XGef functions independently of exchange factor activity to influence RINGO/CDK1 signaling and CPEB activation during Xenopus oocyte maturation

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XGEF FUNCTIONS INDEPENDENTLY OF EXCHANGE FACTOR ACTIVITY TO INFLUENCE RINGO/CDK1 SIGNALING AND CPEB ACTIVATION DURING XENOPUS OOCYTE MATURATION.

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ABSTRACT

XGef functions independently of exchange factor activity and influences RINGO/CDK1 signaling and CPEB activation during *Xenopus* oocyte maturation.

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Metazoan development depends on cytoplasmic polyadenylation, a key mechanism that controls the translation of maternally deposited mRNAs. In *Xenopus laevis* oocytes, CPEB regulates the translation of several developmentally important mRNAs, which drive meiotic progression and the production of fertilizable eggs. Most of our current knowledge of this process, also referred to as oocyte maturation, has been acquired from experiments conducted in *Xenopus laevis* oocytes. Despite over 30 years of research devoted to the exploration of progesterone signaling during maturation, the very early events that occur from progesterone receptor engagement to CPEB activation are not well understood. XGef, a putative Rho family guanine nucleotide exchange factor (GEF), interacts with CPEB and facilitates CPEB activation and timely meiotic progression. To further our understanding of XGef function during meiotic progression, the requirement for exchange factor activity and the activities of several Rho GTPases during maturation were examined. Despite previous reports of XGef activation of Cdc42 in mammalian cell culture, XGef does not stimulate the activation of Cdc42 in maturing *Xenopus* oocytes. Further, Cdc42 activity does not affect CPEB phosphorylation and overexpression of a dominant negative Cdc42 mutant does not affect maturation. Inhibition of Toxin B sensitive Rho GTPases, including Cdc42, Rac1 and Rho A-C, also

fails to affect CPEB activation or meiotic progression. Lastly, the overexpression of XGef exchange deficient point mutants did not affect maturation compared to oocytes overexpressing wildtype XGef. Together, these results suggest that as a facilitator of CPEB activation and meiotic progression, XGef functions independently of exchange factor activity and Rho GTPase activation. Additionally, we found that XGef activity influences the function of RINGO/CDK1, a novel component of the progesterone signaling pathway. XGef inhibition depresses RINGO-induced GVBD, whereas XGef overexpression enhances this process. XGef interacts with RINGO in oocyte extracts and the interaction is direct in vitro. Our protein interaction data, in total, suggest that a XGef/RINGO/MAPK/CPEB complex forms in ovo to facilitate CPEB activation. Lastly, inhibition of RINGO activity directly compromises CPEB phosphorylation during early maturation, which suggests that RINGO/CDK1 directly mediates CPEB-activation.

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LIST OF ABBREVIATIONS

cAMP	3'-5'-cyclic adenosine monophosphate
CDK	Cyclin Dependent Kinase
CPEB	Cytoplasmic polyadenylation element binding protein
CPSF	Cleavage and polyadenylation specificity factor
DH	Dbl Homology
EPAB	Embryonic poly(A) polymerase
GAP	GTPase activating protein
GDI	GTPase dissociation inhibitor
GPCR	G-protein coupled receptor
GST	Glutathione-S-Transferase
GVBD	Germinal vesicle breakdown
HA	Hemagglutinin
IgG	Immunoglobulin G
MAPK	Mitogen activated kinase
MPF	Maturation Promoting Factor
PAK	P21 activated protein kinase
PAP	Poly(A) polymerase
PARN	Poly(A) ribonuclease
PBD	Protein Binding Domain
PBE	Pumilio Binding Element
PG/Prog	Progesterone
PH	Pleckstrin Homology
PKA	cAMP dependent protein kinase A
Plx1	Polo-like kinase 1

Plkk-1	Polo-like kinase kinase 1
PRE	Polyadenylation Response Element
PUM	Pumilio
RBD	Rho Binding Domain
RINGO	Rapid inducer of G2/M progression in oocytes
RRM	RNA recognition motif
XGef	Xenopus guanine nucleotide exchange factor
XPR-1	Xenopus progesterone receptor 1

Chapter I. Introduction

Metazoan development depends on maternally derived cytoplasmic stores within oocytes, which harness all of the elements required to support production of mature ova and cell cycle progression, body axis establishment and cell fate determination within the growing embryo. These important developmental milestones are driven by spatio-temporally controlled protein synthesis (Wickens et al., 2000). Translational regulation of stored maternal mRNAs is especially key during early development when transcription cannot be detected in oocytes and early zygotes. Promptly after fertilization, the zygote undergoes twelve rounds of synchronous cell divisions consisting of only S and M phases. During this Pre-Mid-Blastula Transition (Pre-MBT), the rapidity of these divisions do not allow DNA transcription to occur (Newport and Kirschner, 1982; reviewed in Maller et al., 2001). The cyclic synthesis and destruction of proteins, which drive the meiotic divisions an oocyte must undergo during its transformation into a fertilizable egg, must therefore rely on translational regulation of pre-existing, maternally deposited mRNAs (Nebreda and Ferby, 2000; Mendez and Richter, 2001; Wickens et al., 2000). Cytoplasmic polyadenylation, mediated mRNA 3'UTR cis-elements and trans-acting RNA specificity factors, is a key mechanism of translational regulation during early development in many vertebrates.

The introduction will provide background relevant to the data presented in this thesis. Here I review the process of oogenesis and provide an overview of the progesterone signaling events that drive oocyte maturation, the transformation immature oocytes must undergo to become fertilizable eggs. Additional background is devoted to explaining what is known about the factors that influence the activity of an RNA specificity factor, CPEB, which controls the translation of several developmentally important mRNAs in a polyadenylation-dependent fashion.

A. *Xenopus laevis* oogenesis

The process of oogenesis in most vertebrates involves the formation, development and maturation of an ovum. Oogenesis can be divided into three stages. First, a population of oocytes is established through several rounds of Primordial germ cell (PGC) division in the proliferation stage. In non-mammal vertebrates, in particular, the oocyte undergoes a substantial increase in size during the growth stage. Lastly, in response to a hormone cue, the oocyte undergoes meiotic divisions, a pre-requisite for fertilization. PGCs migrate to the sexually ambiguous gonad during *Xenopus laevis* larval development. The PGCs proliferate and once a population of approximately 10,000 cells is reached, the gonad becomes specified. In *Xenopus laevis* females, PCGs give rise to primary oogonia, from which secondary oogonia are derived (Al-Mukhtar and Webb, 1971). The secondary oogonia undergo four mitotic divisions and the resulting sixteen cells become oocytes (Klock et al., 2004). Over the course of 4 to 8 months, the oocyte expands in size in a growth process divided into six stages (I-VI) according to oocyte diameter. During oogenesis, yolk proteins accumulate, cortical granules and melanosomes are incorporated into the cortex, organization of germ-plasm, mitochondria and maternal mRNAs occurs, and animal/vegetal axis polarity becomes established (Dumont, 1972; Chang et al., 1999).

B. Oocyte maturation

Fully-grown stage VI oocytes are arrested in prophase I of meiosis and remain in this G2-like state for months awaiting a progesterone signal, which induces meiotic progression (reviewed in Ferrell, 1999a; Ferrell, 1999b; Nebreda and Ferby, 2000). Upon progesterone receptor engagement, the oocyte undergoes two consecutive meiotic divisions without an intervening S phase (Newport and Kirschner, 1982). Numerous morphological changes occur during this maturation process. The nuclear

envelope (germinal vesicle) dissociates upon completion of the first meiotic division (MI). This event is referred to as germinal vesicle breakdown (GVBD) and is seen as a white spot on the animal pole, which results from cortical granule displacement. Condensed chromosomes are then separated by the metaphase I spindle and the first polar body is extruded. Without an intervening interphase, the second metaphase spindle forms and the mature egg becomes arrested in metaphase II. Completion of meiosis II does not occur until fertilization (reviewed in Yamashita, 1998).

C. Translational regulation during *Xenopus* oocyte maturation and development

Translational regulation of maternal mRNAs usually occurs at the initation step and depends on the assembly of the translation initiation complex, eIF4F and the small ribosomal subunit, at the mRNA 5' m7GpppN cap. eIF4F consists of eIF4E (cap-binding protein), eIF4A (RNA helicase) and eIF4G (large scaffold). The interactions between eIF4E, eIF4G and poly(A)-binding protein (PABP) bring together the 5' and 3'end, forming a closed-loop structure (Piccioni et al., 2005). Based on this closed-loop model, translational regulation occurs through the interactions between the cap and translation initiation factors (reviewed in Piccioni et al., 2005; Radford et al., 2008; Standart and Minshall, 2008, Wakiyama et al., 2000).

1. mRNA maturation and nuclear polyadenylation

In the nucleus, newly transcribed precursor mRNAs must undergo capping, splicing and nuclear polyadenylation before export as mature mRNA. Only mRNAs that have been modified in this manner can be efficiently translated. Upon transcription, the newly synthesized mRNA is 5' capped with a 7-methyl guanosine residue, which shields against nuclease degradation (reviewed in Wahle and Keller, 1992; Sachs and Wahle, 1993; Manley, 1995). Once transcription is complete, the pre-mRNA undergoes splicing

followed by nuclear polyadenylation, which involves the coordinated association between 3'UTR cis-acting sequences and trans-acting factors. Cleavage and Polyadenylation Specificity Factor (CPSF) binds to an AAUAAA polyadenylation signal hexanucleotide. This hexanucleotide is located 10-30 nucleotides upstream of the actual polyadenylation site. Further downstream is a G/U rich site bound by Cleavage Specificity Factor (CstF) (Takagaki et al., 1992). The mRNA is cleaved to remove the CstF-bound G/U rich site and 100-250 adenines are added to the polyadenylation site by PolyA Polymerase (PAP) (Sheets and Wickens, 1999; Zhao et al., 1999). mRNAs exported from the oocyte nucleus are typically deadenylated and possess shortened poly(A) tails of only 20-40 nucleotides in length. In this deadenylated state, these stored mRNAss are translationally silenced (Huarte et al., 1992).

2. Cytoplasmic polyadenylation

Maternal mRNAs undergo two phases of polyadenylation, the first, described previously, occurs exclusively in the nucleus (Sheets and Wickens 1989). The second polyadenylation event occurs in the cytoplasm and is an important mechanism that regulates the translation of maternal mRNAs in *Xenopus* oocytes (Richter, 1999). Mature mRNAs exported from the nucleus become deadenylated and translationally repressed until a specific moment during oogenesis and/or development when translation is stimulated by cytoplasmic polyadenylation (reviewed in Grey and Wickens, 1998). Maternal mRNAs subject to polyadenylation-induced translation typically contain cis-elements in the 3'UTR, which are bound by trans-factors (Sheets et al., 1994; de Moor et al., 2005). Together, 3'UTR regulatory sequences and trans-acting RNA binding proteins dictate the timing of Poly(A) polymerase elongtion of the poly(A) tail. Poly(A) tails 80 to 250 adenosines in length trigger the recruitment of translation initiation factors (McGrew et al., 1989; Fox et al., 1989; reviewed in Wickens et al., 2000; Gray and

Wickens, 1998; Richter J.D. 1999). Polyadenylation of maternal mRNAs during meiotic progression is regulated by Cytoplasmic Polyadenylation Element (CPE) and Cytoplasmic Polyadenylation Element Binding Protein (CPEB), the most extensively studied mechanism of translational regulation through poly(A) tail length control (Hake and Richter, 1994). CPE/CPEB-mediated regulation of maternal mRNA translation will be presented later in detail.

D. The progesterone receptor

Progesterone released from surrounding follicle cells binds to a cell surface receptor to initiate meiosis resumption in *Xenopus* oocytes. Since progesterone injection into the nucleus or cytoplasm does not induce GVBD, the progesterone receptor is likely membrane bound, facing externally (Maller, 2001). Progesterone signaling has also been proposed to occur through a heterotrimeric G protein-couple receptor (GPCR) to influence adenylyl cyclase (AC) activity (Reviewed in Schmitt and Nebreda, 2002; Hammes, 2004). However, a GPCR has not been identified in *Xenopus* oocytes. Alternatively, progesterone may bind the classic steroid receptor, X-PR1, which Tian and colleagues cloned from Xenopus oocyte cDNA. Increasing evidence suggests that this is indeed the case. Overexpression of X-PR1 in oocytes accelerates maturation, whereas X-PR1 depletion blocks GVBD (Tian et al., 2000; Martinez et al. 2007).

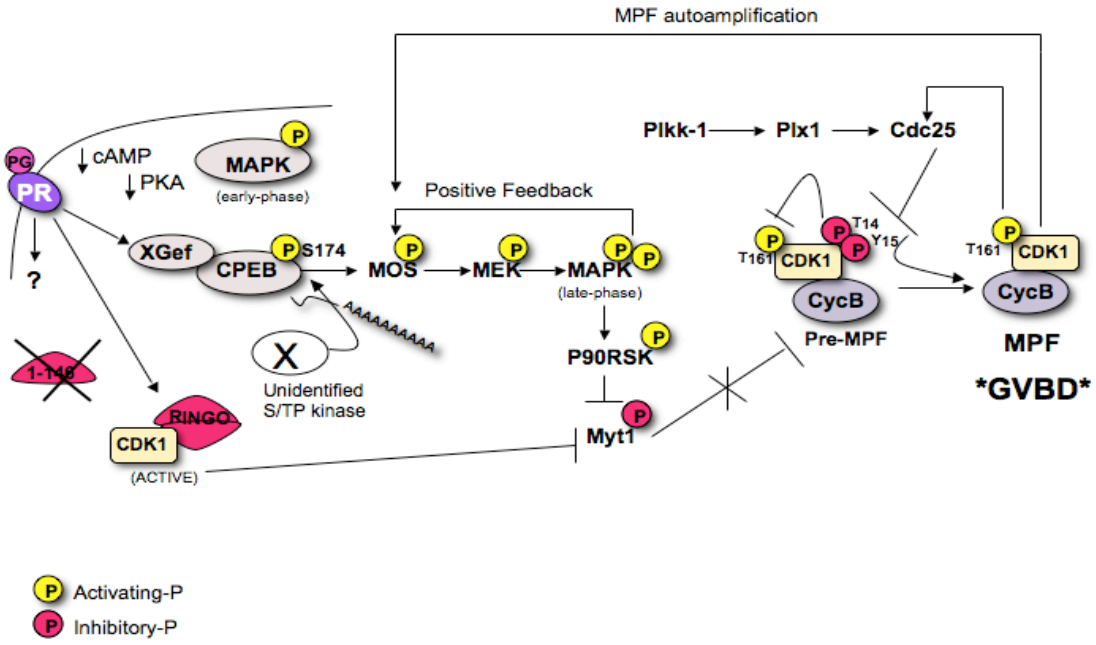
E. Progesterone signaling

1. Overview

Progesterone stimulation, through a non-genomic mechanism, triggers a rapid and transient decrease in cAMP and PKA activity through adenylate cyclase down-regulation (Maller and Krebs, 1997; Maller, 2001). During the first hour of maturation, an atypical Cdk activator, RINGO, must be synthesized (Ferby et al., 1999, Kim and

Richter, 2007; Gutierrez et al., 2007). A low level of MAPK activation is also detectable during this early time in meiosis (Fisher et al., 1999, Fisher et al, 2000). RINGO and MAPK activities are required for the next downstream event, CPEB activation, however, the mechanism is not well understood (Keady et al., 2007; Kim and Richter, 2007). A ser/thr kinase phosphorylates to activate CPEB on Ser174. Although Aurora A kinase is proposed to act as the CPEB activating kinase, its activity cannot be detected in oocytes and mounting evidence suggests that an alternative kinase directs CPEB activation (Mendez et al., 2000; Frank-Vaillant et al., 2000). CPEB activation induces c-Mos mRNA polyadenylation and Mos (MAPKKK) synthesis, initiating a Mos/MEK/MAPK(ERK2) cascade (Posada et al., 1993; Castro et al., 2001). MAPK phosphorylates to activate P90RSK, which phosphorylates to inactivate Myt1, a dual-specificity kinase that catalyzes Cdc2 inhibitory phosphorylations on Thr14 and Tyr15 (Bhatt and Ferrell, 1999). The MAPK cascade effectively blocks Myt1-mediated Cdc2 inhibition to activate the cdc2/cyclinB heterodimer, Maturation Promoting Factor (MPF). In a parallel pathway, Polo-like kinase kinase-1 (Plkk-1) phosphorylates to activate Polo-like kinase 1 (Plx1), which mediates Cdc25 activation, leading to MPF activation through removal of inhibitory Cdc2 phosphorylations (Abrieu et al., 1998; Wang et al.., 2007). MPF activation triggers GVBD and peaks of MPF activity drive MI and MII progression.

Figure 1. Progesterone stimulation of *Xenopus* oocyte maturation



2. The earliest signaling events: a decrease in cAMP and PKA activity

Changes in 3'-5'-cyclic adenosine monophosphate (cAMP) level and cAMP-dependent protein kinase A (PKA) activity are the earliest biochemical events during *Xenopus* oocyte maturation. Minutes after an oocyte is exposed to progesterone, a rapid decrease in cAMP level (40-60% reduction) and PKA activity occur. (Maller and Krebs, 1997; Sadler and Maller, 1981). Elevated PKA activity maintains oocyte G2 arrest since injection of PKA inhibitors, PKA_R or PKI, triggers GVBD without progesterone stimulation (Maller and Krebs, 1977; Sadler and Maller, 1981). Duckworth and colleagues demonstrated that Cdc25C, a phosphatase that removes Cdc2 inhibitory phosphorylations, thereby activating MPF, serves as a physiological PKA substrate. PKA phosphorylates Cdc25C on Ser 287, which creates a 14-3-3 binding site. Cdc25C

interaction with 14-3-3 inactivates the phosphatase through sequestration and/or disruption of Cdc25C/Cdc2 binding. Overexpression of a mutant version of Cdc25C that is immune to PKA inactivation (S287A) can trigger maturation independently of progesterone (Duckworth et al., 2002). Together, these results suggest that PKA is essential for maintaining G2 arrest by enforcing Cdc25C inhibition.

The PKA holoenzyme consists of a regulatory dimer (PKA_R) and two catalytic monomers (PKA_C). The regulatory domains are categorized into two groups, RI and RII. Each group has two isoforms, α and β. PKAc isoforms α, β and γ have also been identified. Each regulatory dimer associates with four cAMP molecules, causing PKAc to dissociate and interact with downstream effectors. When intracellular levels of cAMP are low, the regulatory subunits re-associate with the catalytic subunits, blocking their activity (reviewed in Shibuya, 2003). As additional support of PKA function as a mediator of oocyte meiosis arrest, injection of PKAc also blocks progesterone-induced maturation. Interestingly, numerous research groups have shown that PKA catalytic activity is dispensable towards maintaining meiotic arrest. Overexpression of kinase dead PKAc (K72R) blocks GVBD in response to progesterone. The same inhibition occurs with Mos-induced GVBD. Interestingly, PKAc K72R merely delays constitutively active Cdc25C induction of maturation (Schmitt and Nebreda, 2002). These findings suggest that PKA may possess different functions, in which some steps along the progesterone signaling pathway require PKA catalytic activity, while others do not.

3. RINGO/CDK1: a novel component of progesterone signaling

a. Significance and Discovery

Xenopus RINGO (Rapid Inducer of G2/M progression in Oocytes) synthesis is upregulated shortly after progesterone stimulation (Gutierrez et al., 2007). RINGO protein accumulation and activity as an atypical CDK1 activator are required for the

oocyte maturation process (Ferby et al., 1999, Kim and Richter, 2007; Gutierrez et al., 2006). RINGO was identified in two independent screens for factors that affected the G2/M transition in *Xenopus laevis* oocytes (Ferby et al., 1999; Lenormand et al., 1999). The first used a *X. laevis* total ovary cDNA library to screen for genes that conferred UV damage resistance in a K1 rad- strain of *Saccharomyces pombe*. This particular strain is not resistant to UV irradiation and does not undergo G2 arrest upon UV damage. One clone out of 55,000 transformants rescued the rad- strain, restoring cell cycle arrest similarly to the wildtype Rad+972 strain after irradiation. Overexpression of this gene product in *Xenopus* oocytes triggered GVBD without progesterone signaling. The clone was appropriately named Xenopus Speedy (xSpy) (Leonormand et al., 1999). In the second screen, *Xenopus* oocyte cDNA library inserts were cloned into a FTX5 expression vector and transformed into bacteria. Clones from 150-200 colony pools were purified and used as templates for mRNA *in vitro* transcription. These mRNA batches were then injected into oocytes. The clones ls 26 (RINGO A) and ls 27 (RINGO B), induced GVBD in the absence of progesterone stimulation as well. RINGO A and B are 88% identical and encode 299 and 298 amino acid products, respectively. Both RINGO A and RINGO B function as potent activators of CDK 1 and CDK 2, but are considered atypical due to their dissimilarity to Cyclins (Ferby et al., 1999; Karaiskou et al., 2001; Nebreda, 2006).

b. Translational regulation and protein expression

RINGO mRNA is translationally regulated by cytoplasmic polyadenylation. Padmanabhan and Richter began their investigation of RINGO mRNA translational regulation by first showing that the RINGO 3'UTR possesses cis-elements that repress translation. The RINGO 3'UTR was ligated downstream of a GFP open reading frame (ORF) and injected into immature oocytes. In unstimulated oocytes, GFP was not

expressed. Examination of the RINGO 3'UTR revealed two putative Pumilio Binding Elements (PBEs) UGUAUAAA and UGUAAAUA (Padmanabhan and Richter, 2006). PBEs are bound by either Pumilio-1 or 2 (PUM1, PUM2), which are members of the PUF family of RNA binding proteins initially identified in *Drosophila melanogaster* (reviewed in Wickens et al., 2002). The interaction between PUM2 and the RINGO PBEs was confirmed by an Electrophoretic Mobility Shift Assay (EMSA), in which PUM2 specifically bound to a radiolabeled RINGO 3'UTR probe. Perturbation of PUM2 function also triggered expression of the GFP/RINGO 3'UTR reporter mRNA. Additionally, when PUM2 antibodies were injected into unstimulated oocytes, RINGO synthesis was upregulated in the absence of progesterone. These results suggest that PUM2 regulates RINGO synthesis in *Xenopus* oocytes, silencing RINGO mRNA in G2 arrested oocytes and stimulating translation upon maturation initiation (Padmanabhan and Richter 2005).

Padmanabhan and Richter also found that Deleted for Azoospermia-like (DAZL) and Embryonic Poly A Binding protein (EPAB) are present in the RINGO mRNP. Immunoprecipitation of myc-PUM2 overexpressed in oocytes co-precipitated DAZL and EPAB as well as RINGO mRNA. Interestingly, the components of the RINGO mRNP changed upon progesterone stimulation, in which PUM2 dissociates from the protein complex during maturation. According to their model, RINGO mRNA translation is regulated by PUM2, which binds to 3'UTR PBE elements while associating with DAZL and EPAB to block translation in the immature oocyte. However, in response to progesterone, PUM2 dissociation alleviates the translational repression and allows RINGO synthesis to occur in maturing oocytes (Padmanabhan and Richter, 2005).

RINGO mRNA is present at a constant level from oogenesis to early embryogenesis, although abundance of the 1.3 kb transcript decreases at gastrulation. RINGO protein synthesis is upregulated in response to progesterone stimulation and

was at first presumed absent in immature oocytes (Ferby et al. 1999; Padmanabhan and Richter, 2005). Detection of endogenous RINGO involved immunoblotting or immunoprecipitation of ^{35}S labeled proteins using RINGO antibody affinity purified from the serum of rabbits immunized with the full-length protein. However, immunoprecipitation with this particular antibody proved inefficient and western detection of the endogenous RINGO was poor (Ferby et al., 1999). Gutierrez and colleagues utilized two separate rabbit polyclonal antibodies recognizing N or C terminal RINGO to detect RINGO protein in immature and maturing oocytes. Indeed, immature oocytes express low levels of RINGO protein as well as a 24 kDa truncated fragment, later identified as an N-terminal portion of RINGO. In support of previous findings, progesterone boosts RINGO accumulation. (Gutierrez et al., 2006).

c. RINGO induces GVBD without progesterone stimulation.

RINGO synthesis is required for progesterone stimulation of meiotic progression (Ferby et al., 1999; Kim and Richter, 2007). Ferby and colleagues initially focused their attention on ls26 and ls27 gene products (RINGO A and RINGO B) since oocyte microinjection of mRNAs transcribed from these ORFs induced GVBD without progesterone stimulation (Ferby et al., 1999). RINGO protein injection exerts the same effect. Interestingly, RINGO-induced GVBD is far more rapid than the maturation responses from progesterone or Mos synthesis. Analysis of biochemical markers of meiotic progression support this observation. Surprisingly, the immediate activation of MPF in response to RINGO overexpression bypasses CPEB downstream events, in which CDK1 dephosphorylation (a marker for MPF activation) precedes Mos sythesis and MAPK activation (Ferby et al., 1999; Lenormand et al., 1999). RINGO-induced GVBD also does not depend on Mos synthesis, as shown when Mos depletion in oocytes using Mos-specific AS oligonucleotides does not affect RINGO/CDK1 activity.

However, the rapid GVBD response is likely due to non-physiologically high levels of RINGO protein (and RINGO/CDK complex formation) due to overexpression and protein microinjection (Ferby et al., 1999). Regardless, many groups use this approach to look for changes in downstream markers, which provide clues for biologically significant RINGO-targeted factors that together, facilitate progesterone signaling.

RINGO/CDK1 activity triggers robust MPF activation. Recently, Ruiz and colleagues showed that RINGO/CDK1 catalysis of inhibitory phosphorylations on Myt1 may contribute to rapid MPF activation in oocytes (Ruiz et al., 2008). Myt1 phosphorylates CDK1 on Thr14 and Tyr15 to maintain inactive Pre-MPF complexes and prevent oocyte spontaneous GVBD prior to progesterone stimulation. Through this same mechanism, Myt1 overexpression also blocks progesterone signaling. Upon progesterone exposure, Myt1 undergoes inhibitory hyperphosphorylation, in which serine residues in the non-catalytic carboxy-terminus are phosphorylated (Liu e al., 1999; Wells et al., 1999). Since RINGO phosphorylates Myt1 *in vitro*, Ruiz and colleagues proposed that *in ovo*, RINGO/CDK1 phosphorylates Myt1 on Ser 410, 414 and 444, which are potential RINGO/CDK1 Ser/Thr consensus sites. Interestingly, the Myt1 mutant with Ala mutations Ser410, 414 and 444 (3AP) became immune to RINGO/CDK1 inactivation. Conversely, a version of Myt1 with aspartic acid mutations on these same Ser residues (3DP) became consititutively inactive. Immunoprecipitated Myt1 3AP and 3DP, overexpressed in reticulocyte lysates, were combined with Cyclin B/CDK1 complexes. To measure mutant Myt1 activity, the level of Cyclin B/CDK1 activity was assessed with Histone H1 phosphorylation. As expected, Myt1 WT and 3AP efficiently blocked CyclinB/CDK1 activity, reflected by low Histone H1 phosphorylation. However, the 3DP mutant was not able to inactivate CyclinB/CDK1, as evidenced by high H1 phosphorylation (Ruiz et al., 2008). These results suggest that RINGO/CDK1 facilitates MPF activation through direct catalysis of Myt1 inhibitory phosphorylations.

d. RINGO depletion blocks progesterone signaling

RINGO depletion experiments demonstrate that this atypical CDK activator plays an important role during maturation and support previous RINGO overexpression data (Ferby et al., 1999; Lenormand et al. 1999). Injection of RINGO antisense (AS) oligonucleotides effectively blocks RINGO synthesis and activity, confirmed by northern blotting to detect RINGO mRNA destruction and Histone H1 phosphorylation to gauge RINGO/CDK1 activity. As expected, RINGO depletion with the RINGO AS injection method blocks progesterone induced GVBD (Ferby et al., 1999). In order to place RINGO along the progesterone signaling pathway in *Xenopus* oocytes, various maturation biochemical markers were analyzed after RINGO depletion.

Padmanabhan and colleagues showed that RINGO function occurs early in meiosis, since depletion of endogenous RINGO protein blocks endogenous CPEB phosphorylation. [^{32}P] Orthophosphate labeled oocytes were injected with RINGO AS oligos and then stimulated with progesterone to resume meiosis. Extracts prepared from immature and progesterone treated oocytes were subject to endogenous CPEB immunoprecipitation. Stimulated RINGO AS injected oocytes contained low levels of endogenously phosphorylated CPEB, whereas immunoprecipitated CPEB from control injected oocytes was phosphorylated three hours after progesterone addition. RINGO may therefore mediate CPEB phosphorylation and possibly activation. To pursue this possibility, polyadenylation of a reporter transcript featuring a 3'UTR CPE is typically used to assess CPEB activation. Oocytes were injected with both RINGO AS oligos and a 3'UTR-CPE sequence and stimulated with progesterone to trigger polyadenylation. CPE containing 3'UTR mRNAss were polyadenylated in stimulated oocytes, however, RINGO depletion blocked transcript polyadenylation. These findings suggest that RINGO is intimately involved in CPEB activation and CPEB-mediated polyadenylation-induced translation. CPEB activation causes Embryonic Poly A Binding

Protein (EPAB) to dissociate and bind the poly-A tail, encouraging tail elongation and stability. However, when oocytes were injected with RINGO AS oligonucleotides, EPAB remained CPEB-bound after progesterone stimulation. These findings also support that RINGO function positively influences CPEB activation (Padmanabhan and Richter, 2005).

e. RINGO function as an atypical CDK activator

Upon progesterone stimulation, RINGO protein binds to and activates CDK1 to facilitate oocyte meiotic progression. The Nebreda and Donoghue labs were the first to demonstrate this interaction (Leonormand et al., 1999; Gutierrez et al., 2006). GST-RINGO, overexpressed in oocytes, co-immunoprecipitated endogenous CDK. Affinity purification of recombinant CDK with pSuc13 beads co-precipitated recombinant RINGO in oocytes as well. Interestingly, RINGO binds preferentially to monomeric CDK1, which comprises 90% of the total intracellular CDK1 in oocytes (the other 10% associates with Cyclin B as Pre-MPF complexes) (Leonormand et al., 1999). RINGO association with CDK1 is also Cyclin B independent, suggesting that this separate pool of monomeric CDK1 is available for RINGO-mediated activation. Several groups have shown that in addition to CDK1 association, RINGO is a potent activator of monomeric CDK1 (Ferby et al., 1999; Lenormand et al., 1999; Karaiskou et al., 2001; Dinarina et al., 2005). *Xenopus* oocyte high-speed extracts exhibit robust Histone H1 phosphorylation (an indicator of CDK1 activity) after RINGO addition (Ferby et al., 1999). RINGO also directly activates CDK1 *in vitro* (Karaiskou et al., 2001).

f. Non-conventional mechanisms regulate RINGO/CDK activity.

The mechanisms behind RINGO/CDK1 activation differ from canonical Cyclin/CDK complexes. Whereas CDK1 within the CyclinB/CDK1 dimer must undergo Cdc24C mediated removal of inhibitory phosphoates and CAK phosphorylation on Thr

160 for activation, CDK1 activity when associated with RINGO is independent of these regulatory mechanisms. For example, bacterially expressed GST-CDK1 T160A becomes activated upon RINGO association. Vanadate inhibition of Cdc25C phosphatase activity does not affect RINGO/CDK activation. Additionally, RINGO/CDK complexes are not subject to the same inhibitory mechanisms that regulate Cyclin/CDK activity. Canonical Cyclin/CDK inhibitors such as Wee1, Myt1 P21Cip and P27Kip are ineffective in blocking RINGO/CDK1 activity. Myt1 treatment of F60 *Xenopus* extracts, which contain monomeric CDK1, does not affect CDK1 activation upon RINGO addition. Injection of the CDK1 inhibitor, P21Cip, blocks progesterone-induced maturation, however, RINGO overexpression can still trigger GVBD in oocytes pre-injected with P21Cip. P21Cip recognizes and binds to an MRAIL motif in Cyclins, yet, RINGO lacks this motif and evades P21Cip inhibition (Karaiskou et al., 2001).

4. CPEB

CPEB was discovered in *Xenopus laevis* oocytes as an RNA specificity factor that is required for polyadenylation-induced translation (Hake and Richter, 1994). Immunodepletion of CPEB in egg extracts, containing abundant polyadenylation activity, disrupted poly(A) tail elongation of B4 mRNA, among others (Hake and Richter, 1994 and Stebbins-Boaz et al., 1996). Further, CPEB antibody injection blocks polyadenylation in oocytes in vivo as well as progesterone-induced maturation (Stebbins-Boaz et al., 1996). The 62kDa protein contains two tandem RNA Recognition Motifs (RRM) and a Zinc Finger, consisting of well conserved histidine and cysteine residues, which are both required for CPE binding. The Zinc Finger residues participate in metal ion coordination and are required for RNA binding (Hake et al., 1998). CPEB also contains a PEST domain for proteasome-mediated degradation of CPEB. Approximately 90% of total CPEB is degraded upon GVBD and partial degradation is

required for the polyadenylation-induced translation of cyclin B1 mRNA (Hake and Richter, 1994 and Reverte et al., 2001).

a. CPE/CPEB-dependent cytoplasmic polyadenylation

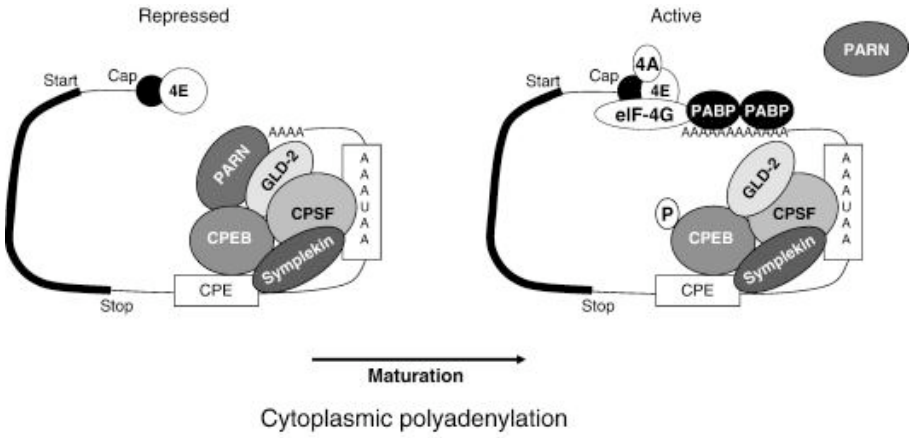
Many mRNAss subject to polyadenylation-induced translation contain a Cytoplasmic Polyadenylation Element (CPE), which are bound by an RNA specificity factor, Cytoplasmic Polyadenylation Element Binding Protein (CPEB). mRNAs such as, c-mos, cyclinsA1, B1 and B2 and cdk2, contain at least one $U_{4-5}AU$ CPE sequence and a AAUAAA polyadenylation signal in the 3'UTR (Sheets et al., 1994; Stebbins-Boaz et al., 1996). The coordination these cis-sequences and trans-acting factors enforce temporal translational regulation of many developmentally important mRNAss.

b. CPEB-mediated polyadenylation requires Poly(A) Ribonuclease (PARN) dissociation.

CPEB acts as both a translational repressor and activator, maintaining translational silencing of several maternally deposited mRNAs in the immature oocyte while playing a key role in activating translation of these same mRNAss at appropriate times during maturation. In the resting oocyte, the CPE of a silenced mRNA is bound by CPEB. CPEB interacts directly with Cleavage and Polyadenylation Specificity Factor (CPSF), which binds to the polyadenylation hexanucleotide (Bilger et al., 1994; Dickson et al., 1999). The poly(A) polymerase, *Xenopus* Germline development 2 (XGld-2), is also present in the mRNP (Barnard et al., 2004). As a constitutively active polymerase, XGld-2 function is overwhelmed by PARN ribonuclease, another component of the factors that mediate mRNA silencing (Kim and Richter, 2006). The scaffold, Symplekin, serves as a molecular platform on which CPEB, CPSF, XGld-2 and PARN are organized (Barnard et al., 2004).

In response to progesterone, CPEB is activated by Ser174 phosphorylation, which enhances its interaction with CPSF and increases CPSF RNA-binding stability (Mendez et al., 2000b). PARN deadenylase dissociates from the mRNP allowing unchecked XGld-2 polymerase activity to stimulate poly(A) tail elongation (Kim and Richter, 2006). The elongating poly(A) tail is bound by ePABP. The mRNA 5' cap is bound by eIF4E with eIF4G in close proximity and as the poly(A) tail lengthens, bound ePABP interacts with eIF4G, facilitating eIF4E/eIF4G association and recruitment of EIF4F and 40S ribosomal subunit for translational activation.

Figure 2. PARN dissociation model



(adapted from Radford et al., 2008)

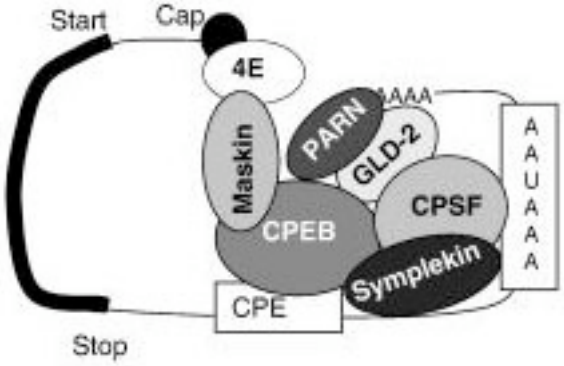
c. CPEB-mediated polyadenylation: Maskin repression model

Although a short poly(A) tail facilitates translational repression, some deadenylated mRNAs are still translated during oogenesis. Since low-level expression of certain factors is sufficient for triggering maturation, complete repression of maternally deposited mRNAss is crucial towards the prevention of spontaneous GVBD.
Maskin was discovered in a search for additional factors that could enforce mRNA silencing (Stebbins-Boaz et al., 1999). This 150 kDa protein is a homologue of

transforming acidic coiled-coil domain protein 3 (TACC3) (Still et al., 1999; Stebbins-Boaz, 1999). Maskin function as a regulator of mRNA translation was unexpected. Compared to the TACC proteins, RNA binding activity is exclusive to Maskin, suggesting that the translational regulation function is not well conserved among these proteins (Stebbins-Boaz et al., 1999). Maskin binds directly to both CPEB and eIF4E. The Maskin/ CPEB interaction is stable through out maturation, however, the Maskin/ eIF4E association is abrogated in maturing oocytes. The model for Maskin mediated translational repression proposes that Maskin is bound to both CPEB and eIF4E in repressed CPE-containing mRNAss. Both Maskin and eIF4G interact with eIF4E on the same site, thus competing for cap protein binding. However, as long as Maskin is associated with eIF4E, eIF4G cannot bind eIF4E and recruit translation initiation factors (Stebbins-Boaz et al., 1999; Cao and Richter, 2002).

Progesterone stimulation of meiosis causes Maskin to dissociate from eIF4E, facilitating translation initiation. The events that trigger Maskin dissociation from eIF4E are not well defined. Upon progesterone stimulation and CPEB activation, the poly(A) tail lengthens and is bound by ePABP, which binds eIF4G. Elongation of the poly(A) tail, ePABP binding of the poly(A) tail and ePABP/ eIF4G association, together, displace Maskin from eIF4E. Maskin is also phosphorylated by Cdk1 on Thr58, Ser152, Ser311, Ser343, Ser453 and Ser638 after progesterone stimulation (Barnard et al., 2005). These phosphorylations have been shown to weaken Maskin affinity for EIF4E, such that EIF4G can out-compete Maskin for EIF4E association and recruit translation initiation factors. Maskin function as a mediator of translational regulation during oogenesis and early oocyte maturation is debatable since the RNA binding factor is not synthesized until late oogenesis and has been shown to regulate the translation of cyclin B1 mRNA during late meiosis as well as mitosis (Cao and Richter, 2002; Groisman et al., 2000; O'Brien et al., 2005).

Figure 3. Maskin repression model



(adapted from Radford et al., 2008)

d. A CPEB code

The relative positioning of the CPE and polyadenylation hexanucleotide (Hex) has also been shown to influence mRNA repression and the timing of translational activation. Piqué and colleagues analyzed the 3'UTR cis-elements of Cyclin B1-5 mRNAs to establish what they call a combinatorial code since each mRNA exhibited not only differences in their number of CPEs and their positioning relative to the polyadenylation hexanucleotide, but also their timing of translation. To test their code theory, 3'UTR variants were made and changes in repression and temporal control over translational activation were compared to their WT counterparts. The transcript was tethered to a firefly luciferase reporter to monitor expression/repression mediated by the downstream cis-elements (Pique et al., 2008; reviewed in Richter, 2008).

First, the number of CPEs affects the degree of repression in prophase I oocytes. Immature oocytes were injected with the 3'UTRs of Cyclin B4 or B5, which contain 3 CPEs each. Both WT B4 and B5 3'UTRs enforced strong repression in immature oocytes with virtually no luciferase expression. However, translational repression

became increasingly weak with each deleted CPE. Second, the degree of translational repression is influenced by the distance between the CPEs, when multiple CPEs are present. There are 62 nucleotides between the two CPEs in the Cyclin B2 3'UTR, which exhibits weak repression in immature oocytes. A decrease in the space between both cis-sequences down to a 22 nucleotide distance, strengthened repression (less luciferase expression). These results suggest that CPE to CPE distance influences transcript silencing. Third, translational activation in response to progesterone is also influenced by CPE and Hex distance. Cyclin B5 is very efficiently translated in response to progesterone and its CPE and Hex are 36 nucleotides apart. However, an increase in distance between the two cis-elements, as seen with Cyclin B2 (48 nucleotides) and Cyclin B1 (121 nucleotides) variant mRNAs, disrupted translation (Pique et al., 2008).

e. Regulatory phosphorylation

As the most proximal upstream signaling event of c-Mos mRNA translation, CPEB Ser174 activating phosphorylation around three hours after meiosis resumption has garnered a great deal of attention. Consequently, kinase activity towards CPEB shortly after progesterone stimulation (within the first hour) has been ignored until recently. His-CPEB is phosphorylated by extracts prepared from oocytes that have been exposed to progesterone for 30 minutes to an hour (Hake lab, unpublished data). Further, these phosphorylation events, in addition to the 3 hour activating phosphorylation, are sensitive to MAPK inhibition. Our lab, among others, have also detected a low level of MAPK activity (Mos independent) that arises just after progesterone stimulation (Fisher et al., 1999, Keady et al., 2007). Additionally, His-CPEB is phosphorylated *in vitro* by MAPK on Thr22, Thr164, Ser184 and Ser248. These results suggest that CPEB activation may require priming phosphorylations catalyzed by MAPK or an alternative Ser/Thr kinase.

CPEB phosphorylation was initially detected as a hyperphosphorylation event in GVBD oocytes (Paris et al., 1991; Hake and Richter, 1994). However, with proposed function as a mediator of c-Mos translation, Mendez and colleagues pursued the possibility that CPEB may be phosphorylated when this early phase mRNA is translated. To detect early CPEB phosphorylation, extracts were prepared from oocytes that were exposed to progesterone for two hours and metabolically labeled with ^{32}P. Endogenous CPEB immunoprecipitation from these oocytes was phosphor-labeled, suggesting that CPEB was phosphorylated during this early time in meiosis. To identify phosphorylated residues, recombinant His-CPEB was incubated in progesterone stimulated oocyte extracts and then subject to tryptic digest and 2D TLC to resolve phosphopeptides. Extracted phosphopeptide analysis by HPLC and peptide sequencing revealed Ser174 phosphorylation (Mendez et al., 2000a).

To test the biological significance of CPEB Ser174 phosphorylation, Ser174 and 180 were alanine mutated to create the CPEB AA mutant. S180 was mutated alongside Ser174 to prevent any additional LDSR phosphorylation that could interfere with Ser174-P analysis. Overexpression of CPEB AA depressed c-Mos 3'UTR-CPE reporter transcript polyadenylation after progesterone stimulation, suggesting that Ser174 phosphorylation was required for c-Mos polyadenylation. In support of these findings, a CPEB DD mutant, which mimicked Ser174 and 180 phosphorylation, triggered polyadenylation without progesterone. Further, Mos inhibition with AS oligonucleotide injection did not affect Ser174 phoshorylation in progesterone stimulated oocytes. Combined, these results suggest that CPEB Ser174 phosphorylation is required for CPE/CPEB mediated polyadenylation of c-Mos mRNA during early maturation (Mendez et al., 2000a).

Since the S/T kinase, Aurora A, has been shown to influence progesterone signaling and accelerate c-Mos translation upon overexpression of the kinase, Mendez

and colleagues pursued the possibility that Aurora A kinase phosphorylated CPEB on Ser174 for activation. Baculovirus expressed Aurora A kinase phosphorylated CPEB WT but not CPEB AA, *in vitro*, suggesting that Ser174 was indeed an Aurora A site. Further, His-CPEB and Aurora A interact in oocyte extracts (Mendez et al., 2000a). For years Aurora kinase was considered the CPEB Ser174 phosphorylating kinase. However, several lines of new evidence suggest otherwise. Recombinant His-CPEB is phosphorylated by extracts prepared from progesterone-stimulated oocytes. Interestingly, extracts from oocytes depleted of Aurora A kinase, through either antisense oligonucleotide injection or immunodepletion approaches, phosphorylate CPEB *in vitro*. CPEB phosphorylation is also unaffected by Aurora A kinase inhibitors. Combined, these findings suggest that an alternative S/TP kinase must be responsible for Ser174 phosphorylation (Frank-Vaillant et al., 2000; Keady et al., 2007).

When the oocyte reaches MI (GVBD), 70-90% of CPEB is degraded (Hake et al., 1994, Reverte et al., 2001). Mendez and colleagues argued that Ser210 phosphorylation by CDK1 is sufficient for this degradation event, although other residues are phosphorylated as well. His-CPEB phosphorylation sites incurred after incubation with GVBD extracts were identified by trypsin digest and phosphopeptide sequencing of individual radioactive “hot” spots after 2D TLC. Serines 138 and 248 were identified through this approach and led to the scrutiny of additional S/TP sites (Ser144, 210, 248 and 423) with regards to their influence on CPEB destruction. A CPEB mutant with alanine mutations on all six of these serine residues (CPEB 6A) was overexpressed in oocytes. This particular CPEB mutant is protected from Ubiquitin Proteasome System (UPS) degradation since CPEB 6A remained stable even at GVBD. However, an A210S substitution in the 6A mutant (5A) caused mutant hyperphosphorylation and degradation at MI, suggesting that Ser210 alone was sufficient for the degradation process. Further, Mendez and colleagues argued that phosphorylation of this S/TP consensus site is likely

catalyzed by either MAPK or Cdc2. Results from *in vitro* phosphorylation of CPEB by recombinant MAPK and Cdc2 suggest that Cdc2 is the candidate kinase responsible for the degradation promoting phosphorylations (*in vitro* phosphorylation with MAPK yielded a single phosphorylation site of unidentifiable origin) (Mendez et al., 2001).

Setoyama and colleagues showed that CPEB must undergo a series of phosphorylations for ubiquitination and degradation by the proteasome. First, CPEB is phosphorylated on Thr125 by Cdc2, which is a pre-requisite to Plx-1 binding and phosphorylation on Ser191. Once phosphorylated on both sites, βTRCP, the F-box protein of the Skp/Cul1/Fbox(SCF)$^{\beta TRCP}$ ubiquitin complex, binds to a $_{190}$TSGFSS$_{195}$ or TSG motif and catalyzes ubiquitin addition to target CPEB for degradation. βTRCP binds specifically to the TSG motif of CPEB, as evidenced by a loss of βTRCP/ TSG binding when the docking motif was mutated with alanine substitutions. Mutants exhibiting disrupted βTRCP binding are also stable at GVBD. A Plx-1 consensus (S-pS/pT-P/X) is found within the TSG and Plx-1 was later confirmed as the kinase responsible for Ser191 phosphorylation-mediated βTRCP/TSG binding. Typically, the Plx-1 docking site must be phosphorylated before binding can occur. CDK1 must phosphorylate Thr125, a S/TP consensus site, within the Plx-1 docking site to facilitate Plx-1 binding. Mutagenesis of Cdc2 and Plx-1 phosphorylation sites also blocks CPEB degradation. Together, with the evidence that CPEB remains stable in MI oocytes when βTRCP binding is abrogated, Thr125 and Ser191 phosphorylation by Cdc2 and Plx-1, respectively, is required for βTRCP ubiquitination of CPEB for degradation at GVBD (Setoyama et al., 2007).

Setoyama and colleagues created a DDS/6A mutant to address the function of the six serine sites of CPEB that Mendez and colleagues used in their degradation studies. The DDS mutation of the TSG mimics phosphorylations that caused constitutive

βTRCP binding in maturing oocytes. Combined with the 6A mutations, CPEB interaction with βTRCP was unaffected and CPEB was still degraded at MI. These findings suggest that the six CPEB phosphorylation sites act independently of those published by Setoyama and colleagues relative to βTRCP-mediated CPEB destruction (Setoyama et al., 2007). Additionally, when Mendez and colleagues deleted the PEST domain, which removes the TSG motif, this mutated version of CPEB is still hyperphosphorylated, but not degraded. Together these findings suggest that at GVBD, CPEB hyperphosphorylation (presumably by CDK1) occurs independently of PEST and TSG motif-mediated βTRCP recruitment and ubiquitination/destruction (Mendez et al., 2002; Setoyama et al., 2007).

f. CPEB-independent polyadenylation-induced translation

Additional cis- and trans-acting elements mediate cytoplasmic polyadenylation besides the CPE and CPEB. Since oocyte maturation and development revolve around mRNA translation timing, mRNAs are categorized (in an oversimplified manner) as early-phase and late-phase mRNAss. Early-phase mRNAs are translated shortly after progesterone stimulation and include RINGO and c-Mos mRNAs, as opposed to late-phase mRNAs, which are translated after Mos synthesis (Ballantyne et al., 1997). Whereas late-phase mRNAss are exclusively polyadenylated in a CPE-dependent manner, some early phase mRNAs rely on CPE-independent polyadenylation mechanisms.

Additional 3'UTR regulatory sequences have been identified in c-mos mRNA that also contribute to Mos synthesis. A Polyadenylation Response Element (PRE) was identified in the c-mos 3'UTR and is bound by Musashi, an RNA binding protein (Charlesworth et al., 2006; Prasad et al., 2008). The PRE and Musashi facilitate CPE

and CPEB-mediated polyadenylation of c-mos mRNA. The RINGO mRNA 3'UTR contains Pumilio Binding Elements (PBEs), which are recognized by Pumilio 2 (PUM2) and together the cis-element and transacting factor regulate RINGO synthesis in a polyadenylation-dependent manner as well (Padmanabhan and Richter, 2006). RINGO and c-mos translational regulation will be discussed in further detail in their respective sections.

g. CPEB homologs

CPEB homologues exist in humans (hCPEB), clams (p82), flies (Orb), zebra fish (Zorba), mice (mCPEB1-4) and worms (CPB1-3 and FOG-1) (Walker et al., 1999; Lantz et al., 1994; Baily-Cuif et al., 1998; Luitjens et al., 2000). Invertebrates possess two CPEB encoding genes, whereas vertebrates possess four different CPEB genes. *Xenopus* CPEB and homologs discussed in this section are considered CPEB1. CPEB 2-4 recognize different RNA binding sites and are involved in diverse biological processes (reviewed in Richter, 2007). High functional conservation exists among CPEB1 proteins, exhibiting dual function as translational repressors and activators. CPEB1 KO mice are viable and develop normally, however, these animals fail to produce fertilizable eggs and are therefore sterile (Tay et al., 2000). Similarly, in worms, CPB-1 represses fem-3 expression to regulate a switch between spermatogenesis to oogenesis (Luitjens et al., 2000). In flies, Orb function is crucial for anterior/posterior and dorsal/ventral axis determination during early development. Anteriorly and posteriorly translated bicoid and oskar mRNAs, respectively, are regulated by Orb mediated repression and polyadenylation-induced translation (Castagnetti and Ephrussi, 2003). CPEB-mediated translational regulation also influences processes beyond oocyte maturation, spermatogenesis and early development. Neuronal CPEB has been shown to influence translation at the synapse in an activity-dependent manner. Controlled

expression of CPE-containing mRNAss influences synaptic plasticity (Wu et al., 1998; reviewed in Klann et al., 2004).

5. XGef

a. Discovery and significance

XGef was identified in a yeast two-hybrid screen for CPEB interactors, in which the amino-terminal half of CPEB, containing a PEST domain and several regulatory phosphorylation sites, was used as bait (Reverte et al., 2003). XGef is a putative Rho family guanine nucleotide exchange factor (GEF) and is required for CPEB activation and timely progression of meiosis. As a putative Rho GEF, we initially proposed that XGef functioned as an exchange factor to activate a specific Rho GTPase during maturation (Reverte et al., 2003; Martinez et al., 2005).

b. Rho GEFs: discovery, protein domains and function

GEFs were first identified in mammals as products of oncogenes that, when expressed in NIH 3T3 mouse fibroblasts, induced transformed foci (Eva and Aaronson, 1985). Virtually all Rho GEFs possess a Dbl Homology (DH) domain, named after the prototype GEF, Dbl. GEF catalytic function resides in the Dbl Homology (DH) domain whereas the C-terminal adjacent Pleckstrin Homology (PH) domain has been shown to facilitate membrane recruitment through phospho-inositide binding. In some cases the PH domain can also facilitate DH-mediated exchange (reviewed in Whitehead, et al., 1997; Lemmon and Ferguson, 2000). Beyond the DH and PH domains, Rho GEFs do not share much sequence similarity. GEFs that share the same binding partners may only be 20% identical in their sequences. DH domain structures from one GEF to the other, however, are very similar in 3D structure, forming a flattened and elongated bundle of 11 alpha helices. The DH domain also contains three conserved regions,

designated CR1, CR2 and CR3, each spanning approximately 10 to 30 amino acids. CR1 and CR3 form the exposed surface of the DH domain and participate in GTPase interaction by forming a binding pocket (reviewed in Schmidt and Hall, 2002; Whitehead et al., 1997, Rossman et al., 2005).

Rho family guanine nucleotide exchange factors (GEFs) activate Rho GTP-binding proteins (Rho GTPases) by catalyzing GDP to GTP exchange. The GEF/GTPase interaction destabilizes the GTPase GDP association, causing GDP release. Intracellular levels of GTP exceed GDP by a ratio of 10 to 1. Due to the surplus of GTP, the GEF-bound GTPase binds GTP and becomes activated (reviewed in Whitehead et al., 1997).

c. Regulation of GEF activity

GEF activity is regulated by a variety of mechanisms. For the prototype GEF, Dbl, the N terminus serves as a regulatory domain and exhibits autoinhibition. An N-terminal alpha helix can bend and block GTPase interaction with the GTP-binding pocket located within the DH domain. However, upon N terminus phosphorylation, the region becomes disordered, allowing GTPase binding and activation (Bi et al., 2001). We have not been able to detect any evidence of XGef phosphorylation or inhibition in this manner in *Xenopus* oocytes (Hake lab, unpublished). GEF activity has also been shown to respond to heterotrimeric G-protein signaling. The G-alpha subunit of heterotrimeric G-Proteins have been shown to bind to the N-terminal region of GEFs, proto-Dbls, beta-1-Pix, and Lbc Rho GEF, to enhance GEF activity. How this stimulatory effect occurs is not well understood (Vanni et al., 2007; Chahdi and Sorokin, 2006; Dutt et al., 2004). GEF function is also mediated by oligomerization through the DH domain. Compromised oligomer formation has been shown to weaken GEF exchange activity of p115 RhoGEF, PDZ-GEF and LARG (Chikumi et al., 2004). XGef has been shown to interact with itself

through the DH domain, however the biological significance of this dimerization or oligomerization is unknown (Martinez et al., 2005).

d. XGef protein domains

Seen in virtually all Rho GEFs, XGef contains a Dbl homology (DH) domain and c-terminal adjacent Pleckstrin homology (PH) domain. A coiled-coil motif is located n-terminal to and partially overlapping with the DH domain (Reverte et al., 2003). Although a function has not been assigned to this particular region of XGef, a coiled-coil in GEF-H1 has been shown to mediate microtubule binding (Krendel et al., 2002). Putative MAPK docking sites have also been identified in XGef, the D-domain and FXF motif. The function of these docking sites as facilitators of MAPK binding has not been confirmed. Finally, the four c-terminal residues conform to the CAAL consensus sequence (CSSL), which is a putative geranyl geranyl isoprenylation signal. Protein geranyl-geranylation has been shown to also facilitate membrane association (Schafer and Rine, 1992).

Figure 4. XGef domains, motifs and homolog alignment

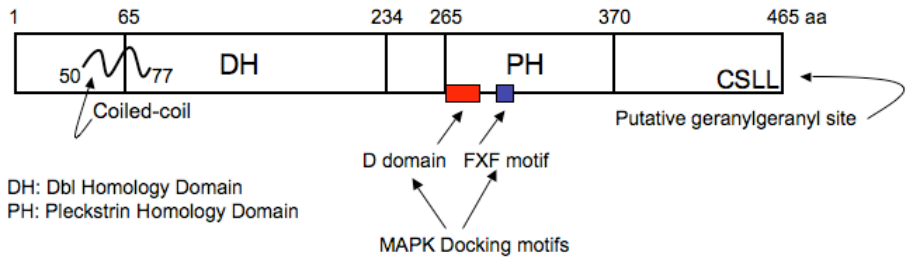


Figure 4. (Continued) Alignment of XGef homologs

e. XGef transcript and protein abundance in oocytes

XGef transcripts accumulate throughout oogenesis, reaching a peak level between stages IV and VI. Transcript levels then remain constant during maturation and early embryo development. XGef protein is expressed during all stages of oogenesis, with the most abundant level of XGef protein detected in stage III oocytes. Protein levels decrease slightly during stages IV through VI and are present at a constant level from maturation to the embryo tailbud stage. XGef expression decreases significantly at the tadpole stage (Reverte et al., 2003).

f. XGef function is required during oocyte maturation

XGef function is required for progesterone-induced oocyte maturation. CPEB typically undergoes activating phosphorylation two to three hours after progesterone stimulation and triggers c-mos polyadenylation and Mos accumulation (Mendez et al., 2000a; Reverte et al., 2003). Interestingly, XGef inhibition disrupts CPEB activation. Experiments utilizing radiolabeled c-mos 3'UTR, a substrate for cytoplasmic polyadenylation, show that injection of XGef neutralizing antibodies perturbs polyadenylation of the labeled mos mRNA, indicative of compromised CPEB activation. Analysis of downstream biochemical markers including Mos synthesis and MAPK activation confirm that meiotic progression is severely depressed in XGef-inhibited oocytes (Reverte et al., 2003). Conversely, XGef overexpression increases the rate of maturation and when the same biochemical markers were analyzed, CPEB phosphorylation was enhanced and an increased amount of Mos was synthesized (Reverte et al., 2003; Martinez et al., 2005). Combined, these results suggest that XGef influences CPEB activation in response to progesterone stimulation.

Data from protein interaction and c-mos mRNP experiments suggest that XGef directly influences CPEB activation. XGef and CPEB interact *in ovo* and during

maturation in an RNA-independent manner (Reverte et al., 2003; Martinez et al., 2005). XGef and CPEB also interact directly *in vitro*. Interestingly, XGef and CPEB are part of a c-mos mRNP. XGef and CPEB immunoprecipitates contain c-mos mRNA, confirmed by RT-PCR analysis of RNA isolated from XGef and CPEB immune complexes. Further, a mutant of XGef (65-360) that cannot bind CPEB, does not exert the same positive influence on maturation compared to wildtype. Oocytes overexpressing XGef 65-360 exhibit depressed maturation kinetics in extracts prepared from the same oocytes contain decreased kinase activity towards CPEB (Martinez et al., 2005). Combined, these results demonstrate that XGef and CPEB are both present in a c-mos mRNP and support the hypothesis that XGef directs CPEB-mediated c-mos polyadenylation.

As a putative Rho GEF, the importance of XGef exchange activity during maturation was tested. A version of XGef with a DH domain deletion was overexpressed in oocytes and the rate of maturation in response to progesterone was analyzed. Oocytes overexpressing this exchange deficient mutant no longer exhibited an accelerated rate of GVBD (Martinez et al., 2005, unpublished). These findings suggest that the accelerated meiotic progression in oocytes overexpressing XGef depends on exchange activity and implicates an XGef specific Rho GTPase.

6. Rho GTPases

GTPases are low molecular weight (~21 kDa) guanine nucleotide binding proteins that serve as molecular switches, cycling between GDP-bound (inactive) and GTP-bound (active) states as well as a transient nucleotide depleted condition (Boguski and McCormick, 1993). As a part of the Ras superfamily, Rho Family GTPases are highly conserved in eukaryotes and participate in diverse signaling pathways to influence many biological processes, ranging from cell proliferation to actin cytoskeleton dynamics (reviewed in Van Aelst and D'Souza-Schorey, 1997). GTPases within a particular family

are structurally very similar and possess a highly conserved G domain fold, which consists of a six stranded β-sheet connected by hydrophilic loops and α-helicies. The most extensively studied Rho Family GTPases include Rho A-C, Rac1 and Cdc42, which participate in an array of signaling pathways that mediate cytoskeletal organization. Cdc42 activity induces actin polymerization and guides filipodia formation and polarized cell growth. Rac1 signaling influences the formation of lamellipodia, which are pleated protrusions involved in cell migration. Rho facilitates stress fiber and tight junction formation, as well as perijunctional actin organization (reviewed in Tapon and Hall, 1997; Hall, 2005). Although known primarily as regulators of actin cytoskeleton dynamics, Rho GTPases have also been shown to participate in signaling processes involving MAPKs (JNK and P38 MAPK), transcription factors (NF-kB) and growth factor stimulation (Serum-response factor) (Hill et al., 1995; Minden et al., 1995; Coso et al., 1995; reviewed in Hall, 2005). Overexpression of Rho GTPases, such as RhoA and Rac1, also induces malignant transformation (Sahai et al., 2001). Rho GTPase signaling may in fact intergrate actin cytoskeletal organization and cell proliferation in response to external stimuli.

Mentioned previously, Rho GTPases are activated by guanine nucleotide exchange factors (GEFs). GTP binding induces conformational changes, allowing the activated GTPase to interact with binding partners. The switch I and switch II regions of Rho GTPases are particularly important not only for the activation process but also Rho GTPase downstream effector binding (reviewed in Hakoshima et al., 2003). First, the switch regions mediate Mg^{2+} ion coordination, mandatory for GTP binding and activation. Second, direct contacts are made between the switch regions and protein interactors. For example, Cdc42 switch I (24-40) and switch II (59-70aa) make direct contacts with the downstream effector, P21 Activated Protein Kinase (PAK). Mutations made within switch regions disrupt this interaction. Similarly, direct contacts are also made between

switch regions and GTPase Dissociation Inhibitors (GDIs), which facilitate GTPase inactivation by binding to GD_bound GTPases to prevent GDP/GTP exchange (Hoffman et al., 2000). Mutation of Cdc42 arginines 66 and 68 block GDI-mediated inhibition (Lin et al., 2003)

Additional mechanisms exist to regulate GTPase function aside from GEF-mediated activation. GTPases can become inactivated through their intrinsic activity of GTP hydrolysis. The rate of inactivation is enhanced by GTPase Activating Proteins (GAPs), which bind to target GTPases to stimulate GTP hydrolysis (Rittinger et al., 1997). Upon GTP hydrolysis, the GTPase becomes GDP bound and inactive.

7. Mos

The Mos proto-oncogene plays an essential role during vertebrate oocyte maturation and functions as a germ-cell specific MAPKKK. Mos is a potent activator of MAPK through a Mos/MEK/MAPK cascade and induces oocyte maturation without progesterone stimulation. Together, these results suggest that Mos acts as an important mediator of MI entry in *Xenopus* oocytes. (Sagata et al., 1989; Sheets et al., 1995; Dupre et al., 2002; Nebreda et al., 1993; Posada et al., 1993; Shibuya and Ruderman, 1993).

Mos is barely detectable in prophase I oocytes and progesterone stimulation induces c-mos mRNA polyadenylation-induced translation and Mos accumulation. CPE and CPEB have been shown to mediate c-mos translation (Mendez et al., 2000a). However, evidence exists that an additional 3'UTR cis-element, the polyadenylation response element (PRE) also controls translation initation of c-mos mRNA (Charlesworth et al., 2002). The c-mos PRE is bound by an RNA binding protein, Musashi, which was originally identified as a translation regulator *Drosophila* (Nakamura et al., 1994; Charlesworth et al., 2006). Mutagenesis of the Musashi AUAGU consensus

and overexpression of dominant negative Musashi depressed, but did not block, c-mos polyadenylation-induced translation. It is important to note that the PRE overlaps the first three nucleotides of the CPE. PRE mutagenesis would therefore potentially perturb CPE function. Charlesworth and colleagues also used a constitutively inactive CPEB mutant (S174,180A) to demonstrate that CPEB activation is dispensable to Mos synthesis, since oocytes overexpressing this mutant of CPEB exhibited normal c-mos polyadenylation. However, their approach did not address the effects of endogenous CPEB activity during this process. Ultimately, both CPE/CPEB and PRE/Musashi are needed for efficient c-mos translation. Disruption of either mechanism diminishes c-mos polyadenylation in response to progesterone, suggesting that both modes of translational regulation are necessary for full, timely mos mRNA translation.

Sustained and robust Mos synthesis relies on positive feedback within the Mos/MEK/MAPK cascade. Howard and colleagues showed that MAPK inhibition, with MAPK-specific phosphatases and chemical inhibitors blocked progesterone-induced Mos accumulation. The methods used to block MAPK activity prevented polyadenylation of an exogenous c-mos 3'UTR transcript, suggesting that MAPK influenced c-mos translation. Additionally, oocytes injected with consitituvely active MEK exhibited robust mos polyadenylation and protein accumulation (Howard et al., 1999). Combined, these results suggest that maintenance of Mos protein relies on the Mos/MEK/MAPK positive feedback mechanism.

Interestingly, Mos may function independently of MAPK activation to mediate MPF activation. Evidence of MAPK-independent Mos activity was first demonstrated in progesterone stimulated, Mos antisense injected oocytes. Mos abalation blocked CDK1 activation in response to progesterone, however the same MPF inhibition did not occur in oocytes pretreated with the MEK/MAPK inhibitor, UO126. These oocytes eventually achieved GVBD without detectable MAPK activation. Unexpectedly, a low level of Mos

protein was detected despite antisense oligo injection. Peter and colleagues argued that the low level of Mos activity was not enough to activate the Mos/MEK/MAPK cascade, but was able to facilitate MPF activation, (through Myt1 phosphorylation) albeit delayed (Peter et al., 2002). Other groups have argued that Mos activation of MPF was MAPK dependent since a complete block of GVBD was reported with the use of MAPK inhibitors, as opposed to a delay in maturation (Fisher et al., 1999; Gross et al., 2000). Further research is required to resolve these discrepancies.

8. *Xenopus* P42 MAPK

Screens for serine/threonine protein kinases activated by growth factors in vertebrates led to the discovery of Mitogen Activated Protein Kinases (MAPKs) (reviewed in Sturgill and Wu, 1991). MAPKs were also identified through screens for mutations that affected intercellular signaling in *S. cerevisiae, C. elegans,* and *D. melanogaster* (reviewed in Ferrell, 1996). Since their discovery, numerous MAPKs and their associated biological processes have been well documented. *Xenopus laevis* oocyte P42 (ERK2) is a member of the ERK subgroup of MAPKs. Mos (MAPKKK) activates MEK1 (MAPKK), which induces P42 (MAPK) activation by catalyzing dual phosphorylations on Thr183 and Tyr185 within a well conserved TXY motif of the kinase domain activation loop (Posada et al., 1993; Nebreda and Hunt, 1993; Shibuya and Ruderman, 1993; Ferrell, 1996). The MAPK activating phosphorylations occur in a two-collision fashion, in which Tyr phosphorylation precedes Thr phosphorylation. The two-step phosphorylation mechanism creates an ultra-sensitive, all or nothing switch-like behavior behind P42 signaling upon progesterone stimulation of *Xenopus* oocyte meiotic progression (Ferrell and Machleder, 1998; Ferrell and Bhatt, 1997).

Contrary to the proposed all or nothing mechanism of MAPK activation, several groups have reported biphasic MAPK activation (Fisher et al., 1999; Fisher et al., 2000;

Keady et al., 2007). Early-Phase MAPK activity was discovered in oocytes that were injected with Mos AS to block Mos synthesis. Interestingly, upon progesterone stimulation, these oocytes contained a low level of mono-phosphorylated MAPK (Y190-P) (Peter et al., 2002). This low level of phosphorylated MAPK can be detected as early as 15 minutes after progesterone stimulation and does not require Mos (Fisher et al., 1999; Peter et al., 2002). The functional significance of this early-phase MAPK-P is not well understood. Interestingly, CPEB activation requires MAPK activity since progesterone stimulation does not trigger CPEB activation in U0126 treated oocytes. MAPK can also phosphorylate CPEB in vitro (Keady et al., 2007). The early-phase MAPK-P may facilitate CPEB activation, however, further experimentation is required to test this hypothesis.

Late-phase MAPK activity is triggered by Mos synthesis and the Mos/MEK/MAPK cascade. References to MAPK activity in the literature typically address Mos and Mos/MEK/MAPK cascade-mediated, late-phase, robust activation. The preferential attention towards Mos-dependent MAPK-P is likely due to the relative ease in detecting abundant phospho-active MAPK at this step of meiotic progression. The robust nature of late-phase MAPK activity occurs through a positive feedback mechanism, in which MAPK activated by the Mos/MEK/MAPK cascade stimulates additional c-mos polyadenylation and Mos synthesis (Matten et al., 1996; Roy et al., 1996; Gotoh et al., 1995). The Mos/MAPK signal is therefore rapidly increased through auto-amplification. A substrate of late-phase active MAPK is P90RSK, 90 kDa ribosomal S6 kinase, which in turn catalyzes Myt1 inhibitory phosphorylations (among other kinases). Through P90RSK, late-phase MAPK signaling contributes to MPF activation (Palmer et al., 1998).

9. Maturation Promoting Factor (MPF)

a. Pre-MPF

A preformed stock of inactive Cdc2/Cyclin B heterodimers (Pre-MPF) is stored in resting oocytes. Only 10% of intracellular Cdc2 is coupled to Cyclin B, whereas the majority of Cdc2 is monomeric (Kobayashi et al., 1991). In the immature oocyte, Pre-MPF cdc2 catalytic activity is inhibited through Myt1 catalyzed phosphorylations on Thr14 and Tyr15 (Mueller et al., 1995). The Cdc25C phosphatase that mediates Thr14 and Tyr15 dephosphorylation, however, remains inactive through PKA-directed Ser287 phosphorylation (Dunphy and Kumagai, 1991; Gautier et al., 1991; Jessus and Ozon, 1993; Duckworth et al., 2002). As long as MPF activity is inhibited, the germinal vesicle remains intact and meiosis resumption is prevented.

b. MPF activation

Robust MPF activity is required for GVBD and completion of the first meiotic division as well Meiosis II progression (Masui and Market, 1971; Iwabushi et al., 2000). MPF activation occurs in two steps. First, a starting, low level, of MPF activity is achieved after progesterone stimulation. Myt1 inactivation by a variety of candidate kinases including, Mos, P90RSK and RINGO/Cdc2 facilitates activation of Pre-MPF complexes and contributes to the starting pool of active MPF (Peter et al., 2002; Palmer et al., 1998 Ruiz et al., 2008). In the prophase oocyte, 90% of total Cdc2 is monomeric and Thr161-phosphorylated by Cdk Activating Kinase (CAK) (De Smedt et al., 2002). Frank-Vaillant and colleagues have shown that Cyclin synthesis occurs upstream of MPF activation and Cyclin B1 mRNA injection alone can induce MPF activation and GVBD without progesterone (Frank-Vaillant et al., 2001). These results suggest that the starting level of MPF activity may also occur through the association between Thr161-

phosphorylated monomeric Cdc2, which also evades Thr14 and Tyr15 inhibitory phosphorylations, and de novo synthesized B-type Cyclins (De Smedt et al., 2002).

Second, multiple factors and pathways converge at MPF activation and participate in an auto-amplification process, which creates a sudden burst of MPF activity (Masui and Markert, 1971; Palmer et al., 1998; Abrieu et al., 1998; Karaiskou et al., 1998). In one pathway, the MAPK cascade activates P90RSK, which then inhibits Myt1 to activate MPF (Palmer et al., 1998). MPF, in turn, stabilizes Mos through Ser3 phosphorylation, whereas Cdc2 inhibition prevents Mos accumulation and MAPK activation (Nebreda et al., 1995; Frank-Vaillant et al., 2001). Together, these results suggest that a Mos/MAPK/MPF positive feedback loop maintains MAPK and MPF activities. In a separate pathway, a Cdc25C/MPF autoamplification loop also exists to trigger immediate MPF activity upon Cdc25 activation. Inactive in the immature oocyte, progesterone stimulation triggers extensive N-terminal regulatory domain phosphorylation of Cdc25C. Multiple kinases have been shown to catalyze Cdc25C activation including, Cdc2/CyclinB and Polo-like Kinase (xPlx-1) (Karaiskou et al., 1998; Nakajima et al., 2003). The functions of some Cdc25C phospho-residues have been identified. Cdc25C T138 phosphorylation facilitates 14-3-3 dissociation, which is required for Cdc25C to associate with Cdc2/Cyclin. Phosphorylation on S285 blocks S287 rephosphorylation, therefore preventing Cdc25C inactivation. Additionally, the PP1 phosphatase has been shown to mediate S287 dephosphorylation, leading to full Cdc25C activity (Margolis et al., 2006). Thus, a starting pool of active MPF can activate Cdc25C. At this time, Plx-1 is also activated and contributes to Cdc25C activation. MPF is then further activated by the active phosphatase.

10. Progesterone stimulates parallel and redundant pathways, which converge on MPF activation.

Although MAPK activity is essential for timely progression of meiosis, Mos and MAPK inhibition experiments have shown that oocytes can still achieve progesterone-induced GVBD without Mos or the Mos/MEK/MAPK cascade (Fisher et al., 1999; Gross et al., 2000). These findings suggested that a parallel and functionally redundant pathway exists to trigger MPF activation and GVBD in response to progesterone. Haccard and Jessus demonstrated that the pathway leading to cyclin B synthesis mediates MPF activation independently of Mos synthesis. Inhibition of both Mos and Cyclin B with antisense oligonucleotide injection blocked progesterone stimulation of GVBD. However, inhibition of either Cyclin B or Mos did not block progesterone-induced maturation. Additionally, maturation in these Mos and Cyclin B inhibited oocytes was rescued by Mos or Cyclin B protein re-injection. Combined, these results suggest that progesterone triggers two pathways that converge upon MPF activation. One pathway activates Mos and the Mos/MEK/MAPK cascade, while the other triggers cyclin B synthesis. Should one pathway become compromised, the other can still activate MPF and induce GVBD (Haccard and Jessus, 2002).

The function of XGef, a putative Rho family guanine nucleotide exchange factor, is necessary for CPEB activation and *Xenopus* oocyte maturation. In this thesis, I first address the necessity of XGef exchange factor function and the activities of several Rho GTPases with regards to CPEB activation and oocyte maturation in response to progesterone. I found that XGef likely functions independently of exchange factor activity. Additionally, the activities of Toxin B sensitive Rho GTPases (Cdc42, RhoA-C, Rac1-3) are not necessary for CPEB activation and oocyte meiotic progression. CPEB activation and oocyte maturation also depend on MAPK activity. Since XGef

immunoprecipitates MAPK in maturing oocytes, I pursue the possibility that an XGef/MAPK interaction bears functional significance. In addition to XGef and MAPK, RINGO synthesis and RINGO/CDK1 activity are required for CPEB activation and oocyte maturation. To further our understanding of how these factors influence early progesterone signaling, I sought out to determine if direct interactions exist between each factor *in vitro*. I found that direct interactions exist between XGef, MAPK and RINGO *in vitro.* I also begin to address the functional importance of an XGef/CPEB/MAPK/RINGO complex that may form in oocytes by disrupting complex formation. Overexpression of an XGef mutant (65-360), which binds exclusively to RINGO, compromises CPEB activation and meiotic progression when overexpressed in oocytes. I propose that XGef 65-360 titrates RINGO/CDK1 from a CPEB activating complex, which is supported by the profound negative effects of overexpressing XGef 65-360. Lastly, I take a closer look at RINGO/CDK1 activity with regards to CPEB activation and demonstrate that RINGO/CDK1 activity may directly influence CPEB activation, which places RINGO/CDK1 as the most proximal upstream event of CPEB activation.

Chapter II. Investigation of XGef exchange factor activity during oocyte maturation

A. Introduction

XGef was identified as a CPEB interactor in a yeast two-hybrid screen. Interestingly, XGef function is required for progesterone-induced CPEB activation and oocyte maturation. Injection of XGef neutralizing antibodies blocked induction of meiotic progression by progesterone (Reverte et al., 2003). This consequence of XGef inhibition is most likely due to compromised CPEB activation in XGef IgG injected oocytes. First, CPEB phosphorylation is severely depressed in oocytes injected with XGef IgG. Second, endogenous XGef inhibition disrupts c-mos polyadenylation and Mos protein synthesis, events that are dependent on CPEB activation. On the other hand, XGef overexpression boosts CPEB phosphorylation and enhances Mos synthesis and the rate of maturation. XGef is undeniably important during early meiotic progression (Reverte et al., 2003; Martinez et al., 2005).

XGef is a putative Rho family guanine nucleotide exchange factor (GEF), complete with Dbl Homology (DH) and Pleckstrin Homology (PH) domains, seen in virtually all Rho GEFs (Reverte et al., 2003; Whitehead, et al., 1997). Since the DH domain catalyzes GDP to GTP exchange for targeted Rho GTPases, a mutant version of XGef lacking the DH domain (XGef Δ65-234) was created to determine if exchange activity was an important component of XGef function during oocyte maturation. Interestingly, oocytes overexpressing the XGef exchange deficient mutant did not exhibit accelerated maturation, as seen with wildtype overexpression. Instead, XGef Δ65-234 injected oocytes behaved similarly to the control. The loss of exchange factor activity did not compromise the function of endogenous XGef, however, the enhancing effect of XGef overexpression seemed to depend on GDP to GTP exchange, suggesting that XGef was activating a Rho GTPase (Martinez et al., 2005).

Here, we first observe Cdc42 and Rac1 activity levels in oocytes overexpressing HA XGef to determine if XGef function may affect the activation of these Rho GTPases. Second, we examined CPEB phosphorylation and meiotic progression in oocytes overexpressing constitutively active and dominant negative Cdc42 to determine if Cdc42 activity influenced these processes. Third, we overexpressed various XGef exchange deficient, DH domain point mutants, and observed progesterone-induced maturation to determine if XGef exchange factor activity affects meiotic progression. Finally, we blocked the activities of Rho GTPases, Cdc42, Rac1-3 and RhoA-C by injected *C. difficile* Toxin B and then observed CPEB phosphorylation and maturation upon progesterone exposure to determine if the activities of the inactivated Rho GTPases are important during meiotic progression.

B. Materials and Methods

1. Oocyte culture

Stage VI oocytes from non-primed *Xenopus laevis* females were collected after enzymatic digestion with 1x Barth's saline containing 0.2 mg/ml Collagenase (type IA, Sigma) and 0.6 U/ml dispase (Roche). Oocytes were then washed and cultured in 1x Barth's. When applicable, oocytes were stimulated with 100 μM progesterone (4-Pregnene-3,20-dione, Sigma).

2. Oocyte injections

mRNAs transcribed from the following plasmids were injected into oocytes (400 ng/μl, 46nl/oocyte): pSP6-HA-Cdc42 WT, pSP6-HA-Cdc42 G12V, pSP6-HA-Cdc42 T17N and pSP6-HA-Globin and pSP6-HA-XGef WT. pSP6-HA-XGef-DH, pSP6-HA-XGef-Y198A, pSP6-HA-XGef T79A/L188A, pSP6HA-XGef-L188A/R194A were made by a previous PhD student, Lei Yuan. In some cases, oocytes were injected with 50 mM GTPγS. For Clostridial Toxin experiments, oocytes were injected with 5 μg/ml Toxin B, which had been reconstituted in 50 mM NaCl, 50 mM Tris pH 7.5, for a final concentration of 12 nM (Calbiochem).

3. Oocyte extracts and immunoblotting

Oocytes were lysed in 3 μl/oocyte extract buffer containing 1xPBS and protease inhibitors (10 ng/μl each chymostatin, pepstatin and aprotinin). For exchange and GST-PBD/RBD pulldown assays, oocytes were lysed in buffer (10 μl/oocyte) containing: 25 mM Tris-HCl pH7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1%NP-40, 1 mM DTT and protease inhibitors. For the His-CPEB phosphorylation assay, oocytes were homogenized in buffer containing 20 mM Hepes pH 7.5, 100 mM NaCl, 10 mM β-glycerophosphate, 5

mM EGTA, 5 mM $MgCl_2$, 100 mM sucrose, 2 mM $NaVaO_4$, 20 mM NaF, 1mM phenylmethylsulfonyl fluoride (PMSF), 1 mM DTT, 2 μM okadaic acid and protease inhibitor cocktail (5ng/μl leupeptin and 10ng/ul each chymostatin, pepstatin, and aprotinin).

4. Antibodies

Primary HA and GST-mouse monoclonal antibodies were each used at a 1:1000 dilution (Covance). Horseradish peroxidase (hrp)-conjugated Mouse monoclonals were detected with anti-mouse secondary (1:10,000, Santa Cruz). Rabbit polyclonal Cdc42 and Rho A antibodies were diluted 1:750 (BD Transduction, Cytoskeleton). Hrp-linked Goat anti-Rabbit secondary antibody was diluted to 1:12,000 (Santa Cruz). The Rac1 primary antibody was raised in chicken and diluted to 1:500 (Ab Cam). Rac1 antibody was recognized by rabbit anti-chicken secondary antibody (1:10,000, Ab Cam). Chemilumenescence detection of hrp-conjugated antibodies was used (Perkin Elmer).

5. Plasmid constructs and cloning

The SP6-HA-Cdc42 G12V mutant was made using the SP6-HA-Cdc42 WT parental vector and site-directed mutagenesis (Strategene) using primers:
For: 5'-CCA GAG GAA CGT TGT CCA CCA GAG GAG CAG AGA GAG C-3'
Rev: 5'-GCT CTC TCT GCT CCT CTG GTC GAC AAC GTT CCT CTG G-3'
The SP6-HA-Cdc42 T17N plasmid was made using the SP6-HA-Cdc42 WT parental vector. Site directed mutagenesis was performed with primers:
For: 5'-GGT GAT GGT GCT GTG GGT AAA AAC TGT CTG CTT ATC TCT TAC AC-3'
Rev: 5'-GTG TAA GAG ATA AGC AGA CAG TTT TTA CCC ACA GCA CCA TCA CC-3'

PCR cycling conditions for SP6-HA-Cdc42 G12V and T17N were: 95°C for 30 seconds; then 16 cycles of 95°C for 30 seconds, 1 min at 55°C and 5 minutes at 68°C.

The SP6-HA-XGef Y198A mutant was made using the parental SP6-HA-XGef WT plasmid and site directed mutagenesis with primers:

For: 5'-CGT TAC TCC GTG TTC TTG AGG CCA CAC ATT ACC-3'

Rev: 5'-GGT AAT GTC TGG CCT CAA GAA CAC GGA GTA ACG-3'

PCR cycling conditions for SP-HA-XGef Y198A mutagenesis were: 95°C for 30 seconds; then 18 cycles of 95°C for 30 seconds, 55°C for 1 minute and 68°C for 6 minutes 30 seconds. *PfuI* DNA polymerase was used for all PCR reactions (Stratagene).

DpnI restriction enzyme (New England Biolabs) was added to each PCR reaction to digest parental template. PCR products were then phenol chloroform extracted and ethanol precipitated. Precipitated PCR products were transformed into XL1-Blue *E. coli* for plasmid propagation and later sequencing.

6. Plasmid Sequencing

Plasmids (265 ng) were first preheated at 85°C for five minutes. Plasmids were then combined with 5 pmol sequencing primer and DTCS mix (4 μl), containing nucleotide labeling dyes and polymerase, in a 10ul final volume. The thermocycler was then programmed to repeat 40 cycles of denaturing at 96°C for 20 seconds, primer annealing at 50°C for 20 seconds and extension at 60°C for 4 minutes. Once the sequencing program was complete, samples were supplemented with 2 μl glycogen and 4 μl of stop solution, containing 1.5 M sodium acetate and 50 mM EDTA pH 8.0. PCR products were precipitated with 60 μl of 100% ethanol, light vortexing and incubation at -20°C for at least 30 minutes. Precipitated PCR products were pelleted by centrifugation at 12,000 rpm for 15 minutes at 4°C. Pelleted material was then washed with 75%

ethanol and re-centrifugation, followed by air-drying. Sample loading solution (Beckman Coulter) was added to the dried pellet and submitted for sequencing.

7. *In vitro* transcription

The following plasmids were linearized with the *Xba*I restriction enzyme and used as templates for *in vitro* transcription using the mMessage mMachine SP6 Kit (Ambion): pSP6-HA-XGef WT, pSP6-HA-XGef-DH, pSP6-HA-XGef-Y198A, pSP6-HA-XGef T79A/L188A, pSP6HA-XGef-L188A/R194A, pSP6-HA-Cdc42 WT, pSP6-HA-Cdc42 G12V, pSP6-HA-Cdc42 T17N and pSP6-HA-Globin. For each *in vitro* transcription reaction, *XbaI* linearized template (1 μg) was combined with NTP Cap (5 mM ATP, CTP, UTP, 1 mM GTP and 4 mM cap analog), Reaction Buffer (salts, buffer, DTT and other ingredients (Ambion)), SP6 RNA polymerase (2 μl) and nuclease free water for a final volume of 20 μl. Reactions were incubated for 2 hours at 37°C. DNA template was digested with DNase I addition and incubation at 37°C for 15 minutes. To purify *in vitro* transcribed mRNA, 0.5 M ammonium acetate and 10 mM EDTA were added to the reaction mixture followed by phenol/chloroform extraction and ethanol precipitation of mRNAs. mRNA yield was determined by spectrophotometric measurement of absorbance at 260 nm. mRNA integrity was analyzed by 1% agarose gel electrophoresis and ethidium bromide staining. Briefly, a 1 μl aliquot of mRNA was combined with gel loading buffer (95% formamide, 0.025% xylene cyanol, 0.025% bromophenol blue, 18 mM EDTA and 0.025% SDS) and dilute ethidium bromide (100 μg/ml) and heated at 80°C for 5 minutes prior to agarose gel electrophoresis.

8. Recombinant protein expression and purification:

GST-PAK PBD

BL21DE *E. coli* cells (Stratagene) were transformed with 10 ng pGEX-2T GST-PAK-PBD and grown in 100 ml of 1 mg/ml Ampicillin/Luria Bertani (LB) broth overnight. Culture volume was expanded to 1 L, grown at 37°C until $O.D_{600}$= 0.600, and induced with 100 μM IPTG. Induced cultures were grown at 30°C for 3.5 hours. After the induction period, cells were pelleted by centrifugation (Sorvall SS-34) at 5,000 rpm, for 10 minutes, at 4°C. Cells were resuspended with 100 ml of Resuspension Buffer containing 50 mM Tris pH 7.5, 20 mM $MgCl_2$ and 150 mM NaCl. Aliquots of 10 ml were made in sarstedt tubes and repelleted and stored at -80°C. To purify protein, pellets were resuspended in buffer containing 50 mM Tris pH 7.5, 20 mM $MgCl_2$, 150 mM NaCl, 5 mM DTT and 0.5% NP40. Resuspended cells were sonicated (output 25%) for three times at 30 second intervals. Sonicated cells were pelleted at 12,000 rpm for 10 minutes at 4°C. Clarified supernatants were combined with 400 μl glutathione bead slurry pre-rinsed in resuspension buffer. GST-PAK-PBD affinity purification occurred at 4°C for 2 hours with end over end rotation. Glutathione-bead bound GST-PAK PBD was then washed with buffer containing 50 mM Tris pH 7.5, 20 mM $MgCl_2$, 150 mM NaCl, 5 mM DTT and 0.5% NP40.

GST-Rhotekin RBD

BL21DE cells were transformed with pGEX-2T-Rhotekin-RBD, described previously. The overnight culture (50 ml) was combined with 450 ml LB/Ampicillin and grown for an hour at 37°C, followed by induction with 600 μM IPTG. Induced cells were grown at 25°C for 16 hours. Cells were then pelleted at 5,000 rpm for 10 minutes, at

4°C. Pellets were stored at -80°C until further use. GST-Rhotekin-RBD purification followed the same procedure as GST-PAK-PBD.

His-CPEB

BL21DE cells were transformed with 50 ng of pET-His-CPEB plasmid. The 1 ml transformation solution was combined with 9 ml LB/ampicillin and cultured overnight at 37°C and shaking at 225 rpm. The overnight culture was then used to inoculate 1 L LB/Ampicillin and grown again at 37°C until an $O.D._{600}$ of 0.600 was reached. His-CPEB expression was induced with 400 μl of 1 M IPTG and incubation at 28°C, for 3.5 hours. After induction, cells were pelleted at 5,000 rpm and 4°C, using a Sorvall SS-34 rotor (200 ml culture/pellet). For purification, pellets were resuspended in Binding Buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH 7.9, 0.25% Tween 20) and sonicated (20-30% output) three times for 30 second intervals. Insoluble proteins were pelleted at 12,000 rpm at 4°C (Sorvall SS-34) for 12 minutes. Clarified supernatants were incubated with 300 μl Ni^+-NTA nickel slurry, pre-washed in Binding Buffer, for 3 hours at 4°C. Nickel beads were washed three times with 5 ml Wash Buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH7.9) to remove nonspecifically bound proteins. Nickel bead-bound His-CPEB was stored on ice until further use.

9. Exchange Activity Assays/GST-tagged effector protein pulldown assays

Extracts prepared from 50 oocytes were incubated with GST-PAK-PBD or GST-Rhotekin-RBD (20 μg) for two hours at 4°C with end over end rotation. Non-specifically bound proteins were washed away with wash buffer containing 25 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$ and 1% NP-40. Bound proteins were eluted with 2x SDS sample loading buffer and boiling. Half of the precipitated proteins (15 μl) were run on

10-12.5% SDS-PAGE. Positive control extracts were treated with GTPγS (0.1 mM), 10 mM EDTA pH 8.0 and 60 mM $MgCl_2$ to irreversibly activate all GTPases.

10. *In vitro* His-CPEB phosphorylation assay

For the *in vitro* His-CPEB phosphorylation assay, oocyte lysates (90 oocytes per reaction) were combined with 3 μCi [γ-^{32}P]ATP and 2.5 μg Nickel Ni^+-NTA bead-tethered His-CPEB, pre-equilibriated in Phosphorylation Buffer containing 10 mM Hepes pH 7.5, 10 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, 1 μM okadaic acid, 1 mM H-89 PKA inhibitor, 50 μM adenosine triphosphate and protease inhibitors. Reactions were conducted at room temperature for 30 minutes and stopped with 25 μl SDS sample loading buffer and boiling. *In vitro* His-CPEB phosphoryation was analyzed by 10% SDS-PAGE and autoradiography.

C. Results

1. HA-XGef overexpression does not enhance Cdc42 or Rac1 activity in maturing oocytes.

XGef function is required for CPEB activation and timely oocyte maturation. HA-XGef overexpression enhances CPEB phosphorylation and rate of GVBD, yet these effects are lost in oocytes overexpressing a guanine exchange mutant version of XGef lacking the DH domain (Δ65-234). These findings suggest that XGef affects meiotic progression by catalyzing GDP to GTP exchange to activate a target Rho GTPase. We have also previously shown that XGef activates Cdc42 in NIH 3T3 mammalian cells (Reverte et al., 2003). Here, we pursued the possibility that XGef influences meiotic progression by activating a small GTPase. To do this, we overexpressed HA-XGef and examined Cdc42 activity in immature and maturing oocytes. We also chose to observe the influence of XGef towards Rac1, as another candidate XGef-specific Rho GTPase. Activation of Cdc42 and Rac1 was measured with a downstream effector pulldown approach, in which extracts were combined with glutathione bead-bound GST-P21 Activated Protein Kinase protein binding domain (GST-PAK-PBD). GST-PAK-PBD encodes residues 67-150 of PAK and bears the minimal sequence required for high affinity interaction with GTP-bound Cdc42 and Rac1-3. In these XGef exchange activity assays, we also co-injected and supplemented extracts with a non-hydrolyzable analog of GTP (GTPγS) to address the potential complications of detecting transient Cdc42 activation. Intrinsic GTPase activity and GTPase interactions with GDIs and GAPs, which enforce inactivation, all contribute to the cyclic nature of Cdc42 activation. However, in the presence of excess intracellular GTPγS, GTPases undergoing guanine nucleotide exchange would most likely associate with the non-hydolyzable analog and became irreversibly activated. GTPγS supplementation

therefore addressed the issue of inefficient GST-PAK PBD pulldown of active Cdc42, in which fleetingly active Cdc42 may have evaded PBD-binding. Oocytes were stimulated with progesterone, lysed at the times indicated, GTPγS treated and combined with glutathione bead-bound GST-PAK-PBD to specifically bind Cdc42 and Rac1-GTP/GTPγS. HA-XGef overexpression in immature and progesterone stimulated oocytes did not affect endogenous Cdc42 or Rac1 activation, evident in the unchanged levels of PAK-PBD precipitated Rho GTPases, compared to HA-Globin control (Figure 5). To address the possibility that our means for detecting endogenous Cdc42 activation may not have been adequately sensitive, we next co-overexpressed HA-Cdc42 and HA-XGef and performed another GST-PAK PBD pulldown with GTPγS injection and lysate treatment, described previously. HA immunoblot analysis of bead-precipitated proteins suggested that HA-XGef overexpression did not enhance HA-Cdc42 activity in immature or maturing oocytes compared to HA-Globin and HA-Cdc42 co-injected oocytes (Figure 6). Altogether, HA-XGef overexpression in oocytes did not stimulate the activation of endogenous Cdc42 or Rac1. The activation of recombinant HA-Cdc42 was also unaffected by HA-XGef overexpression.

2. Cdc42 activity does not affect CPEB phosphorylation or meiosis progression.

Rho GTPases are activated in localized regions within the oocyte. Additionally, specific pools of a Rho GTPase are involved in an array of signaling events, however, changes in total intracellular Rho GTPase activity may be subtle and consequently difficult to detect. We therefore pursued an alternative approach by assessing the significance of Cdc42 activity during progesterone-induced maturation. First, we created constitutively active (Cdc42-G12V) and dominant negative (Cdc42-T17N) mutants of Cdc42. The G12V mutation affects Cdc42 GTP hydrolysis, therefore creating a GTPase

defective mutant that becomes permanently GTP-bound. The T17N mutation has been shown to decrease GTP binding affinity as well as enforce irreversible GEF association, therefore inhibiting endogenous GTPase activity. We next overexpressed these Cdc42 mutants in oocytes, followed by progesterone stimulation to drive G2/M progression. Observation of a GVBD time course revealed that Cdc42 T17N overexpression did not affect maturation compared to the HA-Globin control. Interestingly, G12V blocked maturation completely and oocytes were unable to achieve GVBD due to lysis (Figure 7). To determine if Cdc42 activity affects CPEB phosphorylation, we performed an *in vitro* His-CPEB phosphorylation assay using oocytes overexpressing HA-Cdc42 G12V and HA-Cdc42 T17N. After progesterone stimulation, oocytes were collected at the times indicated, lysed and combined with nickel bead-bound His-CPEB and [γ^{32}P] ATP. Detection of His-CPEB radioactive phosphate incorporation revealed that Cdc42 mutant overexpression did not affect oocyte kinase activity towards His-CPEB (Figure 8). These findings suggest that Cdc42 activity is irrelevant to progesterone signaling during oocyte maturation. However, we cannot rule out the possibility that XGef may activate another Rho GTPase to influence meiotic progression.

3. XGef exchange activity perturbation does not affect progesterone-induced maturation.

Our initial interest in the significance of XGef exchange activity during maturation stemmed from the negative effects imposed by a mutant of XGef lacking the DH domain, which catalyzes nucleotide exchange for Rho GTPase activation. Interestingly, the DH domain also interacts with the adjacent, C-terminal, PH domain and may facilitate proper folding. Removal of the DH domain may therefore compromise other functions besides exchange activity, such as membrane localization. If additional functions were disrupted

by our DH domain deletion, then our previous interpretation of maturation and CPEB phosphorylation data involving this particular mutant may be incorrect. We therefore created various XGef point mutations in the XGef DH domain, which have been shown to abrogate exchange activity in other Rho GEFs. A Y393A mutation in the microtubule binding GEF-H1 decreases DH domain catalytic activity towards RhoA by disrupting GEF/GTPase binding (Krendel et al., 2002). We therefore mutated the analogous residue, Y198A, in XGef. Our XGef R194A mutation is reminiscent of the Trio R1369 mutant, in which the highly conserved hydrophilic amino acid also interacts with E1245 to stabilize the DH domain. Mutagenesis of these stabilizing residues has been shown to block nucleotide exchange and/or GTPase interaction and therefore poses a great consequence to exchange activity (Liu et al., 1998). We also created an XGef-L188A mutant, which is analogous to L1194A of Tiam1, a residue that along with others, is involved in creating an interface between Tiam1 and Rac1. Alanine substitution at L1194 has been shown to depress exchange activity to less than 20% compared to wildtype (Worthylake et al., 2000). Lastly, we mutated the highly conserved L79 in XGef to create the XGef-L79A exchange mutant. Oocyte were injected with each mutant and stimulated with progesterone to resume meiosis. We then monitored the development of GVBD over time and found that overexpression of each exchange point mutant did not affect the rate of maturation compared to oocytes overexpressing HA-XGef WT (Figure 9). Surprisingly, we also did not see a difference in GVBD time course between oocytes overexpressing HA-XGef WT and HA-XGef DH, as previously reported. The oocytes used in the maturation study expressed similar levels of each HA-tagged protein. These results suggest that XGef influence on CPEB activation and meiotic progression is independent of exchange activity.

4. Progesterone-induced maturation and CPEB phosphorylation occur independently of *Clostridium difficile* Toxin B sensitive Rho GTPases.

Previously, we found that constitutively active and dominant negative exchange mutants of Cdc42 did not affect CPEB phosphorylation or oocyte maturation. Further, our exchange assay results showed that XGef did not stimulate the activation of Rho GTPases, Cdc42 and Rac1. XGef exchange point mutant overexpression in oocytes also did not affect progesterone-induced maturation. We next investigated the requirement of Clostridial Toxin B and C3 sensitive Rho GTPases during maturation. First, we injected *Clostridium difficile* Toxin B to inhibit oocyte Cdc42, RhoA-C, Rac1-3, RhoG and TC10, and determine if the function of the affected Rho GTPases was required for progesterone signaling.

As a large clostridial glucosylating toxin, potency depends on glucosyltransferase activity to disrupt Rho GTPase nucleotide exchange and activation. Glucose incorporation on a highly conserved threonine residue required for nucleotide binding and magnesium divalent ion coordination disrupts conformational changes that must occur upon GDP/GTP exchange and GTPase activation. Toxin B efficiently glucosylates Cdc42 and Rac1 on Thr 35 and the analogous residue, Thr 37, of Rho. Upon threonine glucosylation, Rho GTPases are unable to bind their downstream effectors.

Toxin B pre-injected oocytes were treated with progesterone to initiate meiotic progression in the absence of affected Rho GTPase function. At the indicated times, oocytes were lysed and analyzed for kinase activity towards His-CPEB (Figure 10A). Extracts prepared from Toxin B and control injected oocytes that had resumed meiosis for three hours contained equal kinase activity towards His-CPEB, which provided evidence that the activity of Toxin B sensitive Rho GTPases did not influence CPEB phosphorylation. To ensure Toxin B efficacy, extracts prepared in parallel were treated with EDTA to chelate magnesium and force all GTPases to release bound nucleotides.

Extracts were then supplemented with GTPγS and $MgCl_2$ to drive GTPase-GTPγS association and irreversible activation. If Toxin B successfully inhibited the targeted Rho-GTPases, we would not expect them to bind GTPγS upon nucleotide reassociation and associate with GST-PAK-PBD or GST-Rhotekin Rho binding domain (GST-Rhotekin-RBD). These extracts were then combined with GST-PAK-PBD or GST-Rhotekin-RBD, to exclusively bind the activated forms of the Rho GTPases indicated (Figure 10B). GST-PAK-PBD, encoding residues 67-150 of PAK, bear the minimal sequences required for high affinity interaction with GTP-bound Cdc42 and Rac1-3. GST-Rhotekin-RBD, containing residues 7-89 of Rhotekin, binds only active RhoA-C (Ren and Schwartz, 2000). GTPγS binding and activation of Rho GTPases, Cdc42, Rac1 and RhoA, were very efficient in treated extracts prepared from vehicle-injected oocytes (Figure 9B, lanes 1-4). Conversely, Cdc42, Rac1 and RhoA immunoblot analysis of pulldown precipitates revealed potent Rho GTPase inhibition three hours after Toxin B injection (Figure 10B lanes 6-8). Equal amounts of effector proteins were added to each reaction, detected by GST immunoblotting. Lastly, meiotic progression in Toxin B injected oocytes did not differ from control-injected oocytes (Figure 10C).

D. Figures

Figure 5. HA-XGef overexpression does not stimulate the activation of Cdc42 or Rac1 in oocytes. HA-XGef overexpression does not affect endogenous Cdc42 or Rac1 activity in immature and maturing oocytes (n=2). Oocytes were injected with HA-Globin or HA-XGef mRNAs and GTPγS (2.3 mM). Some oocytes were stimulated with progesterone and lysed at the indicated times. Extracts were then supplemented with GTPγS (2 nmoles) and incubated with glutathione bead-bound GST-PAK-PBD. PBD precipitated proteins were analyzed by immunoblotting with Cdc42 and Rac1 antibodies (IB: Cdc42 and IB: Rac1). GST immunoblot analysis revealed GST-PAK-PBD bait protein in each reaction (IB: GST). Input extracts were probed with HA-antibody to detect expression of the indicated proteins (IB: HA). For a positive control, nucleotide depleted extracts were treated with Mg^{2+} and GTPγS to force GTPase re-association with the non-hydrolyzable analog.

Figure 5.

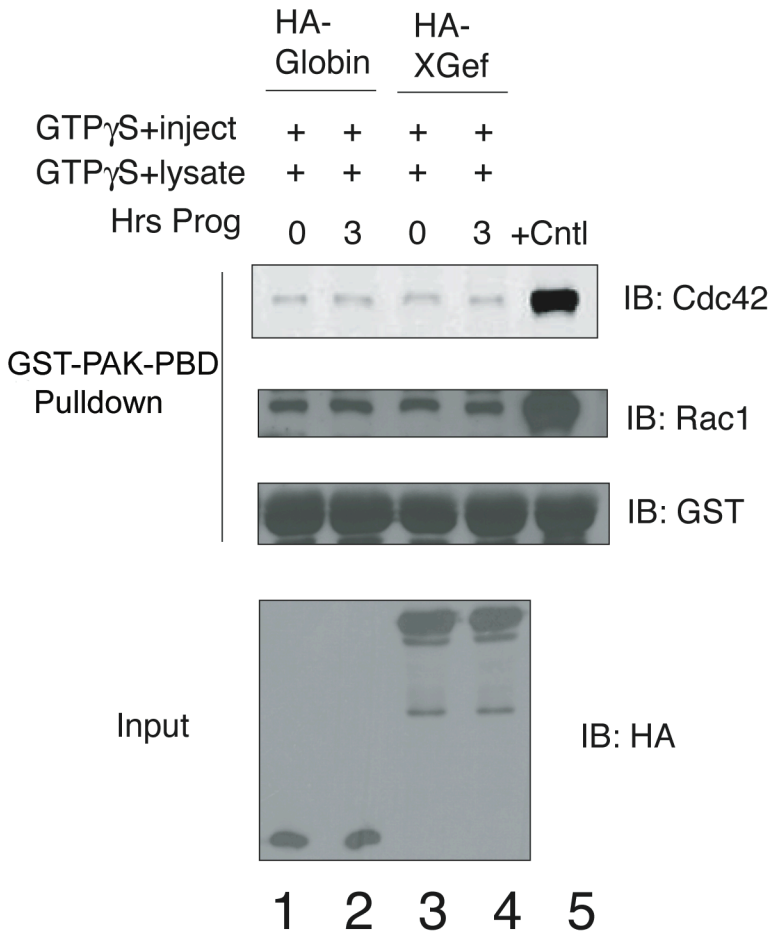

HA-
Globin
HA-
XGef
GTPγS+inject + + + +
GTPγS+lysate + + + +
Hrs Prog 0 3 0 3 +Cntl
GST-PAK-PBD
Pulldown
IB: Cdc42
IB: Rac1
IB: GST
Input
IB: HA
1 2 3 4 5

Figure 6. Recombinant Cdc42 is not activated by HA-XGef overexpression.

A GST-PAK PBD pulldown was performed with oocytes (n=2) co-overexpressing HA-XGef and HA-Cdc42. GTPγS oocyte injection and lysate supplementation were performed in the same manner as the experiment shown in A. PBD precipitated HA-Cdc42-GTP/GTPγS and overexpressed HA-tagged proteins in input extracts were detected by HA immunoblot analysis (IB: HA). GST-PAK PBD levels are shown (IB: GST).

Figure 6.

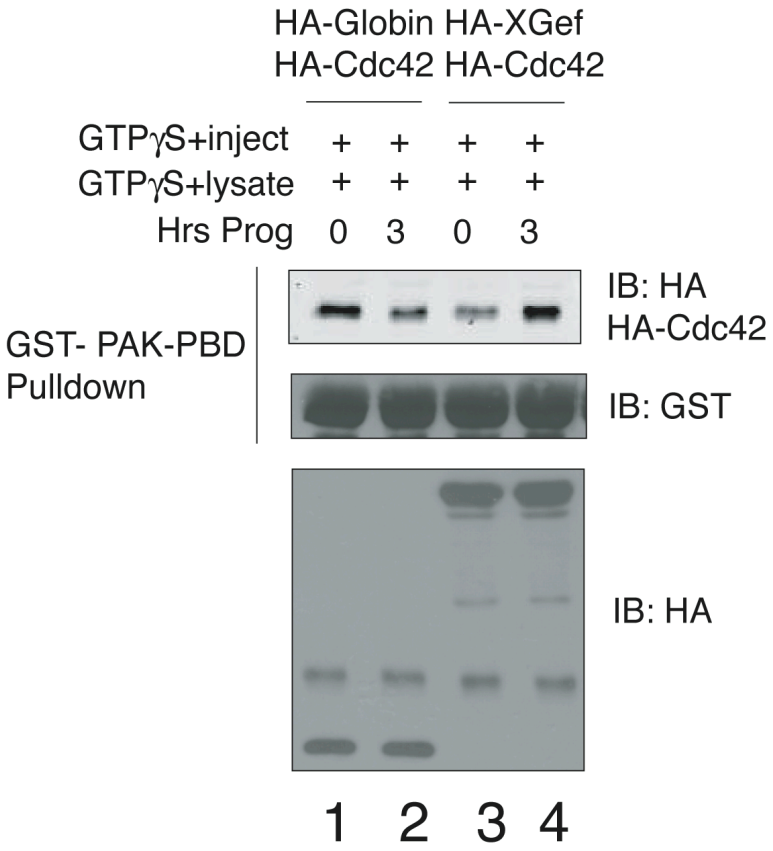

HA-Globin HA-XGef
HA-Cdc42 HA-Cdc42
GTPγS+inject + + + +
GTPγS+lysate + + + +
Hrs Prog 0 3 0 3
GST- PAK-PBD
Pulldown
IB: HA
HA-Cdc42
IB: GST
IB: HA
1 2 3 4

Figure 7. Meiotic progression in oocytes overexpressing constitutively active and dominant negative Cdc42. Oocytes (n=4) were injected with HA-Cdc42 G12V (constitutively active), HA-Cdc42 T17N (dominant negative) or control HA-Globin mRNAs and stimulated with progesterone to trigger meiosis resumption. The effect of Cdc42 mutant overexpression during maturation was monitored in a GVBD time course.

Figure 7.

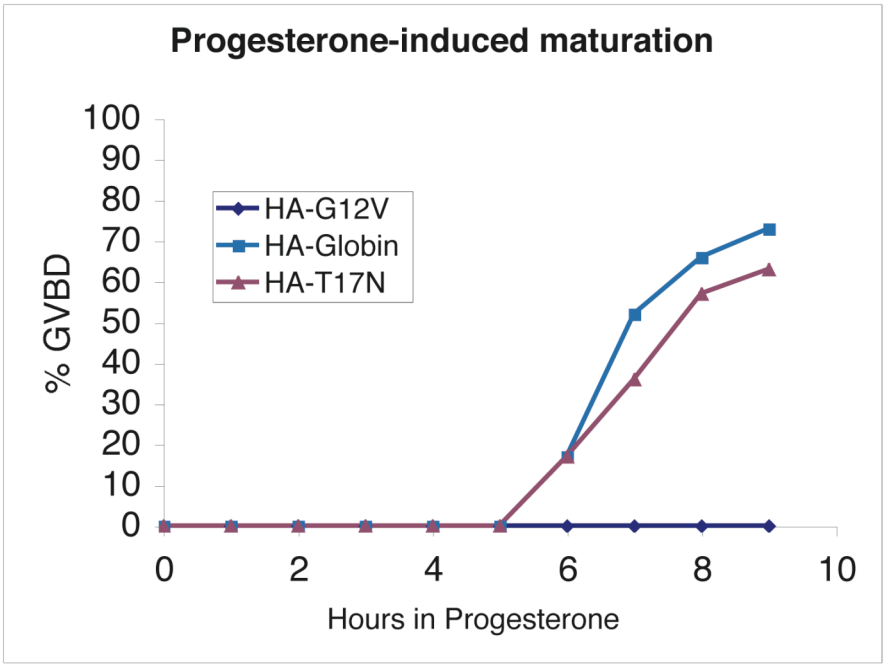

Progesterone-induced maturation
% GVBD
100
90
80
70
60
50
40
30
20
10
0
HA-G12V
HA-Globin
HA-T17N
0
2
4
6
8
10
Hours in Progesterone

Figure 8. Overexpression of constitutively active and dominant negative Cdc42 mutants does not affect His-CPEB phosphorylation. Oocytes (n=3) collected in parallel of the maturation study shown in the previous figure were analyzed in an *in vitro* His-CPEB phosphorylation assay (P32). The amount of His-CPEB added to each reaction is shown (CS). Overexpression of HA-tagged proteins was detected by an HA-immunoblot (IB: HA).

Figure 8.

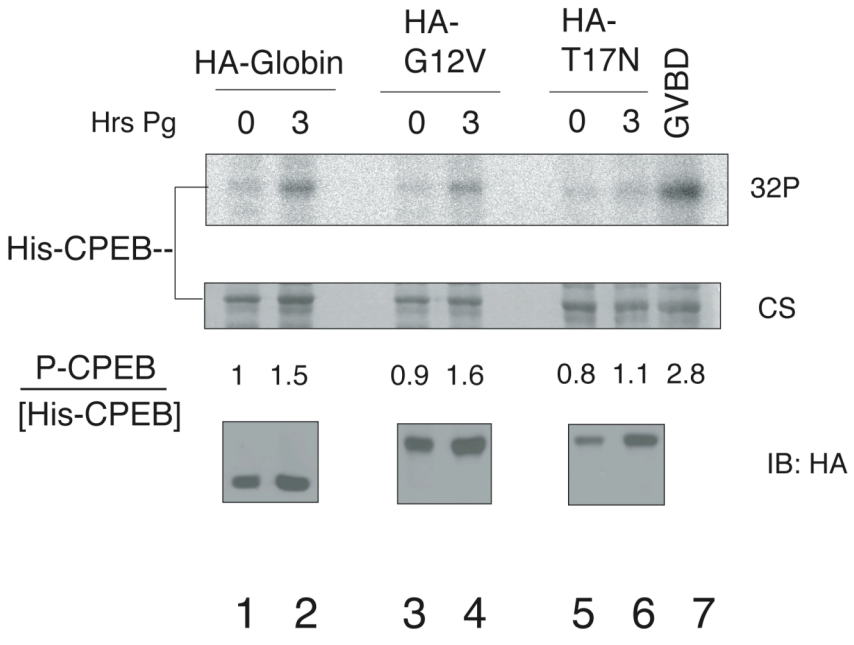
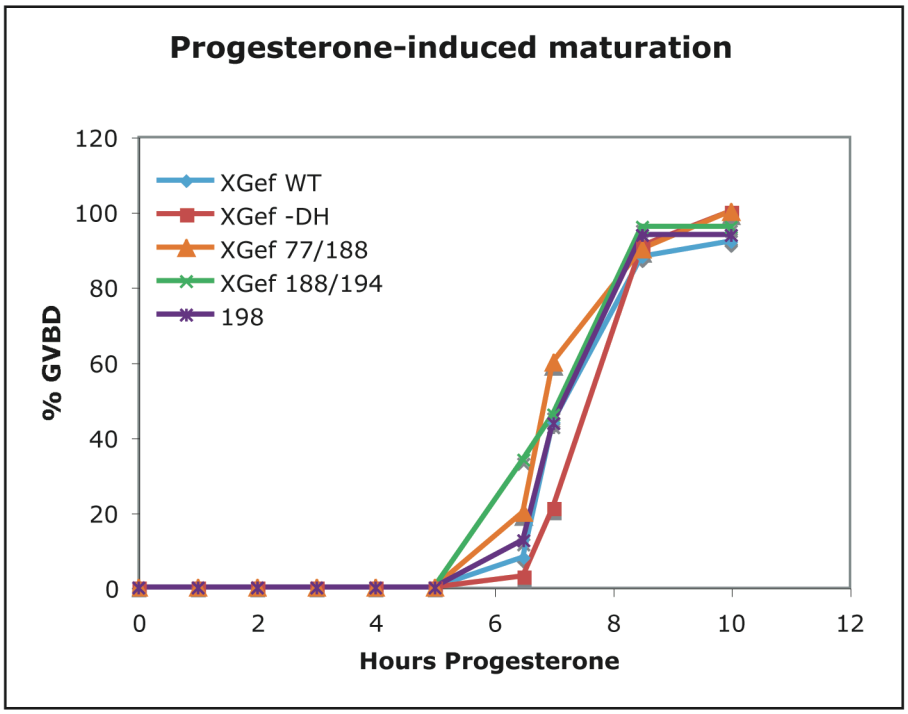
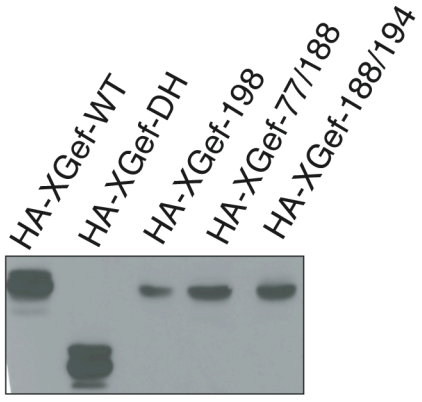

HA-Globin
HA-G12V
HA-T17N
GVBD
Hrs Pg
0 3 0 3 0 3
32P
His-CPEB--
CS
P-CPEB
[His-CPEB]
1 1.5 0.9 1.6 0.8 1.1 2.8
IB: HA
1 2 3 4 5 6 7

Figure 9. Overexpression of XGef DH domain point mutants does not affect progesterone-induced maturation in oocytes. Oocytes were injected with HA-XGef WT, HA-XGef DH, HA-XGef Y198A, HA-XGef T79A/L188A and HA-XGef L188A/R194A mRNAs. After progesterone stimulation, oocytes were monitored for GVBD development. Overexpression of the indicated proteins was analyzed by HA-immunoblot (IB: HA).

Figure 9.

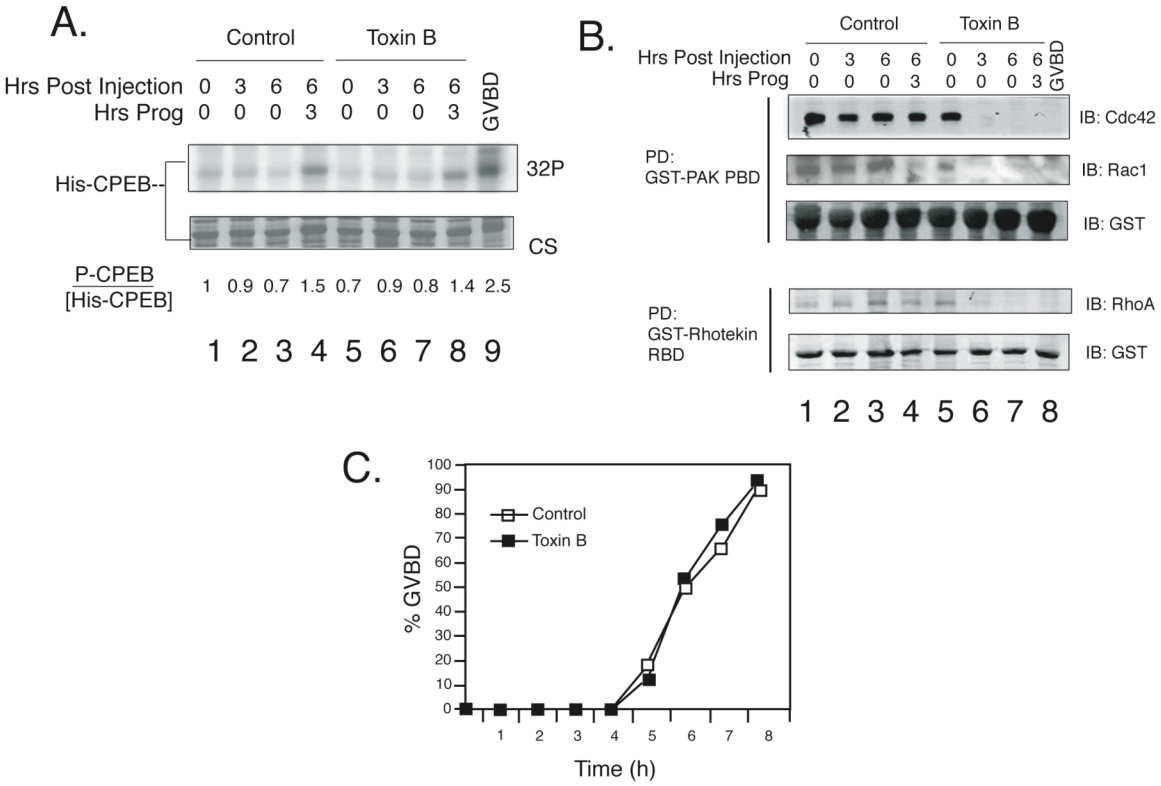




IB: HA

Figure 10. Rho GTPase inhibition with *Clostridium difficile* Toxin B and *Clostridium botulinum* C3 exotransferase does not affect CPEB phosphorylation or progesterone induced maturation. (A) Three hours after Toxin B injection, oocytes (n=3) were stimulated with progesterone and lysed at the indicated times. Extracts were combined with nickel-bead bound His-CPEB and γ^{32}-P[ATP] for an *in vitro* His-CPEB phosphorylation assay (P32). Equivalent levels of His-CPEB were added to each reaction (CS). (B) Extracts prepared from oocytes collected in parallel were then treated to activate all GTPases and incubated with GST-PAK PBD or GST-Rhotekin-RBD to pulldown the indicated active Rho-GTPases. GST-PAK PBD precipitates were probed with Cdc42 and Rac1 antibodies to detect activation of each Rho-GTPase (IB: Cdc42 and IB: Rac1). Active RhoA bound to GST-Rhotekin RBD beads was detected by RhoA immunoblot analysis (IB: RhoA). Bait effector proteins were detected with GST-antibody (GST:IB). (C) Toxin B or DMSO control injected oocytes (n=2) were stimulated with progesterone and monitored for meiotic progression.

Figure 10.


A.
Control
Toxin B
Hrs Post Injection 0 3 6 6 0 3 6 6
Hrs Prog 0 0 0 3 0 0 0 3
GVBD
His-CPEB--
32P
CS
P-CPEB
[His-CPEB]
1 0.9 0.7 1.5 0.7 0.9 0.8 1.4 2.5
1 2 3 4 5 6 7 8 9
B.
Control
Toxin B
Hrs Post Injection 0 3 6 6 0 3 6 6
Hrs Prog 0 0 0 3 0 0 0 3
GVBD
PD:
GST-PAK PBD
IB: Cdc42
IB: Rac1
IB: GST
PD:
GST-Rhotekin
RBD
IB: RhoA
IB: GST
1 2 3 4 5 6 7 8
C.
% GVBD
Control
Toxin B
Time (h)

E. Discussion

1. Overview

As a putative Rho family GEF, we proposed that XGef functions as an exchange factor to activate a Rho GTPase during oocyte meiotic progression, linking Rho GTPase-mediated signaling to the maturation process. XGef activates Cdc42 in mammalian cell culture (Reverte et al., 2003). We therefore proposed that XGef may activate Cdc42 in *Xenopus* oocytes as well. HA-XGef overexpression did not affect endogenous or recombinant Cdc42 activity in maturing oocytes. Progesterone stimulation alone also did not affect Cdc42 activity. A single Rho GTPase can participate in diverse signaling processes, in which a distinct pool becomes activated in response to a particular upstream signal. Our detection of changes in Cdc42 activity during meiotic progression may have been a consequence of these subtle changes in Rho GTPase activity. However, based on our inability to detect fluctuations in Cdc42 activation during maturation, we could not exclude Cdc42 as a participant of the progesterone-signaling pathway. Alternatively, XGef may not activate Cdc42, but rather another Rho GTPase.

Cdc42 activity does not affect CPEB phosphorylation or progesterone-induced oocyte maturation, since overexpression of constitutively active and dominant negative mutants of Cdc42 did not alter these events. Again, we could not rule out a potential role for Cdc42 activity during maturation based on these experiments alone since the endogenous protein may have been functional despite the presence of recombinant Cdc42 mutants. We therefore used *Clostridium difficile* Toxin B to inhibit the Rho GTPases, Rho A-C, Cdc42 and Rac1. Toxin B oocyte injection did not affect CPEB phosphorylation or meiotic progression, suggesting that Toxin B sensitive Rho GTPases were unnecessary for GVBD.

Lastly, we created XGef DH domain point mutants to block exchange activity, in the event that deletion of the entire domain had compromised other functions besides

GDP/GTP exchange, and found that overexpression of various DH domain point mutants behaved similarly to XGef-WT. XGef exchange function is not necessarily irrelevant to the maturation process since the recombinant XGef DH domain point mutants may not have overwhelmed endogenous protein function, which may have masked potential effects. Further research is necessary to characterize XGef function during maturation. It is also important to establish a relationship between Rho GTPase function, the GEFs involved in their activation and the cytoskeletal arrangements and intracellular membrane transport that take place during oocyte meiotic progression.

2. XGef and Cdc42 activities during oocyte maturation

Cdc42 has been shown to mediate G2/M arrest in *Xenopus* oocytes through the activation of its downstream effector *Xenopus* P21-Activated Kinase 2 (XPAK2). Cau and colleagues showed that overexpression of constitutively active Cdc42 (G12V) activates XPAK2 and maintains G2 arrest in the presence of progesterone. Conversely, overexpression of dominant negative Cdc42 (T17N) facilitates progesterone-induced maturation (Cau et al., 2000). The idea of Cdc42-mediated prophase I arrest clearly conflicts with our hypothesis that XGef, a mediator of meiosis resumption, may function as a Cdc42 activator. Further, the effects of Cdc42 activity on maturation presented by Cau and colleagues, were not reproducible in our lab or by others. When we overexpressed Cdc42 G12V in oocytes, early progesterone signaling was unaffected since we were able to detect normal CPEB phosphorylation. However, these oocytes later lysed and we were not able to observe GVBD. We also did not observe any acceleration in oocyte maturation as a result of Cdc42 T17N overexpression. Notably, whereas we injected Cdc42 mutant mRNAs for overexpression, Cau and colleagues injected constitutively active and dominant negative Cdc42 mutant protein, expressed and purified from bacteria. The contrasting overexpression approaches may account for

the disparity in our maturation data. Further research is required to resolve these inconsistencies.

Cdc42 and RhoA activities have been shown to mediate polar body formation in *Xenopus* oocytes. Ect2 is the GEF responsible for activating these Rho GTPases during this asymmetrical cell division. Polar body formation involves Cdc42-mediated out-pocketing of the oocyte surface, an event guided by Rho A-mediated contractile ring placement. As expected, overexpression of dominant negative Ect2 lead to a decrease in Cdc42 and RhoA activity in *Xenopus* oocytes and blocked polar body formation. The same phenomenon occurred upon overexpression of dominant negative Cdc42 (Zhang et al., 2008). The involvement of Cdc42 during polar body formation occurs 2 to 3 hours after GVBD, an event late in meiosis, whereas XGef function has been shown to act very early in meiosis, within the first 3 hours. The differential timing of XGef and Cdc42 activities may explain why we did not observe changes in Cdc42 activity upon progesterone stimulation or XGef overexpression and found that CPEB phosphorylation occurred normally despite the overexpression of constitutively active and dominant negative Cdc42. XGef and Cdc42 are likely involved in completely separate processes at different times in maturation. XGef may act as an exchange factor for a yet to be identified Rho GTPase during early maturation.

3. Rho GTPases and oocyte maturation

During oocyte maturation, dynamic changes occur in cytoskeletal and intracellular membrane organization. Rho GTPase inactivating agents, such as large clostridial toxins, have been used to study the importance of Rho GTPases in progesterone signaling and the extensive cytoskeletal reorganization that occurs during maturation and early development (Kato, 1996). Other groups have also used Toxin B to inhibit several Rho GTPases in *Xenopus* oocytes and major cytoplasmic rearrangements

were reported (Just et al., 1994). Their findings, indeed, support the involvement of Toxin B sensitive Rho GTPases during oocyte maturation. However, we did not see any discernable difference in GVBD in oocytes injected with Toxin B. Since our interest lies in early progesterone signaling and meiotic progression up to GVBD, our results suggest that the targeted Rho GTPases are not required for early signaling or GVBD. Cytoskeletal remodeling continues to occur after the first meiotic division (GVBD) and Toxin B-induced morphological abnormalities reported by other groups may have occurred through the disruption of Rho GTPase activity after GVBD. Additional research is required to identify Rho GTPases that mediate early progesterone signaling. Aside from cytoskeletal changes, Ras superfamily GTPases have been shown to be involved in virtually every stage of intracellular membrane transport during oocyte maturation and egg fertilization. Protein transport from the golgi apparatus to the plasma membrane, cell surface protein recycling through endocytic and exocytic pathways and cortical granule translocation comprise several membrane organization processes mediated by Ras GTPases (reviewed in Gruenberg and Clague, 1992; Ferro-Novick and Novick, 1993). Whether XGef influences the activity of a GTPase that influences some of these processes has not been determined, however, further research of XGef-GTPase partners may create a link between progesterone signaling, XGef mediated CPEB activation and membrane trafficking in maturing oocytes.

4. XGef function independent of exchange factor activity

Previous findings suggested that XGef exchange factor activity may be required during oocyte maturation. Overexpression of an exchange deficient mutant (XGef Δ65-234) no longer accelerated the rate of oocyte maturation in response to progesterone, an effect observed with WT XGef overexpression (Martinez et al., 2005). However, deletion of the entire DH domain may have disrupted proper folding of the XGef protein.

A deletion of this size might compromise other functions, such as membrane localization mediated by the PH domain, and the consequences of overexpressing XGef Δ65-234 would no longer be exclusive to exchange factor activity perturbation. To address this issue, we created various XGef DH domain point mutations. Since the DH domain is very well conserved in structure and function, mutation of residues analogous to those that successfully blocked exchange activity in other related GEFs likely disrupted XGef exchange activity. The effect of these mutants on meiotic progression was the same as wildtype, suggesting that exchange activity is not an important component of XGef function during maturation.

Although characterized by their ability to catalyze GDP/GTP exchange and activate targeted GTP-binding proteins, GEFs also possess functions independent of Rho GTPase activation. Kalirin is a Rho GEF involved in external stimuli-induced morphological changes in neurons. Studies of Kalirin function in an epithelial-derived cell line revealed that its activation of Rac1 and PAK during lamellipodia formation was independent of exchange factor activity. Notably, Kalirin overexpression-mediated lamellipodia formation was not affected by dominant negative Rac1 or catalytically inactive PAK. Exchange deficient Kalirin overexpression induced lamellipodia formation comparable to wildtype. Further, this catalytically inactive Kalirin mutant was also able to activate PAK (Schiller et al., 2005). Together, these results suggest that Kalirin can activate PAK without exchange activity. GEF-independent function has also been found with the Rho GEF, Vav. Mammalian Vav is expressed in hemopoietic cells and is involved in lymphocyte development and activation. Pozo and colleagues showed that Vav function was independent of exchange activity during T-cell spreading. Overexpression of Vav wildtype and the exchange mutant, Vav L213A, both induce T-cell spreading. Interestingly, the Vav R695L mutation, which disrupts a Src Homology 2 (SH2) domain function, blocked Vav-mediated spreading. These findings suggested that

Vav binding with tyrosine phosphorylated protein-binding partners through its SH2 domain, may be required for Vav function. Adaptor function and recruitment of additional factors may have been the function required for Vav-mediated T-cell spreading (Pozo et al., 2003). XGef may also influence oocyte maturation by recruiting factors to CPEB to mediate its activation. Additional research is required to determine if XGef functions as an adaptor protein.

Chapter III. MAPK activity is required for CPEB phosphorylation and binds directly to XGef.

A. Introduction

The early events that directly influence CPEB activation upon progesterone-induced maturation are not well defined. To date, the kinase that phosphorylates to activate CPEB has not been identified. Interestingly, CPEB phosphorylation and timely meiotic progression require MAPK activity. Shortly after progesterone stimulation, a low level of phospho-active MAPK can be detected (Keady et al., 2007). This small pool of active MAPK, which exists independently of the Mos/MEK/MAPK cascade, may influence CPEB activation. MAPK can phosphorylate His-CPEB *in vitro* on residues T22, T164, S184 and S248. Additionally, XGef immunoprecipitates exhibit kinase activity towards His-CPEB in vitro. Immunoblot analysis revealed that phospho-active MAPK co-precipitates with endogenous XGef. Since the precipitated kinase activity is sensitive to UO126, a MEK/MAPK inhibitor, His-CPEB phosphorylation by XGef immune complexes is very likely due to MAPK activity (Keady et al., 2007). Combined, these results suggest that MAPK may phosphorylate CPEB to influence activation *in ovo*.

XGef function is also essential for CPEB activation. Overexpression of a mutant of XGef that is CPEB-binding deficient (XGef 65-360) severely attenuates CPEB activation and rate of oocyte maturation, which suggests that XGef must interact with CPEB for proper activation and timely progression of meiosis (Martinez et al., 2005). Since XGef also interacts directly with MAPK *in vitro* and endogenous XGef co-immunoprecipitates MAPK in extracts prepared from maturing oocytes, we proposed that an XGef/ MAPK/ CPEB complex may exist *in ovo* (Keady et al., 2007). The formation of this complex may facilitate CPEB Ser 174 activating phosphorylation. To investigate the functional significance of XGef/ MAPK/ CPEB complex formation with

regards to CPEB activation, our intention was to observe the consequences of disrupting the XGef/MAPK interaction.

Docking interactions, which exist between MAPK and MAPK interacting proteins, serve as a mechanism for enforcing MAPK signaling fidelity. MAPK interactors often contain a MAPK Docking Domain (D domain) and Dock site for Erk binding FXFP motif (DEF), which are recognized by the Docking Groove in MAPKs. The first MAPK docking site was discovered in Ste7, an *S. cerevisiae* MAPKK (Bardwell et al., 1996). Kinase to substrate interactions are typically considered transient in nature. A surprisingly stable interaction between Ste7 and Fus3, a MAPK, depended on a non-conserved amino terminal extension of Ste7, later named the MAPK Docking Domain (D domain) (Bardwell et al., 1996). The D domain consensus $[K/R]_{2-3}\ X_{1-6}\ [L/I]\ X\ [L/I]$ consists of a cluster of basic residues with turn-forming spaces followed by two or three hydrophobic residues, spaced every other amino acid. Docking mediated interactions involve electrostatic contacts between D domain basic residues and two closely spaced acidic patches within the docking groove of MAPK. D domains have been shown to enforce MAPK-substrate specificity, in which MAPKs from different subgroups prefer distinct D domains. For instance, P38 MAPKs bind D sites containing more consecutive positively charged amino acids. Thus, in addition to tethering capabilities, D domains contribute to substrate specificity (reviewed in Sharrocks et al., 2000; Bardwell et al., 2009).

In addition to the D domain, the Dock site for Erk binding FXFP (DEF) serves as an additional motif that mediates MAPK binding. The DEF motif was discovered through *lin-1* gain of function allele studies in *C. elegans*. Mutations that caused the gain of function phenotype occurred within the *lin-1* gene that encoded the DEF. FXFP mutated Lin-1 transcription factors were no longer negatively regulated by ERK, due to disrupted binding, which led to the synthesis of a gene product that caused lethality (Jacobs et al., 1999). DEF function has been examined extensively through mutagenesis experiments,

which support the importance of the FXFP motif in mediating MAPK binding. Aside from MAPK docking function, the DEF FXFP residues also play a key role in influencing the phosphorylation site selection when multiple S/TP phospho-acceptor consensus sites are present. The Elk-1 transcription factor is phophorylated on Ser 383 by ERK2, however, an Elk-1 mutant lacking its FXFP motif (FQFP) does not undergo ERK2 phosphorylation (Yang et al., 1998a; Yang et al., 1998b). Unlike D domains, which can reside around 100 amino acids upstream or downstream of the phosphoacceptor site, FXFP motifs are almost always 10-20 residues downstream (Sharrocks et al., 2000). The location of DEFs relative to phosphorylation sites may influence kinase site preference on targeted substrates.

MAPK substrates can have a single or multiple D domains and DEF motifs. In some cases, the D domain and DEF motifs function as distinct units. In other cases, they may be functionally interchangeable, forming a modular system that affects MAPK to effector binding (Dimitri et al., 2005). There are numerous theories behind the modularity of D domains and DEF motifs. The D domain may contribute to MAPK:substrate interactions by recruiting the substrate, while the DEF motif stabilizes the interaction. Alternatively, both domains may work together to recruit substrates while the DEF motif enhances the MAPK: substrate interaction. D domain and DEF mutagenesis data suggest that the presence of both is likely to boost binding affinities, while mutation of either domain may or may not inhibit MAPK interaction with its substrate (reviewed in Sharrocks et al., 2000). XGef contains a putative D domain (265-275aa) and DEF (289-291aa), which may potentially mediate MAPK binding.

B. Materials and Methods

1. Oocyte culture

Stage VI oocytes from non-primed *Xenopus laevis* females were collected after enzymatic digestion with 1x Barth's saline containing 0.2 mg/ml Collagenase (type IA, Sigma) and 0.6 U/ml dispase (Roche). Oocytes were then washed and cultured in 1x Barth's. When applicable, oocytes were stimulated with 100uM progesterone (4-Pregnene-3,20-dione, Sigma). For MAPK inhibition experiments, oocytes were incubated 1x Barths containing 50 mM PD98059 (Cell Signal), solubilized in DMSO (Sigma).

2. Oocyte injections

pSP6-HA-XGef WT, pSP6-HA-XGef LA, pSP6-HA-XGef FA and pSP6-HA-XGef LAFA were linearized with Xba1 restriction enzyme. Linear products were used as templates in an *in vitro* transcription reaction (mMessage mMachine Kit, Ambion). Oocytes were injected with 46 nl of 400 ng/μl HA-XGef WT, HA-XGef LA, HA-XGef FA or HA-XGef LAFA mRNA to synthesize exogenous proteins.

3. Oocyte Extracts and Immunoblot Analysis

Oocytes were lysed in 3 μl/oocyte extract buffer containing 1x PBS and protease inhibitors. For PMAPK detection, oocytes were lysed in 3 μl/oocyte extract buffer containing 20 mM Hepes pH 7.5, 100 mM NaCl, 10 mM β-glycerophosphate, 5 mM EGTA, 5 mM $MgCl_2$, 100 mM sucrose, 2 mM $NaVaO_4$, 20 mM NaF, 1mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiotheritol [DTT], 2 μM okadaic acid and protease inhibitor cocktail. For *ex vivo* pulldown assays, oocytes were homogenized in 25 mM Tris pH 7.4, 50 mM NaCl, 1 mM DTT, 0.1% Triton-X, 1 mM PMSF and protease

inhibitors (5ng/μl leupeptin and 10ng/μl each chymostatin, pepstatin, and aprotinin). Once thoroughly homogenized, lysates were centrifuged twice at 13,000 rpm and clarified supernantants were boiled with 2x SDS-Sample loading buffer. Denatured extracts were loaded onto a 10% SDS-Polyacrylamide Gel (25 μg total protein per lane). Resolved proteins were transferred onto PVDF membrane and blocked in 5% Milk/TBST. Hrp and Alexa-fluor-conjugated secondaries were detected with chemilumenescence (Perkin Elmer) and laser scanning at 680 nm (Licor), respectively.

4. Antibodies

HA and His-monoclonal primary antibodies were used at 1:2,000 and 1:1,000 dilutions, respectively (Covance, New England Biolabs). The MAPK primary rabbit polyclonal antibody was used at a 1:1,000 dilution (Cell Signal). Hrp-conjugated anti-mouse and Alexa-fluor 680 anti-mouse secondaries were used to detect HA and His primary antibodies (Santa Cruz). Alexa-fluor 680 conjugated goat anti-rabbit secondary recognized MAPK primary antibody (Molecular Probes).

5. Plasmid Constructs

For the pSP6-HA XGef L273/275A and pGex2T XGef L273/275A constructs, we used the following primers for site directed mutagenesis (Stratagene): Forward: 5'-GCT ATA TCC GGG AGG GTT GGG CCT CAC TTG TAC CAC AGA GTG-3' and Reverse: 5'-CCA CTC TGT GGT ACA AGT GAG GCC CAA CCC TCC CGG ATA TAG C-3'. For the pSP6-HA XGef F289/291A and pGex2T XGef F289/291A plasmids, primers: Forward: 5'-GTT GAA GCA GCC AAT GTT CGC CTT GTT CTC TGA TGT CCT CG-3' and Reverse: 5'-CGA GGA CAT CAG AGA ACA AGG GCA ACA TTC GCT GCT TCA CC-3' were used. The parental vectors containing XGef WT ORF were used and the

PCR conditions were: 95°C for 30 seconds; then 18 cycles of 95°C for 30 seconds, 55°C for 1 min and 68°C for 6 minutes; and finally an additional 10 minutes at 72°C. For the pSP6-HA XGef L273/275A / F289/291A and pGex2T XGef L273/275A / F289/291A quadruple point mutants, the L273/275A mutants were used as a template for site directed mutagenesis PCR. The same F289/291A primers were used to introduce the indicated mutations. PCR conditions were also identical to those described above. For the pGEX2T-XGef ΔD Domain construct the following primers were used: Forward: 5'-CGC AGA TCT TCA GAG CAG GGA GCA ACT CC-3' and Reverse: 5'-CGC TAG CAT ATG GCA TGT CAC ATT CCA GCC CC-3'. pGEX2T-XGef WT template was used in a PCR reaction with the following conditions: 95°C for 1 minute; then 35 cycles of 95°C for 1 min, 56°C for 1 minute and 72°C for 2 minutes 30 seconds; and finally an additional 10 minutes at 72°C. *Pful* DNA polymerase was used for each PCR reaction (Stratagene).

Dpn1 enzyme (New England Biolabs) was added to each PCR reaction to digest parental template. PCR products were then phenol and chloroform extracted and ethanol precipitated. Precipitated PCR products were transformed into XL1-Blue *E.coli* (Stratagene) for plasmid propagation. Plasmids were confirmed by sequencing.

6. Recombinant protein expression and purification

a. His-CPEB

BL21DE cells were transformed with 50 ng of pET-His-CPEB plasmid. The 1 ml transformation solution was combined with 9 ml LB/ampicillin and cultured overnight at 37°C and shaking at 225 rpm. The overnight culture was then used to inoculate 1 L LB/Ampicillin and grown again at 37°C until an $O.D._{600}$ of 0.600 was reached. His-CPEB expression was induced with 400 μl of 1 M IPTG and incubation at 28°C, for 3.5 hours.

After induction, cells were pelleted at 5,000 rpm and 4°C, using a Sorvall SS-34 rotor (200 ml culture/pellet). For purification, pellets were resuspended in Binding Buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH 7.9, 0.25% Tween 20) and sonicated (20-30% output) three times for 30 second intervals. Insoluble proteins were pelleted at 12,000 rpm at 4°C (Sorvall SS-34) for 12 minutes. Clarified supernatants were incubated with 300 μl Ni^{+}-NTA nickel slurry, pre-washed in Binding Buffer, for 3 hours at 4°C. Nickel beads were washed three times with 5 ml Wash Buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH7.9) to remove nonspecifically bound proteins. Ni^{+}-NTA Nickel bead-bound His-CPEB was stored on ice until further use.

b. GST-XGef and GST-XGef mutants

BL21DE cells were transformed with 10-50 ng of pGEX2T (GST), pGEX2T-XGef WT, pGEX2T-XGef LA, pGEX2T-XGef FA or pGEX2T-XGef LAFA plasmid and grown overnight in 10 ml of LB/Ampicillin at 37°C and shaking at 225 rpm. The next day, overnight cultures were expanded 100 fold and grown at 37°C. Once the culture reached an $O.D._{600}$ of 0.600, cells were induced to express the GST-tagged protein with 400 μl of 1 M IPTG and grown at 30°C for 3.5 hours. After induction, cells were pelleted (Sorvall SS-34 rotor) for 10 minutes, at 5,000 rpm and 4°C. Cells were resuspended in 1x PBS, repelleted (250 ml culture/pellet) and stored at -80°C. For purification, pellets were resuspended in 15 ml 1x PBS/0.1% Triton-X and sonicated three times for 30 second intervals. After sonication, lysates were spun at 12,000 rpm at 4°C (sorvall SS-34). Cleared lysates were combined with 200 μl glutathione slurry, which was pre-equilibriated with 1x PBS/0.1% Triton-X, and incubated with end over end rotation at 4°C for 2 hours. After affinity purification, the beads were washed three times with 1 ml of 1x

PBS/0.1% Triton-X. Beads were stored on ice in the same buffer in a 1:1 buffer to bead slurry.

c. His-MAPK

BL21DE cells were transformed with 50 ng of pET-His-MAPK plasmid. The 1 ml transformation solution was combined with 9 ml LB/ampicillin and cultured overnight at 37°C and shaking at 225 rpm. The overnight culture was then used to inoculate 1 L LB/Ampicillin and grown again at 37°C until an $O.D._{600}$ of 0.600 was reached. His-MAPK expression was induced with 400 μl of 1 M IPTG and incubation at 28°C, for 3.5 hours. After induction, cells were pelleted at 5,000 rpm and 4°C, using a Sorvall SS-34 rotor (200 ml culture/pellet). For purification, pellets were resuspended in Binding Buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH 7.9, 0.25% Tween 20) and sonicated (20-30% output) three times for 30 second intervals. Insoluble proteins were pelleted at 12,000 rpm at 4°C (Sorvall SS-34) for 12 minutes. Clarified supernatants were incubated with 300 μl nickel slurry, pre-washed in Binding Buffer, for 3 hours at 4°C. Nickel beads were washed three times with 5 ml Wash Buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH7.9) to remove nonspecifically bound proteins. Nickel-bead bound His-MAPK was eluted with 200 μl of 250 mM Imidazole, 0.5 M NaCl and 20 mM Tris pH 7.9, at room temperature for 30 minutes. Eluted proteins were passed through a centricon column (30,000 dalton molecular weight cut-off) for buffer exchange and concentration of eluted His-MAPK. Solubilized His-MAPK was kept in storage buffer containing 2 mM $MgCl_2$, 25 mM Hepes, 150 mM NaCl and 10% glycerol and frozen at -80°C.

7. *In vitro* protein interaction assay

His-MAPK (2.5 μg/μl) was solubilized in Protein Interaction Buffer containing 20 mM Hepes pH 7.5, 150 mM NaCl, 5 mM EGTA pH 8.0, 5 mM $MgCl_2$, 0.1 M sucrose, 10

mM β-Glycerophosphate, 1 mM DTT, 5% glycerol, 1% NP-40, 1x Protease inhibitor cocktail, and 0.5% BSA. Bead-bound prey proteins were pre-washed with the same buffer. Glutathione bead-bound GST (5 μg), GST-XGef WT (15 μg) or GST-XGef dock mutants (15 μg) were incubated with 4 μg of His-MAPK. Binding reactions took place at 4C for 2 hours with end over end rotation. Bead bound proteins were washed three times in 1 mL of Wash Buffer (Protein Interaction Buffer containing 1% Triton X). Once nonspecifically bound protein was removed, protein complexes were denatured and eluted with 20 μl of 2xSDS-BME sample loading buffer and boiling.

8. *Ex vivo* protein interaction assay

Glutathione bead-bound GST (5 μg), GST-XGef WT (15 μg) or GST-XGef Docking mutants (15 μg), were incubated in immature or GVBD oocyte (n=30) extracts (7.5 μg/μl) at 18°C for 2 hours. Non-specifically bound proteins were washed away with buffer containing 50 mM Tris pH 7.4, 100 mM NaCl, 1mM DTT and 1% Triton-X. Proteins were eluted with 2x SDS-Sample loading buffer and boiling. Eluates were resolved by 10% SDS-PAGE and blotted onto PVDF membrane for PMAPK immunoblot analysis.

9. *In vitro* His-CPEB phosphorylation assay

For the *in vitro* His-CPEB phosphorylation assay, oocyte lysates (90 oocytes pre reaction) were combined with 3 μCi [γ-^{32}P]ATP and 2.5 μg Nickel bead-teathered His-CPEB, pre-equilibriated in Phosphorylation Buffer containing 10 mM Hepes pH 7.5, 10 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, 1 μM okadaic acid, 1 mM H-89 PKA inhibitor, 50 μM adenosine triphosphate and protease inhibitors. Reactions were conducted at room temperature for 30 minutes and stopped with 25 μl SDS sample loading buffer and

boiling. *In vitro* His-CPEB phosphoryation was analyzed by 10% SDS-PAGE and autoradiography.

C. Results

1. MAPK activity influences CPEB phosphorylation and may catalyze priming phosphorylations to facilitate activation.

CPEB phosphorylation upon meiosis resumption requires MAPK (Keady et al., 2007). MAPK also phosphorylates His-CPEB *in vitro*, but not S174, which suggests that MAPK is not responsible for catalyzing the activating phosphorylation on CPEB (Keady et al., 2007). Considering that MAPK activity is required for CPEB activation, we focused our attention on the potential role of the identified phospho-residues and proposed that CPEB undergoes priming phosphorylations catalyzed by MAPK. To test our CPEB priming phosphorylation hypothesis, we stimulated oocytes with progesterone and prepared extracts prior to the time of activation, when priming phosphorylations are likely to occur. Typically, CPEB is activated around 3 hours after progesterone stimulation. However, His-CPEB was also phosphorylated by extracts prepared from oocytes that had resumed meiosis for 30 minutes and 1 hour (Figure 11, ^{32}P). Equivalent amounts of His-CPEB were added to each reaction (Figure 11, CS). Further, the presence of phospho-active MAPK coincided with early His-CPEB phosphorylation, suggesting that MAPK is active when His-CPEB is phosphorylated during this early time in meiosis (Figure 11, IB: PMAPK). If MAPK activity directly influences CPEB phosphorylation during the first hour of maturation, then MAPK inhibition should block these early phosphorylation events. Indeed, progesterone stimulated oocytes pre-treated with MEK inhibitor (PD98059) lost all kinase activity towards His-CPEB *in vitro* (Figure 12A, ^{32}P). As previously reported, His-CPEB was phosphorylated by extracts prepared three hours after progesterone stimulation. (Figure 12A, compare lane 4 and 7). Combined, these results suggest that in addition to influencing CPEB activation, MAPK activity also mediates the earlier, potentially priming, phosphorylation event. Further, the

three hour CPEB phosphorylation event may have been prevented because the preceding phosphorylation event could not occur in the absence of MAPK activity. Oocytes were collected in parallel and probed for phospho-active MAPK (Figure 12B, IB: PMAPK). The PD98059 MEK inhibitor effectively blocked MAPK activation in treated oocytes (Figure 12B, compare lanes 3, 5 and 7 to 2, 4 and 6).

2. XGef interacts directly with MAPK *in vitro* but not through conventional MAPK dock site-mediated binding.

Keady and colleagues showed that XGef immunoprecipitates contain kinase activity towards His-CPEB. Further, the kinase activity was sensitive to a MAPK inhibitor, UO126. MAPK was present in the immunoprecipitated complex, suggesting that XGef and MAPK are present in a complex *in ovo* (Keady et al., 2007). Previous results have also shown that XGef and MAPK activities influence CPEB activation and timely progression of meiosis (Reverte et al., 2003; Martinez et al., 2005; Keady et al., 2007). To further our understanding of how both factors affect CPEB activity, we pursued the possibility that XGef and MAPK interact directly by performing an in vitro protein interaction assay. Further, we mutagenized putative MAPK docking sites, located in the XGef Pleckstrin Homology (PH) domain, to examine the significance of XGef/MAPK binding in oocytes undergoing meiotic progression.

The D domain ($[K/R]_{2-3}\ X_{1-6}\ [L/I]\ X\ [L/I]$) and FXF motif (F-X-F) are two distinct sites within numerous MAPK binding proteins that mediate MAPK interaction. MAPK activators, effectors, and scaffolding proteins often contain at least one MAPK docking site. When both sequences are present, each function to facilitate MAPK binding. Amino acids 265-275 within the XGef PH domain follows the MAPK D domain consensus sequence. An FXF motif (289-291aa) also resides carboxy-terminal to the putative D

domain (Figure 13, top schematic). Electrostatic interactions between the D domain basic residues and the acidic patches within the docking groove on MAPK mediate MAPK to protein interactor binding. We therefore created the LA mutant, in which alanine substitutions were made on leucines 273 and 275, to perturb D domain function. Similarly, both phenylalanines of the FXF motif were replaced with alanines to generate the FA mutant. A mutant harboring both D domain and FXF motif alanine substitutions, designated LAFA, was also made (Figure 13, middle schematic). To determine if the putative D domain and FXF motif indeed functioned as MAPK docking sites, we performed an *in vitro* protein interaction assay using glutathione bead-bound GST-XGef wild type or the assorted GST-tagged docking site mutants combined with soluble His-MAPK. After extensive washing to remove non-specifically bound proteins, we detected the presence of bound His-MAPK through His immunoblot analysis. His-MAPK bound to GST-XGef WT and the indicated GST-XGef dock mutants (LA, FA and LAFA) with equal affinity. His-MAPK did not bind to the GST tag alone (Figure 14). We next decided to use an *ex vivo* approach to examine the interaction between endogenous MAPK, in extracts prepared from maturing oocytes, and bacterially expressed GST-XGef WT, LA, FA and LAFA. Immature or progesterone stimulated oocytes were lysed and extracts were combined with glutathione bead-bound GST-XGef WT, LA, FA or LAFA. Precipitated proteins were probed with MAPK antibody. Wildtype and dock site mutants of XGef precipitated equivalent amounts of endogenous MAPK (Figure 15). Together, these results suggested that the mutagenized sites did not function to facilitate MAPK binding. Alternatively, the mutations may not have sufficiently perturbed dock site function. We next deleted the entire XGef putative D domain (XGef-Dock Deletion) and examined this mutant for disrupted MAPK binding. Glutathione bead-bound GST-XGef WT and GST-XGef Dock Deletion proteins were incubated with soluble His-MAPK. His-immunoblot analysis revealed that GST-XGef WT and GST-XGef Dock Deletion possessed similar

MAPK binding affinities (Figure 16). Analysis of our assorted MAPK docking domain mutants of XGef collectively suggested that the putative D domain and FXF motif do not function to facilitate MAPK binding. Other regions of XGef must mediate the XGef/MAPK interaction. Analysis of meiotic progression in oocytes overexpressing HA-XGef WT, LA, FA and LAFA revealed that the docking mutants did not affect progesterone-induced maturation (Figure 17). However, this finding is not surprising since the alanine point mutations did not disrupt MAPK binding. The MAPK binding sites of XGef have yet to be identified along with the consequences of blocking the XGef/MAPK interaction during meiotic progression.

D. Figures

Figure 11. Early CPEB phosphorylation upon progesterone stimulation.

Oocytes (n=4) were stimulated with progesterone and lysed at the times indicated (Minutes+PG). Extracts were combined with nickel bead-bound His-CPEB and γ^{32}-P [ATP] for an *in vitro* His-CPEB phosphorylation assay (P32). His-CPEB addition to each reaction was quantified by coomassie staining (CS). Parallel extracts were probed with PMAPK antibody (IB: PMAPK).

Figure 11.

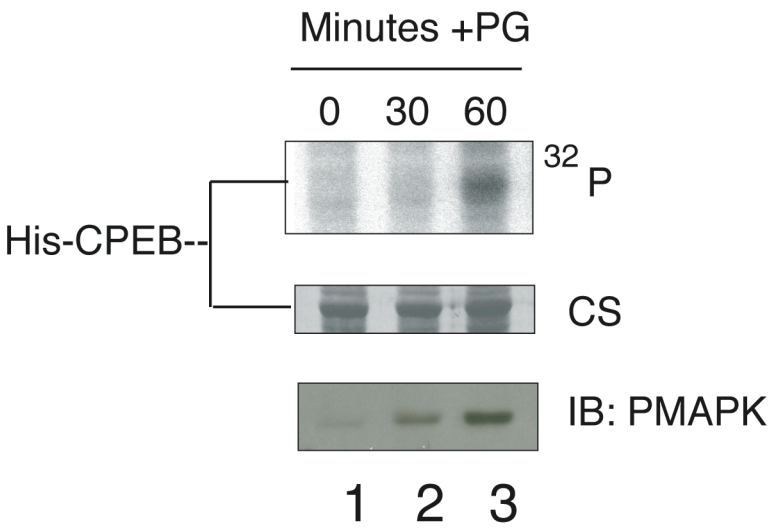
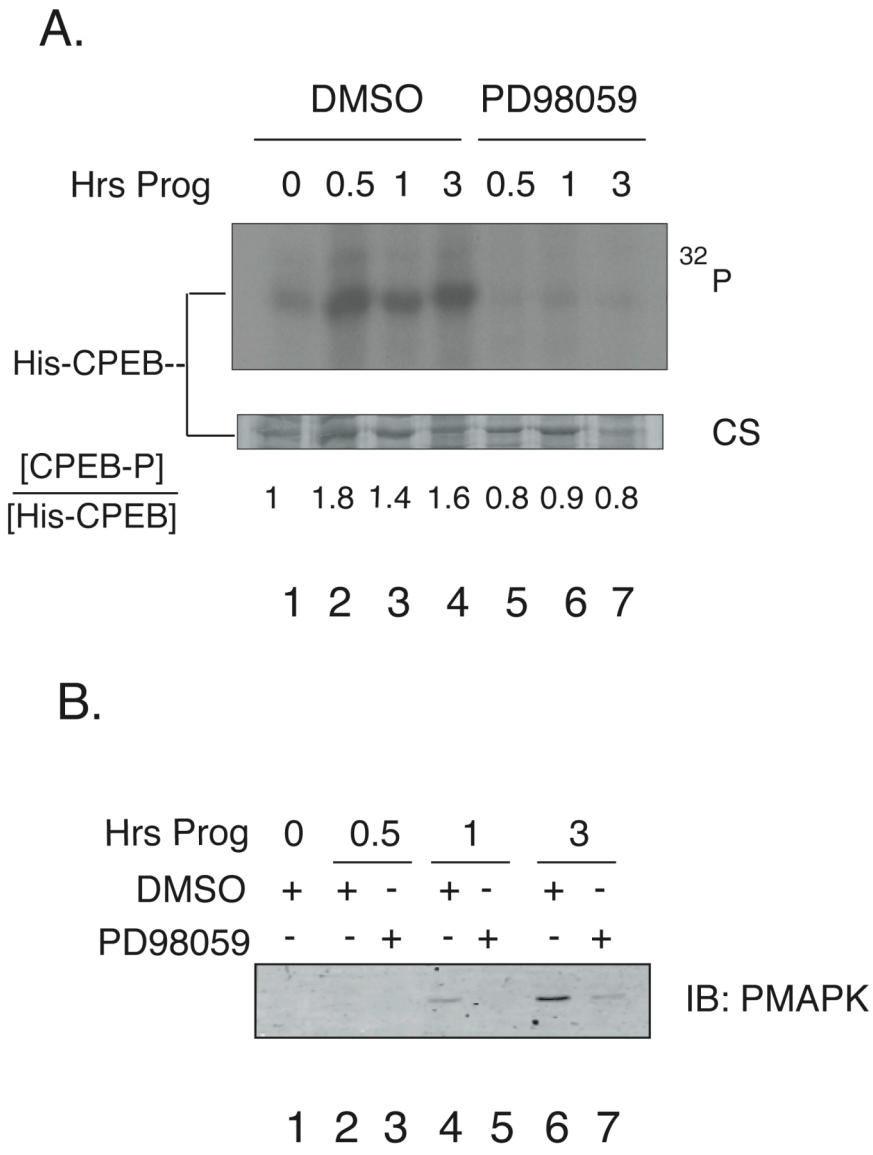


Figure 12. Early CPEB phosphorylation requires MAPK activity.

A. Oocytes were pre-incubated in MAPK inhibitor, PD98059 (50mM), or DMSO control followed by progesterone stimulation. Oocytes were then lysed at the times indicated for an *in vitro* His-CPEB phosphorylation assay (P32). His-CPEB addition to each reaction is shown (CS). B. Parallel extracts were analyzed by PMAPK immunoblotting (IB: PMAPK).

Figure 12.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PD98059
Hrs Prog
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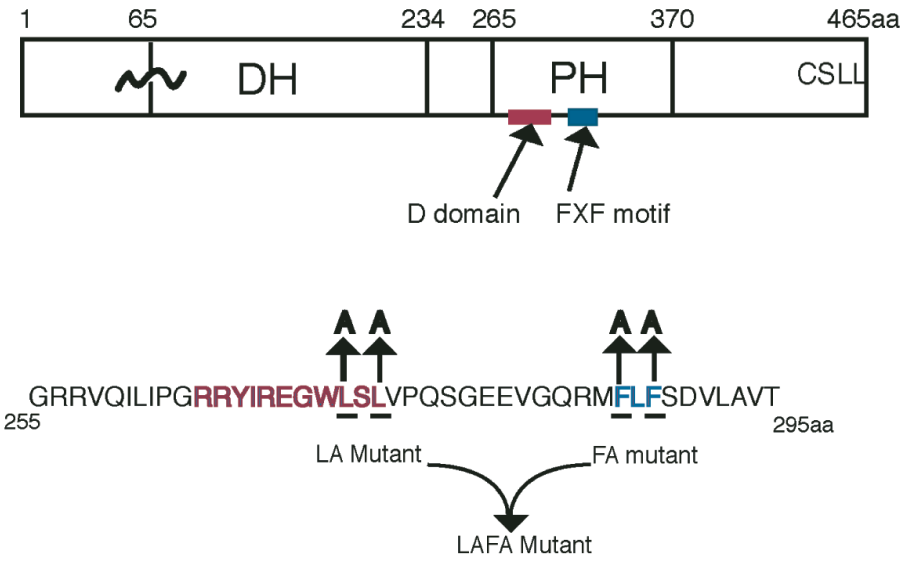
32P
His-CPEB--
CS
[CPEB-P]
[His-CPEB]
1 1.8 1.4 1.6 0.8 0.9 0.8
1 2 3 4 5 6 7
B.
Hrs Prog 0 0.5 1 3
DMSO + + - + - + -
PD98059 - - + - + - +
IB: PMAPK
1 2 3 4 5 6 7

Figure 13. XGef D domain and FXF motif point mutation and alignment schematics. The top schematic of XGef shows the coiled coil (squiggle), Dbl Homology (DH) and Pleckstrin Homology (PH) domains as well as the locations of the putative D domain (red box) and FXF motif (blue box). Amino acids 255 to 295 are shown in the middle schematic with the D domain in red and the FXF motif in blue. Leucine and phenylalanine mutations to alanine for D domain and FXF motif mutagenesis, respectively, are shown (arrows). Clustalw aligned sequences from *X. laevis* and the indicated homologs are shown in the bottom schematic. The red and blue lines indicate the locations of the putative D domains and FXF motifs, respectively. Astericks (*) designate conserved residues. Stacked dotes (:) designate conservative substitutions.

Figure 13.

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*::.::* .** ******* ** .* * * :**:****:*  :
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D Domain FXF motif

Figure 14. GST-XGef D domain and FXF motif mutants bind His-MAPK *in vitro*. Glutathione bead-bound GST XGef WT and assorted MAPK docking domain mutations of XGef were incubated with His-MAPK in an in vitro protein interaction assay (Glutathione PD, IB: His). Equivalent amounts of His-MAPK and GST-tagged proteins were added to each reaction (input, IB: His and CS).

Figure 14.

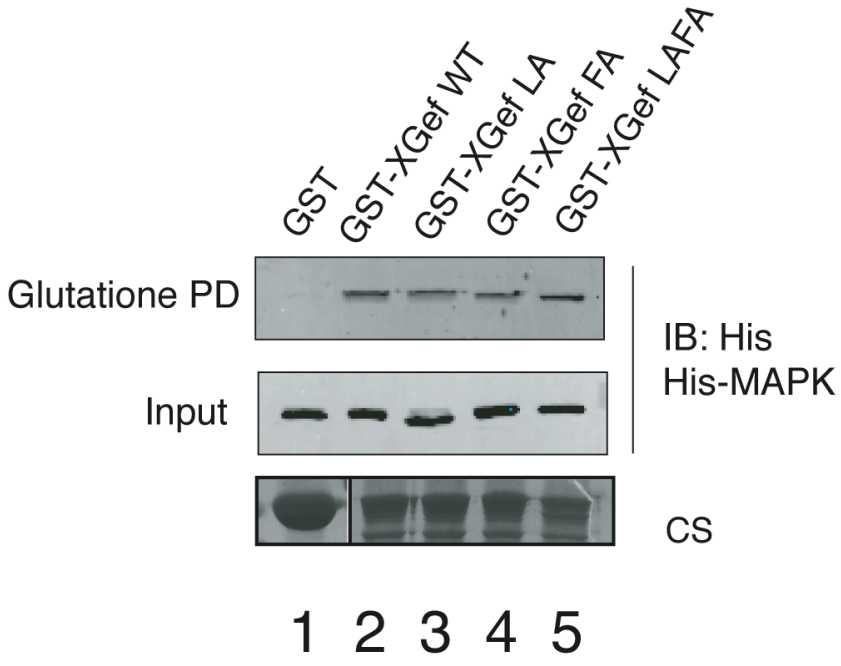

GST
GST-XGef WT
GST-XGef LA
GST-XGef FA
GST-XGef LAFA
Glutatione PD
Input
IB: His
His-MAPK
CS
1 2 3 4 5

Figure 15. GST-XGef D domain and FXF motif mutants associate with MAPK in oocyte extracts. The indicated glutathione immobilized GST-tagged proteins were incubated with immature and progesterone stimulated oocyte extracts. Beads were probed with PMAPK antibody to detect precipitated endogenous phospho-active MAPK (Glutathione PD, IB: PMAPK). Input extracts contained equivalent amounts of PMAPK (Input, IB: PMAPK). Glutathione bead bound proteins are shown (CS).

Figure 15.

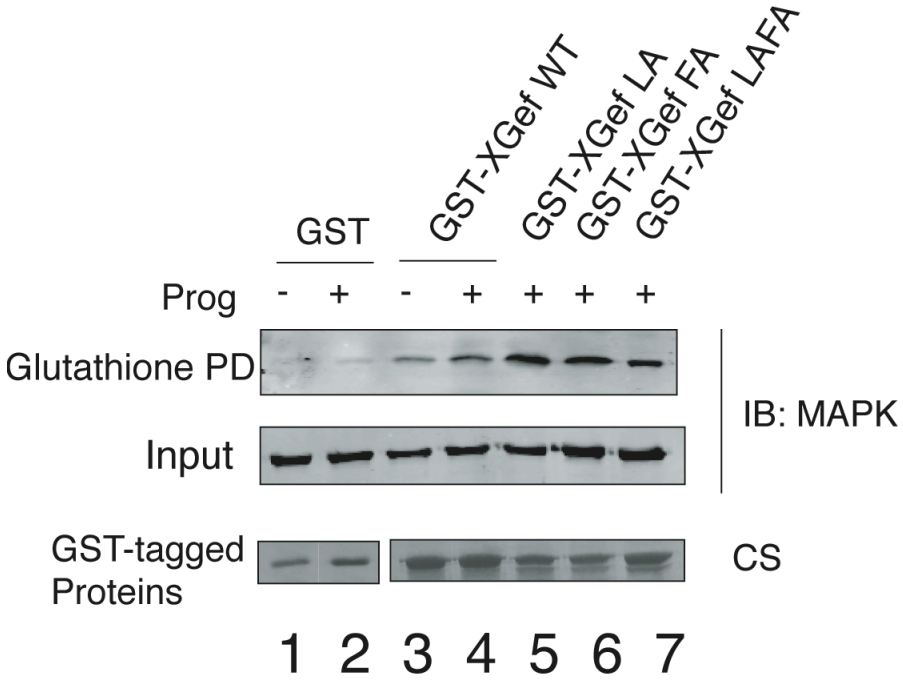

GST
GST-XGef WT
GST-XGef LA
GST-XGef FA
GST-XGef LAFA
Prog
- + - + + + +
Glutathione PD
Input
IB: MAPK
GST-tagged Proteins
CS
1 2 3 4 5 6 7

Figure 16. GST-XGef D domain deletion mutant interacts with His-MAPK *in vitro*. His-MAPK was incubated with bead-bound GST-XGef WT and GST-XGef Dock Deletion in an in vitro protein interaction assay. Bound His-MAPK was detected with His immunoblotting (Glutatione PD, IB: His). Equivalent levels of His-MAPK and GST-tagged proteins were added to each reaction (Input, IB: His and CS).

Figure 16.

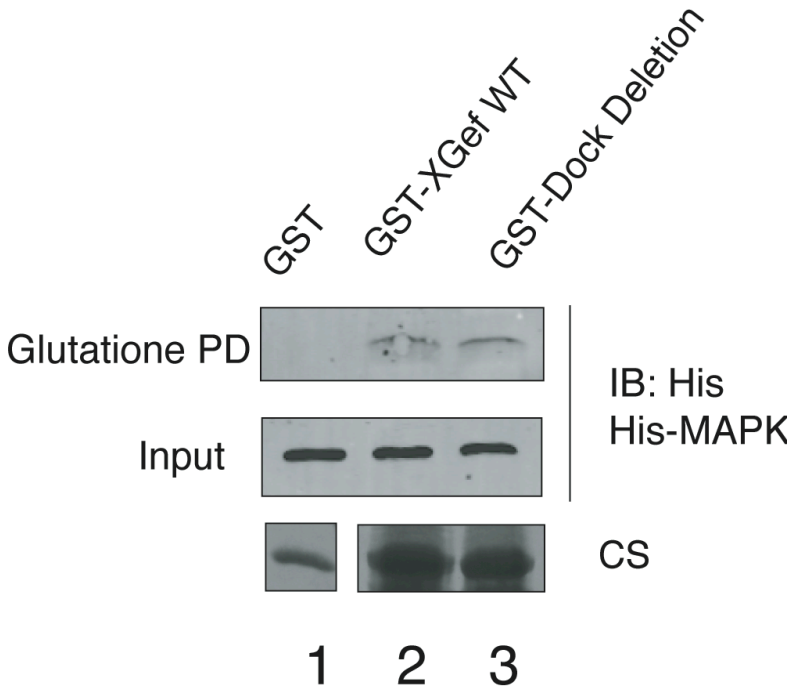
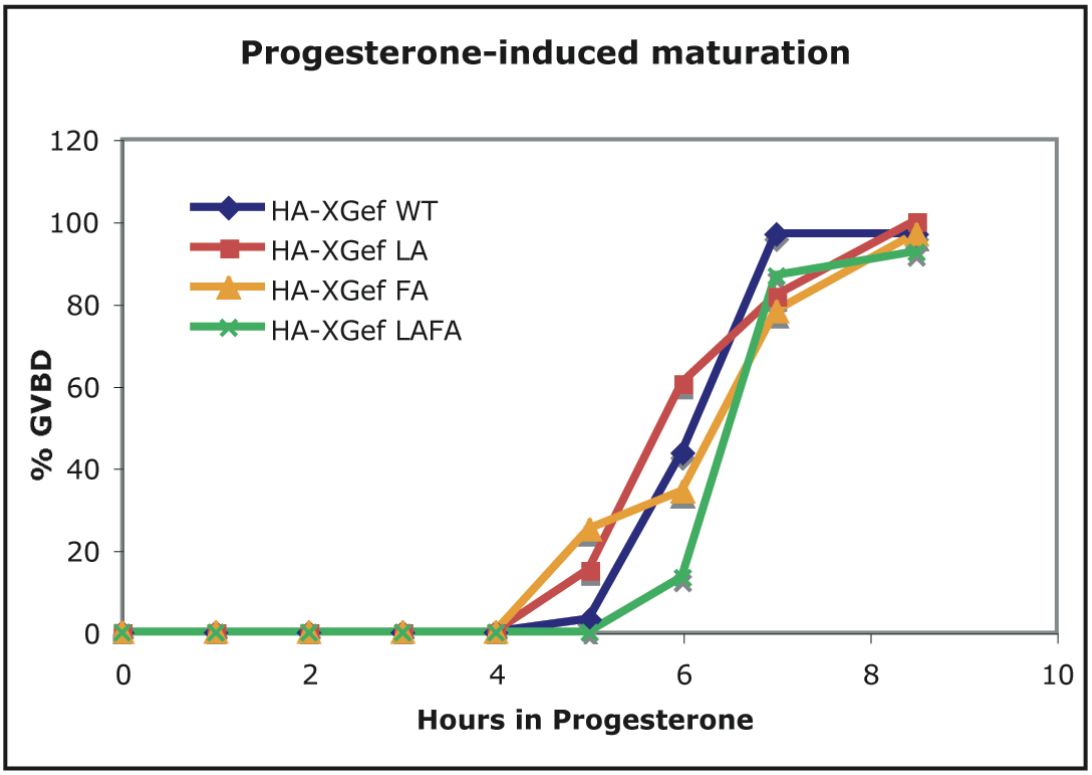

GST
GST-XGef WT
GST-Dock Deletion
Glutatione PD
Input
IB: His
His-MAPK
CS
1
2
3

Figure 17. Maturation time course of oocytes overexpressing HA-XGef LA, FA and LAFA. Oocytes (n=2) were injected with mRNAs encoding HA-XGef WT, LA, FA or LAFA and then stimulated with progesterone to resume meiosis.

Figure 17.


Progesterone-induced maturation
120
100
80
60
40
20
0
% GVBD
HA-XGef WT
HA-XGef LA
HA-XGef FA
HA-XGef LAFA
0
2
4
6
8
10
Hours in Progesterone

E. Discussion

1. Overview

We hypothesize that XGef-mediates MAPK recruitment to CPEB for the catalysis of priming phosphorylations, which facilitate CPEB activation. Here we show that CPEB is phosphorylated during the first hour of oocyte maturation. MAPK is also active at this time. Inhibition of MAPK activity also blocks CPEB phosphorylation. These results suggest that MAPK plays an indirect or direct role in CPEB activation. XGef interacts directly with MAPK. Since XGef interacts with CPEB to facilitate its activation, MAPK recruitment to CPEB by XGef may occur during oocyte maturation. To investigate the functional significance of an XGef/MAPK interaction during meiotic progression, we mutated putative XGef D-domains and FXF motifs, which have been shown to mediate MAPK association in other MAPK interactors. Mutations of the putative MAPK docking sites did not affect XGef interaction with MAPK, which prevented our ability to study the consequences of disrupting the XGef/MAPK interaction.

The XGef putative D domain follows the MAPK docking domain consensus rather loosely. A basic cluster of basic residues are found at the very beginning of the putative XGef D domain, however, only half of the amino acids that should make up the the c-terminal alpha helix have turn forming propensity. According to the consensus, the D domain should also end with hydrophobic residues spaced every other residue, which is not reflected in the XGef putative D domain. The last c-terminal residues of the XGef D domain are hydrophilic with the exception of a hydrophobic serine. Upon comparison of putative D domains of XGef homologs in *Gallus gallus*, *Taeniopygia gattata*, *Xenopus tropicalis*, and *Danio rerio,* a relatively high level of conservation exists among the residues that would constitute the alpha helix (YIREGWL in XGef). Beyond this sequence similarity and complete conservation of the first arginine residue of the

putative D domain, other residues are not conserved. Leucine 275 mutation may not have been effective in disrupting D domain function since this particular residue is not conserved in the other XGef homologs shown in our clustalw alignment (Figure 13, bottom schematic). Similarly, the first Phe of the FXF motif is somewhat conserved. Although mutation of poorly conserved residues within the putative D domain may explain our inability to disrupt function of this MAPK docking site, deletion of the entire D domain did not affect XGef/MAPK binding and strongly suggests that other regions of the XGef protein must mediate MAPK association.

2. Early phase MAPK activity directly or indirectly influences CPEB activation.

The discovery of early-phase MAPK activation during oocyte maturation, which is distinct from the late-phase, Mos-dependent event, supports our priming phosphorylation hypothesis. The vast majority of *Xenopus* oocyte MAPK studies examined late-phase activation, which occurs downstream of CPEB activation. Based on those results alone, CPEB activation dependent on MAPK activity would not make sense. However, Mos depletion experiments revealed that a distinct pool of phospho-active MAPK exists independently of Mos (Fisher et al., 1999; Keady et al., 2007). The concurrent presence of early-phase MAPK activity and CPEB phosphorylation during the first hour of maturation, in conjunction with other data, support the possibility that MAPK may catalyze CPEB priming phosphorylation in vivo. CPEB activation and destruction are both regulated by phosphorylation (Mendez et al., 2002; Setoyama et al., 2007). The potential regulation of CPEB activity through priming phosphorylation adds another component to CPEB regulation through phosphorylation.

Several other lines of evidence suggest that MAPK activity facilitates CPEB activation. Keady and colleagues have shown that MAPK phosphorylates His-CPEB *in vitro* on residues T22, T164, S184 and S248. Since the CPEB S174 activating residue is

not among those phosphorylated in vitro, MAPK is not a likely candidate kinase responsible for catalyzing CPEB activation (Keady et al., 2007). However, since CPEB is phosphorylated during the first hour of maturation and both this phosphorylation event and the subsequent activating phosphorylation are MAPK dependent, it is therefore likely that the physiological significance of the MAPK-directed phosphorylation sites on CPEB may be of priming function for later S174 phosphorylation. We have not analyzed the phoshorylation pattern of His-CPEB phosphorylation by one hour extracts. Identification of phosphorylated residues will allow us to either support or reject the MAPK priming phosphorylation model.

3. Physiological significance of the XGef/ MAPK interaction

GEFs are primarily characterized by GDP/GTP exchange-mediated activation of GTPases. Interestingly, GEFs have also been shown to influence MAPK cascades through exchange activity-dependent and independent means. For example, the Ras GEF, EPAC (Exchange Protein Activated by cAMP), activates JNK (c-jun kinase) in response to high cAMP levels in mammalian HEK-293T cells. EPAC exchange activity is not required for JNK activation. (Hochbaum et al., 2003). Similarly, NET1 (neuroepithelioma transforming gene1) becomes activated in response to DNA damage and activates JNK in an exchange-independent fashion. In addition to SAPKs, GEFs mediate ERK activity as well. In melanoma cells, cAMP levels control CNrasGEF-mediated activation of Ras and ERK1/2 to control menalogenesis (Amsen et al., 2005). Although XGef function and MAPK activity are both involved in the same process of CPEB activation, we have not adequately examined the relationship between XGef function and early-phase MAPK activity. Further research is required to determine if the function of either factor is dependent on the other.

Scaffold proteins typically serve as a platform to which components of a GEF/MAPK signaling cascade can be organized. Unfortunately, direct interactions between GEFs and MAPKs are not well documented. The scaffold JIP4 (JNK-interacting protein 4) recruits Brx GEF, the NFAT5 (Nuclear Factor of Activated T cells 5) transcription factor and P38-MAPK to facilitate P38 activation in immune cells under osmotic stress (Kino et al., 2009). Likewise, JIP2 is a scaffold that recruits Tiam1, Rac1 and components of a MAPK cascade to facilitate P38 activation in mammalian cells (Buchsbaum et al., 2002). MAPKs typically bind to their protein interactors through the recognition of MAPK docking sites (D domain and FXF motif). Mutagenesis of XGef MAPK docking domains suggested that MAPK-binding occurs through other portions of XGef. Interestingly, XGef 65-360 does not interact with MAPK, which suggest that the N-terminal 64 residues and/or C-terminal 105 residues are involved in the interaction. Since XGef 65-360 does not bind CPEB, functional analysis of this particular mutant cannot be used to examine the significance of XGef/MAPK interaction. Further studies will have to be conducted to test the biological significance of XGef and MAPK association and to test the hypothesis that XGef may function as an adaptor protein, recruiting MAPK to CPEB.

Chapter IV. RINGO/CDK1 and XGef function in a common pathway to mediate CPEB activation.

A. Introduction

Translational regulation of maternally deposited mRNAs is vital during *Xenopus* oocyte maturation, a process devoted to the production of fertilizable eggs (Wickens et al., 2000). The translation of many developmentally significant mRNAs, including those that encode Mos and various cyclins is controlled by cytoplasmic polyadenylation. Repressed mRNAs possess a shortened poly(A) tail of 20-50 nucleotides, whereas extension of the poly(A) tail by up to 150 adenines stimulates the recruitment of translation initiation factors and translation ensues. The coordination of cis-elements in the transcript 3'UTR and trans-acting RNA binding factors mediate translation in a polyadenylation-dependent manner (McGrew et al., 1989; Fox et al., 1989; Sheets, 1994; de Moor et al., 2005). The U-rich Cytoplasmic Polyadenylation Element (CPE) is the most extensively studied regulatory element in the 3'UTR involved in the polyadenylation process. The RNA specificity factor, Cytoplasmic Polyadenylation Element Binding Protein (CPEB), binds to the CPE and mediates both translational silencing and activation (Hake and Richter, 1994). In the immature oocyte, CPEB functions as a translational repressor. When the oocyte resumes meiosis in response to progesterone, CPEB becomes activated by serine 174 phosphorylation and stimulates polyadenylation-induced translation (Hake and Richter, 1994; Mendez et al., 2000a).

Progesterone stimulates fully-grown stage VI oocytes to undergo two consecutive meiotic divisions before arresting at metaphase II as an egg awaiting fertilization. Through a non-genomic mechanism, the hormonal cue is likely captured by a classical progesterone receptor, XPR-1 (Tian et al., 2001). Shortly after progesterone exposure, intracellular cAMP levels drop to cause a decrease in PKA activity. A small

pool of MAPK becomes activated and the required synthesis of an atypical CDK activator, Rapid Inducer of G2/M transition in Oocytes (RINGO), also occurs during this early point in maturation (Fisher et al., 1999; Keady et al., 2007; Lenormand et al., 1999; Padmanabhan and Richter, 2006). These events facilitate CPEB activation, which must occur for CPEB-mediated polyadenylation-induced translation of mRNAss that drive meiotic progression. Although the Aurora family kinase, Aurora A, was the initial proposed CPEB activator, mounting evidence suggests that this is not the case. Aurora A is not active during early meiosis at the time of CPEB activation, inhibition of Aurora kinases does not affect oocyte maturation in response to progesterone and CPEB activation occurs in the absence of Aurora A activity (Frank-vaillant et al., 2000; Keady et al., 2007). CPEB activation by an unidentified serine/threonine kinase, mediates c-mos mRNA translation and Mos protein accumulation. As a MAPKKK, Mos initiates the Mos/MEK/MAPK cascade to catalyze robust MAPK activation. The Mos/MAPK pathway is one of several that converge upon MPF activation and germinal vesicle breakdown (GVBD), which marks the completion of the first meiotic division (Sagata et al., 1989; Sheets et al., 1995; Haccard and Jessus, 2002).

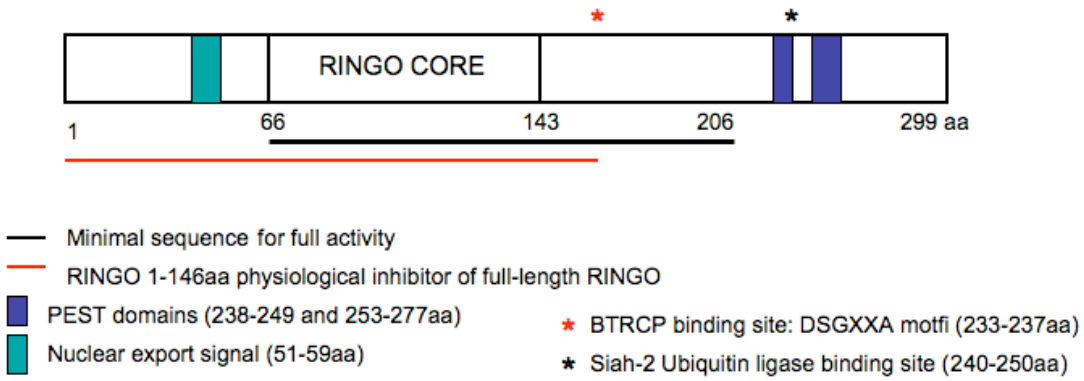
CPEB activation is necessary for the translation of various developmentally significant mRNAs. Although the identity of the kinase that catalyzes CPEB Ser 174 activating phosphorylation remains controversial, several factors have been shown to facilitate the activation event. XGef, a putative Rho family guanine nucleotide exchange factor, was identified as a CPEB-binding protein through a yeast two-hybrid screen. XGef activity is required for CPEB activation. XGef perturbation with activity neutralizing antibodies blocks CPEB activation, c-mos polyadenylation and GVBD (Reverte et al., 2003). Conversely, XGef overexpression enhances these processes. XGef is also a component of an mRNP complex containing c-mos mRNA and CPEB (Martinez et al., 2005). Although XGef function during maturation does not depend on exchange factor

activity, disruption of the XGef-CPEB interaction abrogates CPEB activation, suggesting that XGef influence requires CPEB-binding. CPEB activation also requires MAPK activity. Maturing oocytes pre-treated with the MEK/MAPK inhibitor, UO126, do not exhibit kinase activity towards His-CPEB. c-mos polyadenylation and Mos synthesis are also severely compromised in MAPK-inhibited oocytes. MAPK also phosphorylates His-CPEB *in vitro* on residues T22, T164, S184 and S248 (Keady et al., 2007). Shortly after progesterone stimulation, a low level of phospho-active MAPK arises independently of Mos synthesis. This early MAPK activity may facilitate CPEB activation through the catalysis of phosphorylations that prime CPEB for later activation by a yet to be identified kinase. MAPK also co-immunoprecipitates with endogenous XGef in maturing oocytes. The activities of XGef and MAPK may be coordinated by formation of a complex with CPEB to facilitate activation (Keady et al., 2007). Lastly, the most recent factor implicated in CPEB activation is Rapid Inducer of G2/M in Oocytes (RINGO), which is absolutely required for CPEB activation and progesterone-induced maturation in *Xenopus* oocytes (Ferby et al., 1999; Padmanabhan and Richter, 2006). The mechanism by which RINGO influences CPEB activity is under investigation.

RINGO was isolated in two separate screens for gene products that influenced the G2/M transition and functions as an atypical CDK activator but does not possess any similarity to Cyclins (Ferby et al., 1999; Lenormand et al., 1999). However, the RINGO core, which is required for CDK binding and activation, consists of a stretch of approximately 79 amino acids that form an alpha helical structure similar to the Cyclin Box, which mediates the CDK/Cyclin interaction. The similar secondary structure of the RINGO core and Cyclin Box may explain why RINGO can bind to and activate CDK, despite structural dissimilarity compared to Cyclins. RINGO also has a nuclear export signal (NES) on residues 51-59 and two PEST motifs (238-249aa, 253-277aa). Besides the well-conserved RINGO core, the overall sequence homology of RINGO family

proteins is rather low (Dinarina et al., 2005). Despite sharing only 51-67% sequence identity, a great deal of functional conservation exists among RINGO homologs (Cheng et al., 2005).

Figure 18. RINGO protein domains and motifs



Currently, six mammalian RINGO homologs have been identified. RINGO A1 activates both CDK1 and CDK2 and is highly expressed in mouse testis to mediate G2/M transition in male gametes. RINGO A2 is expressed in mouse and human liver and testis and also mediates cell cycle progression through CDK1 and CDK2 activation. Human RINGO A2 overexpression in *Xenopus* oocytes also triggers a rapid GVBD response like its *Xenopus* counterpart. Similarly, Xenopus RINGO A1 can trigger maturation in Porcine oocytes. Mouse RINGO B is testis specific and human RINGO C is expressed in bone marrow, kidney, liver, placenta and testis. Both function as CDK1 and CDK2 activators. RINGO D and E also function as CDK activators, however, their expression patterns have been not determined (Dinarina et al., 2005; Cheng et al., 2005; Gastwirt et al., 2007).

Overexpression and depletion experiments have demonstrated that RINGO accumulation is required for CPEB activation and progesterone-induced maturation.

RINGO protein is expressed at very low levels in the immature oocyte and progesterone stimulation boosts RINGO accumulation through poladenylation-induced translation of RINGO mRNA. In the immature oocytes, RINGO mRNA translation is repressed by Xenopus Pumilio 2 (PUM2), which binds Pumilio Binding Elements (PBEs) in the 3'UTR. Several proteins interact with RINGO mRNA to form an mRNP. PUM2 also interacts with Deleted for Azoospermia-like (DAZL) and Embryonic Poly A Binding Protein (EPAB). Progesterone stimulation triggers PUM2 dissociation, alleviating translational repression (Padmanabhan and Richter, 2006). Immature oocytes also contain a RINGO N-terminal truncation product called RINGO small and processed (RINGOsp), which includes the first 146 amino acids. RINGOsp is proposed to bind monomeric CDK1 and prevent the full-length counterpart from forming active RINGO/CDK1 complexes. RINGO mRNA or protein injection into oocytes triggers maturation with faster kinetics than progesterone (Gutierrez et al., 2007). The rapid GVBD response is likely due to robust RINGO/CDK1 activity, which boosts MPF activity by providing a starting amount of active CDK1 as well as catalyzing potent Myt1 inactivation (Ruiz et al., 2008). Indeed, analysis of meiotic progression markers revealed that RINGO overexpression induced MPF activation before Mos synthesis and MAPK activation. Endogenous levels of RINGO are far lower than the amounts used in overexpression studies, which explains why the upregulation of RINGO synthesis in response to progesterone does not trigger the same rate of GVBD. Although RINGO overexpression experiments introduce a non-physiological amount of RINGO protein and activity within the oocyte, results from endogenous RINGO perturbation experiments have demonstrated the necessity of this atypical CDK activator towards CPEB activation and oocyte maturation. Injection of RINGO antisense (AS) oligonucleotides effectively blocks RINGO synthesis and activity, confirmed by northern blotting to detect RINGO mRNA destruction and Histone H1 phosphorylation to gauge RINGO/CDK1 activity (Ferby et al., 1999). RINGO depleted oocytes were not

able to mature in response to progesterone. Analysis of biochemical markers revealed that CPEB activation and c-mos polyadenylation were compromised (Padmanabhan and Richter, 2006). These oocytes also lacked Mos protein as well as MAPK and MPF activity. These results suggest that RINGO activity acts upstream of CPEB activation. For CPEB repressed mRNAss, several factors, in addition to CPEB, are assembled to form a Ribonucleoprotein Complex (mRNP) (Martinez et al., 2005; Barnard et al., 2004). ePAB is bound to CPEB in silenced mRNAs. However, CPEB activation causes ePAB to dissociate and bind the elongating poly(A) tail. Interestingly, when oocytes were injected with RINGO AS, ePAB remained CPEB-bound after progesterone stimulation. The persistent CPEB/ePAB interaction after progesterone stimulation in RINGO AS injected oocytes is likely due to disrupted CPEB activation when RINGO is depleted and provides additional evidence of RINGO involvement during CPEB activation (Kim and Richter, 2007).

Oocytes injected with RINGO protein undergo maturation without progesterone stimulation. RINGO-induced maturation is depressed in oocytes pre-injected with XGef neutralizing antibody, yet enhanced by HA-XGef overexpression. XGef and RINGO interact *ex vivo* and the association is direct *in vitro*. These findings suggest that XGef and RINGO may function in a common pathway to influence CPEB activation and meiotic progression. XGef and RINGO each interact directly with MAPK in vitro. Combined with our previous protein interaction data, our results suggest that XGef, MAPK and RINGO may form a complex *in ovo*. To pursue the possibility that formation of an XGef/ MAPK/ RINGO complex facilitates CPEB activation, we used a mutant version of XGef (65-360) that cannot bind CPEB or MAPK, but is capable of RINGO association. Interestingly, HA-XGef (65-360) overexpression depresses RINGO-induced GVBD, likely a consequence of disrupting the formation of this complex. Lastly, we show that RINGO/CDK1 activity may directly influence CPEB phosphorylation. RINGO 1-146

inhibition of the full-length counterpart in extracts prepared from progesterone-stimulated oocytes disrupted early CPEB phosphorylation.

B. Materials and Methods

1. Oocyte culture

Stage VI oocytes from non-primed *Xenopus laevis* females were collected after enzymatic digestion with 1x Barth's saline containing 0.2 mg/ml Collagenase (type IA, Sigma) and 0.6 U/ml dispase. Oocytes were then washed and cultured in 1x Barth's. When applicable, oocytes were stimulated with 100 μM progesterone (4-Pregnene-3,20-dione, SIGMA).

2. Oocyte microinjections

XbaI linearized pSP6-HA-Globin, pSP6-HA-XGef WT and pSP6-HA-XGef 65-360 plasmids served as templates for *in vitro* transcription, performed with the SP6 mMessage mMachine Kit (Ambion). To overexpress various HA-tagged proteins in oocytes, we injected 46 nl of HA-Globin, HA-XGef or HA-XGef 65-360 mRNAs (400 ng/μl). To inhibit endogenous XGef, oocytes were injected with 7.5 ng of XGef IgG. Equivalent amounts of normal rabbit IgG were injected as a control. For RINGO-induced maturation studies, oocytes were injected with 30 ng of MBP-RINGO or the MBP tag (15 ng) alone. For RINGO 1-146 injection experiments, 90 ng of MBP-RINGO 1-146 protein was injected into each oocyte. Micro-injected MBP-tagged proteins were solubilized in 20 mM Tris pH 8.0, 50 mM NaCl and 0.1 mM EDTA. Knockdown of RINGO mRNA was previously described (Ferby et al., 1999). Briefly, oocytes were injected with 100 ng of RINGO sense control oligonucleotide (5'TAG AGA AGA TAA TCG TCA TCT TA-3') or RINGO antisense oligonucleotide (5'ATA TGC TAG AAC CAT TGC TAT GAG A-3').

3. Oocyte Extracts and Immunoblot Analysis

For the detection of recombinant protein expression, oocytes were lysed in extract buffer containing 1x PBS and protease inhibitors (10 ng/μl each chymostatin,

pepstatin and aprotinin). For biochemical marker analysis, oocytes were lysed in 3 μl/oocyte kinase extract buffer containing 20 mM Hepes pH 7.5, 100 mM NaCl, 10 mM β-glycerophosphate, 5 mM EGTA, 5 mM $MgCl_2$, 100 mM sucrose, 2 mM $NaVaO_4$, 20 mM NaF, 1mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiotheritol (DTT), 2 μM okadaic acid and protease inhibitor cocktail. For *ex vivo* pulldown assays, oocytes were lysed in 25 mM Tris pH 7.4, 50 mM NaCl, 1 mM DTT, 0.1% Triton X-100, 1 mM PMSF and protease inhibitors. Lysates were centrifuged twice at 13,000 rpm and clarified supernatants were boiled in 2x Sample Loading Buffer. For *ex vivo* protein interaction assays, oocytes were lysed in 3ul/oocyte extract protein interaction buffer containing 50 mM Tris pH 7.4, 100 mM NaCl, 1 mM DTT, 0.1% Triton-X, 1 mM PMSF and protease inhibitors. Clarified lysates were then applied to bead-bound bait proteins.

For immunoblot analysis, protein extracts were boiled with 2xSDS and β-mercaptoethanol sample loading buffer and subject to 10 or 12.5% SDS-Polyacrylamide Gel Electrophoresis. Resolved proteins (25-50 μg total protein per lane) were blotted onto polyvinylidene difluoride (PVDF) membrane and blocked in 5% Milk/TBST or equal parts 1x PBS and LiCor Blocking Buffer. Blots were then probed with various primary antibodies and their appropriate secondaries. Chemilumenescence (Perkin Elmer) or laser scanning at 680 nM (LiCor) were used to detect antibody-bound proteins.

4. Antibodies

HA, MBP and His-monoclonal antibodies were used at 1:2,000, 1:1,000 and 1:1,000 dilutions, respectively (Covance and New England Biolabs). Primary rabbit polyclonal PMAPK and mouse monoclonal PCNA antibody dilutions were 1:1,000 and 1:2,000, respectively (Cell Signaling and Zymed). HA antibody was detected with hrp-conjugated anti-mouse secondary (Santa Cruz, 1:10,000). MBP and His primary

antibodies were recognized by Alexa-Flour 680 conjugated anti-mouse secondaries (Molecular Probes, 1:10,000). PMAPK and PCNA primaries were recognized by anti-rabbit (Santa Cruz, 1:10,000) and hrp-conjugated anti-mouse secondaries (Santa Cruz, 1:15,000).

5. Plasmid constructs and cloning

For pMalC and pMalC-RINGO 1-146 constructs, we used pMalC-RINGO full-length as the template for PCR amplification. To create the pMalC vector, we used primers: Forward: 5'-CGT CTA GAC GTC GTT TTA CAA CGT CGT GAC TGG-3' Reverse: 5'-GTG TCT AGA GGA TCC GAA TTC TGA AAT CCT TCC-3'.
The 6.6 kb amplified product contained the parental vector, excluding the RINGO coding region. PCR conditions were: 95°C for 5 minutes; then 35 cycles of 95°C for 1 minute, 65°C for 1 minute, and 72°C for 7 minutes; and finally an additional 5 minutes at 72°C. *Pful* turbo DNA polymerase was used (Stratagene). Each primer also introduced an *XbaI* restriction endonuclease site such that the gel extracted PCR product (Zymo Research) could be digested with *XbaI* (New England Biolabs) and ligated for re-circularization. Ligation reactions were performed with 800U T4 DNA ligase (New England Biolabs) in a 20 μl reaction, containing 50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT and 1 mM ATP, at room temperature, overnight. Ligation mixtures (5 μl) were transformed into XL1-Blue *E. coli* (50 μl) for plasmid generation and diagnostic *XbaI* restriction digests.

To create pMalC-RINGO 1-146, we used primers:
Forward: 5'-GCG ATC GCT CAC GCC ACG AGT CTC CTA GTG CCC AGG-3'
Reverse: 5'-GCG ATC GAA GCT TGG CAC TGG CCG TCG TTT TAC AAC G-3'.

The primers were used to amplify the parental vector, excluding nucleotides 439-893 of the RINGO open reading frame. PCR conditions were: 95°C for five minutes; then 35 cycles of 95°C for 1 minute, 68°C for 1 minute, and 72°C for 8 minutes; and finally an additional 5 minutes at 72°C. *PfuI* turbo DNA polymerase was used (Stratagene). Each primer also introduced a *PstI* restriction site, flanking the 7050 bp product. Gel purified product was digested with *PstI* and ligated with T4 DNA ligase in reactions conditions described above.

6. Antibody affinity purification

PVDF strips containing immobilized ~25 μg GST-XGef (bacterial) were first blocked in 5% Milk/TBST for one hour at room temperature. Blocked strips were then incubated with serum collected from His-XGef immunized rabbits (400 μl) for 4 hours at room temperature, followed by TBST washes (1.2 ml) to remove nonspecifically bound immunoglobulins. To elute XGef IgG, strips were incubated with 400 μl of 1 M glycine pH 2.5 for 10 minutes at room temperature follwed by eluate neutralization with 40 μl of 1 M Tris pH 8.0. Eluted XGef IgG was combined with 800 μl of XB buffer, containing 10 mM KCl, 10 mM $CaCl_2$, 100 mM $MgCl_2$, 1 mM Hepes and 5 μM sucrose, and spun through a centricon-30 column to concentrate the XGef IgG. The same procedure was performed for Normal Rabbit IgG purification and concentration.

7. Recombinant protein expression and purification

MBP, MBP-RINGO and MBP-RINGO 1-146aa

BL21DE cells were transformed with 50 ng of pMalC, pMalC-RINGO or pMalC-RINGO 1-146 aa plasmid DNA. The 1ml transformation mixture was brought up to a 10ml final volume with Luria Bertani Broth (LB)/Ampicillin media and grown overnight at

37°C, while shaking at 225 rpm. The overnight culture (3 ml) was then added to 250 ml LB/Ampicillin and grown again at 37°C until OD_{600} = 0.600. Protein expression was induced by 50 μl of 1 M Isopropyl-β-D-thiogalactopyranoside (IPTG). The culture was then brought up to a final volume of 500 mL with LB/Ampicillin. Induction occurred at 23°C for 6 hours. Cells were pelleted at 5,000 rpm for 10 minutes, at 4°C using a sorvall SS-34 rotor. Cells (50 ml culture/pellet) were stored at -80°C. For purification purposes, bacterial pellets were resuspended in 5ml of Lysis Buffer, containing 50 mM Tris pH 7.5, 50 mM NaCl, 5 mM EDTA, 0.1 M PMSF and 1x lysozyme (0.01 μg/μl) and sonicated (30% output) six times for 15 second intervals. After sonication the bacterial resuspension was centrifuged at 12,000 rpm for 12 min at 4C in a Sorvall SS-34 rotor to pellet insoluble material. Clarified extracts were then combined with 250 μl amylose resin, pre-washed with column buffer (50 mM Tris pH7.5, 50 mM NaCl and 5 mM EDTA), and incubated with end over end rotation at 4°C for 1-2 hours. The amylose beads were then washed three times with 5ml Wash Buffer, containing 10 mM Tris pH 7.5, 50 mM NaCl and 5 mM EDTA and 0.1% triton-X, to remove nonspecifically bound proteins. To analyze protein yield and integrity, amylose-MBP-tagged protein slurry (20 μl) was combined with 10 μl of 4X Sample loading buffer and resolved on a 10% SDS-acrylamide gel and coomassie staining. BSA standards ranging from 5-20 μg were also included for yield estimation. Purified MBP-tagged proteins were stored on ice until further use. To elute amylose resin-bound MBP tagged proteins, slurries were incubated in 1.5 ml Elution Buffer (10 mM Tris pH 8.0, 50 mM NaCl, 1 mM DTT, 0.1 mM EDTA and 12 mM Maltose) at room temperature for 30 minutes. Amylose beads were pelleted from the elution solution through centrifugation at 4,000 rpm, at 4°C. Supernatants containing soluble MBP tagged proteins were applied to a centricon-30 column for concentration and buffer exchange. Elution mixtures were passed through the column with

centrifugation at 7,900 rpm, at 4°C (Sorvall SS-34 rotor). After the initial spin, soluble proteins were washed twice with 2ml cold deionized water for desalting. Finally, 2 ml Storage Buffer (20 mM Tris pH 8.0, 50 mM NaCl, 0.1 mM EDTA and 5% glycerol) was passed through the spin column in order to maintain MBP tagged proteins in the appropriate buffer for freezing (-20°C or -80°C) and/or injection. MBP-tagged proteins used for oocyte microinjection were kept in glycerol-free Storage Buffer.

GST, GST-XGef WT and GST-XGef 65-360

BL21DE cells were transformed with 10-50 ng of pGEX2T (GST), pGEX2T-XGef or pGEX2T-XGef 65-360 plasmid and grown overnight in 10 mls of LB/Ampicillin at 37°C and shaking at 225 rpm. The next day, overnight cultures were expanded 100 fold and grown at 37°C. Once the culture reached an $O.D._{600}$ of 0.600, cells were induced to express the GST-tagged protein with 400 μl of 1 M IPTG and grown at 30°C for 3.5 hours. After induction, cells were pelleted (Sorvall SS-34 rotor) for 10 minutes, at 5,000 rpm and 4°C. Cells were resuspended in 1x PBS, repelleted (250 ml culture/pellet) and stored at -80°C. For purification, pellets were resuspended in 15 ml 1x PBS/0.1% Triton-X and sonicated three times for 30 second intervals. After sonication, lysates were spun at 12,000 rpm at 4°C (sorvall SS-34). Clarified lysates were combined with 200 μl glutathione slurry, which was pre-equilibriated with 1x PBS/0.1% Triton-X, and incubated with end over end rotation at 4°C for 2 hours. After affinity purification, the beads were washed three times with 1 ml of 1x PBS/0.1% Triton-X. Beads were stored on ice in the same buffer in a 1:1 buffer to bead slurry.

His-MAPK

BL21DE cells were transformed with 50 ng of pET-His-MAPK plasmid. The 1 ml transformation solution was combined with 9 ml LB/ampicillin and cultured overnight at 37°C and shaking at 225 rpm. The overnight culture was then used to inoculate 1 L LB/Ampicillin and grown again at 37°C until an $O.D._{600}$ of 0.600 was reached. His-MAPK expression was induced with 400 μl of 1 M IPTG and incubation at 28°C, for 3.5 hours. After induction, cells were pelleted at 5,000 rpm and 4°C, using a Sorvall SS-34 rotor (200 ml culture/pellet). For purification, pellets were resuspended in Binding Buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH 7.9, 0.25% Tween 20) and sonicated (20-30% output) three times for 30 second intervals. Insoluble proteins were pelleted at 12,000 rpm at 4°C (Sorvall SS-34) for 12 minutes. Clarified supernatants were incubated with 300 μl Ni^+-NTA nickel slurry, pre-washed in Binding Buffer, for 3 hours at 4°C. Nickel beads were washed three times with 5 ml Wash Buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH7.9) to remove nonspecifically bound proteins. Nickel-bead bound His-MAPK was eluted with 200 μl of 1 mM Imidazole, 0.5 M NaCl and 20 mM Tris pH 7.9, at room temperature for 30 minutes. Eluted proteins were passed through a centricon-30 column for buffer exchange and concentration of eluted His-MAPK. Solubilized His-MAPK was kept in storage buffer containing 2 mM $MgCl_2$, 25 mM Hepes, 150 mM NaCl and 10% glycerol and frozen at -80°C.

His-CPEB

The pET-His-CPEB vector (10 ng) was transformed into BL21 DE *E. coli* cells (50 μl) and cultured overnight in 10 ml LB/Ampicillin. For protein expression, the 1 L LB/Ampicillin was inoculated with the overnight culture and grown to $O.D_{600}$ = 0.6 at 37°C and induced with 400 μl 1 M IPTG. Induction occurred at 20°C for 3.5 hours. Cells

were then pelleted (1 L culture/pellet) at 5,000 rpm, 4°C (Sorvall SS-34) and stored at –80°C. For purification, pellets were resuspended in Binding Buffer and sonicated three times for 30 second intervals. Insoluble material was pelleted through centrifugation at 12,000 rpm for 10 minutes at 4°C (Sorvall SS-34) and supernatants were combined with 300 μl Nickel Ni^{+}-NTA slurry, pre-equilibriated in Binding Buffer. Affinity purification took place at 4°C for 3 hours. Nonspecifically bound proteins were removed by washing three times with 5 ml Wash Buffer. Nickel bead-bound His-CPEB was re-equilibrated in Binding Buffer and stored on ice until further use.

8. *In vitro* direct protein interaction assays

Bait proteins were solubilized in Protein Interaction Buffer containing 20 mM Hepes pH 7.5, 150 mM NaCl, 5 mM EGTA pH 8.0, 5 mM $MgCl_2$, 0.1 M sucrose, 10 mM β-Glycerophosphate, 1 mM DTT, 5% glycerol, 1% NP-40, 1x Protease inhibitor cocktail, and 0.5% BSA. Bead bound prey proteins were pre-washed with the same buffer. For MBP-RINGO/His-MAPK interaction assays, amylose-MBP (2.5 μg) or MBP-RINGO (5 μg) was incubated with 2 ug of soluble His-MAPK. The total buffer volume (excluding bead solid) per reaction was 50 μl. For GST-XGef/His-MAPK interaction assays, GST (5 μg), GST-WT (15 μg) or GST-65-360 (10 μg) was combined with 2 μg of soluble His-MAPK in a 50 μl reaction. GST-XGef/MBP-RINGO interaction assays included glutathione bead bound GST (5 μg), GST-WT (15 μg) or GST-65-360 (10 μg) combined with soluble MBP (2.5 μg) or MBP-RINGO (5 μg). Binding reactions took place at 4°C for 2 hours with end over end rotation. Bead bound proteins were washed three times in 1 mL of Wash Buffer (Protein Interaction Buffer containing 1% Triton X-100). Once nonspecifically bound protein was removed, protein complexes were denatured and eluted with 20 μl of 2xSDS-BME sample loading buffer and boiling.

9. *Ex vivo* protein interaction assays

MBP or MBP-RINGO was expressed and purified from BL21DE *E. coli*, protocol detailed above. MBP-tagged proteins were then re-equilibriated in RINGO Pulldown Buffer, containing 50mM Tris pH 7.4, 100 mM NaCl, 1 mM DTT and 0.1% Triton X-100, to reestablish a 1:1 Bead:Buffer slurry. HA-XGef WT or HA-XGef 65-360 overexpressing oocytes (30 oocytes per reaction) were homogenized in lysis buffer described above. Clarified supernatants were then added to amylose bead-bound MBP (2.5 μg) or MBP-RINGO (5 μg) and incubated at 18°C for 2 hours. Amylose bead protein complexes were washed three times in 500 μl of wash buffer containing 50 mM Tris pH 7.4, 100 mM NaCl, 1 mM DTT and 1% Triton X-100 to remove nonspecifically bound proteins. Precipitated proteins were eluted with 20 μl of 2xSDS-BME sample loading buffer and boiling.

10. Kinase Assays

For the *in vitro* His-CPEB phosphorylation assay, oocyte lysates (90 oocytes pre reaction) were combined with 3 μCi [γ-^{32}P]ATP and 2.5 μg Nickel Ni^{+}-NTA bead-teathered His-CPEB, pre-equilibriated in Phosphorylation Buffer containing 10 mM Hepes pH 7.5, 10 mM MgCl2, 0.1 mM EGTA, 0.5 mM DTT, 1 μM okadaic acid, 1 mM H-89 PKA inhibitor, 50 μM adenosine triphosphate and protease inhibitors. Reactions were conducted at room temperature for 30 minutes and stopped with 25 μl SDS sample loading buffer and boiling. *In vitro* His-CPEB phosphoryation was analyzed by 10% SDS-PAGE and autoradiography. For the H1 kinase assay, extracts from 3 oocytes were (7 μl extract buffer/oocyte) combined with 0.8 μg Histone H1 substrate and 3 μCi [γ-^{32}P]ATP (Perkin Elmer). Reactions took place at room temperature and were stopped

with 20 μl SDS sample loading buffer and boiling. Histone H1 phosphorylation was examined by 12.5% SDS-PAGE and autoradiography.

C. Results

1. XGef function influences RINGO activity.

RINGO synthesis upon progesterone stimulation is required for CPEB activation and oocyte maturation. How RINGO influences CPEB activation and meiotic progression, however, has not been elucidated. Previously, we have shown that XGef function is also essential for CPEB activation and the maturation process. We therefore pursued the possibility that RINGO and XGef functions can facilitate CPEB activation through a common pathway. Initially identified in a screen for factors that triggered G2/M transition in *Xenopus* oocytes, RINGO mRNA or protein injection induces a rapid GVBD response in the absence of progesterone. To determine if XGef function influences RINGO-induced maturation, we observed the GVBD time course of MBP-RINGO injected oocytes, which were first injected with XGef antibody to disrupt endogenous XGef activity. Interestingly, XGef inhibition with neutralizing antibody pre-injection attenuated RINGO-induced maturation compared to the normal rabbit IgG control (Figure 19). We also analyzed extract levels of phospho-active MAPK, a biochemical marker for meiotic progression, in oocytes collected in parallel to the RINGO-induced maturation experiment described above. PMAPK immunoblot analysis of extracts prepared at the indicated times after RINGO injection revealed that perturbation of endogenous XGef activity also compromised MAPK activation during RINGO stimulated GVBD (Figure 20, compare lanes 5 and 12). Together, our results suggested that RINGO-induced maturation required XGef activity. If XGef function was required for RINGO signaling, then XGef overexpression may enhance RINGO activity. We therefore analyzed RINGO-induced maturation in oocytes overexpressing HA-XGef. First, oocytes were injected with HA-XGef mRNA and then re-injected with MBP-RINGO protein to trigger GVBD. RINGO-induced maturation was more rapid in oocytes overexpressing HA-XGef than the HA-Globin control (Figure 21). RINGO-induced maturation rates for

control groups exhibited some variation (Compare Figure 19, NR-IgG and Figure 21, HA-Glb pre-injections) due to contrasting activity levels of MBP-RINGO from different preparations. Altogether, these findings suggested that XGef function facilitated RINGO-induced maturation. Interference of XGef function hindered RINGO mediated maturation, while excess intracellular HA-XGef enhanced RINGO signaling.

2. XGef, MAPK and RINGO form a complex in oocytes.

To pursue the possibility that XGef and RINGO participate in a common pathway to mediate CPEB activation, we performed an *ex vivo* protein interaction assay to determine if XGef and RINGO are present in the same complex in oocytes. Lysates prepared from HA-XGef overexpressing oocytes, in early meiosis, were used in an MBP-RINGO pulldown experiment. After extensive washing to remove non-specifically bound proteins, HA-immunoblotting revealed that HA-XGef bound specifically to MBP-RINGO during early meiosis, but not to MBP beads alone (Figure 22). To determine if the XGef and RINGO interaction was direct, we performed an *in vitro* protein interaction assay and incubated soluble MBP-RINGO with glutathione bead-bound GST and GST-XGef. After probing the precipitates with MBP antibody, we found that MBP-RINGO bound specifically to GST-XGef but not GST alone (Figure 23B). MBP did not bind either GST or GST-XGef (Figure 23A). Altogether, these findings suggest that not only are XGef and RINGO components of a complex in maturing oocytes, but that both factors also interact directly in *in vitro* conditions.

During early meiosis, only a few factors have been identified that exert an effect on CPEB function. In addition to XGef and RINGO, MAPK activity is also required for CPEB activation. Previously, we have shown that kinases are active during the first hour of meiotic progression that can phosphorylate His-CPEB *in vitro*. This CPEB-specific kinase activity is also sensitive to MAPK inhibition. We hypothesized that the early

phosphorylations may serve a priming function to facilitate CPEB activation and that the kinase responsible for the priming phospho-residues was either MAPK itself or another kinase that was influenced by MAPK activity. Interestingly, XGef immunoprecipitates MAPK, suggesting that XGef and MAPK are present in a complex *in ovo*. Before pursing the possibility that an XGef/ RINGO/ MAPK complex exists *in ovo*, we first sought out to determine if XGef and MAPK interact directly. Glutathione bead-bound GST-XGef and soluble His-MAPK were combined in an *in vitro* protein interaction assay. His-MAPK bound specifically to GST-XGef but did not interact with the GST-tag alone (Figure 24, compare lane 1 to lane 2). We next pursued the possibility of a direct interaction between RINGO and MAPK. Indeed, His-immunoblotting of MBP-RINGO pulldown proteins revealed that His-MAPK and MBP-RINGO interacted directly. The MBP tag did not bind His-MAPK (Figure 25, compare lane 1 to lane 2). In total, our protein interaction data suggested that RINGO, XGef and MAPK form a complex *in ovo*.

3. XGef 65-360 may bind and sequester RINGO from a XGef/ RINGO/ MAPK/ CPEB complex, disrupting CPEB activation.

XGef function facilitates CPEB activation and oocyte maturation, however, as a putative Rho family guanine nucleotide exchange factor, XGef influence on maturation is independent of exchange activity. We have previously shown that XGef WT overexpression enhances progesterone signaling and CPEB activation. Interestingly, overexpression of the XGef 65-360 mutant, lacking both amino and carboxy termini and CPEB binding ability, compromised CPEB activation and oocyte maturation. This finding suggested that XGef association with CPEB must occur for CPEB activation. Additionally, our GST-XGef *in vitro* protein interaction data showed that XGef interacted directly with both RINGO and MAPK. In total, both our *ex vivo* and *in vitro* protein interaction data suggested that the proper assembly of an XGef/ CPEB/ MAPK/ RINGO

complex must mediate CPEB activation. To test this hypothesis, we used the XGef 65-360 mutant to disrupt the complex. However, we first had to confirm that the XGef 65-360 mutant could bind at least one protein of the complex otherwise studying an XGef mutant void of any interaction with the factors of interest would prove uninformative. We first performed an *in vitro* protein interaction assay to determine if XGef 65-360 and MAPK could interact directly. Glutathione bead-bound GST, GST-XGef and GST-XGef 65-360 were combined with soluble His-MAPK. His immunoblot analysis of precipitated proteins revealed that GST-XGef 65-360 did not bind to His-MAPK directly (Figure 26). We next performed the same *in vitro* protein interaction assay combining glutathione bead-bound GST-XGef 65-360 with soluble MBP-RINGO. Precipitated proteins were subject to MBP immunoblot analysis and we found that MBP-RINGO bound to GST-XGef 65-360 and WT with equal affinity. MBP-RINGO did not bind GST alone. The soluble MBP tag was not precipitated in any pulldown reactions (Figure 27). We therefore hypothesized that XGef 65-360 may negatively influence meiotic progression by binding and sequestering RINGO from endogenous XGef, MAPK and CPEB, disrupting proper complex association and CPEB activation.

If XGef 65-360 did in fact bind and sequester RINGO from an XGef/ MAPK/ CPEB complex, then the 65-360 mutant should abrogate RINGO function and influence CPEB activation. We next pursued the possibility that the XGef 65-360 mutant interfered with RINGO function by observing RINGO-induced maturation in oocytes overexpressing HA-XGef 65-360. Oocytes were pre-injected with HA-XGef 65-360, HA-XGef WT and HA-Globin mRNAs for translation of exogenous proteins. These oocytes were then re-injected with MBP-RINGO protein to trigger maturation. Interestingly, HA-XGef 65-360 overexpression blocked MBP-RINGO stimulation of GVBD (Figure 28). We next performed an *ex vivo* protein interaction assay to determine if oocyte HA-XGef 65-360 can bind amylose bead-bound MBP-RINGO. Since we had previously shown an

ex vivo interaction between HA-XGef WT and MBP-RINGO, we co-injected HA-XGef and HA-XGef 65-360 mRNAs and repeated the amylose pulldown to compare their relative affinities for MBP-RINGO. HA-XGef 65-360 bound to MBP-RINGO with the same efficiency as HA-XGef WT (Figure 29).

4. RINGO synthesis and activity are required for early CPEB phosphorylation.

RINGO synthesis is upregulated in response to progesterone and accumulation of RINGO protein is required for CPEB activation and meiotic progression. We have previously shown that CPEB phosphorylating kinases are present during the first hour of maturation before CPEB activation, which occurs approximately three hours after progesterone stimulation. To further our understanding of how RINGO influences CPEB activation, we performed an *in vitro* His-CPEB phosphorylation assay to analyze the presence of CPEB phosphorylating kinases in RINGO depleted oocytes, shortly after meiosis resumption. We first injected oocytes with RINGO antisense oligonucleotides, described previously (Ferby et al., 1999). These oocytes were then exposed to progesterone to initiate meiosis and collected at the times indicated for lysis and analysis with an *in vitro* His-CPEB phosphorylation assay. His-CPEB was phosphorylated by extracts prepared from progesterone-stimulated oocytes, however, RINGO depletion blocked only the 3 hour phosphorylation event (Figure 30, compare lanes 4 and 7). Earlier His-CPEB phosphorylation events remained unaffected in the absence of RINGO (compare lanes 2 and 3 with lanes 4 and 5). Oocytes were collected in parallel to the experiment described above and subject to PMAPK immunoblot analysis. RINGO depletion led to a decrease in PMAPK levels during early meiosis (bottom blot compare lanes 3 and 4 to 6 and 7). We next pursued the possibility that RINGO/CDK1 activity is directly involved in CPEB phosphorylation. The N-terminal half of the RINGO protein (1-

146), has been shown to exert regulatory function against its full-length counterpart during G2 arrest by binding CDK1 to form inactive complexes (Gutierrez et al., 2006). Oocytes were stimulated with progesterone and extracts prepared at the indicated times were supplemented with RINGO 1-146 to specifically inhibit endogenous RINGO/CDK1 activity. These extracts were then subjected to an *in vitro* His-CPEB phosphorylation assay. RINGO 1-146 pre-treatment blocked *in vitro* early His-CPEB phosphorylation between 1 to 3 hours after progesterone sitmulation. In the first RINGO inhibition experiment, His-CPEB phosphorylation was compromised in extracts prepared from oocytes that had been stimulated with progesterone for three hours and then supplemented with 1-146 (Figure 31, compare lanes 4 and 7). In this particular experiment, His-CPEB phosphorylating activities present during the first hour of meiosis resumption were not affected by RINGO/Cdc2 activity inhibition (Figure 31, compare lanes 2 and 3 versus 5 and 6). However, in a separate experiment, we found that His-CPEB phosphorylation was negatively affected in 1 hour extracts as a consequence of RINGO activity perturbation (Figure 32, compare lanes 3 and 7). Unlike the previous experiment, the 3 hour His-CPEB phosphorylation remained the same in control and 1-146 treated extracts. Notably, we observed a decline in His-CPEB phosphate incorporation after incubation in RINGO/CDK inhibited, GVBD extracts. A Histone H1 kinase assay was conducted in parallel to show that RINGO 1-146 was an efficacious endogenous RINGO/CDK1 inhibitor. Although the RINGO/CDK1 complex has been shown to efficiently phosphorylate H1 *in vitro*, control and RINGO 1-146 treated extracts prepared from oocytes in early meiosis phosphorylated H1 at a similarly low degree, presumably due to low abundance of RINGO/CDK1 activity at this time in maturation (Figure 32, H1-P). However, since RINGO inactivation blocks MPF activity, which is robust at GVBD, we were able to monitor RINGO 1-146 inhibition of the full-length counterpart by assessing H1 phosphorylation at GVBD. Indeed, RINGO 1-146 treatment

blocked MPF activation as evidenced by low H1 phosphorylation compared to control extracts (Figure 32, compare lanes 5 and 9). We therefore relied on disrupted MPF activation as an indicator of RINGO/CDK1 inhibition in 1-146 supplemented extracts. RINGO/CDK1 inhibition in extracts with pre-existing CPEB phosphorylating activity significantly depressed His-CPEB phosphorylation *in vitro*, which suggests that RINGO/CDK1 directly phosphorylates CPEB at the time of activation. In total, these findings support the requirement of RINGO protein accumulation for CPEB phosphorylation during oocyte maturation. Further, RINGO/CDK1 activity is directly involved in the catalysis of CPEB phosphorylation three hours after progesterone stimulation, when CPEB typically undergoes Ser 174 activating phosphorylation.

D. Figures

Figure 19. Injection of XGef neutralizing antibodies depresses RINGO-induced maturation. Oocytes (n=5) were injected with affinity purified XGef antibody (XGef-IgG) or normal rabbit IgG (NR-IgG) and stimulated to resume meiotic progression with MBP-RINGO microinjection. The graph shown is representative of five independent experiments. Inset: injected XGef and control IgGs were resolved by SDS-PAGE and coomassie stained to determine concentration.

Figure 19.

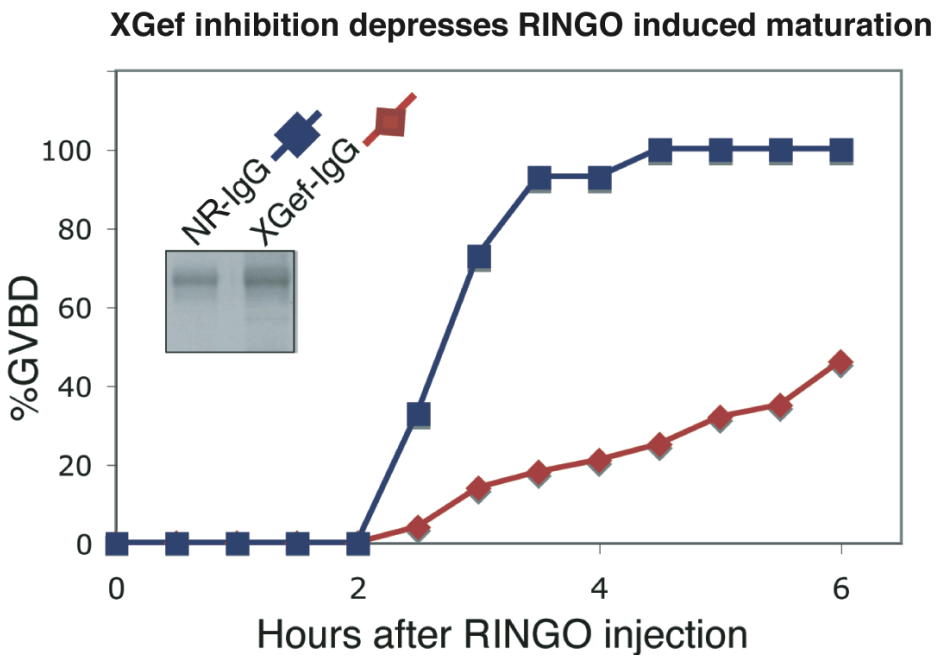

XGef inhibition depresses RINGO induced maturation
NR-IgG
XGef-IgG
%GVBD
100
80
60
40
20
0
0
2
4
6
Hours after RINGO injection

Figure 20. PMAPK immunoblot analysis of RINGO injected oocytes, pre-injected with XGef IgG. The same oocytes shown in the previous XGef IgG maturation experiment were probed for phospho-active MAPK. Protein extracts were analyzed by SDS-PAGE and immunoblotting with PMAPK antibody (IB:PMAPK) or PCNA antibody (IB:PCNA) as a loading control.

Figure 20.

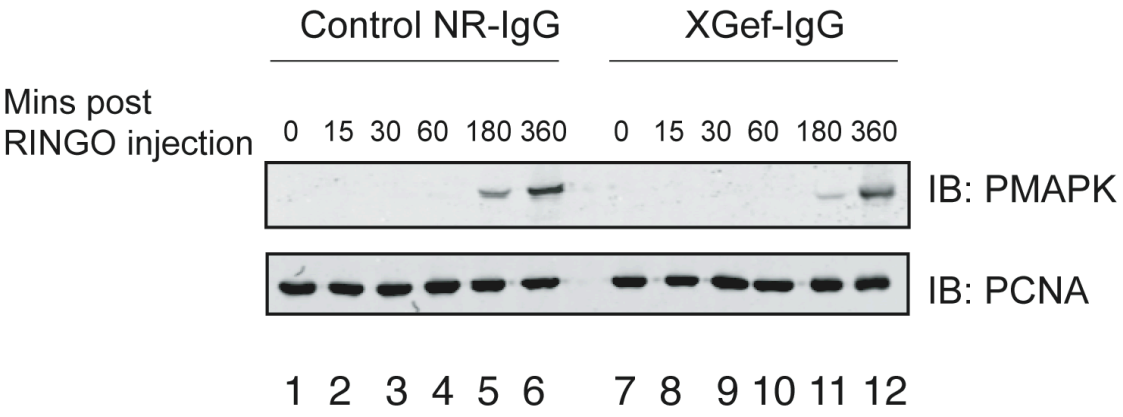

Control NR-IgG
XGef-IgG
Mins post
RINGO injection
0 15 30 60 180 360 0 15 30 60 180 360
IB: PMAPK
IB: PCNA
1 2 3 4 5 6 7 8 9 10 11 12

Figure 21. HA-XGef overexpression enhances RINGO-induced maturation. The effect of HA-XGef or HA-Globin control overexpression on RINGO-induced maturation was analyzed in a GVBD time course (n=3). Inset: the expression of HA-XGef and HA-Globin were detected by HA-antibody immunoblot (IB:HA).

Figure 21.

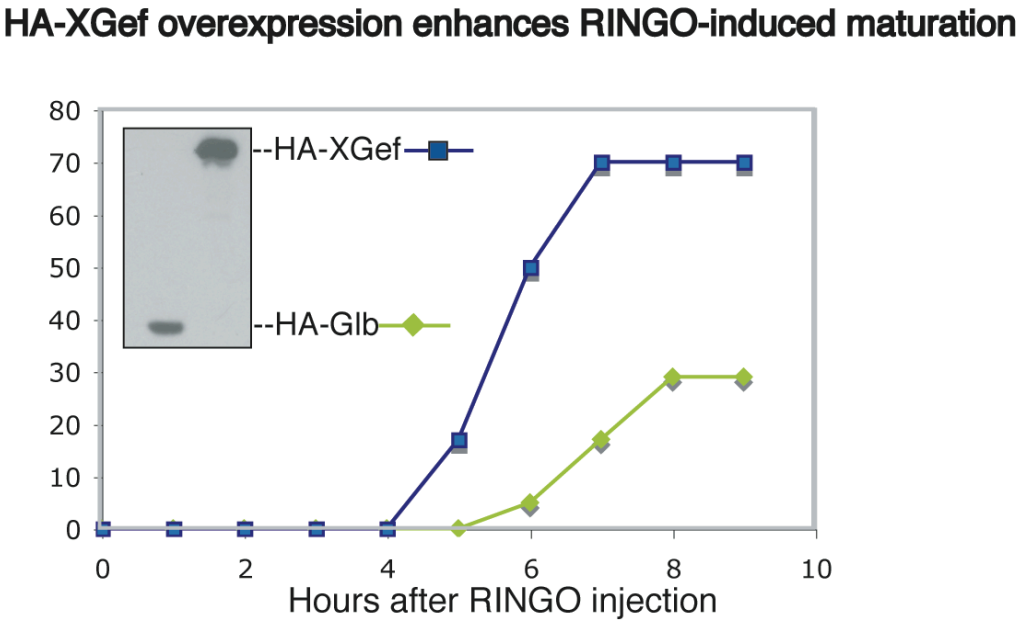

HA-XGef overexpression enhances RINGO-induced maturation
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
Hours after RINGO injection
--HA-XGef
--HA-Glb

Figure 22. HA-XGef and MBP-RINGO interact *ex vivo*.

Oocyte HA-XGef interacts with bacterially expressed MBP-RINGO during early meiosis. Oocytes overexpressing HA-XGef were lysed at the indicated times after progesterone stimulation and incubated with amylose bead-bound MBP-RINGO or MBP alone. Amylose bead pulldown proteins (top and middle panels) and input extracts (bottom panel) were analyzed by SDS-PAGE and HA-antibody western blot analysis.

Figure 22.

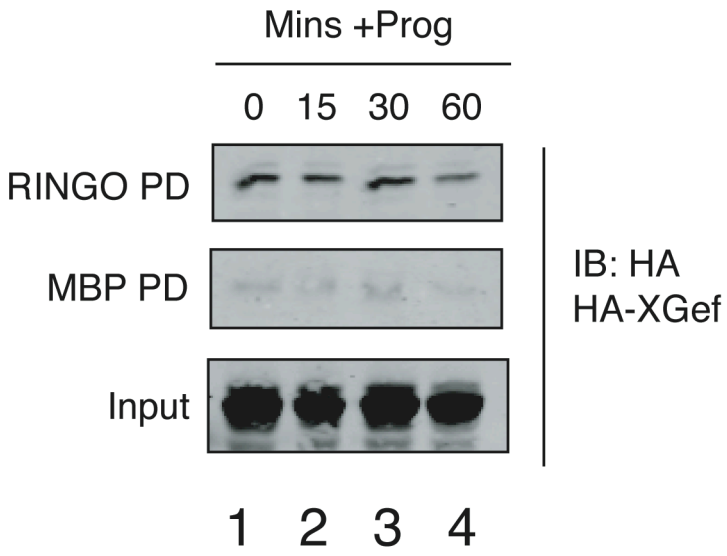


Figure 23. GST-XGef and MBP-RINGO interact directly *in vitro*.

In an *in vitro* protein interaction assay, soluble MBP-RINGO (right-hand blot) and MBP (left-hand blot) were incubated with bacterially expressed, glutathione bead-bound, GST or GST-XGef. Precipitated MBP-tagged proteins were analyzed by immunoblotting with MBP-antibody (IB: MBP). Input MBP and MBP-RINGO are shown (lane 1).

Figure 23.

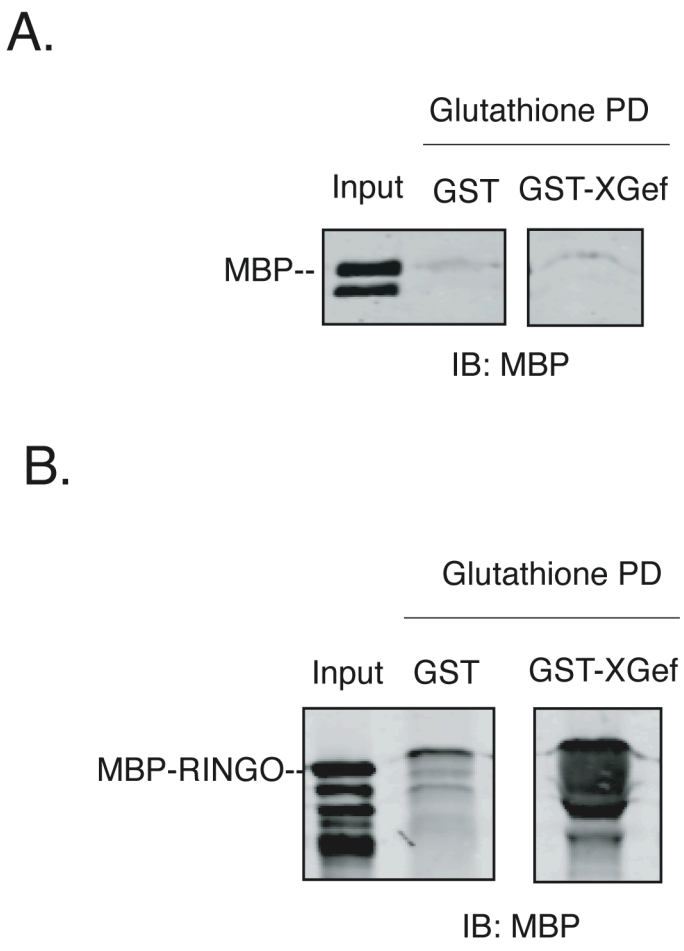

A.
Glutathione PD
Input
GST
GST-XGef
MBP--
IB: MBP
B.
Glutathione PD
Input
GST
GST-XGef
MBP-RINGO--
IB: MBP

Figure 24. GST-XGef and His-MAPK interact directly *in vitro*.

Soluble His-MAPK was combined with glutathione-bound GST or GST-XGef . Bound His-MAPK (top panel) and input His-MAPK (middle panel) were detected with His-antibody (IB: His). Input GST-tagged proteins are shown in the bottom panel (CS).

Figure 24.

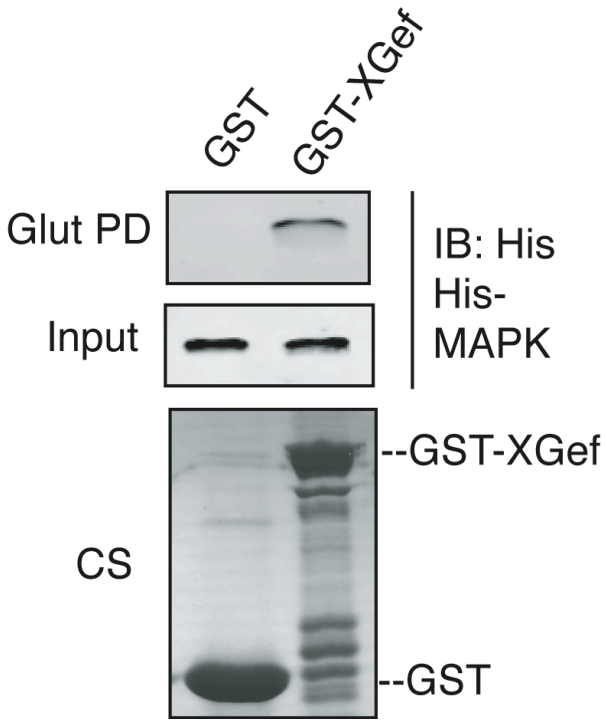


Figure 25. MBP-RINGO and His-MAPK interact directly *in vitro*.

Soluble His-MAPK was incubated with amylose immobilized MBP-RINGO or MBP alone. Bound proteins were analyzed by SDS-PAGE and immunoblotting. Precipitated His-MAPK (top panel) and input His-MAPK (middle panel) were detected with His-antibody (IB: His). Input amylose tethered proteins were resolved and visualized by SDS-PAGE and coomassie staining (CS).

Figure 25.

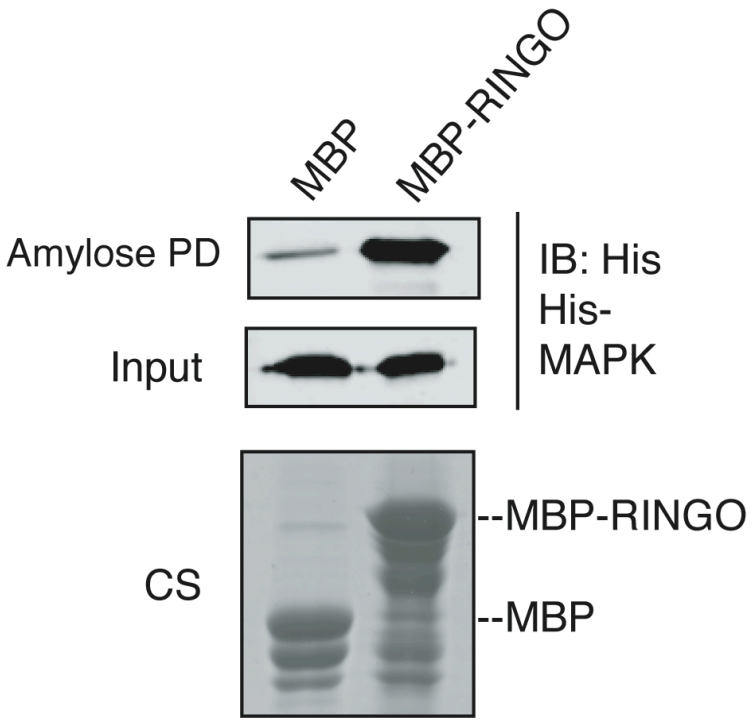

MBP
MBP-RINGO
Amylose PD
Input
IB: His
His-
MAPK
CS
--MBP-RINGO
--MBP

Figure 26. GST-XGef 65-360 does not bind His-MAPK *in vitro*.

Glutathione bead-bound GST-XGef 65-360 was incubated with soluble His-MAPK in a pulldown reaction. Input (middle panel) and bound His-MAPK (top panel) were detected with a His-antibody immunoblot (IB: His). Input GST-tagged proteins were analyzed by SDS-PAGE and coomassie staining (CS).

Figure 26.

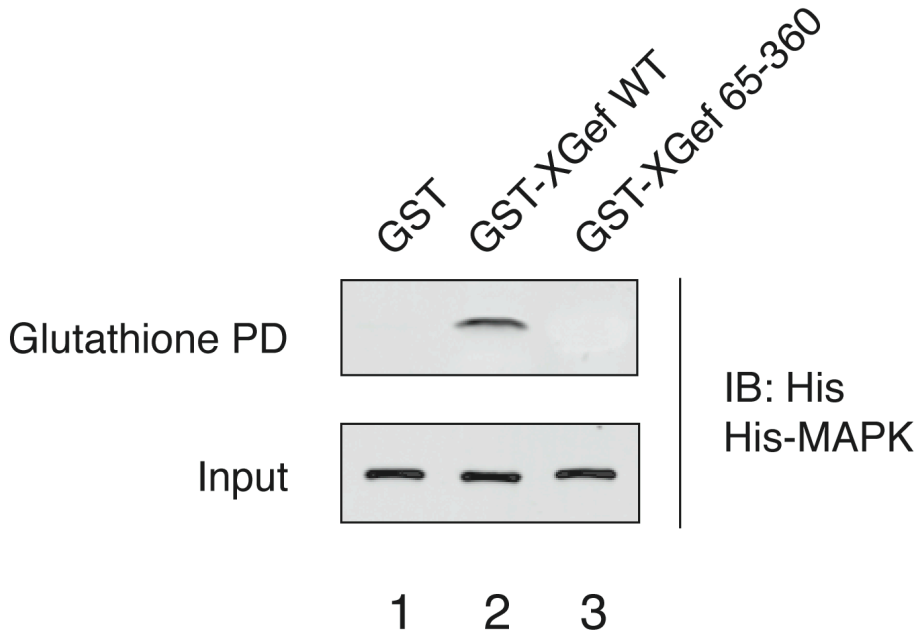

GST
GST-XGef WT
GST-XGef 65-360
Glutathione PD
Input
IB: His
His-MAPK
1
2
3

Figure 27. GST-XGef 65-360 and MBP-RINGO interact directly. Glutathione bead-bound GST-XGef 65-360 and soluble MBP-RINGO were combined in an *in vitro* protein interaction assay. Precipitated MBP-RINGO was detect with MBP immunoblot analysis (Glut PD, IB: MBP). Input MBP and MBP-RINGO (Input, IB: MBP) and GST-tagged proteins (Input, CS) are indicated.

Figure 27.

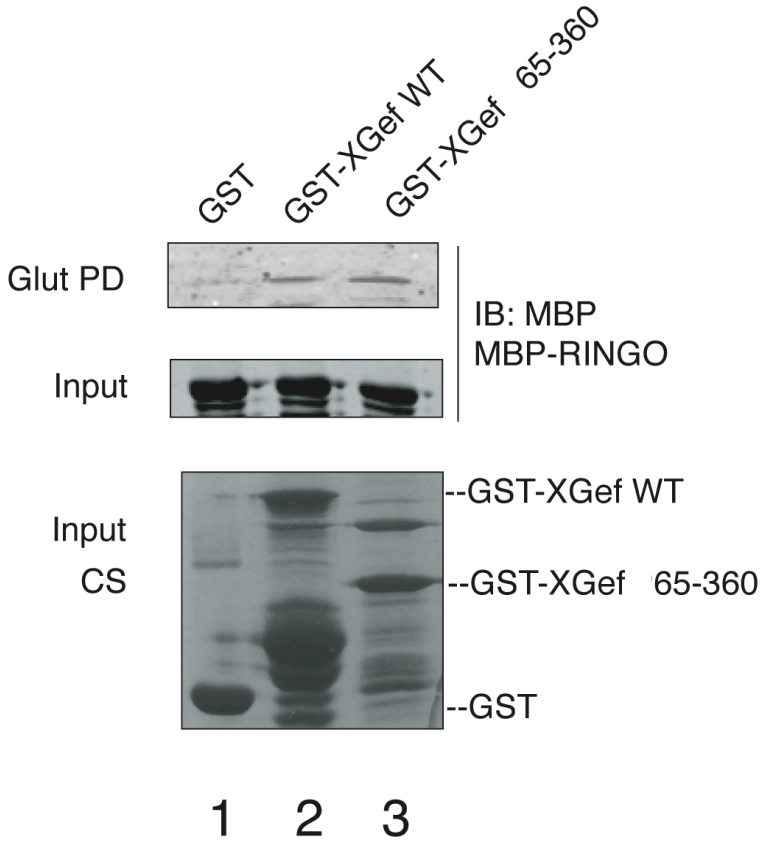

GST
GST-XGef WT
GST-XGef 65-360
Glut PD
Input
IB: MBP
MBP-RINGO
Input
CS
--GST-XGef WT
--GST-XGef 65-360
--GST
1 2 3

Figure 28. XGef 65-360 expression depresses RINGO-induced oocyte maturation. Oocytes overexpressing HA-XGef WT, HA-XGef 65-360 and HA-Globin were injected with MBP-RINGO to trigger maturation. The effects of overexpression of these proteins on RINGO stimulation of meiotic progression were monitored through a GVBD time course. Inset: HA-immunoblotting of HA-tagged proteins overexpressed in oocytes.

Figure 28.

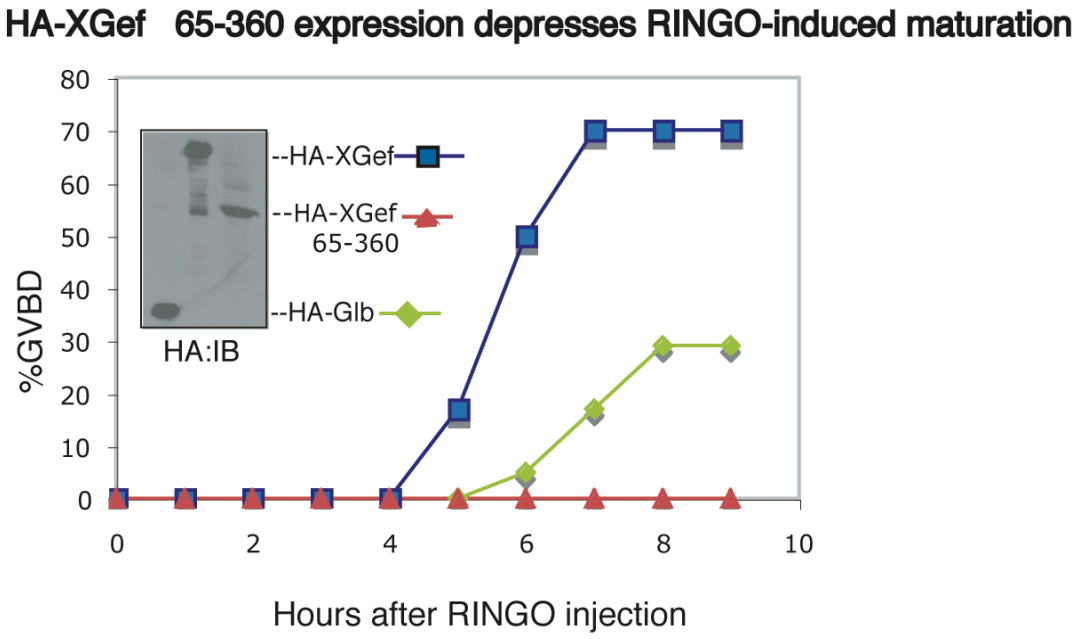

HA-XGef 65-360 expression depresses RINGO-induced maturation
%GVBD
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
Hours after RINGO injection
--HA-XGef
--HA-XGef 65-360
--HA-Glb
HA:IB

Figure 29. XGef 65-360 interacts with RINGO *ex vivo*.

Extracts prepared from oocytes co-injected with HA-XGef WT and HA-XGef 65-360 mRNAs were incubated with amylose bead-bound MBP or MBP-RINGO. Input extracts and pulldown proteins were probed with HA-antibody (IB: HA). Input MBP-tagged proteins were detected by SDS-PAGE and coomassie staining (CS).

Figure 29.

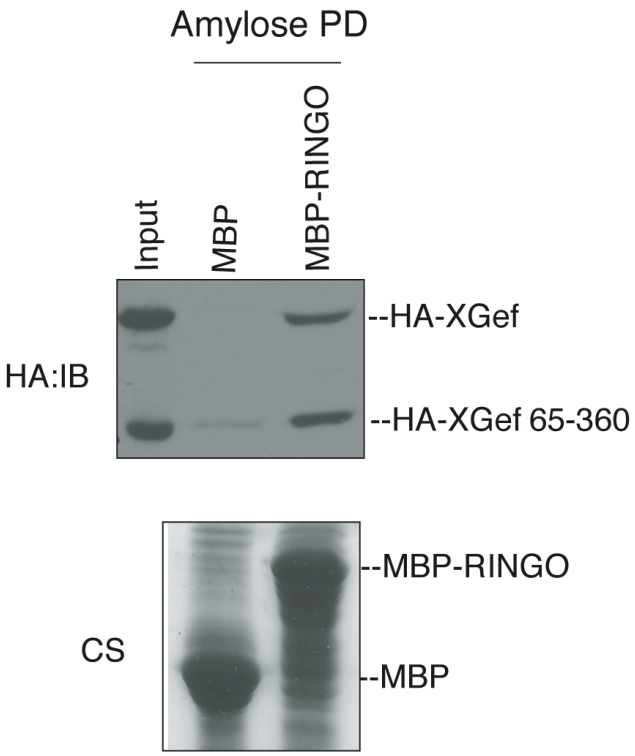

Amylose PD
Input
MBP
MBP-RINGO
HA:IB
--HA-XGef
--HA-XGef 65-360
CS
--MBP-RINGO
--MBP

Figure 30. RINGO depletion disrupts CPEB phosphorylation and MAPK activation. (A) Oocytes (n=3) were injected with 100 ng of RINGO sense or antisense oligonucleotides prior to progesterone stimulation. At the indicated times after maturation initiation (Hrs PG) oocytes were lysed and combined with nickel bead-bound His-CPEB and (^{32}P [ATP] for an *in vitro* His-CPEB phosphorylation assay. Radiolabeled His-CPEB was visualized by SDS-PAGE and autoradiography (^{32}P). Equivalent levels of His-CPEB were added to each reaction (CS). (B) Oocytes collected in parallel of the experiment described in (A) were subject to PMAPK immunoblot analysis (IB: PMAPK). A PCNA antibody was used as a loading control (IB: PCNA).

Figure 30.

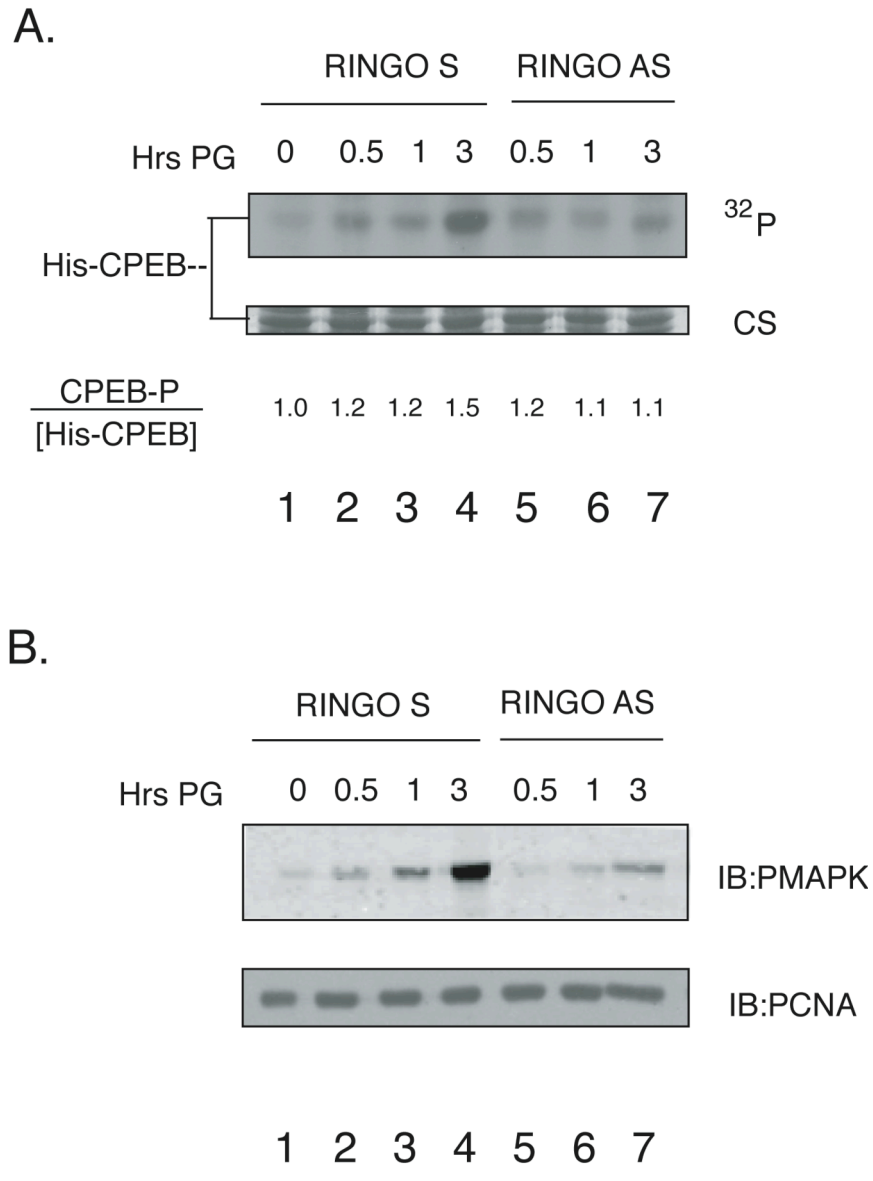

A.
RINGO S
RINGO AS
Hrs PG
0 0.5 1 3 0.5 1 3
His-CPEB--
32P
CS
CPEB-P
[His-CPEB]
1.0 1.2 1.2 1.5 1.2 1.1 1.1
1 2 3 4 5 6 7
B.
RINGO S
RINGO AS
Hrs PG
0 0.5 1 3 0.5 1 3
IB:PMAPK
IB:PCNA
1 2 3 4 5 6 7

Figure 31. RINGO 1-146 perturbation of RINGO activity abrogates CPEB phosphorylation in 3 hour extracts. Oocyte (n=3) extracts were prepared at the indicated times after progesterone stimulation. After pre-treatment with soluble MBP (10 μg) or MBP-RINGO 1-146 (10 μg), extracts were used as a kinase source in an *in vitro* His-CPEB phosphorylation assay (^{32}P). His-CPEB levels in each reaction are indicated (CS).

Figure 31.

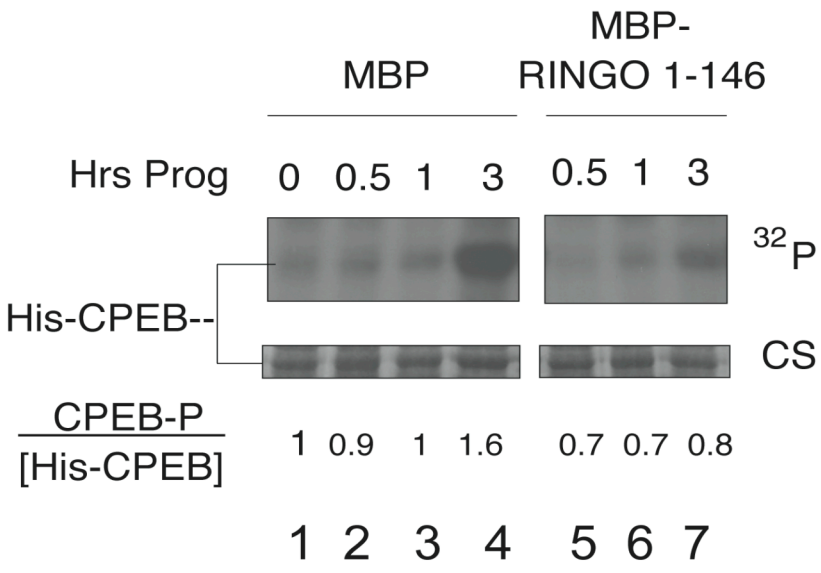
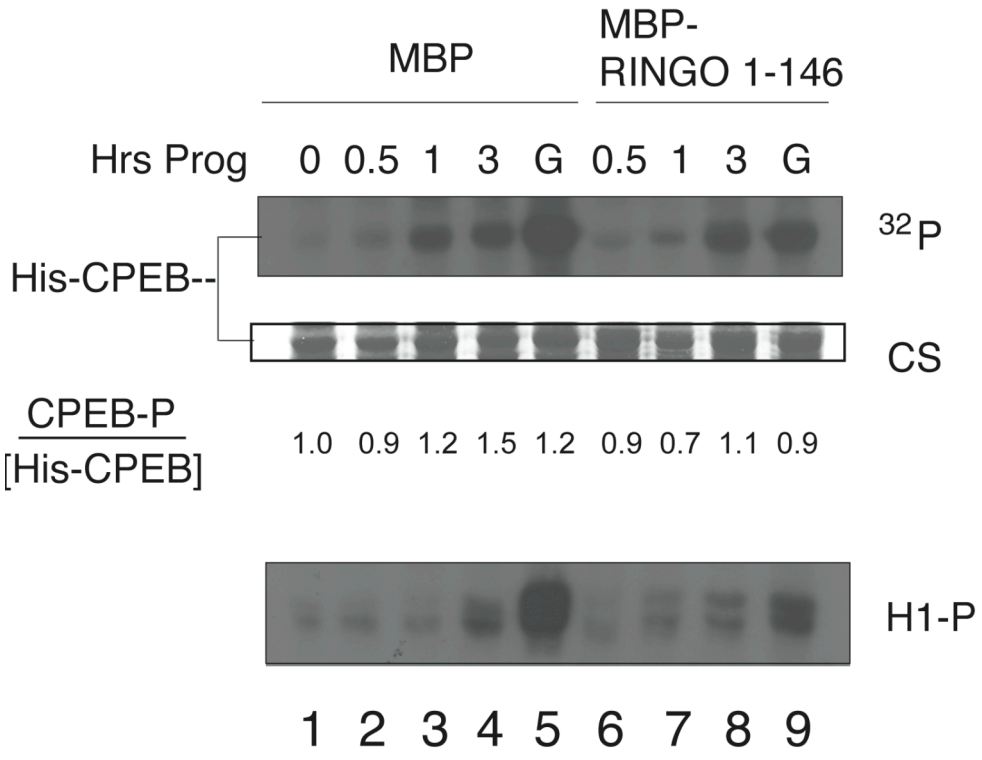

MBP
MBP-
RINGO 1-146
Hrs Prog
0 0.5 1 3
0.5 1 3
32P
His-CPEB--
CS
CPEB-P
[His-CPEB]
1 0.9 1 1.6
0.7 0.7 0.8
1 2 3 4 5 6 7

Figure 32. RINGO 1-146 perturbation of RINGO activity compromises CPEB phosphorylation in 1 hour extracts and at GVBD. Oocyte extracts were prepared at the indicated times after progesterone stimulation. After pre-treatment with soluble MBP (10 μg) or MBP-RINGO 1-146 (10 μg), extracts were used as a kinase source in an *in vitro* His-CPEB phosphorylation assay (^{32}P). His-CPEB levels in each reaction are indicated (CS). Oocytes were collected in parallel for and H1 kinase assay (H1-P).

Figure 32.


MBP
MBP-
RINGO 1-146
Hrs Prog 0 0.5 1 3 G 0.5 1 3 G
His-CPEB--
32P
CS
CPEB-P
[His-CPEB]
1.0 0.9 1.2 1.5 1.2 0.9 0.7 1.1 0.9
H1-P
1 2 3 4 5 6 7 8 9

E. Discussion

1. Overview

We propose that XGef and RINGO function in a common pathway and form a complex with MAPK to mediate CPEB phosphorylation and activation during *Xenopus* oocyte maturation. RINGO induction of GVBD is compromised in oocytes injected with XGef-neutralizing antibodies, whereas HA-XGef overexpression enhances the rate of maturation upon RINGO protein injection. HA-XGef and MBP-RINGO also interact *ex vivo* in maturing oocytes. *In vitro*, XGef, RINGO and MAPK interact directly with one another. In total, our protein interaction data suggest that these factors, all of which have been shown facilitate CPEB activation, may drive this process. We used XGef 65-360, which only binds RINGO, to disrupt the potential XGef/MAPK/RINGO complex. HA-XGef 65-360 overexpression disrupted RINGO-induced GVBD. Lastly, specific inhibition of RINGO/CDK1 with RINGO 1-146 disrupted early CPEB phosphorylation, which suggests that RINGO/CDK1 directly influences CPEB phosphorylation and potentially activation.

2. The cooperative influences of XGef and RINGO/CDK1 on CPEB activation and meiotic progression.

The activities of both XGef and RINGO/CDK1 are important during CPEB activation, since functional disruption of either factor abrogates CPEB activation and oocyte meiotic progression in response to progesterone (Reverte et al., 2003, Martinez et al., 2005; Padmanabhan and Richter, 2006). But how do XGef and RINGO/CDK1 facilitate CPEB activation? We first investigated the relationship between XGef and RINGO/CDK1 functions and then assessed the biological significance of a proposed XGef/MAPK/RINGO complex towards CPEB activation and meiotic progression.

XGef is expressed at a constant level in immature and maturing oocytes, whereas RINGO was initially considered absent before meiosis resumption and only synthesized in response to progesterone signaling (Reverte et al., 2003, Lenormand et al., 1999; Padmanabhan and Richter, 2006). However, in prophase I oocytes, endogenous RINGO evaded detection as a consequence of utilizing poor quality polyclonal antibodies to detect low levels of RINGO protein. Gutierrez and colleagues showed that, indeed, very little RINGO expression took place in G2 arrested oocytes, accompanied by a truncated RINGO product (residues 1-146), which is proposed to block activity of the full-length counterpart (Gutierrez et al., 2007). XGef and RINGO are both expressed in immature and maturing oocytes. Our *ex vivo* protein interaction results suggest that both factors are present in a common complex during this early time in maturation. In order to demonstrate an XGef/RINGO interaction, we were forced to use recombinant versions of each protein since in our experience, as well as those reported by others, the detection of endogenous RINGO protein interactions is challenging due to low protein abundance and/or inadequately sensitive immunoprecipitation and immunoblotting methods. However, our *in vitro* protein interaction data supports our *ex vivo* findings and together suggest that XGef and RINGO interact directly *in ovo*.

To further our understanding of how XGef and RINGO mediate CPEB activation, we asked if the activity of one factor was dependent on the other by assessing RINGO stimulation of GVBD upon XGef functional perturbation and XGef overexpression. Inherent difficulties exist in using the RINGO overexpression approach to piece together signaling events. RINGO overexpression triggers rapid and robust MPF activation, likely due to premature Myt1 inactivation and CDK1 activation, which trip the MPF autoamplification positive feedback loop before Mos synthesis and MAPK activation (Ruiz et al., 2008). Regardless of these limitations, we were still able to use RINGO function as a potent inducer of GVBD to assess RINGO function when XGef was

inhibited or overexpressed. We suspect that XGef function likely occurs upstream of RINGO activity since the inhibition of endogenous XGef with specific antibodies compromises RINGO-induced maturation and HA-XGef overexpression enhances RINGO signaling. Since XGef activity is not sufficient for CPEB activation, whereas RINGO synthesis is absolutely required for this process, XGef does not likely function downstream of RINGO/CDK1.

3. Formation of an XGef/RINGO/MAPK complex may facilitate CPEB activation.

The functional significance of the protein interactions that take place between XGef, RINGO, MAPK and CPEB is undeniable since the disrupted binding between components of this complex prove detrimental to oocyte maturation (Reverte et al., 2003; Martinez et al., 2005; Keady et al., 2007). We used an XGef mutant (65-360) to examine the consequences of disrupting the XGef/ MAPK/ RINGO complex. Overexpression of XGef 65-360, which does not bind CPEB or MAPK, is detrimental to progesterone-driven CPEB phosphorylation and meiotic progression (Martinez et al., 2005). Based on our findings that XGef 65-360 overexpression depresses RINGO-induced GVBD, we propose that this effect is likely due to XGef 65-360 titration of RINGO/CDK1 from CPEB, therefore disrupting CPEB activation. Additionally, the enhancement of both RINGO and progesterone signaling upon HA-XGef overexpression may occur through increased XGef/RINGO/MAPK/CPEB complex formation, which explains the boosted level of activated CPEB and accelerated rate of oocyte maturation with an increase in intracellular XGef.

RINGO function has been shown to require MAPK activity. However, since Mos depletion does not affect RINGO-induced GVBD, early-phase (Mos-independent) MAPK activity must facilitate RINGO function, albeit through an unknown mechanism. GEFs have been shown to facilitate MAPK signaling, sometimes independently of exchange

activity. GEF/MAPK protein complexes, typically organized by scaffolding proteins, have been detected in yeast and mammalian systems. Further research is required to understand if our proposed XGef/MAPK/RINGO complex mediates MAPK-dependent, RINGO activity and CPEB activation.

4. RINGO/CDK1 directly influences CPEB phosphorylation and potentially activation.

Since its discovery in 1999, RINGO/CDK1 has been revealed as a novel component of the progesterone signaling pathway in *Xenopus* oocytes (Ferby et al., 1999; Lenormand et al., 1999; Padmanabhan and Richter, 2006; Kim and Richter, 2007; reviewed in Nebreda, 2006). A mechanism of RINGO/CDK1 function during meiotic progression has been shown to occur through the catalysis of Myt1 inhibitory phosphorylation to facilitate MPF activation (Ruiz et al., 2008). Interestingly, RINGO accumulation and RINGO-mediated CDK1 activation occur shortly after progesterone stimulation, however, Myt1 inhibition occurs later in maturation. A discrepancy in timing of each event is apparent, although the delay between RINGO/CDK1 activation and Myt1 inactivation does not necessarily rule out RINGO/CDK1 function as a Myt1 inhibitor. Besides Myt1, other RINGO/CDK1 substrates have not been well defined. Cheng and colleagues have reported that RINGO/CDK1 complexes exhibit non-canonical substrate preferences and may phosphorylate a separate set of substrates than CyclinB/CDK1 (Cheng et al., 2005). Little speculation has been made about RINGO/CDK1 function with regards to CPEB activation, and we are the first to propose that CPEB may be a RINGO/CDK1 substrate.

RINGO depletion experiments have demonstrated that RINGO synthesis is required for CPEB activation, but do not provide a mechanism for this important event (Padmanabhan and Richter, 2006; Kim and Richter, 2007). Based on our results, we

propose that RINGO/CDK1 directly phosphorylates CPEB and likely controls its activation. Aurora A kinase has been considered the CPEB activating kinase for many years (Andresson and Ruderman, 1998; Mendez et al., 2000a). However, phospho-active AurA is not detected during early meiosis when CPEB becomes activated. The exact timing of Aurora A activity and its involvement in CPEB activation has been controversial. In support of our hypothesis, RINGO depletion experiments clearly show that RINGO/CDK1 influences CPEB phosphorylation and activation. RINGO AS oligonucleotide injection effectively abolished RINGO protein accumulation in maturing oocytes and not only compromised progesterone-induced CPEB phosphorylation, but also c-mos mRNA polyadenylation, which suggested perturbation of CPEB activation as well (Padmanabhan and Richter, 2006). CPEB activation leads to EPAB dissociation and poly(A) tail binding. However, RINGO depletion prevents EPAB dissociation upon progesterone stimulation, which also suggests that CPEB activation is prevented when RINGO accumulation is blocked (Kim and Richter, 2007). Although these experiments do not demonstrate direct involvement, our ability to compromise His-CPEB phosphorylation *in vitro* by treating extracts, harboring progesterone-stimulated kinase activity, with a RINGO/CDK1 specific inhibitor strongly suggests that CPEB phosphorylation and potentially its activation occur directly through RINGO/CDK1 activity.

Abundant kinase activity toward His-CPEB exists in extracts prepared from progesterone treated oocytes, which efficiently phosphorylate His-CPEB *in vitro*, and recapitulate *in ovo* conditions. Here, we demonstrate that CPEB is a direct substrate of RINGO/CDK1 since His-CPEB phosphorylation in extracts prepared from maturing oocytes is compromised as a specific consequence of RINGO/CDK1 activity inhibition. Interestingly, we observed diminished His-CPEB phosphorylation at various times during the first three hours of meiotic progression. This variation may be explained by oocyte

variation from one frog to the other or asynchronous maturation among oocytes from the same frog. Further experimentation is required to address the variability and the biological significance of RINGO/CDK1 catalyzed CPEB phosphorylation in one hour extracts. Surprisingly, RINGO/CDK1 inhibition in GVBD extracts also decreased the level of His-CPEB phosphorylation. CPEB has been shown to serve as a CDK1 substrate and others have demonstrated CDK1-mediated phosphorylation of CPEB at GVBD for degradation (Mendez et al., 2002; Setoyama et al., 2007). Our results would therefore suggest that RINGO/CDK1 is also involved in these late CPEB phosphorylations, however, additional experiments must be conducted to address this possibility.

Although our results strongly suggest RINGO/CDK1 direct involvement in CPEB activation, without phospho-peptide evidence that RINGO/CDK1 inhibition blocks S174 phosphorylation, we cannot rule out the possibility of other CPEB activating kinases. For example, RINGO/CDK1 activity may influence the activity of another kinase, which then phosphorylates to activate CPEB. Addition of the 1-146 inhibitor to extracts may block the activation of this unidentified kinase as a consequence of RINGO/CDK1 inhibition. Future research is required to confirm RINGO/CDK1 as a CPEB activating kinase.

Chapter V. Conclusions and future perspectives

A. Summary of conclusions

XGef was identified as a CPEB interactor through a yeast two-hybrid screen (Reverte et al., 2003). In *Xenopus* oocytes, CPEB activation and timely progesterone-induced maturation are XGef-dependent. XGef inhibition severely attenuates both processes, whereas XGef overexpression enhances the level of CPEB activating phosphorylation and oocyte maturation rate. As a putative Rho family guanine nucleotide exchange factor (GEF), exchange activity and activation of a Rho GTPase, was the proposed mechanism of XGef function during meiotic progression. An exchange deficient mutant of XGef (Δ65-234) no longer boosted CPEB phosphorylation or meiotic progression, which supported the functional significance of exchange activity (Martinez et al., 2005). However, in total, our results suggest that XGef influence on CPEB activation and meiotic progression does not require exchange activity or the activation of Toxin B sensitive Rho GTPases. XGef does not activate Rho GTPases, Cdc42 or Rac1, in *Xenopus* oocytes. Cdc42 activity is also irrelevant to progesterone-induced CPEB activation and GVBD. Results from Toxin B intoxicated oocytes suggest that CPEB activation and oocyte maturation also occur independently of Cdc42, Rho A-C, Rac 1-3, Rho G and TC10. Finally, XGef exchange deficient point mutants behave similarly to wildtype when overexpressed in maturing oocytes.

XGef immunoprecipitates MAPK in maturing oocytes and the interaction is likely direct, according to *in vitro* protein interaction data. MAPK activity is also required for CPEB activation and is proposed to catalyze CPEB priming phosphorylations. We detected CPEB phosphorylation in extracts prepared within an hour of progesterone stimulation, which may represent priming phosphorylations. We pursued the possibility that the XGef/MAPK interaction was of physiological importance during these CPEB

phosphorylation events and oocyte maturation by mutating the putative XGef MAPK docking sites, D domain and FXF motif, which proved ineffective and suggested that other regions of XGef must guide MAPK binding. Our unsuccessful disruption of the XGef/MAPK association prevented further investigation of the functional significance of the interaction.

RINGO, an atypical CDK activator, has become a novel player in the *Xenopus* oocyte progesterone signaling pathway. RINGO synthesis is required for CPEB activation, however through mechanisms unknown. In our ongoing effort to characterize XGef function during maturation, we found that XGef not only interacts with RINGO *in ovo*, but also influences RINGO/CDK1 signaling. XGef inhibition depresses RINGO-induced GVBD, whereas XGef overexpression enhances the maturation response triggered by RINGO protein injection. These findings suggest that XGef and RINGO/CDK1 are likely components of a common pathway that mediate CPEB activation. Additionally, our collective *ex vivo* and *in vivo* protein interaction data suggest that XGef, MAPK and RINGO may form a complex *in ovo* to phosphorylate and activate CPEB during meiotic progression. In support of this model, overexpression of an XGef 65-360, which interacts solely with RINGO, but not MAPK or CPEB, compromises CPEB phosphorylation and meiotic progression. The negative effects of XGef 65-360 may be a consequence of RINGO/CDK1 titration from endogenous XGef/MAPK/CPEB complexes. Lastly, we found that RINGO/CDK1 directly influences early CPEB phosphorylation through the use of RINGO 1-146 inhibitory protein to block endogenous RINGO/CDK1 activity. Formation of the XGef/MAPK/RINGO complex may facilitate RINGO/CDK1 recruitment to CPEB for activation purposes.

B. Future perspectives

1. Characterizing XGef function during *Xenopus* oocyte maturation

The experiments presented in this thesis were performed to further our understanding of the early signaling events that occur between progesterone receptor engagement and CPEB activation. Our efforts have been devoted to characterizing XGef function since its activity is required for CPEB activation and timely progression of oocyte maturation. To date, we have not defined XGef function, catalytic or otherwise. Since the interaction between XGef and CPEB is necessary for CPEB activation and XGef interacts with MAPK and RINGO, factors that also mediate CPEB activation, our understanding of XGef function and early progesterone signaling may be enriched through further investigation of the biological significance of these protein interactions.

a. XGef and early-phase MAPK activity

GEFs have been shown to influence MAPK cascades that mediate a variety of biological processes. The involvement of GEFs in MAPK signaling does not always require exchange activity or activation of a Rho GTPase. EPAC and CNras GEFs have been shown to influence JNK, SAPK and ERK MAPK cascades, respectively, mediating diverse biological processes. XGef function in *Xenopus* oocytes may influence MAPK activity as well. XGef function is clearly an important contributor to CPEB activation (Reverte et al., 2003; Martinez et al., 2005). XGef interacts with MAPK and CPEB activation has been shown to require MAPK activation (Keady et al., 2007). However, what is the relationship between XGef function and early-phase MAPK activation in *Xenopus* oocytes? Could XGef mediate early-phase MAPK activation in *Xenopus* oocytes? To determine if XGef function is required for early-phase MAPK activation, we will look for the presence of phospho-active MAPK shortly after progesterone stimulation in oocytes injected with XGef IgG, to block endogenous XGef activity, and Mos AS

oligonucleotides, to block late-phase MAPK. If we can still detect early-phase MAPK activation in these oocytes, then XGef activity is not required for this early event. However, if early phase-MAPK activity is compromised when endogenous XGef is inhibited, then XGef function lies upstream of early MAPK activation.

XGef immunoprepitates from maturing oocytes have been shown to contain MAPK activity (Keady et al., 2007). These findings suggest that GEFs and the components of a GEF-dependent MAPK cascade may residue in a common complex *in vivo*. As another potential mechanism for mediating early-phase MAPK activity, XGef may recruit MAPK to CPEB for the catalysis of priming phosphorylations, which facilitate CPEB activation. To pursue the possibility that XGef may act as an adaptor protein, we will analyze the consequences of disrupting the XGef/MAPK interaction. XGef putative MAPK docking domains (D domain and FXF motif) did not function as MAPK binding sites. Since XGef 65-360 no longer interacts with MAPK, *in vitro*, the amino and carboxyl-terminal regions flanking the DH and PH domains will be scrutinized for their function as MAPK docking sites. Once we have made a MAPK binding deficient mutant of XGef, we will overexpress the mutant in oocytes and observe CPEB phosphorylation and meiotic progression in response to progesterone. Perturbation of CPEB phosphorylation would suggest that the XGef/MAPK interaction is important for this process and that XGef functions as an adaptor protein, recruiting MAPK to CPEB. MAPK phosphorylates His-CPEB *in vitro* (Keady et al., 2007). If we observe a decrease in His-CPEB phosphorylation upon overexpression of an MAPK-binding deficient XGef mutant, we will also perform phosphopeptide analysis of phospho-His-CPEB to see if the *in vitro* phosphorylated residues bear physiological relevance.

b. XGef and RINGO activities

Little is known about RINGO binding partners. Although RINGO binds CDKs through the RINGO Box domain, additional protein interaction domains have not been characterized (Dinarina et al., 2005). Based on our results, XGef is a novel RINGO interactor and we can only begin to speculate about the purpose of this interaction. XGef interacts directly with CPEB and the interaction is necessary for CPEB activation (Martinez et al., 2005). We have shown that RINGO/CDK1 activity directly influences CPEB phosphorylation and likely activation. Could XGef recruit RINGO/CDK1 to CPEB? To address this possibility we will first identify RINGO binding sites on XGef. Next, we will create a mutant version of XGef that cannot associate with RINGO, overexpress this mutant in oocytes and observe progesterone-stimulated CPEB activation and meiotic progression. If CPEB phosphorylation is abrogated as a result of disrupting the XGef/RINGO interaction, then XGef may indeed recruit RINGO/CDK1 to a CPEB phosphorylating complex to facilitate CPEB activation.

2. CPEB phosphorylation and activation

CPEB activation occurs upon S174 phosphorylation by an unidentified Ser/Thr kinase. Although AurA was proposed to activate CPEB (Mendez et al., 2002), many pieces of evidence strongly suggest that this is not the case (Frank-vaillant et al., 2000; Keady et al., 2007). In addition to CPEB S174 phosphorylation, kinase activity exists within the first hour of progesterone stimulation, to phosphorylate His-CPEB *in vitro*. These findings led us to believe that CPEB may undergo priming phosphorylations. Since MAPK and RINGO/CDK1 activities are required for CPEB activation, could these kinases catalyze priming phosphorylations? We have shown that RINGO/CDK1 is directly involved in CPEB phosphorylation in one hour and three hour progesterone

stimulated extracts. Could RINGO/CDK1 catalyze the S174 CPEB activating phosphorylation?

a. CPEB priming phosphorylation

In some cases, catalysis of an activating phosphorylation may depend on a separate phosphorylation, which "primes" the substrate for the activation. MAPKs and CDKs have been shown to catalyze priming phosphorylations. MAPK phosphorylates Heat Shock Factor-1 (HSF-1) on S307, which is required for S303 phosphorylation by GSK3β and activation the sequence specific transcription factor (Chu et al., 1996). CDK1 phosphorylates Wee1A on S123, which is required for S53 phosphorylation by Polo-like kinase 1 (Plk1) and CDK1 degradation by βTRCP ubiquitin ligase (Watanabe et al., 2005). His-CPEB phosphorylation can be detected within an hour of progesterone stimulation, which we propose to be a CPEB priming phosphorylation event. To determine if this early phosphorylation possesses priming function, we will first perform phosphopeptide analysis of His-CPEB phosphorylated by one hour extracts to identify phosphorylated residues. Next, we will make alanine substitutions at these sites and use this CPEB mutant as a substrate in an *in vitro* phosphorylation experiment with extracts prepared from progesterone stimulated oocytes. Disrupted S174 phosphorylation as a consequence of these mutating these sites would support our hypothesis that CPEB undergoes priming phosphorylations. We will also overexpress, in oocytes, a CPEB mutant with alanine mutations on putative priming phosphorylation sites and observe meiotic progression upon progesterone stimulation. Compromised c-mos polyadenylation (an indicator of CPEB activation) and maturation as a result of overexpressing a non-primed and therefore constitutively inactive CPEB mutant would support our priming hypothesis.

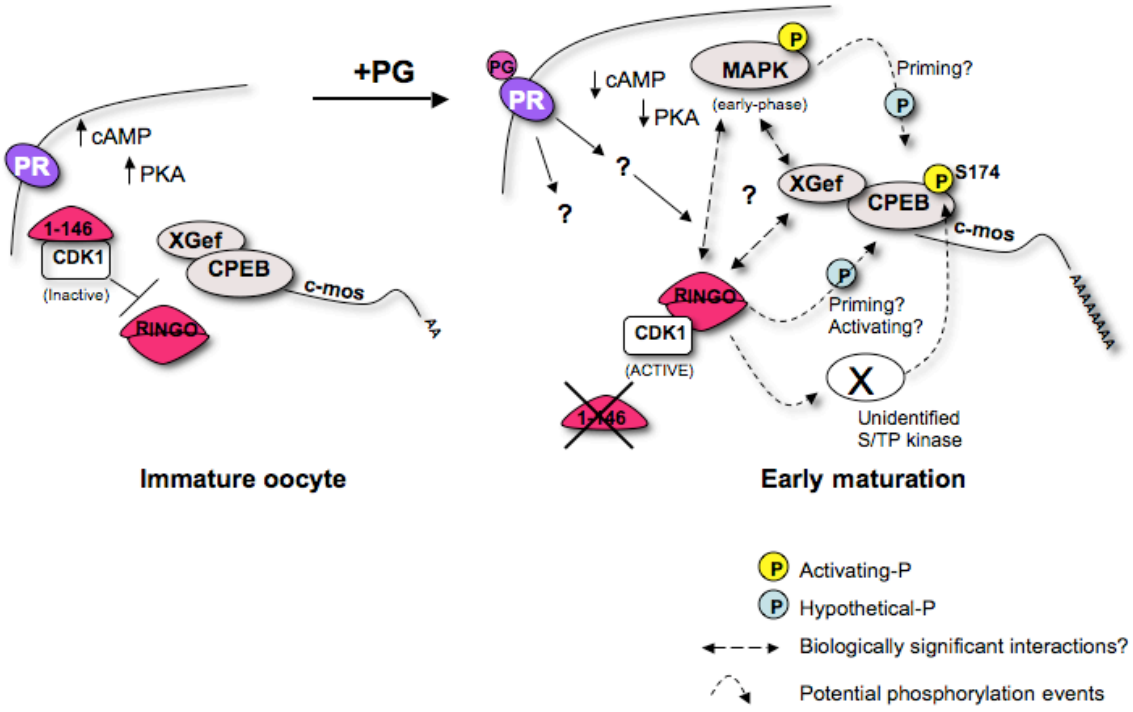
b. RINGO/CDK1 as a CPEB activator

CPEB is a physiological CDK1 substrate in *Xenopus* oocytes. Several studies have shown that CDK1 (of active MPF) phosphorylates CPEB to facilitate its degradation at GVBD, however, with the recent discovery of CDK1 activity through RINGO binding and activation, the possibility of CDK1 directed phosphorylation to mediate CPEB activation has not been pursued. Mendez and colleagues argued that S210 phosphorylation by CDK1 is sufficient for CPEB degradation. However, CPEB is also phosphorylated on five other CDK1 sites (S138, S144, S184, S248 and S423) at GVBD, which are dispensable to degradation (Mendez et al., 2002). What is the functional purpose of these five additional CDK1 phosphorylation sites if they are irrelevant to CPEB degradation? Could these sites be phosphorylated by RINGO/CDK1 for CPEB activation? It is important to note that some phosphorylated residues identified through His-CPEB incubation in GVBD extracts may be taken out of context. Active kinases and/or CPEB phosphorylation sites that arise during early maturation may persist in GVBD extracts, such that several His-CPEB phospho-residues may not be GVBD specific. Our past phosphopeptide analysis data suggest that this is indeed the case. For example, S174-P is detected in GVBD phosphorylated His-CPEB. This issue may explain the Mendez and colleagues identified phosphorylated residues that did not mediate a process specific to GVBD extracts, such as CPEB degradation, and lends support to the possibility that CDK1 sites may exist to affect processes besides CPEB degradation.

RINGO/CDK1 complexes exhibit differential substrate and phosphorylation site preferences compared to CyclinB/CDK1. Using a canonical CDK substrate peptide, KSPRK, Cheng and colleagues showed that RINGO/CDK1 complexes are able to tolerate variation in the P+2 and P+3 residues and therefore less stringent in substrate choice. In substrates with multiple S/TP sites, RINGO/CDK1 complexes phosphorylated

different consensus sites than Cyclin B/CDK1 (Cheng et al., 2005). These findings suggest that CDK1 phosphorylation site preference may change depending on its binding partner, in which RINGO/CDK1 may phosphorylate non-canonical substrates and sites. *Spisula solidissma* CPEB, P82, also undergoes CDK phosphorylation-mediated degradation through the phosphorylation of consensus and non-consensus sites (Thom et al., 2003). We propose that RINGO/CDK1 may phosphorylate S174, which is not a S/TP motif, to activate CPEB. To pursue this possibility, experiments can be conducted to determine if CPEB S174 phosphorylation is blocked when endogenous RINGO/CDK1 activity is inhibited with RINGOsp, the first 146 amino acids of full-length RINGO. Gutierrez and colleagues have shown that RINGO small and processed (RINGOsp) is a physiological inhibitor of the full-length protein (Gutierrrez et al., 2007). We can perform an *in vitro* phosphorylation assay to determine if RINGO/CDK1 directly phosphorylates His-CPEB. If His-CPEB is phosphorylated under these *in vitro* conditions, we will perform phosphopeptide mapping to determine the phosphorylated residues. If RINGO/CDK1 does not phosphorylate His-CPEB *in vitro*, we cannot rule out the possibility of a RINGO/CDK1 catalyzed phosphorylation of CPEB *in ovo*. Other factors may be required for the phosphorylation to occur. RINGO/CDK1 may phosphorylate a kinase, which then phosphorylates to activate CPEB. Identification of the CPEB activating kinase will greatly contribute to our limited knowledge of early progesterone signaling in *Xenopus* oocytes.

Figure 33. Proposed model: early progesterone signaling in *Xenopus* oocytes



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