Genetic disruption of aurora B uncovers an essential role for aurora C during early mammalian development

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SUMMARY
Mitosis is controlled by multiple kinases that drive cell cycle progression and prevent chromosome mis-segregation. Aurora kinase B interacts with survivin, borealin and incenp to form the chromosomal passenger complex (CPC), which is involved in the regulation of microtubule-kinetochore attachments and cytokinesis. Whereas genetic ablation of survivin, borealin or incenp results in early lethality at the morula stage, we show here that aurora B is dispensable for CPC function during early cell divisions and aurora B-null embryos are normally implanted. This is due to a crucial function of aurora C during these early embryonic cycles. Expression of aurora C decreases during late blastocyst stages resulting in post-implantation defects in aurora B-null embryos. These defects correlate with abundant prometaphase figures and apoptotic cell death of the aurora B-deficient inner cell mass. Conditional deletion of aurora B in somatic cells that do not express aurora C results in chromosomal misalignment and lack of chromosome segregation. Re-expression of wild-type, but not kinase-dead, aurora C rescues this defect, suggesting functional overlap between these two kinases. Finally, aurora B-null cells partially arrest in the presence of nocodazole, suggesting that this kinase is not essential for the spindle assembly checkpoint.

KEY WORDS: Aurora kinase B, Aurora kinase C, Cancer target, Mitosis, Mouse development, Spindle assembly checkpoint

INTRODUCTION
Progression through the cell cycle requires the controlled activation of different families of kinases that regulate diverse cellular processes required for cell division. Early work in Drosophila led to the identification of aurora mutants, which carry a loss-of-function mutation in a serine/threonine kinase essential for centrosome separation and the formation of bipolar spindles (Glover et al., 1995). A single aurora protein exists in budding (increase-inploidy 1; Ipl1) or fission (Ark1) yeast, whereas two family members, aurora A and aurora B, are present in worms, flies and frogs. Three different aurora family members, known as aurora A, B and C, exist in mammals (Nigg, 2001). Aurora B and C are close paralogs that probably arose from a cold-blooded vertebrate common ancestor (Brown et al., 2004).

Aurora kinases participate in multiple processes during the mammalian cell cycle (Carmena and Earnshaw, 2003). Aurora A is required for building a bipolar spindle regulating centrosome separation and microtubule dynamics (Barr and Gergely, 2007; Giet et al., 2005). Aurora B belongs to the chromosome passenger complex (CPC) that localizes to the kinetochores from prophase to metaphase and to the central spindle and midbody in cytokinesis (Carmena and Earnshaw, 2003; Ruchaud et al., 2007). Other mammalian CPC proteins include the inner centromere protein incenp, survivin and borealin, which control the targeting, enzymatic activity and stability of aurora B (Ruchaud et al., 2007). The CPC is one of the most upstream regulators of centromere and kinetochore function, being responsible for the recruitment to the kinetochore and centromere of a growing number of proteins, including inner centromeric proteins (Sgo1, Sgo2, MCAK), regulators of the microtubule-kinetochore interaction [such as Ndc80 (HEC1), CENP-E or Plk1 among others] or proteins involved in the spindle assembly checkpoint (SAC; such as Mad2, BubR1 or Mps1) (for a review, see Kelly and Funabiki, 2009). Some of these molecules, including Ndc80, Dam1 and MCAK, are aurora B substrates suggesting a crucial role for the CPC in the destabilization of aberrant microtubule-to-kinetochore attachments and the SAC-dependent delay until these defects are corrected (Nezi and Musacchio, 2009). During cytokinesis, aurora B is localized to the midbody remnant where its local inactivation is crucial for completion of abscission (Guse et al., 2005; Steigemann et al., 2009). Aurora B also participates in mitotic phosphorylation of Ser10 and, probably, Ser 28 in histone H3. These events seem to be necessary for chromosome condensation although the correlation between H3S10 phosphorylation and the condensation of chromosomes is not fully established (Johansen and Johansen, 2006; Nowak and Corces, 2004; Prigent and Dimitrov, 2003). Less is known about aurora C, which can also bind members of the CPC (Li et al., 2004) and its ectopic expression can rescue aurora B loss of function in cultured cells (Sasai et al., 2004; Slattery et al., 2009; Slattery et al., 2008; Yan et al., 2005). Although aurora C is known to have a specific role in spermatogenesis (Dieterich et al., 2007; Kimmins et al., 2007; Tang et al., 2006) and oogenesis (Sharif et al., 2010), its physiological relevance in the regulation of mitosis is not well understood.

In mammals, the CPC seems to be required for early embryonic development and embryos degenerate by E2.5-3.5 in the absence of the CPC components survivin (Uren et al., 2000), incenp (Cutts
et al., 1999) or borealin (Yamanaka et al., 2008). We report here that aurora A-null embryos develop normally during the early cell divisions and die only after implantation, suggesting the presence of normal cell cycles in the early embryonic cell divisions in the absence of aurora B. Interestingly, aurora C is highly expressed during the early cell divisions and it seems to be responsible for CPC function in these pre-implantation embryos. After implantation, aurora B-defective embryos and somatic cells accumulate prometaphase/metaphase figures eventually leading to apoptotic cell death. The cellular defects induced by acute elimination of aurora B in somatic cells can be rescued by re-expressing active aurora C. Finally, we also show that aurora B-null cells can still arrest in the absence of microtubules, suggesting that this protein is not essential for the SAC.

**MATERIALS AND METHODS**

### Aurora B-targeted mice

The conditional targeting construct was assembled by flanking exons 2-6 of the murine *Aurkb* locus withloxP sequences (Fig. 1A). An additional knock-in that expresses the β-galactosidase (*lacZ*) gene was generated (Fig. 1 and see Fig. S1 in the supplementary material) following the FLEX strategy as reported previously (Schnuten et al., 2003). Tg.pCAG-Flpe (Rodriguez et al., 2000) or Tg.CMV-Cre (Schwenk et al., 1995) transgenic mice were used for ubiquitous expression of Flp or Cre recombinases. All animals were maintained in a mixed 129/Sv (25%) x CD1 (25%) x C57BL/6J (50%) background following the animal care standards of the institution (Ethical Committee ISCIII). Genotyping protocols are available upon request (see also Fig. S1 in the supplementary material). Immunohistochemical examination of the tissues and pathologies analyzed were performed using specific antibodies against aurora B (Abcam), aurora C (Invitrogen), phospho-p53 (Ser15; Cell Signaling), γ-H2AX (Ser139; Millipore), active caspase 3 (R&D Systems), cyclin B1 (Millipore), Sox2 (Invitrogen), phospho-p53 (Ser15; Cell Signaling), γ-H2AX (Ser139; Millipore), active caspase 3 (R&D Systems), cyclin B1 (Millipore), Sox2 (Millipore) and GATA4 (Santa Cruz Biotechnology). Other routine histological techniques and the quantification of DNA ploidy in sections was performed as described previously (Garcia-Higuera et al., 2008).

### Culture and transfection of embryos

Fertilized embryos at E15.2-5.2 were collected by flushing the uteri of pregnant females with M2 medium (Sigma) and cultured in vitro in potassium simplex optimized medium (KSPORT; Chemicon International). To generate outgrowths of the inner cell mass, blastocysts were transferred into gelatinized 96-well plates and cultured in embryonic stem (ES) cell medium [DMEM + GlutaMAX, 15% fetal bovine serum (FBS), non-essential amino acids] for several days. Two-cell embryos were incubated with ZM447439 drug (Abbott) and gene expression was quantified with the SuperScript III Platinum Assay Kit (Invitrogen), according to the manufacturer’s instructions, in a BioRad iCycler Real-Time PCR apparatus. Data analysis was performed using the iQ5 v. 2.0 software (BioRad).

### Primary mouse embryo fibroblasts (MEFs)

MEFs were obtained from E14.5 embryos and cultured using routine protocols (Garcia-Higuera et al., 2008). The adenoviruses expressing GFP or the Cre recombinase (Ad5 CMV-Cre) were obtained from the University of Iowa (Iowa City, IA, USA). Infection was carried out for 2 days in cell culture synchronized in G0 by serum deprivation and confluence. DNA content was analyzed by flow cytometry (FACS; Becton-Dickinson). The duration of mitosis was scored by videomicroscopy using Deltavision apparatus. Nocodazole (Sigma) was used at 3.5 μM to completely prevent the formation of microtubules. The mouse aurora C cDNA was amplified by RT-PCR (Superscript II Reverse Transcriptase, Invitrogen) from mouse testis RNA and cloned as GFP- or V5-fusions as described previously (Fernandez-Miranda et al., 2010). The aurora C kinase-dead mutant (K45M) was prepared using the Quick-Change Site Mutagenesis Kit (Invitrogen). The wild-type and kinase-dead (D205A) aurora C cDNAs were described previously (Fernandez-Miranda et al., 2010). For rescue experiments, primary MEFs were first transduced with Cre adeno virus in G0 and 48 hours later they were transfected with GFP-aurora B/C constructs using the Amaxa nucleofection technology (Lonza AG) and seeded in 10% FBS to start a new cell cycle. Mad2 expression was knocked down using specific small interference (si) RNAs from Dharmaco.

### Immunofluorescence and protein analysis

For immunofluorescence, embryos were fixed with cold methanol or 5% paraformaldehyde (PFA), rinsed with M2 medium, washed in PBS containing 0.1% bovine serum albumin (BSA; Sigma) and incubated with 0.1% Triton X-100 for permeabilization. MEFs were fixed with 4% PFA and permeabilized with 0.15% Triton X-100. Embryos or MEFs were then blocked with 3% BSA and incubated with anti-centromere antibody (ACA; Antibodies Incorporated) or primary antibodies against α-tubulin (Sigma), γ-tubulin (Sigma), aurora B (Abcam or BD Biosciences), BubR1 (a gift of S. S. Taylor, University of Manchester, UK), Mad2 [a gift of E. Salmon (University of North Carolina, USA) or K. Wassmann (CNRS, France)], cyclin B1 (Santa Cruz Biotechnology), incenp (a gift of C. Rodriguez, Hospital Puerta del Mar, Spain) and phospho-histone H3 S10 (P-H3; Upstate Biotechnologies). The corresponding secondary antibodies were from Molecular Probes (Invitrogen). Images were obtained using a confocal ultra-spectral microscope (Leica TCS-SP5).

### Immunodetection in protein lysates, cells were lysed in RIPA buffer supplemented with protease and phosphatase inhibitory cocktails (Sigma). Proteins were separated on SDS-PAGE, transferred to nitrocellulose membranes (BioRad), and probed using specific antibodies against aurora B (BD Transduction and Abcam), incenp (Abcam), Mad2 (MBL), GFP (Roche) and α-tubulin (Sigma). For immunoprecipitation, total protein lysates were pre-cleared with protein-G-agarose beads (Amersham) and incubated with mouse anti-GFP (Roche). Immunoprecipitates were then washed three times in RIPA buffer and used for immunodetection.

### RESULTS

#### Generation of aurora B mutant mice

To generate an *Aurkb* null allele we first flanked exons 2-6 with loxP sequences using an frt-neo (neomycin-resistant gene)-fRT cassette for selection purposes as indicated in Fig. 1A. In addition, we also generated a knock-in allele, *Aurkb(Z)*, in which the expression of the endogenous *Aurkb* gene is replaced by *lacZ* transcripts encoding β-galactosidase (Fig. 1B,C and see Fig. S1 in the supplementary material). The neomycin-resistant cassettes were removed by crossing with transgenic mice expressing the FLP recombinase (see Materials and methods) resulting in the *Aurkb(Z)* alleles. Germline deletion of exons was achieved by additional crosses with Tg.CMV-Cre transgenic mice to generate the *Aurkb(−)/−* or *Aurkb(Z) alleles. These two alleles were null for aurora B expression, as detected by immunofluorescence (see below), and led to similar phenotypes.

Lack of one allele of aurora B did not result in major alterations during the proliferation of cultured cells or during development. *Aurkb(+/−)* embryonic fibroblasts proliferated well in culture and did not display obvious defects during cell cycle progression (see Fig. S2 in the supplementary material). Similarly, *Aurkb(+/−)* mice...
developed normally and were fertile. However, a few Aurkb(+/–) males (16.6% incidence) developed oligospermia by 12 months of age (see Fig. S3 in the supplementary material), in agreement with a relevant role of aurora B during spermatogenesis (Kimmins et al., 2007). In addition, Aurkb(+/–) mice displayed a shorter life span owing to an increase in spontaneous pathologies (Fig. 1D). In particular, these mutant mice displayed a significant increase in the number of tumors, including pituitary and liver adenocarcinomas or skin papillomas that were not observed in the control group (Fig. 1E and see Fig. S3 in the supplementary material). Lack of one allele of aurora B resulted in a slight, although not significant, protection against 3-MC-induced fibrosarcomas or DMBA+TPA-induced skin tumors (see Fig. S4 in the supplementary material). These results are similar to those obtained with other models with alteration in specific mitotic regulators suggesting that genomic instability caused by these mutations might increase tumor development in old mice but might be protective against rapid, induced tumors due to proliferative defects (Garcia-Higuera et al., 2008; Weaver and Cleveland, 2009).

**Genetic ablation of aurora B does not disturb early embryonic divisions**

To generate homozygous mutants, we intercrossed Aurkb(+/–) or Aurkb(+/Z) mice and analyzed their progeny. No homozygous mutant was born and no homozygous embryos were observed at
mid gestation, suggesting early embryonic lethality (see Table S1 in the supplementary material). Because genetic ablation of the other CPC components, incenp, borealin or survivin, prevents embryonic cell divisions as early as E2.5 (Cutts et al., 1999; Uren et al., 2000; Yamanaka et al., 2008), we decided to isolate fertilized embryos at E2.0. By this stage, most embryos were at the 4-cell stage (Fig. 2A). After four additional days in culture, wild-type embryos formed normal morulas and blastocysts. Unexpectedly, \( \text{Aurkb}(-/-) \) embryos also formed blastocysts in vitro without any evident sign of decreased size or cellular death. We tested whether this kinase is expressed at these developmental stages and we observed that this protein is detected at the expected cellular structures (centromeres, spindle midzone and cytokinesis bridges) in early \( \text{Aurkb}(+/?) \) [either wild-type or \( \text{Aurkb}(+/–) \)] embryos. Aurora B, however, was not detected in fertilized \( \text{Aurkb}(-/-) \) embryos at the 2-cell stage (see Fig. S5 in the supplementary material) or at later developmental phases (Fig. 2B-D). Thus, the lack of defects in \( \text{Aurkb}(-/-) \) morulas or blastocysts is not a consequence of uncontrolled expression of aurora B or maternal contribution but rather an indication of the dispensability of aurora B during cell division at these stages. Intriguingly, aurora B-null morulas displayed normal phosphorylation of histone H3 (P-H3; Fig. 2B) and the expected localization of CPC components such as incenp, which colocalizes with aurora B in wild-type and aurora B-null mitotic figures \( (n=12 \text{ per genotype; Fig. 2C}) \). After two days in culture, wild-type and \( \text{Aurkb}(-/-) \) blastocysts displayed similar size, cell number and P-H3 staining despite the absence of aurora B in mutant embryos (Fig. 2D and see Fig. S6 in the supplementary material).

**Lack of aurora B prevents proper chromosome segregation in the inner cell mass**

After a few extra days in culture, wild-type blastocysts hatched from the zona pellucida, attached to the culture dish and generated a normal and proliferative (as indicated by P-H3 staining) inner cell mass outgrowth surrounded by trophoblast giant cells (Fig. 3A). Lack of aurora B resulted in a reduced number of trophoblasts with altered nucleus size and morphology (see Fig. S7 in the supplementary material) and deficient ICM outgrowth (Fig. 3A).

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**Fig. 2. Normal development of pre-implantation embryos lacking aurora B.** (A) E2 embryos were isolated from intercrosses between \( \text{Aurkb}(+/–) \) mice, cultured for four additional days and genotyped by PCR. (B) Colocalization of aurora B (AurB) with phospho-histone H3 (P-H3) in mitotic cells of E2.5 pre-implantation embryos. In the absence of aurora B, mitotic cells are also present in the same frequency and display normal staining for P-H3. Arrows indicate P-H3-positive cells. (C) Incenp is also properly localized (arrows) in early embryos in the presence or absence of aurora B. (D) Wild-type and aurora B-null embryos form blastocysts in vitro and display normal mitotic figures (yellow arrows) and cytokinesis bridges (white arrows) despite the absence of aurora B. The \( (+?/) \) genotype indicates either \( (+/+) \) or \( (+/-) \). \( \alpha \)-tubulin (\( \alpha \)-tub) staining is shown in red and DAPI (DNA) in blue. ACA, anticentromeric antigen antibody. Scale bars: 20 \( \mu \)m.
Immunofluorescence of these mutant cells detected numerous ICM cells in prometaphase (PM) or metaphase (M) that were only rarely observed in control cultures (Fig. 3B). Aurora B-deficient cells displayed abnormal spindles with a strong concentration of α-tubulin in the poles and lack of bipolar fibers (Fig. 3C). Multipolar spindles were also frequently observed, suggesting either a defect in organizing bipolar spindles or the presence of polyploid cells. All these mitotic figures displayed misaligned chromosomes (Fig. 3C-E) in the presence of Mad2 (Mad2l1 – Mouse Genome Informatics) and cyclin B1 (Fig. 3D). Finally, incenp is properly localized at the centromeres (arrow), midbodies or cytokinesis bridges (not shown) in wild-type cells, no incenp signal is observed in aurora B-null cells. Scale bars: 10 μm. For these assays, embryos were isolated at E2.5 and cultured in vitro for five (B-E) or eight (A) additional days.

Fig. 3. Lack of aurora B results in mitotic defects in the inner cell mass. (A) Genetic ablation of aurora B results in abnormal inner cell mass (ICM; encircled by dashed line) outgrowths in culture. Whereas Aurkb(−/−) embryos exhibit abundant trophoblast giant cells (TGC), the ICM outgrowth is dramatically reduced and phospho-histone H3 (P-H3) signal is almost undetectable. Error bars represent s.d. (B) Aurora B (green) is present in mitotic and cytokinesis figures in wild-type (arrows and insets) but not in Aurkb(−/−) embryos. In the absence of aurora B, abundant mitotic (white arrowheads) and apoptotic (yellow arrowheads) figures are observed in the ICM outgrowth. M, metaphase; C, cytokinesis. Scale bars: 50 μm. (C) Mitotic distribution in ICM outgrowths. In wild-type cells, aurora B (green) localizes internal to anti-centromeric antibody (ACA) spots (red or white) during prophase (P) or metaphase (M) owing to its localization to the inner centromere. Aurora B moves to the central spindle during telophase (T) or to the cytokinesis bridge during cytokinesis (C). In Aurkb(−/−) embryos, aurora B is not detected and these cells display aberrant bipolar spindles with misaligned chromosomes (white arrow), as well as monopolar or multipolar spindles. Note the presence of multiple poles or isolated α-tubulin spots (yellow arrows). Scale bars: 10 μm. (D) Aurkb(−/−) cells show the presence of Mad2 (green) at the kinetochores during prometaphase (white arrow) or in misaligned chromosomes in incomplete metaphases (yellow arrow). Cyclin B1 is also present in these mitotic figures showing a diffuse expression in some cases accompanied by enrichment at the poles. Scale bars: 10 μm. (E) Although incenp is properly localized at the centromeres (arrow), midbodies or cytokinesis bridges (not shown) in wild-type cells, no incenp signal is observed in aurora B-null cells. Scale bars: 10 μm. For these assays, embryos were isolated at E2.5 and cultured in vitro for five (B-E) or eight (A) additional days.

Genetic ablation of aurora B results in mitotic aberrations and lethality after implantation

Aurora B-negative embryos could be found at distinct post-implantation stages (15.6% at E6.5, 26% at E7.5 and 16% at E9.5; see Table S1 in the supplementary material; note that embryonic structures were absent in a percentage of deciduas suggesting additional dead embryos after implantation). All these implanted aurora B-deficient embryos displayed small size and a progressive reduction in the number of cells (Fig. 4 and see Fig. S8 in the supplementary material). Histological examination of E6.5 embryos revealed defective structures in aurora B-null embryos accompanied with hemorrhage in the ectoplacental cone and frequent edemas and apoptotic cells (Fig. 4A,B). This atrophy did not seem to be due to a defect in specific cell lineages as Sox2-
(epiblast) and Gata4- (primitive endoderm) positive cells were present in both wild-type and mutant embryos (see Fig. S8 in the supplementary material).

Aurora B-null embryos displayed a significant increase in mitotic and apoptotic cells at E6.5-7.5 (Fig. 4B). Many of these cells displayed positive staining for γH2AX (H2afx – Mouse Genome Informatics), p53 (Trp53 – Mouse Genome Informatics) and active caspase 3 (C3A), suggesting DNA damage and activation of the p53 pathway in these cells (Fig. 4C). Interestingly, most of these mitotic figures represented cells in PM and M, including abnormal PM/M figures with misaligned chromosomes (Fig. 4D,E). Most of these mitotic cells displayed a positive signal for cyclin B1, suggesting that anaphase-promoting complex (APC/C)-Cdc20 has not been activated. By E9.5, most embryos were degenerated and only displayed either mitotic or apoptotic cells (see Fig. S8 in the supplementary material).

Aurora C is sufficient to drive mitotic progression during early embryonic development

As lack of the other CPC components, incenp, survivin or borealin, results in early lethality by E2.5 (Cutts et al., 1999; Uren et al., 2000; Yamanaka et al., 2008), we wondered whether aurora B could be dispensable owing to the presence of other aurora kinases during early embryonic development. Indeed, aurora C has been shown previously to bind other CPC components when overexpressed in specific cell lines (Sasai et al., 2004; Slattery et al., 2009; Slattery et al., 2008; Yan et al., 2005). As depicted in Fig. 5A,B, aurora C was detected in early embryos (2-cell stage and morulas) and in testis but it was not expressed in late embryos, as examined by RT-PCR or immunostaining in sections. Further analysis of aurora C expression in adult tissues indicated a significant expression in testis (Fig. 5B), as reported previously (Hu et al., 2000; Kimmins et al., 2007; Tang et al., 2006). Similar data indicating expression of aurora C in early embryos, but not in later stages or most somatic tissues, can be obtained from published expression profiles (Hamatani et al., 2004) and public databases (see Fig. S9 in the supplementary material). Aurora C is also expressed in interphasic brain glial cells but we did not detect significant expression of this protein in most of the other tissues analyzed in a human tissue array (see Fig. S9 in the supplementary material).

Next, we treated embryos with a small molecule inhibitor, ZM447439 (frequently abbreviated as ZM1), that is able to efficiently inhibit both aurora B and C kinases [IC50 aurora B 50
nM; IC_{50} aurora C 250 nM (Girdler et al., 2006)]. Wild-type E1.5 (2-cell) embryos were treated with different ZM1 doses (0.5, 1, 2, 5, 20 and 100 μM) or carrier and analyzed 24 and 48 hours later. As represented in Fig. 5C and Fig. S10 in the supplementary material, ZM1 treatment from 5 to 20 μM resulted in a severe arrest at the 2 to 4-cell stage in nearly all of treated embryos. Importantly, treatment with ZM1 resulted in a phenotype similar to that observed in aurora B-deficient embryos by E7.5, including aberrant prometaphase figures with misaligned chromosomes and tetraploid cells (Fig. 5D). To explore further the possibility that aurora C is responsible for supporting cell division during the morula and early blastocyst stage, we microinjected three short hairpin interfering RNAs (shRNAs) specific for aurora B or aurora C kinases, along with a reporter plasmid that expresses the green fluorescent protein (GFP) fused to histone H2B (Fig. 6A). One day after microinjection, most embryos were at the 2-cell stage and expressed the fusion protein GFP-H2B in the nucleus of these embryonic cells (Fig. 6B). After three days in culture, most embryos injected with aurora C shRNAs or a combination of aurora B and aurora C shRNAs were arrested at the 2-cell stage (Fig. 6B). These embryos displayed a high number of mitotic figures, mostly PM or M by day 3 (Fig. 6C) as previously described in the aurora B-deficient inner cell mass. These abnormal divisions frequently resulted in chromosomal bridges and giant nuclei, suggesting a defect in cytokinesis (Fig. 6D) resulting in an arrest at the 2-4 cell stage (Fig. 6D). Taken together, these results suggest that aurora C, but not aurora B, has a crucial role in driving proper cell division during early embryonic development.

Aurora B is required for chromosome congression but it is not essential for the spindle assembly checkpoint

We took advantage of the conditional AURKBlox allele to analyze the cellular effects of the acute deletion of aurora B in cultured cells that do not express aurora C. Primary AURKBlox/lox MEFs were isolated from E14.5 embryos and cultured in complete medium. Aurora C was not expressed in these mid-gestation embryos or in immortal fibroblasts derived from them (Fig. 5A and see Fig. S9 in the supplementary material). Confluent MEFs were infected with adenoviruses expressing either GFP (AdGFP) or the Cre recombinase (AdCre) in the presence of low serum (0.1% FBS) and seeded in 10% FBS at low confluency 48 hours later (t=0) to allow cells to enter into a new cell cycle (Fig. 7A). Expression of Cre, but not GFP, resulted in an almost complete deletion of exons
cultures by immunofluorescence 48 hours after serum stimulation indicated an efficient elimination of aurora B at the protein level in Cre-infected cells (Fig. 7D). As cells are infected with Cre in G0 and are maintained in G0 for two additional days before entering into G1, this protocol ensures the degradation of residual aurora B proteins, possibly by APC/C-Cdh1 complexes. The residual Aurkb(lox) allele seen in Fig. 7B therefore indicates residual cells in which Cre was not active and Aurora B was still present (as identified by immunofluorescence; not shown) rather than residual aurora B protein in Aurkb(ΔA) cells. Lack of aurora B was accompanied by a massive accumulation of abnormalities, including multiple nuclei, micronuclei and apoptotic figures, as well as lack of cytokinesis (Fig. 7D–F). About 25% of aurora B-deficient cells displayed an accumulation of α-tubulin asters that were either connected to chromosomes in multipolar cells or not connected to chromosomes (‘multiaster’ phenotype; Fig. 7G, arrowheads). The multiple microtubule organizing centers (MTOCs) might also explain monopolar spindles as these figures frequently contain multiple γ-tubulin spots grouped around the single pole (see Fig. S11 in the supplementary material).

Aurora B is essential for the error correction mechanism required for full chromosome alignment in metaphase. During this function, aurora B can generate unattached kinetochores thus resulting in unsatisfied SAC and mitotic arrest. It is, however, currently controversial to what extent aurora B might directly regulate the SAC independent of the error-correction mechanism. We therefore tested mitotic arrest in the presence of 3.5 μM of nocodazole, a condition under which microtubules are not formed. In wild-type MEFs, the duration of mitosis (DOM) was 435±135 minutes in the presence of nocodazole, as detected by videomicroscopy. In the absence of aurora B, the average DOM was 251±43.5 minutes, indicating a clear arrest when comparing with cells treated with DMSO (52±15 minutes) (Fig. 8A). This nocodazole-induced arrest is, however, smaller than that induced in wild-type cells, suggesting certain impairment in building a robust SAC response. The arrest can be partially overcome by Mad2 downregulation, suggesting an SAC-dependent delay both in wild-type and aurora B-deficient cells (Fig. 8A,B). Aurora B-null cells displayed a partial defective localization of BubR1 (Bub1b – Mouse Genome Informatics) to unattached kinetochores in the presence of nocodazole whereas Mad2 was properly localized (Fig. 8C), in agreement with the data in early embryos (Fig. 3). The genetic disruption of aurora B therefore supports the hypothesis that aurora B is not completely essential for the SAC, although it might directly participate in the recruitment of BubR1 to unattached kinetochores.

Aurora C can compensate for the lack of aurora B in somatic cells

We next tested whether the abnormalities caused by the absence of aurora B could be rescued by the expression of aurora C. Aurkb(lox/lox) cells were first infected with AdCre to generate Aurkb(ΔA) cells (day 2 in Fig. 7A) and these cells were then transfected (day 4 in Fig. 7A) with GFP-fusion constructs expressing wild-type aurora B or aurora C or a kinase-dead form of these proteins. As represented in Fig. 9, the elimination of aurora B resulted in an aberrant mitotic distribution with almost inexistent anaphase or telophase figures (Fig. 9A). The expression of exogenous wild-type, but not kinase-dead, aurora B rescued the defects, and Aurkb(ΔA):GFP-AurB WT cultures displayed a normal ratio of cells in anaphase, telophase or cytokinesis (Fig. 9B–D). The exogenous GFP-tagged protein was properly located at cytokinesis.

2-6 [Aurkb(Δ) allele; Fig. 7B]. Aurora B-deficient cells underwent mitosis and accumulated as 4N or >4N DNA content cells after expression of the Cre recombinase (Fig. 7C). Examination of these
bridges in these cells (Fig. 9C). Interestingly, expression of wild-type, but not kinase-dead, aurora C resulted in a similar phenotypic rescue (Fig. 9B-D) and aurora C was able to interact with incenp in these cells (Fig. 9E).

**DISCUSSION**

Mammalian aurora B and C are two closely related paralogs that probably evolved from a duplication event involving the ancestral aurora B/C found in cold-blood vertebrates (Brown et al., 2004). Aurora B is widely expressed in dividing cells. Aurora C, however, displays a very restricted expression pattern with a clear abundance during spermatogenesis and oocyte fertilization (Fig. 5 and see Fig. S7 in the supplementary material). Aurora C deficiency results in viable mice with subfertility defects, such as heterogeneous chromatin condensation, loose acrosomes and blunted sperm heads (Kimmens et al., 2007). Interestingly, aurora C displays frameshift mutations in infertile patients with abnormal spermatozoa characterized by large heads and increased chromosomal content (Dieterich et al., 2007), suggesting a crucial role in mammalian spermatogenesis. Additional loss-of-function studies of aurora C in oocytes suggest specific roles for this protein in meiosis (Sharif et al., 2007; Yang et al., 2007). However, its relevance in CPC function and mitosis in vivo has not been elucidated given the reduced endogenous expression levels in cultured cell lines.

The fact that aurora B-null embryos survive up to post-implantation stages is certainly surprising given the earlier lethality of incenp, survivin or borealin-deficient embryos (Cutts et al., 1999; Uren et al., 2000; Yamanaka et al., 2008) and the crucial roles of the CPC in cell division. During pre-implantation, incenp is properly localized in the absence of aurora B and the phenotypes expected from perturbed CPC function are not observed. However, these embryos are sensitive to aurora B/C inhibitors such as ZM1. Aurora C expression is higher than that of aurora B during these early stages (Fig. 6) and the interference of aurora C results in cell division defects in early zygotes [our data and Lykke-Andersen et al. (Lykke-Andersen et al., 2008)]. The fact that aurora C-null embryos develop normally (Kimmens et al., 2007) might be explained by the presence of maternal aurora C in these embryos. Indeed, the aurora B/C ancestor was recently described to be an essential maternal-effect gene in zebrafish (Yabe et al., 2009). In addition, the mouse genome contains several aurora C loci originally considered to be pseudogenes but that might express functional proteins (Yang et al., 2010) thus making difficult to evaluate a loss-of-function aurora C mutant in this organism. Lack of aurora A results in lethality at the morula/blastocyst stage due to defective formation of a mitotic bipolar spindle, thus confirming the functional differences between aurora A and aurora B/C in vivo. These results suggest that aurora C is the major CPC kinase during these early cell divisions in vivo.

Conditional ablation of aurora B in primary MEFs reveals a crucial role for this kinase in chromosome congression, as previously described (Ditchfield et al., 2003; Hauf et al., 2003; Vader et al., 2007). These mitotic defects can be rescued by expressing wild-type, but not kinase-dead, aurora C. Indeed, aurora C interacts with incenp and seems to act as a CPC component when exogenously expressed (our results) (Sasai et al., 2004; Slattery et al., 2009; Slattery et al., 2008; Yan et al., 2005). These data, along the relevance of aurora C during meiosis (Dieterich et al., 2007; Sharif et al., 2010; Yang et al., 2010) and in the early cell divisions (this work) indicate that aurora C has crucial roles in vivo, at least in germ cells and during early embryo development.
Loss-of-function studies of mammalian aurora B have typically used RNA interference or small-molecule chemical inhibitors (Ditchfield et al., 2003; Hauf et al., 2003; Ruchaud et al., 2007). These studies have the caveat that the elimination of this protein might be incomplete and residual kinase activity might support certain activities. For instance, aurora B-null cells do not reach anaphase, suggesting that previous anaphase figures or abnormal cytokinesis observed upon RNA interference are likely to be due to partial inhibition. Although we cannot discard a minimum trace of aurora B kinase activity in \( \text{Aurkb}^{\Delta/\Delta} \) cells, genetic disruption of aurora B in G0 results in the strongest defects reported so far in the mitosis that follows. Yet, aurora B-null cells display a significant arrest in nocodazole, suggesting that this kinase is not essential for the SAC. As aurora B-deficient cells display...
inefficient localization of BubR1 (Fig. 8) (Ditchfield et al., 2003; Hauf et al., 2003), this kinase might partially contribute, possibly as a separate arm (Morrow et al., 2005), to the SAC response by recruiting BubR1 to unattached kinetochores.

Aurora kinases have recently received much attention owing to their possible involvement in tumor development and their potential therapeutic value (Keen and Taylor, 2004; Lens et al., 2010; Perez de Castro et al., 2008). Whereas initial efforts were focused on aurora A, recent data suggest that aurora B is a relevant cancer target (Girdler et al., 2006; Girdler et al., 2008). In addition, the ability of aurora C to drive CPC function suggests the relevance of this protein in specific cell types. Intriguingly, whereas no point mutations have been reported for aurora B, four miss-sense changes in aurora C (G18E, G53A, E114Q and H210Q) have been found in lung adenocarcinomas (Davies et al., 2005; Forbes et al., 2008). Some of these changes affect the P-loop or activation segment of the kinase domain, a conserved and key functional region known to harbor activating somatic mutations in other kinases in cancer (Davies et al., 2005). Understanding the specific requirements for aurora B/C in different cell types might, therefore, provide useful information for future therapeutic efforts.

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