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Host–pathogen interactions

Pathogens and polymers: Microbe–host interactions illuminate the cytoskeleton

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Intracellular pathogens subvert the host cell cytoskeleton to promote their own survival, replication, and dissemination. Study of these microbes has led to many discoveries about host cell biology, including the identification of cytoskeletal proteins, regulatory pathways, and mechanisms of cytoskeletal function. Actin is a common target of bacterial pathogens, but recent work also highlights the use of microtubules, cytoskeletal motors, intermediate filaments, and septins. The study of pathogen interactions with the cytoskeleton has illuminated key cellular processes such as phagocytosis, macropinocytosis, membrane trafficking, motility, autophagy, and signal transduction.

Introduction

Pathogenic microorganisms offer many advantages for elucidating cytoskeletal function and regulation. They exploit actin, microtubules, septins, and intermediate filaments (IFs) in diverse ways. They provide clear functional read-outs, such as infection efficiency or formation of distinct cytoskeletal structures. Finally, microbes often produce locally focused or exaggerated signals, facilitating the dissection of pathways that might be more diffuse or moderate in the host. Pathogens have helped us assign function to cytoskeletal proteins, discover new regulatory modes, and unravel temporal and mechanistic interplay between factors controlling filament dynamics.

In this review, we discuss four infectious processes that have shed light on the host cytoskeleton. The first is pathogen invasion, which exploits cellular uptake pathways that rely on actin, such as phagocytosis and macropinocytosis. Emerging evidence suggests that microtubules and septins also play roles in distinct entry pathways. The second process is establishment of a replication niche, which subverts cytoskeletal functions that normally operate during membrane trafficking and cellular defense. Third, the actin-based motility (ABM) of pathogens through the cytoplasm mimics vesicle rocketing and has also illuminated host cell migration, whereas pathogen motility on the outer surface of the plasma membrane mimics a receptor tyrosine kinase signaling pathway. Finally, pathogen dissemination is an emerging field for which the endogenous processes are not defined. We draw examples from three decades of cellular microbiology, focusing on recent developments and on cases where pathogens (mostly bacteria) played particularly noteworthy roles in key discoveries.

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Abbreviations used in this paper: ABM, actin-based motility; CDT, *Clostridium difficile* transferase; EPEC, enteropathogenic *Escherichia coli;* EHEC, enterohemorrhagic *Escherichia coli;* GEF, guanine-nucleotide exchange factor; IF, intermediate filament; NPF, nucleation-promoting factor; SCV, *Salmonella*-containing vacuole; T3SS, type 3 secretion system; TCR, T cell antigen receptor.

Bacterial invasion of host cells: Many doors, many keys

Entry pathways converge on actin polymerization.

Diverse bacteria invade nonphagocytic cells by stimulating endogenous uptake processes, such as phagocytosis and macropinocytosis. Actin polymerization is central to both of these processes, driving plasma membrane extensions that engulf external cargo. Invading bacteria use a multitude of signaling molecules upstream of actin polymerization, and thus have contributed broadly to our understanding of actin regulation.

Bacterial invasion pathways have historically been classified into "zipper" and "trigger" categories (Cossart and Sansonetti, 2004). Zipper mechanisms, best studied for *Listeria monocytogenes* and *Yersinia* spp., occur when specialized bacterial surface proteins bind host receptors that signal across the membrane to a phagocytic pathway, producing limited membrane rearrangement closely apposed to the entering bacterium (Fig. 1 A). In trigger mechanisms, exemplified by *Salmonella enterica* serovar Typhimurium (*Salmonella*) and *Shigella flexneri*, the pathogen injects effector proteins across the host membrane, often via the syringe-like type 3 secretion system (T3SS), inducing a bloom of actin-rich membrane ruffles that engulf the bacterium and nearby particles (Fig. 1 B). Study of *Salmonella* invasion established that ruffles directly mediate macropinocytosis, a process in which extracellular cargo is taken up nonselectively, and that ruffles produced by endogenous mechanisms, such as by growth factors, have identical uptake behavior (Francis et al., 1993).

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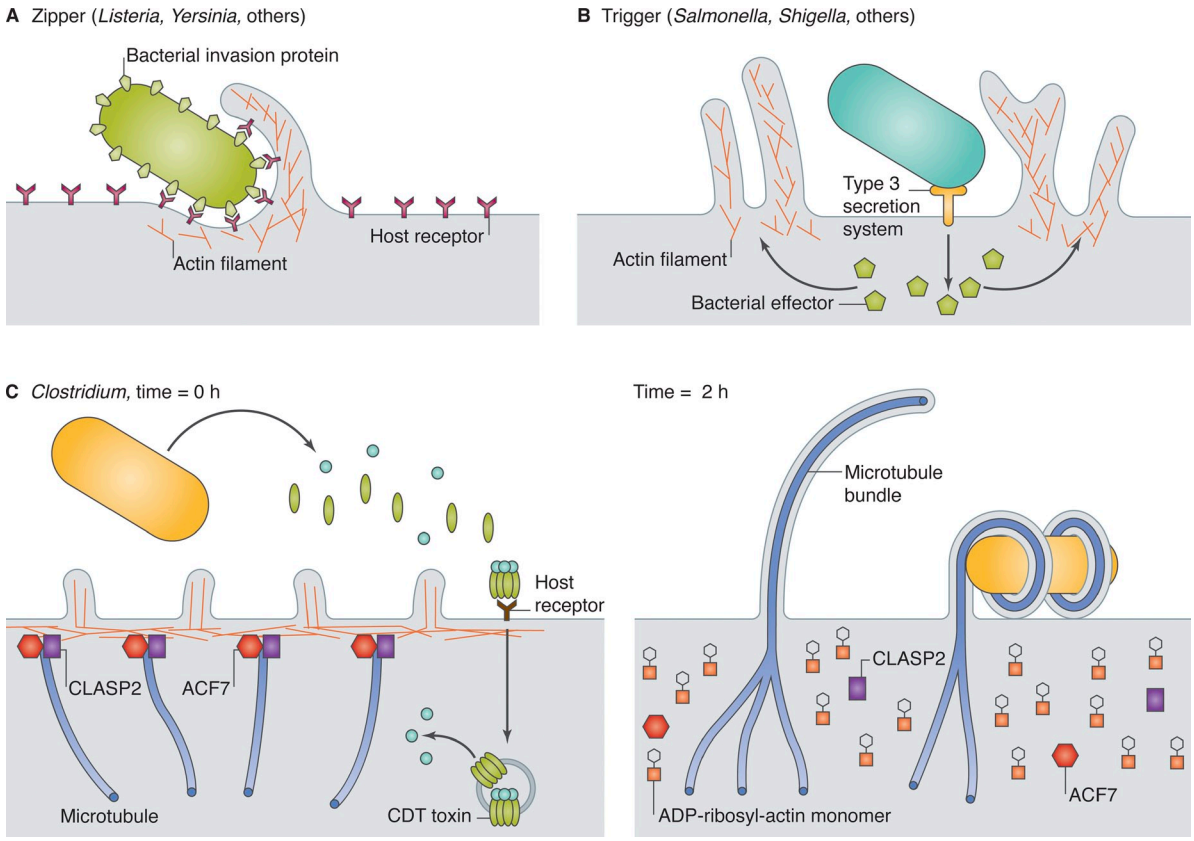

Figure 1. **Bacteria exploit actin and microtubules to promote invasion and adherence.** (A) Zippering bacteria express an invasion protein on their surface, which binds to a host receptor and initiates actin-dependent phagocytosis. (B) Triggering bacteria inject protein effector(s) across the host cell membrane, usually via a T3SS, leading to actin-dependent macropinocytosis. (C) *Clostridium difficile* transferase (CDT), a binary toxin, is endocytosed by intestinal epithelial cells, and the A subunit is released into the cytosol (left). CDT toxin ADP-ribosylates actin, promoting actin filament disassembly, effacement of microvilli, and release of cortical proteins that normally capture and stabilize microtubules. Unrestrained microtubule growth produces cellular extensions that wrap around external bacteria.

Another lesson learned from bacterial entry is the central importance of the Arp2/3 complex in plasma membrane remodeling. The Arp2/3 complex, when activated by nucleation-promoting factors (NPFs), nucleates branched actin networks. NPFs can be recruited to the plasma membrane and activated by Rho-family GTPases, leading to the formation of structures such as ruffles and phagocytic cups. Most invasive pathogens signal to the Arp2/3 complex during entry, although some (notably *Salmonella*) also exploit Arp2/3-independent actin assembly pathways (Hayward and Koronakis, 1999; Zhou et al., 1999; Hänisch et al., 2011). Recent work continues to impart information about the pathways that regulate the Arp2/3 complex. For instance, when the contributions of several NPFs to *Salmonella* invasion were quantified, genetic deletion of N-WASP was found to double expression of the NPF WASH in uninfected cells (Hänisch et al., 2010). This suggests that WASH can partially compensate for the loss of N-WASP and is one of many examples of cytoskeletal plasticity.

A general lesson about actin polymerization is that it requires vigilant modulation to maintain cell function and viability. *Salmonella* illustrates this point, as it injects at least six effectors that control actin rearrangements during entry (Haraga et al., 2008; McGhie et al., 2009). Two effectors (SopE and SopE2) activate Rho GTPases by mimicking host guanine nucleotide exchange factors (GEFs; Hardt et al., 1998; Stender et al., 2000), while another (SopB/SigD) activates GTPases indirectly, via inositol phosphatase activity (Zhou et al., 2001; Terebiznik et al., 2002). Two effectors (SipA and SipC) directly interact with actin, nucleating and stabilizing filaments near the entry site (Hayward and Koronakis, 1999; Zhou et al., 1999; Lilic et al., 2003; McGhie et al., 2004). After a burst of polymerization, a sixth effector (SptP) down-regulates Rho-family proteins by promoting their GTPase activity, restoring normal actin structure at the cell cortex (Fu and Galán, 1999). The balance between Rho activation and inhibition is regulated both spatially (Patel et al., 2009) and temporally (Kubori and Galán, 2003). Thus, triggered entry is not a sudden event like the firing of a gun, but an elaborate, choreographed process.

Is actin the only gatekeeper? Although actin polymerization is critical for uptake processes, roles for other filament networks are being uncovered. Microtubule-dependent bacterial invasion has been reported for *Campylobacter jejuni* and *Citrobacter freundii* (Oelschlaeger et al., 1993), two pathogenic strains of *Escherichia coli* (Donnenberg et al., 1990; Dhakal and Mulvey, 2009), and even the well-studied *Listeria* (Guzman et al., 1995; Kuhn, 1998) and *Salmonella* (Aiastui et al., 2010). Whether actin is also required in some of these cases is unsettled. A precise role for microtubules during entry

has been elusive, in part because the pathway(s) appear to be cell-type and strain specific.

In the past few years, progress has been made toward defining a mechanistic basis for microtubule dependence during bacterial entry. Several toxins made by *Clostridium* spp. were found to induce the temporary formation of long, microtubule-filled projections that entwine bacteria and promote adherence (Fig. 1 C; Schwan et al., 2009). Although *Clostridium* do not invade cells, adherence is a prerequisite of invasion, so microtubule-based projections could promote entry by other pathogens. Interestingly, the toxins affect microtubules indirectly: they ADP-ribosylate actin, leading to actin filament disassembly, followed by release from the cell cortex of proteins that normally capture and stabilize microtubules (CLASP2 and ACF7). For a few hours after toxin application, microtubule-based projections dominate the cell morphology, followed by cell shrinking and rounding. Actin-depolymerizing drugs also induce microtubule-based projections, albeit at lower levels. The exaggerated effect produced by *Clostridium* toxins could provide a tool for dissecting the interplay between actin and microtubules at the cell periphery.

Intermediate filaments and septins might also contribute to bacterial entry. The intermediate filament vimentin has been implicated in invasion by *Salmonella* (Murli et al., 2001) and *Escherichia coli* K1 (Chi et al., 2010). Moreover, depletion of septin-2 reduced invasion efficiency of *Listeria* and *Shigella*, and several septins localized around invading bacteria (Mostowy et al., 2009). For *Listeria*, the effect of septin depletion was specific to one of two receptor-mediated internalization pathways and varied with cell type, suggesting that septins participate in discrete entry pathways.

Cross talk between actin, microtubules, intermediate filaments, and/or septins occurs in many cellular contexts (Rodriguez et al., 2003; Chang and Goldman, 2004; Li and Gundersen, 2008; Gilden and Krummel, 2010; Spiliotis, 2010), complicating the demarcation of roles for each filament type. Further complications arise from findings that blur distinctions between different entry pathways; for instance, zippering bacteria such as *Listeria* exploit the clathrin-mediated endocytic machinery, which is classically associated with smaller cargo (Veiga and Cossart, 2005; Veiga et al., 2007). A systems biology approach, as proposed for viral entry (Damm and Pelkmans, 2006), could help untangle the pathways, and might clarify the variability across cell types. In this approach, RNAi knockdown of numerous host genes is combined with infection by a panel of pathogens to define "functional modules," or host genes that function together during invasion. A broad panel of bacteria could reveal the number of separable entry pathways available in the host and define which cytoskeletal factors participate in each.

Barring the door: Extracellular pathogens disrupt actin to prevent phagocytosis. In contrast to *Salmonella*, which dampens its effects on the cytoskeleton to maintain host cell viability, extracellular bacteria often treat cells more harshly. Many extracellular bacteria deliver toxins into host cells; many of these toxins covalently modify cytoskeletal factors such as actin and Rho-family GTPases, preventing uptake of the pathogen by phagocytic cells. Despite their destructiveness, these toxins' modes of action pertain to endogenous processes. From them, host signaling molecules and post-translational modifications have been identified. For instance, Rac, a member of the Rho family, was discovered as a substrate of the *Clostridium botulinum* C3 toxin (Didsbury et al., 1989), and the toxin was instrumental in elucidating the roles of Rac and Rho as signaling hubs, with Rac controlling membrane ruffles (Ridley et al., 1992) and Rho controlling stress fibers and focal adhesions (Ridley and Hall, 1992). Numerous post-translational modifications—including phosphorylation, glucosylation, adenylylation (AMPylation), ADP-ribosylation, proteolysis, and deamidation—can disable or activate Rho proteins. Both microbial and host proteins use these regulatory modes, as discussed in a recent review (Visvikis et al., 2010).

A newly discovered variation on Rho modification is used by *Photorhabdus luminescens*, which ADP-ribosylates RhoA, preventing GTP hydrolysis and putting RhoA in a constitutively active state (Lang et al., 2010). Previously characterized toxins that ADP-ribosylate Rho proteins, such as C3 toxin, target a different residue and inhibit Rho function. A second *Photorhabdus* toxin ADP-ribosylates actin, preventing β-thymosin from sequestering actin monomers, thus promoting polymerization (Lang et al., 2010). Again, the toxin targets a different residue and has the opposite effect on actin function compared with previously studied toxins. Together, these two *Photorhabdus* toxins promote rampant, disruptive actin rearrangements and inhibit phagocytosis. Although the relevance of these forms of regulation to endogenous processes is not clear, it is interesting that phagocytosis can be blocked by both up- and down-regulating actin polymerization. This emphasizes the theme that actin polymerization must be carefully controlled to produce useful results, and excessive assembly can bring the network to a halt.

Constructing a niche

After invasion, many intracellular pathogens remain within the membrane-bound entry compartment, modifying it to suit their needs. This requires subversion of diverse host pathways to acquire resources for growth while manipulating phagosome maturation to prevent destruction within lysosomes. Again *Salmonella* provides a useful example, as it expresses multiple effectors with overlapping and antagonistic effects on the cytoskeleton (Bakowski et al., 2008; McGhie et al., 2009). We review how study of *Salmonella* led to the identification of a kinesin-interacting partner and potential new roles for cytoskeletal motors in membrane trafficking. We then describe recent insights into IFs and septins, including possible roles in autophagy regulation, gleaned from the study of *Chlamydia trachomatis*, *Shigella*, and others.

***Salmonella* regulates membrane trafficking via kinesin-1 and SKIP.** *Salmonella* replicates in a perinuclear compartment called the *Salmonella*-containing vacuole (SCV), from which membrane tubules called Sifs (*Salmonella*-induced filaments) extend. SCV integrity was known to require microtubules, microtubule motors, and bacterial effectors secreted by the T3SS, including SifA. A key interacting partner of SifA is a host protein of previously unknown function called SKIP (SifA and kinesin-interacting protein; Boucrot et al., 2005). As the name suggests, SKIP interacts with kinesin-1 in vitro and is required

for SCV integrity. However, the role of kinesin in SCV maintenance was puzzling because its recruitment appeared to be regulated both negatively (by SifA; Boucrot et al., 2005) and positively (by another effector, PipB2; Henry et al., 2006). A clue was provided by the discovery that late after infection some SCVs moved toward the cell periphery before dissemination, in a manner dependent on microtubules, kinesin-1, and PipB2 (Szeto et al., 2009). Moreover, in uninfected cells, SKIP was found to promote anterograde movement of late endosomes/lysosomes along microtubules (Jackson et al., 2008; Dumont et al., 2010), possibly through control of membrane tubulation (Ohlson et al., 2008) or scission (Dumont et al., 2010). At a mechanistic level, SKIP binds the late endosomal GTPase Rab9, and SifA might mimic Rab9 (Jackson et al., 2008). A model for SCV maintenance was proposed in which PipB2 recruits inactive kinesin-1 to SCV membranes, where a complex containing kinesin, SKIP, and SifA forms (Fig. 2). SKIP could then activate kinesin, possibly through its interaction with the kinesin light chain, promoting the release of kinesin-associated vesicles (Dumont et al., 2010). The lack of kinesin on SCVs could thus be explained by its rapid dispersal on SCV-derived vesicles. The precise biochemical mechanism of SKIP and kinesin on SCVs remains to be determined. It will be interesting to test this model and to see if analogous mechanisms operate on endogenous SKIP/Rab9-positive compartments.

Another interesting parallel between SCVs and endogenous compartments involves the actin-based motor myosin II, which was unexpectedly implicated in SCV positioning and integrity (Wasylnka et al., 2008). In uninfected cells, myosin II, together with Rab6, contributes to vesicle fission from the Golgi apparatus (Miserey-Lenkei et al., 2010). By analogy, in *Salmonella*-infected cells, myosin II might promote the release of SCV-derived vesicles, perhaps in cooperation with SKIP, kinesin-1, and SifA. The involvement of both myosin and kinesin in SCV-derived vesicle formation could point to new mechanisms of cooperation between actin- and microtubule-based motors.

Filament "cages": Nest or trap? Actin and IFs also contribute to the establishment of replicative niches. In some cases, IFs, alone or with actin, form stabilizing cages around pathogen-containing vacuoles, apparently protecting them from host recognition. For example, *Chlamydia trachomatis* induces relocalization of the IFs vimentin, cytokeratin-8, and cytokeratin-18, as well as actin filaments, to the *Chlamydia* inclusion membrane (Kumar and Valdivia, 2008). IF and actin assembly around the inclusion are interdependent, and disruption of either leads to release of bacteria into the cytoplasm, triggering host defense mechanisms. *Chlamydia* regulates IF assembly in a novel way: the bacterial protease CPAF locally cleaves IF head domains, reducing their cohesiveness. Although the head domain of vimentin is essential for filament assembly in vitro (Herrmann et al., 1996), the IFs cleaved by CPAF form complexes in vitro, and the head domain remains associated with cages (Kumar and Valdivia, 2008). Thus, proteolysis at an unidentified site in the head domain could represent a way to regulate the elasticity of IF networks, allowing cage expansion while maintaining structural integrity.

Protective IF cages also coalesce around other intracellular bacteria. *Salmonella* vacuoles recruit the same three IF proteins as *Chlamydia* (vimentin, cytokeratin-8, and cytokeratin-18), and IF disruption results in SCV dispersal (Guignot and Servin, 2008). Vimentin cages form around *Anaplasma phagocytophilum* inclusions, and a bacterial effector binds vimentin and indirectly promotes survival (Sukumaran et al., 2011), supporting the hypothesis that caging is a protective, pathogen-controlled "nesting" process.

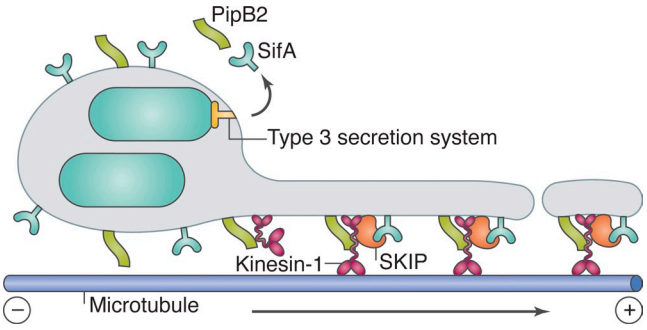

Figure 2. **Model for kinesin-1 and SKIP activity on SCV membranes.** The T3SS-secreted *Salmonella* effectors PipB2 and SifA recruit kinesin-1 and SKIP, respectively, to SCV membranes. It has been proposed that SKIP might then activate kinesin-1 by binding to the kinesin light chain, and that SKIP–kinesin-1 complexes promote tubulation and/or scission of SCV-derived membrane compartments, which are transported toward the plus end of microtubules.

In contrast, a distinct type of cage acts as a host-mediated trap that promotes destruction of the pathogen by autophagy. A proportion of cytosolic *Shigella* become wrapped in septin filaments, in a myosin II–dependent manner, concurrently with acquisition of autophagy markers (Mostowy et al., 2010). Although initial cage assembly requires actin polymerization, an inverse correlation exists between the presence of a cage and productive ABM, and also between myosin activity and motility, indicating that a balance of cytoskeletal forces determines the fate of each bacterium. Because septin cages initially require, but then antagonize actin polymerization, the authors looked for septins around other bacteria that undergo ABM. Septin cages were detected around *Mycobacterium marinum* but not *Listeria* or *Rickettsia conorii*, suggesting that caging is influenced by bacterial factors, which could provide routes to understand its regulation. The connection between septin cages and autophagy suggests a new cellular function for septins. Given that cellular aggresomes, which are vimentin cages surrounding protein aggregates (Wileman, 2007), are also linked to autophagy, it is tempting to speculate that certain filament assemblies simultaneously immobilize cytoplasmic contents and mark them for autophagy. If this is true, then pathogens would be expected to evolve countermeasures to modify filament traps and avoid autophagy. For example, if vimentin acts as an autophagy signal, recruitment of additional filaments (such as cytokeratins or actin) might mask this signal. Intracellular pathogens, by serving as both targets and manipulators of autophagy, are invaluable tools for investigating its regulation.

Tiny rocket scientists: Diverse pathogens "discovered" actin-based motility

Some bacterial species escape from the phagocytic vacuole and replicate freely in the host cytoplasm. A subset of these pathogens expresses factors that trigger actin polymerization against their surface, producing mechanical force that propels them through the cell and facilitates spread to neighboring cells.

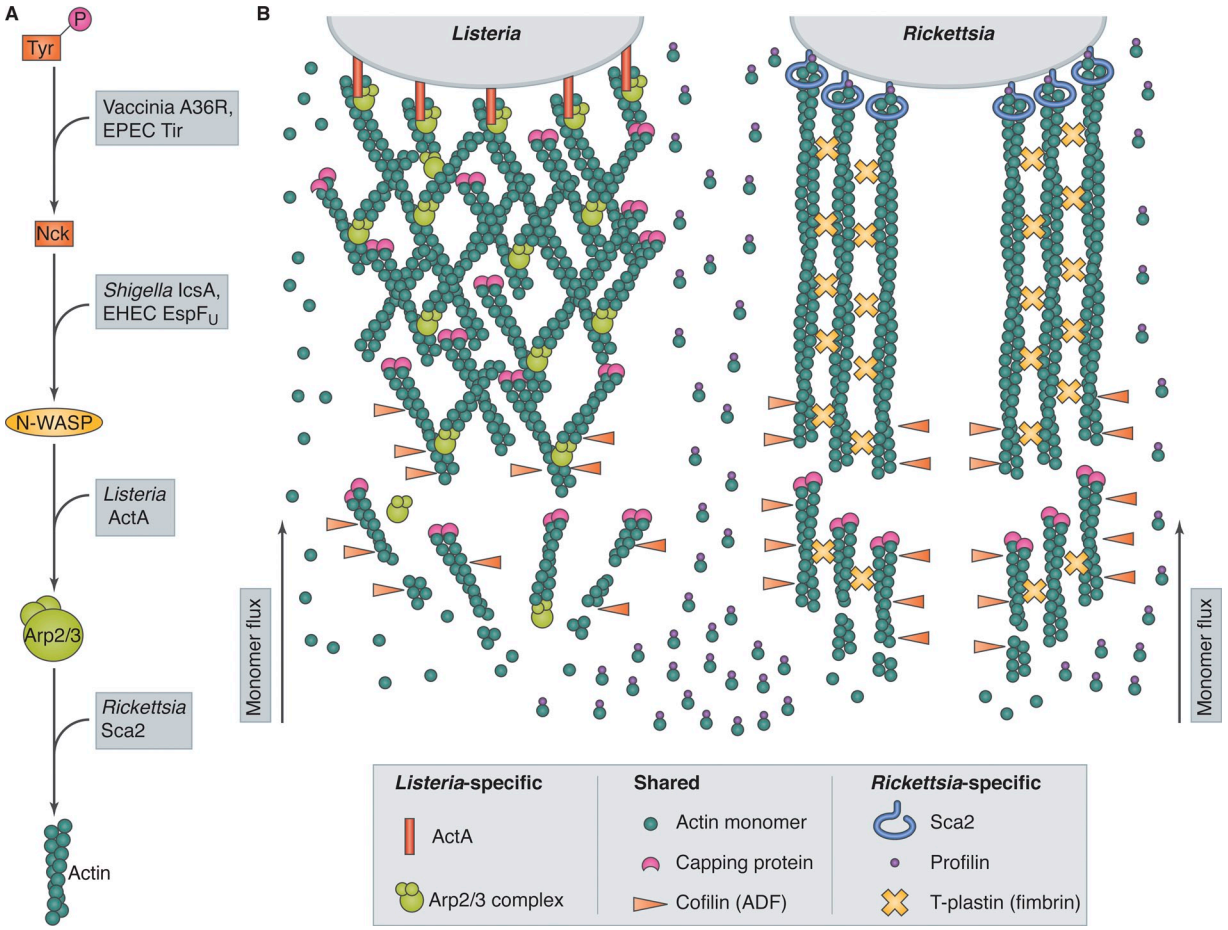

Figure 3. **Pathogens use distinct actin-based motility mechanisms.** (A) Pathogens intercept actin assembly pathways at different levels. Vaccinia virus and EPEC express proteins (A36R and Tir) that mimic host phosphotyrosine motifs to recruit the adaptor protein Nck. *Shigella* and EHEC produce proteins (IcsA and EspF$_U$) that recruit and activate N-WASP. *Listeria* ActA mimics N-WASP to activate the host Arp2/3 complex. *Rickettsia* bypasses host nucleators using the formin-like protein Sca2 to interact directly with actin. (B) The actin-based motilities of *Listeria* and *Rickettsia* have distinct host protein requirements. *Listeria* expresses ActA on its surface, which activates the host Arp2/3 complex, producing branched actin filaments. Actin monomers or profilin–actin complexes can polymerize onto filament ends in *Listeria* tails. *Rickettsia* expresses the formin-like protein Sca2 on its surface, which nucleates unbranched actin filaments and requires profilin for filament elongation. T-plastin is also important for *Rickettsia* tail formation. Both systems require capping protein and cofilin (ADF).

This form of motility evolved independently in *Listeria, Shigella, Rickettsia*, and *Burkholderia*, which each use a distinct mechanism driven primarily by a single bacterial protein (Fig. 3 A), demonstrating that, in contrast to vacuole positioning, ABM may be simpler to achieve. Some viruses—vaccinia and other poxviruses (Cudmore et al., 1995; Dodding and Way, 2009), and a baculovirus (Ohkawa et al., 2010)—also use ABM, although poxviruses trigger it from outside the cell. The study of pathogen ABM, particularly of *Listeria*, revolutionized our understanding of cellular actin-based propulsion, such as vesicle rocketing and lamellipodia-driven cell migration. We review discoveries that established the ABM field, and then highlight recent studies that shed new light on actin dynamics.

Motility through the cytoplasm. Actin's importance for eukaryotic cell migration has been established for decades. It was inferred from the study of *Listeria* actin "comet tails" (Tilney and Portnoy, 1989) that the motive force for lamellipodia protrusion is derived from polymerization itself, rather than myosin activity along a cytoskeletal track (Theriot et al., 1992). However, the mechanisms of actin filament nucleation, organization, and disassembly in lamellipodia were unknown. The comet tails produced by *Listeria* ActA or *Shigella* IcsA were highly similar to each other, suggesting that pathways controlling actin tail morphology were controlled by the host (Kocks et al., 1995). It was clear that identifying host binding partners of ActA or IcsA would advance our understanding of cellular actin dynamics.

The first ActA binding partner identified was the Arp2/3 complex, a conserved seven-subunit complex that was sufficient for actin assembly at the *Listeria* surface (Welch et al., 1997). At the biochemical level, the Arp2/3 complex weakly promoted actin nucleation on its own and was strongly activated by ActA (Welch et al., 1998). It was later determined that *Shigella* also exploits the Arp2/3 complex, but via a distinct mechanism: *Shigella* factors bind and activate host N-WASP, which recruits Arp2/3 and actin (Egile et al., 1999; Leung et al., 2008). The importance of Arp2/3 in cellular processes was quickly recognized, and endogenous proteins such as WASP/N-WASP and WAVE were shown to activate Arp2/3 complex in the same manner as ActA (Goley and Welch, 2006). Collectively, these Arp2/3 activators became

known as NPFs. Additional NPFs, such as WHAMM, WASH, and JMY, continue to be identified and characterized, uncovering new roles for the Arp2/3 complex in processes such as ER-to-Golgi transport and endosome trafficking (Rottner et al., 2010).

Recapitulating Arp2/3 complex–mediated motility of bacteria from purified components requires additional activities besides nucleation. Sustained movement was achieved using the following components: Arp2/3 complex to nucleate filaments; actin-depolymerizing factor (ADF, also known as cofilin) to accelerate depolymerization and maintain the actin monomer pool; and capping protein to prevent nonproductive growth of filaments away from the bacterial surface (Fig. 3 B; Loisel et al., 1999). Profilin, which binds actin monomers and enhances depolymerization from pointed ends, increased the rate of movement but was not strictly required. This landmark study laid the foundation for elucidating the biochemical and biophysical bases of force production by actin assembly, which directly informs the cellular process of vesicle rocketing (Marchand et al., 1995; Merrifield et al., 1999; Taunton et al., 2000). Moreover, the nucleation machinery and signaling molecules involved in ABM are substantially similar to those driving the protrusion of lamellipodia and pseudopodia, although these structures differ from rocketing particles in size and shape (Borisy and Svitkina, 2000; Pollard and Borisy, 2003; Bugyi et al., 2008). Reconstitution of motility also provided a tractable system to dissect the activities of other proteins that regulate actin dynamics. To list just a few examples, it has been used to demonstrate filament capping by twinfilin (Helfer et al., 2006), severing by villin (Revenu et al., 2007), and enhancement of the N-WASP–Arp2/3 interaction by the adaptor protein Grb2 (Carlier et al., 2000).

There is more to learn from pathogens moving through cytoplasm. 22 years after Tilney's micrographs of *Listeria* ABM, the biophysics of its propulsion are still being debated and tested (Mogilner, 2006; Dickinson, 2009). Furthermore, alternative mechanisms of ABM were recently discovered. *Rickettsia* is the first pathogen found to bypass the Arp2/3 complex for ABM (Serio et al., 2010), instead encoding its own formin-like nucleator (Haglund et al., 2010; Kleba et al., 2010). This type of motility requires a different set of host factors compared with *Listeria* and *Shigella* (Fig. 3 B). Specifically, profilin and the actin-bundling protein T-plastin (fimbrin) are critical for *Rickettsia* ABM (Serio et al., 2010). *Rickettsia* may subvert an endogenous motility pathway, as host formins mediate the movement of oocyte chromosomes toward the cortex during meiosis I (Li et al., 2008) and might play a role in ER positioning (Chhabra et al., 2009). Whether *Rickettsia* ABM will prove useful in understanding the transport of cellular cargoes remains to be determined.

Motility across the membrane. A distinct "surfing" form of ABM is used by extracellular vaccinia virus and pathogenic strains of *E. coli*, which intercept receptor tyrosine kinase signaling pathways, resulting in the formation of a moving, actin-rich pedestal beneath the pathogen. Each of these microbes encodes a transmembrane protein that becomes phosphorylated by Src- and Abl-family kinases, leading to recruitment of the adaptor proteins Nck1 and Nck2, which activate N-WASP to stimulate Arp2/3-mediated actin polymerization (Frischknecht et al., 1999; Campellone, 2010). Studies of vaccinia and enteropathogenic *E. coli* (EPEC) demonstrated the physiological relevance of the phosphotyrosine/Nck/N-WASP pathway for signaling to actin assembly. After its elucidation, this pathway was found to be essential in kidney podocytes, signaling through the host receptor nephrin to produce the actin-rich cellular extensions that are critical for kidney filtration function (Jones et al., 2006). Stimulated T cell antigen receptors (TCRs) also induce actin polymerization via Nck, although the pathway uses WASP instead of N-WASP (Barda-Saad et al., 2005). Nck also signals to actin downstream of other receptor tyrosine kinases, including neuronal axon guidance receptors such as Ephrin A4 (Fawcett et al., 2007) and growth factor receptors such as PDGF-R (Rivera et al., 2006; Ruusala et al., 2008), although the downstream pathways and potential roles of Nck are not clearly defined. Nonetheless, pathogen signaling through Nck/N-WASP parallels several endogenous processes.

Study of surfing pathogens has revealed dynamic interplay between Arp2/3, NPFs, and their regulatory partners. Vaccinia virus was used to examine the recruitment and turnover of Nck, N-WASP, WASP-interacting protein (WIP), and Grb2 during motility (Weisswange et al., 2009). Surprisingly, although Nck and WIP are thought to recruit N-WASP, the turnover rate for N-WASP was much slower than for Nck and WIP, implying that other interactions stabilize N-WASP in vaccinia tails. Moreover, N-WASP did not turn over when its ability to stimulate Arp2/3-mediated nucleation was disrupted, suggesting that interaction with the Arp2/3 complex is required to dissociate N-WASP from its binding partners. Presumably, this requirement also applies to N-WASP–mediated nucleation on rocketing vesicles, and possibly to other NPFs, although these hypotheses remain to be tested. These molecular interactions have implications at the level of virus motility. The rate of N-WASP exchange correlated positively with the rate of virus motility, but inversely with the number of tails, illustrating the need to balance speed with stability to achieve productive motility.

Recent work with EPEC has revealed membrane phosphoinositide signals that regulate actin assembly. The EPEC protein that recruits Nck, called Tir, also binds host phosphoinositide 3-kinase (Sason et al., 2009; Selbach et al., 2009) and the inositol-5-phosphatase SHIP2 (Smith et al., 2010). The combined activities of these two enzymes can convert $PI(4,5)P_2$ to $PI(3,4)P_2$, the predominant membrane phosphoinositide in wild-type pedestals. When SHIP2 recruitment is prevented, $PI(3,4,5)P_3$ accumulates instead, and bacteria are associated with multiple, aberrantly long pedestals (Smith et al., 2010), suggesting that $PI(3,4)P_2$ down-regulates signaling after an initial burst of $PI(3,4,5)P_3$-enhanced actin polymerization. The lipid requirements for EPEC pedestal formation might correspond to endogenous processes, particularly to TCR activation (Smith-Garvin et al., 2009) and down-regulation (Smith et al., 2010) and nephrin-mediated signaling (Huber et al., 2003; Zhu et al., 2008). The clear read-out provided by EPEC pedestals could be useful for investigating the mechanisms by which $PI(3,4)P_2$ regulates actin assembly.

Enterohemorrhagic *E. coli* (EHEC), although closely related to EPEC, uses a distinct mechanism of pedestal formation that has illuminated N-WASP regulation. Instead of Nck, EHEC Tir recruits the bacterial effector $EspF_U$, which contains 2–6

repeated sequences that bind and activate N-WASP. Dissection of $EspF_U$ demonstrated the critical role of multivalency in N-WASP activation, as the repeated $EspF_U$ peptides activate efficiently only when they can recruit multiple copies of N-WASP (Sallee et al., 2008). Isolated $EspF_U$ peptides bind N-WASP but do not promote robust actin polymerization (Campellone et al., 2008; Sallee et al., 2008). The importance of oligomerization as a universal mode of NPF regulation is supported by in vitro work showing that dimerized NPF activation domains have ~100 times greater affinity for (Padrick et al., 2008) and activity toward the Arp2/3 complex (Higgs and Pollard, 2000; Padrick et al., 2008) compared with monomeric NPFs. Oligomerization could also explain results in uninfected cells in which artificial clustering of WASP or an upstream binding partner at the plasma membrane stimulated actin polymerization (Castellano et al., 1999; Rivera et al., 2004).

As with cytoplasmic ABM, more remains to be learned from surfing pathogens. For instance, even the well-studied molecule EPEC Tir contains peptides whose effects on actin pedestals are not understood (Campellone, 2010). Intriguingly, both EPEC and host nephrin use secondary mechanisms of actin assembly in addition to the primary phosphotyrosine/Nck-dependent pathway, so additional parallels might exist between the two systems. Finally, EHEC $EspF_U$ can be used to explore how cells generate plasma membrane protrusions independently of tyrosine kinase signaling.

New directions: Pathogen exit and dissemination

Pathogens can quickly consume a host cell's resources and must spread to new cells to continue their life cycle. Although host cell lysis can promote spread, it is often advantageous for microorganisms to exit cells in a controlled, nonlytic manner. Less is known about molecular mechanisms of dissemination compared with entry or intracellular motility, but several exit strategies use the actin cytoskeleton (Fig. 4). It is likely that normal host cell processes are exploited during exit, but these processes are poorly defined. This makes pathogen exit an exciting area for future research.

Bacteria that undergo ABM, such as *Listeria* and *Shigella*, subsequently enter long, plasma membrane–bound protrusions that extend from infected cells into adjacent cells (Tilney and Portnoy, 1989; Kadurugamuwa et al., 1991) and escape into the neighboring cell's cytoplasm (Robbins et al., 1999; Monack and Theriot, 2001). Within protrusions, actin tail filaments become longer and more densely bundled (Sechi et al., 1997; Gouin et al., 1999) and more stable (Robbins et al., 1999). Host adherens junction proteins, such as vinculin (Kadurugamuwa et al., 1991) and cadherins (Sansonetti et al., 1994), have been implicated in protrusion-mediated spread. Because cadherin cytoplasmic domains link to actin filaments via vinculin and other junction components, it was speculated that junction proteins might bind comet tails, altering actin filament organization and promoting the formation and rigidity of protrusions. Analogously, disruption of ezrin, an actin membrane linker that localizes to *Listeria* protrusions but not cytoplasmic tails, results in short, crumpled protrusions (Pust et al., 2005). In contrast, the junction protein Tuba—which has N-WASP binding, scaffolding, and GEF activity—negatively regulates protrusion formation (Rajabian et al., 2009), apparently by promoting cortical tension in epithelial cell layers (Otani et al., 2006; Rajabian et al., 2009). The *Listeria* effector InlC disrupts Tuba–N-WASP binding, relaxing cortical tension and promoting protrusions. Thus, protrusion formation appears to require an initial relaxation of cortical rigidity, followed by promotion of rigidity, presumably by a different set of factors, around the bacterial actin tail.

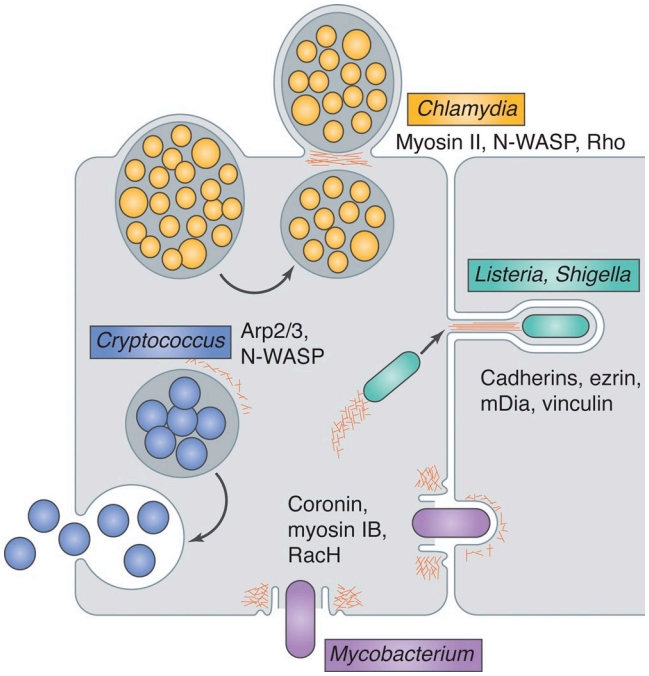

Figure 4. **Exit strategies of diverse intracellular pathogens use actin.** *Chlamydia* (yellow) vacuoles are extruded through a cortical constriction, and the plasma membrane seals around the constriction point in a manner dependent on actin (red), releasing a double-membrane–bound bacterial compartment. The extrusion pathway appears to require myosin II and N-WASP for initiation and Rho for detachment of the extruded vacuole from the host cell. *Listeria* and *Shigella* (green), propelled by actin-based motility, enter plasma membrane protrusions and are taken up by neighboring cells. Cadherins, ezrin, mDia, and vinculin have been implicated in protrusion formation. *Mycobacterium* (purple) exits cells through a plasma membrane break surrounded by a barrel-shaped ejectosome rich in actin, myosin IB, and coronin. In host cells lacking RacH, ejectosomes are not detected and mycobacterial spreading is impaired. *Cryptococcus* (blue) phagosomes fuse with the plasma membrane, and intermittent actin polymerization around the phagosome, apparently mediated by N-WASP and the Arp2/3 complex, inhibits this fusion.

Recently, the formin-family actin nucleators Dia1 and Dia2 were found to support dissemination of *Shigella* (Heindl et al., 2010). As with ezrin, Dia1 and Dia2 localize to actin in protrusions but not to cytoplasmic tails, and disruption of Dia reduces the frequency and length of protrusions. The involvement of formins, which generate long, unbranched actin filaments, is consistent with the parallel filament bundles found in protrusions. Together, the effects of formins, ezrin, vinculin, and cadherin on protrusions but not cytoplasmic tails suggest that a distinct set of actin regulatory factors interacts with motile bacteria after they contact the plasma membrane. Further study is required to determine which factors help build protrusions and how the transition

in actin organization is regulated. Moreover, how these cytoskeletal alterations promote uptake by neighboring cells is unclear. In uninfected cells, uptake of vesicles derived from neighboring cells was observed and was named paracytophagy (Robbins et al., 1999), but paracytophagy's role in cells and its connection to protrusion-mediated spread have not been described.

Another actin-dependent mode of exit is the extrusion of *Chlamydia* inclusions into the extracellular space (Hybiske and Stephens, 2007). Compartments containing a few to hundreds of *Chlamydia*, surrounded by both inclusion and plasma membranes, were observed to balloon out from a constriction point in the host cell, eventually being released into the media. Extrusion requires actin, N-WASP, and myosin II, and the final pinching-off step requires the GTPase Rho. *Chlamydia* have a second, lytic exit strategy, and usually both mechanisms are used with equal frequency. Curiously, the authors found that jasplakinolide, which blocks actin depolymerization but not polymerization, abrogated the lytic strategy and quickly induced extrusions, even early in infection when *Chlamydia* do not normally exit. This suggests that robust actin polymerization is sufficient to induce extrusion, which implies that it is a host-driven process. However, the endogenous function of extrusion, if any, remains a mystery.

A third actin-dependent exit strategy was recently described for *Mycobacterium marinum* and *M. tuberculosis* (Hagedorn et al., 2009). These bacteria, which spend part of their life cycle free in the host cytoplasm, exited host amoebae through a barrel-shaped structure rich in actin, myosin IB, and coronin, which the authors called an ejectosome. Ejection occurred without long membrane extensions, did not require a comet tail or a recipient cell, and took only a few minutes to complete, distinguishing it from protrusion-mediated spread. Ejection resulted in plasma membrane breakage and exit into either the media or an adjacent cell, which formed a phagocytic cup–like structure around the invading bacterium. In spite of membrane rupture, leakage of cellular contents did not occur, apparently due to a tight septum formed by the actin ring. The authors proposed that ejectosomes might have originated as a plasma membrane resealing mechanism. Actin polymerization and actomyosin contraction occur around plasma membrane wounds (Mandato and Bement, 2001), but this process uses myosin II (Mandato and Bement, 2001; Togo and Steinhardt, 2004), whereas myosin II was not detected at ejectosomes. Thus, the connection between ejectosomes and plasma membrane repair pathways requires further investigation.

A membrane-sealing function might also explain the cycles of transient actin polymerization observed around phagosomes containing the fungal pathogen *Cryptococcus neoformans* (Johnston and May, 2010) or bacteria such as *Listeria* (Yam and Theriot, 2004). In the case of *Cryptococcus,* actin "flashes" follow phagosome permeabilization events, in an Arp2/3 complex– and WASP/N-WASP–dependent manner. *Cryptococcus* exits cells by phagosomal fusion with the plasma membrane, and fusion is usually preceded by phagosome permeabilization. In contrast to the above strategies, disruption of actin assembly enhances fungal exit, implying that host cells polymerize actin to limit dissemination. Flashes were also observed on phagosomes containing transferrin-coated beads, on mechanically induced plasma membrane wounds, and at membrane invaginations around particles too large to phagocytose (Yam and Theriot, 2004), demonstrating that flashing is an endogenous process, induced by membrane breakage and possibly by the presence of large internalized particles. Although dynamic actin accumulates around plasma membrane wounds, the role of actin polymerization on phagosomes is less clear. Actin might contribute to membrane repair, or could form a barrier to limit mixing of vesicle contents with cytosol while other repair mechanisms occur. In either case, actin polymerization appears to help maintain the integrity of phagosomes.

Collectively, these reports will lead to further discoveries about the roles of actin in ushering pathogens or other particles out of cells, including insights into membrane resealing. As mentioned in the Introduction, the endogenous pathways exploited during pathogen exit are not defined, and it is currently unknown if other cytoskeletal filaments are involved in these processes.

Future perspectives

Given the numerous ways in which pathogens have contributed to our understanding of the cytoskeleton, it is obvious that future study of pathogen–cytoskeleton interactions will uncover important new insights. In particular, pathogens might reveal clues to the role(s) of actin in the nucleus, an area that is just beginning to be explored (Skarp and Vartiainen, 2010). For instance, baculovirus replication requires nuclear translocation and polymerization of actin (Goley et al., 2006). Furthermore, *Anaplasma phagocytophilum* induces phosphorylation of actin in its host, leading to increased nuclear G-actin and phospho-actin–dependent up-regulation of a host gene required for bacterial survival (Sultana et al., 2010). Pathogens have additional tricks up their sleeves that were not discussed in this review, such as destabilization (Coureuil et al., 2009) or reinforcement (Kim et al., 2009) of intercellular junctions, as well as promotion of host cell motility (Worley et al., 2006), suggesting that insights into these processes will be forthcoming. Advancements will also come from technological improvements, for example in imaging methods, as well as new approaches, such as systems-level analyses and mathematical modeling. In addition to revealing fundamental cellular mechanisms, future studies of the host–pathogen relationship will enhance our understanding of pathogenesis and disease, and may lead to improved diagnostics and treatments for microbial infections.

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