Supporting Information

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SI Materials and Methods

Antibodies and Reagents. Antibody for LegK1 was raised in rabbit by using the N-terminal fragment of 90 amino acids as the antigen. Antibodies for IKKα (#2682), IKKβ (#2678), phospho-IKKα/β (#2697), phospho-JNK (#9251), phospho-p100 (#4810), *p*-Ser-32-IκBα (#2589), and *p*-Ser-32/36-IκBα (#9246) were purchased from Cell Signaling. Anti-His (sc-803), anti-HA (sc-805), anti-IκBα (sc-371), anti-NEMO (sc-8330), anti-p52 (sc-7386), anti-IκBβ, and anti-p50 (sc-7178) antibodies were from Santa Cruz. Anti-actin and anti-Flag M2 antibodies are Sigma products. Cell culture products were from Invitrogen and all other chemicals were Sigma-Aldrich products unless noted.

Cell Culture, Transfection, RNAi, and Luciferase Reporter Assays. HEK 293T, HeLa, and MEF cells were grown in Dulbecco's modified Eagle's medium (HyClone) containing 10% FBS and 2 mM L-glutamine at 37 °C in a 5% CO_2 incubator. U937 cells and THP1 monocytes were cultured in RPMI-1640 containing 10% FBS and grown at 37 °C with 5% CO_2. Vigofect was used for plasmid transfection following the manufacturer's instructions. siRNA oligoes synthesized by Takara Inc. and their sequences were listed Table S1. For siRNA transfection, 100 pmol siRNA was transfected into 1×10^5 cells by using Lipofectamine 2000 (Invitrogen). The second siRNA transfection was done 24 h after the first transfection to achieve higher knockdown efficiency. Cells were maintained for 12 h and then transfected with plasmids when needed. Luciferase activity was determined by using the dual luciferase assay kit (Promega) according to the manufacturer's instructions.

Western Blotting and Immunofluoscence. For western blotting, cells were lysed in the RIPA buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 0.1% SDS, 1% sodium-deoxycholate, and 1% Nonidet P-40 supplemented with a protease inhibitor mixture (Roche Molecular Biochemicals). The total protein concentration was determined by using the Bio-Rad DC protein assay kit. Equal amounts of total proteins were resolved by SDS/PAGE and transferred to immobilon-P membrane (Millipore). The membrane was blocked in 5% low-fat milk in TBS-T [150 mM NaCl, 20 mM Tris-HCl (pH 7.4), and 0.1% Tween-20], and the blots were developed by ECL (GE Healthcare). For p65 staining, 5×10^4 HeLa cells were plated in 24-well formats, and transfected with 2 μg of pEGFP-C1, GFP-LegK1 WT, or GFP-LegK1 K121A plasmids. Twenty-four hours later, cells were fixed for 10 min at room temperature with 4% paraformaldehyde and permeabilized for 10 min with 0.5% Triton X-100 in PBS, followed by staining with p65 antibody (1:200 dilution) and Alexa fluor A546-labeled secondary antibody. As a positive control, HeLa cells transfected with pEGFP-C1 plasmids were stimulated by 10 ng/mL TNFα for 1 h before immunostaining.

Bacterial Culture, Infections, Intracellular Multiplication, and TEM Translocation Assays. *L. pneumophila* strains Lp02 (*thyA*, *hsdR*, and *rpsL*) and Lp03 (Lp02 *dotA*) were cultured on buffered charcoal yeast extract agar supplemented with 0.1 mg/mL thymidine (BCYET). U937 cells were differentiated in medium containing 50 ng/mL PMA for 48 h. For effector translocation, fresh Lp02 or Lp03 single colonies harboring expression plasmid for TEM1-LegAS4, TEM1-LegK1, or TEM1-GST fusion proteins were streaked onto BCYE plates 2 days before infection. Twelve hours before infection, 2×10^5 differentiated U937 cells were plated in 24-well formats without antibiotics. The medium was supplemented with 0.2 mg/mL thymidine-HCl without antibiotics. Bacteria were scraped, diluted in sterile water, and added to cells at an MOI of 10. U937 cells in the 24-well plate were then centrifuged immediately at 250 g for 10 min at 25 °C to enhance bacterial adherence and internalization. Infection was carried out at 37 °C with 5% CO_2 for 2 h. Cells were then washed 3 times with PBS and cultured for another 1 h in the presence of 1 μM CCF2-AM. Effector translocation was examined on a confocal fluorescence microscope to detect the blue fluorescence (~450 nm) and green fluorescence emission (~520 nm) on the excitation at 410 nm. Intracellular multiplication was carried out as previously described [Al-Khodor S, Price CT, Habyarimana F, Kalia A, Abu Kwaik Y (2008) A Dot/Icm-translocated ankyrin protein of *Legionella pneumophila* is required for intracellular proliferation within human macrophages and protozoa. *Mol Microbiol* 70:908–923.] except that Lp02, Lp03, or ΔLegK1 mutant strain was used for infection of differentiated U937 cells.

Purification of Recombinant Proteins. *E. coli* strain BL21 (DE3) harboring expressing plasmids for His-SUMO-LegK1 (WT, KA mutant, or truncations) were grown in LB medium containing 30 μg/mL kanamycin to a density of 1.0 (OD600). Protein expression was induced at 16 °C with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h. Bacteria were harvested and lysed in the buffer containing 20 mM Tris-HCl (pH 7.4), 500 mM NaCl, and 20 mM imidazole. His fusion proteins were purified by affinity chromatography using Ni-NTA beads (Novagen), and eluted with an imidazole gradient from 20 mM to 250 mM in 20 mM Tris-HCl (pH 7.4) and 500 mM NaCl. The proteins were further purified on a Superdex 200 gel filtration column (GE Healthcare). GST-IκBα and GST-p100C were expressed in BL21 (DE3) and purified following a standard GST-fusion protein purification procedure. Protein concentrations were estimated by Coomassie blue staining of SDS/PAGE gels using BSA standards.

Cell-free Reconstitution and in Vitro Kinase Assays. For reconstitution in cell extracts, relevant cells growing to the logarithmic stage were harvested by low-speed centrifugation at 1, 800 × *g* for 10 min at 4 °C. All subsequent steps should be carried out on ice or in the cold room. Cell pellets were washed with ice-cold PBS, and gently resuspended in 5 times the cell pellet volume of the hypotonic buffer [10 mM Hepes (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, and 0.5 mM DTT]. Following 10-min incubation on ice to swell the cells, cells were pelleted by centrifugation at 1, 800 × *g* for 10 min and resuspended in the hypotonic buffer with 2 times of the original cell-pellet volume. Cells were homogenized by 10–15 strokes in a Dounce glass homogenizer (the extent of cell lysis can be checked by trypan blue staining). Cell lysates were centrifuged at 10,000 × *g* for 10 min and the supernatant is further centrifuged at 100,000 × *g* for 1 h. The final supernatants (cell extracts) were aliquoted and frozen in liquid nitrogen and stored at −80 °C. Ten microliters of the cell extracts supplemented with 10 mM ATP and the ATP regeneration system were used for each reconstitution reaction, and approximately 300 ng recombinant TRAF6 or LegK1 were added into the extracts to initiate the NF-κB signaling cascade. Reactions were stopped by SDS loading buffer and subjected to immunoblotting analysis.

For LegK1 in vitro kinase assay, bacterially purified GST-

IκBα or GST-p100C or immunopurified candidate substrates were washed two times with the kinase assay buffer containing 20 mM Hepes (pH 7.4), 150 mM NaCl, and10 mM $MgCl_2$. The substrates were incubated with recombinant LegK1 or other indicated kinases in the kinase assay buffer supplemented with 50 μM ATP at 30 °C for 30 min. For autoradiography, 10 μCi of [γ-^{32}P]-ATP was included in each reaction. Reactions were stopped by SDS loading buffer and subjected to SDS/PAGE electrophoresis followed by immunoblotting analysis or autoradiography.

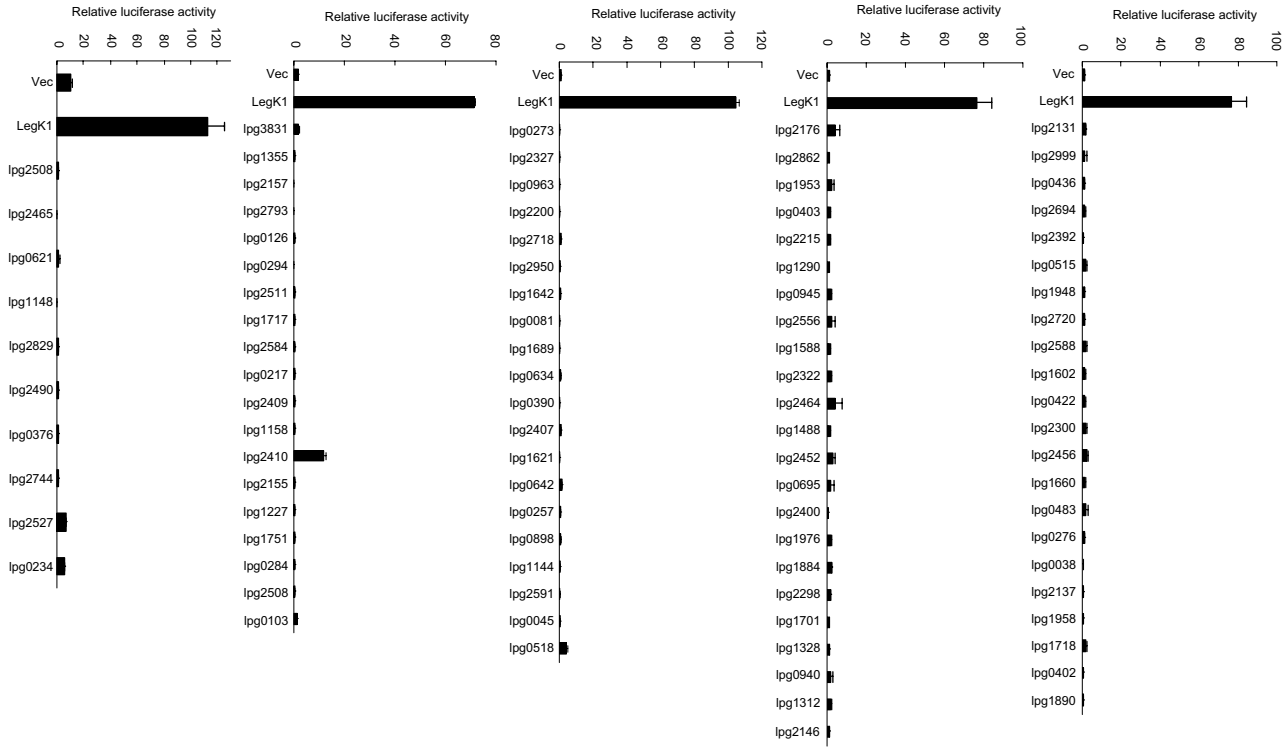

Fig. S1. Luciferase assay screen of potential *Legionella* effectors with the NF-κB-stimulating activity. HEK 293T cells were transfected with NF-κB dual-luciferase reporter plasmids together with an empty vector (Vec), or one of the candidate *Legionella* type IV effectors. ORF name of the candidate *Legionella* effector is used as indicated.

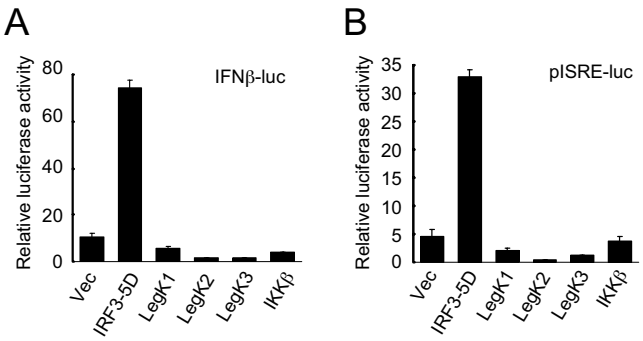

Fig. S2. IFNβ promoter-driven and ISRE luciferase assays of the three *Legionella* Ser/Thr kinases. HEK 293T cells harboring indicated luciferase reporter plasmid were transfected with an empty vector (Vec), or an indicated expression construct. 5D refers to a constitutive active IRF3 mutant (S396D/S398D/S402D/T404D/S405D) serving as a positive control.

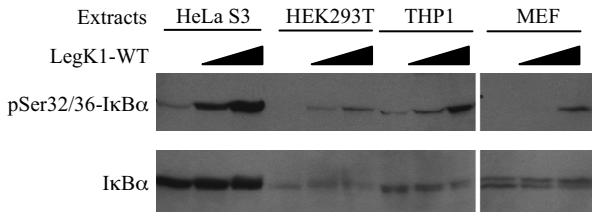

Fig. S3. Activation of the NF-κB pathway by recombinant LegK1 in cell-free extracts from HeLa S3, HEK 293T, THP1, or MEF cells. Shown are immunoblots of total IκBα (*Lower*) and the phosphorylated IκBα (*Upper*) using antibodies as indicated.

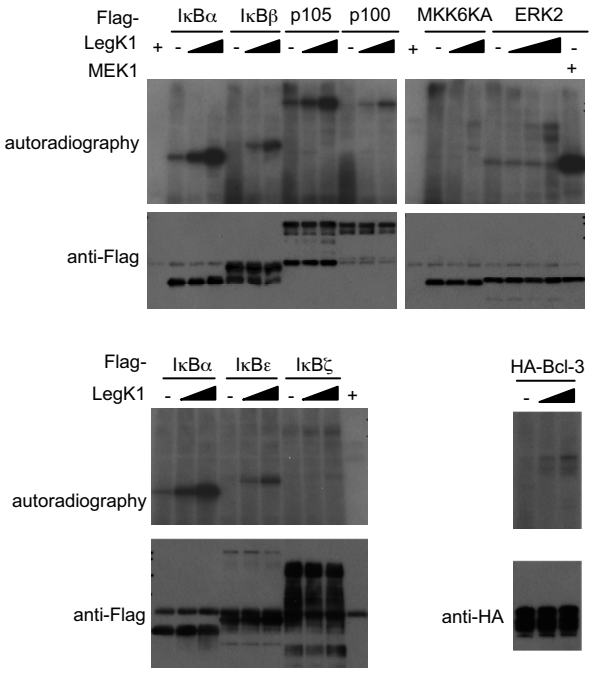

Fig. S4. In vitro phosphorylation of the IκB family of proteins by recombinant LegK1. Each IκB family member was immunopurified from 293T cells and their phosphorylation was analyzed by autoradiography of the SDS/PAGE gel.

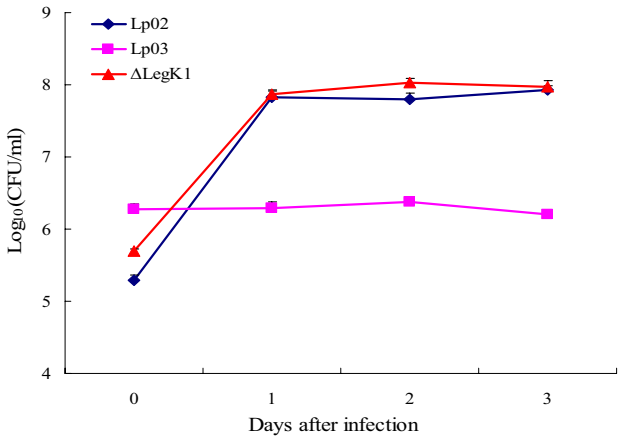

Fig. S5. Intracellular multiplication of *Legionella pneumophila* in differentiated U937 cells. Cell were infected with WT (Lp02), type IV-deficient *DotA* (Lp03), or ΔLegK1 mutant *L. pneumophila* strain at MOI (the multiplicity of infection) of 10. Intracellular growth was monitored by counting the number of bacterial colonies on media plated with cell lysates obtained at the indicated time points after infection. Shown is the representative result obtained from experiments performed in triplicate.

Table S1. Sequences of siRNA oligoes used in this study

Primers	Sense (5′–3′)	Antisense (5′–3′)
hTAK1	UGGCUUAUCUUACACUGGAdTdT	UCCAGUGUAAGAUAAGCCAdTdT
hTRAF2	GAUGUGUCUGCGUAUCUACdTdT	GUAGAUACGCAGACACAUCdTdT
hTRAF6	CCACGAAGAGAUAAUGGAUdTdT	AUCCAUUAUCUCUUCGUGGdTdT
hIKKα	GCAGGCUCUUUCAGGGACAdTdT	UGUCCCUGAAAGAGCCUGCdTdT
hIKKβ	UGGUGAGCUUAAUGAAUGAdTdT	UCAUUCAUUAAGCUCACCAdTdT
β-Gal	AAGGCCAGACGCGAAUUAUdTdT	AUAAUUCGCGUCUGGCCUUdTdT