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 (Kimmins 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

formation. 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 knockdown 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 germinal 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 concomitant with the onset of anaphase both in control oocytes and in all INCENP-depleted oocytes (respective 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 (k_d[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 {k_d[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
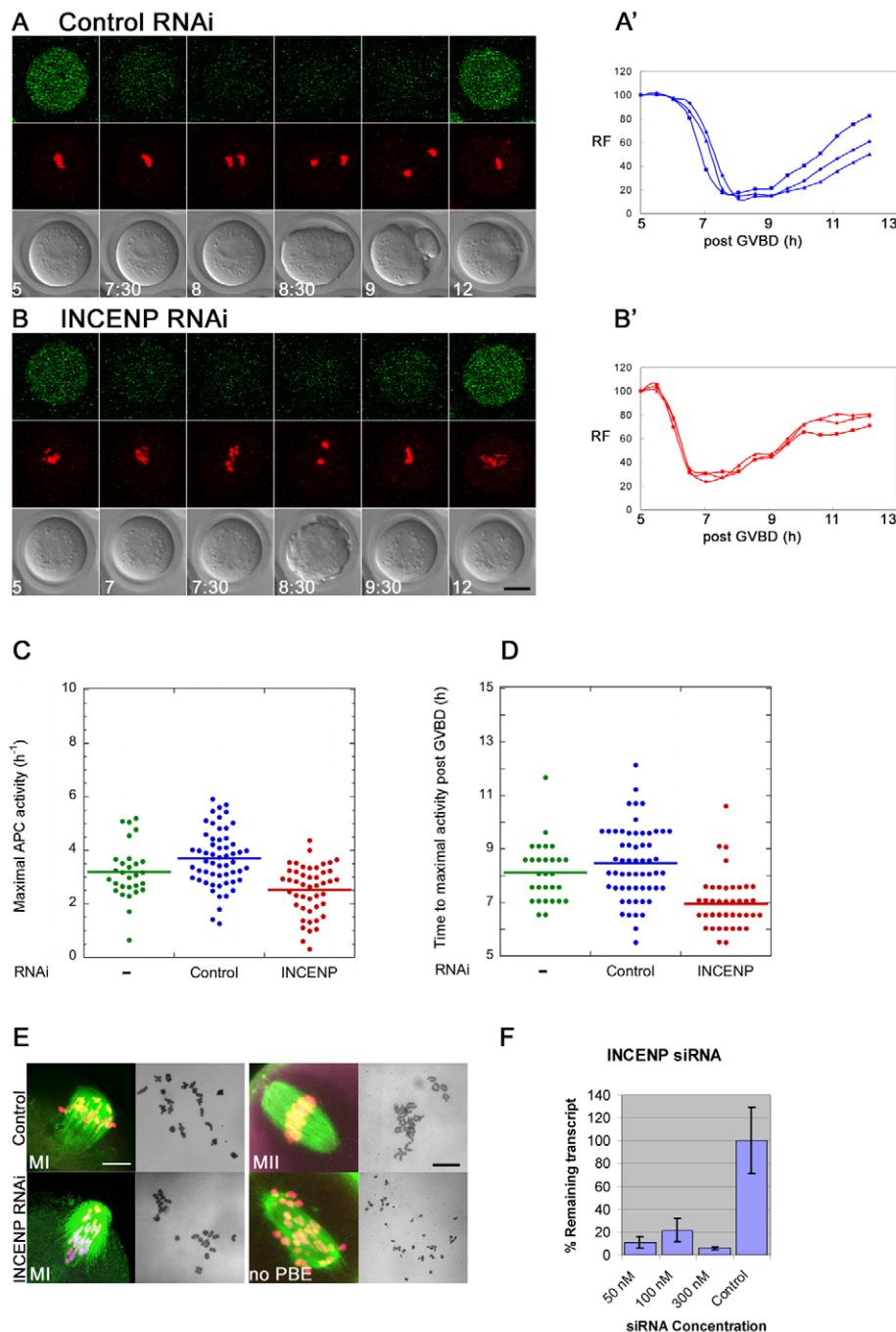
Fig. 1. Depletion of INCENP leads to misaligned chromosomes, advancement of APC/C activation and prevents cytokinesis. (**A,B**) Green fluorescence of Securin–GFP and a digital interference contrast (DIC) image of oocytes is presented for a time-series of a control oocyte injected with an irrelevant siRNA (A) or siRNA against *INCENP*. (B). The scale bar for all time-lapse images represents 50 μm. (**A′,B′**) Quantitation of total fluorescence of Securin–GFP in three representative oocytes showing degradation profiles for RNAi control (blue) and INCENP-RNAi-treated (red) oocytes. 'RF' in this and subsequent figures refers to the relative fluorescence of Securin–GFP (see the Materials and Methods section and the supplementary material for a description of the kinetic parameters). (**C,D**) Maximal APC/C activity and time taken to reach maximal activity, respectively, for every oocyte in the indicated groups. The minus sign (–) denotes oocytes otherwise untreated except for the injection of mRNA encoding Securin–GFP (green). Oocytes labeled 'control' are treated with an irrelevant siRNA. (**E**) Oocytes stained to reveal DNA (red) and microtubules (green) for controls in meiosis I (MI) and meiosis II (MII; upper row) and INCENP RNAi treated at MI and at 10 hours having failed to extrude the PB (lower row). Preparations of spread chromosomes are shown alongside each stained panel. The scale bar for the immunofluorescence images in A and B represents 10 μm and, for the chromosome spreads, 20 μm. (**F**) Knockdown of INCENP transcripts assessed by qrtPCR for the indicated concentrations of siRNA.

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_i<1 nM), a moderate inhibitor of Aurora C (K_i=17 nM) and a poor inhibitor of Aurora A (K_i=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^{-1}±0.2 (n=54) to 1.9 hours^{-1}±0.1 (n=51) or 1.6 hours^{-1}± 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.
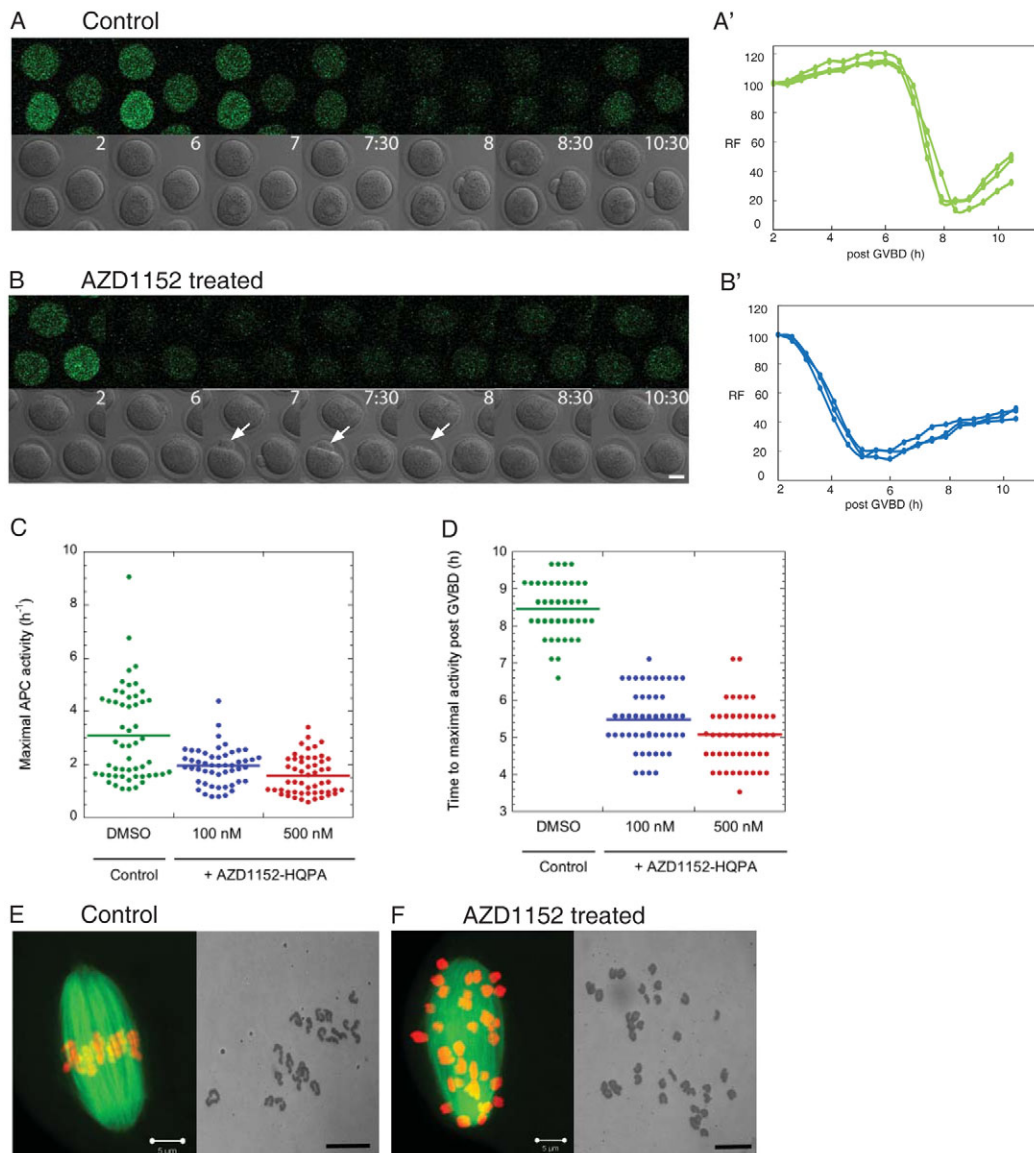
Fig. 2. **Pharmacological inhibition of Aurora kinases B and C phenocopies INCENP depletion.** (**A**,**B**) Green fluorescence of Securin–GFP and a DIC image of oocytes are presented for a time-series of control oocytes (A) or oocytes treated with AZD1152 (B). The scale bar for all time-lapse images 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}±0.3 (*n*=39)} and in those in which it succeeded {k_d[APC]=2.3 hours^{-1}±0.1 (*n*=35)}. These values were comparable to values in the control group of oocytes {k_d[APC]=1.9 hours^{-1}±0.1 (*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}±0.1 (*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; Hauf 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
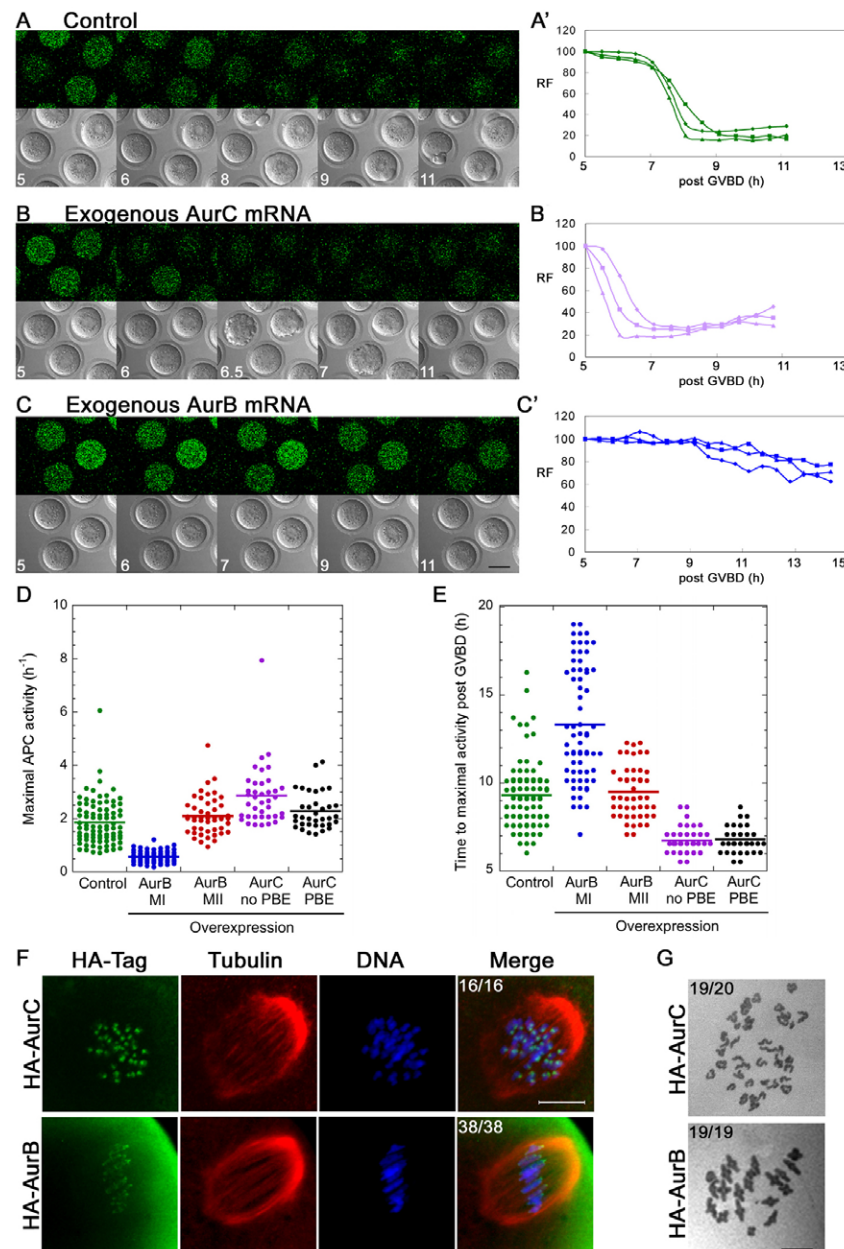

Fig. 3. Overexpression of *AurC* mRNA prevents cytokinesis; overexpression of *AurB* mRNA prevents APC/C activation and leads to arrest with chiasmate dyads. Green fluorescence of Securin–GFP and a DIC image of oocytes are presented for each series in which images are presented at the indicated time-points. (**A**) Time-lapse series of Securin in three representative control oocytes. (**A′**) Quantitation of total fluorescence of Securin–GFP in the three oocytes shown in (A). (**B**) Time-lapse series of Securin in three representative oocytes into which *AurC* mRNA had been injected at 0.5 µg/µl. (**B′**) Quantitation of total fluorescence of Securin–GFP in the three oocytes shown in (B). Note that Securin destruction occurs in all oocytes, although cytokinesis [PB extrusion (PBE)] fails. 58% of oocytes degraded Securin and extruded PBs (black data-points in panels D and E). (**C**) Time-lapse series of Securin in three representative oocytes into which *AurB* mRNA had been injected at 0.5 µg/µl. The scale bar referring to all time-lapse images represents 100 µm. (**C′**) Quantitation of total fluorescence of Securin–GFP in the three oocytes shown in C. Note the slowed destruction of Securin in these oocytes that arrested in MI. 44% of oocytes progressed to MII and showed destruction of Securin (red data-points in panels D and E). (**D**) Values of maximal APC/C activity and (**E**) time to maximal activity post GVBD for every oocyte in all groups analysed. Oocytes expressing synthetic *AurC* mRNA have been divided into two categories, depending upon whether they failed to exclude a polar body (no PBE; purple data-points) or succeeded (PBE; black data-points). Oocytes expressing synthetic *AurB* mRNA have also been divided into two categories, depending upon whether they arrested in MI (MI; blue data-points) or progressed to MII (MII; red data-points). (**F**) Oocytes injected with synthetic mRNA encoding HA-tagged Aurora kinases B or C, fixed 10 hours post GVBD and stained to reveal HA–Aurora-B or HA–Aurora-C (green), tubulin (red) and DNA (blue). Oocytes overexpressing HA–Aurora-C have a meiosis II spindle with some misaligned chromosomes. Oocytes overexpressing HA–Aurora-B have a meiosis I spindle with a chiasmate arrangement of dyads. (**G**) Preparations of chromosome spreads from the indicated number of oocytes that had failed to extrude a PB as a result of expressing exogenous Aurora C (note the 40 univalents), and preparations of chromosome spreads from the indicated number of oocytes that had failed to extrude a PB as a result of expressing exogenous Aurora B (note the 20 chiasmate bivalents). The scale bar for the immunofluorescence images represents 10 µm and, for the chromosome spreads, 20 µm.
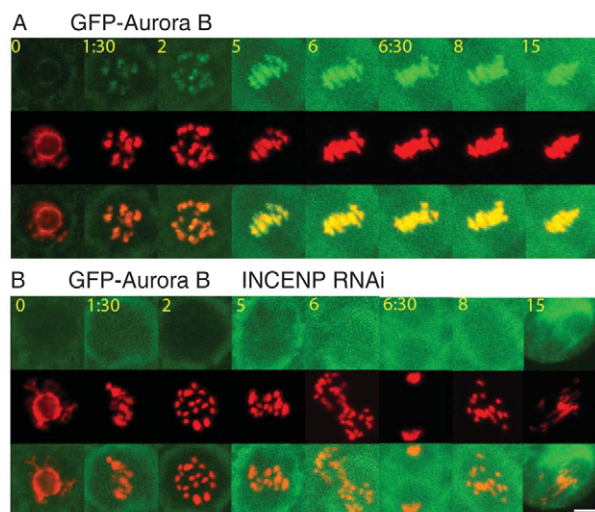
Fig. 4. Depletion of INCENP prevents localization of exogenous Aurora B and suppresses the dominant phenotype of its expression. (A) Time-lapse series showing chromatin (histone-H2B–RFP; red) from oocytes injected with synthetic mRNA encoding Aurora-B–GFP (green). Note the localization of Aurora B to paired homologues held in a chiasmate configuration for at least 10 hours. **(B)** Time-lapse series showing chromatin (histone-H2B–RFP; red) from *INCENP*-RNAi-treated oocytes injected with synthetic mRNA encoding Aurora-B–GFP (green). Note the failure of Aurora B to localize to chromosomes or the central spindle and the completion of meiosis I, as described in the text, in the absence of cytokinesis. Scale bar: 10 μm.

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 function of Aurora B (Sasai et al., 2004; Li et al., 2004). Two lines of evidence from our current study suggest that the form of the CPC containing Aurora C has a subset of functions that differ from the Aurora B form in the first meiotic division of mouse oocytes. First, the two kinases localise differentially in meiosis I: Aurora C is found predominantly along the chromosome arms that hold the homologues together, whereas Aurora B predominates at the centromeric regions – a similar pattern is also seen in spermatocytes (Tang et al., 2006). Only following the resolution of chiasmata do the kinases behave as typical CPC proteins at the mid-zone of the central spindle before moving onto the centromeres of metaphase II chromosomes, as also described recently by Shuda and colleagues (Shuda et al., 2009). The dependence of this localization of the kinase upon its CPC partners is demonstrated by our observation that, following depletion of INCENP, Aurora C fails to localize either to chromatin from GVBD onwards or to the central spindle in anaphase. Second, the arrest in meiosis I with stable levels of

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).

AURKC mutations have been described for humans, where they prevent meiosis I in males, leading to the formation of polyploid, multi-tailed spermatozoa (Dieterich et al., 2007; Dieterich et al., 2009). However, two women have been found who are homozygous for this same *AURKC* 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 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.

Materials and Methods

Oocyte and embryo collection

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 2×10^7 spermatozoa/ml. Before fertilization, zonae 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 2×10^5 spermatozoa/ml). After 30 minutes, the oocytes were pipetted several times to remove loosely attached spermatozoa and then cultured in KSOM.

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-imaging experiments

Time-lapse movies were performed either on a Zeiss Axiovert 200M epifluorescence microscope or a DeltaVision Olympus microscope equipped with 37°C incubator and 5% CO_2 supply, as described previously (Na and Zernicka-Goetz, 2006). The acquisition of digital time-lapse images was controlled by AQM6 (Andor/Kinetic-imaging) and DeltaVision software packages.

Securin–GFP fluorescence was quantitated on each image and normalized at completion of the experiment to assign a 100% value at 5 hours post GVBD. The data sets were used to quantitate the rate of change of Securin and APC/C activity, as described below.

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:

$$\frac{dS}{dt} = k_s M_o - (k'_d + k_d [APC])S \ . \tag{1}$$

Therefore:

$$k_d [APC] = \left(\frac{k_s M_o - \dfrac{dS}{dt}}{S} \right) - k'_d \ , \tag{2}$$

where k_s is the kinetic constant for protein synthesis, M_o is the initial concentration of Securin–GFP mRNA, k_d and k'_d are the rate constants for APC/C-dependent and -independent degradation, respectively, S is the concentration of Securin–GFP at time t and [APC] is the concentration of APC/C. McGuinness and colleagues (McGuinness et al., 2009) showed that mRNA degradation is negligible over the time course of the experiment. In control experiments, where [APC] falls to a low level post-PBE and Securin–GFP fluorescence recovers to ~80% of the initial value, Eqn 1 reduces to:

$$\frac{dS}{dt} = k_s M_o - k'_d S \ . \tag{3}$$

Hence:

$$S = \frac{k'_s M_o}{k'_d} \left(1 - e^{-k'_d t} \right) , \tag{4}$$

at $t = \infty$, $S = S_{max}$, therefore:

$$S = S_{max} \left(1 - e^{-k'_d t} \right) . \tag{5}$$

Fitting of recovery-phase data in this control experiment with Eqn 5 gave values for k'_d (in the range of 0.16 to 0.32 hours^{-1} – of the same order as reported by McGuinness and colleagues of 0.1 hours^{-1} for APC/C-minus cells) and $k_s M_o$ as $S_{max} = k_s M_o / k'_d$. These kinetic parameters were used in Eqn 2 to calculate APC/C activity (k_d[APC]) during oocyte maturation from the levels of S and its rate of change (dS/dt) calculated at each time point. However, in the majority of experiments, where there was substantial APC/C activity post PBE and hence recovery of Securin–GFP to less than 80% of the initial value, typical values of k'_d were used and $k_s M_o$ was calculated from $S_{max} \times k'_d$. The validity of this approach can be seen from Eqn 2, where the S and dS/dt terms dominate, and the absolute values of k'_d and $k_s M_o$ do not significantly affect the calculated values of APC/C activity.

Antibodies, immunostaining and confocal microscopy

The following primary antibodies were used: monoclonal rat anti-tubulin (YL1/2), polyclonal rabbit anti-phospho-H3S10 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 by 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 350862M).

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