SUPPLEMENTARY INFORMATION

Supplemental Figure Legends

Figure S1. RP3 does not induce expression of *CXCL12*. Expression of the noncanonical NF-κB-dependent gene *CXCL12* (Dejardin et al., 2002; Madge et al., 2008) in RP3-expressing MEFs was measured by qRT-PCR and normalized to levels in untreated Control cells that were retrovirally transduced with the MIGR vector alone. Control cells were either untreated (-) or incubated for 24 hrs (+) with mouse LIGHT (10 ng/ml) that activates the noncanonical NF-κB pathway and induces *CXCL12* expression (Dejardin et al., 2002; Madge et al., 2008). Unlike classical NF-κB dependent genes (Figure 1d), RP3 did not induce expression of *CXCL12*; indeed, it appears that RP3 leads to a decrease in basal *CXCL12* expression suggesting that signals elicited by RP3 negatively regulate *CXCL12*.

Figure S2. RP3 does not activate NF-κB in IKKα-deficient cells. Densitometry was performed on EMSAs of nuclear lysates from WT, IKKα$^{-/-}$ and IKKβ$^{-/-}$ MEFs that were stably transduced with either MIGR vector alone (Control; *Empty Bars*) or MIGR expressing RP3 (*Solid Bars*). The Mean Pixel Intensities (MPI) of the upper NF-κB complex (C1) and the lower complex (C2) in each EMSA were determined and these data were normalized to the MPI of an Oct1 DNA-binding band from the same sample. Data are from three separate analyses and represent means ± s.d.

Figure S3. RP3 does not induce expression of *NIK*. Expression of *NIK* in the absence and presence of RP3 was measured by qRT-PCR and normalized to levels in control MEFs that were retrovirally transduced with the MIGR vector alone (Con). Lysates from Control and RP3-expressing MEFs were immunoblotted using the antibodies indicted (*right*). These results demonstrate that NIK protein levels are stabilized in RP3-expressing cells without a concomitant increase in the levels of *NIK* gene expression. Hence, we conclude that the effects of RP3 on NIK levels do not occur via increased gene transcription.

Figure S4. RP3 does not associate with TRAF2, TRAF3, NEMO, IKKα or NIK. (**a**) Lysates from MEFs retrovirally transduced with either MIGR vector alone (Control) or MIGR expressing RP3 were immunoprecipitated (IP) using the antibodies indicated (*top*). Resulting immunoprecipitates were immunoblotted using the antibodies indicated (*left*). A portion of the lysates was retained prior to IP and run on the gel as the input sample (Pre-IP; *lanes 1 and 4*). (**b**) Lysates of RP3-expressing MEFs were immunoprecipitated using either anti-NEMO or anti-RET (*top*) then the samples were immunoblotted using the antibodies depicted (*left*). A Pre-IP sample demonstrating the presence of each protein in the original lysate was run in *lane 1*. These data show that RP3 does not associate with any of the proteins investigated.

Figure S5. RP3-induced PI3K/AKT signaling does not regulate NIK expression. MEFs retrovirally transduced with either MIGR vector alone (Control) or MIGR

expressing RP3 were untreated (Con.) or incubated for 30 minutes with either vehicle (DMSO; 0.1%) or wortmannin (100 nM) then lysates were immunoblotted using the antibodies indicated (*right*). These data show that RP3 induces the phosphorylation of AKT that is inhibited by wortmannin; however, despite a complete loss of phospho-AKT, RP3-induced NIK expression remains intact.

Supplemental Methods

Reagents and Antibodies.

Recombinant mouse LIGHT was from R&D Systems, Inc. (Minneapolis, MN). All antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Wortmannin was from EMD Chemicals (Gibbstown, NJ).

Real Time PCR primers.

All primer sets for qRT-PCR were obtained from Qiagen Inc. (Valencia, Ca). The following sets were used in this study: *Map4K4* (*NIK*) (QT00113582), *CXCL12* (QT00161112), *CCL2* (QT00167832), *TNF* (QT00104006), *CXCL1* (QT00115647), *CSF2 (GM-CSF)* (QT00251286). All qRT-PCR procedures were performed as described in our previous publications (Madge et al., 2008; Solt et al., 2009; Solt et al., 2007).

Immunoprecipitations and Immunoblotting.

Immunoprecipitation (IP) and immunoblotting was performed as previously described (Solt et al., 2009; Solt et al., 2007). Briefly, Cells were lysed at 4°C in 100μl TNT lysis buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, and 1% Triton X-

100) and a complete mini protease inhibitor cocktail (Roche, Indianapolis, IN). Samples were then scraped and harvested into 1.5 ml microcentrifuge tubes, vortexed for 30 seconds then centrifuged (425g / 10 min). For immunoprecipitations, cell extracts were incubated with 2 μg of primary antibody for 1 hour at 4°C followed by incubation (1 hr / 4°C) with 50 μl of a 50% slurry of Protein A/G beads (Pierce; Rockford, IL). A portion of each sample pre-immunoprecipitation (pre-IP; 5%) was retained for analysis. The beads were washed 3 times with lysis buffer then samples were analyzed by SDS-PAGE (10%) followed by immunoblotting.

Densitometry

Densitometry was performed using a Gel-Doc EQ and the QuantityOne software package (Bio-Rad Laboratories, Hercules, CA). Pixel intensity was measured in identical rectangular volumes around each band on EMSAs and a background value in an equal rectangular volume separate from the bands was subtracted to obtain the mean pixel intensity / mm^2 (MPI). The MPI of each NF-κB band was normalized to the MPI of an Oct1 DNA-binding band from the same sample.

Supplemental References

Dejardin E, Droin NM, Delhase M, Haas E, Cao Y, Makris C, Li ZW, Karin M, Ware CF and Green DR. (2002). *Immunity*, 17, 525-35.

Madge LA, Kluger MS, Orange JS and May MJ. (2008). *J Immunol*, 180, 3467-77.

Solt LA, Madge LA and May MJ. (2009). *J Biol Chem*, 284, 27596-608.

Solt LA, Madge LA, Orange JS and May MJ. (2007). *J Biol Chem*, 282, 8724-33.

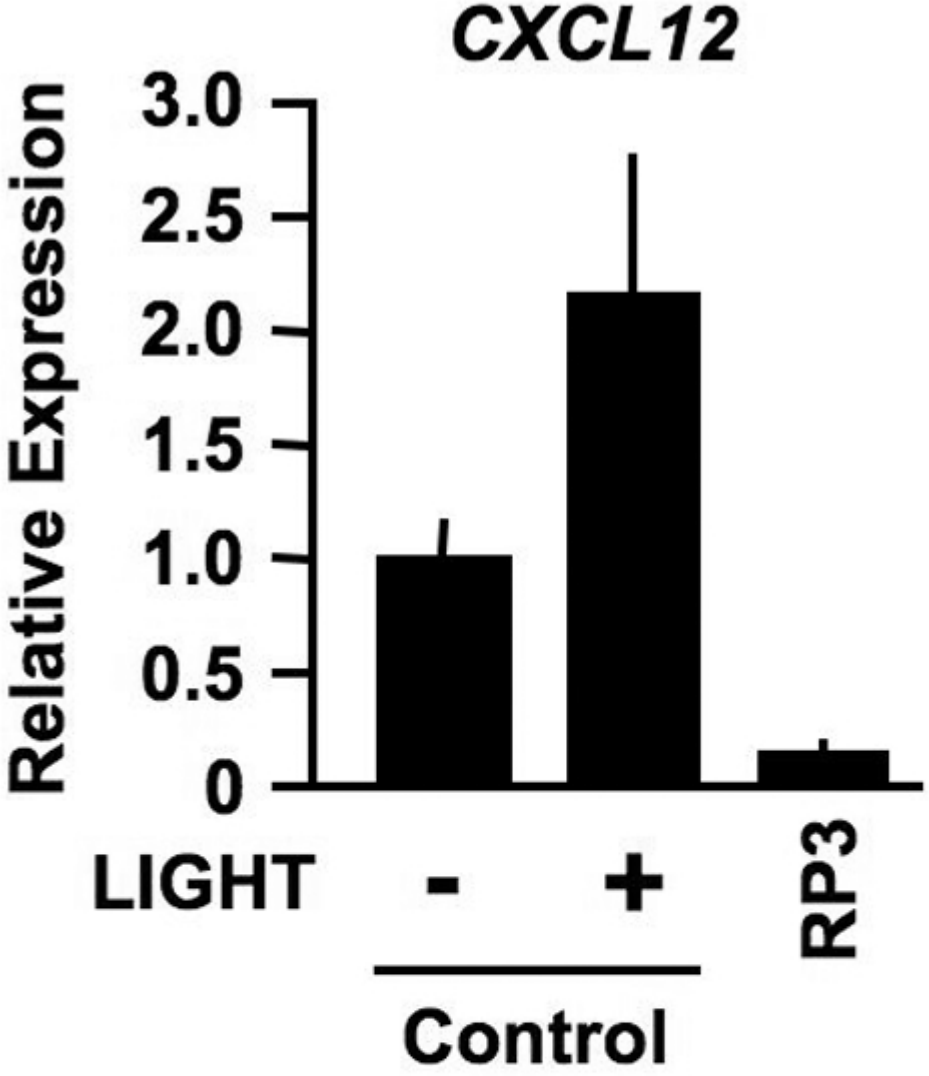
CXCL12
Relative Expression
3.0
2.5
2.0
1.5
1.0
0.5
0
LIGHT
-
+
RP3
Control

Figure S1.

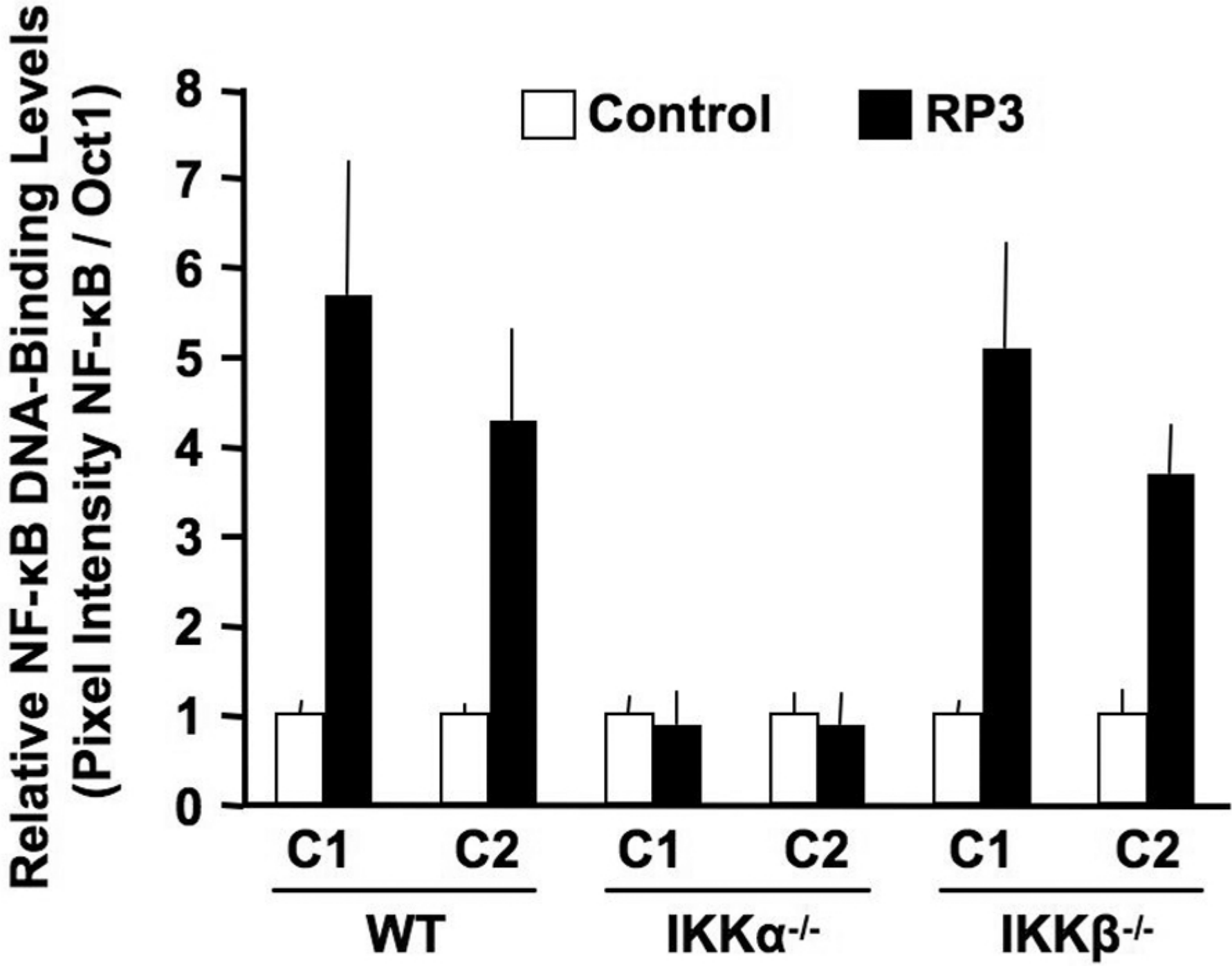
Relative NF-κB DNA-Binding Levels
(Pixel Intensity NF-κB / Oct1)
8
7
6
5
4
3
2
1
0
Control
RP3
C1
C2
C1
C2
C1
C2
WT
IKKα-/-
IKKβ-/-

Figure S2.

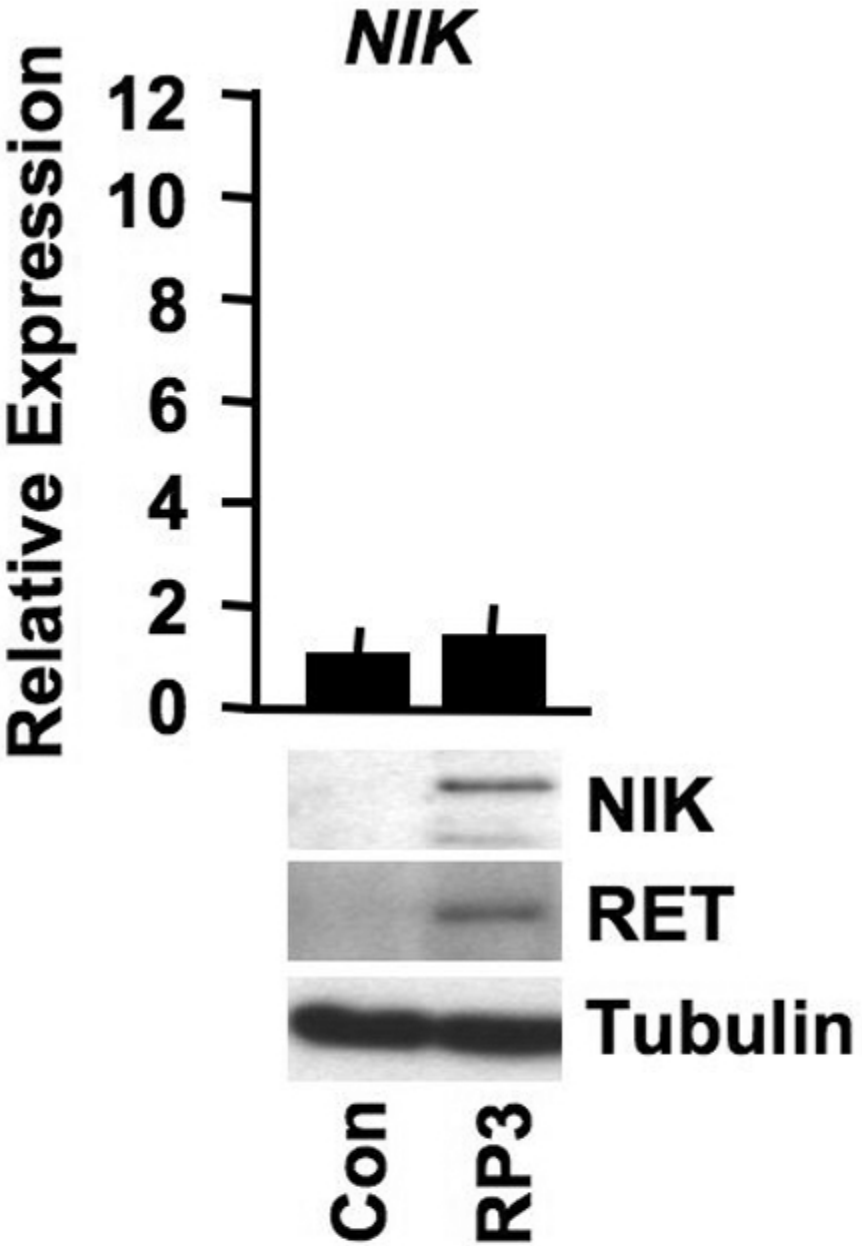

Figure S3.

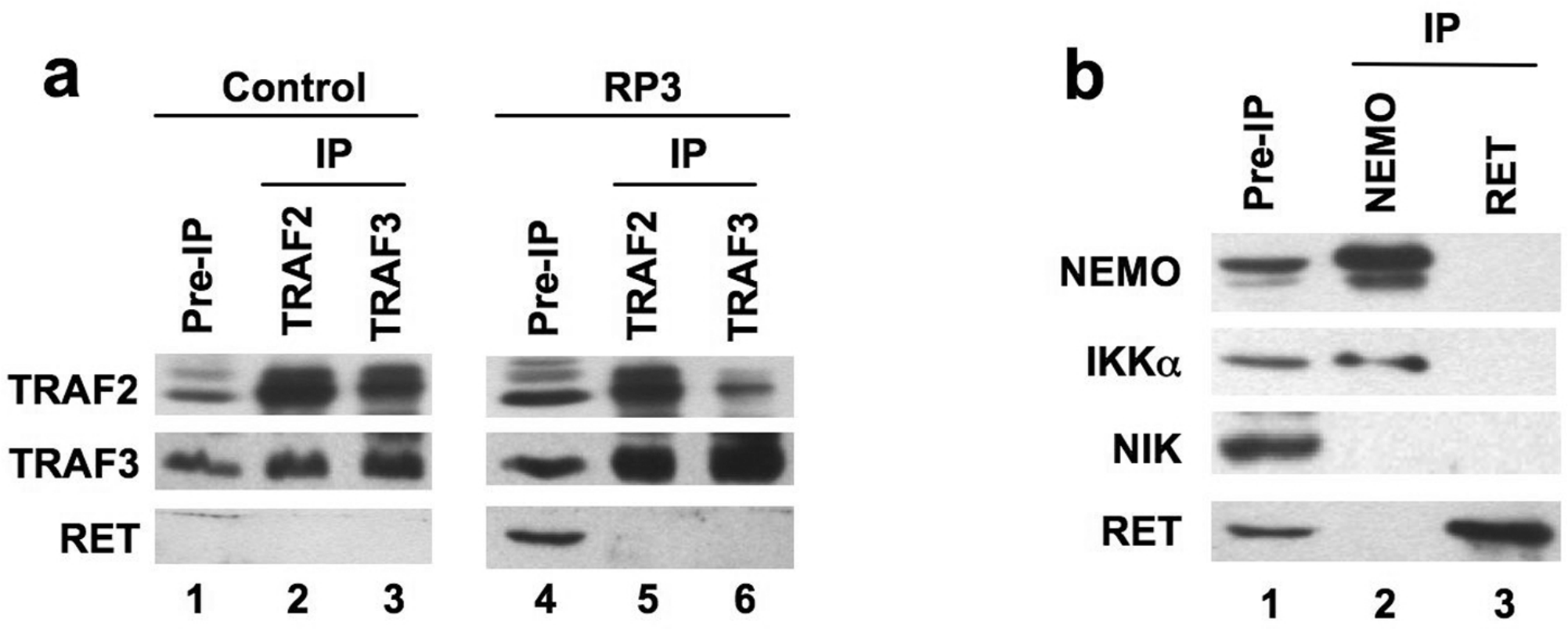
a
Control
RP3
IP
IP
Pre-IP
TRAF2
TRAF3
Pre-IP
TRAF2
TRAF3
TRAF2
TRAF3
RET
1
2
3
4
5
6
b
IP
Pre-IP
NEMO
RET
NEMO
IKKα
NIK
RET
1
2
3

Figure S4.

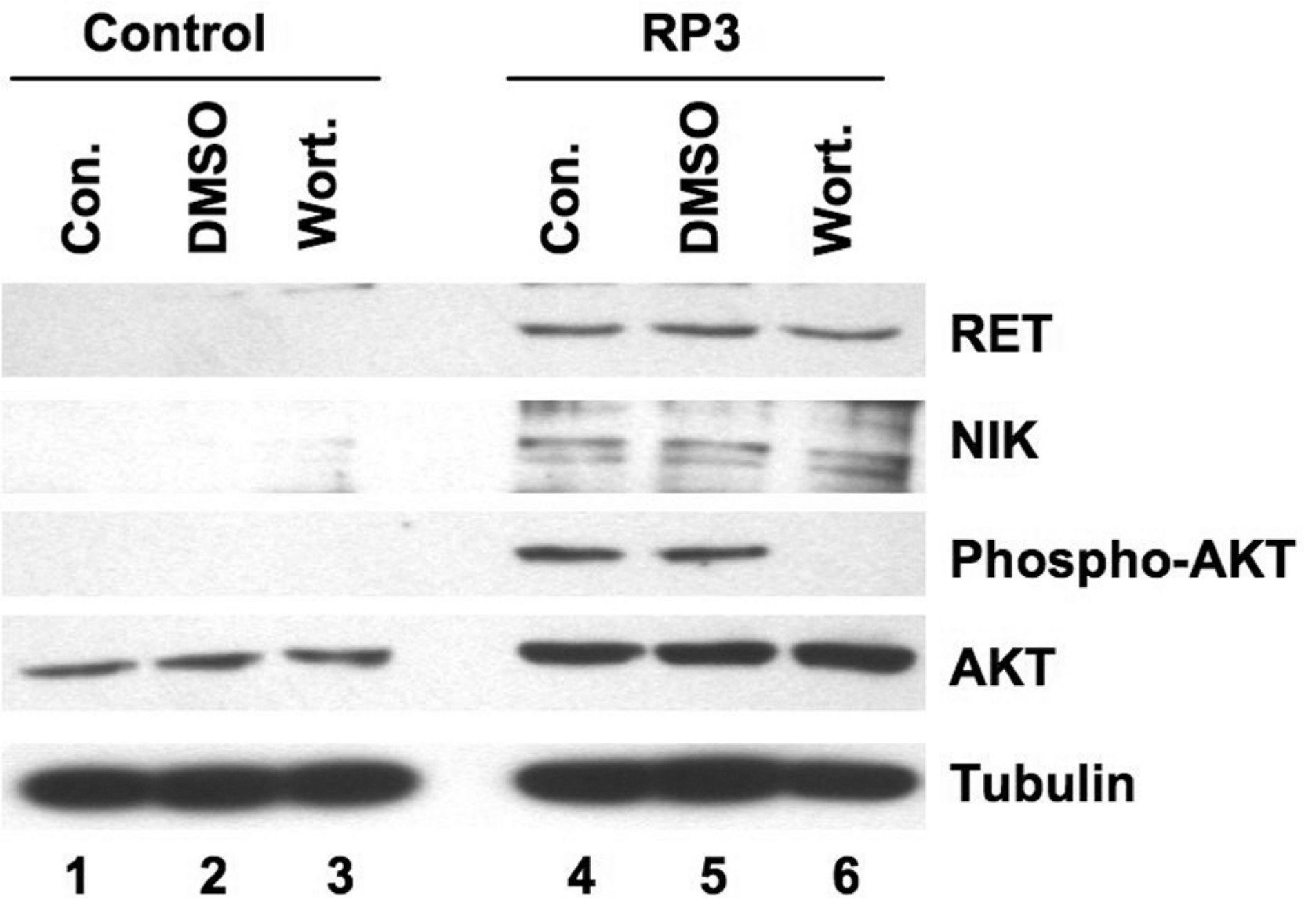
Control
RP3
Con.
DMSO
Wort.
Con.
DMSO
Wort.
RET
NIK
Phospho-AKT
AKT
Tubulin
1
2
3
4
5
6

Figure S5.