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Microbial gasdermins: More than a billion years of pyroptotic-like cell death

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ABSTRACT

In the recent past, the concept of immunity has been extended to eukaryotic and prokaryotic microorganisms, like fungi and bacteria. The latest findings have drawn remarkable evolutionary parallels between metazoan and microbial defense-related genes, unveiling a growing number of shared transkingdom components of immune systems. One such component is the gasdermin family of pore-forming proteins – executioners of a highly inflammatory immune cell death program in mammals, termed pyroptosis. Pyroptotic cell death limits the spread of intracellular pathogens by eliminating infected cells and coordinates the broader inflammatory response to infection. The microbial gasdermins have similarly been implicated in defense-related cell death reactions in fungi, bacteria and archaea. Moreover, the discovery of the molecular regulators of gasdermin cytotoxicity in fungi and bacteria, has established additional evolutionary links to mammalian pyroptotic pathways. Here, we focus on the gasdermin proteins in microorganisms and their role in organismal defense and provide perspective on this remarkable case study in comparative immunology.

1. Introduction

Microorganisms (bacteria, archaea, fungi, protozoa and algae) are ubiquitous on Earth, extremely abundant and diverse, with a mind-blowing upper estimate of a trillion species [1]. 'Engine of terrestrial biogeochemistry' [2], the ecological importance of microbes is difficult to overstate. Fungi and bacteria are among the foundational constituents of the soil microbiome, essential in the cycle of organic carbon and providing resources and services to macroorganisms, especially plants [2–5]. Microbes and host organisms co-evolve forming host-ecosystems or holobionts, redefining immunological self [6–8]. Humans are no exception and are heavily reliant on their microbiota for physiological health [9–11]. Naturally, microorganisms are associated with disease and often studied as pathogens. Yet, most microbes are engaged in competitive and antagonistic relations with other microbes [12,13] and locked into an evolutionary arms race with their own pathogens; bacteriophages in the case of archaea [14] and bacteria [15], or mycoviruses with fungi [16]. The study of these interactions has led to the discovery in fungi and bacteria of defense-dedicated molecular pathways, acting as immune systems [17–19]. The molecular characterization of microbial immune systems can help us understand better the population dynamics of microorganisms, the evolution of immune-related genes and provide potential therapeutic targets and novel strategies in the fight against pathogens.

Regulated cell death (RCD) programs play an important role in immune systems of animals [20], plants [21], fungi [22] and bacteria [23]. The cell death process eliminates damaged or infected cells of the host organism, maintaining homeostasis and limiting the spread of pathogens [24,25]. The RCD programs are thus 'altruistic cell suicide' programs, which operate in both multicellular and unicellular organisms. In unicellular bacteria and archaea, where cell suicide equates organismal death, the immune strategy procures an advantage at population level; by preventing the phage from completing its replication cycle, the dying infected cell protects close kin [26,27]. This anti-viral immune strategy has been named 'abortive infection' (Abi) (Fig. 1) [23]. Hundreds of different Abi-inducing cell death systems have been identified in the recent past, in various bacterial and archaeal genomes [28–30]. Most characterized Abi systems consist of a sensor protein and cell death executioner protein acting downstream of the sensor [23]. The functional units are encoded by adjacent genes, which are genomically clustered with other defense systems in regions termed 'defense islands' [17,31]. The characterization of Abi systems has established a growing

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number of functional analogies and uncovered conserved protein domains between bacterial and mammalian immune systems [32]. Such trans-kingdom evolutionary parallels have also emerged from the study of fungal RCD pathways [19]. Remarkably, these seemingly distant research axes have converged with the discovery of a protein family – the *gasdermins* – controlling immune-related cell death in fungi, bacteria and mammals.

Gasdermins (GSDMs) are a family of pore-forming proteins (PFPs), which have been extensively characterized as the executioners of a highly inflammatory cell death program in mammals, termed *pyroptosis* [33–35]. Pyroptosis is a host-directed cell suicide program, contributing to the clearance of invading pathogens and maintenance of homeostasis [36,37]. Mammalian GSDMs are activated by proteolytic cleavage, following the detection of damage-associated molecular patterns (DAMPs) or pathogen-derived cues (i.e. lipopolysaccharides (LPS), nigericin) [38–40]. The proteolysis removes the inhibitory C-terminal domain of the protein (GSDM-CT) liberating the pore-forming N-terminal domain (GSDM-NT), which oligomerizes puncturing the plasma membrane of the cell (Fig. 2) [41–44]. The GSDMs pores serve as a conduit for cytokines and other signaling molecules, which liberated in the extracellular space, coordinate a broader immune response and inflammation [45,46]. The loss of membrane integrity leads to a loss of osmotic pressure and the entry of water molecules inside the pyroptotic cell, resulting in cell swelling and lysis.

The six identified human GSDMs (GSDMA-GSDME and Pejvakin (PJVK)) are activated by a variety of different proteases in a context-dependent and gasdermin-specific manner [47]. The archetypal member of the family – GSDMD – can be processed by pro-inflammatory caspase-1, − 4, and − 5 in humans (and caspase-11 in mice) (Fig. 2) [39] or activated downstream of caspase-8 [48–50], which also activates GSDMC [51]. Caspase-3 cleaves GSDME [52]. The latter can also be a substrate for a serine protease named granzyme B (GzmB) [53], and granzyme A (GzmA), another member of the granzyme family [54,55], controls the activity of GSDMB [56]. GzmA and GzmB are delivered pre-activated into infected, malfunctioning or malignant cells via secreted lysosomes (cytotoxic granules) by a subset of cytotoxic lymphocytes [57]. Meanwhile, inflammatory caspases can be either directly activated by pathogen-derived molecular cues like LPS (caspases-4, −5, −11) [58,59] or through dedicated intracellular receptors, activating specifically caspase-1 [60]. In both cases, the pyroptotic reaction relies on the formation of large protein assemblies, termed *inflammasomes*, which lead to the downstream cleavage of GSDMs and pro-inflammatory

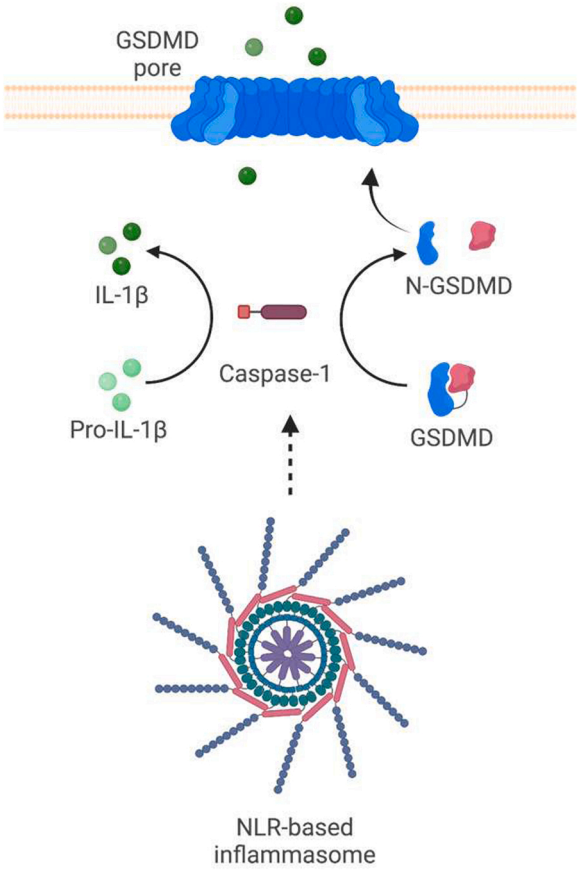

Fig. 2. Caspase-1-dependent activation of GSDMD is central to the canonical pyroptotic pathway in mammals. The activated protease separates the inhibitory and pore-forming domains of GSDMD and processes inflammatory cytokines like IL-1β. The N-terminal domain of GSDMD oligomerizes to form a transmembrane pore, through which the processed cytokines are released. Caspase-1 can be activated by a variety of signaling complexes (inflammasomes) formed by different molecular sensors. NLR-based inflammasomes can directly or indirectly activate caspase-1.

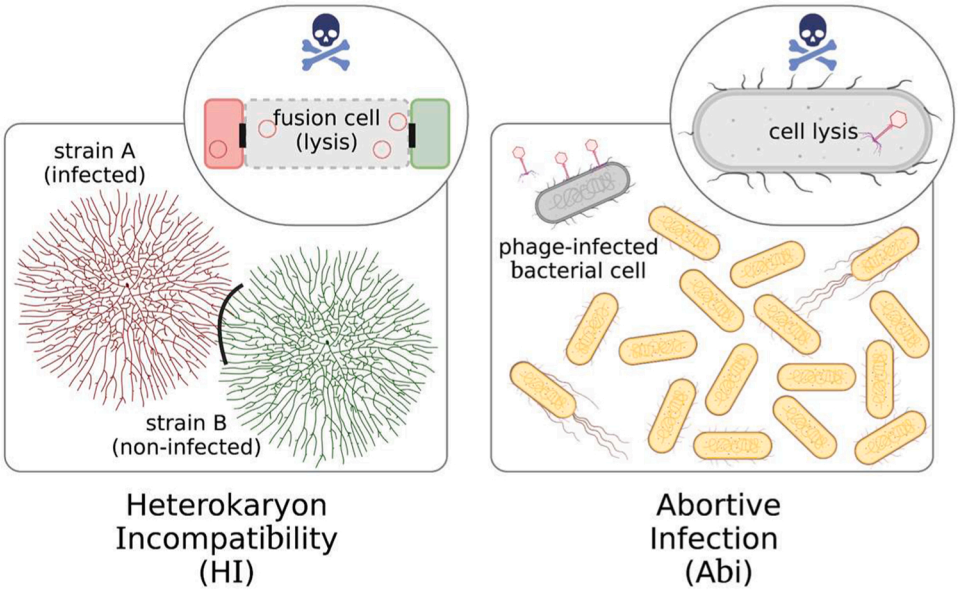

Fig. 1. Heterokaryon incompatibility (HI) and Abortive infection (Abi) are defense-related cell suicide strategies in fungi and bacteria, respectively. The regulated cell death reactions prevent the spread of mycoviruses in fungi and bacteriophages in bacteria. In fungi, HI occurs between genetically incompatible strains of the same species, leading to abortive cell fusion. The allorecognition reaction isolates the two fungal individuals (black line between the red and green strains), while the fusion cells undergo regulated cell death and lysis. The reaction prevents cytoplasmic mixing, which limits the horizontal transmission of mycoviruses (red circles on zoomed panel) and other cytoplasmically transmitted deleterious elements or genome exploitation. In bacteria, Abi acts as a defense reaction on population level. The lysis of bacteriophage-infected cells prevents the multiplication and spread of the phage, protecting the bacterial colony. Both, HI and Abi can be controlled by gasdermin homologs.

cytokines [61–64]. Some of the inflammasome-forming, intracellular receptors controlling the 'canonical pathway' of pyroptosis are multi-domain proteins termed NLRs (Nucleotide-binding site – Leucine-rich repeats or NBS-LRRs proteins, alternatively known as NOD-like receptors) [60,65]. The diverse pathways controlling the mammalian GSDMs and the growing number of roles for pyroptosis underscore the importance of these PFPs in mammalian immunity, and have been reviewed in great details recently [33,36,45,66].

On the other hand, the unexpected discovery of gasdermin-based immune systems in bacteria and fungi indicates that GSDMs and GSDM-mediated immunity are of extremely ancient evolutionary origin, playing a significant role in a variety of species across the three of life. Here, our focus is on the gasdermin family outside of mammals and specifically the involvement of GSDMs in microbial defense systems. First, we briefly review the growing number of experimentally characterized GSDMs in non-mammalian animals, notably fish and corals. Before our attention is turned to an in-depth overview of fungal and bacterial GSDMs. We summarize the current knowledge regarding their biological roles, phylogenetic distribution, mode of regulation and structural features. Opposing microbial GSDMs to mammalian GSDMs, we reflect on the similarities and differences between these distant members of the gasdermin family and conclude by discussing the extremely old origins of GSDMs pore-formation, speculating on the diverse roles it might play in microorganisms.

2. Gasdermin proteins in fish, corals and mollusks

Recent evolutionary analyses of GSDMs in animals (Metazoa) reveal that family members are present in vertebrate and invertebrate species and that GSDM genes have undergone many lineage-specific duplications and deletions [47,67,68]. The evolutionary history of GSDMs in animals reveals that the gene family descends from an ancestral GSDME gene [47]. GSDME genes are conserved throughout major vertebrate phyla, including birds, reptiles, amphibians and fish. Moreover, GSDME-like homologs have been found in invertebrates (also named GSDM*in* for invertebrates), notably corals (Cnidaria) and mollusks (Mollusca) [67,68]. PJVK has emerged as an early duplication of an ancestral GSDME gene (450–500 million years ago (mya)), loosing however its pore-forming ability [47,69]. A second duplication of an ancestral GSDME has occurred ~320 mya, resulting in the ancestor of the human GSDMA gene. GSDMA homologs have been identified in birds and reptiles but not in fish lineages and more recent duplications of GSDMA appear to have resulted in the mammalian specific GSDMB, GSDMC and GSDMD homologs (Fig. 3) [67]. Intriguingly, some large metazoan clades (Ecdysozoa) – arthropods and nematodes – appear to lack identifiable GSDM genes. However, ancestral GSDM genes have been likely lost during the early evolutionary history of Ecdysozoa, as suggested by the discovery of GSDMs in other ancient metazoan lineages like Cnidaria (corals, sea anemones) and Echinodermata (sea urchins) [67,68]. A GSDM homolog is already present in the genome of *Trichoplax adhaerens*, a species from Placozoa, which is one of the most basal eumetazoan clades [68,70,71]. Below, we review the handful of non-mammalian GSDMs, which have been experimentally characterized (Table 1).

A recent functional characterization of coral GSDMs has demonstrated that pyroptotic-like cell death operates in invertebrates and that GSDMs play a role in the innate immune arsenal of Cnidarians [72,73]. A GSDME-like protein from the reef-building *Orbicella faveolata* (OfGSDME) has been shown to induce pyroptosis in human HEK293T cells in a caspase-3-dependent manner [72]. OfGSDME has been identified as a substrate for both OfCASP3 and human CASP3, with the latter being the canonical caspase processing human GSDME [52]. Importantly, Jiang et al. proceed to show that GSDMs mediate a pyroptotic-like cell death *in vivo* in response to bacterial infections in polyps of the coral *Pocillopora damicornis* [72]. Similar findings have recently been reported for a GSDME homolog from the Pacific abalone *Haliotis discus* (Mollusca) [74]. Cleavage of HdGSDME by HdCASP3 leads to pyroptosis and contributes towards the clearing of *Vibrio harveyi* bacterial infections [74].

GSDMs have been also investigated experimentally in some fish

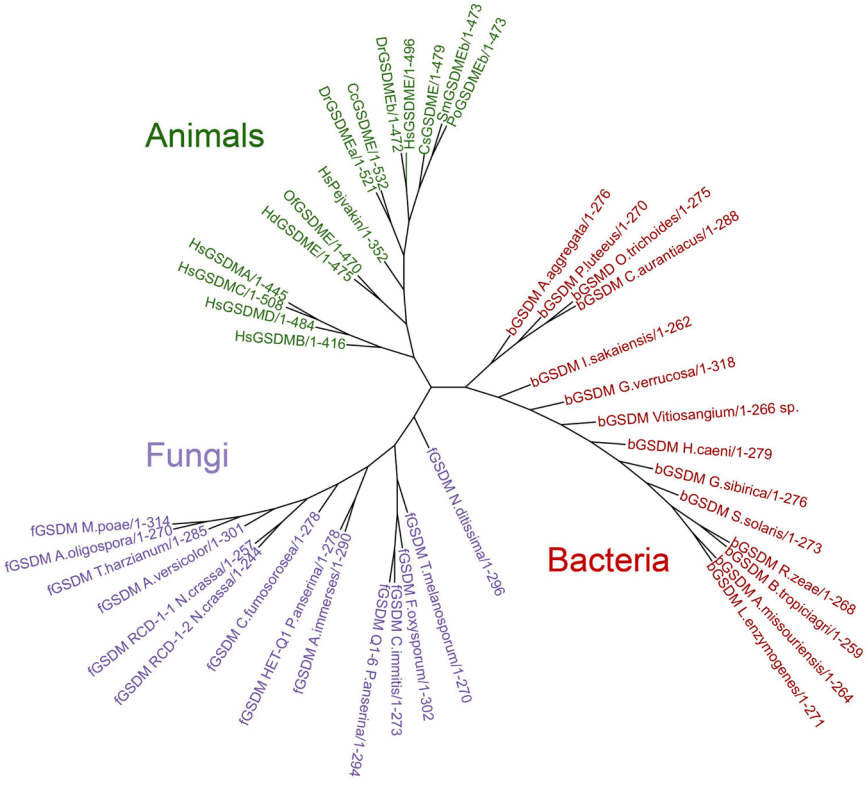

Fig. 3. Maximum-likelihood (ML) phylogenetic tree of select diverse microbial gasdermins (bGSDMs and fGSDMs) and experimentally characterized animal gasdermins. GSDM sequences clustered on three main branches corresponding to the kingdoms, from which they originate. Alignment was performed with Muscle and tree generated with MEGA11. Abbreviations: Cc - *Cyprinus carpio;* Cs - *Cynoglossus semilaevis*; Dr - *Danio rerio*; Hd - *Haliotis discus*; Hs - *Homo sapiens*; Of - *Orbicella faveolata;* Po - *Paralichthys olivaceus; Sm - Scophthalmus maximus.*

Table 1
Experimentally characterized non-mammalian gasdermins.

Name	ID	Species	Role	References
*Of*GSDME	XP_020607257.1	*Orbicella faveolata* (Cnidaria)	Pyroptosis	72
*Hd*GSDME	GIGJ01035958.1	*Haliotis discus* (Mollusca)	Pyroptosis	74
*Cc*GSDME	WAA68623.1	*Cyprinus carpio* (carp)	Pyroptosis	76, 77
*Dr*GSDMEa	XP_005170134.1	*Danio rerio* (zebra fish)	Pyroptosis	78, 79
*Dr*GSDMEb	NP_001001947.1	*Danio rerio*	Pyroptosis	78, 79
*Cs*GSDME	XP_008321525.1	*Cynoglossus semilaevis* (tongue sole)	Pyroptosis	80
*Sm*GSDMEb	XP_035485720.1	*Scophthalmus maximus* (turbot)	Pyroptosis	81
*Po*GSDMEb	XP_019948910.1	*Paralichthys olivaceus* (flounder)	Pyroptosis	85, 86
RCD-1	Q7SBA0.1	*Neurospora crassa* (Fungi)	Allorecognition	104, 105
HET-Q1	B2AXJ5.1	*Podospora anserina* (Fungi)	Allorecognition	114
bGSDMs	GSDM_BRATP	*Bradyrhizobium tropiciagri*	Abi	124, 129, 134
	WP_108071778	*Vitiosangium sp.*		
	WP_157585058	*Runella zeae*		
	WP_057949280	*Lysobacter enzymogenes*		

species, where other key molecular players of pyroptosis – receptors, adaptor proteins and caspases – are conserved [75]. Environmental pollutants, like cadmium (Cd), can induce pyroptosis in fish, notably carp [76]. Another recent study by Zhao et al., demonstrate that carp GSDME mediates antibacterial immunity after proteolytic activation by different caspases and that the N-terminal fragment of the protein forms pores in the plasma membrane of human HEK293T cells [77]. Caspase-dependent GSDME-cleavage, inducing pyroptotic cell death, has been documented in zebrafish [78,79], tongue sole [80] and turbot [81]. Intriguingly, these studies find that some ancestral GSDMEs can be proteolytically processed by the pro-apoptotic caspase-3 [80,82,83], similarly to mammalian GSDME [52], or alternatively, by pro-inflammatory caspases [39]. In zebrafish (*Danio rerio*) for example, a biomedical research model organism, two GSDME variants (GSDMEa and GSDMEb) have been identified as substrates for caspase-19a (caspy2) and caspase-19b, distantly related to human CASP1, and for the pro-apoptotic caspase-3a, caspase-3b and caspase-7 [78,84]. In addition, Chen et al. report that both caspase-8a and caspase-8b can processes the GSDMEb variant in zebrafish but only caspase-8a could cleave GSDMEa [78]. These findings establish parallels with mammalian caspase-8, which is genetically required for the activation of GSDMD in some conditions and shown to process the gasdermin *in vitro* with low affinity [50]. The report by Chen et al. unveils that the identified GSDMEa and GSDMEb in zebrafish exhibit characteristics of both GSDME and GSDMD in mammals. Noteworthy, anti-bacterial role for fish pyroptosis has also been described in zebrafish [79], turbot [81] and Japanese flounder [85,86]. The investigation of pyroptosis in fish thus establishes similarities and differences with mammalian pyroptotic pathways and contributes to elucidate the evolutionary history of gasdermin-dependent immune pathways in early metazoans.

3. Microbial gasdermins

Gasdermin proteins have been recently identified outside Metazoa, in different micro-organisms including fungi (fGSDMs), bacteria (bGSDMs) and archaea [87,88]. Several fungal and bacterial GSDMs have been molecularly characterized, uncovering some striking trans-kingdom similarities, while setting the microbial GSDMs apart from their mammalian counterparts. Importantly, however, both fGSDMs and bGSDMs control defense-related cell suicide, similarly to mammalian GSDMs, extending the immune function of the pore-forming cytotoxic domain to more than a billion years. In this section, we focus on the discovery and characterization of fungal and bacterial gasdermins.

3.1. Gasdermin proteins in fungi

3.1.1. Gasdermin-based allorecognition systems in fungi

In filamentous fungi, colony establishment relies on cellular fusions (*anastomosis*), producing an interconnected web of multicellular thread-like filaments sharing a common cytoplasm and resources [89–91]. Successful cell fusions, producing viable heterokaryons, can also occur between genetically distinct conspecific fungal individuals. However, anastomosis is evaded [92], blocked [93] or results in a regulated cell death reaction and the lysis of the fusion cells [94–96], when the fungal colonies differ at specific genes defining biological individuality. When the conspecific non-self discrimination (or *allorecognition*) occurs at the post-cell fusion stage, resulting in cellular death, the reaction has been termed *heterokaryon (*or *vegetative) incompatibility* (HI or VI) (Fig. 1) and the genes that control it are known as *het* genes [97,98]. The incompatibility reaction prevents cytoplasmic mixing, halting the horizontal spread of deleterious plasmids and mycoviruses between an infected strain and a virus-free strain [99–103]. HI is thus a fungal lifestyle-specific defense reaction (Fig. 1). It is in this context of organismal defense that fGSDMs have been identified and studied. Below, we first review more in depth the two best characterized experimentally fGSDM proteins – RCD-1 and HET-Q1 – encoded in the genomes of the model ascomycete species *Neurospora crassa* and *Podospora anserina*, respectively. Then we proceed to highlight the broad phylogenetic distribution of gasdermins in fungi and their diverse signaling pathways, controlling gasdermin activity beyond allorecognition.

3.1.1.1. RCD-1 from Neurospora crassa. The *regulator of cell death-1* (*rcd-1*) from *Neurospora crassa* is the first identified fGSDM [104,105]. Unlike previously identified *het* genes in *N. crassa*, *rcd-1* controls allorecognition cell death in fusing germinating asexual spores (germlings) (Fig. 4). The regulated cell death reaction has been named after the developmental stage in which it occurs – germling-regulated death (GRD) [106]. The GRD reaction occurs rapidly post-fusion (~20 min) of germlings from the antagonistic genotypes and phenotypically translates with the appearance of strong vacuolization preceding the cell lysis [104,106]. Two incompatible alleles (*rcd-1–1* and *rcd-1–2*) sharing ~55% identity at nucleotide level were uncovered at the *rcd-1* locus in different *N. crassa* strains. The two alleles were shown to be under balanced selection (associated with trans-species polymorphism) with nearly equal number of strains bearing either *rcd-1–1* or *rcd-1–2*. Such hallmarks of molecular evolution have been frequently found on immunity and non-self recognition-dedicated genes in fungi [107,108] and other organisms [109–111].

The *rcd-1–1* and *rcd-1–2* ORFs encode for proteins of 257 and 244 amino acids, respectively [104]. The two variants are highly divergent with only 38% primary sequence identity. Using the HHpred suite [112], RCD-1–1 and RCD-1–2 were exposed as distantly related to GSDMD and the gasdermin family [105]. The homology was limited to the N-terminal pore-forming domain of GSDMD and the two RCD-1 variants appeared to lack an inhibitory C-terminal domain. The molecular characterization of the RCD-1 variants unveiled multiple functional similarities between the fungal proteins and mammalian gasdermins [105]. First, RCD-1–1 and RCD-1–2 showed plasma membrane affinity

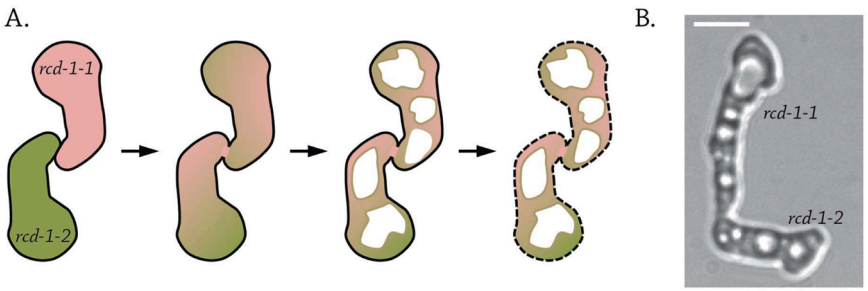

Fig. 4. Germling-regulated death (GRD) induced by the *rcd-1* fGSDM in Neurospora crassa. A. Germinating asexual spores (germlings) of the *rcd-1–1* and the *rcd-1–2* genotypes undergo anastomosis (cell fusion) and cytoplasmic mixing, which result in a rapid vacuolization of the cell pair, followed by cell lysis (dashed line). **B.** Electron micrograph of a lysed *rcd-1–1/rcd-1–2* germlings pair. Scale bar is 2.5 μm.

in vivo and were partially localized near the cell periphery when labeled with fluorescence-emitting proteins like GFP (green fluorescent protein) or mCherry. *In vitro* investigations with recombinant RCD-1 showed that the protein binds to negatively charged phospholipids, notably cardiolipin (CL), phosphatidylserine (PS) and some phosphatidylinositol phosphates (PIPs) [105]. The lipid affinity profile of RCD-1 appeared strikingly similar to the lipid affinity profile of GSDMD [43,113]. In addition, both RCD-1 variants showed a tendency to homo-oligomerize *in vitro* and were uncovered to interact physically when co-expressed in a heterologous set-up of human 293 T cells [105]. The successful reconstitution of the RCD-1–1/RCD-1–2 incompatibility cell death in human 293 T cells further supports the autonomy in cell death induction of this incompatibility system (Fig. 5). However, the precise molecular mechanisms of cross-activation and cell death between the two RCD-1 variants remain to be elucidated.

The characterization of RCD-1 established functional parallels between mammalian and fungal GSDMs and strongly supported the *in silico* uncovered evolutionary link between the two groups. Nonetheless, the exploration of RCD-1 had also led to several open questions about fGSDMs, especially regarding the regulation of their cytotoxic activity. The lack of apparent inhibitory domain, analogous to the inhibitory C-terminal domain of mammalian GSDMS, appeared puzzling. Most of these questions found an answer with the discovery and characterization of another gasdermin-based allorecognition system, encoded in the *het-Q* locus in the genome of the model ascomycete *Podospora anserina*.

3.1.1.2. HET-Q1 from Podospora anserina. Two idiomorphs – non-homologous genes situated in the same locus in different strains – have been found in the *het-Q* locus of *P. anserina* [114]. Approximately half of the investigated strains are from the *het-Q1* genotype and the other half from the *het-Q2* genotype. The *het-Q1* gene encodes a fungal gasdermin protein of 278 amino acid residues, while the *het-Q2* gene encodes a 388 amino-acid-long serine protease from the S8 family [114]. Importantly, it was uncovered that HET-Q1 is proteolytically processed in presence of HET-Q2 during the cell death reaction in *P. anserina*. The HET-Q1 fGSDM loses a ~5-kDa C-terminal fragment. The proteolytic cleavage was dependent on the predicted catalytic triad-defining residues (D35, H105 and S266) of HET-Q2. Transformations of *P. anserina*

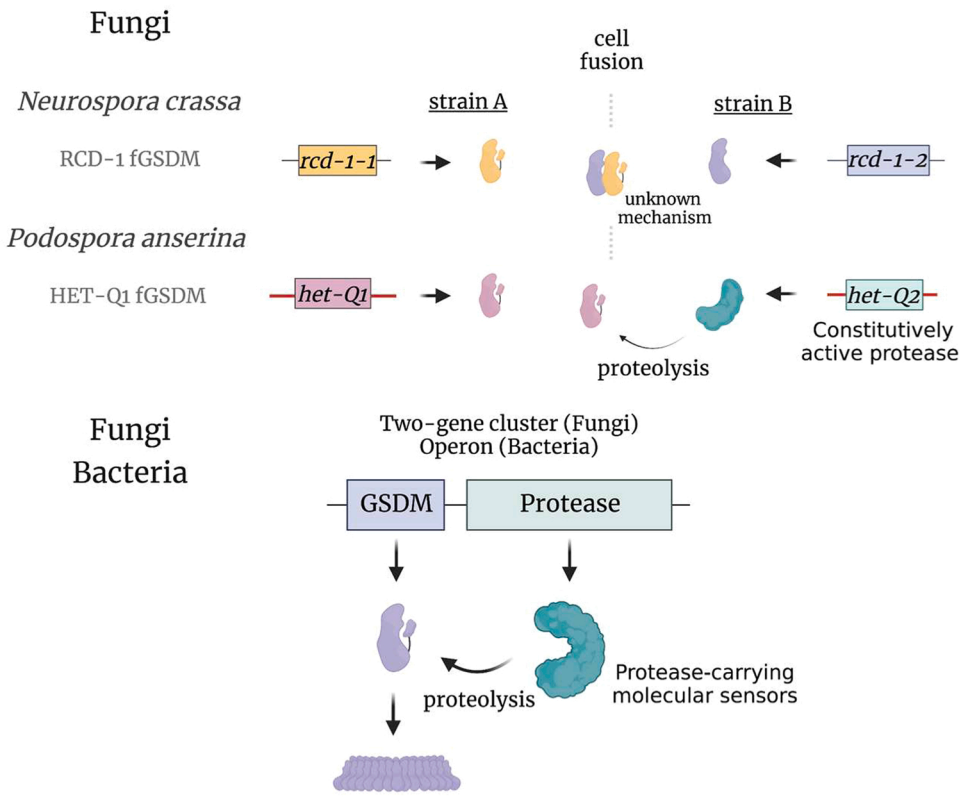

Fig. 5. Cytotoxicity control and regulation of fungal and bacterial gasdermins. Two characterized fGSDMs – *rcd-1* and *het-Q1* – control cell death during heterokaryon incompatibility (HI). Cell fusions (dashed line) between *N. crassa* 'strain A' and 'strain B' expressing different alleles of *rcd-1 – rcd-1–1* or *rcd-1–2* – result in cell death. Experimental data indicates that the antagonistic allelic variants interact physically during the cell death reaction, however the precise molecular mechanisms of cross-activation are currently unknown. The RCD-1–2 variant (violet) appears to lack an inhibitory domain and is abnormally short (244 amino acids), which might be important for the cross-activation reaction with RCD-1–1 (yellow). In *P. anserina*, the HET-Q1 fGSDM is activated by proteolytic cleavage by the HET-Q2 subtilase. The two genes are idiomorphic (located at the same locus in different strains). The HET-Q2 protease appears constitutively active. These GSDM-based allorecognition systems have been proposed to originate from the much broader and preexisting fGSDM pathways, encoded by two-gene clusters in the genomes of hundreds of fungal species. The majority of fGSDM-encoding genes are genomically clustered with protease-encoding genes and similar genomic arrangement has been unveiled for most bGSDMs. The bGSDM/protease operons are situated in bacterial defense islands next to other Abi systems.

with truncated *het-Q1(1−238)* strongly decreased the number of viable transformants, while individual alanine substitution of residues L237 and F238 abolished the proteolytic cleavage of HET-Q1 and the ability of the gasdermin to induce cell death in presence of the HET-Q2 protease [114]. These data strongly suggests that HET-Q2 cleaves HET-Q1 at a P1 amino acid residue F238, removing 40 residues of the C-terminal end of the fGSDM to induce cell death. The reconstitution of the *het-Q1/het-Q2* cell death reaction in two different heterologous systems – the yeast *Saccharomyces cerevisiae* and human HEK 293 T cells – further confirms the direct proteolytic cleavage of the fGSDM by the HET-Q2 protease and as in the case of the antagonistic *rcd-1* alleles, suggests that the *het-Q1*-based incompatibility system is autonomous in cell death induction (Fig. 5) [114].

3.1.2. Distribution and phylogeny of fGSDMs

The two characterized fGSDM (RCD-1 and HET-Q1) belong to a much broader gene family in fungi with ~1900 uncovered members encoded in the genomes of 400 different species [104,114]. Initial analyses of the phylogenetic distribution of fGSDMs have shown that members of the gene family can be found in at least 24 different fungal orders, the vast majority of which belong to the Ascomycota phylum [104]. Almost no fGSDMs were identified in the other main fungal phylum, Basidiomycota, and fGSDMs appeared absent from yeast genomes [104]. The mean number of gasdermin genes per genome was five, while more than a dozen of species carried more than 10 fGSDM genes. The phylogenetic analyses of the uncovered ~1900 fGSDMs showed ~20 well-supported clades, however the fungal gasdermins genealogy did not correlate with species phylogeny [104,114]. Most clades contained gasdermin homologs from phylogenetically distant taxa. Lineage-specific gene expansions were also uncovered, for example in the *Trichoderma* genus. The genomes of several *Trichoderma* species were found to carry close to ~20 fGSDM genes with *T. atroviride* harboring 23 gasdermin homologs. However, only two fGSDM-encoding genes were found in the genome of *T. reesei* [104]. Overall, the scattered phylogenetic distribution and the observed lineage-specific gene expansions suggest that gasdermins are a rapidly evolving multigene family in fungi. A 'birth-and-death' evolutionary model, where genes are frequently duplicated and lost, could explain the fGSDMs genealogy and similar models have been proposed for other fungal [115] and vertebrate immune-related genes [116,117].

3.1.3. Proteolytic regulation of fGSDMs by genomically clustered protease-encoding genes

The discovery of the *het-Q1/het-Q2* allorecognition system, unveiling the proteolytic regulation of the HET-Q1 gasdermin, appeared as a strong indicator that fGSDMs, similarly to their mammalian counterparts, were regulated through proteolysis. Moreover, *in silico* analyses unveiled that the fGSDM-encoding genes are frequently genomically clustered with a protease-encoding gene (Fig. 5) [114]. Nearly 80% of the gasdermin genes were situate in the vicinity (+/- 10-kb region) or adjacently to a protease-encoding gene, forming two-gene clusters. These protease-encoding genes were named *Q2-L* (*het-Q2*-like). In ~60% of the cases, the Q2-Ls carried a subtilase-like serine-S8 protease, similarly to HET-Q2. Approximately one fifth of the Q2-L proteins (17%) carried a CHAT (caspase HetF associated with tetratricopeptide repeats [TPRs]) protease domain, instead of a subtilase-like protease. The latter finding suggests that caspase-like proteases play a role in fungal RCD pathways and regulated some of the fGSDMs.

Unlike HET-Q2 from *P. anserina*, majority of Q2-L proteins were predicted to carry other domains next to the putative protease domain. At least 18 different Q2-L architectures were defined using previously annotated Pfam domains [114,118]. Several superstructure-forming pseudo-repeats (ANK [ankyrin], TPR [tetratricopeptide repeat] and WD40) – previously described as constituents of fungal NOD-like receptors – were represented among the Pfam annotations. These pseudo-repeats-based domains have been proposed to mediate interactions with diverse range of molecules throughout the tree of life and frequently found in molecular sensors [119]. Remarkably, some Q2-L protein architectures were identified as members of the vast group of fungal NLR proteins [120,121]. Several fungal NLRs have been previously characterized during the exploration of fungal allorecognition systems [106,122,123]. However, the abundance, diversity and evolutionary marks associated with the NLR-encoding genes in fungi, have led to the hypothesis that this gene family has much broader role in fungal organismal defense and immunity [19]. Based on these findings, it has been proposed that the Q2-L genes encode molecular receptors, controlling the fGSDMs through proteolytic cleavage [114].

Clavé et al. have demonstrated experimentally that one such two-gene cluster, encoded in the genome of *P. anserina*, works as a functional unit [114]. Specifically, a protein named Q2-L-6 carrying a caspase-like CHAT protease has been demonstrated to process the fGSDM encoded by the neighboring gene and termed Q1–6. The gene cluster *Q2-L-6/Q1–6* has been utilized to engineer an incompatibility system in *P. anserina*, demonstrating the RCD-involvement of the Q2-L proteins. At present, what are the precise molecular signals that activate the Q2-L proteases remain to be elucidated. Yet, the discovery of the *Q2-L* genes in fungi has drawn additional evolutionary links between fGSDMs-dependent RCD and pyroptosis in mammals. Specifically, the uncovered functional link between caspase-like CHAT domains and caspases and the shared role that NLRs play in the proteolytic activation of gasdermins in the fungal and animal kingdoms. These evolutionary parallels have recently been extended to bacteria with the discovery and characterization of bGSDMs.

3.2. Gasdermin proteins in bacteria

bGSDMs have been initially discovered by analyzing the gene content of bacterial *defense islands* [124] – large genomic regions, in which defense-related bacterial genes and different putative Abi systems are clustered [31,125]. At least 50 bGSDMs have been identified, forming a separate clade from their eukaryotic homologs, and spread in bacterial and archaeal genomes. bGSDMs were uncovered in diverse bacterial phyla with majority of species classified either as Proteobacteria (Gram-negative) or Actinobacteria (Gram-positive). Remarkably, gasdermin genes were also identified in the genomes of several cyanobacterial species (i.e. *Nostoc sp.*). Cyanobacteria are among some of the oldest organisms on Earth, estimated to have emerged billions of years ago [126,127]. Johnson et al. have successfully crystalized and solved the structures of three different bGSDMs from *Bradyrhizobium, Vitiosangium* and *Runella* [124]. The structures compared favorably with the pore-forming N-terminal domain of mammalian gasdermins (see 4.1.), confirming the uncovered *in silico* evolutionary link between the microbial and metazoan proteins (Fig. 6). The obtained structures have also revealed the original inhibition mechanism for bGSDMs, which similarly to fGSDMs carry only a short inhibitory C-terminal peptide (Fig. 6). A role in the protein inhibition of some bGSDMs is also played by a palmitoylated cysteine, situated at the extreme N-terminus of the proteins. However, while the post-translationally modified cysteine residue is conserved in majority of bGSDMs, it is absent from some bGSDMs sequences and absent from most fGSDMs.

Approximately 90% (43 of 50) of bGSDMs are genomically clustered with protease-encoding genes. The encoded protease domains belong to different MEROPS families [128] with serine peptidases (trypsin-like and subtilases) and cysteine caspase-like proteases (CHAT and C14) being represented approximately in a 1:3 ratio. Like in fungi, some of the protease domains are part of multidomain proteins, often carrying super-structure forming repeats (TPR, WD40 and LRRs). Johnson et al. demonstrate that upon cleavage by their cognate protease, bGSDMs can oligomerize forming large pores, organized in a mesh-like supra-molecular assemblies [124]. Remarkably, the cytotoxic activity of the bGSDM/protease clusters has been shown to act as an abortive infection (Abi) system [129]. A four-gene operon from *Lysobacter enzymogenes*

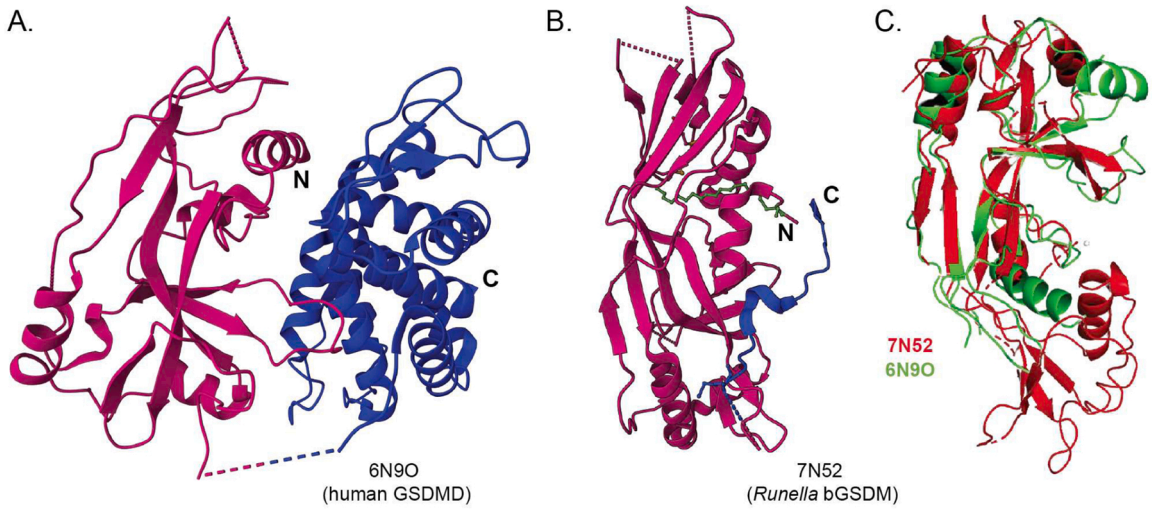

Fig. 6. Structural comparisons between human GSDMD and the bGSDM from *Runella zeae*. Shown are the PDB structures of A. human GSDMD (6N9O) (Liu et al., 2019, Immunity) and B. Runella bGSDM (7N52) (Johnson et al., 2022, Science). The N-terminal pore-forming domain is colored in purple and the inhibitory C-terminal domain in blue. The palmitoyl group on C3 of the bGSDM is colored in green. Dashed lines indicate poorly resolved flexible regions and at the inter-domain limits correspond to loops, in which proteolytic cleavage occurs. C. Structural overlay of the pore-forming domains for the two gasdermins in inactive state, colored in green (GSDMD) and red (bGSDM).

procured anti-phage defense when expressed in *E. coli* against coliphages T4, T5 and T6 [124]. The anti-phage defense reaction relied on the presence in the operon of the bGSDM-encoding gene, showing the essential role that bGSDMs play in mediating Abi [124]. Wein et al. have uncovered that the anti-phage activity of the *Lysobacter* bGSDM system depends on bacterial CARD domains (Caspase activation and recruitment domain); with one such CARD domain integrated at the C-terminus of a bacterial NLR-like protein, encoded by the same operon as the bGSDM [129]. CARDs (~90–100 amino acids) are key players in inflammatory signaling in animals and involved in the proteolytic activation of some mammalian GSDMs [130]. The discovery of CARD-containing bacterial NLR-like proteins, controlling the activation of a bGSDM, suggests that the entire pyroptotic molecular machinery was already present and active in bacteria.

4. Trans-kingdom similarities and differences between gasdermin proteins

While microbial and mammalian GSDMs belong to the same protein superfamily, sharing thus structural and sequence similarity, there are some striking and some more nuanced distinctions in various functional aspects between the two groups of GSDMs, including their inhibitory modes and regulation. Below, we delve into comparisons between these distantly related members of the GSDM family.

4.1. Structure and modes of inhibition

Mammalian GSDM structures have been reported for the auto-inhibited forms of murine GSDMA3 (PDB: 5B5R) [43], murine and human GSDMD (PDB: 6N9O) [131] and human GSDMB (PDB: 7WJQ). The obtained crystal structures show the N-terminal and a globular C-terminal domain connected by 15–20 amino acids linker residues, containing the caspase cleavage site. The cytotoxic N-terminal domains of GSDMD and GSDMA3 (~45% sequence similarity) exhibit high structural similarity, consisting of an extended twisted β-sheet formed by 9 or 10 β-strands in GSDMA3 and GSDMD, respectively [43,131]. The inhibitory domain of these mammalian GSDMs consists of a globular α-helical bundle formed by 11 helices in GSDMA3 and 9 helices in GSDMD, capped in both cases by three consecutive short β-strands [43]. The inhibition mechanism involves a hydrophobic pocket in the inhibitory domain into which docks a β-hairpin-containing loop at the extreme N-terminus of the protein [131]. Meanwhile, fungal and bacterial gasdermins generally present shorter protein sequences, which bear limited homology exclusively with the N-terminal pore-forming domain of mammalian GSDMs [105]. Johnson et al. have solved the crystal structures of three bGSDMs from different species [124]. Strikingly, the bGSDMs exhibited high structural similarity with the twisted β-sheet core of the pore-forming domain of GSDMD and GSDMA3 (Fig. 6). However, as one could have expected form the sequence alignments, bGSDMs differed significantly in their inhibitory mechanism from their mammalian counterparts. The much shorter inhibitory C-terminal domain (~20–40 amino acid residues) of the *Bradyrhizobium* and *Vitiosangium* bGSDMs appears to wrap around the twisted β-sheet core forming the cytotoxic domain of the proteins [124]. Moreover, the authors have identified that a post-translational palmitoylation of a conserved cysteine residue stabilizes the inhibited state of the bGSDMs from *Bradyrhizobium* (PDB: 7N50) and *Vitiosangium* (PDB: 7N51). The conserved cysteine is situated at the extreme N-terminal end (C3 or C4) of some bGSDMs. The 16-carbon fatty acid chain of the palmitoyl group slides into a hydrophobic pocket inside the pore-forming domain, stabilizing the inhibited state of the protein [124]. The conserved cysteine residue has also been identified on some fGSDM sequences, suggesting that post-translational modifications might play a stabilizing role for non-bacterial GSDMs. While molecular models of fGSDMs suggest that the twisted β-sheet core is well conserved [105], high-resolution structural information is lacking for the fungal clade of the microbial GSDMs.

Proteolytically activated GSDMs form ring-like pores. The oligomeric structures of the murine GSDMA3 [132], human GSDMD [46] and GSDMB [133] have been solved by cryogenic electron microscopy (cryo-EM), revealing the conformational changes undergone by the pore-forming domain from inhibited to transmembrane state transition. The 27-fold symmetry structure of GSDMA3 pore (PDB: 6CB8) reveals that the twisted β-sheet core of each GSDMA3 monomer extends in two long β-hairpins. The four transmembrane 'blades' of each GSDMA3 monomer form a 108-stranded anti-parallel β-barrel with a diameter of 180-Å, topped by a cytosolic rim formed by a globular region in GSDMA3-NT [132]. Processed human GSDMD oligomerizes in a similar 33-fold symmetry pores (PDB: 6VFE) with an inner diameter of 215-Å [46]. Several phylogenetically distant bGSDMs have been shown to form pores and pore-like assemblies [124,134]. The inner diameter of the tested bGSDMs ranges from 130-Å – smaller in size than characterized mammalian GSDMs – to impressively large pores with inner diameter of

~400-Å [134]. Johnson et al. have reported a high-resolution cryo-EM structure of a 52-mer bGSDM pore from *Vitiosangium* [134]. The authors have also identified a key role in pore assembly and membrane insertion for the covalently bound palmitoyl at C4 of *Vitiosangium* bGSDM. Remarkably, the regulatory role of palmitoylation for gasdermin pore formation appears conserved in mammals [135,136]. Currently, no high-resolution structural information has been reported for fGSDMs, however RCD-1 fGSDM has been shown to oligomerize and form arc-like and pore-like aggregates *in vitro* [105,114].

Overall, the results indicate that microbial GSDMs, and in particular bGSDMs, can adopt a variety of ring-like pore architectures; similarly to mammalian GSDMs. Future research work should focus on obtaining high-resolution structural data for the cytotoxic pores of fGSDMs. This would allow for in depth trans-kingdom structural comparisons between members of the GSDM family and help elucidate the evolutionary constraints and mechanisms of pore-formation.

4.2. GSDMs activation mode and regulation

A well-established common point between all characterized GSDMs is their mode of activation, consisting of the proteolytic removal of the inhibitory C-terminal domain [87,137]. However, kingdom-specific differences in regard to the type of proteases mediating the proteolytic activation of GSDMs, have been reported [114,124]. These differences are equally reflected in the variety of recognized proteolytic sites, situated on animal GSDMs, bGSDMs and fGSDMs, and likely in the molecular mechanisms of recognition between proteases and GSDMs.

Generally, GSDMs can be processed by cysteine-aspartic proteases – caspases and caspase-like proteins [138]– or various serine proteases [139]. Caspases most often cleave their substrates after an aspartate residue (D), conserved at position P1 or the amino acid residue after which the cleavage occurs [140]. For the archetypal GSDMD, the cleavage site of the inflammatory caspases (CASP1, CASP4, CASP5, CASP11) has been identified with a P1 residue D^{275} in humans and D^{276} in mice [39]. CASP8 can also cleave GSDMD under certain conditions, utilizing the same cleavage site – P4-P1 sequence motif $_{273}$LLSD$_{276}$ and $_{272}$FLTD$_{275}$ in the murine and human variant of the protein, respectively – as the proinflammatory caspases [50]. CASP8 has additionally been found to cleave human GSDMC, after residue D^{240} in response to α-ketoglutarate stimulation [51]. Proapoptotic CASP3 has been found to induce GSDME-dependent pyroptosis, cleaving the human variant of the protein after residue $D^{270,}$ situated at the P1 position of the $_{267}$DMPD$_{270}$ cleavage site [52]. The proteolytic activation of a subset of microbial GSDMs also relies on caspase-like proteins, in bacteria [124] and fungi [114]. The caspase-like peptidases controlling half of bGSDMs, and ~17% of fGSDMs, have been characterized as CHAT domains, a sister clade of animal caspases [141,142]. A recent structure of a TPR-CHAT protein from the bacterium *Desulfonema magnum* has been solved, revealing that the CHAT peptidase domain shares structural similarity with human separase and CASP7 [143]. Using mass spectrometry, Johnson et al. have identified the cleavage site of a CHAT protease processing the bGSDM in *R. zeae*. Unlike the mammalian caspases, generally cleaving after an aspartate residue, in the case of *R. zeae* bGSDM the cleavage site ($_{244}$NRVL$_{247}$) ends with a large hydrophobic residue (L^{247}) [124]. A truncated protein carrying a caspase-like CHAT domain has been shown to cleave the adjacently encoded Q1–6 fGSDM from *P. anserina* [114]. However, no precise cleavage site has been reported in this case. These findings indicate that GSDMs are very ancient substrates of the caspase family. Further investigation of the recognized cleavage sites, situated on the microbial GSDMs, would help us understand better the determinants and evolution of proteolytic specificity in the caspases family.

Majority of fGSDMs (~61%) and some bGSDMs are regulated by serine proteases, often belonging to the MEROPS S8 family (SB clan) of subtilisin-like peptidases [144,145]. In spite of their abundance, only one such subtilase has been explored – HET-Q2 from *P. anserina* – which appears to cleave the HET-Q1 gasdermin at a P1 residue F^{238}, integral to the $_{235}$KVLF$_{238}$ sequence [114]. The HET-Q1 cleavage site has been proposed based on functional and mutational analyses and is yet to be confirmed with mass spectrometry. Some bGSDMs are genomically clustered with genes encoding trypsin-like proteases, belonging to the same family as mammalian granzymes [54]. Trypsin processes its substrates between the carboxyl group of an arginine (R) or lysine (K) residue and the amino group of the adjacent amino acid [146]. Human GZMA has been found to cleave GSDMB after K^{229} and K^{244} amino acid residues with a preference for the latter [56]. Meanwhile, GZMB has been identified to cleave GSDME at the CASP3-specific D^{270} residue, inducing pyroptosis in cancerous cells [53]. These findings indicate that GSDM-controlling serine proteases can recognize divers cleavage sites. Further exploring the proteolytic specificity of these proteases in bacteria and fungi should reveal the extent of this diversity, derive novel P4-P1 consensus motifs, which could help us identify other substrates, processed during pyroptotic-like cell death in microorganisms.

5. The extremely ancient origin of pyroptotic cell death and the many roles of GSDMs

The presence of GSDMs in some of the earliest animal taxa and the established functional similarities between GSDMs throughout Metazoa, suggest that GSDMs and pyroptosis are of extremely old origin, dating back ~800 mya to the last common animal ancestor [147]. The recent discovery of widespread gasdermin homologs in fungi and bacteria suggests that the origin of the gasdermin superfamily, and pyroptotic-like cell death, pre-date the emergence of Metazoa, expanding further the evolutionary timeframe, in which GSDMs have been integral to host defense and cell suicide strategies. During this period of more than a billion years of evolutionary history, metazoan GSDMs appear to have been selected for several other functions like protein secretion [148–150] and direct antibacterial cytotoxicity [41,151], while their controlled membrane disruption features have been integrated in a variety of physiological processes like neutrophil turnover [152], cell differentiation [153] and anti-tumor immunity [56,137,154]. Considering these findings, it would appear plausible that microbial gasdermins have also acquired other biological roles beyond cell suicide execution. Two interesting possibilities for such roles would be their integration into non-canonical secretion pathways and/or direct use as antibacterial cytotoxins.

An important aspect of pyroptosis is the release of proinflammatory cytokines, notably members of the interleukin-1 family (IL-1β and IL-18) [36,38,155]. IL-1β and IL-18 are synthesized as inactive precursors (pro-IL-1β and pro-IL-18), which are proteolytically matured by inflammatory caspases (CASP-1) before release in the extracellular environment through GSDMD transmembrane pores. Remarkably, recent evidence points out that the electrostatic interactions between the negatively charged (acidic) inner side of GSDMD and GSDMA3 pores and the mildly positively charged (basic) surfaces of matured IL-1β and IL-18 contribute to secretory specificity, which is especially important in differentiating between the matured and unmatured variants of these interleukins, with the latter exposing an acidic molecular surface [46,156]. It would thus appear that some gasdermin features have been subject to evolutionary pressure to shape these PFPs as selective secretory conduits, acting as electrostatic filters for molecules in the size range of the pores [46,156]. Other unconventional secretory pathways can rely on transmembrane pores, some of which in bacteria [157,158]. As suggested by Johnson et al., the observed size distribution of *R. zeae* and *Bacteroidetes* bGSDM pores (24–33 nm) could allow for the secretions of moderately large molecules [124]. Considering the high numbers of GSDMs in some fungal species and the uncovered sequence diversity [104,114], a future research axis should certainly explore the potential involvement of these PFPs in unconventional secretion pathways. Obtaining detailed structural data for fGSDM pores, would allow us not only to better understand the evolution of the pore-forming

domain but also offer an overview of the various molecular mechanisms of pore-formation in fungi and bacteria, including those potentially impacting pore secretion selectivity.

An alternative role for some fGSDMs could be their use as antibacterial toxins directly targeting the bacterial plasma membrane from the outside, hence acting similarly to other PFPs with immune roles, often found in the Membrane Attack Complex/Perforin (MACPF) superfamily [159–161]. Noteworthy, direct antibacterial features have already been reported for mammalian GSDMD [41] and GSDMB [151]. Activated GSDMD has been shown to kill *E. coli* cells *in vitro* or when heterologously expressed in bacteria [39,41], while GSDMB can directly target enteroinvasive *Shigella flexneri* [151]. The establishment of bactericidal properties for some fGSDMs could have an important ecological implications as fungal-bacterial interactions are abundant and diverse in nature [162]. One could envisage that some lineage-specific expansions of fGSDMs are driven by niche-specific antagonism with certain bacterial species. Because such interactions could rely on the recognition of specific lipids or other bacteria-derived molecules by the fGSDMs, it appears especially important to learn more about the roles of lipids and the lipid environment for the activity of microbial GSDMs. In addition, cardiolipin – a negatively charged phospholipid frequently found in the plasma membranes of Gram-negative bacteria [163–165] – has been identified in the lipid composition of mitochondrial membranes in *N. crassa* [166]. Considering that the RCD-1 fGSDM variants have been found to bind to cardiolipin [105], it is reasonable to suggest that mitochondria could be a target for some fGSDMs. This hypothesis appears plausible as human GSDME and GSDMD have been found to permeabilize mitochondria [167,168].

The potential roles of microbial GSDMs in secretion or as antibacterial toxins, directly inspired by discoveries about the roles of their mammalian counterparts, appear plausible and further research in this direction, well justified. In addition, some intriguing question remains regarding the possible involvement of microbial GSDMs in various physiological processes in microorganisms, as well as the relations with other programmed and regulated cell death programs. Naturally, it might be difficult to identify potential physiological roles for such GSDMs, especially in case of their involvement and activation being conditioned by specific lifestyle-related processes or highly dependent on the biological context (symbiosis, commensalism, parasitism etc.). While this research axis appears riskier, it offers the possibility of uncovering novel and surprising roles for the gasdermin family in microorganisms, unveiling further how a billion years of evolution throughout the tree of life might have shaped different features of this pore-forming, membrane-permeabilizing protein fold.

6. Conclusion

Pyroptosis and pyroptotic-like cell death radiate through eukaryotic and prokaryotic lineages, diverged billions of years ago, and constitute one of the original cell suicide-based defense strategies. The uncovered evolutionary parallels offer an exciting case study in comparative immunology. Future efforts would elucidate the evolutionary constraints and forces that have shaped the gasdermin superfamily in microbes and allow us to better grasp the impact of pyroptotic-like cell death plays in microbial communities.

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