



Structural basis of GSDME pore formation and its regulation by S-palmitoylation

Gang Du^{a,b,1,2}, Julian F. Ehrmann^{a,b} , Judy Lieberman^{b,c,2} , and Hao Wu^{a,b,2}

Affiliations are included on p. 10.

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Pyroptosis is defined as gasdermin-mediated lytic programmed cell death. Gasdermin E (GSDME), a substrate of the apoptotic caspase-3, can convert apoptosis into pyroptosis, with critical roles in antitumor immunity and chemotherapy-induced tissue damage. Despite its importance, the structural mechanism of GSDME pore formation and its regulation by posttranslational modifications remain largely unknown. Here, we present the cryo-electron microscopy (cryo-EM) structure at 3.16 Å resolution of the human GSDME N-terminal (NT) pore using proteins expressed from mammalian cells. The structure reveals a GSDME-NT pore assembled mainly as a 28-subunit homo-oligomer, and a dramatic conformational rearrangement from the autoinhibited state, with refolding of the two β -hairpins in each monomer to form a membrane-spanning β -barrel with an acidic conduit. Unexpectedly, we identify endogenous S-palmitoylation of C45, C168, and C180, required for membrane binding and pore formation. In addition, extra cryo-EM densities are visible adjacent to the C45 side chain, potentially corresponding to the flexibly linked palmitate chain. Structure-guided mutagenesis demonstrates that these palmitoylation sites synergistically control pore formation. The structure served as a molecular blueprint for analyzing cancer-associated mutations, known to disrupt GSDME function. These mutations cluster at functional hotspots in the oligomerization interfaces, membrane-contact regions, and the β -barrel, where they disrupt pore integrity. Collectively, our findings establish palmitoylation as an obligatory licensing step for membrane binding and pore formation, provide structural visualization of a palmitoylated gasdermin, and reveal how cancer-associated mutations impair pyroptotic function. This structure and these insights will be useful for developing strategies that target GSDME to treat cancer and inflammatory disease.

pyroptosis | gasdermin E | cryo-EM structure | S-palmitoylation | cancer-associated mutations

Pyroptosis is a lytic form of programmed cell death that is critical for host defense against pathogens and has emerged as a key mechanism in inflammation, cancer, inflammatory disease pathogenesis, and chemotherapy-induced toxicity (1–5). The gasdermin (GSDM) family of proteins are the direct effectors of pyroptosis (6–10). Upon activation by inflammatory caspases or other proteases, the gasdermin N-terminal domain (GSDM-NT) oligomerizes to form large, membrane-spanning pores that disrupt cellular homeostasis, leading to the processing and release of proinflammatory cytokines, such as interleukin-1 β (IL-1 β) and interleukin-18 (IL-18), and rapid cell lysis (6–11).

Among the GSDM family members, gasdermin E (GSDME, also known as DFNA5) has garnered particular attention due to its unique role in connecting apoptotic signaling to pyroptosis (5, 12, 13). Unlike gasdermin D (GSDMD), which is primarily activated by inflammatory caspases (caspase-1, -4, -5, and -11) downstream of inflammasome activation (14–16), GSDME is specifically cleaved by caspase-3 following its activation by apoptotic stimuli, thereby converting apoptotic signals into a potent inflammatory pyroptotic response (5, 12). This caspase-3-mediated cleavage of GSDME has been implicated in chemotherapy-induced tissue damage and antitumor immunity, highlighting its dual role as both a mediator of cell death and a modulator of the immune response (5, 17, 18). Despite its biological and clinical importance, the structural basis for GSDME's pore-forming mechanism, its functional regulation by posttranslational modifications, and the molecular consequences of cancer-associated mutations remain incompletely understood.

Structural studies of other gasdermins, gasdermin A3 (GSDMA3), gasdermin B (GSDMB), and GSDMD, have revealed a conserved architecture in which the N-terminal domain undergoes a dramatic conformational rearrangement to form a β -barrel pore (11, 19, 20). However, the GSDME N-terminal domain (GSDME-NT) pore structure has not been solved, limiting our understanding of its unique features. Accumulating evidence suggests that gasdermin activity is regulated by additional layers of control, including lipid

Significance

Gasdermin E (GSDME) links apoptosis to pyroptosis, but the structural mechanism of its pore formation remains unknown. Our cryo-electron microscopy structure of the GSDME pore establishes a general framework for gasdermin-mediated membrane disruption. We identify S-palmitoylation as an obligatory activation checkpoint that targets GSDME to the plasma membrane, revealing a regulatory layer exploitable for therapeutic intervention. The structure provides a molecular roadmap for interpreting cancer-associated GSDME mutations, showing that many variants disrupt pore assembly at defined hotspots—suggesting that pyroptosis loss may serve as an immune evasion mechanism in certain tumors. Collectively, our findings reframe GSDME activation, identify palmitoylation as a potential pharmacological node for modulating pyroptotic cell death, and offer a structural basis for stratifying GSDME mutations across cancers.

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The authors declare no competing interest.

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¹Present address: Institute of Precision Medicine, The First Affiliated Hospital, Sun Yat-Sen University, Guangzhou, Guangdong 510080, China.

²To whom correspondence may be addressed. Email: dugang3@mail.sysu.edu.cn, judy.lieberman@childrens.harvard.edu, or wu@crystal.harvard.edu.

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interactions and posttranslational modifications (21–27). Notably, while GSDMD is known to be regulated by palmitoylation (21, 26–29), the potential for similar modifications in GSDME-NT and their functional significance for pore formation and pyroptotic activity have not been explored. More importantly, the structure of palmitoylated GSDMD has yet to be determined, leaving a critical gap in our understanding of how palmitoylation regulates pyroptosis.

Here, we present the high-resolution cryo-EM structure of the human GSDME pore at 3.16 Å resolution, revealing a 28-subunit homo-oligomer with an architecture that is largely conserved among gasdermins, but also is uniquely adapted to its function. The structure provided a detailed view of the conformational transition from the autoinhibited to the pore-forming state, highlighting the refolding of the two β -hairpins of each subunit that constitute the transmembrane region within the membrane-spanning β -barrel, which has an acidic surface within the pore. Unexpectedly, our structural analysis identified extra densities for a potential palmitoyl chain covalently attached to C45, a modification that we show, together with palmitoylation at C168 and C180, is critical for membrane binding and pyroptotic activity. The structure also identified extensive intersubunit interfaces that stabilize the pore. The structure serves as a molecular blueprint for understanding the loss of function of cancer-associated missense mutations, which cluster at key functional hotspots. Collectively, our findings unveil the structural mechanism of GSDME-mediated pore formation, establish palmitoylation as a central regulator of its function, and provide a framework for understanding its role in health and disease.

Results

Structure Determination of the GSDME Pore. To elucidate the mechanism by which GSDME executes pyroptotic cell death, we sought to determine the high-resolution structure of its pore-forming N-terminal fragment (GSDME-NT). Full-length human GSDME (GSDME-FL) was expressed in Expi293 cells, chosen for their ability to support native posttranslational modifications (Fig. 1A–C and *SI Appendix*, Fig. S1A and B). Upon cleavage by caspase-3 (Fig. 1C), the resulting GSDME-NT forms pores in the presence of liposomes containing phosphatidylcholine (PC), phosphatidylethanolamine (PE), and cardiolipin (CL), as demonstrated by a robust Tb³⁺ leakage assay (Fig. 1D). This finding confirmed that GSDME-NT produced in Expi293 cells is fully functional and capable of forming membrane pores.

For structural studies, we reconstituted GSDME pores *in vitro* by incubating purified GSDME-FL with caspase-3 and liposomes (Fig. 1E). Negative stain EM of the sample revealed the presence of numerous ring-shaped structures with a diameter of approximately 30 nm, consistent with the expected size of gasdermin pores (Fig. 1F). To extract and purify the high-quality pores for cryo-EM analysis, we screened a panel of nonionic detergents (C12E8, C10E9, C12E9, C12E10, LMNG) and an ionic detergent (cholate). C12E8 was identified as the optimal solubilizing agent, yielding stable, high-quality pores (Fig. 1G and *SI Appendix*, Fig. S1C–E). Cryo-EM grids were prepared with high-quality purified pores and screened to obtain high-quality micrographs (Fig. 1H). Single-particle analysis of the Cryo-EM dataset revealed heterogeneity in oligomerization state, with pores exhibiting 27-, 28-, or 29-fold symmetry, among which the 28-fold species was most abundant (*SI Appendix*, Fig. S2A–C). Following extensive 3D classification and refinement using C28 symmetry, a cryo-EM map at an overall resolution of 3.16 Å, allowing for unambiguous model building, was obtained (Fig. 1I and *SI Appendix*, Figs. S2D–F, S3, and S4 and Table S1).

Overall Architecture of the GSDME Pore. The 3.16 Å structure revealed that the GSDME-NT pore is a large, 28-subunit homo-oligomer with an inner diameter of 180 Å, an outer diameter of about 290 Å, and a height of roughly 80 Å (Fig. 2A). The architecture of the GSDME pore shares the fundamental two-domain organization observed in other gasdermin family members: a membrane-inserted β -barrel that spans the lipid bilayer and a globular domain that resides on the cytosolic side of the membrane.

Each GSDME-NT subunit adopts a distinctive fold that resembles a human left hand as shown initially for mouse GSDMA3-NT (19) (Fig. 2B). The globular domain, which forms the bulk of the cytosolic portion, corresponds to the “palm” of the hand. This domain is composed of a mixed α/β structure that is largely conserved from the autoinhibited conformation. The α 1 helix protrudes from the palm like a “thumb,” positioned near the membrane interface. The most striking feature of the pore-forming subunit is the extension of two long β -hairpins, previously termed β -hairpin 1 (HP1) and β -hairpin 2 (HP2) (19), that insert into the membrane. These hairpins, composed of β -strands β 3– β 5 and β 7– β 8 respectively, extend from the globular domain like the “fingers” of a hand. The β 1– β 2 loop, located at the base of the fingers, is positioned at the membrane-proximal region and serves as a “wrist” that participates in initial membrane engagement (Fig. 2B). The 28 subunits assemble into a continuous, antiparallel β -barrel that constitutes the transmembrane region. Each subunit contributes four β -strands (two from each β -hairpin), resulting in a total of 112 β -strands forming the barrel. The β -strands range in length from 15 to 22 residues. The β -barrel is approximately 55 Å in height, sufficient to span the hydrophobic core of a typical plasma membrane (approximately 30 to 40 Å) (Fig. 2B and *SI Appendix*, Fig. S5A). The inner surface of the barrel is lined predominantly by hydrophilic and charged residues, which is consistent with the formation of a water-filled channel.

Conformational Transition Upon Pore Formation. To understand the structural rearrangements that accompany pore formation, we compared the pore structure of GSDME-NT with its full-length autoinhibited state predicted by AlphaFold (AF-O60443-F1, UniProt), which showed the canonical gasdermin architecture with the inhibitory C-terminal domain (GSDME-CT) tightly clasping the N-terminal domain (GSDME-NT) (Fig. 2C). Superposition of the pore-form GSDME-NT onto the N-terminal domain of the autoinhibited full-length structure reveals a dramatic conformational change, with a rmsd of 14.4 Å for 246 aligned C α atoms (Fig. 2D). Importantly, the globular domain remains largely unaltered (rmsd of 1.1 Å for 159 aligned C α atoms), indicating that it serves as a stable scaffold (Fig. 2D). By contrast, the regions that become the two membrane-inserted β -hairpins undergo extensive refolding (Fig. 2D).

The first β -hairpin (HP1) is formed from elements that in the autoinhibited state constitute the β 3– β 3′– β 4– β 5 region. Among this region, β 3′ and β 4, which was previously termed extension domain 1 (ED1) (19) unfold and refold to become part of the long β 3 and the turn in HP1 (Fig. 2E and *SI Appendix*, Fig. S4). Similarly, the second β -hairpin (HP2) arises from the β 7– α 5– β 8′– β 8 region. In the autoinhibited state, the α 5 helix is a key structural element that binds to GSDME-CT and was referred to as extension domain 2 (ED2). Upon cleavage, this α 5 helix and the flanking β -strands straighten out to form the extended β -hairpin of HP2 (Fig. 2E and *SI Appendix*, Fig. S4). These structural transitions are reminiscent of those observed in GSDMA3, GSDMB, and GSDMD (11, 19, 20), suggesting a conserved mechanism of membrane insertion across the gasdermin family.

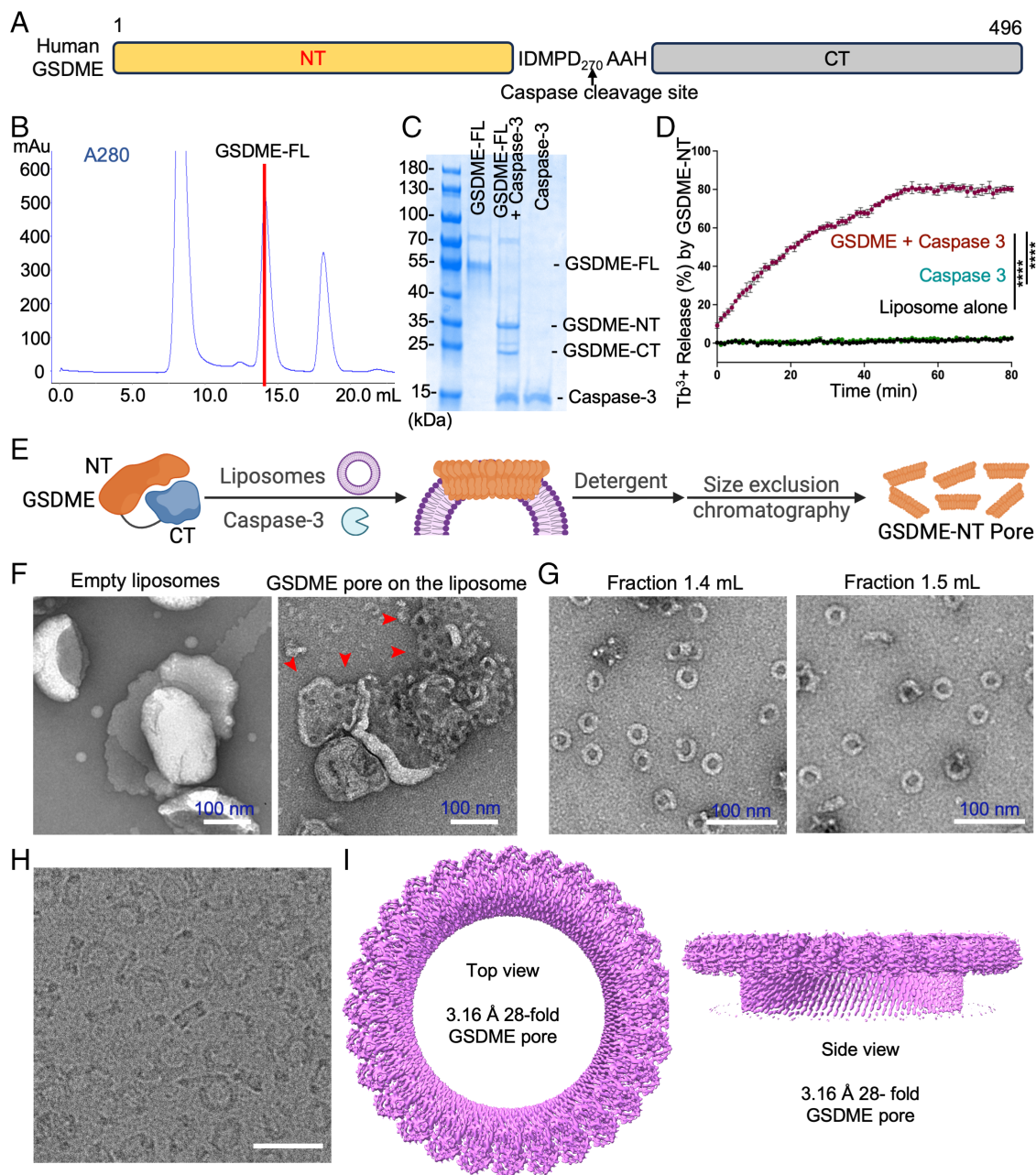


Fig. 1. Reconstitution, purification, and structure determination of GSDME pores. (A) Construct for human GSDME. The arrow at D270 indicates the caspase-3 cleavage site. The C-terminal Flag tag is not shown. (B) Size-exclusion chromatography profiles, and the red line indicates the GSDME peak. (C) SDS-PAGE showing efficient cleavage of full-length GSDME by caspase-3 to generate GSDME-NT and GSDME-CT. (D) Liposome leakage rates for palmitoylated GSDME-NT (cleaved by caspase-3) expressed in Expi293 cells, indicating that GSDME-NT forms pores in liposomes and releases Tb^{3+} . Data are mean $\pm$ SEM of $n = 3$. Statistical analysis was performed using two-way ANOVA; **** $P < 0.0001$. (E) Schematic of GSDME pore reconstitution. (F) Empty liposomes and GSDME-NT pores formed on liposomes. Purified GSDME-FL was incubated with caspase-3 and liposomes at room temperature for 1 h. (Scale bar, 100 nm.) (G) Negative-stain EM images of GSDME-NT pores from the fractions indicated in (SI Appendix, Fig. S1D). (Scale bar, 100 nm.) (H) A cryo-EM image of the GSDME pore sample. (Scale bar, 50 nm.) (I) Overall view of the cryo-EM density map of the 28-fold GSDME pore at 3.16 Å resolution.

An Acidic Pore Conduit Conserved Among Gasdermins. The electrostatic surface potential of the GSDME pore reveals a striking charge distribution that has functional implications for ion and cargo transport (Fig. 3A). The membrane-facing surface, which contacts the lipid bilayer, is enriched in basic residues, facilitating initial electrostatic interactions with the negatively charged inner leaflet of the plasma membrane. Conversely, the luminal surface lining the pore conduit is predominantly acidic, presenting a negative electrostatic potential that extends from the cytosolic entrance through the β -barrel to the extracellular side (Fig. 3A).

We compared this charge distribution to the known structures of GSDMA3 (PDB: 6CB8), GSDMB (PDB: 8ET2), and GSDMD (PDB: 6VFE) pores (11, 19, 20). This analysis revealed that the acidic conduit is a conserved feature among GSDMA3, GSDMD, and GSDME, while the GSDMB pore showed a slightly different charge distribution (Fig. 3A). The negative electrostatic potential of the GSDME pore is dominated by several acidic patches (APs) located on the β -barrel and the globular domain, analogous to the APs regions previously described for GSDMD (11) (Fig. 3A). Structural superposition of a pore-forming subunit of GSDME-NT

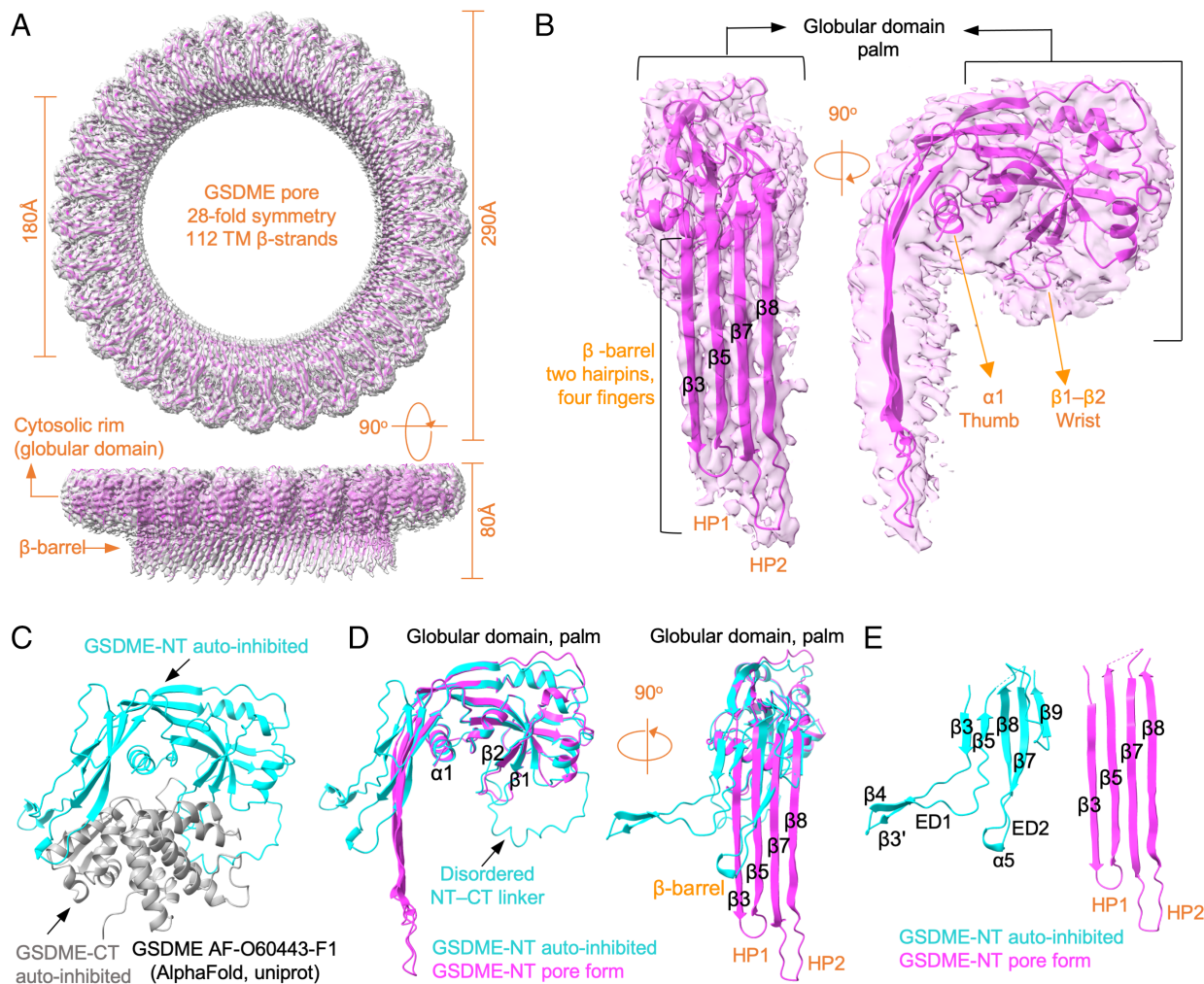


Fig. 2. Architecture and conformational changes of the GSDME pore. (A) Ribbon diagram and dimensions of the 28-subunit human GSDME pore structure fitted into its cryo-EM density map at 3.16 Å resolution. The pore comprises a large transmembrane β -barrel that spans the lipid bilayer, with a globular domain extending into the cytosol. TM, transmembrane. (B) Structure of one pore-forming GSDME subunit fitted into the cryo-EM density map. The structure resembles a human left hand, with the globular domain serving as the “palm,” the $\alpha 1$ helix as the “thumb,” the two membrane-inserted β -hairpins as the “fingers,” and the $\beta 1$ – $\beta 2$ loop as the “wrist.” (C) AlphaFold-predicted full-length autoinhibited structure of GSDME (UniProt). (D) Superposition of the autoinhibited form and the pore form of GSDME-NT. The rmsd of GSDME-NT is 14.44 Å for 246 aligned C α atoms. The rmsd of globular domain of GSDME-NT is 1.1 Å for 159 aligned C α atoms. (E) Structural transitions from the autoinhibited to the pore form involve the formation of two β -hairpins, HP1 and HP2. ED1 and ED2, extension domains 1 and 2, respectively.

with its counterparts from GSDMA3-NT, GSDMB-NT, and GSDMD-NT revealed overall similar conformations. Notably, the palm–finger angle in GSDME-NT is approximately 90°, similar to those in GSDMA3 and GSDMB, whereas GSDMD displays a substantially larger angle of about 120° (*SI Appendix, Fig. S5B*). This conserved acidic environment suggests that the gasdermin pores selectively deter the release of certain cellular factors, such as pro-IL-1 β and pro-IL-18 whose surfaces are negatively charged at physiological pH but allow the release of mature IL-1 β and IL-18 whose surfaces are no longer acidic. Perhaps more importantly for GSDME, whose activation does not necessarily concur with inflammasome-mediated IL-1 β and IL-18 cytokine maturation, the acidic nature of the pore may permit the preferential release of certain damage-associated molecular patterns (DAMPs) such as positively charged HMGB1.

Palmitoylation of GSDME. During model building of the GSDME pore, we observed elongated, albeit noisy extra density extending from the side chain of C45 (*Fig. 3B*), which is located between $\beta 1$ and $\beta 2$ near the membrane-proximal region. We wondered if the density could be due to C45 palmitoylation when expressed in

Expi293 cells but wanted to ensure that it was not due to potential artifacts by high symmetry averaging. We therefore processed these data again without imposing symmetry and masked the four subunits that were most well-resolved in the unaveraged map for focused classifications (*SI Appendix, Fig. S2 and Table S1*). With this approach, we obtained a map that had more consistent resolutions across the different regions of a GSDME protomer (*Fig. 3C*). In addition, we did also capture extra but variable densities stemming from the C45 residue that likely arise from the flexibly linked lipid (*Fig. 3D*), further supporting palmitoylation of GSDME.

To experimentally validate this posttranslational modification, we performed an acyl-biotin exchange (ABE) assay, a well-established method for detecting protein S-acylation (30) (*Fig. 3E*). Using this assay, we assessed the palmitoylation status of GSDME-NT. Wildtype (WT) GSDME-NT showed a strong palmitoylation signal, whereas the signal was markedly reduced in the C45A mutant (*Fig. 3F*), confirming that C45 is a bona fide palmitoylation site. We next investigated whether palmitoylation is required for pore-forming activity. Liposome leakage assays (9, 11, 19, 21) were performed to compare pore formation of GSDME WT and the

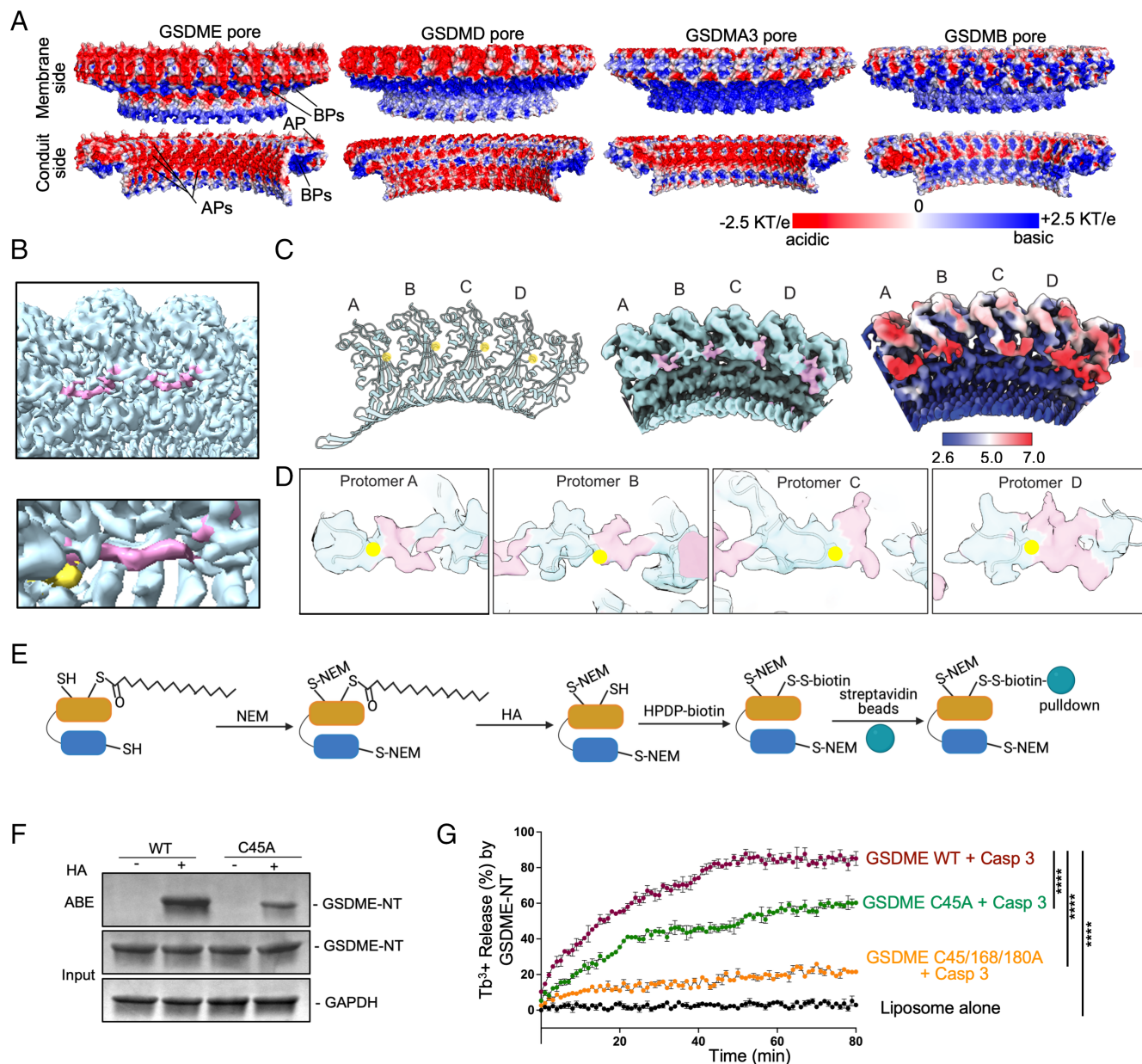


Fig. 3. Pore conduit and palmitoylation of GSDME. (A) Electrostatic surface potential (-2.5 to $+2.5$ kT/e) of the GSDMA3, GSDMB, GSDMD, and GSDME pores. The membrane-bound side is formed by basic patches (BPs), whereas the solvent-exposed side is composed of acidic patches (APs). The GSDMA3 and GSDMD pores show an acidic conduit similar to that of GSDME, whereas the GSDMB pore is slightly different. (B) Cryo-EM density map revealing an extended extra density near C45. Top: Part of the pore density in cyan with the extra density in pink for 2 of the subunits. Bottom: Enlarged view of one of the subunits, with density for C45 marked in yellow. (C) Focus-refined 4 to 5 subunits of GSDME-NT without imposing symmetry, as an atomic model with C45 in yellow, cryo-EM density colored in cyan except for the extra densities near C45 which are in pink, and cryo-EM density colored by resolution in Å. (D) Detailed views of the extra densities near C45 shown in pink for each of the labeled 4 protomers in (B). C45 is shown in yellow while the remaining densities are in cyan. (E) Schematic of ABE assay for detecting protein acylation. NEM, N-ethylmaleimide; HA, hydroxylamine. (F) Palmitoylation of WT and C45A mutant GSDME-NT assessed by ABE. Palmitoylation was reduced in the C45A mutant compared with WT, indicating that GSDME is palmitoylated at C45. (G) Liposome leakage rates for palmitoylated WT and cysteine mutants of GSDME-NT (cleaved by caspase-3) expressed in Expi293 cells. Data are mean $\pm$ SEM $n = 3$. Statistical analysis was performed using two-way ANOVA; **** $P < 0.0001$.

C45A mutant. The C45A mutant showed significantly reduced liposome leakage compared to WT (Fig. 3G). This defect was not due to altered protein expression or cleavage efficiency, as all mutants were expressed at comparable levels and efficiently cleaved by caspase-3. These results indicated that palmitoylation at C45 is critical for the pore-forming function of GSDME-NT.

Palmitoylation Is Critical for GSDME-Mediated Pyroptosis. Given that the C45A mutation only partially reduced palmitoylation (Fig. 3F) and only partially impaired activity (Fig. 3G), we hypothesized that additional palmitoylation sites might exist. To

map these sites, we generated a comprehensive panel of cysteine-to-alanine mutants spanning the entire GSDME-NT sequence and assessed their palmitoylation by ABE. This screen identified C168 and C180 as two additional residues that exhibited a substantial reduction in palmitoylation signal when mutated (Fig. 4A). In addition, ABE showed that GSDME-CT is also palmitoylated at C407 and C408 (SI Appendix, Fig. S5C), which is consistent with a previous finding (13).

To determine the functional relevance of these palmitoylation sites, we expressed WT and mutant GSDME-NT constructs in HEK293T cells and assessed their ability to induce pyroptosis, quantified by

propidium iodide (PI) uptake, lactate dehydrogenase (LDH) release, and cell viability. The C45A, C168A, and C180A single mutants all showed significantly reduced PI uptake, LDH release, and cell viability, compared to WT (Fig. 4B–D). To assess the cumulative effect, we generated a triple mutant (C45/168/180A). ABE analysis confirmed that the triple mutant was completely devoid of palmitoylation (Fig. 4E). Consistent with this, the triple mutant exhibited an even more profound defect in inducing cell death, with reduced PI uptake and LDH release and increased cell viability (Fig. 4F–H). The triple mutation showed almost completely abolished liposome leakage compared to WT (Fig. 3F). Pharmacological inhibition of palmitoylation using 2-bromopalmitate (2-BP) recapitulated the phenotype of the triple mutant, significantly reducing the cytotoxicity of WT GSDME-NT (Fig. 4E–H).

We next examined whether the defect in pyroptosis was due to impaired membrane localization. Confocal microscopy of HEK293T cells expressing mCherry-tagged GSDME-NT constructs revealed that WT GSDME-NT efficiently localized to the plasma membrane

(Fig. 4I). In contrast, the triple mutant maintained a diffuse cytosolic distribution, and 2-BP treatment of WT GSDME-NT similarly prevented membrane association (Fig. 4I). Quantification of membrane localization confirmed these observations (Fig. 4J). These data demonstrate that palmitoylation at triple sites—C45, C168, and C180—is essential for the membrane targeting and the pyroptotic activity of GSDME-NT.

Oligomerization Interface of the GSDME Pore. The high-resolution structure allowed us to delineate the intersubunit interactions that stabilize the 28-fold GSDME pore. Each subunit buries a total surface area of approximately 1,750 Å² with its two neighboring subunits (Fig. 5A and B), a value comparable to those observed for GSDMA3 and GSDMD pores (11, 19). The oligomerization interface can be divided into four distinct patches, designated I through IV, based on the nature and location of the interactions (Fig. 5A and B). Patch I is formed by interactions between the globular domains of adjacent subunits (Fig. 5C). Residues close

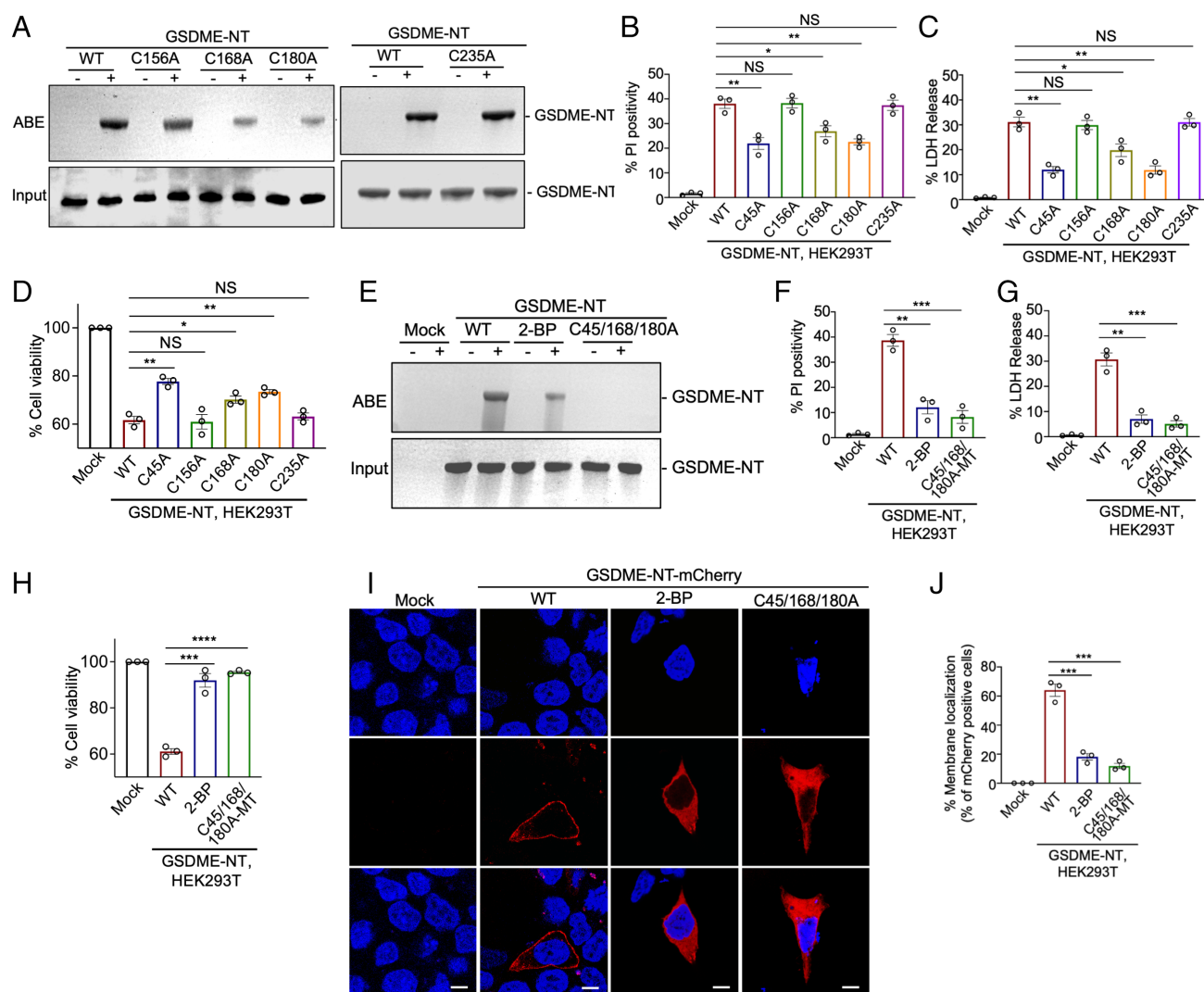


Fig. 4. The palmitoylation of GSDME and its role in pyroptosis. (A) Palmitoylation of WT GSDME-NT and cysteine mutants of GSDME-NT assessed by ABE, revealing palmitoylation at residues C168 and C180. (B–D) PI positivity (B), LDH release (C), and cell viability (D) in HEK293T cells expressing WT or cysteine mutants of GSDME-NT, showing impaired pyroptosis induction by the C45A, C168A, and C180A mutants. (E) ABE assay for palmitoylation of WT GSDME-NT, WT GSDME-NT treated with 2-BP, or the C45/168/180A triple mutant of GSDME-NT, demonstrating complete loss of palmitoylation in the triple mutant. (F–H) PI positivity (F), LDH release (G), and cell viability (H) in HEK293T cells expressing WT GSDME-NT, WT GSDME-NT treated with 2-BP, or the C45/168/180A triple mutant, indicating a marked impairment in pyroptosis induction in the triple mutant or upon 2-BP treatment. (I and J) mCherry fluorescence imaging (I) and quantification (J) of membrane localization of WT GSDME-NT, WT GSDME-NT treated with 2-BP, or the C45/168/180A triple mutant, showing strong defects in membrane association for the triple mutant and upon 2-BP treatment. Scale bar, 5 μm (I). Data are presented as the mean ± SEM of n = 3 (B–D and F–H). Statistics were measured by two-tailed Student's *t* test with NS (nonsignificant) for *P* > 0.05, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

to β 11 strand from one subunit engage with residues in the α 5 helix and the β 2 strand of the neighboring subunit. This patch is characterized by a combination of electrostatic attraction, hydrophobic contacts, and hydrogen bonds. Patch II involves the α 1' helices of one subunit and the β 5' and β 6 strands of neighboring subunits (Fig. 5D). The hydrophobic and electrostatic nature of this patch helps stabilize the oligomeric assembly. Patch III involves additional contacts between the β 6- β 7 and β 8- β 9 loops of one subunit and the β 3 strand of the neighboring subunit (Fig. 5E). Patch IV contains interactions between the β 3 strand of one subunit and the β 8 strand of the adjacent subunit (Fig. 5F). This patch represents the primary lateral interface within the β -barrel. The hydrogen bonding network between the β -strands is extensive, with a two-residue shift per subunit that results in a shear number of 56 for the 28-fold barrel, consistent with the observed twist of the β -strands (SI Appendix, Fig. S6). The presence of multiple, distributed interfaces suggests that the GSDME pore is highly stable once assembled, which is consistent with its function as a lethal membrane channel.

Structural Basis of Cancer-Associated and Previously Generated Mutations. Most cancer cell lines epigenetically repress GSDME expression, but some of the cancers that express GSDME have missense mutations in GSDME that impair pyroptosis (17). These missense mutations in GSDME associated with various human cancer cells have been previously identified in the TCGA cancer genomics database, among which D14Y, D18V, N24D, W44C, R48I, I137T, G199E, P212L, and I217N have been shown to impair pyroptosis activity (17). Additionally, K39A, K40A, and K41A mutations were shown to prevent targeting of GSDME to the plasma membrane and significantly reduce its pyroptotic activity (12). To provide a structural basis for their potential loss of function, we mapped these mutations onto our GSDME pore structure (Fig. 5G and SI Appendix, Fig. S7 A and B). We also analyzed the positions within the GSDME pore structure of additional cancer mutations whose function has not been characterized. These mutations predominantly cluster in three functionally critical regions: the oligomerization interfaces, the membrane contact regions, and the membrane-inserted β -barrel or the globular domain.

Among residues at oligomerization interfaces, D14Y (breast carcinoma), D18V (colon adenocarcinoma), and N24D (colon adenocarcinoma), are situated near the α 1 helix. In the pore structure, D14 in one subunit forms hydrogen bonds with N24 in the adjacent subunit. Similarly, hydrogen bonds are important for the interaction between D18 of one subunit and N26 of the neighboring subunit. The D14Y, D18V, and N24D mutations should disrupt these hydrogen bond interactions, thereby compromising GSDME pore oligomerization and reducing pyroptotic activity. The P212L mutation disrupts the CH- π stacking interaction between the proline ring at position P212 of one subunit and the phenyl ring of F77 in the neighboring subunit and increases the backbone conformational entropy of P212—both effects contributing to a reduced binding affinity (Fig. 5G). Another mutation of GSDME in the TCGA database is S75L in hepatocellular carcinoma and has not been functionally characterized. Structurally, S75 forms a hydrogen bond via its hydroxyl group with D29 in the α 1' helix of the adjacent subunit (Fig. 5I). Thus, the S75L mutation could disrupt this interaction and impairs oligomerization (Fig. 5 H and I and SI Appendix, Fig. S7 C and D). To test this hypothesis, we assessed the function of S75L and found that it led to reduced pyroptotic activity (Fig. 5H and SI Appendix, Fig. S7 C and D).

Among mutations that are likely to affect membrane binding are K39A, K40A, K41A (12), W44C, and R48I. K39, K40, and K41 are located in the β 1 strand near the membrane, and the alanine mutations may reduce the electrostatic attraction of GSDME to the anionic membrane (Fig. 5G). W44C (lung adenocarcinoma) and R48I (uterine corpus endometrioid carcinoma) are situated in the β 1- β 2 loop, also near the membrane (Fig. 5G). Additionally, these residues are adjacent to the palmitoylation site at C45 or the palmitate-binding pocket. Thus, mutations on these residues could potentially interfere with membrane binding directly or through palmitoylation.

Several mutations may disrupt the β -barrel or the globular domain. G199E (uterine corpus endometrioid carcinoma) lies within β 8 and should impair β -hairpin formation during membrane insertion due to its negative charge (Fig. 5G). I217N (lung adenocarcinoma) is located in the β 8- β 9 loop, where it likely disrupts the tight packing and hydrogen bonding network essential for α 1 helix and globular domain stability (Fig. 5G). E134K and I137T (uterine corpus endometrioid carcinoma) are situated in the α 3- α 4 loop, where they also affect the hydrogen bonding network critical for globular domain stability. Specifically, E134 forms a hydrogen bond with D131, and I137 forms a hydrogen bond with G230 (Fig. 5G). Testing the effect of the E134K mutation by expressing GSDME-NT E134K in HEK293T cells resulted in impaired pyroptosis activity, consistent with the critical role of this residue in GSDME pore function (Fig. 5 H and I and SI Appendix, Fig. S7 C and D).

Discussion

In this study, we present the high-resolution cryo-EM structure of the human GSDME pore at 3.16 Å, providing a detailed molecular framework for understanding how GSDME executes pyroptotic cell death. The structure reveals a 28-subunit homo-oligomer that forms a β -barrel pore, establishing GSDME as a member of the gasdermin family that shares a conserved core architecture while exhibiting distinct features. Extra densities attached to C45 in the cryo-EM map offers support for a palmitoyl modification at this site. Beyond structural characterization, our work uncovers an essential role for S-palmitoylation in GSDME function, identifies three critical palmitoylation sites at GSDME-NT, and provides a structural blueprint for interpreting the pathogenicity of cancer-associated mutations. During the preparation of this study, another group independently reported the cryo-EM structure of GSDME-NT and revealed similar conclusions (31).

The GSDME pore structure reveals a dramatic conformational rearrangement accompanying the transition from the autoinhibited, full-length state to the active, membrane-inserted pore. Consistent with studies on GSDMA3, GSDMB, and GSDMD (11, 19, 20), the globular domain of GSDME-NT remains largely unchanged during activation, serving as a stable scaffold from which two long β -hairpins (HP1 and HP2) are deployed. These hairpins refold from α -helical and loop regions (extension domains ED1 and ED2) that, in the autoinhibited state, engage the C-terminal domain through intramolecular interactions. The structural conservation of this refolding mechanism across gasdermin family members suggests a common activation paradigm: Proteolytic cleavage releases the C-terminal inhibitory domain, allowing the N-terminal domain to undergo a concerted unfolding-refolding process that drives membrane insertion. Notably, the GSDME pore exhibits a strong preference for 28-fold symmetry, although lower-abundance species with 27- and 29-fold symmetry are also observed. This heterogeneity may reflect intrinsic flexibility in the oligomerization interface

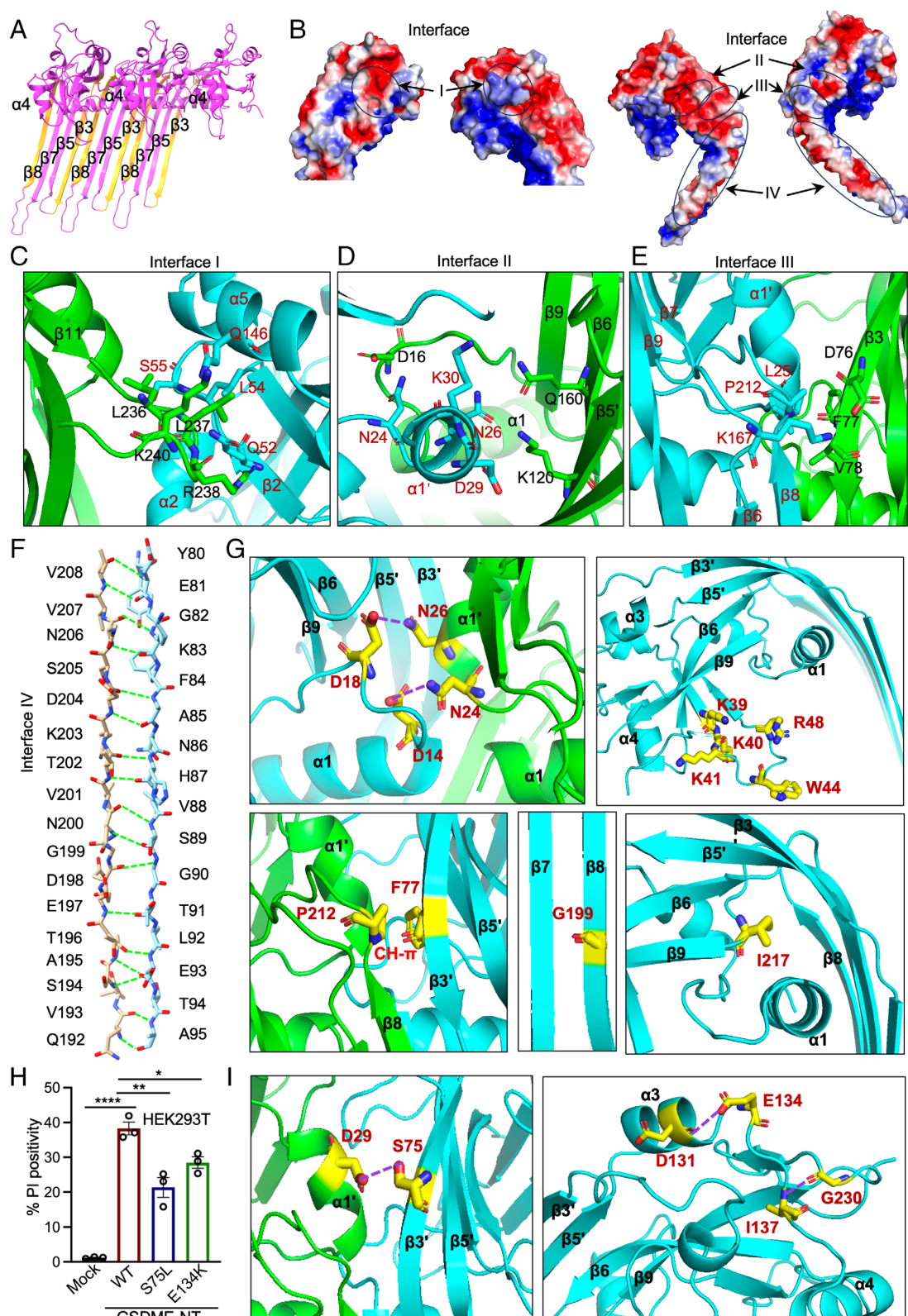


Fig. 5. Mechanism of oligomerization and membrane insertion of GSDME, and variants and mutants of GSDME. (A) Three contiguous subunits in the GSDME pore. Residues in the β -barrel that participate in oligomerization are labeled in yellow. (B) Electrostatic potential map of a GSDME-NT subunit shown in two side views, highlighting the oligomerization interface with four major patches labeled. Each subunit buries a total surface area of approximately $1,750 \text{ \AA}^2$ with its two neighboring subunits. (C–F) Main residues at interface I (C), interface II (D), interface III (E), and interface IV (F). (G) Locations of previously reported cancer-associated missense mutations and mutagenesis-derived mutants mapped onto the GSDME pore. (H) PI positivity of HEK293T cells expressing GSDME-NT with previously unreported cancer-associated missense mutations, showing impaired pyroptosis induced by S75L and E134K. (I) Locations of the unreported cancer-associated missense mutations shown in (H) on the GSDME pore. Data are presented as the mean $\pm$ SEM of $n = 3$ (H). Statistics were measured by two-tailed Student's t test with $*P < 0.05$, $**P < 0.01$, and $****P < 0.0001$.

or be influenced by membrane composition and local lipid environment, potentially affecting pore stability and cargo selectivity. The 28-subunit GSDME pore is nearly identical in size to the 27-subunit GSDMA3 pore (19), larger than the 24-fold GSDMB pore (20), and smaller than the 33-subunit GSDMD pore (11). Consistently, the inner diameter of the GSDME pore (~180 Å) matches that of GSDMA3 (~180 Å), exceeds that of GSDMB (~150 Å), and is smaller than that of GSDMD (~215 Å). These differences likely have functional implications for the size of cargoes that can traverse the pore.

The electrostatic surface of the GSDME pore reveals a striking dichotomy: The membrane-facing surface is enriched in basic residues, facilitating interaction with the negatively charged inner leaflet of the plasma membrane, whereas the luminal surface is predominantly acidic, creating a negatively charged conduit. This acidic environment is conserved in GSDMA3 and GSDMD but is less pronounced in GSDMB, suggesting that gasdermin pores may have evolved to selectively facilitate release of specific cargoes. Notably, mature IL-1 β and IL-18 (the classical pyroptotic cytokines) are positively charged at physiological pH (11) and would therefore be electrostatically favored to traverse the acidic GSDME pore. This observation supports a model in which the charge distribution of the pore lumen contributes to the selective permeability of gasdermin pores, complementing the established role of pore size in determining cargo release.

The identification of palmitoylation at C45, C168, and C180 represents a major advance in understanding GSDME regulation. However, the independent study also reporting the cryo-EM structure of GSDME-NT found no detectable defects for the C168A mutant in their palmitoylation and cell death assays (31). This discrepancy may arise from differences in the sensitivity of the experimental methods used for detection. Consistent with this explanation, our study found that the C168A mutant exhibited a less severe defect in pyroptosis induction than either the C45A or C180A mutants.

The palmitoylation modification of GSDME is essential for membrane targeting, pore formation, and pyroptosis. The three palmitoylation sites function synergistically, as the C45/168/180A mutant completely abolishes palmitoylation and membrane localization, leading to near-complete loss of pyroptotic activity. This finding establishes palmitoylation as a critical regulatory mechanism in gasdermin biology. Palmitoylation is known to regulate protein trafficking, membrane association, and stability; our data indicate that it serves as an essential “licensing” step that primes GSDME-NT for membrane insertion. GSDMD has also been reported to undergo palmitoylation at a juxtamembrane cysteine (C191 in human GSDMD), suggesting a conserved regulatory mechanism across the gasdermin family (21). However, the GSDME palmitoylation sites are located in distinct regions, implying that multiple palmitate anchors may cooperatively orient GSDME-NT at the membrane, facilitating the conformational rearrangements required for β -barrel assembly. Future structural studies of monomeric or prepore GSDME-NT will be required to fully understand how palmitoylation at different sites contributes to membrane engagement, oligomerization, and pore insertion. However, because C45 is predicted by AlphaFold (32) to be near the NT–CT interface in the autoinhibited state, it is possible that palmitoylation may facilitate dissociation of NT and CT after caspase-3 cleavage, in a manner similar to what a GSDMD agonist facilitates to activate GSDMD without cleavage through this weakening of the NT and CT interface.

The functional importance of palmitoylation is further underscored by the cancer-associated mutations W44C and R48I, which lie adjacent to the C45 palmitoylation site and likely disrupt this modification. Collectively, these findings position

palmitoylation as a central hub for gasdermin regulation, with potential therapeutic implications for targeting palmitoyl transferases in gasdermin-driven pathologies.

Our structure serves as a molecular blueprint for understanding how cancer-associated missense mutations impair GSDME function. The mutations analyzed cluster in three critical regions: oligomerization interfaces, membrane-contact regions, and the β -barrel itself. Mutations such as D14Y, D18V, N24D, S75L, and P212L compromise intersubunit hydrogen-bonding networks, reducing oligomerization efficiency and pore formation. Mutations including W44C and R48I localize to the β 1– β 2 loop near the palmitoylation site, suggesting they may interfere with palmitoylation or initial membrane engagement. Finally, mutations such as G199E, I217N, E134K, and I137T disrupt the structural integrity of the β -barrel or globular domain, impairing the conformational rearrangements required for membrane insertion. Several of these mutations (e.g., S75L, E134K) have not been previously functionally characterized; our data demonstrate that they indeed impair pyroptosis, validating the structure-based predictions. This mapping provides a mechanistic framework for interpreting GSDME mutations in cancer and suggests that loss of GSDME function in tumors may represent an immune evasion strategy, given the role of GSDME in antitumor immunity.

The findings presented here have broad implications for understanding gasdermin-mediated cell death and its therapeutic modulation. The structural conservation of the β -barrel and refolding mechanism across gasdermins suggests that small-molecule inhibitors or activators targeting the conserved conformational transition could serve as broad-spectrum modulators of pyroptosis. The identification of palmitoylation as an essential regulatory step opens therapeutic avenues: Inhibitors of palmitoyltransferases, such as 2-bromopalmitate, may suppress GSDME-mediated pyroptosis in chemotherapy-induced toxicity, whereas strategies to enhance palmitoylation might potentiate GSDME-dependent antitumor immunity. Furthermore, the electrostatic properties of the pore lumen raise the possibility of engineering gasdermin pores with altered cargo selectivity, with potential applications in synthetic biology or targeted drug delivery.

Several questions remain for future investigation. First, the palmitoyltransferases responsible for GSDME palmitoylation and the regulatory signals controlling this modification remain unknown; identifying these enzymes will enable mechanistic studies of how palmitoylation is regulated in response to apoptotic stimuli. Second, although our structure reveals the fully assembled pore, the intermediate states of GSDME-NT during membrane insertion remain elusive. Time-resolved structural methods or single-molecule approaches could capture these transient conformations. Third, the functional significance of the acidic pore conduit requires direct testing, for example through mutagenesis of acidic residues to evaluate effects on cytokine release and cargo selectivity. Finally, the *in vivo* relevance of the three distinct palmitoylation sites, particularly in chemotherapy-induced tissue damage and antitumor immunity, should be explored using knock-in mouse models expressing palmitoylation-deficient GSDME.

In summary, our study provides a comprehensive structural and mechanistic framework for GSDME-mediated pyroptosis. The cryo-EM structure of the GSDME pore reveals the molecular basis of its assembly and membrane insertion, establishes palmitoylation as an obligatory regulatory step, and offers a structural foundation for understanding cancer-associated mutations. By defining the architecture of the GSDME pore and uncovering the essential role of palmitoylation, our work advances the understanding of gasdermin biology and opens avenues for therapeutic modulation of pyroptosis in cancer, inflammatory diseases, and chemotherapy-induced toxicity.

Materials and Methods

FLAG-tagged human GSDME was expressed in Expi293 cells and purified using anti-FLAG affinity followed by gel filtration chromatography. GSDME-NT pores were reconstituted in liposomes following cleavage by caspase-3 and solubilized by detergent and further purified. Their structure was solved by cryo-EM. GSDME palmitoylation was detected by acyl-biotin exchange. Mutations were evaluated using cell viability and microscopy-based cytotoxicity assays. Detailed experimental procedures are provided in [SI Appendix](#).

Data, Materials, and Software Availability. The 28-fold averaged and the C1 tetramer cryo-EM density maps have been deposited in the Electron Microscopy Data Bank (EMDB) with accession codes of [EMD-81795](#) (33) and [EMD-81794](#) (34), respectively. The corresponding atomic coordinates have

been deposited in the Protein Data Bank (PDB) with accession codes of [43DQ](#) (35) and [43DP](#) (36), respectively. All other data are included in the manuscript and/or [SI Appendix](#).

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Author affiliations: ^aDepartment of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115; ^bProgram in Cellular and Molecular Medicine, Boston Children's Hospital, Boston, MA 02115; and ^cDepartment of Pediatrics, Harvard Medical School, Boston, MA 02115

Author contributions: G.D., J.L., and H.W. designed research; G.D. and J.F.E. performed research; G.D. and J.F.E. analyzed data; and G.D. and H.W. wrote the paper.

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Supporting Information for

Structural basis of GSDME pore formation and its regulation by S-palmitoylation

Gang Du^{1,2,4,*}, Julian F. Ehrmann^{1,2}, Judy Lieberman^{2,3,*}, Hao Wu^{1,2,*}

¹Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA, USA.

²Program in Cellular and Molecular Medicine, Boston Children's Hospital, Boston, MA, USA.

³Department of Pediatrics, Harvard Medical School, Boston, MA, USA.

⁴Current affiliation: Institute of Precision Medicine, the First Affiliated Hospital, Sun Yat-Sen University, Guangzhou, Guangdong, China.

*Correspondence to Gang Du, Judy Lieberman, and Hao Wu. **Email:** wu@crystal.harvard.edu, judy.lieberman@childrens.harvard.edu, and dugang3@mail.sysu.edu.cn

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- Supplementary Text
- Supplementary Figures S1 to S7
- Supplementary Table S1
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Supplementary Text

Materials and Methods

Cell lines

Expi293F™ cells were sourced from Thermo Fisher Scientific (A14527). Human embryonic kidney 293T (HEK293T) cell lines were purchased from ATCC.

Constructs

Human GSDME-FL, GSDME-NT, and GSDME-CT were amplified by polymerase chain reaction (PCR) and inserted into a pLV vector containing C-terminal mCherry or the pcDNA3.1 vector tagged with C-terminal 3xFLAG using Gibson Assembly Master Mix (New England Biolabs, E2611L). Mutations were generated with the QuikChange II XL site-directed mutagenesis kit (Agilent Technologies, Cat #200521) or the KLD Enzyme Mix (New England Biolabs, M0554S). The human active caspase-3 (29-277) was cloned into pET28a employing Gibson Assembly Master Mix (New England Biolabs, E2611L). All constructs were transformed into DH5α competent cells (NEB, C2987U), followed by mini-prep purification (Qiagen) and confirmation by sequencing.

Mammalian cell culture and transfection

HEK293T cells were maintained in Dulbecco's modified Eagle's medium with glutamine (DMEM, GIBCO, ThermoFisher Scientific) supplemented with 10% fetal bovine serum (FBS, Sigma, TMS-013-B), 1% penicillin/streptomycin (GIBCO, ThermoFisher Scientific). Expi293F cells were maintained in Expi293™ Expression Medium (GIBCO, ThermoFisher Scientific). All cell lines were grown at 37 °C in a humidified incubator with 5% CO₂.

Expression and purification of GSDME proteins in expi293F cells and active caspase-3 in Bacteria

For GSDME expression, the pcDNA3.1-GSDME-FLAG plasmid was transfected into expi293F cells at 2.5×10^6 cells/mL in 1000 mL of Expi293 Expression Medium, using polyethylenimine (PEI, Polysciences, Inc.). Approximately 12 hours post-transfection, the culture was supplemented with 10 mM sodium butyrate and 10 mL of 45% D-(+) glucose solution. After an additional two days, cells were harvested by centrifugation at 4,000 g for 30 min. The resulting pellet was resuspended in buffer A (40 mM HEPES, pH 7.0, 150 mM NaCl) and lysed by sonication (2 seconds on, 8 seconds off, total on-time 3.5 min, 40% amplitude). Lysates were clarified by centrifugation at 40,000 rpm for 1 hour, and the supernatant was incubated with ANTI-FLAG® M2 Affinity Gel (Sigma-Aldrich, A2220-4X25ML) for 2 hours at 4 °C under gentle rotation. Following washing, bound protein was eluted with buffer A containing 150 ng/μL 3xFLAG peptide (Sigma, F4799-25MG). The eluate was concentrated and subjected to size-exclusion chromatography on a Superdex 200 Increase 10/300 GL column (Cytiva) pre-equilibrated with buffer A. Peak fractions were pooled, assessed for purity by SDS-PAGE, and snap-frozen for the following assays. To obtain highly palmitoylated GSDME, expi293F cells were treated with 10 μM Rotenone (Rot, Sigma, R8875-1G) for 4 hours prior to harvest.

For caspase-3 expression, the pET28a-caspase 3 construct was transformed into *E. coli* BL21 (DE3) cells (Agilent Technologies, 230280). Transformed cells were plated and incubated overnight at 37 °C. Single colonies were picked and grown in Luria Broth (LB) medium containing 50 μg/mL kanamycin at 37 °C. Protein expression was induced at an OD₆₀₀ of 0.6 with 500 μM isopropyl β-D-1-thiogalactopyranoside (IPTG), followed by further incubation at 16 °C for 16 hours. Cells were collected by centrifugation at 4,000 g for 30 min, resuspended in buffer A supplemented with 10 mM imidazole, and lysed by sonication. The lysate was cleared by centrifugation at 25,000 g for 45 min, and the supernatant was incubated with Ni-NTA beads for 1 hour at 4 °C. After washing with buffer A containing 10 mM imidazole, the protein was eluted using buffer A supplemented with 500 mM imidazole. The eluted protein was concentrated and further purified on a Superdex 200 Increase 10/300 GL column (Cytiva) equilibrated with buffer A. Peak fractions were collected, verified by SDS-PAGE, and snap-frozen for subsequent assays.

Acyl-biotin exchange (ABE)

ABE was performed based on a previously procedure by Brigidi et al. with modifications (1). Cells were collected by centrifugation and the resulting pellets were resuspended in 0.2 mL of buffer B (50 mM Tris-HCl pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% NP-40) supplemented with 20 mM N-ethylmaleimide (NEM,

Sigma, E3876-5G) and protease inhibitor cocktail. After incubation at 4 °C for 2 hours with gentle rotation, samples were centrifuged at 20,000 g for 20 min. The supernatant was retained, and protein concentrations were determined using the Pierce™ BCA Protein Assay Kit (ThermoFisher, 23225) to ensure equal protein amounts across samples.

Samples were then subjected to methanol-chloroform-water precipitation. Briefly, 800 µL of methanol, 300 µL of chloroform, and 600 µL of ddH₂O were added sequentially to each sample, with thorough vortexing after each addition. Following centrifugation at 16,000 × g for 5 min in a microcentrifuge, the upper aqueous layer was discarded. 800 µL methanol was added to resuspend each pellet, vortexed thoroughly, and centrifuged again at 16,000 × g for 5 min, after which the supernatant was removed as completely as possible. After air-drying for 2 min, this entire precipitation procedure was repeated two additional times.

The dried pellets were dissolved in 0.2 mL of buffer C (50 mM Tris-HCl pH 7.4, 5 mM EDTA, 150 mM NaCl, 1% NP-40, 1% SDS). Complete resuspension was achieved by pipetting or brief water bath sonication and split into two parts (100 µL each). One was treated with 0.7 M hydroxylamine (50% by weight in H₂O, Sigma, 438227-50ML) and another was treated with water. Samples were then incubated at room temperature for 1 hour with gentle rotation. Following another round of methanol-chloroform precipitation (as described above), the pellets were dissolved in buffer C containing 4 mM N-[6-(biotinamido)hexyl]-3'-(2'-pyridyl dithio)propionamide (biotin-HPDP, Cayman Chemical Company, 16459) and incubated at room temperature for 1 hour with gentle rotation. After this step, samples were again subjected to three rounds of methanol-chloroform precipitation.

After drying for 2 min, pellets were resuspended in 100 µL of buffer C, and a certain percentage of each sample was taken out as a loading control. For the remaining samples, the SDS concentration was diluted 10-fold to 0.1% using buffer D (50 mM Tris-HCl pH 7.4, 1 mM EDTA, 150 mM NaCl). Prewashed streptavidin agarose (30 µL, Thermo, 29200) was then added, and the mixture was incubated overnight at 4 °C with rotation. The agarose beads were washed three times with buffer D containing 0.1% NP-40 and 0.1% SDS. Bound proteins were eluted by adding 2× SDS-Laemmli buffer containing β-mercaptoethanol and heating at 98 °C for 5 min. Samples were loaded and run on Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad), followed by transfer and immunoblotting with the appropriate antibodies.

Cell viability and microscopy-based cytotoxicity assays

Lactate dehydrogenase (LDH) release was quantified using LDH-Glo Cytotoxicity Assay kit (Promega, J2380) according to the manufacturer's instructions. Cell viability was assessed by measuring ATP levels using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, G7570) according to the manufacturer's instructions. Luminescence was measured on a SYNERGY microplate reader (Biotek).

For microscopy-based cytotoxicity assessment, cells were seeded in 24-well plates or in CELLview Cell Culture Dishes with four compartments (USA Scientific, 5662-7870) in culture medium containing 1 µg/mL propidium iodide (PI, BD Bioscience, 556463). PI-positive cells were visualized and quantified using either an inverted Nikon Ti2 fluorescence microscope equipped with a 40× objective (N.A. = 1.2) and a stage-top incubator maintaining 37 °C and 5% CO₂, or a Leica TCS SP8 laser scanning confocal microscope fitted with an environmental chamber to sustain 37 °C and 5% CO₂.

Immunoblot analysis

Cells, including debris present in the supernatant, were collected and lysed in lysis buffer consisting of 50 mM Tris-HCl pH 7.4, 150 mM NaCl, and 1% NP-40, supplemented with Halt protease inhibitor cocktail (Sigma, S8830-20TAB). Following a 2-hour incubation at 4 °C with gentle rotation, lysates were clarified by centrifugation at 20,000 g for 20 min. The resulting supernatant was retained, and protein concentrations were determined using the Pierce™ BCA Protein Assay Kit (Thermo, 23225) to ensure equal protein loading across samples. Samples were subjected to SDS-PAGE on 4–15% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad) and transferred onto 0.2 µm PVDF membranes using the iBlot system (Invitrogen). Membranes were then blocked with 5% non-fat milk in Tris-buffered saline with Tween 20 (TBST) for 30 minutes. After blocking, membranes were washed three times with TBST for 5 min each. For anti-FLAG immunoblotting, membranes were incubated with a 1:1000 dilution of mouse monoclonal anti-FLAG® M2-peroxidase (HRP) antibody (Sigma, A8592) for 3 hours at room temperature or overnight at 4 °C. Following three additional washes with TBST (5 min each), signal on the membrane was visualized using the SuperSignal™ West Atto Ultimate Sensitivity Substrate kit (Sigma, A38556). Western blot images were captured on a Bio-Rad ChemiDoc™ MP imaging system.

Gel used for Immunoblot:

4–15% Mini-PROTEAN® TGX™ Precast Protein Gels, 15-well	BioRad	Cat# 4561086
4–15% Mini-PROTEAN® TGX™ Precast Protein Gels, 12-well	BioRad	Cat# 4561085
4–20% Mini-PROTEAN® TGX™ Precast Protein Gels, 15-well	BioRad	Cat# 4561096
4–20% Mini-PROTEAN® TGX™ Precast Protein Gels, 12-well	BioRad	Cat# 4561095

Immunofluorescence assays

HEK293T cells were seeded into CELLview Cell Culture Dishes with four compartments (USA Scientific, 5662-7870). After 24 hours, cells were either pretreated or not with 50 μ M 2-BP (Sigma, 21604-1G) for 30 min, followed by transfection with the appropriate constructs using Lipofectamine 3000 (Invitrogen, L3000008). At 16 h post-transfection, cells were fixed with 500 μ L of 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at 37 °C for 15 min. After washing twice with PBST, cells were incubated with PBST containing DAPI. Images were acquired immediately using a Leica TCS SP8 confocal laser scanning microscope. For quantification, approximately 60 cells were counted per replicate across three independent experiments, and all imaging data were analyzed with the same microscope.

Liposome leakage assay

Liposomes were prepared as previously described (2, 3). Briefly, PC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, 10 mg/mL in chloroform; 60 μ L), PE (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine, 10 mg/mL in chloroform; 110 μ L) and CL (1',3'-bis(1,2-dioleoyl-sn-glycero-3-phospho)-sn-glycerol (sodium salt), 10 mg/mL in chloroform; 150 μ L) were mixed and organic solvent was removed under a stream of nitrogen gas. The resulting lipid film was resuspended in 250 μ L of buffer A (20 mM HEPES pH 7.4, 150 mM NaCl, 50 mM sodium citrate, and 15 mM TbCl₃) for 3 min. To obtain homogeneous liposomes, the suspension was extruded 45 times through a 100 nm Whatman Nuclepore Track-Etched Membrane. The extruded liposomes were then purified by size-exclusion chromatography using a Superose 6 10/300 GL column (Cytiva) equilibrated with buffer B (20 mM HEPES pH 7.4, 150 mM NaCl), which removed external TbCl₃. Fractions eluting near the void volume were pooled to generate a stock of PC/PE/CL liposomes. The purified liposomes were diluted four-fold with buffer C (20 mM HEPES pH 7.4, 150 mM NaCl, 50 μ M dipicolinic acid, DPA). In this assay, leakage of Tb³⁺ from the interior of liposomes is detected as an increase in fluorescence upon binding of Tb³⁺ to DPA. Purified human GSDME (wild-type or mutant) was pre-cleaved with caspase-3 for 1 hour and then added to 384-well plates (Corning 3820) containing PC/PE/CL liposomes. Fluorescence intensity was measured at 545 nm with excitation at 276 nm immediately after mixing and monitored for 80 min using a SYNERGY microplate reader (Biotek). The emission fluorescence at 545 nm prior to protein addition was designated as F₀. After the 80-min measurement, 0.1% Triton X-100 was added to achieve complete Tb³⁺ release, and the mean of the three top fluorescence readings was defined as F₁₀₀. The percentage of Tb³⁺ release at each time point was calculated as: Tb³⁺ release (%) = (F – F₀) × 100 / (F₁₀₀ – F₀).

Reconstitution and purification of GSDME pores

Lipids were prepared at a molar ratio of 1:3 using 1',3'-bis[1,2-dioleoyl-sn-glycerol-3-phospho]-glycerol (cardiolipin, CL) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (PC, Avanti Polar Lipids). After evaporation of solvent chloroform under a stream of nitrogen gas, the lipid film was resuspended in buffer A (20 mM HEPES pH 7.4, 150 mM NaCl) to a final lipid concentration of approximately 5 mM by vigorous vortexing. Unilamellar vesicles were generated by extruding the suspension 45 times through a 100 nm filter (Whatman Nuclepore). For pore formation, purified GSDME was adjusted to a final concentration of approximately 50 μ M and cleaved with caspase-3, followed by the addition of liposomes. The reaction mixture was incubated on ice for 4 hours. To extract GSDME pores, the sample was solubilized by adding 2% detergent. A panel of non-ionic detergents (commercial shorthand CxEy, where Cx denotes the number of carbons in the alkyl chain and Ey the number of ethylene glycol repeats), along with LMNG and the ionic detergent cholate, were screened to identify conditions that yielded stable GSDME pores. C12E8 was selected as the optimal solubilizing agent. Following solubilization, GSDME pores were purified by size-exclusion chromatography using a Superose 6 5/150 GL column (Cytiva) equilibrated with buffer A supplemented with 0.2% C12E8.

Negative staining electron microscopy

Above reconstituted and purified GSDME pore samples were diluted in buffer A (20 mM HEPES pH 7.4, 150 mM NaCl) and applied to glow-discharged Formvar-coated copper grids (Electron Microscopy Sciences) for 60 seconds. Grids were then washed twice with 5 μ L of buffer A, stained with 1% uranyl formate for 60 seconds, and blotted dry. Imaging was performed on a Joel JEM1400 transmission electron microscope operated at 120 keV.

Cryo-EM grids preparation and data collection

For cryo-EM grid preparation, purified GSDME pore samples (3 μ L, 0.7-2.0 mg/mL) were applied to glow-discharged 300-mesh gold lacey carbon grids coated with an ultrathin carbon film (Ted Pella). Grid preparation was performed using a Vitrobot (FEI) set to a blotting force of 0-3, a blotting time of 1-5 s, 100% humidity, and 4 °C. After blotting, grids were immediately plunged into liquid ethane and transferred to liquid nitrogen for storage. Prior to data collection, all grids were pre-screened at the Harvard Cryo-EM Center for Structural Biology (HC²EM) using a Talos Arctica microscope (ThermoFisher) operated at 200 keV and equipped with a Gatan K3 direct electron detector to assess ice quality and particle distribution. Datasets were subsequently collected at HC²EM on a Titan Krios microscope (ThermoFisher) operating at an acceleration voltage of 300 keV, equipped with a BioQuantum K3 Imaging Filter (Gatan, slit width 20 eV). Data collection was performed in super-resolution mode at a magnification of 105,000 magnification (0.825 Å per pixel) with a defocus range of -0.8 to -2.0 μ m. Each image stack consisted of 48 frames, and the total electron dose was 60.0 electrons per Å². Fully automated data collection was carried out using SerialEM 4.1 (4).

Cryo-EM data processing

Computational resources and software for data processing were provided by the SBGrid consortium (5). Raw movies were corrected for gain reference and beam-induced motion using the Relion 3.08 implementation of the MotionCor2 algorithm (6), with two-fold binning with or without dose weighting. Motion-corrected micrographs were imported into cryoSPARC (7), and particles were initially picked using the Blob Picker tool. A total of 3,217,549 particles were selected. Subsequent 2D classification was performed, and particles corresponding to well-defined 2D classes were selected, yielding 881,774 particles. These particles were subjected to 3D classification into four classes using C28 symmetry. Class 1, comprising 48.9% of the particles, was selected for homogeneous refinement followed by local refinement with C28 symmetry, resulting in a final map at 3.22 Å resolution as determined by Fourier shell correlation. Class 2, containing 27.9% of the particles, was refined using non-uniform refinement and local refinement with C28 symmetry, producing a final map at 3.48 Å resolution. To further improve resolution, particles from Class 1 and Class 2 were combined and subjected to non-uniform refinement and local refinement with C28 symmetry, yielding a final map at 3.16 Å resolution.

For processing in C1 to resolve putative lipid densities, 881,774 particles from 2D classifications were subjected to C1 ab initio generation, and subsequent heterogeneous refinement in C1. The best resolved class was further subjected to homogeneous refinement using a mask covering 4-5 subunits, and particles were then further processed by way of 3D classification in C1 with the same mask using 8 total classes with a 3 Å cut-off filter. Local refinement with masking on the most highly populated class yielded a final map consisting of 84,519 particles at 3.46 Å resolution.

Model building and structure analysis

Model building was performed using the density corresponding to GSDME at 3.16 Å resolution, which features 28-fold symmetry. The AlphaFold3-predicted structure of GSDME-NT served as an initial model and was docked into the density map using UCSF ChimeraX (8). Extensive manual remodeling of GSDME-NT was subsequently carried out in Coot (9). The model was refined against the map in real space using PHENIX (10, 11). Model quality was assessed with MolProbity (12). All structural figures were generated with UCSF Chimera and PyMOL.

Quantification and statistical analysis

Statistical significance was calculated using GraphPad Prism 9 (unpaired Student's t-test, <https://www.graphpad.com/scientific-software/prism/>). The number of independent experiments, the statistical significance, and the statistical test used to determine significance are indicated in each figure, figure legend, or method section where quantification is reported.

Supplementary Figures

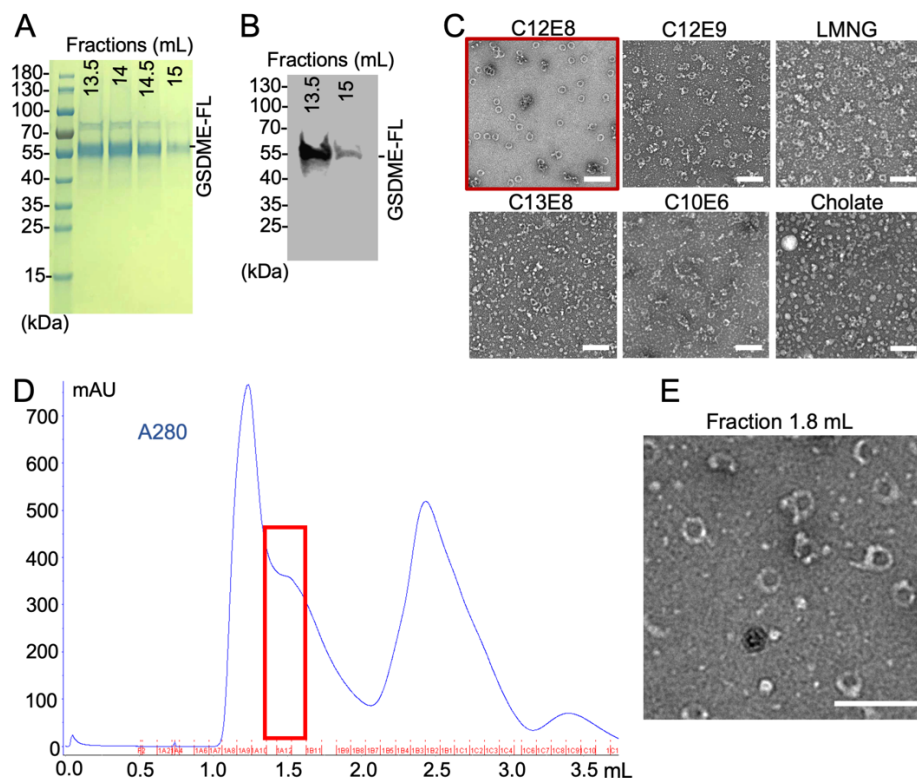


Fig. S1. Reconstitution and purification of GSDME pores. (A) SDS-PAGE analysis of GSDME fractions corresponding to those shown in (Fig. 1B). (B) Western blot of purified GSDME using anti-Flag antibody. (C) Detergent screen. A group of non-ionic detergents (commercial shorthand CxEy; Cx, number of carbons in the alkyl chain; Ey, number of ethylene glycol repeats; and LMNG) and ionic detergents (Cholate) were screened to get stable GSDME pores. C12E8 was selected as the final solubilizing agent. Scale bars, 100 nm. (D) Size-exclusion chromatography profiles from GSDME pore purification following solubilization of liposomes with C12E8 detergent. (E) Negative-stain EM images of GSDME-NT pores from the fractions indicated in (D). Scale bars, 100 nm.

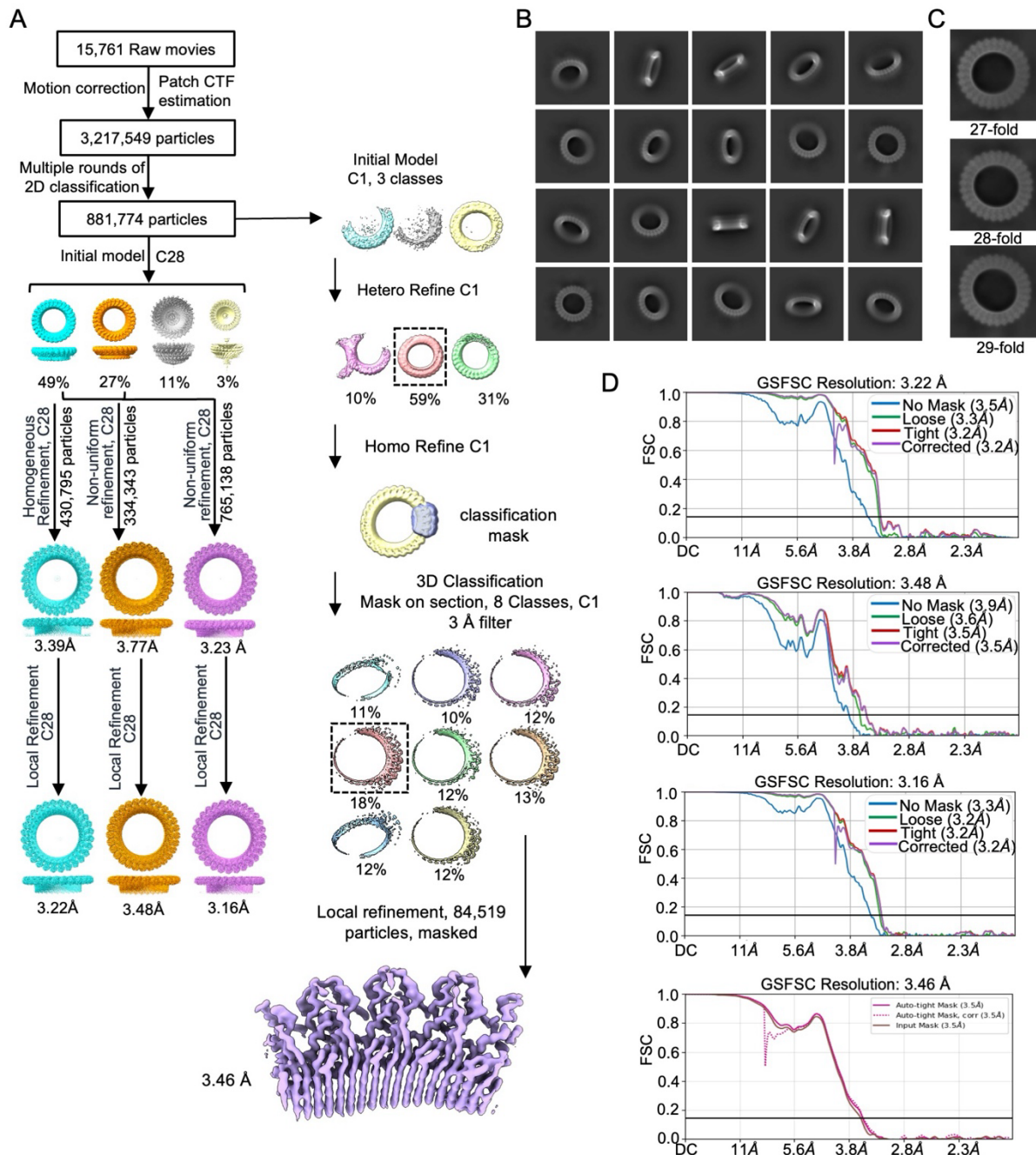


Fig. S2. Cryo-EM data processing for the GSDME pore dataset. (A) Brief flow chart of single-particle cryo-EM data collection and processing for the GSDME pore dataset, showing processing with and without symmetry. (B) Representative 2D class averages of GSDME pores. (C) 2D class averages showing the presence of pores with 27-, 28-, and 29-fold symmetry. (D) Fourier shell correlation (FSC) curves for the cryo-EM structure of the GSDME pore, consistent with the processing workflow in (A).

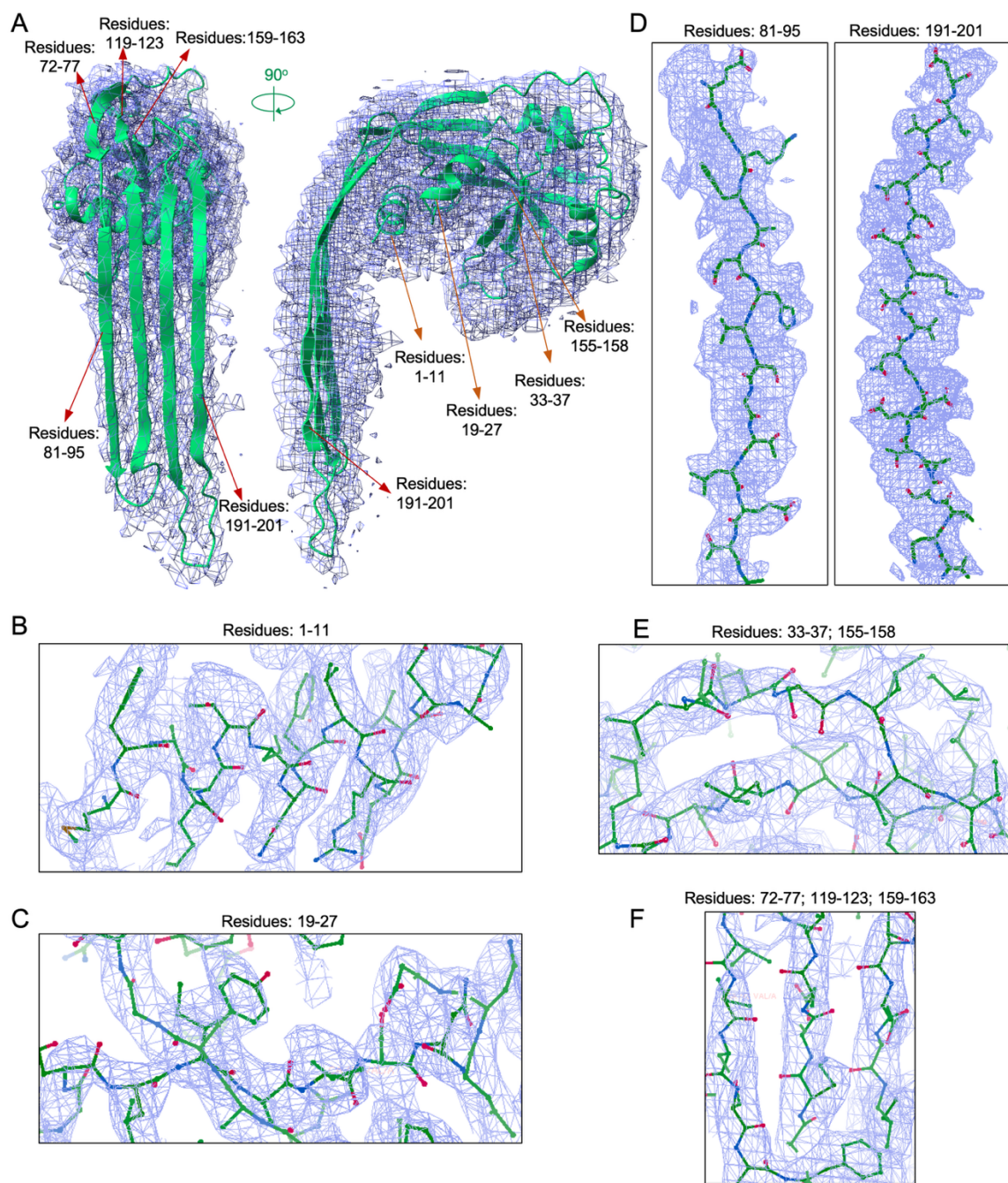


Fig. S3. Analysis of cryo-EM densities and models. (A) A pore-form GSDME subunit fitted into its respective cryo-EM density. Arrows indicate secondary structural elements, with residue numbers shown. (B-F) Close-up views of the pore-form GSDME structure fitted into its cryo-EM density at representative locations denoted by residue numbers in (A).

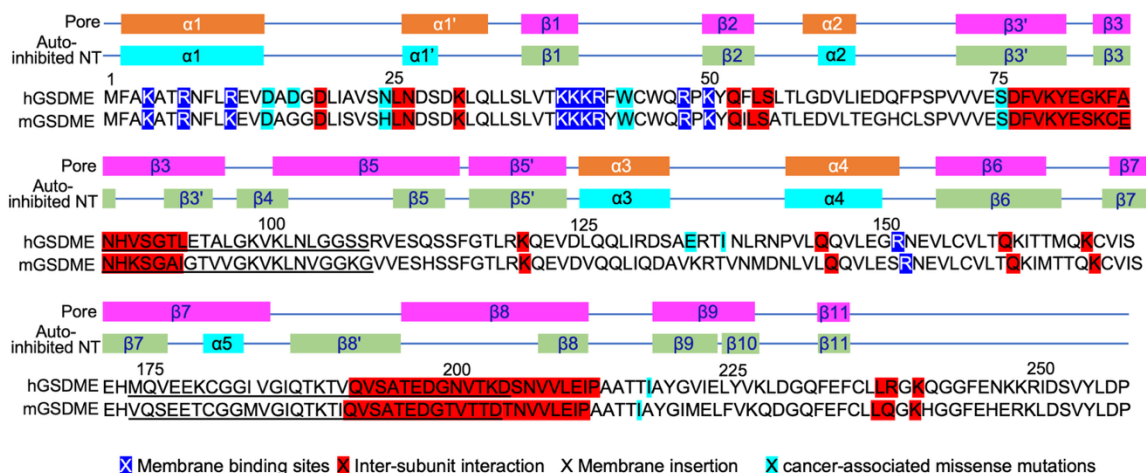


Fig. S4. Sequence alignment of the N-terminal region of human and mouse GSDME. Secondary structures are shown for both the autoinhibited and pore conformations of GSDME. Residues with potential positive charges that mediate acidic membrane lipid binding are highlighted in blue. Red highlighting denotes residues involved in inter-subunit interfaces, while underlined residues indicate those critical for membrane insertion. Cancer-associated missense mutations are highlighted in cyan.

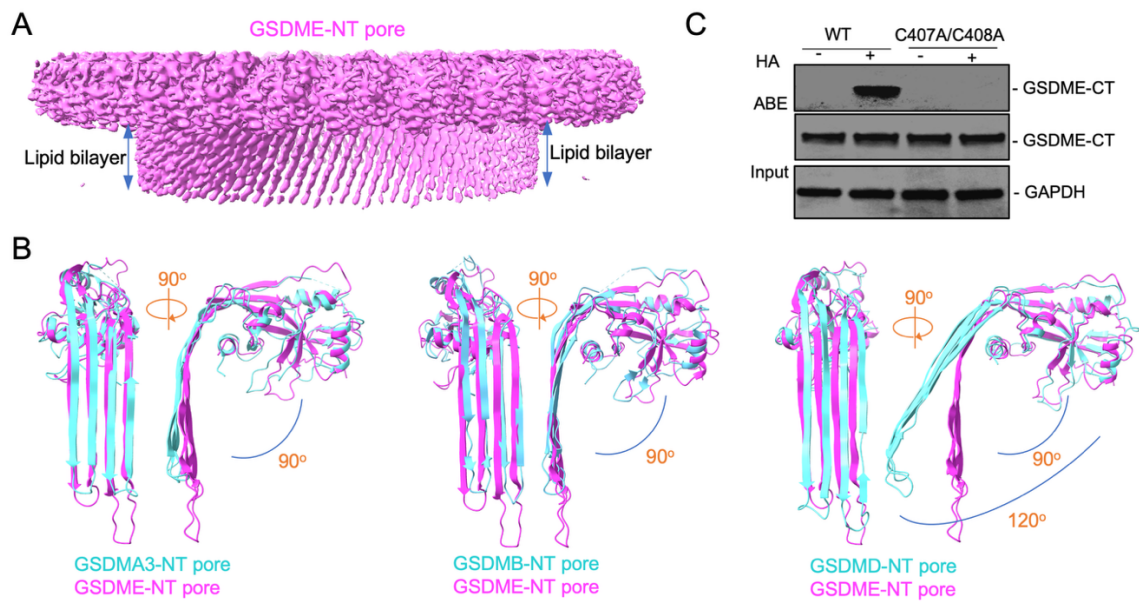


Fig. S5. Comparison of GSDME-NT pore with GSDMA3-NT, GSDMB-NT, and GSDMD-NT pores, and palmitoylation of GSDME-CT. (A) Map of the GSDME-NT pore, with the lipid bilayer indicated. (B) Structural superposition of the pore conformation of GSDME-NT with those of GSDMA3-NT, GSDMB-NT, and GSDMD-NT. (C) ABE assay showed GSDME-CT was palmitoylated at C407 and C408.

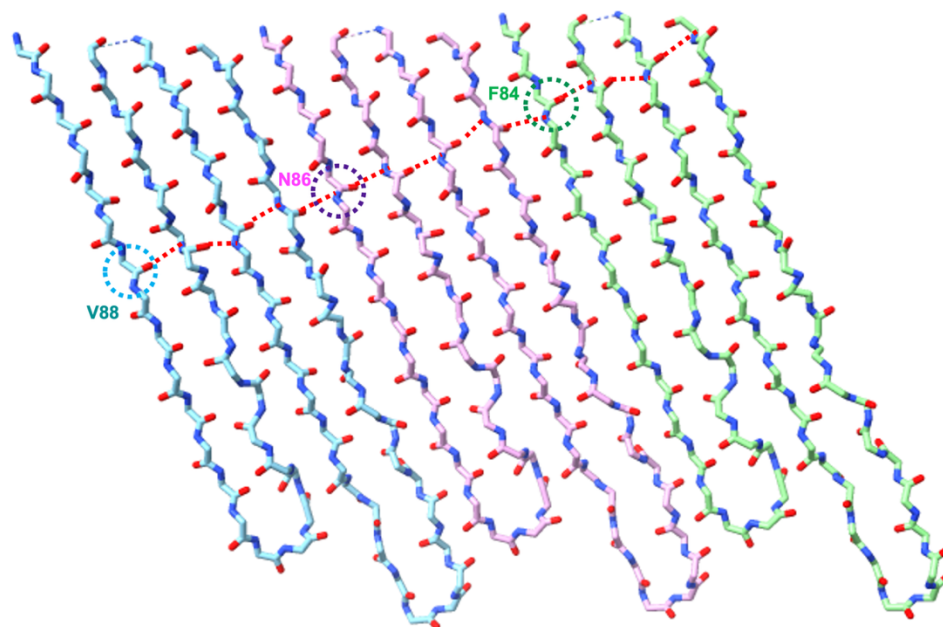


Fig. S6. The hydrogen-bonding pattern in each GSDME subunit is shifted by two residues, resulting in a shear number of 56 for a pore with 28-fold symmetry ($28 \times 2 = 56$).

A

Mutant	Disease	Pyroptosis Ability
D14Y	breast carcinoma	reduced
D18V	colon adenocarcinoma	reduced
N24D	colon adenocarcinoma	reduced
K39A	Mutagenesis, N/A	reduced
K40A	Mutagenesis, N/A	reduced
K41A	Mutagenesis, N/A	reduced
W44C	lung cancer adenocarcinoma	reduced
R48I	uterine corpus endometrioid carcinoma	reduced
S75L	Hepatocellular carcinoma	reduced *
E134K	uterine corpus endometrioid carcinoma	reduced *
I137T	uterine corpus endometrioid carcinoma	reduced
G199E	uterine corpus endometrioid carcinoma	reduced
P212L	melanoma	reduced
I217N	lung cancer adenocarcinoma	reduced

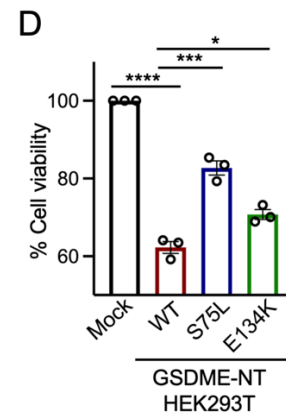
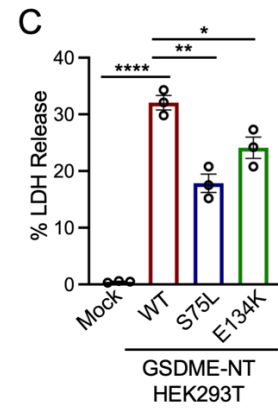
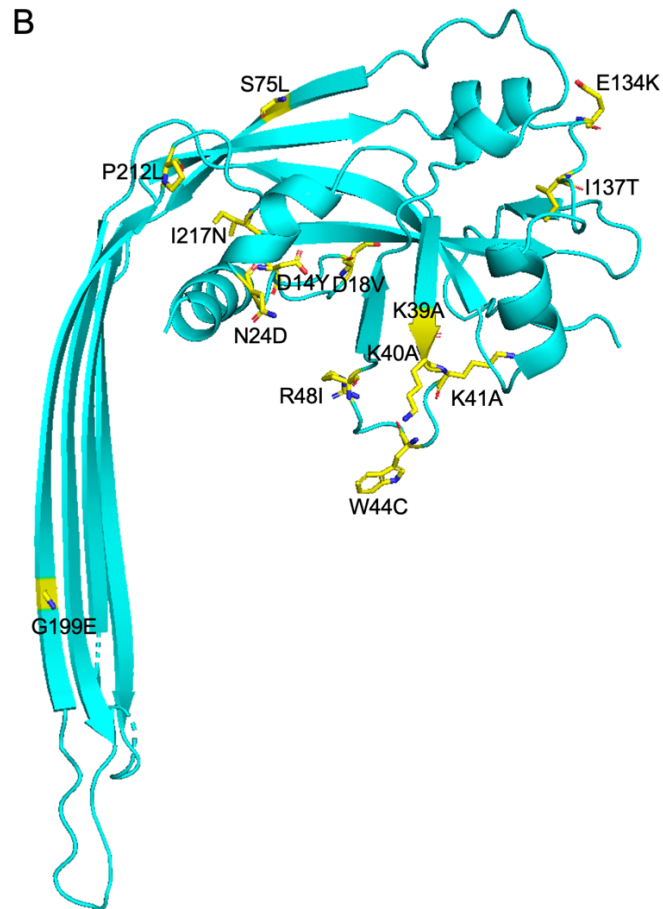


Fig. S7. Variants and mutants of GSDME. (A) Cancer-associated missense mutations from TCGA (<https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga>) and mutagenesis-derived mutants in GSDME-NT, and their pyroptosis-inducing ability. *Denotes functional analysis of the mutants performed in this study. (B) Mapping of cancer-associated missense mutations and mutagenesis-derived mutants within a single subunit of the GSDME-NT pore structure. (C-D) LDH release (C) and cell viability (D) from HEK293T cells expressing GSDME-NT with previously unreported cancer-associated missense mutations, showing impaired pyroptosis induction by S75L and E134K.

Supplementary Table

Table S1. Cryo-EM data collection, refinement and validation statistics.

	GSDME pore	GSDME tetramer
Data collection and processing		
Magnification	130,000	130,000
Voltage (kV)	300	300
Electron exposure (e-/Å ²)	59.46	59.46
Defocus range(m)	-0.8 to-2.0	-0.8 to-2.0
Pixel size (Å)	0.94	0.94
Symmetry imposed	C28	C1
Initial particles (no.)	3,217,549	3,217,549
Final particles (no.)	430,795	84519
Map resolution (Å)	3.22	3.46
FSC threshold	0.143	0.143
Map resolution range (Å)	60.0-3.22	10-2.8
Refinement		
Initial model used	AlphaFold3	AlphaFold3
Model resolution (Å)	3.2	3.9
FSC threshold	0.143/0.5	0.143/0.5
Model resolution range (Å)	45.8 -3.2	
Map sharpening B factor (Å ²)	-128.71	-98.2
Model composition:		
Non-hydrogen atoms	53,900	6,279
Protein residues	6,888	781
Ligands	0	0
B factors (Å ²):		
Protein	90.37	152.9
Ligand	N/A	N/A
R.m.s. deviations:		
Bond lengths (Å)	0.035	0.003
Bond angles (°)	3.554	0.602
Validation:		
MolProbity score	2.33	2.11
Clashscore	10.05	6.63
Poor rotamers (%)	2.76	2.1
Ramachandran plot:		
Favored (%)	92.50	91.55
Allowed(%)	5.42	7.79
Disallowed (%)	2.08	0.66

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