Cell Death and Differentiation (2011) 18, 1279–1288

www.nature.com/cdd

Review

Metacaspases

L Tsiatsiani[1,2,3,4], F Van Breusegem[1,2], P Gallois[5], A Zavialov[6], E Lam[7] and PV Bozhkov*[,8]

Metacaspases are cysteine-dependent proteases found in protozoa, fungi and plants and are distantly related to metazoan caspases. Although metacaspases share structural properties with those of caspases, they lack Asp specificity and cleave their targets after Arg or Lys residues. Studies performed over the past 10 years have demonstrated that metacaspases are multifunctional proteases essential for normal physiology of non-metazoan organisms. This article provides a comprehensive overview of the metacaspase function and molecular regulation during programmed cell death, stress and cell proliferation, as well as an analysis of the first metacaspase-mediated proteolytic pathway. To prevent further misapplication of caspase-specific molecular probes for measuring and inhibiting metacaspase activity, we provide a list of probes suitable for metacaspases.
Cell Death and Differentiation (2011) **18,** 1279–1288; doi:10.1038/cdd.2011.66; published online 20 May 2011

More than a decade ago, an end was put to the until-then frustrating hunt for the presence of caspase orthologues in plants and other non-metazoan organisms. Based on predicted structural homologies with the catalytic domain of caspases, metacaspase sequences were identified in protozoa, fungi and plants.[1] Phylogenetic analysis indicated that eukaryotic caspases, metacaspases and paracaspases are about equally distant from each other and together with the legumains, separases and the bacterial clostripains and gingipains, they are classified within the clan CD of Cys proteases.[2] A common structural feature of this clan is the presence of a caspase/hemoglobinase fold.[3]

Two types of metacaspases can be distinguished. Type I metacaspases have a N-terminal prodomain containing a proline-rich repeat motif and, in plant members, also a zinc-finger motif. Type II metacaspases lack such a prodomain but harbor a linker region between the putative large (p20) and small (p10) subunits. In protozoa and fungi only type I metacaspases are found, whereas plant genomes encode both types with frequent prevalence of type I over type II by gene numbers (Figure 1).

Besides a few exceptions having a catalytic Ser residue instead of a Cys,[4] metacaspases, like caspases have a His-Cys catalytic dyad in their predicted active site, with the Cys residue acting as a nucleophile for substrate peptide bond hydrolysis. For most metacaspases studied so far, enzyme maturation involves autocatalytic processing of the zymogen;[5–8] however, in some cases this step is not necessarily required for proteolytic activation.[9–11] The most striking biochemical feature of all metacaspases that distinguishes them from caspases is a strict Arg and Lys substrate specificity.[5,6,10,12,13]

In *Arabidopsis* studies, metacaspases have been described with two distinct nomenclatures. The Van Breusegem lab used the five-character name AtMC1–AtMC9 (*Arabidopsis thaliana* Metacaspase) whereas the Lam lab used the seven-character name AtMCP1a–AtMCP1c (*A. thaliana* Metacaspase) for the type I and AtMCP2a–AtMCP2f for the type II metacaspases. In order to avoid confusion and for the reasons of simplicity we have decided to establish a uniform nomenclature of *Arabidopsis* metacaspases, namely AtMC1–AtMC9, which we urge to be adopted by other researchers in future publications and discussions (Table 1).

The first decade of metacaspase research documented by more than 130 publications is over now. It has brought exciting information about biological functions of metacaspases and the first attempts to uncover molecular mechanisms of metacaspase action. This period was also marked by vast contradictions within the field, mainly because of different opinions about biochemical and functional relatedness between caspases and metacaspases.[2,14–16] Here we review the essential information related to the biochemistry and function of metacaspases. In addition, we detail some of the important tools that are necessary to specifically dissect the role and activity of this highly conserved group of proteases.

Multifunctionality, Functional Specialization or Redundancy of Metacaspases

As the metacaspase Yca1 (also termed Mca1) was found to be a positive regulator of oxidative stress- and senescence-associated

[1]Department of Plant Systems Biology, VIB, Ghent, Belgium; [2]Department of Plant Biotechnology and Genetics, Ghent University, Ghent, Belgium; [3]Department of Medical Protein Research, VIB, Ghent, Belgium; [4]Department of Biochemistry, Ghent University, Ghent, Belgium; [5]Faculty of Life Sciences, University of Manchester, Manchester, UK; [6]Department of Molecular Biology, Biomedical Center, Swedish University of Agricultural Sciences, Uppsala, Sweden; [7]Department of Plant Biology and Pathology, Rutgers the State University of New Jersey, New Brunswick, NJ, USA and [8]Department of Plant Biology and Forest Genetics, Uppsala BioCentre, Swedish University of Agricultural Sciences, Uppsala, Sweden
*Corresponding author: PV Bozhkov, Department of Plant Biology and Forest Genetics, Uppsala BioCentre, Swedish University of Agricultural Sciences, Box 7080, SE-75007 Uppsala, Sweden. Tel: +46 18 673228; Fax: +46 18 673389; E-mail: peter.bozhkov@slu.se
Keywords: metacaspase; substrate specificity; caspase; cell proliferation; protein aggregation; *in vivo* degradome
Abbreviations: AMC, 7-amino-4-methylcoumarin; ER, endoplasmic reticulum; FB1, fumonisin B-1; fmk, fluoromethylketone; MCA, 4-methylcoumarin-7-amide; 2NA, β-napthylamide; PCD, programmed cell death; SN, staphylococcal nuclease; TSN, Tudor staphylococcal nuclease
Received 10.3.11; revised 18.4.11; accepted 19.4.11; Edited by J Dangl; published online 20.5.11

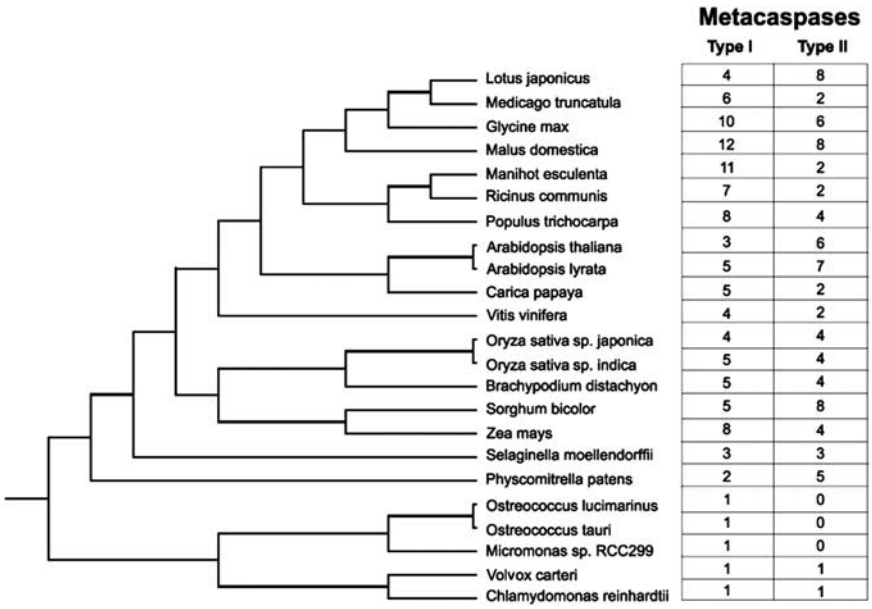

Figure 1 Distribution of type I and II metacaspases within plant genomes

Table 1 The two different *Arabidopsis* metacaspase nomenclatures and the unified one

Gene AGI	Metacaspase type	Van Breusegem lab[5]	Lam lab[12]	Unified
At1g02170	I	AtMC1	AtMCP1b	AtMC1
At4g25110	I	AtMC2	AtMCP1c	AtMC2
At5g64240	I	AtMC3	AtMCP1a	AtMC3
At1g79340	II	AtMC4	AtMCP2d	AtMC4
At1g79330	II	AtMC5	AtMCP2b	AtMC5
At1g79320	II	AtMC6	AtMCP2c	AtMC6
At1g79310	II	AtMC7	AtMCP2a	AtMC7
At1g16420	II	AtMC8	AtMCP2e	AtMC8
At5g04200	II	AtMC9	AtMCP2f	AtMC9

cell deaths in the budding yeast (*Saccharomyces cerevisiae*),[17] a great deal of reverse genetic analysis has been directed to functionally characterize metacaspases in various organisms. This analysis has revealed that cell death regulation is just one of the multifaceted abilities of metacaspases, which can also control other biological processes, either related or unrelated to cell death (Table 2).

The number of metacaspase genes in the genomes of different organisms varies considerably (Figure 1; Table 2; http://merops.sanger.ac.uk/). Such an unequal distribution of metacaspases between different phyla is a good paradigm to study multifunctionality and functional specialization of metacaspases.

A single metacaspase Yca1 of the budding yeast provides a striking example of a multifunctional protein, as apart from cell death activated under various settings, it is also required for cell cycle regulation and clearance of protein aggregates (Table 2).[14] Deletion of the single metacaspase gene *Pca1* of the fission yeast (*Schizosaccharomyces pombe*) suppresses lipotoxic and inositol starvation-induced death.[18,19] LmjMCA, a single metacaspase of *Leishmania major*, is involved in cell cycle regulation[20] and, when overexpressed, stimulates oxidative stress-induced cell death.[21] Further studies using corresponding deletion and inactivation mutants are required to determine whether the single metacaspases present in *L. major* and fission yeast are multifunctional proteins comparable to Yca1.

Most organisms have two or more metacaspase genes (Table 2). There is no evidence yet to indicate significant degree of either specialization or redundancy in the physiological functions of different metacaspase genes expressed in the same organism. Partial redundancy has, however, been demonstrated for three *Trypanosoma brucei* metacaspases during the development of a bloodstream-specific form of the parasite,[22] for two *Podospora anserina* metacaspases in the regulation of senescence-associated death[23] and for two *Aspergillus fumigatus* metacaspases in the resistance to endoplasmic reticulum (ER) stress.[24] Redundancy may not always be the case, as two *Aspergillus nidulans* metacaspases antagonistically control ER stress-induced cell death, one metacaspase (CasA) acting as positive regulator and another as a negative one (CasB).[25] Similarly, a recent study also documented the antagonistic functions of two *Arabidopsis* type I metacaspases, AtMC1 and AtMC2, in mediating hypersensitive response (HR)-associated cell death (Table 2).[26]

Compared with genomes of protozoa and fungi with a single or a few type I metacaspase genes and no type II metacaspase genes, genomes of higher plants encode larger metacaspase families including both type I and type II members; for example, there are nine and twelve metacaspase genes in *A. thaliana* and *Populus trichocarpa*, respectively (Figure 1; http://merops.sanger.ac.uk/). Although the complete range of the roles that metacaspases may serve in plant biology remains to be understood, there is growing evidence that they are required for PCD to take place under diverse settings (Table 2; see following section).

Table 2 Metacaspase functions identified by reverse genetics

Kingdom	Species	Total number of metacaspase genes	Genes with known physiological function	Method	Function	Reference
Protozoa	*Leishmania donovani*	2	*LdMc1, LdMc2*	OE	Positive regulators of oxidative stress-induced cell death	Lee *et al.*[10]
	Leishmania major	1	*LmjMCA*	KO, OE	Cell cycle regulation	Ambit *et al.*[20]
				OE	Positive regulator of oxidative stress-induced cell death	Zalila *et al.*[21]
	Trypanosoma brucei	5	*TbMCA2, TbMCA3, TbMCA5*	RNAi, KO	Required for bloodstream form	Helms *et al.*[22]
	Trypanosoma cruzi	2	*TcMCA5*	OE	Positive regulator of fresh human serum-induced cell death	Kosec *et al.*[34]
Plantae	*Arabidopsis thaliana*	9	*AtMC8*	KO	Required for UVC stress-induced cell death	He *et al.*[30]
			AtMC1, AtMC2	KO, OE, IM	Antagonistically control HR cell death activated by intracellular immune receptors	Coll *et al.*[26]
			AtMC4	KO, OE, IM	Mediates PCD activation by the fungal toxin FB1 and abiotic stress inducers	Watanabe and Lam[32]
	Nicotiana benthamiana	?	*NbMC1*	VIGS	Confers resistance to fungal necrotroph *Colletotrichum destructivum*	Hao *et al.*[66]
	Picea abies	?	*McII-Pa*	RNAi	Required for embryogenesis and associated PCD	Suarez *et al.*[29]
Fungi	*Aspergillus fumigatus*	2	*CasA, CasB*	KO	Confer cytoprotection under ER stress	Richie *et al.*[24]
	Aspergillus nidulans	2	*CasA, CasB*	KO	Antagonistically control ER stress-associated cell death	Colabardini *et al.*[25]
	Candida albicans	1	*CaMCA1*	KO	Required for oxidative stress-induced cell death	Cao *et al.*[28]
	Podospora anserina	2	*PaMca1, PaMca2*	KO	Required for senescence-associated cell death	Hamann *et al.*[23]
	Saccharomyces cerevisiae	1	*Yca1*	KO, OE	Required for oxidative, salt, propolis and osmotic stress-induced cell death	Madeo *et al.*,[27] Chahomchuen *et al.*[67] and de Castro *et al.*[68]
				KO, OE	Required for cell death associated with defects in ubiquitination, DNA replication, mRNA stability and mitochondrial metabolism	Madeo *et al.*[27] and Walter *et al.*[69]
				KO	Required for virus-induced cell death	Ivanovska and Hardwick[70]
				KO	Required for chronological aging-associated death	Herker *et al.*[71]
				KO	Mediates carnitine-dependent lifespan extension	Palermo *et al.*[72]
				KO, IM	Required for clearance of protein aggregates	Lee *et al.*[38]
				KO, IM	Cell cycle regulation	Lee *et al.*[39]
	Schizosaccharomyces pombe	1	*Pca1*	KO	Required for lipotoxic cell death in minimal medium and cell death induced by inositol starvation	Low *et al.*[18] and Guerin *et al.*[19]

Abbreviations: FBI, fumonisin B-1; IM, inactivation mutant; KO, knock out; OE, overexpression; PCD, programmed cell death; RNAi, RNA interference; VIGS, virus-induced gene silencing

Metacaspases in Cell Death

Madeo *et al.*[17] first reported metacaspase-dependent cell death using yeast Yca1 mutant strains subjected to oxidative stress. Since then, a pro-cell death role for Yca1 has been demonstrated in budding yeast cells in response to viral toxins and different types of abiotic and metabolic defect-associated stresses and chronological aging (Table 2).[27] A similar requirement for a metacaspase has been reported in the fission yeast, albeit for a more limited number of cell death conditions, including lipotoxic stress[18] and inositol starvation[19] (Table 2). Further in the fungi kingdom it has been established that both PaMca1 and PaMca2 are required for senescence-associated cell death in *P. anserina*,[23] a single *Candida albicans* metacaspase, CaMCA1, mediates oxidative stress-induced cell death in this pathogenic yeast,[28] whereas one of the two metacaspases, CasA, promotes ER stress-associated cell death in *A. nidulans*[25] (Table 2).

A pro-cell death role of metacaspases has now been extended to protozoa and plants. In *Leishmania* species metacaspases are required for oxidative stress-induced cell death (Table 2).[10,21] In the gymnosperm plant, Norway spruce (*Picea abies*), gene silencing of a type II metacaspase, mcII-Pa, suppressed terminal cell differentiation and PCD in the embryo-suspensor causing developmental arrest at the early stage of embryogenesis.[29] Unexpectedly, PCD phenotypes in metacaspase knockout lines of the model plant *Arabidopsis* have been less forthcoming, possibly due to gene redundancy. In *Arabidopsis*, all nine metacaspase genes are expressed at different levels in various parts of the plant

(our unpublished results). Among these genes, a type II metacaspase gene *AtMC8* is strongly upregulated during oxidative stress. This upregulation requires the gene *Radical-Induced Cell Death1*,[30] which is thought to be a mediator of oxidative stress responses in *Arabidopsis*.[31] Consistent with these observations, *atmc8* knockout lines exhibited reduced cell death triggered by UVC radiation or hydrogen peroxide.[30] Oxidative stress-induced PCD represents a dramatic example of a metacaspase-dependent process that is conserved throughout a long evolutionarily distance, from protozoa to plants (Table 2). Loss of the predominant type II metacaspase in *Arabidopsis*, AtMC4, has been found recently to compromise cell death induction by the fungal toxin fumonisin B-1 (FB1), an incompatible bacterial pathogen, and chemical inducers of oxidative stress in seedlings.[32] This work has further shown that AtMC4 processing from its zymogen is accelerated during activation of cell death by these agents, consistent with its deduced role in orchestrating PCD under these conditions. Lastly, a type I metacaspase AtMC1 was shown to be a positive regulator of the HR cell death in *Arabidopsis*, whereas another type I metacaspase, AtMC2, was found to counteract this pro-cell death effect of AtMC1 (see also review by Coll *et al.*, in this issue).[26] In the case of AtMC2 as antideath regulator, it is interesting to note that its proteolytic activity is apparently not required for this function in *Arabidopsis*. Genetic manipulation of the two metacaspases could nearly eliminate HR activated by plant intracellular immune receptors.[26] To conclude, trans-kingdom conservation of a role for metacaspases in PCD regulation emphasizes that these proteases are a fundamental part of the non-metazoan cell death machinery.

Although several molecular components of PCD pathways have been recently identified in non-metazoan organisms, metacaspase position(s) in these pathways remain elusive. The identification of Tudor staphylococcal nuclease (TSN) as a common substrate for both Norway spruce metacaspase mcII-Pa and human caspase-3 (see below)[33] suggests that metacaspases can execute PCD like effector caspases. Nuclear translocation of metacaspases during cell disassembly in the Norway spruce embryo-suspensors[6] and in the dying epimastigotes of *Trypanosoma cruzi*[34] indirectly supports this scenario. By contrast, the antagonistic control of cell death by two type I metacaspases, such as the one shown for the *Arabidopsis* HR[26] and *A. nidulans* ER stress[25] suggests a possible role in the decision phase.

Noteworthy, in budding yeast approximately 60% of the examples of cell death reported so far are Yca1-independent.[27] For example, Yca1 is not required for PCD during mating,[35] in response to ammonia[36] or induced by human BAX expression.[37] Therefore, it can be expected that further understanding of PCD mechanisms in plants, which have both type I and type II metacaspases, should facilitate a better definition of metacaspase-dependent and -independent pathways.

Metacaspases in Protein Aggregation and ER Stress

Proteome analysis of yeast cells deleted for *Yca1* has revealed a role for this metacaspase in the clearance of insoluble protein aggregates (Table 2).[38] The ablation of *Yca1* was found to direct an accumulation of insoluble protein aggregates during physiological growth conditions, correlated with an increased abundance of vacuolar peptidases and stress-response chaperones. This accumulation of protein aggregates induced the autophagic pathway. Moreover, the normally cytosolic Yca1-GFP was observed to colocalize with the protein aggregation marker Hsp104-mRFP during heat stress and in aged cultures. Deletion of the poly-Q (QQXX) motif in the Yca1 prodomain demonstrated that it was largely responsible for the metacaspase localization in the protein aggregates. Interestingly, catalytic activity of Yca1 was not required for its aggregate-specific localization, representing the first indication that metacaspase could participate in the formation of protease-activation scaffold analogous to signaling platforms such as the apoptosome.[14,38]

These data indicate that metacaspases can function to promote the dissipation of protein aggregates under normal and stress conditions in addition to their role in PCD regulation. It is possible to argue that Δyca1 yeast cells have a reduced level of PCD under stress because of an increased expression of molecular chaperones and of the activation of autophagy, a pro-survival mechanism.[38] This interpretation suggests a possible indirect effect of metacaspase function on PCD regulation, at least in some conditions. However, the poly-Q motif required for aggregate localization is only present in one of the nine *Arabidopsis* metacaspases (AtMC3) and is absent in the other metacaspases shown to regulate PCD, suggesting that this proposition based on observation in yeast might not hold true when investigated in other organisms.

In the human fungal pathogen *A. fumigatus*, deletion of the two metacaspase-encoding genes, *CasA* and *CasB*, leads to hypersensitivity to agents that induce ER stress (Table 2).[24] In contrast, no effect on growth was observed with the ΔcasA/ΔcasB mutant under conditions of a variety of agents that induce oxidative stress. These results indicate that in *A. fumigatus* the metacaspases may be important mediators of survival under ER stress with one possible scenario in their involvement in clearing aggregated proteins in the ER, similar to yeast Yca1. Alternatively, these proteases may be important for cleavage of specific cellular targets that have an important role in the management of ER stress. It would be important to test whether proteolytic activity of CasA and CasB is required for their role for survival under ER stress.

Metacaspases in Cell Proliferation

Ambit *et al.*[20] found that the metacaspase in *L. major*, LmjMCA, exhibits specific subcellular localization. Although it is relatively disperse throughout the cell during interphase, it tends to colocalize with the kinetoplast during mitochondria segregation and it also translocates to the nucleus and mitotic spindle of the parasite during mitosis. Attempts to manipulate LmjMCA levels resulted either in a growth defect with suppression of cytokinesis frequency (overexpression) or inviability (deletion), suggesting that this protein may have an essential function(s) during cell division.[20] However, whether the protease activity of LmjMCA is essential for its role in cell cycle progression in this parasite remains to be clearly demonstrated.

In the budding yeast, loss of Yca1 or its replacement with a point mutation variant in its active site Cys resulted in a delay of the doubling time that correlates with a slower G1/S phase transition.[39] Even more dramatically, loss of Yca1 activity results in the uncoupling of cell cycle arrest at the G2/M checkpoint to nocodazole treatment, which disrupts microtubules important for cytokinesis. Interestingly, the desensitization of cell cycle progression to nocodazole treatments has also been found to result from a loss of caspase-3 activities in human hepatoma cells.[40] These studies thus underscore that metacaspase and caspase could have a common, conserved role in cell cycle progression of eukaryotes.

Measurement and Inhibition of Metacaspase Activities

Owing to the presence of the C14 caspase domain, many efforts initially aimed to demonstrate caspase-like proteolytic activity (namely, proteolysis after an Asp residue) of various metacaspases. Detection of specific proteolytic activity is usually performed by using synthetic tri- or tetrapeptides C-terminally coupled to fluorogenic moieties such as 7-amino-4-methylcoumarin (AMC), 4-methylcoumarin-7-amide (MCA) or β-napthylamide (2NA). Initial reports using caspase substrates VEID-AMC and IETD-AMC monitored differential caspase activity levels in lysates from wild-type, Δyca1 and overexpression yeast strains upon treatment with cell-death promoting agents. The observed activities were abrogated by the broad-range caspase inhibitor zVAD-fluoromethylketone (fmk).[17] Furthermore, metacaspase involvement in the yeast cell death models was investigated after *in vivo* staining for caspase activity by flow cytometry with FITC-labeled VAD-fmk (FITC-VAD-fmk).[17,41–44] Likewise, in the fungus *C. albicans,* it was suggested that the antifungal compound Plagiochin E activated metacaspases based on the appearance of FITC-VAD-fmk labeled cells.[45] In the phytoplankton *Chlamydomonas reinhardtii* and other marine species, such as the diatom *Thalassiosira pseudonana* and the unicellular coccolithore *Emiliania huxlei*, increased caspase-like activity was correlated with increased accumulation of metacaspase mRNA and protein, thereby providing indirect evidence that metacaspases had caspase-like activity.[46–48] Also in plants, silencing of *mcII-Pa* in the Norway spruce embryos reduced the level of the VEIDase caspase-like activity.[29] Overall, these reports suggested that metacaspases might be directly responsible for cellular caspase-like activities in fungi and plants. However, a horde of biochemical studies using recombinant metacaspases or protein extracts from loss- or gain-of-function mutants have now clearly demonstrated that metacaspases are highly specific for Arg or Lys at the P1 position.[4,6,7,9–13,22,23,30,33,49,50] This implies that conclusions, mainly based on a genetic perturbation of metacaspase-encoding genes, that metacaspases have caspase-like proteolytic activities were premature.

To accurately monitor metacaspase activities in cell lysates it is, therefore, essential to use specific metacaspase activity assays that are rooted in substrate specificity and other biochemical characteristics of metacaspases. The determination of the *Arabidopsis* AtMC9 autoprocessing site ITSR(183) (Table 3) was the first indication for a basic amino-acid cleavage preference[5] and steered successful testing of fluorogenic tri- and tetrapeptides with an Arg or Lys residue at the P1 position for cleavage by metacaspases. Based on the autocatalysis of mcII-Pa after KFVK(269) and FESR(188) (Table 3),[6] an equivalent substrate analogue was employed to detect metacaspase activity in embryonic cell extracts of Norway spruce.[33] A tetrapeptide positional scanning synthetic combinatorial library screen indicated that VRPR-AMC (when Arg was fixed at P1) and IISK-AMC (when Lys was fixed at P1) were the preferred synthetic substrates for recombinant *Arabidopsis* AtMC9.[13] Recombinant *T. brucei* TbMCA2 was shown to autocleave at RDAK(55) and ADVK(268) (Table 3) and diverse substrates with Arg or Lys at P1 (GGR, GRR, VRPR, IKLR, IKLK) were efficiently cleaved.[9] Similarly, *Allomyces arbuscula* AMca2 was autoprocessed at GKVR(15) and DDTR(27) (Table 3).[11] Furthermore, peptidyl substrates with Arg at P1 such as GRR-AMC and GGR-AMC were preferentially proteolysed by recombinant metacaspases AtMC1, AtMC4, AtMC5, AtMC8, AtMC9 and TbMCA2,[5,8,9,12,30] by immunoprecipitated metacaspases LdMC1, LdMC2 and LmjMCA from *Leishmania* species,[7,10] and through ectopic overexpression of AtMC1, AtMC4, AtMC5 and Yca1.[8,12]

Table 3 Experimentally shown metacaspase autoprocessing sites and cleavage sites in the substrate proteins

	Metacaspase	Cleaved protein	Cleavage site sequence[a]	P1 position	Reference
Autoprocessing	TbMCA2		RDA**K**GLHG	55	Moss *et al.*[9]
			ADV**K**NTAT	268	Moss *et al.*[9]
	AMca2		GKV**R**DLYG	15	Ojha *et al.*[11]
			DDT**R**STSS	27	Ojha *et al.*[11]
	AtMC9		ITS**R**ALPF	183	Vercammen *et al.*[5]
	AtMC4		AKD**K**SLPL	225	Watanabe and Lam[8]
	mcII-Pa		FES**R**GIHL	188	Bozhkov *et al.*[6]
			KFV**K**VLVT	269	Bozhkov *et al.*[6]
Substrates	AtMC9	AtSerpin	IKL**R**GLLM	351	Vercammen *et al.*[13]
	mcII-Pa	TSN	ASI**R**DLPP	158	Sundström *et al.*[33]
			VLN**R**DVRI	287	Sundström *et al.*[33]
			SNS**K**AIRD	371	Sundström *et al.*[33]
			HSA**R**ESPV	558	Sundström *et al.*[33]
	TbMCA2	EF-Tu	PIV**R**HGSA	172	Moss *et al.*[9]
			SAL**K**ALEG	177	Moss *et al.*[9]

[a]P1 residue is indicated in bold

The substrate specificity of metacaspases towards Arg or Lys at the P1 position in peptide substrates was also consolidated through the identification of a first set of natural substrates and inhibitors. A recombinant serpin-like protein (AtSerpin1) was cleaved within its reactive center loop by recombinant AtMC9 at IKLR(351) (Table 3).[13] During expression of TbMCA2 in *E. coli* cells, the bacterial elongation factor EF-Tu was proteolysed at TPIVR(172) and GSALK(177) (Table 3).[9] Finally, endogenous TSN was cleaved by mcII-Pa at ASIR(158), VLNR(287), SNSK(371) and HSAR(558) during both developmental and oxidative stress-induced PCD in Norway spruce embryos (Table 3).[33]

Arginal protease inhibitors leupeptin and antipain, as well as tosyl-lysyl-chloromethylketone have proven to be potent inhibitors of *Arabidopsis*, *Leishmania* and *Trypanosoma* metacaspases[5,6,9,12] in addition to inhibitory substrate analogues such as FK-trimethylbenzoyloxymethyl ketone, EGR-chloromethyl ketone and GVR-chloromethyl trifluoro acetate.[5,6,11]

Biochemical studies of metacaspases from *Arabidopsis* (AtMC4 and AtMC8), spruce (mcII-Pa), *Leishmania* (LdMC1 and LdMC2) and *Trypanosoma* (TbMCA2) indicate that the majority prefers neutral to slightly basic (7.0–8.5) pH optima for *in vitro* activity assays.[5,6,9,10,30] Until now, AtMC9 is the only reported metacaspase that requires acidic conditions before activation.[5] Noteworthy *in vitro* activities of AtMC4, AtMC5, mcII-Pa, TbMCA2 and the *A. arbuscula* AMca2 depend on high Ca^{2+} concentrations.[5,6,9,12] More recent biochemical studies with recombinant AtMC4 demonstrated that these high Ca^{2+} levels are required to activate the protease activity through induced cleavage at a highly conserved site AKDK(225) (Table 3) that has also been shown to be cleaved in AtMC9.[8] This cleavage was demonstrated to be critical for *in vitro* protease activity of metacaspases[5,8] as well as *in vivo* function of AtMC4 to complement Δyca1 mutant yeast strains in cell death activation.[8]

To conclude, caspase-specific substrates such as YVAD-AMC, DEVD-AMC, VEID-AMC and IETD-AMC (with Asp at P1) are not indicative of metacaspase catalytic activity. This implies that conclusions drawn on the involvement of metacaspases in specific processes from reports based on caspase-specific peptide substrates, should be revisited and it is advised that activity assays tailored towards the specific catalytic characteristics of metacaspases should be used instead. As the level of VEIDase activity was reported to be decreased along with either the loss of *Yca1* in yeast[17] or by suppression of *mcII-Pa* in spruce cells,[29] these results would indicate that cryptic caspase-like proteases may be activated downstream of the metacaspase regulation point. The suppressing effect of pan-caspase inhibitor such as zVAD-fmk and zVEID-fmk on cell death-related phenomena in yeast and spruce cells would support the role for such caspase-like proteases in orchestrating cell death downstream of metacaspases in some of the cell death pathways.[12,17,51] We urge the use of fluorogenic tri- or tetrapeptide substrates (e.g. VRPR-AMC, IISK-AMC, EGR-AMC, GRR-AMC, GGR-AMC and FESR-AMC) with Arg or Lys residues at the P1 position to detect metacaspase activities in cellular extracts or of recombinant metacaspases. Accordingly, the use of caspase inhibitors against metacaspases is inappropriate and should be replaced by arginal protease inhibitors. It should be noted, however, that in addition to metacaspases, these inhibitors can suppress other arginine-specific proteases present in the cells or test tube. Given the variability of metacaspase pH optima and possible Ca^{2+} dependence, it is advisable to screen for proteolytic activity at different levels of pH and different concentrations of Ca^{2+} as well.

In Vivo Metacaspase Degradome: Tudor Staphylococcal Nuclease

Unraveling the identity of natural substrates of metacaspases will give an insight into molecular mechanisms of metacaspase-dependent processes. At present, TSN is the only protein shown to be cleaved by a metacaspase *in vivo*.[33] TSN is an evolutionarily and structurally conserved protein found in all eukaryotes (except for budding yeast). It has an invariant domain architecture including a tandem of four staphylococcal nuclease-like domains (SN), followed by a Tudor domain and the fifth SN domain (Figure 2a). Owing to its complex domain structure, TSN takes part in several fundamental mechanisms of gene regulation in animal cells, including transcription,[52] mRNA splicing[53] and RNA silencing.[54,55] Decrease of *TSN* expression triggers cell death[33,56] and impairs plant viability and stress tolerance.[33,57]

During both developmental and oxidative stress-induced cell death in Norway spruce embryos active metacaspase mcII-Pa cleaves endogenous TSN at four sites. Three sites are situated between SN domains and the fourth site inside SN2 domain (Figure 2a).[33] All cleavage sites contain either Arg or Lys at P1 position, but there was no strict specificity beyond P1 residue (P4-P3-P2 and P1′) (Table 3). In the same study caspase-3-mediated cleavage of human TSN at the canonical cleavage site DAVD/S (Pop and Salvesen[58]) situated between the Tudor and SN5 domains has been revealed in apoptotic cells.[33] A shared *in vivo* target of caspases and metacaspases suggests that plants and animals may have conserved some key proteolytic pathways to compromise cell viability, despite the large differences in morphology and biochemistry of cell death between the two kingdoms. In addition, cleavage of TSN by an effector caspase and a type II metacaspase provides a good model system for comparative analysis of the biochemical properties of caspases and metacaspases *in vivo*.[14] One conclusion from such analysis is that metacaspases appear to be far more liberal than caspases in regards to P4-P3-P2 and P1′ substrate residues (Figure 2a). This conclusion is consistent with earlier results of high-throughput screening of a combinatorial tetrapeptide substrate library with recombinant AtMC9.[13] Future studies of *in vivo* metacaspase degradome might, however, identify more prominent metacaspase specificity towards the aforementioned residues.

The crystal structure of the Tudor-SN5 part of human TSN has been determined to high resolution (Figure 2b).[59] To complete the 3D model of the protein, each of the SN1-4 domains can be reliably reconstructed based on the available crystal structures of staphylococcal nuclease (PDB 1SNC) and human SN5 (PDB 2O4X), for example, the SN2 domain of spruce TSN shown in Figure 2c. The analysis of the metacaspase mcII-Pa cleavage sites in this model reveals

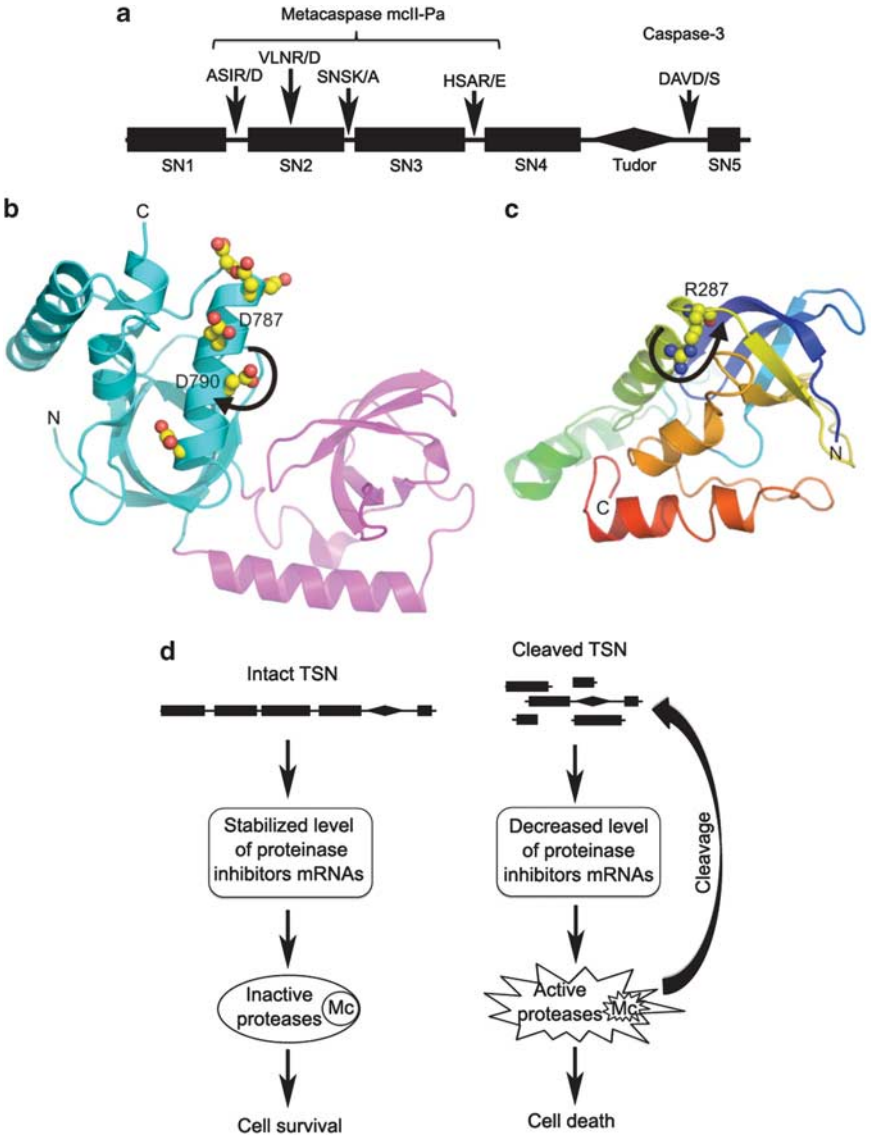

Figure 2 The mechanisms of TSN cleavage during cell death. (**a**) Domain structure of TSN, and location of metacaspase and caspase cleavage sites. (**b** and **c**) Structural preferences for the cleavage of TSN by caspase-3 and metacaspase mcII-Pa. (**b**) Caspase-3 cleaves helix α2 of human TSN. Tudor-SN5 part of human TSN is shown as cartoon with the SN5 and Tudor domains painted in cyan and pink, respectively. The caspase-3 cleavage motif (787-DAVD-790) is located in the middle of helix α2. Three of four residues of the motif, Asp 787 and 790 and Val 789 are accessible to the solvent. The α2 helix exposes six Asp residues (including two Asp residue of the DAVD motif; shown in spheres), creating a highly negatively charged patch on the surface of the helix. The abundance of Asp residues around the cleavage site might facilitate its recognition by caspase-3. (**c**) Metacaspase mcII-Pa cleaves a structured but solvent accessible loop (α2-β8) of the SN2 domain (cartoon painted in rainbow) of spruce TSN. The loop is cleaved after Arg 287 (shown in spheres). The structure of the SN2 domain of spruce TSN was modeled with SwissModel using staphylococcal nuclease (PDB 1SNC) and Tudor-SN5 part of human TSN (PDB 2O4X) as template structures. Arrows in **b** and **c** indicate cleavage sites. Graphics was generated using PyMol. (**d**) A proposed model for pro-cell death role of metacaspase-mediated cleavage of TSN. Mc, metacaspases

that all of them are located in loop regions: three in the loops situated between SN1-4 domains and one in the middle of a structured but solvent accessible loop (α2-β8) of the SN2 domain (Figure 2c). The preference for digesting loops is common for the majority of proteases and, hence, is not surprising. In contrast, the single cleavage site in human TSN for caspase-3 is located in a long helix (α2) of the Tudor-SN5 part (Figure 2b). Although the ability of caspase-3 to cleave α helices has been previously demonstrated,[60] this is not consistent with the structural analysis of the caspase-3–inhibitor complex.[61] In this complex, the inhibitor inserts an extended conformation segment into the active site, suggesting that cleavage sites must either have this conformation or acquire it by unfolding. Interestingly, the caspase-3-cleaved helix α2 of human TSN contains six Asp residues (including two Asp residue of the DAVD motif), creating a highly negatively charged patch on the surface of the helix (Figure 2b). The abundance of Asp residues around the cleavage site might facilitate its recognition by caspase-3. Alternatively, the high density of an uncompensated negative charge might trigger a transient unfolding of the helix, which would facilitate not only the cleavage site recognition but also

its digestion by caspase-3. Cleavage would likely to promote the unfolding of the entire Tudor-SN5 part and subsequent protein aggregation. In contrast, cleavage of spruce TSN in the loop regions (Figure 2c) is likely to generate soluble domains. Therefore, mcII-Pa-mediated cleavage of spruce TSN at multiple sites may provide an alternative mechanism (to a cleavage at a single site within the α2 helix) to ensure efficient proteolytic inactivation of TSN.

As the list of annotated members of the caspase degradome continues to increase, it becomes evident that cleavage of many substrates is just a bystander event, which does not contribute to cell death development.[58] However, cleavage of human TSN by caspase-3 is important for the execution of apoptosis for the following reasons. The cleavage breaks up the hook-like structure formed by the Tudor and SN5 domains. An aromatic cage within the hook binds methyl groups of small nuclear ribonucleoproteins, anchoring TSN to the spliceosome and stimulating mRNA splicing.[59] Accordingly caspase-mediated cleavage of TSN abrogates its stimulatory function in splicing.[33] As TSN is supposed to bridge transcription and splicing by interacting with the components of both processes, the cleavage by effector caspases will also uncouple these processes.

It is more difficult to explain the molecular mechanism underlying pro-cell death role of metacaspase-mediated cleavage of TSN because the molecular role of TSN in plant biology remains poorly understood. Furthermore, plant TSN is a cytoplasmic protein,[33,57] which does not support its involvement in transcription and splicing activation. Frei dit Frey *et al.*[57] have recently demonstrated that TSN confers stress tolerance in *Arabidopsis* through selective stabilization of mRNAs-encoding secreted proteins. Notably, a significant proportion of these proteins are cysteine and serine protease inhibitors, which are known to suppress cell death in plants.[62,63] Although the mechanism of TSN-dependent stabilization of specific mRNAs remains unknown, metacaspase cleavage of TSN molecule in at least four sites should result in deregulation of this mechanism. We propose a model, where intact TSN protein helps to protect cells from death by keeping expression of protease inhibitors at the level sufficient to suppress activation of a subset of cell-death proteases (Figure 2d, left). Contrary, metacaspase-mediated cleavage of TSN abrogates this function and promotes cell death through increased activation of cell-death proteases (including metacaspases; Figure 2d, right). Experimental verification of this model will provide a fruitful avenue for the future research.

Conclusions

It has been approximately 10 years since metacaspases were discovered. During this time, the pendulum has shifted from naive hopes that metacaspases are responsible for cell death events and associated caspase-like activities in plants and other non-metazoans to well-grounded view that metacaspases are a distinct group of enzymes that can regulate (similar to caspases) various cell biological processes, including PCD. We expect that more mechanistic data will be obtained in the near future about metacaspases through integrated efforts of cell and structural biologists and biochemists. This will enable to resolve a number of important issues and controversies, including (but not limited to) (i) molecular conformation of active metacaspase, and differences as well as similarities between type I and II metacaspases; (ii) whether activation of type I metacaspases occurs in multiprotein complexes similar to caspases-2, -8 and -9; (iii) whether the degradome of metacaspases is indeed much larger than that of caspases, considering a weak preference of metacaspases for specific residues located in P4-P2 and P1′ positions in their substrates; (iv) is metacaspase-mediated proteolysis directly responsible for morphological changes that occur during PCD in non-metazoan organisms, which differs significantly from apoptosis in animals; (v) whether metacaspase functions differ in the organisms containing a single metacaspase (e.g. budding or fission yeast) and multiple metacaspases (e.g. *Arabidopsis*) and (vi) how precisely do metacaspases regulate downstream aspartate-specific proteases.

Anticipated discovery of the natural substrates of metacaspases using advanced techniques of protease degradomics[64,65] will assess and further consolidate metacaspase specificity in a broader biological context that will allow a more precise and rational design of synthetic substrates and inhibitors. As metacaspases control vital cellular functions in protozoa, fungi and plants, development of approaches for artificial regulation of metacaspases can find important practical applications in the improvement of growth and disease resistance characteristics.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements. We thank Dr. Martine De Cock for assistance in correcting this manuscript. LT is supported by the VIB International PhD Program. FVB acknowledges the support of Fonds Wetenschappelijk Onderzoek Flanders (grant no. 3G003809N) and Ghent University (Multidisciplinary Research Partnership Ghent Bio-economy). PG is supported by the UK Biotechnology and Biological Sciences Research Council. EL is supported by a Grant (#0744709) from the National Science Foundation of the US.PVB acknowledges the support of the Pehrssons Fund, the Swedish Research Council (VR), the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (Formas) and the Swedish Foundation for Strategic Research (SSF).

1. Uren AG, O'Rourke K, Aravind L, Pisabarro MT, Seshagiri S, Koonin EV *et al.* Identification of paracaspases and metacaspases: two ancient families of caspase-like proteins, one of which plays a key role in MALT lymphoma. *Mol Cell* 2000; **6**: 961–967.
2. Vercammen D, Declercq W, Vandenabeele P, Van Breusegem F. Are metacaspases caspases? *J Cell Biol* 2007; **179**: 375–380.
3. Aravind L, Koonin EV. Classification of caspase-hemoglobinase fold: detection of new families and implications for the origin of eukaryotic separins. *Proteins* 2002; **46**: 355–367.
4. Szallies A, Kubata BK, Duszenko M. A metacaspase of *Trypanosoma brucei* causes loss of respiration competence and clonal death in the yeast *Saccharomyces cerevisiae. FEBS Lett* 2002; **517**: 144–150.
5. Vercammen D, van de Cotte B, De Jaeger G, Eeckhout D, Casteels P, Vandepoele K *et al.* Type II metacaspases Atmc4 and Atmc9 of *Arabidopsis thaliana* cleave substrates after arginine and lysine. *J Biol Chem* 2004; **279**: 45329–45336.
6. Bozhkov PV, Suarez MF, Filonova LH, Daniel G, Zamyatnin AA Jr, Rodriguez-Nieto S *et al.* Cysteine protease mcII-Pa executes programmed cell death during plant embryogenesis. *Proc Natl Acad Sci USA* 2005; **102**: 14463–14468.
7. González IJ, Desponds C, Schaff C, Mottram JC, Fasel N. *Leishmania major* metacaspase can replace yeast metacaspase in programmed cell death and has arginine-specific cysteine peptidase activity. *Int J Parasitol* 2007; **37**: 161–172.

8. Watanabe N, Lam E. Calcium-dependent activation and autolysis of *Arabidopsis* metacaspase 2d. *J Biol Chem* 2011; **286**: 10027–10040.
9. Moss CX, Westrop GD, Juliano L, Coombs GH, Mottram JC. Metacaspase 2 of *Trypanosoma brucei* is a calcium-dependent cysteine peptidase active without processing. *FEBS Lett* 2007; **581**: 5635–5639.
10. Lee N, Gannavaram S, Selvapandiyan A, Debrabant A. Characterization of metacaspases with trypsin-like activity and their putative role in programmed cell death in the protozoan parasite *Leishmania*. *Eukaryot Cell* 2007; **6**: 1745–1757.
11. Ojha M, Cattaneo A, Hugh S, Pawlowski J, Cox JA. Structure, expression and function of *Allomyces arbuscula* CDP II (metacaspase) gene. *Gene* 2010; **457**: 25–34.
12. Watanabe N, Lam E. Two *Arabidopsis* metacaspases AtMCP1b and AtMCP2b are arginine/lysine-specific cysteine proteases and activate apoptosis-like cell death in yeast. *J Biol Chem* 2005; **280**: 14691–14699.
13. Vercammen D, Belenghi B, van de Cotte B, Beunens T, Gavigan JA, De Rycke R *et al.* Serpin1 of *Arabidopsis thaliana* is a suicide inhibitor for metacaspase 9. *J Mol Biol* 2006; **364**: 625–636.
14. Bozhkov PV, Smertenko AP, Zhivotovsky B. Aspasing out metacaspases and caspases: proteases of many trades. *Sci Signal* 2010; **3**: pe48.
15. Carmona-Gutierrez D, Fröhlich K-U, Kroemer G, Madeo F. Metacaspases are caspases. Doubt no more. *Cell Death Differ* 2010; **17**: 377–378.
16. Enoksson M, Salvesen GS. Metacaspases are not caspases – always doubt. *Cell Death Differ* 2010; **17**: 1221.
17. Madeo F, Herker E, Maldener C, Wissing S, Löchelt S, Herlan M *et al.* A caspase-related protease regulates apoptosis in yeast. *Mol Cell* 2002; **9**: 911–917.
18. Low CP, Shui G, Liew LP, Buttner S, Madeo F, Dawes IW *et al.* Caspase-dependent and -independent lipotoxic cell-death pathways in fission yeast. *J Cell Sci* 2008; **121**: 2671–2684.
19. Guerin R, Beauregard PB, Leroux A, Rokeach LA. Calnexin regulates apoptosis induced by inositol starvation in fission yeast. *PLoS One* 2009; **4**: e6244.
20. Ambit A, Fasel N, Coombs GH, Mottram JC. An essential role for the *Leishmania major* metacaspase in cell cycle progression. *Cell Death Differ* 2008; **15**: 113–122.
21. Zalila H, González IJ, El-Fadili AK, Delgado MB, Desponds C, Schaff C *et al.* Processing of metacaspase into a cytoplasmic catalytic domain mediating cell death in *Leishmania major*. *Mol Microbiol* 2011; **79**: 222–239.
22. Helms MJ, Ambit A, Appleton P, Tetley L, Coombs GH, Mottram JC. Bloodstream form *Trypanosoma brucei* depend upon multiple metacaspases associated with RAB11-positive endosomes. *J Cell Sci* 2006; **119**: 1105–1117.
23. Hamann A, Brust D, Osiewacz HD. Deletion of putative apoptosis factors leads to lifespan extension in the fungal ageing model *Podospora anserina*. *Mol Microbiol* 2007; **65**: 948–958.
24. Richie DL, Miley MD, Bhabhra R, Robson GD, Rhodes JC, Askew DS. The *Aspergillus fumigatus* metacaspases CasA and CasB facilitate growth under conditions of endoplasmic reticulum stress. *Mol Microbiol* 2007; **63**: 591–604.
25. Colabardini AC, De Castro PA, De Gouvêa PF, Savoldi M, Malavazi I, Goldman MH *et al.* Involvement of the *Aspergillus nidulans* protein kinase C with farnesol tolerance is related to the unfolded protein response. *Mol Microbiol* 2010; **78**: 1259–1279.
26. Coll NS, Vercammen D, Smidler A, Clover C, Van Breusegem F, Dangl JL *et al.* *Arabidopsis* type I metacaspases control cell death. *Science* 2010; **330**: 1393–1397.
27. Madeo F, Carmona-Gutierrez D, Ring J, Buttner S, Eisenberg T, Kroemer G. Caspase-dependent and caspase-independent cell death pathways in yeast. *Biochem Biophys Res Commun* 2009; **382**: 227–231.
28. Cao Y, Huang S, Dai B, Zhu Z, Lu H, Dong L *et al. Candida albicans* cells lacking CaMCA1-encoded metacaspase show resistance to oxidative stress-induced cell death and change in energy metabolism. *Fungal Genet Biol* 2009; **46**: 183–189.
29. Suarez MF, Filonova LH, Smertenko A, Savenkov EI, Clapham DH, von Arnold S *et al.* Metacaspase-dependent programmed cell death is essential for plant embryogenesis. *Curr Biol* 2004; **14**: R339–R340.
30. He R, Drury GE, Rotari VI, Gordon A, Willer M, Farzaneh T *et al.* Metacaspase-8 modulates programmed cell death induced by ultraviolet light and H_2O_2 in *Arabidopsis*. *J Biol Chem* 2008; **283**: 774–783.
31. Jaspers P, Brosché M, Overmyer K, Kangasjärvi J. The transcription factor interacting protein RCD1 contains a novel conserved domain. *Plant Signal Behavior* 2010; **5**: 78–80.
32. Watanabe N, Lam E. *Arabidopsis* metacaspase 2d is a positive mediator of cell death induced during biotic and abiotic stresses. *Plant J* 2011; e-pub ahead of print 12 March 2011.
33. Sundström JF, Vaculova A, Smertenko AP, Savenkov EI, Golovko A, Minina E *et al.* Tudor staphylococcal nuclease is an evolutionarily conserved component of the programmed cell death degradome. *Nat Cell Biol* 2009; **11**: 1347–1354.
34. Kosec G, Alvarez VE, Aguero F, Sanchez D, Dolinar M, Turk B *et al.* Metacaspases of *Trypanosoma cruzi*: possible candidates for programmed cell death mediators. *Mol Biochem Parasitol* 2006; **145**: 18–28.
35. Zhang N-N, Dudgeon DD, Paliwal S, Levchenko A, Grote E, Cunningham KW. Multiple signaling pathways regulate yeast cell death during the response to mating pheromones. *Mol Biol Cell* 2006; **17**: 3409–3422.
36. Vachova L, Palkova Z. Physiological regulation of yeast cell death in multicellular colonies is triggered by ammonia. *J Cell Biol* 2005; **169**: 711–717.
37. Guscetti F, Nath N, Denko N. Functional characterization of human proapoptotic molecules in yeast *S. cerevisiae*. *FASEB J* 2005; **19**: 464–466.
38. Lee RE, Brunette S, Puente LG, Megeney LA. Metacaspase Yca1 is required for clearance of insoluble protein aggregates. *Proc Natl Acad Sci USA* 2010; **107**: 13348–13353.
39. Lee RE, Puente LG, Kaern M, Megeney LA. A non-death role of the yeast metacaspase: Yca1p alters cell cycle dynamics. *PLoS One* 2008; **3**: e2956.
40. Hsu SL, Yu CT, Yin SC, Tang MJ, Tien AC, Wu YM *et al.* Caspase 3, periodically expressed and activated at G2/M transition, is required for nocodazole-induced mitotic checkpoint. *Apoptosis* 2006; **11**: 765–771.
41. Wadskog I, Maldener C, Proksch A, Madeo F, Adler L. Yeast lacking the SRO7/SOP1-encoded tumor suppressor homologue show increased susceptibility to apoptosis-like cell death on exposure to NaCl stress. *Mol Biol Cell* 2004; **15**: 1436–1444.
42. Guaragnella N, Pereira C, Sousa MJ, Antonacci L, Passarella S, Côrte-Real M *et al.* YCA1 participates in the acetic acid induced yeast programmed cell death also in a manner unrelated to its caspase-like activity. *FEBS Lett* 2006; **580**: 6880–6884.
43. Guaragnella N, Bobba A, Passarella S, Marra E, Giannattasio S. Yeast acetic acid-induced programmed cell death can occur without cytochrome c release which requires metacaspase YCA1. *FEBS Lett* 2010; **584**: 224–228.
44. Sripriya P, Vedantam LV, Podile AR. Involvement of mitochondria and metacaspase elevation in harpin Pss-induced cell death of *Saccharomyces cerevisiae*. *J Cell Biochem* 2009; **107**: 1150–1159.
45. Wu XZ, Chang WQ, Cheng AX, Sun LM, Lou HX. Plagiochin E, an antifungal active macrocyclic bis(bibenzyl), induced apoptosis in *Candida albicans* through a metacaspase-dependent apoptotic pathway. *Biochim Biophys Acta* 2010; **1800**: 439–447.
46. Bidle KD, Haramaty L, Barcelos E, Ramos J, Falkowski P. Viral activation and recruitment of metacaspases in the unicellular coccolithophore, *Emiliania huxleyi*. *Proc Natl Acad Sci USA* 2007; **104**: 6049–6054.
47. Bidle KD, Bender SJ. Iron starvation and culture age activate metacaspases and programmed cell death in the marine diatom *Thalassiosira pseudonana*. *Eukaryot Cell* 2008; **7**: 223–236.
48. Murik O, Kaplan A. Paradoxically, prior acquisition of antioxidant activity enhances oxidative stress-induced cell death. *Environ Microbiol* 2009; **11**: 2301–2309.
49. Belenghi B, Romero-Puertas MC, Vercammen D, Brackenier A, Inzé D, Delledonne M *et al.* Metacaspase activity of *Arabidopsis thaliana* is regulated by S-nitrosylation of a critical cysteine residue. *J Biol Chem* 2007; **282**: 1352–1358.
50. Helmersson A, von Arnold S, Bozhkov PV. The level of free intracellular zinc mediates programmed cell death/cell survival decisions in plant embryos. *Plant Physiol* 2008; **147**: 1158–1167.
51. Bozhkov PV, Filonova LH, Suarez MF, Helmersson A, Smertenko AP, Zhivotovsky B *et al.* VEIDase is a principal caspase-like activity involved in plant programmed cell death and essential for embryonic pattern formation. *Cell Death Differ* 2004; **11**: 175–182.
52. Yang J, Aittomäki S, Pesu M, Carter K, Saarinen J, Kalkkinen N *et al.* Identification of p100 as a coactivator for STAT6 that bridges STAT6 with RNA polymerase II. *EMBO J* 2002; **21**: 4950–4958.
53. Yang J, Välineva T, Hong J, Bu T, Yao Z, Jensen ON *et al.* Transcriptional co-activator protein p100 interacts with snRNP proteins and facilitates the assembly of the spliceosome. *Nucleic Acids Res* 2007; **35**: 4485–4494.
54. Caudy AA, Ketting RF, Hammond SM, Denli AM, Bathoorn AM, Tops BB *et al.* A micrococcal nuclease homologue in RNAi effector complexes. *Nature* 2003; **425**: 411–414.
55. Scadden ADJ. Inosine-containing dsRNA binds a stress-granule-like complex and downregulates gene expression in trans. *Mol Cell* 2007; **28**: 491–500.
56. Tong X, Drapkin R, Yalamanchill R, Mosialos G, Kieff E. The Epstein-Barr virus nuclear protein 2 acidic domain forms a complex with a novel cellular coactivator that can interact with TFIIE. *Mol Cell Biol* 1995; **15**: 4735–4744.
57. Frei dit Frey N, Muller P, Jammes F, Kizis D, Leung J *et al.* The RNA binding protein Tudor-SN is essential for stress tolerance and stabilizes levels of stress-responsive mRNAs encoding secreted proteins in *Arabidopsis*. *Plant Cell* 2010; **22**: 1575–1591.
58. Pop C, Salvesen GS. Human caspases: activation, specificity, and regulation. *J Biol Chem* 2009; **284**: 21777–21781.
59. Shaw N, Zhao M, Cheng C, Xu H, Saarikettu J, Li Y *et al.* The multifunctional human p100 protein 'hooks' methylated ligands. *Nat Struct Mol Biol* 2007; **14**: 779–784.
60. Timmer JC, Zhu W, Pop C, Regan T, Snipas SJ, Eroshkin AM *et al.* Structural and kinetic determinants of protease substrates. *Nat Struct Mol Biol* 2009; **16**: 1101–1108.
61. Riedl SJ, Renatus M, Schwarzenbacher R, Zhou Q, Sun C, Fesik SW *et al.* Structural basis for the inhibition of caspase-3 by XIAP. *Cell* 2001; **104**: 791–800.
62. Solomon M, Belenghi B, Delledonne M, Menachem E, Levine A. The involvement of cysteine proteases and protease inhibitor genes in the regulation of programmed cell death in plants. *Plant Cell* 1999; **11**: 431–444.
63. Coffeen WC, Wolpert TJ. Purification and characterization of serine proteases that exhibit caspase-like activity and are associated with programmed cell death in *Avena sativa*. *Plant Cell* 2004; **16**: 857–873.
64. Agard NJ, Wells JA. Method for the proteomics identification of protease substrates. *Curr Opin Chem Biol* 2009; **13**: 503–509.
65. Impens F, Colaert N, Helsens K, Plasman K, Van Damme P, Vandekerckhove J *et al.* MS-driven protease substrate degradomics. *Proteomics* 2010; **10**: 1284–1296.
66. Hao L, Goodwin PH, Hsiang T. Expression of a metacaspase gene of *Nicotiana benthamiana* after inoculation with *Colletotrichum destructivum* or *Pseudomonas syringae* pv. tomato, and the effect of silencing the gene on the host response. *Plant Cell Rep* 2007; **26**: 1879–1888.

67. Chahomchuen T, Akiyama K, Sekito T, Sugimoto N, Okabe M, Nishimoto S *et al.* Tributyltin induces Yca1p-dependent cell death of yeast *Saccharomyces cerevisiae*. *J Toxicol Sci* 2009; **34**: 541–545.
68. de Castro PA, Savoldi M, Bonatto D, Barros MH, Goldman MH, Berretta AA *et al.* Molecular characterisation of propolis-induced cell death in *Saccharomyces cerevisiae*. *Eukaryot Cell* 2011; **10**: 398–411.
69. Walter D, Matter A, Fahrenkrog B. Bre1p-mediated histone H2B ubiquitylation regulates apoptosis in *Saccharomyces cerevisiae*. *J Cell Sci* 2010; **123**: 1931–1939.
70. Ivanovska I, Hardwick JM. Viruses activate a genetically conserved cell death pathway in a unicellular organism. *J Cell Biol* 2005; **170**: 391–399.
71. Herker E, Jungwirth H, Lehmann KA, Maldener C, Fröhlich KU, Wissing S *et al.* Chronological aging leads to apoptosis in yeast. *J Cell Biol* 2004; **164**: 501–507.
72. Palermo V, Falcone C, Calvani M, Mazzoni C. Acetyl-L-carnitine protects yeast cells from apoptosis and aging and inhibits mitochondrial fission. *Aging Cell* 2010; **9**: 570–579.