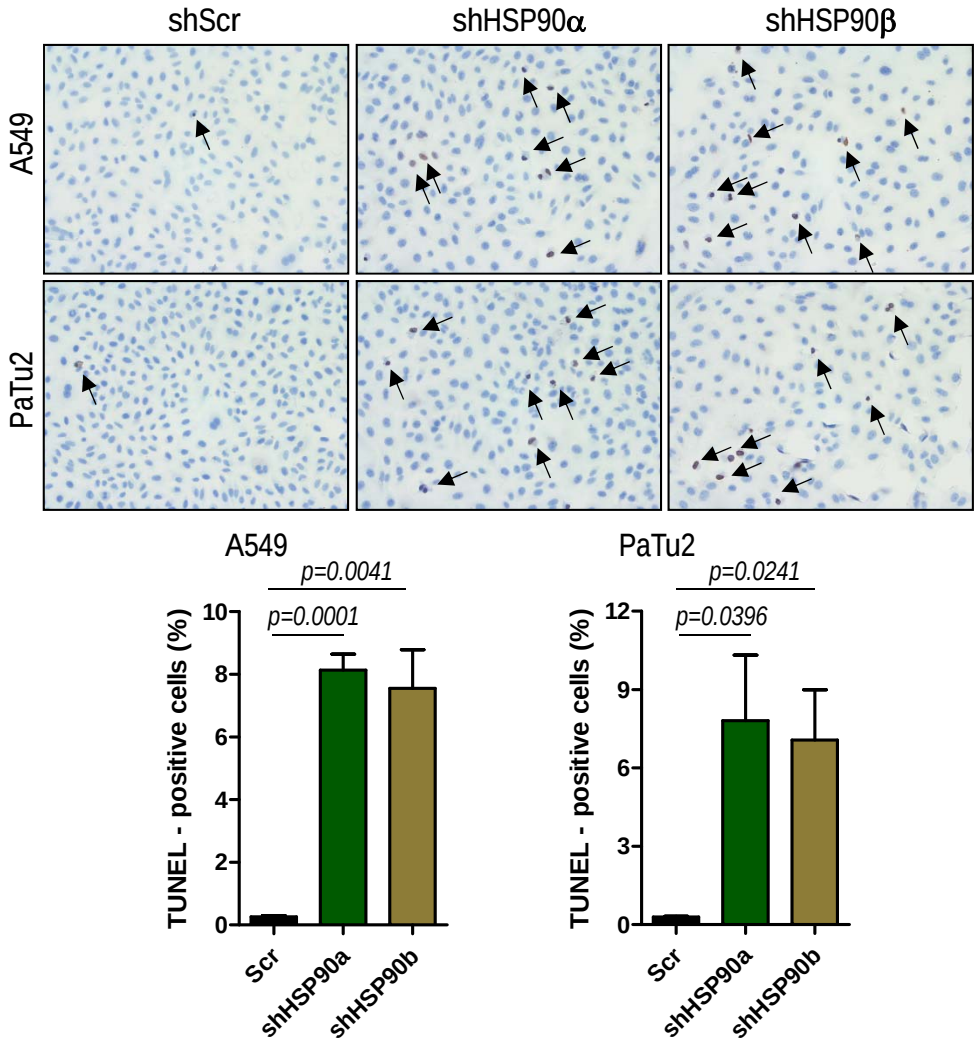
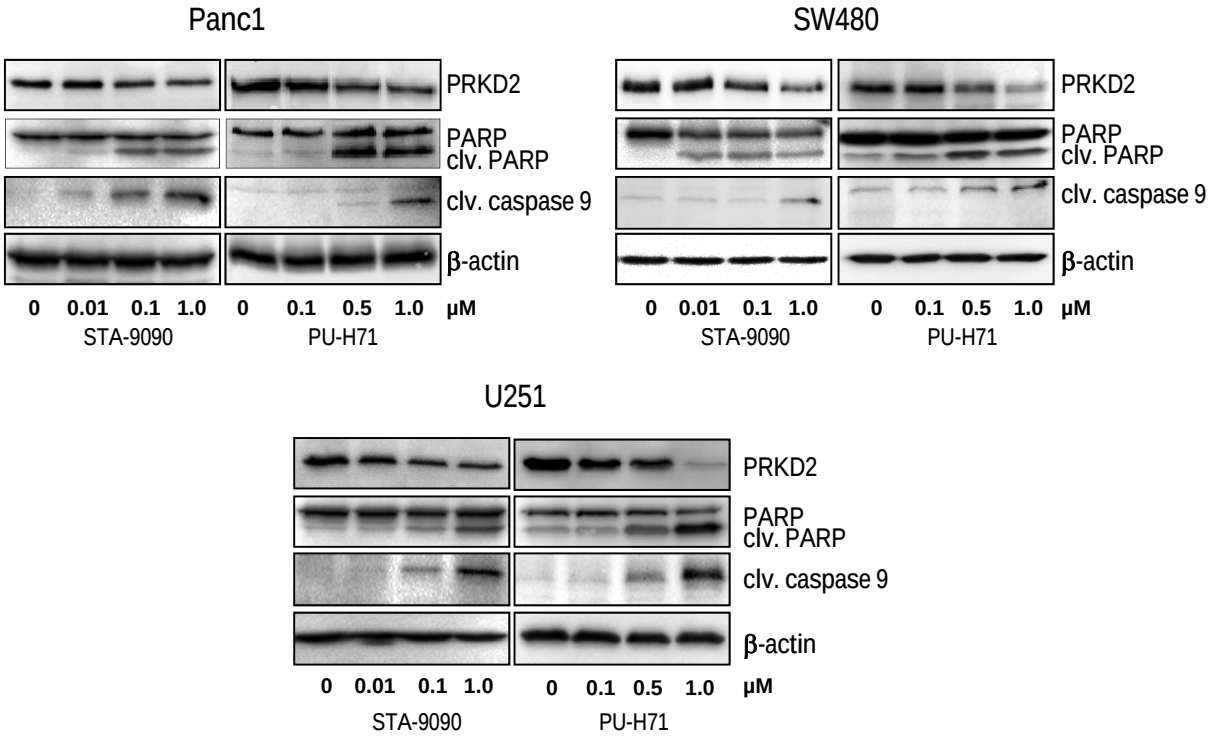
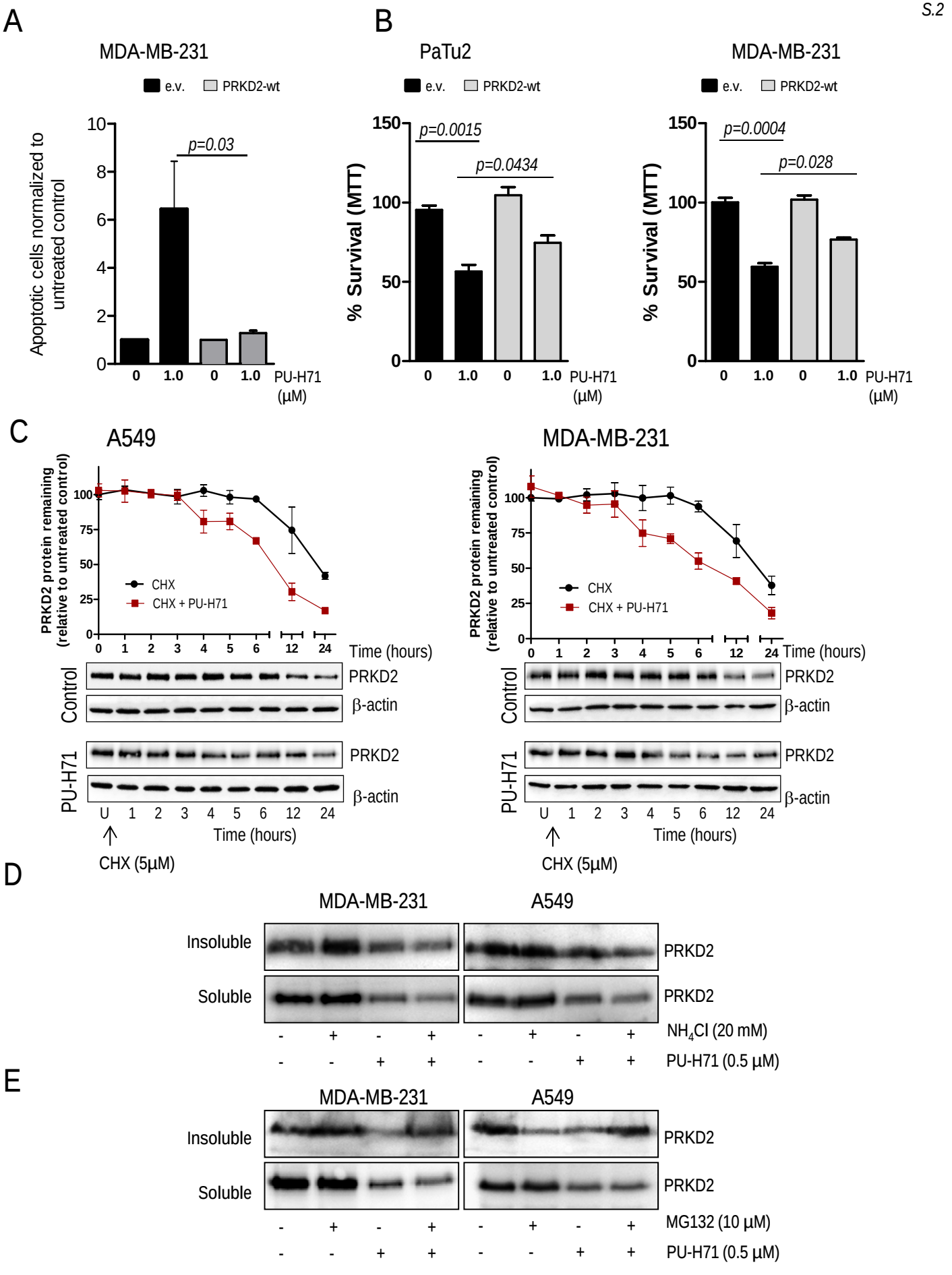
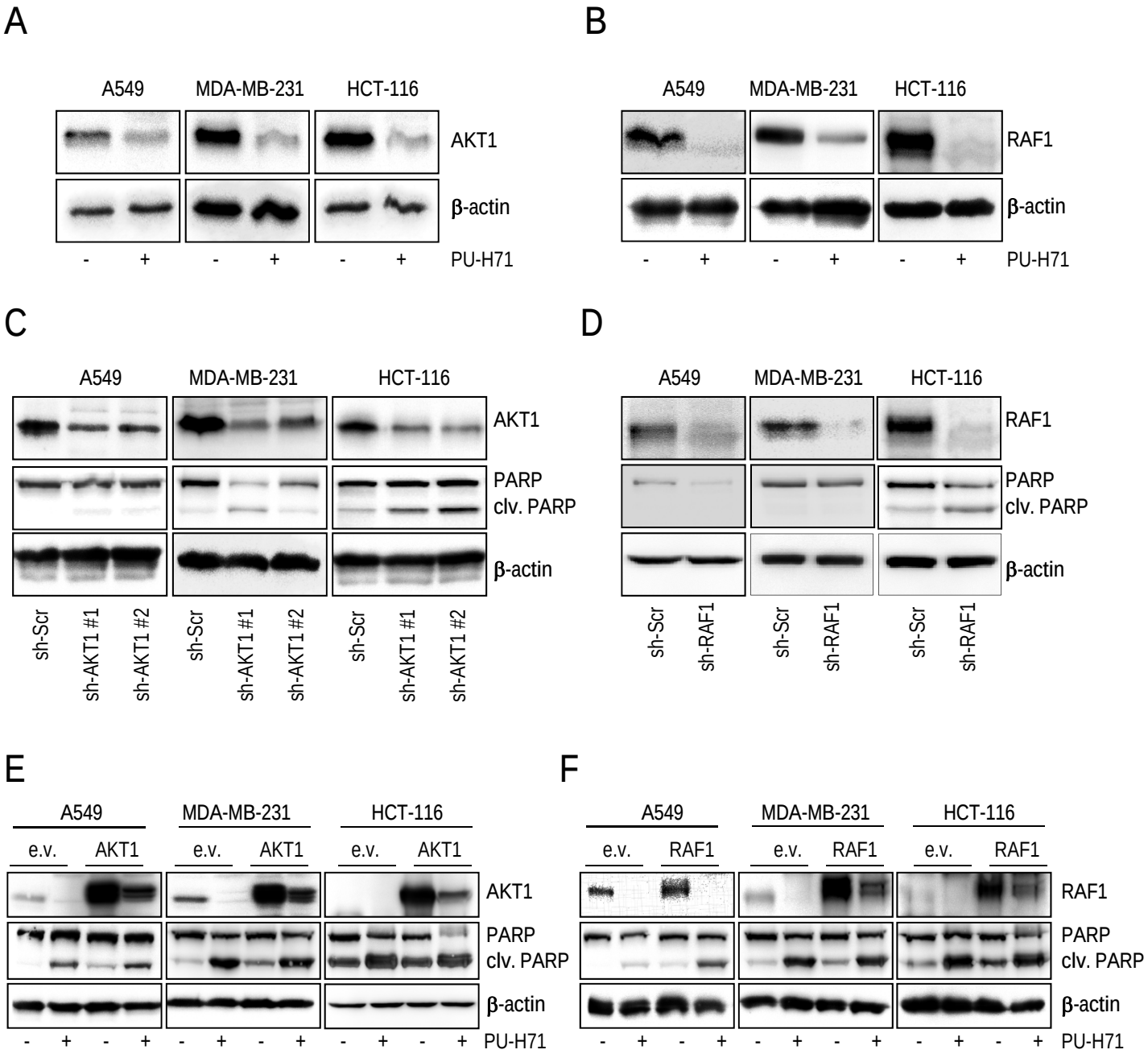
A

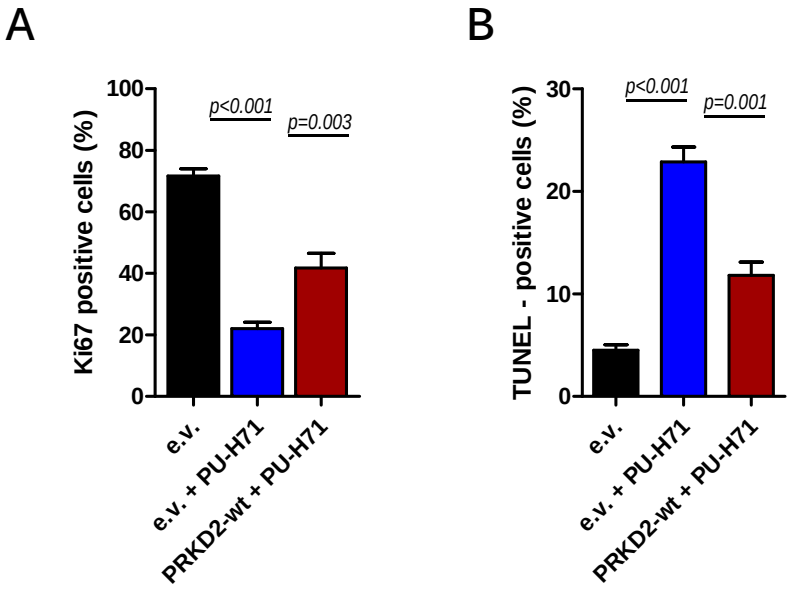

shScr
shHSP90α
shHSP90β
A549
PaTu2
A549
p=0.0041
p=0.0001
TUNEL - positive cells (%)
10
8
6
4
2
0
Scr
shHSP90a
shHSP90b
PaTu2
p=0.0241
p=0.0396
12
9
6
3
0
Scr
shHSP90a
shHSP90b

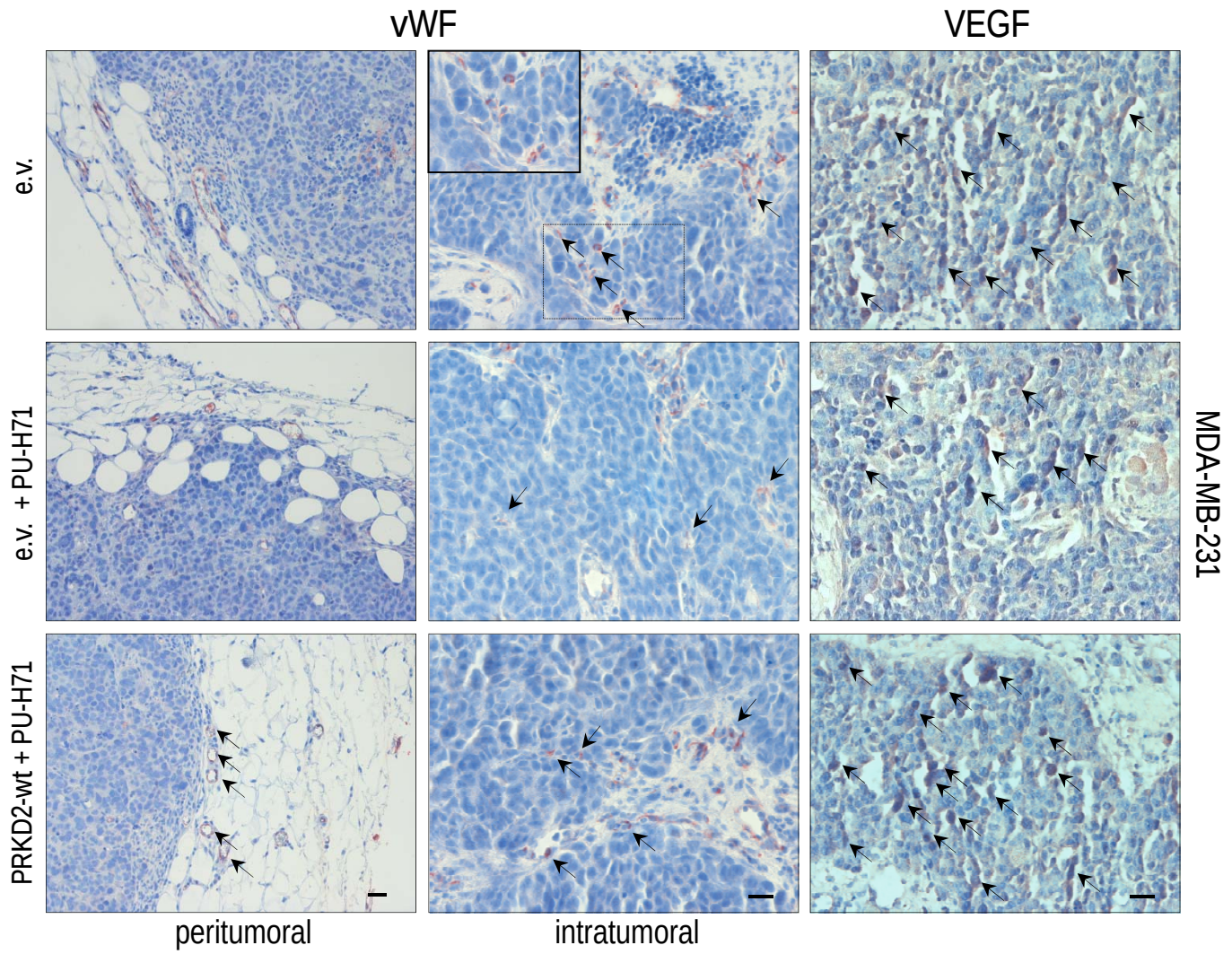
B

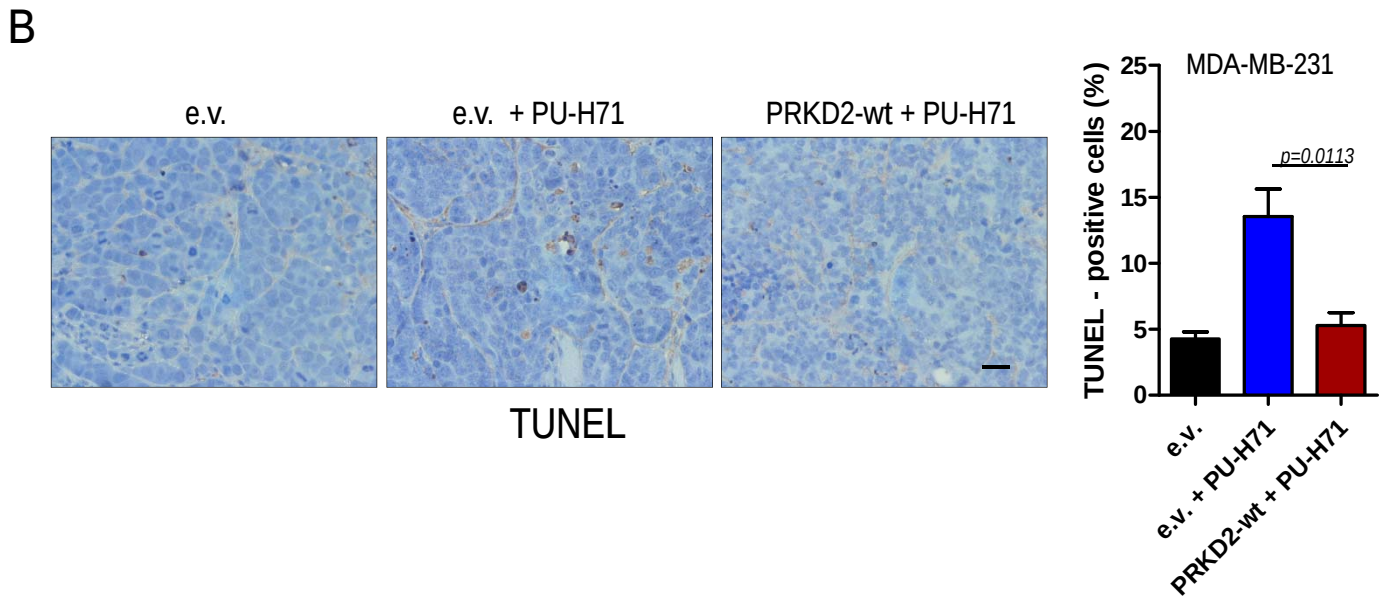

Panc1
PRKD2
PARP
clv. PARP
clv. caspase 9
β-actin
0 0.01 0.1 1.0 0 0.1 0.5 1.0 μM
STA-9090
PU-H71
SW480
PRKD2
PARP
clv. PARP
clv. caspase 9
β-actin
0 0.01 0.1 1.0 0 0.1 0.5 1.0 μM
STA-9090
PU-H71
U251
PRKD2
PARP
clv. PARP
clv. caspase 9
β-actin
0 0.01 0.1 1.0 0 0.1 0.5 1.0 μM
STA-9090
PU-H71


A
MDA-MB-231
e.v.
PRKD2-wt
Apoptotic cells normalized to untreated control
p=0.03
PU-H71 (μM)
B
PaTu2
MDA-MB-231
% Survival (MTT)
p=0.0015
p=0.0434
p=0.0004
p=0.028
C
A549
MDA-MB-231
PRKD2 protein remaining (relative to untreated control)
CHX
CHX + PU-H71
Time (hours)
Control
PU-H71
PRKD2
β-actin
CHX (5μM)
D
Insoluble
Soluble
NH4Cl (20 mM)
PU-H71 (0.5 μM)
E
MG132 (10 μM)

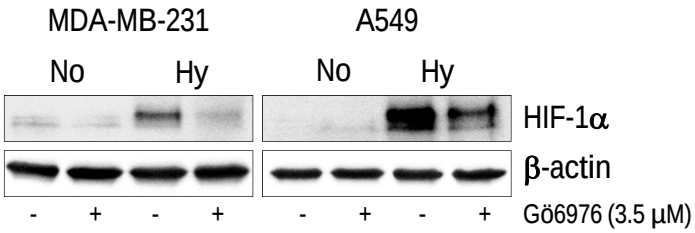

A
A549
MDA-MB-231
HCT-116
AKT1
β-actin
- + - + - +
PU-H71
B
A549
MDA-MB-231
HCT-116
RAF1
β-actin
- + - + - +
PU-H71
C
A549
MDA-MB-231
HCT-116
AKT1
PARP
clv. PARP
β-actin
sh-Scr
sh-AKT1 #1
sh-AKT1 #2
D
A549
MDA-MB-231
HCT-116
RAF1
PARP
clv. PARP
β-actin
sh-Scr
sh-RAF1
E
A549
MDA-MB-231
HCT-116
e.v.
AKT1
AKT1
PARP
clv. PARP
β-actin
- + - + - + - + - + - +
PU-H71
F
A549
MDA-MB-231
HCT-116
e.v.
RAF1
RAF1
PARP
clv. PARP
β-actin
- + - + - + - + - + - +
PU-H71


A
Ki67 positive cells (%)
100
80
60
40
20
0
p<0.001
p=0.003
e.v.
e.v. + PU-H71
PRKD2-wt + PU-H71
B
TUNEL - positive cells (%)
30
20
10
0
p<0.001
p=0.001
e.v.
e.v. + PU-H71
PRKD2-wt + PU-H71

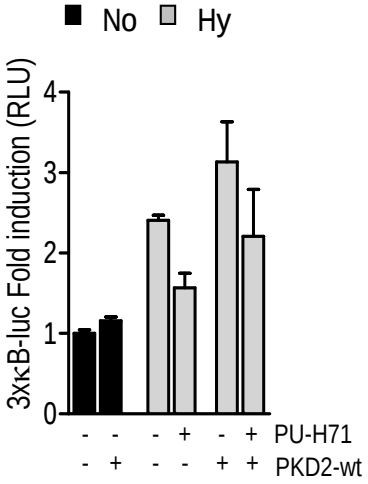

A
vWF
VEGF
e.v.
e.v. + PU-H71
PRKD2-wt + PU-H71
MDA-MB-231
peritumoral
intratumoral


B
e.v.
e.v. + PU-H71
PRKD2-wt + PU-H71
TUNEL
MDA-MB-231
TUNEL - positive cells (%)
25
20
15
10
5
0
p=0.0113
e.v.
e.v. + PU-H71
PRKD2-wt + PU-H71

A


MDA-MB-231
A549
No
Hy
No
Hy
HIF-1α
β-actin
- + - + - + - + Gö6976 (3.5 μM)

B


No
Hy
3κB-luc Fold induction (RLU)
0
1
2
3
4
- - - + - + PU-H71
- + - - + + PKD2-wt

Supplementary Figure legends

Supplementary Figure S1. HSP90 silencing results in augmented apoptosis. A, A549 and PaTu2 cancer cell lines transduced with a non-targeting control shRNA (Scr) or a shRNA-targeting HSP90α or HSP90β were subjected to TUNEL analysis. Quantification of TUNEL-positive is displayed. Error bars represent mean +/- SEM of at least four microscopic fields with 200 cells. **B,** Pancreatic cancer (Panc1), colon cancer (SW480) and glioblastoma (U251) cell lines were incubated with increasing amounts of PU-H71 and STA-9090 as indicated. Western blot analysis with PRKD2, cleaved PARP and anti-cleaved caspase 9 are depicted.

Supplementary Figure S2. Destabilization of PRKD2 is essential for HSP90 inhibition-triggered apoptosis in tumor cells. A, MDA-MB-231 cells transiently transfected with empty vector (e.v.) or PRKD2-wildtype (PRKD2-wt) and incubated with PU-H71 for 24 hours were subjected to Annexin V / Propidium iodide staining. The bar graphs represent the means of Annexin V+/PI- cells (normalized to untreated control). **B,** pancreatic and breast cancer cells were transiently transfected with empty vector (e.v.) or PRKD2-wildtype (PRKD2-wt) before incubation with PU-H71 for 24 hours. MTT assay was conducted. The bar graphs represent the means +/- SEM of cancer cell survival of two independent experiments in quadruplicate. **C**, Immunoblots of PRKD2 protein abundance in control or PU-H71 treated A549 or MDA-MB-231 cells after incubation with 5 µM cycloheximine (CHX) for the indicated periods of time are presented. The relative amount of total PRKD2 to actin was quantified by densitometry by means of ImageJ. The PRKD2 to actin level in untreated cells (U) was defined as 100%. **HSP90 inhibition induces proteasomal degradation of PRKD2. D,** MDA-MB-231 and A549 cancer cell lines were incubated with 20 mM NH_4Cl for 2 h before treatment with PU-H71. Soluble and insoluble protein fractions were prepared and western blot analysis with PRKD2 antibody was conducted. **E,** breast and lung cancer cell lines were pre-treated with MG132 for 2 h before incubation with PU-H71. Soluble and insoluble protein fractions were subjected to SDS-PAGE and membranes were incubated with PRKD2 antibody.

Supplementary Figure S3. Relationship between degradation of AKT1 or RAF1 and killing cancer cells by HSP90 inhibitors. A, A549, MDA-MB-231 and HCT-116 cancer cell lines were incubated with 1 µM PU-H71 for 24 hours. Western blot analysis was conducted with AKT1 and RAf1 antibodies. **B**, Western blot analysis of A549, MDA-MB-231 and HCT-116 cancer cells after shRNA-mediated abrogation of AKT1 or RAF1 was conducted with cleaved PARP, AKT1 and RAF1. **C**, Cancer cell stably stably transduced with empty vector (e.v.), AKT1 or RAF1 were incubated with 1 µM PU-H71. Western blots with cleaved PARP, AKT or RAF1 are presented. β-actin was used as loading control.

Supplementary Figure S4. HSP90 inhibition impairs tumor growth *in vivo* in a PRKD2-dependent manner. A, 1 million MDA-MB-231 breast cancer cells stably transduced with PRKD2 (PRKD2-wt) or empty vector (e.v.) were delivered to chicken CAM. Tumors were allowed to grow for 24 hours subsequent to the ectopic treatment with 1µM PU-H71 or vehicle for the next 48 hours. Quantification of Ki67 positive MDA-MB-231 breast cancer cells is presented. **B,** quantification of TUNEL-positive cells for MDA-MB-231 cancer cell xenografted on chicken CAM is displayed. Error bars represent mean +/- SEM of at least four microscopic fields with 700 cells.

Supplementary Figure S5. PRKD2 overexpression restores tumor viability and vascularization formation in nude mice treated with PU-H71. A, immunohistological analysis with specific antibody against von Willebrand factor (vWF) and VEGF in MDA-MB-231 breast tumors xenografted on nude mice is presented. **B,** Parallel samples subjected to terminal deoxy-nucleotidyl transferase dUTP nick end labeling (TUNEL) are displayed (right panels). Quantification of TUNEL-positive cells belonging to MDA-MB-231 breast tumors xenografted on athymic mice is presented (left panel). Error bars represent mean +/- SEM of at least four microscopic fields with 600 cells. Scale bar indicates 125 µm.

Supplementary Figure S6. Proliferation of colon and breast tumors xenografted in nude mice treated with PU-H71 is restored in a PRKD2-dependent manner. A, immunohistological analysis with specific antibody against Ki67 proliferation marker and PRKD2

in breast tumors xenografted on nude mice is presented. **B,** quantifications of Ki67-positive cells for HCT-116 and MDA-MB-231 are shown. Error bars represent mean +/- SEM of at least four microscopic fields with 600 cells. Scale bar indicates 125 µm.

Supplementary Figure S7. Pharmacologic inhibition or PRKD is sufficient to abrogate hypoxia-induced HIF-1α stabilization. A, A549 lung cancer and MDA-MB-231 breast cancer cells were incubated with 3.5 µM Gö6976 PRKD inhibitor for two hours and further subjected to normoxic or low oxygen atmosphere for 24 hours. Lysates were subjected to western blot analysis with HIF-1α antibody. **B,** MDA-MB-231 breast cancer cells were transduced with control vector (e.v.) or PRKD2 (PRKD2-wt). Stable cells were transfected with 3xκB-luc reporter and further incubated under hypoxic conditions or normoxia for 24 hours in the presence or absence of HSP90 inhibitor. Cell lysates were subjected to luciferase assays. Bars are the means +/- SEM of three independent experiments. No=normoxia; Hy=hypoxia.

Supplementary Materials and Methods

Western blot and immunoprecipitation

Whole cell extracts were prepared using IP lysis buffer containing 10 mMTris-HCl, 5 mM EDTA, 50 mM NaCl, 50 mM NaF and 1% Triton X100 supplemented with Complete Protease inhibitor Cocktail and PhosStop tablets (Roche). Lysates were subjected to SDS-PAGE and proteins transferred to PVDF membranes (Millipore, Massachusetts, USA). Insoluble fractions were prepared using a buffer containing 1.5% SDS and further subjected to 16 cycles sonification (each 30s). For coIP, protein extracts (1 -1.5 mg) were incubated with 2 µg antibody and Protein G_Sepharose (GE Healthcare). Membranes were blocked with 5% non-fat dry milk in phosphate buffered saline (PBS) containing 0.2% Tween 20 and incubated over night at 4°C with specific antibodies. For subsequent washed 0.2% Tween 20 in PBS was used. Bands were quantified by densitometric analysis using ImageJ software (http://imagej/nih/gov/ij). The following antibodies were used: PRKD2 (Bethyl #A300-073A, IP and western blot analysis); PRKD2 (Epitomics #1969-1, IHC); PRKD3 (Bethyl #A300-319A); PRKD1, clone C-20 (Santa Cruz Biotechnology); cleaved PARP (Cell Signaling #9542S); cleaved caspase 9 - Asp 315 (Cell Signaling #9505S); HIF-1α (BD Transduction Laboratories #610959); VEGF-A-165A, clone 6B7 (Abcam #69479); VEGF clone VG-1, (Dako, #M7273); HSP90α (Millipore #AB3466); HSP90α/β, clone H-114 and HSP90β, clone D-19 (Santa Cruz Biotechnology), AKT1, clone H-136 (Santa Cruz Biotechnology) and RAF1 (Cell Signaling # 9422S).

FACS analysis

Annexin-V/propidium iodide (PI) staining was performed via flow cytometry according to the manufacturer's guidelines. Briefly, tumor cells washed in PBS were resuspended in 100 µl of binding buffer and incubated with 2.0 µl of Annexin V-Phycoerythrin (Annexin-V-PE)

(eBiosciences #88-8102-72) for 15 min in the dark at room temperature. Cells were washed in binding buffer and resuspended in 300 µl of the same buffer containing PI. Flow cytometric analysis was performed using FACSCalibur (Becton Dickinson).

Promoter assays

Cancer cells plated in 12-well dishes were transfected with 330 ng of the respective promoter plasmid as indicated in the figure legends using Lipofectamine 2000 (Invitrogen #15338-100) or FugeneHD (Promega #E231A) according to the manufacturer instructions. Luciferase activity was determined using the Dual Luciferase Assay Kit (Promega, Mannheim, Germany). Firefly luciferase units were normalized with Renilla luciferase after co-transfection with 17 ng/well pRL-TK plasmid (Promega, Germany).

Hypoxia experiments

The hypoxic environment was generated using the Anaerocult A Mini Kit (Merck, Darmstadt, Germany #1.01611.0001). Cells were incubated under low oxygen atmosphere for 8-24 hours at 37°C.

ELISA experiments

Experiments were performed using Quantikine human VEGF-A^{165} Immunoassay Kit (R&D Systems #DVE00) according to the manufacturer's instructions.

Cell proliferation assay (MTT)

Cellular proliferation was assessed by means of a colorimetric MTT assay (Sigma, #M5655, USA) measuring the reduction of tetrazolium salts to formazan derivatives by functional mitochondria after 24hours. Measurements were performed with an Epoch reader.