Gasdermin and Gasdermin-Like Pore-Forming Proteins in Invertebrates, Fungi and Bacteria

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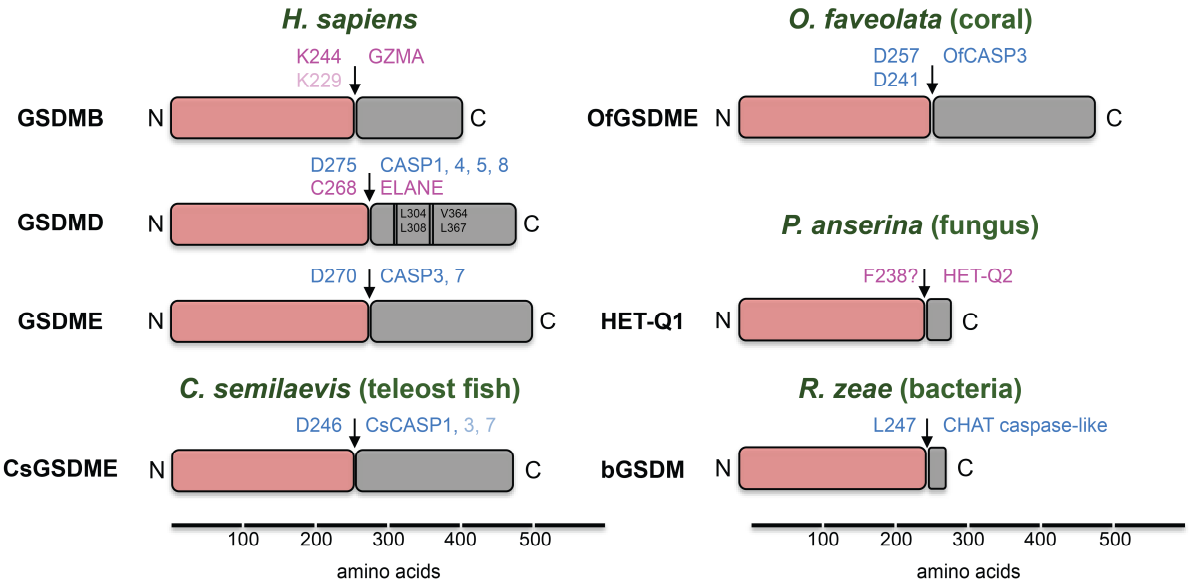
Graphical Abstract



Research highlights

- Gasdermin pore-formation as a key mechanism for cellular suicide
- Gasdermins in early diverged vertebrates and corals
- Fungal gasdermins and their regulation
- Gasdermins control cell death in Bacteria

Abstract

The gasdermin family of pore-forming proteins (PFPs) has recently emerged as key molecular players controlling immune-related cell death in mammals. Characterized mammalian gasdermins are activated through proteolytic cleavage by caspases or serine proteases, which remove an inhibitory carboxy-terminal domain, allowing the pore-formation process. Processed gasdermins form transmembrane pores permeabilizing the plasma membrane, which often results in lytic and inflammatory cell death. While the gasdermin-dependent cell death (*pyroptosis*) has been predominantly characterized in mammals, it now has become clear that gasdermins also control cell death in early vertebrates (teleost fish) and invertebrate animals such as corals (Cnidaria). Moreover, gasdermins and gasdermin-like proteins have been identified and characterized in taxa outside of animals, notably Fungi and Bacteria. Fungal and bacterial gasdermins share many features with mammalian gasdermins including their mode of activation through proteolysis. It has been shown that in some cases the proteolytic activation is executed by evolutionarily related proteases acting downstream of proteins resembling immune receptors controlling necroptosis in mammals. Overall, these findings establish gasdermins and gasdermin-regulated cell death as an extremely ancient mechanism of cellular suicide and build towards an understanding of the evolution of regulated cell death in the context of immunology. Here, we review the broader gasdermin family, focusing on recent discoveries in invertebrates, fungi and bacteria.

Introduction

Pore-formation is a general principle of membrane permeabilization integral to a variety of biological processes (i.e. immunity, virulence, metabolism) and performed by pore-forming proteins (PFPs) or toxins [1,2]. PFPs are ubiquitous throughout the tree of life and with diverse origins [1]. PFPs can be classified based on the secondary structure that the proteins use to permeabilize the lipid bilayer upon pore-formation [3,4]. The α-PFPs use α-helices for membrane insertion, while β-PFPs use β-sheets [5,6]. Although structurally and phylogenetically diverse, PFPs share the ability to transit from a soluble state to a transmembrane and oligomeric state upon activation. Bacteria secrete soluble PFPs that kill other bacterial cells or that are used to invade and colonize a host [7,8]. These PFPs represents virulence factors, which attack the outer layer of the cellular membrane. Eukaryotes (i.e. cnidarians and arachnids) produce PFPs that are integral to their venomous toolkit and are used to attack prey or repel aggressors in a defense reaction [9,10]. Actinoporins are an example of PFPs, and are found in the venom of a variety of sea anemones [11,12]. Mammals have also integrated pore-forming proteins into their immune systems [2,13]. Some mammalian PFPs can target directly the membranes of bacterial or viral pathogens, acting in the extracellular environment of the host [14], or targeting the outer membrane of phagocytosed Gram-negative bacteria [15]. The molecularly characterized PFPs in this category (i.e. *complement* members (C6-C9) [16] and perforin [17,18]), belong to the *membrane attack complex/perforin* (MACPF) superfamily [19]. The MACPF protein superfamily also includes the cholesterol-dependent cytolysins (CDCs) family [20] and pleurotolysine members [21] and is of ancient evolutionary origin with proteins found in eukaryotic, bacterial and archaeal lineages [19,22].

While MACPF members in mammals target cellular surfaces recognized as 'non self' (including damaged host cells), the mammalian immune system equally relies on PFPs integral to regulated cell death (RCD) programs – forms of cellular suicide – protecting the organism by eliminating infected and potentially harmful cells [2,23,24]. One such program of cellular suicide is the *intrinsic apoptotic pathway*, which relies on the pore-forming BAX and BAK proteins from the BCL-2 family

[25,26]. The BCL-2 family consist of ~20 members in mammals, which share a highly similar α-helical fold [27,28]. Activated BAX/BAK proteins oligomerize into the mitochondrial outer membrane [29,30], which disrupts its integrity and leads to release of cytochrome c, triggering downstream activation of pro-apoptotic caspases (cysteine proteases) [31]. Similar to the MACPF superfamily, the BCL-2 family is of ancient evolutionary origin with members identified in early diverging metazoan phyla such as arthropods, mollusks and sponges [32,33].

In mammals, two other RCD pathways lead to permeabilization of the plasma membrane, resulting in inflammatory, immune-related forms of cell death, termed *necroptosis* and *pyroptosis* [2,34–36]. Necroptotic cell death is dependent on permeabilization of the plasma membrane by the MLKL protein (mixed lineage kinase domain-like) [37–39]. MLKL oligomerizes upon phosphorylation, which activates the N-terminal four-helical bundle (4HB) domain of the protein, carrying the cytotoxic activity, and inserts into the lipid bilayer of the plasma membrane [40,41]. The necroptotic pathway appears poorly conserved in Deuterostomia and is not present in Cnidaria [42]. However, MLKL homologs are present in most animal lineages and some viruses, which use decoy MLKL homologs to subvert the necroptotic cell death pathway and evade the mammalian immune system [43]. Furthermore, the pore-forming 4HB domain of MLKL appears homologous to immunity-related, membrane-targeting, cell death-inducing domains in fungi (HeLo and HELL domains) and in plants (RPW8-like coiled coil (CC) domain) [44–47]. The later findings underscore the broad phylogenetic distribution of this pore-forming protein domain.

In this review, we focus on the recently discovered cell death effectors of pyroptosis – the *gasdermin* family of PFPs – and detail the latest findings of gasdermin and gasdermin-like proteins characterized in organisms other than mammals (class Mammalia). Specifically, we will review molecularly characterized gasdermin proteins from invertebrates (Cnidaria), Fungi and Bacteria.

Gasdermins – effectors of pyroptotic cell death in mammals

Pyroptosis is a lytic, pro-inflammatory form of cell death in mammals [34,48]. Gasdermins are a family of pore-forming proteins that play an essential role in pyroptosis by permeabilizing the plasma membrane during the cell death reaction

[49–51]. In humans, there are six gasdermin homologs (GSDMA, GSDMB, GSDMC, GSDMD, GSDME and PJVK – also known as DFNB59), while in mice the gasdermin family comprises 10 members with three GSDMA paralogs (GSDMA1-3), four GSDMC paralogs (GSDMC1-4) but no GSDMB homolog [49]. The gasdermin proteins are differentially expressed in various types of mammalian cells, although they share similar domain organization and a common mechanism of activation. Characterized mammalian gasdermins are two-domain proteins with a pore-forming N-terminal domain and a C-terminal repressor domain, which is removed by proteolytic cleavage to induce pyroptosis [49]. The C-terminal domain of GSDMD has been shown to play a crucial role in the recruitment of active caspases, which bind to it with high affinity [52,53]. Thus, the C-terminal domain of GSDMD is not only essential for inhibition of the cytotoxic activity of the protein but is also a caspase-recruitment module [52,53]. The proteolysis of GSDMD by pro-inflammatory caspase-1 (or -4, -5 (human) and -11 (mouse)) results in a 31-kDa N-terminal fragment (GSDMD-NT) and a 22-kDa C-terminal fragment (GSDMD-CT) [54–56]. GSDMD-NT interacts with negatively charged lipid heads on the inner side of the plasma membrane and oligomerizes to form transmembrane pores [57]. GSDMD pore-formation has been observed *in vitro* in the presence of liposomes and on hydrophilic mica surfaces [57–59] and recently the structure of the GSDMD pore has been obtained using cryo-EM (cryogenic electron microscopy) [60]. The GSDMD pore structure has a 33-mer toroidal pore, featuring a transmembrane β-barrel with inner diameter of ~22-nm (Fig. 1); each GSDMD monomer contributes with two β-hairpins (or four β-strand 'fingers' or 'blades' per monomer) [60]. GSDMD monomers also have a cytosolic globular domain, in addition to the domain (region) forming the transmembrane β-hairpins. The globular domains of the GSDMD molecules form a 40-nm high rim around the pore facing the inside of the cell, thus curving the plasma membrane around the pore towards the extracellular space [60]. GSDMD pore assemblies can have different symmetries – ranging from 31-fold to 34-fold symmetry – and are thus between 10-20% larger than previously characterized 27-fold symmetry GSDMA3 pores (Fig. 1) [61] and even larger than the estimated size of human GSDMB pores, averaging between 13.5-nm and 15.6-nm in pore diameter [62]. GSDMD pore-formation dynamics have been observed with time-lapse atomic force microscopy (AFM),

organizing in time-dependent manner from rearrangements of slit-, arc- and ring-shaped structures that are already inserted in the lipid bilayer [63].

Not all gasdermins are activated by inflammatory caspases. GSDME (DFNA5) is proteolytically processed by the pro-apoptotic caspase-3 (Fig. 2), to induce secondary necrotic cell death in cells undergoing apoptosis [64,65]. Caspase-3-mediated and GSDME-dependent pyroptotic cell death is proposed to play an important role in chemotherapeutic cancer treatments and is responsible for undesired secondary effects [65]. Recently, GSDMC has been identified as a substrate for the 'pro-apoptotic' caspase-8 [66]. CASP8 has been shown to cleave GSDMD in response to *Yersinia* (bacterial) effector protein YopJ, triggering pyroptosis in macrophages [67,68]. In neutrophils the response to *Yersinia* leads to pyroptosis through GSDME cleavage, which is CASP3-dependent [69]. While all three of these gasdermins (GSDMD, GSDME and GSDMC) are caspase substrates, another human gasdermin (GDSMB) has been identified as a substrate of a subtilisin-like serine protease named Granzyme A (GZMA) (Fig. 2) [70]. The protease is delivered into damaged or infected cells by natural killer (NK) lymphocytes, triggering GSDMB-dependent pyroptosis [70]. Interestingly, a different serine protease (ELANE) has been found to cleave GSDMD seven amino acid residues upstream of D275 (the identified caspase cleavage site) to induce lytic cell death in aging neutrophils [71]. Remarkably, some mammalian gasdermins have also been reported as targets of viral proteases [72,73]. For example, human GSDMD can be proteolytically processed after amino acid Q193 by a C3 protease from the Enterovirus 71 (EV71), preventing pyroptosis and evading antiviral immunity [72]. Another cysteine protease of the same family, $C3^{Pro}$ from Seneca Valley virus (SVV), cleaves porcine GSDMD at residue Q277, generating a pyroptosis-inducing pGSDMD(1-277) fragment, which likely plays an important role in viral pathogenesis [73].

Gasdermin proteins in early diverging vertebrates and corals

The gasdermin family of PFPs has mostly been studied in vertebrates and specifically mammals. Phylogenetically, GSDME and PJVK are the two most ancient gasdermin genes in metazoans, found in early diverging vertebrate species (teleost fish) [74] and some invertebrates like corals [49,75]. Three of the gasdermins

(GSDMB, GSDMC, GSDMD) are exclusively found in mammals, while GSDMA is more ancient and is present in birds and reptiles [49]. The recent functional characterization of CsGSDME – a GSDME homolog from the tongue sole *Cynoglossus semilaevis* – has revealed that the gasdermin protein can induce pyroptotic cell death in human HEK293T cells, suggesting that CsGSDME is a pyroptosis executioner in this early group of vertebrates [74]. The N-terminal domain of CsGSDME is essential for the cell death reaction and exhibits bactericidal properties. Unlike the mammalian GSDME, whose activation depends on caspase-3 (CASP3), *C. semilaevis* GSDME is cleaved with highest affinity by CsCASP1 (caspase-1) and much less efficiently by caspase-3 and -7 (Fig. 2) [74]. GSDME homologs are also encoded in the genomes of some invertebrates, notably mollusks (*Lingula anatina* and *Pomacea canaliculata*), sea anemone (*Exaiptasia pallida*) and corals [75]. Jiang *et al*. recently characterized a gasdermin E homolog from the reef-building coral *Orbicella faveolata* [75]. The authors demonstrated that OfGSDME can be proteolytically processed *in vitro* by human capspase-3 and -7 and coral OfCASP3 can induce pyroptotic cell death in human HEK293T cells [75,76]. Similar to previously characterized gasdermin proteins, the cytotoxic activity of OfGSDME has been found to reside in its N-terminal pore-forming domain. In addition, Jiang *et al.* demonstrate that gasdermin E mediates pyroptosis *in vivo* in the coral species *Pocillopora damicornis* in response to bacterial infections. Polyps of *P. damicornis* undergo rapid necrotic cell death reaction when infected with *V. coralliilyticus* bacteria, during which PdGSDME is proteolytically processed in CASP3-dependent manner. These findings show that pyroptosis is an ancient mechanism controlling immune-related cell death in invertebrates and that some pyroptosis-inducing pathways are broadly conserved in divergent metazoan taxa [76].

Gasdermin-like proteins in fungi

Proteins belonging to the gasdermin family have also been identified in another major eukaryotic kingdom – Fungi – in the context of allorecognition-induced cell death [77,78]. Allorecognition (or conspecific non-self discrimination) occurs between genetically distinct fungal individuals (strains) and can manifest through localized regulated cellular death (RCD) of newly formed heterokaryotic

cells, resulting in abortive somatic fusions that prevent cytoplasmic mixing [77,79–81]. Allorecognition-mediated cell death has been shown to serve as a defense mechanism against genome exploitation [82], resource plundering [83] and the spread of cytoplasmic deleterious elements (i.e. mycoviruses and senescence plasmids) [84–86]. The fungal gasdermins have been identified and characterized as cell death executioner proteins in the context of allorecognition in two model ascomycete species, *Neurospora crassa* [78,87] and *Podospora anserina* [88]. Fungal gasdermin-encoding genes are widespread and abundant in the fungal kingdom, especially in the Ascomycota phylum [87,88].

The gasdermin-like protein RCD-1 regulates cell death in the filamentous ascomycete Neurospora crassa

The first identified fungal gasdermin, encoded in the genome of the model ascomycete *N. crassa,* has been named *rcd-1* (*regulator of cell death-1*) [78,87]. While the majority of identified *N. crassa* incompatibility genes appear to be inactive in the earliest developmental stages of somatic growth (spores or *conidia*) [89,90], the co-expression of two different *rcd-1* alleles (*rcd-1-1 and rcd-1-2*) triggers rapid (~20 min post-fusion) lytic cell death in germinating conidia (germlings) and in hyphae [87]. Germling-regulated death (GRD) occurs when conidia of the *rcd-1-1* genotype undergo somatic cell fusion with an *rcd-1-2* strain, resulting in progressively increasing vacuolization and subsequent release of cytoplasmic contents. The uptake of vital dyes (propidium iodide) by *N. crassa* germlings undergoing GRD suggested that plasma membrane integrity is compromised before cytoplasmic leakage [87]. Alleles at the *rcd-1* locus are highly polymorphic, fall into two haplogroups and are associated with genomic re-arrangements in genome analyses of a wild population of *N. crassa*. The two antagonistic alleles (*rcd-1-1* and *rcd-1-2*) show molecular marks of evolution, notably long-term balancing selection and trans-species polymorphism, typically associated with allorecognition genes in fungi [91,92] and immune-related genes in animals [93–95] and plants [96].

The *rcd-1-1* and *rcd-1-2* alleles encode proteins of 257 and 244 amino acids, respectively, and exhibit a low level of sequence identity (38%) when aligned with each other [87]. The homology between the two RCD-1 variants and the mammalian

gasdermins was initially detected in *silico* using primary sequence alignments combined with secondary structure predictions [78]. The fungal gasdermins are evolutionary related to the pore-forming N-terminal domain of mammalian gasdermins and lack regions of similarity to the inhibitory C-terminal domain. Both allelic variants (RCD-1-1 and RCD-1-2) localize to the cell periphery and recombinant RCD-1 binds to negatively charged phospholipids *in vitro* [78]. The lipid-binding profile of fungal gasdermins and mammalian gasdermins are similar and consist of an affinity for cardiolipin (CL), phosphatidylserine (PS) and some phosphoinositides [59,78]. Liposomes containing CL and PS were lysed in presence of RCD-1. RCD-1 also shows a tendency to form oligomers of high molecular size *in vitro* and when observed by electron microscopy, RCD-1 oligomers formed irregularly sized cells resembling a honeycomb, suggesting that the fungal gasdermins might also form pore-like structures [78]. In accordance with these observations, GFP-labeled RCD-1 proteins formed large ordered fluorescent aggregates in *N. crassa* germlings undergoing GRD, indicating that the fungal gasdermins oligomerize *in vivo*. Importantly, co-expression of RCD-1-1 and RCD-1-2 in human 293T induced pyroptotic-like cell death and the appearance of balloon-shaped dead cells [78]. While the precise molecular details of the RCD-1-1/RCD-1-2 allorecognition system remain to be elucidated, it has been determined that the two allelic variants interact during the cell death reaction in the heterologous host (293T cells) [78]. These findings led to a proposed model in which the interaction between RCD-1-1 an RCD-1-2 leads to the formation of cytotoxic pore-like oligomers to permeabilize the plasma membrane and induce cell death. Overall, the molecular characterization of RCD-1 has established an evolutionary relationship between cell death execution modules in fungi and animals, showing that gasdermins operate as PFPs in Opisthokonta (the fungal and animal eukaryotic kingdoms).

HET-Q1 and the proteolytic regulation of the fungal gasdermins

In spite of the functional commonalities between mammalian and fungal gasdermins, it remained unclear what the precise mechanism controlling the cytotoxic activity of fungal gasdermins might be, especially considering that fungal gasdermins lack the inhibitory C-terminal domain found in mammalian gasdermins

and the apparent self-sufficiency of the RCD-1-1/RCD-1-2 cell death system in human 293T cells. A recent study by Clavé *et al.* has shed light on this important question by showing that some fungal gasdermins, similar to their mammalian counterparts, are regulated through proteolytic cleavage [88]. By investigating the genetic determinants of an allorecognition system from *Podospora anserina* (a model ascomycete fungus), the authors identified a fungal gasdermin – HET-Q1 – a 278 amino acid protein whose cytotoxicity is controlled by a subtilisin-like serine protease of the S8 family [97], named HET-Q2. The *het-Q1* and *het-Q2* genes are defined as idiomorphs [98]; different genes encoded in the same locus in the genomes of different strains, with strains being exclusively *het-Q1* or *het-Q2* genotypes. Similar to the GRD reaction in *N. crassa*, co-expression of HET-Q1 and HET-Q2 induced cell death of heterokaryotic cells. The cell death reaction occurred in mature hyphae and was observed via the appearance of a demarcation line (a 'barrage') separating the incompatible *het-Q1* and *het-Q2* strains. The gasdermin HET-Q1 is proteolytically processed during the cell death reaction in a HET-Q2-dependent manner with removal of a ~5-kDa C-terminal fragment [88]. Although the precise cleavage site was not identified, mutational analysis of HET-Q1 showed that two residues – L237 and F238 – abolished cleavage of HET-Q1 and also abolished cell death, while the introduction of a HET-Q1(1-233) truncation variant was sufficient to reduce cell viability. These findings support that the cytotoxicity of HET-Q1 is activated through proteolytic cleavage of an inhibitory C-terminal domain. The HET-Q1/HET-Q2 allorecognition reaction had also been successfully recapitulated in the yeast *Saccharomyces cerevisiae* [88], suggesting that the HET-Q1 gasdermin is the direct substrate of the HET-Q2 serine protease.

Remarkably, genome-mining analyses revealed that ~80% of gasdermin-encoding genes in fungi are situated in the vicinity (10-kbp upstream or downstream) of a protease-encoding *het-Q2*-like gene and often formed *het-Q1*/*het-Q2*-like two-gene clusters [88]. The *het-Q2*-like genes encode multidomain proteins that exhibit a diversity of domain architectures, in which the predicted protease domain was frequently associated with structural repeats like TPR (tetratricopeptide repeat) [99], ANK (ankyrin) [100] and WD40 repeats [101]. Such short tandem-repeated sequences (between 30-45 amino acids) have been documented to form protein domains mediating protein-protein interactions in various kingdoms of life

[102]. Two main types of protease domains have been found associated with the HET-Q2-like proteins: subtilisin-like serine proteases of the S8 family and surprisingly, caspase-like CHAT protease domains [88,103]. Furthermore, some HET-Q2-like protein architectures belonged to the NOD-like family of immune receptors (or NLRs), which have been shown to control pyroptotic cell death in mammals [104] and cell death in other eukaryotes, including plants and fungi [105–107]. Fungal NLR-encoding genes have been previously reported to cluster genomically and to control the cytotoxic activity of downstream effectors, some of which are pore-forming toxins [107–109]. Based on these findings, it has been proposed that the HET-Q2-like proteins represent molecular sensors controlling the cytotoxic activity by proteolytic cleavage of fungal gasdermins [88]. Thus, the *het-Q1*/*het-Q2*-like gene clusters represent molecular pathways controlling immune-related cell death outside of allorecognition and likely in response to heterospecific non-self. Overall, the findings establish an unprecedented evolutionary relationship between immune-related cell death pathways in the animal and fungal kingdoms.

In light of the discovered proteolytic regulation of the fungal gasdermin-like proteins, the *rcd-1-1*/*rcd-1-2* allorecognition system from *N. crassa*, where the co-expression of two different gasdermin alleles triggers cell death [78], is puzzling. Some fungal allorecognition cell death-inducing systems have emerged from pre-existing signaling pathways through selected mutations [110,111], as allorecognition has fitness benefits [79,80]. RCD-1-1 and RCD-1-2 are shorter than the majority of fungal gasdermins, with RCD-1-2 being only 244 amino acids in length and lacking an inhibitory C-terminal domain [78,87]. Such alterations combined with additional factors (mutations) could have led to the emergence of the *rcd-1* incompatibility system. A similar scenario has been proposed for the *het-Q1*/*het-Q2* incompatibility system in *P. anserina*, which could have emerged from a *het-Q1*/*het-Q2*-like gene cluster with the HET-Q2-like protein losing its regulatory, inhibitory domain [88] (Fig. 3). Additional experimental data would be needed regarding the molecular details of fungal gasdermin/protease interactions, recognition and cleavage, to support the latter hypothesis. Although gasdermin homologs are widespread in fungal genomes, their evolutionary history, mechanism of activation and function in allorecognition or immunity has not been fully explored, with the exception of the two incompatibility systems (*rcd-1-1*/*rcd-1-2* and *het-Q1*/*het-Q2*). Further

371 experiments on these two model systems, as well on other gasdermin homologs in
372 fungi, will be fruitful in defining the evolution and function of this enigmatic protein
373 family.
374
375 **Gasdermin-like proteins in Bacteria**
376
377 In addition to fungi, gasdermin-encoding genes have been identified in
378 prokaryotes, notably bacteria [78,87]. A recent study by Johnson *et al*. structurally
379 and functionally characterized bacterial gasdermins, which were encoded in anti-
380 phage defense islands in various bacterial genomes [112]. The authors identified
381 approximately 50 gasdermin sequences from bacterial species, which formed a
382 separate clade from the fungal and mammalian gasdermins. Yet, similar to the fungal
383 gasdermins, the bacterial gasdermin sequences lack an extended inhibitory C-
384 terminal domain. By obtaining crystal structures of two distantly related bacterial
385 gasdermins (sharing only 18% sequence identity), Johnson *et al*. show that in spite of
386 the extreme phylogenetic distance between the eukaryotic and prokaryotic taxa, the
387 N-terminal cytotoxic domain of the bacterial and mammalian gasdermins exhibited
388 remarkable structural homology in their inhibited state [112]. In both cases, a
389 twisted central anti-parallel β-sheet represents the core of the fold, decorated on the
390 periphery by connecting helices and strands, which showed also a good structural
391 overlap between the gasdermin families. The high-resolution molecular structures
392 also revealed the mechanism of auto-inhibition of the bacterial gasdermins, by
393 exposing the role of the short C-terminal fragment, which is positioned in the middle
394 of the twisted central β-sheet, likely stabilizing the inhibited state of the protein. In
395 addition, the structural analyses revealed that some bacterial gasdermins undergo
396 post-translational modification at their extreme N-terminal end, involving a
397 conserved cysteine residue (C3 or C4 in the sequences of the two crystalized
398 bacterial gasdermins). The authors determined that the cysteine sidechain is
399 palmitoylated and that this modification plays an important role in the stability of
400 the protein fold [112]. These findings thus elucidate the minimal requirements for
401 the inhibited inactive state of the bacterial gasdermins. Palmitoylation may also be
402 important in some fungal gasdermins, as they carry a conserved N-terminal cysteine
403 [112].

Genome mining analyses by Johnson *at al.* revealed that the bacterial gasdermin genes, as described for the fungal gasdermin genes, are genomically clustered with protease-encoding genes. Furthermore, several different types of putative protease domains were identified, including caspase-like domains of the CHAT (Pfam ID PF12770) and C14 (PF00656) families and subtilisin-like serine proteases. Some of the predicted protein architectures carrying protease domains, represent bacterial NLRs or NLR-like proteins [112]. Importantly, Johnson *et al*. demonstrate that co-expression of the bacterial gasdermins and their cognate proteases leads to bacterial growth arrest and membrane permeabilization. The cytotoxic activity of the bacterial gasdermins relies on the proteolytic cleavage of the C-terminal inhibitory fragment (Fig. 2), thus triggering the formation of highly ordered ring-like pores. *In vitro*, in presence of liposomes, the ring-like pores assembled into mesh-like supramolecular structures. Surprisingly, the pores formed by the bacterial gasdermins exhibited significantly larger inner diameter (200-300 Å) than the inner diameter of pores formed by the mammalian gasdermins (125-200 Å) [112]. In the future, atomic-level structural characterization of the bacterial pores would be needed to compare in detail the molecular mechanism of pore-formation between the bacterial, fungal and metazoan gasdermins.

Conclusion

The discovery of gasdermin-like pore-forming proteins outside of mammals and vertebrates indicates that the gasdermin family is of extremely old evolutionary origin and likely a core-component of innate immune systems in divergent eukaryotic and prokaryotic taxa. These findings are emerging in the context of the establishment of an elaborate anti-viral (phage) bacterial immune system [113] with several recent studies reporting evolutionary parallels between bacterial and mammalian anti-viral defense systems [114,115]. For example, proteins from the viperin family [114,116] or cyclic GMP-AMP synthase (cGAS) [115] produce antiviral nucleotide-derived metabolites in both bacteria and mammals. Furthermore, prokaryotic anti-viral immune systems rely on a large variety of enzymatic activities and yet to be characterized defense modules [117,118]. The discovery of gasdermins in bacteria – although their anti-phage defense activity is only hypothesized – suggests that some

PFPs are integral to a core of immune-related domains operating in eukaryotes and prokaryotes. In this context, it has recently been proposed that NLR-dependent amyloid-controlled cell death [109], which has been previously characterized in fungi and found to be evolutionary related to mammalian necroptosis [44], operates also in prokaryotes, notably multicellular bacteria [119]. However, additional experimental analyses are needed to determine if the membrane-targeting abilities of the bacterial cell death-inducing BELL domain [119] is analogous to the 4HB domain of the necroptosis executioner protein MLKL [40]. Taken together, these findings indicate that cellular suicide by pore-formation and membrane disruption is a widespread strategy of organismal defense, which can rely on evolutionary related cell death modules, like the gasdermin family, in eukaryotic and prokaryotic lineages. The conserved mode of regulation (proteolysis), which is executed by evolutionary related proteases under the control of homologous receptors, suggest that pyroptotic-like cell death pathways have played an important role throughout a significant part of natural history. Future research will explore the precise molecular cues and mechanisms that trigger gasdermin-dependent cell death in these diverse and distantly-related taxa (fungi and bacteria). Such endeavors will provide valuable details on the biological processes in which gasdermin-dependent pathways are involved, and will be a great contribution to the field of comparative immunology.

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Figures

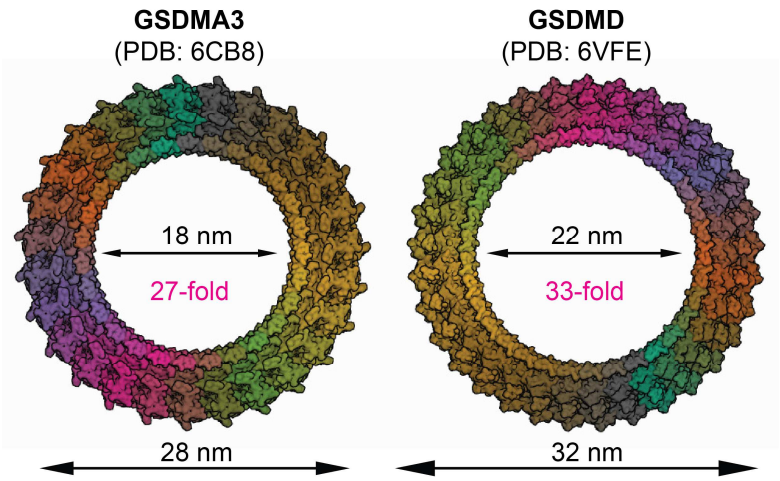

Figure 1. Structures of gasdermin pores formed by murine GSDMA3 (PDB: 6CB8) and human GSDMD (PDB: 6VFE) and obtained by cryo-EM [61]. Different gasdermin monomers are shown in different colors.

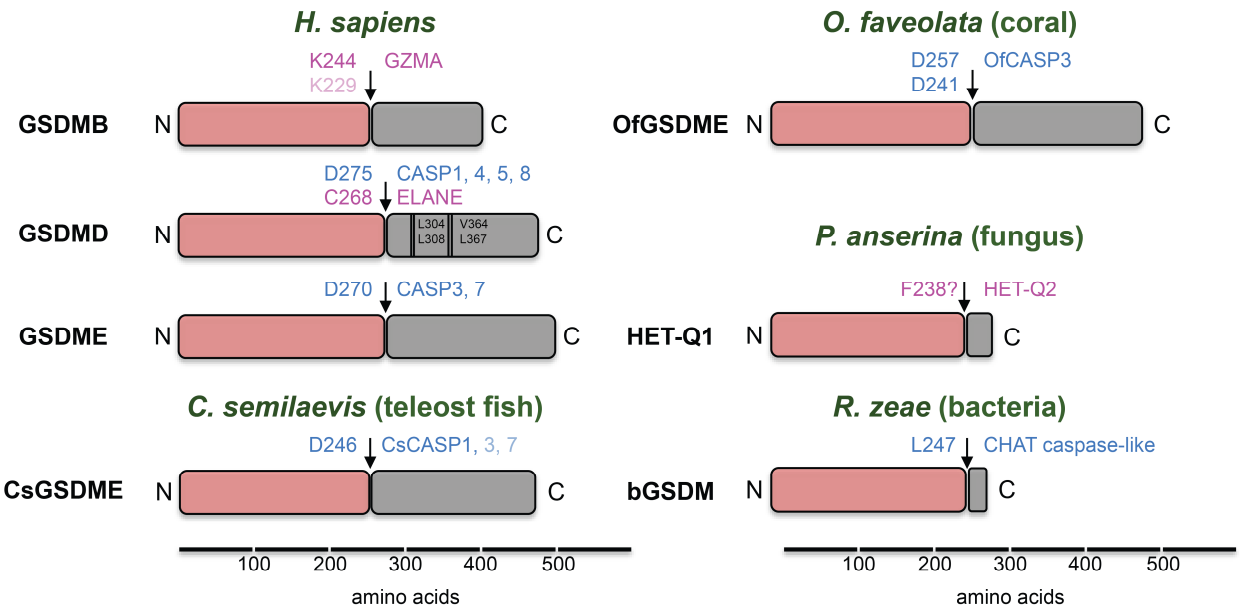

Figure 2. Shown are cartoons of gasdermin proteins from diverse phylogenetic taxa. The N-terminal pore-forming domains are colored in salmon pink. Cleavage sites are indicated with black arrows, flanked by the amino acid residues after which each

gasdermin is proteolytically processed and the proteases responsible for the cleavage. Caspases and caspase-like proteases are shown in dark blue, while serine proteases are shown in fuchsia. Color intensity indicates preferential cleavage sites and/or higher cleavage affinity by a protease. Shown are four key amino acid residues (L304, L308, V364, L367) on GSDMD C-terminal domain, which play a key role in recruitment of caspase-1 and other inflammatory caspases. *H. sapiens* – *Homo sapiens*, *C. semilaevis* – *Cynoglossus semilaevis*, *O. faveolata* – *Orbicella faveolata*, *P. anserina* – *Podospora anserina*, *R. zeae* – *Runella zeae*.

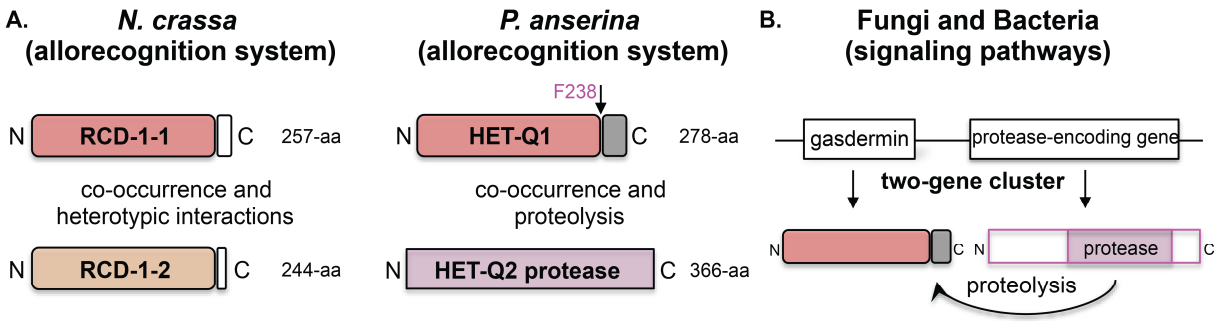

Figure 3. Gasdermin proteins are widespread in fungi and bacteria. A. Gasdermin-based allorecognition systems in fungi. Presently, two different fungal allorecognition systems have been identified in two model fungal organisms – *Neurospora crassa* and *Podospora anserina*. Cartoons represent the cell death-inducing incompatibility factors (RCD-1-1/RCD-1-2 and HET-Q1/HET-Q2), which are expressed by different fungal strains. The incompatibility factors occur in the same cell only when different fungal colonies undergo somatic fusion (co-occurrence). The RCD-1 allorecognition system involves the heterotypic interaction between the two antagonistic allelic variants RCD-1-1 and RCD-1-2. The two variants lack inhibitory C-terminal domains as indicated by white boxes. The gray box shows the inhibitory C-terminal domain of the HET-Q1 gasdermin. **B.** Gasdermin dependent signaling pathways have been uncovered in filamentous fungi and bacteria. The gasdermin-encoding genes are usually clustered with protease-encoding genes and represent functional units.