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Salmonella stimulates pro-inflammatory signalling through p21-activated kinases bypassing innate immune receptors

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Microbial infections are most often countered by inflammatory responses that are initiated through the recognition of conserved microbial products by innate immune receptors and result in pathogen expulsion[1–6]. However, inflammation can also lead to pathology. Tissues such as the intestinal epithelium, which are exposed to microbial products, are therefore subject to stringent negative regulatory mechanisms to prevent signalling through innate immune receptors[6–11]. This presents a challenge to the enteric pathogen Salmonella Typhimurium, which requires intestinal inflammation to compete against the resident microbiota and to acquire the nutrients and electron acceptors that sustain its replication[12,13]. We show here that S. Typhimurium stimulates pro-inflammatory signalling by a unique mechanism initiated by effector proteins that are delivered by its type III protein secretion system. These effectors activate Cdc42 and the p21-activated kinase 1 (PAK1) leading to the recruitment of TNF receptor-associated factor 6 (TRAF6) and mitogen-activated protein kinase kinase kinase 7 (TAK1), and the stimulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) inflammatory signalling. The removal of Cdc42, PAK1, TRAF6 or TAK1 prevented S. Typhimurium from stimulating NF-κB signalling in cultured cells. In addition, oral administration of a highly specific PAK inhibitor blocked Salmonella-induced intestinal inflammation and bacterial replication in the mouse intestine, although it resulted in a significant increase in the bacterial loads in systemic tissues. Thus, S. Typhimurium stimulates inflammatory signalling in the intestinal tract by engaging critical downstream signalling components of innate immune receptors. These findings illustrate the unique balance that emerges from host–pathogen co-evolution, in that pathogen-initiated responses that help pathogen replication are also important to prevent pathogen spread to deeper tissues.

Although inflammation is generally detrimental to bacterial pathogens, stimulation of intestinal inflammation is essential for the enteric pathogen *Salmonella* Typhimurium to compete with the resident microbiota and to have access to critical nutrients that are otherwise unavailable in the uninflamed gut[12,13]. Consequently, *S.* Typhimurium has evolved a mechanism to stimulate inflammation in the intestinal environment through the delivery of a subset of its effector proteins, SopE, SopE2 and SopB, by the type III protein secretion system encoded within its pathogenicity island 1 locus[14–16]. These effectors exert this function by redundantly activating Rho-family GTPases[17–20], which results in transcriptional responses that are very similar to those stimulated by agonists of receptors of the innate immune system[16]. However, the mechanisms by which the pathogen-initiated activation of these GTPases leads to a pro-inflammatory transcriptional response are not completely understood and have been the subject of some controversy[16,21]. It has been proposed that the activation of Rho-family GTPases by the *S.* Typhimurium effectors is sensed as a 'danger-associated molecular pattern' by the innate immune receptor nucleotide-binding oligomerization domain-containing protein 1 (NOD1) through unknown mechanisms[21], which through the stimulation of the associated kinase Rip2[22,23] leads to nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation and pro-inflammatory transcriptional responses. However, previous studies have also shown that depletion of Cdc42 abolishes *S.* Typhimurium stimulation of inflammatory signalling, despite Cdc42 depletion having no effect the ability of *S.* Typhimurium to activate the Rho-family GTPases Rac1 and the resulting actin cytoskeleton rearrangements that lead to bacterial internalization[16,18]. Previous studies have also shown that *S.* Typhimurium stimulates intestinal inflammation in Rip2-deficient mice in a manner that is indistinguishable from wild-type mice[16], even though these animals are completely impaired in NOD1 signalling[22,23]. To clarify these contrasting results, we removed Rip2 from the cell line used in the previous studies[21] using CRISPR–Cas9-mediated genome editing and observed that, contrary to the previous report[21] but consistent with our previous results[16], it had no effect on the ability of *S.* Typhimurium to stimulate NF-κB signalling (Fig. 1a) or to invade cultured cells (Supplementary Fig. 1). As expected from previous reports[22,23], the removal of Rip2 completely abolished NOD receptor signalling (Supplementary Fig. 2). Similarly, NF-κB activation mediated by the transient expression of the *Salmonella* effector SopE was unaffected by the absence of Rip2 (Fig. 1b). In contrast, and consistent with previous observations[16,18], CRISPR–Cas9-mediated removal of Cdc42 completely abolished SopE-stimulated NF-κB signalling (Fig. 1c) and significantly reduced NF-κB activation following wild-type *S.* Typhimurium infection (Supplementary Figs. 3 and 4), although it did not affect TNFα signalling (Supplementary Fig. 5). Although SopE, SopE2 and SopB activate Rho-family GTPases in a functionally redundant manner[18,19], their mechanism of action is different. The highly related effectors SopE and SopE2 are exchange factors for Rho-family GTPases and therefore activate these GTPases directly[17,24]. In contrast, SopB is a phosphoinositide phosphatase that stimulates Rho-family GTPase signalling indirectly by activating endogenous exchange factors through phosphoinositide fluxes[18]. The deletion of *sopB* from *S.* Typhimurium resulted in a significant reduction of the residual NF-κB activation observed in Cdc42-deficient cells after infection with wild-type bacteria (Fig. 1d) indicating that, in the absence of Cdc42, SopB may be able to stimulate NF-κB through

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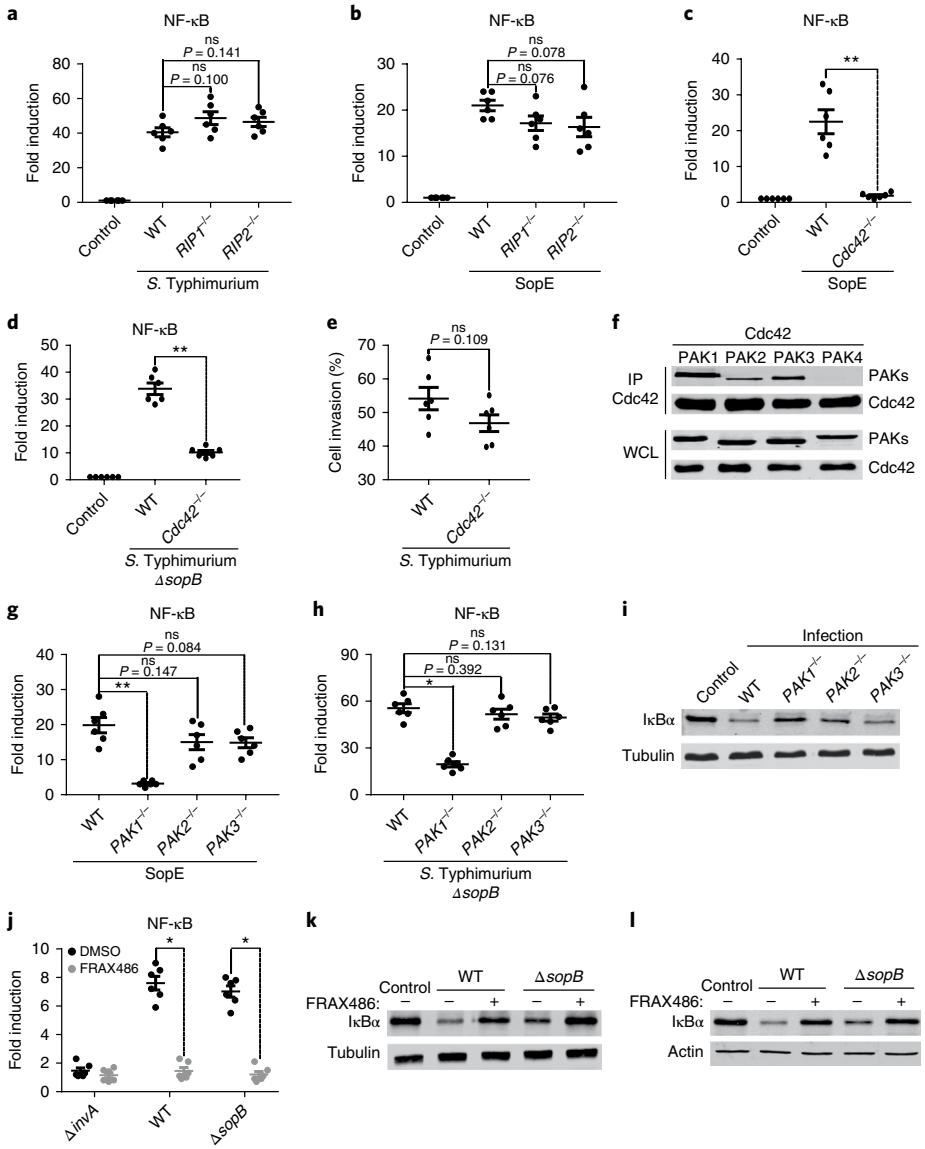

Fig. 1 | *S.* Typhimurium stimulates pro-inflammatory signalling through Cdc42 and its effector kinase PAK1. a,**b**, Rip1 or Rip2 are not required for *S.* Typhimurium stimulation of NF-κB signalling. **a**, HEK293T, Rip1- or Rip2-deficient cells were transfected with a NF-κB luciferase reporter and subsequently infected with *S.* Typhimurium (multiplicity of infection (m.o.i.) = 10). **b**, Alternatively, the same cells were co-transfected with a plasmid expressing the *S.* Typhimurium effector protein SopE. Luciferase activity was measured 8 or 20 h after bacterial infection (**a**) or *sopE* DNA transfection (**b**), respectively. **c**,**d**, NF-κB activation in Cdc42-deficient cells after SopE expression or *S.* Typhimurium infection. HEK293T or Cdc42-deficient cells were transfected with a plasmid expressing the effector protein SopE (**c**) or infected with *S.* Typhimurium Δ*sopB* (**d**; m.o.i. = 10) and the NF-κB activation was measured with a luciferase reporter as indicated above (controls in **a**–**d** are uninfected HEK293T cells). **e**, *S.* Typhimurium invasion of Cdc42-deficient cells. HEK293T or Cdc42-deficient cells were infected with wild-type *S.* Typhimurium (m.o.i. = 10) and the levels of internalized bacteria were determined by the gentamicin protection assay. Data represent the percentage of inoculum that survived the gentamicin treatment due to bacterial internalization and are mean ± s.d. of three independent determinations. **f**, Interaction of Cdc42 with PAK kinases after *S.* Typhimurium infection. HEK293T cells were transiently co-transfected with plasmids encoding FLAG-epitope-tagged Cdc42 and plasmids encoding M45-tagged PAK1, PAK2, PAK3 or PAK4. Eighteen hours after transfection, the cells were infected with *S.* Typhimurium (m.o.i. = 30) for 1 h and the cell lysates were analysed by immunoprecipitation with anti-FLAG and western blotting with anti-M45 and anti-FLAG antibodies. IP, immunoprecipitates; WCL, whole cell lysates. **g**–**i**, NF-κB activation in PAK-deficient cells after SopE expression or *S.* Typhimurium infection. **g**,**h**, HEK293T, PAK1, PAK2 or PAK3-deficient cells were co-transfected with a NF-κB luciferase reporter and a plasmid expressing the *S.* Typhimurium effector protein SopE (**g**) or infected with *S.* Typhimurium Δ*sopB* (**h**; m.o.i. = 10). The luciferase activity was measured 20 and 8 h after *sopE* DNA transfection or bacterial infection, respectively. **i**, Alternatively, the levels of IκBα in *S.* Typhimurium-infected cells were examined by immunoblotting with anti-IκBα and anti-tubulin (as loading control) antibodies. **j**–**l**, Effect of a group I PAKs inhibitor on NF-κB activation after *S.* Typhimurium infection. **j**, Cultured intestinal epithelial Henle-407 cells were transfected with a NF-κB luciferase reporter, treated with the PAK inhibitor FRAX486 (10 μM) or its solvent dimethylsulfoxide (DMSO) for 1 h, then infected (m.o.i. = 10) with wild-type, Δ*invA* (a type III secretion defective mutant) or Δ*sopB* *S.* Typhimurium strains and the luciferase activity was measured 8 h after bacterial infection. **k**,**l**, Alternatively, Henle-407 cells (**k**) or enteroids derived from wild-type C57/BL6 mice (**l**) treated with the PAK inhibitor FRAX486 (10 μM) or its solvent DMSO for 1 h were infected with wild-type *S.* Typhimurium and the levels of IκBα were examined by immunoblotting with anti-IκBα and anti-actin or anti-tubulin (as loading controls) antibodies. **a**–**d**,**g**,**h**,**j**, Data represent fold induction over control and are the mean ± s.d. of three independent measurements. Experiments shown in **f**,**i**,**k**,**l** were independently repeated at least three times with equivalent results. WT, wild-type HEK293T cells; $^{**}P < 0.01$; $^{*}P < 0.05$; ns, not significant; two-tailed Student's *t*-test.

more complex signalling events, leading to the activation of other GTPases. Consistent with previous observations[18], the removal of Cdc42 did not affect the ability of *S.* Typhimurium to stimulate actin-mediated bacterial uptake (Fig. 1e), a phenotype that is dependent on other Rho-family GTPases[18]. Together these results indicate that Cdc42 plays a central role in orchestrating *S.* Typhimurium-mediated inflammatory signalling. These results also indicate that, contrary to previous reports[21], NOD1 signalling is not required for *S.* Typhimurium to stimulate inflammatory signalling in cultured cells. Furthermore, these results show that the presence of internalized *Salmonella* or its ability to modulate actin remodelling and/or Rho-family GTPase activity per se is not sufficient to trigger inflammatory signalling. These observations are also consistent with previous reports indicating that the removal of Rip2[16] or critical components of the inflammasome[16,25] does not impair the ability of *S.* Typhimurium to stimulate intestinal inflammation.

To investigate the mechanisms by which *S.* Typhimurium triggers inflammatory signalling through Rho-family GTPase activation we searched for Cdc42-interacting proteins after *S.* Typhimurium infection. To ensure that the most relevant Cdc42 effectors were identified, we carried out these experiments at a stage of infection when *S.* Typhimurium effectors would be expected to trigger inflammatory signalling[16,26]. We infected a cell line engineered to stably express endogenous levels of FLAG-epitope-tagged Cdc42 and the interacting proteins were identified by affinity purification and liquid chromotography–tandem mass spectrometry (LC-MS/MS) analysis. We readily detected p21-activated kinase 1 (PAK1) and 2 (PAK2) as prominent Cdc42-interacting proteins in *S.* Typhimurium-infected cells, although these interactors were not detected in uninfected cells (Supplementary Tables 1,2 and Supplementary Fig. 6). The interactions between Cdc42 and PAK1 or PAK2 after *Salmonella* infection were confirmed in equivalent experiments conducted in cells expressing epitope-tagged versions of these proteins (Fig. 1f and Supplementary Fig. 7). The PAKs are a family of Cdc42- and Rac-activated serine/threonine kinases involved in signal transduction leading to a variety of cellular responses[27–29]. There are six PAK family members that, on the basis of their structural similarity, can be classified into two groups encompassing PAK1 through 3 (group I) and PAK4 through 6 (group II). Consistent with their potential involvement in the *Salmonell*a-induced responses, the group I PAK kinases PAK1 and PAK2 have been previously linked to inflammatory signalling in the intestine[30,31] and we found that they are both prominently expressed in cultured cells known to mount a pro-inflammatory transcriptional response to *S.* Typhimurium, as well as in the mouse intestine (Supplementary Fig. 8). To investigate the potential involvement of PAKs in the *S.* Typhimurium stimulation of pro-inflammatory signalling, we examined the activation of NF-κB after bacterial infection in CRISPR–Cas9-generated PAK1-, PAK2- or PAK3-deficient cell lines. We found that although the individual removal of the different PAKs did not result in a significant reduction in the ability of wild-type *S.* Typhimurium to stimulate NF-κB signalling (Supplementary Fig. 9), removal of PAK1 resulted in a significant reduction in NF-κB activation after SopE transient expression (Fig. 1g) or *S.* Typhimurium Δ*sopB* infection (Fig. 1h,i). PAK1-deficient cells were unaffected in their response to TNFα (Supplementary Fig. 9) or in STAT3 or Erk activation after *S.* Typhimurium infection (Supplementary Fig. 10). Furthermore, the removal of PAK1, PAK2 or PAK3 did not affect the ability of *S.* Typhimurium to invade these cells (Supplementary Fig. 11). We hypothesized that the more complex signalling stimulated by SopB[32–35], potentially involving other Rho-family GTPases[18], may result in the engagement of multiple PAK family members. We were unable to generate cell lines defective in multiple PAK family members, which is in keeping with their redundant involvement in essential cell biological processes[27–29,36–39]. However, consistent with this hypothesis, the addition of a highly specific inhibitor of group I PAK family members[40] completely abolished the ability of wild-type or *S.* Typhimurium Δ*sopB* to stimulate NF-κB signalling in cultured intestinal epithelial cells (Fig. 1j,k) or in enteroids derived from C57/BL6 mice (Fig. 1l). In contrast, addition of the inhibitor did not affect NF-κB activation after the addition of TNFα or IL1β (Supplementary Fig. 12). These results indicate that Cdc42 orchestrates inflammatory signalling after *Salmonella* infection by engaging its PAK family effectors, particularly PAK1.

Although studies have implicated PAK1 in multiple signalling cascades leading to NF-κB activation[30,31,41], the actual mechanisms by which this kinase participates in this signal transduction pathway are not understood[27–29]. To investigate the mechanisms by which PAK1 contributes to inflammatory signalling stimulated by *S.* Typhimurium, we searched for interacting proteins using the approach described above for Cdc42. We engineered a cell line to stably express endogenous levels of FLAG-epitope-tagged PAK1 and infected these cells with wild-type *S.* Typhimurium. PAK1-interacting proteins were identified by affinity purification and LC-MS/MS analysis as described above. We identified TNF receptor-associated factor 6 (TRAF6) and mitogen-activated protein kinase kinase kinase 7 (TAK1) as prominent PAK1-interacting proteins in infected cells but not in uninfected cells (Supplementary Tables 3,4 and Supplementary Fig. 13). These interactions were verified in equivalent experiments conducted in cells expressing epitope-tagged versions of these proteins (Fig. 2a). Furthermore we found that in the absence of infection, transiently expressed epitope-tagged full-length PAK1 did not interact with TRAF6 (Fig. 2b), consistent with the notion that PAK1 can only engage TRAF6–TAK1 following its Cdc42-mediated activation after bacterial infection. Consistent with this hypothesis, a constitutively active form of PAK1 lacking its auto-inhibitory domain[27–29] readily interacted with TRAF6 in the absence of infection (Fig. 2b). These results indicate that *S.* Typhimurium infection and the subsequent activation of Cdc42 by the bacterial effector proteins leads to the formation of a PAK1–TRAF6–TAK1 complex.

Both TRAF6 and TAK1 are essential components of several immune receptor-initiated pro-inflammatory signalling pathways that lead to NF-κB activation[42,43]. Therefore, to assess their potential role in the *Salmonella*-initiated pro-inflammatory signalling, we generated TRAF6- and TAK1-deficient cell lines using CRISPR–Cas9-mediated genome editing, infected them with wild-type *S.* Typhimurium and examined them for NF-κB activation. We found that the removal of TRAF6 or TAK1 completely abolished NF-κB activation after *S.* Typhimurium infection (Fig. 2c). This phenotype was reversed by the expression of wild-type TRAF6 or TAK1 in the respective defective cell lines (Fig. 2d,e), but not by the expression of a catalytic inactive form of TRAF6 ($TRAF6^{C70A}$) (Fig. 2d). In contrast, the ability of *S.* Typhimurium to invade cells was unaffected in TRAF6- or TAK1-deficient cells (Supplementary Fig. 14). These results indicate that the catalytic activity of both TRAF6 and TAK1 are required for *S.* Typhimurium-mediated pro-inflammatory signalling. Given the known roles of TRAF6 and TAK1 in signal transduction for pathways emanating from innate immune receptors[42,43], these results provide an explanation for the strong similarities observed between the pro-inflammatory transcriptional responses resulting from *S.* Typhimurium infection and those resulting from the stimulation of innate immune receptors[16]. In addition, these results support the notion that *Salmonella* can stimulate innate immune-like outputs in infected cells without the engagement of innate immune receptors[16].

We observed that *S.* Typhimurium infection of cultured cells resulted in the presence of a slower migrating PAK1 band in sodium dodecyl sulphate-polyacrylamide electrophoresis (SDS-PAGE), which we showed to be due to its phosphorylation (Fig. 3a). LC-MS/MS analysis of PAK1 isolated from *S.* Typhimurium-infected cells identified two phosphorylated residues: S220 and

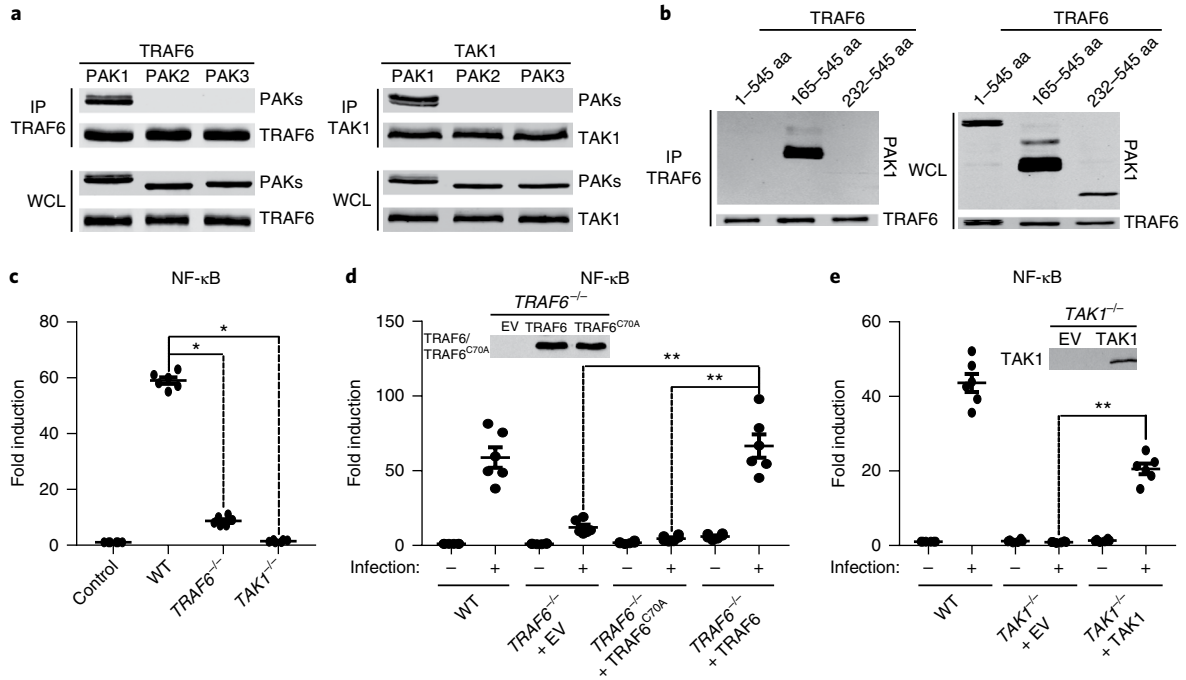

Fig. 2 | PAK1–TRAF6–TAK1 mediates *S.* Typhimurium pro-inflammatory signalling downstream of Cdc42. a, PAK1 forms a complex with TRAF6 and TAK1 after *S.* Typhimurium infection. HEK293T cells were transiently co-transfected with plasmids expressing FLAG-epitope-tagged TRAF6 or TAK1 along with plasmids encoding M45-tagged PAK1, PAK2 or PAK3. Eighteen hours after transfection, cells were infected with *S.* Typhimurium for 1 h (m.o.i. = 30) and the cell lysates were analysed by immunoprecipitation with anti-FLAG and western blotting with anti-M45 and anti-FLAG antibodies. **b**, Interaction between TRAF6 and different PAK1-deletion mutants. HEK293T cells were transiently co-transfected with plasmids expressing FLAG-epitope-tagged TRAF6 along with plasmids encoding M45-tagged full-length (1–545) PAK1 or the indicated deletions. Twenty hours after transfection, cell lysates were analysed by immunoprecipitation with anti-FLAG and western blotting with anti-M45 and anti-FLAG antibodies. **c–e**, NF-κB activation in TRAF6- or TAK1-deficient cells after *S.* Typhimurium infection. **c**, HEK293T and TRAF6- or TAK1-deficient cells were transfected with a NF-κB luciferase reporter and subsequently infected with *S.* Typhimurium (m.o.i. = 10). **d**,**e**, Alternatively, TRAF6- or TAK1-deficient cells stably expressing endogenous levels of the wild-type alleles of TRAF6, its catalytic mutant TRAF6^{C70A} or TAK1 or carrying the empty vector (EV), were infected with *S.* Typhimurium in the same manner. The luciferase activity was measured 8 h after bacterial infection. Data represent fold induction over control and are the mean ± s.d. of three independent measurements. $^{**}P < 0.01$; $^{*}P < 0.05$; two-tailed Student's *t*-test. Experiments in **a**,**b** were independently repeated at least three times with equivalent results.

S223. The latter was enriched in the slower migrating band (Fig. 3b and Supplementary Tables 5,6). Mutagenesis analysis further confirmed that the slower migration of the band was due to the phosphorylation of S223, as infection of cells stably expressing the FLAG-tagged PAK1^{S223A} mutant did not show the presence of the slower migrating band (Fig. 3c) although cells expressing PAK1^{S220A} did (Fig. 3c). PAK1 is regulated by auto-inhibition of its C-terminal catalytic domain by the N-terminal auto-inhibitory domain. The binding of activated Rho-family GTPases results in relief from this auto-inhibition, leading to PAK1 activation and autophosphorylation at multiple amino terminal residues, including S220[27–29]. However, for some phenotypes that may require persistent PAK1 activation, relief from auto-inhibition is not sufficient for PAK1 activity, which requires an additional phosphorylation event at S223 by an exogenous kinase[44]. We found that PAK1^{S223A} is unable to transduce *S.* Typhimurium-initiated NF-κB signalling (Fig. 3d), indicating that S223 phosphorylation is required for PAK1 function in the context of *Salmonella*-induced pro-inflammatory signalling. Importantly, we found that PAK1 S223 phosphorylation did not require its kinase activity (Supplementary Fig. 15) and did not occur in TAK1-deficient cells (Fig. 3e and Supplementary Fig. 15). These observations suggest a model in which the Cdc42-mediated activation of PAK1 (through its autophosphorylation at S220) leads to the recruitment of TRAF6 and TAK1, which subsequently results in the additional phosphorylation of PAK1 at S223 by TAK1. The mechanisms by which TAK1 is activated are not understood but it is probably the result of TRAF6-mediated ubiquitination, given that the catalytic activity of TRAF6 is required for *Salmonella*-induced NF-kB activation (see Fig. 2d). More experiments will be required to clarify some of the details of this signalling pathway.

We investigated the potential relevance of PAK1 signalling in *S.* Typhimurium-induced intestinal inflammation in a mouse model of infection. We first compared the ability of orally administered *S.* Typhimurium to stimulate inflammation and to replicate in the intestinal tract of PAK1-deficient[45] and C57/BL6 control mice. We found that although the production of pro-inflammatory cytokines was reduced in PAK1-deficient animals, the difference did not reach statistical significance (Supplementary Fig. 16). However, the phenotype of PAK1-deficiency was more pronounced in the more simplified experimental system afforded by enteroids derived from PAK1-deficient animals, which showed markedly reduced NF-κB activation after *S.* Typhimurium infection in comparison to enteroroids derived from wild-type animals (Supplementary Fig. 17). We hypothesized that the weak phenotype observed in the deficient animals might be due to compensation for the absence of PAK1 by the highly related kinase PAK2, which shares several redundant activities[36–39]. In fact, we found that PAK2 was also engaged by Cdc42 during *S.* Typhimurium infection (Fig. 1f, Supplementary Tables 1,2 and Supplementary Fig. 6). Because PAK2-defficiency is embryonically lethal, we tested this hypothesis by examining the effect of oral administration of a highly specific inhibitor of group I PAKs[40] on the ability of orally administered wild-type *S.* Typhimurium to stimulate inflammation and replicate in the intestinal tract. We found that oral administration of the inhibitor drastically reduced

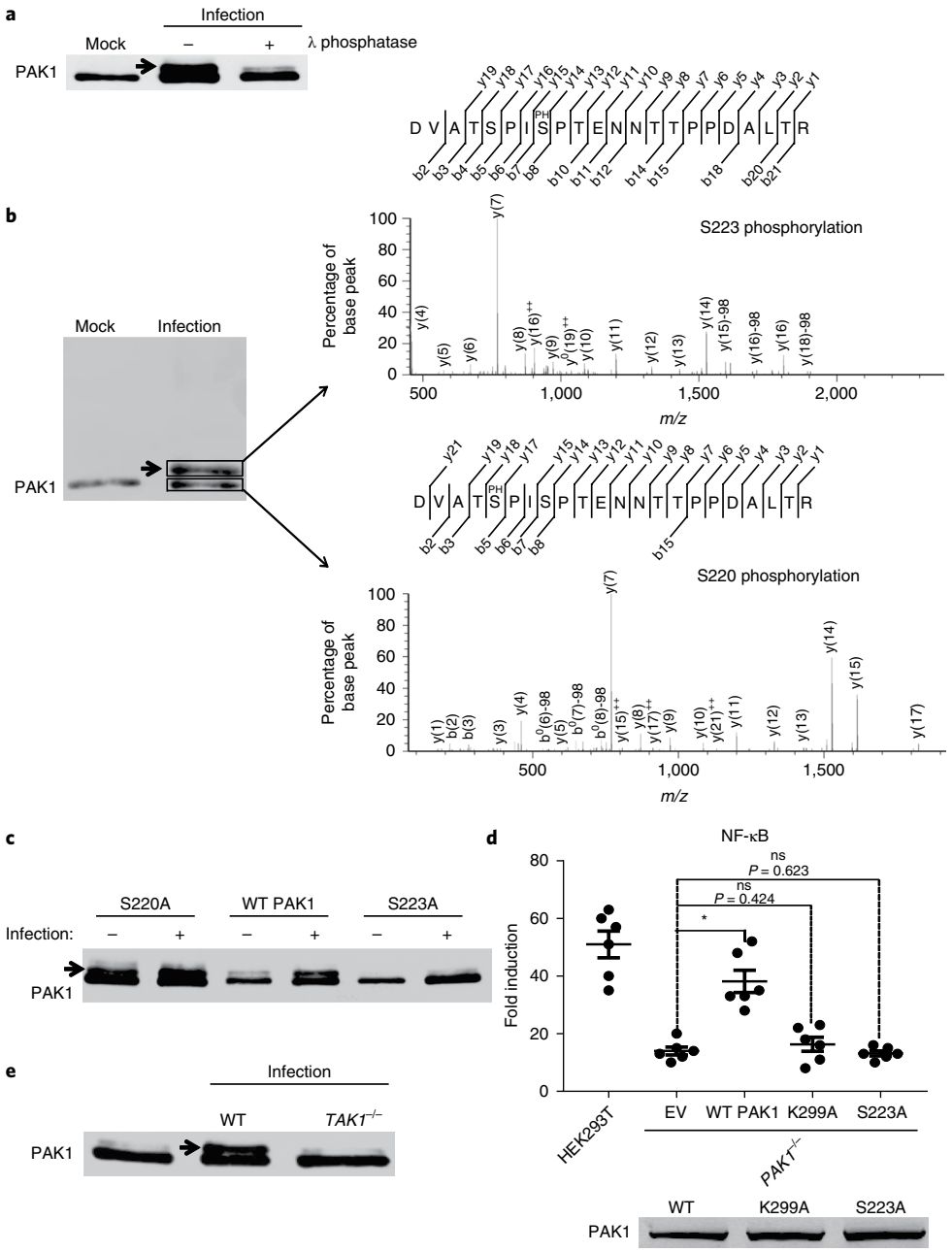

Fig. 3 | *S.* Typhimurium stimulates TAK1-dependent PAK1 phosphorylation, which is essential for pro-inflammatory signalling. a, PAK1 is phosphorylated following *S.* Typhimurium infection. HEK293T cells stably expressing FLAG-tagged PAK1 were infected with wild-type *S.* Typhimurium for 1 h (m.o.i. = 30) and the cell lysates were analysed by immunoblotting with an anti-FLAG antibody before and after λ phosphatase treatment. **b**, Analysis of phosphorylation sites in PAK1 after *S.* Typhimurium infection. PAK1 was isolated from HEK293T cells stably expressing FLAG-tagged PAK1 by immunoprecipitation before and after wild-type *S.* Typhimurium infection (m.o.i. = 30) and separated by SDS-PAGE. The bands corresponding to the slower (phosphorylated) and normal migrating species were subjected to LC-MS/MS analysis to map the phosphorylation sites as indicated in Methods. **c**,**d**, PAK1 S223 phosphorylation is required for *S.* Typhimurium pro-inflammatory signalling. **c**, HEK293T cells stably expressing FLAG-epitope-tagged PAK1 (WT PAK1) or its phosphorylation-site mutants ($PAK1^{S220A}$ or $PAK1^{S223A}$) were infected with *S.* Typhimurium (m.o.i. = 30) and whole cell lysates were analysed by immunoblotting. **d**, Alternatively, HEK293T or PAK1-deficient cells stably expressing wild-type PAK1, its kinase inactive ($PAK1^{K299A}$) or phosphorylation-site mutant ($PAK1^{S223A}$) were infected with *S.* Typhimurium (m.o.i. = 10) and NF-κB activation was measured 8 h after bacterial infection with a luciferase reporter as indicated above**.** Data represent fold induction over control and are the mean ± s.d. of three independent measurements. $^*P < 0.05$; ns, not significant; two-tailed Student's *t*-test. **e**, PAK1 S223 phosphorylation requires TAK1. HEK293T or TAK1-deficient cells stably expressing FLAG-tagged PAK1 were infected with *S.* Typhimurium (m.o.i. = 30) and analysed by immunoblotting with an anti-FLAG antibody. Arrows indicate the position of phosphorylated PAK1 species. Experiments shown in **a**,**b**,**c**,**e** were repeated at least three times with equivalent results.

both the production of pro-inflammatory cytokines (Fig. 4a) and the replication of *S.* Typhimurium in the intestinal tract (Fig. 4b), although the inhibitor itself had no effect on the replication of *S.* Typhimurium in broth culture (Supplementary Fig. 18). The drastic reduction in the inflammatory response to *S.* Typhimurium was also observed in mice that had been pre-treated with streptomycin

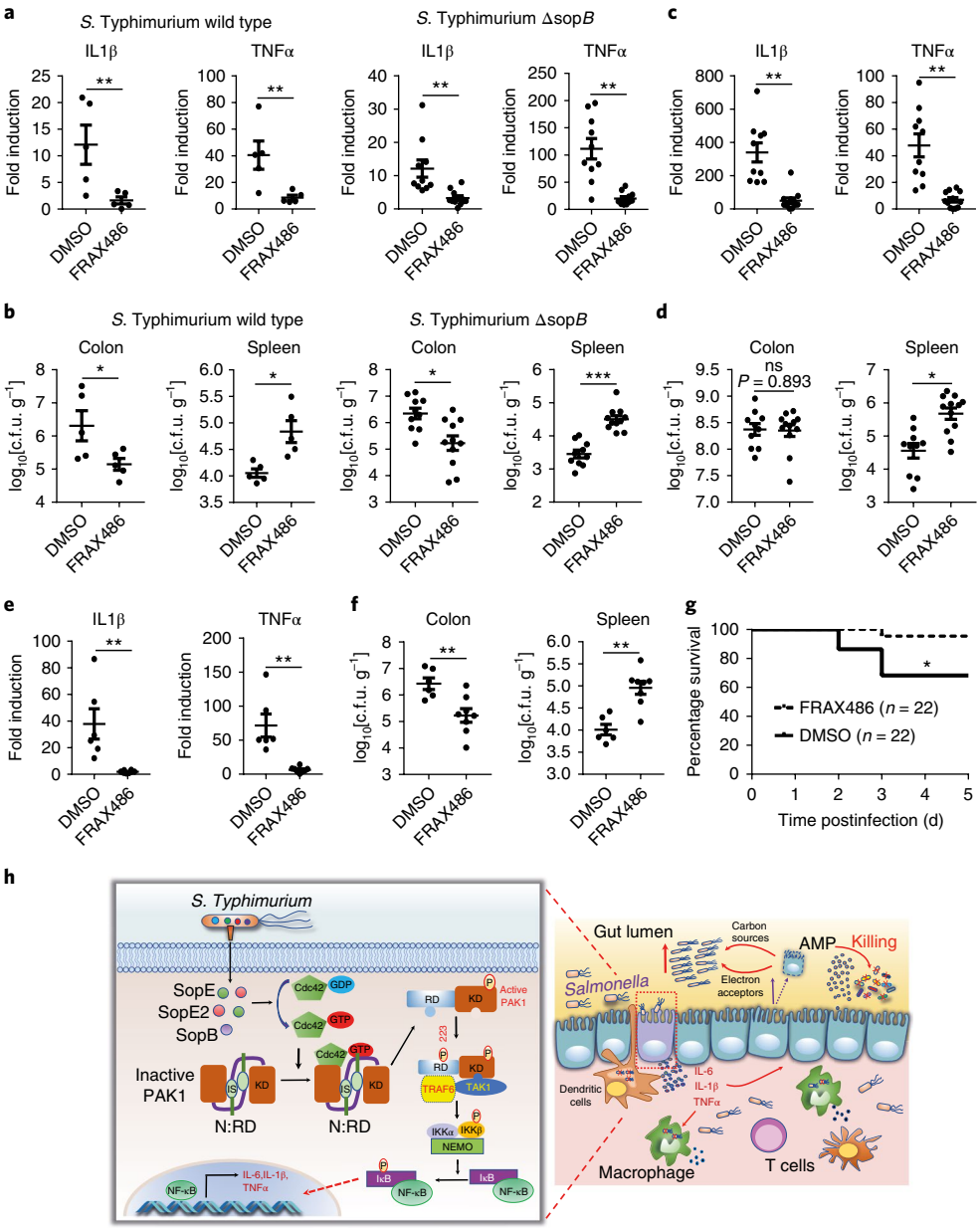

Fig. 4 | PAK-mediated pro-inflammatory signalling is required for *S.* Typhimurium replication within the intestine and for the host response that limits systemic infection. a–**d**, C57/BL6 *Nramp*$^{+/+}$ mice were orally treated with DMSO or the group I PAK inhibitor FRX486 and orally infected with wild-type or Δ*sopB S.* Typhimurium (**a**,**b**). Alternatively, 20 mg streptomycin (to deplete the intestinal microbiota) was orally administered to the mice 24 h before infection (**c**,**d**). **a**,**c**, Four days after infection, the relative levels of pro-inflammatory cytokine mRNAs in the intestine were measured by quantitative real-time PCR. Circles in **a** and **c** represent the relative levels of the indicated cytokines normalized to GAPDH levels and circles in **b** and **d** represent the bacterial loads in the indicated tissues of individual animals. The results are the combination of at least two independent experiments. **e**–**g**, A PAK inhibitor protects animals from intestinal cytokine-mediated death after *Salmonella* infection. C57BL/6 *Nramp*$^{+/+}$ mice were orally treated with DMSO or the group I PAK inhibitor FRX486 and then orally infected with the Δ*pipA* Δ*gogA* Δ*gtgA S.* Typhimurium, a mutant that stimulates an augmented intestinal inflammatory response. **e**, Four days after infection, the relative levels of pro-inflammatory cytokine mRNAs in the intestine were measured by quantitative real-time PCR. Circles in **e** represent the relative levels of the indicated cytokines normalized to GAPDH levels and circles in **f** represent the bacterial loads in the indicated tissues of individual animals. The results are the combination of at least two independent experiments. **g**, The survival of animals at the indicated times was scored as a combination of two independent experiments. **h**, Model of the interaction of *Salmonella* with the intestinal epithelium. Through its type III secretion system encoded within its pathogenicity island 1, *Salmonella* delivers the effector proteins SopE, SopE2 and SopB that activate Cdc42 in a functionally redundant manner. The activation of Cdc42 leads to the formation of a PAK1–TRAF6–TAK1 complex, the phosphorylation of PAK1 at Ser223 and the subsequent activation of NF-κB and production of pro-inflammatory cytokines. The ensuing intestinal inflammation allows *Salmonella* to out-compete the resident microbiota and replicate within the lumen of the intestinal tract. At the same time, the inflammatory response controls the spread of *Salmonella* to deeper tissues. Data represent mean ± s.e.m. *$P < 0.05$; ** < P 0.01; ***$P < 0.001$; two-tailed Student's *t*-test (**a**–**f**); log-rank test (**g**).

to deplete members of the intestinal microbiota (Fig. 4c)[46], except that in this case decreased bacterial replication in the intestine was not observed (Fig. 4d). The latter is consistent with previous studies that have shown that the removal of the competing intestinal microbiota by antibiotic treatment obviates the need for inflammation for *S.* Typhimurium to replicate within intestinal tissues[12]. In contrast, administration of the PAK inhibitor resulted in a significant increase in the number of *S.* Typhimurium colony forming units (c.f.u.) in systemic tissues of both streptomycin-treated (Fig. 4d) and untreated (Fig. 4b) animals. Note that the systemic administration of the PAK1 inhibitor had no effect in the production of pro-inflammatory cytokines and the replication of *S.* Typhimurium in the intestinal tract (Supplementary Fig. 19). These observations indicate that, to be effective, the inhibitor must act locally on the intestinal epithelium. Furthermore, these results are consistent with previous reports indicating that, in the inflamed gut, PAK1 activation is limited to the luminal surface of the intestinal epithelium[30].

To further investigate the role of PAKs in the stimulation of pro-inflammatory responses in the intestine, we made use of a *S.* Typhimurium mutant lacking the type III secretion effector proteins PipA, GogA and GtgA, which dampen the intestinal inflammatory response to *Salmonella* by specifically targeting the NF-κB transcription factors RelA and RelB[47]. In a NRAMP1 (SLC11A1)$^{+/+}$ mouse, this mutant strain exhibits increased lethality that is not due to increased bacterial replication, but to the heightened production of pro-inflammatory cytokines in the gut[47]. We found that the administration of the PAK inhibitor markedly reduced the production of pro-inflammatory cytokines (Fig. 4e) and the bacterial loads (Fig. 4f) in the intestinal tract, although it increased the c.f.u. in systemic tissues (Fig. 4f). More importantly, the addition of the inhibitor protected orally infected animals from death due to a heightened cytokine production as a consequence of infection with this *S.* Typhimurium mutant strain (Fig. 4g). These results indicate that PAKs play a central role in the coordination of the inflammatory response to *S.* Typhimurium in the intestinal tract. They also show that, although the inflammatory response is critically important for the replication of *S.* Typhimurium within the intestine, this response is central for the host to anatomically restrict the pathogen and prevent its access to deeper tissues.

We have described here a pathogen-specific mechanism utilized by *S.* Typhimurium to trigger intestinal inflammation without the engagement of innate immune receptors (Fig. 4h). This mechanism allows *Salmonella* to stimulate a response that shares a high similarity to the responses stimulated by the activation of canonical innate immune receptors, while avoiding the negative regulatory mechanisms that prevent the activation of these receptors in the intestinal tract. This pathogen achieves this remarkable feat by engaging innate immune-signalling pathways downstream from the actual receptors that initiate them. These results are an example of the unique balance that emerges from host–pathogen co-evolution in that pathogen-initiated responses that help pathogen replication are also important to prevent pathogen spread to deeper tissues. The mechanisms described here could help develop anti-pathogen therapeutic strategies by targeting specific host-signalling pathways.

Methods

Plasmids, antibodies and reagents. All of the plasmids used in this paper were generated using the Gibson assembly cloning method[48] as described previously[47]. Antibodies to IκBα (Cat. # 4814S), p-Erk (Cat. # 4370S) and p-STAT3 (Cat. # 9145P), were purchased from Cell Signaling Technology and the anti-FLAG M2 (Cat. # F1804) from Sigma. The monoclonal antibody directed to the M45 epitope was obtained from P. Hearing[49]. The inhibitor of PAK group I, FRAX486 (Cat. # S7807), was purchased from Selleckchem, λ phosphatase (#P0753) from NEB and the Dual-Luciferase Reporter Assay kit (Cat. # E1910) from Promega.

Bacterial strains and growth conditions. The wild-type *Salmonella enteria* serovar Typhimurium strain SL1344[50] and the Δ*sopB*[19], Δ*invA*[51] or Δ*pipA* Δ*gogA* Δ*gtgA*[47] mutant derivatives have been described in earlier works. Bacteria were cultured as described previously[52] under conditions that stimulate the expression of the SPI-1 type III protein secretion system.

Cell culture and bacterial infections. Human intestinal epithelial Henle-407 (obtained from the Roy Curtiss III collection in 1987) and HEK293T cells (American Type Culture Collection) were cultured and infected with bacteria as described in an earlier study[47]. Briefly, 18 h after seeding onto tissue culture plates, cells were infected for the indicated time with various *S.* Typhimurium strains at a m.o.i. as specified in the figure legends. The infected cells were treated with gentamicin (100 μg ml^{-1}) for 1 h. In experiments involving longer infection times, the infected cells were cultured in medium containing a low gentamicin concentration (10 μg ml^{-1}) for the indicated times. All cell lines were routinely tested for the presence of mycoplasma by a standard PCR method. The cells were frequently checked for their morphological features, growth characteristics and functionalities, but were not authenticated by short tandem repeat profiling.

Generation of stable cell lines expressing FLAG-epitope-tagged Cdc42 or PAK1. Stable cell lines (HEK293T) expressing FLAG-epitope-tagged Cdc42 or PAK1 were generated through viral transduction using the plpcx-based retroviral vector (a gift from Walter Mothes Lab). Pseudotyped virus was produced by co-transfecting 1 μg plpcx-FLAG-Cdc42 or plpcx-FLAG-PAK1, 1 μg pGag/Pol and 0.5 μg pVSVG in a 3.5 cm dish of HEK293T cells. Cell-culture supernatants were collected 48 h after transfection and used at a dilution of 1:10 to transduce HEK293T cells. The transduced cells were selected with 1 μg ml^{-1} puromycin (Sigma) for 5–7 d to generate stable HEK293T lines expressing FLAG-epitope-tagged Cdc42 or PAK1.

Generation and infection of mouse intestinal epithelial enteroids. The generation and culture of mouse intestinal epithelial enteroids were performed according to the protocol supplied by STEMCELL Technologies[53]. One hour before infection, enteroids were treated with FRAX486 (10 μM) or the DMSO vehicle alone. Then enteroids were dissociated in 200 μl Dulbecco's phosphate-buffered saline by pipetting up and down at least 50 times. The suspension was centrifuged at 500*g* for 5 min. The supernatant was discarded and the pellets were resuspended in 0.1 ml Dulbecco's modified eagle medium/nutrient mixture F-12 containing *S.* Typhimurium (at an estimated m.o.i. of 10) and incubated at 37 °C for 1 h. After centrifugation (500*g*, 5 min), the supernatants were discarded and the pellets were resuspended in 100 μl SDS lysis buffer. The samples were subjected to western blot analysis.

Generation of CRISPR–Cas9-edited cell lines. The generation of CRISPR–Cas9-edited cell lines was carried out as described in earlier work[54] following standard protocols[55] and using the primers listed in Supplementary Table 7. Briefly, DNA repair templates were designed, containing sequences homologous to the upstream and downstream regions of the target site separated by an insert-containing stop codon in the three reading frames. The repair template and plasmids encoding the guide RNA, Cas9 and a gene encoding puromycin resistance were transfected into HEK293T cells using Lipofectamin 2000. The transfected cells were selected by puromycin treatment for 2 d and the isolated clones were screened by PCR genotyping to identify cells with the inactivated gene of interest (Supplementary Fig. 15) using the primers listed in Supplementary Table 7. In each case, several independently isolated clones were characterized for the relevant phenotypes and found to have indistinguishable behaviour.

Invasion assay. The ability of *S.* Typhimurium to invade cultured cells was evaluated using the previously reported gentamicin protection assay[56].

NF-κB luciferase reporter assay. The NF-κB luciferase reporter assay has been described in earlier work[47]. Briefly, the indicated cells were co-transfected with 20 ng of the pGl3-luc reporter plasmid encoding a NF-κB-responsive element and 20 ng of pRL-actin as an internal control. Cells were subjected to the indicated treatments 18 h after transfection and the luciferase activity was measured in the cell lysates.

Phosphatase treatment of cell lysates. HEK293T cells stably expressing FLAG-tagged PAK1 were seeded onto six-well plates and, ~18 h later, infected with *S.* Typhimurium at a m.o.i. of 10 for 40 min. Cells were then lysed in 0.1% sodium deoxycholate (DOC) in Tris-buffered saline, centrifuged at 14,000 r.p.m. for 15 min at 4 °C and the resulting supernatants were treated with λ phosphatase (800 units) for 2 h at 30 °C. Samples were then analysed by SDS-PAGE and western blotting with antibodies to FLAG.

Quantitative PCR. Quantitative PCR of mRNA in mouse tissues was performed as described previously[47]. Briefly, total RNA from mouse ceca were isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol and reversed transcribed with iScript reverse transcriptase (Bio-Rad). Quantitative PCR was performed using iQ SYBR Green Supermix (Bio-Rad) in an iCycler real-time PCR machine (Bio-Rad). The Primers for quantitative PCR have been described previously[47].

Co-immunoprecipitation assay. HEK293T cells were seeded onto 10-cm dishes and 18–20 h later cells were transfected with 6 µg plasmid DNA encoding the indicated proteins or empty vector as indicated in the figure legends. Twenty-four hours after transfection, cells were either lysed or infected for 1 h with *S.* Typhimurium at a m.o.i. of 10 and then lysed in a lysis buffer (0.5% Triton-X-100; 150 mM NaCl; 20 mM Hepes (pH 7.4); 2 mM egtazic acid; 10 mM NaF and 2 mM dithiothreitol) containing protease inhibitors. After 15 min on ice, the samples were centrifuged for 15 min at 14,000 *g* at 4 °C. The supernatants were incubated with 20 µl prewashed anti-FLAG M2 agarose (50% slurry; Sigma) for 4 h at 4 °C. The immune complexes were collected by centrifugation at 1,500*g* for 3 min, washed three times with 1 ml cold lysis buffer and eluted by adding 0.1 M glycine HCl pH 3.5. Samples were analysed by SDS-PAGE (10% gel) and western blot with antibodies against M45 and FLAG.

Affinity purification and mass spectrometry analysis. Affinity purification of interacting proteins was carried out as described in earlier work[57]. In brief, HEK293T cells stably expressing endogenous levels of FLAG-epitope-tagged Cdc42 or PAK1 were infected with wild-type *S.* Typhimurium or mock infected as indicated in the figure legends. Cells were then lysed in a lysis buffer containing protease inhibitors for 15 min on ice and centrifuged for 15 min at 14,000 *g* at 4 °C. The supernatants were incubated with 20 µl prewashed anti-FLAG M2 agarose (50% slurry) for 4 h at 4 °C. Immune complexes were collected by centrifugation at 1,500*g* for 3 min, washed three times with 1 ml cold lysis buffer and eluted by adding 0.1 M glycine HCl, pH 3.5. To identify the interacting proteins, samples were run on SDS-PAGE gels, the lanes were excised in three slices, subjected to overnight in-gel trypsin digestion and the extracted peptides were subjected to LC-MS/MS analysis as described in earlier work[58]. To identify the phosphorylation sites of PAK1, samples were run on SDS-PAGE gels and the position of the differentially phosphorylated bands was determined by conducting western blot analysis on an aliquot of the same samples. The bands were then excised and processed as indicated above. LC-MS/MS data analysis was carried out using Mascot[59].

Mouse infections. The C57BL/6 SLC11A1$^{+/+}$[47] and C57BL/6 PAK1$^{-/-}$[45] mice have been previously described. Mouse infections were carried out as described in earlier work[47] with some modifications. Briefly, groups of age- and sex-matched C57BL/6 SLC11A1$^{+/+}$ mice were orally treated with the PAK group I inhibitor FRAX486 (1 mg per mouse) or DMSO 1 d before infection. Treated mice were orally infected with the bacterial dose indicated in the figure legends in 100 µl PBS. Twenty-four hours after infection, mice were treated again with the PAK group I inhibitor FRAX486 (1 mg per mouse) or DMSO. The mice were killed at the indicated times. In some experiments, mice were treated orally with Streptomycin (20 mg) one day before infection. The transcription of cytokines and the bacterial loads in the indicated tissues were performed as previously described[47]. The sample sizes were empirically determined to optimize the numbers based on our previous experience with equivalent experiments. The mice were randomly assigned to the experimental groups but experimenters were not blind to the assignment.

Ethics statement. All animal experiments were conducted according to protocols approved by Yale University's Institutional Animal Care and Use Committee (IACUC) under protocol number 2016–07858. The IACUC is governed by applicable Federal and State regulations, including those of the Animal Welfare Act, Public Health Service and the United States Department of Agriculture and is guided by the US Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All data generated or analysed during this study are included in this published article (and the Supplementary Information).

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Author contributions

H.S. and J.E.G. designed the research and analysed data. H.S., J.K. and M.L.-T. performed the research. H.S. and J.E.G. wrote the manuscript with input from all the authors.

Competing interests

The authors declare no competing interests.

Additional information

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Experimental design

1. Sample size

   Describe how sample size was determined.

   Sample sizes were empirically determined to optimize numbers based on our previous experience with equivalent experiments.

2. Data exclusions

   Describe any data exclusions.

   No data were excluded from the analyses.

3. Replication

   Describe the measures taken to verify the reproducibility of the experimental findings.

   All attempts at replicating the results were successful. For each experiment, we repeated at least three times.

4. Randomization

   Describe how samples/organisms/participants were allocated into experimental groups.

   Samples were randomly allocated to different groups.

5. Blinding

   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

   Investigators were not blinded to group allocation during the experiments or to the outcome assessment.

   Note: all in vivo studies must report how sample size was determined and whether blinding and randomization were used.

6. Statistical parameters

   For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

n/a	Confirmed
☐	☒ The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
☐	☒ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒ A statement indicating how many times each experiment was replicated
☐	☒ The statistical test(s) used and whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☐	☒ A description of any assumptions or corrections, such as an adjustment for multiple comparisons
☐	☒ Test values indicating whether an effect is present *Provide confidence intervals or give results of significance tests (e.g. P values) as exact values whenever appropriate and with effect sizes noted.*
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Software

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7. Software

Describe the software used to analyze the data in this study.	GraphPad Prism 7, Photoshop, Mascot.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). *Nature Methods* guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

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8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a third party.	All unique materials used are readily available from the authors.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).	All of antibodies used in this paper were described in "Materials and Methods" and were from commercial sources. And all of them were validated in western blot according to relevant instruction.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.	HEK293T cells obtained from the American Type Culture Collection (ATCC). Henle-407 were from the Roy Curtiss laboratory collection.
b. Describe the method of cell line authentication used.	The cells were frequently checked for their morphological features, growth speed and functionalities, but were not authenticated by short tandem repeat (STR) profiling.
c. Report whether the cell lines were tested for mycoplasma contamination.	All cell lines tested negative for mycoplasma contamination.
d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.	No commonly misidentified cell lines were used.

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide all relevant details on animals and/or animal-derived materials used in the study.	7 to 10-week C57BL/6 mice were used for the study. The tissues were taken based on the relevant policies at the Yale School of Medicine.

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.	The study did not involve human research participants.