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Phosphorylation of the chromodomain changes the binding specificity of Cbx2 for methylated histone H3

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ABSTRACT

The chromatin organizer modifier domain (chromodomain) is present in proteins that contribute to chromatin organization and mediates their binding to methylated histone H3. Despite a high level of sequence conservation, individual chromodomains manifest substantial differences in binding preference for methylated forms of histone H3, suggesting that posttranslational modification of the chromodomain might be an important determinant of binding specificity. We now show that mouse Cbx2 (also known as M33), a homolog of *Drosophila* Polycomb protein, is highly phosphorylated in some cell lines. A low-mobility band of Cbx2 observed on SDS–polyacrylamide gel electrophoresis was thus converted to a higher-mobility band by treatment with alkaline phosphatase. Mass spectrometric analysis revealed serine-42, a conserved amino acid in the chromodomain, as a phosphorylation site of Cbx2. Phosphorylation of the chromodomain of Cbx2 on this residue in vitro resulted in a reduced level of binding to an H3 peptide containing trimethylated lysine-9 as well as an increase in the extent of binding to an H3 peptide containing trimethylated lysine-27, suggesting that such phosphorylation changes the binding specificity of Cbx2 for modified histone H3. Phosphorylation of the chromodomain of Cbx2 may therefore serve as a molecular switch that affects the reading of the histone modification code and thereby controls epigenetic cellular memory.

1. Introduction

Chromatin structure is thought to be an important determinant of the expression state of many genes, representing an epigenetic form of cellular memory that provides continuity in the specific pattern of gene expression from mother to daughter cells. This epigenetic memory is achieved by differential packaging of the genome in a manner dependent on posttranslational modification—such as methylation, acetylation, and phosphorylation—of histones as well as on the effects of such modification on the interaction of histones with other regulatory proteins [1]. Polycomb group (PcG) proteins are among the most well characterized players in such regulation and maintain gene expression in a repressed state [2]. PcG proteins are essential for normal cell proliferation and have been implicated in the maintenance of stem cells [3].

In *Drosophila*, PcG proteins form two major complexes. PC, Ph, Psc, and dRing form Polycomb repressive complex 1 (PRC1) [4], whereas Esc, E(z), Su(z)12, and P55 form PRC2 [5,6]. Vertebrate homologs of PC, the core member of PRC1, contain a chromatin organizer modifier domain (chromodomain), are referred to as chromobox (Cbx) proteins, and include Cbx2, Cbx4, Cbx6, Cbx7, and Cbx8 [7]. The Cbx proteins Cbx1, Cbx3, and Cbx5 are homologs of *Drosophila* heterochromatin protein 1 (HP1). The chromodomain contributes to targeting of proteins to specific regions of chromatin and is present in many proteins that participate in chromatin organization, including Su(var)3-9, Swi6, CHD1 to -9, MSL-3, and MOF in addition to Cbx proteins [8]. The chromodomain of *Drosophila* PC exhibits preferential binding to histone H3 trimethylated at lysine-27 (H3K27me3), a mark that is generated by PRC2, whereas the chromodomain of HP1 recognizes histone H3 trimethylated at lysine-9 (H3K9me3) [9–13]. Despite a high level of sequence conservation, however, the chromodomains of Cbx proteins manifest substantial differences in binding preference [14,15]. For example, Cbx2 and Cbx7 bind to both H3K9me3 and H3K27me3, whereas Cbx4 shows high affinity for H3K9me3 [16]. Together with the

Abbreviations: GST, glutathione S-transferase; ES, embryonic stem; PAGE, polyacrylamide gel electrophoresis; MALDI, matrix-assisted laser desorption–ionization; TOF, time-of-flight; CK2, casein kinase 2; CIAP, calf intestinal alkaline phosphatase.

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finding that mutation of the chromodomain of PC results in the disintegration of PRC1 and loss of its silencing activity [17], these observations suggest that subtle differences in the sequence, conformation, or modification of chromodomains may result in differential affinity for differentially methylated forms of histone such as H3K9me3 and H3K27me3.

Among the Cbx proteins, we have focused on Cbx2 (also known as M33), which has been implicated in maintenance of the inactive X chromosome in the mouse [18]. We now show that a conserved residue in the chromodomain of Cbx2 is phosphorylated in certain cell types. Phosphorylation of this residue resulted in a change in the binding specificity of Cbx2 for methylated histone H3. Our results suggest that the binding specificity of Cbx proteins for methylated histones is regulated by phosphorylation.

2. Materials and methods

2.1. Plasmid constructs and recombinant protein expression

Mouse Cbx2 cDNA was inserted into pCSII and pFLAG vectors (Sigma Aldrich). For generation of pCSII-Cbx2, Cbx2 cDNA was amplified with the forward primer 5′-GAATTCAATGGAGGAGCTGA GCAGCG-3′ and the reverse primer 5′-CTCGAGTCAATAATGCCTCA AGTTGA-3′, digested with *Eco*RI and *Xho*I, and inserted into pCSII. For generation of pFLAG-Cbx2, Cbx2 cDNA was amplified with the forward primer 5′-GAATTCATGGAGGAGCTGAGCAGCGT-3′ and the reverse primer 5′-GGTACCATAATGCCTCAAGTTGAAGA-3′, digested with *Eco*RI and *Kpn*I, and inserted into pFLAG. A cDNA encoding the chromodomain of Cbx2 (amino acids 11–61) was amplified by the polymerase chain reaction with the forward primer 5′-GAATTCA GTCTTCGCCGCCGAGTGC-3′ and the reverse primer 5′-GCGGCCG CCTTCTTCTGGAAGGCTA-3′, digested with *Not*I and *Eco*RI, and inserted into the pGEX4T 3 vector (GE Healthcare). The glutathione S-transferase (GST) fusion protein of the Cbx2 chromodomain encoded by the resulting vector was produced in *Escherichia coli* JM109 and purified from bacterial lysates with the use of glutathione–Sepharose 4B (GE Healthcare).

2.2. Cell culture and transfection

NIH 3T 3, F9, and COS7 cells were cultured under an atmosphere of 5% CO_2 at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Mouse E14 embryonic stem (ES) cells were cultured under an atmosphere of 6% CO_2 at 37 °C in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum. COS7 cells were transfected with pCSII-Cbx2 or pFLAG-Cbx2 with the use of Lipofectamine 2000 (Invitrogen); they were lysed with TNE buffer (150 mM Tris–HCl [pH 7.5], 1 M NaCl, 1 mM EDTA, 1% NP-40) 48 h after transfection, and the lysate was centrifuged at 17,500*g* for 20 min at 4 °C to separate soluble and insoluble fractions. The insoluble fraction was subjected to ultrasonic treatment in SDS sample buffer (50 mM Tris–HCl [pH 6.8], 2% SDS, 10% glycerol, 2.5% 2-mercaptoethanol) and boiled for 5 min.

2.3. Tissue preparation

Tissues from 10-week-old C57BL/6 male mice were homogenized in a solution containing 50 mM Tris–HCl (pH 7.5), 25 mM sucrose, and 1 mM EDTA, and each homogenate was mixed with an equal volume of RIPA buffer (20 mM Tris–HCl [pH 7.5], 150 mM NaCl, 2% NP-40, 1% sodium deoxycholate, 0.2% SDS) before centrifugation at 17,500*g* for 20 min at 4 °C. The resulting supernatant was diluted with SDS sample buffer for analysis by SDS–polyacrylamide gel electrophoresis (PAGE).

2.4. Antibodies and biotinylated peptides

Rabbit polyclonal antibodies specific for Cbx2 were described previously [19], and mouse monoclonal antibodies specific for GST or for HSP90 were obtained from MBL and BD Biosciences, respectively. Biotin-conjugated methylated or unmodified histone H3 peptides were obtained from Millipore.

2.5. Alkaline phosphatase treatment

Alkaline phosphatase treatment was performed as previously described [20]. Cells were lysed in alkaline phosphatase lysis buffer (100 mM Tris–HCl [pH 8.0], 100 mM NaCl, 5 mM $MgCl_2$, 1% SDS, 2 mM phenylmethylsulfonyl fluoride), subjected to ultrasonic treatment, and boiled for 5 min. The lysate was then diluted with alkaline phosphatase buffer (100 mM Tris–HCl [pH 8.0], 100 mM NaCl, 5 mM $MgCl_2$) and incubated at 37 °C with calf intestinal alkaline phosphatase (Takara Biomedicals). The reaction was terminated by the addition of 20 μl of 2 × SDS sample buffer.

2.6. Mass spectrometric analysis

Peptides were prepared according to standard procedures. In brief, protein bands on SDS–PAGE gels were excised and cut into cubes of ~1 mm^3. The gel fragments were washed sequentially with acetonitrile and ammonium bicarbonate, and the protein therein was then subjected to reduction and carbamidomethylation with dithiothreitol and iodoacetamide, respectively. After sequential washing of the gel fragments with methanol, acetonitrile, and ammonium bicarbonate, the protein was treated with trypsin at 37 °C for 16 h. Phosphorylated peptides were selectively concentrated from peptides eluted from the gel with the use of a titanium dioxide column (GL Science), purified with the use of a ZipTip device (Millipore), coupled with α-cyano-4-hydroxycinnamic acid (Sigma Aldrich), and spotted onto a matrix-assisted laser desorption–ionization (MALDI) plate. Analysis was performed by MALDI–time-of-flight (TOF) mass spectrometry with a Shimadzu CFR-plus instrument.

2.7. In vitro phosphorylation

A recombinant GST fusion protein containing the chromodomain of Cbx2 (5 μg) was subjected to in vitro phosphorylation for 1 h at 37 °C in a final volume of 50 μl with 10 U of rat liver casein kinase 2 (CK2) (Promega) in CK2 reaction buffer (25 mM Tris–HCl [pH 7.5], 10 mM $MgCl_2$, 200 mM NaCl, 400 μM ATP).

2.8. Peptide pull-down assays

Peptide "pull-down" assays were performed as described previously [16]. The recombinant GST fusion protein containing the chromodomain of Cbx2 (5 μg), with or without phosphorylation, was incubated for 3 h at 4 °C with biotinylated peptides derived from histone H3 in assay buffer (150 mM KCl, 20 mM HEPES–KOH [pH 7.9], 0.2% Triton X-100), and the avidin-beads (Thermo Scientific) were subsequently washed eight times with assay buffer before analysis by SDS–PAGE.

3. Results

3.1. Cbx2. is highly phosphorylated in cell lines

We examined the expression of Cbx2 in several mouse cell lines and mouse tissues by immunoblot analysis. Cbx2 was abundant in the thymus and liver, but was not detected in the other tissues

examined (Fig. 1A). It was also found to be present in F9 embryonal carcinoma cells, ES cells, and NIH 3T three fibroblasts, but its electrophoretic mobility in these cells was reduced compared with that apparent in the liver (Fig. 1B). The slowly migrating band appeared relatively broad and heterogeneous, suggesting that it might contain multiple modified forms of Cbx2. To test whether this mobility shift might be attributable to phosphorylation of Cbx2 in the cell lines, we treated F9 cell lysate with calf intestinal alkaline phosphatase (CIAP). The broad and slow-migrating Cbx2 band was transformed into a sharper and faster-migrating band after treatment of the lysate with CIAP (Fig. 1C), supporting the notion that phosphorylation of Cbx2 at multiple sites was indeed responsible for the mobility shift. Similar results were obtained for endogenous Cbx2 in NIH 3T 3 cells (Fig. 1D) as well as for exogenous (overexpressed) Cbx2 in COS7 cells (Fig. 1E). These data thus suggested that Cbx2 is highly phosphorylated in cultured mammalian cell lines.

3.2. Phosphorylation of a conserved amino acid in the chromodomain of Cbx2

To identify the phosphorylation sites of Cbx2, we partially purified the protein on the basis of its insolubility. An insoluble fraction prepared from COS7 cells transfected with pFLAG-Cbx2 was subjected to SDS–PAGE, and the band corresponding to Cbx2-FLAG was subjected to in-gel digestion with trypsin (Fig. 2A). Phosphorylated peptides were enriched by column chromatography with titanium dioxide and analyzed by MALDI–TOF mass spectrometry (Fig. 2B). Four principal phosphorylated peptides were identified on the basis of their molecular mass and the presence of neutral loss (Fig. 2B and C). The peptide corresponding to amino acids 40–53 is included within the chromodomain of Cbx2 and contains Ser^{42} as the only serine, threonine, or tyrosine residue. We therefore concluded that Ser^{42}, located in the middle of the chromodomain, is a phosphorylation site of Cbx2. We also identified either Ser^{37} or Ser^{38} as a phosphorylation site in the vicinity of Ser^{42}. The other peptides contain several serine or threonine residues, and we did not determine their phosphorylation sites in this study.

All PC homologs have an NH_2-terminal chromodomain and a COOH-terminal Polycomb repressor box [7]. Comparison of amino acid sequences revealed that Ser^{42} of mouse Cbx2 is conserved at the corresponding position in Cbx2 proteins of other vertebrates such as *Homo sapiens*, *Xenopus laevis*, and *Danio rerio* (Fig. 3A). In other mouse Cbx2 paralogs (Cbx4, -6, -7, and -8) as well as HP1 homologs (Cbx1, -3, and -5), the amino acid at the position corresponding to Ser^{42} of Cbx2 is threonine and the amino acids surrounding this residue are well conserved (Fig. 3B), suggesting that the serine or threonine at this position may undergo phosphorylation in all members of the Cbx family of proteins. Indeed, Cbx1 (also known as HP1β) was recently shown to be phosphorylated at Thr^{51} [21], the residue corresponding to Ser^{42} of Cbx2. In contrast, the amino acid corresponding to Ser^{37} of Cbx2 is not conserved in Cbx6 and Cbx7, and Ser^{38} of Cbx2 is not conserved in any other Cbx protein. Together, these observations suggested that phosphorylation of the chromodomain at the amino acid corresponding to Ser^{42} of Cbx2 might regulate the biological function of this domain.

3.3. Phosphorylation of the chromodomain of Cbx2 affects binding specificity for methylated histone H3

The size or negative charge of the phosphate group attached to Ser^{42} in the groove of the chromodomain might thus be expected to affect the association of Cbx2 with methylated histone H3. Given that the chromodomain of Cbx2 interacts with H3K9me3 and H3K27me3, we examined the effect of Ser^{42} phosphorylation on the association of Cbx2 with unmodified or trimethylated histone H3. For this analysis, we used a recombinant GST fusion protein containing the chromodomain of Cbx2 that had been phosphorylated (or not) at Ser^{42} in vitro by CK2. The unphosphorylated chromodomain of Cbx2 preferentially associated with H3K9me3 rather than with unmodified H3K9 (Fig. 4A and B). However, phos-

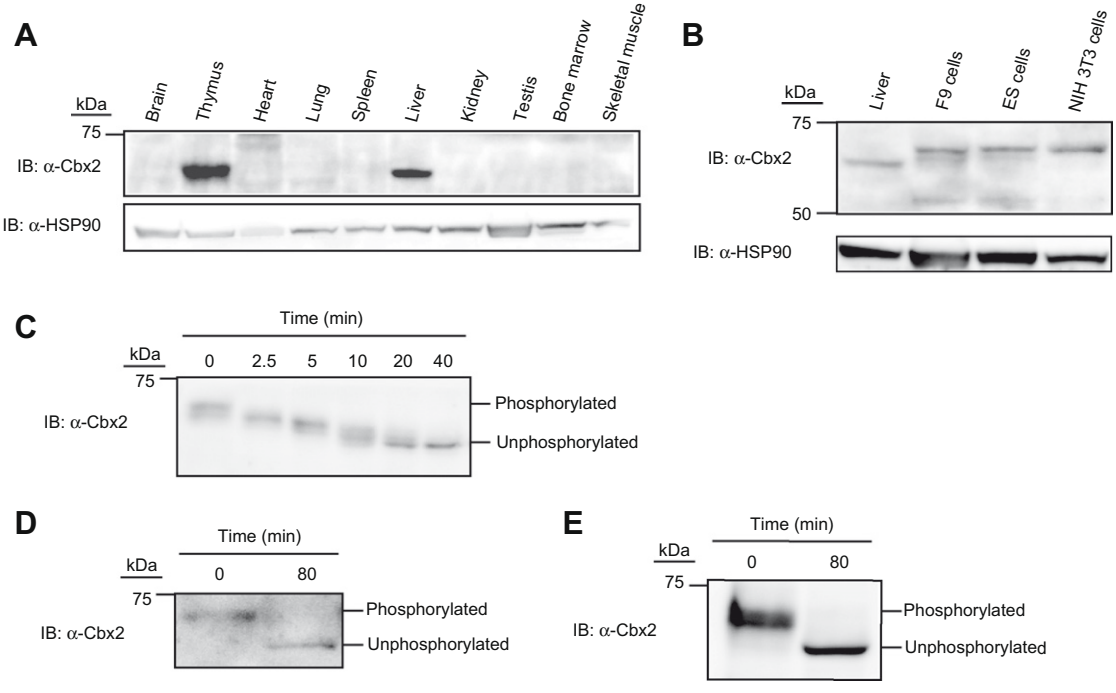
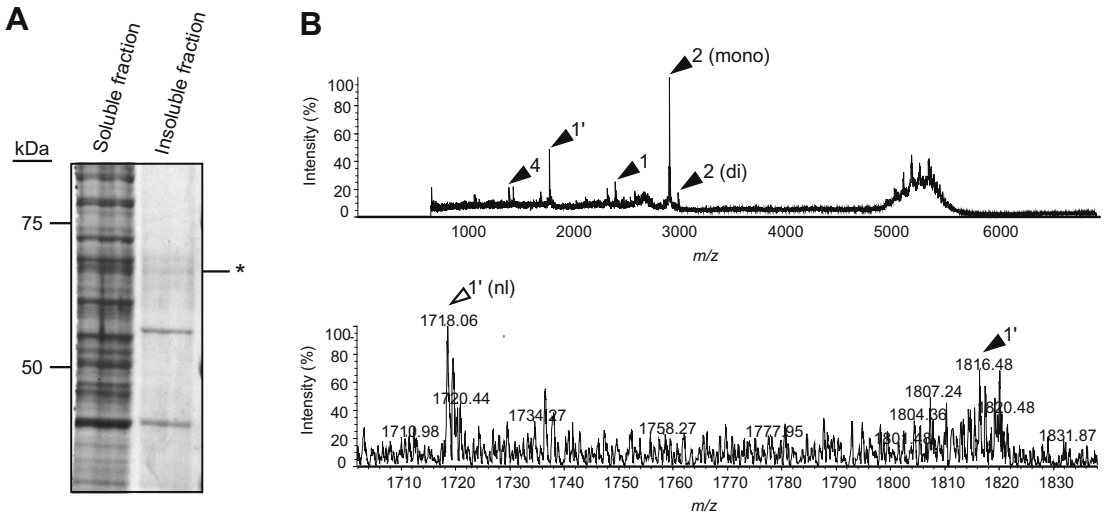


Fig. 1. Expression and phosphorylation of Cbx2 in mouse tissues and cell lines. (A,B) The indicated mouse tissues (A) or cell lines (B) were subjected to immunoblot (IB) analysis with antibodies to (α-) Cbx2 and to HSP90 (loading control). (C–E) Lysates of F9 cells (C), NIH 3T 3 cells (D), or COS7 cells transfected were pCSII-Cbx2 (E) were incubated with CIAP for the indicated times and then subjected to immunoblot analysis with antibodies to Cbx2. The positions of bands corresponding to phosphorylated and unphosphorylated Cbx2 are indicated.



C

Peptide	Mean (*m/z*)	Start residue	End residue	Peptide sequence	No. of phosphorylated residues	Phosphorylated residues
1	2442.4087	35	53	GWSSKHNSWEPEENILDPR	2	Either Ser^{37} or Ser^{38}, and Ser^{42}
1'	1816.8293	40	53	HNSWEPEENILDPR	2	Ser^{42}
2	3034.4282	323	450	GNHSGSPGAQLAPTQELSLQV LDLQSVK	1 or 2	One or any two serines and threonines
3	4932.2661	438	484	PPALSELSTGEENSSSDSDPDS TSLPSAAQNLSVAIQTSQDWKP TR	2	One seine or threonine
4	1434.4943	508	517	ESPTSVGFFNLR	2	One seine or threonines

Fig. 2. Identification of phosphorylated peptides and amino acids of Cbx2 by MALDI–TOF mass spectrometry. (A) SDS–PAGE profile (8% gel) of soluble and insoluble fractions prepared from COS7 cells expressing Cbx2-FLAG. The gel was stained with Coomassie brilliant blue, and the asterisk indicates the band corresponding to Cbx2-FLAG. (B) The band corresponding to Cbx2-FLAG in (A) was excised, subjected to digestion with trypsin, and analyzed by MALDI–TOF mass spectrometry (upper panel); numbered arrowheads indicate phosphopeptides. The post-source decay spectrum of the phosphorylated peptide 1′ (HNSWEPEENILDPR, *m/z* of 1816.8293) is also shown (lower panel); phosphoserine- or phosphothreonine-specific neutral loss (nl) was observed at 1718.06 Da (open arrowhead). (C) Phosphorylated peptides identified for Cbx2-FLAG expressed in COS7 cells.

phorylation of the chromodomain by CK2 abolished this difference in the extent of binding to unmodified or trimethylated forms of H3K9. The unphosphorylated chromodomain of Cbx2 also showed a moderate preference for H3K27me3 rather than for unmodified H3K27, and this preference was increased by CK2 treatment. These results thus suggest that phosphorylation of Ser^{42} in the chromodomain increases the affinity of Cbx2 for H3K27me3 and reduces that for H3K9me3 (Fig. 4C).

4. Discussion

Cell type-specific patterns of gene expression are set during development, and PcG proteins are thought to maintain these expression patterns [1–3]. *Drosophila* PC and its mammalian Cbx homologs share an NH_2-terminal domain (the chromodomain) that mediates association with methylated histones and are integral components of PcG complexes [7]. Despite their high level of sequence similarity, however, the chromodomains of Cbx proteins manifest marked differences in binding preferences for methylated histone H3. We have now found that Cbx2 is highly phosphorylated in a cell type-specific manner, and that phosphorylation of Ser^{42} in the chromodomain results in a change in the binding specificity of Cbx2 for methylated histone H3. The chromodomain of Cbx1 (HP1β) was also recently shown to undergo phosphorylation by CK2 on Thr^{51}, resulting in disruption of a hydrogen-bond network surrounding associated H3K9me3 and thereby reducing the capacity of Cbx1 to bind to chromatin via trimethylated H3K9 [21]. Phosphorylation of the Cbx1 chromodomain on Thr^{51} facilitates mobilization of Cbx1 from chromatin after DNA breakage. These findings suggest that not only the amino acid sequence but also modification of chromodomains may contribute to determination of binding partners. Indeed, the combination of the multiplicity of Cbx proteins and their modifications may allow the interaction of chromodomains with a large number of binding targets.

In contrast to the fixed amino acid sequence of a protein, phosphorylation of specific amino acid residues is inducible and reversible. Phosphorylation of the chromodomain may therefore be subject to signal-dependent regulation and result in a change in binding specificity for modified histones and consequently affect cell fate. We found that the phosphorylation of Cbx2 occurs in a cell type- or tissue-specific manner, suggesting that the binding

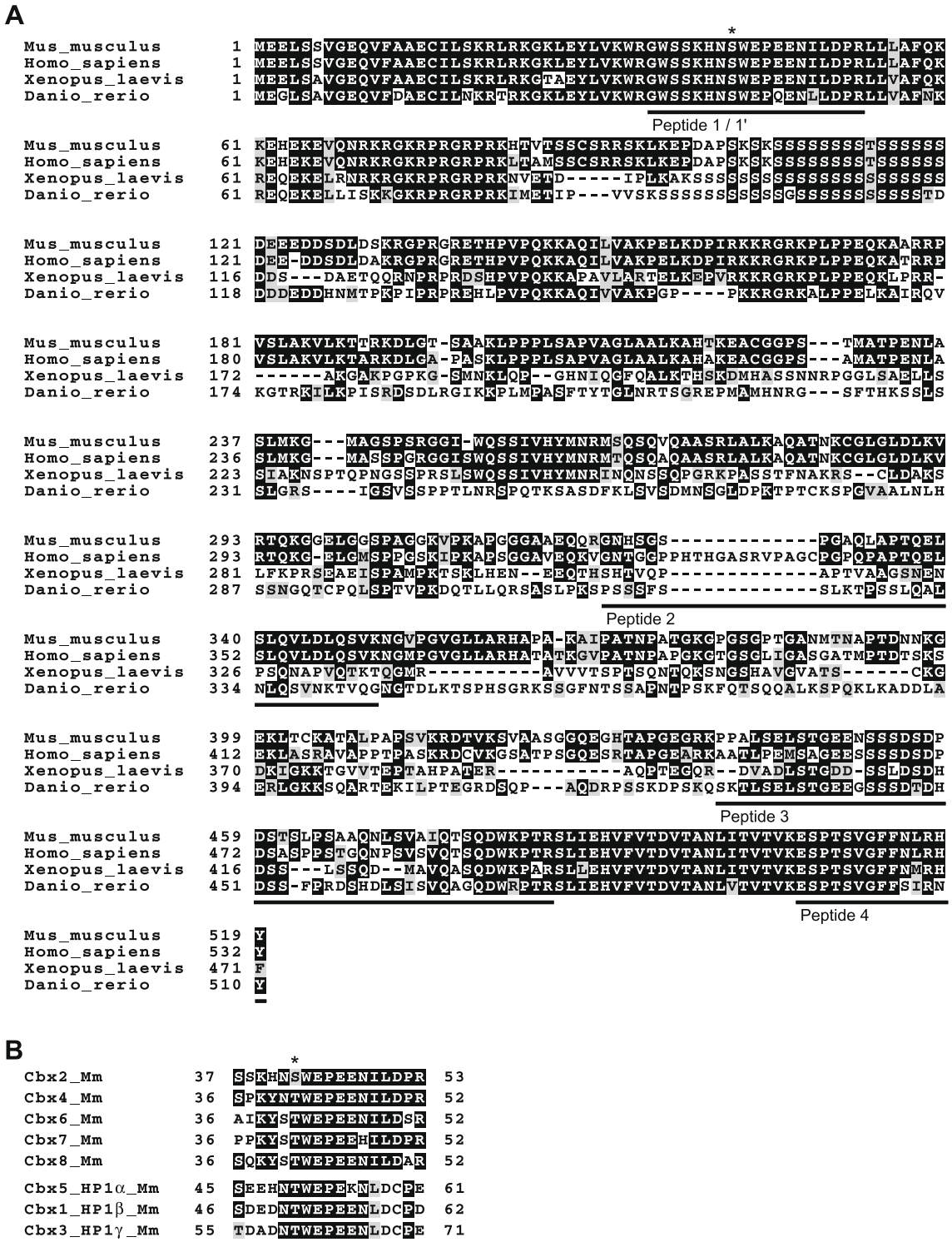


Fig. 3. Comparison of Cbx2-related polypeptide sequences. (A) Alignment of full-length Cbx2 sequences of vertebrates with the use of the CLUSTAL X program. Identical or similar residues appear on a black or gray background, respectively. Numbers to the left of the sequences indicate the position of the leftmost amino acid. Phosphopeptides identified by mass spectrometric analysis are underscored. (B) Sequence alignment of the chromodomain of mouse Cbx proteins that are homologs of *Drosophila* PC or HP1. The asterisk above all sequences indicates the position of the Ser^{42} phosphorylation site of mouse Cbx2. GenBank accession numbers: *Mus musculus* Cbx2, NP_031649; *Homo sapiens* Cbx2, NP_005180; *Danio rerio* Cbx2, AAI64860; *Xenopus laevis* Cbx2, NP_001081900; *Mus musculus* Cbx1, NP_031648.1; *Mus musculus* Cbx3, NP_031650.3; *Mus musculus* Cbx4, NP_031651.2; *Mus musculus* Cbx5, NP_031652.1; *Mus musculus* Cbx6, NP_083039.2; *Mus musculus* Cbx7, NP_659060.1; and *Mus musculus* Cbx8, NP_038954.1.

targets of the Cbx2 chromodomain differ among cells depending on whether Cbx2 is phosphorylated or not. The biological consequences of this phosphorylation during development remain to be elucidated, for instance with knock-in mouse models that harbor a mutation of the *Cbx2* gene that results in the replacement of Ser^{42} with a nonphosphorylatable (alanine) or phosphomimetic (aspartate or glutamate) residue. The kinase (or kinases) responsible for the phosphorylation of Cbx2 in vivo also remains to be identified, although the recombinant Cbx2 chromodomain was found to be effectively phosphorylated by CK2 in vitro. CK2 also phos-

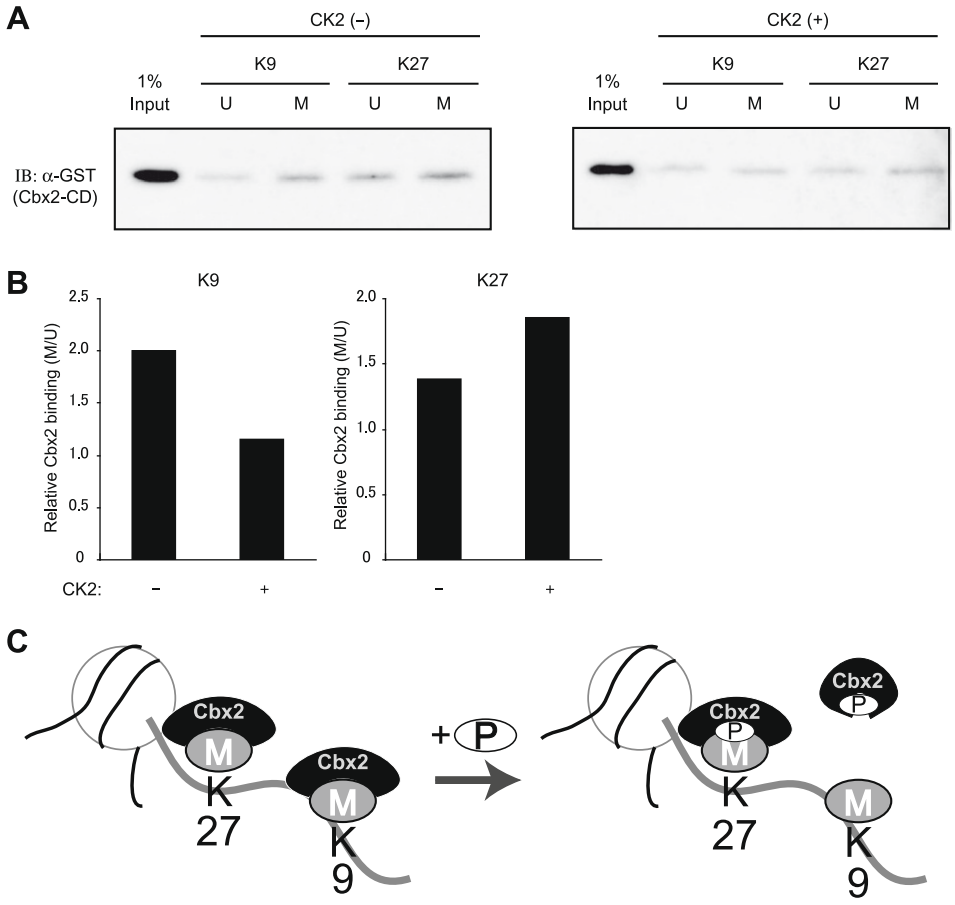


Fig. 4. Phosphorylation-dependent regulation of the binding specificity of the Cbx2 chromodomain for methylated histone H3. (A) A recombinant GST fusion protein containing the chromodomain of Cbx2 (Cbx2-CD) was phosphorylated (or not) with CK2 in vitro and then subjected to a pull-down assay with histone H3 peptides containing unmodified (U) or trimethylated (M) forms of K9 (residues 1 to 21) or K27 (residues 21–44). The pull-down samples, as well as a portion (1%) of the fusion protein input to the binding mixture, were subjected to immunoblot analysis with antibodies to GST. (B) The immunoblots in (A) were subjected to image analysis with ImageJ software, and the extent of Cbx2 binding to each trimethylated peptide normalized by that to the corresponding unmodified peptide was determined. (C) Model for the phosphorylation-dependent regulation of Cbx2 binding specificity for methylated histone H3.

phorylates the corresponding residue (Thr^{51}) in the chromodomain of Cbx1 [21]. In addition, Cbx8 has been shown to associate with CK2 [22].

Cbx2 was found to be phosphorylated in cell lines that proliferate rapidly in culture, whereas it was not phosphorylated in most tissues examined. A kinase associated with the cell cycle might therefore be responsible for phosphorylation of the chromodomain of Cbx2. Alternatively, a phosphatase that reverses the phosphorylation of Cbx2 may be expressed during terminal differentiation. Although examination of additional cell lines and tissues will be necessary to provide further insight into the biological role of phosphorylation of the Cbx2 chromodomain, our finding that phosphorylation of Ser^{42} in this domain changes the binding specificity of Cbx2 for modified histone H3 provides a biochemical basis for further analysis of how this phosphorylation event might contribute to the epigenetic control of cell fate.

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