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Systems-level overview of host protein phosphorylation during *Shigella flexneri* infection revealed by phosphoproteomics

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Running title: Protein phosphorylation during bacterial infection

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ABBREVIATIONS

*Shigella flexneri, S. flexneri; wt, wild-type; interleukin-8, IL-8; Mitogen-activated protein kinase, MAPK; mammalian target of rapamycin, mTOR; liquid chromatography tandem mass spectrometry, LC-MS/MS; Database for Annotation Visualization and Integrated Discovery, DAVID; nuclear pore complex, NPC; mTOR complex 1, mTORC1; mTOR complex 2, mTORC2; regulatory-associated protein of mTOR, Raptor; mammalian LST8/G-protein β-subunit like protein, mLST8/GβL; rapamycin-insensitive companion of mTOR, Rictor; mammalian stress-activated protein kinase interacting protein 1, mSIN1; anaphase-promoting complex/cyclosome, APC/C; inducible Rictor-knockout, iRiKO; mouse embryonic fibroblasts, MEFs; tryptic soy broth, TSB.*
ABSTRACT

The enteroinvasive bacterium *Shigella flexneri* invades the intestinal epithelium of humans. During infection, several injected effector proteins promote bacterial internalization, and interfere with multiple host cell responses. To obtain a systems-level overview of host signaling during infection, we analyzed the global dynamics of protein phosphorylation by LC-MS/MS and identified several hundred of proteins undergoing a phosphorylation change during the first hours of infection. Functional bioinformatic analysis revealed that they were mostly related to the cytoskeleton, transcription, signal transduction, and cell cycle. Fuzzy c-means clustering identified six temporal profiles of phosphorylation and a functional module composed of ATM-phosphorylated proteins related to genotoxic stress. Pathway enrichment analysis defined mTOR as the most overrepresented pathway. We showed that mTOR complex 1 and 2 were required for S6 kinase and AKT activation, respectively. Comparison with a published phosphoproteome of *Salmonella typhimurium*-infected cells revealed a large subset of co-regulated phosphoproteins. Finally, we showed that *S. flexneri* effector OspF affected the phosphorylation of several hundred proteins, thereby demonstrating the wide-reaching impact of a single bacterial effector on the host signaling network.
INTRODUCTION

The enteroinvasive bacterium *Shigella flexneri* invades the intestinal epithelium of humans, causing an acute mucosal inflammation called shigellosis or bacillary dysentery that is responsible for 1.1 million deaths annually [1]. During the infectious process, bacteria use a sophisticated delivery system, the type III secretion apparatus, to inject multiple effector proteins that subvert cellular and immune functions of macrophages and epithelial cells [2]. First, *S. flexneri* crosses the colonic epithelium by transcytosis via M cells [3,4]. Released in the M cell pocket, it invades resident macrophages and induces cell death [5,6]. Dying macrophages release bacteria and several proinflammatory cytokines including IL-1β and IL-18 that contribute to acute intestinal inflammation [7,8]. *S. flexneri* is then able to invade epithelial cells via the basolateral surface, and use them as replication niche [9]. Although infection of epithelial cells represents a key aspect of infection, there is currently no comprehensive model that describes the molecular processes occurring in the first hours of infection.

The entry of *S. flexneri* into epithelial cells is a multi-step process that requires the secretion of effector proteins into the cytoplasm of target cells via type III secretion. Upon contact between the tip complex of the type III apparatus and host cell receptors, including α5β1-integrins and CD44, the secreted proteins IpaB and IpaC insert into the plasma membrane and form a pore into the host membrane through which several effectors translocate [10-14]. Among these, IpaA, IpaB, IpaC, lpgB1, lpgB2, lpgD and VirA act synergistically to induce membrane ruffling and bacterial internalization [2]. Local remodeling of the cell surface depends on the interplay between these effectors and small Rho GTPases, kinases and other regulators of the actin cytoskeleton and microtubules [15,16]. Engulfed bacteria rapidly lyse the membrane of their internalization
vacuole and escape into the cytoplasm where they multiply, and use actin based motility to spread from cell-to-cell [17]. \textit{Shigella}-actin based motility is mediated by the virulence factor IcsA/VirG [18,19]. This autotransporter, which accumulates at one pole of the bacterium, recruits the host protein N-WASP and forms together with vinculin and Arp2/3, a complex that serves as actin polymerization nucleator [20].

Although, epithelial cells are not professional immune cells, they can detect invasion and contribute to acute inflammation by secreting several proinflammatory cytokines such as interleukin-8 (IL-8) and tumor necrosis factor \( \alpha \) [21]. During \textit{S. flexneri} infection, peptidoglycan-derived muramyl peptides are recognized via the receptor Nod1 [22]. This recognition leads to the activation of multiple signaling pathways including NF-\( \kappa \)B and MAP kinase (MAPK). Bacteria manipulate host signaling in infected cells by secreting effectors that affect different signaling pathways. For instance, the effector OspF, which functions as a phosphothreonine lyase, dephosphorylates MAPKs p38 and ERK in the nucleus of infected cells [23,24]. It was proposed that this mechanism leads to reduced histone H3 phosphorylation and selective repression of gene transcription. Expression of the gene encoding for IL-8, a potent chemoattractant for polymorphonuclear cells, is specifically decreased in presence of OspF. A recent study from our laboratory reported a mechanism of cell-cell communication between infected and uninfected bystander cells that restores IL-8 expression in infected cell monolayers, and potentiates inflammation during infection [25].

\textit{S. flexneri} infection of epithelial cells has been well investigated by reductionist approaches that led to important findings regarding specific molecular aspects of infection, and general concepts of infection biology and immunology [26,27]. In addition,
DNA microarrays were instrumental in the systematic identification of genes regulated during infection and the characterization of host cell responses [28,29]. However, these approaches were not well-suited to obtain a systems-level overview of early host signaling during infection.

Protein phosphorylation is the most widespread known post-translational modification. It can either activate or inactivate biological processes, and is commonly used to switch enzyme activity "on" or "off". Protein kinases and phosphatases are abundant in the human genome, giving rise to countless phosphorylation and dephosphorylation events that control the most diverse cellular pathways. Incidentally, the importance of protein phosphorylation has been already described for several aspects of *S. flexneri* infection, and few kinases have been identified [30-33]. Based on this, we reasoned that the complexity of host signaling during *S. flexneri* infection may be addressed by a systematic and unbiased analysis of protein phosphorylation. Here we describe a label-free quantitative phosphoproteomics approach that reveals the dynamics of host protein phosphorylation during infection. Several hundred of proteins undergoing a change in phosphorylation during the first two hours of infection were identified and functionally characterized. Bioinformatic tools were used to recognize six different temporal profiles of phosphorylation, a functional module composed of ATM-phosphorylated proteins related to genotoxic stress, and the central role of mammalian target of rapamycin (mTOR). By comparing our data to a recently published phosphoproteomics analysis of *Salmonella typhimurium* infection [34], we identified a large set of co-regulated phosphoproteins. Finally, we showed that *S. flexneri* effector OspF alters the phosphorylation of several hundred proteins, thereby demonstrating its broad impact during infection.
RESULTS

Label-free quantitative phosphoproteomics reveals the massive impact of *S. flexneri* infection on host protein phosphorylation

To analyze the impact of *S. flexneri* infection on protein phosphorylation in epithelial cells, we used a phosphoproteomics strategy that combines phosphopeptide enrichment and label-free quantification based on liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) (Figure 1A) [35,36]. In three independent experiments, epithelial cells from the HeLa cell line were left untreated or infected with *S. flexneri* for 15, 30, 60 and 120 minutes at a multiplicity of infection of 40 (Figure 1A). Cell lysis, enzymatic proteolysis, phosphopeptide enrichment on titanium dioxide beads, identification and quantification of individual phosphopeptides were performed as described in Methods. A total of 3234 phosphopeptides corresponding to 3785 phosphosites and 1184 phosphorylated proteins were identified (Table S1). Analysis of all detected phosphopeptides revealed that the distribution of phosphorylated peptides was strongly biased towards single and dual phosphorylations (Figure 1B, upper panel). As previously described [37], the large majority of phosphorylation events were found on serine and threonine residues (Figure 1B, lower panel). Correlation analysis based on squared Pearson correlation coefficient $R^2$ between the three biological replicates showed robust reproducibility between independent experiments for all infection conditions (Figure S1A). This was confirmed by data showing that the coefficients of variance calculated for all infection conditions as described in Methods varied between 18% and 29% (Figure S1B), a range of values expected for label-free quantification methods [38,39]. Significant changes in phosphorylation were defined by a q-value (moderated t-test adjusted for multiple testing) cutoff of < 0.01 using the SafeQuant tool.
and a minimum two-fold increase or decrease in peptide phosphorylation between infected and uninfected control cells (Table S1). Based on these criteria, 14.3% of all detected phosphopeptides showed a significant change in phosphorylation after *S. flexneri* infection. A parallel LC-MS/MS analysis, performed before phosphopeptide enrichment (Figure 1A), showed no significant changes in protein amount after infection for significantly altered phosphopeptides (Table S2), demonstrating that changes in phosphopeptide detection reflected true changes in phosphorylation. Phosphorylation events were visible at all time-points with a peak at 30 minutes at which 177 phosphopeptides corresponding to 137 phosphorylated proteins showed an increase in phosphorylation compared to control cells (Figure 1C and 1D). Decreased phosphorylation was also detected at all time-points with a maximum of 200 peptides corresponding to 140 proteins showing reduced phosphorylation 2 hours post-infection (Figure 1C and 1D). For simplification, we defined as phosphoproteome the subset of 334 proteins undergoing a significant change of phosphorylation on at least one phosphopeptide after infection (Table S1). Previous data showing dual phosphorylation of ERK at residues threonine 202 and tyrosine 204 after *S. flexneri* infection [41] were confirmed by our phosphoproteomics data (Table S1). Taken together, these results showed that *S. flexneri* has a massive impact on host protein phosphorylation, and that label free quantitative phosphoproteomics is well suited to reproducibly identify and quantify the phosphorylation changes occurring during infection of epithelial cells.

**Phosphoproteomics reveals new cellular functions affected by infection**

To analyze the biological functions of proteins with altered phosphorylation during *S. flexneri* infection, a gene ontology analysis was performed by using the functional
annotation tool of the Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov/) [42,43]. For enrichment analysis, the whole human genome was taken as background. Biases in the calculation of ontology term enrichment were minimized by comparing the 334 proteins of the altered phosphoproteome to a subset of 334 proteins randomly selected among proteins with unchanged phosphorylation (Figure 2A and Table S3). Results showed a strong comparative enrichment in proteins related to cytoskeleton, cell cycle, small GTPases signaling and cell death. In contrast, proteins associated with RNA processing and mRNA splicing were underrepresented in the phosphoproteome. A systematic literature search was performed to refine the functional annotation of gene ontology terms (Figure 2B, Table S4). This analysis revealed that 40 proteins of the phosphoproteome of *S. flexneri*-infected cells were implicated in the organization and rearrangement of the actin cytoskeleton. This included actin-binding proteins that regulate actin polymerization (i.e. ARPC1, PALLD, CTNN), GTP exchange factors (i.e. ARHGEF7, DOCK9, ARHGEF16 and ARHGEF17), GTPase activating proteins (i.e. ARHGAP29, IQGAP2) and downstream effectors (i.e. BORG5) of the small GTPases Cdc42, Rac and RhoA known to be involved in filopodia, membrane ruffling and stress fiber formation, respectively [44,45]. The comparison with the phosphoproteome of cells infected by a deletion virG mutant (ΔvirG) that cannot perform actin-based motility [19] suggested that proteins undergoing similar phosphorylation changes after both types of infection were important for the cortical actin rearrangements taking place during bacterial internalization (Tables S1 and S5). In contrast, phosphoproteins found exclusively in the wild-type dataset may be either important for actin-based motility or not detected in the LC-MS/MS experiment for technical reasons (Table S5). In addition to proteins regulating the actin cytoskeleton,
proteins associated with the network of microtubules (i.e. MAP1B, MAP7, ELM4) and intermediate filaments (i.e. PLEC, SYNM) were found. These results were expected as it is well documented that S. flexneri induces a profound remodeling of actin and microtubules for bacterial uptake and intra- and intercellular motility [2]. In line with data showing that S. flexneri interferes with cell-cell adhesion [46], the phosphorylation of proteins involved in the assembly of tight junctions in enterocytes (ZO-1 and ZO-2) and the regulation of adherens junctions (CTNA1, CTND1) was altered in infected cells (Table S4). With around 50 members, proteins implicated in signal transduction were also highly represented in the phosphoproteome. They mainly correspond to receptors, adaptors, kinases and phosphatases involved in well-characterized signaling pathways, including EGFR, MAPK, NF-κB, mTOR and PKA. In addition, 24 proteins related to endocytosis (i.e. SH3GL1, DNMBP), exocytosis (i.e. STXB5) and vesicular trafficking (i.e. BET1, Rab7A, WDR44) were also present in the phosphoproteome (Table S4). In line with previous data showing that S. flexneri interferes with host cell cycle [47], we found that infection affected the phosphorylation of regulators of mitotic progression that control spindle dynamics and chromosome separation (i.e. NEK9, BOD1L, PDS5B). To note, the proteins SEPT7 and SEPT9 of the septin family, known to be involved in cytokinesis [48] and more recently, in the formation of septin cages around intracellular bacteria [49,50], were found phosphorylated after S. flexneri infection (Table S4). In agreement with data reporting that cell fate is controlled by opposite signals [51], we found that the phosphorylation of both pro- and anti-apoptotic proteins (i.e. PDCD4, BAG3, PAWR) was altered in infected cells. For the first time, our data showed that the phosphorylation of several proteins (Nup98, Nup50, Nu214 and TPR) from the nuclear pore complex (NPC) was modified after infection. NPCs are large multiprotein channels
embedded in the nuclear envelope that mediate the transport of macromolecules including proteins and RNA between the cytoplasm and the nucleus of eukaryotic cells. The phosphoproteome was graphically illustrated by combining data from the STRING database that assembles protein networks based on known and predicted protein-protein interactions [52], with a functional description of proteins. Only proteins with at least one connection in STRING were represented (Figure 2C). Data showed a complex network with several distinct functional modules containing highly interconnected nodes. As expected, proteins involved in signal transduction were highly connected with members from other groups, including cytoskeleton and cell adhesion.

In order to identify the phosphorylation motifs overrepresented in the phosphoproteome, the Motif-X algorithm [53] was applied to all upregulated phosphopeptides. Six distinctive motif sequences, all phosphorylated on a serine, including the motifs RXRXXS, RRXS, RXXS, SP, SQ and SXE, were extracted (Figure S2). To predict the putative kinases of these motifs, we compared them to those of all known substrates of 107 kinase families [54]. The first three motifs showed striking similarities to substrates of the AGC kinases AKT, PKG/PKA/PKC, RSK and of CamKII (Figure S2). The proline directed SP motif can be phosphorylated by MAPK, CDC2 and CDK whereas the SQ motif is known to be a substrate of ATM/ATR or DNAPK. Finally, the motif SXE remotely resembled a substrate motif of CK2. Although AKT was absent from the phosphoproteome (Table S1), we experimentally confirmed its activation and localization at sites of *S. flexneri* entry (Figure S3A and S3B), as previously reported [55]. The high connectivity of AKT within the STRING graphical representation of the phosphoproteome network suggested that this kinase played a central function during infection (Figure S3C). In particular, AKT was highly connected to proteins associated with the actin cytoskeleton and
apoptosis/survival regulation. Phosphopeptides corresponding to active RSK and ERK were found in the phosphoproteome (Table S1) suggesting that these two kinases may be responsible for the phosphorylation of RXXS and SP motifs during infection, respectively. In addition, phosphorylation changes at the RRXS motif were experimentally confirmed by immunoblotting (Figure S4).

Taken together, these results show that *S. flexneri* infection alters the phosphorylation of a complex network of proteins that are involved in several key aspects of epithelial cell biology. They confirm the large impact of *S. flexneri* infection on the actin cytoskeleton and transcription, and show that numerous proteins related to cell cycle, microtubules, intracellular trafficking and the nuclear pore are also affected during infection.

**Spatio-temporal dynamics of protein phosphorylation during *S. flexneri* infection**

In order to capture the spatial dynamics of phosphorylation during *S. flexneri* infection, we used the human protein atlas (www.proteinatlas.org) database to analyze the subcellular localization of proteins with altered phosphorylation during infection [56]. With 64% coverage of the phosphoproteome, results showed that phosphorylation changes occurred in proteins localized in the cytoplasm, at the plasma membrane, in the nucleus, at the Golgi apparatus and the endoplasmic reticulum (Figure S5A and S5B).

Weak differences were observed over time, indicating for rapid and sustained signaling in different subcellular compartments during infection. Early phosphorylation changes after 15 minutes in the nucleus may result from a fast nuclear translocation of host signaling proteins and/or from a rapid translocation of bacterial effectors to the nucleus of infected cells.
Analysis of the temporal dynamics of individual peptides revealed a large heterogeneity in the patterns of phosphorylation changes after *S. flexneri* infection (Figure S5C). Analysis of all peptides taken individually by fuzzy c-means clustering [57] revealed 6 main profiles (Figure 3A, Table S6). Clusters 1 and 2 corresponded to fast and slow decrease in phosphorylation, respectively. Clusters 3, 4 and 5 corresponded to peptides with fast increase in phosphorylation followed by slow, intermediate and fast decrease, respectively. Cluster 6 grouped peptides showing late phosphorylation with a maximum at 2 hours. A functional analysis indicated that the functional categories were not equally represented in all clusters (Table S6). For instance, proteins associated with the cytoskeleton were overrepresented in clusters 1 and 2. In contrast, proteins involved in the p53 pathway and DNA repair were in majority found in cluster 6. In the same line, the most frequent phospho-motif varied between clusters (Figure 3A). A thorough analysis of cluster 6 revealed that among its 23 members, 15 were phosphorylated on a SQ motif. As this motif is the consensus sequence for the major sensor of DNA double-strand breaks ATM [58,59], we checked the activation profile of this kinase after *S. flexneri* infection by monitoring its phosphorylation at serine 1981. As shown in Figure 3B and 3C, an increase of ATM phosphorylation was observed at 30 minutes and phosphorylation was maximal after two hours, suggesting that several cluster 6-proteins were targets of ATM. This hypothesis was validated by showing that the phosphorylation of 11 proteins from this cluster was sensitive to the ATM inhibitor KU-60019 (Figure S6A and S6B, Table S7). More generally, dynamics analysis of all peptides phosphorylated on a SQ motif after infection revealed also an earlier profile of phosphorylation (Figure S6C), suggesting that another kinase was implicated. In conclusion, analysis of the dynamics of phosphorylation revealed several temporal profiles of phosphorylation.
associated with specific enriched phospho-motifs and distinct functional descriptions. In particular, we identified one group of proteins showing maximal phosphorylation on a SQ motif two hours post-infection. As they were functionally associated with the p53 pathway and DNA repair, our results suggest that they are involved in a genotoxic stress response to infection. More broadly, these results illustrate how phosphorylation dynamics and phospho-motif enrichment data combined with functional annotation can be used to identify and characterize the host pathways regulated during bacterial infection.

mTORC1 and mTORC2 control S6 kinase and AKT phosphorylation respectively

The KEGG database was used to identify the overrepresented pathways in the phosphoproteome of *S. flexneri*-infected cells [60,61]. With 7 KEGG-annotated proteins, the mTOR signaling pathway was the most overrepresented pathway (Figure 4A, Table S8). mTOR is a central serine/threonine protein kinase that regulates cell growth and metabolism, autophagy, the actin cytoskeleton, cell proliferation, cell survival, protein synthesis and transcription [62]. It is found as part of two different protein complexes. mTOR complex 1 (mTORC1) is composed of mTOR, regulatory-associated protein of mTOR (Raptor), mammalian LST8/G-protein β-subunit like protein (mLST8/GβL) and the recently identified partners PRAS40 and DEPTOR [63]. This complex functions as a sensor for nutrient, redox balance and energy, and controls protein synthesis. mTOR complex 2 (mTORC2) is composed of mTOR, rapamycin-insensitive companion of mTOR (Rictor), mLST8, DEPTOR and mammalian stress-activated protein kinase interacting protein 1 (mSIN1) [63]. mTORC2 has been shown to function as an important regulator of the actin cytoskeleton [64]. As indicated on the mTOR signaling map (Figure
components of both mTOR complexes but also regulators and effectors proteins of mTOR, showed altered phosphorylation after *S. flexneri* infection (Table S1). In order to test the implication of mTORC1 during infection, we monitored the effect of rapamycin on the activation of ribosomal S6 kinase, a protein implicated in protein translation known to be a substrate of mTORC1, and found in the phosphoproteome (Table S1). An antibody that recognizes S6 kinase phosphorylated at threonine 389 was used in an immunoblotting experiment. While a strong activation of S6 kinase was confirmed after infection, a short treatment of rapamycin blocked this induction (Figure 4C, upper panel).

The phosphorylation of S6 ribosomal protein, the main substrate of S6 kinase, was also sensitive to rapamycin (Figure 4C, lower panel). In the same condition of drug treatment, rapamycin had no effect on *S. flexneri* entry into cells (Figure 4D). Taken together, these results show that mTORC1 is involved in the early activation of S6 kinase following *S. flexneri* infection, and suggest that it may control protein translation.

In addition to mTORC1, proteins from the mTORC2 pathway were also found in the phosphoproteome (Figure 4B, Table S1). Although this complex is known to control the actin cytoskeleton, inhibition of mTORC2 had no effect on bacterial invasion (Figure 4C). As AKT was activated during *S. flexneri* infection (Figure S3A and S3B) and is a known substrate of mTORC2, we tested whether mTORC2 played a role in the activation of AKT during infection. Interestingly, AKT phosphorylation was blocked by PP242, an inhibitor of both complexes, but unchanged after rapamycin treatment (Figure 4E), showing that mTORC2 alone was implicated in the activation of AKT during infection. This result was confirmed by showing that AKT phosphorylation was abrogated in inducible Rictor-knockout mouse embryonic fibroblasts [65] treated by tamoxifen (Figure 4F). In conclusion, these data show that mTORC1 controls the phosphorylation of S6
kinase whereas mTORC2 is involved in the activation of AKT. As these two kinases control translation and host cell survival, respectively [55,66,67], our study indicates that mTOR plays a critical role in the control of host cell responses to S. flexneri infection.

S. flexneri and S. typhimurium infections trigger common host phosphorylation changes

In order to better characterize the phosphoproteome of S. flexneri-infected cells, we compared it to a recently published phosphoproteome dataset generated from HeLa cells infected by Salmonella typhimurium [34]. This bacterium is also an enteroinvasive pathogen that uses type III secretion to inject multiple effector proteins that facilitate its internalization. However, in contrast to S. flexneri, S. typhimurium remains enclosed in a vacuole where it replicates. Although different quantitative phosphoproteomics methods were employed, the comparison between the phosphoproteomes of cells infected by S. flexneri or S. typhimurium showed an overlap of 62 phosphopeptides corresponding to 57 phosphoproteins (Figure 5A, Table 9). Hierarchical clustering and correlation analysis performed on shared phosphopeptides showed a strong correlation of phosphorylation changes with 61 phosphopeptides co-regulated after both infections (Figure S7A and S7B). As expected, proteins involved in the actin cytoskeleton, microtubules, cell-cell adhesion, cell cycle or gene regulation were similarly regulated during infection by both pathogens (Figure 5B). Taken together, these results identify new common molecular processes triggered by S. flexneri and S. typhimurium in infected epithelial cells, and provide the first large-scale comparison of host signaling after infection by different bacteria.
**OspF has a wide-reaching impact on host protein phosphorylation**

During infection, *S. flexneri* injects several type III effectors that manipulate the signaling pathways controlling the inflammatory response of infected cells. Several mechanisms of action have been characterized based on the enzymatic activity, the structure or the intracellular binding partners of these effectors. For instance, the effector OspF attenuates IL-8 expression via its phosphothreonine lyase activity that irreversibly dephosphorylates MAPKs p38 and ERK on a T-X-Y motif, thereby preventing downstream histone H3 phosphorylation [23]. We experimentally confirmed that p38 and ERK phosphorylation was restored when cells were infected with a deletion mutant of OspF (ΔospF) compared to wild-type-infected cells (Figure 6A). Interestingly, we found that OspF interfered also with the phosphorylation of the kinase RIP2 and the transcription factor CREB (Figure 6A), two proteins involved in the control of inflammation [68,69], suggesting that OspF had a more complex effect on host signaling than expected. To evaluate its impact, the phosphoproteome of HeLa cells infected by ΔospF *S. flexneri* was determined by LC-MS/MS. When we directly compared the phosphorylation changes triggered by ΔospF versus wild-type infection (differential phosphoproteome) by hierarchical clustering, we found 377 peptides with increased phosphorylation and 86 with decreased phosphorylation (Table S10 and Figure 6B), showing that OspF had a massive net negative impact on phosphorylation. As expected, phosphoproteomics data confirmed that OspF strongly dephosphorylated ERK and p38 at residues T202/Y204 and T180/Y182, respectively (Table S10). A systematic search for the T-X-Y motif, known to be the consensus motif for the OspF phosphothreonine lyase activity [70], identified GSK3A as a potential new target. To test this hypothesis, we tested whether a treatment with the ERK and p38 inhibitors, PD98059 and...
SB203580 respectively, abolished all OspF-dependent phosphorylation changes (Table S10). With a q value <0.01 and a minimum 2 fold-ratio, none of the phosphopeptides differentially regulated by OspF (Figure S8A) remained significantly different from wild-type infection after pretreatment with MAPK inhibitors (Figure S8B). In particular, PD98059 and SB203580 completely abolished GSK3A phosphorylation at T201 and Y203 (Figure S8B), indicating that this protein was not a direct substrate of OspF and that this effector affected protein phosphorylation exclusively via its phosphothreonine lyase activity towards ERK and p38. However, because a q-value <0.01 corresponds to a very stringent cutoff, we reasoned that the method employed was likely underestimating the chance to identify inhibitor-insensitive phosphopeptides. Therefore, phosphopeptides with a q-value <0.1 were then considered and sensitivity to the inhibitors was directly tested for one of them. The NPC protein, Nup50, for which a phospho-specific antibody was available, was selected. Its phosphorylation at serine 221 was addressed by immunofluorescence. As expected, wild-type and ΔospF bacteria induced a moderate and strong increase of Nup50 phosphorylation, respectively (Figure S9A). Surprisingly, MAPK inhibitors failed to affect Nup50 phosphorylation in cells infected by ΔospF (Figure S9B). This result showed that, although most OspF-dependent phosphorylation changes were sensitive to MAPK inhibitors, the phosphorylation of at least one protein was independent of ERK and p38. Taken together, these data indicate that p38 and ERK are the main targets of OspF, but suggest also that additional proteins may be directly affected by its phosphothreonine lyase activity.

A STRING network representation of the differential phosphoproteome showed a core network of 136 proteins connected at least once (Figure 6C). Some of these have direct
or indirect connections to ERK and p38. A systematic search for the overrepresented phosphorylation motifs identified among others the SP/TP motif shared by the MAPKs, Cdc2 and Cdk as well as RXXS, which can be phosphorylated by RSK, a kinase identified in the differential phosphoproteome (Figure 6C, Figure S10).

It has been shown that OspF interferes with IL-8 expression in S. flexneri-infected cells [23]. To further characterize the role of this effector during infection, we analyzed the biological functions of the differential phosphoproteome (Figure 6C and 6D, Table S11). In line with an inhibition of gene expression [23], many proteins involved in transcription, chromatin modification and RNA processing were affected by OspF. Phosphorylation changes in several NPC proteins were also observed. In addition, phosphoproteins related to various biological functions including actin cytoskeleton, microtubules, apoptosis and cell cycle were also affected by OspF (Figure 6D). Altogether, these results show that OspF has a massive impact on host protein phosphorylation and illustrate that, by targeting the central hubs p38 and ERK, this effector shuts down a large fraction of the cellular network.
DISCUSSION

For the first time, we used phosphoproteomics to address the dynamics of protein phosphorylation during the first two hours of *S. flexneri* infection in epithelial cells. Using a label-free LC-MS/MS-based approach, we found more than three hundred proteins undergoing a significant change in phosphorylation after infection. This corresponded to 28% of all detected phosphoproteins, showing that infection by the enteroinvasive bacterium *S. flexneri* has a massive impact on host protein phosphorylation. Although label-free LC-MS/MS is not a comprehensive method, our study covered a large portion of the phosphoprotein space and was well-suited to reproducibly identify and quantify host phosphorylation changes after bacterial infection.

Gene ontology analysis and manual functional annotation of the phosphoproteome confirmed the importance of the actin cytoskeleton and microtubules during infection. This result was expected because the first two hours of infection cover the entry mechanism of *S. flexneri* and actin based-motility, two processes highly dependent on actin and microtubule remodeling and controlled by protein phosphorylation [33,71]. The identification of around 50 new phosphoproteins related to the cytoskeleton and the comparison between wild-type and ΔvirG infection open a new avenue to elucidate the mechanisms of bacterial entry and actin-based motility, two critical processes that contribute to successful colonization of the intestinal epithelium in humans.

Further functional analysis of the phosphoproteome showed that the phosphorylation of proteins related to cell cycle was affected by infection. This result was consistent with a report by Iwai et al. showing that the secreted effector IpaB causes cell-cycle arrest of epithelial cells by targeting Mad2L2, an anaphase-promoting complex/cyclosome (APC/C) inhibitor [47]. The addition of Mad2L2 to the STRING network representation of
the phosphoproteome shows that this protein interconnects with CDC23 and BUB1, component and substrate of APC/C respectively, that are important for cell cycle checkpoint enforcement. Interestingly, phosphoproteomics revealed that *S. flexneri* infection alters the phosphorylation of NPC proteins including Nup98, Nup50, Nu214 and TPR. NPCs are composed of around 30 different nucleoporins divided into scaffolding proteins that are important for nuclear pore assembly and maintenance, and peripheral proteins that function directly in nucleo-cytoplasmic transport. Noticeably, all proteins affected by *S. flexneri* infection belong to this second class. Follow up work is required to elucidate how infection impacts nuclear transport, and if trafficking of important host proteins or mRNA is affected during infection. Besides transport, recent studies indicate that peripheral proteins of NPCs can also directly contribute to gene regulation by interacting with chromatin and coupling transcription with mRNA export [72]. It would be of particular interest to investigate whether changes in NPC protein phosphorylation can affect the expression of proinflammatory genes by this mechanism.

To better understand the host phosphorylation dynamics of *S. flexneri* infection and extract useful information regarding the roles of functional modules during infection, we used fuzzy c-means clustering to group all individual phosphopeptide based on the direction and the temporal profiles of phosphorylation changes after infection. Each cluster was then analyzed for the most frequent phospho-motifs and functional annotation of proteins. For clusters showing a fast (cluster 1) or slow reduction (cluster 2) of phosphorylation, the **SP** phospho-motif was highly enriched. As this motif can be phosphorylated by MAPKs, we hypothesize that, for some proteins, the reduction of phosphorylation results from the phosphothreonine lyase activity of OspF on p38 and ERK. Alternatively, a reduction of **SP** phosphorylation can also be explained by the
inhibition of other kinases like CDC2 and CDK. In a non-exclusive manner, the activation 
of phosphatases may also play a role. Remarkably, proteins from cluster 1 were in 
majority involved in the regulation of the actin cytoskeleton, microtubules and cell 
adhesion. In contrast, proteins belonging to cluster 2 were essentially related to 
transcription. Among the other profiles of phosphorylation changes, cluster 6 showed 
maximal phosphorylation two hours post-infection. For this cluster, 14 proteins out of 21 
were phosphorylated on an SQ motif, a known substrate of ATM, ATR and DNAPK. By 
monitoring the activation of ATM at different time-points of infection by immunoblotting, 
we observed a very similar kinetics of activation, suggesting that ATM phosphorylated 
the SQ-containing proteins of cluster 6. This hypothesis was confirmed by showing that 
the ATM inhibitor KU60019 inhibited the phosphorylation of most of them. As these 
proteins were strongly associated with the p53 pathway and DNA repair, we propose 
that they belong to a functional module related to genotoxic stress. This assumption is 
supported by a recent article reporting S. flexneri-induced genotoxic stress and ATM 
activation [73].

Finally, in the clusters showing an increase in phosphorylation, the SP and RXXS 
phospho-motifs were overrepresented. The latter can be phosphorylated by AGC 
kinases such as AKT, PKA, PKC, S6 kinase and the members of the RSK family. 
Whereas our phosphoproteomics data supported the activation of S6 kinase and RSK 
during infection, phosphorylated AKT was not detected. By using an antibody that 
recognizes the active form of AKT, we confirmed its activation after S. flexneri infection 
and localization at entry foci. Although it was reported that AKT activation requires an 
lpgD-dependent production of phosphatidylinositol 5 monophosphate [55] and EGF 
receptor signaling [74], its exact mechanism of activation remains unclear. Here we
found that AKT activation was sensitive to PP242 but not to rapamycin, and was abolished in cells depleted of Rictor, showing clearly that AKT activation was dependent on mTORC2. The implication of mTORC2 was also supported by the phosphoproteomics data showing that both mSin1 and Rictor underwent a change in phosphorylation during *S. flexneri* infection. Further experiments are required to test how mTORC2 is activated during infection. Because AKT controls the survival of infected cells [55,67], our data indicated that mTORC2 was involved in the regulation of cell fate during infection. Interestingly, although mTORC2 can regulate the actin cytoskeleton [64], bacterial entry was not affected by the inhibition of mTORC2. In addition, phosphoproteomics revealed a rapid change in phosphorylation for the two mTORC1 components Raptor and PRAS40, indicating that mTORC1 was implicated in the early phase of infection. This finding was supported by the observation that the phosphorylation of S6 kinase and S6 ribosomal protein observed 15 minutes post infection was sensitive to rapamycin and PP242 treatment. Because these two proteins are known to regulate protein translation, these results suggested that mTORC1 participates to the control of host protein synthesis during *S. flexneri* infection. Interestingly, a recent report by Tattoli et al. shows that *S. flexneri* infection leads to amino acid starvation and to a subsequent down-regulation of mTORC1 activity that induces autophagy [75]. The authors report that amino acid starvation is sensed by GCN2 which becomes phosphorylated within the first hour of infection. Altogether, these two studies support the roles of both mTORC1 and mTORC2, and identify mTOR as a central host player of *S. flexneri* infection. Phosphoproteomics is a method that has been applied only recently to the field of bacterial infection with a first study by Rogers et al. investigating *S. typhimurium*
infection of epithelial cells [34]. As *S. flexneri* and *S. typhimurium* are closely related and both harbor a type III secretion system with several homologue effectors, we compared the respective phosphoproteomes of infected HeLa cells. Although major differences in sample preparation and phosphoproteomics methodology exist between the two studies [76], 61 co-regulated phosphopeptides were discovered. These results demonstrated that *S. flexneri* and *S. typhimurium* induce various common host molecular processes in infected cells. Among the shared phosphopeptides, many belong to proteins related to the cytoskeleton and its regulation. For instance, the protein palladin, strongly phosphorylated after *S. flexneri* and *S. typhimurium* infection, is known to localize at sites of actin remodeling including ruffles and lamellipodia. The protein IQGAP2 interacts with CDC42 and Rac1, two GTPases required for efficient bacterial entry [77,78]. Taken together, these data constitute the first large-scale comparison of host signaling after infection by different pathogenic bacteria. By identifying the common phosphorylation events, this approach allows to define the host pathways that are co-regulated by pathogens, and may facilitate the identification of processes that could be targeted by broad-spectrum drugs in the perspective of treatments against bacterial infections.

As previously described, *S. flexneri* secretes different effectors that manipulate host signaling to finely tune the inflammatory response of infected cells. For instance, it was proposed that OspF blocks the expression of IL-8 by altering the phosphorylation of histone H3 and the access of transcription factors to chromatin [23]. Phosphoproteomics revealed that OspF affects the phosphorylation of more than three hundred proteins, showing that this effector has a wider impact than anticipated. The MAPK inhibitors, PD98059 and SB203580, abolished almost all OspF-dependent phosphorylation changes, suggesting that they resulted from its phosphothreonine lyase activity towards
p38 and ERK. However, the observation that, Nup50 phosphorylation was not sensitive to MAPK inhibitors, suggests that OspF targets additional proteins. In line with an effect of OspF on gene regulation, the phosphorylation of various proteins associated with transcription, chromatin modification and RNA processing was strongly affected. However, the biological functions and the number of proteins impacted by OspF strongly suggested that a reduction of histone H3 phosphorylation was not the only mechanism by which the effector OspF affected IL-8 expression in infected cells. Further functional analysis revealed that phosphoproteins associated with the actin cytoskeleton, microtubules, cell cycle and intracellular transport were also altered by OspF. Altogether, these results illustrate how \textit{S. flexneri} effectively alters a large fraction of the host protein phosphorylation network via a single effector. They also demonstrate that OspF can be used as a molecular tool to discover new targets of MAPKs, and decipher novel downstream regulatory mechanisms.

In summary, we used quantitative phosphoproteomics to investigate host signaling during \textit{S. flexneri} infection of epithelial cells. We found several hundred of proteins undergoing a significant change in phosphorylation during the first two hours of infection. Dynamics studies combined with functional annotation and phospho-motif enrichment demonstrated alterations of proteins involved in different cellular functions, including the cytoskeleton, cell cycle, transcription, and genotoxic stress responses. In addition, we discovered an early signaling function of mTOR during infection, and characterized the impact of OspF on host protein phosphorylation. In conclusion, these data provide the first systems-level overview of host signaling during the first hours of a bacterial infection, and constitute a valuable resource for generating testable hypotheses related to host-pathogen interactions.
METHODS

Reagents and antibodies

PP242 was obtained from Chemdea (#CD0258), Rapamycin from LC Laboratories (#R-5000), KU-60019 (#S1570), SB203580 (#S1076) and PD98059 (#S1177) from Selleck chemicals. Antibodies against MAPK p38 (#9212), phospho-MAPK p38 Thr180/Tyr182 (#4631), ERK (#9102), phospho-ERK (Thr202/Tyr204) (#4377), RIP2 (#4982), phospho-RIP2 Ser176 (#4364), CREB (#9197), phospho-CREB Ser133 (#9198), phospho-AKT Ser473 (#4058), phospho-ATM Ser1981 (#5883), phospho-S6 ribosomal protein Ser235/Ser236 (#4858), phospho-S6K Thr389 (#9205) and Rictor (#2114) were obtained from Cell Signaling Technology. Antibodies against ATM (#05-513) and actin (#MAB1501) were purchased from Millipore. The antibody against vinculin was purchased from Sigma (#V9131). The antibody against NUP50 phosphorylated at Ser221 was kindly provided by Prof. H. Kosako (Tokushima University, Japan).

Cell culture

HeLa CCL-2\textsuperscript{TM} cells were purchased from ATCC and cultured in DMEM, supplemented with 10% FCS, antibiotics and L-glutamine. Inducible Rictor-knockout (iRiKO) mouse embryonic fibroblasts (MEFs) and the corresponding control cells were generously provided by Prof. M. Hall [65]. MEFs were cultured in DMEM, supplemented with 10% FCS, antibiotics and L-glutamine. The knockout of Rictor was induced by culturing the cells for three days in the presence of 1µM Tamoxifen.

Bacterial strains
The M90T S. flexneri and M90T S. flexneri ΔvirG strains were generously provided by Prof. P. Sansonetti (Institut Pasteur, Paris, France). The ospF deletion mutant (ΔospF) was generated by allelic exchange using a modification of the lambda red-mediated gene deletion as previously described [79]. S. flexneri M90T ΔvirG pCK100 (P_{uhpT}::DsRed) was generated by transforming the non-motile S. flexneri strain M90T ΔvirG with the plasmid pCK100. pCK100 contains the fluorescent marker DsRed under the control of the native promoter of the S. flexneri gene uhpT, which is upregulated in presence of glucose-6-phosphate. Briefly, the 251bp promoter region upstream of uhpT was amplified by PCR (Primers used:

GAGAGAGAATGCAGTGCTCGATACCTGGCACT, GCTCTAGAGGGTTACTCCTGAAA TGAATACCT) and ligated into the pMW211 plasmid using BsmI and XbaI.

**Infection assay**

M90T S. flexneri strain was grown in tryptic soy broth (TSB) to exponential growth phase at 37°C and coated with poly-L-lysine. 30 minutes before infection, complete growth medium was replaced by DMEM supplemented with 10 mM Hepes and 2 mM L-glutamine (assay medium). Assay medium (uninfected control) or bacteria were added to 6-well plates at a multiplicity of infection of 40. Infection was initiated by centrifugation of the plates for 5 minutes and their incubation at 37°C for the indicated time periods. Extracellular bacteria were killed by adding gentamycin (50 μg/ml) 30 minutes after infection.

**Microscopy and automated image analysis**
Infection rates were determined by using automated image analysis. Images were automatically acquired with an ImageXpress Micro (Molecular devices, Sunnyvale, USA). At each site, images were acquired at 360 nm, 480 nm, 594 nm and 640 nm to visualize Hoechst, FITC-phalloidin, DsRed-expressing *S. flexneri* and Alexa 647-conjugated secondary antibodies, respectively. Infection rates were determined by image analysis using CellProfiler [80] and MATLAB (The MathWorks, Inc, Natick, USA). The Hoechst staining was used as a mask to automatically identify cell nuclei. The cellular area was defined by extension of the nuclear mask. In parallel, the presence of bacteria within the area of each cell was quantified. Performance of bacterial detection was checked by visual inspection of several images prior to automated processing. In control images (no infection), the algorithm generally classified less than 1% of cells as infected [25].

**SDS-PAGE and immunoblotting**

Uninfected control and infected cells were washed twice in ice cold PBS, lysed in PhosphoSafe™ extraction reagent (Novagen), incubated on ice for 10 minutes, and subsequently centrifuged at 4°C for 20 minutes at 16'000g. BCA Protein Assay kit (Pierce) was used to determine protein concentration. 10-15 μg of protein was subjected to SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes. Immunoblotting was performed using primary antibodies diluted in phosphate buffered saline containing 0.1% tween and 5% bovine serum albumin. HRP-conjugated secondary antibodies were purchased from GE Healthcare or Cell signaling technology. The blots were developed with an enhanced chemiluminescence method (ECL, Pierce).
Immunofluorescence

After fixation in 4% PFA for 10 minutes, cells were permeabilized in 0.3-0.5% Triton X-100 for 10 minutes. Phospho-AKT antibody was incubated overnight at 4°C in PBS followed by a secondary staining using Alexa 647-conjugated secondary antibody (Invitrogen). Additionally, DNA and F-actin were stained for 1 hour at room temperature with Hoechst and FITC-phalloidin (Invitrogen), respectively.

Sample preparation for phosphoproteomics

For each condition, two 6-well plates of HeLa CCL-2™ cells were grown to confluency. Cells were infected as described above. At the indicated time-points, the plates were put on ice and washed twice with ice-cold phosphate-buffered saline (PBS). Samples were then collected in urea solution [8M Urea (AppliChem), 0.1M Ammoniumbicarbonate (Sigma), 0.1% RapiGest (Waters), 1x PhosSTOP (Roche)]. The samples were briefly vortexed, sonicated at 4°C (Hielscher), shaked for 5 minutes on a thermomixer (Eppendorf) and centrifuged for 20 minutes at 4°C and 16'000g. Supernatants were collected and stored at -80°C for further processing. BCA Protein Assay (Pierce) was used to measure protein concentration.

Phosphopeptide enrichment

Disulfide bonds were reduced with tris(2-carboxyethyl)phosphine at a final concentration of 10 mM at 37°C for 1 hour. Free thiols were alkylated with 20 mM iodoacetamide (Sigma) at room temperature for 30 minutes in the dark. The excess of iodoacetamide was quenched with N-acetyl cysteine at a final concentration of 25 mM for 10 minutes at room temperature. Lys-C endopeptidase (Wako) was added to a final enzyme/protein
ratio of 1:200 (w/w) and incubated for 4 hours at 37°C. The solution was subsequently
diluted with 0.1 M ammoniumbicarbonate (Sigma) to a final concentration below 2 M
urea and digested overnight at 37°C with sequencing-grade modified trypsin (Promega)
at a protein-to-enzyme ratio of 50:1. Peptides were desalted on a C18 Sep-Pak cartridge
(Waters) and dried under vacuum. Phosphopeptides were isolated from 2 mg of total
peptide mass with TiO₂ as described previously [35]. Briefly, dried peptides were
dissolved in an 80% acetonitrile (ACN)–2.5% trifluoroacetic acid (TFA) solution
saturated with phthalic acid. Peptides were added to the same amount of equilibrated
TiO₂ (5-µm bead size, GL Science) in a blocked Mobicol spin column (MoBiTec) that
was incubated for 30 minutes with end-over-end rotation. The column was washed twice
with the saturated phthalic acid solution, twice with 80% ACN and 0.1% TFA, and finally
twice with 0.1% TFA. The peptides were eluted with a 0.3 M NH₄OH solution. The pH of
the eluates was adjusted to be below 2.5 with 5% TFA solution and 2 M HCl.
Phosphopeptides were again desalted with microspin C18 cartridges (Harvard
Apparatus).

**LC-MS/MS analysis**

Chromatographic separation of peptides was carried out using an EASY nano-LC
system (Thermo Fisher Scientific), equipped with a heated RP-HPLC column (75 µm x
37 cm) packed in-house with 3 µm C18 resin (Reprosil-AQ Pur, Dr. Maisch). Aliquots of
1 µg total phosphopeptide sample were analyzed per LC-MS/MS run using a linear
gradient ranging from 98% solvent A (0.15% formic acid) and 2% solvent B (98%
acetonitrile, 2% water, 0.15% formic acid) to 30% solvent B over 90 minutes at a flow
rate of 200 nl/min. Mass spectrometry analysis was performed on a dual pressure LTQ-
Orbitrap mass spectrometer equipped with a nanoelectrospray ion source (both Thermo Fisher Scientific). Each MS1 scan (acquired in the Orbitrap) was followed by collision-induced dissociation (CID, acquired in the LTQ) of the 10 most abundant precursor ions with dynamic exclusion for 30 seconds with enabled multistage activation. Total cycle time was approximately 2 s. For MS1, $10^8$ ions were accumulated in the Orbitrap cell over a maximum time of 300 ms and scanned at a resolution of 60,000 FWHM (at 400 m/z). MS2 scans were acquired using the normal scan mode, a target setting of $10^4$ ions, and accumulation time of 50 ms. Singly charged ions and ions with unassigned charge state were excluded from triggering MS2 events. The normalized collision energy was set to 32%, and one microscan was acquired for each spectrum.

**Label-free quantification and database searching**

The acquired raw-files were imported into the Progenesis software tool (Nonlinear Dynamics, Version 4.0) for label-free quantification using the default parameters. MS2 spectra were exported directly from Progenesis in mgf format and searched using the MASCOT algorithm (Matrix Science, Version 2.4) against a decoy database [81] containing normal and reverse sequences of the predicted SwissProt entries of *Homo sapiens* (www.ebi.ac.uk, release date 16/05/2012) and commonly observed contaminants (in total 41,250 sequences) generated using the SequenceReverser tool from the MaxQuant software (Version 1.0.13.13). To identify proteins originating from *S. flexneri*, non phosphopeptide enriched samples were searched against the same database above including predicted SwissProt entries of *S. flexneri* (www.ebi.ac.uk, release date 16/05/2012, in total 49,610 sequences) The precursor ion tolerance was set to 10 ppm and fragment ion tolerance was set to 0.6 Da. The search criteria were set
as follows: full tryptic specificity was required (cleavage after lysine or arginine residues unless followed by proline), 2 missed cleavages were allowed, carbamidomethylation (C) was set as fixed modification and phosphorylation (S,T,Y) or oxidation (M) as a variable modification for TiO2 enriched or not enriched samples, respectively. Finally, the database search results were exported as a xml-file and imported back to the Progenesis software for MS1 feature assignment. For phosphopeptide quantification, a csv-file containing the MS1 peak abundances of all detected features was exported and for not enriched samples, a csv-file containing all protein measurements based on the summed feature intensities of all identified peptides per protein was created. Importantly, the Progenesis software was set that proteins identified by similar sets of peptides are grouped together and that only non-conflicting peptides with specific sequences for single proteins in the database were employed for protein quantification. Both files were further processed using the in-house developed SafeQuant v1.0 R script (unpublished data, available at https://github.com/eahrne/SafeQuant/). In brief, the software sets the identification level False Discovery Rate to 1% (based on the number of decoy protein sequence database hits) and normalizes the identified MS1 peak abundances across all samples. Next, all quantified phosphopeptides/proteins are assigned an abundance ratio and a q-value, per time point. Q-values reflect the quantification level False Discovery Rate of a given ratio cutoff, and are obtained by calculating modified t-statistic p-values [82] and adjusting for multiple testing [83]. The final list of all quantified phosphopeptides/proteins is presented in Table S1/S2. The location of the phosphorylated residues was automatically assigned by MASCOT (score >10). All annotated spectra can be found in supplemental information file 12 (annotated spectra) and, together with the MS raw files and search parameters employed, have been
deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository [84] (data submitted).
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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: CS, CA, AS. Performed the experiments: CS, IS, TT, RD. Analyzed the data: CS, AS, EA, CA, CK. Contributed materials/analysis tools: AS, EA, CK. Wrote the paper: CA, CS.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.
REFERENCES


Figure 1: Phosphoproteomics analysis applied to *S. flexneri* infection

(A) Diagram of the phosphoproteomics protocol applied to *S. flexneri* infection of HeLa cells. (B) Distribution of single, double, triple and quadruple peptide phosphorylation based on 3234 detected phosphopeptides (upper panel). Distribution of serine, threonine and tyrosine phosphorylation based on 3785 detected phospho-sites (lower panel). (C) Dynamics of phosphorylation changes after *S. flexneri* infection of HeLa cells. Data represent the number of phosphopeptides for which a significant increase (in blue) or a decrease (in red) of phosphorylation was observed after *S. flexneri* infection compared to uninfected cells. A minimum two-fold change and a q-value <0.01 were used to select phosphopeptides for quantification. (D) Dynamics of phosphorylation changes at the protein level after *S. flexneri* infection of HeLa cells. Data represent the number of proteins for which an increase of phosphorylation (in blue), a decrease of phosphorylation (in red) or both (in green) was observed after *S. flexneri* infection compared to uninfected cells. All proteins containing at least one phosphopeptide with a minimum two-fold change and a q-value <0.01 were considered for analysis.

Figure 2: Cellular processes affected by *S. flexneri*

(A) Gene ontology analysis of proteins with altered phosphorylation after *S. flexneri* infection performed with DAVID. All proteins (334) containing at least one phosphopeptide with a minimum two-fold change and a q-value <0.01 were considered for analysis (Phosphoproteome, in blue). As comparison, 334 randomly selected proteins containing at least one phosphopeptide with a ratio between 0.67 and 1.5 were
used as reference (Random selection, in red). Default Homo sapiens background was used for gene ontology analysis in DAVID. Full list of GO terms is shown in Table S3.

(B) Literature-based manual annotation of the phosphoproteome. Numbers in brackets indicate the number of corresponding proteins. (C) Graphical representation of the phosphoproteome using STRING (high confidence 0.7) combined with manual functional annotation. Only proteins with at least one connection in STRING are represented.

Figure 3: Dynamics of phosphorylation changes after S. flexneri infection of epithelial cells.

(A) Dynamics of phosphorylation changes for individual phosphopeptides analyzed by fuzzy c-means clustering using the MFuzz algorithm. All individual phosphopeptides showing a significant change at least at one time-point were considered. Fold phosphorylation changes (log Ratio values) obtained for all time-points were normalized to have a standard deviation of 1 and a mean of 0 (z-score). For each cluster, the black line corresponds to an optimal membership of 1. The most overrepresented phospho-motif extracted with the motif-X algorithm was shown for each cluster. (B) Immunoblot of ATM phosphorylation at Ser-1981 in HeLa cells infected for the indicated time periods by S. flexneri. Vinculin was used as a loading control. (C) Densitometric quantification of ATM phosphorylation at Ser-1981 using the gel analysis tool from Fiji [85] (results representative of 2 independent experiments).

Figure 4: mTORC1 and mTORC2 control S6 kinase and AKT phosphorylation respectively
Analysis of the most overrepresented KEGG signaling pathways of the phosphoproteome using DAVID. The dashed line indicates a negative log p-value threshold of 2. (B) Projection of mTOR-related proteins identified by phosphoproteomics onto an mTOR signaling map. (C) mTORC1 is required for S6 kinase and S6 ribosomal protein phosphorylation during S. flexneri infection. HeLa cells were left untreated or pretreated with rapamycin or PP242 for 60 minutes prior to S. flexneri infection (15 min) and analyzed by immunoblotting for the phosphorylation of S6 kinase at residue Thr-389 and of S6 ribosomal protein at Ser-235/236. Actin is used as a loading control. (D) The mTOR inhibitors rapamycin and PP242 do not affect S. flexneri entry. HeLa cells were pretreated either with rapamycin or PP242 at indicated concentrations for one hour, and infected with S. flexneri ΔvirG- pCK100 (P_uhpT::Dsred) for 3 hours. These bacteria, only fluorescent when they are intracellular, form large microcolonies that are effectively detected by automated image analysis. Quantification of the infection rate shows that inhibition of mTORC1 and mTORC2 fails to affect the entry process of S. flexneri into HeLa cells. Results are expressed as the mean ± SD corresponding to three different wells of a 96-well plate and are representative of two independent experiments. (E) S. flexneri infection-induced activation of AKT is mTORC2 dependent. HeLa cells were left untreated or pretreated with rapamycin or PP242 for 60 minutes prior to S. flexneri infection (15 min) and analyzed by immunoblotting for the phosphorylation of AKT at Ser-473. Actin is used as a loading control. (F) mTORC2 component Rictor is required for the phosphorylation of AKT during S. flexneri infection. Control (Ctrl) and inducible-Rictor knockout (iRiKO) mouse embryonic fibroblasts were left untreated or infected with S. flexneri for 15 minutes and analyzed for the expression of Rictor and the level of AKT phosphorylation at Ser-473 by immunoblotting. Inducible Rictor-knockout was obtained...
by tamoxifen pretreatment. AKT is used as loading control. All results were
representative of at least two independent experiments.

Figure 5: S. flexneri and S. typhimurium infections trigger common host phosphorylation changes

(A) Venn diagram showing the overlap of phosphopeptides undergoing significant phosphorylation changes after S. flexneri infection (15 and 30 minutes) and S. typhimurium infection (10 and 20 minutes). For S. typhimurium infection, the phosphorylation events identified in different cellular compartments were compiled. (B) Phosphoproteins undergoing significant phosphorylation changes after both S. flexneri and S. typhimurium infections. Phosphoproteins were grouped according to different biological functions. Arrows indicate whether there is an increase or decrease in phosphorylation.

Figure 6: OspF has a wide-reaching impact on host protein phosphorylation

(A) OspF affects the phosphorylation of several signaling proteins involved in inflammation signaling. Left panels: OspF blocks the activation of p38 and ERK during S. flexneri infection. HeLa cells were left untreated or infected with either S. flexneri wild-type or ∆ospF for 30 minutes. The phosphorylation of p38 and ERK on residues T180/Y182 and T202/Y204 respectively, was analyzed by immunoblotting using the indicated phospho-specific antibodies. Right panels: OspF affects the phosphorylation of RIP2 and CREB on residue S176 and S133 respectively. Cells were left untreated or infected with S. flexneri wild-type or ∆ospF for 30 minutes. The phosphorylation of RIP2 and CREB was analyzed by immunoblotting using the indicated phospho-specific
antibodies. Actin was used as loading control (results representative of 2 independent experiments). (B) Hierarchical clustering analysis of relative peptide intensities obtained from the 3 independent replicates of cells infected for 30 minutes with either *S. flexneri* wild-type or ∆ospF. Phosphopeptides showing a significant change between ∆ospF and wild-type condition (two-fold change, q-value <0.01) were taken for the hierarchical clustering. (C) Graphical representation of the network of phosphoproteins affected by OspF (differential phosphoproteome). Phosphoproteins differentially phosphorylated between wild-type and ∆ospF *S. flexneri* were subjected to STRING (high confidence 0.7). Only proteins with at least one connection are represented. STRING data were combined to manual functional annotation. The known OspF targets p38 (MK14), ERK1 (MK03) and ERK2 (MK01) are highlighted by an increased node size. (D) Manual functional annotation of proteins from the differential phosphoproteome. Numbers in brackets indicate the number of corresponding proteins.
S. flexneri infected cells (15, 30, 60, 120 min)

Cell lysis

Enzymatic proteolysis

Phosphopeptide enrichment

Quantitation and identification of phosphopeptides by LC-MS/MS (Table S1)

Figure 1
Figure 2
Figure 3

A

Cluster 1

Cluster 2

Cluster 3

Cluster 4

Cluster 5

Cluster 6

B

S. flexneri

-15 30 60 120 min

IB:
p-ATM S1981
ATM
vinculin

C

Normalized p-ATM intensity

S. flexneri

0 15 30 60 120 min
Figure 4
### Figure 5

#### Protein Phosphorylation Table

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Figure 6