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Supplementary Materials for

Identification of ROCK1 as an Upstream Activator of the JIP-3 to JNK Signaling Axis in Response to UVB Damage

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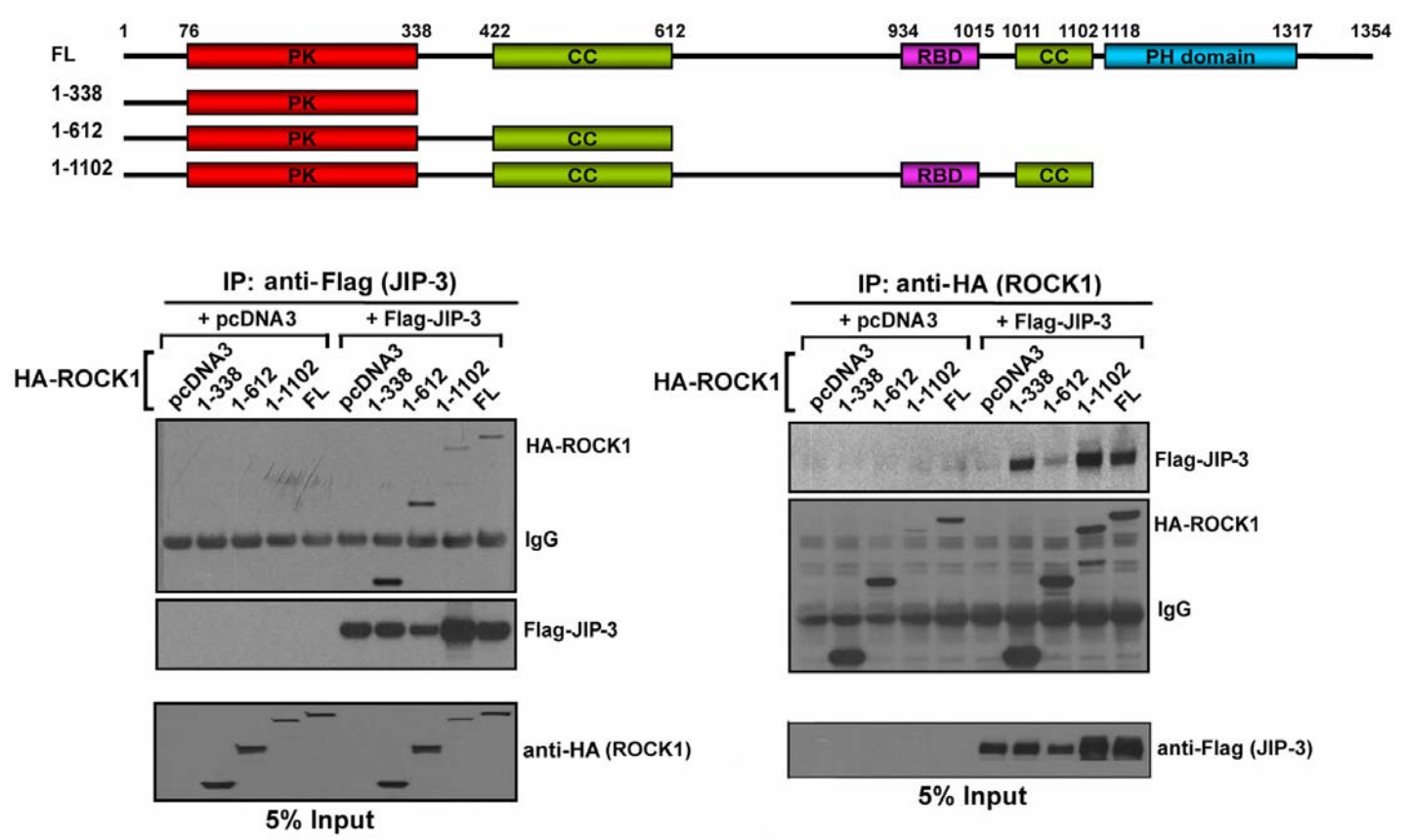

Fig. S1. The kinase domain of ROCK1 is involved in binding to JIP-3. HA-tagged full-length or truncated mutants (1-333, 1-612, and 1-1102) of ROCK1 were cloned into an expression vector (pcDNA3.1), and cotransfected with a plasmid encoding Flag-tagged JIP-3 into 293ET cells. Cells were harvested 36 hours later and subjected to immunoprecipitations with an anti-Flag antibody, to immunoprecipitate JIP-3 (left panel), or an anti-HA antibody, to immunoprecipitate ROCK1 (right panael), and samples were analyzed by Western blotting for the indicated proteins. Domains: PK, protein kinase; CC, coiled-coil domain; RBD, Rho-binding domain; PH, Pleckstrin homology domain.

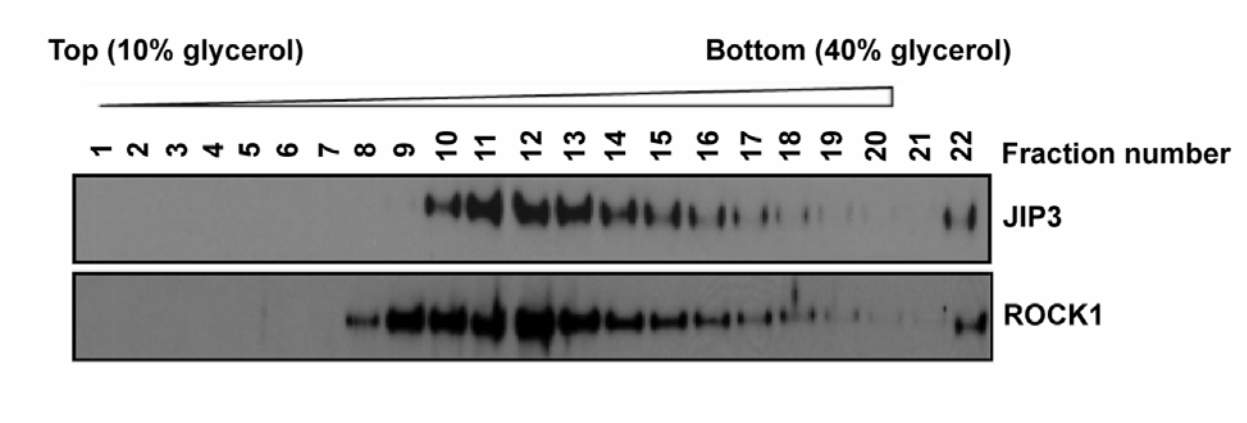

Fig. S2. Glycerol gradient analysis of ROCK1 and JIP-3. A construct encoding His-tagged ROCK1 was transiently transfected into 293ET cells and His-tagged ROCK1 protein was partially purified from on Ni^{2+} columns. The eluate was separated on a 10-40% glycerol gradient followed by ultracentrifugation. 200 μl fractions were collected and each fraction was analyzed by Western blotting with anti-JIP-3 and anti-ROCK1 antibodies.

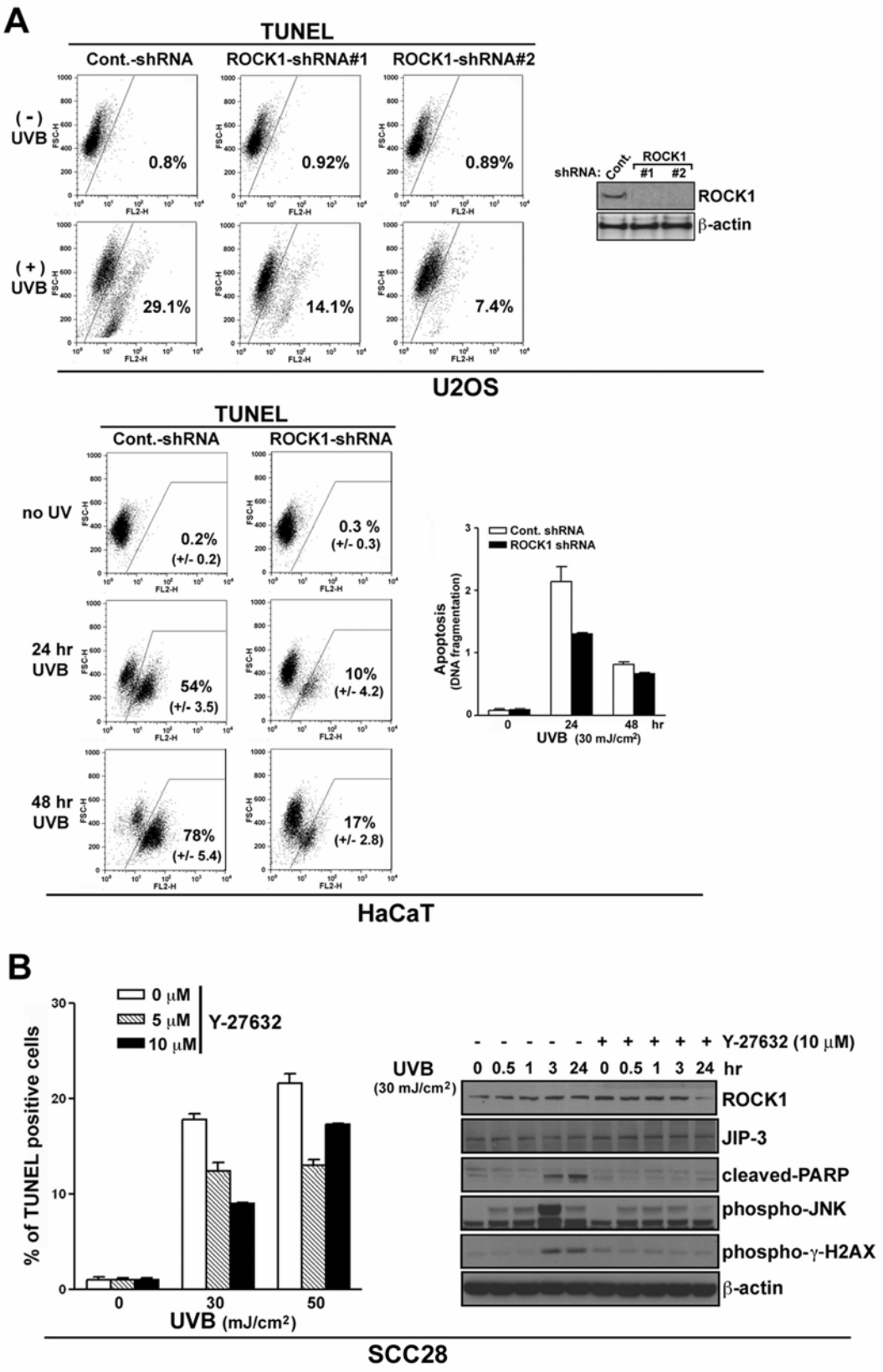
A
TUNEL
Cont.-shRNA
ROCK1-shRNA#1
ROCK1-shRNA#2
(-) UVB
(+) UVB
0.8%
0.92%
0.89%
29.1%
14.1%
7.4%
FSC-H
FL2-H
shRNA: Cont. ROCK1 #1 #2
ROCK1
β-actin
U2OS
TUNEL
Cont.-shRNA
ROCK1-shRNA
no UV
24 hr UVB
48 hr UVB
0.2% (+/- 0.2)
0.3 % (+/- 0.3)
54% (+/- 3.5)
10% (+/- 4.2)
78% (+/- 5.4)
17% (+/- 2.8)
Cont. shRNA
ROCK1 shRNA
Apoptosis (DNA fragmentation)
UVB (30 mJ/cm²)
HaCaT
B
0 μM
5 μM
10 μM
Y-27632
% of TUNEL positive cells
UVB (mJ/cm²)
- - - - - + + + + + Y-27632 (10 μM)
UVB (30 mJ/cm²)
0 0.5 1 3 24 0 0.5 1 3 24 hr
ROCK1
JIP-3
cleaved-PARP
phospho-JNK
phospho-γ-H2AX
β-actin
SCC28

Fig. S3. Depletion of ROCK1 compromises UVB-induced apoptosis. **(A)** U2OS cells and HaCaT cells were stably transfected with ROCK1 shRNAs (#1 or #2) or control shRNA (vector pBabe-shRNA), and irradiated with UVB at 30 mJ/cm^2 for 24 hours or 48 hours. Western blotting was performed to determine the abundance of ROCK1 and β-actin (right panel). Cells were then assessed for apoptosis by TUNEL-FACS (left panel). The amount of DNA fragmentation is presented as the mean ± SEM (n=3). **(B)** Inhibition of ROCK activity by Y-27632 decreases UVB-induced apoptosis in HaCaT cells and abolishes UVB-induced activation of JNK. HaCaT cells were treated with different doses of Y-27632 (0, 5, and 10 μM) and irradiated with UVB at 30 and 50 mJ/cm^2 for 48 hours. The percentage of apoptotic cells was analyzed by TUNEL-FACS and is presented as a graph (left panel). Each data point is presented as the mean ± SEM (n=3). Cell extracts from Y-27632- (10 μM) or DMSO-treated HaCaT cells irradiated with UVB (30 mJ/cm^2) were also analyzed by Western blotting with antibodies against ROCK1, JIP-3, cleaved-PARP, phospho-JNK, β-actin, or phospho-γ-H2AX.

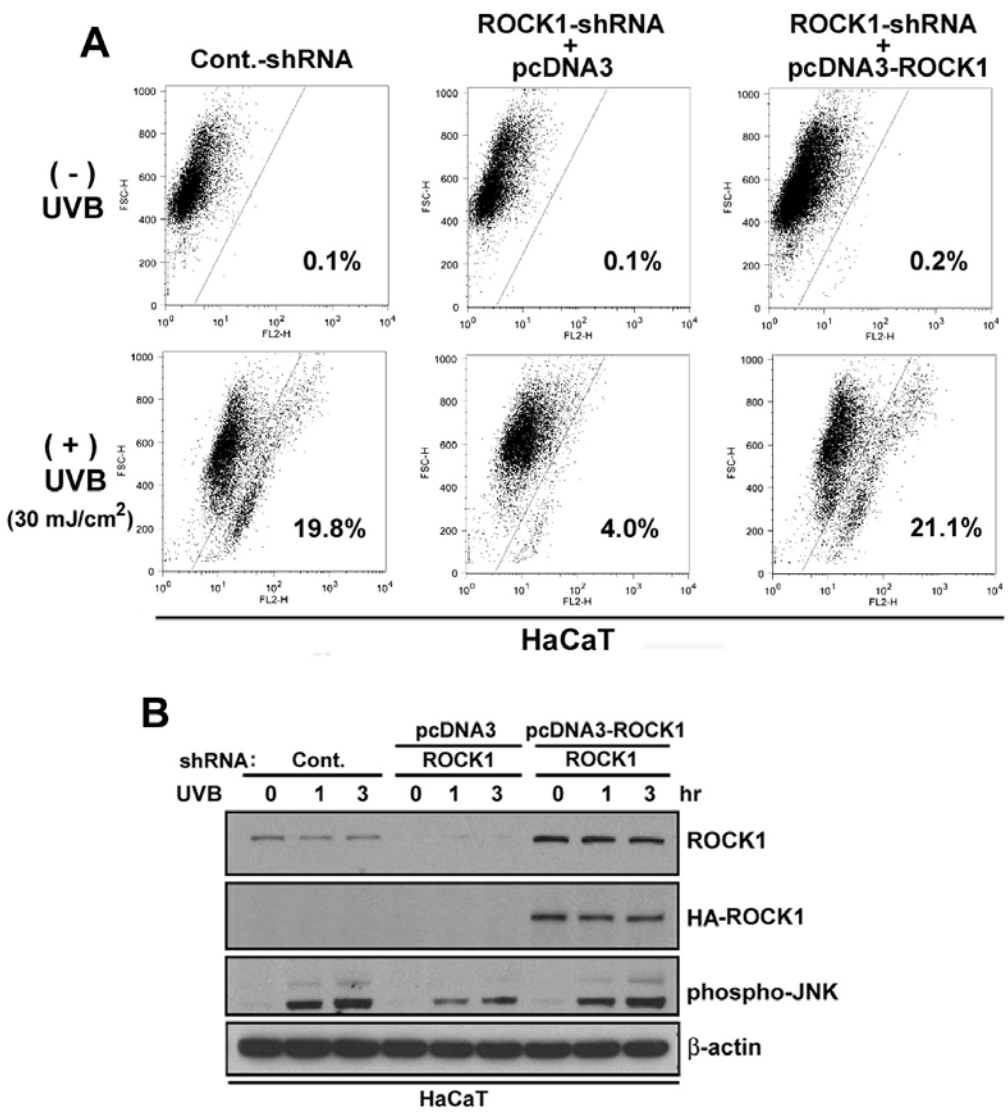

Fig. S4. Ectopic expression of ROCK1 in ROCK1-depleted HACAT cells rescued the ROCK1-suppression phenotype. HaCat cells stably transfected with control-shRNA or ROCK1-shRNA (ROCK1-shRNA #3) were transiently transfected with either pcDNA3 or pcDNA3-HA-ROCK1. The cells were subjected to UVB irradiation (30 mJ/cm^2). (**A**) TUNEL assays: the percentage of TUNEL-positive cells is shown as the mean $\pm$ SEM (n=3). (**B**) Western blot analysis shows that the re-expression of ROCK1 rescues suppression of UVB-induced JNK activation in ROCK1-knockdown cells.