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The RAS Effector RIN1 Modulates Endocytosis via Activation of RAB5 GTPases and ABL Tyrosine Kinases

A dissertation submitted in partial satisfaction of the requirements

for the degree Doctor of Philosophy

in Biological Chemistry

by

Kavitha Balaji

2014

ABSTRACT OF THE DISSERTATION

The RAS Effector RIN1 Modulates Endocytosis via Activation of RAB5 GTPases and ABL Tyrosine Kinases

by

Kavitha Balaji

Doctor of Philosophy in Biological Chemistry

University of California, Los Angeles, 2014

Professor John Colicelli, Chair

Activation of cell surface receptors by ligands leads to the recruitment of a number of signaling molecules that mediate cellular responses. RAS-GTPases are signaling proteins that bring about major changes in cell growth, differentiation, gene expression and cytoskeletal organization upon receptor stimulation. The RAS effector RIN1 is involved in receptor trafficking and signaling. RIN1 is recruited to the receptor following ligand stimulation, but the function of its individual effector domains in this process remained uncharacterized. RIN1 acts as a guanine nucleotide exchange factor (GEF) for the small GTPase RAB5, which is involved in the formation of early endosomes. RIN1 can also directly bind and activate ABL tyrosine kinases, which have been implicated in cellular actin remodeling and receptor trafficking. RIN1 also forms a complex with STAM, an ESCRT complex protein, which favors maturation of receptor-containing endosomes. As all of RIN1's known effectors are involved in modulating receptor trafficking, we resorted to examine the role of individual domains in receptor internalization and fate. Chapters two and three debrief the mechanisms by which RIN1 effectors

function in an integrated manner to regulate receptor trafficking and corresponding cellular responses.

Using domain-specific mutants of RIN1, we show that the RAB5 and ABL signaling pathways mediate opposing functions, maintaining a balance in the route and rate of receptor internalization. RAB5 promotes actin remodeling, EGF induced macropinocytosis and migration, facilitating receptor degradation. ABL kinases, on the other hand, prevent receptor degradation and migration towards EGF. Our study also shows that regulation of RIN1 localization by the 14-3-3 proteins is a major factor in determining signaling intensity. We characterized a novel binding partner of RIN1, namely, BIN1, a BAR domain protein involved in membrane bending.

Intracellular pathogens invade host cells by exploiting host cell surface receptors and signaling pathways. *Listeria monocytogenes* in a food-borne pathogen that binds host MET to invade epithelial cells and modulates RAB5 GTPases at several stages of the infection. The effect of host RAB5 regulators on pathogenesis remained uncharacterized. We analyzed the role of RIN1, a major RAB5-GEF in epithelial cells, in the process of bacterial invasion and spread (chapter four). This study leads the way to determining the mechanism of pathogenesis of several pathogens that depend on host RAB5-GTPases for effective infection progress. Finally, chapter five discusses the significance of our studies from a therapeutic viewpoint in the context of cancers and infectious diseases.

The dissertation of Kavitha Balaji is approved.

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2014

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questions and helping me set up the system in our lab. Dr. Jeff F. Miller and Todd are co-authors in our upcoming publication and I would like to acknowledge them for their inputs. Dr. Enrique Rozengurt and Jim provided us cell lines that helped us perform all the bacterial infection assays. Dr. Sarah D'Orazio gave us prompt support and guidance with the food-borne model of infection and also shipped us several useful bacterial strains. Dr. Kenneth Bradley, Eugene and Charlie have been wonderful collaborators, giving us an opportunity to be a part of their project and helping us with our projects. I would like to acknowledge all the scientific members of Biological Chemistry and other departments for providing us timely support by lending equipment and reagents. Finally, the BC business office, especially Judy, has been extremely helpful with placing purchase orders and tracking them for us.

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KAVITHA BALAJI- BIOGRAPHICAL SKETCH

Summary
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(Expected, June 2014)
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Balaji K, French CT, Miller JF and Colicelli J. ***The RAB5 GEF RIN1 Regulates Multiple Stages in* Listeria monocytogenes *Infection*** (manuscript in revision)

Balaji K, Mooser C, Janson CM, Bliss JM, Hojjat H and Colicelli J. ***RIN1 Orchestrates the Activation of RAB5 GTPases and ABL Tyrosine Kinases to Determine EGFR Fate.*** Journal of Cell Science, 2012, Dec 1; 125(23): 5887-96

Balaji K and Colicelli J. ***RIN1 Regulates Cell Migration through RAB5 GTPases and ABL Tyrosine Kinases.*** Communicative and Integrative Biology, 2013, Sept/Oct; 6(5): e25421

Gillespie E, Ho C, Balaji K, *et al.****, A Selective Inhibitor of Endosomal Trafficking Pathways Exploited by Multiple Toxins and Viruses***. Proc Natl Acad Sci U S A. 2013, Dec 10; 110(50): E4904-12

Research Projects
Role of the RAS effector RIN1 in *Listeria monocytogenes* pathogenesis and host-response
(June 2012-current)
Examined the role played by RIN1 and its effectors in regulating *Listeria monocytogenes* entry and cell-cell spread. **Principal Investigator:** Dr. John Colicelli, Professor, UCLA

Role of RIN1 in EGFR trafficking, signaling and actin remodeling (June 2010-July 2012)
The project uncovered specific roles for RIN1 effectors in EGF dependent actin remodeling and receptor fate determination. **Principal Investigator:** Dr. John Colicelli, Professor, UCLA

Characterization of MAPL loss of function in *D. melanogaster* (April-June, 2010)
Tested and mapped imprecise excision of the MAPL gene (created in *D. melanogaster)*. Set up crosses in order to obtain transgenic strains of *D. Melanogaster* and balance them.
Principal Investigator: Dr. Ming Guo, Professor, UCLA

Testing zinc finger nucleases against the beta-globin gene (January-March, 2010)
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Effect of RIN1 on ABL substrate specificity profile (September-December, 2009)
Tested the processive phosphorylation model of ABL kinase using RAD51 as the substrate. Purified wild type and mutant constructs of RAD51 and used *in vitro* kinase assays to test the working model. **Principal Investigator:** Dr. John Colicelli, Professor, UCLA

Characterization of microRNA-7 over-expressing stable clones of HeLa cells (December 2008- May 2009), Indian Institute of Technology, Madras, India
Studied the effect of miR-7 on chemosensitivity of HeLa cells.

Role of miRs in cancer initiation and progression (May–July, 2008)
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Summer research fellowship (national level), 2007, by Indian Academy of Sciences, Bangalore.

CHAPTER 1. INTRODUCTION

1.1 CELLULAR COMMUNICATION

Communication: a fundamental to survival

Communication is fundamental to survival. Microorganisms, animals, birds, plants and human beings communicate with one another in different ways, albeit with two fundamental components to communication: reception of cues or signals leading to a response.

Cellular responses to environmental cues

Cells receive cues from the external environment, which are then transmitted to intercellular compartments where a cascade of events eventually leads to a change at the molecular level. This basic communication is absolutely essential for all the basic metabolic functions including growth, respiration, nutrient-absorption, building and breakdown of energy, reproduction, secretion of hormones and catalysis by enzymes.

The communication between a cell and its environment occurs through surface proteins called **receptors**.

1.2 RECEPTORS

Cell surface receptors usually span the outer plasma membrane of the cell and have an extracellular component that binds to their cognate ligand(s) and a cytosolic component that recruits several downstream signaling molecules from inside the cell (1). The signaling cascades downstream of receptor activation lead to changes in cell growth, proliferation, division and gene-expression. Cell surface receptors have different functions downstream of ligand binding. Some function as adaptors, leading to a conformational change in the receptor structure, for example, the G-protein coupled receptors (2). Certain receptors function by transporting

molecules and ions across the cell membrane. These receptors either function as a gated channel like the sodium-proton pump (3), or actively bind and transport the cargo, for example, the transferrin receptor (4).

Receptor Tyrosine Kinases

Receptors that possess phosphorylation (kinase) activity to transfer a phosphate group to tyrosine residues of their substrate proteins are termed Receptor Tyrosine Kinases (RTKs). For example, the growth factor receptors such as the Epidermal Growth Factor Receptor (EGFR) and Hepatocyte Growth Factor Receptor (HGFR) are RTKs (5). Binding of growth factor receptors to their respective ligands, for example, EGFR to EGF, Amphiregulin or TGF and HGFR to HGF result in specific cellular responses depending on the ligand and the local concentration of the ligand (6). Following binding of the ligand, the receptor is internalized into the cell by a process called **endocytosis**, following which the receptor is taken through a series of trafficking vesicles to either be degraded or recycled. The fate of the receptor following internalization depends on the ligand, the concentration, activation of specific signaling molecules downstream of the receptor signaling, and so on (6, 7).

1.3 ENDOCYTOSIS

Endocytosis is the process of internalizing cargo into a cell. There are several different mechanisms by which cells can endocytose growth factors, nutrients, salts and other foreign materials (8).

Mechanisms of endocytosis

Clathrin mediated endocytosis (CME) is the predominant mode of internalization of most cell surface receptors, some toxins and plasma membrane components (9). CME involves clathrin, that is recruited to the membrane by the adaptor proteins AP-2 and epsin, which also

serve to bind the cargo proteins, recruiting them to the clathrin coated pits (10, 11). Besides clathrin adaptors, the internalization is dependent on the formation of a clathrin lattice, membrane-bending BAR domain proteins (12) (endophilin and amphiphysin), actin nucleators (ARP2/3), actin-binding proteins (WAVE or WASP complexes and cortactin) (13-16) and dynamin-GTPase mediated pinching of membrane (17). Clathrin mediated endocytosis of growth factor receptors has been shown to favor recycling of receptors downstream of ligand stimulation as against degradation (7). A large number of pathogens have been demonstrated to exploit CME to enter host cells (15, 18-21). The process is also dependent on RAB5 GTPases and signaling molecules such as SRC that mediate phosphorylation events on clathrin (22-26).

Caveolin is an endocytic protein that mediates **caveolar endocytosis**. Caveolin and its adaptor proteins localize in specific membrane domains containing lipid rafts rich in cholesterol (27, 28). Endocytosis via caveolae has been shown to be a major internalization mechanism for receptors, GPI anchored proteins, extracellular ligands, toxins and a few viruses (7, 29-33). Transcytosis across endothelial barriers is one of the major mechanisms utilizing caveolin-mediated endocytosis (34). Caveolin-mediated internalization depends on the GTPase dynamin, actin, RAB5 and cholesterol (31, 35-37). Caveolae also function as signaling platforms for several proteins that are associated with these structures (38, 39). Flotillin-mediated endocytosis resembles caveolar endocytosis, but seen as distinct domains from caveolar pits. This mechanism of internalization is involved in the uptake of GPI-anchored proteins and some toxins (40-42).

Some GPI anchored proteins are internalized via a different cholesterol dependent pathway that by passes RAB5-positive early endosomes and localize in **GPI-anchored- protein enriched early endosomal compartment** (GEEK) (43). There are other pathways, which are specific to certain receptors, such as Il2Rβ, FcεRI and some adeno-associated viruses. These

mechanisms are clathrin-independent and dynamin dependent, but co-localize with CME endosomes (43-48).

Macropinocytosis or fluid phase endocytosis involves bulk internalization of cargo from the surrounding extracellular matrix. This is achieved by global remodeling of the actin cytoskeleton inside the cell that leads to the formation of extensive membrane ruffles, which close-in on themselves or fuse with the plasma membrane (9). This process results in uptake of large volumes of fluid and other particles in the extracellular matrix. This process depends on Rho GTPases (RAC1), PAK, membrane-cholesterol and actin polymerization (49-51). PI3K and RAS can also cause macropinocytosis by activating RAC1 (mostly dynamin independent) (49, 52-56). Recently, we showed that a RAS effector, RIN1, promotes EGF dependent macropinocytosis via its effector RAB5 (57, 58).

Circular dorsal ruffles (CDRs) are formed in certain types of macropinocytic events, usually following stimulation by growth factors like EGF (59-62). The ruffles form in membrane microdomains rich in cholesterol, where EGFR localizes and is rapidly internalized (63, 64). RAB5 has been implicated as a major player in this process (63). A recent study showed that RAS mediated macropinocytosis of proteins is a major route of amino acid supply for protein synthesis to keep up with the intense metabolic needs of cancer cells, giving leads to the significance of macropinocytosis in cancer (65).
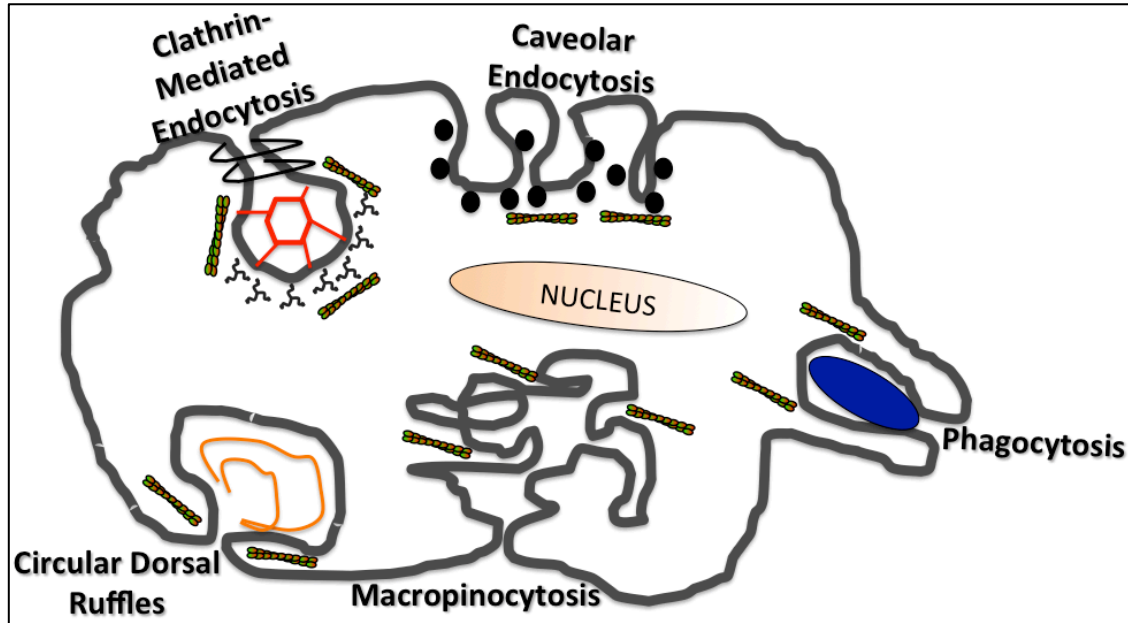
Figure 1. 1 Mechanisms of Endocytosis

Endocytosis of surface receptors can take any of the following major routes to internalization: clathrin-mediated endocytosis, caveolar endocytosis, phagocytosis, macropinocytosis and circular dorsal ruffles. Actin remodeling plays a significant role in all types of endocytosis. There are additional mechanisms that also take part in internalization. The route of internalization depends on the receptor (the cargo), the cell type and the proteins stimulating the internalization process.

1.4 TRAFFICKING OF RECEPTOR TYROSINE KINASES

Once a cell surface receptor has been internalized, it can either be trafficked through the early and late endosomes to the lysosomes, resulting in degradation or recycled back to the plasma membrane from the early endosomes.

Ligand binding and activation of RTKs (EGFR)

In the specific case of receptor tyrosine kinases, for example EGFR, which this research study focuses on, ligand binding typically leads to dimerization and clustering of receptors in a specific membrane domain (66). EGFR also forms heterodimers with other EGFR family members such as ERBB2 (67-69). The receptors that contain an active kinase domain in the

cytosolic tail then undergo auto- and trans-phosphorylation on several tyrosine residues (70-72). These phospho-tyrosines serve as docking sites for several adaptors and signaling molecules that contain an SH2- domain. These include but are not limited to GRB2, CBL, SRC and ABL (73-78). GRB2 is an adaptor protein that recruits SOS, a GEF for the small GTPase RAS and brings the two molecules together, causing RAS activation (79). GRB2 can also recruit CBL to the receptor by an SH3- proline-rich domain interaction (80). ABL is a non-receptor tyrosine kinase that has been implicated widely in actin remodeling both indirectly and by direct actin binding (81, 82). CBL is an E3-ubiquitin ligase and a critical regulator of RTK trafficking to lysosomes for degradation. Interaction with stimulated EGFR leads to tyrosine phosphorylation of CBL by the ABL tyrosine kinases (78, 80, 83-88). Besides its E3 ubiquitin ligase function, CBL recruits and regulates signaling proteins such as CRK, CRKL CAP, PI3K and VAV2 involved in cytoskeletal remodeling. Interactions with its binding partners occur through its SH3 domain and phosphorylated tyrosines (78, 89, 90).

Entry into early endosomes- role of RAB5

Upon recruitment of the adaptor molecules, the receptor is ubiquitinated and ubiquitin-interacting motif (UIM) containing proteins such as EPS15 and Epsin-1 actively recruit EGFR to clathrin coated pits (CCPs) (91-94). The CCPs are then pinched off to form clathrin- coated vesicles (CCVs) by the GTPase dynamin, followed by uncoating of the vesicles (17). Following this step, several membrane fusion events occur which are influenced largely by RAB GTPases and SNARE complex proteins (95-97).

Among the signaling molecules activated by EGFR is RAS. RAS in turn binds to its effectors such as b-RAF and PI3K, activating them and leading to a cascade of signal transduction events in the cell (98). RAS mediates activation of the small GTPase RAB5 through

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its effector RIN1 (RAS and RAB Interactor 1) (99). RAB5 mediates fusion of the vesicles to an EEA1-positive early endosome, a slightly acidic organelle that also serves as a signaling platform for a number of receptors, including EGFR (100-102).

Trafficking to late endosomes and lysosomes

At high concentrations of the ligand, specifically EGF, EGFR is directed from the early endosome to the late endosomes and lysosomes where the receptor is degraded (103). For degradation of the receptor, RAB5 positive early endosomes mature into RAB7 positive late endosomes (104, 105). The ESCRT complex proteins play a significant role in trafficking of the receptor from early endosomes to the lysosomes (106). ESCRT 0 proteins include HRS. The UIM of HRS interacts with the ubiquitin moiety of the cargo and the FYVE domain interacts with the membrane (107). STAM is a part of the ESCRT complex 1 that contains UIM and VHS domains to interact with the ubiquitin moieties on the cargo molecules and with HRS of the ESCRT0 complex respectively (108, 109). The ESCRT II complex consisting of VPS36, VPS22 and VPS25 (110, 111) in turn bridges ESCRT I with ESCRT III, and also contains domains to bind ubiquitin and the membrane. The ESCRT III complex includes VPS20, SNF7, VPS24 and VPS2 and forms a coiled coil structure to make inclusions from the membrane into the late endosome (112-114). At this stage, ubiquitin and other modifications are removed from the sorted cargo, which is concentrated in the intraluminal vesicle bodies. Finally, the ATPase VPS4 pinches off these vesicles to form the multivesicular bodies (MVBs). The ESCRT proteins are recycled once the cargo is enclosed in the MVBs, following the ATPase activity of VPS4 (115).

Finally, the MVBs fuse with the lysosomes that recruit LAMP1, leading to degradation of the cargo (the receptor) molecules, thus down regulating signaling through the receptor (116).

The vesicle transport processes are facilitated by actin and microtubules and fusion by RAB GTPases and SNARE complex proteins (117, 118).

High and low EGF concentrations: Implication on trafficking

The fate of internalized receptor from the early endosomes depends on the concentration of the ligand and the affinity of the ligand to the receptor, which determines the duration of signaling downstream of the growth factor receptor stimulation (6). The concentration of ligand also determines the route by which the receptor is internalized (7).

For example, in the case of EGFR, high EGF (~50-100 ng/ml) concentration has been shown to favor acute signaling, which is down regulated rapidly, followed by EGFR degradation. On the other hand, a low concentration of EGF (1-2 ng/ml) causes prolonged signaling and pronounced recycling of the receptor from the early endosomes (7). RAB4 and RAB11 GTPases are involved in the recycling process (119). Ligands such as TGF-α and Amphiregulin, which have relatively low affinities for EGFR, favor recycling of the receptor as against degradation (6).

1.5 RIN FAMILY PROTEINS

RAS and RAB Interactor 1 (RIN1)

RIN1 was identified as a mammalian cDNA that interferes with RAS function in yeast (120). RIN1 is enriched in the forebrain neurons and is expressed in moderate levels in epithelial cells (121). Its expression is regulated by the transcription factor SNAIL downstream of TGFβ signaling (121). RIN1$^{-/-}$ mice are viable but show neuronal defects. These mice show enhanced long-term potentiation and amygdala-dependent aversive memory (122, 123).

RIN1 domain functions and binding partners

The RAS-association (RA) domain of RIN1 directly binds and interacts specifically with

activated RAS proteins and competes with the RAS effector RAF1 (124). Stimulation of growth factor receptor (RTKs) results in activation of RAS. RIN1 is then recruited to the receptor through its SH2 domain (125) and to RAS by its RAS-association domain. Binding to RAS activates two of its effector functions, one of it being guanine nucleotide exchange factor (GEF) function to the small GTPase RAB5 (99). Activation of RAB5 facilitates receptor endocytosis and early endosome formation (101). The proline-rich and phospho-tyrosine domains of RIN1 can bind the SH3 and SH2 domains respectively of ABL and can activate the ABL tyrosine kinases (126). RIN1 interacts directly with and enhances the catalytic efficiency of the non-receptor tyrosine kinase ABL, resulting in ABL mediated phosphorylation on RIN1 Y_{36}. ABL regulates cytoskeletal dynamics by phosphorylating actin remodeling proteins and directly binding actin (127).

The RA domain of RIN1 also interacts with 14-3-3 proteins, which regulate localization (128). Through its proline-rich domain, RIN1 has been shown to interact with STAM upon EGF stimulation (129). STAM is a component of the ESCRT-1 complex, which mediates late stages of EGFR trafficking to lysosomes.

Functional studies on RIN1

Mutations in the GEF domain of RIN1 can inhibit early-endosomal fusion while over-expression causes enlargement of RAB5-positive endosomes and rapid internalization of stimulated epidermal growth factor receptor (EGFR) (130, 131). RIN1 engages stimulated EGFR through its SH2 domain by binding to a phosphotyrosine ligand on the receptor, and over-expression of RIN1 leads to down regulation of EGFR under high EGF conditions (125). RIN1 also regulates trafficking and signaling of other RTKs like MET, EPHA4 and IR by RAB5 activation (125, 132, 133).

Other RIN family proteins

The other mammalian RIN family proteins have conserved domain structure (SH2-PR-GEF-RA) and have been implicated in receptor trafficking and signaling. RIN2 has been implicated in trafficking of HGFR, integrin and E-cadherin and plays a role in cell adhesion by modulating activation of Rac1 (134, 135). Mutations in RIN2 have been implicated in connective tissue disorder (136-138). RIN3 is enriched in mast cells and plays a role in KIT endocytosis and signaling (139-141).
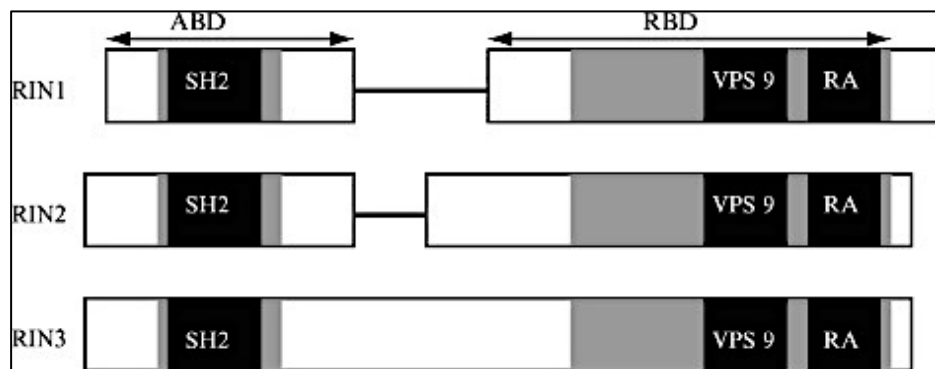
Figure 1. 2 The RIN Family Proteins

The RIN family proteins typically contain a RAS association domain (RA) for binding RAS and a VPS9 domain that acts as a GEF for RAB5. These constitute the RAS binding domain (RBD). The N-terminal of RIN1 can bind and activate the ABL tyrosine kinases and constitutes the ABL binding domain (ABD) (142).

RIN1 in growth factor receptor trafficking and signaling

Although there is ample evidence that RIN1 regulates growth factor receptor trafficking and signaling, the specific role of each of its domains and how their functions integrate has not been fully elucidated. Though the role of actin remodeling in clathrin-mediated endocytosis has been well studied in yeast, the contribution of cytoskeletal remodeling in mammalian cell endocytosis remains unclear. A part of this study focuses on studying trafficking of endogenous EGFR in HeLa cells stably expressing RIN1 or mutants that target specific domains of RIN1.

Chapter two deals with the study of effect of the major effectors of RIN1 in EGFR

internalization and trafficking. We used domain-specific mutants of RIN1, targeting specific interactions and functions to dissect out the effect on receptor internalization and trafficking. In summary, we show that RIN1 effectors RAB5 GTPases and ABL tyrosine kinases play differential roles in initialization of the receptor and regulating the fate of the internalized receptor. RIN1→RAB5 signaling promotes extensive growth factor- dependent actin remodeling, clathrin-independent internalization of the receptor and enhanced degradation of the receptor. This axis promotes fluid phase endocytosis or macropinocytosis of the receptor. RIN1→ABL signaling diminishes internalization of the receptor from the cell surface and enhances recycling of the receptor, indicating a possible clathrin-mediated mechanism of receptor entry into cells (58).

This study also characterized a novel binding partner of RIN1, namely BIN1, a BAR domain protein belonging to the family of amphiphysins, which is involved in membrane bending during endocytosis. BAR domain proteins are an essential component of clathrin-mediated endocytosis (12). RIN1-BIN1 interaction is dependent on the proline-rich domain of RIN1 and is enhanced upon EGF stimulation. Interestingly, RIN3 was shown to interact with BIN1 and also BIN2 to regulate KIT endocytosis in mast cells (139, 140). Overall, our study revealed that an intricate balance between the RIN1 effector functions determines the route of endocytosis of the receptor, the rate of internalization and the fate of the internalized receptor.

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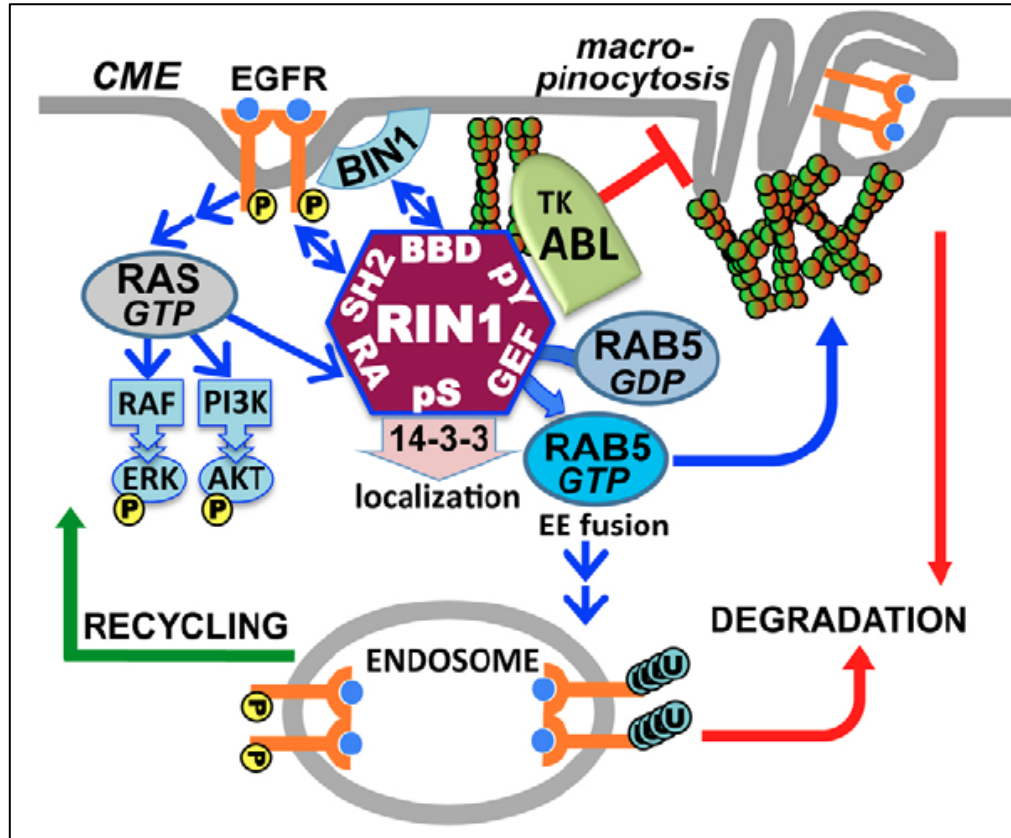
Figure 1. 3 A Model for Role of RIN1 in Growth Factor Receptor Endocytosis

RIN1 is activated by RAS downstream of growth factor stimulation. It in turn activates RAB5 GTPases and ABL tyrosine kinases. RIN1→RAB5 signaling promotes receptor internalization by macropinocytosis and degradation. RIN1→ABL signaling leads to protection of receptor from internalization and facilitates receptor recycling.

In chapter three, we followed-up on the above model and examined the physiological response of cells to specific RIN1 effector functions. We elucidated that RIN1→RAB5 signaling axis, which promotes macropinocytosis also enhances migration towards growth factor. On the other hand, the RIN1→ABL axis inhibits migration towards growth factor over 24 hours. Interestingly, expression of the RIN1^{QM} mutant that is dominant-negative for RIN1's activation of ABL leads to increased RAB5 activation (57).

This study is relevant in the context of a large number of cancers that RIN1 has been implicated. RIN1 expression has been positively correlated in melanoma, gastric

adenocarcinoma, bladder cancer and non-small cell lung cancer (143-146). It would be beneficial to understand the effector functions and specific signaling pathways downstream of RIN1 that play a role in these cancers, as a way to identify potential biomarkers and develop relevant therapeutics.

1.6 ENDOCYTOSIS OF PATHOGENS: SPECIAL CASE OF LARGE CARGO

Several viral and bacterial pathogens exploit host endocytic mechanisms to enter cells. The actin cytoskeleton convincingly plays a major role in most of these processes (147). Interestingly, besides the proteins produced by the pathogens to manipulate host processes, an increasingly large number of host proteins have been coming to light as being involved in the infection process of the pathogens.

Entry into host cells

Pathogens can enter cells using many different kinds of endocytic events that are a part of the host cell. For example, *Salmonella enterica Typhimurium* injects effectors to manipulate the host RHO GTPases to stimulate macropinocytosis and gain entry (148). Its effector SopE acts as a GEF for RAC1 and RAB5, inducing macropinocytosis (148-150). Certain viruses like the hepatitis C virus use clathrin-dependent endocytosis (20) while some others utilize the caveolin machinery to enter mammalian cells. Actin polymerization invariably plays a major role in all of these processes (151).

Clathrin dependent endocytosis of large cargo

Clathrin dependent endocytosis has been characterized in the event of entry of large cargo such as bacteria. It is intriguing that a few pathogens recruit the clathrin machinery as a scaffolding platform to exploit cellular mechanisms that is most favorable to bacterial infection.

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For example, upon *Listeria monocytogenes* infection, isolated clathrin coated pits form around membrane invaginations, instead of the canonical homogenous clathrin coats around the cargo. *L. monocytogenes* requires recruitment of clathrin heavy and light chains along with DAB2, myosin VI, HIP1R, dynamin, cortactin and actin to bring about actin polymerization and myosin induced movement for cellular entry (152). Similarly, enteropathogenic *Escherichia coli* (EPEC) use clathrin platforms to assemble actin pedestals that help them move outside the cell (19). In both these cases, clathrin serves to form isolated clathrin coated pits but not clathrin coated vesicles (15).

Manipulation of host trafficking machinery

Once inside the cells, pathogens manipulate the host trafficking machinery in several different ways to travel to their most favorable cellular compartment. One of the most common reasons for manipulating host vesicle trafficking is to evade the maturation of the pathogen containing vacuole (PCV) or the phagosome, preventing fusion with the host lysosomes (153, 154). Some pathogens can survive in the host lysosomes by altering recruitment of canonical lysosomal proteins (155). *Salmonella enterica Typhimurium* effector SopB is a lipid phosphatase that alters the lipid profile of the plasma membrane and the *Salmonella* containing vacuole (SCV) (156). This action of SopB recruits host RAB5 to the vacuole, which in turn brings the host PI3K VPS34 to the membrane (156). A few pathogens, for example *Legionella pneumophila,* also alter trafficking by modifying host RAB1 in different ways, to direct the PCV to trans-Golgi vesicles to replicate and survive in that compartment (157).

A common feature among all these pathogens is that they commandeer host RAB GTPases to alter trafficking of host vesicles (105). Thus, deciphering regulation of these RAB GTPases by the bacterium is essential to contribute to the understanding of the cellular and

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molecular mechanisms of infection progress. Host GEFs and GAPs regulate a large number of these RABs and these proteins likely play a major role to aid or stop infection progress. Although several bacterial effectors have been characterized on this front, most host regulators of pathogenesis remain unknown. A thorough understanding of host proteins that regulate trafficking machinery in the context of pathogenesis is therefore essential to establish an effective and early treatment regimen.

Listeria monocytogenes

Listeria monocytogenes is a Gram-positive, food-borne pathogen that invades intestine epithelial cells. *L. monocytogenes* ligands InlA and InlB interact with host cell surface receptors E-cadherin and MET (158-160). Both modes of entry depend on clathrin-mediated endocytosis (161). Clathrin adaptor molecules and actin are recruited to the sites of entry as mentioned above (152). Activation of RAB5 and PI3K is required for the entry process (162). *L. monocytogenes* InlB binding to MET mimics binding of the endogenous MET ligand, HGF (163). It activates the RAS signaling pathway and causes signaling downstream of the MEK-ERK pathway. Entry occurs usually at sites of multicellular junctions where the apoptotic cells undergo constant extrusion at the villus tips (164).

L. monocytogenes phagosomal escape and cell spread

Once inside the cell, the bacteria reside in acidic phagosomes. Escape out of the phagosomes, with the help of a cholesterol-dependent-cytolysin LLO (Listeriolysin O), is required to replicate in the cytosol and polymerize host actin (165). *L. monocytogenes* surface protein ActA can directly nucleate host actin polymerization. The bacterium uses this actin-based motility to propel around the host cell (166). The actin based motility helps the bacterium form protrusions into the neighboring cell, which get resolved into double-layered secondary vacuoles

(167). The whole process of phagosomal escape, replication and actin polymerization repeats itself.

L. monocytogenes *GAPDH inhibits RAB5 to allow effective escape*

Upon phagosomal entry, *L. monocytogenes* has to now inactivate RAB5, as the presence of active RAB5 drives fusion of the bacteria-loaded phagosomes with the lysosomes, leading to bacterial degradation (168). To achieve this, *L. monocytogenes* secretes the GAPDH protein that ADP ribosylates host RAB5 (169). This modification makes RAB5 unresponsive to activation by GEFs.

The conundrum of RAB5 regulation in* L. monocytogenes *infection

Thus, *L. monocytogenes* requires active RAB5 for entry, but upon entry it needs to inactivate this host GTPase for it to escape from the phagosome and survive. Upstream host regulators of RAB5 that are involved in this process have not been characterized thus far.

In our study in chapter four, we focused on RIN1, which is one of the major GEFs of RAB5 in epithelial cells and which acts downstream of growth factor receptor stimulation. We determined the function played by RIN1 in *L. monocytogenes* entry and spread. We deciphered two opposing functions played by RIN1 in pathogen entry and spread respectively, as shown by our working model below:

In summary, the study shows that the RAS effector RIN1 activates RAB5 and facilitates *L. monocytogenes* invasion. However, RIN1 inhibits *L. monocytogenes* cell-cell spread. Further investigation of the mechanism showed that signaling through RIN1→RAB5 results in accelerated fusion of phagosome containing bacteria with lysosomes.
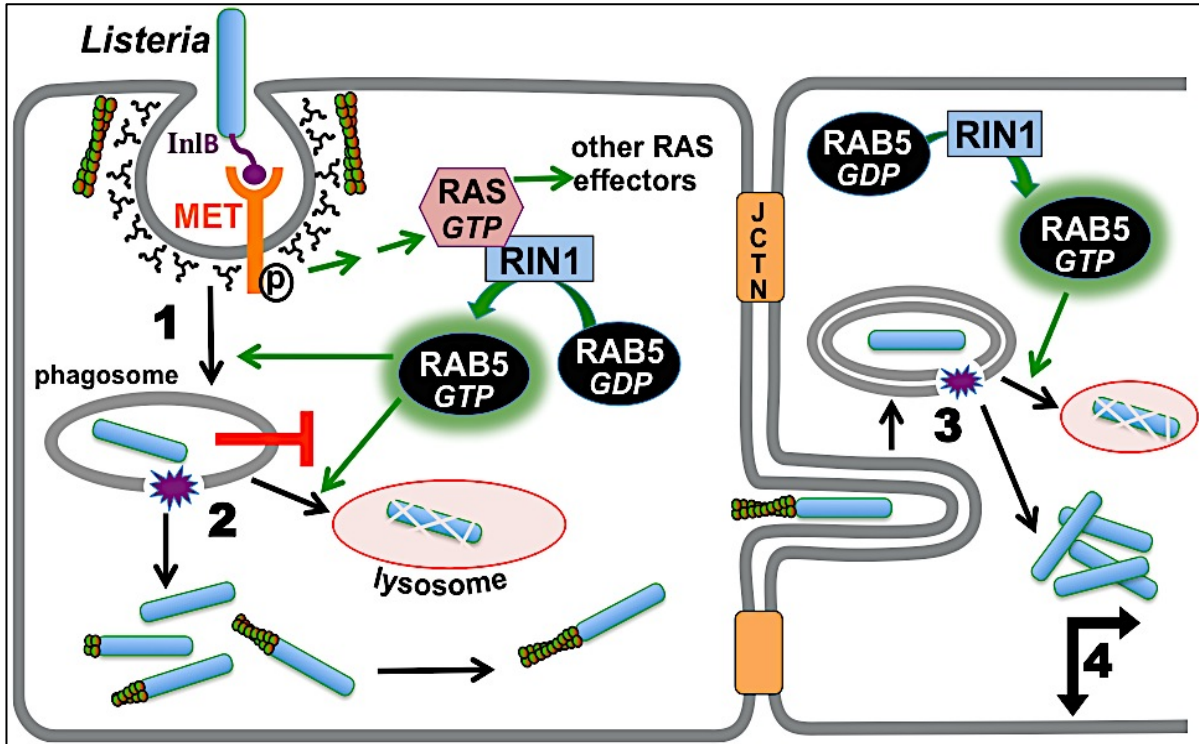
Figure 1. 4 A Model for Role of RIN1 in Infection by *L. monocytogenes*

L. monocytogenes binding to host receptor recruits the clathrin machinery and actin to the site of entry. Activation of RAS leads to RIN1 and hence RAB5 activation, facilitating entry of the pathogen. Downstream of entry, RIN1→RAB5 signaling prevents efficient escape of the bacterium from the phagosome, causing accelerated fusion of the phagosomes with lysosomes. RIN1→RAB5 signaling leads to a more global effect of diminished cell spread of L. monocytogenes.

CHAPTER 2. RIN1 ORCHESTRATES THE ACTIVATION OF RAB5 GTPases AND ABL TYROSINE KINASES TO DETERMINE EGFR FATE

2.1 ABSTRACT

Epidermal growth factor receptor (EGFR) stimulation initiates RAS signaling simultaneously with EGFR internalization. Endocytosed EGFR is then either recycled or degraded. EGFR fate is determined in part by the RAS effector RIN1, a guanine nucleotide exchange factor (GEF) for RAB5 GTPases. EGFR degradation was slowed by RIN1 silencing, enhanced by RIN1 over-expression and accelerated by RIN1 localization to the plasma membrane. RIN1 also directly activates ABL tyrosine kinases, which regulate actin remodeling, a function not previously connected to endocytosis. We report that RIN1-RAB5 signaling favors EGFR down regulation over EGFR recycling, while RIN1-ABL signaling stabilizes EGFR and inhibits macropinocytosis. RIN1^{QM}, a mutant that blocks ABL activation, caused EGF-stimulated membrane ruffling, actin remodeling, dextran uptake and EGFR degradation. An ABL kinase inhibitor phenocopied these effects in cells over-expressing RIN1. EGFR activation also promotes RIN1 interaction with BIN1, a membrane bending protein. These findings suggest that RIN1 orchestrates RAB5 activation, ABL kinase activation and BIN1-recruitment to determine EGFR fate.

2.2 INTRODUCTION

Epidermal growth factor receptor (EGFR) is expressed on multiple cell types and EGFR signaling contributes to cell survival, proliferation and migration. EGF binding to EGFR induces

receptor dimerization that promotes intrinsic tyrosine kinase activity (170). Phospho-tyrosines in the EGFR cytoplasmic region provide docking sites for adaptor proteins that engage RAS activators. This, in turn, triggers signal transduction through RAS effectors, including RAF protein kinases, PI3K lipid kinases and RIN proteins (reviewed in (171)).

EGFR is internalized primarily via clathrin-mediated endocytosis (CME) (172), but may also enter through clathrin-independent endocytosis (CIE) including macropinocytosis (7). RAB5 GTPases are involved in early trafficking steps during both CME and CIE (173, 174). Other common early endocytic components include membrane-bending BAR domain proteins (175). Less is known about the proteins that direct receptors through CME versus CIE pathways.

Endosome-localized EGFR may continue to activate downstream proteins (176), but in a manner that is distinct from plasma membrane localized EGFR signaling (102, 177). Within the endocytic pathway, the engagement of alternate adaptor and effector proteins dictates EGFR signal output (178). Attenuation of endosomal EGFR signaling occurs through dephosphorylation (179) and ligand dissociation (180). Ultimately, internalized EGFR follows one of two possible paths. Recycling endosomes return EGFR to the plasma membrane for reuse (181), a pathway that allows continued EGF detection. The alternative path, favored at high EGF concentrations (7), engages late endosomes (182) and leads to EGFR degradation in the lysosome (6).

Activated EGFR recruits CBL (183), an E3 ubiquitin ligase. CBL-mediated ubiquitination is not required for internalization of EGFR (184, 185), but is a major factor in determining EGFR stability. CBL over-expression increases the EGF-induced degradation of EGFR (186), while a dominant negative CBL mutant can reduce EGF-induced degradation of EGFR (187) and mutation of ubiquitin attachment sites in EGFR disrupts the normal degradation

pathway (185). Sustained poly-ubiquitination by CBL is required for EGFR tethering to HGS and STAM (188, 189) and trafficking to the lysosome (187, 190). The death march of EGFR to lysosomes is negatively regulated by USP8-mediated de-ubiquitination (191).

A key step in regulating EGFR endocytosis is the activation of RAB5 GTPases by guanine nucleotide exchange factors (GEFs) of the VPS9 family, including the RAS effector RIN1 (192). The RAB5 GEF activity of RIN1 promotes early endosome fusion (130), an early event in transit to the lysosome. In addition, the SH2 domain of RIN1 binds to tyrosine phosphorylated EGFR and other RTKs (125, 128), providing a mechanism for prolonged RIN1 engagement through endocytic pathways. During the final stages of endocytosis, an association between RIN1 and STAM has been implicated in promoting RTK degradation through a late endosome to lysosome shunt (129).

RIN1 also binds and activates ABL proteins (ABL1, a.k.a c-Abl, and ABL2, a.k.a. Arg). These closely related non-receptor tyrosine kinases regulate actin remodeling through phosphorylation of actin-associated proteins and through direct F-actin binding (193). Although previous studies have implicated ABL proteins in promoting EGFR phosphorylation (194-196) and actin remodeling has been demonstrated in specialized cases of mammalian cell endocytosis (197), there is no clear mechanism connecting EGF stimulation of EGFR to the activation of ABL and actin remodeling.

We examined whether EGFR endocytosis requires RIN1 signaling through both its RAB5 GTPase and ABL tyrosine kinase pathways. Our results suggest that RIN1 coordinates the activation of RAB5 and ABL to determine the mode of receptor endocytosis, the rate of receptor ubiquitination and degradation, and the amount of receptor recycling. In particular, an excess of RAB5 over ABL signaling triggers membrane ruffling and macropinocytosis with

increased EGFR down regulation. Our data also reveal an EGF-inducible association of RIN1 with BIN1, a membrane bending protein that promotes endocytosis.

2.3 RESULTS

Ligand concentration and RIN1 function determine EGFR ubiquitination and degradation rate

HeLa cells express physiological levels of EGFR and are a well-characterized model for the study of EGFR trafficking (198, 199). Stimulation of HeLa cells using a range of EGF concentrations caused EGFR activation, as assessed by an increase in phosphorylated ERK1 and ERK2, and high concentration EGF elicited a marked down regulation of EGFR (Fig. S1A). Subsequent experiments used EGF at 100 ng/ml (favors degradation) or 1.5 ng/ml (favors recycling), except where indicated. Intermediate EGF concentrations were used when needed to slow EGFR degradation kinetics.

RIN1 over-expression *increased* the EGF-induced degradation rate (Fig. S1B, C), while RIN1 silencing *decreased* the degradation rate (Fig. 1A, Fig. S1C). These results are consistent with a role for RIN1 in determining EGFR stability. EGFR stabilization in RIN1-silenced HeLa cells was less dramatic at a higher EGF concentration (100 ng/ml), leading us to consider the contribution of redundant factors. The closest RIN1 paralogs, RIN2 and RIN3, were undetectable in HeLa cells (Fig. S1D). We did, however, detect RABGEF1 (Rabex5). This protein is the next-closest RAB5-directed GEF family member, as well as a RAS-directed E3 ubiquitin ligase. RABGEF1 silencing enhanced the stability of endogenous EGFR following high concentration EGF treatment (Fig. 1B) and the combined silencing of RIN1 and RABGEF1 caused an even greater stabilization of EGFR (Fig. S1E, F). This is consistent with RIN1 and RABGEF1 both contributing to EGFR down regulation in response to EGF stimulation.

RABGEF1 silenced cells also had an increase in EGF-induced ERK1/2 phosphorylation (Fig. 1B), which is in line with the established role of RABGEF1 in RAS→RAF→MEK→ERK pathway repression through RAS ubiquitination (200, 201). The human RAB5 GEF domain, also called VPS9 domain, family has ten members including RIN1-3 and RABGEF1 (www.ensembl.org). Although we did not examine the six remaining members, our analysis indicates that RIN1 plays a major role in determining EGFR fate in HeLa cells. We next examined whether the increased rate of EGFR degradation in RIN1 over-expressing cells correlated with the rate of receptor ubiquitination following EGF treatment. Indeed, there was a marked increase in ubiquitination at five minutes post-stimulation with 100 ng/ml EGF (Fig. 1C), suggesting that RIN1 induces EGFR degradation at least in part by promoting receptor ubiquitination. The lysosome inhibitor bafilomycin-A stabilized EGFR levels in RIN1 over-expressing cells (Fig. S1G), consistent with a lysosome-mediated mechanism for RIN1-induced EGFR degradation.

Activated RAS can stimulate RIN1's GEF function towards RAB5 in cells over-expressing these components (202). We tested whether an EGFR→RAS→RIN1→RAB5 pathway was operational in control HeLa cells, and found that EGFR stimulation increased endogenous RAB5 (GTP) relative to total RAB5 (Fig. 2A). RIN1 over-expression increased both resting and EGF-stimulated RAB5 (GTP) level but a RIN1 mutant with diminished GEF activity, RIN1^{E574A} (Table 2.1) (128, 131, 169, 200), reduced baseline and EGF-induced RAB5(GTP) to levels below detection (Fig. 2A).
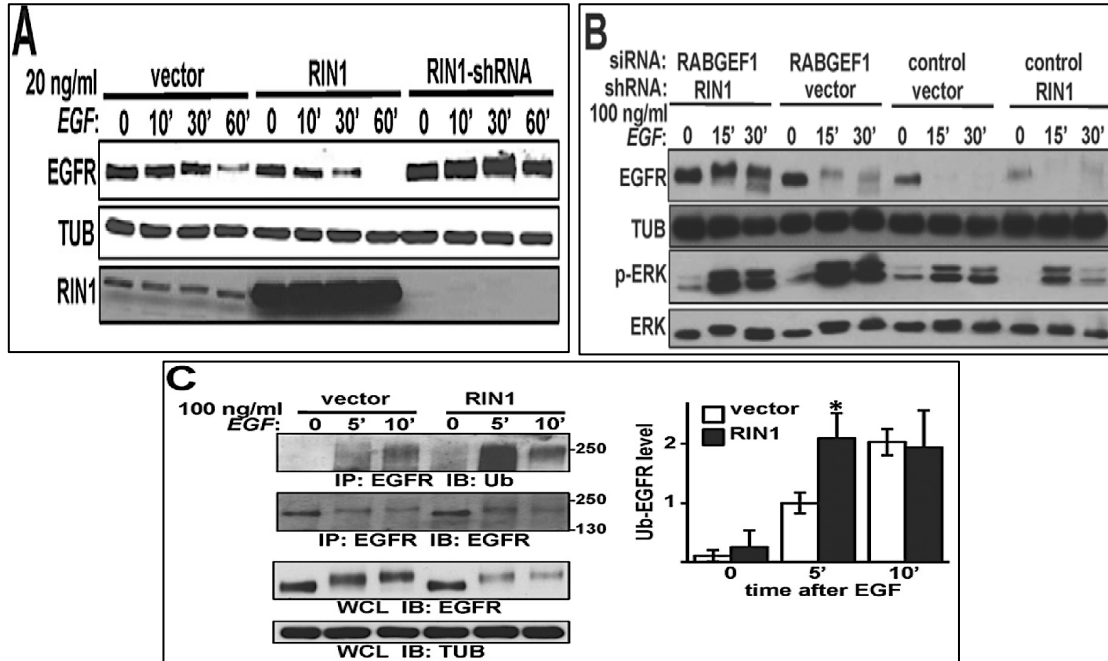
Figure 2. 1 RIN1 promotes EGFR degradation after EGF stimulation.

A. HeLa cells stably transduced with vector, RIN1 or a RIN1-shRNA were treated with 20 ng/ml EGF for the indicated time (min) and lysates immunoblotted for EGFR, RIN1 or alpha-tubulin (TUB). EGFR activation was confirmed by p-ERK immunoblot (data not shown). **B.** Vector and RIN1-shRNA cells transfected with control or RABGEF1 siRNA were treated with 100 ng/ml EGF for the indicated time and lysates immunoblotted as indicated. **C.** Vector and RIN1 transduced HeLa cells were stimulated with 100 ng/ml EGF. EGFR immunoprecipitates were blotted for EGFR or ubiquitin. Whole cell lysates were immunoblotted for EGFR and Tubulin. Ub-EGFR normalized to total immunoprecipitated EGFR using NIH-ImageJ. Mean of three experiments with standard deviations, * p<0.05. EGFR activation was confirmed by p-ERK immunoblot (data not shown).
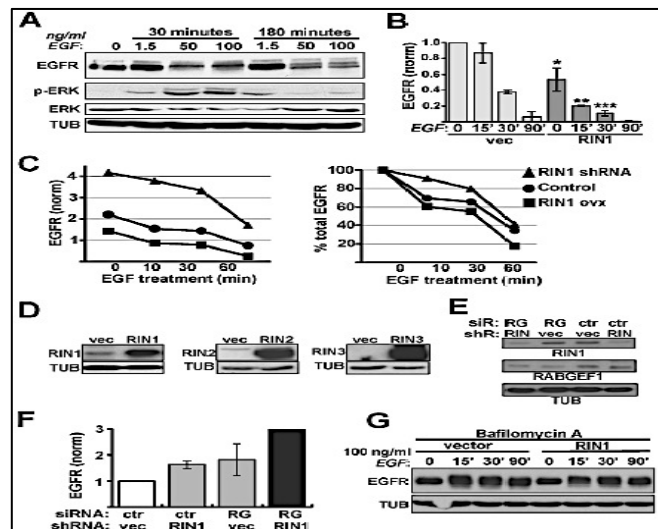
Figure S.2 1 EGF-induced down regulation of EGFR is controlled by RIN1.

A. HeLa cells were stimulated with the indicated concentrations of EGF for the indicated times. Cell lysates were immunoblotted for EGFR, tubulin, total ERK1/2 and p-ERK1/2. **B.** EGFR quantification, normalized to tubulin, in HeLa cells transduced with vector or RIN1 following 100 ng/ml EGF treatment for the indicated times. Mean of three experiments ±SD (*p<0.05, **p<0.005, ***p<0.0005). **C.** Left: EGFR levels from Figure 1A, normalized to tubulin. Right: same data plotted as percent change in EGFR level. **D.** Vector transduced HeLa cells were immunoblotted for endogenous RIN1, RIN2 or RIN3. Cells transfected with RIN1, RIN2 or RIN3 were used as positive controls. **E.** Vector and RIN1-shRNA cells transfected with control or RABGEF1 (RG) siRNA, immunoblotted for RIN1, RABGEF1 or TUB. **F.** Quantification of EGFR levels, 30 minutes post 100 ng/ml EGF stimulation, relative to unstimulated cells, expressed as a fraction of control cells. RIN1 shRNA and RABGEF siRNA data are from three independent experiments. **G.** HeLa cells transduced with vector or RIN1 were pretreated with Bafilomycin A, stimulated with 100 ng/ml EGF for the indicated time, and lysates immunoblotted for EGFR. EGFR activation was confirmed by p-ERK immunoblot (data not shown).

This dominant negative effect suggested that RIN1^{E574A} competes with endogenous RIN1 for efficient activation of RAB5. This analysis does not distinguish among RAB5 paralogs, although RIN1 has been shown to preferentially activate RAB5A (23). Because RIN1^{E574A} binds poorly to RAB5 (131), however, the limiting factor may not be RAB5 itself. These results also reinforce the model that positions RIN1 upstream of RABGEF1 in the activation of RAB5 (200).

RIN1^{E574A} slowed the rate of EGFR degradation following high concentration EGF treatment (Fig. 2B), consistent with a required role for active RAB5 in receptor down regulation. Cells expressing RIN1^{E574A} had smaller endosomes than control or RIN1 cells (Fig. S2), reflecting the contribution of RIN1-RAB5 signaling in early endosome fusion (131). In addition, the RIN1^{E574A} mutant moderately prolonged downstream signaling, as judged by ERK phosphorylation (Fig. 2C). We reasoned that reduced RAB5 activity might favor receptor recycling. Indeed, while EGFR recycling was blocked in cells over-expressing RIN1, recycling was observed at control levels in cells expressing the RIN1^{E574A} mutant (Fig. 2D). These results strongly support the conclusion that RIN1èRAB5 signaling promotes EGFR degradation over recycling.

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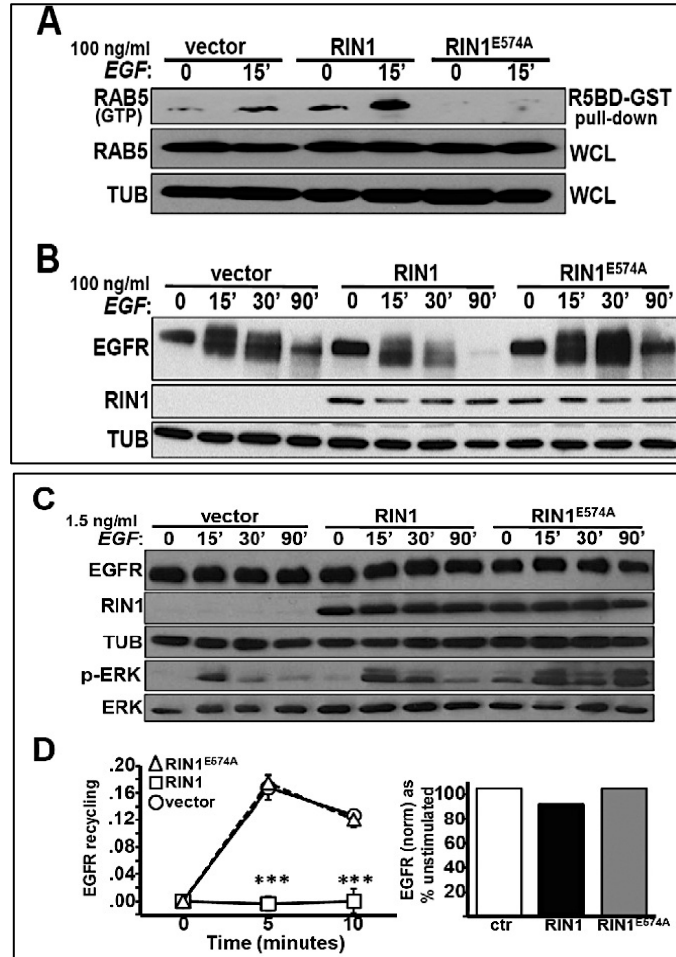
Figure 2. 2 The RIN1→RAB5 signal pathway favors EGFR down regulation.

A. HeLa cells stably transduced with vector, RIN1 or RIN1^{E574A} were treated with 100 ng/ml EGF for 0 or 15 min. Active RAB5 was isolated using a RAB5 binding domain fused to GST (R5BD-GST). Pull down material and whole cell lysate (WCL) were immunoblotted with anti-RAB5 to quantify RAB5(GTP) and total RAB5, respectively. EGFR activation was confirmed by p-ERK immunoblot (data not shown). **B.** Transduced HeLa cells (as in A) were stimulated with 100 ng/ml EGF for the time indicated and lysates immunoblotted for EGFR and RIN1. EGFR activation was confirmed by p-ERK immunoblot (data not shown). **C.** Transduced HeLa cells (as in A) were stimulated with 1.5 ng/ml EGF for the time indicated and lysates immunoblotted for EGFR, RIN1, ERK1/2 and p-ERK1/2. **D.** Left: Transduced HeLa cells were pulsed with 10 ng/ml EGF-Alexa Fluor 647 for 5 minutes. Cells were acid stripped and chased in serum-free medium at 37°C for the time indicated. The data represent fluorescence of acid-stripped recycled EGF-AF647 normalized to bound EGF at time 0 (data from two independent experiments, each performed in duplicate; *** $p<0.0001$). Right: Total EGFR in transduced HeLa cells stimulated with 10 ng/ml EGF for 10 minutes. Final EGFR levels (normalized to tubulin) are shown as a percent of starting EGFR (unstimulated). EGFR activation confirmed by p-ERK immunoblot (data not shown).

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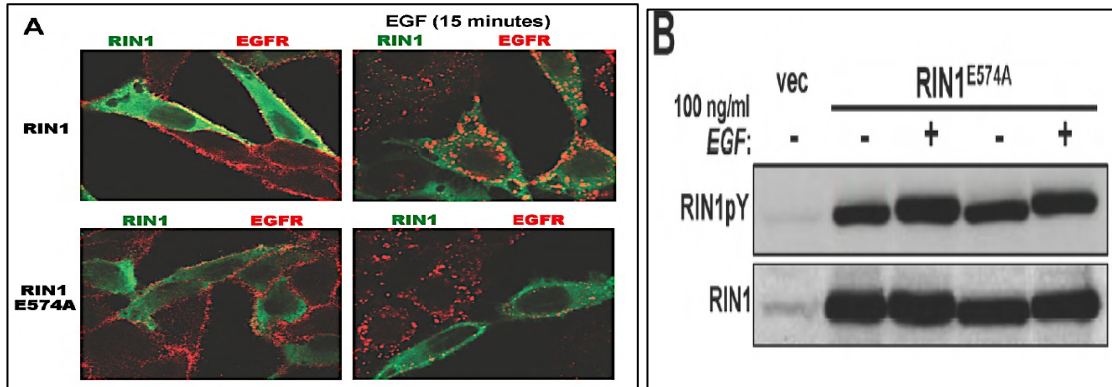
Figure S.2 2 RIN1➔RAB5 signaling regulates early endosome size.

A. HeLa cells stably transduced with RIN1 or RIN1^{E574A} were untreated or stimulated with 100 ng/ml EGF conjugated to AlexaFluor 647 (red) for 15 minutes, then stained for RIN1 (green). Untransduced cells serve as internal controls. **B.** HeLa cells transduced with vector, RIN1 or RIN1^{E574A} were untreated or stimulated with 100 ng/ml EGF for 15 minutes. Cell lysates were immunoblotted for pY-RIN1 and total RIN1. EGFR activation was confirmed by p-ERK immunoblot (data not shown).

RIN1-mediated ABL activation stabilizes EGFR

The RAS effector functions of RIN1 include the activation of ABL tyrosine kinases (126, 127, 203), which regulate actin remodeling (reviewed in (193)). Initial weak binding leads to RIN1 phosphorylation by ABL. RIN1-pY^{36} then binds the ABL SH2 domain, creating a stable interaction that derepresses ABL autoinhibition and increases catalytic efficiency (126, 127). EGF treatment caused a marked increase in the phosphorylation of RIN1 as well as CRKL, an established ABL substrate (Fig. 3A). This is consistent with signal transduction from EGFR through RAS to RIN1, which then directly activates ABL tyrosine kinases concurrent with RAB5 stimulation. Over-expression of wild type RIN1 greatly boosted the pY^{36}-RIN1 signal at all time points, as expected, with a more modest increase in the pY-CRKL signal (Fig. 3A). We next employed a RIN1 mutant that carries Y→F mutations at codon 36 as well as three secondary phosphorylation sites, greatly diminishing the capacity of RIN1 to bind and activate ABL kinases (128) without altering its RAB5 GEF domain (Table 2.1). Expression of this

mutant, RIN1^{QM} (Table 2.1), eliminated the tyrosine phosphorylation signal from endogenous RIN1 and reduced the phosphorylation of CRKL (Fig. 3A). This repression of ABL-mediated phosphorylation was most likely attributable to dominant interference with endogenous RIN1. We observed a modest decrease in ABL1, but not ABL2, in RIN1^{QM} expressing cells (data not shown), and this may have contributed to the diminished pY signal.

Notably, stable RIN1^{QM} cells had a reduced steady state level of EGFR protein (Fig. 3B), which correlated with reduced EGFR mRNA (Fig. S3B). This apparent feedback repression of EGFR transcription may be due to persistent EGFR down regulation (see below). For this reason we focused our analysis primarily on normalized, EGF-induced changes in receptor levels.

Table S1. Characterization of RIN1 mutant proteins used in this work. The RIN1$^{\Delta PR}$ mutant used in these studies (construction described in the Methods section) is similar but not identical to a mutant described in Kong et al., 2007.

Mutant	Defect	Further characterization
RIN1^{E574A}	Reduced GEF activity(Galvis et al., 2009a, Galvis et al., 2009b, Hu et al., 2008) (can not activate RAB5)	Still binds to activated (GTP-bound) RAS (Hu et al., 2008) Still binds to activated (p-Tyr) EGFR (Hu et al., 2008) Still activates ABL tyrosine kinases (*this work*)
RIN1^{QM}	Reduced ABL interaction(Hu et al., 2005, Hu et al., 2008) (does not stimulate ABL)	Still binds activated (GTP-bound) RAS (Hu et al., 2008) Still binds to RAB5^{S34N} (GDP-bound) (Hu et al., 2008)
RIN1^{S351A}	Reduced 14-3-3 interaction(Doppler et al., 2005, Wang et al., 2002, Ziegler et al., 2011) (increased PM localization)	Still binds activated (GTP-bound) RAS (Wang et al., 2002) Still binds to activated (p-Tyr) EGFR (Hu et al., 2008)
RIN1^{R94N}	Reduced EGFR interaction(Barbieri et al., 2003, Hu et al., 2008) (SH2 cannot bind pTyr)	Still binds RAS and RAB5 (SH2 deletion) (Barbieri et al., 2003) Still binds to activated (p-Tyr) EGFR (Hu et al., 2008)
RIN1$^{\Delta PR}$	Reduced STAM binding (Kong et al., 2007) Reduced BIN1 binding (*this work*)	

Table 2. 1 Characterization of RIN1 mutants
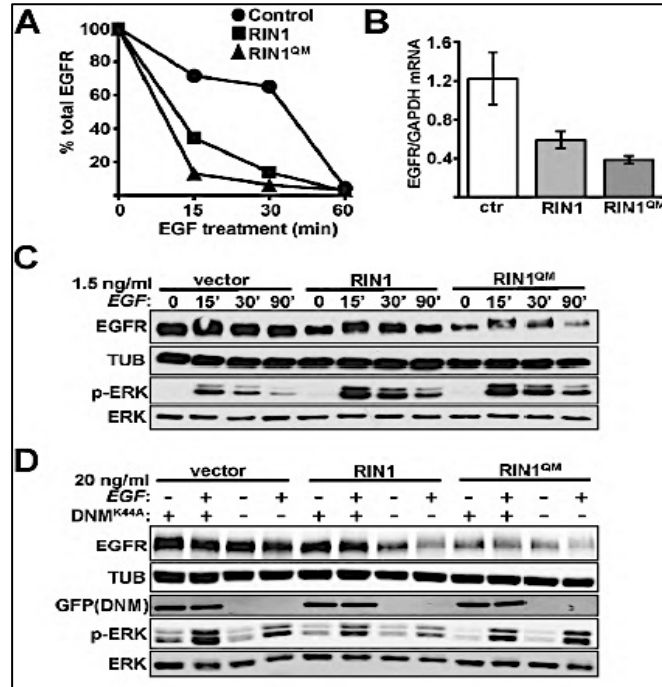
Figure S.2 3 RIN1^{QM} causes accelerated EGFR degradation.

A. Quantification of EGFR degradation rate (Figure 3B data). **B.** EGFR mRNA levels normalized to GAPDH mRNA in vector, RIN1 or RIN1^{QM} HeLa cells. The data represent two independent qRT-PCR experiments, each performed in duplicate. **C.** HeLa cells transduced with vector, RIN1 or RIN1^{QM} were stimulated with 1.5 ng/ml EGF for the indicated times and lysates immunoblotted for EGFR, total ERK1/2 and p-ERK1/2. **D.** Vector, RIN1 or RIN1^{QM} HeLa cells were transfected with empty vector or dominant-negative dynamin (DNM^{K44A}). Cells were stimulated, or not, with 20 ng/ml EGF for 60 minutes and lysates immunoblotted for EGFR, TUB, total ERK1/2, p-ERK1/2 and GFP (dynamin).

Expression of RIN1^{QM} accelerated the rate of ligand-induced EGFR degradation beyond

that resulting from wild type RIN1 over-expression (Figs. 3B and S3A). This finding suggested

that RIN1 functions downstream of EGFR to promote ABL pathways antagonistic to receptor

degradation. We therefore tested the effect of imatinib, an ABL kinase inhibitor, on EGF-

induced EGFR down regulation. Imatinib did not enhance EGFR degradation in control cells,

but did have a pronounced effect in cells over-expressing wild type RIN1 (Fig. 3C). Hence the

more rapid EGFR degradation was likely due to an imbalance between elevated RAB5 activation

(by RIN1^{QM} or RIN1) without a commensurate increase in ABL activation (blocked by QM

mutation or imatinib).
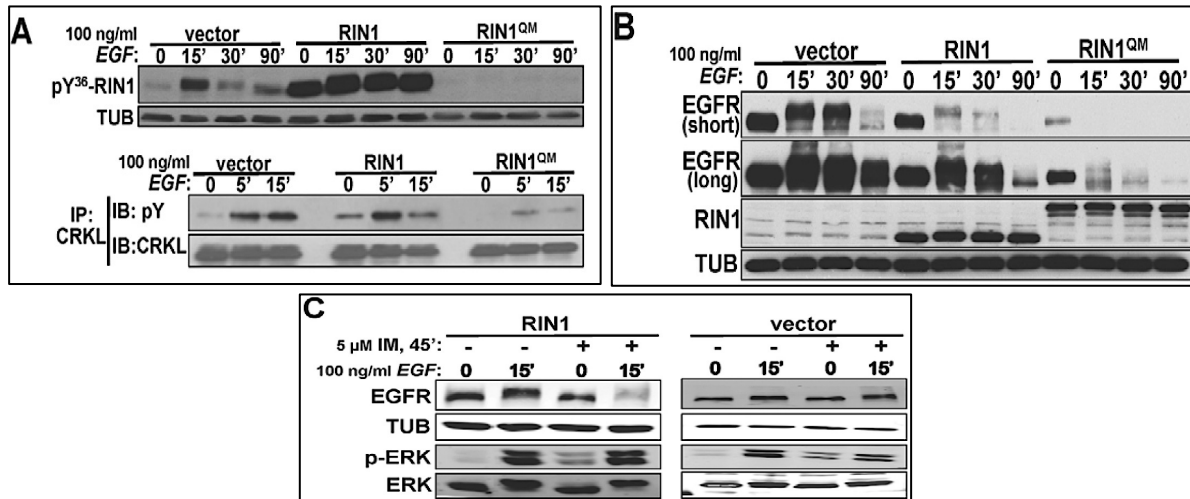
Figure 2. 3 EGF activates RIN1→ABL signaling to regulate EGFR down regulation.

A. Top: HeLa cells transduced with vector, RIN1 or RIN1^{QM} were stimulated with 100 ng/ml
EGF for the indicated times, lysated and immunoblotted with anti-pY^{36}-RIN1. Bottom: Cells
were treated as shown and lysates subjected to anti-CRKL immunoprecipitation. After
immunoblotting this material with anti-pTyr, the blot was stripped and reprobed with anti-
CRKL. EGFR activation confirmed by p-ERK immunoblot (data not shown). **B.** Vector, RIN1 or
RIN1^{QM} transduced HeLa cells were EGF stimulated as in A and lysates immunoblotted. Two
exposures of the EGFR blot are shown. EGFR activation confirmed by p-ERK immunoblot (data
not shown). **C.** HeLa cells stably transduced with vector or RIN1 were pre-treated (or not) with 5
µM imatinib and stimulated (or not) with EGF. Cell lysates were immunoblotted for EGFR,
ERK1/2, p-ERK1/2 and tubulin (TUB).

EGF induced multiple membrane ruffles in RIN1^{QM} expressing cells. These structures

had an underlying F-actin network and were enriched for the RIN1^{QM} protein (Fig. 4A).

Membrane ruffling was not observed in cells over-expressing wild type RIN1 unless they were

pre-treated with the ABL kinase inhibitor imatinib prior to EGF treatment (Fig. 4B). These

structures are reminiscent of those seen in macropinocytosis, a clathrin- and dynamin-

independent endocytosis mechanism favoring receptor degradation and characterized by F-actin

supported membrane ruffles at cholesterol-rich domains (55, 204). To test if the membrane

perturbations we observed were indicative of macropinocytosis, we treated RIN1^{QM} expressing

cells with filipin. Over the course of an hour this cholesterol-stripping drug partially rescued the

basal EGFR down regulation observed in RIN1^{QM} expressing cells (Fig. 5A). These results

suggested that activating RAB5 while blocking ABL kinases (by the QM mutation or by

imatinib), promotes macropinocytosis of EGFR.
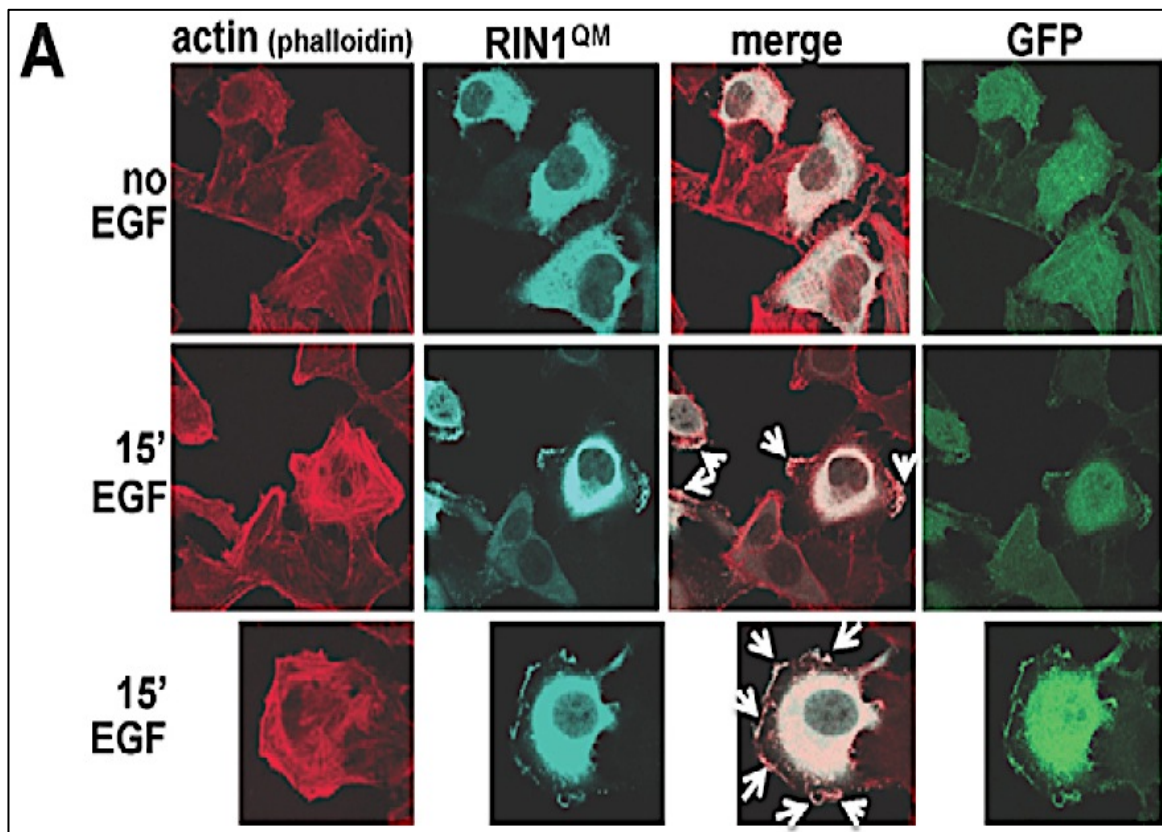
Figure 2. 4 Strong RAB5 activation causes EGF-induced membrane ruffles.

A. HeLa cells transduced with RIN1^{QM} were stimulated or not with100 ng/ml EGF for 15 min.
Fixed and permeabilized cells were stained with phalloidin (red) and anti-RIN1 (light blue).
Arrows point to membrane ruffles. Untransfected cells in the same field (no anti-RIN1 or GFP
signal) serve as internal controls.

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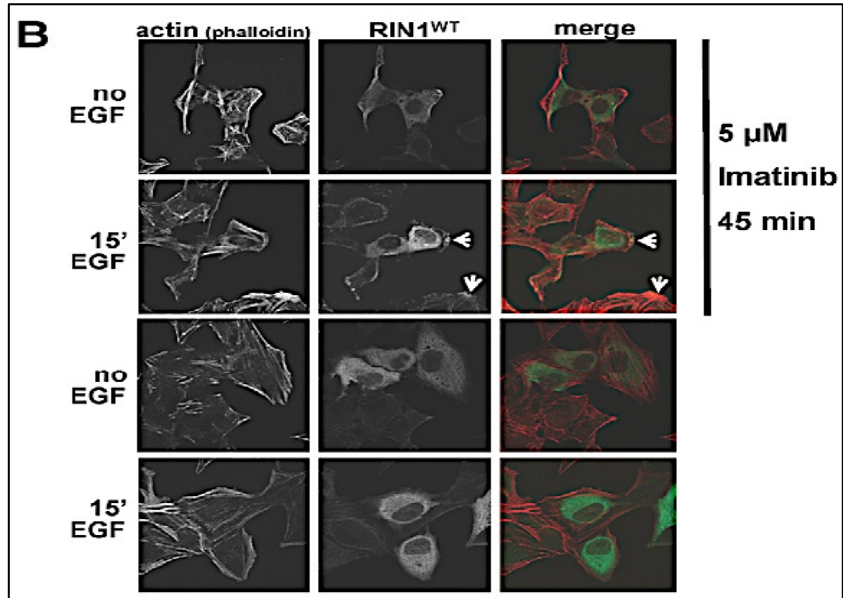
Figure 2. 4 Strong RAB5 activation without ABL activation causes EGF-induced membrane ruffles.

B. RIN1 transduced HeLa cells were pre-treated, or not, with 5 µM imatinib for 45 min. and stimulated, or not, with 100 ng/ml EGF for 15 min. Fixed and permeabilized cells were stained with phalloidin (red) or anti-RIN1 (green). Arrows point to membrane ruffles.

F-actin based membrane ruffles and cholesterol dependence are, together, strongly indicative of macropinocytosis. We next assayed for macropinocytosis-associated large solute uptake. HeLa cells over-expressing wild type RIN1 showed no EGF-induced dextran incorporation. In contrast, RIN1^{QM} cells and RIN1 over-expression cells treated with an ABL inhibitor, both of which showed EGF-induced membrane ruffling, had significant dextran uptake (Fig. 5B). In addition, EGFR stability was increased by dominant negative dynamin (DNM^{K44A}) in cells transduced with vector or RIN1 but not RIN1^{QM} (Fig. 5C and S3D). This dynamin-*in*dependence is also consistent with macropinocytosis in the EGF-stimulated RIN1^{QM} cells. Co-localization of fluorescent dextran and EGF on RIN1^{QM} cells, but not RIN1 cells, indicated that a significant portion of EGFR was entering cells via macropinosomes (Fig. 5D, Fig. S4A).

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Together, these results demonstrate that strong activation of RAB5 by RIN1, without the concomitant activation of ABL tyrosine kinases, leads to EGF-induced macropinocytosis.
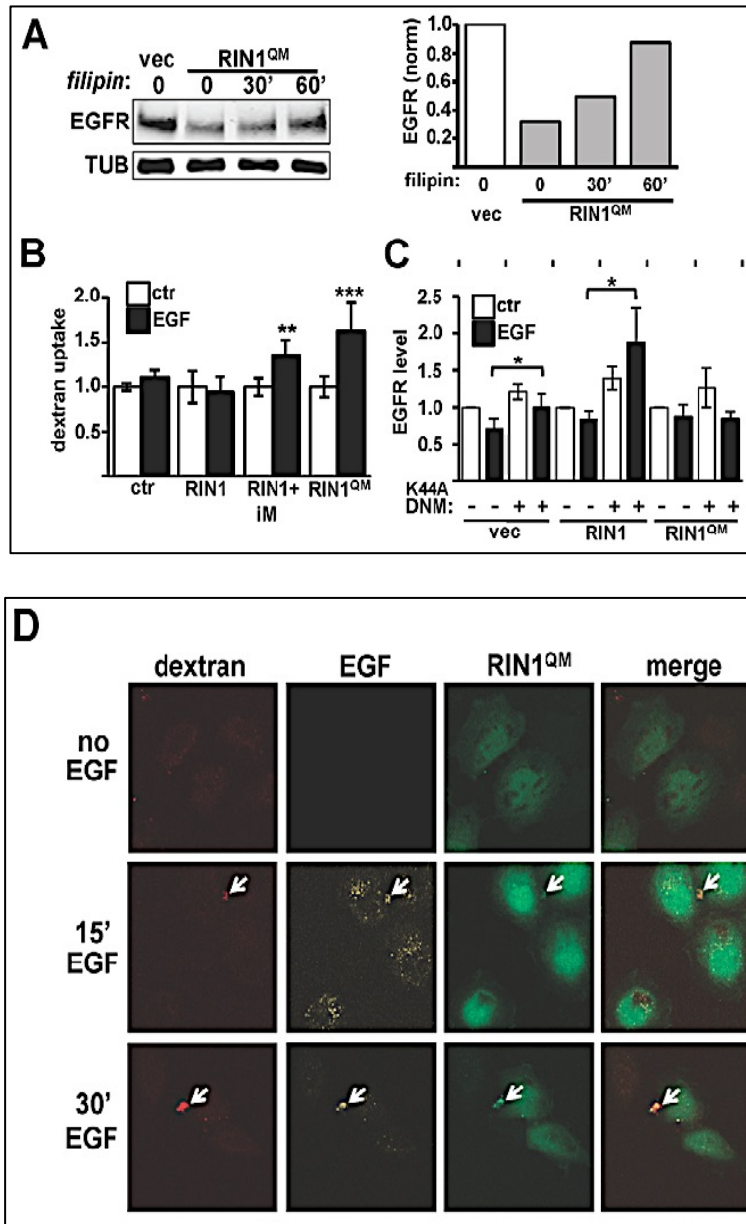
Figure 2. 5 Enhanced RAB5 signaling without ABL signaling induces macropinocytosis.

A. Left: HeLa cell transduced with vector or RIN1^{QM} were treated, or not, with 5 µg/ml filipin for the indicated time and lysates probed with anti-EGFR. Right: EGFR levels normalized to TUB. **B.** Fluorescent dextran uptake in transduced HeLa cells. IM indicates pre-treated with 5 µM imatinib for 45 min. Other samples were mock treated. Means of 5-7 data points ± SD (** p<0.005, *** p<0.0005). **C.** Vector, RIN1 or RIN1^{QM} HeLa cells were transfected with control vector (-) or dominant-negative dynamin (K44A-DNM). EGFR levels, normalized to TUB, were

determined for control and EGF treated (20 ng/ml, 60 min.) cells. Means of three experiments ± SD (*p<0.05). **D.** RIN1^{QM} transduced HeLa cells were pulsed with 20 ng/ml EGF and 1mg/ml Dextran for 5 minutes and chased in serum-free medium for the indicated time. RIN1^{QM} transduced cells are GFP-positive (untransduced cells serve as internal controls). Red=Dextran; Yellow=EGF; Green=GFP.

Because the receptor-targeted E3 ligase CBL plays a critical role in directing activated EGFR through a degradation pathway, we considered whether changes in CBL levels might contribute to the effect of RIN1^{QM} on EGFR stability. RIN1^{QM} cells showed elevated levels of CBL compared to control HeLa cells, while wild type RIN1 over-expressing cells had an intermediate increase in CBL (Fig. S4B). There was also an increase in EGFR-associated CBL in cells over-expressing RIN1 or RIN1^{QM} (Fig. S4C). EGF stimulation had little effect on total CBL protein levels. These findings suggest that RIN1-mediated ABL signaling might normally reduce EGFR degradation in part by de-stabilizing CBL.
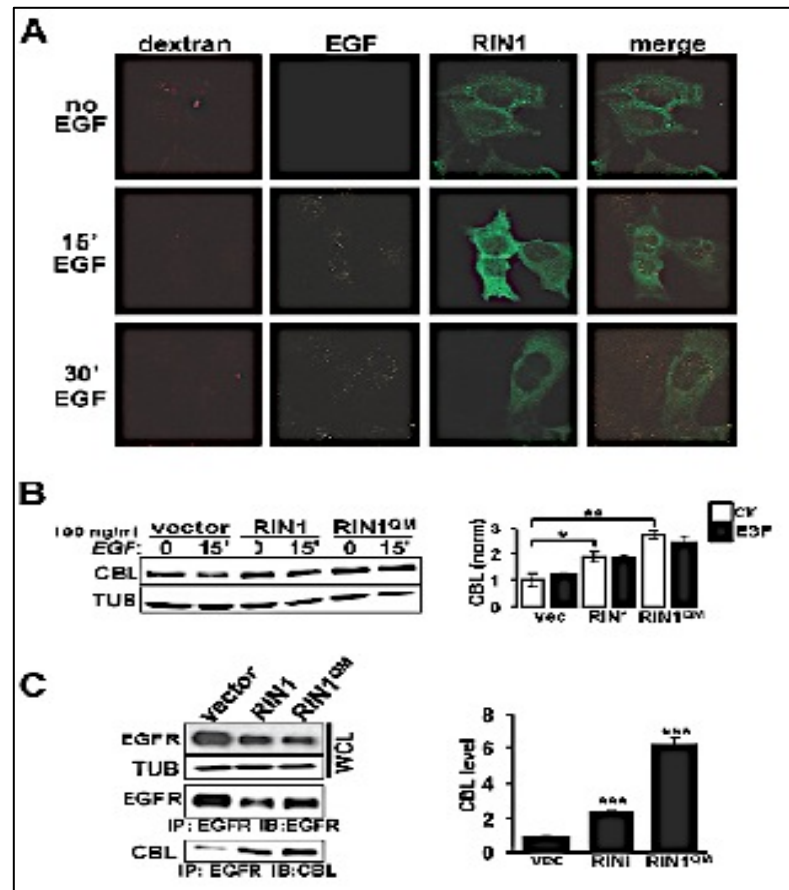
Figure S.2 4 RIN1^{QM} effects in HeLa cells.

A. Control for Figure 5D. HeLa cells transduced with RIN1 were pulsed with 20 ng/ml EGF and 1mg/ml Dextran for 5 minutes, as with RIN1^{QM} cells in Figure 5D and chased in serum-free medium for the indicated time. Untransfected cells serve as internal controls. red=dextran; yellow=EGF, green=RIN1. **B.** Left: Transduced HeLa cells were stimulated, or not, with 100 ng/ml EGF for 15 min. Lysates were immunoblotted with anti-CBL or anti-TUB. Right: CBL levels normalized to TUB (mean of three experiments ± SD). EGFR activation was confirmed by p-ERK immunoblot (data not shown). **C.** Left: Transduced HeLa cells (as in B) were serum-starved overnight and lysates probed for EGFR or TUB. Anti-EGFR immunoprecipitates were blotted with anti-EGFR or anti-CBL. Right: Quantification of CBL associated with EGFR. Mean of three experiments ± SD. *p<0.05; ** p<0.005; *** p<0.0005.

Plasma membrane localization of RIN1 facilitates EGFR down regulation

Post receptor internalization, signaling events continue to take place at endosome membranes where RAS proteins engage effectors, such as RIN1, and where RAB5 proteins are localized by carboxy-terminal prenylation. Translocation of RIN1 between cytoplasmic and membrane compartments is controlled in part by Ser^{351} phosphorylation-dependent binding to 14-3-3 proteins. The RIN1^{S351A} mutant (Table S1) shows reduced 14-3-3 binding and enhanced membrane residence (205) as well as increased association with EGFR (128). We reasoned that greater membrane association should enhance RIN1-mediated effects on EGFR down regulation. Indeed, expression of RIN1^{S351A} increased ligand-induced EGFR down regulation, when compared with cells that over-express wild type RIN1 (Fig. 6A). Even using a low EGF concentration that had little effect on endogenous EGFR levels in control HeLa cells, RIN1^{S351A} expressing cells showed marked receptor down regulation (Fig. S5A). As with RIN1^{QM} cells, RIN1^{S351A} cells had lower basal EGFR protein levels, but they showed no change in the amount of transferrin receptor (Fig. S5B), indicating that RIN1-induced down regulation of basal receptor levels is receptor type specific.

Surprisingly, we observed relatively normal induction of pERK levels following low concentration EGF treatment of cells expressing RIN1^{S351A} (Fig. S5A) or RIN1^{QM} (Fig. S3C),

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despite lower levels of EGFR in these cells. These results suggest that the functions disrupted in these RIN1 mutants are not required for a normal ERK signaling response to EGF, and may even have helped to compensate for lower receptor levels.
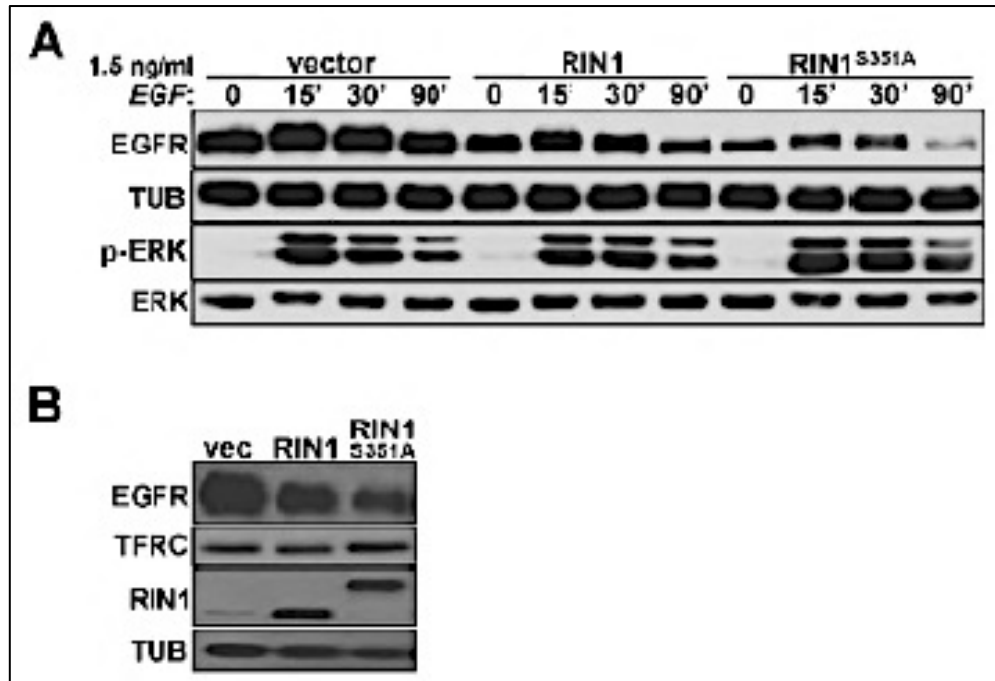
Figure S.2 5 Plasma membrane localization of RIN1 promotes EGFR degradation

A. HeLa cells transduced with vec, RIN1 or RIN1[S351A] were stimulated with 1.5 ng/ml EGF for the indicated times and lysates probed for EGFR, ERK1/2 and p-ERK1/2. **B.** Transduced HeLa cells were immunoblotted for EGFR, transferrin receptor (TFRC) and RIN1.

RIN1 contributes to EGFR fate following activation by ligands other than EGF

EGFR can be activated by several non-EGF ligands with distinct effects on downstream signaling and receptor degradation (reviewed in (206)). Transforming growth factor alpha (TGFα) binds EGFR and triggers many of the same downstream signaling pathways as EGF, including those mediated by phosphorylation of ERK and AKT (207). Once in the early endosome, however, TGFα dissociates from EGFR more readily than EGF, causing most TGFα-activated EGFR to be recycled rather than degraded (6). TGFa levels that gave no discernable EGFR degradation in control cells led to pronounced EGFR down regulation in RIN1 over-

expressing cells and RIN1^{S351A} expressing cells (Fig. 6B), consistent with a RIN1 contribution that is receptor activation dependent but ligand independent.

A surprisingly robust and prolonged pERK signal was observed following TGFa treatment of RIN1 over-expressing cells, compared to control cells (Fig. 6B). This enhanced and sustained burst of pERK was muted in RIN1^{S351A} expressing cells, suggesting that RIN1 translocation from the membrane may be required for normal downstream signaling from TGFa-stimulated EGFR. The increased levels of pERK in RIN1 over-expressing cells correlated with prolonged levels of RAF1-S^{338} phosphorylation (Fig. 6C). This modification is associated with enhanced kinase activity for MEK, which in turn phosphorylates ERK. Reduced dephosphorylation might also contribute to higher pERK levels. This enhanced signaling effect of TGFa seemed to be pathway specific, however, as only normal levels of pAKT were detected in the RIN1 over-expressing cells.

Amphiregulin (AREG) is another physiological ligand with low affinity for EGFR. Compared to EGF, AREG produces less EGFR phosphorylation (208). AREG treatment activates RAS signaling pathways but is not normally accompanied by EGFR degradation, leading instead to the accumulation of cell surface EGFR (208). AREG treatment produced little apparent change in EGFR levels in RIN1 over-expressing cells, but did cause a drop in EGFR levels in RIN1^{S351A} cells (Fig. 6D). Our results suggest that RIN1 signaling, enhanced by plasma membrane localization in the RIN1^{S351A} mutant, promotes degradation of EGFR following activation by a broad range of stimuli.
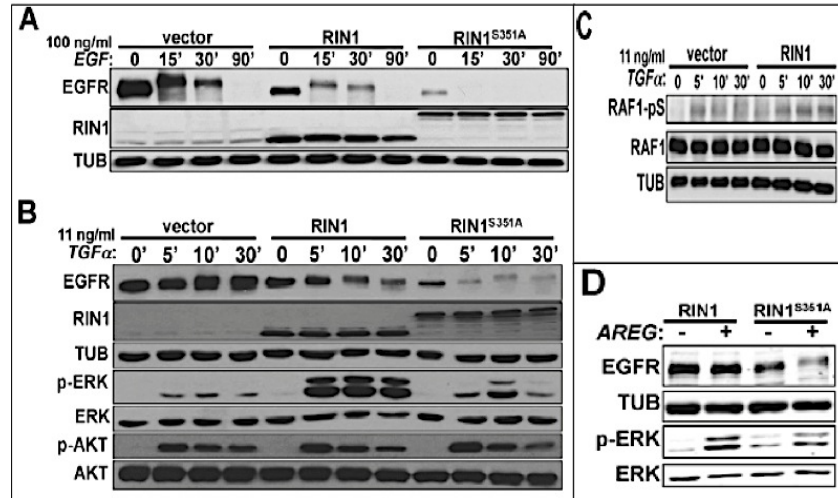
Figure 2. 6 Plasma membrane localization of RIN1 promotes EGFR degradation

A. HeLa cells transduced with vector, RIN1 or RIN1^{S351A} were stimulated with 100 ng/ml EGF for the indicated times, and cell lysates immunoblotted for EGFR and RIN1. EGFR activation confirmed by p-ERK immunoblot (data not shown). **B.** HeLa cells transduced with vector, RIN1 or RIN1^{S351A} were stimulated with 11 ng/ml TGFα for the indicated times, and lysates immunoblotted for EGFR, RIN1, tubulin, total ERK1/2, p-ERK1/2, total AKT and p-AKT. **C.** Transduced HeLa cells were stimulated with 11 ng/ml TGFα for the indicated times, and lysates immunoblotted for total RAF1 and pS^{338}-RAF1. **D.** HeLa cells transduced with RIN1 or RIN1^{S351A} were transfected with a vector (-) or amphiregulin (AREG) construct (+). Cell lysates prepared 48 hours later were immunoblotted for EGFR, tubulin, total ERK1/2 and p-ERK1/2.

Additional RIN1 partners contribute to EGFR signaling

RIN1 encodes a proline rich (PR) sequence with consensus motifs for binding SH3 and/or WW domains (209). Following treatment with high EGF, cells expressing a RIN1 mutant missing the largest proline-rich motif (RIN1$^{\Delta PR}$) showed a pattern of EGFR degradation more similar to control cells than to cells over-expressing wild type RIN1 (Fig. 7A). This result suggested that the RIN1$^{\Delta PR}$ mutant is compromised for interactions that promote EGFR degradation.

The PR domain of RIN1 has been implicated in binding to the SH3 domain of STAM2 (129). STAM proteins are components of ESCRT0 complexes that facilitate receptor transit

from endosomes to multi-vesicular bodies and lysosomes. In agreement with an earlier study using a similar but not identical deletion of the RIN1 proline-rich motif, RIN1$^{\Delta PR}$ was compromised for association with STAM2 (Fig. S6). This may account for the normal EGFR down regulation rate, even when RIN1$^{\Delta PR}$ was expressed at a level similar to that at which wild type RIN1 enhanced EGFR degradation.
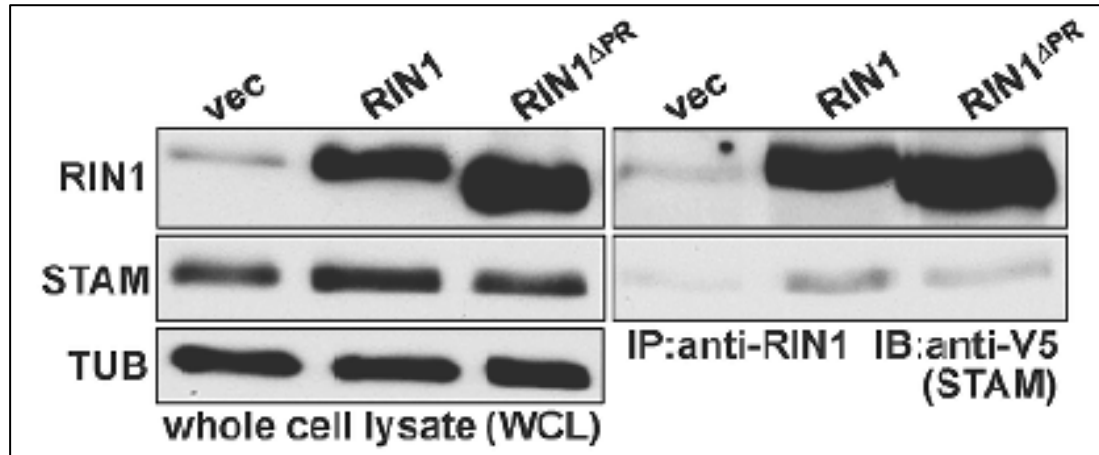
Figure S.2 6 ΔPR mutation reduces the RIN1::STAM interaction.

HeLa cells were transduced with vector, RIN1 or RIN1$^{\Delta PR}$. Cell lysates and RIN1 immunoprecipitates were blotted for V5 (STAM) or RIN1.

A similar PR sequence in RIN3, a RIN1 paralog, has been reported to bind BIN1 (a.k.a. amphiphysin 2) in two-hybrid assays (139). Because BIN1 is a membrane bending protein implicated in trafficking, we tested whether RIN1 also interacts with BIN1. Indeed, the association of RIN1 with BIN1 was readily apparent and was markedly enhanced within five minutes of EGF treatment (Fig. 7B). The RIN1::BIN1 association was reduced by 2.5 fold for the RIN1$^{\Delta PR}$ mutant (Fig. 7B). This is consistent with an interaction between the RIN1 PR domain and the BIN1 SH3 domain, analogous to the proposed interaction of RIN3 with BIN1 (139). These findings suggest a model in which RIN1 recruits BIN1 to activated receptors at an

early stage of endosome formation when the membrane bending properties of BIN1 might aid

the formation or resolution of early endosome structures.
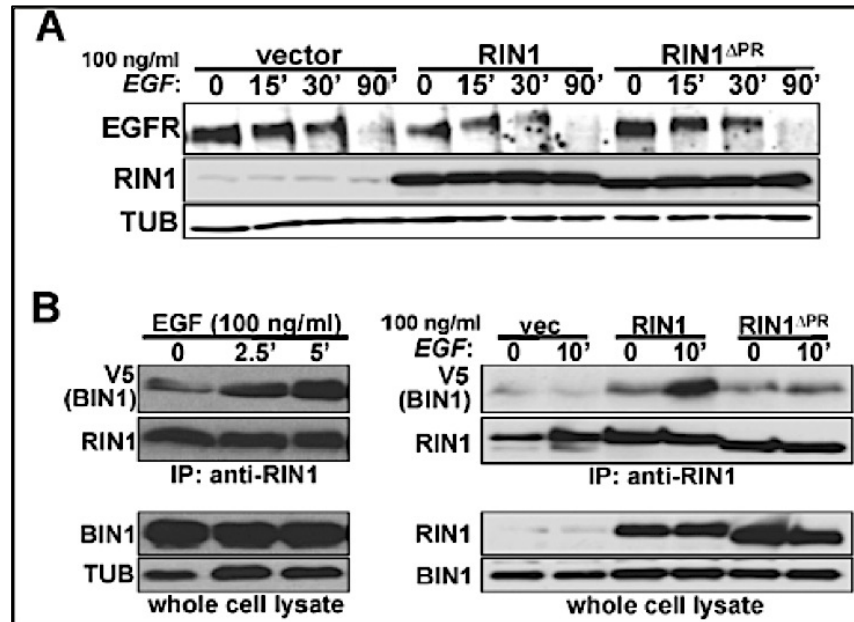
Figure 2. 7 EGF triggers BIN1 recruitment to RIN1.

A. HeLa cells transduced with vector, RIN1 or RIN1$^{\Delta PR}$ were stimulated with 100 ng/ml EGF for time indicated. Cell lysates were immunoblotted for EGFR, RIN1 and TUB. **B.** Left: RIN1 transduced cells were transfected with BIN1-V5 and stimulated with 100 ng/ml EGF for the time indicated. Anti-RIN1 immunoprecipitates and whole cell lysates were blotted for V5 (BIN1) and RIN1. Right: Vector, RIN1 or RIN1$^{\Delta PR}$ cells were stimulated, or not, with EGF (100 ng/ml, 10 min.). Anti-RIN1 immunoprecipitates and whole cell lysates were blotted for V5 (BIN1) and RIN1. RIN1$^{\Delta PR}$ brought down 2.5 fold less BIN1 from unstimulated cells than did wild type RIN1 (normalized to RIN1 in immunoprecipitate; n=3; P < 0.05). For A and B experiments, EGFR activation was confirmed by p-ERK immunoblot (data not shown).

Because the RIN1 SH2 domain mediates binding to activated EGFR (125) and other

receptor tyrosine kinases (128), we next determined the effect of mutating a residue required for

phosphotyrosine binding. Although RIN1^{R94N} has a reduced affinity for activated EGFR (128),

expression of this mutant had little effect on EGFR degradation or pERK levels after high

concentration EGF treatment (Fig. S7A, B). This suggests that RIN1^{R94N} (unlike RIN1^{E574A} and

RIN1^{QM}) does not interfere in a dominant manner with endogenous RIN1, at least in this system.
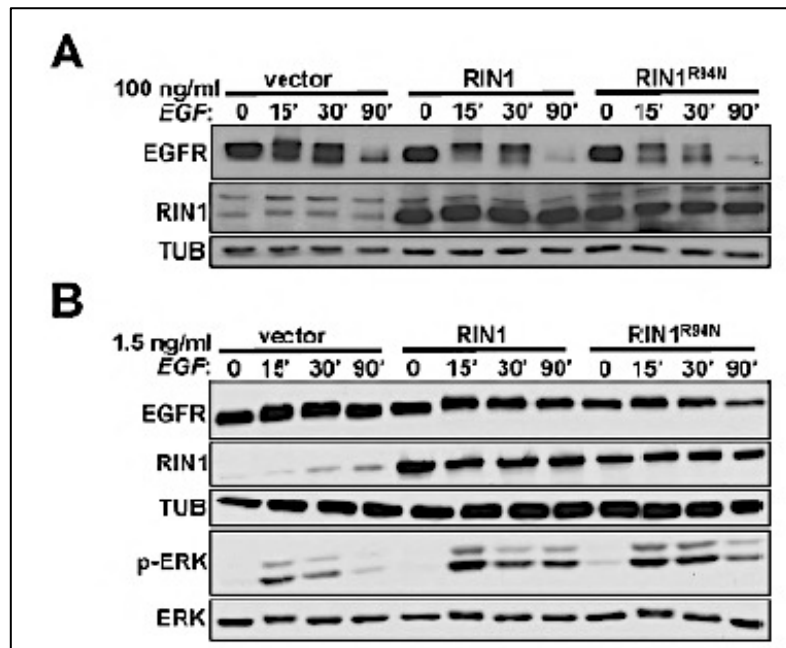
Figure S.2 7 RIN1^{R94N} does not alter EGFR signaling through p-ERK.

A. Vector, RIN1 or RIN1[R94N] HeLa cells were stimulated with 100 ng/ml EGF for the indicated time and lysates immunoblotted for EGFR, RIN1 or TUB. EGFR activation was confirmed by p-ERK immunoblot (data not shown). **B.** Vector, RIN1 or RIN1[R94N] HeLa cells stimulated with 1.5 ng/ml EGF for the indicated time and lysates immunoblotted for EGFR, RIN1, TUB, total ERK1/2 or p-ERK1/2.

2.4 DISCUSSION

RIN1 is a RAS effector protein that regulates endocytosis and signaling of receptor tyrosine kinases such as EGFR. RIN1 encodes a GEF domain that activates RAB5 GTPases governing early endosome traffic (125, 202). A separate RIN1 domain activates ABL tyrosine kinases and actin remodeling, but this had not been implicated in endocytosis. Here we demonstrate that EGF binding to EGFR triggers ABL activation, and we dissect out the unique contributions of RAB5 and ABL during EGFR endocytosis. We propose that a balance of RIN1→RAB5 and RIN1→ABL signaling controls the internalization route and eventual fate of activated receptors (Fig. 8). These studies also suggest that efficient receptor trafficking requires the coordination of RIN1 subcellular localization and BIN1 binding.
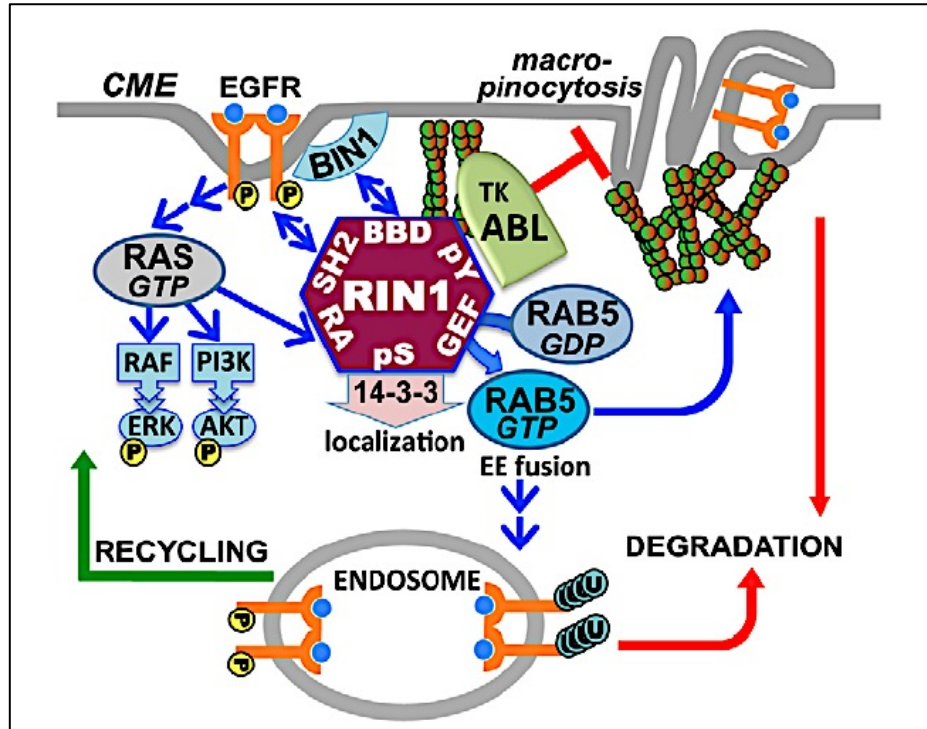
Figure 2. 8 Model of coordinated RIN1 signal pathways in EGFR endocytosis.

Upon EGF stimulation, the RAS effector RIN1 activates RAB5 to drive early endosome (EE) fusion and promote EGFR degradation. Concomitant ABL activation regulates actin remodeling to inhibit macropinocytosis, which otherwise results from unchecked RAB5 signaling. Strong ABL activation in the absence of RAB5 activation favors EGFR stability and recycling. Recruitment of BIN1 to the BIN1 binding domain (BBD) facilitates membrane bending and endocytosis, while 14-3-3 binding inhibits plasma membrane access and endocytosis.

RIN1-ABL signaling regulates the route of EGFR internalization

ABL proteins have been implicated in receptor tyrosine kinase signaling and endocytosis (76). ABL1 directly phosphorylates several receptor tyrosine kinases as well as CAV1 (caveolin) and the ubiquitin ligase CBL (210). The combined effects of ABL activation normally promote EGFR stability (reduced degradation). This may result in part from blocking CBL recruitment and disrupting an EGF-mediated CBL-Abi1 interaction (76, 211). Consistent with a role for ABL in protecting RTKs from down regulation, RIN1^{QM} (mutant that cannot activate ABL) blocked EGF-induced ABL tyrosine kinase activity and accelerated the EGF-induced receptor

degradation rate. We also noted lower EGFR mRNA levels in RIN1^{QM} cells. Regulation of EGFR gene transcription has been previously noted (212), and in this circumstance persistent EGFR down regulation may lead to EGFR gene repression in order to avoid futile cycles of receptor synthesis.

RIN1^{QM} induced membrane ruffling and macropinocytosis, suggesting that elevating RAB5(GTP) levels without a commensurate increase in ABL activity can alter the route of internalization. This interpretation is supported by the fact that treatment of wild type RIN1 over-expressing cells with the ABL inhibitor imatinib phenocopied what was observed in RIN1^{QM} cells. Dextran uptake, EGFR and dextran co-localization, cholesterol dependence and dynamin *in*dpendence all support a macropinocytosis mechanism, which is known to favor receptor degradation over recycling (7). Consistent with our findings, macropinocytosis is promoted by RAB5 (63, 173, 174) and CBL (213) but inhibited by the ABL substrate Abi1 (214). Conversely, blocking RIN1's GEF function (RIN1^{E574A}) restored the EGFR recycling that was greatly diminished in RIN1 over-expression cells. Hence, the coordination of RIN1→ABL and RIN1→RAB5 pathways appears necessary to regulate receptor internalization in a way that balances degradation and recycling.

RIN1 localization and additional partners regulate early stages of receptor endocytosis

Upon RTK stimulation, RIN1 translocates from the cytoplasm to plasma membrane through a mechanism that involves 14-3-3 binding (205) and is similar to the localization control of other RAS effectors such as RAF. The 14-3-3 binding mutant RIN1^{S351A} dramatically enhanced EGFR degradation. This may result from the proximity of RIN1 to RAS, EGFR and the endocytosis machinery, highlighting the importance of properly timed RIN1 localization in endocytosis.

This study also implicates the membrane bending protein BIN1 in early endocytosis by RIN1, because the RIN1::BIN1 interaction was markedly increased by EGF stimulation. The RIN1$^{\Delta PR}$ mutant showed reduced binding to BIN1, suggesting an association through proline-rich (RIN1) to SH3 (BIN1) domains. Recruitment of BIN1 could facilitate membrane deformations associated with early endosome fusion, a function that RIN1 is known to regulate. A report that BIN1 interacts with the RIN family member RIN3 (139) implies a conserved connection between RIN proteins and membrane bending. Notably, a proline-rich segment of RIN1 also interacts with the STAM protein complex that mediates late stage EGFR trafficking and degradation (129). The extent to which BIN1 directly participates in RIN1-mediated receptor trafficking remains uncertain, however, and will require further study.

RIN1 over-expression caused higher CBL levels and more EGFR-associated CBL compared to control cells. The RIN1^{QM} mutant showed even higher levels of total CBL and EGFR-associated CBL, suggesting that ABL activation is not required for – and may actually antagonize - these effects. Aside from its role as a major E3-ubiquitin ligase for receptor tyrosine kinases, CBL functions as an adaptor protein that regulates actin cytoskeleton dynamics (reviewed in (215)). Our results are consistent with CBL participation at multiple levels in RTK endocytosis.

RIN1 plays multiple roles in EGFR trafficking

RTK activation triggers downstream signal transduction through RAS proteins and their effectors including RIN1. Our data demonstrate that RIN1 regulates RTK endocytosis not only through RAB5 activation and RTK binding, but also through its ABL tyrosine kinase activating function. This highlights the need to further define the role played by ABL tyrosine kinases and cytoskeleton remodeling in endocytosis. Importantly, it is the *balance* of RAB5 and ABL

activation that determines receptor fate (Fig. 8). Additional interactions with BIN1 and STAM suggest that RIN1 orchestrates multiple effectors and binding partners to shepherd activated RTKs through successive stages of endocytosis and determine the degree of receptor down-regulation versus recycling. More broadly, our results illustrate the capacity of multi-domain proteins to coordinate seemingly disparate pathways to regulate alternate outcomes.

2.5 MATERIALS AND METHODS

Expression constructs

All RIN1 expression constructs were made in lentivirus vectors. RIN1 wild type, RIN1^{E574A} in pM4-blast^R vector (216); RIN1^{QM} and RIN1^{S351A} were in the M4-IRES-GFP vector (128). Virus production and transduction were performed as previously described (205). To create RIN1$^{\Delta PR}$, 149 base pairs surrounding the proline rich region of RIN1 were removed by restriction digest with KpnI and HindIII and replaced with 89 bp of overlapping oligonucleotides for a precise deletion of 20 codons. The final construct was cloned into pM4-blast^R. For stable RIN1 knockdown, RIN1 shRNA containing lentivirus vector pLKO.1-puro^R was used as described previously (217). The RIN1 mutants and their properties are summarized in Supplemental Table 1.

The RAB5-GTP pull-down construct was created using the Zn^{2+} finger domain of rabenosyn (ZFYVE20), which functions as a RAB5 binding domain (R5BD) (218). The sequence encoding AA 1-40 of human ZFYVE20 was amplified using forward primer 5'-ATGCGCTAGCAGATCTACTAGTATGGCTTCTCTGGACGACCC and reverse primer 5'-ATGCGGATCCTTATCTAGATTCCCCTGAGTGTTCTTCCT. Ligation compatible restriction sites in each primer (XbaI/SpeI and BamHI/BglII) were used for stepwise head-to-tail

additions in pKS bluescript. The 4x concatemer was then inserted into the BamHI - EcoRI sites of pGEX-2T to create pGEX-4xZFYVE (R5BD-GST).

HA-STAM2 (129) (gift of Dr. Philip D. Stahl, Department of Cell Biology and Physiology, Washington University School of Medicine) was used to amplify the STAM2 gene and this was introduced into the pKS vector along with an amino terminal V5 tag, added using adaptor oligos. The final construct V5-STAM2 was introduced into the M4-neo^R lentivirus vector. The BIN1 cDNA was amplified out of pcDNA3-BIN1 (gift of Dr. Corrine Leprince) and introduced into a M4-neo^R-V5 lentivirus vector to obtain M4-neo^R-BIN1-V5 construct. pcDNA3-RIN2-HTM and M4-blast^R-RIN3-Flag tagged constructs were used for transient transfection of RIN2 and RIN3, respectively, in HeLa cells.

Cell culture and reagents

HeLa cells 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). All transfection experiments were performed using Polyfect reagent (Qiagen).

EGF (Invitrogen) was used at 100 ng/ml, 20 ng/ml or 1.5 ng/ml as indicated. TGFα (Invitrogen) was used at 11 ng/ml. pcDNA3-Amphiregulin-Neomycin (gift of Dr. Anson, Stanford University) was transfected into HeLa cells and the cells were selected with 400 μg/ml G418 (Gibco). Filipin (Sigma Aldrich) pretreatment of cells was carried out using 10 μg/ml for 4 or 12 hours at 37°C. Bafilomycin (Sigma Aldrich) treatment was done on cells at 0.25 μM for 1 hour at 37°C. Imatinib treatment was performed at 5 uM for 45 minutes at 37°C.

Growth factor stimulation was performed by serum starving cells overnight followed by addition of serum-free medium with the indicated concentration of the growth factor. The cells were then incubated at 37°C for the indicated time, washed with cold PBS and lysed.

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) (217). Expression levels were evaluated by western blotting. RABGEF1 silencing was achieved by transfection of the targeted or scramble vectors- pcDNA3. Super-Rabex5 shRNA/pCDNA3. Super-scramble shRNA (gift of Dr. Dafna Bar-Sagi, NYU) (200) followed by selection with 4 µg/ml blasticidin in naïve HeLa cells or with 4 µg/ml Blasticidin and 2 µg/ml Puromycin in RIN1-silenced HeLa cells.

RAB5 Pull-down Assay

RAB5-GTP binding domain coated glutathione-sepharose (GE health) beads were prepared by expressing 4xZFYVE-GST (R5BD-GST) in BL21 bacterial cells which were induced with 1 mM IPTG (Fisher) at 30°C for 3 hours. The cells were then pelleted, washed in ice cold PBS and resuspended in lysis buffer (250 mM NaCl, 20 mM Tris pH 8.0, 10% Glycerol, 0.01% Triton-X-100, 1 mM PMSF (Sigma Aldrich), 10 ug/ml Leupeptin (Sigma Aldrich), 1 uM Pepstatin (Sigma Aldrich) and EDTA-free protease inhibitor cocktail tablet (Roche). The lysate was incubated with glutathione sepharose beads (GE Health) for one hour at 4°C. The beads coated with 4X-ZFYVE-GST were then washed three times with the lysis buffer and resuspended in the lysis buffer.

HeLa cells, unstimulated or stimulated, 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, 1 mM Sodium Orthovanadate (Sigma Aldrich) and 10mM $MgCl_2$ to stop the exchange reaction.

1000 ug of total lysate protein was used for pull-down of activated RAB5 and added to 4X-ZFYVE bound glutathione-sepharose beads described above. After overnight incubation at 4°C, the beads were washed with NP-40 lysis buffer (without $MgCl_2$) and boiled in 1X Laemmli loading buffer, followed by gel electrophoresis and immunoblotting for RAB5.

EGFR Recycling Assay

HeLa cells were seeded in duplicate on 6-well plates. The cells were serum starved overnight. Cells were stimulated with EGF conjugated to Alexa Fluor 647 (Invitrogen) at 10 ng/ml, a concentration favoring EGFR recycling over degradation, to allow internalization for 5 minutes at 37°C, then washed with cold medium and stripped twice with 0.2 M sodium acetate pH 4.5 buffer. The cells were rinsed in cold serum-free medium and excess unlabeled EGF and chased at 37°C for the indicated amounts of time. Following the chase, the cells were stripped twice in the Sodium Acetate buffer. Signal from stripped fluorescent EGF (EGF-AF647) from the acid washes and the medium was read on a Wallac 1420 mutilabel counter (PerkinElmer) at 635/665 nm absorption/emission channels.

Dextran Uptake Assays

HeLa cells were plated at 2 x10^4 cells per well in a 96-well plate and serum starved overnight at 37°C. Cells were then pre-treated with control or imatinib containing medium and placed on ice for 30 minutes. This was followed by treatment with serum-free DMEM containing 1 mg/ml 70,000 MW Dextran conjugated to Texas Red (Invitrogen, #D1830), in the presence or absence of 100 ng/ml EGF and 5 µM imatinib. The cells were incubated at 37°C for 30 minutes after which they were placed on ice and quickly rinsed several times in ice-cold PBS. The cells were kept in 100 µl ice cold PBS and fluorescence emission from Dextran uptake was read using a Wallac 1420 mutilabel counter (PerkinElmer) at 560/615 Absorption/Emission channels.

Immunoprecipitation, immunoblotting and immunofluorescence

Antibodies used for immunoblotting and their sources were- EGFR 1:2000 (Santa Cruz, #SC-03), Tubulin 1:5000 (Sigma Aldrich, #T6074-200 ul), RIN1 1:1000 (Mouse mAb, clone #C9E11, Colicelli lab, AbPro), RIN1 1:1000 (Rabbit pAb anti-99, Colicelli lab, 21st century Biochemicals), p-ERK1/2 pY204 1:1500 (Epitomics, #2219-1), ERK1/2 1:5000 (BD, #610123), p-AKT pS473 1:250 (Cell Signaling, #9271-S), AKT 1:1000 (Cell Signaling, #9272), p-Raf1 pS338 1:200 (Millipore, #05-538), c-Raf 1:200 (BD Transduction Laboratories, #R19120), V5 1:2000 (Invitrogen, #R960-25), Transferrin Receptor 1:2000 (Invitrogen, #13-6800), Ubiquitin 1:1000 (Cell Signaling, #3936), CRKL 1:1000 (Santa Cruz, #SC-319), 4G10 1:1000 (Millipore, #05-321), pY^{36} RIN1 (127) 1:1000 (Rabbit pAb, Colicelli Lab, Biosource International), Pan-RAB5 1:1000 (Abcam, #ab18211), CBL 1:1000 (BD, # 610441), c-ABL 1:1000 (Santa Cruz, #SC-131), sheep-anti-mouse-HRP 1:3000 (Amersham Biosciences, #NA931), goat-anti-rabbit-HRP 1:3000 (Kirkegaard and Perry, #4741506), ProteinA-HRP 1:1000 (Invitrogen, #10-1023), goat-anti-rabbit-IRDye 800 1:5000 (Li-Cor Biosciences, #926-32211) and goat-anti-mouse-IRDye 680 1:5000 (Li-Cor Biosciences, #926-32220).

For immunoblotting, proteins were transferred to Nitrocellulose membranes overnight. The membranes were blocked with 5% milk in TBST (0.1% Tween-20) followed by incubation with primary and secondary antibodies at room temperature. The membranes were washed with TBST between the incubations and developed using the ECL plus western blotting reagent (VWR) or scanned using a Li-Cor Odyssey scanner.

For immunoprecipitation experiments, the antibody sources were- EGFR 1:1000 (Santa Cruz, #SC-03), RIN1 1:500 (Rabbit pAb anti-99, Colicelli lab, 21st century Biochemicals) or CRKL 1:1000 (Santa Cruz, #SC-319). 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 1:200 (Mouse mAb, clone #C9E11, Colicelli lab, AbPro), Phalloidin-rhodamine 0.2 uM (Gift of Dr. Margot Quinlan, UCLA), EGF-Alexa Fluor 647 (Invitrogen, #E35351), Dextran-Texas Red 70000 MW (Invitrogen), goat-anti-rabbit Alexa-Fluor 568 1:200 (Invitrogen, #A11036) and goat-anti-mouse Alexa Fluor 647 1:200 (Invitrogen, #A21245). For imaging experiments, 4 x 10^5 cells were seeded on 35 mm MatTek glass bottom tissue culture plates (MatTek corporation). The cells were treated with growth factor as indicated and were 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 EGF internalization experiments, 100 ng/ml EGF-Alexa Fluor 647 was added to cells and bound on ice for 2 hours. The cells were then transferred to 37°C and incubated for 15 minutes, followed by fixation and staining. Cells were then incubated with primary and secondary antibodies at room temperature. Phalloidin was added along with the secondary antibody staining. For EGF-Dextran co-localization studies, cells were pulsed with 20 ng/ml EGF and 1 mg/ml Dextran-Texas Red for five minutes at 37°C. The cells were then chased for the indicated amounts of time at 37°C in serum-free medium, followed by fixation and staining. Confocal microscopy (Zeiss Pascal) was used to image the cells.

RNA isolation and qRT-PCR

RNA was isolated from vector, RIN1 and RIN1^{QM} HeLa cells using the RNeasy mini kit (Qiagen). 1 ug of RNA was converted to cDNA using the SuperScriptIII First-Strand Synthesis SuperMix kit (Invitrogen). qPCR was performed on 2ul of the diluted cDNA (1:5)

template using LightCycler 480 SYBR Green I Master Mix (Roche Applied Science) and

Stratagene MX3000 real-time PCR cycler. The data was analysed using MXPro software

(Agilent Technologies).

The PCR primers used were based on a previous study (219) and are as follows:

EGFR Forward 5'- CTGCGTCTCTTGCCGGAATG -3',

EGFR Reverse 5'- TTGGCTCACCCTCCAGAAGG-3';

GAPDH Forward 5'- TGCACCACCAACTGCTTAGC-3',

GAPDH Reverse 5'- GAGGGGCCATCCACAGTCTT -3'

Microscopy

Fixed and stained cells were examined on a laser scanning confocal microscope (Axiovert

200M, Carl Zeiss LSM 5 Pascal). 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.

CHAPTER 3. RAB5-MEDIATED MIGRATION DIS-ABL-ED

3.1 ABSTRACT

Stimulation of a receptor tyrosine kinase (RTK), such as EGFR, leads to RAS activation followed by RIN1 activation. RIN1, in turn, activates RAB5 family GTPases, as well as ABL tyrosine kinases. As expected, RIN1 expression directly correlates with RAB5-mediated EGFR endocytosis. We previously showed that normal receptor endocytosis and internalized EGFR fate also depend on the ability of RIN1 to concomitantly activate ABL tyrosine kinases, consistent with the established role of ABL kinases in cytoskeleton remodeling and the growing evidence that such remodeling plays a role in endocytic processes. Here we report that growth factor-directed cell migration, a physiological process that involves receptor endocytosis and actin remodeling, also requires the ability of RIN1 to coordinate RAB5 GTPase and ABL tyrosine kinase pathways.

3.2 ROLE OF RIN1 IN REGULATING CELL MIGRATION

RIN1 is a RAS effector protein (120). Through a guanine nucleotide exchange factor (GEF) domain (99), RIN1 activates RAB5 GTPases that promote early endosome maturation (130). In addition, RIN1 directly binds to and activates ABL tyrosine kinases (126, 127). RIN1 associates directly with activated EGFR and binds STAM proteins that escort receptors to lysosomes for degradation (129). RIN1 sub-cellular localization, and hence its availability for interactions, is regulated by binding 14-3-3 proteins that favor cytoplasmic positioning (169).

Overexpression of RIN1 in HeLa cells increased RAB5 activity and enhanced EGFR degradation compared with control cells, while silencing endogenous RIN1 had the opposite effect (58). The RIN1 GEF domain mutant RIN1^{E574A}, which cannot activate RAB5 but can still activate ABL, suppressed RAB5 activity in a dominant negative manner and blocked EGFR degradation while it promoted receptor recycling (58). This confirmed a positive role for RAB5 stimulation in receptor internalization and was consistent with a role for ABL in protecting activated receptors from ubiquitylation and internalization (76).

We further demonstrated that RIN1 undergoes EGF-dependent phosphorylation on Tyr^{36}, emphasizing engagement of the RIN1→ABL pathway following receptor stimulation (58). RIN1^{QM}, a mutant that cannot activate ABL but still activates RAB5, caused dominant suppression of EGF-induced ABL kinase activation and accelerated EGFR degradation (58). HeLa cells expressing this mutant also showed greater RAB5 activation (Fig. 1) than did cells overexpressing wild type RIN1, implying that the RIN1→ABL pathway normally modulates the RIN1→RAB5 pathway during endocytosis.
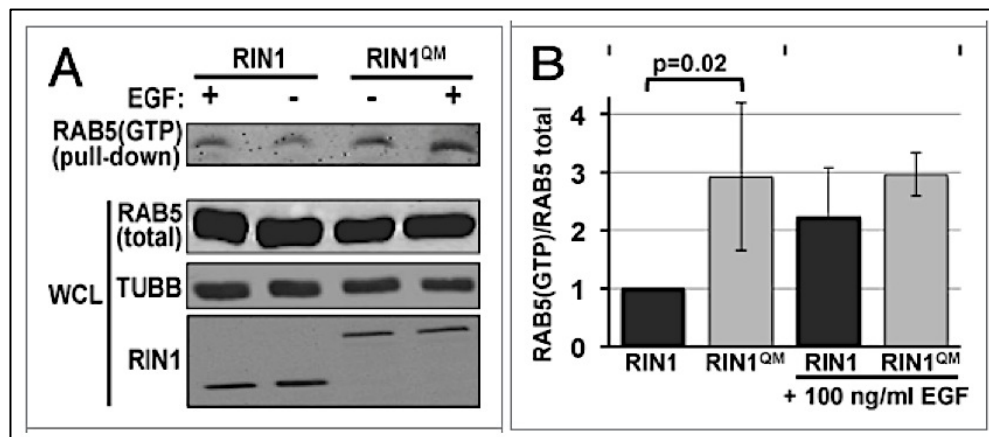
Figure 3. 1 RIN1^{QM} shows enhanced RAB5 Activation.

(**A**, top) Immunoblots of activated RAB5 in RIN1 and RIN1^{QM} HeLa cells untreated (-) or stimulated with 100 ng/ml EGF for 5 min (+). Alternate lanes were left blank to prevent merging of the lanes. Note that EGF addition samples are not alternating. (Bottom) Whole cell lysates of RIN1 and RIN1^{QM} HeLa cells from above, immunoblotted for total RAB5, Tubulin and RIN1.

(**B**) Quantification of relative activated RAB5, normalized to total RAB5 from (**A**). The RIN1^{QM} protein includes multiple carboxy terminal tags that increase it molecular mass.

The influence of RIN1 signaling on EGFR degradation suggested the involvement of CBL, an E3 ubiquitin ligase for EGFR, because receptor ubiquitylation drives sorting and degradation (220). RIN1 overexpression increased CBL levels, consistent with RIN1 promoting EGFR::CBL interactions and accelerated EGFR degradation (58). RIN1^{QM} dominantly blocked ABL kinase activity and enhanced CBL protein recruitment to EGFR, (58) supporting a negative role for RIN1→ABL signaling during CBL recruitment to EGFR.

We noted that RIN1^{QM} cells exhibited EGF-dependent macropinocytosis. In addition, EGF co-localized with macropinocytic membrane ruffles (58) demonstrating that an imbalance in RIN1 effector pathways can shift the mode of RTK endocytosis. Blocking RIN1→RAB5 signaling stabilized EGFR and enhanced recycling, while blocking RIN1→ABL caused enhanced macropinocytosis with increased receptor degradation (58) Notably, RAB5 activation has been linked to macropinocytosis through RAC1 (63, 64, 100).

Because receptor internalization and downregulation are directly relevant to chemotaxis, we examined the physiological significance of coordinated RAB5 and ABL signaling on cell migration toward a growth factor gradient. HeLa cells overexpressing wild type RIN1 showed a reduced rate of migration toward EGF, which was likely due to increased ABL activity (115). Expression of RIN1^{E574A} (activates ABL but not RAB5) also decreased migration toward EGF, compared with cells expressing wild type RIN1 (Fig. 2A).

In contrast, HeLa cells expressing RIN1^{QM} showed significantly higher basal motility (no EGF gradient) compared with wild type RIN1 cells and, despite having less surface EGFR, migration toward EGF was similar to cells expressing wild type RIN1 (Fig. 2B). Hence the loss

of RIN1→ABL signaling, while RIN1→RAB5 signaling persists, can enhance directed cell motility.

This observation is consistent with macropinocytotic membrane ruffling and RAB5 activity being major contributors to cell migration, and suggests a mechanistic connection between RAS and RAC signaling pathways, as previously suggested (63, 64). The macropinocytic and migratory phenotypes we observed also emphasize a role for RIN1 in actin cytoskeleton remodeling, a function well established for ABL tyrosine kinases (81, 83, 221) and strongly implied for RAB5 GTPases (63).
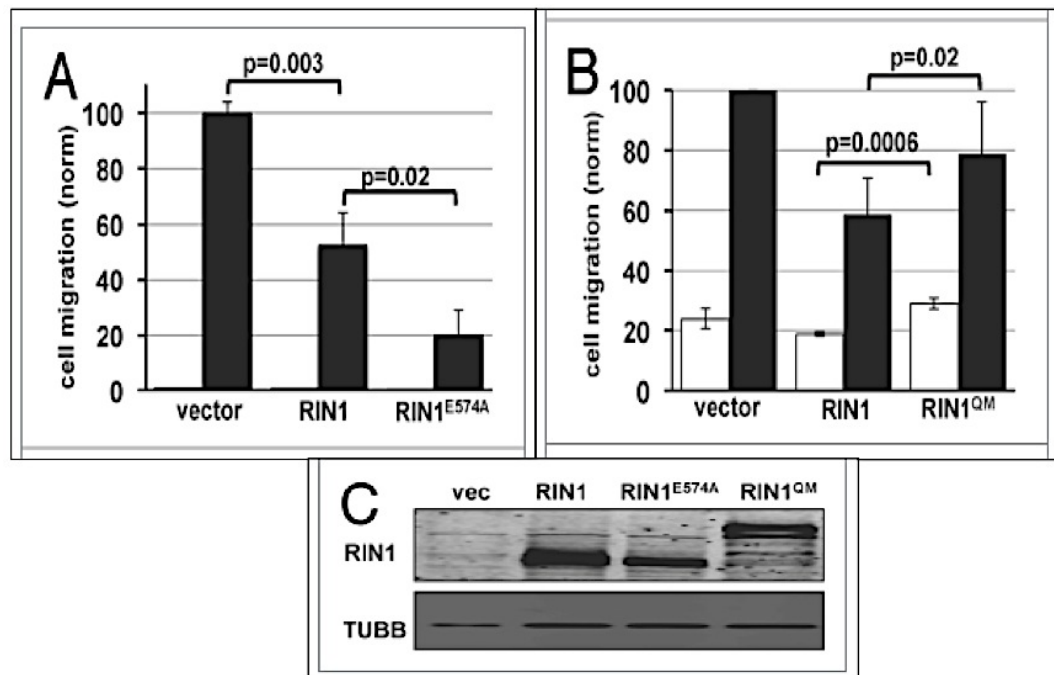
Figure 3. 2 RIN1→RAB5 signaling axis promotes directional migration

(A) Migration across a transwell over one day toward control medium (white bar) or 100 ng/ml EGF medium (black bar) for vector, RIN1 and RIN1E574A HeLa cells. (B) Migration across a transwell, as in (A), for vector, RIN1 and RIN1QM HeLa cells. (C) Immunoblot showing RIN1 protein levels in vector, RIN1, RIN1E574A and RIN1QM HeLa cells.

The role of RIN1 signaling through RAB5 and ABL pathways in cell migration (this work) should be considered in the context of our previous work showing that BIN1, a BAR

domain containing membrane-bending protein, is a RIN1 binding partner (58). RIN1's proline-rich region mediates this interaction, possibly through the BIN1 SH3 domain as reported for in vitro binding of RIN3 to BIN1 (141). EGF stimulation enhanced the RIN1::BIN1 interaction, suggesting that RIN1 recruits BIN1 to facilitate receptor endocytosis during migration. In addition, RIN1 sub-cellular localization appeared to influence receptor internalization, as revealed using RIN1S351A, a mutant with reduced 14-3-3 binding (58). Localized receptor responses are fundamental to efficient directional cell migration. We have proposed a working model to explain the broad integration of signaling pathways through RIN1 following receptor tyrosine kinase activation (58).
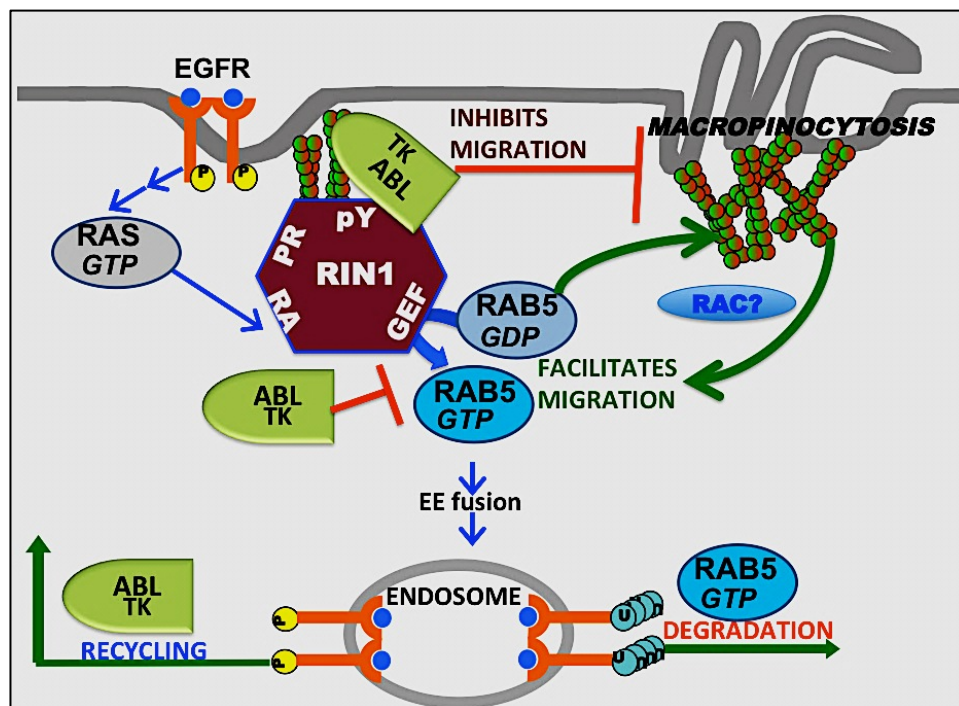
Figure 3. 3 Working model for RIN1's effect on cell migration:

Stimulation by growth factor leads to activation of the receptor. RIN1 is activated downstream of RAS and in turn activates ABL tyrosine kinases and RAB5 GTPases. RAB5 promotes migration of cells towards the growth factor through a likely RAC dependent process. ABL kinases on the other hand diminish migration towards growth factor. Expression of ABL binding mutant of RIN1 shows enhanced RAB5 activation, implicating ABL in regulation of RAB5 activation by RIN1.

Our findings demonstrate a key role for RIN1 as a regulator of membrane trafficking and cytoskeleton remodeling during receptor endocytosis and cell migration. A deeper mechanistic understanding will require examination of other endocytic events in epithelial cells. For example, invasive bacteria, such as *Salmonella* and *Listeria*, enter epithelial cells through macropinocytosis and receptor-mediated phagocytosis, respectively (148, 160, 164, 222). RAB5 is a major player in both these entry mechanisms and functions in subsequent steps required for bacteria replication (156, 162). ABL kinases have also been implicated in the entry of invasive bacteria (223-225). A role for RIN1 during pathogen entry could lead to identification of novel host cell drug targets for combating drug-resistant bacteria. Outside of epithelial cells, RIN1 is found most prominently in mature forebrain neurons where it inhibits certain types of learning (121, 123, 132). A deeper understanding of RIN1 signal orchestration may shed light on synaptic plasticity mechanisms. How RIN1 coordinates GTPase and tyrosine kinase pathways also has direct implications for treatment of cancers with RTK and RAS oncogene involvement.

3.3 MATERIALS AND METHODS

Cell culture and reagents

HeLa cells 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).

Expression constructs

All RIN1 expression constructs were made in lentivirus vectors. RIN1 wild type, RIN1[E574A] were made in in pM4-blast[R] vector (128). In RIN1[QM], Tyrosines 36, 121, 148, and 295 were mutated to phenyalanine. The RIN1[QM] construct was made in the M4-IRES-GFP vector. Virus production and transduction were performed as previously described (169).

The RAB5-GTP pull-down construct was created using the Zn^{2+} finger domain of rabenosyn (ZFYVE20), a RAB5 binding domain. The sequence encoding amino acids 1–40 of human ZFYVE20 was amplified using forward primer 5¢-ATGCGCTAGCAGATCTACTAGTATGGCTTCTCTGGACGACCC and reverse primer 5¢-ATGCGGATCCTTATCTAGATTCCCCTGAGTGTTCTTCCT. Ligation compatible restriction sites in each primer (XbaI/SpeI and BamHI/BglII) were used for stepwise head-to-tail additions in pKS bluescript. The 4x concatemer was then inserted into the BamHI-EcoRI sites of pGEX-2T to create pGEX-4xZFYVE (R5BD-GST).

RAB5 pull-down

Active RAB5 from cells were pulled down using the construct: pGEX-4XZFYVE-GST, purified on glutathione beads as previously described (58). Briefly, $1X10^6$ HeLa cells were seeded overnight per condition. Cells were serum starved for about 18 h. Stimulation was performed with control medium or 100 ng/ml EGF medium. Cells were lysed after the respective times with NP-40 lysis buffer (150 mM Tris pH 8.0, 50 mM NaCl, 1% NP-40) containing 1 mM PMSF, 10 ug/ml Leupeptin, 1 uM Pepstatin and 1 mM Sodium Orthovanadate (Sigma Aldrich) in the presence of 10 mM $MgCl_2$. Lysates were subject to pull-down at 4 °C with 4X-ZFYVE-GST on Glutathione beads in order to enrich for activated RAB5. Beads were then washed with the lysis buffer, boiled in Laemmli loading buffer, followed by gel electrophoresis and analysis of RAB5 by immunoblotting.

For immunoblotting, proteins were transferred to Nitrocellulose membranes overnight. The membranes were blocked with 5% milk in TBST (0.1% Tween-20) followed by incubation with primary and secondary antibodies at room temperature. The membranes were washed with TBST between the incubations and developed using the ECL plus western blotting reagent

(VWR) or scanned using a Li-Cor Odyssey scanner. Quantification of immunoblots was performed using the Li-Cor Odyssey software or ImageJ (NIH). Antibodies used for immunoblotting and their sources were Pan-RAB5 1:1000 (Abcam, #ab18211), Tubulin 1:5000 (Sigma Aldrich, #T6074-200 ul), RIN1 1:1000 (Mouse mAb, clone #C9E11, Colicelli lab, AbPro), sheep-anti-mouse-HRP 1:3000 (Amersham Biosciences, #NA931), goat-anti-rabbit-HRP 1:3000 (Kirkegaard and Perry, #4741506), goat-anti-rabbit-IRDye 800 1:5000 (Li-Cor Biosciences, #926-32211) and goat-anti-mouse-IRDye 680 1:5000 (Li-Cor Biosciences, #926-32220).

Cell migration

$1X10^6$ HeLa cells were seeded in 10 cm plates overnight in triplicate. Cells were serum starved for about 12 h. Boyden chambers (cell culture inserts, 8 μm, BD Falcon) were coated with 10 ug/ml Fibronectin overnight at 4 °C. The cell culture inserts were rinsed once in PBS and placed on 24-well plates, containing either control medium or 100 ng/ml EGF medium. Cells were harvested and seeded at 10^5 cells per ml. Cells were allowed to migrate across the transwell at 37 °C for 24 h. The chambers were then rinsed in PBS and fixed with 4% PFA. Cells that had migrated were stained with crystal violet and counted under the microscope using a hemocytometer.

CHAPTER 4. THE RAB5-GEF FUNCTION OF RIN1 REGULATES MULTIPLE STEPS DURING *Listeria monocytogenes* INFECTION

4.1 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.

4.2 INTRODUCTION

Invasive bacterial pathogens gain entry by manipulating host cell endocytic machinery (19). Bacterial proteins commandeer host factors throughout the infection process, including downstream events controlling survival, replication and spread. Depending on the bacterium, host cell entry is accomplished through macropinocytosis (226), clathrin-dependent endocytosis

(20, 227) or caveolin-mediated uptake (228). The entry processes are predominantly actin-dependent (147), reflecting the need for cytoskeletal remodeling to accommodate large cargo (15). 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 (229). *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 (230)

. In immune-compromised individuals *L. monocytogenes* can cause severe listeriosis with symptoms ranging from gastroenteritis to bacterial meningitis, and mortality rates of approximately 30% (231). In pregnant women, *L. monocytogenes* can lead to spontaneous abortions and neonatal infections (230).

L. monocytogenes uses two surface internalin (Inl) proteins to bind epithelial host cells, and utilizes clathrin-dependent mechanisms to enter these non-professional phagocytes (227). InlA interacts with CDH1 (E-cadherin) (158) while InlB interacts with MET, a receptor tyrosine kinase (232). Either internalin is sufficient for epithelial cell invasion, although both are needed for optimum entry efficiency (233). Engagement with InlB stimulates MET, leading to the recruitment and activation of signal transduction proteins including RAS (160, 163). *L. monocytogenes* infection triggers activation of the downstream RAS effectors PI3K and RAF (163, 234), 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 (162, 235).

Host cells mount a bactericidal response that also employs RAB5, in this case to fusion phagosomes with lysosomes and destroy the internalized bacterium before it can replicate (153, 168). To avoid this fate, *L. monocytogenes* uses a cytolysin to escape the phagosome and enter the cytoplasm (165) where replication takes place. The *L. monocytogenes* surface protein ActA that 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 (167, 236). 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) (168, 169). RAB5 subjugation is essential for *L. monocytogenes* to escape into the cytosol and replicate (168). 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 (58, 205). Through its VPS9 domain, RIN1 functions as a GEF with specificity for RAB5 GTPases, promoting internalization and degradation of activated receptors (99, 132). RIN1 also binds and activates ABL non-receptor tyrosine kinases that regulate actin cytoskeleton remodeling. An intricate balance between these two RIN1 effectors (RAB5 and ABL) determines the rate and route of receptor internalization. (58). The RAB5-GEF activity of RIN1 is exerted independently of RABGEF1 (a.k.a. Rabex5), another RAB5 GEF regulating endocytic processes in the cell (200).

Because *L. monocytogenes* uses a growth factor receptor tyrosine kinase to enter host cells (163), and because RAS proteins signal through RIN1 to regulate RAB5 during endocytosis (58, 99), 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.

4.3 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 (163). 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 (58). 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*

(237). 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 (121). 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 (162) 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} (128), 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 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.
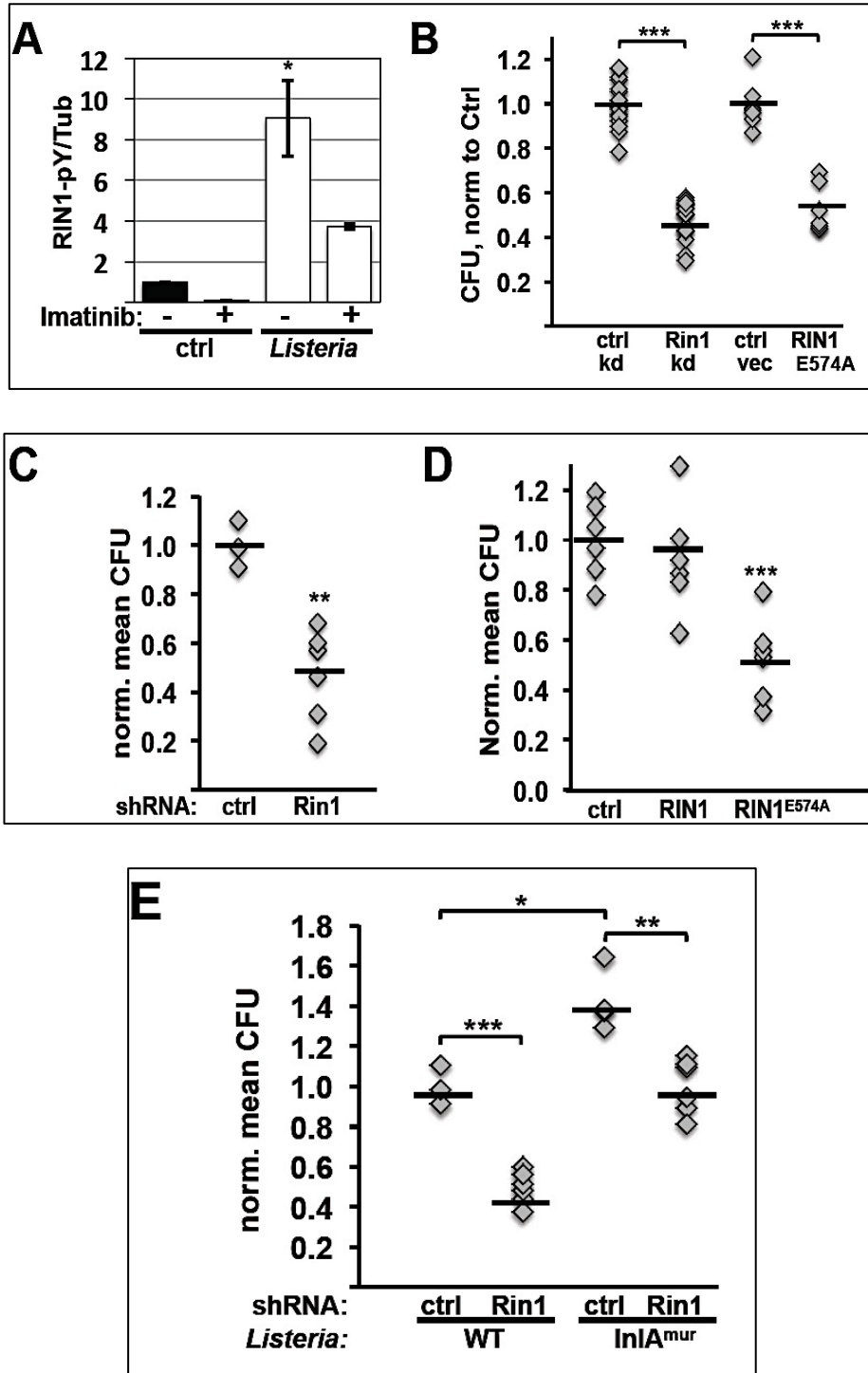
Figure 4. 1 RIN1-to-RAB5 signaling is required for efficient *L. monocytogenes* entry

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, p=1.8x10^{-16}; ctrl-vec and RIN1^{E574A} n=6, 2 independent experiments, p=2.6x10^{-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=2.3x10^{-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=6.7x10^{-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.
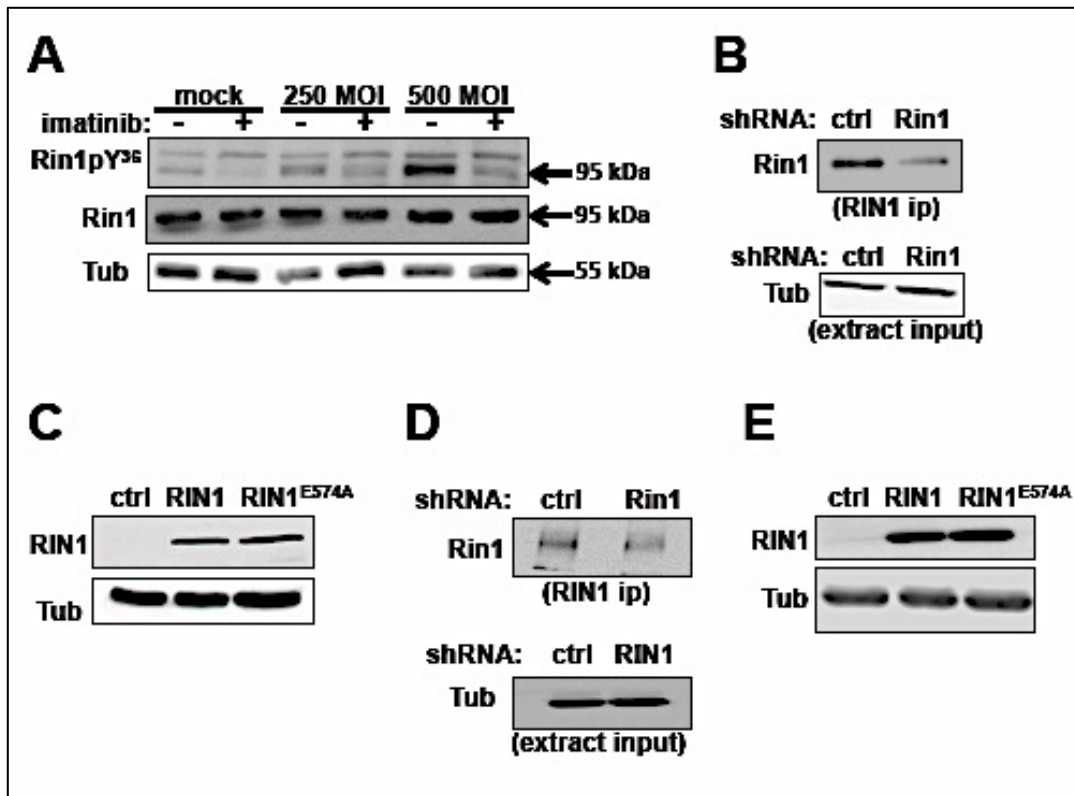
Figure S.4 1 *Listeria monocytogenes* invasion activates RIN1

A. HeLa cells were infected with *L. monocytogenes* at the indicated MOI, or mock infected. Cell lysates prepared 2.5 minutes post infection were blotted for Rin1pY^{36}, total Rin1 or tubulin. **B-E**. Immunoblot evaluation of RIN1 silencing and expression corresponding to IEC-18 cells (B and C) and IEC-6-Cdx2 cells (D and E). Cells were transduced with control, RIN1-shRNA, RIN1 or RIN1^{E574A} vectors as indicated. In panels B and D, RIN1 was first immunoprecipitated from

extracts normalized to total protein and tubulin immunoblot. RIN1 knockdown levels: ~85% in IEC-18 cells, ~60% in IEC-6 Cdx2 cells.
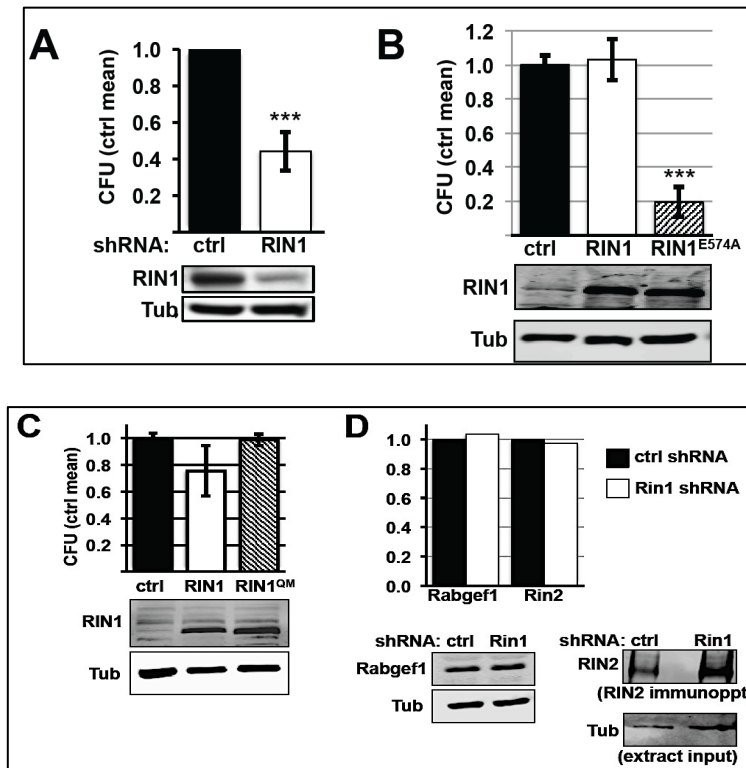
Figure S.4 2 *Listeria monocytogenes* invasion in HeLa cells

A. Control or RIN1 shRNA transduced HeLa cells were infected with *L. monocytogenes* (50 MOI) and extracellular bacteria killed using gentamicin. Cell lysate dilutions were plated on LB-Agar to assay colony-forming units (CFUs) (n=18, p=8.2x10^{-18}). Values are representative of five independent experiments. RIN1 knockdown immunoblot below. **B**. HeLa cells transduced with control, RIN1 or RIN1^{E574A} vectors were infected with *L. monocytogenes* and CFUs obtained as in A (n=3, p=0.0001). Values are representative of two independent experiments. RIN1 expression immunoblot below. **C**. IEC-18 cells transduced with control, RIN1 or RIN1^{QM} vectors were infected with *L. monocytogenes* and CFUs obtained as in A (n=3). Values are representative of two independent experiments. RIN1 expression immunoblot below. D. IEC-6 Cdx2 cells transduced with control or Rin1 shRNA vectors were lysed and probed for RIN1, RABGEF1, Rin2 and tubulin by immunoblot. Protein levels measured by immunoblot (below) and normalized to tubulin. RIN1 knockdown was approximately 60% (data not shown).

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 (158, 160). Because

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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 (238). 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 2A, 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.
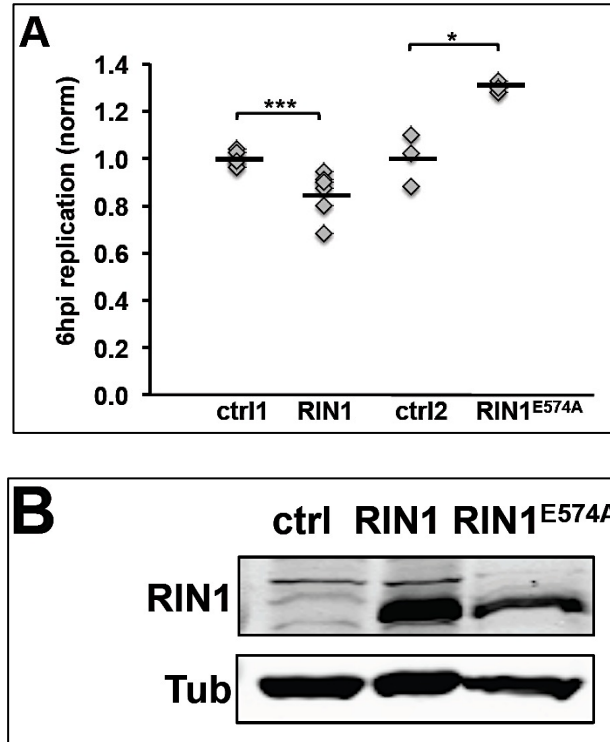
Figure 4.2. RIN1-to-RAB5 signaling inhibits *L. monocytogenes* replication.

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. Intracellular replication was measured as the fold change in CFU (6 hpi vs. 1 hpi) (ctrl1 and RIN1: n=6, p=1x10^{-30}; ctrl2 and RIN1^{E574A}: n=3, p=0.01). Values are representative of two independent experiments. **B.** Immunoblot evaluation of RIN1 expression.

Phagosome escape is a pre-requisite 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. RIN1 over-expression in IEC-18 cells led to lower totals of cytoplasmic actin-coated bacteria as early as three hours post-infection compared to the control group (Figure 3A, 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 (Figure S3).

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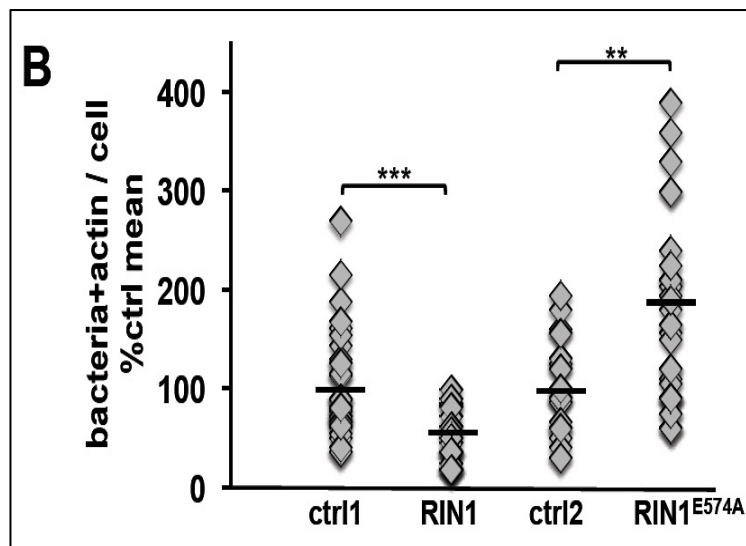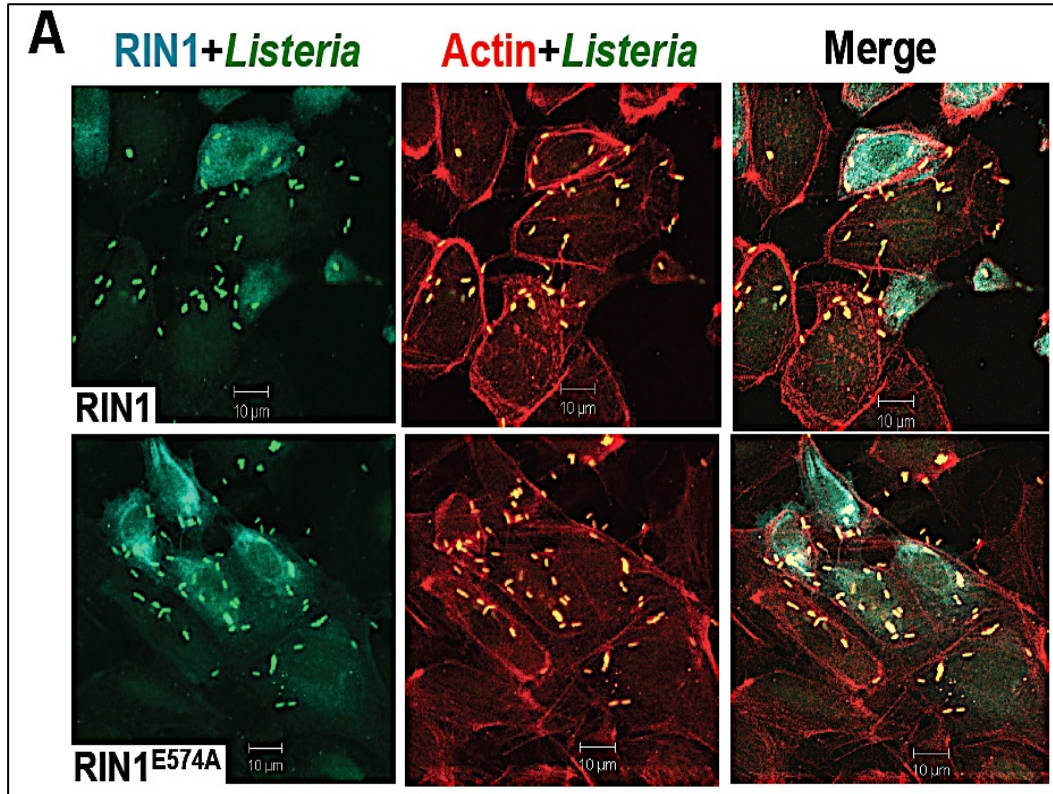
Figure 4. 3 RIN1 inhibits phagosome escape.

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. Values are representative of two independent experiments and normalized to a

percent of the control mean. (ctrl1 n=133, RIN1 n=81, p=4x10^{-6}, ctrl2 n=70, RIN1^{E574A} n=62, p=0.0012).
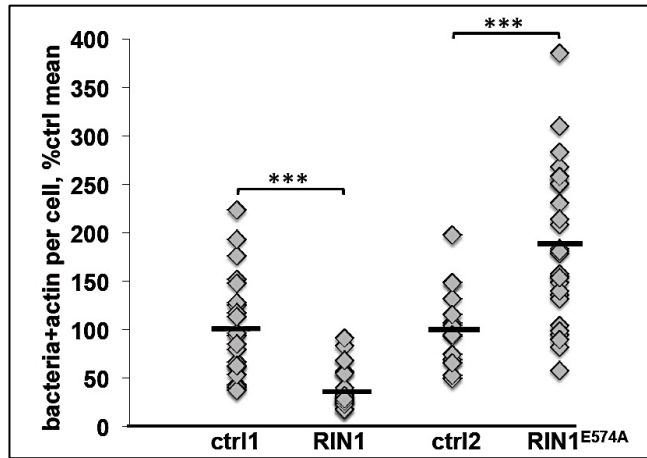
Figure S.4 3 RIN1 inhibits phagosome escape.

IEC-18 cells transduced with control, RIN1 or RIN1^{E574A} vectors were infected with *L. monocytogenes* (20 MOI) and extracellular bacteria killed with gentamicin. Cells were incubated in low gentamicin for 5 hours, fixed and stained for RIN1, actin and *L. monocytogenes* as in Fig. 3A. The number of bacteria co-localized with actin per cell was quantified in a double-blinded manner. Values are representative of two independent experiments. (ctrl1 n=147, RIN1 n=89, p=2.8X10^{-5}, ctrl2 n=45, RIN1^{E574A} n=137, p=0.0005).

Host cells eliminate *L. monocytogenes*-containing phagosomes by fusing them with lysosomes. To test whether RIN1 promotes this RAB5-dependent bactericidal pathway, we quantified the co-localization of bacteria with the lysosomal marker Lamp1 as a measure of lysosomal fusion. 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 4 A, B and C). 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. (169)
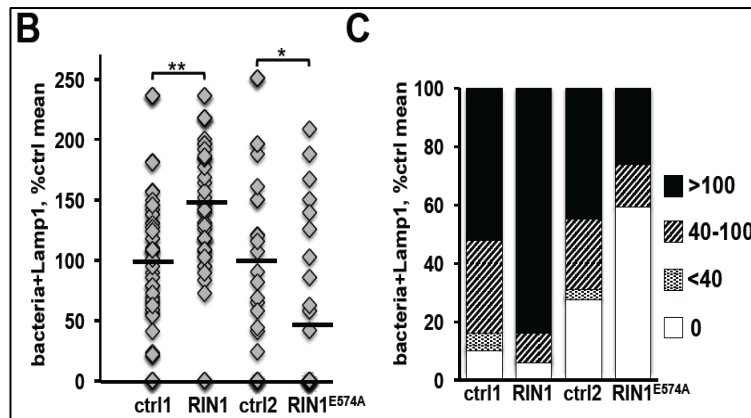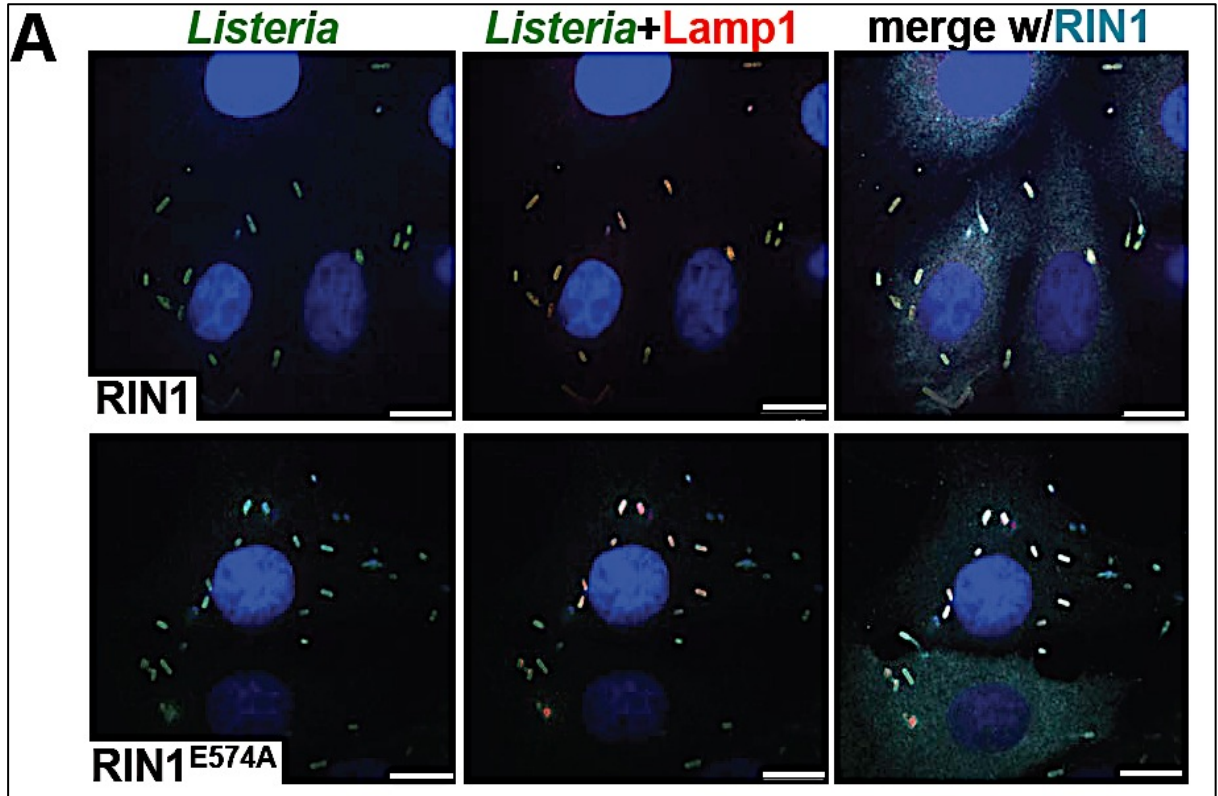
Figure 4. 4 RIN1-to-RAB5 signaling promotes lysosome fusion.

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=9x10^{-4}, ctrl2 n=161, RIN1^{E574A}=105, p=0.03).

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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 5A, Figure S4A). In contrast, RIN1 silencing (Figure S4A) or expression of RIN1^{E574A} (Figure 5A, 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 5B). The ABL-binding mutant of RIN1, RIN1^{QM}, had no effect on replication and plaque formation, when compared with wild type RIN1 (Figure S4B).

Autophagy is a distinct mechanism used by host cells to clear invasive bacteria as well as damaged intracellular organelles (239). *L. monocytogenes* normally evades host cell autophagy during pathogenesis in part by assembling an actin cloak (240, 241). 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 6A, B). 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.
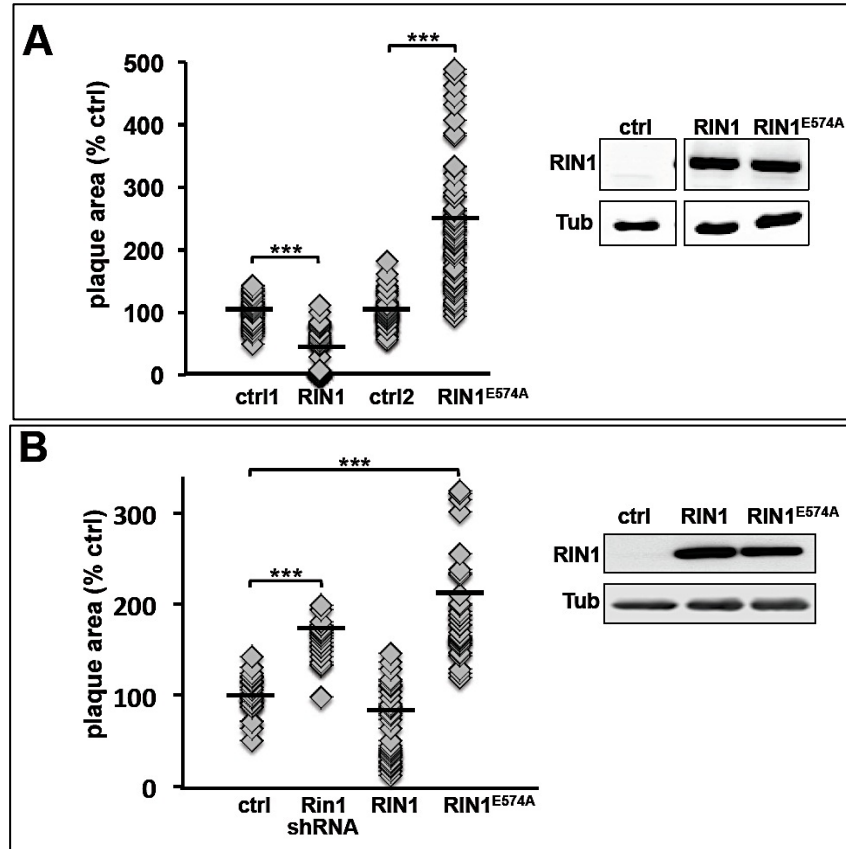
Figure 4. 5 RIN1 inhibits bacteria spread between cells.

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=3x10^{-19}; ctrl2 n=65, RIN1^{E574A} n=75, p= 2x10^{-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=7.1x10^{-13}, RIN1 n=36, p=0.0001, RIN1^{E574A} n=37, p=1.9x10^{-12}). Values were obtained from two independent experiments. Expression confirmed by immunoblot (right).

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 (242),

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.
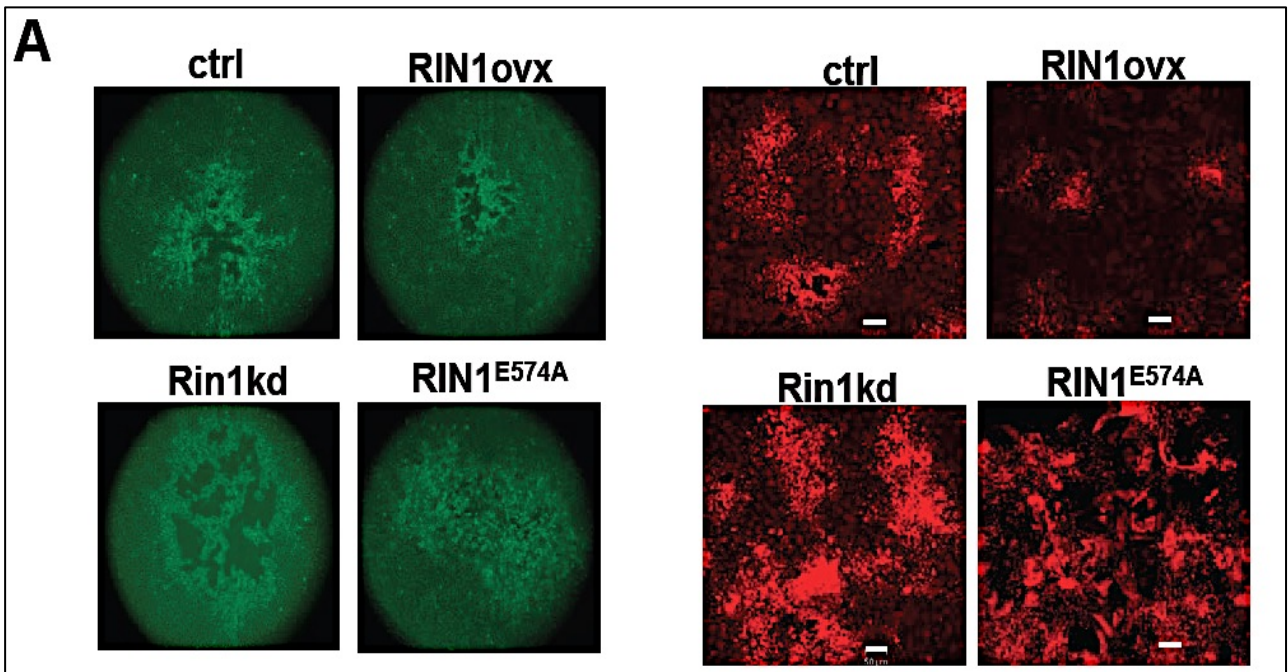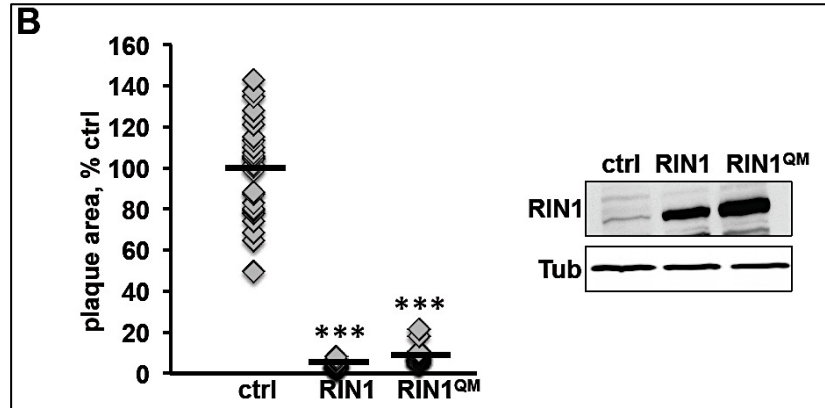
Figure S.4 4 RIN1 inhibits bacteria spread between cells.

RIN1 inhibits cell spread **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-shRNA, RIN1 or RIN1^{E574A} vectors in the presence of 10 μg/ml gentamicin. The monolayer was then overlaid with DMEM+0.7% agarose and incubated for 48 hours. Left: plaques visualized by light microscopy. Right: Cells fixed and stained for *L. monocytogenes,* 8 hpi (red). Scale bar: 50 μm. Rin1 knockdown was ~80% by immunoblot. **B**. Plaque assay performed as in A using IEC-18 cells transduced with control, RIN1 or RIN1^{QM} vector. After 48 hours plaque areas were measured using ImageJ (NIH). Each point is the area of a single plaque, normalized to control mean (ctrl n=26, RIN1 n=23, p=8x10^{-24} RIN1^{QM} n=29, p=9x10^{-27}). Values are representative of two independent experiments. RIN1 expression was confirmed by immunoblot (right).
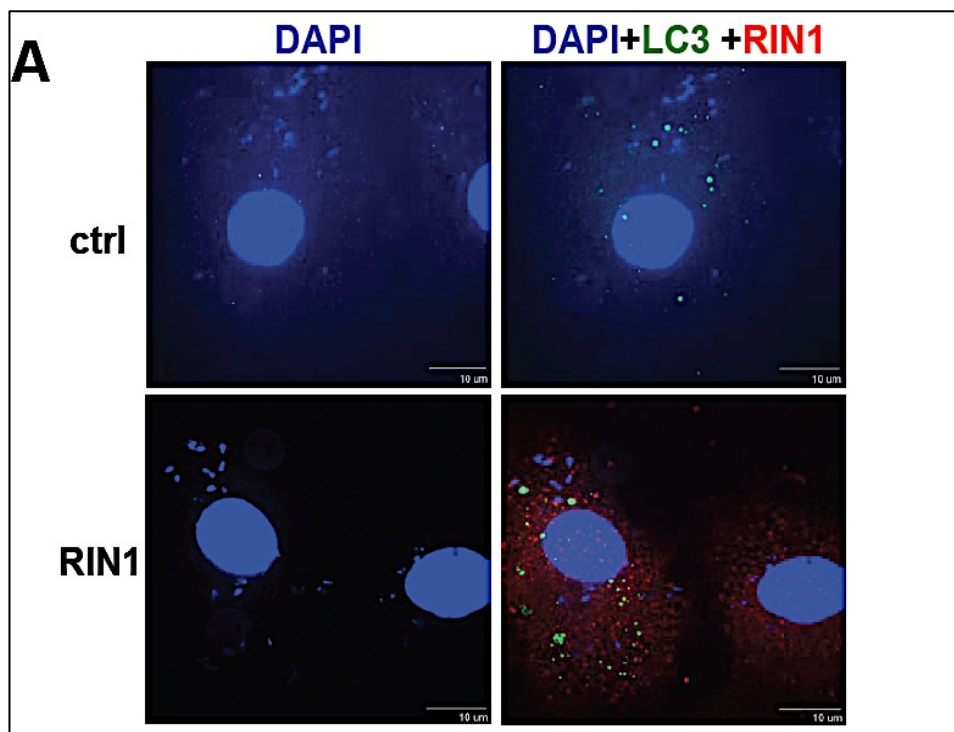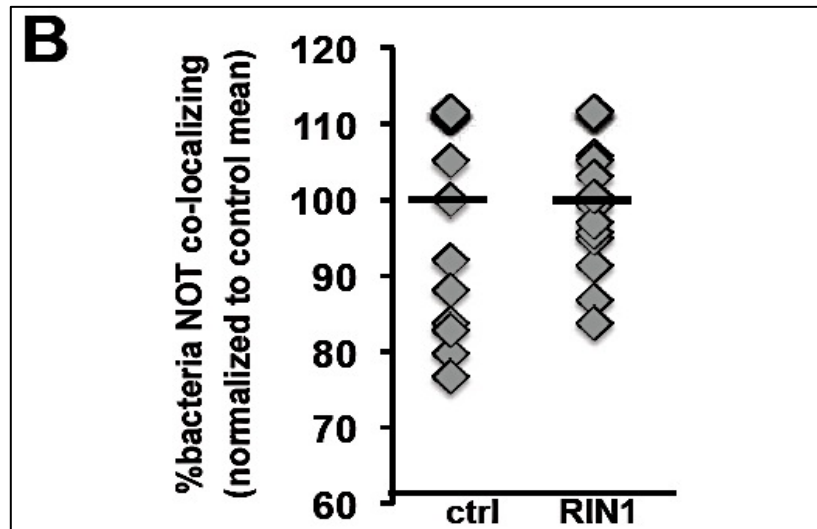
Figure 4. 6. Autophagic clearance is unchanged in RIN1 over-expression cells.

A. IEC-6 Cdx2 cells transduced with control or RIN1 vectors were infected with *L. monocytogenes* (20 MOI) and extracellular bacteria killed with gentamicin. Cells were incubated in low gentamicin medium for 1 hour, fixed and stained for RIN1 (red) and LC3 autophagy marker (green). DAPI was used to stain host cell nuclei and *L. monocytogenes* (blue). Scale bar: 10 μm. **B.** Double blind quantification of bacteria per cell not co-localized with LC3 normalized to control mean. Horizontal bars show mean of each group (n=20).
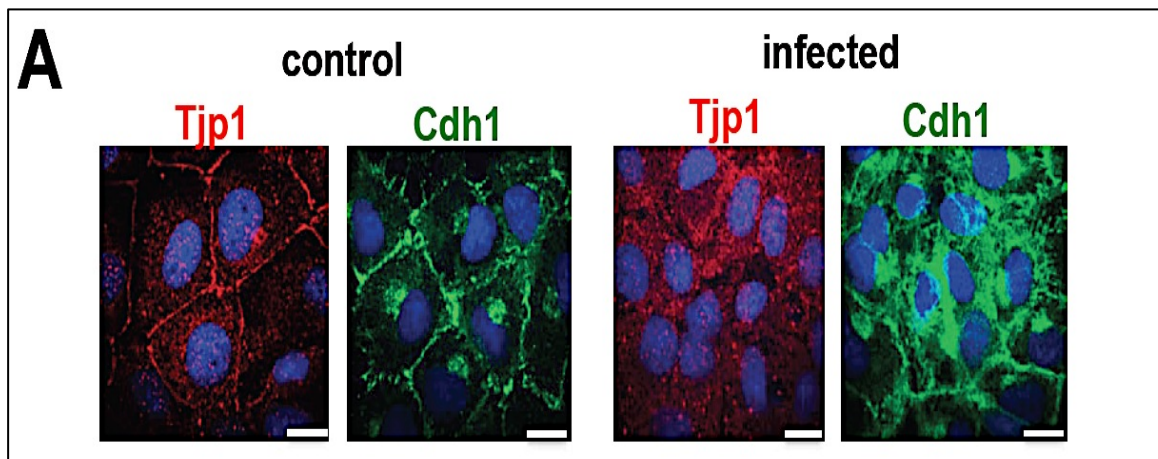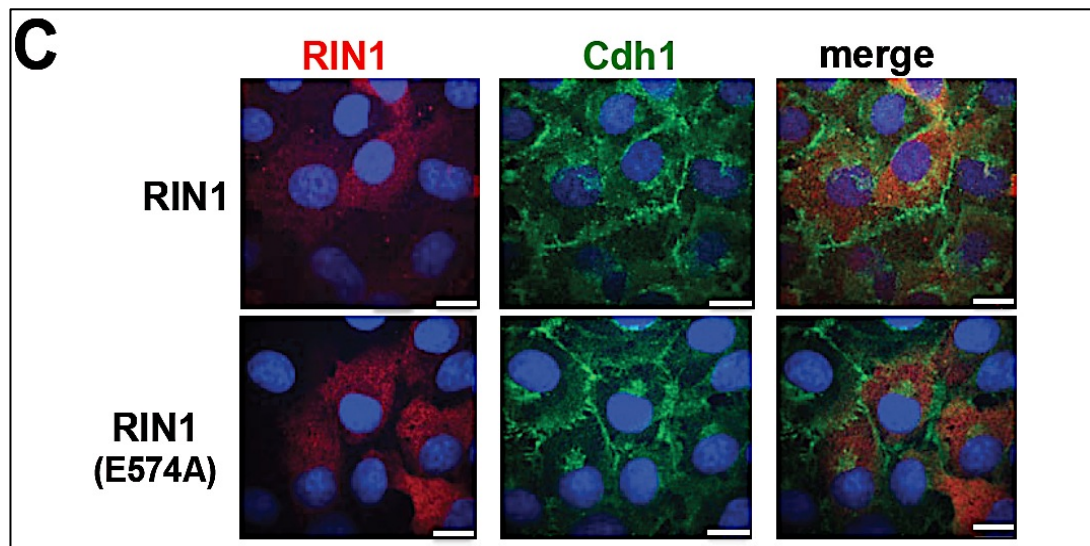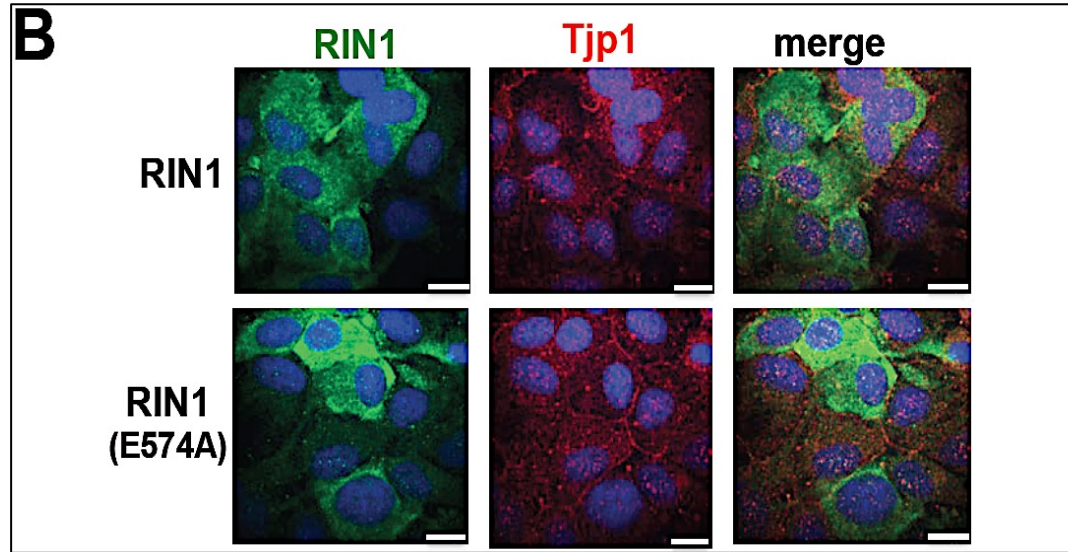
Figure S.4 5 RIN1 does not alter junction organization between IEC-6 Cdx2 cells

A. Cells were infected or not with *L. monocytogenes* (20 MOI, 30 min), fixed and stained for Tjp1 (ZO-1) in red or Cdh1 (E-cadherin) in green. **B**. Cells transduced with RIN1 or RIN1[E574A] were infected with *L. monocytogenes* (20 MOI, 30 min), fixed and stained for RIN1 (green) and Tjp1 (red). **C.** Cells transduced and infected as in B were fixed and stained for RIN1 (red) and Cdh1 (green).
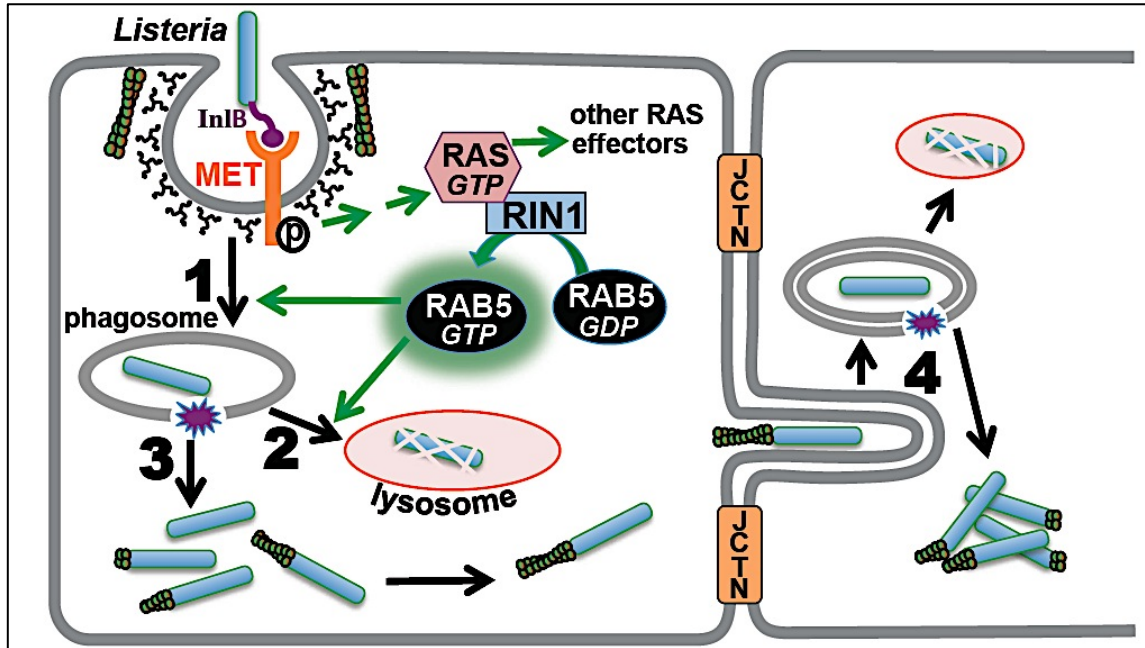
Figure 4. 7. RIN1 is a RAB5 activator that regulates _L. monocytogenes_ infection.

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.

4.4 DISCUSSION

A common feature of bacterial invasion mechanisms is the commandeering of trafficking GTPases, especially RAB5, by pathogen-encoded proteins (105). 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 (152, 243, 244), 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_ (243), but this was not examined further.

During *L. monocytogenes* invasion, InlB-mediated entry is dependent on RAB5 activation (162), but the bacterium must subsequently block RAB5 activation in order to evade clearance by fusion of its phagosome shelter with the lysosome (169). 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 (242), RIN1 alterations had no apparent effect on the stability of cell-junctions. 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 (233, 245). 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 (105). A few pathogens encode their own GEF and/or GAP activities for modulating small-GTPases (226, 246), 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 immune-compromised individuals and pregnant women underscores the need for early treatments that effectively prevent the occurrence of septicemia, bacterial meningitis, spontaneous abortion or stillbirths (247). 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 (248).

4.5 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 (216), (128). Lentivirus generation and transduction were performed as previously described (205). 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) (237) 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. 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. *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) (217). 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:

CCGGCTCCTGTTAGAAGCTGAGTATCTCGAGATACTCAGCTTCTAACAGGAGTTTTT G.

Expression levels were evaluated by western blotting.

Gentamicin Protection Assay

L. monocytogenes (10403S or EGDe) invasion was measured by Gentamicin protection assays. $0.75X10^6$ 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 μg/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, 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.

Immunoprecipitation, immunoblotting and immunofluorescence

Antibodies used for immunoblotting and their sources were- Tubulin (Sigma Aldrich, #T6074-200UL), RIN1 (Mouse mAb, clone #C9E11, Colicelli lab, AbPro), pY^{36} RIN1 (127)

(Rabbit pAb, Colicelli Lab, Biosource International), ZO-1 (Zymed, #61-7300), Rabex-5 (Sigma Aldrich, #R5405), RIN2 (Genetex, #GTX117153), E-Cadherin (BD Transduction Laboratories, #610181), 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 antibody (mouse mAb, clone B9A12, Colicelli Lab) and RIN2 (Rabbit pAb, Colicelli Lab) 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), ZO-1 (Zymed, #61-7300), LAMP1 (Santa Cruz, #SC-19992), phalloidin-rhodamine 0.2 uM (Invitrogen), 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

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). Cells were then incubated with primary and secondary antibodies at room temperature. Fluorescently labeled phalloidin was added along with the secondary antibody staining. 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 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.

CHAPTER 5. CONCLUSION, SIGNIFICANCE AND DISCUSSION

CONCLUSIONS

5.1 RIN1 AND REGULATION OF RAB5 ACTIVITY IN CELLULAR ENDOCYTIC PROCESSES

In growth factor signaling

In this study we show that RIN1 regulates receptor trafficking through ABL and RAB5. RIN1→RAB5 signaling, in the absence of ABL signaling, promotes receptor endocytosis by a mechanism that closely resembles macropinocytosis and promotes degradation. On the other hand, RIN1→ABL signaling in the absence of RAB5 signaling favors recycling of the receptor. An intricate balance between RIN1 effector functions is essential to determine the route and rate of receptor entry and fate upon internalization (58). We also show that RIN1→RAB5 signaling promotes cell migration towards growth factor (57).

These mechanisms point towards RIN1 and its effector interactions being likely therapeutic targets in cancers that exhibit aberrant receptor trafficking.

In pathogenesis

We analyzed the effect of RIN1 signaling on endocytosis of bacteria, using *Listeria monocytogenes* as a model. We show that RIN1→RAB5 signaling facilitates bacterial invasion. Interestingly, RIN1→RAB5 signaling also accelerates fusion of phagosomes to lysosomes leading to faster bacterial clearance in epithelial cells. Influence of RIN1→RAB5 signaling on bacterial invasion and clearance could apply to several other pathogens, which exploit or block RAB5 signaling in the cell.

SIGNIFICANCE AND DISCUSSION

5.2 RECEPTOR TYROSINE KINASES (RTK) IN CANCER

Aberrant expression of Receptor Tyrosine Kinases in cancer

Aberrant expression and trafficking of RTKs has been characterized in a number of cancers. In some cases, the abnormal regulation is due to abnormal trafficking or mutations in signaling proteins downstream of the growth factor receptor (98, 249). However, in a large number of cases, the anomaly is due to point mutations or deletions on the receptor itself, making it a constitutively active kinase or to spontaneously dimerize, independent of growth factor stimulation (67, 69, 250).

Kinase inhibitors in cancer therapy

Kinase inhibitors have long been used as a means for treating cancers bearing aberrant RTK signaling. In the recent times it has come to light that a rather huge subset of these cancers are non-responsive or resistant to these inhibitors. Point mutations in the kinase domain make the receptors resistant to such inhibitors (251, 252).

Targeting downstream signaling molecules

Downstream signaling molecules, example, RAF or mTOR, have turned out to be relevant targets to down regulate signaling from the receptor (98). While small molecule inhibitors are being developed for these downstream signaling molecules, recent focus has turned to vesicle trafficking machinery (253). Receptor internalization and trafficking proteins have been shown to play an essential role in regulating cellular signaling and compartmentalization of signaling responses. Among the trafficking proteins, RAB GTPases and cytoskeletal remodeling have emerged as the crux of the trafficking process (254).

RAB GTPases and regulators as therapeutic targets in cancer

RAB GTPases localize at the plasma membrane or membranes of intracellular organelles and drive vesicle fusion. There are several RAB GTPases that take part in the trafficking process. Depending on their localization, they drive different stages of receptor trafficking (255).

RAB5 is involved in fusion of endocytic vesicles and formation of early endosomes (256). Defect in RAB5 activity affects both endocytosis of the receptor and trafficking to the late endosomes and lysosomes, leading to prolonged signaling. Excess RAB5 activity on the other hand leads to enhanced internalization and degradation of the receptor, down regulating signaling, making it essential to tease out the role of its cellular regulators (257). RIN1 is a prominent RAB5 regulator as seen by evidence that RIN1 is required upstream to recruit the other RAB5-GEFs such as RABEX-5 to down regulate RAS signaling (200). Characterizing further the differential functions of RIN1 in various cancers is essential to effectively utilize the molecule as a therapeutic target.

Role of RIN1 in stimulating macropinocytosis as shown by this study is directly relevant to cancer cells utilizing this internalization mechanism to take in proteins as a source of amino acids (65). This phenomenon, mediated by RAS, is required to meet the metabolic needs of the rapidly proliferating cancer cells. It is likely that this fundamental need for macropinocytic uptake of proteins by certain cancer cells is carried out through the RAS→RAB5→RIN1 pathway. The requirement of RIN1 and its RAB5-GEF function in this process needs to be addressed. If RIN1 is indeed involved in macropinocytic uptake of proteins in cancer cells, then use of inhibitors to abolish the uptake could be considered as an option for development of therapeutics against the specific cancers.

5.3 DISRUPTING HOST-PATHOGEN INTERACTIONS: *AN EFFICIENT THERAPEUTIC REGIMEN*

Antibiotic resistance- a growing problem in bacterial infection

Antibiotic resistance to some broad-spectrum antibiotics has been a common problem among several bacterial pathogens (258-260). Poor patient compliance, prescription errors and defective drugs have led to emergence of multidrug-resistant strains among various classes of pathogenic bacteria.

One of the best methods to deal with such antibiotic resistant strains is to target host-pathogen interactions. Although bacterial proteins have been well characterized in pathogenesis, not many host proteins that are involved in infection are known. Characterizing host-pathogen interactions is the need of the hour to design effective and efficient therapies against pathogens.

Targeting RAB-GTPases in pathogenesis

A large number of pathogens utilize the RAB-GTPases at various stages in the cell to direct the pathogen containing vacuole to a compartment favorable for survival. RAB1, RAB5, RAB35, RAB7 have all been implicated in exploitation by pathogens (261). Interference of RAB-mediated trafficking is likely to serve as an effective therapeutic regimen in various bacterial infections.

RAB5 has specially been implicated in pathogenesis of several bacteria (162, 243, 262) and viruses (242, 263-266). It functions as the gatekeeper of the cell controlling the entry of these pathogens into the host. It seems only fitting to analyze the role of host RAB5 regulators to screen for compounds that disrupt or enhance these interactions.

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5.4 OVERALL SUMMARY AND FUTURE DIRECTIONS

In summary, we have shown the importance of cellular trafficking machinery in growth factor signaling and pathogenesis. This study has also led to a number of impending questions about the differential role of RIN1 effectors in cellular endocytosis and pathogenesis.

Mechanistic studies of RIN1 effectors in receptor trafficking

Differential roles of RIN1's effectors in receptor trafficking has led to impending questions of the precise mechanism by which ABL and RAB5 promote recycling and degradation respectively. ABL effectors that take part in this process remain unknown need to be characterized. ABI1 has previously been characterized in forming differential protein complexes depending on its phosphorylation status by ABL (214, 267). The role of RIN1 in modulating ABI1 complex formation needs to be characterized. RAB5, on the other hand, has also been studied in the context of macropinocytosis, linking RAS activation to RAC1 (64). RIN1 is likely a mediator of this signaling from RAS to RAC1.

Analysis of other RIN family members

A detailed analysis of the other RIN family members such as RIN2 and RIN3 will be beneficial in determining the importance in trafficking in the respective cells that they are enriched in. For example, RIN3 plays a major role in KIT trafficking in mast cells (140) and hence its role in mastocytosis remains to be dissected. Similary, RIN2 has been implicated in cell adhesion by regulating trafficking of E-cadherin (134). The major function of RIN2 in E-cadherin regulation in EMT and receptor trafficking in epithelial cells needs to be addressed.

RAB5 and cytoskeletal remodeling in pathogenesis

Several pathogens rely specifically on RAB5 activation to gain entry into host cells (242). Further examination into the signaling pathways is required to identify the host effectors that

bridge RAB5 activation to cytoskeletal remodeling. Inhibition of pathogen entry can help prevent infection at an earlier stage. This is especially effective in case of infections that affect immuno-compromised individuals and pregnancy-induced immunosuppression.

RIN1→RAB5 signaling in intercellular junctions

Role of RIN family members in regulation of intercellular junctions has to be addressed. RAB5 regulates intercellular junctions in a number of contexts (242). These junctions play a major role both in cancer metastasis and in pathogen cell spread. Regulation by RIN1 needs to be elucidated in various conditions including cell adhesion following division, metastasis and cell spread.

RAB5 activation by small molecules

The above study points towards the possibility of utilizing the RIN1→RAB5 axis as a modulator of EGFR trafficking in ERBB2 or EGFR over-expressing cancers. Determining the effect of RAB5 in internalization of EGFRvIII, a major variant of EGFR, which occurs in glioblastoma (250) would be informative from a therapeutic point of view. Small molecules that activate RAB5 (254) could also likely help in faster degradation of bacteria that manipulate the endosomal maturation pathway. Small molecules that mimic the effect of RAB5 GEFs would be an effective method to target and down regulate growth factor signaling and accelerate pathogen degradation.

RIN1→RAB5 signaling in vaccine vector delivery

Delivery of vaccines against chronic diseases like cancer using attenuated pathogens such as *L. monocytogenes* that efficiently invade host cells has been in the limelight over the last decade (248, 268-270). Our study shows the importance of RIN1→RAB5 signaling downstream

of RAS in bacterial entry. This makes RIN1 a protein of interest whose signaling could be modified to enable efficient delivery of such vectors.

Overall, our study has opened up new avenues to study the RAB5-GEF RIN1 and the roles played by the signaling pathways downstream in receptor trafficking and other cellular endocytic processes. Further mechanistic studies can lead the way to identification of disease-relevant markers and the development of effective therapeutics.

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