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Priority Report

# Translation Inhibitor Pdcd4 Is Targeted for Degradation during Tumor Promotion

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## Abstract

**Inactivation of tumor suppressors is among the rate-limiting steps in carcinogenesis that occur during the tumor promotion stage. The translation inhibitor programmed cell death 4 (Pdcd4) suppresses tumorigenesis and invasion. Although Pdcd4 is not mutationally inactivated in human cancer, the mechanisms controlling Pdcd4 inactivation during tumorigenesis remain elusive. We report that tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate exposure decreases protein levels of Pdcd4 in mouse skin papillomas and keratinocytes as well as in human HEK293 cells. This decrease is attributable to increased proteasomal degradation of Pdcd4 and is mediated by protein kinase C–dependent activation of phosphatidylinositol 3-kinase–Akt–mammalian target of rapamycin–p70$^{S6K}$ and mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase (MEK)–ERK signaling. Both Akt and p70$^{S6K}$ phosphorylate Pdcd4, allowing for binding of the E3-ubiquitin ligase β-TrCP and consequently ubiquitylation. MEK-ERK signaling on the other hand facilitates the subsequent proteasomal degradation. We further show that Pdcd4 protein levels *in vivo* are limiting for tumor formation, establishing Pdcd4 as a haploinsufficient tumor suppressor in Pdcd4-deficient mice. Thus, because endogenous Pdcd4 levels are limiting for tumorigenesis, inhibiting signaling to Pdcd4 degradation may prove a valid strategy for cancer prevention and intervention.** [Cancer Res 2008;68(5):1254–60]

## Introduction

Pdcd4 is a novel suppressor of tumorigenesis, tumor progression, and invasion that acts following initiator/promoter challenge or without challenge (1–3). Although a number of tumor suppressors target transcription, Pdcd4 is the first suppressor found to target translation. Recently, translational dysregulation has been increasingly acknowledged as contributing to carcinogenesis. Moreover, inhibition of translation by mammalian target of rapamycin (mTOR) inhibitors is a promising and widely explored approach for cancer therapies (4). Pdcd4 interacts with translation initiation factors eIF4A and eIF4G to inhibit translation in a mRNA-specific fashion and consequently to inhibit pro-oncogenic events such as activation of activator protein-1 (AP-1)–dependent transcription (5, 6), anchorage-independent growth (5), and invasion (3, 7). The mRNA specificity of Pdcd4 for targeting translation is thought to arise from structural and sequence features of the 5′ untranslated region (8–10).

Inactivation of tumor suppressors contributes to oncogenesis. Although most tumor suppressors, including p53, are mutationally inactivated, others such as p27 and Pdcd4 are not (11, 12). Instead, Pdcd4 expression is down-regulated with progression in a number of human cancer sites such as lung and colon (13–15). Just how Pdcd4 is inactivated during carcinogenesis is unknown.

We report that Akt and p70$^{S6K}$ are activated by the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and are required for degradation of Pdcd4 protein. In addition to Akt-p70$^{S6K}$ signaling, active mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase (MEK)–ERK pathway is essential for tumor promoter–induced down-regulation. Degradation of the tumor suppressor Pdcd4 occurs *in vitro* as well as *in vivo* in papillomas arising during mouse skin carcinogenesis. Furthermore, Pdcd4 expression level is found to predict the susceptibility of mice to tumorigenesis.

## Materials and Methods

**Cell lines and materials.** HEK293 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin/streptomycin, and 2 mmol/L L-glutamine. TPA was purchased from Alexis. 7,12-Dimethylbenz(*a*)anthracene (DMBA), cycloheximide, MG132, Ro31-8425, SP600125, sulfasalazine, and anti–β-actin antibody came from Sigma. PD98059 and SB203580 were from Calbiochem. LY294002, anti–phospho-ERK, anti-ERK, anti–phospho-GSK3β, anti-GSK3β, anti–phospho-S6, and immobilized anti–phospho-Akt substrate antibodies were purchased from Cell Signaling. Anti-rabbit horseradish peroxidase (HRP)–coupled antibody was from Santa Cruz Biotechnology. Nitrocellulose membranes, anti-mouse HRP-coupled antibody, and enhanced chemiluminescence (ECL) solutions were from Amersham Biosciences. Peptide-purified anti-Pdcd4 antibody was described earlier (12).

**Transgenic and Pdcd4-deficient mice.** K14-Pdcd4 transgenic mice were described previously (2). Pdcd4-deficient animals were created using Cre-mediated recombination. A detailed description of the procedure is given in Supplementary Fig. S4. Mice were housed and cared for in accordance with National Cancer Institute-Frederick Animal Care and Use Committee guidelines.

**Immunoprecipitation and Western blotting.** For Western blot analysis, cells were sonicated and then lysed on ice for 30 min in lysis buffer [50 mmol/L Tris-HCl, 150 mmol/L NaCl, 5 mmol/L EDTA, 0.5% NP40, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor mix (Roche)]. For immunoprecipitation, cells were passed through a needle and then lysed on ice for 30 min in immunoprecipitation buffer [50 mmol/L Tris-HCl, 1% NP40, 150 mmol/L NaCl, 1 mmol/L EDTA, 10% glycerol, 1 mmol/L PMSF, protease inhibitor mix, phosphatase inhibitor cocktail 2 (Sigma)]. For Western blot analysis, 50 μg protein were separated. For the immunoprecipitation experiments, 1,000 μg protein and 10 μL anti–Pdcd4 antibody were incubated in 200 μL immunoprecipitation buffer for

**Note:** Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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4 h before 20 μL 50% slurry of Protein G Sepharose fast flow (Sigma) were added overnight. Alternatively, immobilized anti–phospho-Akt substrate antibody was added and incubated overnight. After washing with immunoprecipitation buffer, samples were denatured in 2× loading buffer at 95°C for 5 min, separated on SDS gels, and analyzed using Western blot analysis. Whole-cell lysates were used as loading confirmation for the immunoprecipitation. Proteins were detected using specific antibodies and appropriate secondary antibodies. Then, they were visualized using ECL.

**Animal studies and primary keratinocytes.** Primary keratinocytes were harvested from 2- to 4-d-old postnatal pups as described previously (16).

For the two-stage skin carcinogenesis model, 7- to 8-wk-old mice were initiated with a single topical application of DMBA (100 nmol/0.2 mL acetone) to the shaved backs. Two weeks later, TPA (5 nmol/0.2 mL acetone) was applied twice weekly to the initiated skin for 26 wk. The number of papillomas per mouse (multiplicity) and the total number of mice developing papillomas (incidence) were evaluated biweekly as previously described (2).

**Reverse transcription-PCR and primers.** Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Reverse transcription-PCR (RT-PCR) was done using the RNA-PCR kit (Perkin-Elmer) according to the manufacturer's protocol. RNA (0.25 μg) was used for reverse transcription; for primers and conditions, see Supplementary Text. Amplified products were separated on agarose gels.

**Statistical analysis.** Two-sided *P* values were calculated for papilloma multiplicity by Wilcoxon rank sum test and for tumor incidence by Fisher's exact test.

## Results

**TPA exposure down-regulates Pdcd4 protein levels.** Transgenic expression of Pdcd4 decreases papilloma incidence in the DMBA/TPA two-stage skin carcinogenesis model. The papillomas that formed showed a substantial decrease in Pdcd4 protein levels irrespective of the initial Pdcd4 status (2). To further evaluate the contribution of the tumor promoter exposure to the observed down-regulation, primary keratinocytes were exposed to TPA for 8 hours. Exposure to 50 nmol/L TPA strongly attenuated Pdcd4 protein levels in keratinocytes isolated from both wild-type and transgenic Pdcd4-expressing mice (Fig. 1*A*). To ascertain that this is a general rather than a skin- and mouse-specific mechanism, the untransformed human cell line HEK293 was used for the following experiments. In HEK293 cells, TPA decreased Pdcd4 protein levels in a concentration-dependent manner (Fig. 1*B*) and in a time-dependent manner (Fig. 1*C*). Both phosphatidylinositol 3-kinase (PI3K) and MEK signaling pathways, as measured by GSK3β and ERK phosphorylation status, respectively, were induced by TPA starting at 10 minutes. The activation was transient and decreased toward 8 hours. The downstream effector of the PI3K-Akt-mTOR-$p70^{S6K}$ signaling pathway S6 was phosphorylated as early as 60 minutes after TPA addition and remained phosphorylated. Thus, initial effects of TPA on Pdcd4 coincided with activated PI3K and MEK pathways. In contrast to the activation of the aforementioned pathways, Pdcd4 down-regulation by TPA was not transient as seen when extending the TPA exposures to 24 hours, in which case Pdcd4 levels remained low (Supplementary Fig. S1). This observation rules out a multiphased effect of TPA on Pdcd4.

These findings establish that Pdcd4 protein levels are affected directly and sustained by a tumor promoter (i.e., TPA).

**Pdcd4 protein half-life is shortened by TPA exposure.** To determine if the observed decrease in Pdcd4 is attributable mainly to changes in transcription, cells were exposed to TPA (20 nmol/L) for periods of 30 minutes to 4 hours. Pdcd4 mRNA expression did not change in response to TPA (Fig. 1*D*). To ascertain whether Pdcd4 protein half-life changes in response to TPA, cells were pretreated with 20 nmol/L TPA for 4 hours before new protein synthesis was blocked by addition of the translation inhibitor cycloheximide (10 μmol/L), and the exposures continued for up to 4 hours. As shown in Fig. 2*A* (*left subpanel*), inhibition of translation caused a rapid decrease of Pdcd4 protein in the TPA-treated

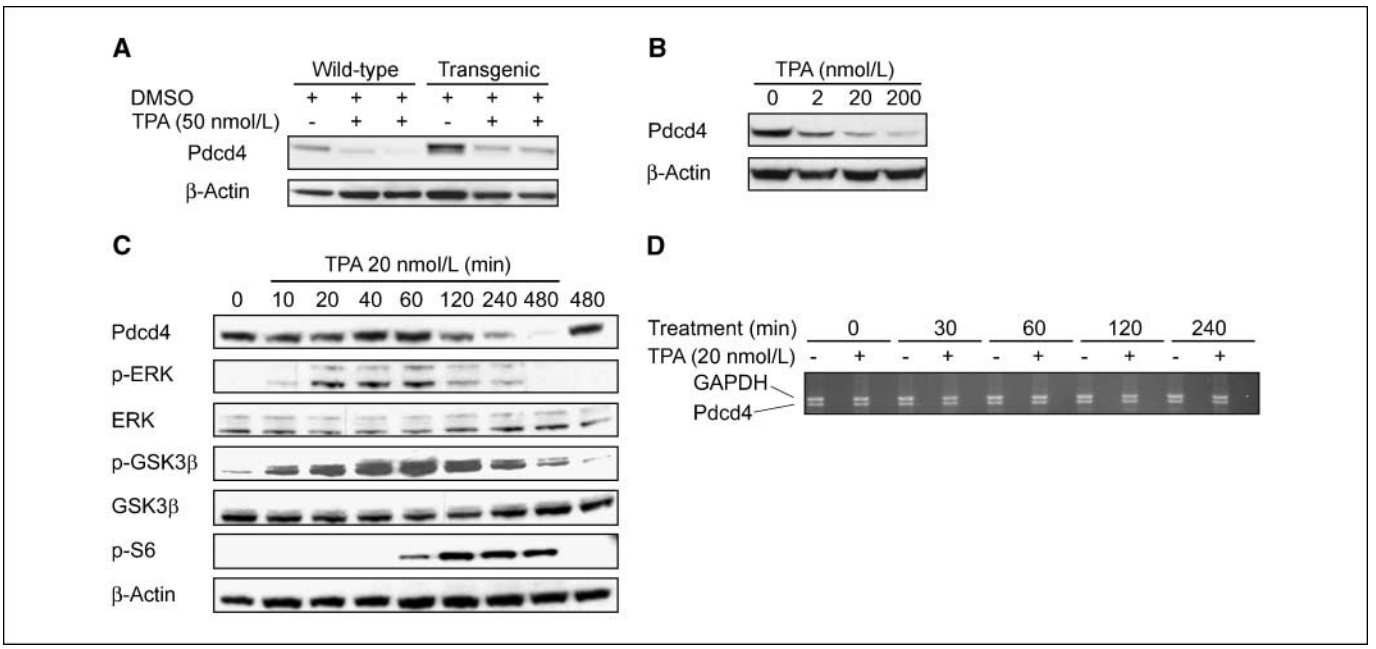


**Figure 1.** TPA exposure regulates Pdcd4 protein levels. *A,* primary basal keratinocytes of wild-type or transgenically Pdcd4-overexpressing mice were exposed to TPA for 8 h. *B,* HEK293 cells were exposed to 2, 20, or 200 nmol/L TPA for 8 h. *C,* HEK293 cells were exposed to TPA (20 nmol/L) for 10 to 480 min. Whole-cell extracts were subjected to Western blot analysis and probed with the indicated antibodies. Blots are representative of at least three independent experiments. *D,* HEK293 cells were exposed to TPA (20 nmol/L) for 30 to 240 min. Pdcd4 expression was assessed by RT-PCR analysis. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as housekeeping gene and loading control.

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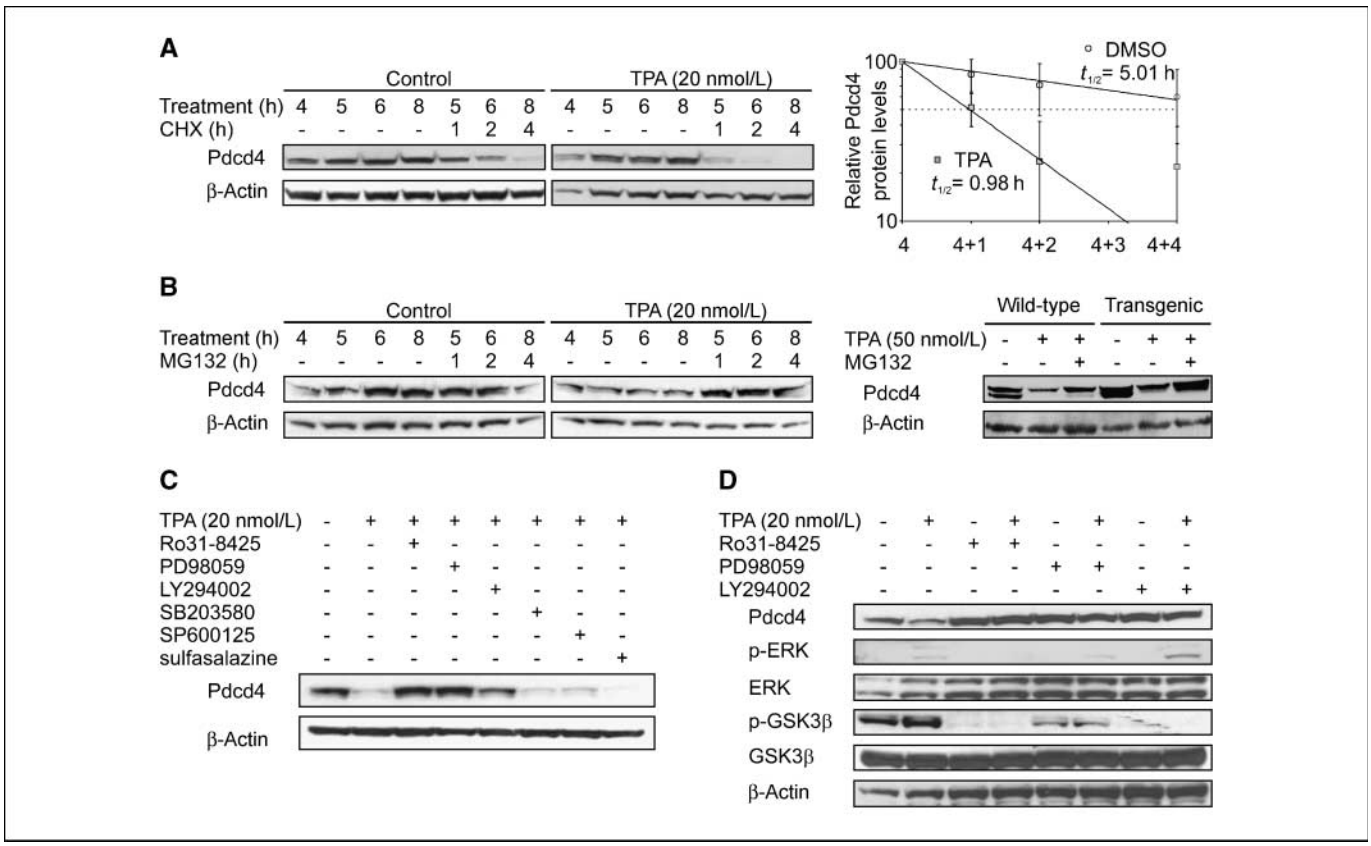


**Figure 2.** TPA exposure regulates Pdcd4 protein levels posttranslationally via increased proteasomal degradation. *A,* HEK293 cells were exposed to TPA (20 nmol/L) for 4 h. Cycloheximide (*CHX*; 10 μmol/L) was added, and incubations continued for 1, 2, or 4 h. Protein data were analyzed densitometrically and Pdcd4 protein levels were normalized to β-actin. Pdcd4 protein half-life was calculated ($n > 3$). *B, left subpanel,* HEK293 cells were exposed to TPA (20 nmol/L) for 4 h. Then, MG132 (10 μmol/L) was added, and incubations continued for 1, 2, or 4 h. *Right subpanel,* primary basal keratinocytes of wild-type or transgenic mice were exposed to TPA for 8 h. Proteasomal degradation was blocked by addition of MG132 (10 μmol/L). *C,* HEK293 cells were preincubated with either pan-PKC inhibitor Ro31-8425 (50 nmol/L), MEK inhibitor PD98059 (50 μmol/L), PI3K inhibitor LY294002 (10 μmol/L), p38 MAPK inhibitor SB203580 (10 μmol/L), JNK inhibitor SP600125 (20 μmol/L), or NF-κB inhibitor sulfasalazine (300 μmol/L) for 30 min. TPA (20 nmol/L) was added and incubations continued for 8 h. *D,* HEK293 cells were preincubated with Ro31-8425 (50 nmol/L), PD98059 (50 μmol/L), or LY294002 (10 μmol/L) for 30 min. TPA (20 nmol/L) was added and incubations continued for 4 h. Whole-cell extracts were subjected to Western blot analysis and probed with the indicated antibodies. Blots are representative of at least three independent experiments.

samples but only a slight decrease in the controls. This translates into a decrease in Pdcd4 protein half-life from ~5 hours under control conditions to <1 hour in response to TPA (Fig. 2*A, right subpanel*). Thus, the TPA-induced decrease in Pdcd4 protein results from a strongly attenuated half-life.

To further characterize the regulatory mechanisms, cells were preincubated with 20 nmol/L TPA for 4 hours before proteasomal degradation was blocked by addition of the proteasome inhibitor MG132 (10 μmol/L), and the exposures continued for up to 4 hours. Pdcd4 protein, which was attenuated by TPA, returned to control levels within 1 hour of inhibition of the proteasome (Fig. 2*B, left subpanel*). Similarly, in primary keratinocytes from both wild-type and transgenic Pdcd4-expressing mice, blocking proteasomal degradation rescued Pdcd4 from TPA-induced down-regulation (Fig. 2*B, right subpanel*).

Thus, TPA exposure down-regulates Pdcd4 protein half-life by enhancing proteasomal degradation.

**TPA exposure regulates Pdcd4 degradation via protein kinase C–mediated activation of PI3K and MEK signaling pathways.** Based on the observation that the decrease in Pdcd4 was accompanied by activation of PI3K and MEK signaling (Fig. 1*C*), we aimed to further characterize the signaling pathways required for TPA-induced Pdcd4 degradation. Therefore, cells were pretreated for 30 minutes with inhibitors of common signal transduction pathways; subsequently, 20 nmol/L TPA was administered and incubations continued for 8 hours. Both the use of the well-characterized inhibitors of pan–protein kinase C (PKC; Ro31-8425, 50 nmol/L) and MEK (PD98059, 50 μmol/L) completely prevented attenuation of Pdcd4 by TPA. Similarly, blocking PI3K signaling with the PI3K inhibitor LY294002 (10 μmol/L) prevented depletion of Pdcd4 protein, albeit not completely. In contrast, inhibition of p38–mitogen-activated protein kinase (MAPK) with SB203580 (10 μmol/L), c-Jun-$NH_2$-kinase (JNK) with SP600125 (20 μmol/L), or nuclear factor-κB (NF-κB) with sulfasalazine (300 μmol/L) did not prevent TPA-induced Pdcd4 degradation (Fig. 2*C*). These results show that Pdcd4 is degraded in response to TPA in a PKC-, PI3K-, and MEK-dependent manner.

To identify the interrelation among PKC, PI3K, and MEK signaling required for TPA-induced Pdcd4 regulation, cells were incubated with the above-mentioned inhibitors of PKC, PI3K, and MEK signaling for 30 minutes before exposure to 20 nmol/L TPA for 4 hours started. The activity of the PI3K pathway, as measured by phosphorylation of GSK3β, was completely blocked by inhibition of either PKCs with Ro31-8425 or PI3K with LY294002, whereas inhibition of MEK signaling with PD98059 had only a minor effect. Conversely, MEK activation, as measured by

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phosphorylation of ERKs, was strongly attenuated by PKC and MEK inhibition, whereas PI3K inhibition did not affect MEK activity (Fig. 2*D*).

Thus, TPA activates PKCs, which subsequently activate PI3K and MEK signaling to facilitate Pdcd4 degradation. Although both PI3K and MEK signaling are required, they seem to be independent of one another.

**PKC-PI3K signaling allows for Pdcd4 phosphorylation, whereas PKC-MEK signaling facilitates proteasomal degradation.** Activation of the PI3K-Akt-mTOR-p70$^{S6K}$ pathway was recently shown to be responsible for phosphorylation (17) and subsequent down-regulation of Pdcd4 in a serum-deprivation-readdition approach (18). Because no phospho-Pdcd4 antibody is currently available, the effect of the phosphorylation status of Pdcd4 in response to TPA was measured by using immobilized anti–phospho-Akt substrate antibody to pull down phosphorylated Pdcd4 out of lysates of TPA-treated cells as previously described (17). To allow for accumulation of phosphorylated Pdcd4, proteasomal degradation was blocked by cotreatment with the proteasome inhibitor MG132. Pretreatment of cells with inhibitors of PKCs (Ro31-8425) or PI3K (LY294002) before incubating with TPA (20 nmol/L) attenuated Pdcd4 phosphorylation (relative density compared with TPA-/MG132-treated cells, 0.65 and 0.44, respectively), whereas inhibition of MEK signaling with PD98059 did not affect phosphorylation (0.98; Fig. 3*A*). As expected, no changes of total Pdcd4 levels in whole-cell extracts were observed due to the continuous presence of the proteasome inhibitor (Fig. 3*A, WCE*). Thus, whereas PKC-PI3K signaling is required, MEK signaling has no effect on the phosphorylation of Pdcd4.

We then addressed the effect of TPA on binding of the specific E3-ubiquitin ligase β-TrCP1 to Pdcd4. Cells were cotreated with proteasome inhibitor MG132 and inhibitors of PKCs (Ro31-8425), PI3K (LY294002), or MEK (PD98059) for 30 minutes before adding TPA for an additional 8 hours. Subsequently, lysates were immunoprecipitated with anti-Pdcd4 antibody to determine binding of β-TrCP1 to Pdcd4. As predicted by the phosphorylation status, binding of β-TrCP1 to Pdcd4 was lowered by inhibitors of PKCs (relative density compared with TPA-/MG132–treated cells, 0.61) and PI3K (0.61), and MEK inhibition (1.04) did not affect β-TrCP1 binding to Pdcd4 (Fig. 3*B*). No changes were observed in β-TrCP1 levels in whole-cell extracts. Consistent pull down was

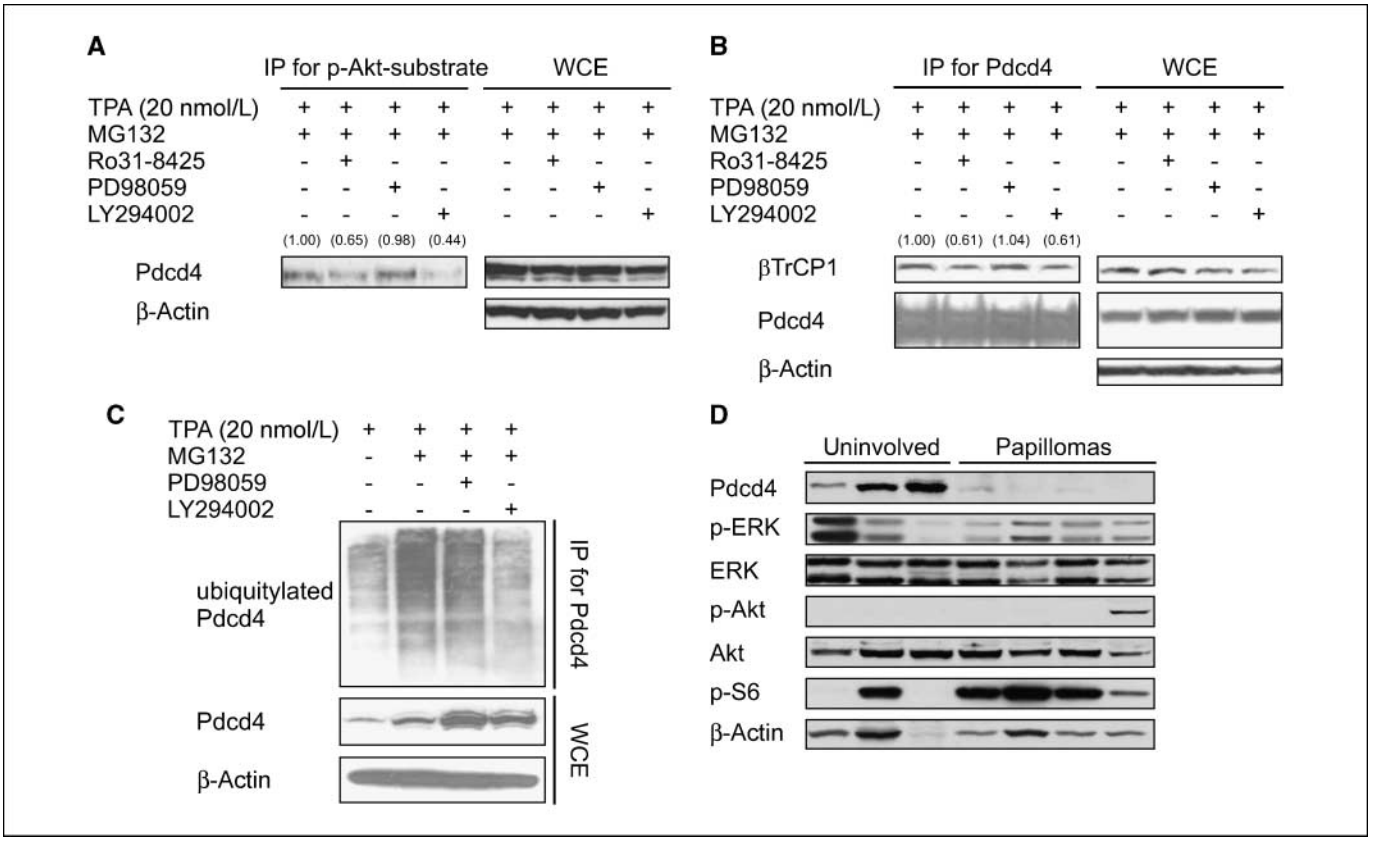


**Figure 3.** TPA exposure induces PI3K-dependent phosphorylation and ubiquitylation of Pdcd4. *A,* HEK293 cells were preincubated with the proteasome inhibitor MG132 (10 μmol/L), and Ro31-8425 (50 nmol/L), PD98059 (50 μmol/L), or LY294002 (10 μmol/L) for 30 min. Subsequently, TPA (20 nmol/L) was added and incubations continued for 8 h. Densitometric analysis of Pdcd4 relative to TPA-/MG132-treated cells in the immunoprecipitation samples is given in parentheses. *B,* HEK293 cells were preincubated with MG132 (10 μmol/L), and Ro31-8425 (50 nmol/L), PD98059 (50 μmol/L), or LY294002 (10 μmol/L) for 30 min. TPA (20 nmol/L) was added and incubations continued for 8 h. Densitometric analysis of β-TrCP1 relative to TPA-/MG132-treated cells in the immunoprecipitation samples is given in parentheses. *C,* HEK293 cells were transfected with a plasmid expressing HA-tagged ubiquitin. On the following day, cells were preincubated with PD98059 (50 μmol/L) or LY294002 (10 μmol/L) for 30 min. TPA (20 nmol/L) was added and incubations continued for 4 h before proteasomal degradation was blocked by addition of MG132 (10 μmol/L). Incubations continued for another 4 h. Whole-cell extracts were immunoprecipitated with anti–phospho-Akt substrate antibody for the detection of phosphorylated Pdcd4 (*A*) and anti-Pdcd4 antibody for the detection of Pdcd4-binding proteins (*B* and *C*), subjected to Western blot analysis and probed with the indicated antibodies. Whole-cell extracts (*WCE*) were used as loading controls. *D,* transgenically Pdcd4-overexpressing mice were treated according to the two-stage skin carcinogenesis protocol with DMBA and TPA. Uninvolved epidermis, that is, epidermis that was treated but did not contain visible papillomas, and papillomas were analyzed. Whole-cell extracts were subjected to Western blot analysis and probed with the indicated antibodies (*D*). Blots are representative of at least three independent experiments.

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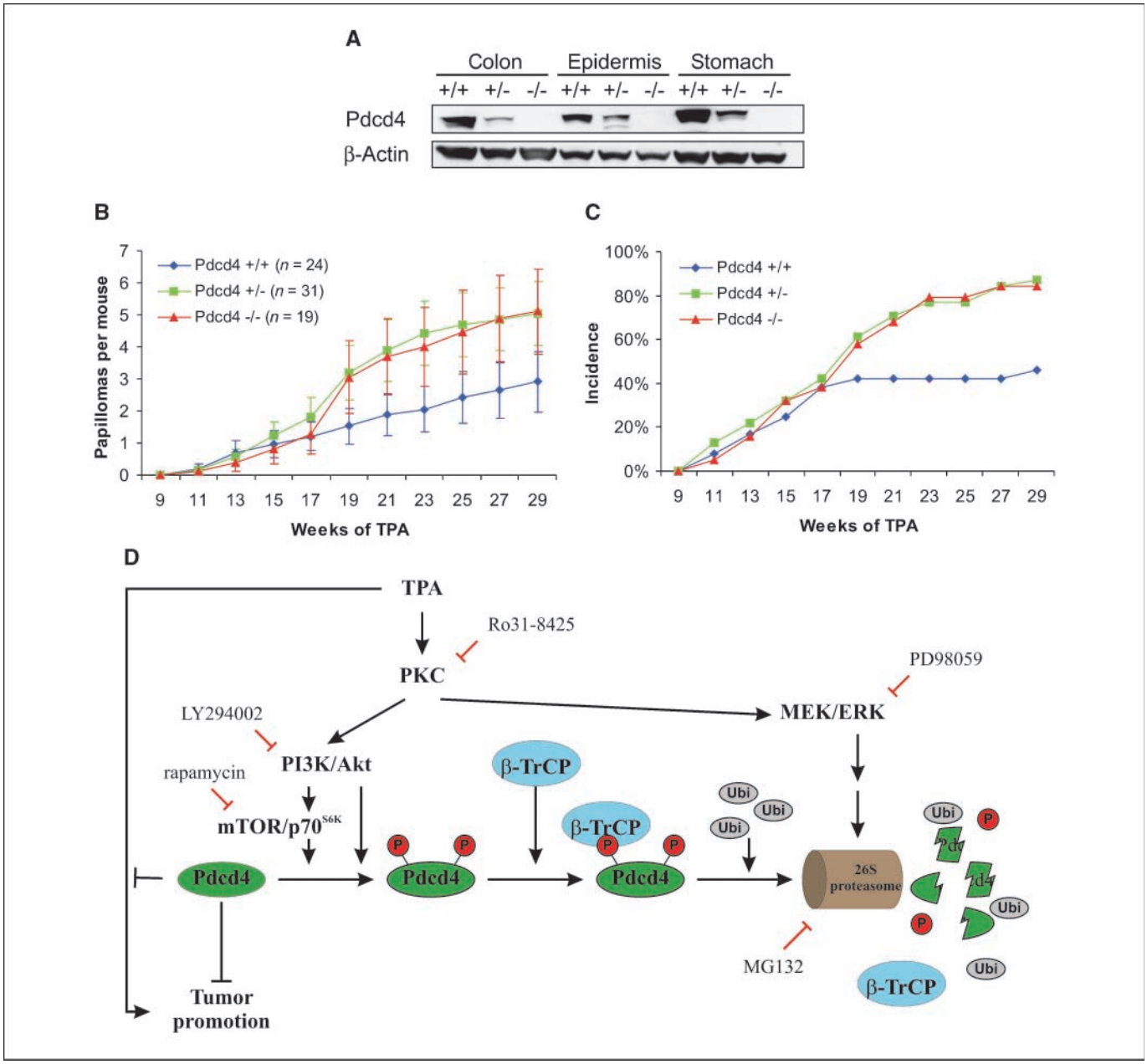


**Figure 4.** Pdcd4 protein levels determine susceptibility to tumor promotion. *A,* Pdcd4 protein status in colon, epidermis, and stomach tissues of wild-type (+/+) mice, and mice heterozygous (+/−) and nullizygous (−/−) for Pdcd4 were analyzed. Whole-cell extracts were subjected to Western blot analysis and probed with the indicated antibodies. Blots are representative of at least three independent experiments. In *B* and *C*, Pdcd4 wild-type (+/+, *blue diamonds*; *n* = 24), heterozygous (+/−, *green squares*; *n* = 31), and nullizygous (−/−, *red triangles*; *n* = 19) mice were treated according to the two-stage skin carcinogenesis protocol with DMBA and TPA. Papilloma formation was monitored for 29 wk. *Points,* papilloma multiplicity (*B*) and incidence (*C*), i.e., the percentage of mice with at least one papilloma; *bars,* SD. *D,* proposed model of TPA-induced Pdcd4 regulation.

verified by detection of similar amounts of Pdcd4/IgG in the immunoprecipitate.

To further characterize the influence of the MEK-ERK pathway, cells were transfected with a plasmid expressing hemagglutinin (HA)-tagged ubiquitin. Cells were pretreated with LY294002 or PD98059 to block PI3K and MEK signaling, respectively, for 30 minutes and then exposed to TPA for 8 hours. Proteasomal degradation was blocked by addition of MG132 after 4 hours. The effects on ubiquitylation were evaluated by immunoprecipitation of Pdcd4 and detection of the HA-tag of the ubiquitin. As predicted, blocking the proteasome was necessary to allow for accumulation of ubiquitylated Pdcd4. TPA strongly increased the amount of ubiquitylated Pdcd4 compared with vehicle control–treated cells. TPA-induced ubiquitylation of Pdcd4 was lowered if PI3K was inhibited. Inhibition of the MEK-ERK pathway, while rescuing Pdcd4 protein from TPA-induced degradation, affected ubiquitylation status only to a minor extent (Fig. 3*C*). Control experiments confirmed that Pdcd4 was phosphorylated, bound by β-TrCP1, and ubiquitylated even if not stimulated by TPA due to the incubation conditions with full serum. TPA, however, vastly increased the level of ubiquitylated Pdcd4 (Supplementary Fig. S3).

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Thus, although phosphorylation of Pdcd4 by Akt and $p70^{S6K}$ marks it for ubiquitylation and is essential for serum- and TPA-induced degradation of Pdcd4, in the case of TPA-induced degradation additional mechanisms enable Pdcd4 degradation. These yet to be identified mechanisms involve the MEK pathway, which apparently acts subsequent to phosphorylation of Pdcd4, binding of the E3-ubiquitin ligase β-TrCP1 and ubiquitylation.

**Endogenous Pdcd4 protein levels determine the susceptibility of mice to tumor formation in the two-stage skin carcinogenesis model.** To ascertain that the regulatory mechanisms described above hold true for the *in vivo* situation, uninvolved epidermis and papillomas that had formed in transgenically Pdcd4-overexpressing mice subjected to the two-stage skin carcinogenesis protocol were analyzed (Fig. 3*D*). Pdcd4 downregulation correlated inversely with active PI3K-mTOR-$p70^{S6K}$ pathway in papillomas. In those papillomas where loss of Pdcd4 did not correlate with active $p70^{S6K}$ as measured by phosphorylation of S6, Akt was phosphorylated (i.e., activated). This suggests that either Akt or $p70^{S6K}$ can act to prime Pdcd4 for degradation *in vivo*. This is consistent with *in vitro* observations in HEK293 cells stably overexpressing constitutively active Akt (Myr-Akt), in which blocking the Akt-pathway downstream of mTOR (i.e., upstream of $p70^{S6K}$) only partially restored Pdcd4 levels (Supplementary Fig. S2). Phosphorylation of ERK as an indicator for MEK activation was detected in all papillomas analyzed, and thus does not seem to be limiting in papillomas. Thus, Akt and/or $p70^{S6K}$ activity seems to be predictive for loss of Pdcd4 in this tumor model, which inherently selects for MEK activation.

To evaluate the biological relevance of Pdcd4 degradation, Pdcd4-deficient mice were analyzed for susceptibility to DMBA/TPA–induced skin carcinogenesis. Heterozygosity and nullizygosity for Pdcd4 resulted in ~50% and complete loss of Pdcd4 protein compared with wild-type, respectively (Fig. 4*A*). In response to the two-stage skin carcinogenesis regimen, both 50% and complete loss of Pdcd4 caused a significant increase in papilloma multiplicity (Fig. 4*B*) from 2.91 tumors per mouse in wild-type animals to 5.03 and 5.11 in heterozygous and nullizygous animals, respectively. Similarly, a significant increase in the incidence was observed between wild-type (46%) and animals heterozygous (87%) or nullizygous (84%) for Pdcd4 (Fig. 4*C*; for statistical analysis, see Supplementary Table S1).

Thus, Pdcd4 null in combination with Pdcd4 transgenic mice define a range of Pdcd4 levels that limit susceptibility to tumorigenesis.

## Discussion

This study elucidates a mechanism by which Pdcd4 is lost during tumorigenesis. Exposure to the tumor promoter TPA decreases protein levels of the tumor suppressor Pdcd4 (see model in Fig. 4*D*). This effect is induced by PKC-mediated activation of PI3K-Akt-mTOR-$p70^{S6K}$ signaling, which leads to phosphorylation of Pdcd4, thereby targeting it for proteasomal degradation. Furthermore, activation of MEK-ERK signaling by TPA is required to facilitate degradation of ubiquitylated Pdcd4. Pdcd4 levels are decisive for carcinogenesis. Low levels of Pdcd4 seem to allow for higher rates of tumor formation (Fig. 4), whereas elevated levels are protective (2).

Pdcd4 inhibits tumorigenesis by inhibiting translation of a specific set of mRNAs. Most studies have concentrated on downstream outcomes of altered Pdcd4 expression. In contrast, little has been known about processes that inactivate this tumor suppressor. Our results indicate that activation of both PI3K and MEK signaling is required to mediate and maintain downregulation of Pdcd4 protein by TPA. Although small changes in mRNA stability and/or transcription were not excluded, the major mechanism of regulation was clearly identified as posttranslational by increased proteasomal degradation. This is consistent with recent findings showing increased proteasomal Pdcd4 degradation in response to serum-deprivation-readdition in a PI3K-mediated, $p70^{S6K1}$-dependent manner (18). These findings now extend to tumor promotion as a mechanism to regulate the tumor suppressor Pdcd4. We show that in addition to $p70^{S6K}$, Akt accounts for a certain proportion of the required phosphorylation (Supplementary Fig. S2). In contrast to serum-deprivation-readdition, TPA-induced degradation of phosphorylated Pdcd4 also requires active MEK-ERK signaling. Although MEK-ERK signaling acts partially to regulate Pdcd4 ubiquitylation, the major effect is to facilitate Pdcd4 degradation downstream of ubiquitylation. This might involve facilitated shuttling of ubiquitylated Pdcd4 to the proteasome or conformational changes in the Pdcd4-ubiquitylation machinery, influencing the accessibility of ubiquitylated Pdcd4 for proteasomal degradation. Although the MEK-ERK role might not be operative in homeostasis (i.e., in response to serum-deprivation-readdition), it seems to be essential in the case of tumor promoter-induced regulation, a mechanism that seems to become permanent in maintaining the tumor phenotype. The DMBA-TPA skin carcinogenesis model selects for activation of Ras signaling and consequently of MEK-ERK signaling (19). In this *in vivo* model, in which MEK-ERK is activated in all papillomas, the level of activation of the MEK-ERK pathway may not be important for the loss of Pdcd4 as long as it exceeds a threshold level needed in combination with Akt $p70^{S6K}$ activation. The level of either activated $p70^{S6K}$ or activated Akt, on the other hand, is predictive for the loss of Pdcd4; that is, Pdcd4 loss correlates with activated $p70^{S6K}$ in most papillomas, whereas in others lack of $p70^{S6K}$ activation seems to be compensated for by increased Akt activation (Fig. 3*D*). This seemingly paradoxical observation is in line with a recent report indicating that blocking mTOR or $p70^{S6K}$ activity activates Akt through an insulin-like growth factor type I receptor–mediated feedback mechanism (20). The inverse correlation between activated MEK signaling and Pdcd4 protein levels in uninvolved epidermis underlines the importance of the MEK-ERK axis in the regulation of Pdcd4. The variability of MEK-ERK activation in uninvolved epidermis may reflect cellular heterogeneity with respect to expression of activated Ras during the clonal selection for activated Ras-expressing cells.

The decreased papilloma incidence in Pdcd4-overexpressing animals (2), coupled with the increased papilloma incidence in Pdcd4-deficient mice, suggests that Pdcd4 protects against tumor induction. The observation that complete and 50% loss of Pdcd4 allowed for similar outcomes in tumor yield and tumor incidence indicates that Pdcd4 protein levels below a certain threshold apparently do not suffice to prevent TPA-induced tumorigenesis and establishes Pdcd4 as a haploinsufficient suppressor. Such is also the case for the cell cycle–regulating tumor suppressor p27 (21). The biological processes that contribute to Pdcd4-attenuated tumorigenesis are not known. We postulate that Pdcd4 inhibits the translation of specific mRNAs that drive tumorigenesis. Some of these mRNA targets are important in AP-1 activation (3), an event required for tumorigenesis. Discovery of the mRNAs selectively targeted by Pdcd4 will facilitate the inquiry into the mechanism by which Pdcd4 suppresses tumorigenesis.

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In summary, our findings suggest that targeting translation initiation by stabilizing Pdcd4 expression might be a promising approach for preventing tumorigenesis. Recent studies using gene therapy delivery of Pdcd4 to mouse lungs indicate efficacy for inducing apoptosis in lung cancer cells (22). As an alternative to delivering Pdcd4, the prevention of Pdcd4 degradation might be envisioned using proteasome inhibition or specific targeting of the E3-ubiquitin ligase β-TrCP1.

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