Supplemental Data

p62 Binding to Protein Kinase C ζ Regulates Tumor Necrosis Factor α-Induced Apoptotic Pathway in Endothelial Cells

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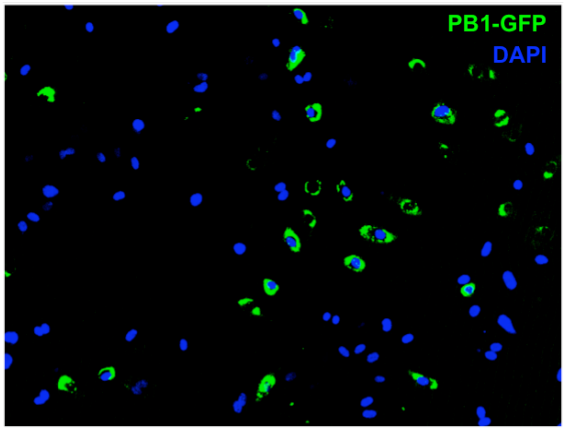
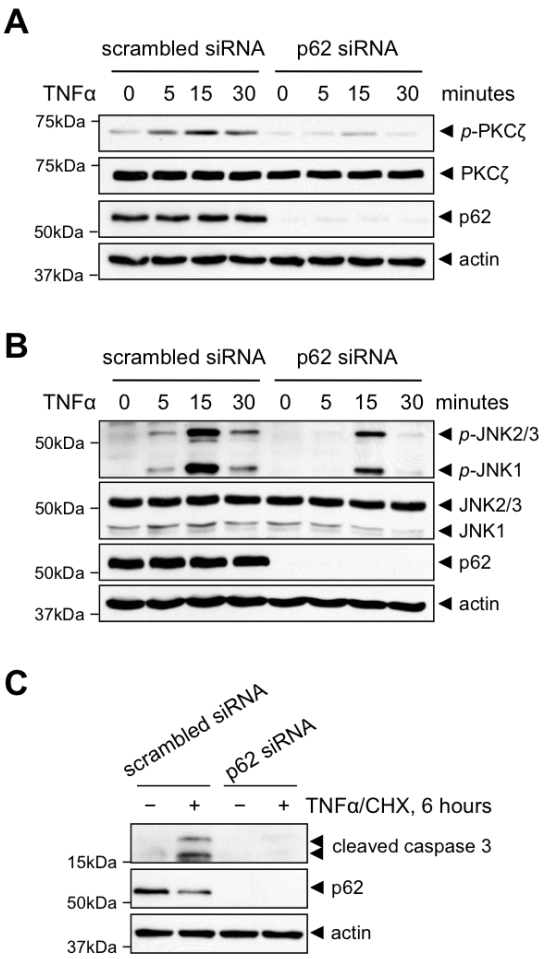


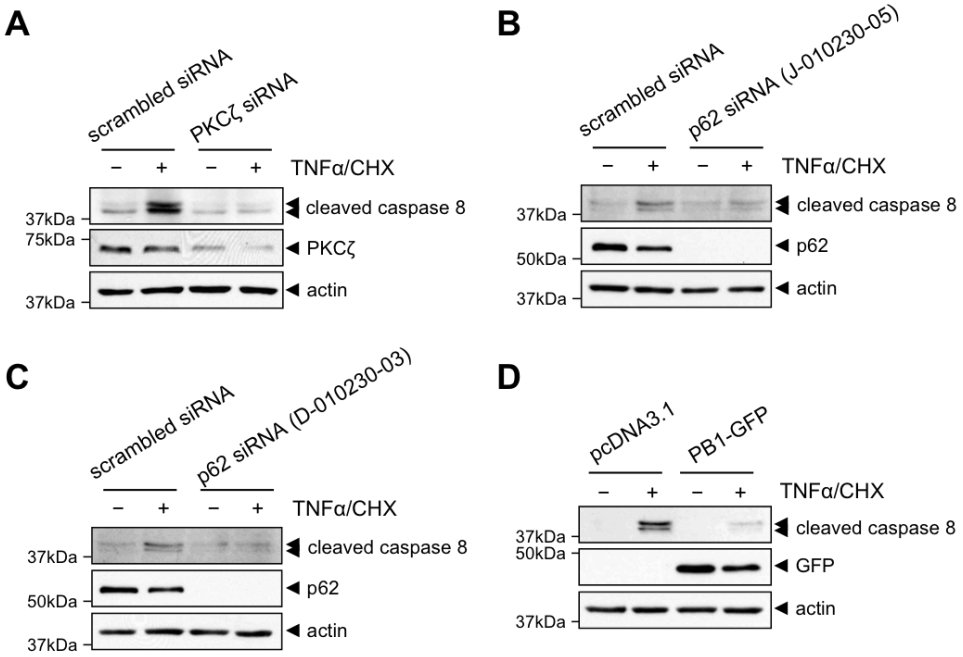
Supplemental Figure I. Transfection of PB1-GFP.

After transfection of PB1-GFP, the expression of GFP was analyzed in randomly selected 5 areas under the Olympus BX51 fluorescent microscope. The representative image is shown.



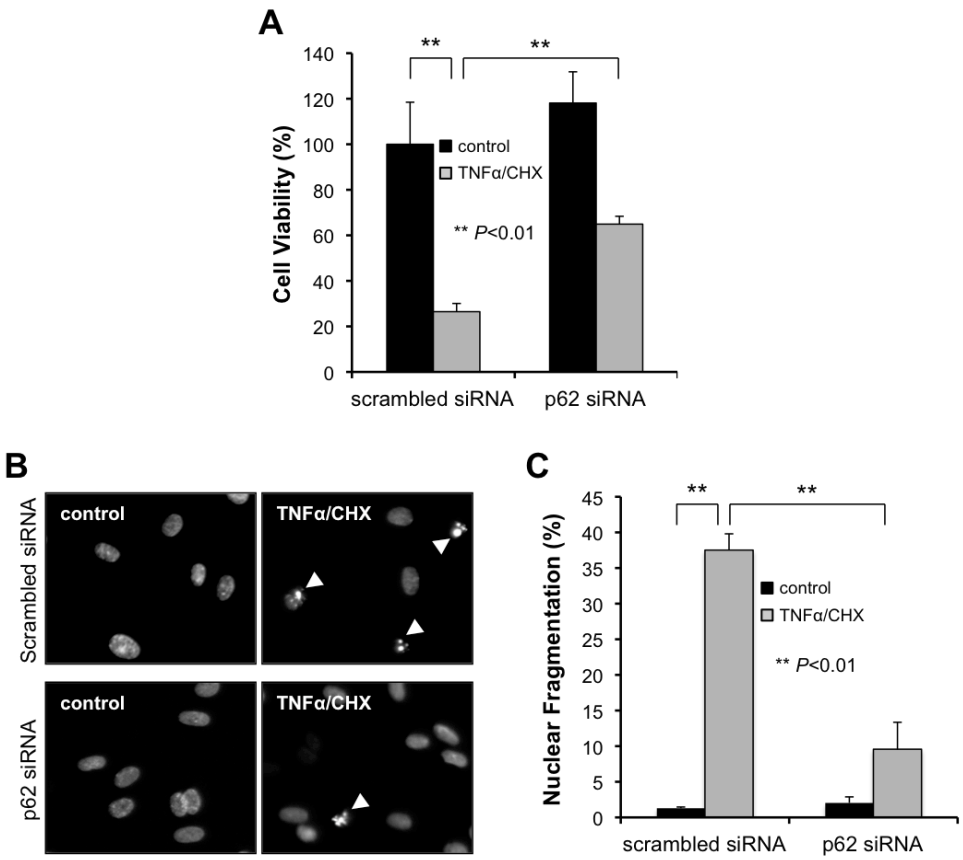
Supplemental Figure II. Inhibitory effect of siRNA-mediated p62 depletion on TNFα-induced PKCζ signaling cascade.

p62 siRNA (Dharmacon RNA Technologies, D-010230-03, 80 nmol/L) was transfected into HUVEC as described in Materials and Methods. The cells were stimulated with TNFα (10 ng/mL) alone or TNFα (10 ng/mL) + CHX (10 μg/mL) for the indicated times and the phosphorylation of PKCζ (A) and JNK (B) and cleavage of caspase 3 (C) were analyzed by western blotting.



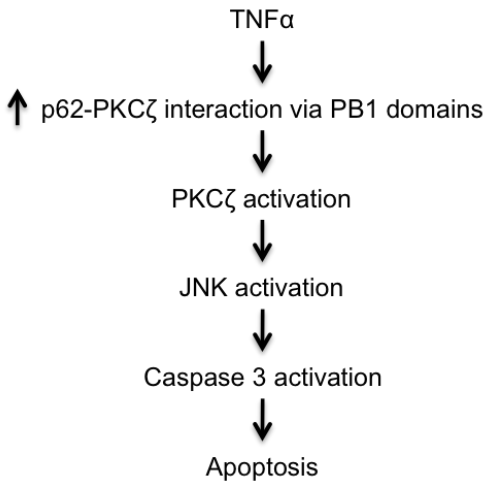
Supplemental Figure III. p62-PKCζ pathway regulates caspase 8 activation in response to TNFα + CHX.

Either PKCζ- (Dharmacon RNA Technologies, L-003526-00, 100 nmol/L; A) or p62- (Dharmacon RNA Technologies, J-010230-05, 80 nmol/L; B or D-010230-03, 80 nmol/L; C) depleted HUVEC were stimulated with TNFα (10 ng/mL) + CHX (10 µg/mL) for 6 hours and the cleavage of caspase 8 was analyzed by western blotting. (D) Either pcDNA3.1- or PB1-GFP-transfected HUVEC were stimulated with TNFα (10 ng/mL) + CHX (10 µg/mL) for 6 hours and the cleavage of caspase 8 was analyzed by western blotting.

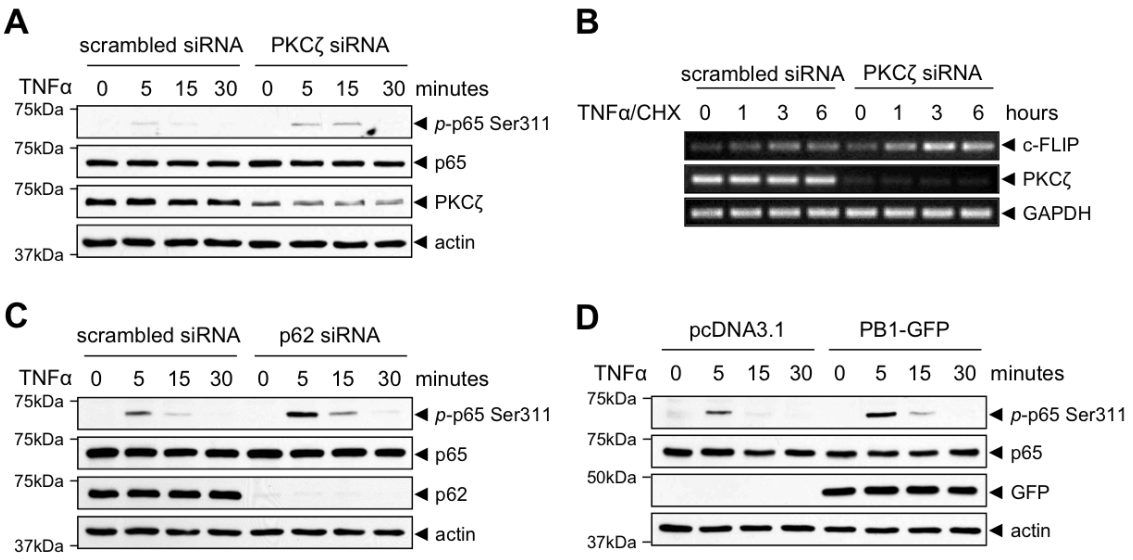


Supplemental Figure IV. Inhibitory effect of siRNA-mediated p62 depletion on TNFα-induced apoptotic cell death.

p62 siRNA (Dharmacon RNA Technologies, D-010230-03, 80 nmol/L) was transfected into HUVEC as described in Materials and Methods. The cells were stimulated with TNFα (10 ng/mL) + CHX (10 μg/mL) for 24 hours and a MTT assay (A), DAPI staining (B; apoptotic cells were indicated by white arrow) and quantification of apoptotic nuclear body (C) were performed as described in Figure 6.



Supplemental Figure V. A model for the requirement of p62 and PKCζ interaction for the PKCζ signaling cascade that leads to endothelial cell apoptosis.



Supplemental Figure VI. PKCζ negatively regulates TNFα-induced phosphorylation of p65 Ser311.

(A) PKCζ (Dharmacon RNA Technologies, L-003526-00, 100 nmol/L) or scrambled siRNA were transfected into HUVEC which were then stimulated with TNFα (10 ng/mL) for the indicated times and phosphorylation of p65 Ser311 was analyzed by western blotting. (B) PKCζ or scrambled siRNA were transfected into HUVEC which were then stimulated with TNFα (10 ng/mL) + CHX (10 μg/mL) for the indicated times and reverse transcription (RT)-PCR was performed using a reverse transcription kit (Promega, Madison, WI) to measure c-FLIP expression. Following primers were used: forward 5′-TAAAACCACCAGCACCACAA-3′ and reverse 5′-CTACGTGTGGCCCGTATCTT-3′ for c-FLIP; forward 5′-CCAGAAGATGGAGGAAGCTG-3′ and reverse 5′-CGTCTACTGGAGGCTCTTGG-3′ for PKCζ; forward 5′-ACGGATTTGGTCGTATTGGG-3′ and reverse 5′-TGATTTTGGAGGGATCTCGC-3′ for GAPDH. (C) p62 (Dharmacon RNA Technologies, J-010230-05, 80 nmol/L) or scrambled siRNA were transfected into HUVEC which were then stimulated with TNFα (10 ng/mL) for the indicated times and phosphorylation of p65 Ser311 was analyzed by western blotting. (D) Either pcDNA3.1 or PB1-GFP transfected HUVEC were stimulated with TNFα (10 ng/mL) for the indicated times and phosphorylation of p65 Ser311 was analyzed by western blotting. Antibody against *p*-p65 (Ser311) was purchased from Novus Biologicals (Littleton, CO); anti-p65 from Cell Signaling Technology.