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XPC deficiency leads to centrosome amplification by inhibiting BRCA1 expression upon cisplatin-mediated DNA damage in human bladder cancer

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ABSTRACT

Xeroderma pigmentosum group C (XPC) is a well-known DNA damage recognition protein. Defects in XPC lead to carcinogenesis and progression of many human cancers. In the current study, we defined a novel, important role of XPC in preventing centrosome amplification during cisplatin-mediated DNA damage response. From experiments with human bladder cancer tissue, urothelial tissue from *Xpc* knockout mice and XPC-silenced cell lines, we found that attenuated XPC expression was associated with increased centrosome amplification in human bladder cancer. A significant increase in centrosome amplification was observed in XPC-silenced cells upon cisplatin treatment. XPC deficiency leads to reduced BRCA1 expression via upregulating its transcriptional repressor, Pit-1. The BRCA1 downregulation results in more DNA double strand breaks accumulation and persistent activation of the ATM-Chk1/Chk2 signaling, resulting in a prolonged G2/M arrest during which centrosome can over-duplicate and lead to centrosome amplification. XPC complementation in silenced cells could reduce Pit-1 expression, increase BRCA1 expression and recover the status of centrosome amplification. Our study reveals a new function for XPC in preventing chromosomal instability, providing new information on cancer chemotherapy and potential clinical significance for cancer management.

1. Introduction

Chromosome instability is widely accepted as a hallmark of human cancer. The centrosome, a major microtubule-organizing center of mammalian cells, plays fundamental roles in organizing both the interphase cytoskeleton and the mitotic spindle. The presence of more than two centrosomes (centrosome amplification, CA) leads to the formation of defective mitotic spindles and, consequentially, chromosome segregation errors. Due to a strong association between CA and aneuploidy, centrosome amplification is believed to be a major cause of chromosome instability in human cancer cells. Studies have shown the frequent occurrence of centrosome amplification in many types of cancers, including breast, lung, head and neck, prostate, colon, brain, liver and bladder cancer [1]. Moreover, studies on bladder cancer indicated that higher degrees of centrosome amplification were strongly associated with higher tumor stage and tumor recurrence [2,3].

Cellular genomic DNA normally sustains continuous damage requiring repair [4]. It is known that a broad range of DNA-damaging agents, including cytostatic drugs and ionizing radiation, can induce centrosome amplification in both malignant and non-transformed cell lines [5–8]. These different types of DNA damage lead to a consequent DNA damage response (DDR), which orchestrates the repair of the damage, halts cell cycle progression and upregulates the expression of genes involved in the repair process. With DNA damage checkpoint, this cell cycle delay is maintained until the DNA damage is repaired or until the cell is driven out of the cell cycle into apoptosis or senescence [9]. It is well established that the following DDR proteins are present at centrosomes, at least during part of the cell cycle: ATR, ATM, CHK1, CHK2, BRCA1, BRCA2 and PARPs [10].

The nucleotide excision repair (NER) pathway is the major DNA

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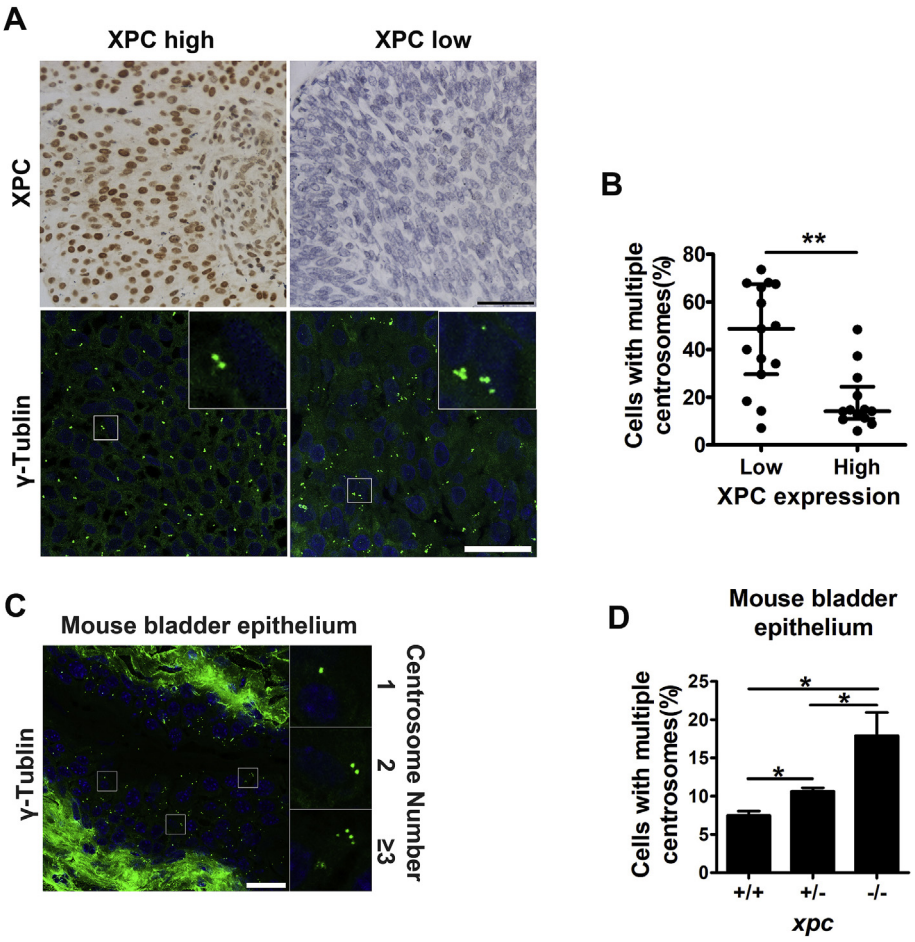

Fig. 1. Centrosome amplification in urinary bladder epithelium correlates negatively with XPC expression level. (A) Representative immunohistochemistry staining of XPC (Upper panel, brown) and immunofluorescence staining of γ-tubulin (lower panel, green); (B) Frequencies of centrosome amplification in bladder cancer tissue are grouped according to XPC expression level and are plotted, data presented as the median (Q1, Q3). (C) Representative immunofluorescence staining of γ-tubulin in mouse bladder epithelium. (D) Quantification of cells with extra centrosomes in each genotype of mouse bladder epithelium. More than 200 cells were counted. data represent the mean ± SEM from three independent experiments. *P < 0.05, **P < 0.01. Scale bar, 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

repair pathway for repairing the bulky DNA damage generated by most environmental factors, such as UV radiation, chemicals, and therapeutic drugs. A functional NER pathway is essential for maintaining genetic integrity and for preventing carcinogenesis [11]. Xeroderma pigmentosum group C (XPC) is a DNA damage recognition protein that plays an important role in the NER process. The XPC protein might also play important roles in other DNA damage responses, including cell cycle checkpoint regulation and apoptosis [12]. XPC defects result in the frequent occurrence of mutations in genomic DNA upon DNA damage [13]. XPC defects also have been found in many types of cancer, including lung and skin cancer [14,15]. However, the relationship between XPC defects and chromosomal aberrations remains unclear.

Human bladder cancer is a heterogeneous neoplasm that presents as a superficial tumor in 80% of cases or as a muscle invasive tumor. Molecular biological research revealed that urothelial carcinoma contains multiple genetic and epigenetic abnormalities, including chromosomal abnormalities, oncogene activation, tumor suppressor gene inactivation and tumor microenvironment alterations [16]. We previously reported that attenuated XPC expression was frequent in bladder cancer tissue and was associated with carcinogenesis and progression of bladder cancer [17,18]. Here, we found that attenuated XPC expression was significantly related to centrosome amplification in bladder cancer tissue in this study. This interesting phenomenon led us to investigate how XPC deficiency affects centrosome amplification.

2. Materials and methods

2.1. Plasmids, lentivirus and cell lines

For plasmid construction, the cDNA of XPC (Origene) was inserted between the SalI and NotI sites of the pLVX-IRES-Puro vector (Clontech). The Quikchange method (QuikChange II Site-Directed Mutagenesis Kit, Agilent) was used to delete coding region of Centrin2 binding motif of XPC (aa847-863). The promoter region of *POU1F1* was inserted between the XhoI and EcoRI sites of the pGL3-basic vector (Promega).

The pLVX-IRES-Puro vector based plasmids were subjected to produce lentivirus particle by using a 2nd generation lentivirus packaging system (pMD2.G and psPAX).

HEK293, T24, 5637, RT4, SW780, J82 and UMUC3 were purchased from the Cell Bank of Chinese Academy of Sciences. All cell lines were cultured by following supplier's instruction.

2.2. Xpc KO mice

The *Xpc* knockout (KO) mice (B6; 129-*Xpc*^{tm1Ecf}/J) were purchased from Jackson Laboratory and were maintained in SPF environment at the laboratory center of Third Military Medical University (TMMU). All procedures of animal experiments were carried out following an animal study protocol approved by TMMU.

2.3. Immunohistochemical staining and evaluation

Cancer tissue samples were acquired from Southwest Hospital of TMMU and the study was approved by the ethics board of Southwest Hospital. Tumors were graded and staged by 2004 WHO criteria and the TNM classification, respectively. Tissues were made into paraffin sections at thickness of 5 μm to detect XPC expression by immunohistohemical staining. The images were captured with a Nikon microscopy (eclipse E600) and XPC expression was scored as described

Table 1
Centrosome hyperamplification, XPC expression and pathological grade/stage in human urothelial carcinoma samples.

Case NO.	centrosome		XPC level	Pathology	
	CH grade	n ≥ 3 (%)		Grade	Stage
1	CH II	5.9	High	PUNLMP	Ta
2	CH II	7.1	Low	High	T2
3	CH II	8.8	High	Low	Ta
4	CH II	10.7	High	PUNLMP	T1
5	CH II	11.4	High	Low	Ta
6	CH II	12.6	High	High	T1
7	CH II	14	High	PUNLMP	T1
8	CH II	14.2	High	High	Ta
9	CH II	14.3	Low	PUNLMP	T1
10	CH II	14.7	High	PUNLMP	Ta
11	CH II	14.9	High	PUNLMP	Ta
12	CH II	18.4	Low	PUNLMP	Ta
13	CH III	20.7	High	PUNLMP	Ta
14	CH III	28.2	High	PUNLMP	T1
15	CH III	29.6	Low	Low	T1
16	CH III	34.1	Low	PUNLMP	T1
17	CH III	36.2	Low	PUNLMP	T1
18	CH III	37.3	High	PUNLMP	T1
19	CH III	40	Low	High	T2
20	CH III	48.4	High	PUNLMP	T1
21	CH III	48.8	Low	High	T3
22	CH III	50	Low	Low	Ta
23	CH III	59.5	Low	High	T1
24	CH III	66	Low	Low	T1
25	CH III	67.5	Low	PUNLMP	Ta
26	CH III	67.9	Low	High	T2
27	CH III	68.1	Low	Low	T2
28	CH III	73.6	Low	PUNLMP	T2

Abbreviation: CH: centrosome hyperamplification, PUNLMP: Papillary neoplasm of low malignant potential, Low: Low-grade papillary carcinoma, High: High-grade papillary carcinoma.

[17].

2.4. Immunofluorescence microscopy

Cells attached on coverslips were fixed with either 4% paraformaldehyde or cold methanol. Tissues are embedded in OCT compound (Sakura Finetek) and cut to a thickness of 8 μm (Leica Biosystems) and then fixed with cold methanol. Cells or cryosections were incubated with primary antibodies overnight at 4 °C and appropriate secondary antibodies (FITC or Cy3 conjugated, ZSGB-BIO) were then incubated. Immunofluorescence images were captured using a Zeiss LSM700 confocal microscope. The degree of centrosome hyperamplification in cancer tissue section was graded as previously described [19,20].

2.5. cDNA preparation and quantitative PCR

Total RNA was extracted with Trizol reagent (Invitrogen) and the cDNA was generated with random primers (GoScript Reverse Transcription Mix, Promega). Quantitative PCR was performed by using the Sso Advanced Universal SYRB Green Supermix (Bio-Rad). The primers used were listed in supplementary material.

2.6. Western blot analysis

Equal amounts of cell lysates (50 μg total protein) were resolved by SDS-PAGE gel and subjected to western blot analysis as previously described [17].

2.7. Cell cycle analysis

Cells were fixed with 70% cold ethanol and incubated at −20 °C for at least overnight. DNA was stained with propidium iodide (50 μg/ml) (Invitrogen) and was detected with a BD Accuri™ C6 Flow Cytometer (Becton Dickinson). The cell cycle distribution was analyzed with FlowJo.

2.8. Luciferase reporter assay

Cells transfected with the pGL3B-hPit-1 constructs (pGL3Basic, pGL3B-hPit-1 − 3732/ + 128, pGL3B-hPit-1 − 863/ + 128), pRL-TK (Promega) was co-transfected to normalize transfection efficiency. Luciferase was measured using the Dual-Luciferase Assay kit (Promega) 48 h after transfection.

2.9. Statistical analysis

Unpaired *t*-test, Mann Whitney test, Kruskul-Wallis test and One-way analysis variance were used to do comparisons, and differences with a P-value < 0.05 were considered significant. All data were analyzed using the statistical tool of GraphPad Prism.

3. Results

3.1. Centrosome amplification in human urothelial carcinoma samples correlates negatively with XPC expression

Based on our previous work, we hypothesized that low XPC expression might contribute to centrosome amplification. To test this hypothesis, centrosome amplification was determined by immunofluorescence staining of γ-tubulin in two panels of bladder cancer tissue selected according to their XPC expression levels. Among the 28 cases selected, 13 cases had high XPC expression, and 15 cases had low XPC expression (Fig. 1A, Upper panel). Centrosome amplification (CA) was detected in all cases (Fig. 1A, Lower panel), and as shown in Table 1, the CA burden was much higher in the XPC low expression panel. Specifically, in the XPC high expression panel, 9 cases (69.2%) were CH II, and 4 cases (30.8%) were CH III, and in the XPC low expression panel, 3 cases (20%) were CH II, and 12 cases (80%) were CH III. When the exact frequency of CA was plotted against the XPC expression panel, the difference was more obvious (Fig. 1B). Together, these results strongly suggest that low XPC expression might contribute to centrosome amplification in bladder cancer.

3.2. XPC deficiency induces centrosome amplification in bladder tissue and bladder cancer cell lines

To determine if XPC deficiency could increase centrosome amplification *in vivo* under physiological conditions, we examined centrosome amplification in the bladder transitional epithelium of *Xpc* knockout mice (Fig. S1). The average CA frequencies were 7.47 ± 1%, 10.63 ± 0.8% and 17.9 ± 5.28% in the urinary epithelia of *Xpc* wild-type, heterozygous and homozygous mice, respectively (Fig. 1C and D).

To determine a direct role for low XPC expression in centrosome amplification, we knocked down XPC in T24 and HEK293 cells by shRNA and in 5637 by siRNA (Fig. 2A), and then monitored centrosome amplification by immunofluorescence staining of γ-tubulin and centrin2 under normal conditions or after DNA damage (Fig. 2B). All of the cell lines showed a basal level of centrosome amplification under normal conditions, centrosome amplification was significantly increased when XPC was knocked down by either shRNA or siRNA. XPC deficiency induced more prominent centrosome amplification upon cisplatin treatment (Fig. 2C). As the main function of centrosomes is to form the mitotic spindle poles during mitosis, we examined the spindle poles by immunofluorescence staining of α-tubulin (Fig. 2D). As expected, we

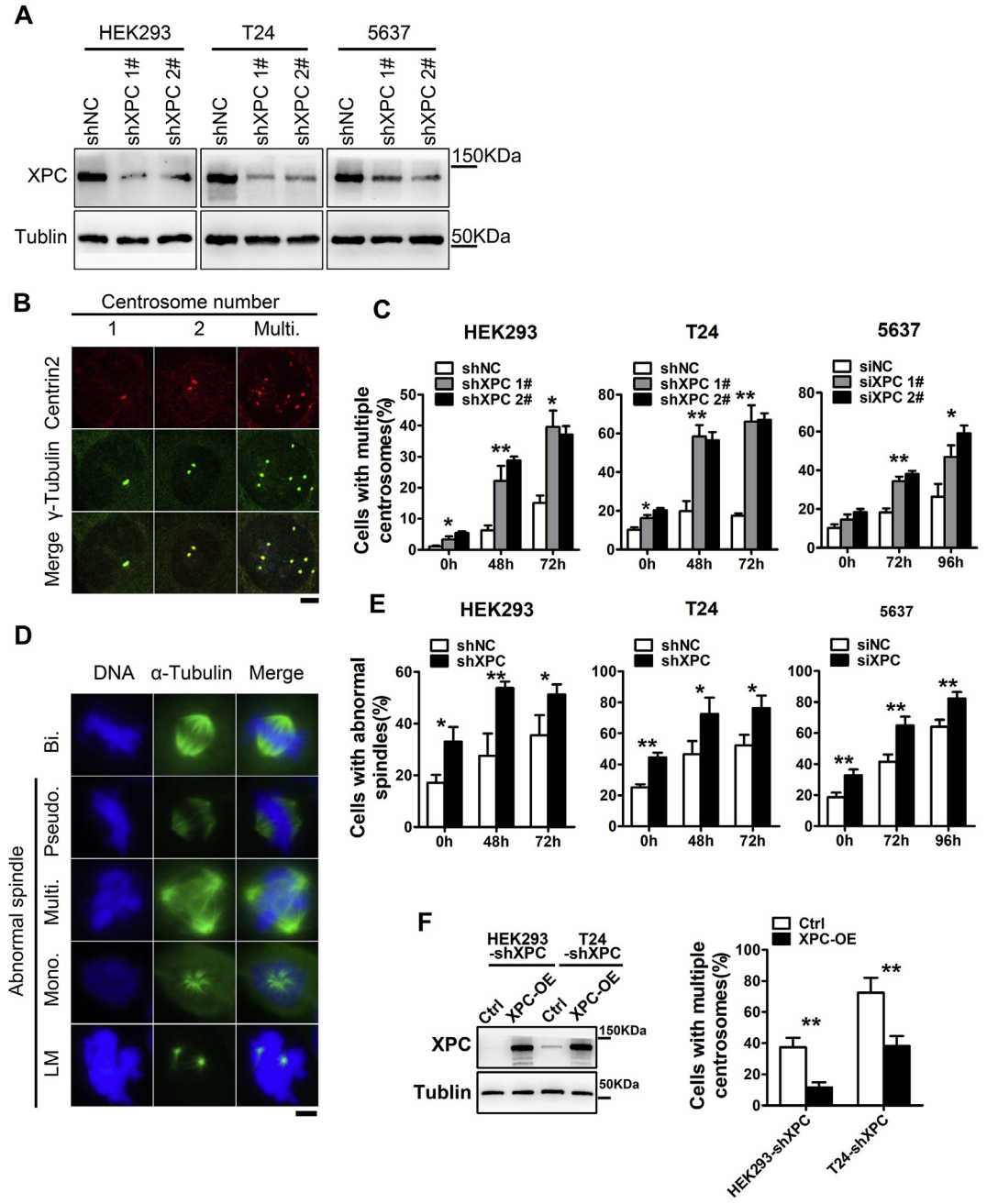

Fig. 2. Loss of XPC induces centrosome amplification and spindle pole defects in human bladder cancer cells. (A) Representative western blotting showing XPC knockdown efficiency in HEK293, T24 and 5637 cells. (B) Representative images showing centrosome morphologies in T24 cells (scale bar, 5 μm). Centrosome was revealed with γ-tubulin (green), centrin2 (red), and nucleus was counterstained with DAPI (blue). (C) Quantification of cells with extra centrosomes at the indicated times post cisplatin (10 μM) treatment. (D) Representative images show spindle in T24 cells. Spindle was revealed by α-tubulin (green) and chromosome was counterstained with DAPI (blue), (scale bar, 5 μm). Abbreviations: Bi., bipolar; Pseudo., pseudo-polar; Multi, multipolar; Mono., monopolar; LM, lack of microtubules. (E) Quantification of cells with spindle pole defect at the indicated time post cisplatin (10 μM) treatment. (F) Restoration of XPC (Left) rescue centrosome amplification in both HEK293-shXPC and T24-shXPC cells (Right). OE: overexpression. Quantification of cells with extra centrosomes when XPC restored after cisplatin (10μM/72h) treatment. For quantification, more than 200 cells for centrosome amplification and more than 100 mitotic phases for spindle per condition were counted, data represent the mean ± SEM from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

observed more spindle pole defects in XPC-deficient cells, both before and after cisplatin treatment (Fig. 2E). To further confirm that the increased centrosome amplification could indeed be ascribed to XPC insufficiency, we rescued the XPC expression and found that centrosome amplification was reduced in both HEK293 and T24 cells (Fig. 2F). Moreover, the centrosome amplification induced by XPC deficiency seems to be independent of NER activity since knockdown of other key components of the NER pathway, including XPA, XPF and Rad23B, did not induce centrosome amplification (Figs. S2A–C). Taken together, we conclude that loss of XPC leads to centrosome amplification under physiological and DNA damage conditions.

3.3. XPC deficiency results in a prolonged G2/M arrest after DNA damage

The potential role for the XPC/centrin2 complex in the coordination of centrosome homeostasis was first addressed by Salisbury JL et al.,

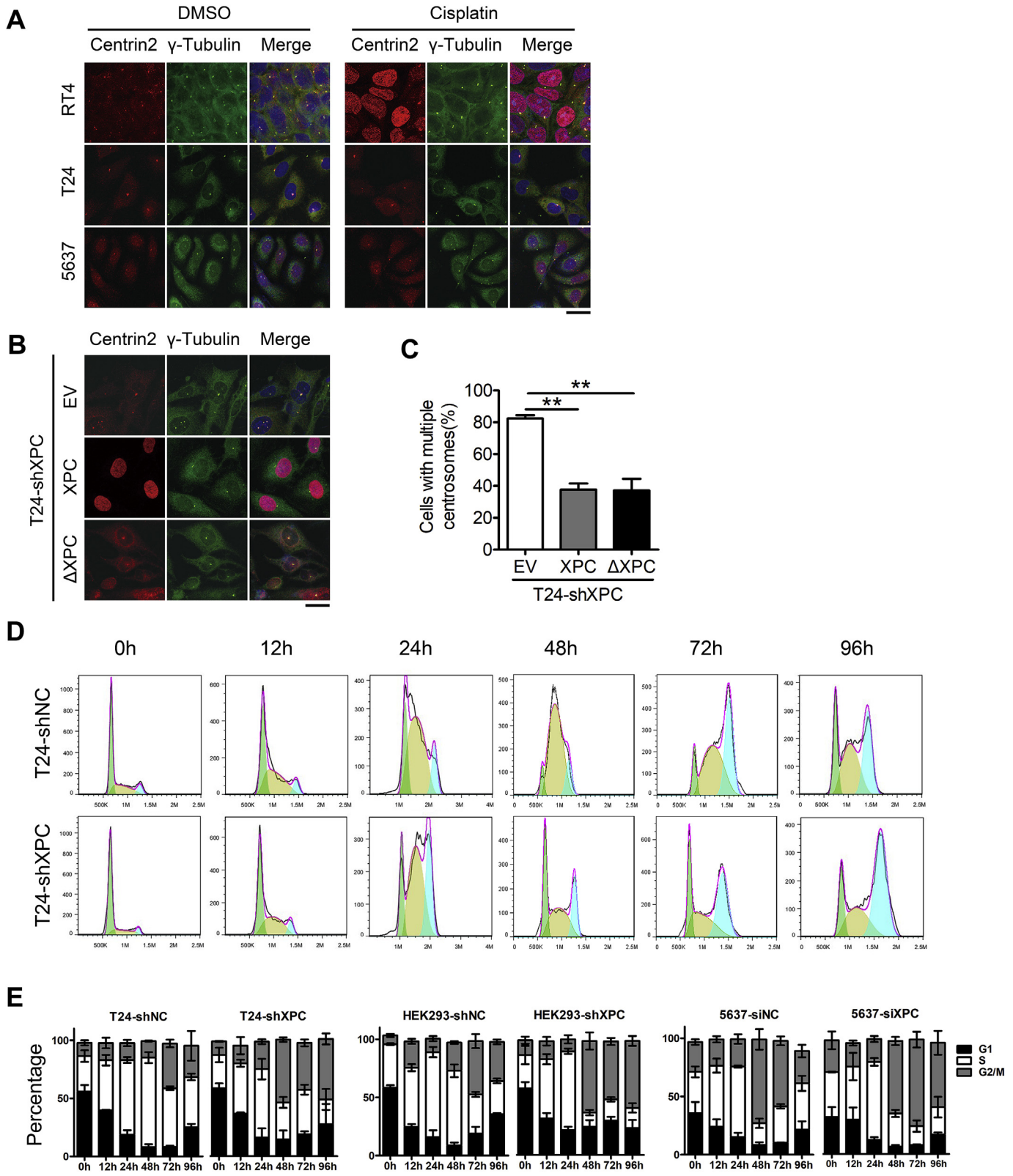

Fig. 3. XPC deficiency results in a prolonged G2/M arrest upon cisplatin treatment. (A) Localization of centrin2 in bladder cancer cell lines before and after DNA damage (scale bar, 20 μm). (B) Localization of centrin2 in T24-shXPC cells when rescued by XPC or ΔXPC upon cisplatin (10μM/72h) treatment (scale bar, 20 μm). (C) Quantification of cells with extra centrosomes in T24-shXPC when XPC or ΔXPC was restored after cisplatin (10μM/72h) treatment. For quantification, more than 200 cells were counted respectively from three independent experiments, data represent the mean ± SEM, **P < 0.01. (D) Representative images show cell cycle profiling of T24-shNC/T24-shXPC cells after 10 μM cisplatin treatment. (E) Quantification of cell cycle profiles in T24, HEK293 and 5637 cells, respectively. Data represent the mean ± SD from three independent experiment.

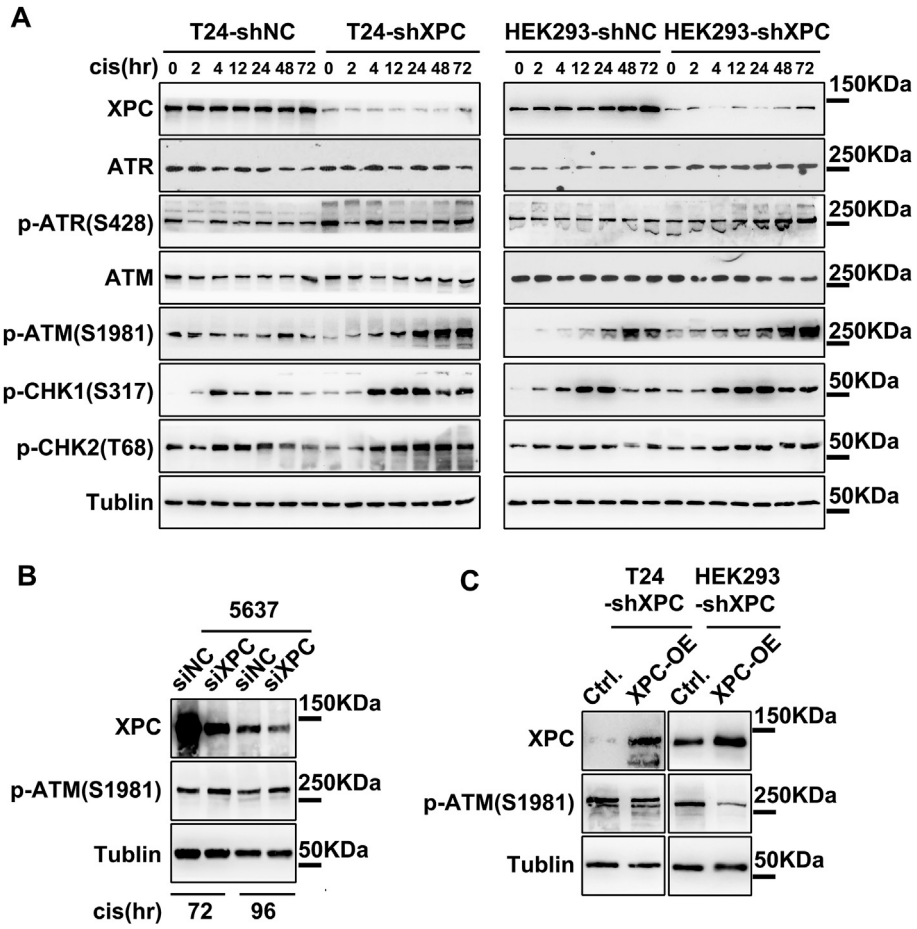

Fig. 4. XPC deficiency hyperactivates the ATM-Chk1/Chk2 signaling pathway upon cisplatin treatment. (A) Cells were treated with 10 μM cisplatin at the indicated times and cell lysates were analyzed by western blotting. (B) 5637 cells that transfected with either scramble or XPC siRNA were treated with 10 μM cisplatin at the indicated times and cell lysates were analyzed by western blotting. (C) Cells which have rescued XPC expression were treated with 10 μM cisplatin for 72h and cell lysates were analyzed by western blotting.

whose study showed that XPC-dependent nuclear sequestration of centrin2 could prevent centrosome amplification upon DNA damage [21]. Here, RT4 cell line which derived from the benign bladder epithelial tumor was presented as a control, our results showed that no significant changes of centrin2 relocalization to nucleus in several bladder cancer cell lines before and after cisplatin treatment except for RT4 cell line (Fig. 3A, S3). Moreover, we rescued XPC and XPC mutant (△XPC) which being deleted centrin2 binding site (aa847-863) in T24-shXPC cells. Both of the rescue could significantly decrease CA although redistribution of centrin2 into nucleus failed in XPC mutant cells (Fig. 3B and C). The result provides a direct evidence that XPC deficiency leading to CA was independent of centin2 in bladder cancer cells. Previous work has suggested that abnormal cell cycle progression may lead to centrosome amplification [5,22,23]. To test whether XPC deficiency affects cell cycle progression, we treated cells with 10 μM cisplatin and examined cell cycle progression. Consistent with many reports, obvious S and/or G2/M arrest was observed in all of the cells analyzed; however, the exact response mode changed after XPC knockdown (Fig. 3D). In T24-shNC cells, a predominant S-phase accumulation was observed followed by a relatively short G2/M accumulation. In contrast, the S-phase accumulation in T24-shXPC cells was relatively transient and was followed by a more prolonged G2/M arrest (Fig. 3E, left panel). Similar results were observed in HEK293 cells (Fig. 3E, middle panel). In 5637 cells, while the S-phase arrest was similar in both cell types, the subsequent G2/M arrest in 5637-siXPC cells was much longer (Fig. 3E, right panel). Thus, these data suggest that XPC deficiency causes a prolonged G2/M arrest during which centrosome can over-duplicate as reported.

3.4. XPC deficiency leads to persistent activation of the ATM-Chk1/Chk2 signaling upon cisplatin-induced DNA damage

In response to DNA damage, a kinase-dependent signaling pathway, which is initiated by the apical kinases (ATM/ATR) and transduced by the mediator kinases (Chk2/Chk1), is activated to overcome the deleterious effects [9]. To dissect the reason for the diminished S-phase arrest and prolonged G2/M arrest seen in XPC-deficient cells, we measured the ATM/ATR-Chk1/Chk2 signaling pathway by western blot (Fig. 4A). For the apical kinases, the level of the active form of ATM (pSer1981) increased remarkably and persisted for a longer time in XPC-deficient cells than in control cells after cisplatin treatment. Knockdown of XPC in 5637 cells also led to hyperactivation of ATM (Fig. 4B). Restoration of XPC rescued p-ATM hyperactivation (Fig. 4C). The active form of ATR (pSer428), in contrast, increased slightly in XPC-deficient cells but persisted similarly in both cell types. The overall expression of ATM in response to cisplatin treatment seemed unaffected upon XPC knockdown, while the overall expression of ATR seemed higher in HEK293 cells when XPC was knockdown. For the downstream checkpoint proteins, the active forms of Chk1 and Chk2 also persisted much longer in XPC-deficient cells (Fig. 4A). To further confirm the role of sustained ATM activation in the prolonged G2/M arrest of XPC-deficient cells, we treated HEK293-shXPC or T24-shXPC cells with KU55933, which specifically inhibits ATM kinase. This treatment caused a remarkable inhibition of ATM phosphorylation at Ser1981 (Fig. 5A) but had no effect on cell survival. Inhibition of ATM led to a significant reduction in the frequencies of cells with centrosome amplification and spindle pole defects after cisplatin treatment (Fig. 5B and C). Consistently, the prolonged G2/M arrest in both HEK293-shXPC and T24-shXPC cells after cisplatin treatment was also relieved by ATM inhibition (Fig. 5D and E). Meantime, after ATM inhibition, the levels of

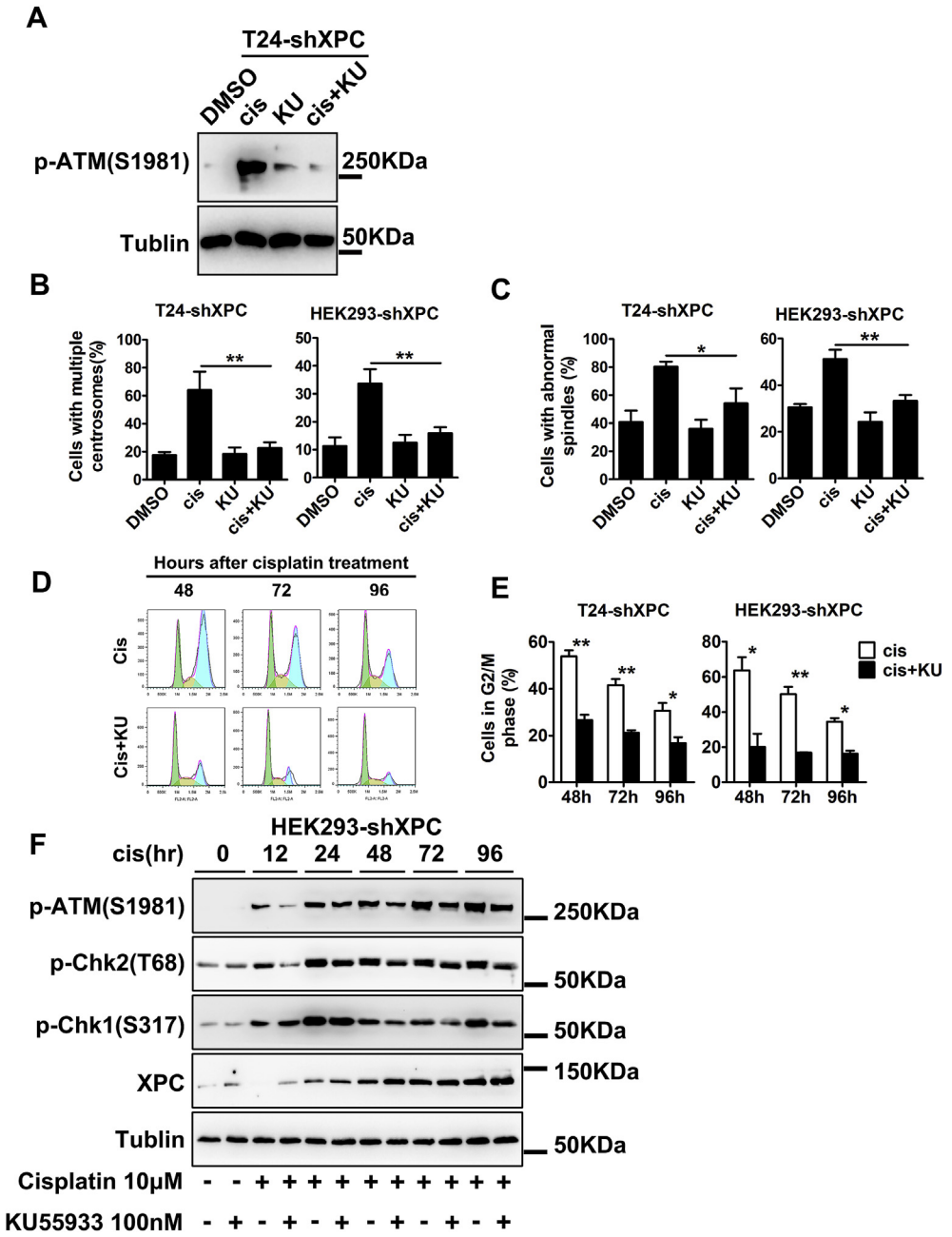

Fig. 5. Inhibition of ATM relieves the centrosome amplification and spindle pole defects in XPC deficient cells. (A) Representative western blotting showing p-ATM inhibition efficiency by KU55933 (KU,100 nM). (B) KU55933 (100 nM) reverse centrosome amplification caused by cisplatin treatment. Quantification of cells with multiple centrosomes upon cisplatin treatment (10μM72h) in the presence or absence of ATM inhibitor KU55933. More than 200 cells per condition were counted from three independent experiment. (C) KU55933 (100 nM) reverses spindle pole defects caused by cisplatin treatment. Quantification of cells with spindle pole defects upon cisplatin treatment (10μM72h) in the presence or absence of ATM inhibitor KU55933. More than 100 mitotic cells were counted for each group. Data represent the mean ± SEM. from three independent experiments, *P < 0.05, **P < 0.01. (D, E) KU55933 (100 nM) relieves G2/M arrest upon cisplatin treatment (10 μM) at the indicated times. Quantification of cells in G2/M phase. Data represent the mean ± SEM from three independent experiments, *P < 0.05, **P < 0.01. (F) Cells were treated with 10 μM cisplatin with or without KU55933 (100 nM) at the indicated times, cell lysates were analyzed by western blotting.

phosphorylated Chk1 and Chk2 were compromised (Fig. 5F). Taken together, these results showed that hyperactivation of ATM is critical to the centrosome amplification induced by cisplatin in XPC-deficient cells.

3.5. XPC deficiency inhibits BRCA1 expression upon cisplatin treatment

To examine the reason why XPC deficiency leads to hyperactivation of the ATM-Chk1/Chk2 signaling pathway, we evaluated the expression of some key DNA-damage response genes, especially DNA double-strand break (DSB) repair genes, in XPC-deficient cells, as cisplatin-induced inter-strand crosslinks might lead to secondary DNA double-strand breaks. We found that XPC deficiency significantly decreased the protein levels of BRCA1 and Rad51 in response to cisplatin treatment in T24 and HEK293 cells during a time-course (Fig. 6A, C) or cisplatin dose-response assay (Fig. 6B, D), although the repression of Rad51 was not as obvious as that of BRCA1. Similar results were obtained in 5637 cells (Fig. 6E). The reduced BRCA1 expression could be ascribed to reduced mRNA expression, as shown by qPCR analysis (Fig. S4). Additionally, restoration of XPC could significantly rescue BRCA1 expression (Fig. 6F). Given the role of BRCA1 in DSB repair, we then tested whether DSBs accumulated more in these XPC-deficient cells. γ-H2AX foci, the classical DSB marker, increased markedly in XPC-deficient cells, as reflected by both the percentage of foci-positive cells and foci numbers (Fig. 6G–I). Moreover, the upstream phosphorylation of ATM (Ser1981) foci increased (Fig. 6J and K), indicating that cells accumulated more DNA damage (DSBs) in XPC-deficient cells. Together, these results indicate that XPC deficiency leads to increased DSB accumulation, which then hyperactivates the ATM-Chk1/Chk2 signaling pathway, leading to a prolonged cell cycle arrest.

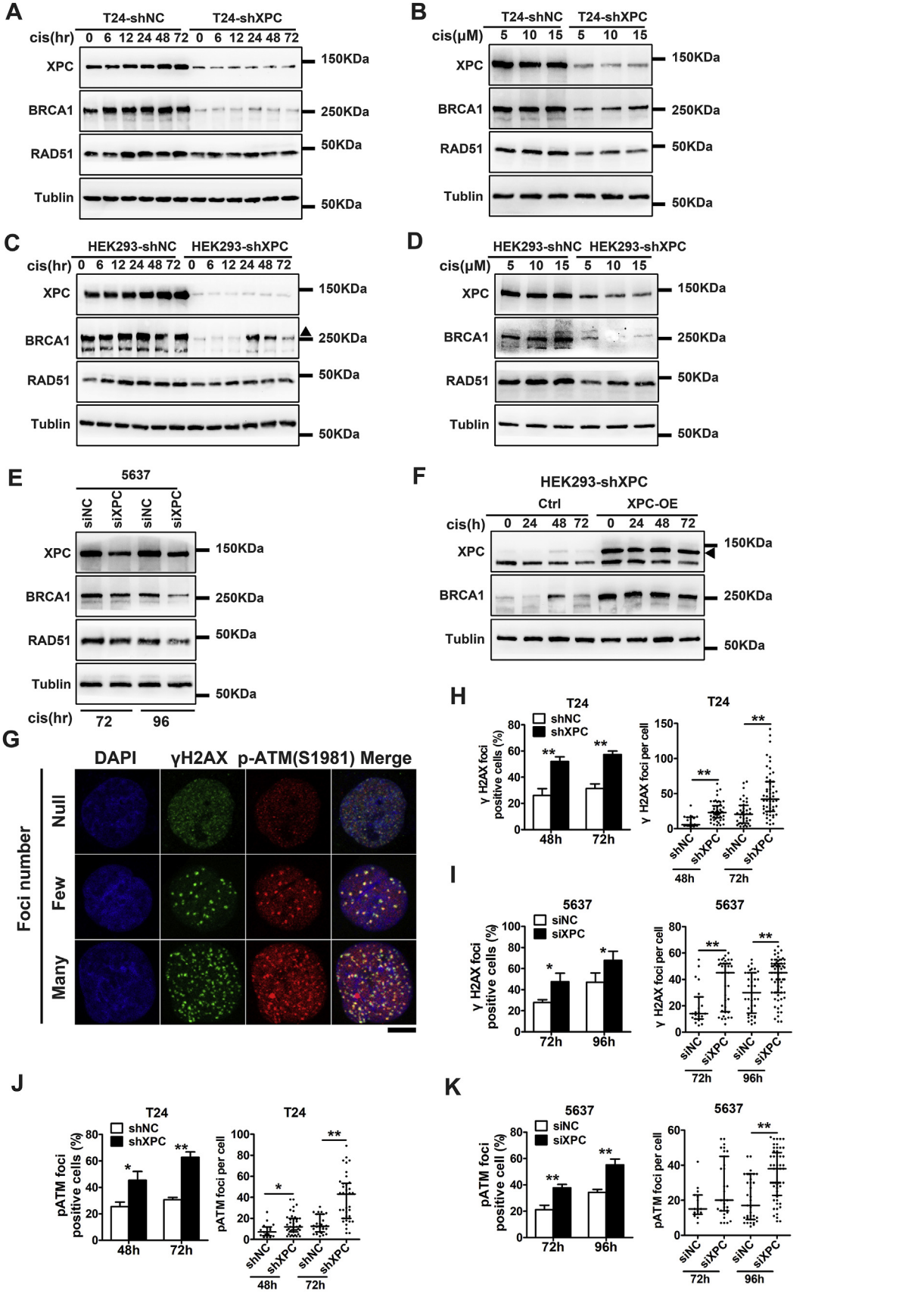

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Fig. 6. XPC deficiency inhibits BRCA1 expression upon cisplatin treatment. (A, C) Cells were treated with 10 μM cisplatin at the indicated times and cell lysates were analyzed by western blotting. (B, D) Cells were treated with cisplatin at the indicated concentrations for 72h and cell lysates were analyzed by western blotting. (E) 5637 cells were transfected with either scramble or XPC siRNA and then treated with 10 μM cisplatin at the indicated times and cell lysates were analyzed by western blotting. (F) Cells were transfected with vector (Control) or XPC overexpression construct and were treated with 10 μM cisplatin at the indicated times and cell lysates were analyzed by western blotting. (G-K) T24 and 5637 cells were treated with cisplatin (10 μM) and. were subjected to immunofluorescence at indicated time. (G) Representative immunofluorescence image of foci-containing cells (scale bar, 10 μm). (H, I) Quantification of cells containing at least five gamma-H2AX foci in T24 and 5637 cells. (J, K) Quantification of cells containing at least five p-ATM (Ser1981) foci in T24 and 5637 cells. More than 200 cells per condition were counted from three independent experiment. Foci positive rate represent as mean ± SEM, foci number per cell represent as median (Q1, Q3). *P < 0.05, **P < 0.01.

3.6. XPC deficiency inhibits BRCA1 expression by upregulating the transcriptional factor, Pit-1

Previous reports have demonstrated that Pit-1 negatively regulates BRCA1 transcription [24]. Therefore, we examined whether XPC influences Pit-1 regulation. Our data showed that XPC deficiency remarkably increased Pit-1 protein level in HEK293 and T24 cells in both time-course and dose-course experiments after cisplatin treatment (Fig. 7A–D). Similar results were observed in 5637 cells (Fig. 7E). Consistently, the Pit-1 mRNA level was also substantially increased after XPC knockdown (Fig. S5), indicating that XPC might regulate Pit-1 at the transcriptional level, rather than post-translationally. To test that, *Pit-1* promoter activity was measured in response to XPC knockdown. We found that XPC deficiency significantly enhanced the Pit-1 promoter activity (pGL3-hPit-1 -863-+128) compared with the control cells (Fig. 7F). In support of this, restoration of XPC can reduce Pit-1 expression with or without cisplatin treatment (Fig. 7G). To further confirm the role of Pit-1 in centrosome amplification, we knocked down Pit-1 in T24-shXPC cells and found that centrosome amplification significantly decreased compared with the control cells (Fig. 7H and I). Also, γ-H2AX foci and p-ATM (Ser1981) foci positive cells reduced (Fig. 7J). At the same time, the upstream hyperactivation of p-ATM (Ser1981) and attenuated BRCA1 expression were also relieved (Fig. 7H). In addition, similar results were obtained in T24 cells when siRNA was used to knock down only XPC compared with simultaneous knock down of XPC and Pit1 (Fig. 7K). Together, these results strongly suggest a signaling pathway in which the upstream factor, XPC, negatively regulates Pit-1 transcription and the latter exerts the same effect on BRCA1.

4. Discussion

Accumulating evidences indicate that centrosome amplification is a common occurrence and is correlated with malignancy degree and poor prognosis in human bladder cancer [2,3,25]. In our current study, attenuated XPC expression was firstly found to be strongly associated with centrosome amplification in bladder urothelial cells of XPC knockout mice and human bladder cancer. A significant increase in centrosome amplification was observed in XPC-silenced cells upon cisplatin treatment. XPC deficiency leads to reduced BRCA1 expression via upregulating its transcriptional repressor, Pit-1. The BRCA1 downregulation results in more DSBs accumulation and persistent activation of the ATM-Chk1/Chk2 signaling, resulting in a prolonged G2/M arrest during which centrosome can over-duplicate.

As for the mechanism of XPC maintaining centrosome homeostasis upon DNA damage, it was reported that XPC prevent CA via translocating centrin2 into nucleus in breast cancer cells [21]. In this study, we found no significant centrin2 relocalization to nucleus in bladder cancer cells upon cisplatin treatment (Fig. 3A, S3). Although rescuing XPC in T24-shXPC cells can lead centrin2 nuclear sequestration, it should be noted that almost four fold higher expression of XPC was artificially produced in rescued cells than T24-shNC cells (Fig. S6). Combined with our previous finding that XPC expression is frequently low in bladder cancer [17], it suggest that only cells which hold higher XPC expression may capable of redistribute centrin2 into nucleus to avoid CA upon DNA damage treatment. We here provide a novel mechanism of XPC in maintaining centrosome homeostasis via cell cycle regulation instead of centrin2 redistribution into nucleus in bladder cancer.

The duplication and separation of centrosomes occur once per cell cycle and proceed in a timely fashion that is coordinated with the cell cycle [26]. Current studies on centrosome amplification suggest that this process can occur as a result of prolonged S or G2/M phases, especially the latter [5,22,23]. Upon DNA damage, signal transduction pathways cause cell cycle arrest which allows time for DNA repair. On the other hand, the sustained cell cycle arrest, which results from extensive DNA damage or DNA repair defects, may facilitate centrosome over-duplication. Accumulating evidences suggest that defects in a number of key genes involved in the DNA damage response could cause centrosome amplification, including Rad51, BRCA1, Mre11, BRCA2, Gadd45, p53, p21 [5,10,27–29]. In this study, we showed that the classical NER factor, XPC, may be involved in the signaling pathway regulating cell cycle arrest and maintaining centrosome homeostasis.

Cisplatin-DNA adducts are cytotoxic for cells that lead to cell cycle arrest or apoptosis. While the major intra-strand cisplatin-DNA adducts are repaired primarily by NER, emerging data on the role of recombination in the repair of inter-strand cross-links is becoming increasingly significant [30,31]. Recent studies indicated that XPC, the classical DNA damage recognition protein in NER, involved in DSB repair [13,32]. In this study, centrosome amplification induced by XPC deficiency seemed to be independent of NER activity since knockdown of other key components of the NER pathway, including XPA, XPF and Rad23B, did not induce centrosome amplification (Fig. S2). Furthermore, γ-H2AX foci, the classical DSB marker, increased markedly in XPC-deficient cells, suggesting the accumulation of DSBs (Fig. 6). Taken together the clue above, we suggest that XPC defects might affect other DNA repair processes upon cisplatin-induced DNA damage. In this study, for the first time we provide evidence that XPC is involved in BRCA1 signaling pathway, which is critical for DSB repair [33]. The BRCA1 downregulation induced by XPC deficiency might lead to more unrepaired DSBs and activation of the ATM-Chk1/Chk2 signaling, resulting in a prolonged G2/M arrest. Moreover, Unlike the high cisplatin sensitivity of XPF or XPA defect cells, XPC deficient cells are resistant to cisplatin treatment (Fig. S2D) [34,35]. The defective apoptotic activity may facilitate generating cells with CA that cannot be efficiently eliminated by apoptosis. On the other hand, it's worthy to note that the ubiquitination activity of BRCA1 is reported to be crucial for the regulation of centrosome number and microtubule aster formation [36–38]. Whether XPC deficiency can affect the ubiquitination targets of BRCA1 is still unknown.

POU class 1 homeobox1 (Pit-1/GHF-1/POU1F1) belongs to the Pit-Oct-Unc (POU) family of transcription factors and was firstly described in the pituitary gland, where it regulates cell differentiation during organogenesis and acts as a transcriptional activator for pituitary gene transcription [39]. Pit-1 is also expressed in non-pituitary cell lines and tissues, such as human breast and hematopoietic lymphoid tissues [40,41]. Previous studies have demonstrated that deregulation of Pit-1 in human breast cancer cells promotes tumor growth and metastasis [42,43]. Here, we report for the first time that XPC negatively regulates Pit-1 transcription. More importantly, centrosome amplification significantly decreased when Pit-1 was knockdown (Fig. 7I). It has been demonstrated that Pit-1 can repress the expression of some key genes involved in DNA repair pathways [24,44]. As we know, proteins

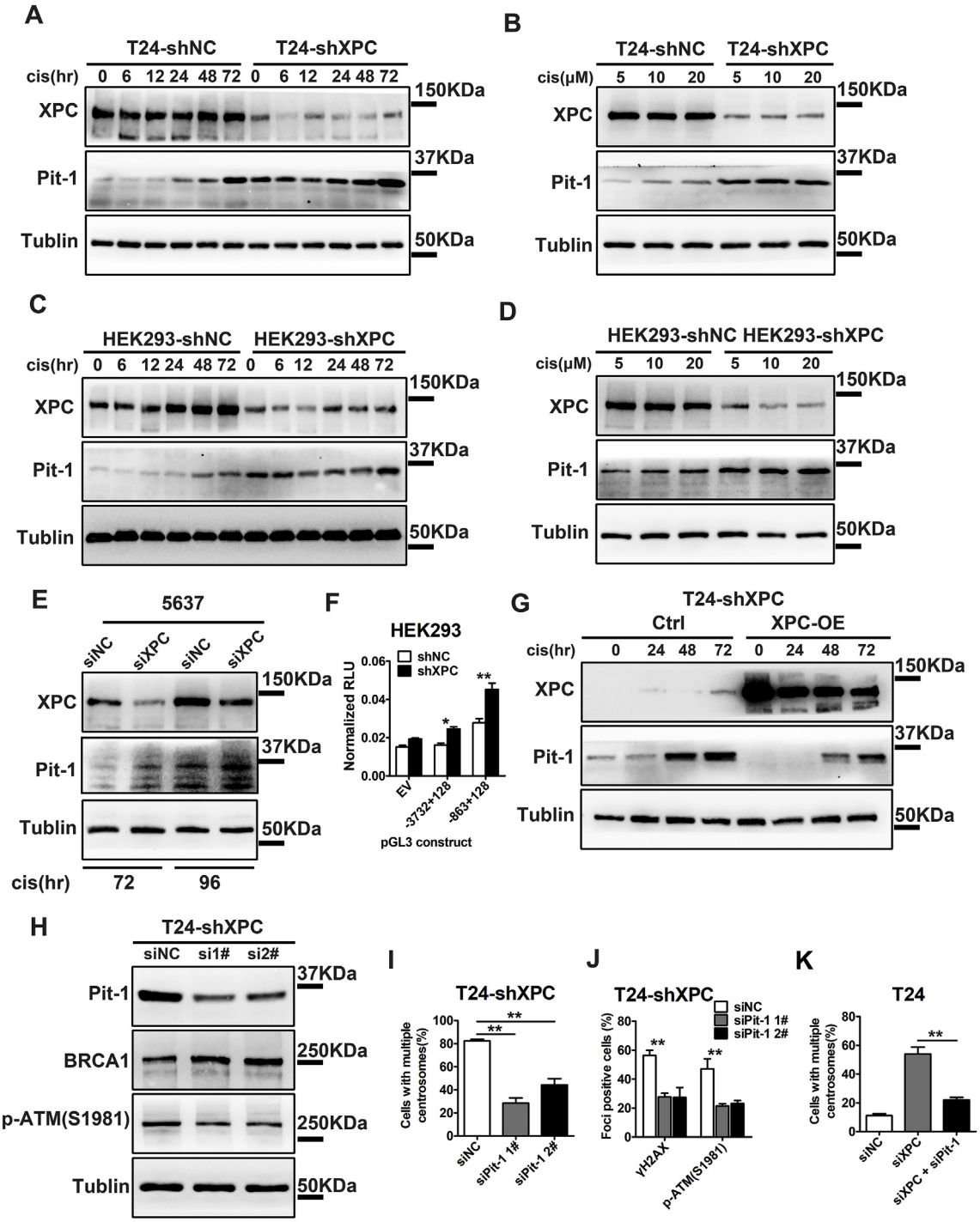

Fig. 7. XPC deficiency inhibits BRCA1 expression by upregulating the transcriptional factor, Pit-1. (A, C) Cells were treated with 10 μM cisplatin at the indicated times and cell lysates were analyzed by western blotting. (B, D) Cells were treated with cisplatin at the indicated concentrations for 72h and cell lysates were analyzed by western blotting. (E) 5637 cells were transfected with either scramble or XPC siRNA and then treated with 10 μM cisplatin at the indicated times and cell lysates were analyzed by western blotting. (F) The Pit-1 promoter fragments that fused to the pGL3Basic vector (pGL3B) were transfected into HEK293 cells. Abbreviation: EV, empty vector. Normalized relative luciferase units (RLU) were calculated, $P < 0.05$, $^{**}P < 0.01$. (G) Cells were transfected with vector (Control) or XPC overexpression construct were treated with 10 μM cisplatin at the indicated times and cell lysates were analyzed by western blotting. (H) Cells were transfected with either scramble or Pit-1 siRNA and then treated with 10 μM cisplatin for 72h and cell lysates were analyzed by western blotting. (I) Cells were treated as in (H). Bar graphs showing fraction of cells with > 2 centrosomes per cell. More than 200 cells per condition were counted from three independent experiment, $^{*}P < 0.05$,$^{**}P < 0.01$. (J) Cells were treated as in (H). Bar graphs showing fraction of foci-positive cells. More than 200 cells per condition were counted from three independent experiment, $^{*}P < 0.05$,$^{**}P < 0.01$. (K) T24 cells were either transfected with XPC siRNA or cotransfected with XPC/Pit-1 siRNA and then treated as in (H). Bar graphs showing fraction of cells with > 2 centrosomes per cell. More than 200 cells per condition were counted from three independent experiment,$^{*}P < 0.05$,$^{**}P < 0.01$.

involved in the DDR are usually stress-induced and return to normal once the stress relieved. In agreement with the facts above, the expression of Pit-1 is low in the early phase of the DDR (0–24h), but gradually increases during the later phase (48–72h) (Fig. 7A, C). This finding suggests that Pit-1 plays a crucial role in returning the DDR-induced proteins back to a normal level once the DNA damage is repaired. Our results showed that XPC deficiency could significantly increase Pit-1 transcription. It is possible that XPC plays a new role in the DDR by promoting the expression of stress-induced proteins. Further studies will be needed to determine the precise mechanism of how XPC regulates Pit-1 at the transcriptional level.

Our previous study indicated that decreased XPC expression, especially coupled with p53 alterations, influences human bladder cancer behavior or outcome [18]. In addition, accumulating evidences suggest that XPC play a vital role in tumor progression and invasion [45–47]. Based on the fact that most human solid malignant tumors usually become more aggressive after recurrence following chemotherapy in clinical observations, our findings provided a new insight that XPC defects may indirectly contribute to tumor invasion and metastatic properties through centrosome amplification and then promote chromosomal instability. In addition, these findings also have potential clinical significance in cancer therapy. As Olaparib (PARP inhibitor) is effective in some cancer patients with BRCA genetic alterations, patients with defective XPC expression will potentially benefit from Olaparib treatment.

In conclusion, we identified a new function of XPC in maintaining centrosome homeostasis beyond its classical role in NER. Our study broadened the role of XPC in the DNA damage response and provide the first demonstration that XPC deficiency leads to DSBs accumulation by downregulating BRCA1 expression via upregulating its transcriptional repressor, Pit-1. Loss of XPC in bladder cancer lead to centrosome amplification and genomic instability and promote tumor progression or even tumor recurrence.

Conflicts of interest

The authors declare no potential conflicts of interest.

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More information is available in supplementary files.

Appendix A. Supplementary data

Supplementary data to this chapter can be found online at https://doi.org/10.1016/j.canlet.2018.12.004.

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