SUPPLEMENTAL INFORMATION

Supplemental Figures and Legends

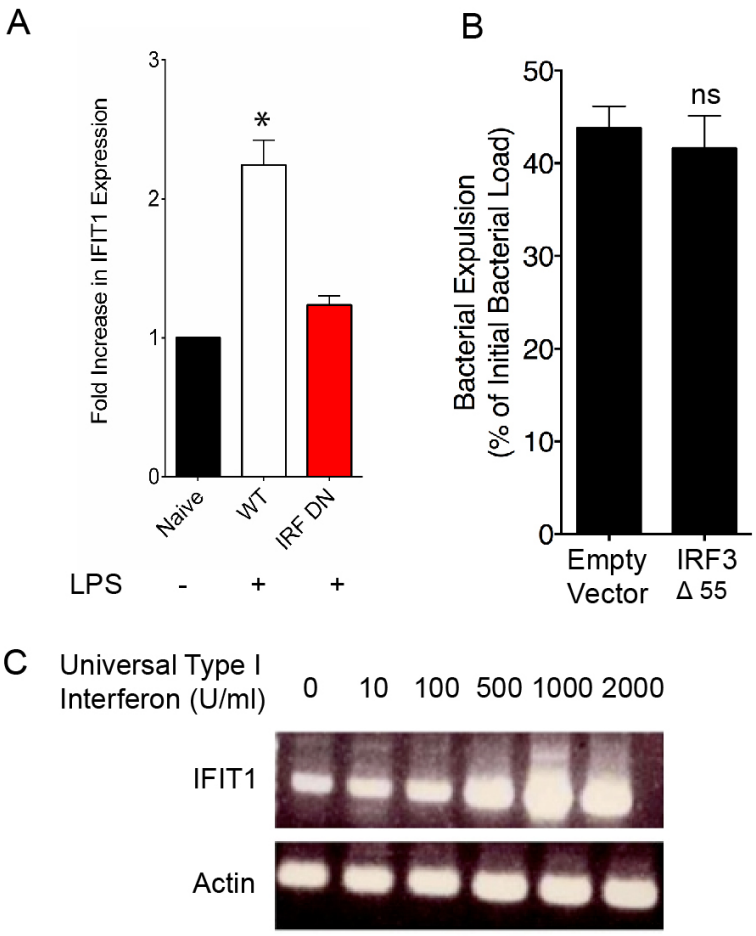

Figure S1, related to Figure 1: Bacterial Expulsion is not a result of interferon responses.

(**A**) RT-PCR amplification of interferon responsive gene IFIT1 in naive BECs, or BECs transfected with vector control or dominant negative mutant of IRF3 shows efficient block of type 1 interferon response by overexpression of IRF3 mutants.

(**B**) Bacterial expulsion from infected BECs transduced with empty vector or a truncated IRF3 dominant negative mutant.

(**C**) RT-PCR amplification of interferon responsive gene IFIT1 shows efficient induction of interferon responses by 1000U universal type I interferon treatment. Actin was used as control for similar number of cells were used.

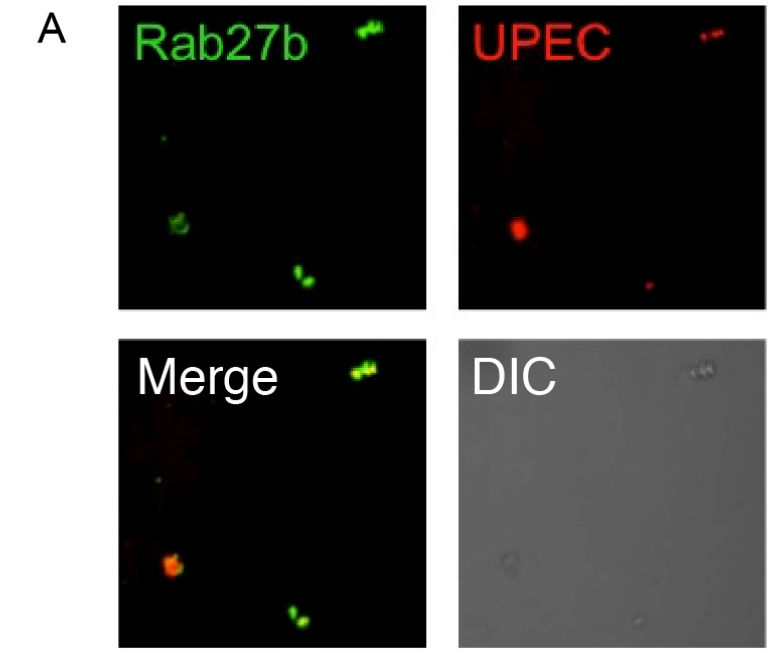
A
Rab27b
UPEC
Merge
DIC
B
IUCV
TCL
IUCV
TCL
Rab27b
LAMP1
EEA1
GM130

Figure S2, related to Figure 2: Magnetic isolation of BCVs.

(**A**) immunofluorescence staining of isolated BCVs shows UPEC (red) are still encased within Rab27b^{+} vesicles (green) and DIC picture shows no other membrane was present in the fractions.

(**B**) western blot probing membrane marker from various subcellular compartments (Rab27b for BCVs; EEA1 for early endosome; LAMP1 for lysosomes; GM130 for golgi) shows the absence of other markers except Rab27b in the BCVs fractions.

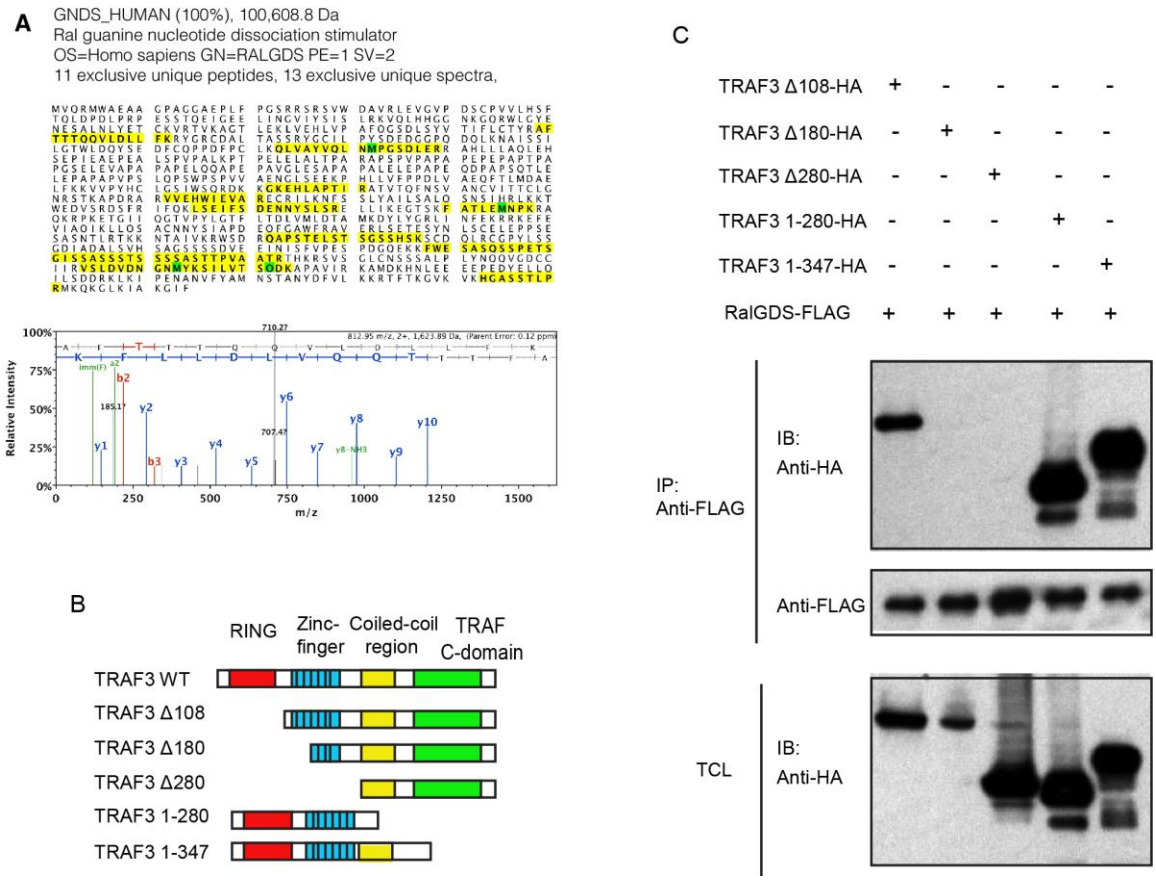

Figure S3, related to Figure 3: The association between RalGDS and TARF3. (**A**) Immunoprecipitates of cell lysate from TRAF3-HA transfected and infected BECs using anti-HA antibody for the pull-down were subjected to mass spectrometry analysis. We observed the presence of several peptides with sequence matching that of RalGDS (upper panel), suggesting that RalGDS was a binding partner to TRAF3. Sample spectra for one of the peptides is also shown in the lower panel. **(B and C)** The interactions of RalGDS with (**C**) different truncated mutant forms of TRAF3 was examined by immunoblotting RalGDS in TRAF3 mutant IP fractions obtained from BECs infected with UPEC, the truncated region of TRAF3 was indicated in (**B**).

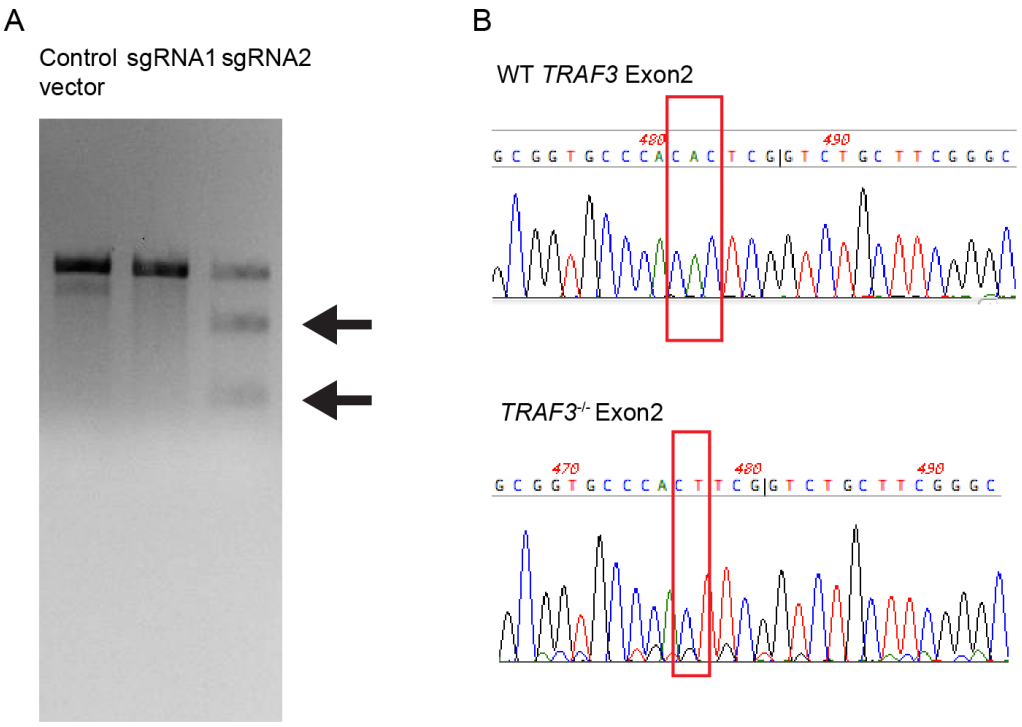

Figure S4, related to Figure 6: CRISPR depletion of TRAF3 in BECs.

(**A**) SURVEYOR assay suggesting that transfection with sgRNA targeting TRAF3 (sgRNA2) generates mutation in the second exon of TRAF3. Scrambled sgRNA sequence and un-effective sgRNA targeting TRAF3 (sgRNA1) were used as controls.

(**B**) Sanger sequencing of the second exon of TRAF3 shows single base pair deletion (red box region) introduced in the *TRAF3*$^{-/-}$ BECs by transfection of sgRNA2 as well as Cas9 protein.

Supplemental Experimental Procedures

Mice

Eight- to ten-week old female C57BL/6, C3H/HeN, C3H/HeJ mice were obtained from the Jackson Laboratory. *Tlr4*$^{-/-}$ (B6.B10ScN-*Tlr4*$^{lps\text{-}del}$/JthJ), *Ticam1*$^{-/-}$ (C57BL/6J-*Ticam1*^{Lps2}/J), and *Myd88*$^{-/-}$ (B6.129P2(SJL)-*Myd88*$^{tm1.1Defr}$/J) were also purchased from Jackson Laboratory and bred at Duke University Medical Center animal care facility. All mouse experiments were performed in accordance with protocol approved by the Duke University Animal Care and Use Committee.

Bacterial strain and cell line

Uropathogenic *E. coli* clinical strain CI5, wild type and Δ*msbB* mutant *E.coli* K12 strain have been described before (Somerville et al., 1996), (Song et al., 2009) and were statically grown overnight in Luria-Bertani broth 24 h prior to infect BECs. The human bladder epithelial cell line 5637 (ATCC HTB-9) was grown in RPMI 1640 (Invitrogen) containing 10% fetal bovine serum (HyClone), GP_2-293 cells (Clontech) and 293T cells were grown in DMEM containing 10% fetal bovine serum. The cells were incubated at 37°C with 5% CO2.

Plasmids

Human TLR4, TRAM, TRIF, and TRAF3 were amplified by PCR followed by insertion into the pCS-2FLAG, or pcDNA-HA, and various lysine mutants was introduced into TRAF3 by Q5 Site−Directed Mutagenesis Kit (NEB) following the vendor's instruction and confirmed by sequencing. hUb-KO was inserted into pRK5-HA and was a gift from

Ted Dawson (Addgene #17603). hUb K6, K11, K27, K29, K33, K48, and K63 mutants was generated using Site−Directed Mutagenesis Kit on the background of hUb-KO to change the arginine residue at site 6, 11, 27, 29 33, 48 or 63 back to lysine. To overexpress various dominant negative mutants of Ubiquitin, Ub-null mutants or wild type human Ubiquitin gene was inserted into plEGFP-N1, and K33R, K48R, or K63R was introduced into wild type ubiquitin gene also by mutagenesis Kit. RalGDS was cloned into pCS2FLAG or pCMV-Myc. RalB, Sec5, or Exo84 was cloned into pEGFP-N1.

Antibody and reagent

For western blot analysis or immunoprecipitation, the following antibodies were used: Rat anti-HA (Roche), Rabbit anti-HA (Cell Signaling), Mouse anti-FLAG M2(Sigma), Rabbit anti-FLAG (Cell Signaling), Rabbit anti-Myc (Cell Signaling), Goat anti-TRIF (R&D), Mouse anti-TRAF3 (G-6) or (C20) (Santa Cruz Biotech), Rabbit anti-TRAF3 (Abcam), Mouse anti-TLR4 (R&D), Rabbit anti-TBK1 (Novus), Rabbit anti-IKKε (Cell Signaling), Rabbit anti-IRF3 (Abcam), Mouse anti-RalGDS (Sigma), Mouse anti-Sec5 (KeraFAST), Mouse anti-Exo70 (KeraFAST), Rabbit anti-Exo84 (Sigma), Mouse anti-RalA (BD Transduction Laboratories), Mouse anti-RalB (Millipore), Mouse anti-Sec8 (BD Transduction Laboratories), Mouse anti-Sec6 (Millipore), Rabbit anti-Rab27b (IBL−America).

Co-infection expulsion assay

Wild type E.coli was introduced with ampicillin resistance whereas Δ*msbB* mutant *E.coli* carry chloramphenicol resistance. BECs were simultaneously infected with WT (MOI

300:1) and Δ*msbB* mutant *E.coli* (MOI 100:1) to ensure each BEC would harbor equal number of both bacteria strain. After one hour infection followed by one hour gentamicin treatment, 50 μL of cell lysate were plated on either ampicillin or chloramphenicol-containing plates to confirm comparable number of each bacterial strain enter the BECs. After incubation in expulsion medium for additional two hours, 50 μL of supernatant were plated on either ampicillin or chloramphenicol-containing plates to quantify each strain being expelled from the BECs and to compare the expelled bacteria number from single strain infected cells.

***In vitro* RNA silencing**

For silencing relevant components in TLR4 pathway, psiRNA vector expressing validated shRNA targeting control Luciferase sequence or human TLR4, MyD88, TRIF, TRAM, TRAF3, TBK1 or IRF3 were purchased InvivoGen. 20 μg DNAs were transfected into $5x10^6$ 5637 BECs using X-fect (Clontech), and Zeocin was added 48 hour later to select transfected cells. The bacterial exocytosis was performed at 7 days post-transfection. TBK1(Silencer® Pre-Designed siRNA, Thermo Fisher, AM51331), IKKε (SMARTpool, Dharmacon Inc., M-003723-02-0005), RalA (SMARTpool, Dharmacon Inc., M-009235-00-0005), RalGDS (SMARTpool, Dharmacon Inc., M-005193-01-0005), were silenced by siRNA targeting four non-overlapping regions, and the siRNAs were transfected into BECs using Lipofectamine 2000 (Invitrogen). To silence other components in Exocyst Complex, shRNA targeting RalA, RalB, Sec5 or Exo84 was inserted into pSuper-Retro-Puro vector and were gifts from Dr. Chris Counter at Duke Medical Center.

Create *TRAF3*$^{-/-}$ BEC

To ablate TRAF3 in the human 5637 BECs, the CRISPR genome editing was performed as described before(Ran et al., 2013). Briefly, the plasmid carrying Cas9 enzyme and guide RNA targeting the Exon2 of human TRAF3 (Sigma HS0000192235, U6-gRNA/EF1a-puro-2A-Cas9-2A-GFP) was transfected into BECs. After selection of positively transfected cells, single cell was prepared and seeded into 96 well plates. Following clonal expansion, standard SURVEYOR assay (Transgenomics, cat. No 706025) was performed to identify the colony that has been successfully edited. The Exon2 region of TRAF3 was then amplified from the positive clone and sequenced to confirm the deletion mutation.

Immunoprecipitation

The plasmids were transfected into $3x10^6$ BECs by X-fect, and 24 hours later, the transfected cells were infected for one hour and lysed. The immunoprecipitations were performed using Co-immunoprecipitation kit (Thermo Scientific), following the instruction from the manufacturer. Briefly, different antibodies were conjugated onto the coupling resin, and mixed with pre-cleared cell lysate. After overnight incubation at 4 degree, the resin was extensively washed with lysis buffer, and the bound proteins were eluted and analyzed by western blot. To pull down endogenous TRAF3 with anti-TRAF3 (Abcam ab76147), $5x10^7$ BECs were used following similar IP procedure.

Immunofluorescence microscopy

BECs were seeded on 22×22 mm glass coverslips and cultured for 48h. After one hour infection of BECs with different bacterial strains, the cells were fixed in 4% paraformaldehyde and permeabilized in blocking buffer (0.1% saponin, 1% fish gelatin, 10% goat serum in PBS) for 30 min at RT. The cells were then incubated with primary antibodies diluted in blocking buffer (100:1) overnight at 4 °C, followed by staining with fluor conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) for 30 min at RT. Coverslips were mounted with Prolong Gold antifade reagent (Molecular Probes) and examined using a Leica SP5 confocal microscope or a Nikon Eclipse TE200 confocal laser scanning instrument.

RalB activation assay

The RalB activation assays were performed using Ral Activation Kit (Millipore), following the instruction. Briefly, the $5x10^7$ BECs were incubated in serum free medium for 24 hours before infection. After 1h infection, the cells were lysed with Mg 2+ rich lysis buffer, and the cell lysate was mixed with recombinant Ral BP1 which is conjugated on agarose beads for 2 hours at 4 degree. After washing, the RalB pulled down by Ral BP1 was analyzed by western blot.

Exocyst Complex Assembly Assay

The exocyst complex assembly was assessed as previously described. Briefly, the naïve or infected BECs were lysed by IP lysis buffer. The Exocyst complex were immunoprecipitated by using antibody against Sec8, and the amount of other exocyst complex component co-IP with Sec8, such as Sec6, was assayed by western blot.

Mass Spectrometry

TRIF, TRAM, or TRAF3-FLAG associated proteins were immunoprecipited by anti-FLAG antibody from $4x10^7$ infected BECs. Loading buffer was added to IP samples and reduced with 10 mM DTT at 70C for 10 min prior to SDS-PAGE separation on a 4-12% bis-tris acrylamide gel (NuPAGE, Invitrogen) with colloidal coomassie staining. Bands corresponding to a band near 100 kDa from TRAF3 IP samples were excised and subjected to standardized in-gel trypsin digestion. Extracted peptides were lyophilized to dryness and resuspended in 12 uL of 0.2% formic acid/2% acetonitrile. Sample was then subjected to chromatographic separation on a Waters NanoAquity UPLC equipped with a 1.7 µm BEH130 C_{18} 75 µm I.D. X 250 mm reversed-phase column. The mobile phase consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. Following a 4 µL injection, peptides were trapped for 3 min on a 5 µm Symmetry C_{18} 180 µm I.D. X 20 mm column at 5 µl/min in 99.9% A. The analytical column was then switched in-line and a linear elution gradient of 5% B to 40% B was performed over 90 min at 400 nL/min. The analytical column was connected to a fused silica PicoTip emitter (New Objective, Cambridge, MA) with a 10 µm tip orifice and coupled to a QExactive Plus mass spectrometer through an electrospray interface operating in a data-dependent mode of acquisition. The instrument was set to acquire a precursor MS scan from *m/z* 50-2000 with MS/MS spectra acquired for the ten most abundant precursor ions. For all experiments, charge dependent CID energy settings were employed and a 120 s dynamic exclusion was employed for previously fragmented precursor ions. Raw LC-MS/MS data files were processed in Proteome Discoverer (Thermo Scientific) and

then submitted to independent Mascot searches (Matrix Science) against an SwissProt database (*Human* taxonomy) for target protein identification.

Statistical analysis

Statistical analyses were performed using GraphPad Prism v.6 (GraphPad Software, La Jolla, CA, USA). Unpaired Student's t-tests were used to determine statistical significance in in vitro assays. For in vivo experiments, Mann-Whitney U test was used. $p<0.05$ was considered statistically significant. Post-test p values are as follows: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Reference

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