The Aurora kinase inhibitor ZM447439 accelerates first meiosis in mouse oocytes by overriding the spindle assembly checkpoint

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Abstract

Previous studies have established that when maturing mouse oocytes are continuously incubated with the Aurora inhibitor ZM447439, meiotic maturation is blocked. In this study, we observe that by altering the time of addition of the inhibitor, oocyte maturation can actually be accelerated by 1 h as measured by the timing of polar body extrusion. ZM447439 also had the ability to overcome a spindle assembly checkpoint (SAC) arrest caused by nocodazole and so rescue polar body extrusion. Consistent with the ability of the SAC to inhibit cyclin B1 degradation by blocking activation of the anaphase-promoting complex, we could also observe a rescue in cyclin B1 degradation when ZM447439 was added to nocodazole-treated oocytes. The acceleration of the first meiotic division by ZM447439, which has not been achieved previously, and its effects on the SAC are all consistent with the proposed mitotic role of Aurora B in activating the SAC. We hypothesize that Aurora kinase activity controls the SAC in meiosis I, despite differences to the mitotic cell cycle division in spindle architecture brought about by the meiotic mono-orientation of sister kinetochores.

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Introduction

There has been much interest in what controls the first meiotic division (meiosis I) in oocytes because it is particularly error prone in humans, resulting in homolog non-disjunction such as trisomy 21 (Hunt & Hassold 2008, Jones 2008). It is also a unique type of chromosome division, where sister kinetochores adopt a side-by-side configuration and consequently are mono-orientated. In meiosis II, and in all other mitotic divisions, sister kinetochores adopt a back-to-back configuration and consequently are bi-orientated (Brar & Amon 2008, Holt & Jones 2009). Such a configuration allows homologous chromosomes to divide reductionally in meiosis I but sister chromatids to divide equationally in meiosis II and mitosis. A further facet of meiosis I is an extremely protracted prometaphase, lasting several hours, during which the spindle assembles for the most part without kinetochore–microtubule attachments (Brunet *et al.* 1999).

In the mitotic cell cycle division, the anaphase-promoting complex/cyclosome (APC) is essential for segregating chromosomes, and its activity is tightly controlled to prevent aneuploidy (Acquaviva & Pines 2006, Peters 2006). Its ubiquitin ligase activity leads, through the 26S proteasome, to degradation of cyclin B1, so reducing CDK1 activity and also to degradation of securin, so freeing separase. The spindle assembly checkpoint (SAC) inhibits the APC during prometaphase

and in so doing prevents aneuploidy (Musacchio & Salmon 2007). Thus, the loss of SAC function in mitosis causes premature APC activity and consequently segregation of sister chromatids before full chromosome congression and bi-orientation, leading to aneuploidy in daughter cells and tumorigenesis (Li *et al.* 2009a).

APC-mediated cyclin B1 and securin degradation are also essential for the disjunction of homologs in maturing mammalian oocytes (Herbert *et al.* 2003, Terret *et al.* 2003, Reis *et al.* 2007). In one very interesting study using the XO mouse, the univalent X chromosome was observed to divide reductionally in oocytes without any block to anaphase (LeMaire-Adkins *et al.* 1997). Consequently, it was concluded that the SAC was absent from female meiosis I. However, more detailed examination of Mad and Bub proteins, which are members of the SAC, has shown that SAC activation is an essential feature of this reductional division. Thus, knock out, knock down, or inhibition of Mad and Bub family members all lead to aneuploidy as a consequence of premature APC activation (Terret *et al.* 2003, Tsurumi *et al.* 2004, Homer *et al.* 2005, 2009, Yin *et al.* 2006, Niaux *et al.* 2007, Li *et al.* 2009b, McGuinness *et al.* 2009).

The high incidence of aneuploidy resulting from mis-segregation of homologs in meiosis I errors is therefore unlikely to be due simply to an absence of the SAC. Nevertheless, the observations on the oocytes of the XO mouse, as well as other studies demonstrating high rates of aneuploidy in the absence of SAC activation

(Reis *et al.* 2007), suggest that the SAC in oocytes may not respond to the same cues as it does in mitotically dividing cells and they may be insensitive or leaky in response to some arrangements of kinetochore–microtubule attachments during meiosis I. Further investigations into how the SAC is controlled and what it responds to in oocytes are therefore worthwhile pursuits.

With respect to the mitotic cell cycle division, in recent years, it has been established that the SAC is switched on through Aurora B kinase, which is a member of the chromosomal passenger complex (Kallio *et al.* 2002, Ditchfield *et al.* 2003, Hauf *et al.* 2003, Dewar *et al.* 2004, Lan *et al.* 2004, Kelly & Funabiki 2009, Liu *et al.* 2009). Aurora B destabilizes incorrect microtubule–kinetochore attachments, and is switched off only when tension across the sister kinetochores, generated by bi-orientation, physically stretches centromeric Aurora B away from the kinetochore (Liu *et al.* 2009). The small molecule inhibitor, ZM447439, has proved useful to study Aurora B kinase activity independent of any structural function within the chromosomal passenger complex (Ditchfield *et al.* 2003). However, ZM447439 is better regarded as a general Aurora inhibitor because it has a similar IC_{50} for both Auroras A and B (Ditchfield *et al.* 2003); and is also likely to inhibit Aurora C, which has sequence homology to Aurora B (Tseng *et al.* 1998) and can rescue cells from loss of Aurora B (Slattery *et al.* 2009). Aurora C is likely to be relevant to meiosis since one recent study has shown that it is the most predominant Aurora kinase in oocytes

(Yang *et al.* 2010). If Aurora kinases function in meiosis as in somatic cell mitosis (Ditchfield *et al.* 2003, Hauf *et al.* 2003, Nilsson *et al.* 2008), ZM447439 should accelerate the passage of oocytes through meiosis I due to an SAC override, generate lagging chromosomes, and raise the incidence of aneuploidy in mature eggs. It should also overcome a SAC-imposed arrest, which could be measured directly in the form of increased APC activity. Although ZM447439 has been used previously on mouse oocytes, none of these observations have been made and instead its main effect is to block polar body extrusion, hence arresting rather than accelerating any passage through meiosis I (Swain *et al.* 2008, Shuda *et al.* 2009, Vogt *et al.* 2009). In this study, we have attempted a further examination of the effects of ZM447439 on mouse oocytes in order to determine whether we could uncover drug effects that would be consistent with similar studies in mitosis.

Results

Early ZM447439 addition causes meiotic failure

Immature dictyate-stage oocytes were cultured with 10 μ M ZM447439 45 min after milrinone washout, at which time about half of oocytes had undergone nuclear envelope breakdown (NEB; Fig. 1A). Time-lapse imaging was used to assess when polar body extrusion had occurred. In controls, the majority of oocytes (94%, $n=68$) extruded their polar bodies, at a time of 8.3 ± 0.7 h after NEB (Fig. 1B), but no oocytes treated with

Figure 1 Continuous ZM447439 exposure blocks meiosis I. (A) Timing of nuclear envelope breakdown after milrinone washout. Cumulative percentage of oocytes undergoing NEB after washout from 1 μ M milrinone ($n=184$). (B) Bright field time lapse of oocytes matured in media with ($n=64$) or without ZM447439 ($n=69$). Polar body extrusion was evident in control oocytes at the time indicated (asterisk), but was aborted in oocytes cultured in ZM447439 (arrowhead). Times are hours:minutes after NEB. (C) Images of chromatin (Hoechst) in live oocytes cultured with ($n=13$) or without ($n=31$) ZM447439 during the period of permanent or aborted polar body extrusion. The red trace shows the location of the plasma membrane. Images are 15 min apart. Scale bars are 50 μ m.

ZM447439 extruded their polar bodies ($n=64$). These observations on ZM447439 are consistent with previous studies that showed at doses between 1.5 and 20 μM , it inhibits polar body extrusion (Swain *et al.* 2008, Shuda *et al.* 2009, Vogt *et al.* 2009).

However, time-lapse imaging revealed oocytes did attempt extrusion of a polar body, albeit incomplete, observed as a protrusion in the plasma membrane, which lasted about 30 min before receding (Fig. 1B). The timing of this protrusion was earlier than controls at 7.3 ± 1.5 h ($P=0.001$) after NEB. To explore this further, we combined bright field and chromosome (Hoechst) imaging to assess for congression and segregation errors during meiosis I. In control oocytes, chromosome congression led to the formation of a metaphase I plate, anaphase onset occurred after migration of the

spindle to the cortex, and this resulted in the archetypical asymmetric division of an oocyte (Fig. 1C; $n=31$; Maro & Verlhac 2002). During these divisions, no lagging chromosomes or other segregation defects were observed, and anaphase onset occurred shortly after the last homologs were aligned on the metaphase plate (Fig. 1C arrows). However, the initial defect in ZM447439 was a failure of homologs to congress. Instead, mal-aligned chromosomes migrated to the oocyte cortex, became spread along the length of the spindle axis, and within 30 min, the extrusion receded back into the oocyte (Fig. 1C). We failed to study these factors further at greater resolution in live oocytes because we found that the increase in illumination needed to image the process actually blocked anaphase (brightness $\propto 1/\text{magnification}^2$).

Figure 2 Prometaphase ZM447439 exposure accelerates meiosis I. (A) Bright field time lapse of oocytes matured in media with or without ZM447439 at the times indicated after NEB. These later ZM447439 additions led to morphologically normal polar bodies (asterisks). (B) Timing of polar body extrusion in oocytes ($n=56-99$) incubated with ZM447439 for the times indicated. Addition of the Aurora kinase inhibitor during prometaphase I (2 and 4 h) accelerated polar body extrusion. (C) In a very small number of oocytes ($n=6$) treated with ZM447439 from 2 h after NEB, the polar bodies that were extruded were later resorbed. For those oocytes affected in this way, timelines are shown (red lines) indicating the period from polar body extrusion to later polar body resorption. Arrow indicates time of ZM447439 addition. (D) Bright field and Hoechst time-lapse images of live oocytes during the process of polar body extrusion after addition of ZM447439 at the times indicated ($n=22$), showing a failure to fully congress chromosomes during prometaphase (arrows) and lagging chromosomes during anaphase (arrowheads). Insets show enlargements of areas of interest. Scale bar is 50 μm .

Late ZM447439 addition causes meiotic acceleration

We wondered whether the block to polar body extrusion would be conserved if 10 μ M ZM447439 was added later in oocyte maturation, 2 or 4 h after NEB. In fact, these later time points did not block polar body extrusion (Fig. 2A and B) but instead accelerated it by more than 1 h (Fig. 2B; $P=0.001$). This acceleration was similar in timing to the attempted polar body extrusion when the inhibitor was added at 0 h (Fig. 1B). ZM447439 addition at 2 h resulted in some resorption of polar bodies, although at this later time, the rate was much lower than observed for aborted polar body at 0 h (11%, $n=57$ vs 75%, $n=64$ at 0 h; Fig. 2C). When the inhibitor was added at 4 h, there was no decline in the polar body extrusion rate versus controls.

Given the opposing outcomes of adding ZM447439 at different times during oocyte maturation, inhibition versus acceleration of polar body extrusion, we wanted to determine whether the first 2 h after NEB represented a period of unique sensitivity to this drug. Therefore, oocytes were incubated in ZM447439 for 2 h after NEB and then washed into drug-free media. Although incubation with ZM447439 during the first 2 h of maturation caused a delay in the timing of extrusion of polar bodies (9.0 ± 0.6 h; Fig. 2B, blue), it did not block polar body extrusion to any extent (Fig. 2B; 97%, $n=56$). Therefore, there appears no critical process during the first 2 h of meiotic resumption that cannot be completed after inhibitor washout. We conclude that the lack of chromosome segregation after 0 h inhibitor addition was likely the primary reason for the block to polar body formation because chromatin in the region of the cleavage furrow has been shown in mitotic studies to be a very potent inhibitor of cytokinesis (Weaver *et al.* 2006, Steigemann *et al.* 2009).

We thought it important to examine how chromosomes segregated during meiosis I in oocytes cultured with the inhibitor at these 2 and 4 h time points. Time-lapse imaging combined with Hoechst staining of chromatin revealed that there was often a failure to achieve homolog congression, with chromatin visible that was never at the spindle equator before anaphase ($n=13/20$; Fig. 2D). Segregation of chromosomes was observed, but unlike controls (Fig. 1C) were often associated with lagging chromosomes or chromatin bridges ($n=15/20$; Fig. 2D). However, in contrast to the 0 h time point, chromosome segregation was associated with cytokinesis and the development of a cleavage furrow resulting in a polar body.

ZM447439 overcomes a nocodazole-imposed SAC arrest

The accelerated passage through meiosis I and the lagging chromosomes at anaphase, after ZM447439 addition at these later time points, would be consistent

with its reported actions on mitotic cells, where it can be used to inhibit Aurora B, and so can overcome a SAC-imposed arrest. This acceleration of meiosis I complements findings in mouse oocytes where, by various nocodazole incubations, the period of meiosis I most sensitive to delay by nocodazole treatment was found to be 6–8 h after NEB (Brunet *et al.* 1999). In this study, we wanted to test whether ZM447439 would overcome a SAC arrest imposed by nocodazole.

Maturing oocytes at 4 h after NEB were cultured with nocodazole, a spindle poison that reduces microtubule stability. A dose of 400 nM was used, the minimal dose needed to block polar body extrusion in the majority of oocytes (Wassmann *et al.* 2003). Time lapse of treated oocytes showed that polar body extrusion was very much inhibited (24%, $n=64$ vs 94%, $n=68$ in vehicle controls; Fig. 3A) and those that did extrude polar bodies did so with greatly delayed timing (11.9 ± 1.5 h; Fig. 3B). Addition of ZM447439 with nocodazole, however, caused a marked rescue of both the rate of polar body extrusion and the timing at which it occurred (55%, 8.9 ± 1.1 h; Fig. 3A and B).

In nocodazole and ZM447439-treated oocytes that had extruded a polar body, chromatin was observed in both the oocyte and polar body (Fig. 4A). However, because of the presence of nocodazole and ZM447439, tubulin staining was weak and sister chromatids were not aligned on what would be expected to be the second meiotic metaphase spindle equator. A morphologically normal metaphase II spindle could be observed, however, if both drugs were washed out after polar body extrusion at 10 h (Fig. 4A). A similar disruption in the formation of a metaphase II spindle was seen when oocytes, matured in ZM447439 alone, were examined

Figure 3 ZM447439 overcomes spindle checkpoint arrest in meiosis I. (A) Bright field time lapse of maturing oocytes cultured in nocodazole with or without ZM447439. Polar body extrusion was blocked by nocodazole but could be rescued (asterisks) by the Aurora kinase inhibitor. (B) Polar body extrusion rates in oocytes cultured in nocodazole ($n=64$), nocodazole and ZM447439 ($n=83$), or vehicle alone ($n=69$). Scale bars are 20 μ m.

Figure 4 Nocodazole and ZM447439 effects on metaphase II spindle formation. (A) Confocal images for DIC, tubulin, and Hoechst in oocytes at 16 h after NEB, which had been matured in nocodazole and ZM447439 as indicated, wash-out of both the agents was at 10 h. The addition of ZM447439 to nocodazole-treated oocytes led to the segregation of chromosomes ($n=13$). A fully formed metaphase II spindle was not observed unless nocodazole and ZM447439 were washed out ($n=20$). (B) Oocytes were treated with or without ZM447439 and were fixed 16 h after NEB, and then stained for tubulin and chromatin. Treatment with ZM447439 resulted in a weakly stained spindle and a lack of congression. Washout of ZM447439 at 10 h after NEB in oocytes that had extruded a polar body recovered a morphologically normal metaphase II spindle with bi-oriented chromosomes congressed at the spindle equator. Scale bar 20 μm .

after polar body extrusion (Fig. 4B). A normal metaphase II spindle was only observed when mature oocytes were washed free of the inhibitor. We surmise that the activity of an Aurora kinase, likely to be Aurora B and/or C, is therefore needed to assemble a bi-oriented metaphase II spindle.

ZM447439 leads to cyclin B1 degradation in the presence of nocodazole

APC activation and consequent cyclin B1 degradation are required for polar body extrusion in mouse oocytes (Ledan *et al.* 2001, Herbert *et al.* 2003, Hyslop *et al.* 2004, Reis *et al.* 2007). Therefore, release from a SAC-imposed meiotic arrest by ZM447439 should lead to cyclin B1 loss. To demonstrate this directly, oocytes were microinjected with cyclin B1–Venus cRNA and imaged for Venus fluorescent protein during maturation. As described previously (Herbert *et al.* 2003, Reis *et al.* 2007), in untreated oocytes, cyclin B1 levels gradually declined during oocyte maturation, reaching a minimum

at the time of first polar body extrusion (Fig. 5). Addition of nocodazole stopped cyclin B1 degradation. However, degradation could be re-initiated when ZM447439 was added, demonstrating that direct release of the SAC by Aurora inhibition, probably Aurora B and/or C, had led to APC activation (Fig. 5).

Aneuploidy associated with ZM447439 addition

All the above data taken together suggest that ZM447439 leads to an acceleration in passage through meiosis I by overcoming the SAC-imposed delay to homolog disjunction; be it a nocodazole-induced delay or delay due to normal prometaphase SAC activity. One consequence of the accelerated passage through meiosis is that oocytes should show some increase in aneuploidy rates (Jones 2008). This is implied by the observation of lagging chromosomes and anaphase bridges in oocytes incubated with ZM447439. Therefore, oocytes were incubated with or without ZM447439 from 4 h after NEB. At 10 h, oocytes that had extruded a polar body were assessed

Figure 5 ZM447439 induces immediate cyclin B1 loss during a checkpoint arrest. Cyclin B1-Venus fluorescence in maturing oocytes injected with cRNA during G2 arrest. Nocodazole and ZM447439 were added at the points indicated. The loss in cyclin B1 fluorescence associated with oocyte maturation could be blocked by nocodazole addition but rescued by further addition of ZM447439. Traces are representative of 35 controls, 50 nocodazole, and 30 nocodazole + ZM447439 oocytes, each from at least two experiments.

by chromosome counts using a monastrol-based *in situ* spreading technique (Fig. 6A; Duncan *et al.* 2009). Low rates of aneuploidy, at consistent levels to other studies, were observed in the absence of ZM447439 (Reis *et al.* 2007, Duncan *et al.* 2009), but oocytes treated with the inhibitor, however, had a significantly higher, 15%, aneuploidy rate (Fig. 6B; $P=0.004$, Fisher's exact test).

Discussion

There have been three previous studies on mouse oocytes examining the effects of the Aurora inhibitor ZM447439 on meiosis I, using a dose range of 1.5–20 μ M (Swain *et al.* 2008, Shuda *et al.* 2009, Vogt *et al.* 2009). Independent of the dose used, the same observation was made in those studies as that made in this study: early ZM447439 addition to oocytes at the time of NEB is associated with a failure of maturing oocytes to complete meiosis I, morphologically evident by the lack of a polar body. Although this is now a consistent finding, it is nonetheless a somewhat disappointing observation, given that the expectation was to observe an acceleration of polar body extrusion. This expectation is based on the acceleration of meiosis I caused by loss of the SAC in oocytes, and the ability of ZM-induced Aurora B inhibition to inhibit the SAC in mitosis.

Investigations using Aurora inhibitors in oocytes of other species have produced differing results. For example, porcine oocyte incubation in ZM447439 results in prophase arrest (Jelinkova & Kubelka 2006). This may be species specific because, similar to mouse oocytes, in bovine oocytes or clam oocytes, VX680 and ZM447439 respectively block meiosis in MI or in interkinesis (George *et al.* 2006, Uzbekova *et al.* 2008). Gadea & Ruderman (2005) demonstrated in cycling *Xenopus* egg extracts that ZM447439 incubation disrupts spindle formation and chromosome condensation but

does not arrest the cell cycle. Likewise, in mitotic cell lines, spindle and chromosome defects are observed but there is no block to cell cycle progression (Ditchfield *et al.* 2003). In the present study, we wished to address the role of Auroras in the SAC response of mouse oocytes. For this reason, the design of this study was adjusted to avoid blocking oocytes in MI. We found that an entirely different response to ZM447439 is uncovered if it is not added at NEB, but a few hours later. With such a design, ZM447439 did not block polar body extrusion, rather it accelerated it. This acceleration in itself suggests that the SAC is abrogated. Furthermore, the shortened meiosis I was associated with lagging chromosomes, anaphase bridges, and a rise in non-disjunction rates, all of which are suggestive of both an override of the SAC and a role for Auroras in ridding erroneous microtubule-kinetochore attachments.

The 15% non-disjunction rates achieved with ZM447439 were not as high as that for the 30% rates achieved by Mad2 knockdown (Homer *et al.* 2005) but nonetheless represent a sixfold increase relative to controls. ZM447439 had the ability to overcome a meiotic arrest caused by nocodazole, a well-established SAC activator. We also demonstrated directly, through real-time loss in cyclin B1, release from SAC arrest when ZM447439 is added following nocodazole. In summary, the results help establish that Aurora kinase activity is important in activation of the SAC, and that the SAC is normally switched on during meiosis I to prevent premature homolog segregation and hence non-disjunction.

ZM447439 inhibition of the Aurora family

There are three members of the Aurora family, all with potential for being inhibited by ZM447439. ZM447439 has been shown directly to inhibit both Auroras A and B (Ditchfield *et al.* 2003), which have distinct cellular functions (Macurek *et al.* 2008, Seki *et al.* 2008). It is likely also to inhibit Aurora C, given this kinase evolved through gene duplication of Aurora B (Brown *et al.* 2004) and can rescue cells from Aurora B knockdown (Slattery *et al.* 2009). Aurora C is predominantly expressed in the testis, where it has an essential and distinct role from Aurora B in late spermatogenesis (Kimmins *et al.* 2007). However, it is also found in many tissues at low levels (Lin *et al.* 2006) and has been shown to be present in oocytes. Indeed, all three Aurora transcripts are present in mouse oocytes (Swain *et al.* 2008, Shuda *et al.* 2009, Yang *et al.* 2010). Although it seems likely that Aurora C is present and functional in oocytes (Yang *et al.* 2010), there appear some contrasting studies on whether Aurora B is also present (Shuda *et al.* 2009, Vogt *et al.* 2009, Yang *et al.* 2010).

Aurora A knockdown arrests mouse oocytes in meiosis I (Solc *et al.* 2008), so it is possible that the addition of ZM447439 to oocytes from NEB onwards is preventing

Figure 6 ZM447439 addition to oocytes during meiosis I results in aneuploidy. (A) The inset shows a flattened Z-stack series of images through a monastrol-treated oocyte; centromeres (CREST) are shown in white and chromatin (Hoechst) in blue. The following images show individual Z-planes from which the inset is comprised; 40 CREST-positive foci are counted in an euploid oocyte. (B) Percentage of non-disjunction rates in oocytes treated with or without ZM447439. Asterisk denotes a significant difference, $P=0.004$ (Fisher's test).

polar body extrusion through Aurora A kinase inhibition. Indeed, one of the major functions of Aurora A is assembling the spindle (Carmena *et al.* 2009). It is, however, likely that the late ZM447439 addition, accelerating passage through meiosis I and overcoming SAC arrest, is through Aurora B or C inhibition, since Aurora A kinase is not involved in this process. Future studies are now needed to distinguish the roles of Auroras B and C in oocytes.

The role of the SAC in oocytes

Interest in the functionality and control of the SAC in oocytes stemmed from the observation that mouse oocytes possessing a univalent X chromosome undergo meiotic maturation with relatively normal timing (LeMaire-Adkins *et al.* 1997). Assuming the SAC to be present in oocytes, one would have anticipated a cell arrest due to a failure in generating tension across the one remaining sister kinetochore pair, and indeed this is observed in the sex-reversed XO male mouse (Kot & Handel 1990, Sutcliffe *et al.* 1991). It would be possible for the SAC to be satisfied if the X chromosome divided

as it does in meiosis II; indeed, such equational divisions can occur in meiosis I when mice lack the synaptonemal complex component SYCP3 (Kouznetsova *et al.* 2007). However, with respect to the XO mouse, the majority of X chromosomes segregate reductionally (LeMaire-Adkins *et al.* 1997, LeMaire-Adkins & Hunt 2000). So why did the lack of tension across the univalent not generate a robust meiotic arrest? It is not because the SAC is absent in oocytes. Many subsequent studies have reported on its presence and functionality in mouse oocytes (Terret *et al.* 2003, Homer *et al.* 2005, 2009, Yin *et al.* 2006, Nault *et al.* 2007, Hupalowska *et al.* 2008, Li *et al.* 2009b, McGuinness *et al.* 2009).

One possibility that would attempt to unify the data is that there are certain types of kinetochore-microtubule attachments that are poorly detected and corrected in oocytes. In addition, end-on microtubule-kinetochore attachments are not formed until very late in meiosis I (Brunet *et al.* 1999). Hence, there is an interest in this study and also in previous studies, in determining whether Auroras have any SAC role in the unusual first division of oocytes. During mitosis, Aurora B activity is thought to be important in correcting merotelic

Figure 7 Model of Aurora and SAC activation in meiosis. Aurora B/C, located at the centromeres, will act to destabilize microtubule–kinetochore interaction and so activate the SAC until the homologous chromosome pair is bi-oriented and under tension, removing centromeric Aurora away from kinetochore substrates.

attachments (Cimini *et al.* 2006, Rosasco-Nitcher *et al.* 2008), which are able to evade the SAC and results in aneuploidy (Cimini *et al.* 2001). Although it still remains possible that certain types of attachment are poorly detected in oocytes, the present observations suggest that Auroras can function in mouse meiosis in much the same way as they do in mitosis with respect to the SAC (Fig. 7). It is tempting to speculate that Aurora C is a meiotic substitute for Aurora B, allowing oocytes to adapt to the unique challenges of meiosis I, such as the mono-orientation of sister kinetochores during meiosis, but that this substitution may also be the source of the leakiness observed in the meiotic SAC.

Materials and Methods

All chemicals were from Sigma–Aldrich unless stated otherwise. ZM447439 (10 mM; Tocris, Ellisville, MO, USA) and nocodazole (400 μ M) in DMSO were used at a dilution of 0.1%.

Oocyte collection

Oocytes were collected from hormonally primed F1 hybrid mice (C57Bl6 females $\times$ CBA males) as described previously (Chang *et al.* 2009). Milrinone (1 μ M) was added to maintain arrest (Tsafiri *et al.* 1996). For long-term incubation, oocytes were cultured in MEM (Gibco) with 20% FCS at 37 °C in 5% CO₂.

Microinjection and imaging

Cyclin B1–Venus cRNA microinjection was performed as previously described (Reis *et al.* 2006). Epifluorescence images were captured using a Nikon Biostation IM fitted with a $\times 20$ objective. Images were analyzed using Metamorph software (Molecular Devices, Sunnyvale, CA, USA). Only oocytes that had undergone NEB within 20 min of the mean NEB time point were used in analysis.

Immunofluorescence

Oocytes were treated for 10 h in 200 μ M monastrol, then fixed in 4% formaldehyde, and permeabilized in Triton X-100 (Holt *et al.* 2010). Immunofluorescence was performed as described previously (Holt *et al.* 2010) using primary antibodies: anti-tubulin (1:400, Invitrogen) and CREST (1:400, Cortex Biochem, San Leandro, CA, USA) supplemented with 1% BSA and 0.2% Tween 20 overnight at 4 °C. Secondary antibodies were Alexa 555- or 633-conjugated (Invitrogen). Oocytes were briefly stained with Hoechst (20 μ g/ml) before mounting on glass slides with Citifluor (Citifluor Ltd, London, UK).

Confocal imaging and aneuploidy analysis

Confocal microscopy was performed using an Olympus FV1000 fitted with a $\times 60$ objective. Z-stacks were compiled with 0.5 μ m intervals. Analysis was performed with FV10-ASW 2.0 Viewer software (Olympus, Tokyo, Japan). Kinetochore counts were made by comparing CREST and Hoechst staining in each Z-plane through a stack of planes encompassing the entire spindle (Fig. 6A). All oocytes that were counted were made anonymous to eliminate the possibility of any observer bias, and confirmed by an independent second count.

Statistical analysis

Fisher's exact test and Student's *t*-test were performed using Minitab15 software (Minitab, State College, PA, USA).

Figure preparation

Figures were assembled using Adobe Illustrator CS4. Images shown in Fig. 1C were generated by creating an outline of the plasma membrane from bright field images using Adobe Photoshop CS4. The trace of the plasma membrane was then applied to the corresponding Hoechst image to create the overlay.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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