

# Protein Arginine Methyltransferase 5 Accelerates Tumor Growth by Arginine Methylation of the Tumor Suppressor Programmed Cell Death 4

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## Abstract

Programmed cell death 4 (PDCD4) has been described as a tumor suppressor, with high expression correlating with better outcomes in a number of cancer types. Yet a substantial number of cancer patients with high *PDCD4* in tumors have poor survival, suggesting that oncogenic pathways may inhibit or change PDCD4 function. Here, we explore the significance of PDCD4 in breast cancer and identify protein arginine methyltransferase 5 (PRMT5) as a cofactor that radically alters PDCD4 function. Specifically, we find that coexpression of PDCD4 and PRMT5 in an orthotopic model of breast cancer causes accelerated tumor growth and that this growth phenotype is dependent on both the catalytic activity of PRMT5 and a site of methylation within the N-terminal region of PDCD4. In agreement with the xenograft model, elevated *PDCD4* expression was found to correlate with worse outcome within the cohort of breast cancer patients whose tumors contain higher levels of *PRMT5*. These results reveal a new cofactor for PDCD4 that alters its tumor suppressor functions and point to the utility of PDCD4/PRMT5 status as both a prognostic biomarker and a potential target for chemotherapy. *Cancer Res*; 71(16); 5579–87. ©2011 AACR.

## Introduction

Although an active area of research in which significant advances have been achieved, breast cancer is a leading cause of mortality among women (1). Breast cancer is a heterogeneous disease that can be difficult to stratify into distinct categories with precise outcomes, especially as improved detection methods allow for diagnosis of early subclinical disease (2). The dilemma of unknown/unquantifiable risk assessments can lead to overtreatment, accompanied by unwanted side effects, or undertreatment, which risks increased recurrence (3). Increasingly, biomarkers, such as hormone receptors, are used to enhance outcome predictions and refine treatment plans. Expanding the arsenal of prognostic biomarkers will help reduce guesswork in treatment plans, leading to better overall survival and improved quality of life.

Programmed cell death 4 (PDCD4) is a promising biomarker that correlates with better outcomes in lung, colon, ovarian,

and esophageal cancer (4–7). PDCD4 is expressed at lower levels in invasive breast carcinoma compared with ductal carcinoma *in situ* or normal samples, indicating there may be loss of PDCD4 during disease progression (8). Experimental models substantiate a tumor suppressor role for PDCD4. In a mouse epithelial cell line, PDCD4 expression reduced phorbol ester-induced transformation (9) and, in transformed cells, PDCD4 expression suppressed anchorage-independent cell growth (10). Transgenic mice expressing epidermal PDCD4 were resistant to chemically induced skin tumors (11), whereas PDCD4 knockout mice developed B-cell lymphoma (12).

Here, we find that *PDCD4* expression in breast cancer correlates with better survival. Yet, as with many biomarkers, there are limitations in using PDCD4 to predict outcome: subsets of patients whose tumors contain elevated *PDCD4* mRNA still experienced poor clinical outcome, indicating the presence of mechanisms that abrogate or change PDCD4 function. Pursuing such a mechanism, we have found a novel PDCD4-interacting partner, PRMT5, which posttranslationally methylates PDCD4. Together, these proteins cause a progrowth tumor phenotype in an orthotopic breast cancer model. Moreover, we have found that *PRMT5* levels are significant in determining long-term survival in *PDCD4*-upregulated breast cancer.

## Materials and Methods

### Constructs

Open reading frames for human PDCD4, PRMT5, and the PDCD4 truncations were cloned into pDONR221. Catalytically dead PRMT5 (PRMT5<sub>cd</sub>) was created by mutation of amino acids glycine 367 and arginine 368 to alanines (13). PDCD4,

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PDCD4 point mutants, and truncations were cloned into pGEX-4T. PRMT5 and PRMT5<sub>cd</sub> were cloned into the pcDNA3.1 nV5/DEST (Invitrogen). PDCD4 and PDCD4<sub>mm</sub> were cloned into the pMIG vector (Addgene plasmid 9044, William Hahn); PRMT5 and PRMT5<sub>cd</sub> were cloned into pMSCVpuro (Clontech) vector.

### Recombinant protein production

BL21/RIL cells (Novagen) transduced with PDCD4-pGEX4T or parental vector were induced by using 0.1 mmol/L isopropyl-1-thio-B-D[*r*]-galactopyranoside for 1 to 3 hours. Pellets were resuspended and sonicated in 1× PBS, 0.4 mmol/L phenylmethylsulfonyl fluoride (PMSF), 5 µg/mL leupeptin and aprotinin, and 0.1% deoxycholate. GST proteins were purified following standard protocol.

### Recombinant PDCD4 pull-downs in cell lysates

A total of 2 to 10 µg of GST proteins were incubated with glutathione resin (GE Healthcare) in binding buffer (20 mmol/L HEPES pH 7.6, 100 mmol/L KCl, 0.5 mmol/L EDTA, 0.25% TritonX100, 20% glycerol) at room temperature for 1 hour. GST–glutathione conjugates were washed and incubated with 100 to 300 µg of cell lysate for 1 hour at room temperature. Pull-downs assessing the eIF4A1 interaction and the pleural effusion samples used methylation buffer (1 mmol/L DTT, 0.25% Triton X-100, 5% glycerol in 1× PBS).

### Preparation of ecotropic retrovirus and MCF7e infections

To prepare virus, HEK293T cells were cotransfected with pCL-Eco plasmid and pMIG or pMSCVpuro in a 1:3 ratio. One day after transfection, the medium was exchanged with fresh media. Medium was collected 48 hours after transfection and filtered through a 0.45-micron filter. Virus was diluted 1:4 into MCF7 media (DME:F12 1:1, 10% FBS, 10 mg/L insulin) and mixed with polybrene (8 µg/mL). This virus mixture was overlaid on 20% to 50% confluent MCF7e cells, which stably express an ecotropic receptor (a kind gift from Dr. Alex Swarbrick, Garvan Institute, Sydney, Australia). Virus media was exchanged for MCF7 media at 24 hours. pMIG-transduced cells were selected by fluorescence-activated cell sorting for green fluorescent protein (GFP)-positive cells, and pMSCVpuro-transduced cells were selected for 2 weeks with 2 µg/mL puromycin. All generated cell lines were found to be insulin independent and subcultured in DME:F12 1:1, 10% FBS.

### Transplantation of transduced MCF7e cells and tumor growth

A total of  $1 \times 10^6$  cells were diluted into 10 µL of Matrigel (BD Bioscience) and kept on ice. Three-week-old recipient nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice were injected with the cell suspension as described (14). Thirty-milligram beeswax pellets containing 15 µg of β-estradiol were implanted s.c. into the interscapular region of recipient mice during the same surgical procedure. Tumors were measured with calipers at 14- to 21-day intervals. Mice were sacrificed by CO<sub>2</sub> asphyxiation and visible tumors collected. For immunoblots, tumor tissue was homogenized in

methylation buffer plus 5 µg/mL leupeptin and aprotinin and 400 µmol/L PMSF. Lysates were clarified by centrifugation at  $20 \text{ k} \times g$  for 15 minutes at 4°C and resulting supernatants were separated by PAGE. Upon sacrifice of mice, lungs, spleen, and liver were dissected and visually inspected for macrometastases. Micrometastases were assessed by detection of GFP-positive cells in tissues by using an Olympus MVX10 dissecting scope with a UV light source.

### V5-immunoprecipitation

HEK293 cells transfected with catalytically active or dead PRMT5 pcDNA3.1 nV5/EXP vectors were lysed in methylation buffer and incubated with V5 antibody (Invitrogen) conjugated to protein A beads. One milligram of lysate was incubated with prebound beads for a minimum of 2 hours at 4°C. Immunoprecipitations were washed 3 times with methylation buffer prior to use in further reactions.

### Human pleural effusion lysate preparation

Pleural effusion samples were collected from consented breast cancer patients under an approved Institutional Review Board protocol. Cells were pelleted from the pleural fluid, and if red blood cells were present, the pellet was resuspended in 135 mmol/L NH<sub>4</sub>Cl, 17 mmol/L Tris pH 7.4 and incubated at 37°C for 5 minutes to remove red blood cells. The remaining cells were pelleted, washed twice with HBSS, resuspended in freezing medium, and stored in liquid nitrogen. Cells were thawed on ice and lysed by dounce homogenization in methylation buffer with protease inhibitors.

### Methyltransferase reactions

A total of 0.5 to 5 µg of GST-tagged protein was incubated with pleural effusion lysate, HEK293 cell lysate, or immunoprecipitated V5-PRMT5 in methylation buffer and 0.5 to 2 µCi <sup>3</sup>H-SAM (Perkin Elmer). Reactions were incubated at 30°C for 1 hour and terminated by addition of SDS sample buffer. Tritiated proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride. For autoradiography, blots were treated with En3Hance Spray (Perkin Elmer) according to manufacturer's recommendations and exposed to film at –80°C for 6 hours to 5 days.

### Antibodies

For immunoblots, α-PDCD4 antibody (Abcam) was used at 1:2,000, whereas α-PRMT5 antibody (Abcam) was used at 1:1,000 and cell lysates were loaded at 2 to 5 µg protein per lane. Anti-V5 antibody (2F11F7; Invitrogen) was used at 1:2,000, antitubulin (YL1/2, Accurate chemical and Scientific Corp.) at 1:2,000, and anti-β-actin (AC-74, Sigma) at 1:2,000 for immunoblots.

### Gene expression and statistical analysis

Gene expression and clinical outcome information were obtained from 2 independent publicly available datasets (15–17). Clinical outcomes from the study by Pawitan and colleagues (16) were obtained from data published in the study by Ivshina and colleagues (17). Data for PRMT5 and PDCD4 were extracted from normalized expression data for each breast tumor sample, and patients were divided into groups on the

basis of the expression of the 2 genes. Each dataset was analyzed separately (see further information in Supplementary Material). Kaplan–Meier survival curves were generated with WINSTAT FOR EXCEL software (R. Fitch Software), and  $P$  values were calculated by log-rank analysis. Values of  $P < 0.05$  were considered significant.

### Cell lines

MCF7e cells were a kind gift of Alex Swarbrick (Garvan Institute, Australia), MDA-MB-453 cells were a gift from Biogen-Idec, and DU4475 cells were obtained from American Type Culture Collection. The cell lines were not further authenticated.

## Results

### Analysis of *PDCD4* as a prognostic marker in breast cancer

To determine the relationship between *PDCD4* expression in breast cancer and patient survival, we evaluated a previously published microarray dataset. This dataset was obtained from tumors less than 5 cm collected from 295 breast cancer patients under the age of 55 at the Netherlands Cancer Institute (15). We stratified this dataset into 2 groups on the basis of *PDCD4* levels, with the "high" group representing those above the median. High expression correlated significantly with better probability of survival ( $P = 0.0014$ ; Fig. 1). Nevertheless, a significant fraction of patients within the high *PDCD4* cohort (~35%, which extrapolates to over 38,000 new patients per year in the United States alone; ref. 1) did not seem to gain benefit from this elevated expression. One explanation for this could be the presence of interacting partners of *PDCD4* that inactivate or change its role as a tumor suppressor.

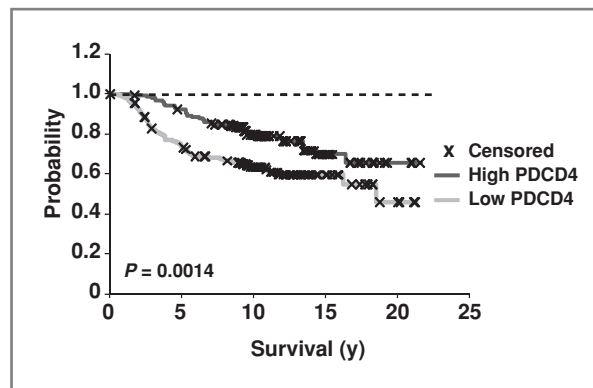


Figure 1. Analysis of *PDCD4* as a prognostic marker in breast cancer. Microarray data from tumors (15) was analyzed with respect to *PDCD4* expression. Patients with tumors expressing *PDCD4* mRNA above the median (high, dark gray) had a significantly higher probability of survival than the other patients (low, light gray).

### Discovery of an interaction between *PDCD4* and *PRMT5* reveals a potential pathway of regulation in breast cancer

To look for new regulatory factors of *PDCD4*, we took a biochemical approach to identify binding partners. We used *Xenopus laevis* egg extract, which has the advantage of limited manipulation to disrupt protein complexes and retains a very high concentration of proteins. A 72-kD protein was consistently recovered from egg extract with recombinant *PDCD4* (Fig. 2A). When analyzed by mass spectrometry, this protein was identified as protein arginine methyltransferase 5 (*PRMT5*; Supplementary Fig. S1). To confirm that *PDCD4* also interacts with human *PRMT5*, lysates were made from a panel

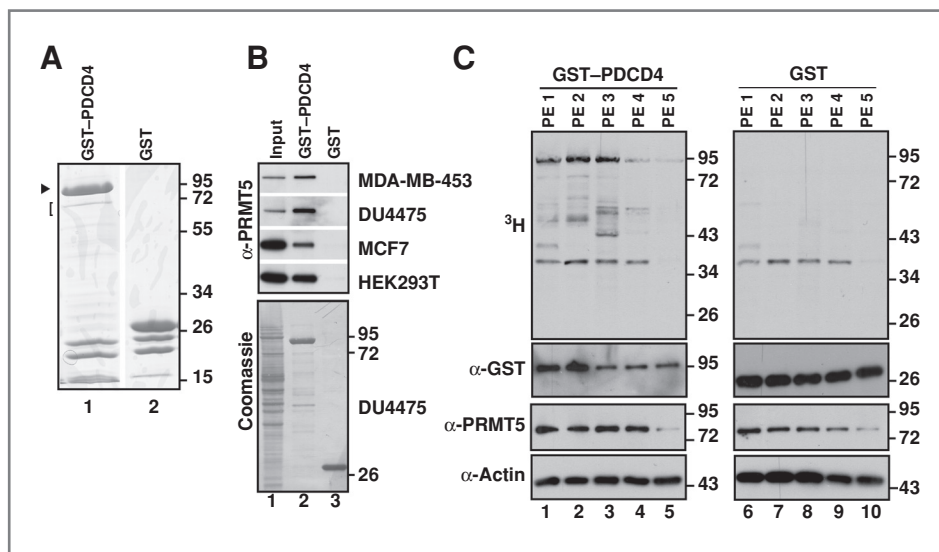


Figure 2. *PRMT5* is a protein partner of *PDCD4*. A, Coomassie-stained gel of proteins associated with GST-*PDCD4* (lane 1; GST-*PDCD4* indicated with arrowhead), compared with background levels of proteins that associate with GST (lane 2), following incubation with *X. laevis* egg extract. The gel was sliced (bracket) to recover a 72-kDa band. Analysis by mass spectrometry identified *PRMT5* in this gel slice. B, top, immunoblot to detect *PRMT5* among proteins associated with GST-*PDCD4* (lane 2) or GST (lane 3), following incubation with lysates from the human cell lines indicated (8.5% to 17% of input material is shown in lane 1); bottom, Coomassie stain of representative immunoblot (DU4475 samples), showing recovery of GST-tagged proteins (lanes 2, 3). C, autoradiograph of a panel of pleural effusion lysates supplemented with  $^3\text{H}$ -SAM and GST-*PDCD4* (left) or GST (right), indicating that *PDCD4* can be targeted for methylation in the context of breast cancer.

of cell lines (representing both estrogen receptor–positive and estrogen receptor–negative breast cancers), and interacting proteins were isolated on an analytical scale. Immunoblotting the proteins retained with GST–PDCD4 confirmed the interaction with human PRMT5 (Fig. 2B).

PRMT5 is a type II methyltransferase and catalyzes addition of a methyl group to both terminal nitrogens of arginine residues (18). Like other posttranslational modifications, methylation can alter protein function, localization, and/or binding partners (18). To assess whether PRMT5 has the potential to be a regulatory factor in the context of breast cancer, we examined a set of clinical breast cancer specimens for protein expression. Again, we surveyed both estrogen receptor–positive and estrogen receptor–negative tumors. This analysis found that PRMT5 is expressed in breast cancer and can vary in level as well as whether it is coexpressed with PDCD4 (Supplementary Fig. S2). To assess whether these proteins interact and have the potential to directly influence each other in the context of breast cancer, breast cancer cells were isolated directly from patients, as pleural effusions. When GST–PDCD4 was incubated with lysates of these cells, PRMT5 was again found to associate (data not shown).

The discovery of a partnership between PRMT5 and PDCD4 raised the question of whether PDCD4 is a target of methylation. To probe the potential for this modification to occur in the context of breast cancer, lysates of tumor cells isolated from fresh pleural effusions were incubated with GST–PDCD4 (Fig. 2C, lanes 1–5) or GST (lanes 6–10), along with tritiated S-adenosylmethionine (SAM) as the methyl donor. Specific labeling of GST–PDCD4 was detected, indicating that PDCD4 is a target of protein methyltransferases present in these lysates. GST–PDCD4 was modified to varying degrees in this assay, underscoring the possibility that the extent to which PDCD4 is targeted by this type of pathway in breast cancer is variable and, in turn, may impact the role of PDCD4 to different extents, depending on the individual context.

### PDCD4 and PRMT5 synergistically enhance tumor growth

Overexpression of PRMT5 transforms NIH3T3 cells and has been found to be upregulated in leukemia, lymphoma, and gastric cancer (13, 19, 20). The presence of PRMT5 in breast cancer, its potential to modulate protein activity, and its reported attributes as a protumor factor prompted us to test whether PRMT5 influences the role of PDCD4 in a tumor context. To do so, MCF7e breast cancer cells (MCF7 cells that ectopically express the mouse ecotropic receptor enabling infection by murine-specific retroviruses) were engineered to express elevated levels of PDCD4 and PRMT5 alone or in concert (Fig. 3A). There were no gross phenotypic changes as a result of increased expression of these proteins, and all 4 cell lines had similar doubling times under normal tissue culture conditions (Fig. 3B).

To assess tumor growth, cells were transplanted orthotopically into NOD/SCID mice that also received estrogen supplementation (see Materials and Methods). Increasing levels of either PDCD4 or PRMT5 alone in the context of MCF7e cells did not significantly alter the growth rate of the tumor (Fig. 3C). However, the PDCD4–PRMT5 coexpressing cells exhibited significantly faster growth as tumors than singly expressing PDCD4, PRMT5, or control cell lines (Fig. 3C). Analysis of lungs, spleen, and liver showed no significant difference in metastasis between cell lines. Immunohistochemical analysis of harvested tumors showed the expected elevated levels of PDCD4 and PRMT5 expression (Supplementary Fig. S3). No significant difference between PDCD4–PRMT5 tumors compared with vector control tumors was found in analysis of activated caspase 3 and Ki67 (Supplementary Fig. S4). In general, however, more necrosis, edema, and vasculature were observed in PDCD4–PRMT5 tumors relative to vector-expressing tumors by hematoxylin and eosin stain (Supplementary Fig. S5).

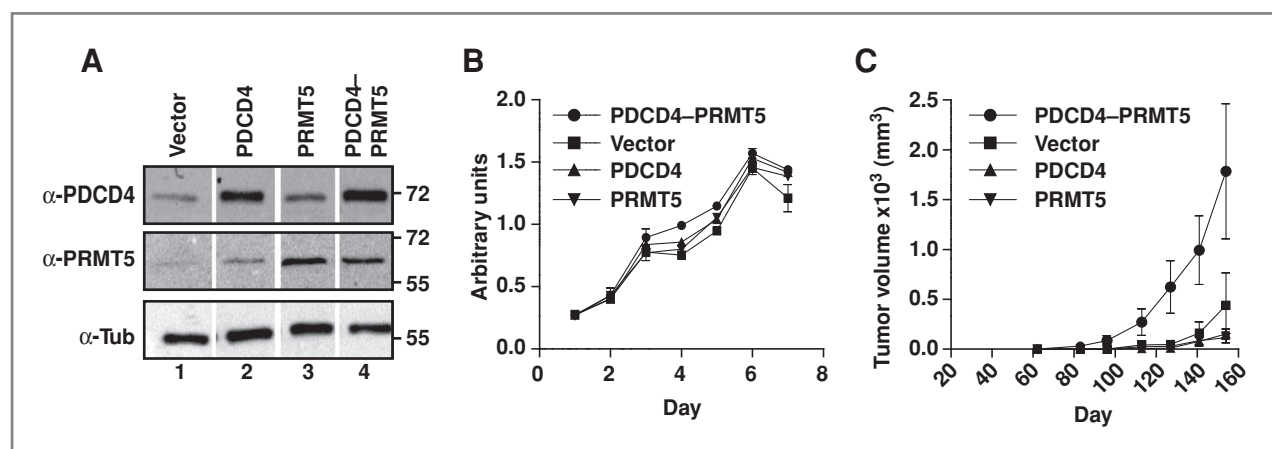


Figure 3. Coexpression of PDCD4 and PRMT5 enhances tumor growth in xenograft model. A, MCF7e cells expressing PDCD4 and PRMT5 or empty constructs, as indicated, were assessed by immunoblot. Tubulin levels were tracked as a loading control. B, cell growth in tissue culture was measured for 7 days by using a WST assay (BioVision). C, the cell panel was transplanted orthotopically and tumor volume was monitored over a 22-week period, with mean numbers and SE graphed. See Supplementary Methods for additional information on xenograft tumor growth.



### PDCD4 is methylated in the N-terminal domain at arginine 110 and is a target of PRMT5

To narrow down the region of methyl modification seen in Fig. 2C, we fragmented PDCD4 into 2 domains. We noted that the N-terminal region contains an arginine-rich subdomain (Fig. 4A). The second region contains the MA3 domains responsible for eIF4A binding (Fig. 4A; refs. 21–23). Purified GST-fusion proteins of full length and both truncation mutants were incubated with cell lysate as an enzyme source and supplemented with  $^3\text{H}$ -SAM. Only full-length protein and the N-terminal fragment were labeled (Fig. 4B). To further map the methylation site of PDCD4, we took a candidate approach. Within the arginine-rich region of PDCD4, R73 and R110 are flanked by glycines, resembling canonical methylation sites (Fig. 4A). These arginines were mutated to lysine, conserving the amino acid charge but disrupting potential arginine methyl acceptor sites. Mutation of R73 had negligible effect, whereas mutation of R110 abolished methylation (Fig. 4C).

To determine whether PRMT5 itself methylates PDCD4, wild-type or PRMT5<sub>cd</sub> were transiently expressed and then immunisolated (Fig. 4D). These enzyme sources were incubated with purified GST-PDCD4 and  $^3\text{H}$ -SAM. Labeled GST-PDCD4 was detected in reactions using wild-type PRMT5 (Fig. 4D, lane 4), showing that PDCD4 can be targeted for methylation by PRMT5. Mutation of R110 again resulted in the absence of methylation (Fig. 4D, lane 7), confirming that this is the acceptor site for methylation by PRMT5. PDCD4 was labeled at greatly reduced levels in reactions by using PRMT5<sub>cd</sub>; this residual activity is likely due to the ability of mutant PRMT5 to homo-oligomerize with its endogenous counterpart (ref. 24 and Fig. 4D, lanes 1 and 2).

### The catalytic activity of PRMT5 and PDCD4-R110 are necessary for enhanced tumor growth

To determine whether PRMT5 enzymatic activity is necessary to promote tumor growth in our breast cancer model system, MCF7e cell lines were generated expressing PRMT5<sub>cd</sub> alone or in conjunction with wild-type PDCD4 (Supplementary Fig. S6A). These cells were transplanted orthotopically and assessed for tumor growth over a 22-week period. As controls, vector only and wild-type PDCD4-PRMT5 cells were also retransplanted. The new injection of the PDCD4-PRMT5 cell line tracked well with the previous PDCD4-PRMT5 tumor growth rate, underscoring the synergistic effect of these 2 proteins (Fig. 5A). Tumor measurements from the second experiment with vector control and PDCD4-PRMT5 were combined with the respective data from the first set to assess growth rates at week 22 and a clear difference in tumor growth was seen between these cell lines (Fig. 5B). The PRMT5<sub>cd</sub>-PDCD4 cells, however, did not show an accelerated growth rate compared with the vector control (Fig. 5A and B), indicating that the enzymatic activity of PRMT5 is necessary for enhanced tumor growth due to coexpression with PDCD4.

To determine whether PDCD4 is the relevant target of PRMT5 in this context or whether PRMT5 is working through a parallel pathway, MCF7e cell lines were generated expressing the methyl mutant PDCD4<sub>mm</sub> (PDCD4 with R110K muta-

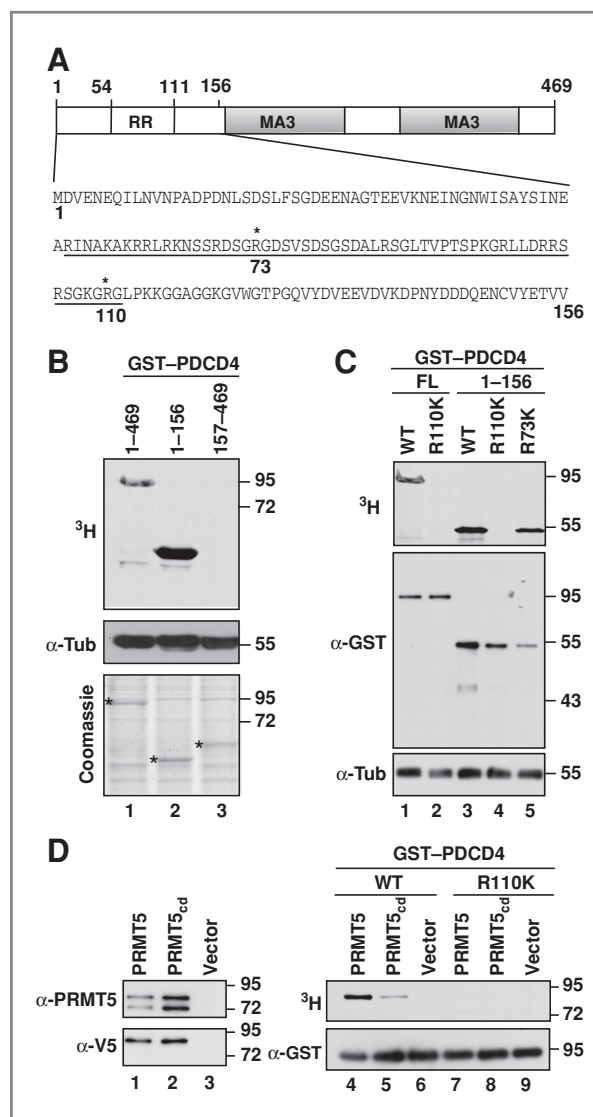


Figure 4. PDCD4 can be methylated in its N-terminal domain and is a target of PRMT5. A, schematic of PDCD4, with arginine-rich (RR) region and MA3 regions indicated. N-terminal sequence is shown with the RR region underlined. B, top, autoradiograph of reactions containing purified GST-PDCD4 (lane 1) or domain fragments (lanes 2, 3), along with HEK293 cell lysate and  $^3\text{H}$ -SAM; middle, immunoblot for tubulin, tracking lysate level in methylation reactions; bottom, total protein in each reaction detected by Coomassie staining; \*, recombinant protein present in each reaction. C, Arg110 and/or Arg73 were mutated to lysines in full-length (FL) PDCD4 or the N-terminal region. These proteins, and wild-type (WT) counterparts, were incubated with cell lysates supplemented with  $^3\text{H}$ -SAM. Top, autoradiography; middle, immunoblot to detect GST fusion proteins; bottom, immunoblot for tubulin to track levels of cell lysate. D, V5-PRMT5, V5-PRMT5<sub>cd</sub> mutant, or control vector were transiently expressed. Left, immunoblots of material immunoprecipitated with V5 antibody and probed with PRMT5 (top) or V5 (bottom) antibody. GST-PDCD4 (lanes 4–6) or GST-PDCD4-R110K (lanes 7–9) were incubated with immunoprecipitates shown in lanes 1–3, along with  $^3\text{H}$ -SAM. Methylation was monitored by autoradiography (top). The presence of recombinant PDCD4 in all reactions was confirmed by immunoblot with anti-GST (bottom).

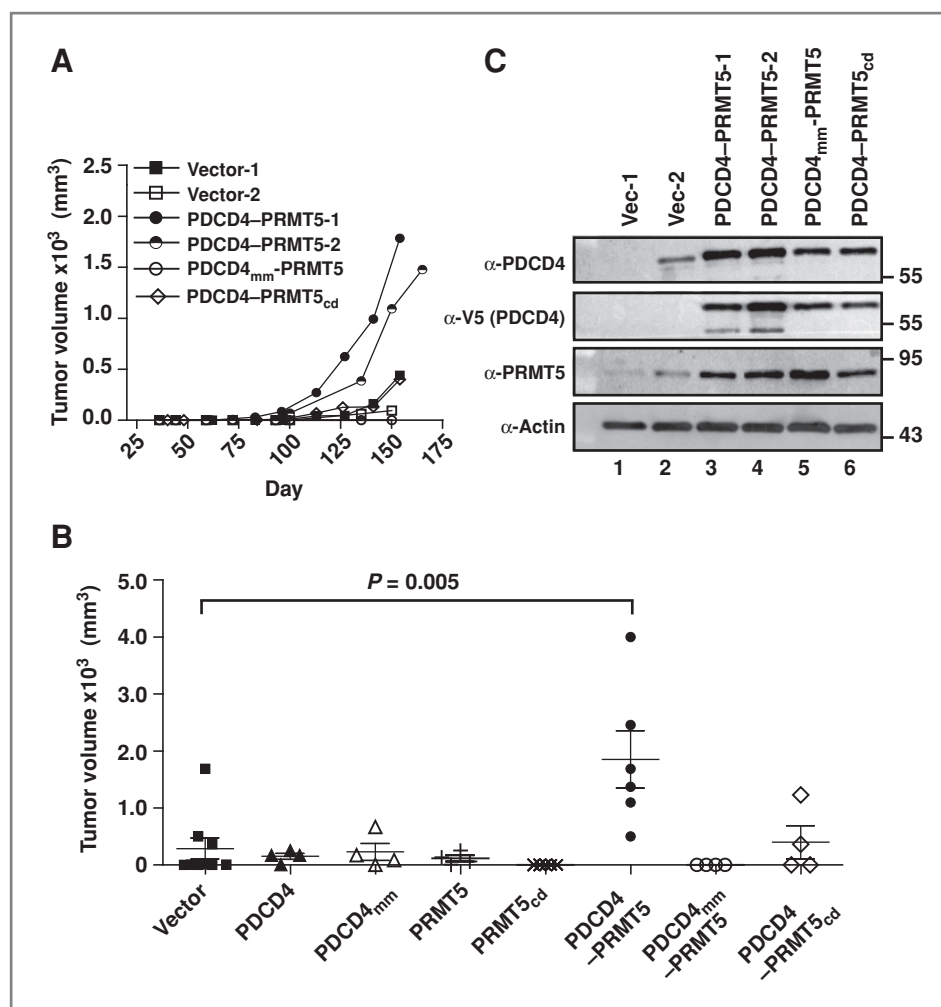


Figure 5. The catalytic activity of PRMT5 and arginine 110 of PDCD4 is necessary for synergistic tumor cell growth.

A, mean tumor growth of PDCD4<sub>mm</sub>-PRMT5 and PDCD4-PRMT5<sub>cd</sub> cell lines is graphed along with repeats of vector only and PDCD4-PRMT5 (denoted "-2"). Data from the first tumor growth experiment (denoted "-1") with these 2 cell lines (Fig. 3) are shown for comparison. B, compiled comparison of tumor volume at 22 weeks, with the mean and SE indicated. The only cells that showed statistically different growth than the vector control cell line were the PDCD4-PRMT5 expressing cells, as indicated. See Supplementary Methods for additional information on xenograft tumor growth. C, representative tumors from (A) were assessed for construct expression by immunoblot.

tion), with or without wild-type PRMT5, and then assessed for tumor growth (Supplementary Fig. S6A; Fig. 5A and B). Tumor growth of PDCD4<sub>mm</sub> with PRMT5 was not significantly different from the control cell line (Fig. 5B). Tumor growth properties in these cohorts are not explained by loss of expression of exogenous constructs, as confirmed at the level of both protein (Fig. 5C) and RNA sequence (Supplementary Fig. S6B). This indicates that methylation of PDCD4 and R110, in particular, is necessary for the enhanced tumor growth observed with coexpression of both wild-type PDCD4 and PRMT5.

Two important properties of PDCD4 are its ability to interact with eIF4A and rapid degradation in response to cell proliferation signals (21, 25, 26). Binding of eIF4A is mediated by 2 MA3 domains that are distal to the methylation site in PDCD4 (Fig. 4A; refs. 27, 28). To determine whether mutation of this methylation site alters eIF4A binding, both GST-PDCD4 and GST-PDCD4<sub>mm</sub> were incubated in cell lysate and then recovered on a glutathione matrix. These samples were probed by immunoblot for eIF4A1, which was found at equal levels (Supplementary

Fig. S7A). Therefore, at the level of methylation that takes place under these assay conditions, an influence on the association of eIF4A is not detectable. We next examined whether increased PRMT5 expression alters the stability of PDCD4. To do so, the MCF7e lines expressing exogenous PDCD4, PDCD4 plus PRMT5, and PDCD4<sub>mm</sub> plus PRMT5 were stimulated with PMA to trigger S6K activation and with cycloheximide to stop new protein synthesis. We then tracked the stability of exogenous PDCD4 over a short time course by probing immunoblots with anti-V5. PRMT5 expression did not seem to alter the stability of PDCD4 (Supplementary Fig. S7B, top and middle panels). Consistent with this, we did not observe a significant difference between wild-type PDCD4 and the methyl mutant (Supplementary Fig. S7B, middle and bottom panels).

#### Clinical data indicate that PRMT5 levels impact the probability of survival when tumors express PDCD4

Our observations that simultaneous expression of elevated PDCD4 and PRMT5 causes accelerated tumor growth in an orthotopic mouse model raised the question of whether this

combination of markers would be useful in a clinical setting. To address this question, we stratified patients with high tumor levels of *PDCD4* into quartiles on the basis of *PRMT5* expression. Each one of these cohorts extrapolates to approximately 25,000 patients a year in the United States (1). We found that the top quartile—in which tumors highly express both *PDCD4* and *PRMT5*—had poor outcomes, similar to the low *PDCD4* cohort. In contrast, with decreases in *PRMT5* expression, the probability of survival increased significantly ( $P = 0.0016$ ). The bottom quartile—high for *PDCD4* and low for *PRMT5*—had remarkably better outcome than the top quartile (Fig. 6A), with a 20-year survival rate of 80% versus 43%. Similar quartile analysis of *PRMT5* alone showed a trend in improved probability of survival, with decreasing *PRMT5*, but was not significant (Fig. 6B). This suggests that the combination of *PRMT5* and *PDCD4* as biomarkers for outcome is potentially useful: High levels of *PDCD4* are protective, unless *PRMT5* is also highly expressed.

To test the reproducibility of these observations, we used another independent breast cancer dataset (17). We selected patients from this cohort on the basis of availability of both clinical follow-up and gene expression data. This resulted in a set of 224 tumors, which we then stratified for *PDCD4* expression, with high defined as those in the top third of expression levels. Patients in this high expression group again had a significantly higher probability of longer disease-free survival (DFS,  $P = 0.0066$ ; Fig. 6C). Yet, within the high *PDCD4*

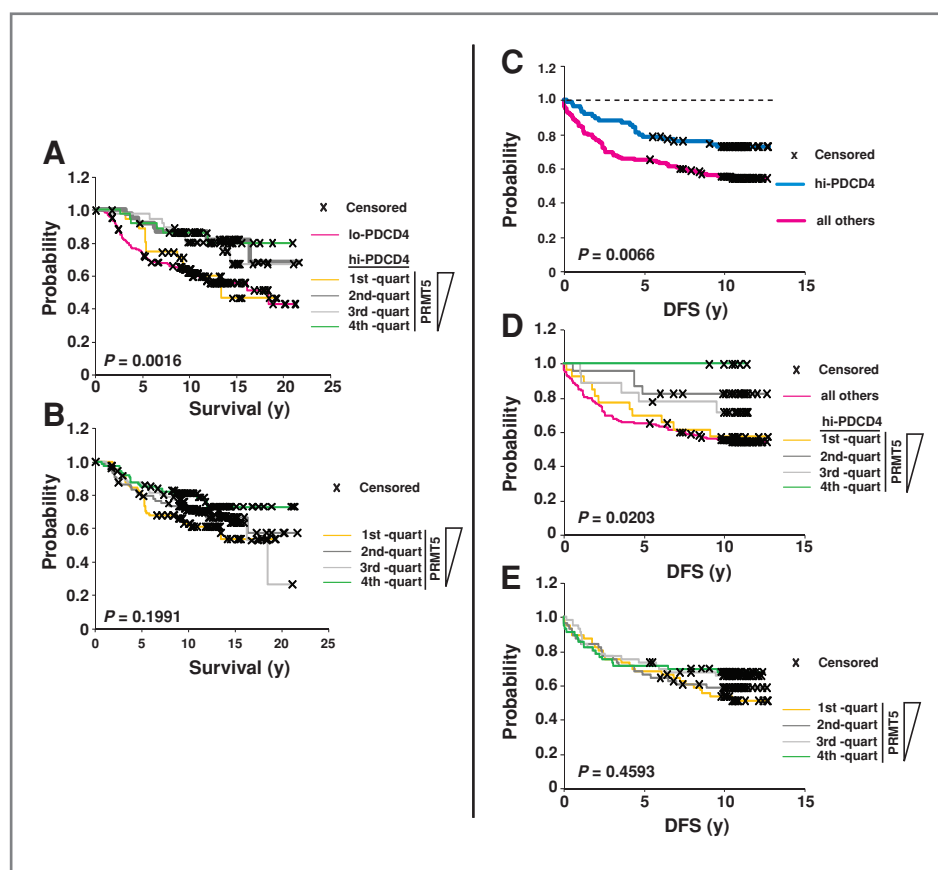
expression group, there was still more than 25% probability of relapse. We then further stratified the *PDCD4* high group by *PRMT5* levels (each subgroup extrapolates to greater than 15,000 patients a year in the United States; ref. 1) and, strikingly, lower levels of *PRMT5* proved similarly to correspond to better outcome in this context ( $P = 0.0203$ ; Fig. 6D), whereas quartile analysis of *PRMT5* alone did not show a correspondence to outcome (Fig. 6E). These data confirm that considering *PRMT5* levels in the context of *PDCD4* could be an effective strategy toward an improved prognostic tool in breast cancer.

## Discussion

*PDCD4* shows promise as a biomarker, with prognostic attributes in a number of cancers (4–7). Here, we further find that *PDCD4* expression is informative with regard to survival of breast cancer patients, in which increased levels correlate with better outcome in 2 large-scale clinical evaluations. *PDCD4* expression alone, however, has limitations in stratifying cancer patient outcomes, as approximately 30% of patients with tumors expressing relatively high *PDCD4* levels have poor survival. This could be due to a potential discrepancy between mRNA and protein levels (26, 29), to environmental factors, or to interacting regulatory pathways.

The discovery here of *PRMT5* as an interacting partner opened a new level of regulation to consider. Neither *PDCD4*

**Figure 6.** Expression of *PRMT5*, in conjunction with *PDCD4*, improves prediction of survival in 2 independent datasets. Breast cancer mRNA expression data were analyzed with respect to both *PDCD4* and *PRMT5* by using the 2 independent datasets (A and B, ref. 15; C–E, ref. 17). A, the high *PDCD4* cohort of patients (Fig. 1; ref. 15) were stratified into quartiles on the basis of *PRMT5* expression, with the first quartile containing the highest levels of *PRMT5* expression. B, patients from the entire dataset were stratified into quartiles on the basis of *PRMT5* expression alone. C–E, analysis of a second dataset of mRNA expression in breast cancer (17). C, this dataset was stratified into thirds on the basis of *PDCD4* expression levels. Patients in the high expression cohort (top one-third) had greater probability of DFS compared with all others. D, the high *PDCD4* cohort was further stratified into quartiles on the basis of *PRMT5* expression; lower *PRMT5* expression corresponded to increased probability of DFS. E, patients stratified into quartiles on the basis of tumor expression of *PRMT5* alone did not show significant differences in survival.



nor PRMT5 affected tumor cell growth when expressed alone in our model system. Although this was somewhat surprising, it may indicate that in a particularly aggressive or advanced tumor context, elevated expression of either protein alone is insufficient to alter tumor properties. In the case of PDCD4, this may be due, at least in part, to endogenous levels of PRMT5. Importantly, the orthotopic tumor model revealed that, together, elevated PDCD4 and PRMT5 expression significantly enhanced tumor growth. This result suggests that PRMT5 not only negates a PDCD4 tumor suppressor function but also works synergistically to promote tumor growth. This progrowth effect occurred only in the tumor context, not in tissue culture, hinting that the combination of proteins activates a pathway that enhances the ability to establish a productive tumor microenvironment. The similar numbers of cells staining for activated caspase-3 and Ki67 in tumors expressing elevated levels of both PDCD4 and PRMT5 versus tumors with vector alone suggest that alteration in apoptosis or proliferation does not account for the larger tumor size in the former case. It will be important to investigate these features at earlier points in tumorigenesis. Changes in early events coordinated between cancer and host cells may accelerate the growth of PDCD4–PRMT5 tumors. The increase in necrosis, edema, and vasculature is consistent with the larger size of the PDCD4–PRMT5 xenograft tumors, but further investigation is needed to determine whether any of these features are critical to the altered tumor growth properties conferred by elevated levels of PDCD4 and PRMT5.

The change in PDCD4 function that occurs in the presence of elevated PRMT5 pointed to a role for posttranslational modification. We found that, indeed, PDCD4 is methylated and further, a PDCD4 methyl mutant expressed with wild-type PRMT5 or PRMT5<sub>cd</sub> expressed with wild-type PDCD4 failed to promote tumor growth. This shows that methylation of PDCD4 by PRMT5 is critical for enhanced tumor growth. The methylation target residue R110 lies near an S6 kinase 1 site (S67) reported to regulate PDCD4 stability (26, 30). Levels of ectopic expression of PDCD4 did not seem to be influenced by the presence of PRMT5 or mutation of R110 (Figs. 3 and 5). Furthermore, under conditions that stimulate PDCD4 degradation, the methyl mutant did not enhance or decrease stability, indicating that methylation of PDCD4 does not help coordinate proteasome targeting.

PDCD4 is thought to exert its tumor suppressor role by regulating translation (21, 25), fitting with a growing theme of translation initiation as a node of regulation in cancer (31). Interestingly, PRMT5 has been shown to influence eIF4E levels (32) and was recently shown to influence ribosomal stability by methylation of ribosomal subunit RPS10 (33). Methylation of PDCD4 may impact its role in translation regulation, although we did not observe a difference in eIF4A1-binding activity when the methylation site was mutated. Another interesting possibility is that, conversely, methylated PDCD4

changes PRMT5 function or specificity. In addition to the recent connection to translation, PRMT5 is known to be involved in transcription (13, 20, 34), in efficient assembly of the spliceosome (35), and in modulation of p53-dependent cell-cycle arrest (32, 36). Methylated PDCD4 potentially accelerates tumor growth by altering these or other functions downstream of PRMT5 (32, 36).

By integrating biochemical and tumor model data, we have found that an elevated level of PRMT5 in conjunction with PDCD4 reverses the tumor suppressive properties of PDCD4. The finding that combined expression analysis of *PDCD4* and *PRMT5* is a powerful prognostic indicator for outcome in breast cancer suggests that these factors could be used as rational, activity-based biomarkers to aid in decisions about how aggressively to treat a breast cancer patient. In the future, chemical inhibition of PRMT5 methyltransferase activity could be used to abrogate the synergism between PDCD4 and PRMT5, potentially unmasking PDCD4 tumor suppressor function in cancers that express both proteins. Finally, although our focus here has been on breast cancer, PDCD4 plays a tumor suppressor role in a wide spectrum of cancers (4–6), raising the possibility that its connection to PRMT5 will be of broad prognostic and, perhaps, therapeutic value.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### Author Contributions

M.A. Powers: designed and performed research, analyzed data, wrote paper; M.M. Fay: designed and performed research, analyzed data, edited paper; R.E. Factor: assessed protein expression and histology in clinical samples and mouse tumor IHC; A.L. Welm: designed research and analyzed data, edited paper; K.S. Ullman: designed research and analyzed data, wrote paper.

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