A bacterial type III secretion-based protein delivery tool for broad applications in cell biology

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Methods enabling the delivery of proteins into eukaryotic cells are essential to address protein functions. Here we propose broad applications to cell biology for a protein delivery tool based on bacterial type III secretion (T3S). We show that bacterial, viral, and human proteins, fused to the N-terminal fragment of the *Yersinia enterocolitica* T3S substrate YopE, are effectively delivered into target cells in a fast and controllable manner via the injectosome of extracellular bacteria. This method enables functional interaction studies by the simultaneous injection of multiple proteins and allows the targeting of proteins to different subcellular locations by use of nanobody-fusion proteins. After delivery, proteins can be freed from the YopE fragment by a T3S-translocated viral protease or fusion to ubiquitin and cleavage by endogenous ubiquitin proteases. Finally, we show that this delivery tool is suitable to inject proteins in living animals and combine it with phosphoproteomics to characterize the systems-level impact of proapoptotic human truncated BID on the cellular network.

Introduction

In cell biology, protein function is addressed by various methods including cDNA transfection, microinjection, and proteofection of purified proteins. Although these methods are informative, they often result in massive overrepresentation of the protein of interest and/or highly heterogeneous cell populations, making functional dynamics studies and -omics approaches difficult to interpret. They can also be costly when used on a large scale.

Bacteria have developed sophisticated nanomachines enabling the delivery of virulence proteins into eukaryotic cells (translocation). The type III secretion (T3S) system of certain gram-negative bacteria functions like a nanosyringe that injects substrate proteins into target cells (Cornelis and Wolf-Watz, 1997; Fig. 1 a). Delivered proteins harbor a short N-terminal secretion signal (Michiels et al., 1990). In bacteria, they bind to chaperones that stabilize them, prevent premature interactions, and favor secretion (Wattiaux and Cornelis, 1993; Gauthier and Finlay, 2003). An ATPase associated with the base of the T3S apparatus participates in directing substrates to be secreted into a thin needle-like structure. These proteins travel unfolded or only partially folded (Feldman et al., 2002) and subsequently refold in the host cell, where they exert their virulence activity toward various host proteins and cellular machineries. Over 100 different effector proteins are known (Mota and Cornelis, 2005), displaying a large repertoire of biochemical activities that modulate the functions of host regulatory molecules.

On a few occasions, immunologists and infection biologists have exploited T3S to deliver hybrid peptides and proteins into target cells. Viral and bacterial epitopes (Sory et al., 1992; Van Damme et al., 1992; Rüssmann et al., 1998, 2003; Chen et al., 2006) as well as peptides from human tumors (Chaux et al., 1992; Rüssmann et al., 1998, 2003; Chen et al., 2006) have been delivered by T3S with the aim of vaccination. *Bordetella pertussis* adenylate cyclase (Sory and Cornelis, 1994), murine DHFR (Feldman et al., 2002), or a phosphorylatable tag (Garica et al., 2006) were used as reporters of translocation to identify the secretion signal requirements for T3S. More recently, an elegant *Yersinia enterocolitica*-based delivery toolbox was developed to investigate Rho-GTPase affecting T3S effectors from *Shigella flexneri* and *Escherichia coli* (Wölke et al., 2011). Functional nanobodies (Blanco-Toribio

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et al., 2010) or nuclear proteins as cre-recombinase and MyoD (Bichsel et al., 2011, 2013) were also delivered inside target cells in vitro, whereas an S. flexneri–based T3S was used to release IL-10 and IL-1 receptor antagonists in vivo (Chamekh et al., 2008). Although these studies were successful, T3S-based protein delivery has never been shown to be applicable to a broad range of eukaryotic cargos, and its utilization in cell biology therefore remained limited.

Here we optimize the delivery of heterologous proteins into target cells via T3S and propose protocols manageable by noninfection biologists. We show that the delivery of bacterial effectors proteins as well as viral and eukaryotic proteins fused to the N-terminal fragment of YopE, a Y. enterocolitica effector with Rho GTPase activating protein (GAP) activity (Von Pawel-Ramlingen et al., 2000). YopE fragment by T3S-translocated tobacco etch virus (TEV) protease or by an ubiquitin-dependent mechanism. Finally, we show that this delivery system is suitable to inject functional eukaryotic proteins in living animals, and that it can be combined with phosphoproteomics to gain new biological insights into the mechanism of apoptosis.

Results

A protein delivery method based on T3S of YopE fusion proteins

We took advantage of Y. enterocolitica-Ysc-T3S to develop an infection protocol that enables effective secretion and delivery of proteins into eukaryotic cells (Fig. 1 a). This method is based on the delivery of proteins fused to the N-terminal fragment of YopE, a Y. enterocolitica effector with Rho GTPase activating protein (GAP) activity (Von Pawel-Ramlingen et al., 2000).
First, the translocation of endogenous effectors was abolished by using a strain deleted for all known effectors named YopH, O, P, E, M, and T (ΔHOPEMT; Iriarte and Cornelis, 1998; Fig. S1 a). Furthermore, this strain was deleted for the aspartate-β-semialdehyde dehydrogenase gene (Δasad) to make it unable to grow in absence of exogenous meso-2,6-diaminopimelic acid (Hoang et al., 1997; Kudryashev et al., 2013) and thereby compatible with biosafety level 1 (Fig. S1 a). The two cloning plasmids, pBad_Si_1 and pBad_Si_2, were optimized for high protein secretion and to facilitate cloning (Fig. S1 b). The N-terminal 138-amino-acid fragment of YopE (YopE_{1–138}), which contains a T3S signal (Feldman et al., 2002) and a chaperone-binding site for the chaperone SycE (Boyd et al., 2000b), is optimal for heterologous T3S substrate translocation (Wölke et al., 2011). Therefore, it was systematically fused to proteins of interest, and SycE was coexpressed. Notably, the Rho-GAP domain of YopE is not in the YopE_{1–138} fragment. The SycE-YopE_{1–138} DNA sequence containing the endogenous promoters of YopE and SycE was cloned from the purified pYV virulence plasmid from Y. enterocolitica (pYV40; Sory et al., 1995; Fig. S1 b). The production of SycE and all YopE_{1–138} fusion proteins was then induced by a rapid temperature shift from growth at room temperature to 37°C (Fig. S2 a). Cloning was further facilitated by adding a multiple cloning site at the 3’ end of YopE_{1–138}, followed by a Myc tag, a 6×His tag and a stop codon (Fig. S1 c).

Protein secretion was first addressed by monitoring the secretion of full-length YopE and YopE_{1–138}-Myc in an in vitro secretion assay (Fig. 1 a and Fig. S2 a). Secretion, artificially induced by a temperature shift to 37°C and deprivation of calcium, was monitored by precipitating proteins from the supernatant and performing a Western blot analysis with an an- ti-YopE antibody (Fig. 1 b). As expected, YopE was secreted via the MOI. As reported for YopE (Isaksson et al., 2009), the production of SycE and all YopE_{1–138} fusion proteins was then induced by a rapid temperature shift from growth at room temperature to 37°C (Fig. S2 a). Cloning was further facilitated by adding a multiple cloning site at the 3’ end of YopE_{1–138}, followed by a Myc tag, a 6×His tag and a stop codon (Fig. S1 c).

Protein translocation into eukaryotic cells was monitored by immunofluorescence staining of Myc after infection of HeLa cells by the strain encoding YopE_{1–138}-Myc. At 2 min, Myc was only detected in bacteria (Fig. 1 c). In contrast, almost all cells were Myc positive at 35 or 60 min. Intensity was homogeneous within the cell population and increasing over time, showing that YopE_{1–138}-Myc accumulated in cells (Fig. 1 c and Fig. S2 b). Interestingly, low MOIs led to weaker levels of protein translocation, but the number of recipient cells was still nearly maximal (Fig. 1 d and Fig. S2 c), indicating that protein levels in target cells was controllable via the MOI. As reported for YopE (Isaksson et al., 2009), YopE_{1–138}-Myc was excluded from the nuclei (Fig. 1 d). Furthermore, in agreement with data showing that Y. enterocolitica can infect different cell types (Boyd et al., 2000a), YopE_{1–138}-Myc was delivered into murine fibroblasts, Jurkat T-lymphocytes, and primary human umbilical vein endothelial cells (Fig. S3 a). Altogether, these data indicated that YopE_{1–138}-Myc is effectively secreted and delivered into cells and that Y. enterocolitica–based delivery is fast, homogenous, and finely controllable.

### Translocation of type III and type IV bacterial effectors

We tested whether translocated heterologous T3S effectors fused to YopE_{1–138} were functionally active in cells. We first monitored the activity of OspF, an S. flexneri effector harboring a phosphothreonine lyase activity that dephosphorylates MAP kinases p38 and ERK (Li et al., 2007). TNF-induced p38 phosphorylation present in uninfected cells or cells infected with YopE_{1–138}-Myc-expressing bacteria was abolished in cells infected by YopE_{1–138}–OspF–expressing bacteria, showing that delivered OspF was fully functional (Fig. 2 a). We also confirmed the effect of translocated YopE_{1–138}-SopB on Akt activation. SopB is a Salmonella enterica effector that protects epithelial cells from apoptosis by inducing a sustained activation of Akt (Norris et al., 1998). Our results show that Akt was activated by YopE_{1–138}–SopB, whereas the translocation of YopE_{1–138}-Myc or YopE_{1–138}-SopE had no effect (Fig. 2 b).

Multiple T3S effectors also target the actin cytoskeleton. The S. enterica SopE effector is a guanine nucleotide exchange factor that interacts with Cdc42, promoting actin cytoskeletal remodeling (Hardt et al., 1998). Although the translocation of YopE_{1–138}-Myc into HeLa cells had no impact, infection with the YopE_{1–138}–SopE–expressing strain induced dramatic changes in the F-actin network (Figs. 2 c and S2 d). These changes were strictly dependent on T3S, as infection by a YopB deletion mutant, unable to form translocation pores in the eukaryotic cell membrane (Håkansson et al., 1996; Fig. S4), had no effect on actin (Figs. 2 c, S2 d, and S3 b). YopE_{1–138}-SopE–induced F-actin foci were detected around 60 s postinfection (p.i.) in most cells (Figs. 2 c and S3 b), whereas at later time points, a characteristic SopE–induced F-actin meshwork (Hardt et al., 1998) was found (Figs. 3 b and S2 d).

Several bacteria use type IV secretion to inject proteins into cells. As recently proposed for a Legionella effector (Rothmeier et al., 2013), we tested whether type IV effectors could also be delivered by our Y. enterocolitica–based protein delivery. The Bartonella henselae full-length BepA, BepA_{305-end}, containing the C-terminal Bid domain, and the Bid domain of BepG, another type IV effector used as control, were cloned into pBad_Si_2. As BepA induces the production of CAMP (Pulliainen et al., 2012), the level of CAMP was measured after infection. Although the translocation of the Bid domain of BepG failed to induce CAMP, full-length BepA and BepA_{305-end} triggered CAMP production in expected amounts (Pulliainen et al., 2012; Fig. 2 d), showing that type IV effectors fused to YopE_{1–138} were effectively translocated and active. Altogether, our results demonstrate that Y. enterocolitica–based protein delivery is suitable for type III and IV effectors.

To explore additional applications in cell biology, we tested whether our tool of T3S-based protein delivery was suitable to address functional interactions of multiple T3S-delivered proteins in cells. We took advantage of the antagonistic activities of the effectors SopE and SptP on the actin cytoskeleton (Stebbins and Galán, 2001). During S. enterica infection, SopE translocation is followed by the translocation of SptP, which then functions as a GAP for Cdc42. Whereas YopE_{1–138}–SopE–Myc alone triggered massive F-actin rearrangements, coinfection with YopE_{1–138}–SptP–expressing bacteria abolished this effect in a dose-dependent manner (Fig. 3, a and b). An anti-Myc staining showed that this inhibition was not caused by a reduced level of translocated YopE_{1–138}–SopE–Myc (Fig. 3, a and c). This result shows that coinfected cells with at least two Y. enterocolitica strains can be used to address functional interactions between translocated proteins.
Translocation of eukaryotic proteins into epithelial cells

To extend the use of *Y. enterocolitica*–based protein delivery to eukaryotic cargos, we monitored the secretion of proteins including cell-cycle proteins (Mad2, CDK1, CDKN2A/INK4A, CDKN2B/INK4B, and CDKN2C/INK4C), apoptosis-related proteins (Bad, FADD, caspase-3 [CASP] p17 and p12, zebrafish BID, and zebrafish t-BID), signaling proteins (TRAF6, TIFA, and the GPCR Go subunit GNA12), an anti-GFP nanobody (VHHGFP4), and a VHHGFP4 fusion construct for targeted protein degradation (Caussinus et al., 2011; Slmb-VHHGFP4; Fig. S5). Although Western blot analysis revealed differences in efficiency, all proteins were secreted by correspondingly engineered bacteria, suggesting that this method allows considerable variability in the size and structure of secreted proteins.

Next, we examined the functional consequences of the translocation of human BH3 interacting-domain death agonist (BID) into HeLa cells. BID is a proapoptotic member of the Bcl-2 protein...
family and a mediator of mitochondrial damage induced by caspase-8. Caspase-8 cleaves BID, and truncated BID (tBID) translocates to mitochondria, where it triggers cytochrome c release (Li et al., 1998). The latter leads to CASP3 activation by cleavage into the 17- and 12-kD subunits. Data showed that translocated YopE1–138-tBID triggered CASP3 (Fig. 4 a) and PARP (Fig. 4 c) cleavage, CASP3 activation (Fig. 4 d) and massive cell death (Fig. 4, e–g). Effects were even more pronounced than after treatment with the apoptosis inducer staurosporine (Fig.4, a and e), strictly dependent on YopB (Fig. 4, c, d, f, and g) and blocked by the pan-caspase inhibitor z-VAD (Fig. 4, d and f). To compare the amounts of translocated proteins to endogenous BID, HeLa cells were lysed with digitonin, which does not lyse bacteria (Lee et al., 1998; Fig. S4, a and c), and analyzed using an anti-BID antibody (Fig. 4 b). Translocated YopE1–138-BID reached the level of endogenous BID, whereas delivered YopE1–138-BID was present in even higher amount (2.5-fold). Altogether, these results show that Y. enterocolitica-based T3S can deliver functional eukaryotic proteins into cells, opening entirely new perspectives for this method in cell biology.

Delivery of fluorescent proteins, nuclear relocalization, and nanobody-dependent targeting of fusion proteins

The delivery of fluorescent proteins can be used to monitor T3S-mediated protein transport or to determine the subcellular localization of translocated fusion proteins. Unfortunately, GFP is a notoriously poor T3S substrate (Jacobi et al., 1998), and use of split GFP was proposed instead (Van Engelenburg and Palmer, 2010). Recently, mCherry fused to an NLS was delivered successfully by a plant pathogenic bacterium to plant cells (Sharma et al., 2013). First, we addressed the ability of the YopE1–138-based tool to deliver GFP into cells. Infection of HeLa cells with YopE1–138-GFP–expressing bacteria revealed a weak cytosolic staining, indicating that GFP proteins were secreted into target cells (Fig. 5 a). To extend these results, we concentrated fluorescent signals into nuclei by adding an NLS (SV40 large T-antigen) to the fusion protein. As predicted, a strong nuclear relocalization of GFP was observed, confirming that YopE-based T3S enables some level of GFP delivery into
cells and that an NLS sequence is efficient to redirect proteins to the nucleus (Figs. 5 a and S3 c). As expected, a ΔyopB mutant failed to inject GFP into cells. For mCherry, protein translocation was also observed, and addition of an NLS enhanced nuclear localization of the fusion protein (Figs. 5 b and S3 d). Altogether, these results show a weak but reproducible translocation of GFP, document successful delivery of mCherry via T3S by a human pathogen in mammalian cells, and confirm the ability of an NLS to relocate proteins to the nucleus.

Besides conventional antibodies, camelids produce antibodies lacking light chains (Hamers-Casterman et al., 1993). The N-terminal antigen-binding domain of these heavy-chain antibodies is known as a nanobody or VHH. Nanobodies, which have successfully been delivered by E. coli via T3S (Blanco-Toribio et al., 2010), constitute a valuable tool to target specific intracellular proteins. We took advantage of a nanobody recognizing EGFP, VHHGFP4 (Saerens et al., 2005), to direct translocation of YopE1–138-VHHGFP4-Myc to the intracellular localization of EGFP, histone 2B-GFP (H2B-GFP; Silljé et al., 2006), and EGFP-Rab2a, stably expressed in different HeLa cell lines. In absence of EGFP, translocated YopE1–138-VHHGFP4-Myc (Fig. 6 a) was detected in the cytoplasm, reflecting the distribution of YopE1–138-Myc (Fig. 1, c and d). In EGFP-expressing cells, the nanobody fusion protein was mostly localized to the cytoplasm, suggesting that the presence of YopE may slightly prevent nuclear localization in some cases. In contrast, in cells expressing H2B-GFP, translocated YopE1–138-VHHGFP4-Myc was exclusively found in nuclei. Interestingly, VHHGFP4-Myc
failed to localize to the nuclei of HeLa cells that did not express H2B-GFP (marked with arrows in Fig. 6 a), attesting the specificity of the interactions. In these cells, a weaker cytosolic staining was observed instead. These results show consistent colocalization between the nanobody fusion protein and the EGFP constructs and illustrate that intracellular targeting of proteins can be achieved by translocated nanobodies. Finally, in an EGFP-Rab2a–expressing cell line, translocated YopE1–138-VHHGFP4-Myc colocalized with EGFP-Rab2a on perinuclear structures (Fig. 6, a and b). Altogether, these data indicate that nanobody fusion proteins can be functionally delivered into eukaryotic cells by T3S and that a fusion partner can be targeted to different subcellular localizations by a specific nanobody.

Intracellular cleavage of the YopE1–138 fragment from T3S-delivered proteins. As the YopE1–138 N-terminal fusion part might prevent some fusion proteins from being fully functional, we implemented the option of cleaving off the YopE1–138 fragment into our Y. enterocolitica–based tool. First, we took advantage of the TEV protease activity, which cleaves proteins at the consensus site ENLYFQS (Carrington and Dougherty, 1988). Two TEV cleavage sites were introduced between YopE 1–138 and two proteins of interest (Fig. 7 a), namely, INK4C and the transcription regulator ET1 (Weber et al., 2002). In addition, the S219V variant of the TEV protease (Kapust et al., 2001), which is resistant to autoinactivation, was fused to YopE1–138, and cells were coinfected with the YopE1–138-proteinX and YopE1–138-TEV–expressing strains. To exclusively lyse human cells and thus restrict analysis to translocated proteins, infected HeLa cells were lysed with digitonin (Fig. S4 c). Western blot analysis with anti-INK4C or anti-Myc antibodies revealed that the YopE1–138-2xTEV-cleavage-sites-Flag-INK4C-MycHis and YopE1–138-2xTEV-cleavage-sites-ET1-Myc were both effectively translocated into cells (Fig. 7, c and e). Remarkably, when cells were coinfected with YopE1–138-TEV–expressing bacteria, the intensity of the band corresponding to the full-length fusion protein was decreased, whereas a second band consistent with the protein of interest plus the N-terminal remnants of the TEV cleavage site (likely one serine) was visible (Fig. 7, c and e). In the case of the YopE1–138-2xTEV-cleavage-sites-Flag-INK4C-MycHis fusion protein, the presence of an additional weaker band was observed (Fig. 7 c). The size of the corresponding protein was compatible with cleavage at the site being closer to the YopE fragment, resulting in S-ENLYFQS-Flag-INK4C-MycHis. A similar pattern was also found when cell lysates were directly incubated overnight with...
purified TEV protease, indicative of TEV-mediated cleavage in injected cells. Although cleavage was not complete, it occurred already 2 h p.i. Furthermore, overnight digestion with purified TEV protease failed to provide a better yield (Fig. 7 c–e). Altogether, these results demonstrate that coinfections with YopE1–138-TEV–expressing bacteria enable the intracellular cleavage of YopE 1–138, and thereby the release of nearly native proteins after T3S.

An alternative approach consisted in incorporating ubiquitin into the fusion protein of interest. Indeed, ubiquitin is processed at its C terminus by a group of endogenous ubiquitin-specific C-terminal proteases (deubiquitinating enzymes, DUBs) and was previously used to monitor cytosolic uptake of proteins fused to transduction domains (Loison et al., 2005). It was shown that C-terminal ubiquitin fusion proteins were freed from ubiquitin upon microinjection into eukaryotic cells (Inomata et al., 2009). This method was tested on the YopE1–138-ubiquitin-Flag-INK4C-MycHis fusion protein. In control cells infected by YopE1–138-Flag-INK4C-MycHis–expressing bacteria, a band corresponding to YopE1–138-Flag-INK4C-MycHis was found, indicative of efficient translocation of the protein (Fig. 7 f). When cells were infected for 1 h with YopE1–138-ubiquitin-Flag-INK4C-MycHis–expressing bacteria, an additional band corresponding to the size of Flag-INK4C-MycHis was visible, indicating that part of the fusion protein was cleaved. This result shows that the introduction of ubiquitin into the fusion protein enables to cleave off the YopE1–138 fragment without a need for an exogenous protease.

**In vivo translocation of z-BIM in zebrafish embryos induces apoptosis.**

An interesting feature of T3S-based protein delivery is its potential use in living animals. We directly tested this opportunity by infecting zebrafish embryos. As apoptosis is an important cellular process that can be easily visualized in vivo, we investigated the impact of the delivery of z-BIM, the best-characterized...
apoptosis inducer in zebrafish (Jette et al., 2008). Although z-BIM has low sequence similarity to human BIM, we found that HeLa cells infected with YopE1–138-z-BIM–expressing bacteria showed a clear reduction of cell number and cell size as well as nuclear condensation (Fig. 8, a and b). In vivo infections of zebrafish embryos were then performed by microinjecting EGFP and YopE1–138-Myc or EGFP and YopE1–138-z-BIM–expressing bacteria into the hindbrain (Benard et al., 2012). Embryos were fixed 5.5 h p.i., permeabilized, and stained for CASP3 p17. Upon infection with the control strain, bacteria were visible in the hindbrain region (Fig. 8 c), but no induction of apoptosis was detected. In contrast, YopE1–138-z-BIM–delivering bacteria induced a strong increase in cleaved CASP3. This observation was confirmed by automated image analysis (Fig. 8 d). This result indicates that translocated YopE1–138-z-BIM is functional in developing zebrafish and validates the use of our T3S tool to deliver eukaryotic proteins into living animals.

Phosphoproteomics reveals the global impact of tBID on protein phosphorylation
T3S-based protein delivery was used to gain new biological insights into the mechanism of apoptosis triggered by tBID. Although tBID is a central player of apoptosis, the systems-level impact of this factor on cellular signaling remains poorly characterized. As phosphorylation is a widespread posttranslational modification, we used a label-free approach of liquid chromatography tandem mass spectrometry (LC-MS/MS)–based phosphoproteomics to determine the impact of translocated tBID on cellular signaling in HeLa cells. The process of Y. enterocolitica infection itself may lead to protein phosphorylation (Schulte et al., 2000; Uliczka et al., 2009).
Therefore, we first determined the background phosphorylation changes triggered by YopE1–138-Myc–encoding bacteria compared with uninfected cells. Phosphopeptides were enriched, quantified, and identified as previously described in Schmutz et al. (2013) and in Materials and methods. A volcano plot representing q-values and phosphorylation ratios between infected and uninfected cells showed that control infection had a significant impact on phosphorylation (Fig. 9 a, left). As this background phosphorylation may interfere with tBID-induced phosphorylation changes, we minimized it by making use of two mutants deleted for the adhesion molecules invasin A (InvA) or YadA, which are known to trigger host signaling (Uliczka et al., 2009). As expected, infection by the ΔHOPEMT asd yadA–deleted strain (encoding Yop1–138-Myc) triggered fewer phosphorylation changes than ΔHOPEMT asd Yop1–138-Myc–delivering bacteria (Fig. 9 a, middle). Interestingly, even fewer changes were observed upon infection with the invA-deleted strain (no more changes with q < 0.04; Fig. 9 a, right), showing that the latter was optimal for system-wide signaling studies. Although translocation efficiency was reduced in absence of InvA (Fig. 9 b), tBID delivery was still able to induce massive cell death (Fig. S3 e). Phosphoproteomics was then performed to measure the impact of tBID delivered by the invA-deleted strain on HeLa cells. Data revealed 240 tBID-dependent phosphorylation changes (q-values < 0.04); 218 phosphopeptides underwent an increase in phosphorylation whereas 22 were less phosphorylated after tBID delivery (Table S4). These phosphopeptides corresponded to 187 different phosphoproteins that we defined as the tBID phosphoproteome. The STRING database was combined with Gene Ontology analysis (Huang da et al., 2008) to create a functional protein-protein interaction network of the tBID phosphoproteome (Fig. 10 a and Table S5). Data showed that phosphorylation of proteins involved in diverse cellular processes are affected by tBID. These include the cytoskeleton (i.e., SPTN2, SPTB2, and FLNB), small GTPase signaling (i.e., DOCK1, DOCK9, and ARHG2), RNA processing (i.e., SF3B2 and SRRM1), and protein synthesis (i.e., IF4B, IF3A, and IF4G1). Interestingly, 12 known CASP3 substrates and 58 putative ones predicted by an in silico tool based on a position-specific scoring matrix approach (Ayyash et al., 2012) were identified in the phosphoproteome of tBID (Fig. 10 b and Table S1). Among known substrates, spectrin (SPTB2) is a cytoskeletal protein that lines the intracellular side of the plasma membrane. Its cleavage by CASP3 leads to membrane collapse and apoptosis (Wang et
A bacteria-based protein delivery tool

• Ittig et al. 923

al., 1998). In the same line, CASP3-mediated cleavage of plectin disrupts the cytoskeleton and induces morphological changes and cell detachment (Liu et al., 2011). Finally, cleavage of ACINU and NUMA1 triggers chromatin condensation and nuclear matrix reorganization. Interestingly, phosphorylation changes of these proteins occurred concomitantly with cell rounding up and nuclei condensation observed after tBID delivery (Fig. 10 c). Altogether, our results show that tBID-induced apoptosis triggers hundreds of phosphorylation events involved in multiple cellular functions and confirms the central role of CASP3 in this process. Besides providing a valuable dataset for apoptosis, these results illustrate how T3S-based protein delivery combined with –omics studies can reveal the systems-level impact of a protein on a cellular network.

Discussion

Here we provide optimized protocols for making use of Y. enterocolitica T3S to inject heterologous type III and IV bacterial effectors as well as viral and mammalian proteins in cells and in developing animals. We show that first effects of translocated proteins can already be observed within seconds after infection, indicating that this method is particularly well suited to study short-lived cellular events that are triggered or controlled by a given protein. As protein translocation via T3S can be temporally controlled, it allows investigating the temporal dynamics of cellular and signaling changes. In contrast to cDNA transfection, T3S-based protein delivery is homogenous within the cell population and can be gradually tuned with the MOI. Unlike proteofection, protein electroporation (Freund et al., 2013), or cell-penetrating peptides, T3S-dependent protein delivery does not require protein purification. We show that the amounts of translocated tBID and BID reach and exceed (2.5-fold) endogenous levels of BID, respectively. According to a deep proteome dataset estimating that HeLa cells contain 4.4 × 10^5 copies of BID per cell (Nagaraj et al., 2011), our results suggest that T3S can inject 10^5–10^6 proteins per cell. We further show that T3S-delivered INK4C can exceed the level of endogenous INK4C by a factor of 5 (Fig. 7, c and f). As deep proteome data indicate that HeLa cells contain 5 times 10^5 copies

Figure 9. Characterization of the cellular impact of T3S-based delivery by phosphoproteomics. (a) Volcano plot representation of the impact of T3S-based delivery on phosphorylation in the presence or in absence of YadA or InvA compared with uninfected cells. HeLa cells were infected for 30 min with the indicated strains at a MOI of 100. Yellow dots represent in all three panels the phosphopeptides associated with a q-value < 0.04 in control infection compared with uninfected cells. Phosphoproteomic analysis was performed in independent triplicates. (b) Digitonin-lysed HeLa cells, infected for 30 min with the indicated strains at an MOI of 100, were analyzed by Western blot analysis with an anti-Myc antibody to detect delivery of YopE1–138-Myc by corresponding strains.
of INK4C per cell (Nagaraj et al., 2011), this suggests that T3S can inject more than $10^6$ proteins per cell. These numbers are compatible with the translocation yield of the *S. typhimurium* T3S substrate SipA ($6 \pm 3 \times 10^3$ molecules per bacterium; Schlumberger et al., 2005) or nanobodies via *E. coli* T3S ($10^5$–$10^6$ molecules per infected cell; Blanco-Toribio et al., 2010).

Interestingly, the described method is not limited to single protein delivery. Coinfection protocols can be performed to inject multiple proteins in cells and study functional interactions. We also show that T3S-based protein delivery is suitable to inject functionally active eukaryotic proteins into living animals.

So far, the use of T3S-based protein delivery was hampered by the difficulties of proteins to pass through the needle of the injectisome. For example, constraints on YopE length and strength of protein folding were previously described for the translocation of DHFR (Feldman et al., 2002) and...
A bacteria-based protein delivery tool • Ittig et al. 925

Materials and methods

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table S3. All E. coli strains were grown on LB agar plates and in LB broth. E. coli Top10 was used for plasmid purification and cloning and E. coli Sm10.2 pir was used for conjugation. Ampicillin was used at 200 µg/ml (Y. enterocolitica) or 100 µg/ml (E. coli) to select for expression vectors. Streptomycin was used at 100 µg/ml to select for suicide vectors. Y. enterocolitica MR540, a non-ampicillin–resistant E40 derivative (Sory and Cornelis, 1994), and all derived strains were routinely grown on brain heart infusion (BHI; Difco). Nalidixic acid (35 µg/ml) was added to all Y. enterocolitica strains. All E. enterocolitica asd strains were additionally supplemented with 100 µg/ml meso-2,6-diaminopimelic acid (mDAP; Sigma-Aldrich).

Construction of plasmids

The pBad_SI2 and pBad_SI_1 plasmids (Fig. S1, a and b) were used to fuse the proteins of interest to the N-terminal 138 amino acids of YopE. pBad_SI2 was constructed by cloning the SycE–YopE1–138 fragment containing the endogenous promoters of YopE and SycE from purified pYV40 into the KpnI–HindIII site of pBad-MychisA (Invitrogen). Additional modifications include the elimination of the Ncol–BglII fragment of pBad-MychisA by digestion, Klenow fragment treatment, religation, and the addition the XbaI–Xhol–BstBI–(HindIII) cleavage sites at the 3′ end of the YopE1–138 fragment (Fig. S1 c). pBad_SI1 is identical to pBad_SI2 except for the insertion of an EGFP marker, amplified from pEGFP-C1 vector (Clontech), into the Ncol–BglII site, under the arabinose inducible promoter.

Full-length genes (Table S3) were amplified with the specific primers listed in Table S2 and cloned into pBad_SI2 or, in case of z-BIM, into pBad_SI1. For genes of bacterial species, purified genomic DNA was used as template (S. flexneri M90T, S. enterica subsp. enterica serovar Typhiurium SL1344, Bartonella henselae ATCC 49882). For human genes, a universal cDNA library (Clontech) was used if not otherwise stated (Table S3). Zebrafish genes were amplified from a zebrafish cDNA library. Ligated plasmids were cloned in E. coli Top10. Sequenced plasmids were electroporated into the desired Y. enterocolitica strain by using the settings of standard E. coli electroporation.

In vitro secretion

Induction of the yop regulon was performed by shifting the bacterial culture to 37°C in BHI supplemented with 4 mg/ml glucose as carbon source and 20 mM sodium oxalate (secretion-permissive conditions; Cornelis et al., 1987). Pellet and supernatant were separated by centrifugation at 20,800 g for 10 min at 4°C. The pellet was taken as total bacteria fraction. Proteins in the supernatant were precipitated with trichloroacetic acid 10% (wt/vol) final for 1 h at 4°C. After centrifugation (20,800 g for 15 min) and elimination of the supernatant, the resulting pellet was washed in ice-cold acetone overnight. The samples were centrifuged again, the supernatant was discarded, and the pellet was air-dried and resuspended in 1x SDS loading buffer. Secreted proteins were analyzed by SDS–PAGE. Typically, each lane was loaded with proteins secreted by 3 × 10^8 bacteria. Detection of specific secreted proteins by immunoblotting was performed using 12.5% SDS-PAGE gels. For detection of proteins in total bacterial extracts, the equivalent of 2 × 10^9 bacteria was loaded per lane, if not stated otherwise. Proteins were also separated on 12.5% SDS-PAGE gels before detection by immunoblotting. Immunoblotting was performed using rat monoclonal antibodies against YopE (13A9; raised against full-length YopE; 1:1,000; Grosdent et al., 2002). In brief, this monoclonal antibody was generated by intraperitoneal injection of 10^7 Y. enterocolitica W22703, grown under secretion inducing condition and mixed with Freund’s adjuvant.
into LOU rats (Bodeus et al., 1990). After 3 mo, the same procedure was repeated. Three days later, the spleen was removed and splenic cells were fused with IR983F azaguanine-resistant cells (Bodeus et al., 1990). Hybrid clones were grown and supernatants were analyzed for the identification of a YopE-detecting antibody. The antibody was pre-absorbed twice overnight against Y. enterocolitica ΔHOPEMT asld to reduce background staining. Detection was performed with secondary antibodies directed against rat antibodies and conjugated to horseradish peroxidase (1:5,000; 3010-05; Southern Biotech), before development with ECL chemiluminescent substrate (LumiGlo, KPL).

Cell culture and infections
HeLa CCL-2, HEK 293T, and Swiss 3T3 fibroblasts were cultured in DMEM supplemented with 10% FCS and 2 mM L-glutamine. Human umbilical vein cells were isolated and cultivated as described previously (Dehio et al., 1997). Jurkat cells were cultured in RPMI 1640 supplemented with 10% FCS and 2mM L-glutamine. Y. enterocolitica were grown in BHI plus additives overnight at RT, diluted in fresh BHI to an OD600 of 0.2 and grown for 2h at RT before a temperature shift to a 37° C waterbath shaker for further 30 min or for 1 h in case of EGFP or mCherry transfection. Finally, bacteria were collected by centrifugation (6,000 rcf, 30 s) and washed once with DMEM supplemented with 10mM Heps and 2mM L-glutamine. Cells, seeded in 96-well (for immunofluorescence) or 6-well (for Western blotting) plates, were infected at indicated MOIs in DMEM supplemented with 10mM Heps and 2mM L-glutamine. After adding bacteria, plates were centrifuged for 1 min at 1,750 rpm and placed at 37°C for indicated time periods. Extracellular bacteria were killed by gentamicin (100 mg/ml) if indicated. In case of immunofluorescence analysis, infection assays were stopped by PFA fixation. For Western blot analysis of cellular proteins, cells were washed and lysed as described in the next paragraph. Z-VAD(OMe)-FMK (zVAD; Santa Cruz) was dissolved at 10mM in DMSO and added at the concentration indicated 1h before the infection.

Western blot analysis of cellular proteins after infection
Cells were infected as described in the previous paragraph, washed twice with ice-cold PBS, and Phosho-safe lysis buffer (Novagen) was added to lyse the cells. After incubation on ice, the cells were centrifuged (16,000 rcf, 25 min, 4°C). Supernatants were collected and analyzed for total protein content by BCA assay (Pierce) before SDS-PAGE and Western blot analysis using anti-p38 (mouse, 1:5,000; NA931V; GE Healthcare), anti-BID (rabbit, 9212; Cell Signaling) and anti-phospho-p38 (Thr180/Tyr182; rabbit, 4631; Cell Signaling) antibodies. Detection was performed with secondary antibodies directed against rabbit antibodies conjugated to horseradish peroxidase (1:5,000; NA934V; GE Healthcare), before development with ECL chemiluminescent substrate (LumiGlo or LumiGlo reserve; KPL).

Western blot analysis of T3S-translocated proteins into infected cells
HeLa cells in 6-well plates were infected at an MOI of 100 as described in the Cell culture and infections paragraph. In coinfection experiments, the two bacterial suspensions were mixed in a tube at a ratio of 1:1 (if not otherwise indicated) and added to the cells at the MOI of 100 for each strain (if not otherwise stated). At the end of the infection assay, cells were washed twice with ice-cold PBS and collected by scraping in ice-cold PBS. After centrifugation (16,000 rcf, 5 min, 4°C), pellets were dissolved in 0.002% digitonin supplemented with a protease inhibitor cocktail (Roche complete; Roche). Dissolved pellets were incubated for 5 min on ice and then centrifuged (16,000 rcf, 25 min, 4°C). Supernatants were collected and analyzed for total protein content by BCA assay (Pierce) before SDS-PAGE and Western blot analysis using an anti-Myc (9E11; mouse, sc-47694; Santa Cruz), anti-BID (rabbit, 2002; Cell Signaling), or anti-INK4C (mouse, 2896; Cell Signaling) antibody. Control lysis was performed with a lysis buffer containing 0.2% Triton (in 20mM Tris, pH 8, 10 mM EDTA, and 100mM NaCl) and repeated sonication at 4°C (Helscher).

Immunofluorescence
Cells, seeded in 96-well plates (Corning), were infected as described in the Cell culture and infections paragraph. After fixation with 4% PFA, they were washed three times with PBS and incubated with 5% goat serum in PBS 0.3% Triton X-100 for 1h at RT. The primary antibody (anti-Myc, 1:100; Santa Cruz) was diluted in PBS with 1% BSA and 0.3% Triton X-100 and cells were incubated overnight at 4°C. Cells were washed four times with PBS before the secondary antibody (AF 488 goat anti-mouse, A11029; 250; Invitrogen), diluted in PBS 1% BSA and 0.3% Triton X-100, was added. If needed, Hoechst DNA staining (2:1500; 62249; Thermo Fisher Scientific) and/or F-actin staining (Dy647-Phalloidin; DyeOmics) were included. In some cases, staining for DNA and/or F-actin was applied directly after PFA fixation. Cells were incubated for 1h at RT, washed three times with PBS and analyzed by automated image analysis as described in the next paragraph. In case of confocal microscopy, 25,000 HeLa CCL-2 cells were seeded per well of an 8-well µ-Slide (80826; Ibidi) and grown to confluence for 24 h before infection. After fixation for 4% PFA in 10 min, cells were permeabilized in blocking buffer (0.3% Triton X-100/5% goat serum) for 1 h. Samples were incubated overnight with an anti-mouse tubulin-β antibody (mouse, MA5-11732, 1:100; Thermo Fisher Scientific) in antibody dilution buffer (1x PBS/1% BSA/0.3% Triton X-100) followed by secondary antibody application using an Alexa Fluor 647–conjugated goat anti-mouse antibody (A21236; Invitrogen) in antibody dilution buffer. In parallel, F-actin and DNA were stained for 1 h at room temperature using FITC-phalloidin (Invitrogen) and Hoechst (62249; Thermo Fisher Scientific), respectively. Mitochondria were stained using Mitotracker (Life Technologies) according to the manufacturer’s instructions. Images were acquired by a Point Scanning Confocal LSM700 Inverted microscope (Carl Zeiss) using a Plan Apo 40x magnification/1.3 NA oil immersion objective and Zen 2010 software. Images were acquired at room temperature in PBS. Filter cubes used were blue (excitation: G 365, dichroic: FT 395, emission: BP 445/50; filter 49 DAPI), green (exc.: BP 470/40; dichroic: FT 495, em.: BP 525/50; filter 38H EGFP), and red (exc.: BP 546/12, dichroic: FT 560, em.: BP 575-640; filter 20 Rhodamine). The confocal stacks were analyzed (maximum intensity projection) using ImageJ software.

Automated microscopy and image analysis
Images were automatically acquired with an ImageXpress Micro automated cellular imaging microscope (Molecular Devices), equipped
with a fast laser autofocus with a precision motorized Z-stage and a Lumencor Spectra X light engine and CFI Super Fluo10x air objective 0.5 NA (Nikon) or CFI Plan Fluor ELWD 20x C 0.45 NA air objective (Nikon). Images were acquired at room temperature and in PBS. Filter cubes (Semrock) were BrightLine, DAPI-5060B (Hoechst); BrightLine, GFP-3035B (EGFP, Alexa Fluor 488, FITC); BrightLine, TXRED-4040B (RFP, mCherry); and BrightLine, CY5-4040A (Alexa Fluor 647, Dy47). Camera used was a CoolSNAP HQ monochrome charge coupled device camera (Photometrics). MetaXpress software controlled all components of the system.

Quantification of anti-Myc staining, EGFP, and mCherry intensities was performed using MetaXpress ( Molecular Devices). Circular regions were manually chosen in the cytoplasm or nucleus excluding cell areas containing bacteria, and used for quantification. Cell number, cell size, nuclear Hoechst intensity staining, and F-actin meshwork were quantified using CellProfiler (Carpenter et al., 2006). In brief, the Hoechst and the actin stainings were used to automatically identify cell nuclei and cell area, respectively. F-actin foci or cables as seen within the actin meshwork triggered by SopE were quantified by specific analysis modules of CellProfiler (see source code files in the Online supplemental material).

CAMP level determination
HeLa cells, seeded in 96-well plates, were infected as described in the cell culture and infections paragraph. Before infection (30 min), cells were incubated with fresh DMEM supplemented with 10 mM Hepes, 2 mM l-glutamine, and 100 µM 3-isobutyl-1-methylxanthine (IBMX; Sigma-Alrich). Gentamicin was added 60 min p.i., and cells were further incubated at 37°C for another 90 min. cAMP concentrations were determined by a competitive ELISA assay performed according to the manufacturer’s instructions (CAMP Biotrak, RPN225; GE Healthcare). As positive control, cells were incubated with chola toxin (C8052; Sigma-Alrich) for 1 h.

Ac-DEVD-amc and resazurin assays
HeLa cells infected at an MOI of 100 for 1 h were lysed with a buffer containing 0.2% Triton (in 20 mM Tris, pH 8, 10 mM EDTA, and 100 mM NaCl) on ice. Cell lysates were mixed 1:1 with 0.2 mM ac-DEVD-amc (Alexis, dissolved at 10 mM in DMSO) in a caspase assay buffer (10 mM Pipes, pH 7.2, 10 mM NaCl, 10% sucrose, 0.1% CHAPS, 1 mM EDTA, and 10 mM DTT) and incubated for 1 h at 37°C before fluorescence was measured at an excitation of 380 nm and an emission of 460 nm.

For the resazurin assay, cells were infected at an MOI of 100. 1 h after the infection, penicillin and streptomycin were added. Cells were further incubated for 23 h. Resazurin was dissolved in PBS at a concentration of 0.1 mg/ml, filter-sterilized and added 1:6 into the assay. After 2 h incubation at 37°C, the fluorescence was measured at an excitation of 528 nm and an emission at 600 nm.

Zebrafish embryo infections, imaging, and automated image quantification
All animal experiments were performed according to approved guidelines. Zebrafish were maintained in standard conditions. Embryos were staged by hours postfertilization at 28.5°C. The wild-type AB/EK and EK/TL zebrafish lines were used in this study. Infection protocol followed guidelines given in Benard et al. (2012). In brief, 12 h after fertilization, embryos were incubated in E3 medium containing 0.2 mM N-phenylthiourea to prevent pigment formation. Then, 2 d postfertilization embryos were anesthetized by 0.2 mg/ml tricaine and aligned on 1% agar plates in E3 using a hair loop tool (Benard et al., 2012). Y. enterocolitica bacteria were grown in a hair loop tool (Benard et al., 2012). with 0.5% arabinose, antibiotics, and mDAP overnight at RT, diluted in fresh BHI with 0.5% arabinose and other additives to an OD600 of 0.2, and grown for 2 h at RT before a temperature shift to 37°C in a water bath shaker for 45 min. Finally, bacteria were collected by centrifugation (6,000 rcf, 30 s) and washed once with PBS. The OD600 was set to 2 in PBS containing mDAP. 1–2 nL of this suspension was injected into the hindbrain of zebrafish embryos using an Femtotjet Microinjector device (Eppendorf) and Femtotips II (Eppendorf). Before injection, the tip of the needle was broken off with fine tweezers. The injection time was set to 0.2 s, the compensation pressure to 15 hPa, and the injection pressure was adjusted between 600 and 800 hPa. Drop size, and thus the inoculum, was checked by microscopy and by control plating. After microinjection, the fish were collected in E3 containing tricaine and incubated for 30 min at 37°C and then for 5 h at 28°C. EGFP bacteria were observed with fluorescence binocular (Leica) 1 h p.i. in zebrafish hindbrains, and embryos that were not properly injected were discarded. At the end of infection, fish were fixed with 2% PFA for 1 h on ice and then incubated with fresh 2% PFA overnight at 4°C. Antibody staining was performed as described previously (Herwig et al., 2011). In brief, embryos were washed four times with PBS 0.1% Tween (PBS-T) for 5 min and permeabilized with PBS-T + 0.5% Triton X-100 for 30 min at RT. Embryos were incubated in blocking solution (PBS-T + TritonX-100, 5% goat serum, and 1% BSA) at 4°C overnight. Cleaved CASP3 antibody (Asp175, 9664; Cell Signaling) was diluted 1:100 in blocking solution and incubated under shaking at 4°C. Fish were washed seven times with PBS-T for 30 min before the secondary antibody (goat anti–rabbit AF647, 1:500; A21244; Invitrogen), diluted in blocking solution, was added and incubated at 4°C overnight. Larvae were washed with PBS-T 30 min at 4°C, incubated overnight, and further washed three or four times. Embryos were mounted in 0.7% low melting agarose (Sigma-Alrich) in 35-mm glass-bottom dishes (MatTek). Confocal images were recorded at RT on a Leica TCS SP5 II point scanning confocal microscope (standard Leica DMI 6000B with adaptive focus, equipped with Leica HyD hybrid detectors and AOBS as a tunable dichroic for all lasers) using a 40x water immersion objective (HCX PLAN APO CS with a Nomarski DIC module). Images were acquired with a Leica TCS SP5 II point scanning confocal microscope (stand Leica DMI 6000B with adaptive focus, equipped with Leica HyD hybrid detectors and AOBS as a tunable dichroic for all lasers) using a 40x water immersion objective (HCX PLAN APO CS with a Nomarski DIC module).

Generation of stable cell lines
To produce the pMDK124 lentiviral vector (a gift of O. Pertz, University of Basel, Basel, Switzerland) encoding EGFP-Rab2A (pSLO52), pMDK124 was digested with EcoRI and BamHI overnight at 37°C and gel purified. PCR was performed with gene-specific primers that contain 15- bp extensions complementary to the vector-digested ends. EGFP-Rab2A was amplified from templates provided by F.A. Barr (Short et al., 2001), pSLO45 (5'-GCCAAGGGCGAGGA-3') and pSLO46 (5'-GATTTCGACGAAA TTCTCAACAGCAGCCGCCCCAG-3') were used to PCR-amplify EGFP-Rab2A. Using the gel-purified PCR products and digested pMDK124, In-Fusion recombination was performed with 200 ng vector and PCR product using In-Fusion enzyme mixture from In-Fusion HD cloning kit (Clontech), incubated 15 min at 50°C, and then transferred to ice. 5 µl of the reaction mixture was transformed into 100 µl...
E. coli DH5α and plated on ampicillin containing LA plates. Helper plasmids pVSV, pMDL, and pRev are gifts from Oliver Perez’s group.

3 × 10⁵ HEK 293T cells were grown in a 10-cm dish and incubated at 37°C, 5% CO₂ for at least 6–8 h. 2.2 µg lentivector viral pMDK124 encoding EGFP-Rab2A, 0.75 µg pVSV, 1.5 µg pMDL, and 0.5 µg pRev in 600 µl DMEM without FCS were then mixed with 25 µl Fugene HD (Promega) in 600 µl DMEM without FCS and incubated for 15 min at RT. The DNA-fugene complex was then added to cells in 5 ml freshly replaced medium. The medium was again replaced the following day. 2 d later, the supernatant of HEK293T containing viruses was collected and filtered through a 0.45-µm membrane filter. Viral containing supernatant (1-ml or 2-ml volumes) were added to HeLa cells in presence of fresh DMEM with 10% FCS to a total volume of 3 ml in a 6-well plate, and polybrene (H9268; Sigma-Aldrich) was added at a final concentration of 5 µg/ml. The medium was exchanged with fresh DMEM with 10% FCS the next day and used for further experiments.

**Sample preparation for phosphoproteomics**

For each biological condition, two 6-well plates of HeLa CCL-2 cells were grown to confluency. Per condition, three independent biological replicates were performed. Cells were infected for 30 or 45 min as described above. The plates were put on ice and washed twice with ice-cold PBS. Samples were then collected in 8 M urea (AppliChem), 0.1 M ammoniumbicarbonate (Sigma-Aldrich), 0.1% RapiGest (Waters), and PhosSTOP (Roche). The samples were briefly vortexed, sonicated at 4°C (Hiescher), shaken for 5 min on a thermomixer, and centrifuged for 20 min at 4°C and 169,000 g. Supernatants were collected and stored at −80°C for further processing. BCA protein assay (Pierce) was used to measure protein concentration.

**Phosphopeptide enrichment**

Disulfide bonds were reduced with tris(2-carboxyethyl)phosphine at a final concentration of 10 mM at 37°C for 1 h. Free thiols were alkylated with 20 mM iodoacetamide (Sigma-Aldrich) at RT for 30 min in the dark. The excess of iodoacetamide was quenched with N-acetyl cysteine at a final concentration of 25 mM for 10 min at room temperature. Lys-C endopeptidase (Wako) was added to a final enzyme/protein ratio of 1:200 (wt/wt) and incubated for 4h at 37°C. The solution was subsequently diluted with 0.1 M ammoniumbicarbonate (Sigma-Aldrich) to a final concentration below 2 M urea and digested overnight at 37°C with sequencing-grade modified trypsin (Promega) at a protein/enzyme ratio of 50:1. Peptides were desalted on a C18 Sep-Pak cartridge (Waters) and dried under vacuum. Phosphopeptides were isolated from 2 mg of total peptide mass with TiO₂ as described previously (Bensimon et al., 2010). In brief, dried peptides were dissolved in an 80% acetonitrile and 2.5% trifluoroacetic acid (TFA) solution saturated with phthalic acid. Peptides were added to the same amount of equilibrated TiO₂ (5-µm bead size; GL Sciences) in a blocked Mobicol spin column (MobiBiTec) that was incubated for 30 min with end-over-end rotation. The column was washed twice with the saturated phthalic acid solution, twice with 80% acetonitrile and 0.1% TFA, and finally twice with 0.1% TFA. The peptides were eluted with a 0.3 M NH₄OH solution. The pH of the eluates was adjusted to be below 2.5 with 5% TFA solution and 2 M HCl. Phosphopeptides were again desalted with microspin C18 cartridges (Harvard Apparatus).

**LC-MS/MS analysis**

Chromatographic separation of peptides was performed using an EASY nano-LC system (Thermo Fisher Scientific), equipped with a heated RP-HPLC column (75 µm × 45 cm) packed in-house with 1.9 µm C18 resin (Reprosil-QP Pur; Dr. Maisch). Aliquots of 1 µg total phosphopeptide samples were analyzed per LC-MS/MS run using a linear gradient ranging from 98% solvent A (0.15% formic acid) and 2% solvent B (98% acetonitrile, 2% water, and 0.15% formic acid) to 30% solvent B over 120 min at a flow rate of 200 nl/min. Mass spectrometry analysis was performed on a dual pressure LTQ-Orbitrap mass spectrometer equipped with a nanoelectrospray ion source (both Thermo Fisher Scientific). Each MS1 scan (acquired with the Orbitrap) was followed by collision-induced dissociation (acquired in the LTQ) of the 20 most abundant precursor ions with dynamic exclusion for 30 s. For phosphopeptide analysis, the 10 most abundant precursor ions were subjected to collision-induced dissociation with enabled multistage activation. Total cycle time was ~2 s. For MS1, 10⁴ ions were accumulated in the Orbitrap cell over a maximum time of 300 ms and scanned at a resolution of 60,000 FWHM (at 400 m/z). MS2 scans were acquired using the normal scan mode, a target setting of 10⁵ ions, and accumulation time of 25 ms. Singly charged ions and ions with unassigned charge state were excluded from triggering MS2 events. The normalized collision energy was set to 32%, and one microscan was acquired for each spectrum.

**Label-free quantification and database searching**

The acquired raw files were imported into the Progenesis software tool (Nonlinear Dynamics, Version 4.0) for label-free quantification using the default parameters. MS2 spectra were exported directly from Progenesis in mgf format and searched using the MASCOT algorithm (version 2.4; Matrix Science) against a decoy database (Perkins et al., 1999) containing normal and reverse sequences of the predicted SwissProt entries of Homo sapiens and commonly observed contaminants (in total 41,250 sequences) generated using the SequenceReverser tool from the MaxQuant software (version 1.0.13.13). To identify proteins originating from Y. enterocolitica, nonphosphopeptide-enriched samples were searched against the same database above including predicted SwissProt entries of Y. enterocolitica. The precursor ion tolerance was set to 10 ppm and fragment ion tolerance was set to 0.6 D. The search criteria were set as follows: full tryptic specificity was required (cleavage after lysine or arginine residues unless followed by proline), two missed cleavages were allowed, and carbamidomethylation (C) was set as fixed modification and phosphorylation (S,T,Y) or oxidation (M) as a variable modification for TiO₂ enriched or not enriched samples, respectively. Finally, the database search results were exported as an xml-file and imported back to the Progenesis software for MS1 feature assignment. For phosphopeptide quantification, a csv file containing the MS1 peak abundances of all detected features was exported and for not enriched samples, a csv file containing all protein measurements based on the summed feature intensities of all identified peptides per protein was created. Importantly, the Progenesis software was set in a way that proteins identified by similar sets of peptides are grouped together and that only nonconflicting peptides with specific sequences for single proteins in the database were used for protein quantification. Both files were further processed using the in-house developed SafeQuant v1.0 R script (see online supplemental material files; also available at https://github.com/eahrne/SafeQuant/). In brief, the software sets the identification level false discovery rate to 1% (based on the number of decoy protein sequence database hits) and normalizes the identified MS1 peak abundances (extracted ion chromatogram [XIC]) across all samples, i.e., the summed XIC of all confidently identified peptide features is scaled to be equal for all LC-MS runs. Next, all quantified phosphoproteptides/proteins are assigned an abundance ratio for each time point, based on the median XIC per time point. The statistical significance of each ratio is given by its q-value (false discovery rate adjusted p-values), obtained by calculating modified t-statistic p-values.


