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Report

Aurora B Defines Its Own Chromosomal Targeting by Opposing the Recruitment of the Phosphatase Scaffold Repo-Man

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Summary

Aurora B is the catalytic subunit of the chromosomal passenger complex (CPC), which coordinates mitotic processes through phosphorylation of key regulatory proteins [1]. In prometaphase, the CPC is enriched at the centromeres to regulate the spindle checkpoint and kinetochore-microtubule interactions. Centromeric CPC binds to histone H3 that is phosphorylated at T3 (H3T3ph) by Aurora B-stimulated Haspin [2–5]. PP1/Repo-Man acts antagonistically to Haspin and dephosphorylates H3T3ph at the chromosome arms but is somehow prevented from causing a net dephosphorylation of centromeric H3T3ph during prometaphase [6, 7]. Here, we show that Aurora B phosphorylates Repo-Man at S893, preventing its recruitment by histones. We also identify PP2A as a mitotic interactor of Repo-Man that dephosphorylates S893 and thereby promotes the targeting of Repo-Man to chromosomes and the dephosphorylation of H3T3ph by PP1. Thus, Repo-Man-associated PP1 and PP2A collaborate to oppose the chromosomal targeting of Aurora B. We propose that the reciprocal feedback regulation of Haspin and Repo-Man by Aurora B generates a robust bistable response that culminates in the centromeric targeting of the CPC during prometaphase.

Results and Discussion

Aurora B Opposes the Targeting of Repo-Man to Chromosomes

The chromosomal passenger complex (CPC) promotes its own accumulation at centromeres during prometaphase [1, 4]. This can be partially explained by the Aurora B-catalyzed phosphorylation of Haspin, which stimulates its ability to phosphorylate H3T3, thereby creating a docking site for the CPC component survivin. However, the phosphatase PP1/Repo-Man is an equally important regulator of mitotic H3T3 phosphorylation and centromeric CPC targeting [6, 7]. Indeed, the loss of PP1/Repo-Man results in the spreading of H3T3ph and Aurora B to the chromosome arms during prometaphase, while an excess of this PP1 holoenzyme causes the precocious loss of centromeric H3T3ph and Aurora B. These data demonstrate that the activity of PP1/Repo-Man must be tightly regulated during mitosis. This regulation includes not only the Cdk1-inhibited association of PP1 with Repo-Man but also a poorly understood chromosomal targeting mechanism of Repo-Man [7–9]. We speculated that Aurora B stimulates its own chromosomal targeting, not only by promoting Haspin-mediated H3T3 phosphorylation but also by opposing H3T3ph dephosphorylation through inhibition of the chromosomal targeting of Repo-Man and associated PP1.

To study the subcellular distribution of endogenous Repo-Man in U2OS osteosarcoma cells, we performed immunofluorescence microscopy with an antibody against the C-terminal residues 761–854 (see Figure S1A available online). Little or no signal was detected after the small interfering RNA (siRNA)-mediated knockdown of Repo-Man, attesting to the specificity of the antibody. Consistent with previous observations [6–9], Repo-Man showed a diffuse staining during prometaphase and metaphase but was largely chromosome associated during anaphase and telophase. Repo-Man sometimes seemed to be excluded from prometaphase chromosomes (Figure S1B), but this is most likely due to the fixation procedure, as Repo-Man was always enriched at chromosomes in detergent-pretreated [6] or live prometaphase cells (Figure S1B). To examine the role of Aurora B in the targeting of Repo-Man to prometaphase chromosomes, we first made use of the Aurora B inhibitors ZM447439 (Figure 1A) and hesperadin (Figure S1C). The inhibition of Aurora B in nocodazole-arrested cells for 30 min resulted in a nearly exclusive chromosomal targeting of endogenous Repo-Man. This retargeting effect was not detected when the cells were preincubated with calyculin A (Figure 1A), a potent inhibitor of PP1, PP2A, and PP2A-like phosphatases, indicating that the chromosomal targeting of Repo-Man not only requires the inhibition of Aurora B but also depends on a dephosphorylation event. The same observations were made for ectopically expressed EGFP-tagged Repo-Man (Figure S1D). The ZM447439-induced chromosomal targeting of Repo-Man was also observed when nocodazole-arrested cells were kept in prometaphase with the proteasome inhibitor AM114 (Figure S1E), indicating that this effect was not due to a precocious mitotic exit resulting from an override of the spindle-assembly checkpoint. ZM447439 treatment resulted in a loss of Aurora B from centromeres (Figure S1F), in accordance with previous findings [4, 10, 11], and this correlated with chromosomal hypertargeting of Repo-Man during prometaphase. As an independent approach to examine the role of chromosome-associated Aurora B in the recruitment of Repo-Man, we performed a knockdown of the mitotic kinesin Mklp2, which is required for the translocation of the CPC to the spindle midzone in anaphase [12]. Aurora B remained associated with the chromosomes during anaphase after the knockdown of Mklp2, and this prevented the association of endogenous (Figure 1B) and ectopically expressed EGFP-Repo-Man (Figure S1G) with the chromosomes. However, the mere addition of ZM447439 was sufficient to rescue this phenotype (Figure 1B), confirming that Aurora B-mediated phosphorylation prevents the recruitment of Repo-Man.

In further agreement with an inhibitory role of Aurora B in the chromosomal targeting of Repo-Man, we found that Repo-Man was barely associated with bleomycin-induced lagging telophase chromosome arms that were located in close proximity to Aurora B at the spindle midzone (Figure 1C). This is in accordance with a report showing that the midzone activation of Aurora B creates an intracellular phosphorylation gradient, with the highest kinase activity at and close to the midzone [13].

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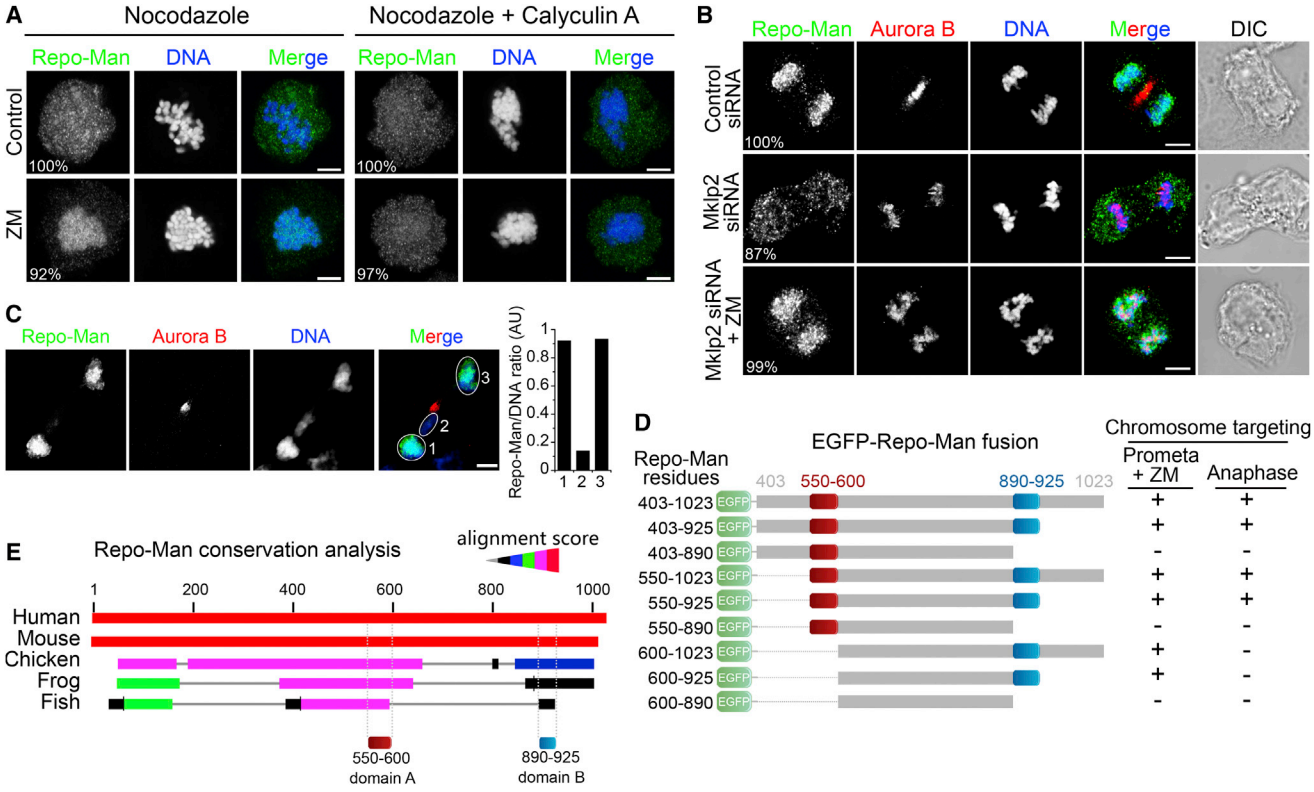

Figure 1. Aurora B Inhibits the Chromosomal Targeting of Repo-Man

(A) Confocal images of fixed nocodazole-arrested U2OS cells after incubation for 30 min with vehicle or 2 μM ZM447439 (ZM), as such or after preincubation of the cells for 30 min with 25 nM calyculin A. Scale bars represent 5 μm. The numbers refer to the percentage of cells with the illustrated phenotype in at least 98 cells in each condition.

(B) U2OS cells were transfected with control or Mklp2 siRNAs. In one condition, the Mklp2 knockdown was combined with an incubation for 30 min with 2 μM ZM447439. Repo-Man, Aurora B, and DNA were visualized in anaphase cells, which were obtained by a 90 min release from a nocodazole arrest. DIC, differential interference contrast. Scale bars represent 5 μm. The numbers refer to the percentage of cells with the illustrated phenotype in at least 86 cells in each condition.

(C) Nocodazole-arrested U2OS cells expressing EGFP-Repo-Man were treated for 60 min with 10 μM bleomycin. Subsequently, the cells were incubated without nocodazole and bleomycin for 2 hr before fixation. Confocal images were taken, and the EGFP/DNA ratio of the indicated chromosome regions was plotted. Scale bars represent 5 μm.

(D) The indicated EGFP-tagged Repo-Man fragments were expressed in U2OS cells and scored for their ability to associate with anaphase chromosomes, which were obtained by a 60 min release from a nocodazole block. In addition, the fusions were examined for their association with prometaphase chromosomes after a 30 min preincubation with 2 μM ZM447439 (ZM).

(E) Protein conservation analysis of Repo-Man with the National Center for Biotechnology Information's Protein Basic Local Alignment Search Tool. The alignment scores are color coded. The positions of domains A and B are indicated below.

To get initial insights into the mechanism by which Aurora B opposes the chromosomal targeting of Repo-Man, we have used deletion mutagenesis to map the fragments of Repo-Man that are required for its chromosomal targeting and that mediate the sensitivity to ZM447439 (Figures 1D and S1H). We started from an EGFP fusion of Repo-Man-(403–1043). This Repo-Man fragment lacks the N-terminal binding sites for importin β and PP1, but it is still properly targeted to anaphase chromosomes [7]. N- and C-terminal deletions identified two Repo-Man domains, comprising residues 550–600 (domain A) and 890–925 (domain B), that were essential for its chromosomal targeting during anaphase. Both domains are phylogenetically conserved in vertebrates (Figure 1E). Intriguingly, domain A was not required for the ZM447439-induced chromosomal targeting of Repo-Man during prometaphase (Figure 1D), indicating that domain B harbors both the chromosome-binding site and the inhibitory Aurora B site.

Phosphorylation by Aurora B Abolishes the Binding of Repo-Man to Histones

The ZM447439-sensitive domain B of Repo-Man only contains S893 as a consensus Aurora B phosphorylation site [14] that is conserved in vertebrates (Figure 2A). Bacterially expressed and purified His-tagged Repo-Man-(865–925), which includes domain B, could be radioactively phosphorylated by purified GST-AuroraB-INCENP (Figure 2B). However, virtually no phosphorylation was detected with the S893D mutant, showing that S893 is an in vitro phosphorylation site for Aurora B. To explore whether S893 is also phosphorylated in intact cells, we raised a phosphoepitope-specific antibody. The affinity-purified antibody revealed a clear phosphorylation of EGFP-Repo-Man that was immunoprecipitated from nocodazole-arrested U2OS cells (Figure 2C). However, no phosphorylation of Repo-Man at S893 was detected in nonsynchronized cells. Neither was a phosphorylation detected in nocodazole-arrested cells that had been preincubated with ZM447439 or

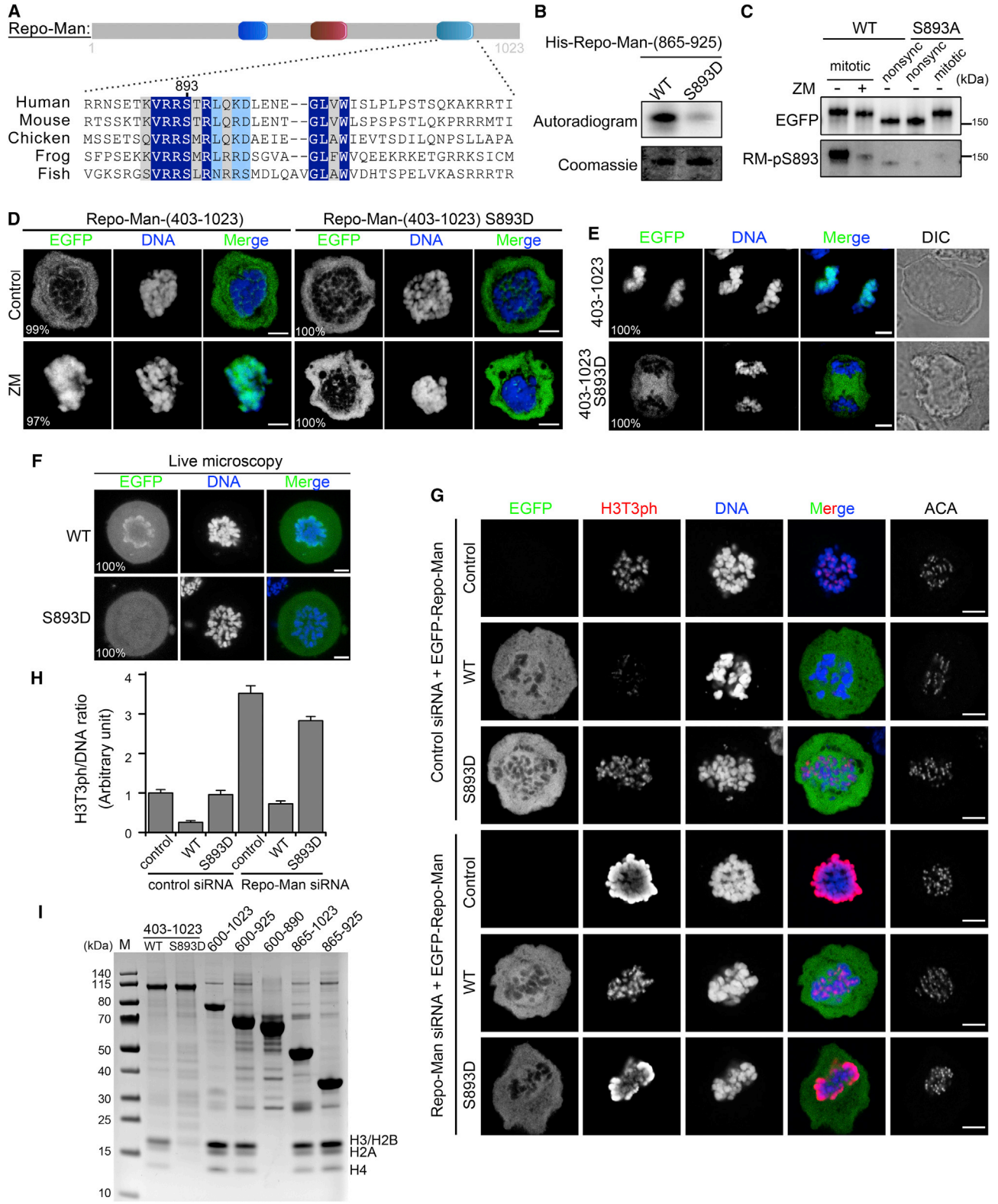

Figure 2. Aurora B-Mediated Phosphorylation of Repo-Man at S893 Disrupts Its Interaction with Histones and Hampers the Dephosphorylation of H3T3ph

(A) Conservation of a fragment of domain B of Repo-Man.

(B) His-Repo-Man-(865–925) wild-type (WT) or the corresponding S893D mutant were phosphorylated with GST-AuroraB-INCENP. The phosphorylation was visualized by autoradiography. The Coomassie staining of the substrates serves as a loading control.

(C) U2OS cells were transfected with full-length EGFP-Repo-Man WT or S893A. EGFP traps were performed from lysates of mitotic shake-off (mitotic) or nonsynchronized (nonsync) cells, pretreated or not with 2 μM ZM447439 (ZM). The figure shows immunoblots with antibodies against EGFP or phospho-S893 of Repo-Man (RM-pS893).

(legend continued on next page)

that expressed the S893A mutant of Repo-Man. Collectively, these data identify S893 of Repo-Man as an Aurora B phosphorylation site in prometaphase cells.

The phosphomimicking S893D mutant of EGFP-tagged Repo-Man-(403–1023) failed to target to prometaphase chromosomes in the presence of ZM447439 (Figure 2D) and was not associated with anaphase chromosomes (Figure 2E). Likewise, the S893D mutant of full-length EGFP-Repo-Man showed no enrichment on prometaphase chromosomes in live cells (Figure 2F). These data represent good evidence that the Aurora B-mediated phosphorylation of S893 prevents the chromosome targeting of Repo-Man. Since the S893D mutant of Repo-Man cannot be targeted to chromosomes, this enabled us to explore the relevance of the chromosome targeting of Repo-Man for the dephosphorylation of H3T3ph by associated PP1. Consistent with our previous data [6], the ectopic expression of EGFP-Repo-Man resulted in the hypophosphorylation of centromeric H3T3 in nocodazole-arrested cells (Figures 2G and 2H). However, this decreased level of H3T3ph was not detected after the expression of the S893D mutant of Repo-Man. Conversely, the knockdown of endogenous Repo-Man caused the hyperphosphorylation and spreading of H3T3 in prometaphase cells. The latter effect could be rescued by the expression of siRNA-resistant EGFP-Repo-Man, but not by the expression of the corresponding S893D mutant. Similar rescue data were obtained for cells in anaphase (Figure S2A). Thus, the dephosphorylation of H3T3ph by PP1/Repo-Man is critically dependent on the chromosome targeting of Repo-Man.

To identify the proteins that mediate the binding of Repo-Man to chromosomes, we performed traps of EGFP fusions of Repo-Man-(403–1023) that were expressed in HEK293T cells. The traps were analyzed by Coomassie staining after SDS-PAGE (Figure 2I). These studies revealed that wild-type Repo-Man, but not the S893D mutant, coprecipitated with nearly stoichiometric amounts of polypeptides that migrated like the core histones. Their identity as core histones was confirmed by immunoblotting (Figure S2C) and is consistent with a previous study that identified histones H2B and H3 in immunoprecipitates of Repo-Man [7]. Deletion mutagenesis revealed that the binding of Repo-Man to histones was dependent on residues 890–925, corresponding to domain B (Figure 2I). We have considered the possibility that the chromosome binding of Repo-Man is also (partially) mediated by DNA. However, Repo-Man does not have a recognizable nucleic acid-binding domain. Also, the prior digestion of the EGFP trap with the nuclease benzonase hydrolyzed the coprecipitated DNA but did not abolish the interaction of EGFP-Repo-Man-(403–1023) with the core histones (Figure S2D). In conclusion, these data suggest that the Aurora B-mediated phosphorylation of Repo-Man at S893 prevents its binding to core histones.

The Chromosome Targeting of Repo-Man Depends on Associated PP2A

To obtain more information on the role of domain A (residues 550–600) in the chromosomal targeting of Repo-Man (Figure 1D), we identified proteins that coprecipitated with EGFP-Repo-Man-(403–1023) from lysates of nocodazole-arrested HEK293T cells. This led to the mass-spectrometric identification of the catalytic subunit of protein phosphatase PP2A (PP2A-C) as a novel mitotic component of the Repo-Man complex (Figure S2B). The presence of PP2A-C was independently confirmed by immunoblotting of EGFP-Repo-Man traps (Figure S2C) and is in accordance with a recent mass-spectrometric analysis of the Repo-Man interactome [8]. PP2A-C also coprecipitated with the S893D mutant of Repo-Man (Figure S2C), indicating that its recruitment is independent of chromosome targeting. To examine whether PP2A is relevant for the chromosome targeting of Repo-Man, we performed knockdown experiments of PP2A-Cα, the major isoform of the catalytic subunit. In cells with a strongly reduced expression of PP2A-Cα, Repo-Man-(550–1023) was no longer associated with anaphase chromosomes (Figure 3A). These results hinted at a stimulatory role of PP2A in the chromosome targeting of Repo-Man and prompted us to explore in more detail the nature and function of Repo-Man-associated PP2A.

PP2A holoenzymes consist of a catalytic C subunit, a scaffolding A subunit, and a variable regulatory B subunit [15]. Immunoblot experiments demonstrated that EGFP-Repo-Man was associated with the C and A subunits of PP2A, as well as with all tested isoforms of the regulatory B56 subunit (Figure 3B). Deletion mutagenesis revealed that residues 550–600, corresponding to domain A, were required for the binding of all three PP2A subunits. Intriguingly, domain A of Repo-Man comprises a region that is conserved in vertebrates (Figure 3C) and is similar to a recently identified direct B56-binding sequence in the kinetochore protein BubR1 [16, 17]. This sequence will be denoted here henceforth as the LSPI-motif, after its most conserved residues (amino acids 590–593 of human Repo-Man). The synthetic peptide Repo-Man-(581–599), comprising the LSPI-motif with its flanking sequences and coupled via BSA to CNBr-activated Sepharose, could be used to bind the PP2A-B56 complex from U2OS cell lysates (Figure 3D), confirming the presence of a PP2A-binding motif. In contrast, PP2A was not retained by BSA-Sepharose. Also, the deletion of the LSPI-motif and its flanking residues (Δ585–599) or the mutation of the LSPI-motif in one (I593A or I593F) or two residues (S591A and I593A, henceforth referred to as the LAPA mutant) abolished the binding of EGFP-Repo-Man-(550–1023) to all three PP2A subunits (Figure 3E). We have subsequently used some of these binding mutants to delineate the contribution of PP2A to the chromosome targeting of Repo-Man. Ectopically expressed EGFP-Repo-Man-(550–1023) with a deleted or mutated LSPI-motif

(D) EGFP-Repo-Man-(403–1023) WT or S893D was expressed in nocodazole-arrested U2OS cells. Confocal images were taken after preincubation with or without 2 μM ZM447439 (ZM) for 30 min. The numbers refer to the percentage of cells with the illustrated phenotype in at least 90 cells in each condition.
(E) Confocal images of fixed U2OS cells in anaphase and expressing EGFP-Repo-Man-(403–1023) WT or S893D. The numbers refer to the percentage of cells with the illustrated phenotype in at least 60 cells in each condition.
(F) Confocal live images in prometaphase-arrested U2OS cells expressing full-length EGFP-Repo-Man WT or S893D. The numbers refer to the percentage of cells with the illustrated phenotype in at least 34 cells in each condition.
(G) EGFP, H3T3ph, and DNA were visualized in fixed prometaphase-arrested U2OS cells in control conditions or after the expression of siRNA-resistant EGFP-Repo-Man WT or S893D. The same images were also obtained after the knockdown of Repo-Man.
(H) Quantification of the H3T3ph/DNA ratios for the conditions illustrated in (G). The results represent means ± SEM for 16–22 cells in each condition.
(I) Cell lysates from HEK293T cells expressing EGFP fusions of the indicated Repo-Man fragments were treated with micrococcal nuclease. Subsequently, the EGFP fusions were trapped and processed for Coomassie staining after SDS-PAGE.

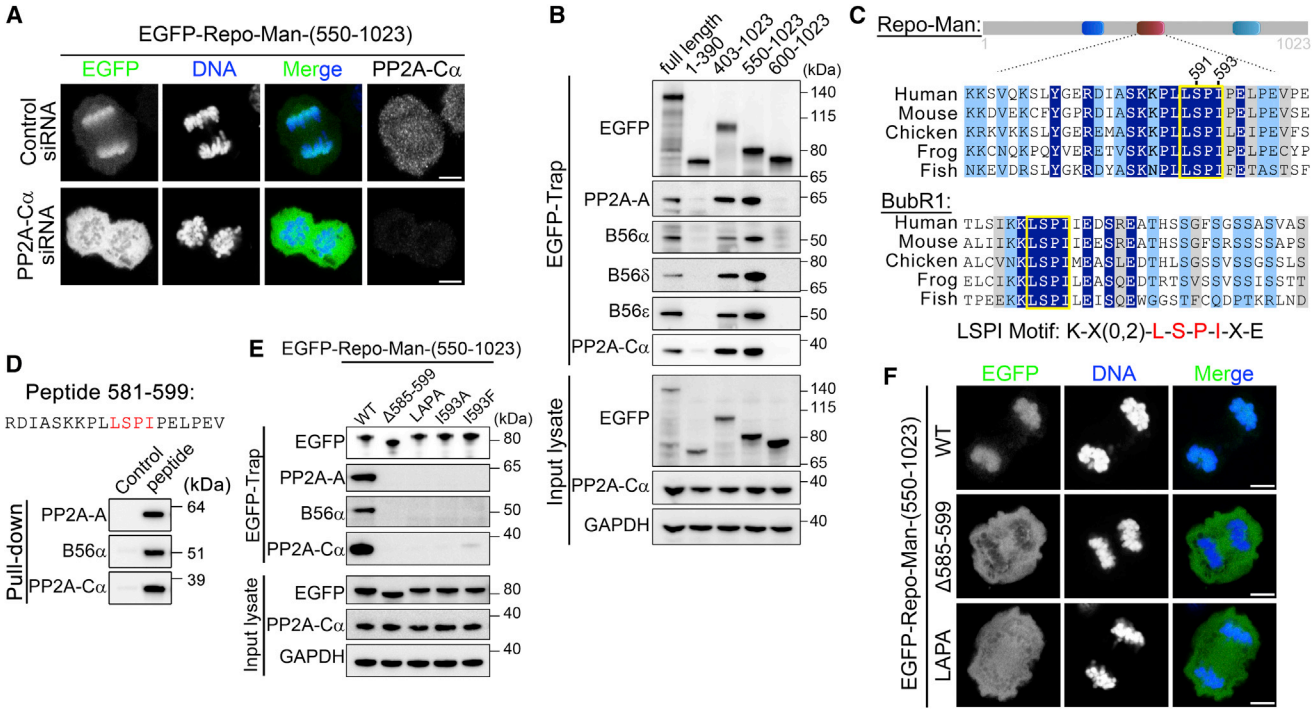

Figure 3. The Chromosome Targeting of Repo-Man Depends on Associated PP2A

(A) Confocal images of fixed U2OS anaphase cells expressing EGFP-Repo-Man-(550–1023) 48 hr after transfection with control or PP2A-Cα siRNAs.

(B) HEK293T cells expressing the indicated EGFP-Repo-Man fusions were nocodazole arrested. The micrococcal-nuclease-treated cell lysates were used for EGFP trapping.

(C) Sequence conservation of a fragment of domain A of Repo-Man. Also shown is the phylogenetic conservation of the PP2A-B56-binding region of BubR1.

(D) The synthetic peptide Repo-Man-(581–599) was coupled to BSA. BSA (control) or peptide-linked BSA (peptide) was subsequently linked to CNBr-activated Sepharose-4B and incubated for 30 min with U2OS cell lysates. The PP2A subunits were visualized by immunoblotting.

(E) HEK293T cells were transfected with EGFP-Repo-Man-(550–1023) WT or the indicated PP2A-binding mutants. The micrococcal-nuclease-treated cell lysates from nocodazole-arrested cells were used for EGFP trapping. The lysates and traps were processed for immunoblotting.

(F) Confocal images of U2OS anaphase cells expressing EGFP-Repo-Man-(550–1023) WT or the indicated PP2A-binding mutants.

was no longer targeted to chromosomes in anaphase (Figure 3F). These data firmly establish the B56-PP2A holoenzyme as an interactor of the LSPI-motif and show that Repo-Man-associated PP2A is essential for its chromosomal targeting.

PP2A and Aurora B Antagonistically Regulate the Phosphorylation of S893

Since the Aurora B-mediated phosphorylation of Repo-Man at S893 hampers its binding to histones, and since PP2A is required for the chromosomal targeting of Repo-Man, this suggested that Repo-Man-associated PP2A acts as a S893 phosphatase. This hypothesis is consistent with our observation that the chromosomal targeting of Repo-Man induced by the inhibition of Aurora B can be rescued with the phosphatase inhibitor calyculin A (Figure 1A). To further test this model, we radioactively phosphorylated bacterially expressed His-tagged Repo-Man-(865–925) at S893 by GST-AuroraB-INCENP (Figure 2B) and subjected it to dephosphorylation by PP2A. As a source of PP2A, we used an immunoprecipitate of EGFP-Repo-Man-(550–1023), which lacks the PP1-binding site (Figure 4A). This immunoprecipitate caused a nearly complete dephosphorylation of S893, which could be blocked with 10 nM okadaic acid, a concentration that selectively inhibits PP2A. However, no dephosphorylation was detected with an immunoprecipitate of a PP2A-binding mutant of this Repo-Man fusion.

To delineate the relative contribution of PP1 and PP2A to the dephosphorylation and chromosome targeting of Repo-Man in prometaphase cells, we made use of PP1-binding (RATA) and PP2A-binding (LAPA) mutants of full-length Repo-Man. Only the LAPA and combined RATA and LAPA mutants were hyperphosphorylated at S893 in nocodazole-arrested cells, as compared to the wild-type protein (Figure 4B), suggesting that this residue is selectively dephosphorylated by PP2A during prometaphase. Consistent with this notion, only the LAPA mutants showed a deficient targeting to prometaphase chromosomes in live cells (Figure 4C). In further agreement with an antagonistic regulation of Repo-Man by Aurora B and PP2A, the LAPA mutants showed a decreased sensitivity to ZM447439-induced chromosome targeting (Figure 4D). This altered sensitivity to ZM447439 was not seen with the RATA mutants. Since the chromosome targeting of Repo-Man is essential for the dephosphorylation of H3T3ph (Figure 2G), this implies that a PP2A-binding mutant of Repo-Man should show a deficient H3T3ph dephosphorylation. Indeed, the ectopic expression of the LAPA mutant did not cause the hypophosphorylation of H3T3 during prometaphase, as detected after expression of wild-type Repo-Man (Figures 4E and 4F). Moreover, the LAPA mutant did not rescue the hyperphosphorylation of H3T3 induced by the knockdown of endogenous Repo-Man. Taken together, our data demonstrate that PP2A acts antagonistically to Aurora B by dephosphorylating

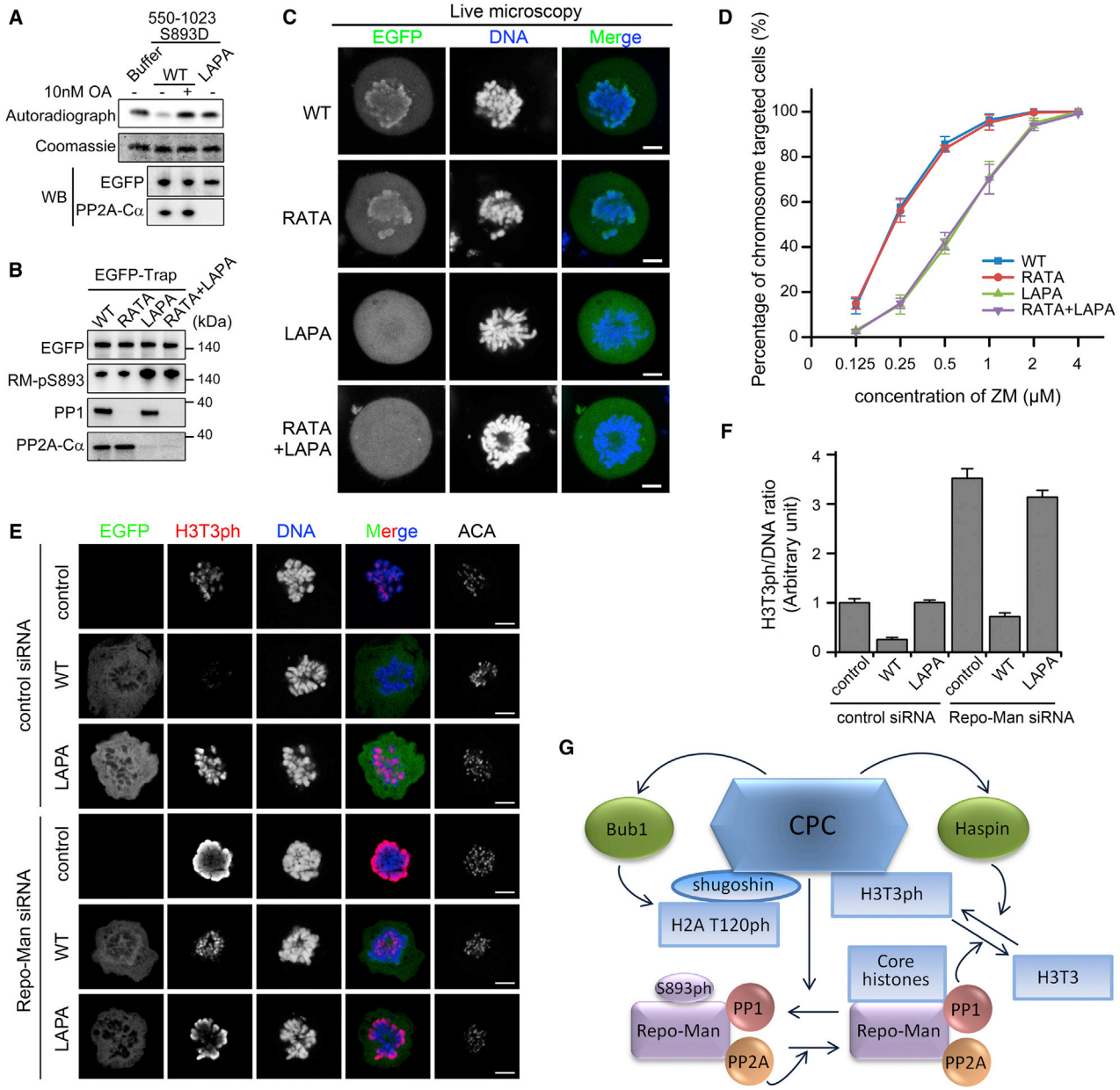

Figure 4. PP2A and Aurora B Antagonistically Regulate the Chromosome Targeting of Repo-Man

(A) U2OS cells were transfected with EGFP-Repo-Man-(550–1023)-S893D or the corresponding LAPA mutant. Micrococcal-nuclease-treated cell lysates prepared from cells collected by mitotic shake-off were used for EGFP traps. The traps were used to dephosphorylate bacterially expressed His-tagged Repo-Man-(865–925) that had been phosphorylated with GST-AuroraB-INCENP. In one condition, okadaic acid (OA) was added. After incubation for 40 min at 30°C, the dephosphorylation reaction was arrested with SDS-sample buffer. The figure shows the Coomassie staining and autoradiograph of the substrate after SDS-PAGE, as well as the amount of added EGFP fusion and PP2A.

(B) U2OS cells were transfected with full-length EGFP-Repo-Man WT or the indicated RATA or LAPA mutants and collected by mitotic shake-off. EGFP traps of the micrococcal-nuclease-pretreated cell lysates were analyzed by immunoblotting. RM-pS893 refers to phosphorylation of Repo-Man at S893.

(C) Confocal images of nocodazole-arrested U2OS cells after the expression of full-length EGFP-Repo-Man WT or the indicated phosphatase-binding mutants. Scale bars represent 5 μm.

(D) Nocodazole-arrested U2OS cells expressing full-length EGFP-Repo-Man WT or the indicated mutants were incubated for 30 min with ZM447439 (ZM) before fixation. DNA was stained with DAPI. The figure shows the percentage of cells where EGFP-Repo-Man was chromosome associated (means ± SEM; three independent experiments, >227 cells per data point).

(E) EGFP, H3T3ph, and DNA images in fixed, prometaphase-arrested U2OS control cells or cells that expressed siRNA-resistant EGFP-Repo-Man WT or LAPA mutant. The images were also obtained after the knockdown of Repo-Man.

(F) Quantification of the H3T3ph/DNA ratio for the conditions illustrated in (E). The results represent means ± SEM for 16–22 cells per condition.

(G) Model of the centromeric targeting of the CPC during prometaphase. The initiation of centromeric CPC targeting stems from Aurora B-stimulated recruitment of Bub1 to the kinetochores, which phosphorylates histone H2A at T120 (H2AT120ph), a docking site for shugoshin, which directly binds to the CPC. This small CPC pool stimulates Haspin-mediated phosphorylation of histone H3 at T3 (H3T3ph), resulting in an amplification of CPC recruitment at the centromeres. In addition, the CPC phosphorylates Repo-Man at S893, which prevents its recruitment to histones and dephosphorylation of H3T3ph by associated PP1.

Repo-Man at S893 during prometaphase, enabling the dephosphorylation of H3T3ph by PP1/Repo-Man. The possibility cannot be excluded that Repo-Man-associated PP2A also directly contributes to the dephosphorylation of H3T3ph during prometaphase. However, its contribution is likely to be minor, since a prometaphase immunoprecipitate of the RATA mutant of Repo-Man could not dephosphorylate H3T3ph, and since mutation of the PP1-docking site alleviated the hypophosphorylation of H3T3 induced by the overexpression of Repo-Man [6].

Conclusions

We have demonstrated here that the chromosomal targeting of Repo-Man is antagonistically regulated by Aurora B and PP2A. The balance between these opposing enzymes regulates the phosphorylation of Repo-Man at S893, which is a negative determinant of histone binding. Until the end of metaphase, Aurora B is enriched at the chromosomes and opposes the recruitment of Repo-Man by phosphorylation of S893. This may explain why Repo-Man is only partially chromosome associated in early mitosis. The translocation of the CPC to the spindle midzone in anaphase reduces its ability to phosphorylate Repo-Man and correlates with an increased chromosomal targeting of Repo-Man. However, it is well established that the targeting of Repo-Man to prometaphase chromosomes is highly dynamic [8, 9, 18], indicating that the balance between Aurora B and Repo-Man-associated PP2A is tightly regulated. This is reminiscent of the antagonism between Aurora B and BubR1-associated B56-PP2A, which dynamically regulates the phosphorylation of Aurora B substrates at the kinetochore and is essential to generate stable and correct kinetechore-microtubule attachments [16, 17, 19]. Here, we have only explored the importance of the chromosome targeting of Repo-Man for the dephosphorylation of H3T3. However, Repo-Man has also established functions in chromosome movement [20], chromosome decondensation [21], and nuclear reassembly [7], but the relevant substrates remain to be identified. We propose that the chromosome targeting of Repo-Man also contributes to the dephosphorylation of other mitotic substrates. Since Repo-Man emerges from our studies as a mitotic scaffold for both PP1 and PP2A, it will be important to understand how these phosphatases are functionally integrated and regulated.

The double-negative feedback regulation from Aurora B to Repo-Man is likely to be an important determinant of the centromeric targeting of the CPC during prometaphase and metaphase, in particular because it coincides with a positive feedback loop from Aurora B to Haspin [4]. Reciprocal feedback regulation of opposing enzymes is indeed well known to generate robust, bistable responses [22]. The centromeric targeting of the CPC also involves its binding to shugoshin via histone H2A that is phosphorylated at T120 by protein kinase Bub1 [23, 24]. The targeting of Bub1 to the kinetochores is stimulated by Aurora B, providing yet another positive feedback loop that autoregulates centromeric CPC targeting. However, the number of shugoshin molecules in mitotic cells is very small compared to that of Aurora B [25], and H2A T120ph already accumulates in prophase [23, 24]. Hence, the shugoshin-mediated recruitment of the CPC is likely to serve only as an initial trigger, to recruit some CPC complexes to the centromeres that then autonomously promote the recruitment of additional CPCs via reciprocal feedback regulation of Haspin and Repo-Man, culminating in the exclusive centromeric accumulation of the H3T3ph-type docking site (Figure 4G).

Supplemental Information

Supplemental Information includes two figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.05.017.

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