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Fucosylation enhances colonization of ticks by *Anaplasma phagocytophilum*

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Abstract

Fucosylated structures participate in a wide range of pathological processes in eukaryotes and prokaryotes. The impact of fucose on microbial pathogenesis, however, has been less appreciated in arthropods of medical relevance. Thus, we used the tick-borne bacterium *Anaplasma phagocytophilum* – the agent of human granulocytic anaplasmosis to understand these processes. Here we show that *A. phagocytophilum* uses α1,3-fucose to colonize ticks. We demonstrate that *A. phagocytophilum* modulates the expression of α1,3-fucosyltransferases and gene silencing

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. α1,3-Fucosyltransferases present in the *I. scapularis* genome.

A. Comparison of signature motifs among several α1,3-fucosyltransferases: *Bm* {EST777694} *Rhipicephalus* (*Boophilus*) *microplus* (cattle tick); *Am* {CAK50252; CAK50261} *Apis mellifera* (honeybee); *Dm* {NP_648754; NP_001036320} *Drosophila melanogaster*; *Aa* {XP_001661902} *Aedes aegypti* (yellow fever mosquito); *Mm* {NP_034372} *Mus musculus* (mouse) and the expressed sequence tag *isfucot* (*isft7*) from *I. scapularis* database {ti|1681614834}.

B. Ten α1,3-fucosyltransferases were detected in the genome of the tick *I. scapularis*. Bioinformatics analysis showed the presence of highly conserved α1,3-fucosyltransferase signature motifs (blue) across all tick α1,3-fucosyltransferases – *isft1* (ISCW004192); *isft2* (ISCW003590); *isft3/9* (ISCW004236); *isft4* (ISCW024758); *isft5* (ISCW024943); *isft6* (ISCW024303); *isft7* (ISCW023318); *isft8* (ISCW024461); *isft10* (ISCW05151).

Fig. S2. siRNA specificity for α1,3-fucosyltransferases associated with *A. phagocytophilum* colonization of ticks.

A. Two siRNAs for *isft1* (blue), *isft2* (red) and *isft3* (green) were designed to determine the importance of these genes during *A. phagocytophilum* acquisition. The siRNAs shown above do not have any similarity with any other gene in the *I. scapularis* genome and are highly specific for *isft1*, *isft2* and *isft3*. Asterisks represent nucleotide similarity between sequences.

B. Two overlapping specific siRNAs for *isft4* (green box) were designed to determine the importance of this gene during *A. phagocytophilum* acquisition. Bold sequence differentiates siRNA-1 from siRNA-2. Yellow highlight indicates primers used for amplication of the *isft4* gene. Red highlight indicates primer used for qPCR detection during experimental analysis.

Table S1. List of primers.

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significantly reduces colonization of tick cells. Acquisition but not transmission of *A. phagocytophilum* was affected when α1,3-fucosyltransferases were silenced during tick feeding. Our results uncover a novel mechanism of pathogen colonization in arthropods. Decoding mechanisms of pathogen invasion in ticks might expedite the development of new strategies to interfere with the life cycle of *A. phagocytophilum*.

Introduction

Glycan structures are present in cells and are added to lipids and protein structures through glycosylation (Staudacher *et al*., 1999; Ma *et al*., 2006; van Kooyk and Rabinovich, 2008). Glycosylation plays a role in enzymatic activity and stability, protein solubility and the immune response. Many of the glycans relevant to these processes contain fucose residues; indeed, the addition of this deoxyhexose appears to be an important step for a number of biological and pathological phenomena (Staudacher *et al*., 1999; Altmann *et al*., 2001; Ma *et al*., 2006). Fucosylation is involved in organogenesis, inflammation, cancer, cell adhesion and microbial pathogenesis; α1,2-fucose forms part of the ABO blood group antigens, whereas α1,3-fucosyl modifications include the mammalian Lewis-type epitopes. In microbial pathogenesis, α1,2-fucose epitopes serve as ligands tethering *Salmonella* and *Campylobacter* to the intestinal mucosa (Ruiz-Palacios *et al*., 2003; Chessa *et al*., 2009). On the other hand, Lewis type mimicry plays a role in gastric colonization by *Helicobacter pylori* (Moran, 2008) and the same function has been suggested for the sialyl Lewis^x expressed on the cell surface of an oral bacterium associated with endocarditis (Hirota *et al*., 1995). Sialic acid binding is required for infectious cell surface receptor recognition by parvovirus (Nam *et al*., 2006), and Norwalk virus – a norovirus that causes non-bacterial gastroenteritis – binds to Lewis type antigens (Hutson *et al*., 2003).

Structural analysis of *N*-glycans from arthropods reveals that these molecules can be fucosylated in three different ways: fucosylation in α1,6 linkage to the innermost *N*-acetylglucosamine (GlcNAc); fucosylation in α1,3 linkage to the proximal GlcNAc residue; and fucosylation in α1,3 linkage to the precursor glycan LacdiNAc (*N*-acetylgalactosamine linked to β1,4- to *N*-acetylglucosamine) to yield a Lewis-like structure (GalNAcβ-1,4{Fucα-1,3}GlcNAcβ-R), found, for instance, on honeybee phospholipase (Staudacher *et al*., 1999; Paschinger *et al*., 2005). The poorly understood role of fucose in arthropods provided further impetus to investigate the importance of this carbohydrate during microbial colonization of ticks. We used the tick-borne pathogen *Anaplasma phagocytophilum* as a model organism to understand fucosylated-mediated processes in arthropods. *A. phagocytophilum* is transmitted by ticks of the genus *Ixodes*, infects neutrophils and causes human granulocytic anaplasmosis – the second most common tick-borne disease in the USA. We hypothesized that *A. phagocytophilum* would serve as a good model of infection because *A. phagocytophilum* resides in *Ixodes scapularis* salivary glands; and requires both sialylation and fucosylation in mammalian cells for cellular invasion (Goodman *et al*., 1999; Herron *et al*., 2000; Yago *et al*., 2003; Reneer *et al*., 2006; 2008; Sarkar *et al*., 2007). Our findings show that *A. phagocytophilum* modulates the expression of three α1,3-fucosyltransferases and uses α1,3-fucosylation to colonize ticks.

Results

Core α1,3-fucosylated structures are present in ticks

To gain a better understanding of the importance of fucose for microbial pathogenesis in arthropods, we studied the colonization of ticks by *A. phagocytophilum*. We first determined whether the tick I. scapularis had fucosylated *N*-glycans on the surface of proteins. Mass spectrometry analysis showed the presence of fucose in engorged nymphal *I. scapularis*

salivary glands (Fig. 1A and B). The *N*-glycan profile revealed oligosaccharides of lower complexity, most notably a series of oligo- and paucimannosidic structures. Only minor amounts of hybrid, bi- and tri-antennary complex glycans were observed in the *N*-glycan spectra. Then we probed salivary gland extracts from engorged nymphal ticks with an anti-horseradish peroxidase (HRP) antibody that recognizes the sugar epitope of core α1,3-fucose to assess whether *N*-glycan structures carrying core α1,3-fucose were present. We detected several proteins carrying core α1,3-fucose residues in *I. scapularis* salivary glands and midguts (Fig. 1C – panel B; lanes SG; MG). Core α1,3-fucose residues were also observed in *Drosophila* (Fig. 1C – panel B; lane Dros). However, core α1,3-fucose residues were not detected in the human promyelocytic cell line HL-60 (Fig. 1C – panel B; lane HL60).

α1,3-Fucose is required for *A. phagocytophilum* colonization of ticks

To determine whether *A. phagocytophilum* would use α1,3-fucosylated glycans to colonize *I. scapularis* ticks, we first searched for putative α1,3-fucosyltransferase homologues in the expressed sequence tag (EST) database of vectorbase. VectorBase (http://www.vectorbase.org) is a bioinformatics resource centre focused on invertebrate vectors of human pathogens (Lawson *et al*., 2009). Bioinformatics analysis revealed the occurrence of one *I. scapularis* EST (ti|1681614834) displaying homology to insect α1,3-fucosyltransferases and encoding conserved motifs for α1,3-fucosyltransferases (Fig. 2A; Fig. S1). This α1,3-fucosyltransferase sequence was later annotated as the corresponding *isft7* gene. To assess whether silencing of α1,3-fucosyltransferases would affect *A. phagocytophilum* colonization of ticks, a corresponding dsRNA was synthesized. The dsRNA covered the core and the conserved motif II region of the *I. scapularis* EST displaying homology to insect α1,3-fucosyltransferases. It was assumed that a dsRNA targeting this ‘conserved’ region would affect the expression of multiple fucosyltransferases. We placed control and α1,3-fucosyltransferase dsRNA-injected *I. scapularis* nymphs on *A. phagocytophilum*-infected mice and allowed naïve ticks to feed for 66–72 h. Changes in fucosylation at the protein level were observed when fucosyltransferases were silenced (Fig. 2B; anti-HRP; lane 2). These results were specific for α1,3-fucosyltransferase silencing because we did not observe any changes in fucosylation when a control *salp25d* dsRNA was used (Fig. 2B; anti-HRP; lane 1). Further, immunoblots of salivary gland protein extracts with anti-Salp25D showed that Salp25D was silenced in the control group (Fig. 2B; anti-Salp25D; lane 1) but not in the α1,3-fucosyltransferase dsRNA treatment (Fig. 2B; anti-Salp25D; lane 2). Immunoblot with rabbit anti-tick immune serum served as a loading control and showed that the protein amounts were similar in control and α1,3-fucosyltransferase dsRNA groups (Fig. 2B; anti-tick; lanes 1 and 2). Engorgement was not affected by silencing of α1,3-fucosyltransferases ($P = 0.78$) (Fig. 2C).

We then determined *A. phagocytophilum* load in mock and dsRNA-injected nymphs. *A. phagocytophilum* load in the salivary glands of dsRNA-injected nymphs was lower when compared with the control treatment (fold difference = 1934; $P = 0.02$) (Fig. 2D). This effect seemed specific for *A. phagocytophilum* because acquisition of the unrelated pathogen *Borrelia burgdorferi* – the agent of Lyme disease – was not dependent on core α1,3-fucosylation ($P = 0.42$) (Fig. 2E). With the data from the ongoing *I. scapularis* genome project now available on vectorbase (Lawson *et al*., 2009), we discovered using TBLASTN that a total of 10 putative fucosyltransferases are present in the *I. scapularis* genome (including the afore-mentioned homologue first identified as an EST and used as a basis for the dsRNA analysis) (Fig. S1B). Therefore, we designed specific siRNAs for the 5′ region of *isft1*, *isft2* and *isft3* (Fig. S2). We chose this region because the N-terminal non-catalytic domain is dissimilar among α1,3-fucosyltransferases (Ma *et al*., 2006). Confocal microscopy indicated that silencing of these α1,3-fucosyltransferases reduced core α1,3-fucosylation in the salivary gland and the midgut of *I. scapularis* ticks when the three siRNAs were used in

combination (Fig. 2H and K). Importantly, core α1,3-fucosylation was not affected when an unrelated gene – *salp25d* – was silenced (Fig. 2G and J).

Transfection of the dsRNA targeting the conserved α1,3-fucosyltransferase sequence into cells originating from *Ixodes ricinus* also decreased the expression of α1,3-fucosyltransferases in the absence (fold difference = 17; $P < 0.01$) and presence of *A. phagocytophilum* – (fold difference = 12; $P < 0.01$) (Fig. 3A). Reduction of α1,3-fucosyltransferase expression significantly reduced the capacity of *A. phagocytophilum* to colonize *I. ricinus* cells (fold difference = 3; $P < 0.05$) (Fig. 3B). These results were confirmed using confocal microscopy, in which *A. phagocytophilum* load was reduced post infection (Fig. 3E). Core 1,3-fucosylation is present in *I. ricinus* (Fig. 3F; lane B); and α1,3-fucosyltransferases in *I. scapularis* and *I. ricinus* share high levels of homology (Fig. 3G).

Transmission of *A. phagocytophilum* does not require α1,3-fucosylation

As α1,3-fucosylation was important for *A. phagocytophilum* acquisition in ticks, we determined if pathogen transmission was also affected. Nymphal ticks were injected with dsRNA targeting either *salp25d* (as control) or the conserved sequence of α1,3-fucosyltransferases. Ticks attached on mice for 24 h displayed low bacterial replication within salivary glands and were not able to transmit *A. phagocytophilum* (data not shown). On the other hand, ticks placed on mice for 72 h had higher levels of *A. phagocytophilum* within the salivary glands and transmitted the pathogen to the mammalian host (Fig. 4B). *A. phagocytophilum* bacterial load during tick transmission was not dependent on core α1,3-fucosylation. Bacterial load in mice was similar at days 5 ($P = 0.26$), 10 ($P = 0.57$) and 15 post infection ($P = 0.29$) (Fig. 4B). Similar to the acquisition studies, *B. burgdorferi* transmission was not dependent on α1,3-fucosylation (Fig. 4C).

A. phagocytophilum increases the expression of specific α1,3-fucosyltransferases in ticks

As a total of 10 α1,3-fucosyltransferase homologues could be defined by TBLASTN searching, we were prompted to investigate which of these genes were important for *A. phagocytophilum* infection. We hypothesized that *A. phagocytophilum* would modulate the expression of target genes for its own benefit. This hypothesis was based on our previous analysis showing that the expression of the tick salivary gland protein gene *salp16* (Sukumaran *et al*., 2006) – an essential gene for *A. phagocytophilum* survival within *I. scapularis* – was dramatically increased in *A. phagocytophilum*-infected ticks during feeding. We did not detect any expression of the tick α1,3-fucosyltransferases *isft5, isft6* and *isft8* during larval acquisition of *A. phagocytophilum* or *isft1*, *isft5*, *isft6* and *isft8* during nymphal transmission of *A. phagocytophilum*. Interestingly, *isft2* and *isft3* at 24 h and *isft1* and *isft2* at 48 h showed increased levels of transcription during *A. phagocytophilum* acquisition by *I. scapularis* (Fig. 5A and B). The increase in transcription for the genes *isft1*, *isft2* and *isft3* was also observed during *A. phagocytophilum* infection of *I. ricinus* cell culture (Fig. 5E). This effect was specific for acquisition, because during transmission of *A. phagocytophilum* by infected nymphs to naïve mice, *isft1*, *isft2* and *isft3* expression levels were comparable to that in uninfected nymphs (Fig. 5C and D).

Silencing of *isft1*, *isft2* and *isft3* reduces *A. phagocytophilum* acquisition during tick infection

As *A. phagocytophilum* infection significantly influenced *isft1*, *isft2* and *isft3* expression, we determined the relative importance of each of these genes for infection of ticks. We silenced each individual gene. The combination of siRNAs for *isft1*, *isft2* and *isft3* was most effective in reducing *A. phagocytophilum* colonization of tick cells (Fig. 6C and G). However, silencing of *isft1*, *isft2* and *isft3* individually also reduced *A. phagocytophilum* colonization (Fig. 6D–F and G). Importantly, the transfection of siRNA specific for *isft4* (Fig. S2B) – an

α1,3-fucosyltransferase that did not show altered expression during *A. phagocytophilum* infection – did not have any effect on *A. phagocytophilum* colonization of ticks (Fig. 6H).

Discussion

A number of human pathogens colonize mammalian cells through fucosylated structures. However, it has been poorly defined how fucosylation contributes to pathogen colonization of arthropods. We studied how the tick-borne pathogen *A. phagocytophilum* colonizes arthropods. We showed that the salivary glands of ticks carry core α1,3-fucosylated structures in *N*-glycans. Fucosylated *N*-glycans were analysed as opposed to fucosylated *O*-glycans structures because system-wide analysis for *O*-glycoproteins remains a challenge due to their structural heterogeneity and the lack of specific tools (Schwientek *et al*., 2007; Hanisch *et al*., 2009). Our results revealed a contrast of fucosylation patterns in mammalian versus arthropod cells. While core α1,3-fucosylation – the common pentasaccharide consisting of two *N*-acetylglucosamine and three mannose residues present in *N*-linked oligosaccharides – was commonly present in arthropods, this sugar structure was not detected in human cells. Further, our mass spectrometry analysis with ticks only revealed the presence of oligosaccharides of lower complexity, as opposed to the presence of complex sugar structures commonly observed in mammalian cells.

In our studies, we reasoned that *A. phagocytophilum* would serve as a valuable model to investigate the contribution of fucosylation for microbial pathogenesis in arthropods because: (i) *A. phagocytophilum* resides in two different locations during its life cycle – neutrophils and *I. scapularis* salivary glands, and (ii) *A. phagocytophilum* uses sialylation and fucosylation to colonize mammalian cells (Goodman *et al*., 1999; Herron *et al*., 2000; Yago *et al*., 2003; Reneer *et al*., 2006; 2008; Sarkar *et al*., 2007). Here we provided strong evidence that *A. phagocytophilum* (i) modulates the expression of three α1,3-fucosyltransferases and (ii) uses α1,3-fucose to colonize ticks during acquisition. These findings seem specific for *A. phagocytophilum* because colonization by *B. burgdorferi* was not affected when α1,3-fucosylated structures were reduced in ticks.

Our findings were somewhat surprising as independent groups have demonstrated that *A. phagocytophilum* uses the tetrasaccharide sialyl Lewis^X to colonize mammalian cells; and sialyl Lewis^X is usually attached to *O*-glycans (Goodman *et al*., 1999; Herron *et al*., 2000; Yago *et al*., 2003; Reneer *et al*., 2006; 2008; Sarkar *et al*., 2007). We suggest that this discrepancy may simply be an evolutionary adaptation acquired by *A. phagocytophilum* to retain optimal cell interaction in both arthropods and mammals. At least two lines of evidence point towards this hypothesis. First, we could not detect the presence of sialic acid in the *N*-glycome of tick salivary glands; such residues are generally rare in invertebrates. On the contrary, our results only revealed the presence of oligosaccharides of lower complexity. A second line of evidence pointing towards *A. phagocytophilum* plasticity is the presence of another type of fucosylation in ticks that is not generally present in mammals – core α1,3-fucosylation. Core α1,3-fucosylation has been described in arthropods, plants and parasites, but it is absent in mice and humans (Ma *et al*., 2006).

In humans, *A. phagocytophilum* uses platelet selectin glycoprotein-1 (PSGL1) to infect neutrophils, thereby, 'hijacking' a molecule involved in the inflammatory response. Both α-2,3-sialylation and α1,3-fucosylation of PSGL1 are essential for successful entry (Goodman *et al*., 1999; Herron *et al*., 2000). Interestingly, *A. phagocytophilum* readily infects *psgl1*-deficient mice demonstrating that murine PSGL1 does not play a role similar to its human counterpart (Carlyon *et al*., 2003). Mice lacking α1,3-fucosylation, however, are refractory to *A. phagocytophilum* infection. *A. phagocytophilum* adhesins required for binding of α1,3-fucosylated receptors during colonization of ticks and mammals have not

been identified. The current model of pathogen–cell interaction suggests that *A. phagocytophilum* expresses at least two adhesins to bind human and mouse receptors (Carlyon *et al*., 2003; Yago *et al*., 2003; Reneer *et al*., 2006; 2008; Sarkar *et al*., 2007; Sarkar *et al*., 2008; Troese *et al*., 2009). One adhesin might bind to α1,3-fucosylated and α2,3-sialylated glycans; the second adhesin may bind to an N-terminal peptide component of human PSGl-1 or a mouse orthologue. It was hypothesized that *A. phagocytophilum* adhesins function cooperatively to achieve maximal binding and tyrosine sulfate residues as well as sialyl Lewis^x on a particular *O*-glycan are of utmost importance to retain optimal cell interaction. Our findings add new features to this model. Our results suggest that *A. phagocytophilum* exploits core α1,3-fucose on *N*-glycans of ticks, rather than Lewis-type antennal fucosylation. Despite all this evidence indicating the importance of *N*-glycan fucosylation for *A. phagocytophilum* colonization of ticks, our results cannot completely rule out the use of *O*-glycans during infection of ticks. The Mucin-type *O*-glycan core 1 structure has been found in the model organism *Drosophila* (Aoki *et al*., 2008). Therefore, this glycan or a variant of this glycan may be present and used by *A. phagocytophilum* during vector infection.

The fucosylated receptor that *A. phagocytophilum* uses to colonize ticks remains unknown. Interestingly, the phenotype related to another important tick protein during *A. phagocytophilum* infection – Salp16 (Sukumaran *et al*., 2006) – may be linked to fucosylation. Salp16 shows potential sites for O- and N-glycosylation in its sequence and glycans may be modified with fucose residues during glycoprotein processing. Similar to the phenotype observed with α1,3-fucosyltransferases in ticks, *salp16* shows upregulation during infection and the silencing of *salp16* reduced *A. phagocytophilum* colonization in tick salivary glands. Future studies should elucidate the intricate mechanisms of *A. phagocytophilum* colonization in ticks, and provide similarities and contrasts with infection of mammalian cells; but fucosylation is certainly a critical element for *A. phagocytophilum* infection of mammals and arthropods, and it would be most interesting to understand these mechanisms.

A better understanding of how *A. phagocytophilum* interferes with α1,3-fucosylation may lead to the development of novel therapeutic interventions against human granulocytic anaplasmosis. The disruption of the *A. phagocytophilum*–α1,3-fucosylation interaction in ticks, the inhibition of tick α1,3-fucosyltransferases, and the development of vaccination against core α1,3-fucosylated proteins based on antibody-mediated killing mechanisms are just a few important strategies to consider when developing novel prophylaxis against *A. phagocytophilum* infection. Our work adds a new facet to the understanding of tick-borne diseases. We provide a better understanding of the glycoconjugates required for *A. phagocytophilum* colonization of the tick *I. scapularis*. Glycans participate in many biological processes such as bacterial cell adhesion, molecular trafficking, receptor activation, signal transduction and endocytosis. These data may be applicable to other tick-borne infections and help to uncover molecular mechanisms that govern the physiology and pathology of rickettsial infections.

Experimental procedures

Ethics statement

Animals were housed in the Animal Resources Facility at Yale University School of Medicine according to the guidelines described under the federal Animal Welfare Regulations Act. Food and water were provided and all animal procedures were approved by the Institutional Animal Care and Use Committee of Yale University.

Tick acquisition and transmission, pathogens and *I. ricinus* cell line

Ixodes scapularis was obtained from the Connecticut Agricultural Experimental Station (New Haven, CT). Ticks were reared at 26°C with 85% relative humidity and a daily photoperiod of 14 h light and 10 h dark. The *I. ricinus* (L.) cell line IRE/CTVM19 (Bell-Sakyi *et al*., 2007) was maintained in L-15 (Leibovitz) medium supplemented with 10% tryptose phosphate broth, 20% fetal calf serum, 2 mM L-glutamine, 100 units ml^{-1} penicillin and 1 μg ml^{-1} streptomycin in ambient air at 30°C. *A. phagocytophilum* strain NCH1 and *B. burgdorferi* strain N40 were used.

To isolate *I. ricinus* α1,3-fucosyltransferases orthologous to those of *I. scapularis*, primers flanking the conserved region of these genes were used. α1,3-Fucosyltransferase 7 PCR products were ligated into pGEM-T Easy vector and 15 clones sequenced. For *I. ricinus* α1,3-fucosyltransferase 2 isolation, *isft2* forward and reverse primers were used to obtain the amplicon. The PCR product was cloned into pGEM-T Easy vector and four clones were sequenced. α1,3-Fucosyltransferase 7 and α1,3-fucosyltransferase 2 were amplified from *I. ricinus* cDNA originated from the IRE/CTVM19 cell line. *I. ricinus* α1,3-fucosyltransferase 7 was 99% identical to the conserved region (nucleotide position 452–561) of *isft7* (XM_023318). α1,3-Fucosyltransferase 2 from *I. ricinus* shared 96% identity (nucleotide position 1–189) with the fucosyltransferase I. *scapularis* 2 (*isft2*) (XM_002401196). All primers used throughout this study are available on Table S1.

Acquisition and transmission studies were carried out as previously described (Pedra *et al*., 2006; Narasimhan *et al*., 2007). Experiments to address acquisition were performed four times with 14 mice used per group and 10–12 ticks per mouse. Pathogen-free *I. scapularis* nymphs were injected through the integument with control or α1,3-fucosyltransferase dsRNA. Ticks were placed on infected mice and allowed to feed for 66–72 h. Salivary glands were analysed in pools of three, and midguts were analysed in pools of two. Results of a representative experiment are shown in the figures. For transmission experiments, naïve larvae were fed on infected C57Bl/6 (*A. phagocytophilum* transmission) or C3H/HeN (*B. burgdorferi* transmission) for 3–5 days and tested for pathogen acquisition – typically 90%. Infected larvae molted to nymphs and nymphs were used for transmission experiments. In experiments to address transmission, six mice were used per group and 8–10 ticks per mouse. Transmission experiments were repeated twice. Infected ticks injected with either *salp25d* or *α1,3-fucosyltransferase* dsRNA were placed on C57Bl/6 (*A. phagocytophilum* transmission) or C3H/HeN (*B. burgdorferi* transmission) mice. Ticks were allowed to feed for 24 or 72 h. Ticks were gently removed, and midguts and salivary glands were dissected and processed in pools of three salivary glands or two midguts for quantitative RT-PCR (qRT-PCR) analysis. A representative experiment is shown in the figures. *A. phagocytophilum* transmission was measured in the peripheral blood of mice at days 5, 10 and 15. *B. burgdorferi* transmission was measured in indicated organs at day 18 post infection.

dsRNA synthesis and injection

The 245 bp fragment of the α1,3-fucosyltransferase EST from *I. scapularis* with similarity to a signature motif for α1,3-fucosyltransferase (described in this study as corresponding to *isft7*) was cloned into the L4440 double T7 Script II vector (Fire *et al*., 1998). Gene-specific primers containing SacI and KpnI restriction sites were used in the PCR reaction. dsRNA complementary to the *isfucot* sequence was synthesized using the Megascript RNAi kit (Ambion, TX) and tick injections were performed as described (Narasimhan *et al*., 2007). Control ticks were injected with dsRNA isolation buffer (Ambion, TX) (mock), *salp25d* dsRNA or *salp25d* siRNA (Narasimhan *et al*., 2007).

siRNA construction and synthesis

siRNA target sites were chosen based on the guidelines available on http://www.ambion.com/techlib/misc/siRNA_design.html. The sequences for *salp25d*, *isft1*, *isft2*, *isft3* and *isft4* were compared with the *I. scapularis* genome (http://www.vectorbase.org) and the NCBI database using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) to eliminate sequences that had homology to unrelated coding sequences. The sequences of two sets of siRNAs for *salp25d*, *isft1*, *isft2*, *isft3* and *isft4* are described in Table S1. siRNAs were synthesized by *in vitro* transcription using the silencer siRNA construction kit (Ambion, TX). siRNAs were injected into the body of ticks as described for dsRNAs. Transfection of dsRNA or siRNAs in *I. ricinus* cells was performed according to RNAiFect transfection guidelines (Qiagen, CA).

dsRNA and siRNA transfection of *I. ricinus* cell lines

Ixodes ricinus cells were cultured *in vitro* as described earlier (Bell-Sakyi *et al*., 2007). Confluent cells were harvested and maintained at 30°C overnight into 12-well cell culture plates at a density of 10^6 cells per well. Tick α1,3-fucosyltransferase dsRNA or siRNA (600 μg) was added to 6 μl of RNAiFect (Qiagen, CA) in 100 μl of L15 (Leibowitz) cell culture medium. This solution was incubated at room temperature for 10 min and diluted to 400 μl of fresh medium. This mixture was added into each well to bring the final concentration of dsRNA or siRNA to 2 μg μl^{-1}. Control cells received equivalent concentration of *salp25d* dsRNA and plates were maintained at 37°C for 66 h. Cells were then harvested and processed for RNA extraction and microscopy.

RNA isolation and PCR

cDNA from ticks, cells and mouse tissues were prepared as described (Pedra *et al*., 2006). Tick samples were normalized to the *I. scapularis actin* gene (AF426178). The genes *B. burgdorferi* N40 flagellin (*flab*) (X75200) and *A. phagocytophilum 16s rDNA* (M73224) were then quantified. Gene-specific primers are described in Table S1. Quantitative PCR (qPCR) was performed using the iQ SYBR green supermix (Bio-Rad, CA).

N-glycan profile in ticks

N-glycan structures were isolated from 60 μg of *I. scapularis* salivary gland extract using a previously described method (Rendić *et al*., 2007a). Briefly, salivary gland proteins were loaded onto a SDS-PAGE gel and stained with Coomassie Blue. Stained proteins were trypsinized and glycopeptides were extracted using 5% formic acid. *N*-glycans were released using *N*-glycosidase A digestion and then purified. Dried *N*-glycans were resuspended in deionized water and used for MALDI–TOF-MS (matrix-assisted laser-desorption ionization–time-of-flight mass spectrometry) analysis. *N*-glycan structures were identified according to the glycan symbol nomenclature established by the Consortium for Functional Glycomics (http://www.functionalglycomics.org). Mass spectra of released glycans were interpreted on the basis of knowledge about invertebrate glycans, their probable adduct status (Na^+ conferring an *m/z* difference of 22) and the different contributions of monosaccharide units to the overall mass of these glycans (203 for *N*-acetylhexosamine, 162 for hexose and 146 for fucose).

Confocal microscopy and Western blot

Confocal microscopy and tick-immune rabbit serum were used as described earlier (Narasimhan *et al*., 2007). Topro-3 iodide (Invitrogen, MD) was used to stain the nucleus, rabbit polyclonal anti-HRP Sigma and rabbit polyclonal anti-*A. phagocytophilum* antibodies were used to stain core α1,3-fucose and *A. phagocytophilum* respectively. Immunoblots to

detect core α1,3-fucose, Salp25D and immunogenic tick proteins were performed as described (Narasimhan *et al*., 2007; Rendić *et al*., 2007b).

Bioinformatics analysis

The whole genome shotgun sequences of *I. scapularis* were searched in vectorbase using the NCBI TBLASTN program for homology to that encoded by the previously identified EST (ti| 1681614834). This EST sequence was described as the sequence corresponding to *isft7*. Ten putative fucosyltransferase reading frames were assembled *in silico* and the sequences 5′ to the homologous regions were examined for an ATG start codon followed by a sequence potentially encoding a transmembrane domain. These 'self-assembled' putative open reading frames were used to design primers for RT-PCR. No obvious start codon could be identified for *isft1*. Accession ID/gene numbers for *isft1* (ISCW004192); *isft2* (ISCW003590); *isft3/9* (ISCW004236); *isft4* (ISCW024758); *isft5* (ISCW024943); *isft6* (ISCW024303); *isft7* (ISCW023318); *isft8* (ISCW024461); *isft10* (ISCW05151) are given.

Statistical analysis

P-values were calculated using experimental and control data using the Student's *t*-test. Statistical significance was denoted by an asterisk and set at $P < 0.05$. Normal distribution was observed throughout our analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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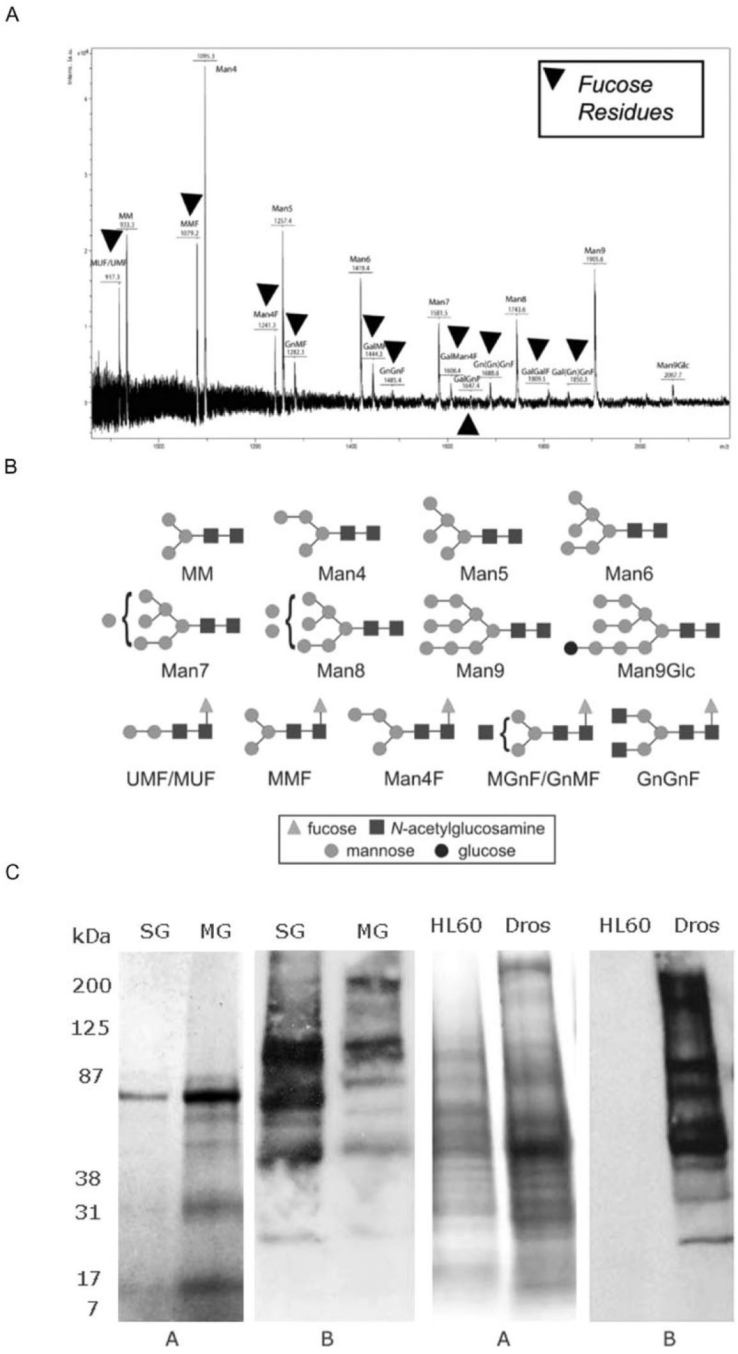

Fig. 1. α1,3-Fucose is present in ticks

A. *N*-glycan structures isolated from *I. scapularis* salivary glands (n = 100) were released by *N*-glycosidase A and *N*-glycan analysis was performed using MALDI–TOF-MS. The relevant structures carrying fucose residues are marked with arrowheads. Glycan *m/z* values and abbreviations are also shown.

B. *N*-glycan structures previously identified in (A) and found in a range of invertebrate organisms are shown according to the glycan symbol nomenclature established by the Consortium for Functional Glycomics (http://www.functionalglycomics.org).

C. Protein extracts (1 μg) from salivary glands (SG) and midguts (MG) of fed *I. scapularis* nymphs, human HL60 (HL60) cells and adult *Drosophila* (Dros) were electrophoresed on 4–

20% gradient SDS-PAGE followed by staining with Coomassie Blue R250 (A). Protein extracts were then immunoblotted with anti-HRP antibody. (B) Anti-HRP antibodies recognized core α1,3-fucose residues in arthropods but not in human cells. A minimum of two independent experiments were performed.

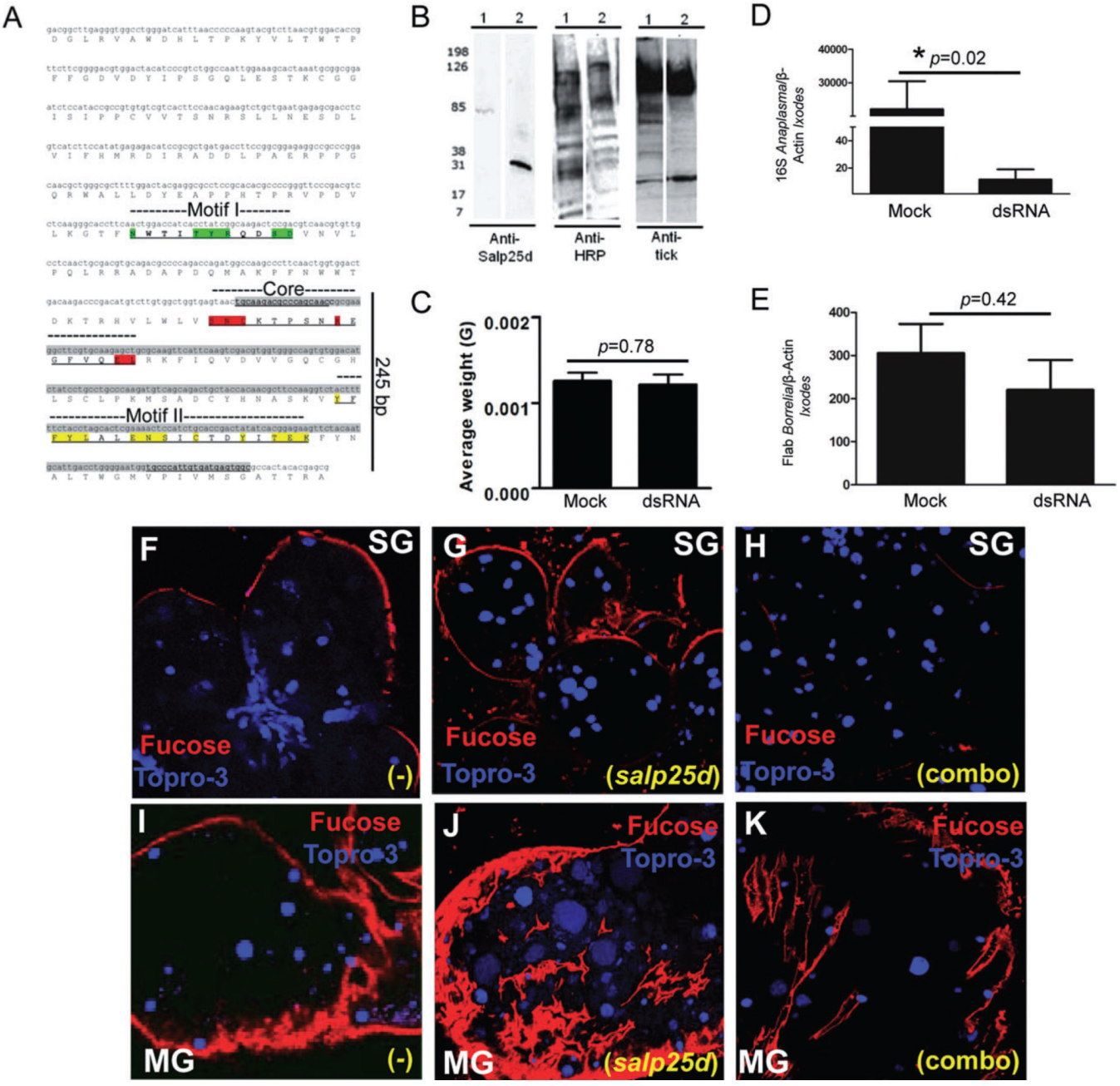

Fig. 2. α1,3-Fucose is required for *A. phagocytophilum* colonization of *I. scapularis* ticks

A. Partial sequence from the *I. scapularis* expressed sequence tag (ti|1681614834). The 245 bp fragment from *I. scapularis* encoding the signature motif for α1,3-fucosyltransferase was cloned into the L4440 RNAi vector and the dsRNA was produced. Grey shade – 245 bp amplicon; grey underlined shade – amplicon primers; green underlined – motif I; red – core motif; yellow – motif II. All motifs for α1,3-fucosyltransferase were identified as previously discussed (Oriol *et al*., 1999; Rendić *et al*., 2007b).

B. Extracts from salivary glands injected with control *salp25d* dsRNA (lane 1) and α1,3-fucosyltransferase dsRNA (lane 2). Salivary glands were immunoblotted with anti-Salp25D, anti-HRP and anti-tick antibodies.

C. Average weight of ticks in mock and α1,3-fucosyltransferase dsRNA treatments is shown.

D and E. (D) *A. phagocytophilum* load in the salivary glands and (E) *B. burgdorferi* presence in the tick midguts were measured during acquisition (66–72 h post attachment). *16s rDNA* and *flab* were used as reference genes to measure bacterial load.

F–K. Confocal microscopy in α1,3-fucosyltransferase siRNA-injected and control ticks fed on naïve mice for 66–72 h. Salivary glands (SG; F–H) and the midgut (MG; I–K) were stained with an antibody that recognizes core α1,3-fucose (anti-HRP). Salivary glands and the midgut of ticks showed reduced presence of core α1,3-fucose when a combination of siRNAs for *isft1*, *isft2* and *isft3* (combo) was injected in ticks (H and K). Non-injected (F and I) and control injected siRNA (G and J) showed abundant presence of core α1,3-fucose in the tissues. Acquisition experiments were repeated four times. Acquisition experiments used 14 mice per group and 10–12 ticks per mouse. Statistical analysis was performed using the Student's *t*-test ($P < 0.05$).

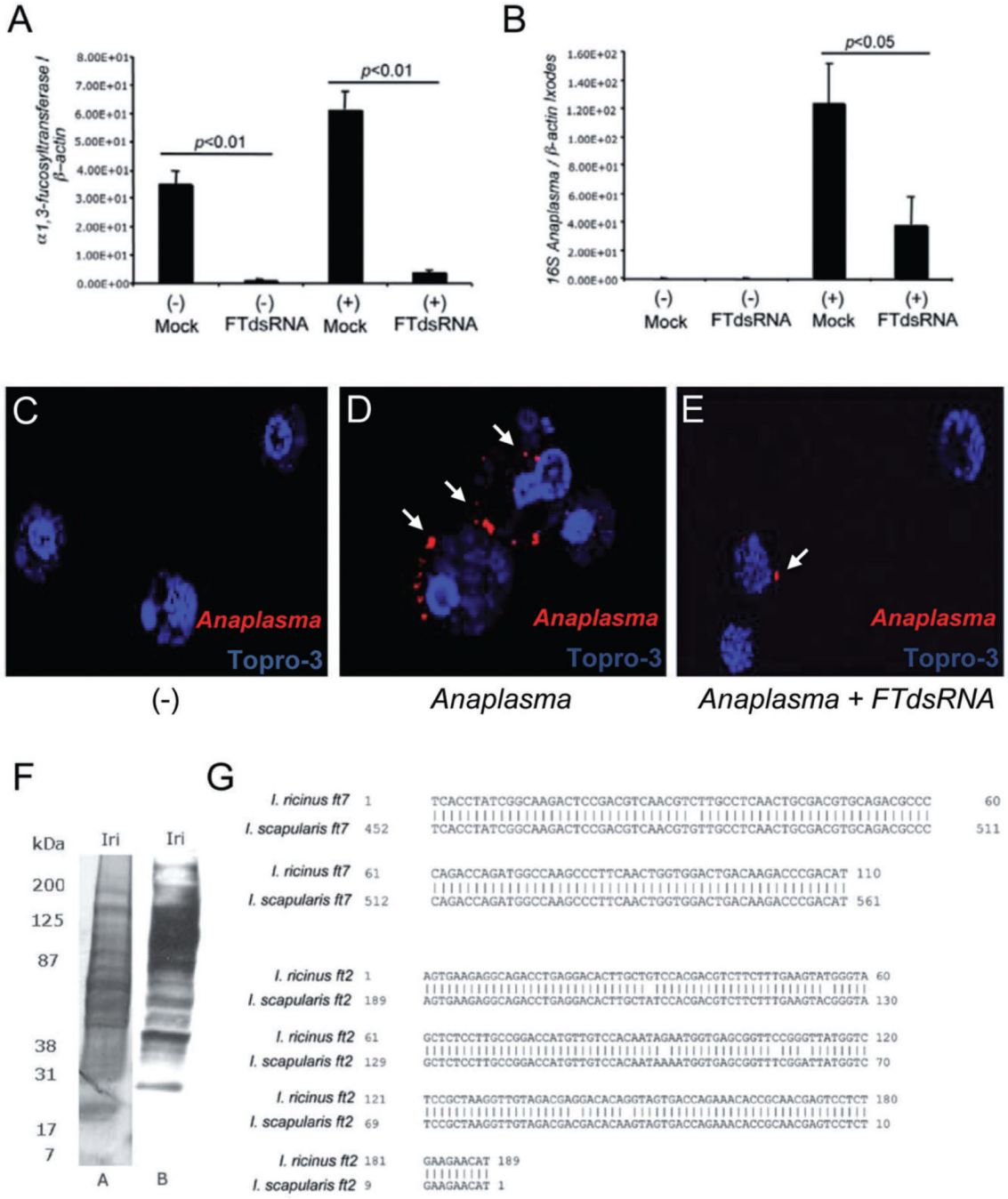

Fig. 3. *A. phagocytophilum* colonizes *I. ricinus* cells by using core α1,3-fucosylation

A. *I. ricinus* IRE/CTVM19 (2×10^5) cells were transfected with the *α1,3-fucosyltransferase* dsRNA and the silencing of *α1,3-fucosyltransferase* was examined in the presence (+) and absence (−) of *A. phagocytophilum*.

B. *A. phagocytophilum* load is shown 72 h post infection in mock and dsRNA-transfected *I. ricinus* cells.

C–E. Mock and dsRNA-transfected *I. ricinus* cells were visualized under the microscope and the presence of *A. phagocytophilum* is shown (arrows).

F. Protein extracts (1 μg) from *I. ricinus* cells (Iri) were electrophoresed and stained with Coomassie Blue R250 (lane A). Protein extracts were immunoblotted with the anti-HRP

antibody (lane B) showing that anti-HRP antibodies recognized core α1,3-fucose residues in *I. ricinus* cells.

G. α1,3-Fucosyltransferase 7 (*ft7*) was amplified from *I. ricinus* cDNA originated from the IRE/CTVM19 cell line. A fragment of 110 bp was cloned into the plasmid pGEM-T Easy Vector and sequenced. This sequence was 99% identical to the conserved region (nucleotide position 452–561) of *I. scapularis fucosyltransferase 7* (*XM_023318*). α1,3-Fucosyltransferase 2 (*ft2*) was amplified from *I. ricinus* cDNA originated from the IRE/CTVM19 cell line. A fragment of 189 bp was cloned into the plasmid pGEM-T Easy Vector and sequenced. This sequence shared 96% identity (nucleotide position 1–189) with the *fucosyltransferase I. scapularis* 2 *(isft2)* (XM_002401196). Cell culture experiments were repeated twice. Statistical analysis was performed using the Student's *t*-test ($P < 0.05$).

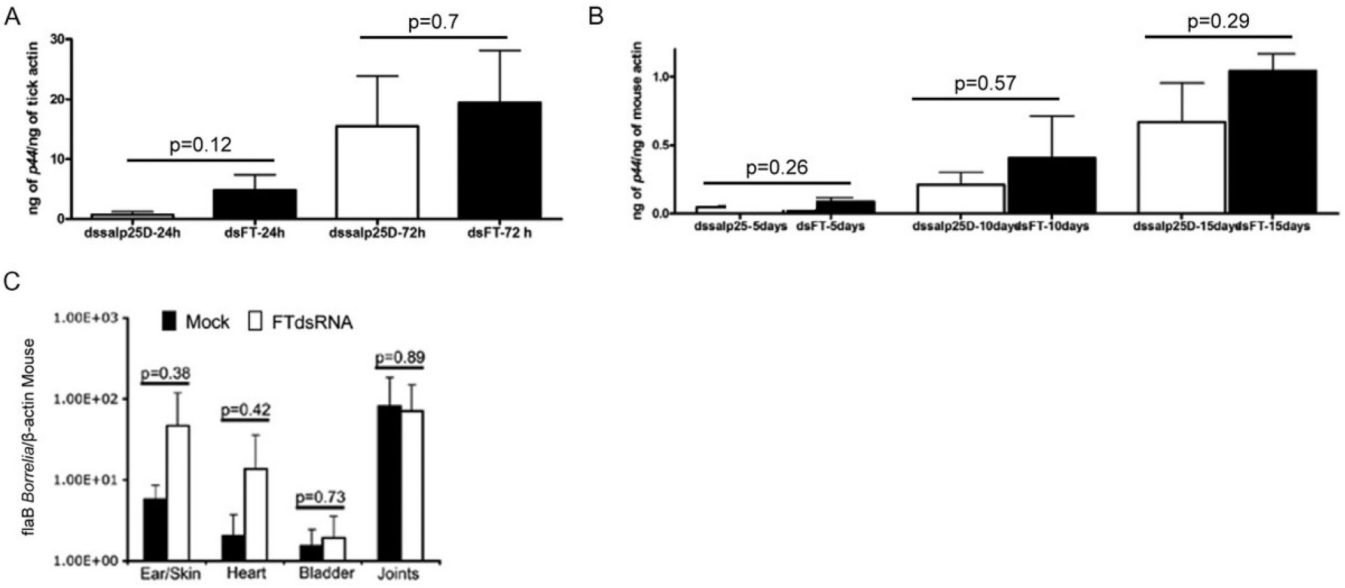

Fig. 4. Transmission of *A. phagocytophilum* does not require α1,3-fucosylation. *I. scapularis* nymphs infected with *A. phagocytophilum* were injected with dsRNA-*salp25d* or dsRNA-α1,3-fucosyltransferase. Ticks were then placed on C57Bl/6 mice for transmission experiments

A. Ticks were removed at 24 or 72 h post feeding and *A. phagocytophilum* load within ticks was measured.

B. *A. phagocytophilum*-infected ticks were removed at 72 h post feeding and transmission of *A. phagocytophilum* at days 5, 10 and 15 in the mouse peripheral blood was measured.

C. *I. scapularis* nymphs infected with *B. burgdorferi* were injected with mock or dsRNA-α1,3-fucosyltransferase. Infected ticks were placed on Balb/c mice during transmission experiments. *B. burgdorferi* infection was measured at day 18 post infection in multiple organs by qPCR using the *flab* gene as a reference. Transmission experiments were repeated twice. Transmission experiments used six mice per group and 8–10 ticks per mouse. Statistical analysis was performed using the Student's *t*-test ($P < 0.05$).

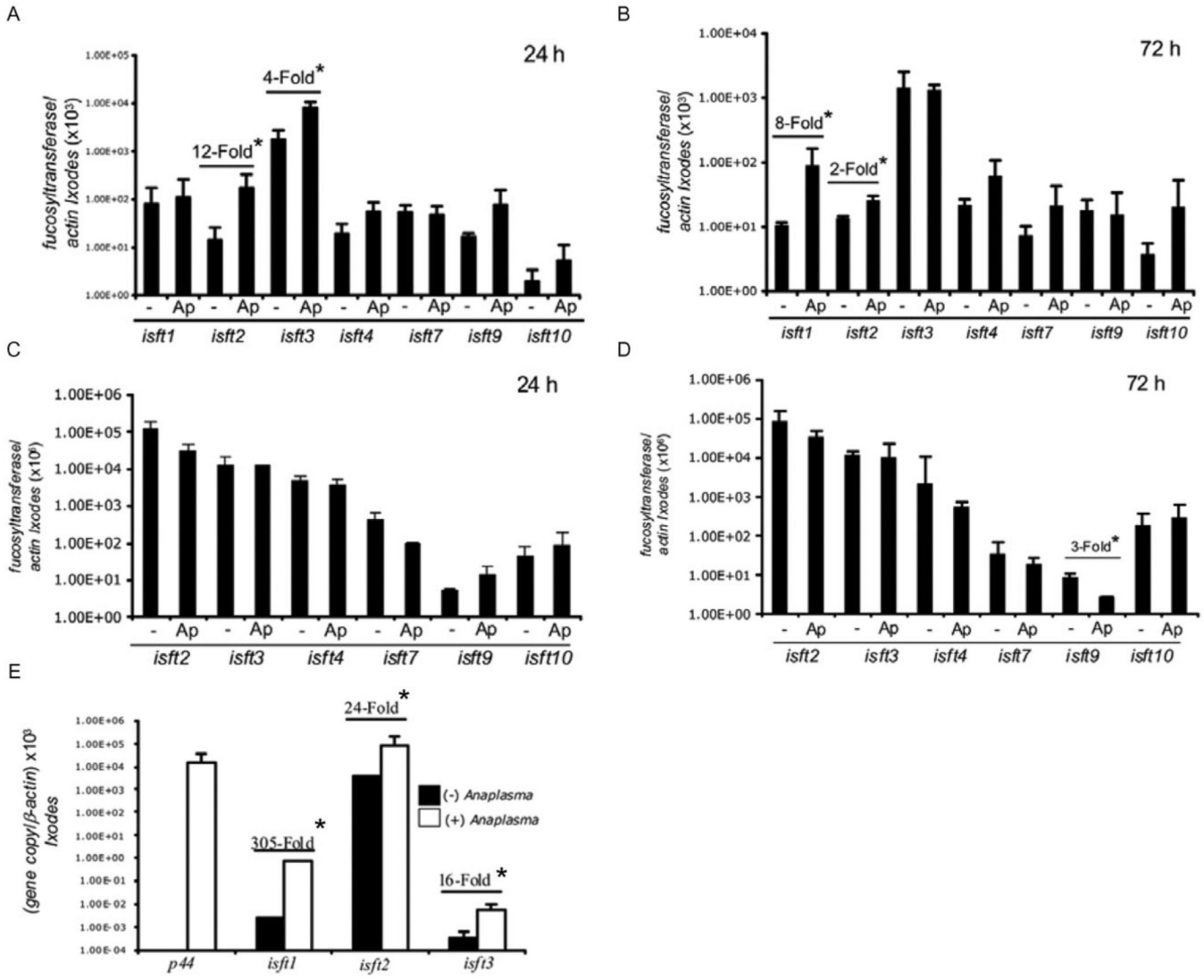

Fig. 5. *A. phagocytophilum* acquisition by *Ixodes* ticks increases *isft1*, *isft2* and *isft3* expression

A–D. Naïve (n = 90) tick larvae were placed on infected and non-infected C57Bl/6 mice for 24 and 72 h. *A. phagocytophilum* load was measured in ticks by qRT-PCR and the data normalized to *actin*. The expression of α1,3-fucosyltransferases was measured in the presence and absence of *A. phagocytophilum* at (A) 24 and (B) 72 h post attachment (*isft5*, *isft6* and *isft8* were not detected). Infected (n = 90) and non-infected nymphal (n = 90) ticks were placed on naïve C57Bl/6 mice for 24 and 72 h. The expression profile of α1,3-fucosyltransferases was measured in the salivary glands of nymphal *I. scapularis* at (C) 24 and (D) 72 h post attachment (*isft1*, *isft5*, *isft6* and *isft8* were not detected).

E. The transcription of α1,3-fucosyltransferases *isft1*, *isft2* and *isft3* was measured in infected and non-infected *I. ricinus* cells. *A. phagocytophilum* load is measured using the expression of the p44 gene as a reference. One representative experiment is shown. Results are expressed as mean plus standard deviation. Statistical analysis was performed using the Student's *t*-test ($P < 0.05$). Statistical significance is demonstrated by an asterisk.

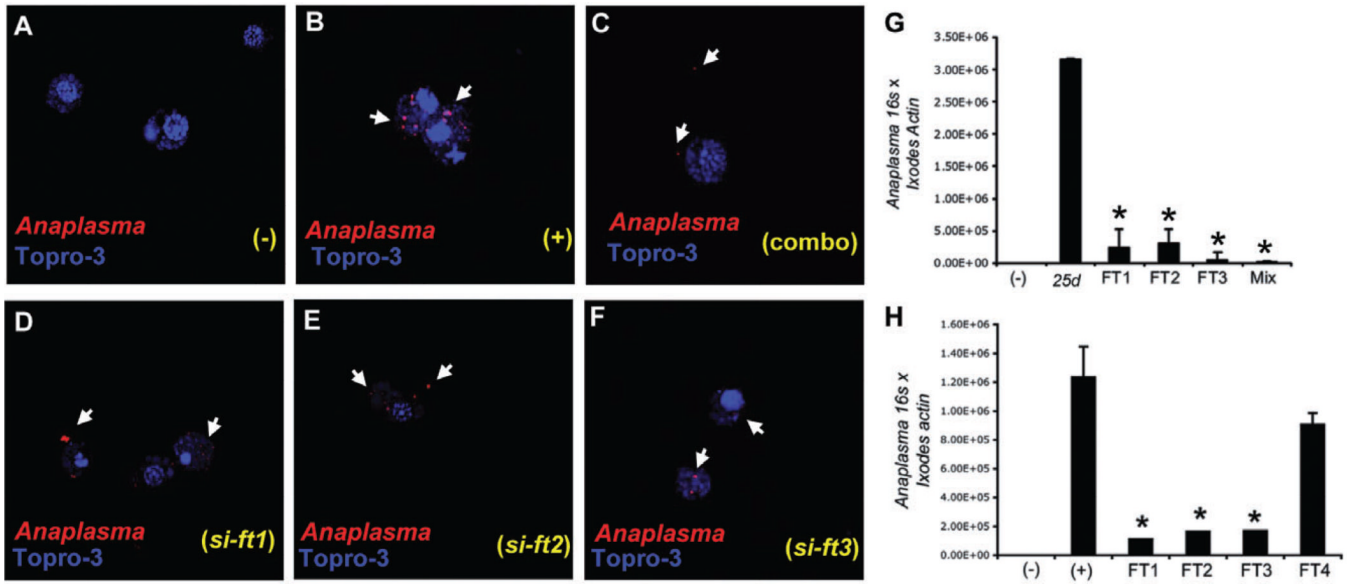

Fig. 6. Silencing of *isft1*, *isft2* and *isft3* by specific siRNAs decreases *A. phagocytophilum* colonization of tick cells. Six hundred ηg of siRNAs specific for α1,3-fucosyltransferase 1 (FT1), α1,3-fucosyltransferase 2 (FT2) and α1,3-fucosyltransferase 3 (FT3) were transfected into *I. ricinus* (1×10^7) cells. Twenty-four hours later, cells were infected with *A. phagocytophilum* [multiplicity of infection (moi) 5]

A–F. Confocal microscopy showing *A. phagocytophilum* in *I. ricinus* cells using a nuclear-specific stain (Topro-3) and an *A. phagocytophilum*-specific antibody. (A) Non-transfected and non-infected cells (B) cells infected with *A. phagocytophilum* and transfected with the *salp25d* control siRNA, and (C) cells infected with *A. phagocytophilum* and transfected with a combination of siRNAs specific for *isft1*, *isft2* and *isft3* as well as (D–F) individual transfections are shown. Arrows show the presence of *A. phagocytophilum*.

G. *A. phagocytophilum 16s rDNA* was measured by qRT-PCR 72 h post infection and compared with the expression of the control siRNA salp25d (*25d*) or a combination of siRNAs for *isft1*, *isft2* and *isft3* (Mix).

H. siRNAs specific for *isft4* did not show any effect on *A. phagocytophilum* infection in *I. ricinus* cells. Results are expressed as mean plus standard deviation. Statistical analysis was performed using the Student's *t*-test (*$P < 0.05$).