PNAS COMMENTARY

Homing in on gasdermins: How fungi regulate cell death

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Members of a protein family named gasdermins (GSDMs) have recently been shown to form membrane pores and thereby induce a lytic type of cell death. In PNAS, Clavé et al. (1) now demonstrate that proteolytic cleavage, the mechanism governing the activation of vertebrate GSDMs, is also conserved in fungi. Intriguingly, the proteases that putatively cleave fungal GSDMs are related to caspases, the proteases mediating GSDM activation in vertebrate cells.

The GSDMs are a protein family with six members in the human system: GSDMs A through E and Pejvakin (PJVK) (2). Prior to 2015, GSDMs were known to play a role in a number of diseases including hearing loss (3, 4) and alopecia (5). Additionally, GSDM genes were known to be silenced in cancer cells (6). However, their molecular function remained unclear. This changed in 2015, when two groups identified human GSDMD as the long-sought mediator of pyroptosis, a lytic, highly inflammatory form of regulated cell death (7, 8). These and subsequent studies clarified that GSDMs are pore-forming proteins that can permeabilize lipid membranes, ultimately inducing membrane rupture (9, 10). Pyroptosis, as an innate immune response, is triggered in a cell-intrinsic manner by cytosolic proteins, which include the evolutionarily ancient NOD-like receptors (NLRs). Vertebrate NLRs can oligomerize into complexes called "inflammmasomes" which serve as activation platforms for caspases, proteases that in turn cleave GSDMs and thereby release their pore-forming N-terminal fragment from the inhibitory C terminus (11). In addition to caspases, GSDMB is cleaved by granzyme A, a protease released by activated cytotoxic T cells into their target cells to induce cell death (12). GSDMA has recently been reported to induce pyroptosis in keratinocytes after cleavage by SpeB, a protease secreted by the bacterium *Streptococcus pyogenes* (13). Excessive pyroptosis promotes sepsis, a deadly condition that can be a consequence of systemic bacterial infection (7). Of note, cells can apparently sustain some amount of GSDM pore formation, and scenarios have been described in which cell lysis following GSDM activation can be averted, for example through increased membrane damage repair (14, 15).

The first nonvertebrate GSDM homolog has been described in fungi, where it is involved in a process called vegetative incompatibility (VI), also known as heterokaryon incompatibility (16, 17). Similar to pyroptosis in vertebrates, VI is a cell-intrinsic response to nonself that has been found in different fungal species including ascomycetes and basidiomycetes. As part of their life cycle, during sexual reproduction fungi can form heterokaryons, cells that contain two genetically distinct nuclei. Fusion of genetically incompatible fungal hyphae triggers VI, leading to the death or growth arrest of affected cells, and ultimately keeping incompatible colonies separate. Among other advantages, maintaining individual identities in fungi is thought to protect against the spread of mycoviruses and resource plundering by aggressive genotypes (16). In contrast to vertebrate pyroptosis, which is a response to heterospecific nonself recognition, VI occurs upon conspecific nonself recognition, also termed allorecognition. While the mechanisms of VI are not yet entirely clear, some of its features have been described. The genetic basis of VI is formed by a number of polymorphic genetic loci, the *het* or *vic* loci. For example, in the ascomycete *Podospora anserina* the het-s/het-S system involves prion-like activation of het-S by het-s, after which het-S inserts into and oligomerizes in membranes, ultimately weakening membrane integrity. Of note, het-S can also be activated by an NLR, NWD2 (18).

Working in *P. anserina*, Clavé et al. (1) find that the polymorphic het-Q locus, an allorecognition determinant, contains one of two alleles, het-Q1 or het-Q2. Genetic complementation and deletion experiments confirm that cells expressing both alleles display reduced viability. Further analyses show that the two alleles encode very different proteins: het-Q2 is similar to subtilisin-like proteases, while het-Q1 is related to RCD-1, a GSDM homolog from *Neurospora crassa* previously characterized by the authors (17). It is then hypothesized that cleavage of het-Q1 by het-Q2 induces cell death, thus mediating het-Q–based VI. Indeed, het-Q1 is cleaved when mixed with het-Q2–containing but not Δhet-Q2 lysates (Fig. 1). Mirroring vertebrate GSDMs, transformants expressing a C-terminally truncated construct of het-Q1 approximately matching the het-Q2 cleavage site exhibit reduced viability. Congruently, the authors observe lytic cell death only when het-Q1 is expressed in the presence of functional het-Q2 in the heterologous fungus *Saccharomyces cerevisiae*. Moreover, cleaved, but not full-length, het-Q1 is found in high-molecular-weight complexes, possibly indicating pore formation. het-Q1 expression also induces cell death in human HEK-293T cells in the presence of functional het-Q2, but not in the absence of het-Q2 or when cleavage is prevented by mutation of the cleavage site in het-Q1. Finally, since the authors previously described genomic clustering of cell-death-regulating genes in fungi, they compare the genomic landscape of 1,884 *P. anserina* het-Q1 homologs across 401 fungal species. In contrast to the idiomorphic constellation in

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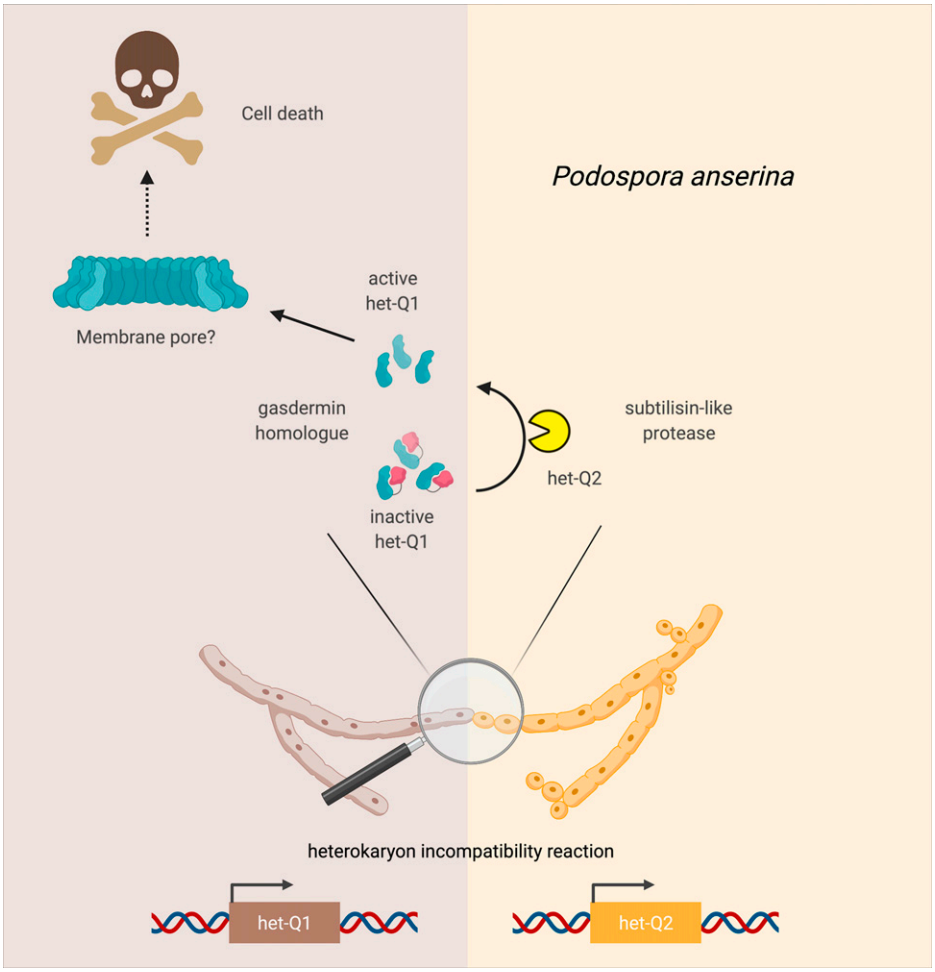

Fig. 1. A schematic view of the heterokaryon incompatibility reaction in *P. anserina*. Fusion of genetically incompatible fungal hyphae triggers cell death due to the expression of the het-Q1 and het-Q2 gene products. het-Q2 encodes a protease that cleaves the cytosolically latent het-Q1 molecule, which subsequently results in cell death. In light of the homology of het-Q1 to GSDM family members, it is reasonable to assume that cleaved het-Q1 molecules assemble into a pore-like structure to execute cell death.

P. anserina, the authors find that for around 80% of these het-Q1 homologs at least one gene encoding a putative protease domain can be located within a proximity of 10 kb. Such a scenario opens the possibility that these genes are part of a heterospecific nonself recognition system employing a dedicated receptor as in vertebrate cells. The authors even identified two such systems encoding distant het-Q1 homologs in *P. anserina*, which were not activated by het-Q2, indicating that different protease specificities could control functionally separate GSDMs. Intriguingly, while most proteases contained the subtilisin-like S8 serine protease domain found in het-Q2, 19% of the 1,884 het-Q1 homologs had at least one protease of the Caspase HetF associated with Tprs (CHAT) family in their vicinity. This family contains caspases, the proteases mediating activation of vertebrate GSDMs. In addition, some of the subtilisin-like proteases contained central P-loop containing NOD domains, a defining feature of NLRs. For one two-gene pair from *P. anserina*, the authors demonstrate that transformation of cells with the GSDM homologue and a truncated protease gene encoding a spontaneously active protease results in cleavage of the GSDM and some cell death as evidenced by a barrage reaction.

Taken together, the findings by Clavé et al. (1) indicate that GSDM-mediated cell death is an ancient defense mechanism and that its regulation by proteolytic cleavage is likely equally old. In this respect it will be interesting to uncover the molecular activation mechanism of RCD-1, a GSDM from *N. crassa* that has previously been characterized by the authors and does not seem to be activated by proteases: RCD-1 is also part of an allorecognition system in which the two alleles *rcd1-1* and *rcd1-2* are exclusive and induce lytic cell death when coexpressed even in human HEK-293T cells (17). The authors found no evidence of cleavage of either protein, but from experiments with inactive chimeric fusion proteins of either RCD1 protein with GFP they conclude that both RCD1 proteins are equally capable of inducing cell death. Recently, another study reported on the structural conservation of GSDMs in bacteria (19): Similar to their fungal and mammalian counterparts, bacterial GSDMs (bGSDMs) are involved in protection against pathogens, activated through cleavage, and they execute their protective function by mediating cell death via their N-terminal domain. As in fungi, the proteases activating bGSDMs are also frequently fused to NACHT domains. bGSDMs are also often found in the genomic vicinity of caspase-like proteins. Of note, the inhibitory C-terminal domain of both bacterial and

fungal GSDMs (5 kDa) is considerably smaller than the corresponding domain in most vertebrate and invertebrate GSDMs (22 kDa) (19). The potency of different GSDMs also appears to vary: While human GSDMs are reported to induce complete lysis, and bacterial GSDMs strongly restrict growth and induce pore formation as measured by propidium iodide uptake, 50% of fungal het-Q1 transformants remained viable when expressing the het-Q1 N terminus in the study by Clavé et al. (1). It will be interesting to find out whether the properties of the individual GSDM molecules or other cellular factors are the cause of these different responses. In addition, the authors' findings raise the possibility that fungal protease-GSDM systems are involved in heterospecific nonself sensing, similar to the corresponding systems in vertebrates. Future research could focus on the triggers that activate these systems.

The conservation of innate immune pathways down to unanticipated evolutionary ages is an exciting research direction that has been fueled by several ground-breaking discoveries in the recent past (20, 21). This line of research will certainly continue to provide intriguing insights into the function and relevance of different defense mechanisms in the future.

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