SUPPLEMENTAL DATA

A neutralizing antibody that blocks delivery of the enzymatic cargo of *Clostridium difficile* toxin TcdB into host cells

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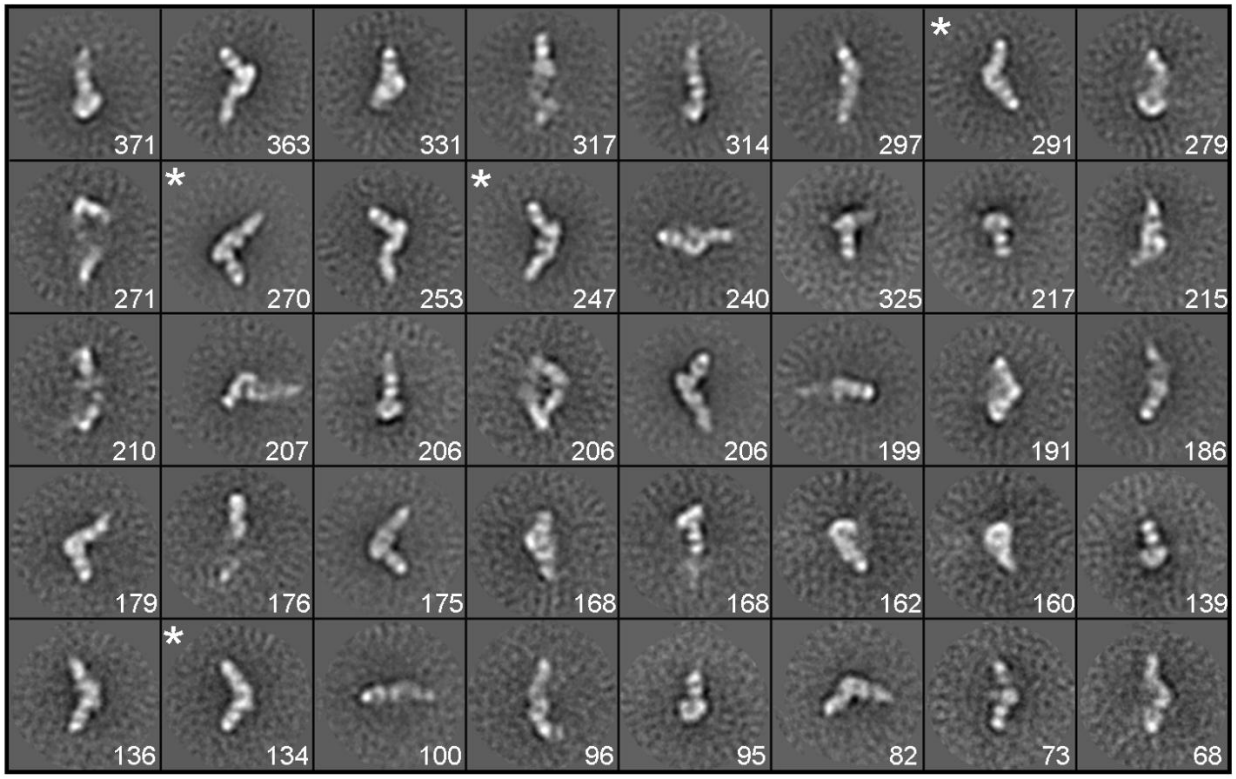

Figure S1. Reference-free 2D class averages (8,301 particles; 40 classes) for $TcdB_{1\text{-}1810}$ in complex with PA41 Fab (negative-stain data). Classes included in Figure 1 are labeled with asterisks, and the total number of particles in each class is in *white*. Side length of each panel is 44.9 nm.

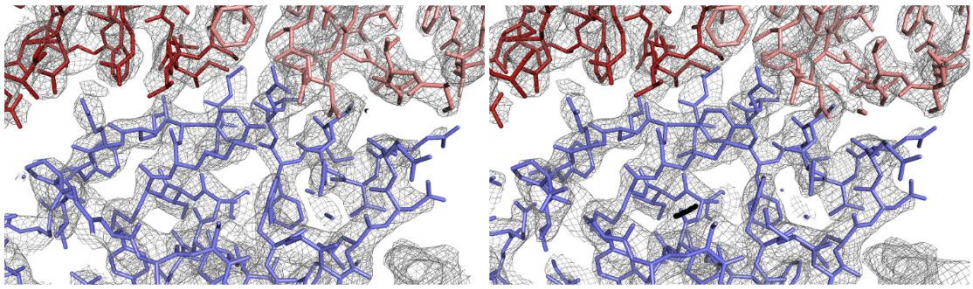

Figure S2. Stereo view of the $2mF_o$-DF_c electron density map superimposed over the coordinates of the PA41 epitope on TcdB-GTD (contour 1.5 σ). TcdB-GTD is shown in *blue*, PA41 Fab heavy chain in *red*, PA41 light chain in *pink*.

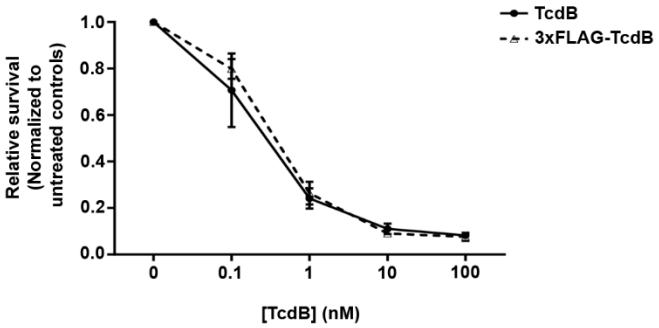

Figure S3. N-terminal 3xFLAG tag does not affect TcdB function. Caco-2 cells were treated with indicated concentrations of TcdB or 3xFLAG-$TcdB_{WT}$ in triplicate. ATP levels were determined using CellTiterGlo and normalized to signal from untreated cells to assess the relative survival of cells post-toxin treatment. Results represent the mean ±SD of three independent experiments.

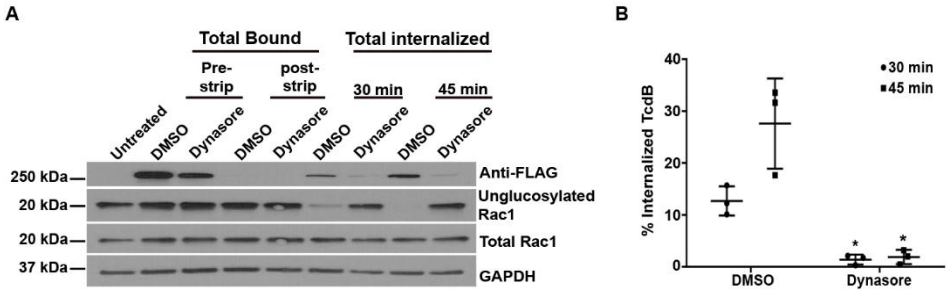

Figure S4. Validation of the TcdB entry assay using dynasore. (A) Caco-2 monolayers were pretreated with either 80 μM dynasore or with an equal amount of DMSO control for 1 h at 37 °C. Cells were switched to 4°C for 1 h and then intoxicated with 25 nM 3xFLAG-TcdB$_{C698A}$ (cleavage-defective mutant of TcdB). Toxins were allowed to bind at 4°C for 1 h and then internalize at 37 °C for the indicated time points. Levels of bound toxin, internalized toxin, unglucosylated Rac1, total Rac1 and GAPDH were assessed as described in Methods. Comparison of bound toxin levels between pre- and post-strip conditions confirms efficient removal of surface-bound toxin by this procedure. (B) Three independent replicates of the experiments shown in (A) were quantified by densitometry. The relative amounts of internalized TcdB were determined by normalizing the internalized toxin signal to that of total bound toxin (pre-strip), and the values were expressed as a percentage. Results reflect the mean ± SD and were analyzed using two-tailed t-test. *$p<0.05$.

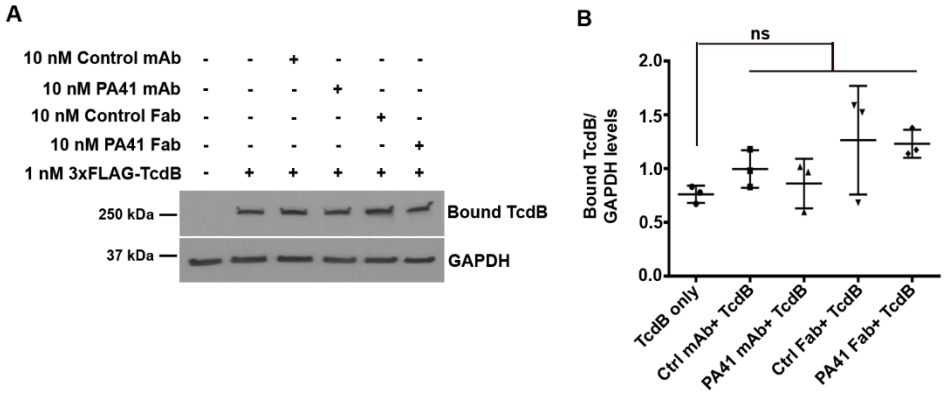

Figure S5. PA41 does not affect TcdB binding to CHO-K1 cells. (A) TcdB binding to CHO-K1 cells in the presence of isotype control or PA41 antibody (mAb or Fab) was assessed as described in SI Methods. Cells that did not receive any treatment and cells that received only toxin were used as controls. (B) Experiments shown in (A) were quantified by densitometry, and relative binding of TcdB to cells was determined by normalizing bound TcdB levels to that of GAPDH. Results reflect the mean ± SD of three independent experiments and were analyzed using one-way ANOVA. p-values were generated using Dunnett's multiple comparisons test. ns, not significant.

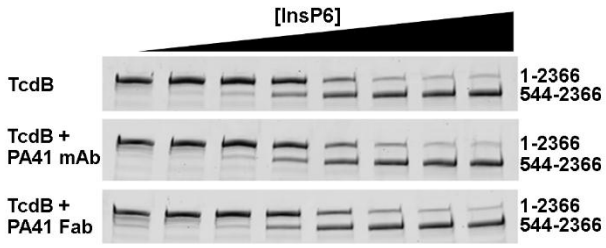

Figure S6. ***In vitro*** **autoprocessing of TcdB holotoxin.** InsP6-dependent autoprocessing of TcdB holotoxin alone (200 nM) or in the presence of equivalent antibody binding sites (200 nM) of PA41 mAb or Fab. Bands corresponding to full length toxin (residues 1-2366) and toxin without GTD (residues 544-2366) are shown. Lane 1 contains no InsP6, and the rest of the lanes contain 0.001-1000 µM InsP6. A representative replicate from three total is shown.

Table S1. Contacting residues in the TcdB-GTD and PA41 Fab complex. Residues with hydrogen bonds are in **bold**, with the rest of the residues involved in non-bonded contacts. Salt bridges are in *italics*. Residues in parentheses are not located in a CDR.

PA41 Fab chain	PA41 Fab Residues	TcdB-GTD Residues
Heavy chain (V_H)		
CDR-H1	Asn-31	Ser-354
	Phe-33	Lys-324, Glu-325
CDR-H2	***Arg-50***	***Glu-325***
	Asn-52	Glu-325
	Ala-57	Glu-325
CDR-H3	**Thr-100**	**Lys-324, Ala-351**
	Ile-101	Tyr-323, Glu-347, Leu-350, Ala-351
	Thr-102	**Lys-322, Tyr-323**
	Ser-103	**Glu-347,** Tyr-323
	Leu-105	Ala-351
Light chain (V_κ)		
CDR-L1	Ser-28	Glu-340
	Val-29	Ser-344
	Gly-30	Glu-340, Glu-341, Ser-344
	Thr-31	**Ser-344**
	Ser-32	**Ser-344**, **Glu-347**
CDR-L2	Phe-50	Glu-347, Ser-348, Ala-351
	Glu-53	**Ser-348**
	(Ser-67)	**Glu-341**
CDR-L3	**Ser-91**	**Glu-347**
	Asn-92	Glu-340, Gln-343
	Lys-93	Glu-340

Table S2.

Sequence alignment of TcdB-GTD subdomain containing the PA41 epitope from multiple strains of *C. difficile* (1). Epitope residues are in *yellow*, with the histidine mutant noted in *green*.

Toxinotype	Ribotype	Strain	Sequence (GTD residues 290-360)
IX		51680	LPGIHPDLFKDINKPDSVKTAVDWEEMQ-LEAIMKYKEYIPEYTSKHFDTLDEEVQSSFESVLASKSDKSEIF
XVII		J9965	LPGIHPDLFKDINKPDSVKTAVDWEEMQ-LEAIMKYKEYIPEYTSKHFDTLDEEVQSSFESVLASKSDKSEIF
XV		R9385	LPGIHPDLFKDINKPDSVKTAVDWEEMQ-LEAIMKYKEYIPEYTSKHFDTLDEEVQSSFESVLASKSNKSEIF
X	036	8864	LPGIHPDLFKDINKPDSVKTAVDWEEMQ-LEAIMKYKEYIPEYTSKHFDTLDEEVQSSFESVLASKSDKSEIF
VIII	017	F1470	LPGIHPDLFKDINKPDSVKTAVDWEEMQ-LEAIMKHKEYIPEYTSKHFDTLDEEVQSSFESVLASKSDKSEIF
XXI		CH6223	LPGIHPDLFKDINKPDSVKTAVDWEEMQ-LEAIMKHKEYIPEYTSKHFDTLDEEVQSSFESVLASKSDKSEIF
IIIa		SE844	LPGIQPDLFESIEKPSSV--TVDFWEMVKLEAIMKYKEYIPGYTSEHFDMLDEEVQSSFESVLASKSDKSEIF
IIIb		R12087	LPGIQPDLFESIEKPSSV--TVDFWEMVKLEAIMKYKEYIPGYTSEHFDMLDEEVQSSFESVLASKSDKSEIF
IIIc		CH6230	LPGIQPDLFESIEKPSSV--TVDFWEMVKLEAIMKYKEYIPGYTSEHFDMLDEEVQSSFESVLASKSDKSEIF
XII		IS25	LPGIQPDLFESIEKPSSV--TVDFWEMTKLEAIMKYKEYIPEYTSEHFDMLDEEVQSSFESVLASKSDKSEIF
XVI		SUC36	LPGIQPDLFESIEKPSSV--TVDFWEMTKLEAIMKYKEYIPGYTSEHFDMLDEEVQSSFESALASKSDKSEIF
VI		51377	LPGIQPDLFESIEKPSSV--TVDFWEMTKLEAIMKYKEYIPGYTSEHFDMLDEEVQSSFESALASKSDKSEIF
VII		57267	LPGIQPDLFESIEKPSSV--TVDFWEMTKLEAIMKYKEYIPGYTSEHFDMLDEEVQSSFESALASKSDKSEIF
IV		55767	LPGIQPDLFESIEKPSSV--TVDFWEMTKLEAIMKYKEYIPGYTSEHFDMLDEEVQSSFESALASKSDKSEIF
	078		LPGIQPDLFESIEKPSSV--TVDFWEMTKLEAIMKYKEYIPGYTSEHFDMLDEEVQSSFESALASKSDKSEIF
	027	R20291	LPGIQPDLFESIEKPSSV--TVDFWEMVKLEAIMKYKEYIPGYTSEHFDMLDEEVQSSFESVLASKSDKSEIF
	027	CD196	LPGIQPDLFESIEKPSSV--TVDFWEMVKLEAIMKYKEYIPGYTSEHFDMLDEEVQSSFESVLASKSDKSEIF
0	003	VPI 10463	LPGIQPDLFESIEKPSSV--TVDFWEMTKLEAIMKYKEYIPEYTSEHFDMLDEEVQSSFESVLASKSDKSEIF
0	003	CD630	LPGIQPDLFESIEKPSSV--TVDFWEMTKLEAIMKYKEYIPEYTSEHFDMLDEEVQSSFESVLASKSDKSEIF

Supplemental Methods

Cell culture. Caco-2 cells (ATCC HTB-37) were maintained in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals), 1% MEM non-essential amino acids (M7145; Sigma), 1% Hepes buffer (15630080; Gibco) and 1% sodium pyruvate (S8636; Sigma). Chinese Hamster Ovary (CHO-K1, ATCC CCL-61) cells were grown in Ham's F-12 medium supplemented with 10% FBS. Dynasore (D7693; Sigma) was dissolved in DMSO to obtain a 25 mM stock and was used at a final concentration of 80 μM. Dynasore experiments were performed under serum-free media conditions as the inhibitor binds to serum proteins and loses activity (2).

Viability assays. Caco-2 cells were seeded at a density of 1,000 cells per well in a 384-well plate and incubated at 37 °C for 48 h. Cells were then challenged with serial dilutions of untagged or N-terminal 3xFLAG-tagged TcdB in triplicate. Cellular ATP was quantified 24 h post intoxication by addition of CellTiter-Glo (G7571; Promega) and used as a measure of cellular viability. Relative cell survival was determined by normalizing the ATP levels of toxin-treated cells to untreated controls.

Cell binding assays. CHO-K1 cells were seeded at a density of 600,000 cells/well in a 6-well plate format and incubated at 37 °C for 24 h. For the binding assay, cells were switched to 4 °C for 1 h and then intoxicated with 1 nM 3xFLAG-TcdB that was preincubated for 30 min on ice with 10-fold excess (10 nM) of isotype control or PA41 antibody (mAb or Fab). Toxin-antibody mixture was allowed to bind cells at 4 °C for 1 h. Media containing unbound toxin were then removed, and cells were washed twice with ice cold PBS. Cells were dislodged by using a cell scraper, collected, and pelleted at 1000 g for 5 min. Additionally, cells that did not receive any treatment and cells that received only toxin were used as controls. Cell pellets were homogenized in lysis buffer (10 mM Tris pH 7.4, 250 mM sucrose, 3 mM imidazole) supplemented with protease inhibitor cocktail (1:100, P8340; Sigma). Lysates were subjected to SDS-PAGE and Western blot analyses. The blot was probed with primary antibodies against TcdB (anti-FLAG, F1804; Sigma) and GAPDH (2118; Cell Signaling). Binding of anti-mouse (7076S; Cell Signaling) or anti-rabbit (7074S; Cell Signaling) HRP-linked secondary antibodies was detected using ECL Western Blotting Substrate (32106; Pierce). Densitometry quantifications were performed using Fiji (3).

***In vitro* autoprocessing assay.** Assays were performed as previously described (4), with minor modifications, using 3xFLAG-$TcdB_{WT}$ (200 nM) either alone or in the presence of equivalent binding sites (200 nM) of PA41 mAb or Fab. Individual reactions containing TcdB and antibody were set up in 20 mM Tris pH 8.0, 100 nM NaCl, 1 mM DTT. Increasing concentrations of $InsP_6$ (0 to 1 mM; Sigma P5681, phytic acid dipotassium salt) were added to the reactions, which were then incubated at 37 °C for 2 h. Reactions were quenched by addition of hot sample buffer and boiled for 2 min, and proteins were separated by SDS-PAGE on a 4-20% polyacrylamide gel and visualized by Coomassie staining. Three independent replicates were performed, with a representative experiment shown in the figure.

Supplemental References

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3. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.-Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P., and Cardona, A. (2012) Fiji: an open-source platform for biological-image analysis. *Nat. Methods*. **9**, 676–682

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