

Regulation of Pancreatic Tumor Cell Proliferation and Chemoresistance by the Histone Methyltransferase Enhancer of Zeste Homologue 2

Andrei V. Ougolkov,¹ Vladimir N. Bilim,² and Daniel D. Billadeau¹

Abstract Purpose: Enhancer of zeste homologue 2 (EZH2), a histone methyltransferase, plays a key role in transcriptional repression through chromatin remodeling. Our objectives were to determine the expression pattern of EZH2 and to assess the anticancer effect of EZH2 depletion in pancreatic cancer cells.

Experimental Design: Immunohistochemistry and cytosolic/nuclear fractionation were done to determine the expression pattern of EZH2 in normal pancreas and human pancreatic tumors. We used RNA interference, Western blotting, reverse transcription-PCR, and chromatin immunoprecipitation to study the effect of EZH2 depletion on pancreatic cancer cell proliferation and survival.

Results: We detected nuclear overexpression of EZH2 in pancreatic cancer cell lines and in 71 of 104 (68%) cases of human pancreatic adenocarcinomas. EZH2 nuclear accumulation was more frequent in poorly differentiated pancreatic adenocarcinomas (31 of 34 cases; $P < 0.001$). We found that genetic depletion of EZH2 results in reexpression of p27^{Kip1} and decreased pancreatic cancer cell proliferation. Moreover, we showed that EZH2 depletion sensitized pancreatic cancer cells to doxorubicin and gemcitabine, which leads to a significant induction of apoptosis, suggesting that the combination of EZH2 inhibitors and standard chemotherapy could be a superior potential treatment for pancreatic cancer.

Conclusions: Our results show nuclear accumulation of EZH2 as a hallmark of poorly differentiated pancreatic adenocarcinoma; identify the tumor suppressor p27^{Kip1} as a new target gene of EZH2; show that EZH2 nuclear overexpression contributes to pancreatic cancer cell proliferation; and suggest EZH2 as a potential therapeutic target for the treatment of pancreatic cancer.

Pancreatic cancer, the fourth leading cause of cancer deaths in the United States, kills more than 30,000 Americans every year. Not only is there no cure but there are also no effective treatments for this disease. The 5-year survival rate for people with pancreatic cancer is 3% (1).

Deciphering the cancer epigenetic code promises to dramatically change our understanding of pancreatic cancer, leading to the discovery of new oncomarkers and targets to develop superior diagnostic and treatment strategies. Recent evidence suggests that epigenetic silencing of tumor suppressor genes plays a significant role in tumor development (2). Epigenetic

control of gene expression occurs in two main ways: either the DNA itself is chemically altered (usually methylation of cytosines by DNA methyltransferases) or the histones, proteins that package DNA into chromatin (the main component of chromosomes), are modified (2). Posttranslational modification of histones determines whether the chromatin is tightly packed, leading to gene repression, or relaxed, leading to gene expression (2). The Polycomb group (PcG) proteins repress gene expression through the formation of multiple, unique complexes, which ultimately lead to the methylation of both histones and DNA (2–4). Specifically, PcG complexes containing the histone methyltransferase enhancer of zeste homologue 2 (EZH2) silence chromatin via methylation of histone H3-lysine 27 (H3-K27; ref. 3). Thus, EZH2 is thought to have the potential to silence genes that could be involved in tumorigenesis. Indeed, *EZH2* gene amplification was first reported in hematologic malignancies (5, 6), and EZH2-catalyzed methylation of H3-K27 is frequently associated with PcG-mediated silencing of tumor suppressor genes, such as *hMLH1*, *ARHI*, and *RASSF1A* in ovarian cancer (7) and *E-cadherin* in gastric cancer (8).

Whereas recent studies suggest EZH2 overexpression as an important factor of prostate (9) and breast (10) carcinoma progression, the expression pattern of EZH2 in human pancreatic cancer and the role of EZH2 in the proliferation, survival, and chemoresistance of pancreatic cancer cells remain

Authors' Affiliations: ¹Division of Oncology Research, Mayo Clinic College of Medicine, Rochester, Minnesota and ²Department of Urology, Yamagata University School of Medicine, Yamagata, Japan

Received 4/18/08; revised 6/16/08; accepted 7/6/08.

Grant support: Mayo Foundation, Specialized Program of Research Excellence in Pancreatic Cancer grant P50 CA102701 (D.D. Billadeau), and a Scholar Award from the Leukemia and Lymphoma Society of America (D.D. Billadeau).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Daniel D. Billadeau, Department of Immunology and Division of Oncology Research, 200 First Street Southwest, Rochester, MN 55905. Phone: 507-266-4334; Fax: 507-266-5146; E-mail: billadeau.daniel@mayo.edu.

©2008 American Association for Cancer Research.
doi:10.1158/1078-0432.CCR-08-1013

Translational Relevance

Our study provides novel insight into the mechanism by which the histone methyltransferase enhancer of zeste homologue 2 (EZH2) contributes to both pancreatic tumor cell proliferation and chemoresistance. We have found EZH2 nuclear accumulation in most pancreatic adenocarcinomas, whereas EZH2 expression is undetectable in normal pancreatic tissue, thus identifying EZH2 as a potential diagnostic oncomarker of pancreatic cancer. Immunohistochemical detection of EZH2 nuclear accumulation in biopsy specimens obtained by pancreatic needle or brush biopsy might be a useful method for pathologic diagnosis of pancreatic cancer. We identify EZH2 as a positive regulator of pancreatic cancer proliferation. Moreover, we show for the first time that RNA interference-mediated gene silencing of EZH2 sensitizes pancreatic cancer cells to doxorubicin and gemcitabine resulting in a significant induction of apoptosis, thus identifying EZH2 as a protein that contributes to pancreatic cancer chemoresistance. Taken together, our findings suggest that the combination of EZH2 inhibitors and standard chemotherapy could be a superior treatment for human pancreatic cancer.

unknown. Here, we show aberrant EZH2 nuclear overexpression in pancreatic cancer cell lines and most pancreatic adenocarcinomas. We show that depletion of EZH2 results in reexpression of the p27^{Kip1} tumor suppressor and decreased pancreatic cancer cell proliferation. Furthermore, for the first time, we show that EZH2 plays a role in pancreatic cancer chemoresistance, suggesting that combination of EZH2 inhibitors with standard chemotherapy could be a superior potential therapy for pancreatic cancer.

Materials and Methods

Reagents, plasmids, and cells. All chemicals were obtained from Sigma. An EZH2-specific targeting short hairpin RNA vector was generated as previously described (11) using the target sequence 5'-GACTCTGAATGCAGTGTCT-3'. All cell lines were obtained from the American Type Culture Collection.

Immunohistochemistry. The Institutional Review Board at the Mayo Clinic approved all studies carried out on human specimens. EZH2 antibody was obtained from BD Biosciences PharMingen. EZH2 immunostaining was done on 104 resected primary pancreatic adenocarcinoma specimens. Two pathologists (A.V.O. and D.D.B.) independently reviewed all cases and classified the tumors as well differentiated ($n = 20$), moderately differentiated ($n = 50$), or poorly differentiated ($n = 34$). For each case, the most representative section reflecting the major features of the primary pancreatic tumor (i.e., histologic type) was selected for immunohistochemical examination to determine the expression of EZH2. Immunohistochemical staining was done as described (12). EZH2 nuclear accumulation was defined as positive staining of more than 10% of cancer cell nuclei throughout the tumor regardless of cytoplasmic staining.

Immunoblot analysis and antibodies. For immunoblots, cells were lysed as described previously (13). Nuclear/cytosolic fractionation was done by the Dignam method (14). Protein sample concentration was quantified and equal amount (50 μ g of whole, nuclear, or cytosolic protein extract) of protein was loaded in each well of SDS-polyacrylamide gel. Cell or tissue extracts were separated by 10%

SDS-PAGE, transferred onto polyvinylidene difluoride membrane (PVDF), and probed as indicated. Antibodies for immunoblot analysis were obtained from the following suppliers: EZH2, poly(ADP-ribose) polymerase, and p27^{Kip1} from BD Biosciences PharMingen; I κ B α from Santa Cruz Biotechnology; trimethyl-K27 histone H3 and histone H3 from Upstate; Cu/Zn superoxide dismutase from Stressgen; and β -actin from Novus. Bound antibodies were detected as previously described.

Reverse transcription-PCR. Expression of mRNA of p27^{Kip1} and MYT1 was determined by reverse transcription-PCR. cDNA was generated from 2 μ g of total RNA by reverse transcription using a Reverse Transcription System Kit (Promega). To quantify the expression in each cDNA sample, the target was amplified by PCR in parallel with an internal control, glyceraldehyde-3-phosphate dehydrogenase. Increased expression of MYT1, EZH2 target gene, was used as a positive control of EZH2 depletion. The following primers were used: p27^{Kip1} upstream, 5'-AGGATGTC AGCGGGAGCCGG-3', and downstream, 5'-CTTCTGGGGCTCTGCTCCA-3'; MYT1 upstream, 5'-ATCCAGTCC-CAGCCTACTT-3' and downstream, 5'-GTCTCCCTCCTG GACCTCAC-3'. Primer pairs that detect glyceraldehyde-3-phosphate dehydrogenase were used as previously described (13). Amplification was done by using a thermal cycling program with varying numbers of cycles for p27^{Kip1} (22 cycles) and MYT1 (22 cycles) in which each cycle consisted of 94°C for 45 s, 58°C for 45 s, and 72°C for 1 min. Amplification for glyceraldehyde-3-phosphate dehydrogenase (22 cycles) was done following a cycling program, in which each cycle consisted of 94°C for 45 s, 50°C for 45 s, and 72°C for 1 min. All PCR products were subjected to electrophoresis through a native 8% polyacrylamide gel and were visualized by staining with ethidium bromide.

Chromatin immunoprecipitation assay. Panc04.03 cells were cross-linked with formaldehyde for 15 min at 25°C, harvested in SDS lysis buffer (Upstate Biotechnology), and sheared to fragment DNA (500-1,000 bp). Samples were then immunoprecipitated with an agarose-conjugated EZH2, trimethyl-histone H3 (Lys²⁷) antibody, acetyl-histone H3 (Lys¹⁴) antibody, or rabbit control IgG at 4°C overnight. Antibodies for chromatin immunoprecipitation analysis were obtained from the following suppliers: trimethyl-H3-K27 and acetyl-H4-K20 from Upstate Biotechnology, and EZH2 from BD PharMingen. Following immunoprecipitation, samples were washed and eluted using the Chromatin Immunoprecipitation Kit (Upstate Biotechnology) according to the manufacturer's instructions. Cross-links were removed at 65°C for 6 h, and immunoprecipitated DNA was purified by phenol/chloroform extraction and ethanol precipitation. In the immunoprecipitated samples, 212 bp of the p27^{Kip1} promoter and 154 bp of the p27^{Kip1} exon 1 were detected by PCR. PCR products were separated on a native 10% polyacrylamide gel and visualized by staining with ethidium bromide.

Statistical analysis. Data were analyzed using the Prism software package (GraphPad, Inc.). Associations between EZH2 nuclear accumulation and degree of tumor differentiation were analyzed by Fisher's exact test for 2 \times 2 contingency tables or by χ^2 test for larger tables. Two-sided tests were used. The χ^2 critical value for 0.05 probability level is 3.841; $\chi^2 > 3.841$ and $P < 0.05$ were considered to indicate statistical significance.

Results

EZH2 accumulates in the nuclei of pancreatic cancer cells. Using immunohistochemical staining for EZH2, we found weak cytoplasmic EZH2 expression in normal human pancreatic ductal and acinar cells (Fig. 1A). Nuclear accumulation of EZH2 was found in well-, moderately, and poorly differentiated adenocarcinomas in 11 of 20 (55%), 29 of 50 (58%), and 31 of 34 (91%) cases, respectively (Fig. 1B-F). EZH2 nuclear accumulation was significantly associated with dedifferentiation of tumors (χ^2 test for independence, 12.296; $P = 0.0021$). It was significantly more frequent in poorly differentiated

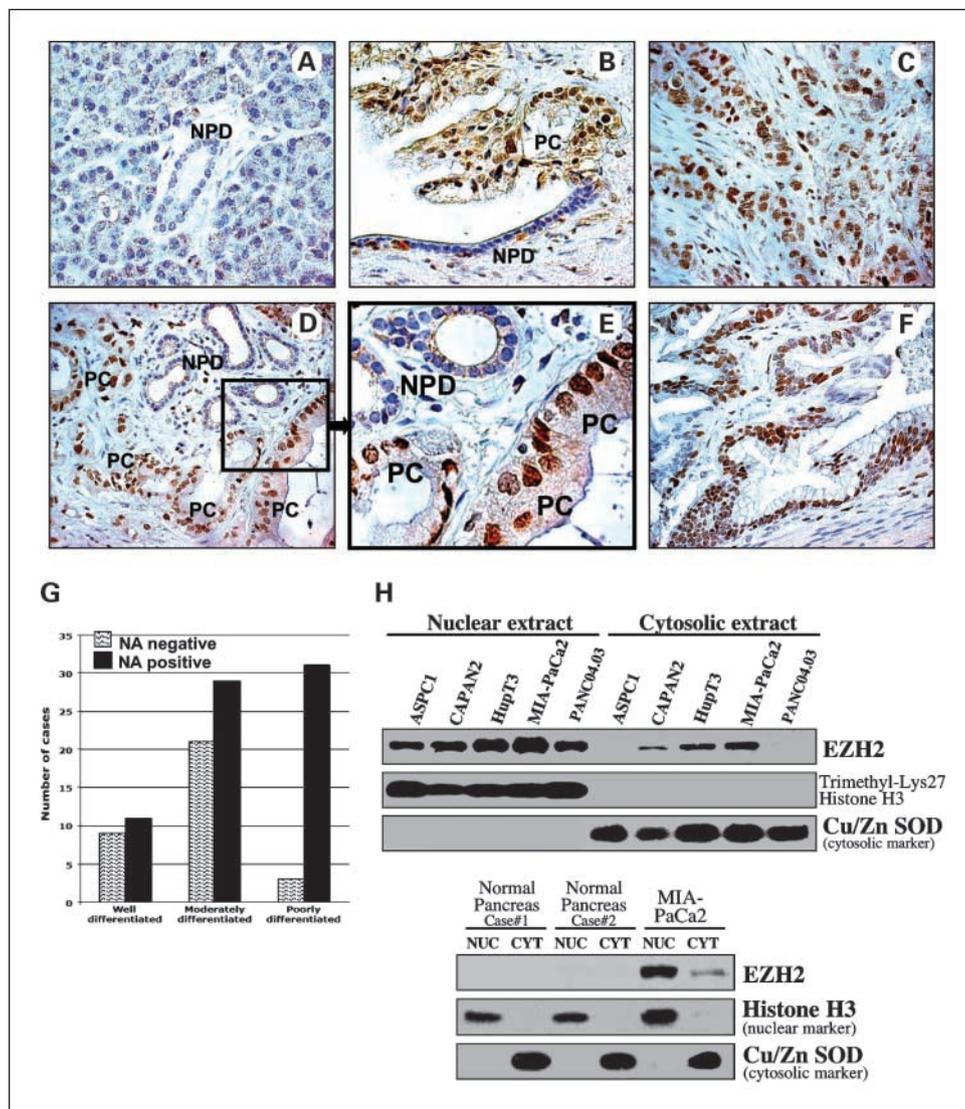


Fig. 1. EZH2 is overexpressed and accumulated in the nuclei of pancreatic cancer cells. *A* to *F*, immunohistochemical analysis of EZH2 expression and localization in normal human pancreas (*A*) and pancreatic adenocarcinoma specimens (*B*–*F*). *A*, normal pancreatic duct (*NPD*) is indicated. *B*, malignant pancreatic duct shows nuclear accumulation of EZH2, whereas adjacent normal pancreatic ductal cells show no nuclear EZH2 staining. *PC*, pancreatic cancer cells. *C*, EZH2 nuclear accumulation in a poorly differentiated pancreatic adenocarcinoma. *D*, nuclear accumulation of EZH2 found in cancer cells of moderately differentiated pancreatic adenocarcinoma but not in adjacent normal pancreatic ductal cells. *E*, higher magnification of the delineated inset in *D*. *F*, nuclear accumulation of EZH2 in cancer cells of well-differentiated pancreatic adenocarcinoma. *G*, distribution of EZH2 staining patterns in pancreatic carcinomas. NA, nuclear accumulation. *H*, equivalent amounts (50 μ g) of nuclear and cytosolic proteins isolated from the indicated pancreatic cancer cell lines and normal human pancreas tissue were separated by SDS-PAGE and immunoblotted.

adenocarcinomas [Fig. 1G; $P = 0.0003$; odds ratio, 7.750 (95% confidence interval, 2.162–27.775); relative risk, 1.614 (95% confidence interval, 1.280–2.034)]. Our results suggest that nuclear accumulation of EZH2 is associated with the loss of pancreatic cancer differentiation.

Using nuclear/cytosolic fractionation, we found nuclear expression of EZH2 in pancreatic cancer cell lines ASPC1, CAPAN2, HupT3, MIA-PaCa2, and Panc04.03 (Fig. 1H, top). In contrast to these findings, EZH2 expression was not detected in normal human pancreatic cells (Fig. 1H, bottom). Thus, nuclear overexpression of EZH2 seems to be a feature of pancreatic cancer cells.

Suppression of EZH2 inhibits pancreatic cancer cell proliferation. Our data identify overexpression of EZH2 in pancreatic cancer cells, but its role in regulating pancreatic cancer cell proliferation and survival is unknown. To address this question, we depleted EZH2 using short hairpin RNA interference (Fig. 2A) and assayed the ability of cells to proliferate using a well-established colorimetric technique [3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium assay] and trypan blue staining. We

observe that transfection of an EZH2-targeting construct (shEZH2) into MIA-PaCa2 and Panc04.03 pancreatic cancer cells resulted in decreased proliferation in these pancreatic tumor cell lines as compared with vector control (shVector)-transfected cells (Fig. 2B and C). We did not observe an increased apoptosis in EZH2-depleted cancer cells (data not shown). These results suggest that EZH2 participates in the regulation of signaling cascades that positively influence pancreatic cancer cell proliferation.

Depletion of EZH2 leads to reexpression of the p27^{Kip1} tumor suppressor gene in pancreatic cancer cells. Using reverse transcription-PCR and immunoblotting, we screened for tumor suppressor genes, which might be reexpressed on depletion of EZH2 in pancreatic cancer cells. Because EZH2 suppression leads to decreased pancreatic cancer cell proliferation, we analyzed the expression levels of the cyclin-dependent kinase inhibitors p15, p16, p21, p27, and p57 in pancreatic cancer cells, as well as the known EZH2 target gene MYT1 as a control for EZH2 suppression. Suppression of EZH2 in MIA-PaCa2 and Panc04.03 resulted in an increase in MYT1 gene expression consistent with the known role of EZH2 in the repression of

MYT1 gene transcription. However, although EZH2 depletion resulted in the reexpression of multiple cyclin-dependent kinase inhibitor genes in several pancreatic cancer cell lines tested (data not shown), only p27^{Kip1} was uniformly and significantly reexpressed at both the transcriptional and protein levels after depletion of EZH2 in all of the pancreatic cancer cell lines examined (Fig. 3A and B, and data not shown). Whereas it has been suggested that loss of p27^{Kip1} protein in human cancer is not related to its gene alterations but mainly resulting from increased degradation of the p27^{Kip1} protein (15, 16), we found that treatment of pancreatic cancer cells with the proteasome inhibitor MG132 did not increase the level of p27^{Kip1} protein expression (Fig. 3C). Our results identify the tumor suppressor p27^{Kip1} as a new target gene of EZH2 in human pancreatic cancer.

EZH2 depletion affects repressive chromatin status, leading to reexpression of p27^{Kip1} in pancreatic cancer cells. EZH2-mediated recruitment of DNA methyltransferases to target gene promoters is one mechanism by which EZH2 can repress gene transcription (4). To investigate whether p27^{Kip1} may be inactivated by DNA hypermethylation through EZH2-mediated recruitment of DNA methyltransferases to the p27^{Kip1} gene in pancreatic cancer cells, we examined the CpG islands located in both the promoter and exon 1 of the p27^{Kip1} gene for hypermethylation by methylation-specific PCR. We did not detect hypermethylation at the p27^{Kip1} promoter or exon 1 in Panc04.03 cancer cells (data not shown), suggesting that reexpression of p27^{Kip1} in EZH2-depleted pancreatic cancer cells is not dependent on the DNA methylation status of the p27^{Kip1} promoter or exon 1.

Opening of chromatin to allow transcription factors to gain access to gene promoters and regulate gene transcription is one of the major functions of histone modifications (i.e., phosphorylation, acetylation, and methylation; ref. 2). PcG complexes containing the histone methyltransferase EZH2 silence chromatin via methylation of histone H3-K27 (3). Methylation H3-K27 is a known feature associated with repressive chromatin status and epigenetic gene silencing (3). To evaluate the possibility that suppression of EZH2 alters its ability to silence the p27^{Kip1} gene through transcriptional repression, we used chromatin immunoprecipitation to assess EZH2 binding and H3-K27 methylation at the p27^{Kip1} promoter and exon 1 in Panc04.03 cancer cells transfected with the shEZH2 or control vector. We found that EZH2 is bound to p27^{Kip1} at exon 1, but not within the p27^{Kip1} promoter region (Fig. 3D). Consistently, EZH2-depleted cancer cells showed a significant decrease in methylation of H3-K27 bound to exon 1 of p27^{Kip1}, but not to its promoter (Fig. 3E, top). It has also been shown that PcG (EED/EZH2) mediated the repression of gene activity by interacting with histone deacetylases (17). Acetylation of H3-K14 is associated with open, actively transcribed genomic regions (18). We found that EZH2-depleted cancer cells show increased acetylation of H3-K14, a marker of active transcription, at exon 1 of p27^{Kip1} (Fig. 3E, bottom). These results suggest that EZH2 regulates p27^{Kip1} expression through the maintenance of inactive chromatin at the p27^{Kip1} gene.

Nuclear overexpression of EZH2 is significantly associated with loss of p27 expression in human pancreatic adenocarcinomas. To test the hypothesis whether EZH2 nuclear overexpression leads to loss of p27^{Kip1} expression in human pancreatic tumors *in vivo*, we have done immunohistochemical analysis of EZH2

and p27^{Kip1} expression in a series of human pancreatic adenocarcinomas. Using a pair of serial sections, we detected nuclear accumulation of EZH2 and loss of p27^{Kip1} expression in pancreatic cancer cells, whereas nuclear accumulation of p27^{Kip1}, but not EZH2, was observed in benign pancreatic lesions (Fig. 3F and G). We found aberrant EZH2 nuclear accumulation and loss of p27^{Kip1} expression in 61% and 67% of pancreatic carcinomas, respectively (Fig. 3F and G). Loss of p27^{Kip1} expression was more frequently found in tumors with nuclear accumulation of EZH2 ($P < 0.001$; Fig. 3F and G) than in tumors without nuclear accumulation of EZH2. Moreover, simultaneous EZH2 nuclear accumulation and loss of p27^{Kip1} expression are associated with the loss of pancreatic cancer differentiation ($P < 0.001$). Consistent with our *in vitro* data, our *in vivo* results show that aberrant nuclear overexpression of EZH2 is significantly associated with loss of p27^{Kip1} expression in human pancreatic cancer cells.

EZH2 depletion affects pancreatic cancer chemoresistance. Pancreatic cancer is less chemosensitive than other common solid malignancies, with partial response to chemotherapy of <10% (19). The role of chemotherapy pancreatic cancer treatment is limited because pancreatic cancer rapidly develops a chemoresistance leading to fatal patient outcome (19, 20). With any chemotherapy regimen, average survival rates are between 3 and 6 months (20). Thus, a better understanding of tumor chemoresistance enables the discovery of novel targets to

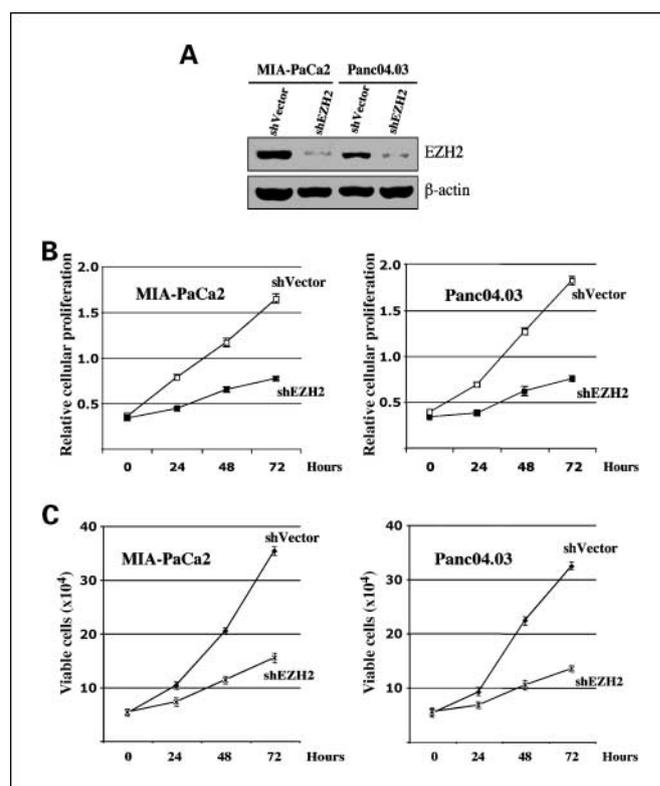


Fig. 2. Suppression of EZH2 inhibits pancreatic cancer cell proliferation. MIA-PaCa2 and Panc04.03 pancreatic cancer cells were transfected with a control vector (shVector) or the shEZH2 silencing vector (shEZH2). **A**, at 48 h posttransfection, the cell pellet was collected and protein was obtained. Cell lysates were separated by SDS-PAGE, transferred onto PVDF membrane, and probed with antibodies to indicated proteins. **B**, relative cell viability was measured by colorimetric 3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay at the indicated times as described (13). **C**, numbers of viable cells were counted by the trypan blue exclusion assay as described (30).

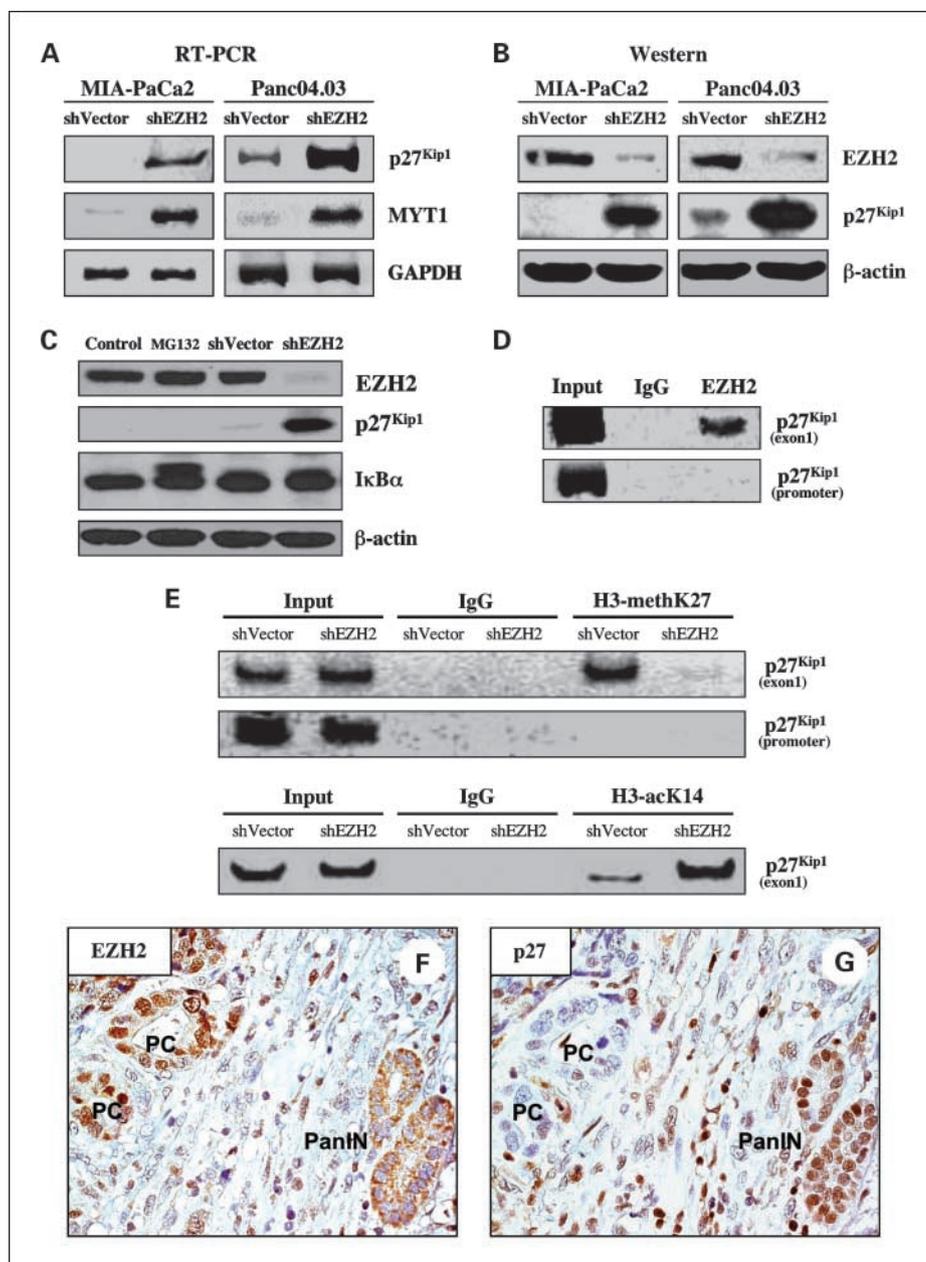


Fig. 3. Depletion of EZH2 leads to reexpression of p27^{Kip1} in pancreatic cancer cells. MIA-PaCa2 and Panc04.03 pancreatic cancer cells were transfected with a control vector or the shEZH2 silencing vector. **A**, at 48 h posttransfection, the cell pellet was collected; mRNA was obtained; and reverse transcription-PCR was done, as described in Materials and Methods. Increased expression of MYT1, a known EZH2 target gene, was used as a positive control. **B**, at 48 h posttransfection, the cell pellet was collected and protein was obtained. Cell lysates were separated by SDS-PAGE, transferred onto PVDF membrane, and probed with antibodies to indicated proteins. **C**, at 48 h posttransfection, Panc04.03 cell pellet was collected. Panc04.03 cancer cells were treated with DMSO (control) or MG132 (10 μ mol/L) for 6 h. Cell lysates were prepared, separated by SDS-PAGE (50 μ g/well), transferred onto PVDF membrane, and immunoblotted as indicated. An increase in I κ B α expression was used as a positive marker of MG132 treatment. **D**, binding of EZH2 to the promoter or exon 1 of p27^{Kip1} gene was assayed in Panc04.03 cancer cells by chromatin immunoprecipitation. **E**, Panc04.03 cancer cells were transfected with a control vector or the shEZH2 silencing vector. At 48 h posttransfection, genomic chromatin fragments were immunoprecipitated with trimethyl-H3-K27 and acetyl-H3-K14 at the p27^{Kip1} promoter or exon 1. PCR analysis on input chromatin (*first two lanes*) confirmed that equal chromatin amounts were used for chromatin immunoprecipitation. **F** and **G**, immunohistochemical staining of a pair of serial sections from the same pancreatic tumor shows nuclear localization of EZH2 (**F**) and loss of p27^{Kip1} expression (**G**) in the same cancer cells, whereas absence of EZH2 nuclear staining but p27^{Kip1} nuclear accumulation has been observed in benign pancreatic lesions (**G** and **F**). PanIN, pancreatic intraepithelial neoplasia.

develop a superior pancreatic cancer therapy. Gemcitabine, a deoxycytidine nucleoside analogue, is currently the standard chemotherapeutic agent used in the treatment of metastatic pancreatic cancer (20). Doxorubicin, an anthracycline antibiotic, has been used as a chemotherapeutic agent in a variety of carcinomas including pancreatic cancer. Interestingly, we found that treatment of Panc04.03 and SU86.86 cells with either doxorubicin or gemcitabine induces the nuclear accumulation of EZH2 in pancreatic cancer cells (Fig. 4A). Moreover, although treatment of pancreatic cancer cells with doxorubicin (1 μ mol/L) or gemcitabine (100 nmol/L) did not induce apoptosis in control transfected cells, EZH2 depletion sensitized cancer cells to doxorubicin and gemcitabine, leading to a significant increase in apoptosis of pancreatic cancer cells (Fig. 4B and C). Our results suggest that nuclear overexpression of EZH2 might participate in pancreatic cancer

chemoresistance and that the combination of EZH2 inhibitors with gemcitabine or doxorubicin could be superior to conventional therapy for pancreatic cancer.

Discussion

Epigenetic silencing of tumor suppressor genes can prevent programmed cell death and lead to uncontrolled proliferation in human tumors (2, 18). Recent studies implicate EZH2, a member of the PcG regulators of gene activity, in the pathogenesis of human cancer, including prostate (9), breast (10), gastric (21), and bladder (22) carcinomas, whereas the role of EZH2 in pancreatic cancer remains unknown.

In the present study, we show aberrant nuclear overexpression of EZH2 in pancreatic cancer cell lines and in 71 of 104 (68%) cases of human pancreatic adenocarcinomas, thus

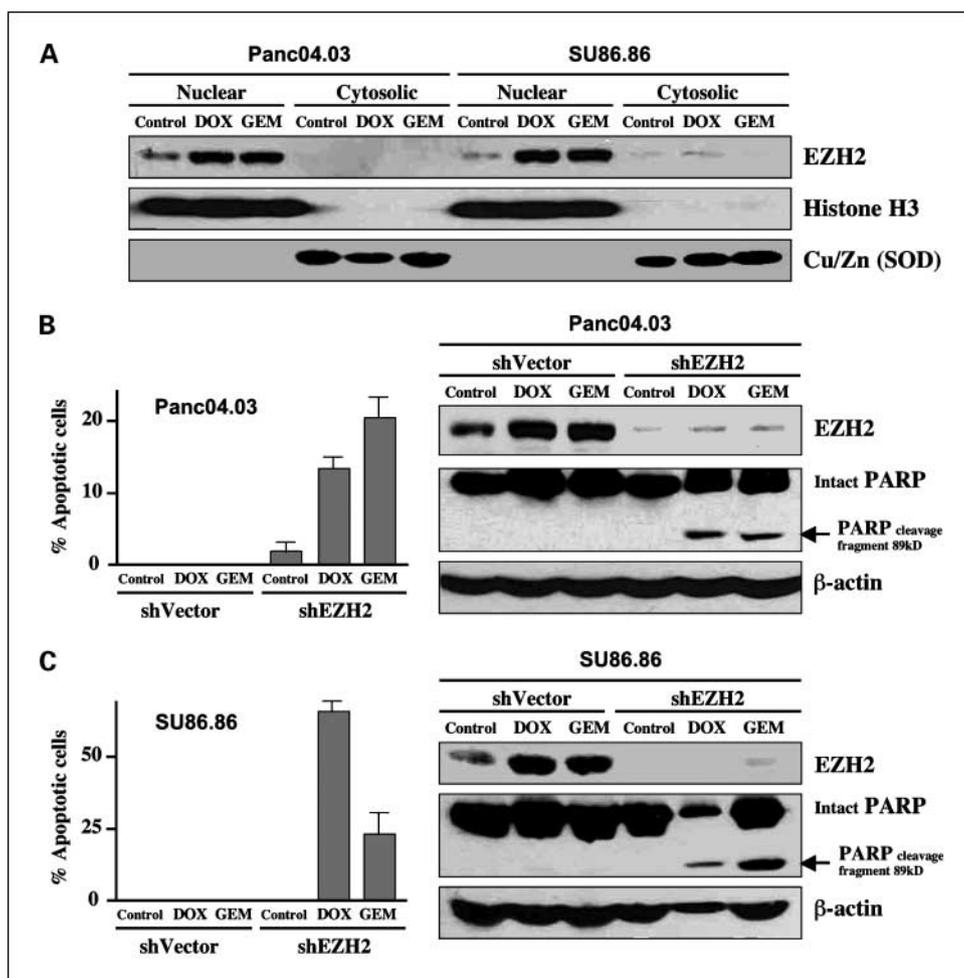
identifying EZH2 as a potential oncomarker in pancreatic cancer. We find that EZH2 nuclear accumulation is strongly associated with poorly differentiated pancreatic adenocarcinoma, suggesting EZH2 as one of the molecular determinants of pancreatic tumor dedifferentiation. Our findings are supported by other studies showing overexpression of EZH2 in advanced prostate (9) and breast (10) cancers, whereas nuclear accumulation of EZH2 was more frequently found in poorly differentiated breast carcinomas (23).

We identify EZH2 as a positive regulator of pancreatic cancer cell proliferation. We find that EZH2 depletion inhibits the proliferation, but not the survival, of pancreatic cancer cells. Our data are in agreement with another study showing that EZH2 knockdown in prostate cancer cells results in decreased proliferation with no effect on cancer cell survival (9). A previous study has also shown that EZH2 contributes to the proliferation of breast cancer cells (23). Our findings of EZH2 nuclear accumulation in most cases (31 of 34) of poorly differentiated pancreatic adenocarcinoma, a highly proliferating tumor fraction, support the hypothesis that EZH2 could play an important role in pancreatic cancer proliferation.

However, it is still unclear how EZH2 controls cancer cell proliferation. To address this question, we performed screening of numerous tumor suppressor genes, which might be reexpressed on depletion of EZH2 in pancreatic cancer cells. We identified the p27^{Kip1} tumor suppressor as a novel EZH2-

regulated gene. We are unaware of any previous report that has assessed the role of EZH2 in the regulation of p27^{Kip1} expression. The cyclin-dependent kinase inhibitor p27^{Kip1} is a tumor suppressor that regulates cell cycle by binding to cyclin-dependent kinases (24). Reduced p27^{Kip1} expression is correlated with poor disease outcome in cases of lung, colon, prostate, ovary, and breast carcinomas (25). A decrease or loss of p27^{Kip1} expression is frequently observed in human pancreatic cancer (26). Although p27^{Kip1} expression seems to be an important prognostic factor in numerous human malignancies, it has been suggested that loss of p27^{Kip1} protein is not related to its gene alterations but mainly resulting from increased degradation of the p27^{Kip1} protein (15, 16). However, we found that treatment of pancreatic cancer cells with the proteasome inhibitor MG132 did not increase the level of p27^{Kip1} protein expression, whereas p27^{Kip1} expression was significantly up-regulated in EZH2-depleted pancreatic cancer cells at both the transcriptional and protein levels. Our findings suggest that EZH2 nuclear overexpression contributes to epigenetic silencing of p27^{Kip1} gene that leads to down-regulation of p27^{Kip1} mRNA and protein levels in pancreatic cancer cells. To confirm our *in vitro* results showing reexpression of p27^{Kip1} in EZH2-depleted cancer cells, we show *in vivo* that EZH2 nuclear accumulation is significantly associated with loss of p27^{Kip1} expression in human pancreatic adenocarcinomas. These results support a speculation that EZH2 might

Fig. 4. EZH2 depletion affects pancreatic cancer chemoresistance. **A**, Panc04.03 and SU86.86 pancreatic cancer cells were treated with DW (control), 1 μ mol/L doxorubicin (DOX), or 100 nmol/L gemcitabine (GEM) for 24 h. Nuclear and cytosolic fractions were prepared, separated by SDS-PAGE (50 μ g/well), transferred onto PVDF membrane, and probed with the indicated antibodies. **B** and **C**, Panc04.03 (**B**) and SU86.86 (**C**) pancreatic cancer cells were treated with DW (control), doxorubicin (1 μ mol/L), or gemcitabine (100 nmol/L) for 48 h. Whole cell lysates were prepared, separated by SDS-PAGE (50 μ g/well), transferred onto PVDF membrane, and immunoblotted as indicated. The percentage of apoptotic cells was determined by Hoechst staining as previously described (31). Columns, mean; bars, SD.



contribute to pancreatic cancer cell proliferation by epigenetic silencing of tumor suppressor genes, in part through suppression of p27^{Kip1}. Whether EZH2 could suppress p27^{Kip1} expression in a similar manner in other human malignancies remains to be determined.

Pancreatic cancer is one of the most drug-resistant tumors (19, 20). Our results identify EZH2 as an important factor of pancreatic cancer cell chemoresistance. We found that EZH2 depletion sensitized cancer cells to doxorubicin and gemcitabine, leading to a significant decrease in survival of pancreatic cancer cells. Our findings are supported by another study showing that loss of EZH2-mediated methylation of H3-K27 resensitizes ovarian cancer cells to cisplatin (7). It has also been shown that trimethylation of H3-K27 is an important prognostic indicator for clinical outcome in patients with pancreatic cancer (27). Increased sensitivity of EZH2-depleted pancreatic cancer cells to doxorubicin and gemcitabine seems to be mediated, in part, by changes in gene expression. We found that EZH2 down-regulates the expression of p27^{Kip1} tumor suppressor in pancreatic cancer cells. Although the speculative role of p27^{Kip1} in pancreatic cancer chemoresistance remains to be investigated,

reduced p27^{Kip1} correlates with poor survival after platinum-based chemotherapy for non-small-cell lung and ovarian carcinomas (28, 29). Because EZH2-mediated H3-K27 methylation is a mark of heterochromatin, it is also possible that loss of chromatin compaction could allow increased DNA damage at lower doxorubicin and gemcitabine doses, leading to a decreased survival in EZH2-depleted pancreatic cancer cells.

In summary, our study provides novel information on the mechanisms underlying the growth and chemoresistance of pancreatic cancer, and urges a demand for the development of pharmacologic inhibitor of EZH2 as a potential anticancer agent for the chemotherapy of pancreatic cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Drs. Jann Sarkaria and Gasper Kitange for their help with methylation analysis, and Darren Riehle for tissue microarray data acquisition.

References

- Jaffee EM, Hruban RH, Canto M, Kern SE. Focus on pancreas cancer. *Cancer Cell* 2002;2:25–8.
- Santos-Rosa H, Caldas C. Chromatin modifier enzymes, the histone code and cancer. *Eur J Cancer* 2005;41:2381–402.
- Cao R, Wang L, Wang H, et al. Role of histone H3 lysine 27 methylation in Polycomb-group silencing. *Science* 2002;298:1039–43.
- Viré E, Brenner C, Deplus R, et al. The Polycomb group protein EZH2 directly controls DNA methylation. *Nature* 2006;439:871–4.
- Cardoso C, Mignon C, Hetet G, Grandchamps B, Fontes M, Colleaux L. The human EZH2 gene: genomic organisation and revised mapping in 7q35 within the critical region for malignant myeloid disorders. *Eur J Hum Genet* 2000;8:174–80.
- Visser HP, Gunster MJ, Kluijn-Nelemans HC, et al. The Polycomb group protein EZH2 is up-regulated in proliferating, cultured human mantle cell lymphoma. *Br J Haematol* 2001;112:950–8.
- Abbosh PH, Montgomery JS, Starkey JA, et al. Dominant-negative histone H3 lysine 27 mutant derepresses silenced tumor suppressor genes and reverses the drug-resistant phenotype in cancer cells. *Cancer Res* 2006;66:5582–91.
- Fujii S, Ochiai A. Enhancer of zeste homolog 2 down-regulates E-cadherin by mediating histone H3 methylation in gastric cancer cells. *Cancer Sci* 2008;99:738–46.
- Varambally S, Dhanasekaran SM, Zhou M, et al. The polycomb group protein EZH2 is involved in progression of prostate cancer. *Nature* 2002;419:624–9.
- Kleer CG, Cao Q, Varambally S, et al. EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells. *Proc Natl Acad Sci U S A* 2003;100:11606–11.
- Zakaria S, Gomes TS, Savoy DN, et al. Differential regulation of TCR-mediated gene transcription by Vav family members. *J Exp Med* 2004;199:429–34.
- Ougolkov A, Zhang B, Yamashita K, et al. Associations among β -TrCP, an E3 ubiquitin ligase receptor, β -catenin, and NF- κ B in colorectal cancer. *J Natl Cancer Inst* 2004;96:1161–70.
- Ougolkov AV, Fernandez-Zapico ME, Savoy DN, Urrutia RA, and Billadeau DD. Glycogen synthase kinase-3 β participates in nuclear factor- κ B-mediated gene transcription and cell survival in pancreatic cancer cells. *Cancer Res* 2005;65:2076–81.
- Dignam JD, Lebovitz RM, and Roeder RG. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res* 1983;11:1475–89.
- Loda M, Cukor B, Tam S, et al. Increased proteasome-dependent degradation of the cyclin-dependent kinase inhibitor p27 in aggressive colorectal carcinomas. *Nat Med* 1997;3:231–4.
- Pagano M, Tam SW, Theodoras AM, et al. Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. *Science* 1995;269:682–5.
- van der Vlag J, Otte AP. Transcriptional repression mediated by the human polycomb-group protein EED involves histone deacetylation. *Nat Genet* 1999;23:474–8.
- Sparmann A, van Lohuizen M. Polycomb silencers control cell fate, development and cancer. *Nat Rev Cancer* 2006;6:846–56.
- Kollmannsberger C, Peters HD, Fink U. Chemotherapy in advanced pancreatic adenocarcinoma. *Cancer Treat Rev* 1998;24:133–56.
- Casper ES, Kelsen DP. Adenocarcinoma of the pancreas: overview of workup and management. *Adv Oncol* 1996;11:17–22.
- Matsukawa Y, Semba S, Kato H, Ito A, Yanagihara K, Yokozaki H. Expression of the enhancer of zeste homolog 2 is correlated with poor prognosis in human gastric cancer. *Cancer Sci* 2006;97:484–91.
- Raman JD, Mongan NP, Tickoo SK, Boorjian SA, Scherr DS, Gudas LJ. Increased expression of the polycomb group gene, EZH2, in transitional cell carcinoma of the bladder. *Clin Cancer Res* 2005;11:8570–6.
- Raaphorst FM, Meijer CJ, Fieret E, et al. Poorly differentiated breast carcinoma is associated with increased expression of the human polycomb group EZH2 gene. *Neoplasia* 2003;5:481–8.
- Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G₁-phase progression. *Genes Dev* 1999;13:1501–12.
- Chu IM, Hengst L, Slingerland JM. The Cdk inhibitor p27 in human cancer: prognostic potential and relevance to anticancer therapy. *Nat Rev Cancer* 2008;8:253–67.
- Hu YX, Watanabe H, Li P, et al. An immunohistochemical analysis of p27 expression in human pancreatic carcinomas. *Pancreas* 2000;21:226–30.
- Wei Y, Xia W, Zhang Z, et al. Loss of trimethylation at lysine 27 of histone H3 is a predictor of poor outcome in breast, ovarian, and pancreatic cancers. *Mol Carcinog* 2008;47:701–6.
- Oshita F, Kameda Y, Nishio K, et al. Increased expression levels of cyclin-dependent kinase inhibitor p27 correlate with good responses to platinum-based chemotherapy in non-small cell lung cancer. *Oncol Rep* 2000;7:491–5.
- Masciullo V, Ferrandina G, Pucci B, et al. p27Kip1 expression is associated with clinical outcome in advanced epithelial ovarian cancer: multivariate analysis. *Clin Cancer Res* 2000;6:4816–22.
- Axanova L, Morré DJ, Morré DM. Growth of LNCaP cells in monoculture and coculture with osteoblasts and response to tNOX inhibitors. *Cancer Lett* 2005;225:35.
- Armstrong DK, Kaufmann SH, Ottaviano YL, et al. Epidermal growth factor-mediated apoptosis of MDA-MB-468 human breast cancer cells. *Cancer Res* 1994;54:5280–3.

Clinical Cancer Research

Regulation of Pancreatic Tumor Cell Proliferation and Chemoresistance by the Histone Methyltransferase Enhancer of Zeste Homologue 2

Andrei V. Ougolkov, Vladimir N. Bilim and Daniel D. Billadeau

Clin Cancer Res 2008;14:6790-6796.

Updated version Access the most recent version of this article at:
<http://clincancerres.aacrjournals.org/content/14/21/6790>

Cited articles This article cites 31 articles, 10 of which you can access for free at:
<http://clincancerres.aacrjournals.org/content/14/21/6790.full#ref-list-1>

Citing articles This article has been cited by 8 HighWire-hosted articles. Access the articles at:
<http://clincancerres.aacrjournals.org/content/14/21/6790.full#related-urls>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link
<http://clincancerres.aacrjournals.org/content/14/21/6790>.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.