Mouse oocytes depend on BubR1 for proper chromosome segregation but not for prophase I arrest

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Mammalian female meiosis is error prone, with rates of meiotic chromosome missegregations strongly increasing towards the end of the reproductive lifespan. A strong reduction of BubR1 has been observed in oocytes of women approaching menopause and in ovaries of aged mice, which led to the hypothesis that a gradual decline of BubR1 contributes to age-related aneuploidization. Here we employ a conditional knockout approach in mouse oocytes to dissect the meiotic roles of BubR1. We show that BubR1 is required for diverse meiotic functions, including persistent spindle assembly checkpoint activity, timing of meiosis I and the establishment of robust kinetochore-microtubule attachments in a meiosis-specific manner, but not prophase I arrest. These data reveal that BubR1 plays a multifaceted role in chromosome segregation during the first meiotic division and suggest that age-related decline of BubR1 is a key determinant of the formation of aneuploid oocytes as women approach menopause.

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Misregulations during the meiotic divisions lead to the generation of aneuploid gametes that can ultimately give rise to aneuploid embryos. In humans, most aneuploidies of autosomes are lethal and result in spontaneous abortions during the first trimester. One example of a viable aneuploidy is trisomy 21, which in most cases is due to the missegregation of chromosome 21 in female meiosis I (ref. 1). Why female meiosis is particularly error prone, and why the rate of missegregations rises sharply with the age of the mother remains poorly understood. One reason put forward is the fact that human oocytes remain arrested for decades in prophase I before entering the meiotic divisions. In addition it has been shown in mice that the cohesin complex holding sister chromatids together, is hardly renewed during oocyte growth and deteriorates with time, leading to precocious sister chromatid separation and destabilization of chiasmata.

As in somatic cells, the spindle assembly checkpoint or SAC verifies whether all the kinetochores are correctly attached to the bipolar spindle, in mouse oocytes, even though SAC control seems to be less stringent in oocytes than in somatic cells and does not seem to recognize a single unattached kinetochore. In mitosis it has been shown that in the case of an unattached kinetochore, the SAC prevents metaphase-to-anaphase transition through the recruitment of SAC proteins to this kinetochore. This leads to the formation of the mitotic checkpoint complex (MCC), consisting of Cdc20, BubR1, Bub3 and Mad2, which inhibits the activation of the anaphase promoting complex/cyclosome (APC/C) and therefore the degradation of securin and cyclin B, the activation of the anaphase promoting complex/cyclosome (APC/C) and thereby the degradation of securin and cyclin B, the activation of separase and anaphase onset.

In meiosis, Bub1, Mps1 and Mad2 have all been shown to be essential for SAC-induced metaphase I arrest by preventing the activation of the APC/C. Furthermore, SAC proteins are required for the correct timing of the extremely long prometaphase I, and meiosis I is accelerated in oocytes without functional Bub1 (ref. 8) or Mps1 (ref. 12) or when heterozygote for Mad2 (ref. 10). BubR1 is essential for the mitotic SAC and recognizes kinetochores that do not harbour tension-generating attachments. BubR1 is an integral part of MCC and in its absence, SAC control is lost and the SAC protein Mad2 is no longer localized to unattached kinetochores. As a result, aneuploid daughter cells are generated.

It was therefore surprising that in mouse oocytes, the knockdown of BubR1 to 25% of endogenous protein levels led to a metaphase I arrest with the SAC protein Mad2 being localized to kinetochores. On the contrary in another BubR1 knockdown study metaphase-to-anaphase transition of meiosis I occurred in an accelerated manner. Furthermore, BubR1 protein was shown to maintain elevated levels of the APC/C activator Cdh1 in prophase I, which is thought to be required for preventing the entry into meiosis I (refs 17,18). Over expression of BubR1 stabilized Cdh1 and led to the accelerated progression through meiosis I in one, but not the other study. Loss of BubR1 was suggested to result in the loss of Cdh1 in prophase I, and as a consequence increased Cdk1 activity leading to spontaneous entry into meiosis and arrest in metaphase. The meiotic role of BubR1 seemed therefore at odds with its role in mitosis for generating the MCC and SAC-induced metaphase arrest.

In mitosis, BubR1 has an additional, SAC-independent role. Kinetochore-localized BubR1 is required for the stabilization of kinetochore–microtubule interactions by counteracting Aurora B phosphorylation through the recruitment of PP2A (refs 19–24). BubR1 may have a similar role in oocyte meiosis I (ref. 17), and its loss may prevent the establishment of correct, stable attachments. Indeed, the previous study suggested that kinetochore–microtubule fibres are diminished upon BubR1 knockdown, but it was not addressed whether the same mechanisms as in mitosis are at work in oocytes to stabilize kinetochore fibres.

In human oocytes, an important decrease in BubR1 protein levels is observed in oocytes from women closer to menopause, compared with younger women. Decline of BubR1 function in combination with cohesin reduction in older oocytes may explain the age-dependent elevated error rates in female meiosis. Therefore it is important to understand how BubR1 controls the first meiotic division in mammalian oocytes. In this study we use a conditional knockout approach to analyse oocytes completely devoid of endogenous BubR1 protein. We show here that BubR1 is required for meiotic SAC control, but not prophase I arrest. We find that BubR1 promotes anaphase onset after complete securin degradation and maturation promoting factor (MPF) inactivation. In addition we identify a meiosis-specific role for BubR1 in establishing a stable spindle, which is independent of Aurora B/C and kinetochore localization of BubR1.

Results

BubR1 is not required for maintaining GV arrest in oocytes. BubR1(+/H) mice harbouring only 10% of endogenous BubR1, due to the introduction of an alternative splicing site, have been generated previously. This BubR1 allele in BubR1(+/H) mice is flanked by LoxP sequences, which allows the generation of a conditional knockout. To analyse oocytes completely devoid of BubR1 protein we crossed BubR1(+/H) mice with a strain expressing the Cre-recombinase from the oocyte-specific Zp3 promoter. Oocytes obtained from BubR1(+/H)/Zp3-Cre+ mice will therefore be called BubR1(+/A) oocytes, and from BubR1(+/+) Zp3-Cre+ mice control oocytes. The efficiency of the knockout was checked by reverse transcription quantitative PCR, using three different primer pairs, and mRNA amounts were assessed relative to mRNA coding for the ribosomal protein Rps16. Fully grown oocytes arrested before entry into meiosis I in prophase I (GV or germinal vesicle) stage, and oocytes entering meiosis I (at GVBD or germinal vesicle breakdown) were compared. No marked differences in mRNA levels were detected between GV stage oocytes from different strain backgrounds, or GV and GVBD oocytes. Importantly, no mRNA coding for full length BubR1 was detected in GV or GVBD BubR1(+/A) oocytes (Fig. 1a), these oocytes are therefore deleted for BubR1. Interestingly, we found that wild-type oocytes harbour ~100 times more BubR1 mRNA than mouse embryonic fibroblasts (MEFs) (Supplementary Fig. 1), indicating that transient knockdown approaches may be inherently difficult to target BubR1 in oocytes.

It was previously shown that BubR1 protein accumulates during meiotic maturation. We compared BubR1 protein levels in oocytes arrested at GV stage with oocytes arrested in metaphase II. Whereas BubR1 was easily detected in metaphase II, protein levels of BubR1 in GV were below detection levels in our hands (Fig. 1b). Therefore we used metaphase II oocytes, which contain much more BubR1 protein to ask whether BubR1 protein can still be detected in BubR1(+/A) oocytes. BubR1 was not detected in BubR1(+/A) but in control oocytes by western blot. Because of the difficulties in obtaining a clear BubR1 signal in GV oocytes by immunofluorescence due to the very low protein amounts and diffuse localization, we analysed oocytes in prometaphase I. BubR1 was not detected in BubR1(+/A) oocytes by immunofluorescence in prometaphase I, when endogenous BubR1 starts to accumulate at the kinetochores in control oocytes (Fig. 1c,d).

We asked whether the loss of BubR1 affected GV arrest and entry into meiosis I (GVBD). BubR1(+/A) oocytes did not enter meiosis I spontaneously in dbcAmp or IBMX-containing medium.
Figure 1 | No BubR1 mRNA or protein is detected in BubR1Δ/Δ oocytes. (a) RNA levels from 20 oocytes were analysed by reverse transcription quantitative PCR normalized to the levels of Rps16 RNA. The position of the three BubR1 primer pairs is indicated in the scheme. Control reactions in which the RT enzyme was omitted were verified to be negative. Graphs show averages normalized to Rps16 and s.e.m. (standard error of the mean) from triplicate samples from a representative experiment. β-Actin was used as an additional internal control. For BubR1 e6-e7 RNA levels, significance of the differences between BubR1+/+ and BubR1Δ/Δ was estimated using Student’s t-test. (mean ± s.e.m.; ***, *** P<0.001; n.s., no signal detected). BubR1 is conditionally deleted in BubR1Δ/Δ oocytes. Note: comparable mRNA levels for BubR1 are found in GV oocytes of two different mouse strains, CD-1 and C57Bl6/Sv129. (b) Western blot of GV stage and metaphase II oocytes. The western blot was repeated three times. (c) Western blot of metaphase II (meta II) control and BubR1Δ/Δ oocytes. The western blot was repeated two times. (d) Chromosomes spreads of prometaphase I control and BubR1Δ/Δ oocytes at GVBD + 3 h, stained for BubR1 (red), kinetochores (Crest, green) and chromosomes (Hoechst, blue). The number of oocytes analysed in four independent experiments is indicated. Scale bar, 5 μm.

Accelerated progression through meiosis I without BubR1. Next, we wanted to establish whether BubR1Δ/Δ oocytes progress beyond metaphase I, unlike BubR1 knockout oocytes17,18, nor were efficiencies of entry or timing different from control oocytes (Fig. 2a–c). BubR1Δ/Δ and wild-type oocytes harboured comparable Cdk1 activity before and after entry into meiosis I (Fig. 2d), demonstrating that APC/C-dependent degradation of mitotic cyclins, which is thought to maintain GV arrest28 is not affected in oocytes without BubR1. Therefore we conclude that BubR1 is not required for prophase I arrest in mouse oocytes.

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A functional SAC is not required for metaphase II arrest. Oocytes undergo the first meiotic division, progress into metaphase II and remain arrested in metaphase II (cytostatic factor, (CSF) arrest) until fertilization occurs. The role of SAC proteins in the establishment of CSF arrest was not entirely clear in oocytes and has not yet been addressed with a clean, genetic approach. In *Xenopus laevis* oocytes, it has been suggested that SAC proteins are required for CSF arrest31–33, whereas in mouse

![Figure 2 | BubR1 is not required for GV arrest in mouse oocytes.](image)

**Figure 2** | **BubR1 is not required for GV arrest in mouse oocytes.** (a) Percentage of the indicated number of GV control and BubR1Δ/Δ oocytes that underwent spontaneous GVBD in the presence of dbcAmp or IBMX in the culture medium, at the indicated times after harvesting, in three independent experiments. (b) Percentage of the indicated number of GV control and BubR1Δ/Δ oocytes that did GVBD within 90 min after release, adjusted to 100%, in three independent experiments. (c) Time of GVBD in control and BubR1Δ/Δ oocytes after release, in three independent experiments. (d) In vitro kinase assays of control and BubR1Δ/Δ oocytes in GV stage and at GVBD, using Histone H1 as a substrate. For quantifications, kinase activity at GV in controls was set to 1 and the mean relative kinase activities of control and BubR1Δ/Δ oocytes at GV and GVBD are shown. Error bars indicate ± s.d. from three independent experiments, using Student’s t-test. (*P<0.05; NS, non-significant.

![Figure 3 | Meiosis I is accelerated and SAC control is impaired without BubR1.](image)

**Figure 3** | **Meiosis I is accelerated and SAC control is impaired without BubR1.** (a) Selected time frames of representative movies of H2B-RFP injected control and BubR1Δ/Δ oocytes undergoing the first meiotic division. Shown are overlays of the stack. Where indicated, mRNA encoding for wild-type (wt) BubR1 was injected at GV stage, or MG132 was added at GVBD + 3 h. Scale bar, 20 μm. DIC, differential interference contrast. The indicated number of oocytes has been analysed in at least three independent experiments. (b) Percentage of the indicated number of control and BubR1Δ/Δ oocytes extruding PBs, in three independent experiments. (c) Time of PB extrusion in the indicated number of control and BubR1Δ/Δ oocytes is adjusted to 100%, in three independent experiments. Nocodazole was added at GVBD + 3 h, where indicated. (d) Representative metaphase II chromosome spreads of a control and a BubR1Δ/Δ oocyte. Kinetochore localization was determined at GVBD + 3 h, adjusted to 100%, in three independent experiments. Prometaphase I spreads at GVBD + 3 h, where indicated. Kinetochores were stained with CreST (green), chromosomes with Propidium iodide (red). Scale bar, 5 μm. The number of spreads analysed in three independent experiments is indicated. (e) Prometaphase I spreads at GVBD + 3 h of control and BubR1Δ/Δ oocytes, stained for Mad2 (red), kinetochores (Crest, green) and chromosomes (Hoechst, blue). Scale bar, 5 μm. The number of spreads analysed in three independent experiments is indicated. (f) BubR1 localization (in green) to kinetochores in metaphase I control and BubR1Δ/Δ oocytes, after wt BubR1 mRNA injection where indicated. BubR1 E406K was injected into BubR1Δ/Δ oocytes, and kinetochore localization was determined at GVBD + 3 h. Oocytes were treated with MG132 at GVBD + 3 h where indicated. Kinetochore localization is visible due to brighter DNA staining. Chromosomes were stained with Hoechst and pseudo-coloured red. Scale bar, 5 μm. The number of spreads analysed in three independent experiments is indicated. (g) Meiotic maturation of control and BubR1Δ/Δ oocytes after the injection of wt BubR1 mRNA at GV stage. The number of oocytes analysed in three independent experiments is indicated. Expression of BubR1 prevents PB extrusion.
oocytes the expression of a dominant negative Bub1 mutant did not perturb CSF arrest. To clarify the SAC’s role in CSF arrest, we asked whether the complete loss of BubR1 affected CSF arrest and release from the arrest. For comparison we additionally analysed CSF arrest in oocytes devoid of functional Mps1 (ref. 12), a kinase essential for SAC control in mitosis and meiosis and upstream of other SAC components. Mps1 mutant oocytes harbour only a mutant version of Mps1, which cannot localize to kinetochores but retains its kinase activity. Control, BubR1 and Mps1 mutant oocytes were induced to undergo meiosis II metaphase-to-anaphase transition with sister chromatid separation.

- **Figure a:** Time in hours:minutes after GVBD for different conditions.
- **Figure b:** Graph showing % No PB and % PB for different conditions.
- **Figure c:** Graph showing % PBE for different conditions.
- **Figure d:** Metaphase II spreads showing control and BubR1 mutant oocytes.
- **Figure e:** Mad2 and Crest staining in control and BubR1 mutant oocytes.
- **Figure f:** BubR1 and BubR1 - DNA staining for control and BubR1 mutant oocytes.
- **Figure g:** Graph showing % of PBE for different conditions.

*Note: The images and graphs are not fully transcribed due to the complexity of the visual content.*
without functional BubR1 or Mps1 are aneuploid, but otherwise we observed no defect in establishing a CSF arrest or release from the arrest, as judged from the presence of single sisters and no dyads in anaphase II (Fig. 4). Hence we conclude that CSF arrest and release per se do not require SAC proteins.

**Loss of BubR1 delays anaphase I onset and PB extrusion.** Interestingly, progression through meiosis I in BubR1Δ/Δ oocytes was accelerated but not as much as observed on the loss of other checkpoint proteins, such as Bub1 (ref. 8) or Mps1 (ref. 12). To directly compare meiotic timing in oocytes without BubR1 to oocytes without Mps1 kinase activity we used reversine to inhibit Mps1 kinase activity in the same strain background35. Addition of reversine to the culture medium after entry into meiosis led to chromosome spreads of control, Mps1Δ/Δ and BubR1Δ/Δ oocytes we asked whether the APC/C is inhibited by the SAC (Fig. 6c). Next, we asked whether chromosome segregation was delayed in oocytes without BubR1. Indeed, simultaneous live imaging of YFP-securin degradation and H2B-RFP-labelled chromosomes showed that anaphase I onset and SAC inactivation8. 

Kinetics and timing of yellow fluorescent protein (YFP)-securin degradation in BubR1Δ/Δ oocytes was indistinguishable from YFP-securin degradation in reversine-treated oocytes, indicating that in both cases the APC/C is not inhibited by the SAC (Fig. 6c). }

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**Figure 4** | **Loss of SAC control does not affect metaphase II CSF arrest.** Chromosome spreads of control, Mps1Δ/Δ and BubR1Δ/Δ oocytes in metaphase II (CSF arrest) and anaphase II. Chromosomes were stained with propidium iodide (red), kinetochores with Crest (green). Scale bar, 5 μm. Schemas of the expected chromosome figures for wild-type oocytes are shown on the right. In metaphase II dyads (paired sister chromatids) are held together by centromeric cohesion, which is illustrated in black. Note that only single sister chromatids are present in anaphase II. The number of oocytes analysed in three independent experiments is indicated.

**Figure 5** | **Reversine treatment phenocopies loss of kinetochore-localized Mps1.** (a) GFP-Mps1 and Histone H2B-RFP (to visualize chromosomes) were expressed in control and reversine-treated oocytes. Reversine was added at GVBD. At GVBD + 3 h, GFP-Mps1 localization was analysed by live imaging, using a spinning disk confocal microscope. Only 1 z-section is shown. Scale bar, 5 μm. The arrowheads indicate GFP-Mps1 localized to the kinetochores in this z-section. 10 oocytes in three independent experiments were analysed. (b) Time of PB extrusion in the indicated number of control and reversine-treated oocytes in three independent experiments. Reversine was added at GVBD, Nocodazole was added at GVBD + 3 h, where indicated. (c) Prometaphase I spreads at GVBD + 2 h 30 min of control and reversine-treated oocytes from GVBD onwards, stained for Mad2 (red), kinetochores (Crest, green) and chromosomes (Hoechst, blue). The number of oocytes analysed in three independent experiments is indicated. Scale bar, 5 μm.
and PB extrusion were significantly delayed in oocytes without BubR1, but not in oocytes treated with reversine (Fig. 6d,e, supplementary Fig. 2). Time from anaphase onset till PB extrusion was the same with or without BubR1 (Fig. 6d, supplementary Fig. 2). The delay in BubR1Δ/Δ oocytes was therefore independent of its role in the SAC, and independent of a potential feedback control for cytokinesis, which has been described recently in mitotic cells37.

We asked whether Cdk1 is inactivated at the same time in oocytes without either Mps1 or BubR1, and whether throughout the supplementary delay in BubR1Δ/Δ oocytes Cdk1 activity remains high or low. APC/C-dependent cyclin B1 degradation to

![Graph showing time of PBE in minutes after GVBD](image)

![Graph showing percentage of PBE](image)

![Graph showing average fluorescence intensity](image)

![Images of control, Reversine, and BubR1Δ/Δ oocytes](image)

![Graph showing time from end of securin degradation until PB extrusion](image)

![Graph showing histone H1 phosphorylation](image)
reduce Cdk1/cyclin B1 activity is required for metaphase-to-anaphase transition. We performed in vitro kinase assays using Histone H1 as a substrate to determine when Cdk1-cyclin B1 is inactivated. In controls, highest Cdk1 activity in meiosis I was observed at GVBD + 7 h, and in both BubR1- and Mps1-deficient oocytes at GVBD + 4 h (Fig. 6f), even though SAC deficient oocytes never reached the same levels of Cdk1/cyclin B1 activity. Checkpoint-deficient oocytes underwent metaphase-to-anaphase transition with much lower overall Cdk1/cyclin B1 activity (Fig. 6f), meaning that in the absence of SAC control less Cdk1/cyclin B1 activity is required for APC/C activation and metaphase-to-anaphase transition. A drop in Cdk1 activity was observed after PB extrusion in control oocytes, and in SAC-devoid oocytes at GVBD + 6 h, at which time PB extrusion had occurred in oocytes without functional Mps1, but not in BubR1+/−/− oocytes (Fig. 6g). This indicates that endogenous cyclin B has been degraded at the same time in both checkpoint mutants, similar to YFP securin. Importantly, during the supplementary delay observed in BubR1+/−/− oocytes, Cdk1 activity remained low (GVBD + 6 h without PB extrusion, Fig. 6g). We conclude that BubR1 has an additional, SAC-independent role for anaphase I onset. Without BubR1, oocytes delay the separation of chromosomes, even though MPF activity is low and securin has been degraded.

Localization of BubR1 to kinetochores in meiosis I. Kinetochore localization in mitosis is downstream of Mps1 and Aurora B kinase activities. Mitotic kinetochore localization of BubR1 is required not only for its role in the SAC, but also for stabilizing kinetochore–microtubule attachments, through recruitment of PP2A-B56 which counteracts Aurora B phosphorylation. To get insights into the different potential roles of BubR1 in meiosis we asked how kinetochore localization of BubR1 is regulated in oocytes. Endogenous BubR1 was localized to kinetochores in prometaphase I, and weakly in metaphase I when kinetochores are attached (Fig. 7a,b). BubR1 signal co-localized with Crest, Fig. 1b), in agreement with the two previous studies. BubR1 localization to kinetochores in prometaphase I depends on Mps1 kinase activity, as the signal disappeared in the presence of reversine (Fig. 7a,b). Importantly, though, the failure to establish stable kinetochore fibres in BubR1+/−/− oocytes was not rescued by simultaneously inhibiting Mps1, or Aurora B/C, unlike in mitosis. This indicates that the formation of stable kinetochore fibres depends on BubR1, but not on its kinetochore localization to counteract Aurora B/C phosphorylation. It was previously shown that the inhibition of Cdk1 activity during meiosis I leads to a delay, but not the abolishment of stable kinetochore fibres. Treatment of oocytes with Cdk or Plk1 inhibitor still allowed the formation of stable microtubule fibres, showing that unlike in mitosis, BubR1 phosphorylation by Plk1

BubR1 is required for the establishment of stable spindles. Now we asked whether BubR1’s role for the establishment of stable kinetochore–microtubule interactions is conserved in meiosis I. Indeed, nearly no cold-stable microtubule fibres attached to kinetochores were detected in oocytes without BubR1 (Fig. 8a,b), in agreement with the previous study. On the other hand inhibition of Mps1 with reversine or Aurora B/C with ZM447439 in control oocytes still allowed the establishment of stable kinetochore fibres (Fig. 8a,b), even though BubR1 was not localized to the kinetochores (Fig. 7a,b). Chromosomes were misaligned in the presence of ZM447439 and upon loss of Mps1 activity, also in agreement with previous literature. The treatment with reversine (Fig. 8a) Importantly, though, the failure to establish stable kinetochore fibres in BubR1+/−/− oocytes was not rescued by simultaneously inhibiting Mps1, or Aurora B/C, unlike in mitosis. This indicates that the formation of stable kinetochore fibres depends on BubR1, but not on its kinetochore localization to counteract Aurora B/C phosphorylation. It was previously shown that the inhibition of Cdk1 activity during meiosis I leads to a delay, but not the abolishment of stable kinetochore fibres. Treatment of oocytes with Cdk or Plk1 inhibitor still allowed the formation of stable microtubule fibres, showing that unlike in mitosis, BubR1 phosphorylation by Plk1

Figure 6 | Loss of BubR1 delays time from securin degradation and Cdk inactivation until anaphase and PB extrusion. (a) Time of PB extrusion in minutes after GVBD in control, reversine (Mps1 inhibitor)-treated and BubR1+/−/− oocytes. Where indicated, reversine was added at GVBD. (b) Time of PB extrusion in hours after GVBD in the indicated number of control, reversine-treated, BubR1+/−/− and reversine-treated BubR1+/−/− oocytes, adjusted to 100%. Reversine was added at GVBD. (c) Quantification of YFP-securin signal in control, reversine-treated (from GVBD onwards) and BubR1+/−/− oocytes during meiotic maturation by live imaging. Representative graphs from three independent experiments are shown and the total number of oocytes analysed is indicated. (d) Selected time frames of representative movies of H2B-RFP and YFP-securin injected control, reversine-treated (from GVBD onwards) and BubR1+/−/− oocytes undergoing the first meiotic division. 10 z-sections of 3 μm were taken to visualize chromosomes, shown are overlays of the stack. Time points were taken every 20 min, time after GVBD is indicated. (Single channels are shown in Supplementary Fig. 3.). DIC, differential interference contrast. Number of oocytes analysed is shown in e. (e) The time from lowest YFP-securin levels to extrusion of the first PB is indicated in minutes in control, reversine-treated and BubR1+/−/− oocytes. (f) Quantification of independent in vitro kinase assays of oocytes 7 h after GVBD (control), or 4 h after GVBD (SAC mutants), using Histone H1 as a substrate. Five control, Mps1+/−/− and BubR1+/−/− oocytes were used for each time point. See Material and Methods section for quantifications. (g) In vitro kinase assays of control, BubR1+/−/− and Mps1+/−/− oocytes at GVBD + 4 h, before and after PB extrusion, and in metaphase II, using Histone H1 as a substrate. Representative kinase assays are shown for each genetic background, and below are the quantifications of at least three independent experiments each. See Material and Methods section for quantifications. Time after GVBD is indicated in hours. — PB, oocytes that have not yet extruded a PB, + PB, oocytes had just extruded a PB (oocytes were selected individually), Meta II, metaphase II. Error bars in a, e, f and g indicate ± s.d. from at least three different experiments, using Student’s t-test (**P<0.0001; ***P<0.001; *P<0.05; NS, non-significant).
Figure 7 | Kinetochore localization of BubR1 in meiosis depends on Mps1 and Aurora B/C kinase activities. (a) Whole-mount immunofluorescence staining to detect endogenous BubR1 in wild-type oocytes at GVBD + 2 h 30 min (early prometaphase I) or GVBD + 7 h (metaphase I). Where indicated, oocytes were treated from GVBD onwards with Mps1 inhibitor (reversine), Aurora B/C inhibitor (ZM447439), Plk1 inhibitor (BI2536) and Cdk inhibitor (flavopiridol), and fixed at GVBD + 2 h 30 min. Oocytes were stained with BubR1 antibody (red) and Hoechst (to visualize chromosomes, blue). All stainings were repeated at least three times in independent experiments, and the number of oocytes analysed is indicated. Scale bar, 5 μm. Inserts show magnifications to better see the kinetochore signal. Scale bar, 5 μm. (b) Quantification of BubR1 antibody staining of oocytes in a, as described in Material and Methods. At least 10 oocytes were analysed per condition, from at least three independent experiments. Error bars indicate mean ± s.d.; ***P<0.0001, by Student’s t-test.
**Figure 8 | Cytosolic BubR1 is required for establishing stable microtubule fibres in meiosis I.** (a) Whole-mount immunofluorescence of control oocytes at 4 h and control and BubR1Δ/Δ oocytes just before anaphase onset. Where indicated, oocytes were treated with reversine, ZM447439, flavopiridol and BI2536, to inhibit Mps1, Aurora B/C, Cdk1 and Plk1 kinases, respectively, from GVBD onwards, except for ZM447439, which was added 2 h before fixation. For staining of stable microtubule fibres, oocytes were cold-treated on ice for 4–5 min before fixation. One representative oocyte is shown for each condition; the number of oocytes analysed from at least three independent experiments is indicated, as well as the number of oocytes with the phenotype shown. For the rescue, mRNA coding for wild-type (wt) BubR1 or the BubR1 E406K mutant was injected into GV oocytes. To obtain oocytes before anaphase I onset, control oocytes were fixed at GVBD + 7 h, except those treated with reversine, which were fixed at GVBD + 4 h. BubR1Δ/Δ oocytes (with or without reversine or ZM447439) were fixed at GVBD + 5 h and BubR1Δ/Δ oocytes + wt or E406K BubR1 mRNA at GVBD + 6 h. A control at GVBD + 4 h is shown as well. Spindles are green (α-tubulin), chromosomes blue (Hoechst), and kinetochores red (Crest serum). Shown are representative overlays of the acquisitions with a spinning disk confocal microscope (20 z-sections every 0.8 μm), from at least three independent experiments. Scale bar, 5 μm. Inserts show zooms of individual z-planes. (b) Quantifications of kinetochores that are attached and shown in (a) as described in Material and Methods. At least three independent experiments were analysed for each condition, and the mean percentage of attached kinetochores of at least 12 oocytes was determined by Student’s t-test. Error bars show mean ± s.d.; ***P < 0.0001, NS, non-significant. KT, kinetochore.)
be addressed whether in meiosis BubR1-associated PP2A targets this and other motor proteins, in a kinetochore-independent manner. We do not think that the supplementary delay of BubR1½/þ oocytes for anaphase I onset is due to a failure to dephosphorylate Knl1 that has been previously phosphorylated by Mps1 (ref. 53), because reversine treatment did not revert the observed delay. Alternatively, PP2A-B56 associated with BubR1 may be important to counteract other phosphorylation events in the cytoplasm or at the spindle that prevent anaphase onset, and in the absence of BubR1, anaphase I onset takes more time because of delayed dephosphorylation of certain substrates that remain to be identified.

How can we explain the discrepancies of our study with the two previous studies on BubR1’s role for maintaining GV arrest? Apart from potential off-target effects that can occur with any RNA interference approach, the knockdown efficiencies can be highly variable between individual oocytes and depend on the amount of morpholino-oligo injected in each oocyte and on the incubation times. Neither study has examined on a single-cell level the variability of the knockdown. Furthermore, our data show that levels of mRNA coding for BubR1 are quite high in oocytes of the two different mouse strains that we compared, relative to MEFS (see also NCBI, UniGene, EST Profile Viewer, for Bub1b) and relative to mRNA levels of actin or RpS16, making a transient knockdown approach extremely difficult. Therefore, we think that the discrepancies for BubR1’s role in GV arrest are due to technical reasons related to the knockdown approach. The differences between our study and the previous transient knockdown studies on BubR1’s role for progression through meiosis I and localizing Mad2 to kinetochores, are also most likely due to the incomplete knockdown of BubR1. The partial depletion of BubR1 may affect the stability of the spindle, but not abolish checkpoint control, in which case oocytes will fail to correctly attach kinetochores and activate the SAC, leading to the observed metaphase arrest with Mad2 at kinetochores described by Homer et al.17. On the other hand, Wei et al.18 reported an accelerated progression through meiosis I, in line with our results. The variability of the phenotypes observed is most likely due to different knockdown efficiencies in the two studies.

A functional SAC should recognize a failure to establish tension-bearing attachments that is due to age-related decline of cohesin, in meiosis, but there are several reasons why this may not be the case (ref. 1): (1) a single univalent may biorient at the meiotic spindle and evade the checkpoint because tension-bearing attachments can be established such as in mitosis, (2) a mono-oriented univalent attached to one pole is not recognized because the meiotic SAC may not sense whether attachments are also under tension from both the poles, (3) the spindle checkpoint is not as sensitive as in mitosis, and a single unattached or not correctly attached kinetochore may not be recognized, (4) activation of the SAC leads only to a transient arrest in metaphase, and ultimately oocytes will exit checkpoint arrest and missegregate chromosomes and (5) the SAC may become weaker with age. Indeed, reduced transcription of the SAC proteins BubR1, Bub1 and Mad2 and reduced protein levels of BubR1 have been observed in aged human oocytes23,24. Whether the SAC is indeed—at least in part—responsible for the observed age-related increase in chromosome missegregations in aged oocytes is a subject of controversy. One previous study was using a senescence accelerated mouse strain, with a premature decline in fertility. Oocytes of these artificially aged mice have troubles aligning their chromosomes at the metaphase plate and meiosis I is accelerated, indicating that the SAC is not fully functional25. On the contrary in another study, oocytes of naturally aged oocytes had a functional checkpoint and did not show an acceleration of anaphase onset, indicating that increased missegregations with age are not due to a missing spindle checkpoint26. Functionality of the checkpoint was examined by treating oocytes with low doses of Nocodazole, and one can argue that even a weakened checkpoint may still respond to Nocodazole, but not recognize a few unattached kinetochores in metaphase I. Oocytes from CBA/Ca mice that also show a premature decline in fertility, missegregate chromosomes at high rates but do not accelerate meiosis I, either31. Importantly, a recent study using standard C57Bl6/J laboratory mice and comparing young with naturally aged mice showed reduced rates of the SAC protein Mad2 at the kinetochores of oocytes from older mice, weakened checkpoint response upon Nocodazole treatment, increased incidence of misaligned chromosomes, but normal timing of meiosis I (ref. 57). The normal timing of meiosis I in these mice and CBA/Ca mice is therefore not necessarily the best indicator for checkpoint functionality: more chromosome misalignments in oocytes of older mice may lead to a weak checkpoint response, which will get overridden, but make it impossible to detect a potential acceleration of meiosis I (ref. 57). A conclusion from these studies is that functionality of the SAC with age is differently affected in different mouse strains, and not all mouse strains are equally suited to study the involvement of the SAC in the increase of missegregation events in oocytes of older mice57. Overall these results suggest that the SAC is functional in oocytes of older mice and still able to generate a delay in anaphase I onset, but that it becomes less efficient with age and this leads to an increase in chromosome missegregations.

To get better insights into how the age-related drop in BubR1 protein levels may affect chromosome segregation in mammalian oocytes it was necessary to analyse oocytes completely devoid of BubR1. This allowed us to distinguish the roles of BubR1 for SAC control, the formation of stable spindle fibres and anaphase onset. SAC control seems to be more permissive in meiosis than mitosis, and therefore less BubR1 in older oocytes will affect chromosome segregation, not only because of its role in the checkpoint, but also because of its checkpoint-independent functions in meiosis. In the light of our study, the observed age-related decline of BubR1 in human oocytes25 in combination with a gradual loss of cohesin3–7 is expected to have severe consequences on chromosome segregation and therefore fertility in humans.

**Methods**

Mouse strains, husbandry and genotyping. Mice were maintained and bred in the animal facility of UMR7622 according to local rules by the French Ministry of Higher Education and Research, and ethical approbation, in compliance with the application of the rule of the ’3 Rs’ (License 5330). The number of mice used was kept as low as possible but high enough to obtain statistically significant results, in agreement with the standards used in the field. Prophase I oocytes were harvested from 9–11-week-old mice as described below. BubR1½/þ mice were generated in a mixed C57Bl6/Sv129 background were crossed with Zp3-Cre (C57Bl6/6-Tg(Zp3-Cre)99Knw/J) mice (The Jackson Laboratories; USA). For every experiment, oocytes from BubR1½/þZp3-Cre−/− mice (BubR1–/−) or oocytes were compared with (corresponding oocytes from) BubR1−/−Zp3-Cre−/− mice (BubR1−/−). For replicate experiments mice from different litters were used. Genotyping was performed as published26 using the following primers: BubR1-1 (forward): 5’-GTA AGT CTA TTT CTC CTG GAT TAA GTA G-3’; BubR1-2 (reverse): 5’-CAT CTG TGG TAC ATA GGT GTG TCT GG-3’; BubR1-3 (reverse): 5’-ATA TTT GCT GAG ATG TCC GTG GGT GGG-3’. Cre presence was determined according to the protocol provided by The Jackson Laboratories. Mps1ΔN/ΔN mice have been characterized previously12. CD-1 (Swiss) mice (Janvier, France) were used for experiments not involving BubR1½/þZp3-Cre−/− mice.

**Reverse transcription semi-quantitative PCR.** Reverse transcription and real time semi-quantitative PCR were carried out as described in ref. 12, from 20 oocytes or 500 ng of RNA from MEF cells, with the following modifications: qPCR reactions were performed in 20 μl using the Brilliant III ultra fast SYBR Green Kit (Agilent Technologies). Primer sequences for BubR1 are: BubR1-e5F: 5’-AGCGA GGGATGGAACGCAAG-3’; BubR1-e6R: 5’-ATTCGATCGCCCGAAGAG-3’; BubR1-e4F: 5’-CAAGGAGAGAGGCCCATATTAT-3’; BubR1-e5R: 5’-TGAACAGG.
Different drugs for this study were used at the following concentrations: Aldrich): 0.3 mM; Flavopiridol (Sigma-Aldrich): 1 mM; MG132 (Sigma-Aldrich): 20 μM; Reversine (Merck Chemicals Ltd.): 0.5 μM; ZM447439 (Santa Cruz): 20 μM; B21536 (Selleckchem): 0.2 μM; Flavopiridol (Sigma-Aldrich): 1 mM; MG132 (Sigma-Aldrich): 20 μM. Drugs were added to the culture medium and for all drugs, the time of addition and duration of treatment are indicated. Because oocytes are cultured in drops covered with mineral oil and because reversine is soluble in oil, mineral oil covering the culture drops contained reversine as well, and untreated controls were cultured in separate dishes.

Chromosome spreads, whole-mount immunofluorescence. Antibody staining of chromosome spreads and fixed oocytes35,36 was done using the following antibodies at the indicated dilutions: BubR1 polyclonal sheep antibody (gift from S. Taylor, 1/50), human CREST auto-immune antibody (Cellion SA, HCT-0100; 1/100), α-tubulin (ab133435) and δ-Tubulin (D1M1) coupled to FITC (Sigma-Aldrich, F2168; 1/100), rabbit polyclonal MCAK-phospho S95 (Abcam, ab74146;1/50) and Mad2 polyclonal rabbit antibody69, 1/100. The following secondary antibodies were used: CY3 anti rabbit (Jackson ImmunoResearch, 711-166-152; 1/200), CY3 anti sheep (Jackson ImmunoResearch, 713-165-147; 1/200) and anti human Alexa 488 (Life Technologies, A-11013; 1/200). Cold-stable spindles were obtained by incubating oocytes on top of an ice-water bath for 5 min before fixation68.

Acquisitions of antibody staining on fixed oocytes were done with an Olympus IX70 Spinning Disk Confocal microscope (100x/1.2 NA objective, triple pass filter set, Sutter excitation filter wheel, MicroMax cooled CCD (charge-coupled device) camera (Princeton Inst.) and Metamorph software59.

In vitro translocation, microinjections and live imaging. In vitro translocation of YFP securin, Histone H2B-RFP, GFP-Mps1 (ref. 12), BubR1 E406K (ref. 61) and wild-type mouse BubR1 (ref. 61) were performed with the Ambion mMessage Machine kit and 1–10 μM of mRNAs purified on RNAeasy columns (Qiagen) were microinjected with Eppendorf micromanipulators on a Nikon Eclipse Ti microinjection stage. BubR1, Mps1 at kinetochores is essential for female mouse meiosis I. Twenty-five to forty centromeres were calculate per cell using mean grey value method. Fluorescence intensity of signals at 4 μm (ref. 58). Incorporation of BrdU in the wild-type control at GVBD experiment, at least 10 oocytes were analysed per condition, from at least three independent experiments, using mice from different litters, and statistical significance was determined with Student’s t-test. In each histogram, values are given as mean ± s.d. For quantification of kinetochore–microtubule attachments (Fig. 4), for each kinetochore visible due to positive staining with Crest serum the attachment status was determined by analysing the individual z-sections. For each oocyte the ratio of attached versus unattached kinetochores was calculated. At least three independent experiments were analysed for each condition, and the mean percentage of attached kinetochores of at least 12 oocytes was determined. Statistical significance was determined with Student’s t-test, error bars indicate mean ± s.d.

Quantifications of YFP-securin degradation (Fig. 6) were done in Image J from original acquisitions of one constant focal plane. The background was subtracted14. For quantifications of kinase assays in Fig. 2d, values were normalized to control at GV, in Fig. 6f, to the control at GVBD + 7 h, and in Fig. 6g to the wild-type control at GVBD + 4 h, which were set to 1. For each reaction, five oocytes were pooled and used for each individual time point. Statistical analysis was performed by Student’s t-test. (NS, non-significant, *P<0.05, **P<0.001, ***P<0.0001). Error bars indicate mean ± s.d.

Oocyte culture and drug treatments. Oocytes were harvested from ovaries of sexually mature female mice and cultured in self-made M2 medium droplets containing dibutylryl cyclic AMP (dbcAMP, Sigma; 200 μM), unless otherwise described. Follicle cells were removed by carefully mouth-pipetting oocytes through a torn-out Pasteur-pipet, with a diameter allowing easy entry of individual oocytes. Only oocytes entering meiosis I within 90 min after release were used for the experiments. Oocyte activation in metaphase II was done by incubating metaphase II oocytes in CaCl2-free M2 medium supplemented with 10 mM SrCl2 (ref. 58). Different drugs for this study were used at the following concentrations: IBMX (14 butyl-biguanine. Sigma-Aldrich): 100 μM; Nocodazole (Sigma-Aldrich): 0.3 μM; Reversine (Merck Chemicals Ltd.): 0.5 μM; ZM447439 (Santa Cruz): 20 μM; B21536 (Selleckchem): 0.2 μM; Flavopiridol (Sigma-Aldrich): 1 mM; MG132 (Sigma-Aldrich): 20 μM. Drugs were added to the culture medium and for all drugs, the time of addition and duration of treatment are indicated. Because oocytes are cultured in drops covered with mineral oil and because reversine is soluble in oil, mineral oil covering the culture drops contained reversine as well, and untreated controls were cultured in separate dishes.

Western blots, kinase assays. Western blots were performed using the indicated number of oocytes at specific stages14. For western blot detection the following antibodies were used: Actin (ab3280, 1/200) and BubR1 (Sheep polyclonal antibody, Jackson ImmunoResearch, 713-165-147; 1/200) and anti human Alexa 488 (Life Technologies, A-11013; 1/200). Cold-stable spindles were obtained by incubating oocytes on top of an ice-water bath for 5 min before fixation68.

Acquisitions of antibody staining on fixed oocytes were done with an Olympus IX70 Spinning Disk Confocal microscope (100x/1.2 NA objective, triple pass filter set, Sutter excitation filter wheel, MicroMax cooled CCD (charge-coupled device) camera (Princeton Inst.) and Metamorph software59.

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