Modular domain swapping among the bacterial cytotoxic necrotizing factor (CNF) family for efficient cargo delivery into mammalian cells

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Running title: *CNF Modular Domain Swapping*

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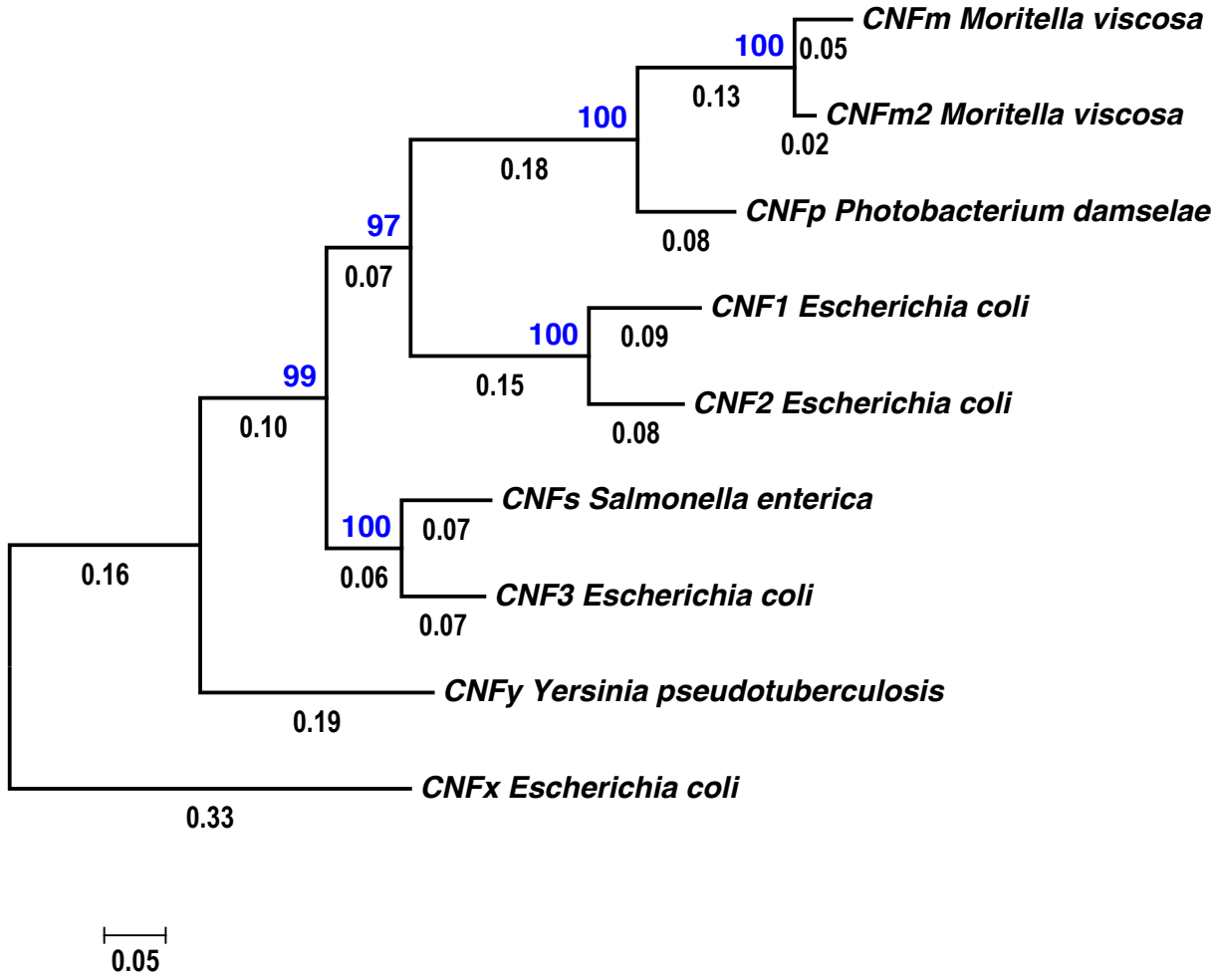
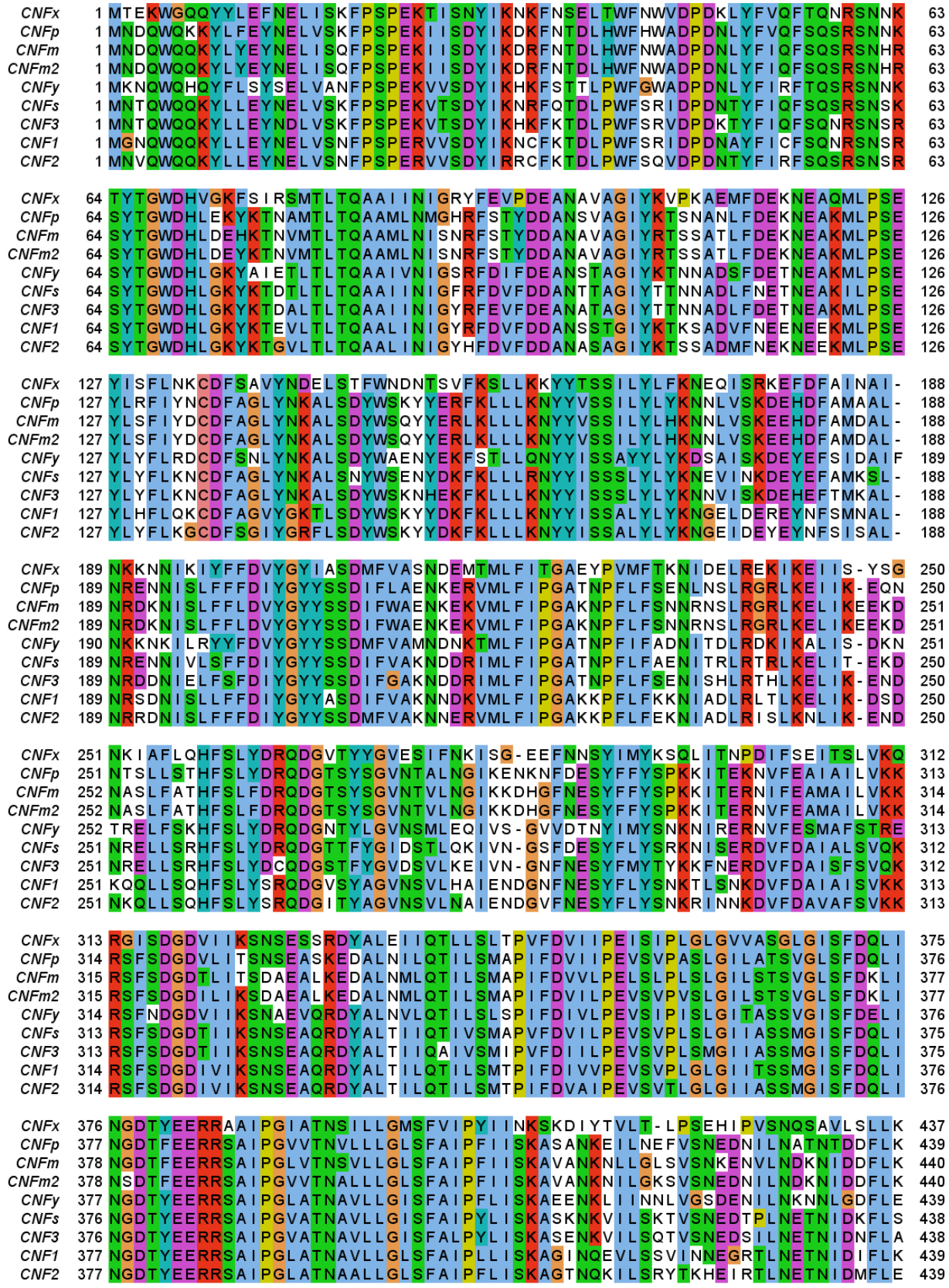
Supplementary Information



Supplementary Figure S1A. Molecular phylogenetic analysis by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (1). The tree with the highest log likelihood (-10668.36) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches (Blue). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (below the branches). The analysis involved 9 amino acid sequences. All positions containing gaps and missing data were eliminated. There was a total of 1004 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (2). NCB Accession # WP045110427 CNFm, NCB Accession # WP075533205 CNFm2, NCB Accession # WP005306733 CNFp, NCB Accession # CAA50007 CNF1, NCB Accession # WP057108870 CNF2, NCB Accession # WP079952502 CNFs, NCB Accession # WP024231387 CNF3, NCB Accession # WP012304286 CNFy, NCB Accession # WP059330985 CNFx.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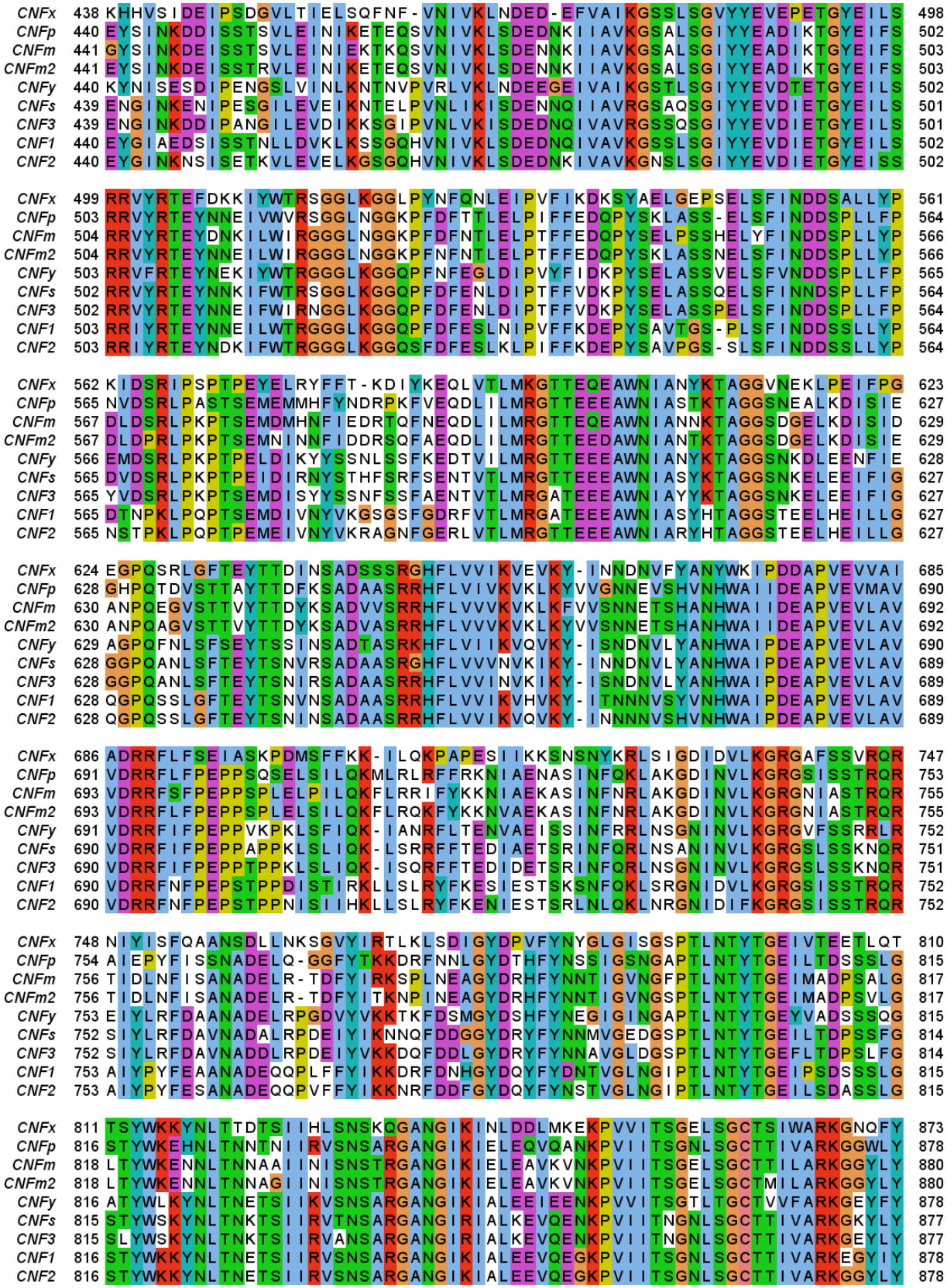
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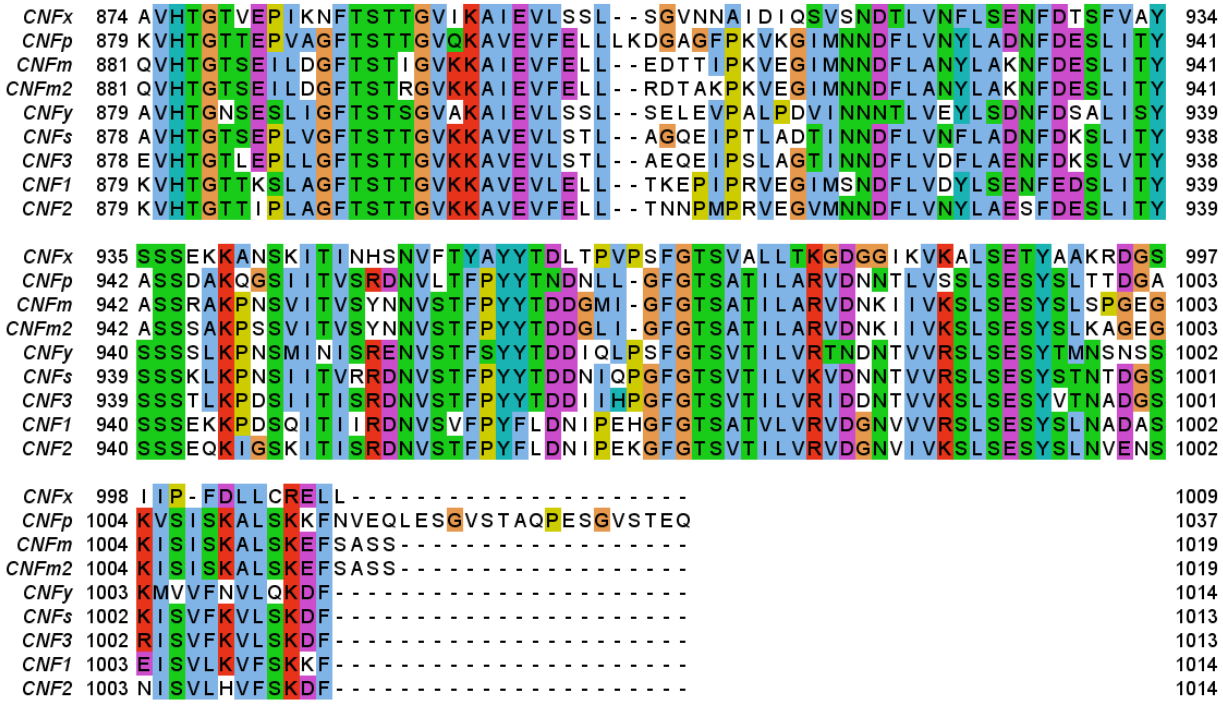


Figure S1B: Amino acid alignment of CNF toxin homologs shown in Supplementary Figure S1A. The alignment was generated using Muscle (39) and visualized using Jalview (40) to color the amino acid residue sequences in Clustal format.

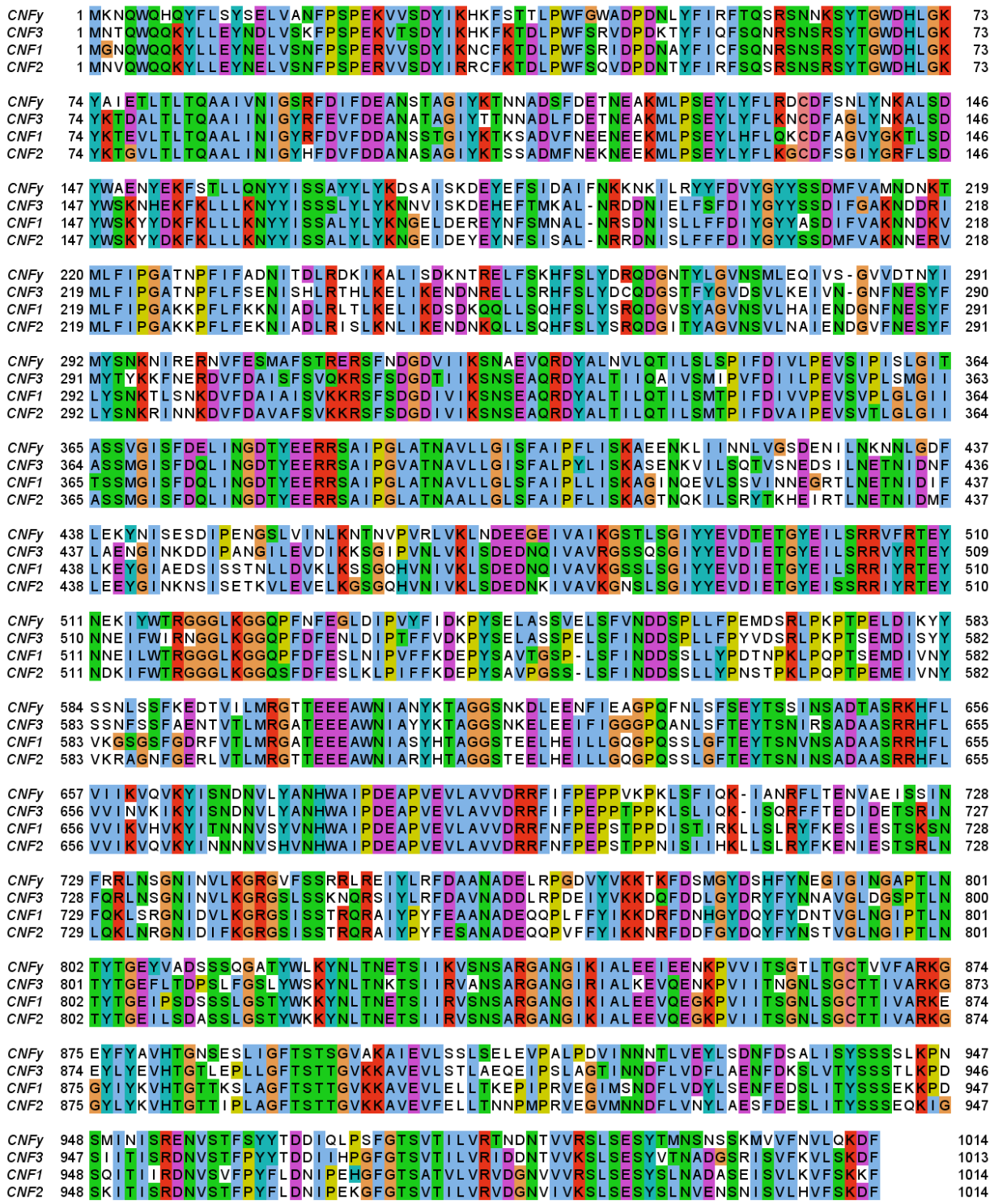
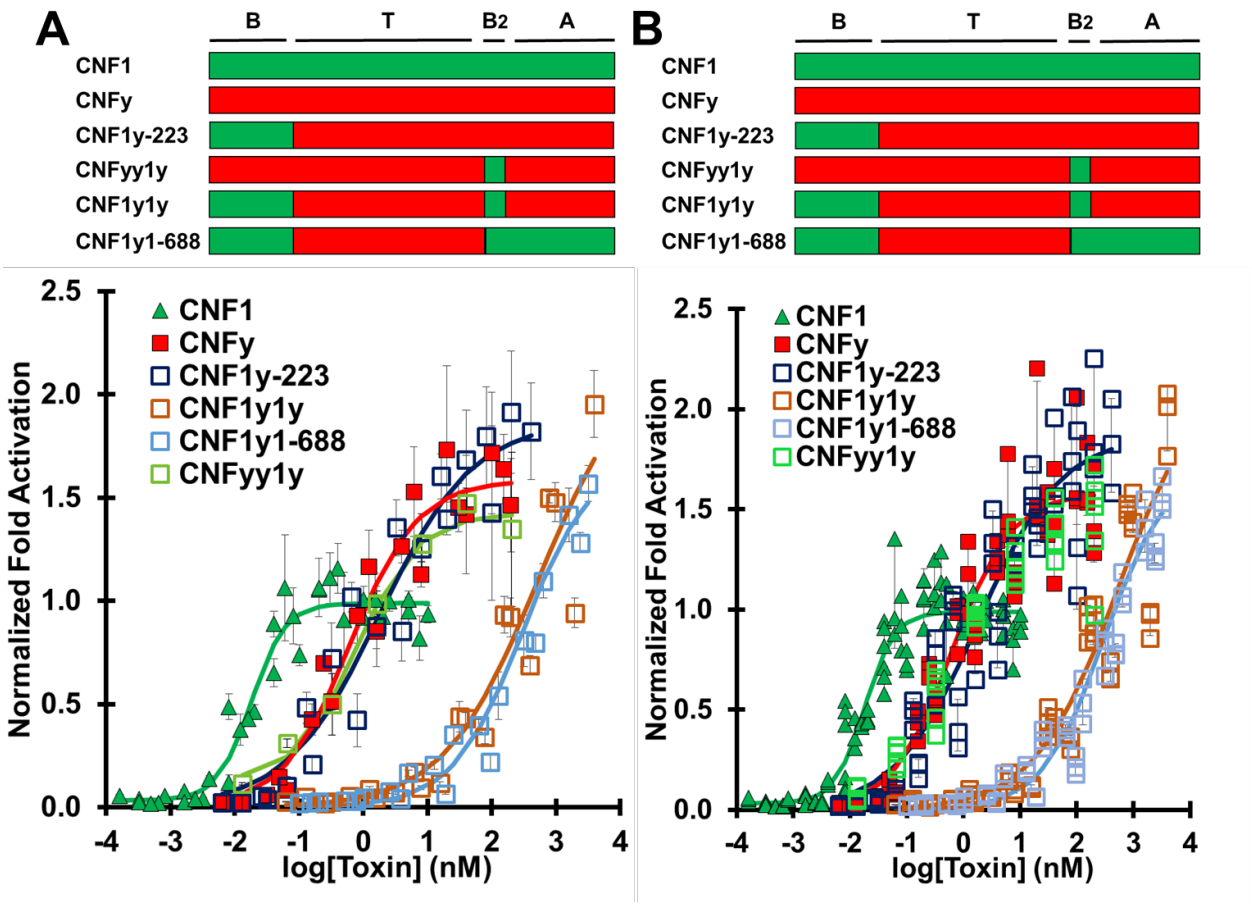
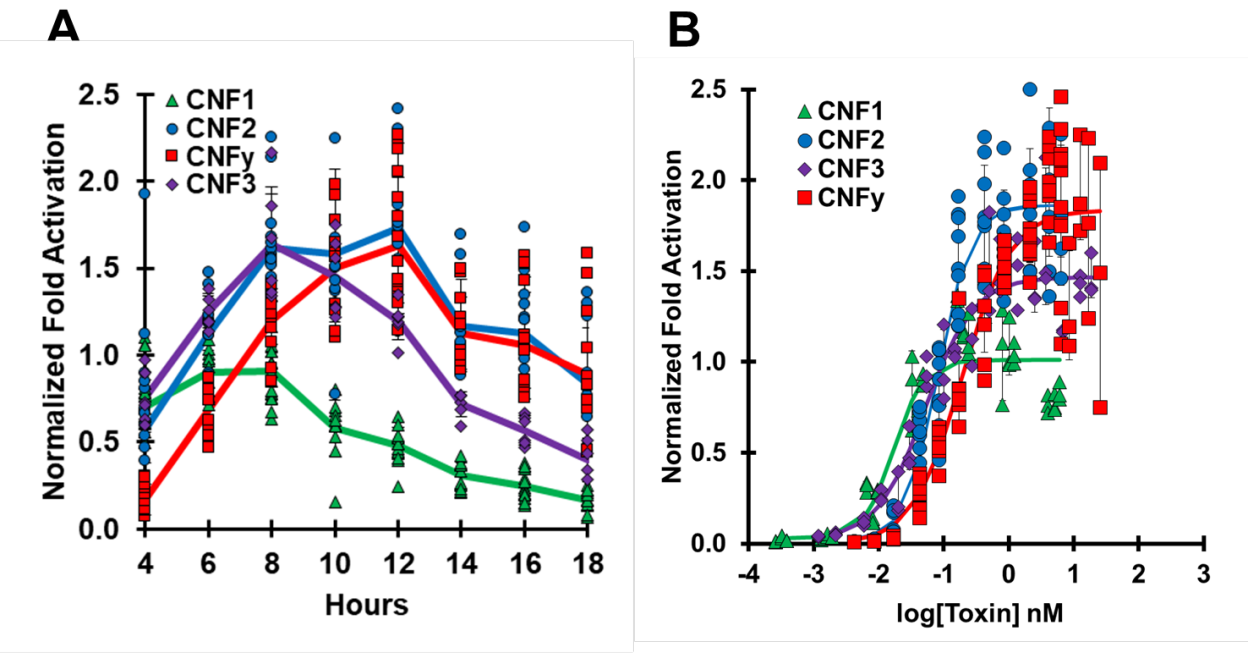


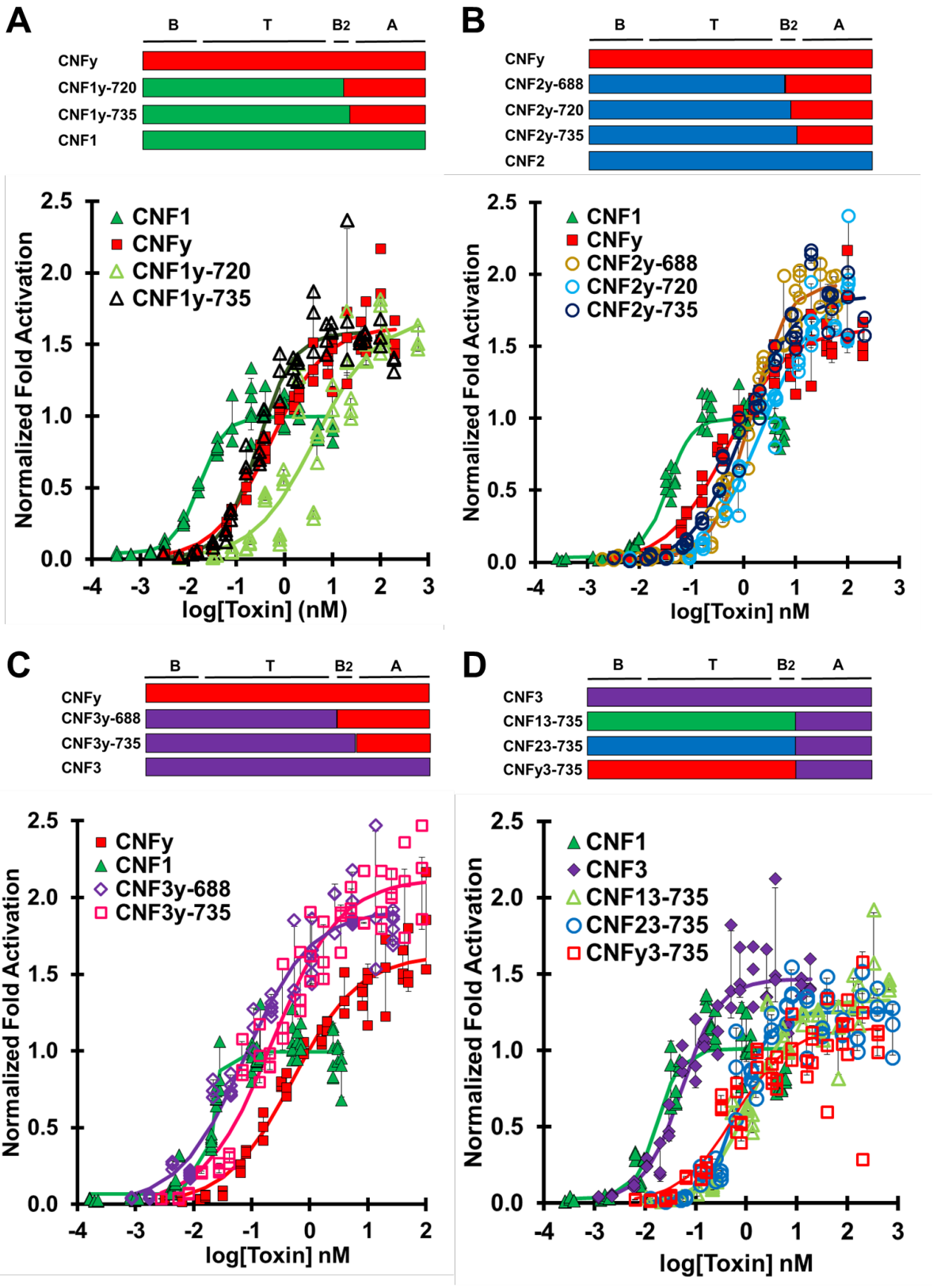
Figure S1C: Amino acid alignment of CNF toxins used in this study. The amino acid sequences of CNF1, CNF2, and CNF3 from *Escherichia coli* and CNFy from *Yersinia pseudotuberculosis* were aligned using Muscle (39) and visualized using Jalview (40) to color the amino acid residue sequences in Clustal format.



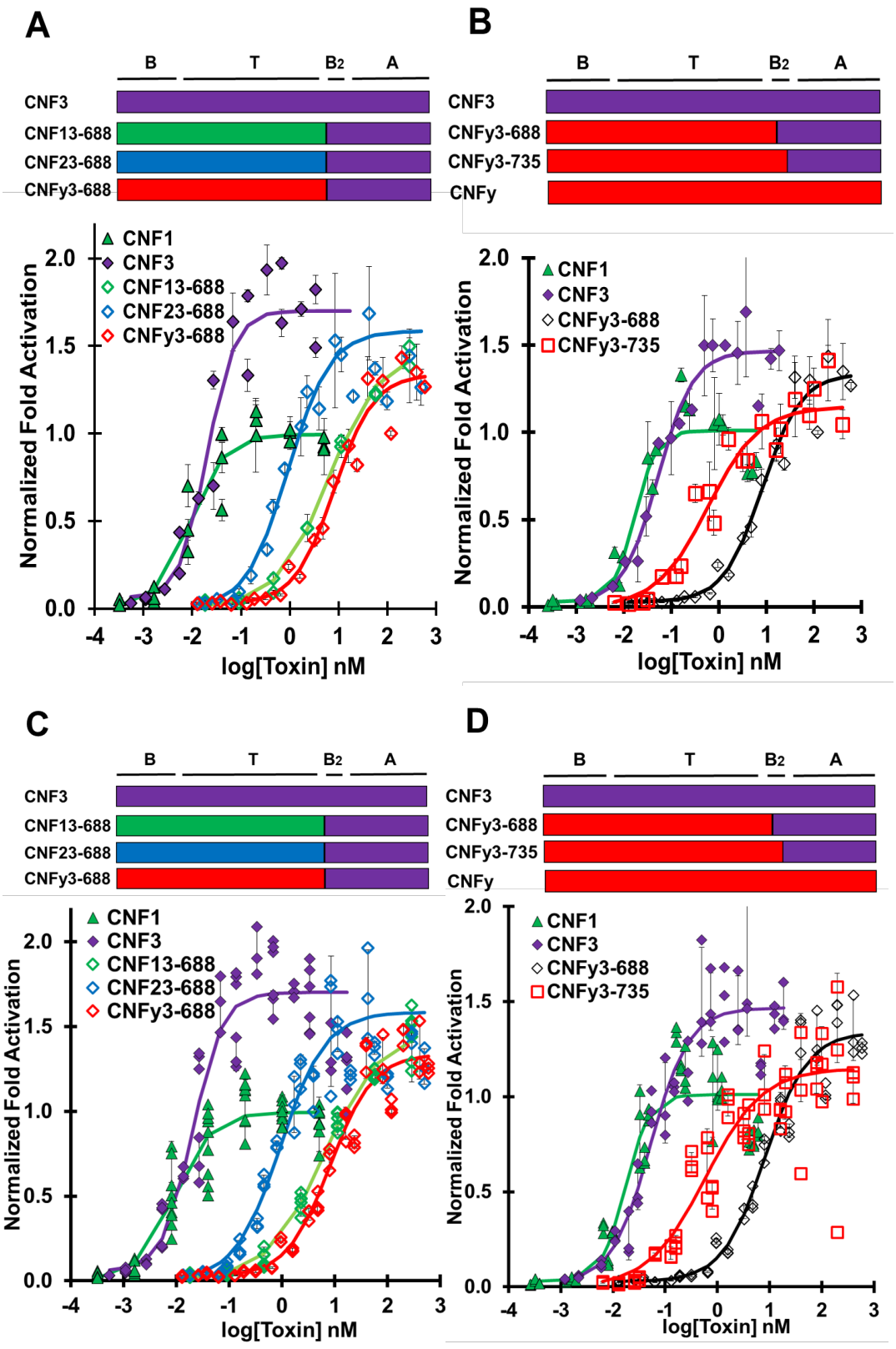
Supplementary Figure S2. Simultaneous exchange of domains B and B_2 of CNFy with that of CNF1 leads to significantly decreased delivery efficiency of by CNFy domain T. HEK293-T cells with reporter plasmids were treated with the indicated toxin at the indicated doses and subjected to SRE-luciferase assay to determine the normalized fold activation relative to CNF1 and untreated cells, as described in Figure 3. (A) Dose response curve comparing CNFy cargo delivered by CNFy translocation domain using the CNF1 receptor binding domains (CNF1 green closed triangles, CNFy red closed squares, CNF1y-223 dark blue open squares, CNF1y1y orange open squares). For comparison, the dose response curve for CNF1y1-688 (pale blue open squares) shown in Figure S9A is also included here. (B) Corresponding scatter plot of all data points used to derive the best-fit lines and mean values show in (A).



Supplementary Figure S3. Corresponding scatter plots of Figure 2. Corresponding scatter plots of all data points used to derive the best-fit lines and mean values show in Figure 2.

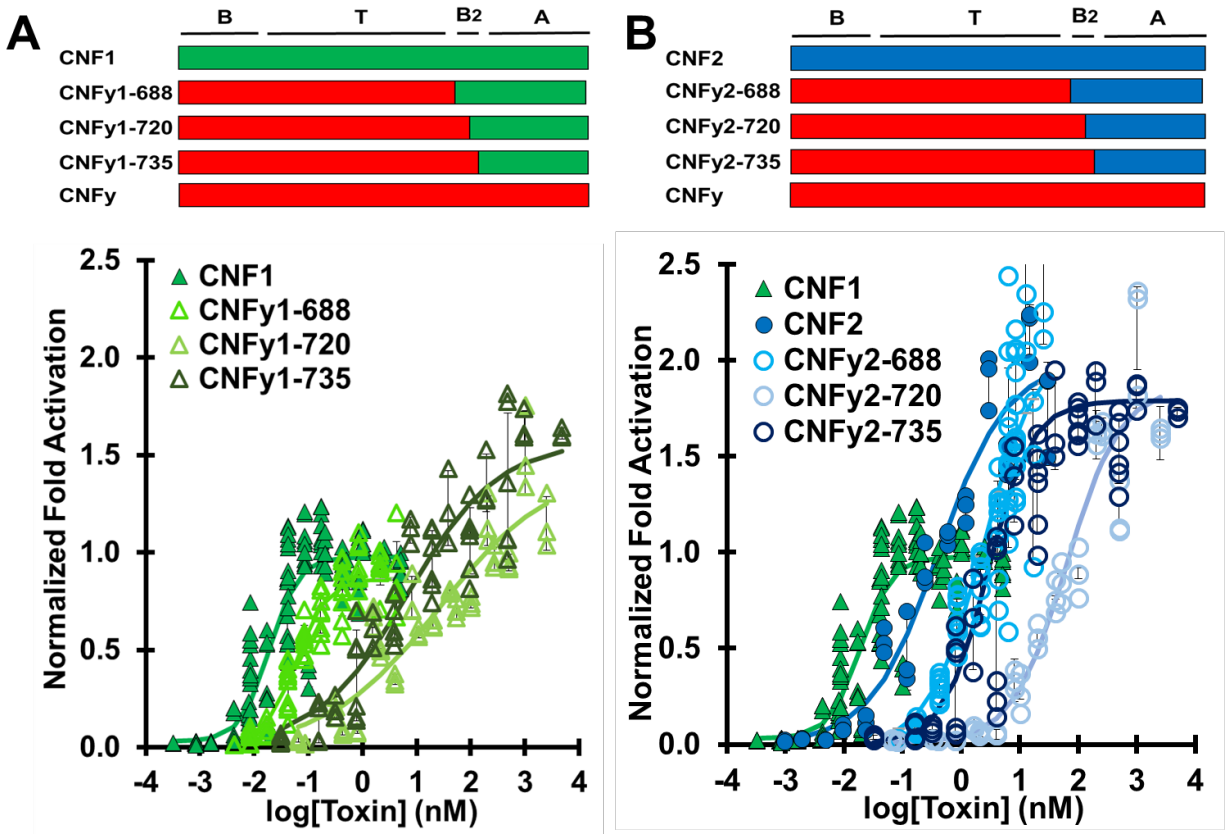


Supplementary Figure S4. Corresponding scatter plots of Figure 3. Corresponding scatter plot of all data points used to derive the best-fit lines and mean values show in main text Figure 3.

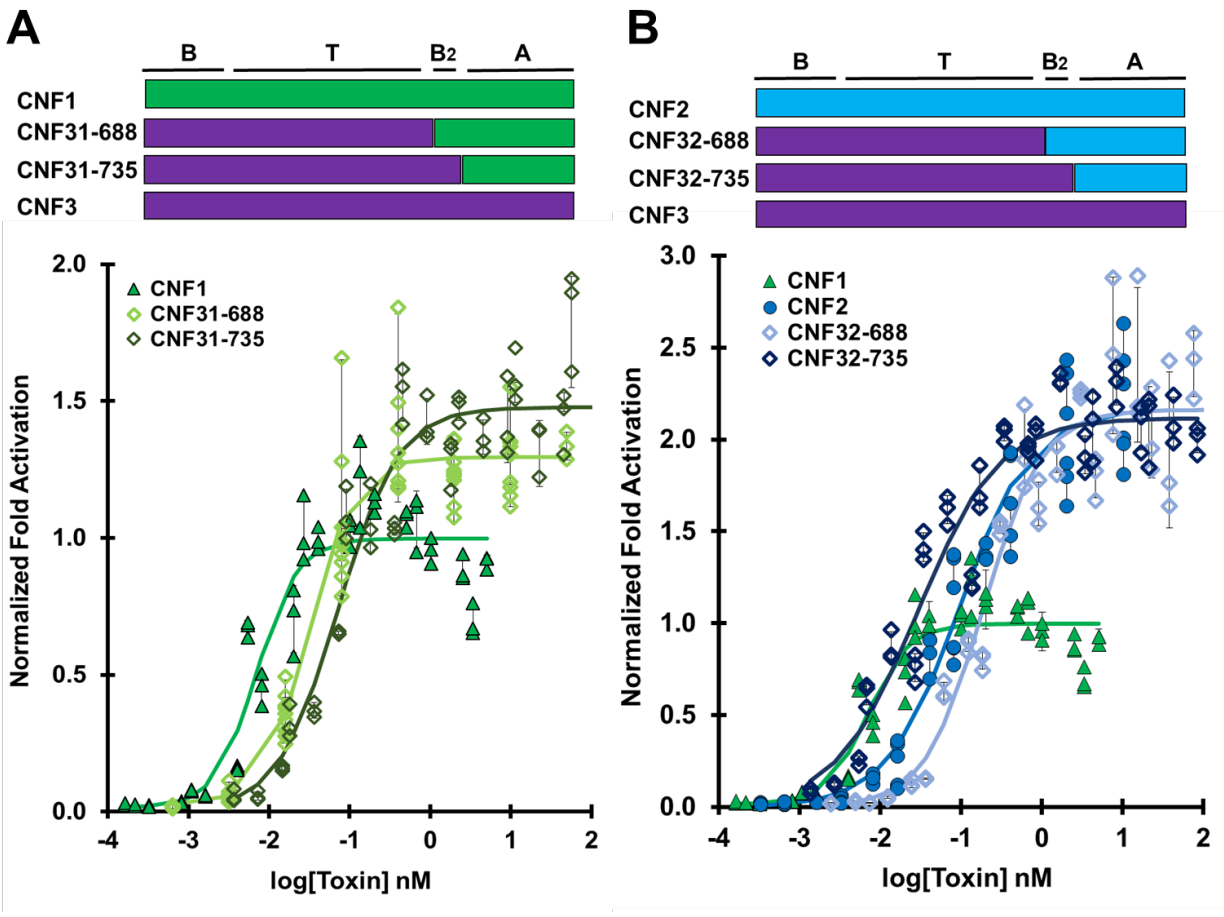


Supplementary Information Figure S5. CNF3 cargo is delivered less efficiently by CNF1, CNF2 and CNFy delivery vehicles, regardless of joining site. HEK293-T cells with reporter plasmids were treated with the indicated toxin at the indicated doses and subjected to SRE-luciferase assay to determine the normalized fold activation relative to CNF1 and untreated cells, as described in Figure 3. (A) Dose

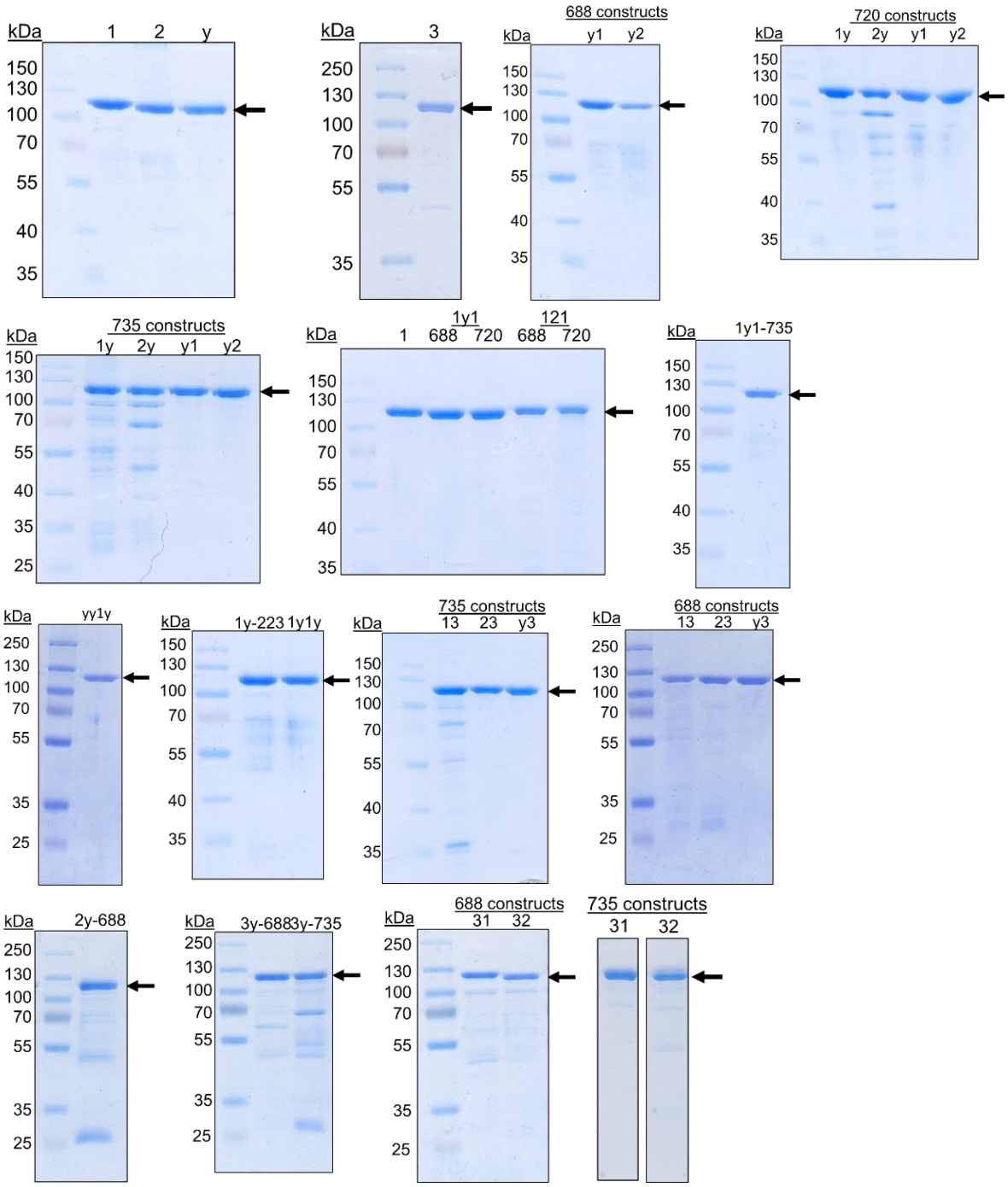
response curves comparing CNF3 cargo delivered by CNF1, CNF2 and CNFy delivery vehicles, joined at amino acid 688 (CNF1 green closed triangles, CNF3 purple closed diamonds, CNF13-688 green open diamonds, CNF23-688 blue open diamonds, CNFy3-688 red open diamonds). (B) Dose response curve comparing CNF3 cargo delivered by CNFy delivery vehicle. Curves are the same as Figure 3D and Supplementary Figure S3A, shown here for comparison. (CNF3 purple closed diamonds, CNFy3-688 black open diamonds, CNFy3-735 red open squares). (C&D) Corresponding scatter plots of all data points used to derive the best-fit lines and mean values show in (A) & (B), respectively.



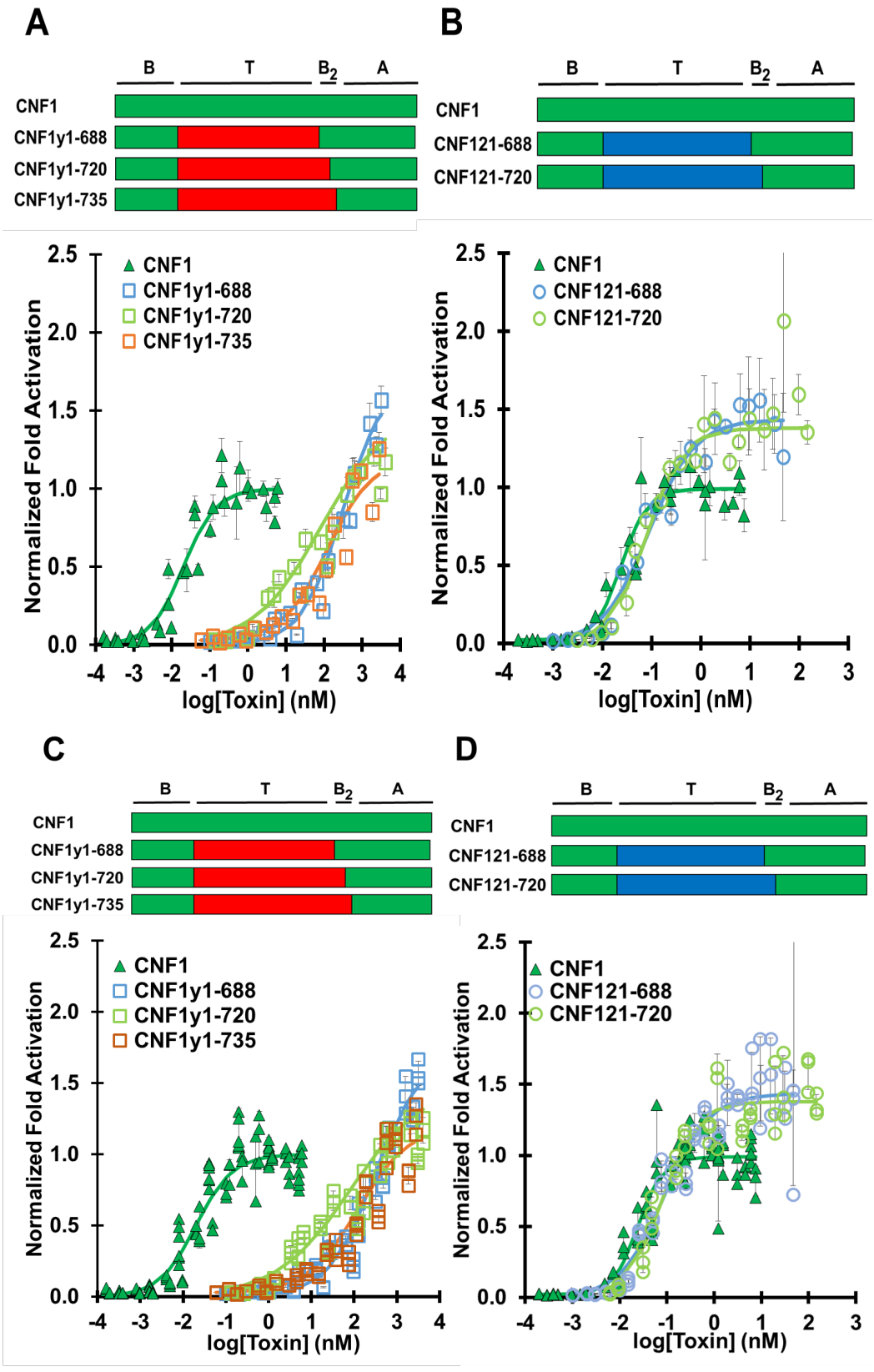
Supplementary Figure S6. Corresponding scatter plots of main text Figure 4. Corresponding scatter plot of all data points used to derive the best-fit lines and mean values show in main text Figure 4.



Supplementary Figure S7. Corresponding scatter plot of main text Figure 5. Corresponding scatter plot of all data points used to derive the best-fit lines and mean values show in main text Figure 5.



Supplementary Figure S8. SDS PAGE Gels of Purified CNF toxins. Purified CNF toxins were diluted to 0.1 µg/µL in a solution of SDS and bromophenol blue dye, then 10 µL of the resulting dilution was loaded and ran on a 10% SDS PAGE gel and visualized by staining with Coomassie Blue. The sizes of the molecular marker bands are listed in kilo-Daltons. Arrows denote the expected protein bands.



Supplementary Figure S9. CNF2 domain T is able to be swapped into CNF1 without loss of efficiency, but utilizing CNFy domain T results in significant drop in efficiency. The receptor-binding domain B and catalytic domain A were previously identified in CNF1, so to generate chimeras swapping domain T, we chose to hold these domains B, and A of CNF1 constant, while exchanging domain T using

the joining sites 223 and 688, 720 or 735. HEK293-T cells with reporter plasmids were treated with the indicated toxin at the indicated doses and subjected to SRE-luciferase assay to determine the normalized fold activation relative to CNF1 and untreated cells, as described in Figure 3. (A) Dose response curve comparing CNF1 cargo delivered by CNF2 translocation domain (CNF1 green closed triangles, CNF121-688 blue open circles, CNF121-720 pale green open circles). (B) Dose response curve comparing CNF1 cargo delivered by CNFy translocation domain (CNF1 green closed triangles, CNF1y1-688 blue open squares, CNF1y1-720 pale green open squares, CNF1y1-735 orange open squares). (C&D) Corresponding scatter plots of all data points used to derive the best-fit lines and mean values show in (A) & (B), respectively.