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Supplemental Data

A Small Molecule Inhibitor

of Inducible Heat Shock Protein 70

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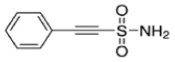
A

2-Phenylethynesulfonamide (PES)

($C_8H_7NO_2S$)



B

Human HSP70

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MAKAAAIGID LGTTYSCVGV FQHGKVEIIA NDQGNRTTPS YVAFTDTERL
IGDAAKNQVA LNPQNTVFDA KRLIGRKFGD PVVQSDMKHW PFQVINDGDK
PKVQVSYKGE TKAFYPEEIS SMVLTKMKEI AEAYLGYPVT NAVITVPAYF
NDSQRQATKD AGVIAGLNVL RIINEPTAAA IAYGLDRTGK GERNVLIFDL
GGGTFDVSIL TIDDGIFEVK ATAGDTHLGG EDFDNRLVNH FVEEFKRKHK
KDISQNKRAV RRLRTACERA KRTLSSSTQA SLEIDSLFEG IDFYTSITRA
RFEELCSDLF RSTLEPVEKA LRDAKLDKAQ IHDLVLVGGS TRIPKVQKLL
QDFFNGRDLN KSINPDEAVA YGAAVQAAIL MGDKSENVQD LLLLDVAPLS
LGLETAGGVM TALIKRNSTI PTKQTQIFTT YSDNQPGVLI QVYEGERAMT
KDNNLLGRFE LSGIPPAPRG VPQIEVTFDI DANGILNVTA TDKSTGKANK
ITITNDKGRL SKEEIERMVQ EAEKYKAEDE VQRERVSAKN ALESYAFNMK
SAVEDEGLKG KISEADKKKV LDKCQEVISW LDANTLAEKD EFEHKRKELE
QVCNPIISGL YQGAGGPGPG GFGAQGPKGG SGSGPTIEEV D
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Human HSC70

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MSKGPAVGID LGTTYSCVGV FQHGKVEIIA NDQGNRTTPS YVAFTDTERL
IGDAAKNQVA MNPTNTVFDA KRLIGRRFDD AVVQSDMKHW PFMVVNDAGR
PKVQVEYKGE TKSFYPEEVS SMVLTKMKEI AEAYLGKTVT NAVVTVPAYF
NDSQRQATKD AGTIAGLNVL RIINEPTAAA IAYGLDEKVG AERNVLIFDL
GGGTFDVSIL TIEDGIFEVK STAGDTHLGG EDFDNRMVNH FIAEFKRKHK
KDISENKRAV RRLRTACERA KRTLSSSTQA SIEIDSLYEG IDFYTSITRA
RFEELNADLF RGTLDPVEKA LRDAKLDKSQ IHDIVLVGGS TRIPKIQKLL
QDFFNGKELN KSINPDEAVA YGAAVQAAIL SGDKSENVQD LLLLDVTPLS
LGIETAGGVM TVLIKRNTTI PTKQTQTFTT YSDNQPGVLI QVYEGERAMT
KDNNLLGKFE LTGIPPAPRG VPQIEVTFDI DANGILNVSA VDKSTGKENK
ITITNDKGRL SKEDIERMVQ EAEKYKAEDE KQRDKVSSKN SLESYAFNMK
ATVEDEKLQG KINDEDKQKI LDKCNEIINW LDKNQTAEKE EFEHQQKELE
KVCNPIITKL YQSAGGMPGG MPGGFPGGGA PPSGGASSGP TIEEVD
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C

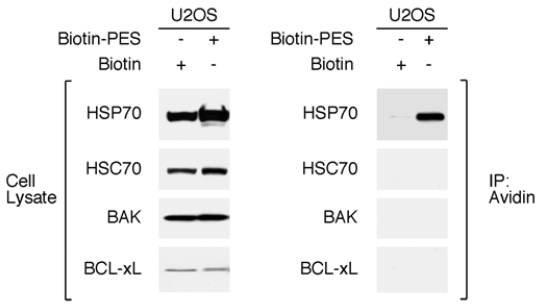

Figure S1. The Interaction of B-PES and HSPs

(A) Chemical structure of PES (from EMD Chemicals, Inc.).

(B) The amino acid sequence of inducible HSP70 (left) and HSC70 (right); the residues detected by mass spectrometry are shown in bold and underlined.

(C) WCE were prepared from U2OS cells following 24 h treatment with 20 μM Biotin or B-PES, and immunoblotted for the proteins as indicated (left). B-PES-containing complexes were captured by NeutrAvidin Resins, and eluted following 100 mM DTT treatment. IP-WB analysis using the indicated antibodies reveals interaction of B-PES with endogenous HSP70, but not endogenous BAK, BCL-xL, or HSC70.

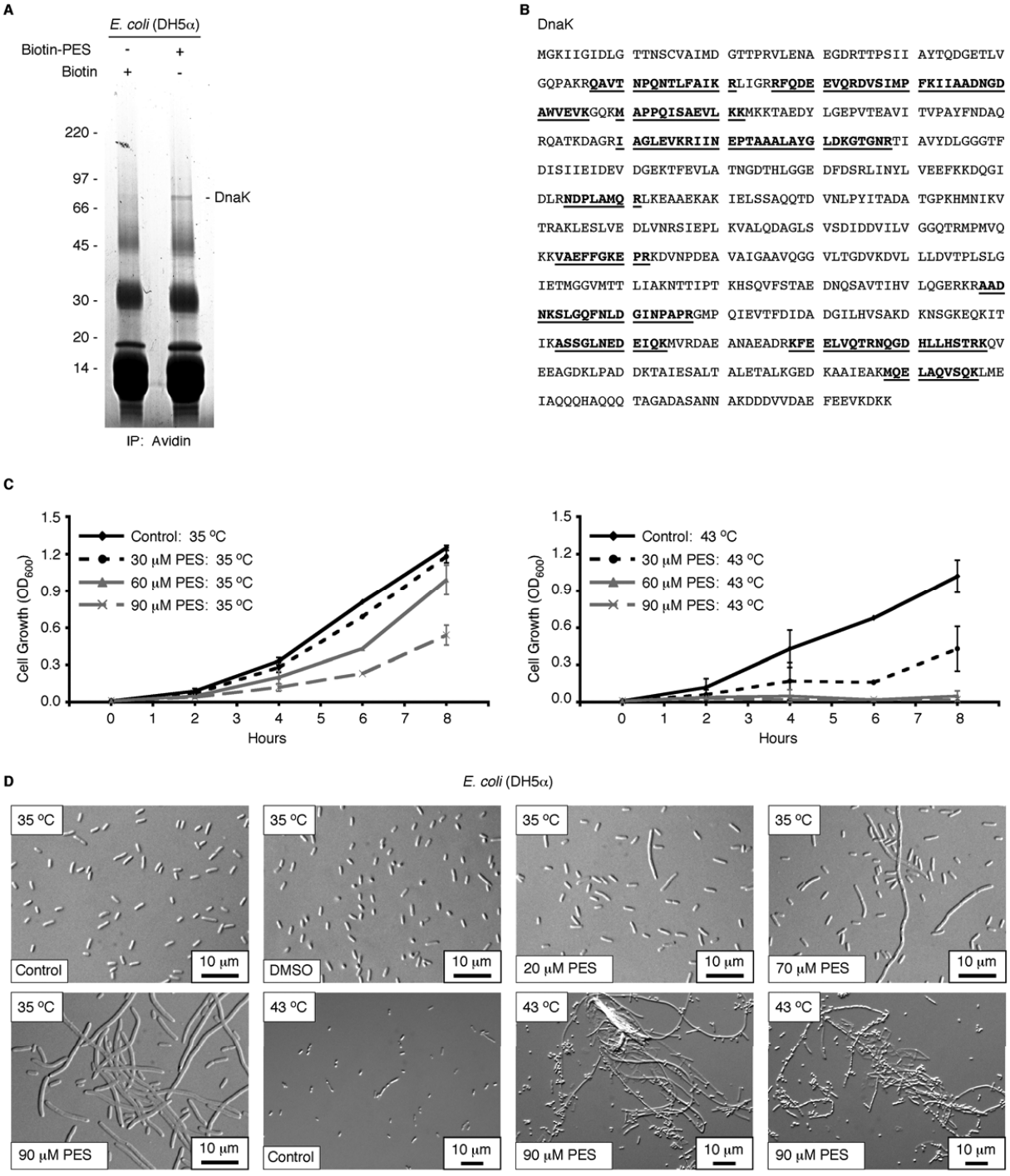
A
E. coli (DH5α)
Biotin-PES - +
Biotin + -
220 -
97 -
66 -
45 -
30 -
20 -
14 -
- DnaK
IP: Avidin
B
DnaK
MGKIIGIDLG TTNSCVAIMD GTTPRVLENA EGDRTTPSII AYTQDGETLV
GQPAKRQAVT NPQNTLFAIK RLIGRRFQDE EVQRDVSIMP FKIIAADNGD
AWVEVKGQKM APPQISAEVL KKMKKTAEDY LGEPVTEAVI TVPAYFNDAQ
RQATKDAGRI AGLEVKRIIN EPTAAALAYG LDKGTGNRTI AVYDLGGGTF
DISIIEIDEV DGEKTFEVLA TNGDTHLGGE DFDSRLINYL VEEFKKDQGI
DLRNDPLAMQ RLKEAAEKAK IELSSAQQTD VNLPYITADA TGPKHMNIKV
TRAKLESLVE DLVNRSIEPL KVALQDAGLS VSDIDDVILV GGQTRMPMVQ
KKVAEFFGKE PRKDVNPDEA VAIGAAVQGG VLTGDVKDVL LLDVTPLSLG
IETMGGVMTT LIAKNTTIPT KHSQVFSTAE DNQSAVTIHV LQGERKRAAD
NKSLGQFNLD GINPAPRGMP QIEVTFDIDA DGILHVSAKD KNSGKEQKIT
IKASSGLNED EIQKMVRDAE ANAEADRKFE ELVQTRNQGD HLLHSTRKQV
EEAGDKLPAD DKTAIESALT ALETALKGED KAAIEAKMQE LAQVSQKLME
IAQQQHAQQQ TAGADASANN AKDDDVVDAE FEEVKDKK
C
Cell Growth (OD600)
Control: 35 °C
30 μM PES: 35 °C
60 μM PES: 35 °C
90 μM PES: 35 °C
Hours
Control: 43 °C
30 μM PES: 43 °C
60 μM PES: 43 °C
90 μM PES: 43 °C
Hours
D
E. coli (DH5α)
35 °C
Control
DMSO
20 μM PES
70 μM PES
90 μM PES
43 °C
10 μm

Figure S2. PES and *E. coli*

(A) Identification of DnaK as Biotin-PES (B-PES) interacting protein in bacteria by liquid chromatography-tandem mass spectrometry, as described in Supplemental Experimental Procedures.

(B) The amino acid sequence of DnaK; the residues detected by mass spectrometry are shown in bold and underlined.

(C) Growth curves of *E. coli* treated with different concentrations of PES either at 35°C or 43°C. Since bacteria with some mutations in DnaK exhibit a thermosensitive phenotype, we treated liquid bacterial cultures with increasing concentrations of PES at temperatures of 35°C or 43°C. Cell proliferation was inhibited in a dose-dependent fashion, especially at 43°C.

(D) Bacterial morphology (phase contrast microscopy) following treatment of the cells with PES at the indicated temperatures. Note evidence of filamentation and aggregation at higher concentrations of PES, especially at 43°C.

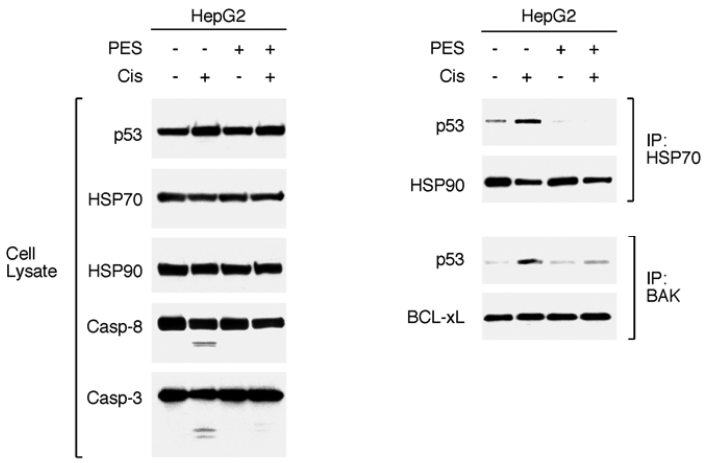

Figure S3. PES Attenuates a p53/BAK Interaction and Inhibits Caspase Activation

Whole cell extracts (WCE) prepared from HepG2 cells that were either untreated or pretreated with PES (20 μM) for 1 h, followed by the addition of 50 μM cisplatin for 14 h. Note evidence of caspase cleavage in cisplatin-treated cells, but not in the presence of PES. Extracts were immunoprecipitated with anti-HSP70 or anti-BAK antibody, and blotted with anti-p53, anti-BCL-xL, or anti-HSP90, as indicated. The data provide evidence that PES inhibits the interaction of p53 with both HSP70 and BAK.

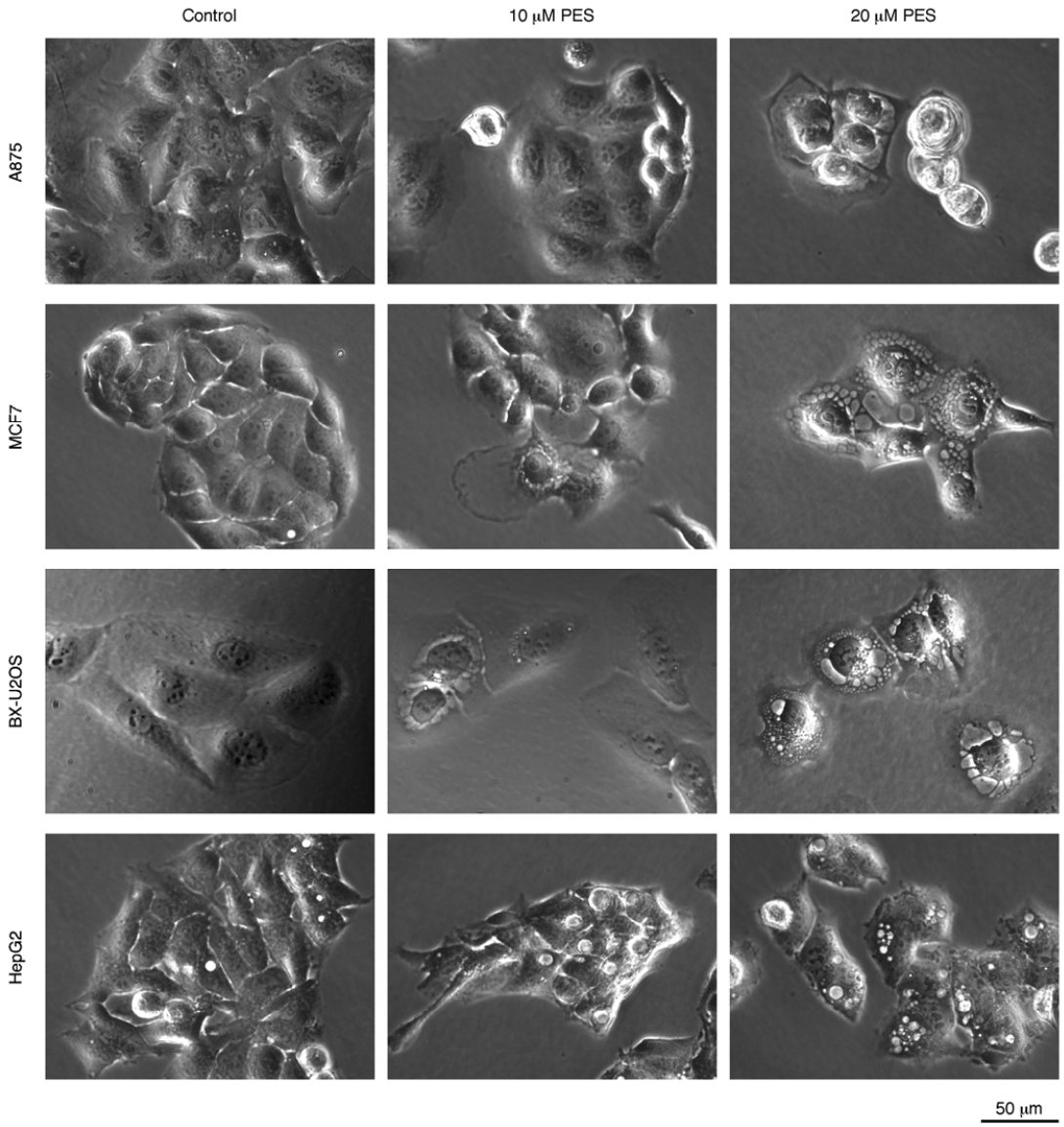

Figure S4. PES Induces Cytoplasmic Vacuolization

Representative phase-contrast images of indicated human cell lines after 24 h of treatment using indicated amount of PES. Note the increased appearance of cytoplasmic vacuoles following increasing dosage of PES exposure.

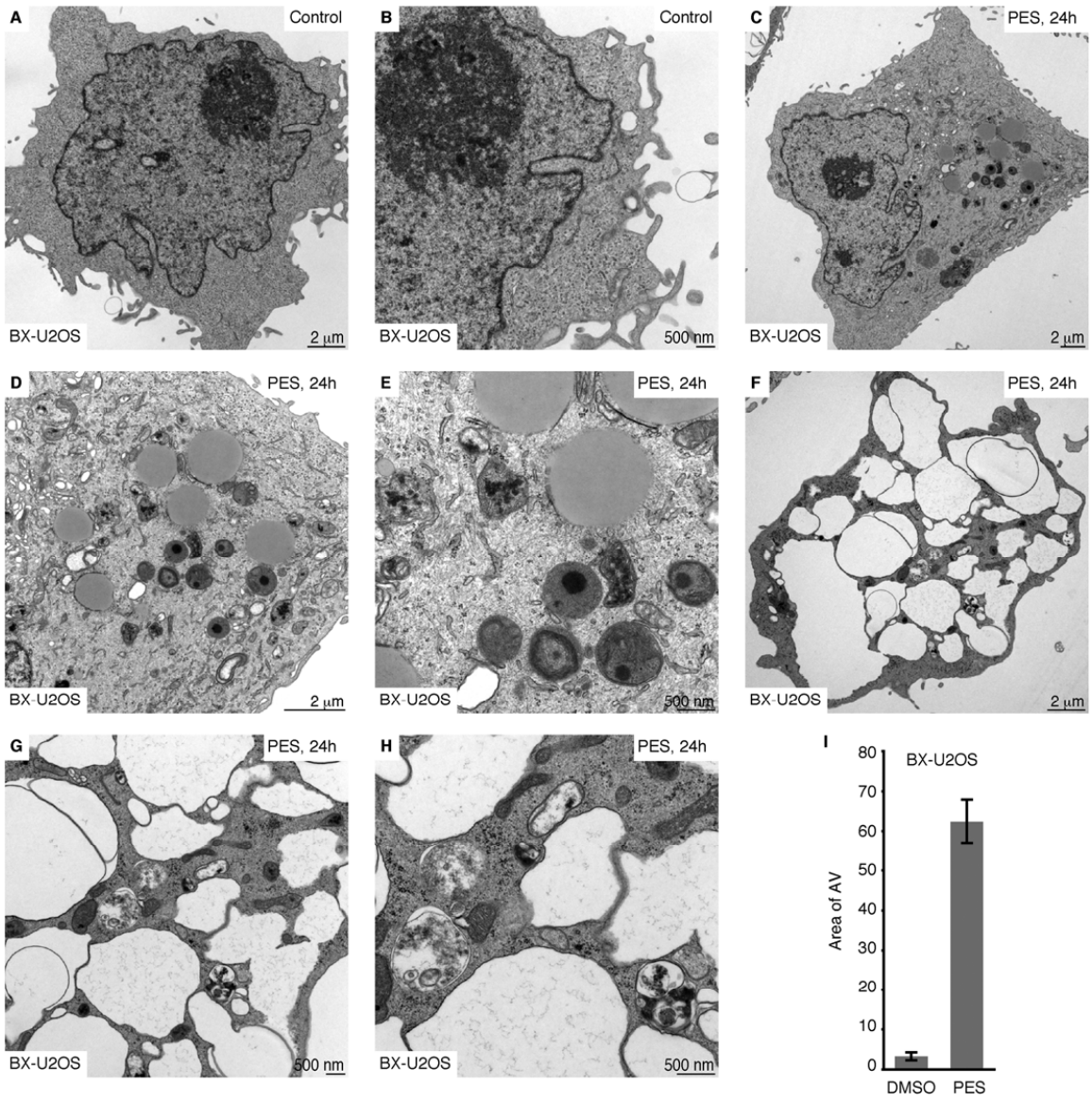

Figure S5. PES Induces Markers of Autophagy

(A-H) Electron micrographs of BX-U2OS cells treated with or without PES (20 μM) for 24 h.

(D) Double membrane autophagic vacuoles (AV) are depicted.

(E) Electron dense AVs containing partially digested cytoplasmic contents are displayed.

(F-H) Numerous large AVs containing partially digested cytoplasmic elements as well as amorphous, membranous, aggregated, or granular masses are shown.

(I) The average area of autophagic vacuoles (AV) per cell calculated with Image J software is indicated. Error bars indicate SD.

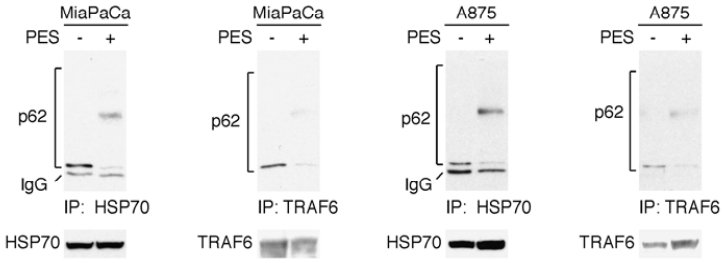

Figure S6. PES Attenuates the Interactions between HSP70 and Monomeric p62/SQSTM1 as well as Between HSP70 and Monomeric TRAF6

IP-WB analyses of WCE from vehicle or PES-treated MiaPaCa pancreatic cells (top panel) and A875 melanoma cells (lower panel). Note that HSP70 binds to the oligomeric forms of p62/SQSTM1 following PES-exposure. In contrast, stable interactions between HSP70 and monomeric p62/SQSTM1 as well as between TRAF6 and monomeric p62/SQSTM1 were noted in vehicle treated cells.

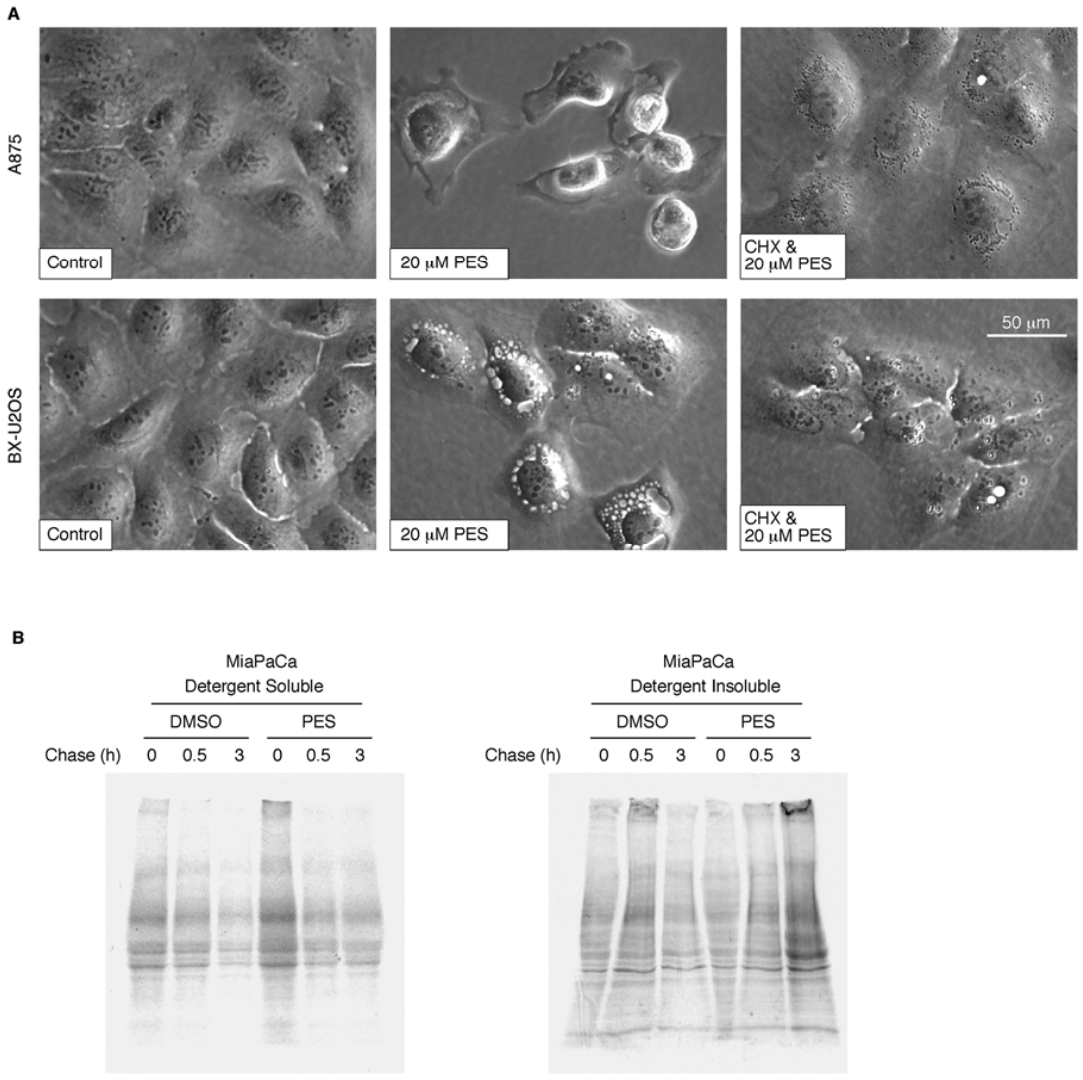

Figure S7. Cycloheximide Reduces PES-Induced Cytoplasmic Vacuolization

(A) Representative phase-contrast images of indicated tumor cell lines pretreated with DMSO or 1 μg/ml of cycloheximide (CHX) for 1 h, followed by the addition of 20 μM PES for 23 h.

(B) The molecular chaperone HSP70 has been implicated in promoting proper folding of nascent polypeptides. Also, increased levels of p62/SQSTM1 have been found to result from either increased synthesis or blockage of autophagy. Since we found that PES inhibits long-lived

protein degradation (Figure 5A), we also evaluated how PES might affect the disposition of newly synthesized proteins. Briefly, MiaPaCa cells were incubated in methionine/cysteine starvation medium for 30 min before adding [^{35}S]methionine/cysteine and DMSO or [^{35}S]methionine/cysteine and 20 μM PES, as indicated, to pulse for 1 h. Following the pulse, cells were immediately chased by complete medium for 0, 0.5, or 3 h, either in the presence of DMSO or 20 μM PES, as indicated. Cells were harvested at indicated time points, lysed in 1% NP40-containing buffer, and fractionated into detergent-soluble and detergent-insoluble preparations. Note the increase in the newly-synthesized proteins in the detergent-insoluble fraction from PES-treated cells apparent 3h following the chase in complete medium.