The chromosome passenger complex is required for fidelity of chromosome transmission and cytokinesis in meiosis of mouse oocytes

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Summary

The existence of two forms of the chromosome passenger complex (CPC) in the mammalian oocyte has meant that its role in female meiosis has remained unclear. Here we use loss- and gain-of function approaches to assess the meiotic functions of one of the shared components of these complexes, INCENP, and of the variable kinase subunits, Aurora B or Aurora C. We show that either the depletion of INCENP or the combined inhibition of Aurora kinases B and C activates the anaphase-promoting complex or cyclosome (APC/C) before chromosomes have properly congressed in meiosis I and also prevents cytokinesis and hence extrusion of the first polar body. Overexpression of Aurora C also advances APC/C activation and results in cytokinesis failure in a high proportion of oocytes, indicative of a dominant effect on CPC function. Together, this points to roles for the meiotic CPC in functions similar to the mitotic roles of the complex: correcting chromosome attachment to microtubules, facilitating the spindle-assembly checkpoint (SAC) function and enabling cytokinesis. Surprisingly, overexpression of Aurora B leads to a failure of APC/C activation, stabilization of securin and consequently a failure of chiasmate chromosomes to resolve – a dominant phenotype that is completely suppressed by depletion of INCENP. Taken together with the differential distribution of Aurora proteins B and C on chiasmate chromosomes, this points to differential functions of the two forms of CPC in regulating the separation of homologous chromosomes in meiosis I.

Key words: Aurora B, Aurora C, Chromosome passenger complex, INCENP, Meiosis, Mouse oocyte

Introduction

The chromosome passenger complex (CPC) comprises the Aurora B kinase (also known as serine/threonine-protein kinase 12) in a complex with INCENP, Survivin (also known as BIRC5) and Borealin (hDasra-B) and is required to regulate the attachments of kinetochores to microtubules, to ensure proper mitotic checkpoint function and for cytokinesis (reviewed by Ruchaud et al., 2007). Unlike other metazoans that have only the B-form of Aurora kinase in the CPC, vertebrates have another form, known as Aurora C (serine/threonine-protein kinase 13), that is expressed at high levels in the germ line and in a number of cancer cells (Bernard et al., 1998; Kimura et al., 1999; Tseng et al., 1998; Hu et al., 2000). The forced overexpression of an Aurora-C-kinase-deficient mutant in cultured cells disrupts the Aurora-B–INCENP complex and induces polyploidy (Chen et al., 2005). Moreover, Aurora C can complement Aurora B function in mitotic cells (Sasai et al., 2004), and its localisation to centromeric regions of chromosomes and then to the spindle midzone in late anaphase indicates that it also acts as a passenger protein (Yan et al., 2005).

The function of Aurora C in the germ lines of disease-free animals is not clear. It has been thought to be redundant with Aurora B in the male germ line because the two enzymes have overlapping distributions in spermatocytes (Tang et al., 2006). It seems possible, however, that Aurora kinases B and C have independent roles in spermatogenesis because overexpression of dominant-negative Aurora B results in abnormal spermatocytes, increased apoptosis and spermatogenic arrest, and Aurora-C-null mice have compromised fertility owing to a variety of sperm defects (Kimmis et al., 2007). Evidence for a yet-uncharacterised role for Aurora C in meiosis I in mammals comes from the formation of tetraploid spermatozoa in sterile human males homozygous for a mutation in the human gene AURKC (Dieterich et al., 2007; Dieterich et al., 2009). In the mouse, AurC−/− females showed sterility, but unfortunately the oocyte phenotype was never examined before the line was lost (P. Sassone-Corsi, personal communication). Several other studies on mouse oocytes have used small-molecule inhibitors of Aurora kinases and shown that these affect meiotic progression in a variety of ways (Uzbekova et al., 2007; Swain et al., 2008; Vogt et al., 2009). However, because these compounds can inhibit all three Aurora kinases, attributing roles to specific enzymes from these studies is difficult. Thus, whether Aurora C has a role in female meiosis and whether its role can be substituted by Aurora B have remained uncertain.

There are several key differences between meiotic and mitotic divisions that offer the possibility for differential behavior of M-phase regulatory molecules. The meiotic maturation of mouse oocytes commences with germinal vesicle breakdown (GVBD), which is followed by chromosome condensation and spindle...
The individualization of chromosomes upon their condensation appears to play an important part in permitting assembly of the first meiotic spindle (Schuh and Ellenberg, 2007). During the prometaphase of the first meiotic division, the spindle migrates to the oocyte cortex to undertake meiosis I in an actin- and Cdc42-dependent process (Halet and Carroll, 2007; Na and Zernicka-Goetz, 2006). Tension is generated at metaphase because homologous chromosomes are held together by chiasmata at the sites of recombination, as opposed to sister chromatids being held together at centromeres, as in mitosis. The chiasmata are resolved when cohesion between chromatid arms, but not centromeres, is lost through the activity of Separase, which is activated through the destruction of its Securin partner mediated by the APC/C (Kudo et al., 2006; McGuinness et al., 2009). Just as in mitosis, the meiotic activation of the APC/C normally does not occur until chromosome bi-orientation has been achieved and, until this time, is kept inactive by the spindle-assembly checkpoint (SAC). Consequently, depletion of the checkpoint protein Mad2 (Homer et al., 2005) or the expression of its dominant-negative form (Wassmann et al., 2003) overcomes the meiotic block induced by nocodazole. Similarly, a dominant-negative Bub1 advances the polar body (PB) extrusion (Tsurumi et al., 2004), as does removal of Bub1 protein in a conditional knockout mouse line (McGuinness et al., 2009). Once the checkpoint is satisfied, dyads, each comprising one parental and one recombinant chromatid, segregate to the opposite poles. The highly asymmetrical positioning of the spindle ensures the extrusion of a small daughter cell from the egg, the first PB.

The scant knowledge about the function of the CPC in female meiosis in mammals has led us to use a series of approaches to modulate expression of its key components during mouse oocyte maturation. Depletion of INCENP or combined inhibition of Aurora kinases B and C demonstrates that CPC function is required to correct chromosome alignment, assure function of the SAC and to enable cytokinesis to extrude the PB. However, two aspects of our data also indicate functional differences between forms of CPC containing either the Aurora B or Aurora C kinase. First, the two Aurora kinases show differential localization on chiasmate chromosomes in meiosis I. Second, we find that expression of exogenous Aurora B stabilises Securin, leading to failure of resolution of chiasmate chromosomes, whereas exogenous expression of Aurora C phenocopies knockout of INCENP.

**Results**

**INCENP is required for chromosome congression in meiosis I and II and for cytokinesis**

To determine the roles of the CPC in female meiosis, we first sought to deplete INCENP. To this end, we used RNAi, previously shown to be effective for the specific downregulation of genes in mouse oocytes and pre-implantation embryos (Wianny and Zernicka-Goetz, 2000; Svoboda et al., 2000). We microinjected oocytes at the germlinal vesicle (GV) stage with various combinations of five different INCENP siRNAs, cultured such injected oocytes with the phosphodiesterase 3 inhibitor milrinone to delay GVBD and allow RNAi to take effect and, after 14 hours, released them to fresh culture medium to determine whether they could undergo normal meiotic maturation. Knockdowns of 95% of transcript levels were confirmed by quantitative rTPCR, with no reduction of levels being observed with siRNAs against scrambled nucleotide, GFP and GAPDH. To follow chromosome behaviour upon INCENP depletion, we used time-lapse microscopy and performed experiments on oocytes that were also injected with histone H2B–EGFP RNA (Hadjantonakis and Papaioannou, 2004).

To examine the destruction dynamics of Securin–GFP (Hagting et al., 2002), oocytes were injected with the relevant mRNA at levels that had no observable effects upon the timing of either GVBD or on extrusion of the PB (Fig. 1A,B). We found that, under these conditions, control RNAi oocytes matured normally. We observed the prometaphase arrays of chromosomes migrating to the cortex at 2 hours, progressing into anaphase at around 7.5 hours, reaching cytokinesis around 9 hours and arresting in metaphase II by 10 hours (Fig. 1A). By contrast, meiotic maturation was perturbed in INCENP RNAi oocytes (Fig. 1B). Although anaphase of meiosis I took place in all INCENP-depleted oocytes, half of these oocytes (49%, n=106) failed to extrude a PB, in comparison with 8% (n=94) of control oocytes. Those INCENP-depleted oocytes that failed to extrude the PB underwent membrane ruffling at 8 hours post GVBD, indicating a failed attempt to organise a contractile furrow.

We observed the expected destruction of Securin that is required to release Separase and mediate removal of the Rec8 cohesin subunit from sister chromatid arms and thus resolve chiasmata (Kudo et al., 2006; Herbert et al., 2003). This occurred concomitantly with the onset of anaphase both in control oocytes and in all INCENP-depleted oocytes (respectively profiles from three representative oocytes are shown in Fig. 1A’ B’). To calculate the rate of change of Securin protein and to determine the value and timing of maximal APC/C activity for each oocyte, we applied a kinetic analysis (McGuinness et al., 2009) (see also Materials and Methods) and expressed APC/C activity as its rate constant (kd[APC]). Maximal APC/C activity was 3.2 hours\(^{-1}\)±0.2 (n=29) or k\(_d\) [APC]=3.7 hours\(^{-1}\)±0.1 (n=57) and occurred between 8.0 hours and 8.5 hours post GVBD, a range of values similar to those described by McGuinness and colleagues (McGuinness et al., 2009). Maximal APC/C activity was slightly lower in INCENP-depleted than in control oocytes {kd[APC]=2.5 hours\(^{-1}\)±0.1 (n=46); Fig. 1C}, and the time of maximal activity was advanced by 1–2 hours (Fig. 1D). Thus, INCENP-depleted oocytes activate the APC/C prematurely and, following Securin destruction, proceed to metaphase II.

The destruction of Securin and progression of anaphase in INCENP-RNAi oocytes suggested that homologues were being separated in meiosis I. However, the temporal advancement of these processes raised the possibility that anaphase might be taking place before chromosomes were properly aligned. This led us to compare the positioning of chromosomes on spindles in fixed preparations of control and INCENP-depleted oocytes at times immediately preceding the predicted metaphase–anaphase transition of meiosis I and at maturation. In meiosis I, the chromosomes in control oocytes adopted a chiasmate arrangement, with their kinetochores being pulled towards the poles. The chromosomes of INCENP-depleted oocytes were much less well aligned at metaphase (Fig. 1E). We also observed that oocytes depleted of INCENP exited metaphase transiently, and sometimes telophase nuclei briefly formed before a spindle reassembled for meiosis II. The metaphase II spindle that formed in such oocytes was populated by numerous chromosome masses that failed to align correctly on a metaphase plate (Fig. 1E). This was in contrast to the tightly aligned chromosomes in control oocytes in meiosis II. When we compared the chromosomes of control and INCENP-depleted oocytes in spread preparations, we found similar sets of chiasmate chromosomes in meiosis I of both types. However, mature oocytes contained 20 univalent chromosomes, whereas the INCENP-depleted oocytes that failed to
extrude a PB had predominantly 40 univalents, with some oocytes showing a few persisting bivalents (Fig. 1E). Thus, the total number of univalents present on spindles of such INCENP-depleted oocytes was consistent with the observed failure of cytokinesis. In summary, the depletion of INCENP allowed oocytes to progress to meiosis II but affected extrusion of the PB. The majority of chromosomes were well condensed and resolved into univalents, indicating that Separase had been activated by destruction of Securin. Thus, chromosomes became scattered along the length of the spindle in both divisions, indicating that depletion of the INCENP passenger complex permitted anaphase before complete congression of bivalents in meiosis I and of univalents in meiosis II.

Inhibition of Aurora B and Aurora C phenocopies INCENP depletion

The ability of approximately one half of the INCENP-RNAi-treated oocytes to undertake cytokinesis suggested to us that we were not fully depleting the target protein, although we were unsuccessful in finding an antibody against INCENP that would allow us to quantitate the level of knockdown. This led us to ask what the consequences might be of pharmacologically inhibiting the function of the CPC in oocytes. Of the many small molecules have been developed as inhibitors of Aurora kinase, most cross-react with both Aurora A (serine/threonine-protein kinase 6) and Aurora B (reviewed in Pollard and Mortimore, 2009). There are, however,
some compounds that show selectivity of inhibition of Aurora B over Aurora A over a considerable concentration range. Therefore, we chose to treat oocytes with one such compound, AZD1152, that is a potent inhibitor of Aurora B (K<sub>i</sub>&lt;1 nM), a moderate inhibitor of Aurora C (K<sub>i</sub>=17 nM) and a poor inhibitor of Aurora A (K<sub>i</sub>=1.4 μM) (Mortlock et al., 2007). As neither this nor any other compound can selectively inhibit Aurora B versus Aurora C, we treated oocytes with AZD1152 at a concentration that we had determined from comparative studies of inhibitors of Aurora kinases in cultured cells should inhibit Aurora proteins B and C, but not Aurora A.

We found that, as with INCENP depletion, inhibition of Aurora B and Aurora C in oocytes with AZD1152 led to degradation of Securin, and this occurred ahead of the time of Securin degradation in control oocytes (compare Fig. 2A and Fig. 2B). The maximal activity of the APC/C was also reduced from 3.1 hours<sup>-1</sup>±0.2 (n=54) to 1.9 hours<sup>-1</sup>±0.1 (n=51) or 1.6 hours<sup>-1</sup>±0.1 (n=53) with 100 nM and 500 nM AZD1152, respectively (Fig. 2C). The reduction in time to progress to maximal APC/C activity was dramatic: from 8.5 hours±0.1 (n=54) in control oocytes to 5.5 hours±0.1 (n=51) and 5.1 hours±0.1 (n=53) in 100 nM and 500 nM AZD1152, respectively (Fig. 2D). Thus, the inhibitor advanced the timing of anaphase by approximately 3 hours.

Just as for INCENP RNAi, treatment with AZD1152 led to a failure of cytokinesis, but this time in all oocytes. We observed that, often, there was strong initiation of ingression of the cleavage furrow to the point at which a structure resembling a PB was formed. However, this structure was transient and in all cases underwent regression (arrows in Fig. 2B). Consistent with this failure of cytokinesis, oocytes in which both Aurora B and Aurora C were inhibited contained 40 univalent chromosomes that were highly scattered on the metaphase II spindle, in contrast to the aligned 20 univalents in control oocytes (Fig. 2E,F). Thus, the response of oocytes to INCENP RNAi and combined chemical inhibition of Aurora kinases B and C is qualitatively similar, but the drug treatment gives a more fully penetrant response.

Fig. 2. Pharmacological inhibition of Aurora kinases B and C phenocopies INCENP depletion. (A,B) Green fluorescence of Securin–GFP and a DIC image of oocytes are presented for a time-series of control oocytes (A) or oocytes treated with AZD1152 (B). The scale bar for all time-lapse images represents 50 μm. Arrows point to the initiation of cytokinesis, followed by subsequent regression of the PB. (A′,B′) Quantitation of total fluorescence of Securin–GFP in three representative oocytes, showing degradation profiles for control oocytes (green) and AZD1152-treated oocytes (blue). (C,D) Maximal APC/C activity and time taken to reach maximal activity, respectively, for every oocyte in the indicated groups. (E) Oocytes stained to reveal DNA (red) and microtubules (green) at 10 hours post GVBD. Preparations of spread chromosomes are shown alongside each stained panel. The scale bar for the immunofluorescence images in E and F represent 5 μm.
Aurora B and Aurora C differ in their dominant effects on meiotic progression upon elevated expression

Aurora B and Aurora C share a high degree of amino acid sequence similarity, they can each phosphorylate histone H3, and, in somatic cells, Aurora C can form complexes with INCENP, the known Aurora B partner, and complement the function of Aurora B (Chen et al., 2005; Sasai et al., 2004; Li et al., 2004). Nevertheless, they have been reported to have differing distributions on chiasmate chromosomes during meiosis I, suggesting that their functions might not be identical. In spermatozoa, Aurora C localises uniquely to the interchromatid axes and chiasmata, whereas Aurora B is at centromeres (Tang et al., 2006). In agreement with previous studies (Shuda et al., 2009), we found a similar distribution of Aurora proteins B and C following expression of the GFP- or HA-tagged kinases in oocytes and by immunostaining of endogenous Aurora kinases B and C (supplementary material Fig. S1A–D). We were, however, only able to detect Aurora B associated with chromosomes in meiosis I and not meiosis II. This localisation of both enzymes was lost following downregulation of INCENP (supplementary material Fig. S2 and also below).

To address whether the differing chromosomal distributions of the two kinases might reflect different functions, we first attempted to downregulate each Aurora by RNAi. We injected various combinations of six Aurora B siRNAs at concentrations sufficient to downregulate specifically greater than 95% of Aurora B RNA but not Aurora A or Aurora C. However, this proved insufficient to eliminate Aurora B protein completely, and it had no observable effects upon meiotic progression (supplementary material Fig. S1F,G). Attempts to downregulate Aurora C by RNAi led to a similar outcome (supplementary material Figs S3, S4). We therefore asked whether, by increasing the concentrations of injected siRNAs, we might be able to achieve a more substantial knockdown. We found that, when we used much higher concentrations of siRNAs than those needed to give specific knockdown of transcripts levels, this led to a SAC-mediated arrest of maturation in meiosis I. However, this turned out to be a nonspecific response of the oocyte to the very high concentrations of siRNA because we observed the same phenotype with all siRNAs used at such concentrations, including those directed against scrambled sequences (supplementary material Fig. S4). Thus, downregulation of these two kinases independently of each other proved impossible in our hands in mouse oocytes.

We therefore next examined whether overexpression of Aurora B or Aurora C, by injecting their synthetic mRNAs, could itself affect meiotic progression. This revealed that overexpression of Aurora C decreased the proportion of oocytes successfully completing maturation (from 84%, n=97, to 58%, n=104). Through time-lapse imaging, we found that the dominant-negative phenotype exhibited by oocytes expressing both endogenous and exogenous AurC mRNA was a failure of cytokinesis. Instead, at the time of cytokinesis in the control oocytes, oocytes overexpressing Aurora C underwent a period of membrane ruffling (Fig. 3A,B). Nevertheless, the Securin–GFP assay indicated that Securin was degraded, both in oocytes that failed and those that succeeded in extruding a PB. This was reflected by the activation of the APC/C to levels similar to those in oocytes expressing endogenous and exogenous Aurora C in which cytokinesis failed \(k_d[APC]=2.8\) hours\(^{-1}\pm0.3\) (n=39) and in those in which it succeeded \(k_d[APC]=2.3\) hours\(^{-1}\pm0.1\) (n=35). These values were comparable to values in the control group of oocytes \(k_d[APC]=1.9\) hours\(^{-1}\pm0.1\) (n=84) (Fig. 3D). Interestingly, this elevation of Aurora C expression led to advancement of maximal APC/C activity in all oocytes (t=6.7±0.1 hours and 6.8±0.1 hours in the two groups) compared with 9.3±0.2 hours in control oocytes (Fig. 3E).

Immunostaining of oocytes that failed to extrude PBI, as a result of this elevation of Aurora C expression, revealed spindles having low levels of chromosome misalignment (one or two univalents misaligned out of 40) yet fully separated univalents (Fig. 3F,G; supplementary material Fig. S5A). Thus, Securin degradation led to the release of Separase and the separation of homologues in meiosis I, but, as a result of cytokinesis failure, such oocytes now contained twice the normal complement of univalents. The similarity of this phenotype to that seen following depletion of INCENP suggested that the elevated levels of Aurora C disrupt the CPC complex and its functions.

By contrast, oocytes injected with a similar concentration of AurB mRNA tended to arrest during maturation (56%, n=134). In those oocytes, degradation of Securin occurred only extremely slowly \(k_d[APC]=0.6\) hours\(^{-1}\pm0.1\) (n=71), with the time to maximal APC/C activity being extended to a mean of 13.3±0.4 hours (Fig. 3C,D). The oocytes that were extensively delayed in meiosis I had bivalent chromosomes that appeared to be under tension, with well-separated kinetochores (Fig. 3F; supplementary material Fig. S5B). To observe the morphology of chromosomes in more detail, we performed preparations of chromosome spreads, which revealed 20 chiasmate bivalents, indicating that the homologues had not separated (Fig. 3G). This is consistent with a requirement for degradation of Securin to release Separase and mediate release of the cojoined chromatid arms in the bivalents (Kudo et al., 2006). The failure to activate the APC/C properly in order to degrade Securin when levels of Aurora B, but not Aurora C, are elevated would therefore be expected to lead to persistence of chiasmate chromosomes. This finding of a different dominant phenotype resulting from the overexpression of Aurora B compared with Aurora C suggests that the Aurora B and Aurora C forms of the CPC have different functions during disjunction of homologues in meiosis I.

Dominant phenotype resulting from Aurora B overexpression is suppressed by INCENP depletion

As Aurora B requires INCENP for its activation and function, we considered whether INCENP was required both for the localization of Aurora B and for the dominant phenotype resulting from its overexpression. To address this, we first examined the localization of ectopic GFP-tagged Aurora B throughout oocyte maturation and the consequences of its expression. We found that GFP–Aurora-B began to localize to chromosomes as they condensed around the time of GVBD and throughout prometaphase. The kinase then retained localization on the chromatin but became more pronounced on the kinetochores of chiasmate chromosomes as they entered a prolonged period of metaphase arrest (5–15 hours post GVBD; Fig. 4A). Of the oocytes injected with mRNA encoding Aurora-B–GFP, 88% (n=16) showed such arrest. When oocytes were co-injected with the same concentration of Aurora-B–GFP mRNA together with siRNA against INCENP, the resulting Aurora-B–GFP no longer localized to any structure in the cell. Moreover, 86% (n=14) no longer arrested at meiosis I and typically entered anaphase 6 hours post GVBD. They transiently entered a telophase-like state at around 6.5 hours but did not undertake cytokinesis before arresting in a metaphase-II-like state but with highly disorganized chromosomes (Fig. 4B). Thus, downregulation of INCENP prevented the association of Aurora B with chromosomes.
and suppressed its dominant phenotype thus allowing the resolution of chiasmata.

**Discussion**

In this study, we have addressed the functions of the two forms of the CPC, containing either Aurora B or Aurora C, in meiosis in the mouse oocyte. This has revealed that, although the CPC is not essential for the execution of the first meiotic division per se, it is required for the fidelity of this process and the subsequent execution of cytokinesis. Loss of the CPC in mouse oocytes as a result of depletion of INCENP led to a failure to correct chromosome misalignment, advancement of APC/C activation and progression through anaphase of meiosis I. Cytokinesis failed and, consequently, oocytes had 40 univalents that also failed to align at metaphase in meiosis II. Thus, the functions of the meiotic CPC appear to mirror those of its mitotic counterpart in correcting mis-attached kinetochores and ensuring SAC activity to control mitotic exit (Ditchfield et al., 2003; Hau‘f et al., 2003; Ciferri et al., 2008). The use of the pharmacological inhibitor of Aurora kinase AZD1152 confirmed the involvement of the CPC in the above cellular processes. This compound is, respectively, 1000 and 100 times more efficacious against Aurora B or Aurora C than against Aurora A (Mortlock et al., 2007) and is thus more selective than compounds previously used to investigate Aurora kinase function in oocyte maturation. Neither VX680, used to treat bovine oocytes (Uzbekova et al., 2007), nor ZM447439, used to treat mouse oocytes (Swain et al., 2008; Vogt et al., 2009; Shuda et al., 2009), shows sufficient selectivity to attribute specific functions to the three different Aurora kinases. Vogt and colleagues (Vogt et al., 2009) suggested that defects in chromosome condensation, congression, chiasmata...
resolution and PB extrusion were due to inhibition of Aurora B, but this conclusion does not take into account the pan-Aurora-kinase-inhibitory properties of this small molecule. Shuda and colleagues (Shuda et al., 2009) concluded that defects in chromosome alignment could result from inhibition of Aurora B because they could be rescued by increasing the expression of this enzyme. However, the dominant phenotype we observe following elevated expression of Aurora B suggests that other explanations are possible.

The existence of two forms of the CPC is a peculiarity of the germ line. In normal tissues, Aurora C appears to be expressed predominantly in the germ line, although it is also present in some cancer cells, where it can compete with Aurora B for binding to INCENP (Chen et al., 2005) and can also complement the mitotic enzyme. However, the dominant phenotype we observe following elevated expression of Aurora B suggests that other explanations are possible.

The differential localization of Aurora C and its choice of partners and spatial behaviour might depend upon similarly subtle differences in their primary sequence. Exactly how their differential localization and functions are achieved necessitates future investigation of the precise partners and/or substrates of Aurora C at its different chromosome locations and of how its choice of partners and spatial behaviour might be influenced by its primary sequence.

Materials and Methods

Oocyte and embryo collection

Oocytes were collected from wild-type F1 (C57BL/6xCBA) females (Charles River) or from females transgenic for histone-H2B–EGFP (Hajantonakis and Papaioannou, 2004) as described previously (Wianny and Zernicka-Goetz, 2000). GV oocytes were collected from ovary tissue in M2 medium supplemented with 4 mg/ml BSA, as described previously (Na and Zernicka-Goetz, 2006). Milrinone (Sigma) was added to collection medium at a final concentration of 100 μM where indicated. For embryos, superovulated F1 females were mated with F1 males. Zygotes were collected 15 hours post treatment with human chorionic gonadotrophin (hCG), in M2 containing 200 IU/ml of hyaluronidase and washed in fresh M2, then cultured in KSOM medium with 4 mg/ml BSA under paraffin oil in an atmosphere of 5% CO2 at 37.5°C (Gray et al., 2004).

Securin and, consequently, unresolved chiasmata following the overexpression of Aurora B, but not of Aurora C, points to the possibility of their differing functions in resolving these recombinant intermediates at the metaphase–anaphase transition of meiosis I. Aurora C would seem to be the main contributor to cytokinesis as increasing its levels to concentrations that would effectively compete with endogenous Aurora C for the binding of INCENP advances APC/C activation and results in some cytokinesis defects. This phenotype strongly resembles that seen following loss of INCENP or combined inactivation of the two kinases. Although we were unable to deplete either Aurora B or C kinase by RNAi in the oocyte, owing to the presence of the maternally inherited protein, this was possible in the zygote, where we found cytokinesis was dependent upon Aurora C rather than Aurora B (data not shown) (Lykke-Andersen et al., 2008) (M. Malumbres, personal communication).

Definitive resolution of the relative functions of Aurora kinases B and C in female meiosis will most likely require reassessment of the phenotypes of genetic knockouts. Previous studies of spermatogenesis in AurC−/− mice (Kimmins et al., 2007) reported defects in sperm morphology but did not fully examine meiosis. Both male and female AurC−/− mice showed sterility, but unfortunately, owing to these fertility problems, this knockout line has been lost and so further examination of the basis of this sterility cannot be undertaken (P. Sassone-Corsi, personal communication).

AURK mutations have been described for humans, where they prevent meiois I in males, leading to the formation of polysperm, multi-tailed spermatozoa (Dieterich et al., 2007; Dieterich et al., 2009). However, two women have been found who are homozygous for this same AURK mutation and yet are fertile (Dieterich et al., 2009). Thus, it is possible that Aurora B might be able to substitute for Aurora C function in female meiosis in the human.

Taken together, our data suggest that Aurora B and Aurora C might have overlapping, yet partially independent, roles in meiosis in the mouse. The different response of oocytes to the overexpression of the two kinases, together with their differential localization, suggests that, in addition to a common set of CPC partners, they might have additional differing partners or alternative substrates. It is noteworthy that two recent papers have shown that the spatial localization of Aurora A and its choice of partners is affected by a single amino acid in its primary sequence – mutating glycine 198 in Aurora A to its asparagine counterpart found at this site in both Aurora B and Aurora C leads to the mutant Aurora A to behave as a passenger protein (Fu et al., 2009; Hans et al., 2009). The differential behavior of Aurora B and Aurora C in meiosis might depend upon similarly subtle differences in their primary sequence. Exactly how their differential localization and functions are achieved necessitates future investigation of the precise partners and/or substrates of Aurora C at its different chromosome locations and of how its choice of partners and spatial behaviour might be influenced by its primary sequence.

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In vitro fertilization
Spermatozoa released from caudae epididymides of F1 males were suspended in 0.5 ml of fertilization medium (Fraser, 1983) containing 4 mg/ml BSA and incubated for 1.5 hours to allow capacitation and a spontaneous acrosome reaction. The sperm concentration was approximately 2 × 10^6 spermatozoa/ml. Before fertilization, zona pellucidae were removed by exposure of oocytes to acidic Tyrode’s solution (pH 2.5) (Nicolson et al., 1975). Oocytes were placed in 100 μl droplets of fertilization medium, and 1 μl of the preincubated sperm suspension was added (final concentration of sperm suspension was approximately 2 × 10^7 spermatozoa/ml). After 30 minutes, the oocytes were pipetted several times to remove loosely attached spermatozoa and then cultured in KSO.

cDNA cloning, mRNA synthesis, siRNA preparation and microinjection
Mouse AurC and AurB were cloned with Superscript One-step RT-PCR for long templates (Invitrogen) using gene-specific primers (supplementary material Table S1). HA-tagged (N-terminus) and GFP-tagged (C-terminus) Aurora C and Aurora B were engineered and subcloned into a RN3P vector for in vitro transcription of mRNA, as described previously (Zernicka-Goetz et al., 1997). siRNA against 5’ and 3’ UTRs of AurC and control siRNAs were designed and purchased from Invitrogen (supplementary material Table S2). Microinjection of mRNA into oocytes was performed as described previously (Na and Zernicka-Goetz, 2006). Annealed siRNAs were dissolved in Dnase, RNase-free water.

Live-lapse imaging experiments
Time-lapse movies were performed either on a Zeiss Axiosvert 200M epifluorescence microscope or a DeltaVision Olympus microscope equipped with 37°C incubator. Time-lapse movies were performed either on a Zeiss Axiovert 200M epifluorescence microscope or a DeltaVision Olympus microscope equipped with 37°C incubator. APC/C activity was calculated from changes in the level of Securin–GFP fluorescence using the analysis devised by McGuinness and colleagues (McGuinness et al., 2009). The acquisition of digital time-lapse images was controlled by AQM6 (Andor/Kinetic-imaging) and DeltaVision software packages.

Calculation of APC/C activity from the dynamics of Securin–GFP levels
APC/C activity was calculated from changes in the level of Securin–GFP fluorescence using the analysis devised by McGuinness and colleagues (McGuinness et al., 2009). In this scheme, the rate of change of Securin–GFP level (dS/dt) is dependent upon two opposing kinetic processes: production through translation from mRNA and degradation through APC/C-dependent and -independent processes:

\[
\frac{dS}{dt} = k_M S - (k'_S + k_{APC})S .
\]

Therefore:

\[
k_{APC} = \frac{k_M - dS }{S - k'_s} .
\]

Hence:

\[
S = S_{max} \left(1 - e^{-\frac{t}{\tau'}}\right) ,
\]

at \(t = \tau'\), \(S = S_{max}\) therefore:

\[
S = S_{max} \left(1 - e^{-\frac{t}{\tau'}}\right) .
\]

Fitting of recovery-phase data in this control experiment with Eqn 5 gave values for \(k'_S\) (in the range of 0.16 to 0.32 hours⁻¹) — of the same order as reported by McGuinness and colleagues (McGuinness et al., 2009) that mRNA degradation is negligible over the time course of the experiment. In control experiments, where [APC] falls to a low level post-PB1 and Securin–GFP fluorescence recovers to ~80% of the initial value, Eqn 1 reduces to:

\[
\frac{dS}{dt} = k_M S - k_S S .
\]

Antibodies, immuno staining and confocal microscopy

The following primary antibodies were used: monoclonal rat anti-tubulin (YL1/2, polyclonal rabbit anti-phospho-HS1S10 and polyclonal rabbit anti-GFP (290) were from Abcam. Monoclonal rat anti-HA (3F10) and monoclonal mouse anti-GFP were from Roche. The antibody against Aurora B was Santa Cruz Biotechnology anti-Ark2 (H-75) and was raised against the N-terminal 75 amino acids specific to human AurB, a region absent from Aurora C. Our in-house antibody against mouse Aurora C was raised in rabbits against peptide amino acids 1–17 from Harlan Sera. Oocytes were first treated with acidic Tyrode’s solution to remove the zona pellucida. Fixation and immunostaining were performed as described previously. Immunofluorescence images were acquired on a Bio-Rad MRC-1024 and an Olympus FV1000 confocal microscope with a ×63 oil-immersion objective.

Chromosome spread assay
Oocytes were first incubated in a hypotonic solution (1% Na Citrate) for 10 minutes, then put on a glass slide, fixed with a drop of freshly made fixative [methanol/acetic acid (3:1)], dried at room temperature and stained with Giemsa (BDH, product 350626M).

We thank Anna Ajduk for help in assaying Aurora B function in the second meiosis, Bernhard Strauss for help in establishing conditions for monitoring Securin degradation and Jonathan Pines for the gift of the Securin–GFP construct; we also thank Helen Bolton for examining the role of Aurora C in embryos and John Crang for help in compiling figures. This work was supported by a Wellcome Trust grant to M.Z.-G. and an MRC Programme Grant to D.M.G. B.S. held a Gates Foundation Studentship of the University of Cambridge. J.N. is supported by a MRC Career Development Fellowship. K.L.-H. was supported by the Alfred Benzon Stipend, Denmark (current address: Institute of Medical Biochemistry, Aarhus University, Denmark). Part of the image acquisition was carried out in the Wellcome Trust-supported light microscopy facility in the University of Sheffield, UK (grant: GR077544/A1A). Confocal microscope facilities in the D.M.G. laboratory were supported by grants from CR-UK and BBBSRC. Deposited in PMC for release after 6 months.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/123/24/4292/DC1

References


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Silencer Select Negative Control #1 siRNA, catalogue number 4390844 (Ambion/Applied Biosystems). Silencer Select GAPDH siRNA (Hs, Mm, Rn), catalogue number 4390849 (Ambion/Applied Biosystems).
Table S2. Effects of exogenous Aurora kinases B and C on polar body extrusion (PBE)*

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<td>81 (84)</td>
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<td>Aurora C mRNA</td>
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<td>44 (42)</td>
<td>60 (58)</td>
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<tr>
<td>Aurora B mRNA</td>
<td>134</td>
<td>75 (56)</td>
<td>59 (44)</td>
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*Aggregated data from two independent experiments.