ANALYSIS OF HUMAN AURORA C KINASE VARIANTS IN OOCYTE MEIOSIS

By

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ABSTRACT OF THE DISSERTATION

Analysis of human Aurora C Kinase variants in oocytes meiosis

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The birth of a healthy child is often a priority for couples, yet many couples will unfortunately suffer from infertility. Aneuploidy is the leading genetic abnormality that causes infertility. Aneuploidies in sperm and eggs arise from errors during chromosome segregation in meiosis. Though many of the critical players are known and mutants characterized, the regulatory components involved in directing accurate chromosome segregation are still not entirely clear. Aurora kinase C is one of the critial players in chromosomes segregation in meiosis and in this thesis, I establish the use of *Aurkc*$^{-/-}$ mouse oocytes as an effective model for studying human AURKC *ex vivo.* By comparing the rescue of the knockout phenotype when hAURKC and mAURKC are expressed in the

oocytes, I show that hAURKC functions similarly in female meiosis as does mAURKC. I also characterized three splice variants of hAURKC in this model after establishing their simultaneous expression in human oocytes. The three splice variants have different abilities and stabilities in meiosis, but promote optimal chromosome segregation when expressed simultaneously during oocyte meiosis. Finally, I used the verified model to characterize the phenotype of three sterility-associated mutations in human *AURKC.* I show through functional assays that the three mutations have differing abilities in meiosis, but all result in a loss of function of the AURKC-CPC, which causes meiotic arrest and polyploid gametes. This work contributes to the field of reproductive biology by providing a tractable model for studying human AURKC and for further analysis of mutants and variants.

Acknowledgements

I would like to first thank my advisor, Dr. Karen Schindler. She accepted me into her lab as a homeless graduate student with nowhere else to go and gave me a chance when no one else would. She turned me from a disorganized graduate student into a thoughtful scientist. She taught me to how to analyze my work and my mistakes thoughtfully and methodically. She turned me into an excellent scientific writer, although that may have been the most painful part for both of us. I can't imagine where I would be had she not offered me the chance to work with her.

Next, I would like to thank my husband, Vincent Tortoriello. He has never known me as anything but a graduate student and somehow still fell in love with me and married me. Without his love and support I would have quit long ago. His free spirit and unending sense of humor rescued me from myself many times over. I love you.

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My younger sister and brother, Chrissy and Dan, though we could not be more different, we are also more alike than we would choose to admit. Thank you both for you love and support. I love you too.

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Finally I want to thank my co-workers in the Schindler lab. Dr. Ahmed Balboula has been a great friend and mentor and always supportive of even the most disastrous experiments. Ally Nguyen who taught me everything I know about maintaining a mouse colony. Amanda Gentilello for always letting me vent and taking breaks for puppy stories and bubble tea. Dr. Suzanne Quartuccio, though we have not overlapped for long you have given me hope for life after grad school and that is a serious gift.

Thank you!

Dedication

To my parents for always being the net to catch me but telling me I didn’t need it.

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Chapter I:

Introduction

The process of embryo formation requires the fusion of two gametes: sperm from the father and an egg from the mother. Proper formation of these sex cells requires a coordinated set of events occurring at just the right time in just the right way to create a cell that contains half of the genetic content required to make an offspring. Meiosis is the critical process involved in gamete formation, where a diploid precursor cell undergoes one round of DNA replication followed by two subsequent divisions resulting in haploid sex cells. One of the most important steps in meiosis is chromosome segregation where complex signaling cascades ensure that the exact complement of DNA is delivered to each daughter cell during division. Errors in this process can result in too many or too few chromosomes, which is a state known as aneuploidy and is the leading genetic abnormality that causes infertility. However, the frequencies of aneuploidies are not equal in males and females. Sperm have an aneuploidy rate of about 5% whereas the rate in oocytes is closer to 20% (Brandriff et al., 1994; Pacchierotti et al., 2007). The majority of these errors occur during meiosis I (MI), which is the reductional phase of meiosis where homologous chromosomes are segregated into separate daughter cells(Hassold and Hunt, 2001). One of the key players in the orchestration of chromosome segregation is the chromosomal passenger complex (CPC), which acts in numerous ways to ensure efficient separation of genetic material. The catalytic subunit of the CPC in meiosis is Aurora kinase C (AURKC). This introduction will review the role of AURKC in meiosis, including its regulators and substrates, as well as its similarities and differences from its mitotic counterpart, Aurora kinase B (AURKB) and the

relation of these factors to female meiosis, fertility, and chromosome segregation.

GENETICS OF REPRODUCTION AND INFERTILITY

The successful act of reproduction is the culmination of many critical processes taking place in step-wise and concurrent fashion during pre- and post-natal development. Pre-natal events include sex determination, gonad development, and gamete formation (in females). Meiotic divisions, hormone signaling, and pubertal development occur after birth. The genes involved in regulating these processes have traditionally been difficult to identify because often times the mutations are embryonic lethal or lead to somatic phenotypes that are difficult to distinguish in mutagenic screens.

The identification of genes involved in reproduction began with the characterization of those required for sex determination such as the SRY locus on the Y chromosome that is required for formation of testis (Berta et al., 1990; Ford et al., 1959; Jacobs and Strong, 1959; Koopman et al., 1990; Sinclair et al., 1990). Sex chromosome syndromes (Klinefelter's, XXY or Turner, XO) are easily identifiable by karyotype, which does not require any detailed genotyping, and patients with these sex chromosome aneuploidies are sterile (Ford et al., 1959; Jacobs and Strong, 1959).

The identification of genetic abnormalities that were not the result of karyosome alterations, made precise functional analysis difficult without the use of sequencing or an effective model system. The use of transgenic mice for

studies began in the 1980's and is now the most common and clinically relevant laboratory animal model used for reproductive research. These studies began with rat and human growth hormone injected into mouse eggs which, after fertilization, grew into abnormally large adult mice (Palmiter et al., 1982; Palmiter et al., 1983). Overexpression studies quickly turned to rescue experiments, where human protein was used to rescue mouse phenotypes. For example, human FSHβ was expressed in transgenic mice lacking endogenous FSHβ and rescued both male and female gonad development defects and infertility (Kumar et al., 1998). The ability of human proteins to function in mouse cells and to rescue mouse phenotypes lead to a boom in the use of transgenic rodents for studying clinically relevant mutations. There is a plethora of mutant mouse lines for a variety of genes that have been and are being studied to understand their relevance to human reproduction and fertility. There are a significant number of mouse lines with mutations in TGFβ superfamily members with defects ranging from uterine and follicle development, to delivery and nursing as well as male gonad development and fertility (Brown et al., 2000; Dong et al., 1996; Matzuk et al., 1994; Matzuk et al., 1995; Mishina et al., 1996; Pangas et al., 2007; Vassalli et al., 1994; Yan et al., 2001). There are mutant mice with spermatogenesis specific defects (Koizumi et al., 2003; Lin et al., 2007; Liu et al., 1998; Ma et al., 2009; Yan et al., 2003). There are also mutant mice with female specific mutations (Andreu-Vieyra et al., 2010; Rajkovic et al., 2004; Yan et al., 2005a). Mutant lines have even been generated in genes required for formation of the gonads in both sexes (Ballow et al., 2006; Pangas et al., 2006; Yang et al.,

2005).

Though orthologous human mutations have not yet been identified for all of these genes, the use of these mutant lines for human health is relevant due to their ability to shed light on the signaling pathways involved in reproduction and fertility. Furthermore, there is direct clinical relevance in studying these mutants because genes leading to infertility without other somatic defects are excellent targets for contraception drugs.

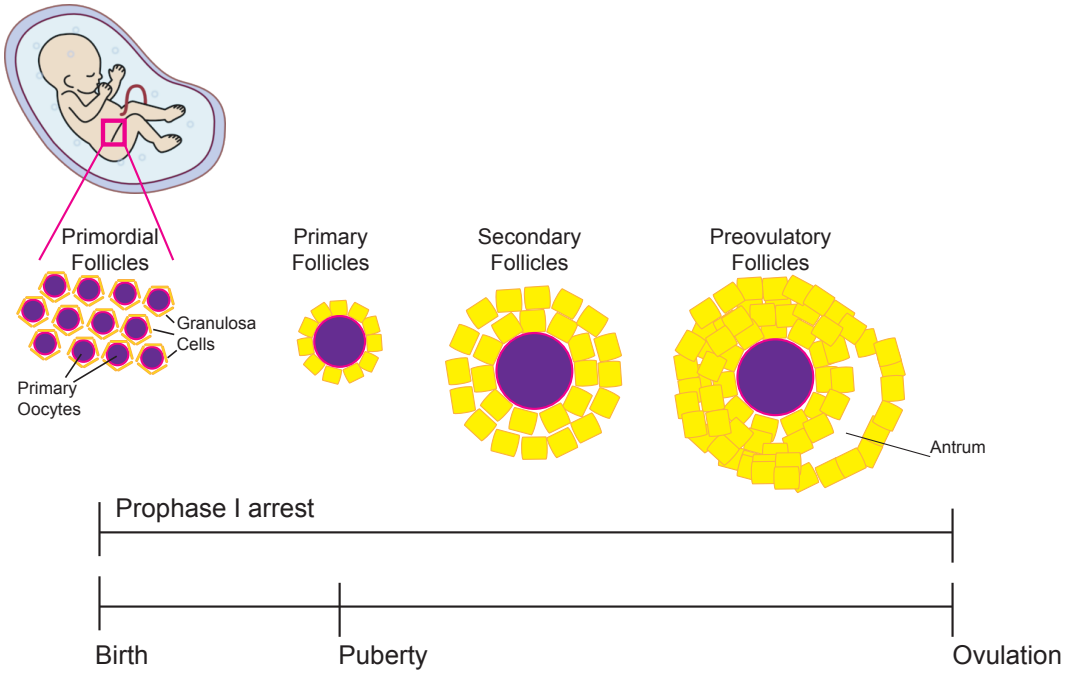
The use of mouse models for studying reproductive defects in humans is well supported in the literature. For example, *BMP15* and *GDF9* mutations are associated with primary ovarian failure and sterility in women (Laissue et al., 2006; Rossetti et al., 2009; Simpson et al., 2014; Wang et al., 2013). However, functional analysis is nearly impossible in humans due to fact that the reserve of primordial follicles is depleted by the time the mutations are identified and the cause is unclear. Use of mouse models for understanding the roles of BMP15 and GDF9 in folliculogenesis is critical to our understanding of these phenotypes in women (Dong et al., 1996; Yan et al., 2001).

Our lab has developed a knockout mouse model for the purposes of studying Aurora kinase C in oocyte meiosis and its relation to fertility and reproduction. Recent genetic studies have identified mutations in *AURKC* associated with sterility in men and the work presented in this thesis develops the *Aurkc*$^{-/-}$ mice as a model to study human AURKC and to characterize these mutations in oocyte meiosis (Ben Khelifa et al., 2012; Dieterich et al., 2007; Dieterich et al., 2009).

FOLLICULAR DEVELOPMENT

During fetal development, female primordial germ cells divide and differentiate into oogonia, which are the diploid precursors of oocytes. Humans are born with approximately 2.5 million primordial follicles (somatic-gametic complexes made up of primary oocytes and granulosa cells, Fig. 1)(Wallace and Kelsey, 2010) and no new primordial follicles develop after birth. Prior to birth, the primary oocytes have already undergone DNA replication and have arrested in the dictyate stage of prophase where they will remain until ovulation. During this time, however, the oocyte will grow and mature with the surrounding granulosa cells and many changes in oocyte size and somatic cell number will occur. Primordial follicles are the smallest follicles and are characterized by flat squamous granulosa cells surrounding the premature oocyte (Fig. 1). As time progresses, the oocyte grows larger and the flat cells become more cuboidal in shape developing into a primary follicle (Fig. 1). Addition of more layers of granulosa cells and a larger oocyte defines secondary follicles while the presence of a fluid filled space (antrum) becomes evident in preovulatory follicles (Fig. 1). Meiosis resumes within the oocyte once luteinizing hormone (LH) signals for ovulation and the follicle is expelled from the ovary into the fallopian tube.(Satoh, 1991; Sawyer et al., 2002; Von Stetina and Orr-Weaver, 2011; Wallace and Kelsey, 2010)

Fig. 1- Follicular Development. Females are born with the full complement of oocytes that they will have for their reproductive lifespan. In the fetal ovary, primordial germ cells develop into oocytes (meiotic female gametes arrested at prophase I) surrounded by granulosa cells that support the development of the oocyte. The cumulus-oocyte complex (COC) is known as the follicle. Primordial follicles are the most immature follicle and make up the entire reserve of fertility at birth. Once puberty begins, waves of primordial follicles begin maturing which involves the growth of the oocyte as well as changes in shape and number of the surrounding granulosa cells. Each cycle, one of these developing follicles will be selected for ovulation, releasing the oocyte into the fallopian tubes.



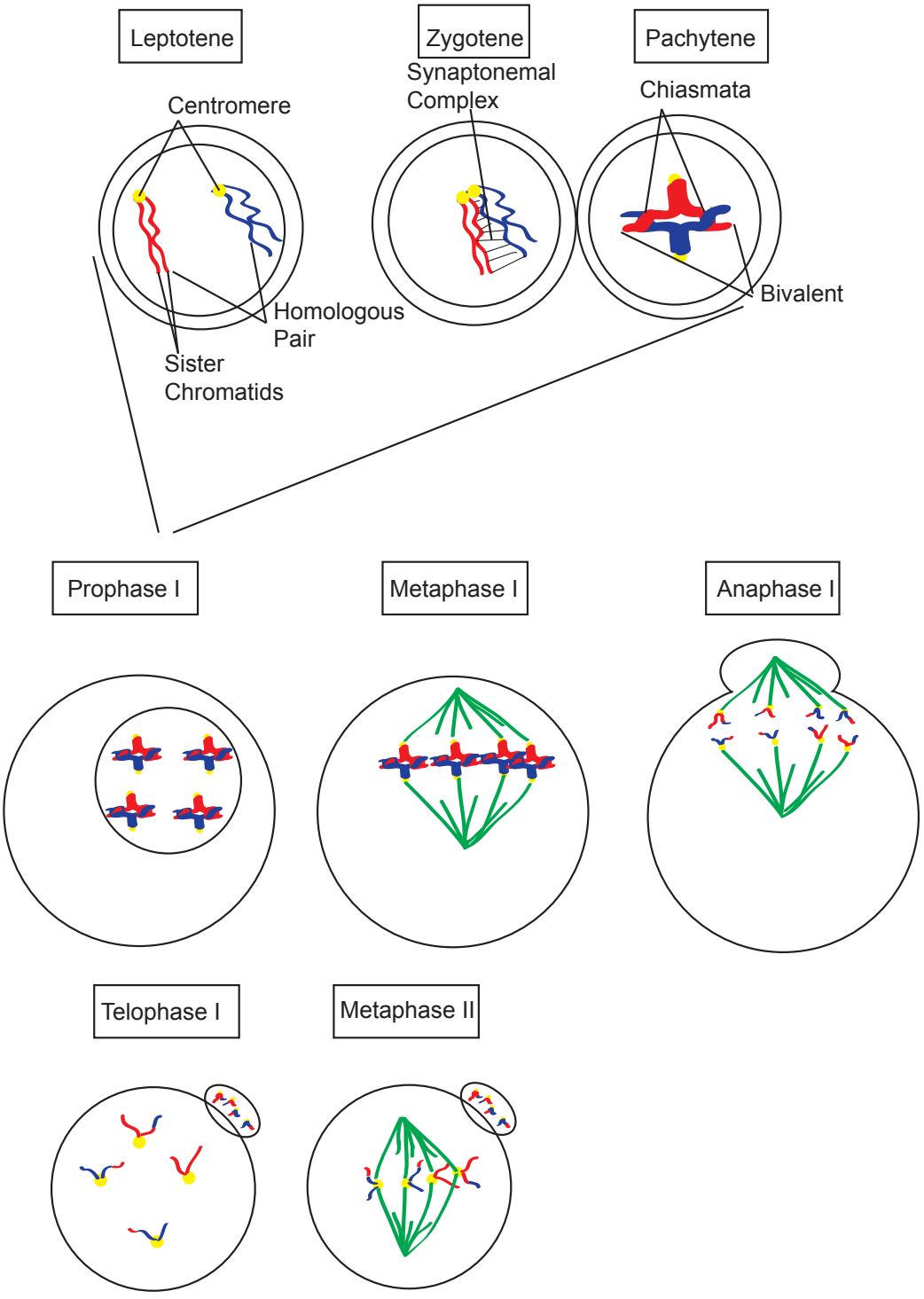
MEIOSIS

Meiosis is the divisional process where a diploid cell (2n) divides twice into four haploid cells (1n) and is a critical step in sexual reproduction. While the general progression of meiosis is similar between males and females, some of the specific regulators, as well as the timing of meiosis, differ between sperm and oocyte production. As previously mentioned, oocytes arrest at prophase I of meiosis and do not complete the process until after fertilization which could be up to 50 years later. Meiosis in sperm takes approximately one day without any arrest.

The first division is called meiosis I (MI) and involves many processes that are critical for sexual reproduction and genetic diversity. Prophase I (Pro I) is the first stage of meiosis and begins with the interlinkage of homologous chromosomes in chiasmata, which are the site of homologous recombination. Homologous recombination involves the formation of double stranded breaks in the DNA which are repaired through the formation of crossovers resulting in increased genetic diversity in the gametes and subsequent offspring (Fig. 2). Oocytes arrest during Pro I after the formation of the chiasmata and will remain arrested until ovulation many years later.

Once meiosis resumes, the cells proceed to metaphase I (Met I), which is characterized by the formation of a bipolar spindle and the alignment of the bivalents at the metaphase plate in preparation for segregation (Fig. 2). Proper alignment of the chromosomes is critical for accurate segregation into opposite

Fig. 2- Oocyte Meiosis. Oocytes arrest during diplotene (known as diakinesis in oocyte) of Pro I, which occurs after pachytene, until ovulation. Once ovulation occurs, the oocytes resume meiosis and progress straight through to Met II where another arrest occurs until the egg (an oocyte that has reached Met II) is fertilized.



daughter cells and relies on the pairs of homologous chromosomes to bi-orient to opposite spindle poles and pairs of sister chromatids to co-orient to the same pole. The bi-orientation of the homologs ensures that daughter cells have one complete copy of the genome while co-orientation of the sisters promotes the segregation of the sister chromatids as a cohesive unit. This orientation is orchestrated by the formation of attachments between the kinetochores of the chromosomes and the microtubules of the spindles (K-MT attachments). The alignment of the bivalents triggers the cell to progress to anaphase I (Ana I) where the chiasmata are resolved and the homologs are separated into two daughter cells in telophase I (Telo I, Fig. 2). In oocytes, this division is asymmetric resulting in one large oocyte and a small polar body that is later discarded. The cells immediately progress to metaphase II (Met II) and the sister chromatids line up and bi-orient at the metaphase plate for segregation in anaphase II (Ana II) and telophase II (Telo II). In oocytes, there is a second arrest once the sisters are aligned at Met II and they do not progress through the final division and extrude the second polar body until after fertilization takes place.

THE AURORA FAMILY OF KINASES

The Aurora kinases were first identified in *Saccharomyces cerevisiae* in a screen for mitotic mutants with chromosome segregation defects and it was named *Ipl1* (*Increase-in-ploidy1)* because when mutated, the cells were

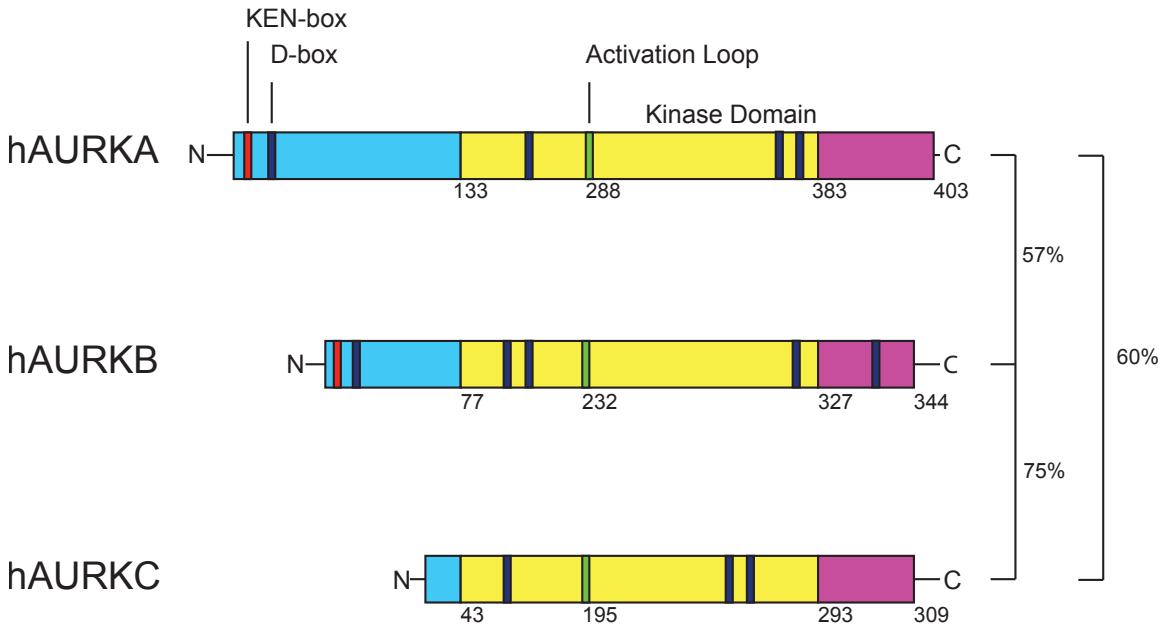
aneuploid after division (Chan and Botstein, 1993; Kimura et al., 1997). Yeast have only the single Aurora kinase present while two (Aurora A and Aurora B) are found in *Drosophila*, *C. elegans*, and *X. laevis* and mammals have three (addition of Aurora C) (Chan and Botstein, 1993; Glover et al., 1995). Aurora kinases are well conserved throughout evolution (Fig. 3) (Kimura et al., 1997; Niwa et al., 1996) and were identified in human diseases and disorders from cancer to infertility (Dieterich et al., 2007; Dutertre et al., 2005; Sasai et al., 2004).

The Auroras are highly conserved serine/threonine kinases that are all involved in chromosome segregation and cell division but in different ways (Fig. 3). The three members exhibit remarkable homology in the kinase domain and critical differences in the N- and C-terminus, which could be responsible for their differing localizations, and functions in M-phase. The subsequent sections will describe the three members of the Aurora family found in mammals.

Aurora Kinase A

AURKA is the most distinct member of the Auroras in sequence, localization, and in function. AURKA is a hot spot for cancer therapy targeting due to its overexpression in many cancers and its oncogenic activity when expressed in cell culture models (Anand et al., 2003; Meraldi et al., 2002; Tatsuka et al., 1998; Vader and Lens, 2008; Zhang et al., 2004). Overexpression of AURKA is associated with multipolar spindles, a hallmark of cancer cell

Fig. 3- The Aurora Family of Kinases. The Auroras are serine/threonine kinases that are highly conserved throughout evolution. Aurka and Aurkb are found in most animals while mammals have the addition of a third, Aurkc.



Adapted from Quartuccio & Schindler 2015, Martinelli et al., 2009, and Umene et al., 2013

mitosis. AURKA is found mainly at the spindle poles and on the microtubules nearest the poles in early M-phase and is important in regulation of centrosome separation and forming a bipolar spindle (Carmena and Earnshaw, 2003; Glover et al., 1995; Honda et al., 2000; Littlepage and Ruderman, 2002). At the poles, AURKA is mainly associated with TPX2 (Bayliss et al., 2003), which also aids in activating AURKA (Bird and Hyman, 2008) and is a substrate of AURKA (Kornev and Taylor, 2010; Kufer et al., 2002; Tsai et al., 2003). Another target of AURKA is Kif11 (Eg5), that is necessary for forcing the centrosomes apart (Barr and Gergely, 2007). It is also hypothesized to play a role in nucleation of microtubules at the centrosomes (Kufer et al., 2003). AURKA is important for stabilizing the microtubules at centrosomes by binding the minus ends of the fibers and opposing the activity of the microtubule destabilizing kinesin, mitotic centromere associated kinesin (MCAK) (Barros et al., 2005; Kinoshita et al., 2005), which is also a substrate of AURKA (Zhang et al., 2008). After the completion of metaphase and the spindle assembly checkpoint (SAC) is satisfied, AURKA function is no longer necessary so its degradation is triggered by ubiquitin-mediated proteasome activity via the anaphase promoting complex and cyclosome (APC/C)(Castro et al., 2002; Honda et al., 2000; Littlepage and Ruderman, 2002; Taguchi et al., 2002).

Aurora Kinase B

AURKB was first identified in humans after a GenBank database search

for clones with high homology to AURKA (Kimura et al., 1998). AURKB shares 74% identity with AURKA but localizes and functions distinctly in mitosis and meiosis (Fig. 3). The N-terminus of AURKB contains a KEN-box destruction motif and several D-boxes (whose role is unclear, Fig. 3), the Ken-box motifs are responsible for targeting the protein for proteasome degradation after metaphase (Nguyen et al., 2005; Stewart and Fang, 2005); however when those motifs are mutated or deleted, the destruction rate of the AURKB is unaffected during oocyte meiosis (Schindler et al., 2012).

AURKB is the sole kinase of the CPC in mitotic cells and therefore performs all of the CPC functions there (see below for elaboration of CPC function in mitosis), it also performs as part of the CPC in meiotic cells but the function there differs from mitosis due to the presence of an additional kinase (see below for elaboration of CPC function in meiosis). The N-terminus of AURKB associates with the CPC by binding to the C-terminal IN-box of INCENP (Adams et al., 2000; Kaitna et al., 2000). This interaction is required for activation of AURKB and causes conformational changes to the kinase enabling autophosphorylation and phosphorylation of INCENP, further activating the complex which is required for the function of the complex in mitosis (Bishop and Schumacher, 2002; Honda et al., 2003; Kang et al., 2001; Sessa et al., 2005; Sugiyama et al., 2002; Yasui et al., 2004).

Substrates of AURKB

AURKB phosphorylates histone H3 at Serine 10 (H3S10) and Serine 28 (H3S28) which is correlated with chromosome condensation, but has many other targets in the cell as well (Adams et al., 2001; Crosio et al., 2002; Giet and Glover, 2001; Goto et al., 2002; Hsu et al., 2000; Murnion et al., 2001; Sugiyama et al., 2002). In this section, I will review the known substrates of AURKB in mitosis and their roles in chromosome segregation.

AURKB is not solely a histone kinase. It phosphorylates the other members of the CPC (INCENP and Survivin) as well (Bishop and Schumacher, 2002; Wheatley et al., 2004). As mentioned above, phosphorylation of INCENP is required for activation of AURKB and is important for recruitment of the CPC to centromeres. Phosphorylation of Survivin is required for mitotic progression and HeLa cells depleted of Survivin by RNAi arrest at prometaphase (Delacour-Larose et al., 2007).

AURKB also phosphorylates a variety of kinetochore proteins that play critical roles in biorientation during prometaphase and metaphase. CENP-A is a a centromere associated histone 3 variant that is targetted by AURKB in mitosis and when mislocalized, results in missegregation of chromosomes (Hayashi et al., 2014; Zeitlin et al., 2001). HEC1 (high express in cancer-1, aka NDC80) is part of the KMN complex (KNL-1 (Kinetochore NuL-1), Mis12 complex, and Ndc80/HEC1) (Cheeseman et al., 2002). HEC1 is part of the microtubule docking site on the kinetochore (Cheeseman et al., 2006), and phosphorylation of this

region by AURKB leads to a decreased affinity for microtubules. There are 6-8 phosphorylation sites in the N-terminus of HEC1 and each successive phosphate decreases the affinity of the protein for the microtubules until they are ultimately released from the kinetochore (Cheeseman et al., 2006; Ciferri et al., 2008; DeLuca et al., 2006; Guimaraes et al., 2008; Miller et al., 2008). MCAK (mitotic centromere-associated kinase) is also localized to kinetochores in mitosis and is a target for AURKB (Andrews et al., 2004; Lan et al., 2004). It plays a similar role to HEC1 where phosphorylation of this kinase results in the depolymerization of microtubules that are erroneously attached to the kinetochore (Sampath et al., 2004; Tulu et al., 2006). Stathmin/OP18 is another substrate that acts in microtubule destabilization and phosphorylation of which results in changes in spindle stability (Andersen et al., 1997; Gadea and Ruderman, 2006; Song et al., 2014). The regulation of error correction by the CPC will be discussed further below.

Shugosin (Sgo2) is a target of AURKB in human cells. Phosphorylation of Sgo2 results in the recruitment of PP2A to centromeres, which aids in protection of centromeric cohesin and spindle assembly (Kitajima et al., 2006; Tanno et al., 2010). Topoisomerase II alpha is also a target of AURKB in human cells and plays a role in condensation of chromatin and decatenation of DNA needed for sister chromatid separation during meiosis (Diaz-Martinez et al., 2006; Lu et al., 2015; Morrison et al., 2002).

Dam1 is a complex in budding yeast that encircles the kinetochore microtubules near the plus ends and four of the subunits of the complex are

targets for AURKB (Cheeseman et al., 2002; Kang et al., 2001). The role of phosphorylation of the Dam1 proteins is not fully understood, but mutation of the sites to an unphosphorylatable alanine results in cell death, while mutation to the phosphomimetic aspartate results in extremely poor growth indicating that the regulation of the phosphorylation status is critical for optimal cell cycle progression (Cheeseman et al., 2002).

Tousled-like kinase-1 (Tlk-1) is another mitotic target of AURKB and is phosphorylated in prophase to prometaphase in C. elegans, which acts to further activate AURKB in an INCENP-dependent manner (Han et al., 2005). The specific role of Tlk-1 in mitosis is not fully understood, but loss of expression of Tlk-1 results in chromosome segregation defects reminiscent of loss of AURKB (Han et al., 2005).

Vimentin, an intermediate filament protein, is also a target of AURKB, phosphorylation of which is required for cleavage furrow formation (Goto et al., 2003; Yasui et al., 2004). Phosphorylation of Vimentin inhibits its formation into filaments and is required for completion of cytokinesis, expression of Vimentin with phospho-sites mutated to alanines results in a long bridge-like structure between daughter cells that fail to separate in HeLa cells (Goto et al., 2003).

There are other proteins in mitotic cells that have been shown to be phosphorylated by AURKB; Myosin II regulatory light chain (Murata-Hori et al., 2000), and MgcTac1GAP (Minoshima et al., 2003) but their relation to chromosome segregation is not clear, so I have decided not to discuss them further in this thesis.

Aurora Kinase C

AURKC is also a catalytic component of the CPC, but is expressed primarily in meiotic cells such as sperm and oocytes as well as preimplantation embryos (Avo Santos et al., 2011; Fellmeth et al., 2015; Tseng et al., 1998). Furthermore it is found overexpressed in some tumors and cancer cell lines indicating its importance in cell cycle regulation though the exact mechanism for of its oncogenic properties are still being investigated(Bernard et al., 1998; Dutertre et al., 2005; Hu et al., 2000; Kimura et al., 1999; Sasai et al., 2004; Tseng et al., 1998). Overexpression of AURKC in mitotic cells acts to disrupt the AURKB-CPC, which induces polyploidy, and is possibly the mechanism through which AURKC functions in cancer formation (Chen et al., 2005). AURKC is also expressed in some somatic tissues such as the placenta and the cells of the pineal gland of rats where it is implicated in circadian regulation (Lin et al., 2006; Price et al., 2009). AURKC appears to be required only for gamete formation in mammals because mouse knockouts and human loss of function mutations result in no gross morphological defects indicating that AURKC is not required for somatic cell mitosis (Dieterich et al., 2007; Kimmins et al., 2007; Schindler et al., 2012). Both AURKB and AURKC are found complexed in the CPC indicating their ability to perform similar functions, however recent work has shown that though they can both compensate for the loss of the other and do have some overlapping functions, they have their own distinct roles to play in meiosis (Balboula and Schindler, 2014; Sasai et al., 2004; Slattery et al., 2009; Yan et al.,

2005b). AURKC plays the primary CPC kinase role in preimplantation embryo divisions in as well because the embryonic genome is not activated until around the 8- cell stage(Braude et al., 1988). Expression of AURKC remains high in human preimplantation embryos until the 8-cell stage where it begins to decline to barely detectable levels in the blastocyst as transcription of *AURKB* begins and expression begins to climb again (Avo Santos et al., 2011).

AURKC shares 75% homology with AURKB and phylogenetic analysis indicates that AURKB and C evolved separately from a gene duplication event that occurred in a cold-blooded vertebrate ancestor (Brown et al., 2004). The N-terminus of AURKC is lacking the KEN-box destruction motifs found in both AURKA and AURKB that target them for APC/C degradation (Fig. 3). Protein stability assays performed in mouse oocytes show that AURKC is significantly more stable that AURKB in meiosis (Schindler et al., 2012). The increased stability of AURKC is potentially a major factor in retaining AURKC function through both meiotic divisions where *AURKB* is largely depleted after Met I; however the mechanism of the stability is not clear.

Another factor contributing to increased AURKC representation in meiosis is the presence of cytoplasmic polyadenylation elements (CPEs) and Dazl-binding sequences in the 3’ UTR that act to recruit ribosomes to the RNA transcripts for increased translation (Schindler et al., 2012). The CPE’s in the UTR of *AURKC* are at least ten times more effective than those in *AURKB*.

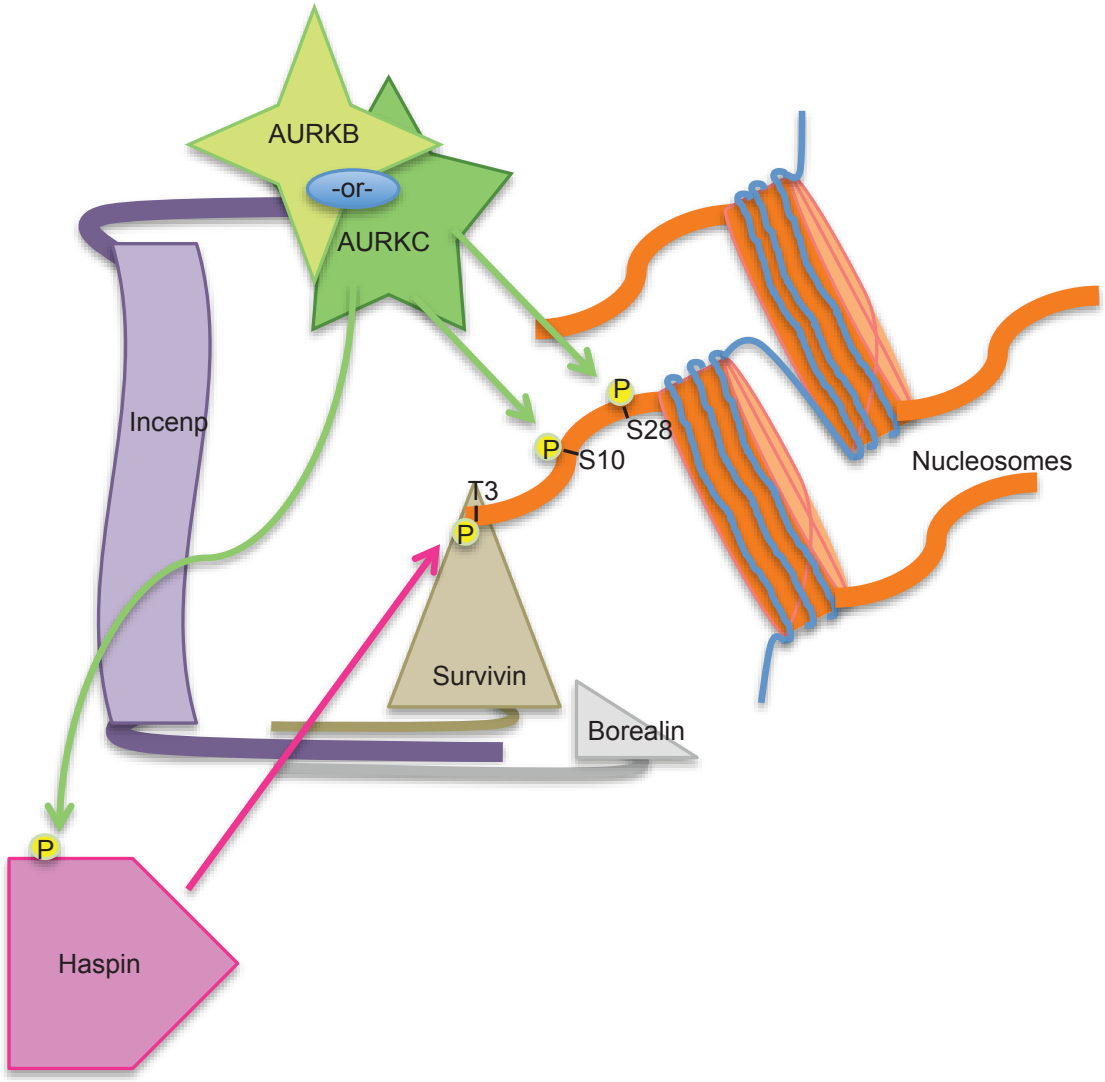
CHROMOSOMAL PASSENGER COMPLEX

The chromosomal passenger complex (CPC) is a collection of proteins that is primarily found on the centromeres of the chromosomes and is involved in the regulation of many cell division processes including chromosome segregation, metaphase alignment, bipolar spindle formation, correcting spindle to chromosome attachment errors, and cytokinesis (Fig. 4). It is a critical upstream regulator of many other chromosome segregation proteins such as the centromere proteins (Sgo1, Sgo2, and MCAK), regulators of microtubule-kinetochore interactions (HEC1, CENP-E, or Plk1), and proteins involved in the spindle assembly checkpoint (Mad2, BubR1, Mps1). This section will review the components, functions, and regulation of the CPC in mitotic cells.

CPC Components and Localization

The CPC is composed of 4 proteins (Fig. 4). INCENP was the first discovered (in a human lymphoma derived cell line) and is the scaffolding subunit that binds the rest of the complex together (Adams et al., 2000; Bolton et al., 2002; Chen et al., 2003; Cooke et al., 1987; Gassmann et al., 2004; Kim et al., 1999; Wheatley et al., 2001). Survivin and Borealin are the targeting subunits and bind to the phosphorylated tails of the histones to bring the complex to the

Fig. 4- The Chromosomal Passenger Complex (CPC). The CPC is made up of four protein subunits and is recruited to the DNA by phosphorylated sites on the histone tales, which are regulated by cellular signaling cascades such as Haspin. The AURKB-CPC is the only version found in mitotic cells, while AURKB and AURKC-CPC are both found in meiosis.



chromatin (Gassmann et al., 2004; Honda et al., 2003; Klein et al., 2006; Sampath et al., 2004). The catalytic subunit is AURKB in mitosis (Kelly and Funabiki, 2009; Ruchaud et al., 2007; Terada et al., 1998; van der Waal et al., 2012) and either AURKB or AURKC in meiosis (Chen et al., 2005; Schindler et al., 2012; Shuda et al., 2009; Swain et al., 2008; Tseng et al., 1998; Yang et al., 2010).

The non-enzymatic subunits (Survivin, Borealin, and INCENP) control the function of the enzyme as well as its stability and localization and activation of the complex involves a positive feedback loop of phosphorylations and conformational changes to both the substrates and the kinase. The activity and presence of all components is critical for the function of the complex. Knockdown of any of the subunits by RNAi delocalizes all the others and disrupts mitotic progression (Adams et al., 2001; Carvalho et al., 2003; Gassmann et al., 2004; Honda et al., 2003; Lens et al., 2003; Vader et al., 2006). Survivin and Borealin bind to the N-terminus of INCENP (residues 1-58 of human INCENP, Fig. 4) (Ainsztein et al., 1998; Gassmann et al., 2004; Honda et al., 2003; Klein et al., 2006; Sessa et al., 2005) while AURKB/C binds to the C-terminal IN-box of INCENP, a conserved region of approximately 60-80 residues (Adams et al., 2000; Chen et al., 2005).

Survivin is a conserved member of the inhibitor of apoptosis protein (IAP) family and bears a single baculovirus IAP repeat (BIR) domain that is responsible for dimerization of Survivin(Chantalat et al., 2000; Gassmann et al., 2004; Honda et al., 2003; Klein et al., 2006; Muchmore et al., 2000; Sampath et al., 2004;

Verdecia et al., 2000). Despite being a member of the IAP family, there is no evidence thus far to implicate Survivin in regulation of apoptosis in oocytes though this function has not been ruled out. Survivin can bind to all three of the other CPC members (INCENP, AURKC, and Borealin) and is a substrate for AURKB (Carvalho et al., 2003; Wheatley et al., 2004). In yeast, Survivin targets the CPC to the centromeres via its interaction with Shugosin (Sgo1), which recognizes the phosphorylation of histone H2A (at Threonine 120 through Bub1 kinase, H2AT120) (Kawashima et al., 2010) and then directs binding of Survivin to proteins at the centromere (Cho and Harrison, 2012; Yoon and Carbon, 1999). In other organisms such as mice, Survivin binds to the histone H3 phosphorylated at Threonine 3 (H3T3) by Haspin kinase to target the CPC to the centromeres (Kelly et al., 2010; Niedzialkowska et al., 2012; Wang et al., 2010; Yamagishi et al., 2010).

Borealin is an *in vitro* target for AURKB and is required for localization of Survivin and the rest of the complex indicating its role in stabilizing the interaction between Survivin and INCENP (Sampath et al., 2004; Vader et al., 2006). It is targeted to centromeres via its interaction with HP1alpha (Liu et al., 2014) and it is possible that this, distinct from Survivin targeting, could be a secondary means of regulating CPC localization.

In mitotic cells, the CPC localization is restricted to the nucleus during G2 and appears along the length of the condensing chromosomes during prophase becoming progressively condensed at the inner centromeres as prometaphase progresses to metaphase (Adams et al., 2001; Crosio et al., 2002; Earnshaw and

Cooke, 1991; Monier et al., 2007; Zeitlin et al., 2001). It relocates to the central spindle during anaphase and then to the midbody during telophase and cytokinesis(Cooke et al., 1987).INCENP is localized at the site of the cleavage furrow, and associates with the contracting furrow(Earnshaw and Cooke, 1991; Eckley et al., 1997). This localization is associated with its role in cytokinesis, and though the CPC is not required for the initiation of division, it is essential for full completion of cytokinesis though the mechanism of how this is accomplished is not entirely clear (Adams et al., 2001; Carvalho et al., 2003; Gassmann et al., 2004; Honda et al., 2003; Schumacher et al., 1998; Tatsuka et al., 1998; Terada et al., 1998). INCENP mutants lacking the C-terminal half of the protein are unable to transfer to the central spindle during metaphase, which disrupts prometaphase chromosome congression, anaphase segregation, and cytokinesis(Mackay et al., 1998). These mutants indicate the requirement for AURKB in this dynamic localization because C-terminal mutants of INCENP cannot associate with AURKB.

In meiotic cells, there is a distinct localization during MI. The CPC is found along the chromosome arms in a characteristic “cross” which is referred to as the inter-chromatid axis (ICA) as well as at the centromeres. The CPC still moves during Ana I and Telo I before returning to the centromeres MII.

Spindle formation

In mitosis, a single pair of centrosomes exist that nucleate microtubules to

form a bipolar spindle. Extra centrosomes create multipolar spindles, which result in errors in chromosome segregation, and are a large contributor to aneuploidy in cancer cells (Ganem et al., 2009; Holland and Cleveland, 2009; Silkworth et al., 2009). Centrosomes are duplicated in S phase concurrent with DNA replication, and then begin to separate in prophase to opposite sides of the nucleus due to the pushing forces of motor proteins such as Eg5 (Blangy et al., 1995; Sawin and Mitchison, 1995; Whitehead et al., 1996). In most cells, spindles are nucleated with their minus ends tethered in the centrosomes located at each pole which are made up of two cylinders called centrioles that are deep within a cloud of pericentriolar material (PCM) (Dumont and Desai, 2012). The plus ends stretch outwards from the centrosomes and trying to make contact with the kinetochores of the chromosomes. This “Search and Capture” type model proposes the microtubules are extremely dynamic and go through multiple cycles of growth and shrinkage, randomly probing the cytoplasm in multiple directions until they hit a kinetochore that they can form a stable attachment with (Kirschner and Mitchison, 1986). The physics of this method orients the chromosomes in the middle of the two poles, which inherently helps in formation of a bipolar spindle.

The self-assembly model proposes a seemingly more disorganized polymerization of microtubules in the absence of centrosomes (as is seen in meiosis in oocytes) and the formation of the bipolar spindle relies more heavily on the functions of motor proteins to bundle and properly orient anti-parallel microtubules (Hyman and Karsenti, 1996). Though this model was proposed to explain how bipolar spindles form in acentrosomal oocytes, recent studies show

that it may play a larger role in mitotic spindle assembly than previously though. Drosophila mutants that lack centrosomes in spermatocytes are still able to form functional spindles, and when centrosomes are laser ablated in primate cells, they also still form bipolar spindles (Bonaccorsi et al., 1998; Hinchcliffe et al., 2001; Khodjakov et al., 2000; Khodjakov and Rieder, 2001; Megraw et al., 2001; Rebollo et al., 2004). These cells are not perfectly normal however, and a majority of them fail to complete cytokinesis due to improperly oriented spindles indicating the role for centrosomes is not in formation of a bipolar spindle, but in orienting that spindle via astral microtubules to the appropriate position at the cell cortex.

Centrosomes are not required for spindle formation, and surprisingly neither are kinetochores or even chromosomes. *Xenopus* egg extracts incubated with DNA coated beads lack kinetochore structures and microtubules nucleated from random sites in the cytoplasm slowly coming together and forming a bipolar spindly (Heald et al., 1996). Furthermore, *fusolo* mutants in Drosophila fail to segregate chromosomes efficiently resulting in frequent secondary spermatocytes lacking any chromosomes. These cells are also still able to form a bipolar spindle and even complete cytokinesis (Bucciarelli et al., 2003). Another interesting point is that AURKB is still found at the spindle midzone in chromosome-free cells indicating that the chromosome localization of AURKB is not a pre-requisite for its localization at the central spindle.

The CPC is critical for the formation of a bipolar spindle as shown by the loss of CPC resulting in spindle assembly defects in Drosophila S2 cells,

Xenopus extracts, or HeLa cells (Adams et al., 2001; Sampath et al., 2004; Tulu et al., 2006). The exact mechanism for the CPC role in spindle assembly is still under investigation, though it is likely through the recruitment of proteins that interact with microtubules. For example, MCAK (described above) is a substrate of AURKB and when the phospho site is mutated to an alanine, *Xenopus* extracts are able to form only monopolar spindles (Ohi et al., 2004). However, when the extracts are completely depleted of MCAK, the spindle is hyperstabilized (Sampath et al., 2004). Similar results were seen in HeLa cell culture, which indicates that the CPC requirement for spindle formation is through inhibition of MCAK microtubule destabilization (Tulu et al., 2006).

Unlike other dividing cells, oocytes spindles develop without the presence of canonical centrosomes, instead microtubules nucleate from multiple microtubule organizing centers (MTOCs) (Albertson and Thomson, 1993; Manandhar et al., 2005; Schuh and Ellenberg, 2007; Szollosi et al., 1972; Theurkauf et al., 1992). In the absence of centrosomes, MTOCs fragment into many smaller MTOC's in the hours surrounding germinal vesicle breakdown (GVBD) and eventually coalesce on opposite sides of the chromatin forming two poles (Clift and Schuh, 2015).

Metaphase Alignment and Error Correction

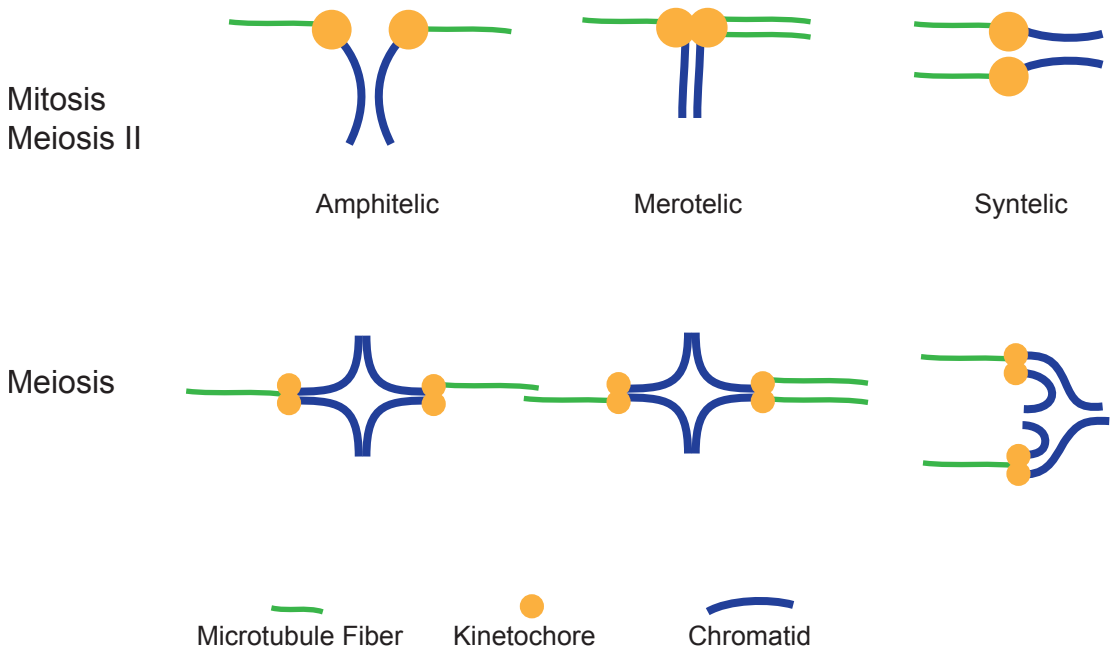
The CPC is a critical regulator of kinetochore microtubule (K-MT) attachments in M-phase, which is linked to the proper biorientation of

chromosomes and alignment at metaphase (Balboula and Schindler, 2014; Brunet et al., 1999; Lampson et al., 2004). In this section I will describe the relationship between error correction and alignment and how the CPC regulates this process through its substrates and binding partners.

Accurate chromosome segregation requires chromosomes to be bioriented at metaphase (one sister connected via spindle fibers to each pole in mitosis or pairs of sisters co-oriented to the same pole in meisois, Fig. 5). Errors in this process do occur and the cell has a checkpoint mechanism (Spindle Assembly Checkpoint described below) that prevents anaphase progression until the errors are corrected to prevent chromosome segregation defects. When the CPC is lost after knockdown of any of the components, there are alignment and segregation defects in mice and *C. elegans* (Kaitna et al., 2002; Rogers et al., 2002; Schumacher et al., 1998; Sharif et al., 2010; Shuda et al., 2009; Speliotes et al., 2000). Inhibition of AURKB using inhibitors or RNAi leads to an increase in misalignment of chromosomes at metaphase in human cell culture (Ditchfield et al., 2003; Hauf et al., 2003). Similar misalignment phenotypes are observed when CPC components are knocked down in meiotic cells (Balboula and Schindler, 2014; Nguyen et al., 2014; Radford et al., 2012; Sharif et al., 2010). This misalignment is accompanied by errors in K-MT attachments (Lampson et al., 2004).

It is easy to image how a chromosome with both pairs of sister kinetochores attached to a single pole (syntelic, Fig. 5) would be pulled out of alignment towards that one pole, where bivalents that are bi-oriented (amphitelic)

Fig. 5 – Erroneous K-MT attachments form in M phase and are corrected by destabilization of microtubules. Amphitelic attachments result from correct attachments of a single kinetochore (or pair of sister kinetochores in MI) to a single pole and the opposite kinetochore to the opposite spindle pole. These attachments result in bioriented chromosomes. Merotelic attachments result from one kinetochore (or pair of sister kinetochores in MI) with attachments to both spindle poles simultaneously and this results in lagging chromosomes in anaphase. Syntelic attachments result when a chromosome is attached to only a single pole and results incorrect chromosome segregation and aneuploidy.



would find themselves pulled equally and line up at the metaphase plate. In budding yeast, Ipl1 (AURKB ortholog) resolves syntelic attachments through a tension sensing mechanism and destabilizes the microtubules there (Tanaka et al., 2002) this mechanism is through phosphorylation of Ndc10 (Ndc80 ortholog, part of the KMN complex at the kinetochore) (Biggins et al., 1999; Cheeseman et al., 2002). Phosphorylation of Hec1 results in a similar microtubule release in human cell culture (DeLuca et al., 2006). Error correction via HEC1 phosphorylation occurs primarily at syntelic attachments indicating that this mechanism is regulated by the lack of tension at the centromere on these chromosomes.

Merotelic attachments (where one kinetochore is attached to both poles simultaneously, Fig. 5) tend to have proper tension across the centromere, which fails to activate the SAC, which can lead to improper segregation and lagging chromosomes at anaphase (Cimini et al., 2002; Cimini et al., 2001; Cimini et al., 2003; Khodjakov et al., 1997; Wise and Brinkley, 1997). AURKB is responsible for destabilizing the extra microtubule attachment and correcting these errors by phosphorylating MCAK (Cimini et al., 2006; Knowlton et al., 2006).

The exact mechanism for this tension sensing is largely unknown; however it is hypothesized to correlate with the proximity of the catalytic subunit of the CPC with substrates on the microtubules (Foley and Kapoor, 2013; Lampson and Cheeseman, 2011; Mitchison and Kirschner, 1984; Nezi and Musacchio, 2009; Nicklas and Ward, 1994; Tanaka, 2010; Yoshida et al., 2015). In MI, the kinetochores of sister chromatids must be treated as one unit so that

the sisters can co-orient while the homologs bi-orient and proper segregation occurs at Met I (Holt and Jones, 2009; Watanabe, 2012). Despite this difference, the substrate proximity model for tension sensing and error correction still holds true except tension is now across the chiasmata holding homologs together, rather than across kinetochores (Jones and Lane, 2013; Lane et al., 2010; Sakuno et al., 2011; Watanabe, 2012).

In mitosis, it is known that the PP2A-B56 phosphatase complex is recruited to kinetochores and counteracts AURKB activity (Foley et al., 2011; Suijkerbuijk et al., 2012). This localization was recently confirmed in mouse oocytes as well (Yoshida et al., 2015). This localization is dependent on the phosphorylation of BUBR1 in both mitosis and meiosis (Elowe et al., 2010; Huang et al., 2008; Kruse et al., 2013; Suijkerbuijk et al., 2012; Yoshida et al., 2015). Creation of phosphomimetic mutants of BUBR1 lead to an increase in PP2A-B56 activity and a decrease in pKnl1 (another member of the KNM complex at kinetochores similar to HEC1) and this resulted in an increase in the number of stable K-MT attachments (Yoshida et al., 2015).

In mouse oocytes, AURKC seems to be primarily responsible for the error correction function of the CPC rather than AURKB which indicates that the microtubule destabilizing substrates of AURKB are also substrates of AURKC (Balboula and Schindler, 2014). This microtubule destabilization function in meiosis is performed by the AURKC-CPC localized along the inter-chromatid axis (ICA) because loss of Haspin results in loss of ICA-CPC, but not kinetochore-CPC, and is accompanied with an increase in erroneous

attachments(Nguyen et al., 2014). This ICA specific function is further supported by studies showing that the CPC localized at kinetochores at Met I is not active (Rattani et al., 2013).

Spindle Assembly Checkpoint (SAC)

The spindle assembly checkpoint (SAC) is a regulatory mechanism that prevents anaphase onset until chromosomes are bi-oriented on the spindle in order to prevent segregation errors and aneuploidy. It involves kinases such as BUB1, BUBR1, and MPS1 as well as protein interactions and complexes involving BUB3, MAD1, and MAD2. These checkpoint proteins are recruited to unattached kinetochores during prometaphase and generate the hold-anaphase signal immediately (Cleveland et al., 2003). This localization leads to the conversion of cytoplasmic MAD2 into a form that binds to Cdc20 (this complex plus BUBR1 is referred to as the Mitotic Checkpoint Complex (MCC)), which inhibits the activation of the APC/C by Cdc20 (De Antoni et al., 2005; Howell et al., 2000; Xia et al., 2004; Yang et al., 2008). The MCC not only sequesters the APC/C activator, Cdc20, but it also binds to APC/C and inhibits its activity directly (Fang et al., 1998; Morrow et al., 2005; Sudakin et al., 2001; Tang et al., 2001). Correct, stabile microtubule attachments at kinetochores displace the MAD1/MAD2 complex, which stops the signal coming from that kinetochore (Buffin et al., 2005; Howell et al., 2001; Sivaram et al., 2009). In mitosis the checkpoint is sensitive to the presence of even a single unattached kinetochore

and will maintain the arrest until the attachment is made (Rieder et al., 1995). As soon as the signal is silenced at all kinetochores, the APC/C will be activated and anaphase will progress (Clute and Pines, 1999; Hagting et al., 2002).

Incorrect attachments result in CPC-mediated depolymerization of the microtubules, which releases spindle fibers allowing kinetochores to form new correct attachments (Pinsky and Biggins, 2005). The time between depolymerization of the incorrect attachment and formation of a new attachment allows for recruitment of SAC proteins to the kinetochore and maintain the signal. Though AURKB in mitosis and AURKC in meiosis contribute to the activation of the SAC through formation of unattached kinetochores, AURKB also plays a role in maintaining the checkpoint through Bub1 kinase by promoting the association of BubR1 with the APC/C (Morrow et al., 2005). BubR1 is a SAC component that helps maintain the “wait anaphase” signal when it is localized to unattached kinetochores; this localization is mediated by Survivin and INCENP (Carvalho et al., 2003; Lens et al., 2003; Vader et al., 2006). The recent evidence from our lab indicating that AURKB is the main regulator of SAC activation in oocyte meiosis indicates that AURKC does not phosphorylate BUB1 in the presence of AURKB (Balboula and Schindler, 2014). The Ndc80 complex is also required for SAC activation though the exact mechanism is unclear it is likely mediated through recruitment of MPS1, Mad1, and Mad2 to unattached kinetochores(Martin-Lluesma et al., 2002; McCleland et al., 2003).

Sister chromatid cohesion

The critical step of meiosis that differentiates it from mitosis is MI where the homologous chromosomes segregate but keep their sister chromatids attached. This is known as the “reductional phase” where the cell transitions from diploid (having two copies of each chromosome) to haploid (having only one of each chromosome). The regulatory processes involved in chromosome segregation during mitosis (where sisters separate during anaphase) are different in MI where the sisters must remain connected until Ana II in order to achieve proper ploidy. Only the cohesion between the homologs can be removed during Ana I so a new layer of regulation must exist in MI to retain cohesion at the centromeres to hold the sisters together.

Cohesin is a multi-protein complex that holds chromatids together from the moment they are formed during S-phase until they are separated in anaphase. Cohesins are made up of four subunits: 2 long structures called SMC proteins (structural maintenance of chromosomes), and 2 non-SMC subunits a kleisin (ring clamp), and a stromalin (required for ring structure) (Haering et al., 2002). RAD21 is the kleisin subunit in vertebrates responsible for holding sisters together in mitosis and is cleaved by separase during anaphase to allow sister chromatids to separate to opposite poles and end up in two different daughter cells(Schleiffer et al., 2003).

In mitosis, cohesins are removed in a step-wise process where the majority of cohesin is released from chromosome arms during prophase due to

phosphorylation of SA2 (the stromalin subunit) by Plk1 (polo-like kinase 1) (Hauf et al., 2005). Loss of AURKB in *X. laevis* extracts and AURKB inhibition in cultured human cell lines reduces the amount of arm cohesin removed during prophase (Losada et al., 2002; Sumara et al., 2002). At this point, the remainder of the cohesin is centromeric and is protected by Sgo1 and its interactions with PP2A (protein phosphatase 2A), which potentially acts to dephosphorylate targets of Plk1 (Kitajima et al., 2006; Riedel et al., 2006; Tang et al., 2006b). Further evidence implicating the CPC in maintenance of sister chromatid cohesion is the fact that the release of cohesion is prevented when AURKB is inhibited in human cell culture (Losada et al., 2002; Sumara et al., 2002).

The remainder of the cohesins are released during anaphase after activation of the APC/C. Activation of the APC/C results in destruction of securin which in turn activates Separase, the protease responsible for cohesin release from chromosomes. This release allows the separation of the sister chromatids into separate daughter cells.

In meiosis, the kleisin role is fulfilled by REC8 (Bannister et al., 2004; Xu et al., 2005), which is protected at centromeres by Shugosin (Sgo2), a known substrate of AURKB, during MI (Resnick et al., 2006). During MI cohesins are found along the chromosomes arms, and restricted only to centromeres in MII. In meiosis, INCENP is necessary for centromeric localization of Sgo2 and may act to bring Sgo2 and AURKB into proximity with each other so that AURKB can phosphorylate and activate Sgo2 (Resnick et al., 2006). In the absence of AURKB, Sgo2 fails to localize to centromeres, however this effect could

potentially be mediated through Bub1 as well, which is also a target of AURKB and required for Sgo2 localization(Dai et al., 2006; Kitajima et al., 2005; Resnick et al., 2006; Riedel et al., 2006).

Condensins are multi-protein complexes very similar to cohesins that also play a role in cohesion. Haspin regulates the ICA localization of the CPC in meiosis and when Haspin is inhibited, cells exhibit a premature loss of sister chromatid cohesion due to a loss of condensin not REC8 (Nguyen et al., 2014). There are also studies in mitotic cells showing that AURKB regulates the localization of condensin I (Bembenek et al., 2013; Collette et al., 2011; Lipp et al., 2007; Nakazawa et al., 2011). Condensin complexes play a role in the condensation of chromatin during prophase and AURKC-CPC has been showed critical for this function (Nguyen et al., 2014).

Cytokinesis

The CPC translocates to the spindle midzone after the metaphase-anaphase transition in mitosis and in meiosis. This dynamic localization is associated with a role in cytokinesis. In mitotic rat kidney cells or C. elegans embryos knockdown of AURKB by expression of a dominant negative mutant or by RNAi respectively, resulted in failure to complete cytokinesis (Schumacher et al., 1998; Terada et al., 1998). INCENP accumulates at the midzone before the formation of the cleavage furrow, even before the recruitment of myosin II for contractile ring formation (Earnshaw and Cooke, 1991; Eckley et al., 1997). It's possible that the CPC plays a direct role in recruitment of the proteins involved in

ring formation. An indirect role is known including phosphorylation of centralspindlin by AURKB that activates Rho GAP, which in turn aids in assembly of the contractile ring (Nishimura and Yonemura, 2006; Petronczki et al., 2007; Piekny et al., 2005; Su et al., 2011; Yuce et al., 2005). It has also been proposed in yeast that AURKB plays a role in the negative regulation of cytokinesis by regulating the localization of anillin-like proteins, which prevents cytokinesis until all chromatids have cleared the midzone (Norden et al., 2006).

The CPC also appears to play another checkpoint role in cytokinesis by preventing abscission until all chromosomes have cleared the cleavage furrow (Norden et al., 2006; Steigemann et al., 2009). This process involves further condensation of chromosome arms as shown in yeast and rat kidney cells through an upregulation of H3S10 and H3S28 phosphorylation (Mora-Bermudez et al., 2007; Neurohr et al., 2011).

The CPC cytokinesis roles thus far appear similar in meiosis where it is also required for cytokinesis completion. In oocytes lacking AURKB and AURKC, the cells are unable to fully extrude a polar body after obvious attempts and retractions (Balboula and Schindler, 2014; Tang et al., 2006a).

CPC Regulation

As previously mentioned, Haspin phosphorylation is required for the localization of the CPC in mitosis (Wang et al., 2011). Haspin is a serine/threonine kinase that is conserved from yeast to humans (Higgins, 2003). Currently, only three substrates of Haspin have been identified (H3T3, H2AS137,

and T57 of CENP-T) (Maiolica et al., 2014). When Haspin is inhibited in mitotic cells, H3T3 phosphorylation is significantly reduced leading to misalignment of metaphase chromosomes (Dai and Higgins, 2005; Dai et al., 2005), loss of chromosome cohesion (Dai et al., 2009), failure to form a bipolar spindle (Dai et al., 2009), delay in anaphase onset, and lagging chromosomes (Dai et al., 2006; De Antoni et al., 2012; Huertas et al., 2012; Markaki et al., 2009; Wang et al., 2012). All of these phenotypes are also associated with a loss of the CPC indicating the major role of Haspin is regulation of CPC localization. H3pT3 is restricted to kinetochores in mitosis where it recruits the CPC via Survivin (Dai et al., 2005; Wang et al., 2010; Yamagishi et al., 2010). This phosphorylation is reversed during anaphase, which is a critical step in relocating the CPC complex from centromeres to the spindle midzone (Bembenek et al., 2013). In meiosis, H3pT3 is along the arms of the bivalents (ICA), but when Haspin is inhibited, the CPC ICA localization is lost, leaving behind only the kinetochore population indicating a second level of regulation for CPC localization in meiosis that is not present in mitosis (Nguyen et al., 2014).

There is evidence in studies of cancer cells that treatment with histone deacetylase (HDAC) inhibitors results in mitotic arrest and phenotypes similar to those seen in AURKB inhibited cells (Li et al., 2006). Another study in PC3 cells showed that AURKB is in fact acetylated during mitosis and that this modification negatively regulates AURKB function (Fadri-Moskwik et al., 2012). Histone acetylation appears to be important for localization of the CPC in oocytes as well. When a histone deacetylase complex component (RBBP7) is knocked down,

there is a loss of all CPC localization (Balboula et al., 2014). The phenotype (chromosome misalignment, improper K-MT attachments, and aneuploidy) can be rescued by overexpression of AURKC indicating that histone acetylation is required for maintaining CPC localization, but probably not for loading it. It is more likely that Haspin is required for loading the CPC since AURKC overexpression does not rescue the Haspin inhibition phenotype (Balboula et al., 2014; Nguyen et al., 2014).

There are many factors that regulate the localization and function of the CPC during mitosis and meiosis without being part of the complex itself. Mitotic kinesin-like protein 2 (Mklp2) acts in mammals to translocate the CPC from its centromeric localization during early M phase, to the central spindle at the onset of anaphase (Gruneberg et al., 2004). Mklp2 also recruits Cdc14A to the central spindle and dephosphorylation of INCENP by Cdc14A may also contribute to relocalization of the CPC to central spindle (Gruneberg et al., 2004; Yuan et al., 2007).

TD-60 (telophase disk 60 kDa) is not part of the core complex of the CPC either, but does localize with the CPC and loss of CPC localization disrupts localizing of TD-60 as well. Evidence shows that TD-60 works with microtubules to activate the kinase activity of AURKB-INCENP (Ruchaud et al., 2007) though the mechanism of this is unclear. The CPC can localize without TD-60 but its activity is greatly reduced and the activation of the complex is dependent on H3T3phos by Haspin (Dai et al., 2005).

KINASE SPECIFIC CPC FUNCTIONS IN MEIOSIS

Both AURKB and AURKC are able to compensate for the loss of the other, which supports the idea that the two kinases have redundant function (Chen et al., 2005; Fernandez-Miranda et al., 2011; Li et al., 2004; Sasai et al., 2004; Schindler et al., 2012). However, this rescue is not complete in meiotic cells indicating they perform non-overlapping roles as well (Balboula and Schindler, 2014; Kimmins et al., 2007; Schindler et al., 2012). The understanding of the kinase specific roles of the CPC in meiosis is still under investigating, but some critical differences have come to light.

AURKB-CPC

In meiotic cells, AURKB-CPC seems primarily responsible for the spindle assembly checkpoint, bipolar spindle formation, and chromosome segregation. In meiosis, AURKB appears to be the main CPC kinase responsible for activating the SAC due to the fact that when AURKC is inhibited in mouse oocytes, the SAC is activated normally, but when both or just AURKB are lost, the cells fail to arrest with K-MT errors ((Balboula and Schindler, 2014), unpublished data).

AURKB-CPC also appears to be the primary CPC kinase responsible for BUB1 localization since there is no change in BUB1 localization in AURKC knockout oocytes, but it is lost when AURKB is also inhibited (Balboula and Schindler, 2014). This localization further supports the role of AURKB-CPC in

being the primary mediator of SAC activity in meiosis.

AURKC-CPC

In human gametes, AURKC is found on the chromosome arms along the inter-chromatid axis (ICA) and at the centromeres at Met I, and at the centromeres at Met II (Avo Santos et al., 2011). When AURKC is lost due to knockout or specific inhibition, AURKB-CPC is found in its place at kinetochores (AURKB is normally found on the spindle in WT oocytes) but there is still an increase in misaligned chromosomes and aneuploidy, reduced pINCENP, and a failure to correct erroneous K-MT attachments indicating these functions are AURKC-specific in meiosis and AURKB is not sufficient for a full rescue (Balboula and Schindler, 2014).

Aurkc$^{-/-}$ oocytes also exhibit an increase in Met I arrest indicating that the SAC is active in these cells supporting the idea that this is mediated by AURKB-CPC (Schindler et al., 2012). H3pS10 is reduced by half but not lost which points to a role in both kinases in phosphorylating this target (Balboula and Schindler, 2014). Mouse oocytes only have cytokinesis failure if both kinases are inhibited indicating that they are both sufficient to perform this function (Balboula and Schindler, 2014).

Aurkc$^{-/-}$ Mice

Kimmins et al. (2007) generated a strain of *Aurkc*$^{-/-}$ mice by neomycin cassette replacement of exons 2-4 in order to study the requirement for AURKC in mouse spermatogenesis. The mice exhibit no morphological or physiological defects other than gamete errors. The KO males produced an average of 3 pups less per litter than their WT littermates and approximately 40% of the males were completely sterile (Kimmins et al., 2007). The sperm presented with abnormal head shapes and chromatin condensation defects. Female *Aurkc*$^{-/-}$though subfertile (2 pups less per litter), there was no significant difference in the number of fully grown oocytes collected from superovulated WT or KO mice (Schindler et al., 2012). The knockout oocytes have a significantly increased incidence of Met I arrest, misaligned chromosomes, and they are slower to reach Ana I than in WT oocytes (Schindler et al., 2012). This phenotype is likely due to an activation of the SAC by the AURKB-CPC in response to the misaligned chromosomes. KO oocytes that are able to progress past the Met I arrest are euploid, and this indicates that these cells had presumably less errors and misalignment of chromosomes at Met I and the SAC was satisfied leading to accurate chromosome segregation while those that arrest would have been aneuploid had they divided.

AURKC DISCOVERY AND VARIANTS

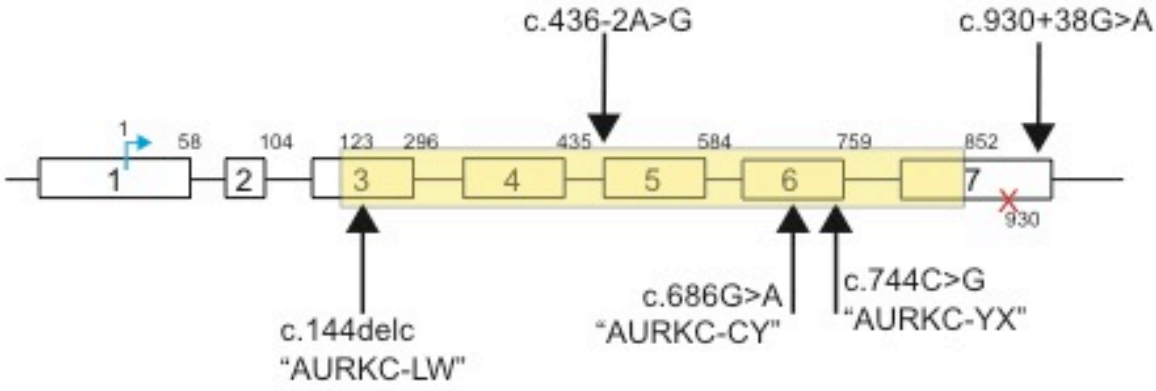
AURKC was originally identified in a kinase expression profile in sperm and egg of mouse and separately in a screen of human placental and testis cDNA libraries (Bernard et al., 1998; Tseng et al., 1998) and is located on chromosome 19 at position q13.43 in humans (Kimura et al., 1999). AURKC is the most recently discovered of the Auroras and localizes and functions similarly to AURKB, but is found mainly in testis (Tseng et al., 1998). There are three splice variants of *AURKC* differing from alternative splicing at the end of exon 1(Bernard et al., 1998; Tseng et al., 1998; Yan et al., 2005c). *AURKC_v1* is the longest transcript; it encodes a protein that is 309 amino acids and has the highest level of expression in the testis relative to other tissues (Yan et al., 2005c). *AURKC_v3* is the shortest, encoding a 275 amino acid protein, and was identified next in a screen of human placental cDNA using probes for an Aurora homolog from *Xenopus laevis* (Bernard et al., 1998). Most recently discovered was *AURKC_v2*, which was found in a screen to isolate and study *AURKC_v3* from human testis (Yan et al., 2005c). This screen was unable to identify *AURKC_v3*, which indicates that three splice variants are not all expressed simultaneously in sperm. *AURKC_v2* encodes a 290-residue protein. Using an *in vitro* kinase assay, it was shown that AURKC_v2 has the ability to phosphorylate MBP supporting the hypothesis that the kinase domain is unaffected due to the alternative splicing. *AURKC_v2* transfected into HeLa cells localized with the CPC similarly to AURKC_v1.

The proteins encoded by these splice variants contain successively shorter N-termini while the rest of the protein (where the catalytic domain lies) remains identical. While these variants are known to exist in testis tissue, it is not known whether they are expressed in oocytes or the relevance of these truncated isoforms.

AURKC STERILITY-ASSOCIATED MUTANTS

A collection of studies have identified five *AURKC* SNP's in a sterile population of North African men presenting with an identical sperm phenotype. The men were recruited to the study due to the homogeneity of their phenotype: large headed sperm (macrozoospermia) with multiple flagella (Dieterich et al., 2007). All of the men had inherited at least one copy of AURKC- c.144delC, which I will refer to as AURKC-LW (Fig. 6). This mutation is a single base pair deletion at nucleotide position 144. They found a prevalence of this mutation of 2.7% in the cohort studied which is nearly double the expected prevalence of the current top genetic causes of male infertility Klinefelter's (1.6%) and Y-chromosome microdeletions (0.23%) (Ounis et al., 2014). They propose that these rates are so high because of a slight reproductive advantage of the mutations in the heterozygous condition. It is possible that cells with *AURKC* mutations bypass the SAC and produce larger numbers of gametes increasing reproduction capability. This would also lead to higher levels of resulting aneuploidy and spontaneous abortion. Two of the patients in their cohort reported that their parents (heterozygotes) had suffered several spontaneous

Fig. 6- Five sterility-associated SNPs' have been identified in infertile men with macrozoospermia. Multiple studies have been done to find genetic causes of infertile men presenting with macrozoospermia, and these studies have found five different mutations occurring within the exons (or borders or exon/intron boundary) of *AURKC*. The blue arrow indicates the ATG start codon and the red X is the location for the stop codon.



abortions supporting this hypothesis (Ounis et al., 2014). It was also proposed that because this mutation leads to the expression of a truncated protein (see below), it could have a dominant negative effect by binding to endogenous AURKB and inhibiting its ability to compensate for the loss of AURKC (Dieterich et al., 2007). However, the mechanism for how this leads to increased fitness and allelic frequency is not clear.

The men identified to have homozygous mutations in *AURKC* are completely sterile with no other physiological or morphological defects. Heterozygous parents are also unaffected and report no trouble conceiving (Dieterich et al., 2009). Two sisters of the sterile men were identified who were homozygous for AURKC-LW (Dieterich et al., 2009). Interestingly, these women had children and reported no trouble conceiving. Though we have no other information such as age at conception, rates of miscarriage, or reproductive lifespan, it is interesting to note that they are not completely sterile as is the phenotype in men. As part of this screen, FISH was also used to analyze the DNA content of the sperm using probes for the X and Y-chromosomes, as well as for chromosome 18. A variety of chromosome make up was observed ranging from 1 copy of each to 4 copies of each. Approximately 50% of the sperm samples exhibited a signal for 1 X and 1 Y chromosome indicating that sisters had not yet been separated via the APC/C and that these cells in fact arrested at Met I (Dieterich et al., 2007). Another 15% and 5% showed 2 copies of each X and Y or 4 copies of chromosome 18 respectively indicating that in these cells the APC/C was activated and sisters had separated and meiotic

progression failed due to cytokinesis failure rather than Met I arrest (Dieterich et al., 2007). This observation was supported by the fact 90% of sperm were mono-nucleated and 10% were binucleate in a second study (Dieterich et al., 2009). This data also agreed with the observed phenotype in mouse oocytes where most cells arrest at Met I, but if the errors are minimal enough to satisfy the SAC, the cells will progress to Met II (Schindler et al., 2012). Multiple ICSI attempts were performed after selection of single sperm that appeared to be of normal size but fertilization failed or the embryo did not develop and no transfers were performed (Ben Khelifa et al., 2011; Ounis et al., 2014).

All of the men identified with the LW mutation were of North African descent (specifically Tunisia, Algeria, and Morocco) indicating that this allele was passed down from a single ancestor and the error is estimated to have occurred between 250 and 650 years ago (Ben Khelifa et al., 2012). A second screen identified a second mutation (AURKC-C229Y) caused by a single base pair substitution in exon 6 (Fig. 6 c.686G>A). This mutation has only been identified as one allele with AURKC-LW in one infertile man so far, however most of the screens performed were sequencing only exon 3 (where the LW mutation occurs) and other AURKC-CY patients may have been missed in those screens. This study also performed FACS analysis on PI stained sperm cells to assess the total DNA content and they found that 100% of the sperm were 4N without any variation further supporting the conclusion that there were no normal sperm present in the semen of these men to select for ICSI (Dieterich et al., 2009).

In a third screen, a new mutation was identified occurring in the 5’ splice

site at exon 5 (Fig. 6, c.436-2A>G) resulting in the loss of exon 5 in the mature mRNA and the protein (Ben Khelifa et al., 2011). This mutation was also identified as a compound heterozygote in two brothers, but not in any other patients to date. And a final screen identified two new mutations occurring simultaneously in 9 new patients (of mixed North African and European descent). The men exhibited an allele of *AURKC* that contained a single base pair substitution in the 3' UTR (Fig. 6, c.930+38G>A, exon 7) as well as a single base pair substitution in exon 6 (Fig. 6, c.744C>G) which I will refer to as AURKC-YX. The YX mutation has not been found without the accompanying UTR mutation. The YX allele was found either homozygous, or compound heterozygous with the LW allele (Ben Khelifa et al., 2012). As part of this study they calculated that AURKC-LW accounts for approximately 85% of the AURKC mutant alleles, YX is 13%, and the two other familial alleles (CY and the alternate splice site at exon 5) make up the remaining 1.5% (Ben Khelifa et al., 2012).

These mutations are of particular interest for many reasons. One of which being that every other mutations resulting in male infertility that is due to a meiotic failure in fact results in azoospermia (lack of sperm) as opposed to macrozoospermia found here. In fact, it was proposed that the invariant phenotype observed in these men are due to severe AURKC mutations where more moderate mutations have not been identified, and are hypothesized to result in azoospermia instead (Ben Khelifa et al., 2012). Screens are underway in azoospermic patients to identify such alleles. Characterizing how these alleles differentially affect meiotic progression can potentially shed light on how other

sterility-associated mutations lead to azoospermia or macrozoospermia.

Characterizing these mutations in meiotic cells can also help us to understand AURKC function as well. These mutations occur in different regions and catalytic subdomains of the protein, which can potentially allow us to identify regional specific effects in chromosome segregation and CPC function. However, the study of these mutations, and human AURKC in general, is not an easy task. There are no meiotic cell lines that can be cultured for ease of study in the lab. Meiosis in sperm takes place in the testis making study of this process difficult, and obtaining human oocyte samples for study is also very difficult because the process is invasive and donors are rare. Therefore, a tractable model for studying human AURKC function *in vivo* is necessary for us to understand the relationship between AURKC and ploidy and fertility as a whole.

CONCLUSION

This thesis proposes the use of the previously developed *Aurkc*$^{-/-}$ mice (Kimmins et al., 2007) as a model for study human AURKC in oocytes due to the ability of hAURKC to functionally compensate for the loss of mAURKC in meiosis. I used this model to characterize the function of three splice variants of hAURKC in meiosis and show that though they function similarly, simultaneous expression of all three is required for optimal error correction and chromosome segregation in MI. Finally, after showing the model is effective for studying variations in AURKC function in meiosis, I characterized the difference in activity

of three of the AURKC mutations described above (AURKC-LW, AURKC-CY, and AURKC-YX). I show that AURKC-LW has a partial dominant negative affect over AURKB, AURKC-CY is a hypomorph that retains partial function which is insufficient for meiotic progression indicating a threshold level of AURKC activity is required in meiosis, and AURKC-YX fails to localize but retains cytoplasmic activity which is insufficient to support chromosome alignment at Met I. I concluded that all three mutants result in male infertility due to a failure of AURKC-CPC function resulting in meiotic failure and cells that are polyploid. As a whole, this work provides a tractable model for studying human AURKC function in meiosis to help further the field of reproductive biology.

Chapter II:

Expression and characterization of three Aurora kinase C splice variants found in human oocytes

I. Preface

This chapter was published, as presented here, in Molecular Human Reproduction, May 2015. My contributions to the project and paper included qRT-PCR analysis and sample preparation, variant construct creation and μinjection, imaging, immunoblotting, data analysis, and writing the manuscript.

II. Abstract

Chromosome segregation is an extensively choreographed process yet errors still occur frequently in female meiosis, leading to implantation failure, miscarriage or offspring with developmental disorders. Aurora kinase C (AURKC) is a component of the chromosome passenger complex and is highly expressed in gametes. Studies in mouse oocytes indicate that AURKC is required to regulate chromosome segregation during meiosis I (MI); however, little is known about the functional significance of AURKC in human oocytes. Three splice variants of AURKC exist in testis tissue. To determine which splice variants human oocytes express, we performed quantitative real time PCR using single oocytes and found expression of all 3 variants. To evaluate the functional differences between the variants, we created GFP-tagged constructs of each variant to express in oocytes from *Aurkc*$^{-/-}$ mice. By quantifying metaphase chromosome alignment, cell cycle progression, phosphorylation of INCENP, and microtubule attachments to kinetochores, we found that AURKC_v1 was the

most capable of the variants at supporting metaphase I chromosome segregation. AURKC_v3 localized to chromosomes properly and supported cell cycle progression to metaphase II, but its inability to correct erroneous microtubule attachments to kinetochores meant that chromosome segregation was not as accurate compared to that caused by the other two variants. Finally when we expressed the 3 variants simultaneously, error correction was more robust than when they were expressed on their own. Therefore, oocytes express three variants of AURKC that are not functionally equivalent in supporting meiosis, but fully complement meiosis when expressed simultaneously.

III. **Introduction**

The process of meiosis involves the formation of haploid gametes from diploid precursor cells. Mistakes in chromosome segregation during meiosis can cause aneuploidy in gametes, which is one of the leading genetic causes of infertility (Brandriff et al., 1994; Hassold et al., 2007; Hassold and Hunt, 2001; Pacchierotti et al., 2007). These errors are sexually dimorphic; the error rate in sperm is ~5% whereas in oocytes the rate can be upwards of 20% (Brandriff et al., 1994; Hassold et al., 2007; Hassold and Hunt, 2001; Pacchierotti et al., 2007). This difference in error rate is not fully understood. Meiotic events in males and females are similar; however, they differ in timing. In sperm, the process occurs continuously and starts at puberty, whereas in oocytes, there is a prolonged arrest at prophase I after homologous recombination during fetal

development, then resumption of meiosis at ovulation, and arrest at metaphase of meiosis II (Met II) prior to fertilization. While the result of chromosome segregation is the same between oocytes and sperm (homologs separate in meiosis I (MI) and sister chromatids separate in meiosis II (MII)), the regulation of these steps may differ. One example of a regulator that appears to function differently in male and female meiosis is Aurora kinase C (AURKC). AURKC is essential for male meiosis, but its requirement in female meiosis is not as stringent (Dieterich et al., 2007).

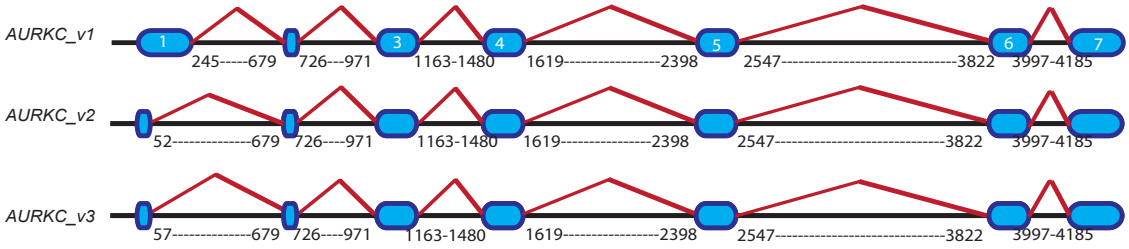
AURKC is a serine/threonine protein kinase that regulates chromosome segregation during meiosis (Glover et al., 1995; Kimmins et al., 2007; Schindler et al., 2012). In mice loss of AURKC leads to subfertility, but the severity of this phenotype is not uniform between males and females. Female *Aurkc*$^{-/-}$ mice produce an average of 2 pups less per litter than their wildtype counterparts, while *Aurkc*$^{-/-}$ males produce 7 pups less per litter and 40% are completely sterile (Kimmins et al., 2007; Schindler et al., 2012). In humans, AURKC is required for male meiosis because the absence of a functional protein is associated with male infertility (Dieterich et al., 2007; Dieterich et al., 2009). Men with *AURKC* mutations present with macrozoospermia that is due to a failure of completing MI. In contrast, two women homozygous for the same sterility-associated mutations reported having no trouble conceiving (Dieterich et al., 2009). Therefore the function or effect of known *AURKC* mutations in females is not known. A tractable model is therefore needed to gain a better understanding of the function of AURKC in female fertility.

In humans, the genomic locus of *AURKC* contains three alternative splice sites located in exon 1 (Fig. 7 A-B) (Bernard et al., 1998; Tseng et al., 1998; Yan et al., 2005c). *AURKC_v1* encodes the longest transcript (Fig. 7 A-B), and is more abundant in the testis relative to other tissues (Yan et al., 2005c). *AURKC_v3*, identified in a screen of human placental cDNA using probes for an Aurora kinase homolog from *Xenopus laevis,* is the shortest (Fig. 7 A-B) (Bernard et al., 1998). *AURKC_v2* was incidentally found in a screen to isolate *AURKC_v3* (Fig. 7 A) from human testis and has *in vitro* kinase activity similar to that of AURKC_v1 (Yan et al., 2005c). The proteins encoded by these splice variants contain identical catalytic domains at the C-terminus but their N-termini are successively shorter (Fig. 7C). While these variants exist in testis tissue, it is not known whether they are expressed in oocytes or if the truncated forms are biologically relevant.

The goals of our study were to determine which variants are expressed in human oocytes and assess their functional relevance in female meiosis. Using quantitative RT-PCR, we observed substantially greater levels of *AURKC* expression in oocytes relative to other cell types (sperm and cumulus cells), and we amplified the 3 splice variants in all oocytes tested. To assess functional differences we demonstrate that oocytes from *Aurkc*$^{-/-}$ mice can be used as a model to study human AURKC function. Finally, we showed that although the splice variants localize similarly and can support meiotic progression, they are not functionally equivalent. We show that when the 3 splice variants are expressed simultaneously in *Aurkc*$^{-/-}$ oocytes, that they support microtubule error

Fig 7- *AURKC* is alternatively spliced. (A) Schematic illustration to show that the 5' end of exon I of *AURKC* contains alternative splice sites. **(B)** Nucleotide sequence of the 5' end of *AURKC* to highlight the alternative start sites for each splice variant. **(C)** Amino acid sequence of each variant. The kinase domain starts at residue 42.

A



B

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AURKC_v1  49 ATCAGTGAGG 59-179 TTCTCCCCAT GAGCTCCCCC 199-239 AAGAGTGTGA
AURKC_v2  49 ATCAGTGAGG 59-179 TTCTCCCCAT GAGCTCCCCC 199-239 AAGAGTGTGA
AURKC_v3  49 ATCAGTGAGG 59-179 TTCTCCCCAT GAGCTCCCCC 199-239 AAGAGTGTGA

AURKC_v1  249-552 CCTCCCCTTC 563-676 TCAGTGGCTA CAGCA
AURKC_v2  249-552 CCTCCCCTTC 563-676 TCAGTGGCTA CAGCA
AURKC_v3  249-552 CCTCCCCTTC 563-676 TCAGTGGCTA CAGCA

AURKC_v1  691-720 GCCATGTGAG 730-965 GTCAGGCGGC
AURKC_v2  691-720 GCCATGTGAG 730-965 GTCAGGCGGC
AURKC_v3  691-720 GCCATGTGAG 730-965 GTCAGGCGGC
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Exon 1 Intron 1 Exon 2 Intron 2 Exon 3 Start Site

C

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AURKC_v1 1 MSSPRAVVQLGKAQPAGEELATANQTAQQPSSPAMRRLTVDDFE...QMASX 309
AURKC_v2 1 ------------------MATANQTAQQPSSPAMRRLTVDDFE...QMASX 290
AURKC_v3 1 --------------------------------MRRLTVDDFE...QMASX 275
```

correction to a greater level than when the variants are expressed alone. Therefore, we conclude that the splice variants of *AURKC* complement each other in female meiosis to optimize the process of MI chromosome segregation. Because oocytes express 3 variants simultaneously and sperm do not, this expression difference could begin to explain why men are more sensitive to *AURKC* mutations than women.

IV. Materials and Methods

Sample Collection

Oocytes and cumulus cells were processed in Cell-to-C_t lysis buffer as recommended (Ambion, #4458236, Grand Island, USA) and stored in liquid nitrogen. Discarded sperm samples were pelleted and washed in PBS prior to RNA collection.

Ethical approval

Human oocyte and cumulus cell samples were obtained from patients undergoing IVF treatments and collected under IRB approval (#20031397) with patient consent. Discarded sperm samples were also collected under IRB approval (#20031397) from couples seeking IVF without a male factor diagnosis.

All animals were maintained following the Rutgers Institutional Animal Use and Care Committee (#11-032) and the National Institutes of Health guidelines.

RNA extraction and cDNA synthesis

cDNA was synthesized from oocytes following the manufacturers protocol for the Cell-to-C_t kit (Ambion, #4458236, Grand Island, USA). Gene-specific reverse transcription was performed using Taqman assays (Applied Biosystems Hs00916672_g1, Hs04184901_m1, Hs00921878_m1, Hs00152930_m1, and Mm99999915_g1, Grand Island, USA) and a High capacity cDNA reverse transcription kit (Applied Biosystems, #4368814, Grand Island, USA). A pre-amplification step was also performed according to the Cell-to-C_t kit. cDNA was synthesized from sperm and cumulus cell samples using the TRIzol Plus RNA purification kit (Ambion, #12183-555, Grand Island, USA) and PureLink RNA mini kit (Invitrogen, #12183-18A, Grand Island, USA) followed by gene-specific RT as above.

Quantitative PCR

Variant-specific Taqman assays were supplied by Applied Biosystems Inc. (Foster City, USA) Hs00916672_g1 (*AURKC_v1*), Hs04184901_m1 (*AURKC_v2*), and Hs00921878_m1 (*AURKC_v3*). Assay Hs00152930_m1 was used as a control to recognize a region shared by the splice variants (between exon 4-5). Assay Mm99999915_g1 was used as endogenous *GAPDH* control. We confirmed Taqman probe specificity by assaying amplification of a cDNA clone for AURKC_v1. We only detected an amplification signal with the AURKC_v1 and the Taqman probe that spans exons 4 and 5 and is shared amongst the 3 variants. AURKC_v2 or AURKC_v3 probes anneal to 5' UTR

sequences that are not included in our cDNA clones precluding their assessment in the same manner. We also note that Applied Biosystems validated these probes for specificity computationally.

For each cDNA sample, duplex reactions were prepared to a final volume of 5 µl in a MicroAmp optical 384-well reaction plate (Applied Biosystems Inc., Grand Island, USA) containing equal amounts of *GAPDH* assay, one of the *AURKC* assays and 1 µl of cDNA. Each reaction was run in triplicate. Reactions were performed on a 7900HT SDS real-time PCR instrument (Applied Biosystems Inc., Grand Island, USA) and the default cycling conditions were used with default dissociation curve settings in the instrument control and data acquisition software (SDS version 2.3, Applied Biosystems Inc., Grand Island, USA). RQ Manager version 1.2 data analysis software (Applied Biosystems Inc., Grand Island, USA) was used with default settings to assign the threshold value for each reaction and results were then exported to Microsoft Excel for statistical analysis. Data were processed using the comparative C_t method as previously described (Livak and Schmittgen, 2001).

Murine oocyte preparation and microinjection

Fully-grown, GV-intact oocytes from 6-9 week old *Aurkc*$^{-/-}$ mice with a 129/SVpas/C57Bl/6 mixed background (Kimmins et al., 2007; Schindler et al., 2012) or CF-1 mice (Harlan Laboratories, #NSACF1, Indianapolis, USA) that were primed (44-48 h before collection) with pregnant mare's serum gonadotropin (PMSG) (Calbiochem #367222) were collected as previously

described (Anger et al., 2005). Oocytes were cultured and matured as previously described (Schindler et al., 2012). The collection and injection medium was bicarbonate-free minimal essential medium (MEM) containing, 25 mM Hepes, pH 7.3, 3 mg/ml polyvinylpyrollidone (MEM/PVP) and 2.5 μM milrinone (Sigma-Aldrich #M4659, St. Louis, USA) to prevent meiotic resumption (Tsafriri et al., 1996). To control for mouse-to-mouse variation, denuded oocytes were kept separate for each mouse and divided equally amongst experimental groups. We injected each oocyte with 250 ng/μl of cRNA, as previously described (Anger et al., 2005). After injection, oocytes were incubated overnight (for MI experiments) or for 3 hours (for MII experiments) in Chatot, Ziomek, and Bavister (CZB) medium containing 2.5 μM milrinone before meiotic resumption was initiated. All culture and *in vitro* meiotic maturation occurred in a humidified incubator with 5% CO_2 in air at 37°C. For Met I analyses, oocytes matured for 7-8 h and for Met II analyses, oocytes matured for 16 h. *In vitro* maturation was conducted in CZB medium without milrinone.

Challenge of kinetochore-microtubule attachment correction

After 7 hours of meiotic maturation, oocytes were incubated in CZB containing 100 μM monastrol (Sigma Aldrich, #M8515, St. Louis, USA) to induce monopolar spindle formation. After 2 hours oocytes were washed out of monastrol-containing media and allowed to recover in CZB containing 5 μM MG132 (Calbiochem, #474791, Darmstadt, Germany) to prevent anaphase onset. After 3 hours, oocytes were incubated for 7 minutes in pre-chilled MEM on

ice to destabilize any unattached microtubules. Oocytes were fixed as described below.

Live Imaging

After microinjection oocytes were treated with 10 µg/ml cycloheximide (Sigma-Aldrich, #C7698-1G, St. Louis, USA). After 1 hour, oocytes were transferred into separate drops of CZB medium with cycloheximide in a 96 well dish (Greiner Bio One, #655892, Monroe, USA). Bright field, GFP (470 nm), and mCherry (585 nm) images were acquired using an EVOS FL Auto Imaging System (Life Technologies, Grand Island, USA) with a 10X objective. The microscope stage was heated to 37°C and 5% CO_2 was maintained using the EVOS Onstage Incubator. Images were acquired every 20 minutes for 10 hours.

Cloning and synthesis of cRNA

The generation of m*Aurkc-Gfp* was previously described (Shuda et al., 2009). The *AURKC_v1* cDNA clone was purchased from Genecopeia (#EX-Q0034-M02-B, Rockville, USA). *AURKC_v2* was cloned via PCR from *AURKC_v1* using a forward primer (5'- GATCGCATGCATGGCTACAG -3'). *AURKC_v3* was obtained from Thermo Scientific (#160-002-F-8, Somerset, USA). All cDNA clones were PCR amplified and ligated into pIVT-EGFP (Igarashi et al., 2007) using SphI and SalI. We performed *in vitro* transcription with a mMessage mMachine kit (Ambion, #AM1344M, Grand Island, USA) as per the

manufacturer's instructions. cRNA was purified using an RNA-Easy purification kit (Qiagen, #74104, Venlo, Netherlands) and eluted in RNase free H_2O.

Immunocytochemistry

After maturation, oocytes were fixed in phosphate buffered saline (PBS) with 2% paraformaldehyde for 20 minutes at room temperature and then washed through blocking buffer (PBS + 0.3% (wt/vol) BSA + 0.01% (vol/vol) Tween-20). Prior to immunostaining, oocytes were permeabilized for 15 min in PBS containing 0.1% (vol/vol) Triton X-100 and 0.3% (wt/vol) BSA. The cells were then washed in blocking buffer (PBS+0.3% BSA+0.01% Tween-20). Immunostaining was performed by incubating in primary antibody; pINCENP [gift from M. Lampson, University of Pennsylvania; (Salimian et al., 2011) 1:1000], acetylated tubulin [Sigma Aldrich, #T7451; 1:1000, St. Louis, USA], CREST [Antibodies Incorporated, #15-234, Davis, USA; 1:30], or Alexa-fluor 488 conjugated alpha-tubulin [Sigma Aldrich, #T9026; 1:100, St. Louis, USA] for 1 hour. After washing, secondary antibodies (anti-rabbit [Life Technologies, #A10042, Grand Island, USA], anti-mouse [Life Technologies, #A10037, Grand Island, USA], anti-human [Life Technologies, #A21091, Grand Island, USA]) were diluted 1:200 in blocking solution and the sample was incubated for 1 h at room temperature. After washing, cells were mounted in VectaShield (Vector Laboratories, #H-1000, Burlingame, USA) with 4', 6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI; Life Technologies #D1306; 1:170, Grand Island, USA). Fluorescence was visualized on a Zeiss 510 Meta laser-scanning confocal

microscope with a 40X objective. The laser power was adjusted to just below saturation relative to the group exhibiting the highest level of signal intensity and all images were scanned at this laser power.

Immunoblotting

Oocytes were lysed in 1% SDS, 1% β-mercaptoethanol, 20% glycerol, and 50 mM Tris-HCl (ph6.8), and denatured at 95°C for 10 min. Proteins were separated by electrophoresis in 10% SDS polyacrylamide precast gel (Bio-Rad, #456-1036, Hercules, USA). Stained proteins of known molecular mass (range 10-250 kDa, Bio-Rad, #161-0376, Hercules, USA) were run simultaneously as standards. The separated polypeptides were transferred to nitrocellulose membranes (Bio Rad, #170-4156, Hercules, USA) using a Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, USA) and then blocked with 2% ECL blocking (Amersham, #RPN418, Pittsburgh, USA) solution in TBS-T (Tris-buffered saline with 0.1% Tween 20) for 1 hour. The membranes were incubated with α-tubulin primary antibody (Sigma-Aldrich, #T-6074, St. Louis, USA; 1:10,000) overnight or GFP primary antibody (Sigma-Aldrich, #G6539, St. Louis, USA; 1:1,000) for 1 hour. After washing with TBS-T five times, the membranes were incubated with a secondary antibody labeled with horseradish peroxidase (GE Healthcare Biosciences, #NA931, Pittsburgh, USA) for 1 hour followed with washing with TBS-T five times. The signals were detected using the ECL Select Western blotting detection reagents (Amersham, #RPN2235, Pittsburgh, USA) following the manufacturers protocol.

Image Analysis

All images were processed using ImageJ software (NIH, Bethesda, USA). Alignment measurements were performed as previously described using the same processing parameters for all images (Lane et al., 2012). In brief, we created a 4 μm box surrounding the metaphase plate that contains all aligned kinetochores. Oocytes containing kinetochores that fall outside of this box were considered “misaligned.” pINCENP intensity measurements were performed by averaging region of interest measurements at 6 kinetochores per oocyte, subtracting an average of 3 background measurements, and normalizing to average GFP expression per cell to account for injection amount. Protein stability measurements were obtained by normalizing all GFP: mCherry ratios for each time point relative to time zero.

Statistical Analysis

JMP Software was used to calculate Spearman Rank Correlation coefficients between AURKC splice variant expression in oocyte samples. One-way ANOVA or linear regression analysis as indicated in the figure legends were used to evaluate the differences between groups using Prism Graphpad software (La Jolla, USA). For experiments analyzing chromosome alignment, a permutation version of the binomial proportions test (Snedecor and Cochran, 1989) was used to analyze differences between groups. Fisher’s Method (Fisher, 1925) was used to combine the results of several independent tests with the same null hypothesis. The Bonferroni correction was used to determine if the

Fisher-Method p-values were significant after multiple testing. $P < 0.05$ was considered significant.

V. **Results**

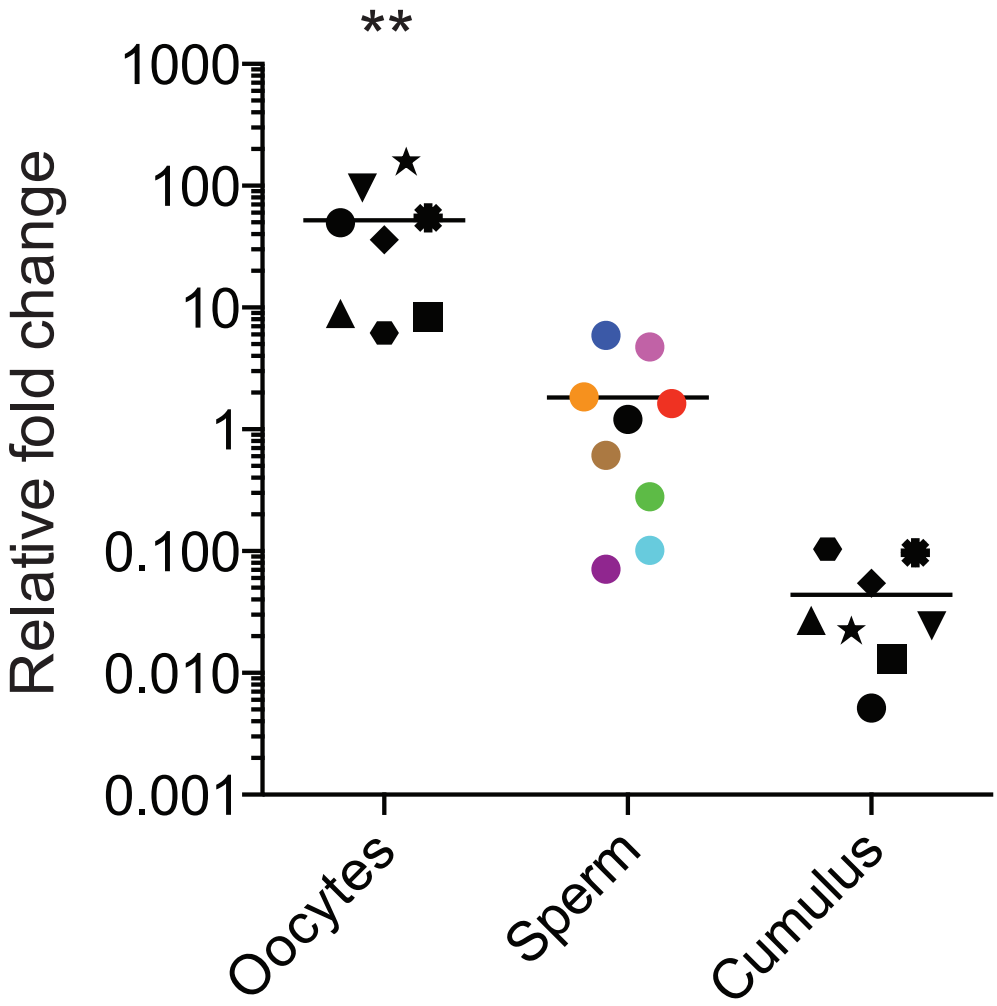
***AURKC* is highly expressed in human oocytes**

High *Aurkc* expression in mice is limited to germ cells (Tseng et al., 1998; Yanai et al., 1997), and its function is important for meiotic cell cycle progression (Balboula and Schindler, 2014; Chen et al., 2005; Hu et al., 2000; Schindler et al., 2012; Tang et al., 2006a; Yang et al., 2010; Yang et al., 2013) and post-meiotic sperm development (Kimmins et al., 2007). However, the expression levels in single oocytes relative to other cell types and the expression of the individual splice variants is not known. To confirm the presence of *AURKC* and to interpret relative quantities of expression in human oocytes, we performed quantitative RT-PCR on cDNA from single oocyte samples, each from a different donor patient with paired cumulus cell samples (Table 1). Human sperm samples were used as controls. After calculating the percent fold change relative to endogenous *GAPDH* message, the data demonstrate the presence of *AURKC* transcript in every oocyte sample tested in agreement with previous findings (Assou et al., 2006; Avo Santos et al., 2011; Grondahl et al., 2010) (Fig. 8).

Table 1 - Human female donor information. Each donor is annotated with a symbol so sample information can be found on each relevant graph in Figs. 8 and 9.

Patient Age (years)	Cell Type	Stage	Symbol
34.1	oocyte cumulus	GV	■
34.4	oocyte cumulus	GV	★
34.7	oocyte cumulus	GV	▼
36.2	oocyte cumulus	GV	▲
37.4	oocyte cumulus	GV	❋
38.3	oocyte cumulus	GV	⬢
38.8	oocyte cumulus	GV	●
40.5	oocyte cumulus	GV	◆

Fig. 8 – *AURKC* is more highly expressed in oocytes than in sperm or cumulus cells. qRT-PCR was performed on human samples to measure total *AURKC* expression using $2^{-\Delta Ct}$. The symbols in oocyte and cumulus represent the individual donors in Table I. The different colors used in the sperm samples represent each donor for ease of identification and comparison with Fig. 9A. The average bar represents the mean level of expression. One-way ANOVA was used to analyze the data. **P=0.005.



Although it is an interesting question to ask, our current sample size was too limited to make correlations between age and expression of *AURKC*. Expression levels were calculated in a range between 6 to 159-fold change greater than the housekeeping gene *GAPDH*. For example, the oocyte from the 34.4 yr old donor had the highest level of AURKC expression and this is 26 fold greater than the lowest expression (38.3 yr old donor).

We detected *AURKC* in cDNA samples from sperm and cumulus cells (Fig. 8). The sperm cDNAs tested expressed *AURKC,* with an average expression level of 2 fold greater than expression of *GAPDH*. This expression level is substantially lower than the expression level in oocytes (Fig. 8). When we compared the average expression level in sperm to the average expression level in oocytes, oocytes contained 64-fold more *AURKC* message than did sperm. Similar to oocytes, the levels of expression of *AURKC* in sperm from donor to donor varied ranging from 6-fold greater to 10-fold lower relative to *GAPDH* expression (Fig. 8). Because the expression level of *AURKC* was higher in oocytes than it was in sperm, these data suggest differential requirements for the protein in male and female meiosis.

We also detected *AURKC* in cumulus cells at levels similar to those previously described (Fig. 8 (Assou et al., 2006)). The expression levels were markedly lower (~1,000 and ~20 fold) compared to levels in oocytes and sperm, respectively, but also varied from patient to patient ranging from 10-fold to 200-fold lower than *GAPDH* expression (Fig. 8). There was no correlation between the abundance of *AURKC* transcript in oocytes to that in cumulus cells from the

same patient (Fig. 8, Spearman Rank Correlation Test, p=0.5). For example, the 34.1 yr old donor had below average expression of *AURKC* in oocytes and cumulus cells while the 40.5 yr old donor had below average expression in the oocyte and above average expression in the cumulus cells.

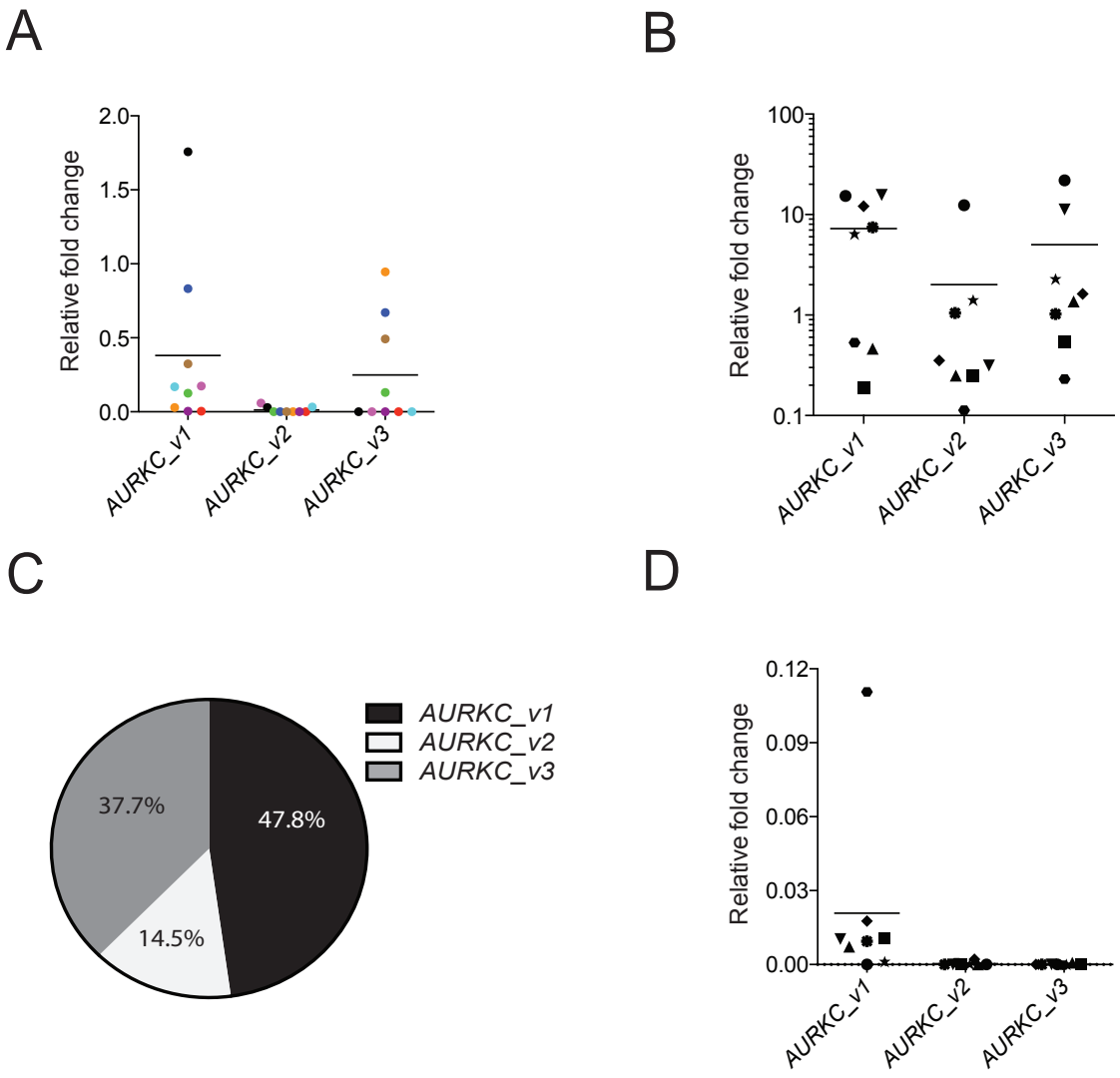
AURKC is expressed at low levels in placental, lung, and some tumor tissue samples (Baldini et al., 2011; Yan et al., 2005c), but not at significant levels in any other mitotic tissues in humans. Its expression in cumulus cells could indicate a more involved role for AURKC in the cumulus-oocyte complex in supporting oocyte growth or meiotic progression.

Human oocytes express 3 variants of *AURKC*

Three *AURKC* splice variants are expressed in human testis tissue (Bernard et al., 1998; Tseng et al., 1998; Yan et al., 2005c). To determine which splice variants are expressed in oocytes, we verified that the variant-specific Taqman assays detect and amplify each transcript. To this end, we generated human sperm cDNA libraries expected to contain the 3 transcripts. The data indicate that *AURKC_v1* was present in all sperm samples tested, whereas *AURKC_v2* and *AURKC_v3* were detected in 3 and 4 of the 9 samples, respectively (Fig. 9A). No sample expressed the three simultaneously.

Next, we used the variant-specific Taqman probes to determine which splice variants were present in the oocyte samples. Each splice variant was detected in every oocyte lysate (Fig. 9B, Table 1). The average expression level for each variant was 7.7, 1.8, and 4.5 fold greater than *GAPDH,* respectively.

Fig. 9 – Oocytes express three splice variants while sperm and cumulus express 1. (A, B, and D) qRT-PCR was performed on human samples to measure variant-specific *AURKC* expression using $2^{-\Delta Ct.}$ The symbols in oocyte (B) and cumulus (D) represent the individual donors in Table I. The different colors used in the sperm samples (A) represent each donor for ease of identification. The average bar represents mean level of expression. **(C)** Average relative expression of splice variants in oocytes using $2^{-\Delta\Delta Ct}$.



Using the comparative C_t method, we determined that *AURKC_v1* was the most abundant, making up approximately 47.8% of the total *AURKC* composition in oocytes, and *AURKC_v2* was expressed at the lowest level, making up approximately 14.5% (Fig. 9 B-C). We note that there was variation amongst expression levels between samples (Fig 9B). Variation of *AURKC_v1* ranged from 5-fold less to 15-fold greater expression than *GAPDH*. Variants 2 and 3 ranged from 9-fold less to 12-fold more and equal to 22 times more than *GAPDH*, respectively. Similar to the results with the amount of total *AURKC* (Fig. 8), there was variation in amounts of each transcript in oocytes from different donors. Even within the same oocyte, there was variability in expression of the 3 variants. For example, the oocyte from the 34.7-yr-old donor expressed above average levels of *AURKC_v1* and *AURKC_v3* but below average levels of *AURKC_v2* (Fig. 9B, Table 1). The significance of the variability from patient to patient is unknown, and outside the scope of this study. All oocytes tested expressed each splice variant while sperm samples most commonly expressed variant 1. This change in expression further supports the model of differential function for AURKC in oocytes and sperm.

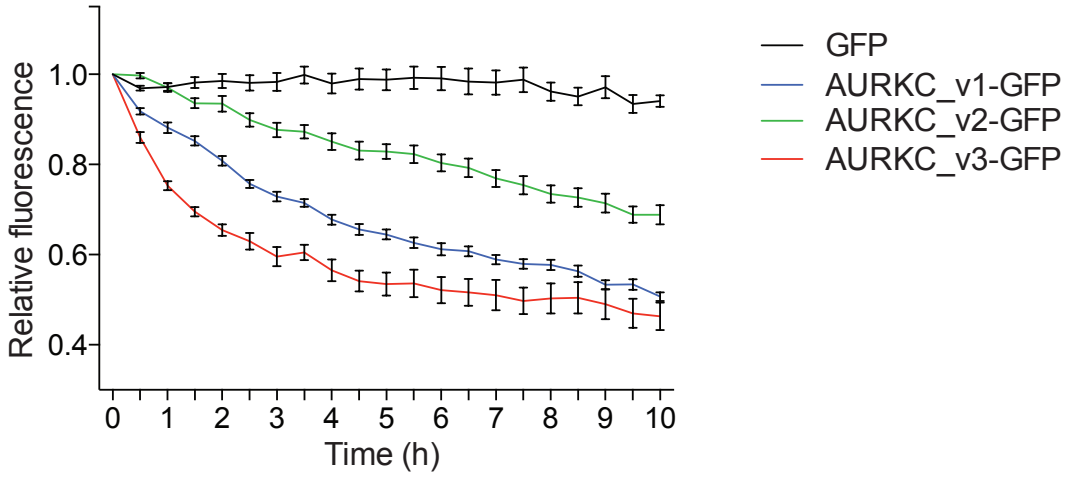
AURKC is expressed at low levels in cumulus cells (Assou et al., 2006); therefore we wanted to investigate the splice variant composition of the mitotic cell counterparts of the oocytes tested. Using the same variant-specific Taqman assays, we detected the expression of *AURKC_v1* in cumulus cell samples (Fig. 9D). Similar to total *AURKC* expression, there was no correlation between variant expression levels in cumulus cells and oocytes from the same patient (Spearman

Rank Correlation Test, p=0.13). Therefore, mitotic cycling cumulus cells expressed *AURKC*, however, they only expressed splice variant 1.

Splice variants are differentially stable during meiosis

Splice variants occur due to natural mutation, but these variants persist only through natural selection if the protein created imparts an advantage on the organism. Therefore, we can predict that the expression of these extra variants of *AURKC* impart some added fitness onto oocytes that is not as necessary in sperm since they do not consistently express all three. It is desirable to conclude this might have to do with the length of time for completion of meiosis in female gametes compared to male gametes. The existence of 3 splice variants of *AURKC* indicates the need for different functions in meiosis but these specific roles are unclear. We hypothesized that the splice variants could have different stability properties. To test this hypothesis, we monitored oocytes from wildtype mice expressing GFP-tagged variants that were treated with cycloheximide via live-imaging and quantified fluorescence over the course of 10 hours. We found that AURKC_v2 was significantly more stable over the time course than either AURKC_v1 or AURKC_v3 (Fig. 10). Because the only difference in these variants is the length of the N-terminus, these results indicate the presence of a destruction motif between residues 1-19 that is lacking in AURKC_v2, and a region that imparts stability between residues 20-34 that AURKC_v3 is lacking (Fig. 7C). This hypothesis is supported by the data showing that the initial destruction (t= 0-2 hours) of AURKC_v3 is faster (slope= -0.1757) than the initial

Fig. 10 – AURKC splice variants differ in stability during meiotic maturation. Fully grown oocytes arrested at prophase of MI from CF-1 mice were co-injected with the indicated *Gfp*-tagged and *mCherry* cRNAs. One hour after cycloheximide addition, fluorescent images were obtained at 30 minute intervals. Data points represent a mean (± SEM) from at least 20 oocytes from two independent experiments. Data were analyzed using linear regression; P<0.0001.



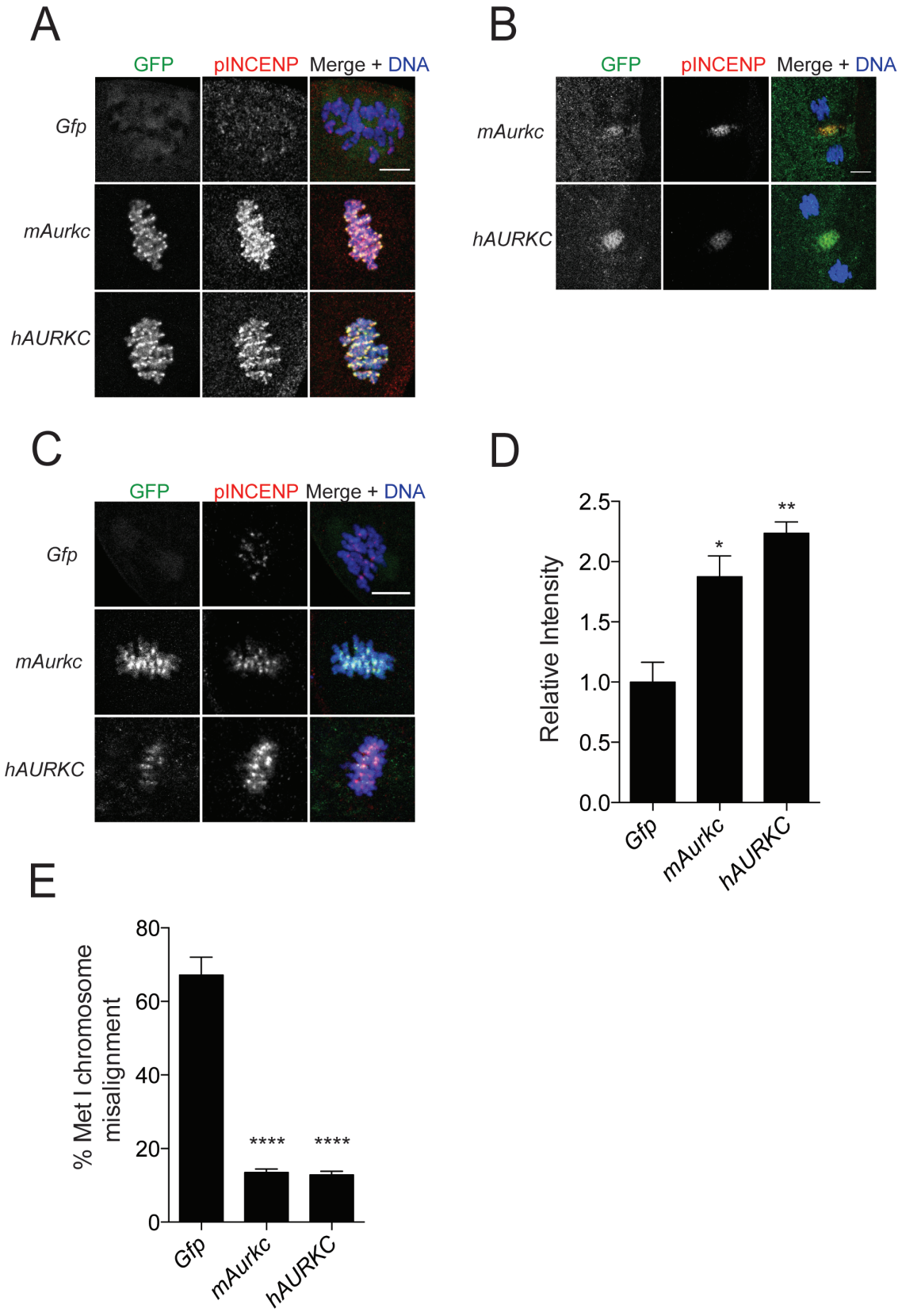
destruction of AURKC_v1 (slope= -0.1149) (Fig. 10). A search for similar motifs has not yielded any significant findings, indicating that these are presently unidentified domains.

Aurkc$^{-/-}$ mouse oocytes as a model for studying human *AURKC* in meiosis

To determine the biological significance of the variants, we first established a model to assay AURKC function. Oocytes from *Aurkc*$^{-/-}$ mice have distinct meiotic phenotypes that are quantifiable and make it a convenient model for studying AURKC function in meiosis. These phenotypes include arrest at metaphase of meiosis I with chromosome misalignment (Schindler et al., 2012), reduced levels of phosphorylated INCENP (the AURKB and AURKC substrate), and increased frequency of errors in kinetochore-microtubule attachments (K-MT attachments) (our unpublished observations). Because human (hAURKC) and mouse (mAURKC) AURKC share 87% homology in the catalytic domain, we hypothesized that hAURKC could complement the defects of oocytes from *Aurkc*$^{-/-}$ mice.

To test this hypothesis, we compared phenotypic rescue of oocytes lacking AURKC that were microinjected with either mouse AURKC or human AURKC_v1 *Gfp*-tagged cRNA. Endogenous and ectopically expressed mAURKC localizes along the interchromatid axis (ICA) and at the inner kinetochore during Met I, moves to the spindle midzone during telophase I (Telo I), and then back to the kinetochore at Met II (Avo Santos et al., 2011; Sharif et al., 2010; Shuda et al., 2009; Yang et al., 2010) (Fig. 11A-C). Ectopically expressed hAURKC had an

Fig. 11 – Human AURKC functionally complements mouse AURKC. Fully grown oocytes arrested at prophase of MI from *Aurkc*$^{-/-}$ mice were injected with the indicated *Gfp*-tagged cRNA and matured to Met I **(A)**, Telo I **(B),** or Met II **(C)** prior to fixation and immunocytochemistry to detect pINCENP (red in merge). (A-C) Representative confocal z-projections of oocytes. DNA was detected by DAPI staining (blue). Detection of GFP is green in the merge. Scale bars represent 10 μm. **(D)** Relative intensity of pINCENP at centromeres from experiments depicted in (A) after normalization with the intensity in the *Gfp* control group at Met I. One-way ANOVA was used to analyze the data and error bars represent the mean (± SEM); **P=0.0043, ****P=0.0005. **(E)** Misaligned chromosomes at Met I were identified as described in Lane *et al.* Each experiment was performed three times using two mice each time. A permutation version of the binomial proportions test was used to analyze differences between groups as described in Materials and Methods section. *P=0.0134, **P=0.0055.


A
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Merge + DNA
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hAURKC
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hAURKC
C
GFP
pINCENP
Merge + DNA
Gfp
mAurkc
hAURKC
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Relative Intensity
2.5
2.0
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hAURKC
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% Met I chromosome misalignment
80
60
40
20
0
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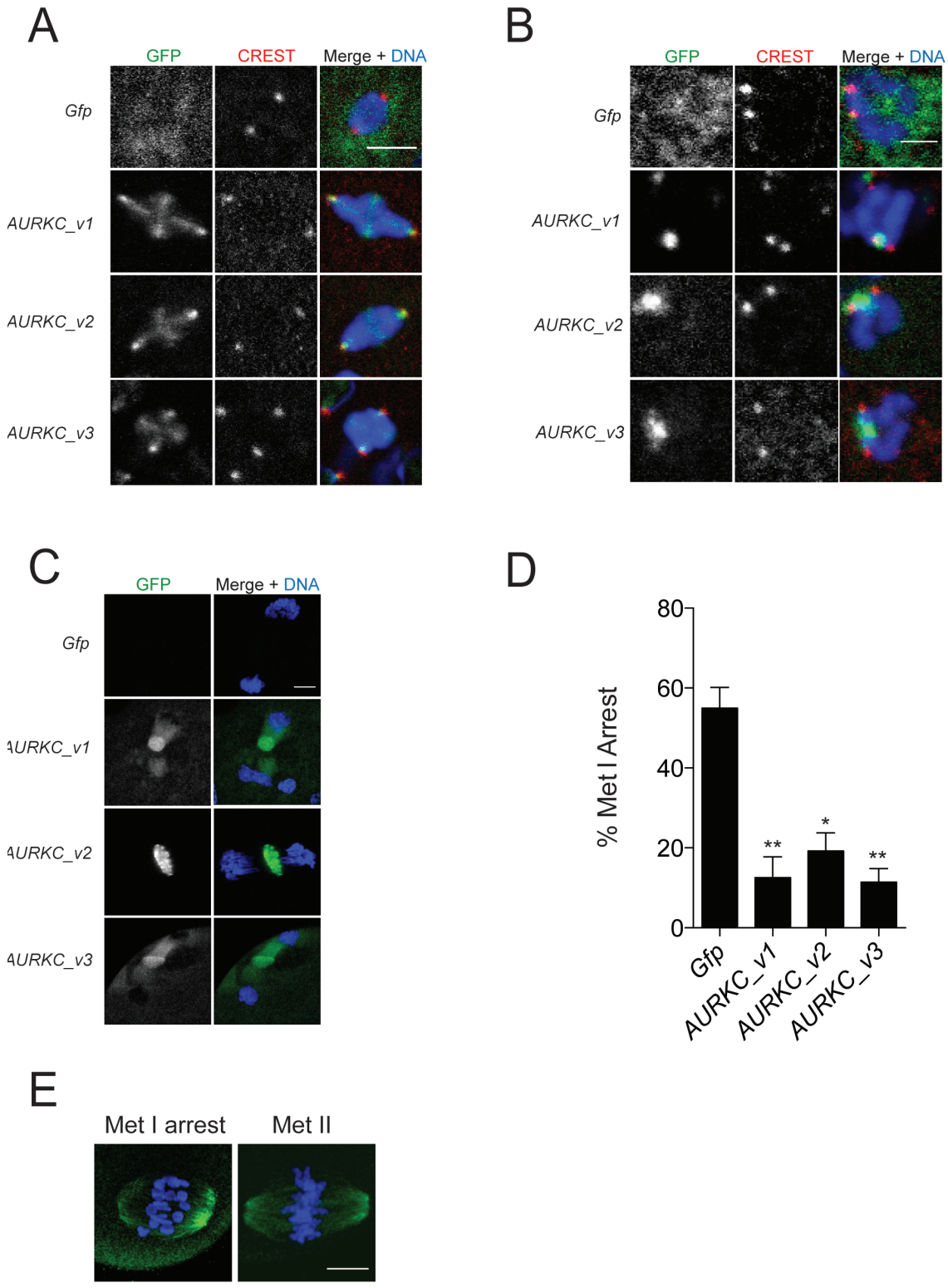
****
Gfp
mAurkc
hAURKC

identical localization as mAURKC when expressed in *Aurkc*$^{-/-}$ oocytes; along the ICA and at kinetochores at Met I, at the spindle midbody at Telo I, and back to kinetochores at Met II (Fig. 11A-C). To confirm that hAURKC is active at this localization, we quantified the relative fluorescence of an antibody that recognizes the phosphorylated form of INCENP (pINCENP) at kinetochores of images obtained via confocal microscopy. Compared to the *Gfp*-injected control, oocytes expressing mAURKC or hAURKC had significantly increased pINCENP levels (~2 fold). Importantly, we found similar levels of INCENP phosphorylation in oocytes injected with *mAurkc* or *hAURKC* indicating that hAURKC phosphorylates mouse INCENP to a similar extent as mAURKC (Fig. 11A-D). Approximately 60% of control-injected *Aurkc*$^{-/-}$ oocytes had chromosomes that were misaligned at the Met I plate (Fig. 11E; (Schindler et al., 2012)). We found that this phenotype can be rescued by overexpression of mouse or human AURKC (Fig. 11E) indicating that hAURKC functionally complements the loss of mAURKC in *Aurkc*$^{-/-}$ oocytes. Therefore we used these assays to assess human AURKC function in female meiosis.

hAURKC **splice variants localize in mouse oocytes and support meiotic progression**

To determine if the N-termini dictate different localization or function, we injected *Gfp*-tagged cRNA for each variant into *Aurkc*$^{-/-}$ oocytes; *Gfp* was injected as a control. At Met I, each variant localized to kinetochores and along the ICA (Fig. 12A). At Met II, localization occurred strictly at kinetochores and did not

Fig. 12- AURKC splice variants have identical localization and support meiotic progression. Fully grown oocytes arrested at prophase of MI from *Aurkc*$^{-/-}$ mice were injected with the indicated cRNA and matured to Met I **(A),** Met II **(B)**, or Telo I **(C)** prior to fixation and immunocytochemistry to detect the outer region of the kinetochore (CREST, red in merge). (A-C) Representative confocal z-projections of oocytes. DNA was detected by DAPI staining (blue). Detection of GFP is green in the merge. **(D and E)** Oocytes were matured for 16 h prior to fixation and immunocytochemistry to detect the spindle (green). After acquiring images on the confocal, Met I arrest was scored by the presence of bivalents (blue). Scale bars represent 10 μm. Each experiment was performed three times using two mice each time. One-way ANOVA was used to analyze the data and error bars represent the mean (± SEM); *P=0.0190, **P=0.0012 and 0.0047, respectively.


A
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AURKC_v2
AURKC_v3
B
GFP
CREST
Merge + DNA
Gfp
AURKC_v1
AURKC_v2
AURKC_v3
C
GFP
Merge + DNA
Gfp
AURKC_v1
AURKC_v2
AURKC_v3
D
% Met I Arrest
80
60
40
20
0
**
*
**
Gfp
AURKC_v1
AURKC_v2
AURKC_v3
E
Met I arrest
Met II

differ between the variants (Fig. 12B). We also noted dynamic localization of the AURKC variants to the spindle midbody during Telo I (Fig. 12C). Therefore, the length of the N-terminus did not affect the localization of AURKC.

Oocytes lacking *Aurkc* do not support meiotic maturation to the same extent as wildtype oocytes (Schindler et al., 2012). As a measure of meiotic progression, we quantified *Aurkc*$^{-/-}$ mouse oocytes injected with cRNA for each variant arrested at Met I after 16 hours of maturation. We found that the three splice variants significantly improved the Met I arrest phenotype witnessed in the control-injected *Aurkc*$^{-/-}$ oocytes (54%) and to the same degree (Fig. 12D). Therefore, the 3 splice variants all localize and support meiotic progression similarly.

AURKC splice variants differ in supporting chromosome segregation

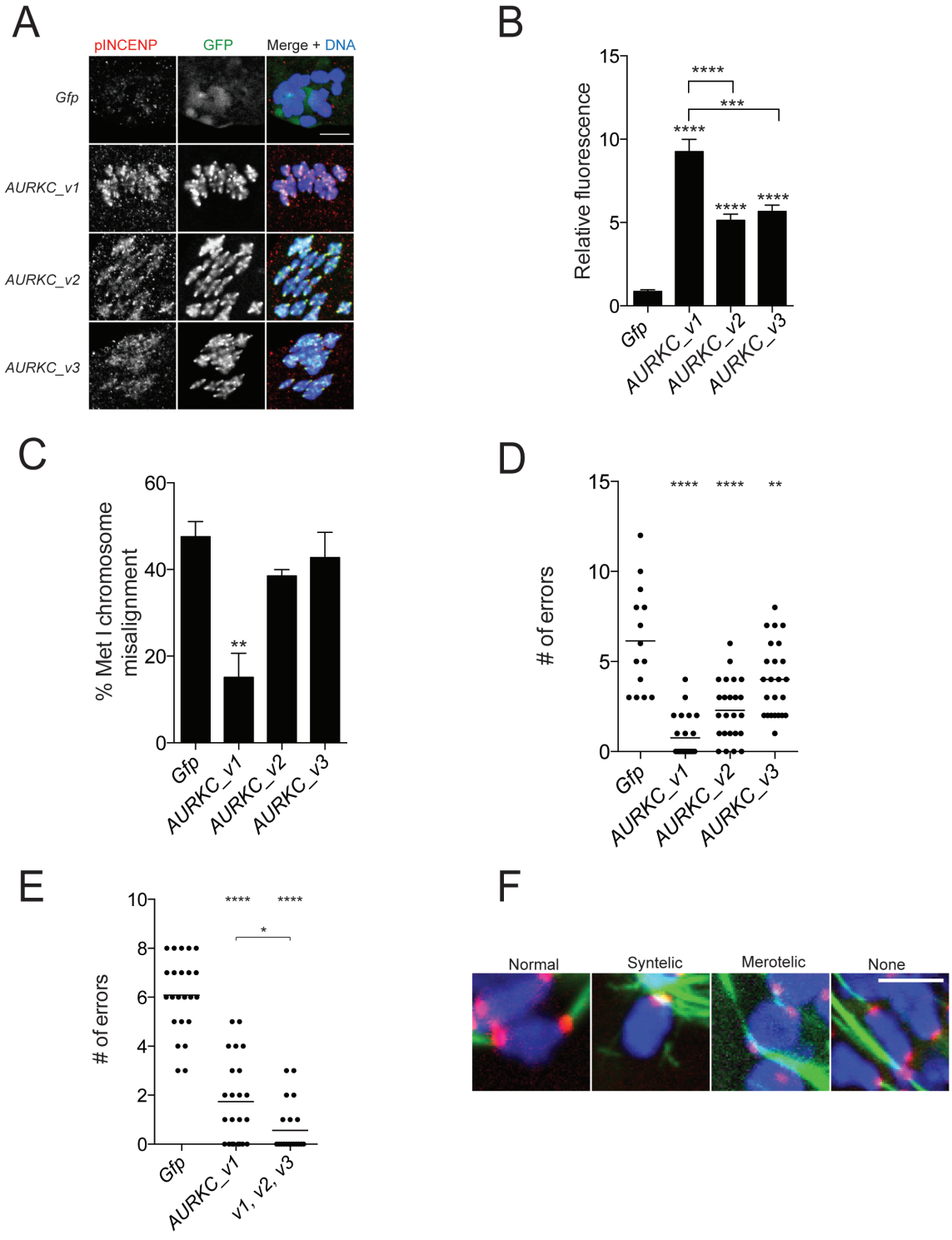
To further investigate the function of the variants during meiosis, we quantified the ability of each variant to phosphorylate INCENP. We injected *Aurkc*$^{-/-}$ oocytes with *Gfp* as a control. Regardless of the variant injected, the fluorescence intensity of pINCENP was significantly higher than in control-injected oocytes (Fig. 13A-B). Furthermore, *AURKC_v1*-injected oocytes had higher pINCENP immunoreactivity than in those injected with *AURKC_v2* or *AURKC_v3* at both Met I (Fig. 13A-B) and Met II (data not shown). These data demonstrate that AURKC_v2 and AURKC_v3 have reduced catalytic activity compared to AURKC_v1. Therefore, the length of the N-terminus appears to influence AURKC's ability to phosphorylate substrates.

AURKC also regulates alignment of chromosomes at the metaphase plate (Balboula and Schindler, 2014; Sharif et al., 2010; Yang et al., 2010). Therefore, we next asked if the GFP-tagged variants rescued Met I chromosome alignment in *Aurkc*$^{-/-}$ mouse oocytes. In the *Gfp*-injected *Aurkc*$^{-/-}$oocytes, Met I chromosome misalignment was observed 48% of the time (Fig. 13C). Despite their ability to progress through meiosis, the variant-injected oocytes did not regulate chromosome alignment to the same extent as one another (Fig. 13C). Chromosomes in *AURKC_v1*-injected oocytes were significantly more aligned than in those oocytes injected with *AURKC_v2* or *AURKC_v3* (Fig. 13C). We also found that neither *AURKC_v2* nor *AURKC_v3*-injected oocytes could significantly improve alignment relative to the *Gfp*-injected control oocytes (Fig. 13C). These data suggest that the reduction of catalytic activity alters chromosome alignment without affecting cell cycle progression.

In order to ensure that these differences in activity were not due to differences in expression of the injected cRNAs, we performed Western Blot analysis on oocytes injected with each cRNA and probed for the GFP tag (Fig. S1A). We found no differences in expression between variants (Fig. S1B).

One of the causes of misaligned chromosomes at the metaphase plate could be incorrect attachments between the kinetochore and the spindle microtubules (K-MT)(Balboula and Schindler, 2014; Brunet et al., 1999; Lampson et al., 2004). A function of AURKC in meiosis is to destabilize the incorrect attachments so that new and correct attachments can form (Balboula and Schindler, 2014). Therefore, we assayed the ability of each variant to correct erroneous K-MT

Fig. 13 - AURKC splice variants differ in catalytic activity. Fully grown oocytes arrested at prophase of MI from *Aurkc*$^{-/-}$ mice were injected with the indicated *Gfp*-tagged cRNA and matured to Met I. **(A–C)** Oocytes were fixed and immunocytochemistry was used to detect pINCENP (red in merge). (A) Representative confocal z-projections. DNA was detected by DAPI staining (blue). Detection of GFP is green in the merge. Scale bars represent 10 μm. (B) Quantification of experiments in (A) after normalization with the intensity in the *Gfp* control group. (C) Quantification of chromosome alignment from experiments in (A) as described in Lane *et al.* and our Materials and Methods section. Each experiment was performed three times using two mice each time. A permutation version of the binomial proportions test was used to analyze differences between groups as described in Materials and Methods section. *P = 0.0057. Variants 2 and 3 were not significantly different from the *Gfp* control (P = 0.7233, and 0.8697, respectively). **(D–F)** After maturation to Met I, oocytes were treated with monastrol for 2 h followed by a 3 h recovery. Prior to fixation, oocytes were incubated in ice-cold medium. After fixation, stable K-MT attachments were detected by immunocytochemistry to visualize spindle fibers (α-tubulin; green) and kinetochores (CREST; red). (D and E) Each data point represents the number of improper K-MT attachments quantified in a single oocyte image. (F) Representative confocal z-projections of types of K-MT attachments. Full images are shown in Supplementary data, Fig. S2. Normal attachments are classified as a chromosome containing two pairs of sister kinetochores attached to opposite poles. Syntelic attachments occur when both pairs of sister kinetochores are attached to the same pole. Merotelic attachments occur when one pair of sister kinetochores is attached to both poles. The scale bar is 5 μm. The experiments were performed twice with a total of four mice (D) or three times with a total of five mice (E). One-way ANOVA was used to analyze the data and error bars represent the mean (+SEM); *P = 0.0143 and 0.0329, respectively, **P = 0.007, ***P =0.0002, ****P < 0.0001.


A
pINCENP
GFP
Merge + DNA
Gfp
AURKC_v1
AURKC_v2
AURKC_v3
B
Relative fluorescence
15
10
5
0
****
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****
****
****
Gfp
AURKC_v1
AURKC_v2
AURKC_v3
C
% Met I chromosome misalignment
60
40
20
0
**
Gfp
AURKC_v1
AURKC_v2
AURKC_v3
D
# of errors
15
10
5
0
****
****
**
Gfp
AURKC_v1
AURKC_v2
AURKC_v3
E
# of errors
10
8
6
4
2
0
****
****
*
Gfp
AURKC_v1
v1, v2, v3
F
Normal
Syntelic
Merotelic
None

attachments in *Aurkc*$^{-/-}$ mouse oocytes. These Met I oocytes were treated with monastrol, an Eg5 kinesin inhibitor, to form 100% incorrect K-MT attachments, and correction was assessed after wash out of monastrol. Control-injected oocytes fixed most of the induced syntelic attachments (Fig. 13D-F), however we never observed an *Aurkc*$^{-/-}$ control oocyte that fixed all of the attachments, consistent with the model that AURKC is required for this activity (Balboula and Schindler, 2014). *AURKC_v3*-injected oocytes also failed to correct all errors and frequently contained oocytes with at least three improper K-MT attachments (Fig. 13D). Some oocytes expressing AURKC_v1 and AURKC_v2 corrected all the syntelic attachments (44% and 16% respectively) while the AURKC_v1 group had no oocytes with greater than three errors (Fig. 13D). Consistent with different levels of catalytic activity, these data demonstrate that the 3 splice variants differ in their ability to correct K-MT attachments.

Because human oocytes express three splice variants simultaneously, we asked if the error correction could be improved further if all three variants were expressed in *Aurkc*$^{-/-}$ oocytes at approximately the same ratio (48%: 15%: 37%) that we find in human oocytes (Fig. 9C). Compared to *Gfp* and variant 1-injected controls, we found significant improvement in the number of oocytes that could fix all of the improper K-MT attachments when oocytes express three variants simultaneously (Fig. 13D-F). These data imply that the three variants complement each others function during meiosis and all three are needed for optimal fidelity of chromosome segregation.

I. Discussion

The presence of AURKC in oocytes was previously determined by gene expression profiling (Assou et al., 2006) and qPCR (Avo Santos et al., 2011), and data from microarray analyses identified *AURKC* transcript in lysates from 15 individual oocytes samples (Grondahl et al., 2010). In this study, we show that *AURKC* expression in single oocyte samples is significantly greater than that in sperm indicating a differential requirement for the transcript in spermatogenesis and oogenesis.

Three AURKC splice variants are expressed in testis and sperm (Bernard et al., 1998; Tseng et al., 1998; Yan et al., 2005c). Here we demonstrate that the three variants are also always found in oocytes (Fig. 9B) while not all sperm samples express the three splice variants simultaneously (Fig. 9A). The differential expression of *AURKC* splice variants in gametes gives credence to the hypothesis that these variants perform different functions in female versus male meiosis. The observation that mutations in *AURKC* are correlated with infertility in men but not women is further proof that AURKC functions differently in oocytes and sperm (Dieterich et al., 2007).

AURKC_v1 is expressed in all oocytes, sperm, and cumulus cells tested (Fig. 9A, B, D), but oocytes express significantly more *AURKC* than the other cell types (Fig. 8). Even the oocyte with the least amount of transcript contained ~8 fold more *AURKC* than the sperm sample with the highest levels of expression (Fig. 8). The prolonged time frame for completion of meiosis in females

compared to males would necessitate a greater abundance of transcripts to account for degradation over time. At the onset of puberty, spermatogenesis occurs daily and takes approximately 2 months to complete and meiosis only takes about 1 day. Oogenesis, on the other hand, begins during fetal development and is not completed until after fertilization taking 12-50 years from start to finish. This prolonged delay is accompanied by transcriptional silencing (Edson et al., 2009; Matzuk and Lamb, 2002). Therefore all RNA messages required for meiosis and early embryogenesis must persist in oocytes for longer than any such transcript in sperm. The stability of these transcripts is imperative for formation of a healthy embryo, and having an abundance of *AURKC* may be advantageous for this process.

Multiple versions of a single gene may impart benefit on oocytes by protecting against non-specific degradation. For example, as cells age, there is an increase in reactive oxygen species released by the mitochondria which in turn will attack methionines, cysteines, and other aromatic residues on proteins (Imlay, 2003). Therefore, having a reserve stock of protein could allow the cell to continue normal function even in the presence of age-related oxidative stress. We hypothesize that this might be one explanation for the presence of multiple AURKC variants.

AURKC is more stable than AURKB in mouse oocytes, which provides sustained Aurora kinase activity to help support meiosis and embryonic mitoses (Schindler et al., 2012). While all the variants can support meiotic progression (Fig. 12D), they are not able to do so with equal efficiency (Fig. 13). Yet, when

they are combined, the overall error correction activity is greater than when any variant is expressed alone (Fig. 13E) indicating a need for the three variants to be expressed concurrently for optimal function. The variants are not found simultaneously in sperm indicating this cumulative effect is not necessary for spermatogenesis. We have not yet examined the function of these variants in older or stressed oocytes, which could further illuminate variable function between the splice variants.

Based on known AURKB mitotic substrates that are also present in mouse oocytes, there are a number of candidate targets of AURKC. The phenotypic differences we observed between the variants could be due different affinities for these substrates. For example, Mitotic centromere-associated kinesin (MCAK) is an AURKB substrate in mitosis. AURKB-activated MCAK is responsible for the depolymerization of plus-ends microtubules to facilitate error correction, primarily merotelic attachments (Cimini et al., 2006; Kline-Smith et al., 2004; Wordeman et al., 2007). MCAK localizes to centromeres in oocytes and therefore it is a likely AURKC substrate (Ems-McClung et al., 2007; Vogt et al., 2010; Zhang et al., 2007). Loss of MCAK in meiotic cells results in delayed chromosome congression and potentially increased errors in K-MT attachments (Illingworth et al., 2010; Vogt et al., 2010). If each variant of AURKC had different affinities for MCAK, error correction at kinetochores would vary which is consistent with our observations. Another candidate substrate at kinetochores in oocytes is HEC1 (DeLuca et al., 2011; Sun et al., 2011), which is involved in bipolar spindle formation and chromosome congression (Gui and Homer, 2012; Sun et al.,

2011). The variants do not show differences in spindle formation but chromosome alignment did vary, making differential affinity for HEC1 a possibility. Due to the relationship of AURKB and the spindle assembly checkpoint protein MAD2 in mitosis, it is likely a downstream effector of AURKC in oocyte meiosis. Just prior to anaphase I, all kinetochores lose MAD2 even when incorrect attachments are still present (Gui and Homer, 2012). If the variants differ in their ability to correct attachments, the SAC would still be satisfied and PBE would occur, consistent with our observations. Therefore, differences in substrate affinity, either to these candidates or unknown meiosis-specific substrates, are a feasible mechanism to explain our results.

Mutations in *AURKC* are linked to male infertility and cancer (Dieterich et al., 2007; Kimura et al., 1999; Sasai et al., 2004; Takahashi et al., 2000). AURKC is a germ-cell specific transcript that has not been well characterized in oocytes. We developed an effective mouse model for studying human AURKC in oocytes. We also found that human oocytes express three splice variants but at different levels and that they functional differently yet additively in meiosis. This could be due to differing stability and activity. Understanding the expression pattern and function of AURKC variants will shed light on the differences between male and female meiosis and genetic causes of infertility (Edson et al., 2009). If causative, this information could ultimately help develop diagnostic tools for better oocyte and embryo selection in the clinic.

Fig. S1 AURKC-GFP splice variants are expressed equally in mouse oocytes. (A) Fully grown oocytes arrested at prophase of meiosis I from CF-1 mice were injected with the indicated Gfp-tagged cRNA and incubated for 16 hours. For each group, 20 oocytes were lysed and probed with the indicated antibody. The panels are images of the same membrane that was stripped and re-probed. **(B)** Quantification of bands in (A).

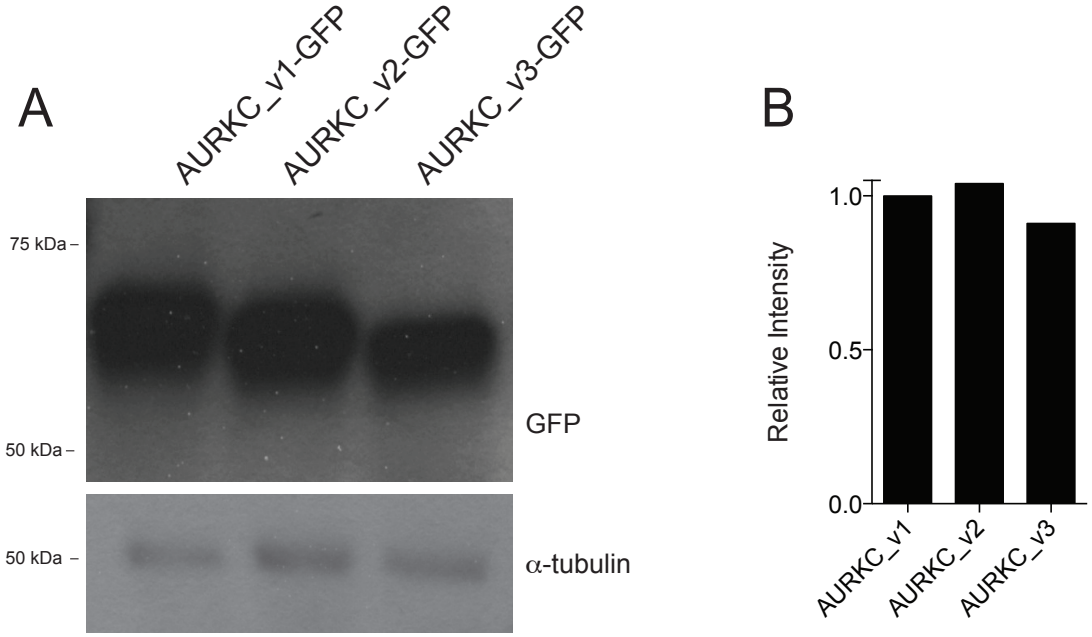
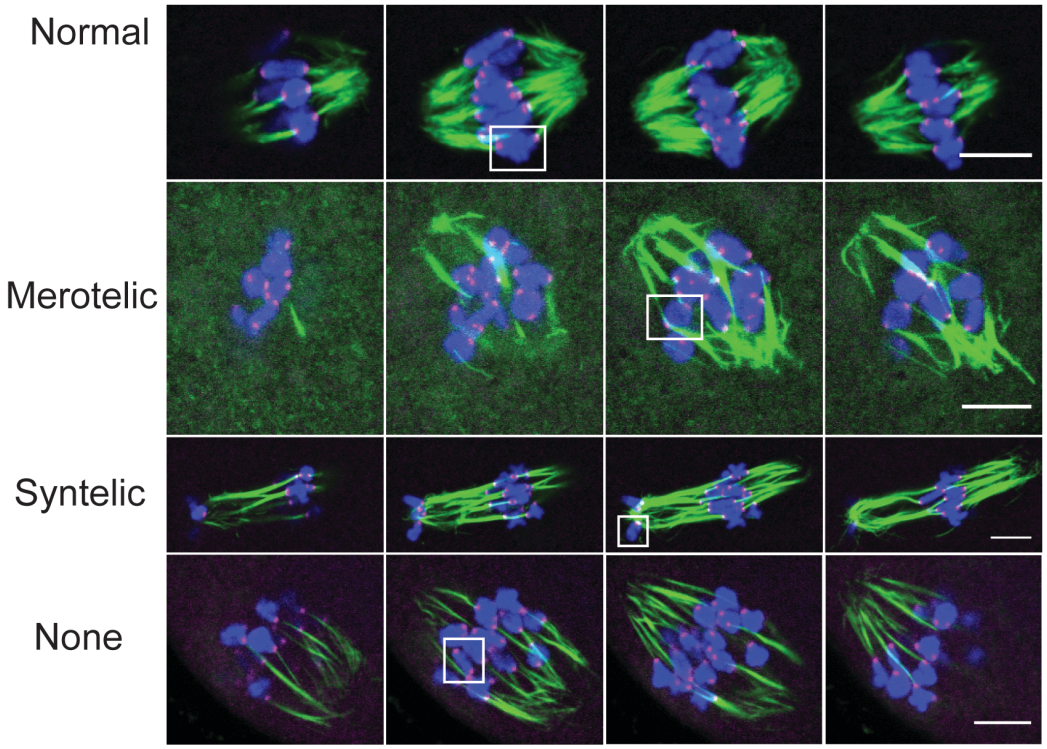


Fig. S2. z-series of representative images from error correction challenge assay. After maturation to Met I, oocytes were treated with monastrol for 2 h followed by a 3 h recovery. Prior to fixation, oocytes were incubated in ice-cold medium. After fixation, stable K-MT attachments were detected by immunocytochemistry to visualize spindle fibers (α-tubulin; green) and kinetochores (CREST; red). Representative confocal z-projections of 3-4 1μm slices of images shown in Fig. 13F (shown here highlighted by white box). Normal attachments are classified as a chromosome containing two pairs of sister kinetochores attached to opposite poles. Syntelic attachments occur when both pairs of sister kinetochores are attached to the same pole. Merotelic attachments occur when one pair of sister kinetochores is attached to both poles. The scale bar is 10 μm.



Chapter III:

Characterization of three sterility-associated Aurora kinase C mutations in oocyte meiosis

I. Abstract

Aneuploidy is the leading genetic cause of infertility and is caused by a failure of accurate chromosome segregation during gamete formation or in the early divisions of embryogenesis. Aurora kinase C (AURKC) is an important regulator of chromosome segregation in meiosis and is essential for formation of euploid sperm in humans. Mutations in AURKC correlate with tetraploid sperm and male sterility resulting from cytokinesis failure in meiosis I. Three of these mutations (AURKC-L49W, AURKC-C229Y, and AURKC-Y248X) occur in the coding region of the gene and are the focus of this study. This study is the first to show, through *ex vivo* analysis in mouse oocytes, the function of human mutant alleles in a mammalian meiotic system. We show here that their activities in meiosis I differ. AURKC-LW is a loss of function mutation, AURKC-CY retains partial function but is insufficient to support cell cycle progression alone, and AURKC-YX retains catalytic ability but is unable to localize and function with the CPC to support chromosome segregation. Finally, we show that these mutations lead to meiotic failure and polyploidy due to a failure in AURKC-CPC function that results in metaphase chromosome misalignment and activation of the spindle assembly checkpoint (SAC). This study helps to understand the function of AURKC in meiosis through analysis of naturally occurring mutations.

II. Introduction

Infertility is a problem for more than 70 million couples worldwide (Ben Khelifa et al., 2011). 7-10% of pregnancies that are clinically recognized have some form of aneuploidy (errors in chromosome number), which is usually incompatible with life, but can also result in birth defects and developmental disabilities. It is currently believed that a large portion of aneuploidies are due at least in part to genetic causes, but there has been great difficulty in identifying these causative genes due to high variability in phenotypes (Dieterich et al., 2007).

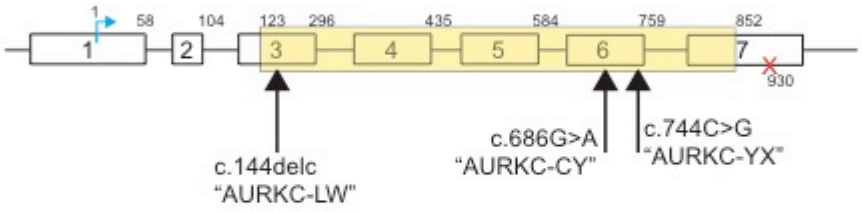
The incidence of aneuploidy is a direct result of errors in chromosome segregation during cell division, many of which occur in meiosis I (MI) (Hassold and Hunt, 2001). The chromosomal passenger complex (CPC) is a complex of proteins that binds centromeres to regulate segregation of chromosomes. The CPC in meiosis consists of an Aurora kinase (B or C, AURKB and AURKC respectively), INCENP, Survivin, and Borealin (Vader et al., 2007). The CPC is responsible for ensuring that cell division does not proceed until the chromosomes are properly aligned and attached to the spindle and that daughter cells receive the appropriate chromosomal complement and that. If any of the components are missing, the cell will arrest at metaphase and cannot proceed through anaphase (Honda et al., 2003; Kimmins et al., 2007; Sharif et al., 2010; Yang et al., 2010). The CPC acts to prevent anaphase in the event of erroneous microtubule attachments to kinetochores, by signalling for the recruitment of

spindle assembly checkpoint (SAC) proteins to the. The SAC senses attachments between the spindle microtubules and the kinetochore complexes at centromeres to ensure proper alignment prior to segregation. Once kinetochores are attached to correct spindle fibers, the SAC will be satisfied, allowing anaphase onset, or preventing it if the attachments are not there (Pinsky BA, 2005). Loss of function mutations in any of the CPC components results in missegregation of chromosomes, metaphase misalignment, cytokinesis failure, and spindle morphology defects (Adams et al., 2000; Bolton et al., 2002; Chen et al., 2003; Cooke et al., 1987; Gassmann et al., 2004; Kim et al., 1999; Wheatley et al., 2001).

Mutations in the coding region of *AURKC* have been identified in infertile men in a small population in North Africa including a few men of African descent living in Europe (Fig. 14) (Dieterich et al., 2007). This study found that 100% of the sperm from these men were tetraploid, and large-headed with multiple flagella (Dieterich et al., 2007; Harbuz et al., 2009). The presence of multiple flagella indicates that while the loss of AURKC resulted in meiotic failure, spermatogenesis was not affected. There were five SNP's identified in total and three of these are located in the coding region of the gene and are the focus of this study (Fig. 14). 85% of these men were homozygous for a single base pair deletion at position 144 which created a frameshift and leucine to tryptophan codon change (*AURKC-144.del.c* referred to here as AURKC-LW) leading to a premature stop codon 19 missense residues later (Fig. 14B-C (Dieterich et al., 2007)). This allele was estimated to have a frequency of 1 in 10,000 in this

Fig. 14- Three sterility-associated mutations in human *AURKC* occur in the coding region. (A) Three mutations occur within the exons (AURK-LW: exon 3, AURKC-CY and AURKC-YX: exon 6). The blue arrow indicates the ATG start codon while the red X indicates the stop codon. The yellow shaded box indicates the catalytic domain. **(B)** AURKC-LW is a single base pair deletion at position 144 resulting in a frameshift. AURKC-CY is a single base pair substitution (G/A) at nucleotide 686. AURKC-YX is a single base pair substitution (C/A) at nucleotide 744. All mutated bases are shown in red. **(C)** The AURKC-LW deletion results in a frameshift mutation resulting in a premature stop codon 23 missense residues later. The AURKC-CY mutation results in a cysteine to tyrosine change at residue 229 in the catalytic domain. AURKC-YX is caused by a tyrosine changed to a stop codon at the end of the kinase domain. All missense residues are shown in red.

A



B

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AURKC-WT   1-140 TCCCCTGGGC   151-680 GCTCTGCTAT   691-740 TTACAGACGC   751-930
AURKC-LW   1-140 TCC-CTGGGC   151-680 GCTCTGCTAT   691-740 TTACAGACGC   751-930
AURKC-CY   1-140 TCCCCTGGGC   151-680 GCTCTACTAT   691-740 TTACAGACGC   751-930
AURKC-YX   1-140 TCCCCTGGGC   151-680 GCTCTGCTAT   691-740 TTAAAGACGC   751-930
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C

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AURKC-WT   1-41 DFEIGRPLGK GKFGNVYLAR LKESHFIVAL   72-221  LWCIGVLCYE LLVGYPPFES ASHSETYRRI 252-309
AURKC-LW   1-41 DFEIGRPWAR GNLGMCTWLG SRKAISLWPX
AURKC-CY   1-41 DFEIGRPLGK GKFGNVYLAR LKESHFIVAL   72-221  LWCIGVLYYE LLVGYPPFES ASHSETYRRI 252-309
AURKC-YX   1-41 DFEIGRPLGK GKFGNVYLAR LKESHFIVAL   72-221  LWCIGVLCYE LLVGYPPFES ASHSETX
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population (Harbuz et al., 2009). Other men were compound heterozygous for *AURKC-LW* and one of the other SNP's, single base pair substitutions at position 686 (G/A) or 744 (C/A), (Fig. 14B-C). The G/A at position 686 causes a cysteine to tyrosine change at residue 229 (AURKC-CY, Fig. 14B-C (Dieterich et al., 2009)), while the C/A at 744 causes a tyrosine to change to a stop codon at residue 258 (AURKC-YX, Fig. 14B-C (Dieterich et al., 2009)). After interviews with family members, the study also found that members heterozygous for any of these mutations were fertile with no reports of conception trouble and that 2 female relatives who were homozygous for AURKC-LW reported conceiving children (Dieterich et al., 2007). No additional information is available regarding age, difficulty conceiving, reproductive lifespan, or rates of miscarriage to provide insight of the effect of these mutations on female fertility.

Recent work has shown that oocytes and sperm express different levels and complements of three AURKC splice variants (Fellmeth et al., 2015). There is also evidence in mice that the function or requirement for AURKC is not equal between males and females. *Aurkc*$^{-/-}$ mice were used to study the requirement for AURKC in spermatogenesis and were found to have no gross morphological defects except for the sperm, but did have reduced litter sizes or were sterile (Kimmins et al., 2007). These sperm exhibited acrosome defects and errors in chromatin condensation. Female *Aurkc*$^{-/-}$ mice are also subfertile though not as severe as the males (Schindler et al., 2012). *Aurkc*$^{-/-}$ oocytes either arrest at metaphase I (MI) with misaligned chromosomes or display defects in cytokinesis (Schindler et al., 2012). These studies indicate that the requirement for AURKC

in male and female gametogenesis is different and understanding the function of this protein in meiosis could lead us to a better understand of fertility and reproduction in humans.

III. Materials and Methods

Ethical approval

All animals were maintained following the Rutgers Institutional Animal Use and Care Committee (#11-032) and the National Institutes of Health guidelines.

Murine oocyte preparation and microinjection

Fully-grown, GV-intact oocytes were collected from 6-9 week old *Aurkc*$^{-/-}$ mice (Kimmins et al., 2007; Schindler et al., 2012) or CF-1 mice (Harlan Laboratories, #NSACF1, Indianapolis, USA) that were pregnant mare's serum gonadotropin (PMSG) (Calbiochem #367222) primed (44-48 h before collection) as previously described (Anger et al., 2005). Oocytes were cultured and matured as previously described (Schindler et al., 2012). The collection and injection medium was bicarbonate-free minimal essential medium (MEM) containing, 25 mM Hepes, pH 7.3, 3 mg/ml polyvinylpyrollidone (MEM/PVP) and 2.5 μM milrinone (Sigma- Aldrich #M4659, St. Louis, USA) to prevent meiotic resumption (Tsafriri et al., 1996). To control for mouse-to-mouse variation, denuded oocytes were kept separate for each mouse and divided equally amongst experimental groups. We injected each oocyte with 0.3pM/μl of cRNA,

as previously described (Anger et al., 2005). After injection, oocytes were incubated overnight (for MI experiments) or 3 hours (for MII experiments) in Chatot, Ziomek, and Bavister (CZB) medium containing 2.5 μM milrinone before meiotic resumption was initiated. All culture and in vitro meiotic maturation occurred in a humidified incubator with 5% CO2 in air at 37°C. For Met I analyses, oocytes matured for 7-8 h and for Met II analyses, oocytes matured for 16 h. In vitro maturation was conducted in CZB medium without milrinone.

Challenge of kinetochore-microtubule attachment correction

After 7 hours of meiotic maturation, oocytes were incubated in CZB containing 100 μM monastrol (Sigma Aldrich, #M8515, St. Louis, USA) to induce monopolar spindle formation. After 2 hours oocytes were washed out of monastrol-containing media and allowed to recover in CZB containing 5 μM MG132 (Calbiochem, #474791, Darmstadt, Germany) to prevent anaphase onset. After 3 hours, oocytes were incubated for 7 minutes in pre-chilled MEM on ice to destabilize unattached microtubules. Oocytes were fixed as described below.

Live Imaging

After microinjection and 3 hours of expression time, oocytes were transferred into separate drops of CZB medium in a 96 well dish (Greiner Bio One, #655892, Monroe, USA). Bright field images were acquired using an EVOS FL Auto Imaging System (Life Technologies, Grand Island, USA) with a 10X

objective. The microscope stage was heated to 37°C and 5% CO_2 was maintained using the EVOS Onstage Incubator. Images were acquired every 20 minutes for 20 hours.

Cloning and synthesis of cRNA

All mutants were cloned using a *AURKC_v1-Gfp* construct (Fellmeth et al., 2015). *AURKC-LW* and *AURKC-YX* were PCR amplified using the AURKC_v1 forward primer previously described and reverse primers to remove the stop codons to allow for GFP expression (AURKC-LW 5'-GATCGTCGACGGGCCACAATGAAATGGC -3' AURKC-YX 5'-GATCGTCGACGTCTCACTGTGGGAGG -3') (Fellmeth et al., 2015). The PCR constructs were then ligated into pIVT-EGFP (Igarashi et al., 2007) using SphI and SalI. *AURKC-CY* was created using the QuikChange Lightening Multi Site-Directed Mutagenesis Kit (Agilent, #210516, Foster City, USA) according to the manufacturer's protocol with a mutagenic primer (5'-GGATTTGTGGTGCATTGGAGTGCTCTACTATGAGCTGCTGGTGGG -3') to mutate the *AURKC-v1-Gfp* construct. We performed *in vitro* transcription with a mMessage mMachine kit (Ambion, #AM1344M, Grand Island, USA) per manufacturer's instructions to make cRNA. cRNA was purified using an RNA-Easy purification kit (Qiagen, #74104, Venlo, Netherlands) and eluted in RNase free H_2O. All constructs were diluted in RNase free H_2O to a concentration of 0.3pM.

Immunocytochemistry

After maturation, oocytes were fixed in phosphate buffered saline (PBS) with 2% paraformaldehyde for 20 minutes (or 4% PFA +0.1% (vol/vol) Triton X-100 for 30 minutes for Survivin staining) at room temperature and then washed through blocking buffer (PBS + 0.3% (wt/vol) BSA + 0.01% (vol/vol) Tween-20). Prior to immunostaining, oocytes were permeabilized for 15 min in PBS containing 0.1% (vol/vol) Triton X-100 and 0.3% (wt/vol) BSA. Cells were washed in blocking buffer (PBS+0.3% BSA+0.01% Tween-20). Immunostaining was performed by incubating in primary antibody; pINCENP [gift from M. Lampson, University of Pennsylvania; (Salimian et al., 2011) 1:1000], acetylated tubulin [Sigma Aldrich, #T7451; 1:1000, St. Louis, USA], CREST [Antibodies Incorporated, #15-234, Davis, USA; 1:30], Survivin [Cell Signaling Technologies, #2808s, 1:500], or Alexa-fluor 488 conjugated alpha-tubulin [Sigma Aldrich, #T9026; 1:100, St. Louis, USA]) for 1 hour. After washing, secondary antibodies (anti-rabbit [Life Technologies, #A10042, Grand Island, USA], anti-mouse [Life Technologies, #A10037, Grand Island, USA], anti-human [Life Technologies, #A21091, Grand Island, USA]) were diluted 1:200 in blocking solution and the sample incubated for 1 h at room temperature. After washing, cells were mounted in VectaShield (Vector Laboratories, #H-1000, Burlingame, USA) with 4', 6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI; Life Technologies #D1306; 1:170, Grand Island, USA). Fluorescence was visualized on a Zeiss 510 Meta laser-scanning confocal microscope with a 40X objective. The laser power

was adjusted to just below saturation relative to the group exhibiting the highest level of signal intensity and all images were scanned at this laser power.

Immunoblotting

Injected oocytes were incubated overnight in CZB with milrinone to allow for protein expression before snap-freezing in MEM at -80C. Oocytes were lysed in 1% SDS, 1% β-mercaptoethanol, 20% glycerol, and 50 mM Tris-HCl (ph6.8), and denatured at 95°C for 10 min. Proteins were separated by electrophoresis in 10% SDS polyacrylamide precast gel (Bio-Rad, #456-1036, Hercules, USA). Stained proteins of known molecular mass (range 10-250 kDa, Bio-Rad, #161-0376, Hercules, USA) were run simultaneously as standards. The separated polypeptides were transferred to nitrocellulose membranes (Bio Rad, #170-4156, Hercules, USA) using a Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, USA) and then blocked with 2% ECL blocking (Amersham, #RPN418, Pittsburgh, USA) solution in TBS-T (Tris-buffered saline with 0.1% Tween 20) for 1 hour. The membranes were incubated with α-tubulin primary antibody (Sigma-Aldrich, #T-6074, St. Louis, USA; 1:10,000) overnight or GFP primary antibody (Sigma-Aldrich, #G6539, St. Louis, USA; 1:1,000) for 1 hour. After washing with TBS-T five times, the membranes were incubated with a secondary antibody labeled with horseradish peroxidase (GE Healthcare Biosciences, #NA931, Pittsburgh, USA) for 1 hour followed with washing with TBS-T five times. The signals were detected using the ECL Select Western blotting detection reagents (Amersham, #RPN2235, Pittsburgh, USA) following the manufacturers protocol.

Ploidy Analysis

After maturation to Met II, oocytes were treated with monastrol for 2 h before fixing as described above. Fixed oocytes were stained with CREST to visualize kinetochores. Images 0.4 μm slices thick were taken via confocal microscopy. Pairs of CREST foci were quantified with a euploid egg expected to contain 20 pairs. Any deviance from 20 was categorized as aneuploid.

Image Analysis

All images were processed using ImageJ software (NIH, Bethesda, USA). Alignment measurements were performed as previously described using the same processing parameters for all images (Lane et al., 2012). pINCENP and Survivin intensity measurements were performed by averaging region of interest (using the free hand tool to trace 6 chromosomes on the DNA channel and measuring the intensity of those regions on the channel of interest) measurements per oocyte, subtracting an average of 3 background measurements, and normalizing to average expression in the GFP control group.

Statistical Analysis

One-way ANOVA was used to evaluate the differences between groups using Prism Graphpad software (La Jolla, USA). For experiments analyzing chromosome alignment, a permutation version of the binomial proportions test (Snedecor and Cochran, 1989) was used to analyze differences between groups.

Fisher's Method (Fisher, 1925) was used to combine the results of several independent tests with the same null hypothesis. The Bonferroni correction was used to determine if the Fisher-Method p-values were significant after multiple testing. $P < 0.05$ was considered significant.

IV. Results

AURKC mutants do not support accurate chromosome segregation or cell cycle progression

The predominant phenotype of men with *AURKC* mutations is errors in MI leading to tetraploid gametes. There is currently no human meiotic system that can be used for characterizing these mutations. We developed a model system using *Aurkc*$^{-/-}$ oocytes to study human AURKC *ex vivo* (Fellmeth et al., 2015). For this study, we generated *Gfp*-tagged cRNA constructs to be injected into *Aurkc*$^{-/-}$ oocytes to characterize the biological significance of the mutations.

To begin this investigation, we first established that the phenotype in the *Aurkc*$^{-/-}$ mouse oocyte model is similar to those observed in humans that express these mutant alleles of AURKC (Fellmeth et al., 2015; Kimmins et al., 2007; Schindler et al., 2012). Oocytes were injected with *Gfp*-tagged cRNA for the mutants, a WT control, or a Gfp-only control and matured 16 hours to Met II. Oocytess were assessed for completion of meiosis I (MI) by the presence of univalent chromosomes, while bivalent chromosomes indicated Met I arrest. In the Gfp control group, we observed arrest at Met I approximately 55% of the

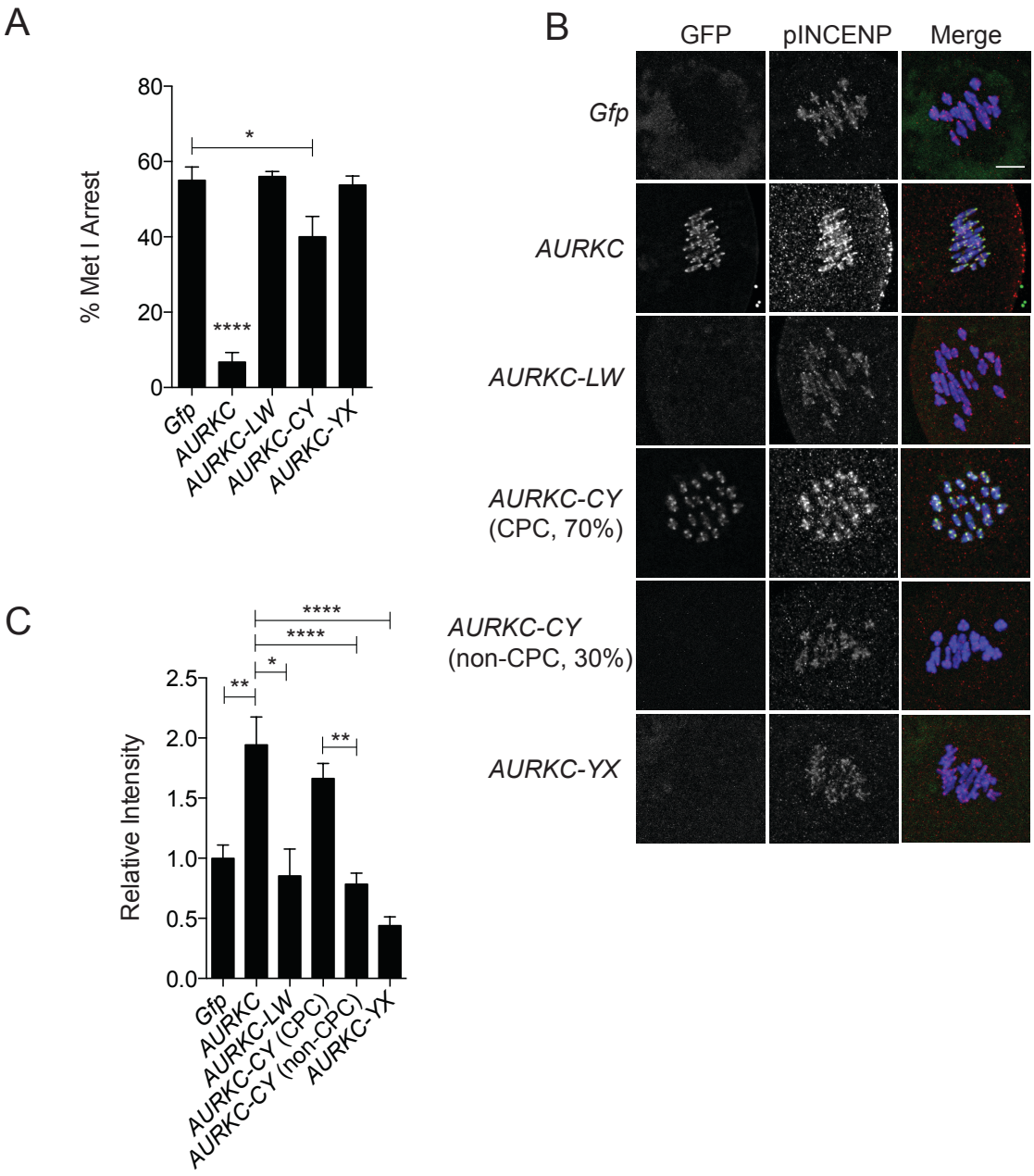
time, which resulted in polyploid eggs (Fig. 15A). This phenotype was rescued by injection with AURKC-WT. *Aurkc*$^{-/-}$ oocytes injected with *AURKC-LW and AURKC-YX* did not rescue the Met I arrest phenotype; however, injection of AURKC-CY did have a partial rescue (40%, P=0.0425, Fig. 15A). The inability to rescue the Met I arrest phenotype due to loss of mouse AURKC mirrors the tetraploid phenotype seen in infertile men. Therefore it is evident from this assessment, that the *Aurkc*$^{-/-}$ oocytes can be used to study the sterility-associated *AURKC* mutations because the polyploid phenotype is similar in human sperm and in mouse oocytes when the mutants are expressed.

To confirm equal expression levels of the mutants in oocytes, we performed Western blot analysis to detect the GFP tag (Fig. S3). GFP signal was observed at the expected sizes (38kDa, 66kDa, and 59kDa). The expression levels varied to the same degree as the molar concentration injected. Due to the size difference resulting in different molar amounts when injected at 500ng/µl, we chose to perform the remainder of the experiments at equal molar concentrations (0.3pmol/µl).

AURKC mutants have localization and catalytic defects

We propose that all three mutations are loss of function mutations due to the loss of the kinase domain in AURKC-LW, the mutation of the critical C229 residue in AURKC-CY, and the loss of the C-terminus of AURKC-YX. This hypothesis is supported by the fact that all men homozygous for these *AURKC*

Fig. 15- Mutations in AURKC fail to rescue meiotic arrest and loss of pINCENP induced by loss of *Aurkc*. Fully grown oocytes arrested at prophase of MI from *Aurkc*$^{-/-}$ mice were injected with the indicated *Gfp*-tagged cRNA and **(A)** matured 18 h to Met II or **(B)** 8 h to Met I (A) and assessed for presence of bivalents indicating Met I arrest, (B) Representative z-projections of images obtained by confocal microscopy. DNA was detected by DAPI staining (blue). Detection of GFP is green in the merge. Immunocytochemistry was performed to detect phosphorylated INCENP (red in merge). **(C)** Relative intensity of pINCENP on chromosomes from experiments depicted in (B) after normalization to the GFP control group at Met I. Scale bars represent 10 μm. Experiments were performed twice each with a total of four mice. All data was analyzed by One-way ANOVA and error bars represent the mean (±SEM); *P=0.0159-0.0425, **P=0.0031-0.0048, ****P<0.0001.


A
% Met I Arrest
80
60
40
20
0
*
****
Gfp
AURKC
AURKC-LW
AURKC-CY
AURKC-YX
B
GFP
pINCENP
Merge
Gfp
AURKC
AURKC-LW
AURKC-CY
(CPC, 70%)
AURKC-CY
(non-CPC, 30%)
AURKC-YX
C
Relative Intensity
2.5
2.0
1.5
1.0
0.5
0.0
****
****
*
**
**
Gfp
AURKC
AURKC-LW
AURKC-CY (CPC)
AURKC-CY (non-CPC)
AURKC-YX

mutations are infertile with an invariant phenotype of polyploidy (Ben Khelifa et al., 2012; Dieterich et al., 2007; Dieterich et al., 2009) and that injection of any of the mutants does not rescue the polyploidy phenotype observed in *Aurkc*$^{-/-}$ oocytes (Fig. 15A).

Because AURKC catalytic activity is required for its localization, we hypohtesized that these loss of function mutants would fail to localize in our system. To test this hypothesis, we injected *Aurkc*$^{-/-}$ oocytes with *Gfp*-tagged cRNA for each mutation as well as a Gfp and a WT control, matured them to Met I, and performed immunocytochemistry to detect pINCENP (the active form of the scaffolding protein in the CPC). We observed no localization of the GFP control in any oocytes indicating that the tag itself does not affect localization of the constructs (Fig. 15B). Wildtype AURKC was found along the interchromatid axis (ICA) and at centromeres at Met I (Fig 15B). AURKC-LW and AURKC-YX failed to localize with the CPC as expected, indicating that they are amorphic. However, AURKC-CY was observed at the ICA and centromeres 70% of the time although greatly reduced compared to wildtype (Fig 15B). For the remaining experiments, the data from the AURKC-CY-injected oocytes were divided into subsets of localized (CPC) or non-localized (non-CPC) oocytes unless otherwise specified. The partial localization of AURKC-CY suggests that this mutant retains partial catalytic activity, consistent with the partial rescue of Met I arrest (Fig. 15A).

To show that the localization failure is accompanied with a loss of active CPC, we quantified the intensity of pINCENP. GFP-control oocytes exhibit a low

level of pINCENP intensity, which is due to the presence of endogenous AURKB-CPC, and this intensity is increased nearly two-fold when AURKC is expressed (Fig. 15B-C). Only the CPC localized AURKC-CY phosphorylated INCENP similar to AURKC (Fig. 15C). The observation that AURKC-CY is able to phosphorylate INCENP when localized suggests that it is a hypomorph rather than a complete loss of function mutation. The inability of AURKC-LW and AURKC-YX to activate the CPC supports the conclusion that they are both loss of function mutations.

AURKC-C229Y is a hypomorph

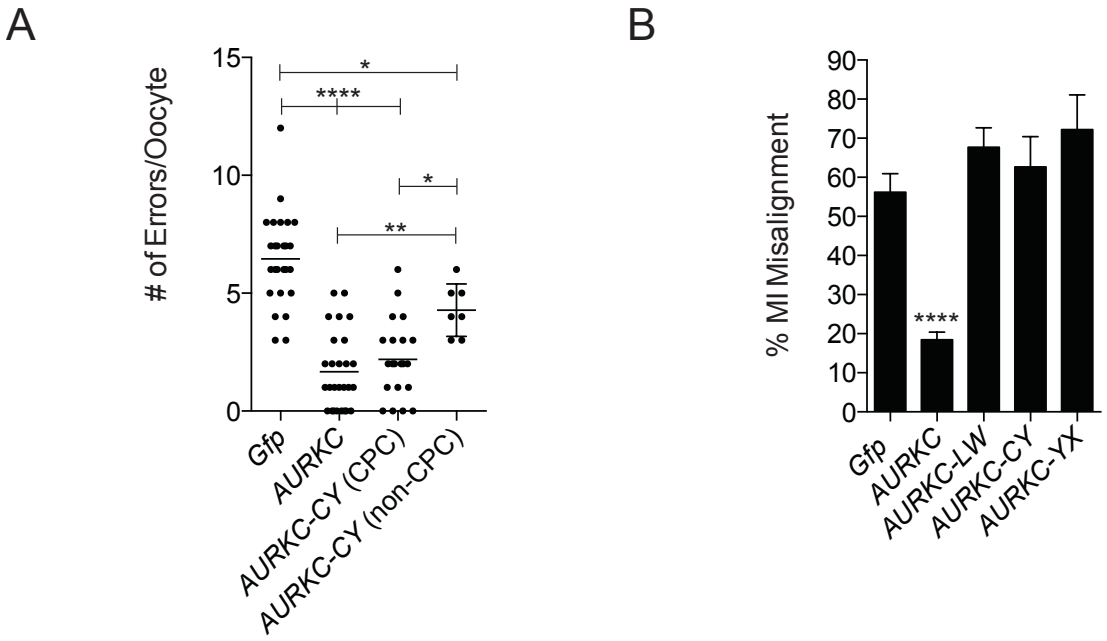
AURKC-LW was the first AURKC mutant identified, and AURKC-CY was found in a second screen (Dieterich et al., 2007; Dieterich et al., 2009). The mutation was found in the compound heterozygous state with an AURKC-LW allele in a single patient (Dieterich et al., 2009). It is possible that other patients with this mutation exist; however many of the early AURKC screens performed only sequenced exon 3 (where AURKC-LW lies). These men also presented with 100% tetraploid sperm with multiple flagella, and there was no variation in the phenotype compared to patients homozygous for AURKC-LW.

The reduced localization of AURKC-CY and its ability to phosphorylate INCENP in most cells suggests that AURKC-CY is a hypermorph. One of the primary functions of AURKC in meiosis is to correct erroneous attachments between kinetochores and spindle microtubules (Balboula and Schindler, 2014). To assess whether this function is affected by the C229Y mutation, we performed

a K-MT correction challenge assay (Fellmeth et al., 2015) where oocytes are matured to Met I and treated with monastrol to induce 100% improper attachments. Monastrol is then washed out of the maturation medium and the oocytes are allowed to recover for 2h. The oocytes are cold treated prior to fixation to destabilize any unattached spindle fibers to enhance visualization of attachments. *Gfp*-injected oocytes corrected slightly more than half of the 20 errors created by monastrol treatment (~7 errors remaining per cell, Fig. 16A) indicating the ability of the AURKB-CPC to partially compensate for this function in *Aurkc*$^{-/-}$ oocytes. Injection of AURKC rescued this defect to ~1.6 errors per cell. AURKC-CY corrected the majority of the syntelic attachments (~2.2 errors per cell, Fig. 16A) in the subset of oocytes where the protein localized and this rate was not significantly different from the correction rate observed in AURKC-injected oocytes.

Another function of the AURKC-CPC in meiosis is regulating the alignment of chromosomes at the metaphase plate (Balboula and Schindler, 2014). There is mounting evidence that maintenance of chromosome alignment at metaphase is correlated with proper biorientation of chromosomes (Balboula and Schindler, 2014; Brunet et al., 1999; Lampson et al., 2004). To assess whether the ability of AURKC-CY to correct attachments results in an ability to also support metaphase alignment, we matured oocytes injected with each construct to Met I and quantified misalignment of chromosomes. Chromosome misalignment was observed in approximately 55% of the Gfp-injected oocytes, which was rescued by injection of AURKC (18% misalignment). Neither AURKC-LW or AURKC-YX

Fig. 16- AURKC-CY is a hypomorph. Fully grown oocytes arrested at prophase of MI from *Aurkc*$^{-/-}$ mice were injected with the indicated *Gfp*-tagged cRNA and matured 8 h to Met I prior to fixation. **(A)** After maturation to Met I, oocytes were treated with monastrol for 2 h followed by a 3 h recovery. Prior to fixation, oocytes were incubated in ice-cold medium. After fixation, stable K-MT attachments were detected by immunocytochemistry to visualize spindle fibers (α-tubulin) and kinetochores (CREST). Each data point represents the number of improper K-MT attachments quantified in a single oocyte image. **(B)** Misaligned chromosomes at Met I were identified as described in methods. Experiments were performed twice using a total of four mice. All data was analyzed by One-way ANOVA and error bars represent the mean (±SEM); *P=0.0193-0.0314, **P=0.0030, ****P<0.0001.



rescued the misalignment of chromosomes. Even though AURKC-CY corrects most of the syntelic attachments (Fig. 16A), this activity did not improve the alignment of chromosomes at Met I in *Aurkc*$^{-/-}$ oocytes in either subset (localized or not, Fig. 16B). The above evidence supports the conclusion that AURKC-CY is a hypomorph. We hypothesize that this allele does not retain sufficient AURKC activity to support meiotic progression when expressed with AURKC-LW in sperm. Future experiments will be aimed at co-expression of AURKC-CY with AURKC-LW to investigate this hypothesis further.

AURKC-L49W is a loss of function mutation with partial dominant negative activity

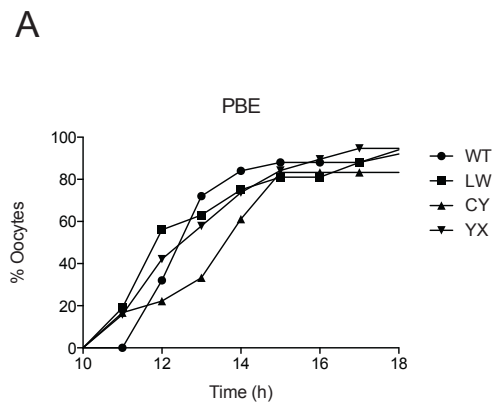
Despite lacking a functional catalytic domain (Fig. 14-15), AURKC-LW is the most frequent genetic cause of male sterility currently identified (1 in 10,000 men) (Ounis et al., 2015). A previous study proposed that if AURKC-LW had a dominant negative affect on AURKC in meiosis, the heterozygous parents of the sterile men would experience higher than normal rates of miscarriage (Ounis et al., 2014). Some evidence from interviews of such couples found that they do report miscarriages (Ounis et al., 2014). We chose to investigate three major functions of the AURK-CPC in meiosis: SAC function, polar body extrusion, and spindle formation. To investigate the potential dominant negative activity of AURKC-LW, we expressed the mutant constructs in WT oocytes and matured them to Met II to quantify polar body extrusion (PBE) as a measure of meiotic progression. There was no significant difference in the time it took for the mutant

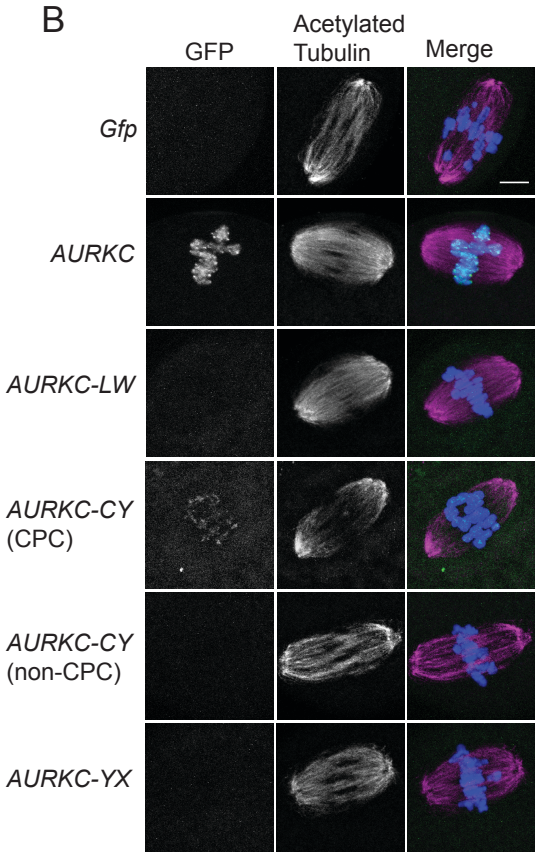
injected oocytes to progress through meiosis (Fig. 17A) compared to WT controls. There was also no difference in the percentage of oocytes that were able to extrude a polar body indicating that none of the mutants have a dominant negative affect in mouse oocytes (Fig. 17A).

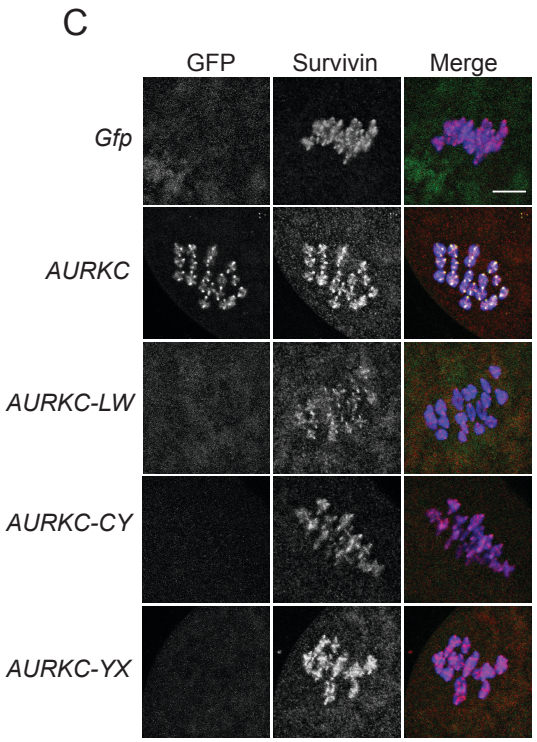
If AURKC-LW blocks function of endogenous AURKB, we would also expect to see defects in formation of a bipolar spindle (Balboula and Schindler, 2014). To test this hypothesis, mutant injected *Aurkc*$^{-/-}$ oocytes were matured to Met I and stained with an acetylated tubulin antibody to detect the spindle. There were no spindle defects observed in any of the oocytes examined indicating the inability to block AURKB-CPC from forming a bipolar spindle (Fig. 17B).

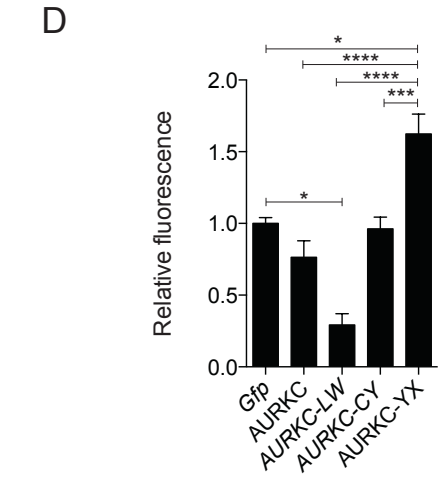
Finally, a loss of CPC localization would be observed if any of the mutants were blocking the function of AURKB in meiosis. In Aurkc-/- oocytes, a dominant negative form of AURKC blocks the localization of endogenous AURKB(Balboula and Schindler, 2014). We matured mutant injected oocytes to Met I and detected Survivin by immunostaining to visualize the localization of the CPC. There was no quantifiable difference in intensity of the Survivin antibody in the GFP, WT, or AURKC-CY groups indicating that these constructs do not have an effect on AURKB-CPC localization (Fig. 18A-B). However, we did observe a significant decrease in Survivin intensity in oocytes injected with AURKC-LW (70% less than GFP control, Fig. 18A-B). These data supports the conclusion that AURKC-LW has dominant negative activity on the function of AURKB by blocking the localization of the CPC.

Fig. 17- AURKC-LW has partial dominant negative affect over AURKB. (A) Fully grown oocytes arrested at prophase of MI from CF-1 mice were injected with the indicated *Gfp*-tagged cRNA and matured 18 h to Met II on the EVOS microscope. Images were taken at 20 min intervals and the percentage of oocytes in the group with a polar body at each time point was counted. **(B-D)** Fully grown oocytes arrested at prophase of MI from *Aurkc*$^{-/-}$ mice were injected with the indicated *Gfp*-tagged cRNA and matured 8 h to Met I. Oocytes were fixed and immunocytochemistry was performed to detect (A) the spindle (acetylated tubulin, purple in the merge) or (B) Survivin. DNA was detected with DAPI (blue in merge) and detection of GFP is green in the merge. (D) Quantification of Survivin intensity from oocytes imaged in (C). Scale bars represent 10 μm. Experiments were performed twice using a total of four mice. All data was analyzed by One-way ANOVA and error bars represent the mean (±SEM); *P=0.0173, **P=0.0072, ***P=0.0003, ****P<0.0001.


A
PBE
% Oocytes
Time (h)
WT
LW
CY
YX


B
GFP
Acetylated Tubulin
Merge
Gfp
AURKC
AURKC-LW
AURKC-CY (CPC)
AURKC-CY (non-CPC)
AURKC-YX


C
GFP
Survivin
Merge
Gfp
AURKC
AURKC-LW
AURKC-CY
AURKC-YX


D
Relative fluorescence
Gfp
AURKC
AURKC-LW
AURKC-CY
AURKC-YX

In this system, we did not observe the expected changes in PBE or spindle morphology associated with a loss of AURKB, but we quantified a significant decrease in Survivin localization on the chromosomes. Therefore we conclude that the N-terminal fragment of AURKC-LW binds AURKB and partially blocks its function in a way that does not inhibit cell cycle progression in WT oocytes.

AURKC-Y248X has mislocalized function

AURKB has a 9-residue consensus sequence (WVRANSRVV) in the C-terminus of the protein that is required for localization of the protein with the CPC in HeLa cell mitosis (Scrittori et al., 2005). This consensus sequence is conserved in AURKC (WVQAHSRRV) and is part of the region lost in to the AURKC-YX truncation. Therefore we hypothesized that the localization failure observed in AURKC-YX injected oocytes (Fig. 15B) was due to a loss of this consensus sequence rather than a loss of function.

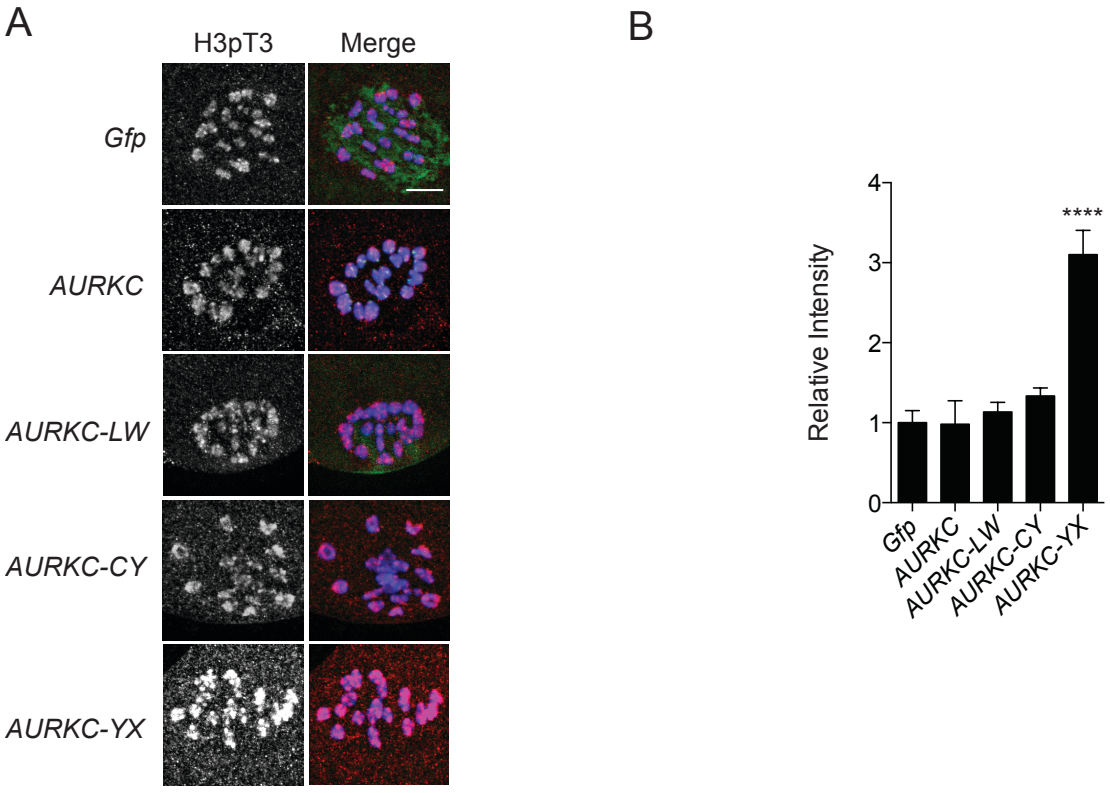
Although the function of AURKC is required for its proper localization with the CPC in meiosis (Balboula and Schindler, 2014), a failure to localize does not mean that the kinase is inactive (Scrittori et al., 2005). We proposed that AURKC-YX might retain catalytic function and act on substrates in the cytoplasm. There is a feedback loop in mitotic cells where Haspin kinase phosphorylates histone 3 at threonine 3 (H3T3), thereby recruiting the CPC to chromosomes (Dai and Higgins, 2005). AURKC then activates via autophosphorylation and feeds

back to activate Haspin further (Dai et al., 2006). The observed increase in Survivin intensity (Fig. 17C-D) is supportive of this hypothesis.

To confirm that the observed increase in Survivin localization in AURKC-YX injected oocytes is due to increased H3pT3, we stained Met I oocytes with an antibody specific for phosphorylated H3T3 to assess Haspin activity (Fig. 18A-B). Control oocytes injected with GFP or WT showed no difference in H3pT3 levels. There was also no observable change in phosphorylation of H3T3 when oocytes were injected with AURKC-LW or AURKC-CY. However, we found that there were significantly higher levels of H3pT3 in oocytes injected with *AURKC-YX* compared to the WT or Gfp control groups (~3 fold increase, Fig. 18A-B). These data supports the hypothesis that AURKC-YX retains catalytic ability despite a failure to localize and that it activates Haspin resulting in increased H3pT3.

The increased intensity of Survivin and H3pT3 immunostaining (Fig. 17C-D, 18) in AURKC-YX –injected oocytes supports the hypothesis that AURKC-YX activates Haspin in the cytoplasm. However, its failure to localize prevents the function of the AURKC-CPC resulting in an inability to support chromosome segregation and meiotic progression (Fig. 15-16). To confirm these findings, we propose using AURKB and Haspin inhibitors on AURKC-YX-injected cells, which should ablate the increase in Survivin and H3pT3. We also propose characterizing a mutant that lacks the C-terminal consensus sequence but retains the rest of the C-terminus of AURKC to confirm that this sequence is required for localization and that this phenotype is due to a localization failure.

Fig. 18- AURKC-YX recruits Survivin through Haspin activation. Fully grown oocytes arrested at prophase of MI from *Aurkc*$^{-/-}$ mice were injected with the indicated *Gfp*-tagged cRNA and matured 8 h to Met I prior to fixation. **(A)** Representative z-projections of oocytes. DNA was detected by DAPI staining (blue). Detection of GFP is green in the merge. Immunocytochemistry was performed to detect H3pT3 (red in merge). **(B)** Relative intensity of H3pT3 on chromosomes from experiments depicted in (A) after normalization to the GFP control group at Met I. Scale bars represent 10 μm. Experiments were performed twice using a total of four mice. All data was analyzed by One-way ANOVA and error bars represent the mean (±SEM); ****P<0.0001.



Discussion:

Men with homozygous mutations in *AURKC* have a completely penetrant phenotype of tetraploid sperm with multiple flagella, acrosome and chromosome condensation defects, and are sterile. Here we report a characterization of three of these mutations and propose that the sterility is caused by a failure of the AURKC-CPC to perform its normal function in MI to support chromosome alignment which leads to metaphase I arrest and polyploidy.

AURKC-LW is an antimorphic allele. AURKC-LW is a mutant that expresses only the N-terminus of the protein and is lacking the entire kinase domain (Fig. 14). AURKC-LW fails to localize to chromosomes with the CPC which is in agreement with our previous study showing that the N-terminus of AURKC does not play a role in localization (Fig. 15B (Fellmeth et al., 2015). Due to its lack of kinase domain, the protein cannot phosphorylate INCENP and activate the CPC resulting in a failure to rescue the Met I chromosome misalignment and arrest in *Aurkc*$^{-/-}$ oocytes (Fig. 15B-C, 16B). However the loss of Survivin localization in AURKC-LW expressing cells supports the conclusion that it has dominant negative activity by inhibiting the ability of the AURKB-CPC from binding to the chromosomes (Fig. 17C-D). We propose further investigating the localization of AURKB in these cells to confirm this phenotype, as well as biochemical studies to assess the binding of the AURKC-LW protein with AURKB.

AURKC-CY is a hypomorph. AURKC-CY has a single base pair substitution at position 686 resulting in a cysteine to tyrosine change at amino acid 229. This position lies within subdomain IX of the kinase domain. This subdomain (helix F) is the critical anchoring point for the catalytic and regulatory spines that stabilize the ATP-binding pocket when the kinase is in its active form (Hanks and Hunter, 1995; Taylor and Kornev, 2011). C229 is adjacent to L228, a residue of the catalytic spine. Changing the side chain at position 229 from a slightly polar cysteine to a highly hydrophobic tyrosine could alter the structure of the spine and therefore the binding pocket. This cysteine residue is highly conserved in many kinases indicating its importance in kinase function.

In humans, AURKC-CY has only been identified in the heterozygous state with AURKC-LW. It was assumed amorphic because these men are infertile and the phenotype is indistinguishable from that of the AURKC-LW homozygous men. However, we found that AURKC-CY retains partial catalytic activity as indicated by its ability to localize (Fig. 15B), phosphorylate INCENP (Fig 15B-C), and correct K-MT attachments (Fig. 16A). Despite these functions it does not support meiotic progression or rescue the misalignment seen in knockout oocytes (Fig. 15A, 16A). These data support the conclusion that AURKC-CY is a hypomorph, but its level of activity is not enough to support cell cycle progression even when overexpressed in oocytes (Fig. 15A, 16B) or at physiological concentrations with an amorphic allele such as AURKC-LW in men (Dieterich et al., 2009).

It is important to note that constructs that are injected into oocytes are overexpressed and this error-correction phenotype may only be possible when the hypermorph is overexpressed. A mouse line with the CY mutation in the endogenous *Aurkc* would be necessary to investigate this phenotype at physiological levels.

AURKC-YX is a hypermorph. AURKC-YX has the most surprising phenotype where it is catalytically active but cannot perform its canonical chromosomal function because it cannot localize with the CPC (Fig. 17C-D, 18). We observed an increase in H3pT3 and Survivin localization on chromosomes compared to WT and Gfp controls (Fig. 17C-D, 18), which indicates that AURKC-YX is activating Haspin via a feedback loop in the cytoplasm. Survivin is likely bringing with it AURKB-CPC causing an increase in the localization of AURKB at the chromosomes but without an efficient AURKB antibody we will need to artificially express AURKB with a fluorescent tag to confirm this hypothesis.

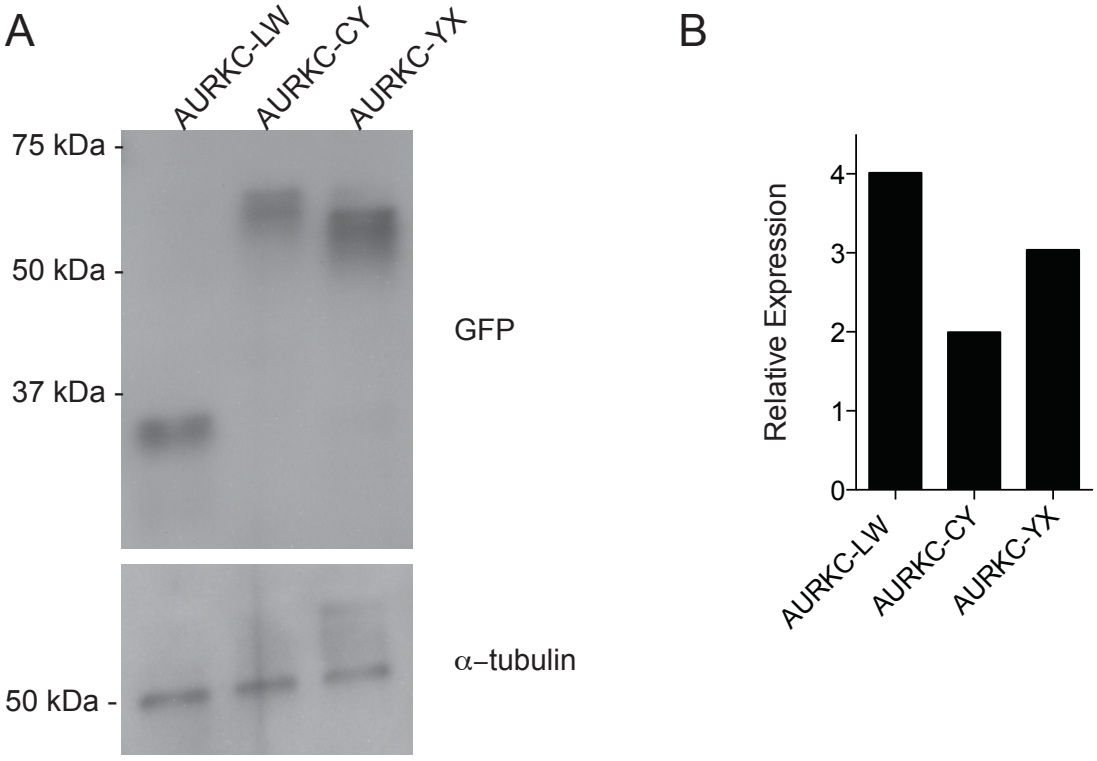
The three mutations presented here exert different effects on multiple events of MI in mouse oocytes. For example, AURKC-LW is a truncated form of the protein and is deficient in localization but has a partial dominant negative effect on AURKB. The AURKC-CY protein retains partial CPC function, but is not sufficient to support meiotic progression indicating that there is a threshold level of AURKC activity required for accurate chromosome segregation. Finally, AURKC-YX fails to localize but is able to act cytoplasmically to activate the Haspin feedback loop and recruit inactive CPC to the chromosomes. However,

the end result of all three mutations is the same: AURKC-CPC fails to function and the cells arrest at Met I with chromosome alignment defects leading to aneuploid gametes. This arrest could be due to activation of the SAC mediated by AURKB-CPC (Balboula and Schindler, 2014). Though AURKB can compensate for the majority of the functions lost in *Aurkc*$^{-/-}$ oocytes, AURKC is more effective at performing the correction of erroneous K-MT attachments (Balboula and Schindler, 2014; Schindler et al., 2012; Shuda et al., 2009). Therefore the loss of a functional AURKC results in an increase in these errors presumably causing the activation of the SAC and Met I arrest. I propose to investigate the SAC-mediated arrest by treating the mutant injected oocytes with reversine, which inhibits the SAC and should rescue the Met I arrest. I also propose to investigate the increase in errors by cold treating Met I oocytes injected with the mutants to compare the K-MT attachments to WT control.

The characterization of these mutants sheds light on the critical function of AURKC in gametogenesis. The explanation of how these mutations differentially affect female meiosis versus male meiosis is still unclear, but it is likely that women with *AURKC* mutations experience some problems with oocyte quality such as higher rates of aneuploidy or shortened reproductive lifespans leading to incompetent primordial follicles that do not mature to eggs. The understanding of how these mutations are causing the sterility phenotype does not provide us with direct treatment for these patients because there are no viable sperm that can be used for ICSI or other IVF treatments (Ben Khelifa et al., 2011; Ounis et al.,

2014), it can however, give couples insight into reasons for infertility and proper counseling on other methods of family planning.

Fig. S3 AURKC-GFP mutants are expressed equal to their molar injection quantity in mouse oocytes. (A) Fully grown oocytes arrested at prophase of MI from CF-1 mice were injected with the indicated Gfp-tagged cRNA and incubated for 16 hours. For each group, 20 oocytes were lysed and probed with the indicated antibody. The panels are images of the same membrane that was stripped and re-probed. **(B)** Quantification of bands in (A).



Chapter IV:

Conclusion and Discussion

The aim of this project was to develop a model for studying human AURKC in meiosis using *Aurkc*$^{-/-}$ mouse oocytes and to use this model for characterization of sterility-associated mutations in AURKC. Through carefully selected assays to assess the multiple functions of AURKC in meiosis and effective use of controls, I have achieved this aim which paves the way for analysis of other *AURKC* mutations, as well as mutations in other components involved in chromosome segregation in meiosis. The assessment of these mutations has also provided us with useful information regarding the function and regulation of AURKC in meiosis that deepens our understanding of chromosome segregation and its relation to reproduction and fertility.

DEVELOPMENT OF AN *Aurkc*$^{-/-}$ MOUSE MODEL FOR THE STUDY OF HUMAN AURKC

The kinase domain of human AURKC shares 87% identity with that of mouse AURKC (see Chapter II). The similarity of the kinase domains alone is not sufficient to conclude that the human protein could function in mouse oocytes. *Aurkc*$^{-/-}$ oocytes exhibit specific phenotypes that are easily assayed using immunocytochemistry and live cell imaging techniques (Balboula and Schindler, 2014; Schindler et al., 2012). I showed in Chapter II that these phenotypes (loss of pINCENP, Met I misalignment, PBE failure, and CPC localization) were rescued by overexpression of hAURKC and that this rescue is equivalent to the rescue by overexpression of mAURKC. The use of *Aurkc*$^{-/-}$ mice has already

proven useful for study the function of AURKC in spermatogenesis and in oogenesis (Balboula and Schindler, 2014; Kimmins et al., 2007; Schindler et al., 2012). By showing that the human ortholog performs the same functions to the same levels when expressed in *Aurkc*$^{-/-}$ oocytes, I have proven that the use of this model can be extended for studying the function of hAURKC allowing us to get a better understanding of the involvement of AURKC in reproduction and fertility.

hAURKC functionally compensates loss of mAURKC in oocyte meiosis

AURKC functions in meiosis through its participation in the CPC. Through immunocytochemistry and confocal imaging, I showed that hAURKC-GFP localized with the mouse CPC in oocytes throughout meiosis and rescued the misalignment of chromosomes at Met I seen in *Aurkc*$^{-/-}$ oocytes (see Ch. II). Through quantification of fluorescence intensity, I showed that hAURKC phosphorylated the mouse substrate (INCENP) indicating that human AURKC has similar affinity for the mouse substrates as mAURKC has. The ability of hAURKC to bind mouse substrates is critical for its function because, as a kinase, AURKC performs these functions by binding to and phosphorylating a variety of proteins within the cell. The phosphorylation by AURKC of proteins such as HEC1 and MCAK are involved in the error-correction function of AURKC which in turn helps to maintain the alignment of chromosomes at metaphase (Andrews et al., 2004; Cheeseman et al., 2002; Lan et al., 2004). The ability of

human AURKC to perform the same functions as mouse AURKC in oocyte meiosis suggests that hAURKC performs these same functions and interactions in human oocytes allowing us to reliably draw conclusions about the clinical relevance of observed phenotypes using this model.

Differences in function of hAURKC variants can be detected in the *Aurkc*$^{-/-}$ oocyte

The inherent effect of microinjecting cRNA constructs causes the resultant proteins to be overexpressed in the oocyte. The only way around this effect is to generate transgenic mouse lines with knock-ins of the construct of choice. Attempts by our lab to do just that have proven ineffective due to difficulties in finding guide RNAs that are specific for the sites of interest owing to large regions of homologous sequence present in AURKC and elsewhere in the mouse genome. At the present time this leaves us with the analysis of overexpression phenotypes, however I propose that these analyses are still valid owing to the fact that we can still easily observe significant differences in the ability of different variants to rescue the *Aurkc*$^{-/-}$ phenotype (see Ch. II). Three splice variants of hAURKC contain the identical catalytic domain, yet still had varying abilities to phosphorylate INCENP and correct erroneous K-MT attachments in *Aurkc*$^{-/-}$ oocytes. Expression of each variant rescued the cell cycle progression defect observed in *Aurkc*$^{-/-}$ oocytes, which supports the hypothesis that the ability to observe differences in pINCENP is a sensitive measure of the variant's affinity for

substrates that may not affect the ability of the kinase to support meiosis. Detection of sensitive phenotype is important for the analysis of proteins such as AURKC that play multiple roles in the cell. Different mutations may only affect subsets of these functions and the ability to detect small changes will lead to more accurate analysis of these variants.

Polyploidy is an orthologous phenotype in mouse and human gametes

The final component of proving the effectiveness of the *Aurkc*$^{-/-}$ oocyte as a model for studying hAURKC was the observation of similar phenotypes, phenologs, seen when mutants were expressed in mouse and human cells. The phenotype observed in men with *AURKC* mutations was meiotic arrest causing polyploidy. I showed in Chapter III that *Aurkc*$^{-/-}$ oocytes expressing mutants of *hAURKC* arrested at Met I and were also polyploid. This is further evidence of the hypothesis stated above that the functions of hAURKC are conserved between mouse and human because the same defects are observed when these functions are disrupted in both species. Phenologs are essential for the translational use of animal models for disease. It is difficult to draw useful conclusions about the relationship between a disease and a protein if the model used does not exhibit the disease phenotype. The fact that mouse oocytes exhibit the meiotic arrest and polyploid phenotype that is observed in human sperm, demonstrates the suitability of this model for studying AURKC.

ANALYSIS OF HUMAN AURKC SPLICE VARIANTS IN OOCYTE MEIOSIS

Prior to this project, the expression of splice variants of human AURKC had only been amplified from testis tissue samples (Bernard et al., 1998; Tseng et al., 1998; Yan et al., 2005c). In Chapter II, I performed qRT-PCR on single oocyte samples to show the expression of three splice variants simultaneously in each cell. Through individual analysis of each variant, I first showed that their abilities in meiosis differ and while AURKC_v2 was the most stable protein, AURKC_v1 was the most effective at supporting meiosis. However, when I expressed all three simultaneously in *Aurkc*$^{-/-}$ oocytes, as is seen in human oocytes, the error correction function was the most efficient. This synergistic effect between splice variants indicates that the different variants are able to correct errors that the others miss. This hypothesis is supported by the observation that the variants are not equally able to phosphorylate INCENP indicating differing affinities for substrates. As mentioned previously, two of these substrates critical for error correction are MCAK and HEC1, and each of these proteins also has differential affinity for correcting certain types of errors (Andrews et al., 2004; Cheeseman et al., 2002; Lan et al., 2004; Zheng et al., 1999). MCAK is more likely to destabilize merotelic attachments while HEC1 is more likely to destabilize syntelic attachments (DeLuca et al., 2006; Kline-Smith et al., 2004). If the variants have different affinities for these substrates, that could lead to the additive effect of error correction observed when they are expressed simultaneously.

This final observation also explains the necessity for three variants in oocyte meiosis, but not why sperm do not express the three simultaneously. It is possible that the prolonged arrest that oocytes undergo from birth to ovulation necessitates redundancies in proteins required for accurate chromosome segregation while this is not necessary in sperm that do not arrest. It is also possible that there are other factors that inherently lead to more errors in oocyte meiosis as opposed to sperm meiosis that requires higher activity of error correction. The increased frequency of aneuploidy in oocytes relative to sperm supports the latter hypothesis while the increased frequency of aneuploidy in older oocytes relative to younger oocytes supports the former (Brandriff et al., 1994; Hassold and Chiu, 1985; Hassold and Hunt, 2009; Hunt, 1998; Pacchierotti et al., 2007). It is likely then a combination of the two effects that requires the expression of multiple variants of AURKC in oocytes.

Human gametes are sexually dimorphic in terms of their rates of aneuploidy as well as their regulation of AURKC function in meiosis (Brandriff et al., 1994; Fellmeth et al., 2015; Kimmins et al., 2007; Pacchierotti et al., 2007; Schindler et al., 2012). Because AURKC plays a direct role in promoting proper ploidy in these cells, it is desirable to attribute the increased aneuploidy to altered AURKC regulation in oocytes. However, the evidence I presented in Chapter II showing that simultaneous expression of splice variants as seen in oocytes promotes increased fidelity of chromosome segregation relative to one variant alone as observed in sperm, argues against this hypothesis. It is still possible that differences in regulation at the protein level cause the aneuploidy rates. The

analysis performed here observed only the expression of RNA transcripts, and not whether those transcripts are recruited for translation. This analysis was beyond the scope of our study at this time for two main reasons. 1) We do not currently have antibodies specific for the different splice variants to allow immunocytochemical analysis of the proteins. 2) I do not have access to a large enough quantity of human oocytes required to run a Western blot that would be able to detect the size differences at the protein level. These analyses are important for understanding the physiological relevance of three splice variants of AURKC in oocytes and should be pursued in the future.

CHARACTERIZATION OF HUMAN AURKC MUTANTS IN MEIOSIS

The establishment of a tractable model for studying human AURKC in meiosis is critical for our understanding of this protein in reproduction. *AURKC* mutations identified in men are difficult to study in the endogenous system. Ejaculated sperm that are easily obtained from donors contain only post-meiotic sperm; making study of the function of AURKC in meiosis limited only to the resultant phenotype and does not allow for any genetic or functional manipulations. Use of an animal model for sperm meiosis results in similar difficulties due to the inability to culture sperm and the problems in manipulating the pre-meiotic spermatogonia. The *Aurkc*$^{-/-}$ mouse oocyte model for hAURKC in meiosis provides us with an easy to culture meiotic cell that addresses the characterization of the function of AURKC. This model does not address

differences in regulation or expression between the sexes, though these differences have yet to be elucidated themselves. At present, the data obtained from this model is able to provide us with clinically relevant information as to the cause of fertility defects observed in patients expressing mutations in *AURKC*. This thesis shows that mutations in *AURKC* resulting in a loss of function of the AURKC-CPC cause polyploidy and meiotic failure due to misaligned chromosomes. This allows clinicians to more adequately counsel patients with these mutations in appropriate family planning methods that do not include IVF or ICSI, which will fail due to the aneuploid state of the sperm.

AURKC-LW has partial DN activity

The *AURKC-LW* allele is expressed at the highest frequency of any other male-sterility mutation in this North African population that has been studied (Dieterich et al., 2009). The proposed explanation for this high frequency is that there may be a slight reproductive advantage due to inhibition of AURKB when the allele is expressed in the heterozygous state (Ounis et al., 2014). In Chapter III, I performed three separate assays to assess different phenotypes of AURKB inhibition: decreased time in MI, spindle defects, and loss of CPC localization. I observed a dominant negative effect only in the localization of the CPC. This observation supports the hypothesis that AURKC-LW has a dominant negative effect over AURKB. However this effect does not alter meiotic progression in our system which does not support the previously set forth hypothesis of producing a

reproductive advantage in the heterozygotes. It is possible that the advantageous phenotype exists only when the allele is expressed in males; a fertility trial using transgenic mice expressing AURKC-LW would be necessary to further investigate this hypothesis.

It is an interesting concept that the functions of the CPC in meiosis can be inhibited independent of each other. The expression of AURKC-LW reduces the localization of the CPC, but does not cause relaxation of the SAC causing a decrease in time to PBE nor does it affect the formation of a bipolar spindle. It would be interesting to identify mutants that block other CPC functions specifically to further analyze the function of these roles in meiosis.

AURKC-CY is a hypomorph

When expressed in *Aurkc*$^{-/-}$ oocytes, AURKC-CY localized with the CPC approximately 70% of the time (see Ch. III), however even in that subset, the localization was not as robust as that observed in oocytes expressing AURKC-WT. Similarly, I observed the ability of the localized subset to correct erroneous K-MT attachments and phosphorylate INCENP, indicating that the localized portion functions similar to WT in MI. I hypothesized that this observed rescue could be an artifact of the overexpression of the protein, however even when overexpressed, AURKC-CY did not support chromosome alignment or meiotic progression leading to polyploidy oocytes similar to the phenotype observed in men.

AURKC-CY has been identified as a compound heterozygote with AURKC-LW in only a single patient thus far, however the sequencing restrictions of some of the screens may have missed other patients with this mutation (Dieterich et al., 2009). It is important to analyze clinically relevant mutations as close to their endogenous state as possible, so I propose the analysis of oocytes expressing *AURKC-CY* with *AURKC-LW* at a 1:1 ratio to confirm the observations from Chapter III.

The identification of a hypermorph of AURKC is compelling for the study of AURKC in general. The ability of AURKC-CY to phosphorylate INCENP and correct errors indicates maintenance of catalytic function albeit reduced. The observations in this study indicate that there is a threshold level of AURKC function that is required to support chromosome segregation and meiotic progression, which is not achieved by AURKC-CY. This allele may prove useful in studying specific functions of AURKC that we are unable to observe in *Aurkc*$^{-/-}$ oocytes due to AURKB compensation. The generation of transgenic mice expressing AURKC-CY would prevent the compensation of AURKB and observed phenotypes would then likely be due solely to a reduction in AURKC-CPC function.

AURKC-YX activates Haspin independent of the CPC

AURKC-YX retains nearly the entire catalytic domain, lacking only the C-terminus of the kinase and protein. The observed localization failure is

hypothesized to be due to the loss of a homologous 9-residue consensus sequence observed in AURKB that was shown in mitosis to be required for localization (Scrittori et al., 2005). The cytoplasmic activity of the mutant supports the hypothesis that this is only a localization defect and does not inhibit kinase function, however similar analysis of a construct lacking only the consensus sequence would be necessary to confirm the phenotype.

AURKC-YX retains catalytic ability as indicated by the observed increase in H3pT3 and Survivin relative to the WT-injected control group (see Ch. III). I propose that AURKC-YX is unable to localize with the CPC, but retains its catalytic ability and is phosphorylating Haspin, which activates it leading to the increased H3pT3 (Dai et al., 2005). Survivin is the CPC subunit that binds H3pT3 and recruits the CPC to the chromosomes (Kelly et al., 2010), and I observed a rise in Survivin similar to the rise in H3pT3. I hypothesize that this CPC is bound to AURKB because the oocytes lack endogenous AURKC and there is no observed GFP localized with the CPC to indicate the localization of the mutant. To investigate this hypothesis, I propose three things. 1) To co-inject *AURKC-YX-Gfp* with *Aurkb-mCherry* to show an increase in AURKB found within the CPC. 2) Treat the AURKC-YX expressing oocytes with ZM447439 (an Aurora B/C inhibitor) to show that the recruitment of Survivin is mediated by Aurora function. 3) Treat AURKC-YX expressing oocytes with 5-iodotubercidin (a Haspin inhibitor) to show that the increased H3pT3 is due to Haspin activation. Confirmation of this phenotype would show that AURKC could function independent from CPC localization, which has not been previously shown.

These observations are interesting because despite the increase in CPC localization on the chromosomes, these oocytes still have misaligned chromosomes at Met I and fail to progress through meiosis. These observations support that hypothesis that although AURKB and C do have some overlapping functions, there are AURKC specific functions that are not rescued by AURKB and the lack of AURKC-CPC is the cause of the phenotype and not simply an overall CPC reduction.

In Chapter III, I analyzed the effect of three AURKC mutations on oocyte meiosis. I observed that although the mutants differ in their abilities in MI, the loss of AURKC-CPC function has only one result: Met I arrest leading to polyploidy which is supported by the phenotypes observed in men (Ben Khelifa et al., 2012; Ben Khelifa et al., 2011; Dieterich et al., 2007; Dieterich et al., 2009). I hypothesize that the Met I misalignment and arrest is due to an error correction failure in these cells and propose to assess the Met I K-MT attachments in mutant-expressing oocytes to quantify the errors present relative to WT oocytes. I expect to see an increase in errors in the mutant-expressing cells. I also hypothesize that these errors result in the activation of the SAC, which is directly causing the arrest phenotype. Treating the cells with Reversine (an MPS1 inhibitor) will block the function of the SAC, and I propose the mutant-expressing cells will no longer arrest without a functional SAC.

Interestingly, this analysis suggests that these mutations are all considered severe, leading to a complete loss of AURKC-CPC function in these cells. It has been hypothesized that moderate to mild mutations in AURKC would

result in azoospermia rather than macrozoospermia identified previously (Ben Khelifa et al., 2012; Ounis et al., 2014). Genetic screens of azoospermic men in the future could identify these less severe alleles and the *Aurkc*$^{-/-}$ oocyte system developed in this thesis would allow for effective characterization of their effect on meiosis.

The vast majority of infertility cases are idiopathic which leaves patients with very few treatment options. The decreasing cost and increasing accuracy of whole exome sequencing, as well as the large library of genetic data available worldwide, is leading to an ease in identifying rare and *de novo* genetic mutations related to fertility and reproduction. This explosion of genetic data will hopefully provide clinicians with more information to identify potentially causative variants in genes associated with infertility and reproductive defects. The work shown in this thesis demonstrates that *Aurkc*$^{-/-}$ mouse oocytes are a tractable model for studying the function of human AURKC in meiosis and use of this model in characterizing *AURKC* mutations illustrates the critical role that AURKC plays in establishing ploidy in gametes through and maintaining fertility and reproductive health.

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