

**Phosphoproteomic analysis of human mitotic chromosomes identified
a chromokinesin KIF4A**

Motoko Takahashi^{1,2}, Kozo Tanaka³, Toshifumi Wakai², and Toru Hirota^{1*}

¹Division of Experimental Pathology, Cancer Institute of the Japanese Foundation for Cancer Research (JFCR), Tokyo 135-8550, Japan; ²Division of Digestive and General Surgery, Niigata University Graduate School of Medical and Dental Sciences, Niigata 951-8112, Japan; ³Department of Molecular Oncology, Institute of Development, Aging and Cancer, Tohoku University, Miyagi 980-8575, Japan

*Corresponding author: thirot@jfc.or.jp

Running title:

Mitotic phosphorylation of KIF4A

Abstract

Protein phosphorylation is the prime post-translational modification to drive cell division. Identification of phosphorylated proteins and their related kinases has uncovered molecular networks underlying mitotic processes, including chromosome assembly. Here we aimed to identify phosphoproteins from a mitotic chromosome-enriched lysate biochemically using mass spectrometry. We employed the Polo-box domain (PBD) of Polo-like kinase to tether phosphorylated proteins in the lysate. Resulting candidates included a number of chromosomal proteins that would not be identified unless using chromosome-enriched fractions. Among them, we focused to a chromokinesin KIF4A which becomes concentrated along the longitudinal axis of mitotic condensed chromatids. We found that KIF4A is phosphorylated specifically during mitosis, depending on the activity of Cdk1 and Aurora B, which turned out to be required for KIF4A to interact with condensin I. The molecular link between KIF4A and condensin raises an interesting possibility that the interaction of two distantly related ATPases is triggered by mitotic phosphorylation.

Transmission of the genome depends on assembly and segregation of chromosomes in mitosis. A series of mitotic events is primarily processed in time and space by the regulated protein phosphorylations that ensure the reversible and dynamic control of mitosis. Phosphorylation typically induces a change in protein conformation and triggers protein complex formation, which affects its function and/or localization. It is therefore crucial to characterize the kinases and their substrates to elucidate molecular grounds of mitotic processes.

Mitotic protein phosphorylation is mediated by a subset of kinases collectively called mitotic kinases, which include cyclin-dependent kinase 1 (Cdk1), Polo-like kinase 1 (Plk1) and Aurora B. Cdk1 is the master kinase that drives mitosis from its entry, whereas Plk1 and Aurora B control assembly and segregation of chromosomes in the presence of Cdk1 activity (7). For example, the first step of mitotic chromosome assembly is initiated by a Cdk1-mediated phosphorylation of the condensin II, the protein complex that plays crucial role in shaping chromosomes. The conserved phosphorylation site is found to be Thr1415 of the CAP-D3 subunit of condensin II,

whose phosphorylation then triggers recruitment of Plk1 to the chromosome axes through binding to CAP-D3 and thereby hyperphosphorylates the condensin II complex (1).

The phospho-proteomics has been a powerful approach that provides comprehensive lists of protein phosphorylation (3, 5, 8). It is not necessarily straightforward, however, to link the information to cellular functional and/or structural significance. In this study, we aimed to study phosphoproteins involved in mitotic chromosome assembly by searching for phosphoproteins specifically in a mitotic chromosome-enriched fraction. We used a phosphoprotein-binding module called the Polo-box domain (PBD) to identify mitotic phosphoproteins. The resulting PBD-associating proteins expectedly included subunits of cohesin and condensin complexes, and also contained a kinesin named KIF4A, which is an axial component of chromosomes. Our data suggest that mitotic phosphorylation of KIF4A facilitates its association with condensin I, which presumably is a protein interaction promoting chromosome assembly in mitosis.

The mitotic kinase Plk1 directly targets its substrate by recognizing a priming phosphorylation (2). A bipartite domain termed the PBD in the carboxyl-terminal half of Plk1 mediates the binding to phospho-serine (pS) or -threonine (pT) meeting the consensus motif, S-pS/pT-P/X, on substrates (Figure 1A). Because this motif includes the consensus for Cdk1-phosphorylation site (pS/pT-P), a screen of PBD-interacting proteins would plausibly identify substrates for Cdk1 as well as for Plk1, as has been suggested previously (5). We generated recombinant PBD encompassing 305-603 amino acids of human Plk1 in bacteria as a GST-fusion protein. We prepared PBD not only wild-type (WT) but also a mutant carrying H538A and K540M (MUT) to disrupt the binding interface (5). PBD were then released from GST and were coupled to NHS beads (Figure 1A).

To obtain mitotic chromosome-enriched lysates, mitotic HeLa cells were collected using double thymidine block and release protocol (18-24 h-treatment with 2 mM thymidine (Sigma-Aldrich), release for 8 h, and 16 h-treatment with thymidine)

followed by 50 ng/mL nocodazole (Sigma-Aldrich) treatment. To prepare chromosome-enriched lysates, cells were treated with a hypotonic buffer consisting of 40: 60 mix (v/v) of PBS and distilled water for 5 min, centrifuged at 5,000 rpm for 5 min at 4°C. The resulting pellets were lysed in the IP buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM MgCl₂, 20 mM β-glycerophosphate, 1 mM DTT, 5% glycerol, 0.1% NP-40) containing 0.1 μM okadaic acid, protease inhibitors (cOmplete™, Mini, EDTA-free, Roche) and 1: 100 (v/v) nuclease (OmniCleave™ endonuclease, ARB-LS) for 20 min on ice. The lysates were obtained as a high-speed supernatant (15,000 rpm, 30 min).

To minimize non-specifically bound proteins, lysates were first incubated with the MUT-PBD columns sequentially for three times, and then incubated with the WT-PBD column (Figure 1B). After being washed with the IP buffer, proteins bound to the WT-PBD column and the first MUT-PBD column (as a negative control) were eluted with 1.2 M NaCl. The samples were resolved by SDS-PAGE followed by silver staining. Bands specifically emerged in the WT-PBD sample were subjected to mass

spectrometry analysis (LC-MS/MS), which identified candidates of phosphorylated chromosomal proteins. We could estimate our experimental setup appropriate as topoisomerase 1 (TOP1) was identified in WT-PBD bound sample as previously reported (5). The candidates interestingly included proteins required for chromosome structure and function (Figure 1B). Immunoblotting analysis confirmed that these proteins indeed bind specifically to WT-PBD. For example, KIF4A is found in WT-PBD bound sample but not in MUT-PBD bound (Figure 1C).

KIF4A is a kinesin superfamily protein also called chromokinesin, based on its characteristic localization to chromatid axes in mitosis (Figure 2A). The function of KIF4A is thought to be involved in shaping mitotic chromosomes, as depletion of KIF4A caused a curly morphological change of chromosome axial structure (Figure 2B; 6, 9). However, KIF4A's mechanistic role in organizing mitotic chromosomes and its regulation are not well understood, and thus we focused our study to KIF4A in further analyses. As shown in the schematic representation (Figure 2C), KIF4A is predicted to have three domains: an N-terminal kinesin-like motor domain, an alpha-helical

coiled-coil stalk domain and a C terminal unstructured tail domain which binds to ‘cargo’ proteins to transport them. Some of the amino acid sequence of KIF4A fits the Cdk1 or Aurora B phosphorylation motifs, including one within the PBD binding motif (Figure 2C).

To examine the phosphorylation status of KIF4A during the cell cycle, synchronous cell populations from early S phase to late M phase were obtained by collecting the cells at 0, 3, 6, 8, 9, 10 h-time points after the release from double thymidine block and were subjected to immunoblotting analysis. Equivalent amounts of total cell extracts were resolved by SDS-PAGE, transferred onto a PVDF membrane and stained with KIF4A antibodies (1: 500, rabbit polyclonal, Bethyl) for over night at 4°C and probed with horseradish peroxidase-labeled secondary antibodies (GE Healthcare). In mitosis-enriched samples (8, 9, 10 h after the release), there appeared a shifted species with slower mobility, suggesting that KIF4A is phosphorylated in mitosis (Figure 2D).

To address which kinase is involved in this phosphorylation(s), we repeated the immunoblotting analysis of mitotic cells after being treated with inhibitors of mitotic kinases, including 9 μ M of Cdk1 inhibitor RO-3306 (Roche), 100 nM of Plk1 inhibitor BI-2536 (Tocris), or 5 μ M of Aurora B inhibitor ZM-447439 (Tocris). Under these conditions, we found that mitotic band shift was partially reversed by Cdk1 and Aurora B inhibitors, suggesting the involvement of these two kinases in mitotic phosphorylation of KIF4A. By contrast, inhibition of Plk1 had less effect on the mitotic mobility of KIF4A (Figure 2D).

Finally we wished to address the effect of these mitotic phosphorylations on KIF4A. As previous studies suggested an interaction of KIF4A with condensin, a protein complex driving mitotic chromosome assembly (6, 9), we asked if Cdk1- or Aurora B-mediated phosphorylation of KIF4A might affect the interaction of KIF4A and condensin. To address this possibility, mitotic cells with or without inhibitor pretreatment were subjected to an immunoprecipitation assay with antibodies to CAP-G subunit of condensin I (antibodies were generated in Abe et al. (1))(Figure 3). To keep

cells in mitotic stages, inhibition of Cdk1 or Aurora B combined with a proteasome inhibitor treatment, 10 μ M of MG132 (Peptide Institute). The electrophoretic mobility shifts of CDC27, phosphorylation of CAP-D3 at Ser1419 and of histone H3 at Ser10 are known to depend on Cdk1, Plk1 and Aurora B, respectively (1), and thus we could verify that these pretreatments efficiently abolished the activities of the targeted kinases. We found that the amount of coimmunoprecipitated KIF4A was significantly decreased by pretreatment of Cdk1 inhibitor and partially by Aurora B inhibitor. These results indicate that the activity of Cdk1 and Aurora B promotes the interaction of KIF4A and condensin I in mitosis (Figure 3).

From chromosome-enriched extracts, we could obtain a list of mitotic chromosomal phosphoproteins, which would not appear if we used total cell extracts (5). Based on cellular phenotype obtained after depleting KIF4A, we extended our study to this chromokinesin, which exemplifies how the molecular information might be able to link to functions. Our data show that KIF4A is phosphorylated dependently on Cdk1 and Aurora B in mitosis, and these phosphorylations facilitate KIF4A and condensin I

interaction. To clarify whether these phosphorylations on KIF4A *per se* promote this interaction requires a detailed identification of phosphorylation sites and generation of mutants to perturb the phosphorylations. Generation of such mutant would also specifically address the cellular significance of this interaction for mitotic chromosome assembly. Aurora B is an essential kinase to shape chromosomes in mitosis and it does so by promoting the loading of chromosomal proteins such as condensin I (4, 10). It would therefore be interesting to address if chromosomal loading of condensin I requires a binding to KIF4A, and if this interaction is promoted by Aurora B activity. Elucidating how the motor activity of KIF4A is involved is a challenging following question in the future, which must provide an as-yet-unappreciated mechanism for chromosome organization.

Acknowledgments

We are grateful to Akira Yasui and Shinichiro Kanno (Tohoku University, Miyagi) for mass spectrometry analysis. Research in the laboratory of T.H. is supported by grants

from the Japan Society for the Promotion of Science (JSPS) and the Ministry of
Education, Culture, Sports and Technology of Japan (MEXT).

References

1. Abe S, Nagasaka K, Hirayama Y, Kozuka-Hata H, Oyama M, Aoyagi Y, Obuse C and Hirota T (2011) The initial phase of chromosome condensation requires Cdk1-mediated phosphorylation of the CAP-D3 subunit of condensin II. *Genes Dev.* 25, 863-874.
2. Elia AE, Rellos P, Haire LF, Chao JW, Ivins FJ, Hoepker K, Mohammad D, Cantley LC, Smerdon SJ and Yaffe MB (2003) The molecular basis for phosphodependent substrate targeting and regulation of Plks by the Polo-box domain. *Cell* 115, 83-95.
3. Hegemann B, Hutchins JR, Hudecz O, Novatchkova M, Rameseder J, Sykora MM, Liu S, Mazanek M, Lénárt P, Hériché JK, Poser I, Kraut N, Hyman AA, Yaffe MB, Mechtler K and Peters JM (2011) Systematic phosphorylation analysis of human mitotic protein complexes. *Sci Signal.* 4, rs12.
4. Lipp JJ, Hirota T, Poser I and Peters JM (2007) Aurora B controls the association of condensin I but not condensin II with mitotic chromosomes. *J Cell Sci.* 120, 1245-1255.
5. Lowery DM, Clauser KR, Hjerrild M, Lim D, Alexander J, Kishi K, Ong SE, Gammeltoft S, Carr SA and Yaffe MB (2007) Proteomic screen defines the Polo-box domain interactome and identifies Rock2 as a Plk1 substrate. *EMBO J.* 26, 2262-2273.
6. Mazumdar M, Sundareshan S and Misteli T (2004) Human chromokinesin KIF4A functions in chromosome condensation and segregation. *J Cell Biol.* 166, 613-620.
7. Nigg EA (2001) Mitotic kinases as regulators of cell division and its checkpoints.

Nat Rev Mol Cell Biol 2, 21-32.

8. Nousiainen M, Silljé HH, Sauer G, Nigg EA and Körner R (2006) Phosphoproteome analysis of the human mitotic spindle. *Proc Natl Acad Sci U S A* 103, 5391-5396.
9. Samejima K, Samejima I, Vagnarelli P, Ogawa H, Vargiu G, Kelly DA, de Lima Alves F, Kerr A, Green LC, Hudson DF, Ohta S, Cooke CA, Farr CJ, Rappsilber J and Earnshaw WC (2012) Mitotic chromosomes are compacted laterally by KIF4 and condensin and axially by topoisomerase II α . *J Cell Biol.* 199, 755-770.
10. Takemoto A, Murayama A, Katano M, Urano T, Furukawa K, Yokoyama S, Yanagisawa J, Hanaoka F and Kimura K (2007) Analysis of the role of Aurora B on the chromosomal targeting of condensin I. *Nucleic Acids Res.* 35, 2403-2412.

Figure Legends

Fig. 1 Identification of phosphoproteins on mitotic chromosomes. **(A)** Schematic diagram of the PBD which recognizes phosphorylated substrates through bipartite PBD-binding (PB) modules. Mutant PBD bearing indicated amino acid substitutions in PB2 does not bind to substrates. **(B)** Experimental strategy for identifying PBD ligands on mitotic chromosomes. WT- and MUT-PBD-bound chromosomal proteins were separated by SDS-PAGE and visualized by silver staining. Mass spectrometric analysis identified some of them as indicated. **(C)** Specific interaction of KIF4A with WT-PBD. Mitotic chromosome-enriched extracts were subjected to a pull down assay with WT- or MUT-PBD. Resulting pull down fractions were immunoblotted for KIF4A.

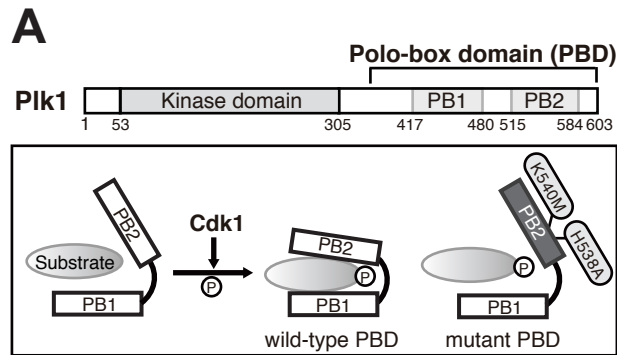
Fig. 2 KIF4A is a chromosomal phosphoprotein required to organize chromosome axis. **(A)** Enrichment of KIF4A to chromosome axes. Logarithmically growing HeLa cells were fixed and stained with KIF4A antibodies and a DNA dye DAPI (4',6-Diamidino-2-phenylindole). Representative cells from indicated phases of the cell cycle are shown. Note that KIF4A predominantly localizes in the nucleus during interphase, and in mitosis, from prophase to telophase, KIF4A become enriched in

chromosome axes, and in the mid-zone during cytokinesis. Scale bar, 10 μm . **(B)**

Chromosome axis formation was disturbed in KIF4A depleted cells. To suppress KIF4A expression HeLa cells were transfected with 50 nM siRNA duplex (5'-UCCACCUCAGGAAUGAGGUUGUGAU-3') using RNAiMAX reagent (Life Technologies). Mitotic cells were hypotonically treated with 75 mM KCl for 30 min at 37 °C, followed by Carnoy's fixation. To process for immunofluorescence microscopy chromosomes spread on glass slides were first neutralized with 1.5 M Tris-HCl (pH 8.5) for 30 min, and stained with TOP2 α antibodies (1: 1000, rabbit polyclonal, Abcam) to probe the chromosome axis. Scale bar, 1 μm . **(C)** Schematic representation of KIF4A. The proposed consensus phosphorylation motifs for Cdk1 and Aurora B and binding motif of PBD are indicated. **(D)** Mitosis-specific phosphorylation of KIF4A is sensitive to Cdk1 and Aurora B inhibitors.

Fig. 3 The activity of Cdk1 and Aurora B promotes the interaction of KIF4A and condensin I in mitosis. Mitotic HeLa cells enriched by inhibiting kinesin 5, Eg5, (7.5 μM S-trityl-L-cysteine) were pretreated with Cdk1, Plk1 or Aurora B inhibitors, and

immunoprecipitated with antibodies to CAP-G, a subunit of condensin I. The efficiencies of inhibitors were validated by CAP-D3 p1419 (for Plk1), CDC27 (Cdk1 and Plk1), H3Ser10 (Aurora B), respectively as in Fig. 2D.



B PBD pull down

