**Salmonella** Disrupts Host Endocytic Trafficking by SopD2-Mediated Inhibition of Rab7

### Graphical Abstract

**Wild-type Salmonella**

- Early Endosome
- Late Endosome
- Rab7
- Lysosome

**ΔSopD Salmonella**

- Early Endosome
- Late Endosome
- Rab7
- Lysosome

**Microtubule-based Trafficking**

- RILP or FYCO1

**S. Containing Vacuole**

**Highlights**

- The effector SopD2 blocks delivery of endocytic cargo to lysosomes
- SopD2 interacts directly with host GTPase Rab7
- SopD2’s N terminus mediates both trafficking suppression and interaction with Rab7
- SopD2 impairs Rab7’s ability to bind cognate effectors RILP and FYCO1

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**In Brief**

Intracellular pathogens can evade host immunity by impairing trafficking of endocytic cargo to lysosomes for degradation. D’Costa et al. show that the effector SopD2 mediates this process in *Salmonella* infection by binding the host regulatory GTPase Rab7 and impairing its ability to recruit dynein- and kinesin-binding effectors RILP and FYCO1.

**Accession Numbers**

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Salmonella Disrupts Host Endocytic Trafficking by SopD2-Mediated Inhibition of Rab7

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SUMMARY

Intracellular bacterial pathogens of a diverse nature share the ability to evade host immunity by impairing trafficking of endocytic cargo to lysosomes for degradation, a process that is poorly understood. Here, we show that the Salmonella enterica type 3 secreted effector SopD2 mediates this process by binding the host regulatory GTPase Rab7 and inhibiting its nucleotide exchange. Consequently, this limits Rab7 interaction with its dynein- and kinesin-binding effectors RILP and FYCO1 and thereby disrupts host-driven regulation of microtubule motors. Our study identifies a bacterial effector capable of directly binding and thereby modulating Rab7 activity and a mechanism of endocytic trafficking disruption that may provide insight into the pathogenesis of other bacteria. Additionally, we provide a powerful tool for the study of Rab7 function, and a potential therapeutic target.

INTRODUCTION

The pathogen Salmonella enterica serovar Typhimurium (S. Typhimurium), a model organism for the study of bacterial pathogenesis, is a significant cause of foodborne gastroenteritis and a causative agent of reactive arthritis (Mead et al., 1999; Yu and Kuipers, 2003). Upon infection, these bacteria invade host cells and reside within membrane-bound Salmonella-containing vacuoles (SCVs), which subsequently undergo interactions with the endocytic pathway. The pathogen, however, is able to manipulate the maturation of SCVs, limiting interactions with lysosomes and ultimately contributing to the establishment of a replicative niche in host cells (Brumell and Grinstein, 2004).

RESULTS

The Effector SopD2 Is Necessary and Sufficient to Block Endocytic Trafficking

To investigate endocytic progression in host cells during infection, we used a fluorescence-based assay that monitors a BODIPY TR-X conjugate of BSA (DQ-Red BSA). Upon delivery to compartments containing active proteases, this self-quenched substrate is cleaved, releasing highly fluorescent peptides. Owing to its broad specificity with respect to lysosomal hydrolases, this assay detects global defects in lysosomal function. To investigate endocytic progression in host cells during infection, we used a fluorescence-based assay that monitors a BODIPY TR-X conjugate of BSA (DQ-Red BSA). Upon delivery to compartments containing active proteases, this self-quenched substrate is cleaved, releasing highly fluorescent peptides. Owing to its broad specificity with respect to lysosomal hydrolases, this assay detects global defects in lysosomal function. Host cell manipulation is mediated by an arsenal of over 30 virulence proteins called effectors (Figueira and Holden, 2012), which are delivered to the host cell cytosol by two independent type 3 secretion systems (T3SSs), encoded by Salmonella pathogenicity island (SPI)-1 and SPI-2. Effectors of the SPI-1 T3SS facilitate bacterial invasion. Subsequently, SPI-2 T3SS effectors mediate processes that promote bacterial survival and replication within SCVs (Brumell and Grinstein, 2004). Among the most prominent yet poorly understood consequences of host cell manipulation by Salmonella and other clinically relevant pathogens is the sustained perturbation of lysosomal function (Buchmeier and Heffron, 1991; Ishibashi and Arai, 1990; Lamothe et al., 2007; Seto et al., 2009). Salmonella can cause a global impairment of host endocytic trafficking to lysosomes. This effect is known to be SPI-2 T3SS dependent (Uchitya et al., 1999; Yu et al., 2002); however, the effector(s) responsible, the host target, and mechanism of action have yet to be elucidated. In this study, we show that the effector SopD2 is both necessary and sufficient to mediate this phenotype and that it functions by directly targeting a key host regulatory GTPase in the endocytic pathway and blocking its function.
Non-infected cells generated bright punctate signals, whereas intensities from infected cells were significantly decreased (Figures S1 B and S1C). The SPI-2 T3SS-defective mutant (ΔssaR) yielded signals equivalent to those emitted by non-infected cells (Figures S1 B and S1C), consistent with findings from previous studies implicating the SPI-2 T3SS (Uchiya et al., 1999; Yu et al., 2002). Cells infected with WT bacteria and subsequently exposed to fluorescent BSA-Cy3 exhibited no difference in probe intensity compared to non-infected cells (Figures S1 D–S1F), suggesting the absence of a defect in internalization.

The SPI-2 T3SS effector SopD2, previously shown to contribute to virulence in a murine systemic infection model (Jiang et al., 2004), localizes to SCVs and late endocytic compartments (Brumell et al., 2003; Jiang et al., 2004). HeLa cells infected with Salmonella lacking SopD2 (ΔsopD2) displayed DQ-Red BSA fluorescence intensities comparable to non-infected cells (Figures 1A and 1B), suggesting a role for this effector in blocking delivery of endocytic contents to lysosomes. This phenotype was also observed in RAW 264.7 macrophages (Figures S2 A and S2B). In contrast, deletion of the effector SopB, whose activity decreases levels of SCV-lysosome fusion at early time points of infection (Bakowski et al., 2010), or the effector SopD, which shares sequence similarity with SopD2, had no effect on DQ-Red BSA fluorescence (Figure 1B). Epithelial cells transfected with GFP-tagged constructs overnight and then pulsed with BSA-Cy3 for 30 min to assess internalization. Quantification of BSA-Cy3 intensity.
a SPI-2 T3SS effector that was recently shown to block M6PR recycling during infection (McGourty et al., 2012). Our findings demonstrate that SifA-mediated inhibition of M6PR recycling, while capable of decreasing cathepsin B activity (McGourty et al., 2012), is not sufficient to suppress overall lysosome function.

We used the Magic Red Cathepsin L Assay Kit to monitor activity of the pH-sensitive protease cathepsin L. This cell-permeant, substrate-based reagent becomes fluorescent upon cleavage. No significant difference in fluorescence intensities was observed between cells infected with WT, ΔsopD2, and ΔssaR deletion mutants (Figures 2A and 2B), suggesting that SopD2 does not affect total cellular lysosomal protease activity.

Expression of SopD2 did not alter internalization of BSA-Cy3 (Figure 1E), and Salmonella-infected cells simultaneously pulsed with DQ-Red BSA and dextran 647 exhibited similar DQ-Red BSA fluorescence intensities when corrected for relative dextran levels (Figures S2C and S2D), ruling out a defect in internalization. Therefore, we further investigated delivery of endosomal cargo to lysosomes. Lysosomes were pre-loaded with dextran 647 prior to transfection and then treated with rhodamine dextran. In GFP-expressing cells, the dextran derivatives colocalized with a Mander’s coefficient of more than 0.5, indicating significant endosome-lysosome fusion, whereas GFP-SopD2 expression resulted in significantly less colocalization (Figures 2C and 2D). Collectively, these observations demonstrate that SopD2 is both necessary and sufficient to impair endocytic trafficking by blocking delivery of endocytic contents to lysosomes.
The Amino Terminus of SopD2 Inhibits Trafficking to Lysosomes

SopD2 is thought to have arisen by gene duplication of the T3SS effector SopD. The two proteins share 43% sequence identity, although they appear to target unique processes in host cells (Brumell et al., 2003). Three-dimensional structures of both effectors were determined by X-ray crystallography (Figure 3A; Figure S3; Table S1). The reconstructed amino terminus of each effector occupied a unique three-dimensional space, whereas extensive homology was established throughout the remainder of the peptides. SopD2’s amino-terminal domain mediates targeting of the protein to late endocytic compartments (Brown et al., 2006; Brumell et al., 2003) (Figure 3B). Using GFP-fused truncations of SopD2, we mapped the domain involved in inhibiting endocytic traffic (Figures 3C and 3D). The first 150 amino acids inhibited DQ-Red BSA fluorescence in a manner comparable to full-length SopD2, while smaller truncations of this region demonstrated a partial effect. In contrast, inhibition was not observed with the carboxyl-terminal portion, which localized to the cytosol.

To assess these findings in the context of infection, we created a series of constructs in the *Salmonella* complementation plasmid pACYC184 representing full-length and N-terminal regions of SopD2 and SopD, as well as chimeric proteins...
SopD2 Disrupts Rab7 Function by Impairing Recruitment of Its Effectors

SopD2 targets to late endocytic compartments where it co-localizes with the small GTPase Rab7 (Brunell et al., 2003) (Figure 4A), previously shown to regulate endocytic traffic and mediate the delivery of endosomal contents to lysosomes (Cantalupo et al., 2001; Feng et al., 1995; Méresse et al., 1995; Progida et al., 2007). Therefore, we hypothesized that SopD2 functions by impairing Rab7 activity. Rab7-dependent endocytic trafficking is mediated by the host effectors RILP (Rab-interacting lysosomal protein) and FYCO1 (FYVE and coiled-coil domain containing protein 1), which are recruited from the cytosol by active (GTP-bound) Rab7 (Cantalupo et al., 2001; Pankiv et al., 2010). This binding promotes recruitment of dynein/dynactin and kinesin, facilitating minus-end- and plus-end-directed vesicle movement, respectively, along microtubules (Jordens et al., 2001; Pankiv et al., 2010). To investigate this process, we used a series of fluorescent probes to visualize active Rab7. GFP-RILPC33 and mCherry-FYCO1 localized to LAMP1-positive endocytic compartments in transfected cells (Figures 4B and S4B), whereas siRNA-mediated Rab7 knockdown resulted in their displacement to the cytosol (Figures S4A, S4D, and S4E). Similarly, cells co-transfected with SopD2 demonstrated a significant relocalization to the cytosol (Figures 4B, 4C, and S4C), suggesting that SopD2 is sufficient to prevent Rab7’s interaction with its effectors.

Next, we examined the impact of SopD2 on Rab7 activity in the context of infection. Cells were infected with Salmonella and transfected with the Rab7 activity probes. SCVs containing WT bacteria displayed low levels of colocalization with RILPC33 and FYCO1 (Figures 4D–4G), whereas levels were significantly higher on SCVs harboring the ΔsopD2 mutant. Furthermore, when the ΔsopD2 Salmonella mutant was used, only complementation with full-length SopD2, its first 150 amino acids, and the chimeric effector with the N-terminal region of SopD2 exhibited decreased levels of SCV colocalization (Figure 5C). This demonstrates the importance of SopD2 in directing vacuolar maturation during infection and indicates that SopD2 disrupts Rab7 activity by impairing its ability to bind its cognate effectors in host cells during infection.

SopD2 Directly Interacts with Rab7 and Perturbs Function

To investigate a possible interaction of SopD2 with Rab7, co-immunoprecipitation experiments were performed using HEK293T cells expressing Flag-SopD2 and RFP-Rab7. SopD2 was observed to interact with Rab7 (Figure 6A) without a distinct preference for either the constitutively active (Q67L) or dominant-negative (T22N) variants. Subsequently, the GFP fusions of SopD2 from Figure 3C were used for domain mapping. By means of pull-down assays, we found that Rab7 binding is mediated by the N-terminal region of SopD2 (Figure 6B), consistent with the ability of this region to both inhibit endocytic trafficking to lysosomes and impair localization of Rab7 effectors RILP and FYCO1. To examine the nature of this interaction, we reconstituted binding in vitro with purified proteins. Using nucleotide-loaded GST-Rab7, we found that the amino terminus of SopD2 preferentially binds Rab7, as compared to its carboxyl terminus, and that this interaction is direct (Figure 6C). This direct association occurred in a manner independent of the activation state of Rab7, consistent with co-immunoprecipitation studies (Figure 6A), and was specific for SopD2, as comparable interactions were not observed with the amino terminus of SopD or full-length SopA (Figures 6C and 6D).

The region of interaction between Rab7 and RILP has been previously established (Wu et al., 2005). This interaction, unlike that of Rab7 and SopD2, occurs in a nucleotide-dependent manner. We utilized mutants of Rab7 known to be impaired in RILP binding (Wu et al., 2005) and assessed their association with SopD2. Co-immunoprecipitations indicated that no single mutation in the Rab7 effector-binding domain diminished binding to SopD2 (Figure 6E). Furthermore, mutation of all four sites essential for RILP binding was not sufficient to impair SopD2 interaction with Rab7. This suggests that SopD2 and RILP interact with Rab7 through different binding sites.

To investigate whether SopD2 directly causes RILP displacement from active Rab7, we performed in vitro competitive binding studies. Regardless of whether Rab7 was preincubated with SopD2 prior to RILP addition, SopD2 was not sufficient to decrease RILP binding (Figure 6F). Collectively, these data suggest that, although SopD2 binds Rab7 directly, it does not cause effector displacement in host cells through a competitive binding mechanism.

The Salmonella T3SS effector SopB has been shown to inhibit the host GTPase Cdc42 by limiting its nucleotide exchange activity (Burkinshaw et al., 2012). Therefore, we examined Rab7 nucleotide exchange using the nucleotide analog N-methylanthraniloyl-guanosine diphosphate (Mant-GDP), which fluoresces optimally when bound to protein. In control experiments, GTP addition resulted in a decrease in fluorescence as Mant-GDP is released via nucleotide exchange (Figure 6G). This effect was suppressed in a dose-dependent manner by SopD2, but not by BSA.

Our data indicate that SopD2 suppresses Rab7 function on SCVs and late endocytic compartments, as SopD2 both impairs Rab7’s ability to recruit its trafficking-related effectors RILP and FYCO1 and prevents the delivery of endocytic contents to lysosomes. We demonstrate that SopD2 directly binds Rab7 in a manner distinct from that of RILP, suggesting that recruitment of host effectors is blocked in a non-competitive manner. We also show that SopD2-mediated Rab7 suppression likely occurs at the level of nucleotide exchange. While GDP-bound Rab7 is normally maintained in the cytosol (Stenmark, 2009), Rab7 remains colocalized with SopD2 on SCVs and late endocytic compartments upon inhibition, consistent with a model whereby SopD2 stabilizes Rab7 on the membrane in its inactive,
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Therefore, we propose that retention of Rab7-GDP on the membrane is caused by SopD2 itself. This would require the bacterial effector to independently associate with the membrane by a Rab7-independent mechanism. Indeed, knockdown of Rab7 did not affect SopD2 targeting to these compartments (Figure S4F).

**DISCUSSION**

The SPI-2 T3SS effector SopD2 has been shown to contribute to S.Typhimurium virulence in a murine systemic infection model (Jiang et al., 2004). Its presence influences the pathogen’s ability to replicate in mice (Jiang et al., 2004), and it expresses at high

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**Figure 4. SopD2 Disrupts Rab7 Function by Impairing Recruitment of Its Endogenous Effectors**

(A) HeLa cells were transfected with GFP-Rab7 (green) and RFP-SopD2 (red). Scale bar, 6 μm.

(B) HeLa cells were transfected with GFP-RILPC33 (green) and either RFP or RFP-SopD2 (red) and were immunostained for LAMP1 (blue). Scale bars, 10 μm.

(C) Quantification of the percentage of cells containing cytosolic RILPC33 or FYCO1.

For quantifications, the means ± SEM are shown.

**Figure 5. Salmonella-Induced Suppression of Endocytic Trafficking to Lysosomes and Impairment of Rab7 Function Is Mediated by the Amino Terminus of SopD2**

For quantifications, the means ± SEM are shown.

(A) Schematic of gene products of the constructs used for infection-based complementation studies in Salmonella. All constructs were generated using the plasmid pACYC184, engineered to contain the promoter region of sopD2. Constructs harbor either full-length effectors, amino-terminal regions, or hybrid effectors.

(B) DQ-Red BSA complementation studies. HeLa cells were infected with GFP-expressing S. Typhimurium strains harboring pACYC184 plasmids for 8 hr and then incubated with DQ-Red BSA for 1 hr. Images were acquired after an additional 4 hr chase. Quantification of the DQ-Red BSA signal, where the genetic background of each strain, either ΔsopD2 or WT SL1344, is indicated on the x axis. Statistical significance of infected versus non-infected populations (indicated by one asterisk) and among infected conditions (one-way ANOVA with Bonferroni post hoc test; indicated by two asterisks) is shown (p < 0.05).

(C) HeLa cells were infected with S. Typhimurium strains harboring pACYC184 complementation plasmids and then transfected with the Rab7 activity probe GFP-RILPC33. Cells were fixed at 10 hr post-infection and immunostained. Quantification of SCV colocalization with RILPC33 is shown. The p values determined to be statistically significant (p < 0.05) were calculated using a one-way ANOVA with Bonferroni post hoc test, and selected comparisons are shown (indicated by one asterisk).

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Figure 6. SopD2 Binds to Rab7 and Inhibits Nucleotide Exchange

(A) Co-immunoprecipitations. HEK293T cells were co-transfected with Flag-SopD2 and RFP-Rab7 variants, and lysates were precipitated with anti-FLAG affinity gel. Elutions were analyzed by western blotting. CA, constitutively active; DN, dominant-negative; RFP, red fluorescent protein.

(B) Pull-down assays. HEK293T cells were transfected with GFP-SopD2 truncations, and lysates were incubated with resin-bound His-Rab7 preloaded with GTPγS. Elutions were analyzed by western blotting.

(C and D) In vitro binding assays. Rab7-bound resin was preloaded with GDP or GTPγS and then incubated with purified effector, either full length or the indicated truncation. Elutions were analyzed by western blotting. Sites of point mutations are indicated, and QM indicates a quadruple mutant (K10A, F45A, D82A, V180A).

(E) Co-immunoprecipitations. HEK293T cells were co-transfected with Flag-SopD2 and RFP-Rab7 CA, mutated in the RILP-binding region, and lysates were precipitated with anti-FLAG affinity gel. Elutions were analyzed by western blotting. Sites of point mutations are indicated, and QM indicates a quadruple mutant (K10A, F45A, D82A, V180A).

(legend continued on next page)
levels relative to other SPI-2 T3SS effectors using tissue culture-based infection models (Xu and Hensel, 2010). However its host target, molecular mechanism, and function in host cells has been poorly understood. In this study, we establish that SopD2 plays a key role in bacterial interference with the endocytic pathway. We propose a model in which SopD2 independently targets SCVs and late endocytic compartments, stabilizes Rab7 on these membranes and functions upstream of effector recruitment to inhibit GTPase cycling (Figure S5A).

Our model suggests several important contributions of SopD2 to Salmonella pathogenesis. By inhibiting Rab7, SopD2 perturbs endocytic trafficking, contributing to the evasion of lysosomal degradation of bacteria. Additionally, the displacement of RILP and FYCO1 on SCVs, as observed in this study, may free host degradation of bacteria. Additionally, the displacement of RILP endocytic trafficking, contributing to the evasion of lysosomal nesin-recruiting effectors (Figure S5 B), thereby promoting the nesin-recruiting effectors (Figure S5B), thereby promoting the formation of Salmonella-induced filaments (Sifs) (Jiang et al., 2004; Schroeder et al., 2011), elongated LAMP-1-positive membrane extensions (García-del Portillo et al., 1993) that are thought to play a role in bacterial cell-to-cell spread (Szető et al., 2009).

Our study characterizes a bacterial effector that has evolved the ability to directly bind and inhibit the host GTPase Rab7, a regulatory switch central to endocytic trafficking, progression, and, ultimately, lysosome function. While deletion of sopD2 in Salmonella resulted in a dramatic restoration of trafficking to lysosomes (Figure 1B), it is likely that multiple effectors contribute to the process of Salmonella-mediated suppression of host lysosome function. Indeed, it has been previously shown that SifA targets the host GTPase Rab9 (Jackson et al., 2008), inhibiting the process of Rab9-dependent M6PR recycling (McGourty et al., 2012). Although SifA is not sufficient to perturb overall levels of lysosome function (Figure 1D), it is capable of impairing the trafficking of selected lysosomal hydrolytic enzymes (McGourty et al., 2012). Therefore, S. Typhimurium has evolved the ability to target two key regulators of late endocytic/lysosomal trafficking.

Salmonella can impair adaptive immune responses by disrupting MHC-II (major histocompatibility complex class II)-associated antigen presentation in dendritic cells in a SPI-2 T3SS-dependent manner (Cheminay et al., 2005). SopD2 was among the effectors implicated in this phenotype (Halici et al., 2008), and given Rab7's role in antigen presentation (Bertram et al., 2002), our data suggest a model whereby SopD2-mediated disruption of endocytic trafficking impedes antigen presentation. Therefore, elucidation of SopD2’s function in manipulating Rab7 activity not only provides a missing link in our understanding of Salmonella’s ability to evade host innate immunity but may also provide a rationale for this pathogen’s ability to target the adaptive immune system.

It is also important to note that SopD2 may harbor domains capable of targeting alternative processes in host cells, as those associated with Rab7 inhibition appear to be restricted to its amino-terminal region. Alternately, Salmonella may use other effectors to complement SopD2 in the attenuation of Rab7. These strategies of utilizing multifunctional effectors or multiple effectors with the same GTPase target to manipulate host cell function are best evidenced by modulation of Rab1 by multiple type 4 secreted effectors from Legionella pneumophila (Ingmundson et al., 2007; Machner and Isberg, 2006, 2007; Müller et al., 2010; Murata et al., 2006).

Rab7 is a critical regulatory switch in the endocytic pathway, playing intrinsic roles in endocytic trafficking and phagosome-lysosome fusion (Cantalupo et al., 2001; Feng et al., 1995; Méresse et al., 1995; Progida et al., 2007). As such, its inhibition represents an important target of bacterial-mediated pathogenesis, as evidenced by Mycobacterium tuberculosis and Burkholderia cenocepacia (Hyynh et al., 2010; Seto et al., 2009). Our study identifies the mechanism of action of a poorly understood bacterial effector, which directly targets this key endocytic regulator and blocks host trafficking. These findings provide not only important insight into the mode of immune evasion by other intracellular pathogens but also structural and functional information that may provide a rationale for both the identification of Rab7-targeting effectors and the use of SopD2 as a target for therapeutic strategies.

EXPERIMENTAL PROCEDURES

Cells
HeLa, HEK293T, and RAW 264.7 cells were obtained from the ATCC and maintained in growth medium (DMEM high glucose supplemented with 10% fetal bovine serum [FBS]) without antibiotics. For infections of epithelial cells, late-log S. Typhimurium cultures were used in a method optimized for bacterial invasion (Steele-Mortimer et al., 1999).

DQ-Red-BSA Loading of Live Cells
HeLa cells seeded in eight-well coverglass chambers were either infected with GFP-expressing S. Typhimurium or transfected. After 8 hr of infection or 12–16 hr of transfection, cells were incubated for 1 hr in growth medium containing DQ Red BSA (0.25 mg/ml), washed with PBS, and incubated in growth medium for 4 hr. For infection of RAW 264.7 mouse macrophages, cells were treated with DQ Red BSA (10 µg/ml) at 16 hr post-infection. For additional details, please refer to the Supplemental Experimental Procedures.

Magic Red Cathepsin L Assay
HeLa cells seeded in eight-well glass-bottom chambers were infected with GFP-expressing S. Typhimurium or transfected. After 8 hr of infection or 12–16 hr of transfection, cells were incubated for 1 hr in growth medium containing DQ Red BSA (0.25 mg/ml), washed with PBS, and incubated in growth medium for 4 hr. For infection of RAW 264.7 mouse macrophages, cells were treated with DQ Red BSA (10 µg/ml) at 16 hr post-infection. For additional details, please refer to the Supplemental Experimental Procedures.

Confocal Microscopy
When indicated, cells were imaged using a Quorum spinning disk microscope with a 63x oil immersion objective (Leica DMI820 inverted fluorescence microscope equipped with a Hamamatsu Back-Thinned EM-CCD camera, spinning disk confocal scan head, and Volocity acquisition software; Improvision). Confocal z-stack images with z steps of 0.3 μm were acquired, and confocal images were analyzed with Volocity software.

(F) Competitive in vitro binding assay. Rab7-bound resin, preloaded with GTPgammaS, was incubated with equivalent amounts of GST-RILPC33 and increasing concentrations of SopD2. Elutions were analyzed by western blotting. The ratio of RILPC33:SopD2 (1:1) is indicated (one asterisk).

(G) Nucleotide exchange assay. Rab7 was loaded with Mant-GDP. Following desalting, exchange reactions were performed supplemented with SopD2 or BSA at the indicated ratios. Reactions were monitored by spectrofluorometry (excitation at 355 nm, emission at 448 nm).
Co-immunoprecipitations
HEK293T cells seeded into 10-cm-diameter tissue culture dishes were co-transfected with Flag-SopD2 and either pmRFP-N1 or RFP-Rab7 variants. At 40 hr post-transfection, cells were lysed, and the resulting lysates were incubated for 2 hr with Anti-FLAG M2 affinity gel. After washing the resin, bound protein was eluted using 1 x SDS-PAGE loading buffer.

In Vitro Binding to Rab7
Recombinant glutathione S-transferase (GST-) and His-tagged proteins, expressed and purified from E. coli, were used for these studies. Nucleotide loading of the Rab7 GTPase was performed using a protocol modified from Jackson et al. (2006), in which His-Rab7 was treated with 0.4 mM GDP or guanosine triphosphate (GTPγS). In vitro binding was performed at 4°C for 2 hr.

Nucleotide Exchange Assay
Purified untagged Rab7 was loaded with 1.5-fold excess of Mant-GDP at room temperature for 1 hr. The reaction was terminated with 10 mM MgCl2 and de-salted using a PD-10 column. Exchange reactions contained 22 μM Rab7 in the presence or absence of SopD2 or BSA. After a 10-min preincubation on ice, reactions were initiated with 200 μM GTP, and data were acquired at 25°C using a microplate spectrofluorometer (excitation at 355 nm, emission at 448 nm).

Statistics
Three independent experiments were performed, except where indicated, and the mean ± SEM is shown in the figures. The p values were calculated using a two-tailed, two-sample, equal-variance Student’s t test, unless otherwise indicated. Where indicated, a one-way ANOVA with Bonferroni post hoc test was used. A p value < 0.05 was determined to be statistically significant and is indicated by an asterisk in the figures.

ACCESSION NUMBERS
Crystal structure data for SopD2 and SopD have been deposited into the Protein Data Bank under accession codes 5CQ9 and 5CPC, respectively.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.07.063,

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