Supplemental Data

Receptor for Activated C Kinase 1 (RACK1) Mediates Activation of Jun N-terminal Kinase (JNK) by Protein Kinase C

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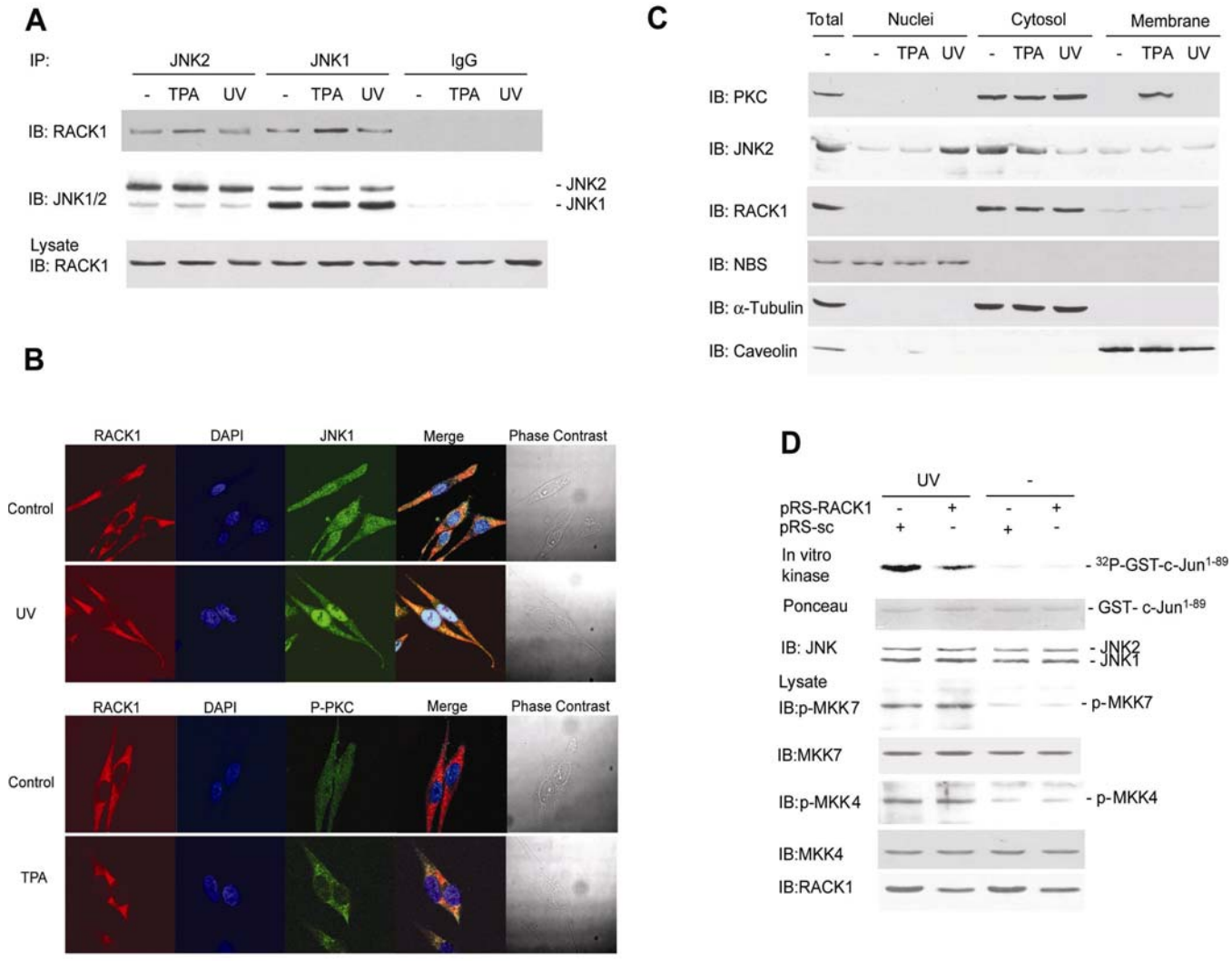

Figure S1

(A) RACK1 interacts with endogenous JNK1 and JNK2. HEK 293T cells were UV irradiated (45 J/m^2) or treated with TPA (20 ng/ml, 60 min). Protein extracts (500 μg) were subjected to IP with anti-JNK1, anti-JNK2, or IgG (used as a control) Ab.

Immunoprecipitates were analyzed by IB with anti-JNK1/2 or anti-RACK1 Ab (upper panels). Lower panel show IB analysis of protein lysates (50 μg) with anti-RACK1 Ab.

(B) RACK1 colocalizes with JNK and PKC. MeWo cells were starved for 12 hr, treated with TPA (50 ng/ml) or UV irradiated, and fixed following 10 min or 30 min, respectively.

(C) RACK1, JNK, and PKC localize primarily in the cytosol. HEK 293 cells were treated as indicated in Figure 2D and subcellular fractions obtained as described in Experimental Procedures. Immunoblot analysis of the different fractions (50 μg) was performed with the indicated antibodies.

(D) siRNA of RACK1 does not affect MKK4/7 activation. MeWo cells were stably transfected with pRS-scramble (pRS-sc) or pRS expressing siRNA for RACK1 (pRS-RACK1). Cells were serum-starved for 12 hr, UV irradiated (45 J/m^2), and harvested after 30 min. JNK in vitro kinase reactions and IB analysis were performed as described in Figure 2B.

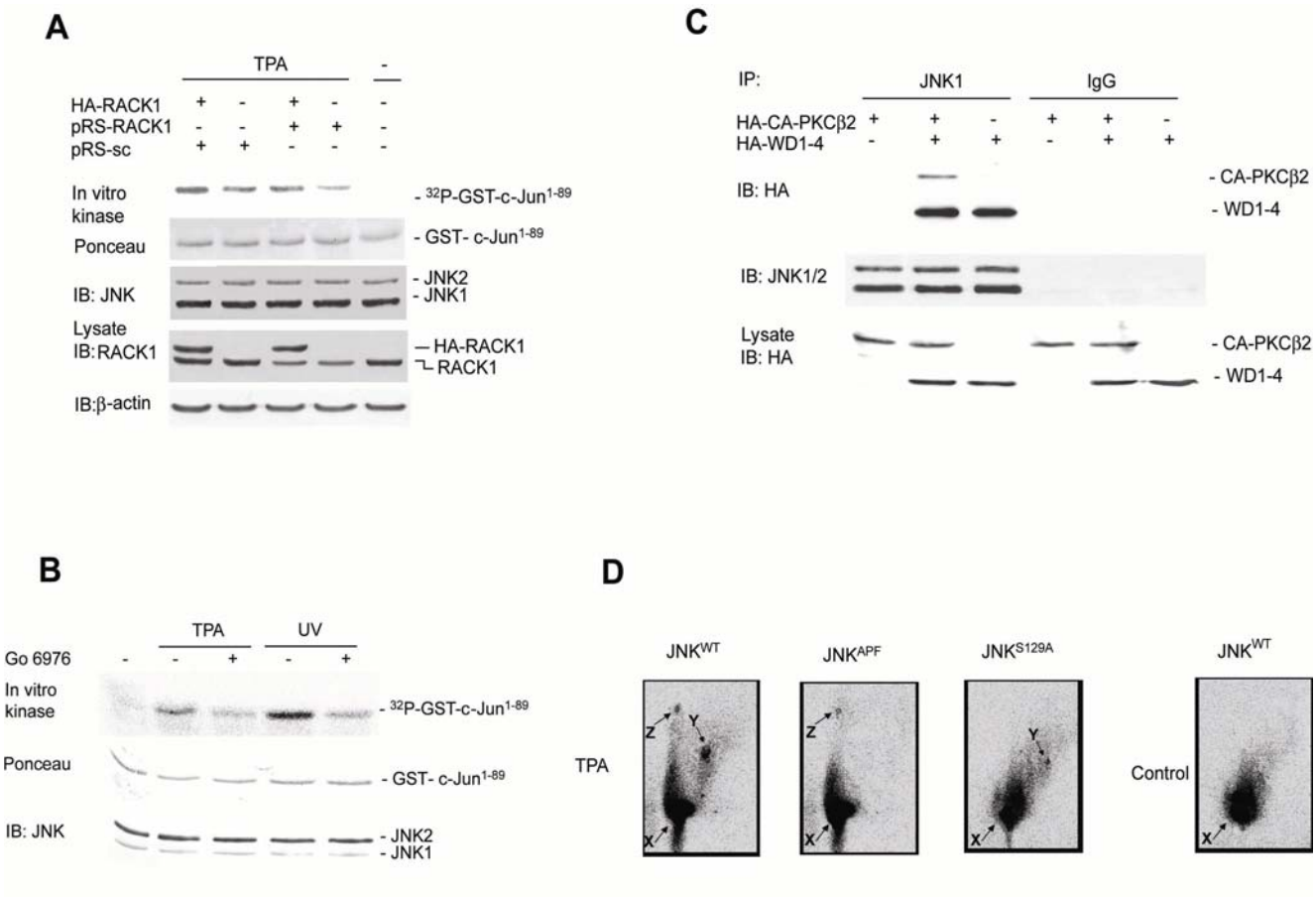

Figure S2

(A) Reconstitution of RACK1 into cells expressing siRNA of RACK1 increases JNK activation upon TPA stimuli. Experiment was performed in HEK 293 cells as described

in Figure 2D except that siRNA used to decrease endogenous RACK1 expression was obtained from the 5'UTR of RACK1. Cells that stably express the siRNA for RACK1 were transiently transfected with pEF-HA-RACK1 or pEF control plasmid followed by treatment with TPA and immunokinase reaction as detailed. siRNA for RACK1 reduces JNK activity (compare lane 4 with lane 2), whereas reconstitution with RACK1 restores JNK activity levels (compare lane 3 with lane 4).

(B) Go6976 blocks JNK2 activation by TPA and UV. MeWo cells were pretreated with Go6976 for 60 min followed by addition of TPA or UV irradiation. JNK in vitro kinase reactions and IB analysis were performed as described in Figure 3A except that an anti-JNK2 Ab was used for the IP.

(C) WD1-4 of RACK1 interacts with JNK and PKC. HEK 293T cells were transfected with 1 μg of pEF plasmids encoding HA-WD1-4 or HA-CA-PKCβII. Whole-cell lysates (500 μg) were subjected to immunoprecipitation (IP) using anti-JNK Ab or IgG (used as a control). Immunoprecipitates were analyzed by immunoblotting (IB) with anti-HA or anti-JNK Ab. Lower panel show IB analysis of protein lysates (80 μg) with anti-HA Ab to monitor protein levels.

(D) Mapping the site of PKC phosphorylation of JNK in vivo.
The experiment was performed as described in Fig. 5A. Cells were treated with TPA as described in experimental procedures. The phosphopeptides X, Y, and Z are indicated.

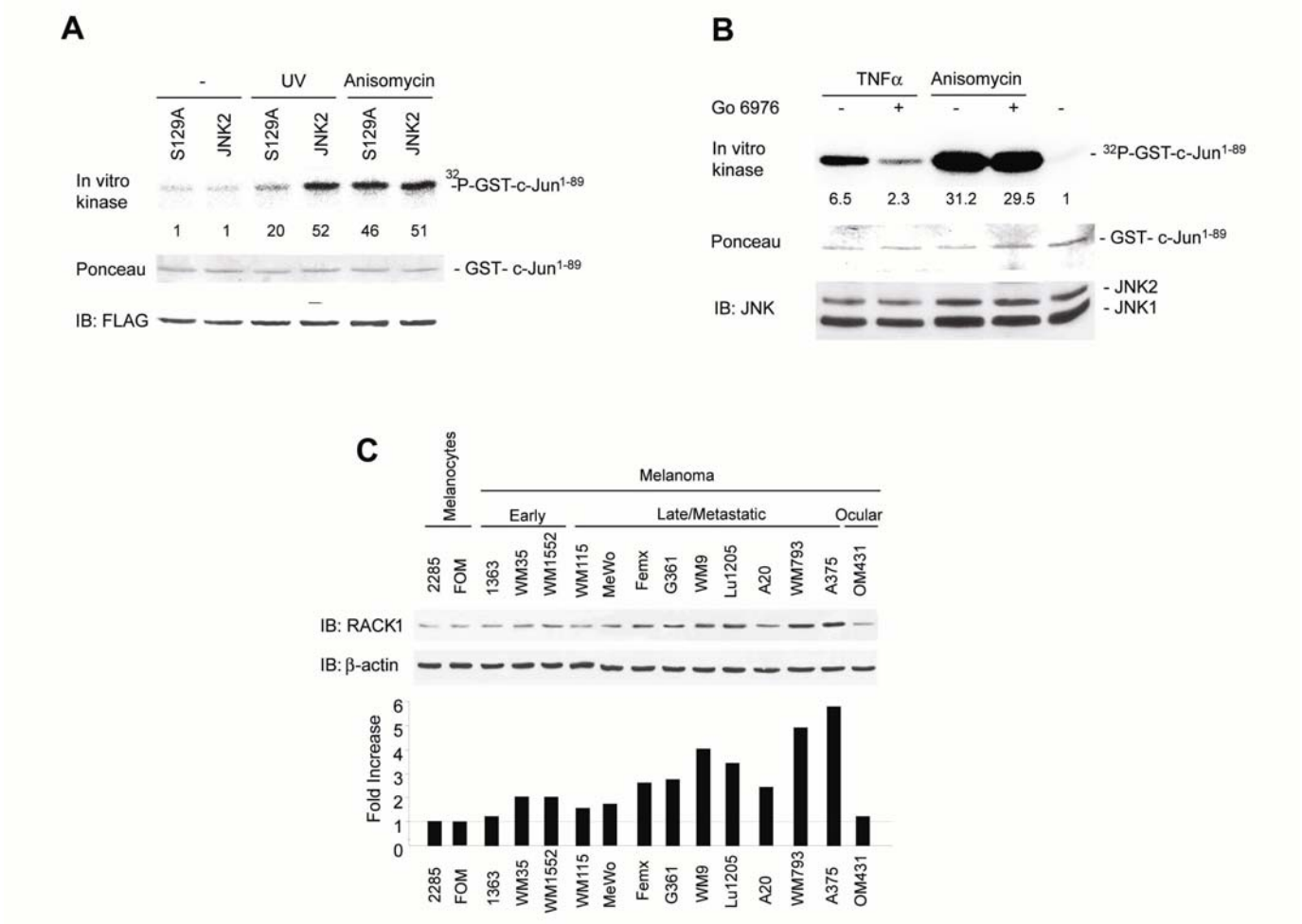

Figure S3

(A) The differences observed in JNK^{wt}/JNK^{S129A} activation by anisomycin and UV is not due to the different strength of these stimuli. The experiment was performed as described in Figure 6B except that 1 μg/ml anisomycin (for 30 min) was used. Quantification of JNK activity is shown.

(B) Anisomycin activates JNK in a PKC-independent manner. HEK 293 cells were pretreated with Go6976 for 60 min followed by addition of TNFα (40 ng/ml, 20 min) or anisomycin (10 μg/ml, 60 min). The JNK in vitro kinase reaction was performed as described in Figure 3A. Quantification of JNK activity is shown.

(C) RACK1 expression is elevated in melanoma cell lines. Protein extracts from normal human melanocytes (2285 and FOM) or the indicated melanoma cell lines (20 μg) were subjected to IB analysis using RACK1 Ab. Graph shows relative RACK1 expression normalized by densitometric analysis of RACK1 and β-actin bands.

Supplemental Experimental Procedures

In Vitro Kinase Assay

JNK immunokinase assays were performed with endogenous or exogenous JNK. When exogenous JNK was used, Flag-JNK2 or Flag-JNK2 mutant forms were transfected into HEK 293T cells. After 48 hr, cells were serum starved for 8-12 hr followed by the corresponding treatment. Flag-JNK2 variants were extracted with TNE buffer (see below), immunopurified with anti-Flag Ab, and subjected to an in vitro kinase assay using GST-Jun$^{1–89}$ as a substrate. Kinase reactions were carried out in 1× kinase buffer (Habelhah et al., 2002) in the presence of 2 μCi [^{32}P]ATP and 25 mM cold ATP for 30 min at 30°C. Reaction mixtures were then separated on SDS–PAGE and transferred to a nitrocellulose membrane, and the phosphorylation states of GST-Jun$^{5–89}$ was detected and quantified using a phosphorimager. Quantification of JNK activity is based on degree of c-Jun phosphorylation relative to the level of JNK in the immunoprecipitates. The same membranes were then used for immunoblotting with anti-phospho-JNK Ab and anti-Flag Ab, and for Ponceau S staining of GST-Jun$^{1–89}$. When endogenous JNK was used, an anti-JNK1 (or anti-JNK2) polyclonal Ab (Santa Cruz) was used for the immunoprecipitation. For the PKC kinase assay His-JNK2 (2 μg) was incubated with 10 ng of a catalytically active fragment of rat brain PKC (BioMol, PA) in 1× kinase buffer in the presence of 2 μCi [^{32}P]ATP and 25 mM cold ATP for 30 min at 30°C. Samples were processed as described for the JNK immunokinase assay.

In Vitro Binding Assay

Bacterially expressed and purified GST and GST-JNK2 bound to glutathione beads were incubated first with 1% BSA in PBS for 1 hr followed by incubation with ^{35}S-labeled CA-PKCβII, RACK1, WD4 or WD5, which were in vitro translated by using the TNT-coupled reticulocyte lysate system (Promega). Beads-bound material was subjected to washes (4×) with buffer A (PBS containing 0.25% NP40, 0.1% β-ME, 2 mM 16 EDTA) before being subjected to separation on SDS-PAGE, transference to nitrocellulose and analyzed using a phosphorimager. For direct interactions assays, GST and GST-JNK2 bound to glutathione beads were incubated first with 1% BSA in PBS for 1 hr followed by incubation with His-RACK1, His-WD1-4, or His-WD5-7. Beads-bound material was washed as described and subjected to separation on SDS-PAGE and immunoblotting with anti-His Ab (Santa Cruz).

Immunoblotting

Cells were harvested and lysed in TNE buffer (20 mM Tris–HCl, [pH 7.5], 350 mM NaCl, 1.0% NP-40, 2 mM EDTA, 2 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 50 mM NaF, 1.0 mM sodium vanadate, 10 mM β-glycerolphosphate and 1× cocktail protease inhibitors) for 30 min on ice followed by centrifugation at 12,500 × g for 20 min at 4°C. Protein samples (50–100 μg protein) were resolved on 10% SDS-PAGE and transferred to nitrocellulose. The primary antibodies were used at dilutions of 1:1,000 to 1:3,000. The secondary antibodies were anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (dilution 1:5,000). Signals were detected using ECL (Amersham Life

Sciences, NJ). Subcellular fractionation was performed as described (Sagawa et al., 2003).

Confocal Microscopy

Cells grown on 22-mm^2 coverslips were fixed in ice-cold methanol for 15 min at -20°C and briefly washed with acetone. The cells were then washed three times (5 min each) in phosphate-buffered saline, followed by further incubation with phosphate-buffered saline supplemented with 5% bovine serum albumin for 30 min. The cells were incubated on 75-µl drops of antibodies (diluted in phosphate-buffered saline [pH 7.4] containing 0.2% bovine serum albumin) for 1 hr at room temperature in a humidity chamber. The cells were washed three times in phosphate-buffered saline (5 min each) before incubation with 75-µl drops of fluorescein- or Texas red-conjugated anti-rabbit or mouse immunoglobulin G (Molecular Probes) diluted (2 µg/ml) in phosphate-buffered saline (pH 7.4) containing 0.2% bovine serum albumin for 60 min at room temperature in a humidity chamber in the dark. The coverslips were mounted on glass slides in Vectashield (Vector Laboratories).

Supplemental References

Habelhah, H., Frew, I.J., Laine, A., Janes, P.W., Relaix, F., Sassoon, D., Bowtell, D.D., and Ronai, Z. (2002). Stress-induced decrease in TRAF2 stability is mediated by Siah2. EMBO J. *21*, 5756–5765.

Sagawa, N., Fujita, H., Banno, Y., Nozawa, Y., Katoh, H. and Kuzumaki N. (2003). Gelsolin suppresses tumorigenicity through inhibiting PKC activation in a human lung cancer cell line, PC10. Br. J. Cancer *88*:606–612.