western blot (below). (C) After treatment with 300nM testosterone for 8 hours, 50 oocytes expressing FLAG-Paxillin, FLAG-ePABP, or nothing (mock) were subjected to immunoprecipitation with FLAG antibody. *Mos* mRNA was extracted and quantified by real-time (above), and approximately 10% of the immunoprecipitation reactions was immunoblotted with FLAG antibody to determine if equivalent amounts of protein were recovered (below). For B and C, the y-axis represents the amount of total oocyte RNA that would contain the amount of *Mos* mRNA observed in each sample.

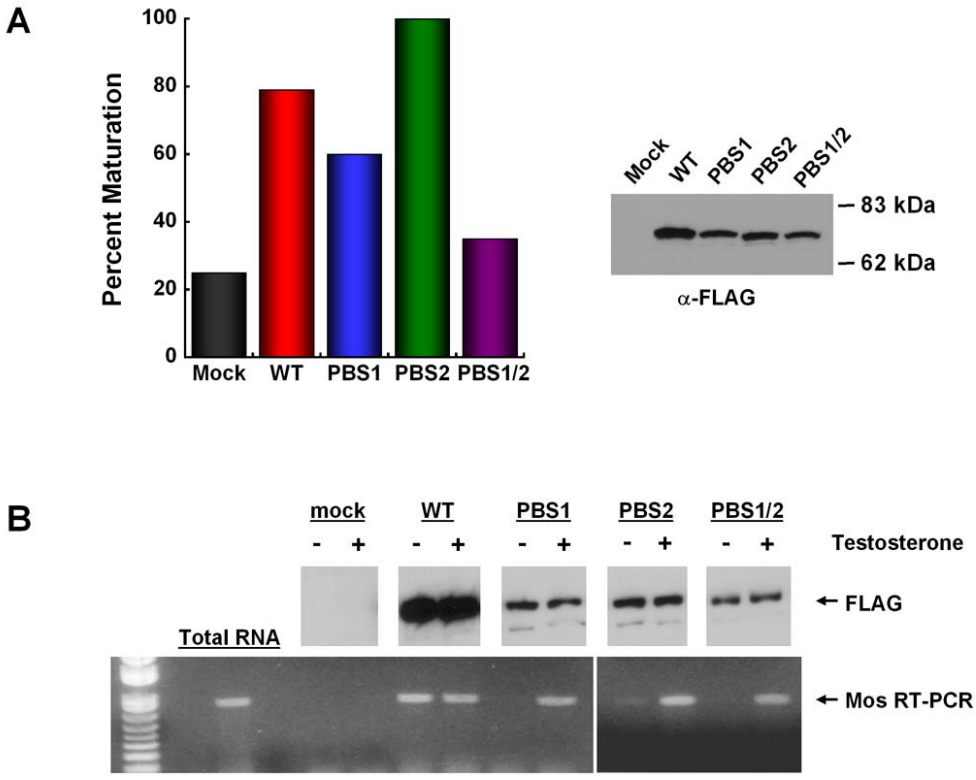


Figure 4-6: Mutation of Paxillin binding sites in PABP1 does not dramatically alter maturation or *Mos* mRNA binding. (A) Oocytes were injected with 4ng cRNAs encoding FLAG-tagged PABP1 wild type (WT) or vehicle control (mock). Similarly, oocytes were injected with cRNAs encoding mutations of PABP1 in which the first PBS was eliminated (PBS1), the second (PBS2), or both (PBS1/2). After 40 hours, oocytes were treated with 100 nM testosterone and maturation was observed at 8 hours. Expression of each of the PABP1 constructs was verified by western blot (α-FLAG). (B) In a separate experiment, oocytes were injected as in (A), and treated +/- 100nM testosterone for 8 hours, immunoprecipitated with FLAG antibody, and RNA was extracted from the immunoprecipitation reactions. Expression of each was verified by western blot (upper) and semi-quantitative RT-PCR for *Mos* was performed. Total oocyte RNA was used as a positive control.

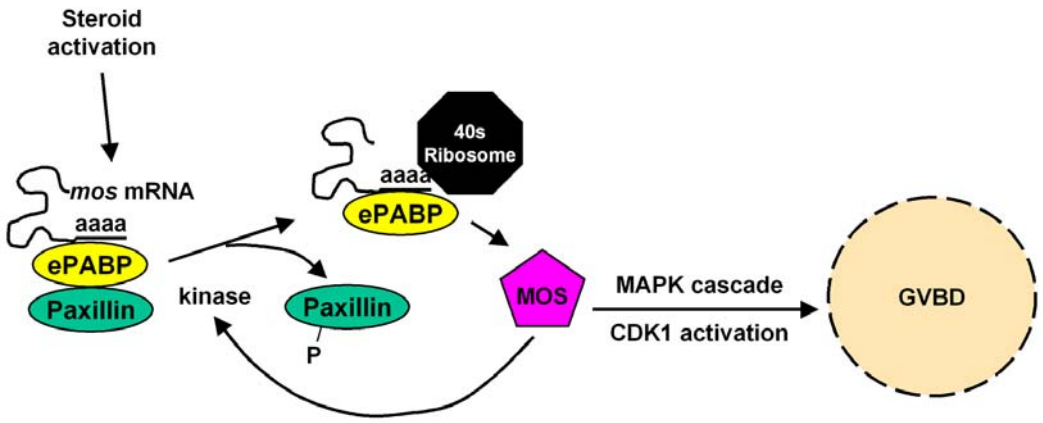


Figure 4-7: Proposed model of a Paxillin-PABP interaction during oocyte maturation. If Paxillin is important for the ability of ePABP to initiate translation in the oocyte, one proposed mechanism would be that Paxillin is either important for the ePABP-mRNA interaction, or it is important for translocating ePABP to the ribosome for translation of its associated cargo. Because phosphorylated Paxillin has not been shown to interact with PABPs (Woods et al., 2005), phosphorylation could be one way in which ePABP-mRNA complexes are delivered to the ribosome. In the case of *Mos* mRNA, it is possible that MOS, or kinases downstream of MOS, cause the phosphorylation of Paxillin, enhancing its ability to dissociate from ePABP, thereby enhancing translation of MOS protein.

Chapter Five

Conclusions and Recommendations

Previous investigations of Paxillin function have focused on a role for Paxillin as a signaling scaffold and have largely attributed regulation of its scaffolding function to tyrosine phosphorylation of Paxillin (Brown and Turner, 2004). Before this study, serine phosphorylation of Paxillin was observed, but no function could be attributed to such phosphorylation (Woodrow et al., 2003). Here, we show that serine phosphorylation of Paxillin is critical to its function as a regulator of oocyte maturation. Specifically, Paxillin appears to be required for steroid-induced accumulation of MOS protein, but its activity is MEK-dependent, suggesting it may play a role in the positive feedback kinase loop that is activated during maturation. However, several questions still remain as to how Paxillin may regulate this important kinase signaling. It is likely that the some of the mechanisms of Paxillin regulation and function that will be elucidated in future oocyte studies will be utilized by somatic cells. In these studies, we briefly examined two hypotheses that may explain how Paxillin regulates oocyte maturation.

Intuitively, it makes sense that because the oocyte is so large, some mechanism must be in place to ensure full activation of the MAPK cascade once the appropriate signals are present to activate it. The all-or-none nature of oocyte maturation indicates that activation of MAPK must be achieved efficiently and completely. As has been described, the MAPK cascade in the oocyte can positively enhance itself, therefore we wish to investigate the possibility that the creation of a MAPK scaffold could increase the

efficiency of MAPK activation by concentrating the components of the cascade to specific locations within these very large cells. Considering Paxillin creates a MAPK scaffold including Raf-MEK-ERK, in mammalian cells, and its activity has been shown by us to be required for MOS accumulation, Paxillin is an obvious candidate for a MAPK scaffold in the oocyte. Interestingly, other studies indicate tyrosine phosphorylation is important for such interactions. In contrast, our studies can only demonstrate an interaction of XeMOS and XePaxillin when overexpressed in COS-7 cells, whereas this association seems to require the serine 107/111 residues. To date, is unclear if XePaxillin interacts with other MAPK components such as MEK or ERK. Therefore, future work will focus on understanding whether Paxillin forms a MAPK scaffold in the oocytes, and if specific residues are required for this activity. In part, this could be accomplished by attempting Paxillin rescue experiments using forms of Paxillin that cannot be tyrosine phosphorylated, as well as determining the order in which Paxillin residues are phosphorylated. Furthermore, if specific motifs that bind MOS can be identified, the effect of ablation of this interaction could be assessed. Additionally, identification of the *in vivo* kinase may also shed some light as to how Paxillin is regulated. Overall, by careful examination of the role tyrosine and serine phosphorylation plays in the oocyte or the ability of Paxillin to bind MAPK cascade components, we may develop a better understanding of the timing and necessity for Paxillin phosphorylation that may apply to several signaling pathways.

Regardless of the whether Paxillin serves as a MAPK scaffold, we must also consider the possibility that Paxillin may function as a general regulator of transcription, such that it promotes the activity of PABPs to initiate translation of polyadenylated

mRNAs. PABP interacts with both initiation complex proteins and Paxillin, but not simultaneously, suggesting this may be one level of regulation of PABP activity (Woods et al., 2005). In these studies, we have only completed preliminary experiments that served to verify that indeed, ePABP is expressed in oocytes, that it can bind mRNAs, and that loss of function appears to mimic the loss of Paxillin. It remains to be determined if loss of Paxillin results in reduced activity of PABPs or if Paxillin is required for the PABP-mRNA interaction. As a result, future studies will focus on developing strategies to better reduce expression of ePABP protein, evaluate the effect on maturation, and determine if the capacity to bind Paxillin plays a part in its ability to initiate translation of polyadenylated messages.

No matter what the mechanism of Paxillin regulation turns out to be, these and future data will certainly provide valuable information about how a complex array of upstream signals can be integrated through Paxillin signaling. In general, oocytes are a superb model system for exploring either MAPK scaffolds or translational activation of polyadenylated mRNAs, since both processes are greatly upregulated during steroid-mediated oocyte maturation and most of the candidate proteins are expressed abundantly. Whether a MAPK scaffold, a regulator of translation, or both, the study of Paxillin in oocytes likely will provide new insight into how Paxillin activity could be regulated in all cells, not just germ cells.

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