Appendix

GSDMD membrane pore formation constitutes the mechanism of pyroptotic cell death

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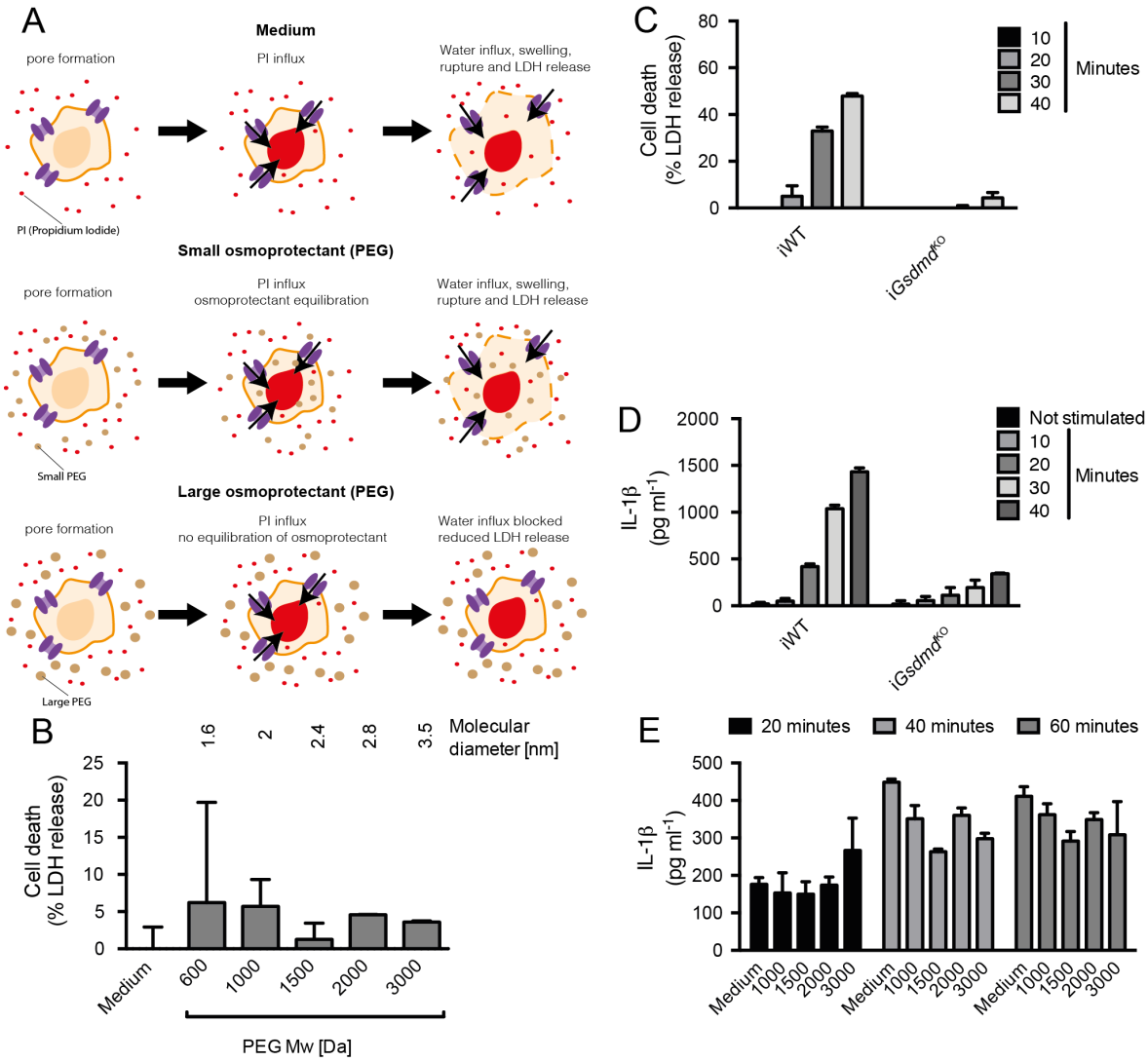
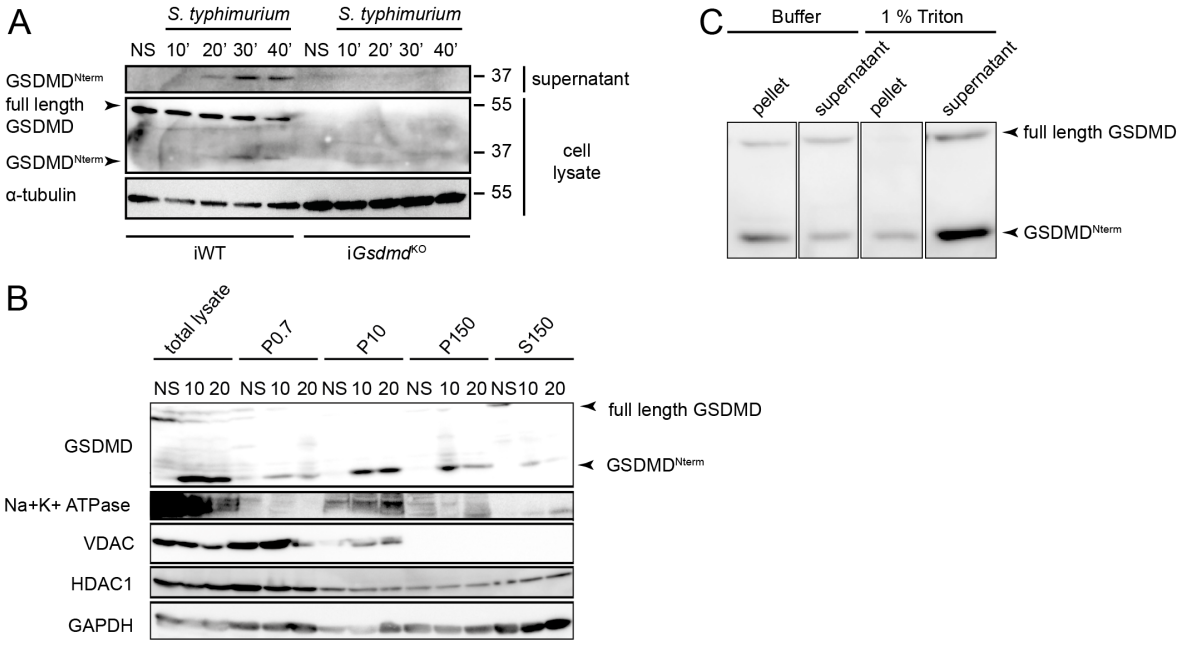


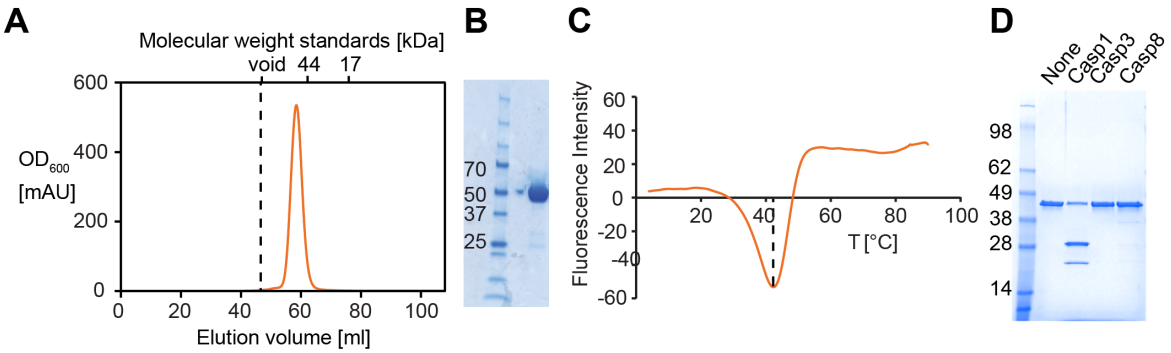
Appendix Figure S1. GSDMD is essential for caspase-1-mediated cell death and cytokine release.

A. Schematic representation of the principle of an osmoprotection assay. **B.** Cell death as measured by LDH release from HEK 293T treated for 8 h with PEGs of increasing molecular weight. **C-D.** LDH and IL-1β release from LPS-primed immortalized wild-type and *Gsdmd*-deficient macrophages infected for 10–40 minutes with log phase *S. typhimurium* (MOI=50). **E.** IL-1β release from LPS-primed primary BMDMs shown in **Fig. 1E–F**. Graphs show mean and s.d. of quadruplicate wells and data are representative of at least three independent experiments.



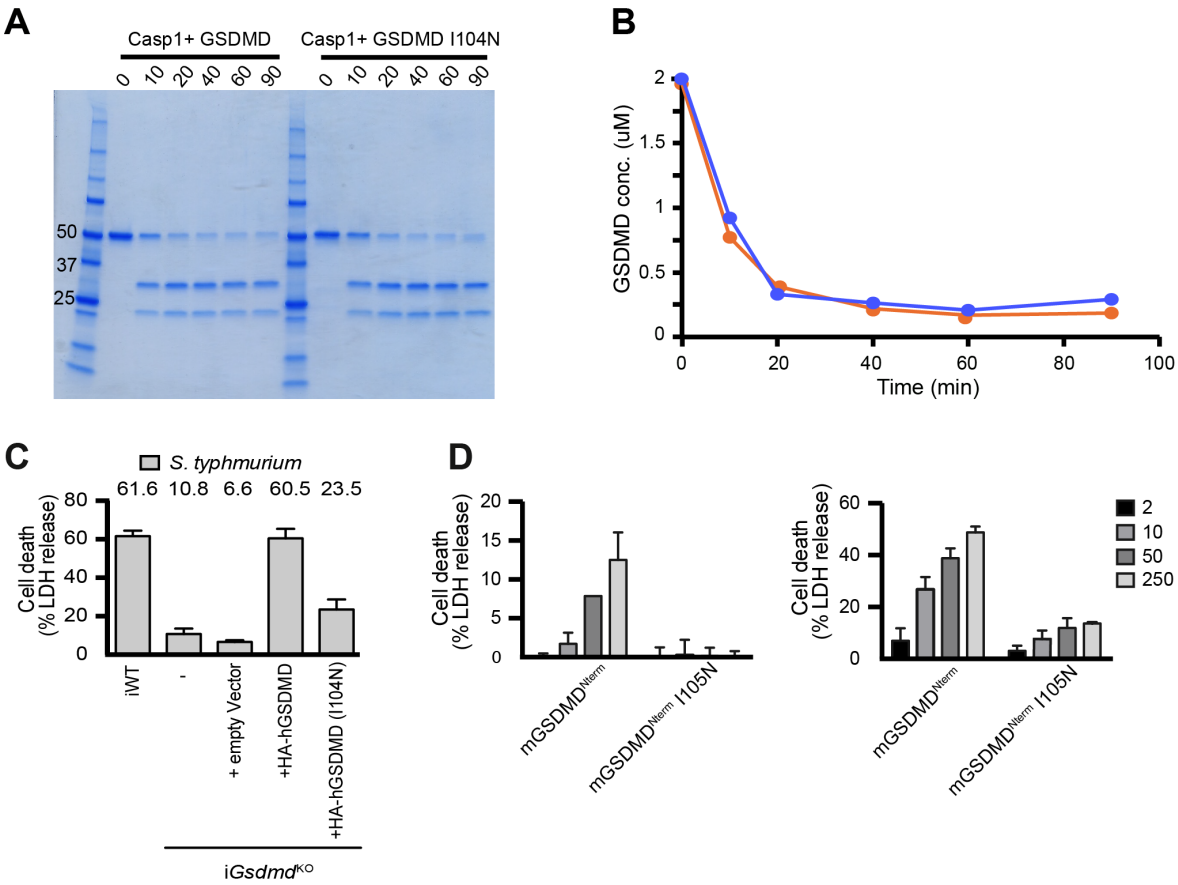
Appendix Figure S2. Membrane targeting of endogenous GSDMD

A. Immunoblot analysis of cleaved Gasdermin-D (GSDMD) in culture supernatants and full length GSDMD, cleaved GSDMD and α-tubulin in the cell lysates of immortalized LPS-primed WT and *Gsdmd*-deficient macrophages left uninfected (NS) or infected for 10–40 minutes with log phase *S. typhimurium* (MOI=50). **B.** Fractionation and immunoblot analysis for Gasdermin-D, Na+K+ ATPase, VDAC (Voltage-dependent anion channel), HDAC1 (Histone deacetylase 1) and GAPDH (Glycerinaldehyd-3-phosphat-Dehydrogenase) of WT macrophages infected for 10 minutes with log phase *S. typhimurium* (MOI=50). Fractionation was carried out as described in the Material and Methods section, but in contrast to Figure 2 the total fraction from equivalent numbers of cells ($2.8x10^6$ cells/lane) instead of equivalent amount of protein was loaded. **C.** Extraction of full-length GSDMD and $GSDMD^{Nterm}$ from isolated membranes of WT macrophages infected for 10 minutes with log phase *S. typhimurium* (MOI=50). Data are representative of 2 (A), 1 (B) and 1 (C) independent experiments.



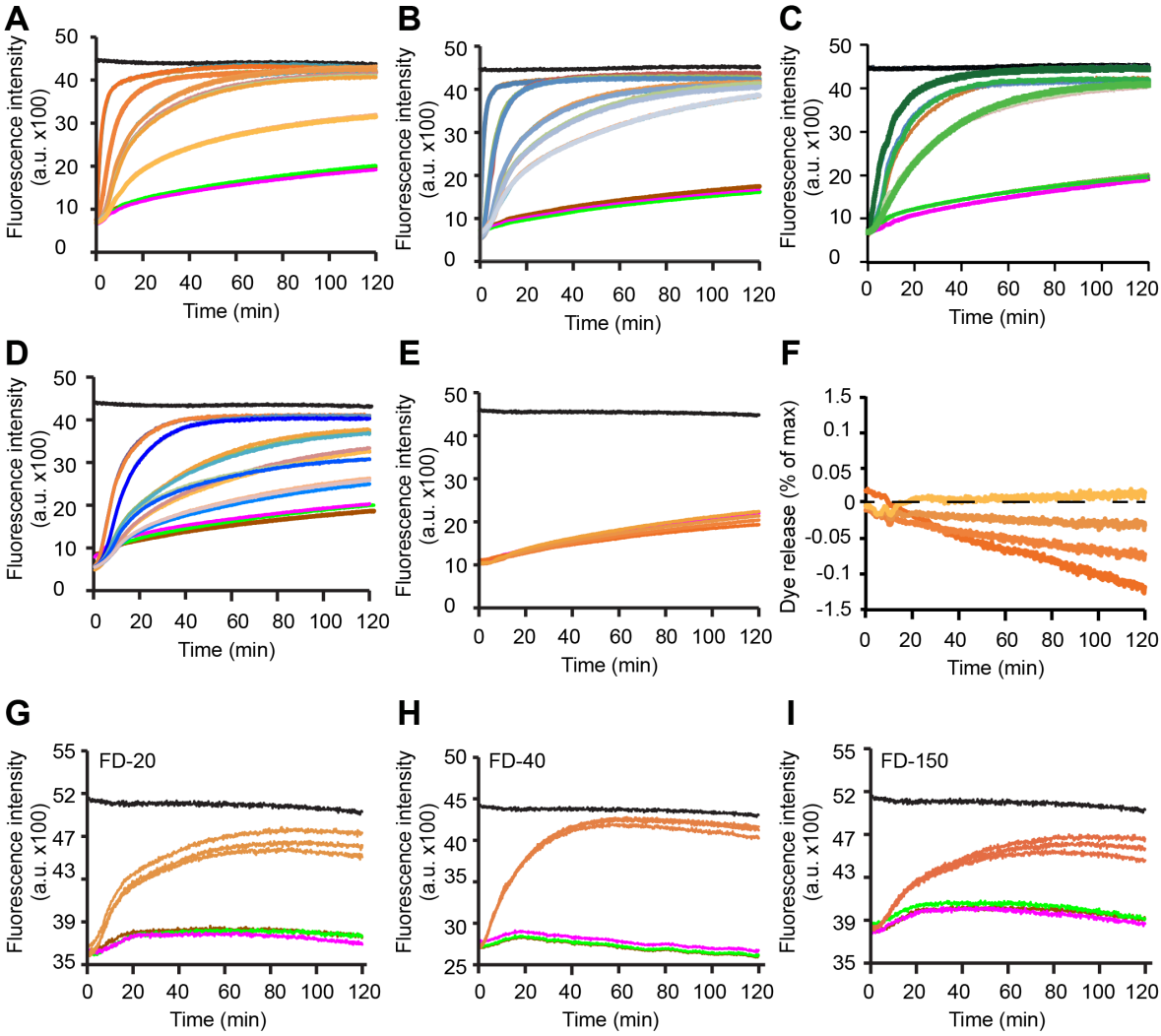
Appendix Figure S3. Purified full-length GSDMD is stable and monomeric in solution

A. Gel filtration profile of purified full-length GSDMD. The chromatogram was recorded using 20 mM Tris buffer, pH 7.5, 50 mM NaCl, 0.5 mM TCEP on a Superdex S75 size exclusion column. The void volume and the molecular weights of a standard calibration curve are indicated. **B.** SDS-PAGE analysis of GSDMD after size exclusion purification. **C.** Thermofluor-assay to assess the stability of GSDMD at the conditions used for the *in vitro* experiments. **D.** SDS-PAGE of 2 μM GSDMD incubated at room temperature with 5 nM of either caspase-1, caspase-3 or caspase-8. Only upon addition of caspase-1, GSDMD was cleaved in into two bands of roughly 30 and 22 kDa.



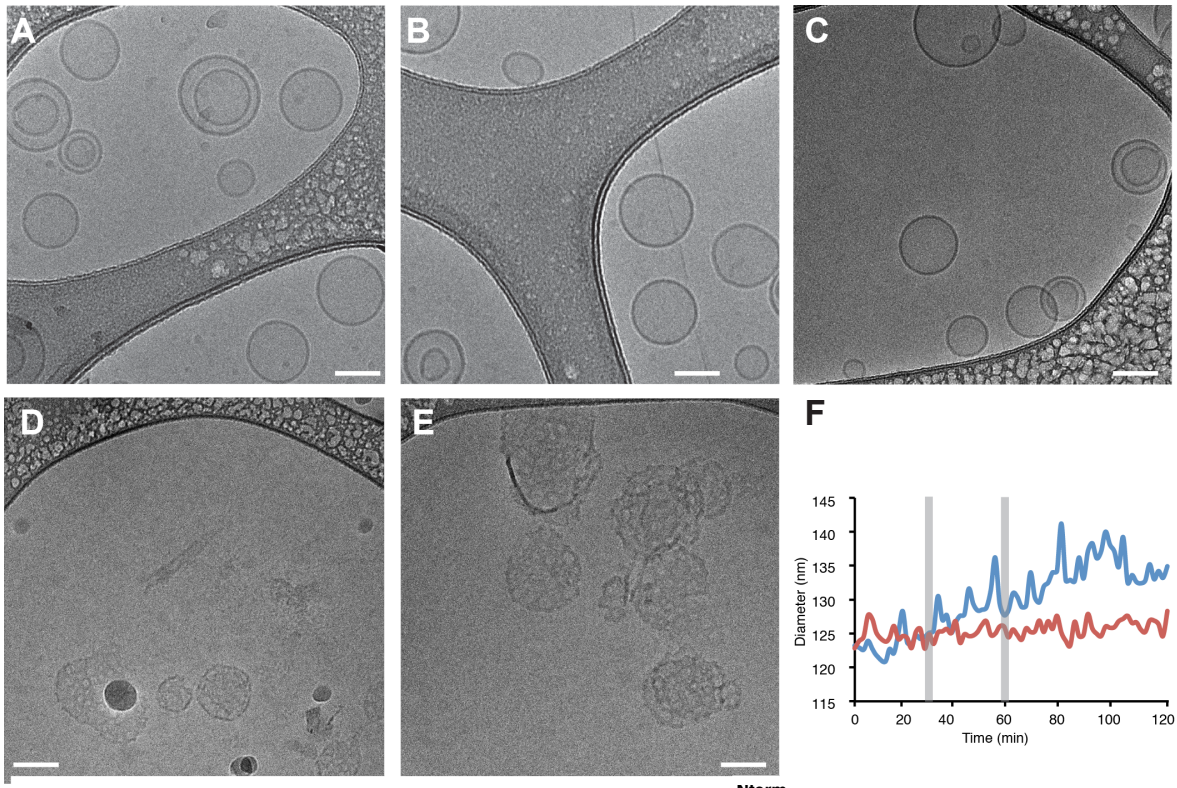
Appendix Figure S4. Cleavage and cell death activity of GSDMD^{I104N}

A. Cleavage of 2 μM human wild-type GSDMD (left) or GSDMD^{I104N} (right) incubated with 5 nM caspase-1 at room temperature. **B**. Cleavage kinetics of GSDMD wild type (orange) and GSDMD^{I104N} (blue), as obtained from quantification of the SDS-PAGE band intensities in panel A with the software ImageJ. **C**. LDH release from immortalized wildtype macrophages or *Gsdmd*^{KO} macrophages, either untransduced or transduced with the indicated constructs in pLJM1-Hygro 7 days post transduction. Transduced cells were selected by Hygromycin treatment. Cells were infected with *S. typhimurium* for 40 minutes. **D.** HEK 293T cells were seeded as described in Figure 1 and transfected with pRetroX tetONE 3G containing the murine GSDMD^{Nterm} or the GSDMD^{I104N} of the protein under a doxycycline inducible promotor. Cells were treated for 8 h (right panel) or 16 h (left panel) with the indicated concentrations of doxycycline. Cell death was analyzed by LDH release. Bars indicate average +/- s.d. of quadruplicate wells. Results are representative of 4 (C) and 2 (D) independent experiments.



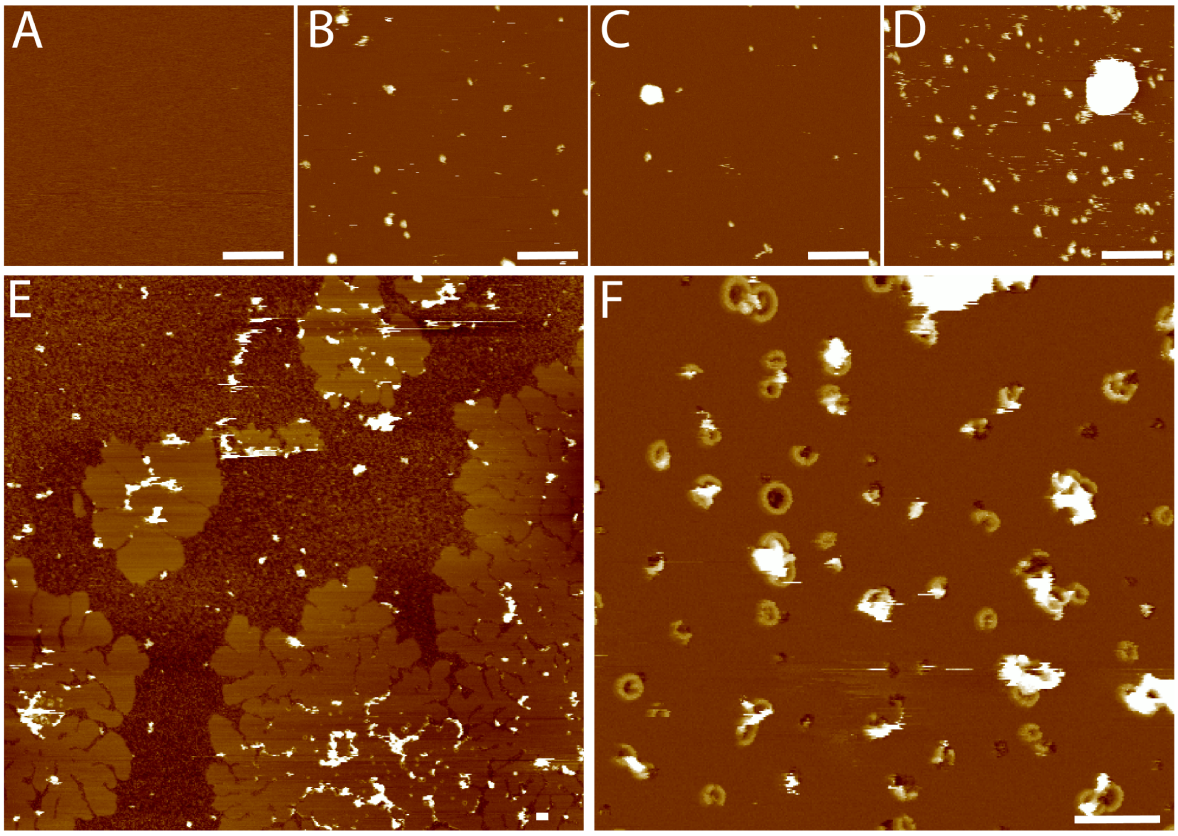
Appendix Figure S5. Experimental raw data of liposome dye release experiments

A–I. Fluorescence intensity time course of dye-loaded liposomes. **A–E.** Triplicate measurements of the experiments described in Figure 4 A–E. The experimental conditions and color code are identical to panels Figure 4 A–E. **F**. Four different reactions where 400 μM 6-carboxifluorescein-loaded liposomes were incubated with GSDMD concentrations of (nM): 2000, 1000, 512, 216 (dark to light orange). **G–I.** Triplicate measurements of the experiments described in Figure 4 G–I. For A–D and G–I, each panel shows the emission intensity for the following control experiments: liposomes (magenta), for liposomes + GSDMD (green) and for liposomes + caspase-1 (brown), as well as the maximum emission intensity for each assay, recorded by adding Triton X-100 to the liposome solution (black).



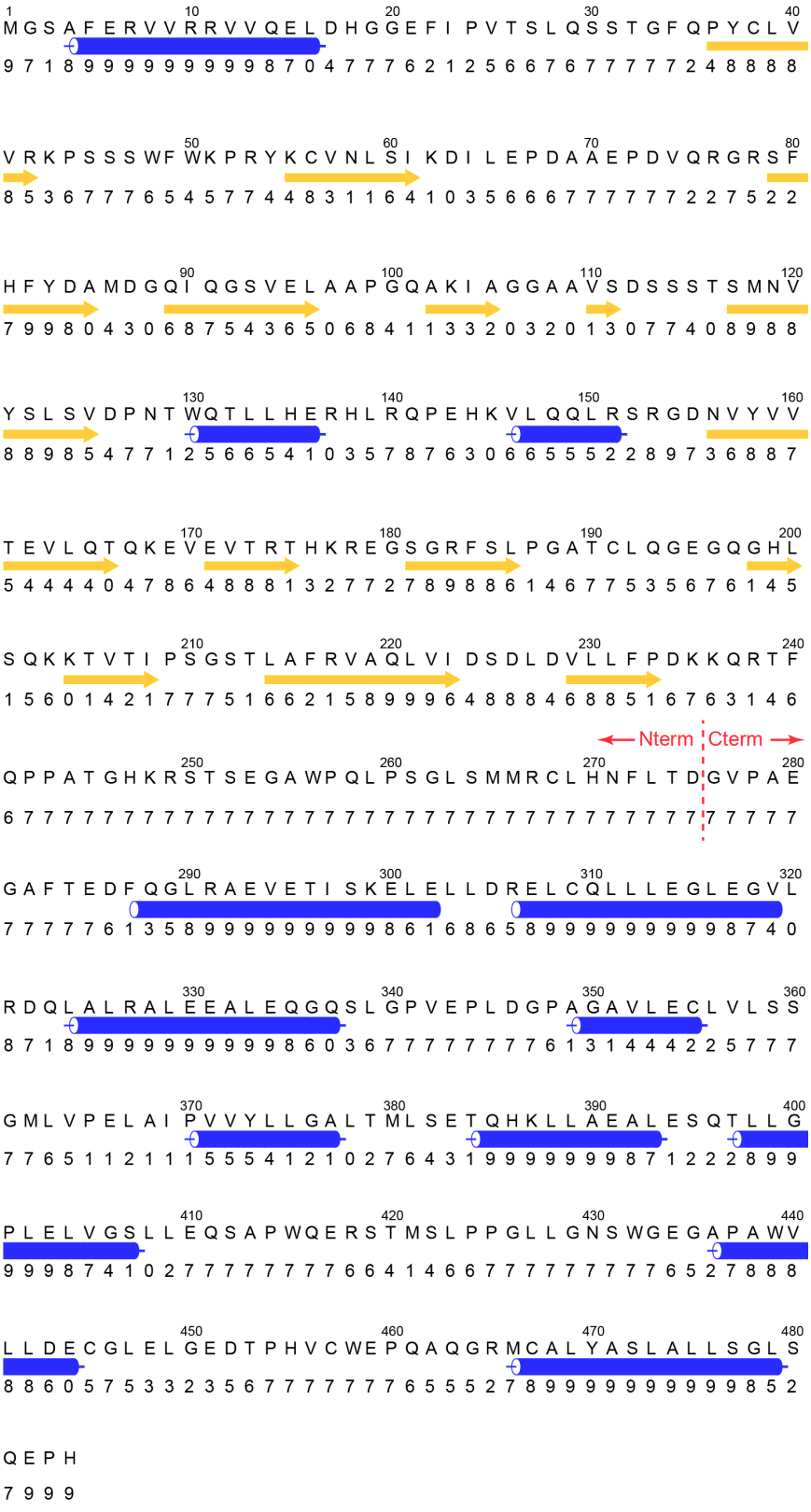
Appendix Figure S6. Time course visualization of GSDMD^{Nterm} pores by cryo-EM.

A. Cryo-EM micrograph of liposomes from *E. coli* polar extract lipids. Scale bar, 80 nm. **B**. Cryo-EM micrograph of 260 μM liposome solution incubated with 2.6 μM of GSDMD in the absence of caspase-1. Scale bar, 80 nm. **C–E**. Cryo-EM micrographs acquired at time points 0 min (C), 30 min (D) and 60 min (E), after incubation of 260 μM liposome solution + 2.6 μM of GSDMD with 48 nM of caspase-1. Scale bars, 80 nm. **F**. Average liposome diameter as determined by time-resolved dynamic light scattering (DLS) measurements at the sample conditions of the cryo-EM experiments (blue) and of the liposome leakage assay (red). Grey bars highlight the 30 and 60 min time points.



Appendix Figure S7. Control experiments showing that full-length GSDMD adsorbed to mica does not assemble into defined oligomeric structures

A. AFM topograph of freshly cleaved mica after incubation of 0.3 µM GSDMD, 0.2 µM caspase-1, or 1 µM GSDMD and 0.2 µM caspase-1 for 90 minutes at 37 °C in buffer solution (50 mM NaCl, 100 mM Hepes, 5 mM TCEP, pH 7.4) the samples were adsorbed onto freshly cleaved mica for 30 minutes and then the mica was rinsed with imaging buffer (150 mM NaCl, 20 mM Hepes, pH 7.8) solution to remove weakly attached molecules. **B**. AFM topograph showing GSDMD adsorbed onto the mica surface as presumably monomers or small oligomers. **C**. AFM topograph showing caspase-1 adsorbed onto the mica surface as presumably monomers or small oligomers. **D**. AFM topograph showing GSDMD and caspase-1 adsorbed onto the mica surface as presumably monomers or small oligomers. **E**. Overview AFM topograph of liposome made from *E. coli* polar lipids extract after being incubated with GSDMD and caspase-1 for 90 min at 37°C. The liposome upon adsorption to the mica support opened showing single membrane patches. **F**. At higher resolution of these membrane patches the AFM topographs show the arc-, slit- and ring-shaped GSDMD oligomers at detail. AFM topographs were taken in buffer solution at room temperature. The full-range color scales of the AFM topographs correspond to a height of 10 nm (A–D) and 22 nm (E–F). Scale bars, 100 nm.



Appendix Figure S8. Secondary structure prediction of human GSDMD.

The prediction was made by Jpred (Drozdetskiy et al., 2015). Below the amino acid sequence of human GSDMD, the predicted secondary structure elements are indicated (α-helix: blue; β-strand: yellow) and the confidence of the prediction (9 = high confidence; 0 = low confidence). The caspase-1-cleavage site between D275 and G276 by is indicated as a red dashed line.