Mps1 promotes rapid centromere accumulation of Aurora B

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Aurora B localization to mitotic centromeres, which is required for proper chromosome alignment during mitosis, relies on Haspin-dependent histone H3 phosphorylation and on Bub1-dependent histone H2A phosphorylation—which interacts with Borealin through a Shugoshin (Sgo) intermediate. We demonstrate that Mps1 stimulates the latter recruitment axis. Mps1 activity enhances H2A-T120ph and is critical for Sgo1 recruitment to centromeres, thereby promoting Aurora B centromere recruitment in early mitosis. Importantly, chromosome biorientation defects caused by Mps1 inhibition are improved by restoring Aurora B centromere recruitment. As Mps1 kinetochore localization reciprocally depends on Aurora B, we propose that this Aurora B-Mps1 recruitment circuitry cooperates with the Aurora B-Haspin feedback loop to ensure rapid centromere accumulation of Aurora B at the onset of mitosis.

Keywords: Mps1; Aurora B; biorientation; centromere

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INTRODUCTION

When cells divide, duplicated chromosomes face the challenge of biorienting on the mitotic spindle to ensure an exact copy of the genome is passed on to the two newly formed daughter cells. Biorientation is assured through destabilization of erroneous kinetochore–microtubule attachments and activation of the mitotic checkpoint, which allows time for error correction [1].

The catalytic subunit of the chromosomal passenger complex (CPC), Aurora B kinase, is a key factor for microtubule attachment error correction [2–5], and impacts on the mitotic checkpoint through destabilization of incorrectly attached microtubules, thereby creating unattached kinetochores that promote formation of the mitotic checkpoint complex required for APC/C inhibition [1]. Moreover, Aurora B can also directly stimulate the mitotic checkpoint at the onset of mitosis via kinetochore recruitment and subsequent activation of the checkpoint kinase Mps1 [6,7]. Finally, Aurora B may also function further downstream in the mitotic checkpoint signalling cascade to maintain the APC/C inhibitory signal [8].

Similar to Aurora B, Mps1 is also a dual function kinase. It is essential for the mitotic checkpoint and for error correction [9–12]. The error correction function of Mps1 has been attributed to the phosphorylation of the CPC subunit Borealin that is required for Aurora B activation [10]. However, Aurora B-independent mechanisms have also been proposed [11,13–15]. Therefore it is unclear exactly how Mps1 promotes error correction.

Localization of Aurora B to mitotic centromeres is important for efficient error correction. It requires Haspin-dependent phosphorylation of histone H3 at Thr3 (H3-T3ph) and Bub1-dependent phosphorylation of histone H2A at Thr120 (H2A-T120ph). H3-T3ph binds the Survivin subunit of the CPC and H2A-T120ph interacts with Borealin through a Shugoshin (Sgo) intermediate [16–18]. The signals upstream of these two converging centromere recruitment pathways are now beginning to emerge. Phosphorylation of Borealin by Cdk1 stimulates the interaction between the CPC and Sgo [19], and Aurora B promotes its own centromere recruitment by reinforcing Haspin activity towards H3-T3ph [20]. We show here that Mps1 and Aurora B cooperate to stimulate the Bub1/H2A-T120ph/Sgo1 recruitment axis, which allows rapid centromere accumulation of the CPC at the onset of mitosis.

RESULTS AND DISCUSSION

Mps1 is needed to establish Aurora B at centromeres

Mps1 is required for Bub1 kinetochore recruitment [14,21]. As Bub1-dependent H2A-T120ph is involved in CPC centromere

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Fig 1 | Mps1 is required to establish Aurora B centromere localization at the onset of mitosis. All quantifications are signal intensities over CREST (centromere staining). (A) IF and quantifications of Aurora B, Aurora B-T232ph and centromeres (CREST) in HeLa cells. Cells were treated with the indicated siRNAs (si-Luc = si-Luciferase) for 48 h followed by 30 min treatment with nocodazole and Reversine (1 μ M) or DMSO. Early mitotic cells were analysed. Graph represents mean ($\pm$ s.d.) of 40 cells (Aurora B) or 20 cells (Aurora B-T232ph) per condition, sampled in four or two independent experiments, respectively. (B) IF and quantification of Aurora B in U2OS cells treated with nocodazole plus DMSO, Reversine (500 nM) or Mps1-IN-1 (10 μ M) for 30 min. Early mitotic cells were analysed. Graph represents mean ($\pm$ s.d.) of 20 cells per condition, sampled in two independent experiments. (C) IF and quantification of Aurora B in HeLa cells treated with nocodazole and MG132 for 1 h, and DMSO or Reversine (1 μ M) for additional 30 min. Graph represents mean ($\pm$ s.d.) of 29 cells per condition, sampled in three independent experiments. Scale bars, 5 μ m. For characterization of inhibitor concentrations and siRNAs see supplementary Fig S1 online. DMSO, dimethyl sulphoxide; IF, immunofluorescence; siRNAs, short interfering RNAs.

recruitment [18], we determined whether Mps1 was needed for Aurora B centromere localization. We allowed cells to enter mitosis without Mps1 activity, using the chemical inhibitors Reversine [15] and Mps1-IN-1 [22], or RNA interference (RNAi)-mediated protein knockdown (Fig 1A,B; supplementary Fig S1A online). Cells were treated with nocodazole to exclude effects of kinetochore-microtubule attachments and, as Mps1 inhibition silences the mitotic checkpoint (supplementary Fig S1C online), we only analysed early prometaphase cells recognized by a dispersed DNA morphology [7].

In the absence of Mps1, Aurora B localized to the chromosomal arms, instead of accumulating at the inner centromere (Fig 1A; supplementary Fig S3C online). The fact that Reversine or Mps1-IN-1 phenocopied Mps1 RNAi indicates that Mps1 activity is required for Aurora B centromere localization (Fig 1A,B). The reduction of centromeric Aurora B corresponded to a decrease in Aurora B-T232 autophosphorylation (Fig 1A). Interestingly, Mps1

activity was mainly required for the establishment of Aurora B localization, because if Mps1 was inhibited during mitosis, when Aurora B had already been accumulated at centromeres, then Aurora B localization was barely affected (Fig 1C; supplementary Video S1 online).

Mps1 allows rapid Aurora B recruitment to centromeres

As Cdk1 activity is important for proper centromere localization of the CPC [19], we wanted to test if the defects in Aurora B recruitment upon Mps1 inhibition were related to reduced Cyclin B levels owing to checkpoint inactivation. First, we depleted the checkpoint protein BubR1 and found it had no effect on Aurora B localization (Fig 1A; supplementary Fig S1B,C online). Second, we used an assay that allowed Aurora B localization to be measured in the absence of Cyclin B degradation. We adapted the protocol of Potapova *et al* [23] and briefly inhibited Cdk1 activity with RO3306 (RO) in cells arrested in mitosis with the Eg5 inhibitor

Fig 2 | Mps1 inhibition induces a delay in Aurora B centromere accumulation. (A) Schematic depiction of Cdk1 reactivation assay. (B) Time lapse analysis of Cdk1 reactivation assay in U2OS (U2OS stably expressing a Tet repressor) cells expressing wt-INCENP-GFP. Cells were treated as depicted in (A), RO3306 was added after 4 min and washed out after 16 min. Per condition, one representative cell out of eight is shown. (C) IF and quantifications of Aurora B and Aurora B-T232ph in HeLa cells. Cells were treated as depicted in (A), and fixed at indicated time points. Graph shows one representative experiment out of two and represents mean ($\pm$ s.e.m.) of 10 cells per time point. (D) Time lapse analysis of FRET in U2OS cells stably expressing a FRET biosensor for Aurora B activity, and treated as depicted in (A). Increased ratio indicates increased FRET sensor phosphorylation. Lines represent means of at least 10 cells ($\pm$ s.e.m.). Colour-coded images are shown for indicated time points. Scale bars, 5 μ m. CREST, centromere staining; DMSO, dimethyl sulphoxide; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; IF, immunofluorescence; STLC, s-trityl-L-cysteine.

Fig 3 | Mps1 regulates the Bub1/H2A-T120ph/Sgo1 pathway for Aurora B centromere localization. All quantifications are signal intensities over CREST (centromere staining) and early mitotic cells were analysed. (A,B,C) IF and quantifications of Bub1, H2A-T120ph, Sgo1 and centromeres (CREST) in UTR (U2OS cells stably expressing a Tet repressor) cells, and UTR cells expressing CB-INCENP or Mis12-Mps1. Expression was induced with doxycyclin. Cells were synchronized in G2 with RO3306, released in a medium containing DMSO, Reversine or Hesperadin, and further treated with nocodazole plus MG132 for 30 min. Data represent means ($\pm$ s.d.) from three independent experiments, each experiment including at least 10 cells per condition. (D) IF of Bub1, Sgo1 and H2A-T120ph in Mis12-Mps1-expressing UTR cells synchronized in G1/S with thymidine and analysed 6 h after thymidine release (enrichment for G2 cells) in the presence or absence of doxycyclin. (E) IF and quantifications of Aurora B in HeLa cells treated with the indicated siRNAs for 48 h followed by half an hour treatment with nocodazole and Reversine. Graph shows one representative experiment out of two and represents mean ($\pm$ s.e.m.) of 11 cells per condition. Scale bars, 5 μ m. DMSO, dimethyl sulphoxide; IF, immunofluorescence; siRNAs, short interfering RNAs; UTR, U2OS cells stably expressing a Tet repressor.

s-trityl-L-cysteine (STLC; in the presence of MG132 to prevent Cyclin B degradation) and then allowed return to a mitotic state upon reactivation of Cdk1 (Fig 2A). As expected, Cdk1 inhibition resulted in a rapid removal of ectopically expressed green fluorescent protein (GFP)-tagged CPC subunit inner centromere protein (INCEP) from centromeres (Fig 2B; supplementary Video S2 online, [23]). This effect of Cdk1 inhibition was reversible because $\sim$ 20 min after RO washout, the centromere localization of INCENP-GFP was fully recovered (Fig 2B; supplementary Video S2 online). When cells were released from Cdk1 inhibition in the presence of Reversine the accumulation of INCENP-GFP at centromeres was significantly delayed (Fig 2B; supplementary Video S2 online). We confirmed these results for endogenous Aurora B in fixed cells and observed a significant delay in recovery of Aurora B and Aurora B-T232ph when Cdk1 was reactivated in the absence of Mps1 activity (Fig 2C). Note that Aurora B did relocalize to centromeres, even in the absence of Mps1 activity, suggesting that Mps1 inhibition merely delays Aurora B recruitment.

To examine the consequences of delayed Aurora B accumulation on substrate phosphorylation, we used a chromatin (H2B)-targeted fluorescence resonance energy transfer (FRET)-based biosensor for Aurora B activity that is sensitive to Aurora B activity coming from the centromere [24,25]. Treatment of mitotic cells with RO/MG132 resulted in decreased sensor phosphorylation, and recovery of Aurora B substrate phosphorylation was delayed when Cdk1 was reactivated in the presence of Reversine/MG132, both in U2OS and HeLa cells (Fig 2D; supplementary Fig S2 online). We noticed that Aurora B-T232ph and sensor phosphorylation were not fully restored in the absence of active Mps1, which could be owing to the requirement of Mps1-dependent Borealin phosphorylation [10]. In conclusion, we demonstrate in different cell lines, both fixed and live, that early in mitosis Mps1 promotes Aurora B centromere recruitment and substrate phosphorylation. The relatively small time window in which Mps1 operates likely explains why the effect of Mps1 on Aurora B centromere localization was not previously detected [10,15].

Mps1 controls the Bub1/H2A/Sgo CPC recruitment axis

Given the importance of the Bub1/H2A-T120ph/Sgo pathway for CPC centromere localization [18], we rationalized that the defects in this pathway could explain the delay in Aurora B centromere accumulation following Mps1 inhibition. While Mps1 activity was dispensable for Haspin-dependent H3-T3ph (supplementary Fig S3A,D online), it was indeed required for Bub1 kinetochore localization, and H2A-T120ph (Fig 3A,B; supplementary Fig S3A online) in mitotic cells [14]. We next determined if the Sgo

intermediate for CPC recruitment was also affected by Mps1 inhibition. While Sgo2 levels were only slightly reduced, Sgo1 centromere localization was highly sensitive to Mps1 inhibition, with Sgo1 localizing over the chromosomal arms instead of to the centromere, much like we observed for Aurora B (Fig 3C; supplementary Fig S3A–C online) [14].

We and others have shown recently that Mps1 localization to kinetochores is Aurora B-dependent [6,7,26]. We therefore tested if Aurora B stimulated its own recruitment to centromeres via Mps1. Aurora B inhibition with Hesperadin caused a reduction in Bub1, H2A-T120ph, Sgo1 and H3-T3ph at kinetochores and centromeres, respectively (Fig 3A–C; supplementary Fig S3D online). To determine whether this was a consequence of reduced Mps1 activity, we restored Mps1 localization to kinetochores using Mis12-Mps1 [7]. Although, as expected, this did not restore H3-T3ph (supplementary Fig S3D online), Bub1 kinetochore localization and H2A-T120ph were improved, and Sgo1 recruitment to centromeres was completely restored (Fig 3A–C). The incomplete rescue of Bub1 kinetochore localization and H2A-T120ph could indicate that Aurora B also affects H2A-T120ph via an additional, Mps1-independent pathway. Indeed, we observed an increase of Bub1 kinetochore localization and H2A-T120ph in the absence of Mps1 activity when Aurora B was restored at centromeres by expressing CB-INCENP ([27]; Fig 3A,B). The complete rescue of Sgo1 recruitment by Mis12-Mps1 in cells with inactive Aurora B suggests that Sgo1 is under control of Mps1 but only partially via Bub1-dependent H2A-T120ph (Fig 3A–C). Indeed, when Bub1 kinetochore localization was restored in cells without active Mps1, using Mis12-Bub1, this did not recover Sgo1 localization (supplementary Fig S4A online). Moreover, expression of Mis12-Mps1 was sufficient to promote Sgo1 centromere localization in G2 cells even though H2A-T120 phosphorylation was absent (Fig 3D). This implies that Mps1 may also signal directly to Sgo1 as has been suggested recently by others [28]. Importantly, RNAi-mediated Sgo1 depletion resulted in a reduction of centromeric Aurora B to a comparable level as was observed after inhibition of Mps1 (Fig 3E), indicating that the reduced Sgo1 levels may indeed be responsible for the impaired Aurora B centromere recruitment after Mps1 inhibition.

Mps1 recruits Aurora B to stimulate error correction

We next tested the significance of the Mps1-Aurora B recruitment circuitry for error correction, a process that relies on Aurora B and Mps1 [2,3,5,10,11]. Chromosome alignment was examined upon inhibition of Mps1 in stable cell lines with inducible expression of CB-INCENP, which renders Aurora B localization insensitive to Mps1 regulation (Fig 4A). Cells that had entered mitosis with

Fig 4 | Mps1-dependent Aurora B centromere recruitment promotes efficient chromosome biorientation. (A) IF of Aurora B and centromeres (CREST) in UTR cells with inducible expression of wt-INCENP-GFP or CB-INCENP-GFP. Cells were treated as in Fig 3. (B) Cells were synchronized in G2 with RO3306 and released to enter mitosis in the presence or absence of Reversine for 60 min. MG132 was added for an additional 30 min to accumulate cells in metaphase. Low expression levels of CB-INCENP at the onset of mitosis were accomplished by a relative short protein induction time (6 h). Graph shows one representative experiment out of two. The number (*n*) of cells analysed per condition is indicated. Scale bars, 5 μ m. (C) Model for the recruitment circuitries that operate between Mps1-Sgo1-Aurora B (black arrows) and Mps1-Bub1-Sgo1-Aurora B (grey arrows) at the beginning of mitosis to ensure robust establishment of the mitotic checkpoint and error correction machineries. DAPI, 4,6-diamidino-2-phenylindole; DMSO, dimethyl sulphoxide; GFP, green fluorescent protein; IF, immunofluorescence; UTR, U2OS cells stably expressing a Tet repressor.

active or inactive Mps1 were scored for chromosome alignment after 30 min treatment with MG132 to allow sufficient time for alignment in control cells (Fig 4B; supplementary Fig S4B online). As expected, inhibition of Mps1 reduced the ability of cells to align their chromosomes. Whereas expression of wt-INCENP did not improve alignment under these conditions, we observed a striking improvement when Aurora B localization to centromeres was restored by CB-INCENP expression (Fig 4A,B; supplementary Fig S4B online). Only mild chromosome misalignments persisted, which might reflect the Aurora B-independent role of Mps1 in chromosome biorientation [11,13–15,28].

CONCLUSION

Our data show that an Aurora B-Mps1 regulatory circuitry promotes timely and robust Aurora B centromere activity at the

onset of mitosis via centromere recruitment of Sgo1, and we suggest that this recruitment circuitry acts together with the Aurora B-Haspin feedback loop [20]. We propose that the mutual dependence of Aurora B and Mps1 for the rapid establishment of Aurora B centromere activity and Mps1 kinetochore activity [7] allows for a fast and coordinated start-up of the error correction and mitotic checkpoint machineries so that both processes are fully functional at the point of nuclear envelope breakdown (Fig 4C).

METHODS

Cell lines and cell culture. Inducible cell lines expressing LAP-Mis12-Mps1 Δ 200, wt-INCENP-GFP and CB-INCENP-GFP were previously described [7]. All inducible cell lines were derived from U2OS cells stably expressing a Tet repressor (UTR) and cultured in Tet-free medium. Protein expression was induced with 1 μ g/ml doxycycline (Sigma-Aldrich) for at least 6 h. HeLa and

U2OS cells were transfected with a pIRESpuro2b vector containing H2B-targeted FRET sensor for Aurora B activity [24] and selected with 1 µg/ml puromycin (Sigma-Aldrich) for stable expression. HeLa and U2OS cells were grown in DMEM supplemented with 6% fetal bovine serum, pen/strep and l-glutamine (2 mM).

Cdk1 reactivation assay. Cells were incubated for 1.5 h with STLC (20 µM, Tocris Bioscience) to arrest cells in mitosis with monopolar spindles. MG132 was added 30 min before addition of RO3306 (10 µM, Enzo Life Sciences). After 10 min, the drug was removed by washing the cells with STLC, MG132 (10 µM, Sigma-Aldrich) and either dimethyl sulphoxide (DMSO), Hesperadin (125 nM, Selleck Chemicals), Mps1-IN-1 (10 µM, gift of Dr N. Grey [22]) or Reversine (Sigma-Aldrich, 500 nM for U2OS cells or 1 µM for HeLa and UTR cells, supplementary Fig S1A online)-containing medium [23]. Cells were either fixed at different time points or recovery was imaged live. Details of live and fixed cell imaging procedures (including FRET sensor experiments), indirect immunofluorescence, antibodies, expression vectors and short interfering RNAs (siRNAs) are available as Supplementary information online.

Chromosome alignment assay. Protein induction was started 3 h before cells were arrested in late G2 by treatment with RO3306 (7.5 µM) for 2.5 h. The relative short protein induction time is important to ensure that the levels of CB-INCENP are still low when cells enter mitosis. Synchronized cells were released to enter mitosis in the presence of Reversine or DMSO. When cells entered mitosis (about 1 h after release) they were treated for 30 min with MG132 before fixation.

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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