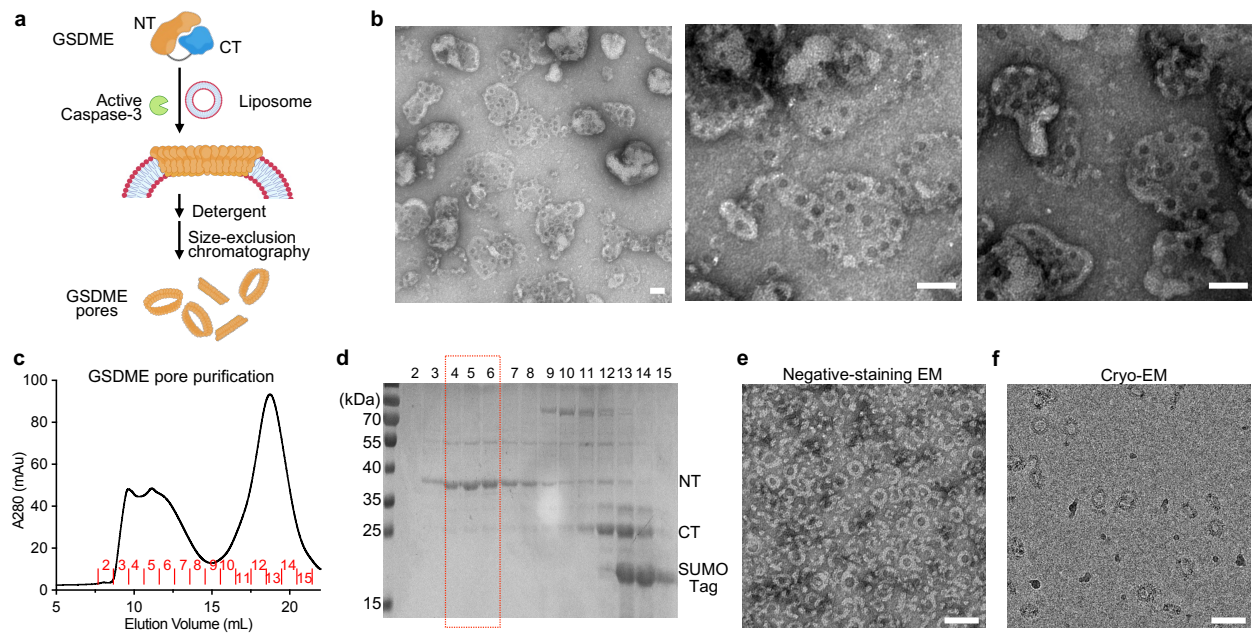
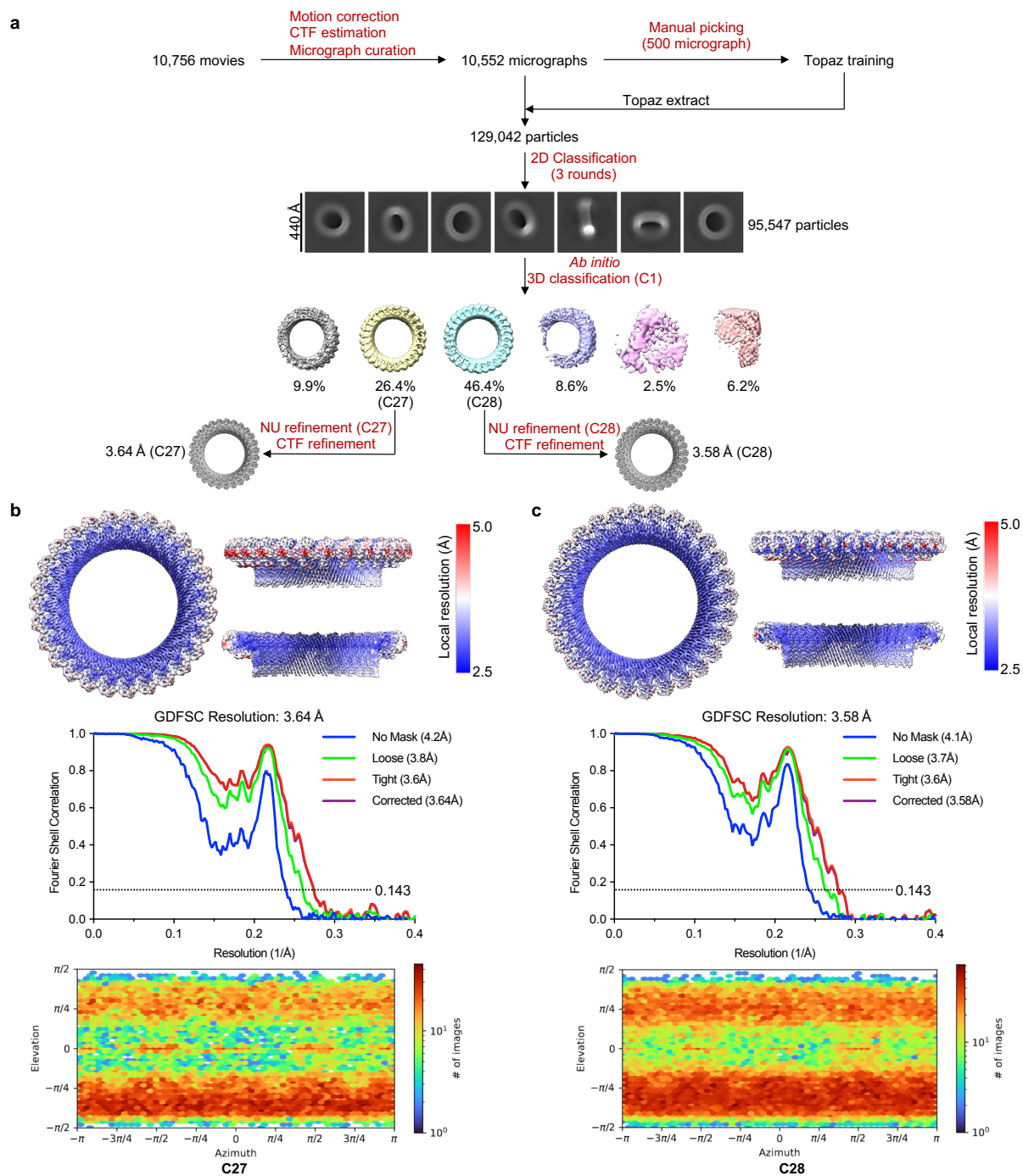


## Supplementary Information

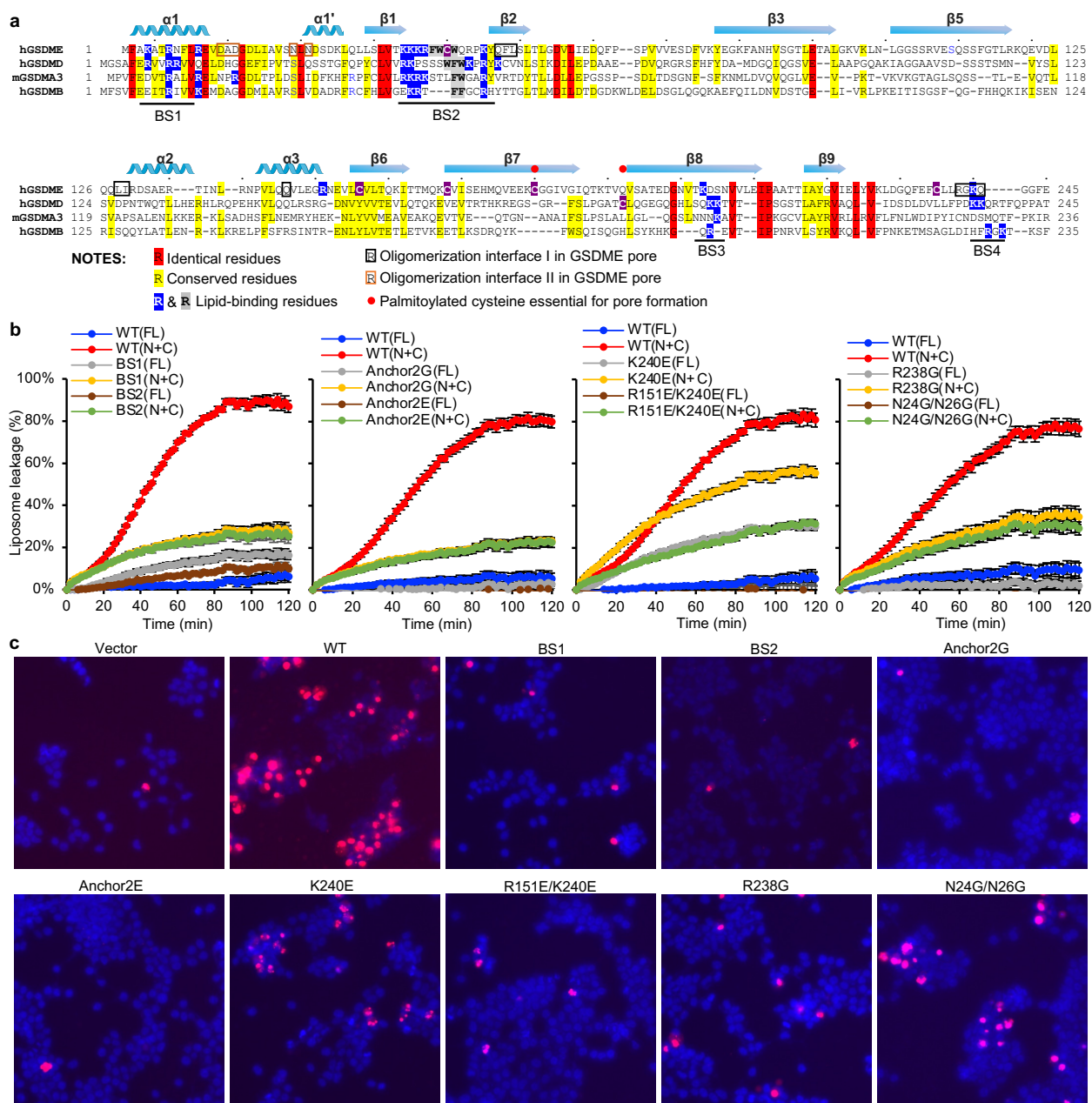


**Supplementary Fig.S1: Reconstitution of GSDME pores in vitro.** **a**, Schematic illustration of GSDME pore reconstitution and purification. Full-length GSDME was cleaved by active caspase-3 to release the pore-forming N-terminal domain (NT), which was reconstituted on liposomes, solubilized by detergent, and purified by size-exclusion chromatography (SEC) for structural analysis. Created in BioRender. Ruan, J. (2026) <https://BioRender.com/5eg7pm8>. **b**, Representative negative-stain electron microscopy (EM) images of liposomes reconstituted with GSDME pores prior to detergent solubilization, showing abundant pore assemblies on membranes. **c**, Size-exclusion chromatography of GSDME pores extracted from liposomes with detergent C12E8. **d**, Coomassie-stained SDS-PAGE analysis of SEC fractions shown in (c). Fractions corresponding to high-molecular-weight pore assemblies (boxed) contain predominantly the GSDME-NT, while GSDME-CT and SUMO tag elute separately. These fractions were pooled and used for cryo-EM analysis. **e**, Representative negative-stain EM image of GSDME pores solubilized in C12E8. **f**, Representative cryo-EM image of GSDME pores. Scale bars, 50 nm. All results were confirmed at least three times as technical replicates.



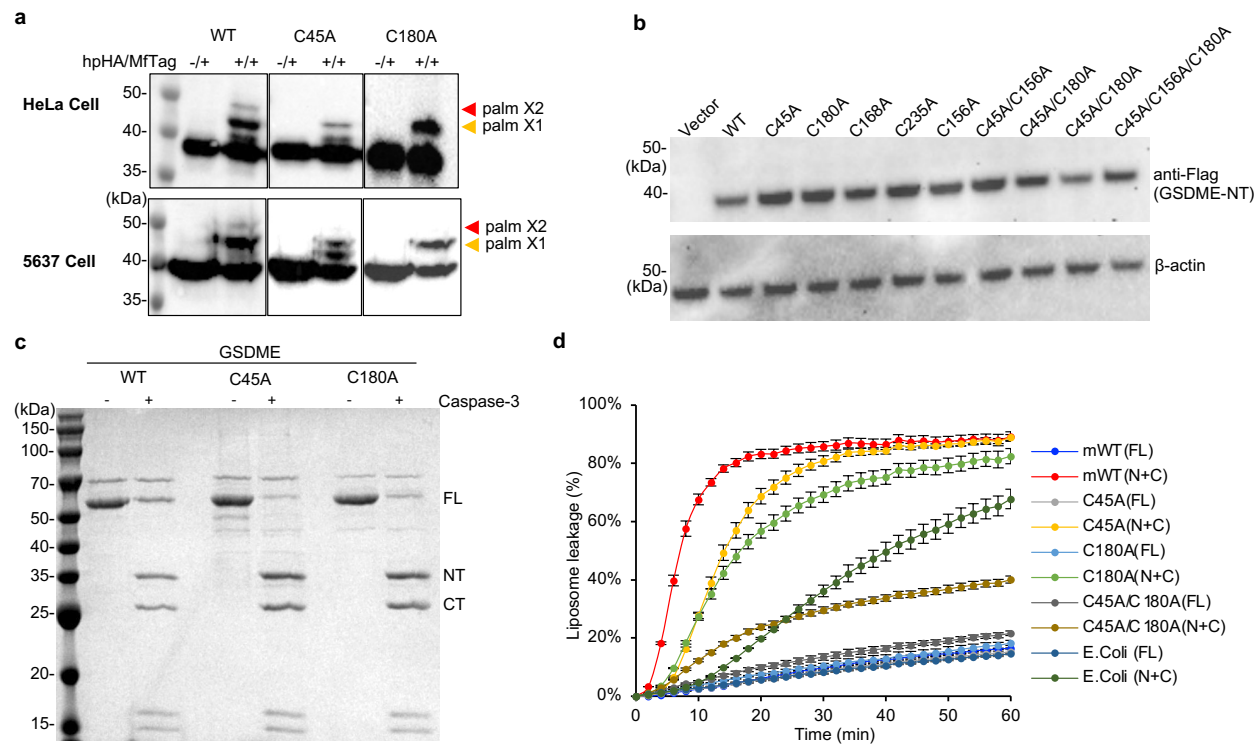
**Supplementary Fig.S2: Local resolution, FSC curves and Guinier plots for the cryo-EM maps of the 27- and 28-fold GSDME pores.** **a**, Workflow for the image processing of cryo-EM data of GSDME pore. 2D averages did not show clear symmetries likely due to the tilted orientation of the particles. 3D classification yielded two major classes of the 27- and 28-fold pores. **b** and **c**, 3D reconstructions of the 27- (**b**) and 28-fold (**c**) GSDME pores, colored according to

local resolution (2.5 – 5.0 Å). Gold-standard Fourier shell correlation (FSC) curve, calculated from two independent half-maps, reveals an overall resolution of 3.64 Å (FSC = 0.143) of the 27-fold pore and 3.58 Å (FSC = 0.143) of the 28-fold pore. Angular distribution calculated in cryoSPARC for both the C27 and C28 GSDME pore particle projections. Heat map shows number of particles for each viewing angle (less = blue, more = red).



**Supplementary Fig.S3: Mechanism of GSDME pore formation.** **a**, Structure-based sequence alignment of the hGSDME, hGSDMD, mGSDMA3, and hGSDMB. Identical residues are in red shade, conserved residues are in yellow shade. Helices and beta strands are shown with symbols. Residues involved in lipid binding are marked with blue and gray shade. Key residues in the oligomerization interfaces are marked with a black (Interface I) and orange (Interface II) squares. Cysteines in GSDME are marked with purple shade and palmitoylated cysteines that facilitate pore formation in GSDME and GSDMD are marked with red dots. **b**, Impairment of the pore-

forming ability of GSDME by mutations in the lipid binding sites and oligomerization interfaces, shown by liposome leakage assay (n = 3 biological replicates, Error bars, mean  $\pm$  SD). FL, full-length; N+C, cleaved by caspase-3p17p12; WT, wild-type; BS1, K4E/R7E/R11E; BS2: K41E/R42E/R47E/K50E; Anchor2G, F43G/W44G/W46G; Anchor2E, F43E/W44E/W46E. **c**, Hoechst (blue)/Propidium iodide (red) double-staining of HEK293T cells expressing wildtype or indicated mutant of GSDME-NT. WT, wild-type; BS1, K4E/R7E/R11E; BS2: K41E/R42E/R47E/K50E; Anchor2G, F43G/W44G/W46G; Anchor2E, F43E/W44E/W46E.



**Supplementary Fig.S4: Palmitoylation of GSDME and its functional consequences.** **a**, Detection of S-palmitoylation of GSDME-NT in different cell types. Wild-type (WT) GSDME-NT and the indicated cysteine mutants (C45A and C180A) were expressed in HeLa and 5637 cells and analyzed using the same MfTag-based palmitoylation assay. The positions of singly (palm  $\times$ 1) and doubly (palm  $\times$ 2) palmitoylated species are indicated. **b**, Immunoblot analysis of WT GSDME-NT and the indicated cysteine mutants expressed in HEK293T cells using an anti-FLAG antibody, with  $\beta$ -actin as a loading control, demonstrating comparable expression levels among constructs. **c**, *In vitro* caspase-3 cleavage of mammalian cell-expressed full-length GSDME (WT, C45A, and C180A). Cleavage reactions were performed in the absence (–) or presence (+) of recombinant caspase-3, and cleavage products corresponding to full-length (FL), N-terminal (NT), and C-terminal (CT) fragments are indicated. **d**, Comparison of pore-forming activity of palmitoylated and non-palmitoylated GSDME-NT measured by liposome leakage assays. Mammalian cell-expressed GSDME-NT (palmitoylated), the indicated cysteine mutants, and *E. coli*-expressed GSDME-NT (non-palmitoylated) were assayed under identical conditions. Data are presented as mean  $\pm$  SD from three biological replicates.

**Supplementary Table S1.** CryoEM data collection and processing.

|                                                     | GSDME pore (27 symmetry)<br>(EMDB: EMD-71549)<br>(PDB: 9PE0) | GSDME pore (28 symmetry)<br>(EMDB: EMD-71544)<br>(PDB: 9PDU) |
|-----------------------------------------------------|--------------------------------------------------------------|--------------------------------------------------------------|
| <b>Data collection and processing</b>               |                                                              |                                                              |
| Magnification                                       |                                                              | 10,552                                                       |
| Voltage (kV)                                        |                                                              | 300                                                          |
| Electron exposure (e <sup>-</sup> /Å <sup>2</sup> ) |                                                              | 50                                                           |
| Defocus range (μm)                                  |                                                              | -0.8~-2.5                                                    |
| Pixel size (Å)                                      |                                                              | 0.926                                                        |
| Initial particles (No.)                             |                                                              | 129,042                                                      |
| Final particles (No.)                               | 17,305                                                       | 44,334                                                       |
| Map Resolution (Å)                                  | 3.64                                                         | 3.58                                                         |
| FSC threshold                                       | 0.143                                                        | 0.143                                                        |
| Map resolution range (Å)                            | 30~3.64                                                      | 30-3.58                                                      |
| <b>Refinement</b>                                   |                                                              |                                                              |
| Model resolution (Å)                                | 3.8                                                          | 3.5                                                          |
| FSC threshold                                       | 0.143                                                        | 0.143                                                        |
| Map sharpening B factor (Å <sup>2</sup> )           | 96.08                                                        | 154.03                                                       |
| Model composition                                   |                                                              |                                                              |
| Non-hydrogen atoms                                  | 51138                                                        | 53004                                                        |
| Protein residues                                    | 6534                                                         | 6776                                                         |
| R.m.s. deviations                                   |                                                              |                                                              |
| Bond lengths (Å)                                    | 0.003                                                        | 0.003                                                        |
| Bond angles (°)                                     | 0.773                                                        | 0.749                                                        |
| Validation                                          |                                                              |                                                              |
| MolProbity score                                    | 2.79                                                         | 3.02                                                         |
| Clashscore                                          | 18.0                                                         | 24.0                                                         |
| Poor rotamers                                       | 0                                                            | 0                                                            |
| Ramachandran plot                                   |                                                              |                                                              |
| Favored (%)                                         | 87.28                                                        | 88.18                                                        |
| Allowed (%)                                         | 12.72                                                        | 11.82                                                        |
| Outlier (%)                                         | 0                                                            | 0                                                            |