# Interactions Between Ticks and Lyme Disease Spirochetes

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## Abstract

*Borrelia burgdorferi* sensu lato causes Lyme borreliosis in a variety of animals and humans. These atypical bacterial pathogens are maintained in a complex enzootic life cycle that primarily involves a vertebrate host and *Ixodes* spp. ticks. In the Northeastern United States, *I. scapularis* is the main vector, while wild rodents serve as the mammalian reservoir host. As *B. burgdorferi* is transmitted only by *I. scapularis* and closely related ticks, the spirochete-tick interactions are thought to be highly specific. Various borrelial and arthropod proteins that directly or indirectly contribute to the natural cycle of *B. burgdorferi* infection have been identified. Discrete molecular interactions between spirochetes and tick components also have been discovered, which often play critical roles in pathogen persistence and transmission by the arthropod vector. This review will focus on the past discoveries and future challenges that are relevant to our understanding of the molecular interactions between *B. burgdorferi* and *Ixodes* ticks. This information will not only impact scientific advancements in the research of tick-transmitted infections but will also contribute to the development of novel preventive measures that interfere with the *B. burgdorferi* life cycle.

## Introduction

*Ixodes* ticks transmit a wide range of infections, including Lyme disease, to animals and humans (Nadelman and Wormser, 1998; Radolf et al., 2012; Nelder et al., 2016; Steere et al., 2016; Stanek and Strle, 2018). Lyme disease is a prominent tick-borne illness (Benach et al., 1983) that features widespread distribution (Mead, 2015) and is caused by a group of spirochetes belonging to the *Borrelia burgdorferi* sensu lato complex (Burgdorfer et al., 1988). Recently, more virulent (albeit less prevalent) strains of *Borrelia* pathogens have been identified, including new clinical isolates of *B. burgdorferi* sensu lato, *B. bissettii*, *B.*

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*mayonii*, and *B. miyamotoi*, which are also transmitted by *Ixodes* ticks and are associated with severe human diseases (Krause et al., 2015; Pritt et al., 2016; Radolf and Samuels, 2021; Rudenko et al., 2016). Globally, the *Borrelia* species that are commonly associated with Lyme disease include *B. burgdorferi* sensu stricto, which is prevalent throughout the United States and Europe, and *B. afzelii* and *B. garinii*, which are distributed throughout Eurasia (Nadelman and Wormser, 1998; Piesman and Gern, 2004; Mead, 2015).

In North America and Europe, Lyme disease spirochetes typically are maintained in nature through a complex tick-rodent infection cycle. Larval ticks acquire the pathogen while feeding on infected wild hosts, such as white-footed mice (Anderson et al., 1987; Anderson, 1989), and then transstadially maintain *B. burgdorferi* before transmitting the pathogen back to a naïve host during the subsequent blood meal (Steere, 2001; Steere et al., 2004). Humans and domesticated animals that develop Lyme disease are incidental hosts and do not play a role in the natural transmission cycle (Radolf et al., 2012).

*B. burgdorferi* does not readily infect most tick species, suggesting that its interactions with *I. scapularis* are highly specific. Limited studies have shed light on these complex pathogen-tick interactions (Munderloh and Kurtti, 1995; Fikrig and Narasimhan, 2006); a recent review summarizes our most current state of knowledge about the interactions between *B. burgdorferi* and *I. scapularis* ticks (Kurokawa et al., 2020). Vector competence, which depends on genetic determinants influencing the vector's ability to transmit a pathogen, also shapes interactions involving the tick, pathogen, and host (de la Fuente et al., 2017). With the advent of genetic tools for creating *Borrelia* mutants (Samuels et al., 2018) (see also Radolf and Samuels, 2021) and the current availability of the *I. scapularis* genome (Gulia-Nuss et al., 2016; Miller et al., 2018), investigators will have increasingly robust tools for studying *Borrelia*-tick interactions, both during the arthropod phase of the spirochete life cycle and at the interface with the mammalian host. A few borrelial proteins already have been demonstrated as prerequisites for spirochete-vector interactions, particularly with receptors in select tick organs, such as the gut or salivary gland (Ramamoorthi et al., 2005; Rosa, 2005; Dunham-Ems et al., 2009; Zhang et al., 2011; Radolf et al., 2012). The adaptation of *Borrelia* to the vector or host also requires the tightly-controlled regulation of bacterial gene expression (Hubner et al., 2001; Caimano et al., 2005; Fisher et al., 2005; Caimano et al., 2007; Samuels, 2011; Caimano et al., 2016; Stevenson and Seshu, 2018). Exciting new studies have elucidated the complexities of gene regulation by *B. burgdorferi* in ticks (Radolf and Samuels, 2021). The RpoN/RpoS pathway is active in feeding nymphs (Caimano et al., 2007) and induces a set of proteins that are mandatory for *B. burgdorferi* transmission from ticks, as well as for host infection (Fisher et al., 2005). Additional proteins and nucleotide factors, like $Rel_{Bbu}$ or 5'-triphosphate-guanosine-3'-diphosphate and 5'-diphosphate-guanosine-3'-diphosphate (collectively known as (p)ppGpp), are required for borrelial persistence, while the Hk1/Rrp1 two-component system and c-di-GMP are required for host transitions and the evasion of tick defenses (Bugrysheva et al., 2015; Caimano et al., 2015; Caimano et al., 2016). More recently, a large number of small noncoding RNAs (sRNAs) were discovered to be encoded in the borrelial genome, some of which are regulators of gene expression (Drecktrah et al., 2018). Future studies of *Borrelia*-tick interactions will allow us to understand the unique adaptive strategies of the bacterium for its survival in an extremely diverse range of host tissues. This information will contribute to the

development of novel preventive measures against borrelial infection. The following sections will review how the molecular interactions of select *B. burgdorferi* and *I. scapularis* gene products support the pathogen's life cycle in nature. We also will comment on recent advances in *Ixodes* genomics, as well as other "omics" technologies (Hasin et al., 2017), and discuss how tick interaction studies may contribute to the development of a transmission-blocking Lyme disease vaccine.

## The *B. burgdorferi* life cycle in ticks

*B. burgdorferi* survives in nature in a tick-mammal infection cycle (Anguita et al., 2003; Pal and Fikrig, 2003; Caimano et al., 2016). Given the lack of transovarial transmission, the pathogen needs to be acquired during one of the life stages of its vector at the time of engorgement on infected mammals, primarily wild rodents (Anderson, 1989; Munderloh and Kurtti, 1995; Pal and Fikrig, 2003). As the arthropod prepares for its blood meal ingestion within the first 12 hours of tick attachment to the host, the transfer of spirochetes to the vector already has begun. The details remain uncertain as to how *B. burgdorferi* transmits between the arthropod and vertebrate host, either by the active chemotactic migration of spirochetes or via passive transfer along with host fluid. During acquisition, spirochetes enter the tick gut from the dermis of an infected host and presumably continue to migrate until the tick is fully engorged, which usually takes 72–96 hours (Piesman et al., 1990; de Silva and Fikrig, 1995). *B. burgdorferi* intimately associates with the midgut tissues within 48 hours of feeding (Yang et al., 2004), and can persist in the gut throughout the life span of the arthropod (Pal et al., 2001). When the tick ingests a subsequent blood meal, the spirochetes multiply in the gut; at this time, an unknown fraction of the *B. burgdorferi* population exits the gut, enters the hemocoel, disseminates towards the salivary glands, and is transmitted through the salivary stream to the new mammalian host (de Silva and Fikrig, 1995; Coleman et al., 1997; Piesman et al., 2001; Schwan and Piesman, 2002; Dunham-Ems et al., 2009). The following section will highlight the varied environments and formidable challenges that *B. burgdorferi* encounters during its entry, long-term persistence, and subsequent transmission through ticks.

### *B. burgdorferi* encounters diverse tick environments

**The feeding gut during acquisition—**When acquired from mammals, *B. burgdorferi* adapts to an entirely new environment in the vector. Despite recent breakthroughs (Smith and Pal, 2014; Smith et al., 2016; Oliva Chavez et al., 2017; Shaw et al., 2017; Shaw et al., 2018), relatively little is known about the immune system of the tick. Nevertheless, the feeding tick gut can be presumed to present a hostile environment, which *B. burgdorferi* encounters immediately following acquisition (Smith et al., 2016) (see also Radolf and Samuels, 2021). As spirochetes migrate into the luminal spaces of the gut and colonize it, they must avoid being digested with the blood meal and also must bypass the tick's innate immune defense mechanisms (Hynes et al., 2005). Phagocytosis allows the tick gut cells to capture constituents of the blood meal, such as cellular remnants and hemoglobin crystals (Sonenshine, 1993), although the ingestion of *B. burgdorferi* is rarely observed. While gastric digestion in ticks is primarily intracellular (Sonenshine, 1993), with intraluminal proteases virtually absent, the degradation of blood meal components could lead to peptides

that have antimicrobial activities (Fogaca et al., 1999; Nakajima et al., 2003). In addition, ticks also may produce conventional antimicrobial peptides in the gut (Broadwater et al., 2002; Hynes et al., 2005). An *I. ricinus* defensin-like gene is upregulated in the gut after infection with *B. burgdorferi* (Grubhoffer et al., 2005); however, the mechanism by which the spirochetes evade this innate immune defense is unknown. *Ixodes* ticks also form a transient acellular peritrophic membrane (PM) that surrounds the blood meal (elaborated upon below). It remains unclear as to how this barrier influences *B. burgdorferi* acquisition and colonization of the gut epithelium in ticks. Invading spirochetes could rapidly colonize the gut epithelial cells prior to the formation of a peritrophic barrier or, alternatively, spirochetes could cross the peritrophic membrane after its formation and colonize the underlying gut epithelium. In either case, the peritrophic barrier could protect the bacteria that are bound to the midgut epithelium against the toxic contents of the lumen.

**The unfed gut**—In its natural cycle, *B. burgdorferi* persists in its arthropod vectors for months, confined to the guts of unfed ticks. *Ixodes* have a relatively long life cycle spanning at least 18 months (Ullmann et al., 2005), a significant part of which is dominated by phases of nutrient deprivation and limited metabolic activity (Sonenshine, 1993). Little is known about the mechanisms by which *B. burgdorferi* persists in unfed ticks (Figure 1), although more recent studies have suggested that the alteration of metabolic pathways may play a critical role in spirochete persistence. For example, studies have shown that the Hk1/Rrp1/c-di-GMP and Rel$_{Bbu}$/(p)ppGpp pathways have essential functions in spirochete adaptation and survival, include a necessary shift in carbon utilization from glucose to glycerol (He et al., 2011; Pappas et al., 2011; Drecktrah et al., 2015; Caimano et al., 2016). Residing within the gut of the poikilotherm, spirochetes also must survive the extremes of seasonal changes, as well as daily temperature fluctuations in temperate climates. The lumen of the unfed gut (Figure 1) is poor in residual nutrients, primarily because gut epithelia store nutrient macromolecules within cellular endosomes, leaving the lumen devoid of nutrients (Sonenshine, 1993). Genes expressed by *B. burgdorferi* in the unfed gut, or in "tick-like" growth conditions *in vitro*, could be associated with the maintenance of the most elemental functions for persistence in the nutrient-depleted and anoxic environment of the arthropod (Ojaimi et al., 2002; Revel et al., 2002). Spirochetes are known to upregulate specific gene products that protect DNA against starvation or oxidative stress-induced damage during the long intermolt period between blood meals (described below). Alternate forms of *B. burgdorferi*, called round bodies (RBs), also have been reported, especially under unfavorable conditions *in vitro* (Brorson and Brorson, 1998; Alban et al., 2000; Brorson and Brorson, 2004; Drecktrah et al., 2015), in ticks *in vivo* (Dunham-Ems et al., 2012), and in incidental hosts *in vivo* (Brorson and Brorson, 1998; Duray et al., 2005).

**Feeding gut during transmission**—Tick attachment to a host initiates a remarkable series of metabolic changes in the gut (Sonenshine, 1993). During the first 36 hours of host attachment, blood enters the tick slowly, and there is little digestive activity (Sauer, 1986; Sonenshine, 1993). Spirochetes in the gut must sense the appropriate physiochemical stimuli before they begin multiplying. Following 24 hours of host attachment, *B. burgdorferi* is principally confined within the lumen of the tick gut; by 72 hours, it is dispersed throughout the entire gut tissue (Pal and Fikrig, 2003). Although the density of *B. burgdorferi* in the gut

increases exponentially as feeding progresses (Piesman et al., 1990; de Silva and Fikrig, 1995), thereby generating a phenotypically diverse population of spirochetes (Ohnishi et al., 2001; Srivastava and de Silva, 2008), the majority of the bacteria do not exit the gut. Only a fraction of spirochetes cross the gut epithelial barrier and disseminate to the hemocoel for transmission to the host via the salivary gland (Coleman et al., 1997). A seminal study provided a detailed mechanistic view of how *B. burgdorferi* travels through the feeding gut during transmission from infected ticks, using an unprecedented biphasic mode of dissemination (Dunham-Ems et al., 2009). In the initial phase of dissemination, the replicating spirochetes in the midgut form networks of nonmotile organisms, which later advance toward the basolateral gut surface via adherence to the resident gut epithelial cells. In the subsequent phase, the spirochetes become motile, invade the gut basement membrane, and migrate to the salivary glands through the hemocoel (Dunham-Ems et al., 2009). Future studies are warranted to decipher the identities of the tick and microbial components, or additional mechanisms that influence *B. burgdorferi* dissemination from the gut. Host-derived plasminogen has been implicated in the efficient dissemination of *Borrelia* within the tick, and in the enhancement of spirochetemia in vertebrate hosts (Coleman et al., 1995; Coleman et al., 1997). As previously mentioned, an ephemeral and porous peritrophic membrane (PM) is formed in the infected tick gut during feeding, which engulfs the blood meal and *B. burgdorferi* in the lumen (Rudzinska et al., 1982; Sonenshine, 1993), and is clearly visible within 36 hours of the onset of feeding. This membrane is a mechanical barrier, and particulate matters from the ingested blood meal can be retained in the lumen proper (Rudzinska et al., 1982). *B. burgdorferi* can be detected in the lumen of the feeding gut (Pal and Fikrig, 2003; Dunham-Ems et al., 2009); however, it is unknown as to how the physical division of the gut luminal spaces and epithelial tissues, as imposed by the peritrophic membrane, influences spirochete transmission. The integrity of the peritrophic membrane, which is essential for *B. burgdorferi* to efficiently colonize the gut epithelium, is influenced by extrinsic factors, such as the gut microbiota, or intrinsic proteins, like the transcription factor STAT (Kariu et al., 2013; Narasimhan et al., 2014b). Although studies have yet to define precisely how *B. burgdorferi* penetrates through the PM, another tick-borne pathogen, *Babesia microti*, is reported to develop a highly specialized organelle, the arrowhead, which helps the microbe to cross the peritrophic barrier of *Ixodes* ticks (Rudzinska et al., 1982). Nevertheless, the peritrophic membrane and its constituents have been shown to influence pathogen persistence or dissemination in many other arthropod-borne pathogens, including *B. burgdorferi* (Pimenta et al., 1997; Beerntsen et al., 2000; Kariu et al., 2013).

**The hemocoel**—During transmission, *B. burgdorferi* needs to traverse the hemocoel to access the salivary glands. In arthropods, the cellular constituents of the hemocoel are dominated by actively phagocytic blood cells or hemocytes that are the primary mediators of the arthropod innate immune responses (Sauer, 1986; Sonenshine, 1993). Little is known about how *B. burgdorferi* travels through the tick hemolymph, avoids phagocytosis and the innate immune defense of hemocytes, and selectively recognizes and enters the salivary gland. Studies indicate that a significant percentage (13 ± 5%) of isolated hemocytes in feeding *I. scapularis* could be associated with disseminated spirochetes (Coleman et al., 1997), although the biological significance of the *Ixodes* hemocyte-spirochete association is

not clear. In another study, when *B. burgdorferi* was experimentally injected into the hemocoels of *I. scapularis* and *Dermacentor variabilis*, a non-competent tick vector of *Borrelia*, the spirochetes readily survived in the *Ixodes* hemolymph, whereas they were rapidly killed by hemocytes in the *D. variabilis* hemolymph, suggesting that *B. burgdorferi* is resistant to killing by *Ixodes* hemocytes (Johns et al., 2001). The recent availability of the *I. ricinus* hemocytome, as analyzed by next-generation sequencing technologies (Kotsyfakis et al., 2015), will contribute to our understanding of *Ixodes* hemocyte biology, including its role in spirochete interactions (Kurokawa et al., 2020). While the number of *B. burgdorferi* in the infected tick gut increases exponentially (Piesman et al., 1990; Piesman et al., 2001), the exact proportion of spirochetes that cross the gut epithelial barrier and systemically disseminate to the salivary gland through the hemocoel is unknown. Studies of infected *I. scapularis* during feeding indicate that approximately 5% and 0.7% of the gut spirochetes can be detected in the hemolymph and salivary glands, respectively (Coleman et al., 1997). Future studies on the kinetics and mechanisms of *B. burgdorferi* dissemination through the *Ixodes* hemocoel should provide important insights into the development of novel inhibitory measures against spirochete transmission from ticks to mammals.

**The salivary gland—**The salivary gland represents the other major tick organ (Sauer, 1986; Sonenshine, 1993) that spirochetes must traverse during transmission to a mammalian host. The saliva from an engorging tick, which is deposited in the tick bite site of the host dermis, plays a pivotal role in pathogen transmission (Simo et al., 2017; Nuttall, 2019). Once *B. burgdorferi* is acquired by the tick vector from an infected host, the pathogen resides in the tick gut and does not infect the salivary glands (Piesman, 1993; Piesman et al., 2001; Pal and Fikrig, 2003; Fikrig and Narasimhan, 2006). The spirochetes migrate from the infected tick gut to the salivary glands during a subsequent blood meal, which lasts for 3–7 days (Piesman, 1993; Piesman et al., 2001; Pal and Fikrig, 2003; Fikrig and Narasimhan, 2006). In comparison, *B. hermsii*, the relapsing fever spirochetes, readily colonize the salivary glands of unfed soft ticks, *Ornithodoros hermsi* (Sonenshine, 1993; Schwan and Piesman, 2002); soft ticks are fast feeders, and these particular spirochetes are transmitted within seconds of vector attachment to a mammalian host (Schwan and Piesman, 2002; Boyle et al., 2014). Regardless of the kinetics of transmission, tick salivary glands potentially act as the second distinct barrier that most arthropod-borne pathogens must cross for efficient transmission. Other tick-borne bacterial diseases, such as *Anaplasma marginale*, require active replication within the salivary gland for effective transmission (Ueti et al., 2007), yet there is no evidence of a similar event for *B. burgdorferi* within the tick. Borrelial spirochetes invade the salivary gland by an undefined mechanism, after which they may be passively carried to the host dermis, along with tick saliva. As several salivary gland genes are upregulated in infected *I. scapularis* nymphs, in comparison to uninfected nymphs (Ribeiro et al., 2006), salivary gland proteins may play an important role in *B. burgdorferi* transmission and infection.

## Regulation of gene expression in ticks

*B. burgdorferi* alters its gene expression profile as it cycles between arthropods and mammals (de Silva and Fikrig, 1997; Anguita et al., 2003; Schwan, 2003; Rosa et al., 2005; Stevenson and Seshu, 2018), and specific genes maintain the spirochete infection cycle in

nature (Coburn and Cugini, 2003; Purser et al., 2003; Grimm et al., 2004b; Pal et al., 2004b; Yang et al., 2004; Revel et al., 2005; Rosa et al., 2005; Li et al., 2007b; Pal et al., 2008b). Gene regulation in *B. burgdorferi* is the subject of several reviews, such as (Samuels, 2011; Caimano et al., 2016; Stevenson and Seshu, 2018) (see also Radolf and Samuels, 2021). The diverse microenvironments within vector tissues likely induce the changes in pathogen gene expression that are required for survival. In contrast to many pathogenic bacteria, *B. burgdorferi* devotes large portions of its genome (more than 8% of its coding genes) toward the production of approximately 150 lipoproteins (Fraser et al., 1997; Casjens et al., 2000; Schutzer et al., 2011), many of which are predicted to be exposed on the bacterial surface and be differentially expressed. A number of studies have attempted to identify *B. burgdorferi* genes that are selectively upregulated in the environments of either unfed or fed ticks *in vivo* (Narasimhan et al., 2002; Kumar et al., 2011; Dunham-Ems et al., 2012; Iyer et al., 2015; Groshong et al., 2017). The comparative microarray study revealed wide-spread global changes in expression of genes encoding lipoproteins, carbon utilization, nutrient uptake, intermediate metabolism and chemotaxis when comparing the transcriptomes of fed larvae and fed nymphs, as well as to those of *B. burgdorferi* cultured *in vitro* and in dialysis membrane chambers in the host (Iyer et al., 2015). This all-encompassing view of the transcriptomic modulation in the spirochetes grown in culture or in the vector provides an appreciable picture of the actual changes in the *in vivo* model of Lyme disease, while dissecting individual mechanisms regulating expression have mostly relied on specific *in vitro* conditions. To that end, a number of microarray and RNA-seq studies have provided indirect but important clues about the regulation and function of *B. burgdorferi* genes in *in vitro* culture models, in some cases partly mimicking tick-specific environments (Narasimhan et al., 2002; Ojaimi et al., 2002; Revel et al., 2002; Brooks et al., 2003; Ojaimi et al., 2003; Tokarz et al., 2004; Fisher et al., 2005; Bugrysheva et al., 2015; Caimano et al., 2015; Drecktrah et al., 2015; Arnold et al., 2016; Arnold et al., 2018; Boyle et al., 2019; Caimano et al., 2019). Temperature changes used to mimic the tick environment (23°C) and incoming blood meal during tick feeding and host environment (37°C) have identified this variable as an important regulator of global gene expression, albeit by unknown mechanisms (Ojaimi et al., 2002). Such studies have important implications, as the temperature in the tick gut changes rapidly during feeding, rising from the ambient arthropod temperature to mammalian body temperature, resulting in the modulation of the expression of genes necessary to establish infection, notably those encoding lipoproteins, especially outer surface lipoprotein C (*ospC*) (Schwan et al., 1995). Another simulation of the conditions of feeding ticks was accomplished by the addition of blood to spirochete cultures, which also induced major remodeling of the transcriptome with some similarities to temperature shifting, but also significant distinctions (Tokarz et al., 2004).

Induction of the RpoN-RpoS alternative sigma factor pathway, where RpoN controls the expression of RpoS (Hübner et al., 2001), is regarded as a key genetic regulatory network in *B. burgdorferi*, particularly during transmission from ticks to the host. The RpoN-RpoS pathway activates many spirochete genes in feeding ticks, implying a role in *B. burgdorferi* transmission through ticks. Both *rpoN* null and *rpoS* null mutants successfully colonized tick guts, but were unable to invade salivary glands or infect the vertebrate host (Fisher et al., 2005; Dunham-Ems et al., 2012). These studies indicate that genes regulated by RpoN and

RpoS are involved in transmission from the vector to the host. The expression of the alternative sigma factor, *rpoS*, is selectively induced during the nymphal blood meal, suggesting a potential role of RpoS as an activator of genes that are relevant to spirochete transmission and the establishment of early infection in a mammalian host (Caimano et al., 2004; Caimano et al., 2007; Dunham-Ems et al., 2012; Caimano et al., 2019). Individual spirochete genes also have been identified that show dramatic expression in ticks, such as *ospA*, *ospB*, *ospC*, *ospD*, *ospE, ospF, elp, bptA, dps, cspA*, and *la7*, and that may play important roles in tick tissue colonization, transmission, and infection of a new host (Schwan et al., 1995; Schwan and Piesman, 2000; Ojaimi et al., 2002; Brooks et al., 2003; Grimm et al., 2004b; Tokarz et al., 2004; Yang et al., 2004; Revel et al., 2005; Bykowski et al., 2007a; Bykowski et al., 2007b; Caimano et al., 2007; Li et al., 2007b; Neelakanta et al., 2007; Pal et al., 2008a). Although many of these genes belong to *B. burgdorferi* paralogous gene families, such as Erps (OspE/F-related proteins), individual members have presented distinct expression patterns in the infected tick during feeding and transmission to the mammalian host. For example, whereas *ospE, ospF,* and *bbk2.11* display progressive induction in the gut while the arthropod is being engorged on the blood meal, certain members of the *elp* family, such as *bbk2.10* and *ospE/F* homolog *p21*, are expressed only at some later points of feeding and dissemination (Hefty et al., 2001).

The two intracellular second messengers guanosine tetraphosphate and pentaphosphate, collectively known as (p)ppGpp, and cyclic-di-GMP (c-di-GMP) are important signaling molecules that globally alter gene expression to adapt *B. burgdorferi* for persistence in the tick (He et al., 2011; Bugrysheva et al., 2015; Caimano et al., 2015; Drecktrah et al., 2015). The levels of (p)ppGpp are controlled by the bifunctional enzyme Rel$_{Bbu}$, which acts as both a synthetase and hydrolase to regulate the stringent response (Fraser et al., 1997; Concepcion and Nelson, 2003; Bugrysheva et al., 2005; Drecktrah et al., 2015). When *B. burgdorferi* is nutrient stressed *in vitro*, conditions used to simulate the tick environment between bloodmeals, levels of (p)ppGpp increase, leading to global changes in gene expression and sRNA levels (Bugrysheva et al., 2015; Drecktrah et al., 2015; Drecktrah et al., 2018).

Genes encoding specific proteins involved in several pathways are targeted for regulation, including carbohydrate metabolism and persistence in the tick, such as the glycerol metabolism (*glp*) operon (Bugrysheva et al., 2015; Drecktrah et al., 2015). The Rel$_{Bbu}$-mediated transcriptional remodeling is important for persistence in the tick as a null mutant is compromised for survival between bloodmeals (Drecktrah et al., 2015). Additionally, the transcriptional regulator DksA also alters gene expression during nutrient stress both cooperatively and independently of (p)ppGpp, targeting similar functional pathways, such as the *glp* operon, and distinct ones as well (Boyle et al., 2019). Together, Rel$_{Bbu}$ and DksA regulate the stringent response to control gene expression to adapt *B. burgdorferi* for persistence in the tick.

A series of studies described the identification and molecular characterization of a c-di-GMP regulatory system, which revealed a critical role in spirochete gene expression, motility, and survival in the tick (Freedman et al., 2010; Pitzer et al., 2011; He et al., 2014; Novak et al., 2014; Mallory et al., 2016; Kostick-Dunn et al., 2018; Zhang et al., 2018). Rrp1, a

diguanulate cyclase of the two-component regulatory system Hk1-Rrp1, synthesizes c-di-GMP in response to as of yet unidentified signals to globally modulate gene expression, including those encoding genes involved in carbon utilization, notably, like the stringent response, the *glp* operon, and surface lipoproteins (Rogers et al., 2009; Sze et al., 2013; Caimano et al., 2015). The *rrp1* mutants unable to synthesize c-di-GMP have a dramatic survival defect in feeding larvae and nymphs, indicating that the c-di-GMP-mediated transcriptional changes likely adapt the spirochete for persistence (He et al., 2011; Kostick et al., 2011; Caimano et al., 2015). At least some of the effects of c-di-GMP on transcription may be mediated by the c-di-GMP binding protein PlzA, and in some strains PlzB (Freedman et al., 2010; Pitzer et al., 2011; He et al., 2014; Kostick-Dunn et al., 2018; Zhang et al., 2018). Overexpression of PlzA partially compensates for the survival defect of *rrp1* mutants (He et al., 2014).

The remarkable convergence of transcriptional regulatory mechanisms on the *glp* operon suggests a finely tuned, central role for these gene products in the tick. This operon is regulated during conditions that mimic the tick environment by both $Rel_{Bbu}$ and DksA, Rrp1 and PlzA, as well as RpoS, the DNA-binding protein BadR, temperature and glycerol (Ojaimi et al., 2003; Caimano et al., 2007; He et al., 2011; Bugrysheva et al., 2015; Caimano et al., 2015; Drecktrah et al., 2015; Arnold et al., 2018; Zhang et al., 2018; Boyle et al., 2019). In fact, mutant strains with compromised glycerol metabolism, likely provided by the bloodmeal or tick itself, show a significant survival defect in ticks (Pappas et al., 2011). The utilization of glycerol within the tick phase of the *B. burgdorferi* life cycle is a prime example of the multiple mechanisms of gene regulation invoked by the spirochete in response to the arthropod environment in order to transit through the enzootic cycle (Caimano et al., 2016).

Overall, a substantial fraction of our current knowledge of gene regulation is supported by the initial microarray and RNA-seq studies, as these significantly contributed to our understanding of differentially expressed *B. burgdorferi* genes that might function in the adaptation of spirochetes to diverse environments *in vivo*. A collection of molecular tools has been developed (Gilbert et al., 2007; Brisson et al., 2012; Samuels et al., 2018), which should foster new studies delineating the regulatory mechanisms of *B. burgdorferi* genes important for interaction and persistence in the tick environment (Alverson et al., 2003; Caimano et al., 2005; Caimano et al., 2007; Lybecker and Samuels, 2007; Xu et al., 2007) (see also Radolf and Samuels, 2021). Further work is certainly needed to understand the intrinsic signal transduction pathways in spirochetes, as well as the details of the environmental factors that influence borrelial gene expression in ticks.

## *B. burgdorferi* genes known to function in ticks

The *B. burgdorferi* genome encodes approximately 1,780 genes that are distributed among a single chromosome and 21 plasmids (Fraser et al., 1997; Casjens et al., 2000; Casjens et al., 2011; Schutzer et al., 2011; Casjens et al., 2012) (see also Radolf and Samuels, 2021). The vast majority of these genes have been annotated as "hypothetical proteins", as they have little resemblance to known proteins in existing databases, and could therefore possibly carry out specialized functions in the spirochete's enzootic life cycle (Fraser et al., 1997;

Casjens et al., 2000). Previous efforts have focused on identifying the borrelial genes that are selectively upregulated at specific phases of the spirochete life cycle. As discussed above, microarray studies of the transcriptome profiling of cultured *B. burgdorferi* could identify genes that exhibit differential expression in response to environmental changes and are relevant to its life cycle and transmission through ticks. Indeed, mutagenesis studies indicate that preferentially regulated *B. burgdorferi* gene products (Grimm et al., 2004b; Pal et al., 2004b; Yang et al., 2004; Li et al., 2007b; Neelakanta et al., 2007; Radolf et al., 2012) could serve important functions in supporting the natural cycle of the spirochete. The following sections describe *B. burgdorferi* genes that are known to be highly expressed in the arthropod vector, many of which are thought to be essential in ticks (Grimm et al., 2004b; Pal et al., 2004b; Yang et al., 2004; Munderloh and Kurtti, 2005; Revel et al., 2005; Rosa et al., 2005; Li et al., 2007a; Li et al., 2007b; Pal et al., 2008a; Radolf et al., 2012; Kung et al., 2013). A recent study utilizing a genome-wide screen also identified a novel set of genes required for *B. burgdorferi* survival in the *Ixodes* vector (Phelan et al., 2019); many of these genes are encoded on plasmids, including *ospAB*, *ospC*, *ospD*, and *bbe16*, or on the chromosome, such as *dps* and *la7*.

## *B. burgdorferi* plasmids

The *B. burgdorferi* genome contains nine circular (cp) and 12 linear (lp) plasmids (Fraser et al., 1997; Casjens et al., 2000) (see also Radolf and Samuels, 2021). Several studies have indicated that specific plasmids are indispensable for *B. burgdorferi* survival in ticks (Purser and Norris, 2000; Grimm et al., 2005; Rosa et al., 2005; Stewart et al., 2005). As noted earlier, microarray studies have demonstrated that, compared to chromosomal genes, the expression of plasmid-encoded genes is more responsive to environmental changes, suggesting that they have pivotal roles in spirochete survival in diverse environments (Ojaimi et al., 2002; Brooks et al., 2003; Tokarz et al., 2004; Caimano et al., 2007; Iyer et al., 2015). *B. burgdorferi* isolates that lack certain plasmids reinforce the roles of plasmid-encoded genes in the spirochete life cycle in nature. While two linear plasmids, lp25 and lp28–1, were required for borrelial persistence in the mammalian host, only lp25 was found to be essential for tick infection (Purser et al., 2003; Grimm et al., 2004a; Strother et al., 2005; Strother and de Silva, 2005). *bbe22* on lp25 encodes for a nicotinamidase that is most likely essential to the biosynthesis of nicotinamide adenine dinucleotide. The complementation of *bbe22* alone was sufficient to restore the ability of spirochetes lacking lp25 to infect mice (Purser et al., 2003), whereas *bbe22* complementation only partially restored tick infectivity (Strother and de Silva, 2005).

*B. burgdorferi* that was deficient in lp28–4 also displayed reduced abilities to persist in ticks and transmit to mice (Strother et al., 2005). The same study also determined that the plasmids lp5, lp28–1, and cp9 were not required for spirochete infectivity in the tick gut. Additionally, *B. burgdorferi* lacking the plasmid lp36 showed no survival defects in ticks, while murine infectivity was significantly compromised (Jewett et al., 2007). Individual genes carried on the same plasmid may have selective host- or vector-specific roles. For example, genes on the plasmid lp54 include *ospA*, which is essential in ticks (Yang et al., 2004), and *dbpAB*, which is important in mice (Liang et al., 2004; Shi et al., 2006; Shi et al., 2008). Microarray studies also indicated the differential expression of genes from many of

the *B. burgdorferi* plasmids, most notably lp54, when spirochetes were grown in the presence or absence of mammal-specific factors, such as blood, or when cultured within a DMC (Brooks et al., 2003; Tokarz et al., 2004; Caimano et al., 2007; Iyer et al., 2015).

### *ospA* and *ospB*

Borrelial genes *ospA* and *ospB* constitute a single operon and encode some of the most abundant outer membrane lipoproteins of *B. burgdorferi*. OspA in particular is shown to interact with multiple host receptors, such as tick receptor TROSPA (Pal et al., 2004a) and plasminogen (Fuchs et al., 1994). Both *ospA* and *ospB* show vector-specific expression in the *Borrelia* life cycle, with both exhibiting upregulation when spirochetes exit an infected mammalian host and migrate to feeding *Ixodes* ticks (de Silva et al., 1996; de Silva and Fikrig, 1997; Schwan and Piesman, 2000; Schwan, 2003; Fikrig et al., 2004; Neelakanta et al., 2007). Recombinant OspA and OspB specifically adhere to the tick gut, suggesting that these outer surface lipoproteins are important for spirochete adaptation within the gut (Pal et al., 2000; Fikrig et al., 2004). The experimental transfer of non-borreliacidal OspA or OspB antibodies into the murine host prevented the tissue adherence of spirochetes in feeding ticks *in vivo*, further implicating these proteins in the colonization of *Borrelia* in the tick gut (Pal et al., 2001). An *ospAB* null mutant has also been generated and used in tick-mouse infection studies (Yang et al., 2004), where it retained full murine infectivity and pathogenicity, but was unable to infect ticks. OspA/B-deficient *B. burgdorferi* migrated from infected mice into ticks, but failed to survive in the feeding tick gut, reinforcing the critical role of the *ospAB* operon in the maintenance of *Borrelia* in ticks. An OspB-deficient *B. burgdorferi* isolate was generated, and infection studies further demonstrated that OspB is also involved in the colonization and persistence of spirochetes in the arthropod vector (Neelakanta et al., 2007). These experiments demonstrate the importance of both OspA and OspB in *B. burgdorferi* colonization in the tick gut.

**ospC**—OspC is a prominent outer membrane lipoprotein of *B. burgdorferi* that is induced in ticks during the transmission of spirochetes from the feeding vector to the host (Schwan et al., 1995). *ospC* is the archetype of RpoS-dependent genes that are associated with tick transmission and host infection (Hubner et al., 2001; Yang et al., 2003; Fisher et al., 2005). Studies with *ospC* deletion mutants have demonstrated that the gene product is essential for infecting the murine host (Grimm et al., 2004b; Pal et al., 2004b; Dunham-Ems et al., 2012). However, the exact role of OspC in the borrelial transmission cycle through ticks warrants further investigation, as there are conflicting results about the phenotype of *ospC* mutants in ticks. In one study, the *ospC* null mutant failed to invade *I. scapularis* salivary glands in significant numbers, and was unable to establish infection in the mammalian host (Pal et al., 2004b). Subsequent studies showed that OspC binds a tick salivary gland protein, Salp15, and that the OspC-tick protein interaction facilitated the establishment of spirochete infection in mice (further discussed below). A study involving *B. afzelii*, which is prevalent in Europe and Asia and is transmitted by *Ixodes* ticks, also supports the notion that OspC is critical for spirochete dissemination from the tick gut to the salivary gland (Fingerle et al., 2007). The complementation of a naturally-occurring *ospC* mutant with a wild-type copy of the *ospC* gene resulted in more efficient migration to the salivary glands (Fingerle et al., 2007). Another group independently created a series of *ospC* mutants and assayed them in

tick-mouse infection studies (Grimm et al., 2004b; Stewart et al., 2006; Tilly et al., 2006; Tilly et al., 2007). Their results showed that OspC is required for establishing infection in the murine host; however, *ospC* deletion had no influence on the migration of *B. burgdorferi* from the tick gut to the salivary gland. A more recent study also demonstrated that *Borrelia* requires one or more RpoS-dependent genes to migrate through the midgut, although OspC function is not required for the process, as it exclusively functions within the mammal (Dunham-Ems et al., 2012). Therefore, results indicating disparate phenotypes of OspC deletion are likely due to the variations in the experimental approaches to tick infection (microinjection versus immersion infection), spirochete detection modalities, and the use of different isolates of *B. burgdorferi* (strain 297 versus B31), all of which may have contributed to the overall divergent results. The kinetics of pathogen dissemination from the tick gut to the salivary gland could also vary among isolates. Finally, host-derived plasminogen, which is required for efficient spirochete dissemination through ticks (Coleman et al., 1995; Coleman et al., 1997), also is known to bind OspC (Lagal et al., 2006; Onder et al., 2012). Nonetheless, further studies are needed to clarify how the specificity of interactions between the same spirochete antigen, such as OspC, with multiple host or vector molecules, such as plasminogen or Salp15, contributes to borrelial infection. OspC has recently been implicated in various additional functions within mammals that support spirochete survival, such as an antiphagocytic factor (Carrasco et al., 2015), binding to the complement component C4b (Caine et al., 2017), or joint invasion and colonization in a strain-specific manner (Lin et al., 2020).

**dps/napA/bicA**—One of the *B. burgdorferi* chromosomal genes that displays regulated expression in the borrelial enzootic cycle is *bb0690*, which encodes a protein similar to the DNA-binding protein of the starved bacteria (Dps) family of proteins (Li et al., 2007b), also known as NapA and BicA (Wang et al., 2012). The protein represents the ortholog of bacterioferritin (Rivera, 2017) fused to a copper-binding metallothionein-like domain, as described in pathogenic Mycobacteria (Gold et al., 2008). *dps* expression is low throughout *B. burgdorferi* infection in mice but is high during tick intermolt periods. Notably, its expression is repressed in spirochetes during recovery from starvation *in vitro* (Drecktrah et al., 2015). Dps-deficient spirochetes retained full murine infectivity, readily migrated to feeding arthropods, and persisted in replete ticks. The mutant, however, failed to survive throughout the extended intermolt periods of resting *I. scapularis*, indicating that Dps is critical to the persistence of spirochetes in unfed ticks (Li et al., 2007b). The protein was later suggested to sequester iron and copper, thus promoting spirochete survival *in vivo* via the detoxification of metal ions, which the pathogen may encounter during its natural life cycle (Wang et al., 2012).

**ospD**—OspD is an outer membrane lipoprotein of *B. burgdorferi* that shows tightly regulated expression in the spirochete life cycle. *ospD*, in general, exhibits elevated expression in ticks as compared to mice (Li et al., 2007a). Two studies examined *ospD* expression in select stages of the tick-mouse infection cycle of *B. burgdorferi* (Li et al., 2007a; Stewart et al., 2008). Although the results from these studies differ in their kinetics of *ospD* induction, both show that *ospD* transcripts are significantly expressed in a narrow time window that corresponds to the phase of tick detachment from the host after feeding. An

OspD-deficient isolate of *B. burgdorferi* was created, and subsequent examination of the mutant phenotype in mice indicated that OspD has no obvious influences on *B. burgdorferi* survival in the murine host, or on spirochete acquisition by ticks (Li et al., 2007a). Recombinant OspD binds to tick gut lysates *in vitro*, suggesting the protein's role in spirochete adherence to tick tissue (Li et al., 2007a). However, while the *ospD* mutant has a reduced capacity for attachment to the tick gut *in vivo*, this does not influence subsequent spirochete transmission during a second blood meal. Taken together, these data suggest that OspD may have a secondary or supportive role in *B. burgdorferi* persistence within ticks (Stewart et al., 2008).

**bptA**—BptA, annotated as *bbe16*, is a lipoprotein that is possibly surface-exposed. *bptA* is located on the plasmid lp25, which has been closely associated with *Borrelia* infectivity (Purser and Norris, 2000; Purser et al., 2003; Strother et al., 2005; Strother and de Silva, 2005). A microarray analysis first identified that *bbe16* is a differentially expressed gene, with upregulation when *B. burgdorferi* was cultivated in DMC, as compared to spirochetes grown in culture similar to the environment of fed ticks (Revel et al., 2002). Later studies showed that *bptA* is selectively expressed in culture conditions mimicking the tick, suggesting its functional role in spirochete persistence in the vector (Revel et al., 2005). *In vivo* analyses of infectious isolates of *bptA* mutants and complemented isolates demonstrated a requirement for BptA in borrelial persistence within ticks (Revel et al., 2005). The exact function of the protein in the *B. burgdorferi* natural cycle, however, remains to be elucidated.

**la7**—*B. burgdorferi* gene *la7*, annotated as *bb0365*, encodes P22, which is also known as lipoprotein LA7 (Lam et al., 1994; Grewe and Nuske, 1996). *la7* is produced in the mammalian host (von Lackum et al., 2007) and is highly upregulated in spirochetes upon their entry into feeding ticks (Pal et al., 2008a). Mutant spirochetes lacking *la7* do not differ from the parental isolates in the establishment of murine infection (Pal et al., 2008a). When naïve ticks engorge on spirochete-infected mice, the *la7* mutant enters ticks, but displays markedly decreased survival within feeding ticks, as compared to wild-type *B. burgdorferi*. LA7 may, therefore, be important for spirochete survival in feeding arthropods. A recent analysis of the high-resolution structure of LA7 will likely contribute to our understanding of the biological function of the protein (Brangulis et al., 2020a).

**Additional genes**—Specific sets of genes, located either on plasmids or the chromosome, show induced expression in ticks; a few of these have already proven to be of functional significance. Many of these proteins may be involved, either directly or indirectly, in interactions with tick molecules, thereby supporting spirochete survival in the vector, as discussed below. Additional borrelial genes have also been identified as being expressed *in vivo*, although they lack essential supportive roles. *B. burgdorferi* mutants were generated with a lack of *luxS* (Hubner et al., 2003; Tilly et al., 2004) or *chbC* (Hubner et al., 2003; Tilly et al., 2004), whose gene products are implicated in a quorum sensing pathway and other potential biochemical roles (Hubner et al., 2003; Tilly et al., 2004) or the transport of the tick cuticle component chitobiose, respectively. The functions of *luxS* or *chbC* are, however, not essential for spirochete persistence in ticks, nor during the natural infection

cycle (Hubner et al., 2003; Tilly et al., 2004). More recently, a CdnL-type CarD regulator in *B. burgdorferi*, termed as LtpA, has been shown to support pathogen survival in ticks (Chen et al., 2018). A *Borrelia* protein annotated as BBE31, the crystal structure of which recently was solved (Brangulis et al., 2020b), is known to aid spirochete movement from the tick gut to the hemolymph during spirochete transmission (Zhang et al., 2011). A surface-exposed borrelial protein, enolase, has been shown to support the interaction of *B. burgdorferi* with plasminogen (Floden et al., 2011; Toledo et al., 2011; Nogueira et al., 2012), contributing to pathogen survival within feeding ticks (Nogueira et al., 2012). Another regulatory protein, annotated as BBD18, is thought to promote spirochete acquisition in ticks, via the regulation of RpoS (Hayes et al., 2014). A polyamine uptake system, termed as PotABCD and encoded by *bb0639–0642*, likely supports the vector phase of borrelial replication and survival (Bontemps-Gallo et al., 2018). The BpuR DNA/RNA-binding protein in *B. burgdorferi* (Jutras et al., 2013), which influences the translation of superoxide dismutase, has been shown to undergo preferential expression in ticks and to enhance spirochete survival in the vector (Jutras et al., 2019). One of the borrelial virulence determinants, annotated as Lmp1, is a surface-exposed and potentially multi-domain protein that is involved in various functions in spirochete infectivity (Yang et al., 2009; Yang et al., 2010). Recent studies indicate that Lmp1 contributes to host-pathogen interactions, including spirochete transmission from ticks to mammals (Zhuang et al., 2018), and possibly spirochete dissemination through tick tissues (Koci et al., 2018), which requires essential contributions from both the N-terminal and middle regions of Lmp1. The gene encoding BBA57 is constitutively expressed during the *Borrelia* infectious cycle involving both mice and ticks (Yang et al., 2013). Although it is unclear whether or how the protein supports *B. burgdorferi* infection in ticks, it is known to facilitate early spirochete infection in mice through the impairment of the host complement and neutrophil-derived antimicrobial defense mechanisms (Bernard et al., 2018).

The recent development of genetic tools has allowed researchers to create specific mutants of *B. burgdorferi* and to identify borrelial proteins that function at different stages in the vector phase of the spirochete life cycle (Radolf et al., 2012; Samuels et al., 2018). As *B. burgdorferi* spends a significant part of its life inside the arthropod, additional genes that are important in ticks are likely to be identified. High-throughput expression analyses, such as quantitative RT-PCR and RNA amplification-based microarray assays, have the potential to reveal genes that are highly expressed in ticks and may perform specialized functions in the complex enzootic life cycle of *B. burgdorferi*. In fact, with an ingenious use of genetic tools and a high-throughput screen, a recent seminal study detailed the identification of a set of novel genes that are required for borrelial survival in *Ixodes* ticks (Phelan et al., 2019). It was shown that many of these essential genes encode proteins of unknown function, membrane-associated proteins, or lipoproteins, which could support host-pathogen interactions or other important aspects of spirochete biology. *B. burgdorferi* harbors a homolog of the hibernation promotion factor, *bb0449*, although the gene function has been characterized as redundant for spirochete survival *in vivo*, including within the tick (Fazzino et al., 2015). The Rel$_{Bbu}$, the RelA/SpoT homolog in *B. burgdorferi*, regulates the stringent response and global gene expression in spirochetes and is required for pathogen survival in the tick (Drecktrah et al., 2015). Finally, the *ssrS* gene encoding the Bb6S RNA of *B.*

*burgdorferi*, which is considered as one of the first regulatory RNAs, has been shown to control the expression of multiple lipoproteins involved in host infectivity (Drecktrah et al., 2020).

## Molecular interfaces: interactions of microbial and vector gene products

Regardless of the nature of the ecological relationship between a microbe and its host, the development of specific molecular interactions is an essential prerequisite for the survival of the microbe *in vivo* (Finlay and Cossart, 1997; Dale and Moran, 2006). Mainly based on the vector-specific expression of limited *B. burgdorferi* gene products, scientists have uncovered a handful of discrete interactions between the spirochete and tick vector that are summarized below. These studies have established that certain borrelial proteins contribute to the maintenance of the spirochete infection cycle within ticks, either directly or via molecular interactions with specific vector receptors.

### Interaction of *B. burgdorferi* with tick gut proteins

**TROSPA—**The tick receptor for OspA (TROSPA), which is predominantly expressed in the gut of *I. scapularis*, specifically interacts with *B. burgdorferi* OspA (Pal et al., 2004a). This receptor-ligand interaction is further explained by the findings that TROSPA co-localizes with OspA in the tick gut *in vivo*, and that OspA specifically bound recombinant TROSPA in a series of *in vitro* assays (Pal et al., 2004a). It was later shown that the N-terminal portion of TROSPA, which contains a putative transmembrane domain, is not involved in the OspA interaction, and that charges on the TROSPA protein influence its interaction with OspA (Figlerowicz et al., 2013). Interestingly, *TROSPA* expression within the tick was upregulated during spirochete infection and decreased during engorgement, suggesting its regulation by extrinsic cues. When uninfected ticks parasitized infected mice that had been passively administered TROSPA antisera, the acquisition of *B. burgdorferi* by the ticks was significantly reduced, as compared to those that fed on infected mice treated with pre-immune sera. TROSPA-immunized ticks also demonstrated a reduced capacity for pathogen transmission. Similarly, the RNA interference of *TROSPA* in nymphal ticks significantly diminished their ability to acquire *B. burgdorferi* from infected mice (Pal et al., 2004). The spirochetes appear to colonize the tick gut by adhering to TROSPA through OspA, which highlights the importance of this interaction in supporting the borrelial infection cycle in ticks. Nevertheless, the native function of TROSPA in the arthropod is currently unknown. *TROSPA* is not consistently expressed in all stages of *I. scapularis*, and its expression is highly influenced by environmental signals, such as blood meal engorgement or *B. burgdorferi* infection. These observations indicate a developmental function, rather than a housekeeping role, of TROSPA in tick biology.

**Dystroglycan-like protein (ISDLP)—***I. scapularis* dystroglycan-like protein (ISDLP) is a *B. burgdorferi*-interacting tick gut protein with similarities to the dystroglycan family of proteins (Coumou et al., 2016). ISDLP is expressed on the gut epithelial surface and is induced during tick feeding and by borrelial infection. Additionally, this tick protein can bind to *B. burgdorferi*, and its knockdown by RNA interference has been shown to impair

pathogen transmission, suggesting an important role in spirochete dissemination from ticks to mice.

**TRE31 and Ixofin3D**—Two tick gut proteins were described that support the dissemination of *B. burgdorferi* from the tick gut, particularly during pathogen transmission to the host. TRE31 was characterized as a tick gut protein that specifically interacts with BBE31, one of the borrelial outer surface lipoproteins (Zhang et al., 2011). The gene is preferentially expressed in the tissues of fed ticks, including the gut, hemolymph, and salivary glands. BBE31 antiserum impaired spirochete migration from the tick gut to the salivary glands, consequently attenuating murine infection by tick-transmitted *B. burgdorferi*. The silencing of *tre31* also lowered the pathogen burden in the tick hemolymph. These data indicate that TRE31 plays a role in enabling spirochete movement by interacting with BBE31. The crystal structure of BBE31 was recently solved, which will be important for a better understanding of the specific ligand-receptor interactions that support *B. burgdorferi* movement in the tick (Brangulis et al., 2020b). Additionally, Ixofin3D represents another tick gut protein that is relevant to pathogen dissemination, with similarities to putative fibronectin type III domain-containing proteins (Narasimhan et al., 2014a). As with TRE31, the antibody or RNA interference-mediated impairment of Ixofin3D resulted in decreased spirochete burdens in the tick salivary glands and in the murine host.

**Dual oxidase**—A dual oxidase in the tick gut (Duox), which is a member of the NADPH oxidase family, is involved in the maintenance of gut microbial homeostasis and the genesis of an acellular gut barrier called the dityrosine network (DTN) (Yang et al., 2014). The DTN develops from the tyrosine cross-linking of the extracellular matrix in *I. scapularis* ticks during blood meal engorgement. This Duox-mediated barrier influences the survival of *B. burgdorferi* in the gut, as an impaired DTN in *duox*-knockdown ticks can result in reduced levels of pathogen persistence within ticks. The absence of complete DTN formation in the tick gut activates a specific tick innate immune pathway, via the induction of nitric oxide synthase, which ultimately reduces the spirochete burden in the arthropods (Yang et al., 2014). Deeper understandings of the tick innate immune responses and the related vector-pathogen interactions, such as the one orchestrated by Duox, along with their contributions to microbial survival, are critical to the development of new interventions against tick-borne infections, including Lyme borreliosis.

**PIXR**—A Reeler domain-containing tick gut protein was described that influences the colonization of *B. burgdorferi* in the tick gut (Narasimhan et al., 2017). The gene product, which was termed "Protein of *I. scapularis* with a Reeler domain" (PIXR), is induced by spirochete infection. Both the knockdown of *pixr* via RNA interference and the antibody-mediated inhibition of PIXR reduced the ability of *B. burgdorferi* to infect the tick gut. Notably, PIXR was shown to be involved in biofilm formation, both *in vitro* and *in vivo*. The impairment of the PIXR function in ticks also had diverse effects on intrinsic and extrinsic elements within the arthropods, including their gut microbiome, metabolome, and immune responses. Additionally, PIXR has a proposed role in tick developmental biology, as the abrogation of its function impaired larval molting (Narasimhan et al., 2017). Subsequent analyses also revealed that the *Ixodes* microbiota is sensitive to anti-tick immunity, with

biofilm formation potentially serving as the microbiome's defensive response to this immunity (Estrada-Pena et al., 2020).

**IsCDA—**One of the first detailed characterizations of the *Ixodes* peritrophic membrane (PM) involved a mass spectrometric analysis of the structure in ticks (Kariu et al., 2013). This revealed information about the *I. scapularis* CDA-like protein, or IsCDA, which is an immunogenic, antigenic, and predominant (60-kDa) component of the PM, with homology to arthropod chitin deacetylase. IsCDA is mainly expressed in the gut and is induced during early tick feeding. Interestingly, the knockdown of *IsCDA* via RNA interference failed to influence PM formation or spirochete persistence, suggesting nonessential roles of the protein in tick biology and host-pathogen interaction. However, IsCDA immunization selectively impaired *B. burgdorferi* survival in the gut, but not the rest of the microbial community, implying that cross-reactive IsCDA antibodies may influence PM functions relevant to spirochete persistence in ticks. It is therefore speculated that the tick PM plays a preferential role in limiting the persistence of *B. burgdorferi* within the vector.

### Interaction of *B. burgdorferi* with tick salivary gland proteins

Since the discovery of immunodominant antigens that are deposited in the host dermis by feeding ticks, *I. scapularis* salivary gland proteins (Salps) have been an intense focus of *Borrelia* research (Das et al., 2000; Das et al., 2001; Francischetti et al., 2005; Ribeiro et al., 2006). Indeed, such so-called saliva-assisted transmission (SAT) (Nuttall, 2019) events have been studied in detail for many other vector-borne diseases, and novel salivary gland vaccine targets have been identified, such as for the leishmaniasis infection (Morris et al., 2001; Valenzuela et al., 2001). Many Salps are soluble, feeding-induced, and co-transmitted to the host along with *B. burgdorferi*; thus, these proteins have an opportunity to influence spirochete migration between the vector and host, either directly or indirectly (Ramamoorthi et al., 2005; Rosa, 2005; Radolf et al., 2012). The generation of host immunity against *I. scapularis* salivary gland proteins, particularly those expressed as early as 24 hours after feeding, is sufficient for the impairment of *B. burgdorferi* transmission between ticks and hosts (Narasimhan et al., 2007). The following are examples of tick Salps that influence spirochete infection.

**Salp15—**As discussed earlier, OspC is a prominent borrelial outer membrane lipoprotein that is swiftly upregulated during tick transmission (Schwan et al., 1995) and binds the tick salivary gland protein Salp15 (Anguita et al., 2002; Ramamoorthi et al., 2005). The OspC-Salp15 interaction has important functional consequences that impact spirochete transmission and pathogenesis in a multipartite manner, involving the pathogen, vector, and host (Ramamoorthi et al., 2005). *B. burgdorferi* carries Salp15 (via OspC binding) during its transmission from the tick salivary gland to the murine host, thereby facilitating *Borrelia* survival within the infected mammal (Anguita et al., 2002). Salp15 specifically binds *B. burgdorferi* OspC and prevents the killing of spirochetes by borreliacidal antibodies *in vitro*. Thus, the coating of the spirochetes via binding to Salp15 in the tick salivary gland could have important implications for pathogen survival in the host. The binding of Salp15 to *Borrelia* could potentially mask the exposure of OspC to components of the host immune system, as OspC is shown to be highly immunogenic and is a target of host borreliacidal

antibodies (Wilske et al., 1993; Fung et al., 1994; Wilske et al., 1996; Mbow et al., 1999; Liang et al., 2002; Wilske, 2005; Buckles et al., 2006; Earnhart et al., 2007; Earnhart and Marconi, 2007b, a). Additionally, by itself, Salp15 carries host immunosuppressive properties by inhibiting CD4^{+} T cell activation (Anguita et al., 2002). Thus, OspC-bound Salp15 provides further benefits to spirochetes, potentially affording protection against immune cells that infiltrate the tick bite site. Interestingly, the OspC-Salp15 interaction could be beneficial to both the vector and pathogen (Ramamoorthi et al., 2005). *B. burgdorferi* induces *salp15* expression in the salivary glands of feeding arthropods; therefore, borrelial infection may provide survival advantages to the arthropod that produces this protein, as it could promote effective feeding via the binding of Salp15 and plasminogen at the bite site (Coleman et al., 1995; Coleman et al., 1997; Ramamoorthi et al., 2005; Lagal et al., 2006).

**Salp25D—**One of the abundant tick Salps, Salp25D (Das et al., 2001), plays an important role in the mammalian host in regards to the acquisition of *B. burgdorferi* by the vector (Narasimhan et al., 2007). The silencing of *salp25D* in *Ixodes* salivary glands has been revealed to block spirochete acquisition by ticks from infected mice. Salp25D functions as an antioxidant; thus, the Salp25D-mediated detoxification of reactive oxygen radicals at the tick-pathogen-host interface potentially provides a survival advantage to *B. burgdorferi* at the bite site in mice.

**Salp20—**In addition to Salp15, which binds to *B. burgdorferi* and contributes to the protection of spirochetes against host immunity, Salp20 has also been shown to protect serum-sensitive strains of *Borrelia* from lysis in *in vitro* assays (Tyson et al., 2007). Accordingly, Salp20 may also protect spirochetes from the host complement system within the vector blood meal and during transmission. Salp20 is related to the *I. scapularis* anti-complement *(Isac)* family of proteins that may block the host alternative complement pathway (Soares et al., 2005; Ribeiro et al., 2006). RNA interference studies have established that *Isac* and related family members of tick proteins promote efficient tick feeding and spirochete growth in ticks (Soares et al., 2005).

**Tick histamine release factor—**Histamine release factor (tHRF) was identified as an *Ixodes* salivary gland protein induced by *B. burgdorferi* infection (Dai et al., 2010). Recombinant tHRF binds host basophils and stimulates histamine release. Tick saliva, from which tHRF is secreted, is known to induce specific host chemokines, including histamine-releasing cytokine, that favor feeding by ticks (Langhansova et al., 2015). As the expression of tHRF varies in correlation to the rapid feeding phase of the tick, it was speculated that the protein contributes to blood meal engorgement and likely influences spirochete transmission from the arthropod. The RNA interference-mediated knockdown of tHRF impaired tick feeding and also decreased *Borrelia* levels in the murine hosts (Dai et al., 2010). Vaccination with tHRF reduced both the tick feeding efficiency and *B. burgdorferi* burden in mice. Additionally, it was reasoned that tHRF in ticks serves to facilitate vascular permeability and to increase blood flow to the tick bite site, ultimately favoring their blood meal engorgement (Dai et al., 2010). These data highlight the vaccine potential of tHRF and its ability to interfere with tick feeding and pathogen transmission.

**Tick salivary lectin pathway inhibitor**—A tick protein designated as tick salivary lectin pathway inhibitor (TSLPI) was found to impair the host complement-mediated killing of *B. burgdorferi* (Schuijt et al., 2011). TSLPI was shown to interfere with the human lectin complement cascade (Wagemakers et al., 2016), which reduced neutrophil phagocytosis and chemotaxis, thus decreasing the lysis of *B. burgdorferi* cells. TSLPI-knockdown ticks, or ticks that fed on TSLPI-vaccinated mice, were unable to transmit the pathogen efficiently. These studies underscore the importance of the lectin complement cascade and associated immune responses (Coumou et al., 2019) in borrelial transmission through *Ixodes* ticks and the establishment of early infection in mammals.

**Additional salivary gland proteins**—In a recent study, researchers identified another *Ixodes* Salp, termed Salp12, which is involved in the vector chemoattractant for the spirochete (Murfin et al., 2019). The gene encoding Salp12 is expressed in the salivary glands and gut. Interference with the function of Salp12 impacted spirochete acquisition by ticks, but not pathogen transmission. In addition to *I. scapularis* Salp15, as detailed in earlier paragraphs, novel Salp15-like immunosuppressant genes were also shown to be produced from the salivary glands of other *Ixodes* ticks, such as *I. persulcatus* (Mori et al., 2010). Additionally, salivary lipocalins that are differentially expressed during infection with *B. afzelii* are thought to support pathogen transmission to the host (Valdes et al., 2016). A panel of immunogenic proteins in the saliva of 24h-fed female *I. scapularis* was also identified, some of which may be involved in interactions with *B. burgdorferi*, consequently influencing spirochete transmission through ticks (Lewis et al., 2015).

## Interactions with additional tick proteins

**Dae2**—A novel antibacterial enzyme was discovered to be acquired by *Ixodes* ticks through lateral gene transfer from a bacterium during the early evolution of ticks and mites. The enzyme, termed Dae2 (domesticated amidase effector 2), shares similarities to peptidoglycan-degrading toxins and specifically limits the proliferation of *B. burgdorferi* in ticks (Chou et al., 2015). Dae2 was also identified as one of the *Ixodes* antimicrobial effector molecules that are downstream of a newly discovered cross-species interferon signaling pathway. This pathway, which is triggered by the mammalian IFNγ that is acquired in the tick blood meal, induces a tick Rho-like GTPase in a STAT-dependent manner, ultimately reducing the levels of *B. burgdorferi* via the induction of select antimicrobial proteins, including Dae2 (Smith et al., 2016).

**Tick antimicrobial peptides (defensin and others)**—Defensins are a well-known class of antimicrobial peptides of innate immunity that are effective against a wide range of pathogens (Ganz, 2003); not surprisingly, *Ixodes* ticks encode multiple defensin-like genes (Hynes et al., 2005; Smith and Pal, 2014; Gulia-Nuss et al., 2016; Smith et al., 2016), some of which are induced in the gut after infection with *B. burgdorferi* (Sonenshine et al., 2002; Rudenko et al., 2005; Smith et al., 2016) (see also Radolf and Samuels, 2021). Two isoforms (Chrudimska et al., 2011) and six novel putative defensins from *I. ricinus* were identified that may have antimicrobial activity (Tonk et al., 2014b). It is unclear whether defensins play a role in controlling *B. burgdorferi* infection in ticks (Smith et al., 2016); however, two chemically-synthesized defensin peptides based on *I. scapularis* tick defensins (Tonk et al.,

2014b), termed as Scapularisin-3 and Scapularisin-6, carry potent anti-*Listeria* and antifungal activities (Tonk et al., 2014a). Codon-substitution models support the neutral evolution of *Ixodes* defensins, resulting in its vast gene expansion (Wang and Zhu, 2011). Other than these defensins, the *Ixodes* tick genome also contains genes that encode a diverse array of antimicrobial proteins or peptides (Smith et al., 2016), some with potential anti-*B. burgdorferi* activities. Tick saliva is reported to possess anti-alarmin effects during the transmission of *Borrelia*, thereby aiding in the establishment of infection in the host (Marchal et al., 2011). Furthermore, events like blood meal digestion in ticks generate novel peptides with antimicrobial activities (Sonenshine, 1993; Kopacek et al., 2010), and the recent deep sequencing analysis of the *I. ricinus* hemocytome suggests additional genes that may encode still more antimicrobial peptides (Kotsyfakis et al., 2015).

Other *Ixodes* tick proteins, even ones that are intracellular, are shown to be induced upon *B. burgdorferi* infection in the vector. One such example is a selenoprotein that is involved in the regulation of oxidative and endoplasmic reticulum stress. However, the mechanisms by which intracellular *Ixodes* proteins support pathogen survival in ticks remain enigmatic (Kumar et al., 2019). Nevertheless, these studies collectively indicate the existence of highly discrete molecular interfaces where specific microbial and vector gene products interact, either directly or indirectly, thus contributing to *B. burgdorferi* survival in a natural enzootic cycle.

## Interface between tick immune system and *B. burgdorferi*

Despite substantial existing knowledge of arthropod innate immunity (Hoffmann et al., 1999; Irving et al., 2001; Hoffmann and Reichhart, 2002; Hoffmann, 2003; Ferrandon et al., 2007; Lemaitre and Hoffmann, 2007; Clayton et al., 2014), the molecular mechanisms by which the *Ixodes* immune system interfaces with and recognizes invading microbes remain poorly understood. Recent studies suggest that *B. burgdorferi* levels in ticks are affected by the independent knockdown of components from specific immune pathways, providing the first evidence of an active interface between the tick immune system and the agents of Lyme disease (Smith et al., 2016; Shaw et al., 2017; Kitsou and Pal, 2018). Research has also revealed that the gut microbiota associates with the *Ixodes* immune system, playing critical and influential roles in arthropod biology, including the ability to maintain and transmit *B. burgdorferi* (Narasimhan et al., 2014b; Narasimhan et al., 2017). These studies highlight novel concepts of tick immune responses that probably involve direct and indirect methods of pathogen recognition, including that of *B. burgdorferi* (Kitsou and Pal, 2018), and therefore incorporate multifaceted interactions between invading microbes and defense responses, as summarized below.

The arthropod immune system likely recognizes microorganisms directly by germ line-encoded, non-rearranging cellular receptors, and controls infection through the rapid generation of microbicidal effector responses (Hoffmann and Reichhart, 2002; Hoffmann, 2003; Ferrandon et al., 2007; Valanne et al., 2011; Myllymaki et al., 2014; Smith and Pal, 2014; Shaw et al., 2018). Recent studies show that the *Ixodes* immune deficiency (IMD) pathway senses microbial molecular patterns, like that of infection-derived lipids, such as 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol and 1-palmitoyl-2-oleoyl diacylglycerol,

or diaminopimelic-type peptidoglycans, which are present in diverse bacterial pathogens including *B. burgdorferi*, ultimately leading to the generation of potent microbicidal responses in ticks (Shaw et al., 2017). The details of such molecular interactions between the tick immune system and spirochete cells or antigens, as well as the identity of any tick receptors that recognize microbial molecules, remain unknown. The tick E3 ubiquitin ligase X-linked inhibitor of apoptosis (XIAP) has recently been demonstrated to act as a molecular rheostat for the IMD pathway (McClure Carroll et al., 2019).

Studies of *Drosophila* and mammals also suggest the existence of alternative immune mechanisms, such as indirect or more host-centric immune recognition pathways, for interactions with virulent bacteria. These indirect immune cascades, including effector-triggered immunity (Cui et al., 2015), can sense pathogens or pathogenic-derived effector molecules that modify a host target molecule (Boyer et al., 2011). While examining the molecular mechanism of *B. burgdorferi* acquisition in *Ixodes* ticks, Smith et al. identified the existence of a novel cross-kingdom signaling cascade, incorporating both the mammalian host and tick vector (Smith et al., 2016). This cascade enables *I. scapularis* to recognize a mammalian cytokine, identified as IFNγ, which arrives in ticks via the ingested blood meal and functions as a potent infection cue by interacting with currently unknown tick receptor(s), thus activating microbicidal responses that limit the proliferation of invading spirochetes (de Silva, 2016; Smith et al., 2016). This novel IFNγ signaling involves the tick STAT protein, which induces an *Ixodes* GTPase (termed as IGTPase), as well as multiple borreliacidal effector molecules, including Dae2 (Chou et al., 2015), thereby reducing *B. burgdorferi* persistence in the arthropod (Smith et al., 2016). More recently, tick extracellular vesicles, resulting from exosome and microvesicle biogenesis, have been proposed to manipulate cross-kingdom signaling networks, potentially impacting pathogen spread and colonization in the host (Chavez et al., 2019). While these indirect or host-centric recognition pathways of infection and interaction between the tick immune system and invading microbe are emerging concepts, additional studies are required to enrich our fundamental knowledge of tick immunobiology and host-pathogen interactions.

## Microbiome interactions

In *Ixodes* ticks, *B. burgdorferi* resides in the gut, which is also inhabited by diverse microbiota, as indicated by a series of recent studies using either laboratory or wild-collected ticks (Narasimhan and Fikrig, 2015; Bonnet et al., 2017; Swei and Kwan, 2017; Couper and Swei, 2018; Greay et al., 2018; Hernandez-Jarguin et al., 2018; Aivelo et al., 2019; Landesman et al., 2019; Tokarz et al., 2019). The role of gut microbiota, its interactions with invading pathogens, and the maintenance of immune homeostasis are now well-established concepts in many organisms (Ley et al., 2008; Molina-Cruz et al., 2008; Round and Mazmanian, 2009; Hooper et al., 2012; Buchon et al., 2013; Kamada et al., 2013). The *Ixodes* gut microbiota was recently described as having an important role in the persistence of tick-borne pathogens like *B. burgdorferi* (Narasimhan et al., 2014b). The presence of pathogens in the tick gut, along with their possible interactions with resident gut microbiota, could impair tick structural barriers, such as the peritrophic matrix, via the modulation of tick proteins and/or specific immune signaling pathways. These complex interactions, involving diverse pathogens, gut microbiota, and tick proteins, essentially shape

the survival of spirochetes in the vector (Narasimhan et al., 2014b; Abraham et al., 2017). Further studies of tick-pathogen and immunome-gut microbiome interactions, particularly how they impact spirochete persistence, will be necessary to enhance our knowledge of tick-borne infections. The use of new experimental approaches, such as dysbiosis (Narasimhan et al., 2014b), aposymbiotic ticks, and the artificial feeder system, including ones recently developed (Oliver et al., 2016; Koci et al., 2018), which can eliminate unwanted host factors and allow an unprecedented degree of empirical manipulation, will be especially valuable for these investigations.

## *Ixodes* genomics

Two separate studies provided initial information about the complete genome sequence of *I. scapularis*, both in ticks (Gulia-Nuss et al., 2016) and an *Ixodes* cell line (Miller et al., 2018). The exploration of the *Ixodes* genome offers new avenues for scientific investigation and should reveal novel features of the microbe-vector interaction. However, further research on this subject largely awaits major developments in gene manipulation tools and the pursuit of additional genome sequencing efforts, which are summarized below.

### Gene manipulation

Stable methods of gene manipulation are well-developed in other arthropod vectors, such as in mosquitoes (Catteruccia et al., 2000; Catteruccia, 2007), and are influenced by the development of advanced genetic tools in the *Drosophila* (Venken and Bellen, 2005). Gene manipulation in ticks remains in its infancy because few of the available genetic tools are readily applicable. RNA interference (RNAi) is now viewed as the only possible gene-silencing technique in ticks (de la Fuente et al., 2007a), although the knockdown effects are transient. Unfortunately, unlike many other arthropods (Bucher et al., 2002; He et al., 2006; Khila and Grbic, 2007), the use of parental RNAi to introduce stable RNAi effects in offspring has brought limited success in ticks. Additional formidable challenges include the lack of information about tick embryology and the tick genome; further progress in these areas would stimulate the development of more advanced gene manipulation methodologies. However, the long life span and slow post-embryonic development of *Ixodes* ticks will pose serious problems for the phenotypic analysis of mutants after the genome has been manipulated. These efforts will be further complicated by the sensitivity of *I. scapularis* embryonic development, including premolt and preoviposition development, to environmental conditions, such as temperature (Heath, 1979; Ogden et al., 2004). Nevertheless, *I. scapularis* cell lines (Munderloh et al., 1994) are available to the research community, which should allow us to optimize the manipulation of the tick genome in cell culture.

**Whole genome sequence**—The *I. scapularis* Genome Project (IGP), a collaborative effort between the international community of tick researchers and genome sequencing centers, was initiated more than a decade ago with the support of the National Institutes of Health (Hill and Wikel, 2005; Pagel Van Zee et al., 2007), with the intention of performing whole genome sequencing of *I. scapularis*. More than 18 million trace reads, representing approximately a five-fold coverage of the genome, have been deposited thus far at the

National Center for Biotechnology Information (NCBI) Trace Archive (Pagel Van Zee et al., 2007). In addition, 20 *Ixodes* bacterial artificial chromosome (BAC) clones, 370,000 BAC-end reads, and more than 80,000 expressed sequence tags (ESTs) have been sequenced. The sequencing, assembly, and annotation of a six-fold coverage of the *Ixodes* genome were expected by 2008, but these efforts took longer due to the large genome size, genomic complexity, and presence of extensive repetitive DNA in *I. scapularis* (Ullmann et al., 2005; Geraci et al., 2007). In 2016, the annotated whole genome sequencing of the *I. scapularis* tick was published (Gulia-Nuss et al., 2016), which was subsequently followed by a draft genome sequence for the *I. scapularis* ISE6 cell line (Miller et al., 2018). The study that addressed the whole genome analysis of ticks annotated approximately 57% of the genome, indicating the presence of at least 20,486 protein-coding genes, with a notable occurrence of gene families associated with tick-host interactions. Despite these seminal efforts, our knowledge of the tick genome remains limited, although more sophisticated sequencing tools are increasingly available and can be utilized to construct a complete tick genome atlas. More recently, high-throughput long-read DNA/RNA sequencing (Amarasinghe et al., 2020), single-cell and single-nucleus RNA-sequencing methods (Ding et al., 2020), and chromosome conformation capture methods (Lieberman-Aiden et al., 2009) have provided unprecedented views of the genome, including that of arthropod vectors, particularly the three-dimensional organization of chromosomes, genomes, and genome-wide chromatin contacts (Dudchenko et al., 2017; Wang et al., 2018), which are recognized as crucial aspects of gene regulation (Bonev et al., 2017). The wealth of information from these genome studies is expected to have a high impact on future gene manipulation technologies and will contribute to our understanding of vector biology, including pathogen survival and transmission through *Ixodes* ticks (de la Fuente et al., 2016).

## *Ixodes* transcriptomics

The availability of next-generation sequencing technologies, such as genome-wide RNA-sequencing (RNA-Seq) (Wang et al., 2009; Stark et al., 2019) and single cell transcriptomics (La Manno, 2019; Skinnider et al., 2019), has provided a unique opportunity to study gene expression in *Ixodes* ticks, including its relevance to vector-pathogen interactions, not only on a high-throughput basis, but also at the levels of the single cell, tissue, or whole organism. Conventional and targeted approaches, like subtractive hybridization (Narasimhan et al., 2017) or quantitative RT-PCR (Smith et al., 2016), have previously been utilized for the identification of tick transcripts that are modulated during borrelial infection. The recent progress of next-generation sequencing, now with capabilities for short or long reads and *de novo* genome transcriptome analysis (Schwarz et al., 2013), has allowed for a robust and global analysis of the *Ixodes* genes that are modulated during the entry, persistence, and exit of *B. burgdorferi* through ticks. Over the past decades, a number of studies have addressed *Ixodes* transcriptomics in the presence or absence of tick-borne pathogens, including in the gut (Perner et al., 2016), salivary glands (Francischetti et al., 2005; McNally et al., 2012; Schwarz et al., 2013), hemocytes (Kotsyfakis et al., 2015), and whole ticks (Charrier et al., 2018) or cell lines (Weisheit et al., 2015; Alberdi et al., 2016; Mansfield et al., 2017), which is important for understanding the unusual biology of ticks, such as their blood meal digestion, development, and host-pathogen interactions. A recent meta-analysis of published

RNA-Seq studies compared the genetic variations between wild strains and laboratory strains of *I. ricinus* (Charrier et al., 2018). Together, these data will enrich our knowledge of the biology of an ancient arthropod vector and will inform the development of novel control strategies against tick bites or tick-transmitted infections.

The integration of transcriptomics data with other available information, like tick biology and proteomics (Ribeiro and Francischetti, 2003), as well as experimental vaccination trials, will aid in the identification of novel tick protective antigens and possibly future vaccines (Contreras et al., 2016; Contreras et al., 2019). This is particularly important, as *Ixodes* ticks can parasitize almost any vertebrate class across the globe and are second only to mosquitoes as vectors of serious human infectious diseases. The tick salivary gland transcriptome (Ribeiro and Francischetti, 2003; Francischetti et al., 2005; Ribeiro et al., 2006) will foster research on the roles of additional tick salivary gland proteins that support *B. burgdorferi* infection and transmission to the host. The salivary gland transcriptome has identified transcripts encoding proteins with signature functional domains, including basic-tail and Kunitz, as well as proline-rich peptides, metalloproteases, and novel proteins with similarities to disintegrins, histamine-binding proteins, anti-complement proteins, and a neuropeptide-like protein (nlp-31) that may have antimicrobial activity (Francischetti et al., 2005). The further characterization of these proteins and their functions will greatly contribute to our understanding of host immunity and the development of vaccines against both ticks and the pathogens that they transmit.

## *Ixodes* proteomics and metabolomics

Similar to transcriptomics, other high-throughput "omics" approaches, such as proteomics and metabolomics, provide useful information about the overall protein diversity and steady-state levels of small molecules in cells or tissues, which is insightful in regards to tick biology and the discovery of vaccine or therapeutic targets (Contreras et al., 2019). High resolution LC-MS/MS (Seger and Salzmann, 2020), iTRAQ (isobaric Tags for Relative and Absolute Quantitation) (Luo and Zhao, 2012), and shotgun proteomics (Lisacek et al., 2006) have allowed for more sensitive and accurate analyses of proteomics and metabolomics in tick-borne infections. These techniques have been applied to the study of pathogens like *B. burgdorferi*, including the characterization of the proteome in the borrelial outer membrane and the classification of protein-protein interactions (Yang et al., 2011; Yang et al., 2018), in addition to tracking the sources of blood meals (Onder et al., 2014) and analyzing the proteome in the tick vector (Di Venere et al., 2015; Iovinella et al., 2016) or in infected tick cells (Weisheit et al., 2015; Grabowski et al., 2016), as well as the immunoproteomic identification of tick antigens (Vu Hai et al., 2013). These experimental approaches have been applied to a system-level analysis of proteome complexity in the *Ixodes* gut and salivary glands (Schwarz et al., 2014), which are the organs relevant to blood meal engorgement, vector interactions, and the colonization of tick-borne pathogens. Another study used iTRAQ to identify at least 12 salivary gland proteins in *I. ricinus* that were modulated in the presence of various *Borrelia* strains (Cotte et al., 2014). As the post-translational modification of proteins is critical to protein function, including host-pathogen interactions, bioanalytical methods for glycobiology were developed, such as those based on mass spectrometry (Vechtova et al., 2018), although they have not yet been used in studies

addressing tick interactions with the spirochete. Proteomic comparative studies of various tick cell lines also were reported, which proved to be indispensable for tick research (Munderloh et al., 1994; Munderloh and Kurtti, 1995), as the tools allow for cell lines to be used as controlled experimental systems in the study of tick-pathogen interactions (Loginov et al., 2019). Using gas chromatography coupled to a mass spectrometry-based approach, a recent study explored the differences between the metabolic profiles of *B. burgdorferi*-infected and naïve *I. scapularis* nymphal ticks during blood meal engorgement (Hoxmeier et al., 2017). Additional studies on tick-pathogen metabolic interactions (see review (Cabezas-Cruz et al., 2019)) are certainly warranted, as they will contribute both to our understanding of the complex interactions between *B. burgdorferi* and *Ixodes* ticks, and to the development of novel interventions against tick-borne infections.

## Interaction-blocking Lyme disease vaccines

During infection in mammals, including humans (Radolf and Samuels, 2021), *B. burgdorferi* enters the skin at the site of the tick bite, typically resulting in a dermal lesion called erythema migrans (Nadelman and Wormser, 1998; Wormser et al., 2006), although almost 30% of human subjects fail to display the lesion. The diagnosis of Lyme disease (Marques, 2015) (see also Radolf and Samuels, 2021), especially during early infection, still remains a challenging task, due to the clinical manifestations it has in common with other febrile diseases, in addition to variations in the immune responses of infected individuals. The available antibiotic treatment usually resolves clinical symptoms, if administered in the early stages of infection. However, persistent or relapsing symptoms (e.g., fatigue, musculoskeletal pain, cognitive difficulties, etc.) later develop in a subset of patients; these symptoms are collectively referred to as chronic Lyme disease or antibiotic treatment-resistant Lyme arthritis, a condition otherwise termed as post-treatment Lyme disease syndrome (PTLDS) (Marques, 2008; Aucott et al., 2013). The pathogenesis and treatment of PTLDS remain obscure (Radolf and Samuels, 2021). These factors, combined with a remarkably high incidence of Lyme disease (Mead, 2015) (see also Radolf and Samuels, 2021), further underscore the need for the development of effective preventive measures, such as vaccines to combat infection (Gomes-Solecki et al., 2020), which is the research focus in many laboratories. Such studies aim to investigate the protective efficacy of potential vaccine candidates in animal models of Lyme borreliosis (Radolf and Samuels, 2021), targeting specific spirochete and/or tick antigens that are essential for pathogen transmission and/or persistence, as exemplified in the following sections (Radolf and Samuels, 2021).

### Antigens based on spirochetes

Understanding the molecular details of the *B. burgdorferi* life cycle, particularly in the arthropod vector, is likely to support the development of novel Lyme disease vaccines that are based on spirochete antigens. This notion is supported by the success and mechanism of protection afforded by the original human vaccine against Lyme disease (Fikrig et al., 1990; Sigal et al., 1998; Steere et al., 1998), as well as many other potential vaccine candidates (for a detailed review, see Radolf and Samuels, 2021).

### Antigens based on tick targets

In addition to bacterial antigens, proteins from the vector also represent promising vaccine candidates (de la Fuente et al., 2007b; Rego et al., 2019). Vaccines targeting tick gut or salivary gland proteins that interact with *Borrelia*, such as Salp15 (Anguita et al., 2002; Ramamoorthi et al., 2005; Dai et al., 2009; Sultana et al., 2016) or TROSPA (Pal et al., 2004a), could inhibit pathogen persistence in ticks and block pathogen transmission to vertebrates. Several *I. scapularis* proteins that impact borrelial persistence and transmission through ticks (Yang et al., 2014; Narasimhan et al., 2017) could be used as novel vaccination strategies. For example, a gut Dual oxidase (Duox) that supports spirochete persistence within ticks was identified as a *B. burgdorferi*-inducible *I. scapularis* gene product (Yang et al., 2014). As previously demonstrated by mosquitoes that were infected with malarial parasites (Kumar et al., 2010), the supportive role of Duox in spirochete survival in ticks was attributed to its involvement in the formation of the dityrosine network, a molecular barrier surrounding the gut lumen. This network has been shown to dampen the epithelial immune response by maintaining homeostasis within the gut microbial community, while also protecting invading pathogens from the vector immune system (Kumar et al., 2010). Therefore, vector proteins like TROSPA or Duox, especially the regions that are unique to ticks, could serve as components of novel transmission-blocking Lyme disease vaccines. Additional *Ixodes* proteins that have been studied as potential anti-tick vaccines have also demonstrated their impacts on the acquisition or transmission of *B. burgdorferi* in ticks, including subolesin (Almazan et al., 2003; Bensaci et al., 2012), TSLPI, a tick mannose-binding lectin inhibitor (Schuijt et al., 2011), and tHRF, a tick histamine release factor (Dai et al., 2010).

Vaccination studies using a combination of tick and spirochete antigens, such as Salp15 and OspA or OspC, were also described as markedly enhancing the protective efficacy of antibodies against *B. burgdorferi* antigens (Dai et al., 2009). A recent review highlighted the current state of knowledge about anti-tick vaccine candidates that can prevent the transmission of tick-borne pathogens (Rego et al., 2019; Bhowmick and Han, 2020).

## Concluding remarks

With more than 800 tick species in existence globally, these ancient ectoparasites have evolved a remarkable tolerance to extreme climates, as well as survivability for prolonged periods, and the abilities to maintain and transmit varied sets of pathogens. *Ixodes* ticks, which arguably transmit the most diverse range of human and animal pathogens amongst all ticks, including a striking array of Lyme borreliosis agents, are remarkably adapted to engorge on a wide collection of vertebrate hosts. The interactions between a distinct set of ligand receptors contributes to the successful persistence of human pathogens, such as *B. burgdorferi*, through a complex life cycle in nature involving *Ixodes* ticks. Studies have established the clear evolution of an intimate relationship between the pathogen and its arthropod vector. *B. burgdorferi* can induce the expression of specific tick genes, such as *TROSPA* (Pal et al., 2004a), *salp15* (Ramamoorthi et al., 2005; Dai et al., 2009), *duox* (Yang et al., 2014), and *pixr* (Narasimhan et al., 2017). Experimental evidence suggests that at least one of these genes, *salp15*, can potentially modulate host immunity in favor of the tick

feeding process; therefore, *B. burgdorferi* infection may provide survival advantages to ticks. Further work is warranted to determine the exact ecological relationship between ticks and *B. burgdorferi*, particularly to ascertain whether it is parasitic or mutualistic. Studies are also needed to address the interactions between *Ixodes* ticks and organisms that have been identified as potential symbionts (Stewart and Bloom, 2020), as well as how polymicrobial interactions shape *B. burgdorferi* infection in ticks. The identification of additional microbial and vector gene products that are actively engaged in spirochete acquisition, maintenance, and transmission through the arthropod should also be explored in future research.

The long life span and slow post-embryonic development of *Ixodes* (Sauer, 1986; Sonenshine, 1993), along with the relatively limited availability of high-resolution genomic information, complicate the application of gene manipulation tools to tick research and hinder the analyses of the tick transcriptome and proteome. Future *Ixodes* studies are required on embryology (Sonenshine, 1993), the three-dimensional configuration of the genome (Kempfer and Pombo, 2020), gene manipulation and transgenesis (Sun et al., 2017), and paratransgenesis (Wilke and Marrelli, 2015), as these would certainly expand upon experimental opportunities and foster a deeper understanding of tick biology and development. Such efforts will also contribute to our knowledge of the molecular interactions between *Borrelia* and tick gene products. Specific questions to be addressed include the molecular details of how *B. burgdorferi* evades tick immunity, the gut digestive activities that occur upon spirochete arrival in feeding ticks, and the mechanism by which the pathogen continues to persist in ticks throughout the intermolt periods that are dominated by temperature extremities and starvation. Studies should also distinguish how *B. burgdorferi* crosses physical barriers, such as the chitinous gut peritrophic membrane and newly-recognized acellular barriers like the dityrosine network, and then selectively migrates through the tick gut, hemocoel, and salivary gland epithelia during transmission to mammals. *Ixodes* and *Borrelia* have probably co-evolved numerous but distinct molecular interfaces that support their coexistence.

Globally, the incidences of tick-borne diseases, including Lyme borreliosis, are increasing. At least 13 new tick-borne pathogens were recognized in the Western Hemisphere during the last 20 years (Paddock et al., 2016). It is likely that ticks evolved robust genetic and epigenetic mechanisms that promote their survival in environmental extremities and their unparalleled tolerance to a wide range of microbial pathogens. Future investigations of the genetic and epigenetic bases of tick vectorial capacity will enrich our knowledge of the interactions between *Ixodes* and diverse pathogens, contributing to a better understanding of tick-borne infections. In particular, more thorough knowledge about the biology of the tick-*B. burgdorferi* interaction (Kurokawa et al., 2020) not only will contribute to the development of preventive measures against Lyme disease but also serve as a paradigm for novel control measures against additional tick-borne infections.

## Acknowledgement

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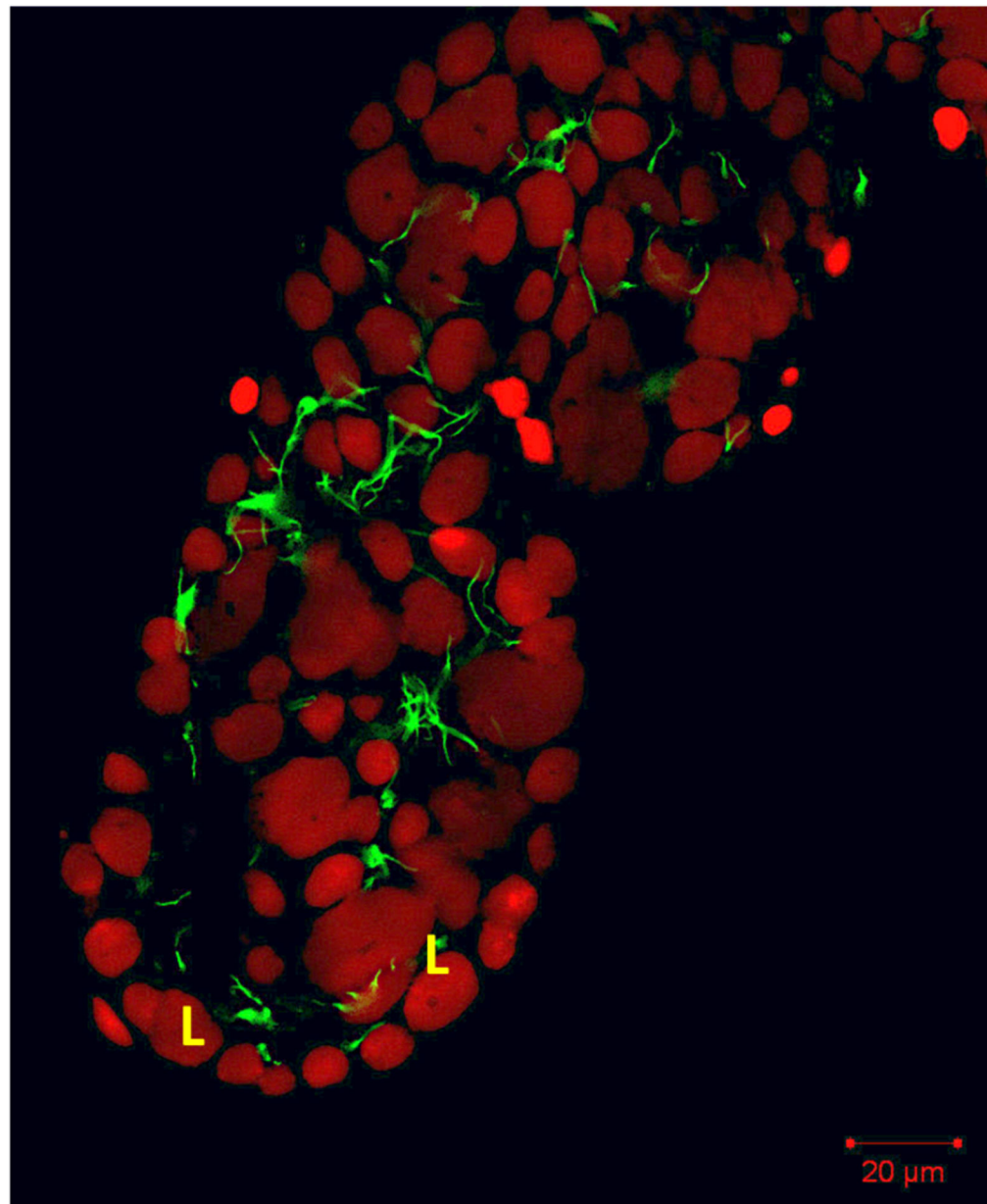
**Figure 1.**

Colonized *B. burgdorferi* within the gut diverticulum of a nymphal *I. scapularis*. The gut of an unfed nymphal *I. scapularis* tick was dissected and processed for confocal immunofluorescence microscopy, as detailed (Pal et al., 2004a). The image shows a portion of the gut diverticulum, where tick nuclei (red) were labeled with propidium iodide, and spirochetes towards the luminal side (green) were detected using FITC-labeled anti-*B. burgdorferi* antibodies. L, Lumen of the gut, Bar = 20 μm.