

Structural basis and regulation of GSDME pore formation

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Reviewers' Comments: version 1

Reviewer #1 (Remarks to the Author):

REDACTED

Response:

We appreciate this important comment. We fully agree that directly testing whether the longer transmembrane β -barrel and comparatively compact palm–finger geometry of GSDME influence membrane curvature or ESCRT-dependent repair in cells would be highly interesting.

However, to our knowledge, even for the extensively studied GSDMD pore, the relationship between specific pore geometry parameters and membrane repair dynamics has not been mechanistically defined. Establishing such a structure–curvature–repair causal chain would require substantial additional investigation (e.g., quantitative membrane curvature measurements, live-cell ESCRT recruitment kinetics, and possibly correlative EM), representing a separate study beyond the scope of the present structural work.

Importantly, our intention was not to claim that we had demonstrated a causal relationship between GSDME pore geometry and membrane repair. Rather, we presented this as a hypothesis motivated by structural observations. We believe that defining the structural parameters of the GSDME pore at near-atomic resolution is a necessary first step toward future functional dissection.

REDACTED

Response:

We agree that this is conceptually valuable and attempted such experiments during revision. Specifically, we tested:

1. Transient expression of full-length GSDME in GSDME-knockout HeLa cells with apoptotic stimulation.
2. Stable reconstitution of GSDME-knockout HeLa cells with wild-type or mutant GSDME.

In both systems, we observed basal GSDME processing in the absence of apoptotic stimulation and variable expression levels among constructs. These factors complicated quantitative comparison of stimulus-dependent pyroptotic responses.

Given these technical limitations, we retained the GSDME-NT-based cellular assays as a controlled and interpretable system to directly interrogate pore-forming determinants independent of upstream activation variability.

Importantly, we strengthened the manuscript by adding:

1. Caspase-3 cleavage assays of mammalian-expressed full-length WT and cysteine mutants, demonstrating comparable cleavage efficiency.
2. Direct comparison of palmitoylated (mammalian-expressed) and non-palmitoylated (*E. coli*-expressed) GSDME-NT in liposome leakage assays.

Together, these data support our mechanistic conclusions regarding pore assembly and palmitoylation without confounding effects from variable activation.

REDACTED

Response:

We thank the reviewer for this careful mechanistic concern. In fact, to directly address whether the C180A phenotype reflects loss of palmitoylation rather than global structural perturbation, we have performed additional analyses in our revised manuscript:

1. Caspase-3 cleavage assays: Mammalian-expressed full-length WT, C45A, and C180A GSDME were incubated with recombinant caspase-3. Cleavage efficiencies were comparable, indicating that C180A does not impair global folding sufficient to affect proteolytic accessibility and palmitoylation does not regulate caspase-3 cleavage.
2. Direct comparison of palmitoylated vs non-palmitoylated protein: We compared pore-forming activity of mammalian-expressed WT (palmitoylated), C45A, C180A, C45A/C180A, and *E. coli*-expressed (non-palmitoylated) GSDME-NT. Under identical liposome leakage conditions, we found that mammalian-expressed WT (palmitoylated) showed highest activity, C180A showed substantial reduction, whereas double mutant and non-palmitoylated *E. coli*-expressed protein were least active. These results demonstrate that palmitoylation enhances pore-forming efficiency, C180 is the principal functional palmitoylation site, and the phenotype is not explained by gross structural destabilization.

While additional substitution variants (e.g., hydrophobic mimetics) could be explored in future studies, we believe current evidence sufficiently establishes the functional contribution of C180 palmitoylation.

Reviewer #2 (Remarks to the Author):

REDACTED

Response:

We thank the reviewer for the careful evaluation of our manuscript and for the constructive feedback during the review process. We are pleased that the revisions have adequately addressed the concerns.

Reviewer #3 (Remarks to the Author):

REDACTED

Response:

We thank the reviewer for the careful evaluation of our manuscript and for the constructive feedback during the review process. We are pleased that the revisions have adequately addressed the concerns.