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Supplemental Information

RACK1 Mediates NLRP3 Inflammasome Activation by Promoting NLRP3 Active Conformation and Inflammasome Assembly

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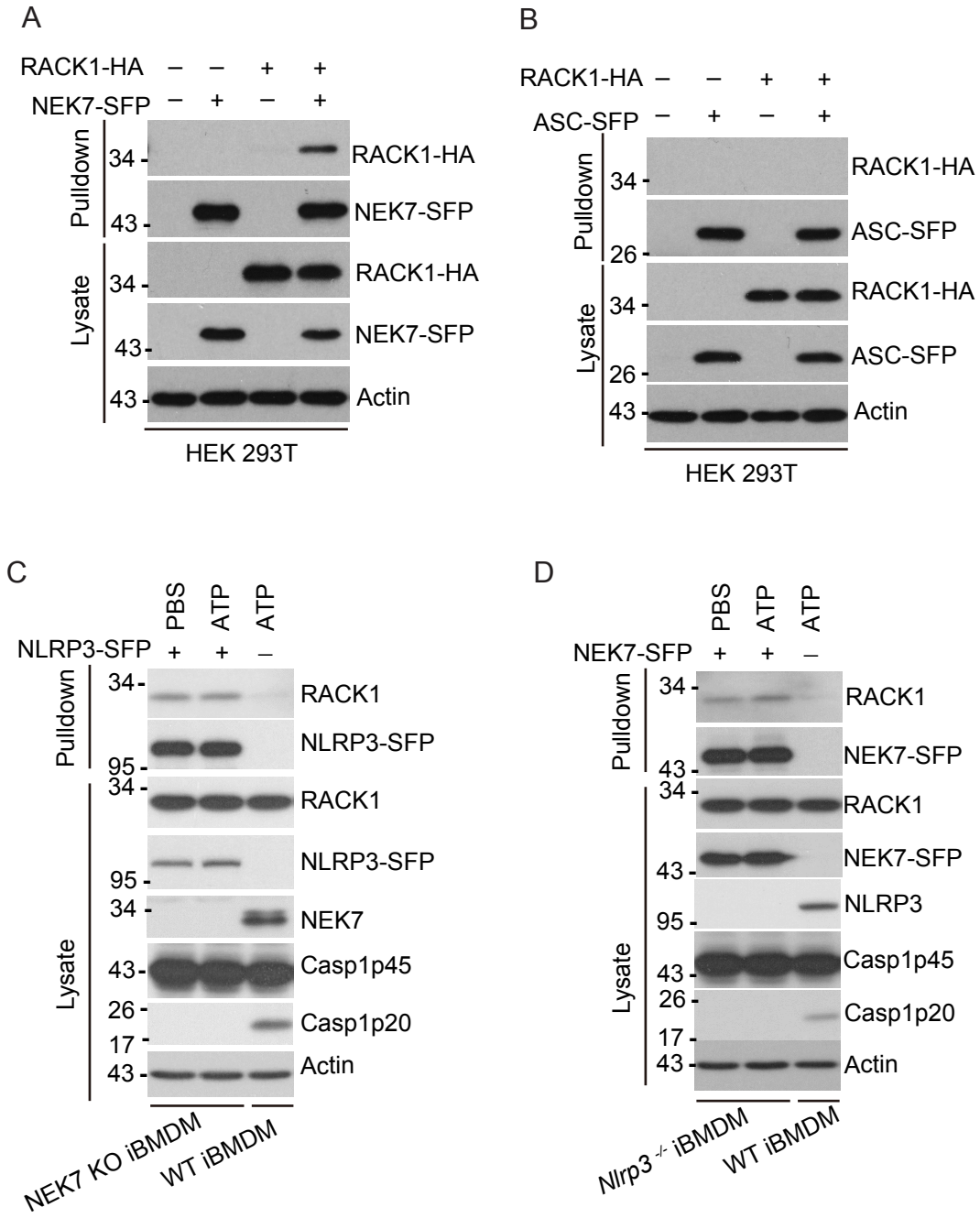

Figure S1. RACK1 interacts with NLRP3 or NEK7 but not ASC. Related to Figure 1. (**A**) Tagged NEK7 (NEK7-SFP), RACK1(RACK1-HA) or both were expressed in HEK293T cells. (**B**) Tagged ASC (ASC-SFP), RACK1-HA, or both were expressed in HEK 293T cells. (**C**) NLRP3-SFP was expressed in NEK7 KO iBMDM after lentiviral transduction. Cells were primed with LPS (200 ng mL^{-1}, 4 h) and then stimulated with PBS (control) or ATP (5 mM, 30 min). (D) NEK7-SFP was expressed in immortalized *Nlrp3* $^{-/-}$ iBMDM after lentiviral transduction. Cells were primed with LPS (200 ng mL^{-1}, 4 h) and then stimulated with PBS (control) or ATP (5 mM, 30 min). Uninfected wild-type (WT) iBMDM were used as a control. SFP-tagged proteins were pulled down by streptavidin beads. The pulldown complexes and cell lysates were analyzed by immunoblotting. Results are representative of three independent experiments.

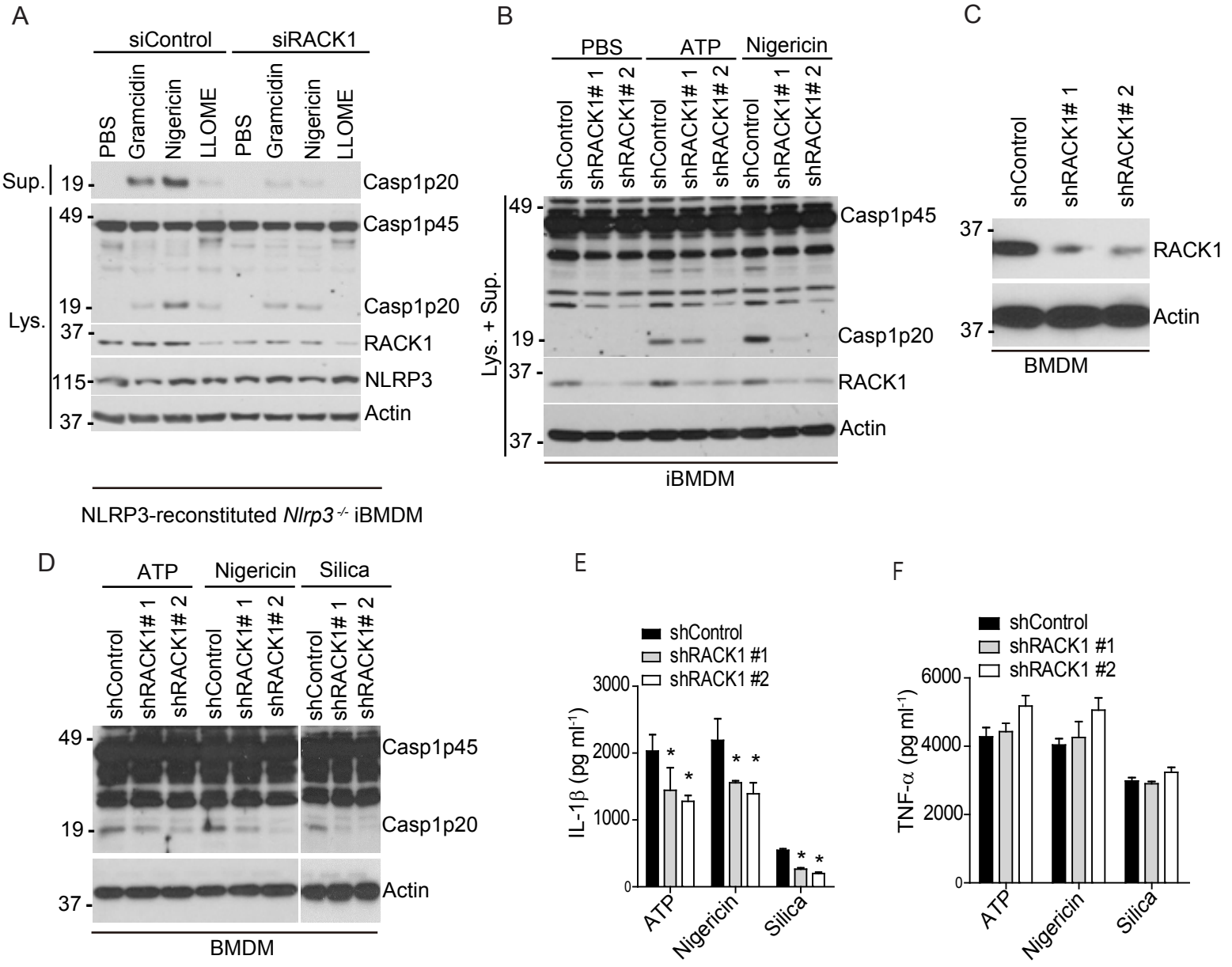

Figure S2. RACK1 is required for NLRP3 inflammasome activation. Related to Figure 2. (**A**) *Nlrp3*$^{-/-}$ iBMDM were reconstituted with NLRP3 by lentiviral transduction. Transduced cells were treated with control siRNA (siControl) or RACK1 siRNA (siRACK1) and then stimulated with PBS (control), gramicidin (0.5 μM, 1 h), nigericin (5 μM, 1 h) or LLOMe (2 μM, 4 h). Culture supernatants (Sup.) and cell lysates (Lys.) were immunoblotted with indicated antibodies. (**B**) iBMDM were treated with control shRNA (shControl), shRACK1 #1 or shRACK1 #2, and then stimulated with LPS (200 ng mL^{-1}, 4 h) plus PBS (control), ATP (5 mM, 30 min) or nigericin (5 μM, 1 h). Mixtures of cuture supernatants and cell lysates were analyzed for caspase-1 activation by immunoblotting. Wild-type BMDM were treated with control shRNA (shControl), shRACK1 #1 or shRACK1 #2, and then stimulated with LPS (200 ng mL^{-1}, 4 h) plus ATP (5 mM, 30 min), nigericin (5 μM, 1 h) or silica (500 μg mL^{-1}, 4 h). Cell lysates were collected and analyzed for RACK1 expression (**C**) or caspase-1 activation (**D**) by immunoblotting. IL-1β (**E**) and TNF-α (**F**) in culture supernatans were analyzed by ELISA. Error bars denote s.d. of triplicate wells. Student's t-test. *p <0.05. Results are representative of three independent experiments.

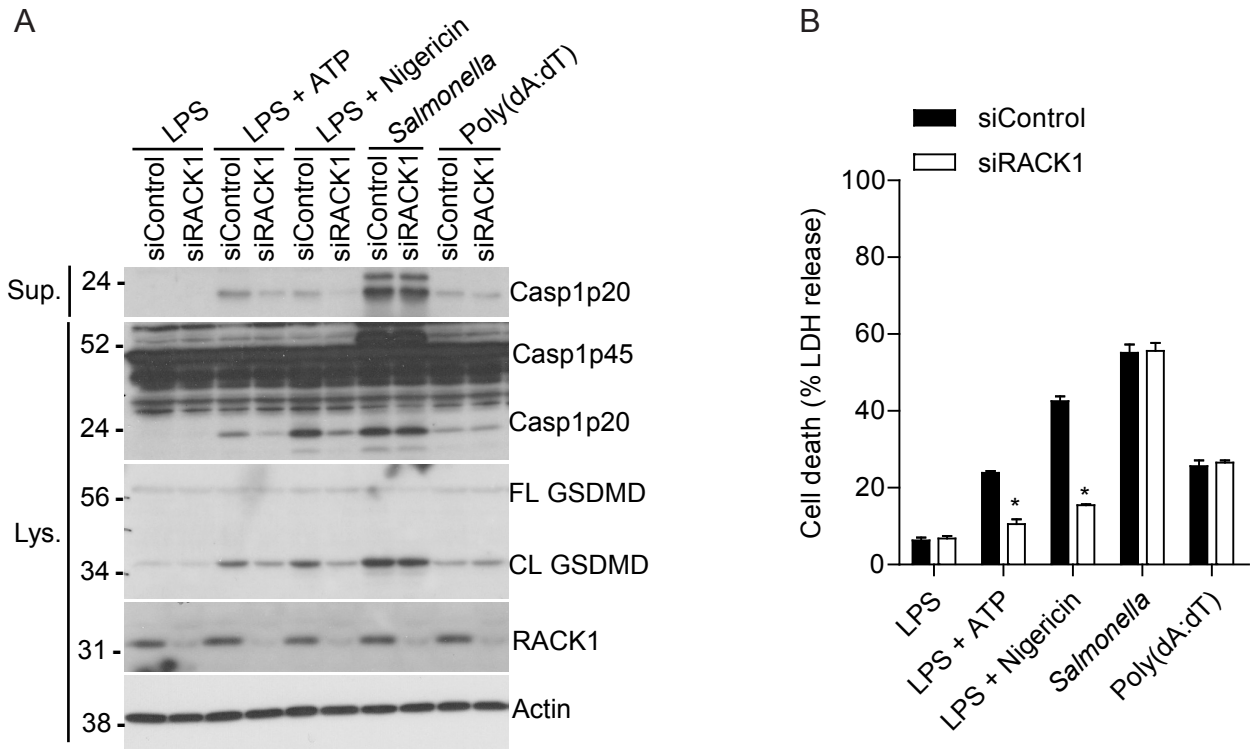

Figure S3. RACK1 specifically promotes NLRP3 stimuli-induced caspase-1 activation, GSDMD cleavage, and cell death. Related to Figure 2. iBMDM were treated with control siRNA (siControl) or RACK1 siRNA (siRACK1) and then stimulated with LPS (200 ng mL^{-1}, 4 h), LPS (200 ng mL^{-1}, 4 h) plus ATP (5 mM, 30 min), LPS (200 ng mL^{-1}, 4 h) plus nigericin (5 μM, 1 h), *Salmonella* (m.o.i=10, 1 h), or Poly(dA:dT) (2 μg mL^{-1}, 4 h). Culture supernatants (Sup.) and cell lysates (Lys.) were immunoblotted with indicated antibodies (**A**). Death of macrophages was measured as the release of LDH into culture supernatants (**B**). Error bars in **B** denote s.d. of triplicate wells. Student's t-test. **p* <0.05. Results are representative of three independent experiments.

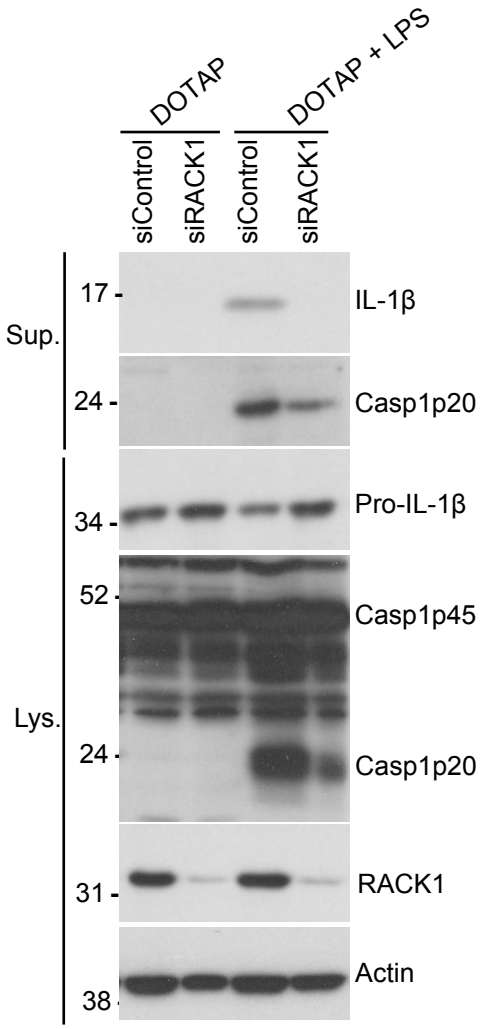

Figure S4. RACK1 knockdown inhibits caspase-1 activation and IL-1β maturation induced by cytosolic LPS in macrophages. Related to Figure 2. Control siRNA (siControl) or RACK1 siRNA (siRACK1)-treated iBMDM were primed with Pam3CSK4 (300 ng mL^{-1}, 6 h) and then transfected with DOTAP alone or DOTAP plus LPS (1 μg mL^{-1}, 4 h). Cell lysates (Lys.) and supernatants (Sup.) were collected and analyzed by immunoblotting with indicated antibodies. Results are representative of three independent experiments.

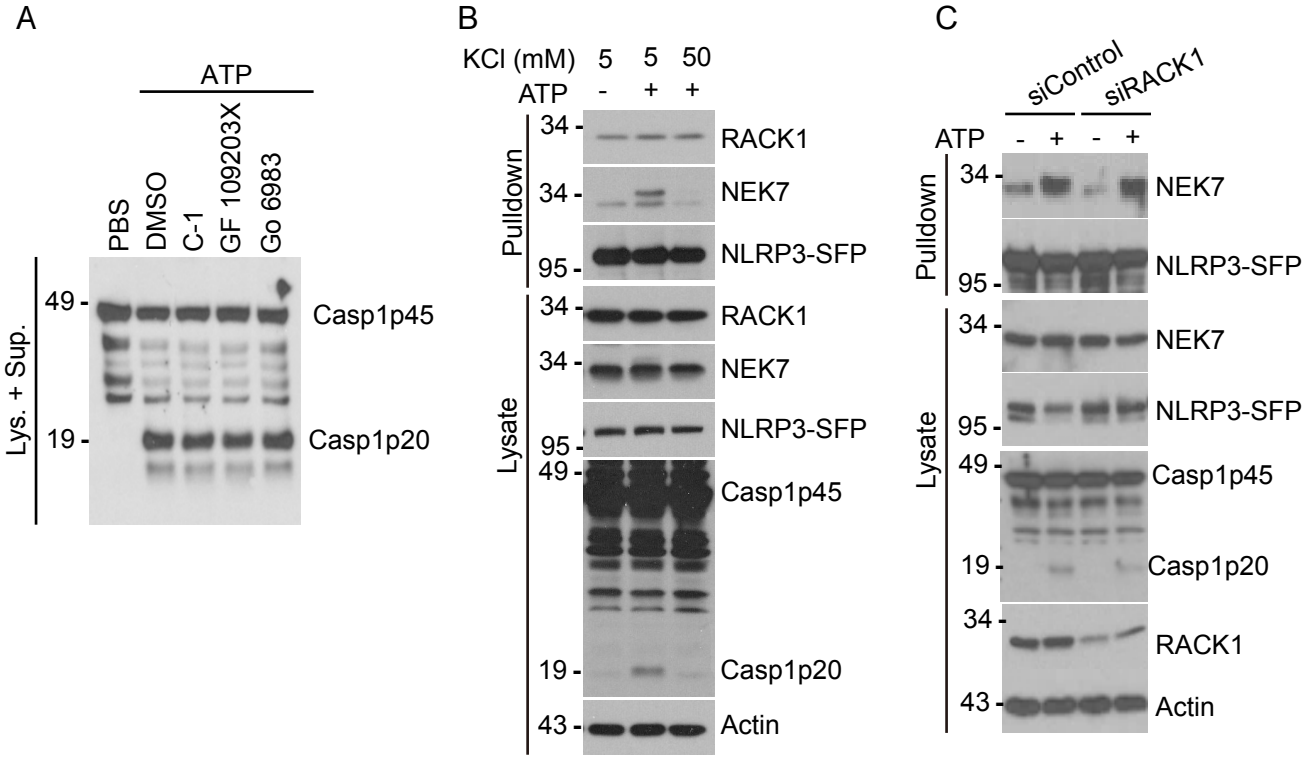

Figure S5. RACK1 promotes NLRP3 inflammasome activation independently of PKC kinase activity and potassium efflux, and RACK1 depletion does not affect the NLRP3-NEK7 interaction. Related to Figure 5. (**A**) LPS-primed BMDM were stimulated with PBS (control) or ATP (5 mM, 30 min) after 1 h-pretreatment with vehicle control (DMSO), PKC inhibitor C-1 (10 μM), GF 109203X (10 μM) or Go 6983 (10 μM). Mixtures of cell lysates and supernatants (Lys. + Sup.) were collected and analyzed for caspase-1 activation. (**B**) LPS-primed NLRP3-reconstituted *Nlrp3*$^{-/-}$ iBMDM were left unstimulated or stimulated with ATP (5 mM, 30 min) in the presence of low extracellular potassium (5 mM) or high extracellular potassium (50 mM). NLRP3-SFP was pulled down by streptavidin beads. The pulldown complexes and cell lysates were analyzed by immunoblotting. (**C**) NLRP3-reconstituted *Nlrp3*$^{-/-}$ iBMDM were treated with control siRNA (siControl) or RACK1 siRNA (siRACK1), and then stimulated with LPS (200 ng mL^{-1}) or LPS (200 ng mL^{-1}) plus ATP (5 mM, 30 min). The NLRP3 pulldown complexes and cell lysates were analyzed by immunoblotting. Results are representative of three independent experiments.

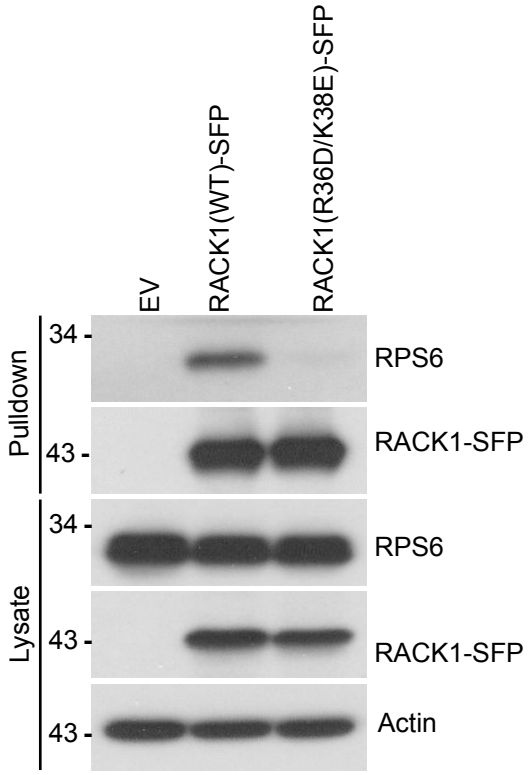

Figure S6. Mutant RACK1 (R36D/K38E) fails to associate with the 40S ribosomal subunit. Related to Figure 5. HEK 293T cells were transfected with empty vector (EV), vector expressing SFP-tagged wild-type RACK1 (WT) or mutant RACK1 (R36D/K38E) for 18 h. RACK1-SFP complexes were pulled down with streptavidin beads and analyzed by immunoblotting with indicated antibodies. Results are representative of two independent experiments.