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Mitotic phosphorylation of MPP8 by cyclin-dependent kinases regulates chromatin dissociation

Makoto Nishigaki [a,b], Yu Kawada [a], Toshinori Misaki [a], Kazuhiro Murata [a], Takahiro Goshima [a], Takahisa Hirokawa [a,c], Chisato Yamada [a], Midori Shimada [a,*], Makoto Nakanishi [a,*]

[a] Department of Cell Biology, Graduate School of Medical Sciences, Nagoya City University, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan
[b] Department of Psychiatry and Cognitive-Behavioral Medicine, Graduate School of Medical Sciences, Nagoya City University, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan
[c] Department of Gastroenterological Surgery, Graduate School of Medical Sciences, Nagoya City University, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan

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ABSTRACT

Repressive epigenetic modifications, DNA methylation at CpG sites and histone H3 lysine 9 (H3K9) methylation, are enriched in heterochromatin, which undergoes drastic changes in structure during mitosis. MPP8 (M phase phosphoprotein 8) has been proposed to regulate positive association between these two repressive modifications, but actual involvement of this protein in changes in the heterochromatin structure during mitosis remains elusive. We demonstrate here that MPP8 predominantly localized to, but dissociated from, chromatin during interphase and early mitosis, respectively. Chromatin dissociation from MPP8 appeared to correlate with the phosphorylation status of MPP8. Experiments using inhibitors of various mitotic kinases demonstrated that the chromatin dissociation of MPP8 during metaphase to anaphase was specifically regulated by cyclin B1-Cdk1. Indeed, cyclin B1-Cdk1 effectively phosphorylated MPP8 in vitro and on STA mutant of MPP8 (all possible sites phosphorylated by Cdk were substituted by alanine) failed to dissociate from chromatin during early mitosis. Taken together, our results indicate that the chromatin association of MPP8 is regulated by Cdk-dependent phosphorylation.

1. Introduction

Heterochromatin was originally defined as an unusually condensed chromatin region forming such structures as centromeres and G bands as seen in cytological studies [1]. Although heterochromatin is usually associated with transcriptional silencing [2], this structure also has other functional properties such as roles in DNA replication timing [3] and sister chromatid cohesion [4]. Methylation of histone H3 at lysine 9 as well as DNA methylation are the modifications that primarily characterize heterochromatin [5]. DNA methylation is known to associate with the methylated state of H3 lysine 9, providing clear in vivo evidence that it is strictly dependent on the presence of H3 lysine 9 methyltransferases in Neuropora [6]. Thus, impaired cooperation of DNA methylation with histone H3 methylation likely affects heterochromatin formation.

The heterochromatin of higher eukaryotes cannot be regarded as a static structure during the course of the cell cycle, but rather undergoes dynamic changes in its structure [7–9]. For example, HP1α (Heterochromatin protein 1), a heterochromatin organizer, recognizes H3 methylation at lysine 9 through its chromodomain, which is important for transporting it to heterochromatin regions [10]. Both H3 tri-methylation at lysine 9 and HP1 are thought to be essential for establishing and maintaining heterochromatin domains. In the G2 phase, HP1α is associated with heterochromatin, but it progressively dissociates from it at the G2-M boundary. This dissociation is dependent on Aurora B-mediated phosphorylation of histone H3 at Ser10 without changes in the level of H3 methylation at lysine 9 [7–10]. Although the exact function of this dissociation remains to be determined, null alleles of HP1 in Drosophila and *swi6* in fission yeast suggest their respective functions in proper mitotic chromosome segregation [11,12].

MPP8, originally identified as a novel M phase phosphoprotein by expression cloning, is composed of two functional domains, an amino-terminal chromodomain and a carboxy-terminal ankyrin domain [13]. Similar to HP1α, MPP8 predominantly localizes at the heterochromatin region during interphase [14] and has an important role in heterochromatin organization through regulation of the interplay between DNA methylation and histone H3 methylation [15]. The MPP8 chromodomain specifically binds to Dnmt3a methylated by G9a or GLP, and to self-methylated GLP.

* Corresponding authors. Fax: +81 52 842 3955.
E-mail addresses: midorism@med.nagoya-cu.ac.jp (M. Shimada), mkt-naka@med.nagoya-cu.ac.jp (M. Nakanishi).

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Since the MPP8 chromodomain forms a dimer, dimeric MPP8 could form a Dnmt3a-MPP8-GLP/G9a complex, suggesting that this complex might cooperate in DNA methylation and H3 lysine 9 methylation in chromatin. Taken together with the essential function of MPP8 as a heterochromatin organizer and the observations regarding HP1, we predicted the dynamic distribution of MPP8 as well as HP1α during the onset of mitosis.

In this report, we demonstrated that MPP8, similar to HP1α, predominantly localized to but dissociated from chromatin during interphase and mitosis, respectively. Importantly, this chromatin release appeared to be regulated at least in part by Cdk-dependent phosphorylation.

2. Materials and methods

2.1. Cell culture and synchronization

HeLa cells and human diploid fibroblasts (MJ90) were grown in DMEM supplemented with 10% FBS. HCT116 cells were cultured in McCoy's 5a medium containing 10% FBS. MJ90 cells were synchronized at G0 by serum starvation for 3 days (in DMEM containing 0.5% FBS) and stimulated with DMEM containing 15% FBS. Cells were harvested at the indicated times after release and cell lysates were subjected to immunoblotting and FACS analysis. HeLa and HCT116 cells were synchronized at prometaphase by treating with nocodazole at a final concentration of 100 ng/ml for 12 h. Mitotically arrested cells were collected by a shake-off of the dishes. For Cdk1 inhibition, cells were treated with 9 μM RO-3306 for 30 min. For Aurora or Plk1 inhibition, cells were treated with 2 μM ZM 447439 for 4 h or with BI 2536 for 2 h, respectively.

2.2. Plasmid constructs and site-specific mutagenesis

The full-length cDNA of wild-type human MPP8 was obtained by RT-PCR and was ligated into eukaryotic expression vector pcDNA3.1 myc His. Point mutations of pcDNA3.1 hMPP8 myc His were generated by inverse PCR with a site-specific mutagenesis kit (Toyobo) using specific primers as follows;

TTGAGGCAGAGAGAAGAGAAAGCCCCAGATGATCTGAAAAAGAAAAAA,
TTTTTTCTTTTTCTTCTTTGGCGCAGTATCTTCTTTTGTCTCACTTTG,
GCCCCGAGAAAGGCTGAGG, CTTCTTTTTCCTCCTGCCTCTG,
GCGCCAAAGGGCCGGAG, TTGGGCAGATACAGGCATCAG,

2.3. Cell cycle analysis

Cells were harvested and fixed with 70% ethanol. They were then washed once with PBS, treated with RNase and stained with propodium iodide (PI). Flow cytometry was performed using a FACS CANTO2 flow cytometer (BD Biosciences).

2.4. Immunoblotting

Chromatin and nuclear soluble fractions, and a whole-cell lysate were prepared as previously described [16]. Antibodies used in this study were as follows; anti-MPP8 (16796-1-AP; Proteintech),

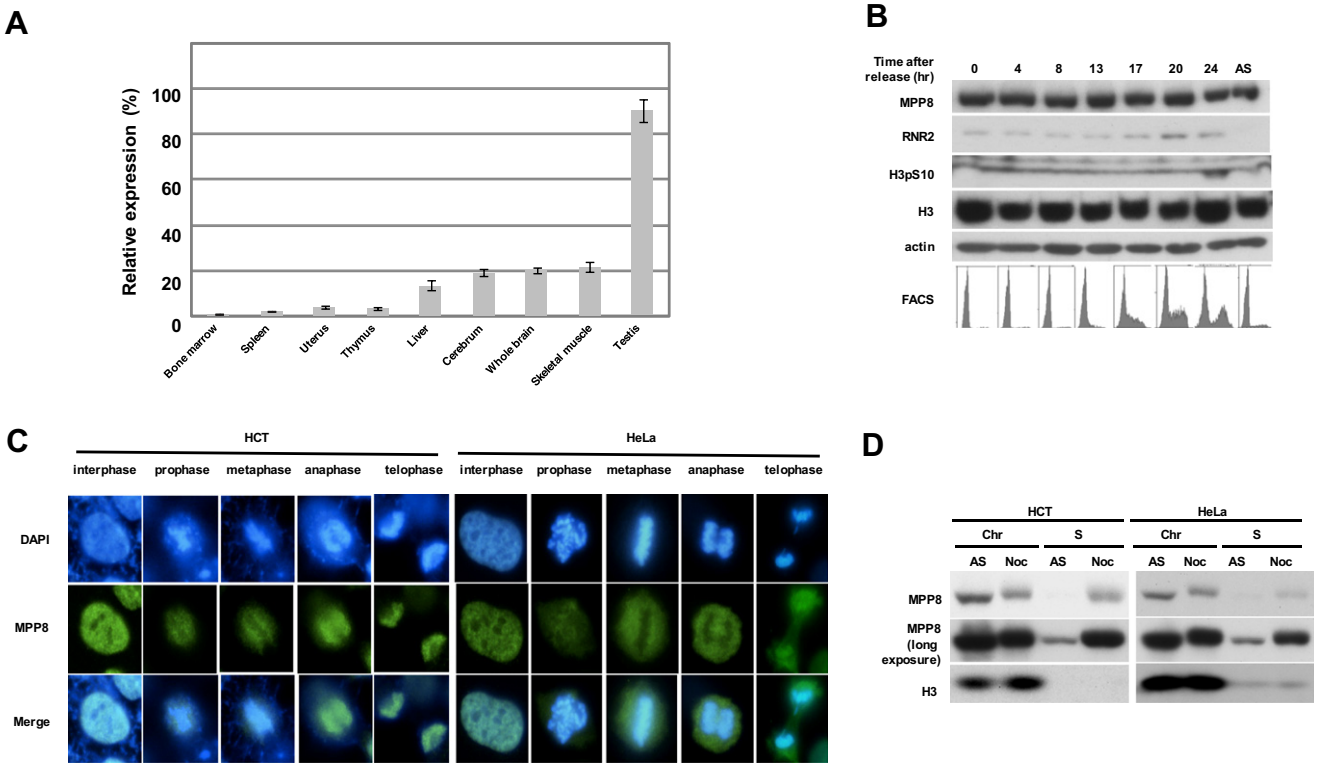

Fig. 1. Tissue-specific expression of MPP8 in mouse and subcellular localization of its protein during cell cycle progression. (A) MPP8 mRNA levels in several tissues of an ICR mouse were detected by quantitative real-time PCR (qRT-PCR). Samples were normalized against levels of beta actin. (B) The level of MPP8 did not vary throughout cell cycle progression. Serum starved MJ90 cells were stimulated by adding serum and were harvested at the indicated times. The cells were prepared for FACS analysis and whole cell lysates were prepared and subjected to immunoblotting with the indicated antibodies. (C) Immunohistochemical analysis of MPP8 in HCT116 cells and HeLa cells. Asynchronously growing HCT116 cells (left) and HeLa cells (right) were fixed and stained with anti-MPP8 antibodies and the nuclei were counterstained by DAPI. Representative images for cell cycle phases are shown. (D) Chromatin fractionation was performed using HCT116 cells (left) and HeLa cells (right) growing synchronously (AS) or synchronized at prometaphase by nocodazole (100 ng/ml, 12 h) (Noc). Chromatin (Chr) and soluble (S) fractions were analyzed by immunoblotting using the indicated antibodies.

anti-phospho-Ser10 histone H3 (06-570; Upstate), anti-phospho-Ser CDKs substrate (2324; Cell Signaling), anti-RNR2 (sc10846; Santa Cruz), anti-Cdk1 (sc54; Santa Cruz), anti-Cyclin B1 (sc245; Santa Cruz), anti-myc (sc40; Santa Cruz) and anti-beta-actin (ab2676-100; Abcam).

2.5. Immunohistochemical analysis

Cells on glass slides were fixed in 4% paraformaldehyde for 10 min at room temperature. Immunofluorescence analyses were performed with anti-MPP8 antibody and the nuclei were counterstained with DAPI as described previously [17].

2.6. Real time PCR

Total RNAs from specific tissues of ICR mice were purchased from UNITECH. Co., Ltd. Primers for the detection of MPP8 transcripts used in this study were as follows; MPP8 TTGGAAGCAGGAGCTTTTGT and TTGCAGTCAGCTCCACATTC, beta Actin AGAAAA TCTGGCACCACACC and AGAGGCGTACAGGGATAGCA.

2.7. Kinase assay

Recombinant-GST-fused MPP8 protein was bacterially expressed and purified with glutathione-sepharose beads (GE). The wild-type and a kinase-dead mutant of cyclin B1-Cdk1 kinase were immunopurified using Cyclin B1 antibodies from Sf9 cells infected with baculoviruses expressing Cyclin B1 and the wild type or kinase-dead mutant of Cdk1. In vitro kinase assays were performed as described [18].

3. Results and discussion

MPP8 is a mitotic phosphoprotein that has recently been reported to mediate interplay between de novo DNA methylation and histone H3K9 methylation [15]. Both DNA methylation and histone H3K9 methylation are involved in the heterochromatin structure, suggesting a function for MPP8 as a heterochromatin organizer. Given that HP1 proteins, a family of heterochromatin organizers, engaged in the dynamic behavior at the G2-M phase transition [19], MPP8 might also exert similar dynamic behavior during the course of the cell cycle and play a role in various cell-cycle-dependent events. To examine the cell-cycle-dependent function of MPP8, we first determined the tissue-specific expression of MPP8 using quantitative PCR. Levels of MPP8 transcripts were extremely varied in our murine model, showing the highest expression in testis (Fig. 1A). We then determined the expression of MPP8 during cell cycle progression. Using normal human fibroblasts synchronized by serum starvation, we found that the expression of MPP8 appeared constant during cell cycle progression although specific expression of RNR2 [20] and phosphorylation of histone H3 at serine 10 [21] were observed at S phase and M phase, respectively, confirming the synchronization of cell cycle progression (Fig. 1B).

We then determined the subcellular localization of MPP8 during M phase. Immunostaining of MPP8 showed a similar signal intensity throughout M phase, confirming its constant expression during the cell cycle. However, MPP8 dissociated from chromatin in early mitosis and re-associated in late mitosis, although its re-association occurred in anaphase in HCT116 cells and in telophase in HeLa cells (Fig. 1C). Consistent with this, cell fractionation

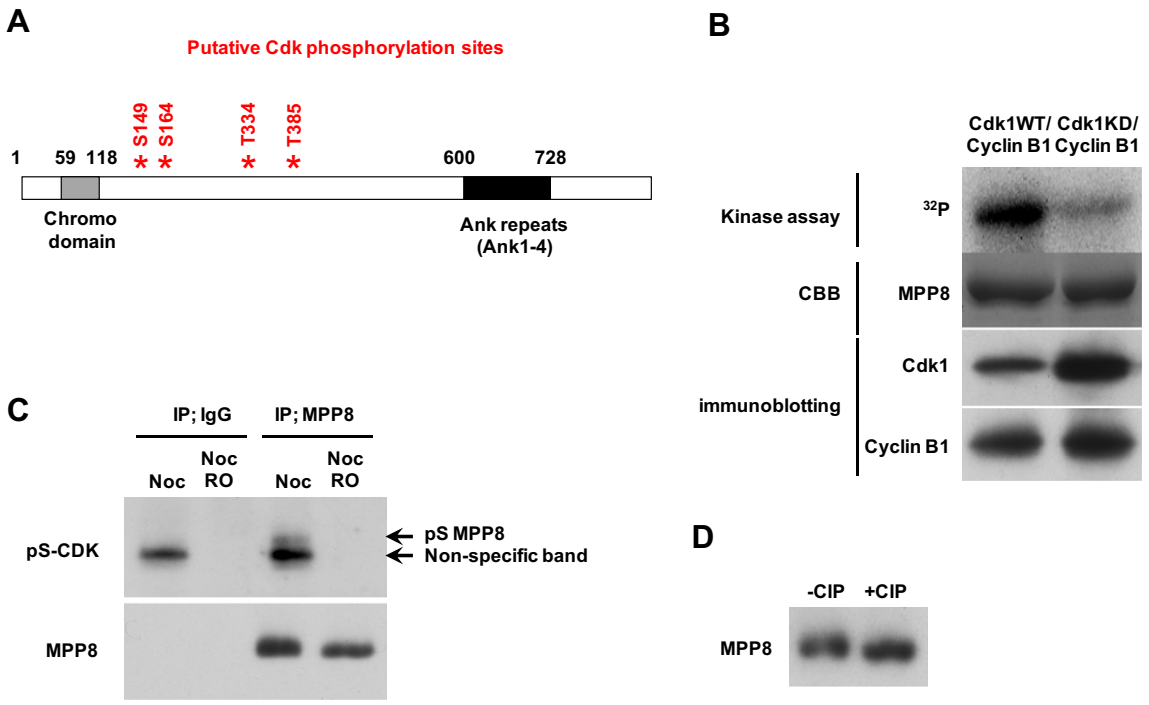

Fig. 2. MPP8 is phosphorylated by cyclin B1-Cdk1 in vitro and vivo (A) Schematic representation of Cdk1 phosphorylation sites on MPP. Putative phosphorylation sites are indicated by an asterisk ∗. (B) Immunopurified wild-type (WT) or kinase-deficient mutant (KD) of Cdk1 and Cyclin B1 kinase was used for in vitro kinase assays using purified recombinant GST-MPP8 as a substrate. Two top panels: products obtained after an in vitro kinase assay were separated by SDS PAGE and visualized by autoradiography (^{32}P) and staining with Coomassie brilliant blue (CBB). Bottom two panels: a reaction mixture without ^{32}P ATP was subjected to immunoblotting using the indicated antibodies. (C) Chromatin fractionation was performed using HCT116 cells treated with nocodazole (Noc) and the resultant chromatin fractions were solubilized and immunoprecipitated with anti-MPP8 antibodies or control IgG. The resultant immunoprecipitates were subjected to immunoblotting using anti-phospho-Ser CDKs substrate antibodies (pS-CDK) and anti-MPP8 antibodies. (D) Chromatin fractions from nocodazole-treated HCT116 cells were incubated with or without calf intestinal phosphatase (CIP) at 37 °C for 2 h. Immunoblotting was performed using anti-MPP8 antibodies. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

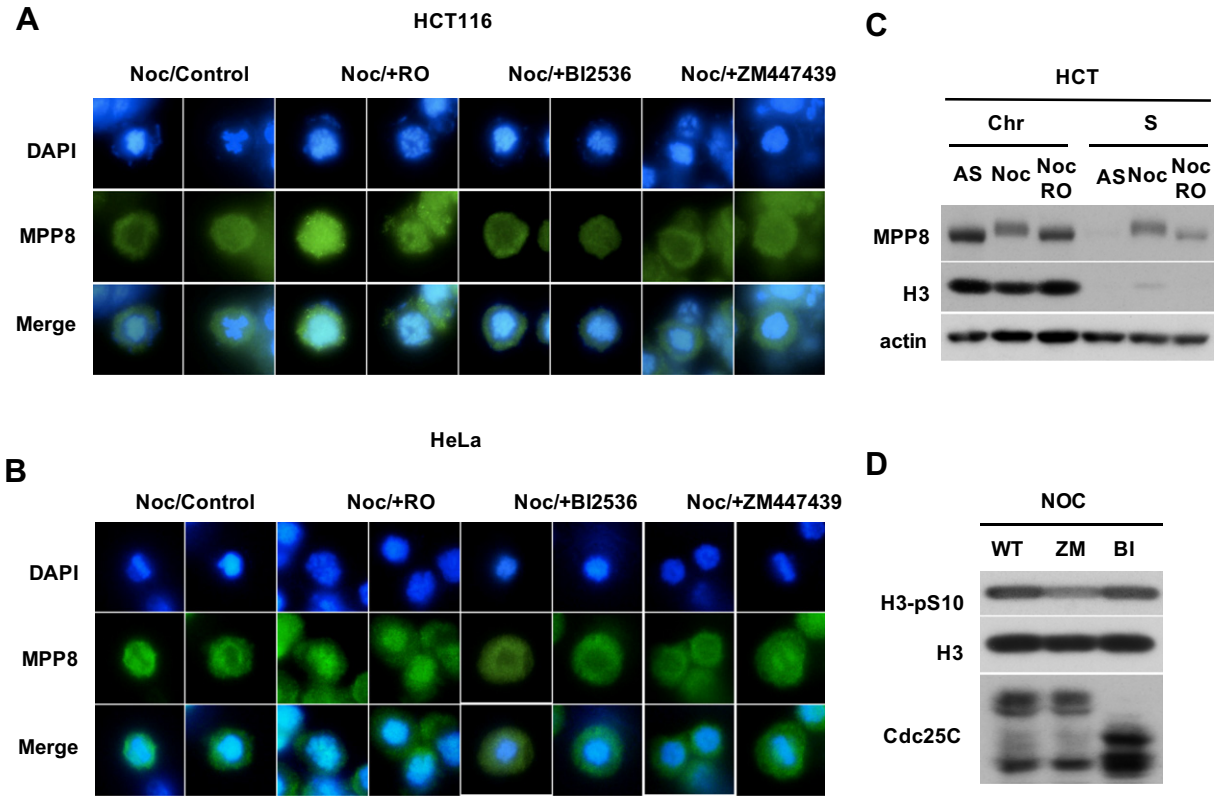

Fig. 3. Inhibition of Cdk1 specifically suppressed dissociation of MPP8 from chromatin during early mitosis. Asynchronously growing HCT116 cells (A) or HeLa cells (B) were treated with nocodazole for 12 h with or without 9 μM RO-3306, 4 μM BI2546 or 2 μM ZM447439 during the last 30 min, 2 h or 4 h, respectively. Mitotic cells were collected by mitotic shake off and spread on the cover glass for immunohistochemistry. (C) Chromatin fractionation was performed from HCT116 cells synchronized by nocodazole with (Noc RO) or without the Cdk1 inhibitor RO-3306 (Noc), or asynchronously growing HCT116 cells (AS). Chromatin (Chr) and soluble (S) fractions were subjected to immunoblotting using the indicated antibodies. (D) HCT116 cells synchronized by nocodazole with or without the 2 μM ZM447439 (ZM) and 4 μM BI2546 (BI) during the last, 2 h or 4 h, respectively. Mitotic cells were collected by mitotic shake-off, chromatin fractionation was performed, and immunoblotting was carried out using the indicated antibodies.

experiments revealed that a significant portion of MPP8 was detected in the soluble fraction collected from cells treated with nocodazole, but not from asynchronized cells (Fig. 1D). In addition, an upshift of the MPP8 band was obvious in cells treated with nocodazole but not in asynchronized cells, suggesting that MPP8 is likely to be phosphorylated during M phase. Taken together, the results indicated that MPP8 was specifically dissociated from chromatin during metaphase to anaphase.

Given that MPP8 is reported to be phosphorylated during M phase [13], we speculated that chromatin dissociation of MPP8 might be regulated by its mitotic phosphorylation. To clarify this point, we first examined whether MPP8 could be phosphorylated in vitro by cyclin B1-Cdk1, one of the major mitotic kinases. There are four putative phosphorylation sites targeted by Cdks in MPP8 (Fig. 2A). Immunopurified cyclin B-Cdk1 expressed in insect cells were incubated with GST-fused recombinant MPP8 produced in *Escherichia coli*. An in vitro kinase assay revealed that wild-type cyclin B1-Cdk1 effectively phosphorylated GST-MPP8, whereas the level of MPP8 phosphorylation was significantly compromised when GST-MPP8 was incubated with the kinase-dead mutant of cyclin B1-Cdk1 (Fig. 2B).

We then examined whether endogenous MPP8 was phosphorylated by Cdks during M phase. MPP8 was immunoprecipitated, using its specific antibodies, from cells treated with nocodazole or nocodazole and RO3306, a Cdk1-specific inhibitor, and then subjected to immunoblotting using anti-phospho-Ser CDKs substrate antibodies. These antibodies specifically recognized MPP8 protein in cells treated with nocodazole, but not with nocodazole and RO3306 (Fig. 2C). In addition, we also found a downshift of the MPP8 band in cells treated with nocodazole and RO3306. To further confirm whether the upshift of the MPP8 band in cells treated with nocodazole was due to phosphorylation, we treated immunopurified MPP8 from nocodazole-exposed cells with calf intestinal phosphatase. We found a clear downshift of the MPP8 band in the CIP-treated samples (Fig. 2D). Taken together, MPP8 was phosphorylated specifically during M phase by cyclin B1-Cdk1.

We then examined whether chromatin dissociation of MPP8 during metaphase and anaphase was regulated by its Cdk1-dependent phosphorylation. Immunostaining of MPP8 revealed that although dissociation of MPP8 was obvious in prometaphase HCT116 cells treated with nocodazole, this dissociation was strongly suppressed when cells were simultaneously treated with RO3306, but not with BI2536, a PLK specific inhibitor, or with ZM447439, an Aurora kinase inhibitor (Fig. 3A). A similar dissociation of MPP8 was observed in prometaphase in HeLa cells (Fig. 3B). Consistent with this, the amount of MPP8 in the soluble fraction from nocodazole-treated cells was reduced when HCT116 cells were simultaneously treated with RO3306 (Fig. 3C). Inhibition of Aurora and Plk kinases was confirmed by suppression of H3 pS10 and phospho-Cdc25C, respectively (Fig. 3D). Thus, these results suggested that chromatin dissociation of MPP8 during metaphase and anaphase was regulated by its phosphorylation by cyclin B1-Cdk1.

Finally, we examined whether the Cdk phosphorylation site mutant of MPP8 retained chromatin binding during early mitosis in order to further confirm the Cdk1-dependent dissociation of MPP8 from chromatin. We substituted serine or threonine residues in four putative phosphorylation sites of MPP8 by replacing them with alanine (STA mutant) and expressed the mutant in HeLa cells (Fig. 4A). Importantly, immunostaining of ectopically expressed

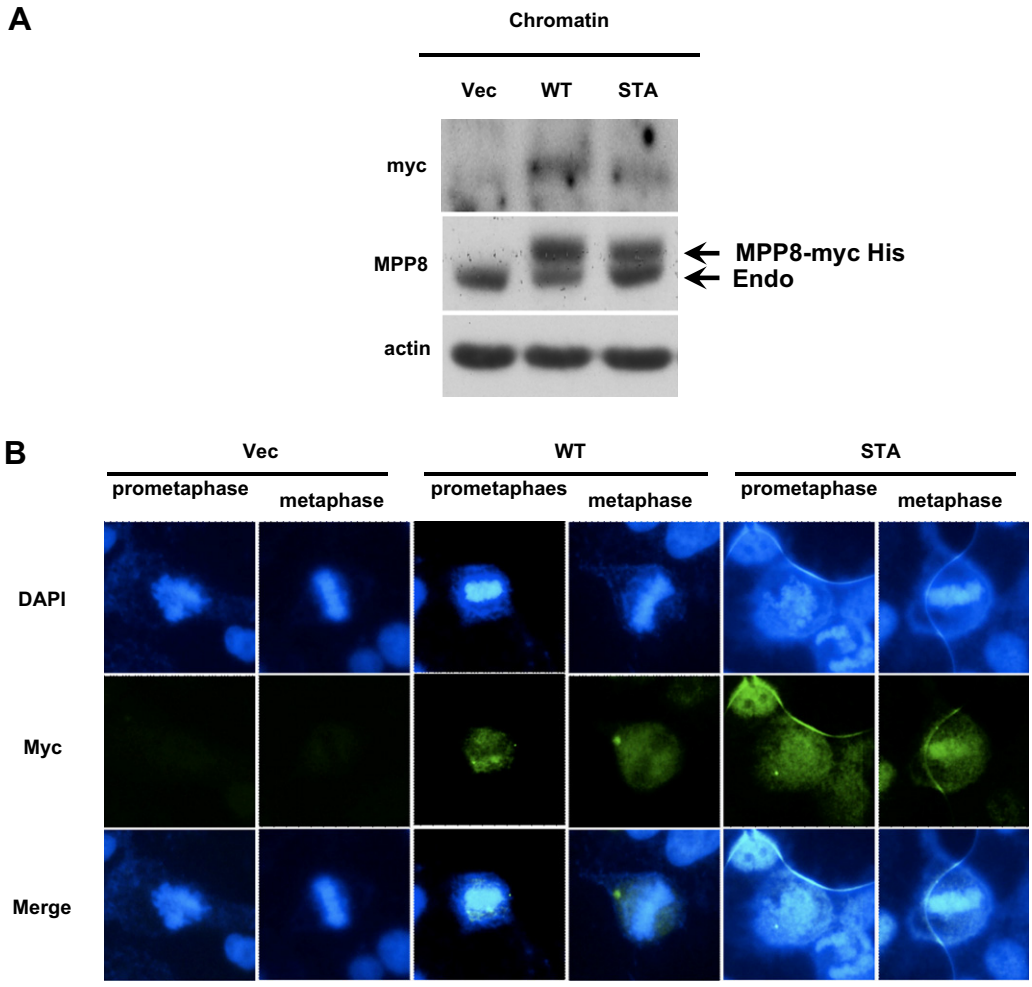

Fig. 4. MPP8 with mutations at CDK phosphorylation sites localized on metaphase chromosomes. (A) Asynchronously growing HeLa cells were transiently transfected with expression vectors expressing MPP8 WT, MPP8 STA (S149A, S164A, T334A and T385A) or empty vector. Three days after transfection, the cells were collected for chromatin fractionation and immunoblotting was performed using the indicated antibodies. (B) The transfected cells described in (A) were fixed and stained with anti-myc monoclonal antibody and the nuclei were counterstained with DAPI. Representative images during prometaphase to metaphase are shown.

MPP8 revealed that although wild-type MPP8 dissociated from chromatin during early mitosis, its STA mutant retained chromatin during this period (Fig. 4B). Thus, the results suggested that Cdk1-mediated phosphorylation of MPP8 regulated its chromatin dissociation during early mitosis.

In summary, our results clearly demonstrated that chromatin localization of MPP8 was regulated by cyclin B1-Cdk1-dependent phosphorylation during early mitosis. As to the physiological role of MPP8 dissociation from mitotic chromatin, removal of MPP8 might be important for enhancing the accessibility of factors essential for mediating proper chromosomal condensation and segregation during early mitosis. In this respect, a similar mitotic dissociation of HP1α from chromosomal arms was proposed to be essential for maintaining the proper structure of mitotic chromosomes [7–9], although dissociation of HP1α from mitotic chromatin was mainly regulated by Aurora B-mediated H3 phosphorylation at Ser 10. This dissociation might also include further modifications of H3-tail (H3 acetylation at lysine 14) [10,22]. In this regard, the binding of MPP8 chromodomain to methylated H3K9 is also reduced in the presence of H3pS10 [23]. Thus, chromatin dissociation of heterochromatin organizers during early mitosis may be commonly required for maintaining a proper heterochromatin structure during the course of the cell cycle regardless of the regulatory mechanisms.

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