



aPKC ζ promotes gallbladder cancer tumorigenesis and gemcitabine resistance by competing with Nrf2 for binding to Keap1

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ABSTRACT

Gallbladder cancer (GBC) is a highly malignant bile duct cancer with poor prognosis characterized by its insensitivity to chemotherapy. Emerging evidence indicates that cytoprotective antioxidation is involved in drug resistance of various cancers; however, the underlying molecular mechanisms remain obscure. Here, we demonstrated that atypical protein kinase C ζ (aPKC ζ) mediated reactive oxygen species (ROS) inhibition in a kinase-independent manner, which played a crucial role in tumorigenesis and chemoresistance. Mechanistically, we found that aPKC ζ facilitated nuclear factor erythroid 2-related factor 2 (Nrf2) accumulation, nuclear translocation and activated its target genes by competing with Nrf2 for binding to Kelch-like ECH-associated protein 1 (Keap1) through a highly conserved DLL motif. In addition, the aPKC ζ -Keap1 interaction was required for antioxidant effect, cell growth and gemcitabine resistance in GBC. Importantly, we further confirmed that aPKC ζ was frequently upregulated and correlated with poor prognosis in patients with GBC. Collectively, our findings suggested that aPKC ζ positively modulated the Keap1-Nrf2 pathway to enhance GBC growth and gemcitabine resistance, implying that the aPKC ζ -Keap1-Nrf2 axis may be a potential approach to overcome the drug resistance for the treatment of GBC.

1. Introduction

Gallbladder cancer (GBC), a primary malignancy of the biliary tract, is the sixth most common gastrointestinal cancer and has a 5-year survival rate of 5% [1,2]. Although radical resection is the most promising potential curative approach for patients, less than 10% of patients are considered candidates for resection because of advanced stage disease [3,4]. Gemcitabine has been widely used for the treatment of GBC; however, the response rate of only 36% leaves much to be desired [5]. Therefore, it is urgently required to identify the molecular mechanisms responsible for chemotherapy resistance in GBC.

Maintenance of reactive oxygen species (ROS) homeostasis is essential for cell survival. In recent years, growing evidence shows that ROS dysregulation is involved in the development of multiple diseases,

including cancers [6,7]. In order to survive in complex internal and external environments, cancer cells have developed effective antioxidant system to limit the excessive accumulation of ROS [8]. Indeed, conventional chemotherapeutic drugs often utilize elevated ROS to eliminate cancer cells [9]. The upregulation of antioxidant capacity in cancer cells can confer drug resistance. Thus, dissecting such drug-resistant molecular mechanisms may be potential therapeutic targets to overcome the resistance.

The Kelch-like ECH-associated protein 1 (Keap1)-nuclear factor erythroid-2-related factor 2 (Nrf2) complex is the major regulator of cytoprotective responses to endogenous and exogenous stresses caused by ROS and electrophiles [10]. Nrf2, as the key signaling protein of the pathway, mediates the expression of a series of oxidative stress-related genes that maintain cellular redox balance [11]. Under normal

Abbreviations: AJCC, American joint committee on cancer; ARE, antioxidant response element; aPKC, atypical protein kinase C; Bax, Bcl-2-associated X; Bcl2, B-cell lymphoma 2; CCA, Cholangiocarcinoma; CCK-8, Cell Counting Kit-8; Co-IP, Co-Immunoprecipitation; Ect2, Epithelial cell transforming sequence 2; GBC, Gallbladder cancer; IHC, Immunohistochemistry; Keap1, Kelch-like ECH-associated protein 1; Nrf2, Nuclear factor erythroid 2-related factor 2; OS, overall survival; ROS, Reactive oxygen species

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physiological conditions, Nrf2 activity is tightly regulated by Keap1 and promoted its ubiquitin-dependent proteasomal degradation in the cytoplasm; however, oxidative stresses prevent the Keap1-induced degradation of Nrf2, which leads to Nrf2's accumulation, nuclear translocation and subsequently transcriptional activation of its downstream target genes such as heme oxygenase 1 (HMOX1), NADPH quinone oxidoreductase 1 (NQO1), and glutamate-cysteine ligase (GCL) [12]. Recent studies suggest that the dysfunctional Keap1-Nrf2 interaction may be involved in drug metabolism and increasing the resistance to chemotherapy [10]. However, the precise molecular mechanisms underlying the Keap1-Nrf2 pathway and chemoresistance remain largely unknown.

Atypical protein kinase C ι (aPKC ι), one of the protein kinase C (PKC) isozymes, has emerged as an important oncogene in various cancers [13]. We previously demonstrated that aPKC ι was overexpressed and correlated with poor clinical outcome in cholangiocarcinoma (CCA) and drove CCA cells invasion and metastasis in vitro and in vivo [14]. Moreover, aPKC ι was also found to function in conferring resistance to chemotherapy of human leukemia cells [15]. The above reports have expanded the concept that aPKC ι is a particularly attractive therapeutic target for cancer treatment. Unfortunately, how aPKC ι functions in GBC has not been extensively studied.

In this study, we found that a previously unknown fundamental function of aPKC ι is to compete with Nrf2 for binding to Keap1. We further demonstrated that aPKC ι -Keap1-Nrf2 signaling is critical for promoting cell tumorigenesis and gemcitabine resistance in GBC.

2. Materials and methods

2.1. Cell lines

Human GBC cell lines NOZ and GBC-SD were generously provided by Prof. Yingbin Liu, Xinhua Hospital, Shanghai Jiao Tong University School of Medicine, China. These cells were maintained in William's medium or RPMI 1640 (both from Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco), respectively. All cell lines were authenticated, mycoplasma-free and cultured at 37 °C in a humidified incubator containing 5% CO $_2$.

2.2. Plasmids construction

The pFlag-PRKCI and pMyc-Keap1 plasmids were purchased from ViGene Biosciences (Shandong, China). All 5 truncated Keap1 mutants were constructed as previously described [16]. The PRKCI and Keap1 deletion mutants were subcloned into the pENTER vector using an appropriate restriction enzyme. Site-directed mutagenesis was performed to generate DLL²⁵⁷, DLM³⁴¹ and DLK³⁸⁰ mutants of PRKCI by using the QuickMutation™ Kit (Beyotime, Hangzhou, China) with full-length PRKCI expressing plasmid as a template. The plasmids used in this study are listed in Supplementary Table S1. All mutants were validated by sequencing.

2.3. Western blotting

Briefly, cells or tissues were lysed by ice-cold RIPA Lysis Buffer plus protease and a phosphatase inhibitor cocktail (Beyotime, Hangzhou, China). The cytoplasmic and nuclear fractions were isolated from cells by using the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, Hangzhou, China) according to the manufacturer's protocol. Equal amount of proteins were analyzed on 10% SDS-PAGE gels. β -actin was used as a loading control for cytoplasmic fraction, whereas LaminB was used as a loading control for the nuclear fraction. All blots were visualized by ECL (Boster, Wuhan, China). The intensity of bands was evaluated by Image Lab (Bio-Rad, California, USA). The experiments were repeated independently for three times. The antibodies used in this study are listed in Supplementary Table S2.

2.4. Co-immunoprecipitation

Co-immunoprecipitation (Co-IP) assay was performed to analyze protein-protein interactions. In brief, the cells were lysed by RIPA buffer and divided into parallel groups named input or IPs. Then, the primary antibody or IgG was added to the lysates for incubation overnight at 4 °C. Subsequently, the Protein A + G agarose beads were added to the mixture at 4 °C for 3 h. After centrifugation at 15,000 \times g for 15 min, the beads were collected and washed 5 times with RIPA buffer. The immunoblotting was performed with the indicated antibodies as previously reported.

2.5. Patients and specimens

In total, 72 human GBC tissues and paired normal gallbladder tissues (5 cm distant from tumor) were collected from patients undergoing resection at the Department of Biliary and Pancreatic Surgery, Tongji Hospital (Wuhan, China) between January 2009 and December 2016. Ethical approval for the use of human samples was obtained from the Tongji Hospital Research Ethical Committee. None of the patients had received any adjuvant therapy before surgery. All cases were diagnosed by two independent pathologists. The GBC samples were staged according to the 7th edition of the American Joint Committee on Cancer (AJCC) Cancer Staging Manual. The detailed clinicopathological characteristics of the 72 patients with GBC are listed in Supplementary Table S3.

2.6. Immunohistochemistry (IHC)

GBC samples or xenograft tumor tissues were fixed in 4% paraformaldehyde, paraffin embedded, and sectioned. The expression of aPKC ι , Nrf2 and Keap1 was detected by immunohistochemistry as previously reported [17]. The positively stained cells were scored, with scores ranging from 0 to 12. The total score \leq 4 was considered as low expression and $>$ 4 as high expression [18].

2.7. Animal study

Six-week-old female BALB/c-nude mice were used in all animal experiments and housed under specific pathogen-free (SPF) conditions in Central Animal Laboratory, Tongji Medical College. For the first animal experiment, 20 mice were randomly divided into four groups (n = 5 per group). A total of 2 \times 10⁶ NOZ cells transfected with lentivirus empty vector, aPKC ι overexpression, si-neg, or si-aPKC ι vectors were injected subcutaneously in the upper back of mice, respectively. For the second animal experiment, the same number of mice were randomly divided into 2 groups (n = 10 per group). A total of 2 \times 10⁶ NOZ cells transfected with lentiviral si-neg or si-aPKC ι vectors were injected subcutaneously in the upper back of mice, respectively. One week later, each group was randomly regrouped two subgroups (n = 5 per group) to receive intraperitoneal injection of gemcitabine (15 mg/kg) or 0.9% sodium chloride (NS) every 3 days. The diameter of tumors and the weight of the mice were measured every 3 days. The volume of tumors was calculated using the formula: 1/2 (length \times width²). All mice were sacrificed 3 weeks later, and the tumors were dissected out for immunohistochemistry, western blot assay or quantitative real-time PCR (qPCR). All animal experiments were conducted according to the ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines and were approved by the Committee on the Ethics of Animal Experiments of the Tongji Medical College, HUST.

Additional experimental procedures are provided in detail in the Supplementary data.

2.8. Statistical analyses

Statistical analyses were performed using SPSS 22.0 software (IBM

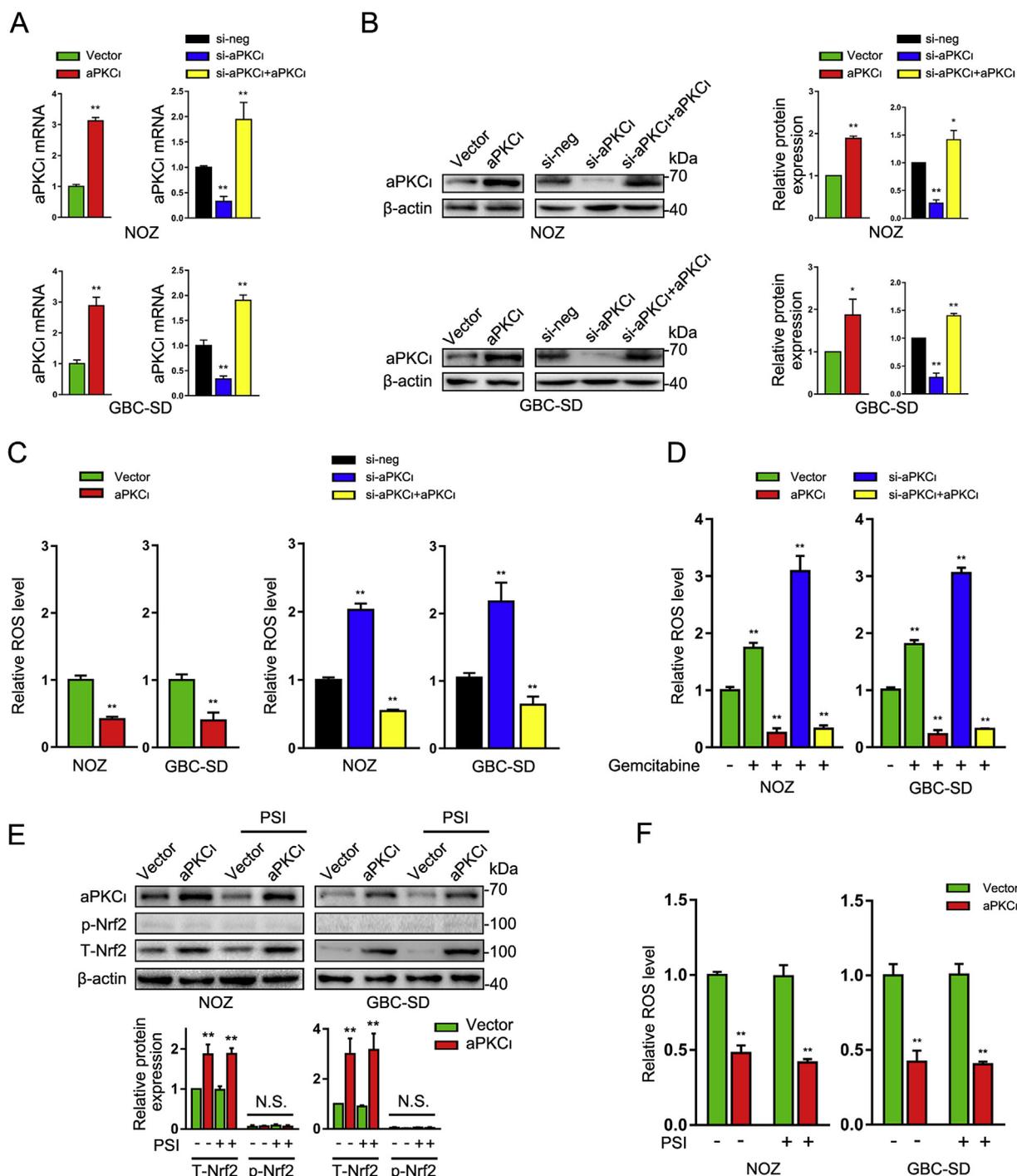


Fig. 1. aPKC1 inhibits ROS in a kinase-independent manner. (A–B) qPCR and western blotting were performed to detect the expression levels of aPKC1 in NOZ and GBC-SD cells with lentivirus-mediated aPKC1 overexpression (aPKC1)/knockdown (si-aPKC1) or re-expression. β -actin was used as a loading control. neg, negative control. N.S. no significance. (C–D) ROS levels were measured by DCFH-DA in GBC cells with the treatment of aPKC1 overexpression, knockdown, re-expression or gemcitabine. (E) Protein levels of aPKC1, phosphorylated-Nrf2 (p-Nrf2) and Total Nrf2 (T-Nrf2) were tested in GBC cells after aPKC1 overexpression with or without PSI treatments (10 μ M). (F) Relative ROS levels were detected in GBC cells after aPKC1 overexpression with or without PSI treatments (10 μ M). * $P < 0.05$, ** $P < 0.01$. Data are derived from three independent experiments and presented as means \pm SDs.

SPSS, Armonk, NY, USA) or GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA). The results were presented as mean \pm standard deviation (SD). Quantitative data were analyzed by two-tailed independent Student's t tests and analysis of variance. Categorical variables were compared using chi-square tests or Fisher exact tests. Clinical correlations were analyzed using χ^2 tests, and survival analysis was conducted by the Kaplan-Meier method with log-rank tests. Differences with P values of less than 0.05 were considered statistically

significant.

3. Results

3.1. aPKC1 inhibits ROS in a kinase-independent manner

To investigate a possible functional link between aPKC1 and ROS, we first stably established ectopic aPKC1 expression or knockdown GBC

cell lines NOZ and GBC-SD (Fig. 1A and B), which were used in the subsequent experiments. The ectopic expression of aPKC ζ significantly reduced the cellular ROS levels in GBC cells. Conversely, aPKC ζ silencing triggered the accumulation of ROS. Interestingly, restoring exogenous aPKC ζ expression reversed the effects of aPKC ζ knockdown, suggesting that aPKC ζ may act as an antioxidative factor (Fig. 1C). Importantly, we further found that aPKC ζ overexpression also attenuated gemcitabine-induced ROS production. aPKC ζ deletion elevated the cellular ROS levels under this condition, which was reversed by the expression of exogenous aPKC ζ (Fig. 1D). Therefore, these data showed that aPKC ζ suppressed the intracellular ROS to control cytoprotective response under both normal and oxidative stress conditions.

Previous studies indicated that aPKC(s) involved in oxidant stress response by phosphorylation of Nrf2 [19]; however, we found that aPKC ζ overexpression significantly changed ROS levels in GBC cells treated with PSI, an aPKC peptide inhibitor, while there was weak and no obvious alteration of phosphorylated Nrf2 (Fig. 1E and F). Interestingly, aPKC ζ overexpression led to significant accumulation of the total Nrf2 protein (Fig. 1E). Of note, we also found that PSI reduced the expression levels of phosphorylated aPKC ζ , but the total aPKC ζ , phosphorylated Nrf2 and total Nrf2 proteins had no significant change. The phosphorylation of epithelial cell transforming sequence 2 (Ect2), an aPKC substrate [20,21], was indeed inhibited by PSI, while there was no obvious alteration of the total Ect2 protein (Fig. S1A). More importantly, we constructed Flag-tagged wild-type (WT), constitutively active catalytic domain (CAT) and kinase-inactive (KI) mutants of aPKC ζ as previously reported [22]. The data showed that these mutants had minimal impact on the expression levels of phosphorylated Nrf2 (Figs. S1B and C). Together, these results suggest that aPKC ζ -mediated antioxidant effect was independent of its well-studied kinase function.

3.2. aPKC ζ promotes Nrf2 accumulation and activates antioxidative signaling

Given that the Keap1-Nrf2 complex is the key regulator of antioxidative signaling, we determined whether aPKC ζ induced ROS inhibition through the Keap1-Nrf2 pathway. Our data showed that aPKC ζ did not affect the protein expression of Keap1 in GBC cells (Fig. 2A). However, ectopic aPKC ζ expression significantly increased the protein levels of Nrf2. aPKC ζ knockdown led to reduction in the level of the Nrf2 protein, which was reversed by restoring aPKC ζ expression (Fig. 2A). Interestingly, the mRNA levels of both Keap1 and Nrf2 were not altered by aPKC ζ expression (Fig. 2B). These results indicated that aPKC ζ positively modulated Nrf2 accumulation, and the process did not occur at the transcriptional level.

Next, we further investigated whether aPKC ζ regulated the intracellular distribution of the Nrf2 protein. Western blotting results showed that aPKC ζ promoted Nrf2 nuclear location, while aPKC ζ knockdown prevented the transposition (Fig. 2C). In contrast, there was no obvious change in the level of the Keap1 protein in both the cytoplasm and nucleus. Previous studies demonstrated that nuclear Nrf2 can bind to the antioxidant response element (ARE) and then activate the downstream genes [23]. To confirm the transcriptional activity of Nrf2, qPCR was performed to detect the mRNA levels of well-established downstream genes such as *HMOX1*, *NQO1*, *GCLC*, *GCLM* and *FTH1*. The ectopic aPKC ζ expression showed an estimated 2.3–3.8 fold increase in GBC cells, whereas aPKC ζ depletion reduced the expression of these genes by 50% (Fig. 2D). Notably, re-expression of aPKC ζ reversed the effect of aPKC ζ knockdown. In addition, we further found that aPKC ζ -mediated ROS inhibition can be abolished by Nrf2 knockdown (Fig. 2E and F). Therefore, Nrf2 is required for aPKC ζ -induced antioxidative signaling. Collectively, the above findings suggest that aPKC ζ promotes Nrf2 accumulation, nuclear location, and lowers the ROS levels.

3.3. aPKC ζ competes with Nrf2 for Keap1 binding through the DLL motif

Previous studies have reported that Keap1 could bind with Nrf2 and promote its degradation by the ubiquitin proteasome pathway [24]. In this study, we found that the proteasome inhibitor MG132 completely rescued aPKC ζ knockdown-induced reduction of Nrf2 (Fig. 3A and S2A). The data revealed that aPKC ζ might inhibit ubiquitin-proteasome-mediated degradation of Nrf2. Indeed, we further confirmed that ectopic aPKC ζ expression significantly reduced the ubiquitination of the endogenous Nrf2 in GBC cells. Conversely, aPKC ζ knockdown increased the level of ubiquitin-Nrf2 (Fig. 3B and S2B). Interestingly, it has also been demonstrated that the degradation of Nrf2 may be induced in a Keap1-independent manner [25]. These observations prompted us to investigate whether aPKC ζ -induced Nrf2 accumulation is associated with Keap1. The results indicated that Keap1 knockdown did not affect the protein expression of aPKC ζ , while abolished aPKC ζ -induced Nrf2 accumulation (Fig. 3C and S2C). Moreover, there was no significant alteration of p62 protein, reported as a negative regulator of Keap1, after aPKC ζ or Keap1 knockdown (Fig. 3C). Therefore, we speculated that aPKC ζ may compete with Nrf2 to bind with Keap1. To validate the hypothesis, we conducted immunoprecipitation experiment to analyze the interaction between aPKC ζ and the Keap1-Nrf2 complex by using Flag-aPKC ζ and Keap1-Myc plasmids. Under normal cellular conditions, Flag-aPKC ζ was immunoprecipitated by Keap1-Myc. Conversely, Keap1-Myc was detected in the Flag-immunoprecipitates (Fig. 3D). It has previously been reported that stress-induced reduction of Keap1-Nrf2 was the main mechanism of Nrf2 activation [26,27]. Thus, we further analyzed whether aPKC ζ was involved in regulating the process under oxidative stresses induced by gemcitabine. Interestingly, the binding amount of aPKC ζ and Keap1 proteins was significantly increased, accompanying with a decreased interaction of Keap1 and Nrf2 (Fig. 3E and S2D). To further validate that aPKC ζ can compete with Nrf2 for Keap1 binding, in vitro translation and immunoprecipitation experiments were performed. The equal amount of Nrf2 and Keap1 proteins were incubated together with different doses of aPKC ζ protein. Interestingly, the binding amount of Nrf2 and Keap1 was gradually decreased with an increased interaction of aPKC ζ and Keap1 (Fig. 3F). More importantly, aPKC ζ overexpression reduced, while aPKC ζ knockdown increased, the Keap1-Nrf2 complex in GBC cells. The Keap1-aPKC ζ complex exhibited an opposite result (Fig. S2E and F). These data suggested that aPKC ζ disrupted the formation of the Keap1-Nrf2 complex by competing with Nrf2 for Keap1 binding.

Next, we investigated the mechanisms underlying aPKC ζ -mediated dysregulation of the Keap1-Nrf2 system. Previous studies demonstrated that Nrf2 contained seven functional domains, including ETGE and DLG motifs, which are known as Keap1 binding sites [10]. Besides Nrf2, recent studies have identified other proteins with ETGE or similar motifs that can bind with Keap1 [28–30]. Indeed, Fukutomi and colleagues demonstrated that the DLG motif needs to be much more extended than the classical motifs [31]. Surprisingly, we found that 252-QDFDLL-257 was highly conserved from chicken to human, as a related DLG motif in Nrf2, within aPKC ζ . Thus, we generated a series of mutants, in which the QDFDLL was deleted, or DLL motif was changed to DLA. Moreover, two other DLM³⁴¹ and DLK³⁸⁰ motifs were also mutated with DLA (Fig. 3G). The results indicated that both deletion mutant and DLL²⁵⁷ mutant were unable to interact with Keap1. However, DLM³⁴¹ and DLK³⁸⁰ mutants have no obvious effect on this interaction (Fig. 3H). Therefore, the DLL²⁵⁷ motif of aPKC ζ is required for its molecular recognition of Keap1.

To identify the specific regions that are involved in the interaction of aPKC ζ and Keap1, we performed immunoprecipitation assays with a series of deletion mutants of Keap1 in HEK293T cells. The results showed that the Keap1 N-terminal deletion mutants (D1, D2 and D3) and C-terminal deletion mutant D5 bound with aPKC ζ , whereas DGR deletion mutant (D4) failed to interact with aPKC ζ , indicating that the DGR domain is required for Keap1 to bind with aPKC ζ (Fig. 3I and J). It

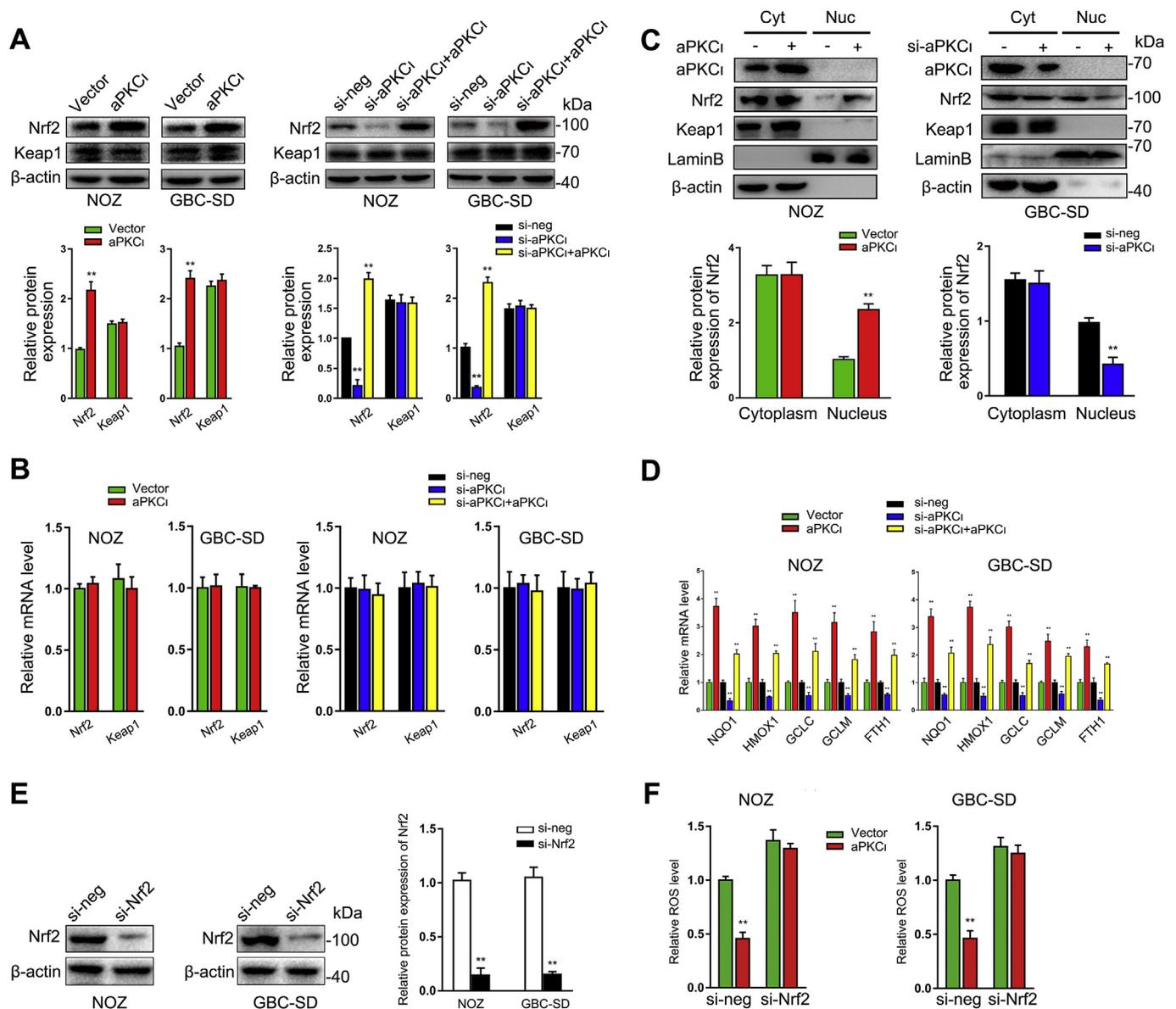


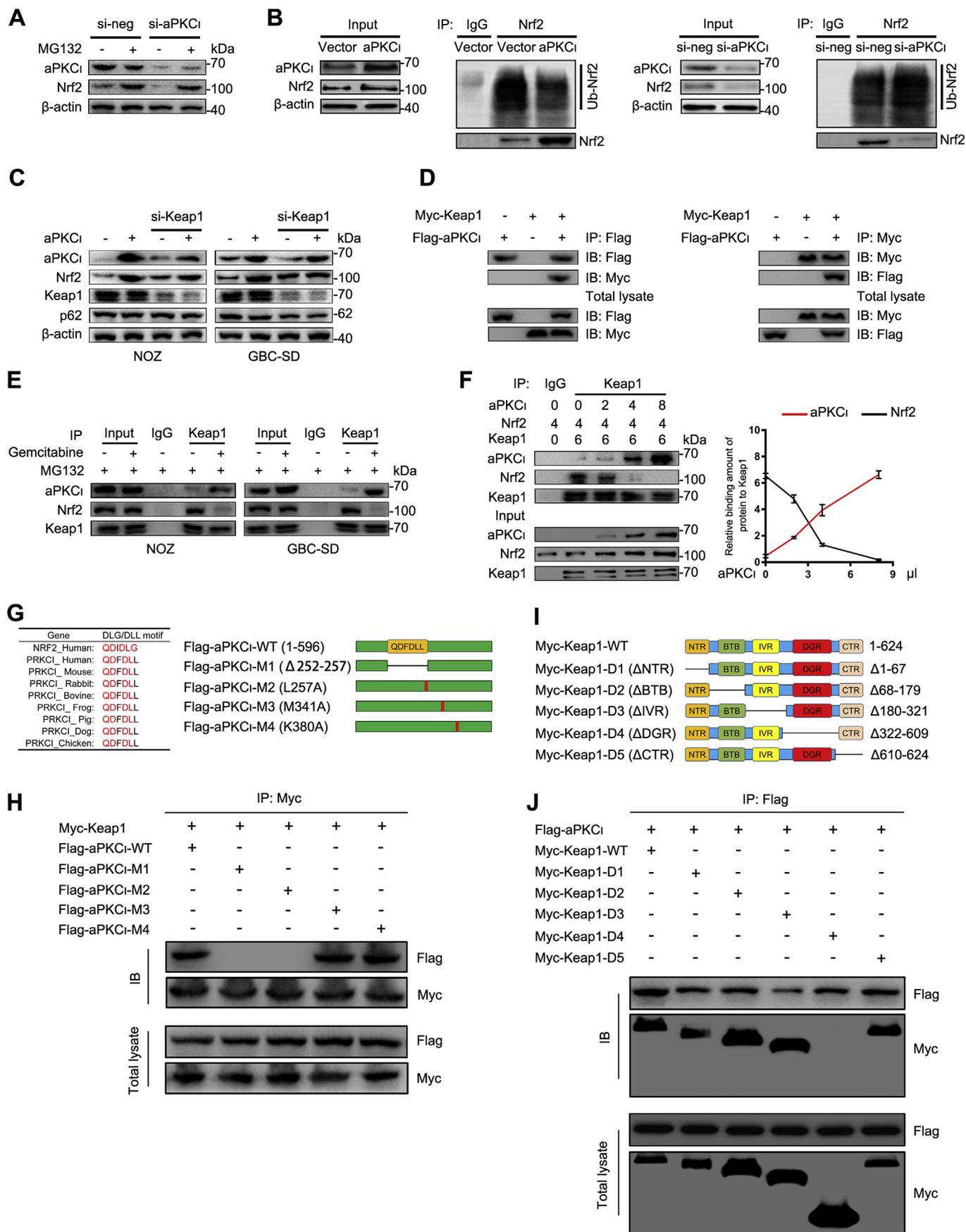
Fig. 2. aPKC_i promotes Nrf2 accumulation and activates antioxidative signaling. (A–B) The protein and mRNA levels of Nrf2 and Keap1 were determined in GBC cells with indicated treatments. (C) Western blotting was used to test the intracellular distribution of the Nrf2 protein after aPKC_i overexpression or knockdown. LaminB and β -actin were used as loading control for the nucleus and cytoplasm, respectively. Cyt, cytoplasm. Nuc, nucleus. (D) The mRNA levels of Nrf2 target genes were measured in the indicated GBC cell lines. (E) The protein levels of Nrf2 were measured in GBC cells after Nrf2 knockdown. (F) Relative ROS levels were detected in aPKC_i overexpression GBC cells with or without Nrf2 knockdown. $^{**}P < 0.01$. Data are derived from three independent experiments and presented as means \pm SDs.

is worth noting that previous studies have identified the DGR domain is essential to maintain the interaction of Keap1 and Nrf2 [32,33]. These results therefore suggest that aPKC_i competes with Nrf2 to bind with Keap1 through the DLL motif.

3.4. aPKC_i promotes GBC cells tumorigenesis both in vivo and in vitro

Based on the above findings, we sought to further investigate whether aPKC_i led to phenotypic changes in GBC cells. The Cell Counting Kit-8 (CCK-8) assay revealed that ectopic aPKC_i expression dramatically enhanced the GBC cells proliferation compared with the cells transfected with empty vector. Conversely, aPKC_i silencing significantly suppressed the cells proliferation ability, which was reversed by re-expression of aPKC_i (Fig. 4A). Consistently, aPKC_i overexpression increased, while aPKC_i knockdown inhibited, GBC cells growth as demonstrated by a soft agar growth assay. Recovery of exogenous aPKC_i

expression eliminated the effect of aPKC_i deficiency (Fig. 4B). In vivo tumorigenicity assays, we further confirmed that the xenograft tumors grew more rapidly in the aPKC_i overexpression group than that in the empty vector group. aPKC_i depletion reduced the volume and weight of xenograft tumors compared with those of the negative control group (Fig. 4C and D). Western blotting and immunostaining results confirmed that aPKC_i affected the expression of Nrf2, but not Keap1, in xenograft tumors (Fig. 4E and S3A and B). In addition, Nrf2 mRNA level showed no obvious alteration; however, its target genes mRNA levels, such as *HMOX1*, *NQO1*, *GCLC*, *GCLM* and *FTH1*, were significantly increased in the aPKC_i overexpression group. Consistently, aPKC_i knockdown effectively inhibited the expression of these genes except Nrf2 (Fig. 4F). Together, our data suggest that aPKC_i promotes GBC cells tumorigenesis both in vivo and in vitro.



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Fig. 3. aPKC ζ competes with Nrf2 for Keap1 binding through the DLL motif. (A) The protein levels of aPKC ζ and Nrf2 were determined by western blotting in negative control or aPKC ζ knockdown NOZ cells with or without MG132 treatments (20 μ M for 6 h). (B) The ubiquitinated Nrf2 (Ub-Nrf2) was measured by IP in NOZ cells after aPKC ζ overexpression or knockdown. (C) The protein levels of aPKC ζ , Nrf2, Keap1 and p62 were examined in aPKC ζ overexpression GBC cells with or without Keap1 knockdown. (D) Co-IP assays were performed to detect the interaction between Flag-tagged aPKC ζ and Myc-tagged Keap1 in HEK293T cells. (E) The interaction between aPKC ζ and Keap1 was determined by Co-IP in GBC cells with the treatment of gemcitabine for 24 h. MG132 (20 μ M) was added 6 h before cells were collected. (F) The interaction among aPKC ζ , Nrf2 and Keap1 was examined by Co-IP in vitro translation systems with aPKC ζ (0, 2, 4, or 8 μ l), Nrf2 (4 μ l), and Keap1 (6 μ l). (G) Left panel, sequence alignment of Keap1-recognizing motif in aPKC ζ from chicken to human. Right panel, schematic description showed the aPKC ζ wild-type or mutants. (H) Co-IP assays were used to evaluate the interaction of aPKC ζ and Keap1 in HEK293T cells transfected with indicated plasmids. (I) Diagrams of the Keap1 wild-type or deletion mutants. (J) The interaction of aPKC ζ with Keap1 wild-type or deletion mutants was analyzed by Co-IP assays in HEK293T cells transfected with indicated plasmids.

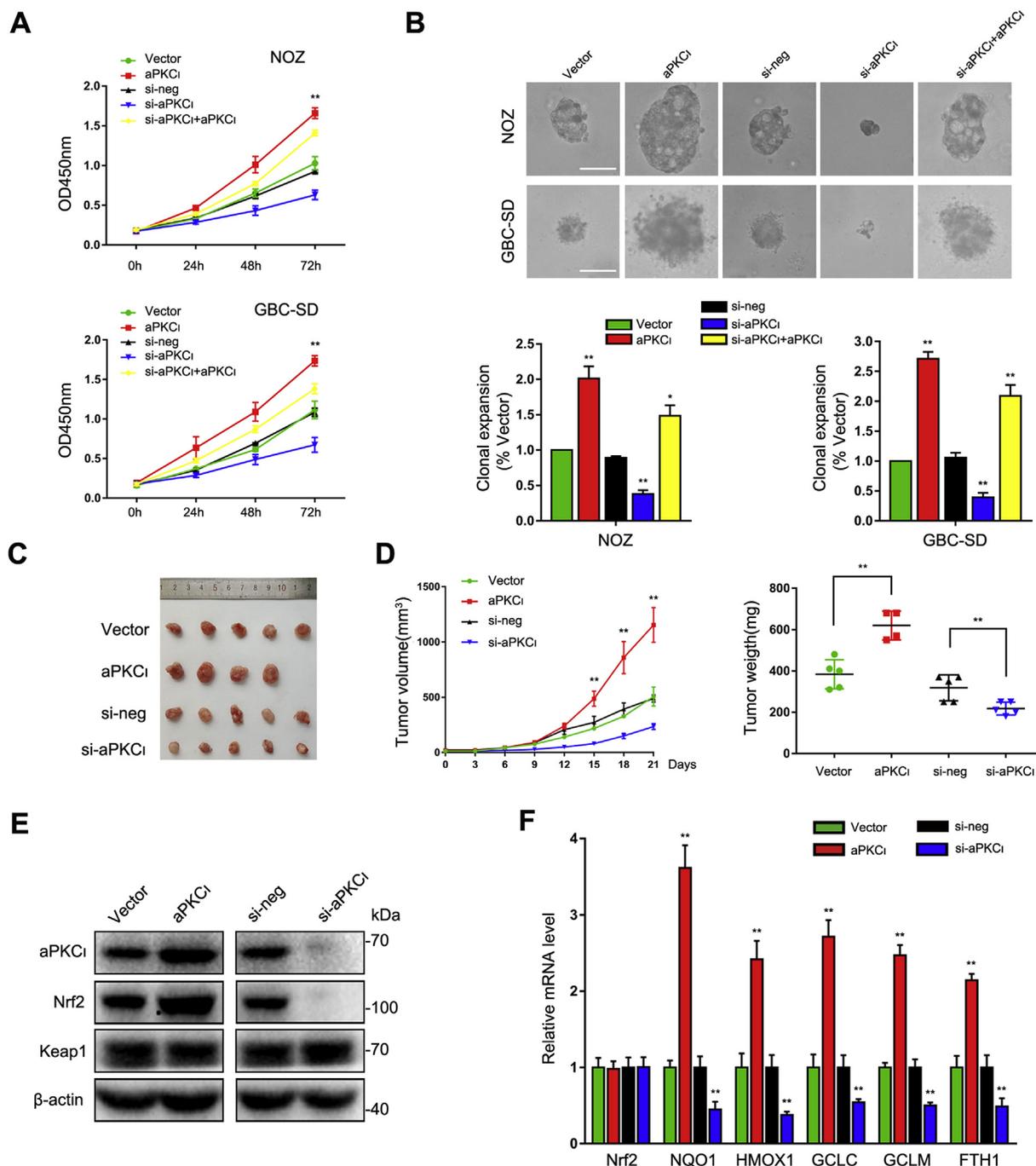


Fig. 4. aPKC ζ promotes GBC cells tumorigenesis both in vivo and in vitro. (A) Cell proliferation ability was analyzed by the CCK-8 assay in GBC cells with indicated treatments. (B) Anchorage-independent growth was evaluated by soft agar growth assay in the indicated GBC cells. Scale bar, 50 μ m. (C) Images of subcutaneously transplanted tumors from the nude mice injected with NOZ cells following the indicated treatments (n = 5 per group). (D) Tumor volume and tumor weight of NOZ xenografts with indicated treatments. (E) Expression of aPKC ζ , Nrf2 and Keap1 proteins in samples derived from xenograft tumors were measured by western blotting. (F) The mRNA levels of Nrf2 and its target genes in samples derived from xenograft tumors were analyzed by qPCR. *P < 0.05, **P < 0.01. Data are derived from three independent experiments and presented as means \pm SDs.

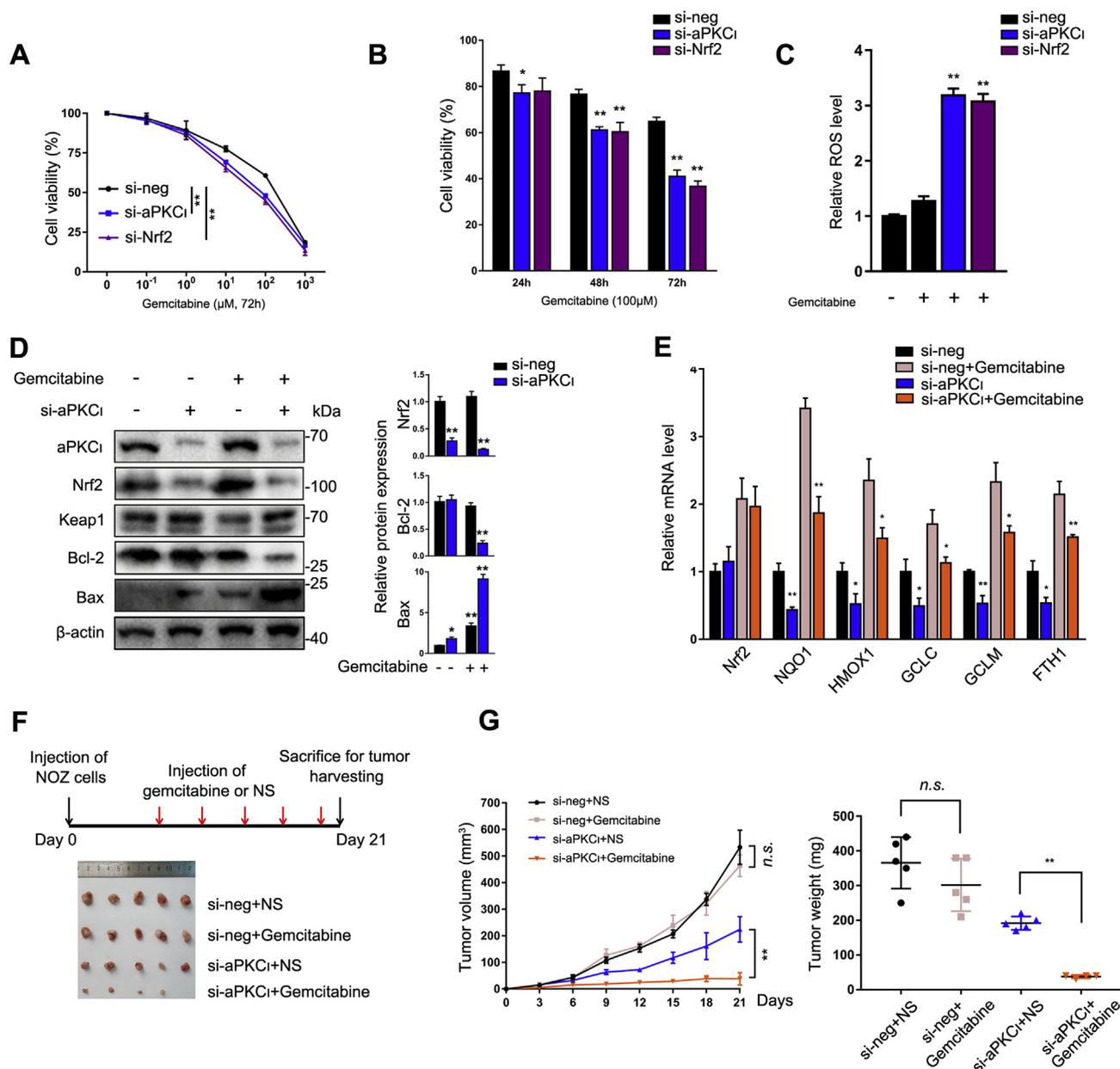


Fig. 5. aPKC-mediated ROS inhibition enhances gemcitabine resistance in GBC. (A) The cell viability was measured by the CCK-8 assay in NOZ cells. (B) The cell viability of NOZ cells with indicated treatment was evaluated at 24, 48 and 72 h. (C) Relative ROS levels were determined by DCFH-DA in the indicated NOZ cells in the presence or absence of gemcitabine. (D) Expression levels of Nrf2, Keap1, Bcl-2 and Bax proteins were detected by western blotting assay in NOZ cells with or without gemcitabine treatment. (E) The mRNA levels of Nrf2 and its target genes were measured by qPCR in NOZ cells with the indicated treatments. (F) Negative control and aPKC_i knockdown NOZ cells were subcutaneously injected into the upper back of nude mice with or without gemcitabine treatment (n = 5 per group). NS, sodium chloride. (G) Tumor volume and tumor weight of NOZ xenografts with indicated treatments. *P < 0.05, **P < 0.01. Data are derived from three independent experiments and presented as means ± SDs.

3.5. aPKC_i-mediated ROS inhibition enhances gemcitabine resistance in GBC

Next, we further investigated whether aPKC_i would influence the sensitivity of GBC cells to commonly used chemotherapeutic drugs gemcitabine. Given that ROS has been reported to participate in chemoresistance, we speculated that aPKC_i may involve in this process. We treated aPKC_i or Nrf2 silencing GBC cells with various concentrations of gemcitabine for 72 h to analyze the cell viability by CCK-8 assays. Indeed, when aPKC_i was knocked down, the cells exhibited more sensitivity to gemcitabine than the negative control group, with a dramatically decreased of IC₅₀ (Fig. 5A and S4A). Then, we examined the viability of the two GBC cell lines with the indicated dose of

gemcitabine for 24, 48 and 72 h. The results showed that the sensitivity of the cells to gemcitabine increased with time in the aPKC_i knockdown group (Fig. 5B and S4B). Notably, we also observed similar results that the loss of Nrf2 could help GBC cells to overcome the obstacle of chemoresistance. Moreover, we found that aPKC_i or Nrf2 depletion led to ROS accumulation in gemcitabine-treated cells (Fig. 5C and S4C). Consistently, aPKC_i deficiency decreased the Nrf2 protein and its target genes expression levels in cells treated with gemcitabine (Fig. 5D-E and S4D and E). Furthermore, B-cell lymphoma 2 (Bcl2) protein was decreased, while Bcl-2-associated X (Bax) protein was increased after aPKC_i knockdown in GBC cells treated with gemcitabine (Fig. 5D and S4D). In vivo, gemcitabine had slight suppression effect on the growth of xenograft tumors in the negative control group. However, when

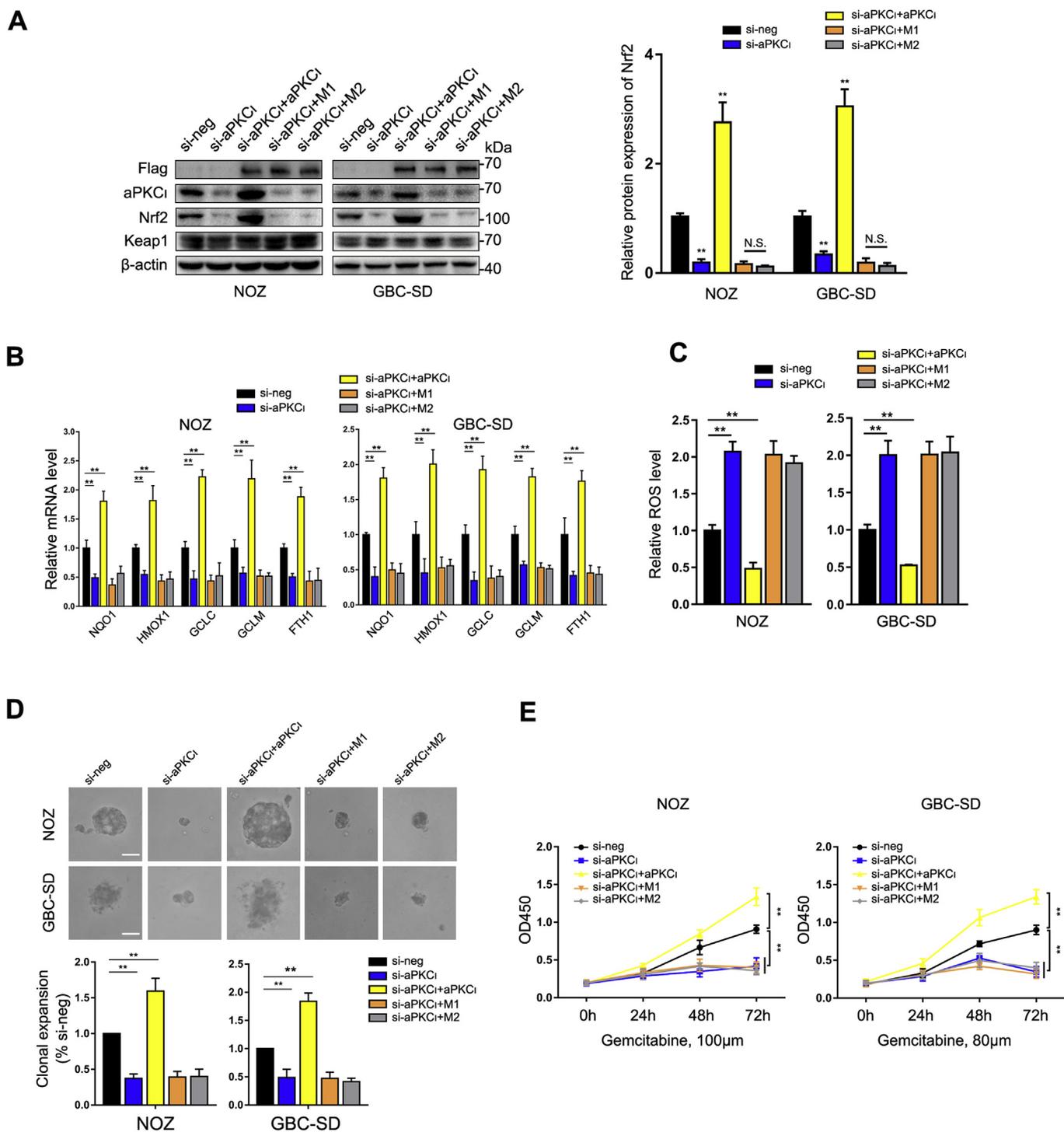


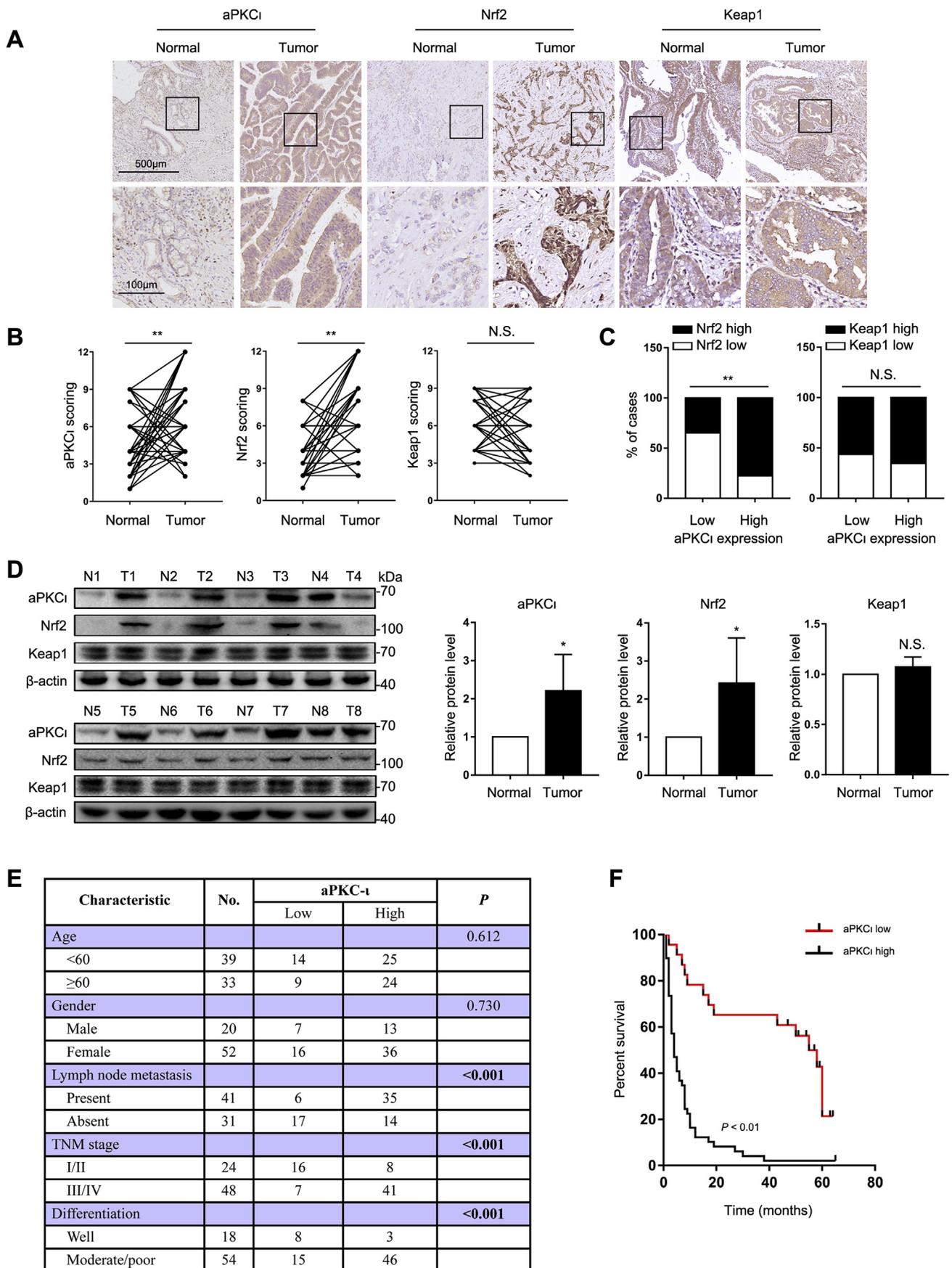
Fig. 6. The DLL motif is required for aPKC_i-mediated ROS inhibition, cell growth and gemcitabine resistance. (A) The expression levels of aPKC_i, Nrf2 and Keap1 proteins were determined in GBC cells after aPKC_i knockdown, re-expression, DLL deletion or missense mutant, as indicated. N.S. no significance. (B) The mRNA levels of Nrf2 target genes were measured by qPCR in GBC cells with the indicated treatments. (C) Relative ROS levels were detected in GBC cells with the indicated treatments. (D) GBC cells with indicated treatments were subjected to soft agar growth assay. Scale bar, 50 μm. (E) Cell proliferation was assessed by CCK-8 in GBC cells with gemcitabine treatment. ***P* < 0.01. Data are derived from three independent experiments and presented as means ± SDs.

aPKC_i was depleted, the tumors were significantly repressed in the presence of gemcitabine (Fig. 5F and G). Therefore, aPKC_i improves the resistance of GBC cells to gemcitabine through ROS inhibition.

3.6. The DLL motif is required for aPKC_i-mediated ROS inhibition, cell growth and gemcitabine resistance

To further validate whether the aPKC_i-Keap1 interaction through

the DLL motif is associated with these biological functions, aPKC_i-WT (wild-type), aPKC_i-DLL²⁵⁷ deletion mutant (M1) and aPKC_i-DLL²⁵⁷ mutant (M2) vectors were transfected into aPKC_i-deficient cells. We found that re-expression of aPKC_i-WT, but not mutant M1 or M2, reversed the reduction of the level of Nrf2 protein and its target genes induced by aPKC_i knockdown (Fig. 6A and B). In addition, we examined the intracellular ROS levels in aPKC_i-deficient cells with the above vectors. As shown in Fig. 6C, mutant M1 or M2 did not reduce the



(caption on next page)

Fig. 7. aPKC ζ is frequently upregulated and correlated with poor prognosis in patients with GBC. (A) Representative images of IHC staining of aPKC ζ , Nrf2 and Keap1 expression in 72 GBC samples and pair-matched normal tissues. (B) Quantification of aPKC ζ , Nrf2 and Keap1 expression levels in 72 GBC samples and pair-matched normal tissues. (C) The histogram showed the correlation of low or high aPKC ζ expression with the expression levels of Nrf2 and Keap1. (D) The protein levels of aPKC ζ , Nrf2 and Keap1 in 8 representative GBC samples (T) and pair-matched normal tissues (N) were evaluated by western blotting. (E) Correlation between aPKC ζ and clinicopathologic characteristics in patients with GBC. The clinical stage was according to 7th AJCC staging criteria. (F) Kaplan-Meier analysis showed the correlation between aPKC ζ expression and the overall survival of patients with GBC. * $P < 0.05$, ** $P < 0.01$.

intracellular ROS levels in both NOZ and GBC-SD cells. Likewise, mutant M1 or M2 had no obvious effect on cell growth when aPKC ζ was silenced (Fig. 6D). Consistently, aPKC ζ -WT rescued the defects of cell proliferation in aPKC ζ -deficient cells treated with gemcitabine; however, transfection with mutant M1 or M2 failed to produce this effect (Fig. 6E). These results suggest that the aPKC ζ -Keap1 interaction through the DLL motif is required for cell growth, ROS inhibition, and chemoresistance in GBC cells.

3.7. aPKC ζ is frequently upregulated and correlated with poor prognosis in patients with GBC

Finally, we further investigated the correlation among aPKC ζ , Nrf2, and Keap1 in 72 GBC specimens. IHC analysis showed that the expression levels of aPKC ζ and Nrf2 were significantly higher in GBC tissues than in pair-matched normal tissues. There was no obvious alteration of Keap1 expression levels in GBC samples (Fig. 7A and B). 77.6% (38 cases) of specimens with higher aPKC ζ (49 cases) tended to express higher Nrf2, while 65.2% (15 cases) of specimens with lower aPKC ζ (23 cases) exhibited lower Nrf2. However, there was no significant correlation between aPKC ζ and Keap1 (Fig. 7C). Consistently, the upregulation of aPKC ζ and Nrf2 was further examined at the protein level in representative 8 paired GBC tissues (Fig. 7D). In addition, we also found that the mRNA levels of Nrf2 target genes increased in GBC tissues (Fig. S5). The expression level of aPKC ζ was significantly associated with advanced TNM stage ($\chi^2 = 19.965$, $P < 0.001$), lymph node metastasis ($\chi^2 = 13.125$, $P < 0.001$), and poor tumor differentiation ($\chi^2 = 29.154$, $P < 0.001$) in GBC (Fig. 7E). Kaplan-Meier analysis indicated that patients with high aPKC ζ expression exhibited a shorter overall survival (OS) than those with low aPKC ζ expression (Fig. 7F). Multivariate Cox regression analyses indicated that aPKC ζ expression was an independent prognostic factor for OS in patients with GBC (Supplementary Table S4). Therefore, these observations suggested that aPKC ζ was frequently upregulated and associated with poor prognosis in patients with GBC. The overexpression of Nrf2 protein and its target genes may be dependent, at least in part, on the elevation of aPKC ζ in GBC samples.

4. Discussion

Chemotherapy resistance is a major obstacle to the effective treatment of cancer. Although recent evidence has shown that the elevated expression of aPKC ζ is correlated with chemoresistance in various human cancers [34], the molecular mechanisms that drive the enhanced tumorigenic potential and drug resistance of GBC cells remain enigmatic. Here, we provide novel evidence that aPKC ζ competes with Nrf2 for binding to Keap1, which leads to Nrf2 nuclear accumulation and ROS inhibition in GBC cells (Fig. 8). Our data may provide an explanation of how aPKC ζ exerts oncogenic functions in GBC. In addition, this ability of aPKC ζ has been further demonstrated to be associated with resistance to gemcitabine in GBC, which is notorious for its insensitivity to chemotherapy.

As a distinct member of the protein kinase C family, aPKC ζ has been reported to be a particularly attractive therapeutic target for cancer treatment [13,35]. We and other researchers have demonstrated that aPKC ζ drove multiple cancer cells invasion and transformed growth in vitro and in vivo [14,36,37]. Recent studies also showed that aPKC ζ maintained a stem-like phenotype in lung squamous cell carcinoma

through autonomous Hedgehog (Hh) signaling and served as a potential therapeutic strategy for Hh mutations that confer resistance [38]. All abovementioned studies focused on the regulation of cell polarization and kinase activity of aPKC ζ , while interestingly, we found that aPKC ζ functioned as an antioxidative factor in a kinase-independent manner. Our findings were supported by other studies that aPKC ζ promoted neuronal differentiation in a manner that did not depend on kinase activity [39]. More importantly, we further demonstrated that this function of aPKC ζ was involved in drug resistance and cell growth.

Nrf2 has been regarded as one of the main orchestrators of the antioxidant response pathway. For the canonical Keap1-Nrf2 system, also known as “hinge and latch” model, previous studies have reported that Keap1 homodimer interacts with monomeric Nrf2 through DLG and ETGE motifs [40,41]. This binding model facilitates optimal positioning of the lysine residues between the two ubiquitin-conjugated motifs [23]. The modification of cysteine residues in Keap1 can be induced by oxidative stress, which causes a conformational change of Keap1 that further reduces the ubiquitination and degradation of Nrf2 [42]. On the other hand, accumulating lines of evidence demonstrate that there is cross talk between Keap1-Nrf2 and other proteins, such as PALB2, P62, iASPP2 and DPP3, which can disrupt the normal Keap1-Nrf2 signaling and associate with malignant progression [16,43–45]. These proteins can bind with either Keap1 or Nrf2 through Keap1-binding motifs (DLG or ETGE motifs). In this study, we found that aPKC ζ interacted with Keap1 through the DLL motif, a similar set in Nrf2, which is highly conserved from poultry to human in aPKC ζ . Indeed, we have detected the protein complex of aPKC ζ -Keap1-Nrf2 in GBC cell lines. Furthermore, aPKC ζ can elevate Keap1-Nrf2 signaling under both basal conditions and stressed conditions. The data showed that the binding amount of aPKC ζ and Keap1 was dramatically elevated with the treatment of gemcitabine. It is also noteworthy that increased aPKC ζ -Keap1 interaction was accompanied by a significant reduction of Keap1-Nrf2 interaction under oxidative stresses. According to the “hinge and latch” model, gemcitabine-induced oxidative stress may modify the specific cysteine residues of Keap1, which leads to conformational change of Keap1 resulting in the dissociation of Nrf2 from Keap1. It is propitious for the binding of aPKC ζ and Keap1, and consequently, newly synthesized Nrf2 proteins bypass Keap1. These observations indicate that revealing the regulatory mechanisms in the Keap1-Nrf2 system may provide potential opportunities for pharmacological intervention in different cellular conditions.

Although previous studies have reported that phosphorylation of Nrf2 by PKC facilitated the dissociation of Nrf2 from Keap1 [19,46], the main enzyme and molecular mechanism underlying the interaction were not investigated. In contrast, the kinase-independent functions of aPKC, including aPKC ζ , have been reported in multiple signaling pathways [39,47–49]. In this study, we have investigated that aPKC ζ can promote nuclear translocation of Nrf2, but not phosphorylated Nrf2, to activate cytoprotective gene expression. Mechanistically, we constructed a series of mutants to demonstrate how aPKC ζ disrupts the Keap1-Nrf2 system. Interestingly, we found that aPKC ζ may act as a “competitive” binder of Keap1. On one hand, Keap1 knockdown has no effect on the expression of aPKC ζ . Conversely, aPKC ζ depletion also does not result in the change of Keap1. This suggests that Keap1 is not a substrate of aPKC ζ . On the other hand, our data show that aPKC ζ overexpression increased, while knockdown decreased, the protein level of Nrf2. However, the mRNA level of Nrf2 has not significant alteration with aPKC ζ overexpression or knockdown. Therefore, we

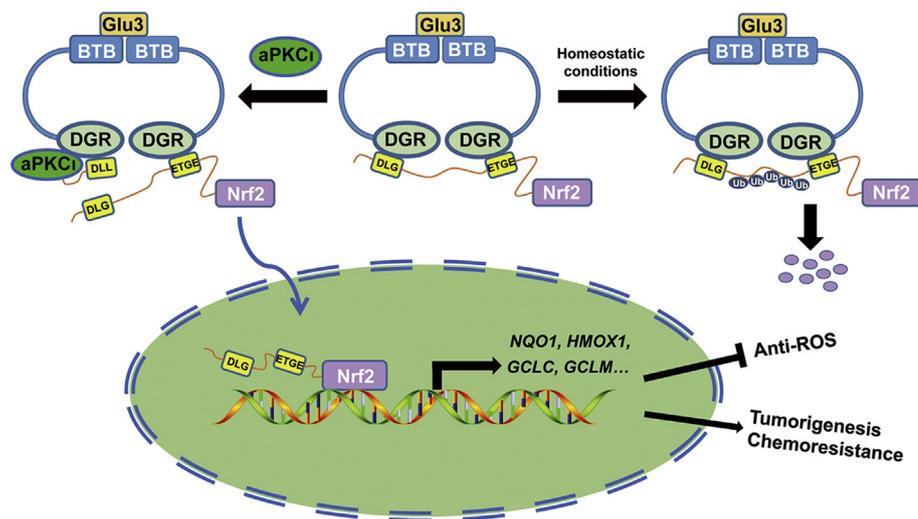


Fig. 8. Schematic summary of how aPKC ι induces ROS inhibition and promotes cell tumorigenesis and gemcitabine resistance in GBC. aPKC ι competes with Nrf2 for binding to Keap1, which leads to Nrf2 nuclear accumulation and activation of its target genes; this ultimately induces ROS inhibition, tumor growth, and drug resistance.

speculated that aPKC ι might modulate Nrf2 protein did not occur at the transcriptional level. Indeed, we found that aPKC ι regulated the protein stability of Nrf2 and inhibited its proteasome-mediated degradation. Although DLG motif is weaker than ETGE motif, both DLG and ETGE motifs are required for the Keap1-dependent degradation of Nrf2. In addition, a recent study has shown that the interaction between Keap1 and Nrf2 is dynamic. It contains two distinct conformations and follows a cycle: “open”, in which Nrf2 binds one Keap1 molecule through ETGE, and “closed”, in which Nrf2 interacts with the Keap1 dimer through DLG and ETGE [42,44]. Of note, it has been reported that when the ETGE motif bound with Keap1 singly, Nrf2 would not be ubiquitinated by the E3-ubiquitin ligase [50]. When the conformations changed from an open to a closed, Nrf2 was polyubiquitinated and then subsequently released for degradation by the proteasome [42]. Then, aPKC ι will be placed in a better position to bind with the regenerated free Keap1 dimer through the DLL motif, which leads to the newly synthesized Nrf2 accumulation and nuclear translocation. Thus, it is not surprising that aPKC ι regulates the Keap1-Nrf2 system by competing with Nrf2.

It is also noteworthy that elevated aPKC ι expression was further confirmed in human gallbladder cancer specimens. Although lack of high throughput sequencing data, our results showed that the upregulation of Nrf2 protein and its target genes may be at least in part dependent on the elevation of aPKC ι in GBC samples. The overexpression of aPKC ι significantly correlates with poor prognosis in patients with GBC. Furthermore, it is widely accepted that sustained increase in ROS leads to develop chemoresistance, which may be presumably due to the abnormalities of the Keap1-Nrf2 pathway. Importantly, elevated aPKC ι can induce drug resistance in GBC cell lines, indicating that aPKC ι may be a potential and an attractive therapeutic target for GBC. However, whether aPKC ι modulates mitochondrial ROS in a similar or distinct manner is yet to be delineated. Further studies are required to understand how to target the aPKC ι -Keap1-Nrf2 pathway to improve the efficacy of treatment for GBC.

In conclusion, our study demonstrated the importance of elevated aPKC ι in promoting the tumorigenesis and gemcitabine resistance through competitive interaction with Nrf2 for binding to Keap1 in GBC cells. Accordingly, our findings expand our knowledge of the functions of aPKC ι in cancer, especially its role as an anti-ROS factor is kinase-independent. These results provide important insights into the development of new, effective therapeutic approaches to overcome drug resistance for the treatment of GBC.

Conflicts of interest

The authors declare no potential conflicts of interest.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.redox.2019.101149>.

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