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***Salmonella typhimurium* usurps the scaffold protein IQGAP1 to manipulate Rac1 and MAP kinase signaling**

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Synopsis

Salmonella typhimurium invades eukaryotic cells by re-arranging the host cell cytoskeleton. However, the precise mechanisms by which *Salmonella* induces cytoskeletal changes remain undefined. IQGAP1 is a scaffold protein that binds multiple proteins, including actin, the Rho GTPases Rac1 and Cdc42, and components of the mitogen activated protein kinase (MAPK) pathway. We have previously shown that optimal invasion of *Salmonella* into HeLa cells requires IQGAP1. Here, we use IQGAP1-null mouse embryonic fibroblasts (MEFs) and selected, well-characterized IQGAP1 mutant constructs to dissect the molecular determinants of *Salmonella* invasion. Knockout of IQGAP1 expression reduced *Salmonella* invasion into MEFs by 75%. Reconstituting IQGAP1-null MEFs with wild-type IQGAP1 completely rescued invasion. By contrast, reconstituting IQGAP1-null cells with mutant IQGAP1 constructs that specifically lack binding to either Cdc42 or Rac1 (termed IQGAP1 MK24), actin, MAPK kinase (MEK) or extracellular-regulated kinase (ERK) partially restored *Salmonella* entry. Cell-permeable inhibitors of Rac1 activation or MAPK signaling reduced *Salmonella* invasion into control cells by 50%, but had no effect on bacterial entry into IQGAP1-null MEFs. Importantly, the ability of IQGAP1 MK24 to promote *Salmonella* invasion into IQGAP1-null cells was abrogated by chemical inhibition of MAPK signaling. Collectively, these data imply that the scaffolding function of IQGAP1, which integrates Rac1 and MAPK signaling, is usurped by *Salmonella* to invade fibroblasts and suggest that IQGAP1 may be a potential therapeutic target for *Salmonella* pathogenesis.

Introduction

Salmonella typhimurium is a highly virulent, gram-negative pathogen that causes severe systemic disease, including gastroenteritis and typhoid fever in humans [1, 2]. During infection, *Salmonella* usurps host cell signaling pathways, particularly those that regulate the actin cytoskeleton [3, 4]. *Salmonella* is equipped with a type three secretion system (T3SS)

that injects host cells with several bacterial proteins [5]. These include SopE and SopE2, which mimic the function of guanine nucleotide exchange factors (GEFs) and activate the Rho GTPases Rac1 and Cdc42 by stimulating the exchange of GDP for GTP [6, 7]. Active Rac1 and Cdc42 induce the activation of the neuronal Wiskott Aldrich Syndrome protein (N-WASP), the WASP family member 2 (WAVE2) and the actin-related protein (Arp2/3) complex, which triggers actin polymerization and membrane ruffling [8–10]. The generation of membrane ruffles substantially facilitates bacterial invasion into host cells [3, 4]. After entry, *Salmonella* inactivates Rac1 and Cdc42 using SptP, a GTPase activating protein (GAP) that helps restore the host cell's original cytoskeletal architecture [3]. While it is generally accepted that Rho GTPases participate in *Salmonella* invasion, the exact roles of Rac1 and Cdc42 during *Salmonella* uptake are unclear. For example, Chen *et al.* [11] reported decreased *Salmonella* invasion into COS-1 cells expressing a dominant negative Cdc42 construct, suggesting that Cdc42 is the pivotal GTPase manipulated during host cell invasion. However, the same group showed that *Salmonella* invasion into COS-2 fibroblasts and intestinal Henle 407 cells was abrogated following siRNA-mediated knockdown of Rac1, but not Cdc42, indicating that Rac1 is the more important small GTPase for *Salmonella* entry [12]. Another group observed that siRNA-mediated knockdown of Rac1 and Cdc42 had no significant effect on *Salmonella* invasion into human foreskin fibroblasts [13]. While some of the discrepant data have been ascribed to differences among cell types, these studies indicate that the mechanisms underlying Rac1 and Cdc42 function in *Salmonella* pathogenesis are incompletely understood.

The mitogen activated protein kinase (MAPK) pathway relays extracellular signals to various intracellular targets, including the actin cytoskeleton [14–16]. The most extensively studied module of the MAPK pathway is the MAPK kinase/extracellular-regulated kinase (MEK/ERK) cascade. In this cascade, extracellular stimuli induce activation of the small GTPase Ras, which activates B-Raf. B-Raf then phosphorylates and activates MEK, resulting in phosphorylation of ERK [16]. The MEK/ERK pathway regulates cell adhesion and motility, processes that are governed by changes in the actin cytoskeleton [14]. Importantly, *Salmonella* stimulates MAPK activation in host cells [17–19] and treatment of cells with the MEK inhibitor PD98059 reduces *Salmonella* uptake [13, 19]. These findings suggest that *Salmonella* may also target the actin cytoskeleton via the MAPK cascade to achieve infection, although the precise mechanism by which this occurs is unknown.

IQGAP1 is a ubiquitously expressed 189-kDa protein that is a pivotal element of cytoskeletal architecture and function [20, 21]. IQGAP1 crosslinks actin filaments [22, 23] and influences actin assembly both by virtue of its association with actin, N-WASP and the Arp 2/3 complex [24] and by modulating the active state of Rac1 and Cdc42 [25, 26]. Despite its name, IQGAP1 is not a GAP, but preferentially binds to activated Rac1 and Cdc42, stabilizing the GTPases in their active forms [26, 27]. In addition, IQGAP1 binds to numerous other proteins, including actin, calmodulin and growth factor receptors [28]. It has become apparent that IQGAP1 functions as a scaffold, integrating diverse signaling pathways [28]. For example, IQGAP1 binds to and regulates the activation of B-Raf [29], MEK [30] and ERK [31], thereby facilitating MAPK signaling. A recently uncovered role for IQGAP1 is in microbial pathogenesis [32]. Published evidence has demonstrated that

Salmonella manipulates IQGAP1 to invade HeLa cells [33] and enteropathogenic *Escherichia coli* (EPEC) requires IQGAP1 to form actin pedestals in host cells [34]. Moreover, the *Salmonella* effector SseI binds directly to IQGAP1 and exploits IQGAP1 to reduce macrophage motility, thereby promoting chronic infection [35]. In order to further elucidate the molecular mechanisms by which *Salmonella* enters host cells, we reconstituted IQGAP1-null mouse embryonic fibroblasts (MEFs) with selected IQGAP1 mutant constructs that lack binding to specific target proteins. Using this approach, we report a novel IQGAP1-dependent mechanism for *Salmonella* invasion that involves both Rac1 and MAPK signaling.

Experimental

Reagents

All cell culture reagents were obtained from Invitrogen. The IQGAP1 polyclonal antibody has been previously described [36]. Monoclonal antibodies used were anti-Rac1, anti-Cdc42 (BD Biosciences), anti-ERK, anti-phospho-ERK (Cell Signaling) and anti-beta-tubulin (Sigma). Anti-mouse and anti-rabbit secondary antibodies (conjugated to horseradish peroxidase) were obtained from GE Healthcare. Fluorescent polystyrene beads were purchased from Polysciences. Bovine collagen solution and bovine serum albumin (BSA) were obtained from Sigma and Invitrogen, respectively. The enhanced green fluorescent protein (pEGFP) vector was obtained from Clontech. The mutant IQGAP1 constructs that lack binding to Rac1/Cdc42 (IQGAP1 MK24), actin (IQGAP1-G75Q), ERK (IQGAP1 WW) or MEK (IQGAP1 IQ) have been described previously [37–39]. The mutant IQGAP1 construct that binds neither Rac1/Cdc42 nor actin (IQGAP1-G75Q MK24) was prepared in two steps. Initially, PCR was performed using IQGAP1 in pcDNA3 as a template, with the following primers:

GCTGTACAAGTCCGCCGACGAGGTTGACG (forward) and TTCAGCAAGATCACTGTGGTAAG (reverse). The forward primer included the BSRG1 restriction site. The PCR product was cut with BSRG1 and Pac1 restriction enzymes. pEGFP-IQGAP1 was digested with BSRG1 and Pac1 and the PCR product was ligated into the pEGFP-IQGAP1 plasmid, yielding pEGFP-IQGAP1-G75Q. IQGAP1-G75Q MK24 was prepared by ligating the Pac1/Spe1 fragment of pcDNA3-IQGAP1 MK24 into the Pac1/Spe1 digested product of pEGFP-IQGAP1-G75Q. The sequence of all constructs was confirmed by dye-terminator sequencing. The constitutively active (Q61L) Rac1 and Cdc42 constructs were a generous gift from Anne Ridley (King's College, London, UK). Trans-IT transfection reagent was purchased from Mirus Bio. Glutathione-S-transferase (GST)-tagged fragments of the Cdc42/Rac interacting binding region of p21 activated kinase (GST-PAK-CRIB) and the GTPase binding domain of WASP (GST-WASP-GBD) were purified from bacterial lysates as described previously [25]. Chemical inhibitors of Rac1 (NSC23766) and MEK1 (PD98059) were purchased from Calbiochem. Guanosine triphosphate gamma S (GTPγS) was obtained from Cytoskeleton.

Cell culture and transfection

MEFs were obtained from IQGAP1-knockout mice and control mice, and immortalized as described [29]. MEFs were cultured in Dulbecco's modified Eagle's medium supplemented

with 10% (v/v) fetal bovine serum and 5% (v/v) antibiotic solution (Invitrogen). Wild-type and mutant IQGAP1 cDNA constructs were transiently transfected into IQGAP1-null MEFs using Trans-IT transfection reagent according to the manufacturer's instructions. To optimize protein expression, the transfection was performed twice on consecutive days.

Bacterial quantification

Ds-Red-tagged *Salmonella* (strain SL1344, a gift from Lynn Bry, Brigham and Women's Hospital, Boston, MA) was used for all experiments. Bacteria were grown for 16 h at 37°C with shaking prior to subculture at a dilution of 1:10 in fresh Luria-Bertani medium and incubated at 37°C with shaking until an optical density (at 600 nm) of 0.5 was reached. The culture was serially diluted and plated in triplicate onto MacConkey agar plates and incubated at 37°C overnight. The number of *Salmonella* present per mL of culture was determined based on calculations using the number of colony-forming units and the dilution factor.

Gentamycin protection assay

Salmonella invasion was quantified using a gentamycin protection assay [33]. MEFs were infected with *Salmonella* at a multiplicity of infection (MOI) of 30 for 30 min. Cells were then washed with phosphate buffered saline (PBS) and incubated with gentamycin (Gibco) (50 µg/mL) to kill extracellular bacteria. After extensive washing, cells were lysed in 1% Triton X-100. The number of bacteria was quantified by serial dilution, as described above.

Immunocytochemical analysis of *Salmonella* invasion

MEFs grown on glass coverslips were infected with DS-Red-tagged *Salmonella* at an MOI of 30 for 30 min, then incubated with gentamycin (50 µg/mL) to kill extracellular bacteria [40]. After extensive washing, cells were fixed in 4% paraformaldehyde (Fisher Scientific) for 20 min, permeabilized in 0.1% Triton X, and stained with Alexa-647 phalloidin (Molecular Probes) to visualize actin. Coverslips were washed with PBS, mounted and analyzed using a Zeiss spinning disk confocal microscope and SlideBook software. *Salmonella* invasion was assessed by (1) counting the number of cells containing one or more intracellular bacteria and (2) by quantifying the average number of intracellular bacteria per cell. At least 50 cells from a minimum of three fields of view were analyzed per experiment. Moreover, each experiment was performed at least three times.

Bead internalization assay

Fluorescein isothiocyanate (FITC)-conjugated polystyrene beads (2 µm diameter) were coated with fibrillar collagen or 3% (v/v) BSA essentially as previously described [41, 42]. MEFs cultured in 6-well plates were incubated overnight with coated beads at a ratio of 6 beads per cell, to allow binding and internalization of the beads. BSA-coated beads bind to the cell surface and are phagocytosed in a non-specific manner, while collagen-coated beads are taken up through cell surface $\beta 1$ integrins. Extracellular fluorescence was quenched with trypan blue; the fluorescence from internalized beads was analyzed by flow cytometry. Internalization was quantified based on the percentage of cells with one or more internalized beads. Approximately 10,000 cells were analyzed in each experiment for each condition.

Rac1 and Cdc42 GTPase assay and ERK activation assay

Assays to determine the amounts of active Rac1 and Cdc42 were performed essentially as previously described [25, 26, 43]. Following *Salmonella* infection, control and IQGAP1-null MEFs were lysed in buffer A (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EGTA and 1% Triton X-100) containing protease inhibitors. Equal amounts of protein lysate were incubated with GST-PAK-CRIB (for Rac1) or GST-WASP-GBD (for Cdc42) for 3 h at 4°C. Glutathione beads were used to pull down active, GTP-bound Rac1 or Cdc42 and washed with buffer A. Samples were resolved with SDS-PAGE and immunoblotted for Rac1 or Cdc42. In some experiments, protein lysates were pre-incubated with GTP γ S as a positive control. To determine the amount of active ERK, control and IQGAP1-null MEFs were lysed and equal amounts of protein lysate were resolved with SDS-PAGE and immunoblotted for phospho-ERK.

Statistical analysis

Statistical analysis was performed using SPSS software. One-way analysis of variance (ANOVA) and Bonferroni post-hoc multiple comparison tests were used to test the effect of IQGAP1 expression on bacterial invasion. Statistical significance was set at $p < 0.05$.

Results

Maximal invasion of *Salmonella* into MEFs requires IQGAP1

To further our understanding of the molecular basis of *Salmonella* infection, we used cells from IQGAP1-null mice. Knockout of IQGAP1 was verified by Western blotting (Fig. 1A). To test the hypothesis that IQGAP1 is required for optimal *Salmonella* invasion, MEFs from control (+/+) and IQGAP1-null (-/-) mice were infected with DS-Red-tagged *Salmonella*. Microscopic analysis revealed that control MEFs harbored considerably more intracellular *Salmonella* than IQGAP1-null MEFs (Fig. 1B). The extent of *Salmonella* invasion was quantified by three different methods. (In all assays, control MEFs were compared to IQGAP1-null MEFs). First, the mean number of intracellular bacteria was evaluated by confocal microscopy (Fig. 1D). Second, the percentage of cells harboring at least one intracellular *Salmonella* was determined (Fig. 1E). Third, we performed a gentamycin protection assay in which cells were infected with *Salmonella*, followed by incubation with gentamycin for 2 h to kill extracellular bacteria. Cells were then lysed, plated onto agar plates and colony-forming units were counted to determine numbers of intracellular *Salmonella* (Fig. 1F). All three assays yielded virtually identical results: *Salmonella* invasion into IQGAP1-knockout cells was 75% lower than that into control cells (Figs. 1D, 1E, 1F). The experimental approach (using DS-Red-tagged *Salmonella*) was validated against a dual-color immunofluorescence assay that differentially stains extracellular and intracellular bacteria [33, 44]; essentially identical results were obtained using the two assays (data not shown).

If the reduced entry of *Salmonella* is due to the absence of IQGAP1, one would anticipate that restoring IQGAP1 in IQGAP1-null cells would rescue *Salmonella* entry. To test this hypothesis, IQGAP1-null MEFs were reconstituted with GFP-tagged full-length IQGAP1 (Fig. 1A, 1C). (Previous studies from both our group and other investigators have shown

that the GFP tag does not interfere with IQGAP1 function [33, 45].) Reconstitution of IQGAP1-null MEFs with wild-type IQGAP1 restored *Salmonella* invasion to that seen in control MEFs when evaluated by confocal microscopy (Figs. 1D, 1E). In the gentamycin protection assay, while wild-type IQGAP1 also promoted *Salmonella* infection, this was consistently (albeit not statistically significant) less than that into control cells (Fig. 1F). The last observation can be explained by the incomplete (~50%) transfection efficiency of the IQGAP1-null MEFs (data not shown). Collectively, these data validate the importance of IQGAP1 for efficient *Salmonella* invasion into MEFs.

Our findings may result from specific targeting of IQGAP1 by *Salmonella* during invasion. Alternatively, IQGAP1 may be an essential component of the phagocytosis signaling machinery and the reduced *Salmonella* uptake may be due to a general reduction of phagocytosis. In order to discriminate between these possibilities, we compared phagocytosis of beads by cells with IQGAP1 to cells without IQGAP1. MEFs were incubated with FITC-conjugated beads coated with BSA and bead internalization was quantified by flow cytometric analysis of bead fluorescence. The number of beads phagocytosed into control MEFs was indistinguishable from that into IQGAP1-null MEFs (Fig. 1G). In addition, we analyzed beads coated with fibrillar collagen to engage the $\beta 1$ integrin receptors [46]. Control and IQGAP1-null MEFs internalized similar numbers of beads (Fig. 1G). These data indicate that IQGAP1 is a specific target for *Salmonella* invasion, but is not of central importance for the non-specific phagocytosis of beads.

***Salmonella* invasion into MEFs is partially dependent on binding of IQGAP1 to Rac1/Cdc42**

The Rho GTPases Rac1 and Cdc42 are exploited by *Salmonella* to effect entry into host cells [3, 4, 6], but the mechanism by which this occurs is incompletely understood. To directly evaluate the role of IQGAP1 in the manipulation of Rac1 and Cdc42 by *Salmonella*, we reconstituted IQGAP1-null MEFs with an IQGAP1 construct, termed IQGAP1 MK24, that specifically lacks binding to Rac1 and Cdc42 [37] (Fig. 2B). Successful reconstitution with GFP-tagged IQGAP1 MK24 was visualized by both Western blotting and confocal microscopy (Fig. 2A, 2C). IQGAP1 MK24 significantly augmented *Salmonella* entry into IQGAP1-null MEFs, but reached only $53.9 \pm 7.7\%$ (mean $\pm$ S.E.M., $n=3$) of that with control cells (Fig. 2D). To assess the role of IQGAP1 in the manipulation of actin by *Salmonella*, we reconstituted IQGAP1-null MEFs with an IQGAP1 point mutant construct, termed IQGAP1-G75Q (Fig. 2A, 2B), that specifically lacks binding to actin [38]. As was observed with IQGAP1 MK24, IQGAP1-G75Q partially rescued *Salmonella* entry into IQGAP1-null MEFs (Fig. 2D). A third mutant IQGAP1 construct, termed IQGAP1-G75Q MK24, binds neither actin nor Rac1/Cdc42 (Fig. 2B). This double mutant construct also partially ($50.8 \pm 7.7\%$, mean $\pm$ S.E.M., $n=3$) rescued *Salmonella* infection (Fig. 2D). Interestingly, all three mutant IQGAP1 constructs promoted invasion of *Salmonella* by the same magnitude (to ~50% of control cells), suggesting that IQGAP1, Rac1/Cdc42 and actin are part of a common pathway targeted by *Salmonella* during invasion.

Salmonella invasion into MEFs requires the interaction of Rac1, but not Cdc42, with IQGAP1

IQGAP1 MK24 binds neither Rac1 nor Cdc42 [37], therefore it is not possible to determine whether Rac1 and/or Cdc42 are needed for optimal *Salmonella* uptake. In order to dissect the relative contributions of the two GTPases in *Salmonella* invasion, we employed two complementary strategies: a cell-permeable Rac1 inhibitor (NSC23766) and constitutively active Rac1 and Cdc42. Chemical inhibition of Rac1 activation reduced *Salmonella* entry into control MEFs by $52.5 \pm 12.4\%$ (mean $\pm$ S.E.M., $n=3$, $p<0.05$) (Fig. 3A). Pull-down assays of active Rac1 (using GST-PAK-CRIB, which binds only GTP-Rac1) confirmed that NSC23766 abrogated *Salmonella*-induced Rac1 activation without changing levels of total Rac1 (Fig. 3B). Importantly, no active Rac1 was detected in IQGAP1-null cells infected with *Salmonella* (Fig. 3B). Notably, the number of *Salmonella* that entered control MEFs incubated with the Rac1 inhibitor (Fig. 3A) was virtually identical to that observed with IQGAP1-null MEFs reconstituted with IQGAP1 MK24 (Fig. 2D), suggesting that *Salmonella* modulates the interaction between Rac1 and IQGAP1 to invade host cells. Consistent with this hypothesis, NSC23766 had no effect on *Salmonella* entry into IQGAP1-null MEFs (Fig. 3A). No activation of Cdc42 by *Salmonella* was detected, regardless of the presence or absence of IQGAP1 (Fig. 3C). The positive control with the non-hydrolyzable GTP γ S verifies that the assay detects active Cdc42. Collectively, these results, which validate the importance of Rac1 activation in *Salmonella* invasion into host cells, reveal that IQGAP1 is required for *Salmonella* to usurp Rac1 function to promote entry.

The second approach to dissect the roles of Rac1 and Cdc42 employed constitutively active (Q61L) forms of the Rho GTPases. Cells transfected with the Q61L constructs were identified by cell morphology: Q61L Rac1 induced membrane ruffling, while cells expressing Q61L Cdc42 had visibly enhanced formation of actin-rich cell extensions (Fig. 3D). Neither constitutively active Rac1 nor constitutively active Cdc42 significantly altered *Salmonella* uptake into control MEFs (Fig. 3D, 3E). *Salmonella* invasion into IQGAP1-null MEFs was significantly augmented by transfection of Q61L Rac1, reaching $69.5 \pm 8.8\%$ (mean $\pm$ S.E.M., $n=3$, $p<0.05$) of levels observed in control MEFs (Fig. 3D, 3E). By contrast, constitutively active Cdc42 had no significant effect on *Salmonella* invasion into IQGAP1-null MEFs. These data reinforce the concept that optimal *Salmonella* infection requires the binding of Rac1, but not Cdc42, to IQGAP1.

Salmonella invasion into MEFs is partially dependent on binding of MEK and ERK to IQGAP1

The data shown above reveal that the interactions of Rac1 and actin with IQGAP1 comprise only part of the mechanism by which *Salmonella* usurps IQGAP1 function to enter host cells. We therefore set out to identify other IQGAP1-regulated pathways that account for the decreased uptake of *Salmonella* into IQGAP1-null cells. We chose to explore the MEK/ERK module of the MAPK pathway for several reasons. ERK is activated upon *Salmonella* infection [17] and inhibition of MEK/ERK signaling attenuates *Salmonella* uptake into eukaryotic cells [13, 19]. Moreover, IQGAP1 binds and modulates the activity of both MEK and ERK [30, 31]. We therefore hypothesized that *Salmonella* may usurp the IQGAP1-dependent activation of MEK/ERK. We again employed a multi-faceted approach, using

both selected, discrete IQGAP1 mutant constructs and a cell-permeable MAPK inhibitor. We used two well-characterized IQGAP1 mutant constructs; one termed IQGAP1^{IQ} that does not bind MEK [30], the other termed IQGAP1^{WW} that selectively lacks binding to ERK [31] (Fig. 2B). *Salmonella* invasion into IQGAP1-null MEFs reconstituted with IQGAP1^{IQ} or IQGAP1^{WW} (Fig. 4A) was evaluated by confocal microscopy. IQGAP1^{IQ} and IQGAP1^{WW} each partially rescued *Salmonella* invasion to $54.6 \pm 7.6\%$ and $57.5 \pm 12.5\%$ (mean $\pm$ S.E.M., $n=3$, $p<0.05$), respectively, of that seen in control MEFs (Fig. 4B & C). These data suggest that the efficiency of *Salmonella* entry is partly dependent on MEK and ERK binding to IQGAP1. The MEK inhibitor PD98059 reduced *Salmonella* invasion into control MEFs by $42.5 \pm 9.0\%$ (mean $\pm$ S.E.M., $n=3$, $p<0.05$) (Fig. 4C). Importantly, PD98059 did not impair *Salmonella* invasion into IQGAP1-null MEFs (Fig. 4C). These observations imply that IQGAP1 is necessary for *Salmonella* to usurp MAPK signaling. This postulate is supported by the finding that IQGAP1^{IQ} or IQGAP1^{WW} partially rescues *Salmonella* invasion into MEFs despite treatment with PD98059 (Fig. 4C).

To confirm the integrated role of MAPK and IQGAP1 in *Salmonella* infection, we evaluated activation of the MAPK cascade by measuring levels of phospho-ERK by Western blotting. Uninfected MEFs, both $+/+$ and $-/-$, have very low levels of active ERK (Fig. 4D). Infection of control cells with *Salmonella* markedly increased phospho-ERK, without altering total ERK. The ability of *Salmonella* to activate ERK was significantly attenuated in IQGAP1-null MEFs (Fig. 4D, E). Importantly, ERK activation by *Salmonella* was completely rescued by reconstituting IQGAP1-null MEFs with full-length IQGAP1. In contrast, reconstitution of IQGAP1-null cells with IQGAP1^{IQ} or IQGAP1^{WW} failed to significantly increase activation of the MAPK pathway (Fig. 4D, 4E). Collectively, these data show that MAPK signaling is exploited by *Salmonella* during invasion into MEFs, and that this function is mediated through IQGAP1.

IQGAP1 integrates Rac1 and MAPK signaling for *Salmonella* entry

In order to dissect the functional roles of IQGAP1, Rac1, MEK and ERK during *Salmonella* invasion, we pre-treated control MEFs, IQGAP1-null MEFs and IQGAP1-null MEFs reconstituted with IQGAP1^{MK24} with the MEK inhibitor PD98059. *Salmonella* invasion into IQGAP1-null MEFs reconstituted with the non-Rac1-binding IQGAP1^{MK24} construct was essentially identical to that observed in control MEFs in which MEK signaling had been blocked with PD98059 (Fig. 5A). Importantly, PD98059 significantly reduces *Salmonella* entry into IQGAP1-null MEFs expressing IQGAP1^{MK24}, suggesting that the binding of MAPK and Rac1 to IQGAP1 are additive. Moreover, concurrent disruption of Rac1 binding to IQGAP1 (with IQGAP1^{MK24}) and MAPK signaling (with PD98059) reduces *Salmonella* infection by the same magnitude as elimination of IQGAP1 (Fig. 5A). Collectively, these data indicate that *Salmonella* usurps both Rac1 and MAPK signaling to achieve invasion into MEFs and that these activities require IQGAP1.

Discussion

Invasion into eukaryotic cells is an essential component of microbial pathogenesis, but the molecular mechanisms underlying infection are incompletely understood. Taking advantage

of the availability of IQGAP1-null cells that we generated [29], we observe that the absence of IQGAP1 impairs entry of *Salmonella* into host cells by 75%. Verification that *Salmonella* requires IQGAP1 for efficient entry is provided by our data that invasion is completely restored when IQGAP1-null cells are reconstituted with wild type IQGAP1. Uptake of beads occurs normally in cells lacking IQGAP1, indicating that our observation is not merely a non-specific effect of IQGAP1 on phagocytosis.

IQGAP1 binds to and regulates a diverse variety of proteins, thereby co-ordinating several fundamental cellular activities [28]. A number of IQGAP1 binding partners link pathogenic microbes to invasion of host cells [32]. In order to dissect out the host cell pathways that are regulated by *Salmonella* via IQGAP1, we used a series of well-characterized mutant IQGAP1 constructs. The first construct examined was IQGAP1 MK24, which is unable to bind Rac1 or Cdc42 and maintain them in the active state [37]. This was selected both because the *Salmonella* effectors SopE and SopE2 facilitate invasion by activating Rac1 and Cdc42 in the host cell [3, 4, 6] and because IQGAP1 maintains Rac1 and Cdc42 in their active, GTP-bound forms [25–27]. Reconstitution of IQGAP1-null MEFs with IQGAP1 MK24 or transfection with constitutively active Rac1 increased *Salmonella* entry to 50% of that into control cells. Consistent with this observation, a selective Rac1 inhibitor reduced by 50% *Salmonella* invasion into control cells, but had no influence on entry into IQGAP1-null cells. Moreover, the presence of IQGAP1 in cells incubated with *Salmonella* was required to detect active Rac1. The uniformity of the results observed with these different strategies strongly suggests that IQGAP1 is required for *Salmonella* to fully usurp Rac1 function to infect host cells. Our analyses also identified some differences between Cdc42 and Rac1. Unlike Rac1, constitutively active Cdc42 failed to increase *Salmonella* invasion into IQGAP1-null cells, suggesting that *Salmonella* does not target interactions of IQGAP1 with Cdc42 during infection of MEFs.

Reconstitution of IQGAP1-null MEFs with IQGAP1-G75Q, which lacks actin binding, reveals that optimal *Salmonella* infection also requires association of actin with IQGAP1. The ability of IQGAP1 to cross-link actin filaments is enhanced by Rho GTPases [23] and one interpretation of our data is that *Salmonella* manipulates IQGAP1 to link Rac1 to actin. However, because the effects of IQGAP1 MK24 and IQGAP1-G75Q could be independent, we generated IQGAP1-G75Q MK24 that binds neither Rac1 nor actin. Invasion of *Salmonella* into IQGAP1-null MEFs reconstituted with the double mutant IQGAP1-G75Q MK24 was essentially the same as that into cells reconstituted with each of the single mutant constructs, validating that IQGAP1, Rac1 and actin share a common pathway targeted by *Salmonella* during infection of MEFs. Thus, our data suggest that IQGAP1 serves as a scaffold that links Rac1 activation to actin assembly required for membrane ruffling and *Salmonella* internalization.

Interactions of Rac1 with IQGAP1 account for only part of the *Salmonella* invasion defect observed with IQGAP1-null cells. Therefore, a separate IQGAP1-associated pathway is likely also targeted by *Salmonella* during entry into MEFs. In addition to Rho GTPases, *Salmonella* usurps other host cell functions to effect entry. One of these is the MAPK pathway. *Salmonella* stimulates the activity of MEK and ERK in human intestinal cells [17, 18] and macrophages [19]. Because IQGAP1 is a MAPK scaffold [30, 31], we reconstituted

IQGAP1-null cells with IQGAP1^{IQ} and IQGAP1^{WW}, which lack binding to MEK and ERK, respectively. Disruption of IQGAP1 binding to MEK or to ERK yielded *Salmonella* invasion 45% lower than that measured with wild type IQGAP1. We also observed that the MEK/ERK inhibitor PD98059 reduced *Salmonella* invasion into control MEFs by 45%, which is the same magnitude of attenuation as that recently reported with human foreskin fibroblasts [13]. In contrast, PD98059 had no effect on *Salmonella* entry into IQGAP1-null cells, regardless of whether they were reconstituted with IQGAP1^{IQ}, IQGAP1^{WW} or neither. Consistent with these findings, the ability of *Salmonella* to activate ERK is significantly attenuated in MEFs lacking IQGAP1. ERK activation by *Salmonella* is completely restored when IQGAP1-null cells were reconstituted with wild type IQGAP1, but no rescue was seen with IQGAP1 constructs that lack MEK or ERK binding. Collectively these data strongly suggest that an interaction of IQGAP1 with MEK and ERK is necessary for *Salmonella* to usurp MAPK signaling, implying that IQGAP1 may link *Salmonella* infection to MAPK activation.

One of the most striking results from the present study is that Rac1 and MAPK signaling are additive in terms of their contributions to *Salmonella* invasion into MEFs. This is substantiated by the finding that concurrent disruption of Rac1 and MAPK signaling reduces *Salmonella* invasion by 75%, while inhibition of Rac1 alone or MAPK alone reduces invasion by 45%. Interestingly, simultaneous inhibition of Rac1 and MAPK signaling attenuates *Salmonella* infection to the same extent as complete absence of IQGAP1. This observation is congruent with accumulating evidence that IQGAP1 is a scaffold that integrates multiple intracellular signaling pathways [28, 47]. Scaffold proteins control specificity and spatio-temporal aspects of cell function [48, 49]. The participation of scaffolds in yeast and mammalian cell function has been the focus of considerable attention [14–16]. While scaffolds are less recognized in bacterial infection, a recent paper showed that the *E. coli* T3SS effector EspG assembles a signaling complex containing ADP-ribosylation factor and p21-activated protein kinase [50]. To our knowledge, the present study is the first to identify a host cell protein scaffold that links three endogenous proteins in two distinct signaling pathways that are usurped by *Salmonella* to infect cells. Based on our data, we propose a model in which *Salmonella* exploits the Rac1-, actin-, MEK- and ERK-binding properties of IQGAP1, allowing efficient infection (Fig 5B). Disruption of the interaction between Rac1 and IQGAP1, actin and IQGAP1 or chemical inhibition of Rac1 activation, reduces *Salmonella* uptake into MEFs, but the pathogen continues to exploit interactions of IQGAP1 with MEK and ERK to achieve invasion (Fig. 5B). Similarly, disruption of the interaction of MEK or ERK with IQGAP1 reduces *Salmonella* invasion, but partial uptake is observed by virtue of ongoing interactions between Rac1 and IQGAP1 (Fig. 5B). Finally, simultaneous suppression of Rac1 and MAPK signaling (and the greatest inhibition of *Salmonella* infection) is observed following complete loss of IQGAP1 expression (Fig. 5B). In conclusion, this study identifies IQGAP1 as an integrator of Rac1- and MAPK-dependent signaling usurped by *Salmonella* to achieve infection in the host. Further research into the molecular determinants of microbial pathogenesis is essential for the development of novel therapeutic agents for infectious diseases.

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Fig. 1. Optimal *Salmonella* invasion requires IQGAP1

A. Equal amounts of protein lysate obtained from control MEFs (+/+), IQGAP1-null MEFs (-/-) and IQGAP1-null MEFs reconstituted with full-length GFP-IQGAP1 (WT) were resolved by SDS-PAGE. Western blots were probed (immunoblot, IB) with anti-IQGAP1 and anti-tubulin (loading control) antibodies. Compared to endogenous IQGAP1, GFP-IQGAP1 (WT) exhibits reduced migration on SDS-PAGE due to the mass of the GFP tag. Data are representative of 3 independent experiments. **B.** Control (+/+) and IQGAP1-null (-/-) MEFs were infected with DS-Red-tagged *Salmonella* at an MOI of 30 for 30 min,

incubated with gentamycin for 2 h and washed extensively to remove extracellular bacteria. Cells were then fixed and stained for F-actin using Alexa-647 phalloidin. Representative confocal micrographs are shown, indicating intracellular *Salmonella* (left panels) and F-actin (center panels). Merged image (right panels) represents a composite of both channels (*Salmonella* in red and F-actin in blue). Bar=10 μ m. **C.** IQGAP1-null MEFs were reconstituted with GFP-tagged full-length IQGAP1 prior to infection with DS-Red-tagged *Salmonella*. Confocal micrograph illustrates GFP-IQGAP1 (far-left panel), intracellular *Salmonella* (center-left panel) and F-actin (center-right panel). Merged image (far-right panel) represents a composite of all three channels (GFP-IQGAP1 in green, *Salmonella* in red and F-actin in blue). Yellow indicates colocalization. Representative images are shown. Bar=10 μ m. **D.** Histograms depict the average number of intracellular *Salmonella* per cell in control (+/+), IQGAP1-null (-/-) and IQGAP1-null MEFs reconstituted with wild-type IQGAP1 (WT). Data, expressed as mean $\pm$ S.E.M. with control MEFs set at 1, were obtained from three independent experiments. *, $p<0.05$. **E.** Histograms depict the percentage of cells showing at least one intracellular *Salmonella* in control (+/+), IQGAP1-null (-/-) and IQGAP1-null MEFs reconstituted with wild-type IQGAP1 (WT). Data, expressed as mean $\pm$ S.E.M. with control MEFs set at 100%, were obtained from three independent experiments. *, $p<0.05$. **F.** Control (+/+), IQGAP1-null (-/-) and IQGAP1-null MEFs reconstituted with wild-type IQGAP1 (WT) were infected with *Salmonella* at an MOI of 30 for 30 min, incubated with gentamycin for 2 h and lysed, and the number of intracellular *Salmonella* were quantified (based on the number of colony forming units, CFU). Data, expressed as mean $\pm$ S.E.M. with control MEFs set at 1, were obtained from three independent experiments, each performed in triplicate. *, $p<0.05$. **G.** Control (+/+) and IQGAP1-null (-/-) MEFs were incubated with FITC-conjugated beads coated with either bovine serum albumin (BSA beads) or with collagen (Collagen beads). The number of cells with internalized bead(s) was evaluated by flow cytometry. Histograms depict the percentage of cells showing at least one intracellular bead. Data, expressed as mean $\pm$ S.E.M., were obtained from three independent experiments.

Fig. 2. *Salmonella* invasion is partially dependent on IQGAP1 binding to Rac1/Cdc42

A. Equal amounts of protein lysate obtained from control (+/+) MEFs, IQGAP1-null MEFs (-/-), IQGAP1-null MEFs transfected with GFP-tagged IQGAP1 MK24 (ΔMK24), IQGAP1-G75Q (G75Q) or IQGAP1-G75Q MK24 (G75QΔMK24) were resolved by SDS-PAGE. Western blots were probed (immunoblot, IB) with anti-IQGAP1 and anti-tubulin (loading control) antibodies. Data are representative of 3 independent experiments. **B.** Schematic illustration of the IQGAP1 constructs used in this study. From top to bottom: WT, wild-type IQGAP1 containing the calponin homology domain (CHD), the WW domain

(WW), the IQ region (IQ) and Ras GTPase-activating protein (GAP)-related domain (GRD);

MK24, deletion of the Rac1/Cdc42 binding region; G75Q, point mutation of the actin-binding site of IQGAP1; G75Q MK24, deletion of the Rac1/Cdc42-binding region and point mutation of the actin-binding site of IQGAP1; IQ, deletion of the MEK-binding region; WW, deletion of the ERK-binding region. **C.** IQGAP1-null MEFs were transfected with GFP-IQGAP1 MK24 prior to infection with DS-Red-tagged *Salmonella*. A representative confocal micrograph shows GFP-IQGAP1 MK24 (far-left panel), intracellular *Salmonella* (center-left panel) and F-actin (center-right panel). Merged image (far-right panel) represents a composite of all three channels (GFP-IQGAP1 MK24 in green, *Salmonella* in red and F-actin in blue). Bar=10 μ m. **D.** Histograms depict the average number of intracellular *Salmonella* per cell in control (+/+) MEFs, IQGAP1-null (-/-) MEFs and IQGAP1-null MEFs transfected with GFP-IQGAP1 MK24 (MK24), IQGAP1-G75Q (G75Q) or IQGAP1-G75Q MK24 (G75Q MK24). Data, expressed as mean $\pm$ S.E.M. with control (+/+) MEFs set at 1, were obtained from three independent experiments. *, significantly different from control (+/+) MEFs ($p<0.05$). †, significantly different from IQGAP1-null (-/-) MEFs ($p<0.05$).

Fig. 3. *Salmonella* utilizes IQGAP1 interactions with Rac1, but not Cdc42 to invade MEFs
A. Control (+/+) and IQGAP1-null (-/-) MEFs were pre-incubated with vehicle or the Rac1 inhibitor NSC23766 for 30 min and then infected with DS-Red-tagged *Salmonella* at an MOI of 30 for 30 min. Histograms depict the average number of intracellular *Salmonella* per cell in control (+/+, white bars) and IQGAP1-null (-/-, black bars) MEFs. Data, expressed as mean±S.E.M. with control (+/+) MEFs treated with vehicle set at 1, were obtained from three independent experiments. *, significantly different from control (+/+) MEFs treated with vehicle ($p<0.05$). †, significantly different from IQGAP1-null (-/-) MEFs treated with vehicle ($p<0.05$).

vehicle and from IQGAP1-null MEFs treated with NSC23766 ($p<0.05$). **B.** Equal amounts of protein lysate were prepared from control (+/+) and IQGAP1-null (-/-) MEFs infected with *Salmonella* at an MOI of 30 for 30 min. Samples were resolved by Western blotting and probed with anti-Rac1 antibody (total Rac1). Equal amounts of protein lysate were also incubated with GST-PAK-CRIB as described in Materials and Methods. Complexes were collected with glutathione-Sepharose and resolved by SDS-PAGE. Western blots were probed with anti-Rac1 antibody (active Rac1). **C.** Equal amounts of protein lysate were prepared from control (+/+) and IQGAP1-null (-/-) MEFs infected with *Salmonella* at an MOI of 30 for 30 min. Western blots were probed with anti-Cdc42 antibody (total Cdc42). Equal amounts of protein were also incubated with GST-WASP-GBD as described in Materials and Methods. Complexes were collected with glutathione-Sepharose and resolved by SDS-PAGE. Samples were resolved by Western blotting and probed with anti-Cdc42 antibody (active Cdc42). As a positive control, protein lysates were pre-incubated with guanosine triphosphate gamma S (GTP γ S) prior to probing with anti-Cdc42 antibody (far-right panels). **D.** Control (+/+) and IQGAP1-null (-/-) MEFs were transfected with constitutively active (Q61L) Rac1 or constitutively active (Q61L) Cdc42 prior to infection with DS-Red-tagged *Salmonella* at an MOI of 30 for 30 min. Representative confocal micrographs are shown, indicating intracellular *Salmonella* (left panels) and F-actin (center panels). Merged image (right panels) represents a composite of both channels (*Salmonella* in red, F-actin in blue). Bar=10 μ m. **E.** Histograms depict the average number of intracellular *Salmonella* per cell in control MEFs (+/+, white bars) and IQGAP1-null MEFs (-/-, black bars), which were either untransfected, transfected with constitutively active (Q61L) Rac1 or transfected with constitutively active (Q61L) Cdc42. Data, expressed as mean $\pm$ S.E.M. with untransfected control (+/+) MEFs set at 1, were obtained from three independent experiments. *, significantly different from control (+/+) MEFs ($p<0.05$). †, significantly different from IQGAP1-null (-/-) MEFs, either untransfected or transfected with Q61L Cdc42 ($p<0.05$).

Fig. 4. *Salmonella* invasion is partially dependent on MEK and ERK binding to IQGAP1

A. Equal amounts of protein lysate obtained from control MEFs (+/+), IQGAP1-null MEFs (-/-) as well as IQGAP1-null MEFs transfected with GFP-IQGAP1 ΔIQ (ΔIQ) or GFP-IQGAP1 ΔWW (ΔWW) were resolved by SDS-PAGE. Western blots were probed (immunoblot, IB) with anti-IQGAP1 and anti-tubulin (loading control) antibodies. Data are representative of 3 independent experiments. **B.** IQGAP1-null MEFs transfected with GFP-IQGAP1 ΔIQ (upper panels) or GFP-IQGAP1 ΔWW (lower panels) were infected with DS-Red-tagged *Salmonella* at an MOI of 30 for 30 min, incubated with gentamycin for 2 h and

washed extensively to remove extracellular bacteria. Cells were then fixed and stained for F-actin. Confocal micrographs illustrate GFP-IQGAP1 IQ (upper row, far-left panel) and GFP-IQGAP1 WW (lower row, far-left panel), intracellular *Salmonella* (center-left panels) and F-actin (center-right panels). Merged image (far-right panels) represents a composite of all three channels (GFP-IQGAP1 IQ and GFP-IQGAP1 WW in green, *Salmonella* in red and F-actin in blue). Representative images are shown. Bar=10 μ m. **C.** Histograms depict the average number of intracellular *Salmonella* per cell in control (+/+), IQGAP1-null (-/-) and IQGAP1-null MEFs transfected with GFP-IQGAP1 IQ (IQ) or GFP-IQGAP1 WW (WW) constructs. Where indicated, cells were pre-incubated with vehicle or the MEK1 inhibitor PD98059 for 30 min prior to *Salmonella* infection. Data, expressed as mean $\pm$ S.E.M. with (+/+) MEFs incubated with vehicle set at 1, were obtained from three independent experiments. *, significantly different from control (+/+) MEFs treated with vehicle ($p<0.05$). †, significantly different from IQGAP1-null (-/-) MEFs reconstituted with GFP-IQGAP1 IQ or with GFP-IQGAP1 WW ($p<0.05$). **D.** Control (+/+) and IQGAP1-null (-/-) MEFs were uninfected (-) or infected (+) with *Salmonella* at an MOI of 30 for 30 min. In addition, IQGAP1-null MEFs transfected with full-length GFP-IQGAP1 (WT), GFP-IQGAP1 IQ (IQ) or GFP-IQGAP1 WW (WW) were infected with *Salmonella*. Equal amounts of protein lysate were resolved by SDS-PAGE. Western blots were probed (immunoblot, IB) with anti-phospho-ERK (pERK) antibodies, then stripped and reprobed with anti-ERK antibodies. Data are representative of three independent experiments. **E.** The amounts of phospho-ERK in cells infected with *Salmonella* in the experiments depicted in panel D were quantified by densitometry. Data, expressed as mean $\pm$ S.E.M., were obtained from three independent experiments. *, significantly different ($p<0.05$) from both control (+/+) MEFs and IQGAP1-null MEFs reconstituted with full-length IQGAP1 (WT).

Fig. 5. *Salmonella* targets IQGAP1 function for entry into MEFs in a Rac1- and MAPK-dependent manner

A. Control (+/+), IQGAP1-null (-/-) MEFs and IQGAP1-null MEFs reconstituted with GFP-IQGAP1 MK24 were pre-incubated with vehicle or the MEK1 inhibitor PD98059. After 30 min, cells were infected with DS-Red-tagged *Salmonella* at an MOI of 30 for 30 min. Histograms depict the average number of intracellular *Salmonella* per cell in control (+/+), IQGAP1-null (-/-) and IQGAP1-null MEFs reconstituted with GFP-IQGAP1 MK24 (ΔMK24). Data, expressed as mean±S.E.M. with (+/+) MEFs incubated with vehicle set at 1, were obtained from three independent experiments. *, significantly different from control

(+/+) MEFs treated with vehicle ($p<0.05$). †, significantly different from IQGAP1-null (-/-) MEFs reconstituted with GFP-IQGAP1 MK24 and control (+/+) MEFs treated with PD98059 ($p<0.05$). **B.** Proposed model by which IQGAP1 integrates Rac1- and MAPK-signaling during *Salmonella* invasion into MEFs. a, IQGAP1 binds to Rac1, actin, MEK and ERK, facilitating efficient entry of *Salmonella* into control (+/+) MEFs. Hatched areas indicate sites of *Salmonella* entry at nascent membrane ruffles. b, Disruption of Rac1 binding to IQGAP1 or inhibition of Rac1 activation (with NSC23766) reduces the ability of *Salmonella* to usurp Rac1 function, and consequently, to enter the host cell. Disruption of actin binding to IQGAP1 also precludes optimal *Salmonella* uptake. MAPK signaling is unaffected under these conditions, allowing partial *Salmonella* invasion. c, Disruption of MEK or ERK binding to IQGAP1 or inhibition of MAPK signaling with PD98059 partially reduces *Salmonella* invasion. Rac1 signaling is unaffected under these conditions, allowing partial *Salmonella* invasion. d, Loss of IQGAP1 expression abrogates both Rac1- and MAPK-mediated signaling required for efficient *Salmonella* invasion.