PNAS

Supporting Information for

Palmitoylation at a conserved cysteine residue facilitates gasdermin D-mediated pyroptosis and cytokine release

Zhonghua Liu[a,b,1,2], Sai Li[a,b,1] , Chuanping Wang[a], Kaylynn J. Vidmar[a], Syrena Bracey[a] , Ling Li[c], Belinda Willard[c] , Masaru Miyagi[d], Tong Lan[e] , Bryan C. Dickinson[e] , Abdullah Osme[a,3], Theresa T. Pizarro[a] , and Tsan Sam Xiao[a,2]

Author affiliations:

[a] Department of Pathology, Case Western Reserve University, Cleveland, OH 44106, USA.
[b] Ministry of Education Key Laboratory for Membraneless Organelles and Cellular Dynamics, Hefei National Research Center for Physical Sciences at the Microscale, Division of Life Sciences and Medicine, University of Science and Technology of China, Hefei, Anhui 230027, China.
[c] Proteomics and Metabolic Core, Lerner Research Institute, Cleveland Clinic, Cleveland, OH 44196, USA.
[d] Department of Pharmacology, Case Western Reserve University, Cleveland, OH 44106, USA.
[e] Department of Chemistry, University of Chicago, Chicago, IL 60637, USA.

[1] Z.L. and S.L. contributed equally to this work.
[2] To whom correspondence may be addressed. Email: xixiaolu@mail.ustc.edu.cn or tsx@case.edu.
[3] Present address: Department of Pathology, Heersink School of Medicine, University of Alabama at Birmingham, Birmingham, AL 35249.

Table of Contents:

Fig. S1. Inhibition of palmitoylation dose-dependently suppresses GSDMD-mediated pyroptosis and cytokine release.
Fig. S2. The N-terminal domains of gasdermins are palmitoylated.
Fig. S3. C191A mutation delays the kinetics of LDH release.
Fig. S4. Expression of ZDHHC palmitoyltransferases in THP-1 cells.
Table S1. Reagents or Resources
Table S2. Oligonucleotide Sequences
References

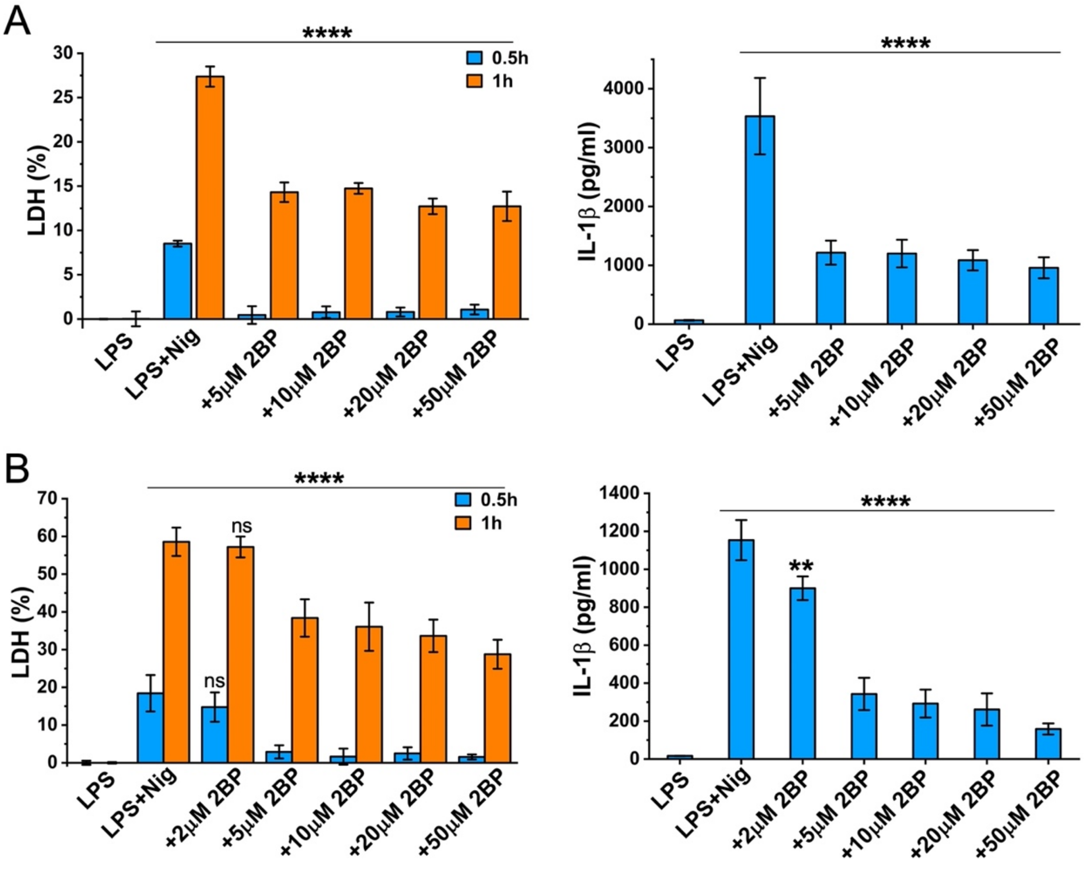

Fig. S1. Inhibition of palmitoylation dose-dependently suppresses GSDMD-mediated pyroptosis and cytokine release.

(*A*) LDH release (left) and IL-1β secretion (right) were measured from THP-1 cells treated with 1 μg/ml LPS for 4 hours followed by 20 μM nigericin for the specified time, in the presence of increasing concentrations of 2BP.

(*B*) LDH release (left) and IL-1β secretion (right) were measured from iBMDM cells treated with 1 μg/ml LPS for 4 hours followed by 20 μM nigericin for the specified time, in the presence of increasing concentrations of 2BP.

Data are plotted as mean ± standard deviation from at least three independent experiments. Statistical analyses were performed using two-way (left panels) or one-way (right panels) ANOVA with Bonferroni's multiple comparisons test versus the "LPS+Nig" samples. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001, ns: not significant.

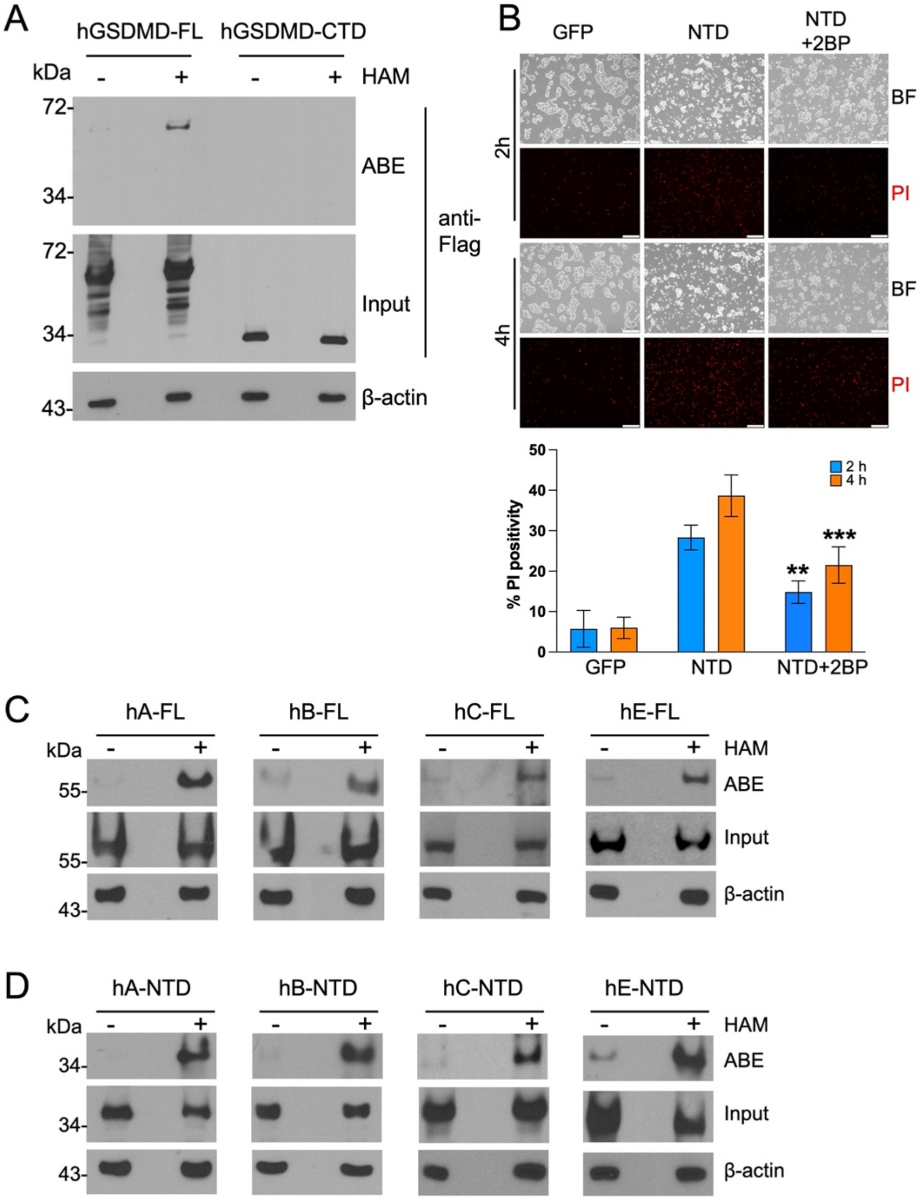

Fig. S2. The N-terminal domains of gasdermins are palmitoylated.
(*A*) FLAG-tagged full-length ("FL") and C-terminal domain ("CTD") from human GSDMD expressed in HEK293T cells were subjected to ABE assays.
(*B*) HEK293T cells expressing hGSDMD-NTD in a tetracycline inducible system was treated with tetracycline and stained with propidium iodide at 2 and 4 hours post induction. The scale bars denote 200 μm. Quantitation of the PI positive cells is shown below the images. Data are plotted as mean ± standard deviation from at least three independent experiments. Statistical analyses were performed using two-way ANOVA with Bonferroni's multiple comparisons test versus the "NTD" samples. *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$.
(*C*) ABE assays were performed with FLAG-tagged full-length human gasdermin proteins expressed in HEK293T cells. "hA", "hB", "hC", and "hE" represent hGSDMA, hGSDMB, hGSDMC, and hGSDME, respectively.
(*D*) ABE assays were performed with FLAG-tagged human gasdermin N-terminal domains expressed in HEK293T cells.

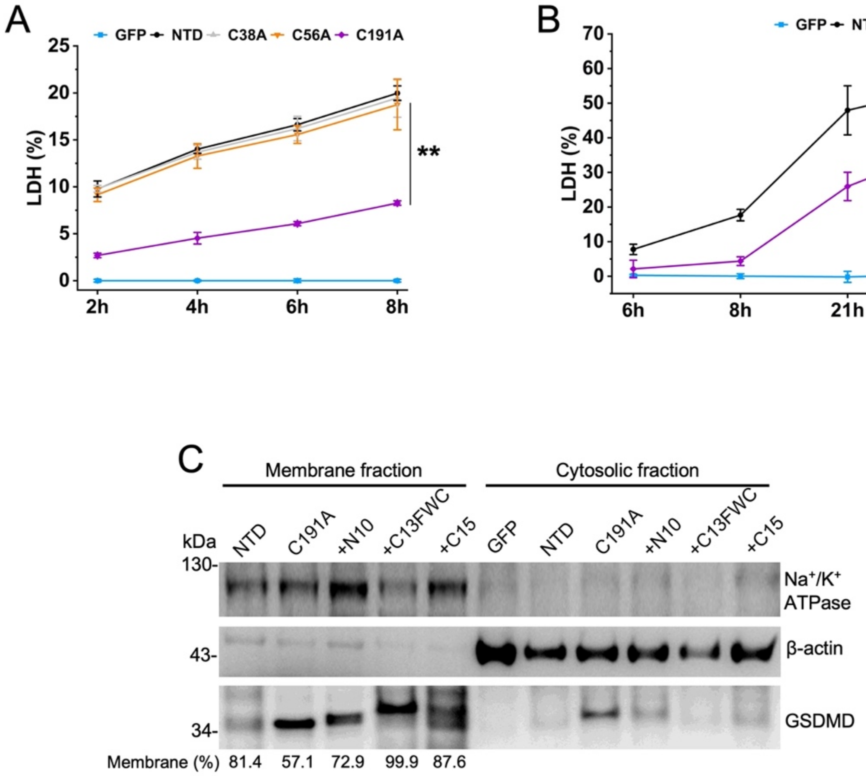

Fig. S3. C191A mutation delays the kinetics of LDH release.

(*A*) Time course of LDH release from HEK293T cells expressing the wildtype or mutant hGSDMD-NTD in a tetracycline-inducible system.

(*B*) Time course of LDH release from HEK293T cells expressing the wildtype or C191A mutant hGSDMD-NTD in a transient expression system.

Data are plotted as mean ± standard deviation from at least three independent experiments. Statistical analyses were performed using two-tailed student t test, compared with the ‘‘NTD’’ samples. **P* < 0.05, ***P* < 0.01.

(*C*) Analysis of the membrane or cytosolic fractions of HEK293T cells expressing the wildtype hGSDMD-NTD (“NTD”), C191A mutant (“C191A”), or the C191A mutant chimera forms containing exogenous palmitoylation sequences (“N10”, “C13FWC”, or “C15”) from human or yeast proteins. The percentage of GSDMD in the membrane fractions for the NTD, C191A, and C191A chimeras is marked.

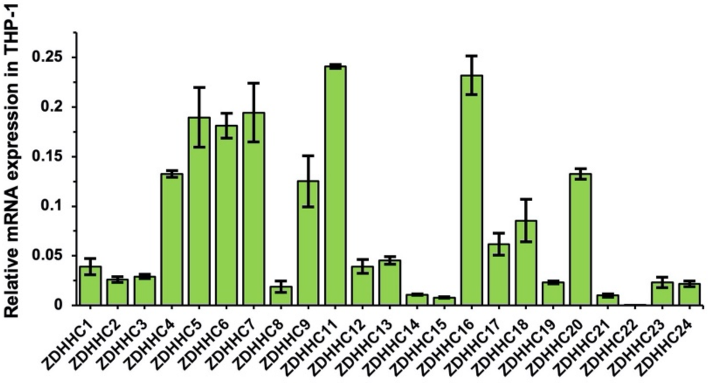

Fig. S4. Expression of ZDHHC palmitoyltransferases in THP-1 cells.
Real time-PCR analysis of ZDHHC expression in THP-1 cells after 1 μg/ml LPS priming for 4 hours. The relative mRNA expression level was calculated and normalized to GAPDH. Data are plotted as mean ± standard deviation from at least three independent experiments.

Table S1. Reagent or Resources

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Monoclonal anti-mouse GSDMDC1 (A-7)	Santa Cruz Biotech	sc-393656; RRID: AB_2728694
Monoclonal anti-human β-actin	Santa Cruz Biotech	sc-47778; *RRID*: AB_626632
Monoclonal anti-Flag M2	Millipore Sigma	F3165; RRID: AB_259529
Monoclonal anti-human GSDMD	Cell Signaling	Cat#97558; RRID: AB_2864253
Polyclonal anti-Na, K-ATPase	Cell Signaling	Cat#3010; RRID: AB_2060983
Bacterial and Virus Strains		
BL21-CodonPlus (DE3)-RIPL	Agilent Technologies	Cat#230280
NEB 5-alpha	New England Biolabs	Cat# C2987I
Chemicals, Peptides, and Recombinant Proteins		
Dulbecco’s Modified Eagle Medium (DMEM)	Gibco	Cat#11965084
RPMI 1640	Gibco	Cat#11875085
Fetal bovine serum (FBS)	Gemini BioProducts	Cat#900-208
Penicillin and streptomycin	Gibco	Cat#15140122
2-Mercaptoethanol	Thermo Fisher Scientific	Cat#125472500
Nigericin	MilliporeSigma	Cat#N7143
Phorbol 12-myristate 13-acetate (PMA)	Thermo Fisher Scientific	Cat#J63916-MB
LPS	MilliporeSigma	Cat#L2630
Propidium iodide	Thermo Fisher Scientific	Cat#P1304MP
Polybrene	MilliporeSigma	Cat#TR-1003
Penicillin and streptomycin	GIBCO	Cat#15140122
Blasticidin S Hydrochloride	RPI	Cat#3513-03-9
Polyethylenimine (PEI) max	Polysciences	Cat# 24765
Calcium Chloride	Quality Biological	Cat#351-130-721
HEPES	Thermo Fisher Scientific	Cat#BP310-1kg
Triton X-100	Millipore Sigma	Cat#T9284-500ml
2-Bromohexadecanoic acid (2BP)	Millipore Sigma	Cat#238422-10G
Cyano-myracrylamide (CMA)	Bryan Dickinson (1)	N/A
ProLong Gold antifade reagent with DAPI	Thermo Fisher Scientific	Cat#P36931
Triethanolamine (TEA)	Millipore Sigma	Cat#T58300
Protease inhibitor cocktail	Roche	Cat#04693132001
Benzonase	Novagen	Cat#70664-3
N-ethylmaleimide	Thermo Fisher Scientific	Cat#23030
Biotin-HPDP	APExBio	Cat#A808
Streptavidin agarose beads	Thermo Fisher Scientific	Cat#15942

Critical Commercial Assays		
Cytotoxicity Detection Kit (LDH)	Roche	Cat#04744934001
LDH cytotoxicity assay kit	Thermo Fisher Scientific	Cat#88953
IL-1 beta/IL-1F2 quantiGlo ELISA Kit	R&D systems	Cat#QLB00B
Mouse IL-1 beta/IL-1F2 DuoSet ELISA Kit	R&D systems	Cat#DY401
BCA Protein Assay Kit	Pierce	Cat#23227
ProcartaPlex^{TM} Mouse Th1/Th2 Cytokine Panel, 11plex	Thermo Fisher Scientific	Cat#EPX110-10810-901
RNeasy Mini Kit	Qiagen	Cat#74104
iScript Reverse Transcription Supermix for RT-qPCR	Bio-Rad	Cat#170-8840
iQ SYBR Green Supermix	Bio-Rad	Cat#170-8880
Experimental Models: Cell Lines		
HEK293T	ATCC	Cat#CRL-3216
THP-1	ATCC	Cat#TIB-202
iBMDM	Eicke Latz (2)	N/A
Experimental Models: Organisms/Strains		
Mouse: C57BL/6	The Jackson Laboratory	JAX: 000664
Mouse: C57BL/6J-*Gsdmd*^{em1Vnce}/J	The Jackson Laboratory	JAX: 032663
Oligonucleotides		
qRT-PCR and mutagenesis primer sequences	See Table S2	N/A
Recombinant DNA		
pCDNA4/TO-human full-length GSDMA-2xFlag	This paper	N/A
pCDNA4/TO-human N-terminal domain GSDMA (1-247aa)-2xFlag	This paper	N/A
pCDNA4/TO-human full-length GSDMB-2xFlag	This paper	N/A
pCDNA4/TO-human N-terminal domain GSDMB (1-248aa)-2xFlag	This paper	N/A
pCDNA4/TO-human full-length GSDMC-2xFlag	This paper	N/A
pCDNA4/TO-human N-terminal domain GSDMC (1-249aa)-2xFlag	This paper	N/A
pCDNA4/TO-human full-length GSDMD-2xFlag	This paper	N/A
pCDNA4/TO-human N-terminal domain GSDMD (1-245aa)-2xFlag	This paper	N/A
pCDNA4/TO-human N-terminal domain GSDMD (1-245aa)-2xFlag harboring F5A mutation	This paper	N/A
pCDNA4/TO-mouse N-terminal domain GSDMD (1-245aa) harboring F5A mutation	This paper	N/A
pCDNA4/TO-human N-terminal domain GSDMD (1-245aa) harboring C38A mutation	This paper	N/A

pCDNA4/TO-human N-terminal domain GSDMD (1-245aa)-2xFlag harboring C56A mutation	This paper	N/A
pCDNA4/TO-human N-terminal domain GSDMD (1-245aa)-2xFlag harboring C191A mutation	This paper	N/A
pCDNA4/TO-human N-terminal domain GSDMD (1-245aa) harboring C191A mutation and 10 residues (MLCCMRRTKQ) from protein hGAP43 added to the N-terminus (N10)	This paper	N/A
pCDNA4/TO-human N-terminal domain GSDMD (1-245aa) harboring C191A mutation and 15 residues (MATKPRWYRIWNFWC) from protein GAP1p added to the C-terminus (C15)	This paper	N/A
pCDNA4/TO-human N-terminal domain GSDMD (1-245aa) harboring C191A mutation and 13 residues (DHEPKTFWDKFWN) from protein Can1p with extra 3 amino acids (FWC) added to the C-terminus (C13+FWC)	This paper	N/A
pCDNA4/TO-human full-length GSDME-2xFlag	This paper	N/A
pCDNA4/TO-human N-terminal domain GSDME (1-270aa)-2xFlag	This paper	N/A
pEGFP-human N terminal domain GSDMD (1-245aa)	Derek Abbott (3)	N/A
pEGFP-human N terminal domain GSDMD (1-245aa) harboring C191A mutation	Derek Abbott (3)	N/A
pEF-Bos-HA-DHHC expression constructs	Masaki Fukata (4)	N/A
Software and Algorithms		
Microsoft Excel	Microsoft Corporation	Version 16.16.8
Origin	OriginLab	Version 2021b
Prism	GraphPad Software	Version 10.2.3
Other		
Lab-Tek 8-well Chamber Slide	Nunc Inc.	Cat#177402

Table S2. Oligonucleotide Sequences.

Name	Sequence (5’->3’)
qRT-PCR for GAPDH Forward	GTCTCCTCTGACTTCAACAGCG
qRT-PCR for GAPDH Reverse	ACCACCCTGTTGCTGTAGCCAA
hGSDMD-NTD (1-245aa) F5A Forward *	GGTCGGCCgcTGAGCGGGTAGTCCGGAGAGTGGTCCAG
hGSDMD-NTD (1-245aa) F5A Reverse *	CCCGCTCAgcGGCCGACCCCATGGATCCGAGCTCGGTAC
mGSDMD-NTD (1-245aa) F5A Forward *	TCCATGCCATCGGCCgcTGAGAAAGTGGTCAAGAATGTGATC
mGSDMD-NTD (1-245aa) F5A Reverse *	AgcGGCCGATGGCATGGATCCGAGCTCGGT
hGSDMD-NTD (1-245aa) C38A Forward *	AGCCCTACgcCCTGGTGGTTAGGAAGCCCTCAAGCTCAT
hGSDMD-NTD (1-245aa) C38A Reverse *	ACCACCAGGgcGTAGGGCTGGAAGCCAGTGGAGCTCTGC
hGSDMD-NTD (1-245aa) C56A Forward *	GTTATAAGgcTGTCAACCTGTCTATCAAGGACATCCTG
hGSDMD-NTD (1-245aa) C56A Reverse *	AGGTTGACAgcCTTATAACGGGGTTTCCAGAACCATGAG
hGSDMD-NTD (1-245aa) C191A Forward *	GAGCCACGgcaTTGCAGGGTGAGGGCCAGGGCCATCTG
hGSDMD-NTD (1-245aa) C191A Reverse *	CCCTGCAAtgcCGTGGCTCCGGGCAGGGAAAACCGGCC
hGSDMD-NTD (1-245aa) N10 Forward	tgttgcatgcgacgtacgaagcagATGGGGTCGGCCTTTGAGCGGGTAGTCC
hGSDMD-NTD (1-245aa) N10 Reverse	cgtacgtcgcatgcaacatagcatGGATCCGAGCTCGGTACCAAGCTTAAGT
hGSDMD-NTD (1-245aa) C15 Forward	atcggatatggaacttctggtgtGACTACAAAGACGATGACGACAAGGAT
hGSDMD-NTD (1-245aa) C15 Reverse	accatcgcggcttagttgccatCTCGAGTGTCGCGGGTGGCTGGAAGGT
hGSDMD-NTD (1-245aa) C13+FWC Forward	ggacttctggaacttctggtgtGACTACAAAGACGATGACGACAAGGAT
hGSDMD-NTD (1-245aa) C13+FWC Reverse	cagaaggtcttcggctcatgatcCTCGAGTGTCGCGGGTGGCTGGAAGGT
hGSDMA-NTD (1-247aa) Forward	TAAAGCCCGGGCGGATCCATGACCATGTTTGAAAATGTC

hGSDMA-NTD (1-247aa) Reverse	CGTCTTTGTAGTCCTCGAGTGCCTGGATAAGGATGACCTTC
hGSDMA-FL Reverse	TCGTCTTTGTAGTCCTCGAGGGAGGCCTTGGTAAGCTGCTG
hGSDMB-NTD (1-248aa) Forward	TAAAGCCCGGGCGGGATCCATGTTCAGCGTATTTGAGGA
hGSDMB-NTD (1-248aa) Reverse	CGTCTTTGTAGTCCTCGAGCGAACCCAAAGACTTTCCT
hGSDMB-FL Reverse	CGTCTTTGTAGTCCTCGAGGGAAGAGACAGAGGTAGGCC
hGSDMC-NTD (1-249aa) Forward	TAAAGCCCGGGCGGGATCCATGCCCTCCATGTTGGAACGC
hGSDMC-NTD (1-249aa) Reverse	CGTCTTTGTAGTCCTCGAGGCCTACCATTTCGGAAATTT
hGSDMC-FL Reverse	CGTCTTTGTAGTCCTCGAGGGCCTCAGCCAGCTGCTGCAG
hGSDMD-NTD (1-245aa) Forward	CGGGATCCATGGGGTCGGCCTTTGAGCGG
hGSDMD-NTD (1-245aa) Reverse	CCGCTCGAGTGTCGCGGGTGGCTGGAAGGTC
hGSDMD-FL Reverse	CCGCTCGAGGTGGGGCTCCTGGCTCAGTC
hGSDME-NTD (1-270aa) Forward	CGGGATCCATGTTTGCCAAAGCAACCAGGAAT
hGSDME-NTD (1-270aa) Reverse	CCGCTCGAGATCTGGCATGTCTATGAATGC
hGSDME-FL Reverse	CCGCTCGAGTGAATGTTCTCTGCCTAAAGC

* Red-colored nucleotides mark the mutated codons.

References

1. S.-A. Azizi, *et al.*, Development of an Acrylamide-Based Inhibitor of Protein *S* -Acylation. *ACS Chem. Biol.* **16**, 1546–1556 (2021).

2. A. Stutz, G. L. Horvath, B. G. Monks, E. Latz, ASC speck formation as a readout for inflammasome activation. *Methods in molecular biology (Clifton, N.J.)* **1040**, 91–101 (2013).

3. J. K. Rathkey, *et al.*, Chemical disruption of the pyroptotic pore-forming protein gasdermin D inhibits inflammatory cell death and sepsis. *Science immunology* **3**, eaat2738 (2018).

4. M. Fukata, Y. Fukata, H. Adesnik, R. A. Nicoll, D. S. Bredt, Identification of PSD-95 Palmitoylating Enzymes. *Neuron* **44**, 987–996 (2004).