SUPPLEMENTARY DATA

A Naturally Generated Decoy of the Prostate Apoptosis Response-4 Protein Overcomes Therapy Resistance in Tumors

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Running Title: Par-4 Fragment Overcomes Therapy-resistance

Key words: Par-4, Therapy-resistance, Apoptosis

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The authors declare no potential conflicts of interest.

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Supplementary Figures

Figure S1

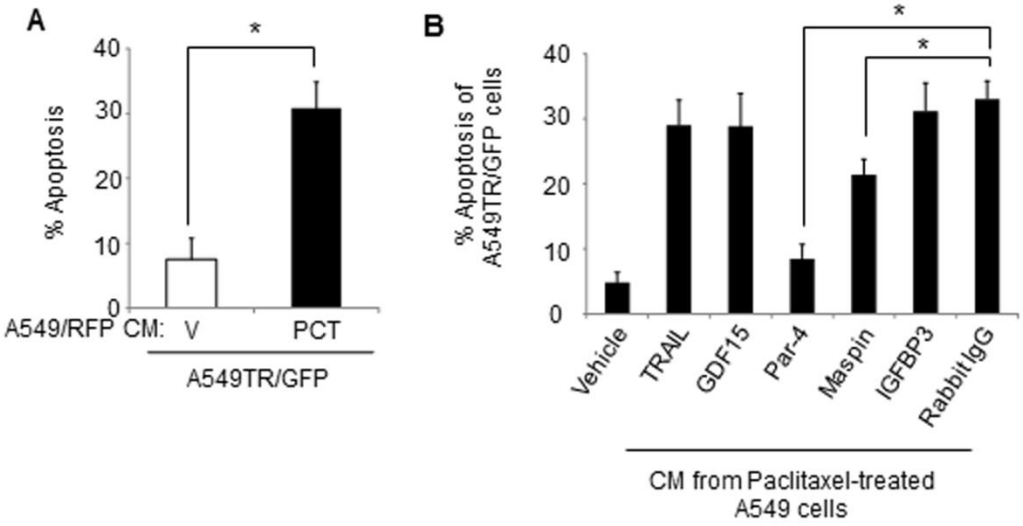

Figure S1. Paclitaxel induces paracrine apoptosis in Paclitaxel-resistant cells by inducing the secretion of a Par-4-like secreted factor.

A. CM from Paclitaxel-treated A549/RFP sensitive cells induces apoptosis in Paclitaxal-resistant A549TR/GFP cells. A549/RFP cells (1 x 10^6) were treated with Paclitaxel (PCT, 25 nM) or vehicle (V) for 24 h. The CM from the cells was transferred to untreated A549TR/GFP cells, and 24 h later, the A549TR/GFP cells were scored for apoptosis by ICC for active caspase 3.

B. Unbiased screen links apoptosis to Par-4-like factor. A549 cells were treated with Paclitaxel (25 nM) for 16 h. The CM from these cells was transferred to A549TR cells for 24 h in the presence of antibodies against TRAIL, GDF15, Par-4, Maspin, IGFBP3 or control IgG. The cells were stained and scored for apotosis by ICC for active caspase 3.

A and B. Data shown represent mean of three independent experiments $\pm$ SD. Asterisk (*) indicates statistical significance ($P < 0.001$) based on Student's t test.

Figure S2

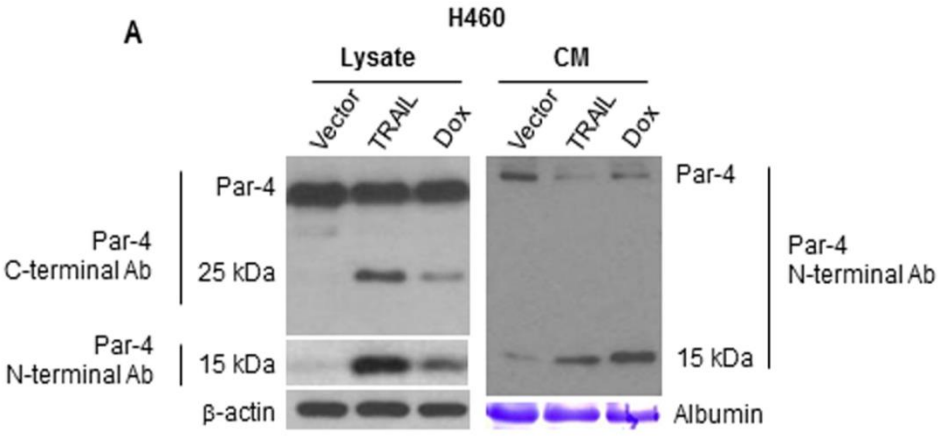
A
H460
Lysate
CM
Vector
TRAIL
Dox
Vector
TRAIL
Dox
Par-4
Par-4
C-terminal Ab
25 kDa
Par-4
N-terminal Ab
15 kDa
β-actin
Par-4
15 kDa
Albumin
Par-4
N-terminal Ab

Figure S2.

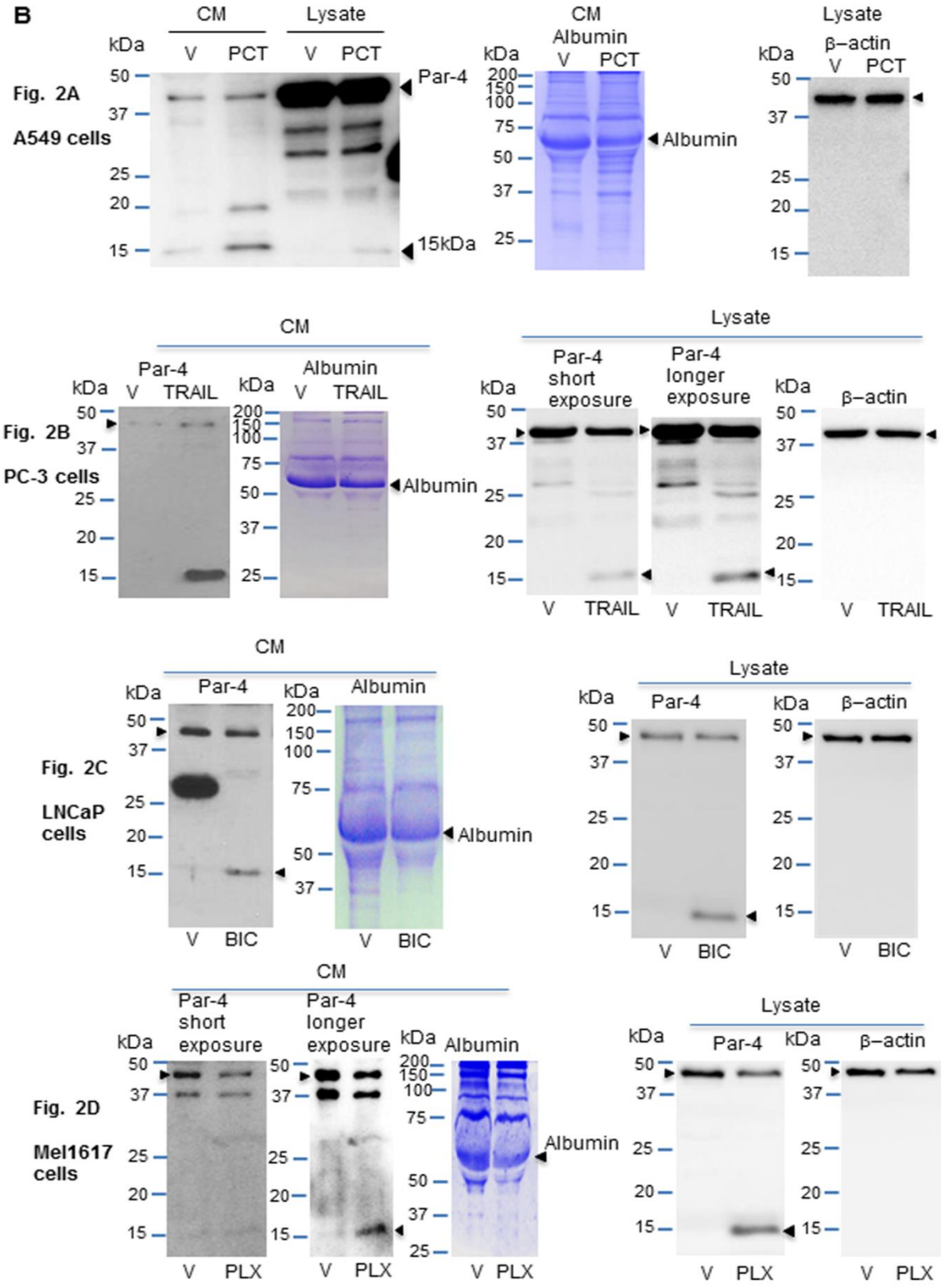
B
CM
Lysate
kDa
V
PCT
Par-4
15kDa
Fig. 2A
A549 cells
CM
Albumin
Albumin
Lysate
β–actin
Fig. 2B
PC-3 cells
Par-4
TRAIL
Par-4 short exposure
Par-4 longer exposure
Fig. 2C
LNCaP cells
BIC
Fig. 2D
Mel1617 cells
PLX

Figure S2. Par-4 is cleaved to produce the 15 kDa fragment.

A. **Effect of TRAIL or Doxorubicin**. H460 cells were treated with TRAIL (100 ng/ml) for 6 h, or Doxorubicin for 16 h. The CM and whole cell lysates were subjected to Western blot analysis with the Par-4 C-terminal antibody (Ab), Par-4 N-terminal Ab or β-actin antibody. Albumin in the CM was detected by Coomassie blue staining of the SDS-PAGE gel and used as protein loading control.

B. **Images corresponding to Figure 2 showing larger portions of each gel**. Images of Coomassie blue stained proteins or immunoblots probed with Par-4 antibody and re-probed with actin antibody are shown.

Figure S3

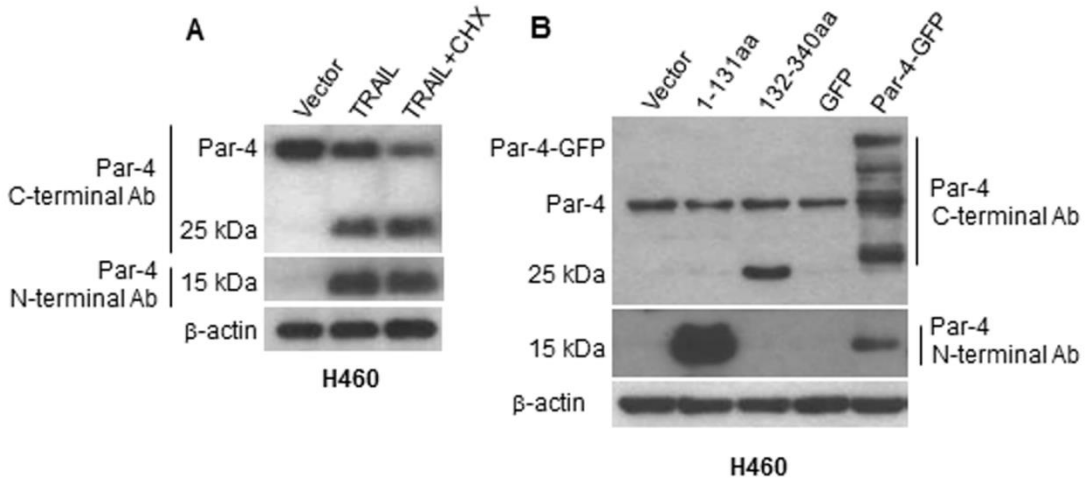

Figure S3. The 15 kDa fragment is produced by cleavage of Par-4 at D131.

A. Production of the 15 kDa and 25 kDa fragments was not dependent on new gene expression. H460 cells were pre-treated with cycloheximide (CHX, 20 μg/ml) for 30 minutes and then treated with TRAIL (100 ng/ml) for 6 h. Whole-cell lysates were subjected to Western blot analysis.

B. Identification of the 15 kDa and 25 kDa bands as the products of 1-131aa and 132-340aa, respectively, of Par-4. H460 cells were transfected with expression constructs for 1-131aa Par-4, 132-340aa Par-4, GFP, or Par-4-CT-GFP, or vector for control, and whole-cell lysates were subjected to Western blot analysis.

A and B. Western blot analysis was performed with the Par-4 C-terminal antibody (Ab), Par-4 N-terminal Ab or β-actin antibody.

Figure S4

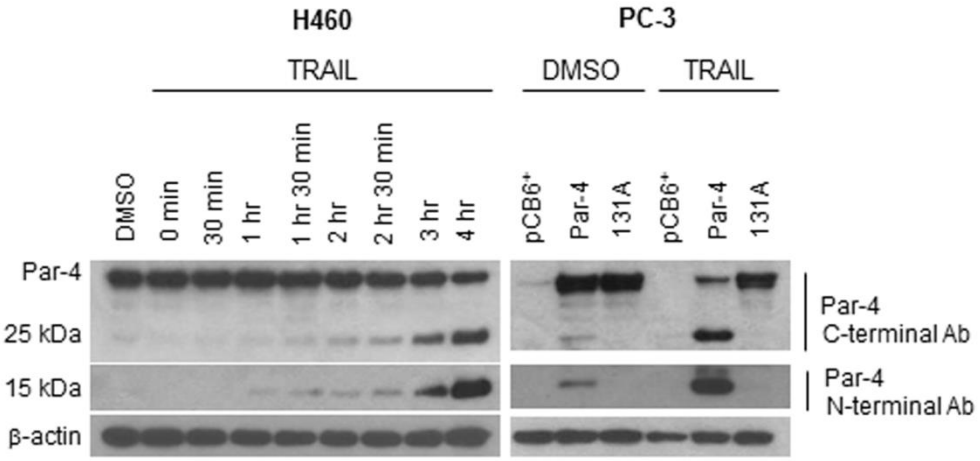

Figure S4. Par-4 cleavage occurs at the D131 residue in a caspase-dependent manner. TRAIL induces apoptosis in cancer cells by activation of the caspase 8/caspase 3 pathway. TRAIL-sensitive H460 cells (left panel) were treated with TRAIL (100 ng/ml) and whole cell lysates were collected at 30 min time intervals up to 4 h and subjected to Western blot analysis. TRAIL-sensitive PC-3 cells (right panel) were transfected with expression constructs for Par-4, Par-4/131A mutant or pCB6+ vector for 24 h and subsequently treated with TRAIL for 6 h. Whole cell lysates were prepared and subjected to Western blot analysis with the Par-4 C-terminal antibody (Ab), Par-4 N-terminal Ab or β-actin antibody.

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Figure S5

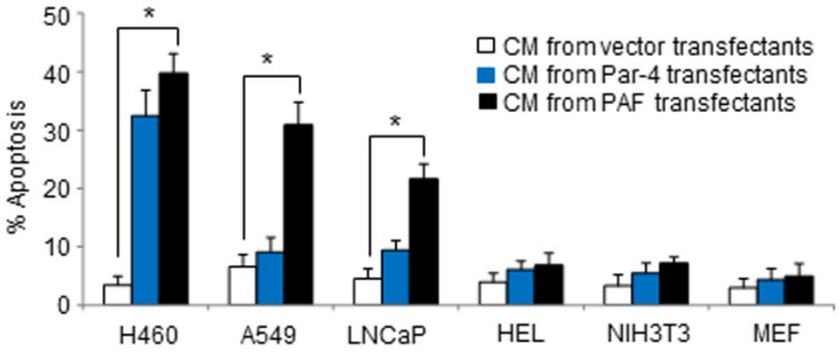

Figure S5. PAF induces apoptosis specifically in cancer cells and not in normal cells. Mouse fibroblasts were transfected with expression constructs for Par-4, PAF or vector. The CM was applied to H460, A549, and LNCaP cancer cells and normal HEL cells, NIH 3T3 and MEFs. After 24 h the cells were scored for apoptosis by ICC for active caspase 3. Data shown represent mean of three independent experiments $\pm$ SD. Asterisk (*) indicates statistical significance ($P < 0.001$) based on Student's t test.

Figure S6

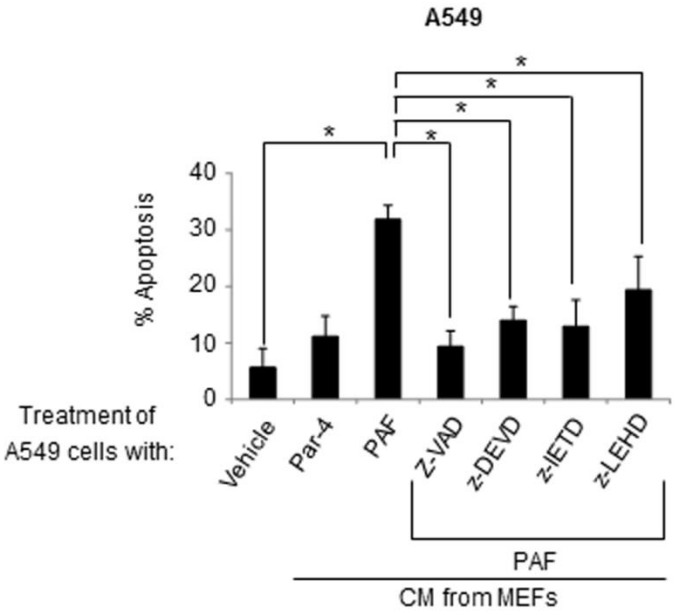

Figure S6. PAF induced apoptosis is dependent on caspase 8 and caspase 3. MEFs were transfected with vector, expression constructs for Par-4 or PAF. The CM from vector or Par-4 transfected cells, or CM from PAF transfected cells that was incubated with pan-caspase inhibitor (z-VAD-fmk), or inhibitors of caspase-3, caspase-8, or caspase-9, respectively, was applied to A549 cells for 24 h and the cells were scored for apoptosis. Data shown represent mean of three independent experiments $\pm$ SD. Asterisk (*) indicates statistical significance ($P < 0.001$) based on Student's t test.

Figure S7

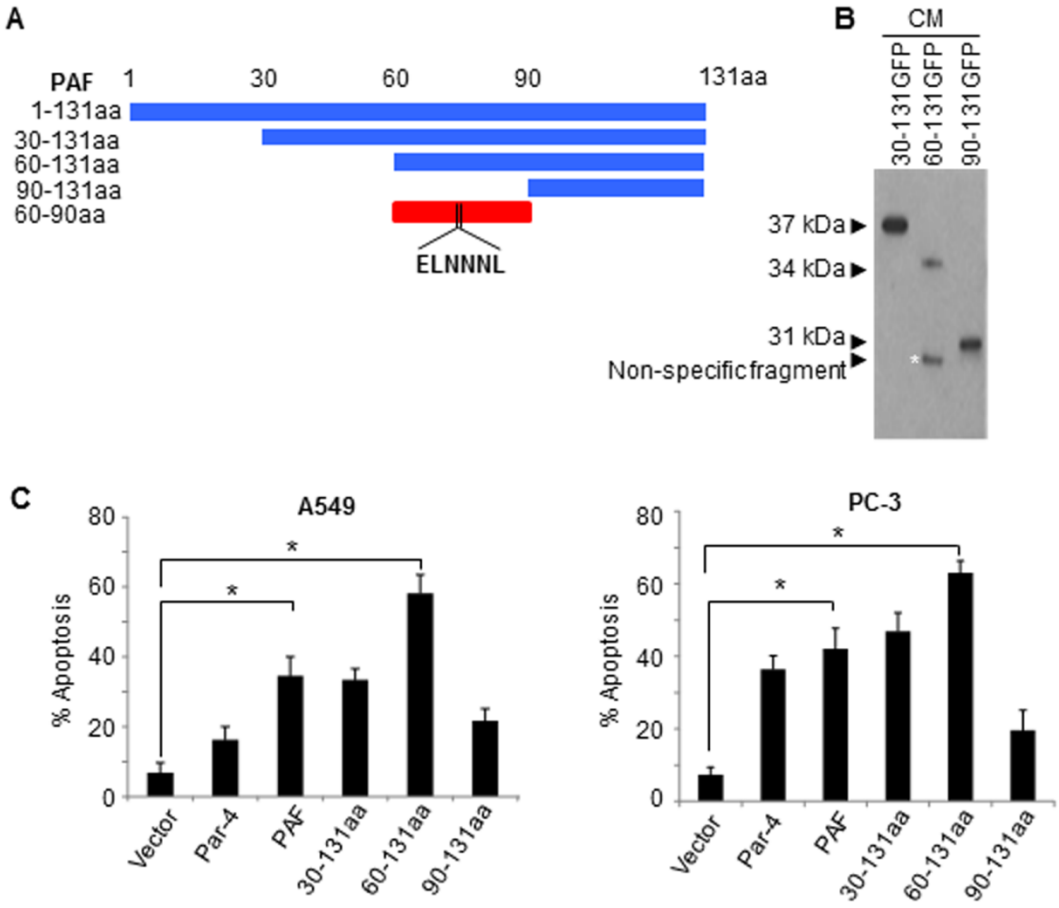

Figure S7. Structure-function analysis for the minimum domain of PAF essential for apoptosis induction.

A. Diagrammatic illustration of PAF (1-131aa) and its mutants (blue) and the minimal domain 60-90aa that is essential for apoptosis (red).

B. MEFs were transfected with GFP-tagged Par-4, PAF or deletion mutants of PAF and the CM was examined for expression of the mutants by Western blot analysis.

C. The CM from the transfectants was applied to A549 or PC-3 cells for 24 h, and the cells were scored for apoptosis by ICC for active caspase 3. Data shown represent mean of three independent experiments $\pm$ SD. Asterisk (*) indicates statistical significance ($P < 0.001$) based on Student's t test.

Figure S8

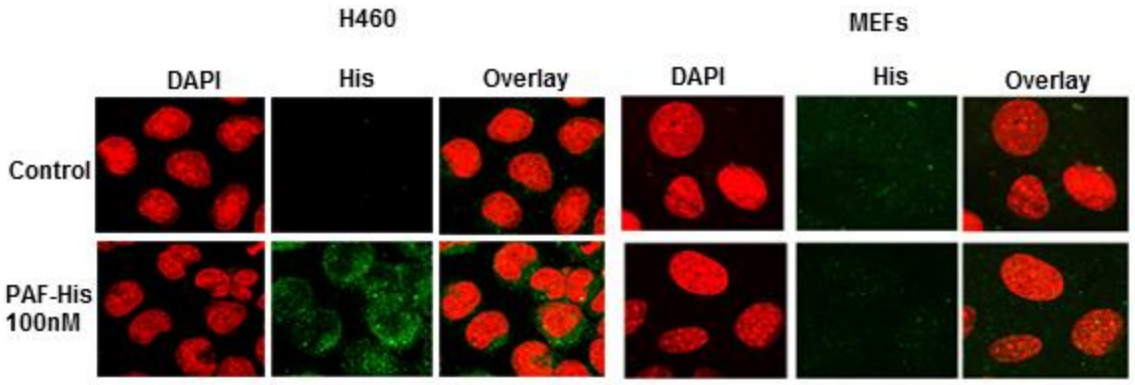

Figure S8. Extracellular PAF selectively enters cancer cells, not normal cells. H460 or MEF cells were treated with His-tagged PAF (100 nM) or heat-inactivated PAF (100 nM) (Control), and the intracellular presence of the His-tagged protein was examined by ICC and confocal microscopy. Image magnification, 40X.

Figure S9

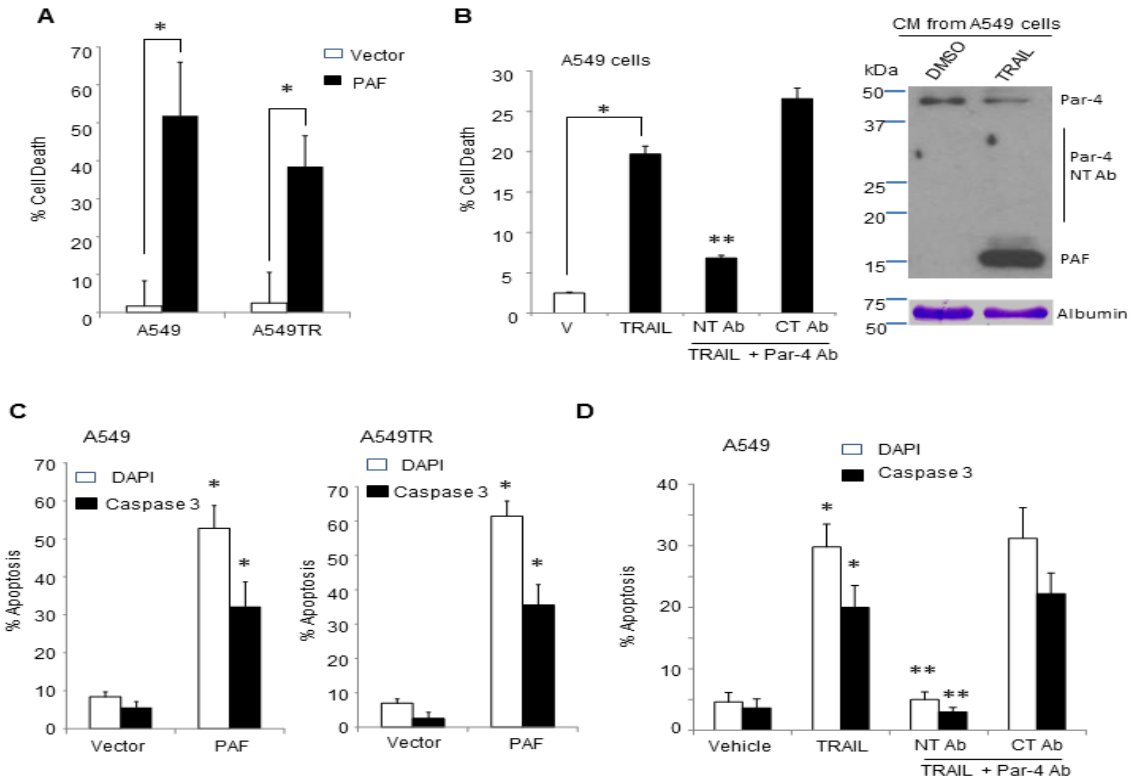

Figure S9. Reproducibility of cell death/apoptosis assays.

A and B. Cell death/growth inhibition analysis by automated MTS method.

A. A549 and A549TR cells were transfected with vector or PAF and cell death was quantified by MTS assays in three independent experiments.

B. A549 cells were treated with vehicle (v, DMSO) or TRAIL (100 ng/ml) alone or in the presence of the Par-4 NT or CT antibody (Ab) and cell death was quantified by MTS assays carried out in three replicates (left panel). The CM from cells treated with DMSO or TRAIL was examined by Western blot analysis (right panel).

C and D. Cell death/apoptosis by DAPI and caspase 3 ICC.

C. A549 and A549TR cells were transfected with vector or PAF and cell death was quantified by DAPI and active caspase 3 ICC in three independent experiments.

D. A549 cells were treated with DMSO (Vehicle) or TRAIL (100 ng/ml) alone or in the presence of the Par-4 NT or CT antibody (Ab) and cell death was quantified by DAPI and active caspase 3 ICC in three independent experiments.

Panels A-D. Mean $\pm$ SD from three experiments (performed as indicated above) are shown. Asterisk (*) indicates statistical significance ($P < 0.001$ based on Student's t test) of the data with PAF or TRAIL relative to vector or vehicle control, respectively. **Panel D.** Asterisks (**) indicate statistical significance ($P < 0.001$ based on Student's t test) of the data with Par-4 NT antibody relative to Par-4 CT antibody.