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High expression of *PRKDC* promotes breast cancer cell growth via *p38 MAPK* signaling and is associated with poor survival

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Abstract

Background: DNA-Dependent Protein Kinase Catalytic Subunit (*PRKDC*), a key component of the DNA damage repair pathway, is associated with chemotherapy resistance and tumor progression.

Methods: Here we analyzed transcriptome data of ~2,000 breast cancer patients and performed functional studies in vitro to investigate the function of *PRKDC* in breast cancer.

Results: Our results revealed overexpression of *PRKDC* in multiple breast cancer subtypes. Consistent with patients' data, overexpression of *PRKDC* was also observed in breast cancer cell lines compared to normal breast epithelial cells. Knockdown of *PRKDC* in MCF-7 and T47D breast cancer cell lines resulted in proliferation inhibition, reduced colony formation and G2/M cell cycle arrest. Furthermore, we showed that *PRKDC* knockdown induced proliferation inhibition through activation of p38 MAPK, but not ERK MAPK, signaling pathway in breast cancer cells. Blockage of p38 MAPK signaling could largely rescue proliferation inhibition and cell cycle arrest induced by *PRKDC* knockdown. Moreover, we analyzed gene expression and clinical data from six independent breast cancer cohorts containing ~1,000 patients. In all cohorts, our results consistently showed that high expression of *PRKDC* was significantly associated with poor survival in both treated and untreated breast cancer patients.

Yan Zhang and Wei-kang Yang have contributed equally to this work.

Conclusion: Together, our results suggest that high expression of *PRKDC* facilitates breast cancer cell growth via regulation of p38 MAPK signaling, and is a prognostic marker for poor survival in breast cancer patients.

KEYWORDS
breast cancer, cell cycle, cell proliferation, p38 MAPK, PRKDC

1 | INTRODUCTION

In mammalian cells, specific DNA repair pathways have evolved to protect them from different types of damages and to maintain genomic integrity (Hsu, Zhang, & Chen, 2012; Jackson & Bartek, 2009). On the other hand, however, these pathways are often aberrantly exploited by cancer cells to tolerate the high levels of basal DNA damage they receive, resulting in accumulation of mutations and carcinogenesis (Hsu et al., 2012; Jackson & Bartek, 2009; Jeggo, Pearl, & Carr, 2016; Macheret & Halazonetis, 2015). In the particular case of breast cancer, higher levels of DNA adduct and oxidative base lesions have been observed in patients versus controls, and recurrent lesions in DNA damage response genes were detected across all the molecular subtypes, including genes linked to defective damage repair (e.g., *BRCA1* and *ARID1A*) and cell cycle checkpoint (e.g., *TP53* and *PTEN*). Reversal of these genetic lesions strongly affects tumor cell proliferation and survival, consistent with aberrant activation of DNA damage pathway being important to the initiation and maintenance of malignancy (Kotsantis, Jones, Higgs, & Petermann, 2015; Lee et al., 2011; Macheret & Halazonetis, 2015; Puigvert, Sanjiv, & Helleday, 2016; Yin et al., 2019).

DNA-Dependent Protein Kinase Catalytic Subunit (*PRKDC,* OMIM accession number: 600899) is a key component of the nonhomologous end-joining pathway for DNA damage response and double-strand break repair (Holgersson, Erdal, Nilsson, Lewensohn, & Kanter, 2004; Sun et al., 2017). It is classified as a member of the phosphatidyl inositol 3-kinase-like (*PIKK*) protein kinase group, along with some well-known DNA damage response kinase such as Telangiectasia Mutated (*ATM*) and Rad3-related (*ATR*). In response to DNA damage stimuli, *PRKDC* is recruited by DNA double-strand breaks, where it forms the *DNAPK* complex with DNA-binding *Ku70/80* heterodimer to facilitate the repair of DNA lesions (Holgersson et al., 2004; Sun et al., 2017; Xing, Wu, Vaporciyan, Spitz, & Gu, 2008). Aberrant expression or mutations of the gene has been observed in multiple human cancers and now there is growing interest in understanding the molecular mechanisms by which *PRKDC* promotes tumor initiation and progression. For example, overexpression of *PRKDC* has been observed in several cancer types, such as liver cancer, lung cancer, and lymphoma, and has been associated with more advanced tumor grade and faster progression (Cornell et al., 2015; Hsu et al., 2012; Stronach et al., 2011; Sun et al., 2017). Given its primary role in DNA damage response, *PRKDC* has been suggested to play an important role in tumor cell resistance to chemo- and radiotherapies. In addition, it has been shown that *PRKDC* also plays a critical role in regulating cell cycle and chromosomal segregation, which might all promote tumorigenesis.

In the present study, we examined the expression of *PRKDC* in different breast cancer subtypes and cell lines to explore its putative role as a prognostic biomarker. We also performed *PRKDC* knockdown followed by functional assays to determine the essentiality of *PRKDC* to breast cancer cells and the underlying molecular mechanisms. Finally, we investigated the association between *PRKDC* expression and overall survival of breast cancer patients using data from six independent cohorts. Our results revealed the important roles of *PRKDC* overexpression in breast cancer and supported its role as for development of novel therapies.

2 | MATERIALS AND METHODS

2.1 | Ethical compliance

This study was approved by an ethics committee of Shenzhen Longhua District Maternity & Child Healthcare Hospital.

2.2 | Bioinformatics analyses

Gene expression profiles of *PRKDC* (Reference Sequence: NG_023435.1) in 144 normal breast samples and 1,992 breast carcinoma samples were obtained from the METABRIC (Molecular Taxonomy of Breast Cancer) project (http://www.ebi.ac.uk/ega/studies/EGAS00000000083) using Oncomine. The predictive value of *PRKDC* on overall survival of breast cancer patients were analyzed using six independent cohorts including HER2-negative invasive breast cancers with neoadjuvant taxane-anthracycline chemotherapy (GSE250255),

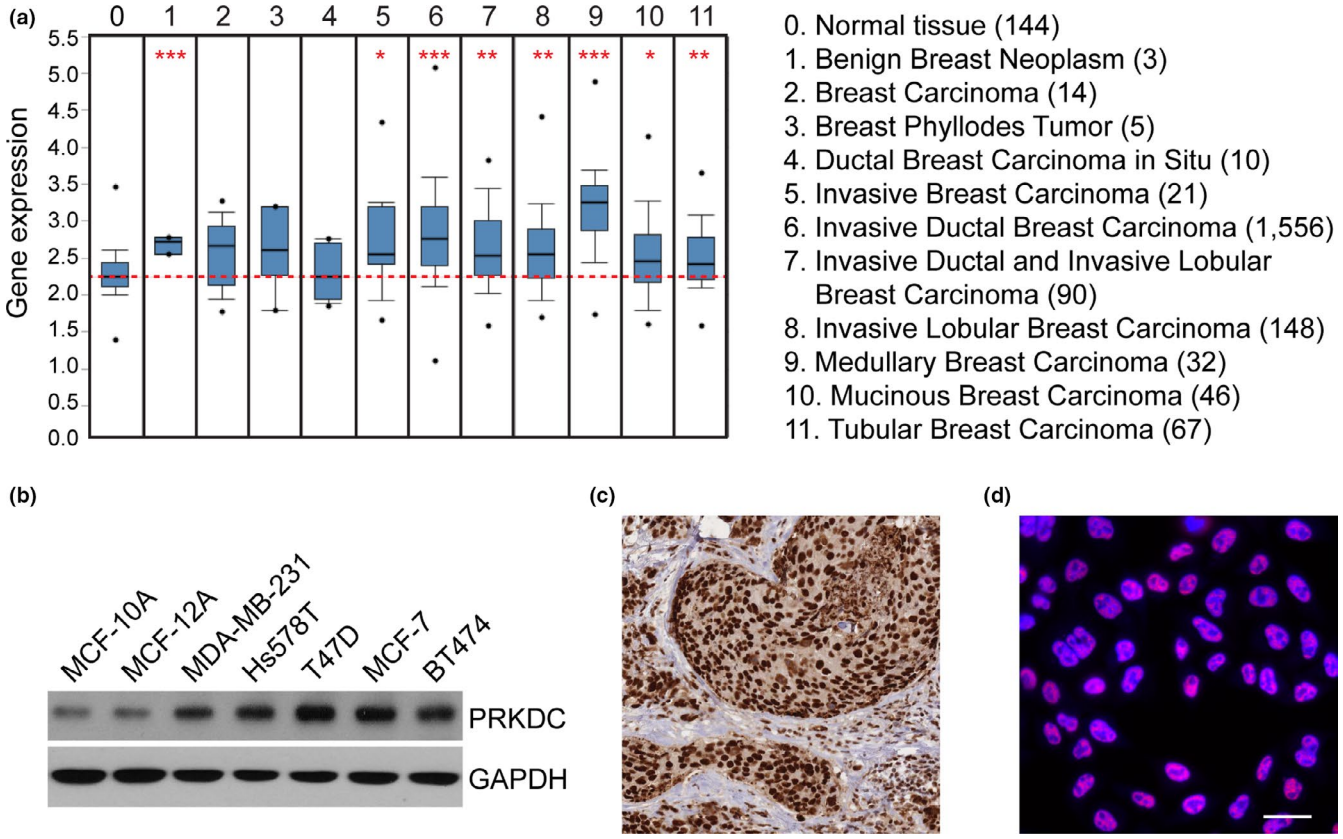

FIGURE 1 Overexpression expression of *PRKDC* [#]in breast cancer patients. (a) Expression of *PRKDC* in normal breast tissue and different breast tumor tissues are shown. Center lines show the means, box limits indicate the 25th and 75th percentiles, and whiskers extend to minimum and maximum values. (b) Western blot of *PRKDC* expression in different breast epithelial cell lines and breast cancer cell lines are shown. (c) A representative immunohistochemistry staining of *PRKDC* in breast tumor sample is shown. Data were obtained from human protein atlas database (Lobular carcinoma (M-85203)). (d) Immunofluorescent staining of *PRKDC* (red) and DAPI (blue) in MCF-7 cell line is shown. Scale bar indicates 25 µm. ***$p < .001$; **$p < .01$; *$p < .05$. [#]Reference Sequence: NG_023435.1

early breast cancer patients treated with adjuvant therapy (GSE1456), untreated primary breast tumors (GSE2990), untreated primary breast tumors (GSE11121), invasive adenocarcinoma breast cancers (GSE31448) and patients with breast-conserving treatment (GSE30682). Patients from each cohort were separated into high and low *PRKDC* expression groups and Kaplan–Meier survival analyses were performed to compare the differences in overall survival. The Immunohistochemistry staining of *PRKDC* in breast tumor sample was obtained from human protein atlas database (https://www.proteinatlas.org/).

2.3 | Cell culture

Breast cancer cell lines MDA-MB-231, Hs578T, T47D, MCF-7, and BT474 were obtained from Shanghai Institute of Cell Bank. Normal breast epithelial cell lines MCF-10A and MCF-12A were obtained from American Type Culture Collection. Cells were cultured in DMEM (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Gibco) in an incubator of 5% CO2 at 37°C.

2.4 | Cell proliferation assay and colony formation

cells were seeded at 5,000 cells/well in a 96-well plate and Cell proliferation assay was performed using CellTiter-Glo® Luminescent Cell Viability Assay (Promega) as per manufacturer's instructions. Briefly, 100 µl of CellTiter-Glo® reagent were added to each well is added to each well and incubated at 37°C. The plates were read using a fluorescence GloMax™ 96 Microplate Luminometer (Promega). For colony formation assays, cells infected with shRNAs were seeded in semisolid agar medium (2,000 cells/well) in a 6-well plate in triplicate. After 14 days, colonies were stained with crystal violet (Sigma-Aldrich) and counted using an inverted microscope. Data present mean ± SEM of three replicates.

2.5 | Cell cycle assay

Cells were fixed in 75% ethanol at 4°C overnight, washed twice with PBS, and then incubated with PI solution (Promega) as per manufacturer's instructions. Flow cytometry

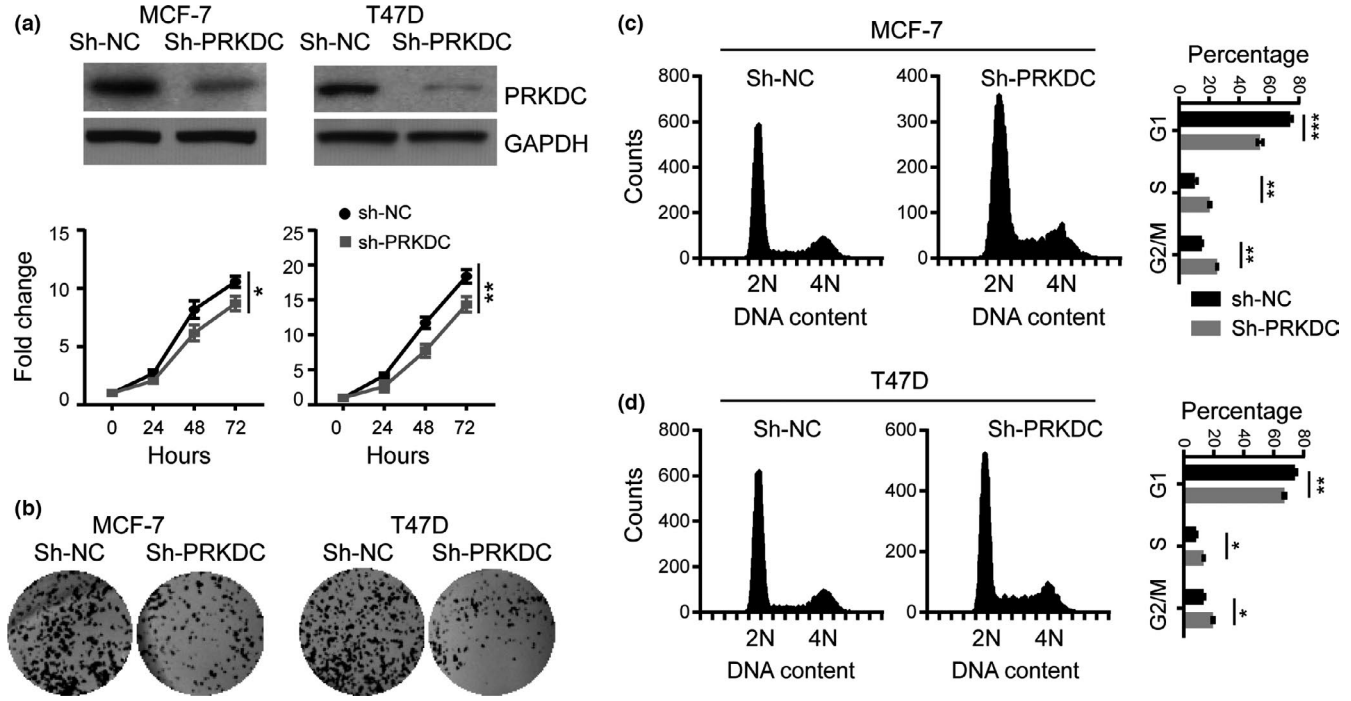

FIGURE 2 Knockdown of *PRKDC* leads to proliferation inhibition and mitotic arrest. (a) Western blot of *PRKDC* and the proliferation curves of different cell lines transfected with control or *PRKDC* shRNAs are shown. (b) Colony formation assay 14 days post transfection with control or *PRKDC* shRNAs are shown. (c) MCF-7 and (d) T47D cells were transfected with control or *PRKDC* siRNAs for 24 hr and then stained with PI for analysis of cell cycle distribution. Quantitation of cells at different cell cycle phases are shown. Data present mean ± SEM of three replicates. ***$p < .001$; **$p < .01$; *$p < .05$

analyses were performed using a FACSCalibur flow cytometer (BD Biosciences) and FlowJo 7.0 software was used for cell-cycle analysis.

2.6 | PRKDC knockdown

Sh-*PRKDC* target sequence (a) CCGGTAAAGATCCTA ATTCTA; (b) GAAAGG.

AGGTTCTAAACTACT or none-target sequence (a) CGCGATTAAGATGTCCTTATG; (b) GCTACGTGAAT ATAGACCATA were cloned into pLKO.1 lentiviral plasmid. Briefly, shRNA-encoding plasmids were cotransfected with VSV-G envelop plasmid and psPAX packaging plasmid into 293 T cells using lipofectamine 2000 (Thermo Fisher Scientific) as per manufacturer's instructions. Growth media was changed the following day and lentivirus-containing supernatants were harvested 3 days after transfection, filtered and used to infect cells in the presence of 4 μg/ml polybrene (Sigma-Aldrich).

2.7 | Western blot

The following primary antibodies were used in western blot: anti-*PRKDC* (1:1,000, Cell Signaling), anti-γH2AX (1:2000, Cell Signaling), anti-p38 (1:1,000, Cell Signaling) anti-p-p38 (1:1,000, Cell Signaling), anti-ERK (1:1,000, Cell Signaling), anti-p-ERK (1:1,000, Cell Signaling), anti-GAPDH (1:5,000, Sigma-Aldrich). For immunoblots, whole cell lysates were prepared using RIPA buffer (Sigma-Aldrich). Approximately 10 μg protein were loaded and separated by SDS page, and then transferred to a PVDF membrane (Millipore). Proteins were incubated with primary antibodies at 4°C overnight and horseradish peroxidase-conjugated secondary antibodies (1:10,000, Sigma-Aldrich) for 1 hr at room temperature.

2.8 | Immunofluorescence staining

Cells were then fixed with 4% paraformaldehyde for 15 min and treated with 0.1% Triton X-100 for 15 min at room temperature. Cells were incubated with anti-PRKDC antibody (1:200, Cell Signaling) in the presence of 1% BSA at room temperature for 1 hr. Subsequently, the cells were washed 3 × 5 min with PBST, and incubated at room temperature for 1 hr with cy3-labeled anti-IgG antibody (Cell Signaling, Danvers, MA, USA). Cells were washed 3 × 5 min with PBST and nuclei were stained with DAPI.

2.9 | Statistical analysis

All statistical analysis was performed using PRISM 6 (GraphPad). Statistical significance of differences among data sets were determined using unpaired *t* test. Survival

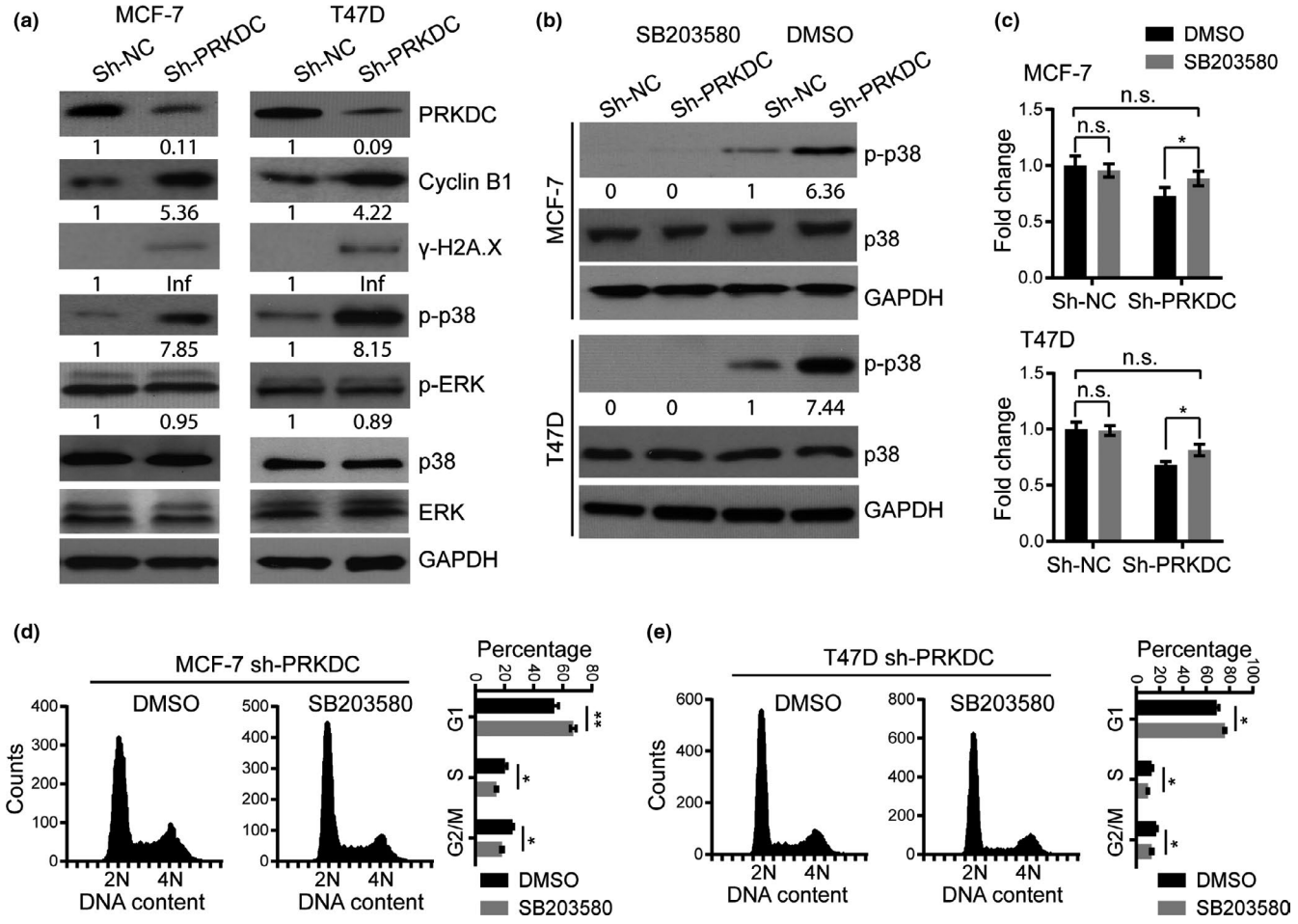

FIGURE 3 Knockdown of *PRKDC* activates p38 MAPK signaling pathway. (a) Western blot of key cell cycle, DNA damage and signaling transduction genes in cells transfected with control or *PRKDC* shRNAs are shown. The ratio between groups is shown below the blot. Inf, infinity. The ratios of phosphorylation proteins have been adjusted by the abundance of total proteins. Quantification of western blot is performed using ImageJ. (b) Western blot of p-p38 in cells treated with p38 phosphorylation inhibitor SB203580 or DMSO are shown. (c) The fold change of cell number following treatment with SB203580 or DMSO for 24 hr are shown. Sh-NC treated with DMSO was set as 1. Data present mean ± *SEM* of three replicates. $*p < .05$. (d) MCF-7 and (e) T47D cells with *PRKDC* knockdown were treated with SB203580 or DMSO for 24 hr and then stained with PI for analysis of cell cycle distribution. Quantitation of cells at different cell cycle phases are shown. Data present mean ± SEM of three replicates. $**p < .01$; $*p < .05$

curves were constructed with the Kaplan–Meier method and compared using log-rank test.

3 | RESULTS

3.1 | Overexpression of *PRKDC* in breast cancers

To determine the expression level of *PRKDC* in breast cancers, we reanalyzed transcriptome data of 144 normal breast samples and 1,992 breast carcinoma samples from the METABRIC (Molecular Taxonomy of Breast Cancer) project (Curtis et al., 2012). The relative expression of *PRKDC* mRNA in normal breast samples and 11 different breast cancer subtypes are shown in Figure 1a. Our results revealed consistent overexpression of *PRKDC* in all breast cancer subtypes compared to normal breast tissues (although the difference was not significant in some cancer types due to small sample size and patient-to-patient variations). We further investigated *PRKDC* protein expression in normal breast epithelial cell lines (MCF-10A and MCF-12A) and breast cancer cell lines (MDA-MB-231, Hs578T, T47D, MCF-7, and BT474). Consistent with the breast cancer patient data, elevated *PRKDC* expression in breast cancer cell lines were observed compared to normal breast epithelial cell lines (Figure 1b), supporting its oncogenic roles in promoting breast cancer tumorigenesis. Furthermore, as shown in Figure 1c,d, immunohistochemistry and immunofluorescence staining detected strong *PRKDC* signals within the nucleus of primary breast cancer cells and MCF-7 cell line, respectively, providing a

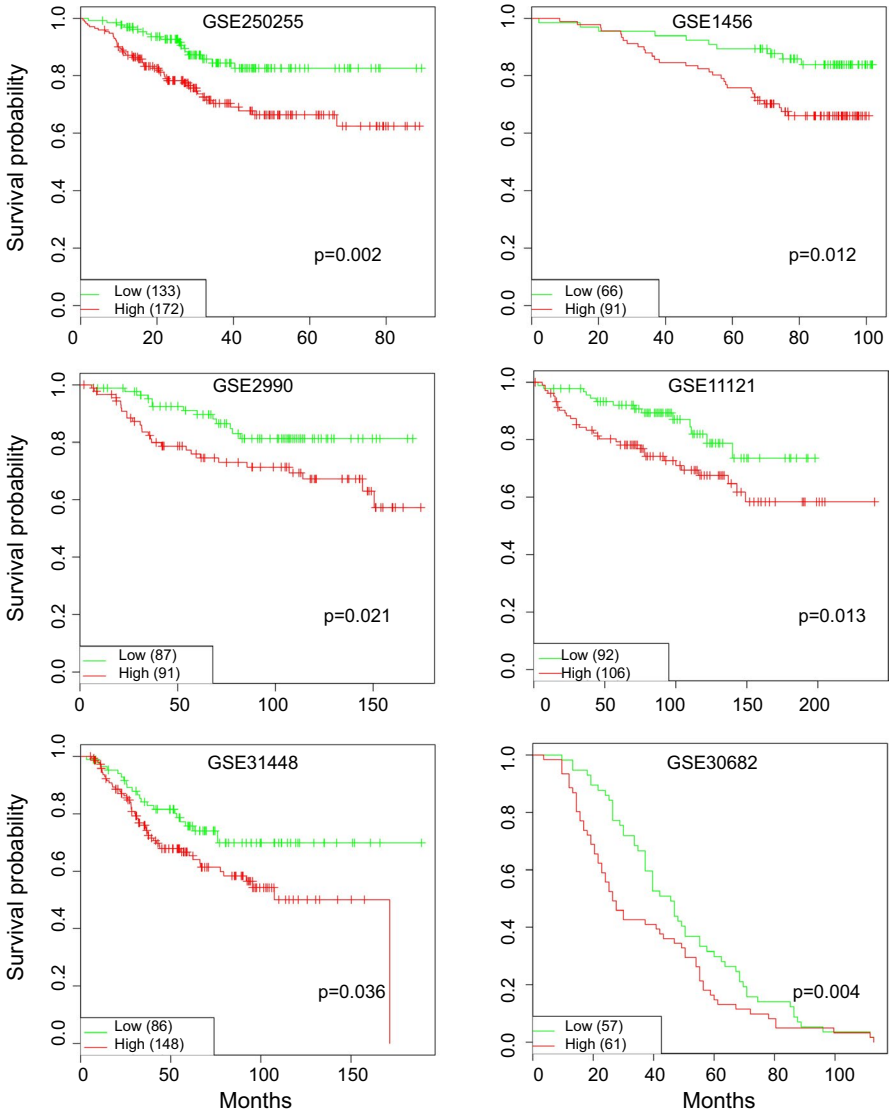

FIGURE 4 High expression of *PRKDC* associates with poor survival in breast cancer patients. Kaplan–Meier plots show overall survival of patients with relatively high or low expression of *PRKDC*

fundamental rationale for targeted cancer therapies. Our results thus suggested that *PRKDC* is frequently overexpressed in breast cancer cells.

3.2 | Knockdown of *PRKDC* results in proliferation inhibition of breast cancer cells

To determine the function of *PRKDC* in breast cancer cells, we selected two breast cancer cell lines with high *PRKDC* expression, MCF-7 and T47D. Cells were infected with *PRKDC* shRNA or control shRNA-encoding lentivirus and efficient knockdown of *PRKDC* protein expression was observed in both cell lines (Figure 2a, all $p < .05$). Of note, we observed a significant decrease in cell proliferation in both cell lines after *PRKDC* knockdown, indicative of its critical role in regulating breast cancer growth (Figure 2a). We further analyzed the colony formation ability of control shRNA or *PRKDC* shRNA-transfected cells. After incubation for 14 days, our results revealed potent reduction in the number of colonies formed upon loss of *PRKDC* in both cell lines (Figure 2b). Moreover, we investigated cell cycle in these cells using Propidium Iodide (PI) staining and flow cytometry. As shown in Figure 2c,d, loss of *PRKDC* resulted in a prolonged mitotic arrest in both cell lines, suggesting that *PRKDC* is required G2/M cell cycle transition. To rule out the possibility of off-targets effect, we have repeated the above analysis using an independent set of shRNAs, and the results remained the same (Figure S1). In contrast, we did not observe any significant cell death induced by *PRKDC* knockdown.

Together, these data established *PRKDC* as a putative driver of breast cancer survival and proliferation.

3.3 | Knockdown of *PRKDC* activates p38 MAPK signaling pathway

Consistent with mitotic arrest being a major defect after *PRKDC* knockdown, western blot analysis revealed elevated

levels of mitotic marker Cyclin B1 in both MCF-7 and T47D cells transfected with *PRKDC* shRNA versus control shRNA (Figure 3a). Moreover, we observed significant accumulation of DNA double-strand break marker γ-H2A.X in *PRKDC* knockdown cells, demonstrating an increase in DNA damage upon *PRKDC* depletion (Figure 3a). Previous studies suggested that activation of p38 MAPK signaling and ERK MAPK signaling pathways by DNA damage stimuli (e.g., ionizing radiation, UV, chemotherapeutic drugs) can also lead to the induction of G2/M cell cycle checkpoint and cell death (Thornton & Rincon, 2009; Wagner & Nebreda, 2009). Of note, as shown in Figure 3a, our results showed that *PRKDC* knockdown significantly enhanced the activity of p38 MAPK signaling pathway as evidenced by enhanced phosphorylation of p38 protein. In contrast, however, no significant change in ERK phosphorylation was observed, indicating that *PRKDC* depletion only activates p38 MAPK signaling pathway.

To further examine whether *PRKDC* depletion induced cell cycle arrest via p38 MAPK signaling pathways, we treated *PRKDC* knockdown cells with a specific p38 MAPK inhibitor SB203580. As shown in Figure 3b, treatment with SB203580 fully abolished p38 phosphorylation in both MCF-7 and T47D cell lines transfected with different shRNAs. Inhibition of p38 MAPK signaling had no influence on the growth of cells transfected with control shRNA (Figure 3b). However, in striking contrast, it significantly enhanced the growth of cells treated with *PRKDC* shRNA, suggesting that activation of p38 MAPK contributed to the proliferation inhibition by *PRKDC* knockdown (Figure 3c). Moreover, p38 MAPK inhibition largely abolished cell cycle arrest induced by *PRKDC* knockdown in both cell lines (Figure 3d,e). Similar results were observed when an independent set of shRNA targeting *PRKDC* was used (Figure S2). Therefore, we suggested that activation of p38 MAPK signaling is involved in *PRKDC* knockdown-induced defects in cell proliferation and cell cycle progression.

3.4 | High *PRKDC* expression is a prognostic marker for poor survival in breast cancer

In support of the above-mentioned findings, we further examined whether *PRKDC* expression is associated with survival of breast cancer patients with or without treatment. To this end, we collected gene expression profiles from six independent large cohorts of breast cancer patients, including (a) 305 cases of HER2-negative invasive breast cancers with neoadjuvant taxane-anthracycline chemotherapy (GSE250255); (b) 157 cases of early breast cancer patients treated with adjuvant therapy (GSE1456); (c) 178 cases of untreated primary breast tumors (GSE2990); (d) 198 cases of untreated primary breast tumors (GSE11121); (e) 234 cases of invasive adenocarcinoma breast cancers (GSE31448); (f) 118 cases of patients with breast-conserving treatment (GSE30682). Expression profiles of *PRKDC* were determined in different patients, who were separated into two groups based on median *PRKDC* expression in the cohort, and Kaplan–Meier survival analyses were performed to compare the differences in overall survival of patients with high *PRKDC* expression versus those with low *PRKDC* expression. As shown in Figure 4, strikingly, our results revealed that high expression of *PRKDC* was significantly associated with poor survival through all six cohorts. Of note, the effect was independent of treatment status, providing strong evidence supporting high expression of *PRKDC* as a prognostic marker for survival and drug response. Together, our results suggest that overexpression of *PRKDC* is involved in tumor progression and is strongly associated with worse outcomes of breast cancer patients.

4 | DISCUSSION

In the current study, we systematically investigated the expression and function of *PRKDC* in breast cancer. Through integrative analysis of transcriptome data of thousands of breast cancer patients, our results revealed that *PRKDC* expression is frequently overexpressed in different breast cancer subtypes and it is a robust prognostic marker for poor survival in patients. Moreover, we showed that *PRKDC* depletion induced proliferation inhibition, defective colony formation, and G2/M cell cycle arrest. One putative mechanism involved activation of p38 MAPK signaling pathway, such that chemical inhibition of the pathway largely augmented the proliferation inhibition and mitotic arrest induced by *PRKDC* knockdown. Together, our results established the role of *PRKDC* as a putative driver of breast cancer and provided a rationale for the development of targeted therapies.

In line with our results, previous studies suggested that *PRKDC* SNPs act in association with *Ku70/80* SNPs to increase the risk of breast cancer (Fu et al., 2003). There is also growing evidence supporting the association between overexpression of *PRKDC* and advanced tumor stage, lymph node metastasis, treatment-resistance, and poor patient survival in multiple cancers (Cornell et al., 2015; Shimomura, Takasaki, Nomura, Hayashi, & Senda, 2013; Stronach et al., 2011; Sun et al., 2017). Our previous results also identified frequent copy number gain and overexpression of *PRKDC* in gastric cancers. Given the strong association between aberrant *PRKDC* expression and cancer incidence, it might play an important role in driving tumorigenesis, however, the underlying mechanisms are largely elusive. Notably, it has been shown that *PRKDC* might play a role in enhancing the expression and stability of oncogene *MYC*, which induces DNA double strand breaks (An et al., 2008;

Z. Zhou et al., 2014). Moreover, previous chromatin immunoprecipitation experiments show that *PRKDC* colocalizes with *ATM* at DNA double-strand breaks (Nonredundant functions of *ATM* and DNA-PKcs in response to DNA double-strand breaks). It might induce *ATM* phosphorylation at multiple sites and result in inhibition of *ATM* kinase activity (Stronach et al., 2011; Zhou et al., 2017).We thus suggest that overexpression of *PRKDC* might serve as a critical mechanism to inactivate *ATM* and p38 MAPK signaling pathways upon DNA damage stimuli to overcome cell death and cell cycle checkpoints. Herein, we showed that *PRKDC* depletion specifically activated p38 MAPK signaling pathway, which was responsible for proliferation inhibition and cell cycle arrest, as the p38 MAPK inhibitor SB203580 rescued all the phenotypical changes induced by *PRKDC* knockdown. It has been shown that p38 MAPK signaling plays a critical role in DNA damage response, cell cycle checkpoints and cell death, acting as both activator or suppressor of cell proliferation and tumorigenesis, depending on the cellular context. The oncogenic or tumor suppressor potential of this signaling pathway has long been aroused the interest to evaluating p38 as a novel target for cancer therapy. Accordingly, p38 MAPK signaling may serve as a putative drug target in cancers with overexpression of *PRKDC*. Consistent with this notion, our results showed that loss of *PRKDC* could cause the persistence of DNA damage that might activate p38 MAPK signaling. Deregulation of mitosis and damage response mediated by *PRKDC* overexpression might cause continual cycling and accumulation of genetic aberrations ultimately leading to genomic instability and cancer.

Collectively, our results suggest that breast cancer cells might become highly reliant on *PRKDC* for survival. In line with this, as shown in Figure 4, we found robust evidence supporting the potential role of *PRKDC* as a biomarker for predicting cancer prognosis and chemotherapeutic outcomes in the clinical setting. Accordingly, chemical activation of p38 MAPK signaling as a new approach for enhancing chemosensitivity of breast cancer cell with high *PRKDC* expression. As current anticancer chemotherapies and radiotherapies are mainly designed to kill rapidly dividing cancer cells by damaging genomic DNA, inhibition of damage repair pathways may sensitize tumor cells to the therapies, a conception important for developing novel targets for personalized medicine (Hsu et al., 2012; Kotsantis et al., 2015; Puigvert et al., 2016). In light of this, *PRKDC* has been the focus of a number of small molecule studies. These inhibitors may be used as a monotherapy but mostly they enhance the therapeutic effects in combination with chemotherapies or radiotherapies (Davidson, Amrein, Panasci, & Aloyz, 2013). For example, NU7441 is amongst the most selective molecule which exhibited strong radiosensitization effects without causing significant cellular toxicity (Shinohara et al., 2005). Further investigation of *PRKDC* and its complementary signaling pathways should clarify the detailed mechanism underlying their functions in breast cancer and provide new insights into the development of novel prognostic biomarkers and molecular targets for breast cancer therapy.

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CONFLICTS OF INTEREST

The authors claim no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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