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***Borrelia burgdorferi* chemotaxis toward tick protein Salp12 contributes to acquisition**

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Abstract

Lyme disease is a common tick-borne infection caused by the spirochete *Borrelia burgdorferi* sensu stricto (s.s.). *B. burgdorferi* s.s. may utilize chemotaxis, the directional migration towards or away from a chemical stimulus, for transmission, acquisition, and infection. However, the specific signals recognized by the spirochete for these events have not been defined. In this study, we identify an *Ixodes scapularis* salivary gland protein, Salp12, that is a chemoattractant for the spirochete. We demonstrate that Salp12 is expressed in the *I. scapularis* salivary glands and midgut and expression is not impacted by *B. burgdorferi* s.s. infection. Knockdown of Salp12 in the salivary glands or passive immunization against Salp12 reduces acquisition of the spirochete by ticks but acquisition is not completely prevented. Knockdown does not impact transmission of *B. burgdorferi* s.s. This work suggests a new role for chemotaxis in acquisition of the spirochete and suggests that recognition of Salp12 contributes to this phenomenon.

Graphical Abstract

Keywords

Borrelia burgdorferi sensu stricto; chemotaxis; acquisition; *Ixodes scapularis*; tick; Lyme disease

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Introduction:

Borrelia burgdorferi sensu stricto (s.s.), the causative agent of Lyme disease, is one of the most prevalent arthropod-transmitted pathogens in the United States and Europe (Kuehn 2013, Hinckley, Connally et al. 2014). There is currently no preventative human vaccine for Lyme disease, and although it is treatable with antibiotics, long lasting symptoms can occur. Additionally, treatment places a significant burden on the medical system (Adrion, Aucott et al. 2015). Therefore, identifying new vaccine and therapeutic targets is warranted, and the elucidation of crucial interaction points between the tick host and *B. burgdorferi* s.s. has the potential to uncover novel targets for vaccine or therapeutic development.

In the United States, *Ixodes scapularis* ticks are the most common vector of *B. burgdorferi* s.s. *I. scapularis* ticks feed on small mammals and can acquire the spirochete during the first two life stages, the larva and nymph. Infected ticks then transmit the spirochete during subsequent feedings, as a nymph or adult. Both nymphs and adults may feed on human hosts and transmit the spirochete. To complete the infectious cycle, *B. burgdorferi* s.s. must perform distinct migration patterns through animal and tick tissues for successful transmission, infection, and acquisition. Uninfected ticks acquire *B. burgdorferi* s.s. when feeding on infected animals, and during acquisition, the spirochetes present in mammalian skin enter the tick midgut as the tick takes a blood meal (Bockenstedt, Gonzalez et al. 2014). The spirochetes are then maintained in the tick gut during molting. During transmission, spirochetes residing in a tick's midgut migrate to the tick's salivary glands, where they exit the tick along with saliva (de Silva and Fikrig 1995). After being deposited in mammalian skin, *B. burgdorferi* s.s. establishes an infection at the bite site and disseminates to other organs (Barthold, Persing et al. 1991, Pahl, Kuhlbrandt et al. 1999). Many steps of these processes are facilitated by or improved by tick proteins. For example, tick protein Salp15 binds to the *B. burgdorferi* s.s. protein OspC and protects the spirochete from antibody mediated killing (Ramamoorthi, Narasimhan et al. 2005) and knocking down Salp15 or vaccinating animals against Salp15 reduces transmission of the spirochete (Dai, Wang et al. 2009). Additionally, Salp15 is anti-inflammatory and inhibits CD4+ T cells contributing to tick feeding and vector-host-pathogen interactions (Anguita, Ramamoorthi et al. 2002).

Bacteria can perform directed migration patterns through chemotaxis, the directional migration of cells toward or away from a chemical stimulus. Chemotaxis in *B. burgdorferi* s.s. has been shown to be important for spirochete growth in ticks (Novak, Sekar et al. 2016), transmission from the tick (Sze, Zhang et al. 2012, Novak, Sekar et al. 2016, Xu, Sultan et al. 2017), and infection of mammals (Lin, Gao et al. 2012, Sze, Zhang et al. 2012, Novak, Sekar et al. 2016, Xu, Sultan et al. 2017). Although it has not been directly shown, several lines of evidence suggest that acquisition of the spirochete by the tick host might be facilitated by chemotaxis. When infected and uninfected ticks co-feed on an animal, there is acquisition of the spirochete by nearby ticks prior to disseminated infection (Gern and Rais 1996, Patrican 1997, States, Huang et al. 2017). Additionally, when a nymph feeds on an infected mouse, spirochete levels around the tick bite site decrease concurrent with an increase in spirochetes in the tick (Hodzic, Borjesson et al. 2001). Finally, intravital imaging of spirochetes during tick feeding detected spirochetes migrating into the tick bite site and

disappearing (Bockenstedt, Gonzalez et al. 2014). Together these data suggest that spirochetes may migrate toward the tick bite site for acquisition.

Although it is known that chemotaxis is critical for *B. burgdorferi* s.s., chemoattractants and chemorepellents have not been identified in the infectious cycle. *In vitro* studies have shown that sugars and amino acids, such as N-Acetylglucosamine and glutamate, are chemoattractants for *B. burgdorferi* s.s. (Bakker, Li et al. 2007). Additionally, rabbit serum (Shi, Yang et al. 1998) and tick salivary gland extract (SGE, ground filtered salivary glands) (Shih, Chao et al. 2002) have been shown to be chemoattractants for *B. burgdorferi* s.s. It is likely that spirochete recognition of serum components functions in dissemination of the spirochete during infection. Recognition of tick salivary gland components could facilitate transmission or acquisition of the spirochete by the tick host. In transmission, the spirochete may recognize salivary gland components to migrate from the midgut to the salivary glands. In acquisition, spirochetes in mammalian skin could recognize components of tick saliva to migrate to the tick bite site, where they are ingested by the tick. During feeding, ticks secrete a large quantity of saliva that facilitates the feeding process (Kazimírová and Štibrániová 2013). As tick saliva is made within the salivary glands, SGE and saliva likely contain overlapping repertoires of molecules and therefore chemoattractants.

The specific chemotactic exchanges that occur between the spirochete and the tick are of particular interest because of their potential as therapeutic targets. Previous work has demonstrated that blocking tick-spirochete interactions that function in transmission reduces spirochete burden in the tick and mammal (Ramamoorthi, Narasimhan et al. 2005, Dai, Wang et al. 2009, Zhang, Zhang et al. 2011, Narasimhan, Coumou et al. 2014, Coumou, Narasimhan et al. 2016). Therefore, blocking chemotactic interactions that are critical for transmission or acquisition would likely reduce or prevent these processes. Interactions that are essential for transmission could be targeted in human or companion animal vaccines, as preventing transmission is protective. Interactions that facilitate acquisition could be targeted for environmental control strategies. This study defines a chemoattractant molecule present in tick SGE and saliva and establishes that recognition of this molecule is involved in acquisition *in vivo*.

Materials and Methods:

Bacterial strains.

B. burgdorferi s.s. strains were obtained from MA Motaleb (Table 1) (Motaleb, Miller et al. 2005, Motaleb, Miller et al. 2007). *B. burgdorferi* s.s. was grown without aeration in BSK-H (Sigma-Aldrich) at 32°C and were frozen at -80°C in BSK-H supplemented with 20% glycerol. *Escherichia coli* Top 10 cells (ThermoFisher Scientific) were used for cloning (Table 2) and were grown at 37°C with aeration in Lysogeny Broth supplemented with ampicillin (100 µg/mL).

Animals.

Rabbits and mice were housed and handled under the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal protocol was approved

by the Yale University's Institutional Animal Care and Use Committee (protocol number 2016-07941, approved December 20, 2016). All animal experiments were performed according to Yale University standards in a biosafety level 2 facility. For all mouse experiments, female, 5–10 weeks of age, C3H/HEJ mice were used (Jackson Laboratory, RRID:IMSR_JAX:000659). For tick saliva collection, New Zealand White rabbits were used (Charles River Laboratories, strain 571).

Ticks.

I. scapularis larvae and adults were acquired from the CDC, and larvae were used to generate nymphs. Briefly, 100 larval ticks were placed on naïve mice and allowed to feed to repletion. For infected nymphs, *B. burgdorferi* s.s. infected mice were used, see experiments below. Fed larvae were collected and stored until molted, approximately 6 weeks. Ticks were maintained at 23°C with 85% relative humidity with 14-hour light, 10-hour dark photoperiod. To limit variability of ticks, all ticks (i.e. both control and test samples) within a single experiment were from the same egg clutch.

Cell Lines.

Schneider's *Drosophila* Line 2 cells purchased from ATCC (CVCL_Z232) were used for protein expression and purification. Cells were grown in Schneider's medium (Sigma-Aldrich) with 10% FBS at 25°C. For maintenance of plasmids, cells were selected with 200 µg/mL Hygromycin B (Invitrogen). Cells were authenticated by ATCC prior to purchase. Additionally, authentication of the cell line during growth was confirmed by regular morphology checks by microscopy.

Collection of tick products.

Salivary gland extract (SGE) was obtained by feeding nymphs for 48 hours, dissecting, rinsing in PBS three times, and placing the salivary glands in a fresh tube of PBS. Prior to collection, salivary glands were visually inspected for any cross-contaminating midgut tissues. For capillary tube assays, salivary glands from ~40 nymphs were pooled, and for fractionation, salivary glands from ~100 nymphs were pooled. The pooled salivary glands were homogenized using a hand-grinder, filtered through a 0.2-µm filter, and were stored at –80°C until used.

Tick saliva was collected from adult female ticks as previously described (Anguita, Ramamoorthi et al. 2002). Briefly, adult female and male ticks were placed on rabbits and collected after feeding to repletion. Female ticks were immobilized on microscope slides with double-sided tape. Tick mouth parts were placed inside capillary tubes, and 1–3 µL of 50 mg/mL pilocarpine was applied. Saliva was collected for 2–3 hours, pooled and stored at –80°C.

Capillary tube chemotaxis assays.

Chemotaxis assays were performed using a capillary tube chemotaxis assay (Motaleb, Miller et al. 2007). *B. burgdorferi* s.s. cells were grown to logarithmic phase ($1 \times 10^{7-8}$ cells/mL), collected by centrifugation, and re-suspended in motility buffer to a concentration of 1×10^7 cells/mL. 200 µL of the suspension was added to microcentrifuge tubes and covered with

parafilm. Capillary tubes containing chemoattractants or buffer were placed into the suspension and incubated at 32°C for 2 hr. Capillary tubes were removed and the liquid from the tube collected by centrifugation. Bacteria from the capillary tubes were enumerated by extracting DNA using the DNeasy Blood and Tissue Kit (Qiagen) and qPCR. Chemotaxis results are reported as the relative chemotactic response, the number of cells/mL in the test capillary tube divided by the number of cells/mL in the buffer control capillary tube. In each experiment, 3 technical replicates were performed, and the experiment was repeated at least 5 times. For SGE, 50 µL of SGE or fractionated SGE (~100 µg/mL protein) was prepared as described above and diluted in 500 µL of motility buffer. For testing purified proteins or bovine serum albumin, 50 µL of 50 µg/mL was diluted in 500 µL of motility buffer. Purified proteins were also tested at half this concentration and showed similar results (data not shown). The empty vector control was purified and diluted in the same ratio as the purified protein. For tick saliva, 40 µL was diluted in 500 µL motility buffer. These solutions were used to fill uncoated Micro-hematocrit Capillary Tubes (ThermoFisher Scientific).

Removing proteins and degrading sugars from SGE.

To remove proteins from SGE, 50 µL of SGE was diluted to 70 µL with PBS and was treated with 30 µL proteinase K (QIAGEN, >600 mAU/mL) (Edwinson, Widmer et al. 2016) at 55°C overnight. The proteinase K was then heat inactivated and the digested solution was rinsed and concentrated to 50 µL using a 3 kDa spin column (Millipore). We confirmed that the heat inactivation conditions were sufficient to remove proteinase K activity. Removal of proteins was confirmed by silver staining of SDS-PAGE gels (Invitrogen). Sugars were degraded using sodium-metaperiodate (Edwinson, Widmer et al. 2016). 50 µL of SGE was diluted to 100 µL with PBS and was treated with 100 µL of 100 mM sodium metaperiodate (pH 7.2) at 4°C overnight. Remaining sodium metaperiodate was inactivated by adding 100 µL of 260mM glycerol. The solution was rinsed and concentrated to 50 µL using a 3 kDa spin column, which also allows for passthrough of small molecules and reacted sodium metaperiodate. Removal of intact sugars was confirmed by blotting (Kropec, Maira-Litran et al. 2005) and phenol-sulfuric acid method (Masuko, Minami et al. 2005). It is important to note that sodium-metaperiodate may also oxidize and therefore degrade other compounds.

Protein fractionation and identification.

SGE was fractionated using anion exchange medium pressure chromatography (NGC, Bio-rad). SGE was diluted in 10mM NaCl, pH 7.0 for column binding. Proteins were salted-off the column using 1M NaCl, pH 7.0, and 3 mL fractions were collected. Fractions were concentrated using a 3 kDa spin column and were re-suspended in PBS for chemotaxis assays and mass spectrometry analysis.

Proteins from fractionated pools processed for bottom-up proteomics using high resolution LC-MS/MS of a Waters NanoACQUITY UPLC coupled to a Q-Exactive Plus mass spectrometer. Mass spectral data were analyzed with Mascot Search Engine (Matrix Science, Boston, MA) against the tick protein database (VectorBase). Significant protein hits were those with a Mascot expectation value of 0.05 (equivalent to a 95% confidence identification) (<https://www.ncbi.nlm.nih.gov/BLAST/tutorial/Altschul-1.html>) and represented by at least 2 unique peptides. These analyses resulted in 86 and 168 significant

protein identifications, within two biological replicate fractionated pools. The intersection (70 shared proteins) of these two replicates were compared to a database of adult tick saliva proteins (Kim, Tirloni et al. 2016) to obtain 15 putative targets. These targets were assessed for secretion signals, resulting in 5 possible proteins of interest. One protein was selected for additional analysis based on our ability to clone and express the intact protein.

Bioinformatic analysis of Salp12.

The protein sequences were analyzed using blastp (<https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins>) to identify homologs, InterPro (<https://www.ebi.ac.uk/interpro/>) (Finn, Attwood et al. 2017) to identify domains, and SignalP 4.1 (<http://www.cbs.dtu.dk/services/SignalP/>) (Petersen, Brunak et al. 2011) to identify signal sequences. Alignment of Salp12 to human co-lipase was done using MUSCLE (<https://www.ebi.ac.uk/Tools/msa/muscle/>).

Protein Purification.

For cloning of tick proteins, cDNA from ticks fed for 48 hours was produced as described above. The transcript was cloned into pMT/V5-His A using primers specific to the gene (Table 4). The resulting plasmid (Table 1) was transfected into *Drosophila* S2 cells using Effectene (Quiagen). For expression, cells were grown in Insect Express medium (Lonza) and induced by addition of copper sulfate (500 μ M). The cell supernatant was collected after 7 days and the protein was purified using a Talon column (Clontech). The eluted protein was concentrated using a 3 kDa filter (Millipore) and checked by Western blot with anti-V5 (Thermo Fisher Scientific) and anti-His (Thermo Fisher Scientific) antibodies. Mosquito protein AAEL010228 was produced and purified from S2 cells similarly (Table 1, S5). Mosquito RNA was extracted from salivary glands of *Aedes aegypti* mosquitoes and used to produce cDNA for cloning. Gels of the purification can be seen in Figure S3. Gels were run with the Precision Plus Protein Dual Color Standards (Bio-Rad), and stained with either Simply Blue Safe Stain (Thermo Fisher Scientific) or SilverQuest Silver Staining (Invitrogen).

Tick expression studies.

For gene expression studies, ticks were placed on mice and allowed to feed for 24, 48, or 72 hours. Ticks were then removed and dissected to collect salivary glands and midguts. To prevent cross contamination of the salivary glands and midguts, the organs were visually inspected for inappropriate tissues and rinsed three times prior to collection. Organs from 2–3 ticks were pooled for RNA extraction using the RNeasy Kit (Quiagen), reverse transcribed using iScript (Bio-rad), and used for qPCR.

Immunofluorescence assay.

IFA was performed similarly to previously described methods (Ramamoorthi, Narasimhan et al. 2005, Narasimhan, Coumou et al. 2014). *B. burgdorferi* s.s. cultures were incubated with glass coverslips for 3 days under normal growth conditions in a 24-well tissue culture plate. Coverslips were removed and airdried, and spirochetes were fixed using 4% paraformaldehyde. Coverslips were blocked with 1% bovine serum albumin and were incubated with 50 μ g of Salp12 overnight at 4°C. Control coverslips for detecting Salp12

alone were incubated with Salp12 overnight at 4°C and then blocked. Salp12 was detected using a mouse anti-V5 antibody (Invitrogen, 36–7500) and a goat anti-mouse antibody conjugated to Alexa Fluor 555 (Life Technologies, A-21147). *B. burgdorferi* s.s. was detected using a BacTrace® anti-*Borrelia* antibody conjugated to FITC (Kirkegaard and Perry Laboratories, 5330–0064). Coverslips were mounted using Prolong® Gold anti-fade reagent (Invitrogen) and were viewed at 20x magnification using an Axiovert 200M microscope (ZEISS). Imaging acquisition and processing was performed using ZEN software (ZEISS).

RNAi silencing of Salp12.

Knockdown of Salp12 and subsequent transmission and acquisition studies were performed as previously described (Pedra, Narasimhan et al. 2006). Briefly, dsRNA was amplified using primers specific to the sequence (Table 4) and cloned into the T7 script II vector (Fire, Xu et al. 1998). Complimentary dsRNA was synthesized *in vitro* using the Megascript RNAi kit (Ambion). *I. scapularis* nymphs, clean or *B. burgdorferi* s.s. infected, were injected in the body with ds *salp12* or ds *gfp* control (6 nl, 3×10^{12} molecules) (Schuijt, Coumou et al. 2011). To determine knockdown efficiency, ticks were fed on mice for 24 hours, and samples were collected and analyzed as described above.

Passive immunization.

Antibodies against Salp12 and ovalbumin were produced in New Zealand white rabbits at Cocalico Biologicals, Inc. Rabbits, 4–6 weeks old, were immunized subcutaneously with 30 µg of protein in complete Freud's adjuvant and boosted twice, every 3 weeks. Test bleeds were collected from the ear vein at 2 weeks and after the final boost. After the final boost, rabbits were euthanized and the serum was collected by cardiac puncture. Recognition of purified Salp12 or ovalbumin was performed by Western blot and ELISA (data not shown). To passively immunize mice, 200 µL of whole serum injected intraperitoneally 24 hours prior to tick placement and at the time of tick placement.

Acquisition and transmission studies.

For acquisition studies, mice were infected subcutaneously with $1 \times 10^{4-5}$ spirochetes and used for studies 14–21 days post infection. Uninfected and injected ticks were placed and allowed to feed to repletion. Ticks were assessed for time to repletion and weight. Ticks were dissected to collect salivary glands and midguts. RNA was collected as described above and used for qPCR. For time course experiments, ticks were removed at 24 hours post placement or collected after repletion and incubated for 7 days in normal storage conditions. Ticks were dissected to collect salivary glands and midguts for RNA extraction and qPCR.

For transmission studies, 5 *B. burgdorferi* s.s. infected and injected ticks were placed on each mouse (5 total per experimental condition) and allowed to feed to repletion. Fed ticks were assessed for time to repletion and weight. To monitor infection, skin samples were collected at 3, 5, 7, and 14 days. At 21 days, mice were euthanized, and skin, bladder, heart, and joints were collected. *B. burgdorferi* s.s. burden in mouse tissues was determined by DNA extraction using the DNeasy Blood and Tissue Kit (Quiagen) and qPCR.

Absolute quantification qPCR.

All qPCR results were obtained by absolute quantification qPCR. The qPCR reactions used 1 µL of template DNA with primers specific to the gene (Table 4) in SYBR Green (Invitrogen). Quantification of copy number was performed using a standard curve for each gene.

Statistics.

All details regarding biological and technical replicates can be found in the figure legends. Details regarding the statistical tests used for each experiment can be found in both the results section and figure legends. Bar graphs and dot plots show the mean and SEM. Significance was defined as $p < 0.05$, and specific p-values can be found in both the text and figures. To assess distribution of the data, all data sets were analyzed in both the variable and outcome using the Shapiro-Wilk normality test performed in PRISM. Data sets with normal distribution were assessed by ANOVA in R. Data sets with non-normal distribution were assessed by Mann-Whitney tests, performed in PRISM. Significance of proteomics hits were assessed using Mascot Search Engine criteria.

Data Availability.

The mass spectrometry proteomic data are available via the ProteomeXchange Consortium and the PRIDE partner repository (<http://www.proteomexchange.org/>) under the identifier PXD011273.

Results:

Proteins in tick salivary glands and saliva are chemoattractants for *B. burgdorferi* s.s.

To assess which components of tick salivary glands are chemoattractants for the spirochete, we removed specific compounds from SGE and tested these solutions in *in vitro* assays. Small molecules, such as sugar monomers, dipeptides, or ions, are common chemoattractants for bacteria (Porter, Wadhams et al. 2011). Therefore, we tested whether small molecules in SGE are chemoattractants by assessing *B. burgdorferi* s.s. chemotaxis towards small molecule (<3 kDa) or large molecule (>3 kDa) fractions (Fig. 1A). We examined chemotaxis *in vitro* using a capillary tube assay with our putative chemoattractants relative to a buffer control (Bakker, Li et al. 2007, Motaleb, Miller et al. 2007). Bacterial numbers were enumerated using absolute quantification qPCR, and data are shown as the relative chemotactic response (number of cells in the chemoattractant tube divided by the number of cells in the buffer control tube). Assays were performed with a wildtype, non-infectious isolate, B31A, and a chemotaxis deficient mutant, B31A *cheX* (Table 1) (Motaleb, Miller et al. 2005), to confirm that migration toward putative chemoattracts was due to chemotaxis. Wildtype *B. burgdorferi* s.s. but not the *cheX* mutant migrated toward the large molecule fraction of SGE (ANOVA, $df=3$, $p=0.02$), indicating small molecules within SGE are not the chemoattractant detected in the *in vitro* assay.

Because sugars, such as N-acetyl glucosamine, are chemoattractants for *B. burgdorferi* s.s. (Bakker, Li et al. 2007), we next assessed if sugars and carbohydrates present in SGE are chemoattractants for *B. burgdorferi* s.s.. Sugars in SGE were degraded using a sodium-

metaperiodate treatment (Edwinson, Widmer et al. 2016), and the absence of intact sugar molecules was confirmed by blotting (Kropec, Maira-Litran et al. 2005) and the phenol-sulfuric acid method (Masuko, Minami et al. 2005) (Fig. S1 A, B). *B. burgdorferi* s.s. migration toward sodium-metaperiodate treated SGE was statistically significant (ANOVA, $df=2$, $p=0.02$), indicating sugars in SGE are not a significant chemoattractant as measured by the *in vitro* assay (Fig. 1B).

Another major component of a tick salivary gland is protein (Chmela, Kotál et al. 2016). We therefore tested whether proteins in SGE are chemoattractants by removing proteins using proteinase K (Edwinson, Widmer et al. 2016) and testing the chemotactic response (Fig. 1B). Removal of proteins was confirmed by silver staining of protein gels (Invitrogen) (Fig. S1C). *B. burgdorferi* s.s. did not migrate toward the proteinase K treated SGE, demonstrating that this treatment removed the chemoattractant present in SGE. Therefore, proteins are the chemoattractant molecules present SGE. Because amino acids, such as glutamate (Bakker, Li et al. 2007), are known to be chemoattractants for *B. burgdorferi* s.s., we assessed if this interaction is specific to proteins within SGE. We tested migration of *B. burgdorferi* s.s. toward proteins not expected to function as chemoattractants, mosquito salivary protein AAEL010228 (Conway, Londono-Renteria et al. 2016) and bovine serum albumin (Fig. 1C). These proteins were not chemoattractants for *B. burgdorferi* s.s., indicating that the chemotactic response of *B. burgdorferi* s.s. is specific to proteins in tick SGE.

As tick saliva and tick salivary glands overlap in proteins and sugars, we assessed if proteins in tick saliva are chemoattractants for *B. burgdorferi* s.s. Similarly, we found that *B. burgdorferi* s.s. migrated toward tick saliva and that proteins, but not sugars, within saliva are chemoattractants (Fig. 1D, ANOVA, $df=3$, $p<0.02$).

Tick protein Salp12 is an attractant.

To determine which proteins in tick SGE and saliva are chemoattractants for *B. burgdorferi* s.s., we fractionated the proteins and tested the fractions in an *in vitro* capillary tube chemotaxis assay. Because tick saliva can only be collected in small amounts and at low concentrations, we utilized SGE for these experiments. Using anion exchange medium pressure chromatography, we fractionated SGE into 4 fractions (Fig. S2). *B. burgdorferi* s.s. seemed migrated toward one of these fractions, Fraction 3, more consistently than other fractions and had the largest difference between wildtype and *cheX* mutant *B. burgdorferi* s.s. (Fig. 2). This was not a statistically significant difference.

Proteomic analysis of Fraction 3 revealed 70 significant hits shared by two replicate protein fractions (Table S1). As this is a large number of hits, we narrowed this list by comparing the significant hits to a database of proteins known to be in tick saliva (Kim, Tirloni et al. 2016). Because tick saliva is produced in the salivary glands and also contains chemoattractant proteins, it is likely that the chemoattractants identified in tick saliva and SGE are overlapping. However, this comparison is more likely to identify proteins involved in acquisition, as tick saliva is encountered by the spirochete at the tick bite site during tick feeding. During transmission, spirochetes exit the tick with the tick saliva but there is not an obvious gradient of tick saliva that would be encountered in this process. This comparison

yielded 15 proteins (Table 2). We then prioritized this list by significance of the hit and the presence of a secretion signal sequence to yield 5 candidates. The presence of a secretion signal sequence indicates that the protein could be secreted outside of the salivary gland cells at higher concentrations, which may make the protein better signal for chemotaxis. Due to its small size and our ability to purify it, we selected the protein from transcript ISCW024249 for testing. *B. burgdorferi* s.s. migration toward the recombinant protein was significant, indicating this protein is a chemoattractant for the spirochete (ANOVA, df=3, p=0.04, Fig. 3A). The spirochete failed to migrate toward purified empty vector, containing only the protein tag. Additionally, specificity of this interaction is further supported by the previously mentioned lack of migration toward an irrelevant protein, AAEL010228, produced in the same manner (Fig. 1C).

The B31A strains used in our *in vitro* experiments are noninfectious, laboratory adapted strains that genetically differ from infectious isolates. Therefore, it is possible that the chemotactic response of infectious isolates might differ. To assess if the protein from transcript ISCW024249 is also a chemoattractant for infectious *B. burgdorferi* s.s., we tested the protein in the capillary tube chemotaxis assay using an infectious background, B31A3 and B31A3 *cheX* (Table 1) (Fig. 3B). As expected, wild type *B. burgdorferi* s.s. B31A3 migrated toward the purified protein from transcript ISCW024249, but the *cheX* mutant did not (ANOVA, df=2, p=0.03). This demonstrates that the protein is a chemoattractant for an infectious isolate of *B. burgdorferi* s.s. and may contribute to the *in vivo* infectious cycle of the spirochete.

The protein encoded by transcript ISCW024249 is a small, 12.18 kDa protein, not previously assessed for function (Fig. S3). Analysis of the protein by blastp yielded proteins of undefined function and human colipase-like proteins (Table 3). Human colipase is a small, 10kDa, cofactor that functions to aid pancreatic lipase in the digestion and absorption of lipids and lipid soluble nutrients (Lowe 1997). However, further analysis of the ISCW024249 protein by InterPro did not reveal any predicted functions or known domains, including colipase domains, and alignment of the ISCW024249 protein to known colipases did not yield significant homology (data not shown). Therefore, we hypothesize that this protein is sufficiently different that it is likely to have a distinct function from human colipase. As there is not significant homology of this protein to proteins of known function, we will name the protein according to the nomenclature used for other *I. scapularis* salivary proteins (Salp), and throughout this manuscript the protein will be termed Salp12. This protein does not share homology with other previously identified Salp proteins (Das, Banerjee et al. 2001).

As other tick salivary gland proteins have been shown to bind *B. burgdorferi* s.s., we assessed if we could detect binding between Salp12 and the spirochete in an *in vitro* immunofluorescence assay (IFA) similar to IFAs previously used for Salp15 (Ramamoorthi, Narasimhan et al. 2005) and Ixofin3D (Narasimhan, Coumou et al. 2014). In this experiment, *in vitro* grown *B. burgdorferi* s.s. B31A was incubated with Salp12 and protein localization was detected through fluorescent antibodies (Fig. S4). We were unable to detect any binding between the spirochete and Salp12. Although some Salp proteins bind *B. burgdorferi* s.s., it is not surprising that binding was not detected between Salp12 and the

spirochete. *In vivo* chemotactic interactions are transient in nature, and therefore, interactions between *B. burgdorferi* s.s. and Salp12 may not be stable enough to be identified by an IFA. It is also possible that Salp12 interacts with secreted spirochete proteins that are then able to serve as chemoattractants or that the protein is cleaved by proteases prior to binding that prevent visualization.

Salp12 is expressed in the midguts and salivary glands of *I. scapularis* ticks.

To gain a better understanding of Salp12 expression and its role within the tick, we assessed *salp12* mRNA expression during the course of feeding in *I. scapularis* nymphs. *salp12* was detectable in both salivary glands and midguts throughout feeding, and expression in tick midguts was significantly higher than expression in tick salivary glands at every time point (Fig. 4A) (Mann-Whitney, $df=2$, $p=0.002$). To determine whether the expression of *salp12* is altered by the presence of *B. burgdorferi* s.s., we assessed *salp12* expression in infected and uninfected nymphs. These data show no difference in *salp12* expression between uninfected and *B. burgdorferi* s.s. infected salivary glands (Fig. 4B) or midguts (Fig. 4C).

Salp12 contributes to *B. burgdorferi* s.s. acquisition.

To assess if *B. burgdorferi* s.s. chemotaxis towards Salp12 is involved in acquisition, we performed RNAi knockdown experiments with *I. scapularis* nymphs (Fig. 5). Ticks received thoracic injection of dsRNA to knockdown expression in the salivary glands, and ticks were injected with ds *salp12* for knockdown or ds *gfp* as a control. RNAi knockdown of *salp12* was not complete and resulted in a 50% decrease of transcript levels in the salivary glands at 24 hours of feeding (Fig. 5A) (ANOVA, $df=1$, $p<0.05$). Uninfected nymphs were injected and fed to repletion on *B. burgdorferi* s.s. B31A3 infected mice. We first assessed if knockdown of *salp12* impacted tick feeding, which could alter acquisition. There were no significant differences in tick feeding, as measured by engorgement weight (Fig. 5B) and time to repletion (Fig. 5C). *B. burgdorferi* s.s. burdens in replete ticks injected with ds *salp12* trended lower than ds *gfp* infected ticks, but this was not statistically significant (Fig. 5D, Mann-Whitney test, $df=1$, $p=0.15$).

There are two distinct steps for *B. burgdorferi* s.s. acquisition. Spirochetes enter the tick midgut during tick feeding and then colonize the midgut to be maintained during molting. Therefore, to gain a more nuanced view of the acquisition process, we assessed the impact of Salp12 on spirochete entry (after 24 hours of tick attachment) and on colonization (7 days post detachment) in *I. scapularis* nymphs (Fig. 5E). The 24 hour timepoint was chosen because Salp12 is most highly expressed at 24 hours (Fig. 4A) and would therefore likely have the largest impact at this time. Spirochete entry into the ds *salp12* injected ticks trended lower than in ds *gfp* injected ticks at 24 hours, but this was not statistically significant (Mann-Whitney test). By seven days post detachment, the spirochete burden in the midgut was the same between control and ds *salp12* injected ticks. The trend in the data suggests that Salp12 contributes to spirochete entry but does not impact maintenance or growth of the spirochete in the tick midgut after entry.

To circumvent the incomplete knockdown of Salp12 by RNAi, we utilized passive immunization to block Salp12-spirochete interactions and assessed acquisition *I. scapularis*

nymphs (Fig. 6). Antibodies against Salp12 or control antibodies against ovalbumin were transferred into mice infected with *B. burgdorferi* s.s. B31A3, and acquisition was assessed. Ticks fed on mice immunized against Salp12 had a decrease in spirochete entry at 24 hours (Mann-Whitney test, $df=2$, $p<0.03$). There was also a decrease in spirochete burden at 7 days post detachment (not statistically significant, Mann-Whitney test), indicating that colonization of the spirochete is affected. Similar to the RNAi experiments, these data suggest that Salp12 contributes to spirochete entry during tick feeding but blocking Salp12 interactions with the spirochete does not completely prevent acquisition.

Chemotaxis Toward Salp12 is not involved in transmission of the spirochete.

Because Salp12 has a role in acquisition and signals for acquisition and transmission are likely different, we hypothesized that Salp12 would not have a role in transmission. To confirm this, we performed transmission studies with RNAi knockdown ticks using thoracic injection. There were no differences in tick feeding in ds *salp12* injected ticks compared to control ticks, as measured engorgement weight (Fig. 7A) and time to repletion (Fig. 7B). Replete ticks that were injected with ds *salp12* had significantly different burdens of *B. burgdorferi* s.s. in their midguts at repletion (Fig. 7C, Mann-Whitney test, $df=2$, $p<0.0001$). However, this difference was driven by one tick sample, indicating that transmission would likely not differ between ds *salp12* injected and control ticks. Differences in *B. burgdorferi* s.s. burden did not impact the level of transmission to mice, as measured by *B. burgdorferi* s.s. levels in the skin, heart, bladder, and joints (Fig. 7D, E). These data support that chemotaxis toward salivary gland Salp12 is not involved in transmission.

As there were differences in the level of spirochetes in replete nymphs, we next assessed if migration of the spirochete during transmission was impacted by knockdown of *salp12*. During feeding, spirochetes grow within the tick midgut for 36 hours and then migrate to the salivary glands. From the tick salivary glands, spirochetes are able to exit with the tick saliva. Because Salp12 is present in the tick salivary glands and midgut, it is possible that the spirochete is recognizing the protein for egress from the midgut or invasion of the salivary glands. If chemotaxis toward Salp12 is involved in transmission, a decrease in spirochete burden in the salivary glands would be observed. Knockdown experiments showed that levels of *B. burgdorferi* s.s. in the salivary glands were unchanged (Fig. 7G), indicating that knockdown of salivary gland Salp12 does not impact invasion of the salivary glands by the spirochete or migration through the tick. However, *B. burgdorferi* s.s. levels in the tick midgut trended higher than in control ticks (not statistically significant, Mann-Whitney test, Fig. 7F), indicating a possible role in egress or growth in the midgut. As spirochetes are still able to migrate to the tick salivary glands and infect mice, chemotaxis toward salivary gland Salp12 does not significantly contribute to transmission of the spirochete.

Discussion:

Our fractionation experiments demonstrate that proteins, and not sugars or other small molecules, are the chemoattractant compounds contributing to *B. burgdorferi* s.s. migration in the capillary tube chemotaxis assay and that Salp12 is a chemoattractant for *B. burgdorferi* s.s. However, it is important to note that there is likely a minimum necessary

concentration of chemoattractants to identify a response in the capillary tube assay. Therefore, it is possible that other chemoattract compounds may be present in *I. scapularis* SGE, fractionated SGE, and saliva that were not identified by this *in vitro* assay. Further analysis of higher concentrations of these fractions or in more sensitive assays may identify other chemoattracts. It is also of note that while *B. burgdorferi* s.s. migrated toward solutions of purified Salp12 in a reproducible and statistically significant manner, *B. burgdorferi* s.s. migration toward the SGE fraction containing Salp12 was modest and not statistically significant. This is likely due in part to the fact that a lower concentration of Salp12 would be in the protein fraction compared to the solution of purified Salp12.

Our data demonstrate that Salp12 is sufficient to induce chemotaxis of *B. burgdorferi* s.s. *in vitro*, and Salp12 contributes to spirochete entry during acquisition. However, knockdown of Salp12 or passive immunization against Salp12 did not completely prevent acquisition of the spirochete. For the RNAi experiments, this is at least partially due to incomplete knockdown of the protein, as RNAi only reduced transcription by 50%. Continued acquisition of the spirochete could also be explained by the presence of an additional chemoattractant in the tick saliva or a chemoattractant produced by wounding from tick feeding. Moreover, some acquisition likely occurs through passive processes, such as the spirochetes already residing within the tick bite site or migrating through the bite site at random.

Our RNAi and passive immunization studies differed in spirochete burden at 7 days post detachment. Spirochete colonization trended lower in ticks fed on mice passively immunized against Salp12 than on control mice, but this trend was not identified in RNAi experiments. This difference is likely due to passive immunization affecting Salp12 function in the midgut, where RNAi injections only target Salp12 in the salivary glands. In passive immunization, Salp12 antibodies will enter the tick with the blood meal and likely impair midgut Salp12, in addition to Salp12 in the skin. This indicates that midgut Salp12 may influence the midgut environment. It is unlikely that this phenotype is dependent on chemotaxis toward Salp12 because the trend was not observed in RNAi experiments.

As expected, our transmission data support that chemotaxis toward Salp12 does not play a significant role in transmission of the spirochete. However, we identified that Salp12 may affect egress from the tick midgut or growth within the midgut during transmission. As thoracic injection specifically decreases mRNA expression in the tick salivary glands and *salp12* levels are unchanged in the midgut, it is unclear how growth or egress are affected by RNAi injections. It is possible that changes in Salp12 expression in the salivary glands could alter global expression of tick proteins, which in turn alters the midgut environment. Together with the findings that Salp12 contributes to spirochete colonization after acquisition and that *salp12* is expressed in the midgut, these data suggest that Salp12 likely shapes the midgut environment.

Although the spirochete encounters Salp12 during acquisition and transmission, migration of the spirochete is only altered in acquisition, which highlights that the spirochete is sensing different signals for these processes. This is supported by the fact that although the environment within the tick is similar during acquisition and transmission, spirochetes migrate to the tick salivary glands during transmission but not acquisition. The difference in

spirochete response to a similar environment is likely due to divergent gene expression by the spirochete (de Silva and Fikrig 1997).

Identifying a protein as a chemoattractant is quite surprising, as chemoattractants identified for other bacteria are small molecules, such as sugars and short peptides. Additionally, the bacterial receptors for chemotaxis, methyl-accepting chemotaxis proteins (MCPs), have binding pockets that accommodate smaller molecules, and MCPs are localized to the inner-membrane of bacteria. The presence of the bacterial outer-membrane prevents direct interaction of the MCPs with extracellular molecules. The B31A strains used in this study have five predicted MCPs (Fraser, Casjens et al. 1997), all with structural similarity to previously identified MCPs (Fraser, Casjens et al. 1997, Xu, Raddi et al. 2011), and two of these MCPs have been shown to localize to the inner-membrane (Xu, Raddi et al. 2011). Therefore, *B. burgdorferi* s.s. must utilize a mechanism different from canonical systems for recognition of a large protein. There are two possible hypotheses. One is that the tick protein is autoproteolytic or cleaved by proteases, and the peptides are able to interact with the MCPs directly. A second possibility is that the intact tick protein interacts with an additional receptor to transduce a secondary signal to the MCPs. This hypothesis is supported by studies in other bacterial species that have demonstrated MCPs can respond to external chemical stimuli by binding internal signals (Ni, Huang et al. 2015, Machuca, Liu et al. 2016).

Conclusion:

Overall, our study suggests that chemotaxis contributes to acquisition in the infectious cycle of *B. burgdorferi* s.s. We hypothesize that Salp12 is deposited into the skin as part of the tick saliva, where it is recognized by resident *B. burgdorferi* s.s. This recognition contributes to migration of the spirochete into the tick feeding site, and the spirochete enters the tick along with the blood meal. However, our data also suggest that multiple signals may be recognized by the spirochete for acquisition, such as additional tick molecules or cues from wounding. It is possible that identification of additional chemoattractants and targeting these molecules in combination with Salp12 may effectively block pathogen acquisition. This strategy could be used for environmental control of the infectious cycle of vector-borne diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

SGE	Salivary gland extract
Salp12	<i>Ixodes scapularis</i> salivary gland protein of 12kDa

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Figure 1. *I. scapularis* salivary gland and saliva proteins are chemoattractants for *B. burgdorferi*.

The wild type (WT, black bars) and chemotaxis mutant (*cheX*, grey bars) of the *B. burgdorferi* isolate B31A were tested for *in vitro* chemotaxis towards potential chemoattractants. Data is shown as the relative chemotactic response (number of cells responding to the test sample divided by the number of cells responding to the buffer control). Chemotaxis was tested for *I. scapularis* salivary gland extract (SGE) (A, B), non-tick proteins (C) bovine serum albumin (BSA) and mosquito protein (AAEL010228), and *I. scapularis* tick saliva (TS) (D). SGE was separated into fractions greater than and less than 3 kDa (A). SGE and TS were also treated with sodium-metaperiodate (SMP) to remove sugars and proteinase K (PK) to remove proteins (B, D) to assess of which molecules are chemoattractants. Data are the average of 5 biological replicates each containing 3 technical replicates. Significance was tested using ANOVA.

Figure 2. Identification of chemoattractant fractions.

I. scapularis salivary gland proteins were fractionated using anion exchange medium pressure liquid chromatography. Fractions were tested using the *in vitro* chemotaxis assay for the *B. burgdorferi* isolate B31A, wild type (WT, black bars) and chemotaxis mutant (*cheX*, grey bars). Data are the average of 2 biological replicates each containing 3 technical replicates, and statistical significance was not identified using ANOVA.

Figure 3. The *I. scapularis* protein from isolate ISCW024249 is a chemoattractant for *B. burgdorferi*.

The recombinant *I. scapularis* protein from transcript ISCW024249 and an empty vector control were tested in the *in vitro* chemotaxis assay with the *B. burgdorferi* isolate B31A (A), wild type (WT, black bars) and chemotaxis mutant (*cheX*, grey bars). The protein was also tested with the infectious *B. burgdorferi* isolate B31A3 (B). Significance was tested using ANOVA.

Figure 4. *I. scapularis* protein Salp12 is expressed throughout tick feeding in both the salivary glands and midgut.

I. scapularis ticks were collected at 24, 48, or 72 hours of feeding and assessed for expression of Salp12 relative to tick actin in their salivary glands (SG, circles) or midguts (MG, squares) using absolute quantification qPCR. Expression levels were compared between tick organs (A). Transcript expression was also compared for SG (B) and MG (C) between uninfected (black) and *B. burgdorferi* infected (grey) samples. Each panel shows a representative experiment, one of two biological replicates. For each experiment at least five ticks were collected per time point. Significance was tested using a Mann-Whitney test.

Figure 5. Knockdown of Salp12 expression decreases *B. burgdorferi* acquisition by *I. scapularis* ticks.

Protein expression was knocked down in *I. scapularis* salivary glands using RNAi with *ds salp12* (white) or control dsRNA, *ds gfp* (black), which was measured in *I. scapularis* salivary glands (SG) and midgut (MG) (A). Knock down *I. scapularis* ticks were fed on *B. burgdorferi* infected mice to assess acquisition of the spirochete. The impact of *salp12* knockdown on tick feeding was measured by engorgement weight (B) and time to repletion (C). *B. burgdorferi* burden in replete tick midguts (D) was determined using absolute quantification qPCR of *flaB* relative to *tick actin*. Analysis of acquisition during the course of tick feeding (E) was determined by assess *B. burgdorferi* burden in the midgut at 24 hours and at 7 days post detachment. Data (A-D) is the average of 2 biological replicates, and panel E shows the average of 3 biological replicates. For B and C, statistical significance

was tested using ANOVA, and for A, D, and E, statistical significance was tested using a Mann-Whitney test.

Figure 6. Passive immunization against Salp12 decreases *B. burgdorferi* acquisition of by *I. scapularis* ticks.

Mice infected with *B. burgdorferi* were passively immunized with rabbit antibodies against Salp12 (black circles) or control antibodies against ovalbumin (OVA, white circles).

Uninfected *I. scapularis* ticks were fed on *B. burgdorferi* infected mice and removed at 24 hours or allowed to feed to repletion and incubated for 7 days. *B. burgdorferi* burden in tick midguts was measured by absolute quantification qPCR of *flaB* relative to *tick actin*. The graph shows an average of two biological replicates. Statistical significance was assessed using a Mann-Whitney test.

Figure 7. Knockdown of Salp12 expression does not alter *B. burgdorferi* transmission.

Expression of Salp12 in *I. scapularis* salivary glands was knocked down by RNAi with *ds salp12* (black), and control ticks were exposed to *ds gfp* (white). *I. scapularis* feeding was measured by tick engorgement weight (A) and time to repletion (B), and significance was not detected using ANOVA. *B. burgdorferi* burden in replete *I. scapularis* ticks (C) was measured by absolute quantification qPCR of *flaB* relative to *tick actin*, and significance was not detected using ANOVA. Transmission from knockdown *I. scapularis* ticks was measured by qPCR of *flaB* relative to *mouse actin* in mouse skin (D) or organs (E), significance was not detected using a Mann-Whitney test. Assessment of *B. burgdorferi* burden during the course of feeding was measured by absolute quantification qPCR of tick midguts (F) and salivary glands (G), significance was not detected using a Mann-Whitney test. Data for tick feeding and *B. burgdorferi* burden shows the average of two biological replicates (A-C, F-

G). Data for transmission experiments (D-E) is shown by a representative experiment, one of two biological replicates.

Table 1.**Bacterial Strains and Plasmids^a**

Bacterial Strain Name	Description
<i>B. burgdorferi</i> s.s. B31	Non-infectious derivative of strain B31A3
<i>B. burgdorferi</i> s.s. B31:: <i>cheX</i>	<i>B. burgdorferi</i> s.s. B31 with knockout mutation of the <i>cheX</i> gene (Motaleb, Miller et al. 2005)
<i>B. burgdorferi</i> s.s. B31A3	Infectious strain B31A3
<i>B. burgdorferi</i> s.s. B31:: <i>cheX</i>	<i>B. burgdorferi</i> s.s. B31A3 with knockout mutation of the <i>cheX</i> gene (Motaleb et. al, unpublished)
<i>E. coli</i> Top 10	<i>Escherichia coli</i> strain used for cloning
Plasmid Name	Description
pMT/V5-His A	Plasmid used for cloning
pMT/V5-His A:: <i>salp12</i>	pMT/v5-His A containing <i>salp12</i>
pMT/V5-His A::AAEL010228	pMT/V5-His A containing <i>AAEL010228</i>

^aTable of bacterial strains and plasmids used in this study.

Table 2.Tick proteins identified by protein fractionation and comparison to tick saliva protein database^a

Protein ID ^b	Predicted Function and Transcript Name ^c	Signal Sequence ^d
B7Q407	Heme lipoprotein, ISCW021710	Yes
B7Q9F1	Protein disulfide isomerase, ISCW010827	Yes
B7Q406	Hemelipoglycoprotein, ISCW021709	Yes
B7P4U1	Protein disulfide isomerase, ISCW016161	Yes
B7PFA2	Is6 putative, ISCW024249	Yes
B7PEV0	Chaperonin subunit, ISCW017824	No
B7QI01	Hsp90 protein, ISCW014265	No
B7QGE3	Hemelipoglycoprotein, ISCW012424	No
B7QJH6	Alpha-actinin, ISCW013566	No
B7PAR6	Heat shock protein, ISCW017456	No
B7PBG2	Actin putative, ISCW024111	No
B7P1Q2	Myosin heavy chain, ISCW001340	No
B7PQC5	60S acidic ribosomal protein P1, ISCW006538	No
Q8MVB0	Truncated secreted metalloprotease	No
B7P1Z8	Heat shock protein, ISCW016090	No

^aTable of identified proteins shared in two proteomics analyses of chemoattractant *I. scapularis* tick protein fractions and a *I. scapularis* tick saliva protein database (Kim, Tirloni et al. 2016)

^bID of protein as found in the VectorBase database

^cPredicted function based on bioinformatics analysis of VectorBase and the transcript name from the database

^dPresence (Yes) or absence (No) of a signal sequence as detected by SignalP software

Table 3.BLAST hits for the protein from transcript ISCW024249^a

Accession Number ^b	Predicted Function ^c	Percent Identity ^d	E-value ^e	Bit Score ^f
AAY66501.1	putative secreted salivary gland protein [<i>Ixodes scapularis</i>]	47.8%	5.1E-10	62.4
CAX51408.1	hypothetical protein [<i>Opisthacanthus cayaporum</i>]	30.6%	6.7E-09	59.3
EEC14111.1	secreted salivary gland protein, putative [<i>Ixodes scapularis</i>]	40.6%	3.9E-06	52
KPM07042.1	hypothetical protein QR98_0055240 [<i>Sarcoptes scabiei</i>]	34.6%	2.1E-04	47.8
XP_023241637.1	colipase-like [<i>Centruroides sculpturatus</i>] uncharacterized protein LOC106813686	31.1%	2.9E-04	47
XP_014673379.1	[<i>Priapulus caudatus</i>] uncharacterized protein LOC111634649	33.3%	3.8E-04	47.4
XP_023235249.1	[<i>Centruroides sculpturatus</i>]	33.3%	4.9E-04	46.6
OTF74413.1	hypothetical protein BLA29_000290 [<i>Euroglyphus maynei</i>]	32.3%	2.0E-03	45.4
EEC14112.1	secreted protein, putative [<i>Ixodes scapularis</i>]	31.4%	5.0E-03	43.5
XP_020024523.1	colipase-like [<i>Castor canadensis</i>]	36.5%	7.0E-03	43.1

^aTable of the top ten BLASTP hits for the protein of transcript ISCW024249^bAccession number of protein as found in GenBank^cPredicted function based on the submission to GenBank. The species that the hit came from is found in the brackets.^dPercent identity of the protein hit to the protein of transcript ISCW024249^eE-value of the match between the hit and the protein of transcript ISCW024249^fBit score of the match between the hit and the protein of transcript ISCW024249

Table 4.**Primers**

Primer Name^a	Sequence^b
<i>Salp12</i> _cloning_F	GAT AT CGACATGGCCGCGTGGCCGGATGACGAT AA
<i>Salp12</i> _cloning_R	CTCGAGGTCGTCCTTATCTTCGTCGGTGTTT
010228_F	CTCGCTCGGGAGATCTAGGCCAACTCCCGAAGACGATGGTGGTACCA
010228_R	GCCCTCTAGACTCGAGTGATCTATTGGAAGCAACGCAAGACG
<i>Salp12</i> _qPCR_F	AAGGTTCTCGTAGTCCTCTACTT
<i>Salp12</i> _qPCR_R	TGGCCACTTGCTGTATGTTAT
tick_actin_qPCR_F	GGCGACGTAGCAG
tick_actin_qPCR_R	GGTATCGTGCTCGACTC
mouse_actin_qPCR_F	AGCGGGAAATCGTGCGTG
mouse_actin_qPCR_R	CAGGGTACATGGTGGTGCC
flaB_qPCR_F	TTCAATCAGGTAACGGCACA
flaB_qPCR_R	GACGCTTGAGACCCTGAAAG
dsRNA_GFP_F	TAATACGACTCACTATAGGGGCGACGTAAACGGCCACAAGTT
dsRNA_GFP_R	TAATACGACTCACTATAGGGAGAGGGTCTTGTAGTTGCCGTC
dsRNA_ <i>Salp12</i> _F	TAATACGACTCACTATAGGGAGACAGGACCACGAGGACAGG
dsRNA_ <i>Salp12</i> _R	TAATACGACTCACTATAGGGAGATTAACGTGACACTGGGCAGG

^a Descriptive name of the primer that includes the gene name and use.

^b Nucleic acid sequence of the primer.