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By Gina Marie Borgo

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Abstract

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Bacteria in the genus *Rickettsia* are arthropod-borne obligate intracellular microbes that can cause a spectrum of diseases in humans with manifestations ranging from mild to severe. Following invasion of host cells, *Rickettsia* must escape from the membrane-bound vacuole to gain access to the cytosol, where they reside. In the cytosol, bacteria must avoid detection and degradation by host pathways such as autophagy. They also undergo actin-based motility and initiate cell-cell spread to infect new cells. Although we have a mechanistic understanding of invasion, actin-based motility, and cell-cell spread, how *Rickettsia* interacts with and manipulates host membranes is poorly understood. In particular, *Rickettsia* genomes encode factors predicted to interact with and mediate rupture of host membranes, such as phospholipases and hemolysins, but very little is known about how these proteins function during infection. In this dissertation, I describe the characterization of a conserved *Rickettsia* phospholipase, Pat1, to address key unanswered questions about the role of this membrane targeting enzyme in the *Rickettsia* intracellular life cycle and in pathogenesis. I investigated the role of *Rickettsia* Pat1 by characterizing the phenotype of a *Rickettsia parkeri* mutant with a transposon insertion in the *pat1* gene. I found that Pat1 is critical *Rickettsia* factor for efficient escape from the vacuole into the cytosol, both following invasion and during cell-cell spread. This provides genetic evidence to support a long-held hypothesis that phospholipases mediate *Rickettsia* vacuolar escape. Pat1 is also important for preventing association of the bacteria with damaged membranes marked by galectin-3 and for initial targeting by autophagy via the autophagy adapter NDP52. Pat1 is also important for avoiding autophagy that occurred on bacteria not associated with damaged membranes and involved targeting by host polyubiquitin and the autophagy cargo adaptor p62. Moreover, Pat1 is critical for actin-based motility and escape from the secondary vacuole, two processes related to cell-cell spread. Although Pat1 does not affect growth inside tissue culture cells, it is required for virulence in a mouse model of infection. Altogether, the data presented in this dissertation suggest Pat1 is important at multiple steps of the *Rickettsia* life cycle that involve manipulating host membranes. This work also contributes more generally to our understanding of the role of bacterial

patatin-like phospholipases in the host-microbe interaction. Future work on *Rickettsia* Pat1 will further define the mechanistic details of Pat1 function during infection, as well as how Pat1 activity is regulated, how it cooperates with other bacterial and host proteins to allow bacteria to efficiently access the cytosol, and what role it plays in animal infection.

Table of Contents

List of Figures

List of Abbreviations

AG	Ancestral Group *Rickettsia*
BMDMs	Bone marrow-derived macrophages (murine)
CRD	Carbohydrate recognition domains
Gal	Galectin
HMECs	Human microvascular endothelial cells
i.v.	Intravenous infection
LAMP-1	Lysosomal-associated membrane protein 1
LC3	Microtubule-associated protein-1 light chain 3
LLO	Listeriolysin O
NDP52	Nuclear dot protein 52
NPFs	Nucleation promoting factors
PFU	Plaque forming units
Pat	Patatin-like phospholipases of *Rickettsia*
PI	Phosphatidylinositol (PI)
PI(3)P	Phosphatidylinositol 3-phosphate
$PI(4,5)P_2$	Phosphatidylinositol 4,5-bisphosphate
PLA2	Phospholipase A2
PLC	Phospholipase C
PLD	*Rickettsia* phospholipase D
PLP	Patatin-like phospholipase
SFG	Spotted Fever Group *Rickettsia*
TEM	Transmission electron microscopy
TG	Typhus Group *Rickettsia*
TRG	Transition Group *Rickettsia*
T3SS	Type 3 secretion system

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Chapter 1

Introduction

Rickettsia are Gram-negative, obligate intracellular bacteria that replicate in hematophagous arthropods, such as ticks, fleas, and body lice. *Rickettsia* species are divided into four phylogenetic groups: ancestral group (AG), typhus group (TG), transition group (TRG) and spotted-fever group (SFG) (1), and within these groups, species are surprisingly diverse in terms of lifecycle, vector manipulation, and pathogenicity (2-4). This chapter will focus on TG and SFG *Rickettsia*, because these two groups contain important human pathogens and have been the primary focus of research on *Rickettsia* pathogenesis. The TG group of *Rickettsia* includes *R. prowazekii* (causes epidemic typhus) and *R. typhi* (causes murine or endemic typhus), which are transmitted by inoculation of contaminated feces from body lice or fleas (4). *R. prowazekii* is a pathogen of historical significance (5) and continues to be a risk for biosecurity (6,7) as well as for populations living in crowded or unhygienic conditions (8-11). The SFG *Rickettsia* include *R. rickettsii* (causes Rocky Mountain spotted fever) and *R. parkeri* (causes *R. parkeri* rickettsiosis), which are both human pathogens that are transmitted through the bite of an infected tick (4). Infections with SFG *Rickettsia* species are on the rise (12,13), likely due to a combination of increased awareness (14), climate change (15), changes in tick distribution (16-18), and increased interaction between ticks and humans (15). Despite the most pathogenic species of TG and SFG *Rickettsia* being identified over 100 years ago (19-22), fundamental questions about *Rickettsia* biology and virulence remain unanswered.

Similarities and differences in the intracellular life cycles of *Rickettsia* species

Both TG and SFG *Rickettsia* target endothelial cells (23-25) and macrophages (26,27) during human infection. The main steps of the life cycle in host cells involve invasion, vacuolar escape, replication, and dissemination (or spread to new cells) (2,3, 24). Although all *Rickettsia* species are obligate intracellular bacteria, there are many similarities and differences between how TG and SFG *Rickettsia* interact with host cells (**Figure 1.1**)

The *Rickettsia* life cycle begins with invasion of the host cell. For invasion, the outer membrane protein OmpB has been shown to bind host receptor Ku70, triggering a signaling cascade that facilitates actin polymerization at the invasion site (28,29). Although OmpB-dependent invasion has not been investigated in TG *Rickettsia*, the conservation of OmpB across all *Rickettsia* genomes (30,31) suggests a conserved entry mechanism**.** For TG *Rickettsia*, a secreted protein RalF has also been identified as critical for invasion by enriching phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) at the invasion site via interaction with the GTPase Arf6 (32). OmpA is another outer membrane protein that was recently shown to interact with fibroblast growth factor receptor-1 to mediate caveolin-1-dependent invasion (33).

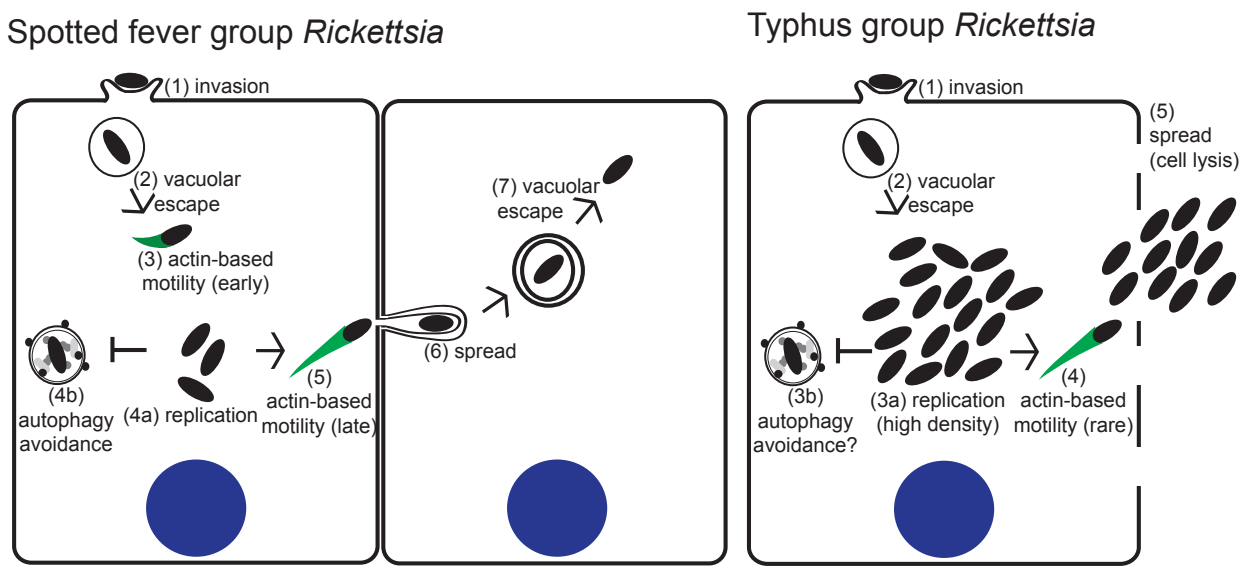

Figure 1.1 Cellular life cycle of SFG and TG *Rickettsia*. The intracellular life cycles of SFG (left) and TG (right) *Rickettsia* species. SFG *Rickettsia* (1) invade host cells, (2) escape from the vacuole, and (3) undergo an early phase of actin-based motility. (4a) Bacteria replicate in the cytosol and (4b) avoid detection by anti-bacterial autophagy. Later stages of infection involve (5) a second phase of actin-based motility that positions the bacteria at the cell periphery, (6) protrusion formation and internalization into a neighboring cell, and (7) escape from the secondary vacuole. In contrast, TG *Rickettsia* species may or may not undergo actin-based motility and (3a) reach high bacterial density during replication. (3b) Autophagy has not been studied in the context of TG *Rickettsia* and avoidance strategies are unknown. (4) Infrequent actin-based motility can be detected for *R. typhi* and (5) bacteria exit the cell by host cell lysis.

Following invasion, both TG and SFG *Rickettsia* enter into a membrane-bound phagosome, termed a vacuole. Escape of bacteria from the vacuole into the cytosol, where bacteria replicate, is an important step in infection and the subject of much of this thesis. This step will therefore be described in more detail below. Once bacteria escape into the cytosol, as obligate intracellular microbes, they require metabolic support of the host cell for their growth. Reconstruction of *Rickettsia* metabolic networks demonstrated similar metabolic requirements from the host cell for TG and SFG *Rickettsia* (34,35), suggesting similar metabolic parasitism of the host despite differences seen in bacterial density. Some of the above differences are related to truncation or absence of specific genes, but due to most *Rickettsia* proteins being uncharacterized in both TG and SFG species, it is unclear which bacterial factors are critical for survival of all species versus factors that mediate the observed differences between species.

In addition to growth in the cytosol, SFG *Rickettsia* hijack the host cytoskeleton to undergo actin-based motility (discussed in more detail below) (36-40) and begin to spread to neighboring cells early in infection through a non-lytic process called cell-cell spread (40-42). In contrast, TG *Rickettsia* rarely undergo actin-based motility (36,37,43), grow to high density within cells, and lyse the cell to release bacteria to infect new cells (44,45).

Genetics have aided identification of key genes important for the *Rickettsia* intracellular life cycle

Despite DNA sequencing technology improving the ability to discover new *Rickettsia* species and sequence their genomes, understanding the molecular mechanisms of *Rickettsia* host cell manipulation has lagged in comparison with other model organisms due to limited availability of genetic tools. Targeted mutagenesis remains possible but challenging (46-48). Forward genetics strategies have shown the most promise for understanding how bacterial proteins interact with the host, facilitate infection, and contribute to virulence. Transposon mutagenesis has been executed in *R. prowazekii* (49,50), *R. rickettsii* (39,51), *R. parkeri* (52), and *R. conorii* (53), and mutants have yielded unexpected insight into both bacterial and host biology. For example, although actin-based motility is a strategy used by other cytosolic bacteria, forward genetics has revealed that *R. parkeri* and likely other SFG species are unique in having two distinct phases of actin-based motility mediated by two different actin nucleators (39,54). Recently, the genes involved in *R. conorii* O-antigen synthesis were identified and shown to be important for pathogenesis and to be the *Rickettsia* target of Weil-Felix serology (53). Investigation of an *ompB* mutant, identified by forward genetics, revealed that OmpB protein blocks ubiquitination of bacterial surface proteins (55). These examples highlight that *Rickettsia* is an ideal system for understanding the delicate balance between parasite and host and understanding *Rickettsia* biology can reveal critical details to important biological processes.

Vacuolar escape is mediated by pore-forming proteins and phospholipases

One poorly understood part of the *Rickettsia* life cycle, mentioned above, is bacterial escape from the vacuole (here called the primary vacuole) following invasion. For SFG *Rickettsia,* bacteria also need to escape from the vacuole following cell-cell spread (here called the secondary vacuole) (2). Vacuolar escape is critical for

successful infection, yet the mechanisms of membrane rupture during *Rickettsia* infection have largely been unexplored. This thesis focuses on understanding the role of *Rickettsia* phospholipases in vacuolar escape and how *Rickettsia* uses phospholipases to manipulate host membranes during infection. Although genes encoding phospholipases are conserved in *Rickettsia* genomes, their function during infection remains enigmatic and poorly defined. Using genetics and microscopy approaches, as described in Chapter 2, I have characterized the contribution of a bacterial phospholipase from the human pathogen, *R. parkeri*, during infection of endothelial cells.

In order to survive intracellularly, *Rickettsia* and other intracellular bacterial pathogens that grow in the cytosol, such as *Listeria monocytogenes* and *Shigella flexineri*, must escape from the plasma membrane-derived primary vacuole following host cell invasion. Escape typically occurs shortly after invasion of cells and bacteria can often be detected in the cytosol by 30 min post infection (mpi) (56-61). These kinetics suggest the factors that mediate escape are in a race against host cell processes to deliver bacteria to the cytosol.

The process of escape is thought to be driven mainly by bacterial proteins that directly interact with the vacuolar membrane. Recent studies have revealed that subversion of host pathways also contributes to successful escape. For example, *L. monocytogenes* escapes the primary vacuole using a cholesterol-dependent cytolysin, listeriolysin O (*hly*/LLO), and two phospholipase C (PLC) enzymes, PlcA and PlcB (62). LLO is required for virulence in mouse models, (63,64), escape from the primary vacuole in murine cell lines (64-66), and is sufficient to mediate escape when expressed in the soil bacterium *Bacillus subtlilis* (67). LLO monomers bind cholesterol on membranes and oligomerize before inserting into the membrane to form a transmembrane pore (68), disrupting the ion gradient needed for vacuolar maturation (57,58,69). Interestingly, LLO can facilitate growth in a vacuole, revealing additional complexity to LLO as a mediator of adaptation to the intracellular niche (70-73). The above data suggests that LLO functions in escape by directly targeting membranes of the *Listeria*-containing vacuole and interrupting normal vacuolar trafficking to the lysosome.

L. monocytogenes PlcB, a broad range PLC, plays an important role in LLO-independent escape in human cell lines (64,73,74). PlcA, a phosphoatidylinositol (PI)-specifc phospholipase, makes a minor contribution to primary vacuolar escape with LLO and PlcB (75,76). Although how these proteins lead to complete vacuolar breakdown is not known, the paradigm of *L. monocytogenes* vacuolar escape demonstrates the rapid action and efficient coordination of bacterial proteins that drive this process.

In contrast to the above example, *S. flexneri* escapes from the primary vacuole using translocators/effectors IpaB and IpaC (77-79), which are required for pore formation with the type 3 secretion system (T3SS) (80,81 IpaB and IpaC can form complexes that insert into membranes (82), and IpaD is required for IpaB/C pore formation (83). IpgD, a ($PI(4,5)P_2$ phosphatase, recruits infection-associated macropinosomes enriched in components of recycling and exocytic pathways to the *Shigella*-containing vacuole, a process that might help move membrane remnants away from the bacteria (84,85). Interestingly, the *S. flexneri* T3SS translocon appears to be sufficient for cytosolic access (86), suggesting that vacuolar rupture can occur with

IpaB/C pore formation independent of any additional effector delivery. Although *S. flexneri* escape is similar to *L. monocytogenes* in that it involves pore formation and manipulation of trafficking, the mechanism of the bacterial proteins involved in these processes is distinct. *Rickettsia* genomes encode factors that could work analogously to proteins described in *Shigella* and *Listeria* escape and little is known about which proteins facilitate membranolytic activity during infection.

***Rickettsia* genomes encode putative hemolysins and phospholipases predicted to mediate vacuolar escape**

Manipulation of host membranes is critical to intracellular adaptation. In the context of *Rickettsia* infection, phospholipids present not only a physical barrier, but also a potential target for modulating host cell processes. All sequenced *Rickettsia* genomes encode proteins such as hemolysins and phospholipases that are predicted to function in membrane manipulation (87). TlyA and TlyC are annotated as hemolysins (88). Hemolytic activity has been demonstrated for TG *Rickettsia* with heterologous expression of TlyC in a hemolysin-negative strain of *Proteus mirabilis* (89). *Salmonella typhimurium* expressing *R. prowazekii* TlyC are found free in the cytosol more frequently than WT *S. typhimurium* (88). Given the central role that pore-forming proteins play in vacuolar escape of other cytosolic bacteria, it seems likely that pore formation, either through TlyA/C or the type 4 secretion system, contributes to vacuolar escape for *Rickettsia* species.

In addition to pore-forming proteins, *Rickettsia* genomes encode at least two phospholipases (87) that are classified by the site of phospholipid cleavage (90). The gene encoding phospholipase D (PLD) is conserved in all *Rickettsia* genomes (87,88). PLD is expressed in both TG and SFG *Rickettsia* (91) and is sufficient to mediate vacuolar escape of *S. typhimurium* expressing PLD (88). The *pld* gene is one of the few that has been deleted by homologous recombination to investigate its function (47). The *R. prowazekii pld* mutant demonstrated no significant differences in vacuolar escape or growth inside RAW 264.7 cells, but the *pld* mutant was attenuated in a guinea pig model (47). These results confirm that additional *Rickettsia* proteins must facilitate vacuolar escape.

Phospholipase A2 (PLA2) enzymes have also been proposed to function in vacuolar escape. Patatin-like (Pat) PLA2 enzyme Pat1 is encoded in all *Rickettsia* genomes, and Pat2 is encoded in TG *Rickettsia* genomes and a small number of SFG genomes (87,92). The putative PLA2 activity of Pat1 and Pat2 is based on the N-terminal patatin-like phospholipase (PLP) domain. PLPs were first described in potatoes (93) and PLP domains have been identified in proteins from bacteria, fungi, plants, and animals (94-97). All PLPs have a Ser-Asp catalytic dyad required for enzymatic activity, a conserved hydrolase motif (Gly-X-Ser-X-Gly) and a Gly-rich oxyanion hole (92,98) and these structural features are shared with calcium-independent PLA2 (iPLA2) and cytosolic PLA2 (cPLA2) (96). Bacterial PLPs share many structural features with eukaryotic PLPs and do not share homology with other bacterial lipases (94). Importantly, PLPs are enriched in bacteria that are symbionts and pathogens (94), suggesting they are important mediators of the host-microbe interaction.

Pat1 and Pat2 from TG *Rickettsia* have been demonstrated to be cytotoxic when expressed in yeast (99) and both proteins can be detected in the cytosol of mammalian

host cells during infection (99,100). Mutation of catalytic residues important for PLA2 activity prevented cytotoxicity (99,100), indicating that PLA2 activity is required. Both Pat1 and Pat2 display enhanced enzymatic activity *in vitro* in the presence of Vero cell lysate or bovine liver superoxide dismutase (SOD) (99-101), consistent with these proteins functioning inside of host cells. Pre-treatment of bacteria with either anti-Pat1 or anti-Pat2 antibodies reduced the number of infected cells and increased colocalization with endosomal/lysosomal marker lysosomal-associated membrane protein 1 (LAMP-1) compared to incubation with pre-immune serum (100), although the difference in LAMP-1 colocalization was not significant. Because antibody pre-treatment was used in these studies, the antibodies are likely blocking Pat1 or Pat 2 that is associated with the bacteria, not Pat1 or Pat2 that is secreted when bacteria are inside host cells. No studies that investigate Pat1 function in SFG *Rickettsia* have been published to date.

Bacterial PLP function has, however, been investigated for other intracellular as well as extracellular pathogens. The PLP VipD from *Legionella pneumophila* targets mitochondrial membranes and induces the release of cytochrome C and activation of caspase 3 (102). VipD also interferes with endocytic trafficking by binding Rab5 and Rab22, but this is mediated by the C-terminal domain, not the PLP domain or lipase activity (103). Another PLP, ExoU from *Pseudomonas aeruginosa*, is a potent cytotoxin that has been well characterized during infection (104). Notably, ExoU homologs (~18% amino acid identity) are found in *Rickettsia* species *R. prowazekii*, *R. typhi*, *R. belli*, and *R. massile* (99,101). *P. aeruginosa* ExoU is associated with rapid cytotoxicity in cell culture and accelerated lung damage in animal models and patients (105-107 ExoU cytotoxic activity is ablated by mutation of the Ser-Asp dyad or iPLA2/cPLA2 inhibitors, consistent with PLP-dependent cytotoxicity (108,109). ExoU-mediated release of arachidonic acid can also modulate inflammation via release of eicosanoids and enhanced recruitment of neutrophils (110,111). These examples demonstrate the potential for PLPs to mediate manipulation of multiple host cell processes like membrane disruption, trafficking, and eicosanoid signaling.

We are just beginning to understand bacterial PLP function, and investigations into the role of *Rickettsia* PLPs during infection have been limited. In Chapter 2 of this thesis, I address the function of Pat1 during *R. parkeri* infection of endothelial cells by comparing a *pat1* mutant to wild type bacteria at key events in the life cycle. Studying how *Rickettsia* uses PLPs to interact with host cells will help further our understanding of how membrane targeting proteins facilitate adaptation to the host and can potentially reveal underlying mechanisms for how PLPs can facilitate both symbiosis and virulence.

Host cells can sense membrane damage by invading bacteria

The ability of host cells to recognize damage associated with an invading pathogen has emerged as a strategy for cells to detect intracellular pathogens. One type of damage caused by pathogens, particularly those that escape from vacuoles into the cytosol, is damage to surrounding membranes. Damage to host membranes is detected in part by host galectin proteins (112,113), which are β-galactoside-binding proteins that have been identified in both vertebrates and invertebrates (114). Galectins contain up to two carbohydrate recognition domains (CRD) and can be classified into one of three groups: prototypical galectins that have one CRD that forms homodimers;

chimera-type galectins that have a single CRD with an N-terminal peptide enriched with Gly, Tyr, and Pro; and tandem-repeat galectins that have two CRD domains connected by a peptide linker (114-116). Galectins localize to the cytosol, nucleus, and extracellular space and each galectin CRD recognizes different glycans (117). Binding to the disaccharide N-acetyllactosamine (LaNAc, type I and type II) is conserved, and specificity is determined by affinity to branching, repeating, or modified glycans (116-119). The CRD has sites that can interact with other proteins, such as the C-terminal CRD of galectin-8 binding the autophagy receptor nuclear dot protein 52 (NDP52) (120). Thus, galectins can bind both carbohydrate ligands and protein partners.

Galectins play an important role in responding to infection, as they can directly bind pathogens and sense pathogen-associated damage. For example, the CRD of galectin-3 can directly bind glycans on lipopolysaccharide from *Klebsiella pneumoniae* and the N-terminal tail can bind to lipid A from *Salmonella Minnesota R7* (121). In addition to directly binding bacteria, glycans normally displayed on the outside of the cell and sequestered in the lumen of plasma membrane-derived vesicles (such as primary and secondary vacuoles) can be detected by cytosolic galectins upon damage to the membrane. *Mycobacterium tuberculosis* was the first bacterium shown to have galectin-3 accumulate around the bacteria-containing vacuole (122). In addition, galectin-3 has now been shown to mark ruptured vacuoles containing the pathogens *S. flexneri* (123, 124), *L. monocytogenes* (124,125), *S. typhimurium* (126), *L. pneumophila* (127), *Streptococcus pyogenes* (128), and *Coxiella burnetti* (129). Use of cell lines lacking galectin-3 ligands (124, 126) or inhibitors of N-glycan synthesis (125) have demonstrated the recruitment of galectin-3 to the bacteria-containing vacuole requires host glycans, and galectins are recruited to sites of damage on endosomes or lysosomes in the absence of infection (126, 127), both consistent with galectin binding to glycans exposed on host membranes, not bacteria. Additionally, galectin-8 can be recruited to damage on bacteria-containing vacuoles of *S. typhimurium*, *S. flexneri*, *L. monocytogenes* (126), *C. burnetti* (126), and *S. pyogenes* (128), and galectin-8 appears particularly important for controlling bacterial replication (discussed more below) (126, 128). Although galectins were known to play a role in adaptive and innate immune responses to pathogens (116, 130), these studies suggest an additional function of specific cytosolic galectins as sentinels for endocytic and lysosomal damage. Galectins have thus emerged as important detectors of danger and coordinators of cellular responses. Because membrane damage to the bacteria-containing vacuoles is required for intracellular survival, it will be interesting to explore the strategies pathogens use to subvert targeting by galectins.

Host cells can target bacteria and bacteria-containing vacuoles by autophagy (xenophagy)

Autophagy is an intracellular degradation pathway that is critical to cell homeostasis (131,132). It is also an innate immune response for pathogen elimination (this process is also called xenophagy; here I will refer to it as autophagy) (133-135) During infection, bacteria can be marked with polyubiquitin (136,137). Polyubiquitin-positive bacteria recruit autophagy receptors like p62/SQSTM1 (138,139) and NDP52 (139,140). Autophagy receptors recruit microtubule-associated protein-1 light chain 3

(LC3), leading to autophagosome formation (138,140) and fusion with lysosomes (133-135). *R. parkeri* uses OmpB and methylation to evade autophagy (55,141). OmpB blocks ubiquitination of outer membrane proteins, such as OmpA, and is required for evading autophagy in macrophages (55,141). Two *R. parkeri* protein-lysine methyltransferases (PKMTs) were recently shown to methylate Lys on bacterial outer membrane proteins, further protecting *R. parkeri* from polyubiquitination and targeting by autophagy (141,142). Thus, intracellular pathogens must subvert autophagy to avoid detection and degradation by the host.

Components of the autophagy machinery can also be recruited to bacteria-containing vacuoles (113). Membrane remnants marked by galectin-3 also colocalize with polyubiquitin (123,143), p62, and LC3 (123), and galectin-8 directly interacts with NDP52 (126) and the E3 ligase parkin (128). This observation suggests that membrane remnants can be targeted for degradation via autophagy. Interestingly, galectin-8 recruitment is specifically associated with an anti-bacterial response whereas galectin-3 supports bacterial replication. In particular, by altering host glycan composition, recruitment of galectin-3 to vacuoles damaged during *L. monocytogenes* infection was shown to downregulate autophagy (125). In addition, higher recruitment of galectin-3 to *S. pyogenes* vacuoles protected bacteria from degradation by preventing galectin-8-mediated recruitment of the E3 ligase parkin (128). In contrast, recent examples for *C. burnetti* and *S. typhimurium* suggest that recruitment of the autophagy machinery to repair damage on the vacuolar membranes can be beneficial by maintaining vacuolar integrity (129,144). It seems likely that galectins play a role in recruiting host factors for membrane repair in this context.

***Rickettsia* hijacks the host cytoskeleton for actin-based motility**

Actin-based motility is a process by which bacteria hijacking the host actin machinery by polymerizing actin on their surface and harnessing the force of polymerization to move around the cell (145). One common mechanism for actin-based motility is to activate the host actin-related protein Arp2/3 complex to form actin tails consisting of branched filaments (146). In this process, bacterial mimics of nucleation promoting factors (NPFs) activate the host Arp2/3 complex. Bacterial NPFs include *L. monocytogenes* ActA (147,148), *B. thailandensis* BimA (149), and *R. parkeri* RickA (54,150) proteins. *S. flexneri* also hijacks the Arp2/3 complex using a bacterial protein called IcsA that recruits a host NPF, N-WASP (151). A second, distinct mechanism for actin-based motility is to directly polymerize actin to form actin tails consisting of bundled filaments. For example, BimA from other *Burkholderia spp.*, *B. mallei* and *B. psuedomallei*, form actin tails by mimicking host Ena/VASP actin polymerases (152). *R. parkeri* also directly polymerizes actin using the bacterial Sca2 protein, which mimics host formins (54,153,154). Interestingly, although *R. parkeri* uses two distinct pathways for actin-based motility (involving RickA and Sca2), it is unclear whether these different mechanisms are interchangeable. It is also unclear why there is variability in which *Rickettsia* species undergo actin-based motility.

A key role for actin-based motility is cell-cell spread (54,62,155-158), and both *L. monocytogenes* and *S. flexneri* use actin-based motility for propulsion into the plasma membrane that results in the formation of long protrusions containing bacteria (42,65, 159,160). For *L.* monocytogenes, actin-based motility also plays an important role in

autophagy avoidance by moving bacteria away from LC3-positive membranes (161,162). Although the role of Sca2 in SFG *Rickettsia* spread has been demonstrated using *sca2* mutants (39,54), it is unknown if the main function of RickA is also in cell-cell spread. SFG *Rickettsia* undergo actin-based motility using both RickA and Sca2 in both mammalian (54) and tick cells (163), but RickA was important for spread in tick cells (163), compared to both RickA and Sca2 being important for spread in mammalian cells (54). In animal models, *sca2* mutants are attenuated (39,164) and impaired in dissemination in a mouse intradermal model (164) but not a tick model (163). These results suggest that the contribution of actin-based motility or actin manipulation by these factors may differ depending on the host or cell type.

The plasma membrane is manipulated for spread and ruptured for escape from the secondary vacuole

Once bacteria reach the plasma membrane, manipulation of the host cytoskeleton is critical for bacterial cell-cell spread, a complex, multistep process that involves protrusion formation/engulfment and escape from the secondary vacuole. For *L. monocytogenes* and *S. flexneri*, actin-based motility into the plasma membranes leads to formation of a long protrusion containing a bacterium with its associated actin tail (165-166). In contrast, *R. parkeri* stops actin-based motility at the cell cortex, then forms a short protrusion (42). In addition to actin-based motility, protrusion formation and resolution requires further manipulation of the cytoskeleton at cell junctions. For example, *L. monocytogenes* protrusion formation is facilitated by internalin C (InlC) blocking the interaction between Tuba and N-WASP at apical tight junctions, and interference with cortical tension could promote protrusion formation by relieving membrane stiffness (167). InlC also interferes with Tuba interaction with COPII proteins Sec31A and Sec13 (168). *R. parkeri* protrusion engulfment utilizes a secreted bacterial effector, Sca4, that binds vinculin, interfering with the normal vinculin-α-catenin interactions at adherens junctions (42). For *S. flexneri*, IpaC manipulates cell tension by interacting with β-catenin at the membrane (169). Together, these results demonstrate the two-fold contribution of cytoskeletal disruption in promoting spread (powering motility and modulating membrane tension), revealing that although there is a conserved strategy for cell-cell spread, the outcome is achieved by diverse underlying mechanisms that target distinct host pathways.

Beyond cytoskeletal proteins, other host factors at the plasma membrane are targeted by pathogens to promote spread and protrusion engulfment. LLO can cause local membrane damage in protrusions, exposing the phospholipid phosphatidylserine to bind to TIM-4 receptors on macrophages and promoting spread by exploiting efferocytosis (170). *S. flexneri* protrusion engulfment and subsequent vacuolar escape requires T3SS mediated activation of tyrosine kinase signaling in the protrusion (165). Tyrosine kinase signaling likely activates the class II phosphatidylinositol 3-phosphate (PI(3)P) kinase, PIK3C2A, at the protrusion membrane, which enriches PI(3)P in the protrusion membrane (171). Although it is unclear what role PI(3)P enrichment plays in facilitating protrusion formation, these results suggest that direct manipulation of membrane phospholipids can facilitate spread.

Cell-cell spread culminates with escape from a double membrane secondary vacuole. This escape event follows similar principles as escape from the primary

vacuole. *L. monocytogenes* utilizes the same proteins for secondary vacuole escape as primary escape, including LLO (172), PlcA (172), and PlcB (62,172). For *S. flexneri*, escape from the secondary vacuole involves the T3SS and translocators/effectors IpaB and IpaC (165,173). Escape from the secondary vacuole is facilitated by an additional protein, IcsB, which has been shown to have acyl-transferase activity towards membrane-associated proteins (174-176). These results suggest that *Rickettsia* might also use similar factors to escape from the primary and secondary vacuole.

Summary

Rickettsia encounters host membranes at critical times throughout its life cycle. Phospholipases are prime candidates to facilitate the interaction between bacteria and membranes. The *Rickettsia* life cycle as described above involves targeted manipulation of host cell processes. What role phospholipases such as Pat1 play in the different steps of the life cycle is not clear. In chapter 2, I investigate the role of Pat1 during infection by determining how loss of Pat1 affects the ability of bacteria to invade, escape from the vacuole, replicate, and spread. Studying how phospholipases contribute to intracellular adaptation, as I describe in Chapter 2, will further our understanding of both *Rickettsia* biology and the molecular mechanisms governing membrane manipulation in both symbiotic and parasitic relationships.

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Chapter 2

Characterization of a *pat1::tn* mutant reveals multiple roles for Pat1 in the *R. parkeri* life cycle

Introduction

Spotted fever group (SFG) *Rickettsia* are Gram-negative, obligate intracellular bacteria that infect tick vectors and can be transmitted by tick bites to vertebrate hosts (1). SFG *Rickettsia* that can infect humans include *R. rickettsii*, the causative agent of Rocky Mountain spotted fever, a disease characterized by high fever, neurological symptoms, organ failure, and occasional fatality (2–4). SFG *Rickettsia* also include species such as *R. parkeri*, which causes milder eschar-associated rickettsiosis characterized by lower fever and an eschar at the site of the tick bite yet has not been documented to cause fatality (3,5,6). Because *R. parkeri* can be studied under BSL2 conditions, it is emerging as a model for understanding the molecular determinants of SFG *Rickettsia* pathogenicity.

R. parkeri targets endothelial cells (7–9) as well as macrophages (5,7–11) during infection in humans and animal models. The intracellular life cycle of *R. parkeri* and other SFG *Rickettsia* begins with invasion of a host cell, followed by escape from the primary vacuole into the cytosol, where bacteria replicate (12,13). Bacteria then initiate actin-based motility and move to the plasma membrane, where they enter into protrusions that are engulfed into neighboring cells, necessitating another escape event from a double-membrane secondary vacuole into the cytosol and completing the life cycle (12,13).

Other bacteria with a similar life cycle utilize pore-forming proteins and phospholipases to escape from the primary and/or secondary vacuole. For example, *Listeria monocytogenes* utilizes the cholesterol dependent cytolysin listeriolysin O (LLO) (14–19) and *Shigella flexneri* uses the IpaB-IpaC translocon to form pores that facilitate membrane rupture (20–25). *L. monocytogenes* also uses two phospholipase C enzymes, PlcA and PlcB, to escape from primary and secondary vacuoles and for LLO-independent escape in epithelial cells (15,26–30). It is likely that *Rickettsia* also utilizes at least one protein that can directly disrupt the vacuolar membrane to mediate escape.

SFG *Rickettsia* genomes encode two types of phospholipase enzymes, phospholipase D (PLD) and up to two patatin-like phospholipase A2 (PLA2) enzymes (Pat1 and Pat2) (13,31–33). PLD is dispensable for escape, as a *pld* mutant in *R. prowazekii* showed no delay in vacuolar escape (34), even though exogenous PLD expression in *Salmonella enterica* was sufficient to facilitate escape (35). This suggests that other bacterial factors contribute to this process. PLA2 enzymes have been proposed to perform a role in escape. PLA2 activity from *R. prowazekii* was demonstrated to target host phospholipids throughout infection (36,37). Furthermore, pretreatment of bacteria with either a PLA2 inhibitor or anti-Pat1 or anti-Pat2 antibodies reduced plaque number for both *R. rickettsii* (38–40) and *R. typhi* (40,41) and increased colocalization of *R. typhi* with the lysosomal marker LAMP-1 (41). This suggests that Pat1 and Pat2 are important for infection and vacuolar escape prior to trafficking to the lysosome. Nevertheless, the precise role of phospholipases in rickettsial vacuolar escape remain unclear.

Phospholipase activity and escape from the vacuole may also be important to enable downstream events including actin-based motility as well as avoidance of targeting by host damage-response and autophagy pathways. With regard to the latter processes, membrane damage to the bacteria-containing vacuole can expose glycans

internalized from the host cell surface that are recognized by host cytosolic glycan-binding galectin (Gal) proteins (42–47). In particular, Gal3 and Gal8 can target damaged vacuolar compartments during infection with cytosolic *L. monocytogenes* (43,44,47) and *S. flexneri* (43–45), as well during infection with bacteria that typically reside in membrane-bound compartments such as *Legionella pneumophila* (46), *S. enterica* (43,44), *Coxiella burnetti* (48), and *Mycobacterium tuberculosis* (49). Importantly, membrane remnants marked by Gal3 and Gal8 are also positive for polyubiquitin (45,49,50), autophagy adaptors p62/Sequestome 1 (SQSTM1; hereafter referred to as p62) (45,49), nuclear dot protein 52 (NDP52)/calcium-binding and coiled-coil domain 2 (CALCOCO2; hereafter referred to as NDP52) (44,48), and microtubule-associated protein 1A/1B-light chain 3 (LC3) (44,45,48,49). Thus, membrane rupture is important for pathogens to gain access to the cytosol and is also critical for host cell detection of invading pathogens that can be linked to other anti-bacterial processes such as autophagy. Bacteria free in the cytosol can also be directly targeted by autophagy through conjugation of ubiquitin to bacterial substrates (51–53). Polyubiquitin on the bacterial surface is linked to anti-bacterial autophagy through selective cargo receptors (54) such as p62 (55–57) and NDP52 (56,58,59). These bind both ubiquitin and LC3 (53,55,57–59), which marks nascent and mature autophagosomal membranes (53,60,61). Bacterial phospholipases may facilitate autophagy avoidance by promoting escape from damaged membranes, or through manipulation of phospholipids needed for autophagosome formation, such as with *L. monocytogenes* PlcA targeting of phosphatidylinositol 3-phosphate (PI(3)P) to block LC3 lipidation (62,63). However, it remains unknown if *Rickettsia* utilizes phospholipases to evade autophagy.

To better understand the role of PLA2 enzymes during SFG *Rickettsia* infection, we characterized a *R. parkeri* mutant with a transposon insertion in the single PLA2-encoding gene *pat1*. We found that Pat1 is critical throughout infection for escaping host membranes, avoiding targeting by autophagy, and spreading to neighboring cells. These results suggest that Pat1 is a key bacterial factor involved in interacting with host membranes and avoiding detection in host cells.

Results:

Pat1 is important for infection of host cells and contributes to virulence in mice

To determine the role of Pat1 during infection and virulence, we used a *R. parkeri* mutant with a transposon insertion in the *pat1* gene (*pat1::tn*) that was previously isolated in a screen for mutants that cause small plaque size (64). We first complemented the *pat1::tn* mutation by generating a strain (*pat1::tn pat1*$^{+}$) that also contains a second transposon encoding full length *pat1* plus the intergenic regions immediately 5’ and 3’ to the gene predicted to contain the native promoter and terminator (**Figure 2.1A**). Using an antibody we generated that recognizes *R. parkeri* Pat1 by western blotting, we observed a band at the predicted molecular weight for Pat1 in WT bacteria, no corresponding band in the *pat1::tn* mutant, and a restoration of the band in the *pat1::tn pat1*$^{+}$ complemented mutant. This indicates that Pat1 protein is absent in the mutant, suggesting it is a null mutant, and that protein expression is fully restored in the complemented strain (**Figure 2.1B**). Because the *pat1::tn* mutant was initially identified based on its small-plaque phenotype, we next compared plaques sizes of WT, mutant, and complemented mutant strains. Compared with WT, the *pat1::tn*

mutant showed significantly smaller plaques, and plaque size was rescued in the complemented mutant (**Figure 2.2A; Figure 2.1C**). This demonstrates that the observed reduction in plaque size is caused by loss of *pat1*.

To further determine if Pat1 plays a role in bacterial replication, growth curves measuring plaque-forming units (PFU) were performed in two cell types, African green monkey Vero cells and human microvascular endothelial cells (HMECs). There were no differences in bacterial replication kinetics for WT, *pat1::tn* and *pat1::tn pat1*$^{+}$ complemented strains in HMECs (**Figure 2.1D),** or WT and *pat1::tn* bacteria in Vero cells (**Figure 2.2B**). These data indicate that the transposon disruption of *pat1* interferes with some part(s) of the bacterial life cycle but not intracellular growth.

We next examined the contribution of Pat1 to virulence *in vivo* using mice lacking the receptors for IFN-I (*Ifnar1*) or IFN-γ (*Ifngr1*) (*Ifnar1*$^{-/-}$ *Ifngr1*$^{-/-}$ mice), which succumb to infection with WT *R. parkeri* and can be used to investigate the importance of bacterial genes to virulence (65), T.P. Burke, C.J. Tran, P. Engstrom, D.R. Glasner, D.A. Espinosa, E. Harris, M.D. Welch, *eLife*, in press). Mice infected intravenously (i.v.) with WT *Rickettsia* at 5x10^{6} PFU showed a rapid drop in temperature and body weight following infection (**Figure 2.2C, D**) and did not survive past day 8 (**Figure 2.1E**). In contrast, mice infected i.v. with the *pat1::tn* mutant maintained a steady temperature following infection (**Figure 2.2C**), showed an initial drop in weight that stabilized around 2 weeks post infection before increasing (**Figure 2.2D**), and the majority survived until the end of the experiment (day 40) (**Figure 2.1E**). These results indicate that Pat1 is an important virulence factor in an animal model.

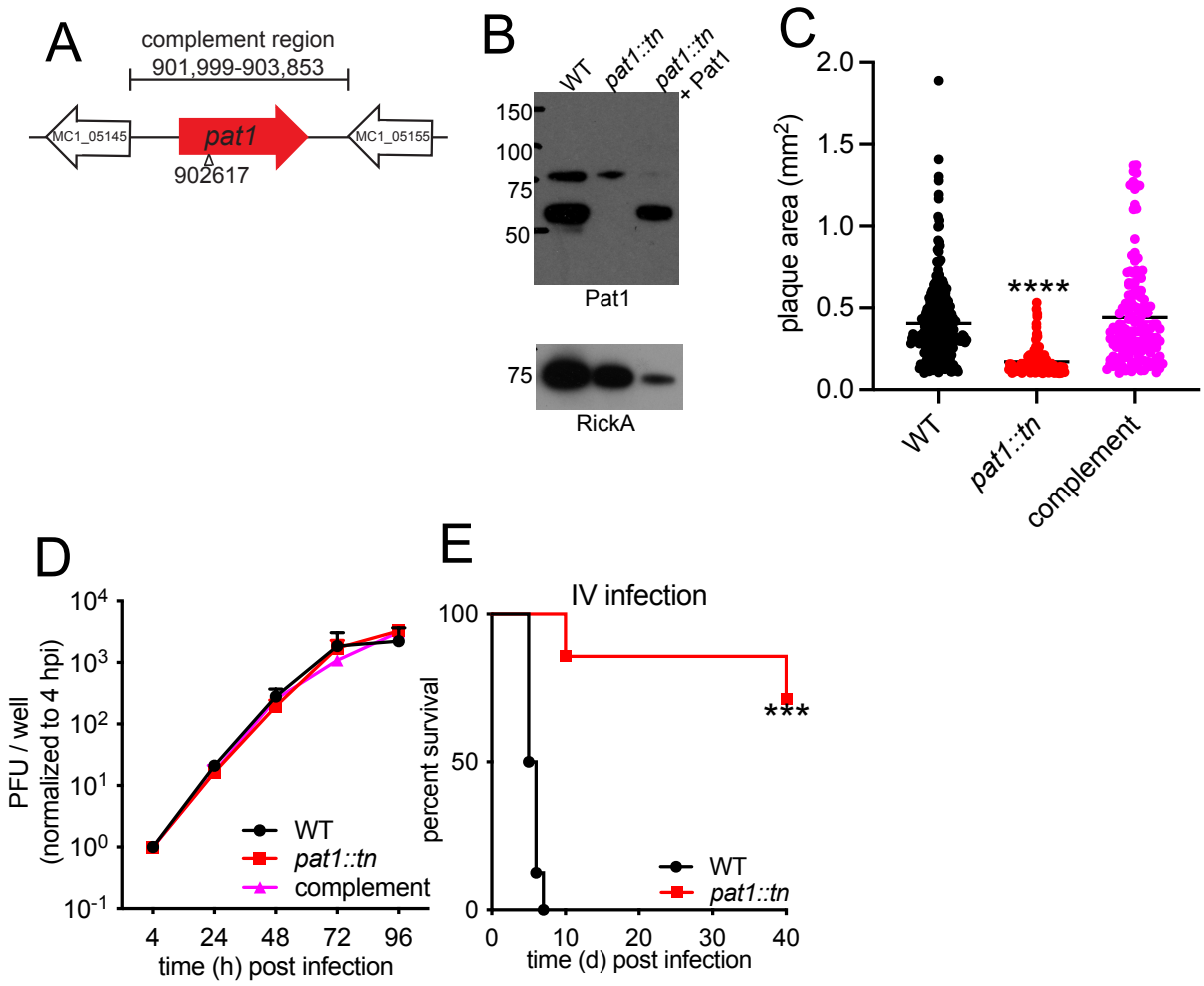

Figure 2.1 Pat1 is important for infection in cells and in mice. (A) Genomic locus of *pat1.* Triangle represents transposon insertion site with genome position. Genes upstream and downstream are included to show intergenic regions included for complementation. Nucelotide numbers indicate the position in the genome that was used. (B) Western blot of purified *R. parkeri* strains, WT, *pat1::tn*, and complemented strain (*pat1::tn pat1*$^{+}$); RickA was used as a loading control. (C) Plaque area in Vero cells infected with WT, *pat1::tn*, and complemented strain. (D) Growth curve of WT, *pat1::tn*, and complemented strain in HMECs (n=3). (E) Survival of *Ifnar*$^{-/-}$*Ifngr*$^{-/-}$ mice infected intravenously (i.v.) with 5x10^{6} WT or *pat1::tn* mutant (n=6 mice for WT, n=8 mice for *pat1::tn*, data represents 2 independent experiments). Data in (C) and (D) are mean ± SEM; ****p<0.0001 relative to WT (one way ANOVA). Data in (E) were analyzed using a log-rank (Mantel-Cox) test ***p<0.001.

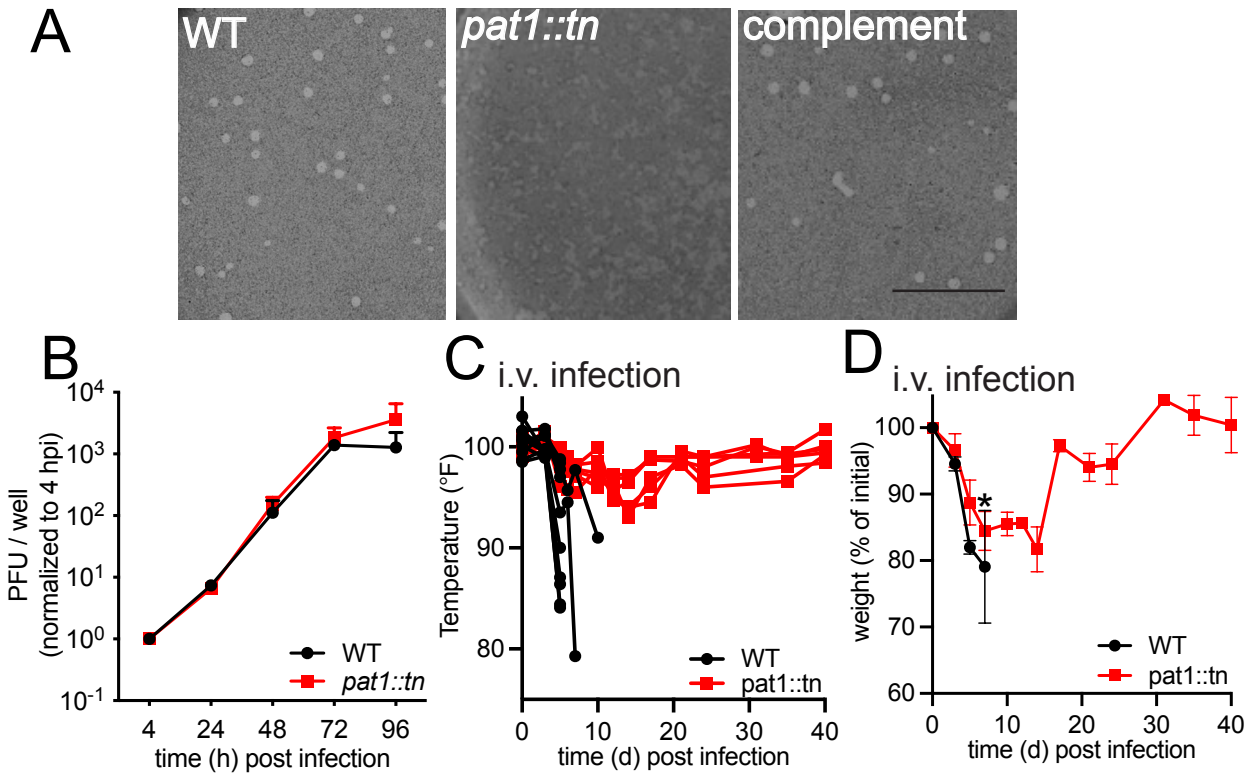

Figure 2.2 Supplemental data related to Figure 2.1, Pat1 is important for infection in cells and in mice. (A) Images of plaques stained with neutral red at 6 dpi. Scale bar 10 mm. (B) Growth curve of WT and *pat1::tn* bacteria in Vero cells (n=3). (C) Temperature changes over time in i.v. infection of *Ifnar*$^{-/-}$*Ifngr*$^{-/-}$ mice with 5x10^{6} WT or *pat1::tn* mutant bacteria; graphs represent data from individual mice. (D) Weight change over time expressed as percent change from initial weight in i.v. infection of *Ifnar*$^{-/-}$*Ifngr*$^{-/-}$ mice with 5x10^{6} WT or *pat1::tn* mutant bacteria. All data are mean ± SEM. Data in (B) were not signicantly different (unpaired t test). Data in (D) were analyzed using a two way ANOVA from 0 to 7 dpi.

Pat1 promotes efficient escape from the vacuole post-invasion

Because *R. typhi* Pat1 and Pat2 had been previously implicated in membrane rupture (41), we sought to determine if the *R. parkeri pat1::tn* mutant was impaired in its ability to escape from the primary vacuole during infection. To evaluate the role of Pat1 in vacuolar escape, we used transmission electron microscopy (TEM) to investigate whether there were host membranes around intracellular bacteria 1 h post infection (hpi) in HMECs. This time point was chosen because most bacteria had invaded cells (**Figure 2.4A**), and prior studies reported escape from the vacuole by 30 min post infection (mpi) for *R. typhi* (41), *R. prowazekii* (34), *R conorii* (66), *L. monocytogenes* (67) and *S. flexneri* (68). At this timepoint, significantly more WT bacteria were found free in the cytosol (74%) compared with the *pat1::tn* mutant (38%) (**Figure 2.3A, B)**. Moreover, significantly fewer WT bacteria were found within membranes (25% in single membranes, 1% in double membranes) in comparison with the *pat1::tn* mutant (50% in single membranes, 12% within double membranes). This suggests that Pat1 facilitates escape from membranes following invasion.

We further hypothesized that the increased localization of the *pat1::tn* mutant within membranes may impair access to the cytosol, particularly to the pool of actin, and would therefore interfere with actin-based motility. To test this hypothesis, we quantified the percentage of bacteria with actin tails at 30 mpi and 1 hpi. Approximately 3-4% of WT bacteria were associated with actin tails, in keeping with previous reports (69,70). The frequency of *pat1::tn* mutant association with actin tails was half that of WT at both time points (**Figure 2.3B**). These results demonstrate that failure of the *pat1::tn* mutant to escape from the vacuole can impact actin-based motility. To confirm that the reduced frequency of actin-based motility resulted from bacteria being trapped within membranes, we used hypotonic shock (alternating treatment with hypertonic and then hypotonic solutions) to lyse primary vacuoles (71,72) and deliver bacteria to the cytosol. When cells infected with WT bacteria were subjected to hypotonic shock at 5 mpi, there was no significant increase in the percentage of bacteria with actin tails at 30 mpi, suggesting that WT bacteria optimally access the cytosol following invasion (**Figure 2.3C**). In contrast, hypotonic shock significantly increased the percentage of *pat1::tn* mutant bacteria with actin tails (**Figure 2.3C**). These results confirm that reduced frequency of actin-based motility in the *pat1::tn* mutant is due to entrapment in the primary vacuole.

Pat1 antagonizes targeting by autophagy

The presence of a marked fraction (12%) of *pat1::tn* mutant bacteria in double-membrane compartments at 1 hpi could not be fully explained by failure to escape from the vacuole, suggesting the possibility that bacteria are targeted by host cell autophagy. Because an initial step of anti-bacterial autophagy is recognition and ubiquitylation of the bacterial surface (52), we first tested for bacterial association with polyubiquitin in infected HMECs at 0-2 hpi. Whereas fewer than 2% of WT bacteria were polyubiquitin-positive from 0-2 hpi (**Figure 2.5A, B**), the percentage of polyubiquitin-positive *pat1::tn* mutant bacteria was significantly higher and increased (from about 6% at 0 hpi to about 16% at 1 hpi), before falling slightly (**Figure 2.5A, B)**.

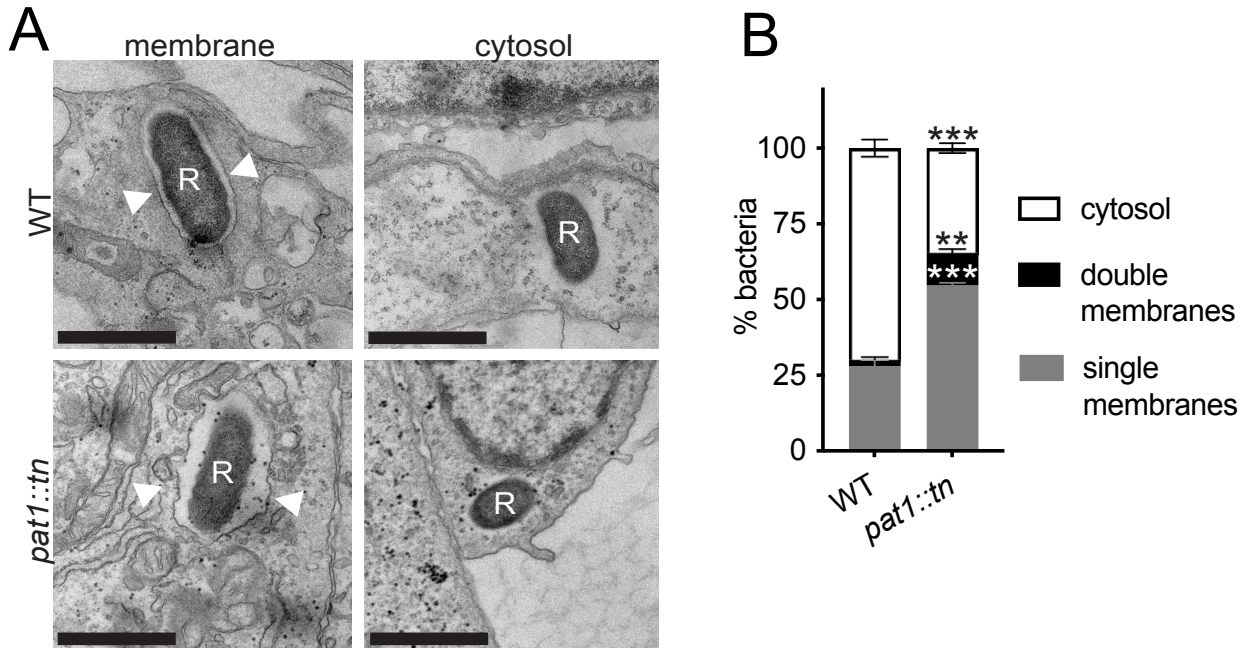

Figure 2.3 Pat1 facilitates escape from single and double membrane compartments following invasion. (A) TEM images of WT and *pat1::tn* mutant bacteria in HMECs at 1 hpi. "R" indicates *R. parkeri* and arrowheads point to continuous membrane surrounding the bacteria. Scale bar 1 μm. (B) Quantification of single and double membrane-bound or cytosolic bacteria (WT=80 bacteria, *pat1::tn*=88 bacteria, n=3 independent experiments). Data in (B) are mean ± SEM; ***p<0.001 **p<0.01 relative to WT (unpaired t test).

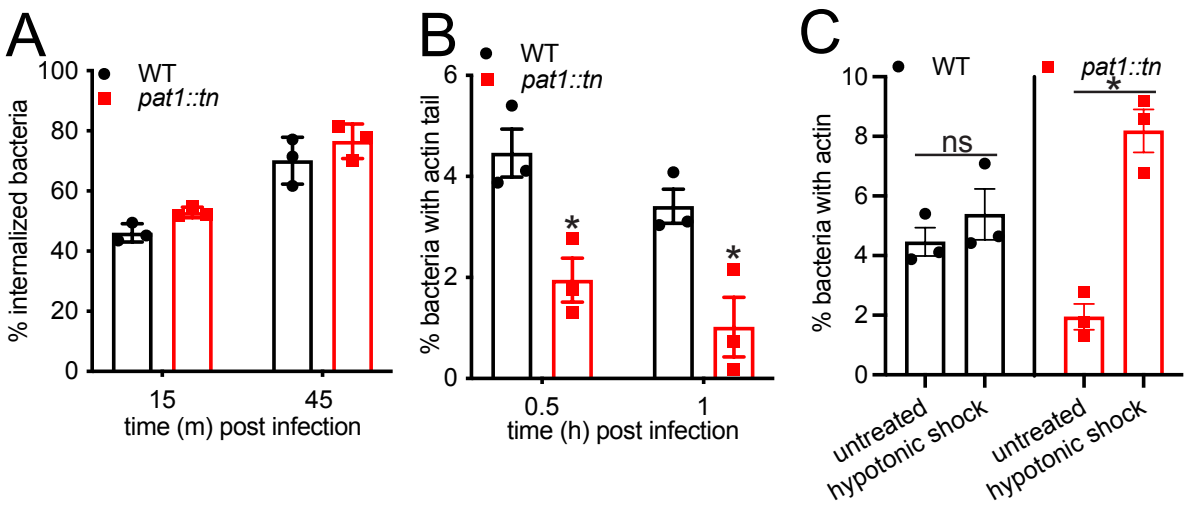

Figure 2.4 Supplemental data related to Figure 2.3, Pat1 facilitates escape from single and double membrane compartments following invasion. (A) Percent of bacteria internalized at 15 mpi and 45 mpi. (B) Percent of bacteria with actin tails at 30 mpi and 1 hpi. (C) Percent of bacteria with actin in untreated cells or cells that have undergone hypotonic shock treatment to lyse vacuoles. All data represents n=3 independent experiments. Data in (A) and (B) are mean ± SEM; *p<0.05 relative to WT (unpaired t test) and data in (C) are mean ± SEM; *p<0.01 relative to untreated (paired t test).

Complementation of the *pat1::tn* mutant reduced the percent of polyubiquitin-positive bacteria to levels seen with WT (**Figure 2.6A**). This suggested that Pat1 reduces recognition of bacteria by the host ubiquitylation machinery.

To further examine whether polyubiquitin-positive bacteria were targeted by the autophagy machinery, we examined the recruitment of autophagy receptors p62 and NDP52, as well as the autophagosome protein LC3 at 1 hpi, the time point with the most polyubiquitin-positive bacteria. Compared with WT (fewer than 2% stained with these markers), markedly more of the *pat1::tn* mutant were positive for p62 (10%) and NDP52 (6%) (**Figure 2.5C, D**). Moreover, more of the *pat1::tn* mutant bacteria colocalized with LC3 at 1 and 2 hpi (**Figure 2.5E**). Interestingly, the increased recruitment of LC3 to the *pat1::tn* mutant preceded increased colocalization of the mutant with LAMP-1, a marker for late endosomal and lysosomal compartments (**Figure 2.6B, C**). These results suggest that Pat1 is important for counteracting the recruitment of autophagy adaptors and targeting to autophagosomes and lysosomes.

Because there is evidence that Pat1 is secreted into the host cell (41), we also sought to further ascertain whether Pat1 counteracts ubiquitylation and targeting by the autophagy machinery by acting locally on the bacterium producing the protein, and/or by acting at a distance on other bacteria. To test this, we co-infected HMECs with WT bacteria expressing 2xTagBFP and *pat1::tn* mutant bacteria, and quantified colocalization of *pat1::tn* bacteria with polyubiquitin, NDP52, and p62. The *pat1::tn* mutant exhibited significantly reduced colocalization with polyubiquitin and p62 (but not NDP52) in co-infected cells compared with cells infected with the *pat1::tn* mutant only (**Figure 2.6D**). These results suggest that Pat1 is secreted and can function at a distance to reduce bacterial targeting with polyubiquitin and p62.

Pat1 antagonizes bacterial association with damaged membranes that recruit galectin-3 and NDP52

It remained unclear whether polyubiquitin and the autophagy machinery were associated with bacteria free in the cytosol or those enclosed in damaged vacuolar membranes. To determine whether polyubiquitin, NDP52, and p62 were present at damaged vacuoles at 1 hpi, we quantified the percentage of bacteria staining for polyubiquitin, NDP52, or p62, that also stained for Gal3 as a marker of damaged membranes (43). WT bacteria staining positive for polyubiquitin, p62, or NDP52 did not colocalize with Gal3, and only a small fraction (0.5%) of WT bacteria stained at all with Gal3 (**Figure 2.7A-B**). For the *pat1::tn* mutant, a significantly higher fraction of those staining for NDP52 (~50%) stained for Gal3 (**Figure 2.7A, C**). Only a small portion (5%) stained for p62 and none of those positive for polyubiquitin stained for Gal3) (**Figure 2.7A, C**). Although significantly more *pat1::tn* mutant bacteria than WT stained for Gal3, the overall percentage (2%) remained small (**Figure 2.7A, B)**. Interestingly, although the *pat1::tn* mutant was more frequently associated with Gal3, we observed fewer clusters of Gal3-positive membranes in *pat1::tn* mutant cells (**Figure 2.8A, B**), consistent with reduced overall membrane damage compared with cells infected with WT bacteria. To further confirm whether NDP52 colocalized with bacteria associated with damaged membranes, we tested if release from membranes by hypotonic shock treatment

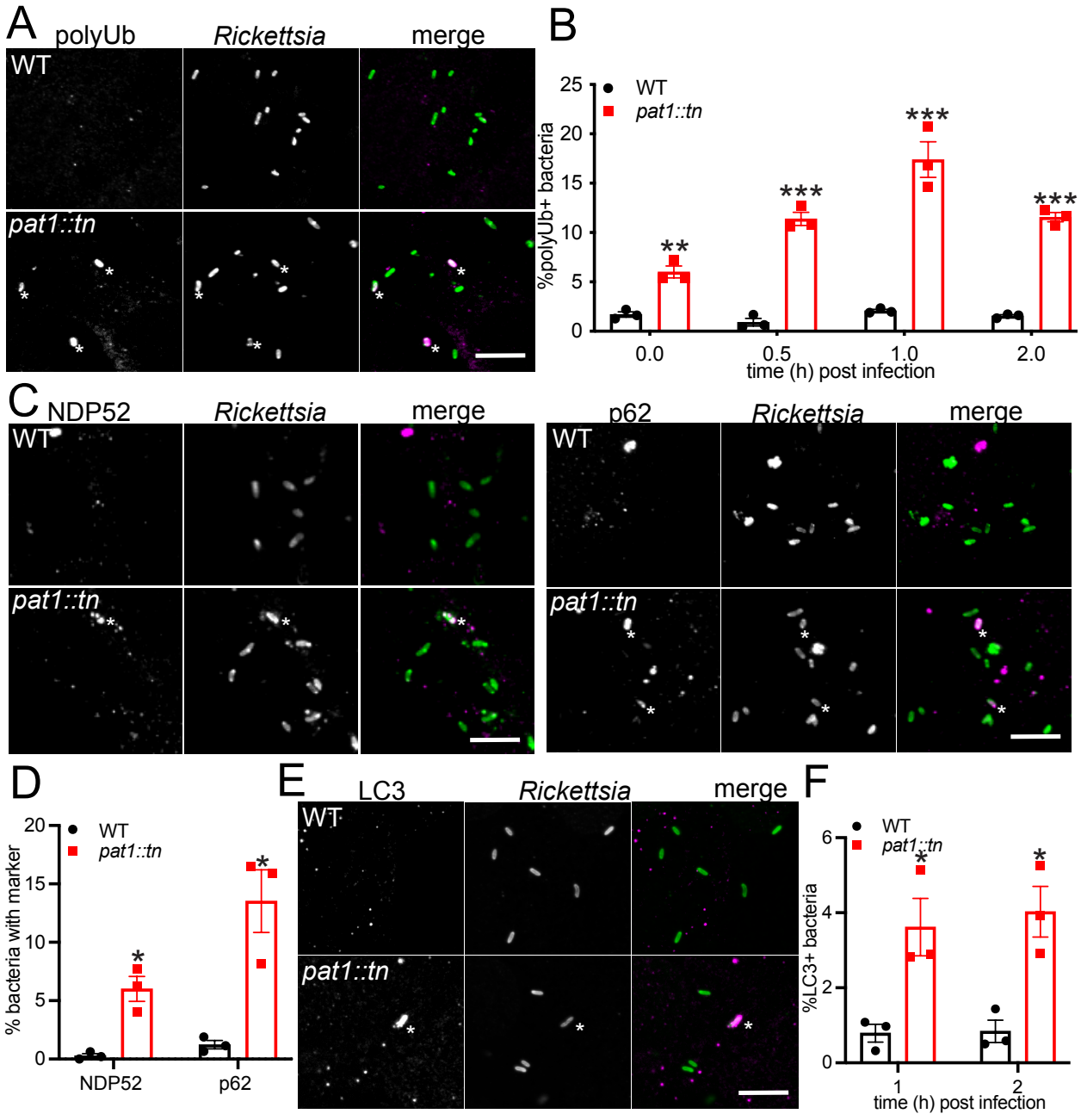

Figure 2.5 Pat1 enables evasion of recognition by autophagy. (A) Images of polyubiquitin (polyUb; magenta) in HMECs infected with WT and *pat1::tn* bacteria (green) (asterisk denotes colocalization between bacterium and polyUb). (B) Percentage of polyUb-positive bacteria at the indicated time points. (C) Images of autophagy adaptors NDP52 (left; magenta) and p62 (right; magenta) in WT and *pat1::tn* (green) infected HMECs (*denotes colocalization between bacterium and adaptor). (D) Percentage of bacteria staining for NDP52 or p62 at 1 hpi. (E) Images of LC3 (magenta) in HMECs infected with WT and *pat1::tn* bacteria (green) (*denotes colocalization between bacterium and LC3). (F) Percentage of bacteria staining for LC3 at 1 hpi. All data represents n=3 independent experiments. Data in (B, D, F) are mean ± SEM;

***p<0.001 **p<0.01*p<0.05 relative to WT (unpaired t test). Scale bars in (A, C, E) are 5 μm.

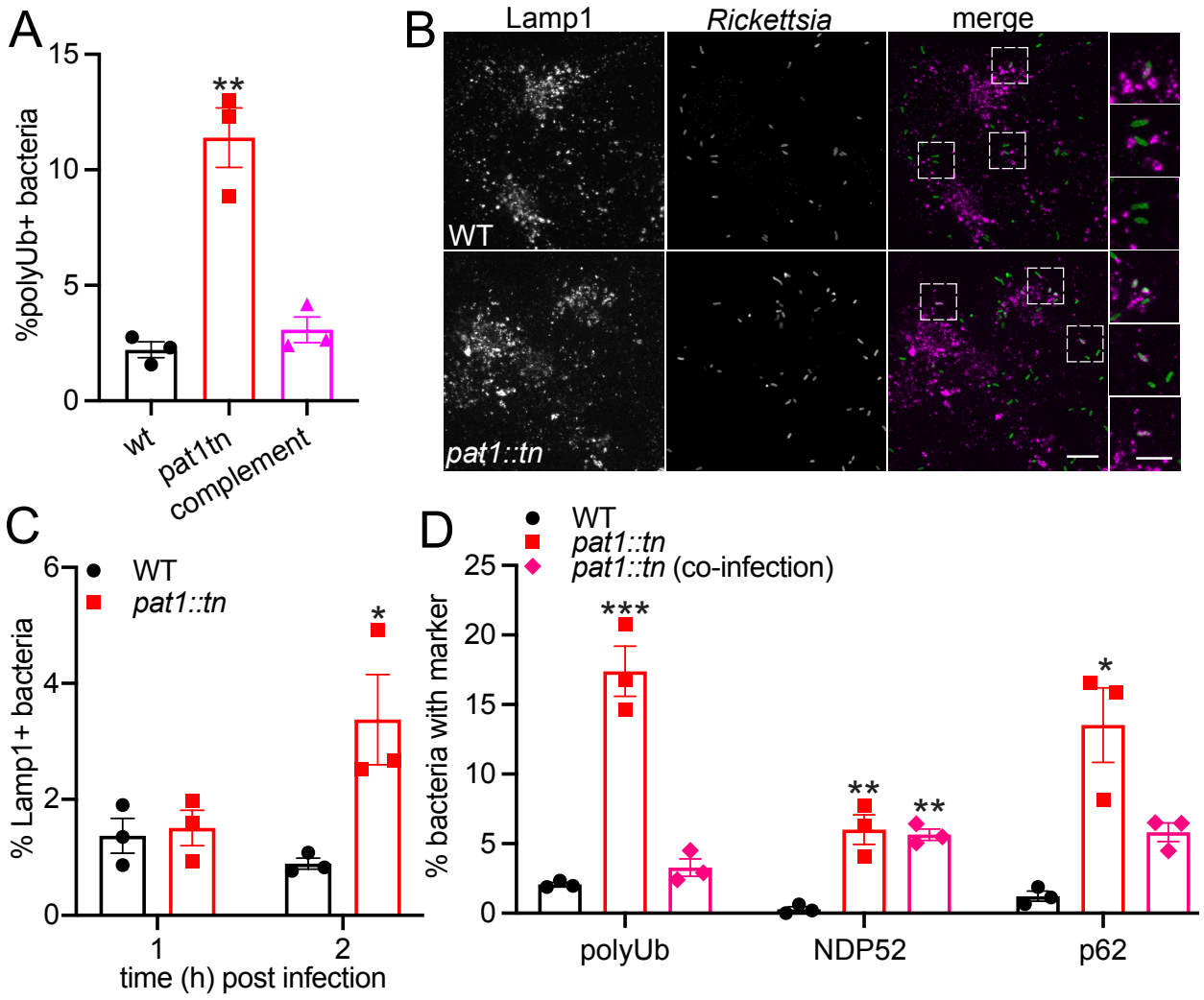

Figure 2.6 Supplemental data related to Figure 2.5, Pat1 enables evasion of recognition by autophagy. (A) Percent PolyUb-positive bacteria in HMECs infected with WT, *pat1::tn,* or complemented mutant at 1 hpi. (B) Images of LAMP-1 (magenta) in HMECs infected with WT or *pat1::tn* bacteria (green) at 2 hpi. Boxes indicate insets on right. (C) Quantification of 1 hpi (images not shown) and 2 hpi (B). (D) Percentage colocalization of bacteria with polyUb, NDP52, and p62 in HMECs infected with WT, *pat1::tn* mutant, or co-infected with WT and *pat1::tn* mutant. For co-infections, quantification is for *pat1::tn* bacteria only. All data represents n=3 independent experiments. Data in (A) and (D) are mean ± SEM; ***p<0.001 **p<0.01 *p<0.05 relative to WT (one way ANOVA). Data in (C) are mean ± SEM; *p<0.05 relative to WT (unpaired t test). Scale bar for B is 10 μm, inset 3 μm.

reduced colocalization with NDP52. Cells infected with WT and *pat1::tn* bacteria were subjected to hypotonic shock treatment at 5 mpi, and then at 30 mpi, we quantified the number of bacteria that colocalized with NDP52. Fewer than 1% of WT bacteria colocalized with NDP52 in untreated or hypotonic shock treated cells (**Figure 2.7D**). In contrast, hypotonic shock significantly reduced the percent colocalization of the *pat1::tn* mutant with NDP52 (from ~6% in untreated cells to ~1% in treated cells) (**Figure 2.7D**). Together, these results support the conclusion that Pat1 promotes efficient escape from damaged vacuolar membranes and enables avoidance of targeting by NDP52.

Pat1 facilitates actin-based motility and spread into neighboring cells late in infection

Although Pat1 was not essential for normal bacterial replication kinetics, it was important for normal plaque size, suggesting that Pat1 may function in cell-cell spread. To initially assess if Pat1 is important for spread, we used an infectious focus assay, in which the number of infected host cells per focus of infection is quantified at 28 hpi to measure spread efficiency (69,73). Compared with WT bacteria (~4.5 cells per focus), the *pat1::tn* mutant infected significantly fewer cells (~3.5 cells per focus) (**Figure 2.9A, B**). This suggested that Pat1 is important for spread. To further assess cell-cell spread, we carried out a "mixed cell" assay in which "primary" cells stably expressing a plasma-membrane marker (TagRFP-T-farnesyl) were infected for 1 h, detached from the plate, and mixed with unlabeled "secondary" cells (**Figure 2.9D).** The percent of bacteria in the primary cell and secondary cell were quantified at 32 hpi (**Figure 2.9C, D**). In this assay, ~50% of WT bacteria were found in primary cells and 50% had spread into secondary cells (**Figure 2.9E**). In contrast, ~85% of *pat1::tn* mutant bacteria were in primary cells and only ~15% were found in secondary cells. This confirms that Pat1 is important for cell-cell spread.

Because our data indicated that Pat1 facilitates cell-cell spread, we wanted to further investigate whether impaired spread might be due to differences in the fraction of bacteria undergoing actin-based motility, which is known to contribute to spread (69,70). We found the *pat1::tn* mutant formed significantly fewer actin tails compared to WT bacteria at 24 hpi and 48 hpi (**Figure 2.9F, G**), suggesting fewer bacteria initiated actin-based motility. Complementation of the *pat1::tn* mutant restored the frequency of actin tail formation to WT levels (**Figure 2.10A, B**). In the mixed cell assay, which distinguishes between primary and secondary cells, ~6% of WT bacteria in the primary cell recruited actin, mostly as actin tails but also as actin "clouds" surrounding the bacteria, compared with ~1% of *pat1::tn* mutant bacteria (**Figure 2.9C**). Differences between WT and *part1::tn* bacteria in the secondary cell could not be discerned (**Figure 2.9C**). The observed differences between WT and the *pat1::tn* mutant were not due to differences in the localization of the *R. parkeri* protein Sca2, which is important for actin-based motility and cell-cell spread (69,74) (**Figure 2.10D**). Taken together, these results suggest that Pat1 is important for the frequency of bacterial actin-based motility, and hence bacterial spread to neighboring cells.

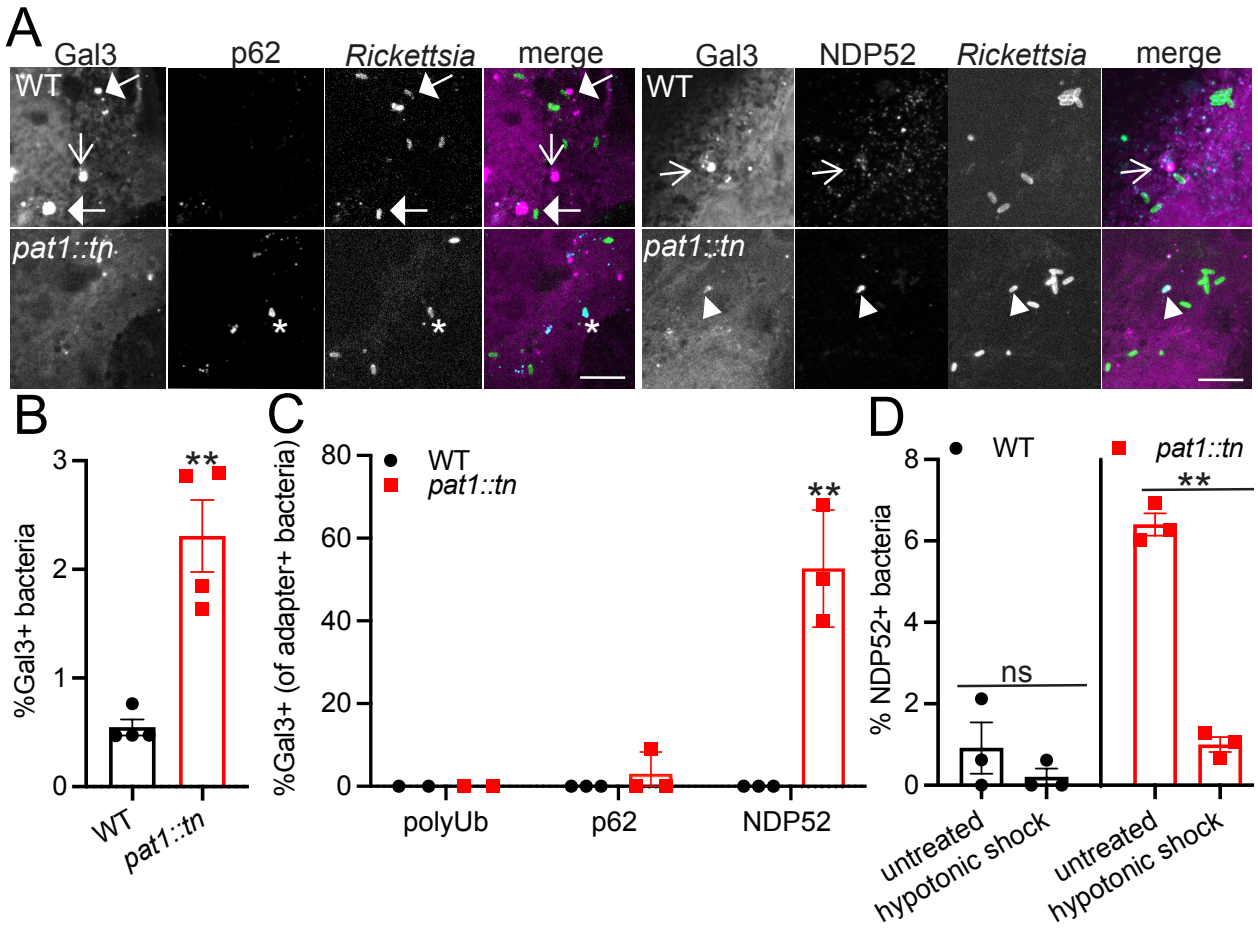

Figure 2.7 Pat1 is important for avoiding bacterial association with damaged membranes. Images of Gal3 (magenta) and adaptors p62 (left; cyan) and NDP52 (right; cyan) in HMECs infected with WT or *pat1::tn* bacteria (green) at 1 hpi. Arrows indicate large Gal3 positive clusters near bacteria, asterisk indicates bacteria that are adaptor positive and Gal3 negative, arrowheads indicate colocalization between all three signals. (B) Percentage of bacteria positive for Gal3 (n=4). (C) Percent adaptor positive bacteria that are also positive for Gal3 (n=2 for polyUb and n=3 for p62 and NDP52). (D) Percent of bacteria positive for NDP52 in untreated cells or cells that undergo hypotonic lysis of vesicles (n=3). Data in (B) and (C) are mean ± SEM; **p<0.01 relative to WT (unpaired t test). Data in (D) are mean ± SEM; **p<0.01 relative to untreated (paired t test). Scale bar for (A) is 5 μm.

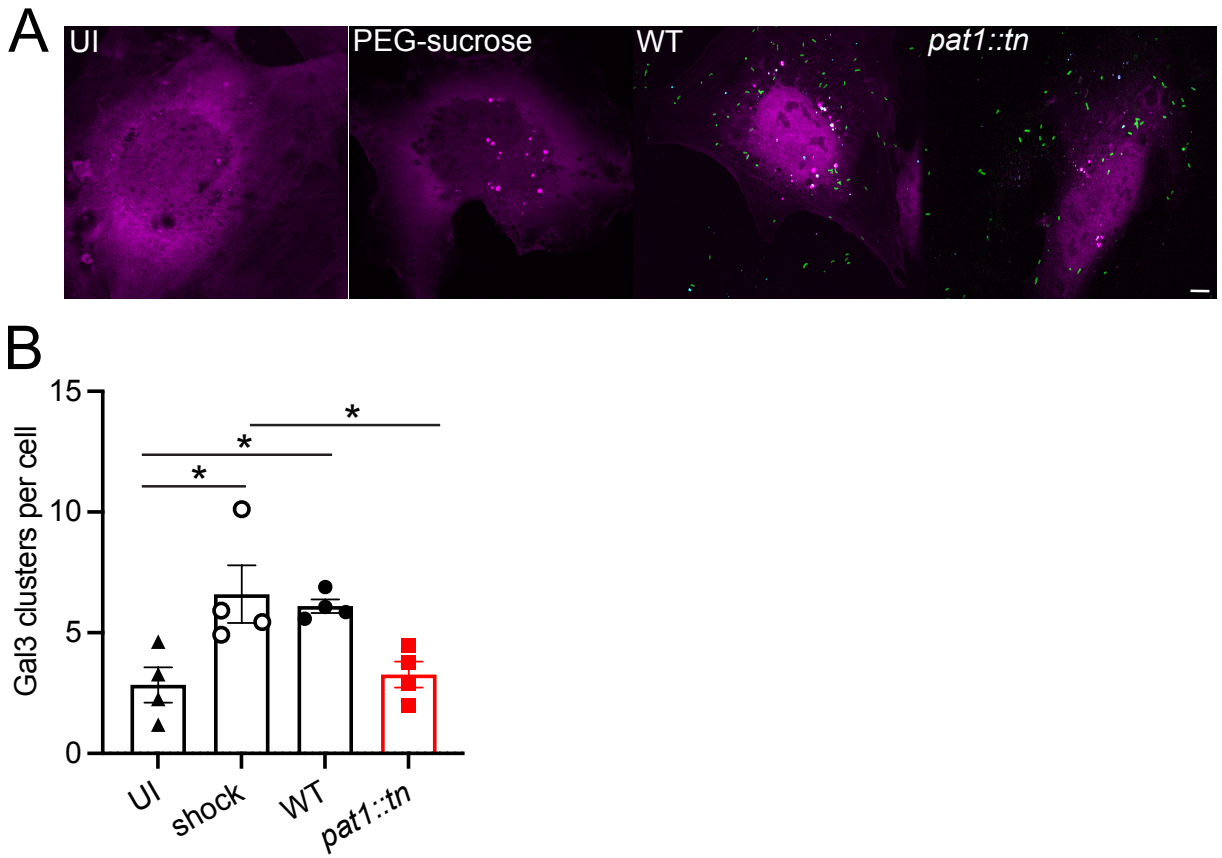

Figure 2.8 Supplemental data related to Figure 2.7, Pat1 is important for avoiding bacterial association with damaged membranes. (A) Images of Gal3 (magenta) in HMECs that are uninfected (UI), undergo sterile lysis of vesicles (hypotonic shock; PEG-sucrose), WT-infected, and *pat1::tn* mutant (green) infected at 1 hpi. Infected panels are also stained for NDP52 (cyan). Scale bar 5 μm. (B) Number of Gal3 clusters per cell (n=4). Data in (B) are mean ± SEM; *p>0.01 (one way ANOVA, mulitple comparisons with Tukey post hoc test).

Pat1 is important for avoiding double membranes during cell-cell spread

Because Pat1 plays an important role escaping the primary vacuole following invasion, we hypothesized that Pat1 also plays a role in escaping the secondary vacuole following cell-cell spread. To test this, we imaged infected HMECs by TEM at 48 hpi and quantified the percent of intracellular bacteria free in the cytosol or within membranes. Significantly more *pat1::tn* mutant bacteria were surrounded by double membranes (~60%) in comparison with WT bacteria (~25%) (**Figure 2.11A, B**). The double membranes we observed were often discontinuous, with the mutant remaining mostly enclosed and WT bacteria having very few surrounding membrane fragments. This suggests that Pat1 plays a role in escaping from membranes later in infection.

We next sought to further distinguish whether bacteria surrounded by double membranes were in secondary vacuoles that result from cell-cell spread, or other double-membrane structures such as autophagosomes. We used the mixed cell assay described above, in which infected primary cells stably expressing TagRFP-T-farnesyl were infected for 1 h and then mixed with uninfected and unlabeled secondary cells (**Figure 2.10D**). Fewer than 1% of WT bacteria that spread from primary into secondary cells were colocalized with the plasma membrane marker from the primary cell (**Figure 2.11C**), suggesting that these bacteria had escaped the secondary vacuole. In contrast, of the *pat1::tn* mutant bacteria that spread into secondary cells, ~12% colocalized with the plasma membrane marker from the primary cell (**Figure 2.11C**). These results suggest that a significant fraction of double-membrane structures seen in the TEM images are secondary vacuoles and confirm that Pat1 is important for escaping from these vacuoles.

To further examine whether some of the double membranes seen surrounding bacteria by TEM were also due to targeting by autophagy, we assessed whether bacteria colocalized with polyubiquitin, p62 or NDP52 at 48 hpi. Significantly more of the *pat1::tn* mutant colocalized with p62 and NDP52 than WT, although the overall percentages were low in all cases (**Figure 2.11D**). Moreover, the percentage of bacteria that colocalized with these markers was lower than at 1 hpi (compare with **Figure 2.5B, D**). Interestingly, polyubiquitin labeling was not significantly different between WT and *pat1::tn* mutant bacteria, suggesting p62 and NDP52 were not being recruited by polyubiquitin. These data demonstrate that late in infection, in addition to facilitating escape from the secondary vacuole, Pat1 contributes to avoidance of autophagy.

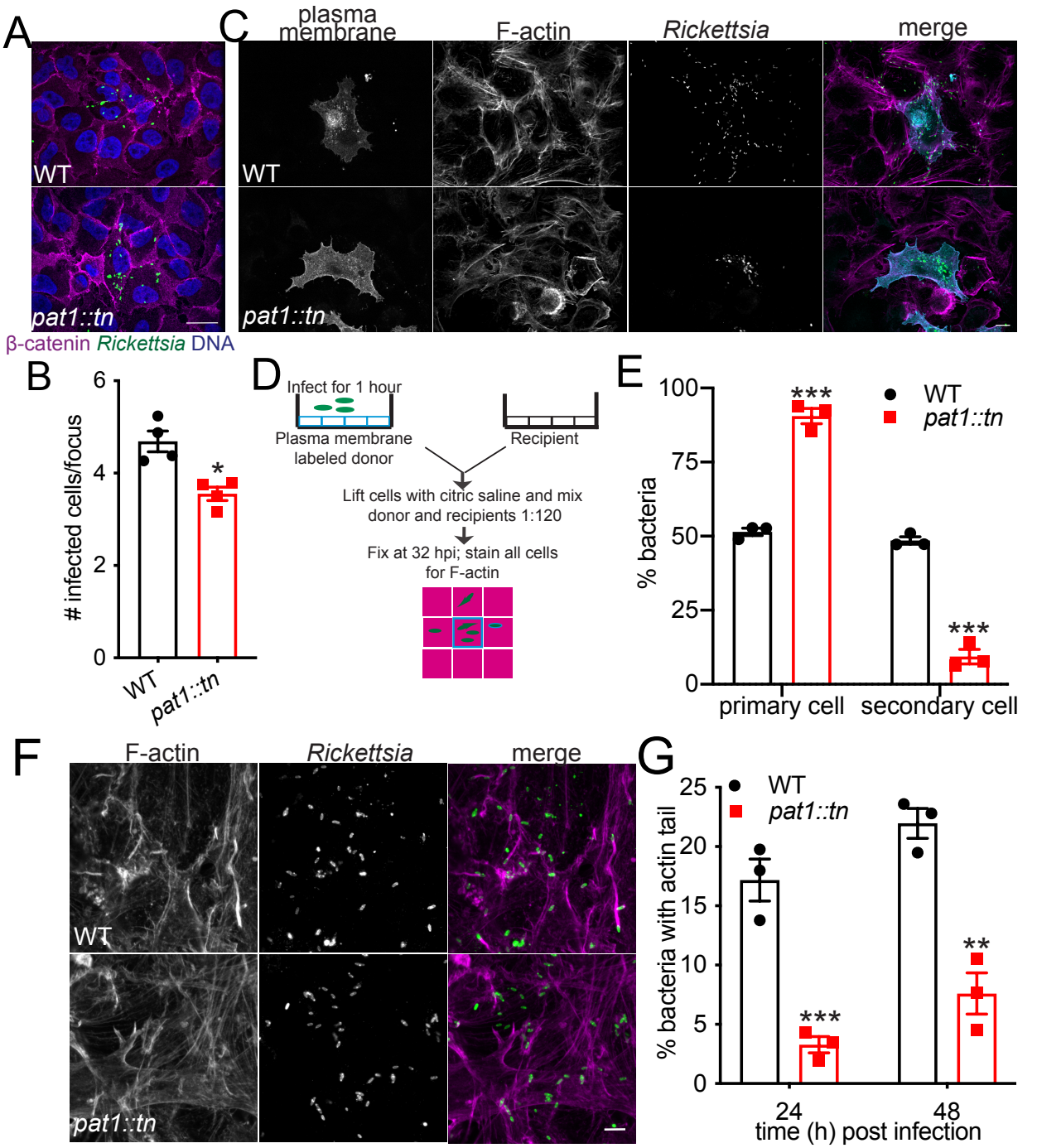

Figure 2.9 Pat1 is important for cell-cell spread and facilitates actin-based motility. (A) Images of infectious foci formed by WT or *pat1::tn* mutant in A549 cells at 28 hpi (magenta, β-catenin; green, bacteria; blue, nuclei)(n= 4). Scale bar for 10 μm. (B) Quantification of (A). (C) Images of mixed cell assay depicted in (D) showing plasma membrane (A549-TRTF; magenta), F-actin (green), and bacteria (blue) (n=3). (E) Percent bacteria in primary and secondary cells quantified from (C). (F) Images of actin tails (F-actin; magenta) and bacteria (green) in HMECs (n=3). (G) Percent of bacteria

with actin tails at 24 hpi and 48 hpi in HMECs. Scale bar is 5 μm. All data are mean ± SEM; ***p<0.001 **p<0.01 *p<0.05 relative to WT (unpaired t test).

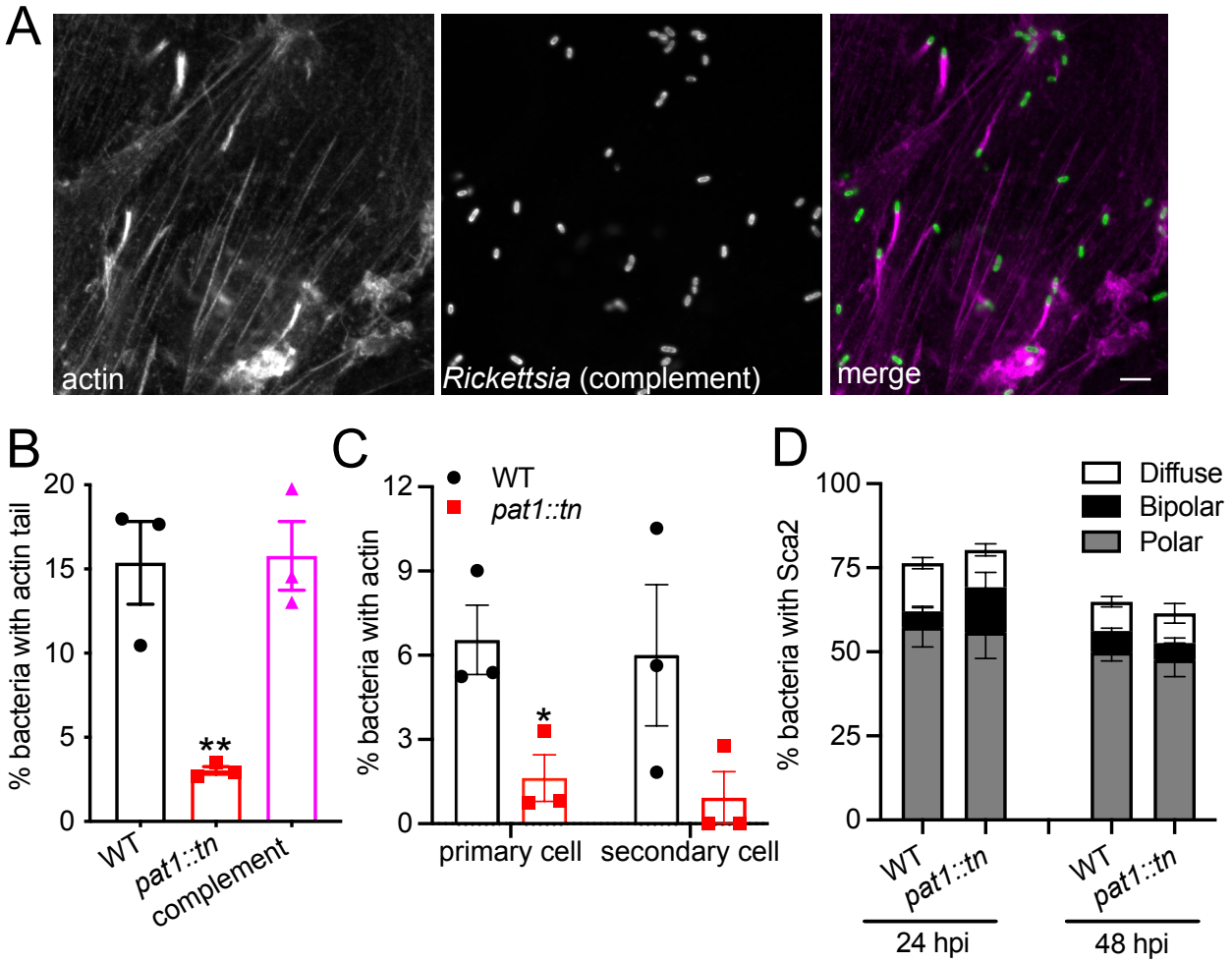

Figure 2.10 Supplemental data related to Figure 2.9, Pat1 is important for cell-cell spread and facilitates actin-based motility. (A) Images of actin tails in the complemented mutant (F-actin, magenta; bacteria, green) in HMECs at 48 hpi. Scale bar 5 μm. (WT and *pat1::tn* images represented in figure 2.9F.) (B) Percentage of bacteria with actin tails for the indicated strains. (C) Percentage of bacteria with actin tails in primary and secondary cells, related to Figure 2.9C. (D) Percentage of bacteria with Sca2 with the indicated distributions in WT and *pat1::tn* mutant bacteria. All data represents n=3. Data in (B) are mean ± SEM; **p<0.01 relative to WT (one way ANOVA). Data in (C) are mean ± SEM; *p<0.05 relative to WT (unpaired t test).

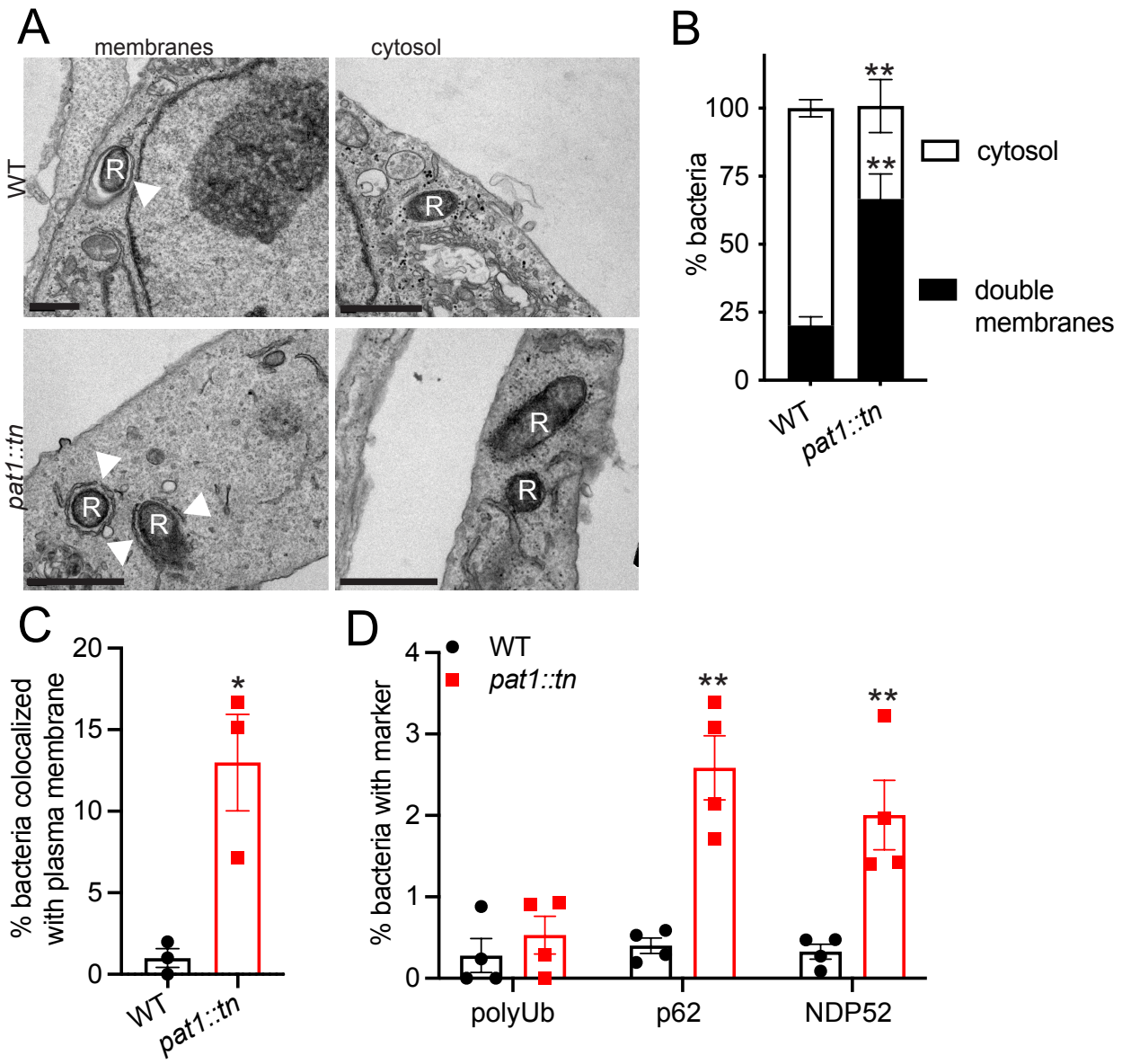

Figure 2.11 Pat1 is important for escape from the secondary vacuole. (A) TEM images of WT and *pat1::tn* mutant bacteria in HMECs at 48 hpi. "R" indicates *R. parkeri* and arrowheads point to continuous membrane surrounding the bacteria. Scale bar 1 μm. (B) Percentage of bacteria in double membrane compartments or in the cytosol (WT=120 bacteria, *pat1::tn* n=112 bacteria, n=3). (C) Percentage of bacteria in the secondary cell that colocalize with the plasma membrane from the primary cell, in mixed cell assays from Figure 2.9 (C) at 32 hpi (n=3). (D) Percentage of WT and *pat1::tn* mutant bacteria colocalizing with polyUb (n=3), p62 (n=4), and NDP52 (n=4) at 48 hpi in HMECs. All data are mean ± SEM; **p<0.01 *p<0.05 relative to WT (unpaired t test).

Discussion

The ability of *Rickettsia* to escape and avoid host membranes meant to sequester bacteria from the cytosol is a critical facet of their life cycle. Here, we demonstrate that the *R. parkeri* patatin-like phospholipase Pat1 enables bacterial escape from host membranes throughout infection. Pat1 mediates efficient exit from primary vacuoles following invasion, helping *R. parkeri* avoid detection by host galectins and autophagy adaptor NDP52. Pat1 further enables cytosolic bacteria to avoid recruitment of polyubiquitin and autophagy adaptor p62. As infection progresses, Pat1 facilitates spread into neighboring cells and escape from the secondary vacuole. Altogether, these data suggest Pat1 is important at multiple steps of the *Rickettsia* life cycle that involve manipulating host membranes.

Our genetic data indicate that Pat1 mediates escape from both single and double membrane compartments in host cells. At early time points, *pat1::tn* mutant bacteria were more frequently surrounded by single membranes following invasion, likely to be primary vacuoles derived from the host cell plasma membrane. Consistent with a failure to fully escape the primary vacuole, the *pat1::tn* mutant also showed significantly reduced frequency of actin-based motility and increased trafficking to LAMP-1-positive compartments. We also found the *pat1::tn* mutant had increased localization to double membrane structures at later time points when bacteria are spreading to neighboring cells. These structures are likely to be secondary vacuoles, as only a small portion colocalized with autophagy adaptors p62 or NDP52. Pat1 was previously suggested as a candidate for escape from the vacuole due to its phospholipase activity (33,41) and the observation that *R. typhi* pre-treated with anti-Pat1 antibody (which could block surface-associated by not secreted Pat1) caused increased colocalization with LAMP-1 (41). Our results provide genetic confirmation of this role. Several other bacterial phospholipases mediate membrane rupture (75), including *L. monocytogenes* PLCs (15,29,75,76), *Clostridium perfrinogens* alpha-toxin (a PLC) (75,77–79), and *Psuedomonas aeruginosa* ExoU (75,80). Similarly, lecithin:cholesterol acyltransferase (LCAT) enzymes from pathogenic protists, including *Plasmodium berghei* phospholipase (PbPL) (81,82) and *Toxoplasma gondii* TgLCAT (83), have PLA2 and acyl transferase activity (81,83) that facilitate break down the parasitophorous vacuole. Phospholipases are also used by nonenveloped viruses to breech the endosome (84,85), including parvovirus capsid protein VP1 which his PLA2 activity that is essential for capsid translocation from the endosome to the cytosol (85,86), and host PLA2 group XVI which is recruited by picornaviruses to endosomes for genome translocation (85,87). Thus, the role of Pat in vacuolar breakdown and escape represents a common strategy employed by many intracellular pathogens.

Despite its importance in escaping from primary and secondary vacuoles, Pat1 is not important for growth in the cell lines we tested, suggesting that the *pat1::tn* mutant retains some ability to rupture vacuolar membranes and gain access to nutrients in the cytosol. Consistent with this notion, the *pat1::tn* mutant colocalizes more frequently with damaged membranes marked by Gal3. *L. monocytogenes* PLC enzymes have overlapping function in escape (15,26,29) and double phospholipase mutants of PlcA and PlcB show more severe defects in escape (15,26,29) and growth (26,62). Pat1 must also share functional redundancy with other proteins. Based on our mechanistic understanding of vacuolar escape for *L. monocytogenes* and *S. flexneri* (19,76,88) and

the data presented here, we would expect the other *Rickettsia* factor(s) involved in escape to cause membrane damage and possibly manipulate trafficking of the vacuole. The *Rickettsia* protein TlyC, a putative hemolysin (35,89), could function analogously to LLO. Pat2, a second PLP, may also have an overlapping role with Pat1 in species like *R. typhi* (33,41). In addition to the membranolytic proteins, Risk-1, a phosphatidylinositol 3-kinase, was recently reported to manipulate early trafficking events important for invasion, vacuolar escape, and autophagy (90). *R. parkeri* has multiple proteins with potential to synergize with Pat1 during vacuolar escape. At least one of these factors can damage vacuolar membranes, but Pat1 is important for efficient escape that allows bacteria to escape the vacuole shortly after rupture.

We observed that Pat1 plays a role in avoiding targeting by autophagy following invasion. One role of Pat1 is to enable efficient escape from damaged membrane remnants marked by host Gal proteins that are subsequently targeted by autophagy. Consistent with this, we observed that the *pat1::tn* mutant colocalizes more frequently with NDP52 and Gal3. The detection of membrane damage by Gal proteins and subsequent recruitment of autophagy is a potential obstacle for bacteria trying to access the cytosol. Interestingly, Gal3 promotes replication by suppressing autophagy during *L. monocytogenes* infection (42,47) and preventing recruitment of Gal8 and parkin during Group A *Streptococcus* infection (42,91). Our results found that WT bacteria rarely associated with damaged membranes, but whether differential recruitment of Gal proteins leads to different infection outcomes in *Rickettsia* remains unknown. Altogether, these results suggest that *R. parkeri* avoids association with vacuolar rupture through rapid escape, allowing bacteria to evade initial targeting by autophagy associated with membrane damage.

Pat1 also played a role in avoiding polyubiquitylation, p62 recruitment, and targeting by autophagy for bacteria that were not associated with damaged membranes. Thus, Pat1 may augment other autophagy-avoidance mechanisms, including OmpB-mediated shielding of bacterial surface from polyubiquitylation and lysine methylation of OmpB (92,93). Pat1 might function in a similar manner to PlcA from *L. monocytogenes*, which reduces PI(3)P levels to block autophagosome formation and stall autophagy (62,63). Both Pat1 and PlcA/B are secreted and can act at a distance, as we observed that a *R. parkeri pat1* mutant can be rescued from targeting by autophagy by co-infection with WT bacteria, similar the rescue of a *plcA/B* mutant by WT *L. monocytogenes* (63). Thus, secreted Pat1 might also target early and/or regulatory aspects of autophagy.

We further found that Pat1 is important for cell-cell spread, including in late actin-based motility and escape from the secondary vacuole (the latter is discussed above). The *pat1::tn* mutant formed fewer actin tails and exhibited reduced spread into neighboring cells when compared with WT, consistent with the known role for motility in cell-cell spread of SFG *Rickettsia* (69,70,74). One key contribution of Pat1 to actin-based motility is to mediate escape from the vacuole, allowing recruitment of the host actin machinery to the surface of the bacteria. However, it remains possible that Pat1 targeting of phosphoinositides (PIs) might also affect actin-based motility, as PIs regulate actin dynamics (94–96) by influencing the activity of actin-binding proteins (97,98).-Moreover, Pat1 targeting of PIs at the plasma membrane could directly contribute to protrusion dynamics during cell-cell spread. PIs can recruit proteins

involved in membrane curvature, (i.e. BAR proteins), and endocytic pathways (clathrin-mediated endocytosis) to the plasma membrane (96,99–102) and these processes have been shown to mediate protrusion resolution for *L. monocytogenes* and *S. flexneri* (103,104). Pat1-mediated local membrane damage might also promote spread, as *L. monocytogenes* LLO-mediated membrane damage in the protrusion has been shown to enable exploitation of efferocytosis for spread (105). Thus, Pat1 may play multiple roles in cell-cell spread.

Our data demonstrate that *R. parkeri* Pat1 plays an important role throughout the intracellular life cycle. However, it remains unclear whether Pat1 primarily mediates membrane damage or Pat1 whether Pat1 also performs other functions during infection. For example, Pat1 phospholipase activity could contribute to both vacuolar breakdown and the release of bioactive lipids such as eicosanoids derived from arachidonic acid. Our data suggests Pat1 may exert both local effects on vacuolar escape and global effects on other processes such as autophagy and actin-based motility. Membranes are critical hubs of signaling and protein-protein interactions and *R. parkeri*, like other intracellular pathogens, has likely evolved diverse ways of manipulating membranes. Further studies of Pat1 function promise to elucidate how PLA2 enzymes facilitate microbial adaptation to host cells and could reveal previously unappreciated strategies of membrane manipulation by obligate intracellular and other pathogens.

Materials and methods

Mammalian cell lines

Mammalian cell lines were obtained from the UC Berkeley Cell Culture Facility and grown at 37˚C and 5% CO_2. Vero cells (African green monkey kidney epithelial cells) were grown in DMEM with high glucose (4.5 g/L) (Gibco; 11965-092) and 2% FBS (GemCell; 100500) for culturing or 5% FBS for plaque assays (described below). A549 cells (human lung epithelial cells) were grown in DMEM (Gibco, 11965-092) with high glucose (4.5 g/L) and 10% FBS (ATLAS; catalog number F-0500-A). HMEC-1 cells (human microvascular endothelial cells) were grown in MCDB 131 media (Sigma, M8537) supplemented with 10% FBS (HyClone; catalog number SH30088), 10 mM L-glutamine (Sigma, M8537), 10 ng/ml epidermal growth factor (Corning; catalogue number 354001), 1 ug/mL hydrocortisone (Spectrum Chemical, CO137), and 1.18 mg/mL sodium bicarbonate. A549 cells stability expressing a farnesyl tagged TagRFP-T (A549-TRTF) to mark the plasma membrane were described previously (73) and were maintained in the A549 media described above.

R. parkeri strains and bacterial isolation

R. parkeri Portsmouth strain (WT) was provided by Dr. Christopher Paddock (Centers for Disease Control and Prevention). The *pat1::tn* mutant was generated from this strain as described previously (64).

To make the complemented pat1::tn *pat1*+ mutant, we first constructed the pMW1650-Spec-*pat1*) complementation plasmid. Nucleotides 901,999-903,853 from *R. parkeri* genomic DNA were amplified by PCR and subcloned into pMW1650-Spec that had been linearized with PstI (New England Bioloabs; R3140S). The amplified sequence contained a predicted promoter upstream of *pat1* (determined using SoftBerry, BPROM prediction of bacterial promoters (106) and several predicted transcriptional terminators (determined using WebGeSTer DB) (107). Small scale electroporations were performed as previously described for pMW1650 (64) to generate *Rickettsia* strains containing pMW1650-Spec plasmids. A spectinomycin overlay of Vero media (5% FBS) with 0.5% agarose and 50 µM spectinomycin was added to the cells. Individual plaques were picked, resuspended in 200 µl BHI, and expanded in Vero cells in a T25 flask rocked at 37˚C for 30 min. 50 µM spectinomycin was added and the flasks were placed at 33˚C and monitored for plaque formation. This process of bead disruption and bacteria isolation was repeated in T75 flasks to generate frozen stocks ("bead preps") for screening candidate plaques by PCR, plaque size, and Pat1 expression by western blot. For PCR, we confirmed (1) the original transposon using primers for the rifampicin resistance cassette, (2) presence of new transposon using primers for spectinomycin resistance cassette, and (3) the presence of *pat1::tn and* WT *pat1*. Following screening, T175 flasks were infected to purify bacterial stocks (30% prep, described below).

R. parkeri strains were purified by infecting confluent Vero cells in T175 flasks at an MOI of 0.05. Flasks were monitored for plaque formation and harvested when 70-80% of the cells in the flask were rounding, typically 5-7 d after infection. Cells were scraped and pelleted at 12,000 x g for 30 min at 4˚C. The pelleted cells were resuspended in ice-cold K36 buffer and transferred to a Dounce homogenizer. Repeated douncing of 60-80 strokes released intracellular bacteria and the dounced cells and bacteria were centrifuged at 200 x g for 5 min at 4˚C. The supernatant containing the bacteria was overlaid on a 30% MD-76R solution and

centrifuged at 18,000 rpm for 30 min at 4˚C in a SW-28 rotor to further separate host cell components from bacteria. Bacterial pellets were resuspended in BHI and stored at -80˚C. Purified bacteria are referred to as "30% preparations" below.

Plaque assays and growth curves

To determine the titer of purified bacteria, media was aspirated from Vero cells grown in 6-well plates and 200 µl of bacteria diluted in Vero media (10^{-3}-10^{-8}) were added to each well. Plates were rocked at 37˚C for 30 min then overlaid with 3 ml of Vero media (5% FBS) and 0.5% agarose. Plaques were counted 5-7 d post infection to determine pfu/mL. For imaging plaques, neutral red (Sigma, N6264) was overlaid (0.01% final concentration) with Vero media (2% FBS) and 0.5% agarose and imaged the next day.

Growth curves were carried out following infection of HMECs or Vero cells at an MOI of 0.01 in 24 well plates. At each time point, media was aspirated from individual wells, cells were washed 2X with sterile deionized water, 1 ml of sterile deionized water was added, and cells were lysed by repeated pipetting. Three serial dilutions of the supernatant from lysed cells in Vero media, totaling 1 ml each, were added in duplicate to confluent Vero cells in 12 well plates. Plates were spun at 300 x g for 5 min at room temperature and incubated at 33˚C overnight. The next day, media was aspirated and 2 ml of Vero media (5% FBS) and 0.5% agarose was overlaid in each well. Once plaques were visible, an overlay with neutral red was done as described above. Because of differences in timing of plaque formation for the WT and *pat1::tn* mutant strains, plaque counts for WT and complemented *pat1::tn* plaques were usually at ~5 d post infection and *pat1::tn* plaques were counted at ~7 d post infection.

Pat1 expression and antibody generation

The DNA sequence encoding full length Pat1 (AA 1-490; nucleotide 1-1473) was amplified from *R. parkeri* genomic DNA by PCR and subcloned into a pET1 vector containing an N-terminal 6x His-tag, maltose binding protein (MBP) tag, and TEV cleavage site (Addgene plasmid 29656). The resulting plasmid, pET-M1-6xHis-MBP-TEV-Pat1 was transformed into *E. coli* strain BL21 codon plus RIL-Cam^r (DE3) (UC Berkeley QB3 Macrolab). Expression of 6xHis-MBP-TEV-Pat1 was induced with 1 mM IPTG for 1 h at 37˚C. Bacteria were pelleted by spinning at 4000 rpm for 30 min at 4˚C and the pellet was resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 1 mM EDTA) supplemented with 1 µg/ml each leupeptin (MilliporeSigma; catalogue number L2884), pepstatin (MilliporeSigma; catalogue number P5318), and chymostatin (MilliporeSigma; catalog number E16), and 1 mM phenylmethylsulfonyl fluoride (PMSF, MilliporeSigma; 52332). Bacteria were flash frozen in liquid nitrogen and stored at -80˚C until purification. Bacterial cultures were thawed quickly and kept on ice/cold for remaining purification. Lysozyme (Sigma; catalogue number L4919) was added to a final concentration of 1 mg/ml and incubated for 15 min on ice. Bacterial pellets were subjected to 8 cycles of sonication at 30% power for 12 s bursts, followed by rest on ice for 30 s. Lysed bacteria were spun at 13,000 rpm for 30 min at 4˚C. The supernatant was passed three times over a column of 10 ml of amylose resin (New England Biolabs; catalogue number E8031L). The column was washed with wash buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl) by passing 15 column volumes. Bound protein was eluted by adding 2-3 column volumes of elution buffer (50 mM Tris-HCL, pH 8.0, 300 mM NaCl, 0.5 mM DTT, 10 mM maltose) to the column and collecting 500 µl fractions. Fractions were checked for eluted

protein by both Bradford assay and SDS-PAGE and fractions with high concentration of protein and a single band at the expected molecular weight for MBP-Pat1 were pooled and concentrated.

To generate rabbit anti-Pat1 antibodies, 1.7 mg of purified MBP-Pat1 was sent to Pocono Rabbit Farm and Laboratory and immunization was carried out following their 91 day custom antibody production protocol, then extended for an additional 6 weeks for an additional boost and bleed before final exsanguination.

To affinity purify anti-Pat1 antibodies, full length *pat1* was subcloned into the pSMT3 plasmid to make pSMT3-6x-His-SUMO-Pat1. Expression of 6x-His-SUMO-Pat1 was induced as described above. Pellets were resuspended in lysis buffer (20 mM Tris-HCL, pH 8.0,300 mM NaCl, 10 mM imidazole) supplemented with protease inhibitors PMSF and LPC as described above. Bacteria were lysed as described above and supernatant was incubated with 2.0 ml of Ni-NTA resin and rotated for 1 hr at 4˚C. The column was washed with wash buffer (20 mM Tris-HCL, pH 8, 300 mM NaCl, 30 mM imidazole) and protein was eluted from the column in 500 µl aliquots with 2 column volumes of elution buffer (50 mM NaH_2PO_4, pH 8, 300mM NaCl 250 mM imidazole). In addition, the same protocol was followed to purify 6x-His-SUMO. Purified 6x-His-SUMO or 6x-His-SUMO-Pat1 were coupled to NHS-activated Sepharose 4 fast flow resin (GE Healthcare; catalogue number 17-0906-01) in ligand coupling buffer (200 mM $NaHCO_3$, pH 8.3, 500 mM NaCl) for 2-4 h at RT. To remove anti-SUMO antibodies, the resin containing 6x-His-Sumo was incubated with 10 mL anti-Pat1 serum diluted in binding buffer (20mM Tris-HCL, pH 7.5) and incubated with rotation for 2 h at 4˚C. The flow through was collected and added to the resin containing 6x-His-SUMO-Pat1 and was incubated at 4˚C for 4 h with rotation. Bound antibody was eluted using 100 mM glycine, pH 2.5, into 1M Tris-HCL, pH 8.8, to neutralize to pH 7.5.

Bacterial infections for imaging

Infections were carried out in 24 well plates unless otherwise noted. For immunofluorescence microscopy, 24 well plates containing 12 mm sterile coverslips were used. HMECs were seeded at $2.5x10^5$ cells/well and infected 36-48 h later. A549 cells were seeded at $1.2x10^5$ cells/well and infected 24 h later. For timepoints from 0-2 hpi, an MOI of 3-5 was used for all cell types, and for 24-48 hpi, an MOI of 0.01-0.05 was used. For the infectious focus assay, an MOI of 0.001 was used. To infect cells, a 30% preparation of *R. parkeri* was thawed on ice prior to infection and immediately diluted into fresh media on ice. Cell media was aspirated, the well was washed once with 1x phosphate-buffered saline (PBS; Gibco; catalogue number 10010049), 0.5 mL of bacteria in media was added per well and the plate was spun at 300 x g for 5 min at RT. Warm media was added following centrifugation and infected cells were incubated at 33˚C in 5% CO2.

For Gal3 imaging experiments, pmCherry-N1-Gal3 was transfected into cells 24 hours after seeding on to coverslips using Lipofectamine LTX (Invitrogen, catalogue number A12621). Plasmid and transfection reagent was added to HMEC media and incubated at 37˚C overnight. Next morning, the wells were washed 2x with PBS and replaced with fresh, warm HMEC media. Cells were visually examined to confirm 80-100% confluency and the presence of Gal3 expressing cells. Infections were performed a few hours later.

The mixed cell assay was adapted from (73), A549-TRTF cells and unlabeled A549 cells were seeded into 12-well plates at a density of $3x10^5$ cells/ml and grown overnight. The following day, A549-TRTF cells were infected at an MOI of 5 as described above. Cells were incubated at 33˚C for 1 h. Both infected A549-RFP-T-Farn cells and unlabeled A549 cells were detached by adding warm citric saline (135 mM KCl, 15 mM sodium citrate) and incubating for 5 min at 37˚C. Cells were gently resuspended by pipetting up and down and adding to media, then pelleted. Cells were washed twice with A549 media and resuspended in A549 media WITH 10 µg/ml gentamycin to kill extracellular bacteria. Infected A549-TRTF and unlabeled cells were mixed at a ratio of 1:120, plated on coverslips in a 24 well plate, and incubated in a humidified secondary container at 33˚C until 32 hpi.

Hypotonic shock treatment was adapted from (72). Briefly, HMECs were infected as stated above and incubated for 5 min at 37˚C, at which point media was exchanged with a hypertonic solution (10% PEG-1000, 0.5 M sucrose in PBS) and incubated at 37˚C for 10 min. Wells were washed gently once with the hypotonic solution (60% PBS), incubated in hypotonic solution at 37˚C for 3 min, then incubated in isotonic media (cell media) for 15 min at 37˚C.

Immunofluorescence microscopy

All coverslips were fixed for 10 min in fresh 4% paraformaldehyde (Ted Pella; catalogue number 18505) at room temperature. Coverslips were washed 3x with PBS pH 7.4 and stored at 4˚C until staining. All incubations were done at RT unless otherwise noted and all coverslips were mounted in Prolong Gold antifade (Invitrogen; catalogue number P36930) and sealed with nail polish after drying.

Primary antibodies used to stain *Rickettsia* were rabbit anti-*Rickettsia* I7205 (1:300; (108)); gift from T. Hackstadt), rabbit anti-*Rickettsia* OmpB (1:1000; (92)), and mouse anti-*Rickettsia* 14-13 (1:400; (108)); gift from T. Hackstadt). Primary antibodies were incubated with coverslips for 30 min. Coverslips were washed and the following secondary antibodies were added for 30 min, protected from light: goat anti-rabbit Alexa 488 (1:400; Invitrogen; catalogue number A11008), goat anti-rabbit Alexa 404 (1:150; Invitrogen; catalogue number A31556), goat anti-mouse Alexa 488 (1:400; Invitrogen; catalogue number A11001), goat anti-mouse Alexa 404 (1:150; Invitrogen; catalogue number A31553).

To quantify colocalization with polyubiquitin and autophagy adapters, cells were permeabilized with 0.5% triton-X100, washed three times with PBS. Primary antibodies were added for 30 min -1 h at the following dilutions: mouse anti-polyubiquitin FK1 (1:250; EMD Millipore; catalogue number 04-262), guinea pig anti-p62 (1:500; Fitzgerald; catalogue number 20R-PP001), mouse anti-NDP52 (1:300; Novus Biologicals; catalogue number H00010241-B01P). Cells were post-fixed in 100% methanol at RT for 5 min for staining with rabbit polyclonal anti-LC3 (1:250; Novus Biologicals; catalogue number NB100-2220SS) and mouse anti-human Lamp1 (1:25; BD Bioscience, catalogue number 555801). After incubation, coverslips were washed three times with PBS and the following secondary antibodies were added for 30 min and protected from the light: goat anti-mouse Alexa 568 (1:500; Invitrogen; catalogue number A11004), goat anti-mouse Alexa 488 (1:400; Invitrogen; catalogue number A11001), anti-guinea pig Alexa 568 (1:500; Invitrogen; catalogue number A11075), and anti-guinea pig Alexa

488 (1:400; Invitrogen; catalogue number A11073). Coverslips were then washed three times with PBS.

To quantify the percent of bacteria with actin tails, cells were permeabilized with 0.5% triton-X100 for 5 min then washed three times with PBS. *Rickettsia* were stained with either anti-*Rickettsia* 14-13 or anti-*Rickettsia* I7205 as described above. After staining for *Rickettsia,* actin was stained with phalloidin-568 (1:500; Life Technologies; catalogue number A12380).

To quantify the percent of bacteria with actin tails in the mixed cell assay, cells were permeabilized with 0.1% triton-X100 for 5 min then washed three times with PBS. All antibodies were incubated with coverslips for 30 minutes. *Rickettsia* was detected with the primary antibody mouse anti-*Rickettsia* 14-13 and the secondary antibody goat anti-mouse Alexa 404 as described above. After staining for *Rickettsia,* actin was stained with phalloidin-488 (1:400; Life Technologies; catalogue number P3457).

To quantify the number of infectious foci, cells were permeabilized with 0.05% triton-X100 for 5 min, washed three times with PBS, and blocked with PBS containing 2% BSA for 1 h. Coverslips were incubated with anti-β-catenin (1:200; BD Bioscience; catalog number 610153) for 1 h at room temperature then washed three times with PBS, followed by incubation with goat anti-mouse Alexa-568 (1:500; Invitrogen; catalogue number A11004) for 30 min protected from the light. *Rickettsia* was detected with anti-I7205 for 30 min and goat anti-rabbit Alexa 488 as described above. Nuclei were stained with hoechst (1:10,000; Thermo Scientific; catalogue number 62249) for 15 minutes.

Transmission electron microscopy

HMEC-1 cells were seeded into 6 well plates ($1x10^6$ cells per well) and grown for 36 h. Media was aspirated and 2.5 ml of bacteria in media at an MOI of 5 were added. The plates were spun at 300 x g for 5 min at room temperature then 2.5 ml of warm HMEC-1 media was added to each well and the plates were placed at 33˚C. Time points were taken by aspirating media, washing the well with 1x PBS, and fixing the cells in fixative (2% paraformaldehyde, 2% glutaraldehyde in 0.05M cacodylate buffer, pH 7.2) for 45 min at RT. Cells were scraped and pelleted in microcentrifuge tubes and stored in fresh fixative at 4˚C until embedding. Samples were embedded in 2% low melt agarose and placed in 2% glutaraldehyde in 1M cacodylate buffer, pH 7.2 and stored at 4˚C overnight. The next day, samples were post-fixed with 1% osmium tetraoxide and 1.6% potassium ferricyanide, then dehydrated in increasing concentrations of ice cold ethanol (70%-100% EtOH). Samples were embedded in Epon 812 resin (11.75g Eponate 12, 6.25g dodecenyl succinic anhydride,7g nadic methyl anhydride, 0.375 ml of the accelerator benzyldimethylamine was added during the dehydration step) and stained with 2% uranyl acetate and lead citrate. Images were captured with a FEI Tecani 12 transmission electron microscope and analyzed manually to determine the total number of intracellular bacteria and their respective localizations within the cell.

Mouse Studies

Animal research was conducted under a protocol approved by the University of California, Berkeley Institutional Animal Care and Use Committee (IACUC) in compliance with the Animal Welfare Act and other federal statutes relating to animals and experiments using animals

(Welch lab animal use protocol AUP-2016-02-8426). Mice were between 8 and 20 weeks old at the time of initial infection. Mice were selected for experiments based on their availability, regardless of sex. All mice were of the C57BL/6J background and carried mutations in the genes encoding the receptors for IFN-I (*Ifnar*) and IFN-γ (*Ifngr*) (*Ifnar-/-Ifngr-/-*) (described in (65)), and were healthy at the time of infection. For infections, *R. parkeri* was prepared by diluting 30% prep bacteria into 1 ml cold sterile PBS, centrifuging the bacteria at 12,000 x *g* for 1 min (Eppendorf 5430 centrifuge) and resuspending in cold sterile PBS to the desired concentration (5×10^6 pfu/ mL for intravenous infection or 1×10^6 pfu/mL for intradermal infections). The bacterial suspensions were kept on ice during injections. For intravenous infections, the mice were exposed to a heat lamp while in their cages for approximately 5 min and then each mouse was moved to a mouse restrainer (Braintree, TB-150 STD). The tail was sterilized with 70% ethanol and 200 µl bacterial suspensions were injected using 30.5-gauge needles into the lateral tail vein. For intradermal infections, mice were anaesthetized with 2.5% isoflurane via inhalation. The right flank of each mouse was shaved with a hair trimmer (Braintree CLP-41590), wiped with 70% ethanol, and 50 µl of bacterial suspension in PBS was injected intradermally using a 30.5-gauge needle. Mice were monitored for ~3 min until they were fully awake. Body temperatures were monitored using a rodent rectal thermometer (BrainTree Scientific, RET-3).

Statistics

The statistical parameters and significance are reported in the figure legends. Data were considered to be statistically significant when $P < 0.05$, as determined by an unpaired Student's *t*-test, a one-way ANOVA with either multiple comparisons or comparison to WT bacteria, a two-way ANOVA, or a log-rank (Mantel-Cox) test. Differences were determined to be statistically significant when $P < 0.05$. Asterisks denote statistical significance as: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$, compared with the indicated controls. Statistical analyses were performed using GraphPad PRISM v.9.

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Chapter 3

Future Directions

The results in Chapter 2 provide a foundation for understanding Pat1 function. Beyond these initial studies establishing that Pat1 is important throughout infection, there are many directions that future research can take to answer the following outstanding questions. Where does Pat1 function? What are Pat1's targets in the host cell? How is potential cytotoxicity regulated? Is phospholipase activity required for Pat1 function? Are there downstream effects of Pat1 hydrolytic activity? How interchangeable are Pat1 and Pat2 among *Rickettsia* species? Investigating these questions will fill in major gaps of knowledge for *Rickettsia* biology and further our understanding of phospholipase function during infection.

Where does Pat1 function? What are Pat1's targets in the host cell? How is potential cytotoxicity regulated?

Developing more detailed models of Pat1 function will require a better understanding of its localization and cellular targets. One of the main functions proposed for Pat1 is vacuolar escape (1). Our transmission electron microscopy data are consistent with Pat1 playing a role in escaping host membranes. However, we still do not know whether Pat1 localizes to *Rickettsia*-containing vacuoles or if Pat1 interacts with host membranes during infection.

Pat1 from *R. typhi* has been detected in the cytosol of infected host cells. In addition, immunofluorescence microscopy images show Pat1 staining consistent with Pat1 secretion into the cytosol (1). In preliminary studies, I examined Pat1 localization upon exogenous overexpression in uninfected mammalian cells. My preliminary results (**Figure 3.1A**) show localization to the cell periphery in a pattern that overlaps with a fluorescent plasma membrane marker. This localization is consistent with Pat1 targeting host cell membranes. In contrast, my preliminary results from imaging of Pat1 during infection have only yielded one example suggestive of Pat1 being localized within a membrane (**Figure 3.1B**). Surprisingly, Pat1 is abundant within bacteria at late timepoints of 72 hpi (**Figure 3.1B**). A similar phenotype is seen with *L. monocytogenes* PlcB, where large intracellular stores of protein are found in bacteria, but this localization pattern is restricted to a small portion of the bacterial population (3,4). It is difficult to imagine a model in which Pat1 facilitates vacuolar escape without localizing to the vacuolar membrane, but if Pat1 is expressed at low levels, it may be difficult to visualize by immunofluorescence microscopy. Because my results indicate that Pat1 can be detected by western blotting, and prior results suggest the same for Pat1 and Pat2 from *R. typhi* (1), fractionation experiments will be a good starting point to test if Pat1 associates with cellular membranes.

Our ability to complement the *pat1::tn* mutant will also allow us to utilize other approaches for detecting secreted Pat1 during infection. For example, *pat1::tn* can be complemented with genes expressing Pat1 tagged with fluorescent or other reporters, such as Fluorescence-Activating and absorption-Shifting Tag (FAST) or LOV (light-oxygen-voltage) sensing domains, to detect secreted bacterial proteins, which have shown promise with live-cell based approaches of tracking secretion of proteins in a bacterial population (5-8). As an alternative, our lab has previously used a TEM-1-based

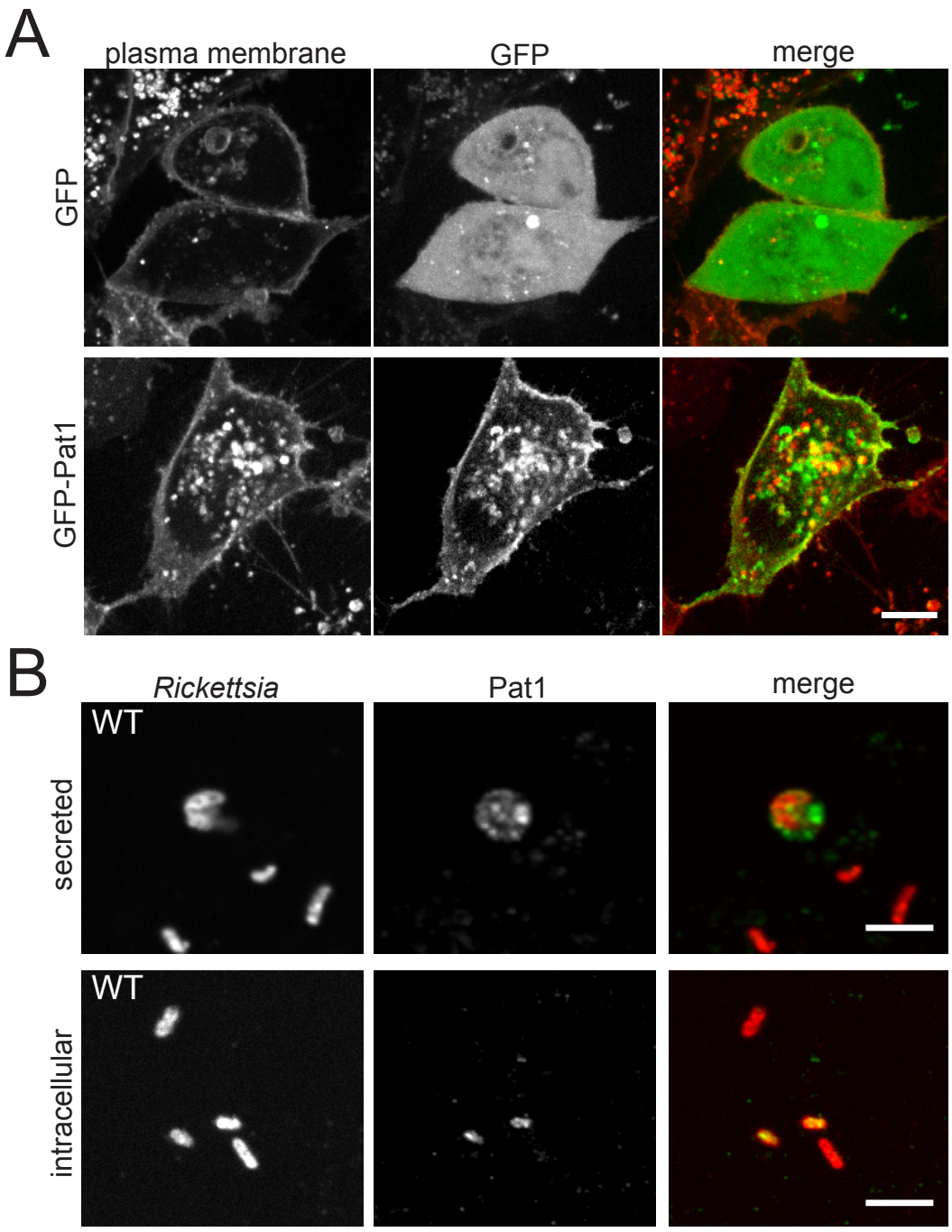

Figure 3.1 Localization of exogenously-expressed Pat1 in host cells (A) A549 cells expressing the plasma-membrane marker TagRFP-T-Farn (A549-TRTF) (red) transfected with GFP (top row; green) or GFP-Pat1 (second row; green). Scale bar 5 μm. (B) Pat1 localization during infection of HMEC cells with WT *R. parkeri*. Top row - Pat1 staining (green) consistent with secretion into a membrane bound compartment at 30 min post infection. Bottom row - Pat1 staining (green) within bacteria (red) at 72 hpi. Scale bar 2 μm.

β-lacatmase assay to demonstrate secretion (9). However, this approach does not reveal spatial information or variability in the population of individual bacteria.

Another important question for understanding Pat1 function is, how is Pat1 regulated? What role does compartmentalization (above) versus regulation (transcriptional, translational, or post-translational) play? Without regulation, Pat1 could lyse the host cell by disrupting the plasma membrane, destroying the replicative niche *Rickettsia* requires (1). Biochemical experiments demonstrate that Pat1 enzymatic activity is only observed in the presence of host cell lysates (1), suggesting that host factors may be an important regulator of PLA2 activity. For the related PLA2 ExoU from *P. aeruginosa*, the structural requirements for activation, membrane binding, phospholipase activity, and cytotoxicity have been investigated by biochemical and imaging approaches (10-12). Similar approaches could reveal regulatory mechanisms for Pat1. In addition, knowing how Pat1 is regulated can inform construction of mutants with inducible/controllable expression, a helpful tool in trying to assess function at different times in the life cycle.

Is phospholipase activity required for Pat1 function and are there downstream effects of Pat1 hydrolytic activity?

Pat1 enzymatic activity has been demonstrated *in vitro* and there is compelling evidence to suggest that Pat1 can generate lipid second messengers. Previous studies detected free fatty acid released from *Rickettsia* infected cells, an increase in cyclooxygenase-2 (COX-2) expression (13,14) and prostaglandin synthesis (15,16). Does Pat1 mediate the above observations of PLA2 activity during infection? Is free fatty acid release and prostaglandin synthesis dependent on host and/or bacterial PLA2 activity? What is the functional consequence of free fatty acid release and prostaglandin synthesis on infection/disease (discussed more below)? The *pat1::tn* mutant is an ideal tool to test the hypothesis that *Rickettsia* PLA2 activity modulates the cellular responses to infection through the release of lipid second messengers.

To further understand the role of phospholipase activity, in preliminary studies I performed a complementation experiment in the *pat1::tn* mutant by introducing a *pat1* gene coding for an enzyme in which the Ser residue required for enzymatic activity is mutated to Ala (17,18). My preliminary results suggest that this mutant forms small plaques similar to the *pat1::tn* mutant (**Figure 3.2**), suggesting that phospholipase activity is required for Pat1 function. This mutant will need further characterization and will provide a useful tool for investigating which phenotypes observed in Chapter 2 require phospholipase activity, and for future studies on the role of enzymatic activity during infection.

To augment this mutagenesis approach, it will also be interesting to characterize Pat1-dependent phospholipase activity during infection using lipidomics approaches, work that is already underway. First, mass spectrometry of lipid profiles from uninfected, WT and *pat1::tn*-mutant-infected cells has the potential to reveal details regarding lipid dynamics in the context of infection. By comparing cells infected with WT versus the *pat1::tn* mutant, we will also be able to determine if there are changes in cellular lipids indicative of PLA2 activity, such as an increase in lysophospholipids and free fatty acids. This would suggest that *Rickettsia* PLA2 activity is driving changes in cellular

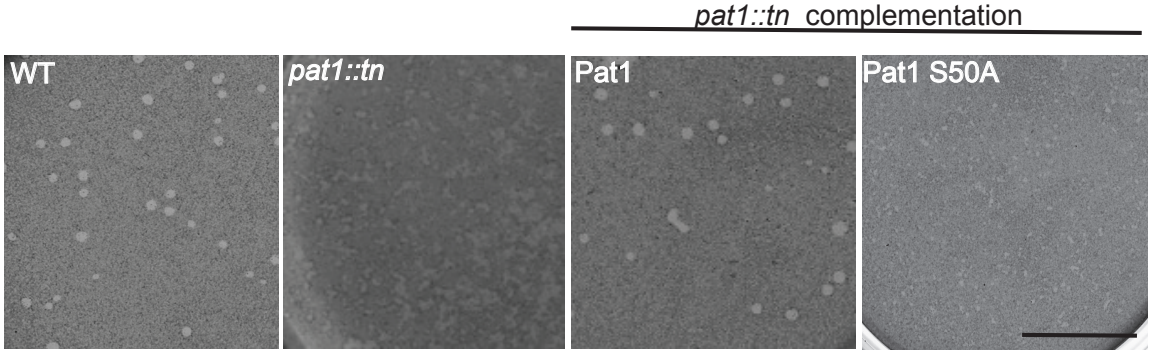

Figure 3.2 Complementation of the *pat1::tn* mutant with Pat1 lacking catalytic activity retains a small plaque phenotype Plaque assay stained by neutral red at 6 d post infection. Scale bar 10 mm

Figure 3.2 Complementation of the *pat1::tn* mutant with *pat1* encoding an enzyme with a mutated active site retains a small plaque phenotype. Plaques were stained with neutral red at 6 d post infection. Scale bar 10 mm.

lipids during infection. Second, the production of eicosanoids prostaglandin E2 (PGE2) and prostaglandin I2 (PGI2) has been demonstrated during *Rickettsia* infection (15,16), but it is unknown if *Rickettsia* or host PLA2 enzymes are driving the release of arachidonic acid for eicosanoid synthesis. *R. parkeri* Pat1 is the single PLA2 enzyme in this species, and comparison of eicosanoid production during infection with WT and *pat1* mutants will help address the outstanding question in the field about the dependence of eicosanoid production on bacterial phospholipases. Notably, the *P. aeruginosa* PLA2 enzyme, ExoU, mediates eicosanoid synthesis that directly contributes to inflammatory responses tied to pathogenicity (18,19). Eicosanoid synthesis can have major impacts on immunity (21, 22), inflammation (22-25), and vascular function (23,25,26), and thus represents an underexplored process that may be impacted by *Rickettsia* infection and disease. The work described in this thesis provides new tools for answering these longstanding questions in the field.

How interchangeable are Pat1 and Pat2 among *Rickettsia* species?

The *pat2* gene has been lost in most *Rickettsia* species and evidence of *pat2* gene fragments can be found in *Rickettsia* genomes lacking *pat2* (1,18). Due to these observed differences in distribution of *pat1* and *pat2* genes, and the divergence between Pat1 and Pat2 protein sequences, it has been proposed that Pat1 and Pat2 have distinct functions during infection (1). Pat2 conservation in TG *Rickettsia* species is notable considering TG *Rickettsia* spread by host cell lysis. However, there is no evidence outside of genomic comparisons to support the hypothesis that Pat2 influences host lysis and bacterial exit. In addition, analysis of Pat1 sequences from different *Rickettsia* species shows unexpected divergence in length and sequence in the C-terminal region downstream of the patatin-like phospholipase domain (1). Altogether, these observations suggest the role of Pat1 and Pat2 in host cells is complex and can't be predicted from gene distribution or protein sequence.

Our ability to complement the *pat1::tn* mutant is a potential way to begin to address whether there are different biological functions associated with Pat1 or Pat2 in

different *Rickettsia* species. Complementing the *R. parkeri pat1::tn* mutant with *pat1* genes from other species, either as full-length sequences or as chimeras, may reveal critical sequence determinants and structure-function relationships. For example, the variable region in the C-terminus of Pat1 sequences could be altering localization or regulation, similar to what has been seen for *P. aeruginosa* ExoU (10). In addition, we can investigate whether inserting *pat2* into the *pat1::tn* mutant is sufficient to alter the exit strategy of *R. parkeri* from cell-cell spread to cell lysis, which would support the model that TG *Rickettsia* uses Pat2 to escape host cells.

Another compelling hypothesis for the diversity in Pat1 sequences is that the divergence represents adaptation to a variety of hosts (i.e. ticks versus mammals) or cell types (i.e. endothelial versus macrophages). My preliminary data has demonstrated restricted bacterial growth of the *pat1::tn* mutant in murine primary bone marrow-derived macrophages (BMDMs) (**Figure 3.3**), suggesting Pat1 may play an important role in this cell type.

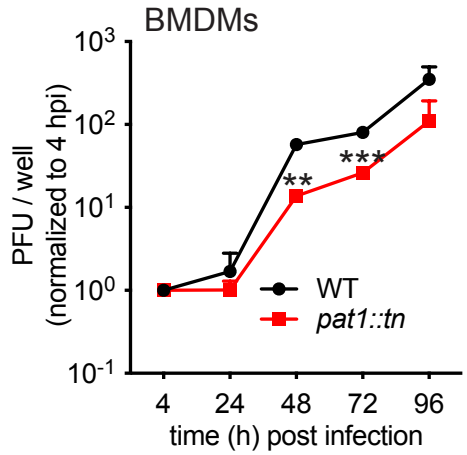

Figure 3.3 Pat1 is important for *R. parkeri* growth in primary murine BMDMs. Growth curves of WT and *pat1::tn* mutant bacteria from 4-96 hpi as measured by plaque forming units (PFU). Data are mean +/- SEM, n=3. Statistical comparisons were by an unpaired T-test; **p<0.01, ***p<0.001.

Cell type differences in bacterial proteins involved in vacuolar escape have been demonstrated for *L. monocytogenes*. LLO is dispensable for vacuolar escape in multiple human cell lines (27-30). These differences are thought to be due to differences in host factors, such as trafficking or membrane composition (30), not the bacterial proteins. Because the variable region in Pat1 sequences is uncharacterized, first addressing how the variable region determines localization or function will be important to support the hypothesis that the variable region determines cell-type specific targets or expression. My preliminary data demonstrates the feasibility of exogenous expression of GFP-Pat1 in mammalian cells to investigate localization (**Figure 3.1**). A logical next step would be to express truncation mutants to investigate the minimal sequences needed for plasma membrane localization, or to express chimeras to investigate how variable regions from Pat1 in other SFG *Rickettsia* species affect localization. Results from these experiments can inform construction of additional Pat1 mutants that can be further characterized during infection or in different cell types and hosts.

Long-term impact

Phospholipases are important mediators of the host-microbe interaction, yet their function in obligate intracellular pathogens remains undefined. With the development of genetic tools for *Rickettsia*, the field is now equipped to dive deeper into mechanistic investigations of bacterial phospholipase proteins. Studying *Rickettsia* PLA2 function will further our understanding of intracellular strategies of phospholipid manipulation for both symbiosis and pathogenesis. Achieving a mechanistic understanding of Pat1 activity and its interaction with membranes could also be of practical importance in areas such as drug delivery into the cytosol of mammalian cells (31), industrial applications (oil degumming and industrial food production) (32,33), and development of therapeutics for inflammatory conditions (34). Continued investigation of *Rickettsia* PLA2 enzymes will contribute to our understanding of bacterial phospholipases during infection and more broadly to PLA2 biochemistry and function.

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