CELL CYCLE REGULATION DURING MAMMALIAN FEMALE MEIOSIS: CHARACTERIZATION OF THE MASTL-ENSA/ARPP19 PATHWAY IN MOUSE OOCYTES

by

Lauren Matthews

A dissertation submitted to Johns Hopkins University in conformity with the requirements for the degree of Doctor of Philosophy

Baltimore, Maryland

August 2014
ABSTRACT

Mammalian oocytes undergo two successive rounds of division without an intervening S-phase to form a haploid gamete. Oocytes are arrested in prophase I in the ovary until the time of ovulation when the oocyte exits from this arrest and progresses through meiosis I. The egg then arrests at metaphase of meiosis II until fertilization when the second meiotic division is completed. The progression from prophase I to metaphase II, known as meiotic maturation, must be carefully regulated in order to successfully form a haploid gamete. The MASTL-ENSA/ARPP19 pathway has emerged as an important regulator of M-phase entry and progression. The kinase, MASTL (microtubule-associated serine/threonine kinase-like), phosphorylates its two substrates, ENSA (alpha-endosulfine) and ARPP19 (cAMP-regulated phosphoprotein-19), which can then inhibit the activity of the protein phosphatase PP2A. Inhibition of PP2A maintains the phosphorylated state of CDK1 substrates, thus allowing progression into and/or maintenance of an M-phase state. The work in this thesis characterizes the MASTL-ENSA/ARPP19 pathway during meiotic maturation in mouse oocytes. ENSA is an abundant protein in mouse oocytes and was expressed by prophase I oocytes, metaphase II eggs and early embryos. ARPP19 was not detected in lysates of prophase I oocytes or early embryos, but was detected in lysates of metaphase II eggs. The kinase, MASTL, and its substrate, ENSA, both play a key role in the progression from prophase I arrest into M-phase of meiosis I. The majority of MASTL-deficient and ENSA-deficient oocytes failed to exit from prophase I arrest. This function of ENSA in oocytes is dependent on PP2A, and specifically on the regulatory subunit PPP2R2D (also known as B55δ). Treatment of ENSA-deficient oocytes with okadaic acid to inhibit PP2A or
knockdown of the PPP2R2D subunit rescued the meiotic progression defect, with these oocytes being able to exit from prophase I arrest. These data are evidence of a role for MASTL and ENSA in regulating meiotic maturation in mammalian oocytes and have important implications for our understanding of the molecular regulation of mammalian female meiosis.

Thesis readers:
Janice Evans, Ph.D. – Advisor
Daniela Drummond-Barbosa, Ph.D.
Alan Scott, Ph.D.
Geraldine Seydoux, Ph.D.
ACKNOWLEDGMENTS

I would like to thank my thesis advisor, Dr. Janice Evans, for her support and scientific guidance. I appreciate her enthusiasm for reproductive biology and I have enjoyed learning from her over the past six years. I would also like to thank my thesis advisory committee members, Dr. Daniela Drummond-Barbosa, Dr. Michael Matunis, Dr. Alan Scott and Dr. Geraldine Seydoux, and my alternate thesis readers, Dr. Michael Matunis and Dr. DeLisa Fairweather, for their input and support.

A big thank you to the BMB department and staff for creating a supportive environment and for making my journey as a Ph.D. student a little bit easier. I have enjoyed working in such a collaborative department and I am thankful for the assistance and support I have received from the BMB faculty. I am especially grateful for Ann Lawler and Chip Hawkins from the Transgenic Core Facility for their assistance and guidance with microinjection and microinjection needles over the past few years.

I would like to thank the past and present members of the Evans lab, especially Lauren McGinnis and Hyo Lee, who I have had the pleasure of working with over the past six years. It has been wonderful to work with these two ladies and I am thankful for all of the laughs we have shared. I would like to thank my classmates, Ulli Hain and Mindy Graham, as well as the other BMB students for their support and encouragement.

I would like to thank my family and friends for their endless support, love and encouragement. I would not be where I am today without all of these wonderful, supportive people in my life. I am thankful to my parents and my siblings, Michael and Kimberly, for all of the love, joy and support they bring to my life. Last but not least, I
would like to thank my husband, Josh, for his unconditional love and support. I dedicate this thesis to Josh and to my family.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>iv</td>
</tr>
<tr>
<td>List of Tables</td>
<td>viii</td>
</tr>
<tr>
<td>List of Figures</td>
<td>ix</td>
</tr>
<tr>
<td><strong>Chapter 1: Literature Review</strong></td>
<td>1</td>
</tr>
<tr>
<td>I. Cell cycle regulation of mammalian female meiosis</td>
<td>1</td>
</tr>
<tr>
<td>II. Cell cycle regulation by MASTL, ENSA and ARPP19</td>
<td>11</td>
</tr>
<tr>
<td>III. Introduction to the Research Presented in this Thesis</td>
<td>28</td>
</tr>
<tr>
<td>IV. References</td>
<td>30</td>
</tr>
<tr>
<td>V. Tables and Figures</td>
<td>48</td>
</tr>
<tr>
<td><strong>Chapter 2: Characterization of the MASTL-ENSA/ARPP19 pathway during meiotic maturation in mouse oocytes</strong></td>
<td>55</td>
</tr>
<tr>
<td>I. Introduction</td>
<td>55</td>
</tr>
<tr>
<td>II. Materials &amp; Methods</td>
<td>59</td>
</tr>
<tr>
<td>III. Results</td>
<td>74</td>
</tr>
<tr>
<td>IV. Discussion</td>
<td>80</td>
</tr>
<tr>
<td>V. References</td>
<td>88</td>
</tr>
<tr>
<td>VI. Tables and Figures</td>
<td>96</td>
</tr>
</tbody>
</table>
Chapter 3: Characterization of α-endosulfine (ENSA) during meiotic maturation: ENSA regulates exit from prophase I arrest in mouse oocytes ...................................................121

I. Introduction ......................................................................................................121
II. Materials & Methods.......................................................................................126
III. Results............................................................................................................133
IV. Discussion......................................................................................................138
V. References......................................................................................................145
VI. Tables and Figures.........................................................................................156

Chapter 4: Conclusions and Discussion.................................................................173

Curriculum Vitae ............................................................................................................190
LIST OF TABLES

Table 1.1: Overview of species-specific nomenclature for the MASTL/Greatwall pathway components .................................................................48

Table 2.1: Primer sequences for cloning of *Ensa* and *Arpp19* into the PGEX-4T-1 vector ...........................................................96

Table 2.2: Primer sequences for knockdown assessment by RT-PCR ..........97

Table 2.3: Primer sequences for detection of *Ensa* and *Arpp19* transcript variants ..........98

Table 2.4: Summary of experiments assessing knockdown of *Arpp19* at the RNA level in mouse oocytes .................................................................99

Table 2.5: Comparison of quantitative studies assessing ENSA and ARPP19 expression in cultured cells, other abundant proteins in mouse oocytes and the amount of ENSA per mouse oocyte .................................................................100

Table 3.1: Primer sequences for cloning of *Ensa* and *Arpp19* into the PGEX-4T-1 vector .................................................................156

Table 3.2: Primer sequences for knockdown assessment by RT-PCR ...............157
LIST OF FIGURES

Figure 1.1: Overview of mammalian female meiosis .......................................................49

Figure 1.2: Molecular regulation of prophase I arrest .......................................................51

Figure 1.3: The MASTL/Greatwall pathway as a regulator of mitosis .........................53

Figure 2.1: MASTL is expressed by mouse oocytes ......................................................102

Figure 2.2: Knockdown of Mastl in mouse oocytes .......................................................104

Figure 2.3: Ability to exit from prophase I arrest is impaired in MASTL-deficient oocytes ..........................................................................................................................................106

Figure 2.4: Assessment of MASTL knockdown in individual oocytes .........................108

Figure 2.5: Sequence alignment of mouse ENSA and ARPP19 .....................................110

Figure 2.6: Assessment of specificity of anti-ENSA and anti-ARPP19 antibodies using recombinant proteins .......................................................................................................112

Figure 2.7: Characterization of MASTL substrates, ENSA and ARPP19, in mouse oocytes and somatic cultured cells ..................................................................................114

Figure 2.8: Knockdown of Arpp19 in mouse oocytes and 3T3 cells .........................116

Figure 2.9: Quantification of ENSA in mouse oocytes .................................................119

Figure 3.1: ENSA is expressed by mouse oocytes .........................................................158

Figure 3.2: Knockdown of Ensa in mouse oocytes .........................................................160

Figure 3.3: Ability to exit from prophase I arrest is impaired in ENSA-deficient oocytes .
Figure 3.4: Anti-ENSA immunofluorescence in mouse oocytes .....................................164

Figure 3.5: Progression through meiosis in control and Ensa siRNA-injected oocytes ......

Figure 3.6: Suppression of PP2A activity restores the ability of Ensa siRNA-injected oocytes to exit from prophase I arrest and progress through meiosis I .....................169

Figure 3.7: Knockdown of the PP2A-B55δ regulatory subunit (Ppp2r2d) restores the ability of Ensa siRNA-injected oocytes to exit from prophase I arrest and progress through meiosis I .................................................................171
CHAPTER 1: LITERATURE REVIEW

I. CELL CYCLE REGULATION OF MAMMALIAN FEMALE MEIOSIS

I.A. Overview of mammalian female meiosis

During meiosis, cells undergo two consecutive cell divisions without an intervening S-phase to form a haploid gamete. In the female gamete, these divisions are carefully regulated both temporally and spatially. Each division is asymmetric, resulting in a large oocyte and a small polar body (Brunet and Maro 2005). An overview of mammalian female meiosis is provided in Figure 1.1.

In the adult ovary, the mammalian oocyte develops in a compartment called the ovarian follicle, and the oocyte itself is arrested in prophase of meiosis I. This arrest persists until the time of ovulation, when the oocyte exits from this arrest and completes the first meiotic division. The egg then arrests at the metaphase II stage. The mammalian female gamete is referred to as an oocyte until meiosis I is completed and is then referred to as an egg. The process whereby the metaphase II egg is formed from the prophase I oocyte is referred to as meiotic maturation or oocyte maturation (Conti, Hsieh et al. 2012). Metaphase II arrest is maintained until fertilization occurs; during the egg-to-embryo transition the fertilizing sperm triggers completion of the second meiotic division and formation of the haploid gamete (Nader, Kulkarni et al. 2013).
I.B. Oogenesis and arrest at the prophase I stage

Mammalian oocytes originate from primordial germ cells in the fetal ovary during the process of oogenesis. Each primary oocyte is surrounded by epithelial cells that form the ovarian follicle, which at this stage is referred to as a primordial follicle (Jamnongjit and Hammes 2005). Following DNA replication, meiosis begins in the fetal ovary. Oocytes progress through the leptotene, zygotene and pachytene stages where chromosome pairing and genetic recombination by crossing over occurs, and arrest at the diplotene stage of prophase I. Prophase I arrest extends from fetal life through adulthood and can last for last for months to years depending on the species (Sagata 1996; Mehlmann 2005; Oktem and Urman 2010; Tripathi, Kumar et al. 2010; Holt, Lane et al. 2013).

During the majority of this period of prophase I arrest, the oocyte exists in a quiescent state. The primordial follicle pool provides a source of developing follicles and oocytes during the reproductive lifespan. The mechanism for the initiation of follicular growth from the primordial follicle stage is not completely understood, but is known to occur over a prolonged period of time (McGee and Hsueh 2000; Adhikari and Liu 2014). During this process, a subset of primordial follicles enter the growing follicle pool where the oocyte increases in size and accumulates RNA transcripts required for meiotic progression and early embryonic development (Moore, Lintern-Moore et al. 1974; Sorensen and Wassarman 1976; Holt, Lane et al. 2013). The oocyte is now referred to as “meiotically competent” and can respond to the hormonal cues that regulate meiotic resumption (Holt, Lane et al. 2013). The development of meiotic competence occurs
around the same time as antrum formation within the ovarian follicle. During follicular growth, the follicular somatic cells divide to form multiple layers and a fluid-filled antral cavity is formed. Early antral follicles have a small antral cavity; follicles at this stage can be recruited for further growth to the antral or preovulatory stage in response to gonadotropin signaling (discussed below in Section I.D.). The mature ovarian follicle consists of two main somatic cell populations: cumulus cells that surround the oocyte and granulosa cells that line the ovarian follicle (Mehlmann 2005). The oocyte itself is surrounded by the zona pellucida, a glycoprotein coat that is secreted by the oocyte (Picton, Briggs et al. 1998). Prophase I arrest is maintained during this entire period of preovulatory growth; the prophase I-arrested oocyte is characterized by a prominent germinal vesicle (GV) or nucleus and is also referred to as the germinal vesicle-intact (GVI) oocyte (Holt, Lane et al. 2013).

I.C. Molecular regulation of prophase I arrest

The molecular regulation of prophase I arrest requires coordination between the oocyte itself and the somatic cells of the ovarian follicle. This process, involving the cyclic nucleotide second messengers, cyclic AMP (cAMP) and cyclic GMP (cGMP), regulates the oocyte cell cycle machinery resulting in maintenance of arrest at the prophase I stage. The molecular regulation of prophase I arrest is summarized in Figure 1.2. High levels of cAMP within the oocyte are critical for the maintenance of prophase I arrest. cAMP is produced within the oocyte by adenylyl cyclase (AC), which is stimulated by a $G_\text{s}$-linked G-protein coupled receptor. In the mouse, the $G_\text{s}$-linked G-
protein coupled receptor, GPR3, is necessary and sufficient for maintenance of meiotic arrest and resumption. Mouse oocytes lacking GPR3 exit from prophase I arrest and resume meiosis in the absence of normal physiological cues (Mehlmann, Saeki et al. 2004; Hinckley, Vaccari et al. 2005; Ledent, Demeestere et al. 2005; Mehlmann 2005; Conti, Hsieh et al. 2012). There is evidence to suggest that GPR3 also regulates meiotic arrest in human and *Xenopus* oocytes (Deng, Lang et al. 2008; DiLuigi, Weitzman et al. 2008). Another GPR isoform, GPR12, plays a similar role to GPR3 in rat oocytes (Hinckley, Vaccari et al. 2005). The adenylyl cyclase isoform, AC3, is also essential for maintenance of prophase I arrest and mice lacking AC3 cannot maintain prophase I arrest within the ovarian follicle (Horner, Livera et al. 2003). While the oocyte itself is capable of producing the cAMP required for maintenance of prophase I arrest, removal of the oocyte from the ovarian follicle results in meiotic resumption.

Coordination between the oocyte and the somatic cells of the ovarian follicle is centered on the regulation of phosphodiesterases that hydrolyze cAMP. Mouse oocytes express the phosphodiesterases, PDE3A, and *Pde3a*-null oocytes have elevated cAMP levels and do not undergo meiotic maturation (Masciarelli, Horner et al. 2004; Vaccari, Horner et al. 2008). Recent studies have shown that the PDE3A inhibitor, cGMP, passes from the cumulus cells to the oocyte via gap junctions. Granulosa cells express natriuretic peptide precursor type C (NPPC), which acts on the NPPC receptor, NPR2, in the neighboring cumulus cells to promote cGMP production. This communication between the follicular somatic cells and the oocyte is bidirectional; oocyte-derived paracrine factors promote expression of *Npr2* mRNA in cumulus cells (Zhang, Su et al. 2010; Wigglesworth, Lee et al. 2013). In vitro, meiotic arrest of oocytes isolated from ovarian
follicles can be sustained by maintenance of high cAMP levels using cAMP analogs, such as dibutyryl cAMP (dbcAMP), or phosphodiesterase inhibitors, 3-isobutyl-1-methylxanthine (IBMX) or milrinone (Conti, Andersen et al. 2002).

Prophase I arrest is analogous to the G2 stage of the mitotic cell cycle. Like the G2-M transition in mitotic cells, exit from prophase I arrest is triggered by CDK1 activity (also known as maturation-promoting factor, MPF) (Holt, Lane et al. 2013). MPF was initially characterized in frog oocytes and was named maturation-promoting factor based on the ability of this factor to induce oocyte meiotic maturation (Masui and Markert 1971). Elevated levels of cAMP within the oocyte activate protein kinase A (PKA), which suppresses CDK1 activity through two mechanisms (Schindler 2011; Conti, Hsieh et al. 2012). Firstly, PKA phosphorylates the kinases MYT1 and WEE1 (in mouse previously known as WEE1B, now known as WEE2), which inhibit CDK1 through phosphorylation on two inhibitory residues, threonine 14 and tyrosine 15. PKA can also phosphorylate and inhibit the activity of the phosphatase CDC25; when active, CDC25 can activate CDK1 through removal of inhibitory phosphates (Han, Chen et al. 2005; Pirino, Wescott et al. 2009; Oh, Han et al. 2010; Schindler 2011). In mouse oocytes, CDC25B is the CDC25 isoform required for prophase I arrest and meiotic resumption. Cdc25b-null mice are sterile and CDC25A or CDC25C are not able to compensate for the lack of CDC25B (Lincoln, Wickramasinghe et al. 2002). By activating a negative regulator of CDK1 activity (WEE1 and MYT1) and inactivating a positive regulator of CDK1 activity (CDC25), PKA keeps CDK1 activity low in the prophase I-arrested oocyte.
CDK1 activity requires the binding of the regulatory subunit cyclin B. A secondary mechanism for the regulation of CDK1 activity involves regulation of levels of cyclin B. Regulated synthesis and degradation of cyclin B1 ensures that CDK1 activity is limited until required (Polanski, Ledan et al. 1998; Ledan, Polanski et al. 2001; Schindler 2011). Proteasomal degradation is driven by the anaphase-promoting complex/cyclosome (APC/C), a multi-subunit E3 ubiquitin ligase complex that targets mitotic proteins for degradation. The APC/C coactivator proteins, CDC20 or FZR1 (also known as CDH1), are critical for the activity of APC/C complex. Oocytes lacking FZR1 prematurely exit from prophase I arrest due to increased cyclin B levels (Reis, Chang et al. 2006; Holt, Tran et al. 2011; Jones 2011). In mouse oocytes, the phosphatase CDC14B also promotes FZR1 binding to the APC, and CDC14B-deficient oocytes undergo premature meiotic maturation (Schindler and Schultz 2009).

I.D. Molecular regulation of exit from prophase I arrest and progression through meiosis

Meiosis resumption occurs in response to gonadotropin hormone signaling. A surge of lutenizing hormone (LH) activates a series of signaling cascades that culminate in meiotic resumption and ovulation. The oocyte does not possess gonadotropin receptors, so hormone signaling acts on the somatic follicular cells to regulate this process (Amsterdam, Koch et al. 1975; Dekel, Galiani et al. 1988; Zhang, Ouyang et al. 2009). The action of gonadotropins on the somatic follicle cells ultimately results in a decrease in cAMP levels within the oocyte through several mechanisms. In response to
the LH surge, cGMP influx into the oocyte is reduced due to decreased NPR2 activity and ligand availability (NPPC), as well as closure of gap junctions between the oocyte and granulosa cells (Norris, Ratzan et al. 2009; Vaccari, Weeks et al. 2009; Robinson, Zhang et al. 2012). Decreased cGMP levels allow PDE3A activity to increase and results in a drop in cAMP levels within the oocyte.

This drop in cAMP levels within the oocyte leads to decreased PKA activity. As PKA activity decreases, the inhibition of CDK1 through WEE1, MYT1 and CDC25 also decreases, and CDK1 activity begins to rise. Without PKA activity, the kinases WEE1 and MYT1 are now inactive and no longer phosphorylate CDK1 on the inhibitory residues threonine 14 and tyrosine 15 (Han, Chen et al. 2005; Pirino, Wescott et al. 2009; Oh, Han et al. 2010). Active CDC25 phosphatase (specifically CDC25B in mouse oocytes) can then activate CDK1 (Lincoln, Wickramasinghe et al. 2002). At the same time, cytoplasmic cyclin B1 translocates to the nucleus to form active CDK1-cyclin B1 complexes. As CDK1 activity rises, the activity of the APC/C is decreased, allowing for cyclin B1 accumulation (Marangos and Carroll 2004; Reis, Chang et al. 2006; Holt, Weaver et al. 2010; Polanski, Homer et al. 2012).

The activation of CDK1 triggers phosphorylation of CDK1 substrates, leading to M-phase initiation. Phosphorylation of these M-phase substrates results in nuclear envelope breakdown, chromosome condensation, and spindle assembly (Philpott and Yew 2008). Some examples of CDK1 substrates include the CDK1 regulators, WEE1 and MYT1, and microtubule-associated proteins, such as kinesin-related motor proteins and microtubule-binding proteins (Nigg 2001). Phosphorylation of nuclear lamins by CDK1 leads to destabilization of the nuclear envelope structure leading to one of the first
observable hallmarks of M-phase entry, nuclear envelope breakdown (Peter, Nakagawa et al. 1990; Enserink and Kolodner 2010; Adhikari, Zheng et al. 2012). In the mouse oocyte the process of nuclear envelope breakdown is referred to as germinal vesicle breakdown (GVBD) (Figure 1.1).

During the first meiotic division, homologous chromosomes divide, giving rise to a large egg and a small polar body. At this stage, sister chromatids remain attached and will separate during the second meiotic division (Holt and Jones 2009). After completion of meiosis I, oocytes directly enter into metaphase II arrest without an intervening S-phase. The mammalian female gamete is referred to as an oocyte until meiosis I is completed and is then referred to as the metaphase II egg. In the brief period between meiosis I and meiosis II, CDK1 activity only partly declines before progression into meiosis II (Hampl and Eppig 1995; Holt, Lane et al. 2013)

I.E. Maintenance of metaphase II arrest

The metaphase II-arrested egg has high CDK1 (MPF) activity and a fully formed meiotic spindle. Metaphase II arrest can last for several hours and elevated MPF activity is a unique feature of this arrest in oocytes; in mitotic cells APC/C activity would result in cyclin B degradation and mitotic exit (Madgwick and Jones 2007). Inhibition of APC/C activity and maintenance of high levels of MPF activity is critical for maintenance of metaphase II arrest. During metaphase II arrest in mouse oocytes, APC/C-dependent cyclin B degradation does occur to some extent, and continuous

Metaphase II arrest is maintained by the activity of a cytoplasmic factor called cytostatic factor (Masui and Markert 1971). The full characterization of CSF activity and the molecular basis for the maintenance of metaphase II arrest is still ongoing, although some important components have been identified. The c-Mos-MAPK pathway appears to be a regulator of this process and depletion of c-Mos from *Xenopus* oocytes caused a loss of arrest (Sagata, Watanabe et al. 1989; Madgwick and Jones 2007). Oocytes from *c-Mos* knockout mice can maintain metaphase II arrest for two to four hours before undergoing parthenogenetic activation suggesting c-Mos is important to arrest maintenance, but not the initial establishment (Colledge, Carlton et al. 1994; Hashimoto, Watanabe et al. 1994; Verlhac, Kubiak et al. 1996; Madgwick and Jones 2007). Emi2 (early mitotic inhibitor 1-related protein) is another regulator of metaphase II arrest that acts as an APC/C inhibitor (Wu and Kornbluth 2008). Emi2 is critical for maintenance of metaphase II arrest in mouse oocytes, as depletion of Emi2 resulted in an inability to establish arrest or premature meiotic exit (Madgwick, Hansen et al. 2006; Shoji, Yoshida et al. 2006; Madgwick and Jones 2007).

**I.F. Exit from metaphase II arrest and completion of meiosis II**

Metaphase II arrest is maintained until the time of fertilization. If fertilization occurs, the fertilizing sperm triggers a rise in intracellular calcium resulting in exit from metaphase II and completion of meiosis (Jones 2004). This egg-to-embryo transition is
also referred to as egg activation. In mammals, sperm fusion delivers a sperm-specific form of phospholipase C (PLCζ), triggering an IP3-dependent release of calcium from endoplasmic reticulum stores. Calcium release activates calmodulin-dependent protein kinase II (CaMKII) (Markoulaki, Matson et al. 2003; Wakai, Vanderheyden et al. 2011). CaMKII activates a number of downstream signaling cascades, including activation of WEE1B, decreased mitogen-activated protein kinase (MAPK) signaling and activation of the APC/C. These signaling cascades trigger the hallmark events of egg activation, including meiotic resumption and progression into the first embryonic division (Krauchunas and Wolfner 2013; Nader, Kulkarni et al. 2013).
II. CELL CYCLE REGULATION BY MASTL, ENSA AND ARPP19

II.A. Balancing kinase and phosphatase activity during mitosis

Mitotic entry and progression depends on the phosphorylation of key M-phase substrates by cyclin-dependent kinase 1 (CDK1; also called CDC2). (NOTE: Species-specific nomenclature rules are followed when discussing studies in different species. Mouse nomenclature guidelines are used as the default for general discussions of genes/proteins. An overview of species-specific nomenclature rules is provided in Table 1.1.) Mammals express multiple CDKs, but CDK1 is the only CDK required for progression through M-phase (Santamaria, Barrière et al. 2007; Álvarez-Fernández and Malumbres 2014). While it has long been appreciated that activation of CDK1 is sufficient to drive mitotic progression, more recent studies have revealed that inhibition of the anti-mitotic protein phosphatases is also required. Phosphorylation of key mitotic phosphoproteins leads to progression into M-phase and inhibition of the dephosphorylation of these CDK1-substrates during M-phase is critical to maintain their net phosphorylation status. The balance between kinase activity and phosphatase activity ensures that M-phase substrates that are phosphorylated remain phosphorylated until M-phase exit (Johnson and Kornbluth 2012).

Dephosphorylation of CDK1 substrates is performed by protein phosphatases. The main phosphatase of interest for this literature review is PP2A. PP2A is a heterotrimer composed of a catalytic (C) subunit, a scaffold (A) subunit and a regulatory (B) subunit (Virshup and Shenolikar 2009). In mammals, there are two PP2A catalytic
(C) subunits (PPP2CA and PPP2CB) and two scaffold (A) subunits (PPP2R1A and PPP2R1B). Four families of regulatory subunits have been identified, B (B55; PPP2R2A, -B, -C, -D), B' (PPP2R5A, -B, -C, -D, -E), B'' (B56; PPP2R3A, -B, -C) and B''' (striatins).

Each regulatory subunit family has multiple isoforms and in combination with the different catalytic and scaffold subunits, it is possible to form a large number of potential PP2A heterotrimer combinations (Shi 2009; Virshup and Shenolikar 2009; Johnson and Kornbluth 2012).

Studies in *Xenopus* egg extracts revealed that protein phosphatases active against mitotic phosphoproteins were active during interphase, but inactive during mitosis (Mochida and Hunt 2007; Mochida, Ikeo et al. 2009). PP2A associated with the regulatory subunit B55δ was identified as the specific phosphatase of interest in *Xenopus* extracts; depletion of PP2A-B55δ from interphase extracts resulted in accelerated entry into mitosis, while adding PP2A-B55δ to extracts delayed or suppressed mitotic entry (Mochida, Ikeo et al. 2009). Genetic studies in *Drosophila* also showed mitotic defects in mutants of the single fly B55 isoform, *twins* (Mayer-Jaekel, Ohkura et al. 1994).

While these studies established that inhibition of protein phosphatase activity is required for mitotic entry and progression, they did not address the mechanism of this inhibition. The mechanism of PP2A inhibition centers around the MASTL/Greatwall pathway discussed below in Section II.B.
II.B. Discovery of the MASTL/Greatwall pathway as a regulator of cell cycle progression

Greatwall (Gwl), a serine/threonine protein kinase, was originally identified in *Drosophila* as a regulator of mitotic progression (Yu, Fleming et al. 2004). Greatwall is the name used for the kinase in *Drosophila* and *Xenopus*. The official name for this kinase is MASTL (microtubule-associated serine/threonine kinase-like) in mouse [MGI:1914371] and human [HGNC:19042] (see also Table 1.1). Mutations in *greatwall* resulted in chromosome condensation defects and delays in cell cycle progression in *Drosophila* larval neuroblasts. A fraction of cells were arrested at the G2/M transition indicating that nuclear envelope breakdown was delayed and mitotic entry did not occur normally. The gene was named *greatwall* because the mutant phenotype suggested a role in protecting chromosome structure (Yu, Fleming et al. 2004). Studies in *Xenopus* egg extracts extended these findings and demonstrated that Greatwall is required for M-phase entry and maintenance. Depletion of Greatwall from arrested CSF extracts (arrested in M-phase) resulted in M-phase exit, and depletion from cycling interphase extracts prevented entry into M-phase (Yu, Zhao et al. 2006). Activated Greatwall was able to accelerate the G2/M transition in cycling extracts, demonstrating that Greatwall is sufficient to cause premature onset of mitosis. Addition of activated Greatwall mimicked the effects of the phosphatase inhibitor okadaic acid, leading to a model where Greatwall inhibits an unknown phosphatase that is critical for M-phase (Zhao, Haccard et al. 2008).
II.C. MASTL/Greatwall functions as an inhibitor of PP2A

The phosphatase regulated by Greatwall was identified to be PP2A with B55δ the regulatory subunit. These studies, performed in *Xenopus* egg extracts, linked Greatwall to the PP2A pathway described above in Section II.B. PP2A-dependent dephosphorylation of MPF substrates was increased in Greatwall-depleted extracts (Castilho, Williams et al. 2009; Vigneron, Brioudes et al. 2009). Removal of PP2A-B55δ rescued the defect in mitotic entry phenotype seen in Greatwall-depleted extracts (Castilho, Williams et al. 2009). Greatwall itself is activated by MPF, providing a mechanism to couple MPF activation and PP2A inactivation during M-phase (Zhao, Haccard et al. 2008). One study reported that Greatwall itself is part of MPF; MPF activity is undetectable (by assessment of phosphorylation of M-phase substrates) in starfish oocytes when Greatwall is inhibited, despite normal Cdk1-cyclin B activity. The authors propose that MPF is made up of both kinases, with one regulating mitotic entry and the other ensuring inhibition of PP2A-B55 activity (Hara, Abe et al. 2012).

As noted above in Section II.B and in Table 1.1, MASTL is the mammalian ortholog of Greatwall (Burgess, Vigneron et al. 2010; Voets and Wolthuis 2010). MASTL depletion by RNAi in human cell lines resulted in a G2 delay. MASTL-deficient cells that were able to enter mitosis displayed defects in chromosome condensation, spindle formation and cytokinesis. Despite normal CDK1 activity, the phosphorylation of CDK1-substrates is decreased in MASTL-deficient cells (Voets and Wolthuis 2010). The mitotic defects observed in MASTL-deficient cells could be
rescued by suppression of PP2A using siRNA knockdown of PP2A or the phosphatase inhibitor, okadaic acid (Burgess, Vigneron et al. 2010).

The MASTL pathway has also been examined in the mouse. Mastl-knockout mice displayed embryonic lethality (Álvarez-Fernández, Sánchez-Martínez et al. 2013). The study used conditional knockout mouse embryonic fibroblasts (MEFs) generated using G0-arrested MEFs with a loxP-flanked Mastl gene and adenovirus-driven Cre recombinase expression to explore the function of MASTL in mouse cells. Cells were stimulated with serum to reenter the cell cycle and mitotic entry and progression was examined. Surprisingly, mitotic entry was unaffected in Mastl-deficient MEFs compared to controls (Álvarez-Fernández, Sánchez-Martínez et al. 2013). Mitotic entry was impaired when MASTL/Greatwall function was inhibited in Drosophila, Xenopus and human cells, and it is unclear whether the lack of mitotic entry phenotype observed in the mouse is due to a species-specific difference in MASTL function or to the particular cellular context and experimental manipulation used in this study (Yu, Fleming et al. 2004; Yu, Zhao et al. 2006; Zhao, Haccard et al. 2008; Castilho, Williams et al. 2009; Burgess, Vigneron et al. 2010; Voets and Wolthuis 2010). The majority of Mastl-deficient MEFs did display defects in mitotic progression, including defects in chromosome condensation, pro-metaphase arrest and chromosome segregation. In addition, Mastl-deficient cells displayed reduced levels of phosphorylated CDK substrates, which suggests that PP2A function was unopposed in these Mastl-deficient cells. siRNA-mediated knockdown of the B55 regulatory subunits of PP2A rescued the defect in phosphorylation of CDK substrates, as well as the mitotic defect in chromosome condensation (Álvarez-Fernández, Sánchez-Martínez et al. 2013).
In mammalian cells, there is some evidence to suggest that the MASTL pathway serves to inhibit PP2A in complex with regulatory subunits other than the B55δ subunit (gene name is PPP2R2D) identified in *Xenopus*. Two regulatory subunits, PPP2R2D/B55δ and PPP2R2A/B55α, have been implicated in the regulation of mitotic exit in experiments using human and mouse cells (Manchado, Guillamot et al. 2010; Hegarat, Vesely et al. 2014). One study found that both PPP2R2A/B55α and PPP2R2B/B55β regulate mitotic exit in human cells, although this study was not looking specifically at the MASTL pathway during mitotic exit (Schmitz, Held et al. 2010). In the mouse, knockdown of four B55 regulatory subunits (B55α, β, γ, δ) rescued mitotic defects observed in MASTL-deficient cells (Álvarez-Fernández, Sánchez-Martínez et al. 2013). Knockdown of individual B55 regulatory subunits was not performed, so it is unclear whether the MASTL pathway in the mouse regulates PP2A in complexes with all four of these regulatory subunits.

II.D. The localization of MASTL/Greatwall during mitosis

In human, starfish and *Drosophila* cells, MASTL/Greatwall is localized to the nucleus during interphase (Yu, Fleming et al. 2004; Voets and Wolthuis 2010; Hara, Abe et al. 2012). In human cells, following nuclear envelope breakdown, MASTL becomes cytoplasmic with a fraction of protein localized to the centrosomes, mitotic spindle poles and spindle microtubules. During telophase, MASTL localized to the cytokinetic cleavage furrow (Burgess, Vigneron et al. 2010; Voets and Wolthuis 2010). In *Drosophila* early embryos, Greatwall becomes cytoplasmic following nuclear envelope
breakdown, but the centrosome, spindle and spindle mid-body localization was not observed (Wang, Galan et al. 2013).

MASTL/Greatwall function requires these dynamic cell cycle-regulated changes in localization, based on the finding that interference with this pattern of localization results in mitotic defects (Álvarez-Fernández, Sánchez-Martínez et al. 2013; Wang, Galan et al. 2013). Expression of a mutant form of human MASTL with a deleted nuclear localization signals (NLS) in a MASTL-deficient background could rescue some, but not all mitotic defects observed in these cells (Álvarez-Fernández, Sánchez-Martínez et al. 2013). Similar results were observed in studies in Drosophila cells (Wang, Galan et al. 2013). These results indicate that nuclear localization is important for MASTL/Greatwall function during mitosis. MASTL/Greatwall requires access to the nucleus during interphase and is shuttled to the cytoplasm during prophase, right before nuclear envelope breakdown occurs. The nuclear export of MASTL/Greatwall occurs shortly before nuclear envelope breakdown and after the nuclear accumulation of cyclin B (Álvarez-Fernández, Sánchez-Martínez et al. 2013; Wang, Galan et al. 2013). CDK1 phosphorylation of MASTL appears to be required for nuclear export based on the finding that mutation of the MASTL CDK1-phosphorylation site inhibits nuclear export (Álvarez-Fernández, Sánchez-Martínez et al. 2013). These studies present a model where MASTL/Greatwall is activated by CDK1 phosphorylation in the nucleus and is then exported to the cytoplasm where it acts to inhibit PP2A-B55 (Álvarez-Fernández, Sánchez-Martínez et al. 2013; Wang, Galan et al. 2013).
II.E. Identification of MASTL/Greatwall substrates, ENSA and ARPP19

Biochemical and genetic data have implicated MASTL/Greatwall as a negative regulator of PP2A activity, but Greatwall was not shown to phosphorylate PP2A directly (Virshup and Kaldis 2010). The mechanism for Greatwall inhibition of PP2A was addressed in two separate studies that used *Xenopus* extracts to identify Greatwall substrates (Gharbi-Ayachi, Labbé et al. 2010; Mochida, Maslen et al. 2010). Two small proteins, cAMP-regulated phosphoprotein-19 (Arpp19) and alpha-endosulfine (Ensa), were identified as substrates of Greatwall by incubating active Greatwall with *Xenopus* extracts and analyzing phosphorylated proteins by mass spectrometry. Greatwall-phosphorylated Ensa was able to bind PP2A-B55δ, but did not bind to other regulatory or catalytic subunits suggesting that the interaction with PP2A-B55δ is specific (Mochida, Maslen et al. 2010). Addition of phosphorylated Ensa or Arpp19 (phosphorylated *in vitro* by Greatwall) to interphase extracts resulted in rapid mitotic entry (Gharbi-Ayachi, Labbé et al. 2010). An overview of the MASTL/Greatwall pathway is provided in Figure 1.3.

While these two different studies characterized the role of Ensa and Arpp19 in *Xenopus* extracts, they differed in their conclusions about the physiological relevance of the two substrates. In one study, depletion of Ensa from cycling *Xenopus* extracts inhibited M-phase entry (Mochida, Maslen et al. 2010), while the other found that immunodepletion of Arpp19, but not Ensa, abolished entry in M-phase (Gharbi-Ayachi, Labbé et al. 2010; Mochida, Maslen et al. 2010). Interestingly, addition of
phosphorylated Arpp19 or Ensa was able to rescue the mitotic entry phenotype seen in Greatwall-depleted extracts (Gharbi-Ayachi, Labbé et al. 2010).

ENSA and ARPP19 are part of an evolutionarily conserved family of cAMP-regulated phosphoproteins (Dulubova, Horiuchi et al. 2001). Both proteins were previously identified, although their role as MASTL/Greatwall substrates and inhibitors of PP2A activity was unknown until 2010 (Mochida, Ikeo et al. 2009; Gharbi-Ayachi, Labbé et al. 2010). ARPP19 was initially characterized in the mouse fetal brain as a potential neurotransmitter-mediated signaling molecule (Girault, Shalaby et al. 1988; Girault, Raisman-Vozari et al. 1989; Girault, Horiuchi et al. 1990) Alpha-endosulfine (ENSA) was originally identified in rat and pig brain samples and named based on its potential role as a regulator of a K\textsubscript{ATP}-coupled sulfonylurea receptor, which is targeted by a class of anti-diabetic drugs called sulfonylureas (Virsolvy-Vergine, Leray et al. 1992; Peyrollier, Héron et al. 1996; Virsolvy-Vergine, Salazar et al. 1996; Heron, Virsolvy et al. 1998). Interestingly, the Drosophila protein endos (an overview of the nomenclature used for MASTL/Greatwall substrates in different species is provided in Section II.F and Table 1.1) was identified as a regulator of oocyte meiotic maturation prior to its identification as a MASTL/Greatwall substrate (Von Stetina, Tranguch et al. 2008). The role of endos in Drosophila oocytes is discussed in greater detail in Section II.H.

II.F. MASTL/Greatwall substrates in different species

Different species express one or two MASTL/Greatwall substrates. An overview of species-specific nomenclature for the MASTL/Greatwall substrates is provided in
Table 1.1. *Drosophila* has a single gene encoding a Greatwall substrate called endos (Von Stetina, Tranguch et al. 2008; Kim, Bucciarelli et al. 2012). The starfish, *Patiria pectinifera*, also has a single ENSA/ARPP19 ortholog (Hara, Abe et al. 2012). *C. elegans* do not have an obvious Greatwall ortholog, but have a ENSA/ARPP19 ortholog that can be phosphorylated in vitro by *Drosophila* or *Xenopus* Greatwall (Kim, Bucciarelli et al. 2012). Mouse, human and *Xenopus* possess two substrates, ENSA and ARPP19 (Gharbi-Ayachi, Labbé et al. 2010; Mochida, Maslen et al. 2010). Interestingly, the pig Ensa gene has a premature stop codon and cells do not express a functional protein (Li, Kang et al. 2013). ENSA and ARPP19 share a high level of sequence similarity at the amino acid level. In the mouse, the amino acid sequence for ARPP19 and ENSA is 76% identical. These proteins are also highly conserved between species; for example, mouse ENSA and *Drosophila* Endos share 47% identity, and mouse and *Xenopus* Ensa are 85% identical.

Budding yeast also possesses components of the MASTL/Greatwall signaling pathway. *Saccharomyces cerevisiae* have a MASTL/Greatwall ortholog, Rim15, and two Rim15 substrates, Igo1 and Igo2. Rim15 is part of a nutrient-sensing pathway that regulates entry into quiescence (Talarek, Cameroni et al. 2010; Luo, Talarek et al. 2011; Juanes, Khoueiry et al. 2013). Igo1/2 promote activation of PP2A/Cdc55 (PP2A in complex with the Cdc55 regulatory subunit), the yeast PP2A phosphatase that is homologous to mammalian PP2A/B55. In yeast, PP2A/Cdc55 serves as a positive regulator of mitotic entry (Talarek, Cameroni et al. 2010; Bontron, Jaquenoud et al. 2013; Juanes, Khoueiry et al. 2013). Budding yeast also has two proteins, Zds1 and Zds2, that act as inhibitors of PP2A in a manner similar to ENSA and ARPP19. Zds1 and Zds2
have no sequence similarity to Igo1 and Igo2 and are part of two distinct signaling pathways in yeast (Bi and Pringle 1996; Yu, Jiang et al. 1996; Yasutis, Vignali et al. 2010).

II.G. Function of the MASTL/Greatwall substrates, ENSA and ARPP19, in species with two MASTL/Greatwall substrates

As discussed above in Section II.E, the two studies that identified Ensa and Arpp19 as Greatwall substrates reached different conclusions about the physiological relevance of the two substrates in *Xenopus* (Gharbi-Ayachi, Labbé et al. 2010; Mochida, Maslen et al. 2010; Haccard and Jessus 2011). An argument has been made in *Xenopus* that based on protein concentrations, dynamics of phosphorylation and depletion phenotype, that Arpp19 is the physiological Greatwall substrate (Haccard and Jessus 2011). Recent studies in *Xenopus* have focused on the role of Arpp19, but have not excluded a role for Ensa. Expression of a dominant-negative, non-phosphorylatable form of Arpp19 in *Xenopus* oocytes inhibited exit from prophase I arrest (Dupre, Buffin et al. 2013). It is unclear whether this dominant-negative form of Arpp19 could also impair Ensa function in this context. Arpp19 was also identified as a substrate of protein kinase A (PKA) in *Xenopus* oocytes. As discussed in Section I.C, PKA is an important regulator of prophase I arrest in oocytes. Arpp19 phosphorylated by PKA at serine 109 was shown to regulate the process of meiotic resumption from prophase I arrest (Dupre, Daldello et al. 2014). It is possible that Arpp19 is the main physiologically relevant substrate in *Xenopus*, but future studies should examine a potential role for Ensa in order to determine
any role that Ensa plays during mitotic and meiotic cell divisions. One recent study examined Ensa phosphorylation patterns at the Greatwall (serine 67), PKA (serine 109) and CDK1 (threonine/serine 28) phosphorylation sites in *Xenopus* oocytes at the prophase I and metaphase II stages, but did not look at the *in vivo* functional significance of these phosphorylation events (Mochida 2013).

There is also disagreement about the specific mitotic roles of ENSA and ARPP19 from experiments in human cells. In one study, depletion of ARPP19 from HeLa cells using siRNA-mediated knockdown resulted in reduced numbers of mitotic cells compared to controls suggesting that ARPP19 promotes mitotic entry (Gharbi-Ayachi, Labbé et al. 2010). In another study, also using HeLa cells, ARPP19 was not detected by immunoblotting and siRNA-mediated knockdown of ARPP19 had no effect on mitotic progression. This study used the dephosphorylation of PRC1, a component of the anaphase central spindle, as a readout of mitotic progression; temporal regulation of PRC1 dephosphorylation is necessary for chromosome segregation. Depletion of MASTL or ENSA by siRNA-mediated knockdown did alter the dephosphorylation of PRC1 and the authors suggest that ENSA is the major target of MASTL in HeLa cells (Cundell, Bastos et al. 2013).

It remains to be determined whether ENSA and ARPP19 are functionally redundant or serve distinct functions during M-phase. In yeast, the Rim15 substrates, Igo1 and Igo2, are functionally redundant and deletion of both Igo1 and Igo2 is required for a defective G₀ entry phenotype (Talarek, Cameroni et al. 2010). Zds1 and Zds2 are also functionally redundant and only the *zds1Δ/zds2Δ* double mutant shows defects in G₂ delay (Bi and Pringle 1996; Yu, Jiang et al. 1996).
II.H. The MASTL pathway during female meiosis

The MASTL/Greatwall pathway functions in oocytes of several species, including *Xenopus, Drosophila*, starfish and sea urchin (Archambault, Zhao et al. 2007; Von Stetina, Tranguch et al. 2008; Zhao, Haccard et al. 2008; Yamamoto, Blake-Hodek et al. 2011; Hara, Abe et al. 2012; Kim, Bucciarelli et al. 2012; Dupre, Buffin et al. 2013; Okumura, Morita et al. 2014). In *Xenopus* oocytes, injection of wild-type Greatwall or a hyperactive form of Greatwall was able to induce meiotic maturation to the same the extent as the normal meiotic maturation signal, progesterone (Zhao, Haccard et al. 2008; Yamamoto, Blake-Hodek et al. 2011). Arpp19 has been implicated as a critical regulator of meiotic maturation in *Xenopus* oocytes, but the role of Ensa has not been directly examined (discussed above in Section II.G.) (Dupre, Buffin et al. 2013; Dupre, Daldello et al. 2014).

In starfish oocytes, injection of Greatwall function-blocking antibodies resulted in a slight delay in nuclear envelope breakdown upon addition of the maturation-inducing signal (1-methyladenine), but did not impede the overall ability of oocytes to resume meiosis (Hara, Abe et al. 2012). However, when the analogous experiment was performed using Arpp19 function-blocking antibodies, nuclear envelope breakdown was inhibited. These results suggest that Arpp19, but not Greatwall, is required for resumption of meiotic maturation in starfish oocytes. This interpretation assumes that the Greatwall and ARPP19 antibodies have roughly equivalent function-blocking ability. It appears that in starfish oocytes, Cdk1-cyclin B can directly phosphorylate Arpp19 (as
noted above and in Table 1.1, there is one Greatwall substrate in starfish, which is referred to as Arpp19); this phosphorylation converts Arpp19 to a PP2A-B55 inhibitor without Greatwall phosphorylation. While entry into M-phase occurred without Greatwall phosphorylation, Greatwall was required for successful meiotic completion. In oocytes injected with a neutralizing antibody against Greatwall, Arpp19 phosphorylation at the Greatwall phosphorylation sites was blocked and chromosome segregation failure was observed (Okumura, Morita et al. 2014).

In *Drosophila*, the Greatwall substrate, Endos, is required for oocyte meiotic maturation (Von Stetina, Tranguch et al. 2008; Kim, Bucciarelli et al. 2012). The majority of endos mutant oocytes arrested at the prophase I stage and oocytes that progressed to the metaphase I stage displayed severe spindle defects. In addition, these oocytes have reduced levels of M-phase phosphoproteins despite normal levels of Cdk1 activity. The function of Endos appears to be evolutionarily conserved and expression of human ENSA rescued the meiotic maturation defects in endos mutant oocytes (Von Stetina, Tranguch et al. 2008). Like Endos, Greatwall is also required for meiotic maturation in fly oocytes and *Greatwall* mutant oocytes displayed meiotic defects (Archambault, Zhao et al. 2007; Kim, Bucciarelli et al. 2012). Greatwall phosphorylation of Endos at serine 68 is required for meiotic maturation and expression of a non-phosphorylatable form of Endos could not rescue meiotic maturation defects. Interestingly, heterozygosity for *twins* (the fly’s sole PP2A-B55 subunit) suppressed meiotic maturation defects observed in *Greatwall* mutants, but not in endos mutants. These results suggest that Greatwall functions to oppose PP2A-B55 activity in oocytes, but Endos may also have additional functions beyond regulation of PP2A-B55 activity.
(Kim, Bucciarelli et al. 2012). One possible explanation may be that Endos was shown to interact with the E3 ligase Elgi and may mediate oocyte protein dynamics through this interaction (Von Stetina, Tranguch et al. 2008).

There are limited data about the role of MASTL pathway during mammalian female meiosis and a recent study examined the MASTL pathway in pig oocytes (Li, Kang et al. 2013). In MASTL-knockdown porcine oocytes, approximately 55% of oocytes arrested at prophase I (germinal vesicle-intact stage) or germinal vesicle breakdown stage compared to approximately 18% of control oocytes. Of the MASTL-deficient oocytes that were able to exit from prophase I arrest, 65% had spindle, chromosome condensation and congression defects. Overexpression of porcine MASTL resulted in meiotic exit while oocytes were still in arrest-maintaining culture conditions. The porcine *Ensa* gene has a premature stop codon and ENSA is not produced suggesting that ARPP19 is the sole MASTL substrate in the pig (Li, Kang et al. 2013).

II.I. The MASTL/Greatwall pathway during mitotic exit

The MASTL/Greatwall pathway is critical for the temporal regulation of mitotic exit and interference with this pathway leads to chromosome segregation defects and cytokinesis failure in human cultured cells (Cundell, Bastos et al. 2013). It is assumed that the MASTL/Greatwall pathway must be reset to allow exit from M-phase and progression into the next cell cycle, but the molecular mechanisms underlying this aspect of mitotic exit is unclear. Recent studies have attempted to identify the phosphatases responsible for the dephosphorylation of MASTL/Greatwall and its substrates at mitotic
exit. In human cells, depletion of the B55α and δ regulatory subunits of PP2A inhibited the dephosphorylation of MASTL at the CDK1-phosphorylation site at the time of mitotic exit suggesting that both PP2A-B55α and PP2A-B55δ regulate the dephosphorylation of MASTL (Hegarat, Vesely et al. 2014). Although these results initially appeared to be counterintuitive, the same PP2A-B55 phosphatase that the MASTL/Greatwall pathway inhibits during mitosis is also responsible for shutting off this pathway to allow mitotic exit (Hegarat, Vesely et al. 2014; Williams, Filter et al. 2014).

The mechanism for dephosphorylation of the MASTL/Greatwall substrates, ENSA and ARPP19 is less clear. The MASTL/Greatwall phosphorylation site is highly conserved between ENSA and ARPP19 and the antibodies used in these studies could not distinguish between the two substrates (Hegarat, Vesely et al. 2014). One study using human HeLa cells and in vitro phosphatase assays found that ENSA/ARPP19 were dephosphorylated by the phosphatase Fcp1, a RNA polymerase II C-terminal tail domain phosphatase that was recently implicated in the regulation of mitotic exit (Visconti, Palazzo et al. 2012; Hegarat, Vesely et al. 2014). A second study performed using Drosophila cell extracts, refuted these findings and instead identified PP2A-B55 as the main phosphatase regulating Endos dephosphorylation. The kinetic parameters of the interaction of phosphorylated Endos (pEndos) and PP2A-B55 help explain how pEndos can be both an inhibitor and substrate of PP2A-B55; pEndos binds tightly to the PP2A-B55 heterotrimer at its active site, but is slowly dephosphorylated compared to other PP2A-B55 substrates (Williams, Filter et al. 2014). In addition, it is estimated that there is a 5:1 ratio of Endos (0.5-1 µM) to B55 (0.1-0.25 µM) in Drosophila S2 cells and
*Xenopus* cell extracts and this molar excess of Endos allows it to function as a potent stoichiometric inhibitor of PP2A-B55. The model presented by this work predicts that at the time of mitotic exit, Greatwall is dephosphorylated and can no longer phosphorylate and activate Endos. The levels of pEndos will then decline as PP2A-B55 dephosphorylates pEndos and this pool of phosphorylated protein is not replenished (Williams, Filter et al. 2014).
III. INTRODUCTION TO THE RESEARCH PRESENTED IN THIS THESIS

This literature review has provided an overview of the molecular regulation of mammalian female meiosis and of cell cycle regulation by the MASTL-ENSA/ARPP19 signaling pathway. The goal of this thesis research is to characterize the MASTL-ENSA/ARPP19 pathway in mouse oocytes during meiotic maturation. Progression through mammalian female meiosis is staggered with long periods of arrests (at the prophase I and metaphase II stages) and extended periods of M-phase. In mammalian oocytes, M-phase lasts for hours compared to minutes in a typical mitotic cell. The unique temporal dynamics of mammalian female meiosis may create a cellular context where the inhibition of PP2A activity during M-phase is especially critical. We therefore sought to characterize the MASTL-ENSA/ARPP19 in mouse oocytes in order to provide insights into the regulation of oocyte biology and reproduction.

While there are few data to date that directly address the function of the MASTL-ENSA/ARPP19 pathway in mammalian oocytes, it is known that inhibition of protein phosphatase activity results in meiotic resumption from prophase I arrest. Most of these studies were performed with the phosphatase inhibitor okadaic acid. Okadaic acid inhibits PP2A and to a lesser extent PP1, with a 100-fold higher potency for PP2A inhibition (MacKintosh, Beattie et al. 1990; Shi 2009). After removal from the ovarian follicle, mouse oocytes will remain arrested in prophase I arrest in culture in the presence of dbcAMP, or the phosphodiesterase inhibitors, IBMX or milrinone, which act to maintain high cAMP levels and PKA activity (Conti, Andersen et al. 2002). Treatment with okadaic acid results in germinal vesicle breakdown and meiotic resumption even in
the presence of dbcAMP, IBMX or milrinone (Rime and Ozon 1990; Alexandre, Van Cauwenberge et al. 1991; Schwartz and Schultz 1991). These results suggest that inhibition of PP2A (and PP1, which may also be inhibited to some extent) is sufficient for CDK1 activation and meiotic entry in mouse oocytes.

This thesis explores the role of the kinase, MASTL (Chapter 2), and the MASTL substrates, ENSA and ARPP19 (Chapter 2 and Chapter 3), during meiotic maturation in mouse oocytes. Importantly, this work also addresses the question of whether ENSA is a functional MASTL substrate in species with two substrates (Chapter 3). It is unclear whether the functions of ENSA and ARPP19 are species-specific and cell-context specific and previous studies have reached different conclusions about whether both substrates are functional (discussed in detail in Section II.G.). Examination of the MASTL-ENSA/ARPP19 pathway in mouse oocytes provides an important new dimension to our understanding of the molecular regulation of mammalian female meiosis.
IV. REFERENCES


Adhikari, D., W. Zheng, et al. (2012). "Cdk1, but not Cdk2, is the sole Cdk that is essential and sufficient to drive resumption of meiosis in mouse oocytes." Human Molecular Genetics 21: 2476-2484.


Proceedings of the National Academy of Sciences of the United States of America

85(20): 7790-7794.


Journal of Cell Biology 60(2): 416-422.

Nader, N., R. P. Kulkarni, et al. (2013). "How to make a good egg!: The need for 
remodeling of oocyte Ca(2+) signaling to mediate the egg-to-embryo transition." 
Cell Calcium 53(1): 41-54.


Nixon, V. L., M. Levasseur, et al. (2002). "Ca(2+) oscillations promote APC/C- 
dependent cyclin B1 degradation during metaphase arrest and completion of 
meiosis in fertilizing mouse eggs." Current Biology 12(9): 746-750.

Norris, R. P., W. J. Ratzan, et al. (2009). "Cyclic GMP from the surrounding somatic 
cells regulates cyclic AMP and meiosis in the mouse oocyte." Development 

Oh, J. S., S. J. Han, et al. (2010). "Wee1B, Myt1, and Cdc25 function in distinct 
compartmentsof the mouse oocyte to control meiotic resumption." The Journal of 
Cell Biology 188(2): 199-207.


### Table 1.1: Overview of species-specific nomenclature for the MASTL/Greatwall pathway components

<table>
<thead>
<tr>
<th>Species</th>
<th>MASTL/Greatwall gene name</th>
<th>MASTL/Greatwall protein name</th>
<th>MASTL/Greatwall substrates gene name</th>
<th>MASTL/Greatwall substrates protein name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>MASTL</td>
<td>MASTL</td>
<td>ENSA, ARPP19</td>
<td>ENSA, ARPP19</td>
</tr>
<tr>
<td>Mouse</td>
<td>Mastl</td>
<td>MASTL</td>
<td>Ensa, Arpp19</td>
<td>ENSA, ARPP19</td>
</tr>
<tr>
<td>Pig</td>
<td>Mastl</td>
<td>MASTL</td>
<td>Arpp19</td>
<td>ARPP19</td>
</tr>
<tr>
<td>Xenopus</td>
<td>Greatwall</td>
<td>Greatwall</td>
<td>Ensa, Arpp19</td>
<td>Ensa, Arpp19</td>
</tr>
<tr>
<td>Drosophila</td>
<td>greatwall</td>
<td>Greatwall</td>
<td>endos</td>
<td>Endos</td>
</tr>
<tr>
<td>Starfish*</td>
<td>Greatwall</td>
<td>Greatwall</td>
<td>Arpp19</td>
<td>Arpp19</td>
</tr>
</tbody>
</table>

- Species-specific nomenclature rules are followed when discussing studies in different species. This table uses MASTL/Greatwall and the MASTL/Greatwall substrates as an example of these nomenclature rules.

- Mouse nomenclature guidelines are used as the default for general discussions of genes/proteins.

* Nomenclature rules for *Xenopus* are used for discussion of starfish genes/proteins.
Figure 1.1: Overview of mammalian female meiosis

The oocyte is arrested in prophase of the first meiotic division in the ovary from before birth until the time of ovulation. Prophase I arrest is characterized by the presence of an intact germinal vesicle (GV) and oocytes at this stage are also referred to as germinal vesicle-intact (GVI). During ovulation, meiosis resumes and the oocyte exits from prophase I arrest. Following germinal vesicle breakdown (GVBD), the oocyte DNA condenses and chromosomes align on the metaphase plate to form the meiotic spindle. The oocyte divides completing the first meiotic division to form a small polar body (first polar body) and a large egg, and arrests at the metaphase II stage. The mammalian female gamete is referred to as an oocyte until meiosis I is completed and is then referred to as the metaphase II egg. This process is referred to as meiotic maturation or the oocyte-to-egg transition. The egg remains arrested at the metaphase II stage until fertilization occurs. The fertilizing sperm triggers cell cycle resumption and the second polar body is emitted. This process is referred to as egg activation or the egg-to-embryo transition.
Prophase I Oocyte → GVBD → Metaphase I Oocyte → Metaphase II Egg

Meiotic Maturation (oocyte-to-egg transition)

Metaphase II Egg → Zygote

Egg Activation (egg-to-embryo transition)
Within the ovarian follicle, the oocyte is surrounded by two somatic cell types, granulosa cells and cumulus cells. A Gs-coupled G-protein receptor (GPR3 in the mouse) on the oocyte cell membrane activates adenylyl cyclase (AC) resulting in production of cAMP. High levels of cAMP are critical for the maintenance of prophase I arrest. The phosphodiesterase, PDE3A, can hydrolyze cAMP and phosphodiesterase activity must be inhibited to maintain high levels of cAMP. This inhibition of PDE3A is accomplished by signaling from the somatic cells of the ovarian follicle. Granulosa cells express natriuetic peptide precursor type C (NPPC), which acts on the NPPC receptor, NPR2, in the neighboring cumulus cells to promote cyclic GMP (cGMP) production. cGMP passes from the cumulus cells to the oocyte via gap junctions and inhibits the phosphodiesterase, PDE3A, thereby maintaining elevated cAMP levels within the oocyte. cAMP activates protein kinase A (PKA), which phosphorylates and activates the kinases WEE1 and MYT1. WEE/MYT1 phosphorylates CDK1 on two inhibitory residues, threonine 14 and tyrosine 15, leading to CDK1 inactivation. PKA also phosphorylates and inactivates the phosphatase CDC25B, preventing the removal of inhibitory phosphates from CDK1. Inhibition of CDK1/cyclin B activity ultimately results in meiotic arrest. Figure adapted from (Mehlmann 2005) and (Holt, Lane et al. 2013).
Figure 1.3: The MASTL/Greatwall pathway as a regulator of mitosis

M-phase substrates are phosphorylated by CDK1/cyclin B (MPF). These phosphorylation events can be removed by the protein phosphatase, PP2A. The MASTL/Greatwall pathway serves to maintain the net phosphorylation status of MPF substrates by inhibiting PP2A activity during M-phase. MPF phosphorylates and activates MASTL/Greatwall, which then phosphorylates its two small phosphoprotein substrates, ENSA and ARPP19. The phosphorylated forms of ENSA and ARPP19 can then directly interact with and inhibit PP2A function during M-phase. Figure adapted from (Virshup and Kaldis 2010).
I. INTRODUCTION

Mammalian oocytes progress through meiosis in a carefully regulated manner in order to form a haploid gamete that is capable of supporting embryonic development. Mammalian oocytes are arrested in prophase of meiosis I until the time of ovulation when the oocyte exits from this arrest, completes the first meiotic division and arrests at the metaphase II stage. Exit from prophase I arrest and progression through pro-metaphase and metaphase I takes several hours in the mammalian oocyte compared to minutes in the typical mitotic cell. This transition from the prophase I oocyte to the metaphase II egg is referred to as meiotic maturation or oocyte maturation. Metaphase II arrest is maintained until the time of fertilization when the second meiotic division is completed and the haploid female gamete is formed. Arrest at the metaphase II stage can also be maintained for several hours before the fertilizing sperm triggers M-phase exit and progression into the first embryonic mitotic division (Conti, Hsieh et al. 2012).

During M-phase, cyclin-dependent kinase 1 (CDK1), complexed with cyclin B, phosphorylates numerous substrates leading to entry into and progression through M-phase. The CDK1/cyclin B complex is also referred to as MPF (M-phase promoting factor or maturation promoting factor) based on its critical role in regulating M-phase entry (Masui and Markert 1971). In the prophase I-arrested oocyte, protein kinase A
(PKA) maintains low levels of CDK1 activity. PKA phosphorylates and activates the kinases MYT1 and WEE1, which inhibit CDK1 through phosphorylation on two inhibitory residues, threonine 14 and tyrosine 15. PKA also phosphorylates and inhibits the phosphatase CDC25, which can activate CDK1 through removal inhibitory phosphates. During prophase I arrest, the activity of PKA decreases, leading to CDC25 activation and dephosphorylation of CDK1 (Han, Chen et al. 2005; Pirino, Wescott et al. 2009; Oh, Han et al. 2010). Active CDK1 can then phosphorylate key M-phase substrates, and progression through meiosis I occurs. The progression from prophase I into metaphase I in oocytes is analogous to the G2–M transition in mitotic cells.

During M-phase, both CDK1 activity and inhibition of protein phosphatases that dephosphorylate CDK1 substrates are required to maintain the phosphorylation status of M-phase substrates. Studies in Drosophila and Xenopus identified PP2A as the key phosphatase involved in the dephosphorylation of M-phase substrates (Mayer-Jaekel, Ohkura et al. 1994; Mochida and Hunt 2007; Mochida, Ikeo et al. 2009). PP2A functions as a heterotrimic holoenzyme, with a catalytic/C subunit, a scaffold/A subunit, and a regulatory/B subunit (Virshup and Shenolikar 2009). PP2A in complex with the regulatory subunit B55δ was identified as the specific PP2A heterotrimer that dephosphorylates MPF substrates in Xenopus extracts. Mitotic entry was accelerated when PP2A-B55δ was immunodepleted from interphase extracts and addition of PP2A-B55δ suppressed mitotic entry (Mochida, Ikeo et al. 2009). These studies established a model for cell cycle entry and progression that requires not only CDK1 activity, but also maintenance of the phosphorylation of CDK1 substrates through inhibition of PP2A.
Inhibition of PP2A activity during M-phase is mediated by the kinase, MASTL/Greatwall (microtubule-associated serine/threonine kinase-like is the official name in mouse and human; Greatwall is the kinase name in *Drosophila* and *Xenopus*). Depletion of Greatwall from cycling *Xenopus* extracts prevented entry into M-phase (Yu, Zhao et al. 2006). In human cells, knockdown of MASTL resulted in a delayed G2 to M-phase transition. MASTL-deficient cells that entered M-phase displayed numerous mitotic defects, including defective chromosome condensation, spindle formation and cytokinesis (Burgess, Vigneron et al. 2010; Voets and Wolthuis 2010). The MASTL/Greatwall pathway has been shown to function in numerous species including *Xenopus*, *Drosophila*, starfish, human, mouse and pig (Yu, Fleming et al. 2004; Yu, Zhao et al. 2006; Zhao, Haccard et al. 2008; Burgess, Vigneron et al. 2010; Voets and Wolthuis 2010; Hara, Abe et al. 2012; Álvarez-Fernández, Sánchez-Martínez et al. 2013; Li, Kang et al. 2013; Okumura, Morita et al. 2014). Budding yeast has a MASTL/Gwl ortholog, Rim 15, and two Rim15 substrates, Igo1 and Igo2, that function as part of nutrient-sensing pathway that regulates entry into quiescence (Talarek, Cameroni et al. 2010; Luo, Talarek et al. 2011; Bontron, Jaquenoud et al. 2012; Bontron, Jaquenoud et al. 2013).

In the current model for the MASTL/Greatwall pathway, CDK1 phosphorylates and activates MASTL/Greatwall, which then phosphorylates its two substrates, ENSA and ARPP19. The phosphorylated forms of ENSA and ARPP19 can then interact with and inhibit the phosphatase activity of PP2A-B55δ (Gharbi-Ayachi, Labbé et al. 2010; Mochida, Maslen et al. 2010). Some species, including *Drosophila*, starfish and pig, only have one MASTL substrate, while *Xenopus*, human and mouse express both ENSA and ARPP19 (Von Stetina, Tranguch et al. 2008; Gharbi-Ayachi, Labbé et al. 2010; Mochida,
Maslen et al. 2010; Hara, Abe et al. 2012; Kim, Bucciarelli et al. 2012; Li, Kang et al. 2013) [Note: We attempt to use species-specific gene/mRNA/protein nomenclature when discussing ENSA and ARPP19 in different species; for example, ENSA and ARPP19 in mouse or human, Ensa and Arpp19 in *Xenopus* and Endos in *Drosophila*. The mouse/human nomenclature is used by default with appropriate nomenclature used when discussing findings from a specific species]. It is unclear whether ENSA and ARPP19 are functionally redundant or if the two substrates have distinct roles in the regulation of M-phase entry and progression. One study found that immunodepletion of Ensa from *Xenopus* extracts inhibited M-phase entry, while another study found that depletion of Arpp19, but not Ensa, resulted in a M-phase entry defect (Gharbi-Ayachi, Labbé et al. 2010; Mochida, Maslen et al. 2010). More recent studies in *Xenopus* have focused on a role for Arpp19 and have not addressed a potential physiological role for Ensa (Dupre, Buffin et al. 2013; Dupre, Daldello et al. 2014). This discrepancy also extends to human cells where one group determined that depletion of ARPP19 resulted in defective mitotic entry, while another group did not detect ARPP19 in cell lysates by immunoblotting and did not observe a phenotype with ARPP19 knockdown (Gharbi-Ayachi, Labbé et al. 2010; Cundell, Bastos et al. 2013). In budding yeast, deletion of both Igo1 and Igo2 is required for a G0 entry phenotype with neither single mutant displaying this phenotype (Talarek, Cameroni et al. 2010).

This work explores the role of the MASTL-ENSA/ARPP19 pathway during meiotic maturation in mouse oocytes. We first determined whether the known components of the pathway are expressed in mouse oocytes and whether this pathway regulates entry into meiotic M-phase in the mouse. Importantly, this work builds upon
previous findings that the MASTL pathway is critical for entry into and progression through M-phase in other cellular contexts.

II. MATERIALS AND METHODS

Oocyte collection, maturation, and culture

Animals were used in accordance with the guidelines of the Johns Hopkins University Animal Care and Use Committee. Germinal vesicle-intact (GVI), prophase I-arrested oocytes were collected from 6-8 week old CF-1 mice (Harlan; Indianapolis, IN). Oocytes were collected in Whitten's-HEPES medium (109.5 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 5.5 mM glucose, 0.23 mM pyruvic acid, 4.8 mM lactic acid hemicalcium salt (Whitten 1971)) supplemented with 7 mM NaHCO₃, 15 mM HEPES (hereafter referred to as Whitten's-HEPES) and 0.05% polyvinyl alcohol (PVA, Sigma, St. Louis, MO). Dibutyryl cAMP (dbcAMP, 0.25 mM) or milrinone (2.5 μM) was added to culture medium to maintain prophase I arrest (Cho, Stern et al. 1974). Ovaries were punctured with syringe needles to release oocyte-cumulus complexes from ovarian follicles, and cumulus cells were dissociated from oocytes by pipetting oocyte-cumulus complexes through a thin-bore pipette. Oocytes were transferred to Whitten's medium with 22 mM NaHCO₃ (hereafter referred to as Whitten's-Bicarbonate medium) containing 0.05% PVA and 0.25 mM dbcAMP or 2.5 μM milrinone for culture. Oocytes were cultured at 37°C in a humidified atmosphere of 5% CO₂ in air. For microinjection of siRNA (details below), oocytes were transferred to EmbryoMax® KSOM + amino acids with D-Glucose (MR-106-D, Millipore; Billerica, MA) (hereafter referred to as...
KSOM) supplemented with 0.25 mM dbcAMP or 2.5 μM milrinone. Microinjected oocytes were cultured in KSOM medium following microinjection and during in vitro meiotic maturation.

**Collection of mature metaphase II eggs and in vitro fertilization**

Metaphase II-arrested eggs were collected from 6-8 week old superovulated CF-1 mice at 13 hours after hCG injection as previously described (McAvey, Wortzman et al. 2002). Cumulus cells were removed by brief incubation in Whitten’s HEPES media containing 15 mg/ml BSA (EmbryoGro™ Bovine Albumin, Low Endotoxin, Embryo Transfer Grade, MP Biomedicals: Solon, OH) and 0.02% Type IV-S hyaluronidase (Sigma).

For in vitro fertilization, the zona pellucida (ZP) was removed by briefly incubating the oocytes in acidic culture medium-compatible buffer (~10 seconds; 10 mM HEPES, 1 mM NaH2PO4, 0.8 mM MgSO4, 5.4 mM KCl, 116.4 mM NaCl, pH 1.5). ZP-free eggs were allowed to recover for 45-60 minutes in Whitten’s-Bicarbonate medium containing 15 mg/ml BSA under oil at 37°C in a humidified atmosphere of 5% CO2 in air. In vitro fertilization of ZP-free eggs was performed as described previously (Evans, Schultz et al. 1995; McAvey, Wortzman et al. 2002). Briefly, sperm was retrieved from one epididymis of a CD-1 retired breeder male mouse (Harlan). The epididymal tissue was minced in a 125 μl drop of Whitten’s-Bicarbonate medium containing 15 mg/mL BSA. After 10-15 minutes, the tissue was removed from the medium and sperm were pipetted to the bottom of a tube containing 750 μl of Whitten’s-Bicarbonate medium containing 15 mg/ml BSA (referred to as swim-up culture). After 45 minutes, 220 μl was
removed from the top of the swim-up culture and placed in a separate tube. The sperm were cultured for a total of 3 hours in Whitten’s-Bicarbonate medium containing 15 mg/mL BSA to allow sperm to undergo capacitation and spontaneous acrosome reaction.

Ten ZP-free eggs per 10 µl drop were inseminated for 1.5 hours with a sperm concentration of 150,000 sperm/ml. After this 1.5 hour insemination, eggs were gently washed to remove loosely bound sperm and were cultured for a total of 9 hours or 18 hours in WB/PVA. Fertilization was confirmed by assessing presence of the second polar body at 2 hours and presence of pronuclei at 5-6 hours. Embryos were washed with WB/PVA to remove BSA and were lysed in SDS-PAGE sample buffer (3% SDS, 10% glycerol, 0.02% bromophenol blue, 4% 2-mercaptoethanol, 65 mM Tris-HCl, pH 6.8) for immunoblot analysis.

**Bacterially-expressed recombinant proteins**

cDNA fragments encoding the entire coding region of mouse *Ensa* (NM_019561.2) and *Arpp19* (NM_021548.4) were generated by polymerase chain reaction (PCR) from oocyte cDNA using Phusion™ High-Fidelity DNA polymerase (New England Biolabs; Ipswich, MA). Cloning primer sequences are provided in Table 2.1. The resulting PCR products were gel-purified (QIAquick Gel Extraction kit; Qiagen; Valencia, CA), digested with *Bam* HI and *Sal* I (New England Biolabs), and cloned into pGEX-4T-1 (Amersham-Pharmacia Biotech; Piscataway, NJ) according to standard protocols. The resulting plasmids (pGEX-4T-ENSA; pGEX-4T-ARPP19) were verified by DNA sequencing. Plasmids were transformed into *E. coli* BL21 cells (Stratagene; La Jolla, CA). Transformed cells were induced to express GST fusion proteins with 0.5 mM IPTG
(Sigma) at 37°C for 4 hours. Cell lysates were prepared by sonication in cold PBS containing 1 µg/ml leupeptin, 1 µg/ml aprotinin and 1 mM AESBF (Sigma). The lysate was centrifuged twice (20,000 x g, 10 minutes each), and GST fusion proteins were purified by affinity chromatography on a glutathione column, eluting with 10 mM of reduced glutathione in 10 mM Tris-HCl, pH 7.5. The purified protein was dialyzed against PBS. Protein concentration was determined using the micro BCA assay (Thermo Scientific; Rockford, IL), and purity was confirmed by examining the protein on silver-stained SDS-PAGE gels. *Drosophila* GST-Endos (plasmid gift of Drummond-Barbosa lab) was expressed and purified as described above for GST-ENSA and GST-ARPP19 except induction was performed at 30°C for 3 hours (Drummond-Barbosa and Spradling 2004).

For examination of proteins on silver-stained gels, SDS-PAGE was performed as described below (“SDS-PAGE and Immunoblotting” section) except the 4% 2-mercaptoethanol was omitted from the sample buffer. Silver staining was performed by fixing the SDS-PAGE gel in a solution of 40% ethanol, 12% acetic acid and 0.5 ml of 37% formaldehyde for 60 minutes, washing three times for 10-20 minutes in 50% ethanol and pretreating gel in 0.2 g/L of sodium thiosulfate (Na₂S₂O₃-5H₂O) for 60 seconds. The gel was rinsed three times for 20 seconds each with distilled water and stained with a solution of 2 g/L silver nitrate (AgNO₃) and 0.75 ml of 37% formaldehyde for 20 minutes. The gel was then rinsed twice with distilled water for 10 seconds each and developed with a solution of 60 g/L sodium carbonate (Na₂CO₃), 0.5 ml of 37% formaldehyde and 0.004 g/L of sodium thiosulfate (Na₂S₂O₃-5H₂O) for 5-10 minutes (when bands reach desired intensity). Gels were rinsed with the fixing solution (40% ethanol, 12% acetic
acid and 0.5 ml of 37% formaldehyde) for 10 minutes to stop the developing reaction and washed with 50% methanol for 20 minutes. Gels were scanned using the HP LaserJet 3390 scanner, and images were saved as TIFF files. Gels have a faint haze on the top half of the gel from approximately 55 kD to the top of the gel. This haze occurred in multiple experiments in all lanes and was independent of sample type and sample concentration (see Figure 2.9 with 20, 40 and 60 ng of GST-Endos and GST-ENSA). For GST-ENSA, we observed a dominant upper Mr protein band and a secondary lower Mr protein band. The lower band is likely a product of incomplete translation or proteolysis. The sum of the intensity of both bands was quantified for all experiments (see “Immunoblot analysis of ENSA protein levels” section below for further details) since both bands cross-reacted with the anti-ENSA antibody and were part of the total protein concentration determined by the BCA assay.

**Antibodies**

The MASTL rabbit monoclonal antibody (D3J4Y; catalog #12069; produced against the amino-terminus peptide of human MASTL) used in immunoblotting and immunofluorescence studies was obtained from Cell Signaling Technologies (Danvers, MA). The ENSA rabbit polyclonal antibody (catalog #sc-135145; made against full-length human ENSA) used in immunoblotting studies was obtained from Santa Cruz Biotechnology (Dallas, TX).

Two anti-ARPP19 antibodies were used in these studies. An anti-ARPP19 rabbit polyclonal antibody (catalog #11678-1-AP; made against full-length human ARPP19, Genbank no: BC003418) was obtained from ProteinTech Group (Chicago, IL). A custom
anti-ARPP19 goat polyclonal antibody (EB12183) was produced by Everest Biotech (Oxfordshire, UK). EB12183 was developed using the peptide C-PAAAPDKTEVTGDH, which is specific to ARPP19 and is not found in ENSA. A sequence alignment of ENSA and ARPP19 is shown in Figure 2.5 with the peptide sequence used to make the ARPP19 antibody highlighted in yellow.

The anti-actin mouse monoclonal antibody used in immunoblotting studies was obtained from Sigma (catalog # A-1978).

**Lysis of cultured cells**

Mouse NIH 3T3, mouse 308 keratinocytes or human HeLa cells were scraped into SDS-PAGE sample buffer (3% SDS, 10% glycerol, 0.02% bromophenol blue, 4% 2-mercaptoethanol, 65 mM Tris-HCl, pH 6.8). Cells were lysed in 300 µl or 800 µl of SDS-PAGE sample buffer for a 35 mm well (one well of a 6-well plate) or 100 mm plate respectively. The lysates were then recovered from the dish, transferred to an Eppendorf tube, and sonicated on ice three times for 10 seconds each. Lysates were aliquoted to avoid multiple freeze/thaw cycles and stored at -20°C. Protein concentration was determined using the Amido Black assay. Five microliters of each lysate was spotted on a nitrocellulose membrane along with five BSA standards (0, 0.25, 1, 2, 10 µg). The nitrocellulose membrane was incubated in 50% Methanol/H2O for 5 minutes, amido black stain for 5 minutes (0.1% Amido black in 30% methanol/10% acetic acid), and washed 3 times in 50% methanol/H2O until the membrane was white again. The membrane was dried for 30 minutes and each spot was eluted in 0.1 NaOH for 30 minutes. Protein
concentrations of the cell lysates were determined by reading the OD$_{600}$ and generating a standard curve using the BSA standards.

**SDS-PAGE and Immunoblotting**

For immunoblot analysis, purified recombinant proteins, and lysates of cultured cells, oocytes, eggs, or embryos were suspended in SDS-PAGE sample buffer (3% SDS, 10% glycerol, 0.02% bromophenol blue, 4% 2-mercaptoethanol, 65 mM Tris-HCl, pH 6.8) and heated for 10 minutes at 95°C. Proteins were separated on a 10% or 12.5% SDS-gel and transferred to an Immobilon-P PVDF membrane (Millipore, Billerica, MA).

For anti-MASTL immunoblots, the membrane was blocked for 2 hours in TBS containing 0.1% Tween-20 (Sigma) (hereafter referred to as TBS-T) plus 5% dry milk. The membrane was then incubated with an anti-MASTL antibody (Cell Signaling Technology) diluted to a concentration of 0.4 µg/ml in TBS-T containing 5% BSA and 0.02% NaN$_3$ overnight at 4°C, washed three times with TBS-T, then incubated with 0.3 µg/ml goat anti-rabbit IgG-horseradish peroxidase (HRP) (Jackson Immunoresearch, West Grove, PA) in TBS-T plus 3% BSA for 2 hours, followed by three washes with TBS-T.

For anti-ENSA immunoblots, the membrane was blocked overnight at room temperature in 10% cold-water fish gelatin (Sigma) in PBS containing 0.1% Tween-20 (Sigma) (hereafter referred to as PBS-T). The membrane was then incubated with an anti-ENSA antibody (Santa Cruz Biotechnology) diluted to a concentration of 0.5 µg/ml in PBS-T containing 5% BSA and 0.02% NaN$_3$ for 2-3 hours, washed three times with PBS-T, then incubated with 0.3 µg/ml goat anti-rabbit IgG-horseradish peroxidase (HRP)
(Jackson Immunoresearch, West Grove, PA) in PBS-T plus 3% BSA for 1.5-2 hours, followed by three washes with PBS-T.

For anti-ARPP19 immunoblots using the custom ARPP19 antibody from Everest Biotech, the membrane was blocked for 2-3 hours in 10% cold-water fish gelatin (Sigma) in PBS-T. The membrane was then incubated with an anti-ARPP19 antibody (Everest Biotech) diluted to a concentration of 1 µg/ml in PBS-T containing 5% BSA and 0.02% NaN₃) overnight at 4°C, washed three times with PBS-T, then incubated with 0.3 µg/ml donkey anti-goat IgG-horseradish peroxidase (HRP) (Jackson Immunoresearch, West Grove, PA) in PBS-T plus 3% BSA for 1.5-2 hours, followed by three washes with PBS-T.

For anti-ARPP19 immunoblots using the ProteinTech Group antibody, the membrane was blocked overnight at 4°C in TBS-T plus 5% dry milk. The membrane was then incubated with an anti-ARPP19 antibody (ProteinTech Group) diluted to a concentration of 0.6 µg/ml in TBS-T containing 3% BSA and 0.02% NaN₃) for 2-3 hours, washed three times with TBS-T, then incubated with 0.3 µg/ml goat anti-rabbit IgG-horseradish peroxidase (HRP) (Jackson Immunoresearch, West Grove, PA) in TBS-T plus 3% BSA for 1.5-2 hours, followed by three washes with TBS-T.

For anti-actin immunoblots, the membrane was blocked in PBS-T plus 5% dry milk overnight at 4°C. The membrane was then incubated with an anti-actin antibody (Sigma) diluted to a concentration of 0.1 µg/ml in PBS-T containing 5% BSA and 0.02% NaN₃) for 1 hour, washed three times with PBS-T, then incubated with 0.3 µg/ml goat anti-mouse IgG-horseradish peroxidase (HRP) (Jackson Immunoresearch, West Grove, PA) in PBS-T plus 3% BSA for 1 hour, followed by three washes with PBS-T.
For detection, the membrane was incubated with Supersignal® West Pico Chemiluminescent substrate (Pierce Chemical Company; Rockford, IL) and exposed to X-ray film. X-ray film was scanned using the HP LaserJet 3390 scanner, and images were saved as TIFF files. ImageJ software (http://rsb.info.nih.gov/ij/) was used to analyze band intensity. The rectangular selection tool was used to select each band and peak intensity was determined. The area under each peak was calculated as a measure of band intensity.

**Immunoblot quantification of ENSA protein levels**

For each experiment, one to two oocyte lysates (between 26 to 45 oocytes per lysate) along with a range of amounts of recombinant GST-ENSA (15, 20, 25, 30, 40, 50, 60 ng) were separated by SDS-PAGE as described above and transferred to an Immobilon-FL PVDF membrane (Millipore). The membrane was then incubated with the anti-ENSA antibody (0.3 µg/ml, diluted 1:685 in a 1:1 solution of PBS and Odyssey® Block Buffer) for 1 hour, washed four times in PBS-T (5 minutes each wash), incubated with goat anti-rabbit IRDye® 800CW secondary antibody (diluted 1:20,000 in a 1:1 solution of PBS and Odyssey® Block Buffer) for 1 hour, washed four times in PBS-T (5 minutes each wash) and washed once in PBS. The membrane was then scanned using the Odyssey® CLx Infrared Imaging System (Licor; Lincoln, NE) at an intensity setting between 6.5 and 7.5. ImageJ software (http://rsb.info.nih.gov/ij/) was used to analyze band intensity as described above. For the GST-ENSA standards, the two bands that cross-reacted with the anti-ENSA antibody were quantified and added together to obtain
a total band intensity for each sample. A standard curve was generated using the seven known amounts of GST-ENSA and the linear equation for the best-fit line.

**Immunofluorescence**

For immunofluorescence analysis, ZP-free oocytes were prepared by briefly incubating the oocytes in acidic culture medium-compatible buffer (~10 seconds; 10 mM HEPES, 1 mM NaH2PO4, 0.8 mM MgSO4, 5.4 mM KCl, 116.4 mM NaCl, pH 1.5). ZP-free oocytes were fixed for 30 minutes at 37°C in 4% paraformaldehyde (Sigma) in 130 mM KCl, 25 mM HEPES, 3 mM MgCl2, 0.06% Triton-X (pH 7.4). Fixed oocytes were briefly washed in PBS, permeabilized in PBS containing 0.1% Triton X-100 (Sigma) for 15 minutes, then incubated for 1 hour in immunofluorescence (IF) blocking buffer (PBS containing 1% BSA). Following blocking, oocytes were incubated with MASTL primary antibody diluted in IF blocking buffer overnight (10.6 µg/ml). The oocytes were then washed in IF blocking buffer, and incubated for 1-1.5 hours in secondary antibody diluted in blocking buffer (7.5 µg/ml goat-anti-rabbit IgG-FITC) (Jackson Immunoresearch, West Grove, PA) followed by three washes in IF blocking buffer. Oocytes were mounted on slides in VectaShield mounting medium (Vector Laboratories) containing 0.75 µg/ml DAPI. Microscopic imaging was performed on a Zeiss Axio Observer Z1 microscope with AxioCam MRm Rev3 camera, ApoTome optical sectioning, and AxioVision software (Carl Zeiss, Inc.; Jena, Germany).

In order to allow comparison of images, exposure time and display settings (brightness and contrast) were standardized for each experiment. Fluorescence intensity was quantified using ImageJ software (http://rsb.info.nih.gov/ij/). For each oocyte, the
rectangular selection tool was used to draw a rectangle within the germinal vesicle and the integrated density of the germinal vesicle was determined. A rectangle of the same size was also drawn in the area outside of the cell (background) and the background integrated density was calculated. The corrected total cell fluorescence (CTCF) was calculated by subtracting the background integrated density from the integrated density of the germinal vesicle. Each CTCF was normalized to the mean CTCF of the control group. Oocytes were categorized into eight groups based on the normalized CTCF (0-0.25, 0.26-0.50, 0.51-0.75, 0.76-1.0, 1.01-1.25, 1.26-1.50, 1.51-1.75, 1.76-2.0).

**RNAi-mediated knockdown in mouse oocytes**

ON-TARGETplus SMARTpool siRNA targeting mouse *Mastl* or *Arpp19* (Dharmacon, Waltham, MA) was resuspended according to manufacturer's instructions to a concentration of 100 µM in four volumes of RNase-free water and one volume of 5X siRNA Buffer (Dharmacon, B-002000-UB-100). The ON-TARGETplus control pool (Dharmacon, D-001810-10-20) was used as a negative control (the siRNA sequences used for the negative control pool are proprietary and are not provided by the manufacturer). The 100 µM siRNA stocks were diluted to a final concentration for microinjection in 1X siRNA buffer. For *Mastl* knockdown, 20 µM and 50 µM of *Mastl* siRNA were tested, with 50 µM used for most experiments shown here. For *Arpp19* siRNA injection, two different siRNA pools were tested. The first pool (*Arpp19*-targeting siRNA-#1) was used in two experiments at a concentration of 20 µM. The second pool was used in five total experiments at doses of 20 µM (four experiments), 40 µM (2 experiments) and 80 µM (1 experiment). The negative control siRNA pool
concentration was matched to the experimental group for each experiment. ZP-intact prophase I oocytes were injected using a Nikon Eclipse TE 2000-5 microscope (Melville, NY) equipped with an Eppendorf FemtoJet® (Hamburg, Germany), using injection pressure \((p_i)\) of 100-200 hPa, injection time \((t_i)\) of 0.1-0.2 seconds, and compensation pressure \((p_c)\) of 0 hPa. Injection was considered successful upon observation of cytoplasmic recoiling following needle insertion and post-injection dispersal of the siRNA solution into the oocyte cytoplasm. siRNA-injected oocytes were cultured in KSOM medium containing 0.25 mM milrinone for 44-48 hours, with transfer to fresh medium after 24 hours.

**RNA isolation, cDNA synthesis, and semi-quantitative RT-PCR**

Total RNA was isolated from siRNA-injected oocytes or control by lysing 20 prophase I oocytes in 200 µl Trizol (Invitrogen) for 5 minutes at room temperature. This mixture was chloroform-extracted, then the RNA-containing aqueous phase was supplemented with a final concentration of 1 µg/ml glycogen (Invitrogen) and subjected to isopropanol precipitation overnight at -20°C, after which the RNA was pelleted by centrifugation (15 minutes, 14,000 x g, 4°C). The pellet was washed with 70% ethanol, air dried, and then suspended in 8 µl of RNase-free water. The RNA concentration was determined by measuring the OD\(_{260}\). First-strand cDNA was synthesized from total RNA with random hexamer primers, SuperScript Reverse Transcriptase III (Invitrogen), and RNaseOUT RNase Inhibitor (Invitrogen), according to the manufacturer's protocol, after which the cDNA was treated with *E. coli* RNase H (New England Biolabs; Ipswich, MA).
To assess RNA knockdown, RT-PCR using gene-specific primers was performed; primer sequences are provided in Table 2.2. Tissue plasminogen activator (gene symbol Plat) was used as a positive control, as previously described for mouse oocytes (Svoboda, Stein et al. 2000). For semi-quantitative PCR, the amount of PCR product was assessed for a range of cycle numbers, ensuring that the reaction was in the linear range and not saturated. PCR products were separated on agarose gels, which were scanned using the FujiFilm FLA-7000 imaging system (FujiFilm, Valhalla, NY). FLA-7000 image files were exported to the MultiGauge program (FujiFilm) and saved as TIFF files. ImageJ software (http://rsb.info.nih.gov/ij/) was used to analyze band intensity. The rectangular selection tool was used to select each band and peak intensity was determined. The area under each peak was calculated as a measure of band intensity. Band intensity for Mastl or Arpp19 primer-amplified products was normalized to those for the positive control (Plat) primer set. Transcript levels in oocytes injected with target-specific siRNAs (Mastl, Arpp19) were expressed relative to those levels in oocytes injected with negative control siRNA.

Primers were designed to distinguish between the two Ensa transcript isoforms (NM_019561.2, isoform a, encodes NP_062507.1 and NM_001021383.1, isoform b, encodes NP_001021383.1) and the two Arpp19 transcript isoforms (NM_021548.4, isoform 1, encodes NP_067523.1 and NM_001136127.1, isoform 2, encodes NP_001136127.1). Primer sequences are provided in Table 2.3. RT-PCR using oocyte cDNA was performed to assess the presence of the Ensa and Arpp19 transcript variants. The two Ensa transcripts encode proteins that are identical except that isoform a has an additional four amino acids at the C-terminus. For ARPP19, isoform 1 and isoform 2
have an identical C-terminal region and isoform 2 has an additional 33 amino acid region at the N-terminus. Figure 2.5 provides a multiple sequence alignment of the four ENSA and ARPP19 isoforms using Clustal W (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

**Assessment of germinal vesicle breakdown**

siRNA-injected oocytes were washed through six drops of KSOM to wash away the milrinone (Cho, Stern et al. 1974). For observation of progress through germinal vesicle breakdown (also known as nuclear envelope breakdown), oocytes were cultured in KSOM, with observation at 30-minute intervals for 5 hours by dissecting microscope for the presence of an intact germinal vesicle. Data are presented as the percentage of oocytes having undergone germinal vesicle-breakdown (GVBD) over time.

**RNAi mediated knockdown in NIH-3T3 cells**

These experiments were performed in collaboration with Anita Ramachandran; AR performed cell culture work, transfection, RNA extraction and cDNA synthesis; LM performed RT-PCR, protein extraction and immunoblotting. NIH 3T3 cells were seeded in a 6-well plate at a density of 2x10^5 cells per well in 1X Dulbecco’s Modified Eagle Medium (DMEM; Gibco, Life Technologies; Grand Island, NY) plus 10% Fetal Bovine Serum (FBS) the evening before transfection to allow for a confluency of ~50% at the time of transfection. The next morning, 3 µl of Lipofectamine 2000 (Invitrogen) was mixed with 97 µl of Opti-MEM® reduced serum medium (Gibco, Life Technologies) (volume for each well) and let sit for 5 minutes. In a separate tube, 100 pmoles of *Arpp19*-targeting siRNA was mixed with 100 µl of Opti-MEM® reduced serum medium
(Gibco, Life Technologies) for each well. For each control well, the lipofectamine mixture was mixed with 100 µl of Opti-MEM® reduced serum medium (Gibco, Life Technologies). For each siRNA-transfected well, the lipofectamine mixture was mixed with the siRNA mixture and let sit for 20 minutes at room temperature. The 200 µl mixture (lipofectamine only for control and lipofectamine plus siRNA for siRNA-transfected cells) was mixed to 1.8 ml of Opti-MEM® reduced serum medium (Gibco, Life Technologies) and added to each well for 4 hours at 37°C. Following transfection, cells were washed and cultured in DMEM plus 10% FBS for 44 hours at 37°C to allow knockdown.

After 44 hours, cells were washed with 1X PBS and lysed for total protein or RNA extraction. For RNA extraction, the PerfectPure RNA cell and tissue kit was used according to manufacturer’s instructions (Five Prime; Gaithersburg, MD). First-strand cDNA was synthesized using the iScript cDNA synthesis kit according to manufacturer’s instructions (Bio-Rad). RT-PCR was performed as described above. For protein extraction, 300 µl of 2x SDS-PAGE sample buffer (3% SDS, 10% glycerol, 0.02% bromophenol blue, 4% 2-mercaptoethanol, 65 mM Tris-HCl, pH 6.8) was added to each well. Cells were separated from the plate using a cell scraper and pipetted into a microcentrifuge tube. Each tube was sonicated on ice three times for 10-15 seconds each and stored at -20°C for immunblot analysis as described above.
III. RESULTS

The MASTL-ENSA/ARPP19 pathway is critical for maintenance of M-phase in numerous mitotic and meiotic cell types. To determine if this pathway was functioning in mouse oocytes during meiotic maturation, we first confirmed that MASTL was expressed by mouse oocytes at the RNA and protein level. The Mastl transcript was detected in oocyte cDNA by RT-PCR using gene-specific primers (Figure 2.1A). MASTL protein was detected in a lysate of 80 germinal vesicle-intact (GVI), prophase I-arrested oocytes (lane 1, Figure 2.1B), as well as positive control cell lysates from mouse 308 keratinocytes (lanes 2-3) and human HeLa cells (lanes 4-5).

We used RNAi-mediated knockdown to assess the function of MASTL in mouse oocytes. Prophase I-arrested oocytes were microinjected with negative control siRNA (control oocytes) or Mastl-targeting siRNA and cultured in the presence of milrinone to maintain prophase I arrest for 44-48 hours to allow knockdown. Milrinone is a phosphodiesterase inhibitor that maintains high levels of cyclic AMP and PKA signaling in the oocyte allowing maintenance of meiotic arrest at the prophase I stage. Two siRNA doses were tested for knockdown at the RNA level, 20 µM and 50 µM. The extent of knockdown was dependent on the dose of siRNA injected and using the 20 µM dose, Mastl mRNA levels were decreased by 64 ± 11% compared to controls (three experiments; data not shown). Mastl mRNA levels were decreased by 84 ± 3% in Mastl siRNA-injected oocytes compared to controls following the 44-48 hours culture period using the 50 µM siRNA dose (Figure 2.2A; three experiments; range 81-89%). Based on the higher extent of knockdown, the 50 µM dose was used for all other experiments. Following the 44-48 hour culture period, MASTL protein levels were decreased by 95%
(Figure 2.2B; one experiment) or to undetectable levels (Figure 2.2C; two experiments with one representative experiment shown) in oocytes injected with 50 µM Mastl-targeting siRNA as compared to controls.

MASTL-deficient oocytes were assessed for the ability to undergo exit from prophase I arrest. Oocytes were placed in culture conditions to allow meiotic maturation by washing the oocytes through drops of milrinone-free media and assessed at 30-minute intervals for 5 hours for germinal vesicle breakdown. On average, 71% of control oocytes exited from prophase I arrest during the 5-hour period compared to 28% of MASTL-deficient oocytes (Figure 2.3; three experiments). This observed defect in the exit from prophase I arrest suggests that MASTL regulates this process in mouse oocytes.

While the majority of MASTL-deficient oocytes remained arrested at the prophase I stage during the 5-hour experiment period, 28% of oocytes were able to exit from prophase I arrest. We hypothesized that the extent of MASTL knockdown might be variable within this population of MASTL-deficient oocytes allowing some oocytes to exit from prophase I arrest. To compare MASTL protein levels in Mastl siRNA-injected oocytes between individual oocytes, we used immunofluorescence using an anti-MASTL antibody (Figure 2.4A). MASTL was mainly localized to the germinal vesicle, but was apparently excluded from the nucleolus in both control (Figure 2.4A, Panels i-iii) and Mastl siRNA-injected (Figure 2.4A, Panels iv-xii; three representative images shown) oocytes. Fluorescence intensity was quantified and normalized to the mean of the control group (control group normalized to 1; Figure 2.4B). The mean normalized fluorescence intensity was 1.0 ± 0.04 (mean ± standard error of the mean) for the control group (white bars; n = 53) and 0.31 ± 0.04 for the Mastl siRNA-injected group (black bars; n = 46).
To illustrate the oocyte-to-oocyte variability in more detail, levels of anti-MASTL signal in individual oocytes were categorized into eight groups based on the normalized fluorescence intensity. The majority of Mastl siRNA-injected oocytes had a normalized fluorescence intensity between 0 - 0.25 and 0.26 - 0.50, although some Mastl siRNA-injected oocytes did have a higher fluorescence intensity. This result indicates that the extent of MASTL knockdown was variable between individual oocytes, which may explain why a subset of oocytes was able to exit from prophase I arrest.

In the current model for MASTL function during M-phase in mitotic cells, MASTL functions in a signaling pathway where it phosphorylates its small phosphoprotein substrates that then directly inhibit PP2A-B55. To further elucidate the role of MASTL signaling during oocyte meiotic maturation, we investigated the expression of MASTL substrates in mouse oocytes. Mouse has two genes encoding MASTL substrates, ENSA and ARPP19; these genes encode closely related proteins that are 76% identical at the amino acid level (sequence alignment provided in Figure 2.5). Ensa and Arpp19 each have two transcript variants encoding two protein isoforms. Using transcript-specific primers, we were able to detect all four transcript variants by RT-PCR using mouse oocyte cDNA (Ensa isoform a [NM_019561.2], Ensa isoform b [NM_001021383.1], Arpp19 isoform 1 [NM_021548.4], and Arpp19 isoform 2 [NM_001136127.1]; data not shown). We used recombinant GST-ENSA and GST-ARPP19 in immunoblotting studies to assess the specificity of the anti-ENSA and anti-ARPP19 antibodies used in these studies (Figure 2.6). The anti-ENSA antibody did not cross-react with recombinant ARPP19 (Figure 2.6A, lanes 4-6) and was specific for recombinant ENSA (Figure 2.6A, lanes 1-3). The ProteinTech Group anti-ARPP19 antibody was not
specific for recombinant ARPP19 and cross-reacted with both recombinant ARPP19 (Figure 2.6B, lanes 4-6) and recombinant ENSA (Figure 2.6B, lanes 1-3). A custom ARPP19 antibody was developed using a peptide sequence specific to ARPP19 as an antigen (peptide sequence is highlighted in Figure 2.5). This custom ARPP19 antibody proved to be specific for recombinant ARPP19 (Figure 2.6C, lanes 3-4) and did not react with recombinant ENSA (Figure 2.6C, lanes 1-2).

ENSA and ARPP19 expression were assessed by immunoblotting in protein samples of mouse prophase I oocytes, metaphase II eggs, early embryos, recombinant protein, and cultured cell lysates. First, to determine the optimal amount of total protein needed to detect ENSA and ARPP19 by immunoblot in mouse NIH 3T3, mouse 308, and human HeLa cell lysates, a range of cell lysate amounts was compared (Figure 2.7A and 2.7B). ARPP19 was detected in 2 µg (total protein) of NIH 3T3 cells (Figure 2.7A, lanes 1-3), 308 cells (Figure 2.7A, lanes 4-6) and HeLa cells (Figure 2.7A, lanes 7-9). ENSA was detected in 3 µg (total protein) of NIH 3T3 cells (Figure 2.7B, lanes 1-3), 3 µg of 308 cells (Figure 2.7B, lanes 4-6), and 7.5 µg of HeLa cells (Figure 2.7B, lanes 7-9).

ARPP19 was detected in lysates of 100 metaphase II eggs (Figure 2.7C, lane 2; 2.5 µg total protein), but was not detected in lysates of 100 germinal vesicle-intact (GVI), prophase I-arrested oocytes (GVIs; Figure 2.7C, lane 1; 2.5 µg total protein). ARPP19 was detected more strongly in 2 µg of cultured cell lysates (NIH 3T3, 308, and HeLa; Figure 2.7C, lanes 7-9) than in a lysate of 2.5 µg (total protein) of metaphase II eggs (100 metaphase II eggs; Figure 2.7C, lane 2). We also assessed ARPP19 levels in early mouse embryos to determine whether the maternal Arpp19 transcript is translated in early embryos. The early mouse embryo is transcriptionally inactive and depends on stored
maternal transcripts until the time of zygotic genome activation (occurring around the late one-cell stage in mice) (Li, Zheng et al. 2010). One-cell embryo lysates were prepared at nine hours or 18 hours after in vitro fertilization. These time points were chosen based on previous studies examining polysome recruitment and transcription of maternal transcripts during early murine embryonic development (Cascio and Wassarman 1982; Howlett and Bolton 1985; Moore, Ayabe et al. 1996; Wang and Latham 1997; Wang and Latham 2000; Backs, Stein et al. 2010). ARPP19 was not detected in 100 nine-hour one-cell embryos (Figure 2.7C, lane 3; 2.5 µg total protein) or in 100 18-hour one-cell embryos (data not shown; 2.5 µg total protein).

ENS A was detected in lysates of 20 germinal vesicle-intact (GVI), prophase I-arrested oocytes (Figure 2.7D, lane 1; 0.5 µg total protein), 20 metaphase II eggs (Figure 2.7D, lane 2; 0.5 µg total protein), 20 nine-hour one-cell embryos (Figure 2.7D, lane 3; 0.5 µg total protein), and 20 18-hour one-cell embryos (data not shown). For ENSA, the band intensity for the oocyte, egg and embryo lysates (0.5 µg total protein) was greater than the band intensity for 3 µg (total protein) of NIH 3T3 cells (Figure 2.7D, lane 7), 3 µg of 308 cells (Figure 2.7D, lane 8), and 7.5 µg of HeLa cells (Figure 2.7D, lane 9).

Although ARPP19 protein was not detected in mouse oocytes, we nevertheless attempted to use RNAi-mediated knockdown to assess any possible function of ARPP19 in mouse oocytes. We were interested in exploring the function of both ENSA (study of ENSA function is in Chapter 3) and ARPP19 in a cellular context with two MASTL substrates to determine whether both substrates are functional during meiotic maturation; however, these attempts to knockdown Arpp19 were unsuccessful (Figure 2.8A; Table 2.4). Arpp19 mRNA levels were decreased by 26 ± 5% in Arpp19 siRNA-injected
oocytes compared to controls (Figure 2.8A) in nine experiments using two different Arpp19-targeting siRNA pools and three different concentrations (see Table 2.4 for a summary of experiments). We also attempted to knockdown Arpp19 in mouse NIH 3T3 cells using the second Arpp19-targeting siRNA pool. Arpp19 mRNA levels were unaffected in Arpp19 siRNA-transfected cells compared to controls (Figure 2.8B). There was a small reduction in ARPP19 protein levels (8%) in Arpp19 siRNA-transfected cells compared to controls (Figure 2.8C).

We sought to extend the results in Figure 2.7D, and examine the abundance of ENSA in mouse oocytes in more detail. As shown above, ENSA was easily detected in a lysate of 0.5 µg total protein from germinal vesicle-intact (GVI), prophase I-arrested oocytes (Figure 2.7D, lane 1), with the band detected in oocyte lysates being comparable to or even darker than the bands detected in samples of 3-7.5 µg protein from NIH 3T3 cells (3 µg total protein; Figure 2.7D, lane 7), 308 cells (3 µg total protein; Figure 2.7D, lane 8), and HeLa cells (7.5 µg total protein; Figure 2.7D, lane 9). To get a better estimate of the amount of ENSA protein in mouse oocytes, we undertook quantitative immunoblotting studies using the Licor Odyssey® CLX Infrared Imaging System (Figure 2.9), using methods based on those used to quantify the amounts of specific keratins in basal keratinocytes (Feng, Zhang et al. 2012). First, we assessed the purity of the recombinant GST-ENSA protein used for these quantitative immunoblots by silver staining (Figure 2.9A). Samples of another purified recombinant protein, Drosophila GST-Endos, which was expressed and purified at a different time, were included as a control. For both GST-Endos and GST-ENSA, there was a dominant upper Mr protein band and a secondary lower Mr protein band that was likely the result of incomplete
translation or proteolysis. Both the upper and lower \( M_r \) bands cross-reacted with the anti-ENSA antibody in immunoblots (Figures 2.6A, 2.7D and 2.9B). A faint haze was present on the top half of the resolving gel from \( M_r = \sim 50,000 \) to the top of the gel. This haze occurred in multiple experiments in all lanes. While it is possible that this haze is proteinaceous, it was independent of sample type and sample concentration and did not increase in intensity in lanes with a higher concentration of protein. In the representative experiment shown in Figure 2.9B, a lysate of 35 germinal vesicle-intact (GVI), prophase I-arrested oocytes (Figure 2.9B, lane 1) and seven known amounts of recombinant GST-ENSA (Figure 2.9B, lanes 2-8; 15-60 ng) were assessed by immunoblotting using an anti-ENSA antibody. Fluorescence band intensity was quantified and GST-ENSA samples were used to construct a standard curve (Figure 2.9C). Based on these analyses and on the assumption that the protein present in the GST-ENSA sample was close to 100% pure GST-ENSA, the amount of ENSA per mouse oocyte was estimated to be 0.85 ± 0.05 ng.

**IV. DISCUSSION**

This study identifies the MASTL pathway as a critical regulator of M-phase entry during meiotic maturation in mouse oocytes. MASTL-deficient oocytes were impaired in their ability to exit from prophase I arrest with the majority of oocytes remaining arrested at the prophase I stage. The phenotype observed in MASTL-deficient oocytes is similar to phenotypes observed when the MASTL-ENSA/ARPP19 pathway is disrupted in pig, *Xenopus* and *Drosophila* oocytes (Archambault, Zhao et al. 2007; Zhao, Haccard et al.
2008; Kim, Bucciarelli et al. 2012; Li, Kang et al. 2013). In MASTL-deficient pig oocytes, a subset of oocytes arrested at the prophase I stage or during germinal vesicle breakdown with oocytes that progressed beyond the prophase I stage showing numerous meiotic progression defects, including spindle, chromosome condensation and DNA alignment defects (Li, Kang et al. 2013). Injection of active Greatwall into *Xenopus* oocytes induced meiotic maturation in the absence of the physiological signal for maturation (progesterone) (Zhao, Haccard et al. 2008). *endos* mutant *Drosophila* oocytes display prolonged arrest at the prophase I stage with the majority of oocytes failing to initiate progression to metaphase I (Von Stetina, Tranguch et al. 2008). The meiotic maturation defect observed in MASTL-deficient mouse oocytes in this study, as well as the defects previously observed in pig, *Xenopus* and *Drosophila* oocytes, are suggestive of an inability to maintain phosphorylation of M-phase substrates due to unopposed PP2A activity. The link between the ENSA and PP2A in mouse oocytes is explored in Chapter 3.

While the phenotype observed in MASTL-deficient oocytes in this study is similar to previous studies, there are also some distinct differences. These differences mainly center on whether interference with MASTL-ENSA/ARPP19 pathway results in defective M-phase entry and/or defects in progression through M-phase. *Drosophila* oocytes expressing mutant forms of *Greatwall* displayed defects in meiotic maturation, including spindle and chromosome defects; however, these oocytes appear to exit from prophase I arrest and progress into meiosis normally (Archambault, Zhao et al. 2007; Kim, Bucciarelli et al. 2012). The role of MASTL in M-phase entry in the mouse was explored in G2-arrested *Mastl*-conditional knockout cells created using adenovirus driven
Cre recombinase expression in mouse embryonic fibroblasts with a loxP-flanked *Mastl* allele. Serum was used to stimulate the cell cycle reentry and cells progressed from G2 to M phase normally. However, the majority of cells arrested in mitosis and displayed chromosome condensation and segregation defects (Álvarez-Fernández, Sánchez-Martínez et al. 2013). There is also recent evidence for different requirements for the Greatwall pathway during meiotic entry and meiotic progression in the starfish, *Patiria pectinifera*. In starfish oocytes, Greatwall is not essential for M-phase entry, but the sole starfish Greatwall substrate, Arpp19, is required for nuclear envelope breakdown even in the absence of Greatwall (Hara, Abe et al. 2012; Okumura, Morita et al. 2014). In the starfish, it appears that a Greatwall-independent pathway for Cdk1/cyclin B activation exists and Cdk1/cyclin B can directly phosphorylate Arpp19 converting Arpp19 into a PP2A-B55 inhibitor. Phosphorylation of Arpp19 by Cdk1/cyclin B was not sufficient for progression through M-phase and Greatwall phosphorylation of Arpp19 is still required for proper M-phase completion and chromosome segregation (Okumura, Morita et al. 2014). Differences in the requirement for MASTL/Greatwall during M-phase entry and M-phase progression may be explained by differences in cellular context, including cell type and species of origin. The method of MASTL/Greatwall depletion is also likely to be important and cells may be able to enter M-phase, but not successfully progress through mitosis with low MASTL levels. These studies highlight the nuances of the MASTL/Greatwall pathway and suggest that MASTL/Greatwall is not essential for M-phase entry in all cell types and that some cells can enter M-phase without MASTL/Greatwall.
The majority of *Mastl* siRNA-injected mouse oocytes remained arrested at the prophase I stage, but a portion of oocytes (28%) was able to exit from prophase I arrest. We predicted that this ability of some oocytes to exit from prophase I arrest was due to oocyte-to-oocyte variability in the extent of MASTL knockdown. We did observe variation in the extent of MASTL knockdown using immunofluorescence assessment of MASTL protein levels in a population of siRNA-injected oocytes. The residual MASTL protein in some oocytes may have been sufficient to inhibit PP2A activity allowing maintenance of CDK1 phosphorylation events and progression from prophase I into metaphase.

Our study is the first to examine the presence of the two MASTL substrates, ENSA and ARPP19, in cells from two mammalian species, human and mouse. The presence of ENSA and ARPP19 was recently assessed in pig oocytes and it was determined that the pig *Ensa* gene contains a premature stop codon and functional protein is not expressed (Li, Kang et al. 2013). There have been no studies to date looking at the expression of ENSA and ARPP19 in mouse cells. We detected both ENSA and ARPP19 in human and mouse cell lysates (mouse NIH 3T3 cells, mouse 308 cells, and human HeLa cells). Previous work in the field disagreed about whether ARPP19 was expressed by HeLa cells with one paper detecting a mitotic defect phenotype after siRNA knockdown of ARPP19 in HeLa cells (Gharbi-Ayachi, Labbé et al. 2010), and another paper being unable to detect ARPP19 by immunoblotting in HeLa cell lysates and observing no phenotype after transfection with ARPP19-targeting siRNA (Gharbi-Ayachi, Labbé et al. 2010; Cundell, Bastos et al. 2013). These differences in the ability to detect ARPP19 protein in HeLa cells may be due to differences in HeLa cell lines or the use of
different reagents (mainly primary antibody) for detection. Both studies used full-length recombinant ARPP19 as antigens to produce polyclonal antibodies, but characterization of the specificity of these antibodies is not reported (Gharbi-Ayachi, Labbé et al. 2010; Cundell, Bastos et al. 2013). Our data here with two different antibodies made to ARPP19 highlight the importance of assessing the specificity of primary antibodies for ENSA or ARPP19. These proteins are 76% identical at the amino acid level, and one commercially available antibody for ARPP19 we tested cross-reacted with both mouse ENSA and mouse ARPP19.

ENSA and ARPP19 expression was examined during meiotic maturation and early embryogenesis using lysates of mouse prophase I oocytes, metaphase II eggs and early embryos. ARPP19 was not detected in oocyte or embryo lysates and low levels were detected in metaphase II eggs (2.5 µg total protein in each lysate) compared to similar amounts of cultured cell lysates (2 µg total protein mouse NIH 3T3 cells, mouse 308 cells and human HeLa cells). ARPP19 expression may be regulated during meiotic maturation and fertilization with protein levels only rising in the metaphase II egg. ENSA was detected at all three stages in lysates of only 20 cells (0.5 µg total protein). We attempted to examine the function of ARPP19 in mouse oocytes, but we were unable to successfully knockdown Arpp19 at the transcript level. Chapter 3 focuses on the characterization of ENSA function during meiotic maturation. We demonstrate a role for ENSA in the exit from prophase I arrest similar to the phenotype observed in MASTL-deficient oocytes. Based on comparisons of band intensities of ENSA and ARPP19 in oocytes to band intensities of known amounts of recombinant proteins (Figure 7C, D), ENSA appears to be more abundant than ARPP19 in mouse oocytes. Interestingly, in the
two human cancer cell lines, ENSA is more abundant than ARPP19 (Table 2.5; 1.99 - 3.75 x 10⁵ copies per cell for ENSA and 1.35 x 10³ - 2.02 x 10⁴ copies per cell for ARPP19) (Beck, Schmidt et al. 2011; Nagaraj, Wisniewski et al. 2011). Additionally, we were only able to detect ARPP19 in metaphase II eggs, but not in prophase I oocytes or early embryos. It is possible that ARPP19 regulates M-phase maintenance during metaphase II arrest, while ENSA regulates M-phase entry and progression during other stages of meiotic maturation. Future studies should address whether ARPP19 regulates the maintenance of metaphase II arrest and if ENSA also plays a role in this process.

The amount of ENSA protein per oocyte was estimated to be 0.85 ± 0.05 ng (3.84 x 10¹⁰ copies per cell, Table 2.5) using quantitative immunoblotting studies (Figure 2.9) and assuming that the purified recombinant GST-ENSA used in quantitative immunoblotting studies is 100% pure. While this may be an overestimate for the purity of recombinant GST-ENSA, even if GST-ENSA was only 50% pure, the amount of ENSA per mouse oocyte would still be an impressive 0.43 ng. Another example of an abundant protein in mouse oocytes is the RNA-binding protein, MSY2, with 0.45 ng of protein or 7.12 x 10⁹ copies per cell detected per oocyte (Table 2.5) (Yu, Hecht et al. 2001). To give the reader some context, the number of copies per mouse oocyte of the cell cycle proteins CDK1, cyclin B1, CDC25C and WEE1 was estimated to be 1.43 x 10⁷, 9.55 x 10⁷, 8.00 x 10⁷, 4.01 x 10⁷, respectively (Table 2.5) (Kanatsu-Shinohara, Schultz et al. 2000). CDK1, CDC25C and WEE1 are enzymes and cyclin B1 is an enzyme regulatory subunit. ENSA has a somewhat different action, functioning as a stoichiometric inhibitor of PP2A, and it is possible that ENSA is needed at higher concentrations in mouse oocytes.
Quantitative proteomic studies in cultured cells also provide information about the abundance of ENSA and ARPP19 in two other cell types, HeLa cells and U2OS cells, which are human osteosarcoma cells. These data are summarized in Table 2.5. For ENSA, there are $3.84 \times 10^{10}$ copies per mouse oocyte, $3.75 \times 10^5$ copies per HeLa cell, and $1.99 \times 10^5$ copies per U2OS cell (Beck, Schmidt et al. 2011; Nagaraj, Wisniewski et al. 2011). The observation that ENSA appears to be more abundant in mouse oocytes than in cultured cells is not solely due to the larger volume of a mouse oocyte, based on conversion of these copies per cell values to molar concentrations (Table 2.5). ENSA levels may be higher in mouse oocytes than in cultured cells due to the temporal requirements for M-phase entry and maintenance during mammalian female meiosis. For example, in mouse oocytes, metaphase I lasts for hours compared to minutes in a normal mitotic cell. Arrest at the metaphase II stage is also unique to mammalian female meiosis. High levels of ENSA may be critical for efficient inhibition of PP2A activity during these extended periods of M-phase. Only ENSA that is phosphorylated by MASTL/Greatwall is able to function as a PP2A inhibitor (Mochida, Maslen et al. 2010; Mochida 2013). Estimates for the proportion of cellular ENSA that is phosphorylated during M-phase range from approximately 50% in HeLa cells to nearly 100% in *Xenopus* egg extracts (Mochida, Maslen et al. 2010; Cundell, Bastos et al. 2013; Williams, Filter et al. 2014).

Phosphorylated ENSA acts as a stoichiometric inhibitor of PP2A-B55 by binding to the phosphatase active site and there is evidence from other cell types that ENSA is present at a 5:1 molar ratio compared to PP2A-B55 (Cundell, Bastos et al. 2013; Williams, Filter et al. 2014). Although only approximate concentrations are presented, a similar ratio of Ensa to PP2A-B55 was reported in studies using *Xenopus* egg extracts.
with Ensa present at ~150 – 300 nM and PP2A-B55δ at ~50 – 70 nM (Mochida, Maslen et al. 2010). The cellular requirement for an excess of ENSA is explained in part by a recent study that found that ENSA functions as a PP2A-B55 inhibitor, but can also be dephosphorylated by PP2A-B55 to facilitate M-phase exit (Williams, Filter et al. 2014). This work presents a model where phosphorylated ENSA binds tightly to PP2A-B55 blocking the phosphatase active site. PP2A-B55 can also dephosphorylate ENSA, but this is a slow dephosphorylation event compared to the kinetic parameters for the dephosphorylation of other PP2A-B55 substrates. During M-phase, the kinase MASTL/Greatwall continues to phosphorylate ENSA, so the pool of phosphorylated ENSA is replenished even if dephosphorylation by PP2A-B55 is occurring at the slow rate. During M-phase exit, MASTL/Greatwall is no longer active and levels of phosphorylated ENSA fall allowing M-phase exit (Williams, Filter et al. 2014). While a 5:1 molar ratio of ENSA to PP2A-B55 was observed in mitotic cultured cells and Xenopus extracts (Mochida, Maslen et al. 2010; Cundell, Bastos et al. 2013; Williams, Filter et al. 2014), it is possible that even higher ratios of ENSA to PP2A-B55 are required by mammalian oocytes to maintain the phosphorylation status of M-phase substrates during the extended periods of meiotic M-phase.

This chapter demonstrates a role for MASTL during the exit from prophase I arrest in mammalian oocytes and examines the expression of the MASTL substrates, ENSA and ARPP19, during meiotic maturation and early embryogenesis. Chapter 3 will extend these studies by examining the role of ENSA during meiotic maturation in mouse oocytes.


VI. TABLES AND FIGURES

Table 2.1: Primer sequences for cloning of *Ensa* and *Arpp19* into the PGEX-4T-1 vector

<table>
<thead>
<tr>
<th>Description</th>
<th>Sequence (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse <em>Ensa</em> (NM_019561.2) forward primer for cloning full-length <em>Ensa</em>,</td>
<td>5’- GCGGATCCATGTCCCAGAAACAAGAAGA-3’</td>
</tr>
<tr>
<td>for expression of ENSA as a GST fusion protein; includes a Bam HI site</td>
<td></td>
</tr>
<tr>
<td>Mouse <em>Ensa</em> (NM_019561.2) reverse primer for cloning full-length <em>Ensa</em>,</td>
<td>5’- ACGCGTCGACTCACTCAACTTGGCCACCCG-3’</td>
</tr>
<tr>
<td>for expression of ENSA as a GST fusion protein; includes a Sal I site</td>
<td></td>
</tr>
<tr>
<td>Mouse <em>Arpp19</em> (NM_021548.4) forward primer for cloning full-length <em>Arpp19</em>,</td>
<td>5’- GCGGATCCATGTCCGCGGAAGTCCCCGA-3’</td>
</tr>
<tr>
<td>for expression of ARPP19 as a GST fusion protein; includes a Bam HI site</td>
<td></td>
</tr>
<tr>
<td>Mouse <em>Arpp19</em> (NM_021548.4) reverse primer for cloning full-length <em>Arpp19</em>,</td>
<td>5’- ACGCGTCGACTCAGCCAGCCAGCTTGCTAG-3’</td>
</tr>
<tr>
<td>for expression of ARPP19 as a GST fusion protein; includes a Sal I site</td>
<td></td>
</tr>
</tbody>
</table>
### Table 2.2: Primer sequences for knockdown assessment by RT-PCR

<table>
<thead>
<tr>
<th>Description</th>
<th>Sequence (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse <em>Mastl</em> forward primer</td>
<td>5’-TCCCTCAAAGGCAGCCAAAAGC-3’</td>
</tr>
<tr>
<td>Mouse <em>Mastl</em> reverse primer</td>
<td>5’-GCAGGACCATGGGCTGTACC-3’</td>
</tr>
<tr>
<td>Mouse <em>Arpp19</em> forward primer</td>
<td>5’-AGCGTCTGCGGGAGGAGGACA-3’</td>
</tr>
<tr>
<td>Mouse <em>Arpp19</em> reverse primer</td>
<td>5’-AGCAACCAGGGAGGTTACAG-3’</td>
</tr>
<tr>
<td>Mouse <em>Plat</em> forward primer</td>
<td>5’-CATGGGCAAAGCTACAG-3’</td>
</tr>
<tr>
<td>Mouse <em>Plat</em> reverse primer</td>
<td>5’-CAGAGAAAGATTGAGACGAT-3’</td>
</tr>
</tbody>
</table>
Table 2.3: Primer sequences for detection of *Ensa* and *Arpp19* transcript variants

<table>
<thead>
<tr>
<th>Description</th>
<th>Sequence (5' → 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse <em>Ensa</em> (NM_019561.2) forward primer for detection of transcript</td>
<td>5'-ATGTCAGAAACAAGAAGAAG-3'</td>
</tr>
<tr>
<td>variant a</td>
<td></td>
</tr>
<tr>
<td>Mouse <em>Ensa</em> (NM_019561.2) reverse primer for detection of transcript</td>
<td>5'-TCACTCAACTTGGCCACCCG-3'</td>
</tr>
<tr>
<td>variant a</td>
<td></td>
</tr>
<tr>
<td>Mouse <em>Ensa</em> (NM_001021383.1) forward primer for detection of transcript</td>
<td>5'-GAAAACCTGCGGAGGAGACC-3'</td>
</tr>
<tr>
<td>variant b</td>
<td></td>
</tr>
<tr>
<td>Mouse <em>Ensa</em> (NM_001021383.1) reverse primer for detection of transcript</td>
<td>5'-AGCTGCAGCCTCGCACTCCT-3'</td>
</tr>
<tr>
<td>variant b</td>
<td></td>
</tr>
<tr>
<td>Mouse <em>Arpp19</em> (NM_021548.4) forward primer for detection of transcript</td>
<td>5'-TGTCGTCACAAGCGGGCTGTCG-3'</td>
</tr>
<tr>
<td>variant 1</td>
<td></td>
</tr>
<tr>
<td>Mouse <em>Arpp19</em> (NM_021548.4) reverse primer for detection of transcript</td>
<td>5'-AGCAACCAGGGACCGGTTCG-3'</td>
</tr>
<tr>
<td>variant 1</td>
<td></td>
</tr>
<tr>
<td>Mouse <em>Arpp19</em> (NM_001136127.1) forward primer for detection of transcript</td>
<td>5'-AGAGGAAGTGGCTCGGCGATCG-3'</td>
</tr>
<tr>
<td>variant 2</td>
<td></td>
</tr>
<tr>
<td>Mouse <em>Arpp19</em> (NM_001136127.1) reverse primer for detection of transcript</td>
<td>5'-AGCAACCAGGGACCGGTTCG-3'</td>
</tr>
</tbody>
</table>
Table 2.4: Summary of experiments assessing knockdown of *Arpp19* at the RNA level in mouse oocytes

<table>
<thead>
<tr>
<th>Experiment number</th>
<th>siRNA pool</th>
<th>siRNA dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>20 µM</td>
</tr>
<tr>
<td>1</td>
<td>Pool #1</td>
<td>23%</td>
</tr>
<tr>
<td>2</td>
<td>Pool #1</td>
<td>25%</td>
</tr>
<tr>
<td>3</td>
<td>Pool #2</td>
<td>53%</td>
</tr>
<tr>
<td>4</td>
<td>Pool #2</td>
<td>49%</td>
</tr>
<tr>
<td>5</td>
<td>Pool #2</td>
<td>20%</td>
</tr>
<tr>
<td>6</td>
<td>Pool #2</td>
<td>10%</td>
</tr>
<tr>
<td>7</td>
<td>Pool #2</td>
<td></td>
</tr>
</tbody>
</table>

Average of all experiments is 26 ± 5% (mean ± standard error of the mean).
Table 2.5: Comparison of quantitative studies assessing ENSA and ARPP19 expression in cultured cells, other abundant proteins in mouse oocytes and the amount of ENSA per mouse oocyte

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ENSA</td>
<td>Mouse oocytes</td>
<td>Quantitative immunoblot</td>
<td>0.85[^1]</td>
<td>6.37 x 10[^14]</td>
<td>2.37 x 10[^3]</td>
<td>3.84 x 10[^10]</td>
<td>Figure 2.9</td>
</tr>
<tr>
<td>MSY2</td>
<td>Mouse oocytes</td>
<td>Quantitative immunoblot</td>
<td>0.45</td>
<td>1.81 x 10[^14]</td>
<td>4.41 x 10[^5]</td>
<td>7.12 x 10[^9]</td>
<td>(Yu, Hecht et al. 2001)</td>
</tr>
</tbody>
</table>

[^1]: Data provided from the referenced study is highlighted in gray; all other numbers were calculated as described below.
1 – This value was calculated based on the assumption that the protein present in the GST-ENSA sample, used for the standard curve, is close to 100% pure GST-ENSA (further details provided in Figure 2.9C and in Results and Discussion text).

2 – Moles of protein was calculated by dividing the amount of protein in grams by the molecular weight of protein in daltons (grams/mole) or by dividing copies per cell by Avogadro’s number. Protein molecular weights were calculated using the amino acid sequence and the ExPASy molecular weight calculator tool (http://web.expasy.org/compute_pi/).

3 – Molarity was calculated by dividing number of moles by the cell volume in liters. Cell volume was calculated using the cell radius. For mouse oocytes a radius of 40 µM was used to calculate cell volume, for HeLa cells a radius of 10.5 µM was used (Milo, Jorgensen et al. 2010) and for U2OS cells a radius of 10 µM was used (Milo, Jorgensen et al. 2010; Milo, Jorgensen et al. 2010; Beck, Schmidt et al. 2011)

4 – Copies per cell was calculated by multiplying the number of moles by Avogadro’s number.

5 – This paper provides values for both copies per cell and number of moles. The number of moles is calculated using a formula that accounts spectral counts of each protein. The number of moles was re-calculated using the formulas provided above to allow comparison of data from different data sets.
Figure 2.1: MASTL is expressed by mouse oocytes

Panel A: RT-PCR of oocyte cDNA using Mastl-specific primers demonstrating detection of the Mastl transcript in mouse oocytes. Panel B: Anti-MASTL immunoblot of 80 prophase I-arrested, germinal vesicle intact (GVI) oocytes (lane 1, 2 µg total protein). Mouse 308 (lane 2, 3.8 µg; lane 3, 19 µg total protein) and human HeLa (lane 4, 12.9 µg; lane 5, 25.8 µg total protein) whole cell lysates (WCL) were loaded as positive controls. The band of interest is marked by a black arrowhead.
A

Mastl

B

<table>
<thead>
<tr>
<th>80 GVIs</th>
<th>308 WCL</th>
<th>HeLa WCL</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
</tbody>
</table>

1  2  3  4  5
Figure 2.2: Knockdown of *Mastl* in mouse oocytes

Prophase I-arrested oocytes were injected with 50 µM negative control siRNA or *Mastl*-targeting siRNA and cultured for 44-48 hours in the presence of milrinone to maintain prophase I arrest and allow time for knockdown. Panel A: RT-PCR analysis of *Mastl* transcript levels. Lanes 1-2 show the positive control PCR product (transcripts for tissue plasminogen activator, *Plat*) for negative control siRNA-injected oocytes (lane 1) and *Mastl* siRNA-injected oocytes (lane 2). Lanes 3-4 show the *Mastl* PCR product for negative control siRNA-injected oocytes (lane 3) and *Mastl* siRNA-injected oocytes (lane 4). *Mastl* mRNA levels were decreased by 84 ± 3% in *Mastl* siRNA-injected oocytes compared to controls (three experiments, with one representative experiment shown; range 81-89%). Panel B and C: Anti-MASTL immunoblot of negative control siRNA-injected oocytes (lane 1) and *Mastl* siRNA-injected oocytes (lane 2) (75 oocytes per lane). Mouse NIH 3T3 (lanes 3 and 4) and mouse 308 (lanes 5 and 6) whole cell lysates (WCL) were loaded as a positive control. MASTL protein was decreased by 95% in *Mastl* siRNA-injected oocytes compared to controls in one experiment (Panel B). MASTL protein levels were decreased to undetectable levels in *Mastl* siRNA-injected oocytes compared to controls in two experiments (Panel C, one representative experiment shown) (three total experiments; asterisk indicates background band). The band of interest is marked by a black arrowhead.
Figure 2.3: Ability to exit from prophase I arrest is impaired in MASTL-deficient oocytes

Assessment of germinal vesicle breakdown in negative control siRNA-injected oocytes (n = 184 oocytes; open circles) and Mastl siRNA-injected oocytes (n = 206 oocytes; gray squares). Prophase I-arrested oocytes were injected with 50 µM negative control siRNA or Mastl-targeting siRNA and cultured for 44-48 hours in the presence of milrinone to maintain prophase I arrest and allow time for knockdown. These data are pooled from three replicate experiments and error bars show standard errors of the mean.
Figure 2.4: Assessment of MASTL knockdown in individual oocytes

Panel A: Immunofluorescence analysis of MASTL levels in negative control siRNA-injected (control) and *Mastl* siRNA-injected oocytes. Images show prophase I-arrested oocytes from the control group (Panels i-iii) and the *Mastl* siRNA-injected group (Panels iv-xii) stained with an anti-MASTL antibody (ii, v, viii, xi) and DAPI (iii, vi, ix, xii) to label DNA. MASTL was mainly localized to the germinal vesicle and excluded from the nucleolus. Three representative images are shown of *Mastl* siRNA-injected oocytes to demonstrate oocyte-to-oocyte variability in MASTL fluorescence intensity. Scale bar (shown in Panel i) equals 10 µm. Panel B: Quantification of MASTL fluorescence intensity in negative control-injected (control; white bars; n=53) and *Mastl* siRNA-injected oocytes (*Mastl*-siRNA; black bars, n=46). Fluorescence intensity was measured within the germinal vesicle for each individual oocyte and was normalized to the mean fluorescence intensity of the control group. The mean normalized fluorescence intensity was 1.0 ± 0.04 (mean ± standard error of the mean) for the control group and 0.31 ± 0.04 for the *Mastl* siRNA-injected group. Oocytes were categorized into eight groups (oocyte count shown on the y-axis) based on the normalized fluorescence intensity (0-0.25, 0.26-0.50, 0.51-0.75, 0.76-1.0, 1.01-1.25, 1.26-1.50, 1.51-1.75, 1.76-2.0).
A

<table>
<thead>
<tr>
<th>Merge</th>
<th>Mastl siRNA injected</th>
</tr>
</thead>
<tbody>
<tr>
<td>i</td>
<td>iv</td>
</tr>
<tr>
<td>ii</td>
<td>v</td>
</tr>
<tr>
<td>iii</td>
<td>vi</td>
</tr>
<tr>
<td>iv</td>
<td>vii</td>
</tr>
<tr>
<td>v</td>
<td>viii</td>
</tr>
<tr>
<td>vi</td>
<td>ix</td>
</tr>
<tr>
<td>vii</td>
<td>x</td>
</tr>
</tbody>
</table>

B

![Chart showing Normalized Fluorescence Intensity vs Count (number of oocytes)](chart.png)

- **Neg. Control**
- **Mastl siRNA**

Normalized Fluorescence Intensity

Count (number of oocytes)
Figure 2.5: Sequence alignment of mouse ENSA and ARPP19

Multiple sequence alignment of the two ENSA isoforms (isoform a, NP_062507.1 and isoform b, NP_001021383.1) and the two ARPP19 isoforms (isoform 1, NP_067523.1 and isoform 2, NP_001136127.1) using Clustal-W (http://www.ebi.ac.uk/Tools/msa/clustalw2). The two ENSA isoforms are identical except that isoform a has an additional four amino acids at the C-terminus. ARPP19 isoform 1 and isoform 2 have an identical C-terminal region and isoform 2 has an additional 33 amino acid region at the N-terminus. The sequence highlighted in yellow was used to produce a custom anti-ARPP19 goat polyclonal antibody, EB12183 (produced by Everest Biotech, Oxfordshire, UK). EB12183 was developed using the peptide C-PAAAPDKTEVTGDH, which is specific to ARPP19 and is not found in ENSA.
ENSA – isoform a
ENSA – isoform b
ARPP19 – isoform 1
ARPP19 – isoform 2

---

ENSA – isoform a
ENSA – isoform b
ARPP19 – isoform 1
ARPP19 – isoform 2

---

ENSA – isoform a
ENSA – isoform b
ARPP19 – isoform 1
ARPP19 – isoform 2

---
Figure 2.6: Assessment of specificity of anti-ENSA and anti-ARPP19 antibodies using recombinant proteins

Purified recombinant GST-ENSA and GST-ARPP19 were used to assess antibody specificity by immunoblotting. Panel A: Anti-ENSA immunoblot of GST-ENSA (lanes 1-3) and GST-ARPP19 (lanes 4-6) (25, 50, 75 ng). This anti-ENSA antibody appears to be specific for mouse ENSA and did not cross-react with mouse ARPP19 under these conditions. The lower Mr band in the GST-ENSA samples is likely a product of incomplete translation or proteolysis. Panel B: Anti-ARPP19 (ProteinTech Group) immunoblot of GST-ENSA (lanes 1-3) and GST-ARPP19 (lanes 4-6) (25, 50, 75 ng). This anti-ARPP19 antibody was not specific for mouse ARPP19 and cross-reacted with both mouse ENSA and ARPP19 under these conditions. Panel C: Anti-ARPP19 (custom antibody from Everest Biotech) immunoblot of GST-ENSA (lanes 1-2) and GST-ARPP19 (lanes 3-4) (30, 50 ng). This anti-ARPP19 antibody was specific for mouse ARPP19 and did not cross-react with mouse ENSA under these conditions.
A

<table>
<thead>
<tr>
<th>GST-ENSA</th>
<th>GST-ARPP19</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>75</td>
<td>75</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>GST-ENSA</th>
<th>GST-ARPP19</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>75</td>
<td>75</td>
</tr>
</tbody>
</table>

C

<table>
<thead>
<tr>
<th>GST-ENSA</th>
<th>GST-ARPP19</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>
Figure 2.7: Characterization of MASTL substrates, ENSA and ARPP19, in mouse oocytes and somatic cultured cells

Anti-ARPP19 and anti-ENSA immunoblots were performed to determine the amount of NIH 3T3, 308 and HeLa cell lysate needed to detect ARPP19 and ENSA (Panels A and B). Panel A: Anti-ARPP19 immunoblot of three amounts of mouse NIH 3T3 (lanes 1-3; 2, 3, 5 µg), mouse 308 (lanes 4-6; 2, 3, 5 µg) and human HeLa (lanes 7-9: 2.5, 5, 7.5 µg) whole cell lysates. Open arrows indicate the band of interest. Panel B: Anti-ENSA immunoblot of three amounts of NIH 3T3 (lanes 1-3; 2, 3, 5 µg), 308 (lanes 4-6; 2, 3, 5 µg) and HeLa (lanes 7-9; 5, 7.5, 10 µg) whole cell lysates. Open arrows indicate the band of interest. Panel C: Anti-ARPP19 immunoblot of 100 prophase I germinal vesicle-intact oocytes (lane 1; GVIs; 2.5 µg), 100 metaphase II eggs (lane 2; MIIs; 2.5 µg), 100 1-cell embryos (lane 3; Emb.; 2.5 µg), GST-ARPP19 (lanes 4-6; 10, 20, 30 ng), NIH 3T3 cells (lane 7; 2 µg), 308 cells (lane 8; 2 µg), and HeLa cells (lane 9; 2 µg). Open arrows indicate the band of interest in the cell lysate lanes; black arrows indicate the band of interest in the recombinant protein lanes. Panel D: Anti-ENSA immunoblot of 20 prophase I germinal vesicle-intact oocytes (lane 1; GVIs; 0.5 µg), 20 metaphase II eggs (lane 2; MIIs; 0.5 µg), 20 1-cell embryos (lane 3; Emb.; 0.5 µg), GST-ENSA (lanes 4-6; 10, 15, 25 ng), NIH 3T3 cells (lane 7; 3 µg), 308 cells (lane 8; 3 µg), and HeLa cells (lane 9; 7.5 µg). The lower Mr band in the GST-ENSA samples is likely a product of incomplete translation or proteolysis. Open arrows indicate the band of interest in the cell lysate lanes; black arrows indicate the band of interest in the recombinant protein lanes.
Figure 2.8: Knockdown of Arpp19 in mouse oocytes and 3T3 cells

Panel A: Prophase I-arrested oocytes were injected with negative control siRNA or Arpp19-targeting siRNA and cultured for 44-48 hours in the presence of dbcAMP to maintain prophase I arrest and allow time for knockdown. Arpp19 transcript levels were analyzed by RT-PCR. Lanes 1-2 show the positive control PCR product (transcripts for tissue plasminogen activator, Plat) for negative control siRNA-injected oocytes (lane 1) and Arpp19 siRNA-injected oocytes (lane 2). Lanes 3-4 show the Arpp19 PCR product for negative control siRNA-injected oocytes (lane 3) and Arpp19 siRNA-injected oocytes (lane 4). mRNA levels were decreased by 26 ± 5% in Arpp19 siRNA-injected oocytes compared to controls (nine experiments using two different Arpp19-targeting siRNA pools and three concentrations of Arpp19 siRNA (20, 40, 80 µM), range 20-53%; see Table 2.4 for a summary of experiments). Panels B-D: NIH 3T3 cells were transfected with lipofectamine only (control) or 20 µM Arpp19-targeting siRNA and cultured for 44 hours to allow time for knockdown. RNA and protein were harvested and used for RT-PCR (Panel B) and immunoblot (Panel C, D) analysis. This experiment was performed one time and was not repeated due to lack of substantial knockdown in both oocytes and 3T3 cells using this Arpp19-targeting siRNA pool. Panel B: RT-PCR analysis of Arpp19 transcript levels. Lanes 1-2 show the positive control PCR product (Plat) for control 3T3 cells (lane 1) and Arpp19 siRNA-transfected 3T3 cells (lane 2). Lanes 3-4 show the Arpp19 PCR product for control 3T3 cells (lane 3) and Arpp19 siRNA-transfected 3T3 cells (lane 4). Arpp19 mRNA levels were unaffected in Arpp19 siRNA-transfected 3T3 cells compared to controls. Panel C: Anti-actin immunoblot of four amounts of control 3T3 cells (lanes 1-4) and Arpp19 siRNA-transfected 3T3 cells (lanes 5-8). Panel D: anti-
ARPP19 immunoblot of four amounts of control 3T3 cells (lanes 1-4) and *Arpp19* siRNA-transfected 3T3 cells (lanes 5-8). ARPP19 levels were normalized to actin as a loading control. ARPP19 levels were decreased by approximately 8% in *Arpp19* siRNA-transfected 3T3 cells compared to controls. Open arrows indicate the band of interest.
Figure 2.9: Quantification of ENSA in mouse oocytes

Panel A: Silver-stained gel showing 20, 40 and 60 ng of purified recombinant *Drosophila* GST-Endos (lanes 1-3) and mouse GST-ENSA (lanes 4-6) proteins. For both recombinant proteins, we observed an upper Mr dominant band (GST-ENSA, open arrow) and a lower Mr secondary band (GST-ENSA, closed arrow). The lower band is likely a product of incomplete translation or proteolysis. A faint haze was present on the top half of the gel, from Mr = ~50,000 to the top of the gel. This haze occurred in multiple experiments in all lanes and was independent of sample type and sample concentration. Panel B: Anti-ENSA Odyssey immunoblot of 35 germinal vesicle-intact, prophase I-arrested oocytes (GVIs; lane 1) and seven known amounts of GST-ENSA (lanes 2-8; 15-60 ng). The lower Mr band in the GST-ENSA samples is likely a product of incomplete translation or proteolysis. The sum of the intensity of both bands was quantified for all experiments since both bands cross-reacted with the anti-ENSA antibody and contributed to the total protein concentration determined by the BCA assay. Panel C: Fluorescence band intensity was quantified and GST-ENSA samples were used to construct a standard curve. The equation and $r^2$ value for the best-fit line are shown on the standard curve. With the assumption that the protein present in the GST-ENSA sample is close to 100% pure GST-ENSA, the amount of ENSA per mouse oocyte was estimated to be $0.85 \pm 0.05$ ng (the band intensity for 35 oocyte lysate is 3531.33; experiment repeated three times with one representative experiment shown).
CHAPTER 3: CHARACTERIZATION OF $\alpha$-ENDOSULFINE (ENSA) DURING MEIOTIC MATURATION: ENSA REGULATES EXIT FROM PROPHASE I ARREST IN MOUSE OOCYTES


I. INTRODUCTION

The progression of the mammalian oocyte through meiosis is carefully regulated. This regulation is essential to ensure the coordination of cell cycle progression (also known as nuclear maturation) with a series of events that mediate the acquisition of competence to undergo the egg-to-embryo transition upon fertilization (also known as egg activation) and to progress into embryogenesis (Berrios and Bedford 1979; Kubiak 1989; Ajduk, Malagocki et al. 2008; Chiba 2011). Mammalian female meiosis occurs in a very staggered fashion. The oocyte in the ovarian follicle is arrested at prophase of meiosis I; this arrest occurs before birth, and lasts for weeks to years, depending on the species. Upon ovulation, the oocyte undergoes a transition known as meiotic maturation (also called oocyte maturation), characterized by exit from prophase I arrest and progression through meiosis I (Conti, Hsieh et al. 2012). This process, particularly pro-metaphase and metaphase, takes several hours. The oocyte then emits the first polar body and proceeds to an arrest at metaphase II. The second meiotic division occurs after this metaphase II arrest if fertilization occurs; the fertilizing sperm triggers exit from M-phase,
progression to the first embryonic mitosis, and other events of the egg-to-embryo transition.

The phosphorylation status of key substrates of cyclin-dependent kinase 1 (CDK1; also known as CDC2 or CDC2A) is crucial for the M-phase state. Active CDK1, associated with its regulatory subunit cyclin B, phosphorylates various substrates, leading to events associated with entry into M-phase. Inhibition of the dephosphorylation of CDK1 substrates during M-phase is critical, and then the dephosphorylation of these CDK1 substrates is required for exit from M-phase, allowing a "reset" back to an interphase state. Such regulation of M-phase phosphoproteins is likely highly significant for mammalian oocytes, which have to regulate extended metaphase states during meiosis I and II, with these multi-hour M-phase states sharply contrasting the minutes-long M-phase in the typical somatic cell.

In mammalian oocytes, meiotic resumption from prophase I arrest is analogous to a G2-to-M transition in mitotic cells. CDK1 activity is low in prophase I-arrested oocytes as a result of protein kinase A (PKA) action on the kinases MYT1 and WEE1 (in the mouse, previously WEE1B, now known as WEE2), and the phosphatase CDC25 (CDC25B in mouse) (Han, Chen et al. 2005; Pirino, Wescott et al. 2009; Oh, Han et al. 2010; Schindler 2011). WEE2 and MYT1 phosphorylate CDK1 on two inhibitory residues, T14 and Y15. Inhibition of CDC25 by PKA reinforces this inactive state of CDK1 (Pirino, Wescott et al. 2009). Also downstream from PKA is the phosphatase CDC14B and the anaphase-promoting complex/cyclosome (APC/C), with functions in maintaining prophase I arrest by regulating cyclin B levels (Polanski, Ledan et al. 1998; Ledan, Polanski et al. 2001; Reis, Chang et al. 2006; Schindler and Schultz 2009; Holt,
Tran et al. 2011; Schindler 2011). Exit from prophase I is mediated by decreased PKA activity, contributing to activation of CDC25B, which in turn dephosphorylates of CDK1. Active CDK1 then phosphorylates its M-phase substrates, and progression into M-phase is observed with the occurrence of breakdown of the nuclear envelope of the germinal vesicle (known as GVBD).

A key phosphatase involved in the dephosphorylation of M-phase substrates is PP2A, as identified by immunodepletion studies in *Xenopus* egg extracts and genetic studies in *Drosophila* (Mayer-Jaekel, Ohkura et al. 1994; Mochida, Ikeo et al. 2009). PP2A refers to a broad class of heterotrimeric holoenzymes, composed of a catalytic/C subunit, a scaffold/A subunit, and a regulatory/B subunit (Virshup and Shenolikar 2009). The kinase known as MASTL or Greatwall is at the intersection between CDK1 and PP2A. (Note: MASTL [microtubule-associated serine/threonine kinase-like] is the official name in mouse and human; this kinase is Greatwall in *Drosophila*, and Greatwall is the name used in most *Xenopus* studies.) Immunodepletion studies in *Xenopus* egg extracts identified the specific PP2A form that is inhibited by MASTL/Greatwall as PP2A with the regulatory/B subunit B55δ (MGI symbol, PPP2R2D), consistent with data showing that *Drosophila* mutants lacking this regulatory/B subunit of PP2A have low phosphatase activity toward certain CDK1 substrates (Mayer-Jaekel, Ohkura et al. 1994; Castilho, Williams et al. 2009; Vigneron, Brioudes et al. 2009; Lorca, Bernis et al. 2010; Voets and Wolthuis 2010). Taken together, these data have produced the model that MASTL/Greatwall activity, through its inhibition of PP2A activity, contributes to maintenance of phosphorylated M-phase substrates (Castilho, Williams et al. 2009; Vigneron, Brioudes et al. 2009; Burgess, Vigneron et al. 2010; Goldberg 2010).
MASTL/Greatwall achieves this inhibition of PP2A through intermediary proteins, ENSA (α-endosulfine) and ARPP19 (cyclic adenosine monophosphate-regulated phosphoprotein 19). ENSA and ARPP19 are substrates of MASTL/Greatwall (Gharbi-Ayachi, Labbe et al. 2010; Mochida, Maslen et al. 2010). The phosphorylated forms of these MASTL/Greatwall substrates bind to PP2A-B55δ/PPP2R2D, inhibiting PP2A-mediated dephosphorylation M-phase phosphoproteins (Figure 1.3) (Gharbi-Ayachi, Labbe et al. 2010; Mochida, Maslen et al. 2010). In fact, based on this, MASTL/Greatwall, in addition to CDK1 and cyclin B, has been proposed to be a component of M-phase-Promoting Factor (MPF) activity (Hara, Abe et al. 2012). This pathway is conserved in a wide range of organisms. The related *Drosophila* protein Endos is a substrate of Greatwall, and the starfish *Patiria pectinifera* has a similar, single ENSA/ARPP19 ortholog (Rangone, Wegel et al. 2011; Hara, Abe et al. 2012; Kim, Bucciarelli et al. 2012). *Saccharomyces cerevisiae* also has components of this system. The yeast endosulfines Igo1 and Igo2 are substrates of Rim15, the yeast MASTL/Greatwall; this Rim15-Igo1/2 pathway regulates entry into G0 (Talarek, Cameroni et al. 2010; Bontron, Jaquenoud et al. 2012; Juanes, Khoueiry et al. 2013). Additionally, the yeast proteins Zds1 and Zds2 (unrelated to Igo1/2) function as inhibitors of PP2A (Bi and Pringle 1996; Yu, Jiang et al. 1996; Yasutis, Vignali et al. 2010; Yasutis and Kozminski 2013).

This work on mammalian meiotic maturation addresses aspects of this model, building on work in non-mammalian species, and puts the model in context with more recent findings in mammalian cells. As shown in Chapter 2, MASTL regulates the exit from prophase I arrest in mouse oocytes. Studies presented here address the fundamental
questions of whether ENSA has a role in murine female meiosis, and, additionally, if ENSA is a physiologically relevant protein in cell cycle regulation in this cellular context and in a species that has the two MASTL/Greatwall substrates. Specifically, we tested the hypothesis that mouse ENSA would play a role in some aspect(s) of regulation of meiotic M-phase in oocytes. This is of significance to the field, as there are some differences in data regarding which protein, ENSA or ARPP19, is phosphorylated by Greatwall/MASTL in various types of *Xenopus* egg extracts, and on which protein functions in cells (Gharbi-Ayachi, Labbe et al. 2010; Mochida, Maslen et al. 2010; Haccard and Jessus 2011; Cundell, Bastos et al. 2013; Dupre, Buffin et al. 2013). Results on the effects of depletion of ARPP19 or ENSA differ in studies using HeLa cells (Gharbi-Ayachi, Labbe et al. 2010; Cundell, Bastos et al. 2013). In the context of oocyte meiotic maturation, ARPP19 has been implicated as a key regulator in *Xenopus* oocytes, as has Endos in *Drosophila* oocytes (Von Stetina, Tranguch et al. 2008; Kim, Bucciarelli et al. 2012; Dupre, Buffin et al. 2013). As shown in Chapter 2, ENSA is abundant in mouse prophase I oocytes and metaphase II eggs, while ARPP19 was not detected in mouse oocytes and was detected at low levels in metaphase II eggs. The kinase MASTL/Greatwall has functions in starfish and porcine oocytes, although these species only have one MASTL/Greatwall substrate protein, as does *Drosophila* (Hara, Abe et al. 2012; Li, Kang et al. 2013). (The pig genome has genes encoding *Ensa* and *Arpp19*, but the *Ensa* gene homolog is a non-functional pseudogene, with a premature stop codon (Li, Kang et al. 2013).) The data presented here on mouse oocyte meiotic maturation contribute to this body of knowledge, as well as address some key issues in the field,
having revealed a function for ENSA in mammalian oocyte meiotic maturation in a specific pathway with PP2A with the regulatory subunit PPP2R2D/B55δ.

II. MATERIALS AND METHODS

Oocyte collection, maturation, and culture

Animals were used in accordance with the guidelines of the Johns Hopkins University Animal Care and Use Committee. Germinal vesicle-intact (GVI) (prophase I-arrested) oocytes were collected from 6-8 week old CF-1 mice (Harlan; Indianapolis, IN). Mice were injected with 5 or 10 I.U. of pregnant mare serum gonadotropin (PMSG) 46-48 hours prior to oocyte collection for experiments were oocytes were matured to the metaphase II stage. Oocytes were collected in Whitten's-HEPES medium (109.5 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 5.5 mM glucose, 0.23 mM pyruvic acid, 4.8 mM lactic acid hemicalcium salt (Whitten 1971)) supplemented with 7 mM NaHCO₃, 15 mM HEPES (hereafter referred to as Whitten's-HEPES) and 0.05% polyvinyl alcohol (PVA; Sigma, St. Louis, MO). Dibutyryl cAMP (dbcAMP, 0.25 mM) was added to culture medium to maintain prophase I arrest (Cho, Stern et al. 1974). Ovaries were punctured with syringe needles to release oocyte-cumulus complexes from ovarian follicles, and cumulus cells were dissociated from oocytes by pipetting oocyte-cumulus complexes through a thin-bore pipette. Oocytes were transferred to Whitten's medium with 22 mM NaHCO₃ (hereafter referred to as Whitten's-Bicarbonate medium) containing 0.05% PVA and 0.25 mM dbcAMP for culture. Oocytes were cultured in a
humidified atmosphere of 5% CO₂ in air. For microinjection of siRNA (details below),
oocytes were transferred to EmbryoMax® KSOM + amino acids with D-Glucose (MR-
106-D, Millipore; Billerica, MA) (hereafter referred to as KSOM) supplemented with
0.25 mM dbcAMP. Microinjected oocytes were cultured in KSOM medium following
microinjection and during in meiotic maturation.

Bacterially-expressed recombinant ENSA and ARPP19

cDNA fragments encoding the entire coding region of mouse Ensa (NM_019561.2)
and Arpp19 (NM_021548.4) were generated by polymerase chain reaction (PCR) from
oocyte cDNA using Phusion™ High-Fidelity DNA polymerase (New England Biolabs;
Ipswich, MA). Cloning primer sequences are provided in Table 3.1. The resulting PCR
products were gel-purified (QIAquick Gel Extraction kit; Qiagen; Valencia, CA),
digested with BamHI and SalI (New England Biolabs), and cloned into pGEX-4T-1
(Amersham-Pharmacia Biotech; Piscataway, NJ) according to standard protocols. The
resulting plasmid (pGEX-4T-ENSA) was verified by DNA sequencing. Plasmids were
transformed into E. coli BL21 cells (Stratagene; La Jolla, CA). Transformed cells were
induced to express GST fusion proteins with 0.5 mM IPTG (Sigma) at 37°C for 4 hours.
Cell lysates were prepared by sonication in cold PBS containing 1 µg/ml leupeptin, 1
µg/ml aprotinin and 1 mM AESBF (Sigma). The lysate was centrifuged twice (20,000 x
g, 10 minutes each), and GST fusion proteins were purified by affinity chromatography
on a glutathione column, eluting with 10 mM of reduced glutathione in 10 mM Tris-HCl,
pH 7.5. The purified protein was dialyzed against PBS. Protein concentration was
determined using the micro BCA assay (Thermo Scientific; Rockford, IL), and purity was
confirmed by examining the protein on silver-stained SDS-PAGE gels.

**Immunoblotting**

For immunoblot analysis, purified recombinant proteins or oocyte lysates were suspended in SDS-PAGE sample buffer (3% SDS, 10% glycerol, 0.02% bromophenol blue, 4% 2-mercaptoethanol, 65 mM Tris-HCl, pH 6.8) and heated for 5 min at 95°C. Proteins were separated on a 12.5% SDS-gel and transferred to an Immobilon-P PVDF membrane (Millipore, Billerica, MA). The membrane was blocked overnight in 10% cold-water fish gelatin (Sigma) in PBS containing 0.1% Tween-20 (Sigma) (hereafter referred to as PBS-T). The membrane was then incubated with an anti-ENSA antibody (Santa Cruz Biotechnology, catalog #sc-135145; rabbit polyclonal IgG made against full-length human ENSA) diluted to a concentration of 0.5 µg/ml in PBS-T containing 3% BSA and 0.02% NaN₃ for 3 hours, washed three times with PBS-T, then incubated with 0.3 µg/ml goat anti-rabbit IgG-horseradish peroxidase (HRP) (Jackson Immunoresearch, West Grove, PA) in PBS-T plus 3% BSA for 1.5-2 hours, followed by three washes with PBS-T. For detection, the membrane was incubated with Supersignal® West Pico Chemiluminescent substrate (Pierce Chemical Company; Rockford, IL) and exposed to X-ray film. X-ray film was scanned using the HP LaserJet 3390 scanner, and images were saved as TIFF files. ImageJ software (http://rsb.info.nih.gov/ij/) was used to analyze band intensity. The rectangular selection tool was used to select each band and peak intensity was determined. The area under each peak was calculated as a measure of band intensity. Alternatively, for some experiments, the membrane was then incubated with the anti-ENSA antibody (0.3 µg/ml, diluted 1:685 in a 1:1 solution of PBS and
Odyssey® Block Buffer) for 1 hour, washed four times in PBS-T (5 minutes each wash), incubated with goat anti-rabbit IRDye® 800CW secondary antibody (diluted 1:20,000 in a 1:1 solution of PBS and Odyssey® Block Buffer) for 1 hour, washed four times in PBS-T (5 minutes each wash) and washed once in PBS. The membrane was then scanned using the Odyssey® CLx Infrared Imaging System (Licor; Lincoln, NE) at an intensity setting between 6.5 and 7.5.

**Immunofluorescence**

Immunofluorescence experiments used a mouse monoclonal anti-α-tubulin antibody (clone 12G10 (Thazhath, Liu et al. 2002); developed by Joseph Frankel and E. Marlo Nelsen, obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA) to label the meiotic spindle, a rabbit polyclonal anti-lamin B antibody (Chaudhary and Courvalin 1993), gift of the lab of Mike Matunis, Department of Biochemistry and Molecular Biology, Johns Hopkins School of Public Health) to label the nuclear envelope, and a rabbit polyclonal anti-ENSA antibody (Santa Cruz Biotechnology, sc-135145; made against full-length ENSA of human origin). For immunofluorescence analysis, zona pellucida (ZP)-free oocytes were prepared by briefly incubating the oocytes in acidic culture medium-compatible buffer (~10 seconds; 10 mM HEPES, 1 mM NaH2PO4, 0.8 mM MgSO4, 5.4 mM KCl, 116.4 mM NaCl, pH 1.5). ZP-free oocytes were fixed for 30 minutes at 37°C in 4% paraformaldehyde (Sigma) in 130 mM KCl, 25 mM HEPES, 3 mM MgCl2, 0.06% Triton-X (pH 7.4). Fixed oocytes were briefly washed in PBS, then permeabilized (PBS containing 0.1% Triton X-100 (Sigma);
15 minutes), then incubated for 1 hour or overnight in immunofluorescence (IF) blocking buffer (PBS containing 1% BSA). Following blocking, oocytes were incubated with primary antibody diluted in IF blocking buffer for 1-1.5 hours (anti-tubulin, 10.6 µg/ml; anti-lamin B, diluted 1:1500; anti-ENSA, 1.3 µg/ml). For anti-ENSA immunofluorescence, controls included use of non-immune rabbit IgG, or anti-ENSA antibody that had been pre-incubated with a 20X molar excess of GST-ENSA for 3 hours. The oocytes were then washed in IF blocking buffer, and incubated for 1-1.5 hours in secondary antibody diluted in blocking buffer (7.5 µg/ml goat-anti-mouse IgG-FITC; 7.5 µg/ml goat-anti-rabbit IgG-FITC; 5 µg/ml goat-anti-rabbit IgG-Dylight-488) (Jackson Immunoresearch, West Grove, PA) followed by three washes in IF blocking buffer. Oocytes were mounted on slides in VectaShield mounting medium (Vector Laboratories) containing 0.75 µg/ml DAPI. Microscopic imaging was performed on a Zeiss Axio Observer Z1 microscope with AxioCam MRm Rev3 camera, ApoTome optical sectioning, and AxioVision software (Carl Zeiss, Inc.; Jena, Germany).

RNAi-mediated knockdown

ON-TARGETplus SMARTpool siRNA targeting mouse Ensa or Ppp2r2d (Dharmacon, Waltham, MA) was resuspended according to manufacturer's instructions, to a concentration of 100 µM in four volumes of RNase-free water and one volume of 5X siRNA Buffer (Dharmacon, B-002000-UB-100). The ON-TARGETplus control pool (Dharmacon, D-001810-10-20) was used as a negative control. The 100 µM siRNA stocks were diluted to a 20 µM in 1X siRNA buffer for microinjection, or, for double knockdown of Ensa and Ppp2r2d, siRNA stocks were 10 µM each. ZP-intact oocytes
were injected using a Nikon Eclipse TE 2000-5 microscope (Melville, NY) equipped with an Eppendorf FemtoJet® (Hamburg, Germany), using injection pressure ($p_i$) of 100-200 hPa, injection time ($t_i$) of 0.1-0.2 seconds, and compensation pressure ($p_c$) of 0 hPa. Injection was considered successful upon observation of cytoplasmic recoiling following needle insertion and post-injection dispersal of the siRNA solution into the oocyte cytoplasm. siRNA-injected oocytes were cultured in KSOM medium containing 0.25 mM dbcAMP for 44-48 hours, with transfer to fresh medium after 24 hours. For experiments assessing meiotic maturation to metaphase II, siRNA-injected oocytes were cultured for 36 hours prior to the initiation of maturation.

**RNA isolation, cDNA synthesis, and semi-quantitative RT-PCR**

Total RNA was isolated from siRNA-injected oocytes by lysing 20 prophase I oocytes in 200 µl Trizol (Invitrogen) for 5 minutes at room temperature. This mixture was chloroform-extracted, then the RNA-containing aqueous phase was supplemented with a final concentration of 1 µg/ml glycogen (Invitrogen) and subjected to isopropanol precipitation overnight at -20°C, after which the RNA was pelleted by centrifugation (15 minutes, 14,000 x g, 4°C). The pellet was washed with 70% ethanol, air-dried, and then suspended in 8 µl of RNase-free water. The RNA concentration was determined by measuring the OD$_{260}$. First-strand cDNA was synthesized from total RNA with random hexamer primers, SuperScript Reverse Transcriptase III (Invitrogen), and RNaseOUT RNase Inhibitor (Invitrogen), according to the manufacturer's protocol, after which the cDNA was treated with *E. coli* RNase H (New England Biolabs; Ipswich, MA).
To assess RNA knockdown, RT-PCR using gene-specific primers was performed; primer sequences are provided in Table 3.2. Tissue plasminogen activator (gene symbol \textit{Plat}) was used as a positive control, as previously described for mouse oocytes (Svoboda, Stein et al. 2000). For semi-quantitative PCR, the amount of PCR product was assessed for a range of cycle numbers, ensuring that the reaction was in the linear range and not saturated. PCR products were separated on agarose gels, which were scanned using the FujiFilm FLA-7000 imaging system (FujiFilm, Valhalla, NY). FLA-7000 image files were exported to the MultiGauge program (FujiFilm) and saved as TIFF files. ImageJ software (\url{http://rsb.info.nih.gov/ij/}) was used to analyze band intensity. The rectangular selection tool was used to select each band and peak intensity was determined. The area under each peak was calculated as a measure of band intensity. Band intensity for \textit{Ensa}, \textit{Arpp19}, or \textit{Ppp2r2d} primer-amplified products was normalized to those for the positive control (\textit{Plat}) primer set. Transcript levels in oocytes injected with target-specific siRNAs (\textit{Ensa}, \textit{Ppp2r2d}) were expressed relative to those levels in oocytes injected with negative control siRNA.

\textit{In vitro meiotic maturation}

siRNA-injected oocytes were washed through six drops of KSOM to wash away the dbcAMP (Cho, Stern et al. 1974). For observation of progress through germinal vesicle breakdown (also known as nuclear envelope breakdown), oocytes were cultured in KSOM, with observation at 30-minute intervals for 5 hours by dissecting microscope for the presence of an intact germinal vesicle. Data are presented as the percentage of oocytes having undergone germinal vesicle-breakdown (GVBD) over time. For \textit{in vitro
maturation to the metaphase II stage, oocytes were cultured for 14-15 hours and assessed for first polar body emission by observing with a dissecting microscope.

For a subset of experiments, oocytes were treated with the phosphatase inhibitor okadaic acid. Okadaic acid (Santa Cruz Biotechnology, sc-3513) was suspended in dimethyl sulfoxide (DMSO) to a 2 mM stock concentration and stored at -80°C; this 2 mM stock was diluted to 2.5 µM in KSOM medium. This dose was chosen based on past studies of okadaic acid treatment of mouse oocytes (Rime and Ozon 1990; Schwartz and Schultz 1991; Hampl and Eppig 1995). These studies had four experimental groups: negative control siRNA-injected oocytes, negative control siRNA-injected oocytes treated with 2.5 µM okadaic acid, Ensa siRNA-injected oocytes, and Ensa siRNA-injected oocytes treated with 2.5 µM okadaic acid. For treatment with okadaic acid, oocytes in KSOM medium containing 0.25 mM of dbcAMP were washed through six drops of KSOM and then transferred to KSOM with 2.5 µM okadaic acid. Oocytes were assessed at 30-minute intervals for the presence of an intact germinal vesicle as described above.

III. RESULTS

Immunoblotting was used to assess the specificity of the anti-ENSA antibody used in these studies; these data are shown in Chapter 2 in Figure 2.6A. This anti-ENSA antibody bound to recombinant mouse ENSA (GST-ENSA; Figure 2.6A, lanes 1-3), and did not cross-react with recombinant mouse ARPP19 (GST-ARPP19; Figure 2.6A, lanes 4-6); mouse ARPP19 has 76% amino acid identity with mouse ENSA. As also shown in
Chapter 2 (Figure 2.7D and 2.9B), this anti-ENSA antibody detected ENSA in lysates of prophase I mouse oocytes with by immunoblotting (Figure 3.1A, lanes 1-2; lysates of 26 and 40 oocytes, respectively), as well as positive control purified GST-ENSA (Figure 3.1A, lanes 3-5).

To assess the function of ENSA in mouse oocytes, we utilized RNAi-mediated knockdown to generate Ensa-deficient oocytes. Oocytes were microinjected with negative control siRNA (hereafter referred to as "control oocytes") or with Ensa-targeting siRNA, and then cultured in conditions to maintain prophase I arrest, allowing RNAi-mediated knockdown and protein turnover to occur. Following a 44-48 h culture period, Ensa mRNA levels were decreased by $67 \pm 11\%$ in Ensa siRNA-injected oocytes compared with controls (five experiments; range 39-91%; Figure 3.2A). Arpp19 mRNA levels were unaffected by the Ensa-targeting siRNA (Figure 3.2B), in agreement with our BLAST searches revealing that the Ensa-targeting siRNA did not have any regions of homology to Arpp19. ENSA protein levels were decreased by $80 \pm 6\%$ in Ensa siRNA-injected oocytes as compared with controls, as detected by immunoblotting (five experiments; range 71-97%; Figure 3.2C).

In testing this anti-ENSA antibody in immunofluorescence experiments, we observed staining of prophase I oocytes, and this staining was largely lost in oocytes incubated with anti-ENSA antibody that was pre-incubated with a 20-fold molar excess of GST-ENSA (Figure 3.4), but oocytes that were injected with the Ensa-targeting siRNA still were labeled by the anti-ENSA antibody (data not shown). With this result raising questions about the utility of this particular antibody in immunofluorescence applications, we did not pursue immunofluorescence studies further here.
ENSA-deficient oocytes were examined for the ability to progress through meiotic maturation. To assess exit from prophase I arrest, oocytes were placed in culture conditions that support progression from prophase I arrest (i.e., absence of dbcAMP), and were observed at 30-minute intervals over a 5 h culture period for germinal vesicle breakdown (also called nuclear envelope breakdown). Over this time period, an average of 60% of control oocytes underwent germinal vesicle breakdown compared with only 23% of ENSA-deficient oocytes (Figure 3.3A). These data suggested that ENSA deficiency impaired the ability of mouse oocytes to exit from prophase I arrest.

To assess the meiotic status of these oocytes in more detail, we examined lamin B immunofluorescence staining of Ensa siRNA-injected and control oocytes after the 5 h of culture in conditions that support exit from prophase I arrest (Figure 3.5A). After this 5 h of culture, oocytes from the negative control siRNA and Ensa siRNA experimental groups were sorted into two classifications for these immunofluorescence studies: prophase I (based on the presence a germinal vesicle) or pro-metaphase I (based on the absence of a germinal vesicle). In the oocytes classified as prophase I, anti-lamin B immunofluorescence showed strong labeling of the nuclear lamina oocytes, and no apparent differences were detected between Ensa siRNA-injected (Figure 3.5A, panels vii-ix) and control (Figure 3.5A, panels i-iii) oocytes. While the majority of Ensa siRNA-injected oocytes were arrested at the prophase I stage after 5 h (average 77%, Figure 3.5D), a subset of Ensa siRNA-injected oocytes did show evidence of germinal vesicle loss. Normal nuclear lamina breakdown and chromosome condensation was observed in these Ensa siRNA-injected oocytes (Figure 3.5A, panels x-xii), with pro-metaphase I control oocytes having very similar appearances (Figure 3.5A, panels iv-vi).
Thus, the subset of Ensa siRNA-injected oocytes that exited from prophase I arrest appeared comparable to control oocytes at this stage, able to progress normally from prophase I arrest into meiosis I.

A separate series of experiments assessed completion of meiosis I (i.e., emission of the first polar body) and progression to metaphase II arrest in control and Ensa siRNA-injected oocytes (Figure 3.5B-D). At 14 h after initiation of meiotic maturation (i.e., culture in conditions that support exit from prophase I arrest), 76% (81/106) of control oocytes and 33% (34/104) of Ensa siRNA-injected oocytes had exited from prophase I arrest and undergone GVBD (Figure 3.5B). Of this subset that exited from prophase I arrest, 56% (45/81) of control oocytes and 50% (17/34) of ENSA-deficient oocytes progressed through meiosis I, as evidenced by the presence of the first polar body (Figure 3.5C). These oocytes that emitted the first polar body were confirmed to have metaphase II spindles, with comparable spindle morphology and chromosome alignment in both the negative control siRNA-injected oocytes and the Ensa siRNA-injected oocytes (Figure 3.5D). There was no evidence of parthenogenetic exit from metaphase II arrest during this in vitro culture period in oocytes injected with either the negative control siRNA or the Ensa siRNA.

To test the hypothesis that ENSA was functioning to inhibit PP2A activity in mouse oocytes, we first used okadaic acid treatment to inhibit PP2A activity in control and Ensa siRNA-injected oocytes, assessing the abilities of these oocytes to exit from prophase I arrest (Figure 3.6A). As also shown above (Figure 3.3A), Ensa siRNA-injected oocytes were impaired in their ability to exit from prophase I arrest as compared to controls. However, Ensa siRNA-injected oocytes that were treated with 2.5 µM
okadaic acid progressed out of prophase I arrest to extents comparable to control oocytes (Figure 3.6).

This result was rationale for more detailed examination of PP2A. We targeted the PP2A B55δ regulatory subunit, Ppp2r2d, for siRNA-mediated knockdown in mouse oocytes. Following a 44-48 h culture period to allow for knockdown, Ppp2r2d mRNA levels were decreased by 80 ± 5% in Ppp2r2d siRNA-injected oocytes as compared to controls (three experiments; range 73-90%; Figure 3.7A). These Ppp2r2d-siRNA-injected oocytes did not exit prophase I arrest during this 44-48 h culture period in conditions that support maintenance of prophase I arrest (i.e., inclusion of 0.25 mM dbcAMP in the culture medium, data not shown). Considering that prophase I oocytes treated with or injected with okadaic acid will exit from prophase I arrest even in the presence of agents that maintain high protein kinase A activity, this result suggested that the PPP2R2D/B55δ regulatory subunit is not the only PP2A regulatory subunit required for the G2-M progression in mouse oocytes (Rime and Ozon 1990; Gavin, Tsukitani et al. 1991; Schwartz and Schultz 1991).

Subsequent experiments assessed whether the Ppp2r2d deficiency would rescue the ability of Ensa-siRNA-injected oocytes to exit from prophase I arrest. As shown above (Figure 3.3A), Ensa-siRNA-injected oocytes in these experiments showed reduced exit from prophase I arrest as compared to controls (Figure 3.7B). Ppp2r2d-deficient oocytes exited from prophase I arrest to extents similar to control oocytes. Most significantly, the extent of germinal vesicle breakdown was similar to controls in oocytes injected with both Ensa-targeting and Ppp2r2d-targeting siRNA. Thus, the inability of Ensa-siRNA-injected oocytes to exit from prophase I arrest was rescued when Ppp2r2d
was also knocked down, indicating that ENSA acts in a pathway with PP2A containing the PPP2R2D/B55δ regulatory subunit during meiosis I in mouse oocytes.

**IV. DISCUSSION**

The data in this chapter extend the work presented in Chapter 2, and define a physiological role for ENSA in mouse oocytes. Knockdown of ENSA in mouse oocytes results in defective progression out of prophase I arrest. The defect in progression into M-phase of meiosis I in ENSA-deficient mouse oocytes has similarities to *Drosophila* oocytes, with *endos* mutant oocytes having a prolonged prophase I arrest and delayed progression to metaphase I (Von Stetina, Tranguch et al. 2008). However, differences between *Ensa* siRNA-injected mouse oocytes and *endos* mutant *Drosophila* oocytes should be noted as well. *endos* mutant *Drosophila* oocytes that progressed past prophase I show abnormalities in metaphase I spindle organization and chromosome congression (Von Stetina, Tranguch et al. 2008). Moreover, Endos deficiency has more deleterious effects on *Drosophila* female meiosis than in other cell divisions, including male meiosis, leading to speculation that Endos has additional functions in female meiosis in *Drosophila* (Kim, Bucciarelli et al. 2012). In our study, while the majority of *Ensa* siRNA-injected mouse oocytes remain arrested at the prophase I stage, a subset of *Ensa* siRNA-injected oocytes undergo normal meiotic maturation, progressing into meiosis I and to the metaphase II arrest stage. Obviously, an explanation for our results is that there is oocyte-to-oocyte variability in the extent of knockdown of *Ensa* RNA and/or
ENSA protein. A subset of *Ensa* siRNA-injected oocytes here may have been able to undergo germinal vesicle breakdown because in these particular oocytes, sufficient amounts of ENSA persisted to support exit from prophase I arrest. We attempted to test this hypothesis and examine knockdown in individual oocytes by immunofluorescence (Figure 3.4), but found that *Ensa* siRNA-injected oocytes still showed some labeling with the anti-ENSA antibody (data not shown). Since immunoblotting shows clear knockdown of ENSA protein in *Ensa* siRNA-injected oocytes (Figure 3.3), this result makes us question the utility of this particular anti-ENSA antibody in immunofluorescence applications. An additional possibility is that in the context of ENSA deficiency, this polyclonal anti-ENSA antibody is able to cross-react with ARPP19 (even though this anti-ENSA antibody does not cross-react with purified ARPP19 in immunoblots; Figure 3.2). Even though ARPP19 was not detected by immunoblotting in lysates of 100 prophase I oocytes (Figure 2.7C; 2.5 µg of total protein), it is still possible that some ARPP19 protein is present in mouse oocytes at this stage.

Demonstration of this functionality of ENSA in mouse oocytes is relevant to the discussion of whether ENSA and/or ARPP19 are physiologically relevant substrates for MASTL/Greatwall. This question is obviously not an issue in the species that have only one MASTL/Greatwall substrate, such as *Drosophila* with Endos. The case has been made, based on data in *Xenopus* related to protein concentrations, phosphorylation dynamics, and effects of depletion, that ARPP19 is the physiological MASTL/Greatwall substrate (Haccard and Jessus 2011). Consistent with this model, expression of a non-phosphorylatable form of ARPP19 in *Xenopus* prophase I oocytes results in defects in exit from prophase I arrest, suggesting that this non-phosphorylatable form of ARPP19
acts as a dominant-negative in this system, although it is unknown if this dominant-negative ARPP19 would have any effects on the function of ENSA (Dupre, Buffin et al. 2013). Additional studies using injected ARPP19 show how exogenously introduced ARPP19 can affect normal Xenopus oocyte maturation parameters; these actions were characterized in wild-type Xenopus oocytes with endogenous ARPP19 and ENSA (Dupre, Daldello et al. 2014). With regard to the physiological relevance of ENSA versus ARPP19 in mammalian cells, one study observes that ARPP19 knockdown in HeLa cells produces defects in mitotic progression, whereas another study reports that siRNA-mediated depletion of ARPP19 had little effect in one specific molecular aspect of cell cycle progression, and additionally, that ARPP19 was not detected in HeLa cells (Gharbi-Ayachi, Labbé et al. 2010; Cundell, Bastos et al. 2013). This is potentially explained by differences in the efficiency of siRNA-mediated knockdown or lab-to-lab differences in HeLa lines, but nonetheless highlights the potential variability in pathways utilizing ENSA or ARPP19. Budding yeast is another system in which there are two substrates of the yeast MASTL/Greatwall (Rim15), known as Igo1 and Igo2 (Talarek, Cameroni et al. 2010; Bontron, Jaquenoud et al. 2012; Juanes, Khoueiry et al. 2013). This pathway in yeast regulates entry into G0, and deletion of both Igo1 and Igo2 is required to produce a defect in G0 entry, with igo1Δ and igo2Δ single mutants not having this phenotype (Talarek, Cameroni et al. 2010). Budding yeast has two proteins, Zds1 and Zds2 with no sequence homology to Igo1/2 but with functional similarity to ENSA and ARPP19, serving as inhibitors of PP2A (Yasutis, Vignali et al. 2010; Yasutis and Kozminski 2013). Like Igo1 and Igo2, Zds1 and Zds2 also appear to be redundant to each other, with zds1Δ/zds1Δ double mutants, but not zds1Δ or zds1Δ single mutants, showing a defect in
G2 delay (Bi and Pringle 1996; Yu, Jiang et al. 1996). This is suggestive of functional specialization between cell types arising in metazoans that have both ENSA and ARPP19.

There is strong evidence for the MASTL/Greatwall pathway functioning in oocytes of multiple species, including *Xenopus*, *Drosophila*, starfish, and pig (Archambault, Zhao et al. 2007; Von Stetina, Tranguch et al. 2008; Zhao, Haccard et al. 2008; Yamamoto, Blake-Hodek et al. 2011; Hara, Abe et al. 2012; Kim, Bucciarelli et al. 2012; Dupre, Buffin et al. 2013; Li, Kang et al. 2013). Starfish and *Drosophila* have only one MASTL/Greatwall substrate, and the pig *ENSA* gene has a premature stop codon, such that porcine cells would not express functional ENSA protein (Li, Kang et al. 2013).

As shown in Chapter 2, in mouse oocytes ENSA is detected throughout meiotic maturation (prophase I oocytes and metaphase II eggs), while ARPP19 was only detected at low levels in metaphase II stage eggs. *Xenopus* oocytes may rely solely or primarily on ARPP19 to regulate exit from prophase I arrest, while the data here clearly indicate that ENSA is important for exit from prophase I arrest in mouse oocytes (Haccard and Jessus 2011; Dupre, Buffin et al. 2013). Such differences between species are not unprecedented, with one example in female meiosis being the phosphatase calcineurin. Calcineurin is important for exit from metaphase II arrest in *Xenopus*, exit from metaphase I arrest in an ascidian species, and progression of *Drosophila* oocytes through meiosis, whereas there is no evidence for calcineurin functioning in exit from metaphase II arrest in mouse (Mochida and Hunt 2007; Suzuki, Suzuki et al. 2010; Takeo, Hawley et al. 2010; Levasseur, Dumollard et al. 2013).

The defect in meiotic resumption with ENSA deficiency in mouse oocytes, analogous to a G2-to-M transition in mitotic cells, differs from what is observed in mouse
embryonic fibroblasts (MEFs) depleted of MASTL through a conditional knockout approach (Álvarez-Fernández, Sánchez-Martínez et al. 2013). G0-arrested MEFs with a loxP-flanked Mastl allele were infected with adenovirus to drive expression of Cre recombinase, then allowed to re-enter the cell cycle. These Mastl-deficient MEFs showed no differences in several markers of mitotic entry as compared to control cells, although defects in progression through mitosis were detected (Álvarez-Fernández, Sánchez-Martínez et al. 2013). These differences between MASTL-deficient MEFs and ENSA-deficient mouse oocytes may reflect fine-tuned functions of this pathway in these two cellular contexts.

The prophase I arrest phenotype in ENSA-deficient mouse oocytes was rescued by inhibition of PP2A using okadaic acid or knockdown of the PP2A regulatory subunit Ppp2r2d. Thus, the data here are consistent with the model coming from Xenopus studies that ENSA functions to inhibit PP2A with the regulatory subunit PPP2R2D/B55δ, and in turn suggests that this activity is important during the exit from prophase I arrest in mouse oocytes. An additional observation in our studies is that Ppp2r2d siRNA-injected oocytes maintain prophase I arrest for ~48 h in the presence of dbcAMP, and then undergo germinal vesicle breakdown only upon removal of dbcAMP. In contrast, okadaic acid-treated oocytes exit from prophase I arrest and progress into meiosis I even in the presence of dbcAMP or the phosphodiesterase inhibitor IBMX (Rime and Ozon 1990; Gavin, Tsukitani et al. 1991; Schwartz and Schultz 1991). Since okadaic acid treatment would inhibit all PP2A forms, these results suggest that multiple PP2A forms function in maintenance of prophase I arrest, and that the PPP2R2D/B55δ regulatory subunit is dispensable during prophase I arrest. Our data on the involvement of
PPP2R2D in this ENSA-dependent pathway in oocytes are also interesting in light of findings from various mammalian cell types, showing that two regulatory subunits, PPP2R2D/B55δ and PPP2R2A/B55α, are involved in the regulation of mitotic exit in two different mammalian systems, and that mitotic progression defects in Mastl-deficient MEFs are rescued by depletion of all four B55 regulatory subunits (Manchado, Guillamot et al. 2010; Álvarez-Fernández, Sánchez-Martínez et al. 2013; Hegarat, Vesely et al. 2014). Our data here identify PP2A-B55δ specifically as the phosphatase functioning in the MASTL-ENSA/ARPP19 pathway in this particular mammalian cell type, the prophase I mouse oocyte.

The metaphase II arrest stage and exit from this arrest are likely to involve diverse phosphatase activities of various types of PP2A heterotrimers. The model summarized in Figure 1.3 and other data predict that exit from metaphase II arrest would involve ceasing inhibition of PP2A to allow PP2A-mediated dephosphorylation of CDK1 M-phase substrates (Manchado, Guillamot et al. 2010; Hegarat, Vesely et al. 2014). With regard to data here, the subset of Ensa siRNA-injected oocytes that progress to metaphase II arrest do not show evidence of parthenogenetic exit from metaphase II arrest. This may hint that ENSA is dispensable for metaphase II arrest, but this result should be interpreted conservatively, as these Ensa siRNA-injected oocytes were not subjected to extended culture, such as was used in studies of parthenogenesis in Mos-null oocytes or of parthenogenetic exit from metaphase II with increased times after ovulation or start of in vitro maturation (Colledge, Carlton et al. 1994; Hashimoto, Watanabe et al. 1994; Xu, Abbott et al. 1997; Abbott, Xu et al. 1998). Knockdown of MASTL in metaphase II pig eggs results in a moderate extent of exit from metaphase II arrest over an 18 h culture
period (Li, Kang et al. 2013). Data in Chapter 2 showed that ARPP19 is also detected at low levels in metaphase II eggs and further studies to assess the roles of MASTL and ENSA and/or ARPP19 in regulating PP2A during exit from metaphase II arrest will be valuable. Metaphase II arrest also entails specific PP2A functions, although with different PP2A types. PP2A with a B56 regulatory subunit localizes to centromeres in metaphase II mouse eggs, with function in protection of centromeric cohesion (Chambon, Touati et al. 2013). Fine-tuning of CDK1 activity during metaphase II arrest is achieved through a feedback loop with combined action of CDK1 and PP2A (in *Xenopus* eggs, with a B56β/ε regulatory subunit (Isoda, Sako et al. 2011)) on EMI2, an inhibitor of the anaphase-promoting complex/cyclosome (Wu, Guo et al. 2007; Wu and Kornbluth 2008; Isoda, Sako et al. 2011). Data from studies using okadaic acid to inhibit PP2A and FTY720 to activate PP2A provide evidence that this same mechanism functions in metaphase II mouse eggs (Chung, Jennings et al. 2011).

In summary, this study demonstrated that exit from prophase I arrest is impaired in ENSA-deficient oocytes. Our data further indicate that ENSA functions to inhibit PP2A-B55δ during exit from prophase I arrest in mouse oocytes. This adds a new dimension to our understanding of the molecular regulation of mammalian female meiosis, extending recent work showing that MASTL deficiency in pig oocytes also impairs meiotic progression, with added potential relevance to human oocyte biology, as mouse and human have genes encoding both *Arpp19* and *Ensa* (Li, Kang et al. 2013).
V. REFERENCES


VI. TABLES AND FIGURES

Table 3.1: Primer sequences for cloning of *Ensa* and *Arpp19* into the PGEX-4T-1 vector

<table>
<thead>
<tr>
<th>Description</th>
<th>Sequence (5' → 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mouse Ensa</strong> (NM_019561.2) forward primer for cloning full-length <em>Ensa</em>, for expression of ENSA as a GST fusion protein; includes a <em>Bam</em> HI site</td>
<td>5'-GCGGATCCATGTCCCAGAAACAAGAAGA-3'</td>
</tr>
<tr>
<td><strong>Mouse Ensa</strong> (NM_019561.2) reverse primer for cloning full-length <em>Ensa</em>, for expression of ENSA as a GST fusion protein; includes a <em>Sal</em> I site</td>
<td>5'-ACGCGTCGACTCACTCAACTTGGCCACCCCG-3'</td>
</tr>
<tr>
<td><strong>Mouse Arpp19</strong> (NM_021548.4) forward primer for cloning full-length <em>Arpp19</em>, for expression of ARPP19 as a GST fusion protein; includes a <em>Bam</em> HI site</td>
<td>5'-GCGGATCCATGTCCGCGGAAGTCCCCGA-3'</td>
</tr>
<tr>
<td><strong>Mouse Arpp19</strong> (NM_021548.4) reverse primer for cloning full-length <em>Arpp19</em>, for expression of ARPP19 as a GST fusion protein; includes a <em>Sal</em> I site</td>
<td>5'-ACGCGTCGACTCAGCCAGCCAGCTTGCTAG-3'</td>
</tr>
</tbody>
</table>
Table 3.2: Primer sequences for knockdown assessment by RT-PCR

<table>
<thead>
<tr>
<th>Description</th>
<th>Sequence (5' → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse <em>Ensa</em> forward primer</td>
<td>5’-GAAAACCCCTGCGAGGAGACCG-3’</td>
</tr>
<tr>
<td>Mouse <em>Ensa</em> reverse primer</td>
<td>5’-CCCGCAAGCTTGCTGGTGAC-3’</td>
</tr>
<tr>
<td>Mouse <em>Arpp19</em> forward primer</td>
<td>5’-AGCGTCTGCGAGAGACCGAACAAG-3’</td>
</tr>
<tr>
<td>Mouse <em>Arpp19</em> reverse primer</td>
<td>5’-AGCAACCAGGGACGGTTTCCG-3’</td>
</tr>
<tr>
<td>Mouse <em>Ppp2r2d</em> forward primer</td>
<td>5’-CGGCACACGCGTTCCAATA-3’</td>
</tr>
<tr>
<td>Mouse <em>Ppp2r2d</em> reverse primer</td>
<td>5’-TGGCATGCTGTCACATAGG-3’</td>
</tr>
<tr>
<td>Mouse <em>Plat</em> forward primer</td>
<td>5’-CATGGGCAAGCGTTACACAG-3’</td>
</tr>
<tr>
<td>Mouse <em>Plat</em> reverse primer</td>
<td>5’-CAGAGAAGAATGGGAGACGAT-3’</td>
</tr>
</tbody>
</table>
Figure 3.1: ENSA is expressed by mouse oocytes

Anti-ENSA immunoblot of lysates of 26 and 40 mouse oocytes (lanes 1-2) alongside positive control samples of 30 ng, 40 ng and 50 ng of GST-ENSA (lanes 3-5). The lower Mr band in the GST-ENSA sample is likely either a product of proteolysis or incomplete translation.
Figure 3.2: Knockdown of *Ensa* in mouse oocytes

Prophase I-arrested oocytes were injected with 20 µM negative control siRNA or *Ensa*-targeting siRNA and cultured for 44-48 hours in the presence of dbcAMP to maintain prophase I arrest and allow time for knockdown. Panel A: RT-PCR analysis of *Ensa* transcript levels. Lanes 1-2 show the positive control PCR product (tissue plasminogen activator, *Plat*) for negative control siRNA-injected oocytes (lane 1) and *Ensa* siRNA-injected oocytes (lane 2). Lanes 3-4 show the *Ensa* PCR product for negative control siRNA-injected oocytes (lane 3) and *Ensa* siRNA-injected oocytes (lane 4). *Ensa* mRNA levels were decreased by 67 ± 11% in *Ensa* siRNA-injected oocytes compared to controls (five experiments, with one representative experiment shown here; range 39-91%). Panel B: RT-PCR analysis of *Arpp19* transcript levels. Lanes 1-2 show the positive control *Plat* PCR product for negative control siRNA-injected oocytes (lane 1) and *Ensa* siRNA-injected oocytes (lane 2). Lanes 3-4 show the *Arpp19* PCR product for negative control siRNA-injected oocytes (lane 3) and *Ensa* siRNA-injected oocytes (lane 4). Panel C: Anti-ENSA immunoblot of negative control siRNA-injected oocytes (lane 1) and *Ensa* siRNA-injected oocytes (lane 2) (20 oocytes per lane). GST-ENSA (20 ng) was loaded in lane 3 as a positive control. ENSA protein levels were decreased by 80 ± 6% in *Ensa* siRNA-injected oocytes as compared to controls (five experiments, with one representative experiment shown here; range 71-97%).
A
Control (Plat)

B
Control (Plat)

C
Negative control
Ensa-siRNA
GST-ENSA
Figure 3.3: Ability to exit from prophase I arrest is impaired in ENSA-deficient oocytes

Assessment of germinal vesicle breakdown in negative control siRNA-injected oocytes (n = 149 oocytes; gray triangles) and Ensa siRNA-injected oocytes (n = 174 oocytes; open circles). Prophase I-arrested oocytes were injected with 20 µM negative control siRNA or Ensa-targeting siRNA and cultured for 44-48 hours in the presence of dbcAMP to maintain prophase I arrest and allow time for knockdown. These data are pooled from four replicate experiments, and error bars show standard errors of the mean.
Figure 3.4: Anti-ENSA immunofluorescence in mouse oocytes

Images show prophase I oocytes stained with an anti-ENSA antibody (Panels B, E, H) and with DAPI (Panels C, F, I) (scale bar = 10 µm). In Panels D-F, the anti-ENSA antibody was pre-incubated with a 20-fold molar excess of GST-ENSA in the absorption control group. As an additional control, non-immune rabbit IgG was used in place of primary antibody for the non-immune IgG control group (Panels G-I). Staining was largely lost in oocytes incubated with anti-ENSA antibody was pre-incubated GST-ENSA. However, oocytes that were injected with the Ensa-targeting siRNA still showed some labeling with the anti-ENSA antibody (data not shown). Since immunoblotting shows clear knockdown of ENSA protein in oocytes injected with Ensa-targeting siRNA (Figure 3.2), this result makes us question the utility of this particular antibody in immunofluorescence applications and these studies were not pursued further.
Figure 3.5: Progression through meiosis in control and Ensa siRNA-injected oocytes

Panel A: Immunofluorescence analysis of nuclear lamina in oocytes fixed at 5 hours after initiation of meiotic maturation (i.e., removal of dbcAMP). After this 5 hours of culture, oocytes from the negative control siRNA and Ensa siRNA experimental groups were separated into two classifications: prophase I (based on the presence of a germinal vesicle) or pro-metaphase I (based on the absence of a germinal vesicle). As shown in Figure 3.3 and addressed in the text, while the majority of Ensa siRNA-injected oocytes were arrested at the prophase I stage after 5 hours, a subset of oocytes showed evidence of germinal vesicle loss. Images show prophase I oocytes from the control group (panels i-iii) and the Ensa siRNA-injected group (panels vii-ix) and pro-metaphase I oocytes from the control group (panels iv-vi) and the Ensa siRNA-injected group (panels x-xii) stained with an anti-lamin B antibody (ii, v, viii, xi) and DAPI (iii, vi, ix, xii) to label DNA. Scale bar (shown in Panel i) = 10 µm. Panel B: Meiotic progression at 14 hours after initiation of meiotic maturation (i.e., removal of dbcAMP). Control and Ensa siRNA-injected oocytes were classified as being prophase I (ProI, based on the presence of a germinal vesicle; open bar), GVBD/no PB1 (based on the absence of a germinal vesicle and first polar body; gray bar), or having the first polar body (PB1; black bar). Panel C: Meiotic progression at 14 hours after initiation of meiotic maturation in the subset of oocytes that exited from prophase I arrest. Control and Ensa siRNA-injected oocytes were classified as being GVBD/no PB1 (absence of a first polar body; gray bar), or having the first polar body (PB1; black bar). Panel D: Immunofluorescence analysis of the metaphase II spindle in oocytes fixed at 14 hours after initiation of meiotic maturation. Images show oocytes from the control group (panels i-iii) and the Ensa siRNA-injected
group (panels iv-vi) stained with an anti-α-tubulin antibody (ii, v) and DAPI (iii, vi) to label DNA. Scale bar (shown in Panel i) = 10 µm.
A

Merge Lamin B DNA

Prophase I

Pro-metaphase I

Negative control siRNA-injected

Ensa siRNA-injected

B

Percentage of oocytes (%)

0 20 40 60 80 100

Neg. Control

Ensa

n = 106

n = 104

81 oocytes underwent GVBD

70

34 oocytes underwent GVBD

25

36

17

45

17

C

Percentage of oocytes that underwent GVBD (%)

0 20 40 60 80 100

Neg. Control

Ensa

n = 81

n = 34

36

17

45

17

D

Merge Tubulin DNA

Metaphase II

Negative control siRNA-injected

Ensa siRNA-injected
Figure 3.6: Suppression of PP2A activity restores the ability of *Ensa* siRNA-injected oocytes to exit from prophase I arrest and progress through meiosis I

Assessment of germinal vesicle breakdown in negative control siRNA-injected oocytes, untreated (gray triangles) and treated with 2.5 µM okadaic acid (black squares), and *Ensa* siRNA-injected oocytes, untreated (open circles) and treated with 2.5 µM okadaic acid (open squares). These data are pooled from two replicate experiments (63-77 total oocytes per experimental group), and error bars show the high and low values for these two experiments.
Figure 3.7: Knockdown of the PP2A-B55δ regulatory subunit (Ppp2r2d) restores the ability of Ensa siRNA-injected oocytes to exit from prophase I arrest and progress through meiosis I

Prophase I-arrested oocytes were injected with 20 µM negative control siRNA, Ensa-targeting siRNA, Ppp2r2d-targeting siRNA or Ensa-targeting + Ppp2r2d-targeting siRNA and cultured for 44-48 hours in the presence of dbcAMP to maintain prophase I arrest and allow time for knockdown. Panel A: RT-PCR analysis of Ppp2r2d transcript levels. Lanes 1-2 show the positive control Plat PCR product for negative control siRNA-injected oocytes (lane 1) and Ppp2r2d siRNA-injected oocytes (lane 2). Lanes 3-4 show the Ppp2r2d PCR product for negative control siRNA-injected oocytes (lane 3) and Ppp2r2d siRNA-injected oocytes (lane 4). Ppp2r2d mRNA levels were decreased by 80 ± 5% in Ppp2r2d siRNA-injected oocytes as compared to controls (three experiments, with one representative experiment shown here; range 73-90%). Panel B: Assessment of germinal vesicle breakdown in negative control siRNA-injected oocytes (gray triangles), Ensa siRNA-injected oocytes (open circles), Ppp2r2d siRNA-injected oocytes (open squares), and Ensa + Ppp2r2d siRNA-injected oocytes (black diamonds). These data are pooled from three replicate experiments (115-128 oocytes per experimental group), and error bars show standard errors of the mean.
I. Overview

The MASTL-ENSA/ARPP19 signaling pathway has emerged as an important regulator of M-phase entry and progression. Studies in Xenopus extracts presented a model where the kinase, Greatwall (Note: Greatwall is the name used in Xenopus and Drosophila; in human and mouse the kinase is referred to as MASTL), can phosphorylate two substrates, Ensa (alpha-endosulfine) and Arpp19 (cAMP-regulated phosphoprotein-19), which can then inhibit the activity of the protein phosphatase, PP2A (Gharbi-Ayachi, Labbé et al. 2010; Mochida, Maslen et al. 2010). When active, PP2A can dephosphorylate CDK1 substrates. Inhibition of PP2A allows the net phosphorylation status of CDK1 substrates to be maintained during M-phase. This inhibition of PP2A activity is critical for mitotic progression and interference with the MASTL/Greatwall pathway results in mitotic entry and progression defects (Yu, Zhao et al. 2006; Zhao, Haccard et al. 2008; Vigneron, Brioudes et al. 2009).

This maintenance of the M-phase state is especially critical during mammalian female meiosis with extended periods of M-phase during meiosis I and meiosis II. For example, arrest at the prophase I stage can last for days up to decades depending on the species. Progression through metaphase I, as well as arrest at the metaphase II stage, can last for hours and the M-phase state must be maintained during these periods. (Conti, Andersen et al. 2002). This staggered nature of mammalian female meiosis presents
unique challenges for the regulation of M-phase entry and maintenance. It is well known that CDK1 activity is critical for the regulation of M-phase entry and progression during mammalian female meiosis. The data in this thesis support the model established through studies in *Xenopus* and *Drosophila*, with evidence that in mouse oocytes the MASTL-ENSA/ARPP19 pathway acts to inhibit PP2A activity during M-phase. This body of work extends our understanding of the regulation of CDK1 signaling during mammalian female meiosis and establishes the MASTL-ENSA/ARPP19 pathway as a key regulator of the exit from prophase I arrest.

II. Insights into the physiological relevance of the two MASTL substrates, ENSA and ARPP19, in mouse oocytes and cultured cells

Chapter 2 examines the expression of the known components of the MASTL-ENSA/ARPP19 pathway. In humans, mouse and *Xenopus*, MASTL/Greatwall has two known substrates, ENSA and ARPP19. Other species, such as *Drosophila*, only have one MASTL/Greatwall substrate. In species with two MASTL substrates there is disagreement about the functional significance of these substrates, with some studies detecting a function for ARPP19, while others not (Gharbi-Ayachi, Labbé et al. 2010; Cundell, Bastos et al. 2013). The function of the MASTL substrates is discussed in more detail in Section III. Previous studies also disagree about whether both substrates are even expressed in human HeLa cells (Gharbi-Ayachi, Labbé et al. 2010; Cundell, Bastos et al. 2013). We detect both ENSA and ARPP19 in lysates of mouse NIH 3T3 cells,
mouse 308 cells and human HeLa cells, demonstrating that both MASTL substrates are expressed in the human and mouse cultured cells examined (Figure 2.7). These findings address an outstanding question in the field and provide rationale to explore the function of both ENSA and ARPP19 in future studies.

Chapter 2 also examines the expression of ENSA and ARPP19 in mouse prophase I oocytes, metaphase II eggs and early embryos. ENSA is detected in the three stages of meiotic maturation and early embryogenesis that are examined here (Figure 2.7D). In contrast, ARPP19 is detected at low levels in metaphase II eggs, but is not detected in prophase I oocytes or early embryos (Figure 2.7C). Although the amount of ARPP19 was not quantified, ARPP19 appears to be less abundant than ENSA at the metaphase II stage based on immunoblotting studies (Figure 2.7C, D). These patterns of expression suggest that ENSA is the main MASTL substrate regulating meiosis I in mouse oocytes and provide rationale to explore the potentially unique functions of ENSA and ARPP19 during meiosis II (discussed in more detail in section V).

ENSA appears to be present in mouse oocytes with an estimated abundance of up to 3.84 x 10^{10} copies per oocyte (Figure 2.9, Table 2.5). The abundance of ENSA in mouse oocytes is supported by studies in other cell types that have estimated that ENSA is present at a 5:1 molar ratio compared to PP2A-B55 (Cundell, Bastos et al. 2013; Williams, Filter et al. 2014). ENSA inhibits PP2A-B55 by binding to its active site and an excess of ENSA is likely required for efficient inhibition of PP2A-B55 activity during M-phase. While a 5:1 molar ratio of ENSA to PP2A-B55 was observed in mitotic cells, it is possible that an even higher concentration of ENSA is required in mouse oocytes to inhibit PP2A-B55 during the extended periods of meiotic M-phase.
III. The MASTL-ENSA/ARPP19 pathway in mouse oocytes: Implications for the molecular regulation of exit from prophase I arrest

Work in Chapter 2 and Chapter 3 explores the roles of MASTL and ENSA during meiotic maturation. MASTL and ENSA regulate the exit from prophase I arrest with the majority of MASTL-deficient and ENSA-deficient oocytes being unable to exit from prophase I arrest. Attempts to knockdown ARPP19 in mouse oocytes were unsuccessful (discussed below in section V) and it is unknown whether ARPP19 plays a role in the exit from prophase I arrest. The majority (~70-80%) of both MASTL-deficient and ENSA-deficient oocytes are unable to exit from prophase I arrest (Figures 2.3 and 3.3). The role of MASTL has been explored in oocytes from another mammalian species, the pig. MASTL-deficient porcine oocytes are also impaired in their ability to exit from prophase I arrest with ~55% of oocytes arresting at the prophase I stage or germinal vesicle breakdown stage compared to approximately 18% of control oocytes. The majority of oocytes (65%) that exit from prophase I arrest display spindle defects at the metaphase I or metaphase II stage (Li, Kang et al. 2013). In our studies, we observe a defect in M-phase entry during meiosis I, but do not observe defects in meiotic progression in the ENSA-deficient oocytes that exited from prophase I arrest; Ensa siRNA-injected oocytes that exit from prophase I arrest are able to successfully complete meiosis and arrest at the metaphase II stage to an extent similar to control oocytes (Figure 3.5).

Differences in the observed phenotypes between our studies and the studies of pig oocytes could be explained by differences in the extent of knockdown. Residual MASTL protein in porcine MASTL-deficient oocytes could allow oocytes to exit from prophase I...
arrest, but lead to arrest at a later stage of meiotic maturation. It is possible that fewer oocytes would exit from prophase I arrest with a higher extent of knockdown and lower residual levels of MASTL. These differences in the effects of MASTL pathway disruption between mouse and pig oocytes may also be due to differences in the regulation of meiotic M-phase entry and progression between the two species.

Interestingly, pig Ensa has a premature stop codon and functional protein is not expressed, suggesting that ARPP19 is the sole MASTL substrate in pig oocytes (Li, Kang et al. 2013). Our studies demonstrate that ENSA is a physiologically relevant substrate in mouse oocytes and the lack of ENSA protein in pig oocytes implies some differences in the regulation of meiotic maturation exist in these different mammalian species.

The main phenotype observed in MASTL-deficient and ENSA-deficient oocytes is the inability to exit from prophase I arrest. This phenotype is reminiscent of that found in studies of other cell cycle regulators during meiosis in mouse oocytes. Cdk1-null oocytes are unable to exit from prophase I arrest (Adhikari, Zheng et al. 2012). The phosphatase CDC25B, a CDK1 regulator that removes inhibitory phosphates from CDK1 allowing CDK1 activation and entry into M-phase (Figure 1.2), is also essential for exit from prophase I arrest in mouse oocytes (Lincoln, Wickramasinghe et al. 2002). While CDC25B acts upstream of CDK1 activation and MASTL/ENSA are downstream of active CDK1, interference with these regulators results in similar impairments in meiotic maturation and ultimately oocytes are unable to exit from prophase I arrest. The work in this thesis supports the recent paradigm shift in our understanding of the regulation of CDK1 activity; both CDK1 activation and inhibition of phosphatases that remove phosphates from CDK1 substrates are required for M-phase entry (Mochida and Hunt
2007; Mochida, Ikeo et al. 2009; Johnson and Kornbluth 2012). Our studies highlight that inhibition of PP2A activity is just as critical as CDK1 activity during the exit from prophase I arrest.

Chapter 3 shows that suppression of PP2A activity using the phosphatase inhibitor, okadaic acid, or knockdown of the B55δ regulatory subunit of PP2A (gene name is PPP2R2D) rescues the meiotic maturation defect observed in ENSA-deficient oocytes. ENSA-deficient oocytes treated with okadaic acid or injected with Ppp2r2d-targeting siRNA are able to exit from prophase I arrest to extents similar to control oocytes (Figures 3.6 and 3.7). These data are consistent with the model established in Xenopus and suggest that PPP2R2D/B55δ is the main PP2A regulatory subunit regulated by ENSA in mouse oocytes (Mochida, Ikeo et al. 2009; Gharbi-Ayachi, Labbé et al. 2010; Mochida, Maslen et al. 2010). Depletion of four B55 regulatory subunits (α, β, γ, δ) was shown to rescue mitotic progression defects in Mastl-deficient mouse embryonic fibroblasts (Álvarez-Fernández, Sánchez-Martínez et al. 2013), but our studies are the first to specifically identify PP2A-B55δ as the phosphatase functioning the MASTL-ENSA/ARPP19 pathway in a mammalian cell type.
V. Unresolved questions and future directions

One of the major unresolved questions in this field in species that have two MASTL substrates is whether ENSA and ARPP19 are functionally redundant or serve distinct cellular roles. Mouse, human and *Xenopus* express both ENSA and ARPP19 and the cellular roles of these substrates is unresolved. Our studies show a clear physiological role for ENSA in mouse oocytes and future studies should explore the role of ARPP19 in mouse oocytes. Attempts here to knockdown ARPP19 using two different *Arpp19*-targeting siRNA pools were unsuccessful (Figure 2.8), but a morpholino-based approach or conditional knockout oocytes could be used to address the function of ARPP19 during meiotic maturation and fertilization. Embryonic stem cells expressing a modified *Arpp19* allele that can be used to create conditional knockout mice are available from the Knockout Mouse Project Repository (NIH Knockout Mouse Project (KOMP); www.komp.org). A comparison of the functions of ENSA and ARPP19 in mouse oocytes would provide insight into the role of MASTL substrates in species with two MASTL/Greatwall substrates.

The majority of ENSA-deficient oocytes are unable to exit from prophase I arrest, however a portion of ENSA-deficient oocytes are able to exit from prophase I arrest and progress to the metaphase II stage (Figure 3.3). Incomplete knockdown and the presence of residual ENSA protein in *Ensa* siRNA-injected oocytes may explain why some oocytes are able to exit from prophase I arrest. It is also possible that ARPP19 is able to compensate for the lack of ENSA protein in some ENSA-deficient oocytes. ENSA appears to be more abundant than ARPP19 in mouse oocytes, with ARPP19 only being
detected at low levels in metaphase II eggs (Figure 2.7). ENSA may be the main physiological MASTL substrate that regulates the exit from prophase I arrest due to its abundance in mouse oocytes; it is possible that expression of ARPP19 at comparable levels could compensate for the lack of ENSA protein in ENSA-deficient oocytes. This hypothesis could be addressed by overexpression of ARPP19 in ENSA-deficient oocytes to test if ARPP19 could rescue the meiotic maturation defect observed in ENSA-deficient oocytes. There is evidence that MASTL/Greatwall substrates can serve redundant functions from studies showing *Drosophila* and *Xenopus* substrates are interchangeable and can function to inhibit PP2A activity in *in vitro* assays. The single *Drosophila* Greatwall substrate, Endos, can inhibit PP2A when added to *Xenopus* extracts (Kim, Bucciarelli et al. 2012).

Future studies could also explore differences between the molecular regulation of meiosis I and meiosis II. A subset of *Ensa* siRNA-injected oocytes progressed to the metaphase II stage and was able to maintain metaphase II arrest (Figure 3.5). However, these results should be interpreted cautiously as these oocytes may not truly be ENSA-deficient and prolonged maintenance of metaphase II arrest and exit from metaphase II arrest were not assessed. ARPP19 is not detected in lysates of prophase I oocytes and is detected at low levels in lysates of metaphase II eggs. This expression pattern raises the possibility that ARPP19 may function to regulate M-phase during the second meiotic division; future studies could examine the function of ARPP19 during metaphase II arrest and the second meiotic division. ENSA and ARPP19 levels could be manipulated in the metaphase II egg directly by injection of *Ensa*-targeting or *Arpp19*-targeting siRNA into metaphase II eggs. The maintenance of metaphase II arrest, completion of meiosis II and
progression into embryogenesis could be examined in ENSA-deficient and ARPP19-deficient metaphase II eggs. These studies would also address the possibility that ENSA could be the main MASTL substrate regulating M-phase entry and progression during meiosis I, while ARPP19 could be a physiologically relevant MASTL substrate during meiosis II.

The function of ENSA and ARPP19 is regulated by phosphorylation, both by MASTL/Greatwall, as well as other mitotic kinases. The MASTL/Greatwall phosphorylation sites for ENSA and ARPP19 are highly conserved and have been studied in *Xenopus* and *Drosophila* (Gharbi-Ayachi, Labbé et al. 2010; Mochida, Maslen et al. 2010; Kim, Bucciarelli et al. 2012; Dupre, Buffin et al. 2013). To explore the functional significance of ENSA and/or ARPP19 phosphorylation by MASTL during mammalian female meiosis, the putative MASTL phosphorylation sites for ENSA and ARPP19 could be mutated. Expression of non-phosphorylatable forms (mutate phosphorylation site to alanine) or phospho-mimetic forms (mutate phosphorylation site to aspartic acid or glutamic acid) of ENSA and ARPP19 in a deficient background would provide insights into the temporal requirements for ENSA/ARPP19 phosphorylation and dephosphorylation. The ENSA and ARPP19 phosphorylation sites for other mitotic kinases, such as protein kinase A and CDK1/cyclin B, have been studied in other species including *Xenopus* and starfish (Dupre, Buffin et al. 2013; Dupre, Daldello et al. 2014; Okumura, Morita et al. 2014); these conserved phosphorylation sites could also be mutated to examine the role of multiple ENSA and ARPP19 phosphorylation events in mouse oocytes. Examination of the regulation of ENSA and ARPP19 function by
phosphorylation by multiple kinases would provide insights into how these small phosphoproteins can coordinate signals from numerous signaling pathways.

VI. Broader significance of this work

This dissertation research provides new insights into the MASTL-ENSA/ARPP19 pathway and the regulation of entry into M-phase during mammalian meiotic maturation. These studies have important implications for our understanding of oocyte biology, as well as human health. Cell cycle deregulation and aberrant cell proliferation is a hallmark feature of cancers. An understanding of cell cycle regulation is critical for advancements in the field of cancer biology and development of new cancer therapies (Williams and Stoeber 2011; Diaz-Moralli, Tarrado-Castellarnau et al. 2013). The MASTL-ENSA/ARPP19 pathway is a key regulator of cell cycle entry and progression, and pathway components are considered as attractive candidates for the development of cancer therapies (Ma and Poon 2011). In a study in mice and cultured cells, the inhibition of MASTL was explored as part of a combination therapy to target the process of mitotic exit in tumor cells (Manchado, Guillamot et al. 2010). Another study tested the hypothesis that loss of ENSA/MASTL function could lead to cancer by exploring the expression of ENSA in human liver and breast cancer samples (Chen, Kuo et al. 2013). The MASTL-ENSA/ARPP19 pathway may also play a role in the development of other human diseases. While the mechanism for pathogenesis is unknown, a single amino acid change in human MASTL is associated with autosomal dominant inherited form of
thrombocytopenia, a disease characterized by low platelet count (Gandhi, Cummings et al. 2003; Johnson, Gandhi et al. 2009).

This thesis research identifies the MASTL-ENSA/ARPP19 pathway as a regulator of mammalian female meiosis and provides new insights into the regulation of fertility and potential causes of infertility in humans. Infertility, which is defined as the failure to conceive after 12 months of regular, unprotected intercourse, affects approximately 9-15% of couples or an estimated 70 million couples worldwide (Boivin, Bunting et al. 2007; Ombelet, Cooke et al. 2008; Ata and Seli 2010). For about 30-40% of these couples, no identifiable cause for infertility is found and these couples' infertility is classified as unexplained (Ray, Shah et al. 2012). Basic scientific research on normal oocyte biology is critical to aid our understanding of the etiology of infertility.

The MASTL-ENSA/ARPP19 pathway is a critical regulator of meiotic maturation, a process that is essential for the development of a mature female gamete, as evidenced by data in this thesis. While it is difficult to estimate the overall incidence, there are some reports of maturation arrest defects in human infertility patients (Levran, Farhi et al. 2002). The molecular basis of these maturation defects is unknown, but is similar to the defects seen in Cdk1-null, Cdc25b-null, MASTL-deficient and ENSA-deficient mouse oocytes (Lincoln, Wickramasinghe et al. 2002; Adhikari, Zheng et al. 2012). While the MASTL-ENSA/ARPP19 pathway is essential in both mitotic and meiotic cells and complete loss of function mutations would likely result in embryonic lethality, it is interesting to note that clinical infertility cases similar to the meiotic maturation defects observed for MASTL-deficient and ENSA-deficient oocytes do exist. Importantly, humans, like the mouse and unlike the pig, appear to express both MASTL substrates,
ENSA and ARPP19. Humans are predicted to express one *Arpp19* isoform and eight
*Ensa* isoforms; while little is known about the expression of these eight *Ensa* isoforms, it
is possible that human oocytes could express an oocyte-specific *Ensa* isoform. Future
studies could examine this possibility by examining the expression of these *Arpp19* and
*Ensa* isoforms in oocytes from humans or non-human primates.
VII. REFERENCES

Adhikari, D., W. Zheng, et al. (2012). "Cdk1, but not Cdk2, is the sole Cdk that is essential and sufficient to drive resumption of meiosis in mouse oocytes." Human Molecular Genetics 21: 2476-2484.


CURRICULUM VITAE

Lauren M. Matthews, M.Sc.

Johns Hopkins University, Bloomberg School of Public Health
Department of Biochemistry and Molecular Biology
615 N. Wolfe Street, Room E8607
Baltimore, MD 21205
Mobile phone: (518) 956-3530
Email: laurenmatthews8@gmail.com

EDUCATION

Ph.D. candidate, Johns Hopkins University, Bloomberg School of Public Health,
Department of Biochemistry & Molecular Biology, Division of Reproductive Biology,
August 2008 – Current
Anticipated completion date: August 2014
Thesis advisor: Janice P. Evans, Ph.D.
Thesis title: Cell cycle regulation during mammalian female meiosis: Characterization of
the MASTL-ENSA/ARPP19 pathway in mouse oocytes

Master of Science in Medicine, Reproductive Health and Human Genetics,
University of Sydney, Australia, Department of Obstetrics & Gynaecology,
March 2005 – June 2007
Thesis title: Dendritic cells in the eutopic endometrium during the menstrual cycle in
normal women and women with endometriosis

Bachelor of Science, Cum Laude, Cornell University, College of Agriculture and Life
Sciences, August 2001 – January 2005
Major: Animal Science, GPA 3.9; Cumulative GPA 3.6

Exchange student program, University of Sydney, Australia, March 2004 – November
2004

CERTIFICATE

Certificate in Maternal & Child Health, Johns Hopkins Bloomberg School of Public
Health, Department of Population, Family and Reproductive Health, 2009-2012
• Acquired knowledge and concepts in women’s and children’s clinical health,
reproductive biology and women’s health policy and programs
RESEARCH EXPERIENCE

Ph.D. candidate, *Johns Hopkins Bloomberg School of Public Health, Department of Biochemistry & Molecular Biology, Division of Reproductive Biology*, August 2008 – Current

- Conducted basic research on egg biology with implications for female reproductive health; this work was published in a peer-reviewed journal and presented at an international scientific conference.
- Published 2 collaborative papers in peer-reviewed journals.
- Completed 5 research rotations in the Department of Biochemistry & Molecular Biology:
  - Rotation 1: Mapping the SUMO-interacting motif of RAD51, Advisor: Michael Matunis, Ph.D.
  - Rotation 2: Modulation of myosin-II mediated cortical dynamics in female meiosis, Advisor: Janice P. Evans, Ph.D.
  - Rotation 3: Development of a seminiferous tubule culture system to study the function of the stem spermatogonial niche, Advisor: William Wright, Ph.D.
  - Rotation 4: Analysis of microRNA regulation of Keratin 17, Advisor: Pierre Coulombe, Ph.D.
  - Rotation 5: The down regulation of superoxide dismutases under anaerobic conditions in *Saccharomyces cerevisiae*, Advisor: Valeria Culotta, Ph.D.


- Conducted clinical research on immune function in women with endometriosis. Reported for the first time that dendritic cell populations in women with endometriosis are altered compared to controls.
- Published 2 first-author and 3 collaborative papers in peer-reviewed journals and presented research at 2 international scientific conferences.
- Organized and oversaw a departmental monthly journal club.

Medical Scientist, *Fertility First, Centre for Reproductive Health*, Sydney, Australia, March 2006 – January 2008

- Performed clinical services and diagnostic testing. Worked directly with patients and clinical staff to ensure a high level of medical care.
- Conducted research on the effect of lifestyle modifications on fertility that was published in a peer-reviewed journal.


- Worked independently to conduct research on osteoarthritis and hip dysplasia.
- Compiled data for laboratory’s new microarray project.
HONORS & AWARDS

Training Grant recipient, *Johns Hopkins University Reproductive Biology Training Grant, National Institute of Child Health & Development, 2009-2011*

Undergraduate Academic Award, *American Society of Animal Science, 2004*

PUBLICATIONS
(Maiden name Lauren M. Schulke)


PROFESSIONAL AFFILIATIONS

Association for Women in Science
Society for the Study of Reproduction
Fertility Society of Australia
Scientists in Reproductive Technology
Asia Pacific Endometriosis Alliance

CONFERENCES AND PRESENTATIONS

Gordon Research Conference: Fertilization & Activation of Development, July 2009 (attendee) and 2012 (poster presentation)
Poster presentation: Meiotic maturation in mammalian oocytes: MASTL, ENSA and ARPP19 as candidates to regulate M-phase

10th World Conference of Endometriosis, March 2008
Poster presentation: Uterine dendritic cell populations are altered in women with endometriosis compared to controls

University of Sydney, “From Cell to Society” Health Research Conference, November 2006
Presentation: Dendritic cells in eutopic endometrium during the menstrual cycle in women with and without endometriosis

LEADERSHIP EXPERIENCE

Science Outreach Program volunteer, Johns Hopkins University, April 2014 – August 2014
• Developed lesson plans for a summer science program for 3rd and 4th grade Baltimore city students.
• Organized and taught these classes to students.
• Revised lesson plans throughout the summer to reflect the needs and learning styles of the students.

STEM specialist in the Classroom volunteer, Maryland Business Roundtable for Education, 2013 – current
• Program brings STEM professionals to Maryland high schools to strengthen STEM teaching and to inspire students to pursue STEM careers.
• Completed the STEM specialist training program and will begin classroom visits in September 2014.

Research!America Science communication workshop participant, October 2013
• Received instruction and practical experience communicating with different audiences, including the general public, the media and policy makers.

Manuscript reviewer, Molecular Reproduction and Development, 2012 – current
• Critically reviewed manuscripts and provided feedback to authors.

Grading Assistant, Fundamentals of Reproductive Biology, Johns Hopkins University, 2009 – 2012
• Graded exams for class of approximately 100 graduate students.

Teaching Assistant, Domestic Animal Biology I, Cornell University, 2003
• Organized and conducted weekly laboratory sessions and monthly review sessions for class of approximately 150 undergraduate students (in groups of 20-30 students).

Extension Educator, Cornell Cooperative Extension Center, 1999 – 2001
• Planned and implemented educational programs for both children and adults about a wide range of agricultural and environmental topics.

OTHER EMPLOYMENT EXPERIENCE

Pharmacy Assistant - Oxford Pharmacy, Sydney, Australia, 2004-2006
Veterinary Technician - Harborside Veterinary Hospital, Halesite, NY, 2002-2004
Assistant Retail Manager - The Athlete’s Foot, Lake Grove, NY, 1999-2002