Supplemental Information

Materials and Methods

Western blots. YAMC or HeLa cells were seeded in a six well plate and incubated overnight at 33 °C or 37 °C. Cells were intoxicated with 10 nM TcdA or TcdB for 3 or 1 hours, respectively, washed, and then lysed. Antibodies against TcdA (ab19953, Abcam), TcdB (20B3) (34), glucosylated Rac1 (clone 23A8, EMD Millipore), or total Rac1 (clone 102, BD Biosciences) were used to probe the lysates and were detected with anti-mouse HRP (7076, Cell Signaling).

Live/Dead assay. HeLa cells were seeded in a 96 well plate and incubated overnight at 33 °C or 37 °C. Cells were challenged with 10 nM TcdA, 100 pM TcdA, 10 nM TcdB, or 10 pM TcdB and incubated at 33 °C or 37 °C. Brightfield images were captured on an Olympus IX73 equipped with a Q color 3 camera at 1 h, 4 h, and 18 h post-intoxication. One hour prior to the 18 h acquisition, cells were washed with PBS and treated with LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells (ThermoFisher, L3224) for 30 min at room temperature. Images were acquired using the fluorescein and rhodamine filter sets on the Olympus IX73.

Figure Legends

Fig. S1. TcdA and TcdB bind YAMC cells and glucosylate Rac1 similarly at 33 °C and 37 °C. YAMC cells were seeded overnight at 33 °C or 37 °C and challenged with A, 10 nM TcdA for 3 hours or B, 10 nM TcdB for 1 hour. Cells were washed, lysed, and lysates were immunoblotted for TcdA, TcdB, non-glucosylated Rac1, and total Rac1.

Fig. S2. TcdA and TcdB bind HeLa cells and glucosylate Rac1 similarly at 33 °C and 37 °C. HeLa cells were seeded overnight at 33 °C or 37 °C and challenged with A, 10 nM TcdA for 3 hours or B, 10 nM TcdB for 1 hour. Cells were washed, lysed, and lysates were immunoblotted for TcdB, non-glucosylated Rac1, and total Rac1.

Fig. S3. TcdA and TcdB induce similar cytopathic effects and cytotoxicity at 33 °C and 37 °C. HeLa cells were plated and incubated overnight at 33 °C or 37 °C, intoxicated with TcdA (10 nM, 100 pM), TcdB (10 nM, 10 pM), or mock buffer and incubated at 33 °C or 37 °C. Cell rounding was determined by brightfield imaging after an overnight exposure. Cytotoxicity was assessed by addition of a live-dead indicator prior to analyzing the overnight exposure.

Fig. S4. TcdB D286/288N induces ROS production and cell death similarly to wild-type TcdB. A, YAMCs were tested for ROS production in response to TcdB (black bars) or TcdB D286/288N (gray bars) after 6 h using a fluorescent ROS reporter. Statistical analysis by two-way ANOVA revealed no statistical significance between TcdB and TcdB D286/288N. B, Viability of YAMCs was assessed in response to TcdA (white bars), TcdA D285/287N (light gray bars), TcdB (black bars), and TcdB D286/288N (dark gray bars) after 18 hours. Data represent the average of three experiments performed in triplicate. Error bars represent the standard deviation of the means.

Fig. S5. Simultaneous intoxication with TcdA and TcdB results in cell death more similar to TcdB. Viability of YAMCs challenged with 10 nM TcdA (white bars), 10 nM TcdB (black bars), or 10 nM TcdA + 10 nM TcdB (gray bars) was assessed 18 hours after intoxication. Data

represent the average of three experiments performed in triplicate. Error bars represent the standard deviation of the means.

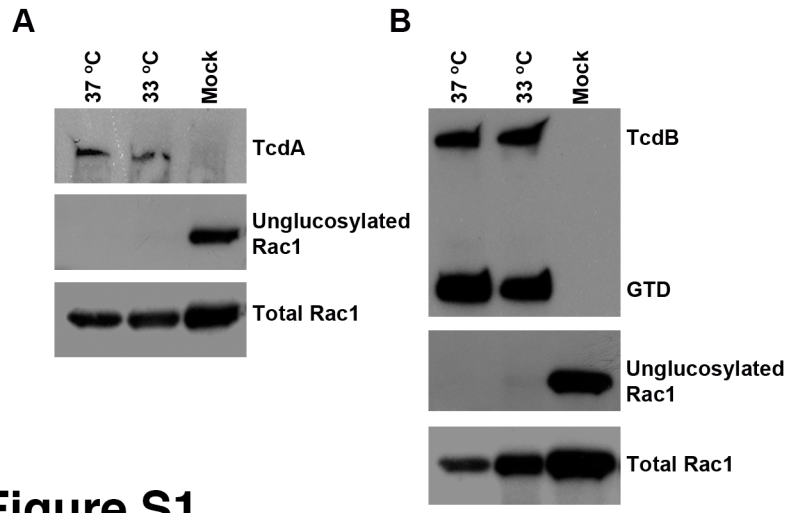

Figure S1

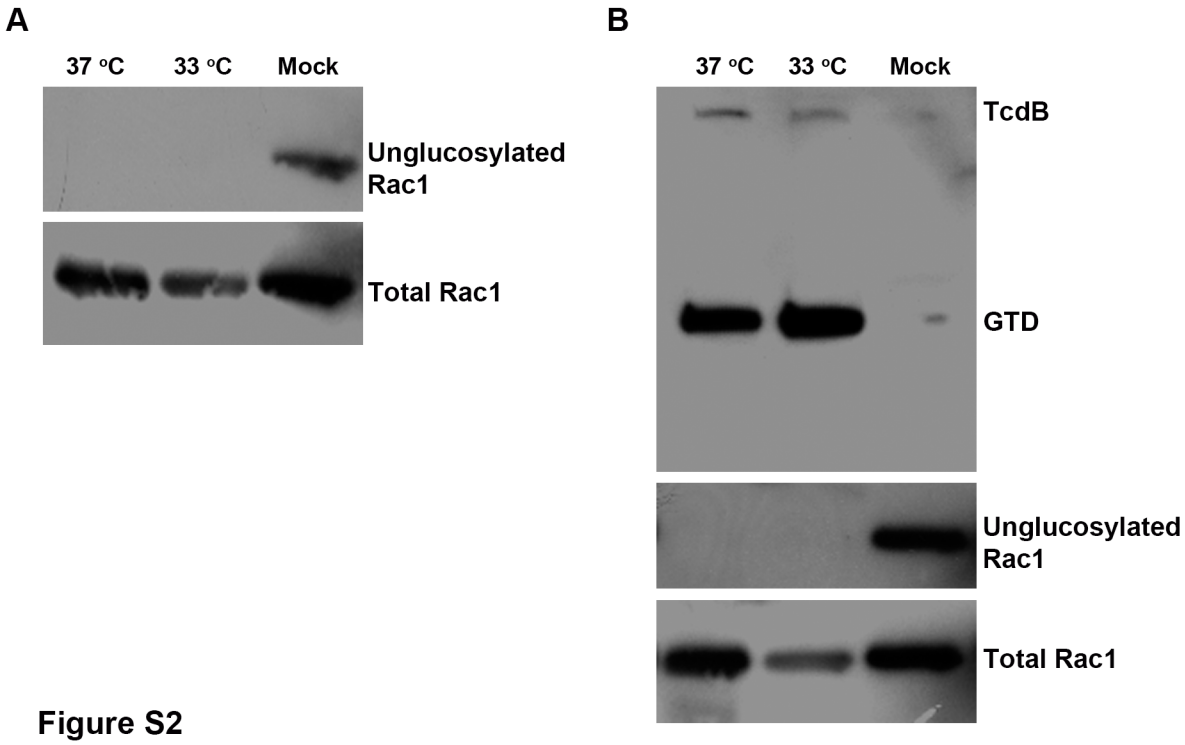

Figure S2

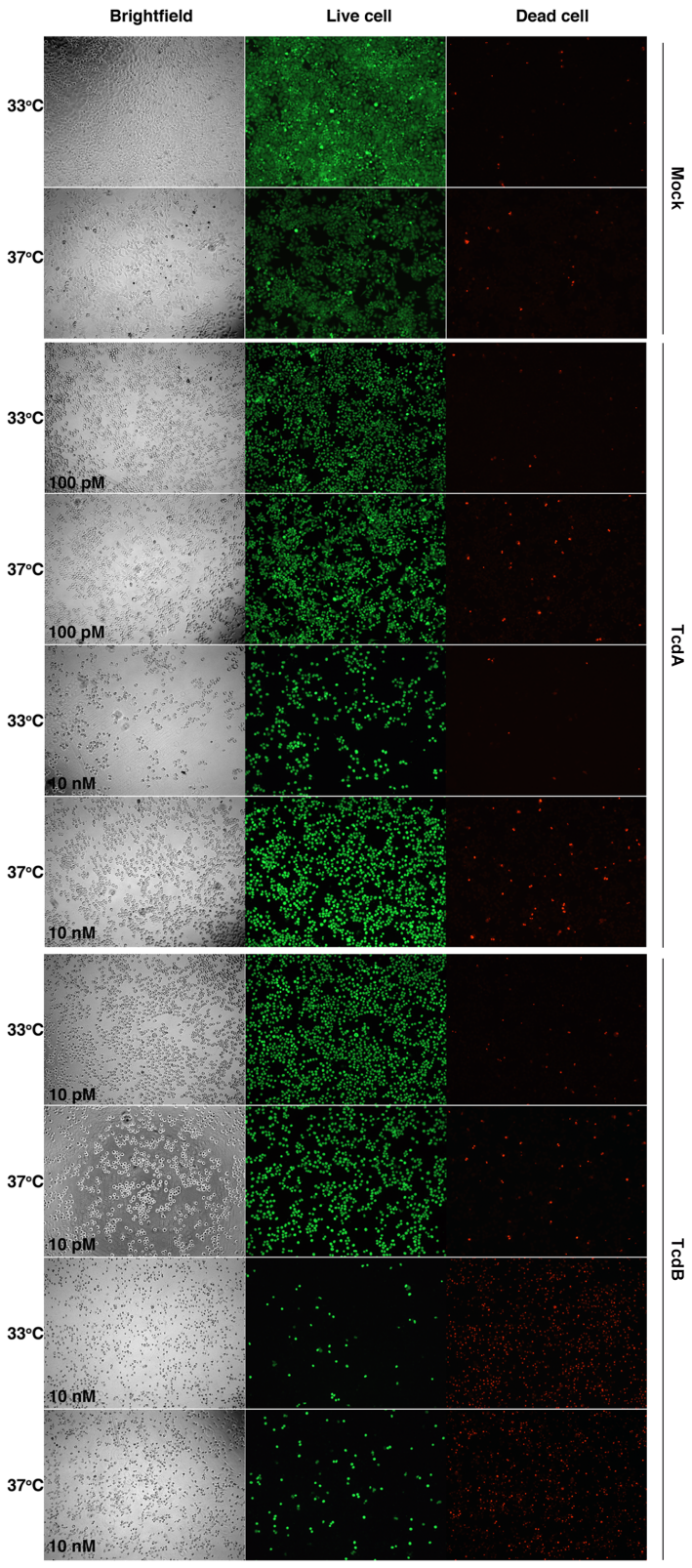
Brightfield
Live cell
Dead cell
33°C
37°C
Mock
33°C
100 pM
37°C
100 pM
33°C
10 nM
37°C
10 nM
TcdA
33°C
10 pM
37°C
10 pM
33°C
10 nM
37°C
10 nM
TcdB

Figure S3

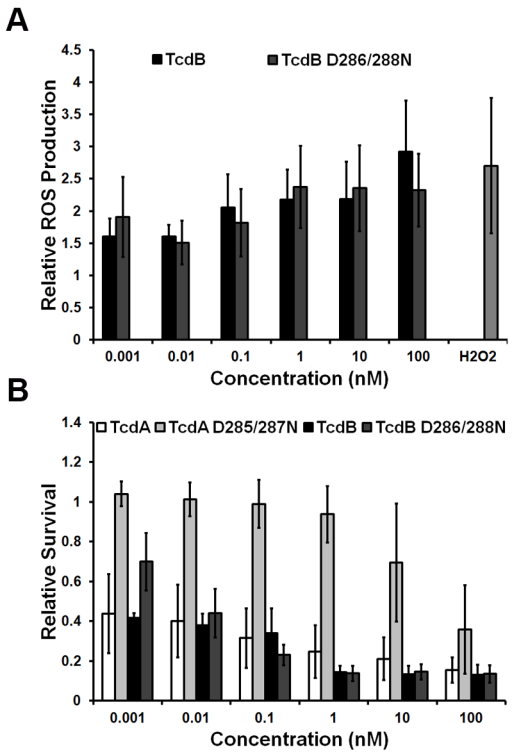
A
TcdB
TcdB D286/288N
Relative ROS Production
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0.001
0.01
0.1
1
10
100
H2O2
Concentration (nM)
B
TcdA
TcdA D285/287N
TcdB
TcdB D286/288N
Relative Survival
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.001
0.01
0.1
1
10
100
Concentration (nM)

Figure S4

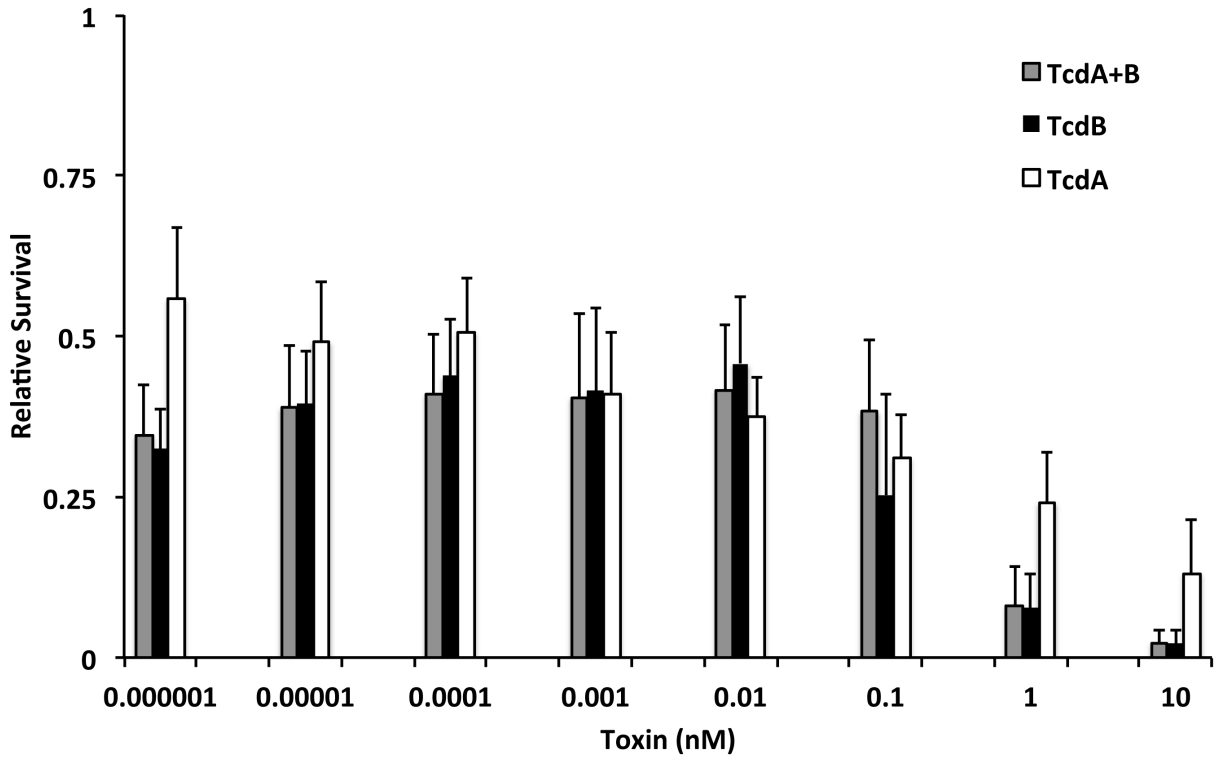
1
0.75
0.5
0.25
0
Relative Survival
TcdA+B
TcdB
TcdA
0.000001
0.00001
0.0001
0.001
0.01
0.1
1
10
Toxin (nM)

Figure S5