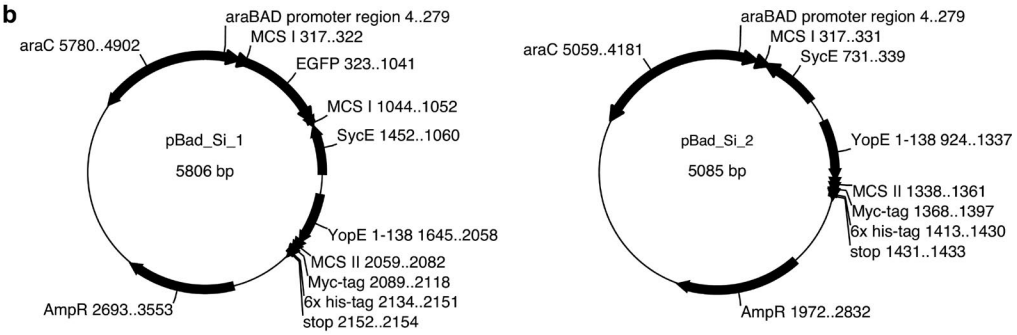
a

Bacterial strain	Relevant characteristics	Reference	Biosafety level
Yersinia enterocolitica ΔyopH,O,P,E,M,T	MRS40 pIML421 [$yopH_{\Delta 1-352}$, $yopO_{\Delta 65-558}$, $yopP_{23}$, $yopE_{21}$, $yopM_{23}$, $yopT_{135}$]	Iriarte and Cornelis, 1998	2
Yersinia enterocolitica ΔyopH,O,P,E,M,T Δasd	MRS40 $asd_{\Delta 292-610}$, pIML421 [$yopH_{\Delta 1-352}$, $yopO_{\Delta 65-558}$, $yopP_{23}$, $yopE_{21}$, $yopM_{23}$, $yopT_{135}$]	Kudryashev et al., 2013	1
Yersinia enterocolitica ΔyopH,O,P,E,M,T ΔyopB	MRS40 pIML421 [$yopB_{\Delta 89-217}$, $yopH_{\Delta 1-352}$, $yopO_{\Delta 65-558}$, $yopP_{23}$, $yopE_{21}$, $yopM_{23}$, $yopT_{135}$]	unpublished data	2

b



c

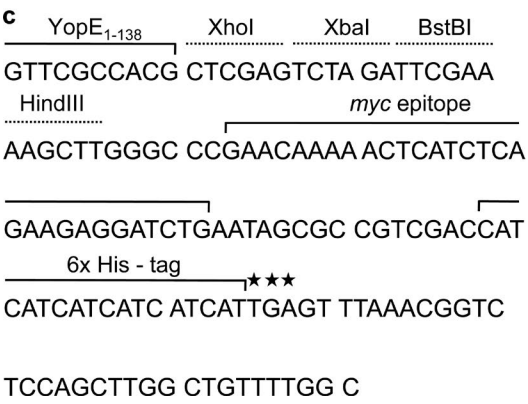


Figure S1. **Description of the T3S-based delivery toolbox.** (a) Description of the *Y. enterocolitica* strains used in this study. (b) Vector maps of the cloning plasmids used to generate the $YopE_{1-138}$ fusion constructs. The chaperone SycE and the $YopE_{1-138}$ fusion are under the native *Y. enterocolitica* promoter. The two plasmids differ only in the presence of an arabinose-inducible EGFP. (c) Sequence of the multiple cloning site located directly after the $YopE_{1-138}$ fragment.

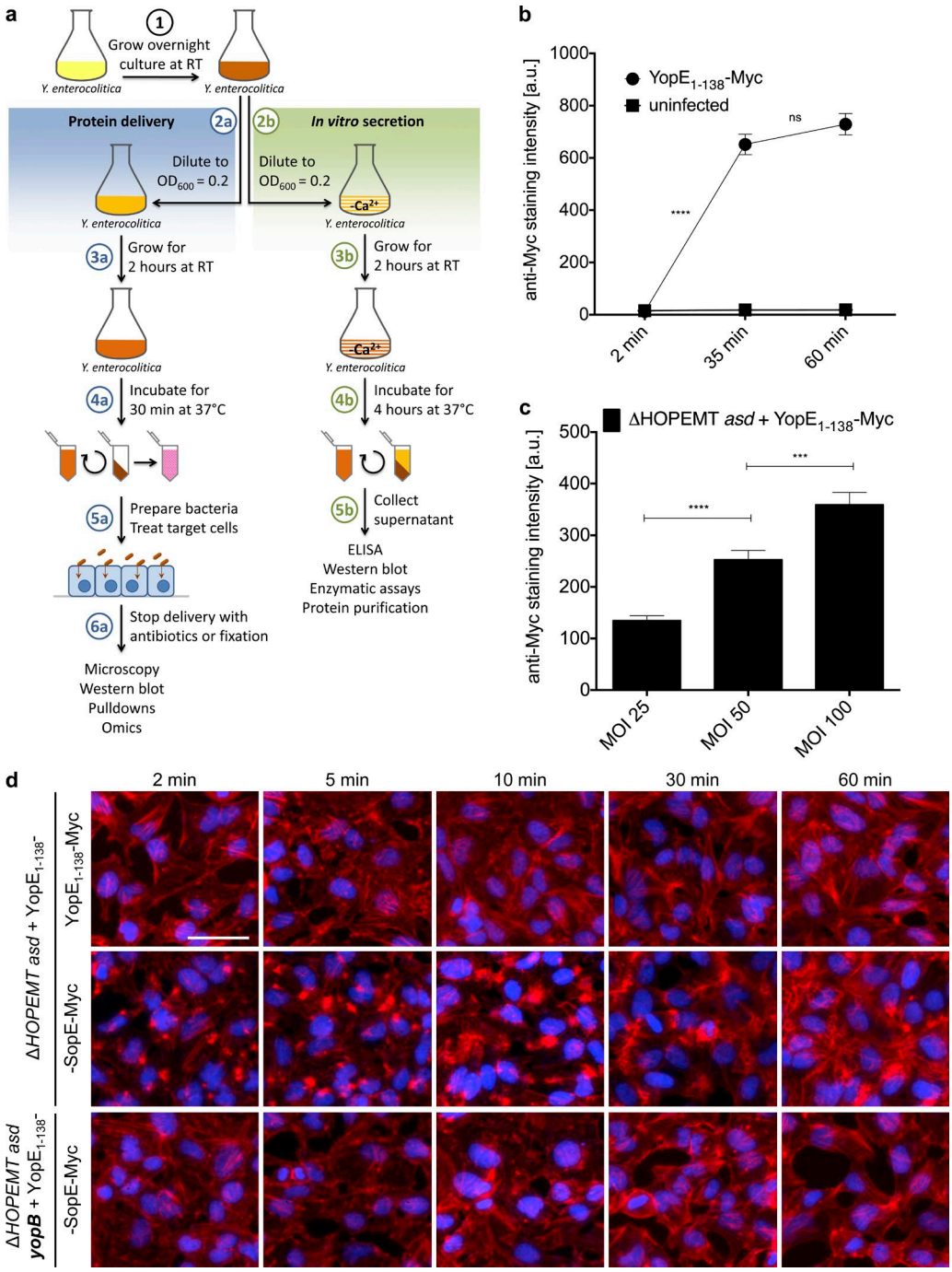


Figure S2. **Protocol and kinetics the T3S-based delivery toolbox.** (a) Schematic representation of the standard secretion and infection procedure. (b) Quantification of anti-Myc immunofluorescence staining intensity within HeLa cells as shown in Fig. 1 C. Data correspond to the mean of $n = 60$ cells per condition, and error bars indicate standard errors of the mean. Statistical analysis was performed using a Mann-Whitney test (****, $P < 0.0001$; ns, not significant). (c) Quantification of anti-Myc immunofluorescence staining intensity within HeLa cells as shown in Fig. 1 D. Data correspond to the mean of $n = 30$ cells per condition, and error bars indicate standard errors of the mean. Statistical analysis was performed using a Mann-Whitney test (***, $P < 0.001$; ****, $P < 0.0001$). (d) Translocated SopE induces a dramatic remodelling of F-actin. HeLa cells were infected with the indicated strains at an MOI of 100 for the indicated time periods. After fixation, cells were stained for nuclei (blue) and F-actin (red). Bar, 50 µm.

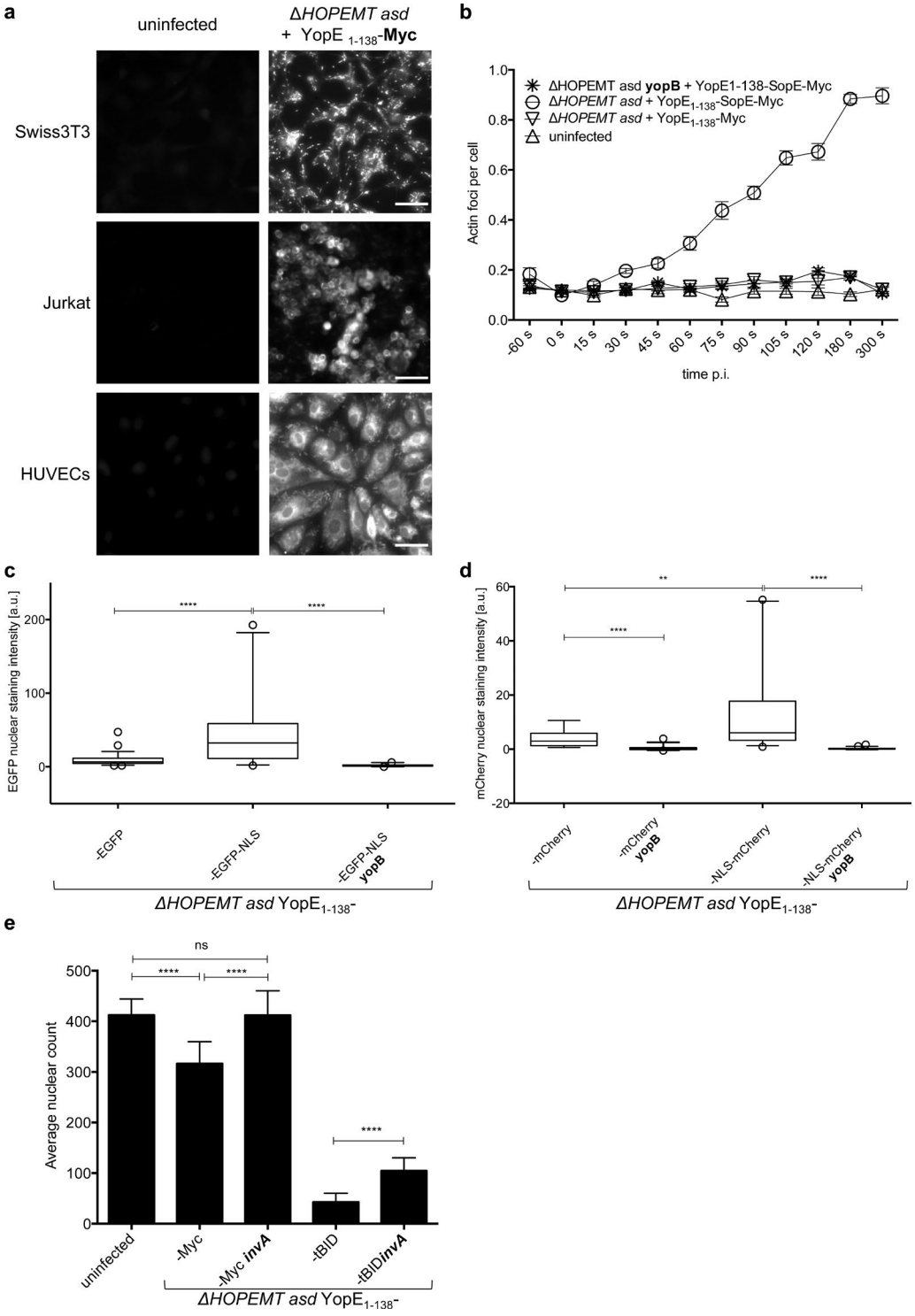


Figure S3. **Spectrum of target cells and quantifications of T3S-based delivery.** (a) T3S-based protein delivery into various cell lines and primary cells. Anti-Myc immunofluorescence staining of Swiss 3T3 fibroblasts, Jurkat, and human umbilical vein cells infected or not for 1 h at MOIs 200, 25, and 400, respectively, with the strain delivering $YopE_{1-138}$-Myc. Bars, 50 µm. (b) Automated quantification of actin foci per cell in images as in Fig. 2 C. Automated image analysis was performed on $n = 18$ images per condition and time point. Error bars indicate standard errors of the mean. (c and d) Quantification of EGFP or mCherry fluorescence intensity, respectively, in the nuclei of images as in Fig. 5 (A or B). A 5–95%ile boxplot is shown. Statistical analysis was performed using a Mann-Whitney test (**, $P < 0.01$; ****, $P < 0.0001$). (e) Delivery of t-BID by *invA* strain leads to a strong reduction in cell number, assessed by counting of nuclei. HeLa cells were infected for 60 min with the indicated strains at an MOI of 100. Automated image analysis was performed on $n = 27$ images per condition. Error bars indicate standard errors of the mean. Statistical analysis was performed using a Mann-Whitney test (****, $P < 0.0001$; ns, not significant).

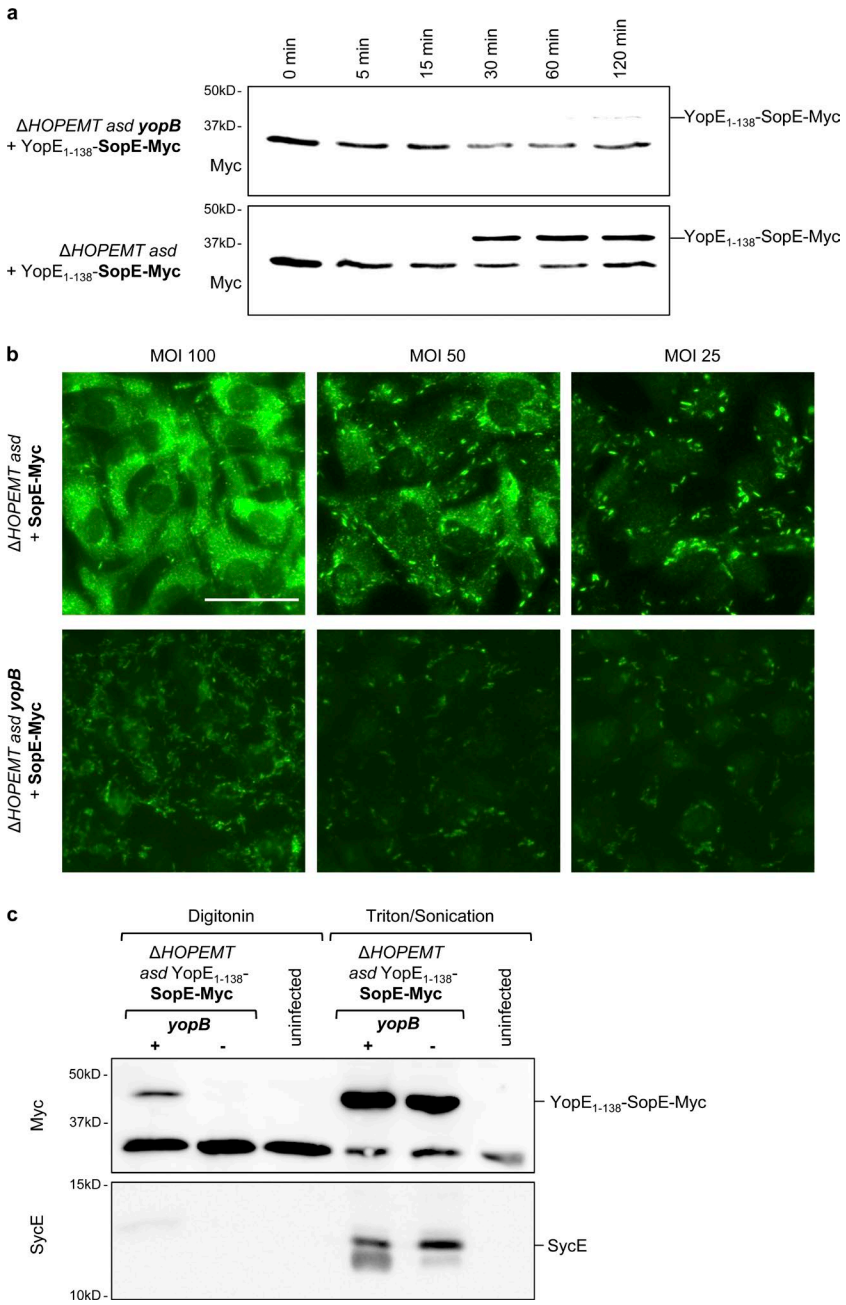


Figure S4. **T3S-dependent delivery of bacterial effectors into eukaryotic cells.** (a) Digitonin-lysed HeLa cells infected at MOI 100 for indicated time periods with Δ*HOPEMT asd* or Δ*HOPEMT asd* Δ*yopB* strains expressing $YopE_{1-138}$-SopE-Myc were analyzed by Western blot with an anti-Myc antibody. (b) Anti-Myc immunofluorescence staining on HeLa cells infected with the indicated strains at different MOIs for 1 h. Gentamicin was added 30 min p.i. The anti-Myc staining is shown in green. Bar, 50 µm. (c) HeLa cells infected at MOI 100 for 1 h with Δ*HOPEMT asd* or Δ*HOPEMT asd* Δ*yopB* strains expressing $YopE_{1-138}$-SopE-Myc were lysed with digitonin or by a combination of Triton and sonication and were analyzed by Western blot with an anti-Myc or an antibody against the bacteria cytosolic chaperone SycE (anti-SycE).

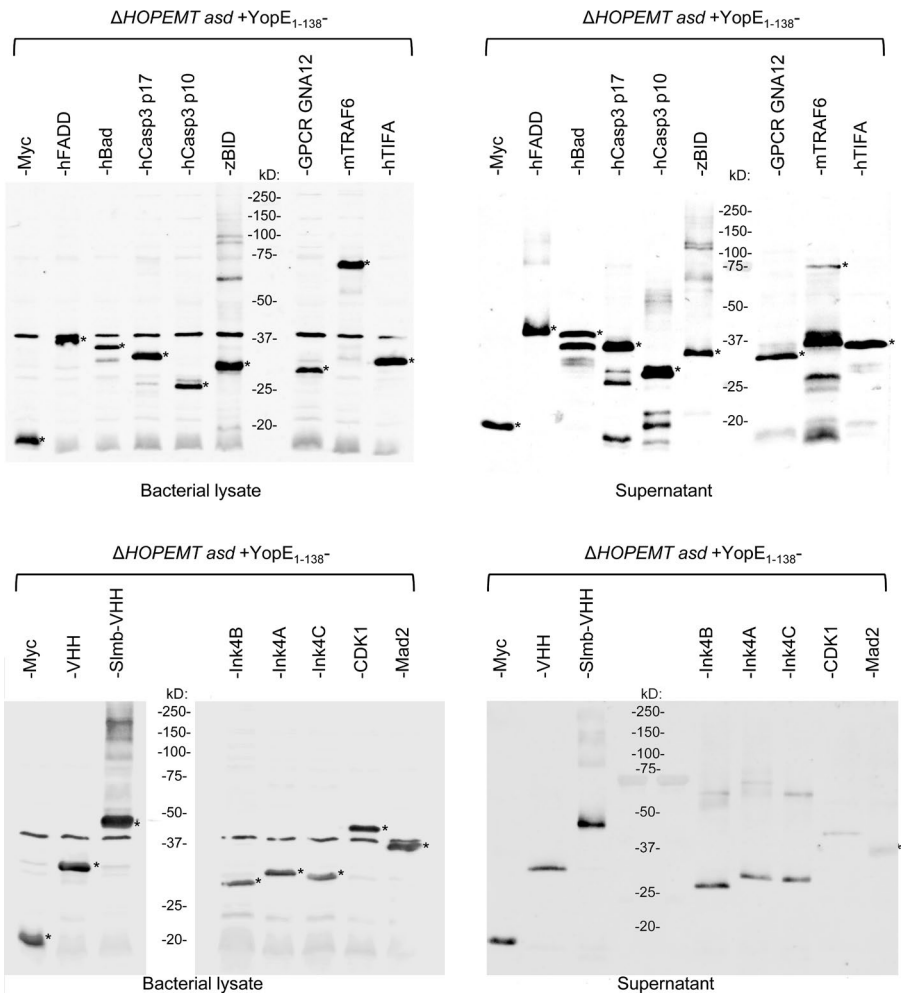


Figure S5. **T3S-dependent secretion of eukaryotic proteins.** In vitro secretion of indicated strains revealed by Western blot analysis using an anti-YopE antibody. As a control for protein expression, bacterial lysates of corresponding strains are shown. Asterisks mark the proteins of interest.

Table S1. **Known and predicted caspase-3 substrates from the tBID phosphoproteome**

Protein	Caspase-3 substrate status[a]
SPTB2	known
PAK2	known
TAOK1	known
ACINU	known
DBNL	known
IF4H	known
IF4G1	known
IF4B	known
HS90B	known
ICAL	known
NUMA1	known
PARG	known
PERQ2	predicted
CTND1	predicted
DOCK9	predicted
AHNK	predicted
AMPD2	predicted
TB182	predicted
SMG1	predicted
MYO9B	predicted
MYO1E	predicted
PKHG3	predicted
E41L1	predicted
TRRAP	predicted
ACINU	predicted
LIMC1	predicted
DOK1	predicted
DOCK1	predicted
NOP14	predicted
SPTN2	predicted
AKA12	predicted
CP131	predicted
SYMPK	predicted
CIRBP	predicted
MYCB2	predicted
FA21A	predicted
LS14A	predicted
M3K2	predicted
GCFC2	predicted
P2R3A	predicted
PEAK1	predicted
GAB2	predicted
PAIRB	predicted
INF2	predicted
SPAG1	predicted
NFAC4	predicted
ARHGA	predicted
RSF1	predicted
JIP4	predicted
IQGA2	predicted
ACACA	predicted
NGAP	predicted
NCOA7	predicted
CUX1	predicted
NAV1	predicted
CIC	predicted
PKP4	predicted
EF2K	predicted
GAPD1	predicted
PDE3A	predicted

Table S1. **Known and predicted caspase-3 substrates from the tBID phosphoproteome** *(Continued)*

Protein	Caspase-3 substrate status[a]
HECD1	predicted
OXSR1	predicted
PLEC	predicted
DVL3	predicted
ZFHX3	predicted
RBBP6	predicted
ZEP1	predicted
MEF2D	predicted
SI1L1	predicted
FLNB	predicted

[a]Ayyash et al., 2012.

Table S2. **List of primers used in this study**

Primer No.: Si_	Sequence (5′–3′)
285	CATACCATGGGAGTGAGCAAGGGCGAG
286	GGAAGATCTTTACTTGTACAGCTCGTCCAT
287	CGGGGTACCTCAACTAAATGACCGTGGTG
288	GTTAAAGCTTTTCGAATCTAGACTCGAGCGTGGCGAACTGGTC
296	CAGTCTCGAGACTAACATAACACTATCCACCCAG
297	GTTAAAGCTTTCAGGAGGCATTCTGAAG
306	GTTAAAGCTTGGAGGCATTCTGAAGATACTTATT
307	CAGTCTCGAGCAAATACAGAGCTTCTATCACTCAG
308	GTTAAAGCTTTCAAGATGTGATTAATGAAGAAATG
317	CAGTTTCGAACCCATAAAAAAGCCCTGTC
318	GTTAAAGCTTCTACTCTATCATCAAACGATAAAATGG
324	CAGTCTCGAGTTCACTCAAGAAACGCAAA
341	CGTATCTAGAAAAATGATGAAAATGGAGACTG
342	GTTAAAGCTTTTAGCTGGAGACGGTGAC
346	CAGTCTCGAGTTCCAGATCCCAGAGTTTG
347	GTTAAAGCTTTCACTGGGAGGGGG
351	CAGTCTCGAGCTCGAGTTATCTACTCATAGAAACTACTTTTGCAG
352	CGCGGATCCTCAGTGTCTCTGCGGCATTA
353	CATTTATTCCTCCTAGTTAGTCACAGCAACTGCTGCTCCTTTC
354	GAAAGGAGCAGCAGTTGCTGTGACTAACTAGGAGGAATAAATG
355	CGATTCACGGATTGCTTTCTCATTATTCCCTCCAGGTACTA
356	TAGTACCTGGAGGGAATAATGAGAAAGCAATCCGTGAATCG
357	CGTATCTAGACGGCTTTAAGTGCGACATTC
364	CGTATCTAGACTAAAGTATGAGGAGAGAAAATTGAA
365	GTTAAAGCTTTCAGCTTGCCGTCGT
367	CGTATCTAGAGACCCGTTCCTGGTGC
369	CGTATCTAGACCCCCCAAGAAGAAGC
373	GTTAAAGCTTGCTGGAGACGGTGACC
386	CGTATCTAGATCAGGACGCTTCGGAGGTAG
387	CGTATCTAGAATGGACTGTGAGGTCAACAA
389	CGTATCTAGAGGCAACCGCAGCA
391	GTTAAAGCTTTCAGTCCATCCCATTTCTG
403	CGTATCTAGATCTGGAATATCCCTGGACA
406	GTTAAAGCTTGTCTGTCTCAATGCCACAGT
410	CAGTCTCGAGATGTCCGGGGTGGTG
413	CAGTTTCGAATCACTGCAGCATGATGTC
417	CAGTCTCGAGAGTGGTGTTGATGATGACATG
420	CAGTTTCGAATTAGTGATAAAAATAGAGTTCTTTTGTGAG
425	GTTAAAGCTTTTACACCTTGCGCTTCTTCTTGGGCGGGCTGGAGACGGTGAC
428	CGTATCTAGAATGGACTTCAACAGGAACTTT
429	CGTATCTAGAGGACATAGTCCACCAGCG
430	GTTAAAGCTTTCAGTTGGATCCGAAAAAC
433	CGTATCTAGAGAATTAAAAAAAACACTCATCCCA
434	CGTATCTAGACCAAAGGCAAAAGCAAAAA
435	GTTAAAGCTTTTAGCTAGCCATGGCAAGC
436	CGTATCTAGAATGCCCCGCCCC
437	GTTAAAGCTTCTACCCACCGTACTCGTCAAT
438	CGTATCTAGAATGTCTGACACGTCCAGAGAG
439	GTTAAAGCTTTCATCTTCTTCGCAGGAAAAAG
445	CGCGGATCCTTATGGGTTCTCACAGCAAAA
446	CATTTATTCCTCCTAGTTAGTCAAGGCAACAGCCAATCAAGAG
447	CTCTTGATTGGCTGTTGCCTTGACTAACTAGGAGGAATAAATG
448	TTGATTGCAGTGACATGGTGCATTATTCCCTCCAGGTACTA
449	TAGTACCTGGAGGGAATAATGCACCATGTCACTGCAATCAA
450	CGTATCTAGATAGCCGCAGATGTTGGTATG
451	CGTATCTAGAGATCAAGTCCAACTGGTGG
452	CGTATCTAGAGCTAGCCCTAGGCGTACGACTAGTGATCAAGTCCAACTGGTGG
453	CCTAGCTAGCATGGTGAGCAAGGGCG
454	CATGACTAGTCTTGTACAGCTCGTCCATGC
463	CAGTCTCGAGGAAAGCTTGTTTAAGGGGC
464	CAGTTTCGAATTAGCGACGGCGACG

Table S2. **List of primers used in this study** *(Continued)*

Primer No.: Si_	Sequence (5′–3′)
476	GTTAAAGCTTTTACTTGTACAGCTCGTCCAT
477	CGTATCTAGAGTGAGCAAGGGCGAG
478	CAGTCTCGAGATGGAAGATTATACCAAAATAGAGAAA
479	GTTAAAGCTTCTACATCTTCTTAATCTGATTGTCCA
482	CGTATCTAGAATGGCGCTGCAGCT
483	GTTAAAGCTTTCAGTCATTGACAGGAATTTTG
486	CGTATCTAGAATGGAGCCGGCGGCG
487	GTTAAAGCTTTCAATCGGGGATGTCTG
492	CGTATCTAGAATGCGCGAGGAGAACAAGGG
493	GTTAAAGCTTTCAGTCCCCTGTGGCTGTGC
494	CGTATCTAGAATGGCCGAGCCTTG
495	GTTAAAGCTTTTATTGAAGATTTGTGGCTCC
504	CGTATCTAGAGAAAATCTGTATTTTCAAAGTGAAAATCTGTATTTTCAAAGTATGCCCCGCCCC
505	GTTAAAGCTTCCCACCGTACTCGTCAATTC
508	CGTATCTAGAGAAAATCTGTATTTTCAAAGTGAAAATCTGTATTTTCAAAGTATGGCCGAGCCTTG
509	GTTAAAGCTTTTGAAGATTTGTGGCTCCC
511	CGTATCTAGAGAAAATCTGTATTTTCAAAGTGAAAATCTGTATTTTCAAAGTGTGAGCAAGGGCGAG
512	CGTATCTAGAGAAAATCTGTATTTTCAAAGTGAAAATCTGTATTTTCAAAGTCCGCCGAAAAAAAAACGTAAAGTTGTGAGCAAGGGCGAG
513	GTTAAAGCTTTTAAACTTTACGTTTTTTTTTCGGCGGCTTGTACAGCTCGTCCAT
515	CGTATCTAGAGAAAATCTGTATTTTCAAAGTGAAAATCTGTATTTTCAAAGTGATTATAAAGATGATGATGATAAAATGGCCGAGCCTTG
558	CGTATCTAGAATGACCAGTTTTGAAGATGC
559	GTTAAAGCTTTCATGACTCATTTTCATCCAT
561	CGTATCTAGAATGAGTCTCTTAAACTGTGAGAACAG
562	GTTAAAGCTTCTACACCCCCGCATCA
585	CAGTCTCGAGATGCAGATCTTCGTCAAGAC
586	GTTAAAGCTTGCTAGCTTCGAAACCACCACGTAGACGTAAGAC
588	CAGTTTCGAAGATTATAAAGATGATGATGATAAAATGGCCGAGCCTTG

Three additional tables are provided online. Table S3 shows the list of strains used in the study. Table S4 shows quantification of protein phosphorylation by phosphoproteomics. Table S5 shows ontology analysis of the tBID phosphoproteome.

All source codes for computational methods are available online, including CellProfiler pipelines and the SafeQuant algorithm. CellProfiler (Version 2.1.1) pipelines for the different analyses can be found as .cppipe files (text files) in the online supplemental material. The Actin_meshwork.cppipe file corresponds to an analysis module used to quantify the actin meshwork induced by, e.g., SopE. It is based on actin staining intensity. The Count_nuclei_actin_foci.cppipe pipeline allows analysis of the number of actin foci per cell. The Count_nuclei_cell_size_nobinning.cppipie pileline allows analysis of the number of nuclei per image as well as the size of the cells, whereas the Count_Nuclei.cppipe pipeline simply counts nuclei per image. The Zebrafish_INF_PIPE.cppipe pipeline identifies bacterial spots and generates around each bacterial spot a circle with a radius of 10 pixels, where CASP3 p17 staining intensity was measured. The SafeQuant v1.0 R or v2.1 script (also available at https://github.com/eahrne/SafeQuant/) was used to process the Progenesis analysis results further to obtain protein relative abundances.

References

Ayyash, M., H. Tamimi, and Y. Ashhab. 2012. Developing a powerful in silico tool for the discovery of novel caspase-3 substrates: a preliminary screening of the human proteome. *BMC Bioinformatics.* 13:14. http://dx.doi.org/10.1186/1471-2105-13-14

Iriarte, M., and G.R. Cornelis. 1998. YopT, a new *Yersinia* Yop effector protein, affects the cytoskeleton of host cells. *Mol. Microbiol.* 29:915–929. http://dx.doi.org/10.1046/j.1365-2958.1998.00992.x

Kudryashev, M., M. Stenta, S. Schmelz, M. Amstutz, U. Wiesand, D. Castaño-Díez, M.T. Degiacomi, S. Münnich, C.K. Bleck, J. Kowal, et al. 2013. In situ structural analysis of the *Yersinia enterocolitica* injectisome. *eLife.* 2:e00792. http://dx.doi.org/10.7554/eLife.00792