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The RAB5-GEF Function of RIN1 Regulates Multiple Steps During *Listeria monocytogenes* Infection

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Abstract

Listeria monocytogenes is a food-borne pathogenic bacterium that invades intestinal epithelial cells through a phagocytic pathway that relies on activation of host cell RAB5 GTPases. *L. monocytogenes* must subsequently inhibit RAB5, however, in order to escape lysosome-mediated destruction. Relatively little is known about upstream RAB5 regulators during *L. monocytogenes* entry and phagosome escape processes in epithelial cells. Here we identify RIN1, a RAS effector and RAB5-directed GEF, as a host cell factor in *L. monocytogenes* infection. RIN1 is rapidly engaged following *L. monocytogenes* infection and is required for efficient invasion of intestinal epithelial cells. RIN1-mediated RAB5 activation later facilitates the fusion of phagosomes with lysosomes, promoting clearance of bacteria from the host cell. These results suggest that RIN1 is a host cell regulator that performs counterbalancing functions during early and late stages of *L. monocytogenes* infection, ultimately favoring pathogen clearance.

Keywords

Listeria monocytogenes; MET; RIN1; RAB5; phagosome

INTRODUCTION

Invasive bacterial pathogens gain entry by manipulating host cell endocytic machinery (1). Bacterial proteins commandeer host proteins, referred to as host cell factors, throughout the infection process. A variety of host cell factors are required for entry, downstream events controlling survival, replication and spread. Depending on the pathogen, host cell entry is accomplished through macropinocytosis (2), clathrin-dependent endocytosis (3, 4) or caveolin-mediated uptake (5). The entry processes are predominantly actin-dependent (6),

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reflecting the need for cytoskeletal remodeling to accommodate large cargo (7). Although various genera of invasive bacteria employ distinct sets of host cell factors for entry into host cells, there is a striking commonality in their reliance on small GTPases (8, 9).

Listeria monocytogenes is a Gram-positive, food-borne pathogen that invades intestinal epithelial cells, spreads laterally through the gut epithelium and traverses the intestinal barrier, eventually disseminating to distal organs (10). In immune-compromised individuals *L. monocytogenes* can cause severe listeriosis with symptoms ranging from gastroenteritis to bacterial meningitis, and mortality rates of approximately 30% (11). In pregnant women, *L. monocytogenes* can lead to spontaneous abortions and neonatal infections (10).

L. monocytogenes uses two surface internalin (Inl) proteins to bind epithelial host cells, and utilizes clathrin-dependent mechanisms to enter these non-professional phagocytes (4). InlA interacts with CDH1 (E-cadherin) (12) while InlB interacts with MET, a receptor tyrosine kinase (13). Either internalin is sufficient for epithelial cell invasion, although both are needed for optimum entry efficiency (14). Engagement with InlB stimulates MET, leading to the recruitment and activation of signal transduction proteins including RAS (15, 16). *L. monocytogenes* infection triggers activation of the downstream RAS effectors PI3K and RAF (15, 17), but other RAS effectors commonly activated following MET stimulation have not been studied in this context. *L. monocytogenes* attachment also triggers RAB5 activation through an unknown mechanism, a step required for efficient internalization by receptor-mediated phagocytosis (18, 19).

Host cells mount a bactericidal response that also employs RAB5, in this case to fuse phagosomes with lysosomes and destroy the internalized bacterium before it can replicate (20, 21). To avoid this fate, *L. monocytogenes* uses a cytolysin to escape the phagosome and enter the cytoplasm (22) where replication takes place. The *L. monocytogenes* surface protein ActA promotes actin polymerization to cloak bacteria from the host autophagic clearance systems and to propel bacteria through the cytoplasm to facilitate protrusive entry into adjacent cells (23, 24). To allow time for phagosomal escape, the *L. monocytogenes* GAPDH protein ADP ribosylates RAB5, rendering this GTPase non-responsive to activation by guanine nucleotide exchange factors (GEFs) (21, 25). RAB5 subjugation is essential for *L. monocytogenes* to escape into the cytosol and replicate (21). Hence, while RAB5 facilitates invasion it subsequently promotes bacterial killing in phagolysosomes. This requires *L. monocytogenes* to switch from promoting to suppressing RAB5 activity for a successful infection. Little is known, however, about the role of host cell RAB5 regulators during *L. monocytogenes* invasion and spread.

RIN1 is a RAS effector involved in receptor tyrosine kinase endocytosis and trafficking (26, 27). Through its VPS9 domain, RIN1 functions as a GEF with specificity for RAB5 GTPases, promoting internalization and degradation of activated receptors (28–30). RIN1 also binds and activates ABL non-receptor tyrosine kinases that regulate actin cytoskeleton remodeling (31). An intricate balance between these two RIN1 effectors (RAB5 and ABL) determines the rate and route of receptor internalization. (27, 32). The RAB5-GEF activity of RIN1 is exerted independently of RABGEF1 (a.k.a. Rabex5), another RAB5 GEF regulating endocytic processes in the cell (33).

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Because *L. monocytogenes* uses a growth factor receptor tyrosine kinase to enter host cells (15, 34), and because RAS proteins signal through RIN1 to regulate RAB5 during endocytosis (27, 29), we examined whether RIN1 functions as a host cell factor for *L. monocytogenes*. We found that RIN1 facilitates *L. monocytogenes* intestinal epithelial cell entry through its RAB5-GEF function and that loss of RIN1 impaired invasion. RIN1 plays a strikingly different role post-invasion by accelerating RAB5-dependent fusion of *L. monocytogenes* containing phagosomes with lysosomes.

RESULTS

MET-mediated *L. monocytogenes* invasion is facilitated by RIN1-mediated activation of RAB5

Engagement of *L. monocytogenes* InlB with host cell MET stimulates receptor tyrosine kinase activity, leading to activation of RAS proteins and the downstream MAP kinase cascade (15). We tested whether *L. monocytogenes* binding also activates the RAS effector RIN1 in HeLa cells, a human cervical cancer cell line widely used as a model for epithelial cell invasion. Following growth factor stimulation by RAS, the RIN1 protein becomes phosphorylated by ABL tyrosine kinases (27). We observed the same signaling mark (RIN1-pY^{36}) as early as 2.5 minutes following addition of *L. monocytogenes* to HeLa cells (Figure 1A, Figure S1A). Phosphorylation was ABL-dependent, as judged by reduced phosphorylation in the presence of the ABL-specific kinase inhibitor imatinib. This result suggests that RIN1 signaling is engaged early in the process of host cell invasion by InlB binding to MET.

We next sought to determine whether RIN1 was necessary for cell invasion by *L. monocytogenes*. For infection assays, we initially used IEC-18, a rat intestine epithelial cell line that resembles epithelial cells of the intestinal barrier that *L. monocytogenes* invades *in vivo* (35). Rin1 silencing significantly reduced the efficiency at which *L. monocytogenes* invaded IEC-18 cells (Figure 1B, Figure S1B). A similar effect was seen using IEC-6 Cdx2, a rat intestinal epithelial cell line that stably over-expresses the homeobox domain transcription factor, Cdx2 (36). These cells show enhanced polarization with tight and adherens junctions, making them an especially useful model of an intestinal epithelium. Rin1-silenced IEC-6 Cdx2 cells were also compromised in their ability to be invaded by *L. monocytogenes* (Figure 1C, Figure S1D). The same effect of RIN1 silencing was seen in HeLa cells (Figure S2A). These findings implicate RIN1 as a host cell factor employed during *L. monocytogenes* invasion.

Given the established requirement for RAB5 activation during *L. monocytogenes* invasion (18) we next tested whether the RAB5-GEF function of RIN1 contributes to invasion efficiency. Expression of a GEF domain mutant that prevents RIN1-mediated RAB5 activation, $RIN1^{E574A}$, significantly decreased *L. monocytogenes* invasion in all three cell lines tested (Figures 1B, 1D and Figures. S1C, S1E, S2B). In contrast, a mutation that disrupts RIN1 signaling through ABL tyrosine kinases, $RIN1^{QM}$ (37), did not affect *L. monocytogenes* entry into IEC-18 cells (Figure S2C). To rule out the possibility that other RAB5-GEFs were reduced as a side effect of RIN1 knockdown, we assessed the levels of Rabgef1, a ubiquitous RAB5 GEF also called Rabex5, and Rin2, a RIN1 paralog. Protein

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levels of Rabgef1 and Rin2 were unaltered in Rin1 silenced IEC-6 Cdx2 cells (Figure S2D). These results suggest that MET-dependent *L. monocytogenes* entry is facilitated by RIN1-mediated activation of RAB5. Notably, RIN1 over-expression had little effect on *L. monocytogenes* entry into these cells (Figs. 1D, S2B, S2C), possibly because another host cell factor is rate limiting for invasion.

Having shown that RAB5 activation by RIN1 in epithelial cells is required for efficient *L. monocytogenes* invasion, we reasoned that RIN1 and RAB5 could potentially be recruited to the host cell surface and phagosomes during bacterial entry. We visualized RIN1 and Rab5 at 50 and 80 minutes post infection (mpi). We observed co-localization of both proteins with bacteria during early (50 mpi) as well as later (80 mpi) stages of entry into IEC-6 Cdx2 cells expressing wild type RIN1 (Figure. 2A, C). Recruitment of Rab5 was diminished, however, in IEC-6 Cdx2 cells expressing $RIN1^{E574A}$ (Figure. 2B, C). These observations provide further support for the role of RIN1 as a host cell factor needed for efficient RAB5 activation during *L. monocytogenes* entry into epithelial cells.

Dual receptor-mediated entry also relies on host cell RIN1

L. monocytogenes entry into mammalian cells is mediated by two distinct ligands, InlA and InlB, which bind to host cell CDH1 (E-cadherin) and MET, respectively (12, 16). Because of sequence variation between human CDH1 and rodent Cdh1 proteins, *L. monocytogenes* InlA does not recognize mouse or rat epithelial cells. In order to examine the course of *L. monocytogenes* invasion involving both host surface receptors, as occurs during the normal course of infection, we utilized a murinized version of the bacterium (*L. monocytogenes* $InlA^{mur}$). This previously characterized strain shows enhanced affinity to mouse and rat Cdh1 and consequently more efficient internalization (38). As expected, $InlA^{mur}$ *L. monocytogenes* invaded IEC-6 Cdx2 cells more efficiently than the wild type strain (Figure 1E). Rin1 silencing still decreased invasion efficiency, however, demonstrating the importance of RIN1 signaling even when both host cell receptors are available and compatible.

RIN1 accelerates the host cell bactericidal response

Following *L. monocytogenes* entry, host cells mount a bactericidal response that includes fusing phagosomes to bacteria-degrading lysosomes through RAB5-mediated trafficking. *L. monocytogenes* avoids this fate by inhibiting host cell RAB5, and by escaping the phagosome to replicate in the cytoplasm. We postulated that RIN1 might promote the bactericidal response through enhanced RAB5 activity. Indeed, normalizing for differences in cell entry efficiency, RIN1 over-expression led to a small but significant decrease in *L. monocytogenes* escape to the cytoplasm and replication at 6 hours post-infection in IEC-18 cells (Figure 3A, B). In contrast, expression of the GEF-mutant $RIN1^{E574A}$ led to an increase in cytoplasmic *L. monocytogenes*, confirming that the effects of RIN1 in this assay are RAB5-dependent. Phagosome escape is a prerequisite for *L. monocytogenes* actin polymerization and actin-based motility. We used co-localization of *L. monocytogenes* with actin as a measure of phagosome escape and replication. Although RIN1 over-expression did not appear to alter the rate of actin coating in IEC-18 cells, it did lead to lower totals of cytoplasmic actin-coated bacteria as early as three hours post-infection compared to the

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control group (Figure 4A, B). Conversely, the RIN1^{E574A} mutant increased the number of actin-coated bacteria, supporting our hypothesis that RAB5 activation by RIN1 contributes to the host cell strategy for preventing *L. monocytogenes* escape and replication. The same effect was seen at six hours post infection (data not shown).

Host cells eliminate *L. monocytogenes*-containing phagosomes by fusing them with lysosomes, a process initiated by phagosome acquision of the lysosomal marker Lamp1. To test whether RIN1 promotes this RAB5-dependent bactericidal pathway, therefore, we quantified the co-localization of bacteria with Lamp1 at 2.5 hpi as an early measure of *L. monocytogenes* clearance. RIN1 over-expression increased co-localization of *L. monocytogenes* with Lamp1 in IEC-18 cells, compared to control cells, whereas expression of the GEF-defective mutant RIN1^{E574A} caused a decrease in the fraction of intracellular bacteria co-localized with Lamp1 (Figure 5 A, B and C, S3A). The fraction of Lamp1-negative *L. monocytogenes* in these images may represent early escapers to the cytoplasm, but this could not be verified without actin staining.

To examine whether Lamp1-positive bacteria went on to fuse with lysosomes, we looked at co-localization of GFP-*L. monocytogenes* with dextran-Texas red-loaded lysosomes (Figure S3B). The co-localization of bacteria with fluorescent dextran confirmed that at least a portion of the internalized *L. monocytogenes* were undergoing lysosome fusion in these cells. Hence, RIN1 signaling through RAB5 increases the fusion of bacteria-laden phagosomes with host cell lysosomes, counteracting the effect of *L. monocytogenes* proteins that block RAB5 activation to improve escape efficiency. (25)

Autophagy, an intracellular destruction mechanism distinct from Lamp1-marked lysosome uptake, provides another way for host cells to clear invasive bacteria (39). *L. monocytogenes* normally evades autophagy during pathogenesis, at least in part by assembling an actin cloak (40, 41). We examined whether autophagy contributed to the RIN1-mediated bactericidal response by examining co-localization of *L. monocytogenes* with the autophagy marker LC3 in infected IEC-6 Cdx2 cells over-expressing RIN1 (Figure S3C and D). There was little discernable co-localization of *L. monocytogenes* with LC3, suggesting that autophagy plays an insignificant role in rapid clearance of *L. monocytogenes* by RIN1 signaling.

Cell-cell spread of *L. monocytogenes* is impeded by the RIN1-to-RAB5-mediated bactericidal response

We expected that changes in the rate of phagosome escape would be reflected in the efficiency of cell-cell spread by *L. monocytogenes*. This was tested by plaque assay following *L. monocytogenes* infection. RIN1 over-expressing IEC-18 cells produced plaques of smaller average area than control, implicating a defect in *L. monocytogenes* cell-cell spread (Figure 6A, Figure S4A). In contrast, Rin1 silencing (Figure S4A) or expression of RIN1^{E574A} (Figure 6A, Figure S4A) led to larger plaques compared to control cells, demonstrating that the bactericidal contribution of RIN1 signal transduction results in a cell-spread defect following *L. monocytogenes* infection. This result was confirmed in IEC-6 Cdx2 cells (Figure 6B). The ABL-binding mutant of RIN1, RIN1^{QM}, had no effect on replication and plaque formation, when compared with wild type RIN1 (Figure S4B).

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Intercellular attachments, such as tight junctions, play an important role in maintaining polarity and establishing the barrier function of intestinal epithelia. Junction slackening is required for efficient *L. monocytogenes* spread from primary host cells to adjacent cells. Because tight junction organization and turnover are regulated by RAB5-dependent mechanisms (42), we tested the possibility that the cell-spread defects we observed in RIN1 modified cells were, at least in part, due to altered junctions. A disruption of tight and adherens junction organization was readily apparent following *L. monocytogenes* infection (Figure S5A). Staining of collagen-attached IEC-6 Cdx2 cells for the tight junction associated protein Tjp1 (ZO-1) and adherens junction protein Cdh1 (E-Cadherin) revealed no organizational remodeling in cells over-expressing RIN1 or RIN1^{E574A} (Figure S5B, C). Examination of IEC-6 Cdx2 cells attached to Matrigel or fibronectin matrix also showed no difference in tight junction organization (data not shown). Total levels of the junction proteins Tjp1 and Cdh1 remained unaltered upon RIN1 over-expression or expression of RIN1^{E574A} (data not shown), suggesting that the *L. monocytogenes* cell-spread defects we observed were primarily due to alterations in the RAB5-dependent host cell bactericidal response.

DISCUSSION

A common feature of bacterial invasion mechanisms is the commandeering of trafficking GTPases, especially RAB5, by pathogen-encoded proteins (9). In addition, multiple pathogens are known to inhibit RAB5 activity in order to evade lysosome-mediated degradation. Although there has been some progress in characterizing host protein involvement during these stages of infection (43–45), little is known about the role played by host cell regulators of RAB GTPases during infection. A recent report showed that RIN1 over-expression can partially rescue the effects of RAB5 inactivation during macrophage entry by *Pseudomonas aeruginosa* (44), but this was not examined further.

During *L. monocytogenes* invasion, InlB-mediated entry is dependent on RAB5 activation (18), but the bacterium must subsequently block RAB5 activation in order to evade clearance by fusion of its phagosome shelter with the lysosome (25). Any defect in this critical switch from RAB5 activation to inactivation can derail the course of infection. In this study we characterized the contributions of RIN1, an epithelial cell RAS effector and RAB5-GEF, during *L. monocytogenes* invasion, bactericidal escape and cell-cell spread. Importantly, we validate the role of RIN1 using silencing of the endogenous gene and by specifically disabling the RIN1-GEF function.

Our results suggest a model in which MET-dependent invasion of intestinal epithelial cells by *L. monocytogenes* rapidly engages RIN1, and RIN1-GEF activity is required for efficient entry (Figure 7). At later time points, however, RAB5 activation by RIN1 facilitates *L. monocytogenes* clearance from the cell by driving the fusion of bacteria-loaded phagosomes with degrading lysosomes. Even a partial escape from this bactericidal response can provide the pathogen an opportunity to replicate and spread to adjacent cells. Somewhat surprisingly, although RAB5 has been implicated in formation and turn over of tight junctions (42), RIN1 alterations had no apparent effect on the stability of cell-junctions.

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Future studies should evaluate the potential effect of other RAB5 regulators on cell junction stability during *L. monocytogenes* infection.

InlB-dependent invasion by *L. monocytogenes* is particularly relevant during the establishment of fetoplacental listeriosis and for traversing the blood brain barrier (14, 46). The pronounced and multi-stage contributions of RIN1 during *L. monocytogenes* infection of an intestinal epithelium model system strongly implicate RIN1 as a host cell factor for *in vivo* infections by *L. monocytogenes*. We note that numerous other bacteria utilize RAB5 to enter host cells, or they modulate RAB5 activity while directing pathogen-containing vesicles to cellular compartments favorable for pathogen replication and propagation (9). A few pathogens encode their own GEF and/or GAP activities for modulating small-GTPases (2, 47), but most appear to rely on host cell components. Based on our findings, RIN1 should be considered as a potential host cell factor for this larger group of pathogens.

The increased incidence of listeriosis in immunecompromised individuals and pregnant women underscores the need for early treatments that effectively prevent the occurrence of septicemia, bacterial meningitis, spontaneous abortion or stillbirths (48). This necessitates the identification and validation of host cell regulatory proteins required during pathogen infection. Based on its multiple levels of involvement at vulnerable stages of infection, RIN1 would appear to be a promising candidate for this approach. Finally, a thorough understanding of the host proteins that mediate internalization and trafficking is critically important for developing *L. monocytogenes* as a tumor vaccine vector (49).

MATERIALS AND METHODS

Expression constructs

All RIN1 expression constructs were made in lentivirus vectors. RIN1 wild type, RIN1^{E574A} and RIN1^{QM} in pM4-blast^{R} vector (50), (37). Lentivirus generation and transduction were performed as previously described (26). For stable Rin1 knockdown, mRin1 shRNA (Sigma Aldrich) in pM4-blast^{R} was used.

Cell culture and reagents

HeLa cells and rat Intestine Epithelial Cells (IEC-18 and IEC-6 Cdx2, from Enrique Rozengurt and James Sinnett-Smith, UCLA Dept. Medicine) (35) were cultured in DMEM (Media Tech) with 10% Fetal Bovine Serum (Hyclone) and 1% Penicillin Streptomycin (Invitrogen). HeLa cells stably expressing M4-blast^{R} constructs were established by lentivirus infection followed by selection with 4 μg/ml blasticidin (Invitrogen). Lentivirus expressing LC3-EGFP construct for autophagy was obtained as a kind gift from Dr. Jeff F. Miller's laboratory (UCLA). The virus was titered to an MOI=10 and cells were selected for infection using 2 μg/ml puromycin. 10 μg/ml fibronectin (Sigma Aldrich) or 100 μg/ml collagen (Sigma Aldrich) were used to coat the culture dishes and cover slips as indicated. *L. monocytogenes* strain 10403S was used in most experiments performed. EGDe strain was used to compare murinized *L. monocytogenes* (from Dr. Sarah D'Orazio, U. Kentucky College of Medicine) with the matched wild type strain. GFP-*L. monocytogenes* strain

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10403S (from Dr. Jeff F. Miller, UCLA) was used to observe co-localization with dextran. *L. monocytogenes* strains were grown at 30°C.

Gene Silencing

HeLa cells with stable silencing of RIN1 were established by infection with pLKO.1-shRIN1-puro^R and selection with 2 μg/ml puromycin (Invivogen) (51). IEC-18 and IEC-6 Cdx2 cells were subject to stable silencing of Rin1 using Rin1 shRNA obtained from PLKO-mRin1 1680 shRNA-puro^R vector (Sigma) and cloned into pM4-Blast^R with a U6 promoter. The shRNA sequence is: CCGGCTCCTGTTAGAAGCTGAGTATCTCGAGATACTCAGCTTCTAACAGGAGTTTTTG.

Expression levels were evaluated by western blotting.

Gentamicin Protection Assay

L. monocytogenes (10403S or EGDe) invasion was measured by Gentamicin protection assays. 0.75×10^6 cells were plated on fibronectin coated 6-well plates. Cells were infected with the indicated MOI of *L. monocytogenes* for 60 minutes at 37°C, 5% CO_2. Extracellular bacteria were killed for one hour with 150 μg/ml gentamicin (Sigma Aldrich) at 37°C, 5% CO_2. Cells were trypsinized and lysed in 0.1% Triton, in the presence of DNase and appropriate dilutions of the lysate were plated on LB-Agar plates. Agar plates were stored at 30°C for about 20 hours before colony counts were obtained. For replication assays, cells were transferred to 10 μg/ml gentamicin following killing of extracellular bacteria and lysed at 3 hours or 6 hours post-infection in order to obtain colony counts as described above. All statistical analyses were performed using Student's t-test.

Plaque Assays to Assess Cell-Spread

To measure *L. monocytogenes* cell spread, wild type IEC-18 or IEC-6 Cdx2 cells were infected with *L. monocytogenes* at MOI=50 for one hour as described above. Extracellular bacteria were killed using 150 ug/ml gentamicin. Cells were trypsinized and added to a confluent monolayer of wild type or mutant cells at a ratio of 1:10 in the presence of 10 μg/ml gentamicin, followed by a three hour incubation at 37°C, 5% CO_2. The cells were washed and an overlay of 0.7% Agarose, 1X DMEM and 10 μg/ml gentamicin was laid on the monolayer. After 48 hours of incubation at 37°C, plaques were visualized and plaque areas measured using ImageJ software (NIH). All statistical analyses were performed using Student's t-test.

Preparation of Whole Cell Lysates for Immunoprecipitation and Immunoblotting

HeLa, IEC-18 or IEC-6 Cdx2 cells were lysed in NP-40 lysis buffer (150 mM NaCl, 50 mM Tris pH 8.0, 1% NP-40) containing 1 mM PMSF, 10 μg/ml leupeptin, 1 μM pepstatin and 1 mM sodium orthovanadate (Sigma Aldrich). 50 ug of the lysates were prepared in 5X SDS Laemmlli buffer and boiled for 5 minutes, before loading on an 8% SDS PAGE.

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Immunoprecipitation, immunoblotting and immunofluorescence

Antibodies used for immunoblotting and their sources were- Tubulin (Sigma Aldrich, #T6074-200UL), human RIN1 (Mouse mAb, clone #C9E11, Colicelli lab, AbPro), mouse Rin1 (mouse mAb, Colicelli lab), pY^{36} RIN1 (31) (Rabbit pAb, Colicelli Lab, Biosource International), Rabex-5 (Sigma Aldrich, #R5405), RIN2 (Genetex, #GTX117153), sheep-anti-mouse-HRP (Amersham Biosciences, #NA931), goat-anti-rabbit-IRDye 800 (Li-Cor Biosciences, #926-32211) and goat-anti-mouse-IRDye 680 (Li-Cor Biosciences, #926-32220).

For immunoblotting, proteins were transferred to Nitrocellulose membranes overnight. The membranes were blocked with 5% milk in PBST (0.1% Tween-20) followed by incubation with primary and secondary antibodies at room temperature. The membranes were washed with PBST between incubations. Imaging and quantification were done using a Li-Cor Odyssey scanner.

For immunoprecipitation experiments, mRin1 (mouse mAb, clone B9A12, Colicelli Lab) and RIN2 (Rabbit pAb, Colicelli Lab) antibodies were used. Cells were lysed in NP-40 lysis buffer containing protease and phosphatase inhibitors. Lysates were incubated with the antibody and protein A- agarose (Fisher) beads overnight at 4°C. Following incubation, beads were washed in lysis buffer, boiled in SDS sample loading buffer and run on 8% SDS PAGE, followed by immunoblotting.

The antibody sources for immunofluorescence experiments were- RIN1 (Mouse mAb, clone #C9E11, Colicelli lab, AbPro), RIN1 (Sigma, #HPA035491), *L. monocytogenes* (Abcam, #ab35132), pan-RAB5 (Abcam, #ab18211), ZO-1 (Zymed, #61-7300), Lamp1 (Santa Cruz, #SC-19992), phalloidin-rhodamine 0.2 uM (Invitrogen), 70,000 MW dextran-Texas red, lysine fixable (Invitrogen, #D1864), E-Cadherin (BD Transduction Laboratories, #610181), goat-anti-rabbit Alexa-Fluor 568 (Invitrogen, #A11036) and goat-anti-mouse Alexa Fluor 647 (Invitrogen, #A21245), goat-anti-mouse Alexa Fluor 488 (Invitrogen, #A11001), goat-anti-rat Alexa Fluor 647 (Invitrogen, #A21247). For imaging experiments, 0.75×10^6 cells were plated on fibronectin or collagen coated cover slips overnight. The cells were infected with *L. monocytogenes* as indicated, followed by killing of extracellular bacteria with 150 ug/ml gentamicin and incubation for the described times at 10 ug/ml gentamicin. The cells were washed and fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton-X-100 detergent, quenched in 50 mM NH_4Cl and blocked in 10% goat serum (Gibco). For RAB5 staining experiments, cells were fixed and permeabilized in methanol for 5 minutes at −20°C and the rest of the procedure was followed as specified above. Cells were then incubated with primary and secondary antibodies at room temperature. Fluorescently labeled phalloidin was added along with the secondary antibody staining. DAPI staining was performed for 5 minutes following secondary antibody incubation. Confocal microscopy (Zeiss Pascal) was used to image the cells.

Microscopy

Fixed and stained cells were examined on a laser scanning confocal microscope (Axiovert 200M, Carl Zeiss LSM 5 Pascal) or spinning disc confocal microscope (Yokagawa CSU-22

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spinning disc, Hamamatsu C9100-13 EMCCD camera, Zeiss Aiovert 200M). Cells were imaged with a plan/neofluar 100x oil lens, NA 1.3 (Carl Zeiss) and 8-bit digital images were captured using a cooled charge-coupled device (transmitted light channel for lsm5 camera, Zeiss). LSM 5 Pascal software (release version 4.2 sp1) was used to process the images. All images were quantified in a double-blinded fashion and all statistical analyses were performed using Student's t-test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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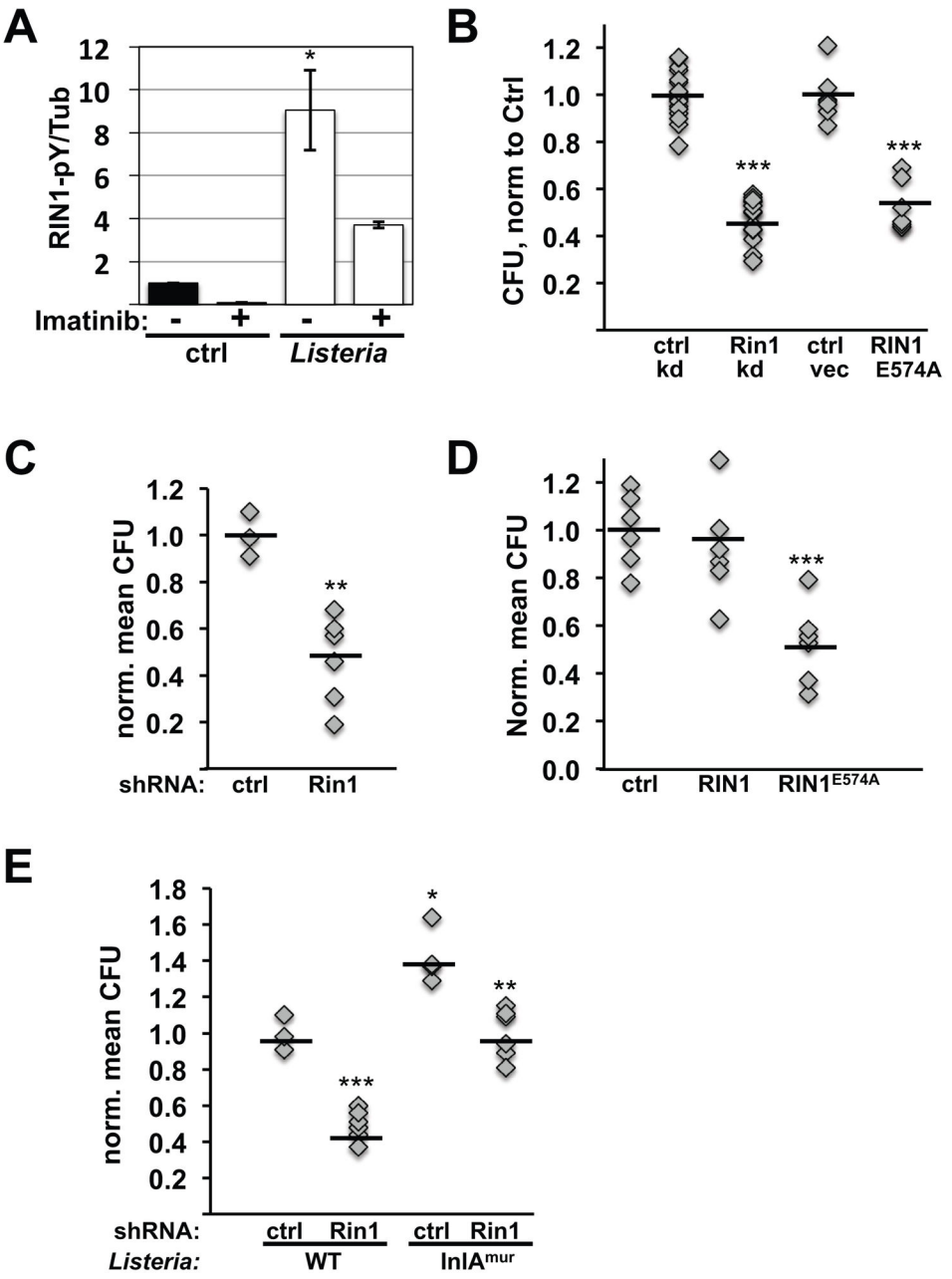

FIGURE 1. RIN1-to-RAB5 signaling is required for efficient *L. monocytogenes* entry into epithelial cells

A. HeLa cells were infected with *L. monocytogenes* (500 MOI; 2.5 min) and lysates probed with anti-RIN1pY^{36}, anti-RIN1 and anti-tubulin (Tub). A short time and high MOI were used to detect the transient phosphorylation that occurs upon infection. RIN1pY^{36} levels were normalized to total RIN1 and tubulin (Figure S1). The ABL tyrosine kinase inhibitor imatinib was used at 5μM. n=2 experiments, p=0.03. **B.** Control, Rin1 shRNA (Rin1 kd) or RIN1^{E574A} transduced IEC-18 cells were infected at 50 MOI for 1 hour and extracellular bacteria killed using gentamicin. Cell lysate dilutions were plated to determine colony-forming units (CFUs) (ctrl-kd n=17, Rin1-kd n=16, 4 independent experiments,

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$p=1.8\times10^{-16}$; ctrl-vec and $RIN1^{E574A}$ n=6, 2 independent experiments, $p=2.6\times10^{-6}$). **C.** Invasion assay (as in B) using IEC-6 Cdx2 cells transduced with control or Rin1-shRNA. Cell lysate dilutions were plated to obtain CFUs (ctrl n=3, Rin1-kd n=6, p=0.003). **D.** IEC6-Cdx2 cells transduced with control, RIN1 or $RIN1^{E574A}$ vectors were subjected to invasion assays as in B and CFUs determined (ctrl n=9, RIN1 and $RIN1^{E574A}$ n=10, 2 independent experiments, $p=2.3\times10^{-6}$). **E.** Control or Rin1-shRNA transduced IEC-6 Cdx2 cells were infected (10 MOI) with wild type or $InlA^{mur}$ *L. monocytogenes* and extracellular bacteria killed using gentamicin. Cell lysate dilutions were plated to obtain CFUs (ctrl n=4, Rin1-kd n=6; wild type *L. monocytogenes* $p=6.7\times10^{-5}$, $InlA^{mur}$ p=0.002). $InlA^{mur}$ *L. monocytogenes* invaded more efficiently than wild type (p=0.009). Note: Rin1=rat protein; RIN1=human protein. (*$p \leq 0.05$, **$p \leq 0.005$, ***$p \leq 0.0005$)

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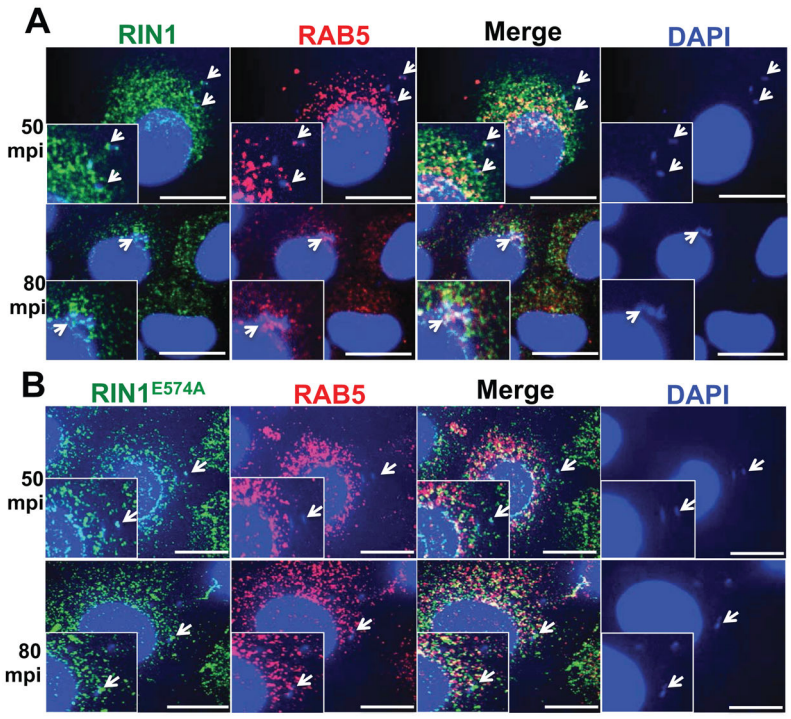

C

Percent of bacteria co-localized with RAB5		
transfected gene	50 mpi	80 mpi
RIN1	82.3 (n=56)	68.8 (n=45)
RIN1^{E574A}	18.7 (n=37)	33.0 (n=71)

FIGURE 2. RIN1 and RAB5 are recruited to *L. monocytogenes* during invasion

A and B. IEC-6 Cdx2 cells transduced with RIN1 (**A**) or RIN1^{E574A} (**B**) were plated on collagen, infected with *L. monocytogenes* at MOI 100 for 50 or 80 minutes at 37°C. The cells were washed thoroughly, fixed and stained for RIN1 (green) and RAB5 (red). Host cell nuclei and *L. monocytogenes* were visualized by DAPI (blue). Arrows show RAB5 and RIN1 recruitment to *L. monocytogenes.* Insets show magnified versions of the regions pointed by the arrows. Scale bar: 10 μm. **C.** Quantification of percentage total bacteria co-localizing with RAB5 at each time point in RIN1 and RIN1^{E574A} IEC-6 Cdx2 cells, representative of two independent experiments.

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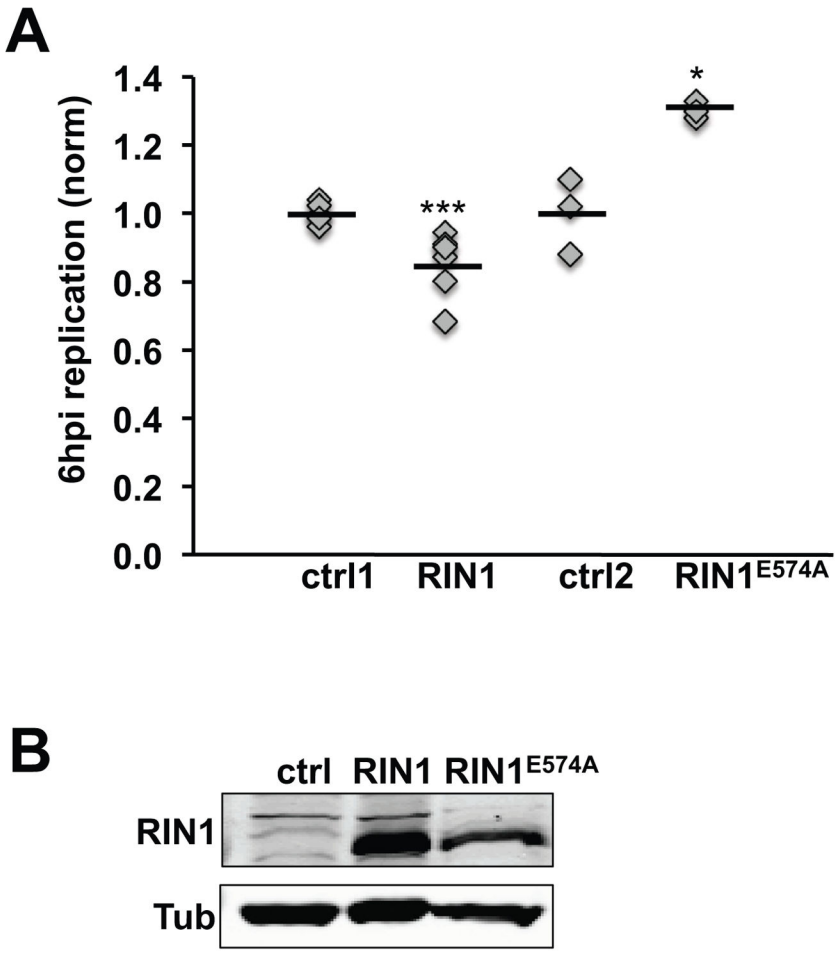

FIGURE 3. RIN1-to-RAB5 signaling inhibits *L. monocytogenes* replication

A. IEC-18 cells transduced with control, RIN1 or RIN1^{E574A} were infected with *L. monocytogenes* (50 MOI) after which extracellular bacteria were killed with gentamicin. At 1 hour post-infection (hpi) cells were either lysed or incubated in medium with low gentamicin before lysing at 6 hpi. Dilutions of the lysates were plated to obtain CFUs. Intracellular replication was measured as the fold change in CFU (6 hpi vs. 1 hpi) (ctrl1 and RIN1: n=6, $p=1\times10^{-30}$; ctrl2 and RIN1^{E574A}: n=3, p=0.01). Values are representative of two independent experiments. **B.** Immunoblot evaluation of RIN1 expression. (*$p \leq 0.05$, **$p \leq 0.005$, ***$p \leq 0.0005$)

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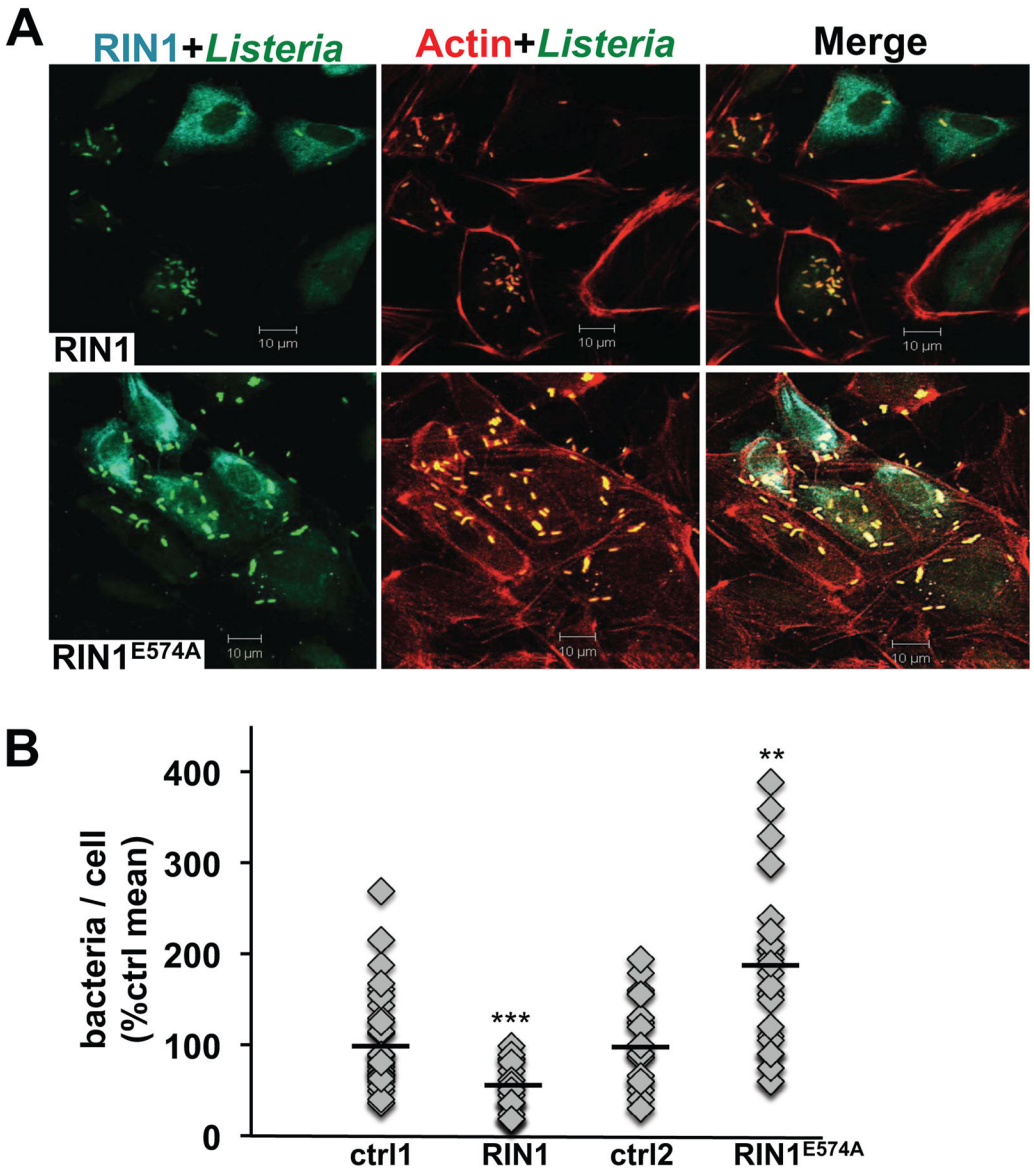

FIGURE 4. RIN1 inhibits phagosome escape

A. IEC-18 cells transduced with control, RIN1 or RIN1^{E574A} vector were infected with *L. monocytogenes* (20 MOI) and extracellular bacteria killed with gentamicin, then incubated in medium with low gentamicin for 2 hours. Fixed cells were stained for RIN1 (cyan), actin (red) and *L. monocytogenes* (green). Scale bar: 10 μm. **B.** Quantified total bacteria co-localized with actin per cell (actin coating correlates with cytoplasmic localization). Values are representative of two independent experiments and normalized to percent of the control mean. (ctrl1 n=133, RIN1 n=81, $p=4 \times 10^{-6}$, ctrl2 n=70, RIN1^{E574A} n=62, p=0.0012; *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.0005)

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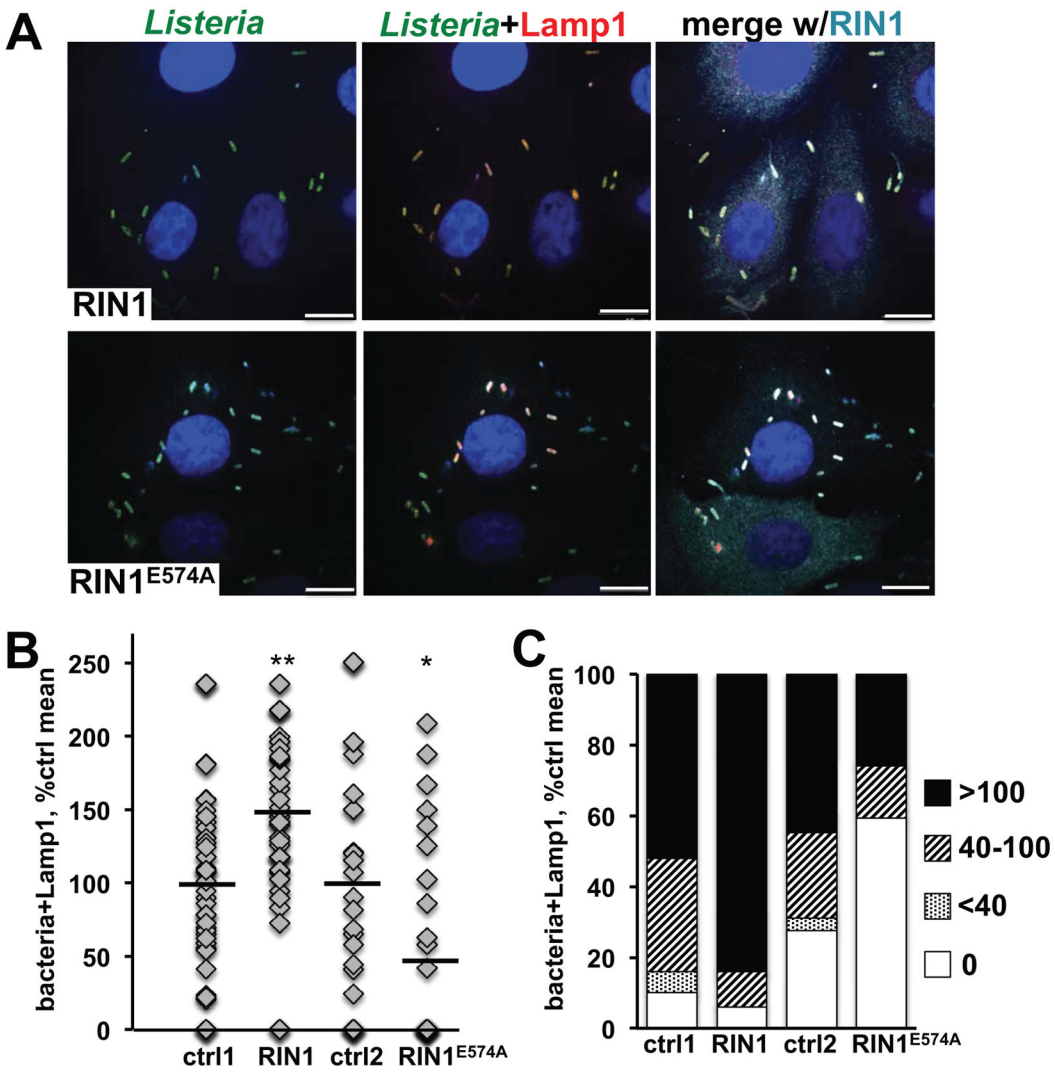

FIGURE 5. RIN1-to-RAB5 signaling promotes lysosome fusion

A. IEC-18 cells transduced with control, RIN1 or RIN1^{E574A} vectors were infected with *L. monocytogenes* (20 MOI) and extracellular bacteria killed with gentamicin followed by incubation in medium with low gentamicin for 1.5 hours. Fixed cells were stained for RIN1 (cyan), *L. monocytogenes* (green) and Lamp1 (red). Nuclei were stained with DAPI (blue). Scale bar: 10 μm. **B.** and **C.** Quantification of lysosome-localized bacteria in infected IEC-18 cells. **B.** Each point is the percentage of total bacteria co-localizing with Lamp1 in a given field. **C.** Each bar is the percent co-localization in distribution categories. Values are representative of two independent experiments and normalized to percent of control mean (ctrl1 n=147, RIN1 n=124, $p=9\times10^{-4}$, ctrl2 n=161, RIN1^{E574A}=105, p=0.03; *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.0005)

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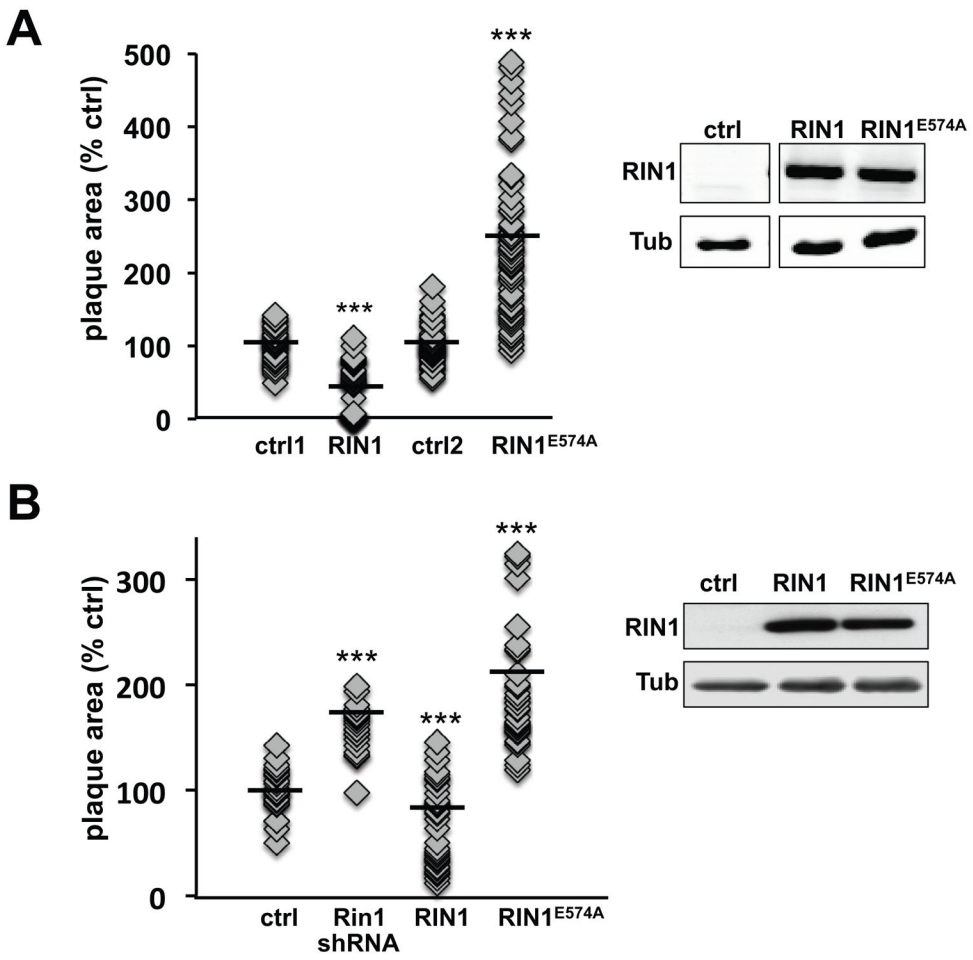

FIGURE 6. RIN1 inhibits bacteria spread between cells

A. IEC-18 cells infected with *L. monocytogenes* (50 MOI) were trypsinized and transferred to a monolayer of IEC-18 cells transduced with control, RIN1 or RIN1^{E574A} vectors in low gentamicin medium and overlaid with DMEM+0.7% agarose. After 48 hours plaque areas were measured using ImageJ (NIH). Each point is a single plaque area normalized to percent of control mean (ctrl1 n=55, RIN1 n=50, p=3×10^{-19}; ctrl2 n=65, RIN1^{E574A} n=75, p=2×10^{-19}). Values were obtained from two independent experiments. RIN1 expression confirmed by immunoblot (right). **B.** Plaque assay as described in A but using IEC-6 Cdx2 cells transduced with control, Rin1-shRNA, RIN1 or RIN1^{E574A} vector (ctrl n=29, Rin1-kd n=21, p=7.1×10^{-13}, RIN1 n=36, p=0.0001, RIN1^{E574A} n=37, p=1.9×10^{-12}). Values were obtained from two independent experiments. Expression confirmed by immunoblot (right). (*p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.0005)

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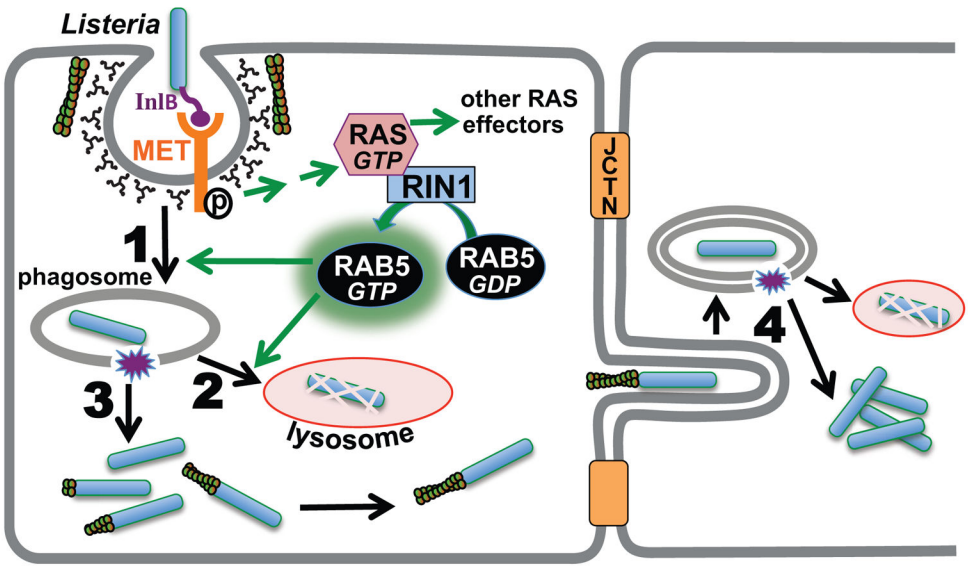

FIGURE 7. RIN1 is a RAB5 activator that controls the course of *L. monocytogenes* infection

InlB binding to MET initiates clathrin-mediated invasion while also triggering activation of RAS and its effectors. RIN1 activates RAB5 by guanine nucleotide exchange. RAB5 facilitates *L. monocytogenes* internalization (1) but also promotes the fusion of phagosome-encased bacteria with lysosomes (2). This step clears bacteria before its escape and replication in the cytoplasm (3) and blocks spread to adjacent cells (4). Disrupting RIN1 function (silencing or GEF-interfering mutant) lowers invasion efficiency, but impedes the bactericidal response.