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The EHEC-host interactome reveals novel targets for the translocated intimin receptor

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Enterohemorrhagic *E. coli* (EHEC) manipulate their human host through at least 39 effector proteins which hijack host processes through direct protein-protein interactions (PPIs). To identify their protein targets in the host cells, we performed yeast two-hybrid screens, allowing us to find 48 high-confidence protein-protein interactions between 15 EHEC effectors and 47 human host proteins. In comparison to other bacteria and viruses we found that EHEC effectors bind more frequently to hub proteins as well as to proteins that participate in a higher number of protein complexes. The data set includes six new interactions that involve the translocated intimin receptor (TIR), namely HPCAL1, HPCAL4, NCALD, ARRB1, PDE6D, and STK16. We compared these TIR interactions in EHEC and enteropathogenic *E. coli* (EPEC) and found that five interactions were conserved. Notably, the conserved interactions included those of serine/threonine kinase 16 (STK16), hippocalcin-like 1 (HPCAL1) as well as neurocalcin-delta (NCALD). These proteins co-localize with the infection sites of EPEC. Furthermore, our results suggest putative functions of poorly characterized effectors (EspJ, EspY1). In particular, we observed that EspJ is connected to the microtubule system while EspY1 appears to be involved in apoptosis/cell cycle regulation.

Enterohemorrhagic E. coli (EHEC) are pathogenic *E. coli* that produce shiga toxins similar to *Shigella dysenteriae*[1,2]. Aside from bloody and non-bloody self-limiting diarrhea, EHEC can cause so-called he molytic uremic syndrome (HUS), a disease that is characterized by hemolytic anemia, acute and occasionally chronic renal failure as well as thrombocytopenia. The public health impact of EHEC infections is considerable[3,4] as a consequence of their low infectious dose (<100 colony forming units) and the corresponding frequency of systemic complications in patients. EHEC colonization utilizes a type three-secretion system (T3SS) and up to 62 putative effector proteins. The T3SS system directly translocates at least 39 effectors into the cytoplasm of their human host cells[5]. The combined action of these effectors results in brush border remodeling and the formation of attaching and effacing (A/E) lesions in the small intestine that are typical for EHEC and enteropathogenic *E. coli* (EPEC) infections. Furthermore, effectors manipulate several host cell signaling pathways to allow successful colonization of the host. Especially the assembly of pedestals through actin-cytoskeleton reorganization is considered important for host cell colonization, a step that is substantially conditioned by the translocated intimin receptor (TIR)[6]. TIR is involved in several distinct cellular processes besides actin pedestal assembly, such as down-regulation of Map-dependent filopodia formation and suppression of inflammatory cytokine production[6–9]. Furthermore, TIR interacts with numerous host cellular targets such as 14-3-3tau, alpha-actinin, cortacin, CK18, IQGAP1, IRTKS, IRSp53, Nck, PI3K, talin, vinculin and AnxA2[10–21] as reviewed in ref 7. Only recently two novel interactions with SHP-1 and SHP-2 were published[8,9]. Although several other novel EHEC - host protein interactions have been described that involve the effectors EspG, EspZ, NleB and NleF[8,9,22–26]

our knowledge of interactions between effector and host cell proteins is far from complete. To fill this gap we mapped the first comprehensive, experimentally derived network of binary EHEC-host interactions which allowed us to investigate the interactions between the translocated intimin receptor TIR and its human targets in more detail.

Results and Discussion

The EHEC effector-host protein interaction map. In 2006 Tobe et al. identified 62 putative EHEC effector proteins through computational analysis and experimentally confirmed that 39 effectors were indeed translocated into host cells[5]. While several already have been demonstrated to modify certain host functions (reviewed in ref 7), many effector proteins remain functionally poorly characterized. To investigate the global landscape of effector-host interactions we systematically screened 34 of the 39 effectors verified by Tobe et al. using a well established, semi-automated comprehensive Yeast 2-Hybrid (Y2H) screening protocol[27,28] (Figure 1, Supplementary Table S1). Each effector was fused to the GAL4-DBD (DNA binding domain of the GAL4 transcription factor) on both its N- and C-terminus. Similarly, we built two different prey screening libraries from human open reading frame (ORFeome) collections[29–31]. Each consisted of a single pool of over 10,000 individual human protein-coding ORFs that were either fused C- or N-terminally to the AD (activation domain) of GAL4. The N- and C-terminally-tagged effector bait constructs were then screened individually against both ORFeome libraries to increase the detection sensitivity[32,33] as well as against a cDNA prey library (Human Universal, Clontech). These screens resulted in 1,025 positive yeast colonies from which we successfully retrieved 328 unique EHEC bait-human prey pairs. We removed 207 pairs with prey sequences that were not located in the protein coding region of genes (3′-UTR artifacts), interactions involving randomly activating bait constructs (i.e. baits that returned exclusively multiple non-reproducible hits), or interactions including prey proteins known to interact non-selectively with many different bait proteins. In the latter case we excluded prey constructs known to bind to more than five bait proteins[28] (Schwarz et al., unpublished). Out of the remaining 121 interactions 86 were detected only once, i.e., a prey could be assigned to the known bait protein only from one unique positive yeast colony. Using pairwise Y2H assays we retested all 121 pairs that had human full-length ORF constructs. We cloned 78 out of 90 human interactors into prey plasmids and were able to reproduce 35 out of 82 tested PPIs (42.7%). All 23 pairs that had two or more colonies in the screens were retested positive; pairs with only a single colony had a success rate of 20.7%. In the final dataset, we included all PPIs that were isolated multiple times, as well as those that were independently reproduced. In total, our approach yielded 48 individual EHEC-host PPIs of high confidence (Table 1 and for more details Supplementary Table S2) where the majority of 36 PPIs was identified by using a C-terminal ORFeome prey library. In comparison, the N-terminal ORFeome prey library and the cDNA prey libraries isolated clearly fewer interactions (seven PPIs each). Only two interactions were found in all the different ORFeome prey libraries (NleF with Caspase 9 and dihydrofolate reductase).

To generate a combined protein interaction network including the 48 newly found interacions, we extensively reviewed the existing literature and curated 130 effector-host interactions, including 76 EHEC-host protein interactions. Furthermore, we found a few interactions that were inferred from protein complexes although no experimental evidence for a direct interaction appears to be available.

The combined network is shown in Figure 2 (for a complete list of EHEC-host interactions and references see Supplementary Table S3). We found that five out of the discovered 48 pathogen-host

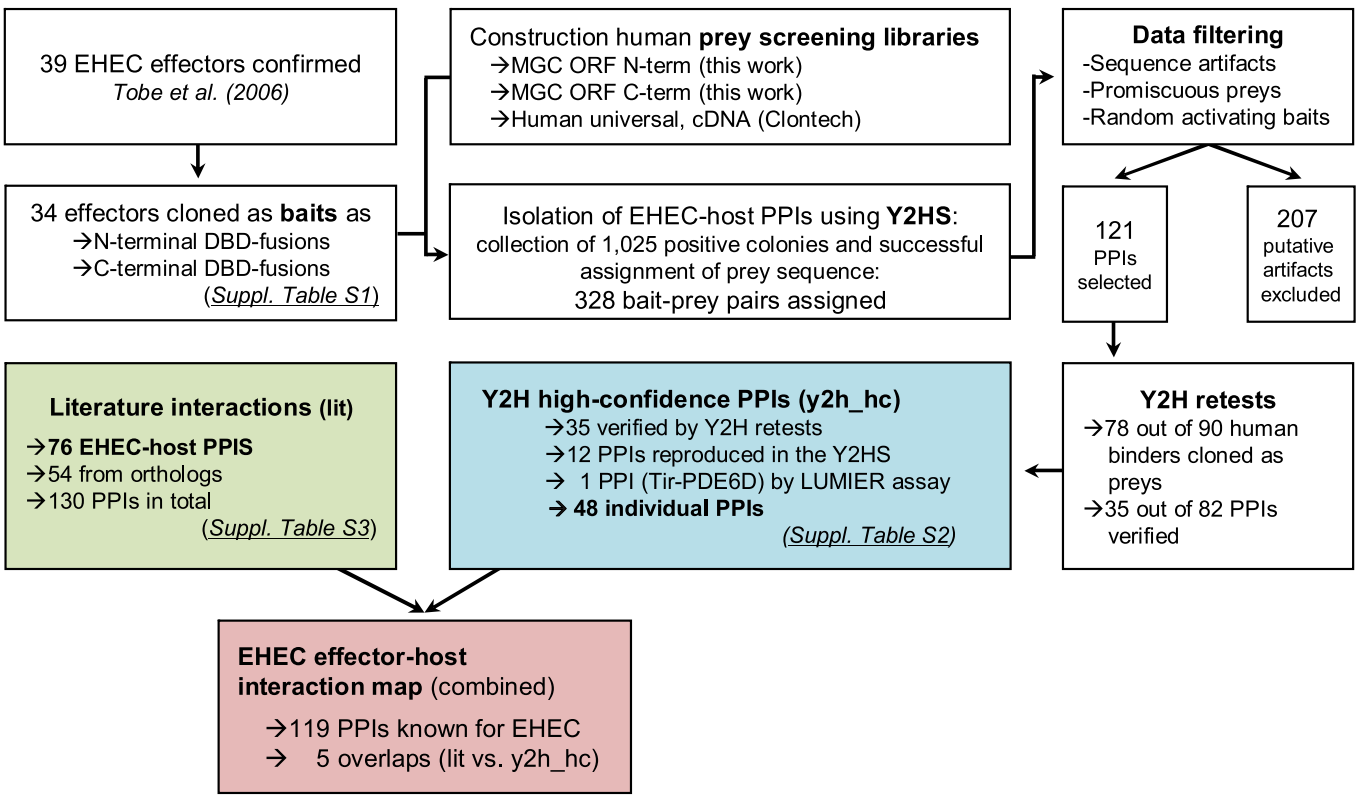

Figure 1 | Interaction screening workflow. Workflow of our procedure to find interactions between EHEC effector and human host proteins. See main text for a detailed explanation.

Table 1 | All high confidence interactions found in this study. Previously published interactions are in bold with their source citations given under "Ref"

EHEC effector		Interacting human protein			
Name	Sakai gene id	Name	GeneID	Protein names	Ref
EspB	ECs4554	RBCK1	10616	RanBP-type and C3HC4-type zinc finger-containing protein 1	
EspB	ECs4554	STK16	8576	Serine/threonine-protein kinase 16	
EspF1	ECs4550	MAD2L2	10459	Mitotic spindle assembly checkpoint protein MAD2B	
EspF1	ECs4550	SNX33	257364	Sorting nexin-33	
EspF1	**ECs4550**	**SNX9**	**51429**	**Sorting nexin-9**	35
EspG	ECs4590	"Hs.658052"	UGID:2727370	N/A	
EspG	ECs4590	NMI	9111	N-myc-interactor	
EspJ	ECs2714	CENPH	64946	Centromere protein H	
EspJ	ECs2714	IFT20	90410	Intraflagellar transport protein 20 homolog	
EspJ	ECs2714	MRFAP1L1	114932	MORF4 family-associated protein 1-like 1	
EspJ	ECs2714	RIC8A	60626	Synembryn-A	
EspO1-1	ECs1567	FEM1B	10116	Protein fem-1 homolog B	
EspY1	ECs0061	CAPN3	825	Calpain-3	
EspY1	ECs0061	CDKN2AIPNL	91368	CDKN2AIP N-terminal-like protein	
EspY1	ECs0061	CLK1	1195	Dual specificity protein kinase CLK1	
EspY1	ECs0061	DNAJC14	85406	DnaJ homolog subfamily C member 14	
EspY1	ECs0061	PCID2	55795	PCI domain-containing protein 2	
EspY1	ECs0061	PIH1D1	55011	PIH1 domain-containing protein 1	
EspY1	ECs0061	PSMC1	5700	26S protease regulatory subunit 4	
EspY1	ECs0061	ZNHIT1	10467	Zinc finger HIT domain-containing protein 1	
Map	ECs4562	RHPN1	114822	Rhophilin-1	
Map	**ECs4562**	**SLC9A3R2**	**9351**	**Na(+)/H(+) exchange regulatory cofactor NHE-RF2 (NHERF-2)**	34
NleA	ECs1812	DSCR4	10281	Down syndrome critical region protein 4	
NleA	ECs1812	FRMD3	257019	FERM domain-containing protein 3	
NleA	ECs1812	PENK	5179	Proenkephalin-A	
NleA	ECs1812	PTP4A1	7803	Protein tyrosine phosphatase type IVA 1	
NleB1	ECs3857	DRG2	1819	Developmentally-regulated GTP-binding protein 2	
NleB1	ECs3857	LRRC18	474354	Leucine-rich repeat-containing protein 18	
NleB1	ECs3857	POLR2E	5434	DNA-directed RNA polymerases I, II, and III subunit RPABC1	
NleC	ECs0847	C8orf71	26138	Putative uncharacterized protein encoded by LINC00588	
NleC	ECs0847	CTDSPL2	51496	CTD small phosphatase-like protein 2	
NleD	ECs0850	METTL2A	339175	Methyltransferase-like protein 2A	
NleF	**ECs1815**	**CASP9**	**842**	**Caspase-9**	23
NleF	ECs1815	DHFR	1719	Dihydrofolate reductase	
NleF	ECs1815	ERI3	79033	ERI1 exoribonuclease 3	
NleF	ECs1815	HMGN2	3151	Non-histone chromosomal protein HMG-17	
NleF	ECs1815	LMO4	8543	LIM domain transcription factor LMO4	
NleF	ECs1815	TRNT1	51095	CCA tRNA nucleotidyltransferase 1, mitochondrial	
NleH1-2	ECs1814	UFC1	51506	Ubiquitin-fold modifier-conjugating enzyme 1	
TccP	ECs2715	ZNF626	199777	Zinc finger protein 626	
Tir	ECs4561	ARRB1	408	Beta-arrestin-1	
Tir	**ECs4561**	**BAIAP2**	**10458**	**Brain-specific angiogenesis inhibitor 1-associated protein 2**	15
Tir	**ECs4561**	**BAIAP2L1**	**55971**	**Brain-specific angiogenesis inhibitor 1-associated protein 2-like protein 1**	14
Tir	ECs4561	HPCAL1	3241	Hippocalcin-like protein 1	
Tir	ECs4561	HPCAL4	51440	Hippocalcin-like protein 4	
Tir	ECs4561	NCALD	83988	Neurocalcin-delta	
Tir	ECs4561	PDE6D	5147	Retinal rod rhodopsin-sensitive cGMP 3',5'-cyclic phosphodiesterase subunit delta	
Tir	ECs4561	STK16	8576	Serine/threonine-protein kinase 16	

pairs were previously published (Tir-BAIAP2L1 (=IRTKS)[14], Tir-BAIAP2 (=IRSp53)[15], Map-NHERF2 (=SLC9A3R2)[34], EspF-SNX9[35] and NleF-CASP9[23]).

From the literature, EHEC-host interactions are known for 18 out of 39 effector proteins described by Tobe et al. Our screens allowed us to find PPIs that involved 15 out of 39 effectors including EspJ and EspY1 that previously were not reported to interact with the host. While cellular targets of the remaining 13 effectors, EspB, EspF, EspG, EspO, Map, NleA, NleB; NleC, NleD, NleF, NleH, TccP (EspFu) and TIR, were previously known, we determined 31 novel interactions.

A global analysis of EHEC-host interactions compared to other pathogens. To compare proteins targeted by EHEC with those of other pathogens, we compared our data to 8,297 protein interactions between proteins of *B. anthracis, F. tularensis* and *Y. pestis* and 3,363 human host proteins[36]. Furthermore, we compiled a set of 3,156 interactions between viral proteins of the Epstein-Barr, HIV,

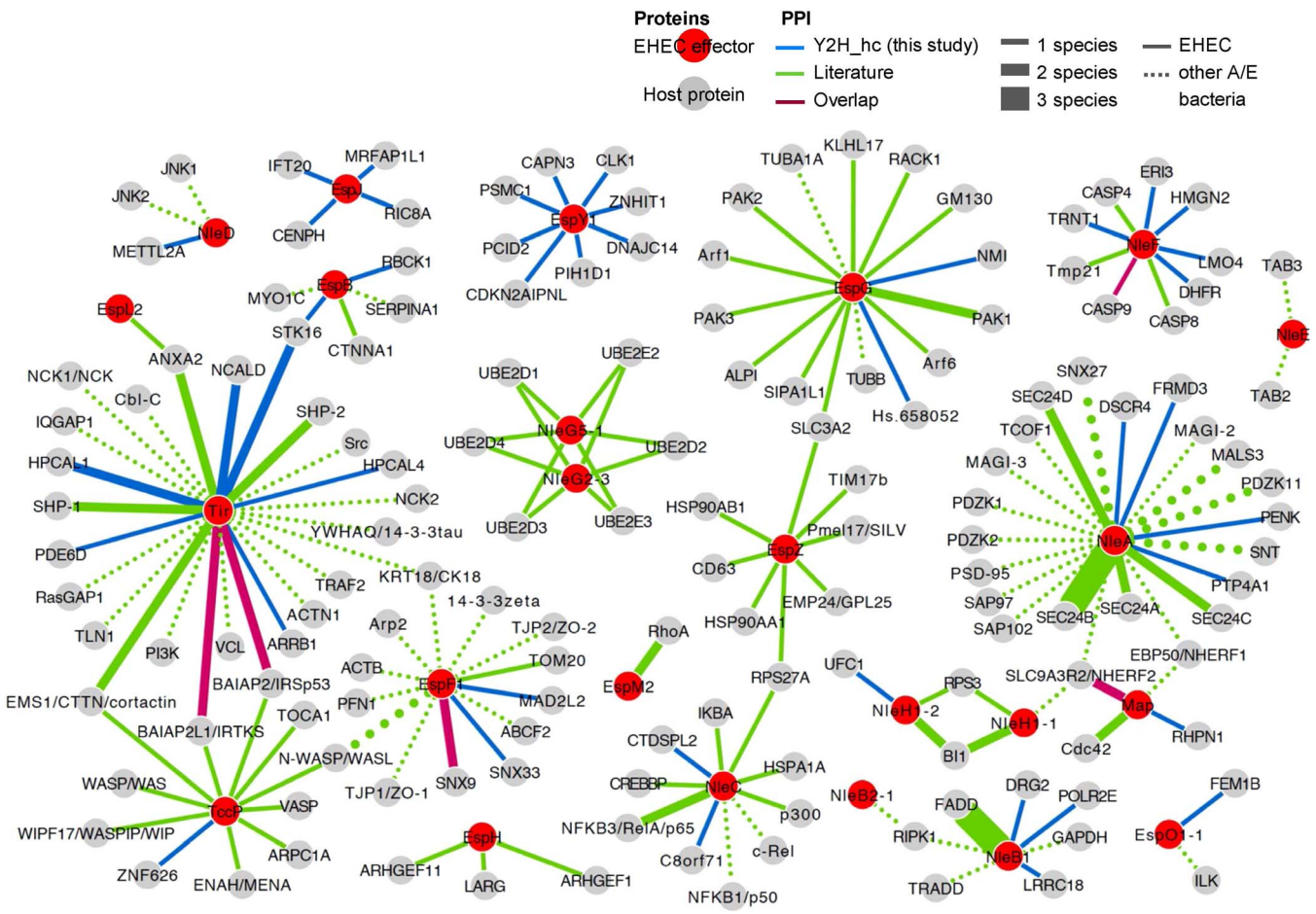

Figure 2 | EHEC effector - host protein interaction map. Combined network showing the 48 interactions detected in this study as well as 119 literature-curated interactions between EHEC effector and human host proteins. Furthermore, we added interactions between human host and effector proteins of various A/E pathogens that have orthologs with EHEC effector proteins. For a list of all interactions see Table 1 and Supplementary Table S3.

Hepatitis, Herpes, Influenza, Papilloma and Vaccinia virus and 1,778 human host proteins[37]. We find that many human host proteins were targeted by both bacterial and viral proteins (Figure 3A).

To investigate the impact on pathways, we determined the enrichment of proteins targeted by EHEC effectors in pathways that we collected from the molecular signature database[38]. We observed a significant enrichment of a small set of mostly signaling as well as infection-related pathways (Figure 3B).

In general, the majority of human target proteins interact with one effector protein, a characteristic that is shared with bacterial and viral targets (Figure 4A). Next, we wondered whether pathogen proteins interact with highly connected human proteins (hubs) or rather focus on less frequently interacting proteins in the human interactome. Figure 4B indicates that bacterial as well as viral proteins are predominately enriched in groups of highly connected host proteins. In comparison, targets of EHEC effector proteins surprisingly show a significantly stronger enrichment signal. Calculating shortest path lengths (Figure 4C) we observed that proteins targeted by EHEC effector proteins had shorter paths to other human proteins than the corresponding targets of other bacteria and of viruses (Student's t-test, $p < 10^{-20}$). A similar trend for binding to highly connected proteins is apparent in the enrichment of targeted proteins in protein complexes, as shown in Figure 4D. In comparison to bacterial and viral targets we observed that targets of EHEC effector proteins appear significantly more frequently in protein complexes.

EspY1 interacts with proteins involved in apoptosis and cell cycle regulation. While neither a function nor a human interacting protein was previously known for EspY1, we detected eight novel interactions that involved this effector protein. EspY1 appears to be primarily involved in apoptosis and cell cycle regulation as these functions are shared among its host targets calpain-3, 26S protease regulatory subunit 4, and PCI domain-containing protein 2. Such interactions may complement other EHEC effector proteins that target host proteins known to block apoptosis while assuring host cell survival. For instance, NleF interacts with caspases 4,8, and 9[23], while NleH1/NleH2 inhibit the anti-apoptotic protein Bax inhibitor-1 (BI-1[39]). Furthermore, NleD cleaves JNK1 and JNK2, potentially affecting AP-1 activity in EHEC and EPEC-infected cells[40].

EspJ and its interactions with the cytoskeleton and phagocytosis. EspJ is another poorly characterized effector that we found to interact with four proteins in our screens. Manually reviewing the function of its binding partners we found that EspJ was functionally linked to the cytoskeleton in three cases: (i) EspJ binds to the guanine nucleotide exchange factor Synembryn-A (RIC8A) that may be involved in signaling and the cytoskeleton[41] (ii) Furthermore, EspJ interacts with intraflagellar transport protein 20 homolog (IFT20) that may play a role in the trafficking of ciliary membrane proteins from the Golgi complex to the cilium[42]. (iii) EspJ also interacts with centromere protein H (CENHP), a component of the nucleosome-associated complex that plays a role in assembly of kinetochore proteins, mitotic progression and chromosome segregation[43–45].

EspJ was shown to inhibit FCYR and CR3-mediated phagocytosis (immune globulin and complement system-mediated, respectively)[46], thereby avoiding the internalization of the bacterial cell

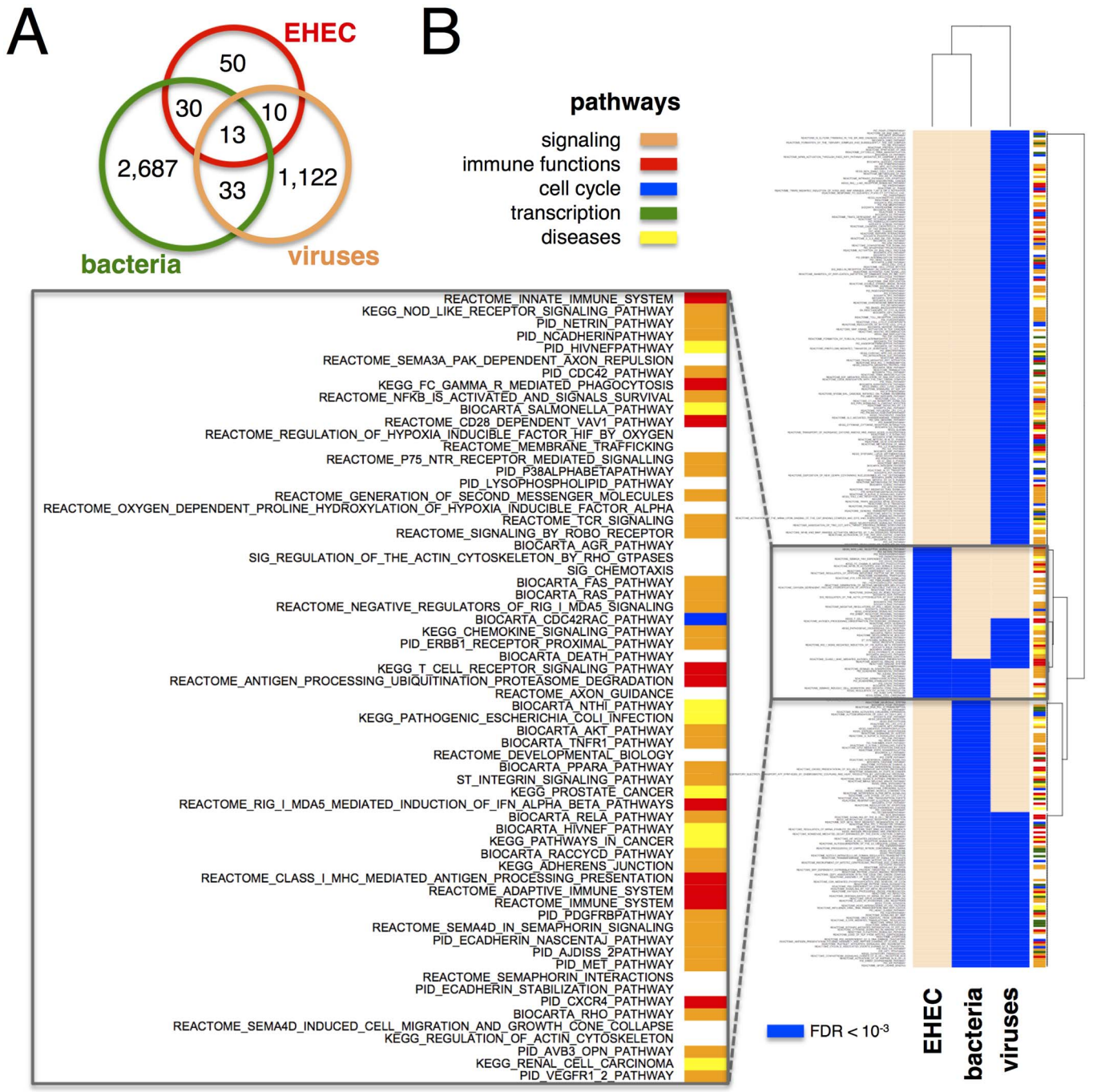

Figure 3 | **Pathway comparison of pathogen-host interaction networks.** (a) Overlap of various host-pathogen interactions. Numbers indicate host proteins targeted by the pathogen group. (b) Pathways that were significantly enriched among human EHEC targets (Fisher's exact test, FDR $< 10^{-3}$). In particular, we observed a small set of mostly signalling pathways that were significantly targeted by EHEC effector proteins.

and degradation by macrophages. However, the mechanism as well as any human binding partners that could explain these observations remained unknown. Another effector, EspH, is known to counteract FCYR phagocytosis by binding to Rho-GEFs (ARHGEF11, ARHGEF1, LARG) while blocking Rho activation through actin cytoskeletal remodeling[46]. Finally, EspF, inhibits phagocytosis probably through its actin cytoskeleton-related binding partners (actin, profilin, sorting nexin 9; reviewed in ref 7). The microtubule system is essential for optimal FCYR-mediated phagocytosis[47]. We speculate that EspJ may specifically affect the microtubule system to block phagocytosis while EspH and EspF interfere with such processes through targeting the actin cytoskeleton.

EspO1 and EspF interact with cell cycle regulators and apoptosis proteins. *EspO1-1*: The *Shigella flexneri* effector OspE (an ortholog of EspO1-1) was shown to bind to integrin-linked kinase (ILK) at focal adhesions while reinforcing host cell adherence to the basement membrane[48,49]. As for EHEC EspO1-1 we discovered another interaction with fem-1 homolog b (Fem1b), a component of an E3 ubiquitin-protein ligase complex acting as substrate recognition subunit[50]. Fem1b interacts with the checkpoint kinase CHEK1 to sense replication stress[51] and can act as a proapoptotic protein in cancer cells[52–54]. EHEC EspO1-1 may be another candidate that could act anti-apoptotically through its Fem1b interaction. In particular, targeting of the ubiquitin system by type III secreted

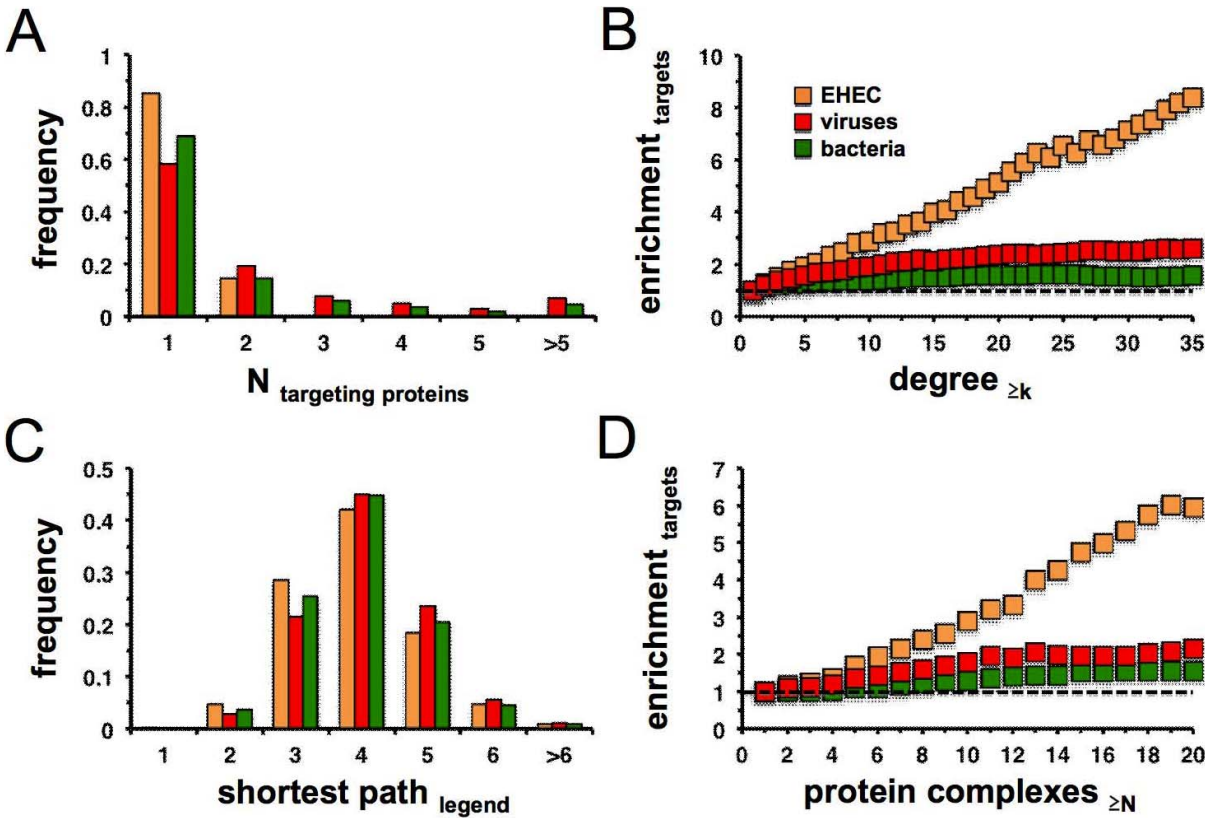

Figure 4 | Topological characteristics of EHEC effector – host protein interactions. (a) Counting the number of EHEC effector – host protein interactions, we found that the vast majority of host proteins were targeted by one EHEC effector protein. Such an observation resembles targets of viruses and bacteria. (b) We calculated the enrichment of targeted proteins as a function of their numbers of interaction partners in a human interaction network. Focusing on targets of viruses and bacteria, respectively, targeted host proteins were predominately enriched in groups of highly connected proteins. In comparison, targets of EHEC effector proteins showed a significantly reinforced enrichment signal. (c) Proteins that were targeted by EHEC effector proteins had shorter paths to other human proteins than the corresponding targets of bacteria and viruses (Student's t-test, $p < 10^{-30}$). (d) We determined the enrichment of targeted proteins as a function of their appearance in different protein complexes. In comparison to bacterial and viral targets EHEC effector proteins interact with host proteins that occurred in an increasing number of complexes.

factors is a common theme in pathogenesis of many bacteria (reviewed in ref 55).

EspF1: EspF1 is a highly multifunctional effector that localizes to mitochondria, the cytosol, nucleus, apical and lateral membrane and tight junctions[56]. EPEC EspF1 interacts with 14-3-3-zeta, ABCF2, actin, ARP2, CK-18, N-WASP, profilin, ZO-1/ZO-2 and SNX9 (sorting nexin 9). These interactions potentially allow EPEC to disrupt mitochondria, tight junctions, nucleolus, intermediate filaments, PI3K-dependent phagocytosis, and membrane remodeling. Such processes are involved in apoptosis and counteract phagocytosis[7,35,57–62]. Specifically, we found that EHEC EspF1 interacts with sorting nexin 33 (SNX33) and mitotic arrest deficient like 2 (MAD2L2) as well as with SNX9, an interaction that was previously found for EPEC and EHEC EspF. Sorting nexins are a large evolutionary conserved protein family involved in cargo sorting through the endosomal network[63]. SNX9 and SNX33 - both members of the SNX9 subfamily - are (together with SNX18) required for progression and completion of mitosis[64]. This role in cell cycle regulation is in line with EspF's binding to MAD2L2, a protein participating in the spindle assembly checkpoint[65], translesional DNA synthesis in S phase cells[66,67] and in Cdc20 homolog 1 (Cdh1) regulation[68].

Notably, MAD2L2 is also targeted by the *Shigella* effector IpaB, leading to a cell cycle arrest in the G2/M phase[69], a cellular event that is also induced during EPEC infection[70].

TIR interactions in vitro and localization in vivo. To validate the quality of our data we focused on interactions isolated for the translocated intimin receptor (TIR). Our Y2H screen found eight human proteins interacting with TIR, namely arrestin beta 1 (ARRB1), BAI1-associated protein 2 (BAIAP2), BAI1-associated protein 2-like 1 (BAIAP2L1), hippocalcin-like 1 (HPCAL1), neurocalcin delta (NCALD), serine/threonine kinase 16 (STK16), hippocalcin like 4 (HPCAL4) and phosphodiesterase 6D (PDE6D).

Interaction validation: To validate, we tested these interactions using LUMIER assays[71,72] (Figure 5A): The effector proteins were purified using a Protein A tag and co-purified binding partners fused to luciferase were detected by luminescence. This confirmed all Y2H interactions, including the interactions of TIR with BAIAP2 and BAIAP2L1 that have been previously found with EHEC and EPEC TIR[14,15].

Five interactions are conserved in EHEC and EPEC: Since the TIRs of EHEC and EPEC share 58% of their amino acid sequence[73] and carry out similar though not identical functions in these bacteria, we wondered if the isolated TIR_{EHEC} interactors would also bind TIR_{EPEC}. We used the N- and C-terminal cytosolic domains and a full-length construct of TIR to compare TIR of EHEC and EPEC (Figure 5B). We used BAIAP2 and BAIAP2L1 as positive controls as they are known to interact with both TIR_{EPEC} and TIR_{EHEC}[14,15]. HPCAL1, NCALD and STK16 interacted with TIRs from both EPEC and EHEC, representing novel conserved targets of TIR. However, we could not detect any interactions between TIR and ARRB1 and HPCAL4 in EPEC.

HPCAL1, NCALD and STK16 co-localize with EPEC infection sites in COS-7 cells. To test the *in vivo*-relevance of the conserved

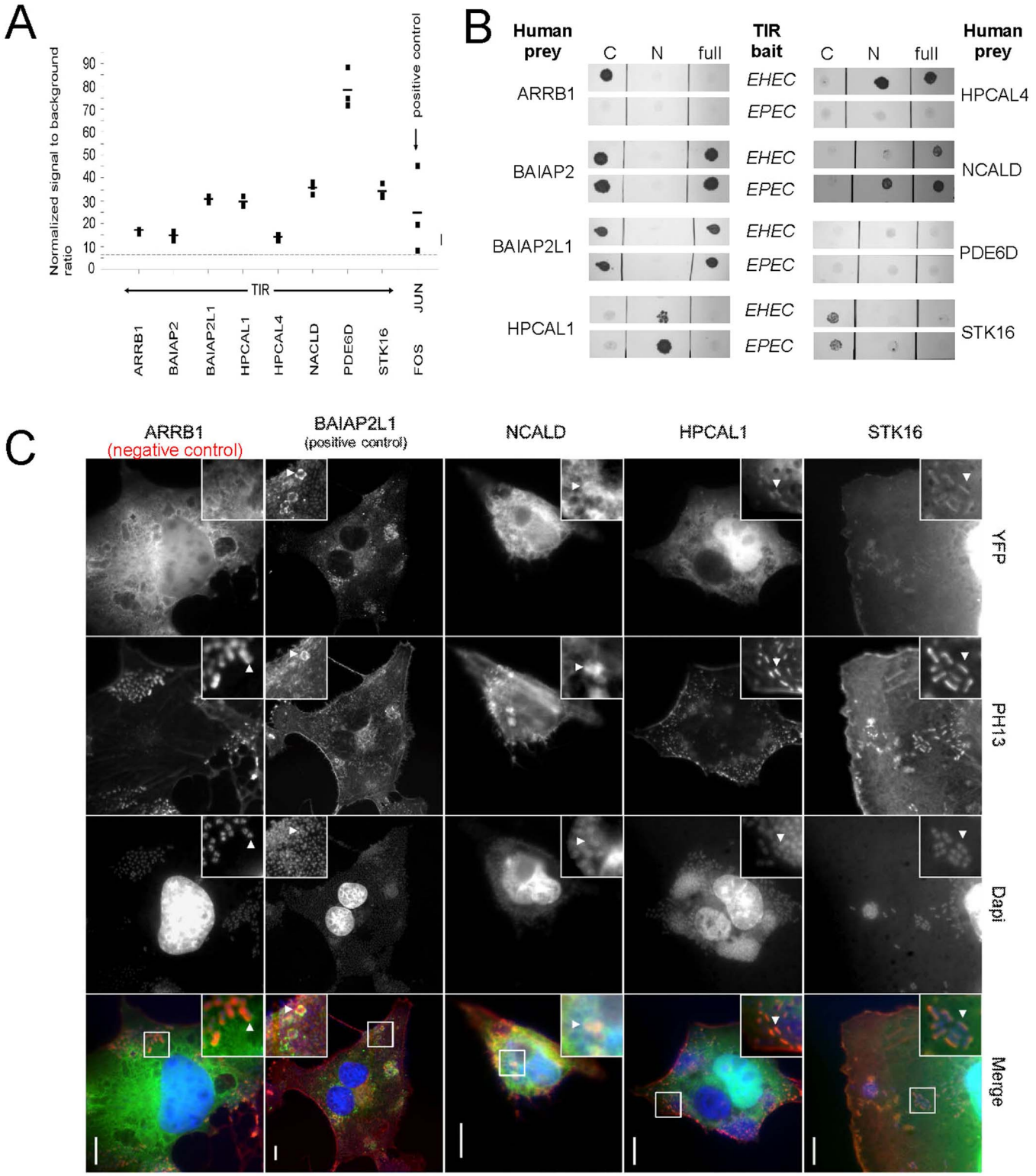

Figure 5 | Validation and conservation of TIR-host interactions. (a) TIR_{EHEC} interactions that were detected in our Y2H screens were confirmed by LUMIER assays, using full-length TIR as protein A fusion and the human test partners co-purified as luciferase-tagged fusions. Black squares represent individual measurements of the co-purified luciferase by luminescence, while their averages are shown as black horizontal bars. We considered values above the dashed threshold line (signal to background ratio >6) as a positive binding signal. As a positive control we used JUN/FOS. (b) Comparison of homologous interactions of TIR in EHEC and EPEC by pairwise Y2H tests. In particular, we probed full-length constructs (full) as well as the N- and C-terminal cytosolic domains (not shown) as baits against human preys. (c) We tested the co-localization of TIR interactors with EPEC infection sites on COS-7 cells and used BAIAP2L1 that interacts with TIR as positive control. Co-localization sites are indicated by white arrows. Specifically, we fused (YFP) human binders C-terminally to YFP and used (PH13) F-actin staining to visualize pedestals and (Dapi) DNA staining.

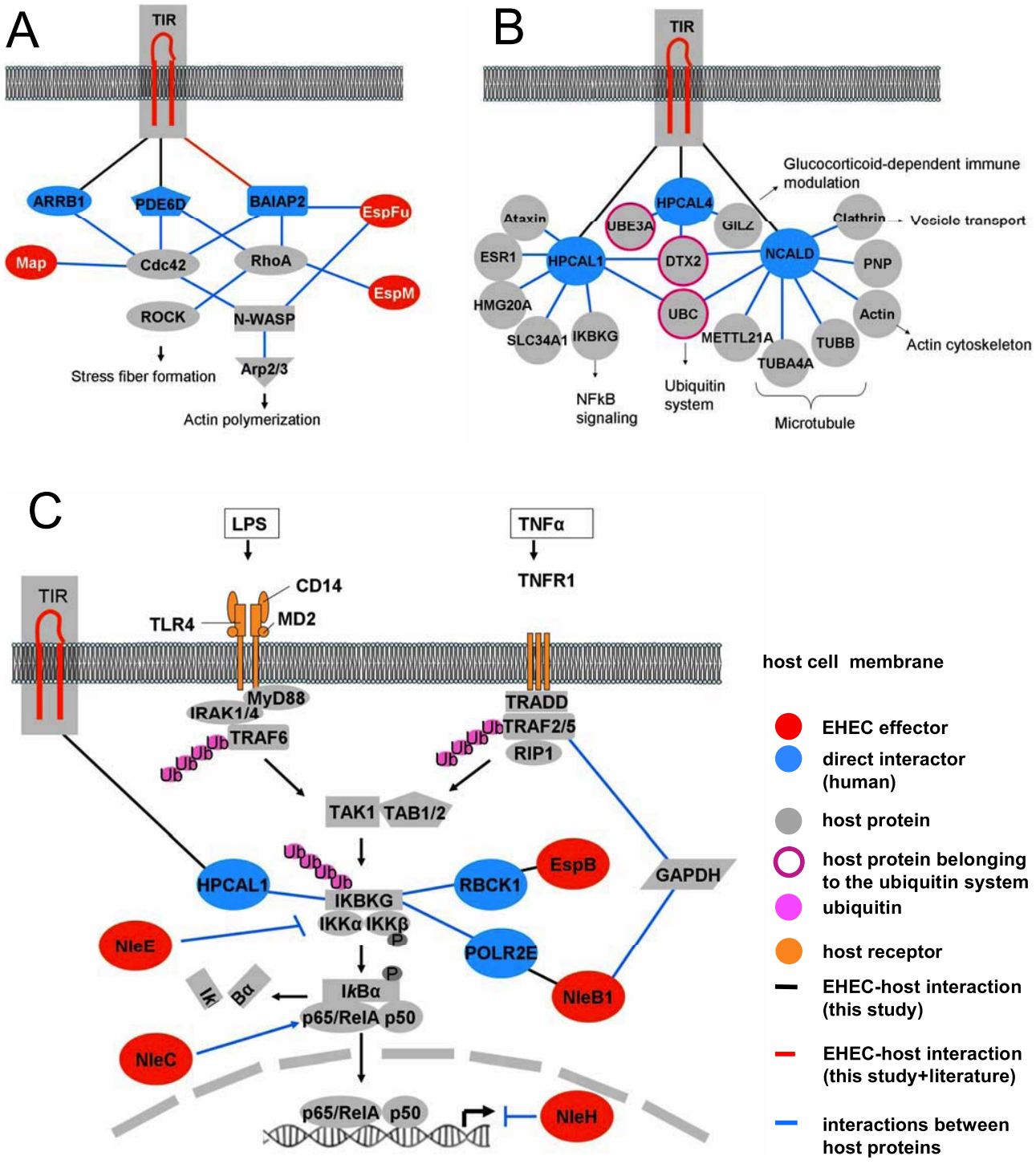

Figure 6 | Tir subnetworks. (a) TIR and other effectors are highly interconnected with human proteins. Detected, published and detected/published interactions are depicted in black, blue and red, respectively. BAIAP2L1 (not depicted) also interacts with EspFu and TIR and thus plays a homologous role as BAIAP2. (b) EHEC TIR directly targets three calcium binding proteins of the VILIP family. Possible targeted pathways are indicated. (c) IKBKG (=NEMO) is indirectly targeted by three effector proteins, TIR, EspB and NleB1. In addition, the NF-kB pathway is targeted by several effector proteins such as the metalloprotease NleC. Furthermore, NleH inhibits NF-kB signaling, and NleB1 targets glyceraldehyde 3-phosphate dehydrogenase (GAPDH) while interfering with the signaling cascade through TRAF2. For previously published interactions see Suppl. Table S3.

TIR_{EPEC} interactors we expressed the human binders as YFP-tagged fusion proteins in COS-7 cells and infected the cells with EPEC afterwards. Subsequently, we assessed co-localization of the YFP constructs with the infection sites. As expected, BAIAP2L1 (Figure 5C) and BAIAP2 (data not shown) clearly co-localized with EPEC infection sites[14,15]. As for HPCAL1, STK16 and NCALD, that interact with TIR_{EPEC}, we found that only HPCAL1 and STK16 clearly co-localized with EPEC infection sites despite stronger signals obtained for BAIAP2 and BAIAP2L1. In case of NCALD, the signal seems to be enhanced in the vicinity of EPEC infection sites, but not directly where bacteria and TIR are located. The localization is somewhat reminiscent of NHERF2 and Annexin

A2, which also localize to membrane areas surrounding the bacteria[21]. As expected, ARRB1 and HPCAL4 (not shown) do not co-localize with EPEC infection sites, as they interact specifically with TIR_{EHEC}.

In summary, we discovered another group of TIR_{EHEC} interactors, including three calcium binders of the VILIP family, namely HPCAL1, HPCAL4 and NCALD with highly similar amino acid sequences (62% identity). Although mainly known as proteins involved in neuronal signaling in the central nervous system[74,75], each gene is expressed in tissues of the digestive tract[76,77]. The interactions of TIR_{EHEC} with the three calcium binding proteins are noteworthy, since the roles of Ca^{2+} signaling during EPEC infection are contentious[78]. Some studies found evidence supporting a role of Ca^{2+} in pedestal formation[16,79,80], others did not[81]. The interactions of TIR are summarized in Figure 6, including previously published interactions.

Conclusions

In this study we determined 43 novel and 5 previously published pathogen-host interactions of EHEC effector proteins. Including data from the literature, our network comparisons showed that EHEC effectors attack the host interactome more specifically than bacteria and viruses.

The quality of the interactome was exemplarily confirmed for six novel TIR interactions by LUMIER assay. Furthermore, we provide evidence for the biological relevance of three interactions, involving NCALD, HPCAL1 and STK16, based on their recruitment to EPEC infection sites.

Notably, these three TIR interactors are small calcium binding proteins that are composed of four consecutive tandem repeated EF-hand motifs. They all belong to the superfamily of neuronal calcium sensors[82,83] and were found in TIR-dependent signaling complexes, in addition to AnxA2[21], IQGAP and calmodulin[16]. Early upon pathogen contact, microvilli effacement is induced, which was also shown to involve calcium signaling and calcium binding proteins such as calpain[84]. Calcium has been implicated in microvilli- effacement and disruption[84,85] but requires detailed further analysis for a better understanding of EHEC/EPEC pathogenicity.

Methods

Cloning. Effector ORFs of EHEC[5] were cloned from *E. coli* O157:H7 str. Sakai DNA using the Gateway® Technology (Invitrogen) (see Supplementary Table S1). Each effector was amplified in a two-step PCR using gene specific primers and the universal second primers attB1 and attB2 for the attachment of the Gateway attB sites. Primer design, PCR and cloning followed manufacturer's instructions. Resulting PCR products were shuttled into the pDONR221 entry vector via Gateway® BP reactions. Correctness of entry clones was confirmed by sequencing. Then the ORFs were shuttled into Gateway-compatible pGBT9 and pGBKCg plasmids[27,32] using an LR reaction (Invitrogen). For the LUMIER tests, TIR_{EHEC} was shuttled into pTREX-dest30-ntPrA[72]. Human ORFs of the TIR binders were shuttled from entry clones (collections[29–31]) into pcDNA3-Rluc-GW[72] and for the infection/localization assay into pEYFP-N1[86].

For the amplification of the TIR_{EHEC} amino-and carboxy-terminal fragments the gene specific primers 5′-A GGC TCC ACC ATG AAA ATA ACA AAC TAT ATA CTG CC-3′ (forw) + 5′-C TGG GTG GAT YCA ACT TTC CCT CCC GTA AAG-3′ (rev) and 5′-A GGC TCC ACC ATG GGC GGG CAG CAG TTT ATT TGG-3′ (forw) + 5′-C TGG GTG GAT YCA TTT CGA TGC ATT TAC CAT TGA G-3′ (rev) were used, respectively.

TIR_{EPEC} was cloned from *E. coli* O127:H6 str. E2348/69. The amino- and carboxy terminal regions as well as full length constructs were amplified using the gene specific primer pairs 5′-A GGC TCC ACC ATG CCT ATT GGT AAC CTT GG-3′ (forw) + 5′-C TGG GTG GAT YCA ATC GGT GGT TGT AGG ATC-3′ (rev), 5′-A GGC TCC ACC ATG GAA AGC AAT GCA CAG GCG C-3′ (forw) + 5′-C TGG GTG GAT YCA AAC GAA ACG TAC TGG TCC C-3 (rev) and 5′-A GGC TCC ACC ATG CCT ATT GGT AAC CTT GG-3′ (forw) + 5′-C TGG GTG GAT YCA AAC GAA ACG TAC TGG TCC C-3′ (rev), respectively. Depending on whether a stop is required or not Y can be either T or C (stop or no stop). The second PCR and cloning were performed as described for the EHEC effectors.

Y2H prey libraries. The Mammalian Gene Collection (MGC)[30] and the human ORF collection from the German Center for Genome Research (RZPD)[29–31] were shuttled as pools into pGAD424-GW (Clontech, Gateway-compatible variant) and pGADCg (Clontech[32]), using an LR reaction. After counter selection in *E. coli* DH10B prey plasmids were then transfected into yeast strain Y187 (Clontech). The cDNA prey library was purchased from Clontech (Human Universal, Clontech 638874).

Y2H screening. Y2H pool screening was done as described in refs 27, 28 (Figure 1).

Pairwise Y2H tests. In binary Y2H assays, a single bait and a single prey plasmid were transformed in the bait and prey yeast strains, CG-1945 and Y187, respectively (Clontech). Bait and prey were mated on YPDA plates at 30°C over night or for 3 h at 100 rpm in liquid YPDA containing 40% PEG. Diploids were harvested and grown in ΔLW medium for 2 days and then plated on selective agar plates (ΔLWH). To adjust stringency, various concentrations ranging from 0 to 150 mM of 3-amino-1, 2, 4-triazole were added to the selective agar plates. To check for auto-activation all baits were tested against Y187 harboring empty prey vector (negative control). Details can be found in ref 87.

LUMIER assay. Luciferase based pull down assays were performed as described in ref 23.

Infection assay. Transfection of the YFP constructs, infection assays, and image analysis was performed as described in ref 15.

Computational analysis. *Human protein interactions.* We utilized a total of 28,627 high quality protein interactions between 8,495 human proteins from the HINT database[88], accounting for both binary and co-complex interactions.

Human canonical pathway information. As a source of canonical pathway information, we used 1,320 pathways from the Molecular Signatures Database[38] that were largely obtained from the NCI Pathway Interaction Database, Reactome and Biocarta.

Bacteria-host and Virus-host protein interactions. As for Bacteria we utilized 2,989 interactions of *B. anthracis*, 1,347 interactions of *F. tularensis* and 3,961 interactions of *Y. pestis* with human host proteins[36]. Utilizing the IntAct database[37], we collected 330 interactions of the Epstein-Barr virus, 557 interactions of the HIV virus, 766 interactions of the Hepatitis virus, 332 interactions of the Herpes virus, 756 interactions of the Influenza virus, 238 interactions from the Papilloma virus and 177 interactions of the Vaccinia virus with human host proteins.

Human protein complexes. We utilized 1,843 protein complexes in *H. sapiens* from the CORUM database[89], that collects information about experimentally determined protein complexes from the literature.

Enrichment analysis as a function of degree. We grouped human proteins according to their number of interactions in an underlying human protein interaction network. We represented each group by $N_{\geq k}$ proteins that had at least k interactions and calculated the number of targeted proteins i, $N_{i,\geq k}$ in each group. Randomly picking targeted genes we defined $E_{i,\geq k} = \frac{N_{i,k}}{N^r_{i,k}}$ as their enrichment where $N^r_{i,\geq k}$ was the corresponding random number of targeted proteins among all $N_{i,\geq k}$ proteins. After averaging E_i over 10,000 randomizations $E_i > 1$ pointed to an enrichment and *vice versa*, while $E_i \sim 1$ indicated a random process[90].

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Author contributions

The experiments were performed by S.B., S.A., G.S and M.A.S. Experiments were designed and conceived by S.B., M.K., P.U., T.S. and P.A. Data was analyzed by S.B., R.H., A.C., S.W. and F.S. The paper was written by S.B., R.H., P.U., S.W. and M.K.

Additional information

Note The protein interactions from this publication have been submitted to the IMEx (http://www.imexconsortium.org) consortium through the IntAct database[37] and assigned the identifier IM-23549.

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