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Plk1 Phosphorylation of Orc2 and Hbo1 Contributes to Gemcitabine Resistance in Pancreatic Cancer

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Abstract

Although gemcitabine is the standard chemotherapeutic drug for treatment of pancreatic cancer, almost all patients eventually develop resistance to this agent. Previous studies identified Polo-like kinase 1 (Plk1) as the mediator of gemcitabine resistance, but the molecular mechanism remains unknown. In this study, we show that Plk1 phosphorylation of Orc2 and Hbo1 mediates the resistance to gemcitabine. We show that the level of Plk1 expression positively correlates with gemcitabine resistance, both in pancreatic cancer cells and xenograft tumors. Overexpression of Plk1 increases gemcitabine resistance, while inhibition of Plk1 sensitizes pancreatic cancer cells to gemcitabine treatment. To validate our findings, we show that inhibition of Plk1 sensitizes tumors to gemcitabine treatment in a mouse xenograft study. Mechanistically, we find that Plk1 phosphorylation of Orc2 maintains DNA replication on gemcitabine treatment. Furthermore, Plk1 phosphorylation of Hbo1 transcriptionally increases cFos expression and consequently elevates its target multidrug resistance 1 (MDR1), which was previously reported to confer chemotherapeutic drug resistance. Knockdown of cFos or MDR1 sensitizes gemcitabine-resistant cells to gemcitabine treatment. Finally, pancreatic cancer cells expressing Plk1-unphosphorylatable mutants of Orc2 or Hbo1 are more sensitive to gemcitabine than cells expressing wild-type Orc2 or Hbo1. In short, our study provides a mechanism for Plk1-mediated gemcitabine resistance, suggesting that Plk1 is a promising target for treatment of gemcitabine-resistant pancreatic cancer. *Mol Cancer Ther*; 12(1); 58–68. ©2012 AACR.

Introduction

The pancreas is a unique organ that has both exocrine and endocrine compartments. Pancreatic cancer is the fourth leading cause of cancer-related death in the United States, with a 5-year survival rate of less than 6%. More than 90% of pancreatic cancers arise from the exocrine portion of the pancreas and are pancreatic ductal adenocarcinomas. Because of a lack of early cancer-related symptoms, patients with pancreatic cancer are often diagnosed at an advanced stage (1, 2).

Gemcitabine, a deoxycytidine analogue, is the standard chemotherapy treatment for advanced pancreatic cancer. Gemcitabine can directly incorporate into DNA or inhibit ribonucleotide reductase to prevent DNA replication and, thus, tumor cell growth (3). However, almost all patients

have either primary or eventually gain secondary resistance to gemcitabine treatment. The major causes for resistance can be summarized into 3 aspects: failure of gemcitabine uptake through hENT1 transporter, decrease of effective drug dose by enzyme metabolism, and gain of resistance to cellular stresses or apoptosis. Because of potential improved cytotoxicity, several combination therapies of gemcitabine plus additional agents are being tested in clinical trials. So far, gemcitabine with erlotinib, an epidermal growth factor receptor tyrosine kinase inhibitor, is the only U.S. Food and Drug Administration-approved combination treatment. This regimen has a modest effect, which can prolong median overall survival for less than 2 weeks (4). Thus, understanding the molecular events that occur during the development of gemcitabine resistance will lead to improvement of pancreatic cancer treatments.

Polo-like kinase 1 (Plk1) is a well-studied serine and threonine protein kinase. It plays important roles in cell proliferation, such as mitotic entry, centrosome maturation, kinetochore-microtubule attachment, chromosome segregation, and cellular checkpoint adaptation (5–12). Although evidence of *Plk1* as an oncogene is scarce, it is overexpressed in various tumors. In addition, the level of Plk1 correlates with poor prognosis of cancer patients (13). Inhibition of Plk1 induces mitotic arrest, followed by cell death in cancer cells, but not in normal cells (14). A recent study suggests that Plk1 might be a mediator for gemcitabine resistance in pancreatic cancer. Among

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approximately 40 potential target genes, *Plk1* was the only gene that distinguished gemcitabine-sensitive versus -resistant tumors (15). Our previous work indicates that Plk1 phosphorylates origin recognition complex 2 (Orc2) to promote DNA replication under replication stress such as the one induced by gemcitabine treatment (16). We also reported that Plk1 regulates prereplicative complex (pre-RC) formation through phosphorylation of histone acetyltransferase binding to the Orc1 (Hbo1; ref. 17). Therefore, we hypothesize that Plk1-associated kinase activity toward Orc2 and Hbo1 drives DNA replication in the presence of gemcitabine, eventually contributing to development of gemcitabine resistance in pancreatic cancer.

To test our central hypothesis, we first investigated the correlation between Plk1 expression and gemcitabine resistance with a combination of different pancreatic cell lines, xenograft mice and pancreatic cancer patient tissues. Our data suggest that Plk1 overexpression correlates with gemcitabine resistance in pancreatic cancer cells and xenograft tumors. Inhibition of Plk1 activity significantly enhances the antitumor effect of gemcitabine in a Panc-1 xenograft model. Mechanistically, we found that Plk1 phosphorylation of Orc2 at the origin of DNA replication is increased on gemcitabine treatment, and that cells expressing a Plk1-unphosphorylatable mutant of Orc2 are more sensitive to gemcitabine treatment. Surprisingly, gemcitabine treatment decreases Hbo1 at the replication origin but recruits it to the promoter of cFos, an AP-1 transcription factor. We further show that Hbo1 phosphorylation by Plk1 upregulates the transcriptional expression of cFos, consequently resulting in an elevation of its target multidrug resistance 1 (MDR1). Knockdown of cFos or MDR1 sensitizes gemcitabine-resistant cells to gemcitabine treatment. Taken together, our findings define an important signaling pathway of gemcitabine resistance in pancreatic cancer, suggesting a novel strategy to treat gemcitabine-resistant pancreatic cancer.

Materials and Methods

Chemicals

BI2536 was purchased from Symansis NZ Ltd, New Zealand. Gemcitabine (Cat. 3259) was purchased from Tocris Bioscience (Fig. 2B and C).

Mouse xenograft model

Panc-1 cells (5×10^6 cells per mouse) were mixed with an equal volume of Matrigel (Collaborative Biomedical Products) and inoculated into the right flank of athymic nude mice (Harlan Laboratories). One week later, the animals were randomized into treatment and control groups of 5 mice each. BI2536 was dissolved in 0.1 N HCl, diluted with 0.9% NaCl, and injected into the tail vein twice weekly for 6 weeks. Gemcitabine was dissolved in 0.9% NaCl, diluted with 0.9% NaCl, and injected into the tail vein twice weekly for 6 weeks. Tumor volumes, estimated from the formula: $V = L \times W^2/2$ (V , mm³; L , mm; W , mm), were measured on alternate days with digital calipers.

Statistical analysis

A standard 2-tailed unpaired Student *t* test was used to calculate differences between samples. One-way ANOVA was used to determine statistically significant differences from the mean in the xenograft study.

Cell culture, transfection, and RNAi

The Panc-1 and BxPC-3 cells were purchased from the American Type Culture Collection. Cells were initially grown and multiple aliquots were stored at -180°C for future use as required. Cells were purchased more than 6 months ago and were not further tested or authenticated by the authors. Panc-1 cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% FBS, 100 units/mL penicillin, and 100 units/mL streptomycin at 37°C in 5% CO₂. BxPC-3 cells were cultured in RPMI1640 medium. HPDE6 cells were cultured in keratinocyte medium (Invitrogen). Plasmid DNA was transfected with MegaTran (Origene) as described by the manufacturer. The cFos (sc-29221) and MDR1 (sc-29395) siRNAs were purchased from Santa Cruz Biotechnology and transfected with Oligofectamine (Invitrogen) as described by the manufacturer.

Western blotting

After cells were lysed in AMI lysis buffer (Active Motif), proteins were detected by Western blotting with antibodies against Plk1 (sc-17783; Santa Cruz Biotechnology), β -actin (A5441; Sigma), and PARP (AB3565; Millipore).

Immunohistochemistry

After murine or human paraffin-embedded slides were deparaffinized and rehydrated, antigens were retrieved in antigen unmasking solution (Vector Laboratories) with a 2100-Retriever (Pierce & Warriner Laboratories). Samples were then incubated with primary antibodies against Plk1 (08544; Upstate) and Ki-67 (ab16667; Abcam) or subjected to TUNEL assay (11684817910; Roche).

Cell viability assay

Cells were grown in 96-well plates, and viable cell numbers were determined with the CellTiter-Glo Luminescent Cell Viability Assay kit (Promega) as described by the manufacturer.

Combination index

IC₅₀ and combination index of gemcitabine and BI2536 were calculated as indicated in (18). Combination index more than 1 indicates antagonism; combination index less than 1 indicates synergy; and combination index equal to 1 indicates an additive effect.

BrdU labeling assay

BrdU-labeling assays were conducted with a kit from Roche (Cat. 11170376001) according to the manufacturer's instructions.

TUNEL assay

TUNEL assays were conducted with a kit from Roche (Cat.11684817910) according to the manufacturer's instructions. Details of isolation of cells from xenograft tumors, chromatin immunoprecipitation (ChIP), and quantitative real-time PCR (qRT-PCR) analysis are described in supplementary material.

Results

Plk1 is overexpressed in pancreatic tumors

To follow the expression of Plk1 protein in pancreatic ductal adenocarcinoma, we conducted immunohistochemical (IHC) staining of Plk1 on a pancreatic tissue microarray ($n = 140$) that included normal pancreas, cancer-adjacent tissue, and pancreatic ductal adenocarcinoma. We found that nearly 80% of the tumors had positive Plk1 staining (60% with strong staining and 20% with weak staining), while only 20% of the cancer-adjacent tissue had positive Plk1 staining, and 80% to 90% of normal tissues had no Plk1 staining (Fig. 1). The difference of Plk1 protein expression between normal and cancer tissues is statistically significant. Taken together, these data suggest that Plk1 is significantly differently expressed between normal and cancerous tissue.

Plk1 expression correlates with gemcitabine resistance *in vitro* and *in vivo*

To investigate whether the elevated Plk1 protein levels in pancreatic tumor samples correlates with an active role of Plk1 in gemcitabine resistance of pancreatic cancer, we measured the IC_{50} values of gemcitabine in 4 different pancreatic cell lines, HPDE6 (an immortalized human pancreatic epithelial cell line), BxPC-3 (human pancreatic cancer cell line), Panc-1 (human pancreatic cancer cell line), and AsPC-1 (human pancreatic cancer cell line). HPDE6 and BxPC-3 had low IC_{50} values, thus representing gemcitabine-sensitive cell lines, while Panc-1 and AsPC-1 cells had high IC_{50} values, thus possessing the gemcitabine-resistant property (Supplementary Table S1). To compare Plk1 levels in cell lines with different gemcitabine sensitivities and to avoid the possibility of comparing cells at different cell cycle stages or with different proliferation rates, we synchronized the cells at S phase with thymidine or at M phase with nocodazole, followed by Western blotting. Within the same stage of the cell cycle, Panc-1 cells had the most abundant Plk1 protein, while HPDE6 cells had the lowest level of (Fig. 2A), positively correlating with the gemcitabine IC_{50} values of these cell lines (Supplementary Table S1).

To determine whether the sensitivity to gemcitabine is influenced by Plk1 activity, we measured the combination index of gemcitabine and BI2536 (an ATP competitive inhibitor that specifically inhibits Plk1 kinase activity) as described in the materials and methods section (Fig. 2B and C). The IC_{50} value of gemcitabine for Panc-1 cells was 1284 nmol/L (Table 1). However, the IC_{50} of gemcitabine

was reduced to 44 nmol/L when the cells were treated in combination with 2 nmol/L BI2536. The combination index was calculated to be 0.3 (Table 1), which is less than 1, suggesting a synergistic effect between gemcitabine and BI2536.

Because Panc-1 cells have the highest Plk1 level and are most resistant to gemcitabine, we treated Panc-1 cells with gemcitabine, BI2536 alone, or gemcitabine in combination with BI2536. We followed cell death by Western blot of cleaved-PARP protein. PARP is a poly ADP-ribose polymerase that facilitates the survival of cells. Cleavage of PARP disassembles cellular structure and serves as a marker for cellular apoptosis. Gemcitabine or BI2536 alone had a minimal effect on cell death, but inhibition of Plk1 activity by BI2536 enhanced gemcitabine-induced cellular apoptosis (Fig. 2D). Consistent with the result in Fig 2D, the combination of gemcitabine and BI2536 led to significantly reduced cell survival compared with gemcitabine or BI2536 alone (Supplementary Fig. S1A), indicating that inhibition of Plk1 sensitizes gemcitabine-resistant cells to the chemotherapy. To further expand our observation, we also repeated this experiment in AsPC-1 cells, which is a gemcitabine-resistant cell line, and detected similar enhanced cellular apoptosis by inhibition of Plk1 activity using BI2536 in the presence of gemcitabine (Supplementary Fig. S1C and D). To confirm this observation, we overexpressed Plk1 in HPDE6 cells, which have the lowest Plk1 protein level. Plk1 overexpression induced gemcitabine resistance as shown by a decreased cleaved-PARP protein level compared with control samples (Fig. 2E). In conclusion, the level of Plk1 protein influences responsiveness to gemcitabine treatment in pancreatic cells.

With the aim to better assess the correlation between Plk1 protein expression and gemcitabine sensitivity, we examined the Plk1 protein level in xenograft tumors. Briefly, animals bearing subcutaneous pancreatic tumors derived from Panc-1 cells were treated with 40 mg/kg gemcitabine. After 8 weeks of treatment, the tumors showed different responses to gemcitabine. We repeated this experiment for several times, and observed a similar trend that the Plk1 protein expression level is relatively lower in tumors with smaller volume, indicating that these tumors are more sensitive to gemcitabine treatment (Fig. 2F–I). Cyclin A protein levels in these tumors are similar, suggesting that Plk1 protein expression levels were compared in tumor cells with a similar cell-cycle distribution. Two substrates of Plk1 that might be involved in Plk1-mediated gemcitabine resistance were also measured. Neither Orc2 nor Hbo1 protein levels show any obvious differences among these tumors; therefore, it is unlikely that gemcitabine resistance is more related to the protein levels of the Orc2 and Hbo1 (Fig. 2I). More importantly, to assess the significance of this correlation, we quantified the Plk1 protein levels (Fig. 2I) and measured the correlation efficiency between Plk1 expression signal intensity and tumor volume by Pearson product-moment

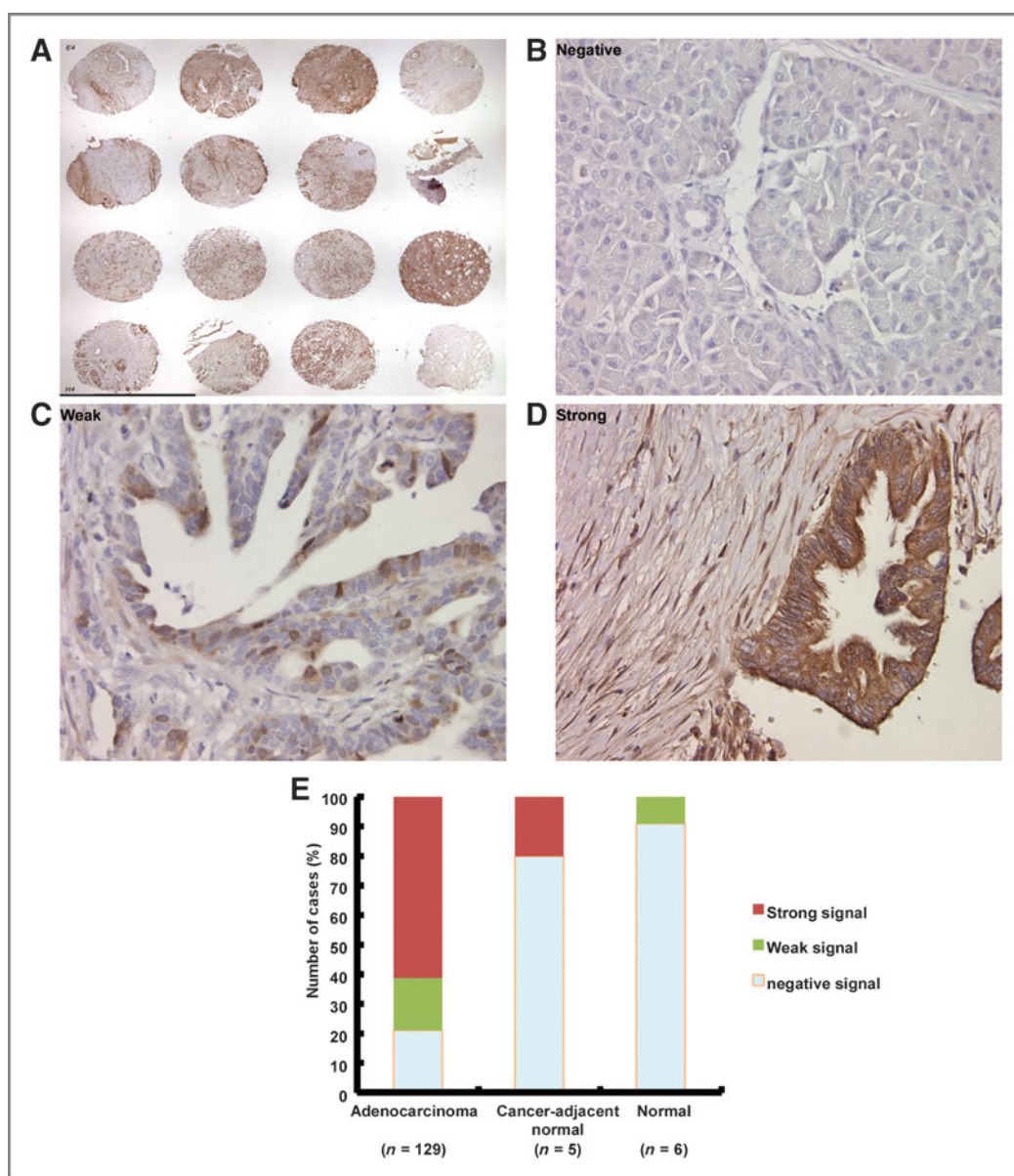


Figure 1. Plk1 is overexpressed in pancreatic cancer tissues. A, representative images of immunohistochemical staining of a pancreatic cancer tissue microarray with an anti-Plk1 antibody. This tissue microarray includes pancreatic adenocarcinomas, cancer adjacent tissues, and normal pancreatic tissues. B, a representative image of Plk1 immunohistochemical staining of normal pancreatic tissue. C, a representative image of weak Plk1 immunohistochemical staining of pancreatic adenocarcinoma tissue. D, a representative image of strong Plk1 immunohistochemical staining of pancreatic adenocarcinoma tissue. E, quantification of Plk1 immunohistochemical staining in the pancreatic cancer tissue microarray. (The difference between normal and cancer pancreatic tissue samples is significant, $P < 0.05$).

correlation coefficient analysis (Fig. 2J). The value of Pearson product-moment correlation coefficient (R) was equal to 0.90 with P values less than 0.05, suggesting a highly linear correlation between Plk1 protein expression and tumor volume. We also compared protein expression level of Plk1 between control untreated tumors and gemcitabine-treated tumors. Three untreated control tumors all showed high levels of Plk1 expression; while the gemcitabine-sensitive tumors tended to have lower levels of Plk1, the gemcitabine-resistant

tumors retained high levels of Plk1 (Fig. 2K). The decrease of Plk1 levels can be because of individual host-tumor interaction or the heterogeneity of cultured cancer cells, but either case supports a role of high levels Plk1 in maintaining gemcitabine resistance. These results are consistent with our observations in the cell-based experiments, providing additional evidence to support a functional correlation between Plk1 protein expression and gemcitabine resistance in pancreatic cancer.

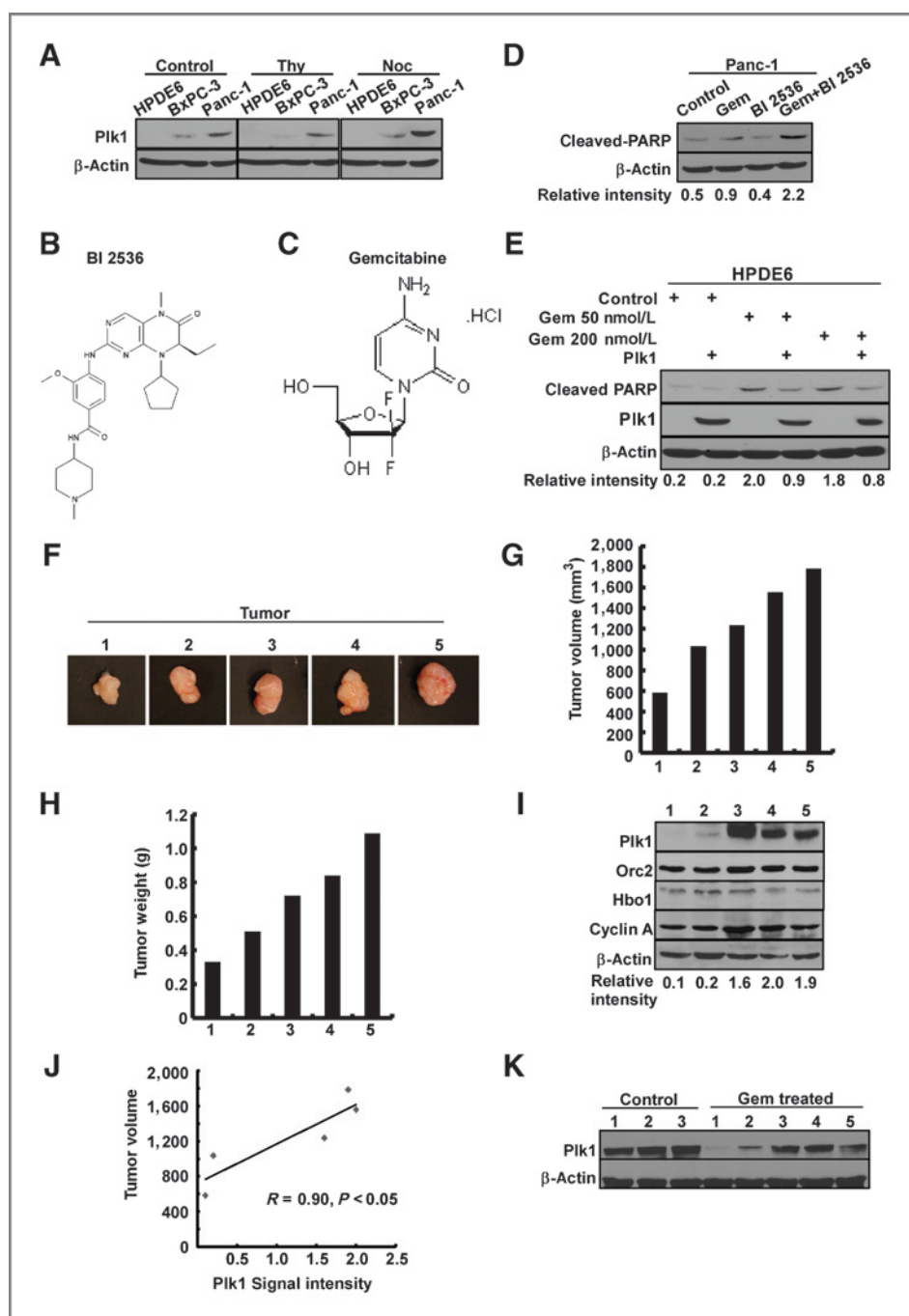


Figure 2. Plk1 expression correlates with gemcitabine resistance in cells and *in vivo*. **A**, HPDE6, BxPC-3, and Panc-1 cells were synchronized by thymidine (24 hours) or nocodazole (10 hours) treatment, then whole-cell lysates were extracted and analyzed by anti-Plk1 and anti-β-actin Western blots. **B**, Panc-1 cells were treated with gemcitabine (100 nmol/L), BI2536 (1 nmol/L), or both for 72 hours, followed by Western blotting. **C**, structure of BI2536. **D**, structure of gemcitabine. **E**, HPDE6 cells were infected with lentivirus to overexpress Plk1. Twelve hours after infection, cells were treated with gemcitabine (50 or 200 nmol/L) for 24 hours and harvested for Western blotting. **F**, Panc-1-derived xenograft tumors from nude mice that had been treated with gemcitabine (40 mg/kg) twice a week for 8 weeks. **G**, volumes of tumors in **F**. **H**, weights of tumors in **F**. **I**, proteins were extracted from the tumors as in **F** and analyzed by Western blots. **J**, the correlational efficiency between Plk1 expression signal intensity in **I** and tumor volume is measured by Pearson product-moment correlation coefficient analysis ($R, 0.90; P < 0.05$). **K**, proteins were extracted from gemcitabine-treated or -untreated Panc-1-derived xenograft tumors and analyzed by Western blots.

Inhibition of Plk1 sensitizes pancreatic tumors to gemcitabine treatment *in vivo*

To test if elevated Plk1-associated kinase activity in pancreatic cancer contributes to induction of gemcitabine resistance *in vivo*, we next examined the effects of gemcitabine and BI2536, alone or in combination, on subcutaneous pancreatic tumors. As indicated in Fig. 3A, neither treatment with gemcitabine alone nor BI2536 alone significantly prevented tumor growth, likely due to the relatively low concentrations of the

drugs used in this study. In striking contrast, a combination of the same doses of gemcitabine and BI2536 strongly inhibited tumor growth, suggesting that inhibition of Plk1 sensitizes pancreatic tumors to gemcitabine treatment.

Apoptosis and cell proliferation were further examined by TUNEL and Ki-67 staining on tumor sections prepared from these xenograft tumors by the end of the study. As shown in Fig. 3B, the combination of gemcitabine and BI2536 significantly increased cell

Table 1. The IC₅₀ values of gemcitabine and BI2536 in Panc-1-cells

Drugs	IC ₅₀ (nmol/L)	Notes
BI2536	3.68	
Gemcitabine	1,284.07	
Gemcitabine (in combination with 2 nmol/L BI2536)	44.35	Combination index = 0.3

death compared with control or monotherapy groups. Although control tumors showed moderately faster cell proliferation, the 4 groups of tumors did not have a statistically significant different rate of cell proliferation as shown by Ki67 staining (Fig. 3C). These data suggest inhibition of Plk1 enhances the efficacy of gemcitabine mainly by promoting cell death.

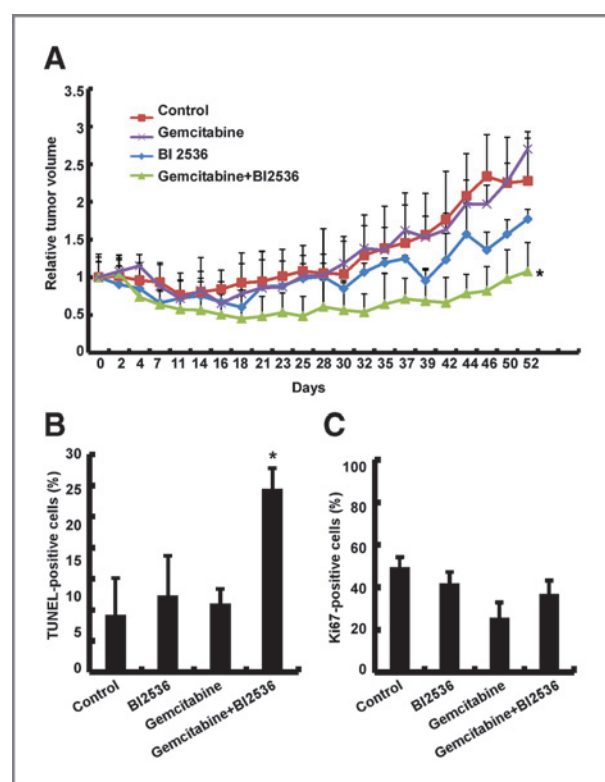


Figure 3. Inhibition of Plk1 sensitizes xenograft tumors to gemcitabine treatment. A, Panc-1 cells (5×10^6) were inoculated into flanks of female nude mice. One week after inoculation, the mice were treated with gemcitabine (40 mg/kg), BI2536 (15 mg/kg), or a combination of both drugs, and the relative sizes of the tumors in each group were plotted against the number of days ($P < 0.01$). B, *in situ* TUNEL assay of the xenograft tumors. The average percentages of TUNEL-positive cells from multiple tumor sections were calculated to assess the degree of cell death ($P < 0.05$). C, immunohistochemistry staining of Ki-67 of xenograft tumors. The average percentages of Ki-67-positive cells from multiple tumor sections were calculated to assess cell proliferation.

Inhibition of Plk1 counteracts gemcitabine resistance in cells

To confirm that Plk1 confers gemcitabine resistance in pancreatic cancer, we isolated tumor cells from Panc-1-derived xenograft tumors with different resistance levels to gemcitabine (Supplementary Fig. S2A–D). Fourteen tumors were used to generate the sublines, 8 sublines were generated. As shown in Fig. 4A, consistent with our previous observations, the gemcitabine-sensitive tumors (#6) had a lower level of Plk1. Interestingly, the p-Orc2 level increased 3-fold for tumor #19 compared with tumor #6, and increased by 4-fold for tumor #21 compared with tumor #6, which correlated with the high expression of Plk1 (Fig. 4A). *In vitro* measurement of the gemcitabine IC₅₀ values of these tumor cells further confirmed their gemcitabine resistance (Fig. 4B). Cells derived from tumors 19 and 21 had higher gemcitabine IC₅₀ values *in vitro* corresponding to their larger tumor sizes *in vivo*. Because of these elevated Plk1 levels and higher gemcitabine IC₅₀ values, we further treated those gemcitabine-resistant tumor cells with gemcitabine *in vitro*, alone or in combination with BI2536. Both gemcitabine-resistant tumor cells became more gemcitabine sensitive in the presence of a low dosage of BI2536 (Fig. 4C, 4D), while the same dosage of BI2536 alone did not significantly affect the cell viability (Supplementary Fig. S2 E and F). Finally, we further confirmed that the gemcitabine-resistant tumor cells are more sensitive to inhibition of Plk1 on the basis of the more rapid response of these cells to BI2536 treatment as measured by pH3 staining (Histone H3 is specifically phosphorylated at Ser10 during mitosis, which can serve as a mitotic marker) and FACS analysis (Supplementary Fig. S2G and H). These analyses support the notion that Plk1 confers the gemcitabine resistance of pancreatic cancer.

Plk1 phosphorylation of Orc2 promotes DNA replication in the presence of gemcitabine

Next, we dissected the mechanism for Plk1-associated gemcitabine resistance in pancreatic cancer. Orc2 is a key component of the pre-RC complex, which plays important roles in initiation of DNA replication (19). We recently reported that Plk1 phosphorylation of Orc2 promotes DNA replication under various stress conditions (16). Because gemcitabine-resistant pancreatic tumors have elevated Orc2 phosphorylation, we hypothesize that Plk1 phosphorylation of Orc2 is a driving force for cell proliferation in the presence of gemcitabine in pancreatic cancer.

We first asked whether Plk1-mediated Orc2 phosphorylation is enhanced at the replication origin by the DNA replication stress induced by gemcitabine. Toward this end, we conducted ChIP experiments with antibodies against Orc2 and p-Orc2, and examined the well-defined Orc2-associated DNA replication origin (MCM4). As shown in Fig. 5A, we were able to detect the signal by Orc2 antibody on the MCM4 origin (~500 genomic units) and the signal by phospho-Orc2 antibody (~200 genomic units)

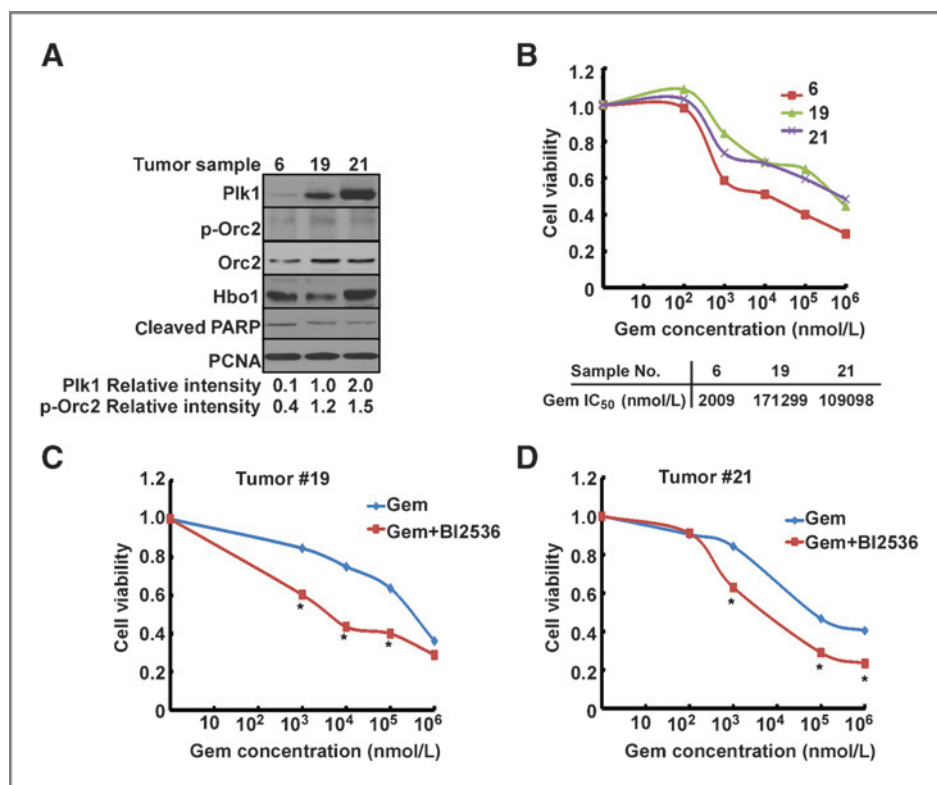


Figure 4. Inhibition of Plk1 counteracts the gemcitabine resistance in cells derived from gemcitabine-resistant tumors. **A**, Panc-1-derived xenograft tumors were treated as in Fig. 3A to generate tumors with different sensitivities to gemcitabine. Part of each tumor sample was subjected to protein extraction for Western blots. **B**, cells isolated from the tumors as in **A** were grown in 96-well plates and cell viability was assessed. **C** and **D**, isolated gemcitabine-resistant cells (#19 and #20) were grown in 96-well plates, treated with gemcitabine alone or in combination with BI2536, and cell viability was assessed ($P < 0.05$).

in control cells without gemcitabine treatment. After gemcitabine treatment, the signal by Orc2 antibody remained about the same, but the signal by the p-Orc2 antibody increased to approximately 450 genomic units, an approximately 2-fold increase compared with untreated cells (Fig. 5A). This observation supports our hypothesis that Plk1-mediated phosphorylation of Orc2 at the replication origin is elevated in response to gemcitabine treatment.

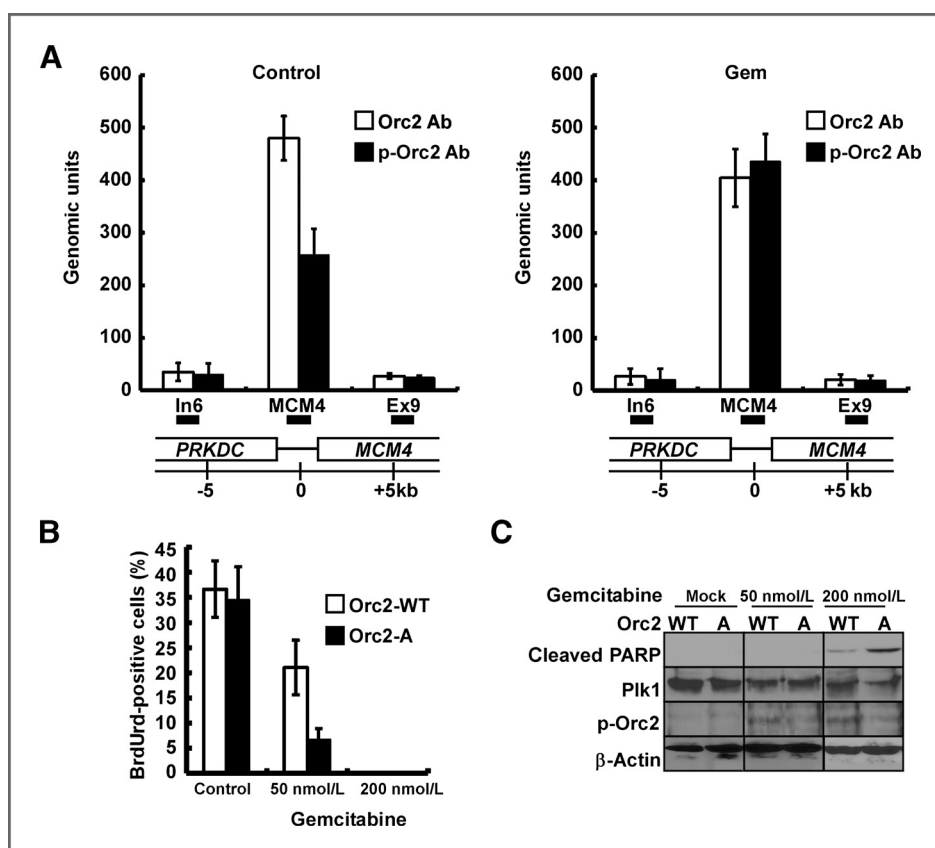
To further evaluate the significance of this phosphorylation event in resistance to gemcitabine in pancreatic cancer, Panc-1 cells expressing Orc2-WT or Orc2-A (Plk1 unphosphorylatable mutant, S188A) were treated with gemcitabine and subjected to BrdU labeling assays (Supplementary Fig. S3A). Without gemcitabine treatment, Orc2-WT and Orc2-A cells displayed similar incorporation of BrdU. However, Orc2-A-expressing cells showed reduced DNA replication compared with Orc2-WT cells upon gemcitabine treatment (Fig. 5B). This result suggests that Panc-1 cells expressing the Orc2-A mutant are more sensitive to gemcitabine treatment, indicating that Plk1 phosphorylation of Orc2 maintains DNA replication capacity in the presence of gemcitabine. Treating Panc-1 cells with BI2536 also decreases p-Orc2 level (Supplementary Fig. S1B). To further confirm this notion, we examined cell death in these two populations upon gemcitabine treatment. Cells expressing the Orc2-A mutant showed increased cell death compared with cells expressing Orc2-WT, suggesting that Plk1 phosphorylation of Orc2 is, indeed, one mechanism for increased gemcitabine resistance in pancreatic cancer (Fig. 5C).

Plk1 phosphorylation of Hbo1 increases cFos, consequently elevating its target, MDR1

Hbo1, the enzyme responsible for histone H4 acetylation, is a core subunit of a protein complex comprised of JADE1/2/3 paralogs, hEaf6 and ING5. This complex interacts with the Mcm helicase and is essential for DNA replication to occur during S phase (20). Recently, it was reported that Hbo1 is recruited to the promoter of AP-1 and serves as a coactivator to increase AP-1 transcription in response to environmental stress (21). More importantly, the expression level of Hbo1 is high in various human carcinomas (22). Previous studies in our laboratory showed that Plk1 phosphorylation of Hbo1 positively regulates its acetylation activity to promote DNA replication (17). Given these facts, we next asked whether Plk1 phosphorylation of Hbo1 also contributes to gemcitabine resistance in pancreatic cancer.

To test this hypothesis, we first conducted BrdU-labeling assays with cells expressing Hbo1-WT or Hbo1-A mutant (Plk1 unphosphorylatable mutant, S57A; Supplementary Fig. S3B). As shown in Supplementary Fig. S3C, cells expressing Hbo1-A are more sensitive to gemcitabine treatment as compared with cells expressing Hbo1-WT. As a result of this sensitivity, cells expressing Hbo1-A showed increased cell death as indicated by an increased cleaved-PARP protein level after gemcitabine treatment (Supplementary Fig. S3D). These data suggest that Plk1 phosphorylation of Hbo1 also plays a role in gemcitabine resistance of pancreatic cancer.

Figure 5. Plk1 phosphorylation of Orc2 promotes DNA replication in the presence of gemcitabine. **A**, Panc-1 cells were treated with gemcitabine (50 $\mu\text{mol/L}$) for 12 hours and then subjected to ChIP analysis. The qRT-PCR was carried out with DNA that was extracted from chromatin precipitated with antibodies against Orc2 or p-Orc2. The MCM4 locus represents an established replication origin (29). In6 is the region 6-kb upstream of the MCM4 locus, and Ex9 is the region 5-kb downstream of the MCM4 locus. **B**, Panc-1 cells were transfected with Orc2-WT or Orc2-A constructs and synchronized by a double thymidine block (DTB, 16-hour thymidine block, 8 hours of release, followed by a second thymidine block for 16 hours) protocol. After release from the second thymidine block, cells were treated with gemcitabine (50 or 200 nmol/L) for 24 hours, followed by BrdU labeling assay. **C**, whole-cell lysates from Panc-1 cells treated as in B were subjected to Western blots.



To dissect the mechanism of this observation, we first examined the occupancy of endogenous Hbo1 at the replication origin upon gemcitabine treatment by ChIP analysis. To our surprise, Hbo1 was significantly decreased from the replication origin after gemcitabine treatment (Supplementary Fig. S3E). Instead, we observed that Hbo1 accumulated at the promoter of *cFos* (Fig. 6A), an AP-1 transcription factor. This observation is consistent with the previous report that Hbo1 is recruited at the *cFos* promoter site to serve as a transcriptional coactivator upon stress (21). Therefore, it is possible that Hbo1 at the *cFos* promoter activates *cFos* gene transcription. As the induction of *cFos* by therapeutic drug treatment is involved in the acquisition of drug resistance (23–25), we then examined whether *cFos* transcription is induced by gemcitabine treatment. As shown in Fig. 6B, the expression level of *cFos* was increased by 2.5-fold after 1-hour gemcitabine treatment. Further, overexpression of Hbo1-WT, but not Hbo1-A, significantly amplified the induction of *cFos* (20-fold) on gemcitabine treatment (Fig. 6C), suggesting phosphorylation of Hbo1 contributes to the induction of *cFos* by gemcitabine treatment.

To test whether the elevated *cFos* contributes to gemcitabine resistance in pancreatic cancer, we next examined its transcriptional target, MDR1, as MDR1 has been reported to mediate drug resistance by exporting drugs out of cells (24, 26, 27). Consistent with the results in Fig. 6C, we observed that overexpression of Hbo1-WT, but not

Hbo1-A, also amplified the induction of MDR1 (3-fold) upon gemcitabine treatment (Fig. 6D). Moreover, the expression levels of *cFos* and MDR1 were higher in gemcitabine-resistant cells than in gemcitabine-sensitive cells (Fig. 6E and F), suggesting a possible role of the *cFos*-MDR1 pathway in gemcitabine resistance. To confirm the contribution of Plk1 phosphorylation of Hbo1 on the elevation of *cFos* and MDR1, we treated the gemcitabine-resistant cells with BI2536 and examined the expression of *cFos* and MDR1. As shown in Fig. 6G and H, inhibition of Plk1 decreased *cFos* expression by 50% and MDR1 expression by 40%, confirming the role of Plk1 phosphorylation of Hbo1 on the elevation of *cFos* and MDR1 levels. Finally, to test whether elevated expressions of *cFos* and MDR1 confer gemcitabine resistance, we employed RNAi to knock down their expression in gemcitabine-resistant cells (Supplementary Fig. S4), and found that the IC_{50} of gemcitabine was reduced from 171.2 to 14.9 $\mu\text{mol/L}$ after *cFos* RNAi transfection and to 1.0 $\mu\text{mol/L}$ after MDR1 RNAi transfection (Fig. 6I). These data suggest that elevated *cFos* and MDR1 maintain the gemcitabine resistance in pancreatic cancer.

Finally, to test the contribution of these two phosphorylation events to gemcitabine resistance, Panc-1 cells were synchronized, transfected, and treated with or without gemcitabine. As shown in Fig. 6J, Panc-1 cells expressing Hbo1-A alone showed less cleaved-PARP protein level than cells expressing both Hbo1-A and

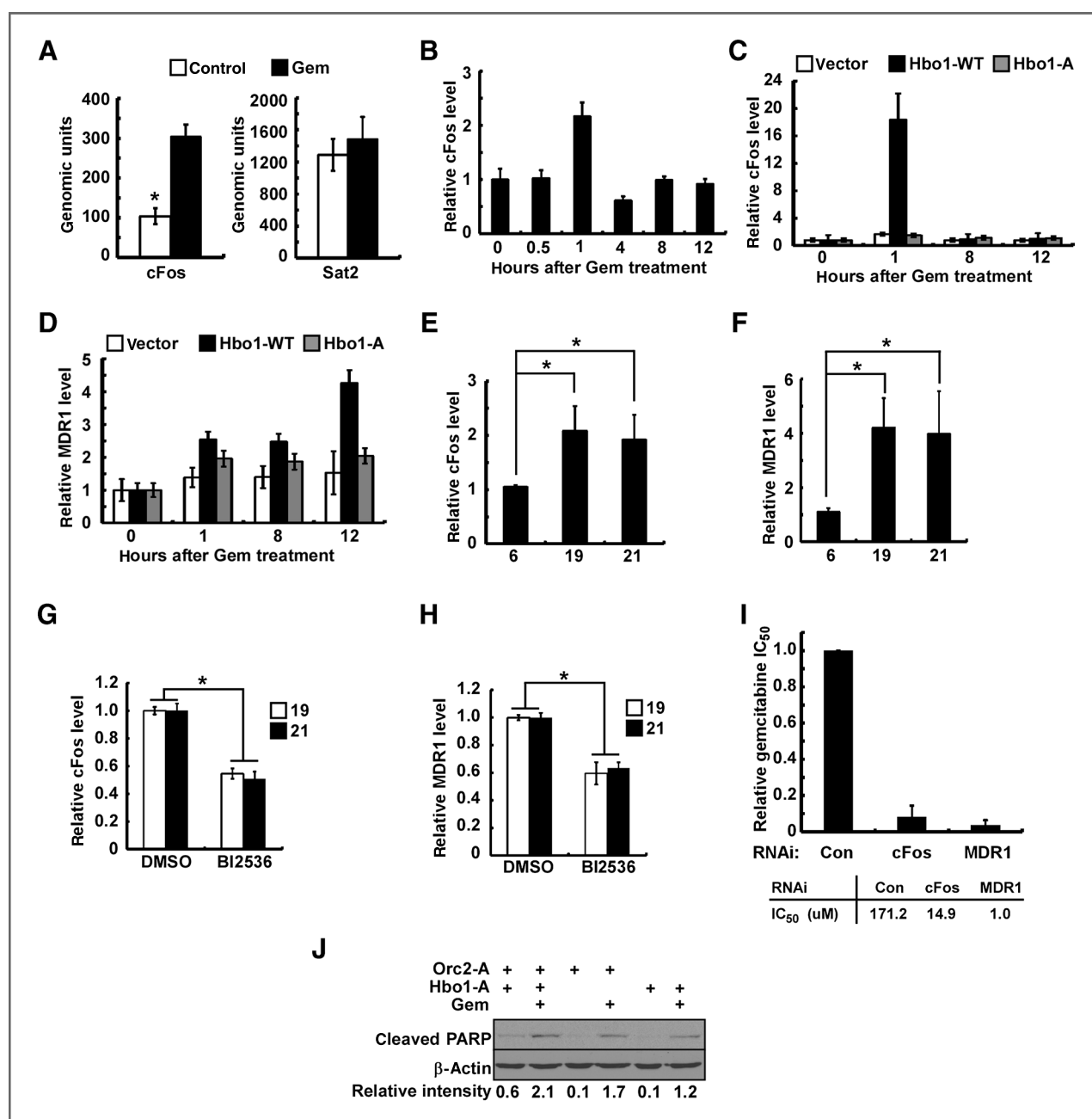


Figure 6. Plk1 phosphorylation of Hbo1 increases cFos expression and consequently elevates its target, MDR1. **A**, Panc-1 cells were treated with gemcitabine (50 μ M) for 12 hours and then subjected to ChIP analysis with anti-Hbo1 antibody. qRT-PCR was carried out with DNA extracted from precipitated chromatin to examine the association of Hbo1 on the promoters of cFos and Sat2 ($P < 0.05$). Hbo1 localization on the promoter of Sat2 is not induced by stress and, thus, serves as a negative control for this experiment (#21). **B**, Panc-1 cells were treated with gemcitabine and harvested at different times after treatment. The mRNA level of cFos from each sample was quantified by qRT-PCR and normalized to a mock control. **C** and **D**, Panc-1 cells were transfected with Flag-Hbo1-WT, Flag-Hbo1-S57A, or Flag vector alone, treated with gemcitabine, and harvested at different times after treatment. The mRNA level of cFos (**C**) and MDR1 (**D**) for each sample was examined by qRT-PCR ($P < 0.05$). The mRNA levels of cFos (**E**) and MDR1 (**F**) in the gemcitabine-sensitive Panc-1 cell line (#6) and gemcitabine-resistant Panc-1 cell lines (#19 and #21) were examined by qRT-PCR. **G** and **H**, after gemcitabine-resistant Panc-1 cells (#19 and #21) were treated with BI2536 (500 nmol/L) for 4 hours, the mRNA levels of cFos (**G**) and MDR1 (**H**) were examined by qRT-PCR ($P < 0.05$). **I**, gemcitabine-resistant Panc-1 cells (#19) were transfected with cFos or MDR1 siRNA for 24 hours and the IC₅₀ values of gemcitabine were determined. **J**, Panc-1 cells were synchronized by double thymidine block protocol, then transfected with Flag-Hbo1-A, GFP-Orc2-A, or both, then treated with or without gemcitabine (50 nmol/L) for 24 hours, and subjected to Western blot analysis.

Orc2-A mutants in response to gemcitabine treatment. Cells expressing Orc2-A alone has a similar cleaved-PARP protein expression level as cells expressing both

mutants. This result suggests that Plk1 phosphorylation of Orc2 might be a dominant mechanism for Plk1-mediated gemcitabine resistance.

Discussion

Gemcitabine is the current standard chemotherapy for pancreatic cancer, a deadly disease. However, only 30% of patients benefit from this agent, and among those, almost all will become resistant, usually within 3 to 4 months. In this study, we have investigated the putative mechanisms of gemcitabine resistance in pancreatic tumors. We found that Plk1 phosphorylation of Orc2 and Hbo1 mediates gemcitabine resistance and that inhibition of Plk1 sensitizes pancreatic tumor cells to gemcitabine treatment *in vitro* and *in vivo*. Plk1 blockade may represent a novel avenue for treatment of gemcitabine-resistant pancreatic cancer.

Plk1 is a well-established regulator of many mitosis-related events. However, our recent work suggests that Plk1 might also have functions in interphase events, such as DNA replication. For example, we showed the involvement of Plk1 in promotion of DNA synthesis by phosphorylation of Orc2 under replication stress. Moreover, elevated levels of Plk1 and phospho-Orc2 in pancreatic tumors are correlated with gemcitabine resistance (Figs. 2 and 3). Increased phosphorylation of Orc2 at the replication origin on gemcitabine treatment maintains DNA replication for cell survival (Fig. 5A and B). As a consequence, pancreatic cancer cells expressing the Plk1-unphosphorylatable mutant of Orc2 became more sensitive to gemcitabine treatment (Fig. 5C), suggesting that Plk1-mediated phosphorylation of Orc2 is one mechanism that contributes to gemcitabine resistance of pancreatic cancer.

We acknowledge that Plk1 likely regulates cellular responses to gemcitabine treatment via multiple mechanisms. We also reported previously that Hbo1 is phosphorylated by Plk1 to regulate DNA replication (17). To our surprise, we observed a decrease of Hbo1 at the replication origin upon gemcitabine treatment (Supplementary Fig. S3E). Instead, Hbo1 accumulated at the promoter region of cFos, an AP-1 transcription factor (Fig. 6A), which is consistent with the recent report of Hbo1 recruitment to the AP-1 promoter under environmental stress and the overall role of Hbo1 in regulating the p53 pathway (21, 28). These observations suggest that Hbo1 might contribute to gemcitabine resistance independent of its role in DNA replication. To support this notion, we found that cFos and its target MDR1 were significantly induced by gemcitabine treatment in a manner dependent on Plk1 phosphorylation of Hbo1 (Fig. 6B–D). The elevation of cFos transcription is likely due to the accumulation of Hbo1 at the cFos promoter on gemcitabine treatment (Fig. 6A). In the gemcitabine-resistant Panc-1 cells that we isolated from xenograft tumors, both cFos and MDR1 expression were elevated (Fig. 6E, F). Significantly, knock-down of cFos or MDR1 sensitizes these gemcitabine-resistant cells to gemcitabine treatment. Additional genes in the p53 pathway that are regulated by Hbo1 may also have a role in gemcitabine resistance, but our data support one mechanism of Hbo1-mediated resistance in which overexpressed Plk1 in pancreatic tumors phosphorylates

Hbo1 to elevate cFos and its target MDR1, eventually contributing to gemcitabine resistance.

Combining these results with the data of Orc2 described above, we propose a model that Plk1 phosphorylates Orc2 and Hbo1 to mediate gemcitabine resistance in pancreatic cancer. As shown in Fig. 3, inhibition of Plk1 kinase activity significantly enhanced gemcitabine sensitivity in pancreatic cancer. Gemcitabine treatment alone did not significantly increase cell death or block cell proliferation in the Panc-1 cell-based xenograft study, mirroring gemcitabine resistance found in the clinical setting. However, inhibition of Plk1 in combination with gemcitabine significantly increased cell death and prevented tumor growth, indicating that Plk1 activity is critical for the development of resistance to gemcitabine. Overexpression of Plk1 in HPDE6 cells, which are sensitive to gemcitabine treatment (Supplementary Table S1), induced resistance of HPDE6 cells to gemcitabine (Fig. 2E). This result suggests a potential role of Plk1 in secondary resistance to gemcitabine. Further, we showed that tumors maintaining high Plk1 levels on gemcitabine treatment were resistant to gemcitabine, and that tumors with decreased Plk1 levels on gemcitabine treatment were sensitive to gemcitabine (Fig. 2K), indicating that the response of Plk1 level on gemcitabine treatment can be used to predict the efficacy of gemcitabine in the treatment of pancreatic cancer. By examining a potential mechanism of gemcitabine resistance of pancreatic cancer, our study provides a novel rationale for molecularly targeting Plk1 in the treatment of this deadly disease.

Disclosure of Potential Conflicts of Interest

Steven Rice received a Commercial Research Grant Entity from the Howard Hughes Medical Institute Undergraduate Summer Research Grant Relationship (Minor \$10,000 or less). No potential conflicts of interest were disclosed by the other authors.

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