Supporting Information (SI):

Supporting Appendix

The chaperonin TRiC/CCT is essential for the action of bacterial glycosylating protein toxins like *Clostridium difficile* toxins A and B

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SI: Materials and Methods

Materials, bacterial strains and plasmids

Restriction enzymes, T4 DNA ligase and Phusion® High-Fidelity DNA polymerase were from New England Biolabs (Ipswich, MA, USA). UDP-[^{14}C]glucose and UDP-[^{14}C]*N*-acetylglucosamine were from Biotrend (Cologne, Germany), [^{32}P]α-NAD and $^{86}Rb^{+}$ were from Perkin-Elmer Life Sciences (Rodgau, Germany). ATP and ADP were from Sigma-Aldrich (St-Louis, MO, USA). *Escherichia coli* BL21 (DE3) CodonPlus (Stratagene) was used for protein

expression of pET28a (Novagen, Madison WI, USA) vector constructs. *E. coli* TG1 was used for cloning and protein expression of pGEX-2TGL vector (GE Healthcare, Freiburg Germany) constructs. CCT4 and CCT5 ORF cDNA clones in pDONR223 vectors (Invitrogen) were obtained via the human ORFeome V5.1 collection (Open Biosystems), and were provided by the BIOSS toolbox Freiburg (Germany). HSF1A was from Axon Medchem (Groningen, The Netherlands). and radicicol from *D. chlamydosporia* was from Sigma-Aldrich. HSP70 and HSP90 proteins were from Enzo Life Sciences (Lörrach, Germany), recombinant GST-CCT5 was provided by Abnova (Taipei City, Taiwan). Diphtheria toxin (DT) from *Corynebacterium diphtheriae* was acquired from BioAcademia (Osaka, Japan).

Cloning Genes for bacterial expression

CCT4 and CCT5 genes were amplified with Phusion® High-Fidelity DNA polymerase from pDONOR233 cDNA clones with oligonucleotide primers (Table S1) with additional restriction sites for BamHI and SalI. The genes were ligated into a predigested pET-28a vector with an introduced Tobacco Etch Virus (TEV) protease cleavage site. All sequences of corresponding plasmids were confirmed by sequencing (GATC inc., Konstanz, Germany).

Recombinant protein expression

E. coli transformed with the desired plasmid was grown in Luria-Bertani broth supplemented with the corresponding antibiotics on a shaker at 37°C until A_{600}= 0.8. Protein expression was induced by 1 mM isopropyl-*β*-*D*-thioglactopyranoside (Roth, Karlsruhe) for 4-5 h at 22 °C for pET28-based plasmids, and 0.2 mM isopropyl-*β*-*D*-thiogalactopyranoside for 5 h at 37°C for pGEX-based plasmids. Bacterial cells were harvested by centrifugation at 6,000 *g* for 15 min and resuspended in lysis buffer [50 mM Tris-HCl pH 7.4, 150 mM NaCl, 25 mM imidazole, 30 µg DNaseI, 1 mM *β*-mercaptoethanol and protease inhibitor cocktail (Roche, Mannheim, Germany)]. In case of CCT4 and CCT5 purification, cells were resuspended in a buffer, containing 20 mM HEPES pH 7.4, 300 mM NaCl, 15 mM $MgCl_2$, 10 mM imidazole, 10% glycerol, 2 mM DTT, 1 mM ATP, 30 µg DNaseI and protease inhibitor cocktail. Cell lysis was

achieved by ultrasonic treatment. The cleared lysates were purified via chromatography on glutathione-Sepharose beads (Amersham Biosciences, Piscataway, NJ, USA) or Protino® Ni-IDA Resin (Macherey-Nagel, Düren, Germany) with Fast Flow columns, according to manufactures' instructions. Bound proteins were eluted with 10 mM reduced glutathione, 0.5 M imidazole or thrombin treatment depending on the construct used. Thrombin was removed by binding to a benzamidine-Sepharose (GE Healthcare) beads. Further purification and removal of small-molecule weight components was achieved by Sephadex G-25 columns (GE Healthcare).

2D gel electrophoresis

Jurkat cells were lysed in a buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl and protease inhibitor cocktail. Lysate was subjected treatment with SetA, and UDP-glucose (radiolabeled and unlabeled) in a buffer containing 50 mM Hepes pH 7.4, 2 mM $MgCl_2$, 1 mM $MnCl_2$ for 60 min at 30°C. Total reaction volume was 40 µl. Aceton precipitation was performed and the precipitated lysate was solubilized in a buffer containing 8 M urea, 2 M thiourea, 2% CHAPS, 60 mM DTT and 0.5% IPG buffer pH 4-7 (GE Healthcare). A volume of 360 µl was loaded on an IPG dry Strip (pH 4-7, 18 cm, GE Healthcare). IPG strips were rehydrated for 10 h at room temperature. Isoelectric focusing was performed on an IPGphor isoelectric focusing system (Pharmacia Biotech, Piscataway, NJ, USA with the following parameters: Current 50 µA per strip, step and hold 500 V with 0.5 kVh, gradient 1,000 V with 0.8 kVh, gradient 8,000 V with 7 kVh and step and hold 8,000 V with 16 kVh. IPG Strips were then equilibrated for 10 min in a buffer containing 50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS with the addition of 65 mM DTT and 10 min with the addition of 135 mM iodoacetamide (IAA). The IPG strip was then subjected to SDS-PAGE.

Mass spectrometry

After 2D SDS-PAGE excised gel spots were destained with 30% acetonitrile (ACN), shrunk with 100% ACN and dried in a vacuum concentrator. The Proteins were then digested with trypsin (0.1 µg per gel spot) overnight at 37 °C in 0.1 M NH_4HCO_3, pH 8.0. Extraction of peptides from the gel matrix was performed with 5% formic acid. Extracted peptides were analyzed by nanoLC-MS/MS on an Orbitrap Fusion (Thermo Scientific) mass spectrometer equipped with an EASY-Spray Ion Source and coupled to an EASY-nLC 1000 (Thermo Scientific). Peptides were loaded on a trapping column (2 cm x 75 µm ID, PepMap C18, 3 µm particles, 100 Å pore size) and separated on an EASY-Spray column (25 cm x 75 µm ID, PepMap C18, 2 µm particles, 100 Å pore size) with a 30-minute linear gradient from 3% to 40% acetonitrile and 0.1% formic acid. Both MS and MS/MS scans were acquired in the Orbitrap analyzer with a resolution of 60,000 for MS scans and 15,000 for MS/MS scans. A TopSpeed method that performs HCD on doubly protonated peptides and HCD and ETD on higher protonated peptides was applied; singly charged precursors were excluded from selection. EASY-IC was used for internal calibration. Raw data were processed with Mascot Distiller 2.5 and database searching was performed with Mascot Server 2.5 against UniProt database of human proteins (Database: 1_UniProt_Human 2014_07, 88,949 sequences, Fig. S1C). The following parameters were applied: peptide mass tolerance: 10 ppm, MS/MS mass tolerance: 0.6 Da, enzyme: trypsin; variable modifications: oxidation (M) and acetyl (protein N-term).

Surface plasmon resonance spectrometry

$TcdB^{GT}$ was covalently immobilized via amine-coupling on a CM-5 sensor chip (GE Healthcare) after EDC/NHS activation according to manufacturer's instructions. Coupling was achieved in a 10 mM acetate buffer of pH 3.7. Interaction analysis was conducted at 25°C using a Biacore X100 biosensor (GE Healthcare) in a running buffer containing 10 mM HEPES pH 7.4, 150 mM KCl, 2 mM MgCl2, and 0.01% Tween-20. Single pulse of 0.5 M NaCl for 100 seconds was

used for sensor regeneration. Binding affinities were calculated using the Biacore X100 evaluation software.

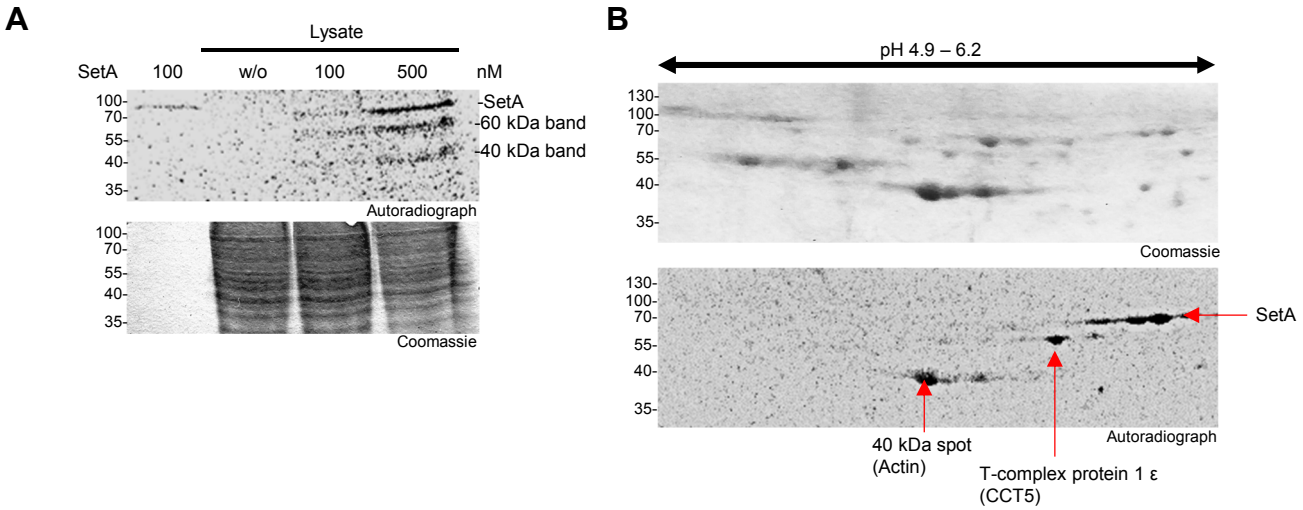
$^{86}Rb^{+}$ release assay

CHO-K1 cells (2 x 10^5 cells) were seeded in 24-well culture plates containing complete medium (Ham`s F-12 + 10% FCS) together with $^{86}Rb^{+}$ (0.5 µCi/ml) and incubated for 48 h. After incubation cells were washed with two times with PBS and incubated for 1 h at 37°C with DMSO, HSF1A (50 µM and 100 µM) containing complete medium. Cells were then cooled to 4°C and recombinant TcdB was added in a concentration of 0.5 nM and incubated for another 1 h to allow the toxin to bind to the cells. Toxin-containing medium was then removed and membrane insertion of the toxin was induced via addition of fresh pre-warmed medium (F-12 with 20 mM Hepes pH 8.0 or F-12 with 20 mM MES pH 4.8) for 5 min. The cells were then cooled again to 4°C and incubated for 40 min. $^{86}Rb^{+}$ efflux was measured via transfer of the medium for liquid scintillation counting.

Autoproteolytic cleavage of TcdB

Recombinant TcdB (0.5 µM) was incubated for 1 h at 37 °C together with 0.1 mM inositol hexakisphosphate (InsP6), 1 mM DTT, HSF1A (50 µM or 100 µM) or DMSO. Autoproteolytic cleavage of TcdB was analyzed via SDS-PAGE and Coomassie staining.

SI Figures



C

#	Accession	Score	Sequence number	Description
1	P48643|TCPE_HUMAN	1299	29	T-complex protein 1 subunit epsilon (CCT5)
2	P10809|CH60_HUMAN	237	9	60 kDa heat shock protein, mitochondrial
3	P04264|K2C1_HUMAN	177	7	Keratin, type II cytoskeletal 1
4	Q13283|G3BP1_HUMAN	63	2	Ras GTPase-activating protein-binding protein 1
5	Q8TF72|SHRM3_HUMAN	39	1	Protein Shroom3

Database: UniProt: *Homo sapiens*

Fig. S1. Modification of a 60 kDa protein from lysates by *Legionella* glycosyltransferase SetA. (A) Autoradiograph and Coomassie staining of SDS-PAGE of *in vitro* glucosylation of Jurkat cell lysate (100 µg) by the *Legionella pneumophila* glucosyltransferase SetA using UDP-[^{14}C]glucose. Besides SetA autoglucosylation, two distinct bands at 60 kDa and 40 kDa were glucosylated. (B) Coomassie and autoradiograph of 2D SDS-PAGE of Jurkat lysate (300 µg), treated with SetA (500 nM) and UDP-[^{14}C]glucose. The pH scale indicates isoelectric point ranges of proteins separated by isoelectric focusing. (C) Mass spectrometry results of 60 kDa Protein of 2D SDS-PAGE shown in B. The Protein with the highest Mascot score was T-complex protein 1 subunit epsilon (Unitprot: P48643|TCPE_HUMAN). The Score is −10log(P), where P is the probability that the observed match is a random event. Scores greater than 31 are significant ($p < 0.05$).

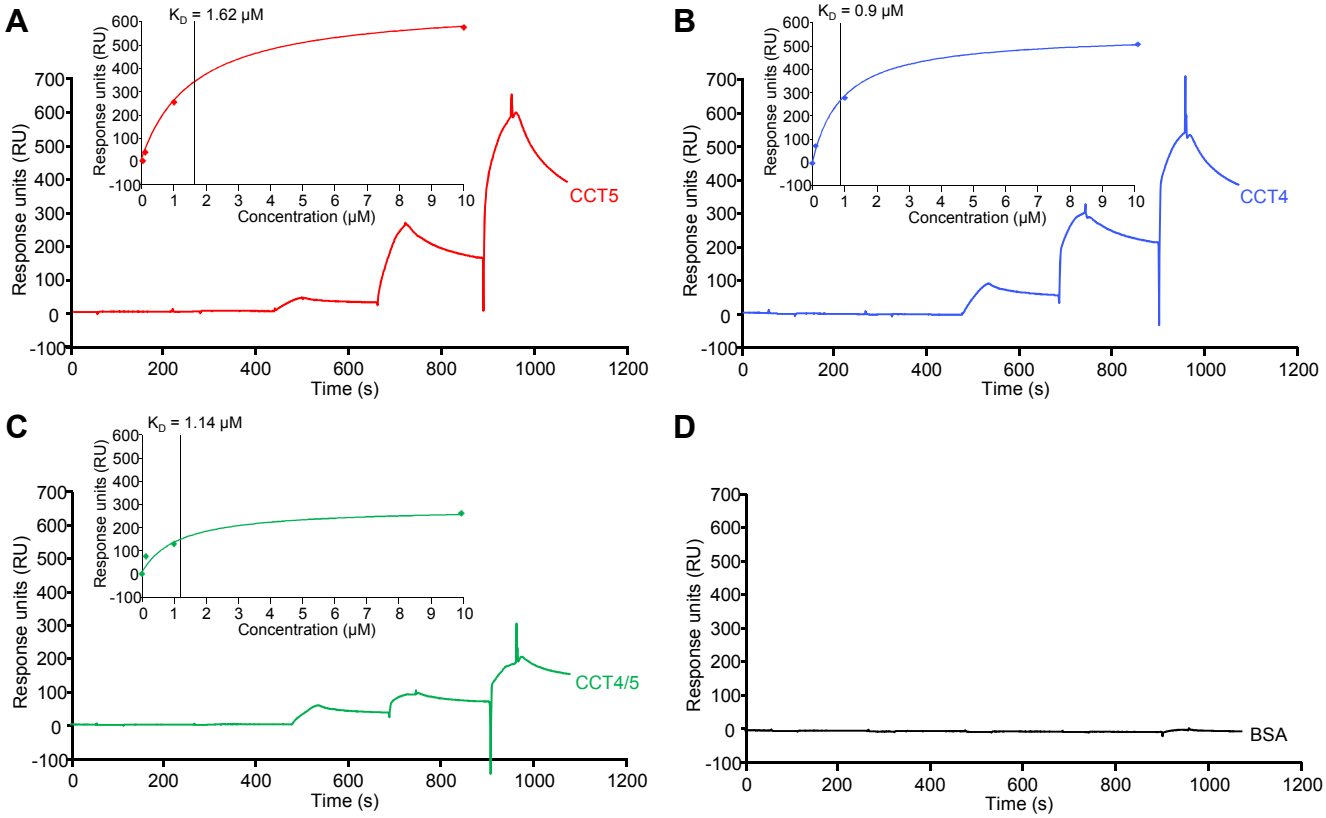


Fig. S2. TcdB^{GT} interacts with subunits of the TRiC/CCT complex. (A-D) Surface plasmon resonance spectroscopy affinity determination of the interaction of TcdB^{GT} with CCT-subunits. Increasing concentrations of CCT5 (A), CCT4 (B), and a complex of CCT4/5 (C) were run over a TcdB^{GT}-immobilized surface sensor chip. Increasing BSA-concentrations were used as control (D).

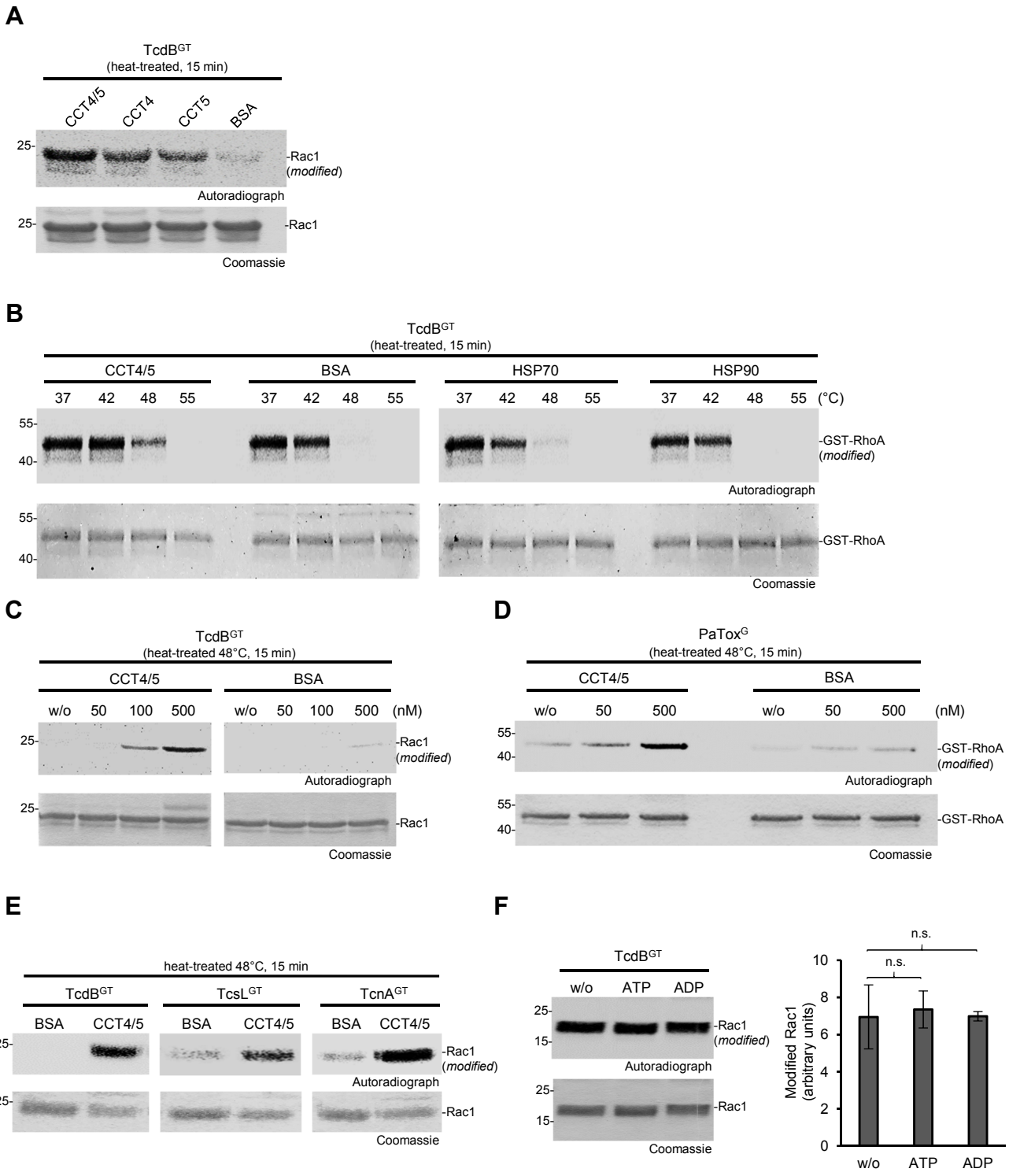


Fig. S3. Activity of various glycosyltransferase toxins are recovered by CCT4/5 after heat treatment. Autoradiograph and Coomassie of SDS-PAGE of recovery assays with heat-treated (t= 15 min) glycosyltransferases. (A) After TcdB^{GT} heat treatment BSA, CCT4, CCT5 or CCT4/5 (1:1 ratio) were added together with ATP (0.5 mM) for TcdB^{GT} activity recovery (1 h, 30 °C). (B) Heat-treated TcdB^{GT} (100 nM, temperatures are indicated) was incubated in the presence of either CCT4/5 (100 nM) or BSA (100 nM), HSP70 (100 nM) or HSP90 (100 nM) in a buffer containing 50 mM Hepes pH 7.4, 2 mM $MgCl_2$, 1 mM $MnCl_2$ and 1 M KCl and 0.5 mM ATP for 1 h at 30°C. Subsequently, recovery of TcdB^{GT} activity was determined by glycosylation of GST-RhoA using UDP-[^{14}C]glucose. (C) Recovery assay of heat-treated TcdB^{GT} with Rac1 (1 µg) instead of GST-RhoA. (D) Autoradiograph and Coomassie-stained SDS-PAGE of the CCT4/5-dependent recovery of the glycosyltransferase activity of Rho-modifying enzyme domain of *Photorhabdus asymbiotica* toxin PaTox^{G} after heat treatment (48°C, 15 min). BSA served as control. Concentrations of CCT4/5 and of BSA as indicated. (E) Recovery assays with different clostridial glycosyltransferases. *C. sordellii* lethal toxin (TcsL) and *C. novyi* alpha toxin (TcnA) (100 nM each) were analyzed in an *in vitro* glycosylation assay after heat treatment. BSA or CCT4/5 (each 200 nM) was added for recovery (1 h, 30°C). For TcnA^{GT} mediated glycosylation of Rac1 radiolabeled UDP-[^{14}C]GlcNAc (10 µM) was used as donor-substrate instead of UDP-[^{14}C]glucose (10 µM). (F, *Left*) Effect of addition of nucleotides (0.5 mM each) to the glycosyltransferase reaction of TcdB^{GT} (200 nM) and Rac1 (1 µg). (F, *Right*) Quantification of autoradiograph is shown. Values are average ± SD (n = 2). Student's t-test was applied for statistical comparison. n.s. = not significant, $p > 0.05$.

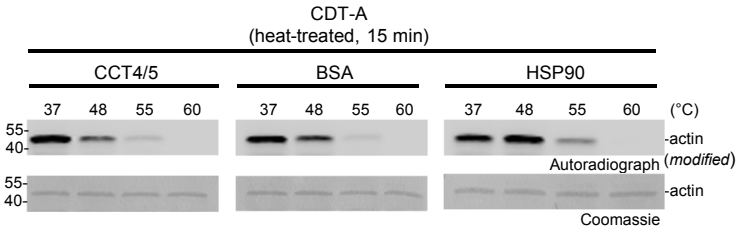


Fig. S4. CDT-A mediated ADP-ribosylation of actin after heat treatment and recovery. Autoradiograph and Coomassie staining of a SDS-PAGE after CDT-A (100 nM) heat treatment at different temperatures for 15 min. For the recovery reaction CCT4/5, BSA and HSP90 (100 nM each) and ATP (0.5 mM) were added for 1 h at 30°C. The enzymatic activity was measured *via* ADP-ribosylation of actin with radiolabeled [^{32}P]NAD (10 mM) as donor substrate.

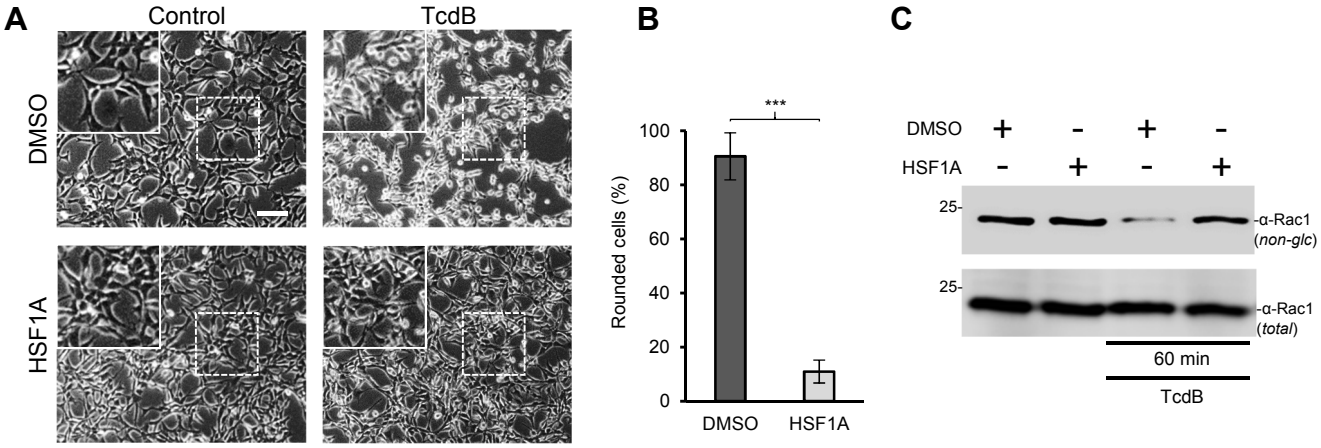


Fig. S5. HSF1A inhibits TcdB-mediated intoxication of MEF cells. (A) DIC micrographs of MEF cells pretreated with 100 µM HSF1A or DMSO as control for 1 h before intoxication with TcdB (5 pM). Pictures were taken after 60 min of intoxication. (Scale bar, 100 µm.) (*Insets*) Magnifications from dotted areas. (B) Quantification of TcdB-intoxicated MEF cells after 60 min pretreatment with DMSO or HSF1A. Percentage of rounded cells per picture (>500 cells) is given as mean ± SD (n = 8). Student's t-test was applied for statistical comparison. ***, $p < 0.001$. (C) DMSO or HSF1A (100 µM) pretreated MEF cells were incubated for 60 min with or without addition of TcdB (5 pM). Cell lysates were analyzed by Western blot with the glycosylation specific anti-Rac1 antibody (MAB102) which cannot recognize glucosylated Rac1. Anti-Rac1 (23A8) antibody was used as input control.

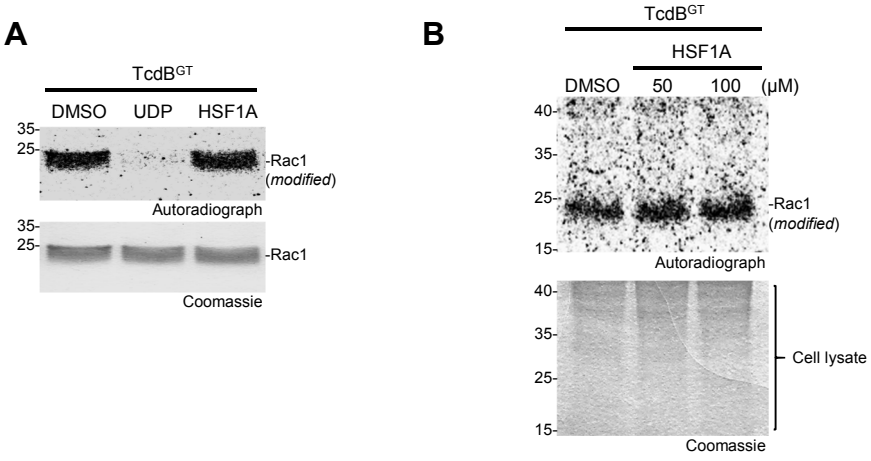


Fig. S6. HSF1A does not interfere with TcdB^{GT} mediated Rac1 glucosylation *in vitro*. (A) Autoradiograph and Coomassie staining of SDS-PAGE of Rac1 (1 µg) incubated with TcdB^{GT} (1 nM) in presence of [^{14}C]-labeled UDP-glucose with addition of DMSO, UDP (100 µM) or HSF1A (100 µM). (B) Addition of DMSO and HSF1A to an *in vitro* glucosylation reaction of HeLa lysate together with TcdB^{GT}. Coomassie gel was used as input control.

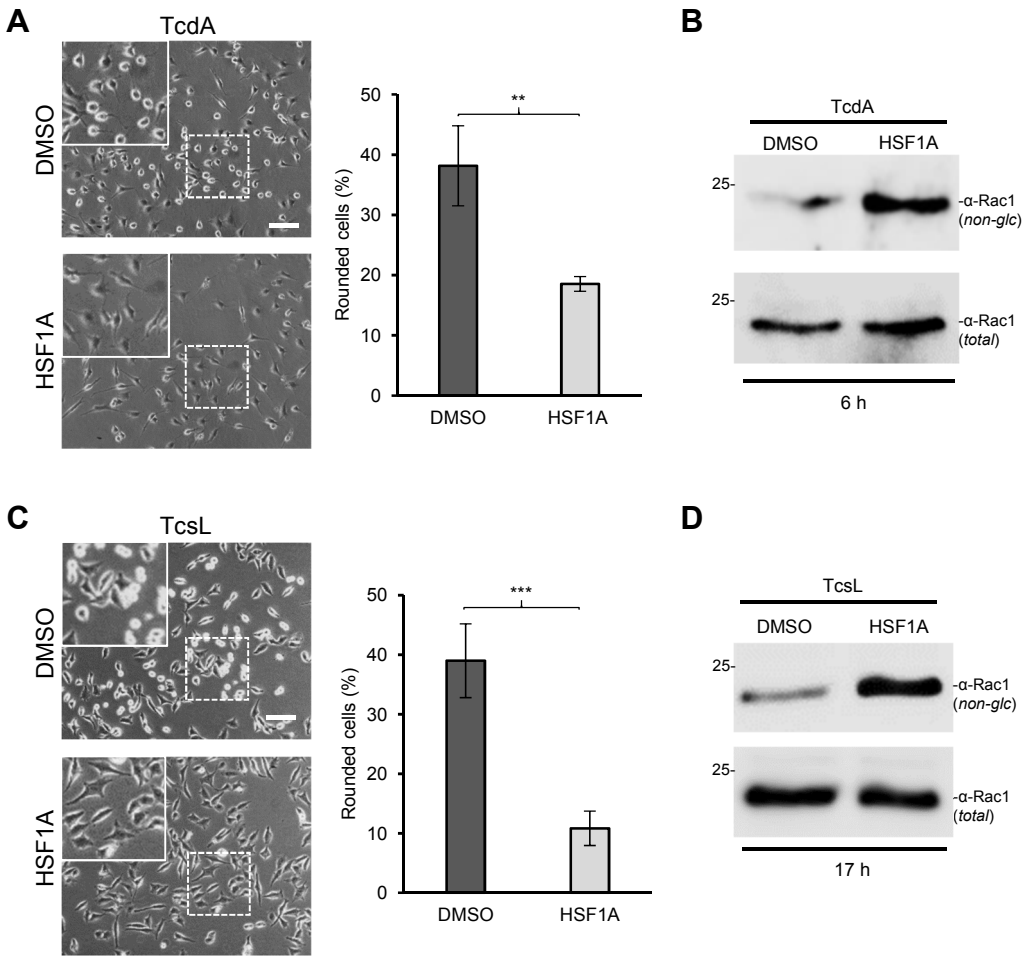


Fig. S7. HSF1A inhibits *C. difficile* TcdA and *C. sordellii* TcsL mediated intoxication of HeLa cells. (A, *Left*) HeLa cells were pretreated with 100 µM HSF1A or DMSO (control) for 1 h before intoxication with TcdA (0.5 nM). Pictures were taken after 6 h. (Scale bar, 100 µm.) (*Insets*) Magnification of dotted areas. (A, *Right*) Percentage of rounded cells per picture (>100 cells) is given as mean ± SD (n = 3). Student's t-test was applied for statistical comparison. **, $p < 0.01$. (B) Western blot analysis of TcdA intoxication of DMSO- or HSF1A-pretreated HeLa cells with anti-Rac1 (MAB102) antibody, which cannot recognize glycosylated Rac1. (C, *Left*) HeLa cells were pretreated with 50 µM HSF1A or DMSO (control) for 1 h before intoxication with TcdL (0.5 nM). Pictures were taken after 17 h. (Scale bar, 100 µm.) (*Insets*) Magnification of dotted areas. *(*C, *Right*) Percentage of rounded cells per picture (>100 cells) is given as mean ± SD (n = 5). Student's t-test was applied for statistical comparison. ***, $p < 0.001$. (D) DMSO or HSF1A (50 µM) pretreated HeLa cells (1 h) were incubated for 17 h with TcsL (0.5 nM). Cell lysates were analyzed *via* Western blot with anti-Rac1 (MAB102) antibody.

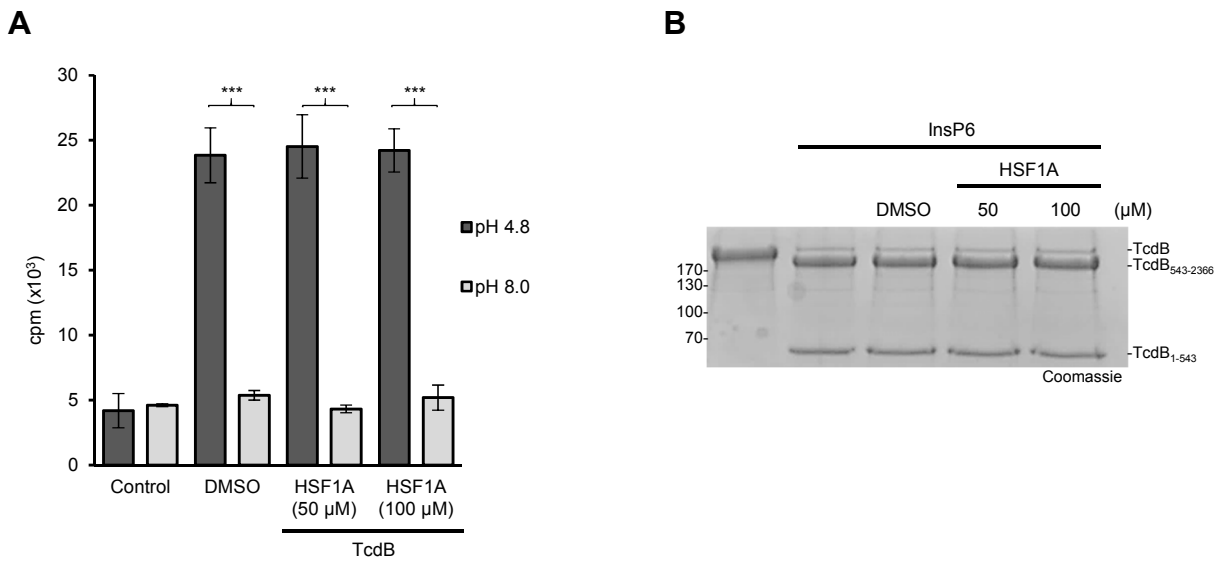


Fig. S8. HSF1A does not affect pore formation or autocatalytic cleavage of TcdB. (A) $^{86}Rb^{+}$ release assay. CHO-cells were preloaded with $^{86}Rb^{+}$ and preincubated with HSF1A for 1 h at 37°C. TcdB (0.5 nM) was added after cooling the cells to 4°C. Pore formation of TcdB was induced by an acidic pH-shift (pH 4.8) for 5 min and extracellular $^{86}Rb^{+}$ was measured (counts per minute, cpm). Error bars indicate ± SD (n = 3). Student's t-test was applied for statistical comparison. ***, $p < 0.001$. (B) SDS-PAGE of TcdB after induction of autoproteolytic cleavage of the toxin with InsP6 (0.1 nM) and addition of HSF1A (50 and 100 µM) for 1 h at 37°C. In the absence of InsP6 no autocleavage is observed.

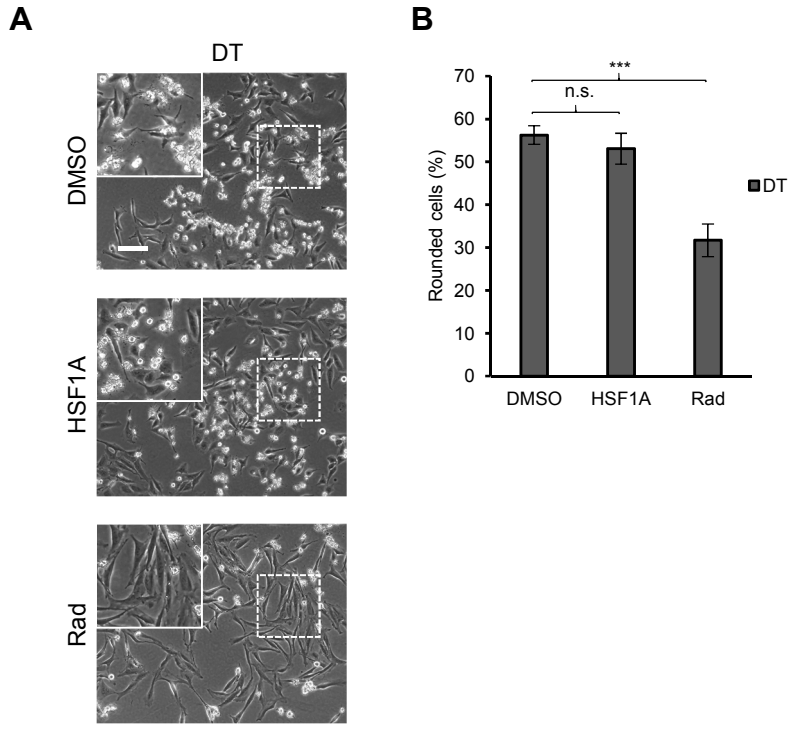


Fig. S9. HSF1A does not inhibit Diphtheria Toxin (DT) mediated intoxication of HeLa cells. (A) HeLa cells were pretreated with 100 µM HSF1A or with 50 µM radicicol (Rad) for 1 h at 37°C before intoxication. DMSO was used as control. The cells were intoxicated with 1 nM Diphtheria Toxin DT from *Corynebacterium diphtheriae*. Pictures were taken after 5 h. (Scale bar, 100 µm.) (*Insets*) Magnifications of dotted areas. (B) Percentage of rounded cells per picture (>150 cells) is given as mean ± SD (n = 8). Student's t-test was applied for statistical comparison. ***, $p < 0.001$; n.s. = not significant, $p > 0.05$.

SI Table

Supplementary table S1: oligonucleotides used in this study

#	name	sequence (5’ to 3’)
oligoTJ346	fw BamHI hCCT5	TCAAGGGATCCATGGCGTCCATGGGGACCCTCG
oligoTJ347	rv hCCT5 SalI	GTTGAGTCGACTCATTCTTCAGATTCTCCAGGC
oligoTJ348	fw BamHI hCCT4	TCAAGGGATCCATGCCCGAGAATGTGGCACCCC
oligoTJ349	rv hCCT4 SalI	GTTGAGTCGACTTATCGAGTGTTTACCACATCATC