Punching holes in cellular membranes : biology and evolution of gasdermins

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Authors: Elke De Schutter[1,2,3], Ria Roelandt[1,2], Franck B. Riquet[1,2,4], Guy Van Camp[3,5], Andy Wullaert[1,2,6] and Peter Vandenabeele[1,2,7]

Affiliations:

[1] VIB Center for Inflammation Research, 9052 Ghent, Belgium

[2] Department of Biomedical Molecular Biology, Ghent University, 9052 Ghent, Belgium

[3] Center of Medical Genetics, University of Antwerp and Antwerp University Hospital, Prins Boudewijnlaan 43/6, BE-2650 Edegem, Antwerp Belgium.

[4] Université de Lille, Lille, France

[5] Center for Oncological Research, University of Antwerp and Antwerp University Hospital, Universiteitsplein 1, BE-2610 Wilrijk, Antwerp Belgium.

[6] Department of Internal Medicine and Pediatrics, Ghent University, Ghent, Belgium.

[7] Methusalem program CEDAR-IC, Ghent University, Ghent, Belgium.

*Correspondence: Peter.Vandenabeele@irc.vib-ugent.be (P. Vandenabeele)

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Abstract

The Gasdermin (GSDM) family has evolved as six gene clusters (*GSDMA-E* and *Pejvakin*), which are characterized by a unique N-terminal domain (N-GSDM). Except for Pejvakin, the N-GSDM domain is capable of executing plasma membrane permeabilization. Pending on the cell death modality, several protease- and kinase-dependent mechanisms directly regulate the activity of GSDME and GSDMD, two widely expressed and best-studied GSDMs. We provide a systematic overview of all GSDMs in terms of biological function, tissue expression, activation, regulation and structure. In-depth phylogenetic analysis reveals that *GSDM* genes show many gene duplications and deletions suggesting strong evolutionary forces and a unique position of the *Pejvakin* gene associated with the occurrence of complex inner ear development in Vertebrates.

Gasdermins: same same but different

The human genome contains six **gasdermin** (GSDM) genes: *GSDMA-E* and *Pejvakin* (*PJVK*), located on 4 different chromosomes (Table 1). The mouse genome lacks a *GSDMB* orthologue, but repetitive duplication events resulted in three *Gsdma* genes (*Gsdma1*, *Gsdma2* and *Gsdma3*), four *Gsdmc* genes (*Gsdmc1*, *Gsdmc2*, *Gsdmc3* and *Gsdmc4*) and single genes for *Gsdmd*, *Gsdme* and *Pjvk,* raising questions about functional differences between gasdermins and which evolutionary selective forces have driven gene losses and amplifications. The gasdermins, originally coined according to their expression pattern along gastrointestinal tract and skin (dermis) [1,2], were until recently considered as orphan genes with unknown physiological functions, though some members have been associated with skin diseases such as alopecia [3,4], with asthma [5–8], hearing loss [9,10] and cancer [1,11–16]. Since several members of the gasdermin gene family were shown to execute plasma membrane permeabilization during different forms of **regulated necrosis** [17–21], GSDMs recently gained a lot of interest regarding their role in inflammation and host defense.

All GSDMs (except PJVK) consist of N-terminal (N-GSDM) and C-terminal domain (C-GSDM) connected by a linker region. Structural insights in the activation and pore-forming mechanisms of N-GSDM domains are largely based on the structures of GSDMA3 [22,23] and GSDMD [24]. The pore-forming mechanism involves three steps: interdomain proteolytic cleavage releasing N-GSDM from the autoinhibitory C-GSDM domain (Figure 1); phospholipid-mediated recruitment of the N-GSDM domain to the plasma membrane (Table 1); and finally oligomerization and **pore formation** leading to plasma membrane permeabilization. Nevertheless, it is still unclear whether this three step model applies for all

GSDMs. For example, there is no experimental evidence for proteolytic cleavage of GSDMA, implying other mechanisms of activation.

Since the discovery that particular GSDMs are implicated in the execution of different cell death modalities, their activation has been proposed as a marker of **pyroptosis** [25]. However, by doing so, "pyroptosis" becomes a rather generic term. One can have inflammasome-mediated activation of GSDMD by caspase-1/4/5/11 [18,19], chemotherapy-induced activation of GSDME by caspase-3 [21,26–28] or natural killer cell-induced activation of GSDME by granzyme-B [29], all leading plasma membrane permeabilization without signs of apoptosis. Additionally, to narrow down all GSDM-mediated cell death modalities to "pyroptosis" may also become confusing in cases of GSDME-mediated **secondary necrosis** following **apoptosis** (apoptosis-driven secondary necrosis) [17] and GSDMD-mediated release of NETs during **NETosis** [30,31].

In this review, we outline the differential expression of GSDM proteins in various tissues, showing ubiquitous expression of GSDME. Furthermore, we report on inflammasome dependent and independent cellular conditions leading to GSDM activation as well as on checkpoints involving proteolysis, phosphorylation and exosome formation that prevent N-GSDM cytotoxicity. Finally, we performed an in-depth phylogenetic analysis of the gasdermin family in many species, in order to understand possible evolutionary forces driving *GSDM* gene loss and amplification. Altogether, the evolutionary emergence of multiple *GSDM* genes and the restricted expression pattern of some of them reflect their crucial role in particular cell types in an organism living a life full of challenges.

Gasdermins: executioners on the necrotic battle field

All gasdermins but PJVK share the feature that (over)expression of their N-GSDM domain causes plasma membrane permeabilization [22]. In case of PJVK the N-GSDM domain is directly followed by a small C-terminal domain containing a zinc finger domain with an unknown function (Figure 1A) [10,32]. In contrast, GSDMA-E comprise clear two-domain arrangements consisting of the cytotoxic N-GSDM domain separated from an autoinhibitory C-GSDM domain by a flexible hinge region with highly conserved aspartate residues, making them potential substrates for aspartate-specific proteases such as caspases and granzymes. More information on the mechanisms of autoinhibition and release of N-GSDM from C-GSDM is provided in Box 1. GSDME cleavage by caspase-3 at D270 generates an N-GSDME fragment that causes membrane permeabilization during apoptosis-driven secondary necrosis [17] occurring after apoptotic features such as membrane blebbing, PS exposure and DNA fragmentation. However, GSDME does not explain all cases of membrane permeabilization following apoptosis. In some cells apoptosis-driven secondary necrosis occurs independently of GSDME, such as in immortalised *Gsdme*$^{-/-}$ macrophages [33], human T cells and monocytes [34], suggesting redundant mechanisms. Recently, the ill-characterized nerve injury-induced protein 1 (NINJ1), a cell surface protein, was shown to be essential for plasma membrane rupture following apoptosis-driven secondary necrosis, pyroptosis and necroptosis [35].

Canonical and **non-canonical** inflammasome activation of caspase-1/11 (mouse) or caspase-1/4/5 (human) leads to proteolytic activation of GSDMD [18,19,36–38] and the consecutive release of pro-inflammatory cytokines such as IL-1β [39], linking inflammasome-mediated GSDMD activation with pyroptosis. Next to caspase-1/11, recent studies in mouse

macrophages revealed that in conditions of TAK1 and IKK inhibition (such as by YopJ during Yersinia infection), also caspase-8 directly activates GSDMD initiating pyroptosis [40–42] in a RIPK1 kinase activity dependent [40] or independent way [43]. This illustrates a proteolytic convergence during pyroptosis execution. However, in cancer cell lines treated with chemotherapeutic drugs, caspase-3-mediated cleavage of GSDME can directly proceed to plasma membrane permeabilization without inducting apoptotic features such as blebbing, suggesting that also GSDME can trigger **primary necrosis** [21,26–28]. Likewise, granzyme B from killer cells can directly activate GSDME resulting in direct pyroptotic death of tumor cells rather than apoptosis-driven secondary necrosis [29].

While GSDMD-mediated pyroptosis in macrophages and neutrophils is associated with release of inflammasome substrates such as processed IL-1β [30,39,44], GSDMD activation in neutrophils via non-canonical inflammasome mediated cytosolic sensing of LPS or Gram-negative bacteria results in the release of neutrophil extracellular traps (NETs) [30]. Alternatively, in PMA-stimulated human neutrophils, ELANE (elastase from neutrophils) proteolytically activates GSDMD resulting in NETosis [31]. Also cathepsin G following serpin inhibition can function as backup for GSDMD activation in neutrophils and monocytes [45]. Furthermore, caspase-8–dependent GSDMD activation in macrophages provides host defense against *Yersinia* infection [46]. The fact that both GSDME (caspase-3, granzyme B) and GSDMD (caspase-1/4/5/11, caspase-8, ELANE, cathepsin G) can be activated by multiple proteases and directly cause plasma membrane permeabilization represents a redundant backup mechanism for pyroptosis to ensure necrotic death and consecutive release of cytokines, chemokines and DAMPs eliciting a strong immune response during infection, inflammation and anti-cancer responses [29].

In contrast to GSDME and –D, full size human GSDMB is capable of promoting pyroptosis by activating caspase-4 through interaction with the CARD domain, while the same caspase-4 also proteolytically inactivates GSDMB [47]. As such, GSDMB-mediated activation of caspase-4 may represent a mechanism for triggering non-canonical inflammasome activation and pyroptosis in humans, but also a dampening mechanism. Recently, GSDMB was shown to mediate pyroptosis after cleavage by granzyme A delivered by natural killer cells [48] and caspase-1 [8].

Like their relatives, overexpression of the N-terminus of GSMDA or -C is cytotoxic [8,22]. Cancer cells expressing PDL1-induced GSDMC switch from chemotherapy- and TNF/cycloheximide-induced apoptosis to pyroptosis which is due to caspase-8-mediated generation of a cytotoxic N-GSDMC [20]. In contrast to other GSDMs, GSDMC is cleaved by caspase-8 at D365 within its C-GSDM domain instead of the hinge region (Figure 1A). With regard the physiological functions and upstream activating pathways of GSDMA, we are still groping in the dark (Table 1). In that respect, next to proteolytic cleavage by caspases, granzymes, cathepsins or ELANE, GSDMs might be activated by other mechanisms including gain-of-function mutations or splicing mechanisms. Indeed, gain-of-function mutations in mGSDMA3 and hGSDME associated with alopecia and hearing loss, respectively, apparently disrupt the C-GSDM domain and its autoinhibitory function resulting in cell death following transfection in Human Embryonic Kidney (HEK)293T cells [19,32]. Similarly, different splice variants of hGSDMB are associated with asthma [6], cancer [14] and multiple sclerosis [49], suggesting that GSDMB activity next to proteases might be regulated by alternative splicing as well.

GSDMs show various expression patterns in human tissues

The various GSDMs show very different expression profiles in tissues, cell types and subcellular localizations, suggesting functions restricted to particular cells and organelles. Both GSDMD (inflammasome-mediated pyroptosis) and GSDME (apoptosis-driven secondary necrosis or pyroptosis) are widely expressed in many tissues and cell types (Figure 2, Figure S1 and Table S1). However, despite their ubiquitous expression, *Gsdmd*$^{-/-}$ and *Gsdme*$^{-/-}$ mice lack a spontaneous phenotype. This suggests a specific role during various challenges such as infection and cancer, which is supported by the high expression of GSDMD at sites of pathogen entry in humans such as the respiratory tract, the gastrointestinal tract and the urogenital system (Figure 2). In addition, GSDME expression was shown to increase macrophage-mediated phagocytosis and the number and function of tumour-infiltrating natural-killer and CD8+ T lymphocytes, thereby suppressing tumour growth [29]. The restricted expression pattern of GSDMA and -C in the skin (GSDMA) or in lung, buccal mucosa, esophagus and stomach (GSDMC) (Figure 2) may also be associated with particular challenge conditions. Moreover, some GSDMs are highly induced during conditions of cellular stress. GSDME expression is transcriptionally induced after dexamethasone treatment [50] and GSDMC expression is elevated *via* the immune checkpoint ligand PD-L1 under conditions of hypoxic stress [20]. Except from immunohistochemistry data of a limited amount of cell types (Table S1) and early studies distinguishing GSDM expression between differentiating (GSDMD, -C), differentiated (GSDMA, -C) and proliferating (GSDMB) esophagus and stomach epithelium [12], profound knowledge about GSDM expression in particular cell types is still lacking.

Gasdermins target different organelle membranes

At subcellular level, GSDM proteins during homeostasis are associated with the cytosol (GSDMA and –E; GSDMB to a lesser extent), nucleoplasm (GSDMB and –D; GSDMA to a lesser extent) and mitochondria (GSDMD and PJVK) [51] (data available from v20.proteinatlas.org). The physiological relevance of the distinct subcellular locations of the GSDM family members and whether it represents their processed form or not is currently unknown. N-GSDM domains of GSDMA3, -D, -E interact with negatively charged phosphoinositides at the inner leaflet of the plasma membrane, but also with the acidic lipid cardiolipin as revealed by binding of N-GSDM to phospholipid strips and membrane mimicking liposomes [21,22,52,53]. Cardiolipin under conditions of cellular stress is exposed at the outer membrane leaflet of bacteria and, in accordance with the endosymbiotic origin of mitochondria, also at the outer mitochondrial membrane. Indeed, it was shown that N-GSDMA, N-GSDMD and N-GSDME target mitochondria facilitating cyt c release [54,55]. Likewise, during LPS-induced NETosis, N-GSDMD in a caspase-11 dependent manner is recruited to the nuclear envelop [30], suggesting that N-GSDMD may participate in nuclear envelop permeabilization allowing release of nuclear DNA. During PMA-induced NETosis, N-GSDMD targets ELANE-containing granules close to the plasma membrane, thereby releasing elastase in the cytosol and propagating plasma membrane permeabilization and release of NETs [31]. Similarly, N-GSDME generated by caspase-3 creates a positive feedback loop expediting apoptosis by facilitating mitochondrial cyt c release leading to apoptosome formation, further propagating caspase-3-mediated GSDME activation and plasma membrane targeting [54]. However, GSDM organelle targeting can be uncoupled from pyroptotic cell death as well. In NLRP3-activated neutrophils, N-GSDMD targets granules resulting in elastase release and inducing formation of $LC3^{+}$ autophagosomes, without

targeting the plasma membrane nor facilitating lytic death [44]. Finally, N-GSDM activation is associated with **autophagy**, a cytoprotective adaptation mechanism to various forms of cellular stress. Expression of N-GSDMA3 and N-GSDMD in HEK293T cells resulted in an increase of the autophagic marker LC3-II next to mitochondria with decreased mitochondrial membrane potential [56], reflecting a possible role in mitophagy. These examples suggest that organelle targeting by GSDMs may precede eventual plasma membrane permeabilization or constitute an adaptive response following cellular stress. Another member of the GSDM family is PJVK that does not induce cell death but fulfils specialized functions in the homeostasis and adaptive responses following peroxisomal stress, explaining its localization at peroxisomal membranes [57,58]. Peroxisomal dynamics are indeed affected in PJVK knockout mice [57] as a result of impaired **pexophagy** [59], a peroxisome-specific form of autophagy [60].

Checkpoints of the cytotoxic function of N-GSDM by specific proteolysis, phosphorylation and exosome formation

Release of C-GSDM is not sufficient for oligomerization of N-GSDM, suggesting that additional regulatory mechanisms are implicated. Indeed, specific proteolysis and phosphorylation events within the N-terminal GSDM domain result in inactivation of their pore-forming function, providing an extra checkpoint functioning as a safeguard mechanism. Caspase-3 cleaves GSDMB and GSDMD at evolutionary conserved D91 and D87 residues, respectively [61,62], thereby generating an inactive p20 fragment instead of a membrane permeabilizing p30 N-GSDM domain (Figure 1A). The inactivating caspase-3 cleavage site is only present in the inflammasome-associated GSDMD and –B proteins, but not in GSDMA, -C

and –E (Figure S2). As such, active caspase-3 generated during apoptotic conditions, may provide a conserved mechanism to prevent GSDMD-mediated pyroptosis and GSDMB's contribution to non-canonical caspase-4 activation [47], allowing apoptosis to occur instead of pyroptosis. This bias towards promoting apoptosis while preventing pyroptosis may favor a cellular fate that results in containment and phagocytic uptake of the cellular corpse, forming an additional mechanism how apoptosis contributes to anti-inflammatory mechanisms by preventing pyroptosis. Similarly, enterovirus 71 (EV71) disrupts N-GSDMD activity by cleavage at the conserved residue Q193 by the viral protease 3C, showing that pathogens may conduct a similar strategy to repress inflammatory and antiviral responses [63]. In that respect, active N-GSDMD was shown to prevent EV71 replication in host cells [63].

Another mechanism inactivating the cytotoxicity of particular GSDMs is by phosphorylation at Thr6 in hGSDME or Thr8 in hGSDMA, preventing oligomerization of their N-terminal domains [54]. This kinase-sensitive threonine residue is only present and highly conserved in GSDMA, -B, -E and PJVK but absent in GSDMC and –D (Figure S2), suggesting that both regulatory mechanisms (inactivating proteolysis and phosphorylation) are shared by some but not all GSDM family members (Figure 1A).

Finally, ESCRT-mediated exosome formation established another protective mechanism against N-GSDM-mediated cell death [64]. Ca^{2+} influx through GSDMD pores, which is one of the first GSDMD-dependent events occurring during the pyroptotic process [65], triggers ESCRT-III proteins to repair the damaged plasma membrane by shedding the perforated plasma membrane areas as exosomes and thus removing the GSDMD pores [64]. In this scenario, only when the ESCRT-III machinery is inhibited or is overpowered by too many

GSDMD pores, a cell will ultimately undergo necrotic cell death. The interaction between GSDMs and ESCRT-mediated protection mechanisms [64] may fine tune release of pro-inflammatory intracellular factors and may even represent a reversible way of GSDM activation. In conclusion, certain GSDMs share highly conserved residues that reflect similar mechanisms of autoinhibition based on hydrophobic interaction between N- and C-terminal domains (GSDMA,-C,-D,-E) (Box 1 and Figure 1) and similar mechanisms of recruitment to plasma membranes (GSDMA-E) (Table 1). Several mechanisms of negative regulation provided by phosphorylation (GSDMA,-B,-E), by alternative proteolytic cleavage within the cytotoxic N-GSDM domain (GSDMB,D) (Figure 1A) and by exosome formation *via* the ESCRT mechanism (GSDMD) serve as back up mechanism to dampen cell death. Also in case of MLKL-induced necroptosis [66,67] and bacterial toxins [68], ESCRT-III dependent detoxification mechanisms have been reported.

Phylogenetic analysis reveals a strong evolutionary variation in GSDM genes

Most gasdermins operate as final executioner molecules of different cell death modalities (apoptosis-driven secondary necrosis, pyroptosis, NETosis). This puts them in the frontline of selective pressure during infection and may explain some remarkable findings in the phylogenetic analysis such as sporadic GSDM gene ablations and numerous gene duplications (Figure 3). The global picture reveals that *GSDME* genes were found in all animals starting with the phylum of Cnidaria (hydroids, jellyfish, anemones, corals), the superphylum of Lophotrochozoa (molluscs, brachiopods, but not in annelids), and Deuterostomata (echinoderms, hemichordates and chordates). Apparently, *GSDM*-like genes are absent in the whole superphylum of Ecdysozoa including arthropods and nematods. This

almost ubiquitous presence of *GSDME* is probably related to its function as an executioner of apoptosis-driven secondary necrosis and pyroptosis. Indeed, biochemical and cellular studies revealed that coral GSDME is activated by caspase-3 cleavage and elicits pyroptosis following bacterial infection [69], representing the most ancestral function of GSDMs.

The PJVK gene emerged first in the subphylum of the Vertebrata, starting with the Cyclostomata (lamprey) and is found ubiquitously in fish, amphibians, reptiles, birds and mammalians. The phylogenetic tree reveals that it occurred by gene duplication from the ancestral *GSDME* gene. PJVK differs from other GSDMs in the sense that it has no pore forming capacity, while it acquired a novel unrelated function in peroxisome homeostasis [57]. *PJVK* mutants (a.o. DFNB59) in humans have been associated with noise-induced ROS-damage of hair cells and auditory neurons due to non-functional **pexophagy** [57,58]. This function of PJVK in hair cells and auditory neurons coincides with the evolution of a complex inner ear system in vertebrates, starting with the cyclostomes (lampreys) [70,71].

The next bifurcation in the evolution of the GSDM family is the occurrence of the *GSDMA* gene cluster in a few species of fish, and reptile, bird and mammalian species, while apparently lacking in amphibians. The *GSDMB* gene, located on the same chromosome as *GSDMA* gene, occurred by gene duplication of the *GSDMA* gene within marsupials (Metatheria) and placentals (Eutheria), together with two other gasdermin genes, *GSDMC* and *GSDMD*. This implies that fish, amphibians, reptiles, birds and platypus (an ancestral egg laying mammalian) lack the prototype inflammasome-activated GSDMD. In platypus a *GSDMD* gene has been annotated but appears in the phylogenetic cluster of *GSDMA* genes, suggesting it may result from a *GSDMA* gene duplication. That fish, amphibians, reptiles, birds and platypus lack GSDMD suggests that inflammasome-dependent pyroptosis in these

283 clades may be accomplished by GSDME alone or in combination with GSDMA. Indeed, in
284 case of fish (teleosts) it was reported that fish GSDME during infection and tissue damage
285 can be activated both by caspase-1 representing inflammasome-mediated activation leading
286 to pyroptosis and by caspase-3, representing an executioner role in pyroptosis and
287 apoptosis-driven secondary necrosis [72–74]. Similar double functions of GSDME during
288 pyroptosis and apoptosis may occur also in other clades lacking GSDMD (cnidarians,
289 molluscs, echinoderms, hemichordates, lampreys), as was shown experimentally in case of a
290 coral species [69].

291 In marsupials (Metatheria) and placentals (Eutheria) an additional bifurcation of *GSDMA*
292 genes led to the occurrence of the *GSDMB* gene. GSDMB is involved in regulating non-
293 canonical pyroptosis as a direct activator of caspase-4, but is also negatively regulated by the
294 latter [47]. In opossum, an explosion of *GSDMA* genes occurred, most of which annotate
295 within the *GSDMB* phylogenetic cluster, and may therefore in fact belong to the latter class.

296 The occurrence of *GSDMB, GSDMC* and *GSDMD* genes in marsupials and placentals, and the
297 many gene amplifications of *GSDMA* and *GSDMC* in partiular species (such as mice, but not
298 in rats) argue for a strong evolutionary pressure favouring duplication and amplification of
299 these genes. On the other hand, some orders and species completely lost *GSDMB, GSDMC*
300 and *GSDMD* genes. There is an apparent loss of the *GSDMC* gene in several mammals that
301 returned independently to the sea, representing a possible example of parallel evolution.
302 Whales, but not dolphins, walrus and earless seals independently lost GSDMC, possibly as an
303 adaptation to a different pathogen exposure associated with the return to sea life in which
304 GSDMC-mediated responses may have been counter selected. However, this gene loss did

not happen in the sea lions, fur seals and sea otter, questioning the general applicability of this return to the sea hypothesis.

In the monophyletic clade of rodents, lagomorphs, treeshrews, colugos and primates (Euarchontoglires) *GSDMB* is absent in the branch that includes mice, rats and rabbits, while it is present in the branch that delivered flying lemurs, tree shrew and primates, [68]. In mice, but not in rats or rabbits, *Gsdma* duplicated twice (*Gsdma1, Gsdma2, Gsdma3*) and *Gsdmc* duplicated even thrice (*Gsdmc1, Gsdmc2, Gsdmc3, Gsdmc4*). While the selective forces responsible for these gene losses and multiple gene duplications remain elusive, they feed the speculation that they may be associated with particular exposure to infectious microorganisms or viruses. In line with this hypothesis, GSDMA and –C are mainly expressed at sites of pathogen entry such as skin (GSDMA) and esophagus, stomach, cervix and vagina (GSDMC) (Figure 2). Some species (not all) of the Chiroptera (microbats) have lost the prototype pyroptotic *GSDMD*. Therefore it is tempting to speculate that absence (reptiles, birds) or loss (microbats) of *GSDMD*, although potentially compensated by other GSDMs, may explain why both birds and bats function as primary reservoirs for zoonotic viruses such as influenza A virus in birds and coronaviruses, hepaciviruses, pegiviruses and Ebola virus in bats [75,76]. Dampened Nlrp3 inflammasome responses have been hypothesized as an immunological explanation why bats can host many viruses without apparent pathological consequences for the host [77]. The absence of GSDMD may allow propagation of viruses without devastating immune responses in these reservoir species, facilitating viral transmission to other species [77].

Altogether, our phylogenetic analysis suggests that from gene duplication events in the Mammalia, except for the Prototheria, have evolved an extended set of gasdermin genes on

top of the G*SDME* and *PJVK* genes: GSDMB by duplication of GSDMA in the same gene cluster, and further duplication of *GSDMC and GSDMD* in a next gene cluster. Although occurring in different phylogenetic clusters, both GSDMB and GSDMD are implicated in regulation of inflammasome-mediated pyroptosis, the former as an amplifier of caspase-4 activation [42] and the latter as the executioner of pyroptosis.

Most likely, evolution provided redundancy in the GSDM gene family to ensure pyroptotic cell death following cellular stress and infection, and the generation of an immunogenic environment to cope with associated threats. This implies that GSDM membrane targeting mechanisms may have a primary role as conduit for intra- and intercellular signalling following stress and infection preceding the cell death process. In that respect specific marking of organelles for pexophagy, **mitophagy** or **nucleophagy**, may be considered when studying the non-cell death related functions of GSDM family members. Furthermore, the high conservation of aspartate cleavage sites in the hinge region between the N-GSDM pore forming domain and the C-GSDM regulatory domain emphasizes the importance of caspase-dependent cleavage in their evolutionary selective function (Figure S2). The same applies for the highly conserved protective threonine-residue (Figure S2), reflecting the need for a tight regulation of these deadly proteins.

Concluding remarks

Functional GSDME was shown already in corals [69], suggesting that it fulfilled ancestral functions as final executioner of apoptosis-driven secondary necrosis and pyroptosis. The first gene amplification with the occurrence of the *PJVK* gene in Cyclostomes and all higher

Vertebrates illustrates a second set of functions of GSDM family proteins in adaptive responses following organelle stress, marking stressed peroxisomes [57,59]. In more complex organisms in the animal kingdom starting from the Vertebrates, a combination of the need for specific execution mechanisms in particular cell types and their localization in particular organelles such as nucleus, mitochondria, granules, autophagosomes and peroxisomes (Table 1) may be reflected by the amplification of gasdermin genes. The organelle-specific functions need further research to reveal the molecular mechanisms implicated. The critical importance of GSDM activation is reflected by the fact that the two most common GSDME and GSDMD are a point of convergence for activation by different proteases (caspases, elastases, granzymes, cathepsins) as a point of integration of adaptive responses following infection or cellular stress, and explaining the high conservation of cleavage sites in the hinge region between the N-GSDM membrane permeabilizing domain and the C-GSDM regulatory domain (Figure S2). Moreover, additional checkpoints of GSDM functioning include negative regulation by phosphorylation of conserved threonine residues (GSDMA, -B, -E and PJVK) and proteolytic inactivation by caspase-3 or viral protease 3C (GSDMB and –D), reflecting the need for fine-tuning and dampening after activation [54,61–64]. Also the functional interaction with ESCRT-III reflects the need for a dampening system following GSDM activation [64].

Bearing in mind that particular gene ablation and extensive *GSDM* duplications have occurred in particular taxa (some rodents, microbats and mammals returned to the sea) (Figure 3) may reflect a high evolutionary pressure associated with new habitats that have shaped species-specific balances of GSDMs, but also illustrates the high redundancy of some GSDM members compensating the loss. The restricted expression pattern of some GSDM family members in normal conditions might be misleading and may hide important adaptive

functions of GSDMA, -B, and -C during infection and cellular stress, as was recently shown for GSDMC showing upregulated expression and execution of pyroptosis-like cell death during hypoxic stress [20].

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Glossary Box

Autophagy: A biological process that involves the enzymatic breakdown of a cell's cytoplasm or cytoplasmic components (such as damaged or unneeded organelles or proteins) within the lysosomes of the same cell.

Canonical inflammasome activation: Canonical inflammasome activation involves cytosolic detection of pathogen-associated molecular patterns (PAMPs) or damage/danger-associated molecular patterns (DAMPs), followed by the formation of inflammasome complexes leading to caspase-1 dependent processing of GSDMD as well as the pro-forms of IL-1β and IL-18, culminating in pyroptosis and cytokine secretion.

Gasdermin: Gasdermin (GSDM) is a member of the gasdermin protein family, characterized by a conserved gasdermin-domain at the N-terminal end (N-GSDM). Release of N-GSDM from the autoinhibitory C-terminal end (C-GSDM) by specific proteolysis or other yet to be determined mechanisms results in organelle membrane translocation and plasma membrane recruitment and permeabilization, contributing to necrotic cell death modalities.

Mitophagy: Type of specialized macroautophagy that selectively recognizes damaged or stressed mitochondria and targets them to lysosomes for degradation.

NETosis: Regulated necrotic cell death fate characterized in neutrophils following contact with PAMPs leading to the release of neutrophil extracellular traps (NETs) consisting of decondensed chromatin and granular contents to the extracellular space ensnaring extracellular pathogens.

Non-canonical inflammasome activation: Non-canonical inflammasome activation involves cytosolic detection of LPS derived from a Gram-negative infection leading to activation of caspase-11 in mice and caspase-4/5 in humans, after which caspase-4/5/11 directly cleaves

GSDMD and initiates pyroptosis without the need for caspase-1 activity. Caspase-1 is activated secondary to GSDMD pore formation and subsequently facilitates maturation and secretion of IL-1β and IL-18.

Nucleophagy: Type of specialized macroautophagy that selectively recognizes damaged or stressed nuclear envelopes and targets them to lysosomes for degradation.

Pexophagy: Type of specialized macroautophagy that selectively recognizes damaged or stressed peroxisomes and targets them to lysosomes for degradation.

Pore formation: Membranous conformational changes resulting in membrane permeabilization due to amphipathic interaction of membrane targeting proteins or peptides with cellular (plasma) membranes.

Primary necrosis: Immediate regulated necrotic cell death fate without preliminary signs of apoptosis as opposed to apoptosis-driven secondary necrosis.

Pyroptosis: Current definition: GSDM-mediated cell death. Former definition: Regulated primary necrotic cell death fate associated with infection and induced by canonical or non-canonical inflammasome activation resulting in caspase-1/4/5/11-mediated activation of GSDMD as well as IL-1β and IL-18 maturation and secretion.

Regulated necrosis: Necrotic cell death fate involving active mechanisms of plasma membrane permeabilization such as protease-dependent gasdermin activation (secondary necrosis, pyroptosis), kinase-dependent MLKL activation (necroptosis) or lipid peroxidation (ferroptosis). The morphology is characterized by cellular swelling (oncosis) and plasma membrane permeabilization.

Secondary necrosis: Regulated necrotic cell death fate following caspase-dependent apoptosis. Occurs *in vitro* and *in vivo* in the absence of phagocytic cell capacity. Recently, plasma membrane permeabilization during secondary necrosis has been associated with caspase-3-mediated GSDME activation.

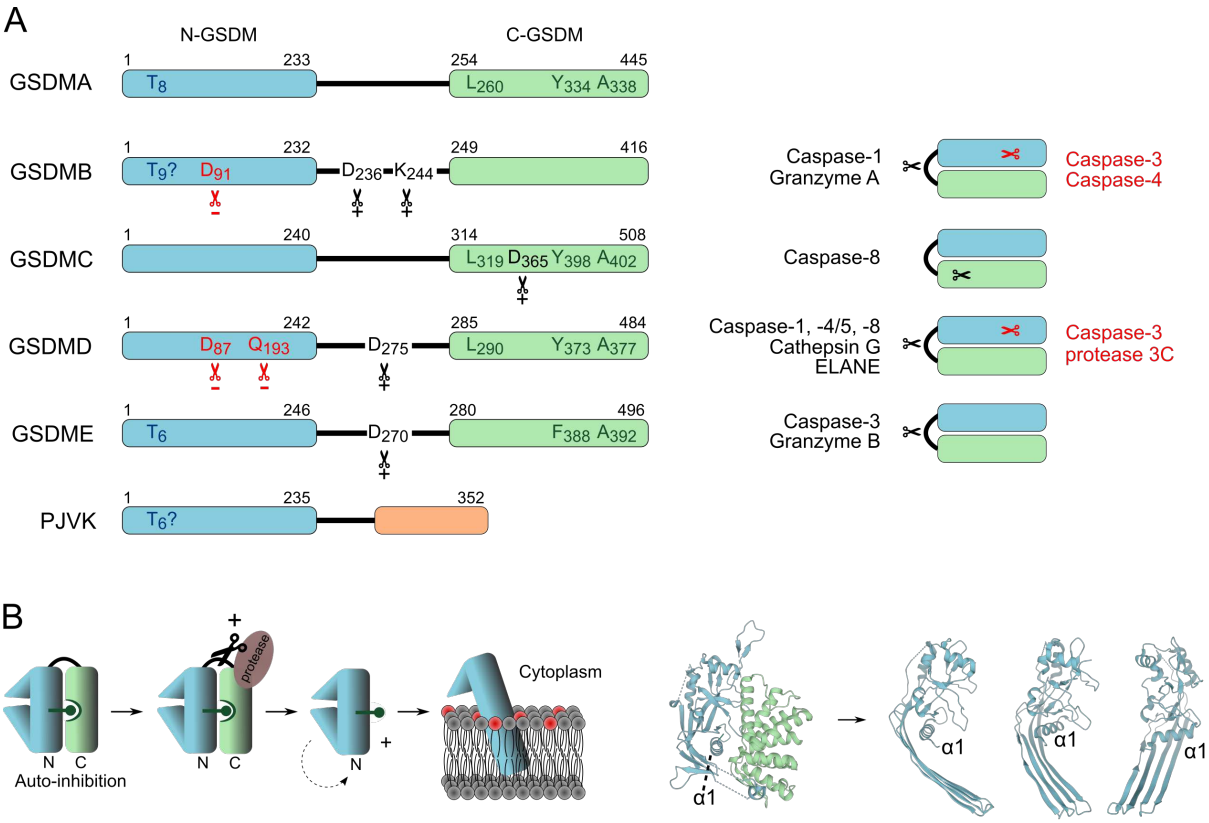
Box 1

Mechanisms of auto-inhibition and release of N-GSDM from C-GSDM

The N- and C-terminal domains of unprocessed GSDM are kept in a closed autoinhibitory conformation. The crystal structure of GSDMA3 revealed that auto-inhibition is provided by two fitting hydrophobic interfaces and two regions of hydrogen bonds between N-GSDMA3 and C-GSDMA3 [22,78]. The hydrophobic interaction residues are highly conserved in the gasdermin family (GSDMA: L260, Y334, A338; GSDMA3: L270, Y344, A348; GSDMC: L319, Y398, A402; GSDMD: L290, Y373, A377; GSDME: F388, A392) (Figure 1A). Mutation of these residues even resulted in cytotoxicity of full-length GSDMA, -A3, -C, -D and -E after transient transfection in HEK293T cells [22], suggesting that the mechanism of auto-inhibition is shared between these members of the gasdermin family. For GSDMA, GSDMD and GSDME, the release of the auto-inhibitory C-terminal domain is required because the full-length proteins are not able to bind negatively charged phospholipids including phosphoinositides and cardiolipin [21,22,53]. However, the membrane recruitment mechanisms of GSDMB and PJVK are very different. N-GSDMB cannot bind cardiolipin but instead targets phosphoinositides and sulfatide (Table 1). Moreover, the GSDMB C-terminal domain is not auto-inhibitory because it lacks the self-inhibitory hydrophobic residues mentioned above [62,78], allowing a more open conformation in its unprocessed form. As shown for GSDMA3,

release from C-GSDM facilitates a drastic conformational change of N-GSDM, resulting in an open, elongated structure characterized by a large β-sheet composed of four intact β-strands (Figure 1B), crucial for membrane insertion [23]. Electrostatic binding to negatively charged phospholipids is conducted by a positively charged pocket between the α1 helix and inserting β-sheet of the open conformation that is shielded by C-GSDM in the closed conformation. Basic arginine and lysine residues (R9, R13 in GSDMA3) in the α1 helix are responsible for cardiolipin binding and are conserved among all GSDMs, including PJVK [22,23,78]. Nevertheless, this positively charged patch cannot explain the distinct binding of GSDMs to various lipids suggesting that other not yet defined lipid binding sites may be present or that distinct patches formed by oligomerization are required for membrane targeting.

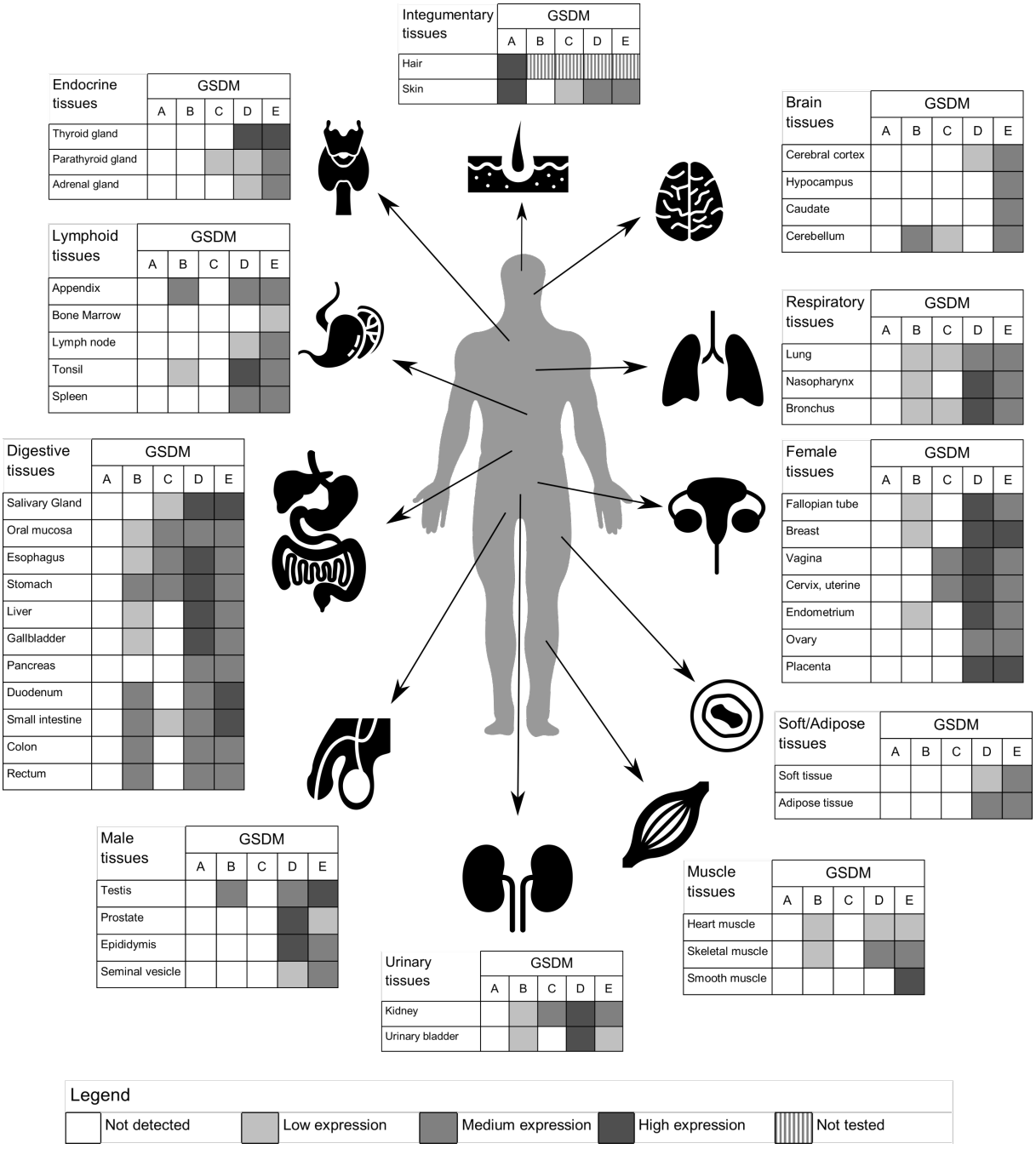
Proteolytic cleavage in the hinge region (GSDMD and –E) or in C-GSDM (GSDMC) in order to expose N-GSDM requires docking of a protease on the unprocessed closed form of GSDM. In case of GSDMD the mechanism has been explored and involves an additional hydrophobic groove provided by a set of highly conserved residues in C-GSDMD (L304, L308, V364 and L367) (Figure S3). These hydrophobic residues are crucial for docking of activated caspases-1/4/11 through its small enzymatic domain (p10) followed by cleavage in the hinge region ($FLTD_{275}$) [79]. Sequence alignment between GSDMD and other GSDMs reveals that this hydrophobic docking station for caspase-1/4/11 in C-GSDMD apparently is a unique feature of GSDMD (Figure S3). Therefore, proteolytic activation of GSDMD by other proteases such as caspase-8, ELANE and cathepsin G (Table 1) probably involves other yet unrevealed protease docking stations in GSDMD and other GSDMs.



669

670 **Figure 1: Schematic overview of the conserved structure and regulatory residues of the**
671 **GSDM proteins.** Sequences of the putative GSDMA-E and PJVK homologs were aligned using
672 Clustal Omega (v1.2.4) and adapted in JalView (v2.10.5). The schematic overview is based on
673 the crystal structures of GSDMA3 (PDB: 5B5R) [22] and N-GSDMA3 (PDB: 6CB8) [23]. (A)
674 GSDMA-E contain a membrane permeabilizing domain (N-GSDM) (blue) and inhibitory
675 domain (C-GSDM) (green). In case of PJVK the latter is replaced by a zinc finger domain
676 (orange). Interaction between N-GSDM and C-GSDM is provided by conserved hydrophobic
677 residues (dark green) forming a hydrophobic groove in C-GSDM (green). Phosphorylation of
678 Thr6 inhibits membrane permeabilization by GSDME [54]. A conserved Threonine residue
679 (dark blue) is found as well in GSDMA, -B and PJVK, but in case of the latter two this is a
680 putative regulatory site based on location and indicated by '?'. Activating (black) and
681 inactivating (red) cleavage sites are indicated by scissors with '+' and '-' symbols,
682 respectively. Inactivating cleavage sites D91 and D87, are conserved in GSDMB (caspase-3, -

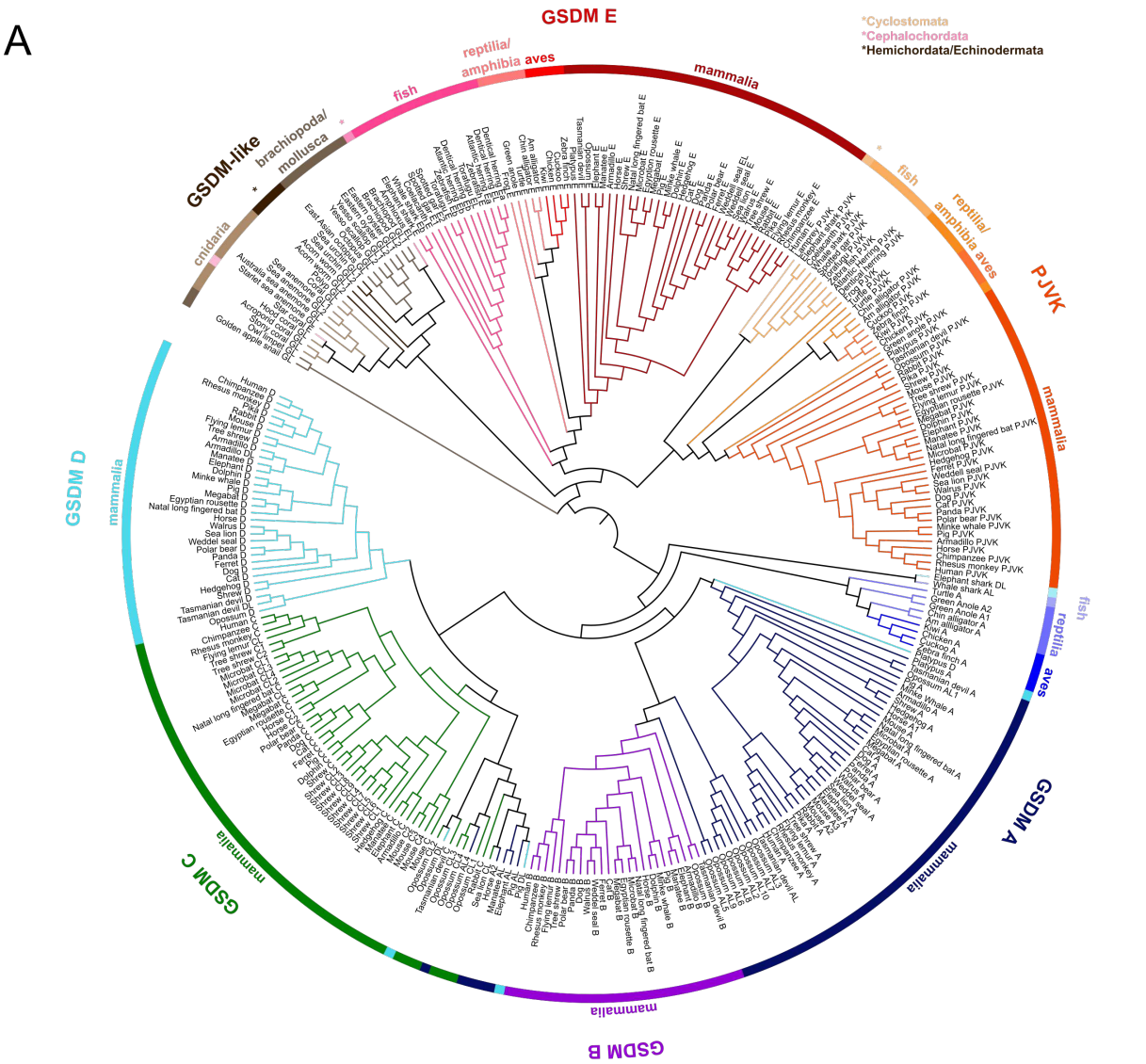
683 4) and –D (caspase-3), respectively. Similarly, viral protease 3C cleaves GSDMD at the
684 conserved site Q193. GSDMB, -D and –E are proteolytically activated by cleavage in the
685 hinge region. GSDMB is cleaved by caspase-1 and granzyme A at D236 and K244,
686 respectively. Human caspase-1/4/5/8 cleave GSDMD at D275. Nor the human ELANE
687 cleavage site C268 nor cathepsin G cleavage site L273 in GSDMD are conserved. Both human
688 caspase-3 and granzyme B cleave GSDME at D270. In addition, caspase-8 activates GSDMC at
689 D365 within C-GSDMC. (B) N-GSDM destabilizes the plasma membrane after interaction of
690 basic residues in the α1 helix with negatively charged phospholipids (red).

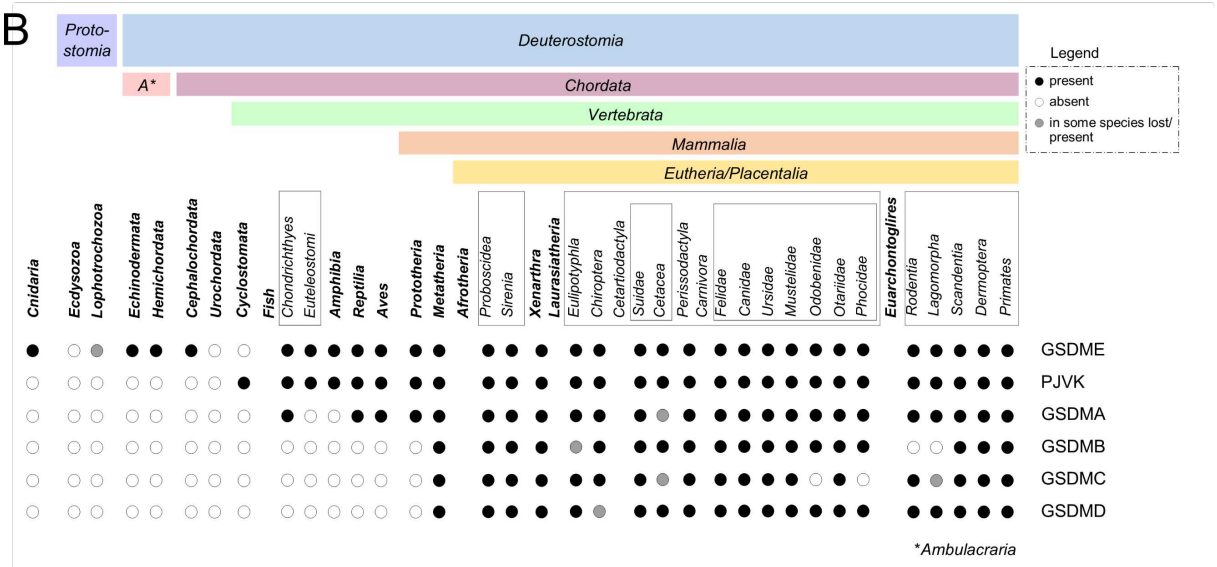


691

692 **Figure 2: Protein expression overview of GSDMA-E in humans according to The Human**
693 **Protein Atlas.** Grayscale represents weighted and arbitrary annotation of cellular protein
694 levels based on immunohistochemistry staining of tissues (intensity and relative fraction of
695 positive cells) as described by the Human protein Atlas. Processing of the 3,3'-
696 diaminobenzidine substrate by HRP linked to the secondary antibodies resulted in brown
697 staining and the sections were furthermore counterstained with haematoxylin to enable

visualization of microscopical features. All images of tissues stained by immunohistochemistry were annotated manually and can be found at v20.proteinatlas.org.


A
GSDM E
*Cyclostomata
*Cephalochordata
*Hemichordata/Echinodermata
fish
reptilia/
amphibia
aves
mammalia
brachiopoda/
mollusca
GSDM-like
cnidaria
PJVK
GSDM A
GSDM B
GSDM C
GSDM D


B
Proto-
stomia
Deuterostomia
A*
Chordata
Vertebrata
Mammalia
Eutheria/Placentalia
Legend
present
absent
in some species lost/
present
Cnidaria
Ecdysozoa
Lophotrochozoa
Echinodermata
Hemichordata
Cephalochordata
Urochordata
Cyclostomata
Fish
Chondrichthyes
Euteleostomi
Amphibia
Reptilia
Aves
Prototheria
Metatheria
Afrotheria
Proboscidea
Sirenia
Xenarthra
Laurasiatheria
Eulipotyphia
Chiroptera
Cetartiodactyla
Suidae
Cetacea
Perissodactyla
Carnivora
Felidae
Canidae
Ursidae
Mustelidae
Odobenidae
Otariidae
Phocidae
Euarchontoglires
Rodentia
Lagomorpha
Scandentia
Dermoptera
Primates
GSDME
PJVK
GSDMA
GSDMB
GSDMC
GSDMD
*Ambulacraria

700

Figure 3: Phylogenetic analysis of GSDMA-E and PJVK Homologs. (A) A phylogenetic analysis was done on the publicly available genome assemblies of the indicated species for the presence or absence of GSDMA-E and PJVK proteins by utilizing the BLASTP algorithm against the predicted proteomes of these species. The presence or absence of these proteins was validated by a BLAST search of conserved sequences against the genome assemblies in combination with an evaluation of the completeness of the genomic context in the ENSEMBL, NCBI, and UCSC genome browsers. Species in each clade from which the genomes were investigated can be found in Table S2. If all of the above-mentioned analyses were negative, a gene was considered absent. The protein sequences of the putative GSDMA-E and PJVK homologs were aligned using Clustal Omega (v1.2.4) and the data are presented as unrooted circular phylogenetic tree by maximum likelihood using Mega (Molecular Evolutionary Genetics Analysis v.10.2.4). Final phylogenetic tree was edited with iTOL (Interactive Tree Of Life v5.7). (B) Presence and absence of GSDMA-E and PJVK homologs in the Animal Kingdom based on the phylogenetic tree.

	GSDMA	GSDMB	GSDMC	GSDMD	GSDME	PJVK	References
Chromosomal location							
Human	*GSDMA*: chr17q21.1	*GSDMB:* chr17q21.1	*GSDMC*: chr8q24.21	GSDMD: chr8q24.3	*GSDME*: chr7p15.3	*PJVK*: chr2q31.2	
Mouse	*Gsdma1, Gsdma2, Gsdma3*: chr11D	-	*Gsdmc1, Gsdmc2, Gsdmc3, Gsdmc4*: chr15D1	*Gsdmd*: ChrD3	*Gsdme*: chr6B2.3	*Pjvk*: chr2.3	
Domain							
Gasdermin N (N-GSDM)	+	+	+	+	+	+	[2,32]
Gasdermin_C (C-GSDM)	+	+	+	+	+	-	[32]
Zinc finger	-	-	-	-	-	+	[32]
Cytotoxicity							
Full length	-	-	-	-	-	-	[8,17,22,53,54]
N-GSDM	+	+	+	+	+	-	[8,17,22,53,54]
Activating proteolytic cleavage	ND	Caspase-1 Granzyme A	Caspase-8	Caspase-1 Caspase-4/5 Caspase-8 Cathepsin G ELANE	Caspase-3 Granzyme B	ND	[8,17,40–42,45,48,53,80,81,18–21,28,29,31,37]
Membrane targeting	Plasma membrane	ND	ND	Plasma membrane Nucleus Mitochondria Neutrophil granules $LC3^+$ autophagosomes	Plasma membrane Mitochondria	Peroxisomes	[22,31,32,37,44,52–55,82]
Lipid binding							
Full length	-	Phosphoinositides Phosphatidic acid	ND	-	-	ND	[21,22,53,62]

			Phosphatidylglycerol sulfatide					
	N-GSDM	Phosphoinositides Cardiolipin Phosphatidic acid Phospatidylserine	Phosphoinositides Phosphatidic acid Phosphatidylglycerol sulfatide	ND	Phosphoinositides Cardiolipin Phosphatidic acid	Phosphoinositides Cardiolipin Phosphatidylserine	ND	[21,22,53,62]

715

716 **Table 1: Biological and biochemical properties of the GSDM protein family.** ND: not determined.