Ixodes scapularis salivary gland protein, P11, facilitates migration of *Anaplasma phagocytophilum* from the tick gut to salivary glands

Lei Liu [1], Sukanya Narasimhan [1], Jianfeng Dai [1], Lili Zhang [1],

Gong Cheng [1] and Erol Fikrig [1,2]

[1] Section of Infectious Diseases, Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut 06520, USA; [2] Howard Hughes Medical Institute, Chevy Chase, Maryland 20815, USA

Supplementary information

SI Materials and Methods

Cell lines and mice

The human promyelocytic leukemia cell line (HL-60) (ATCC, Manassas, VA) was maintained and infected with *A. phagocytophilum* as described previously (1, 2) and the *I. ricinus* (L.) cell line IRE/CTVM19 (3) was maintained as described earlier (4). 4 - 6 week old female C3H/HeJ and C3H/SCID mice were purchased from The Jackson Laboratory and all animal experiments were performed according to the protocols approved by the Institutional Animal Care and Use Committee at the Yale University School of Medicine.

Infection of C3H/HeJ mice with *A. phagocytophilum*

The *A. phagocytophilum* isolate NCH-1 (5) was maintained through serial passage of infected blood in C3H/SCID mice (6). C3H/HeJ mice were injected intraperitoneally with 100 µl of pooled *A. phagocytophilum*-infected anticoagulated blood and infection was confirmed on day 6 as described earlier (7). In all acquisition experiments, ticks were allowed to engorge upon infected mice on day 7, a time coincident with maximal infection of *A. phagocytophilum* in the peripheral blood.

Ixodes scapularis ticks

I. scapularis nymphs and larvae were obtained from a tick colony at the Connecticut Agricultural Experiment Station in New Haven CT, USA. Ticks were maintained at 23°C and 85% relative humidity under a 14 hour light, 10 hour dark photoperiod. To generate *A. phagocytophilum*–infected *Ixodes scapularis* nymphs, the larvae were fed to repletion on *A. phagocytophilum*–infected C3H/HeJ mice and molted into nymphs. 10% of the molted nymphs from each infection group were individually tested by PCR to confirm infection and to determine infection prevalence (7). To produce uninfected nymphs, larvae engorged on uninfected mice and allowed to molt.

Analysis of genes differentially expressed upon *A. phagocytophilum* infection of *I. scapularis* nymphs.

A subset array of 200 secreted salivary gland genes (8) was probed with amplified RNA generated using the Amino Allyl MessageAmp aRNA Amplification and Labeling kit (Ambion Inc, TX) from 3 biological replicates of *A. phagocytophilum*-infected and uninfected 25-30 pooled nymphal *I. scapularis* salivary glands, and labeled with Cy3 and Cy5 (CyDye Post-Labeling Reactive Dyes, Amersham Biosciences, NJ). Hybridization and data analysis was performed as described earlier (8).

RNAi

p11 was amplified from engorged nymphal salivary gland cDNA using the primers p11RNAiF and p11RNAiR containing T7 polymerase promoter sequence as listed in Supplementary Table 2. *p11* dsRNA and *salp16* dsRNA to knock-down *p11* and *salp16* gene expression respectively, was prepared as described (7). 5 nL (5×10^{12}

molecules/μL) *p11* or *salp16* dsRNA was injected into the idiosoma of un-infected or *A. phagocytophilum*-infected nymphal ticks as described earlier. The control ticks (Mock) received 5 nL of the injection buffer (10 mM Tris-HCl, pH 7.5, and 1 mM EDTA).

A. phagocytophilum acquisition

For the acquisition experiments, control ticks (buffer-injected) and ticks injected with *p11* dsRNA were allowed to feed on *A. phagocytophilum*-infected C3H/HeJ mice for 72 h. 5 mice were used in each group (experimental and control/mock), and 10 ticks were placed on each mouse. cDNA was made from salivary glands, guts and hemolymph (pool of 3 ticks) of fed ticks and used to assess *p11* and *16S rRNA* gene expression levels by quantitative RT-PCR. For the time course acquisition experiments, mock and *p11* dsRNA–injected ticks were fed for 24, 48, and 72 h on *A. phagocytophilum*–infected C3H/HeJ mice, and salivary glands, guts and hemolymph from each group were collected at each time point and analyzed in pools of three ticks. To determine the levels of *A. phagocytophilum* in the salivary glands, gut and hemolymph in mock and *p11* dsRNA–injected ticks at day 3 post-detachment from the host, the engorged ticks were maintained in an incubator at 23°C with 85% relative humidity, and dissected on day 3 post-detachment.

A. phagocytophilum survival in ticks and transmission

To examine whether P11 could also impair *A. phagocytophilum* survival in *I. scapularis* ticks, *A. phagocytophilum*-infected nymphal ticks were injected with *p11* dsRNA, allowed to rest for 72 h, and the *A. phagocytophilum* load was examined by

qPCR. To examine the role of P11 in the transmission of *A. phagocytophilum* from ticks to mammalian host, *A. phagocytophilum*–infected and P11-deficient or control nymphs were allowed to feed to repletion on each of 10 naive C3H/HeJ mice (3-4 ticks each mouse). The mice were bled on days 3, 4, 5, 6, 7, 8, 9 and 10, after tick detachment. Total genomic DNA was isolated from the peripheral blood and *A. phagocytophilum* burden measured by qPCR as described below. The murine skin was also collected on day 3, 7 and 14 to check the pathogen load by qPCR.

RNA extraction, cDNA synthesis, quantitative RT-PCR, and genomic DNA isolation

Total RNA was extracted from the salivary glands, hemolymph and gut samples using RNeasy kit (QIAGEN, CA), cDNA synthesized and quantitative PCR performed using the iQ SYBR Green Supermix (Bio-Rad Laboratories, CA) on an iCycler Real-Time Detection System (Bio-Rad Laboratories, CA). The salivary gland and gut cDNA levels were normalized to the *I. scapularis β-actin* gene, and the levels of *16S rRNA* gene computed by extrapolation from the standard curve (iCycler IQ software version 3.1; Bio-Rad Laboratories, CA). Mouse peripheral blood genomic DNA was prepared as described earlier (7), and 16S rDNA levels as a measure of *A. phagocytophilum* burden computed after normalization to the mouse *β-actin* gene. The sequence-specific primers for the *p11* gene (p11qF and p11qR), *A. phagocytophilum 16S rRNA* gene (16S F and 16S R), mouse *actin* (mActinF and mActinR), tick *actin* (tActinF and tActinR) are listed in Supplementary Table 2. qPCR primers for human *actin* (9) and *salp16* gene were as described previously (7).

Protein expression and preparation of polyclonal antibody

Full length of *p11* was amplified using primers rP11F and rP11R (Supplementary Table S2), cloned into pGEX6P-2 bacterial expression vector (GE Healthcare, NJ) and recombinant P11protein purified according to the manufacturor's protocol. The glutathione S-transferase fusion tag was cleaved with PreScission Protease (GE Healthcare, NJ). Polyclonal antibodies against bacterially expressed P11 (henceforth referred to as rP11), was generated in rabbits using standard protocols. Full length *p11* was also amplified using primers rP11-DES F and rP11-DES R (Supplementary Table S2) and cloned into pMT/V5/His vector (Invitrogen, CA) for *Drosophila* S2 expression. P11 was expressed in a *Drosophila* S2 cells (Invitrogen, CA) and purified using Talon affinity chromatography (Clontech Laboratories, Inc, CA) and henceforth referred to as rP11-DES. rP11-DES was utilized for functional studies.

Immunoblots

Salivary glands (10 pooled samples) isolated from the mock and P11-deficient ticks were resuspended in sterile PBS with protease inhibitors (protease inhibitor cocktail; Roche, CA) and homogenized. Equal amounts of salivary gland protein (10 μg) from mock or *p11* dsRNA–injected ticks were electrophoresed on an SDS/12% polyacrylamide gel and processed for immunoblotting. The immunoblots were probed with recombinant rP11 antisera. A duplicate immunoblot was probed with recombinant Salp25D antisera (10) or anti-actin antibody (Sigma-Aldrich, MO). Te detect the existence of P11 in tick hemolymph, 50 *A. phagocytophilum* infected fed ticks were dissected and hemolymph was collected and electrophoresised on SDS/12%

polyacrylamid gel and probed with rabbit anti-rP11 sera. To ascertain the secretory nature of P11, 20 µl of adult tick saliva (10 ng/µl) was electrophoresised on an SDS/12% polyacrylamide gel and probed with rabbit anti-rP11 sera. The bound antibodies were detected using horseradish peroxidase–conjugated secondary antibodies (Sigma-Aldrich, MO), and blots developed using the enhanced chemiluminescence Western Blotting Detection System (GE Healthcare, NJ).

In vivo **detection of** ***A. phagocytophilum*** **in tick tissues by RNA-in situ hybridization**

RNA probes were designed based on *A. phagocytophilum 16S rDNA* sequence. Primers probF1 and probR1 were used to generate sense strand probe, primers probF2 and probeR2 were used to generate antisense strand probe (Supplementary Table S2). The sizes of *A. phagocytophilum* specific probes were 411 bp. The RNA probes were generated and labeled with Alexa Fluor 594 by FISHTag^{TM} RNA Multicolor Kit (Invitorgen, CA) according to manufacture's protocols. Tick tissues (salivary gland, midgut and hemolymph) were fixed on slides with paraformaldehyde solution (4% in PBS, USB Corporation, USA) for 30 min, and then incubated with permeabilization buffer (0.1% Triton X100, 0.05% Tween 20, in PBS) for 30 min. The slides were put in 70% ethanol and processed for hybridization. The probe was denatured at 75^{o}C for 10 min and annealed on ice for 10 min. The samples were dehydrated by washing in a series of ethanol dilutions (2 min each in 70%, 80%, 95% and 100%), and then air-dried. 5 µL of the denatured probe was then applied to the samples, coverslip placed on the probe and incubated at 37^{o}C overnight in a humidified chamber, protected from light. The coverslips were gently removed by flushing with 4X SSC and slides washed as follows:

2X SSC, 50% formamide at 39^{o}C for 5 min (three times); 2X SSC at 39^{o}C for 5 min (three times); 1X SSC at room temperature once for 10 min; 4X SSC at room temperature once for 10 min. The samples were counterstained with DAPI or TOPRO3 (Invitrogen, CA) for 5 min at room temperature and then washed in 4X SSC at room temperature for 5 min prior to visualization by fluorescence microscopy (Carl Zeiss MicroImaging, Inc, Germany).

Blocking *A. phagocytophilum* migration in ticks by anti-P11 antibodies

Purified rabbit anti-rP11 IgG (500 ng/tick) was injected into the hemocoel of naïve nymphal ticks and ticks fed on *A. phagocytophilum*-infected mouse as described above. Normal rabbit IgG was injected in control ticks. Ticks were collected after feeding for 2 and 3 days. The salivary glands, guts and hemolymph were dissected for qPCR analysis as described above. To further confirm the influence of anti-P11 antibodies, groups of 5 *A. phagocytophilum*-infected C3H mice were passively immunized with normal rabbit serum (as control), or 200 μl of anti-P11 antiserum, respectively. 24 h after immunization, 8 naïve nymphal ticks were placed on each mouse. 72 h after feeding, engorged ticks were collected and dissected as described above.

P11 over-expression in HL-60 cells and *I. ricinus* tick cells

p11 was amplified using primers p11CAG F and p11CAG R (Supplementary Table S2) and cloned into pCAG expression vector (11). The recombinant plasmid was transfected into HL-60 cells using the Nucleofector Kit V (Lonza, Germany) and *I. ricinus* tick cell using the Effectene transfection reagent (Qiagen, CA) according to the

manufacturor's instruction. The cells were infected with *A. phagocytophilum* at 2 days post-transfection.

To test whether P11 facilitate *A. phagocytophilum* infection *in vitro*, 1 μg of purified recombinant rP11-DES was incubated with *A. phagocytophilum* enriched from infected HL-60 cells for 1 hr at 37^{o}C. *A. phagocytophilum* was pelleted by centrifugation, and free rP11-DES was removed by wash the bacteria with PBS. The bacterial was used to infect *I. ricinus* tick cells and HL-60 cell. The pathogen burden was determined by qPCR at different time point.

Binding assay

To assess P11 binding to *A. phagocytophilum*, 1.0 μg of rP11-DES protein was coated overnight at 4°C in 0.1 M sodium carbonate buffer (pH 9.5) in 96-well plates. The wells were blocked with 3% BSA/PBS, and incubated with *A. phagocytophilum* enriched from infected HL60 cells by low-speed centrifugation (500 *g*) followed by high-speed centrifugation (15,000 *g*) (8, 15 and 30 μg of total protein per well). Bound *A. phagocytophilum* was detected using anti-*Anaplasma* antibody and HRP-conjugated anti-rabbit IgG followed by microwell peroxidase substrate (Kirkegaard & Perry Laboratories, MD). To rule out artifacts due to coating of rP11-DES on the plate, *A. phagocytophilum* enriched from infected HL60 cells was coated on the wells (25 μg of total protein per well). The wells were blocked with 3% BSA/PBS, and incubated with different amounts of rP11-DES (0.1, 0.5 and 1.0 μg). Bound P11 was detected using the mouse anti-V5 antibody and HRP-conjugated anti-mouse IgG followed by microwell peroxidase

substrate as described above. Another tick protein tHRF (tick Histamine Release Factor) (12), which was also purified from *Drosophila* S2 cells, was used as control.

For *in vitro* image analysis, *I. ricinus* tick cells were infected by *A. phagocytophilum* described as above. Tick cells were fixed with 4% paraformaldehyde in 1xPBS for 15 min at room temperature, and then rinse briefly twice with PBS. The fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 15 min. 1 μg of purified recombinant rP11-DES was incubated with permeabilized tick cells for 60 min at 37 °C. Another tick protein, tHRF was used as control. Washed cells briefly twice with PBS, then incubated with blocking buffer (1% BSA in PBS) for 30 min at room temperature. Mouse anti-P11 antibody and rabbit anti-*Anaplasma* antibody was diluted in blocking buffer and incubated with slides for 60 min at room temperature. Washed the cells 3 times with blocking buffer and incubated with diluted Alexa-488 labeled anti-mouse IgG and Alexa-555 labeled anti-rabbit IgG (Invitrogen, CA) for 60 min at room temperature in dark. Wash the cells 3 times with blocking buffer and counterstained the cells with TOPRO3 (Invitrogen, CA) and viewed under a LSM510 scanning laser confocal microscope (Carl Zeiss MicroImaging, Inc, Germany).

Immunofluroscence staining of tick hemocytes

A. phagocytophilum infected tick hemocytes were collected on microslids as described above. Hemocytes were fixed with 4% paraformaldehyde in 1xPBS for 15 min at room temperature, and then rinse briefly twice with PBS. One group of the fixed cells was permeabilized with 0.1% Triton X-100 in PBS for 15 min and another group cells were not permeabilized. Washed cells briefly twice with PBS, then incubated with

blocking buffer (1% BSA in PBS) for 30 min at room temperature. Anti-*Anaplasma* antibody was diluted in blocking buffer and incubated with slides for 60 min at room temperature. Wash the cells 3 times with blocking buffer and incubated with diluted Alexa-488 labeled secondary antibody (Invitrogen, CA) for 60 min at room temperature in dark. Washed the cells 3 times with blocking buffer and counterstained the cells with TOPRO3 (Invitrogen, CA) and viewed under confocal microscopy.

Statistical analysis

Significance of differences observed in experimental and control groups was analyzed using GraphPad Prism version 4.00 (GraphPad Software, CA). A two-tailed Student's *t* test was utilized to compare the mean values, and $p < 0.05$ was considered significant.

Supplementary Reference

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Supplementary Tables

Table S1. *Ixodes scapularis* genes that were differentially expressed in *A. phagocytophilum*-infected salivary glands

Gene ID	Fold Increase	Fold Decrease	Potential Function
AF483740		5.0	Putative 10.2 kDa secreted protein
AF483712		10.0	Salp10-like
AF483696		4.8	Collagen-like secreted protein
AF209919		23.8	Salp26A-like
AF483665		1.7	Anticoagulant protein
AF483719	3.0		Disintegrin like
AF483743		3.0	Histamine binding protein
DQ066011/ p11*	2.0		Putative secreted salivary protein
DQ066005	3.0		Putative secreted salivary protein
DQ065996	2.0		Putative secreted salivary protein
AF483714	1.5		Putative protease inhibitor
AF483736	5.0		Unknown function
AF483695		3.0	Collagen-like secreted protein
AF483742	10.0		Histamine binding protein
AF209915	5.0		Salp16
AF483720	2.0		Thrombospondin

Table S2. Primers used in this study.

Primer Name	Sequrence
p11RNAiF	5′-TAATACGACTCACTATAGGGTTCACCGTTGTCGTGGCAGC-3′
p11RNAiR	5′-TAATACGACTCACTATAGGGTTACAACACGTGCTCGCGGGC-3′
p11qF	5′-AAAAGACACTGTGTGCAGCG-3′
p11qR	5′-CCCACTTTTCCAGGAGGTTT -3′
16S F	5′ GGTGAGTAATGCATAGGAATC -3′
16S R	5′-GCTCATCTAATAGCGATAAATC -3′
mActinF	5′-AGCGGGAAATCGTGCGTG -3′
mActinR	5′-CAGGGTACATGGTGGTGCC -3′
tActinF	5′-GGCGACGTAGCAG -3′
tActinR	5′-GGTATCGTGCTCGACTC -3′
rP11F	5′-AGAATTCCCTACAGCGAACATTTGAGG-3′
rP11R	5′-AGCGGCCGCTTACAACACGTGCTCGC-3′
rP11-DES F	5′-AGAATTCTTACAGCGATACATTTGAG-3′
rP11-DES R	5′-AGCGGCCGCCAACACGTGCTCGCGGC-3′
probF1	5’-TAATACGACTCACTATAGGGTTATCTCTGTGTTGTAGC-3’
probR1	5’-TTGTAGTCACCATTGTAG-3’
probF2	5’-TTATCTCTGTGTTGTAGC-3’
probR2	5’-TAATACGACTCACTATAGGG TTGTAGTCACCATTGTAG -3’
p11CAG F	5’-TAATACGACTCACTATAGGG TTGTAGTCACCATTGTAG -3’
p11CAG R	5′- CCCGGGACAACACGTGCTCGCGGCC -3′

Supplementary Figure and Legend

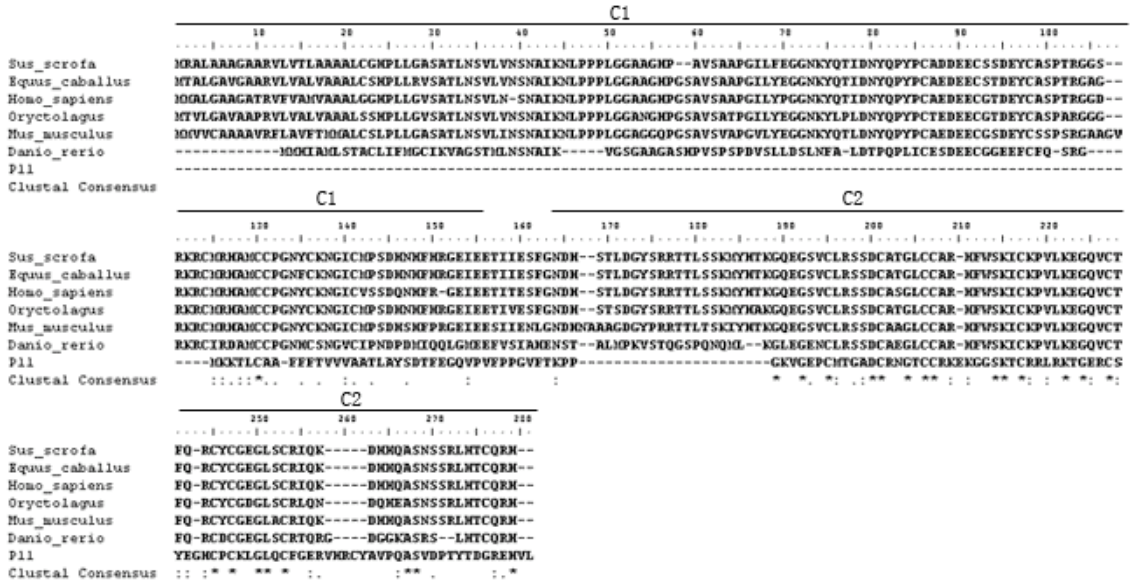

Figure S1: **Clustal alignment of P11 with Dickkopf (DKK) related proteins from other organisms.** *Sus scrofa* (XP_001926307); *Equus caballus* (XP_001503344); *Homo sapiens* (NP_036374); *Oryctolagus cuniculus* (NP_001076206); *Mus musculus* (NP_034181); *Danio rerio* (BAA82135) and P11 (AAY66648). C1 and C2 represent the N-terminal and C-terminal cysteine rich domains of DKK, respectively.

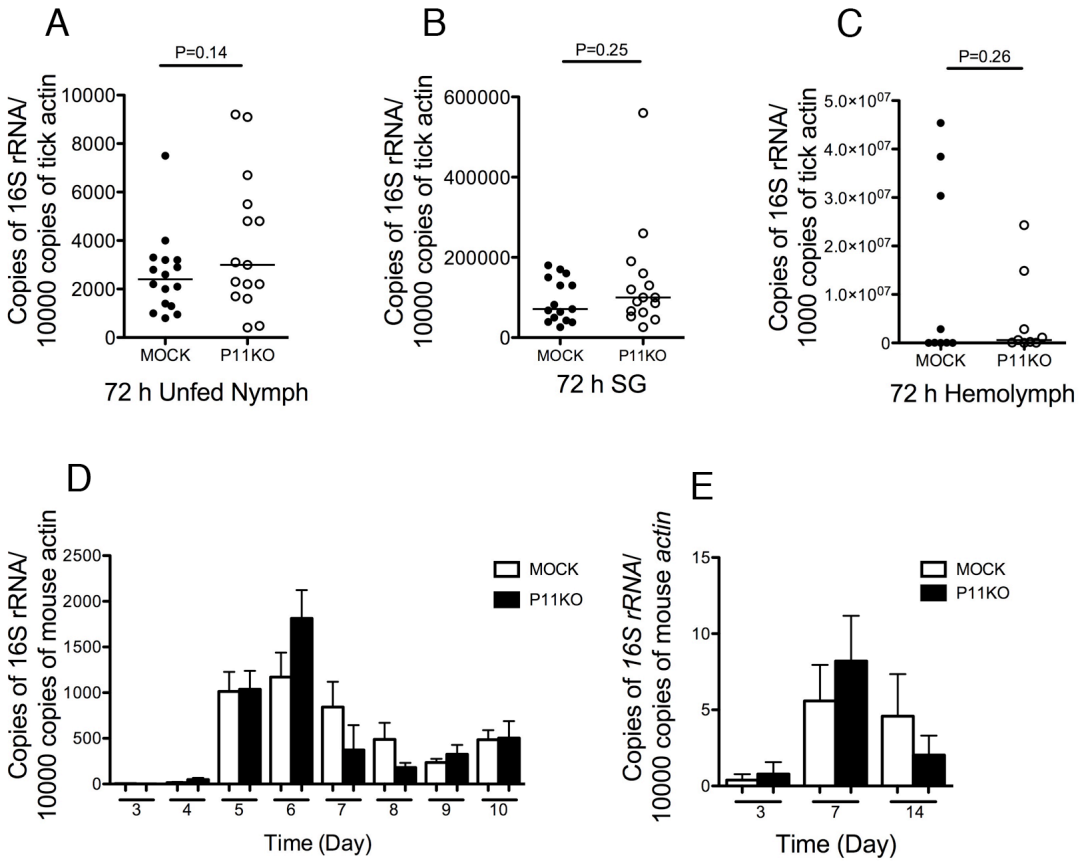

Figure S2: **Survival and transmission of *A. phagocytophilum* is not affected in P11-deficient ticks.** The levels of *A. phagocytophilum 16S rRNA* transcripts in *p11* silenced (P11KO): (A) unfed nymphal whole ticks; (B) salivary gland (SG); and (C) Hemolymph compared to that in control group of ticks (MOCK), after infected nymphs were fed on naïve C3H mice. (D) MOCK or *p11* deficient (P11KO) infected nymphs were fed on naïve C3H mice and *A. phagocytophilum* burden in mice blood and (E) skin, assessed at different time points after tick detachment. Error bars show means ± SEM. The horizontal line represents the medians and *p* values were indicated. Three independent experiments yielded similar results.

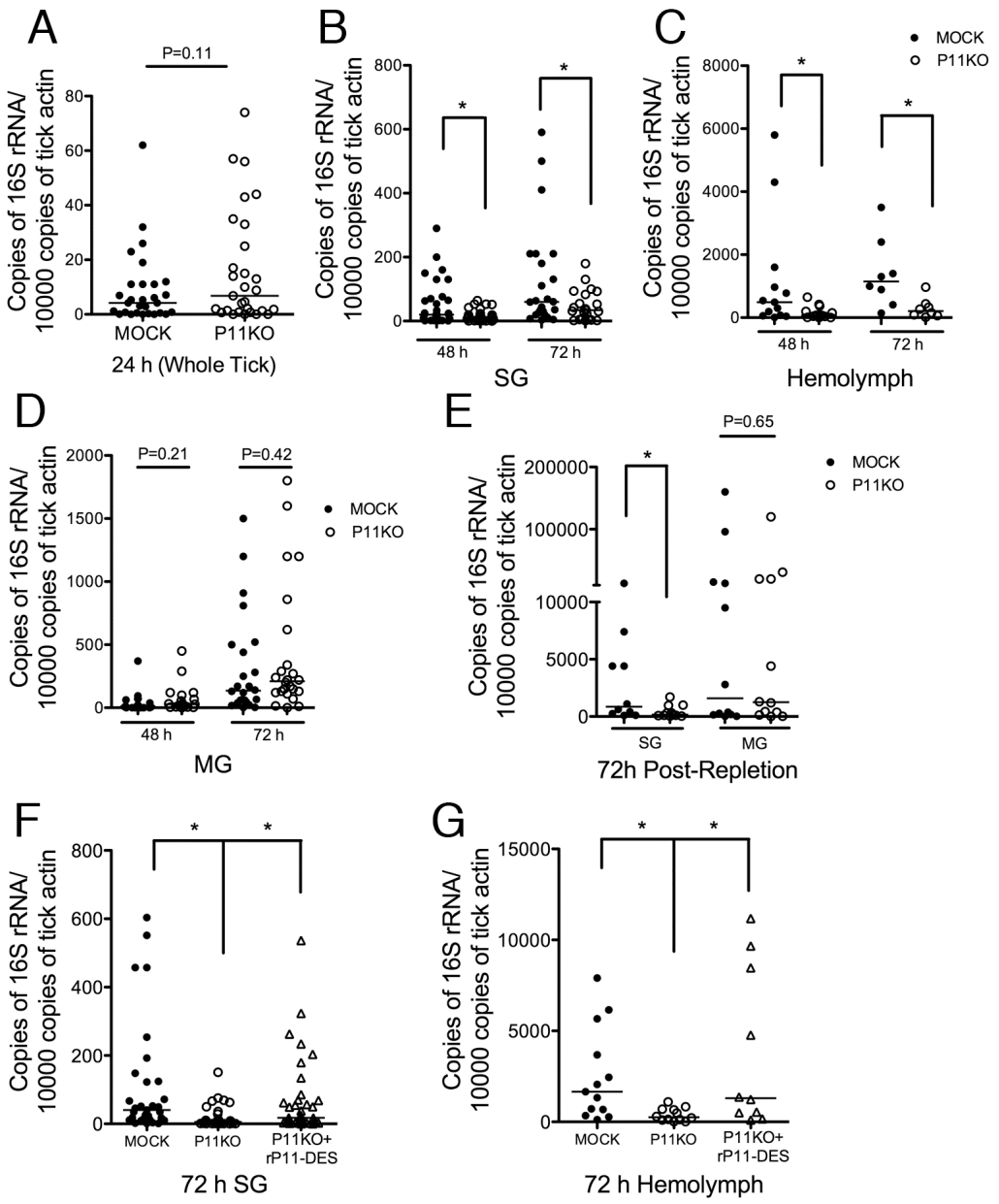

Figure S3: ***A. phagocytophilum* requires P11 for migration from tick midgut to salivary gland through hemolymph.** A time course assessment of the levels of *A. phagocytophilum 16S rRNA* transcripts within the salivary gland (SG), hemolymph and midgut (MG) of P11-deficient (P11KO) and mock ticks (MOCK) fed on infected mice for 24, 48 and 72 h. (A) *A. phagocytophilum* burden at 24 h after feeding in whole ticks.

A. phagocytophilum load at 48 and 72 h after feeding in: (B) salivary glands; (C) hemolymph; and (D) midguts. (E) *A. phagocytophilum 16S rRNA* transcripts at 72 h post-engorgement (6 days after feeding) in tick salivary glands (SG) and midguts (MG). The levels of *A. phagocytophilum 16S rRNA* transcripts in *p11* dsRNA and rP11-DES injected ticks: (F) Salivary glands (SG) and (G) Hemolymph. The horizontal line represents the medians. * $p < 0.05$. Three independent experiments yielded similar results.

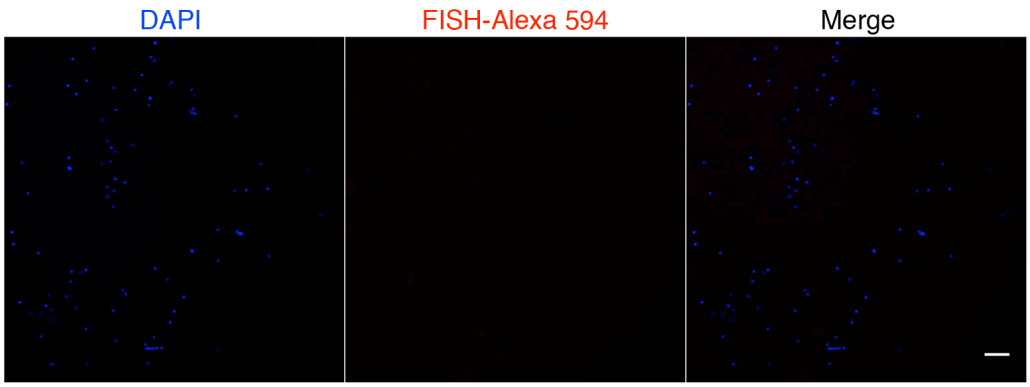

Figure S4: ***A. phagocytophilum* infection in tick hemocytes.** RNA-FISH images of uninfected tick hemocytes, no *A. phagocytophilum* was detected. Scale bar represent 50 μm.

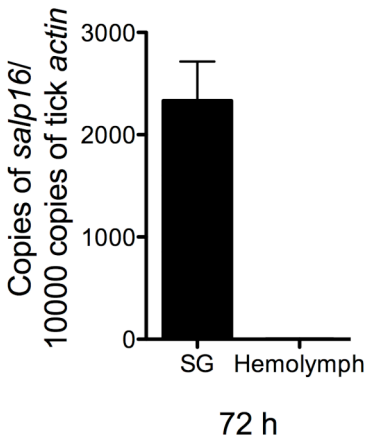

Figure S5: ***salp16* expression in tick hemolymph.** qPCR analysis of *slap16* mRNA level in tick salivary glands and hemolymph. Error bars show means ± SEM. Three independent experiments yielded similar results.

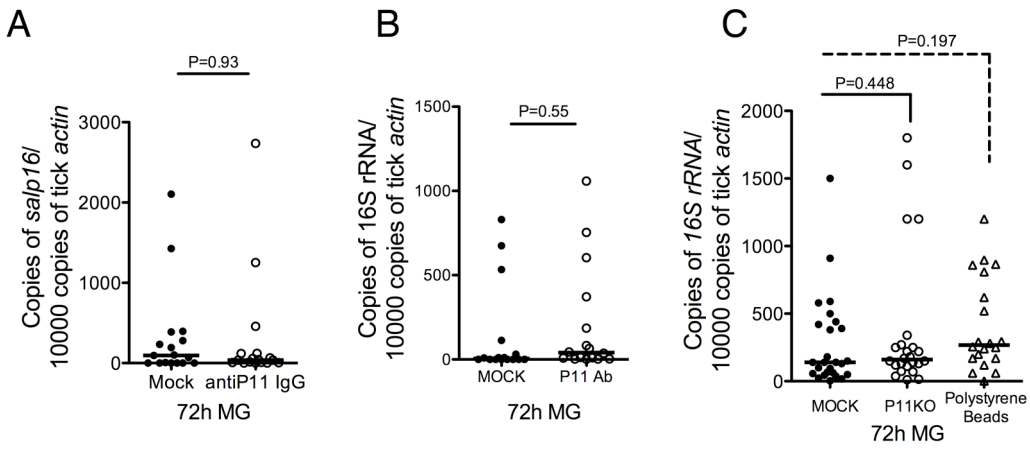

Figure S6: **P11 antiserum and polystyrene beads have no effect on *A. phagocytophilum* infection of tick midgut.** Normal rabbit IgG (MOCK) or purified rabbit anti-P11 IgG (P11 Ab) was injected into the hemocoel and ticks fed on *A. phagocytophilum* infected mice. (A) The levels of *A. phagocytophilum 16S rRNA* transcripts in midgut. (B) *A. phagocytophilum* infected C3H/HeJ mice were passively immunized by normal rabbit IgG (MOCK) or purified rabbit anti-P11 IgG (P11 Ab) and the levels of *A. phagocytophilum 16S rRNA* was measured in tick midgut after feeding for 72 h. (C) Polystyrene beads was injected into the hemocoel and ticks fed on *A. phagocytophilum* infected mice and levels of *A. phagocytophilum 16S rRNA* transcripts in midgut assessed. The horizontal line represents the medians. Three independent experiments yielded similar results.

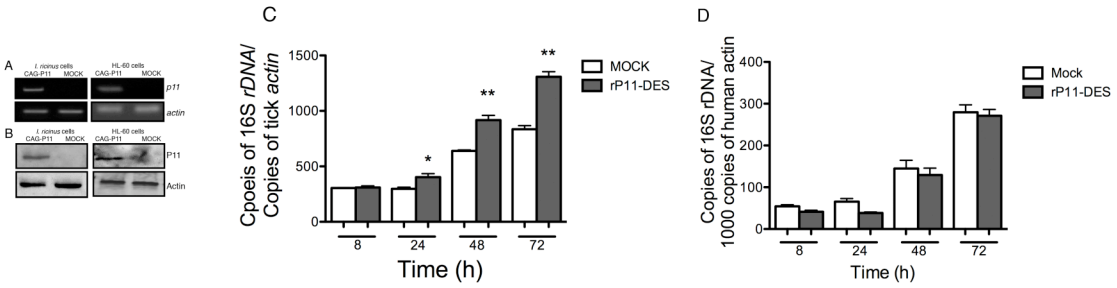

Figure S7: **P11 facilitates *A. phagocytophilum* infection by binding to the bacteria.** Expression of *p11* in *I. ricinus* tick cells and HL-60 cells were assessed by (A) reverse transcript-PCR and (B) western blot. qPCR analysis results showed that recombinant rP11-DES could facilitate *A. phagocytophilum* infection of (C) *I. ricinus* tick cells but not (D) HL60 cells *in vitro*.

Author contributions

L.L., S.N., J.D., and L.Z. designed research; L.L., J.D. and L.Z. performed research; L.L., S.N., J.D., L.Z., G.C., and E.F. analyzed data, L.L, S.N and E.F. wrote the paper.