INVENTORY OF SUPPLEMENTAL INFORMATION

Figure S1 related to figure 1

Figure S2 related to figure 3

Figure S3 related to figure 4

Figure S4 related to figure 5

Figure S5 related to figure 6

Figure S6 related to figure 7

Movies S1-3

The Supplemental Information contains 6 supplemental figures, 3 movies and their legends, experimental procedures and references pertaining to them.

S1 shows that cycloheximide has no effect on efferocytosis by S2 cells and shows co-immunoprecipitation of HA-PALLFL and HA-PALLΔF with RpS6-V5, which supports the data presented in figure 1 to further strengthen our conclusion that RpS6 and PALL physically interact.

S2 describes the making strategy of the *pall* knock-out null allele that we generated by homologous recombination and its characterization in apoptotic cell clearance. We used this knock-out allele in the genetic interactions presented in figure 3, in which a loss of function of RpS6 rescues the $pall_{ko}$ apoptotic cell clearance defect, providing evidence that RpS6 acts as a substrate of PALL *in vivo*.

S3 shows that other ribosomal proteins of the small ribosome sub-unit do not play any roles in efferocytosis in S2 cells or *in vivo*, in support of figure 4

S4 shows F-actin staining phenotypes and F-Actin levels quantification of Mock-treated and *pall*-RNAi S2 cells that support the F-actin staining phenotypes of figure 5, as well as the *pall* RNAi efficiency in S2 cells.

S5 shows total RAC staining in Mock, *pall* and *RpS6* RNAi-treated S2 cells, and our characterization of the anti-active mammalian RAC1 antibody that strictly recognizes the

active form of *Drosophila* RAC2, supporting data in figure 6 that PALL and RpS6 regulate RAC2 level and activity.

S6 shows that PALL does not play a role in bacterial clearance and describes its NES signals and amino-acid sequences of various mutant forms of PALL in support of figure 7.

The 3 supplemental movies 1-3 are 3D-reconstruction animations that support figure panels 7A-C, respectively.

SUPPLEMENTAL INFORMATION

SUPPLEMENTAL FIGURES

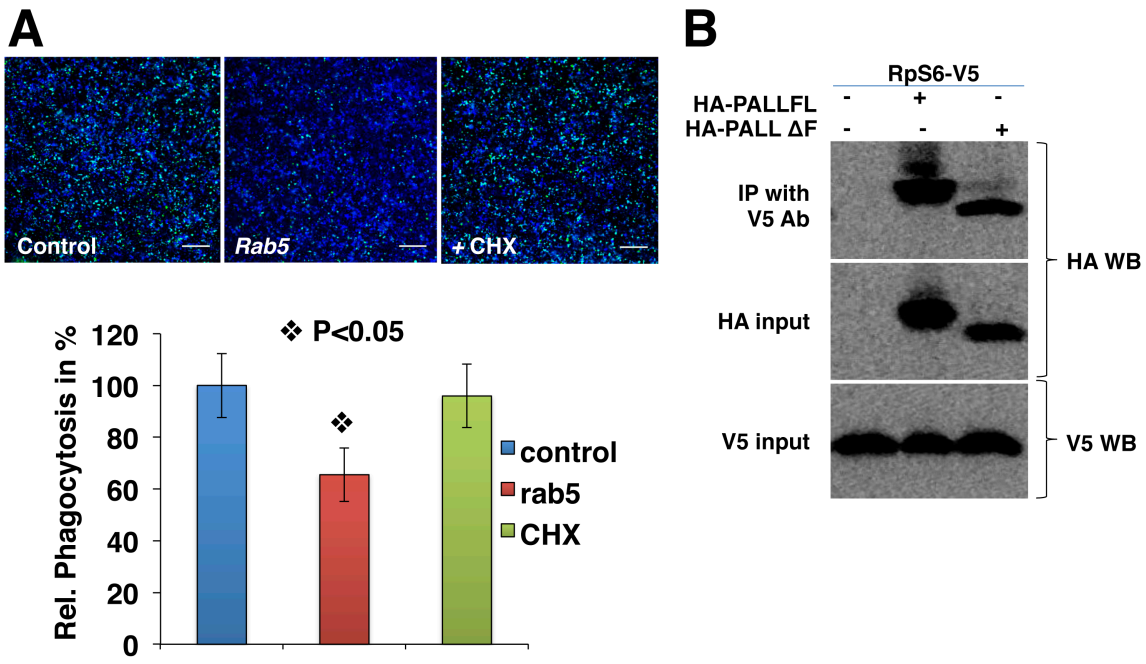
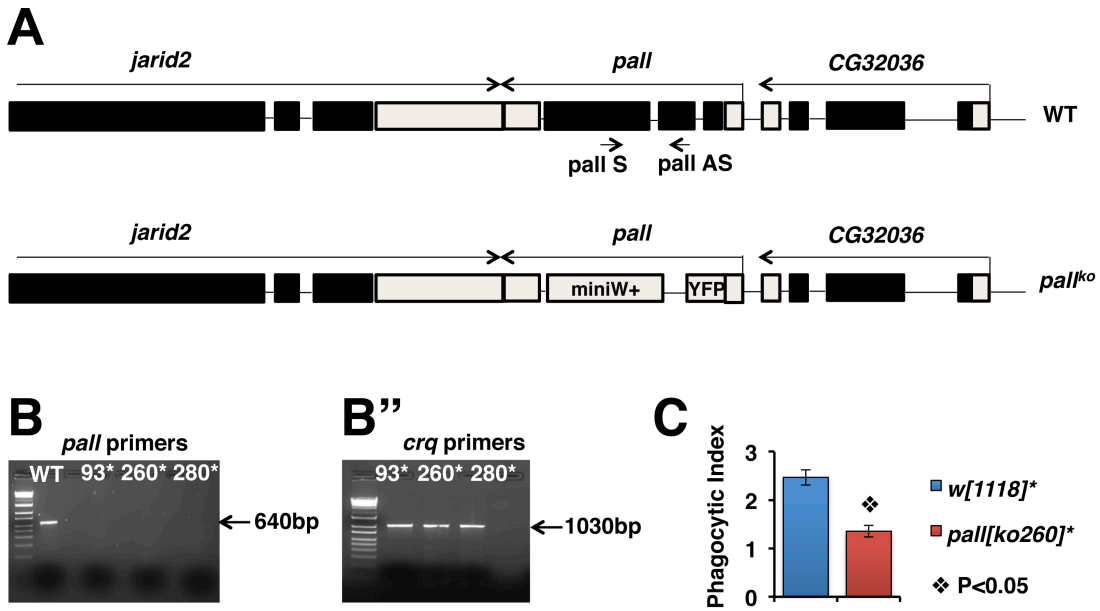


Figure S1, related to figure 1



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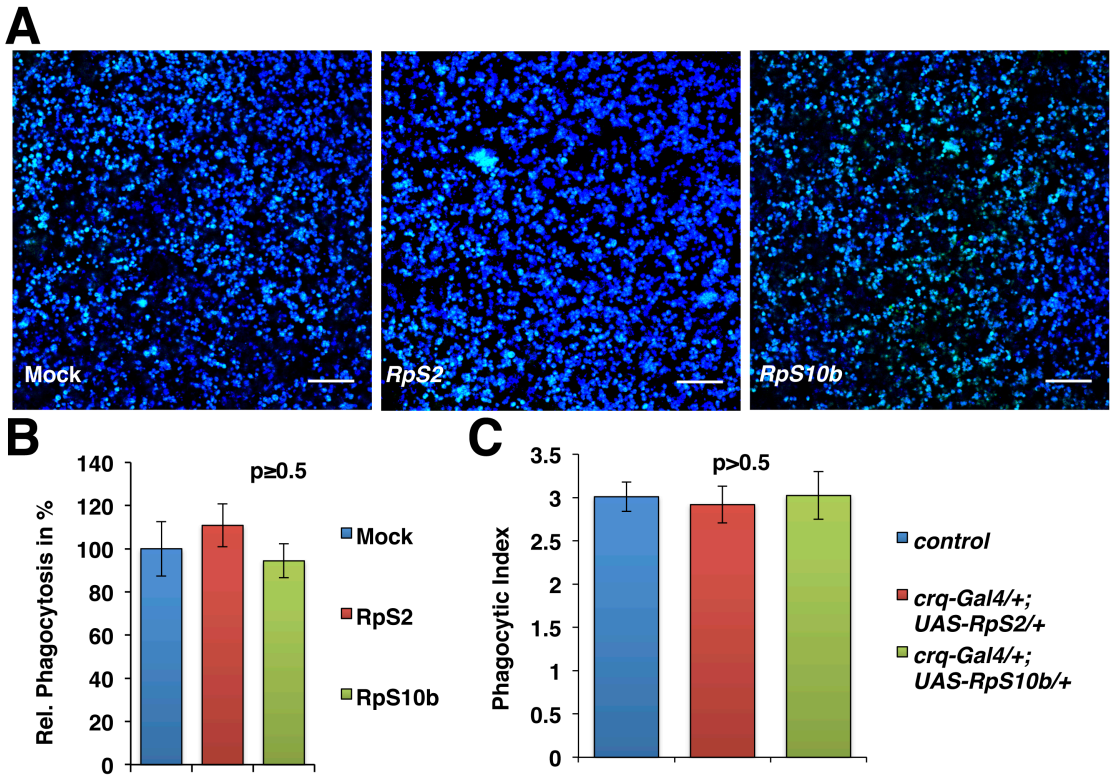

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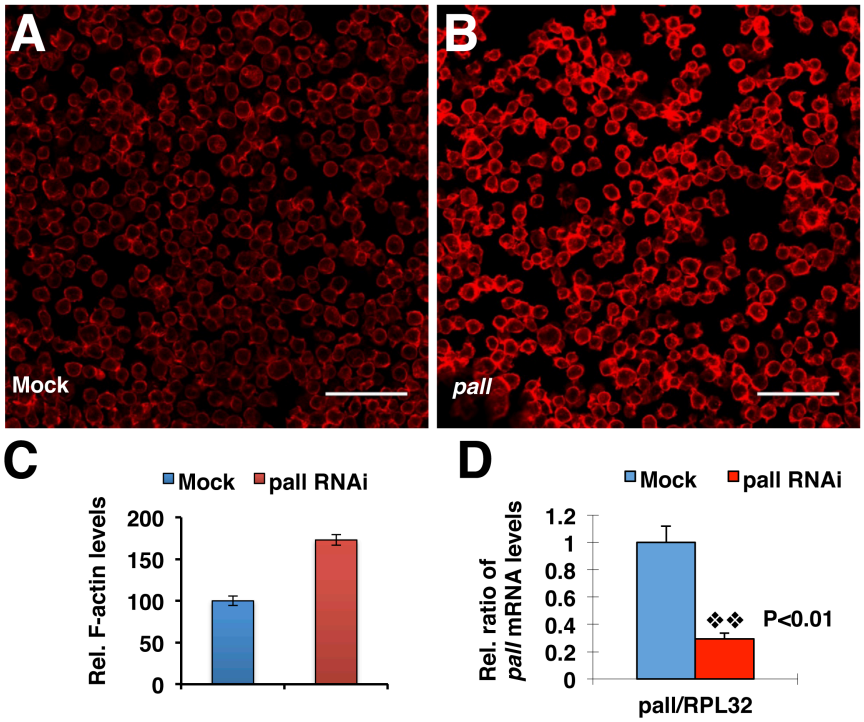

Figure S4, related to figure 5

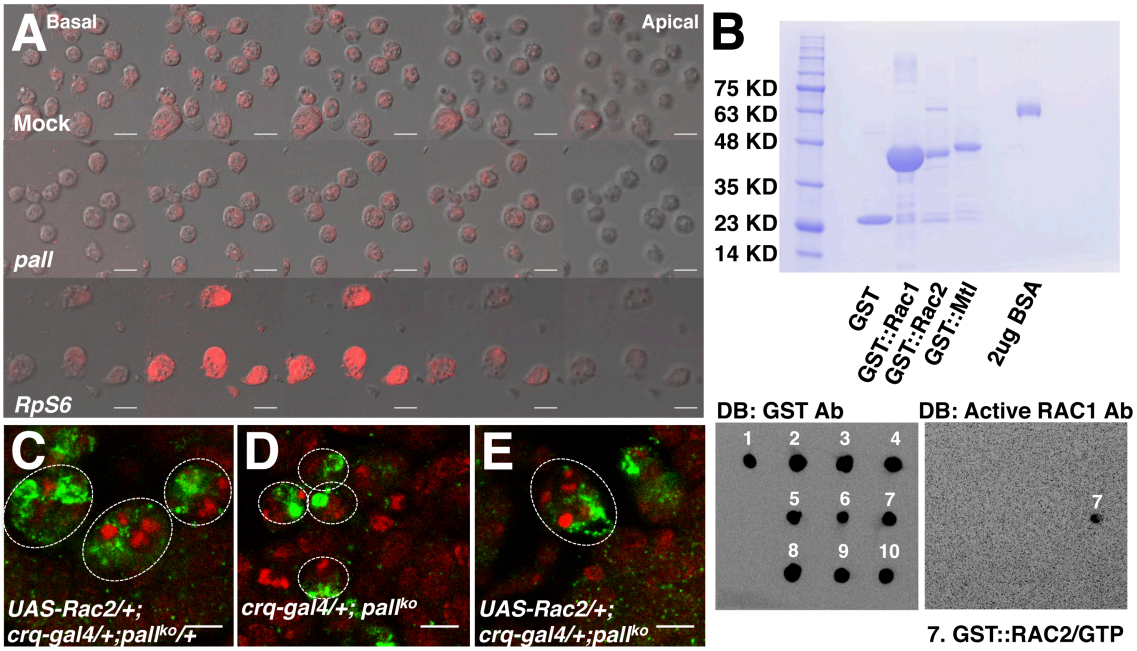

Figure S5, related to figure 6

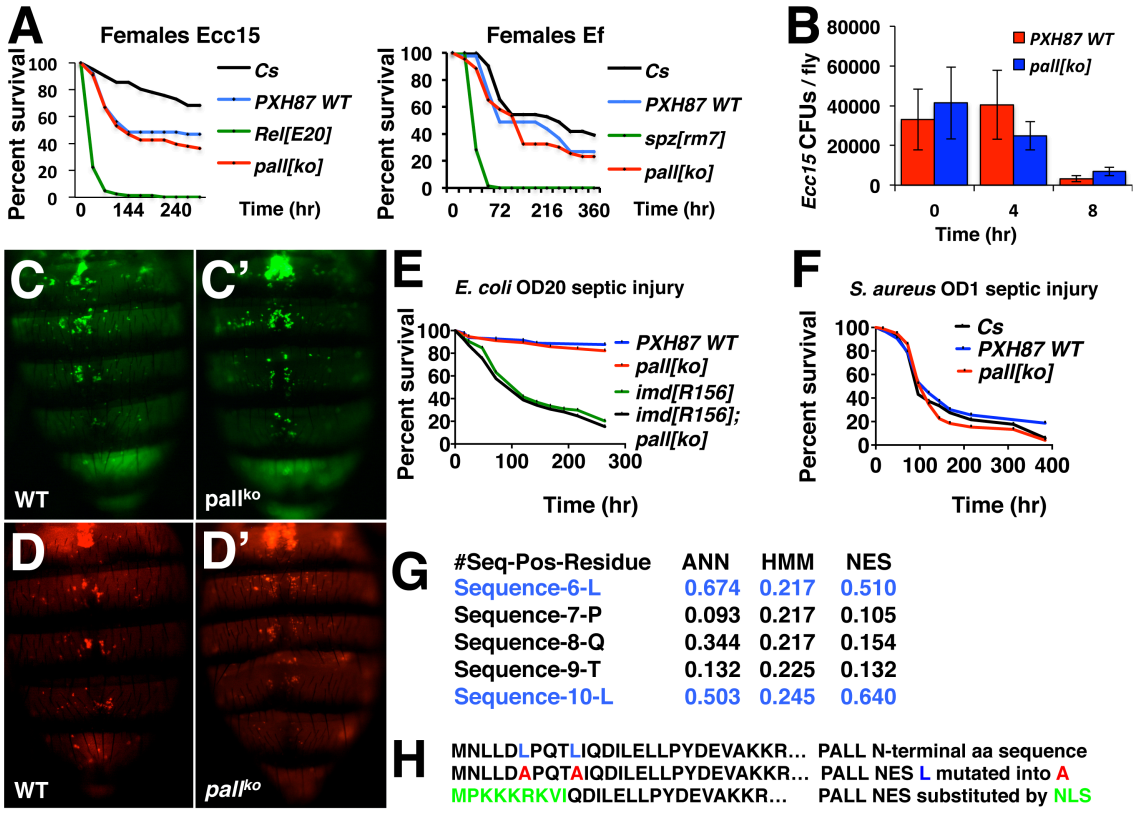

Figure S6, related to figure 7

SUPPLEMENTAL FIGURE LEGENDS

Figure S1 related to figure 1. HA-PALLFL and HA-PALLΔF co-immunoprecipitate with RpS6-V5.

(**A**) Phagocytosis of ACs by mock-, *Rab5* RNAi- and CHX-treated S2 cells and corresponding quantification graph. Live cells are blue, engulfed FITC-labeled ACs are green. Scale bars represent 100 μm. (**B**) Crude protein extracts of transiently transfected S2 cells expressing RpS6-V5 and HA-PALLFL or HA-PALLΔF were immunoprecipitated with V5 Ab and blotted with HA Ab. HA-PALLFL and HA-PALLΔF co-immunoprecipitate with RpS6-V5. Inputs are given by WBs on crude

extracts in which the expression of RpS6-V5 and the expression of HA-PALLFL and HA-PALLΔF are detected with V5 and HA Abs, respectively.

Figure S2 related to figure 3. Characterization of *pallbearer knock-out* mutant flies

(**A**) Schematic of the *pall* targeting strategy with primer sets used. (**B-B"**) Three independent *pall* knock-out alleles have been confirmed by PCR. There are no PCR products using *pall*-specific primers (**B**). *crq* primers were used as a PCR control for DNA template (**B"**). (**C**) Graphs show the mean PIs ± SEM of macrophages for each genotype. ❖ is for p values <0.05.

Figure S3 related to figure 4. RpS2 and RpS10b are not required for AC clearance

(**A**) Phagocytosis of ACs by mock-, *RpS2 and RpS10b* RNAi-treated S2 cells. Live cells are blue, engulfed FITC-labeled ACs are green. **Scale bars represent 100 μm.** (**B**) Quantification of experiments in (**A**) given in % of relative phagocytosis when compared to the mock control set at 100%. (**C**) Graph shows the mean PIs ± SEM of macrophages for each genotype.

Figure S4 related to figure 5. Actin phenotypes of mock, *pall* and *RpS6* RNAi-treated S2 cells

(**A-B**) Confocal micrographs of Mock- (**A**), and *pall* RNAi-treated (**B**) S2 cells that were stained with phalloidin. Z-stack images collected through the cells are shown that were collected at 1.74 μm intervals (basal membrane is in contact with the glass slide). Scale bars correspond to 10 μm. (**C**) Graph shows the relative level of F-actin in Mock and *pall*

RNAi-treated S2 cells. Mock-treated S2 cells were normalized at 100%. Results are the mean of 3 independent experiments (**D**) Graph shows the ratio of *pall* mRNA expression in *pall* RNAi-treated S2 cells when compared to mock-treated control S2 cells, as determined by QPCR. *RpL32* was used as an internal control. ❖❖ is for p value <0.01.

Figure S5 related to figure 6. RAC staining phenotypes of mock, *pall* and *RpS6* RNAi-treated S2 cells and RpS6 phagocytosis phenotype rescue by RAC2 overexpression

(**A**) Confocal micrographs of Mock-, *pall* RNAi- and *RpS6* RNAi-treated S2 cells that were stained with a mammalian RAC1 Ab that recognizes *Drosophila* Rac proteins. Z stack images taken through the cells are shown that were collected at 1.74 μm intervals. Scale bars correspond to 20 μm. (**B**) Characterization of the specificity of the anti-active mammalian Rac1 antibody for the *Drosophila* active form of Rac2. Coomassie staining of gel loaded with purified GST alone, GST::Rac1, GST::Rac2, and GST::Mtl (compared to 2 μg of BSA) showing bands at their appropriate molecular weights. Dot blots with purified (1) GST, (2) GST::Rac1, (3) GST::Rac1/GDP (4) GST::Rac1/GTP, (5) GST::Rac2, (6) GST::Rac2/GDP, (7) GST::Rac2/GTP, (8) GST::Mtl, (9) GST::Mtl/GDP, (10) GST::Mtl/GTP blotted with either the GST antibody or the anti-active mammalian Rac1 antibody that only recognizes the GTP-loaded active form of *Drosophila* Rac2 (7) (**C-E**) Confocal micrographs of *UAS-rac2*/+;*crq-gal4*/+;*pallko*/+ heterozygous control (**C**), *crq-gal4*/+;$pall^{ko}$ mutant (**D**) and *UAS-Rac2*/+;*crq-gal4*/+;$pall^{ko}$ rescued (**E**) macrophages. Macrophages were stained with CRQ Ab (green) and the ACs with 7-AAD (red). Dotted white circles outline the size of single macrophages. Scale bars are 5 μm.

Figure S6 related to figure 7. PALL is specific to efferocytosis and its specificity is driven by its nuclear export in response to AC binding

(A) Survival rates of *pall*^{ko} mutant flies after septic injury with either Gram-negative *Erwinia carotovora carotovora 15* (*Ecc15*) or Gram-positive *Enterococcus faecalis* (Ef) were compared to that of wild-type flies of the same background than the *pall*^{ko} mutant (PXH87 WT control), wild-type flies (Canton S, Cs), and immune deficient flies (*Rel*^{E20} lacks a functional Imd pathway (Hedengren et al., 1999); *spz*^{rm7} lacks a functional Toll pathway (Stein and Nusslein-Volhard, 1992)). *pall*^{ko} flies and *PXH87* flies die at similar rates, whereas the *imd*^{R156} hypomorph mutant flies (Peng et al., 2005) die significantly faster ($p<0.001$) indicating that *pall*^{ko} flies are not susceptible to infection. (**B)** Bacterial persistence was assayed by plating fly homogenates at 3 different time-points after septic injury with a bacterial pellet of *Ecc15* (OD_{600}=200). No significant difference in the elimination of *Ecc15* was detected between control (*PXH87*) and *pall*^{ko} flies, indicating that *pall*^{ko} mutant hemocytes are fully differentiated and capable of eliminating bacteria. (C) *pall*^{ko} mutant flies were injected with pHrodo red *E. coli* or *S. aureus* (molecular probes) and the engulfment of bacteria in fully mature phagosomes was monitored after 3 hrs as in (Cuttell et al., 2008). (**C-D'**) *In vivo* phagocytosis assay by injection of Alexa 488 *E. coli* (**C-C'**) and pHrodo-red *S. aureus* (**D-D'**) into wild-type control flies (*PXH87*)(**C and D**) and *pall*^{ko} flies (**C' and D'**), respectively. (**E**) Percent survival over time of *PXH87* control, *pall*^{ko} and *imd*^{r156} single and double mutant flies after septic injury with a low dose of *E. coli*. (**F**) Percent survival over time of *Canton S* and *PXH87* control flies, and of *pall*^{ko} homozygous male flies after septic injury with *S. aureus*. (**G**) NetNES

1.1-predicted NES signal scores in *pall* amino-acid sequence. (**H**) N-terminal amino-acid sequences of wild type PALL with L residues of NES signals in blue, the NES mutated version of PALL where L residues were mutated into A (red), and of the NLS-PALL version where the NES was replaced by an NLS sequence (green).

Movies S1-3 related to figure 7A-C: 3D reconstruction animations that support figure panels 7A-C, respectively.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Plasmid constructs

The cDNA insert of the expressed sequence tagged (*EST*) *LD31286* encoding full-length *RpS6* was digested using the *XbaI and BglII* restriction enzymes. 100 ng of the insert was ligated overnight at room temperature with 50 ng of *XbaI/BglII* digested *pUAST* vector, using 5 units of T4 DNA ligase in a 20 µl final volume. 0.2 µl of this ligation was transformed into One Shot TOP10 bacteria (Invitrogen/Life Technologies) using a water bath, and following manufacturer's instructions. Transformed bacteria were grown in 1 ml of SOC medium (Invitrogen) in a bacterial shaker at 37°C, and subsequently plated onto LB agar plates containing 100 µg/ml of ampicillin, and incubated overnight at 37°C. Single colonies were picked and grown in 2 ml of LB medium containing 50 µg/ml of ampicillin. Plasmid DNA was prepared using the Qiagen mini-prep kit (Qiagen). Individual clones were identified by restriction map analysis and gel electrophoresis. For immunoprecipitation experiments, full-length *pallbearer* and F-box deleted-*pallbearer* cDNA sequences were cloned into *pAHW,* while *skpA* was cloned into *pMT/V5-His*

(Invitrogen), as previously described in (Silva et al., 2007). Full-length *RpS6,* serine residues mutated into alanine-*RpS6* and FLAG- tagged full-length *RpS6* were amplified by PCR from *LD31286* cDNAs, cloned first into *Topo-pENTR* vector (Invitrogen) and subsequently into *pMT-DES48* vector (Invitrogen). Full-length *RpS6* was also digested using *KpnI and XbaI* restriction enzymes, cloned into the sites of pAc 5.1 /V5-HisA (Invitrogen) digested by *KpnI and XbaI*. For Ends-out gene targeting experiments, 3-kb regions upstream of *pall* was digested by *AgeI*, 3-kb regions downstream of *pall* was digested by *XhoI*/*StuI,* cloned into the targeting vector pXH87 digested by *KpnI* and *AgeI*, and *XhoI*/*StuI* restriction enzyme combinations, respectively. For the ubiqutination assay, the ubiqutin cDNA was amplified from pUAST-UB construct (a kind gift from Jin Jiang, UT Southwestern Medical Center, USA) and digested using *KpnI and XbaI* restriction enzymes, cloned into the sites of pAc 5.1 /V5-HisA (Invitrogen) digested by *KpnI and XbaI*. Primers used are supplied below (see primer sets for constructs and PCR)**.**

The cDNA inserts of the expressed sequence tagged (*EST*) *LD34217, GM13874* and *AT17867* encoding full-length *rac1*, *rac2* and *mtl1,* respectively, were digested using the *BamHI* and *XhoI* restriction enzymes. 100 ng of the insert was ligated overnight at room temperature with 50 ng of *BamHI / XhoI* digested *pGEX4T3* vector, using 5 units of T4 DNA ligase in a 20 µl final volume. 0.2 µl of this ligation was transformed into One Shot TOP10 bacteria (Invitrogen/Life Technologies) using a water bath, and following manufacturer's instructions. Transformed bacteria were grown in 1 ml of SOC medium (Invitrogen) in a bacterial shaker at 37°C, and subsequently plated onto LB agar plates containing 100 µg/ml of ampicillin, and incubated overnight at 37°C. Single colonies

were picked and grown in 2 ml of LB medium containing 50 μg/ml of ampicillin. Plasmid DNA was prepared using the Qiagen mini-prep kit (Qiagen). Individual clones were identified by restriction map analysis and gel electrophoresis (see primer sets for constructs and PCR)

***In vivo* Phagocytosis Assays**

Stage 13 embryos were fixed and stained with CRQ Ab (made in rabbit) alone, as previously described (Franc et al., 1996) or with both CRQ and GFP Abs (mouse monoclonal Ab, GIBCO) at 1:1,000 and 1:4,000 dilutions, respectively. Rabbit or mouse fluorescein-coupled and rabbit Cy5-coupled Abs (Jackson Laboratories) were used as secondaries at a 1:1,000 dilution. 7-AAD staining was performed as in (Franc et al., 1999). Stained embryos were mounted in Vectashield medium (Vector laboratories) and observed on a Leica SP5 confocal microscope equipped with a Leica DMI6000 microscope with10X, 20X, 40X, and 63X objectives. Images were collected with the LAS AF software and processed with Adobe Photoshop 4.0 or Image J 1.34 g softwares. In overexpression experiments, the FLYORF UAS lines F000781 (RpS2 ORF-3xHA), F001085 (RpS10b ORF-3xHA) and F001268 (RpS17 ORF)(Bischof et al., 2013) were crossed to *crq-gal4* lines, embryos were collected and their macrophages were assessed for their phagocytosis phenotypes as described above.

For the bacterial phagocytosis assays, bacteria were prepared according to supplier's instructions and 3-5 days old adult flies of appropriate genotypes were injected in their thorax with 69 nl of Alexa 488-labelled *E. coli* or *S. aureus* (Molecular Probes/ Technologies), followed after 3 hrs by an injection of 69 nl of a 0.4 % Trypan blue

solution (Sigma) to quench the fluorescence of bacteria that had not been fully ingested. Images of the flies abdomen were then taken using a Leica DFC300FX camera and a Leica MZFLIII binocular fluorescent microscope and are representative of at least three experiments. Experiments carried out with pH-sensitive pHrodo-red *E coli* and *S. aureus* (Molecular Probes/Life Technologies) were performed similarly without the need for Trypan blue injection and quenching.

Phagocytosis Index

Phagocytosis indices (PIs) were quantified as previously (Silva et al., 2007). In general, PIs presented in graphs are average PIs calculated from confocal images taken on five embryos per genotype; from these embryos, three confocal image stacks of 16 sections through macrophages per embryo were taken at 63X. Standard error from the mean (SEMs) were derived from the PIs calculated from all five embryos per genotype. p values are indicated in the figures that were calculated using the ANOVA method.

Preparation of Apoptotic Cells

Exponentially grown S2 cells were treated with 0.25 μg/ml of actinomycin D (Sigma) for 18 hrs, fixed in 10% formaldehyde/serum-free Schneider medium (SFM), washed, and resuspended in 1 ml of SFM at a concentration equivalent to 8–10x10^6 cells/ml. Lyophilized FITC isomer (Molecular Probes) was resuspended to 100 μl/ml in DMSO; 25 μl was freshly added to 1 ml of ACs, incubated for 1 hr at room temperature, and washed twice in SFM.

S2 cells Phagocytosis Assays

Exponentially growing S2 cells were plated at a density of ~2.5-7.2x10^3 cells/mm^2 in 96-well plate. ACs were added to live S2 cells at a ratio of 10:1; this ratio is a live cell to live cell ratio prior to apoptosis induction. Cells were incubated for 5 hrs or overnight, counterstained with 25 μM Cell Tracker Blue CMAC (Molecular Probes) for 1 hr, and washed in PBS. 50 or 100 μl of 0.4 % Trypan blue solution (Sigma) was added in each well, respectively. In inhibition experiments, Rapamycin was used at a final concentration of 50 nM and CHX at 100μM.

RNA interference experiments

Amplicons were amplified from single fly DNA preparation. Briefly, one fly is placed in a 0.5 ml tube and mashed-up for 5 to 10 seconds with a pipette tip containing 50 μl of squishing buffer (10 mM Tris-Cl pH 8.2, 1 mM EDTA, 25 mM NaCl, and 200 μg/ml Proteinase K), incubate at 25-37°C for 20-30 minutes. Inactivate the Proteinase K by heating to 95°C for 1-2 minutes. Typically use 1 μl of the DNA prep in a 10-15 μl reaction volume. In all reactions, a *crq* primer set was used as an internal control for the presence of DNA. PCR cycles were as follows: 94°C for 3 min, followed by 94°C for 45 s, 57°C for 30 s, and 72°C for 45 s for 30 cycles, and a 10 min extension at 72°C. 15 μl of each PCR reaction were loaded onto a 2 % agarose/TBE (Tris/Borate/EDTA) gel and their products were separated, visualized and photographed on a UVP Bioimaging system. Primer sets were generated at Integrated DNA Technologies and their sequences are supplied below. PCR amplicons for *rab5, RpS6*, *pall*, *RpS2*, *RpS10b* and *RpS17* RNAi experiments were amplified using primer sequences corresponding to DRSC31655,

DRSC37989, DRSC36242, DRSC03614, DRSC32573 and DRSC11271 amplicons, respectively, which can be found on the DRSC public web site at http://flyrnai.org/. These are predicted to have no off-targets (using 19bp sequence comparison). Double-stranded RNAs were produced using 1 μg of amplicon and the T7 Megascript RNAi kit following Ambion's instructions. RNAi experiments were performed on exponentially growing cells, following the DRSC bathing protocol in 96-well plates (http://flyrnai.org/). S2 cell phagocytosis assays were performed after 3 days of RNAi, as described above. *Rab5* RNAi-treated S2 cells served as a control, as we previously showed that *Rab5* knock-down inhibits AC engulfment by S2 cells (see **Fig. 5B** and (Cuttell et al., 2008)).

Primer sets for RNAi

RpS10b S: 5' TAATACGACTCACTATAGGGGATCCTCTCCGGTCTTGGAG 3'

RpS10b AS: 5' TAATACGACTCACTATAGGGGTTAAGGAGCAGTTCGCCTG3'

RpS2 S: 5' TAATACGACTCACTATAGGGGAAGTTGCCGGGTTCC3'

RpS2 AS: 5' TAATACGACTCACTATAGGGGGCAAGATCAAGTCTTTGG3'

RpS17 S: 5' TAATACGACTCACTATAGGGTAGTTGCGACGACCAAAGT3'

RpS17 AS: 5' TAATACGACTCACTATAGGGGCCGCCTGCGTCACT3'

Primer sets for constructs and PCR

crq S: 5' GCCACCGATGCTTGCAGAT 3'

crq AS: 5' AGCCGAATATGATTCCGTACTG 3'

pall_S: 5' CCGTATGTTCCCATCCAGGTG 3'

pall_AS *AgeI*: 5' CCACCGGTCGAGCGGAGCGATTATAGTCG 3'

pall_S *StuI*: 5’ CCAGGCCTCCCTCCAGATGGTGTAGCTGG 3’

pall_AS *XhoI*: 5’ GGCTCGAGGCGTGAACTCGAACTCATCAC 3’

RpS6 S *KpnI*: 5’ CCGGTACCATGGGACAGGTTGTGGAGGCC 3’

RpS6 AS *XbaI*: 5’ CCTCTAGACTTCTTGTCGCTGGAGACAGAG 3’

RpS6 S: 5’ CACCATGGGACAGGTTGTGGAGGC 3’

RpS6 AS: 5’ CTTCTTGTCGCTGGAGACAG 3’

RpS6 S *BglII*: 5’ CCAGATCTATGGGACAGGTTGTGGAGGC 3’

RpS6 AS *XbaI*: 5’ CCTCTAGATTACTTCTTGTCGCTGGAGAC 3’

RpS6 AS mutant: 5’

CTTCTTGTCGGCGGAGACAGCGCTCTTGGCCTCGCGAATGGCGGCAGCACGG

CGGCG 3’

RpS6 AS FLAG: 5’

TTACTTGTCATCGTCTTTGTAGTCCTTCTTGTCGCTGGAGACAGA 3’

ub S *KpnI:* 5’ CCGGTACCATGCAGATGTTCGTGAAGACC 3’

ub AS *XbaI:* 5’ CCTCTAGATTACCCACCTCTGAGACGGAG 3’

rac1 S *BamHI*: 5’ CCGGATCCATGCAGGCGATCAAGTGCGT3’

rac1 AS *XhoI*: 5’GGCTCGAGTTAGAGCAGGGCGCACTTGC3’

rac2 S *BamHI*: 5’ CCGGATCCATGCAGGCCATCAAGTGTGT3’

rac2 AS *XhoI*: 5’GGCTCGAGTTAGAGCAGGGCGCACTTGT3’

mtl1 S *BamHI*: 5’ CCGGATCCATGTCAACCGGAAGGCCCAT3’

mtl AS *XhoI*: 5’GGCTCGAGTTACATTATTAAACACTTTCGC3’

RpS10b S: 5’ TAATACGACTCACTATAGGGGATCCTCTCCGGTCTTGGAG 3’

RpS10b AS: 5’ TAATACGACTCACTATAGGGGTTAAGGAGCAGTTCGCCTG3’

RpS2 S: 5’ TAATACGACTCACTATAGGGGAAGTTGCCGGGTTCC3’

RpS2 AS: 5’ TAATACGACTCACTATAGGGGGCAAGATCAAGTCTTTGG3’

Sample preparation for MS

To each of the samples 60 μl of 8 M Urea, 100 mM Tris, pH 8.5 were added to solubilize the protein. The subsequent mixture was then reduced by adding 0.3 μl of 1M TCEP (for a final concentration of 5 mM TCEP) and incubated at room temperature. To alkylate, 1.2 μl of Iodoacetamide (10 mM final concentration) was added and the samples were subsequently incubated at room temperature while in the dark for 15 minutes. The addition of 180 μl of 100 mM Tris pH 8.5 diluted the solutions to 2 M Urea. Calcium chloride (100 mM) was then added (2.4 μl) for a final concentration of 1 mM CaCl2. Trypsin (0.5 μg/μl) was added in the amount of 7.0 μl. The resulting mixtures were then shaken for 18 hours and incubated in the dark at 37 ºC. To neutralize 13.5 μl of Formic Acid (90 %) was added for a final concentration of 5 % Formic Acid. The tubes were centrifuged for 30 minutes at 2ºC on a table-top centrifuge.

Multidimensional Protein Identification Technology (MudPIT)

Upon completion of the digestion, the proteins were pressure-loaded onto a fused silica capillary desalting column containing 3 cm of 5-μm strong cation exchange (SCX) followed by 3 cm of 5-μm C18 (reverse phase or RP material) packed into an undeactivated 250-μm i.d capillary. Using 1.5 ml of buffer A (95% water, 5% acetonitrile, and 0.1 % formic acid) the desalting columns were washed overnight. Following the desalting process, a 100-μm i.d capillary consisting of a 10-μm laser

pulled tip packed with 10 cm 3-μm Aqua C18 material (Phenomenex, Ventura, CA) was attached to the filter union (desalting column– filter union–analytical column) and the entire split-column (desalting column–filter union–analytical column) was placed in line with an Agilent 1100 quaternary HPLC (Palo Alto, CA) and analyzed using a modified 6-step separation described previously (Washburn et al., 2001). The buffer solutions used were 5 % acetonitrile/0.1 % formic acid (buffer A), 80 % acetonitrile/0.1 % formic acid (buffer B), and 500 mM ammonium acetate/5 % acetonitrile/0.1 % formic acid (buffer C). Step 1 consisted of a 90 min gradient from 0-100 % buffer B. Steps 2-5 had the following profile: 3 min of 100% buffer A, 2 min of X % buffer C, a 10 min gradient from 0-15% buffer B, and a 97 min gradient from 15-45 % buffer B. The 2 min buffer C percentages (X) were 20, 40, 60, 80 % respectively for the 6-step analysis. The final step, the gradient contained: 3 min of 100 % buffer A, 20 min of 100 % buffer C, a 10 min gradient from 0-15 % buffer B, and a 107 min gradient from 15-70 % buffer B. As peptides eluted from the microcapillary column, they were electrosprayed directly into an LTQ 2-dimensional ion trap mass spectrometer (ThermoFinnigan, Palo Alto, CA) with the application of a distal 2.4 kV spray voltage. A cycle of one full-scan mass spectrum (400-1400 m/z) followed by 8 data-dependent MS/MS spectra at a 35 % normalized collision energy was repeated continuously throughout each step of the multidimensional separation. Application of massspectrometer-scan functions and HPLC solvent gradients were controlled by the Xcalibur datasystem.

Analysis of Tandem Mass Spectra

As each step was executed, its spectra were recorded to a RAW file. This data was then converted into .ms2 format through the use of RawXtract (Version 1.9). From the .ms2 files, poor quality spectra were removed from the dataset using an automated spectral quality assessment algorithm (Bern et al., 2004). MS/MS spectra remaining after filtering were searched with the SEQUEST™ algorithm against the NCBI *Drosophila* protein database concatenated to a decoy database in which the sequence for each entry in the original database was reversed (Peng et al., 2003). All searches were parallelized and performed on a Beowulf computer cluster consisting of 100 1.2 GHz Athlon CPUs (Sadygov et al., 2002). No enzyme specificity was considered for any search. SEQUEST results were assembled and filtered using the DTASelect (version 2.0) program (Cociorva et al., 2007; Tabb et al., 2002). DTASelect 2.0 uses a linear discriminant analysis to dynamically set XCorr and DeltaCN thresholds for the entire dataset to achieve a user-specified false positive rate. False positive rates are estimated by the program from the number and quality of spectral matches to the decoy database.

Infection experiments and bacterial persistence

Bacterial infections were performed by perforating the thorax of 20 flies of each genotype with a thin needle previously dipped into a concentrated bacterial culture of *Erwinia carotovora carotovora 15 (Ecc15), Enterococcus faecalis* (*Ef*), *E. coli* or *S. aureus*. Flies dead and alive were counted every day to monitor survival, and the experiments carried out on three separate occasions. For bacterial persistence assays, three groups of five flies infected with *Ecc15 or Ef* were homogenized in 1mL PBS and plated on Rifampicin LB agar plates with a spiral plater for each time-point. CFUs were

then calculated based on serial dilutions of the homogenates. Results are representative of at least three experiments. Error bars represent SEM values, and p values were > 0.05 both in log-rank/Mantel-Haenszel and Wilcoxon Gehan tests.

Real Time-Quantitative PCR (RT-qPCR)

10^7 RNAi–treated cells were pelleted by centrifugation and total mRNAs were extracted with the RNeasy Mini Kit (Qiagen) following the supplier's protocol. RNAs were eluted in 30 µl of RNase-free water. DNA contaminants were removed from the RNA extracts with a DNA-free™ Kit (Life Technologies). First-strand cDNA syntheses were performed from 1µg of RNAs in 20 µl reverse transcription reactions using the MuLV Reverse Transcriptase (Applied Biosystems). Ten-fold dilution series of *RpL32* (internal control) and *pall* cloned cDNAs (copies/µl) were prepared to establish a standard curve. 2 µl of reverse-transcribed cDNAs and the diluted cloned cDNAs were used in 20 µl QPCR reactions with primers at 400 nM each and 10 µl of 2X iTaq Universal SYBR Green supermix (BioRad).

Primer sequences for detection of *RpL32* and *pall* were as follows:

RpL32 Forward, 5'-GGCCCAAGATCGTGAAGAAG-3',

RpL32 Reverse, 5'-TTTGTGCGACAGCTTAGCATATC-3',

pall Forward, 5'-ACGCAGGAACCACATTCTATCC-3',

pall Reverse, 5'-CAGCATCGAGATCCGTGTTTC-3'.

RT-qPCR cycles were as follows: 95°C for 3 min for one cycle; 95°C for 30 s, 60°C for 45 s for 50 cycles; Melt Curve: 95°C for 60 s, Melt curve from 55-94.5°C. Reverse

transcribed cDNA samples were processed in triplicates, diluted cloned cDNAs were processed in duplicates.

PALL translocation assay

pAHW-PALL, pAHW-NLSPALL and pAHW-NESPALL were transiently transfected into S2 cells with Effectene (QIAGEN) according to the supplier's instructions. 72 hrs after transfection, the cells were incubated for 5 hrs with FITC labeled ACs. Immunostaining with HA Ab was performed following the DGRC standard immunostaining protocol (https://dgrc.cgb.indiana.edu/Protocols?tab=cells). Rat monoclonal HA Ab (*Roche*, Indianapolis, IN) was used at 1:500. TRITC donkey anti–rat secondary Ab (Jackson Laboratories) was used at 1:1,000. DRAQ5™ (BioStatus Limited) was used at 5 µmol. Stained S2 cells were mounted in Vectashield medium (Vector laboratories) and observed on a Leica SP5 confocal microscope equipped with a Leica DMI6000 microscope with10X, 20X, 40X, and 63X objectives. Images were collected with the LAS AF software and processed with Adobe Photoshop 4.0 or Image J 1.34 g softwares.

S2 cells bacterial phagocytosis assays

pAWG-PALL was transiently transfected into S2 cells with Effectene (QIAGEN). After 72 hours, transfected S2 cells were plated at a density of $1x10^6$ cells/well in 96-well plate. Cells were incubated for 5 hrs with 100 µl of pHrodo™ *E. coli* BioParticles or pHrodo™ *S. aureus* BioParticles (Molecular probes), mounted in Vectashield medium (Vector laboratories) and observed on a Leica SP5 confocal microscope equipped with a Leica

DMI6000 microscope with10X, 20X, 40X, and 63X objectives. Images were collected with the LAS AF software and processed with Adobe Photoshop 4.0 or Image J 1.34 g softwares.

Actin staining and RAC immunostainings

For actin immunostaining, staged embryos were fixed in 4% methanol–free formaldehyde, devitellinized and stained with both CRQ (made in rat) and F-actin (made in mouse A-2066; Sigma, St Louis, MO) Abs at 1:200 dilutions. Donkey fluorescein-coupled anti-mouse and Donkey TRITC-coupled anti-rat secondary Abs (Jackson Laboratories) were used at a 1:1000 dilution. For actin staining in S2 cells, we used the Invitrogen phalloidin staining protocol. For RAC and active RAC immunostainings, RNAi experiments were performed on exponentially growing cells with RNAiCarrier (EPOCH) according to the supplier's instructions using *mock control*, *pall* and *RpS6* dsRNAs. S2 cell-immunostainings were performed after 3 days of RNAi following the DGRC standard immunostaining protocol found at https://dgrc.cgb.indiana.edu/Protocols?tab=cells. RAC (23A8, Thermo Scientific) and active RAC1 (NewEast Bioscience) monoclonal Abs were used at a 1:500 dilution overnight at 4℃. TRITC goat anti–mouse secondary Abs (Jackson ImmunoResearch Laboratories) were used at a 1:1,000 dilution. Stained embryos and S2 cells were mounted in Vectashield medium (Vector laboratories) and observed on a Leica SP5 confocal microscope equipped with a Leica DMI6000 microscope with 10X, 20X, 40X, and 63X objectives. Images were collected with the LAS AF software and processed with Adobe Photoshop 4.0 or Image J 1.34 g softwares.

Characterization of the anti-active mammalian RAC1 antibody against the *Drosophila* RACs

Recombinant GST-RAC1, GST-RAC2 and GST-MTL1 proteins were expressed in BL21 (DE3) bacteria and purified with glutathione-Sepharose beads (GE healthcare) according to the supplier's instructions. In brief, an overnight culture was prepared from a single colony of transformed bacteria in 50ml of LB containing glucose and ampicillin. This was used to inoculate a 450ml culture in LB containing ampicillin that was grown while shaking at 37℃ until the OD at 600nm reached 0.6. GST fusion protein expression was then induced by adding IPTG to a final concentration of 0.5mM and further incubating the culture for 6hrs at 25℃. The bacteria were then pelleted at 5,000 g for 5 minutes at 4 ℃, resuspended in 30ml of ice-cold PBS containing protease inhibitors and lysed by mild sonication. Triton X-100 was then added at a final concentration of 1% on ice for 5 minutes. After centrifugation at 14,000 g for 30 minutes at 4℃, the supernatant was mixed with a 80% suspension of Glutathione-agarose beads (600ul, GE healthcare) and incubated with mild shaking at 4℃ overnight. The beads were pelleted by centrifugation at 500 g for 5 minutes, washed twice with ten volumes of lysis buffer (1% TritonX-100 in PBS), then 6 times with ten volumes of 20 mM Tris-HCl at pH.8.0, 150 mM NaCl. The Glutathione beads with bound GST-fusion protein were further incubated for 20 min at RT in 500 µl of GST elution buffer (GE healthcare) at a ratio of 1:1 (w/w), with constant but mild mixing on a platform shaker. The elution was repeated one more time and the eluates pooled. The eluates were dialyzed overnight at 4°C in dialysis buffer (50mM Tris pH 7.5, 1mM DTT, 200mM NaCl, 5mM MgCl2, 1% NP40, 10% glycerol, 1mM PMSF).

The amount of proteins present was evaluated on a SDS page by comparison with 2 μg of BSA after Coomassie staining. 10 μg of GST-RAC1, GST-RAC2 and GST-MTL1 were then loaded with either 1mM GDP or 0.1mM GTP (final concentration) in dialysis buffer containing 10 mM EDTA for 15 min at 30℃ and the reaction stopped with 60mM $MgCl_2$. 0.5 μg of each protein were then deposited to a PVDF membrane for dot blotting. Antibodies were used as follows: mouse monoclonal anti-active RAC1 1:1000 (NewEast Biosciences), rabbit polyclonal anti-GST 1:2000 (Sigma). Anti-mouse or rabbit HRP-coupled secondary antibodies (Jackson Laboratories) were used as secondary antibodies at a 1:10,000 dilution, followed by ECL detection following supplier's instructions (Pierce).

Similar experiments were performed with mammalian RAC1 Ab (23A8, Thermo Scientific), which recognizes *Drosophila* Rac1 and Rac2, but not Mtl (***data not shown***).

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