Cell. Mol. Life Sci. (2016) 73:1103–1116
DOI 10.1007/s00018-015-2041-9

Cellular and Molecular Life Sciences

ORIGINAL ARTICLE

MALT1 is not alone after all: identification of novel paracaspases

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Received: 11 May 2015 / Revised: 18 August 2015 / Accepted: 7 September 2015 / Published online: 16 September 2015

Abstract Paracaspases and metacaspases are two families of caspase-like proteins identified in 2000. Up until now paracaspases were considered a single gene family with one known non-metazoan paracaspase in the slime mold *Dictyostelium* and a single animal paracaspase called MALT1. Human MALT1 is a critical signaling component in many innate and adaptive immunity pathways that drive inflammation, and when it is overly active, it can also cause certain forms of cancer. Here, we report the identification and functional analysis of two new vertebrate paracaspases, PCASP2 and PCASP3. Functional characterization indicates that both scaffold and protease functions are conserved across the three vertebrate paralogs. This redundancy might explain the loss of two of the paralogs in mammals and one in *Xenopus*. Several of the vertebrate paracaspases currently have incorrect or ambiguous annotations. We propose to annotate them accordingly as PCASP1, PCASP2, and PCASP3 similar to the caspase gene nomenclature. A comprehensive search in other metazoans and in non-metazoan species identified additional new paracaspases. We also discovered the first animal metacaspase in the sponge *Amphimedon*. Comparative analysis of the active site suggests that paracaspases constitute one of the several subclasses of metacaspases that have evolved several times independently.

Keywords NF-κB · CYLD · Protease · MALT1 · Evolution · Zebrafish · Chicken · Xenopus

Introduction

Paracaspases and metacaspases received their names due to sequence similarity to caspases (cysteine-dependent aspartate proteases) [1]. The names metacaspase and paracaspase are somewhat a misnomer because metacaspases cleave specifically after arginine or lysine [2], whereas paracaspases cleave after arginine [3]. All three classes of proteases have a similar three-dimensional structure, with a caspase fold consisting of a six-stranded beta sheet, five alpha helices, and four active site loops [4]. The distribution of the three families points to an interesting evolutionary history: Caspases are exclusive to metazoans, and members of several modern caspase families are found in metazoan species that diverged early from our lineage, such as *Trichoplax* and sponges. Paracaspases as well seem to be almost exclusive to metazoans, with a few members found outside of the metazoan lineage, namely *Dictyostelium* pcp [1] and rhizobacteria [5]. On the other hand, metacaspases are not found in metazoans [6] but are present in all other eukaryotic phyla and many different domain architectures in prokaryotes [7]. One possibility is that the common ancestor of caspases and paracaspases was introduced into an ancestral organism by horizontal gene transfer, and that this organism lost its metacaspases [5].

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Electronic supplementary material The online version of this article (doi:10.1007/s00018-015-2041-9) contains supplementary material, which is available to authorized users.

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The best-known paracaspase is the mammalian MALT1, which received its name because it was originally discovered in lymphomas of mucosa-associated lymphoid tissue [8]. Aberrant MALT1 activity is also essential for a certain type of B-cell lymphoma called ABC-DLBCL (activated B-cell-like diffuse large B-cell lymphoma) [9, 10]. The phenotypes of two independent knockout mice and of naturally occurring inactivating mutations in humans indicate that its major role is in B- and T-cell antigen receptor signaling [11–13]. MALT1 has also been implicated in numerous other signaling pathways mediating innate and adaptive immune responses [14]. All the known signaling pathways involving MALT1 act via a so-called CBM complex consisting of a specific CARD family protein (CARD9, CARD10, CARD11), BCL10, and MALT1 [15]. MALT1 is activated by forced proximity, possibly by formation of filamentous structures of activated CARD11 [16]. MALT1 consists of several distinct domains: an N-terminal death domain followed by two immunoglobulin-like domains (Ig1, Ig2), the caspase-like (paracaspase) domain, another immunoglobulin-like domain (Ig3), and a C-terminal unstructured region. The structures of the N-terminal death domain and the two immunoglobulin domains indicate that Ig1 and Ig2 contribute to oligomerization of MALT1, whereas the death domain sticks out from the structure [17]. No interaction partner for the MALT1 death domain has been identified, but it appears to stabilize the interaction between MALT1 and Bcl10, which interacts with Ig1 [18]. Loss of the death domain also results in spontaneous activation and de-stabilization of MALT1, indicating that the death domain plays a role in negative regulation [19]. The structures of the caspase-like and Ig3 domains have also been determined. The third immunoglobulin-like domain folds into a structure that is very similar to the gamma-adaptin (EAR) peptide-binding domain [20, 21]. Changes in both domains upon substrate binding indicate that Ig3 might be involved in regulating the protease activity [22]. Recently, it was shown that ubiquitination of the Ig3 domain is critical for protease activity, further strengthening the notion that this domain is intimately involved in the protease function [23]. Interestingly, proper folding and recombinant expression of the caspase-like domain appear to depend on Ig3 [3, 20, 21]. Despite being called a paracaspase, MALT1's protease activity was not discovered until much later, when it was found to cleave the anti-inflammatory deubiquitinase A20 (TNFAIP3) [24] and the MALT1 interaction partner Bcl10 [25]. Later, other MALT1 substrates were identified, namely the deubiquitinase CYLD [26], the NF-κB inhibitor RelB [27], and the RNases Zc3h12a (aka MCPIP or regnase-1) [28], and Roquin [29], which are all implicated in the regulation of pro-inflammatory gene expression. These findings indicate that MALT1 protease activity has a major role in inflammatory signaling. Moreover, it was recently reported that autoprocessing of MALT1 is critical for its signaling function [19]. As there is only one human paracaspase and it has no close homologs, MALT1 protease activity has turned into an attractive druggable target for a wide range of inflammatory diseases and certain forms of cancer [30, 31]. The function of paracaspase in non-mammalian species has been poorly studied, but a recent investigation of the distant *Dictyostelium* homolog, which contains the caspase-like protease domain but lacks all other domains found in MALT1, revealed a role in osmotic regulation via vacuolar expansion [32].

Surprisingly, though MALT1 has a major role in NF-κB activation in mammalian innate and adaptive immunity, its protein domain structure is highly conserved much further back. Adaptive immunity arose somewhere in the early evolution of the jawed vertebrates, with lampreys possessing an adaptive-like immune system and cells related to B- and T-cells [33]. However, the MALT1-like domain composition is much older than that of the paracaspase homolog F22D3.6 of the nematode *Caenorhabditis elegans* which lacks NF-κB. This indicates that there may be yet unknown functions of the animal paracaspase that have been conserved since the earliest multicellular metazoans evolved. By delineating the evolutionary history of this protein family, we provide a first step in understanding its function.

Materials and methods

Identification of new metazoan and non-metazoan paracaspases

In a first step, paracaspase genes and protein sequences were retrieved from the NCBI Entrez Gene database using three queries: (1) "MALT1"[gene] to get a list of all MALT1 annotated genes, (2) "mucosa associated lymphoid tissue" to identify homologs not annotated as MALT1 but referred to as MALT, e.g., in the gene description, and (3) "paracaspase*" to find paracaspases without a MALT(1) reference. The results of these queries and the resultant reference list of paracaspases are in Table S1. The synteny of the vertebrate paracaspases was investigated in the UCSC genome browser by looking at the genomic region of each locus based on the information in Entrez Gene. Secondly, by a combination of BLASTP (NCBI), BLAT (UCSC, JGI), and HMM (HMMER) searches, we identified new paracaspases in the genomes of *Ciona intestinalis, Branchiostoma floridae*, *Nematostella vectensis*, *Amphimedon queenslandica*, *Mnemiopsis leidyi*, *Monosiga brevicollis*, *Salpingoeca rosetta*, *Capsaspora owczarzaki*, *Stereum hirsutum*, *Dictyostelium discoideum*,

Dictyostelium purpureum, and many bacterial species (Table S2). Sequence identity was 90 % between the amphioxus ZNF592-like gene downstream of the amphioxus paracaspase and human and zebrafish ZNF592 downstream of pcasp3. Sequence identity was 74 % between amphioxus ZNF592 and human and zebrafish ZNF532 downstream of pcasp1.

Cloning of novel paracaspases from different species

Plasmids of the cloned genes were deposited in the LMBP plasmid collection (http://bccm.belspo.be/about-us/bccm-lmbp): *Xenopus tropicalis* paracaspase-1 (LMBP: 8955), chicken paracaspase-2 (LMBP: 9374), zebrafish paracaspase-1 (LMBP: 6461), zebrafish paracaspase-3 (LMBP: 8823), *C. elegans* type 1 paracaspase (LMBP: 8336), *Nematostella* type 1 paracaspase (LMBP: 8516), *Nematostella* type 2 paracaspase (LMBP: 8824), and *Trichoplax* type 2 paracaspase (LMBP: 9118).

Analysis of functional conservation

To investigate functional conservation, N-terminal ETV6 HLH fusion constructs (pCDNA3-Flag-HLH-) were made with *Xenopus tropicalis* PCASP1 (LMBP: 9569), chicken PCASP2 (LMBP: 9575), Zebrafish PCASP1 (LMBP: 9568) and PCASP3 (LMBP: 9573), *C. elegans* type 1 PCASP (LMBP 9570), and *Trichoplax* type 2 PCASP (LMBP: 9574). These novel fusion proteins and the human HLH-MALT1 fusion (LMBP: 6102) were co-transfected with pCAGGS-E-CYLD wild-type (LMBP: 6613) or R324A mutant (LMBP: 6645) in MALT1 knockout (KO) HEK293T cells using the calcium phosphate method. The MALT1 KO HEK293T cells were generated by transient transfection of pre-designed TALENs (clone pair H43180, TALEN library resource, Seoul National University, Korea) and subsequent single clone isolation by limiting dilution. Several clones were selected based on lack of MALT1 protein expression as determined by Western blot (anti-MALT1, EP603Y, Abcam) and subsequent functional analysis. For all experiments in this paper, clone #69 was used. Detection of cleaved CYLD was done with the E10 antibody (Santa Cruz Biotechnology) recognizing the C-terminal 70 kDa cleavage band. Expression of the fused paracaspases was determined with anti-Flag (F-3165, Sigma). All Western blots were developed on an Odyssey scanner (LI-COR). For analysis of downstream signaling, a firefly luciferase NF-κB reporter (LMBP: 3249) and actin promoter-driven β-galactosidase expression plasmid (LMBP: 4341) were co-transfected with the fused paracaspases in three independent transfections per set-up. Luciferase expression was normalized per sample based on β-galactosidase expression, and a 95 % confidence interval (Student's t distribution) was calculated with LibreOffice Calc. For further characterization of the A20 cleavage site, wild-type (LMBP: 3778), R439 K (LMBP: 6085), and L435G (LMBP: 5692) mutants of human A20 were co-transfected with API2-MALT1 wild-type (LMBP: 5537) or catalytic cysteine mutant (LMBP: 5538). Cleaved A20 was detected with anti-E-tag antibodies.

Phylogenetic analysis of paracaspases

Protein sequences were aligned with MUSCLE. Only regions with sequence conservation were used for phylogeny, e.g., all domains for type 1 paracaspases but only the caspase-like domain for the analysis of non-metazoan sequences. Neighbor-Joining trees were constructed with ClustalX2 and Bayesian inference using MrBayes. The resulting trees were visualized with Dendroscope.

Results

A reference set of paracaspase genes

The human paracaspase gene was first described in 1999 and named MALT1 or MLT due to its role in mucosa-associated lymphoid tissue (MALT) lymphoma [8, 34]. The name paracaspase family was first used in 2000 to describe a novel family of caspase-related proteins, including MALT1 and a similar sequence from *C. elegans* (F22D3.6) and *Dictyostelium discoideum* (PCP) [1]. To our knowledge, most of the metazoan genome sequences in the literature and sequence databases contain a single paracaspase gene, which has been annotated as MALT1 and named after the human ortholog. The paracaspase in multicellular animals typically consists of a single death domain and two immunoglobulin domains preceding the caspase-like or peptidase C14 domain, in contrast to the *Dictyostelium* PCP, which contains only a caspase-like domain. In this study, we investigated both the metazoan and the pre-metazoan ancestry and evolution of paracaspases. A reference set of paracaspase genes was compiled for this evolutionary study. Entrez Gene database searches for annotated MALT1 paracaspase genes retrieved 174 genes currently annotated as MALT1 or referred to as MALT or paracaspase (Table S1). Of the 174 annotated MALT1 genes, 153 are vertebrate, of which 72 are mammalian. Remarkably, non-mammalian vertebrates (e.g., chicken, alligator, frog, coelacanth, zebrafish) seem to have up to three MALT1-like genes, one of which is often annotated as malt1. The two other genes, and sometimes all three, currently have symbols beginning with LOC + -GeneID, indicating uncertain function and orthology. They were retrieved based on their gene description "mucosa-

associated lymphoid tissue lymphoma translocation protein 1-like." Except for the nematode *C. elegans* (F22D3.6) and the amoeba *Dictyostelium* (PCP) paracaspases, not much is known about invertebrate and non-metazoan paracaspases [1, 32]. We found several paracaspase-like genes in bilaterian invertebrates: sea urchin (*Strongylocentrotus*, an echinoderm), sea slug (*Aplysia*, a mollusc), *Brugia* (another nematode), and arthropods such as tick (*Ixodes*), honey bee (*Apis*), and jewel wasp (*Nasonia*), but not in fruit fly (*Drosophila*). Non-bilaterian MALT1-like genes from animals are also annotated in Entrez Gene, e.g., from the fresh-water polyp *Hydra vulgaris* (LOC100198709) and one gene similar to MALT1 in the placozoan *Trichoplax adhaerens* (TRIADDRAFT_54622). The *C. elegans* and *Dictyostelium* paracaspases were not retrieved by the Entrez Gene queries but were added to the paracaspase reference list. Unexpectedly, two bacterial genes, from *Spirosoma linguale* (hypothetical protein Slin_6462) and *Desulfotalea psychrophila* (paracaspase DP1212), were found based on the presence of a similar caspase-like, peptidase C14 domain. These were also included for further analysis.

Conserved synteny of three vertebrate paracaspases

To determine orthology and paralogy among the vertebrate paracaspases, we compared their genomic organization. Based on conserved gene syntenies, three different vertebrate paracaspases can be distinguished (Fig. 1). The current paracaspase gene annotations are not consistent. Therefore, we propose to annotate these paracaspases in a more uniform way (PCASP*n*, Table 1) like the naming of caspases (CASP*n*) and metacaspases (MC*n*) [35, 36]: paracaspase-1 (PCASP1), paracaspase-2 (PCASP2) and paracaspase-3 (PCASP3). Upstream of paracaspase-1 (PCASP1) are alpha-kinase 2 (ALPK2), miR-122, and the E3 ubiquitin protein ligase gene NEDD4L. Downstream genes are zinc finger protein 532 (ZNF532), SEC11 homolog C (SEC11C), and retina and anterior neural fold homeobox gene RAX. In mammals, PCASP1 is now commonly named MALT1 for its role in MALT lymphoma patients. However, the PCASP1 gene in chicken corresponds to LOC426852 instead of the currently annotated MALT1 gene on chicken chromosome 10, where the ortholog of PCASP3 is located. A second paracaspase with conserved synteny, which we named PCASP2, also has a retina and anterior neural fold homeobox gene (RAX2) downstream, followed by the megakaryocyte-associated tyrosine kinase MATK. Upstream genes are mitochondrial ribosomal protein L54 (MRPL54), amyloid beta precursor protein-binding family A member 3 (APBA3), and tight junction protein 3 (TJP3). Apparently, mammalian genomes have lost PCASP2, and frogs also seem to have lost this gene independently, as evidenced by the *Xenopus tropicalis* genome. In chicken (*Gallus gallus*), PCASP2 is located on chromosome 28 and is known as LOC429451 (RefSeq: NP_001026745). Zebrafish (*Danio rerio*) PCASP2, known as malt1a (RefSeq: NP_694508), is located on chromosome 22, and of the flanking genes only mrpl54 is shared by PCASP2 orthologs. The rest of the genomic region has a different organization. PCASP3 is not present in mammalian genomes but is present in other vertebrate genomes. The chicken PCASP3, currently annotated as MALT1, is not the true ortholog of the human MALT1 or PCASP1 but a paralog. It is orthologous to the other vertebrate PCASP3 genes of turtle (LOC101940395), frog (LOC100497102), and zebrafish (si:dkey-77f17.1). PCASP1 genes are orthologs and are present in all vertebrates. Similarly, the vertebrate PCASP2 genes are orthologs, but mammals and *Xenopus* have lost PCASP2. PCASP3 orthologs seem to be present in all vertebrates but mammals. The three vertebrate paracaspases PCASP1, PCASP2, and PCASP3 are paralogs.

Fig. 1 Conserved synteny of the three vertebrate paracaspases. ▶ **a** Paracaspase-1 (PCASP1), known as MALT1 in mammals, is conserved in all vertebrate species and has shared gene synteny, except for zebrafish. **b** Paracaspase-2 (PCASP2) is missing in mammals (e.g., human) and the frog *Xenopus tropicalis*. **c** Also paracaspase-3 (PCASP3) has been lost in mammals but is present in the other vertebrate lineages with conserved synteny

Identification of additional new metazoan paracaspases

By a combination of BLA(S)T and HMM searches, we identified putative new paracaspases in both metazoan and non-metazoan species (Fig. 2; Table S2). Paracaspases with death and Ig domains, such as PCASP1, PCASP2 and PCASP3, will be referred to as type 1 paracaspases, and those without death and Ig domains, such as *Dictyostelium* pcp, will be classified as type 2 (Fig. 2). The phylum Chordata consists of vertebrates, urochordates, and cephalochordates. To determine when the three type 1 paracaspases arose, we first examined the genomes of the urochordate *Ciona intestinalis* (vase tunicate) and cephalochordate *Branchiostoma floridae* (lancelet, also known as amphioxus). *Ciona intestinalis* has only one paracaspase (LOC100185957). In amphioxus, two paracaspase genes were found in the same genomic region, both flanked downstream by a znf592-like gene similar to the znf592 gene that is downstream of the vertebrate paracaspase-3. The longest predicted transcripts (JGI fgenesh2 models) of these amphioxus paracaspases share 97 % protein sequence identity and are most likely the result of a

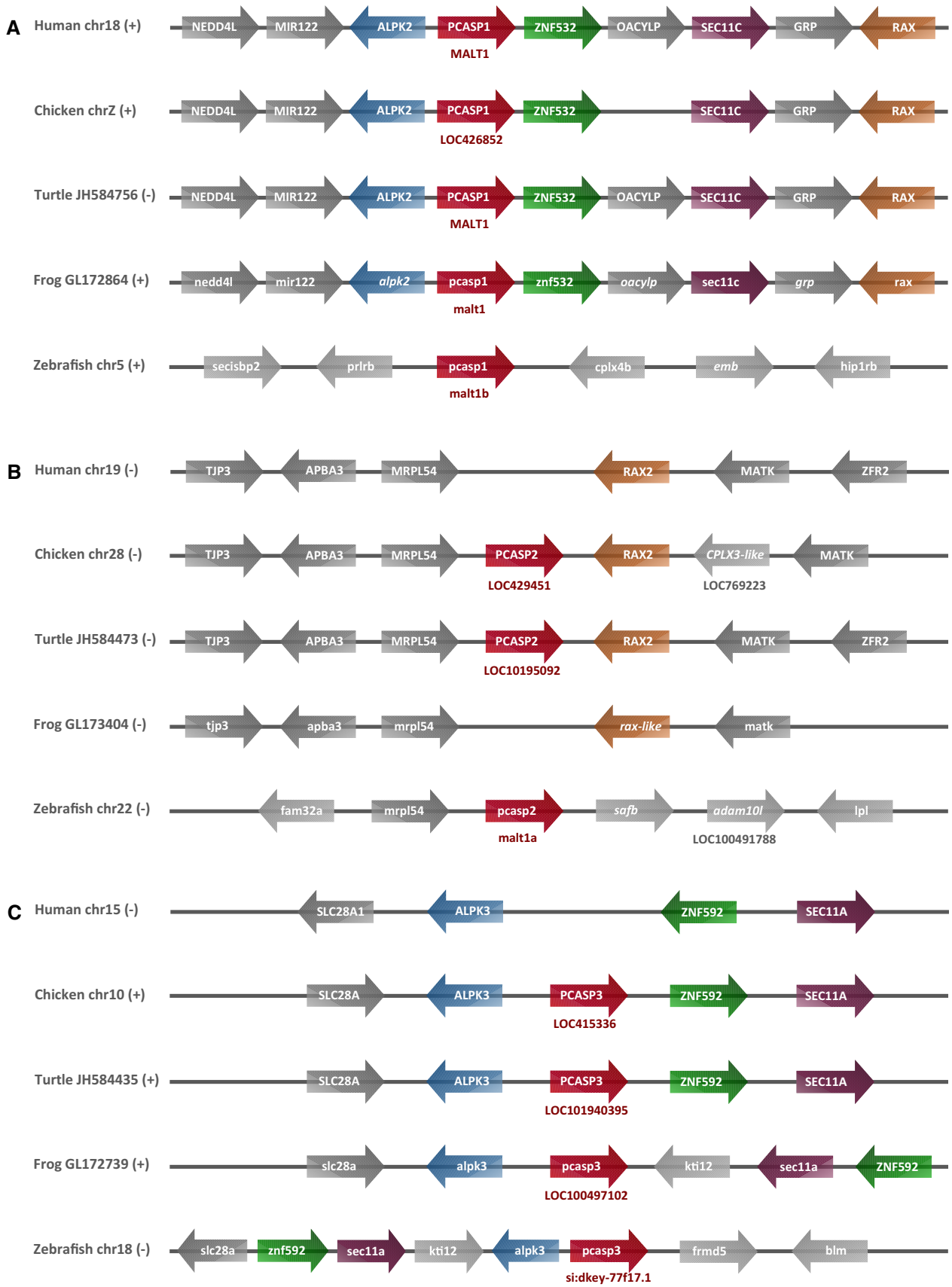

A
Human chr18 (+)
NEDD4L
MIR122
ALPK2
PCASP1
ZNF532
OACYLP
SEC11C
GRP
RAX
MALT1
Chicken chrZ (+)
NEDD4L
MIR122
ALPK2
PCASP1
ZNF532
SEC11C
GRP
RAX
LOC426852
Turtle JH584756 (-)
NEDD4L
MIR122
ALPK2
PCASP1
ZNF532
OACYLP
SEC11C
GRP
RAX
MALT1
Frog GL172864 (+)
nedd4l
mir122
alpk2
pcasp1
znf532
oacylp
sec11c
grp
rax
malt1
Zebrafish chr5 (+)
secisbp2
prlrb
pcasp1
cplx4b
emb
hip1rb
malt1b
B
Human chr19 (-)
TJP3
APBA3
MRPL54
RAX2
MATK
ZFR2
Chicken chr28 (-)
TJP3
APBA3
MRPL54
PCASP2
RAX2
CPLX3-like
MATK
LOC429451
LOC769223
Turtle JH584473 (-)
TJP3
APBA3
MRPL54
PCASP2
RAX2
MATK
ZFR2
LOC10195092
Frog GL173404 (-)
tjp3
apba3
mrpl54
rax-like
matk
Zebrafish chr22 (-)
fam32a
mrpl54
pcasp2
safb
adam10l
lpl
malt1a
LOC100491788
C
Human chr15 (-)
SLC28A1
ALPK3
ZNF592
SEC11A
Chicken chr10 (+)
SLC28A
ALPK3
PCASP3
ZNF592
SEC11A
LOC415336
Turtle JH584435 (+)
SLC28A
ALPK3
PCASP3
ZNF592
SEC11A
LOC101940395
Frog GL172739 (+)
slc28a
alpk3
pcasp3
kti12
sec11a
ZNF592
LOC100497102
Zebrafish chr18 (-)
slc28a
znf592
sec11a
kti12
alpk3
pcasp3
frmd5
blm
si:dkey-77f17.1

Table 1 Proposed annotation for the vertebrate paracaspase paralogs

Species	Gene ID	Old annotation	New annotation
Human (*Homo sapiens*)	10892	MALT1	PCASP1
Mouse (*Mus musculus*)	240354	Malt1	Pcasp1
Chicken (*Gallus gallus*)	426852	LOC426852	PCASP1
	429451	LOC429451	PCASP2
	415336	MALT1	PCASP3
Turtle (*Chrysemys picta*)	101936385	MALT1	PCASP1
	101950925	LOC101950925	PCASP2
	101940395	LOC101940395	PCASP3
Frog (*Xenopus tropicalis*)	100493493	malt1	pcasp1
	100497102	LOC100497102	pcasp3
Zebrafish (*Danio rerio*)	561914	malt1b	pcasp1
	259196	malt1a	pcasp2
	571242	si:dkey-77f17.1	pcasp3

lineage-specific duplication. Next, we examined the genome of the sea urchin *Strongylocentrotus purpuratus*, an echinoderm that is considered an important outgroup for the chordates in the superphylum Deuterostomia [37]. Two paracaspase hits were found. The first sea urchin gene LOC590194 (XP_794899) corresponds only to the N-terminal part containing the death domain and two Ig domains. The second gene, LOC574947 (XP_780448), encodes only part of the caspase-like domain. Using the current sea urchin genome assembly, it was not possible to predict the full-length paracaspase, but based on these two partial sequences, sea urchin likely has one type 1 paracaspase. These findings suggest that the deuterostome ancestor had only one type 1 paracaspase containing the same domains as the three vertebrate type 1 paracaspase paralogs, and that duplications that occurred in the ancestry of vertebrates led to these three paracaspase paralogs (Fig. 2). Furthermore, since the vertebrate PCASP3 orthologs are flanked by the znf592 gene and amphioxus shares this synteny, PCASP3 seems to be the original copy. This hypothesis is further supported by subsequent phylogenetic analysis.

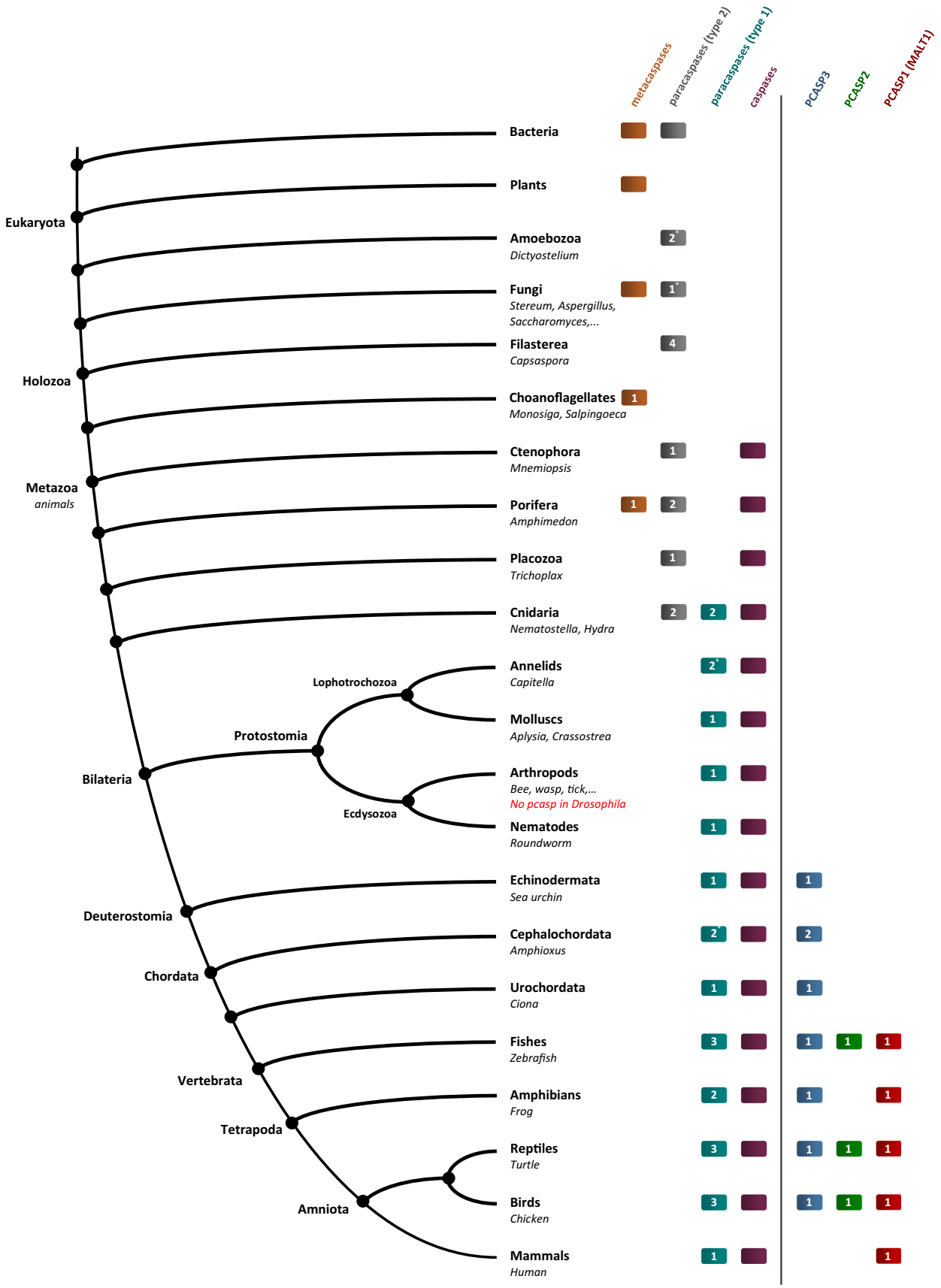
The Bilateria, animals with bilateral symmetry and three germ layers, are divided into the three superphyla of Deuterostomia, Ecdysozoa, and Lophotrochozoa, of which the latter two are grouped in the clade Protostomia. Arthropods and nematodes belong to the Ecdysozoa. The Lophotrochozoa contain annelids, molluscs, and plathelminthes. We compared the paracaspases in several protostomian species to deuterostomian and non-bilaterian paracaspases (Fig. 2). We found a single type 1 paracaspase in most arthropods (e.g., bee, wasp, body louse, water flea, and tick), in nematodes (e.g., *Caenorhabditis*, *Brugia*, *Trichinella*), in molluscs (e.g., *Aplysia*, *Crassostrea*), and two in the annelid *Capitella* (Fig. 2; Tables S1, S2, Fig. S1). There were no paracaspases in fruit fly.

Fig. 2 Paracaspases, metacaspases, and caspases in the tree of life. ▶ The presence is indicated by a *colored gene box*. If the number of genes is known from this study, it is shown in the *box*. An *asterisk* indicates a finding within a specific species of the lineage. Paracaspases are divided into type 1 (with additional Ig domains) and type 2 (without Ig domains). In deuterostomians, type 1 paracaspases are further subdivided into PCASP1 (MALT1), PCASP2, and PCASP3

The presence of a similar type 1 paracaspase in all three bilaterian superphyla suggests that the bilaterian ancestor had such a paracaspase containing the three types of domains (death, Ig and caspase-like). This bilaterian paracaspase (type 1) differs from the non-metazoan paracaspase (type 2) found in the slime mold *Dictyostelium*. *Dictyostelium* pcp is much smaller and contains only the caspase-like domain. To further unravel the evolutionary events resulting in this difference, we first looked for paracaspases in non-bilaterian animals and then in close relatives of the Metazoa. We investigated the genomes of several basal animals: *Nematostella vectensis* and *Hydra magnipapillata* of the phylum Cnidaria, *Trichoplax adhaerens* of the phylum Placozoa, *Amphimedon queenslandica* of the phylum Porifera, and *Mnemiopsis leidyi* of the phylum Ctenophora (Fig. 2). We found four paracaspases in *Nematostella* and three in *Hydra* (Tables S1, S2; Fig. S2, S3). The type 1 paracaspase with the additional death and Ig domains and the type 2 paracaspase with only the caspase-like domain without Ig domains are both represented in cnidarians. Cnidaria are considered a sister group of the Bilateria. The other basal metazoan genomes of Placozoa, Porifera, and Ctenophora encode only type 2 paracaspases (Table S2). We identified one type 2 paracaspase in *Trichoplax adhaerens* and one in *Mnemiopsis leidyi* with only a caspase-like domain. In *Amphimedon queenslandica*, we found three putative paracaspases. Two of them also have only a caspase-like domain (type 2) but, remarkably, a third contains several WD40 repeat domains preceding the caspase-like domain, which is typical of prokaryotic metacaspases [7]. These data indicate that the longer type 1 paracaspase arose in the cnidarian–bilaterian last common ancestor from an ancestral type 2 form that gained death and Ig domains. The earliest metazoan animals have the type 2 paracaspase with only the caspase-like domain.

Exploring the structural and functional conservation of animal paracaspases

Structural information exists for the death domain (PDB: 2G7R), the first and second Ig domain (PDB: 3K0W), and the paracaspase and Ig-like domain (PDB: 3UO8, 3V55) of


metacaspases
paracaspases (type 2)
paracaspases (type 1)
caspases
PCASP3
PCASP2
PCASP1 (MALT1)
Bacteria
Plants
Amoebozoa
Dictyostelium
Fungi
Stereum, Aspergillus, Saccharomyces,...
Filasterea
Capsaspora
Choanoflagellates
Monosiga, Salpingoeca
Ctenophora
Mnemiopsis
Porifera
Amphimedon
Placozoa
Trichoplax
Cnidaria
Nematostella, Hydra
Annelids
Capitella
Molluscs
Aplysia, Crassostrea
Arthropods
Bee, wasp, tick,...
No pcasp in Drosophila
Nematodes
Roundworm
Echinodermata
Sea urchin
Cephalochordata
Amphioxus
Urochordata
Ciona
Fishes
Zebrafish
Amphibians
Frog
Reptiles
Turtle
Birds
Chicken
Mammals
Human
Eukaryota
Holozoa
Metazoa
animals
Bilateria
Lophotrochozoa
Protostomia
Ecdysozoa
Deuterostomia
Chordata
Vertebrata
Tetrapoda
Amniota

human PCASP1 (MALT1) [20, 21]. Structures of metacaspases and caspases have also been published [4, 38, 39]. To gain insights into potentially conserved functional mechanisms of the new vertebrate paracaspases PCASP2 and PCASP3 and non-vertebrate paracaspases, we compared the structurally important sequence motifs (Fig. 3). PCASP1 (MALT1) cleaves substrates with an arginine at the P1 position, such as A20, Bcl10, and CYLD (Fig. S4), in contrast to the aspartic acid used by caspases. The critical residues forming the active site are three acidic residues (D365, D462, and E500 based on the residue numbers of human MALT1) responsible for six of the ten hydrogen bonds contacting the positively charged arginine [20]. These are fully conserved in both vertebrate and invertebrate paracaspases (Fig. 3). Five other important residues in the active site are also highly conserved: P362, H415, G416, C464, and A498. These findings suggest that the functions and mechanisms of these different vertebrate and invertebrate paracaspases are similar. In basal animals and non-metazoans, the first and second acidic residues, D365 and D462, are also fully conserved in all paracaspases, even in metacaspases. The fourth motif, containing the third acidic residue (structural loop L4), shows more variations. Paracaspases usually have an arginine in the third motif (DxCR), whereas metacaspases in most cases have a histidine (DxCH). However, exceptions clearly exist for both (Fig. 3), and as the rest of the sequence shows less sequence identity, it is not always evident whether to classify a protein as a metacaspase or a paracaspase.

Our current knowledge of paracaspase-interacting molecules and substrates is primarily based on PCASP1 (MALT1) studies. But because PCASP1 is a vertebrate-specific paracaspase, it is uncertain if these interacting molecules and substrates are also functionally conserved in other paracaspases throughout metazoan evolution. We investigated the evolutionary conservation of 10 molecules reported to interact with PCASP1: BCL10, CARD9, CARD10 (CARMA3), CARD11 (CARMA1), CARD14 (CARMA2), CYLD, RELB, TRAF6, TNFAIP3 (A20), and ZC3H12A (MCPIP1 or Regnase-1) (Table S3). Most of them do not have non-vertebrate orthologs, except for the cleavage substrate CYLD and the PCASP1-interacting protein TRAF6, both of which have orthologs in invertebrates dating back even before bilaterians, as evidenced by a cyld and a traf6 gene in the sea anemone *Nematostella vectensis*. *Nematostella* also seem to have a BCL10 ortholog, which is absent in protostomians.

Cleavage of the evolutionary conserved substrate CYLD by different paracaspases was experimentally validated. We cloned *Xenopus tropicalis* PCASP1, Chicken PCASP2, Zebrafish PCASP1 and PCASP3, *C. elegans* type 1 PCASP, and *Trichoplax adherens* type 2 PCASP with an N-terminal fusion of the activating ETV6 HLH domain [19]. The fused paracaspase constructs were co-transfected with wild-type human CYLD or the R324A mutant in HEK293T cells. To ensure that the protease activity occurred directly downstream of the transfected fusion proteins and not via activation of endogenous MALT1, the experiments were performed with MALT1-deficient HEK293T cells. If the wild-type CYLD is cleaved and the R324A mutant is not, this shows a conserved substrate specificity since human MALT1 only cleaves CYLD at R324. As is apparent from the CYLD cleavage patterns, all types of vertebrate paracaspases cleave human CYLD after R324, indicating a functional conservation of the protease across the vertebrate paralogs (Fig. 4a). We have no explanation for the poor performance of XtPCASP1, but perhaps XtPCASP3 is the dominant variant in this organism. The fused paracaspases were also co-transfected with an NF-κB firefly luciferase reporter plasmid and β-galactosidase driven by an actin promoter as transfection level control (Fig. 4b). NF-κB activation indicates conserved interaction with critical downstream proteins, like TRAF6. In analogy with the protease function, we can see NF-κB activation by all activated vertebrate paracaspases.

Identification of new non-metazoan paracaspases

Outside the animal kingdom not much is known about paracaspases and their evolutionary history. A single paracaspase named pcp was found in the slime mold *Dictyostelium*. In two Rhizobiales species of the Alphaproteobacteria, putative paracaspases have been detected: one in Rhizobia, and up to seven in *Mesorhizobium loti* [40]. We continued our paracaspase search by including the newly identified metazoan paracaspases as query, and started by analyzing the recently published genome sequences of unicellular relatives of multicellular animals. Only one relevant hit was found in the choanoflagellates *Monosiga brevicollis* and *Salpingoeca rosetta* (Fig. 2; Table S2, Fig. S6), which were both included for subsequent phylogenetic analyses. However, in both protein sequences, consisting of only a Peptidase_C14 (caspase-like) domain, we found the conserved motif DxCH, which is a typical metacaspase feature rather than the DxCR motif of paracaspases [1]. Notably, a hypothetical protein (PTSG_12389) was found in *S. rosetta*: it contains the GHG and DxCR paracaspase motifs but in an N-terminal region, where no caspase-like or other domain was predicted.

In the genome of the filasterean *Capsaspora owczarzaki*, another close unicellular relative of metazoans besides choanoflagellates, four putative type 2 paracaspases were found (Fig. 2;Table S2; Fig. S7). One has the consensus GHG and DxCR motifs (CAOG_07085). Two have one or more amino acid variations but still a DxxR motif

(CAOG_05990 and CAOG_04287) and both have a COG0790 region C-terminally composed of Tetratrico Peptide Repeats (TPR) or Sel1-like repeats. The protein RefSeq XP_004348112 of the CAOG_04287 gene has only a partial CASc (caspase-like) domain, but further domain analysis with Smart and Pfam predicted a much larger Peptidase_C14 region. This finding points to the presence of two caspase-like domains supported by a GHS/DASR motif in the first domain and a GHG/DWCQ motif in the second domain. In the fourth *Capsaspora* hit (CAOG_06568), only the GHS motif is present in the caspase-like domain. Multiple sequence alignment with paracaspases indicates the loss of the other DxCR motif mutated in SAAA. No metacaspase was found in *Capsaspora*.

In the fungal kingdom, only metacaspases have been described, such as *Saccharomyces* Yca1, *Candida* MCA1,s and *Aspergillus* CasA and CasB [41]. However, in the mushroom *Stereum hirsutum*, we found one putative paracaspase (STEHIDRAFT_135948) with a DxCR motif in addition to several *Stereum* metacaspases (Fig. 2; Table S2; Fig. S8). Remarkably, no other paracaspase was found in the fungal sequences currently available at GenBank.

The *Dictyostelium purpureum* genome encodes three paracaspases (Fig. 2; Table S2; Fig. S7). DICPUDRAFT_83788 is the ortholog of the known *Dictyostelium discoideum* pcp. The other two paralogs, DICPUDRAFT_79158 (pcp2) and DICPUDRAFT_147756 (pcp3), are more similar to a second, new paracaspase we found in *Dictyostelium discoideum*, DDB_G0277689, which we named pcp2. All *Dictyostelium* paracaspases have the consensus DxCR motif, except for DICPUDRAFT_79158 (pcp2), in which the Asp is replaced by a

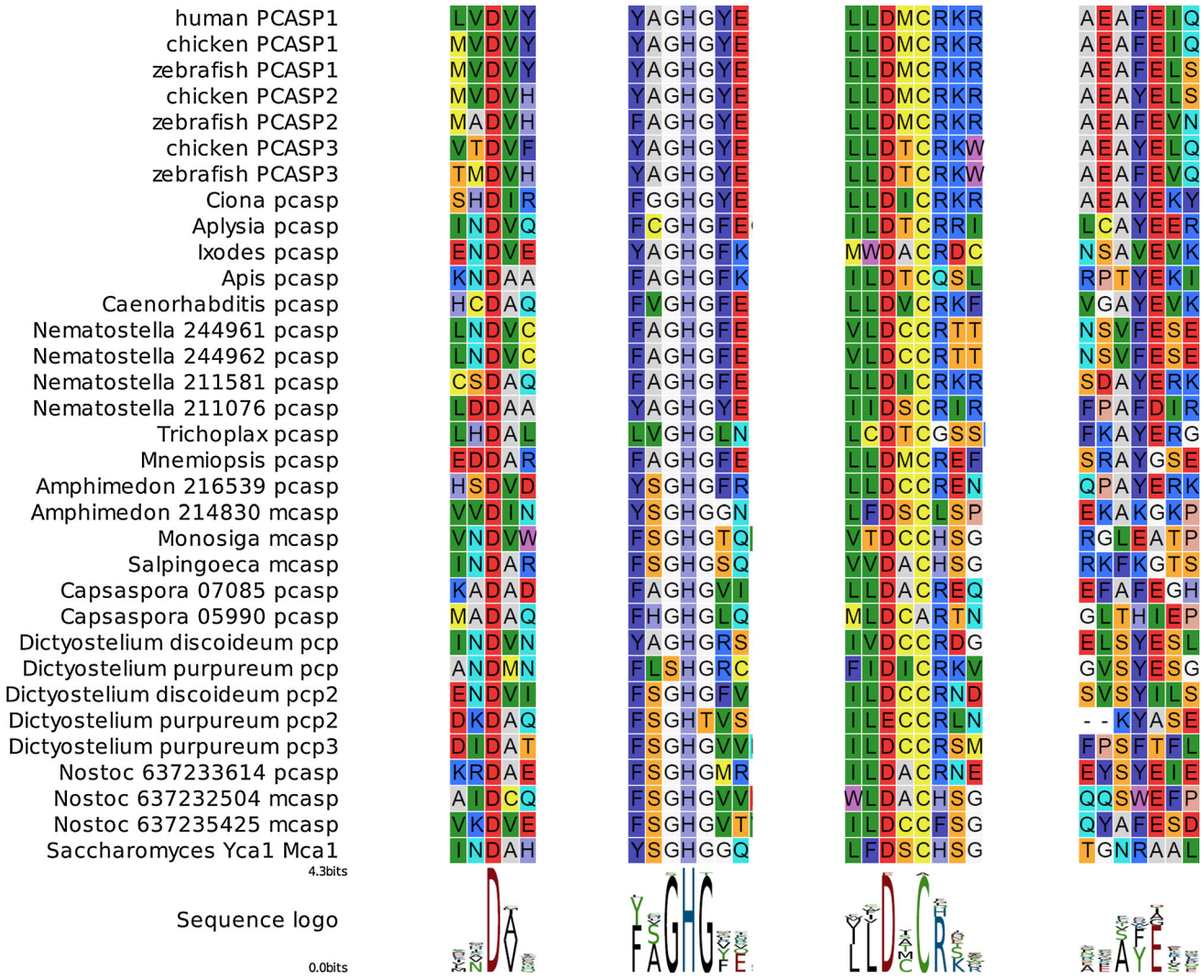


Fig. 3 Alignment of the paracaspase regions involved in the active site. The substrate amino acid P1-Arg is coordinated by three crucial acidic residues, indicated by an *asterisk in the red box*, which are fully conserved in the three vertebrate paracaspases and the invertebrate paracaspases. Other highly conserved residues important for substrate recognition are also indicated by an *asterisk* or by the preferred amino acid in the *yellow box*

Glu, producing an ECCR motif. No metacaspase was found in *Dictyostelium*.

To our knowledge, paracaspases outside the animal kingdom have been found only in *Dictyostelium* and some Alphaproteobacteria belonging to the rhizobacteria [5]. The origin and evolution of paracaspases and metacaspases in the Amoebozoa and Opisthokont (Fungi/Metazoa) groups is unclear for several reasons: the finding of new paracaspases but the absence of metacaspases in *Capsaspora* and *Dictyostelium*, the presence of a putative metacaspase in choanoflagellates but the absence of paracaspases, and the presence of a single paracaspase and several metacaspases in the fungus *Stereum*. We continued our search in other eukaryotes and prokaryotes. As expected, only

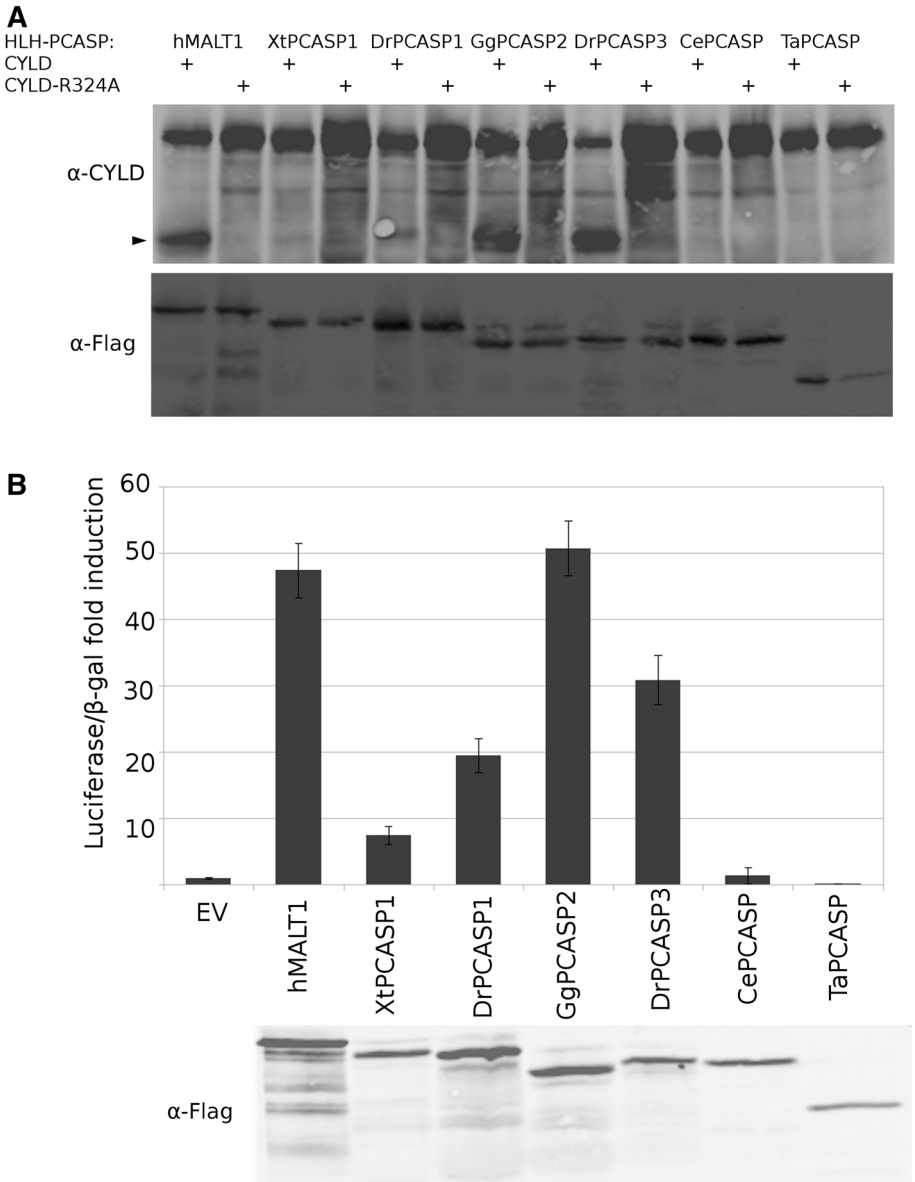


Fig. 4 Functional characterization of activated HLH-PCASP fusion proteins. **a** Cleavage of human CYLD by vertebrate paracaspases. Flag-tagged HLH-paracaspase expression constructs were co-transfected with wild-type human CYLD or the R324A mutant in MALT1-deficient HEK293T cells. Lysates were subjected to Western blotting and immunodetection with anti-CYLD. A CYLD C-terminal fragment (*black arrowhead*; 70 kDa band) appears with all types of vertebrate paracaspases. None of the invertebrate paracaspases tested showed CYLD cleavage. Expression of the paracaspase constructs is determined by detection of the Flag-tag. hMALT1: human MALT1; XtPCASP1: Xenopus tropicalis PCASP1; DrPCASP1: Danio rerio PCASP1; GgPCASP2: Gallus gallus PCASP2; DrPCASP3: Danio rerio PCASP3; CePCASP: Caenorhabditis elegans PCASP; TaPCASP: Trichoplax adhaerens PCASP. **b** NF-κB luciferase assay. *Top* values represent β-gal adjusted luciferase values as fold-induction compared to empty vector control. *Error bars* indicate 95 % confidence intervals (Student's *t* distribution). All types of vertebrate paracaspases are able to activate NF-κB. *Bottom* Western blot with anti-flag reveals paracaspase expression levels in the luciferase assay lysates

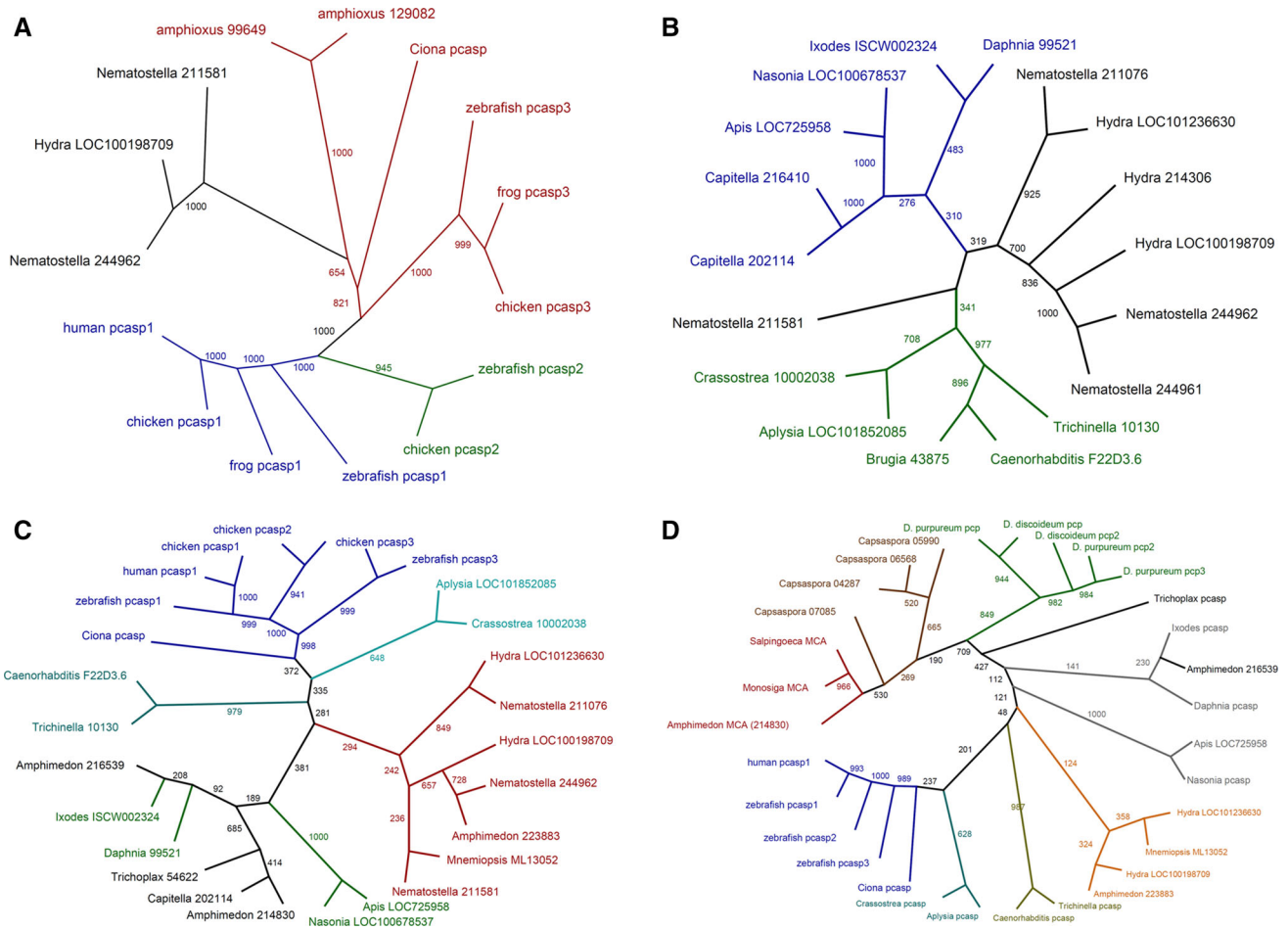


Fig. 5 Phylogenetic analyses of **a** deuterostomian and cnidarian paracaspases, **b** protostomian and cnidarian paracaspases, **c** metazoan paracaspases, and **d** paracaspases and metacaspases of metazoans and closely related non-metazoan species

metacaspases and no paracaspases were found in plants. However, unexpectedly, we did find besides the paracaspases described in rhizobacteria other putative paracaspases in many bacterial phyla, including Bacteroidetes, Cyanobacteria, and many Proteobacteria (Table S2; Fig. S9, S10). All have the highly conserved DxCR paracaspase motif. A genome-wide comparative study in 2010 identified 58 putative metacaspases in Cyanobacteria [42]. Several of these putative metacaspases have motifs that differ from the typical metacaspase motifs containing the active site residues, *e.g.* DxCF instead of DxCH. Remarkably, some have the DxCR motif, which is conserved in nearly all paracaspases, suggesting they are paracaspases rather than metacaspases.

Phylogenetic analysis of the expanded paracaspase family

Both annotated and putative new paracaspases were used for in-depth phylogenetic analyses, together with some representative metacaspases as outgroup. Four different types of phylogenetic analyses were performed. In each successive analysis, more distantly related sequences from less related species were added and analyzed by both the neighbor-joining (NJ) method (Fig. 5) and Bayesian inference (Fig. S11). The first analysis on deuterostomian and cnidarian paracaspases (Fig. 5a; Fig. S11A) supports the paralogy of the three vertebrate paracaspases and the closer relationship of paracaspase-3 to the paracaspases of *Ciona*, amphioxus, and Cnidaria. The two amphioxus paracaspases are paralogs resulting from a lineage-specific gene duplication. The chordate ancestor possessed a single paracaspase gene that duplicated early in vertebrate evolution to generate three vertebrate paracaspase paralogs. Amphibians lost paracaspase-2 again, and mammals lost both paracaspases-2 and -3. Figure 5b and Fig. S11B show a second phylogenetic analysis that includes protostomian and cnidarian paracaspases. Paracaspases fall within clades according to their phyla of molluscs (*Aplysia* and *Crassostrea*), nematodes (*Caenorhabditis*, *Brugia* and

Trichinella) and arthropods (*Daphnia*, *Ixodes*, *Nasonia*, *Apis*). But unexpectedly, mollusc paracaspases are more similar to nematode paracaspases, while both paracaspases from the annelid *Capitella* are more similar to the arthropod paracaspases. The *Capitella* paracaspases seem to be paralogs resulting from a lineage-specific duplication.

Phylogenetic analysis of all metazoan paracaspases (Fig. 5c; Fig. S11C) corroborates our previous findings. The mollusc paracaspases are more similar to the *Ciona* and vertebrate paracaspases, in contrast to arthropod paracaspases, which are in a separate clade. The cnidarian paracaspases from *Hydra* and *Nematostella* form a separate clade, indicating that there were lineage-specific duplications also in Cnidaria. One *Amphimedon* paracaspase (223883) is in the same clade as these cnidarian paracaspases and can be considered an ortholog. The two other *Amphimedon* paracaspase-like sequences we found are different. *Amphimedon* paracaspase 216539 is orthologous to the arthropod paracaspases. Arthropod paracaspases seem to have a different evolutionary origin from the mollusc and vertebrate paracaspases. The different paracaspases in *Amphimedon* suggest that paralogs existed in basal metazoans, one of which is orthologous to mollusc and vertebrate paracaspases, whereas the other is orthologous to the arthropod paracaspases. The third putative paracaspase with WD40 repeat domains in *Amphimedon* (Fig. S5) can be considered a metacaspase, as evidenced by the fourth phylogenetic analysis. WD40 domains are typically found in metacaspases. This is corroborated by both the Neighbor-Joining tree (Fig. 5d) and the Bayesian consensus tree (Fig. S11D), in which the *Amphimedon* metacaspase forms a clade with the two choanoflagellate metacaspases we identified in *Monosiga* and *Salpingoeca*. No other animal metacaspase has been described before. The typical His in the DxCH motif of metacaspases is replaced by a Leu in the DSCL motif of *Amphimedon* metacaspase, which could indicate loss of a typical metacaspase function or a variation towards another type of metacaspase. Finally, the phylogenetic analysis suggests that the different paracaspases of *Dictyostelium* and those of *Capsaspora* are probably lineage-specific duplications and can be considered paralogs.

Discussion

Until recently only a single paracaspase had been described in animals, and it was thought that the metacaspases had been lost in all animals. In this study, we show that the modern type 1 paracaspases containing a death domain and Ig domains in addition to the caspase-like domain arose in the cnidarian–bilaterian last common ancestor. Mammals have only PCASP1 (MALT1), but other vertebrates have up to three paralogs of these paracaspases, of which PCASP3 is the parent gene. This type of paracaspase is not present in more basal metazoans (Porifera, Placozoa, Ctenophora), which possess a type 2 paracaspase containing only a caspase-like domain. The paracaspase family we studied can now be annotated more appropriately based on the phylogenetic profile, genomic synteny, and domain composition. The availability of sequenced genomes has enabled us to have a good understanding of the vertebrate evolution of paracaspases. In contrast, we have probably not been able to sample the full diversity of paracaspases in invertebrates, and even less so in pre-metazoan evolution. Nevertheless, we identified new invertebrate paracaspases and non-metazoan paracaspases in *Capsaspora*, *Dictyostelium*, *Stereum*, and many bacteria. It is still unknown whether paracaspases (and true caspases) originated by a horizontal gene transfer event before the last common ancestor of the unikonts (the same paracaspase origin in *Dictyostelium* and metazoa) or have been transferred from bacteria to eukaryotes on multiple occasions. The finding of the new paracaspases described here leads to two other hypotheses. There are now examples of both prokaryote and eukaryote species having both para- and metacaspases. Eukaryotes having either metacaspases or paracaspases could be the result of losing one or the other. The second perhaps even more likely new hypothesis is that metacaspases and paracaspases can switch into the other protein type by a few amino acid modifications. This could explain why bacteria and the fungus *Stereum* have a paracaspase similar to the metacaspases they possess, while in other species such as *Dictyostelium*, *Capsaspora*, and choanoflagellates, the caspase-like protein sequences evolved into what we identify as either a paracaspase or a metacaspase. In this model, paracaspases could simply be a subclass of the metacaspase family and might have evolved several times independently, e.g., in *Capsaspora* and *Dictyostelium*, while in choanoflagellates, the typical metacaspase was retained. Even in our extended study of the paracaspase family, we could not identify a likely evolutionary event in which the true caspases evolved from a paracaspase (or metacaspase), but if the urmetazoan ancestor possessed a 'meta/para' caspase-like protein, one copy might have diverged into the metazoan paracaspase while another copy diverged into the later metazoan caspase. In contrast to the true caspases, which evolved considerably in the metazoan lineage by adopting different domain compositions and specialized cleavage site specificities, both the paracaspase active site and its domain composition remained remarkably conserved. This indicates an ancient role of the paracaspase protease. One candidate mechanism that could have acted to conserve the paracaspases is the recently discovered interaction with the autophagy receptor p62/SQSTM [43] and the protease-

dependent role of MALT1 in mTOR signaling [44, 45]. Most known MALT1 substrates are not conserved as far back as the type 1 paracaspases, excluding them from being the cause of conservation. Conservation of currently known substrate cleavage sites stops at the early vertebrates and no obviously conserved cleavage site has so far been discovered in invertebrates (Fig S4). A20 has had conserved functions as far back as amphioxus [46], but little is known about older homologs of A20. For A20, even closely related species as human and mouse display two distinct paracaspase cleavage sites [24], and the cleavage site has been lost at several independent events (Fig S4). The human A20 cleavage site, which is conserved since the earliest mammals (Fig S4), depends on a pentapeptide with a P5 Leucine (Fig S5). This is different from all other currently known substrates which are defined by a tetrapeptide sequence. The A20 GASR tetrapeptide has however been shown to be a very poor MALT1 substrate [3]. Interestingly, loss of the first cleavage site results in two alternative sites, which could explain the evolutionary flexibility of this site (Fig. S5). This illustrates that even if the currently known site is not conserved, the paracaspase–substrate interaction might be older and occur at a different site. The two oldest currently known paracaspase substrates are the paracaspase itself (MALT1 autoprocessing) and CYLD (Table S3). Intriguingly, the MALT1 autoprocessing site is conserved throughout mammals and is not found in other PCASP1 homologs. We do not know the strength of the selection pressure to keep this autoprocessing site. There is, for example, a SNP in human MALT1 (rs147414021) causing an un-cleavable R149Q mutation. CYLD can be found as a conserved protein as far back as *Nematostella*, dating it back to when type 1 paracaspase arose. CYLD is also well established in *Drosophila* models, where it regulates JNK and NF-κB signaling as in humans. Intriguingly, the CYLD in *Drosophila*, which lacks paracaspase, approximates the C-terminal fragment after MALT1 cleavage of CYLD in humans. Because most of the known substrates of paracaspase evolved later than the paracaspase itself, it is likely that the substrates evolved to get cleaved, while the paracaspase remained relatively unchanged.

The existence of three functionally conserved paracaspase paralogs in non-mammalian vertebrates highlights the need for care when non-mammalian model organisms are used to study MALT1 function. It would be interesting to establish the co-evolution of upstream activating signals, substrates, interactors, and paracaspase as it gets co-opted from its original unknown function to its current dominant function in mammalian adaptive immunity. By N-terminal fusions to an HLH domain, we have bypassed all upstream events and focused on the downstream scaffold and protease functions of the paracaspases. A cautionary note on the interpretations from this method is that while positive results provide strong evidence for conserved functions in a cellular context, negative results are harder to interpret. It could very well be that paracaspases, like for example the *C. elegans* type 1 paracaspase, have a MALT1-like activity in its native cellular environment but that (currently unknown) critical host proteins required for optimal activation are too divergent in a human cell environment. An important future follow-up would be to complement our results with biochemical characterizations and investigations of the paracaspases in their native cellular environment. It was recently discovered that the zebrafish Bcl10 is functionally conserved and that CARMA proteins and Bcl10 can induce NF-κB in the zebrafish cell line PAC2 [47]. It will be interesting to see which paracaspase paralog(s) participate in the CBM complex(es) in the PAC2 cell model, since this could tell us whether the paralogs are also redundant in their upstream signaling under native conditions. In addition, finding the most distantly related paracaspases with MALT1-like activities could provide structure–function insights through correlation of conserved sequence segments and activity. In particular, it would be interesting to study the invertebrate ortholog PCASP3 proteins from molluscs, sea urchins, lancelets, and tunicates to determine how far back the MALT1-like activities are conserved. It will also be interesting to look even further back when more invertebrate genome data make it possible to identify closely related invertebrate type 1 paracaspase homologs lacking MALT1-like activities. Also the known paracaspase substrates could be used for further evolutionary functional studies. Due to its highly conserved cleavage site (Fig. S4), observation of the cleaved Bcl10 neo-epitope should be an excellent tool to study the role of paracaspase activity in the early evolution of adaptive immunity in bony fish and sharks. Identification of the oldest substrates could also be a way to find the most critical or "core" cleavage events downstream of paracaspase activation.

Acknowledgments We thank Dr. Amin Bredan for editing the manuscript. *Danio rero* paracaspase-1 was obtained from Dr. Mathijs Baens (KU Leuven, Belgium), *Xenopus tropicalis* cDNA was obtained from Dr. Hong Thi Tran (Ghent university, Belgium), *Danio rerio* cDNA from prof. Kris Vleminckx (Ghent university, Belgium), *C. elegans* cDNA from prof. Bart Braeckman (Ghent university, Belgium), and *Nematostella vectensis* cDNA from Srividhya Sundararaman (Ghent university, Belgium). Chicken spleen was kindly provided by Dr. Alexander Van Parys (Ghent university, Belgium).

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