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

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\textbf{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 AURK is required to regulate chromosome segregation during meiosis I; 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 three variants. To evaluate the functional differences between the variants, we created green fluorescent protein-tagged constructs of each variant to express in oocytes from Aurkc\textsuperscript{-/-} mice. By quantifying metaphase chromosome alignment, cell cycle progression, phosphorylation of INCENP and microtubule attachments to kinetochores, we found that AURKC\textsubscript{v1} was the most capable of the variants at supporting metaphase I chromosome segregation. AURKC\textsubscript{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 with the other two variants. Finally, when we expressed the three 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.

\textbf{Key words:} Aurora kinase C / female fertility / meiosis / oocyte maturation

\section*{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 and Hunt, 2001; Hassold et al., 2007; 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 and Hunt, 2001; Hassold et al., 2007; Pacchierotti et al., 2007). This difference in error rates 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\textsuperscript{-/-} mice produce an average of two pups less per litter than their wildtype counterparts, while Aurkc\textsuperscript{-/-} males produce seven 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, 2009). Men with AURKC mutations present with macrozoospermia that is due to a
failure to complete 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 identified 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.1A and B) (Bernard et al., 1998; Tseng et al., 1998; Yan et al., 2005). AURKC_v1 encodes the longest transcript (Fig.1A and B), and is more abundant in the testis relative to other tissues (Yan et al., 2005). AURKC_v3, identified in a screen of human placental cDNA using probes for an Aurora kinase homolog from Xenopus laevis, is the shortest (Fig. 1A and B) (Bernard et al., 1998). AURKC_v2 was incidentally found in a screen to isolate AURKC_v3 (Fig. 1A) from human testis and has in vitro kinase activity similar to that of AURKC_v1 (Yan et al., 2005). The proteins encoded by these splice variants contain identical catalytic domains at the C-terminus but their N-termini are successively shorter (Fig.1C). 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 to assess their functional relevance in female meiosis. Using quantitative RT-PCR (qRT-PCR), we observed substantially greater levels of AURKC expression in oocytes relative to other cell types (sperm and cumulus cells), and we amplified the three splice variants in all oocytes tested. To assess functional differences, we demonstrated 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 three splice variants are expressed simultaneously in Aurkc^{−/−} oocytes, that they support microtubule error 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 three variants simultaneously and sperm do not, this expression difference could begin to explain why men are more sensitive to AURKC mutations than women.

### Materials and Methods

#### Sample collection

Oocytes and cumulus cells were processed in Cell-to-Ct lysis buffer as recommended (Ambion, #4 458 236, Grand Island, USA) and stored in liquid nitrogen. Discarded sperm samples were pelleted and washed in phosphate-buffered saline (PBS) prior to RNA collection.
Ethical approval

Human oocyte and cumulus cell samples were obtained from patients undergoing IVF treatments and collected under Internal Review Board (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 (#I-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 kit (Ambion, #4 458 236). 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, #4 368 814, Grand Island, USA). A pre-amplification step was also performed according to the Cell-to-C kit. cDNA was synthesized from sperm and cumulus cell samples using the TRizol Plus RNA purification kit (Ambion, #12183-55S) and PureLink RNA mini kit (Invitrogen, #12183-1BA, 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 exons 4 and 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 three 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 these probes were validated for specificity computationally by Applied Biosystems.

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 Ct method as previously described (Livak and Schmittgen, 2001).

Murine oocyte preparation and microinjection

Fully-grown, GV-intact oocytes from 6- to 9-week old Aurkc−/− mice with a 129/SvPas/C57Bl/6 mixed background (Kimmis et al., 2007; Schindler et al., 2012) or CF-1 mice (Harlan Laboratories, #NSAF1, Indianapolis, USA) that were primed (44–48 h before collection) with pregnant mare’s serum gonadotrophin (Calbiochem #367 222, Darmstadt, Germany) 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 polyvinylpyrrolidone (MEM/PVP) and 2.5 μM milrinone (Sigma-Aldrich #M4659, St. Louis, USA) to prevent meiotic resumption (Tsafri 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 Mil experiments) or for 3 h (for Mil 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 metaphase of meiosis I (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 h of meiotic maturation, oocytes were incubated in CZB containing 100 μM monastrol (Sigma-Aldrich, #M8515) to induce monopolar spindle formation. After 2 h oocytes were washed out of the monastrol-containing media and allowed to recover in CZB containing 5 μM MG132 (Calbiochem, #47 4791) to prevent anaphase onset. After 3 h, oocytes were incubated for 7 min 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). After 1 h, oocytes were transferred into separate drops of CZB medium with cycloheximide in a 96 well dish (Greiner Bio One, #655 892, Monroe, USA). Bright field, green fluorescent protein-tagged (GFP) (470 nm), and mCherry (585 nm) images were acquired using an EVOS FL Auto Imaging System (Life Technologies, Grand Island, USA) with a 10× objective. The microscope stage was heated to 37 °C and 5% CO2 was maintained using the EVOS Onstage Incubator. Images were acquired every 20 min for 10 h.

Cloning and synthesis of cRNA

The generation of mAurkc-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 Spol and Sail. We performed in vitro transcription with a mMessage mMachine kit (Ambion, #AM1344M) as per the manufacturer’s instructions. cRNA was purified using an RNA-Easy purification kit (Qiagen, #74 104, Venlo, Netherlands) and eluted in RNase free H2O.

Immunocytochemistry

After maturation, oocytes were fixed in PBS with 2% paraformaldehyde for 20 min 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; phosphorylated form of INCENP (pINCENP) [gift from M. Lampson, University of Pennsylvania (Sali et al., 1996)] using Sphl and Sail. We performed in vitro transcription with a mMessage mMachine kit (Ambion, #AM1344M) as per the manufacturer’s instructions. cRNA was purified using an RNA-Easy purification kit (Qiagen, #74 104, Venlo, Netherlands) and eluted in RNase free H2O.
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 (pH 6.8), 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) were run simultaneously as standards. The separated polyepptides were transferred to nitrocellulose membranes (Bio-Rad, #170-4156) using a Trans-Blot Turbo Transfer System (Bio-Rad) and then blocked with 2% ECL blocking solution (Amersham, #RPN418, Pittsburgh, USA) solution in TBS-T (Tris-buffered saline with 0.1% Tween 20) for 1 h. The membranes were incubated with α-tubulin primary antibody (Sigma-Aldrich, #T-6074; 1:10 000) overnight or GFP primary antibody (Sigma-Aldrich, #G6539; 1:1000) for 1 h. 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 h followed with washing with TBS-T five times. The signals were detected using the ECL Select western blotting detection reagents (Amersham, #RP2235) 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 six kinetochores per oocyte, subtracting an average of three 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 (LaJolla, 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.

**Results**

AURKC is highly expressed in human oocytes

High Aurkc expression in mice is limited to germ cells (Yana et al., 1997; Tseng et al., 1998), and its function is important for meiotic cell cycle progression (Hu et al., 2000; Chen et al., 2005; Tang et al., 2006; Yang et al., 2010; Schindler et al., 2012; Yang et al., 2013; Balboul and Schindler, 2014) 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 qRT-PCR on cDNA from single oocyte samples, each from a different donor patient with paired cumulus cell samples (Table I). 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; Grondahl et al., 2010; Avo Santos et al., 2011) (Fig. 2). Although it is an interesting question to ask, our current sample size was too limited to make correlations between age and expression of AURKC.

We detected AURKC in cDNA samples from sperm and cumulus cells (Fig. 2). 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. 2). 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. 2). 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 (Assou et al., 2006). The expression levels were markedly lower (~1000 and ~20-fold) compared with levels in oocytes and sperm, respectively (Fig. 2). There was no correlation between the abundance of AURKC transcript in oocytes to that in cumulus cells from the same patient (Fig. 2, Spearman Rank Correlation Test, P = 0.5). AURKC is expressed at low levels in placental, lung and some tumor tissue samples (Yan et al., 2005; Baldini et al., 2011), 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 three variants of AURKC**

Three AURKC splice variants are expressed in human testis tissue (Bernard et al., 1998; Tseng et al., 1998; Yan et al., 2005). 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 three transcripts. The data indicate that AURKC_v1 was present in all sperm samples tested, whereas AURKC_v2 and AURKC_v3 were detected in three and four of the nine samples, respectively (Fig. 3A). No sample expressed the three variants 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. 3B, Table I). The average expression level for each variant was 7.7, 1.8 and 4.5 fold greater than GAPDH, respectively. Using the comparative Ct method, we determined that AURKC_v1 was the most abundant, making up ~47.8% of the total AURKC composition in oocytes and AURKC_v2 was expressed at the lowest level, making up ~14.5% (Fig. 3B and C). We note that there was variation amongst expression levels between samples (Fig. 3B). 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. 2), there was variation in the amounts of each transcript in oocytes from different donors. Even within the same oocyte, there was variability in expression of the three variants. For example, the oocyte from the 34.7-year-old donor expressed above average levels of AURKC_v1 and AURKC_v3 but below average levels of AURKC_v2 (Fig. 3B, Table I). 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. 3D). 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**

The existence of three 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 and treated with cycloheximide, via live-imaging and quantified fluorescence over the course of 10 h. We found that AURKC_v2 was significantly more stable over the time course than either AURKC_v1 or AURKC_v3 (Fig. 4). 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. 1C). This hypothesis is supported by the data showing that the initial destruction (\( t = 0–2 \) h) of AURKC_v3 is faster (slope = 0.1757) than the initial destruction of AURKC_v1 (slope = 0.1149) (Fig. 4). 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 MI with chromosome misalignment (Schindler et al., 2012), reduced levels of pINCENP (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 the 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 (Shuda et al., 2009; Sharif et al., 2010; Yang et al., 2010; Avo Santos et al., 2011) (Fig. 5A–C). Ectopically expressed hAURKC had an identical localization as mAURKC when expressed in Aurkc^2/2 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. 5A–C). To confirm that hAURKC is active at this localization, we quantified the relative fluorescence of an antibody that recognizes the pINCENP at kinetochores of images obtained via confocal microscopy. Compared with 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. 5A–D). Approximately 60% of control-injected Aurkc^-/- oocytes had chromosomes that were misaligned at the Met I plate [Fig. 5E; (Schindler et al., 2012)]. We found that this phenotype can be rescued by overexpression of mouse or human AURKC (Fig. 5E), 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.

Figure 3  Oocytes express three splice variants while sperm and cumulus cells express 1. (A, B and D) qRT-PCR was performed on human samples to measure variant-specific AURKC expression using 2^−ΔΔCt. 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 the mean level of expression. (C) Average relative expression of splice variants in oocytes using 2^−ΔΔCt.

Figure 4  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 min 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.
To determine if the N-termini dictate different localization or function, we injected cRNA of Gfp-tagged constructs 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. 6A). At Met II, localization occurred strictly at kinetochores and did not differ between the variants (Fig. 6B). We also noted dynamic localization of the AURKC variants to the spindle midbody during Telo I (Fig. 6C). 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<sup>−/−</sup> mouse oocytes injected with cRNA for each variant arrested at Met I after 16 h of maturation. We found that the three splice variants significantly improved the Met I arrest phenotype witnessed in the control-injected Aurkc<sup>−/−</sup> oocytes (54%) and to the same degree (Fig. 6D). Therefore, the three 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<sup>−/−</sup> 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. 7A and B). Furthermore, AURKC<sub>v1</sub>-injected oocytes had higher pINCENP immunoreactivity.
Figure 7 AURKC splice variants differ in catalytic activity. Fully grown oocytes arrested at prophase of M1 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 occur when both pairs of sister kinetochores are 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.
than in those injected with AURKC_v2 or AURKC_v3 at both Met I (Fig. 7A and B) and Met II (data not shown). These data demonstrate that AURKC_v2 and AURKC_v3 have reduced catalytic activity compared with AURKC_v1. Therefore, the length of the N-terminus appears to influence the ability of AURKC to phosphorylate substrates.

AURKC also regulates alignment of chromosomes at the metaphase plate (Shafir et al., 2010; Yang et al., 2010; Balboula and Schindler, 2014). 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. 7C). Despite their ability to progress through meiosis, the variant-injected oocytes did not regulate chromosome alignment to the same extent as one another (Fig. 7C). Chromosomes in AURKC_v1-injected oocytes were significantly more aligned than in those in oocytes injected with AURKC_v2 or AURKC_v3 (Fig. 7C). We also found that neither AURKC_v2 nor AURKC_v3-injected oocytes could significantly improve alignment relative to the Gfp-injected control oocytes (Fig. 7C). 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 (Supplementary data, Fig. S1A). We found no differences in expression between variants (Supplementary data, 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) (Brunet et al., 1999; Lampson et al., 2004; Balboula and Schindler, 2014). 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 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. 7D–F, Supplementary data, Fig. S2); 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. 7D, Supplementary data, Fig. S2). 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. 7D, Supplementary data, Fig. S2). Consistent with different levels of catalytic activity, these data demonstrate that the three 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. 3C). Compared with Gfp and variant one-injected controls, we found significant improvement in the number of oocytes that could fix all of the improper K-MT attachments when oocytes express the three variants simultaneously (Fig. 7E, Supplementary data, Fig. S2). These data imply that the three variants complement each other in function during meiosis and all three are needed for optimal fidelity of chromosome segregation.

**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., 2005). Here we demonstrate that the three variants are also always found in oocytes (Fig. 3B) while not all sperm samples express the three splice variants simultaneously (Fig. 3A). 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 (Dietrich et al., 2007).

AURKC_v1 is expressed in all oocytes, sperm and cumulus cells tested (Fig. 3A, B and D), but oocytes express significantly more AURKC than the other cell types (Fig. 2). 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. 2). The prolonged time frame for completion of meiosis in females compared with males would necessitate a greater abundance of transcripts to account for degradation over time. At the onset of puberty, spermatogenesis occurs daily and takes ~2 months to complete and meiosis only takes ~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 (Matzuk and Lamb, 2002; Edson et al., 2009). 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 a 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. 6D), they are not able to do so with equal efficiency (Fig. 7). Yet, when they are combined, the overall error correction activity is greater than when any variant is expressed alone (Fig. 7E), 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.
Better oocyte and embryo selection in the clinic. This information could ultimately help develop diagnostic tools for female meiosis and genetic causes of infertility (Edson et al., 2009). If causal- tive, this information could ultimately help develop diagnostic tools for better oocyte and embryo selection in the clinic.

Based on known AURKB mitotic substrates that are present in mouse oocytes, there are a number of candidate targets of AURKC. The phenotypic differences we observed between the variants could be due to 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 (Kline-Smith et al., 2004; Cimini et al., 2006; Wordeman et al., 2007). MCAK localizes to centromeres in oocytes and therefore it is a likely AURKB substrate (Ems-McClung et al., 2007; Zhang et al., 2007; Vogt et al., 2010). 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 AURKB 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 (Sun et al., 2011; Gui and Homer, 2012). 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 AURKB 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 checkpoint would still be satisfied and polar body extrusion would occur, consistent with our observations. Therefore, differences in substrate affinity, either to these candidates or unknown meiosis-specific substrates, is a feasible mechanism to explain our results.

Mutations in AURKC are linked to male infertility and cancer (Kimura et al., 1999; Takahashi et al., 2000; Sasai et al., 2004; Dieterich et al., 2007). 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 function 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 causal- tive, this information could ultimately help develop diagnostic tools for better oocyte and embryo selection in the clinic.

**Supplementary data**

Supplementary data are available at http://molehr.oxfordjournals.org/.

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**Authors’ roles**

J.E.F., K.S. and N.R.T. conceived and designed the experiments. J.E.F. and C.E.R. performed the experiments. J.E.F., D.G., N.R.T. and K.S. analyzed the data. K.S., R.T.S. and N.R.T. contributed reagents, materials or analysis tools. J.E.F. and K.S. wrote the paper.

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**Conflicts of interest**

None declared.

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