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The molecular mechanism and evolutionary divergence of caspase 3/7-regulated gasdermin E activation

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Abstract

Caspase (CASP) is a family of proteases involved in cleavage and activation of gasdermin, the executor of pyroptosis. In human, CASP3 and CASP7 recognize the same consensus motif DxxD, which is present in gasdermin E (GSDME). However, human GSDME is cleaved by CASP3 but not by CASP7. The underlying mechanism of this observation is unclear. In this study, we identified a pyroptotic pufferfish GSDME that was cleaved by both pufferfish CASP3/7 and human CASP3/7. Domain swapping between pufferfish and human CASP and GSDME showed that the GSDME C-terminus and the CASP7 p10 subunit determined the cleavability of GSDME by CASP7. p10 contains a key residue that governs CASP7 substrate discrimination. This key residue is highly conserved in vertebrate CASP3 and in most vertebrate (except mammalian) CASP7. In mammals, the key residue is conserved in non-primate (e.g., mouse) but not in primate. However, mouse CASP7 cleaved human GSDME but not mouse GSDME. These findings revealed the molecular mechanism of CASP7 substrate discrimination and the divergence of CASP3/7-mediated GSDME activation in vertebrate. These results also suggested that mutation-mediated functional alteration of CASP probably enabled the divergence and specialization of different CASP members in the regulation of complex cellular activities in mammals.

eLife assessment

This **important** study elucidates the molecular divergence of caspase 3 and 7 in the vertebrate lineage. **Convincing** biochemical and mutational data provide evidence that in humans, caspase 7 has lost the ability to cleave gasdermin E due to changes in a key residue, S234. The diversification and specialization of gasdermins such as gasdermin E in humans compared to early vertebrates such as teleosts may enable each human gasdermin molecule to have more restricted and tightly regulated physiological functions in different cell death pathways.

Introduction

Pyroptosis represents a form of programmed cell death that provokes robust inflammatory immune response (Bergsbaken, Fink, & Cookson, 2009; Tsuchiya et al., 2019). Gasdermin (GSDM) serves as the direct executioner of pyroptosis. GSDM forms transmembrane pores to permeabilize the cytoplasmic membrane, which leads to the release of pro-inflammatory cytokines and, if these transmembrane pores persist, pyroptosis (Kovacs & Miao, 2017; J. Shi, Gao, & Shao, 2017; Xia et al., 2021). Human has six GSDM (HsGSDM) family members, HsGSDMA-E, and HsPJVK (Tamura et al., 2007). All HsGSDMs (except for HsPJVK) adopt a two-domain architecture, the N-terminal (NT) pore-forming domain and the C-terminal (CT) autoinhibitory domain (De Schutter et al., 2021; Shao, 2021). Proteolytic cleavage of HsGSDM to remove the autoinhibitory CT domain enables the binding of the lipophilic NT fragment to the cell membrane, where the NT fragments oligomerize and form transmembrane pores to induce osmotic cell lysis (Kuang et al., 2017; X. Liu et al., 2016).

Among the signaling pathways that activate GSDM-mediated pyroptosis, caspase (CASP) 1/4/5/11-mediated GSDMD activation is well documented. In human, HsGSDMD is specifically cleaved by the inflammatory HsCASP1 through multiple inflammasome signaling pathways. HsCASP4/5 also cleave HsGSDMD in response to lipopolysaccharide (LPS) stimulation. After cleavage by HsCASP1/4/5, the pore-forming HsGSDMD-NT fragment becomes unconstrained and triggers pyroptosis (He et al., 2015; Kayagaki et al., 2015; J. Shi et al., 2015). Structural and biochemical analysis reveal that HsCASP1/4 βIII/βIII' sheet forms an exosite that interacts with the hydrophobic pocket of HsGSDMD-CT domain, rendering HsGSDMD cleavage independent of CASP recognition of the tetrapeptide motif FLTD (K. Wang et al., 2020). When the tetrapeptide FLTD is mutated to AAAD, HsCASP1/4 cleavage of HsGSDMD is not affected. The residues that form the hydrophobic pocket of HsGSDMD-CT domain are not conserved in HsGSDME, suggesting that HsGSDME cannot be cleaved by HsCASP1/4 via interaction with an exosite (Z. Liu et al., 2020).

Actually, human HsGSDME is specifically cleaved by HsCASP3 at the consensus motif DMPD, which is required for HsCASP3-mediated HsGSDME proteolysis. Mutation of either the Asp residue of the DMPD tetrapeptide motif leads to cleavage resistance of HsGSDME (Rogers et al., 2017; Wang et al., 2017), indicating that HsCASP3-mediated HsGSDME activation paradigm is distinct from that of HsGSDMD. It is intriguing that although HsCASP7 shares the same recognition motif (DxxD) with HsCASP3, HsCASP7 cannot cleave HsGSDME (Agniswamy, Fang, & Weber, 2009; Slee, Adrain, & Martin, 2001). The molecular mechanism underlying the HsCASP3/7 cleavage discrimination of HsGSDME is unknown.

Historically, HsCASP3 and HsCASP7, which are 54% identical, are known to possess similar structure and share overlapping protein substrate repertoire, and are considered to have functionally redundant roles in regulating cell death (Crawford & Wells, 2011; Kumar, 2007; Y. Shi, 2002). Both HsCASP3 and HsCASP7 consist of two polypeptide subunits named p20 and p10 (1:1 ratio), which assemble to form active heterotetramer (Timmer & Salvesen, 2007). HsCASP3/7 contain highly conserved QACRG and SHG motifs in p20 subunit, and SWR and GSWF motifs in p10 subunit, which participate in the catalytic reaction and substrate binding, respectively (Boyce, Degterev, & Yuan, 2004; Cohen, 1997). In the apoptosis signaling pathway, death receptor-mediated extrinsic pathway and mitochondria-mediated intrinsic pathway ultimately converge to the activation of HsCASP3/7 (Budd, Tenneti, Lishnak, & Lipton, 2000; Wajant, 2002). Active HsCASP3/7 cleave a series of protein substrates, including poly(ADP-ribose) polymerase and DNA fragmentation factor 45, which causes chromatin fragmentation and leads to apoptosis (Erener et al., 2012; Wolf, Schuler, Echeverri, & Green, 1999; Zheng et al., 1998). Although HsCASP3 and HsCASP7 exert almost indistinguishable proteolytic specificity to certain polypeptides, there exist functional differences between these two HsCASPs in cleaving some substrates (Brentnall,

Rodriguez-Menocal, De Guevara, Cepero, & Boise, 2013; Demon et al., 2009). For instance, HsCASP3 cleaves Bid much more efficiently than HsCASP7, while HsCASP7 cleaves cochaperone p23 more efficiently than HsCASP3 (Slee et al., 2001; Walsh et al., 2008). These findings support the notion that HsCASP3 and HsCASP7 are enzymatically similar but functionally non-redundant.

Different from HsGSDME that is specifically cleaved by HsCASP3, teleost GSDME is cleaved via various modes (Angosto-Bazarra et al., 2022; Yuan, Jiang, Qin, & Sun, 2022). In this study, we identified a functional GSDME (named TrGSDME) from pufferfish *Takifugu rubripes*. We found that TrGSDME was specifically cleaved by both HsCASP3/7 and TrCASP3/7, whereas HsGSDME was cleaved by TrCASP3/7 and HsCASP3, but not by HsCASP7. We examined the underlying mechanism of this observation. We found that the GSDME-CT and the CASP7 p10 were critical for CASP7 cleavage of GSDME. By a series of site-directed mutagenesis, we discovered a previously unrecognized key residue (S234 in HsCASP7) in p10 that was responsible for the discriminative cleavage of HsGSDME by HsCASP7.

Results

Pufferfish GSDME is specifically cleaved by both human and fish CASP3/7

It has long been observed that although human caspase (HsCASP) 3 and 7 share the same consensus recognition motif DxxD, HsCASP3, but not HsCASP7, cleaves human GSDME (HsGSDME) (at the site of DMPD) (Poreba, Strozyk, Salvesen, & Drag, 2013; Wang et al., 2017). The underlying molecular mechanism is unknown. In this study, we found that a pufferfish *Takifugu rubripes* GSDME (designated TrGSDME), which belongs to the GSDMEa lineage of teleost GSDME (**Figure-figure supplement 1**), was specifically cleaved by both HsCASP3 and 7 (**Figure 1A and B**). This intriguing observation promoted us to explore the mechanism of HsCASP7 substrate discrimination. We first examined whether TrGSDME could be cleaved by pufferfish CASP (TrCASP) 3/7. The active forms of TrCASP3/7 were prepared (**Figure 1C**), both exhibited high proteolytic specificity and activity towards the tetrapeptide DEVD (**Figure 1D and E**), which is the conserved recognition motif of HsCASP3/7. When incubated with TrGSDME, both TrCASP3 and TrCASP7 cleaved TrGSDME into the NT and CT fragments, similar to that cleaved by HsCASP3/7, in a dose dependent manner (**Figure 1F and G**). Accordingly, TrCASP3/7-mediated TrGSDME cleavage was inhibited by the CASP3 inhibitor (Z-DEVD-FMK) and the pan-CASP inhibitor (Z-VAD-FMK) (**Figure 1H**). Based on the molecular mass of the cleaved NT and CT products, we inferred that the tetrapeptide motif ^{255}DAVD^{258} in the vicinity of the linker region of TrGSDME may be the recognition site of CASP3/7. Indeed, the D255R and D258A mutants of TrGSDME were resistant to TrCASP3/7 and HsCASP3/7 cleavage (**Figure 1I and J**, **Figure 1-figure supplement 2**). Taken together, these results demonstrated that TrGSDME was cleaved by fish and human CASP3/7 in a manner that depended on the specific sequence of DAVD (**Figure 1K**).

CASP3/7-cleaved TrGSDME is functionally activated and induces pyroptosis

We next examined whether TrGSDME, like HsGSDME, is a functional pyroptosis inducer. For this purpose, HEK293T cells were transfected with mCherry-tagged full length (FL) or NT/CT domain of TrGSDME. The results revealed that TrGSDME-FL and -CT were abundantly expressed in the cells, whereas TrGSDME-NT expression was barely detectable (**Figure 2A**, **Figure 2-figure supplement 1**). No significant morphological change or LDH release was observed in cells expressing TrGSDME-FL or -CT (**Figure 2B and C**). By contrast, cells expressing TrGSDME-NT showed necrotic cell death with massive LDH release and positive Sytox Green staining (**Figure 2B**

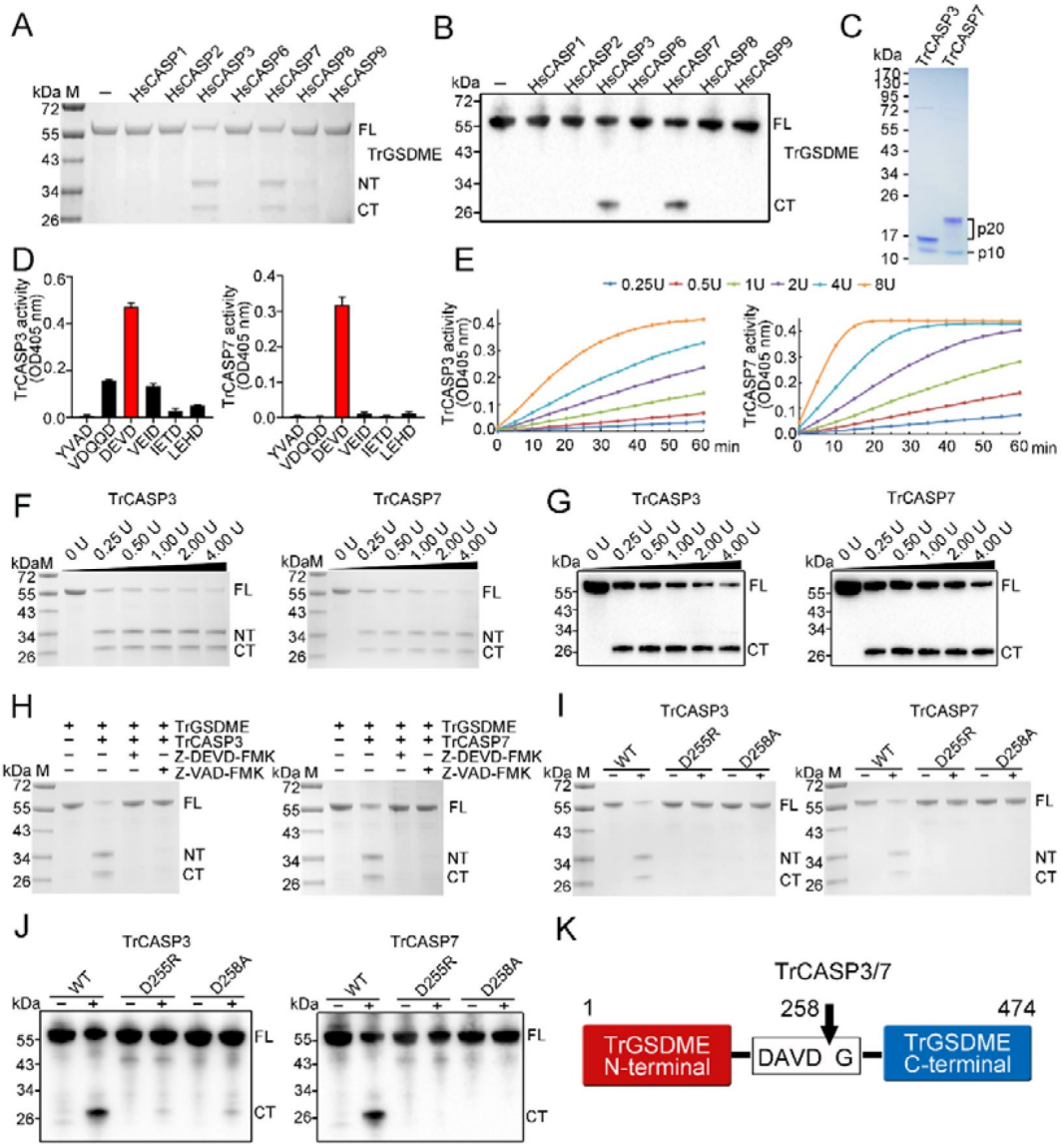

Figure 1.

Cleavage of TrGSDME by caspases.

(A, B) TrGSDME was treated with 1 U of different HsCASPs for 2 h and then subjected to SDS-PAGE (A) and immunoblotting (B). **(C)** SDS-PAGE analysis of purified TrCASP3/7. The p10 and p20 subunits are indicated. **(D)** TrCASP3/7 cleavage of different colorimetric CASP substrates was monitored by measuring released pNA. The values are the means ± SD of three replicates. **(E)** TrCASP3/7 (0.25-8U) were incubated with Ac-DEVD-pNA, and time-dependent release of pNA was measured. **(F, G)** SDS-PAGE (F) and immunoblotting (G) analysis of TrGSDME cleavage by TrCASP3/7 (0.25-4U). **(H)** TrGSDME cleavage by TrCASP3/7 was determined in the presence or absence of 20 μM Z-DEVD-FMK or Z-VAD-FMK. **(I, J)** TrGSDME wild type (WT) and mutants (D255R or D258A) were incubated with TrCASP3/7 for 2 h, and the cleavage was determined by SDS-PAGE (I) and immunoblotting (J). **(K)** A schematic of TrGSDME cleavage by TrCASP3/7. The arrow indicates cleavage site. For all panels, FL, full length; NT, N-terminal fragment; CT, C-terminal fragment.

and C, **Figure 2-figure supplement 2**). TrGSDME-NT-induced cell death exhibited osmotic cell membrane swelling, a typical feature of pyroptosis (**Figure 2D**). Time-lapse imaging showed that TrGSDME-NT triggered rapid cell swelling and membrane rupture, which led to release of cytoplasmic contents and eventually cell death (**Figure 2E**, **Figure 1**-video 1). To examine the pyroptotic activity of CASP3/7-cleaved TrGSDME, TrCASP3/7 and TrGSDME were overexpressed in HEK293T cells (**Figure 2F and G**). The cells expressing TrCASP3, TrCASP7 or TrGSDME alone had no apparent morphological change or LDH release, whereas the cells co-expressing TrGSDME and TrCASP3 or TrGSDME and TrCASP7 underwent pyroptosis, accompanying with massive LDH release, membrane rupture, and TrGSDME cleavage (**Figure 2G-I**, **Figure 2-figure supplement 3A**). Consistently, the presence of the CASP3 inhibitor and pan-CASP inhibitor hampered TrGSDME-mediated pyroptosis (**Figure 2J and K**). Mutation of the cleavage site (D255R and D258A) inhibited pyroptosis and significantly decreased LDH release and TrGSDME cleavage (**Figure 2L and M**, **Figure 2-figure supplement 3B** and **C**). These results indicated that TrCASP3/7 cleavage was required to activate TrGSDME-mediated pyroptosis.

GSDME-CT domain mediates the recognition by CASP7

Since it is known that human GSDME is cleaved by CASP3 but not by CASP7, the observation that TrGSDME was cleaved by both human and fish CASP3/7 intrigued us to explore the underlying mechanism. We found that unlike TrCASP1, both HsCASP3 and 7 exhibited proteolytic activity towards the consensus CASP3/7 recognition motif, DMPD, in HsGSDME, but HsCASP7 failed to cleave HsGSDME (**Figure 3A and B**, **Figure 3-figure supplement 1** and 2). Structure analysis revealed that compared to TrGSDME, HsGSDME possesses two additional regions with the possibility to form a loop (261-266 aa) and an α-helix (281-296 aa), respectively (**Figure 3C**). We tested whether deletion of these two regions could confer HsCASP7 cleavage on HsGSDME. The results showed that similar to the wild type HsGSDME, the loop deletion mutant (Δ261-266) and the α-helix deletion mutant (Δ281-296) were cleaved by HsCASP3 but not by HsCASP7 (**Figure 3D**). Since the NT and CT domains play different roles in GSDM structure maintenance, we constructed GSDME chimeras consisting of HsGSDME-NT plus TrGSDME-CT (named HsNT-TrCT), or TrGSDME-NT plus HsGSDME-CT (named TrNT-HsCT) (**Figure 3E**). Compared with wild type HsGSDME and TrGSDME, chimeric HsNT-TrCT was cleaved by HsCASP3/7, whereas TrNT-HsCT was cleaved only by HsCASP3 (**Figure 3F-I**), suggesting that the CT domain determined the cleavability of GSDME by HsCASP7.

The p10 subunit determines the substrate specificity of CASP7

Since as shown above, unlike HsCASP7, TrCASP7 was able to cleave HsGSDME (**Figure 3H**), we compared the sequences of TrCASP7 and HsCASP7. The two CASPs share 66.24% sequence identity. In these CASPs, the catalytic motifs SHG and QACRG in the p20 subunit and the substrate binding motifs SWR and GSWF in the p10 subunit are conserved (**Figure 4A**). To explore their substrate discrimination mechanism, we constructed CASP7 chimeras consisting of HsCASP7 p20 plus TrCASP7 p10 (named Hsp20-Trp10) or TrCASP7 p20 plus HsCASP7 p10 (named Trp20-Hsp10) (**Figure 4B**, **Figure 4-figure supplement 1A**). Compared to the wild types (HsCASP7 and TrCASP7), the chimeras exhibited comparable enzymatic activities towards the tetrapeptide substrates DAVD and DMPD (**Figure 4C**). Like the wild types, both chimeras cleaved TrGSDME and HsNT-TrCT (**Figure 4D and E**). By contrast, the Hsp20-Trp10 chimera cleaved HsGSDME, whereas the Trp20-Hsp10 chimera did not cleave HsGSDME (**Figure 4F**). Similar cleavage pattern was observed towards TrNT-HsCT (**Figure 4G**). These observations indicated that the p10 subunit dictated the cleavage specificity of CASP7 toward GSDME.

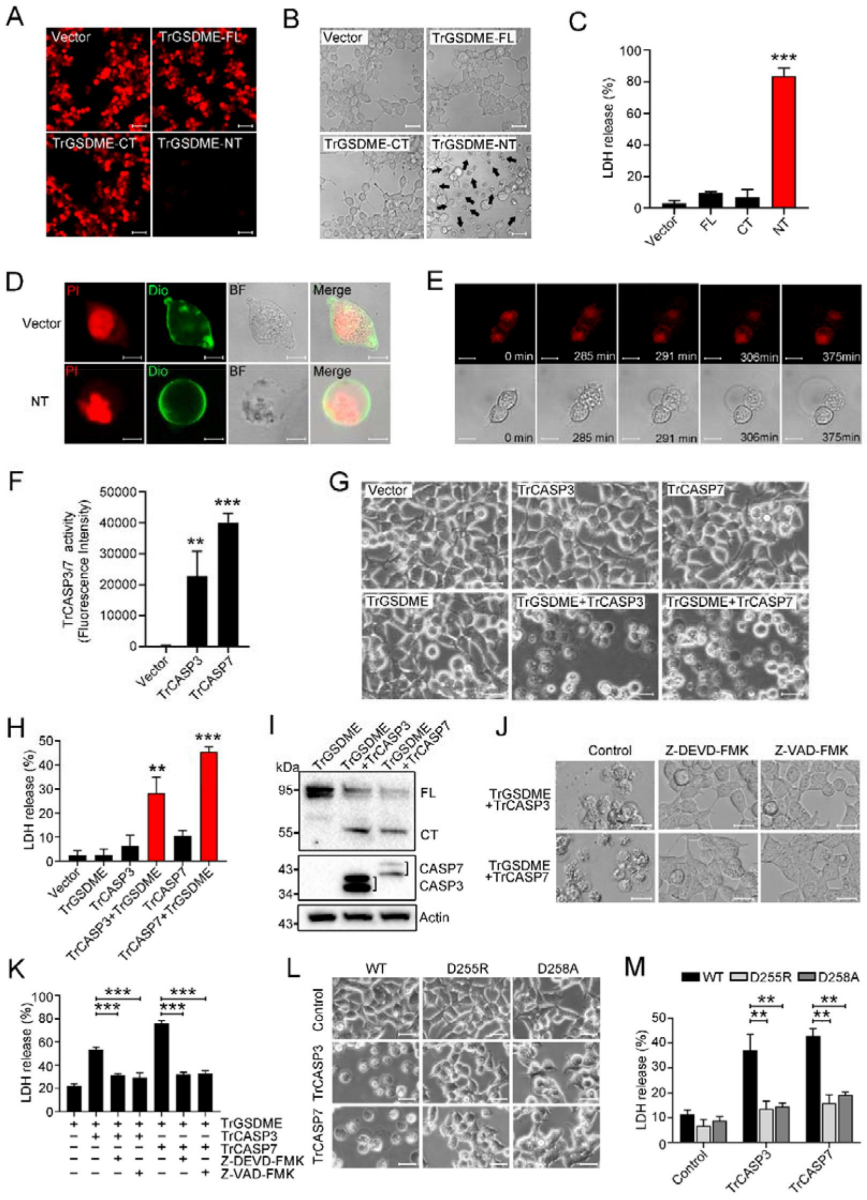

Figure 2.

The pyroptotic activity of TrGSDME.

(A-C) HEK293T cells were transfected with C-terminally mCherry-tagged TrGSDME full length (FL) or truncate (NT or CT) for 24 h, and then analyzed for TrGSDME expression (A), morphological change (B), and LDH release (C). Scale bars, 50 μm (A) and 20 μm (B). **(D)** HEK293T cells were transfected with the backbone vector or vector expressing Myc-tagged TrGSDME-NT for 24 h. The cell nuclei and membrane were stained with PI and DiO, respectively. Scale bar, 10 μm. **(E)** HEK293T cells were transfected with mCherry-tagged TrGSDME-NT for 16 h, and the progression of cell death was shown by time-lapse microscopic imaging. Scale bar, 10 μm. **(F)** HEK293T cells were transfected with TrCASP3/7 for 24 h, and the proteolytic activity of TrCASP3/7 was assessed by treatment with fluorogenic Ac-DEVD-AFC. **(G)** Phase-contrast images of HEK293T cells transfected with the indicated vectors for 24 h. Scale bar, 20 μm. **(H, I)** LDH release (H) and TrGSDME cleavage and TrCASP3/7 expression (I) in the above transfected cells were determined. For panel (I), FL, full-length; CT, C-terminal fragment. **(J, K)** HEK293T cells co-expressing TrGSDME and TrCASP3/7 for 24 h in the presence or absence (control) of 10 μM Z-VAD-FMK or Z-DEVD-FMK were subjected to microscopy (J) and LDH measurement (K). Scale bar, 20 μm. **(L, M)** HEK293T cells expressing TrCASP3/7 plus TrGSDME or TrCASP3/7 plus the D255R/D258A mutant were subjected to microscopy (L) and LDH measurement (M). Scale bar, 20 μm. For panels (C, F, H, K and M), values are the means of three experimental replicates and shown as means ±SD. $^{**}P < 0.01$, $^{***}P < 0.001$.

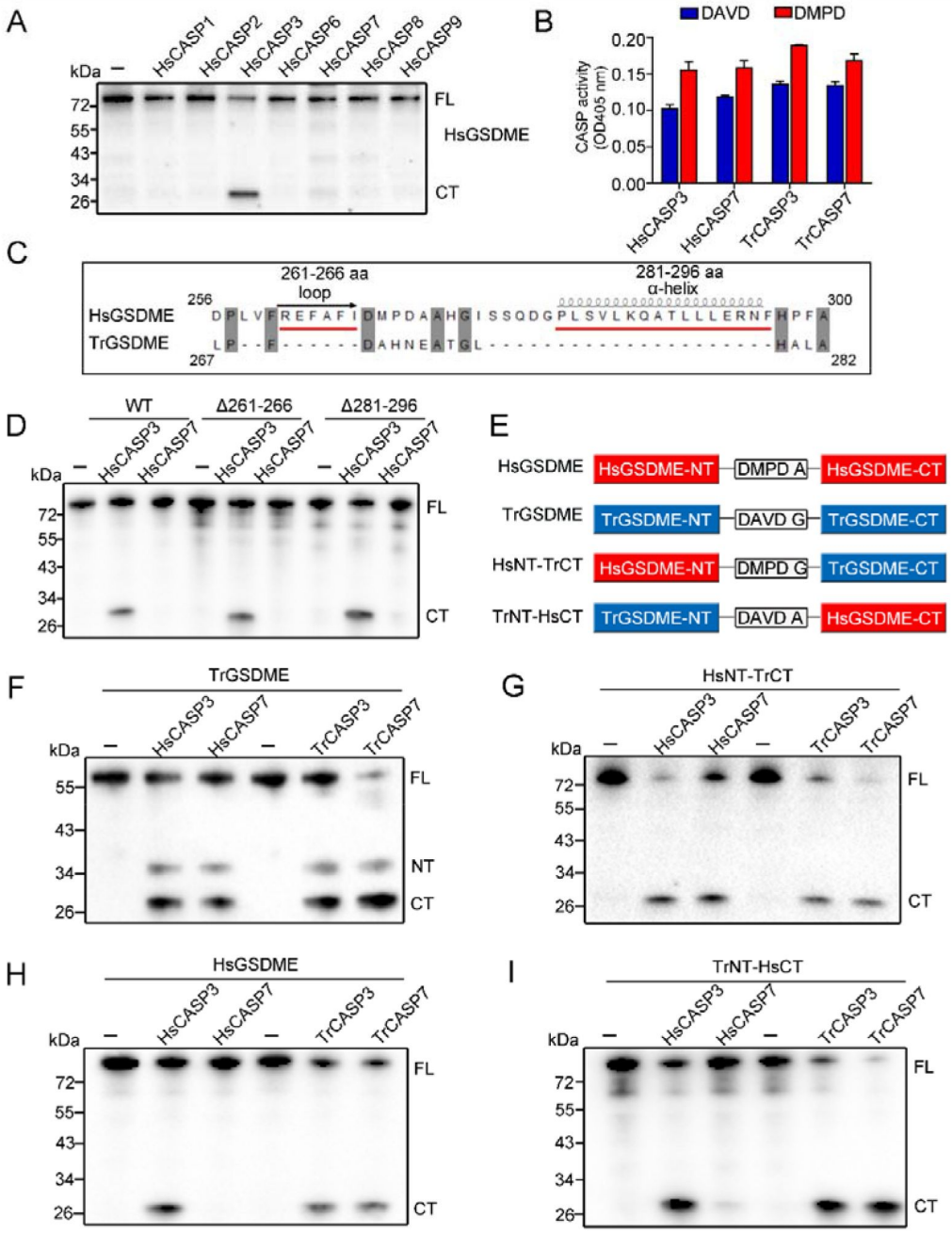

Figure 3.

GSDME-CT domain determines the recognition by CASP7.

(A) HsGSDME was treated with 1 U of various HsCASPs for 2 h, and the products were analyzed by immunoblotting with anti-HsGSDME-CT antibody. **(B)** HsCASP3/7 and TrCASP3/7 were incubated with Ac-DAVD-pNA or Ac-DMPD-pNA at 37°C for 30 min, and the proteolytic activity was determined. Data are expressed as the means ±SD of three replicates. **(C)** Structure analysis of the linker region from HsGSDME and TrGSDME with moue GSDMA3 (PDB:5b5r) as the template. Two regions that may form α-helix and loop are indicated. **(D)** HsGSDME wild type (WT) and mutants (Δ261-266 and Δ281-296) were treated with or without 1 U of HsCASP3/7 for 2 h, and the cleaved fragments were subjected to immunoblotting with anti-HsGSDME-CT antibody. **(E)** Schematics of the chimeric GSDME constructs. **(F-I)** TrGSDME (F), HsNT-TrCT (G), HsGSDME (H) and TrNT-HsCT (I) were incubated with 1 U of TrCASP3/7 or HsCASP3/7 for 2 h, and the cleavage was determined by immunoblotting with anti-TrGSDME or anti-HsGSDME-CT antibodies. For panels (A, D, and F-I): FL, full length; NT, N-terminal fragment; CT, C-terminal fragment.

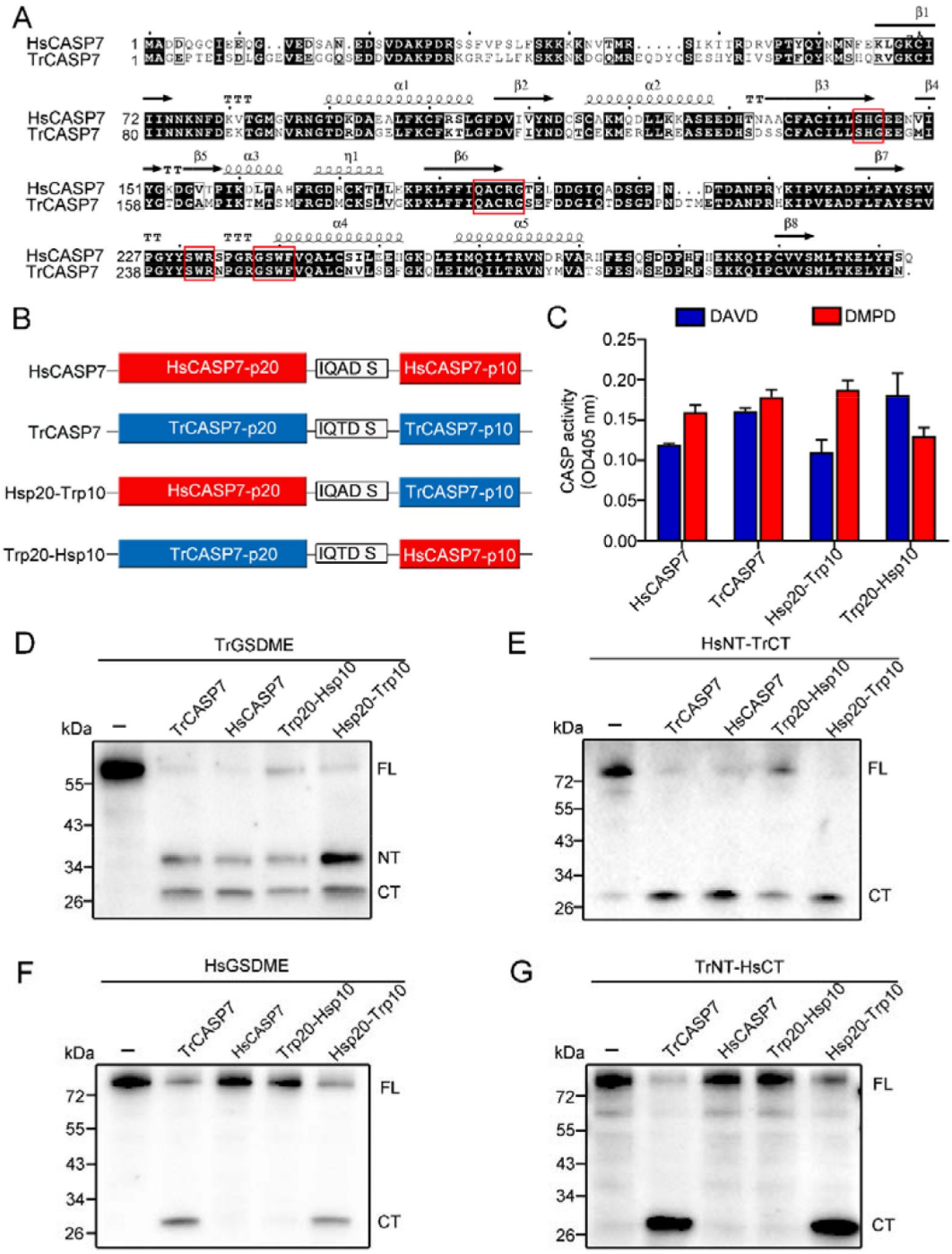

Figure 4.

The p10 subunit of CASP7 is essential for discrimination on GSDME.

(A) Sequence alignment of HsCASP7 and TrCASP7 with HsCASP7 (PDB:1K86) as the template. α-helices and β-strands are indicated. The motifs involved in catalytic reaction (SHG and QACRG) and substrate binding (SWR and GSWF) are boxed in red.**(B)** Schematics of CASP7 chimeras. **(C)** The proteolytic activities of TrCASP7, HsCASP7 and their chimeras were determined by cleaving Ac-DAVD-pNA and Ac-DMPD-pNA. Data are expressed as the means ± SD of three replicates. **(D-G)** TrGSDME (D), HsNT-TrCT (E), HsGSDME (F) or TrNT-HsCT (G) were incubated with TrCASP7, HsCASP7 and their chimeras for 2 h. The products were assessed by immunoblotting with anti-TrGSDME or anti-HsGSDME-CT antibodies. For panels (D-G): FL, full length; NT, N-terminal fragment; CT, C-terminal fragment.

The S234 of p10 is the key to HsCASP7 discrimination on HsGSDME

Comparative analysis of the p10 sequences of TrCASP7 and HsCASP7 identified 13 non-conserved residues. To examine their potential involvement in GSDME cleavage, a series of site-directed mutagenesis was performed to swap the non-conserved residues between HsCASP7 p10 and TrCASP7 p10 (**Figure 5A**, **Figure 4-figure supplement 1B** and C). Of the 13 swaps created, S234N conferred on HsCASP7 the ability to cleave HsGSDME (**Figure 5B**), whereas its corresponding swap in TrCASP7 (N245S) markedly reduced the ability of TrCASP7 to cleave HsGSDME (**Figure 5C**). None of the 13 swaps affected the ability of HsCASP7 or TrCASP7 to cleave TrGSDME (**Figure 5D**, E). HsCASP7-S234N cleaved HsGSDME in a dose-dependent manner (**Figure 5F**), and the cleavage was not enhanced by the additional mutation of S247N&I248V (**Figure 5G**). Additionally, HsCASP7 and HsCASP7-S234N exhibited similar cleavage capacities against other CASP7 substrates, such as PARP1 and gelsolin (**Figure 5-figure supplement 1**) (Erener et al., 2012; Walsh et al., 2008). Previous studies showed that human CASP1/4 cleaved GSDMD through exosite interaction (Z. Liu et al., 2020; K. Wang et al., 2020). However, this exosite is not conserved in HsCASP7, in which the corresponding region forms a coil, not a β sheet (**Figure 5-figure supplement 1**). Structural modeling showed that the residues Q276, D278, and H283 are close to the region corresponding to the HsCASP1/4 exosite (**Figure 5-figure supplement 1**). Since these three residues are also not conserved between HsCASP7 p10 subunit and TrCASP7 p10 subunit, they were selected for mutagenesis to examine whether there existed an exosite interaction between HsCASP7 and HsGSDME. The mutation results showed that, similar to the wild type, the mutant Q276W&D278E&H283S was unable to cleave HsGSDME (**Figure 5G**), suggesting that, unlike human GSDMD, HsGSDME cleavage by CASPs probably did not involve exosite interaction. In contrast to HsGSDME, TrGSDME cleavage by HsCASP7 was not affected by the mutation of S234N, S234N plus S247N&I248V, or Q276W&D278E&H283S (**Figure 5H**).

All the above observed GSDME cleavages by CASP7 were verified in a cellular system. When co-expressed in HEK293T cells, HsGSDME was cleaved by TrCASP7 but not by HsCASP7, while TrGSDME was cleaved by both TrCASP7 and HsCASP7 (**Figure 5-figure supplement 3**). Additionally, both HsGSDME and TrGSDME were cleaved by HsCASP7-S234N as well as TrCASP7-N245S (**Figure 5I and J**). An apparent dose effect was observed in the cleavage of HsGSDME by HsCASP7-S234N (**Figure 5K**).

Divergent and GSDME-independent evolution of CASP7 leads to the loss of GSDME-activation function in mammalian CASP7

Given the importance of N234 in p10 for HsCASP7 cleavage of HsGSDME, we analyzed the sequence conservation of this locus in CASP3/7. For CASP3, a highly conserved Asn residue (corresponding to HsCASP7 S234) was found immediately after the SWR motif in vertebrates (**Figure 6A and B**). In HsCASP3, this conserved Asn is at position 208. We examined whether this residue was functionally essential to HsCASP3. Compared to the wild type, the N208S mutant exhibited much weaker cleavage of HsGSDME (**Figure 6C**, **Figure 4-figure supplement 1D**). Similar weakened cleavage was also observed in HEK293T cells co-expressing HsCASP3-N208S and HsGSDME (**Figure 6D**). These results indicated that the Asn residue was also critical for HsCASP3 cleavage of HsGSDME.

For CASP7, an Asn at the position corresponding to HsCASP7 S234 is highly conserved in teleost, amphibians, reptiles and birds, but not in mammals (**Figure 6A**). In mammals, the corresponding Asn is present in most non-primate species, such as mouse *Mus musculus* and bovine *Bos taurus*, but is replaced by Ser in primate, such as human *H. sapiens*, gorillas *Gorilla gorilla*, and chimpanzee *Pan troglodytes* (**Figure 6E**, **Figure 6-figure supplement 1**). We examined whether mouse CASP7 (MmCASP7) could cleave GSDME. The results showed that MmCASP7 cleaved HsGSDME in a dose-dependent manner, and the cleaving ability was abrogated by N234S mutation (**Figure 6G and H**, **Figure 4-figure supplement 1E**). By contrast, neither MmCASP7 nor MmCASP7-N234S was able to cleave mouse GSDME (MmGSDME) (**Figure 6I**).

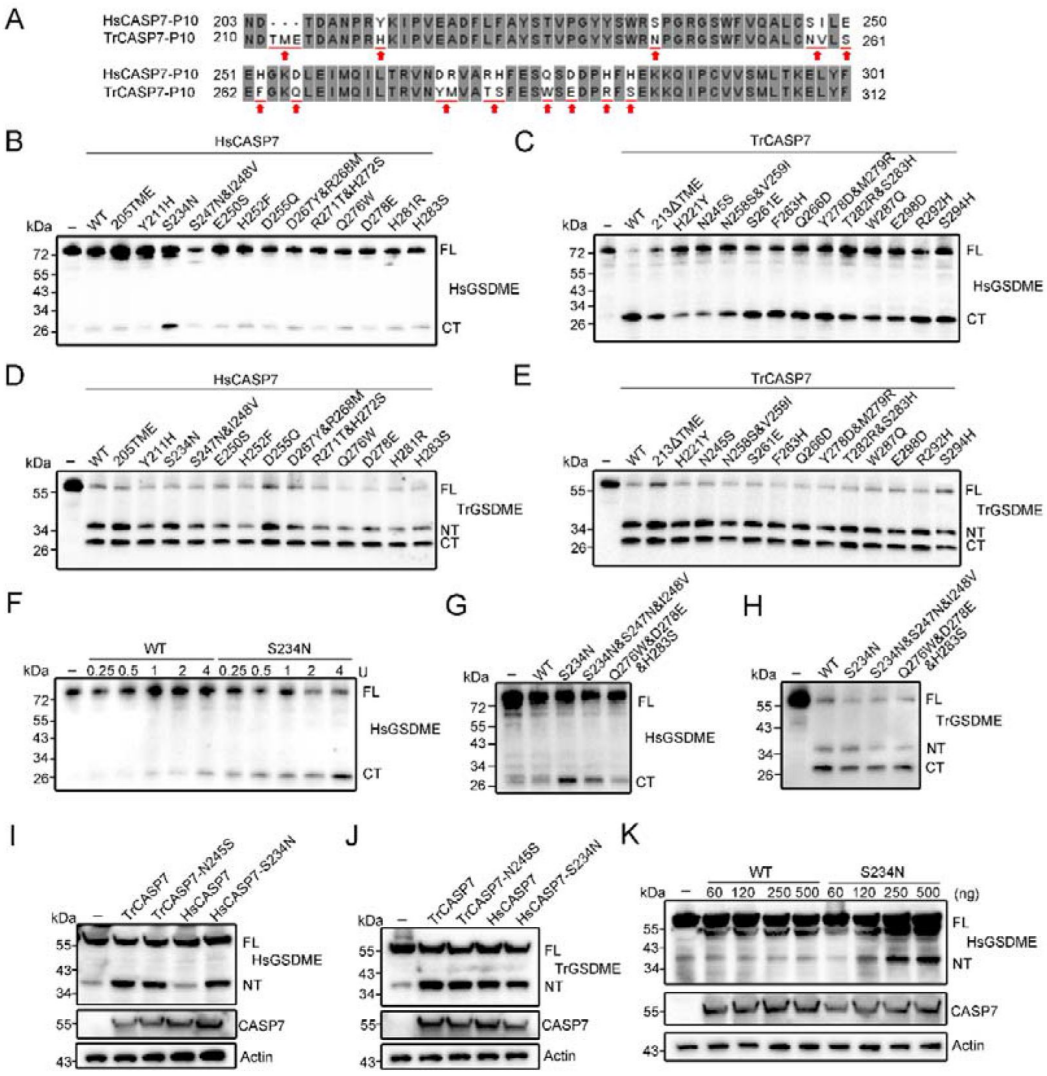

Figure 5.

Functional importance of the non-conserved residues in the p10 of HsCASP7 and TrCASP7.

(A) Sequence alignment of the p10 region in HsCASP7 and TrCASP7. The arrows indicate non-conserved residues. **(B, C)** HsGSDME was treated with wild type (WT) or mutant HsCASP7 (B) or TrCASP7 (C) for 2 h. The cleavage was determined by immunoblotting with anti-HsGSDME-CT antibody. 205TME, HsCASP7 with TME insertion at position 205; 213ΔTME, TrCASP7 with TME deleted at position 213. **(D, E)** TrGSDME was incubated with wild type (WT) or mutant HsCASP7 (D) or TrCASP7 (E) for 2 h. The cleavage was determined by immunoblotting with anti-TrGSDME antibody. **(F)** HsGSDME was incubated with HsCASP7 or the S234N mutant (0.25-4U) for 2 h. HsGSDME cleavage was analyzed as above. **(G, H)** HsGSDME (G) and TrGSDME (H) were treated with wild type or mutant HsCASP7 for 2 h. The cleavage was determined as above. **(I, J)** TrGSDME (I) or HsGSDME (J) was co-expressed with wild type or mutant TrCASP7/HsCASP7 for 24 h. GSDME cleavage, CASP7 and β-actin were determined as above. **(K)** HsGSDME was co-expressed with HsCASP7 or HsCASP7-S234N for 48 h. Cleavage of GSDME was detected as above. For panels (B-K): FL, full length; NT, N-terminal fragment; CT, C-terminal fragment.

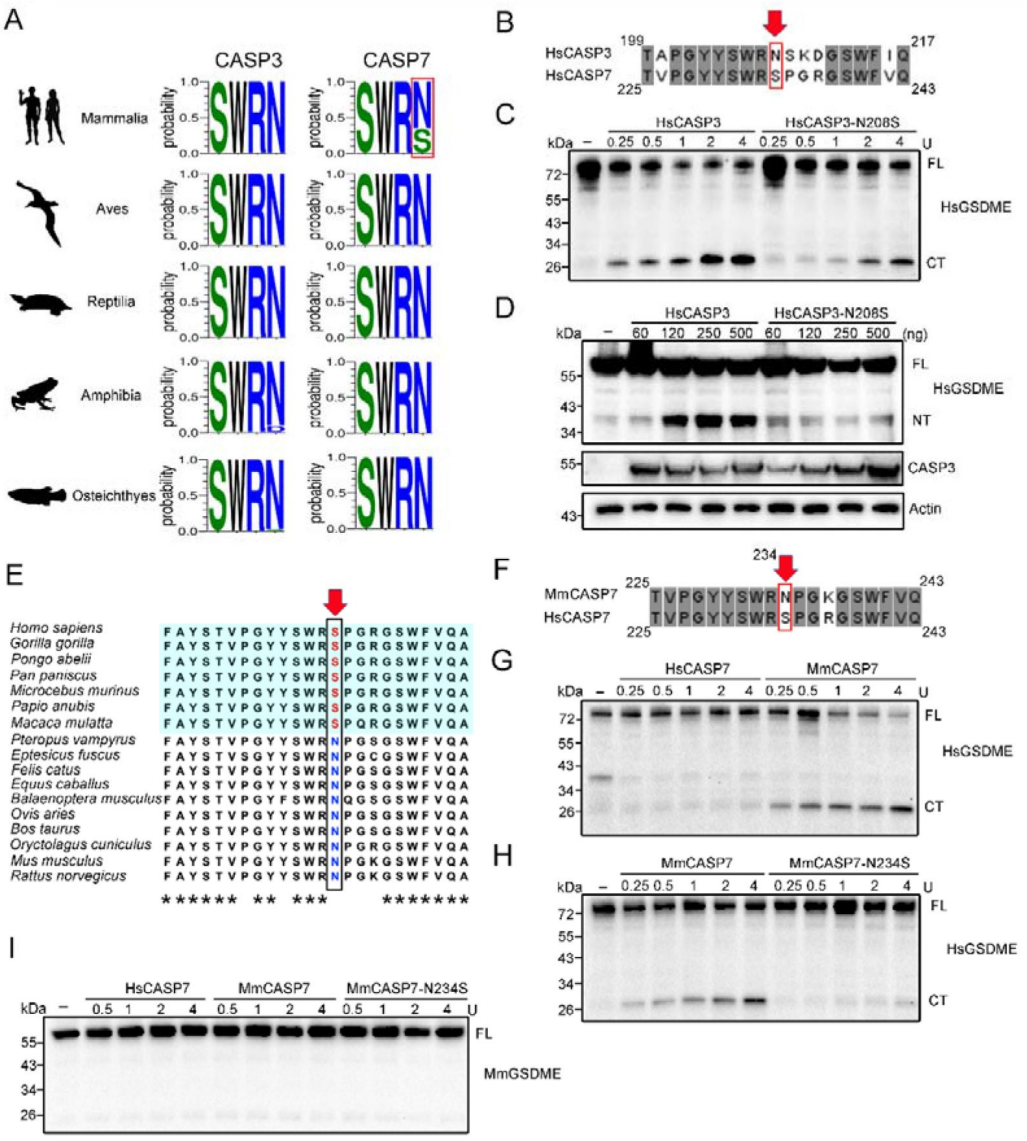

Figure 6.

Conservation and functional importance of S/N234 in CASP3/7.

(A) Weblogo analysis of the conservation of S/N234 in CASP3/7 in Mammalia, Aves, Reptilia, Amphibia and Osteichthyes. **(B)** Sequence alignment of HsCASP3/7. The S/N residues are indicated by red arrow. **(C)** HsGSDME was treated with HsCASP3 or HsCASP3-N208S for 2 h, and then subjected to immunoblotting with anti-HsGSDME-CT antibody. **(D)** HsGSDME was co-expressed in HEK293T cells with different doses of HsCASP3 or HsCASP3-N208S for 24 h. The cell lysates were immunoblotted to detect HsGSDME, CASP3, and β-actin. **(E)** Sequence alignment of the S/N234 region in the CASP7 of primate (shaded cyan) and non-primate mammals. The S/N residues are indicated by red arrow. Asterisks indicate identical residues. **(F)** Alignment of human and mouse CASP7 sequences. The S/N234 residues are indicated by arrow. **(G)** HsGSDME was treated with different units of HsCASP7 or MmCASP7 for 2 h, and the cleavage was assessed by immunoblotting with anti-HsGSDME-CT antibody. **(H)** HsGSDME was incubated with different units of MmCASP7 or its mutant (N234S) for 2 h, and HsGSDME cleavage was analyzed as above. **(I)** MmCASP7 was treated with HsCASP7, MmCASP7 or MmCASP7-N234S for 2 h. The cleavage was determined by immunoblotting with anti-MmGSDME-NT antibody. For panels (C, D, and G-I), FL, full length; NT, N-terminal fragment; CT, C-terminal fragment.

Mouse CASP3 (MmCASP3), however, cleaved MmGSDME efficiently (**Figure 6-figure supplement 2**). These results indicated the existence of an intra-species barrier for GSDME cleavage by CASP7 in some mammals, suggesting independent evolutions of CASP7 and GSDME.

Discussion

GSDME is an ancient member of the GSDM family existing ubiquitously in vertebrate from teleost to mammals (Broz, Pelegrin, & Shao, 2020; De Schutter et al., 2021). In human, GSDME is specifically cleaved by CASP3 at the consensus motif DMPD, but it is not cleaved by CASP7, which recognizes the same DxxD motif. CASP3 cleavage releases the pyroptosis-inducing NT fragment from the association of the inhibitory CT fragment and switches cell death from apoptosis to pyroptosis (Rogers et al., 2017; Wang et al., 2017). In this study, we found that different from human GSDME, a pufferfish GSDME belonging to the teleost GSDMEa lineage was specifically cleaved by both CASP3 and CASP7 at the site of DAVD to liberate the pyroptosis-inducing NT fragment. In teleost, there generally exist two GSDME orthologs, named GSDMEa and GSDMEb. Comparative study on the functions of GSDMEa and GSDMEb is scarce, and it remains to be explored whether clear physiological roles are played by these two orthologs in fish. As executors of pyroptosis, teleost GSDMEs are activated via different mechanisms. In tongue sole *Cynoglossus semilaevis*, GSDMEb is preferably cleaved by CASP1 to trigger pyroptosis (Jiang, Gu, Zhao, & Sun, 2019); in zebrafish *Danio rerio*, GSDMEb is activated by caspy2 (CASP5 homolog) through the NLR family pyrin domain containing 3 (NLRP3) inflammasome signaling pathway (Li et al., 2020; Z. Wang et al., 2020). These observations indicate that similar to mammalian GSDMD, teleost GSDMEb is activated by inflammatory CASPs. In contrast, teleost GSDMEa is cleaved mainly by apoptotic CASPs. In zebrafish, GSDMEa is cleaved by CASP3 to generate the pyroptotic NT fragment (Wang et al., 2017). In turbot *Scophthalmus maximus*, GSDMEa is bi-directionally regulated by CASP3/7, which activate GSDMEa, and CASP6, which inactivates GSDMEa (Xu, Jiang, Yu, Yuan, & Sun, 2022). In common carp *Cyprinus carpio haematopterus*, GSDMEa is cleaved by CASP3 and induces pyroptosis (Zhao et al., 2022).The complicated scenario of GSDME-mediated pyroptosis signaling in fish is likely due to the reason that, unlike mammals that have multiple GSDM members to induce pyroptosis under different conditions, fish have only GSDME to induce pyroptotic cell death. Regulation by different CASPs may represent a mechanism that enables fish GSDME to execute the orders of different pyroptotic signals.

It is intriguing that, although HsCASP3 and 7 were indistinguishable in proteolysis towards the consensus tetrapeptide, they differed remarkably in the cleavage of HsGSDME and TrGSDME. HsCASP3, but not HsCASP7, cleaved HsGSDME, whereas both HsCASP3 and 7 cleaved TrGSDME at the same site. NT/CT domain swapping between HsGSDME and TrGSDME showed that chimeric GSDMEs containing the TrGSDME-CT domain were cleaved by human as well as pufferfish CASP7 regardless of the source of the NT, whereas chimeric GSDMEs containing the HsGSDME-CT domain were resistant to HsCASP7. These results indicate that the CT domain, which is well accepted to exert an inhibitory effect on the pore-forming NT domain (Z. Liu et al., 2019; J. Shi et al., 2015), is the target of HsCASP7 discrimination, and hence determines the cleavability of GSDME by HsCASP7. Recently, Wang and Liu studied the molecular mechanism of human GSDMD recognition by CASP1/4, and showed that GSDMD-CT interacted with CASP1/4 exosites through binding to a hydrophobic pocket, which enhanced the recognition by CASP1/4 and contributed to tetrapeptide sequence-independent cleavage of GSDMD (Z. Liu et al., 2020; K. Wang et al., 2020). Different from GSDMD, we found that for TrGSDME, mutation of either the P4 (D255) or P1 (D258) residue of the consensus motif ^{255}DAVD^{258} made it resistant to CASP3/7, implying a lack of DAVD-independent cleavage mechanism. Similarly, a previous study observed that human and mouse GSDME harboring cleavage site (P4 or P1) mutation resisted CASP3 cleavage (Wang et al., 2017). These results suggest that, compared to GSDMD, GSDME has a distinct enzyme-substrate engagement mode that, as demonstrated in the present study, involves GSDME-CT.

Unlike HsCASP7, which was unable to cleave HsGSDME, TrCASP7 effectively cleaved HsGSDME. The swapping of p20 between HsCASP7 and TrCASP7 revealed that the two catalytic motifs in p20, i.e., SHG and QACRG, were not involved in HsCASP7 discrimination on GSDME. SWR and GSWF are known to be responsible for CASP substrate binding (Chai et al., 2001; Riedl et al., 2001). However, in our study, we found that these two motifs are conserved in human and pufferfish CASP7, and they had no apparent effect on the substrate discrimination of HsCASP7. By contrast, the chimeric HsCASP7 containing the p10 subunit of TrCASP7 acquired the ability to cleave HsGSDME, indicating an important role of p10. By a series of residue swapping and mutation analyses, we discovered that the residue in the position of 245 (in TrCASP7) or 234 (in HsCASP7) of the p10 subunit was the key that determined CASP7 cleavage of HsGSDME. Sequence analysis revealed that N245 is highly conserved in the CASP7 of teleost, amphibians, reptiles and birds. In contrast, this Asn residue was changed to Ser in the CASP7 of mammals, especially primates, resulting in divergence of CASP7 from CASP3 in substrate recognition and cleavage. Since, unlike non-mammalian vertebrates, mammals possess multiple pyroptotic GSDM members (GSDMA, B, C, D, and E), the functional divergence of CASP7 from CASP3 may have occurred as a result of specific and non-redundant regulation of programmed cell death mediated by different executor molecules. One of the physiological consequences is that CASP7 frees itself from the GSDME-mediated pathway and thus is able to be engaged in other cellular processes. Another physiological consequence is that GSDME activation is limited to CASP3 cleavage, thus restricting GSDME activity to situations more specific, such as that inducing CASP3 activation. More physiological consequences of CASP3/7 divergence in GSDME activation need to be explored in future studies. With the exception of primates, the conserved Asn is present in a large number of mammals, including mice. Mouse CASP7 was able to cleave HsGSDME, further supporting the importance of the Asn in CASP7 recognition and cleavage. The inability of mouse CASP7 to cleave mouse GSDME is consistent with a previous report that CASP7 deletion had little effect on mouse GSDME-mediated pyroptosis (Sarhan et al., 2018). These results suggest mutually independent evolution of GSDME and CASP7 in mice, which likely has distanced GSDME and CASP7 from each other. Similarly, human CASP7 and GSDME may also have undergone independent evolution, which leads to the disengagement of GSDME from the substrate relationship with CASP7.

In conclusion, we identified a teleost GSDME that can be cleaved by fish and human CASP3/7 to trigger pyroptosis. The GSDME-CT domain and the CASP7 p10 subunit are critical in the determination of CASP7 cleavage of GSDME. Within the p10 subunit, a single residue plays a key role in CASP7 substrate recognition and cleavage. Our results reveal the molecular basis of the functional divergence of CASP7 and CASP3, and suggest separate evolutions of CASP7 and GSDME in mammals. These findings add new insights into CASP-regulated GSDME activation in lower and higher vertebrates.

Materials and Methods

Animal, ethics, and cell line

Clinically healthy pufferfish (*Takifugu rubripes*) were obtained from a local fish farm. In the laboratory, the fish were maintained at 19-20°C in aerated seawater as reported previously (Xu et al., 2022). For euthanization, the fish were immersed in excess tricaine methane sulfonate (Sigma, St. Louis, MO). The animal experiments were approved by the Ethics Committee of institute of Oceanology, Chinese Academy of Sciences. HEK293T cells (ATCC, Rockville, MD, USA) were cultured in DMED medium (Corning, NY, USA) supplemented with 10% fetal bovine serum (ExCell Bio, Shanghai, China) at 37°C in a 5% CO2 incubator.

Sequence analysis

The sequences of 742 CASP3 and 758 CASP7 used in this study were downloaded from NCBI Orthologs. TrCASP7 and HsCASP7 sequences were aligned with Clustal W program (*www.ebi.ac.uk/clustalw/*) and visualized with ESPript 3.0 (*http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi/*) (Robert & Gouet, 2014). Conservation of S/N234 in CASP3/7 was analyzed via Weblogo3 (*https://weblogo.threeplusone.com/*) (Crooks, Hon, Chandonia, & Brenner, 2004), and the sequences of CASP3/7 used are shown in **Table 1** and **Table 2**. The phylogenetic analysis of GSDME was performed as reported previously (Xu et al., 2022). The phylogenetic relationship of major mammalian clades was estimated based on the Mammals birth-death node-dated completed trees in VertLife (Upham, Esselstyn, & Jetz, 2019). The phylogenetic tree was subsequently viewed and edited in iTOL (Letunic & Bork, 2021). The icons representing phylogeny clades were retrieved from the PhyloPic (*http://www.phylopic.org/*), with the detailed credentials provided in **Table 3**.

Antibodies and immunoblotting

Monoclonal anti-mouse (ab215191) and anti-human (ab221843) GSDME antibodies were purchased from Abcam (Cambridge, MA). Antibodies against β-actin (AC026), Flag (AE005), Myc (AE010), His (AE003), and mCherry (AE002) were purchased from Abclonal (Wuhang, China). Mouse polyclonal antibody against TrGSDME was prepared as reported previously (Xu et al., 2022). Anti-serum (1:1000 dilution) was used for immunoblotting. Immunoblotting was performed as reported previously (Zhao & Sun, 2022). Briefly, the samples were fractionated in 12% SDS-polyacrylamide gels (GenScript, Piscataway, NJ, USA). The proteins were transferred to NC membranes, immunobloted with appropriate antibodies and visualized using an ECL kit (Sparkjade Biotechnology Co. Ltd., Shandong, China).

Gene cloning and mutagenesis

Total RNA was extracted from pufferfish kidney using the FastPure Cell/Tissue Total RNA Isolation Kit V2 (Vazyme Biotech Co. Ltd., Nanjing, China). cDNA synthesis was performed with Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, United States). The coding sequences (CDSs) of pufferfish GSDME (Accession number XP_029701077.1) and CASP3/7 were amplified by PCR. The CDSs of mouse GSDME and CASP3/7 were synthesized by Sangon Biotech (Shanghai, China). Site-directed mutagenesis was performed using Hieff Mut Site-Directed Mutagenesis Kit (Yeasen, Shanghai, China). Truncates of TrGSDME were created as reported previously (Xu et al., 2022) and subcloned into pmCherry-N1 (Clontech, Mountain View, CA, USA). For the construction of GSDME and CASP7 chimera, the CDSs of GSDME NT/CT and CASP7 p10/p20 were obtained by PCR and ligated. The constructs of the truncate and chimeric proteins are shown in **Table 4**. All sequences/constructs were verified by sequencing analysis. The primers used are listed in **Table 5**.

Plasmids and transient expression

For expression in mammalian cell expression, the CDSs of GSDME (pufferfish, human and mouse) were subcloned into pCS2-3×Flag (Wang et al., 2017) or pmCherry-N1 and CDSs encoding CASP (pufferfish, human and mouse) were subcloned into p-CMV-Myc (Clontech, Mountain View, CA, USA) or pCS2-Myc (Wang et al., 2017). All plasmids were prepared with endotoxin-free plasmid kit (Sparkjade Biotechnology Co. Ltd., Shandong, China). For transient expression, HEK293T cells were cultured in 96- or 24-well plates (Corning, NY, USA) overnight, and then transfected with 100 ng/well (96-well plates) or 500 ng/well (24-well plates) indicated plasmids using Liopfectamine 3000 (Invitrogen, USA) for 24 h or as specified. For GSDME processing, the plasmids of GSDME and CASP were co-transfected in HEK293T cells as described above and cultured continuously for 24 h or as specified. Cells were harvested and lysed with RIPA Lysis Buffer (Beyotime, Shanghai, China) for immunoblotting. The primer used are shown in **Table 5**.

Class	Species	Accession Number
Mammalia	*Homo sapiens*	P42574
	Mus musculus	P70677
	Sus scrofa	Q95ND5
	Canis lupus familiaris	Q8MKI5
	Saimiri boliviensis	NP_001266895.1
	Equus caballus	NP_001157433.1
	Mesocricetus auratus	Q60431
	Pan troglodytes	XP_009446811.1
	Ovis aries	XP_014960045.1
	Capra hircus	NP_001273018.1
	Pan paniscus	XP_003823509.1
	Ailuropoda melanoleuca	XP_002914062.1
	Oryctolagus cuniculus	Q8MJC3
	Loxodonta africana	XP_023410784.1
	Bos taurus	Q08DY9
	Macaca fascicularis	Q2PFV2
	Delphinapterus leucas	XP_022426661.1
	Aotus nancymaae	XP_012327995.1
	Vulpes vulpes	XP_025874057.1

Table 1.

The CASP3 sequences used for WebLogo analysis in this study.

	Heterocephalus glaber	EHB15384.1
	Bos mutus grunniens	XP_010834058.1
	Balaenoptera acutorostrata	XP_007177921.2
	Odobenus rosmarus divergens	XP_012422221.1
	Tursiops truncatus	XP_033704658.1
	Saimiri boliviensis	AAV74267.1
	Theropithecus gelada	XP_025242051.1
	Rhinopithecus roxellana	XP_010376231.2
	Chlorocebus sabaeus	XP_007998582.1
	Arvicanthis niloticus	XP_034376775.1
	Castor canadensis	JAV41953.1
	Urocitellus parryii	XP_026256383.1
	Neogale vison	XP_044081570.1
	Lynx rufus	XP_046947013.1
	Canis lupus dingo	XP_025327613.1
	Balaenoptera acutorostrata scammoni	XP_007177921.1
	Balaenoptera musculus	XP_036694636.1
	Vicugna pacos	XP_006198038.1
	Camelus bactrianus	XP_010951604.1
	Eptesicus fuscus	XP_008150114.1
	Pteropus alecto	XP_006916432.1
	Ochotona princeps	XP_004579072.1
	Equus quagga	XP_046504640.1
	Talpa occidentalis	XP_037382298.1
	Manis pentadactyla	XP_036750218.1
	Gracilinanus agilis	XP_044536929.1
Aves	*Corvus hawaiiensis*	XP_048160132.1
	Taeniopygia guttata	XP_030127125.3

Table 1. (continued)

Pyrgilauda ruficollis	XP_041335801.1
Hirundo rustica	XP_039921773.1
Onychostruthus taczanowskii	XP_041283907.1
Passer montanus	XP_039555833.1
Molothrus ater	XP_036238378.1
Oxyura jamaicensis	XP_035179722.1
Melopsittacus undulatus	XP_030904860.1
Strigops habroptila	XP_030349011.1
Aythya fuligula	XP_032043173.1
Lonchura striata domestica	OWK64686.1
Geospiza fortis	XP_005415590.1
Camarhynchus parvulus	XP_030803986.1
Serinus canaria	XP_030094646.1
Manacus vitellinus	XP_008923018.1
Zonotrichia albicollis	XP_005483896.1
Apteryx rowi	XP_025939844.1
Numida meleagris	XP_021249041.1
Gallus gallus	NP_990056.1
Tyto alba	XP_042663613.1
Lagopus leucura	XP_042726563.1
Pygoscelis adeliae	XP_009328950.1
Calypte anna	XP_030305365.1
Aquila chrysaetos chrysaetos	XP_029861401.1
Haliaeetus leucocephalus	XP_010568301.1
Tyto alba	XP_032854868.1
Hirundo rustica	XP_039921766.1
Cygnus atratus	XP_035413755.1
Lepidothrix coronata	XP_017664322.1

Table 1. (continued)

	Amazona aestiva	KQK77628.1
	Chaetura pelagica	XP_010000752.1
	Antrostomus carolinensis	XP_010171751.1
	Nipponia nippon	XP_009473093.1
	Pelecanus crispus	XP_009479867.1
	Leptosomus discolor	XP_009958677.1
	Dryobates pubescens	XP_009909179.1
	Manacus vitellinus	XP_008923018.1
	Parus major	XP_015479949.1
	Falco rusticolus	XP_037254769.1
	Numida meleagris	XP_021249038.1
Reptilia	*Pelodiscus sinensis*	XP_006128559.1
	Gopherus evgoodei	XP_030419000.1
	Chelonia mydas	XP_043402007.1
	Dermochelys coriacea	XP_043369528.1
	Mauremys reevesii	XP_039396469.1
	Terrapene carolina triunguis	XP_029766549.1
	Trachemys scripta elegans	XP_034627282.1
	Python bivittatus	XP_025018785.1
	Pogona vitticeps	XP_020658385.1
	Zootoca vivipara	XP_034967628.1
	Podarcis muralis	XP_028600040.1
	Sceloporus undulatus	XP_042323800.1
	Lacerta agilis	XP_033017209.1
	Varanus komodoensis	XP_044305785.1
	Notechis scutatus	XP_026522951.1
	Pantherophis guttatus	XP_034257179.1
	Protobothrops mucrosquamatus	XP_015687286.1

Table 1. (continued)

	Eremias argus	QCQ80689.1
	Sceloporus undulatus	XP_042323800.1
	Gekko japonicus	XP_015272894.1
	Lacerta agilis	XP_033017209.1
	Notechis scutatus	XP_026522951.1
	Pseudonaja textilis	XP_026553662.1
	Sphaerodactylus townsendi	XP_048364960.1
	Thamnophis sirtalis	XP_013910221.1
	Crotalus tigris	XP_039186723.1
	Thamnophis elegans	XP_032080611.1
	Crocodylus porosus	XP_019390228.1
	Chelonoidis abingdonii	XP_032656285.1
	Chrysemys picta bellii	XP_005282030.1
	Mauremys mutica	XP_044873973.1
Amphibia	*Xenopus laevis*	NP_001081225.1
	Bufo gargarizans	XP_044145804.1
	Rana temporaria	XP_040189928.1
	Bufo bufo	XP_040274658.1
	Geotrypetes seraphini	XP_033799250.1
	Microcaecilia unicolor	XP_030047368.1
	Nanorana parkeri	XP_018421176.1
	Cynops orientalis	AFN55260.1
	Xenopus tropicalis	NP_001120900.1
	Rhinatrema bivittatum	XP_029441076.1
	Geotrypetes seraphini	XP_033799250.1
Osteichthyes	*Paralichthys olivaceus*	XP_019958716.1
	Danio rerio	AWP39888.1
	Dicentrarchus labrax	ABC70997.1

Table 1. (continued)

Larimichthys crocea	NP_001290322.1
Scophthalmus maximus	AVW89178.1
Cynoglossus semilaevis	XP_016894801.1
Cyprinus carpio	XP_018965718.1
Cyprinodon variegatus	XP_015245088.1
Cyprinodon tularosa	XP_038127805.1
Takifugu rubripes	NP_001027871.1
Salmo salar	NP_001133393.1
Oncorhynchus mykiss	BAU69680.1
Monopterus albus	XP_020451082.1
Nothobranchius furzeri	KAF7225424.1
Miichthys miiuy	AHG06618.1
Anguilla japonica	AYC61977.1
Scleropages Formosus	XP_018591577.1
Poecilia reticulata	XP_008417983.1
Poecilia formosa	XP_007549682.1
Oplegnathus fasciatus	AFM09714.1
Collichthys lucidus	TKS72762.1
Notothenia coriiceps	XP_010794822.1
Poeciliopsis prolifica	JAO80483.1
Haplochromis burtoni	XP_005944370.2
Cyprinus carpio	AGU12796.1
Cyprinus carpio	AGU12795.1
Electrophorus electricus	XP_026884624.2
Amphiprion melanopus	AEA08874.1
Archocentrus centrarchus	XP_030591190.1
Bagarius yarrelli	TSN95700.1
Oryzias melastigma	XP_024138660.1

Table 1. (continued)

	Oryzias latipes	NP_001098140.1
	Siniperca chuatsi	ADK47519.1
	Kryptolebias marmoratus	XP_017278585.1
	Astyanax mexicanus	XP_022539261.1
	Miichthys miiuy	AHG06618.1
	Xiphophorus couchianus	XP_027872889.1
	Oreochromis niloticus	ADJ57601.1
	Lutjanus peru	QBY35776.1
	Mugil incilis	QDK54780.1

Table 1. (continued)

Class	Species	Accession Number
Mammalia	*Homo sapiens*	P55210
	Mus musculus	P97864
	Mesocricetus auratus	P55214
	Bos taurus	XP_002698555.1
	Pan troglodytes	XP_003825650.1
	Macaca fascicularis	XP_011822901.1
	Oryctolagus cuniculus	XP_002718761.1
	Ovis aries	XP_042094975.1
	Capra hircus	XP_005698554.1
	Ailuropoda melanoleuca	XP_034519188.1
	Saimiri boliviensis boliviensis	XP_034519188.1
	Equus caballus	XP_014588811.1
	Delphinapterus leucas	XP_022424428.1
	Sus scrofa	XP_020928977.1
	Pan paniscus	XP_003825650.1
	Loxodonta africana	XP_010587277.1
	Canis lupus familiaris	XP_005637795.1
	Oryctolagus cuniculus	XP_002718761.1
	Vulpes vulpes	XP_025849552.1
	Heterocephalus glaber	XP_004838949.1
	Urocitellus parryii	XP_026244955.1
	Equus asinus	XP_014723324.1
	Tachyglossus aculeatus	XP_038614071.1
	Camelus bactrianus	XP_010968130.1
	Balaenoptera acutorostrata scammoni	XP_007173176.1
	Myodes glareolus	XP_048279394.1
	Dipodomys spectabilis	XP_042545561.1

Table 2.

The CASP7 sequences used for the WebLogo analysis in this study.

	Delphinapterus leucas	XP_022424428.1
	Eptesicus fuscus	XP_028005288.1
	Bison bison bison	XP_010857610.1
	Pteropus alecto	XP_006920912.1
	Rhinopithecus bieti	XP_017747992.1
	Balaenoptera musculus	XP_036685506.1
	Saimiri boliviensis boliviensis	XP_010331177.1
	Myotis brandtii	XP_005874576.1
	Ursus americanus	XP_045659135.1
	Neogale vison	XP_044096500.1
	Vulpes lagopus	XP_041601933.1
	Equus quagga	XP_046510739.1
	Oryctolagus cuniculus	XP_002718761.1
	Galemys pyrenaicus	KAG8517409.1
	Manis pentadactyla	XP_036754817.1
	Gracilinanus agilis	XP_044521530.1
Aves	*Corvus hawaiiensis*	XP_048166914.1
	Taeniopygia guttata	XP_030132303.3
	Pyrgilauda ruficollis	XP_041317496.1
	Hirundo rustica	XP_039927077.1
	Onychostruthus taczanowskii	XP_041271849.1
	Passer montanus	XP_039565350.1
	Molothrus ater	XP_036241577.1
	Oxyura jamaicensis	XP_035186324.1
	Turdus rufiventris	KAF4791228.1
	Melopsittacus undulatus	XP_005154576.1
	Strigops habroptila	XP_030344254.1
	Aythya fuligula	XP_032047208.1

Table 2. (continued)

Lonchura striata domestica	XP_021381698.1
Geospiza fortis	XP_005416425.1
Camarhynchus parvulus	XP_030807062.1
Serinus canaria	XP_009085424.1
Manacus vitellinus	XP_029814357.1
Zonotrichia albicollis	XP_005481147.1
Apteryx rowi	XP_025925619.1
Numida meleagris	XP_021255526.1
Gallus gallus	XP_040530979.1
Tyto alba	XP_032844696.2
Lagopus leucura	XP_042719653.1
Pygoscelis adeliae	XP_009324216.1
Corvus brachyrhynchos	XP_008632648.1
Calypte anna	XP_030309482.1
Apteryx mantelli mantelli	XP_013797196.1
Apaloderma vittatum	XP_009876249.1
Haliaeetus leucocephalus	XP_010563034.1
Tyto alba	XP_032844693.2
Lonchura striata domestica	OWK56987.1
Cygnus atratus	XP_035403725.1
Cyanistes caeruleus	XP_023785639.1
Amazona aestiva	KQK80332.1
Mesitornis unicolor	XP_010180183.1
Melopsittacus undulatus	XP_005154576.1
Athene cunicularia	XP_026707509.1
Camarhynchus parvulus	XP_030807062.1
Gallus gallus	XP_421764.3
Chaetura pelagica	XP_010006222.1

Table 2. (continued)

	Ficedula albicollis	XP_005048826.1
	Serinus canaria	XP_009085424.1
	Cuculus canorus	XP_009568871.1
	Corvus cornix cornix	XP_039410202.1
	Nipponia nippon	XP_009474328.1
	Pelecanus crispus	XP_009483464.1
	Balearica regulorum gibbericeps	XP_010295946.1
	Manacus vitellinus	XP_017924402.1
	Phalacrocorax carbo	XP_009508983.1
	Parus major	XP_015489222.1
	Falco rusticolus	XP_037256573.1
	Corvus hawaiiensis	XP_048166914.1
Reptilia	*Pelodiscus sinensis*	XP_006135034.1
	Gopherus evgoodei	XP_030424754.1
	Chelonia mydas	XP_037760318.1
	Dermochelys coriacea	XP_038265833.1
	Mauremys reevesii	XP_039402417.1
	Terrapene carolina triunguis	XP_026506928.1
	Trachemys scripta elegans	XP_034632909.1
	Python bivittatus	XP_007441954.1
	Pogona vitticeps	XP_020635566.1
	Zootoca vivipara	XP_034988803.1
	Podarcis muralis	XP_028586060.1
	Sceloporus undulatus	XP_042313006.1
	Anolis carolinensis	XP_008112942.1
	Varanus komodoensis	KAF7253514.1
	Lacerta agilis	XP_033005524.1
	Notechis scutatus	XP_026526250.1

Table 2. (continued)

	Notechis scutatus	XP_026526259.1
	Pantherophis guttatus	XP_034296056.1
	Python bivittatus	XP_007441954.1
	Protobothrops mucrosquamatus	XP_015676047.1
	Zootoca vivipara	XP_034988803.1
	Podarcis muralis	XP_028586060.1
	Sceloporus undulatus	XP_042313006.1
	Anolis carolinensis	XP_003223489.1
	Varanus komodoensis	KAF7253514.1
	Varanus komodoensis	XP_044295172.1
	Lacerta agilis	XP_033005524.1
	Notechis scutatus	XP_026526250.1
	Pantherophis guttatus	XP_034296056.1
	Protobothrops mucrosquamatus	XP_015676047.1
	Pseudonaja textilis	XP_026560589.1
	Sphaerodactylus townsendi	XP_048360798.1
	Crotalus tigris	XP_039208641.1
	Thamnophis elegans	XP_032081709.1
	Chelonoidis abingdonii	XP_032631043.1
	Mauremys mutica	XP_044881213.1
	Chrysemys picta bellii	XP_042711209.1
	Trachemys scripta elegans	XP_034632910.1
	Crocodylus porosus	XP_019411759.1
Amphibia	*Xenopus laevis*	BAA94748.1
	Lithobates catesbeianus	ACO51875.1
	Bufo gargarizans	XP_044152860.1
	Rana temporaria	XP_040217514.1
	Bufo bufo	XP_040291577.1

Table 2. (continued)

	Geotrypetes seraphini	XP_033799427.1
	Microcaecilia unicolor	XP_030058570.1
	Nanorana parkeri	XP_018419981.1
	Cynops orientalis	AFN55259.1
	Xenopus tropicalis	NP_001016299.1
	Xenopus tropicalis	CAJ82745.1
	Rhinatrema bivittatum	XP_029466303.1
Osteichthyes	*Paralichthys olivaceus*	XP_019965790.1
	Danio rerio	AWP39893.1
	Dicentrarchus labrax	CBN81450.1
	Larimichthys crocea	XP_010740374.1
	Scophthalmus maximus	XP_047183439.1
	Cynoglossus semilaevis	XP_024912944.1
	Oncorhynchus kisutch	XP_020329675.1
	Cyprinus carpio	XP_042591656.1
	Cyprinodon variegatus	XP_015245976.1
	Fundulus heteroclitus	XP_012710613.2
	Cyprinodon tularosa	XP_029690759.1
	Takifugu rubripes	XP_029690759.1
	Salmo salar	XP_014012537.2
	Oncorhynchus mykiss	QWC93456.1
	Ictalurus punctatus	XP_017338665.1
	Monopterus albus	XP_020460887.1
	Nothobranchius furzeri	KAF7204369.1
	Alosa alosa	XP_048088911.1
	Alosa sapidissima	XP_041939757.1
	Anabarilius grahami	ROI81868.1
	Salvelinus alpinus	XP_023842909.1

Table 2. (continued)

Salvelinus alpinus	XP_023861943.1
Gadus morhua	XP_030195895.1
Labrus bergylta	XP_020506858.1
Toxotes jaculatrix	XP_040923277.1
Collichthys lucidus	TKS89629.1
Notothenia coriiceps	XP_010765636.1
Melanotaenia boesemani	XP_041830322.1
Clarias magur	KAF5896512.1
Anabas testudineus	XP_026207209.1
Chelmon rostratus	XP_041817955.1
Anguilla anguilla	XP_035260198.1
Trematomus bernacchii	XP_033975792.1
Pimephales promelas	KAG1954935.1
Bagarius yarrelli	TSK14754.1
Seriola dumerili	XP_022598445.1
Xiphophorus hellerii	XP_032429481.1
Sebastes umbrosus	XP_037611938.1
Parambassis ranga	XP_028285718.1
Oryzias melastigma	KAF6724972.1
Megalops cyprinoides	XP_036387401.1
Salarias fasciatus	XP_029954337.1
Salvelinus namaycush	XP_038866282.1

Table 2. (continued)

Icon	Figure Origin
Monotremata	Becky-Barnes
Didelphimorphia	Daniel-Stadtmauer
Diprotodontia	Gavin-Prideaux
Afrosoricida	Mo-Hassan
Macroscelidea	uncredited
Pilosa	Xavier-A-Jenkins
Primates	T-Michael-Keesey
Rodentia	Jiro-Wada
Lagomorpha	Margot-Michaud
Eulipotyphla	Becky-Barnes
Perissodactyla	Mercedes-Yrayzoz-vectorized-by-T-Michael-Keesey
Artiodactyla	DFoidl-modified-by-T-Michael-Keesey
Carnivora	Chlo-Schmidt
Pholidota	Steven-Traver
Chiroptera	Margot-Michaud
Homo sapiens	NASA
Avian	Ferran Sayol
Reptilia	Gabriela Palomo-Munoz
Amphibia	Yusan Yang
Actinopterygii	Milton Tan

Table 3.

The credits for the pictures used in this study.

Truncate/chimeric protein	Amino acid sequence
TrGSDME-NT	1-258 aa of TrGSDME
TrGSDME-CT	259-474 aa of TrGSDME
HsNT-TrCT	1-270 aa of HsGSDME + 259-274 aa of TrGSMDE
TrNT-HsCT	1-258 aa of TrGSDME + 271-496 aa of HsGSDME
Hsp20-Trp10	1-198 aa of HsCASP7 + 207-313 aa of TrCASP7
Trp20-Hsp10	1-206 aa of TrCASP7 + 199-303 aa of HsCASP7

Table 4.

The constructs of the truncate and chimeric proteins.

Primer	Sequence (5’-3’)
Clone primers	
TrCASP3 forward	ATGTCGGCCAACGGACC
TrCASP3 reverse	GGAAAAATACATCTCTTTGGTCAGC
TrCASP7 forward	ATGCAGATGGCTGGAGAACC
TrCASP7 reverse	GTTAAAGTACAGTTCTTTTGTCAGCATCG
TrGSDME forward	ATGTTTTCCAAGGCCACGG
TrGSDME reverse	ATCGATAAAATCCGTTTCAGACTTTG
Recombiantion primers	
TrCASP3 forward	TAAGAAGGAGATATACATATGATGT CGGCCAACGGACC
TrCASP3 reverse	GTGGTGGTGGTGGTGCTCGAGGGA AAAATACATCTCTTTGGTCAGC
TrCASP7 forward	TAAGAAGGAGATATACATATGATGCA GATGGCTGGAGAACC
TrCASP7 reverse	GTGGTGGTGGTGGTGCTCGAGGTTAA AGTACAGTTCTTTTGTCAGCATCG

Table 5.

Primers used in this study.

TrGSDME forward	TAAGAAGGAGATATACATATGATGTTT TCCAAGGCCACGG
TrGSDME reverse	GTGGTGGTGGTGGTGCTCGAGATCGA TAAAATCCGTTTCAGACTTTG
TrGSDME-D255R forward	TGGGAATCCCCGAGAGCGGTGGATGG TCGTTGTCT
TrGSDME-D255R reverse	AGACAACGACCATCCACCGCTCTCGG GGATTCCCA
TrGSDME-D258A forward	AGACAACGACCATCCACCGCTCTCGG GGATTCCCA
TrGSDME-D258A reverse	AACGACCATCCACCGCTCTCGGGGATT CCCA
HsNT-TrCT forward	TAAGAAGGAGATATACATATGATGTT TGCCAAAGCAACCAGG
HsNT-TrCT reverse	GTGGTGGTGGTGGTGCTCGAGATCG ATAAAATCCGTTTCAGACTTTG
TrNT-HsCT forward	TAAGAAGGAGATATACATATGATGTT TTCCAAGGCCACGG
TrNT-HsCT reverse	GTGGTGGTGGTGGTGCTCGAGTGAA TGTTCTCTGCCTAAAGC
Hsp20-Trp10 forward	TAAGAAGGAGATATACATATGATGG CAGATGATCAGGGCTG
Hsp20-Trp10 reverse	GTGGTGGTGGTGGTGCTCGAGGTTA AAGTACAGTTCTTTTGTCAGCATC
Trp20-Hsp10 forward	TAAGAAGGAGATATACATATGATGG CTGGAGAACCCACTGAG
Trp20-Hsp10 reverse	GTGGTGGTGGTGGTGCTCGAGTTGA CTGAAGTAGAGTTCCTTGGTGA

Table 5. (continued)

MmGSDME-N234S forward	GTTATTACTCATGGAGGAGCCCAGG GAAAG
MmGSDME-N234S reverse	CTTTCCCTGGGCTCCTCCATGAGTA ATAAC
HsGSDME-Δ261-266 forward	CCAGGATGGACATCCATTTGCGGAG CTGC
HsGSDME-Δ261-266 reverse	CAAATGGATGTCCATCCTGGGAAGAT ATCCCAT
HsGSDME-Δ281-296 forward	CCTGGTCTTT GACATGCCAGATGCTG CGCA
HsGSDME-Δ281-296 reverse	CTGGCATGTCAAAGACCAGGGGGTCC AGGTAGA
Overexpression preimers	
TrGSDME-FL forward	TCAGATCTCGAGCTCAAGCTTATGTTT TCCAAGGCCACGG
TrGSDME-FL reverse	CATGGTGGCGACCGGTGGATCATCGA TAAAATCCGTTTCAGACTTTG
TrGSDME-NT forward	TCAGATCTCGAGCTCAAGCTTATGTTT TCCAAGGCCACGG
TrGSDME-NT reverse	CATGGTGGCGACCGGTGGATCGTCTAC AGCATCAGGAGACTCC
TrGSDME-CT forward	TCAGATCTCGAGCTCAAGCTTATGGGCC GGTGCCTGGA
TrGSDME-CT reverse	CATGGTGGCGACCGGTGGATCATCGA TAAAATCCGTTTCAGACTTTG
TrCASP7 forward	TTCTGAAGAGGACTTGAATTCAATGG CTGGAGAACCCACTGAG
TrCASP7 reverse	ACGACTCACTATAGTTCTAGATCAGT

Table 5. (continued)

	TAAAGTACAGTTCTTTTGTCAGC
TrCASP3 forward	TTCTGAAGAGGACTTGAATTCAAATG
	TCGGCCAACGGACC
TrCASP3 reverse	ACGACTCACTATAGTTCTAGAGGAA
	AAATACATCTCTTTGGTCAGC
HsGSDME forward	CAAGCTTGCGGCCGCGAATTCAATGT
	TTGCCAAAGCAACCAGG
HsGSDME reverse	ACGACTCACTATAGTTCTAGATCATG
	AATGTTCTCTGCCTAAAGC
HsCASP7 forward	TTCTGAAGAGGACTTGAATTCAATGG
	CAGATGATCAGGGCTG
HsCASP7 reverse	ACGACTCACTATAGTTCTAGACTATTG
	ACTGAAGTAGAGTTCCTTGGTG
HsCASP3 forward	TTCTGAAGAGGACTTGAATTCAATGG
	AGAACACTGAAAACTCAGTGG
HsCASP3 reverse	ACGACTCACTATAGTTCTAGATTAGTG
	ATAAAAATAGAGTTCTTTTGTGAGC
MmCASP7 forward	TTCTGAAGAGGACTTGAATTCAATGA
	CCGATGATCAGGACTGTGC
MmCASP7 reverse	ACGACTCACTATAGTTCTAGATCAAC
	GGCTGAAGTACAGCTCTTTGG

Table 5. (continued)

Protein purification

Recombinant GSDMEs and CASPs are all soluble and were purified as described previously (Jiang, Zhou, Sun, Zhang, & Sun, 2020; Xu et al., 2022). Briefly, the CDSs of TrGSDME and CASP variants were each cloned into pET30a (+), and the CDSs of HsGSDME, MmGSDME, and chimeric GSDME were each cloned into pET28a-SUMO. The recombinant plasmids were introduced into *Escherichia coli* Transetta (DE3) (TransGen, Beijing, China) by transformation. The transformants were cultured in Luria broth (LB) at 37℃ until logarithmic growth phase. Isopropyl-β-D-thiogalactopyranoside (0.3 mM) was added to the medium, followed by incubation at 16℃ for 20 h. Bacteria were harvested and lysed, and the supernatant was collected for protein purification with Ni-NTA columns (GE Healthcare, Uppsala, Sweden). The proteins were dialyzed with PBS at 4 °C and concentrated.

CASP activity

To measure their proteolytic activity, recombinant CASPs were incubated separately with various colorimetric substrates at 37℃ for 2 h as described previously (Jiang et al., 2020; Xu et al., 2022), and then monitored for released ρNA at OD405. To compare their substrate preference, TrCASP3/7 and HsCASP3/7 were incubated with Ac-DAVD-ρNA or Ac-DMPD-ρNA (Science Peptide Biological Technology Co., Ltd, shanghai, China) at 37℃ for 30 min, and the released ρNA was measured at OD405. The CASP activity in cells transfected TrCASP3/7 was determined as described previously (Xu et al., 2022).

GSDME cleavage by CASPs

GSDME cleavage assay was performed as described previously (Jiang et al., 2020; Xu et al., 2022). Briefly, recombinant GSDME was incubated with 1 U of recombinant HsCASP1, 2, 3, 6, 7, 8 and 9 (Enzo Life Sciences, Villeurbanne, France) at 37℃ for 2 h in the reaction buffer (50 mM Hepes (pH 7.5), 3 mM EDTA, 150 mM NaCl, 0.005% (v/v) Tween 20, and 10 mM DTT). TrGSDME, HsGSDME or their chimeras were treated with 1 U of TrCASP3/7, HsCASP3/7, or their chimeras for 2 h as above in reaction buffer. After incubation, the samples were boiled in 5 x Loading Buffer (GenScript, Piscataway, NJ, USA) and subjected to SDS-PAGE or immunoblotting with indicated antibody.

Cell death examination

Cell death examination by microscopy was performed as reported previously (Jiang et al., 2020; Xu et al., 2022). Briefly, HEK293T cells were plated into 35 mm glass-bottom culture dishes (Nest Biotechnology, Wuxi, China) at about 60% confluency and subjected to the indicated treatment for 24 h. To examine cell death morphology, propidium iodide (PI) (Invitrogen, Carlsbad, CA, USA) and DiO (Solarbio, Beijing, China) were added to the culture medium, and the cells were observed with a Carl Zeiss LSM 710 confocal microscope (Carl Zeiss, Jena, Germany). To video the cell death process, the cells were transfected with 1 μg pmCherry-N1 vector expressing TrGSDME-NT for 16 h, and cell death was recorded using the above microscope. Cell death measured by lactate dehydrogenase (LDH) assay was performed as reported previously (Xu et al., 2022).

Statistical analysis

The Student's t test and one-way analysis of variance (ANOVA) were used for comparisons between groups. Statistical analysis was performed with GraphPad Prism 7 software. Statistical significance was defined as $P < 0.05$.

Acknowledgements

We thank Prof. Feng Shao (National institute of Biological Sciences, Beijing, China) for providing human CASP3/7 and GSDME expression vectors. This work was supported by the grants from the Science & Technology Innovation Project of Laoshan Laboratory (LSKJ202203000), the Youth Innovation Promotion Association CAS (2021204), the National Natural Science Foundation of China (41876175), and the Taishan Scholar Program of Shandong Province (2018 and 2021).

Author Contributions

Hang Xu: Methodology; Investigation; Visualization; Writing—original draft.;**Zihao Yuan**: Investigation. **Kunpeng Qin**: Investigation; **Shuai Jiang**: Conceptualization; Methodology; Supervision; Writing—review & editing. **Li Sun**: Conceptualization; Supervision; Writing—review & editing.

Competing Interest Statement

The authors declare that they have no competing interests.

Legends for Figure 1-video 1

Figure 1-video 1 (separate file). TrGSDME-NT induces pyroptosis of HEK293T cells. HEK293T cells were transfected with the vector expressing mCherry-tagged TrGSDME-NT, and time-lapse images of the cells were recorded with a confocal microscope.

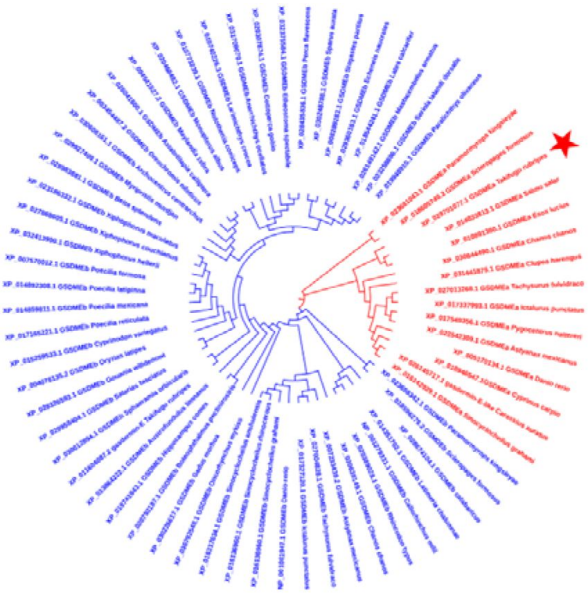

Figure 1-figure supplement 1.

Phylogenetic analysis of the TrGSDME used in this study.

The phylogenetic tree was constructed using 15 fish GSDMEa (red) and 51 fish GSDMEb (blue) collected from NCBI databank. The TrGSDME used in this study is indicated by a red star.

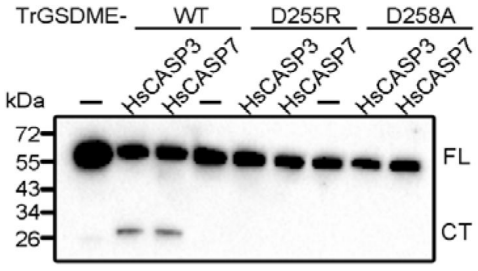

Figure 1-figure supplement 2.

Cleavage of TrGSDME mutants by HsCASP3/7.

TrGSDME wild type (WT) and mutants (D255R and D258A) were treated with or without (-) HsCASP3/7 for 2 h, and the cleavage was determined by immunoblotting with anti-His antibody. FL, full length; CT, cleaved C-terminal region.

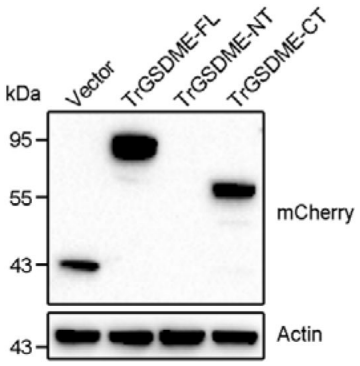

Figure 2-figure supplement 1.

Ectopic expression of TrGSDME-FL and truncates.

HEK293T cells were transfected with the backbone vector or the vector expressing mCherry-tagged TrGSDME full length (FL) or truncates (NT or CT) for 24 h. The cell lysate was then immunoblotted with antibody against mCherry or β-actin.

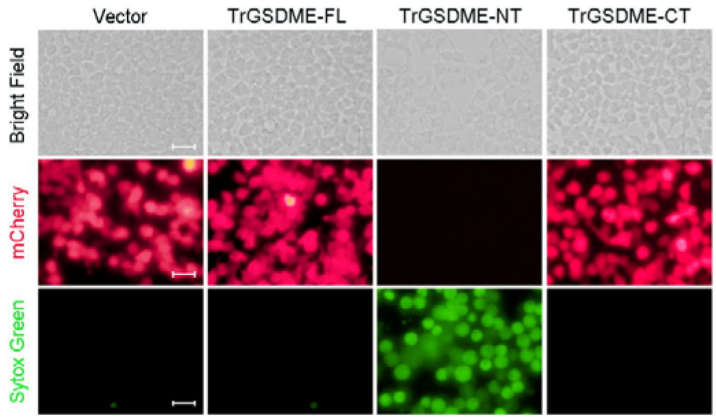

Figure 2-figure supplement 2.

The cell death-inducing ability of TrGSDME-FL and truncates.

HEK293T cells were transfected with the backbone vector or the vector expressing mCherry-tagged TrGSDME full length (FL) or truncate (NT or CT) for 24 h. The cells were then stained with Sytox Green and examined with a microscope. Scale bar, 30 μm.

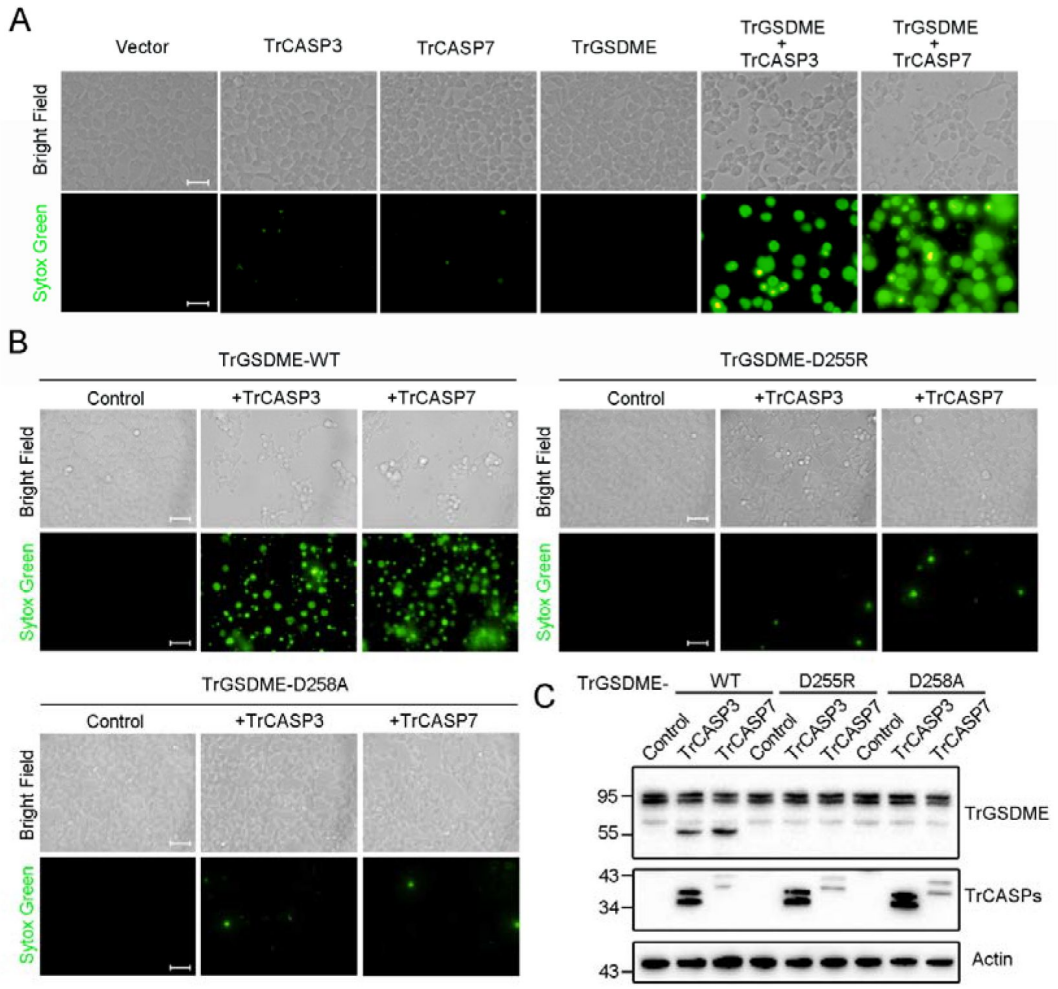

Figure 2-figure supplement 3.

Activation of TrGSDME-mediated pyroptosis by TrCASP3/7.

(A) HEK293T cells were transfected with the backbone vector or the vector expressing the indicated protein for 24 h and then stained with Sytox Green. Scale bars, 30 μm. **(B, C)** HEK293T cells expressing wild type (WT) or mutant TrGSDME together with or without (control) TrCASP3 or TrCASP7 were subjected to Sytox Green staining (B) and TrGSDME cleavage analysis by immunoblot with antibodies against mCherry (upper), Myc (middle), and actin (lower) (C). Scale bar, 30 μm.

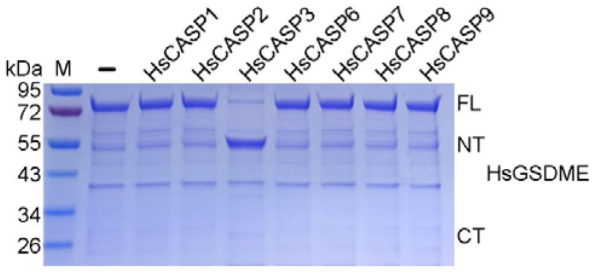

Figure 3-figure supplement 1.

Cleavage of HsGSDME by caspases.

Recombinant HsGSDME was incubated with or without (-) HsCASP1, 2, 3, 6, 7, 8, and 9 for 2 h, and the cleavage was determined by SDS-PAGE. M, molecular markers. FL, full length; NT, N-terminal fragment; CT, C-terminal fragment.

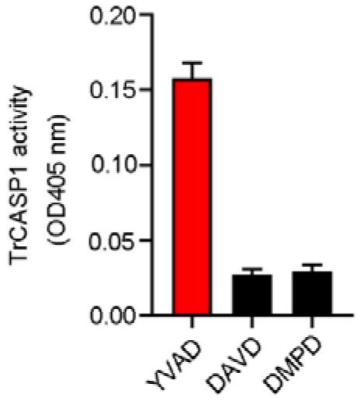

Figure 3-figure supplement 2.

Proteolytic activity of TrCASP1.

TrCASP1 was incubated with colorimetric Ac-YVAD-ρNA, Ac-DAVD-ρNA, and Ac-DMPD-ρNA for 1 h, and the cleavage was determined by measuring the release of ρNA. Values are the means of triplicate experiments and shown as means ±SD..

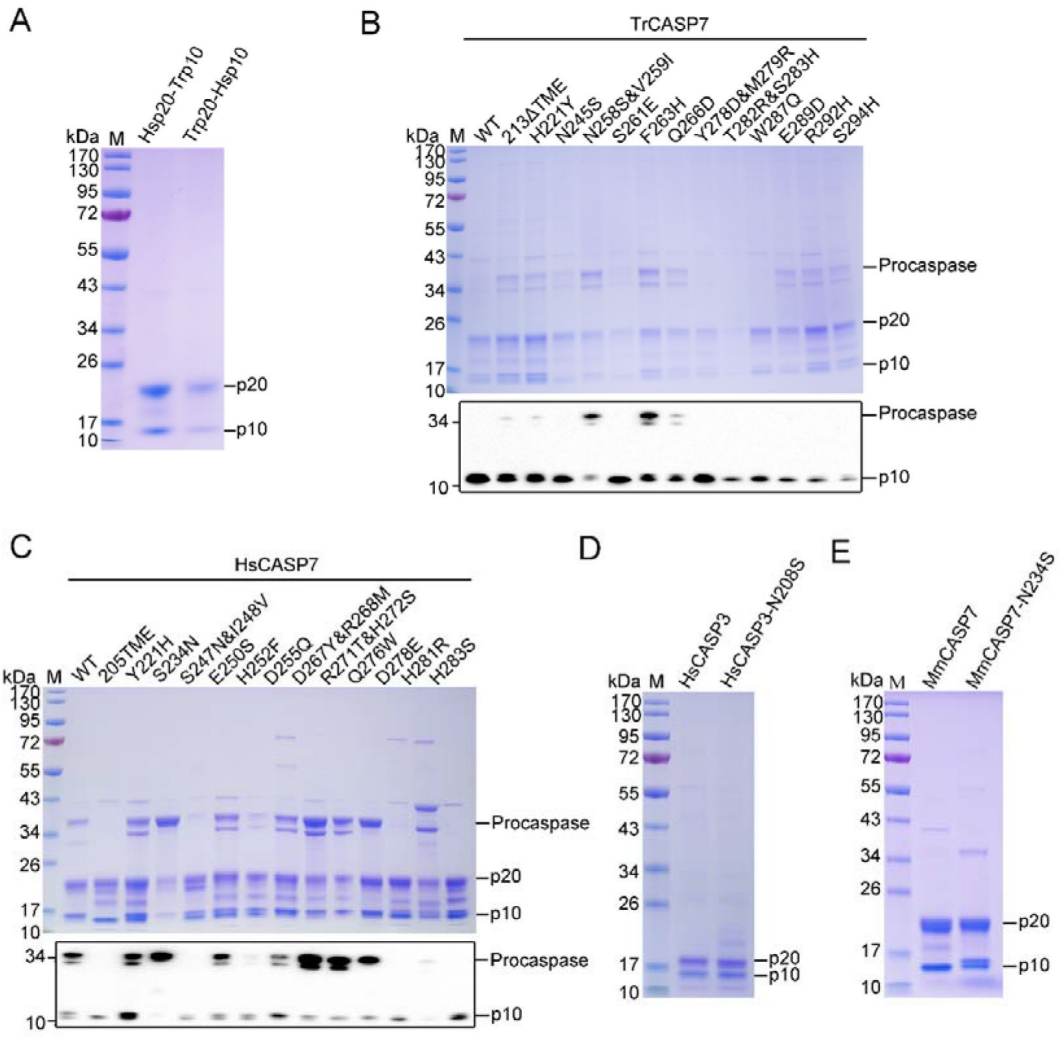

Figure 4-figure supplement 1.

Preparation of recombinant proteins.

(A) SDS-PAGE analysis of purified Hsp20-Trp10 and Trp20-Hsp10. **(B, C)** Purified TrCASP7 (B) and HsCASP7 (C) were subjected to SDS-PAGE (upper panel) and immunoblotting with anti-His tag antibody (lower panel). **(D)** HsCASP3 and its mutant (N208S) were analyzed by SDS-PAGE. **(E)** SDS-PAGE analysis of purified MmCASP7 and its mutant (N234S). In all panels, the p10 and p20 subunits are indicated.

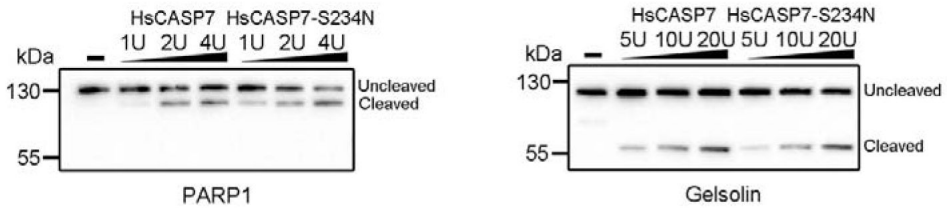

Figure 5-figure supplement 1.

Cleavage of poly (ADP-ribose) polymerase 1 (PARP1) and gelsolin by HsCASP7.

HEK293T cells were transfected with C-terminal Flag-tagged PARP1 (left panel) or gelsolin (right panel) for 24 h. The cell lysates were incubated with or without (-) different units of HsCASP7 and HsCASP7-S234N for 2 h and then subjected to immunoblotting with anti-Flag antibody.

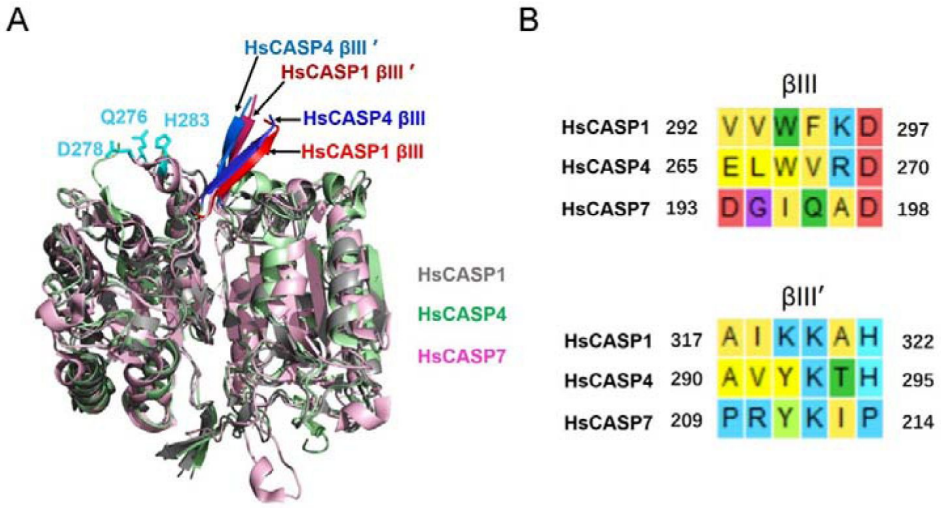

Figure 5-figure supplement 2.

Structural analysis of HsCASP1/4/7.

(A) The structures of HsCASP1 (PDB: 6kn0, gray), HsCASP4 (PDB: 6kmz, green), and HsCASP7 (PDB: 6kn0, purple) are superimposed. The βIII/βIII' sheets of HsCASP1/4 are indicated by arrows; the residues Q276, D278, and H283 of HsCASP7 are shown in cyan. **(B)** The sequence alignment of HsCASP1/4 βIII/βIII' sheet with the corresponding region of HsCASP7.

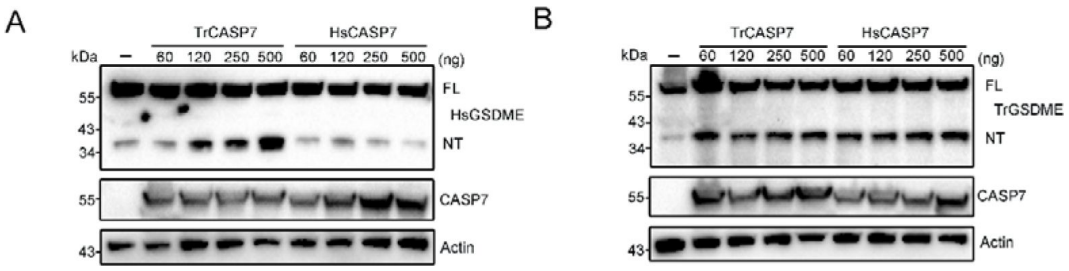

Figure 5-figure supplement 3.

GSDME cleavage by CASP7 in cellular system.

TrGSDME (A) or HsGSDME (B) was co-expressed with different doses of TrCASP7/HsCASP7 for 48 h in HEK293T cells. The expression/cleavage of TrGSDME/HsGSDME and the expression of TrCASP7/HsCASP7 were determined by immunoblotting. β-actin was used as a loading control.

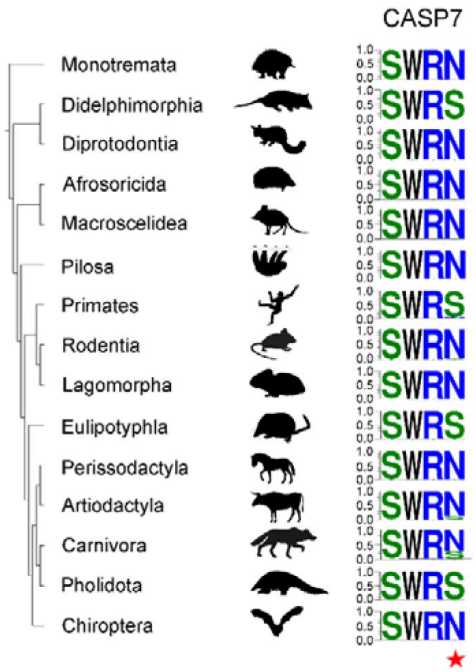

Figure 6-figure supplement 1.

Conservation of S/N234 in mammalian CASP7.

The four conserved amino acids are shown in LOGOs, with height representing the relative proportion of the corresponding amino acid at that position. The S/N234 is presented as the fourth LOGO and indicated with a red star.

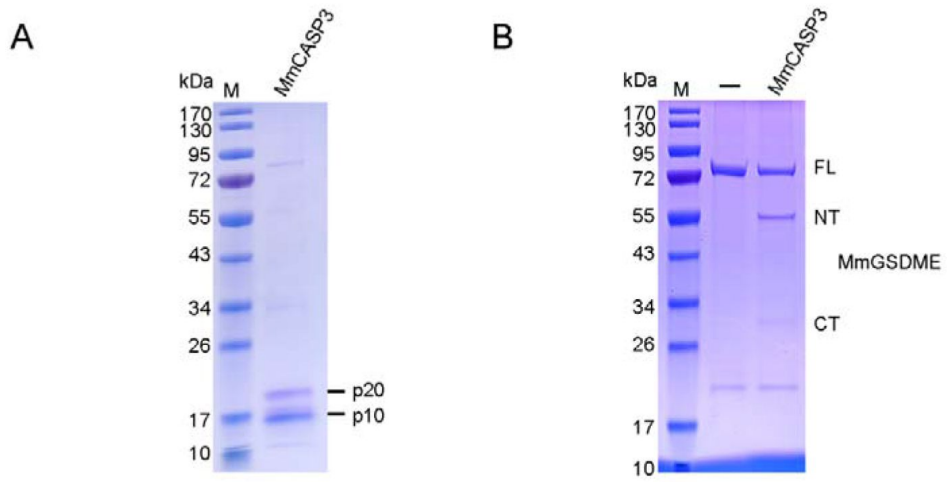

Figure 6-figure supplement 2.

The cleavage of MmGSDME by MmCASP3.

(A) SDS-PAGE analysis of purified MmCASP3. **(B)** MmGSDME was treated with 1U of MmCASP3 for 2 h and then subjected to SDS-PAGE.

References

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Reviewer #1 (Public Review):

Summary
In this study, Xu et al. provide insights into the substrate divergence of caspase 3 and 7 (CASP3 and CASP7) for gasdermin E (GSDME) cleavage and activation during evolution in vertebrates. Using a diverse set of biochemical assays, domain swapping, site-directed

mutagenesis, and bioinformatics tools, the authors demonstrate that the human GSDME C-terminal region and the S234 residue of human CASP7 are the key determinants that impede the cleavage of human GSDME by human CASP7. Their findings suggest that mutations affecting the function of caspases have enabled the functional divergence of distinct caspase family members to specialize in controlling complicated cellular functions in mammals.

Strengths
The authors made an important contribution to the field by demonstrating how human CASP7 has functionally diverged to lose the ability to cleave GSDME and showing that reverse-mutations in CASP7 can restore GSDME cleavage. The use of multiple methods to support their conclusions strengthens the authors' findings. The unbiased mutagenesis screen performed to identify S234 in huCASP7 as the determinant of its GSDME cleavability is also a strength.

Weaknesses
While the authors employed a comprehensive experimental setup to investigate the CASP7-mediated GSDME cleavage across evolution, future studies will be required to fully understand the physiological implications of this evolutionary divergence.

https://doi.org/10.7554/eLife.89974.2.sa1

Reviewer #2 (Public Review):

The authors wanted to address the differential processing of GSDME by caspase 3 and 7, finding that while in humans GSDME is only processed by CASP3, Takifugu GSDME, and other mammalian can be processed by CASP3 and 7. This is due to a change in a residue in the human CAPS7 active site that abrogates GSDME cleavage. This phenomenon is present in humans and other primates, but not in other mammals such as cats or rodents. This study sheds light on the evolutionary changes inside CASP7, using sequences from different species. Although the study is somehow interesting and elegantly provides strong evidence of this observation, it lacks the physiological relevance of this finding, i.e. on human side, mouse side, and fish what are the consequences of CASP3/7 vs CASP3 cleavage of GSDME.

Fish also present a duplication of GSDME gene and Takifugu present GSDMEa and GSDMEb. It is not clear in the whole study if when referring to TrGSDME is the a or b. This should be stated in the text and discussed in the differential function of both GSDME in fish physiology (i.e. PMIDs: 34252476, 32111733 or 36685536).

https://doi.org/10.7554/eLife.89974.2.sa0

Author Response

The following is the authors' response to the original reviews.

> ***eLife assessment***
>
> *This important study elucidates the molecular divergence of caspase 3 and 7 in the vertebrate lineage. Convincing biochemical and mutational data provide evidence that in humans, caspase 7 has lost the ability to cleave gasdermin E due to changes in a key residue, S234. However, the physiological relevance of the findings is incomplete and requires further experimental work.*
>
> ***Public Reviews:***
>
> ***Reviewer #1 (Public Review):***

Summary

In this study, Xu et al. provide insights into the substrate divergence of CASP3 and CASP7 for GSDME cleavage and activation during vertebrate evolution vertebrates. Using biochemical assays, domain swapping, site-directed mutagenesis, and bioinformatics tools, the authors demonstrate that the human GSDME C-terminal region and the S234 residue of human CASP7 are the key determinants that impede the cleavage of human GSDME by human CASP7.

Strengths

The authors made an important contribution to the field by demonstrating how human CASP7 has functionally diverged to lose the ability to cleave GSDME and showing that reverse-mutations in CASP7 can restore GSDME cleavage. The use of multiple methods to support their conclusions strengthens the authors' findings. The unbiased mutagenesis screen performed to identify S234 in huCASP7 as the determinant of its GSDME cleavability is also a strength.

Weaknesses

While the authors utilized an in-depth experimental setup to understand the CASP7-mediated GSDME cleavage across evolution, the physiological relevance of their findings are not assessed in detail. Additional methodology information should also be provided.

Specific recommendations for the authors

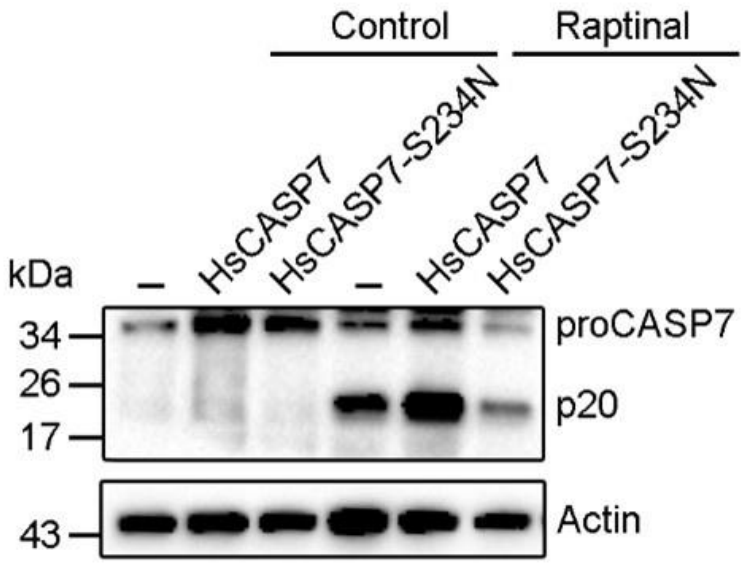
(1) The authors should expand their evaluation of the physiological relevance by assessing GSDME cleavage by the human CASP7 S234N mutant in response to triggers such as etoposide or VSV, which are known to induce CASP3 to cleave GSDME (PMID: 28045099). The authors could also test whether the human CASP7 S234N mutation affects substrate preference beyond human GSDME by testing cleavage of mouse GSDME and other CASP3 and CASP7 substrates in this mutant.

(1) The physiological relevance was discussed in the revised manuscript (lines 328-340). Our study revealed the molecular mechanism underlying the divergence of CASP3- and CASP7-mediated GSDME activation in vertebrate. One of the physiological consequences is that in humans, CASP7 no longer directly participates in GSDME-mediated cell death, which enables CASP7 to be engaged in other cellular processes. Another physiological consequence is that GSDME activation is limited to CASP3 cleavage, thus restricting GSDME activity to situations more specific, such as that inducing CASP3 activation. The divergence and specialization of the physiological functions of different CASPs are consistent with and possibly conducive to the development of refined regulations of the sophisticated human GSDM pathways, which are executed by multiple GSDM members (A , B, C, D, and E), rather than by GSDME solely in teleost, such as Takifugu. More physiological consequences of CASP3/7 divergence in GSDME activation need to be explored in future studies.

With respect to the reviewer's suggestion of assessing GSDME cleavage by the human CASP7 S234N mutant in response to triggers such as etoposide or VSV: (i) CASP7 S234N is a creation of our study, not a natural human product, hence its response to CASP7 triggers cannot happen under normal physiological conditions except in the case of application, such as medical application, which is not the aim of our study. (ii) CASP3/7 activators (such as raptinal) induced robust activation of the endogenous CASP3 (Heimer et al., Cell Death Dis. 2019;10:556) and CASP7 (Author response image 1, below) in human cells. Since CASP3 is the natural activator of GSDME, the presence of the triggers inevitably activates GSDME via CASP3. Hence, under this condition, it will be difficult to examine the effect of CASP7 S234N.

Author response image 1.

HsCASP7 activation by raptinal. HEK293T cells were transfected with the empty vector (-), or the vector expressing HsCASP7 or HsCASP7-S234N for 24 h. The cells were then treated with or without (control) 5 μM raptinal for 4 h. The cells were lysed, and the lysates were blotted with anti-CASP7 antibody.



(2) As suggested by the reviewer, the cleavage of other CASP7 substrates, i.e., poly (ADP-ribose) polymerase 1 (PARP1) and gelsolin, by HsCASP7 and S234N mutant was determined. The results showed that HsCASP7 and HsCASP7-S234N exhibited similar cleavage capacities. Figure 5-figure supplement 1 and lines 212-214.

> *(2) It would also be interesting to examine the GSDME structure in different species to gain insight into the nature of mouse GSDME, which cannot be cleaved by either mouse or human CASP7.*

Because the three-dimensional structure of GSDME is not solved, we are unable to explore the structural mechanism underlying the GSDME cleavage by caspase. Since our results showed that the C-terminal domain was essential for caspase-mediated cleavage of GSDME, it is likely that the C-terminal domain of mouse GSDME may possess some specific features that render it to resist mouse and human CASP7.

> *(3) The evolutionary analysis does not explain why mammalian CASP7 evolved independently to acquire an amino acid change (N234 to S234) in the substrate-binding motif. Since it is difficult to experimentally identify why a functional divergence occurs, it would be beneficial for the authors to speculate on how CASP7 may have acquired functional divergence in mammals; potentially this occurred because of functional redundancies in cell death pathways, for example.*

According to the reviewer's suggestion, a speculation was added. Lines 328-340.

> *(4) For the recombinant proteins produced for these analyses, it would be helpful to know whether size-exclusion chromatography was used to purify these proteins and whether these purified proteins are soluble. Additionally, the SDS-PAGE in Figure S1B and C show multiple bands for recombinant mutants of TrCASP7 and HsCASP7. Performing protein ID to confirm that the detected bands belong to the respective proteins would be beneficial.*

The recombinant proteins in this study are soluble and purified by Ni-NTA affinity chromatography. Size-exclusion chromatography was not used in protein purification.

For the SDS-PAGE in Figure 4-figure supplement 1B and C (Figure S1B and C in the previous submission), the multiple bands are most likely due to the activation cleavage of the TrCASP7 and HsCASP7 variants, which can result in multiple bands, including p10 and p20. According to the reviewer's suggestion, the cleaved p10 was verified by immunoblotting. Figure 4-figure supplement 1B and C.

> *(5) For Figures 3C and 4A, it would be helpful to mention what parameters or PDB files were used to attribute these secondary structural features to the proteins. In particular, in Figure 3C, residues 261-266 are displayed as a β-strand; however, the well-known α-model represents this region as a loop. Providing the parameters used for these callouts could explain this difference.*

For Figure 3C, in the revised manuscript, we used the structure of mouse GSDMA3 (PDB: 5b5r) for the structural analysis of HsGSDME. As indicated by the reviewer, the region of 261-266 is a loop. The description was revised in lines 172 and 174, Figure 3C and Figure 3C legend.

For Figure 4A, the alignment of CASP7 was constructed by using Esprit (https://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi) with human CASP7 (PDB:1k86) as the template. The description was revised in the Figure legend.

> *(6) Were divergent sequences selected for the sequence alignment analyses (particularly in Figure 6A)? The selection of sequences can directly influence the outcome of the amino acid residues in each position, and using diverse sequences can reduce the impact of the number of sequences on the LOGO in each phylogenetic group.*

In Figure 6A, the sequences were selected without bias. For Mammalia, 45 CASP3 and 43 CASP7 were selected; for Aves, 41 CASP3 and 52 CASP7 were selected; for Reptilia, 31CASP3 and 39 CASP7 were selected; for Amphibia, 11 CASP3 and 12 CASP7 were selected; for Osteichthyes, 40 CASP3 and 43 CASP7 were selected. The sequence information was shown in Table 1 and Table 2.

> *(7) For clarity, it would help if the authors provided additional rationale for the selection of residues for mutagenesis, such as selecting Q276, D278, and H283 as exosite residues, when the CASP7 PDB structures (4jr2, 3ibf, and 1k86) suggest that these residues are enriched with loop elements rather than the β sheets expected to facilitate substrate recognition in exosites for caspases (PMID: 32109412). It is possible that the inability to form β-sheets around these positions might indicate the absence of an exosite in CASP7, which further supports the functional effect of the exosite mutations performed.*

According to the suggestion, the rationale for the selection of residues for mutagenesis was added (lines 216-222). Unlike the exosite in HsCASP1/4, which is located in a β sheet, the Q276, D278, and H283 of HsCASP7 are located in a loop region (Figure 5-figure supplement 2),

which may explain the mutation results and the absence of an exosite in HsCASP7 as suggested by the reviewer.

> ***Reviewer #2 (Public Review):***
>
> *The authors wanted to address the differential processing of GSDME by caspase 3 and 7, finding that while in humans GSDME is only processed by CASP3, Takifugu GSDME, and other mammalian can be processed by CASP3 and 7. This is due to a change in a residue in the human CAPS7 active site that abrogates GSDME cleavage. This phenomenon is present in humans and other primates, but not in other mammals such as cats or rodents. This study sheds light on the evolutionary changes inside CASP7, using sequences from different species. Although the study is somehow interesting and elegantly provides strong evidence of this observation, it lacks the physiological relevance of this finding, i.e. on human side, mouse side, and fish what are the consequences of CASP3/7 vs CASP3 cleavage of GSDME.*

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> *Fish also present a duplication of GSDME gene and Takifugu present GSDMEa and GSDMEb. It is not clear in the whole study if when referring to TrGSDME is the a or b. This should be stated in the text and discussed in the differential function of both GSDME in fish physiology (i.e. PMIDs: 34252476, 32111733 or 36685536).*

The TrGSDME used in this study belongs to the GSDMEa lineage of teleost GSDME. The relevant information was added. Figure 1-figure supplement 1 and lines 119, 271, 274-276, 287 and 288.

> ***Recommendations for the authors:***
>
> ***Reviewer #1 (Recommendations For The Authors):***
>
> *(1) For the chimeric and truncated constructs, such as HsNT-TrCT, TrNT-HsCT, Hsp20-Trp10, Trp20-Hsp10, etc., the authors should provide a table denoting which amino acids were taken from each protein to create the fusion or truncation.*

According to the reviewer's suggestion, the information of the truncate/chimeric proteins was provided in Table 4.

> *(2) Both reviewers agree that functional physiological experiments are needed to increase the significance of the work. Specifically, the physiological relevance of these findings can be assessed by using western blotting to monitor GSDME cleavage by the human CASP7 S234N mutant compared with wild type CASP7 in response to triggers such as etoposide or VSV, which are known to induce CASP3 to cleave GSDME (PMID: 28045099).*

> *Additionally, the authors can assess cell death in HEK293 cells, HEK293 cells transfected with TrGSDME, HEK293 cells expressing TrCASP3/7 plus TrGSDME, and TrCASP3/7 plus the D255R/D258A mutant. These cells can be stimulated, and pyroptosis can be assessed by using ELISA to measure the release of the cytoplasmic enzyme LDH as well as IL-1β and IL-18, and the percentage of cell death (PI+ positive cells) may also be assessed.*

(1) With respect to the physiological relevance, please see the above reply to Reviewer 1's comment of "Specific recommendations for the authors, 1".

(2) As shown in our results (Fig. 2), co-expression of TrCASP3/7 and TrGSDME in HEK293T cells induced robust cell death without the need of any stimulation, as evidenced by LDH release and TrGSDME cleavage. In the revised manuscript, similar experiments were performed as suggested, and cell death was assessed by Sytox Green staining (Figure 2-figure supplement 3A and B) and immunoblot to detect the cleavage of both wild type and mutant TrGSDME (Figure 2-figure supplement 3C). The results confirmed the results of Figure 2.

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *Abstract:*
>
> *Although the authors try to summarize the principal results of this study, please rewrite the abstract section to make it easier to follow and to empathise the implications of their results.*

We have modified the Abstract as suggested by the reviewer.

> *Introduction:*
>
> *The authors do not mention anything about the implication of the inflammasome activation to get pyroptosis by GSDM cleave by inflammatory caspases. Please consider including this in the introduction section as they do in the discussion section.*

The introduction was modified according to the reviewer's suggestion. Lines 58-61.

> *From the results section the authors name the human GSDM as HsGSDM and the human CASP as HsCASP, maybe the author could use the same nomenclature in the introduction section. The same for the fish GSDM (Tr) and CASP.*

According to the reviewer's suggestion, the same nomenclature was used in the introduction.

> *Line 39. Remove the word necrotic.*

"necrotic" was removed .

> *Line 42. Change channels by pores. In the manuscript, change channels by pores overall.*

"channels" was replaced by "pores".

> *Line 42: Include that: by these pores can be released the proinflammatory cytokines and if these pores are not solved then pyroptosis occurs. Please rephrase this statement.*

According to the reviewer's suggestion, the sentence was rephrased. Lines 46-48.

> *Line 45. GSDMF is not an approved gene name, its official nomenclature is PJVK (Uniprot Q0ZLH3). Please use PJVK instead GSDMF.*

GSDMF was changed to PJVK.

> *Line 103: Can the authors explain better the molecular determinant?*

The sentence was revised, line 109.

> *Results:*
>
> *Line 110: Reference for this statement. The reference for this statement was added in line 116.*
>
> *Figure 1A, B: Concentration or units used of HsCASP?*

The unit (1 U) of HsCASPs was added to the figure legend (line 661).

> *Line 113: Add Hs or Tr after CASP would be helpful to follow the story.*

“CASP” was changed to “HsCASP”.

> *Fig 1D: Why the authors do not use the DMPD tetrapeptide (HsGSDME CASP3 cut site) in this assay? Comparing with the data obtained in Fig 3B the TrCASP3 activity is going to be very closer to that obtained for VEID o VDQQD in the CASP3 panel.*

The purpose of Figure 1D was to determine the cleavage preference of TrCASPs. For this purpose, a series of commercially available CASP substrates were used, including DEVD, which is commonly used as a testing substrate for CASP3. Figure 3B was to compare the cleavage of HsCASP3/7 and TrCASP3/7 specifically against the motifs from TrGSDME (DAVD) and HsGSDME (DMPD).

Figure 1D and Figure 3B are different experiments and were performed under different conditions. In Figure 1D, CASP3 was incubated with the commercial substrates at 37 ℃ for 2 h, while in Figure 3B, CASP3/7 were incubated with non-commercial DAVD (motif from TrGSDME) and DMPD (motif from HsGSDME) at 37 ℃ for 30 min. More experimental details were added to Materials and Methods, lines 443 and 447.

> *Fig 1H: What is the concentration used of the inhibitors?*

The concentration (20 μM) was added to the figure legend (line 669).

> *Does the Hs CASP3/7 fail to cleave the TrGSDME mutants (D255R and D258A)? the authors do not show this result so they cannot assume that HsCASP3/7 cleave that sequence (although this is to be expected).*

The result of HsCASP3/7 cleavage of the TrGSDME mutants was added as Figure 1-figure supplement 2 and described in Results, line 133.

> *Line 132-133: Can the author specify where is placed the mCherry tag? In the N terminal or C terminal portion of the different engineered proteins?*

The mCherry tag is attached to the C-terminus. Figure 2 legend (line 676).

> *Fig 2A: Although is quite clear, a column histogram showing the quantification is going to be helpful.*

The expression of TrGSDME-FL, -NT and -CT was determined by Western blot, and the result was added as Figure 2-figure supplement 1.

> *Fig 2A, B, C: After how many hours of expression are the pictures taken? Can the authors show a Western blot showing that the expression of the different constructions is similar?*

The time was added to Figure 2 legend and Materials and Methods (line 466). The expression of TrGSDME-FL, -NT and -CT was determined by Western blot, and the result was added as Figure 2-figure supplement 1.

> *Fig 2C: Another helpful assay can be to measure the YO-PRO or another small dye internalization, to complete the LDH data.*

According the reviewer's suggestion, in addition to LDH release, Sytox Green was also used to detect cell death. The result was added as Figure 2-figure supplement 2 and described in Results, line 146.

> *Fig 2C: In the figure y axe change LHD by LDH.*

The word was corrected.

> *Fig 2D: Change HKE293T by HEK293T in the caption.*

The word was corrected.

> *Fig 2G: Please add the concentration used with the two plasmids co-transfection. A Western blot showing CASP3/7 expression vs TrGSDME is missing. Is that assay after 24h? please specify better the methodology.*

The concentration of plasmid used in co-transfection and the time post transfection were added to the Materials and Methods (lines 422 and 424). In addition, the expression of CASP3/7 was added to Figure 2I.

> *Fig 2 J, K: Change HKE293T by HEK293T in the figure caption. The concentration of the caspase inhibitors is missing. Depending on the concentration used, these inhibitors used could provoke toxicity on the cells by themselves.*

The word was corrected in the figure caption. The inhibitor concentration (10 μM) was added to the figure legend (line 690).

> *Line 151: TrCASP3/7 instead of CASP3/7*

CASP3/7 was changed to TrCASP3/7.

> *Fig 3A, 3B: Please add the units used of the HsCASP*

The unit was added to the figure legends (lines 697).

> *Fig 3A: Can the authors add the SDS-PAGE to see the Nt terminal portion as has been done in Fig 1A? Maybe in a supplementary figure.*

The SDS-PAGE was added as Figure 3-figure supplement 1.

> *Fig 3B: If the authors could add some data about the caspase activity using any other CASP such as CASP2, CASP1 to compare the activity data with CASP3 and CASP7 would be helpful.*

The proteolytic activity of TrCASP1 was provided as Figure 3-figure supplement 2.

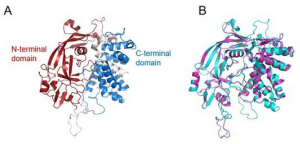
> *Fig 3C: To state this (Line 160), the authors should use another prediction software to reach a consensus with the sequences of the first analysis. In fact, what happens when GSDME is modelled 3-dimensionally by comparing it to crystalized structures such as mouse GSDMA? If the authors add an arrow indicating where the Nt terminal portion ends and where Ct portion begins would make the figure clearer.*

According to the suggestions of both reviewers, in the revised manuscript, we used mouse GSDMA3 (PDB: 5b5r) for the structural analysis of HsGSDME, which showed that the 261-266 region of HsGSDME was a loop. As a result, Figure 3C was revised. Relevant change in Results: lines 172 and 174.

As suggested by the reviewer, we modelled the three-dimensional structure of HsGSDME by using SWISS-MODEL with mouse GSDMA3 as the template (Author response image 2, below).

Author response image 2.

The three-dimensional structure model of HsGSDME. (A) The structure of HsGSDME was modeled by using mouse GSDMA3 (MmGSDMA3) as the template. The N-terminal domain (1-246 aa) and the C-terminal domain (279-468 aa) of HsGSDME are shown in red and blue, respectively. (B) The superposed structure of HsGSDME (cyan) and MmGSDMA3 (purple).



> *Fig 3F: if this is an immunoblotting why NT can be seen? In other Western blots only the CT is detected, why? The use of the TrGSDME mouse polyclonal needs more details (is a purify Ab, was produced for this study, what are the dilution used...)*

Since the anti-TrGSDME antibody was generated using the full-length TrGSDME, it reacted with both the N-terminal and the C-terminal fragments of TrGSDME in Figure 3F. In Figure 3G, the GSDME chimera contained only TrGSDME-CT, so only the CT fragment was detected by anti-TrGSDME antibody. More information on antibody preparation and immunoblot was added to “Materials and Methods” (lines 390 and 391).

> *Fig 4B: Can the authors show in which amino acid the p20 finish for each CASP? (Similarly, as they have done in panel 3E)*

Fig 4B was revised as suggested.

> *Fig 5F: With 4 units of WT CASP7 the authors show a HsGSDME Ct in the same proportion than when the S234N mutant is used (at lower concentrations). How do the authors explain this?*

The result showed that the cleavage by 4U of HsCASP7 was comparable to the cleavage by 0.25U of HsCASP7-S234N, indicating that S234 mutation increased the cleavage ability of HsCASP7 by 16 folds.

> *Line 203: Can the authors show an alignment between this region of casp1/4 and 7? Maybe in supplementary figures.*

As reported by Wang et. al (PMID: 32109412), the βIII/βIII' sheet of CASP1/4 forms the exosite critical for GSDMD recognition. The structural comparison among HsCASP1/4/7 and the sequence alignment of HsCASP1/4 βIII/βIII' region with its corresponding region in HsCASP7 were added as Figure 5-figure supplement 2.

> *Line 205: A mutation including S234N with the exosite mutations (S234+Q276W+D278E+H283S) is required to support this statement.*

The sentence of "suggesting that, unlike human GSDMD, HsGSDME cleavage by CASPs probably did not involve exosite interaction" was deleted in the revised manuscript.

> *Fig 5I, 5J: which is the amount of HsGSDME and TrGSDME? I would place these figures in supplementary material.*

The protein expression of TrGSDME/HsGSDME was shown in the figure. Fig 5I and 5J were moved to Figure 5-figure supplement 3.

> *Line 218: I would specify that this importance is in HUMAN CASP7 to cleavage Human GSDME.*

"CASP7" and "GSDME" were changed to "HsCASP7" and "HsGSDME", respectively.

> *Fig 6C: 4 units is the amount of S234N mutant needed to see an optimal HsGSDME cleavage in Fig 5F.*

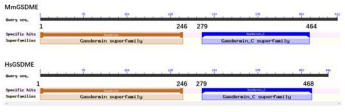
In Figure 6C, the cleavage efficacy of HsCASP3-N208S was apparently decreased compared to that of HsCASP3, and 4U of HsCASP3-N208S was roughly equivalent to 1U of HsCASP3 in cleavage efficacy. In Figure 5F, cleavage by 4U of HsCASP7 was comparable to the cleavage by 0.25U of HsCASP7-S234N. Together, these results confirmed the critical role of S234/N208 in HsCASP3/7 cleavage of HsGSDM.

> *Fig 6I: Could be the fact that the mouse GSDME has a longer Ct than human GSDME affect the interaction with CASP7? Less accessible to the cut site? Needs a positive control of mouse GSDME with mouse Caspase 3.*

Although mouse GSDME (MmGSDME) (512 aa) is larger than HsGSDME (496 aa), the length of the C-terminal domain of MmGSDME (186 aa) is comparable to that of HsGSDME (190 aa).

Author response image 3.

Conserved domain analysis of mouse (upper) and human (lower) GSDME.



As suggested by the reviewer, the cleavage of MmGSDME by mouse caspase-3 (MmCASP3) was added as Figure 6-figure supplement 2 and described in Results, lines 258.

> *Material and Methods:*
>
> *-Overall, concentrations or amounts used in this study regarding the active enzyme or plasmids used are missing and need to be added.*

The missing concentrations of the enzymes and plasmids were added in Material and Methods (lines 421, 453, 457, and 470) or figure legends (Figure 1 and 3).

> *-It would be helpful if the authors label in the immunoblotting panels what is the GSDME that they are using. (Hs GSDME FL...).*

As suggested, the labels were added to Figures 1A ,1B, and 3.

> *-Add the units of enzyme used.*

The units of enzyme were added to figure legends (Figure 1A, 3A, 3D, and 3F) or Material and Methods (lines 453 and 457).

> *The GSDME sequence obtained for Takifugu after amplification of the RNA extracted should be shown and specified (GSDMEa or GSDMEb). From which tissue was the RNA extracted?*

The details were added to Materials and Methods (lines 398 and 402).