Supplementary data for manuscript entitled:

Pathogen-derived effectors trigger protective immunity via the modification of RhoGTPases and activation of IMD or Rip1 and Rip2-dependent defense responses

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Supplementary Methods

Fly strains. Stocks used were HSP70-Gal4,tubulin-Gal80^{ts} (Cronin et al., 2009) and GMR-Gal4, Oregon R (WT) and Rac2Δ (Rac2 Delta) obtained from Bloomington Fly Center. Drosomycin-GFP reporter flies (Ferrandon et al., 1998) was kindly provided by D. Ferrandon. *imd* mutant flies, *imd*^{1} and *imd*^{Shadok}, were provided by JM Reichhart. The *Myd88* fly mutant (DmMyd88 c03881) was a gift from JL Imler. Amino acids 691 to 1014 of the C-terminus of CNF1 (CNF1^{CT}) were cloned and the inactive C-terminus (CNF1^{CS}) generated by mutagenesis of C866S. The CNF1^{CT}, CNF1^{CS}, Rac2^{L16} and Rac2^{N17} flies were generated using the plasmids pUAST-RFA-CNF1-CT, -CNF1-CS, -Rac2^{L61} and -Rac2^{N17}. These constructs were injected into w- flies to generate transgenic flies at Bestgene Inc (Chino Hills, CA). CNF1^{CS} transgenic lines were balanced over C(1)Dx yf FM7 Act-GFP .

DNA cloning and quantitative Real-Time PCR. *Drosophila* Rac2 and Dorsal gene were cloned from S2 cells cDNA into the pENTR plasmid in order to use the Gateway® technology (Invitrogen). QuikChange Site-directed Mutagenesis (Stratagene) was used to generate the Q61L, Q61E, G12V, Y40C, F37A and T17N mutation of Rac2. The Rac2^{L61}ΔCAAX mutant was generated by inserting a premature stop codon (C189 stop). Flag, mRFP, GFP (*Drosophila* Genomics Resource Center, Bloomington, IN) or Bioease^{TM} (Invitrogen) plasmids were generated using Clonase LR recombination (Invitrogen). The CNF1 catalytic domain or the mutant (C866S) were PCR amplified from the pET28a-CNF1-C and pET28a-CNF1-C-C866S (Buetow et al., 2001) and cloned in the pUAST-RFA (Kondo et al., 2006) in order to generate pUAST-RFA-CNF1-CT and pUAST-RFA-CNF1-CS. qRT-PCR were performed by Realplex2 (Eppendorf) instrument using SYBRGreen RT and QPCR Kit (Applied Biosystems) according to manufacturer's instructions. Primer sequences are available upon request. The Myc tagged human Rac2 mutant constructs were kindly provided by Gerard Gacon (Institute Cochin, Paris)

Cell culture and transfections. *Drosophila* S2 cells were maintained in Schneider's medium (Invitrogen) and HEK 293T in DMEM (Invitrogen) both supplemented with

10% FBS, 100U/ml penicillin, and 100µg/ml streptomycin. Transfections were performed with calcium phosphate transfection reagent (Invitrogen). S2-derived stable cell lines were generated by cotransfection with pCoBlast plasmid (Invitrogen) and selected using Blasticidin. For CNF1 catalytic domain expression in S2 cells, pUAST-RFA-CNF1-CT and pUAST-RFA-CNF1-CS were co-transfected with the pMT-GAL4 and 500μM $CuSO_4$ induction was performed for 24h before lysis.

In vitro **Drosophila Rac2 modification by CNF1.** GST-TEV-Rac2 protein was produced in BL21 *E. coli* using the pGEX-dRac2WT (DGRC) plasmid and purified using agarose-glutathione beads. Rac2 elution was performed using the rTEV protease (Invitrogen). The CNF1 modification of Rac2 was performed as described previously for RhoA (Flatau et al., 1997). Samples of native Rac2 or Rac2 modified by CNF1 were analyzed by Mass Spectrometry (Taplin Facility, Harvard University).

Epifluorescence and Confocal microscopy. 18h hours after transfection *Drosophila* S2 or HEK293T cells were fixed with 3% paraformaldehyde. Coverslips were incubated with rabbit polyclonal anti-myc (BD Bioscience), mouse monoclonal anti-flag M2 (Sigma) or rabbit polyclonal anti-IMD (provided by JM Reichhart) antibodies and incubated for 45min with appropriate secondary antibody Alexa-488 or -546 (Invitrogen). Coverslips were mounted using Mowiol and imaged using a Nikon TE2000U inverted microscope or a Zeiss LSM510 Meta confocal microscope and Openlab software.

HEK 293T RNAi experiments. For RNAi experiments cells were transfected with pools of siRNAs to Rip1 (RIP) and Rip2 (RICK) as indicated in the figure legend. Pools were obtained from Santa Cruz Biotechnology or ON-TARGETplus SMART pool targeting Human RIPK1(#8737) and Human RIPK2(#8767) (Dharmacon) and transfected using Darmafect2 reagent (Dharmacon). Quantity of siRNA in the single transfection was complemented by the equivalent amount of NT siRNA to maintain equivalent total siRNA. Cells were incubated 72h before lysis and RNAi efficiency was verified by Immunoblot. RNAi treated HEK293T cells were also functionally validated as follows: siRNAs versus RIP1 were confirmed by showing their ability to inhibit IL-8 expression induced by TNF-α and siRNAs versus RICK/RIP2 were confirmed by showing their

ability to inhibit NOD2-induced IL-8 expression (**Supplementary Figure SF6**). HEK-293T cells were stimulated either with 20ng/ml TNF-α (Invivogen) for 5h before lysis or by co-transfection with pEGFPC1-NOD2 (gift from D. Podolsky (MGH).

NF-κB-luciferase reporter assay. Dual luciferase reporter assays for NF-κB activation were performed in HEK-293T cells as previously described (Stuart et al., 2005).

Flag-Rip1 and Flag-Rip2 immunoprecipitation of Myc-hRac2 mutants

$5x10^6$ HEK 293T cells were transfected using lipofectamine 2000 (Invitrogen) with Flag tagged Rip1 or Rip2 and Myc-hRac2L61 or Myc-hRac2N17 plasmids, as indicated in the figure legend, for 16h before lysis with buffer 2 (LB2) (20mM Tris ph7.5, 100 mM NaCl, 10mM MgCl2, 0.5% Bridj-58). After pre-clearing the lysates with 20μl of Dynabeads®-ProteinG (Invitrogen) for 20 min, 1μg of anti-Flag (clone M2, sigma) was added and incubated for 16h at 4°C. 30μl of Dynabeads®-ProteinG were then added to each condition for 4h before washing 3 times with iced cold LB2. 30μl of 2x laemmli buffer was added to the beads, samples were processed for Western blotting.

GST-Rac2 Pull Down of IMD

The purification of the GST-Rac2 was performed by elution with reduced forms of glutathione and gel filtration purification. The CNF1 modification of GST-Rac2 was performed using 20μg of GST-Rac2 and CNF1-modified as described previously. Agarose-glutathione beads were added to each condition for 20 min in order to generate agarose beads coated with native or CNF1-modified GST-Rac2. These beads were washed 3 times with LB1 then 50μg of protein from a S2 cell lysates were added to the beads and incubated at 4°C for 4h. After three washes with iced cold LB1, 30μl of 2x laemmli buffer was added to the beads. Samples were processed for Western blotting.

Bioease™ based pull down assay. A stable cell line was generated with $Rac2^{L61}$ fused to a Bioease™ tag (hereafter referred to as $^{Bioease}Rac2^{L61}$) and whose expression was under the control of a copper sulphate ($CuSO_4$)-inducible metallothionein promoter (Basile et al., 2007; de Boer et al., 2003). Stable S2 derived cell lines expressing either ^{Bioease}LacZ (Invitrogen), $^{Bioease}Rac2^{N17}$ or $^{Bioease}Rac2^{L61}$ were induced 48h with 500µM $CuSO_4$ before

lysis. 10^8 cells were lysed with buffer 1 (LB1) (25 mM Tris pH 7,5, 100 mM NaCl, 0.1% TX100, 0.1% NP 40, 1mM DTT, 1mM PMSF) and the clarified lysates were purified for 1h at 4ºC on Dynabeads MyOneC1 Streptavidin conjugated beads. Beads were washed six times in LB1 and resuspended in 50μl of laemmli buffer. Proteins were processed for immunoblotting. 2% of pre-purification total lysate was loaded onto the gel (Input). ^{Bioease}Rac2 proteins were visualized using a Streptavidin-HRP conjugate and IMD using an anti-IMD antibody (JM Reichhart, **Supplementary Figure SF4**).

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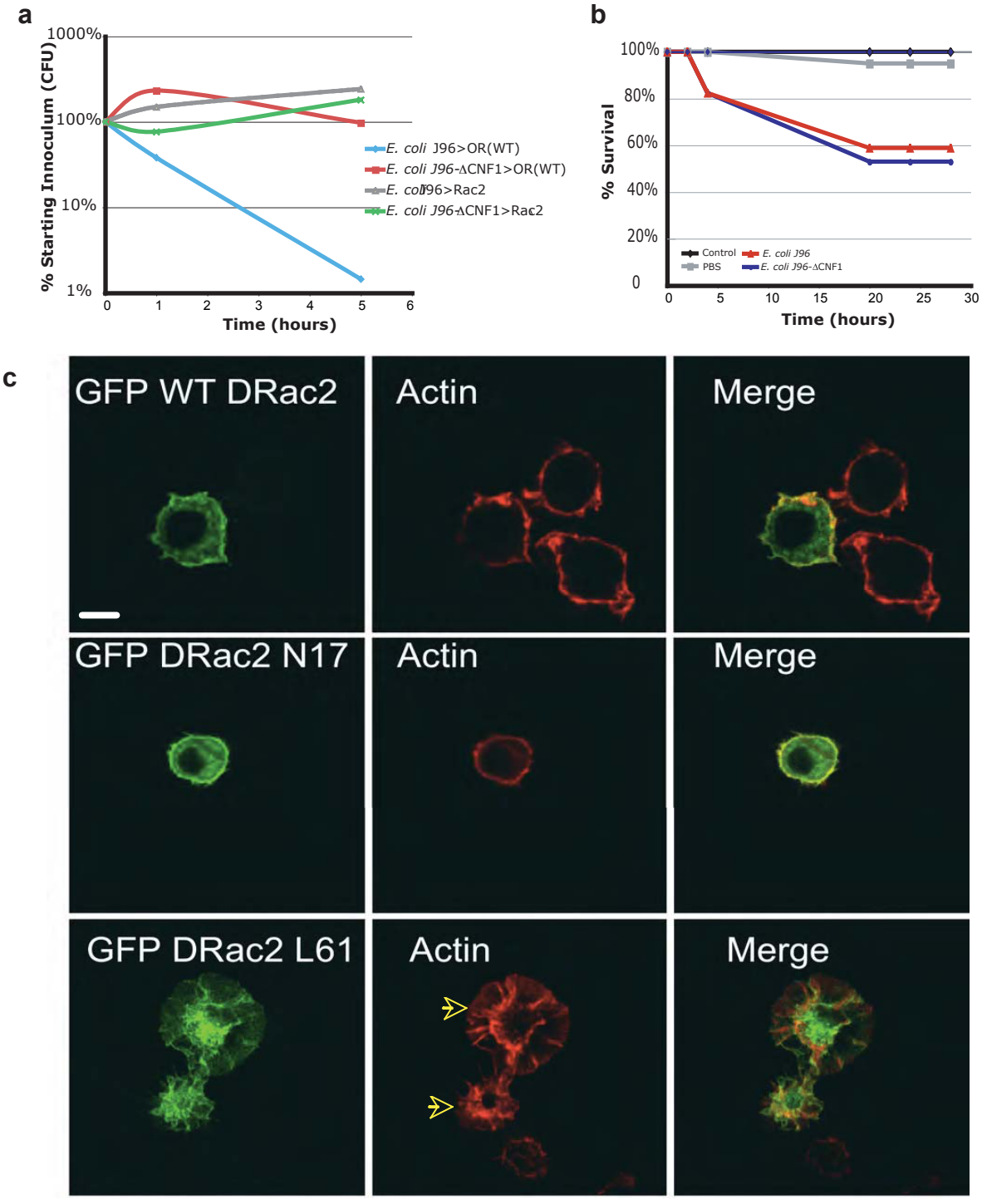


Supplementary Figure SF1. Role of Rac2 in ETI

a & b. Infection of Rac2Δ flies with E. coli J96 and J96-ΔCNF1

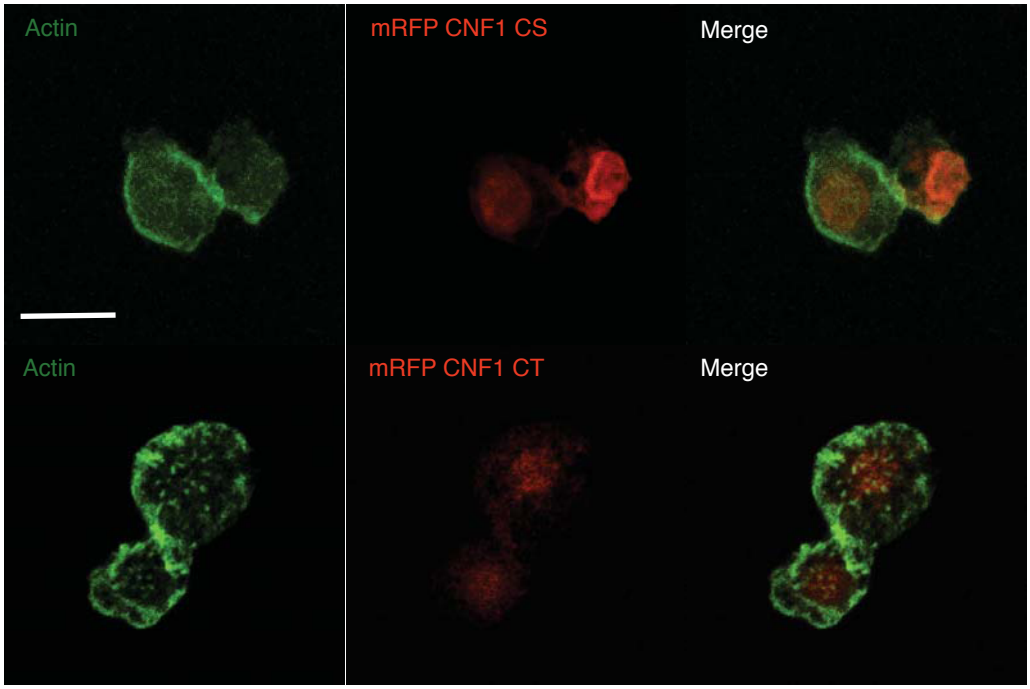
a. Oregon R (WT) and Rac2Δ fles were infected with *E. coli* J96 and J96-ΔCNF1 and bacterial loads determined by colony counting at the indicated times (data mean of three or more flies in each group and representative of two or more experiments).

b. Survival of Rac2Δ fles infected with *E. coli* J96 and J96-ΔCNF1.

c. Characterisation of Drosphila Rac2 mutants.

GFP-tagged WT Rac2 and N17 and L61 Rac2 mutants were expressed in S2 and cell morphology visualized by F-actin staining. Arrowed head, lamellopodia formation and membrane ruffles

a



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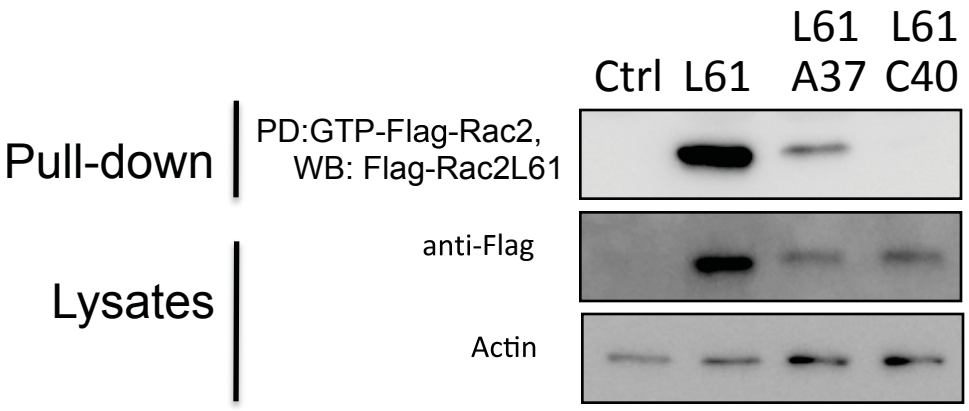
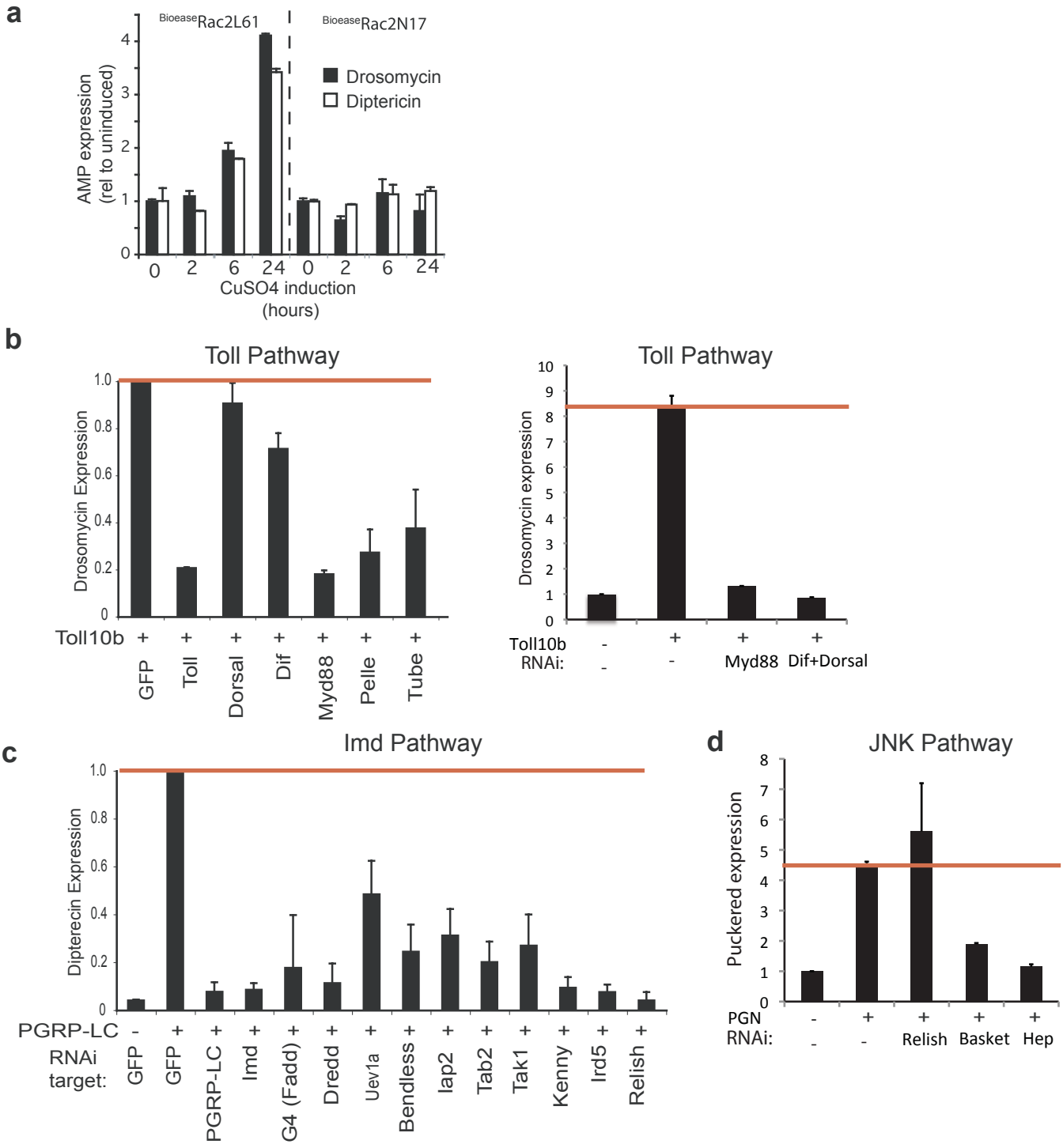
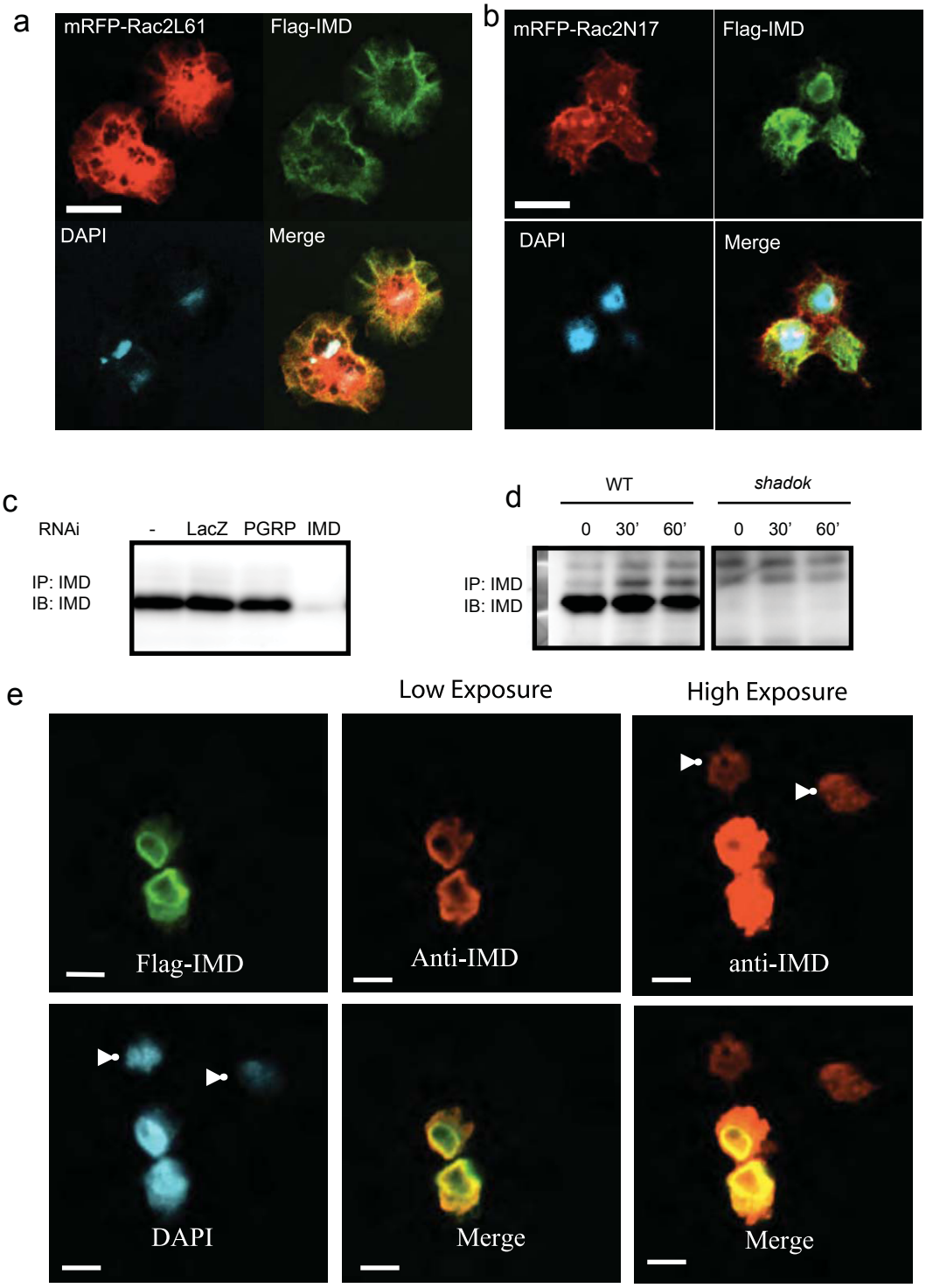


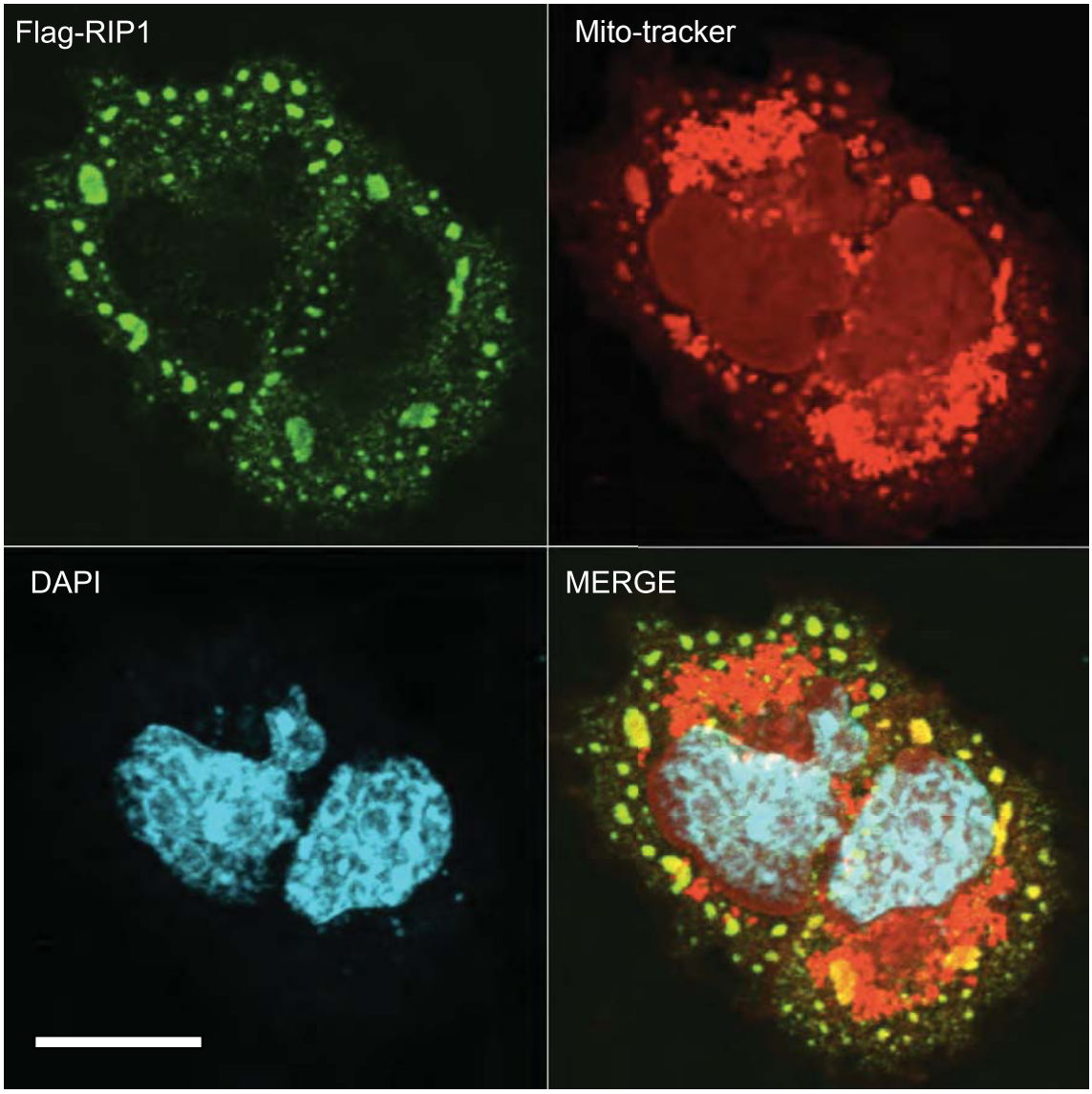
Supplementary Figure SF2. Characterization of the cytoskeletal changes induced in S2 cells by CNF1.
(a) S2 cells were transfected with mRFP-CNF1 catalytically active (CNF1CT) or the inactive point mutant and the cell morphology monitored by phalloidin staining. Red, CNF1; green, phalloidin. Note focal adhesions in CNF1CT transfectants.
(b) GST-PAK pull-down of the effector domain mutants of activated Rac2, Ctrl lane corresponds to untransfected cells.



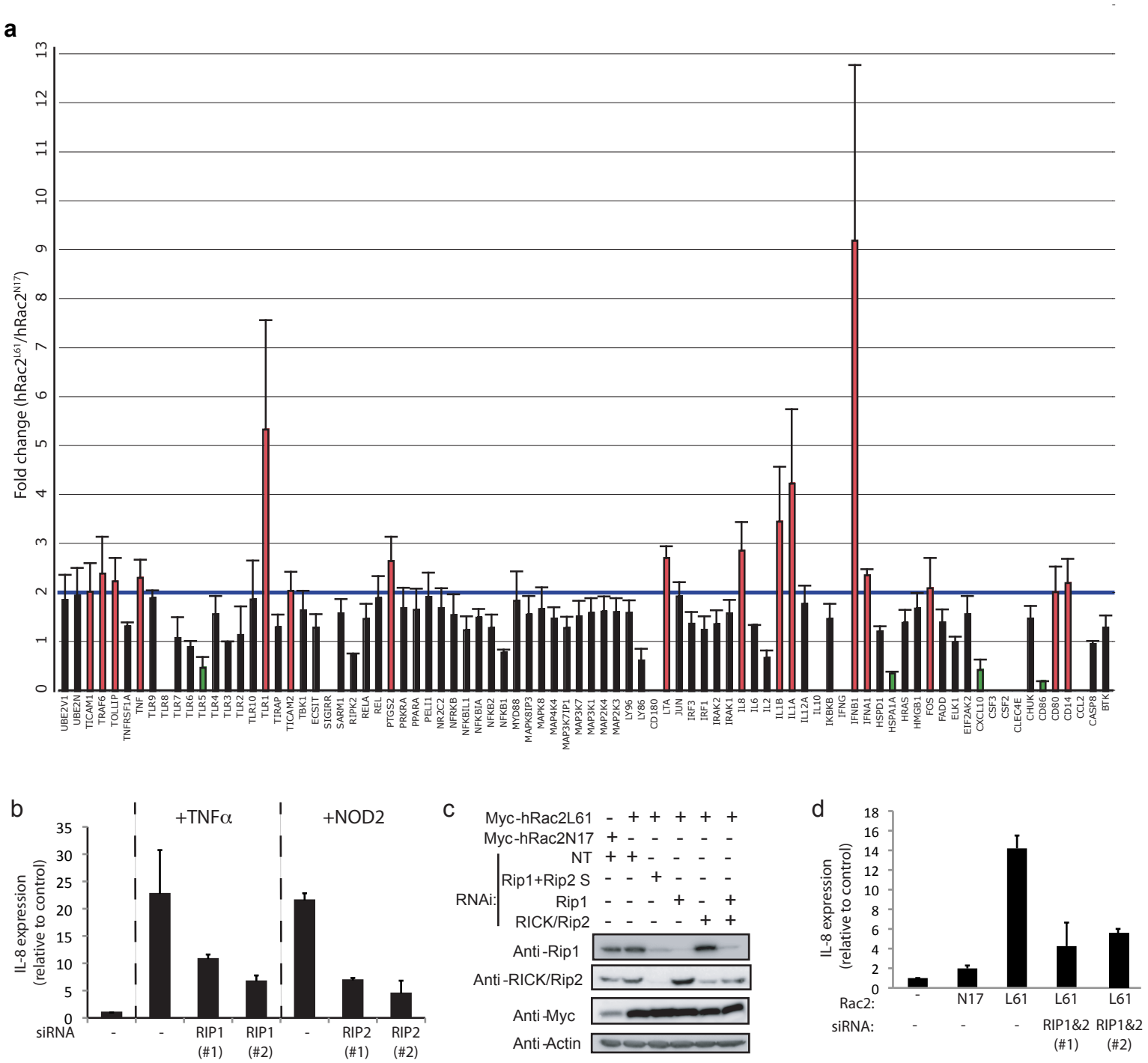
Supplementry Figure SF3. Validation of RNAi epistasis screening approach.
(a) Time course of AMP induction using S2BioeasRac2L61 and S2BioeaseRac2N17 used for the RNAi screen. Rac2 expression was induced by CuSO4 and AMP expression determined by QRT-PCR after indicated times. (b,c & d).Validation of RNAi collection. b)Efficacy of RNAis targeting the Toll pathway component indicated was determined by monitoring Drosomycin expression in S2 cells stably expressing Toll10b treated with the indicated dsRNA. (Data expressed as %Toll10b expressing cells treated with GFP control RNAi). (c) Efficacy of RNAis targeting the IMD pathway components was determined by monitoring Diptericin in S2 cells stably expressing PGRP-LC treated with the indicated dsRNA. (Data expressed as % PGRP-LC treated with control RNAi).
(d) Efficiency of silencing of the JNK pathway components was determined by monitoring Puckered expression in S2 cells treated with RNAi as stated and stimulated with PGN.



Supplementary Figure SF4 . Localization of Flag-IMD and Rac2 and IMD antibody controls. (**a & b**) Immunofluorescent microscopy of S2 cells cotransfected with mRFP-Rac2L61or mRFP-Rac2N17 (red) and Flag-IMD and stained for Flag using a mouse monoclonal antibody (green). Scale bar 10μm. (**c & d**) IP and immunoblot of IMD. (**c**) S2* cells treated with RNAi targeting PGRP, IMD or LacZ (control). S2 cells were primed with 1uM Ecdysone for 24 hours prior to stimulation with peptidoglycan for 10 minutes. (**d**) WT (Drosomycin-GFP, Diptericin-LacZ reporter) or *shadok*(IMD mutant) flies were pricked with *E.coli* for the stated time prior to harvesting. (**e**) Immunofluorescence of S2 cells transfected with Flag-IMD and stained for Flag using a mouse monoclonal antibody (green) and endogenous IMD using a rabbit antisera (red). Low exposure images are shown to demonstrate nuclear localization of ectopically expressed IMD and high exposure shows endogenous protein in the nuclei of untransfected cells (arrowheads). Nuclei are shown in blue (scale bar 10μm).



Supplementary Figure SF5. Rip1 localization to mitochondria. Flag-Rip1 was transfected into HEK293T cells and visualized using an anti-Flag antibody. Mitochondria were stained using mito-tracker dye. Scale bar 10μm



Supplementary Figure SF6.

a. SupperArray T2 ProfilerTM PCR Arrays of Rac2L61-regulated genes. SuperArrays were used to screen for changes in innate immune genes triggered in HEK 293T cells expressing the hRac2 mutants, hRac2L61 or hRac2N17 (mean+/-range of duplicates). >2 fold upregulated genes, red and >2 fold downregulated, green. Changes in selected genes were then confirmed by QRT-PCR (Figure 4).

b-d. Validation of Rip1 and RICK/Rip2 siRNAs. b. Functional validation of pathway inactivation. RIP1 siRNAs inhibit TNFalpha and RIP2 siRNAs inhibit NOD2 induced immune activation (Santa Cruz, #1 and Dharmacon, #2) **c.** WB confirming RNAi mediated gene silencing using Santa Cruz (S) or Dharmacon siRNA pools. **d.** Rac2L61 mediated IL-8 induction is decreased by both Santa Cruz (#1) and Dharmacon (#2) siRNAs

Gene name	Sense	Reverse
PGRP-LC	TTGGCAGCATCGCCCTGACC	CTTGCGTAGAGCGCATCCGC
TAK1	CCTCAGCTGCAGGTTCC	TCAACCGCCTGCTCATTC
Relish	GTTGCATCCATTTCCGATCT	ATTCTGGATGCCCAAAATGA
IAP2	ACAACTCCTCCATCGATACC	GAGCACAAGCGCTTTTTTC
imd	TCTCTAATATTGCCATGCATTT	CGCTGCCCTCCACCG
BG4 (Fadd)	TCGCAGAATCTGGGACTTC	GAGATTGGTTCGCGACG
Dredd	TGCTAACATTCCGGTGAAAC	ACTACTTGCCGCATATCG
Uev1a	TCGCTGCTGTTCCCTTTACT	GGAAAACGAACCACCATCAT
Bendless	TATGTATATCCCGCCCCAGA	CAGATCCGGACCATATTGCT
Tab2	CTGCGTGGGATATGGGAAA	GGTGCGACATCGTTCAGT
Kenny	CAGGACATCGGATTGAATTTC	GACGAAGAGTCATTCGTTATC
Ird5	GGGTTCAGGTATTTTGTAGTCA	AGAGGATCCGGAGGATAAC
Toll	TCCTTTCCCGTGGATCATC	CCAGATCAACTCCCCGTTT
Dorsal	CCGTGTATATCTCATCCAGTT	TTGCAACAAGAGCAATATACAC
Dif	TGGCACTCATTTTCTGACTTA	GCCACAAATTGCGACCAC
Myd88	CTGGATATCGTCGCAGAC	ACTCGGTGGCCCATTCC
Pelle	CCGAGGCTGATGCTAAAC	GGGCGTCAGCAACTCAA
Tube	TGATTCGCGGAATGTTAGG	CCCTGCCCGGCCTGT
Cactus	CGTCGTCGTTTTGTTGATAG	AACAAGCAAAAGGAATTTGCC
Basket	ACTATTCCTTGGGCACCTGA	CCGATATGCTCCTTGCTCTC
Hemipterous	AGCAATAGCGGTGACTGTCC	ACCCAATTGAATTCCACAGC

Supplementary table ST1: Primer sequences used to generate the validated *Drosophila* innate immune RNAi collection