CBX8 interacts with chromatin PTEN and is involved in regulating mitotic progression

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Abstract

Objectives: Besides its role in regulating phosphatidylinositol-3 kinase (PI3K) signaling in the cytosol, PTEN also has a nuclear function. In this study, we attempted to understand the mechanism of chromatin PTEN in suppressing chromosomal instability during cell division.

Materials and methods: Immunocoprecipitation, ectopic expression, and deletional analyses were used to identify the physical interaction between Chromobox Homolog protein 8 (CBX8) and PTEN, as well as the functional domain(s) of PTEN mediating the interaction. Cell synchronization followed by immunoblotting was employed to study cell cycle regulation of CBX8 and the functional interaction between chromatin PTEN and CBX8. Small interfering RNAs (siRNAs) were used to study the role of PTEN and CBX8 in modulating histone epigenetic markers during the cell cycle.

Results: Polycomb group (PcG) proteins including CBX8 function to repress gene expression in a wide range of organisms including mammals. We recently showed that PTEN interacted with CBX8, a component of Polycomb Repressing Complex 1 (PRC1), and that CBX8 co-localized with PTEN in the nucleus. CBX8 levels were high, coinciding with its phosphorylation in mitosis. Phosphorylation of CBX8 was associated with monoubiquitinated PTEN and phosphorylated-BubR1 on chromatin. Moreover, CBX8 played an important role in cell proliferation and mitotic progression. Significantly, downregulation of either PTEN or CBX8 induced H3K27Me3 epigenetic marker in mitotic cells.

Conclusion: CBX8 is a new component that physically interacts with chromatin PTEN, playing an important role in regulating mitotic progression.

1 INTRODUCTION

Phosphatase and tensin homolog (PTEN) is one of the best characterized tumour suppressors because it is mutated at a high frequency in a variety of human malignancies and inherited PTEN mutations cause cancer-susceptibility conditions.1–4 Biochemically, PTEN dephosphorylates the lipid second messenger phosphatidylinositol 3,4,5-trisphosphate to generate phosphatidylinositol 3,4-bisphosphate and, by doing so, antagonizes the PI3K/Akt signalling pathway. PTEN protein levels and its activity in the cell profoundly influence cell growth, survival, and tumour susceptibility.5,6 In the past several years, we have focused on studying the nuclear
function of PTEN. We have shown that PTEN plays a role in the maintenance of chromosomal stability by modulating DNA repair and anaphase-promoting complex/cyclosome (APC/C) activity.

Extensive studies in the past have shown that Polycomb group (PcG) proteins including CBXs function to repress gene expression in a wide range of organisms including mammals. Chromodomain-containing CBXs are a group of structurally conserved proteins, known to be integral components of Polycomb Repressive Complex 1 (PRC1). The CBX family proteins are named after the Chromobox located at its N terminus, which primarily recognizes and binds to trimethylated lysine 27 of histone H3 (H3K27Me3). The CBX family proteins also share a conserve domain termed C box at the C-terminus. Different from CBX2, CBX4, CBX6, and CBX8, CBX7 does not have C box and is expressed primarily in pluripotent cells. Other than Chromodomain and C box, CBXs share little resemblance in their structures. It has been postulated that the differences in domain structures among CBXs may allow them to function in regulating gene expression in a tissue-specific and/or temporal manner.

PcG proteins play an important role in maintaining the repressed transcriptional states and chromosomal architectures during differentiation and cell cycle progression. Extensive studies have shown that the PRC1 complex interacts with both interphasic chromatin and mitotic chromosomes. It has been shown that the residence time of PcG proteins on the mitotic chromatin is about 300-fold longer than that of interphasic chromatin, suggestive of a drastically different binding dynamics of PRC1 proteins with chromatin between interphase and mitosis. However, it remains relatively unclear how PRC1 complex is maintained on mitotic chromosomes and what molecular component(s) mediates their interaction with chromosomes during mitosis.

Recent studies have revealed that histone methylation is a reversible process. Methylation of specific histone residues and the extent of methylation (mono-, di-, and/or trimethylation) on the same residues are either critical for regulating gene expression or marks of heterochromatin and euchromatin in various organisms. Extensive studies have shown that the PRC1 complex interacts with both interphasic chromatin and mitotic chromosomes. It has been shown that the residence time of PcG proteins on the mitotic chromatin is about 300-fold longer than that of interphasic chromatin, suggestive of a drastically different binding dynamics of PRC1 proteins with chromatin between interphase and mitosis. However, it remains relatively unclear how PRC1 complex is maintained on mitotic chromosomes and what molecular component(s) mediates their interaction with chromosomes during mitosis.

Recent studies have revealed that histone methylation is a reversible process. Methylation of specific histone residues and the extent of methylation (mono-, di-, and/or trimethylation) on the same residues are either critical for regulating gene expression or marks of heterochromatin and euchromatin in various organisms. It is well also known that histone methylations play a crucial role in cell cycle progression and cell division. Recent studies have shown that PTEN expression is correlated with trimethylation of Lysine 27 on histone H3. However, it remains unclear how chromatin PTEN may regulate histone modifications, thus modulating epigenetics during the cell cycle. Mitosis is a unique cell cycle phase in which duplicated chromosomes are highly condensed. This high order of chromatin compaction is particularly important for accurate chromosomal segregation and genomic stability maintenance during cell division. Because histones, primarily as scaffold proteins, function to facilitate the formation of high order structures of chromatin, we propose that the methylation status (and/or its extent) of specific lysine residues on histones is crucial for mitotic progression and chromosomal stability during cell division.

Upon mitotic entry, a protein entity termed mitotic checkpoint complex (MCC) is formed, regulating chromosomal congression and segregation. MCC consists of core spindle checkpoint proteins including Cdc20, Mad2, BubR1, and Bub3. MCC can be assembled during mitosis without kinetochore localization. We have previously shown that PTEN accumulates in the nucleus during mitosis and that it is also a component of MCC. To further elucidate the mechanism by which nuclear PTEN functions as important molecule in the maintenance of chromosomal stability during cell division, we have recently focused on PRC1 components as they are known to repress gene expression and may play a role in mitotic progression. We identified CBX8 as a new PTEN-interacting protein. Among the family member of CBXs, CBX8 displays the strongest interaction with PTEN and MCC. CBX8 expression is high and phosphorylated during mitosis. In addition, whereas ectopic expression of CBX8 reduces H3K27Me3 silencing CBX8 or PTEN greatly induces the epigenetic marker in mitotic cells.

2 | MATERIALS AND METHODS

2.1 | Cell culture and transfection

HEK293T (human embryonic kidney) and HeLa (human cervical carcinoma) cell lines obtained from the American Type Culture Collection were cultured in DMEM supplemented with 10% foetal bovine serum (FBS, Invitrogen) and antibiotics (100 µg/ml of penicillin and 50 µg/ml of streptomycin sulphate, Invitrogen) at 37°C under 5% CO2. Transfection of individual cell lines was achieved with either Lipofectamine 2000 (Invitrogen) or Fugene HD (Roche Diagnostics) following the manufacturers’ protocol. Transfection efficiency was estimated to be between 80% and 100% in all cases through co-transfecting a GFP expressing plasmid (Data not shown).

2.2 | Plasmids and siRNAs

CBX expression plasmids were obtained from Dr Guoxiang Chen’s laboratory at Shanghai Jiao-Tong University. Various PTEN and deletion expression constructs were as described in our earlier studies. Human ON-TARGETplus SMARTpool siRNA oligonucleotides that specifically target CBX8 (5’-CUCCUCUUGCUCCAGCCCUU-3’, 5’-GGAAAGGACGCAUGGAAUA-3’, 5’-GGCCUUCGAAACAUGGGUU-3’, 5’-GCCUUUGAAACAUGGGUU-3’, and 5’-GAGAGUGAGCGUGACGCUU-3’) and PTEN (5’-CGAUAGCAUUGCAGAUAA-3’, 5’-GAUCGAGCAUAACAAAUA-3’, 5’-GACUAGACUUGACCUUA-3’, 5’-GAUCUUGACCAUUAGCUUA-3’, and 5’-CCAUAGAUA UUGACAAAGA-3’) were purchased from Dharmacon. Pool of siRNAs was transfected into cells with Dharmafect I according to the protocol provided by the supplier. Briefly, cells seeded at 50% confluence in an antibiotic-free culture medium were transfected with siRNA duplexes at a final concentration of 100 nM for 24 h. siRNA controls which include two nucleotide changes from the target sequences were designed as described in previously and used as negative control for transfection.
2.3 | Cell cycle synchronization

HeLa cells were synchronized at the mitosis by thymidine-nocodazole block as described previously. Briefly, cells were treated with 2 mM thymidine (Sigma-Aldrich) for 24 h followed by a 3 h release; these cells were then treated with nocodazole (50 ng/ml; Sigma-Aldrich) for another 18 h. Mitotic shake-off cells were obtained from gentle tapping of cell culture dishes. For nocodazole release, cells were washed three times with 1X PBS, then split onto cell culture dishes with complete medium. Cells were collected at various time points after release.

2.4 | Protein extraction and immunoblotting

Total cell lysates were prepared in a buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% IGEPAL, 0.1% SDS, and 0.5% sodium deoxycholate] supplemented with a mixture of protease and phosphatase inhibitors. Protein concentrations were measured using the bicinchoninic acid protein assay reagent kit (Pierce Chemical Co). Equal amounts (20ug) of protein lysates from various samples were used for SDS–PAGE analysis followed by immunoblotting. Antibodies to cyclin B1 (12231), cyclin A2 (4656), cyclin D3 (2936), vinculin (13901), Eg5 (14404), Histone H3 K27 Me3 (9733), Histone H2A K11 Ub1 (8240), phospho-Histone H3 (Ser 10) (3377), DYKKDDDK (FLAG Tag) (14793), BubR1 (4116), CBX8 (14696), PARP-1 (9542), α-Tubulin (2144), Histone H2A (12349), phospho-Histone H2A (2577) and β-Actin (4970) were purchased from Cell Signaling Technology. Antibodies to MAD2 (A300-301A) and PTEN (A300-700A) were obtained from Bethyl laboratories. Specific signals on immunoblots (polyvinylidene difluoride) were visualized using enhanced chemiluminescence (Super-Signal, Pierce Chemical Co.).

2.5 | Immunoprecipitation

Cells were lysed in TBSN buffer [20 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.5% NP-40, 5 mM EGTA, 1.5 mM EDTA, 0.5 mM Na3VO4, and 20 mM β-Glycerol phosphate]. The cell lysates were clarified by centrifugation at 15 000 x g for 20 min at 4°C. Cleared lysates (1 mg) were added to FLAG M2 agarose (Sigma) followed by incubation in the TBSN buffer for 1 h at 4°C. After incubation, resins were thoroughly washed with the binding buffer and proteins bound to resin eluted in the SDS-PAGE sample buffer. A fraction of eluted sample was also analysed by SDS-PAGE.

2.6 | Lambda phosphatase assay

Cells were harvested and pellets were lysed in lambda phosphatase lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1 mM EGTA, 1 mM dithiothreitol, 2 mM MnCl2, 0.01% Brij35, 0.5% NP-40, and protease inhibitor) for 30 min at 4°C. Lysates were incubated with or without 30 U of λ-phosphatase 1 h at room temperature.

2.7 | Cell proliferation assay

Cells were seeded onto 96-well plates (2 x 10^3 cells per well) and transfected with siControl or siCBX8 for 24 h. Proliferation was measured using a Cell Counting kit-8 (Dojindo Molecular Technologies) with the water-soluble tetrazolium salt WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] as a substrate.

2.8 | Immunofluorescence microscopy

Fluorescence microscopy was performed essentially as described. Briefly, cells were fixed with 4% paraformaldehyde (w/v) for 30 min, washed 3 times in PBS-T (PBS-Tween 0.1%), and permeabilized with 0.1% Triton X-100 in PBS before blocking with 4% Bovine serum albumin (BSA) in PBS-T for 30 min. Primary antibodies were incubated for 1 h, and secondary antibodies conjugated to Alexa-Fluor 488 and 555 were incubated for 30 min at room temperature in 4% BSA. DAPI staining was performed for 10 min in PBS. After washing, coverslips were dried and mounted on glass slides. Fluorescence microscopy was performed using a Leica Confocal Laser Scanning Microscopes SP5 and images were analysed by Image J.

2.9 | Statistical analysis

Each experiment was performed at least three times. The data were plotted as the mean ± SD Student’s t test was used for all comparisons. A P value of less than 0.05 was considered statistically significant.

3 | RESULTS

3.1 | PTEN interacts with phosphorylated CBX8

We have previously shown that nuclear PTEN plays an important role during mitosis. We have also observed that downregulation of PTEN via RNAi enhances the formation of MCC during the cell cycle. To understand the molecular basis by which PTEN mediates mitotic progression, we first determined the kinetics of MCC components during mitotic progression after PTEN silencing. We observed that in the absence of PTEN, MCC components including Bub1, Cdc20, Mad2, and Bub3 were either inactivated or decreased slower in PTEN-depleted cells than in control cells (Figure 1A). Specifically, Cdc20 degradation was significantly delayed after PTEN silencing (Figure 1A and Fig. S1A). Consistent with this observation,
both Mad2 and Bub3 also displayed high basal levels in the absence of PTEN. Moreover, BubR1 inactivation (dephosphorylation) was slowed down in cells with PTEN silencing, and a significant amount of sumoylated BubR1 was detected in PTEN-silenced cells but not in control cells (Figure 1A). These observations strongly suggest that PTEN regulates mitotic progression partly through regulating the MCC complex.

To understand the molecular mechanism by which chromatin PTEN regulates mitosis, we attempted to identify new gene products that interacted with PTEN. We first focused on chromobox homolog proteins (CBXs) as recent studies show that they play an important role in regulating the cell cycle, as well as chromosomal structures.\(^\text{45,46}\) We transfected HEK293T cells with expression plasmid constructs encoding FLAG-tagged CBX2, CBX4, CBX6, CBX7, or CBX8. Equal amounts of cell lysates transfected with various CBX constructs were immunoprecipitated with the anti-FLAG antibody. FLAG immunoprecipitants, along with cell lysate inputs, were blotted with antibodies to PTEN, BubR1, Cdc20, Mad2, and FLAG. We observed that CBX8 pulled down PTEN, as well as MCC components (BubR1, Cdc20, and Mad2), more efficiently than did with other CBX family members (Figure 1B), suggesting that CBX8 physically interacts with PTEN and MCC. To further confirm the physical interaction between CBX8 and PTEN, protein lysates were prepared from mitotic cells (rounded up) and asynchronous cells (AS) and immunoprecipitated with the CBX8 antibody or with IgG. We observed that CBX8 immunoprecipitants specifically pulled down PTEN and BubR1 and that mitotic CBX8 was more efficient in precipitating PTEN and BubR1 (Figure 1C). We also noted that mitotic CBX8 displayed a slow mobility on the denaturing gel compared with the interphase one.

We next determined if the electrophoretic mobility shift of mitotic CBX8 is due to phosphorylation. Extracts of mitotic cells treated with or without λ-phosphatase were blotted for CBX8. We observed that mitotic CBX8 with a slow mobility was collapsed to the interphase form after λ-phosphatase treatment (Figure 2A). As a control, no effect of the phosphatase treatment on the mobility was observed with vinculin. These results thus confirm that CBX8 is phosphorylated during mitosis. To determine whether dephosphorylation of CBX8 coincided with the mitotic exit, mitotic cells were released into the cell cycle and cell lysates collected at various times post-release were blotted for CBX8, as well as for cell cycle components. As expected, mitotic exit was manifested by the disappearance of cyclin B1, phospho-H3, and cyclin A2 (Figure 2B). Likewise, BubR1 was dephosphorylated and rapidly degraded as well. Mitotic exit was also accompanied by an increase in cyclin D3 (Figure 2B), a G1 phase marker. Further, we observed that CBX8 was dephosphorylated and degraded in a manner similar to that of BubR1 during the

![Figure 1](image-url)

**Figure 1** Phosphatase and tensin homolog regulates MCC and mitotic progression. (A) HeLa cells were transfected with PTEN-specific siRNAs or control siRNAs for 24 h and then treated with nocodazole (Noc) for 16 h, after which mitotic cells were collected by shake-off. After washing, mitotic cells were released into the cell cycle. At various times post-release, cells were lysed and equal amounts of cell lysates were blotted for PTEN and MCC components including BubR1, Cdc20, Mad2 and Bub3. Modified (sumoylated or phosphorylated) forms of BubR1 are indicated. HeLa cell line was used here because it has been widely recognized for studying cell cycle regulation, largely because of ease of collecting mitotic cells via shake-off. (B) HEK293T cells were transfected for 24 h with plasmid constructs expressing FLAG-tagged CBX2, CBX4, CBX6, CBX7 or CBX8. After transfection, cell lysates were immunoprecipitated with the anti-FLAG antibody. FLAG immunoprecipitants, along with lysate inputs, were blotted with the anti-FLAG antibody and antibodies to BubR1, Cdc20, Mad2 and PTEN. HEK293T cells were used here because these cells are amenable to transfection. (C) HeLa cells were treated with nocodazole for 16 h, after which mitotic cells were collected by shake-off. Cell lysates of asynchronously (AS) cells and mitotic cells (rounded up, R. up) were immunoprecipitated with the anti-CBX8 antibody or with control IgG. CBX8 Immunoprecipitants, along with lysate inputs, were blotted for CBX8, BubR1 and PTEN.
To define the domain of PTEN that mediated the interaction with CBX8, we made a series of PTEN deletion constructs as shown in Figure 3A. Full-length PTEN and its deletion constructs were sub-cloned as GST-fusion constructs. We co-transfected HEK293T cells for 24 h with FLAG-CBX8 construct and a GST-PTEN construct (or individual PTEN deletion constructs). Equal amounts of cell lysates from various transfections were immunoprecipitated with the FLAG antibody. FLAG immunoprecipitates and the lysates containing unbound antigen were washed and released into the cell cycle for various times as indicated. Cell lysates were blotted for CBX8, BubR1, cyclin B1, phosphorylated H3 (p-H3), cyclin A2, cyclin D3 and β-actin transition into the interphase (Figure 2B and Fig. S1B). Combined, these results strongly suggest that CBX8 phosphorylation may facilitate the binding of both PTEN and BubR1, playing an essential role in regulating mitotic progression.

3.2 | Both C2 and C-terminal domain of PTEN mediate the interaction between PTEN and CBX8

Phosphatase and tensin homolog is essential for cell proliferation.\textsuperscript{48,49} Our previous studies have shown that PTEN is required for proper mitotic timing and cell cycle progression by regulating MCC.\textsuperscript{39,40,43} To determine whether CBX8, a PTEN-interacting protein, affected cell proliferation, we transfected cells with CBX8 siRNAs, or control siRNAs, and then measured cell proliferation rate. We observed that cells transfected with CBX8 siRNAs displayed a significantly reduced cell proliferation when they were compared with that of the control cells (Figure 4A). We then investigated the effect of ectopic expression of CBX8 on mitosis. Cells transfected with a plasmid expressing FLAG-tagged CBX8, or empty vector, were treated with or without nocodazole. Asynchronized and mitotic cells were analysed for a panel of mitotic markers, as well as two histone modification markers. As expected, nocodazole treatment greatly enriched mitotic markers including cyclin B1, Eg5, and phospho-H3 (Figure 4B and Fig. S1C). However, ectopic expression of CBX8 decreased phospho-H3 signals, as well as other mitotic markers such as cyclin B and Eg5, suggesting that CBX8 may promote mitotic exit. We also noticed that nocodazole treatment suppressed levels of H3K27Me3.
and H2AK119Ub1 but enhanced H3K79Me3 (Figure 4B, Fig. S1C and Fig. S2) and that expression of CBX8 further reduced the level of H3K27Me3 in mitotic cells (Figure 4B and Fig. S1C), suggesting that CBX8 may have an effect on histone H3 modification.

To further determine whether CBX8 had an impact on histone modifications, we first measured levels of H3K27Me3 and H2AK119Ub1 during mitotic release. We observed that whereas H2AK119Ub1 levels were low in mitotic cells H3K27Me3 levels were undetectable (Figure 5A). However, during mitotic release, both histone modification markers increased albeit with different kinetics. H2AK119Ub1 increased in a pattern similar to that of cyclin D3, suggesting that its modification is associated with G1 phase progression. On the contrary, H3K27Me3 signals rapidly increased after mitotic exit, peaking around 4-h post-mitotic release. Mitotic release was efficient as shown by various cell cycle markers.

Phosphatase and tensin homolog is known to have a role in regulating chromatin dynamics and gene expression. To determine the underlying mechanism by which nuclear PTEN and CBX8 regulate chromatin remodelling and gene expression during mitosis, we transfected cells with PTEN siRNAs and/or CBX8 siRNAs for 24 h followed by nocodazole treatment for 16 h. Mitotic cells were analysed for H3K27Me3 and H2AK119Ub1, and downregulation of either PTEN or CBX8 resulted in a significant induction of H3K27Me3, suggesting that PTEN and CBX8 suppress this epigenetic modification during mitosis. Supporting this, downregulation of both PTEN and CBX8 further increased H3K27Me3 signals (Figure 5B, Fig. S1D and Fig. S2). On the contrary, H2AK119Ub1 signals were only slightly reduced after downregulation of PTEN, but not CBX8. Further, silencing of either
PTEN or CBX8 increased phosphorylated H2A, suggesting that these proteins may play a role in protecting cells from DNA damage during mitosis.\textsuperscript{52,53}

Based on our studies, we propose the following model that depicts the regulation of chromatin modifications by PTEN and CBX8 (Figure 6). As part of MCC, PTEN recruits phosphorylated CBX8 during early mitosis; MCC-associated PTEN/CBX8 negatively regulates trimethylation of H3K27, which appears to be an important step in mitotic progression and exit.

4 | DISCUSSION

It is well known that PTEN also resides in the nucleus, functioning in the maintenance of genomic stability in the cell cycle.\textsuperscript{4,7,10,39,40,43} In the past, great efforts have been made to understand the molecular basis by which nuclear PTEN regulates gene expression, chromatin dynamics, and chromosomal stability.\textsuperscript{51,54} However, it remains largely unclear regarding how PTEN functions in the nucleus. Here, we show that PTEN interacts with CBX8, a Chromobox protein.
It is known that CBX family proteins are integral components of Polycomb Repressive Complexes 1 and 2 (PRC1 and PRC2) and are required for targeting PRC1 to the chromatin. The observation that PTEN interacts with CBX8 suggests that chromatin PTEN may be involved in suppressing gene expression, especially during mitosis. Given that PRC1 and PRC2 are required for maintaining the stemness of embryonic stem cells and many types of adult stem cells, it is conceivable that deregulated activities of PRC1 and PRC2 would have detrimental effects in development, as well as in malignant transformation and tumour development. This line of research may shed light on PTEN’s additional role in functioning as a tumour suppressor because of its potential role in modulating activities of PRC1 and PRC2.

Among CBX family proteins, CBX8 exhibits the highest affinity with PTEN. Although CBX family members share Chromodomain and Polycomb Repressive box, they display specificities in either undifferentiated and/or differentiated stem cells, which is likely though an interplay between compositional diversity of PRC1 and PRC2. We have demonstrated that CBX8 primarily interacts with the C2 domain of PTEN with potential assistance of C-tail (Figure 3A–C). PTEN-C2 domain is post-translationally modified such as phosphorylation. It is conceivable that phosphorylation plays an important role in the regulation of CBX8 function. Supporting this, we have observed that CBX8 is also phosphorylated during mitosis and dephosphorylated after entering G1 (Figure 2B). Furthermore, CBX8 phosphorylation by PIM1 has been described during PIM1-induced senescence. It is conceivable that phosphorylation plays an important role in the regulation of CBX8 function.

The MCC plays an important role in mitosis by binding to the kinetochore, regulating chromosomal congression and segregation. Centromeres are specialized domains of heterochromatin that provide the foundation for forming the kinetochores during mitosis. Centromeric heterochromatin is rather unique as it contains CENP-A (a histone H3 variant), specific histone modifications, and cohesins, as well as checkpoint components including MCC in mitosis. The fact that CBX8 specifically pulldown BubR1, Cdc20 and Mad2 supports the notion that it may have a function in chromosomal congression and segregation, as well as mitotic exit.

Several studies have shown that CBX8 expression is correlated with tumour progression and metastasis, which is consistent with its role in the maintenance of stemness. CBX8 was significantly overexpressed in chemoresistant colon cancer tissues which appears to be due to upregulation of LGR5. It is known that LGR5 is a G protein-coupled receptor 5 rich containing leucine-rich repeat and functioning as a candidate marker of cancer stem cell populations. It has also been shown that aberrant expression of LGR5 is a major alteration in human malignancies due primarily to upregulation of canonical Wnt/β-catenin signalling. Consistent with this notion, CBX8 overexpression is observed in hepatocellular carcinoma, correlating with poor outcome and it efficiently activated Akt/β-catenin signalling. Here we demonstrate that CBX8 physically interacts with PTEN, a protein integrally involved in negative regulation of the PI3K/Akt signalling. On the contrary, our studies strongly suggest a role of CBX8 in mediating a nuclear role of PTEN during mitotic progression. Obviously, further studies are needed to fully understand how CBX8 regulates chromatin compaction and suppresses gene expression during mitosis and how dysregulated CBX8 activities may promote transformation and tumour development.

Post-translational modifications of histone tails are crucial for normal mitotic entry and progression. For example, it is well known that histone H3S10 is heavily phosphorylated during mitosis and that this phosphorylation may play essential role in regulating molecular processes mediated by H3K9 methylation in vivo. In addition, histone acetylation is required for proper chromosome condensation in the mammalian oocytes as HDAC inhibitors induce chromosome abnormalities by interfering with chromosome-microtubule interactions and/or sister chromatid segregation. In the current study, we have shown that downregulation of PTEN and/or CBX8 greatly induces H3K27Me3 signals in mitotic cells, strongly suggesting that these proteins are important in suppressing molecular pathways leading to trimethylation of H3K27 residue. Given that mitotic cells contain condensed chromatin and largely transcriptionally inactive and that H3K27Me3 is an important epigenetic marker for transcriptional suppression, it is rather surprising to observe that H3K27Me3 signals are low in mitosis (Figure 5 and Fig. S2). It is known that CBX family proteins are integral component of PRC1, recognizing and binding to the modified H3K27Me3. EZH2 is a histone methyltransferase, functioning as a catalytic component of PRC2. In fact, EZH2 catalyses trimethylation of histone H3 at Lys 27 (H3K27Me3), regulating gene expression through epigenetic machinery. Our discovery that CBX8 negatively regulates H3K27Me3 suggests that there is crosstalk between PRC1 and PRC2. Further, given that PTEN physically interacts with CBX8 and that PTEN suppresses H3K27Me3, it is reasonable to propose that chromatin PTEN may play a major role in modulating epigenetic machinery during cell division.

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Figure 6 A schematic model that depicts regulation of chromatin modifications by PTEN and CBX8 during mitotic progression. PTEN and phosphorylated CBX8 interact with MCC and negatively regulates trimethylation of histone H3K27 during mitosis. Reduced levels of H3K27Me3 are necessary for mitotic progression and exit. H3K27Me3 levels are high in G1.
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CONFLICT OF INTEREST STATEMENT
We declare no conflicts of interest with the current study.

AUTHOR CONTRIBUTIONS
B. H. C., T. M. C., W. D.: Formal analysis; B. H. C., T. M. C., Z. K., W. D.: Investigation; B. H. C., T. M. C., Z. K., W. D.: Methodology; B. H. C., W. D.: Conceptualization; W. D.: Supervision; B. H. C., W. D.: Writing—review and editing; W. D.: Funding acquisition.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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