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The current *Salmonella*–host interactome

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Abstract

Salmonella bacteria cause millions of infections and thousands of deaths every year. This pathogen has an unusually broad host range including humans, animals, and even plants. During infection, *Salmonella* expresses a variety of virulence factors and effectors that are delivered into the host cell triggering cellular responses through protein–protein interactions (PPIs) with host cell proteins which make the pathogen's invasion and replication possible. To speed up proteomic efforts in elucidating *Salmonella*–host interactomes, we carried out a survey of the currently published *Salmonella*–host PPI. Such a list can serve as the gold standard for computational models aimed at predicting *Salmonella*–host interactomes through integration of large-scale biological data sources. Manual literature and database search of >2200 journal articles and >100 databases resulted in a gold standard list of currently 62 PPI, including primarily interactions of *Salmonella* proteins with human and mouse proteins. Only six of these interactions were directly retrievable from PPI databases and 16 were highlighted in databases featuring literature extracts. Thus, the literature survey resulted in the most complete interactome available to date for *Salmonella.* Pathway analysis using Ingenuity and Broad Gene Set Enrichment Analysis (GSEA) software revealed among general pathways such as MAPK signaling in particular those related to cell death as well as cell morphology, turnover, and interactions, in addition to response to not only *Salmonella* but also other pathogenic – viral and bacterial – infections. The list of interactions is available at http://www.shiprec.org/indicationslist.htm

Keywords

Interactome; Pathway analysis; Protein–protein interaction; *Salmonella*; Subnetwork analysis

1 Introduction

Salmonella are Gram-negative bacterial pathogens that are capable of infecting a wide range of animals including humans and farm animals such as cows, chicken, and pigs as well as

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pets such as reptiles, and even plants. *Salmonella* is a facultative endopathogen and the causative agent of various human diseases, reaching from enteritis to typhoid fever. According to the world health organization, Salmonellosis is the most frequent food-borne disease with around 1.5 billion infections world-wide yearly (http://www.who.int/mediacentre/factsheets/fs139/en/). Disease in mammals usually occurs by oral ingestion of contaminated food or water. Systemic infection of animals and humans depends on the ability of the bacteria to survive the harsh conditions of the gastric tract before entering intestinal epithelial and subsequently other host cells. After entering the small intestine, *Salmonella* traverses the intestinal mucous layer and can invade nonphagocytic enterocytes of the intestinal epithelium by bacterial-mediated endocytosis. Once the epithelial barrier has been breached, *Salmonella* can enter intestinal macrophages, sensing the phagosomal environment and activating various virulence mechanisms in order to survive in the microbicidal environment of the host cells.

Salmonella replicates within host cells in a membrane-bounded compartment, the *Salmonella*-containing vacuoles (SCVs). Intravacuolar bacterial replication depends on tightly controlled interactions with host cell vesicular compartments [1]. *Salmonella* type III secretion effector proteins subvert trafficking events and alter vacuole positioning by acting on host cell actin filaments, microtubule motors, and components of the Golgi complex. *Salmonella* replicates in SCVs in both nonphagocytic epithelial cells and macrophages. Once positioned, maturation is stalled and bacterial replication is initiated.

Salmonella encodes two distinct type III secretion systems (TTSSs) on chromosomal *Salmonella* pathogenicity islands 1 and 2 (SPI1 and SPI2). These two secretion systems are very well characterized compared with other known *Salmonella* secretion systems which contribute to virulence as well. Although the TTSS-1 inserts into the host cell membrane and translocates *Salmonella* effectors into the host cell, the TTSS-2 translocates effectors across the SCV membrane into the host cytosol. The majority of TTSS-1 translocated effectors promote actin cytoskeletal modification and rearrangements to force bacterial internalization [2]. Other TTSS-1 and -2 translocated effectors trigger various host pathways and act on location and maturation of the SCV, *Salmonella*-induced filaments (Sifs) formation as well as *Salmonella* replication, escape from the SCV, systemic spread and function to manipulate the host innate and adaptive immune response [3, 4].

Understanding the precise mechanisms for the communication between *Salmonella* and its hosts requires taking a system-wide view and determining the network of interactions between the *Salmonella* proteins and the host proteins. The use of system-wide approaches to study infectious diseases, and thus the protein–protein interaction (PPI) networks mediating the communication between pathogen and host, is expected to yield new approaches to design treatment strategies. Identification of the interactions allows inference of common proteins targeted by pathogens in host signal transduction and metabolic pathways [5–8]. Alternatively, alternate paths circumventing the pathogen disrupted paths in signal transduction pathways, can be identified through pathway analysis [9]. The information on *Salmonella* interactions can then be exploited for drug discovery. However, establishing *Salmonella*–host interactomes is in its infancy.

The identification of global networks of PPI has been accelerated by the development of new high-throughput technologies such as two-hybrid assays [10] and affinity purifications followed by mass spectrometry [11]. Thus, a vast amount of PPI data has been collected for a number of different organisms, but not yet for *Salmonella* proteins, neither within *Salmonella*, nor across its different hosts. Because of *Salmonella*'s broad host range, it is a particularly interesting question to what extent the different interactomes between *Salmonella* and its hosts overlap or differ, requiring essentially several interactomes to be

determined. It is unlikely that multiple interactomes will be fully discovered through experimental proteomic efforts alone. Instead, integration with available data and transfer learning from one host to another host organism through computational methods will be particularly suitable in this case to speed up the studies of the multiple interactomes to be determined. Indeed, numerous in silico methods have been developed to predict PPI, both for intraspecies as well as interspecies interactome scenarios [12]. The most successful methods integrate multiple biological databases through machine-learning approaches such as supervised classification [13]. The PPI prediction task is cast as a binary classification problem, where the two classes are "interact" and "does not interact." In order to develop the models to differentiate between the two classes, a so-called "gold standard" set is required in which pairs of proteins are labeled as interacting pairs based on experimental evidence. It is the goal of this review to survey the literature as well as PPI-related databases to develop such a gold standard. This review focuses on the known host–protein interaction partners for *Salmonella* TTSS-1 and -2 translocated effectors as well as their functions. We will discuss the impacts of the interactions at different stages of *Salmonella* infections. Finally, we will discuss the use of this knowledge for interactome modeling and drug development through pathway analysis.

2 Gathering the current *Salmonella*–host interactome: Literature search is essential

Thirty six pairwise interactions between *Salmonella* and host proteins in total have been reviewed previously by Haraga et al. 2008 [14], McGhie et al. 2009 [15], and Heffron et al. 2011 [16]. To expand and update the current *Salmonella*–host interactome, here, we describe the screening of more than 2200 journal articles, followed by survey of the retrieved papers, and over 100 databases (Supporting Information Table 1). This resulted in a data set of 62 known *Salmonella*–host PPI, of which the previously reviewed 36 pairs are a subset (Table 1, Fig. 1). The new, expanded data set now includes 22 *Salmonella* effectors and 57 direct and three indirect interactions (SipC-Cyto-keratin-8, SipA-T-plastin, SsaB-Hook3). The resulting interactome of 62 pairs represents the most complete *Salmonella*–host interactome available to date and its functional relevance is described in subsequent sections. The details of the manual curation process and its conclusions are provided below.

The literature search initially utilized PubMed's Mesh database to search abstracts using multiple keywords (e.g. *Salmonella* effector+bind/degrade/cleave). However, a parallel basic search in PubMed revealed that the Mesh database was missing numerous relevant publications. We therefore entered individual *Salmonella* effector protein gene names and abbreviation (e.g. SopE) into the search field of PubMed and manually inspected the resulting publications. Each article was deemed relevant based on its title and abstract; the full-text article was retrieved and was searched for the occurrence of specific keywords such as "bind," "interacts," "directly interacts," "cleaves," and the effector's name/abbreviation. Relevant articles were stored using the PMID reference code. This was repeated for all *Salmonella* effector proteins. In total, more than 2200 articles were screened. All papers that passed this first stage were then scrutinized for evidence supporting a direct interaction by thoroughly examining results and methodology. For papers passing this stage, the following data were extracted from the article to be incorporated into the overall data set: experimental method(s), strain of *Salmonella* and host organism studied. If multiple journal articles confirmed the same result, these findings were also extracted for incorporation into the data set.

The database search began with a Google search for "protein–protein interaction databases" which retrieved the "Jena Protein-Protein Interaction Website (PPI): Databases," listing 112 databases and data collection tools for searching PPI (http://ppi.fli-leibniz.de/

jcb_ppi_databases.html [last update of website: January 5, 2011]). Cross-comparison with other relevant hits from the Google search indicated that this listing is comprehensive and we tested each of the databases listed. The results of this database search are summarized in Supporting Information Table 1. In addition, "PSICQUIC VIEW" was used to search several databases including intact, dip, bind, string, and apid simultaneously (http://www.ebi.ac.uk/Tools/webservices/psicquic/view/main.xhtml). The UniProt database (http://www.uniprot.org/) was also used extensively since it not only includes links to IntAct's "binary interactions" fields but also provides functional information and links. The database most directly related to pathogen–host interactions was the PIG database (http://molvis.vbi.vt.edu/pig/) but is restricted to Human interspecies interactions, and retrieves the same *Salmonella*–host interactions as when using PSICQUIC.

The exhaustive search of both publications and databases revealed an important conclusion related to the rate-limiting steps in developing pathogen–host interactomes: listing interactions in the databases and not integrating different databases is the current bottleneck in obtaining pathogen-–host interactomes, as demonstrated here for *Salmonella*. Only 16 of the 62 interactions gathered through the literature search can also be found in PPI databases. Furthermore, of these 16, only 6 are listed in the databases DIP, IntAct, PIG, and/or BIND and are thus retrievable in an automated fashion. In contrast, the other ten are merely found in the descriptions of the UniProt database (www.uniprot.org). This demonstrates the need for manual curation of thousands of articles to obtain a reliable interactome for *Salmonella*. This is significant because it is unlike the intraspecies interactions e.g. between all human proteins or all yeast proteins. Therefore, integration of information listed in different databases is essential because each database has a different coverage and there is sufficient diversity across databases. For example, the BIANA platform provides one such capability to retrieve interactions across many relevant databases through a single interface [17]. The only pathogen for which listing of interactions in databases is not the rate-limiting step is HIV-1, for which thousands of PPI are listed in several databases, most comprehensively in the HIV-1, Human Protein Interaction Database (http://www.ncbi.nlm.nih.gov/RefSeq/HIVInteractions/index.html).

The gathering of reported PPI is an essential step in computer-assisted PPI prediction for training and for validation, representing the "gold standard" from which the algorithms learn to distinguish interacting from noninteracting protein pairs. Incorporating these literature-curated PPI in computational methods requires further processing, since the publications reporting the experimental work usually do not use standard identifiers and the genes derived are therefore often ambiguous. Many computational techniques (such as the network analysis described in Section 4) use information from various databases and to associate any properties from these to an interaction involving such genes will not be possible without an appropriate identifier. We therefore mapped the host proteins into gene symbols through the Entrez Gene mapping tool. Further problems arise while converting gene-level interactions to protein-level interactions since a single gene may have multiple gene products. We used the most popular protein symbol. Finally, not all of the interactions reported in the literature were with human proteins. Therefore, Table 1 lists the respective organism from which the host protein was derived. For simplicity, we mapped the protein names to the respective human gene symbols (column 4 in Table 1). The data set obtained based on the literature retrieved interactions and removing ambiguities in identifiers as much as possible using only human gene symbols and human protein symbols is available in excel format in Supporting Information 2 and in csv format at www.shiprec.org under the "Technologies" tab, direct link: http://www.shiprec.org/indicationslist.htm.

3 Specific impacts of *Salmonella*–host PPIs at different stages of Salmonella infection

Below, we propose a brief overview of the functional roles of the interactions in the gold standard.

3.1 *Salmonella* invasion of host cells

Upon contact with the host cell *Salmonella* inserts the SPI-1-encoded TTSS into the host cell membrane to translocate effectors into the cytosol of the targeted cell. Focusing on this secretion system, several effectors are necessary to promote *Salmonella* uptake by modifying the actin cytoskeleton, membrane ruffling, and bacterial engulfment. SipA, SopA, SopB, SopD, and SopE2 play a role in the process of *Salmonella* invasion of epithelial cells [18]. SopE and SopE2 are guanine nucleotide exchange factors (GEFs) which activate small Rho GTPases [19]. Both effectors bind and activate Cdc42 with SopE2 being a more efficient GEF for Cdc42 than SopE. Only SopE has been shown to efficiently activate Rac1 although a weak binding of SopE2 to Rac1 was observed [20–22]. The activation of these GTPases stimulates actin polymerization. Two other effectors, SipA and SipC, which directly bind F-actin are involved in *Salmonella* internalization [23, 24]. The C-terminal domain of SipC harbors the F-actin binding site, nucleates actin polymerization, and bundles F-actin. It has been shown that SipC is necessary for *Salmonella* invasion, whereas SipA enhances its nucleation and bundling efficiency [25]. It is supposed that SipA achieves localization of membrane ruffles thereby restricting them to the site of *Salmonella*–cell contact [23]. SipC binds to the filament protein Cytokeratin-18 as well as Exo70, a component of the exocyst complex [26, 27], resulting in activation of the Arp2/3 complex to stimulate actin polymerization and recruitment of exocytic vesicles to the sites of bacterial internalization possibly to supply membranes for macropinocytosis.

After internalization of *Salmonella*, SptP functions to reverse the process of membrane ruffling in order to establish normal cytoskeleton arrangement. The N-terminal domain of SptP possesses GAP activity and thereby inactivates Rac1 and Cdc42, resulting in the downregulation of signaling through those GTPases [28, 29].

3.2 The SCV, Sif formation, and *Salmonella* replication

After engulfment of bacteria within the SVC, it has been reported that the SCV acquires several endocytic markers. One of the early markers is the GTPase Rab5 which is recruited by SopE in the GTP-bound form and is required for the fusion of the SCV membrane with early endosomes [30, 31]. The SCV membrane is enriched with the GTPase Rab7 which participates in maturation of phagosomes and is involved in late endocytic transport. Rab7 interacts with RILP which can associate with the minus-end-directed dynein–dynactin motor complex, leading to the transport of the vesicle along microtubules toward the cell center. SifA may uncouple Rab7 from RILP which may facilitate the extension of tubules from the SCV [32]. Moreover, the SCV membrane is modified by SseJ activity leading to esterification of membrane cholesterol. This process is stimulated by the interaction of SseJ with the GTPase RhoA [33]. In order to prevent exposure to microbicidal compounds, *Salmonella* is able to prevent the fusion of SCVs with lysosomes. This may be achieved by the binding of SipC to TassC [34] and the inactivation of Hook3 by this *Salmonella* effector [35], followed by inhibiting the recruitment of the dynein motor complex (SifA–Rab7 interaction) [36]. The establishment of an intermediate filament network comprising Cytokeratin, Vimentin, and the adaptor protein 14-3-3 around the SCV is necessary to ensure localization of the SCV close to the nucleus [37]. SipC binds Cytokeratin-18 directly and Cytokeratin-8 indirectly [26] and SspH2 binds 14-3-3γ [38], but the precise functions of these interactions remain unclear.

Upon localization of the SCV close to the nucleus and the Golgi in epithelial cells [39], *Salmonella* replicates and Sifs are formed. Although there is evidence that *Salmonella* interacts with the Golgi in macrophages as well, an association of the SCV with the Golgi could not be observed in macrophages [39]. Sifs are membranous tubular structures extending from the SCV along microtubules to the cell periphery. The Sifs are thought to play a role in nutrient acquisition, movement of bacteria from cell to cell and may provide *Salmonella* with more space for replication. Upon extension of tubules, vesicles are observed at the cell periphery. Several effectors are thought to contribute to tubulation of the SCV membrane, the extension of the Sifs, and location of endocytic compartments to the cell periphery. Among those effectors, SifA and PipB2 take on a central role. PipB2 binds KLC, a subunit of the plus end-directed kinesin-1 microtubule motor complex that drives transport of cargo to the cell periphery, and thereby recruits kinesin-1 to the SCV membrane [40]. SifA, which is anchored to the SCV membrane by its C-terminal prenylated CaaX motif [41], binds SKIP [42, 43]. SKIP itself interacts with KLC [42]. Recent data suggest that SKIP does not downregulate the recruitment of kinesin-1 to the SCV as believed previously [42] but instead activates kinesin-1 and leads to its expulsion from vesicles [44]. SifA antagonizes Rab9 for binding SKIP and the fact that SifA-SKIP binding is tighter than Rab9-SKIP binding thus may enable SifA to recruit SKIP to the SCV membrane [45]. The interaction of the N-terminal domain of SifA with the C-terminal PH-domain of SKIP requires residues W197 and E201 of SifA's WxxxE motif and SifA may thus mimic the mammalian GTPases [45]. The ability of SifA to bind Rab7 contributes to the formation of tubules.

Another activity of SifA is to interact with the GDPbound form of RhoA. Although this interaction does not trigger nucleotide exchange [46], SifA seems to promote RhoA-family GTPase signaling pathways on the phagosome membrane [47]. Moreover, the interaction of SseJ, which localizes to the SCV membrane [48] with GTP-bound RhoA and RhoC, is speculated to induce tubulation of the SCV membrane [38, 47]. Furthermore, it has been shown that SNX3 (sorting nexin 3) is essential for the formation of tubules and that SopB-mediated recruitment of Rab5 to the SCV is required for SNX3 tubulation. SNX3 tubules are formed within 30–60 min postinfection and go along with the recruitment of LAMP1 and Rab7 which is impaired when SXN3 is depleted [49]. It is very likely that so far unknown factors contribute to endosomal tubulation as well.

In epithelial cells, SseG and SseF play a role in keeping the SCV in the Golgi region [39, 50]. However, it is unclear how the interaction of SseG and SseF with Desmoplakin, Caprin-1, TIP60, and junction plakoglobin relate to this function. Rather, the SseF–TIP60 interaction appears to play a role in *Salmonella* replication in macrophages (see below). Moreover, TIP60 is generally known to be a multifunctional enzyme involved in diverse processes, including cell cycle, apoptosis, signaling, and DNA repair [51]. Caprin-1 has been shown to have a role in cell proliferation as the suppression of Caprin-1 expression resulted in the prolongation of the cell cycle [52]. Thus, it is unlikely that the interaction of SseG with Caprin-1 can be connected with SseG's role in SCV positioning. Finally, SeeG interacts with Desmoplakin and SseF with junction plakoglobin [38]. Both host proteins are part of the desmosome, with a major role in cell–cell adhesion [53]. Thus, SseG and SseF may influence processes associated with the desmosome and are likely to have multiple functions in *Salmonella* pathogenicity.

An actin network is built around the SCV [54] referred to as vacuole-associated actin polymerizations (VAPs). SteC has Raf-like activity and was shown to be essential for the establishment of VAP and F-actin remodeling [55]. A number of effectors seem to help in inhibiting the formation of VAP. Both SseI and SspH2 bind Filamin A through their N-terminus and thereby preventing the crosslinking of F-actin by Filamin dimers [56]. The C-

terminal domain of SspH2 interacts with Profilin-1 and thus prevents the interaction of Profilin-1 with G-actin in order to inhibit polymerization [56]. The C-terminal ADP-ribosyltransferase domain of SpvB modifies G-actin at residue Arg177 through which actin polymerization is inhibited, most likely by a steric mechanism. Activity of SpvB leads to reduced VAP formation as well as disruption of the cytoskeleton and apoptosis [57, 58].

Besides reversing the cytoskeleton to its normal shape after *Salmonella* entry, SptP dephosphorylates vasolin-containing protein (VCP), an AAA ATPase family protein, by its C-terminal tyrosine phosphatase activity. Dephosphorylation of VCP enhances replication of bacteria in the SCV [59]. Another effector that contributes to *Salmonella* replication is SseF which binds to TIP60 and thereby enhances the histone acetyltransferase activity of TIP60. Knockdown of TIP60 expression in macrophages resulted in a reduced replication of *Salmonella* [60]. Interaction of SopA with HsRMA1 results in ubiquitination of SopA [61]. Monoubiquitinated SopA either directly or in cooperation with an unknown host factor facilitates *Salmonella* escape from SCVs into the cytoplasm of host cells [61]. Polyubiquitinated SopA is degraded by the proteasome.

3.3 Systemic infection

Salmonella can spread within its host leaving the intestine moving via the bloodstream or the lymphatic vessels to other organs like the spleen and the liver. The only effector that is known to contribute to this process is SseI although it has been shown that SseI alone is not the only *Salmonella* protein responsible for systemic infection. SseI-deficient *Salmonella* can still but to a lesser extent spread through the bloodstream and mutation of the TTSS-2 completely abolished *Salmonella* ability to spread systemically. Interaction of SseI with the LIM-domain of TRIP6 enhances dissemination of CD18+*Salmonella*-containing cells most likely by blocking TRIP6 in inhibiting cell motility through interaction with the Rac pathway [62]. IQGAP1 is another protein that is bound by SseI. This interaction facilitates the maintenance of chronic systemic infection by inhibiting migration of dendritic cells to the sites of infection thus keeping the host from clearing *Salmonella* from systemic sites of infection [63].

3.4 Activation and regulation of immune response

Salmonella manipulates and evades the host innate and adaptive immune response by a diverse set of mechanisms involving several *Salmonella* effectors and affecting inflammatory response, antigen (AG) presentation and T cells. Although at least six effectors that are exclusively translocated via TTSS-1 trigger inflammatory responses, effectors that require TTSS-2 or TTSS-1 and -2 for their translocation function to dampen the innate and adaptive immune response. It may be assumed that *Salmonella* first attracts polymorphonuclear neutrophils (PMNs) which the bacteria invade and then by manipulation of the host immune system escapes host defense and uses host cells for systemic spread in order to manifest a chronic or long-term infection [64].

Activation of the inflammatory response occurs through several mechanisms. The GEF activity of SopE and SopE2 causes exchange of GDP by GTP in Cdc42 and thereby activates this small GTPase [19, 65]. This leads to the activation of MAPK pathways, which in turn activate transcription factors resulting in the production of cytokines like IL-8 [19, 66]. Consequently, PMNs are attracted to the site of infection. Other *Salmonella* effectors that induce PMN migration and activate inflammatory response are SipA and SopA. SipA is cleaved into its two functional domains by host caspase-3. The C-terminal domain of SipA directly binds F-actin (see above) and the N-terminal domain is responsible for inducing PMN migration across the intestinal epithelium by triggering MAPK pathways leading to cytokine secretion [67]. SopA, a HECT-like E3 ubiquitin ligase, has been shown to induce

PMN migration. This function has been attributed to its ubiquitin ligase activity [68]. Moreover, SopA contains two functional caspase-3 recognition motifs which are in close proximity to each other [67]. Mutation of both motifs renders SopA insensitive to cleavage by caspase-3 and leads to a reduced induction of PMN migration [67]. Beside the functional caspase-3 cleavage sites of SipA and SopA, other *Salmonella* effectors that comprise putative caspase-3 recognition motifs are AvrA, SopB, SifA, SopD, SptP, and SpvB [67]. Thus, *Salmonella* may exploit the host to cleave effector proteins into functional domains necessary for their activity.

Downregulation and inhibition of immune response is accomplished by *Salmonella* targeting several host proteins to inhibit MAPK signaling and/or activation of NF-kB using the effectors AvrA, SseL, SspH1, SptP, SseI, and SpvC. AvrA causes inhibition of JNK and NF-kB signaling through acetylation of MKK4 and MKK7 [69, 70] and inhibits NF-kB activation by deubiquitination of IkBa [71]. In cells, IkBa binds NF-kB and thereby inhibits this transcription factor. Ubiquitination of IkBa results in its degradation by the host proteasome and an increase of unbound active NF-kB [71]. Therefore, deubiquitination of IkBa by AvrA leads to increased levels of this NF-kB inhibitor protein. The function of SseL in interacting with IkBa is assumed to be the same as for AvrA [72]. Another host protein that has been recently identified to be targeted by SseL is OSBP1 [38]. The LLR-domain of the E3 ubiquitin ligase SspH1 interacts with PKN1. It is proposed that due to this interaction NF-kB is inhibited [73]. As described above, SptP resembles GAP activity which leads to the inactivation of Cdc42 and Rac1. Thus, SptP downregulates MAPK signaling. Moreover, the tyrosine phosphatase activity of SptP is involved in reversing MAPK activation [74]. Both the GAP and the tyrosine phosphatase activity of SptP lead to inhibition of Raf-induced ERK activation [75]. SseI blocks NF-kB signaling through a novel mechanism involving at least Nod1 and Nod2 [16]. SpvC interacts with a phosphorylated MAPK1-derived peptide that contains the TXY motif whose phosphorylation at T and Y is necessary for kinase activity [76, 77]. Due to its phosphothreonine lysase activity, SpvC inactivates the MAP kinases ERK and p38 thereby inhibiting MAPK signaling downstream of p38 and MAPK1 [78]. Moreover, a *Salmonella* strain overexpressing SpvC has been shown to reduce cytokine release from infected cells [77].

Interference with AG presentation by dendritic cells through MHC-I and MHC-2 molecules is proposed to be caused by the TTSS-2 translocated effectors SifA, SspH2, SlrP, PipB2, SopD2 and to a lesser extent by SseF and SseG. This activity allows *Salmonella* to suppress AG-dependent T-cell proliferation [79–81]. AG presentation to T cells is reduced by either direct or indirect ubiquitination of MHC-II by *Salmonella* effectors [82]. The only *Salmonella*–host interaction that is known so far and could contribute to the inhibition of AG-presentation is between SlrP and ERdj3. This interaction may cause the accumulation of unfolded proteins in the ER and thereby disturb AG presentation by MHC-I [83, 84].

3.5 Induction and inhibition of apoptosis

Salmonella both dampens and activates apoptosis of host cells. Induction of cell death may allow bacteria to evade host cells in order to infect new cells and the killing of cells may aim at reducing the host immune response. On the other hand, the prolongation of cell survival may provide *Salmonella* with a safe environment for replication, and permit systemic spread and manipulation of host defense. The following mechanisms are at play. SipB induces apoptosis of macrophages through its interaction with caspase-1. Interaction results in caspase-1 activation which induces apoptosis. Moreover, activation of caspase-1 leads to maturation of IL-1β, a caspase-1 substrate known to be an endogenous pyrogen, and thereby may contribute to the inflammatory response [85]. SipB has also been shown to induce apoptosis in a caspase-1 independent way which involves caspase-2, -3, -6, -8 and mitochondrial cytochrome *C* [86]. Another effector, SlrP, is thought to promote cell death

by interacting with Thioredoxin 1 in the cytosol and the chaperone ERdj3 in the ER. SlrP-mediated ubiquitination of Thioredoxin 1 could cause a reduced redox activity of the target protein and its degradation [87]. Interaction with ERdj3 may lead to the accumulation of unfolded proteins in the ER [83]. The ADP-ribosyltransferase activity of SpvB may additionally contribute to cell death through disruption of cytoskeletal structures (see above) [58]. SseL plays a role in *Salmonella*-induced cytotoxicity in macrophages [88]. On the other hand, AvrA targeting MKK4 and MKK7 inhibits JNK signaling and thus slows down apoptosis [69, 70]. The proposed SpvC-mediated inhibition of p38 may have the same effect (see above).

4 Pathway and subnetwork analysis of host proteins

Next, we performed pathway and network analyses with the human genes implicated directly or by inference from other hosts (such as mouse) in interactions with *Salmonella* proteins using two complementary approaches.

In the first approach, pathway enrichment was calculated based on multiple pathway data sources using software GSEA (http://www.broadinstitute.org/gsea/index.jsp) on July 21, 2011 (reference: http://www.ncbi.nlm.nih.gov/pubmed/16199517). The software integrates several pathway sources to perform gene set enrichment analysis, which provides comprehensive insight on the enriched pathways in our gene set. These data sources include KEGG (http://www.genome.jp/kegg/), BioCarta (http://www.biocarta.-com), Reactome (http://www.reactome.org/), Signal transduction knowledge environment (ST, http://stke.sciencemag.org/) and Signaling gateway (SIG, http://www.grt.kyushu-u.ac.jp/spad/menu.html). The pathways with *P*-value less than 1×10^{-4} are summarized in Table 2. As expected from the large number of interactions involving actin and actin regulatory proteins, the pathways “Genes related to regulation of the actin cytoskeleton” and “Regulation of actin cytoskeleton” are among the most significantly targeted pathway. The analysis further revealed interference of *Salmonella* especially with pathways related to cell shape, cell growth, and interactions with other cells. This includes, for example, the pathways, Adherens junction, Neurotrophin signaling pathway, Focal adhesion, Genes involved in Sema4D-induced cell migration and growth-cone collapse, Genes involved in Apoptotic cleavage of cell adhesion proteins, Agrin in Postsynaptic Differentiation, Genes involved in Axon guidance, Integrin Signaling Pathway, Calpain and friends in Cell spread and mCalpain and friends in Cell motility. Numerous pathways are involved in immune response to pathogens, such as Pathogenic *Escherichia coli* infection, Genes involved in TRAF6-Mediated Induction of the antiviral cytokine IFN-αη cascade, Genes involved in Toll-Like Receptor 3 (TLR3) Cascade, How does *Salmonella* hijack a cell, Epithelial cell signaling in *Helicobacter pylori* infection, Genes involved in Toll Receptor Cascades, Toll-like receptor signaling pathway, and HIV-I Nef: negative effector of Fas and TNF. The occurrence of different signaling pathways related to pathogenic processes of various species supports the notion that there are conserved mechanisms of communication, even for pathogens as diverse as viruses and bacteria. The remaining over-represented pathways are frequently generic, such as general signaling mechanisms, such as Pathways in cancer, Ras Signaling Pathway, Genes involved in G α (12/13) signaling events and MAPK signaling pathway. Particularly interesting is the retrieval of Phospholipids as signaling intermediaries because numerous lipidation events are known to modulate *Salmonella* interactions with hosts [89] and phosphoinositide signaling plays an important role in *Salmonella* invasion [90].

In a second approach, Ingenuity Pathway Analysis (IPA, http://www.ingenuity.com/) was carried out. For a given canonical signaling pathway in IPA, Fisher’s exact test was performed to measure the probability (*P*-value) that the pathway is randomly selected. To control the error rate, the *P*-values were corrected by Benjamini–Hochberg method [91] and

those pathways whose *P*-values were less than 1.0×10^{-5} (or score >5, here score=−log *P*) were defined as the significant pathways. The results are summarized in Supporting Information Table 3. A mapping of the human genes involved in the respective pathways, together with the *Salmonella*-human PPI targeting them, is provided in Supporting Information Table 4. This analysis supports the conclusion that current host targets of *Salmonella* effectors affect numerous ubiquitous signaling pathways through interactions with hub proteins involved in crosstalk among different pathways.

The IPA analysis was then used to investigate subnet-work enrichment in the resulting global network. To this end, the genes of interest were first overlaid onto a global molecular network developed based on the Ingenuity Pathways Knowledge Base and then subnetworks for these genes were extracted from the global network based on their connectivity using the algorithm developed by IPA (http://www.ncbi.nlm.nih.gov/pubmed/16136080). Similar to pathway analysis in IPA, for each subnetwork, IPA computed a score based on the *P*-value calculated by Fisher's exact test and the *P*-value indicated the likelihood of the genes in the subnetwork were found by chance. Additionally, the top three biological functions according to the IPA score were assigned to each network. We identified the enriched subnetworks in our gene set if a given subnetwork has one score higher than 5. The results are summarized in Table 3. A mapping of the *Salmonella* proteins involved in interactions with the proteins in these subnetworks is provided in Supporting Information Table 5. Five subnetworks were identified. It is particularly interesting to examine the topranked functional terms associated with these networks: (i) Cell Death, Cellular Assembly and Organization, Skeletal and Muscular System Development and Function, (ii) Cellular Assembly and Organization, Gastrointestinal Disease, Genetic Disorder, (iii) Cancer, Cellular Development, Cellular Growth and Proliferation, (iv) Cardiovascular System Development and Function, Cell-To-Cell Signaling and Interaction, Cell Signaling, and (v) Increased Levels of CRP, Cell Morphology, Cellular Development.

Subnetwork 1 demonstrates the impact *Salmonella* has on "cellular assembly and organization" and "cell death." Much of the actin skeleton modulations are related to this, in addition to direct induction or suppression of apoptosis, described in Section 3, above. This includes, in particular, the roles of SipA and SipC in actin polymerization and bundling, the proposed role of SseJ to induce SCV tubulation through interacting with RhoC, the function of SpvB to disrupt the cytoskeleton and promote apoptosis, the fact that the interaction of SspH2 with profilin inhibits actin polymerization and that the SipB–caspase-1 interaction induces apoptosis. The interactions between SseF and JUP and between SseL and OSBP may fit into this subnetwork by influencing cytoskeletal rearrangement and membrane dynamics. This is supported by the finding that JUP is part of the desmosome [53] and the manipulation of immune response is a proposed role for OSBP based on its involvement in MAPK signaling [92]. Subnetwork 2 represents the disruptive nature of the epithelial cells being infected with *Salmonella*. Specifically, there are several host proteins that have been shown or proposed to have a role in *Salmonella* invasion and internalization when interacting with the identified *Salmonella* protein-binding partner. These are Cytokeratin-8 and -18, Exo70, F-actin, and Cdc42 with the *Salmonella* proteins SipC, SopB, SopE, and SopE2. The interaction of SptP with Cdc42 also fits into this subnetwork because it results in the reversal of membrane ruffles after bacterial internalization. As the interaction of SseI and SspH2 with Filamin A is thought to prevent VAP formation, this can be categorized under the same top function, namely "cellular assembly and organization" as the interactions for subnetwork 2 listed above. The other focus host proteins within subnetwork 2 most likely fall into the function "gastrointestinal disease" based on their roles in MAPK signaling, inhibition of immune response, and cell death. The function of the interaction between SseG and DSP may be the same as for the interaction between SseF and JUP listed in subnetwork 1.

The general aspects of cell growth that also come out in the numerous generic pathways targeted, including those involved in cancer, are represented by subnetwork 3. Subnetworks 4 and 5 are related to the cell morphology and interactions aspect that is also observed by the very large number of related pathways being targeted. Presumably, much of *Salmonella* survival is related to the turnover of the infected cell and how loosely it is embedded in its native tissue. Cells that shed frequently and are degraded rapidly will automatically remove *Salmonella*-infected cells, limiting the establishment of an infection. On the other hand, it may be that rapid degradation of *Salmonella*-containing cells may conversely also contribute to a higher rate of infection. Bacteria inside cells are spread within the host and their release from the cells enables *Salmonella* to infect new cells.

5 Significance of the data set

This review provides the most complete *Salmonella*–host interactome to date. It stratified several previously reviewed interactions as indirect and added numerous new interactions, so that the current set of interactions includes 62 pairs. These pairs were derived from careful analysis of the primary literature and we consider the labels therefore of high quality. Although the primary literature and experimental evidence given for every PPI listed here has been carefully examined, the level of confidence, that a reported interaction is true, varies depending on the nature of the performed experiments as well as the amount of evidence given. To assist the reader in making their own judgments, the experiments done supporting an interaction are also listed in Table 1. It is important to note, however, that there is inherent bias in the choice of experiments and proteins investigated. Thus, identification of novel PPI focuses often on the proteins that are already known or thought to be interesting key molecules in pathogenicity, both on the *Salmonella* and on the host side. For example, most studies to date have focused on *Salmonella* proteins translocated via the bacterial TTSS-1 and TTSS-2 and many host proteins are those that are known key signaling molecules. A systematic high-throughput investigation of *Salmonella*–host PPI has not yet been carried to our knowledge. Moreover, any experimental approach depends on the nature of the protein itself. Thus, most data available for *Salmonella* are for proteins which are comparatively easy to handle, e.g. soluble proteins, as opposed to membrane proteins.

Despite this bias, the data set of known *Salmonella*–host PPI presented here is the most complete set of interactions available and makes it suitable to assist computational modeling. It can be used as a training set in a machine-learning approach and can serve to validate models predicting the pathogen–host interactome. In comparison to predicting intraspecies PPI, where much work has been done especially for the model organism yeast [93–95] and for human [96–99], the prediction of pathogen–host interactions is still in its infancy. Current study in interspecies PPI prediction includes domain profile-based approaches [5, 100], interolog- and homology-based approaches [6, 101, 102], and structural similarity-based approaches [103]. Supervised classification-based approaches have so far only been applied to the HIV–human interactome due to the availability of the data [12, 104]. It is significant to note that the homology-based models for bacteria use PPI databases such as DIP and iPFAM. The work presented here for *Salmonella* demonstrates that these machine readable databases lack many of the published known interactions (we only found 10% of the already published direct *Salmonella*–host interactions in PPI databases). This creates two problems. First, such approaches will suffer from false positives due to the large number of general domain overlap in proteins including those that do not interact. At the same time, they will likely miss critical interactions because bacteria and host have diverged greatly so that their genome's similarity is generally low. The few cross-species pairs listed in databases cannot provide sufficient numbers to compensate for this loss in information due to sequence divergence. The only current computational approach to model *Salmonella*–host interactions uses such a homology-based approach [101]. Thus, our new data set opens

the door to greatly improving the prospects for accurate prediction of the *Salmonella*–host interactome, by enabling application of machine-learning approaches to a larger data set and allowing the integration of multiple information sources, rather than basing predictions on homology alone.

Modeling the pathogen–host interactome can have a range of diverse objectives. It helps to acquire new insights into the communication between the two organisms, to get a better understanding of the infection process on a molecular level, to make comparison of different pathogen–host interactomes possible and last but not least modeling can push the rational development of drugs and vaccines to fight diseases. Computational machine-learning methods to model host–pathogen PPI and the host response in general will likely have great impact here. For example, modeling the host response to *Brucella melitensis*, *Mycobacterium avium* Paratuberculosis, and *Salmonella enterica* Typhimurium has provided strategies to develop vaccines on a rational basis [105]. Protein network analysis combined with tissue expression for human proteins and experimental validation of predicted interactions using GeneChip data was used to delineate the interactions between the gastric cancer causing pathogen *H. pylori* and the human [106]. In order to identify common strategies of pathogens and discover conserved host target proteins during infection, experimentally identified or computationally predicted interactomes have been compared [105, 107, 108]. The validity of this strategy for future *Salmonella* studies can also be found from the retrieval of a number of pathways related to other pathogens, including viruses. Thus, the present analysis presents an important milestone in the mapping of *Salmonella*–host interactions to the functional consequences of these interactions in human signal transduction pathways.

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Abbreviations

AG	antigen
GEF	guanine nucleotide exchange factor
PMN	polymorphonuclear neutrophil
PPI	protein–protein interaction
SCV	*Salmonella*-containing vacuole
Sifs	*Salmonella* induced filaments
SPI	*Salmonella* pathogenicity island
TTSS	type III secretion system
VAP	vacuole-associated actin polymerization
VCP	vasolin-containing protein

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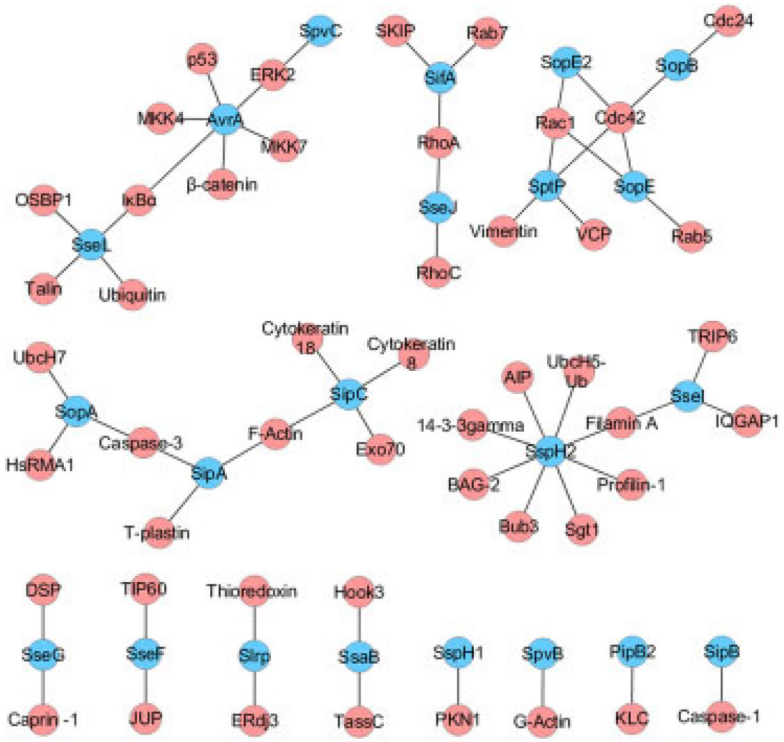

Figure 1.

Current *Salmonella*–host interactome. The image was created using cytoscape software (www.cytoscape.org). Blue, *Salmonella* proteins; red, host proteins.

Table 1

Salmonella –host PPI data set

***Salmonella* protein**	**Description**	**Host protein**	**Human gene symbol**	**Host organism**	**Experiment**	**Reference**	**Database listed**
AvrA	Avirulence Protein A	p53	TP53	Eukaryotic cells	Immunoprecipitation	[109]	
		ERK2	MAPK1	NA	Yeast 2-hybrid assay	[70]	
		MKK7*a)b)*	MAP2K7	NA	Yeast 2-hybrid assay	[70]	
		MKK4*a)b)*	MAP2K4	Mammalian	Acetylation	[69]	
		IκBα (ubiquitinated)*a)b)*	NFKBIA	Human	Deubiquitination	[71]	
		β-Catenin (ubiquitinated)*a)b)*	CTNNB1	Human	Deubiquitination	[71]	
SipA or SspA	*Salmonella* Invasion Protein A	F-actin*a)b)c)*	ACTA1*d)*	Human, *E. coli*, rabbit, mouse	Pull-down assay, cosedimentation, immunofluorescence	[23, 25, 110]	UniProt
		T-plastin*a)b)c)e)*	PLS3	Human	Yeast 2-hybrid screen, pull-down assay	[111]	
		Caspase-3	CASP3	Human	Caspase-3 cleavage assay, immunoblot	[67]	
SipB	*Salmonella* Invasion Protein B	Caspase-1*c)*	CASP1	Mouse, *E. coli*	Western blotting, pull-down assays, immunoblotting	[85, 112]	IntAct, UniProt
SipC or SspC	*Salmonella* Invasion Protein C	F-Actin*a)b)c)*	ACTA1*d)*	Human	Pull-down assay, coimmunoprecipitation	[24]	UniProt
		Cytokeratin 8*c)e)*	KRT8				
		Cytokeratin 18*c)*	KRT18	Human	Yeast-2 hybrid assay	[26, 37]	
		Exo70	EXOC7	Human	Yeast-2 hybrid assay, pull-down assay	[27]	
SopA	*Salmonella* Outer Protein A	UbcH7 (UBE2L3; UBCE7)	UBE2L3	Human	Pull-down assay, fluorescence polarization	[113]	dip
		Caspase-3	CASP3	Mouse	Caspase-3 cleavage assay	[67]	
		HsRMA1*a)b)*	RNF5	Human	Yeast 2-hybrid assay, pull-down assay	[61]	
SopB or SigD	*Salmonella* Outer Protein B	Cdc24	CDC24	*Saccharomyces cerevisiae*	Pull-down assay	[114]	
		Cdc42*b)*	CDC42	Human	Pull-down assay, immunoprecipitation, colocalization	[114, 115]	
SopE	*Salmonella* Outer Protein E	Cdc42*a)b)c)*	CDC42	Human	Hela cDNA library screening, bacteriophage plaque-binding assay, Surface plasmon resonance analysis, X-ray crystallography	[19–21]	Intact, PIG, BIND, UniProt
		Rac1*a)b)c)*	RAC1	Human	Hela cDNA library screening, bacteriophage plaque-binding assay, Surface plasmon resonance analysis	[19, 20]	

Salmonella protein	Description	Host protein	Human gene symbol	Host organism	Experiment	Reference	Database listed
		Rab5*c)*	RAB5A	Mouse	Pull-down assay, ELISA, competition ELISA	[30, 31, 116]	
SopE2	*Salmonella* Outer Protein E2	Cdc42*a)b)c)*	CDC42	NA	Surface plasmon resonance analysis, HSQC-NMR spectroscopy	[20, 22]	
		Rac1*c)*	RAC1	NA	Surface plasmon resonance analysis	[20]	
SptP	*Salmonella* Protein Tyrosine Phosphatase	Rac1*a)b)c)*	RAC1	Human	Pull-down assay, Gel-filtration, X-ray crystallography	[28, 117]	Intact, PIG, UniProt
		VCP*b)*	VCP	Pig, human	Pull-down assay, immunoprecipitation	[59]	
		Vimentin*a)b)*	VIM	Human	Yeast-2 hybrid, pull-down assay	[74]	
		Cdc42*a)b)*	CDC42	Human, *S. cerevisiae*	Activation assay	[28, 29]	
PipB2	NA	Kinesin Light Chain (KLC)*a)b)c)*	KLC1	Mouse, human	Yeast 2-hybrid assay, pull-down assay, immunoprecipitation	[40]	UniProt
SifA	Sifs A	SKIP (PLEKHM2; KIAA0842)*a)b)c)*	PLEKHM2	Human	Yeast 2-hybrid assay, pull-down assay, immunoprecipitation, Copurification, isothermal titration calorimetry, colocalization studies, X-ray crystallography	[42, 43, 47]	Dip, BIND, PIG, UniProt
		Rab7*a)c)*	RAB7A	NA	Pull-down assay	[32]	
		RhoA	RHOA	NA	Surface plasmon resonance analysis	[46]	
SpiC or SsaB	*Salmonella* Pathogenecity Island Protein C	Hook3*a)b)c)e)*	HOOK3	Mouse	Pull-down assay, coimmunoprecipitation (yeast-2-hybrid and pull down with recombinant Hook3 was not successful)	[35]	UniProt
		TassC*a)b)*	NIPSNAP3A	Human, mouse	Yeast-2-hybrid, co-purification (in vitro and in vivo)	[34]	UniProt
SpvB	*Salmonella* Plasmid Virulence Protein B	G-Actin*a)b)c)*	ACTA1*d)*	Mouse	Copurification, mass spectrometry	[57, 58]	
SseF	Secretion System Effector F	TIP60 acetyltransferase	KAT5	Human	Yeast 2-hybrid screen, (pull-down assay performed but the results are not shown)	[60]	
		Junction Plakoglobin	JUP	Human	SILAC (stable isotope labeling of amino acids in cell culture)	[38]	
SseG	Secretion System Effector G	Desmoplakin	DSP	Human	SILAC	[38]	
		Caprin-1	CAPRIN1	Human	SILAC	[38]	
SseI or SrfH	Secretion System Effector I	TRIP6*a)b)c)*	TRIP6	Human, mouse	Yeast 2-hybrid assay, fluorescence microscopy, localization studies, immunoprecipitation	[62, 63] could not reproduce interaction may be due to using	UniProt

Salmonella protein	Description	Host protein	Human gene symbol	Host organism	Experiment	Reference	Database listed
						a different *Salmonella* strain	
		Filamin A[a)b)c)]	FLNA	Mouse	Yeast 2-hybrid assay	[56]	
		IQGAP1[b)]	IQGAP1	Mouse	Copurification, immunoprecipitation, colocalization studies	[63]	UniProt
SseJ	Secretion System Effector J	RhoA	RHOA	Human	Yeast-2-hybrid assay, immunoprecipitation, size-exclusion chromatography, Gel filtration analyses	[33, 47]	UniProt
		RhoC	RHOC	Human	Yeast 2-hybrid assay SILAC	[38, 47]	
SseL	Secretion System Effector L	Ubiquitin A, B and C (mono- and polyubiquitinated proteins)[c)]	UBA52 UBB UBC	Human	Yeast 2-hybrid assay, coimmunoprecipitation	[88]	
		IκBα[a)b)]	NFKBIA	Human, mouse	Deubiquitination	[72]	
		Oxysterol-binding protein 1	OSBP	Human	SILAC, coimmunoprecipitation	[38]	
		Talin-1	TLN1	Human	SILAC	[38]	
SspH2	*Salmonella*- Secreted Protein H2	Filamin A[a)b)c)]	FLNA	Mouse	Yeast 2-hybrid assay, colocalization	[56]	
		Profilin-1[a)b)c)]	PFN1	Mouse, human	Yeast 2-hybrid assay, pull-down assay	[56]	
		UbcH5-Ub conjugate	UBE2D1	Human	Heteronuclear 2-D-NMR spectroscopy	[118]	
		Sgt1	SUGT1	Human	SILAC, coimmunoprecipitation	[38]	
		AIP	AIP	Human	SILAC	[38]	
		Bub3	BUB3	Human	SILAC, coimmunoprecipitation	[38]	
		14-3-3γ	YWHAG	Human	SILAC	[38]	
		BAG regulator 2	BAG2	Human	SILAC	[38]	
Slrp	*Salmonella* Leucine-Rich Repeat Protein	Thioredoxin 1[a)]	TXN	Human, mammalian	Yeast 2-hybrid screen, copurification, coimmunoprecipitation	[87]	UniProt
		ERdj3	DNAJB11	Human	Yeast 2-hybrid screen, pull-down assay, coimmunoprecipitation, colocalization	[83]	
SspH1	Salmonella- secreted protein H1	PKN1[a)b)c)]	PKN1	Human	Yeast 2-hybrid screen, coimmunoprecipitation	[73]	UniProt
SpvC		MAPK1 (PRKM1; ERK2; PRKM2)	MAPK1	Human	Crystallography	[76]	DIP

The *Salmonella* effectors are translocated by TTSS-1: SipA, SipB, SipC, **SipD**, SopA, SopB, **SopD**, SopE, SopE2 TTSS-1 and -2: AvrA, **GtgE**, **PagJ**, SlrP, SpvC, **SpvD**, SptP, **SseK1**, SspH1, **SteA**, **SteB** TTSS-2: **CigR**, **GogB**, **GtgA**, **PipB**, PipB2, SifA, **SifB**, **SopD2**, SpiC, SpvB, **SseB**, **SseC**, **SseD**, SseF, SseG, SseI, SseJ, **SseK2**, **SseK3**, SseL, SspH2, **SteC**, **SteD**. *Salmonella* effectors where no protein-binding partner is known and that are consequently not listed here are given in bold face.

[a)]Reviewed in [15].

[b] Reviewed by Heffron et al. 2011 [16].

[c] Reviewed in [14].

[d] For the network analysis, ACTA2, ACTB, ACTC1, ACTG1, and ACTG2 were also included in addition to ACTA1.

[e] Indirect. SipA and T-plastin complex is mediated by F-actin; this complex cannot be formed in the absence of actin; Cytokeratin-18 bind to Cytokeratin-8; SpiC–Hook3 interaction may be indirect as speculated by [35].

Table 2

Pathways overrepresented in the 54 proteins based on multiple data sources

Description	*P*-value	Data source
Adherens junction	5.52×10^{-8}	KEGG
Role of MAL in Rho-Mediated Activation of SRF	1.88×10^{-7}	Biocarta
Fas Signaling Pathway	1.91×10^{-7}	ST
Neurotrophin signaling pathway	3.10×10^{-6}	KEGG
Pathogenic *E. coli* infection	3.88×10^{-6}	KEGG
Genes related to regulation of the actin cytoskeleton	4.80×10^{-6}	SIG
Colorectal cancer	5.20×10^{-6}	KEGG
Calpain and friends in Cell spread	7.32×10^{-6}	BIOCARTA
Focal adhesion	1.26×10^{-5}	REACTOME
Erk and PI-3 Kinase Are Necessary for Collagen Binding in Corneal Epithelia	2.46×10^{-5}	Biocarta
Genes involved in Sema4Dinduced cell migration and growth-cone collapse	2.46×10^{-5}	REACTOME
Granule Cell Survival Pathway is a specific case of more general PAC1 Receptor Pathway	2.91×10^{-5}	ST
Influence of Ras and Rho proteins on G1 to S Transition	3.43×10^{-5}	Biocarta
Amyotrophic lateral sclerosis (ALS)	3.84×10^{-5}	KEGG
Genes involved in TRAF6-Mediated Induction of the antiviral cytokine IFN-αη cascade	3.84×10^{-5}	REACTOME
Genes involved in Sema4D in semaphoring signaling	5.35×10^{-5}	REACTOME
Genes involved in Apoptotic cleavage of cell adhesion proteins	5.91×10^{-5}	REACTOME
Genes involved in Toll-Like Receptor 3 (TLR3) Cascade	6.49×10^{-5}	REACTOME
NOD-like receptor signaling pathway	8.25×10^{-5}	KEGG
Genes related to CD40 signaling	9.03×10^{-5}	SIG
How does salmonella hijack a cell	1.01×10^{-4}	Biocarta
Genes involved in Semaphorin interactions	1.11×10^{-4}	REACTOME
Agrin in Postsynaptic Differentiation	1.28×10^{-4}	Biocarta
Epithelial cell signaling in *H. pylori* infection	1.29×10^{-4}	KEGG
Regulation of actin cytoskeleton	1.54×10^{-4}	KEGG
Genes involved in Axon guidance	1.55×10^{-4}	REACTOME
Integrin Signaling Pathway	1.58×10^{-4}	Biocarta
JNK MAPK Pathway	1.58×10^{-4}	ST
Role of PI3K subunit p85 in regulation of Actin Organization and Cell Migration	1.96×10^{-4}	Biocarta
Leukocyte transendothelial migration	2.04×10^{-4}	KEGG
Arrhythmogenic right ventricular cardiomyopathy (ARVC)	2.18×10^{-4}	KEGG
Integrin Signaling Pathway	2.46×10^{-4}	ST
Genes involved in p75 NTR receptor-mediated signaling	3.11×10^{-4}	REACTOME
Keratinocyte Differentiation	3.36×10^{-4}	Biocarta
Genes involved in Toll Receptor Cascades	3.89×10^{-4}	Biocarta
MAPKinase Signaling Pathway	4.10×10^{-4}	Biocarta
Trefoil Factors Initiate Mucosal Healing	4.52×10^{-4}	Biocarta

Description	*P*-value	Data source
Apoptotic Signaling in Response to DNA Damage	5.21×10^{-4}	Biocarta
Pathways in cancer	5.47×10^{-4}	KEGG
Ras Signaling Pathway	5.96×10^{-4}	Biocarta
Genes involved in G α (12/13) signaling events	6.23×10^{-4}	REACTOME
MAPK signaling pathway	6.48×10^{-4}	KEGG
Genes involved in Further platelet releasate	6.78×10^{-4}	REACTOME
Genes involved in Smooth Muscle Contraction	6.78×10^{-4}	REACTOME
mCalpain and friends in Cell motility	7.66×10^{-4}	Biocarta
HIV-I Nef: Negative effector of Fas and TNF	8.18×10^{-4}	Biocarta
Toll-like receptor signaling pathway	8.51×10^{-4}	KEGG
Phospholipids as signaling intermediaries	9.65×10^{-4}	Biocarta

Table 3

Subnetworks over-represented in the 54 proteins

ID	Molecules in subnetwork	Score[a)]	Number of focus molecules[b)]	Top functions
1	**ACTA1**[c)], **ACTA2**, **ACTB**, **ACTC1**, **ACTG1**, **ACTG2**, Actin, Alpha actin, Alpha Actinin, Alpha catenin, atypical protein kinase C, Cadherin, **CASP1**, **CASP3**, ERK1/2, Fgf, G-Actin, Hsp27, Hsp90, Ige, **JUP**, **KAT5**, **OSBP**, **PFN1**, Phosphatidylinositol4,5 kinase, Pld, PP2A, Profilin, **RAB9A**, Ras homolog, Rho gdi, **RHOC**, RhoGap, Rock, Rsk	29	14	Cell Death, Cellular Assembly and Organization, Skeletal and Muscular System Development and Function
2	14-3-3, Alcohol group acceptor phosphotransferase, BAG5, BCR, Caspase, CD3, Cdc2, **CDC42**, **DSP**, Ephb, **EXOC7**, F Actin, Fcer1, Filamin, **FLNA**, IQGAP, **IQGAP1**, **KRT8**, **KRT18**, Laminin, **MAP2K4**, **MAP2K7**, MAP2K4/7, MAP3K, Mek, NFkB (complex), Pak, Pkg, **PKN1**, Raf, Rap1, Sapk, TCR, **TXN**, VAV	24	12	Cellular Assembly and Organization, Gastrointestinal Disease, Genetic Disorder
3	26s Proteasome, **AIP**, Akt, APC/APC2, Calmodulin, Cbp/p300, Ck2, **CTNNB1**, **DNAJB11**, FSH, Gpcr, hCG, Histone h3, Histone h4, Hsp70, Ifn gamma, IgG, Ikb, IL12 (complex), Insulin, Interferon alpha, Jnk, Lh, Pka, PLC, **PLS3**, RNA polymerase II, **TP53**, **UBB**, **UBC**, **UBE2D1**, **UBE2L3**, Ubiquitin, **VCP**, **YWHAG**	20	11	Cancer, Cellular Development, Cellular Growth and Proliferation
4	AMPK, Ap1, Calpain, Collagen type I, Collagen type IV, ERK, Estrogen Receptor, Fibrinogen, Focal adhesion kinase, G protein beta gamma, Gef, IFN Beta, IL1, Integrin, LDL, Mapk, **MAPK1**, **NFKBIA**, P38 MAPK, p85 (pik3r), Pdgf (complex), PDGF BB, PI3 K (complex), Pkc(s), **RAB5A**, **RAB7A**, Rac, **RAC1**, Ras, **RHOA**, STAT5a/b, Tgf beta, **TLN1**, **TRIP6**, Vegf	14	8	Cardiovascular System Development and Function, Cell-To-Cell Signaling and Interaction, Cell Signaling
5	AFAP1L2, AGT, AKIRIN2, ARL6, ARL4C, **BUB3**, C11orf1, **CAPRIN1**, CES2, COX7A2, GIPC2, HNF4A, HNRNPA0, **HOOK3**, IL6, IRF6, LPP, MRPL44, **NIPSNAP3A**, POLR3G, PRCP, PTK2, PTK7, REG1A, SBNO2, SETDB1, SLC14A2, SLC16A6, **SUGT1**, TDO2, TXNDC9, **UBA52**, UTP3, XPNPEP2, ZFP64	10	6	Increased Levels of CRP[d)], Cell Morphology, Cellular Development

[a)] Scores were log-transformed by the *P*-values, which were calculated by Fisher's exact test.

[b)] The number of focus molecules is the number of the genes with the bold font.

[c)] The genes with the bold front are these genes existed in our gene set.

[d)] CRP is abbreviated for c-reactive protein.