Supplementary information, Data S1

Materials and Methods

Reagents and antibodies

TNFα, IL-1β and IFNγ (R&D systems); Cycloheximide and BMS-345541 (Sigma); MG132 (Biomol); mouse monoclonal antibodies against Flag, HA and β-actin (Sigma), Myc (Cell Signal Technology), IKKα, p50 and Ubiquitin (Santa Cruz Biotechnology); rabbit polyclonal antibodies against IKKγ, p65 (Santa Cruz Biotechnology), IκBβ (Cell Signal Technology) were purchased from the indicated manufacturers. Mouse anti-MIB1, anti-IκBα and anti-p100 antisera were raised against recombinant human MIB1 (286-889), IκBα and p100 proteins respectively.

Constructs

NF-κB and IRF1 promoter luciferase reporter constructs were previously described [15]. Mammalian expression plasmids for Flag- or HA-tagged human MIB1 and its mutants were constructed by standard molecular biology techniques. Targeting vector TK-Neo-USER and two packaging vectors, AAV-RC and Helper, were previously described [16].

Expression Cloning

The expression clones encoding ubiquitin-related enzymes on GFC-Transfection Arrays were purchased from Origene. The clones were transfected together with an NF-κB luciferase reporter plasmid into 293 cells. Sixteen hours after transfection, reporter assays were performed to identify clones that could markedly activate the NF-κB reporter.

Genetic knockout in human somatic cells

The genetic knockout in human colon cancer HCT116 cells were performed following a previously described method [13]. The targeting vector was constructed based on the TK-Neo-USER plasmid. The 293T cells (~1×10^7) were transfected with the targeting construct (3 μg) and two packaging vectors (AAV-RC, Helper; 3 μg each) to make recombinant adeno-associated viruses (rAAVs). HCT116 cells (~8×10^5) were infected with virus stock for 48 hr and were then split into a series of 96-well plates with limited dilution methods in order to obtain single clones. Two weeks after G418 (0.4 mg/ml) selection, hundreds of clones were screened with designed primers. The positive recombinant clones were amplified to 2×10^5 cells for infection with Adeno-cre virus. The infected cells were split and diluted into 96-well plates in order to obtain single clones. Two-weeks later, the clones were screened by PCR with proper primers for one allele disrupted positive clones. Subsequently, the second recombination was performed through infection of the one allele disrupted clones (8×10^5) with rAAV stock again. Screening of the second allele disrupted clones was then performed similarly. Western blot analysis was performed to confirm the deficiency of expression of targeted gene in

the two-allele knockout cells.

Transfection and reporter assays

The 293 cells (~1×10^5) were seeded on 24-well dishes and transfected the following day by standard calcium phosphate precipitation. In the same experiment, empty control plasmid was added to ensure that each transfection receives the same amount of total DNA. To normalize for transfection efficiency, 0.02 μg of pRL-TK *Renilla* luciferase reporter plasmid was added to each transfection. Luciferase assays were performed using a dual-specific luciferse assay kit (Promega). Firefly luciferase activities were normalized based on *Renilla* luciferase activities.

Coimmunoprecipitation and immunoblot analysis

Transfected 293 cells from each 100 mm dish were lysed in 1 ml lysis buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1% Triton, 1 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF). For each immunoprecipitation, 0.8 ml aliquots of lysates were incubated with 1 μl polyclonal anti-p65, anti-MIB1 or anti-IκBα, or 0.5 μg monoclonal antibody against the epitope tag, and 25 μl of a 1:1 slurry of Protein G Sepharose (GE healthcare) for at least 1 hr. The sepharose beads were washed three times with 1 ml of lysis buffer containing 500 mM NaCl. The precipitates were fractionated on SDS-PAGE. Immunoblot analysis was performed with the indicated antibodies.

Quantitative real-time PCR

Total RNA was extracted from cultured cells with TRIzol reagent (Roche) and was reverse-transcribed with M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Real-time PCR was performed using iQ SYBR Green Supermix and analysed on C1000 Thermal Cycler (Bio-Rad). PCR was done with the following primers: GAPDH, 5'-GACAAGCTTCCCGTTCTCAG-3' and 5'-GAGTCA ACGGATTTGGTCGT-3'; IκBα, 5'-CGGGCTGAAGAAGGAGCGGC-3' and 5'-AC GAGTCCCCGTCCTCGGTG-3'; cIAP1, 5'-CAGACACATGCAGCTCGAATGAG-3' and 5'-CACCTCAAGCCACCATCACAAC-3'; ICAM1, 5'-TCAGTGTGACCGCAG AGGACGA-3' and 5'-TTGGGCGCCGGAAAGCTGTAGAT-3'; TNFα, 5'-GCCGCA TCGCCGTCTCCTAC-3' and 5'-CCTCAGCCCCCTCTGGGGTC-3'; Rantes, 5'-GGC AGCCCTCGCTGTCATCC-3' and 5'-GCAGCAGGGTGTGGTGTCCG-3'; CXCL10, 5'-GGTGAGAAGAGATGTCTGAATCC-3' and 5'-GTCCATCCTTGGAAGCACTG CA-3'.