Translation inhibitor Pdcd4 is targeted for degradation during tumor promotion

Supplementary Information

Supplementary Fig. S1.

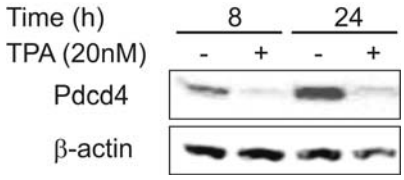

Fig. S1. HEK293 cells were exposed to TPA (20nM) for 8 or 24h. Whole cell extracts were subjected to Western blot analysis and probed with the indicated antibodies. Blots are representative for at least 3 independent experiments.

Supplementary Fig. S2.

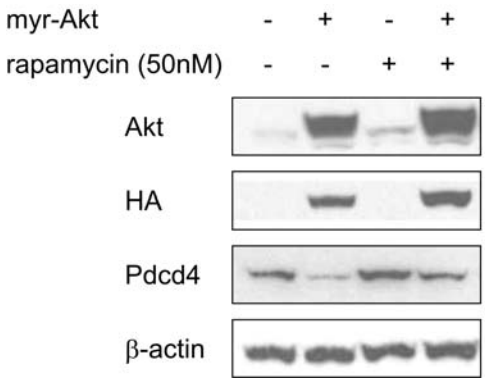

Fig. S2. Akt-mediated Pdcd4 degradation can be partially blocked by mTOR inhibition. HEK293 cells stably expressing either control vector or HA-tag-containing Myr-Akt expression vector were created by retroviral infection (for details see Supplementary Text). Cells were serum starved for 24h and then treated with mTOR-inhibitor rapamycin (50nM) for 8h. Whole cell extracts were subjected to Western blot analysis. Expression of Akt was verified by detection of Akt and of the HA-tag of the Myr-Akt construct using antibodies described in Supplementary Text. Pdcd4 expression was detected using a previously published antibody against Pdcd4 (1). Blots are representative for at least 3 independent experiments.

Supplementary Fig. S3.

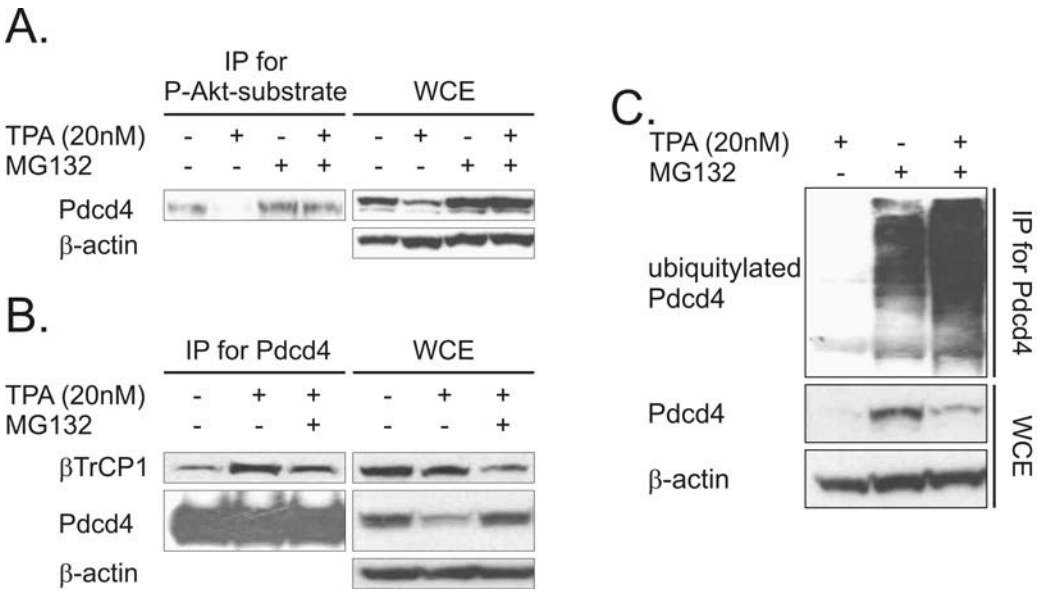

Fig. S3. TPA exposure induces PI3K-dependent phosphorylation and ubiquitylation of Pdcd4 – vehicle and TPA-alone controls.

A, HEK293 cells were pre-incubated with the proteasome inhibitor MG132 for 30min. Subsequently, TPA (20nM) was added and incubations continued for 8h. Pdcd4 is phosphorylated in vehicle alone conditions due to full serum conditions. TPA strongly attenuates total Pdcd4 protein and consequently phosphorylated Pdcd4. Blocking the proteasome increases both total and phosphorylated Pdcd4 in TPA-treated cells. *B,* HEK293 cells were pre-incubated with MG132 (10μM) for 30min. TPA (20nM) was added and incubations continued for 8h. β-TrCP1-binding to Pdcd4 increases in response to TPA. While there is a slight decrease in TPA-induced binding of β-TrCP1 to Pdcd4 upon inhibition of the proteasome, this can be attributed to slightly lower levels of total β-TrCP1 in the whole cell extract. *C,* HEK293 cells were transfected with a plasmid expressing HA-tagged ubiquitin. On the following day, cells were incubated with TPA

(20nM) for 4h before proteasomal degradation was blocked by addition of MG132 (10μM). Incubations continued for another 4h. TPA induces rapid degradation of Pdcd4 and does not allow for accumulation of ubiquitylated Pdcd4 when the proteasome is active. Inhibition of the proteasome allows for accumulation of ubiquitylated Pdcd4 under control and to a larger extend under TPA-treated conditions. 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. Blots are representative for at least 3 independent experiments.

Supplementary Fig. S4.

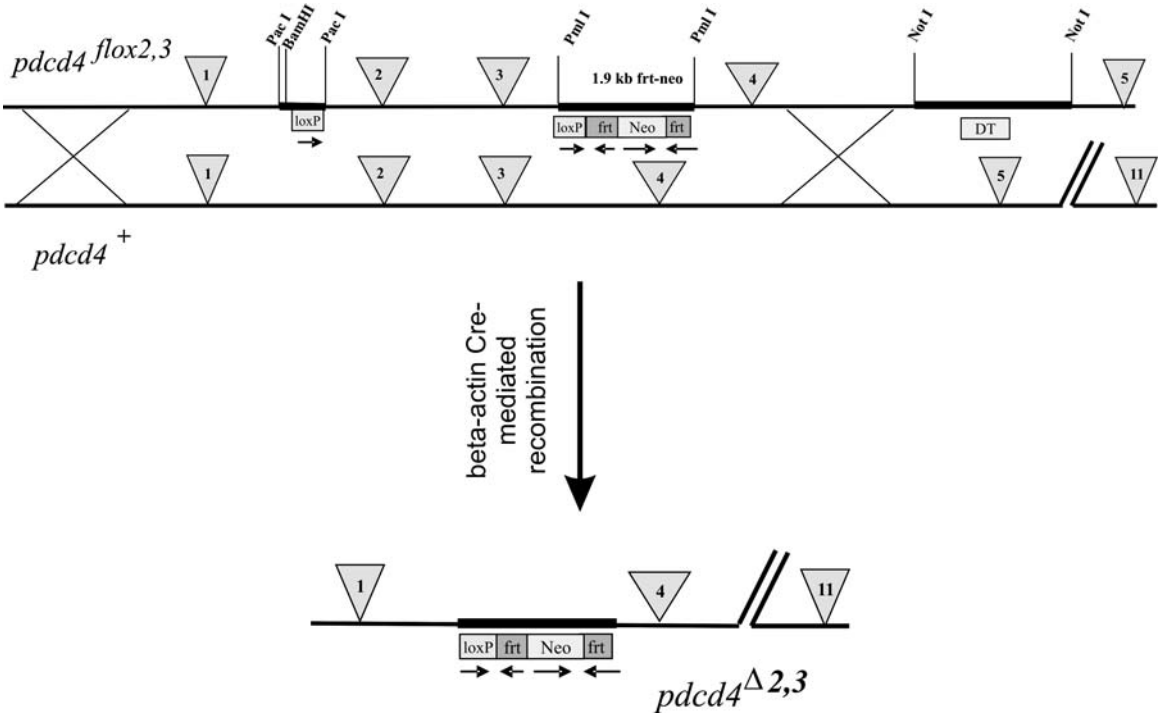

Fig. S4. Generation of Pdcd4-deficient mice. Generating the pdcd4 null alleles using Cre-mediated recombination. The mouse *pdcd4* gene exists as a single gene with 11 exons spanning 21Kbp. Ablation of exons 2 and 3 from the *pdcd4* gene will cause a frameshift mutation of the *pdcd4* transcript creating a stop codon at amino acid 16. The targeting allele was constructed from genomic *pdcd4* isolated form bac clones. The targeting allele contains two *frt* sites (flanking the neo marker) for Flp mediated recombination and two *loxP* sites (flanking exons 2 and 3) for cre-mediated homologous recombination. The targeting allele was cloned on a mixed 129 / C57Bl/6 background. Cre-mediated recombination was achieved by crossing target allele bearing mice with CD-1 beta-actin cre mice.

Supplementary Fig. S5.

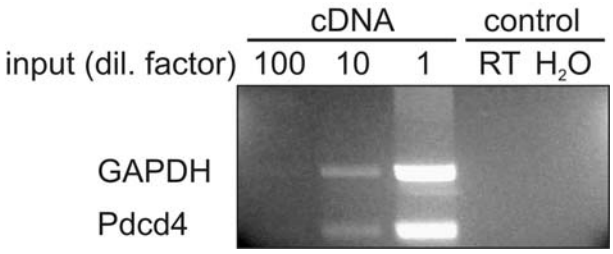

Fig. S5. Validation of the RT-PCR conditions. To validate that the RT-PCR conditions used were suitable to allow for the conclusions drawn, mRNA from HEK293 cells was reverse transcribed. The resulting cDNA either undiluted (dil. factor: 1), diluted 10- or 100-fold in H_2O was used for PCR (25 cycles). The resulting products were separated on an agarose gel and visualized with ethidium bromide. An RT control and a water control were added. As seen in lanes 1, 2 and 3, the PCR at 25 cycles allows for determination of changes at the mRNA level both for Pdcd4 and GAPDH. The RT- and water controls ruled out unspecific effects and contaminations.

Supplementary Table S1.

Table S1. Pdcd4-deficiency increases both papilloma multiplicity and papilloma incidence.

Pdcd4 status	+/+	+/-	-/-
Multiplicity	2.91	5.03¶	5.11¶
Incidence	46%	87%‖	84%‖

NOTE: Mouse skin was initiated with a single application of DMBA (100nmol), followed by twice weekly application of TPA (5nmol/0.2mL acetone) for 29 weeks. Changes in papilloma multiplicity and incidence between mice Pdcd4 hetero- (+/-) and nullizygous (-/-) mice were compared to respective wild-type (+/+) littermates at 29 weeks. Two-sided *P* values were calculated for papilloma multiplicity by Wilcoxon rank sum test and for tumor incidence by Fisher's exact test.

¶ $p<0.05$ and $p=0.07$ for comparison between +/+ and +/- and +/+ and -/-, respectively.

‖ $p<0.01$ and $p<0.05$ for comparison between +/+ and +/- and +/+ and -/-, respectively.

Supplementary Text

RT-PCR conditions and primers. 0.25μg RNA were used for reverse transcription. Pdcd4 (primer 1: TAA TCA GTG CAA GCG AAA TTA AGG AA; primer 2: CCT TTC CCA GAT CTG GAC CGC CTA TC) and GAPDH (primer 1: TGA AGG TCG GAG TCA ACG GAT TTG GT; primer 2: CAT GTG GGC CAT GAG GTC CAC CAC) fragments were amplified at 25 cycles (15sec, 94°C; 30sec, 55°C; 45sec, 72°C).

Generation of stable cell lines. pSRαMSVtkneo and pSRαMSVtkneo-Myr-Akt, kindly provided by P.A. Dennis, were described previously (2, 3). Plasmids were transfected into RetroPack PT67 cells, and packaged virus was collected using established methods (Clontech). HEK293 cells stably expressing either the control vector or Myr-Akt were created via retroviral infection. Stable clones were selected for using DMEM medium containing 10% FBS, 4,500mg/l D-glucose, 2mM L-glutamine, 110mg/l sodium pyruvate, 25μg/ml gentamicin, and 1mg/ml G418. Following two weeks of selection, resistant clones were pooled and reseeded in selective media for maintenance and further experimentation. For verification of the expression anti-Akt-antibody (Cell Signaling) and anti-HA-antibody (Roche) were used.

Supplementary References

1. Jansen AP, Camalier CE, Stark C, Colburn NH. Characterization of programmed cell death 4 in multiple human cancers reveals a novel enhancer of drug sensitivity. Mol Cancer Ther 2004;3(2):103-10.
2. Ahmed NN, Grimes HL, Bellacosa A, Chan TO, Tsichlis PN. Transduction of interleukin-2 antiapoptotic and proliferative signals via Akt protein kinase. Proc Natl Acad Sci U S A 1997;94(8):3627-32.

3. Muller AJ, Young JC, Pendergast AM, *et al.* BCR first exon sequences specifically activate the BCR/ABL tyrosine kinase oncogene of Philadelphia chromosome-positive human leukemias. Mol Cell Biol 1991;11(4):1785-92.