Supporting Information

Narasimamurthy et al. 10.1073/pnas.1209965109

SI Materials and Methods

Reagents. Mouse TNF-α (T7539) and DMSO (D2650) were purchased from Sigma. IKK2 inhibitor MLN120B was purchased from Millennium Pharmaceuticals; 8Br-cAMP (203800), H89 (371963), forskolin (344270), and isoproterenol (420355) were obtained from Calbiochem. Prostaglandin E2 (14750) and IBMX (I-1036) were purchased from Caymen Chemicals and A.G. Scientific Inc., respectively. Lentiviral vector to knockdown *AKIP1* was purchased from Open Biosystems (RMM4534).

Antibodies. The antibodies against IκBα (C-21, sc-371), phospho-IKK2 (sc-2681), VASP (sc-13975), p65 (sc-8008), and adenylyl cyclase III (sc-588) were purchased from Santa Cruz Biotechnology. IKK2 was detected by using an antibody from Biosource. Anti-α-tubulin (T6074) and anti-FLAG (F-1804) antibodies were purchased from Sigma. The antibodies against phospho-IκBα S32/36 (9246), phospho-VASP S157 (3111), and phospho-p65 S276 (3037) were obtained from Cell Signaling Technology. Anti-TBP (TATA Binding Protein, ab51841-100) antibody was purchased from Abcam. The HRP conjugated secondary antibodies to detect mouse IgG (sc-2005) and rabbit IgG (NA934V) were purchased from Santa Cruz Biotechnology and GE Health care, respectively.

Transfection. Fibroblasts were seeded into a 60-mm dish one day before transfection, and cells were transfected with indicated plasmids by X-tremeGene HP reagent (Roche) according to manufacturer's protocol. Forty-eight hours after transfection, GFP positive cells were sorted by FACS analysis and collected for mRNA extraction; 293T cells were seeded into a six-well plate one day before transfection, and cells were transfected with indicated plasmids by Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol.

Western Blotting, Densitometric Analysis, and Coimmunoprecipitation. Cells were lysed in lysis buffer [150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1% Nonidet P-40, and 0.5% deoxycholic acid] containing protease inhibitors (Complete; Roche) and phosphatase inhibitors (PhosStop; Roche). Protein samples were loaded and separated on NuPAGE (Invitrogen) 4–12% Bis-Tris gradient gel by electrophoresis and transferred on PVDF membrane (Immobilon-P; Millipore). After blocking in PBS containing 0.1% Tween-20 and 5% nonfat milk, membranes were probed with indicated primary antibodies and appropriate secondary antibodies conjugated with HRP. Signals were detected with ECL reagents from Denville Scientific, Inc. (Hyglo) and GE Healthcare. Densitometric analysis of the protein bands obtained by Western blot was performed using the ImageJ (National Institutes of Health) software. Relative value of the protein was shown after normalizing it against the appropriate loading control; here the value of the untreated WT cell sample was set as 1 and other samples were calculated correspondingly to compare ratios. For coimmunoprecipitation, 293T cells transfected with either Flag-CRY1 or GFP, then 48 h later, cells were lysed in a lysis buffer [Tris-HCl at pH 8.0 (50 mM), NaCl (250 mM), and Nonidet P-40 (0.5%)] containing protease and phosphatase inhibitors. After preclearing with Dynabeads (Invitrogen) for 1 h, lysates were added with Dynabeads cross-linked to either nonspecific IgG or adenylyl cyclase III, and allowed to rotate overnight at 4 °C. Then, beads were collected and washed thrice in lysis buffer. Proteins from beads were extracted with LDS sample buffer (NuPAGE; Invitrogen) and performed Western blot.

RNA Extraction, Reverse Transcription, and Quantitative Real-Time PCR. To isolate RNA, cells were harvested in TRIzol reagent (Invitrogen) and RNA was isolated according to manufacturer's protocol. Reverse transcription was performed using the SuperScript III kit (Invitrogen) with either 0.5 or 1.0 μg of total RNA and oligo(dT) for 50 min at 50 °C, followed by 5 min at 85 °C. Quantitative real-time PCR was performed using ABI Prism 7700 (Applied Biosystems) sequence-detection system with SYBR Green (Applied Biosystems). *Actin* was used as an internal control gene to normalize against the target genes. The following qRT–CR primer pairs were used to detect expression of various mouse genes:

IL-6: TAGTCCTTCCTACCCCAATTTCC and TTGGTCCTTAGCCACTCCTTC
TNF-α: TCTCATGCACCACCATCAAGGACT and TGACCACTCTCCCTTTGCAGAACT,
IκBα: CGC TTG GTG GAC GAT CG and TTGCTCGTACTCCTCGTCCTTC
iNOS: GGCAGCCTGTGAGACCTTTG and GCATTGGAAGTGAAGCGTTTC
Lcn2: TGGCCCTGAGTGTCATGTG and CTCTTGTAGCTCATAGATGGTGC
SAA1: TTCCATGCTCGGGGAACTATG and TCTGTAGTGATTGGGGTCCTTG
SAA2: TGGCTGGAAAGATGGAGACAA and AAAGCTCTCTCTTGCATCACTG
Cxcl1: CTGGGATTCACCTCAAGAACATC and CAGGGTCAAGGCAAGCCTC
AKIP1: ACCCTTAGCTCTTCCTTCGGA and CGGTAGACATGGGTTGCTCC
Actin: GATATCGCTGCGCTGGTC and CCTCGTCACCCACATAGGAG

Bone Marrow Transplantation. Bone marrow hematopoietic progenitor cells were harvested from femur and tibia of WT or *Cry1*$^{-/-}$*:Cry2*$^{-/-}$ mice and injected via tail vein into lethally irradiated B6.129S4*Il2rgtm1Wjl*/J mice (Jackson Laboratories) (1100 rads; Cobalt-60 source) with a cell dose of 10^6 mononuclear cells. Efficient reconstitution with donor bone marrow cells was confirmed (see also Fig. 2*A* and Fig. S2 *A–D*). Eight weeks after transplantation, BMT mice were injected with PBS or LPS (5 mg/kg of body weight) (see text in *Results* section).

Quantification of TNF-α and IL-6 by ELISA. Protein levels of TNF-α and IL-6 in the serum of mice and in the supernatants of the fibroblast or BMDM cells were measured according to the manufacturer instructions (555268 and 555240 BD OptEIA; BD Bioscience).

Quantification of cAMP by ELISA. Fibroblasts were seeded into a 12-well plate and 12 h later, cells were treated with 500 μM of IBMX, a phosphodiesterase inhibitor, for 30 min. Then the cells were either left untreated (control) or treated with DMSO or forskolin (10 μM) for 30 min and lysed in cell lysis buffer. Lysates were treated according to the manufacturer's instructions (KGE002B; R&D Systems) and measured cAMP levels using a 96-well plate reader (HTS 7000 PLUS; Perkin-Elmer).

Lentiviral Vectors. The LV-GFP and LV-GFP-IκBαM were derived from p156RRLsinPPTPGK-eGFP-PRE vector, wherein

the transgene is driven by the mouse phosphoglycerate kinase (PGK) promoter. For sh-RNA-mediated knockdown of *AKIP1* lentiviral vectors purchased from Open Biosystems was applied.

Isolation of Nuclear and Cytoplasmic Fractions. Nuclear and cytoplasmic fractions of WT and *Cry1*$^{-/-}$:*Cry2*$^{-/-}$ fibroblasts were isolated according to the manufacturer's instructions (NE-PER, Thermo Scientific).

p65 Binding Activity Assay. p65 binding activity was performed on an ELISA-based assay according to the manufacturer's instructions (Active Motif, TransAM NF-κB p65). Briefly, nuclear extracts (5 μg) from WT and *Cry1*$^{-/-}$:*Cry2*$^{-/-}$ fibroblasts were incubated for 1 h in the wells of a NF–κB consensus binding sequence oligonucleotide-coated 96-well plate. Specificity for p65 was achieved by incubation with anti-p65 primary antibodies for 1 h. HRP-conjugated secondary antibodies were used for the detection of p65 bound to the κB sequences. The p65 binding activity was measured by using a colorimetric plate reader (HTS 7000 PLUS; Perkin-Elmer) at 450 nm. Competitor assays were performed by incubation of nuclear lysates with excess (unbound) of either WT or mutated consensus NF–κB oligonucleotides.

Glosensor Assay. The 293T cells were transfected with 125 ng of pGlosensor 22F cAMP plasmid (E2301; Promega) together with either 125 ng of pcDNA3.1 (filler plasmid) or Flag-CRY1 plasmid per well of a 96-well plate. Twenty four hours after transfection, cells were incubated with CO_2-independent medium (18045; Invitrogen) containing Glosensor cAMP reagent (E1291; Promega) for 90 min at room temperature (RT) and then added 500 μM of IBMX, an inhibitor phosphodiesterase, to the medium and allowed for 30 min at RT. Then the cells were treated either with vehicle, forskolin (10 μM), PGE2 (10 μM), or isoproterenol (10 μM), and immediately the kinetics of cAMP turnover was measured in a 96-well plate reader (Infinite M200; Tecan) for 30 min at RT.

Statistical Analysis. Results are expressed as either mean ± SD or mean ± SEM from an appropriate number of samples as indicated in the figure legends. Student's *t* test was used to determine statistical significance.

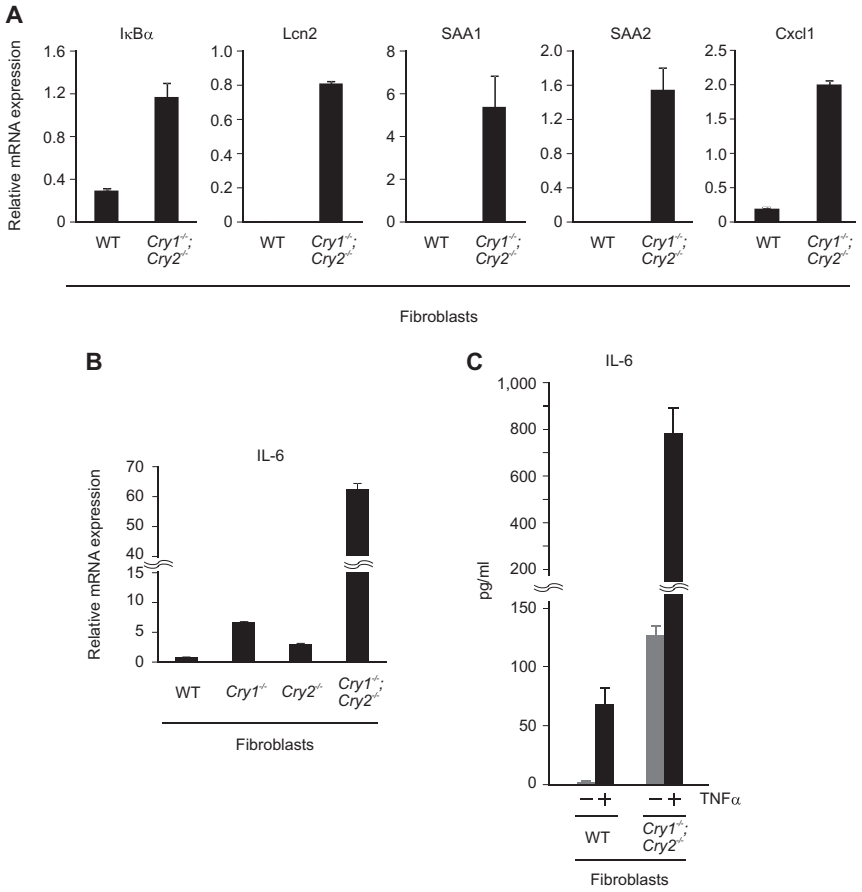

Fig. S1. Absence of *Cry* leads to constitutive expression of inflammatory genes in *Cry1*$^{-/-}$;*Cry2*$^{-/-}$ fibroblasts compared with WT cells. (*A*) Quantification of mRNA levels of indicated genes by qRT-PCR in WT and *Cry1*$^{-/-}$;*Cry2*$^{-/-}$ fibroblasts are shown. Data are mean ± SD (n = 3). (*B*) Estimation of *IL-6* mRNA expression in WT, *Cry1*$^{-/-}$, *Cry2*$^{-/-}$, and *Cry1*$^{-/-}$;*Cry2*$^{-/-}$ fibroblasts by qRT-PCR. Data are mean ± SD (n = 3). (*C*) WT and *Cry1*$^{-/-}$;*Cry2*$^{-/-}$ fibroblasts were treated with or without TNF-α (10 ng/mL) for 12 h and the supernatants were analyzed for IL-6 protein levels by ELISA. Data are mean ± SD (n = 3).

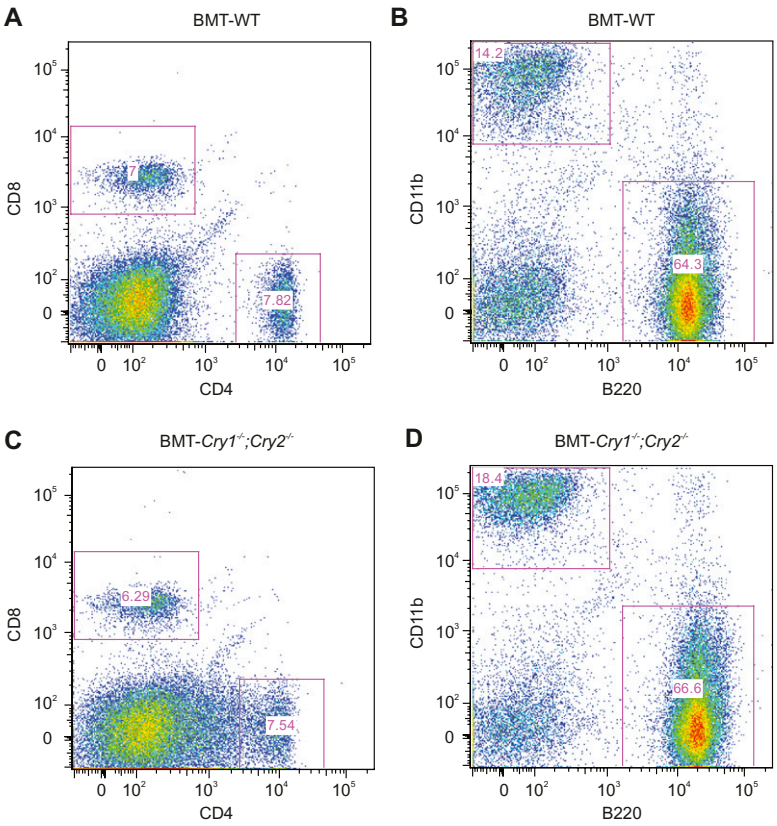

Fig. S2. Reconstitution analysis of bone marrow transplanted mice. (*A–D*) Reconstitution analysis by FACS of blood lymphocytes derived from recipient mice, B6.129S4-Il2rgtm1Wjl/J, 8 wk after injection with bone marrow of either WT or $Cry1^{-/-};Cry2^{-/-}$ donor mice are shown for one representative animal each. (*A* and *B*) (BMT-WT) and (*C* and *D*) (BMT-$Cry1^{-/-};Cry2^{-/-}$) show the population of donor-derived CD4 and CD8 positive T cells, B220 positive B cells, and also the CD11b positive monocytes and macrophages of WT and $Cry1^{-/-};Cry2^{-/-}$ bone marrow–reconstituted mice.

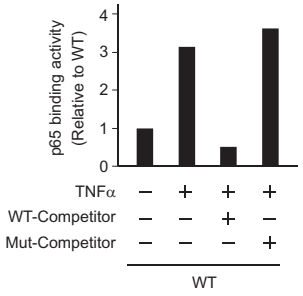

Fig. S3. ELISA for p65 binding activity in fibroblasts. Nuclear extracts (5 μg) from untreated or TNF-α (10 ng/mL, 30 min) treated WT fibroblasts were analyzed for p65 binding activity by ELISA. Competition assay was performed with wild-type or mutated NF–κB consensus oligonucleotide to confirm specificity of p65 binding to NF–κB consensus binding sequence. Value of untreated WT p65 binding activity is set to 1.

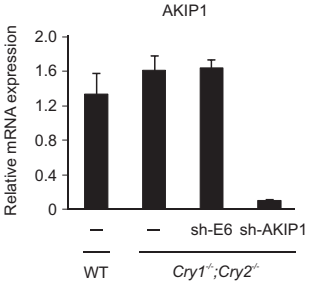

Fig. S4. Analysis of *AKIP1* knockdown in $Cry1^{-/-};Cry2^{-/-}$ fibroblasts. RNA extracted from $Cry1^{-/-};Cry2^{-/-}$ fibroblasts infected with lentivirus expressing sh-RNA against a nonspecific target gene *E6* or against *AKIP1* were analyzed for *AKIP1* expression by qRT-PCR. Data are mean ± SD (n = 3).