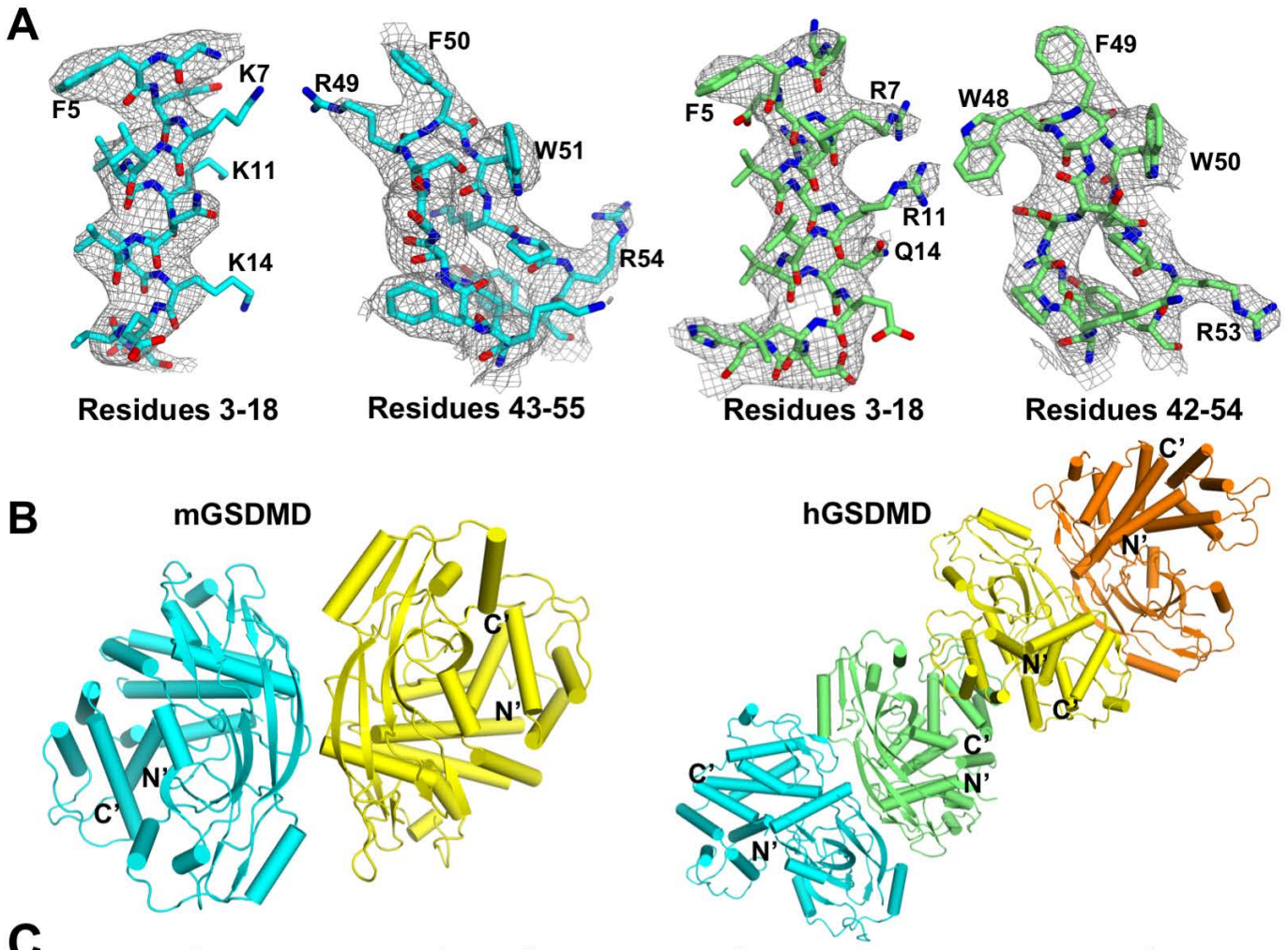
Fig. S1, Liu et al.



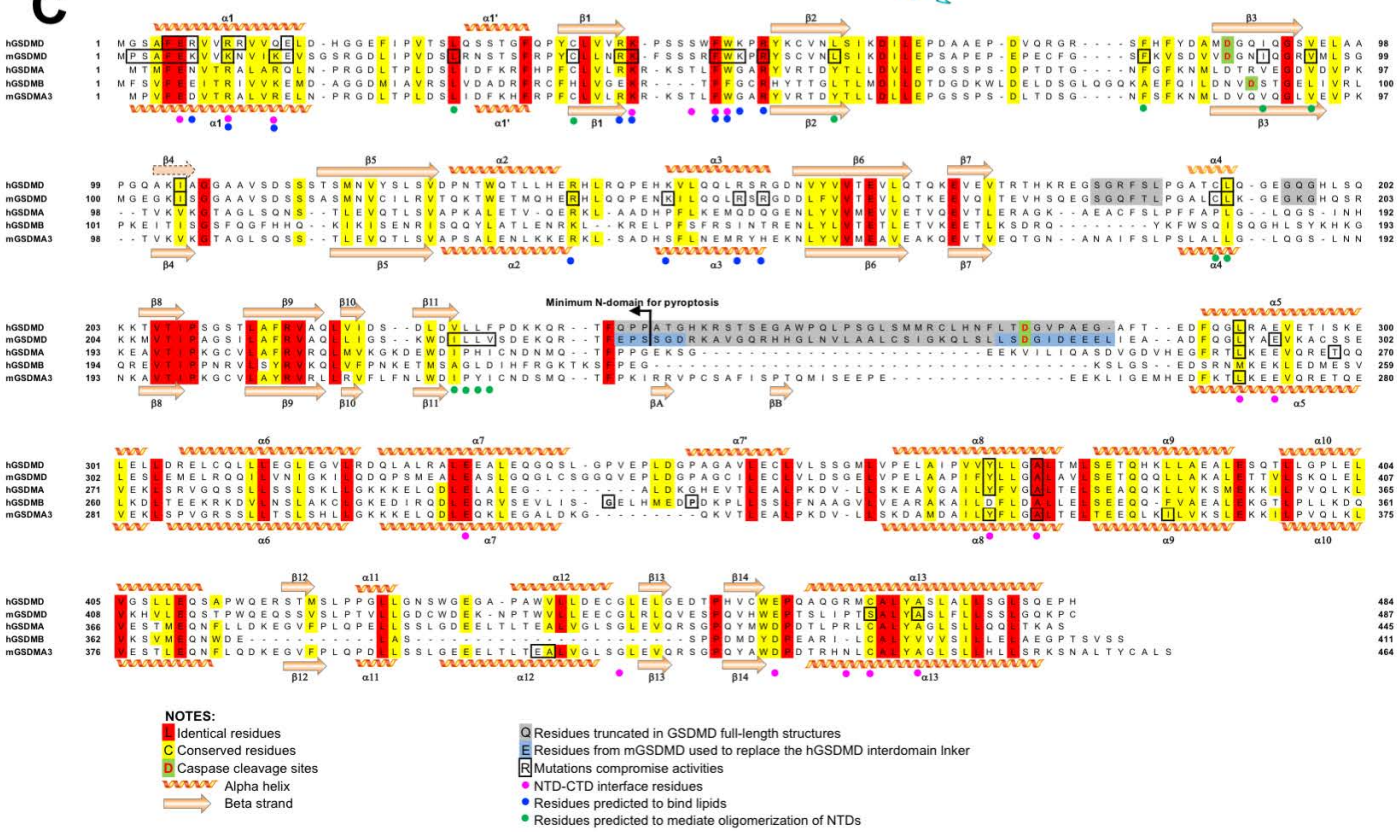


Figure S1. Electron density maps and packing of mGSDMD and hGSDMD in the crystals. Related to Figure 1.

(A) Electron-density maps (2*F*o-*F*c) for the mGSDMD (left, cyan) and hGSDMD (right, lime) contoured at 1σ are shown.

(B) The two mGSDMD molecules in the crystallographic asymmetric unit are shown in cyan and yellow on the left, and the four hGSDMD molecules in the crystal lattice are show in cyan, lime, yellow, and orange on the right. The N' and C' termini of each molecule are marked.

(C) Sequence alignment of the hGSDMD, mGSDMD, hGSDMA, hGSDMB, and mGSDMA3. Identical residues are in red shade, conserved residues are in yellow shade, and caspase cleavage sites are in green shade and red letters. Helices and beta strands are shown with symbols. Residues truncated in the full-length GSDMD expression constructs for crystallization are marked with gray shade, except that residues from mGSDMD that replace the hGSDMD interdomain linker are in light-blue shade. Mutations of residues that compromise GSDMD function are marked with a black square. Residues mediating NTD-CTD domain interactions are marked with magenta dots, those predicted to bind lipids with blue dots, and those predicted to mediate oligomerization with green dots.

Fig. S2, Liu et al.

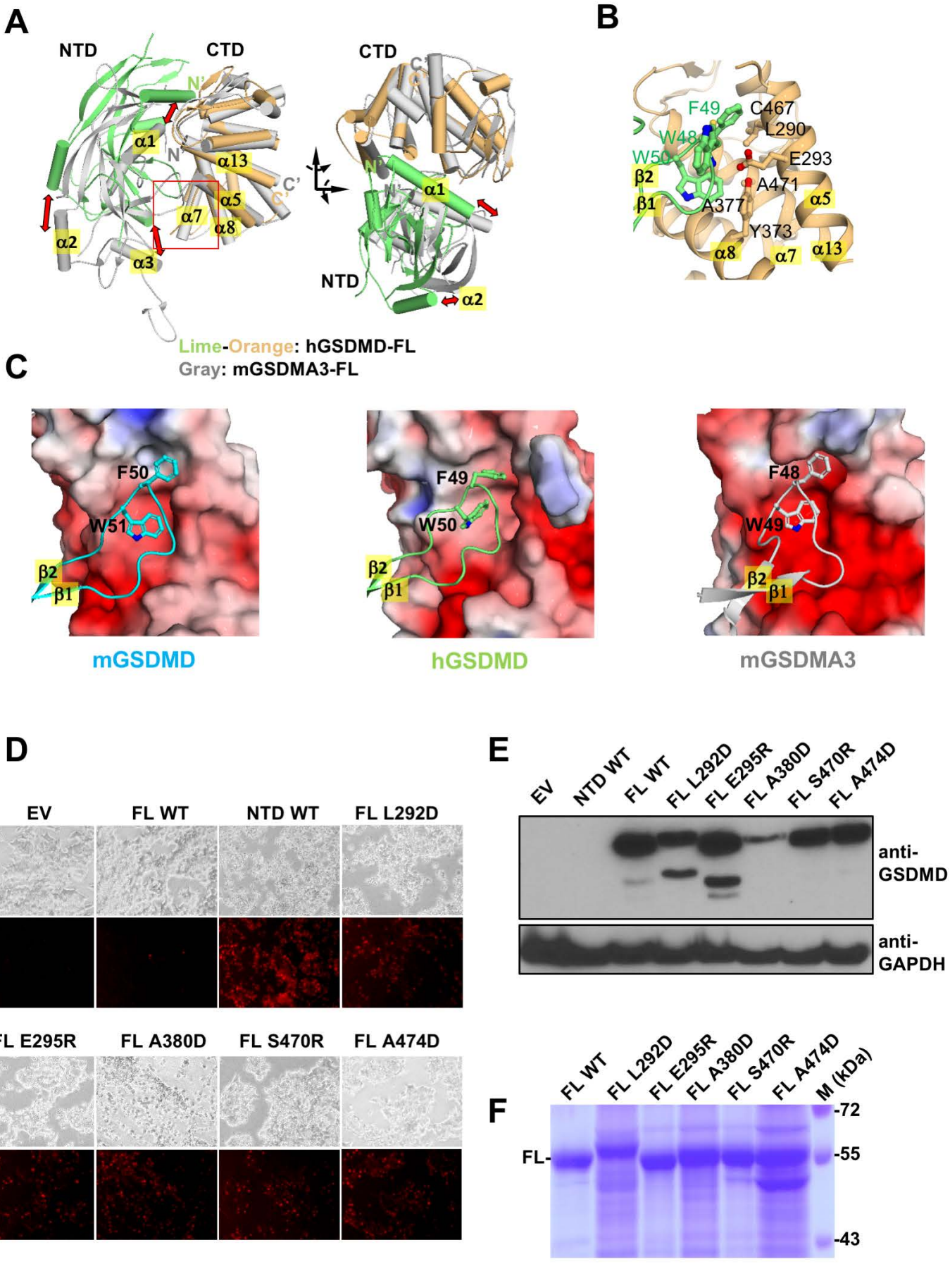


Figure S2. The NTD-CTD interfaces for mGSDMD, hGSDMD and mGSDMA3. Related to Figure 2.

(A) Superposition of the full-length hGSDMD structure (NTD in lime and CTD in orange) and the full-length mGSDMA3 structure (gray) through their respective CTDs. Shifts of their NTDs are indicated with red arrows.

(B) The domain interface near the β1-β2 loop of hGSDMD with the NTD in lime and CTD in orange. Residues participating in NTD-CTD interface are shown in ball-and-sticks.

(C) Close-up views of the mGSDMD (left), hGSDMD (middle), and mGSDMA3 (right) NTD-CTD interfaces at their β1-β2 loops. The two aromatic residues at each interface are shown as ball-and-sticks, and the CTDs are shown as electrostatic charge surface. The view is similar to that in Figure S2B.

(D) Propidium iodide uptake upon expression of full-length wild type, NTD wild type and full-length mutant mGSDMD. “EV” is empty vector control. Bright field and fluorescent images of the same cells are shown on the top and bottom panels, respectively.

(E) Expression of mGSDMD-NTD and wild type or mutant mGSDMD-FL proteins in HEK293T cells.

(F) The purified wild type mGSDMD-FL or mutant mGSDMD-FL harboring mutations at the CTDs are used in the liposome leakage assay. Location for the full-length (“FL”) protein is marked.

Fig.S3, Liu et al.

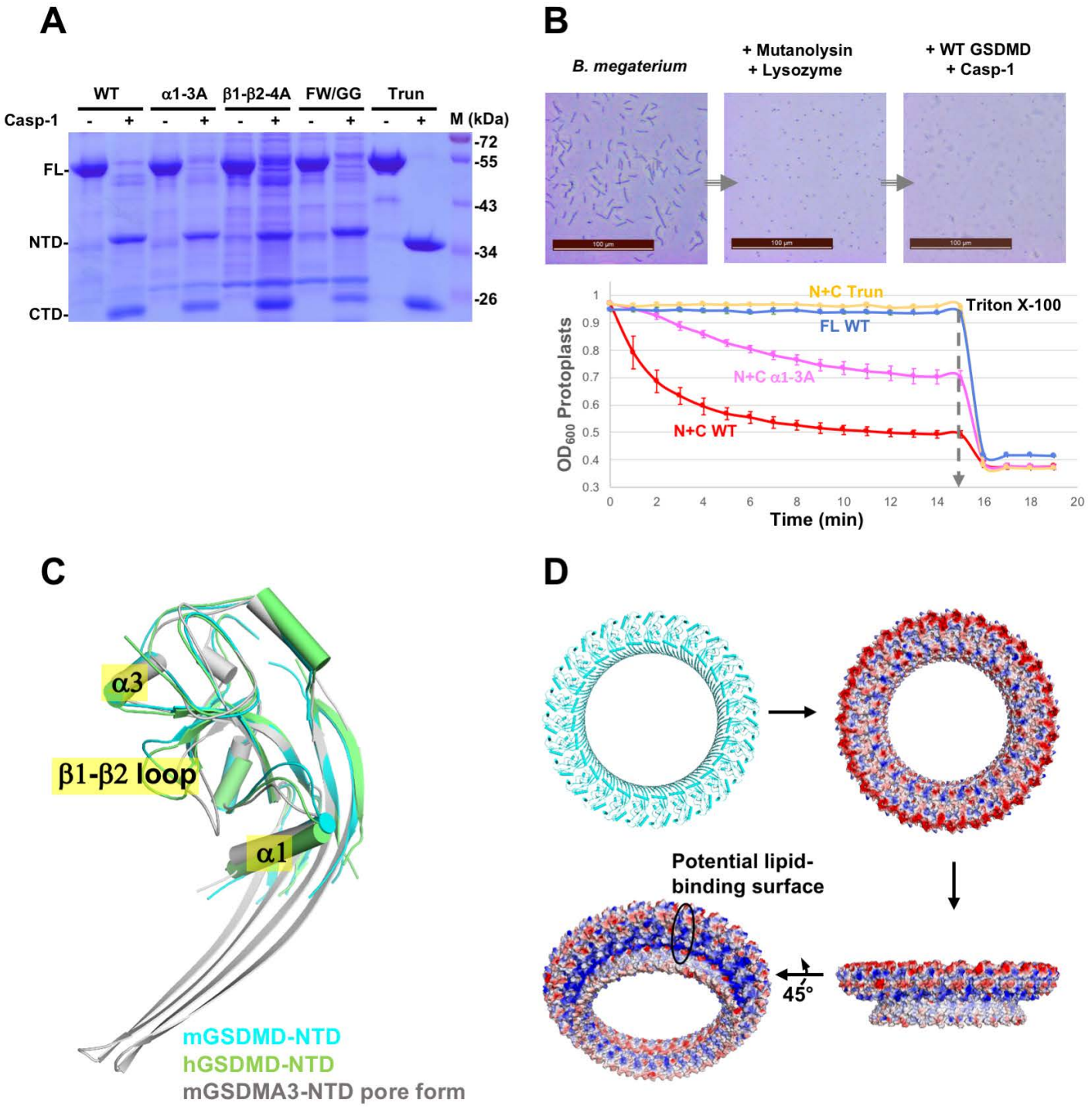


Figure S3. Lipid-binding and pore formation by the mGSDMD-NTD. Related to Figure 3.

(A) The purified wild type mGSDMD-FL or mutant mGSDMD-FL harboring mutations at the NTDs are cleaved by caspase-1 and used in the liposome leakage and protoplast lysis assays. “Trun” denotes mGSDMD harboring truncations of residues 182-187, 197-199 and 248-273. Locations for the full-length (“FL”), NTD, and CTD are marked.

(B) Protoplasts of *Bacillus megaterium* generated through mutanolysin and lysozyme treatment were incubated with mGSDMD. Images of *B. megaterium* with or without treatment are shown on the top panel. Lysis of the protoplasts was monitored through OD_{600} measurements. GSDMD protein samples were added at time 0, and 0.1% Triton X-100 was added at 15-minute, marked by a gray arrow. “FL WT” is full-length wild type mGSDMD without caspase-1 cleavage; “N+C α1-3A” is the full-length α1-3A mutant cleaved by caspase-1; “N+C WT” is the full-length wild type protein cleaved by caspase-1; and “N+C Trun” is the truncated full-length protein cleaved by caspase-1. Data shown are representative of three independent experiments.

(C) Superposition of the GSDMD-NTD with mGSDMA3-NTD in the pore form. The mGSDMD-NTD, hGSDMD-NTD and mGSDMA3-NTD are colored cyan, lime, and gray, respectively.

(D) A model of the mGSDMD-NTD pore is shown in ribbons and electrostatic charge surface. A positively-charged surface patch on the outer rim of the pore is marked, and is composed of the α1 helix, β1-β2 loop, and α3 helix.

Fig.S4, Liu et al.

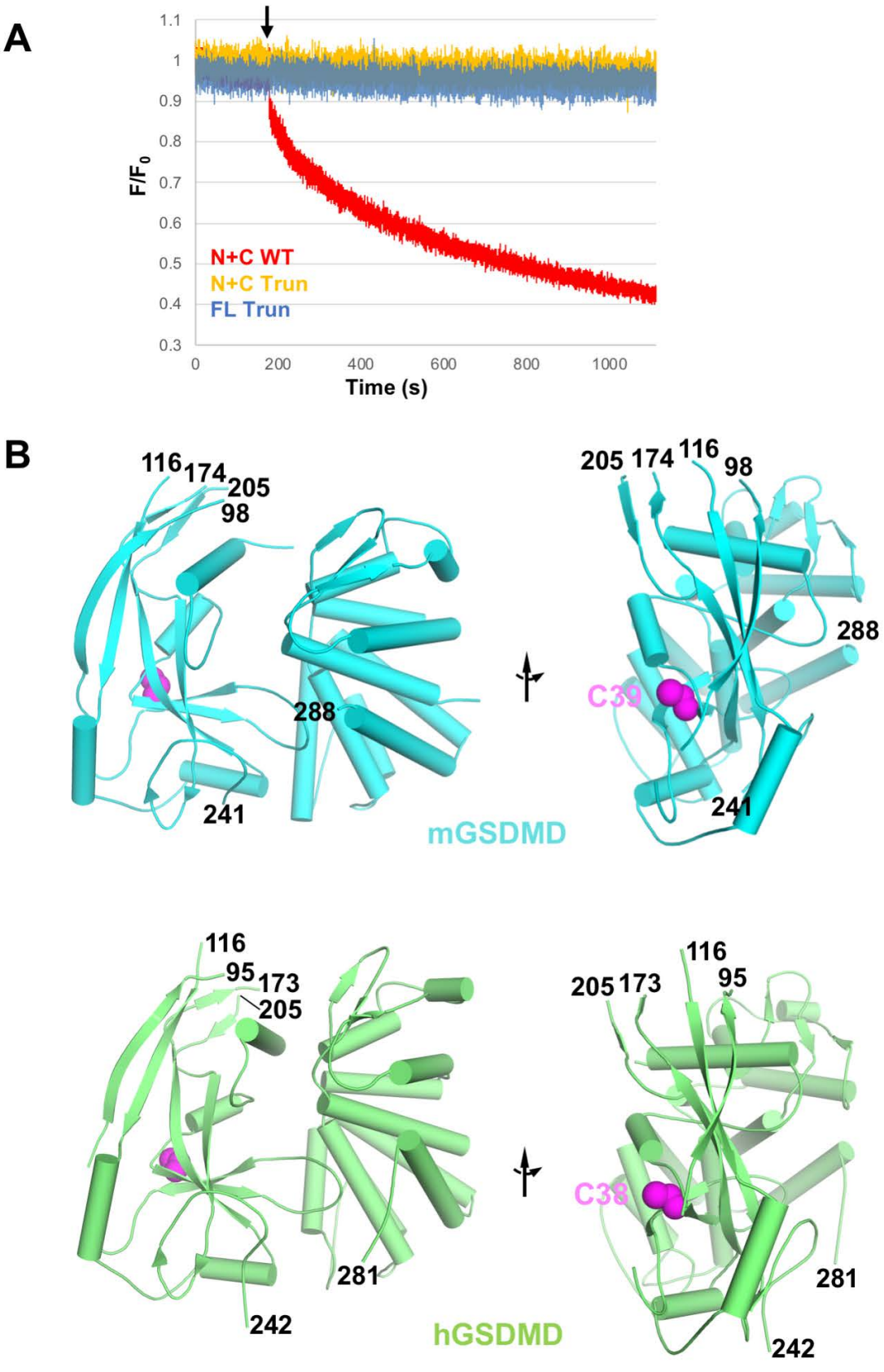


Figure S4. The truncated mGSDMD lost its ability to induce leakage of liposomes. Related to Figure 4.

(A) The ability of the wild type and truncated mGSDMDs to induce liposome leakage was monitored through measuring the quench of the $[Tb(DPA)_3]^{3-}$ fluorescent complex inside liposomes by external EDTA. The “N+C WT” denotes the wild type mGSDMD cleaved by caspase-1. The “N+C Trun” denotes the truncated mGSDMD cleaved by caspase-1. The ”FL Trun” denotes the truncated mGSDMD without caspase-1 cleavage. “F” stands for fluorescence at each time point, whereas ”F_0” stands for fluorescence in the first second when no mGSDMD was present. The black arrows mark the time point when mGSDMD samples were added to the liposomes. Data shown are representative of three independent experiments.

(B) The mGSDMD structure is shown in cyan ribbons on the top panel in two different views, and the hGSDMD structure is shown in lime on the lower panel. C39 from mGSDMD and C38 from hGSDMD are shown as magenta spheres. Residue numbers at the boundaries of the disordered regions for both structures are marked.

Table S1. X-ray Diffraction Data Collection and Refinement Statistics. Related to Figure 1.

	mGSDMD	hGSDMD
Data Collection		
Spacegroup	$P2_1$	$P2_1$
Unit cell (a, b, c) (Å)	66.4, 86.8, 83.5	58.6, 105.6, 151.8
(α, β, γ) (°)	90, 95.3, 90	90, 94.5, 90
Wavelength (Å)	1.0332	1.0332
Resolution (Å) (Last shell)	46.0-3.3 (3.4-3.3)	49.5-3.5 (3.6-3.5)
No of reflections (total/unique)	50453/14323	73185/23251
Completeness (%) (Last shell)	99.6 (99.7)	99.1 (99.3)
$\langle I/\sigma(I)\rangle$ (Last shell)	15.7 (1.2)	10.2 (1.4)
R_{meas} (%)[a] (Last shell)	5.9 (152.2)	8.6 (167.4)
CC ½ (%)[b] (Last shell)	99.9 (70.1)	99.7 (60.5)
Refinement		
Number of atoms (protein/solvent)	5954/0	11604/0
Rmsd bonds length (Å)/angles (°)	0.004/0.76	0.003/0.77
R_{work}/ R_{free} (%) [c]	27.7/32.1	31.5/34.8
Ramachandran plot favored/disallowed [d]	92.0/0.0	91.4/0.0
PDB ID	6N9N	6N9O

[a] $R_{meas} = \Sigma_h \{N_h /[N_h -1]\}^{1/2} \Sigma_i |I_i(h) - \langle I(h)\rangle | / \Sigma_h\Sigma_i I_i(h)$, where $I_i(h)$ and $\langle I(h)\rangle$ are the ith and mean measurement of the intensity of reflection *h*, and N_h is the multiplicity .

[b] $CC_{1/2} = \Sigma (x - \langle x\rangle) (y-\langle y\rangle) / [\Sigma (x - \langle x\rangle)^2 \Sigma (y-\langle y\rangle)^2]^{1/2}$ where x and y are randomly split half datasets. This is the Pearson's correlation coefficient of randomly split half datasets (Karplus and Diederichs, 2012).

[c] $R_{work} = \Sigma_h||F_{obs}(h)|-|F_{calc}(h)|| / \Sigma_h|F_{obs}(h)|$, where $F_{obs}(h)$ and $F_{calc}(h)$ are the observed and calculated structure factors, respectively; R_{free} is the R value obtained for a test set of reflections consisting of a randomly selected 5% subset of the data set excluded from refinement.

[d] Values from the Molprobity server (http://molprobity.biochem.duke.edu/).

Table S2. Oligonucleotides used in this study. Related to STAR Methods and the Key Resources Table.

Oligonucleotides	SOURCE	IDENTIFIER
hGSDMD-del181-186 and 196-198 Forward: TGCTTGCAGGGTGAGCATCTGAGCCAGAAGAAGAC	This paper	N/A
hGSDMD-del181-186 and 196-198 Reverse: CGTGGCTCCGGGGCCCTCCCGCTTGTGGGTGCGCG	This paper	N/A
hGSDMD-del247-272 Forward: CTGACAGATGGGGTCCCTGCGGAGGGG	This paper	N/A
hGSDMD-del247-272 Reverse: GCCTGTCGCGGGTGGCTGGAAGGTCCT	This paper	N/A
hGSDMD-mGSDMD linker Forward: GATGGGATTGATGAGGAGGAATTAGCGTTCACTGAAGACTTCC AGGGCCTACGG	This paper	N/A
hGSDMD-mGSDMD linker Reverse: TGACAGGTCACCTGAGGAGGGCTCGAAGGTCCTCTGCTTCTTA TCCGGGAAGAG	This paper	N/A
mGSDMD-del248-273 Forward: CGGGATCCGGGATTGATGAGGAGGAATTAAT	This paper	N/A
mGSDMD-del248-273 Reverse: GTCACCTGAGGAGGGCTCAAAGGTCCTC	This paper	N/A
mGSDMD-del182-187 and 197-199 Forward: TGCTTGAAGGGTGAACACCAAAGCCGGAAGAAGAT	This paper	N/A
mGSDMD-del182-187 and 197-199 Reverse: TAAAGCTCCAGGGCCCTCTTGGCTGTGGACCTCAGT	This paper	N/A
mGSDMD-FL L292D Forward *: CGACTATGCTGAGGTGAAGGCTTGCTC	This paper	N/A
mGSDMD-FL L292D Reverse: CCCTGGAAGTCTGCCGCCTCAATTAAT	This paper	N/A
mGSDMD-FL E295R Forward *: CGGGTGAAGGCTTGCTCCTCAGAACTGGAGAG	This paper	N/A
mGSDMD-FL E295R Reverse: AGCATACAGGCCCTGGAAGTCTGCCGCCTC	This paper	N/A
mGSDMD-FL A380D Forward *: GGGAGATCTGGCTGTGCTGAGTGAAAC	This paper	N/A
mGSDMD-FL A380D Reverse: AGCAGGTAGAAGATAGGGGCTGCGAGT	This paper	N/A

mGSDMD-FL S470R Forward *: CGTGCGCTCTATGCCTCCCTGTTCCTATTGT	This paper	N/A
mGSDMD-FL S470R Reverse: TGTGGGGATCAGAGACGTTGGTTCCCAGTG	This paper	N/A
mGSDMD-FL A474D Forward *: ACTCCCTGTTCCTATTGTCAAGTCTAGGCC	This paper	N/A
mGSDMD-FL A474D Reverse *: CATAGAGCGCACTTGTGGGGATCAGAGACG	This paper	N/A
mGSDMD-NTD K7/K10/K14 AAA Forward *: GAGGCAGTGGTCGCGAATGTGATCGCGGAGGTAAGCGGCAGC AGAGGCGATCTCAT	This paper	N/A
mGSDMD-NTD K7/K10/K14 AAA Reverse: AAAGGCCGATGGCATGGATCCGAGCTCGGT	This paper	N/A
mGSDMD-NTD R43/K44 AA Forward *: AACGCGGCATTTTCAAGCTCAAGGTTCTGG	This paper	N/A
mGSDMD-NTD R43/K44 AA Reverse *: GCTTGAAAATGCCGCGTTCAGAAGGCAGTAGGGCCTGAAGCT	This paper	N/A
mGSDMD-NTD K52/R54 AA Forward *: TGGGCACCCGCTTATTCATGTGTCAACCTGTCAATCAAG	This paper	N/A
mGSDMD-NTD K52/R54 AA Reverse *: GAATAAGCGGGTGCCCAGAACCTTGAGCTTGAAAAT	This paper	N/A
mGSDMD-NTD F50G Forward *: CCCTGGAAACCCCGTTATTCATGTGTC	This paper	N/A
mGSDMD-NTD F50G Reverse: CCTTGAGCTTGAAAATTTCCTGTTCAG	This paper	N/A
mGSDMD-NTD W51G Forward: AAACCCCGTTATTCATGTGTCAACCTG	This paper	N/A
mGSDMD-NTD W51G Reverse *: ACCGAACCTTGAGCTTGAAAATTTCCT	This paper	N/A
mGSDMD-NTD F50G/W51G Reverse *: CCCTCCCCTTGAGCTTGAAAATTTCCTGTT	This paper	N/A
mGSDMD-NTD L29A Forward *: TGGACAGCGCGCGGAACTCCACCAGCTTCAGG	This paper	N/A
mGSDMD-NTD L29A Reverse *: CCGCGCGCTGTCCACCGGAATGAGATCGCCTCTGCT	This paper	N/A
mGSDMD-NTD L60G Forward *: TGTGTCAACGGGTCAATCAAGGACATCCTGGAGCCC	This paper	N/A

mGSDMD-NTD L60G Reverse *: TGATTGACCCGTTGACACATGAATAACGGGGTTTCC	This paper	N/A
mGSDMD-NTD F81D Forward *: TTTGGCTCCGACAAAGTCTCTGATGTCGTCGATGGG	This paper	N/A
mGSDMD-NTD F81D Reverse *: AGACTTTGTCGGAGCCAAAACACTCCGGTTCTGGTT	This paper	N/A
mGSDMD-NTD I91D Forward *: GATGGGAACGATCAGGGCAGAGTGATGTTGTCAGGC	This paper	N/A
mGSDMD-NTD I91D Reverse *: TCTGCCCTGATCGTTCCCATCGACGACATCAGAGAC	This paper	N/A
mGSDMD-NTD V95D Forward *: AGGGCAGAGACATGTTGTCAGGCATGGGAGAAGGG	This paper	N/A
mGSDMD-NTD V95D Reverse *: ACAACATGTCTCTGCCCTGAATGTTCCCATCGACG	This paper	N/A
mGSDMD-NTDL193D Forward *: GACAAGGGTGAAGGCAAGGGCCACCAAAG	This paper	N/A
mGSDMD-NTD L193D Reverse: GCATAAAGCTCCAGGCAGCGTAAACTG	This paper	N/A
mGSDMD-NTD 230ILLV233/AAAA Forward *: GATGCCGCTGCCGCCTCAGATGAGAAACAGAGGACCTTTG	This paper	N/A
mGSDMD-NTD 230ILLV233/AAAA Reverse *: TGAGGCGGCAGCGGCATCCCATTTAGAGCCAATAAGCAGT	This paper	N/A

* Red-colored nucleotides mark the mutated codons.