



The “Biological Weapons” of *Ehrlichia chaffeensis*: Novel Molecules and Mechanisms to Subjugate Host Cells

Yasuko Rikihisa *

Laboratory of Molecular, Cellular, and Environmental Rickettsiology, Department of Veterinary Biosciences, College of Veterinary Medicine, Infectious Diseases Institute, The Ohio State University, Columbus, OH, United States

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*Correspondence:

Yasuko Rikihisa
rikihisa.1@osu.edu

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Ehrlichia chaffeensis is an obligatory intracellular bacterium that causes human monocytic ehrlichiosis, an emerging, potentially fatal tick-borne infectious disease. The bacterium enters human cells via the binding of its unique outer-membrane invasin EtpE to the cognate receptor DNase X on the host-cell plasma membrane; this triggers actin polymerization and filopodia formation at the site of *E. chaffeensis* binding, and blocks activation of phagocyte NADPH oxidase that catalyzes the generation of microbicidal reactive oxygen species. Subsequently, the bacterium replicates by hijacking/dysregulating host-cell functions using Type IV secretion effectors. For example, the *Ehrlichia* translocated factor (Etf)-1 enters mitochondria and inhibits mitochondria-mediated apoptosis of host cells. Etf-1 also induces autophagy mediated by the small GTPase RAB5, the result being the liberation of catabolites for proliferation inside host cells. Moreover, Etf-2 competes with the RAB5 GTPase-activating protein, for binding to RAB5-GTP on the surface of *E. chaffeensis* inclusions, which blocks GTP hydrolysis and consequently prevents the fusion of inclusions with host-cell lysosomes. Etf-3 binds ferritin light chain to induce ferritinophagy to obtain intracellular iron. To enable *E. chaffeensis* to rapidly adapt to the host environment and proliferate, the bacterium must acquire host membrane cholesterol and glycerophospholipids for the purpose of producing large amounts of its own membrane. Future studies on the arsenal of unique *Ehrlichia* molecules and their interplay with host-cell components will undoubtedly advance our understanding of the molecular mechanisms of obligatory intracellular infection and may identify hitherto unrecognized signaling pathways of human hosts. Such data could be exploited for development of treatment and control measures for ehrlichiosis as well as other ailments that potentially could involve the same host-cell signaling pathways that are appropriated by *E. chaffeensis*.

Keywords: *Ehrlichia chaffeensis*, invasin, ROS, type IV secretion effector, RAB5, autophagy, ferritinophagy, membrane cholesterol

1 INTRODUCTION

Ehrlichia chaffeensis is a tick-borne Gram-negative obligatory intracellular bacterium of the family *Anaplasmataceae* in the order Rickettsiales. Infection causes severe flu-like febrile disease called human monocytic ehrlichiosis (HME), which is often accompanied by hematologic abnormalities and signs similar to those of hepatitis (Dawson et al., 1991; Paddock and Childs, 2003). Tick-borne diseases are on the rise (Biggs et al., 2016; Madison-Antenucci et al., 2020; Alkhishe et al., 2021). Discovered in 1986 (Maeda et al., 1987), HME is currently among the most prevalent life-threatening tick-borne zoonoses (Adams et al., 2017). HME diagnosis is challenging, as early signs and symptoms are indistinct or mimic other illnesses. No HME vaccine exists, and the only effective therapy is the broad-spectrum antibiotic doxycycline. However, treatment is often delayed (or even not initiated) owing to misdiagnosis or comorbidity with an unrelated underlying illness or injury, stress, immunosuppression, and/or co-infection with other tick-borne pathogens, which collectively can lead to severe complications or death, with a mortality rate of 1–5% among different populations (Paddock and Childs, 2003). *Ehrlichia* spp. also can negatively impact livestock agro-economics and working and companion animals, as the various species and strains of *Ehrlichia* can cause severe and potentially fatal diseases in animals (Rikihisa, 1991).

Ehrlichia chaffeensis replicates within monocytes and macrophages, which are primary immune cells that recognize pathogen-associated molecular patterns (PAMPs) to unleash potent innate antimicrobial defenses. As a survival strategy, *E. chaffeensis* has lost genes encoding major PAMPs such as lipopolysaccharide, peptidoglycan, flagella, and common pili (Lin and Rikihisa, 2003). It has a single small (1.18 Mbp) circular genome that lacks most genes for amino-acid biosynthesis and intermediary metabolism (Dunning Hotopp et al., 2006); consequently, the bacterium depends on host cells for these molecules. Molecular and cellular research on *E. chaffeensis* has revealed unique mechanisms that mediate its parasitism. Foremost is the *E. chaffeensis* invasin EtpE (entry-triggering protein of *Ehrlichia*), which binds its cognate host-cell surface receptor DNase X, thereby inducing its internalization. This occurs without eliciting host-derived signals that normally would activate the phagocyte NADPH oxidase 2 (NOX2) complex, that catalyzes the production of microbicidal reactive oxygen species (ROS) from molecular oxygen (Mohan Kumar et al., 2013; Teymournejad et al., 2017). Once internalized, the bacterium replicates within a membrane-bound compartment (inclusion); this is also secluded from components of the NOX2 complex (Lin and Rikihisa, 2007).

Ehrlichia chaffeensis inclusions rapidly fuse with host-cell early endosomes, thereby acquiring early-endosome markers including the small GTPase RAB5 and its effectors such as early endosome antigen 1, VPS34, and Rabankyrin-5. This facilitates subsequent fusion with other early endosomes that contain transferrin receptor (TfR). Exogenous iron-loaded transferrin (Tf) enters into inclusions through the TfR-Tf endosome recycling pathway (Barnewall et al., 1997). Recent

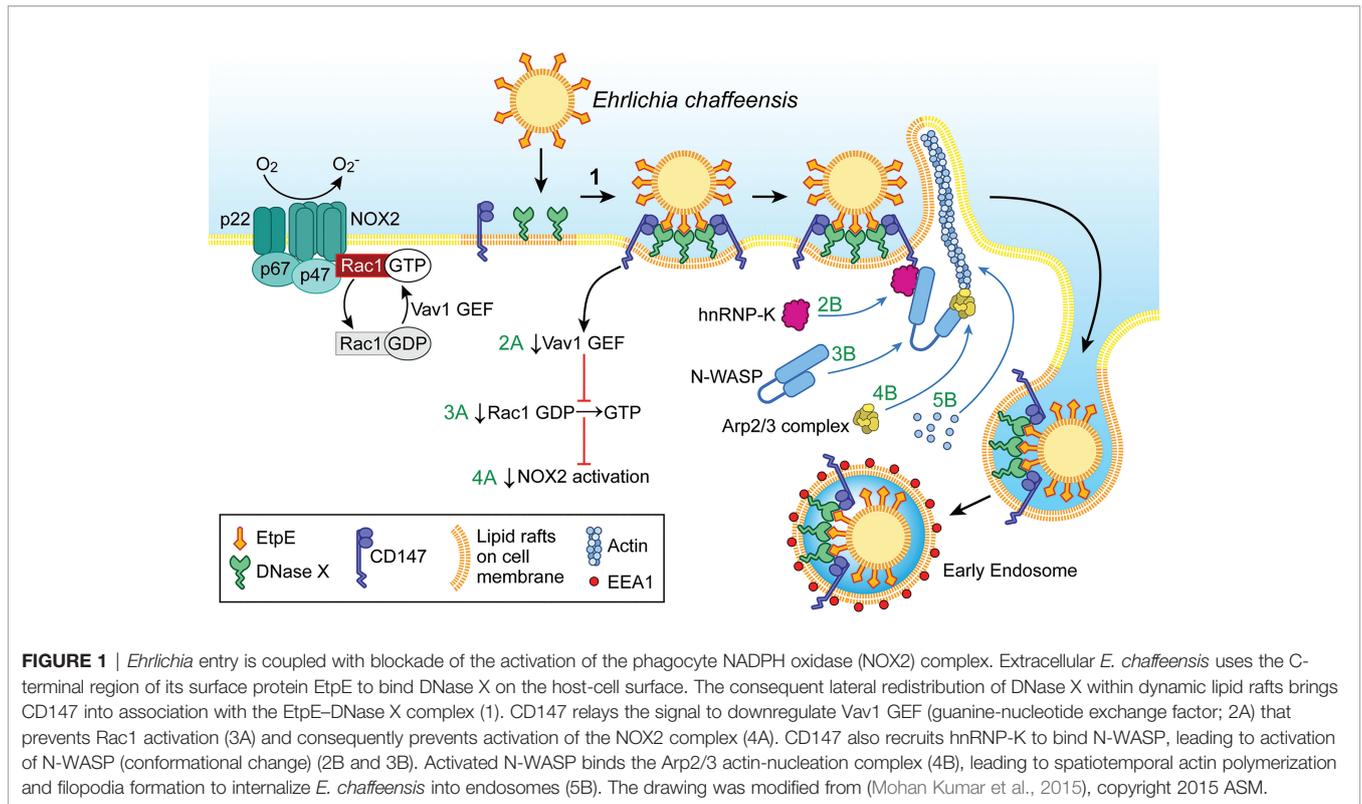
studies revealed that the inclusions have features of the early amphisome, which is the vesicular compartment formed by fusion of early endosomes with early autophagosomes, as ATG5, but not LC3 or ATG14L, was also found in inclusions (Lin et al., 2016). This review primarily focuses on recent findings pertaining to invasin, Type IV secretion system (T4SS) effectors, and host-cell membrane lipids that are acquired by *E. chaffeensis*. Readers are referred to several informative reviews for discussion of other aspects of *E. chaffeensis* (Paddock and Childs, 2003; Rikihisa, 2010; McBride and Walker, 2011; Rikihisa, 2011; Rikihisa, 2015; McClure et al., 2017; Byerly et al., 2021).

2 MOLECULES UNIQUE TO *E. CHAFFEENSIS* THAT FACILITATE ENTRY INTO HOST CELLS AND SUBSEQUENT INTRACELLULAR REPLICATION

2.1 *Ehrlichia* Entry Is Coupled With Blockade of the Activation of the NOX2 Complex

As an obligatory intracellular bacterium, *E. chaffeensis* cannot survive without entry into permissive host cells. To enter host monocytes and macrophages, *E. chaffeensis* uses the C-terminus of its unique outer-membrane protein, EtpE-C, to directly bind the host-cell DNase X (DNASE1like1), a cell-surface glycosylphosphatidylinositol-anchored receptor that senses extracellular DNA (Mohan Kumar et al., 2013) (**Figure 1**). Actin polymerization is not required for *E. chaffeensis* binding to host cells but is necessary for entry, and thus entry can be inhibited by cytochalasin D (Mohan Kumar et al., 2015).

EtpE-C-induced actin polymerization is dependent on DNase X as well as activation of the actin nucleation-promoting factor neuronal Wiskott-Aldrich Syndrome protein (N-WASP) (Mohan Kumar et al., 2015). The N-WASP inhibitor wiskostatin or overexpression of the WA domain of N-WASP, which exerts a dominant-negative effect on N-WASP, inhibits actin polymerization and *E. chaffeensis* entry (Mohan Kumar et al., 2015). How does EtpE-C binding to DNase X on the macrophage surface activate cytoplasmic signaling? EtpE-C binding to DNase X on the macrophage surface recruits three factors, namely the type I transmembrane glycoprotein CD147 (basigin/extracellular matrix metalloproteinase inducer), cytoplasmic heterogeneous nuclear ribonucleoprotein K (hnRNP-K), and N-WASP, to facilitate actin polymerization at the site of *E. chaffeensis* binding (Mohan Kumar et al., 2015) (**Figure 1**). CD147 is the key molecule to relay signals started with DNase X membrane receptor to the inside of the cell. Thus, the extracellular bone marrow-derived macrophages from *CD147^{fllox/fllox}-Lyz2-Cre* mice, in which *Cre* expression (driven by the *Lyz2* promoter) is used for myeloid cell-specific *CD147* knockout, are significantly less susceptible to *E. chaffeensis* infection (Teymournejad and Rikihisa, 2020). hnRNP-K binds N-WASP and activates the Arp2/3 complex to nucleate actin



polymerization *in vitro* (Yoo et al., 2006), and the intracellular nanoantibody clone #47 (iAB-47), which binds and confines hnRNP-K to the nucleus (Inoue et al., 2007), potently blocks *E. chaffeensis* entry (Mohan Kumar et al., 2015). Entry requires host-cell energy but not *Ehrlichia* energy, as demonstrated by the fact that latex beads coated with recombinant EtpE-C could bind DNase X and enter phagocytes as well as non-phagocytic cells that are permissive to *E. chaffeensis* infection (Mohan Kumar et al., 2013).

Phagocytes, such as monocytes and neutrophils, produce powerful NADPH oxidase (NOX2 complex), a multicomponent enzyme composed of a membrane-bound heterodimeric cytochrome b558 component (gp91^{phox} [NOX2] and p22^{phox}), three cytoplasmic subunits (p67^{phox}, p47^{phox}, and p40^{phox}), and the small GTPase Rac1 or Rac2 (Panday et al., 2015). In resting phagocytes, these NOX2 components are dissociated and hence the enzyme is inactive. Phagocyte-activating agents such as phorbol myristate acetate (PMA), invading pathogens, or an *N*-formyl peptide can induce rapid assembly of all NOX2 components into a holoenzyme that catalyzes the production of superoxide anion (O₂⁻) from molecular oxygen (Debeurme et al., 2010). O₂⁻ is secreted extracellularly and into the lumen of phagosomes and serves as starting material for the production of microbicidal reactive oxygen species (ROS), including hydrogen peroxide (H₂O₂), oxidized halogens, hydroxyl radicals, and singlet oxygen (Gabig and Babior, 1981). Paradoxically, *E. chaffeensis* isolated from host cells is quite sensitive to ROS, and infectivity decreases rapidly when bacteria are exposed to ROS (Lin and Rikihisa, 2007). In fact, the *E. chaffeensis* genome lacks genes

encoding enzymes that facilitate ROS detoxification, free-radical scavenging, repair of ROS-induced damage, and the oxidative stress response (Dunning Hotopp et al., 2006; Lin and Rikihisa, 2007). How, then, does *E. chaffeensis* prevent or overcome ROS assault by host macrophages? Remarkably, unlike most other bacteria, *E. chaffeensis* does not induce ROS production in human monocytes and rapidly blocks O₂⁻ generation induced by PMA (Lin and Rikihisa, 2007). This inhibition is specific to monocytes, as *E. chaffeensis* cannot block ROS production by PMA-stimulated neutrophils, and a host cell-surface protein is required (Lin and Rikihisa, 2007). This surface protein was later revealed to be DNase X, as inhibition of NOX2-complex activation could be initiated by the binding of EtpE-C to DNase X (Teymournejad et al., 2017) (**Figure 1**). Thus, DNase X-mediated entry and ROS blockade are coupled to ensure *E. chaffeensis* survival during entry. However, neutrophils do not express DNase X (Teymournejad et al., 2017), which is likely the primary reason why *E. chaffeensis* neither infects neutrophils nor blocks activation of the NOX2 complex. The binding of *E. chaffeensis* or of recombinant EtpE-C-coated beads to DNase X can trigger activation of N-WASP (Mohan Kumar et al., 2015). However, N-WASP activation is not involved in the blockade of ROS production initiated by *E. chaffeensis* or EtpE-C binding to DNase X (Teymournejad et al., 2017). Rac GTPases act as binary switches for the activation of NOX2 (Seifert et al., 1986; Roberts et al., 1999; Zhao et al., 2003; Bokoch and Zhao, 2006). Two Rac isoforms exist, namely Rac1 and Rac2, and Rac2 is the predominant isoform in human neutrophils, whereas Rac1 predominates in monocytes, the latter accounting for 90% of

cellular Rac (Zhao et al., 2003). For Rac activation, GTP-for-GDP exchange is facilitated by a membrane-localized, Rac-specific guanine-nucleotide exchange factor (GEF) (Bokoch et al., 1994), and Rac becomes inactivated upon GTP hydrolysis catalyzed by a GTPase-activating protein specific for Rac (Geiszt et al., 2001). Vav1 is a hemopoiesis-specific Rho/Rac guanine-nucleotide exchange factor that plays a prominent role in adhesion-mediated suppression of ROS generation in phagocytes (Zhao et al., 2003). Engagement of EtpE-C with DNase X triggers CD147-dependent suppression of the PMA-induced activation of Vav1 (Teymournejad and Rikihisa, 2020) (**Figure 1**). Consequently, *E. chaffeensis* and EtpE-C, upon binding DNase X, block Rac1 activation (Teymournejad and Rikihisa, 2020) (**Figure 1**). Actin polymerization led by Rac/wave activation is a well-known mechanism for the entry of several intracellular bacteria including *Listeria*, *Yersinia*, *Salmonella*, and *Chlamydia* into non-phagocytes (Alrutz et al., 2001; Carabeo et al., 2004; Bosse et al., 2007; Humphreys et al., 2013). However, *E. chaffeensis* does not utilize this mode of entry (Mohan Kumar et al., 2015) to colonize phagocytes, as Rac-dependent actin polymerization and entry would activate phagocyte NOX2 as well.

Immunization of mice and dogs with recombinant EtpE-C significantly inhibits *E. chaffeensis* infection *via* intraperitoneal or infected-tick challenge (Mohan Kumar et al., 2013; Budachetri et al., 2020). Thus, EtpE-C could be included in a candidate vaccine to counter tick-transmitted ehrlichiosis.

2.2 Functions of *E. chaffeensis* T4SS Effectors

The T4SS can transfer bacterial proteins or nucleoprotein complexes across the membrane of eukaryotic cells (Alvarez-Martinez and Christie, 2009). The T4SS has several ancestral lineages including the archetype *virB/virD* system of *Agrobacterium tumefaciens* and the *dot/icm* system of *Legionella pneumophila*, sometimes referred to as T4aSS and T4bSS, respectively (Alvarez-Martinez and Christie, 2009). All members of the order Rickettsiales, which includes *E. chaffeensis*, have T4aSS (Gillespie et al., 2010). T4SS functions through its effectors/substrates. To date, three T4SS effectors have been experimentally demonstrated, namely *Ehrlichia* translocated factor (Etf)-1, -2, and -3 (Liu et al., 2012; Lin et al., 2016; Yan et al., 2018; Yan et al., 2021).

Etf-1, -2, and -3 directly bind the *E. chaffeensis* T4SS coupling ATPase VirD4 and are then transferred from the bacterium into the host-cell cytoplasm by crossing three membranes (inner and outer *Ehrlichia* membranes, and inclusion membrane) (Liu et al., 2012; Lin et al., 2016; Yan et al., 2018; Yan et al., 2021). Each Etf is required for *E. chaffeensis* infection, as downregulation of any *Etf* gene by electroporation of *E. chaffeensis* with an individual *Etf*-specific antisense peptide nucleic acid significantly reduces the expression of the corresponding mRNA and hence the bacteria's ability to infect host cells (Sharma et al., 2017; Yan et al., 2018; Yan et al., 2021). This type of inhibition could be trans-complemented by ectopic expression of the corresponding GFP-coupled Etf in host cells, underscoring the critical roles of the three T4SS effectors in *E. chaffeensis* replication (Sharma et al., 2017; Yan et al., 2018; Yan et al., 2021). Characteristics of the three T4SS effectors of *E. chaffeensis* is listed in **Table 1**.

2.2.1 Etf-1 Inhibits Host-Cell Apoptosis

E. chaffeensis inhibits host-cell apoptosis to maximize bacterial proliferation inside host cells (Liu et al., 2011; Liu et al., 2012). Etf-1 is highly upregulated during early exponential growth of *E. chaffeensis* in human monocytes (Liu et al., 2012). In Etf-1-transfected mammalian cells, Etf-1 was found to localize to mitochondria and inhibit apoptosis induced by the treatment with etoposide (Liu et al., 2012) (**Figure 2**). Moreover, in similar experiments with yeast, Etf-1 also localized to mitochondria and inhibited apoptosis induced by heterologous expression of human Bax (Liu et al., 2012). The N-terminal 24 amino-acid residues of Etf-1, especially residue K23, play a critical role in mitochondrial targeting of Etf-1, as deletion mutation of this residue significantly decreased Etf-1 localization to mitochondria (Zhang et al., 2021). The mitochondrial matrix protein manganese superoxide dismutase (MnSOD) maintains a basal level of ROS in cells by scavenging O₂⁻ and is essential for maintaining aerobic life (Holley et al., 2012). The MnSOD level was found to increase in *E. chaffeensis*-infected cells or Etf-1-transfected cells, and the amount of ROS in infected or Etf-1-transfected cells was significantly lower than that in uninfected or control plasmid-transfected cells (Liu et al., 2012; Yan et al., 2021). These data suggest that, by upregulating mitochondrial MnSOD, Etf-1 serves as an antioxidant to prevent ROS-induced

TABLE 1 | Characteristics of Type IV secretion effectors from *E. chaffeensis*.

Effector	Amino acid residues	C-terminal residues (basic residues <u>underlined</u>)	Protein motifs	Subcellular localization/functions
Etf-1	380	<u>K</u> HFSNPGK <u>V</u> H <u>A</u> R	Near N-terminal mitochondria localization signal	Mitochondria, bacterial inclusions/ Inhibits mitochondria-mediated apoptosis Upregulates MnSOD
Etf-2	264	H <u>A</u> RQACGRFF <u>B</u> R	An Arg finger and a Gln finger of Tre2-Bub2-Cdc16 domain	Binds Beclin 1 and induces autophagy Early endosomes and bacterial inclusions/ Binds RAB5-GTP and blocks RABGAP5 engagement with RAB5-GTP
Etf-3	621	<u>R</u> LSEIFSAL <u>R</u> T <u>I</u> A <u>R</u>	*	Ferritinophagolysosomes/ Binds ferritin light chain and induces ferritinophagy

*Research concerning this motif is ongoing.

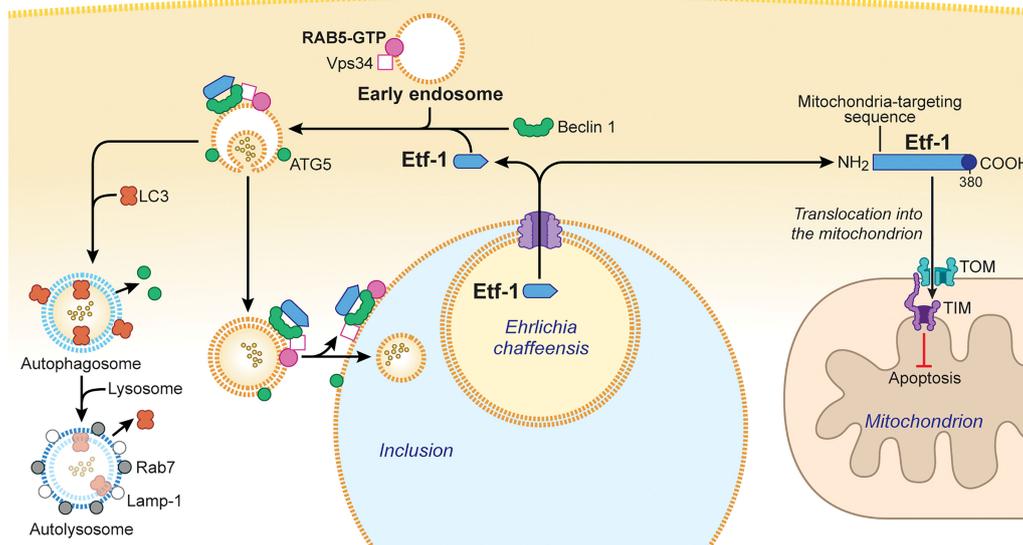


FIGURE 2 | *Ehrlichia chaffeensis* Etf-1 localizes to mitochondria to block apoptosis of host cells. Alternatively, Etf-1 binds to the Beclin 1–VPS34–RAB5-GTP complex and induces RAB5-regulated autophagy. (Right) Etf-1 is depicted in blue with the putative T4SS signal depicted in dark blue. Etf-1 has a mitochondria-targeting presequence and localizes to mitochondria. Mitochondria-localized Etf-1 blocks apoptosis of eukaryotic host cells by preventing loss of mitochondrial membrane potential. TOM: transporter outer membrane complex; TIM: transporter inner membrane complex. (Left) Etf-1 binds the Beclin 1–VPS34–RAB5-GTP complex and induces RAB5-regulated autophagy. Etf-1 autophagosomes are recruited to *E. chaffeensis* inclusions and deliver captured host cytoplasmic contents. If not recruited to inclusions, Etf-1 autophagosomes mature to autolysosomes, in which captured substrates are degraded and catabolites are released to the cytoplasmic to promote bacterial proliferation. The drawing was modified from (Rikihisa, 2019), copyright 2017 Taylor & Francis, and from (Rikihisa, 2017), copyright 2017 Springer.

cellular damage and apoptosis to allow intracellular infection (Liu et al., 2012).

To verify the functions of intracellular Etf-1 and investigate the possibility that Etf-1 could be used as a therapeutic target, Etf-1-specific nanobodies were developed by immunizing a llama (Zhang et al., 2021). One particular nanobody could form a stable complex with Etf-1 and thereby block the mitochondrial localization of Etf-1 (Zhang et al., 2021). Intracellular expression of this anti-Etf-1 nanobody inhibited three activities of Etf-1 and *E. chaffeensis*: upregulation of mitochondrial MnSOD, reduction of intracellular ROS, and inhibition of apoptosis (Zhang et al., 2021). Conjugation of this nanobody to cyclized cell-permeable peptide 12 facilitated effective entrance into mammalian cells, where it abrogated the blockade of apoptosis caused by *E. chaffeensis* and inhibited infection by *E. chaffeensis* in cultured cells and in a mouse model of severe combined immunodeficiency (Zhang et al., 2021). Thus, in principle, intracellular nanobodies that interfere with T4SS effector functions could be developed as research tools as well as therapeutic agents.

2.2.2 Etf-1 Induces RAB5-Regulated Autophagy

Autophagy is the process by which eukaryotic cells routinely degrade cellular components to ensure homeostasis and is considered a part of the innate immune response that clears a variety of intracellular pathogens (Levine et al., 2011; Deretic,

2012). However, intracellular replication of *E. chaffeensis* is enhanced by the autophagy inducer rapamycin and inhibited by the autophagy inhibitor 3-methyl adenine (Lin et al., 2016). Use of Spautin-1 (a cell-permeable inhibitor of the autophagy regulator Beclin 1), Beclin 1 small interfering RNA, or mouse bone marrow-derived macrophages from *atg5^{fllox/fllox}-Lyz2-Cre* mice (in which *Lyz2* promoter-driven *Cre* expression is used for myeloid cell-specific *Atg5* knockout) demonstrated that autophagy not only enhances ehrlichial infection but also is required for *E. chaffeensis* replication (Lin et al., 2016). In fact, *E. chaffeensis* induces a unique type of cellular autophagy to recycle host-cell catabolites for use during its replication (Lin et al., 2016). *E. chaffeensis*-induced autophagy is independent of the general cellular ubiquitination pathways as well as the canonical autophagy pathway involving MTOR, ULK1, and AMPK (Lin et al., 2016). Etf-1 binds Beclin 1 and VPS34 and activates the class III PtdIns3K (phosphatidylinositol 3-kinase) complex, which is an essential component and master regulator of autophagy initiation (Figure 2), but this Etf-1 complex does not recruit the endoplasmic reticulum resident ATG14L, unlike Ats-1 of *Anaplasma phagocytophilum*, that also binds Beclin 1 and VPS34 and induces autophagy (Niu et al., 2010). Rather, the Etf-1-Beclin 1 complex recruits RAB5-GTP (Lin et al., 2016) (Figure 2). This type of autophagy is referred to as “RAB5-regulated autophagy” (Ravikumar et al., 2008), as constitutively active RAB5 induces autophagy by binding to the RAB5 effector VPS34, which binds Beclin 1 and hence the class III PtdIns3K

complex. Expansion of a polyglutamine tract within the Huntingtin protein due to the mutation causes its accumulation and aggregation in the cytoplasm, leading to the neurodegenerative genetic disorder Huntington's disease (Raspe et al., 2009). The mutant Huntingtin protein, is poorly degraded in proteasomes but can be degraded *via* RAB5-regulated autophagy (Ravikumar et al., 2008). Etf-1-induced RAB5-regulated autophagy was found to clear an aggregation-prone mutant Huntingtin protein in a class III PtdIns3K-dependent manner (Lin et al., 2016).

During the exponential growth stage of *E. chaffeensis*, the concentrations of free/cytoplasmic L-glutamine and L-glutamate in infected human monocytes increase substantially, making them available for ehrlichial growth (Lin et al., 2016). Indeed, host cell-preincorporated radioactive L-glutamine could be readily taken up by *E. chaffeensis* in an autophagy-dependent manner, and the human cytoplasmic autophagy cargo protein GAPDH could be delivered into *E. chaffeensis* inclusions as well (Lin et al., 2016). In addition to several early-endosome markers, Etf-1 and the early autophagosome marker ATG5 (but not LC3) are present on the membrane of *E. chaffeensis* inclusions (Barnewall et al., 1997; Mott et al., 1999; Lin et al., 2016) (Figure 2), and thus the inclusions can be considered as large amphisomes formed by fusion of early endosomes and early autophagosomes. Etf-1-induced autophagy releases host-cell small-molecule catabolites into the host cytoplasm to provide nutrients (e.g., amino acids) to *E. chaffeensis*. Furthermore, Etf-1-induced autophagy creates a host cytoplasmic space for *E. chaffeensis* to grow without lysing host cells.

How are the two competing functions of Etf-1 distributed within *E. chaffeensis*-infected cells? The translocase of the outer membrane of mitochondria (TOM) complex is the main pore for the import of nuclear-encoded proteins into mitochondria, and mitochondrial membrane potential is required for import (Pfanner and Truscott, 2002). The majority of Etf-1 targets mitochondria during the early stage of infection when mitochondrial membrane potential is maximal. As infection progresses, Etf-1 is diverted to autophagosomes as mitochondria begin to lose membrane potential (Wurm et al., 2011). This suggests that host-cell physiologic conditions during infection influence the distribution of Etf-1 between mitochondria and autophagosomes, consequently affecting *E. chaffeensis* growth.

Although Etf-1 interacts with RAB5-GTP *via* Beclin 1 and localizes to *E. chaffeensis* inclusions, inhibition of lysosome fusion with inclusions by keeping RAB5 on inclusions, requires another T4SS effector, Etf-2, because Etf-1-GFP vesicles mature to autolysosomes (Lin et al., 2016).

2.2.3 Etf-2 Prevents Lysosomal Fusion of *E. chaffeensis* Inclusions

Ehrlichia chaffeensis sequesters the regulator of endosomal traffic, RAB5, on its membrane-bound inclusions to avoid being routed to host-cell phagolysosomes (Barnewall et al., 1997; Mott et al., 1999). How is RAB5 sequestered on the ehrlichial inclusion membrane? The answer is *via* its association with Etf-2. Etf-2 directly binds RAB5-GTP on the membrane of early endosomes and of

E. chaffeensis-containing inclusions (Yan et al., 2018) (Figure 3). A yeast two-hybrid assay and a microscale thermophoresis assay revealed that Etf-2 binds tightly to RAB5-GTP but not RAB5-GDP (Yan et al., 2018). This is because Etf-2 contains two conserved motifs of RAB GAP Tre2-Bub2-Cdc16 domain, namely an Arg finger and a Gln finger, although it lacks RAB5-specific GAP activity (Yan et al., 2018). Thus, Etf-2 binding to RAB5-GTP blocks RAB5-GTP engagement with RABGAP5 (Figure 3), and consequently RAB5-GTP hydrolysis is delayed on *E. chaffeensis* inclusions (Yan et al., 2018).

2.2.4 Etf-3 Induces Ferritinophagy

Ehrlichia is an obligate aerobe that requires the electron transport chain, thus iron, because its glycolytic pathway is incomplete and it lacks ATP-ADP translocase, unlike *Rickettsia* and *Chlamydia* (Dunning Hotopp et al., 2006). *Ehrlichia chaffeensis* lacks the siderophore biosynthesis pathway and Fe³⁺ uptake regulator (Dunning Hotopp et al., 2006). Instead, *Ehrlichia* acquires iron from the host-cell labile cellular iron pool, and pretreating human monocytes with deferoxamine, a membrane-permeable chelator of this iron pool, blocks *E. chaffeensis* infection (Barnewall and Rikihisa, 1994). *Ehrlichia* enhances host-cell iron uptake *via* upregulating TfR mRNA (Barnewall et al., 1999) and acquires iron from holoTf, as *E. chaffeensis* endosomes intersect with TfR-recycling endosomes and are slightly acidic—enough to release iron from holoTf (Barnewall et al., 1997). In fact, treatment of macrophages with interferon- γ downregulates TfR mRNA and almost completely inhibits *Ehrlichia* infection, and addition of holoTf abrogates this inhibition (Barnewall and Rikihisa, 1994). However, TfR mRNA levels return to basal level after 24 h post-infection, when bacterial exponential growth begins (Barnewall et al., 1999); at that time, treatment with interferon- γ can no longer inhibit infection (Barnewall and Rikihisa, 1994). How, then, does exponentially growing *Ehrlichia* acquire iron from host cells? The answer is Etf-3, which binds directly and tightly to ferritin (the primary eukaryotic cytoplasmic iron storage protein) and thereby induces ferritinophagy, a selective form of autophagy by recruiting NCOA4 (nuclear receptor coactivator 4), a cargo receptor that mediates ferritinophagy, and LC3, an autophagosome biogenesis protein (Yan et al., 2021) (Figure 4). Etf-3-induced ferritinophagy causes ferritin degradation and significantly increases the labile cellular iron pool, which can feed *E. chaffeensis* (Figure 4). Indeed, an increase in cellular ferritin by adding ferric ammonium citrate to the culture medium, or overexpression of Etf-3 or NCOA4, enhances *E. chaffeensis* proliferation, whereas knockdown of Etf-3 in *Ehrlichia* *via* transfection with a plasmid encoding an Etf-3 antisense peptide nucleic acid inhibits *Ehrlichia* proliferation (Yan et al., 2021).

Excessive ferritinophagy induces the generation of toxic ROS, which could presumably kill both *Ehrlichia* and host cells. During *Ehrlichia* proliferation, however, there is concomitant upregulation of *Ehrlichia* Fe-superoxide dismutase, the gene that is co-regulated with the *Ehrlichia* T4SS operon, and increase in mitochondrial MnSOD in response to the co-secreted Etf-1 (Yan et al., 2021).

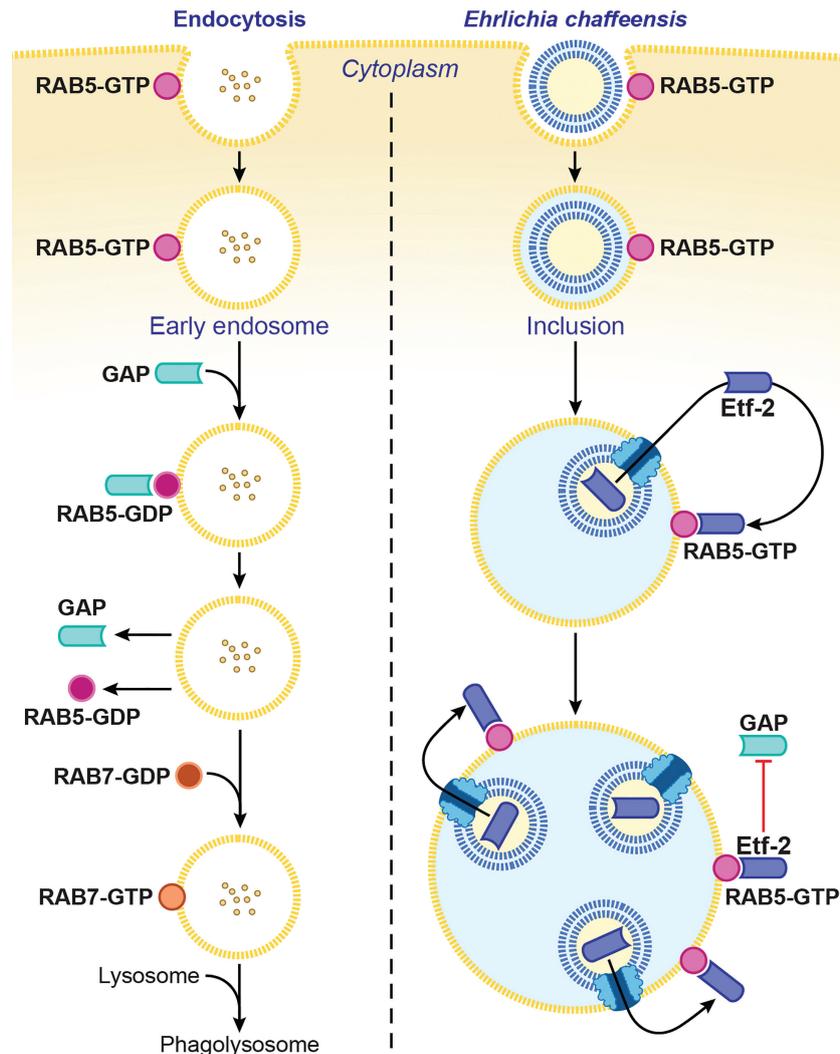


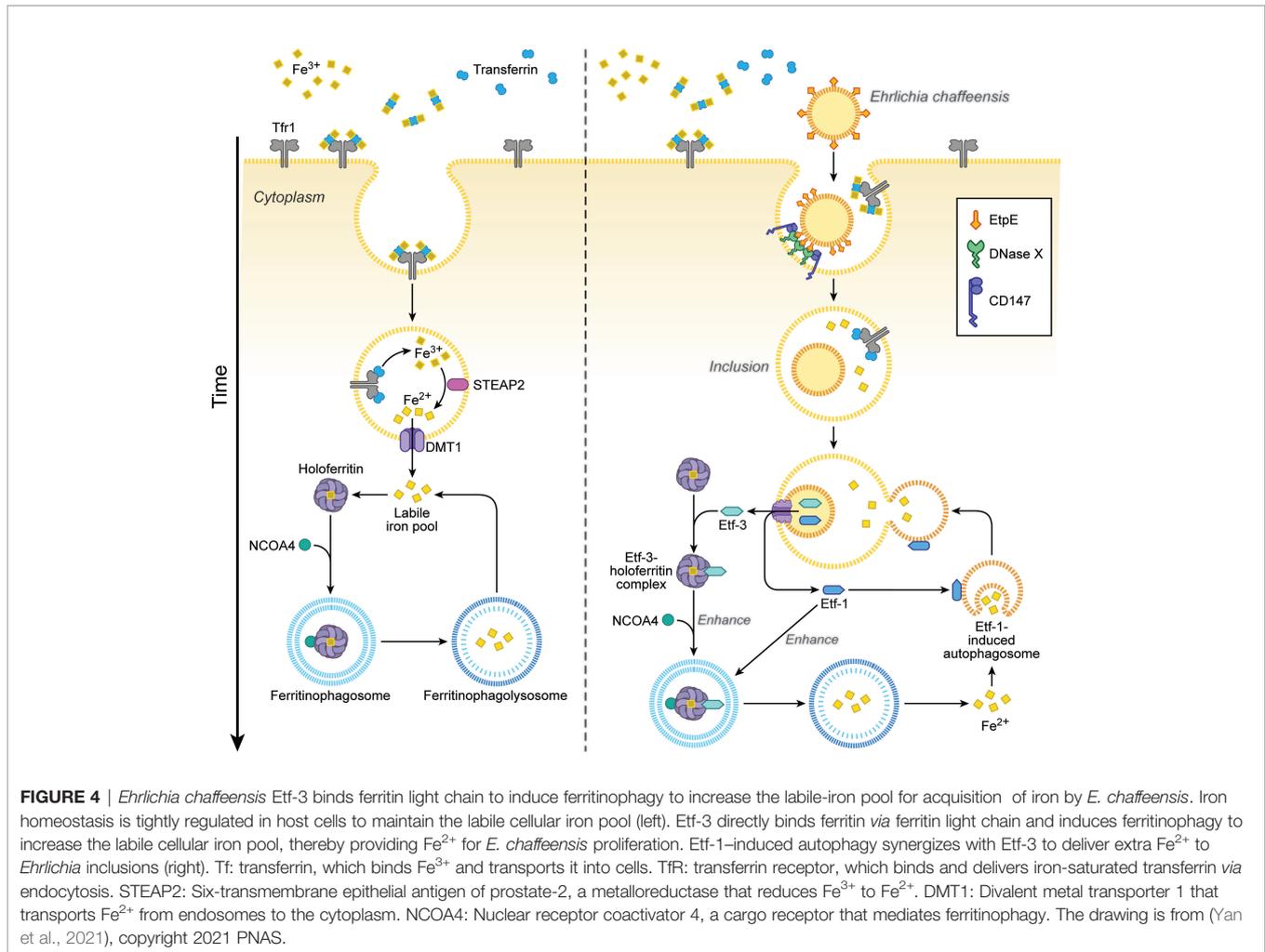
FIGURE 3 | *Ehrlichia chaffeensis* Etf-2 binds RAB5-GTP and blocks RABGAP5 from acting on RAB5. RAB5-GTP hydrolysis by the RAB5-specific GAP is required for endosome maturation and lysosomal fusion with *E. chaffeensis* inclusions via binding to RAB5-GTP and competitively blocking RABGAP5 from acting on RAB5 on the inclusion surface (right). The drawing is from (Yan et al., 2018), copyright 2018 PNAS.

Consequently, despite enhanced ferritinophagy, cellular ROS levels are reduced in *Ehrlichia*-infected cells compared with uninfected cells (Yan et al., 2021). Thus, *Ehrlichia* robs host-cell iron sequestered in ferritin without killing the host cell.

3 HIJACKING HOST MEMBRANE LIPIDS (CHOLESTEROL AND GLYCEROPHOSPHOLIPID) BY *E. CHAFFEENSIS*

The *E. chaffeensis* cell membrane is cholesterol-rich (Lin and Rikihisa, 2003), but the bacterium cannot synthesize cholesterol

and partially lacks genes for glycerophospholipid biosynthesis (Lin et al., 2020). As small Gram-negative bacteria, *Ehrlichia* spp. require abundant membrane lipids for rapid intracellular proliferation. Thus, *E. chaffeensis* must acquire these membrane lipids from host cells. Furthermore, by incorporating eukaryotic lipids such as phosphatidylcholine and cholesterol, *E. chaffeensis* mimics the eukaryotic plasma membrane and, by doing so, adapts to the cellular environment of the host. Indeed, exogenous 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD)-phosphatidylcholine, Bodipy-phosphatidylethanolamine, and Bodipy (TopFluor)-cholesterol are rapidly trafficked to ehrlichia inclusions in infected cells (Figure 5). DiI (3,3'-diiododecylindocarbocyanine)-prelabeled host-cell membranes are unidirectionally trafficked to *Ehrlichia* inclusions and the



bacterial membrane (Figure 5), but DiI-prelabeled *Ehrlichia* membranes are not reversibly trafficked to host-cell membranes (Lin et al., 2020). The trafficking of host-cell membranes to *Ehrlichia* inclusions is dependent on both the host endocytic and autophagic pathways as well as bacterial protein synthesis, as the respective inhibitors block the trafficking of DiI-labeled host membranes to *Ehrlichia* as well as infection (Lin et al., 2020). Cryosections of infected cells show numerous membranous vesicles inside *Ehrlichia* inclusions as well as multivesicular bodies docked on the inclusion surface, both of which can be labeled by GFP-tagged 2xFYVE protein that binds to phosphatidylinositol 3-phosphate, a product of PtdIns3K activity (Lin et al., 2020). Focused ion-beam scanning electron microscopy of infected cells has validated the existence of numerous membranous structures inside bacterial inclusions (Lin et al., 2020). These results support the notion that *Ehrlichia* inclusions are amphisomes formed through fusion of early endosomes, multivesicular bodies, and early autophagosomes induced by Etf-1, and they provide the host-cell membrane glycerophospholipids and cholesterol necessary for bacterial proliferation.

4 FUTURE DIRECTIONS

Ehrlichia species propagate via perpetual transmission between ticks and mammalian hosts and can proliferate in each of these two distinct environments. Owing to multiple technical limitations, little is known about the bacterial components that enable *Ehrlichia* to thrive throughout this lifecycle. Recently, Himer1 transposon mutagenesis was successfully applied to *E. chaffeensis* as well as other *Ehrlichia* species, and functional knockout mutants have been cloned (Cheng et al., 2013; Bekebrede et al., 2020). Moreover, the application of targeted mutagenesis techniques to *Rickettsia* and *Ehrlichia* species is on the horizon (McClure et al., 2017). Combined with advanced analysis of functional genes in ticks along with molecular and cellular techniques to manipulate ticks, it is expected that *Ehrlichia* Himer1 transposon insertional mutant libraries will facilitate this line of investigation. Further experimental discoveries of bacterial factors and their functions during the natural life cycle of *Ehrlichia*—in which humans are merely accidental hosts—are expected to reveal the remarkable molecular evolution of these

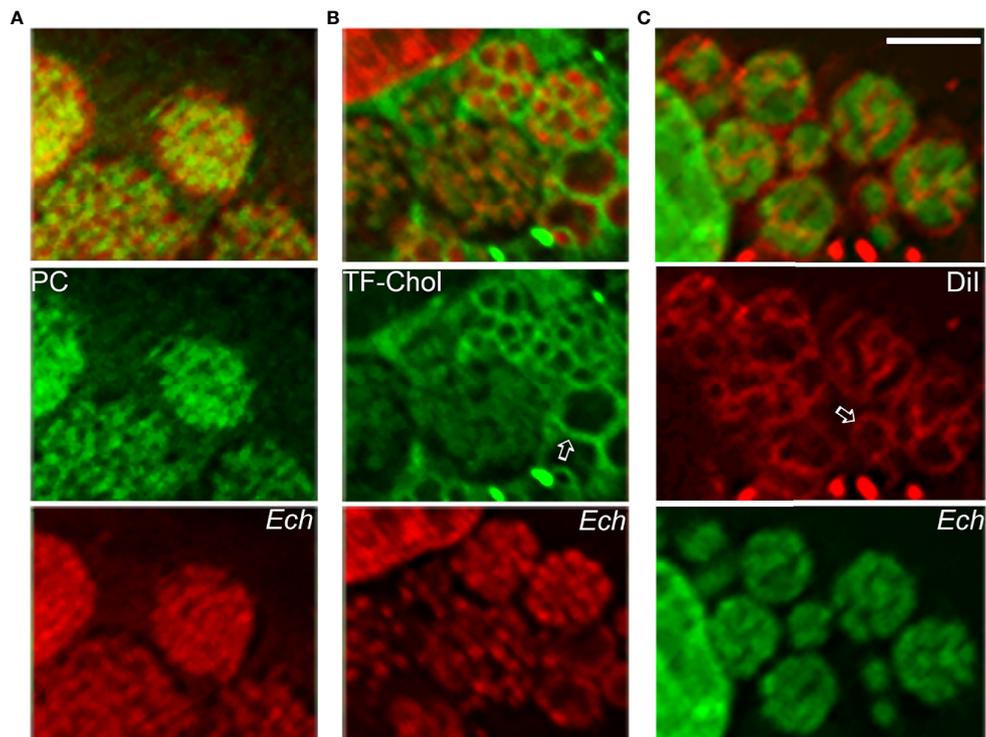


FIGURE 5 | Host-cell membrane lipids (cholesterol and glycerophospholipids) are trafficked to the membrane of *E. chaffeensis* and its inclusions. NBD-phosphatidylcholine (**A**), TopFluor-cholesterol (**B**), or Dil (**C**) is added to *E. chaffeensis*-infected RF/6A cells to monitor the intracellular distribution of the fluorescent lipids. DNAs of bacteria and host are stained with Hoechst 33342 (pseudocolored green and red, respectively). Note labeling of densely packed intraluminal *E. chaffeensis* (*Ech*, small cocci stained with Hoechst 33342) with the three fluorescent lipids (**A–C**) and labeling of the bacterial inclusion membrane (open arrows) and intraluminal membranes with TopFluor-cholesterol (**B**) and Dil (**C**). Bar: 3 μ m. The drawing was modified from (Lin et al., 2020), copyright 2020 PNAS.

tick-borne pathogens and inform the development of effective therapeutic strategies and preventative measures for diseases caused by *Ehrlichia* species.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this review and has approved it for publication.

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