Current Biology 21, 766–773, May 10, 2011 ©2011 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2011.03.047

Report

PP1/Repo-Man Dephosphorylates Mitotic Histone H3 at T3 and Regulates Chromosomal Aurora B Targeting

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Summary

The transient mitotic histone H3 phosphorylation by various protein kinases regulates chromosome condensation and segregation, but the counteracting phosphatases have been poorly characterized [1–8]. We show here that PP1γ is the major histone H3 phosphatase acting on the mitotically phosphorylated (ph) residues H3T3ph, H3S10ph, H3T11ph, and H3S28ph. In addition, we identify Repo-Man, a chromosome-bound interactor of PP1γ [9], as a selective regulator of H3T3ph and H3T11ph dephosphorylation. Repo-Man promotes H3T11ph dephosphorylation by an indirect mechanism but directly and specifically targets H3T3ph for dephosphorylation by associated PP1γ. The PP1γ/Repo-Man complex opposes the protein kinase Haspin-mediated spreading of H3T3ph to the chromosome arms until metaphase and catalyzes the net dephosphorylation of H3T3ph at the end of mitosis. Consistent with these findings, Repo-Man modulates in a PP1-dependent manner the H3T3ph-regulated chromosomal targeting of Aurora kinase B and its substrate MCAK. Our study defines a novel mechanism by which PP1 counteracts Aurora B.

Results and Discussion

PP1γ Is a Major Mitotic Histone H3 Phosphatase

In vertebrates, most dephosphorylations at the end of mitosis are catalyzed by the protein Ser/Thr phosphatases PP1 and PP2A [10]. However, at least 200 distinct PP1 holoenzymes and approximately 70 PP2A holoenzymes exist, each with their own set of substrates, and for most mitotic phosphoproteins it is not known which phosphatase holoenzyme catalyzes their dephosphorylation during mitosis. This also applies to histone H3, one of the most abundant mitotic phosphoproteins. Indeed, there are no published data on the nature of the mitotic phosphatases that dephosphorylate phosphorylated (ph) H3T3 and H3T11ph. Initial evidence, mostly based on genetic research conducted in yeast, suggests a key role for PP1 in the dephosphorylation of H3S10ph and H3S28ph, but the involved PP1 targeting subunits have not yet been identified [5].

We have characterized histone H3 phosphatases in U2OS osteosarcoma cells. Using phosphoepitope-specific antibodies with a validated specificity and sensitivity (see Figures S1A and S1B available online), we first examined the global phosphorylation of histone H3 in cells that were nonsynchronized, synchronized in either G_1/S or prometaphase, or released from a prometaphase arrest (Figure 1A). Histone H3 was only measurably phosphorylated during mitosis and was completely dephosphorylated again at the end of mitosis (Figure 1A), in the period between the degradation of cyclin B1 and Aurora A during metaphase and anaphase, respectively [11]. The dephosphorylation of H3T3ph and H3S28ph occurred earlier than that of H3S10ph and H3T11ph and closely followed the degradation of cyclin B1.

The mere incubation of cell lysates from prometaphase-arrested cells resulted in a rapid dephosphorylation of histone H3 (Figures S1C and S1D). No dephosphorylation was detected in the presence of 1 μM microcystin-LR, a potent inhibitor of protein Ser/Thr phosphatases PP1 and PP2A and the PP2A-like phosphatases PP4–6 (Figure S1C). Conversely, the dephosphorylation of histone H3 was hardly affected by 50 nM okadaic acid, which inhibits PP2A(-like) phosphatases but not PP1, hinting at an important role for PP1 as a mitotic histone H3 phosphatase. Accordingly, the dephosphorylation of histone H3 was considerably delayed (H3T3ph, H3S28ph) or even completely blocked (H3S10ph, H3T11ph) by the addition of 15 μM of the central domain of NIPP1 (residues 143–224), a highly specific inhibitor of PP1 [12]. Also, the dephosphorylation of histone H3 occurred much more slowly in lysates prepared from U2OS cells after the small interfering RNA (siRNA)-mediated knockdown of all PP1 isoforms (Figure S1D). To further characterize PP1 as a histone phosphatase, we isolated histones from mitotically arrested U2OS cells and used them as in vitro substrates for the purified catalytic subunit of PP1 (Figure S1E). Histone H3 turned out to be an excellent substrate and was completely dephosphorylated within 15 min by low nanomolar concentrations of PP1. The dephosphorylation of H3T3ph and H3S28ph required about 10 times less PP1 than that of H3S10ph and H3T11ph, which may explain why the dephosphorylation of the former residues is incompletely blocked by the addition of NIPP1 (Figure S1C) or after the knockdown of PP1 (Figure S1D). Collectively, these data identify PP1 as an important histone H3 phosphatase in mitotic lysates.

To delineate the role of PP1 as a mitotic histone H3 phosphatase in intact cells, we performed isoform-specific knockdowns of PP1α, PP1β, and PP1γ with previously validated siRNAs [13]. The knockdown of PP1γ prevented the dephosphorylation of all examined histone H3 sites during a release from a prometaphase arrest (Figure 1B). A deficiency of PP1α had similar but less-pronounced effects, whereas the knockdown of PP1β did not affect histone dephosphorylation. It is possible that the hampered histone dephosphorylation in PP1α-deficient cells was indirectly caused by the associated delay in G_2/M, as detected by fluorescence-activated cell sorting (FACS) analysis (Figure S1F), which can be explained by key functions of PP1α in centrosome maturation and separation [10]. However, no cell-cycle delay (Figure S1F) or mitotic-arrest phenotype [14] was detected after the knockdown of PP1γ. Thus, histone dephosphorylation at the end of mitosis appears to be largely mediated by PP1γ, but a (minor) contribution of PP1α cannot be excluded.

Repo-Man Regulates the Dephosphorylation of H3T3ph and H3T11ph

PP1 is controlled by PP1 interacting proteins (PIPs), which often act as substrate-targeting subunits [15]. To identify one

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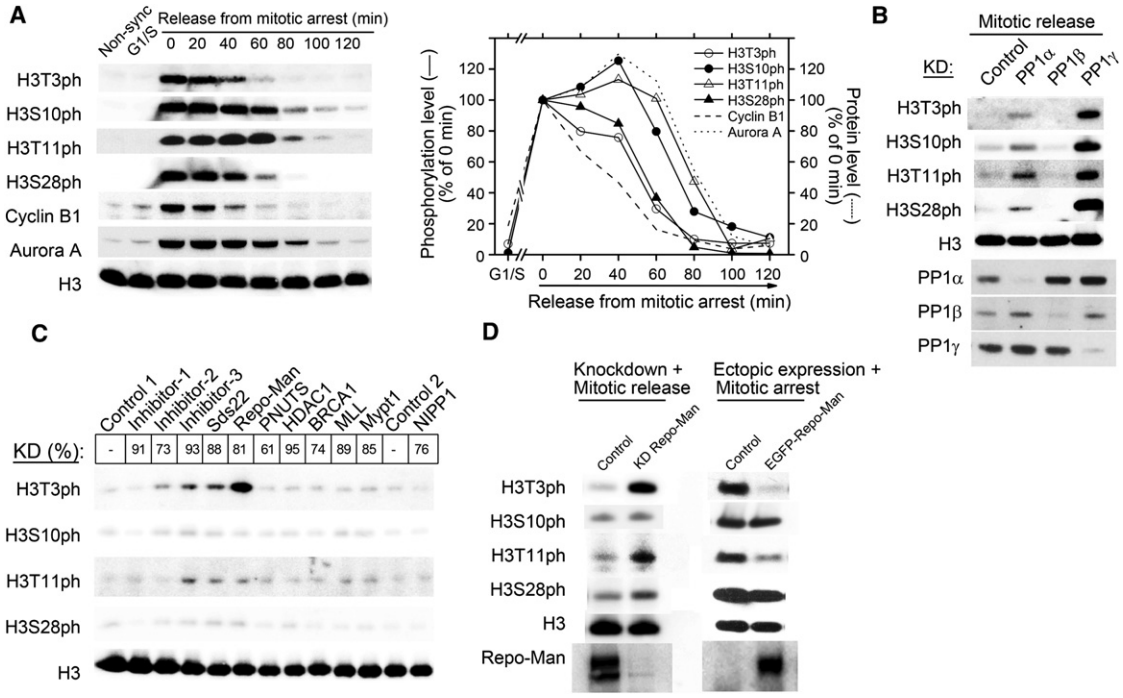

Figure 1. Identification of PP1γ/Repo-Man as a Mitotic Histone H3 Phosphatase

(A) The left panel shows immunostainings of phosphorylated histone H3 residues (T3, S10, T11, and S28), cyclin B1, Aurora A, and histone H3 in U2OS cells. The cells were not synchronized (nonsync), arrested in G_1/S by a double thymidine block (G_1/S), arrested in prometaphase by a sequential thymidine-nocodazole block and shake off (0 min), or released from a prometaphase arrest by incubation in nocodazole-free medium for the indicated times (20–120 min). The samples were also probed for histone H3, cyclin B1, and Aurora A levels. The quantification of the blots, expressed as a percentage of the values during mitotic arrest (0 min), is shown (right).

(B) Knockdown (KD) of PP1α, PP1β, or PP1γ was achieved by isoform-specific small interfering RNAs (siRNAs), as verified by immunoblotting (bottom lanes). At 48 hr following the knockdown of the indicated PP1 isoforms, U2OS cells were arrested in prometaphase and released again for 90 min by the removal of nocodazole (mitotic release), and histone H3 dephosphorylation was analyzed.

(C) A siRNA screen for mitotic histone H3 targeting subunits of PP1. Following the siRNA-mediated knockdown of the indicated PP1 interactors, U2OS cells were arrested in prometaphase, released for 90 min, and probed for histone H3 phosphorylation. Controls 1 and 2 refer to control siRNAs. The numbers above represent the percentage of KD, determined to be 61%–95% by RT-qPCR.

(D) Histone H3 phosphorylation in mitotically released cells (90 min) after transfection with control or Repo-Man siRNA (left). The KD of Repo-Man was verified by immunoblotting (bottom lane). Histone H3 phosphorylation in prometaphase-arrested cells after transfection with empty vector (control) or EGFP-tagged Repo-Man (wild-type, WT) is shown (right). The expression of EGFP-tagged Repo-Man was quantified with EGFP-directed antibodies (bottom lane). See also Figure S1.

or more PIPs that direct PP1 to histone H3, we performed a limited siRNA screen of abundant or chromatin-associated PIPs and searched for inhibitory effects on histone H3 dephosphorylation during a release from a prometaphase arrest (Figure 1C). The most striking effects were seen in cells that were deficient for Repo-Man (*re*cruits *P*P1 *o*nto *m*itotic chromatin at *an*aphase), a well-established interactor of PP1γ and mitotic chromosomes [9]. Indeed, knockdown of Repo-Man with a previously validated siRNA [16] caused an inhibition of the dephosphorylation of H3T3ph and H3T11ph but had no effect on H3S10ph and H3S28ph (Figures 1C and 1D). Similar data were obtained with unrelated SMARTpool siRNAs (data not shown), arguing against off-target effects. The selectivity of this effect is good evidence that it cannot be attributed to an arrest in mitosis, which, moreover, could not be detected by FACS analysis (Figure S1G). In further agreement with a key role for Repo-Man as a regulator of mitotic histone H3 dephosphorylation, we found that its ectopic expression decreased the level of H3T3ph and H3T11ph in prometaphase-arrested cells but had no effect on H3S10ph and H3S28ph (Figure 1D). Our results show that Repo-Man selectively regulates the mitotic level of H3T3ph and H3T11ph.

To further differentiate between direct and indirect effects on H3T3ph and H3T11ph dephosphorylation, we immunoprecipitated the Repo-Man complex from prometaphase-arrested cells (Figure 2A) and examined its ability to dephosphorylate purified mitotic histone H3 (Figure 2B). The Repo-Man immunoprecipitate rapidly dephosphorylated H3T3ph but was unable to dephosphorylate H3S10ph, H3T11ph, or H3S28ph. In contrast, a dephosphorylation of all histone H3 sites was seen after prior trypsinolysis of the holoenzyme, which releases the free C-terminally nicked but fully active catalytic subunit of PP1 (Figure 2C). The best substrates of the trypsin-released catalytic subunit were H3T3ph and H3S28ph (Figure 2B), in accordance with their superior substrate quality for purified PP1 (Figure S1E). The latter findings show that Repo-Man acts as a substrate specifier and restricts the histone H3 phosphatase activity of PP1 to H3T3ph. The inability of the Repo-Man complex to dephosphorylate H3T11ph in vitro suggests that this site is unlikely to be a direct substrate. Because Repo-Man-associated PP1 dephosphorylates and inactivates protein kinase ATM [16], an upstream regulator of the H3T11 kinase Chk1 during DNA damage [17], we speculate that a similar type of regulation

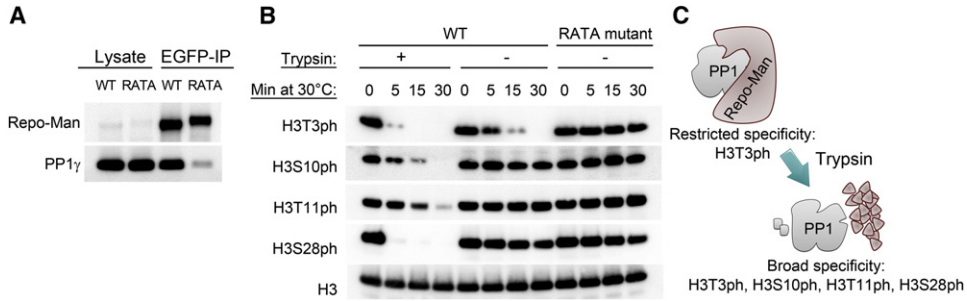

Figure 2. Repo-Man Targets PP1γ to Histone H3T3ph

(A) After the ectopic expression of either EGFP-tagged Repo-Man (WT) or Repo-Man RATA (RATA), the fusions were immunoprecipitated from prometaphase-arrested cells with EGFP-directed antibodies. The blot shows the Repo-Man fusions and PP1γ in the lysates and immunoprecipitates.

(B) Aliquots of the immunoprecipitated EGFP fusions, before (−) and after (+) trypsinolysis, were incubated for the indicated times with phosphorylated histones extracted from mitotically arrested cells, and histone H3 dephosphorylation was followed by immunoblotting.

(C) Illustration showing how trypsinolysis of the Repo-Man holoenzyme releases a C-terminally nicked free catalytic subunit with broad substrate specificity.

applies to the mitotic phosphorylation of H3T11. An indirect role of PP1γ/Repo-Man in mitotic H3T11ph dephosphorylation can also explain why this site is dephosphorylated within a time frame that differs from that of the direct target H3T3ph (Figure 1A). Importantly, H3T3ph was not dephosphorylated by an immunoprecipitate of Repo-Man that was mutated in its PP1-binding RVTF sequence (mutation of RVTF to RATA) and, hence, contained little PP1 (Figures 2A and 2B). This shows that the dephosphorylation of H3T3ph is mediated by PP1, and not by another phosphatase that may have coprecipitated with Repo-Man.

PP1γ/Repo-Man Dephosphorylates H3T3ph throughout Mitosis

We subsequently used confocal fluorescence microscopy to investigate how the phosphorylation of H3T3 is affected at different mitotic stages by modulating the level of PP1γ or Repo-Man. U2OS cells that were synchronized with a combined thymidine and nocodazole block and were then released showed a maximal phosphorylation of H3T3 during metaphase, but this phosphorylation was largely reversed during anaphase and became undetectable during telophase (Figure 3A). The knockdown of either PP1γ or Repo-Man resulted in a hyperphosphorylation of H3T3 from prometaphase until telophase that was often associated with the formation of chromosome bridges during chromosome segregation (Figure 3A). Quantification of H3T3ph using Z stacks showed a knockdown-induced increase to approximately 250% in prometaphase and metaphase, which became even more pronounced during anaphase and telophase, in particular following Repo-Man knockdown (Figure 3C). The smaller effects of a knockdown of PP1γ are likely due to functional redundancy between PP1 isoforms and the ability of Repo-Man to interact with both PP1γ and PP1α [9].

The expression of EGFP-tagged Repo-Man decreased the phosphorylation of H3T3 from prometaphase to anaphase by at least 75% (Figures 3B and 3C). As expected, the expression of Repo-Man RATA did not have a significant effect on H3T3 phosphorylation. In accordance with published data [9], EGFP-tagged Repo-Man, but not EGFP, partially colocalized with the chromosomes during prometaphase and metaphase and became strongly associated with the chromosomes in anaphase and telophase. The association of EGFP-tagged Repo-Man with prometaphase chromosomes was confirmed by fluorescence analysis of cells that were pretreated with Triton X-100 to remove soluble Repo-Man (Figure 3D). Because the expression of Repo-Man also promoted H3T3ph dephosphorylation during (pro)metaphase, its enhanced association with the chromosomes during anaphase does not appear to be a prerequisite for H3T3ph dephosphorylation. This is reminiscent of Aurora B, which keeps histone H3 phosphorylated at S10 and S28 until anaphase without being specifically enriched at the chromosome arms during (pro)metaphase [18, 19]. Repo-Man RATA showed a perichromosomal staining during metaphase and early anaphase and only became entirely chromosome associated during late anaphase and telophase, indicating that the initial recruitment of Repo-Man to the chromosomes is regulated by associated PP1.

Repo-Man Regulates the Chromosomal Targeting of Aurora B and MCAK

In prophase, the Aurora B-containing chromosome passenger complex (CPC) is detected along the chromosome arms but then progressively moves to the inner centromeres during (pro)metaphase [19]. The chromosomal targeting of CPC is regulated through phosphorylation of H3T3 by protein kinase Haspin, which creates a docking site for the CPC component survivin [4, 7, 8]. Because a loss of Haspin reduces the phosphorylation of H3T3 and the recruitment of Aurora B at centromeres (Figure S2A and [4, 7, 8]), we reasoned that overexpression of the counteracting H3T3 phosphatase should have a similar effect. Indeed, during prometaphase Aurora B was enriched at the centromeres, but this localization was lost upon expression of EGFP-tagged Repo-Man (Figures 4A and 4B). Instead, Aurora B showed a more diffuse distribution. The expression of EGFP-tagged Repo-Man RATA had a much smaller effect on the centromeric targeting of Aurora B, illustrating yet again the key mediatory role of PP1. Immunoblot analysis showed that the more diffuse staining of Aurora B after the expression of EGFP-tagged Repo-Man cannot be explained by the loss of the Aurora B protein from the chromatin-enriched fraction (Figure 4C), indicating that the Aurora B complex remains associated with this fraction by a H3T3ph-independent mechanism [18]. It is known that the binding of the Aurora B complex to the chromosomes is also (partially) mediated by Shughosin, which binds to histone H2A phosphorylated on T120 [20]. However, this mechanism

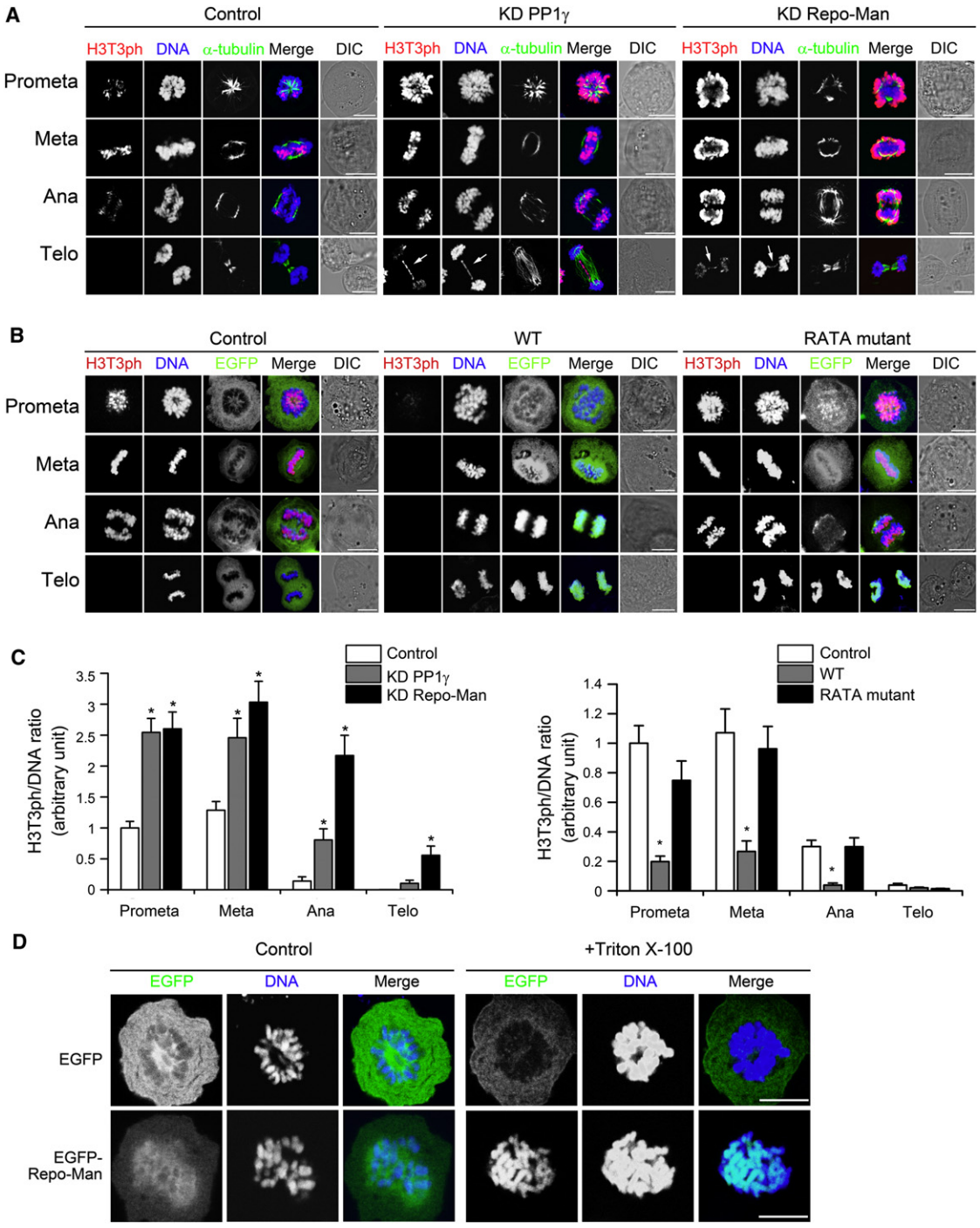

Figure 3. PP1γ and Repo-Man Regulate Histone H3T3 Phosphorylation at Different Stages of Mitosis

(A) After transfection with control, PP1γ, or Repo-Man siRNA, U2OS cells were arrested in mitosis and then released 1–2 hr before fixation. The figure shows confocal images of cells at distinct mitotic stages. The following abbreviations are used: DIC, differential interference contrast; prometa, prometaphase; meta, metaphase; ana, anaphase; telo, telophase. The arrows point at chromosome bridges. Scale bars represent 10 μm.

(B) A similar experiment was performed after the ectopic expression of EGFP (control), EGFP-tagged Repo-Man (WT), or EGFP-tagged Repo-Man RATA (RATA mutant).

(C) Z stack quantification of the level of H3T3 phosphorylation at different mitotic phases for the experiments described in (A) (left) and (B) (right). The data represent the means ± standard error (SE) for 10–16 cells in each condition, obtained from three different experiments. *$p < 0.01$, Student's t test.

(D) Prometaphase-arrested U2OS cells transiently expressing EGFP or EGFP-tagged Repo-Man were fixed before and after incubation for 3 min with 0.1% Triton X-100 and used for the visualization of DNA and EGFP by confocal microscopy. Scale bars represent 10 μm.

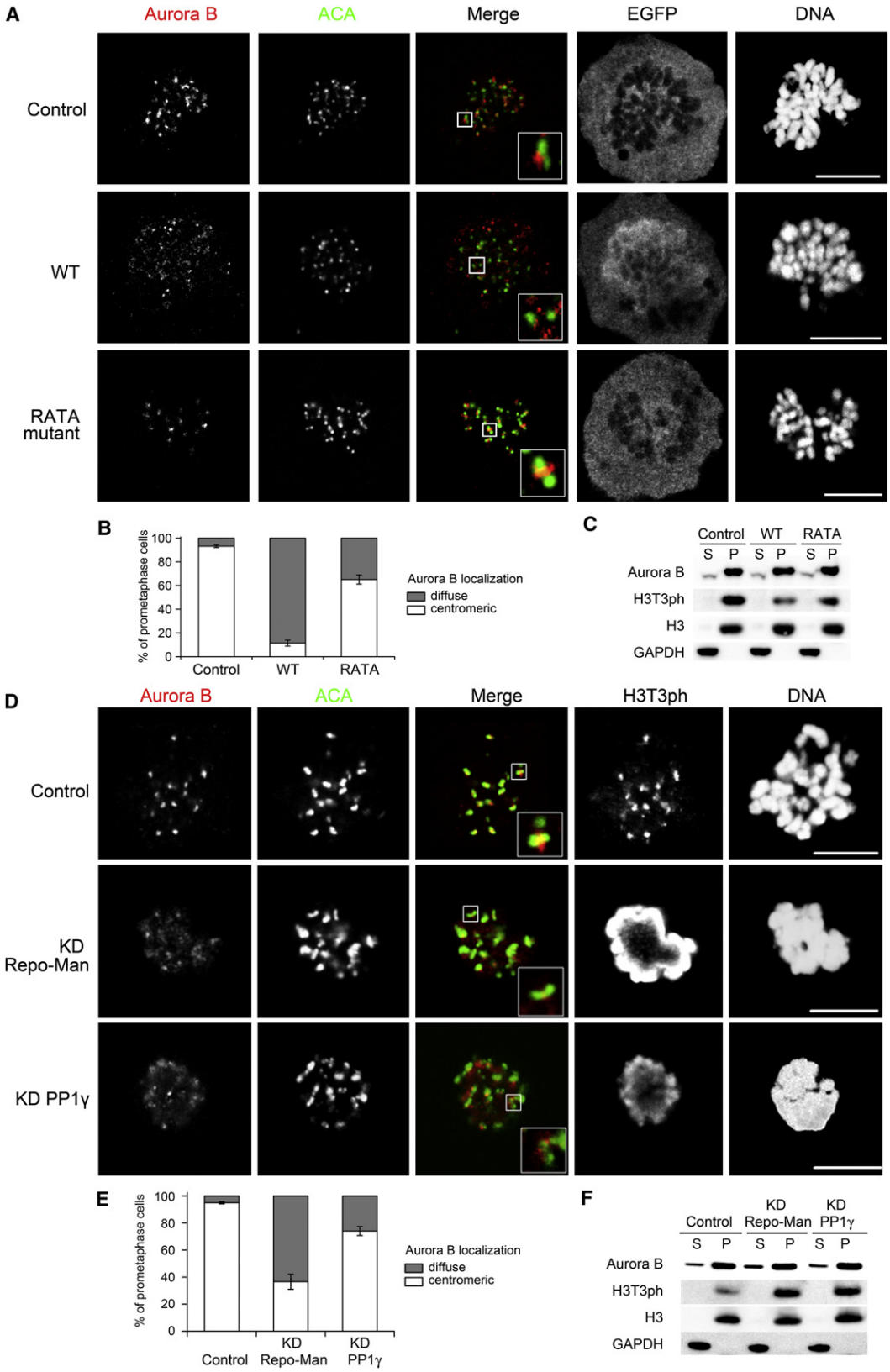

Figure 4. Repo-Man Regulates the Chromosomal Localization of Aurora B

(A) U2OS cells transfected with constructs encoding EGFP (control), EGFP-tagged Repo-Man (WT), or EGFP-tagged Repo-Man RATA (RATA mutant) were allowed to recover for 1 hr from a prometaphase block and were fixed. The figure shows confocal (immuno)fluorescence images of prometaphase cells. ACA denotes anticentromere antibody (kinetochore marker). Scale bars represent 10 μm.

does not appear to account for the diffuse localization of Aurora B in the absence of Repo-Man, because the centromeric localization of Shughosin 1 was not affected by changes in the concentration of Repo-Man (Figure S2).

Intriguingly, the knockdown of Repo-Man or PP1γ also caused a more diffuse staining of Aurora B (Figures 4D and 4E), which remained associated with the chromatin-enriched fraction (Figure 4F). This can be rationalized by the spreading of the H3T3ph-dependent docking of the Aurora B complex along the chromosome arms. Consistent with this notion, both H3T3ph and Aurora B were detected along the chromosome arms of prometaphase spreads in Repo-Man-deficient cells (Figure S3). Thus, both a decreased and an increased concentration of Repo-Man result in a reduced centromeric targeting of the Aurora B complex by either the loss or spreading of the H3T3ph docking site, respectively. Likewise, both deficiency and overexpression of Haspin have been reported to result in a reduced centromeric targeting of Aurora B [7].

One of the key substrates of Aurora B is the mitotic microtubule-depolymerizing kinesin MCAK, of which the targeting to the inner centromeres and chromosome arms is regulated by Aurora B [21, 22]. To explore whether the contribution of Repo-Man to the chromosomal targeting of Aurora B is functionally important, we examined the effects of an altered Repo-Man level on the localization of MCAK in prometaphase cells. The expression of EGFP-tagged Repo-Man severely reduced the centromeric targeting of MCAK (Figure S4A). A similar displacement of MCAK was detected in Haspin-deficient cells (Figure S4B; [7]). These findings confirm that the H3T3ph-dependent chromosomal localization of Aurora B is required for the phosphorylation and targeting of MCAK. In further agreement with this conclusion, we found that the spreading of H3T3ph and Aurora B to the chromosome arms in Repo-Man-deficient cells (Figure 4D and Figure S3) was associated with a more diffuse distribution of MCAK (Figure S4C). It therefore appears that Repo-Man regulates chromosomal MCAK targeting by controlling Aurora B localization.

Rescue of the Haspin-Deficient Phenotype by the Codepletion of Repo-Man

Collectively, the obtained data strongly suggest that PP1γ/Repo-Man acts antagonistically to Haspin in regulating the mitotic level of H3T3ph. In further agreement with this view, we found that the severely decreased mitotic H3T3 phosphorylation and skewed centromeric Aurora B targeting in Haspin-deficient cells could be partially rescued by the simultaneous codepletion of Repo-Man, as shown by both immunoblotting (Figure 5A) and immunofluorescence analysis (Figures 5B and 5C). Only 10% of the Haspin-deficient cells showed clear H3T3 phosphorylation. In contrast, 56% of the cells showed H3T3 phosphorylation after the codepletion of Haspin and Repo-Man. However, the response was rather heterogenous in that 33% of the cells showed a normal, centromeric H3T3 phosphorylation and Aurora B localization, whereas 23% displayed an increased, diffuse H3T3 phosphorylation, as well as a more diffuse Aurora B distribution, similar to that seen in Repo-Man-deficient cells (Figure 5C). This heterogeneity is likely to be due to variations in the efficiency of the knockdowns, resulting in distinct activity ratios of Haspin and PP1γ/Repo-Man. Finally, we point out that Repo-Man-deficient cells had a normal Haspin level, showing that a loss of Repo-Man does not affect H3T3 phosphorylation by depleting Haspin (Figure 5A).

Conclusions

We have demonstrated here that Repo-Man functions as a histone H3T3ph-specifying subunit of PP1γ. The PP1γ/Repo-Man complex opposes the spreading of H3T3 phosphorylation to the chromosome arms during the early phases of mitosis and catalyzes the net dephosphorylation of H3T3ph at the end of mitosis. In doing so, the Repo-Man phosphatase contributes to the centromeric enrichment of the Aurora B complex in (pro)metaphase (Figure 5D). Intriguingly, other PP1 holoenzymes counteract Aurora B by complementary mechanisms [23–25], i.e., by dephosphorylating and inactivating Aurora B or by dephosphorylating kinetochore-associated substrates. We also found that PP1γ/Repo-Man is indirectly implicated in the dephosphorylation of H3T11ph. In addition, PP1γ dephosphorylates H3S10ph, H3T11ph, and H3S28ph at the end of mitosis, but the involved targeting subunits remain to be identified.

Experimental Procedures

Unless otherwise indicated, a prometaphase arrest was induced by culturing U2OS cells consecutively for 24 hr with 2 mM thymidine, 2 hr without thymidine, and 16 hr with 100 ng/ml nocodazole. The arrested cells were harvested by shake off. A mitotic release was induced by culturing the arrested cells for 60–120 min in medium without nocodazole. To produce chromosome spreads, we treated mitotically arrested cells for 15 min at 37°C with 75 mM KCl before fixation. Images with a single optical section were acquired at 21°C with a Zeiss 510 META laser-scanning confocal microscope equipped with a Plan Neofluar 40 × 1.3 NA oil differential interference contrast (DIC) objective (for Figure 3) or Plan Apochromat 63 × 1.40 NA oil DIC objective (for Figure 4 and Figure 5). Images were deconvoluted with Zeiss 510 image software. Final images were processed and assembled using Photoshop CS3 (Adobe). Brightness and contrast were adjusted using only linear operations applied to the entire image. For quantification, Z stack scans were performed through each cell for 13–16 sections with 1 μm intervals. Signals of H3T3ph and DNA were processed with ImageJ 1.43u software (National Institutes of Health) using the "sum slices" feature of z project. After subtraction of the background signal, the H3T3ph/DNA ratio was calculated and plotted with Origin 8.1 software (OriginLab software).

Supplemental Information

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2011.03.047.

Acknowledgments

We thank Laura Trinkle-Mulcahy for the gift of a Repo-Man construct, Linda Wordeman for the gift of MCAK antibodies, and Elke Van Ael for critical comments on the manuscript. Annemie Hoogmartens, Tine Jaspers, Nicole Sente, and Gerd Van der Hoeven provided technical assistance. This work was supported by the Fund for Scientific Research-Flanders (grant

(B) In each condition, 71–100 cells from three independent experiments were classified visually according to the diffuse or mainly centromeric localization of Aurora B. The results are represented as means ± SE.
(C) Immunoblots from (A) stained for Aurora B, histone H3, and GAPDH in the soluble (S) and chromatin-enriched (P) fraction.
(D) Similar experiment as in (A), but after transfection of U2OS cells with control, Repo-Man, or PP1γ siRNA.
(E) Quantification of the Aurora B distribution pattern after knockdown of Repo-Man or PP1γ, as in (B).
(F) Immunoblots after knockdown of Repo-Man or PP1γ, as in (C). See also Figures S2–S4.

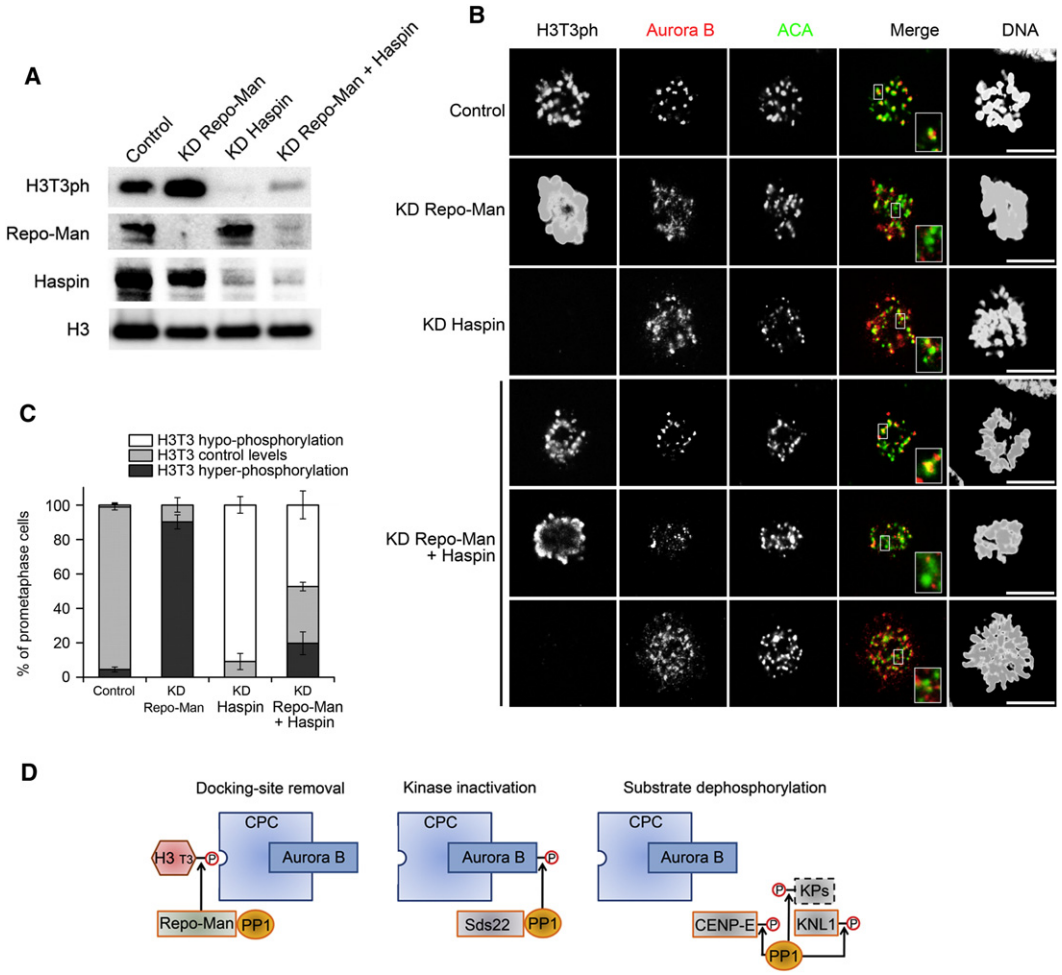

Figure 5. Rescue of the Phenotype Associated with Haspin Deficiency by Codepletion of Repo-Man

(A) Immunoblots of lysates after KD of Repo-Man and/or Haspin in U2OS cells arrested in prometaphase.

(B) Confocal images of prometaphase-arrested cells after knockdown with the indicated siRNAs. Scale bars represent 10 μm.

(C) The percentage of occurrence of the H3T3ph phenotype (hypophosphorylation, normal phosphorylation, diffuse hyperphosphorylation) after knockdown with the indicated siRNAs was visually scored for 204–451 cells (three different experiments) in each condition. The results are represented as means ± SE.

(D) Three distinct mechanisms by which PP1 opposes Aurora B. PP1γ/Repo-Man erases the Aurora B docking site H3T3ph (left). PP1/Sds22 dephosphorylates and inactivates Aurora B near kinetochores (middle). Tension across attached sister kinetochores separates Aurora B from its kinetochore substrates, resulting in the dephosphorylation of KNL1 and CENP-E and their recruitment of PP1 to dephosphorylate other kinetochore proteins (KPs, right).

G.0478.08), a Flemish Concerted Research Action (GOA 10/16), the Prime Minister's office (IAP-P6/28P), and the cell imaging core of the K.U. Leuven. B.L. holds a postdoctoral fellowship of the Fund for Scientific Research-Flanders.

Received: January 5, 2011
Revised: February 18, 2011
Accepted: March 16, 2011
Published online: April 21, 2011

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