Inhibition of the deubiquitinase USP9x induces pre-B cell homeobox 1 (PBX1) degradation and thereby stimulates prostate cancer cell apoptosis

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Running Title: The USP9x/PBX1 axis in prostate cancer chemoresistance

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Abstract
Chemoresistance is a leading obstacle in effective management of advanced prostate cancer (PCa). A better understanding of the molecular mechanisms involved in PCa chemoresistance could improve treatment of patients with PCa. In the present study, using immunohistochemical, -chemistry, and -precipitation assays with cells from individuals with benign or malignant prostate cancer or established PCa cell lines, we found that the oncogenic transcription factor pre-B-cell leukemia homeobox-1 (PBX1) promotes PCa cell proliferation and confers to resistance against common anti-cancer drugs such...
as doxorubicin and cisplatin. We observed that genetic PBX1 knockdown abrogates this resistance, and further experiments revealed that PBX1 stability was modulated by the ubiquitin-proteasomal pathway. To directly probe the impact of this pathway on PBX1 activity, we screened for PBX1-specific deubiquitinases (Dubs) and found that ubiquitin-specific peptidase 9 X-linked (USP9x) interacted with and stabilized PBX1 protein by attenuating its Lys48-linked polyubiquitination. Moreover, the USP9x inhibitor WP1130 markedly induced PBX1 degradation and promoted PCa cell apoptosis. The results in this study indicate that PBX1 confers to PCa chemoresistance and identify USP9x as a Dub of PBX1. We concluded that targeting the USP9x/PBX1 axis could be a potential therapeutic strategy for managing advanced prostate cancer.

Introduction
Prostate cancer (PCa) is a malignant disease developed in prostate, a gland in the male reproductive system. The epidemiological studies reveal that PCa is one of the most common cancers in men and one of the leading causes of cancer-related deaths worldwide (1). Several treatment modalities have been developed against prostate cancers, including androgen deprivation therapy (ADT), localized radiotherapy and chemotherapy. Currently, ADT is the standard frontline therapy for advanced PCa patients; nevertheless, most of them will eventually develop resistance and these castration-resistant PCa patients rely on chemotherapy. Unfortunately, this regimen only prolongs modest survival of PCa patients, of which many acquire chemoresistance and eventually evolve to a fatal clinical outcome (1). It is widely believed that molecular and genetic events are key players in the resistance but they are not well defined.

Pre-B-cell leukemia homeobox-1 (PBX1), a member of the TALE (three-amino-acid loop extension) family of atypical homeodomain proteins, is cloned from pre-B cell leukemia (2), our recent study demonstrated that it upregulates the transcription of ring finger protein 6 and contributes to leukemia chemoresistance (3). However, more and more evidence shows that PBX1 is dysregulated and contributes to proliferation, survival, metastasis and chemoresistance in various solid tumors, including breast, lung, gastric and ovarian cancers. For example, high expression of PBX1 drives breast cancer proliferation and metastasis by regulating the estrogen receptor transcriptional response to epidermal growth factor signaling (4). In gastric carcinomas (5) and non-small cell lung cancers (6), PBX1 promotes epithelial to mesenchymal transition that is associated with chemoresistance. Although there are less studied on PBX1 in PCa, a recent study revealed that PBX3, a homolog of PBX1, is a putative biomarker of aggressive PCa (7). There are two PBX1 transcripts: PBX1a and PBX1b, between which the only difference is that PBX1b lacks the 7th exon (8), but their biological functions remain very similar as a transcription factor.

Protein stability is modulated by two
major metabolizing pathways in eukaryotes, one is the autophagy-lysosomal pathway, and the other is the ubiquitin-proteasomal pathway (UPP). The turnover of most transcription factors is processed via UPP (9). Proteasomal degradation depends on protein ubiquitination, a process in which a ubiquitin chain is attached to a specific lysine residue at the substrate protein to be degraded. Notably, protein ubiquitination is a dynamic process and the covalent ubiquitin molecules could be removed by certain deubiquitinases (Dubs). Ubiquitin-specific protease 9, X-linked (USP9x), one of the most studies Dubs, has been found to prevent ubiquitination from diverse protein substrates (10,11). In the present study, we found that USP9x stabilizes PBX1 by preventing its polyubiquitination and confers to PCa proliferation. Moreover, we found that inhibition of the USP9x/PBX1 axis overcomes PCa chemoresistance.

RESULTS
PBX1 is highly expressed in PCa tissues and promotes PCa cell proliferation

PBX1 has been demonstrated to confer to chemoresistance in both breast cancer (12) and leukemia (3), but it was not well known in PCa. To figure out the expression profile of PBX1 in PCa, a panel of prostate tissues from patients with benign prostate hyperplasia (BPH), prostatic intraepithelial neoplasia (PIN) or primary PCa was evaluated by immunohistochemical (IHC) assay. As shown in Fig. 1A, compared with the BPH and PIN samples, PCa expressed a higher level of PBX1. In comparison with the positive rate of PBX1 (33.3%) in BPH, it was raised to more than 70% in PCa and the difference was significantly (Fig. 1B). Subsequently, we evaluated PBX1 in PCa cell lines, as shown in Fig. 1C, except DU145, other three cell lines including PC3, 22RV1 and PC-3M expressed a high level of PBX1.

Because PBX1 was highly expressed in PCa tissues and cell lines, and its homolog PBX3 is a putative progressive biomarker in PCa (7), we wondered whether PBX1 was important for PCa cell proliferation and survival. To this end, we used DU145 that lacks PBX1 and PC3 that express PBX1 for next study. DU145 cells were transfected with either PBX1 isoform (PBX1a or PBX1b) followed by proliferation evaluation. The results showed that ectopic expression of both PBX1a (Fig. 1D and E) and PBX1b (Fig. 1F and G) promoted DU145 cell proliferation, while PBX1 knockdown by siRNA suppressed the proliferation of PC3 cells (Fig. 1H and I). Therefore, PBX1 promotes PCa cell proliferation.

PBX1 expression is associated with PCa chemoresistance

To find out the therapeutic implications of PBX1 in PCa, we next evaluated the significance of PBX1 in anti-cancer treatment. PC3 that expresses high PBX1 and DU145 that lacks PBX1 were treated with cisplatin (CDDP) or doxorubicin (DOX), two typical cytotoxic anti-cancer drugs that are also used for advanced PCa, followed by measurement of the cleavage of PARP and Caspase-3, two hallmarks of apoptosis, by immunoblotting assay. As shown in Fig. 2A, both doxorubicin and cisplatin
induced marked cleavage of PARP and Caspase-3 in DU145 but not in PC3 cells, indicating that PC3 might resist to these two drugs. To confirm this finding, four PCa cell lines including PC3, DU145, 22RV1 and PC-3M were treated with doxorubicin or cisplatin at increased concentrations or extended incubation time. Consistent with the above findings, both drugs induced PARP and Caspase-3 cleavage in DU145 in a concentration- and time-dependent manner (Fig. 2B), but no apoptotic caspase-3 cleavage in PBX1-expressing PCa cells including PC3, 22RV1 and PC-3M at the same concentrations of each drug (Fig. 2C). These results were further confirmed by Annexin V-FITC/propidium iodide staining and flow cytometric analyses. As shown in Fig. 2D, PC3 cells displayed no response to neither drug, in contrast, the apoptotic fractions (Annexin V positive cells) in DU145 were significantly increased by both drugs. Therefore, these results showed PBX1 expression might play a critical role in PCa chemoresistance.

Next, we wondered whether PBX1 directly contributed to PCa chemoresistance. To this end, PBX1 was overexpressed in drug-sensitive DU145 or knocked down from drug-resistant PC3 cells followed by drug treatment and analyses on cell viability and apoptosis. As shown in Fig. 3A and B, knockdown of PBX1 sensitized PC3 cells to doxorubicin in terms of inhibition on cell viability and induction of PARP cleavage that indicated PCa cell apoptosis (Fig. 3C and D). Consistent with this finding, when PBX1a or PBX1b was over-expressed, DU145 cells became resistant. As expected, there were more viable cells in DU145 with ectopic PBX1 at the same treatments (Fig. 3E and F). Furthermore, PARP was less cleaved at the same treatment when PBX1a or PBX1b was introduced to DU145 cells (Fig. 3G and H). Therefore, PBX1 confers to PCa chemoresistance.

**PBX1 protein stability is modulated by the ubiquitin-proteasome pathway**

The above results have clearly demonstrated that PBX1 is a critical factor in PCa chemoresistance, targeting at PBX1 degradation will be a potential therapeutic strategy for PCa treatment. Because most transcription factors (such as c-Maf, p53, and NF-κB) are processed via the UPP pathway (13-15), we wondered whether PBX1 stability could be modulated by UPP. To this end, we first measured PBX1 in HEK293T cells, followed by treatment with MG132, one of the typical proteasomal inhibitors, or Bafilomycin A1 (BMA1), one of the typical inhibitors of lysosomes. The IB assays showed that PBX1 was accumulated by MG132 but not by BMA1 (Fig. 4A), suggesting PBX1 was processed in proteasomes. To confirm this finding, PBX1 was ectopically expressed in HEK293T cells followed by exposure to MG132 at a concentration-dependent manner. As shown in Fig. 4B, PBX1 protein was steadily increased following increase of MG132. Furthermore, endogenous PBX1 in PC3 cells was also increased following the treatment of bortezomib, another typical and widely used proteasomal inhibitor (Fig. 4C). In the preliminary study we found that HEK293T cells were sensitive to doxorubicin, and we wondered whether PBX1 degradation by doxorubicin in HEK293T cells could be prevented by proteasomal inhibitors. As expected, doxorubicin-downregulated PBX1 was stabilized by bortezomib (Fig. 4D).
Moreover, MG132 increased the polyubiquitination level of PBX1 (Fig. 4E). Therefore, these results collectively demonstrated that PBX1 protein stability was modulated by UPP.

**USP9x deubiquitinates and stabilizes PBX1**

Protein ubiquitination is a dynamic process in which the protein substrate is regulated by continuous ubiquitination and deubiquitination mainly involving ubiquitinating enzymes and deubiquitylating enzymes (Dubs), respectively. The presence of approximately 100 Dubs in human cells illustrates the intrinsic specificity for particular cellular substrates. To identify the specific Dub(s) responsible for PBX1 deubiquitination and stability, a screen examining a total of 62 mammalian Dubs was performed in HEK293T cells which were co-transfected HA-PBX1 and individual Dubs for 36 hrs, followed by measurement of PBX1 protein levels. Among all Dubs examined, USP9x was found to be the most potent one in stabilizing PBX1 protein (Fig. 5A). Subsequent studies showed that USP9x increased ectopic PBX1 protein (Fig. 5B) and endogenous PBX1 in PC3 cells (Fig. 5C) in a concentration-dependent manner. This effect on PBX1 stability by USP9x was also time-dependent (Fig. 5D). Moreover, when USP9x was knocked down by siRNA from PC3 cells, PBX1 was consistently downregulated (Fig. 5E). Furthermore, when PBX1 synthesis *de novo* was inhibited by cycloheximide, additional expression of USP9x strikingly delayed PBX1 degradation (Fig. 5F and G). Consistently, when USP9x was knocked down by siRNA followed by CHX treatment, PBX1 degradation was rapidly processed (Fig. 5H and I).

Interestingly, USP9x knockdown decreased PBX1 could be rescued by MG132, a typical proteasome inhibitor, in PC3 cells (Fig. 6A). Because USP9x is a deubiquitinase of PBX1, we wondered whether USP9x could decrease the polyubiquitination level of PBX1. To this end, we first evaluated the interaction between USP9x and PBX1 in both HEK293T and PC3 cells. As shown in Fig. 6B and C, IP and IB assays demonstrated that PBX1 and USP9x interacted with each other at both exogenous and endogenous contexts. Next, we wondered whether USP9x could reduce the ubiquitination levels of PBX1. The results showed that overexpression of USP9x significantly decreased the ubiquitination levels of PBX1 in PCa cells, and when USP9x was knocked down by its specific siRNA, the polyubiquitination level on PBX1 was markedly increased (Fig. 5D), which suggested that USP9x modulates PBX1 ubiquitination. It is known that there are two major types of ubiquitination: K48- and K63-linked ubiquitin chain. To find out which ubiquitination could be modulated by USP9x, plasmids of PBX1, USP9x and K48-Ub or K63 Ub were co-transfected into HEK293T cells, followed by IP/IB assays. The results showed that PBX1 could be ubiquitinated at both K48- and K63-linked manners, and both ubiquitin chains on PBX1 could be abolished by USP9x (Fig. 5E). Because K48-linked ubiquitination is a marker for proteasomal degradation and K63-linked ubiquitination modifies protein function, this result suggested that USP9x not only stabilizes PBX1 but also modulates
its biological function, which was consistent to a previous report that USP9x possesses protease activity for both K48- and K63-linked ubiquitin (16). Therefore, all the above results collectively demonstrated that USP9x was a deubiquitinase that stabilized PBX1 by decreasing its polyubiquitination level.

**Inhibition of the USP9x/PBX1 axis sensitizes PCa cells to apoptosis**

Because PBX1 has been found to promote PCa cell proliferation and confer to chemoresistance and its inhibition restores drug sensitivity (Fig. 1-3), we subsequently tested the effects on PCa cell apoptosis by inhibition of USP9x using its specific small molecule inhibitor WP1130. Firstly, we evaluated the suppressive action of WP1130 on PBX1 function as a transcription factor. To this end, a PBX1-recognition element-driven luciferase reporter was constructed as described previously (3). After co-transfection with the PBX1.Luci, PBX1 with or without USP9x for 24 hrs, HEK293T cells were treated with WP1130 for another 24 hrs followed by luciferase assays. The result showed that USP9x markedly increased PBX1–driven luciferase activity in association with raised PBX1 protein levels (Fig. 7A), indicating that the transcriptional activity PBX1 was augmented. Notably, treatment of WP1130 significantly inhibited PBX1.Luci activity along with decreased PBX1 and USP9x (Fig. 7A). These findings thus collectively suggested that inhibition of USP9x led to PBX1 degradation and decreased its transcriptional activity. Next we measured the apoptotic effects of on PCa cell lines upon USP9x inhibition. As shown in Fig. 7B, WP1130 induced cleavage of PARP at 5 μM in PBX1-deficient DU145 cells, while less PARP was cleaved even at 10 μM when PBX1 was overexpressed (Fig. 7C). Consistent with these findings, WP1130 at 10 μM or cisplatin at 40 μM only induced marginal cleavage of PARP in PBX1-expressing PC3 cells (Fig. 7D and E), however, when this cell line was treated with WP1130 in combination with cisplatin, PARP cleavage was significantly induced (Fig. 7E). These results therefore suggested that targeting at the USP9x/PBX1 axis could be a potential therapeutic modality to overcome chemoresistance in PCa patients.

**Discussion**

The above study demonstrated that as an oncogenic transcription factor PBX1 is highly expressed in most PCa tissues, promotes PCa cell proliferation and confers to chemoresistance against representative anti-cancer drugs. Moreover, we found for the first time that USP9x is a deubiquitinase in stabilizing PBX1 by inhibiting its polyubiquitination. We also demonstrated that targeting the USP9x/PBX1 axis could be a potential modality to overcome PCa chemoresistance.

Resistance is a major obstacle to success with chemotherapy in advanced PCa, but the underlying mechanisms are not well known. Evidence has shown that chemoresistance of advanced PCa might be associated with the presence of cancer stem cells that regenerate tumorigenicity, overactivated androgen receptor signaling that promotes PCa...
cell proliferation, ATP-binding cassette proteins that pump out drugs outside the cancer cells, epithelial-mesenchymal transition (EMT) that confers to cancer cell migratory and invasive ability (17,18). However, PCa cells are highly heterogeneous (1), even in prostate tumors with similar pathology (19). Therefore, in specific subsets of chemoresistant PCa patients, unique expression of certain genes might play a key role. In the present study, we found that PBX1 expression in prostate tissues is increased following the malignancy from BPH, PIN to PCa. Although we could not provide the PBX1 expression data from normal prostate tissues, PBX1 was found to be positive in 1/3 of BPH patients, which rose to around 54% in PIN patients, and it was found in more than 70% of PCa patients, suggesting that PBX1 expression level might be involved in progress of PCa. We also found PBX1 is highly expressed in 3/4 PCa cell lines. Moreover, PBX1 expression determines PCa cell proliferation and drug sensitivity. Ectopic expression of PBX1 promotes PCa cell proliferation and confers to resistance to cytotoxic anti-cancer drugs, in contrast, knockdown of PBX1 restores drug sensitivity. Although it is originally identified from pre-B leukemia cells (2), PBX1 is now also reported in other cancers such as lung, breast and ovarian cancers to promote cancer cell proliferation, survival, and metastasis. PBX1 acts as a stem cell reprogramming factor in regulating long-term hematopoietic stem cells by maintaining their self-renewal and quiescence (20) but also driving stemness of ovarian cancer stem cells (12) and initiating leukemogenesis (21). It is also believed as a pioneer factor promoting a transcriptional program favorable to breast cancer progression (22). Furthermore, PBX1 has been extensively involved in promoting EMT in various cancers in collaboration with other transcription factors such as ZEB2 (23) and PREP1 (6). Therefore, there is no doubt that PBX1 promotes PCa cell proliferation, survival and chemoresistance. Actually, a recent study showed that ovarian cancer chemoresistance relies on PBX1 overexpression (12). Our study recent study has demonstrated that PBX1 promotes the expression of oncogenic RNF6 thus increasing leukemia chemoresistance to doxorubicin (3). Based on previous reports and our present study, PBX1 is believed to confer to chemoresistance in PCa cells.

In the present study, we also found that PBX1 stability is modulated by the ubiquitin proteasomal system because PBX1 protein could be polyubiquitinated and its degradation could be prevented by inhibition of proteasomes but not lysosomes. Moreover, the Dub USP9x interacts with PBX1 and abolishes its polyubiquitination level by reducing its K48-linked ubiquitin chain thus leading to PBX1 stabilization. Previous studies showed that USP9x ablates K48-linked ubiquitination of ERG, another oncogenic transcription factor (24). In the present study, we also found that USP9x abolishes K63-linked ubiquitination from PBX1. The K48- and K63-linked ubiquitination forms have been reported in the same proteins, such as PTEN and ErbB2 (25), but the function may vary dependent on the specific ubiquitination types. It is well
known that K48-linked polyubiquitination results in substrate degradation, while the K63-linked modification usually alters the function of the target proteins. In BCR-ABL expressing chronic myeloid leukemia cells (CML), the USP9x inhibitor WP1130 leads to increased K63-linked ubiquitination of BCR-ABL that results in BCR-ABL accumulation in aggresomes in which it fails to conduct signal transduction therefore CML cells undergo apoptosis (16). In Ca cells, we found USP9x downregulates both K48- and K63-linked ubiquitination levels of PBX1, suggesting these two modifications might enhance PBX1 oncogenicity, while inhibition of USP9x suppresses its activity. As shown in Fig. 6, WP1130 leads to PBX1 degradation and reverses Ca cell chemoresistance. Notably, we also found that WP1130 leads to DU145 cell apoptosis although this cell line does not express PBX1. The possible reason could be that USP9x has diverse oncogenic substrate proteins and these proteins also promote Ca cell survival and proliferation. Actually, oncoproteins such as MCL-1 (26) and YAP1 (27) are also substrates of USP9x, and these two oncoproteins also highly expressed in Ca and promote Ca cell proliferation and survival (28,29). These findings further demonstrated that USP9x plays a critical role in promoting Ca cell proliferation, survival and chemoresistance. Inhibition of USP9x could directly lead to apoptosis of Ca cells that does not express PBX1, however, in PBX1-expressing Ca cells, inhibition of USP9x could help overcome their chemoresistance because WP1130 sensitizes PC3 cells to cisplatin at the same concentrations as shown in Fig. 6.

In conclusion, the present study for the first time highlights the role of PBX1 in Ca chemoresistance and identifies USP9x as a Dub of PBX1. By stabilizing PBX1, USP9x also promotes Ca cell proliferation. Moreover, inhibition of USP9x sensitizes PBX1-expressing Ca cell apoptosis. Therefore, the present study suggests that targeting at the USP9x/PBX1 axis could be a potential modality to overcome Ca chemoresistance due to the high expression of PBX1.

EXPERIMENTAL PROCEDURES

Prostate tissues

Prostate tissues were obtained from patients with BPH, PIN or Ca when patients made their first visits to Department of Urology, Jinling Hospital, Nanjing Jinling Hospital from May, 2016 to April of 2018. The usage of these specimens for the present study was informed to each patient and a signed content was obtained from each patient. The collection and use of prostate tissues in the present study was approved by the Review Board of Medical Ethics of Jinling Hospital in accordance with the Declaration of Helsinki.

Cell culture and chemicals

Human Ca cell lines (PC3 and DU145) were purchased from American Type Culture Collection (Washington, DC, USA). Cell lines 22RV1 and PC-3M were obtained from China Center for Type Culture Collection (Wuhan, China); Human embryonic kidney (HEK293T) cell line was provided by Dr. Michael Moran, The University of Toronto, Canada. Ca cells were cultured in RPMI-1640 medium. HEK293T cells were maintained in Dulbecco's high glucose modified Eagle's medium. All the media
were supplemented with 10% fetal bovine serum, 100 μg/ml of penicillin, and 100 units/ml of streptomycin.

Doxorubicin was purchased from Sangon Biotech Co., Ltd. (Shanghai, Shanghai, China). Cisplatin was purchased from Jiangsu Hansoh Pharmaceutical Group Co., Ltd (Lianyungang, Jiangsu, China). WP1130 was purchased from Selleck Chemicals (Houston, TX, USA). MG132 and Bortezomib were purchased from Chemcatch (Shanghai, Shanghai, China) and Adamas Chemicals (Shanghai, Shanghai, China), respectively.

**Antibodies and plasmids**

Rabbit anti-PBX1, anti-Caspase3, anti-Cleaved-Caspase3, and anti-PARP antibodies were obtained from Cell Signaling Technology, Inc., (Danvers, MA, USA). Anti-HA, anti-Flag and anti-Myc monoclonal antibodies were obtained from Medical & Biological Laboratories Co., Ltd. (Tokyo, Japan). A mouse anti-GAPDH antibody was purchased from Abgent (Suzhou, Jiangsu, China). A mouse anti-V5 antibody was purchased from Huabio Antibodies (Hangzhou, Zhejiang, China). The rabbit anti-USP9x antibody was obtained from Proteintech Group Co., (Wuhan, Hubei, China).

The PBX1a and ubiquitin (Ub) plasmids were prepared as described previously (3) and subcloned into a pcDNA3.1 vector carrying an HA, Flag or Myc tag. The V5-USP9x plasmid was generously provided by Dr. Guoqiang Xu (Soochow University). The PBX1b plasmid was purchased from General Biosystems, Inc., (Morrisville, NC, USA).

**Immunohistochemistry (IHC)**

All tissues were embedded by paraffin waxes and were sliced to 5-μM-thick sections with a rotary microtome (Leica Biosystems, Wetzlar, Germany) and mounted onto slides. Immunostaining was conducted using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) following the manufacturer’s instructions. Briefly, slides were deparaffinized, rehydrated, and treated with citric acid solution before being applied for IHC. After blocking the endogenous peroxidase activity by preincubation in 3% hydrogen peroxide solution, the slides were incubated in blocking solution (PBS, 3% bovine serum albumin) and sequentially incubated with anti-PBX1 antibody (Abcam, MA, USA). The sections were counterstained with hematoxylin (Wuhan Service Biotechnology Co., LTD, Wuhan, China) for nuclear staining. To evaluate the expression levels of the protein, the intensity of staining was scored according to a semiquantitative 4-grade scale as follows: 0, none (no positive tumor cells); 1, weak (<33% positive tumor cells); 2, moderate (33–67% positive tumor cells); 3, strong (>67% positive tumor cells). An intensity score of ≥2 was considered as high expression, whereas < 2 was regarded as low expression.

**Immunoblotting (IB)**

Cell lysates were prepared as described previously (30). After concentration determination, equal amounts (40 μg) of total proteins were resolved by sodium dodecylsulfate polyacrylamide gel electrophoresis, followed by IB analyses with specific antibodies.

**Immunoprecipitation (IP)**

Twenty four hours after transfected with appropriate plasmids, cells were treated with MG132 for another 12 h. Cell lysates were incubated with indicated antibodies overnight at 4°C, followed by incubation with protein A+G sepharose beads (Beyotime Institute of Biotechnology, Nantong, Jiangsu, China) for 4 h. The proteins
were then subjected to the IB assay with appropriate antibodies.

**Cycloheximide (CHX) chase assay**

HEK293T cells were transfected with HA-PBX1 and V5-USP9x plasmids for 24 h before being treated with CHX (100 µg/ml) for 0 to 12 h. Whole cell lysates were prepared for the IB assay with specific antibodies.

**Luciferase assay**

The RNF6 promoter containing a PBX1 recognition element (PBX1.Luci) was cloned into a pGL4 luciferase report system (Promega, Madison, WI, USA) as described previously (3). To examine the effect of USP9x on PBX1 transcriptional activity, the PBX1.Luci and HA-PBX1a were co-transfected into HEK293T cells. Thirty six hours later, luciferase activity was analyzed with the Bright-Glo substrate (Promega) as described previously (3).

**Plasmid transfection**

Prostate cancer lines were transfected with individual expressing plasmids including pcDNA3.1-PBX1a, -PBX1b, or -USP9x by lipofectamine 2000® (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer’s instructions. HEK293T cells were transfected with specific plasmids as needed by using polyethyleneimine (Sigma, St. Louis, MO, USA) as described previously. (30) The appropriate amounts of plasmids were determined to result in a transfection efficiency about 70-80% when cells were split for further studies.

**siRNA transfection**

Small interfering RNAs (siRNAs) were transfected into PC3 or DU145 cells by Lipofectamine® 2000 as shown above. The specific small interfering RNA sequences targeting PBX1 were as follows: siPBX1#1 5’- CCA TCA CAG ACC AGA GTT T -3’, si-PBX1#2 5’- GAA TGA AGC CCT TGT T -3’, si-PBX1#3 5’- GGA AGA GAC GGA ATT TCA A -3’. The specific sequence targeting USP9x was si-USP9x 5’- ACA CGA UGC UUU AGA AUU UTT -3’. All the siRNAs were purchased from RiboBio Inc., (Guangzhou, Guangdong, China).

**Cell viability**

Cell viability was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as described previously (31) after transfected with specific plasmids or drug treatment.

**Flow cytometric analysis**

After treatment with appropriate drugs with or without plasmids transfection, PCa cells were stained with Annexin V and PI staining (MultiSciences Biotech Co. Ltd, Hangzhou, Zhejiang, China) for 5 min in dark, followed by analyses on a flow cytometer (FACS Calibur®; Becton Dickinson, Franklin Lakes, NJ, USA) as described previously (31).

**Densitometric analyses**

Densitometric analyses of western blots in the protein stability were performed as described previously (3) by using the ImageJ® software developed by National Health Institute of USA.

**Statistics**

All experiments were performed at least 3 times except for the IHC studies. Statistical analyses between the control and the experiment groups were analyzed by the student’s t test and the p value < 0.05 was considered statistically significant.
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Conflict of Interest Statement
The authors declare that the research was conducted in absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authorship Contributions
XM, XX and XZ designed the study; PL, YH, YL, YZ, BC, and ZZ conducted experiments; JL, GS, XZ, and XM analyzed data; XM and YL wrote the manuscript.

Availability of Data and Materials
All data generated or analyzed during this study are included in this published article.

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Abbreviations
ADT, androgen deprivation therapy; BMA1, Bafilomycin A1; CDDP, cisplatin; CML, chronic myeloid leukemia; CRPC, castration-resistant prostate cancer; DOX, doxorubicin; Dub, deubiquitinase; EMT, epithelial to mesenchymal transition; IHC: immunohistochemistry; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide; PBX1, pre-B-cell leukemia homeobox-1; PCa, prostate cancer; TALE, three-amino-acid loop extension; UPP, ubiquitin-proteasome pathway; USP9x, ubiquitin-specific protease 9, x-linked

**Figure Legends**

**Fig. 1.** PBX1 is overexpressed in prostate cancer tissues and confers to chemoresistance. (A) Representative immunohistochemical analyses of PBX1 in benign prostate hyperplasia (BPH), prostatic intraepithelial neoplasia (PIN) and primary prostate cancer (PCa) tissues. (B) The rate of PBX1 expression in BPH, PIN and PCa specimens. (C) DU145, PC3, PC-3M and 22RV1 cell lysates were subjected to IB assay to detect PBX1 expression. (D) PC3 and DU145 cells were treated with doxorubicin (DOX) or cisplatin (CDDP) at indicated concentrations for 24 h, whole cell lysates were subjected to IB with specific antibodies against PARP or cleaved caspase-3. (E) DU145 cells were treated with DOX or CDDP with indicated concentrations and incubation time. Whole cell lysates were applied for IB assay. (F) PBX1-expressing PC3, PC-3M and 22RV1 cells were treated with DOX or CDDP at increasing concentrations for 24 h before being harvested for IB assays against PBX1, PARP, and Caspase-3. (G) DU145 and PC3 cells were treated with DOX and CDDP at increasing concentrations for 24 hrs, followed by Annexin V-FITC and PI staining and flow cytometric assays.

**Fig. 2.** PBX1 promotes prostate cancer cell proliferation. (A, B) An HA-PBX1a plasmid was transfected into DU145 cells for 48 hrs before cell lysates were prepared for IB assays (A). Cell proliferation was assessed by MTT assay at 0, 24, 48 and 72 hrs (B). (C, D) DU145 cells were transfected with a Myc-PBX1b plasmids for 48 hrs. Cells were harvested for IB assays (C), and cell proliferation was measured by MTT assay at 0, 24, 48 and 72 hrs (D). (E, F) PBX1 was knocked down in PC3 cells by specific siPBX1, followed by IB assays (E). Cell proliferation was evaluated by MTT assay. Compared with control, *P <0.05, **P <0.01, ***P <0.001.

**Fig. 3.** Knockdown of PBX1 sensitizes PC3 cells to doxorubicin. (A, B) siRNAs of PBX1 and negative control (NC) were transfected into PC3 cells for 48 hrs before cell lysates were prepared for IB assay of PBX1 (A). Cells were then treated with doxorubicin (DOX) for 24 hrs at indicated concentrations, cell viability was then detected by MTT assays (B). (C, D) siPBX1 was transfected into PC3 cells for 24 hrs, followed by DOX treatment for another 24 hrs. Cell lysates were subjected to IB assays with PARP and PBX1 antibodies (C), and Image J evaluation of cleaved PARP (D). (E, F) Plasmids of Myc-PBX1a and Myc-PBX1b were transfected into DU145 cells for 36 hrs followed by DOX treatment for another 24 hrs. PBX1 was measured by IB assays (E) and cell viability was assessed by MTT assay (F). (G, H) The PBX1a (G) or PBX1b (H) plasmids were transfected into DU145 cells for 36 hrs, followed by DOX treatment. The total cell lysates were subjected to IB assays. Compared with control, *P <0.05, **P <0.01, ***P <0.001.
Fig. 4. **PBX1 is degraded through the ubiquitin-proteasome pathway.** (A) HEK293T cells were transfected with HA-PBX1a plasmids for 24 hrs, followed by DMSO, MG132 and Bafilomycin A1 (BMA) treatment for 12 hrs. The cell lysates were subjected to IB assays. (B) HEK293T cells were transfected with an HA-PBX1a plasmid for 24 hrs, followed by MG132 treatment for 6 hrs, followed by IB assays with anti-HA antibody. (C) PC3 cells were treated with Bafilomycin A1 (BMA), chloroquine (CHQ), MG132 and bortezomib (BZ), respectively, for 12 hrs, followed by cell lysate preparation and IB assays. (D) HEK293T cells were transfected with HA-PBX1a plasmids for 24 hrs, followed by the treatment of DOX and BZ for 12 hrs and cell lysates were subjected to IB. (E) HA-PBX1a and Flag-Ub plasmids were co-transfected into HEK293T cells for 24 hrs, followed by the treatment of MG132 for another 12 hrs. Cell lysates were subjected to the immunoprecipitation (IP) and IB assays.

Fig. 5. **USP9x stabilizes PBX1 in prostate cancer cells.** (A) HA-PBX1 plasmid was co-transfected with individual Dub plasmids for 36 hrs, followed by cell lysate preparation and IB assays. (B) HA-PBX1 and USP9x plasmids were co-transfected into HEK293T cells for 36 hrs, and then cell lysates were subjected to IB assays. (C) A USP9x plasmid was transfected into HEK293T cells for 48 hrs, followed by IB assays. (D) HA-PBX1 and USP9x plasmids were co-transfected into HEK293T cells for the indicated periods followed by IB assays. (E) USP9x was knocked down from PC3 cells by its specific siRNA with increasing concentrations for 48 hrs, followed by IB assays. (F) PC3 cells were transfected with USP9x plasmids for 24 hrs, followed by cycloheximide (CHX) treatment for indicated periods, and IB assays. (G) The quantitative analyses on PBX1 stability from F by using the ImageJ® software. (H) 22RV1 cells were transfected with siUSP9x for 48 hrs, followed by cycloheximide (CHX) treatment for indicated periods, and IB assays. (I) The quantitative analyses on PBX1 stability from H by using the ImageJ® software.

Fig. 6. **USP9x interacts with PBX1 and decreases its polyubiquitination level.** (A) USP9x was knocked down by siUSP9x for 48 hrs, followed by MG132 and bortezomib (BZ) treatment for 12 hrs. The cell lysates were subjected to IB assays. (B) HA-PBX1 and USP9x plasmids were co-transfected into HEK293T cells for 48 hrs followed by the IP/IB assays. (C) PC3 cells were transfected with a myc-PBX1a plasmid for 24 hrs, followed by MG132 treatment for another 12 hrs. Cell lysates were subjected to the IP/IB assays. (D) PCa cells were transfected with USP9x plasmids or siUSP9x for 48 hrs, cell lysates were subjected to the IP/IB assays. (E) Myc-PBX1, HA-K48-Ub, HA-K63-Ub and V5-USP9x plasmids were co-transfected into HEK293T cells. Thirty six hours later, cells were subjected to the IP/IB assays.

Fig. 7. **Inhibition of USP9x sensitizes prostate cancer cells to cisplatin.** (A) HEK293T cells were co-transfected with HA-PBX1, USP9x, PBX1.Luci and β-gal plasmids for 36 hrs, followed by WP1130 treatment for another 12 hrs. The luciferase activity was evaluated and cell lysates were prepared for IB assays. (B) DU145 cells
were treated with WP1130 for 24 hrs, followed by cell lysate preparation and IB assays. (C) PBX1a or PBX1b plasmids were transfected into DU145 cells for 36 hrs, followed by WP1130 treatment for another 12 hrs before being applied to IB assays. (D) PC3 cells were treated with WP1130 for 24 hrs, followed by IB assays. (E) PC3 cells were co-treated with cisplatin and/or WP1130 with indicated concentrations for 24 hrs. Cell lysates were applied to IB assays for indicated protein expression.
Figure 1

A) Images of BPH, PIN, and PCa.

B) Table showing the distribution of Low and High percentages:

| Tissue  | Low | High | % of High |
|---------|-----|------|-----------|
| BPH     | 6   | 3    | 33.3      |
| PIN     | 6   | 7    | 53.8      |
| PCa     | 12  | 29   | 70.7      |

C) Western blots of PBX1a, PBX1b, USP9X, and GAPDH

D) Western blots of Pro-PARP, Cle-PARP, GAPDH, PBX1a, PBX1b, Cle-Cas3, and GAPDH for PC3 and DU145.

E) Western blots of Pro-PARP, Cle-PARP, GAPDH, Cle-Cas3, and GAPDH for DU145.

F) Western blots of PBX1a, PBX1b, Pro-PARP, Cle-PARP, Pro-Cas3, Cle-Cas3, and GAPDH for PC3, PC-3M, and 22RV1.

G) Flow cytometry results showing the distribution of Propidium Iodide and Annexin V-FITC for DU145 and PC3.
Figure 2
Figure 3
Figure 4
Figure 5

A

B

C

D

E

F

G

H

I

21
Figure 6
Figure 7
Inhibition of the deubiquitinase USP9x induces pre-B cell homeobox 1 (PBX1) degradation and thereby stimulates prostate cancer cell apoptosis

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