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Mps1 at kinetochores is essential for female mouse meiosis I

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SUMMARY

In female meiosis, chromosome missegregations lead to the generation of aneuploid oocytes and can cause the development of trisomies or infertility. Because mammalian female meiosis I is error prone, the full functionality of control mechanisms, such as the spindle assembly checkpoint (SAC), has been put into question. The SAC monitors the correct orientation, microtubule occupancy and tension on proteinaceous structures named kinetochores. Although it has been shown previously that the SAC exists in meiosis I, where attachments are monopolar, the role of microtubule occupancy for silencing the SAC and the importance of certain essential SAC components, such as the kinase Mps1, are unknown in mammalian oocytes. Using a conditional loss-of-function approach, we address the role of Mps1 in meiotic progression and checkpoint control in meiosis I. Our data demonstrate that kinetochore localization of Mps1 is required for the proper timing of prometaphase and is essential for SAC control, chromosome alignment and aurora C localization in meiosis I. The absence of Mps1 from kinetochores severely impairs chromosome segregation in oocyte meiosis I and, therefore, fertility in mice. In addition, we settle a long-standing question in showing that kinetochore-microtubule attachments are present in prometaphase I at a time when most of the SAC protein Mad2 disappears from kinetochores.

KEY WORDS: Chromosome segregation, Kinetochore, Meiosis I, Mouse oocytes, Mps1 (Ttk), Spindle assembly checkpoint

INTRODUCTION

In mitosis, the spindle assembly checkpoint (SAC) verifies during each cell division whether all kinetochores are properly attached to the opposite poles of the bipolar spindle and under tension before anaphase takes place. Core SAC proteins include monopolar spindle 1 (Mps1; also known as Ttk), mitotic arrest deficient (Mad1, Mad2), budding uninhibited by benomyl (Bub1, Bub3) and Mad3/Bub1 related (BubR1; also known as Bub1b), but more proteins are either directly or indirectly required for SAC control (Musacchio and Salmon, 2007). SAC components are localized to the unattached kinetochore and are involved in the detection of missing attachment and tension, whereupon a 'wait-anaphase' signal is generated (Rieder et al., 1994). This signal prevents SAC inactivation and therefore metaphase-to-anaphase transition by inhibiting the E3 ubiquitin ligase anaphase-promoting complex/cyclosome (APC/C) (Musacchio and Salmon, 2007). Furthermore, the SAC components Mad2, BubR1 and Mps1 have a timer function in mitosis (Meraldi et al., 2004; Sliedrecht et al., 2010; Tighe et al., 2008) that is independent of their kinetochore localization (Maciejowski et al., 2010; Malureanu et al., 2009; Meraldi et al., 2004) and can be separated from SAC function in the case of BubR1 (Rahmani et al., 2009). Absence of this timer leads to a reduction in the time from nuclear envelope breakdown to anaphase onset.

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Mps1 is a dual specificity kinase (Lauze et al., 1995) that is conserved from yeast to man. In mammalian cells, studies using chemical inhibitors, antibody injections or RNA interference have shown that Mps1 is required for proper SAC control and also during unperturbed mitosis (Liu et al., 2003; Sliedrecht et al., 2010; Stucke et al., 2002; Tighe et al., 2008). Mps1 dimerizes and trans-autophosphorylates, and inhibition of Mps1 kinase activity increases its localization to kinetochores, suggesting that auto phosphorylation is required to release Mps1 from kinetochores and for anaphase onset (Hewitt et al., 2010; Jelluma et al., 2010). Mps1 is a key regulator in the signaling cascade that generates the mitotic checkpoint complex (MCC), which comprises BubR1, Cdc20, Bub3 and Mad2, to inhibit the APC/C (Musacchio and Salmon, 2007). Inhibitory MCC complexes can also be generated in the cytoplasm in interphase and early mitosis that are independent of kinetochores (Malureanu et al., 2009; Meraldi et al., 2004; Sudakin et al., 2001). The kinetochore localization of Mps1 is therefore not required to produce this cytosolic MCC, which fulfills the timer function of the SAC to delay prometaphase in mitosis (Maciejowski et al., 2010).

Mps1 is required for correct chromosome alignment in mitotic cells. Synthelic attachments (both kinetochores are attached to the same pole) are corrected through an error correction pathway that involves Mps1 and aurora B kinase (Aurkb). In the absence of aurora B or Mps1, chromosomes that are not correctly attached are not aligned at the metaphase plate and are instead found at the poles. In human cells, full aurora B activity has been shown to depend on the presence of MPS1 and the phosphorylation of borealin (CDCA8 – Human Gene Nomenclature Committee) by MPS1 (Bourhis et al., 2009; Jelluma et al., 2008; Kwiatkowski et al., 2010), but phosphorylation of aurora B substrates does not depend on Mps1 kinase activity (Hewitt et al., 2010; Maciejowski et al., 2010; Santaguida et al., 2010). Mps1 and aurora B kinase might not function in a linear pathway (Lan and Cleveland, 2010) and may phosphorylate certain substrates concomitantly.

Meiosis I is a specialized cell division: homologous chromosomes (rather than sister chromatids, as in mitosis) that are held together by chiasmata (sites at which recombination has occurred) are segregated to opposite poles. We and others have shown previously that the SAC exists in mammalian meiosis I and leads to a transient cell cycle arrest in metaphase I. The SAC proteins Mad2 and Bub1 prevent precocious metaphase-to-anaphase transition and, therefore, chromosome missegregations (Brunet et al., 2003; Homer et al., 2005; McGuinness et al., 2009; Niault et al., 2007; Wassmann et al., 2003). Zebrafish homozygous for a hypomorphic *mps1* mutant cannot maintain the correct chromosome number throughout meiotic divisions (Poss et al., 2004). Interestingly, meiosis seems to be much more sensitive to reduced Mps1 function than mitosis. Furthermore, female meiotic divisions are more affected than male (Poss et al., 2004). In *Drosophila melanogaster* oocytes, loss of Mps1 (Ald – FlyBase) leads to segregation errors due to a failure to mono-orient achiasmate chromosomes and to maintain chiasmata (Gilliland et al., 2007). The role that Mps1 plays in mammalian meiosis I is unknown. Here, we present evidence that Mps1 is essential for meiosis I in mouse. Kinetochore localization of Mps1 is required for correct timing of prometaphase, to prevent precocious APC/C activation, for SAC control, correct aurora C (Aurkc) localization and chromosome alignment. Furthermore, we clarify that recruitment of the SAC protein Mad2 to unattached kinetochores correlates with microtubule occupancy.

MATERIALS AND METHODS

Mouse strains and genotyping

Zp3-Cre [*C57BL/6-Tg(Zp3-Cre)93Knw/J*] breeding pairs were obtained from Jackson Laboratories. *Mps1*$^{F/F}$ mice have a 129/SV and C57/BL6 mixed background. For experiments in Fig. 1C, CD-1 (Swiss) mice were used (Janvier). Transgenic mice were tagged with small ear tags (Harvard Apparatus). Genotyping was performed as described (Dobles et al., 2000) using the following primers: Mps1-1, 5′-CCTGGTAGTCTACCCATC-CTCCTGCTC-3′; Mps1-2, 5′-GACACAGACATGGTTGGAGAGTC-CTGAG-3′; and Mps1-3, 5′-GAATACCGAATGAGCGAAAAGCCCC-3′; primers Mps1-1 and Mps1-2 amplify both the wild-type and the floxed *Mps1* allele, whereas Mps1-1 and Mps1-3 amplify only the *ΔNMps1* allele. Presence of Cre was determined using the protocol provided by Jackson Laboratories.

Oocyte culture

Oocytes were removed from ovaries of adult female mice at 9-16 weeks of age in self-made M2 medium containing 100 μg/ml dibutyryl cyclic AMP (dbcAMP; Sigma). Fully grown GV stage oocytes were removed from surrounding follicle cells by careful mouth pipetting into drops of medium kept under embryo culture-certified mineral oil (Sigma). Oocytes were kept in an incubator without CO_2 at 38°C. Oocytes were released from inhibitory dbcAMP through at least three washes in M2 medium without dbcAMP. GVBD was scored 60 and 90 minutes after release, and only oocytes that entered meiosis I within 90 minutes were used in experiments. PB extrusion was observed 7-9 hours after GVBD in at least 85% of mature oocytes. Nocodazole was used at a final concentration of 200 nM.

Reverse transcription semi-quantitative PCR

Reverse transcription was carried out on 20 oocytes from *Mps1*$^{F/F}$ (control) or *Mps1*$^{F/F}$ *Zp3-Cre*$^{+}$ mice that had been disrupted by vortexing for 5 seconds at maximum speed. Reactions were performed with SuperScript III reverse transcriptase (Invitrogen) and random hexanucleotides for 1 hour at 50°C according to the manufacturer's protocol in a 16 μl total volume supplemented with 150 ng/μl BSA. Real-time semi-quantitative PCR (qPCR) was carried out on a Stratagene Mx3005p using the Brilliant II SYBR Green Kit (Stratagene) according to the manufacturer's instructions. Briefly, 4 μl of reverse transcribed samples were used in 25 μl qPCR reactions. End products from qPCR amplifications were run on a 1% agarose gel and stained with ethidium bromide, or data were computed as previously described (Batsche et al., 2006). Primer sequences are: Mps1-a, 5′-ACTACTTCCAGATGGCCAGGGAAA-3′; Mps1-b, 5′-TCTGGAG-GTGTAAGTTACGCATGG-3′; Mps1-c, 5′-AGGCTGACAAAGAGTC-ACCACCAA-3′; Mps1-d, 5′-TTGATGATGGACAGGCAGGTGGAA-3′; Mps1-e, 5′-GCAGCAGTGTGACGATTGATTCCA-3′; RPS16-F, 5′-AATGGGCTCATCAAGGTGAACGGA-3′; RPS16-R, 5′-TTCACACG-GACCCGAATATCCACA-3′.

Reverse transcription (RT)-PCR

Mouse kidneys were harvested and flash frozen. mRNA was obtained from kidneys homogenized in TRI reagent (Sigma), followed by phenol-chloroform extraction. Oocytes were harvested in M2 medium and flash frozen. For RT reactions, 1 μg of total RNA from kidneys or lysates from 20 oocytes were used (SuperScript II, Invitrogen) according to the manufacturer's protocol, using oligo$(dT)_{12\text{-}18}$. The following primers were used to amplify and PCR clone (Expand High Fidelity^{PLUS} PCR system, Roche) full-length *Mps1* or *ΔNMps1*: forward primer, 5′-CGCCTC-GAGCGGCCGCCATGGAGGCTGAAGAGTTAATTG-3′; and reverse primer, 5′-GAAGTAAGGATCCTTTCTTTCTCTCTTTTTGTCAAA-3′.

Plasmids

A pRN3EGFP-C1 plasmid (gift from S. Louvet, UMR7622, Paris, France) was obtained by subcloning EGFP-C1 with its polylinker (Clontech) into pRN3. Mouse *Mps1* and *ΔNMps1* were cloned into pRN3EGFP-C1 with *Xho*I and *Bam*HI to obtain GFP N-terminal-tagged Mps1.

In vitro transcription and microinjections

All mRNAs were transcribed using the T3 mMessage mMachine Kit (Ambion) according to the manufacturer's protocol. mRNAs were resuspended in RNAse-free water. Microinjections were performed on GV stage-arrested oocytes in M2 medium containing dbcAMP. Injection needles were self-made (Narishige micropipette puller) and holding pipettes were from Eppendorf. Injections into the cytoplasm of GV oocytes were performed with a FemtoJet microinjector (Eppendorf) with constant flow settings. After injection, oocytes were left to recover before release into M2 medium.

Live imaging of oocytes

Live microscopy was performed on a Leica SP5/AOBS confocal microscope with a Plan APO 63×/1.4 NA objective, as indicated. A Leica DMI6000B microscope (Plan APO 40×/1.25 NA objective) coupled to a Sutter filter wheel (Roper Scientific) and a Yokogawa CSU-X1-M1 spinning disk, or a motorized inverted Nikon TE2000E microscope (Plan APO 20×/0.75 NA objective) with PrecisExite high power LED fluorescence [LED array module (LAM) 1, 400/465; LAM 2, 585] were used as indicated. All imaging stations were equipped with a temperature chamber (Life Imaging Services). The Leica DMI6000B and the Nikon TE2000E microscopes were equipped with a Märzhäuser Scanning Stage and a Photometrics CoolSNAP HQ2 camera, and were controlled by MetaMorph software (Molecular Devices). The same results (but with different resolutions) were obtained using the different imaging stations. All experiments were repeated at least three times.

Area calculations and quantitations

The area occupied by chromosomes in Fig. 5B was determined with ImageJ (NIH) from live imaging of oocytes extruding PBs perpendicular to the microscope. The ratio R (area occupied by oocyte/area occupied by PB) was determined to address whether PBs were of abnormal size. R=8-13 in controls of the mouse strain used in this study; R≤6 was considered to indicate an abnormally large PB. For securin quantitations, the YFP signal was quantitated with ImageJ from original acquisitions of one focal plane, and the mean background was subtracted.

Chromosome spreads and immunofluorescence

Chromosome spreads of metaphase II-arrested oocytes and Mad2 kinetochore staining on fixed oocytes were performed as described previously (Niault et al., 2007; Wassmann et al., 2003). For Mad2 staining

on chromosome spreads, spreads were washed with PBS, blocked in PBS containing 3% BSA and incubated with 1:100 rabbit anti-Mad2 antibody overnight.

Immunofluorescence studies to visualize kinetochore-bound microtubule fibers were performed after removal of the zona pellucida with Tyrode's acidic solution (Kubiak et al., 1992). After recuperation, oocytes were incubated at 38°C in 0.2 mM $CaCl_2$ in 400 mM PIPES for 5 minutes, before fixation in BRB80 buffer (Bonifacino et al., 2010) containing 0.2% formaldehyde and 0.2% Triton X-100. Oocytes were fixed for 1 hour, washed three times in PBS, and left overnight at 4°C in PBS containing 3% BSA and 0.1% Triton X-100. For detection of aurora C, oocytes were fixed in 2% paraformaldehyde containing 0.3% BSA in PBS.

For immunofluorescence staining of kinetochores and spindles, fixed oocytes were incubated with 1:50 Crest (human centromere auto-antibody) HCT-100 (Cellon SA) and 1:700 DM1A (Sigma) primary antibodies overnight at 4°C in a humid chamber. For detection of aurora C, oocytes were incubated with 1:30 anti-aurora B rabbit antibody (Abcam 61188), which detects a region conserved between aurora B and C. As a negative control, we used aurora B antibody directed against the N-terminal region that is not conserved in aurora C (anti-AIM1, BD Transduction Laboratories), as published (Yang et al., 2010). As secondary antibodies, anti-human Cy3 (Interchim), anti-rabbit Cy3 (Interchim), anti-human Alexa 488 (Molecular Probes) and anti-mouse Alexa 488 (Molecular Probes) were used at 1:200.

Chromosomes were stained for 10 minutes with 5 μg/ml Hoechst 33342 (Sigma) or with 2 μg/ml propidium iodide (Molecular Probes). Citifluor AF-1 mounting medium was used. All experiments were repeated at least three times in independent experiments.

Confocal microscopy and image analysis of fixed oocytes

Acquisitions for Fig. 6A were performed on a Leica SP5/AOBS confocal microscope with a 63×/1.32 NA objective and 5× zoom, an image size of 512×512 pixels and a line average of 3; step size was 0.13 μm. Compiled three-dimensional image data sets were deconvolved using a non-linear iterative method based on the classical maximum likelihood estimation method algorithm. Volocity Visualization (PerkinElmer) was used for high-resolution volume rendering of the three channels. For Mad2 immunofluorescence, acquisitions were performed as above on a Leica SP5/AOBS confocal microscope with a 63×/1.32 NA objective, 4× zoom, an image size of 1024×1024 pixels and a line average of 4; step size was 0.5 μm and images were not deconvolved.

RESULTS

Generation of a mouse strain that harbors a mutant form of Mps1 specifically in oocytes

The kinetochore-binding domain of Mps1 is crucial for its correct localization and recruitment of core SAC components to unattached kinetochores in mammalian cells, and thereby for a proper checkpoint response (Liu et al., 2003; Maciejowski et al., 2010; Santaguida et al., 2010; Stucke et al., 2004; Tighe et al., 2008; Xu et al., 2009). To obtain cells that are defective for the role of Mps1 in the SAC response, a mouse strain harboring a Cre-recombinase-dependent deletion in the kinetochore-binding domain was constructed (*Mps1*$^{F/F}$) (S.Z.X., F. Foijer, Y. Yue, S. Davis, A. Schulze-Lutum, E. Kregel, J. Jonkers and P.K.S., unpublished). Exons 3 and 4 were flanked by loxP sites (the *Mps1* floxed allele), and Cre-mediated recombination resulted in a mutant allele, *ΔNMps1*, that encodes a form of Mps1 with a 107 amino acid deletion (Fig. 1A). ΔNMps1 was expressed in mouse oocytes exclusively using the oocyte-specific zona pellucida glycoprotein 3-*Cre* (*Zp3-Cre*) driver (Lewandoski et al., 1997). Efficient deletion was confirmed by genotyping the heterozygous offspring of *Mps1*$^{F/+}$ *Zp3-Cre*$^{+}$ females crossed with *Mps1*$^{F/F}$ *Zp3-Cre*$^{+}$ males (see Fig. S1A in the supplementary material). *ΔNMps1* was cloned from kidneys and sequenced, confirming the deletion of amino acids 47-154 (see Fig. S1B in the supplementary material). In *Mps1*$^{F/F}$ *Zp3-Cre*$^{+}$ oocytes, mRNA encoding ΔNMps1 protein was transcribed at levels comparable to wild-type *Mps1*, as determined by RT-qPCR (Fig. 1B). To address whether ΔNMps1 protein is stable in oocytes, germinal vesicle (GV) stage oocytes (which are in prophase I) were injected with mRNAs encoding GFP-Mps1 and GFP-ΔNMps1, and released to enter the first meiotic division. Both GFP-tagged wild-type Mps1 and GFP-ΔNMps1 were stably expressed in mouse oocytes, showing that mouse ΔNMps1 is not degraded (Fig. 1C).

Live confocal imaging allowed us to visualize GFP-Mps1 at kinetochores in early meiosis I. GFP-ΔNMps1 was not localized to unattached kinetochores in *Mps1*$^{F/F}$ *Zp3-Cre*$^{+}$ prometaphase I oocytes, demonstrating that the deleted domain is indeed required for the kinetochore localization of Mps1 (Fig. 1D). GFP-ΔNMps1 was still localized to kinetochores in control oocytes, albeit at very low levels (Fig. 1D). This is explained by dimerization of ΔNMps1 with endogenous Mps1 at kinetochores, confirming recent findings that Mps1 forms dimers (Hewitt et al., 2010).

Full-length Mps1 is required for fertility and correct chromosome segregation in meiosis I

To address the in vivo importance of kinetochore-localized Mps1 for generating healthy fertilizable oocytes, female *Mps1*$^{F/F}$ *Zp3-Cre*$^{+}$ mice were crossed with male *Mps1*$^{F/F}$ or *Mps1*$^{F/F}$ *Zp3-Cre*$^{+}$ mice that had been shown previously to produce offspring. We observed a dramatic decrease in the fertility of *Mps1*$^{F/F}$ *Zp3-Cre*$^{+}$ female mice, with the average number of pups per litter dropping from 6.5 to 0.7 (Fig. 2A). Genotyping of live pups revealed that all had a wild-type *Mps1* allele remaining (data not shown). This confirms that Mps1 is essential for embryonic development, similar to other SAC components (Schvartzman et al., 2010). The fact that some live births were obtained from female mice harboring only oocytes without kinetochore-localized Mps1 indicates that the meiotic divisions seem to succeed in a small fraction of oocytes.

To gain insights into the role of Mps1 in meiosis, we analyzed meiotic maturation in *Mps1*$^{F/F}$ *Zp3-Cre*$^{+}$ oocytes. GV stage oocytes that are in meiotic prophase I are obtained from adult female mice. Oocytes were induced to undergo meiotic maturation in vitro to determine when polar body (PB) extrusion takes place, which is indicative of the metaphase-to-anaphase transition of meiosis I. Ovaries of *Mps1*$^{F/F}$ *Zp3-Cre*$^{+}$ mice contained the same numbers of GV stage oocytes as *Mps1*$^{F/F}$ mice, and *Mps1*$^{F/F}$ *Zp3-Cre*$^{+}$ oocytes entered meiosis I with the same efficiency and timing as control oocytes (data not shown). However, anaphase I onset was dramatically accelerated by an average of 2.5 hours, with some oocytes extruding PBs as early as 4.5 hours after GV breakdown (GVBD) (Fig. 2B). Kinetochore localization of Mps1 is therefore important for proper prometaphase timing in meiosis I. We analyzed the ploidy of oocytes that underwent meiosis I and were arrested in metaphase of meiosis II awaiting fertilization. Approximately 70% of *Mps1*$^{F/F}$ *Zp3-Cre*$^{+}$ oocytes were aneuploid, with both hyperploidies and hypoploidies, as compared with less than 10% in *Mps1*$^{F/F}$ controls (Fig. 2C,D). About 30% of *Mps1*$^{F/F}$ *Zp3-Cre*$^{+}$ oocytes had normal ploidy and should therefore be fertilizable. This explains why rare live births were observed from *Mps1*$^{F/F}$ *Zp3-Cre*$^{+}$ mothers.

To address whether the observed phenotypes are indeed due to loss of Mps1 function, rescue experiments with full-length GFP-tagged Mps1 were performed. mRNAs encoding Mps1 were injected into control and *Mps1*$^{F/F}$ *Zp3-Cre*$^{+}$ oocytes. As expected, injection of *Mps1* mRNA delayed PB extrusion in control oocytes.

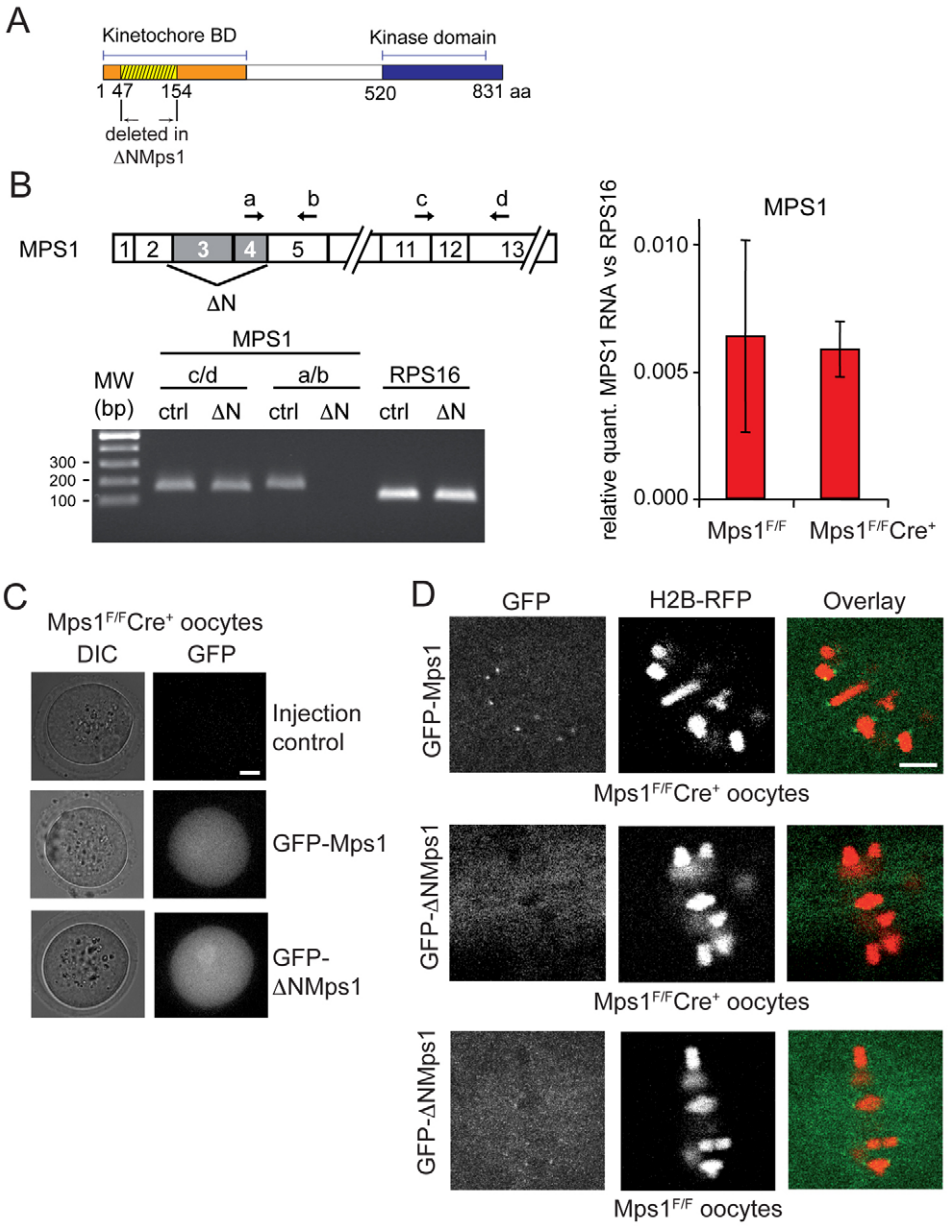

Fig. 1. Conditional expression of a *ΔNMps1* mutant allele specifically in oocytes. (**A**) ΔNMps1, which harbors a 107 amino acid deletion within the conserved kinetochore-localization domain. (**B**) ΔNMps1 is expressed conditionally in mouse oocytes owing to the insertion of loxP sites flanking exons 3 and 4 and expression of Cre recombinase under the control of the oocyte-specific *Zp3* promoter. RT-qPCR shows that the *ΔNMps1* allele is transcribed at levels as high as wild-type *Mps1* in oocytes. Arrows indicate primers used for qPCR; primers that detect mRNA encoding the ribosomal protein Rps16 provided an internal control in the same qPCR. The bar chart shows the amount of *Mps1* mRNA relative to that of *Rps16* in oocytes of the indicated genotypes. Error bars indicate ± s.e.m. from three independent experiments. (**C**) GFP-tagged wild-type Mps1 and ΔNMps1 are stably expressed in mouse oocytes. Oocytes were injected with the indicated GFP-Mps1-encoding mRNAs and released to enter meiosis I. Still images show the GFP fluorescence of oocytes in prometaphase I. (**D**) Confocal microscopy in live oocytes shows that only GFP-tagged wild-type mouse Mps1, and not ΔNMps1, is localized to kinetochores in early meiosis I in *Mps1$^{F/F}$ Zp3-Cre^{+}* oocytes. ΔNMps1 is localized weakly to kinetochores in *Mps1$^{F/F}$* control oocytes. One *z*-section is shown, and the GFP signal is only visible on kinetochores that are in the corresponding *z*-section. At least 20 oocytes were analyzed in each of three independent experiments. *Mps1$^{F/F}$Cre^{+}* indicates *Mps1$^{F/F}$ Zp3-Cre^{+}*. Scale bars: 20 μm in C; 5 μm in D.

In *Mps1$^{F/F}$ Zp3-Cre^{+}* oocytes, GFP-Mps1 rescued the timing defect and PB extrusion, as observed by time-lapse imaging of histone H2B-RFP co-injected oocytes (Fig. 3A and see Fig. S2A in the supplementary material). We conclude that the phenotypes observed are indeed due to the loss of Mps1 function in *Mps1$^{F/F}$ Zp3-Cre^{+}* oocytes.

Next, we addressed whether ΔNMps1 is still functional. If ΔNMps1 were still functional, we would expect that its overexpression in wild-type oocytes would lead to a delay in PB extrusion, similar to that observed upon overexpression of full-length Mps1. ΔNMps1 dimerizes with endogenous Mps1 at the kinetochore and should be able to fulfill its function there. Indeed, expression of GFP-ΔNMps1 led to a delay in PB extrusion in control oocytes, but, importantly, not in *Mps1$^{F/F}$ Zp3-Cre^{+}* oocytes (Fig. 3B and see Fig. S2B in the supplementary material). This result demonstrates that ΔNMps1 is still functional in the presence of endogenous Mps1, and strongly suggests that at least transient kinetochore localization of Mps1 is required for the correct timing of prometaphase I.

Mps1$^{F/F}$ Zp3-Cre^{+} oocytes are defective in SAC control and activate APC/C precociously

We next asked whether kinetochore localization of Mps1 is required for proper SAC control in meiosis I. Low doses of nocodazole (200 nM) did not cause cell cycle arrest in *Mps1$^{F/F}$ Zp3-Cre^{+}* oocytes, even though control oocytes arrested for the length of the experiment (Fig. 4A). Importantly, all *Mps1$^{F/F}$ Zp3-Cre^{+}* oocytes extruded PBs (Fig. 4A) and were highly aneuploid (data not shown).

We have shown previously that Mad2 is recruited to unattached kinetochores early in meiosis I, presumably preventing premature APC/C activation (Wassmann et al., 2003). Indeed, no Mad2 staining on kinetochores was observed in untreated *Mps1$^{F/F}$ Zp3-Cre^{+}* oocytes (Fig. 4B,C). This confirms that kinetochore localization of Mad2 depends on Mps1, and suggests that the accelerated meiotic timing is due to the failure of SAC components to localize to unattached kinetochores.

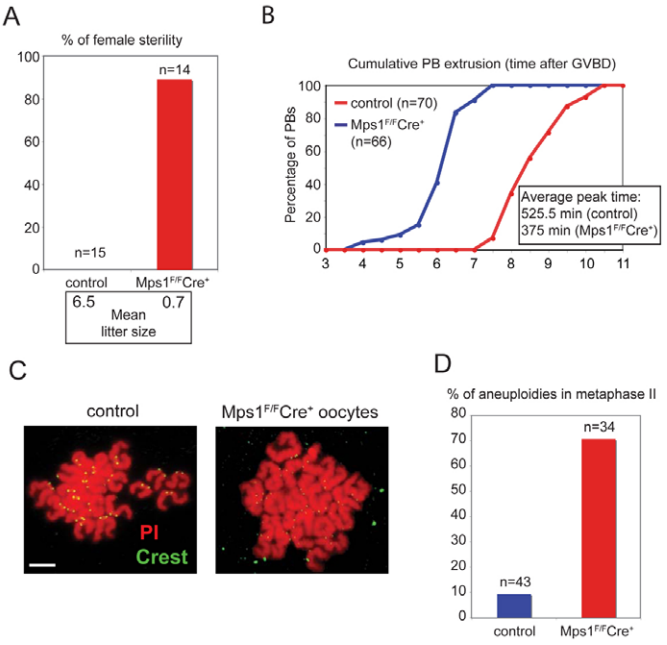

Fig. 2. Female *Mps1$^{F/F}$ Zp3-Cre^{+}* mice show a drop in fertility and harbor oocytes that progress precociously through meiosis I and are highly aneuploid. (**A**) Female *Mps1$^{F/F}$ Zp3-Cre^{+}* mice were crossed with confirmed-fertile *Mps1$^{F/F}$* males. The mean litter size, the percentage of females that were sterile and the number of crosses (*n*) are indicated. (**B**) In vitro maturation of *Mps1$^{F/F}$* and *Mps1$^{F/F}$ Zp3-Cre^{+}* oocytes. Cumulative polar body (PB) extrusion times after release from germinal vesicle (GV) arrest and synchronization at GV breakdown (GVBD) are shown. PB extrusion times were determined visually by observation of oocytes under the microscope. (**C**) Examples of chromosome spreads of *Mps1$^{F/F}$* and *Mps1$^{F/F}$ Zp3-Cre^{+}* oocytes used to determine ploidy. Chromosomes are in red (propidium iodide), kinetochores in green (Crest). Scale bar: 5 μm. The *Mps1$^{F/F}$* control oocyte contains 20 chromosomes, whereas the *Mps1$^{F/F}$ Zp3-Cre^{+}* oocyte shown here has 22 chromosomes. (**D**) The percentage of aneuploid oocytes in metaphase II, as determined by chromosome spreads.

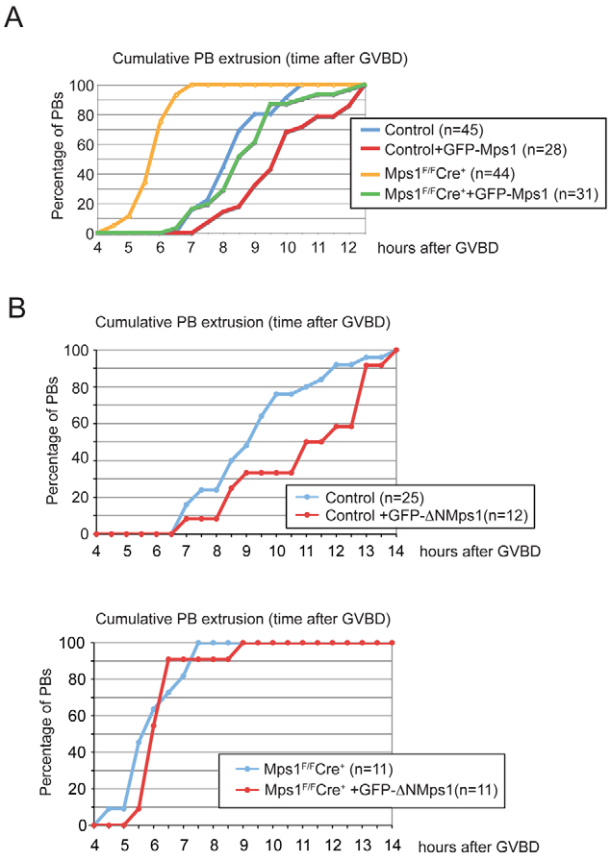

Fig. 3. Rescue of *Mps1$^{F/F}$ Zp3-Cre^{+}* oocytes upon expression of GFP-Mps1 but not GFP-ΔNMps1. (**A**) Cumulative PB extrusion times in control and *Mps1$^{F/F}$ Zp3-Cre^{+}* mouse oocytes expressing GFP-Mps1. *GFP-Mps1* mRNA was injected into GV stage oocytes before release and visual observation. The total number of oocytes analyzed is indicated (*n*). (**B**) As in A, except that *GFP-ΔNMps1* mRNA was injected into GV stage oocytes of the indicated genotypes.

Premature anaphase I onset in *Mps1$^{F/F}$ Zp3-Cre^{+}* oocytes suggests that the APC/C is activated too early. The separase (Espl1) inhibitor securin (Pttg1 – Mouse Genome Informatics) is a substrate of the APC/C that has to be degraded before anaphase I onset to permit separation of chromosomes by separase-dependent cleavage of cohesin (Herbert et al., 2003; Kudo et al., 2006; Terret et al., 2003). When we analyzed the degradation of exogenously expressed YFP-tagged securin in live oocytes, degradation onset was significantly advanced in *Mps1$^{F/F}$ Zp3-Cre^{+}* oocytes compared with controls (Fig. 4D). This indicates that the APC/C has indeed been activated precociously, confirming that kinetochore-localized Mps1 is required to maintain the APC/C in an inactive state until all chromosomes are aligned at the metaphase plate in meiosis I.

Chromosome alignment and segregation in *Mps1$^{F/F}$ Zp3-Cre^{+}* oocytes

We determined how chromosome segregation takes place in *Mps1$^{F/F}$ Zp3-Cre^{+}* oocytes by injecting oocytes with histone H2B-RFP to follow chromosomes by time-lapse microscopy. We could distinguish two predominant phenotypes: anaphase I onset occurred with the majority of chromosomes aligned but with some chromosomes remaining at the spindle poles (phenotype 'A'; Fig. 5A,B and see Movies 1 and 2 and Fig. S3A in the supplementary material); or anaphase I onset took place at a time when the chromosomes had not yet reached the metaphase plate and were scattered along the spindle and at poles (phenotype 'B'; Fig. 5A,B and see Movie 3 and Fig. S3A in the supplementary material). Lagging chromosomes were frequently seen in both phenotypes. We performed live imaging of *Mps1$^{F/F}$ Zp3-Cre^{+}* and control oocytes injected with histone H2B-RFP and β-tubulin-GFP to visualize chromosomes and spindles, respectively. Except for the accelerated timing and aberrant chromosome segregation, no obvious defects in the assembly of a bipolar spindle were observed in oocytes of phenotype A (data not shown) or B (see Fig. S3B and Movies 4 and 5 in the supplementary material).

During meiotic maturation, scattered chromosomes are observed when bipolarization of the spindle takes place (see Movie 4 in the supplementary material for an example). We examined whether variations in the time between bipolarization of the spindle and anaphase I onset could explain the two different phenotypes observed. Indeed, in both control and *Mps1$^{F/F}$ Zp3-Cre^{+}* oocytes, the time of bipolarization was highly variable (Fig. 5C). Importantly, anaphase I onset took place in most control oocytes between 3 and 4 hours after bipolarization, whereas in *Mps1$^{F/F}$ Zp3-Cre^{+}* oocytes

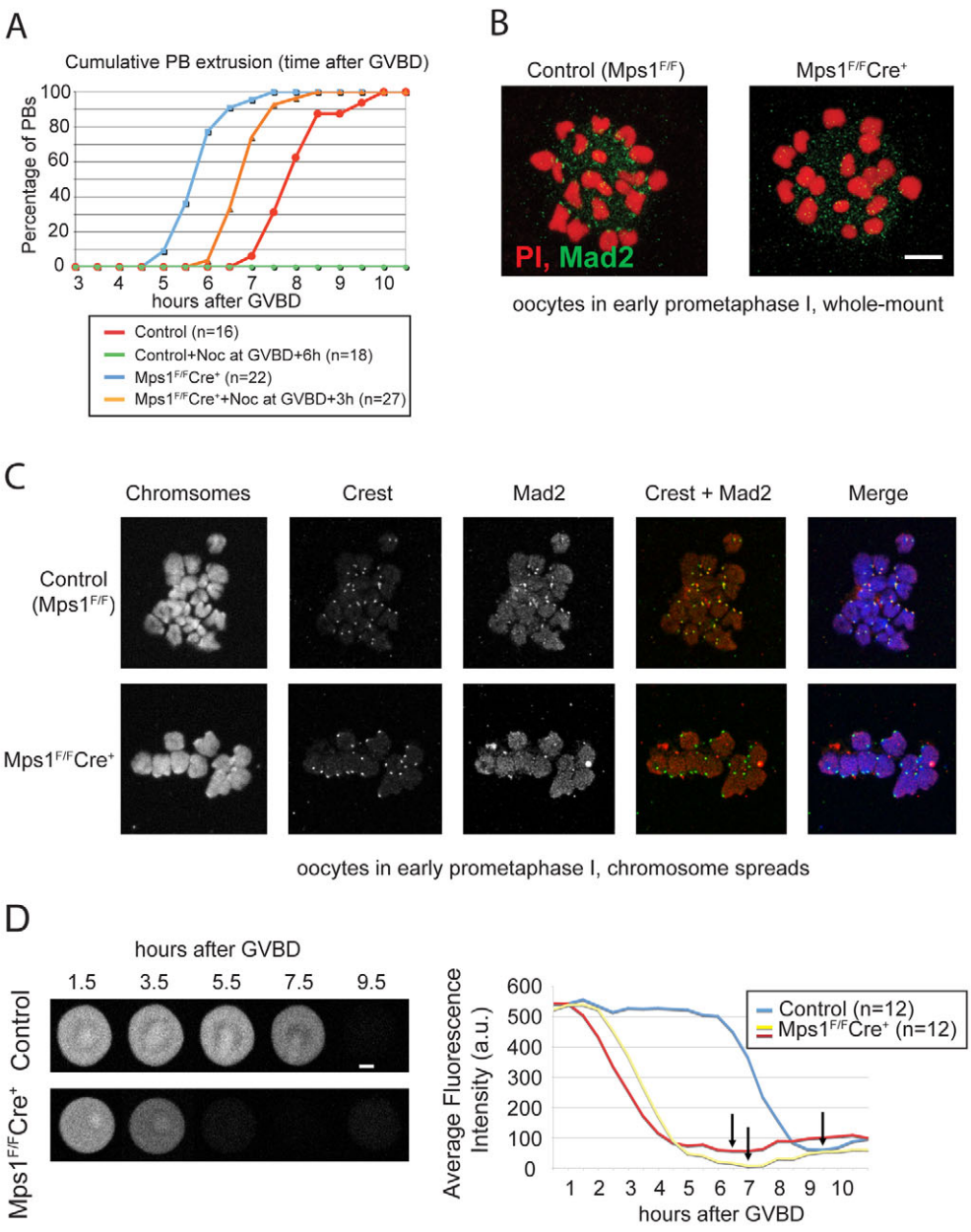

Fig. 4. Mad2 is lost from kinetochores in *Mps1*$^{F/F}$ *Zp3-Cre*$^{+}$ oocytes and SAC control in meiosis I is impaired. (**A**) Control and *Mps1*$^{F/F}$ *Zp3-Cre*$^{+}$ mouse oocytes were treated with 200 nM nocodazole at 6 and 3 hours after GVBD, respectively. Oocytes were examined visually to score GVBD and PB extrusion times. Cumulative PB extrusion times are shown. (**B**) Mad2 immunofluorescence (polyclonal rabbit antibody, green) in fixed control (GVBD + 3.5 hours) and *Mps1*$^{F/F}$ *Zp3-Cre*$^{+}$ (GVBD + 1.5 hours) oocytes, without nocodazole treatment. Chromosomes were stained with propidium iodide (red). At least 20 oocytes were analyzed in each of three different experiments. (**C**) As in B, except that chromosome spreads were performed. Kinetochores were stained with Crest serum (green). (**D**) *YFP*-securin mRNA was injected into oocytes of the indicated genotype. Oocytes were released 1-2 hours after injection, and acquisitions were started immediately after GVBD (one *z*-section), every 30 minutes. Still images are shown from a representative movie with representative quantitations of YFP-securin fluorescence levels in oocytes of the indicated genotype. PB extrusion times are indicated with arrows. The total number of oocytes analyzed is indicated (*n*). Scale bars: 5 μm in B; 20 μm in D.

the time between bipolarization and anaphase I onset was reduced to 1-2 hours (Fig. 5C). Therefore, in some *Mps1*$^{F/F}$ *Zp3-Cre*$^{+}$ oocytes bipolarization takes place only shortly before anaphase onset, whereas in other oocytes bipolarization occurs 2 hours before the metaphase-to-anaphase transition. This might explain why scattered chromosomes are observed in some, but not all, *Mps1*$^{F/F}$ *Zp3-Cre*$^{+}$ oocytes. Furthermore, *Mps1*$^{F/F}$ *Zp3-Cre*$^{+}$ oocytes formed enlarged PBs at increased frequencies (~20% compared with at most 3% in the controls; see Fig. S4 in the supplementary material). This indicates that chromosome segregation takes place prematurely, before spindle migration to the cortex.

Localization of Mad2 to kinetochores correlates with microtubule occupancy in meiosis I in control oocytes

The failure to correctly align chromosomes in *Mps1*$^{F/F}$ *Zp3-Cre*$^{+}$ oocytes could be due to kinetochores not being attached by 4.5 hours after GVBD, when PB extrusion starts to take place. *Mps1*$^{F/F}$ *Zp3-Cre*$^{+}$ oocytes might also fail to correct synthelic attachments of chromosome pairs in meiosis I. To determine why chromosomes are not aligned in *Mps1*$^{F/F}$ *Zp3-Cre*$^{+}$ oocytes, we addressed whether kinetochores are attached before anaphase I onset in *Mps1*$^{F/F}$ *Zp3-Cre*$^{+}$ oocytes. First, we aimed to establish when exactly kinetochore-microtubule interactions take place in control oocytes. The SAC protein Mad2 is localized to unattached kinetochores until around 4 hours after GVBD (Fig. 4B) (Wassmann et al., 2003). Later, during meiotic maturation, only very weak endogenous Mad2 staining on some kinetochores can be detected (Wassmann et al., 2003). It has not been shown whether the loss of most of the Mad2 correlates with kinetochore occupancy in meiosis I. On the contrary, stable attachments and the formation of kinetochore fibers were detected only at 7 hours after GVBD in metaphase I in a previous study by transmission electron microscopy (Brunet et al., 1999). We show here by immunofluorescence staining of kinetochores, chromosomes and spindles (after $CaCl_2$ treatment to visualize stable microtubules

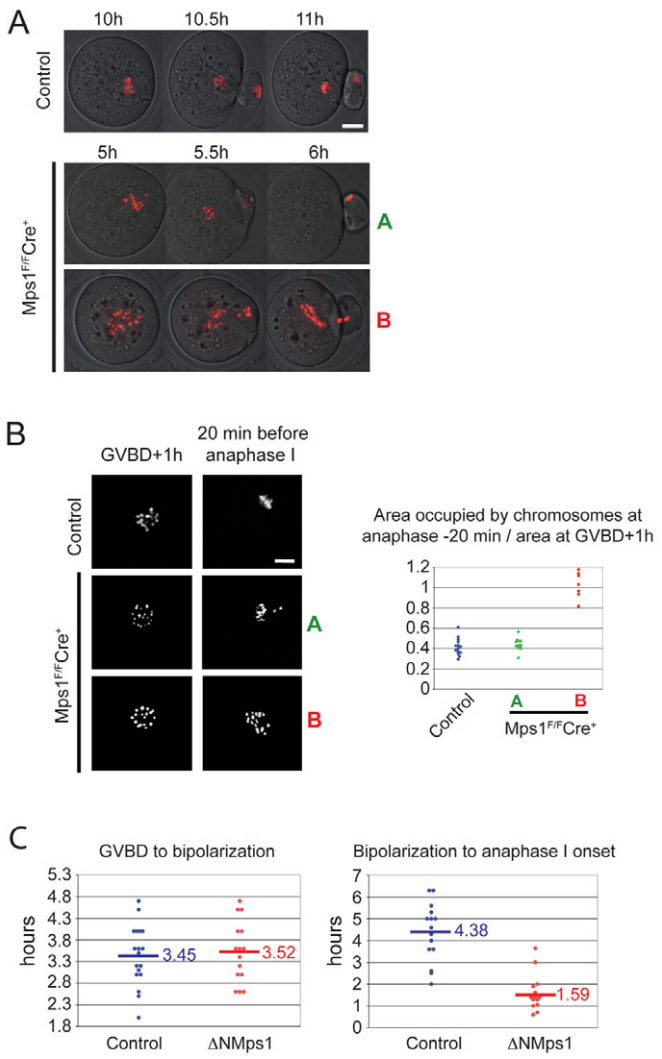

Fig. 5. Premature anaphase I onset and chromosome missegregations in *Mps1$^{F/F}$ Zp3-Cre^{+}* oocytes. (**A**) Mouse oocytes were injected with histone H2B-RFP. Collapsed still images of 13 *z*-sections, overlaid with a DIC picture acquired by confocal live imaging 30 minutes before and after anaphase onset are shown. Chromosomes are in red. Two distinct phenotypes ('A' and 'B') can be observed. Note that in phenotype A, chromosomes in the oocyte were lost from the focal plane in anaphase I (for the entire movie, see Movie 2 in the supplementary material). (**B**) The area occupied by chromosomes calculated at GVBD + 1 hour and at 20 minutes before anaphase I onset after live imaging by video microscopy. Eight *z*-sections were collapsed. *Mps1$^{F/F}$ Zp3-Cre^{+}* oocytes with phenotype B occupy a large area, whereas oocytes of the phenotype A align most chromosomes on the metaphase plate, but show single chromosomes at poles. Each dot in the graph corresponds to one oocyte that has been analyzed by live video microscopy (see also Fig. S3 in the supplementary material). (**C**) Oocytes were injected at the GV stage with mRNAs encoding histone H2B-RFP and β-tubulin-GFP to visualize chromosomes and spindles. Time of bipolarization (when the two poles of the spindle become visible) and anaphase I onset were scored by live video microscopy. Each dot represents one oocyte and the bars indicate the respective mean times. Scale bars: 20 μm.

only) that microtubule attachments are present at most kinetochores 4 or more hours after GVBD (Fig. 6 and see Movies 6-9 in the supplementary material), but not before (data not shown). Therefore, localization of Mad2 to kinetochores is strong as long as no microtubule-kinetochore interactions are present, and Mad2 disappears upon attachment of kinetochores in control oocytes. It is important to note that kinetochore attachments seem more robust at 7 than 4 hours after GVBD. Kinetochore-microtubule attachments are also evident 4.75 hours after GVBD in *Mps1$^{F/F}$ Zp3-Cre^{+}* oocytes (Fig. 6 and see Movies 10 and 11 in the supplementary material). This indicates that premature metaphase-to-anaphase transition in mutant oocytes occurs with most chromosomes attached to the spindle via their kinetochores. Therefore, the failure to align chromosomes at the metaphase plate might at least in part be due to a failure to correct synthelic attachments of paired chromosomes.

Mps1 is required for chromosome alignment and aurora C localization in meiosis I

If chromosomes are already attached before anaphase onset in *Mps1$^{F/F}$ Zp3-Cre^{+}* oocytes, the presence of chromosomes at poles is either due to a shortened prometaphase I, which does not leave enough time to get chromosomes from the poles back to the metaphase plate, or indicates a more direct role of Mps1 in chromosome alignment. We asked whether prolonging meiosis I would allow the establishment of a proper metaphase plate in *Mps1$^{F/F}$ Zp3-Cre^{+}* oocytes. We inhibited separase by expressing an N-terminal deletion construct of securin (Δ91Securin) (Herbert et al., 2003), which is not recognized by the APC/C and is therefore stable. Both control and *Mps1$^{F/F}$ Zp3-Cre^{+}* oocytes remained arrested in metaphase I upon *Δ91Securin* mRNA injection. Importantly, mainly phenotype A *Mps1$^{F/F}$ Zp3-Cre^{+}* oocytes, with some chromosomes at the poles and the formation of a metaphase plate, were observed under these conditions, and hardly any phenotype B oocytes, with chromosomes scattered along the spindle (Fig. 7A,B and see Fig. S5 in the supplementary material). Therefore, we conclude that the establishment of a metaphase plate can be rescued by reversing the timing defect in *Mps1$^{F/F}$ Zp3-Cre^{+}* oocytes, but that this is not sufficient to move chromosomes that are trapped at the poles back to the metaphase plate. This indicates that Mps1 has a role in chromosome alignment in meiosis I that can be separated from its role in preventing precocious anaphase I onset.

It has been shown that the kinases aurora B in mitosis and aurora C in mouse oocyte meiosis are required for the correct alignment of chromosomes (Yang et al., 2010). In mouse oocyte meiosis I, aurora B is not expressed, whereas its homolog aurora C is (Yang et al., 2010). Expression of a kinase-deficient aurora C mutant leads to an acceleration of meiosis I and to chromosome segregation with scattered chromosomes (Yang et al., 2010), reminiscent of what we observe in *Mps1$^{F/F}$ Zp3-Cre^{+}* oocytes. Therefore, we asked whether aurora C is mislocalized in *Mps1$^{F/F}$ Zp3-Cre^{+}* oocytes. Indeed, in the absence of kinetochore-localized Mps1, aurora C staining of chromosomes in prometaphase I and at the spindle midzone in anaphase I was lost (Fig. 8A,B). Our results strongly suggest that, in prometaphase I, mislocalization of aurora C in *Mps1$^{F/F}$ Zp3-Cre^{+}* oocytes is responsible for the chromosome misalignment phenotype observed.

DISCUSSION

Even though sisters are attached in a monopolar fashion in meiosis I, the SAC is functional in mammalian oocytes. With knockdown experiments (siRNA and morpholino oligos), dominant-negative mutants and genetic approaches, the roles of the mitotic SAC components Mad2 and Bub1 in meiosis I SAC arrest have been demonstrated (Homer et al., 2005; McGuinness et al., 2009; Niault

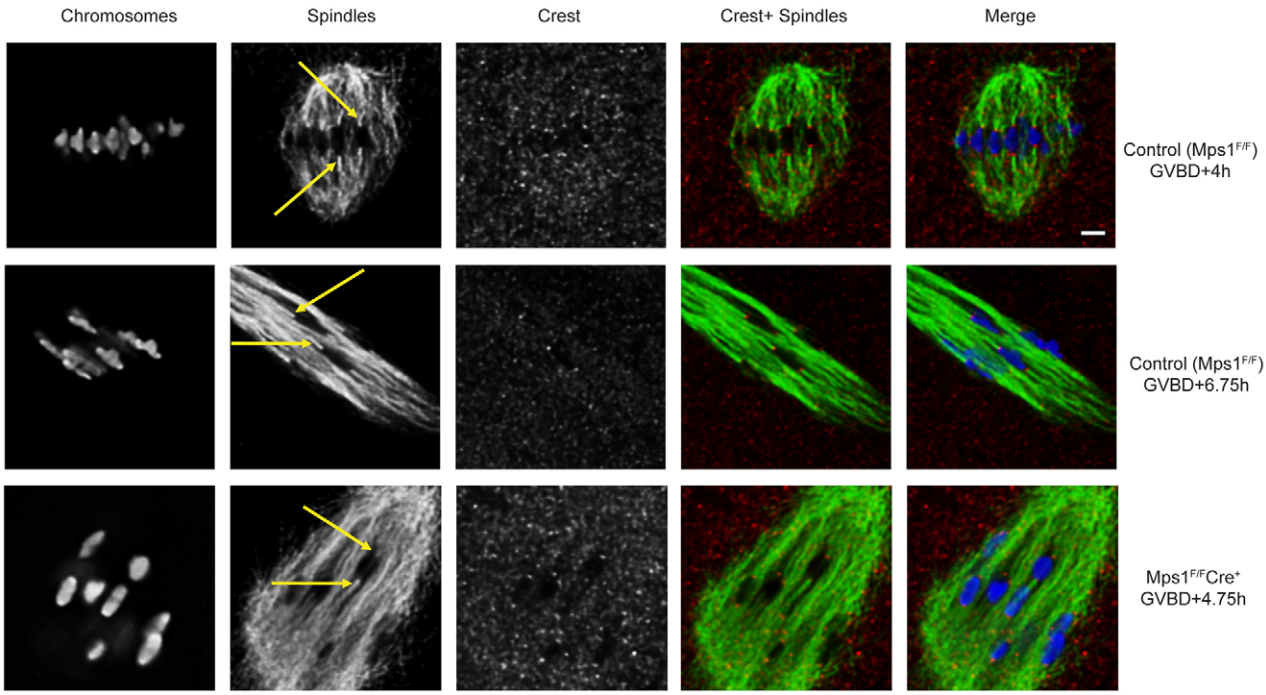

Fig. 6. Stable microtubule fibers are attached to kinetochores early in prometaphase I. Visualization of microtubules attached to kinetochores at the indicated time points after GVBD. $CaCl_2$-treated mouse oocytes were fixed and immunostained with anti-α-tubulin (spindles, green) and Crest (red) antibodies. Chromosomes were stained with Hoechst (blue). The merge shows all three channels of one *z*-section. Scale bar: 5 μm. Microtubules with end-on kinetochore attachment are indicated by arrows. At least 25 oocytes were analyzed in each of three different experiments.

et al., 2007; Wassmann et al., 2003). The role of Mps1 for SAC control in meiosis I has been obscure in vertebrate oocytes. Using a conditional loss-of-function approach in mouse oocytes, we demonstrate here that the SAC kinase Mps1 is indeed essential for SAC control in meiosis I. A 107 amino acid deletion in the kinetochore-binding domain of Mps1 prevents localization of Mps1 to unattached kinetochores, but only in oocytes that do not harbor endogenous Mps1, providing in vivo confirmation of the homodimerization of Mps1 at kinetochores (Hewitt et al., 2010). Truncated Mps1 is still functional, as it leads to a delay in PB extrusion in oocytes harboring endogenous Mps1. The downstream effector of Mps1, Mad2, is not localized to kinetochores of *Mps1*$^{F/F}$ *Zp3-Cre*$^{+}$ oocytes early in meiosis I, and anaphase onset and APC/C activation occur precociously.

Loss of Mps1 leads to a shortening by 2.5 hours on average of prometaphase I after kinetochore attachment. We propose that timing of early prometaphase I, before kinetochore attachment, requires additional regulatory events. SAC control becomes important once Cdc20-associated APC/C is active, in accordance with a recent study of APC/C activity in mouse oocytes (McGuinness et al., 2009). Acceleration of anaphase I by a few hours has severe consequences: missegregation events occur at such high rates that few fertilizable

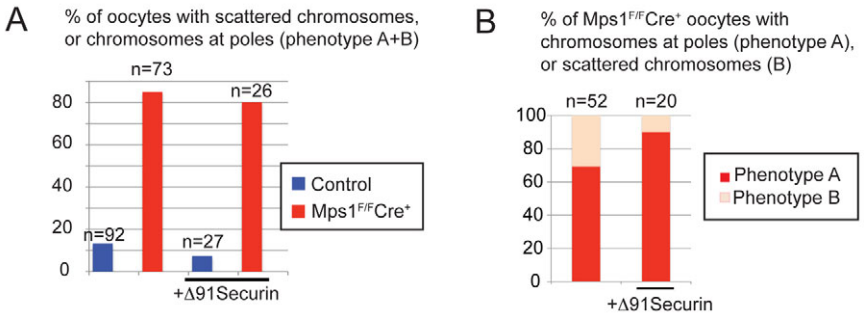

Fig. 7. Prolongation of a shortened prometaphase resulting from precocious APC/C degradation does not rescue chromosome alignment in *Mps1*$^{F/F}$ *Zp3-Cre*$^{+}$ oocytes. (**A**) GV stage control and *Mps1*$^{F/F}$ *Zp3-Cre*$^{+}$ mouse oocytes were injected with mRNAs encoding Δ91Securin and histone H2B-RFP to visualize chromosomes, and released. The number of oocytes analyzed is indicated (*n*). Shown is the percentage of oocytes of the indicated genotype with unaligned chromosomes (phenotypes A and B) at a time when control oocytes are in metaphase I (7.5 hours after GVBD). (**B**) Percentage of *Mps1*$^{F/F}$ *Zp3-Cre*$^{+}$ oocytes with unaligned chromosomes from panel B, with either phenotype A (formation of a metaphase plate but with some chromosomes at poles) or phenotype B (scattered chromosomes and no metaphase plate visible). Data are pooled from three independent experiments.

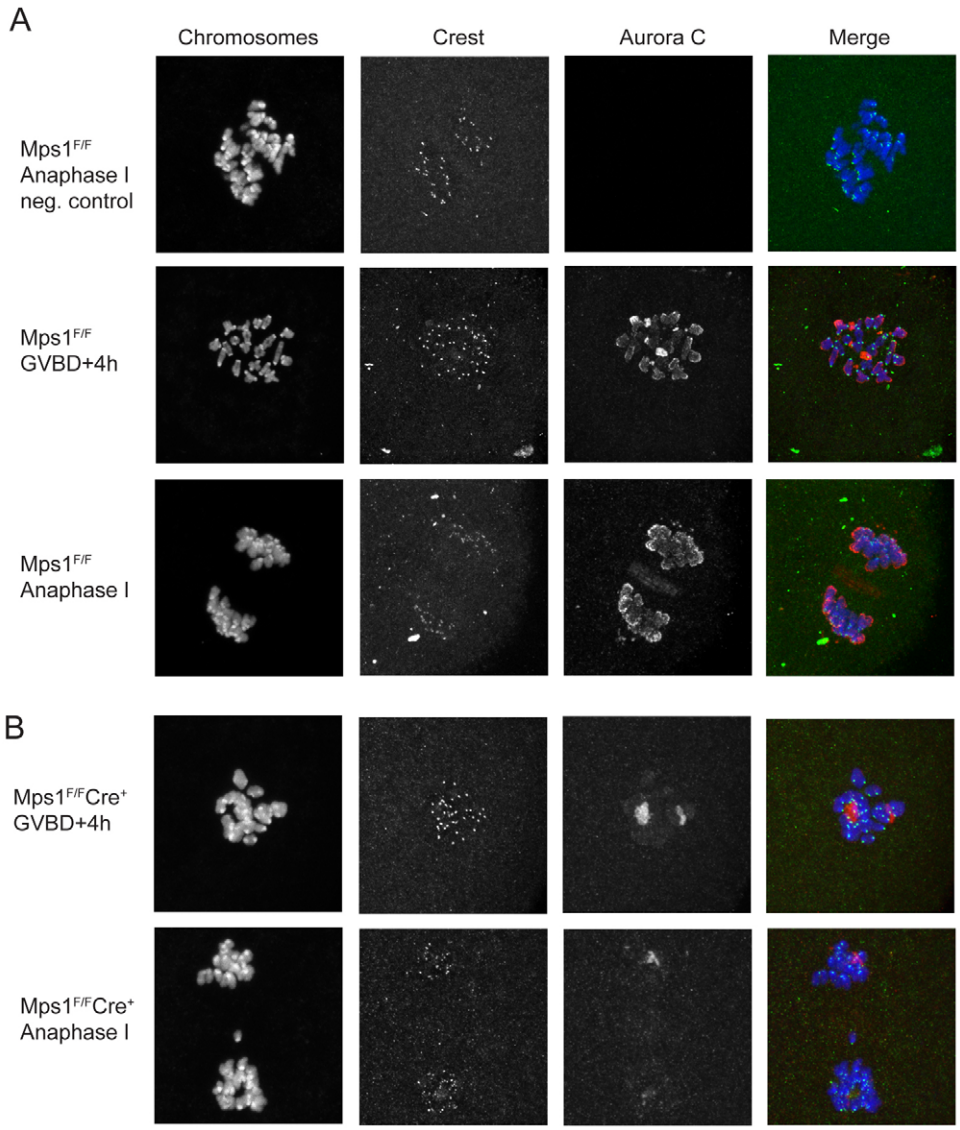

Fig. 8. Aurora C localization to chromosomes depends on kinetochore localization of Mps1 in meiosis I. Confocal microscopy on fixed (**A**) control and (**B**) *Mps1$^{F/F}$ Zp3-Cre^{+}* mouse oocytes at the indicated stages of meiosis I stained with Hoechst (blue), Crest serum (green) and aurora B antibody that recognizes both aurora B and C (red). Shown are the single channels of collapsed *z*-sections (nine) and the merged image. Note that the patchy aurora C staining at GVBD + 4 hours, which is not associated with chromosomes, remains the same in the control and *Mps1$^{F/F}$ Zp3-Cre^{+}* oocytes.

oocytes with the correct ploidy remain, leading to a dramatic reduction in fertility rates of female mice harboring Mps1-deficient oocytes. We asked whether the observed phenotypes are due to the shortened prometaphase I and impaired SAC control, or whether Mps1 has additional roles. In mitosis, Mps1 plays a role in chromosome alignment and error correction via phosphorylation of borealin and/or downstream of aurora B, or via recruitment of CENP-E (Lan and Cleveland, 2010). We propose that even though attachments are monopolar, Mps1 has at least an indirect role in meiosis I to convert synthelic attachments into amphitelic attachments. In the absence of Mps1, chromosomes attached to only one pole cannot be detached and therefore remain at the poles, even when prometaphase I is artificially prolonged. Indeed, we were able to show that correct localization of aurora C kinase depends on kinetochore-localized Mps1, suggesting that *Mps1$^{F/F}$ Zp3-Cre^{+}* oocytes are defective in correcting erroneous attachments via aurora C. Future work will elucidate how Mps1 promotes chromosome alignment in meiosis I in mammalian oocytes, and whether this is different from its role in mitosis.

Loss of Bub1 in mouse meiosis I leads to the precocious loss of sister chromatid cohesion in nearly a third of all chromosomes, indicating that Bub1 is at least partially required for maintaining centromeric cohesion in meiosis I (McGuinness et al., 2009). We did not observe premature sister chromatid separation in *Mps1$^{F/F}$ Zp3-Cre^{+}* oocytes (Fig. 2C), indicating that the role of Bub1 in protecting centromeric cohesion does not require Mps1 at kinetochores.

We have shown previously that Mad2 is localized to unattached kinetochores early in meiosis I, and persists until around 4 hours after GVBD (McGuinness et al., 2009; Wassmann et al., 2003), at which time most of the Mad2 disappears. It has also been shown that stable kinetochore-microtubule fibers are established only in metaphase I, ~7 hours after GVBD (Brunet et al., 1999), which is difficult to reconcile with the fact that most of the Mad2 has already gone at 4 hours after GVBD. Chromosome arms interact laterally with microtubules in early meiosis I, which would not inactivate a kinetochore-mediated SAC. Here we demonstrate that, at the time when most of the Mad2 is lost from kinetochores, end-on kinetochore-microtubule interactions are present. It might be that kinetochore-microtubule interactions are as yet not very stable, as not all kinetochores are correctly mono-oriented, and this might explain why they had not been detected in a previous study by electron microscopy (Brunet et al., 1999). GFP-Mps1 (data not shown) and endogenous Mad2 (Wassmann et al., 2003) remain

detectable, albeit at low levels, on some presumably misoriented kinetochores in wild-type oocytes until metaphase I. Therefore, the SAC is required to prevent meiosis I anaphase onset before kinetochores are correctly attached. The high error rate of the first meiotic division in oocytes that lack functional Mps1 can be explained by the fact that attachments are still unstable and error prone at 4 hours after GVBD, with some chromosomes at the poles, which is when Mps1-deficient oocytes start to extrude PBs.

In conclusion, our study demonstrates the essential roles that Mps1 plays during the first meiotic division in mammalian oocytes. Mps1 is required for SAC control, for correct timing of prometaphase I and for chromosome alignment. The SAC becomes important in prolonging prometaphase I from the time when the meiotic spindle bipolarizes and when kinetochore-microtubule interactions are first established. The SAC ensures that the metaphase-to-anaphase transition of meiosis I does not occur before strong and stable kinetochore fibers are formed. In the absence of SAC control, precocious APC/C activation leads to the missegregation events observed in oocytes without correctly localized Mps1 (this study) or without Bub1 (McGuinness et al., 2009). The timer function of Mps1 requires at least a transient localization to kinetochores in meiosis. In addition, we show here that Mps1 has an additional role in chromosome alignment in meiosis I, through aurora C localization.

We are still far from a complete understanding of the molecular mechanisms at work in mammalian oocytes. Future studies will elucidate exactly how the SAC can distinguish between monopolar attachments in meiosis I and bipolar attachments in mitosis, and how SAC control and error correction are connected in meiosis I. Such studies will help us to gain a better understanding of how the SAC works in meiosis I and why meiosis I is so error prone in mammalian oocytes.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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