Review

Calpain chronicle—an enzyme family under multidisciplinary characterization

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Abstract: Calpain is an intracellular Ca^{2+}-dependent cysteine protease (EC 3.4.22.17; Clan CA, family C02) discovered in 1964. It was also called CANP (Ca^{2+}-activated neutral protease) as well as CASF, CDP, KAF, *etc.* until 1990. Calpains are found in almost all eukaryotes and a few bacteria, but not in archaebacteria. Calpains have a limited proteolytic activity, and function to transform or modulate their substrates' structures and activities; they are therefore called, "modulator proteases." In the human genome, 15 genes—*CAPN1*, *CAPN2*, *etc.*—encode a calpain-like protease domain. Their products are calpain homologs with divergent structures and various combinations of functional domains, including Ca^{2+}-binding and microtubule-interaction domains. Genetic studies have linked calpain deficiencies to a variety of defects in many different organisms, including lethality, muscular dystrophies, gastropathy, and diabetes. This review of the study of calpains focuses especially on recent findings about their structure–function relationships. These discoveries have been greatly aided by the development of 3D structural studies and genetic models.

Keywords: calpain, muscular dystrophy, calpainopathy, gastropathy, intracellular proteolysis, calcium ion

Introduction

An enzyme corresponding to calpain was first described in 1964 by Gordon Guroff (1933–1999).[1),2)] Over the next several years, calpain was reported with various names and in various contexts by other researchers, including Edmond H. Fischer (1920–), Edwin G. Krebs (1918–2009),[3),4)] Darrel E. Goll (1936–2008),[5)] and Yasutomi Nishizuka (1932–2004).[6)] In 1978, Kazutomo Imahori (1920–) and his colleagues purified chicken calpain to homogeneity and named it CANP (calcium-activated neutral protease).[7)] This marked the beginning of serious, focused studies of calpain, and this protease was soon established as one of the most important and intriguing enzymes in the cell.[8)–10)] That same year, an endogenous calpain-specific inhibitor protein was reported by Takashi Murachi (1926–1990) and his colleagues.[11)] They proposed the names calpain in place of CANP and calpastatin for its inhibitor protein in 1981.[12)] In 1984, Koichi Suzuki (1939–2010) and his colleagues revealed the complete primary structure of the calpain catalytic subunit,[13)] and calpain studies turned toward structure–function analyses. For more than ten years, both CANP and calpain were used in the literature. This complication was resolved by Suzuki in accordance with Imahori in 1990. He proposed the usage of calpain as an authorized name at the 8th International Conference

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Abbreviations: aa: amino acid(s); aar: amino acid residue(s); C2 domain: PKC conserved domain 2; C2L domain: C2 domain-like domain; CANP: calcium-activated neutral protease; *CAPN*: calpain gene; *CAPNS*: calpain regulatory small subunit gene; CASF: Ca^{2+}-activated sarcoplasmic factor; CBS-1 and -2: Ca^{2+}-binding site in PC1 and PC2, respectively; CL: calpain catalytic large subunit; CysPc: calpain-like cysteine protease sequence motif defined in the conserved domain database of the National Center for Biotechnology Information (cd00044); GR domain: Gly-rich domain; KAF: kinase activating factor; MIT motif: microtubule interacting and transport motif; NIDDM: noninsulin-dependent diabetes mellitus (type 2 diabetes); PC1 and PC2 domains: calpain protease core domains 1 and 2, respectively; PEF: penta-EF-hand; PEF(L) and PEF(S) domains: PEF domains in calpain catalytic large subunit and regulatory small subunit, respectively; PKC: protein kinase C; QTL: quantitative trait locus.

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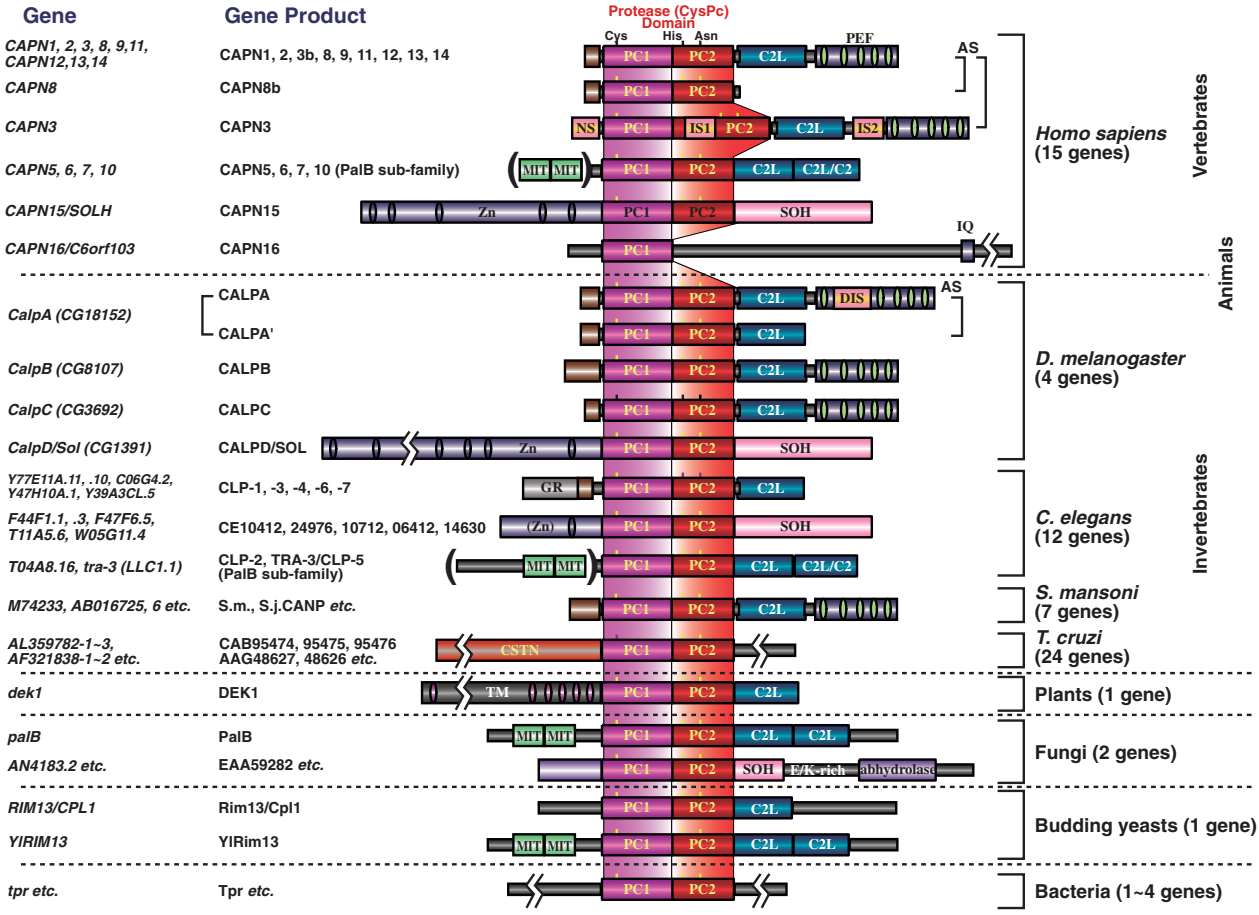


Fig. 1. Schematic structures of calpain superfamily members.

Calpain homologs have been identified in almost all eukaryotes and in some bacteria. Historically, there are several nomenclatures for domains. To avoid confusion in this review, the domains are called by the abbreviation corresponding to the structure, *i.e.*, PC1 (protease core domain 1), PC2 (protease core domain 2), C2L (C2 domain-like), PEF (penta-EF-hand) *etc.* Symbols: N, N-terminal region; PEF(L) and PEF(S), PEF domains of large catalytic and small regulatory subunits, respectively; GR, glycine-rich hydrophobic domain; AS, alternative splicing products; NS/IS1/IS2, CAPN3-characteristic sequences; C2, C2 domain; MIT, microtubule-interacting-and-transport motif; Zn, Zn-finger-containing domain; SOH, SOL-homology domain; DIS, CALPA-specific insertion sequence; TM, transmembrane domain; CSTN, calpastatin-like domain; IQ, a motif interactive with calmodulin.

on Proteolysis and Protein Turnover.[14] Since Suzuki's 1984 paper,[13] cDNA and genomic cloning studies have exploded, and thousands of calpains and related molecules were identified—among them 15 human calpain genes, which are now called *CAPNn* ($n = 1, 2, 3$, and *5–16*), which is short for calpain.[15] There are two genes for small regulatory subunits, *CAPNS1* and *CAPNS2*, short for calpain small subunit, and one for calpastatin, *CAST*.

Calpains (EC 3.4.22.17; Clan CA, family C02) constitute a distinct group of intracellular cysteine proteases found in almost all eukaryotes and a few bacteria (Fig. 1; for other reviews, see the two landmark reviews in 1991[16] and 2003[17] and later reviews[18)–49)]). Calpains are first defined as cytosolic proteases exhibiting Ca^{2+}-dependent proteolytic activity at a neutral pH. Most calpains are intracellular, and their activity is strictly regulated, as is the case with other intracellular proteolytic systems, such as the ubiquitin–proteasome system,[50] the autophagy–lysosome system,[51] and caspases[52] (Fig. 2). Caspases proteolyze substrates sequence-specifically, mostly for apoptosis, and they are activated by signal-dependent oligomerization. The former two systems act as "erasers" that degrade and eliminate substrate proteins. In contrast, calpains act in proteolytic processing, rather than degradation, *i.e.*, calpains proteolyze their substrates at a limited number of sites to transform or modulate their substrates' structures and activities. Therefore,

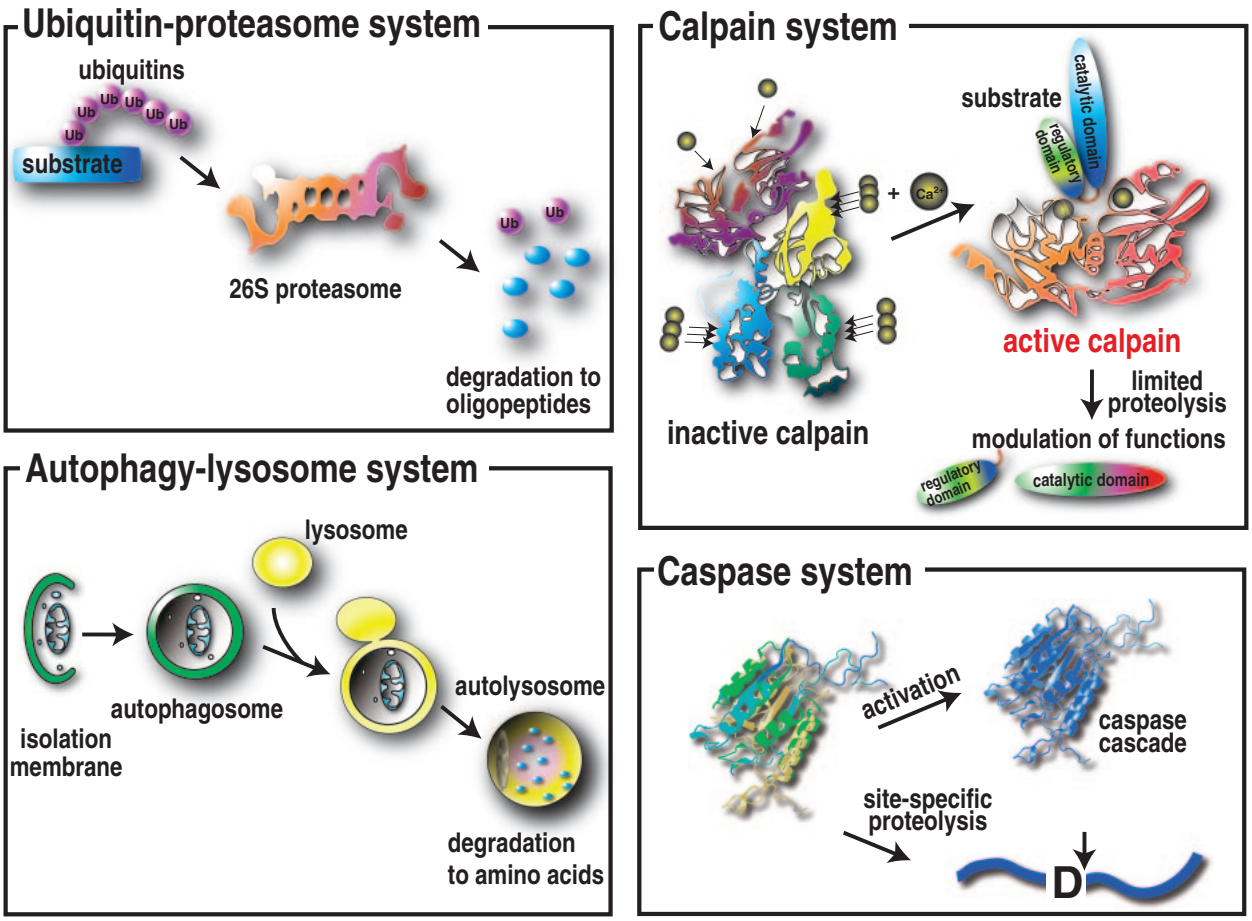


Fig. 2. Major intracellular proteolytic systems.
The ubiquitin–proteasome system degrades and eliminates specific substrate proteins in an ubiquitin-tagging system consisting of more than 1,000 ubiquitin ligases. The autophagy–lysosome system primarily degrades non-specific cell components, including proteins and micro-organisms, by compartmentalization by isolation membranes. The caspase system functions mainly for apoptosis. In contrast, calpains primarily use proteolytic processing, rather than degradation, to modulate or modify their substrates' activity, specificity, longevity, localization, and structure.

calpain is referred to as an intracellular "modulator" protease.[53),54)] Ubiquitin and autophagy systems are regulated in various steps mediated by many proteins, but calpain is distinctive in that a functional entity rests on a single molecule or two.

The importance of the calpains' physiological roles is reflected in the variety of defects caused by compromised calpain function in different tissues and organisms, including embryonic lethality (disruption of mouse *Capn2*),[55)] muscular dystrophies (mutations in human *CAPN3*),[56)] gastropathy (mutated *Capn8*),[57)] lissencephaly[58)] and tumorigenesis[59)] (indirect defects in calpain functions), impaired neurogenesis in flies,[60)] deficient sex determination in nematodes,[61)] defects in aleurone cell development in maize,[62)] and alkaline stress susceptibility in fungi[63)] and yeasts.[64),65)] These effects will be described in more detail in the following sections.

Mammalian gene products can be written as non-italic upper-case letters of their gene name, according to the international nomenclature system for a gene product; for example, mouse *Capn1* and *Capn2* produce CAPN1 and CAPN2, respectively. Although recently mammalian calpain gene products are often written as calpain-1, calpain-2, *etc.*, this nomenclature is misleading as described below ("calpain" is also used for enzyme names), so we will use the above formal nomenclature (CAPN1, CAPN2, *etc.*) throughout this review. Some calpains were previously called by independent names, such as μCL and p94. In this review, to clarify old and new mammalian calpain nomenclatures, we will note the

old after the current names: *e.g.*, CAPN1/μCL and CAPN3/p94.

1. A research history of calpain and its definition

Because of the scope of this review, it is important to define calpain, and the complexities of defining calpain are best explained in the context of its research history.[66)–68)]

In 1964, Guroff partially purified a Ca^{2+}-dependent neutral protease from rat brain, which afterwards turned out to be calpain.[1)] In the same year, Meyer, Fischer and Krebs described the activation of rabbit skeletal muscle phosphorylase b kinase by Ca^{2+} and partially purified the responsible protein, which they called KAF (kinase activating factor).[4)] Four years later, Huston and Krebs,[3)] and Drummond and Duncan[69)] independently reported that this protein was a Ca^{2+}-dependent protease, although they concluded that the proteolytic activation of phosphorylase b kinase was unlikely to be physiological. Krebs and Fishers' main interest then moved to phosphorylation and its cascade, for which they were awarded a Nobel Prize in 1992.[70),71)] Since then, Krebs has changed his mind about the physiological importance of proteolytic activation. Indeed, one of calpain's most important physiological functions is to modulate substrate protein functions by limited proteolysis, and this property must be considered in the definition—or at least, the classification—of calpain.

In 1972, Darrel E. Goll and his colleagues identified a factor that Ca^{2+}-dependently removes the Z-lines of skeletal muscle, and named it CASF (Ca^{2+}-activated sarcoplasmic factor), which also turned out to be calpain.[5)] This study is the beginning of research on calpain involved in postmortem tenderization of meat.[72)] In 1977, Yasutomi Nishizuka and his colleagues identified calpain as a protein kinase C (PKC) activating factor,[6)] and several calpain studies were done in line with PKC.[73)–76)] In 1978, Imahori and his colleagues for the first time in the world purified chicken calpain to homogeneity and called it CANP. Following this remarkable study, Imahori's group established calpain research field by publishing important studies on enzymology of calpain such as substrate specificity,[77)] purification from human muscle,[78)] inhibitors,[79),80)] autolysis,[8),81)] and activation.[82),83)]

Calpain purification studies (*ca.* 90% purity in 1976,[84)] and to homogeneity in 1978[7)]) revealed that functional native calpain is composed of large (*ca.* 80 kDa) and small (*ca.* 30 kDa) subunits, which is another important property of calpain. The chicken μ/m-calpain catalytic subunit cDNA was cloned in 1984,[13)] and since then many cDNAs and genomic DNAs corresponding to various calpain species have been cloned and sequenced. Little is known about the nature of calpain proteins as enzymes, however, and most calpains are known only by their sequence.

The most extensively studied calpains are the major ubiquitous mammalian μ- and m-calpains, and the major ubiquitous calpain in chicken, μ/m-calpain.[14)] These are called "conventional" calpains in the wake of PKC;[85)] all others are termed "unconventional" calpains. The chicken μ/m-calpain has properties placing itself as an intermediate of μ- and m-calpains.[7),77),86)] Its catalytic subunit was shown to be an ortholog of mammalian CAPN11 (see also 6.3.3).[87)]

Among the amino acid (aa) sequences available, there is a group of peptidases that possess a protease domain that is significantly similar to one another but distinct from that of other peptidases.[88)] A search for "CysPC" in the conserved domain database of NCBI[89)] extracts the sequences of almost all the calpain homologs from various living organisms, including plants, fungi, yeast, and even bacteria. Calpains belong to the papain superfamily of cysteine proteases and have weak similarity to papains and cysteine cathepsins, although it is clearly less significant than the similarities between calpains. In this superfamily, calpain may evolutionarily be the oldest branch.[89)]

Considering this situation, it is reasonable to define calpain mainly by the aa sequence in relation to the most-characterized mammalian μ- and m-calpains. Therefore, this review uses the following simple but clear definition: *calpains are defined as proteins that have aa sequences significantly similar to the protease domain of the human μ-calpain large subunit* (replacing μ-calpain with m-calpain in this definition would give the same result). Additional characteristics reflect calpain classifications: the conventional, classical, non-classical, ubiquitous, and/or tissue-specific calpains should be discussed separately when considering their physiological functions. In other words, sequence similarity does not necessarily reflect functional similarities between calpain species.

According to this definition, humans have 15 genes that encode calpains (Figs. 3 and 4). In other species, *Schistosoma mansoni* (Fig. 5), *Caenorhabditis elegans* (Fig. 6), *Anopheles gambiae* (Fig. 7), *Drosophila melanogaster* (Fig. 7), *Arabidopsis thaliana* (Fig. 1), *Emericella* (*Aspergillus*) *nidulans*

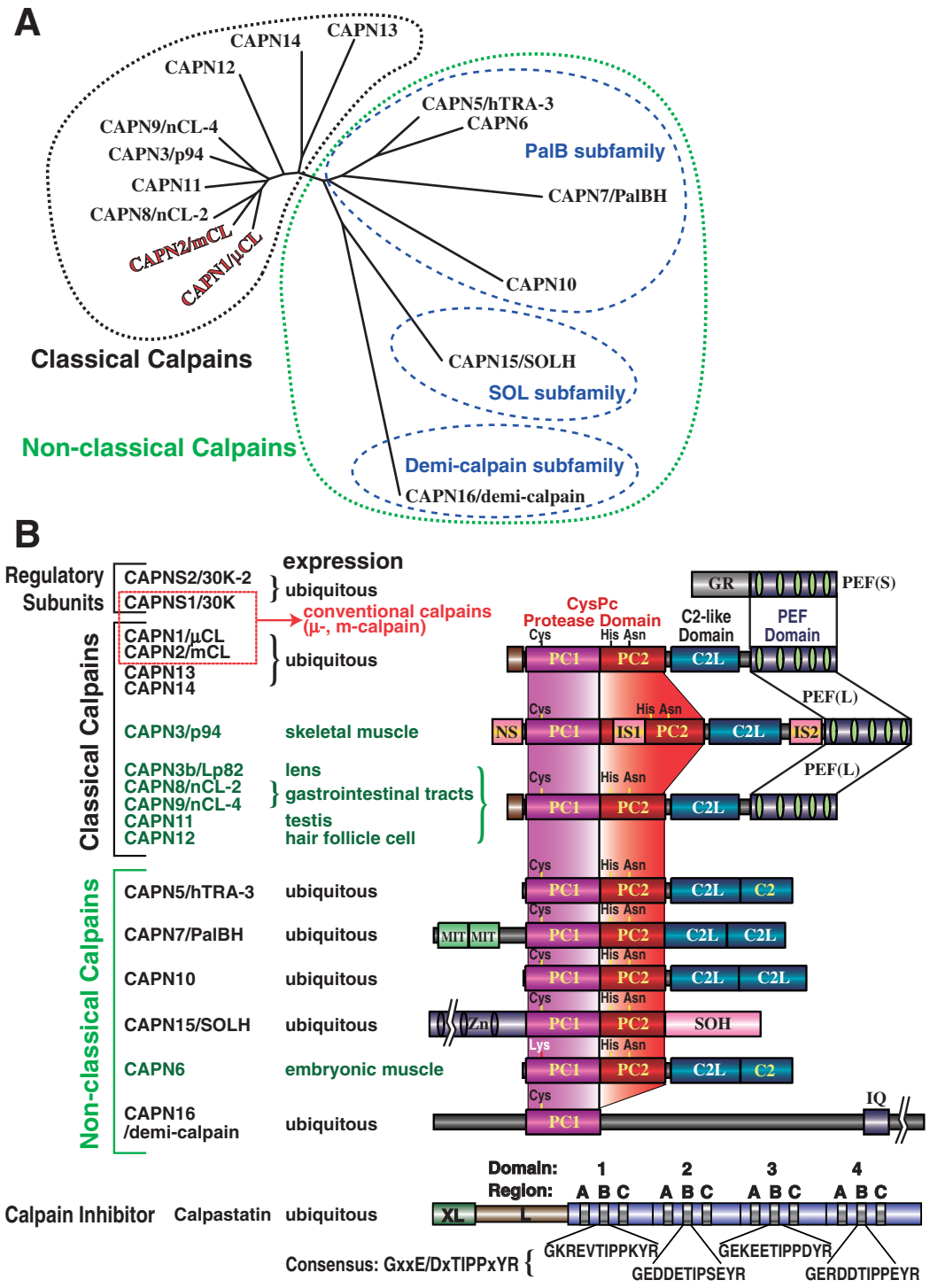


Fig. 3. Phylogenetic tree and schematic structures of human calpain-related molecules.
A. Phylogenetic tree of human calpains. Method: The tree was drawn by the neighbor-joining/bootstrap method after aligning all the sequences using MAFFT v6.240 (at http://align.genome.jp/mafft/, strategy: E-INS-i). Atypical calpains are more divergent than classical calpains. The PalB subfamily consists of the strict PalB group, the TRA-3 group, and the CAPN10 group. B. Schematic structures: Black and green letters indicate ubiquitous and tissue/organ-specific calpains, respectively. See also Fig. 4. Symbols: L and XL, N-terminal and extended N-terminal regions of calpastatin. See Fig. 1 legend for other symbols.

Gene	Chromosome location	Gene product	Aliases	Protease activity[a]	Domains[b] C2L[c]	C2	PEF	Expression	Phenotype of gene deficiency in mice	Note
Catalytic subunits										
CAPN1	11q13	CAPN1	μ-calpain large subunit (μCL), μ80K, μCANP/calpain-I large subunit, calpain-1	+	+	-	+	ubiquitous	platelet dysfunction	μ-calpain dimerizes with CAPNS1/30K
CAPN2	1q41-q42	CAPN2	m-calpain large subunit (mCL), m80K, mCANP/calpain-II large subunit, calpain-2	+	+	-	+	ubiquitous (except for erythrocytes)	embryonic lethal	m-calpain dimerizes with CAPNS1/30K
CAPN3	15q15.1-q21.1	CAPN3	p94, calpain-3, calpain-3a, nCL-1	+	+	-	+	skeletal muscle	muscular dystrophy	
		(CAPN3b, ...)	Lp82, Lp85, etc.	+	+	-	+	lens, retina		termination codon in human first exon
		CAPN3c, …	p94:Δex15, Tp36, Mp18, Up84 etc.	+	+	-	+	ubiquitous		Ups, Tps, and Mps are transcribed from an alternative promoter
CAPN5	11q14	CAPN5	hTRA-3, nCL-3, calpain-5	+	+	+	-	ubiquitous (abundant in testis and brain)	sudden death ?	nematode TRA-3 homolog
CAPN6	Xq23	CAPN6	calpamodulin, CANPX, calpain-6	-	+	+	-	embryonic muscles, placenta	n.d.	nematode TRA-3 homolog; the active site Cys->Lys in all mammals, and His->Tyr in mice and below; authentic C-H-N sequences in birds and below
CAPN7	3p24	CAPN7	PalBH, calpain-7	+	++	-	-	ubiquitous	n.d.	*Aspergillus* PalB and yeast Cpl1 homolog
CAPN8	1q41	CAPN8	nCL-2, calpain-8, calpain-8a	+	+	-	+	gastrointestinal tracts	stress-induced gastric ulcer	G-calpain is a complex of CAPN8 and CAPN9
		CAPN8b	nCL-2'	+	-	-	-	gastrointestinal tracts		
		CAPN8c	nCL-2:Δex10,17~fs	+	+	-	+/-	gastrointestinal tracts		
CAPN9	1q42.11-q42.3	CAPN9	nCL-4, calpain-9, calpain-9a	+	+	-	+	gastrointestinal tracts	stress-induced gastric ulcer	G-calpain is a complex of CAPN8 and CAPN9
		CAPN9b	nCL-4:Δex8	+	+	-	+	gastrointestinal tracts		
CAPN10	2q37.3	CAPN10	calpain-10, calpain-10a	+	++	-	-	ubiquitous	no significant phenotype	SNP is associated with NIDDM
		CAPN10b, -10c, …		n.d.	+/-	-	-			
CAPN11	6p12	CAPN11	calpain-11	n.d.	+	-	-	testis	n.d.	
CAPN12	19q13.2	CAPN12	calpain-12a	n.d.	+	-	+	hair follicle	n.d.	
		CAPN12b, 12c, …		n.d.	+	-	+/-			
CAPN13	2p22-p21	CAPN13	calpain-13	n.d.	+	-	+	ubiquitous	n.d.	
CAPN14	2p23.1-p21	CAPN14	calpain-14	n.d.	+	-	+	ubiquitous	n.d.	
CAPN15/SOLH	16p13.3	CAPN15	SOLH, calpain-15	n.d.	-	-	-	ubiquitous	n.d.	*Drosophila* SOL homolog
CAPN16/C6orf103	6q24.3	CAPN16	Demi-calpain, C6orf103, calpain-16	-	-	-	-	ubiquitous	n.d.	contains only PC1 domain
Regulatory subunits										
CAPNS1	19q13.1	CAPNS1[d]	CANP/calpain small subunit, 30K, css1, CAPNS1, CAPN4, calpain-s1	-	-	-	+	ubiquitous	embryonic lethal	regulatory subunit for CAPN1/μCL and CAPN2/mCL
CAPNS2	16q12.2	CAPNS2[d]	calpain small subunit 2, 30K-2, css2, CAPNS2, calpain-s2	-	-	-	+	ubiquitous	n.d.	intron-less gene; *CAPNS2* overlaps with *LPCAT2* (lysophosphatidylcholine acyltransferase 2)
Calpain inhibitor										
CAST	5q15	calpastatin	CANP inhibitor	-	-	-	-	ubiquitous	excitotoxicity in neuronal cells	specific inhibitor for μ- and m- calpains; many splicing variants exist

[a] + indicates that the molecule was experimentally shown to have protease activity, and - means that it does not have the active-site cysteine, asparagine, and/or histidine residues.

[b] + or - indicates that the molecule has or does not have a corresponding domain, respectively, and +/- indicates that it has one or more but fewer than five EF-hand motifs.

[c] Abbreviations: n.d., not yet determined/done; CANPX, calpain on chromosome X; nCL, novel calpain large subunit; LGMD2A, limb-girdle muscular dystrophy type 2A; TRA-3, transform genotypic hermaphrodites; PalB, phosphatase mutants: loss in activity at alkaline pH but normal or increased activity at acidic pH; PalBH, PalB homolog; SNP, single nucleotide polymorphism; NIDDM, non-insulin-dependent diabetes mellitus; SOL, small optic lobes; SOLH, SOL homolog.

[d] Note that these gene products are not "calpain" according to the definition of this review.

Fig. 4. Human calpain-related genes.
See also Fig. 3.

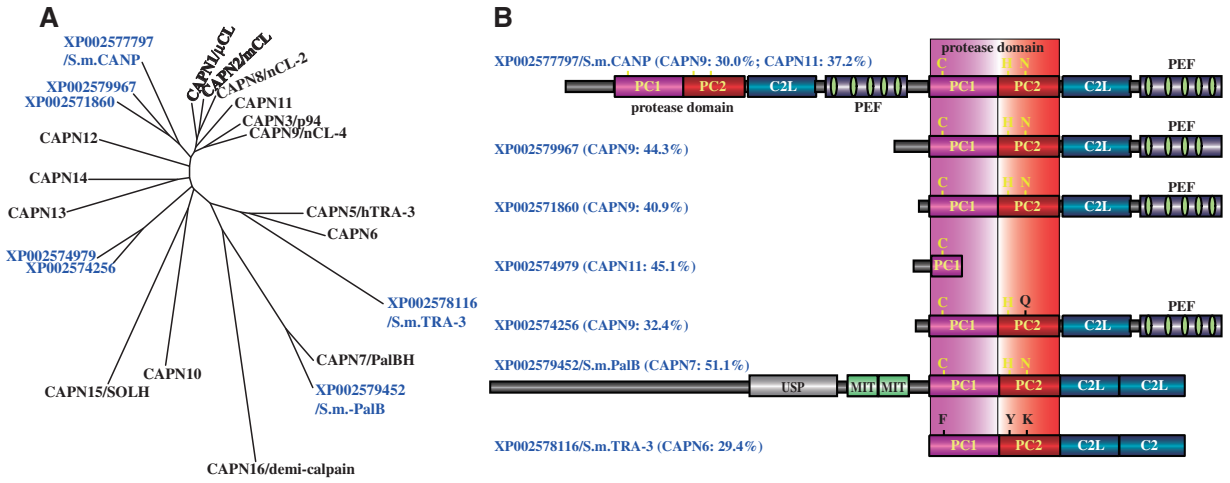


Fig. 5. Phylogenetic tree and schematic structures of *S. mansoni* calpains.
A. Phylogenetic tree of *S. mansoni* and human calpains. See Fig. 3A for methods. B. Schematic structures. The human calpain most similar to each *S. mansoni* calpain is indicated in parentheses, with the aa sequence identity. The idnentity is given for each of the tandem classical calpain structures in XP_002577797. USP: ubiquitin-specific peptidase. See Fig. 1 legend for other symbols.

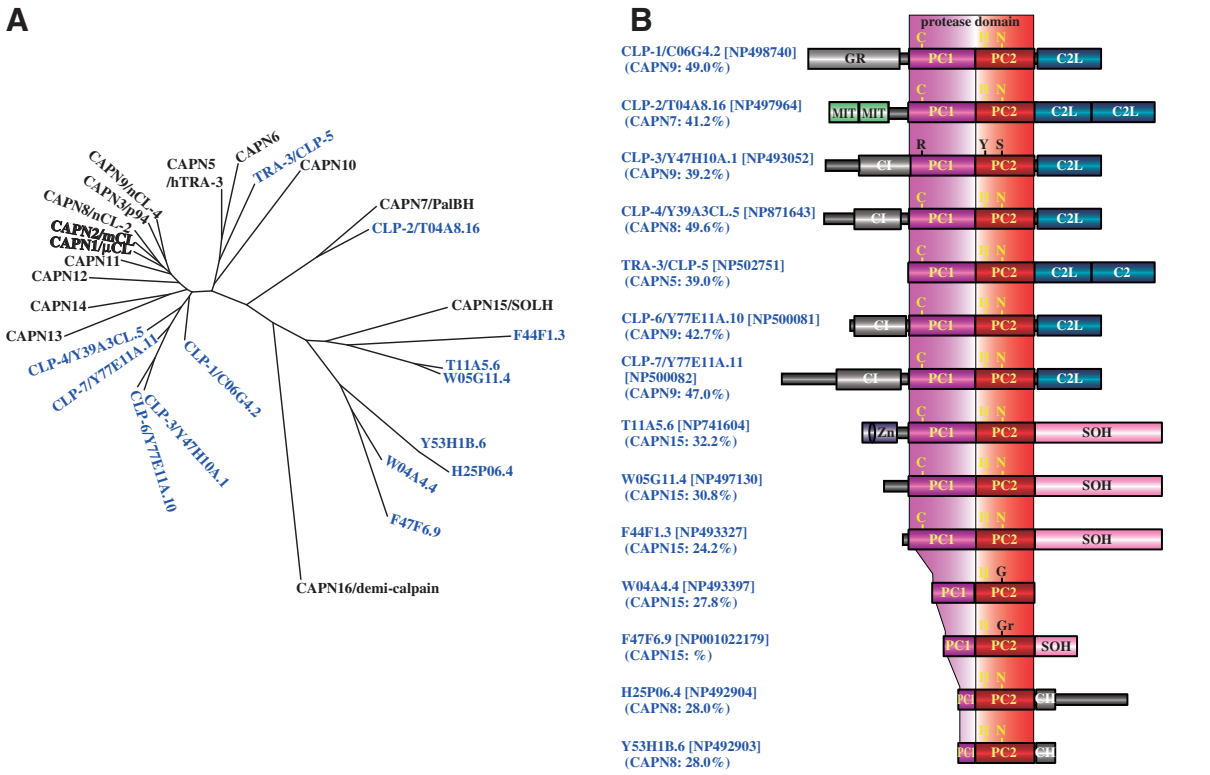


Fig. 6. Phylogenetic tree and schematic structures of *C. elegans* calpains.
A. Phylogenetic tree of *C. elegans* and human calpains. See Fig. 3A for methods. B. Schematic structures. The human calpain most similar to each *C. elegans* calpain is indicated in parentheses, with the aa sequence identity. CI and CII stand for conserved regions I and II with unknown function, respectively. See Fig. 1 legend for other symbols.

(Fig. 1), and *Saccharomyces cerevisiae* (Fig. 1) have 7, 14, 7, 4, 1, 2, and 1 calpain genes, respectively. There is no calpain gene in *Encephalitozoon* or *Schizosaccharomyces pombe*. In prokaryotes, 53 calpains from 42 bacteria were found among the 914 completely sequenced microbial genomes present in

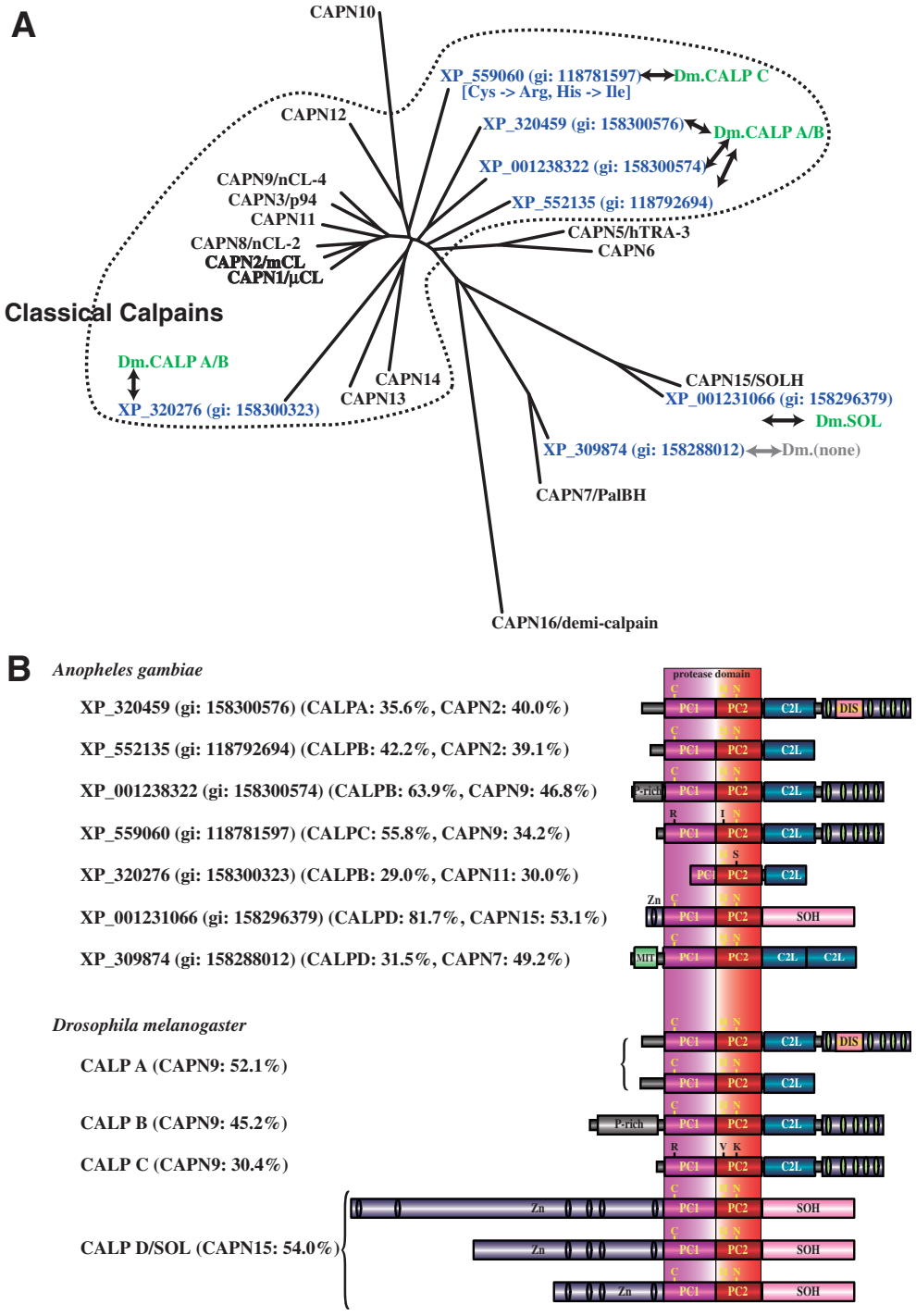


Fig. 7. Phylogenetic tree and schematic structures of insect calpains.
A. Phylogenetic tree of *A. gambiae* and human calpains. See Fig. 3A for methods. *D. melanogaster* (DM) homologs are shown in green letters. B. Schematic structures. The human calpain most similar to each insect calpain is indicated in parentheses, along with the aa sequence identity. The *D. melanogaster* calpain most similar to *A. gambiae* calpain is also indicated. P-rich: Pro-rich region. DIS: *Drosophila*-type insertion sequence. See Fig. 1 legend for other symbols.

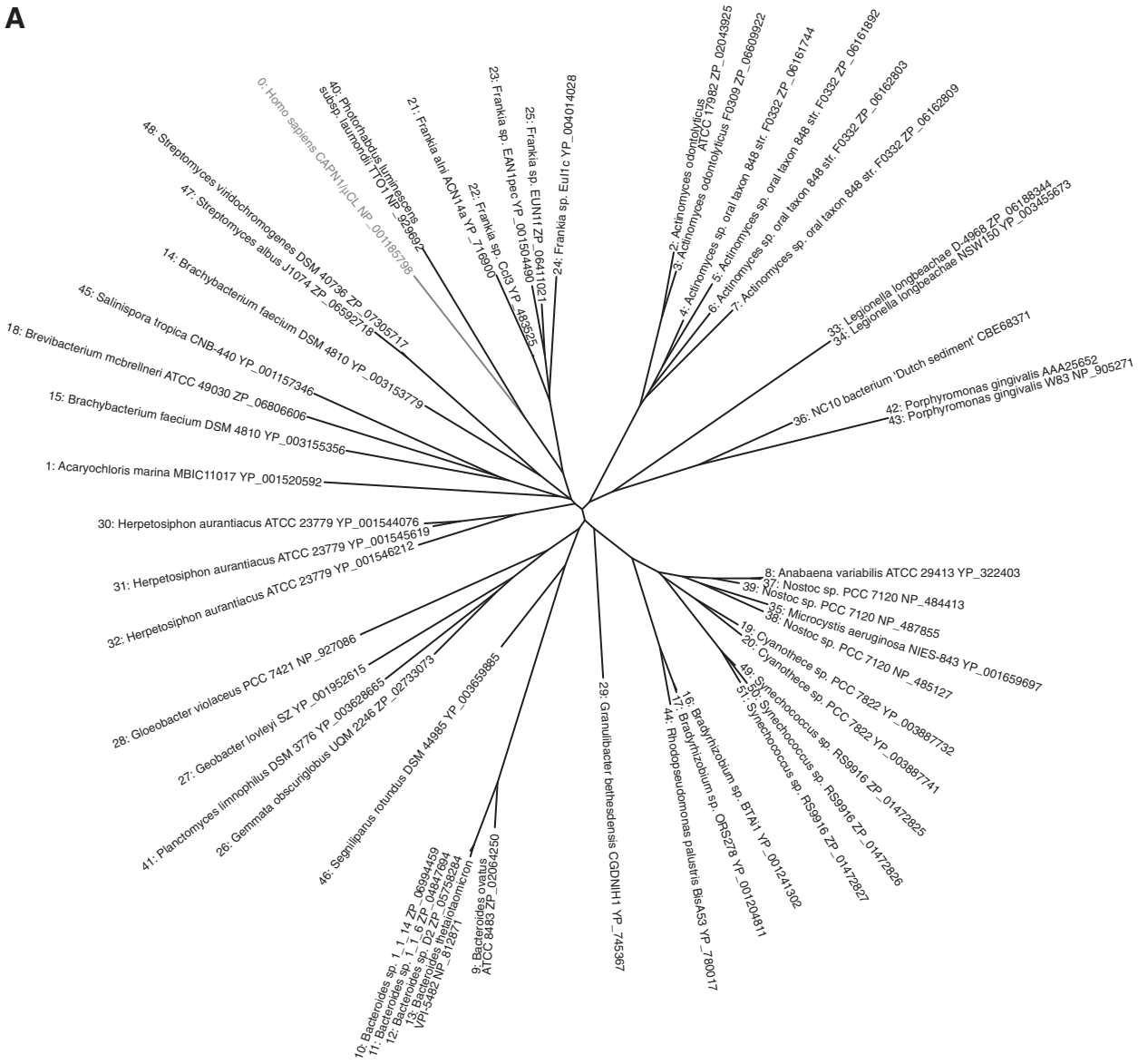


Fig. 8. *Continued on next page.*

the database so far. Each of the bacterial species has 1–4 calpain genes (Fig. 8). However, most genome-sequenced bacteria, including *Escherichia coli* or any of the archeabacteria, do not have calpain genes.

2. Conventional calpain—the basics

2.1. Domain structures of conventional calpains. The conventional calpains, μ- and m-calpains, are heterodimers consisting of a common small regulatory subunit (CAPNS1/30K; *ca.* 30 kDa) and a distinct, large (*ca.* 80 kDa) μ- or m-calpain catalytic subunit (CAPN1/μCL or CAPN2/mCL, respectively) (Fig. 3). Calpain-I is an old alternative name for μ-calpain, named as an enzyme, and it is not the same as calpain-1, which is the name often used for a gene product, a subunit of an enzyme. Instead, calpain-I is a heterodimer of CAPN1/μCL and CAPNS1/30K. The distinction is similar for calpain-II and calpain-2.

CAPN1/μCL and CAPN2/mCL have approximately 60% aa identity. Accordingly, they have almost indistinguishable substrate and inhibitor specificities, and both are almost ubiquitously expressed. However, they differ greatly in their *in vitro*

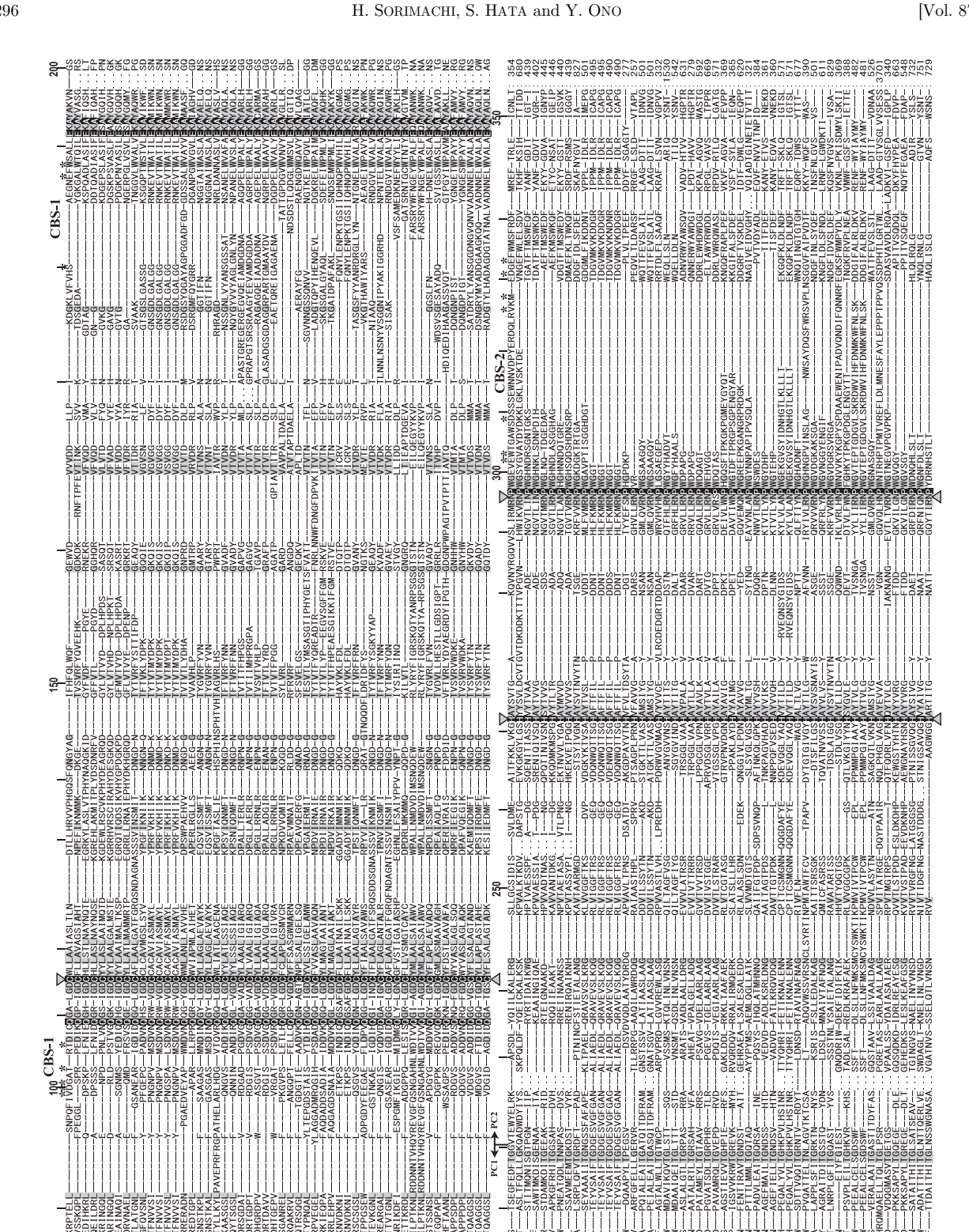


Fig. 8. *Continued on next page.*

Fig. 8. Phylogenetic tree and sequence alignment of bacterial calpains.
A. Phylogenetic tree of bacterial calpains and human CAPN1/μCL. See Fig. 3A for methods. B. Sequence alignment of the bacterial calpain protease domains and human CAPN1/μCL. Only the sequences of the protease domains are compared, with the start and end aa residue numbers indicated, as other sequences and lengths are somewhat divergent. Reversed fonts and gray shadow indicate residues conserved in all sequences or more than half, respectively. Triangles: active site residues. Asterisks: residues involved in Ca^{2+}-binding of the protease domain of human CAPN1/μCL. Sequences: 0: *H. sapiens* NP_001185798; 1: *Acaryochloris marina MBIC11017* YP_001520592; 2: *Actinomyces odontolyticus ATCC 17982* ZP_02043925; 3: *Actinomyces odontolyticus F0309* ZP_06609922; 4: *Actinomyces sp. oral taxon 848 str. F0332* ZP_06161744; 5: *Actinomyces sp. oral taxon 848 str. F0332* ZP_06161892; 6: *Actinomyces sp. oral taxon 848 str. F0332* ZP_06162803; 7: *Actinomyces sp. oral taxon 848 str. F0332* ZP_06162809; 8: *Anabaena variabilis ATCC 29413* YP_322403; 9: *Bacteroides ovatus ATCC 8483* ZP_02064250; 10: *Bacteroides sp. 1_1_14* ZP_06994459; 11: *Bacteroides sp. 1_1_6* ZP_04847694; 12: *Bacteroides sp. D2* ZP_05758284; 13: *Bacteroides thetaiotaomicron VPI-5482* NP_812871; 14: *Brachybacterium faecium DSM 4810* YP_003153779; 15: *Brachybacterium faecium DSM 4810* YP_003155356; 16: *Bradyrhizobium sp. BTAi1* YP_001241302; 17: *Bradyrhizobium sp. ORS278* YP_001204811; 18: *Brevibacterium mcbrellneri ATCC 49030* ZP_06806606; 19: *Cyanothece sp. PCC 7822* YP_003887732; 20: *Cyanothece sp. PCC 7822* YP_003887741; 21: *Frankia alni ACN14a* YP_716900; 22: *Frankia sp. CcI3* YP_483525; 23: *Frankia sp. EAN1pec* YP_001504490; 24: *Frankia sp. EuI1c* YP_004014028; 25: *Frankia sp. EUN1f* ZP_06411021; 26: *Gemmata obscuriglobus UQM 2246* ZP_02733073; 27: *Geobacter lovleyi SZ* YP_001952615; 28: *Gloeobacter violaceus PCC 7421* NP_927086; 29: *Granulibacter bethesdensis CGDNIH1* YP_745367; 30: *Herpetosiphon aurantiacus ATCC 23779* YP_001544076; 31: *Herpetosiphon aurantiacus ATCC 23779* YP_001545619; 32: *Herpetosiphon aurantiacus ATCC 23779* YP_001546212; 33: *Legionella longbeachae D-4968* ZP_06188344; 34: *Legionella longbeachae NSW150* YP_003455673; 35: *Microcystis aeruginosa NIES-843* YP_001659697; 36: *NC10 bacterium 'Dutch sediment'* CBE68371; 37: *Nostoc sp. PCC 7120* NP_484413; 38: *Nostoc sp. PCC 7120* NP_485127; 39: *Nostoc sp. PCC 7120* NP_487855; 40: *Photorhabdus luminescens subsp. laumondii TTO1* NP_929692; 41: *Planctomyces limnophilus DSM 3776* YP_003628665; 42: *Porphyromonas gingivalis* AAA25652; 43: *Porphyromonas gingivalis W83* NP_905271; 44: *Rhodopseudomonas palustris BisA53* YP_780017; 45: *Salinispora tropica CNB-440* YP_001157346; 46: *Segniliparus rotundus DSM 44985* YP_003659885; 47: *Streptomyces albus J1074* ZP_06592718; 48: *Streptomyces viridochromogenes DSM 40736* ZP_07305717; 49: *Synechococcus sp. RS9916* ZP_01472825; 50: *Synechococcus sp. RS9916* ZP_01472826; 51: *Synechococcus sp. RS9916* ZP_01472827.

Ca^{2+}-requirement (μM versus mM) for proteolytic activity. Their functions are fundamental and essential, and are implicated in the regulation of signal transduction systems,[90),91)] cell motility,[21),92)] membrane repair,[93),94)] and apoptosis,[95),96)] although their precise roles in these events remain elusive.

The catalytic and regulatory subunits of conventional calpains can be divided into several domains (Fig. 3). The N-terminal anchor α-helix of the catalytic large subunit (also called anchor region or domain I) is autolyzed when activated by Ca^{2+}, resulting in its ability to function at a lower Ca^{2+} concentration,[81),97)–99)] with a different substrate specificity,[100)] and subunit dissociation in some cases[100)–103)] but not others.[104)–106)] Therefore, autolysis of this anchor helix is one of the important regulatory mechanisms for calpain activity.

In isolation, m-calpain's protease (CysPc) domain (also called domain II), has Ca^{2+}-dependent protease activity.[107)] 3D structural analyses in the absence of Ca^{2+} showed that the CysPc domain is further divided into two core domains, PC1 (also called D-I or subdomain IIa) and PC2 (D-II or IIb) (Fig. 3). These two core domains fold into a single functional CysPc domain when bound to Ca^{2+} (Fig. 9).[108)–113)] In other words, calpain remains structurally inactive in the absence of Ca^{2+}. This is reasonable, because calpain resides in the cytosol in direct contact with many other proteins, and its activity must be strictly regulated.

Ca^{2+} also binds to C2 domain-like (C2L) domain (also called domain III)[114)] and penta-EF-hand (PEF) domain (domain IV) of the large catalytic subunit (PEF(L) domain), and that of the small regulatory subunit (PEF(S), also called domain VI).[115),116)] While C2L and the C2 domains have no sequence similarity, their 3D-structure is very similar (β-sandwich structure composed of eight anti-parallel β-strands). Two PEF domains (PEF(L) and PEF(S)) each contain five EF-hand motifs.[48),117)–119)] The fifth EF-hand motif of each domain contributes to heterodimer formation.[120)] It should be noted that the N-terminal anchor helix is in contact with the second EF-hand motif (EF-2) of PEF(S) of CAPNS1/30K, and the interaction is broken either by Ca^{2+}-binding to the EF-2 or by autolysis of the N-terminus upon activation.

Gly-rich (GR) domain (also called domain V) in the N-terminus of CAPNS1/30K contains hydrophobic Gly-clusters, most of which are autolyzed during activation. This domain was invisible in the 3D structure, indicating a very "soft" structure. In humans, the *CAPNS2* gene encodes a regulatory subunit homolog composed of GR and PEF(S) domains, whose physiological roles remain unclear.[121)]

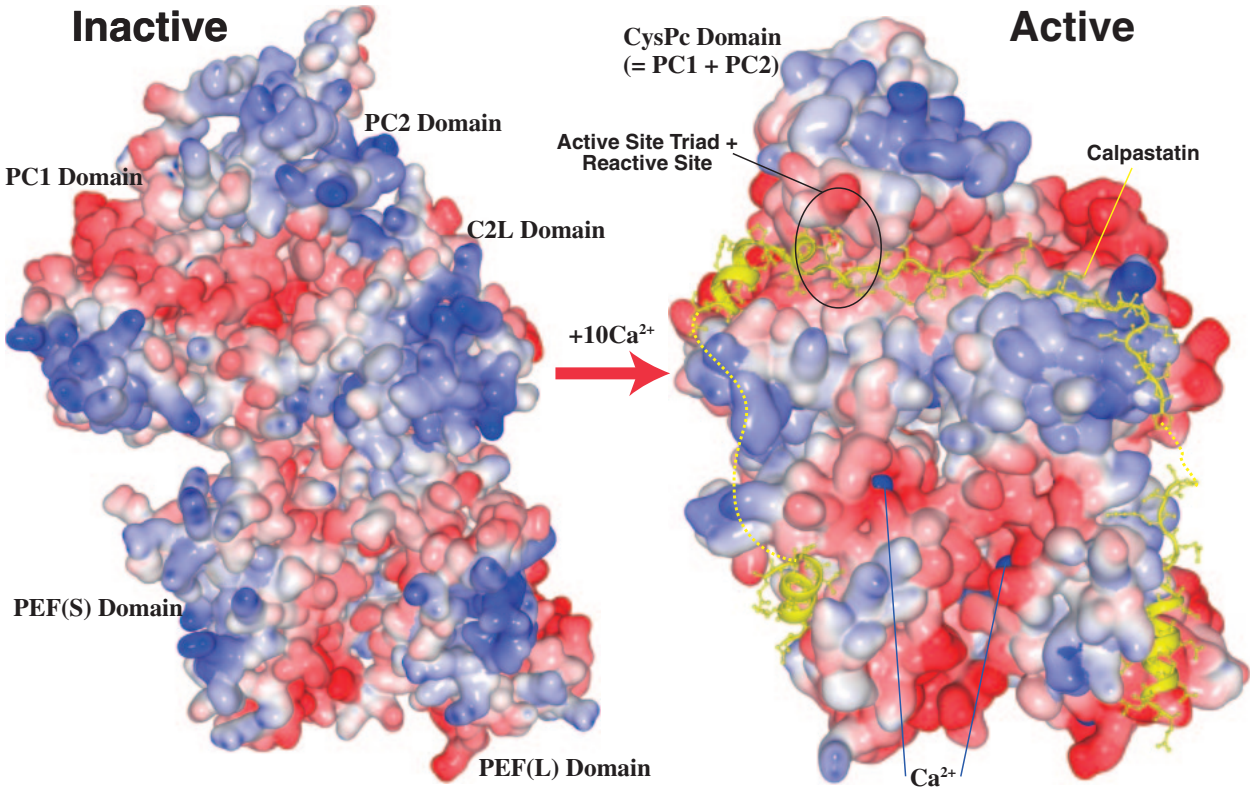


Fig. 9. Schematic of the 3D structure of inactive and active m-calpain.
Surface-type schematic 3D structures of inactive (Ca^{2+} free) and active (Ca^{2+}- and calpastatin-bound) forms of m-calpain using PDB data, 1KFX[109] and 3DF0.[111] The oligopeptides represented by the yellow ribbon + ball-and-stick indicate portions of calpastatin bound to active m-calpain. The dotted lines indicate portions that were too mobile for the 3D structure to be determined. The active protease domain (CysPc) is formed by the fusion of core domains PC1 and PC2 upon the binding of one Ca^{2+} to each of the core domains. The active site is circled in black. Blue balls represent Ca^{2+} (not all are visible).

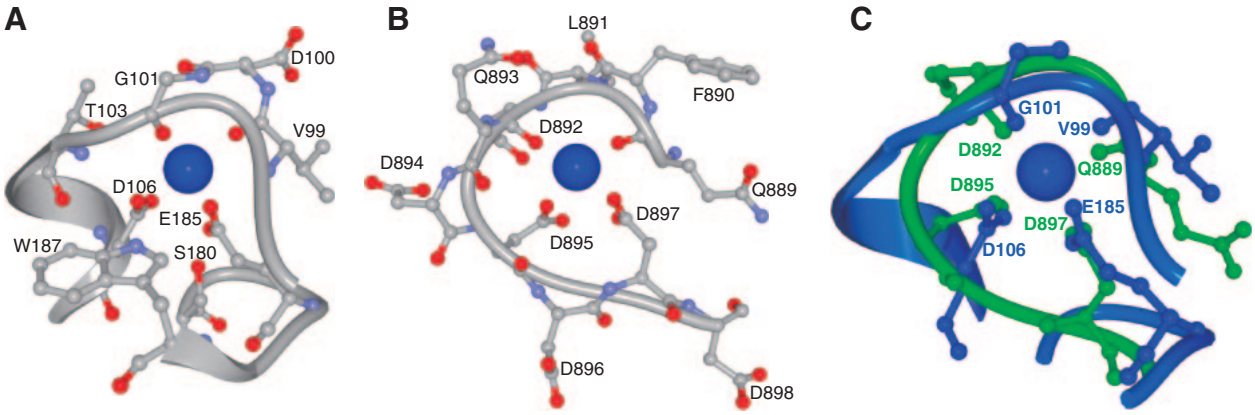


Fig. 10. A comparison of 3D structures of the Ca^{2+}-binding sites of the CAPN1/μCL protease domain IIa and SLO1 (Ca-bowl).
Ca^{2+}-binding sites in (A) the CAPN1/μCL protease domain (2R9F) and (B) the high-conductance voltage- and Ca^{2+}-activated K^+ channel (BK or SLO1 channel) (3MT5). (C) The structures were superimposed for comparison.[126]

2.2. Activation and substrates. Ca^{2+} is required for the activation of conventional calpains, raising the question of which part of the calpain molecule contains the Ca^{2+}-binding site that provokes calpain activation. It was previously believed that PEF domains were responsible for activating calpain

in response to Ca^{2+}, as they are the only Ca^{2+}-binding sites visible in the primary structure.[13] However, the 3D structure of calpain revealed that the PEF domains are somewhat distant from the protease domain (Fig. 9). CD spectrum measurements found no significant changes in calpain's secondary structure upon activation by Ca^{2+} binding.[122] Biochemical analysis of recombinant m-calpain protease domains revealed that they respond directly to Ca^{2+}.[107] Finally, the 3D structures of these domains showed that Ca^{2+} binds directly to PC1 and PC2, to activate conventional calpain (Fig. 9).[112),113]

A number of substrates have been reported for calpains, including protein kinases, phosphatases, phospholipases, cytoskeletal proteins, membrane proteins, cytokines, transcription factors, lens proteins, and calmodulin-binding proteins, but few of them have been shown to be physiological. Due to its complexity and "finesse", the exact rules governing calpain substrate specificity remain incomplete, although bioinformatics approaches revealed some cleavage-site preferences[123),124] (see also http://calpain.org). Substrate 3D structures also seem to be important factors for many cases.

2.3. Novelties deduced from the calpain 3D structure. The classical calpains CAPN1/μCL, CAPN2/mCL, and CAPN9/nCL-4 have highly conserved protease domains (59–71% aa identity); however, the Ca^{2+}-bound 3D structures of these domains reveal distinguishing features.[112),113),125]

First, comparing the m-calpain protease domains in the presence and absence of Ca^{2+}, all the α-helices and β-strands are conserved, consistent with previous CD spectrum results.[122] Both PC1 structures can be aligned very closely (Cα root-mean-squared deviation (r.m.s.d.) = 1.72 Å), whereas those of PC2 are moderately aligned (r.m.s.d. = 3.78 Å). As predicted from PC2's Ca^{2+}-unbound 3D structure, Ca^{2+} binding would cause it to be rotated and translated by 50° and 6 Å, if the alignment of PC2 is fixed. Surprisingly, a Ca^{2+}-induced Ca^{2+}-binding site (CBS-1 and CBS-2) is generated in each core domain, and Ca^{2+} binding to these sites causes the above-described PC2 rotation and translation for activation. These Ca^{2+}-binding sites have a novel and unique structure. Only recently, one structure was shown to have similarity with this site: the 3D structure of the "Ca^{2+}-bowl" of the high-conductance voltage- and Ca^{2+}-activated K^+ (BK or SLO1) channel has a similar Ca^{2+}-binding geometry to the μ-calpain CBS-1, although they share no similarity in their primary sequences (Fig. 10).[126]

Second, the active-site cleft is deeper and narrower than that of papain. Due to this constraint, the substrate must be in a fully extended conformation with its backbone stretched; this was verified by the 3D structures of the Ca^{2+}-bound active μ-calpain protease domain co-crystallized with leupeptin or E64.[127] This finding explains calpain's preference for proteolyzing inter-domain unstructured regions.

Third, although the active protease domains of CAPN1, 2, and 9 are highly similar, a reversible intrinsic inhibitory/safety mechanism exists only for CAPN2/mCL. W106, which is adjacent to the active-site C105 of CAPN2/mCL, positions to interfere with substrates' access to the active site; in contrast, this mechanism does not occur in rat CAPN1/μCL.[113] Moreover, CAPN9/nCL-4 has a novel intrinsic inhibitory mechanism, *i.e.*, the misalignment of the catalytic residues.[125] These findings together show that the activation/latency mechanisms of calpains, regardless of their highly conserved sequences, are somewhat specialized for each molecule, and are controlled by inter-domain interactions. In the whole 3D structure of m-calpain,[110),111] the above-mentioned W106 mechanism[113] was not found, indicating that inter-domain interactions impose extra controls over molecule-specific mechanisms.

Small-angle X-ray scattering analysis revealed direct semi-micro images of both active and inactive whole calpain molecules in the presence of E64,[105] showing the following: in the presence of 100 µM Ca^{2+} and E64, human erythrocyte μ-calpain did not aggregate and had similar dimensions as in the absence of Ca^{2+} (maximal diameter = 120→130 Å, gyration radius = 36→39 Å). This was consistent with the previous CD spectrum results and with the 3D structure of CAPNS1/30K, whose conformation changes little in the presence or absence of Ca^{2+}.[117),119] On the other hand, a small but significant change was observed in the protease domain; it appeared to compact somewhat and partly detach from C2L domain.

In other words, upon activation, the protease domain dynamically changes its conformation without changing its secondary structure. This feature was finally confirmed when the whole m-calpain 3D structure was determined by co-crystallization with calpastatin repeat 1 or 4 (see 2.6) and Ca^{2+},[110),111] revealing one of the mechanisms used by calpastatin to inhibit conventional calpain activity. G174/G613 of rat calpastatin repeat 1/4, respectively, which is near the conserved TIPPXYR (179–185/618–624) motif in the reactive site of calpastatin, forces a

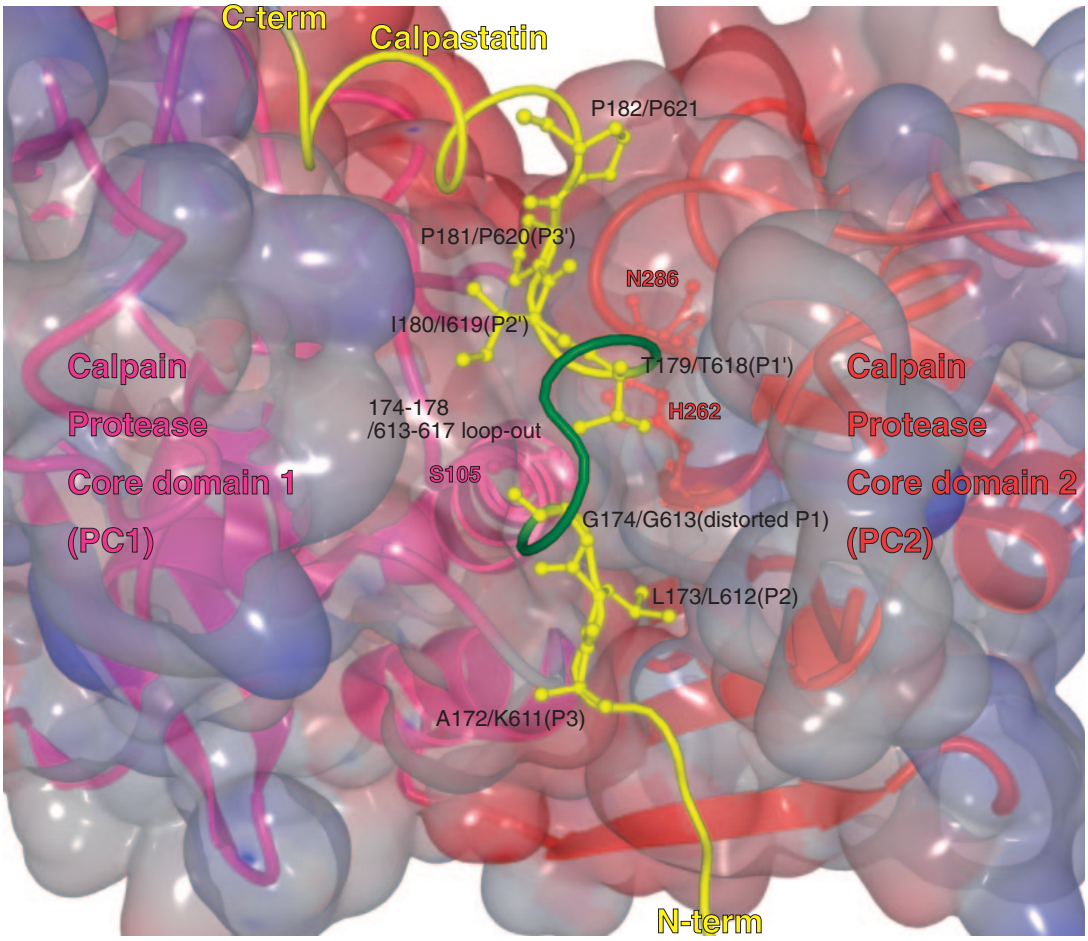


Fig. 11. Calpastatin region B binds and inhibits calpain.
Enlarged view of the interaction between calpastatin (yellow) and the catalytic cleft in the protease core domain PC1 (pink)–PC2 (red) of m-calpain (the 3D structure is from 3DF0). Calpastatin binds in the substrate orientation indicated by positions P3 to P3′. At P1, calpastatin distorts from the substrate path and projects residues 174–178 or 613–617, which form a kink between the P2 and P1′ anchor sites.

kink (G174-IKE-G178/G613-ERD-D617) between its flanking residues, L173/L612 at the catalytic S2 site and T179/T618 at S1′, to loop-out not to interact with the active site of calpain. A172/K611, L173/L612, T179/T618, I180/I619, and P181/P620 fit into the S3, S2, S1′, S2′, and S3′ pocket of calpain's active site, respectively (Fig. 11).[110),111)] Therefore, calpastatin interacts tightly with calpain, but no proteolysis occurs. The importance of this "kink" in resisting proteolysis by calpain was confirmed by calpastatin mutagenesis studies. The deletion of K176, E177, or both, or the insertion of Phe between L612–G613, allows mutant calpastatins to be proteolyzed by calpain.[110),111)]

2.4. Genetic studies of conventional calpains. The first calpain studies using genetically modified mice were reported in 2000, when Arthur *et al.* and Zimmerman *et al.* independently demonstrated that disrupting the mouse gene (*Capns1*) for the conventional calpain regulatory subunit CAPNS1/30K causes embryonic lethality before E11.5.[128),129)] These reports showed that conventional calpains were necessary for mammalian life, which greatly encouraged researchers struggling to understand this enigmatic enzyme. The disruption of CAPNS1/30K causes the down-regulation of both the CAPN1/μCL and CAPN2/mCL proteins, indicating that CAPNS1/30K is required for the stable presence of both calpain catalytic subunits *in vivo*, and probably functions as an intramolecular chaperone. *In vitro*, CAPN2/mCL alone (without CAPNS1/30K) shows full proteolytic activity after being denatured and

then renatured by a long incubation with PEG or GroE.[130] In cells, unfolded calpain large subunits are probably degraded by other proteases before they form active conformations.

Disrupting mouse *Capn1* or *Capn2* leads to contrasting results: *Capn2*$^{-/-}$ mice die before the blastocyst stage, whereas *Capn1*$^{-/-}$ mice appear normal and are fertile.[55),131] This suggests that μ- and m-calpain differ in their function and/or expression level, at least at specific developmental stage(s). *Capns1*$^{-/-}$ embryonic stem (ES) cell growth and adhesion were not noticeably different from those of wild-type ES cells, while *Capn2*$^{-/-}$ ES cells could not be obtained even after extensive screening.[55]

Cells from *Capns1*$^{-/-}$ mice served as ideal tools to unequivocally demonstrate the role of calpain in specific cellular events: Dourdin *et al.* showed that calpain is required for cell migration;[132] Mellgren *et al.* showed that calpain is required for the rapid, Ca^{2+}-dependent repair of wounded plasma membrane;[133] and Demarchi *et al.* showed that calpain is required for macroautophagy.[134]

2.5. Expression of conventional calpains. Although *CAPN1* and *CAPN2* shows ubiquitous distribution, their gene expression patterns are slightly different in that *CAPN1* is rather constantly expressed whereas expression of *CAPN2* varies depending on tissues.[135),136] The promoter region of *CAPN2* lacks typical promoter elements such as TATA and CAAT boxes and has ca. 70% GC content in the −300 to −20 region with several potential GC boxes.[136] The upstream region contains at least four tandemly reiterated regulatory sequences, each of which negatively regulates *CAPN2* expression.[136] These features are common to several house-keeping genes such as adenosine deaminase[137] and hypoxanthine phosphoribosyltransferase,[138] and support that *CAPN2* gene is a typical house-keeping gene that shows ubiquitous and relatively low-level expression. On the other hand, *CAPN2* promoter region also has potential AP-1 binding site, which is responsible for phorbol ester-mediated induction of gene expression.[139),140] Indeed, proerythroblastic K562 cell line cells were reported to show upregulation of CAPN2/mCL expression by phorbol 12-myristate 13-acetate.[141]

Although *CAPN1* gene has not been examined in literature, human genomic sequence showed that *CAPN1* has features similar to those of *CAPN2*, *i.e.*, no significant TATA and CAAT boxes with *ca.* 70% GC content in the −1000 to −1 region and with potential AP-1 binding site at the −80 position. Analysis of *CAPNS1* gene again revealed similar features:[142] the promoter region has G/C-rich sequences with several G-C boxes but without TATA or CAT box. Thus, three genes encoding subunits of the conventional calpains are all regarded to be regulated in the same manner as other house-keeping genes.

2.6. Structure and function of calpastatin. Calpastatin is the only endogenous inhibitor protein for the conventional calpains. It is highly effective and specific, and does not inhibit any other enzymes. Calpastatin has an inhibitor unit that is repeated four times (Fig. 3B), and each unit inhibits one calpain molecule—although their inhibitory efficiencies vary.[143)–145] Calpastatin inhibits both μ- and m-calpains with similar efficiencies. Among other calpain homologs, CAPN8/nCL-2 and CAPN9/nCL-4, but not CAPN3/p94, are inhibited by calpastatin *in vitro*.[53),146),147]

Disruption of *Cast*, the mouse gene for calpastatin, does not produce a significant phenotype under normal, unstressed conditions.[148] This suggests that conventional calpains are not normally activated dynamically, and that calpastatin is dispensable as a safety for calpain regulation. In accordance with this idea, intra-hippocampal injection of kainic acid (KA), which causes apoptotic neuronal cell death by excitotoxicity, resulted in significantly more DNA fragmentation in *Cast*$^{-/-}$ mice than in wild-type mice.[148] Moreover, the KA effect was reduced in transgenic (Tg) mice overexpressing calpastatin in neuronal cells, whereas Tg mice overexpressing the baculoviral caspase inhibitor p35 showed no change.[149] These results indicated that kainic acid-induced apoptotic neuronal cell death is mediated by the conventional calpains, and that caspases are not involved in this process.

Spencer and colleagues developed Tg mice that overexpress calpastatin in muscles.[150),151] These mice appear healthy, without observable body or muscle mass changes or gross physiological, morphological, or behavioral defects. These mice were crossed with *mdx* mice, which have a nonsense *Dmd* mutation that causes mild muscular dystrophic phenotypes due to a lack of dystrophin; the dystrophic phenotype was significantly ameliorated in the resulting calpastatin-overexpressing *mdx* mice.[150] Even in wild-type mice, calpastatin overexpression slows muscle atrophy during muscle unloading.[151]

Studies using Tg mice also impact on food science, *e.g.*, the postmortem tenderization of muscles. Both calpastatin-overexpressing[152] and *Capn1*$^{-/-}$ mice[153]

showed reduced postmortem proteolysis of muscle proteins. Thus, calpain system has drawn attention to one of the targets for meat quality control.

3. Classification of calpain superfamily members

3.1. Classical and non-classical calpains. Calpains have a wide variety of domains in addition to their protease domain, such as C2L and PEF domains (Fig. 1). The calpain superfamily is divided into several subfamilies according to the structures of these additional domains. Since the human conventional calpain catalytic subunits (CAPN1/μCL and CAPN2/mCL) are the reference point for calpain structure, calpains having a similar domain structure are called "classical" calpains (Fig. 3) in contrast to "non-classical" ones described below.[46),154)] Classical calpains are defined as those having C2L and PEF domains in addition to the CysPc domain. Accordingly, non-classical calpains are those having not both of C2L and PEF domains. Among the 15 human calpains, nine are classical and six are non-classical (Fig. 3).

Human classical calpains include CAPN1/μCL, CAPN2/mCL, CAPN3/p94, CAPN8/nCL-2, CAPN9/nCL-4, and CAPN11–14, most of which are conserved among vertebrates; fishes have a duplicate set of most of them.[87)] In invertebrates, only a few classical calpains have been identified. The blood fluke *S. mansoni* has four (Fig. 5),[155)] the fruit fly *D. melanogaster* has three: CALPA/Dm-calpain, CALPB, and CALPC,[34)] and the African malaria mosquito *A. gambiae* has five (Fig. 7). No classical calpain homologs have been found in *C. elegans* (Fig. 6), trypanosomes,[156)] plants, fungi, or *S. cerevisiae*.

Non-classical calpain CysPc domains have aa identities with each other ranging from <30% to >75%, and they may have functional domains on the N- and/or C-terminal sides of CysPc domain (Figs. 1 and 3). These include transmembrane (TM), Zn-finger, and conserved domains with unknown functions such as SOH (SOL homology) domain. This cohort also includes several alternatively spliced products of classical calpain genes, such as *Capn8* and *CalpA* (Fig. 7B). Thus, non-classical calpains probably function differently from classical calpains, and not all are Ca^{2+}-dependent. These features, together with the organization of mammalian calpain genes,[157)] strongly suggest that calpain molecules were generated evolutionarily by combining an ancestral calpain-type cysteine protease gene with genes encoding other functions.

3.2. Subfamilies. Non-classical calpains contain several subfamilies grouped according to their additional domain structures. The PalB subfamily is the most evolutionarily conserved, and is found in humans, yeasts, fungi, protists, all insects except *Drosophila*, and nematodes, but not plants (Fig. 12).[63),64),158)–161)] PalB homologs commonly contain two C2L domains in tandem, each of which diverge greatly or moderately from that of conventional calpains, and have a conserved microtubule-interacting and transport (MIT) motif(s) at the N-terminus (Fig. 3).

Human CAPN10, the representative product of *CAPN10* from the longest transcript,[162)] also has both conserved and diverged C2L domains in succession at the C-terminus. The CAPN10 homologs are only found in vertebrates, and have no MIT domain, but they can be included in the PalB subfamily (Fig. 3).[46)]

TRA-3 is found in nematodes and vertebrates, but not in insects or lower organisms (Fig. 13). Compared with classical calpains, TRA-3 homologs have a C2 domain at the C-terminus, instead of a PEF domain (Figs. 3 and 13). Therefore, TRA-3 homologs can also be included in the PalB subfamily, with its structural consensus of two tandem C2L/C2 domains at the C-terminus. TRA-3 homologs, like CAPN10 homologs, have no MIT domain.

The evolutionarily conserved SOL subfamily occurs in almost all animals, including humans, insects, and nematodes, and also in green algae. SOL homolog structures are characterized by having various numbers of Zn-finger motifs in the N-terminal domain. They also share an SOH domain (Figs. 1 and 3).

Plant calpains, called phytocalpains,[163)] were first described in 2002.[62)] The maize calpain homolog, DEK1 (defective kernel 1), is involved in aleurone cell development. DEK1 homologs are found in various other plants, including rice plants and *Arabidopsis*.[164)–166)] They have one C2L domain at the C-terminus and a TM domain at the N-terminus. A calpain that is highly similar to DEK1 is also found in *Tetrahymena thermophila* (Fig. 14A). Some of the nematode calpains (CLP-3, -4, -6, and -7) have a domain structure (CysPc-C2L-COOH) similar to that of DEK1, but they do not have a TM domain (Fig. 6). These calpains are all grouped as the DEK1 subfamily.

The first member of the calpain bacterial subfamily was reported in 1992 from *Porphyromonas gingivalis*; it is called tpr (thiol protease).[167)] Sub-

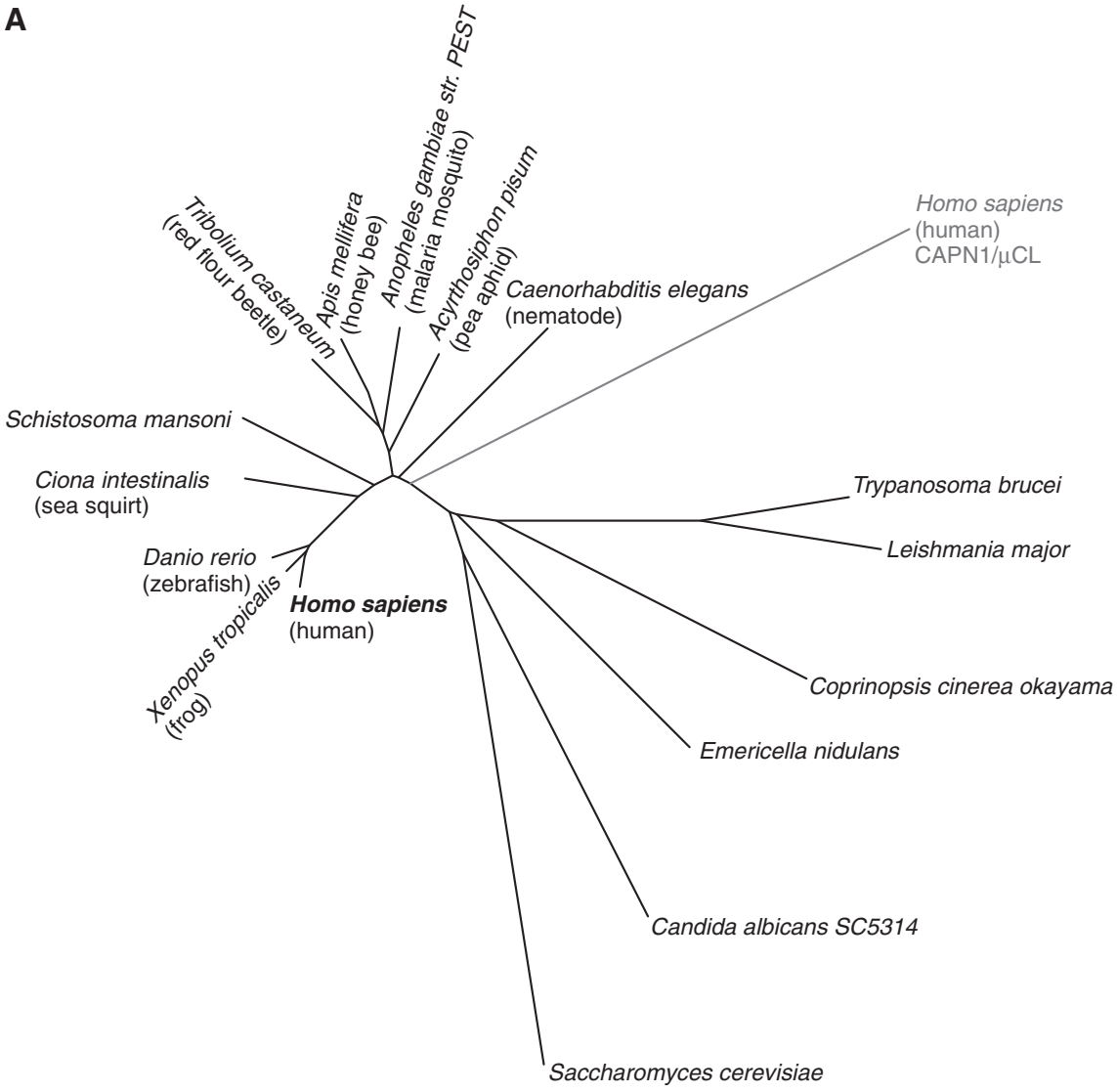


Fig. 12. *Continued on next page.*

sequent bacterial genome projects revealed several bacterial calpain homologs. These are the most divergent calpain species, sharing similarity only in the protease domain (Fig. 8). Although *E. coli* was once reported to have a calpain-like protease,[168] its sequence rather belongs instead to the narrowly defined papain superfamily (clan CA-C1). In summary, all the calpain subfamily consensus structures are shown in Fig. 14B.

3.3. Ubiquitous and tissue-specific calpains. In addition to their structural features, mammalian calpains are also independently categorized according to their tissue and organ distribution.[169] CAPN1/μCL, CAPN2/mCL, CAPN5/hTRA-3, CAPN7/PalBH, CAPN10, CAPN13, and CAPN15/SOLH are ubiquitously expressed, whereas CAPN3/p94, CAPN8/nCL-2, CAPN9/nCL-4, CAPN6, CAPN11, and CAPN12 are expressed in specific tissues or organs (Figs. 3 and 4). Widely accepted assumption is that ubiquitous calpains play fundamental roles in all cells, whereas tissue-specific calpains have tissue-specific roles. In fact, defects in ubiquitous calpains may be lethal, as in *Capn2*$^{-/-}$ mice,[55] whereas defects in tissue-specific calpains may cause tissue-specific diseases, like the muscular dystrophy caused by defective *CAPN3*.[56]

At the same time, under various disease/damaged conditions such as muscular dystrophies,

Fig. 12. *Continued on next page.*

Fig. 12. Phylogenetic tree and sequence alignment of PalB calpains.
A. Phylogenetic tree of strict PalB calpains and human CAPN1/μCL. See Fig. 3A for methods. B. Sequence alignment of PalBs and human CAPN1/μCL (the start and end aa residue numbers are indicated). Triangles: active site residues. Asterisks: residues involved in Ca^{2+}-binding of the protease domain of CAPN1/μCL. Arrows: domain boundaries. Deletion (−) and multiple (+) residue positions are indicated. Residue numbers of human CAPN1/μCL are given above the sequences. Residues conserved in all the sequences, all except human CAPN1/μCL, and in more than half the sequences are indicated by black reversed fonts, gray reversed fonts, and gray shadow, respectively. Sequences: 0: *H. sapiens* (human) CAPN1/μCL (NP_005177); 1: *H. sapiens* CAPN7/PalBH (NP_055111); 2: *X. tropicalis* (frog) (NP_998853); 3: *D. rerio* (zebrafish) (NP_001128580); 4: *Ciona intestinalis* (sea squirt) (XP_002121253); 5: *Tribolium castaneum* (red flour beetle) (XP_967682); 6: *Apis mellifera* (honey bee) (XP_001121978); 7: *Acyrthosiphon pisum* (pea aphid) (XP_001945029); 8: *A. gambiae* str. PEST (malaria mosquito) (XP_309874); 9: *S. mansoni* (XP_002579452); 10: *C. elegans* (NP_497964); 11: *T. brucei* TREU927 (XP_828540); 12: *L. major* (CAJ06528); 13: *Coprinopsis cinerea* okayama7#130 (common ink cap) (EAU91907); 14: *E. nidulans* FGSC-A4 (XP_657860); 15: *C. albicans* SC5314 (EAL03290); 16: *S. cerevisiae* (Q03792).

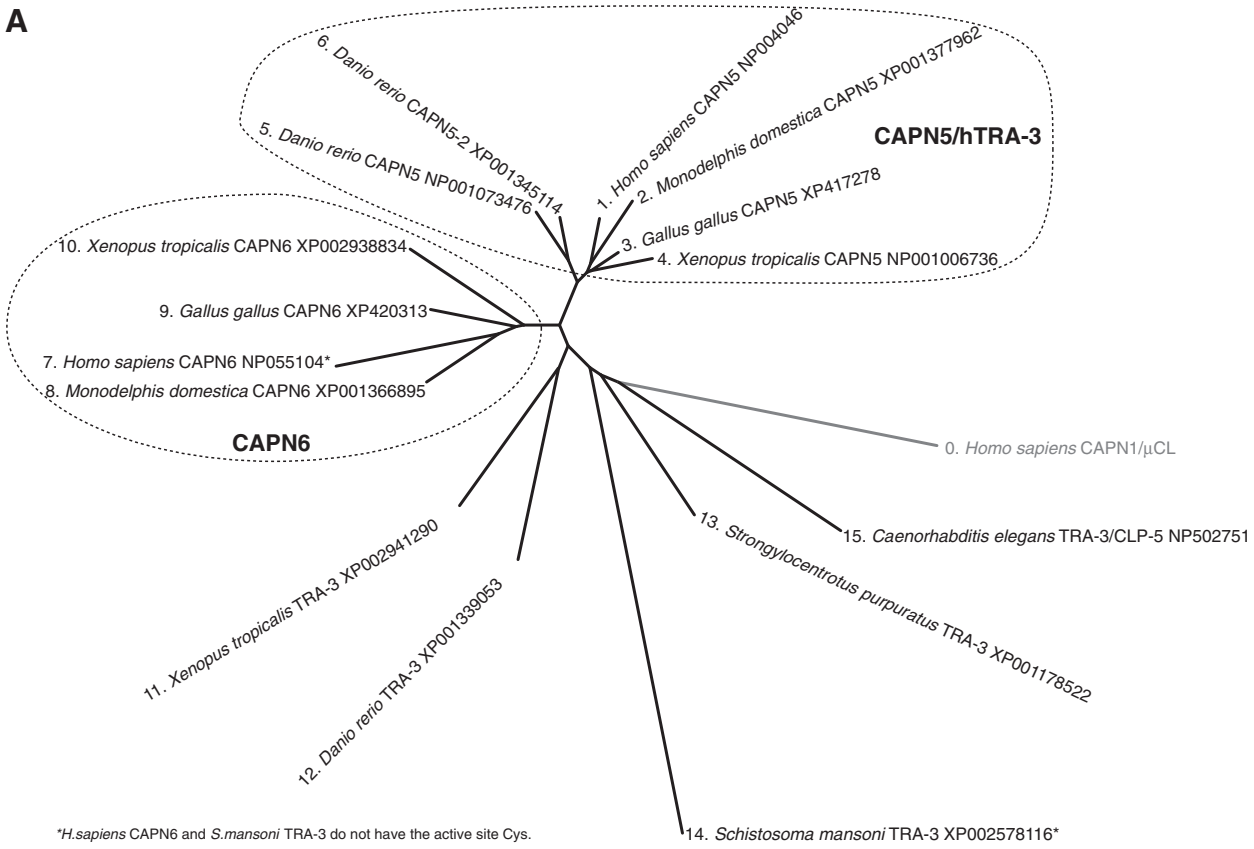


Fig. 13. *Continued on next page.*

cardiomyopathies, traumatic ischemia, and lissencephaly, conventional calpains tend to be overactivated—probably because the intracellular Ca^{2+} homeostasis is compromised by the disease—and that overactivity often exacerbates the disease. Conventional calpain inhibitors are currently used to prevent the progression of such diseases, a therapeutic technique first applied by Suzuki and colleagues.[58),79),170),171)]

3.4. Expression of tissue-specific calpains. Tissue-specificity of tissue-specific calpains should be regulated by their promoter regions. Human, mouse and rat *CAPN3*/*Capn3* upstream regions have highly conserved structures containing TATA-box and several E-boxes, which probably are responsible for muscle-specific expression of *CAPN3*/*Capn3*.[172)] Intriguingly, *CAPN3*/*Capn3* has an alternative upstream promoter, which drives ubiquitous expression of Up86 *etc.* starting from an alternative initial exon with slight abundance in the brain and blood cells.[173)–175)] Moreover, rodent *Capn3* has additional alternative promoter and initial exon between exons

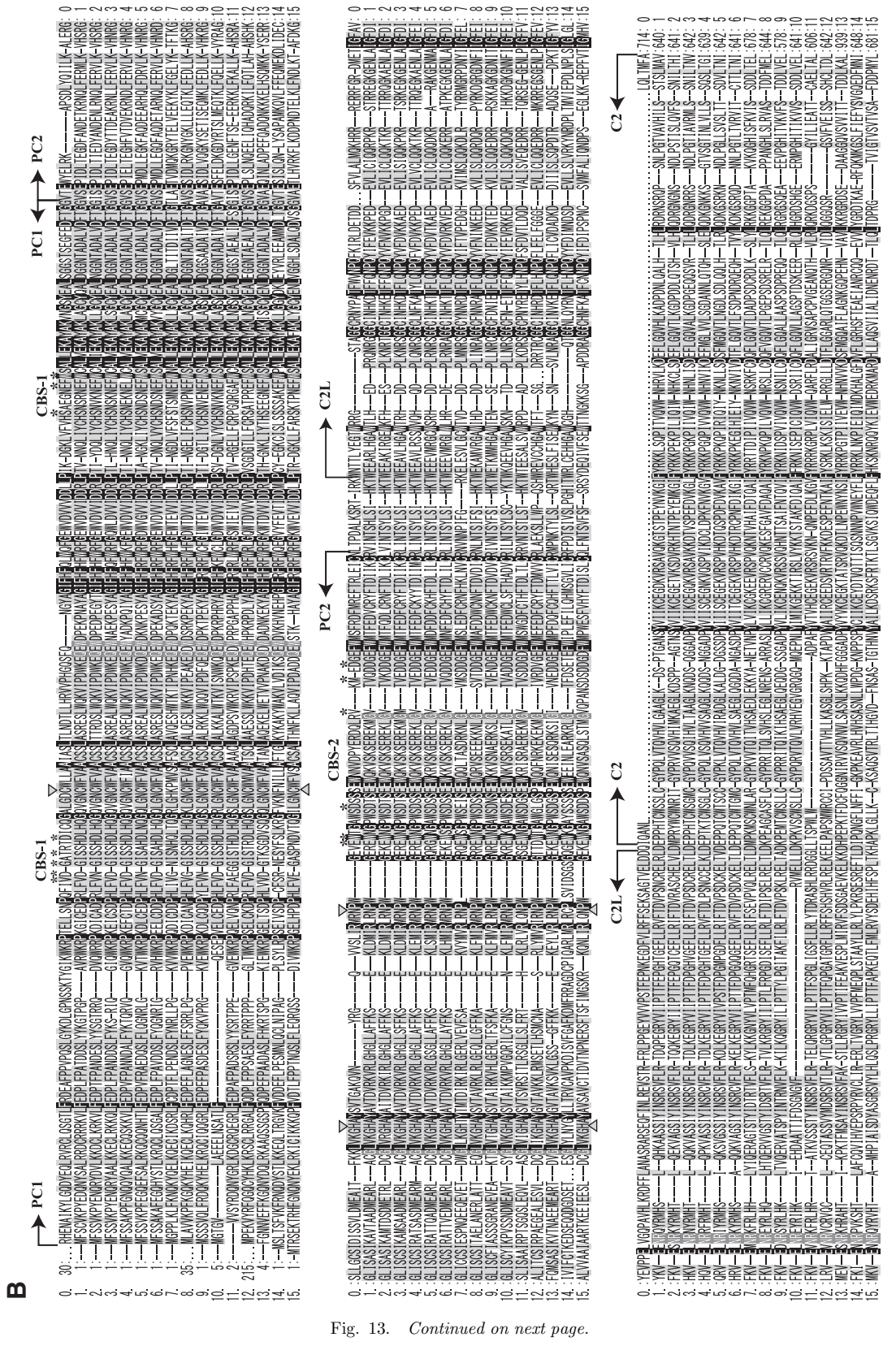


Fig. 13. *Continued on next page.*

Fig. 13. Phylogenetic tree and sequence alignment of TRA-3 calpains.
A. Phylogenetic tree of TRA-3 calpains and human CAPN1/μCL. See Fig. 3A for methods. B. Sequence alignment of TRA-3 and human CAPN1/μCL (the start and end aa residue numbers are indicated). Sequences: 0: *H. sapiens* CAPN1/μCL NP005177; 1: *H. sapiens* CAPN5 NP004046; 2: *M. domestica* CAPN5 XP001377962; 3: *G. gallus* CAPN5 XP417278; 4: *X. tropicalis* CAPN5 NP001006736; 5: *D. rerio* CAPN5 NP001073476; 6: *D. rerio* CAPN5-2 XP001345114; 7: *H. sapiens* CAPN6 NP055104; 8: *M. domestica* CAPN6 XP001366895; 9: *G. gallus* CAPN6 XP420313; 10: *X. tropicalis* CAPN6 XP002938834; 11: *X. tropicalis* TRA-3 XP002941290; 12: *D. rerio* TRA-3 XP001339053; 13: *Strongylocentrotus purpuratus* CAPN5 XP001178522; 14: *S. mansoni* XP002578116; 15: *C. elegans* TRA-3/CLP-5 NP502751. For other explanation, see Fig. 12B.

1 and 2, and these are responsible for lens-specific expression of alternative splicing products of *Capn3* such as Lp82 and Rt88.[176),177)] However, human *CAPN3* lost lens-specific products by in-frame termination in exon 1′.[178)] *Capn3* expression is also noted in testis.[173)]

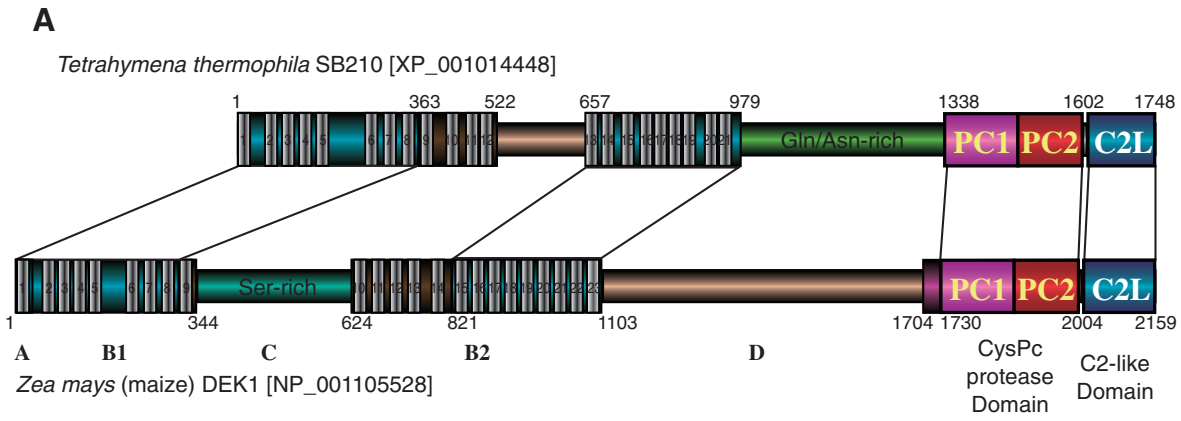
The promoter region of *Capn8* contains two TATA boxes with several GATA boxes, which are probably responsible for stomach expression of *Capn8*.[157)] Some cAMP responsive elements is also found in this region. Notably, *CAPN8* and *CAPN2* are close to each other on human chromosome 1q41 (mouse: chromosome 1), and their transcripts have overlapping complementary sequences, suggesting possible interaction with each other.[157)] In addition, *Capn8* has additional exon in the middle, which generates an alternative splicing product, CAPN8b/nCL-2′. Its physiological relevance, however, is elusive.[157),179)] As for other tissue-specific calpain genes, their upstream sequences have not been analyzed in the detail.

4. Unconventional calpain functions in health and disease

4.1. Skeletal muscle-specific calpain. The first tissue-specific calpain, CAPN3/p94, was found in 1989.[180)] It is expressed predominantly in skeletal muscle. This classical calpain is approximately 50% identical to CAPN1/μCL and CAPN2/mCL (Fig. 3). However, it contains three additional characteristic regions NS, IS1, and IS2, located at the N-terminus, in PC2 domain, and between C2L and PEF domains, respectively. These regions give CAPN3/p94 some unique features.

The most characteristic property of CAPN3/p94 is its extremely rapid autodegradation—its half-life *in vitro* is less than 10 minutes—starting with autolysis in the NS and IS1 regions.[181)–183)] This autodegradative activity depends on both IS1 and IS2, and conventional calpain inhibitors such as calpastatin and E64 have little effect on it.[181)] Surprisingly, this autodegradation occurs Na^+-dependently in the absence of Ca^{2+}, establishing CAPN3/p94 as the first example of an intracellular Na^+-dependent enzyme.[54)] The physiological relevance of CAPN3/p94's Na^+-dependency is still unclear, but its substrate specificities differ when it is activated by Ca^{2+} versus Na^+. For example, LIM domain-binding protein 3, tropomyosin α-1 chain, and α-actinin-3 are candidate *in vivo* substrates for Na^+-activated CAPN3/p94, whereas troponins T and I and Ras-specific guanine nucleotide-releasing factor 2 are for Ca^{2+} activation.[54)] Furthermore, CAPN3/p94 possesses a nuclear localization-signal-like sequence in the IS2 region, and is sometimes found in the nucleus as well as the cytosol.[181)] CAPN3/p94 binds specifically to the N2A and M-line regions of the gigantic filamentous muscle protein connectin/titin ($Mr > 3{,}000$ kDa), and the binding site for N2A connectin/titin is the N-terminal vicinity of the IS2 region.[184)] Its autodegradative activity is almost completely suppressed *in vivo*, most probably by binding to N2A connectin/titin. This region also aligns important subcellular structures of muscle, such as the sarcoplasmic reticulum and T-tubules, and therefore represent the potential space for CAPN3/p94 to function.[183)]

In 1995, *CAPN3* mutations were shown to be responsible for limb-girdle muscular dystrophy type 2A (LGMD2A), also called calpainopathy, by an analysis of families of La Réunion Island and by positional cloning.[56),185)] *Capn3*$^{-/-}$ mice recapitulate a human calpainopathy-like phenotype,[186),187)] although milder, indicating that calpainopathy is indeed caused by *CAPN3* defects.[186)] Calpainopathy appears to be primarily caused by compromised CAPN3/p94 protease activity, rather than by damaged structural properties.[188)] This was confirmed by CAPN3/p94 knock-in (*Capn3*$^{CS/CS}$) mice, which express a structurally intact but inactive CAPN3/p94:C129S mutant, and also present a muscular dystrophy phenotype.[189)] Intriguingly, however, *Capn3*$^{CS/CS}$ mice have a less severe phenotype than *Capn3*$^{-/-}$ mice, indicating that the proteolytically inactive CAPN3/p94 retains some function, probably involving its structural properties.[189)]



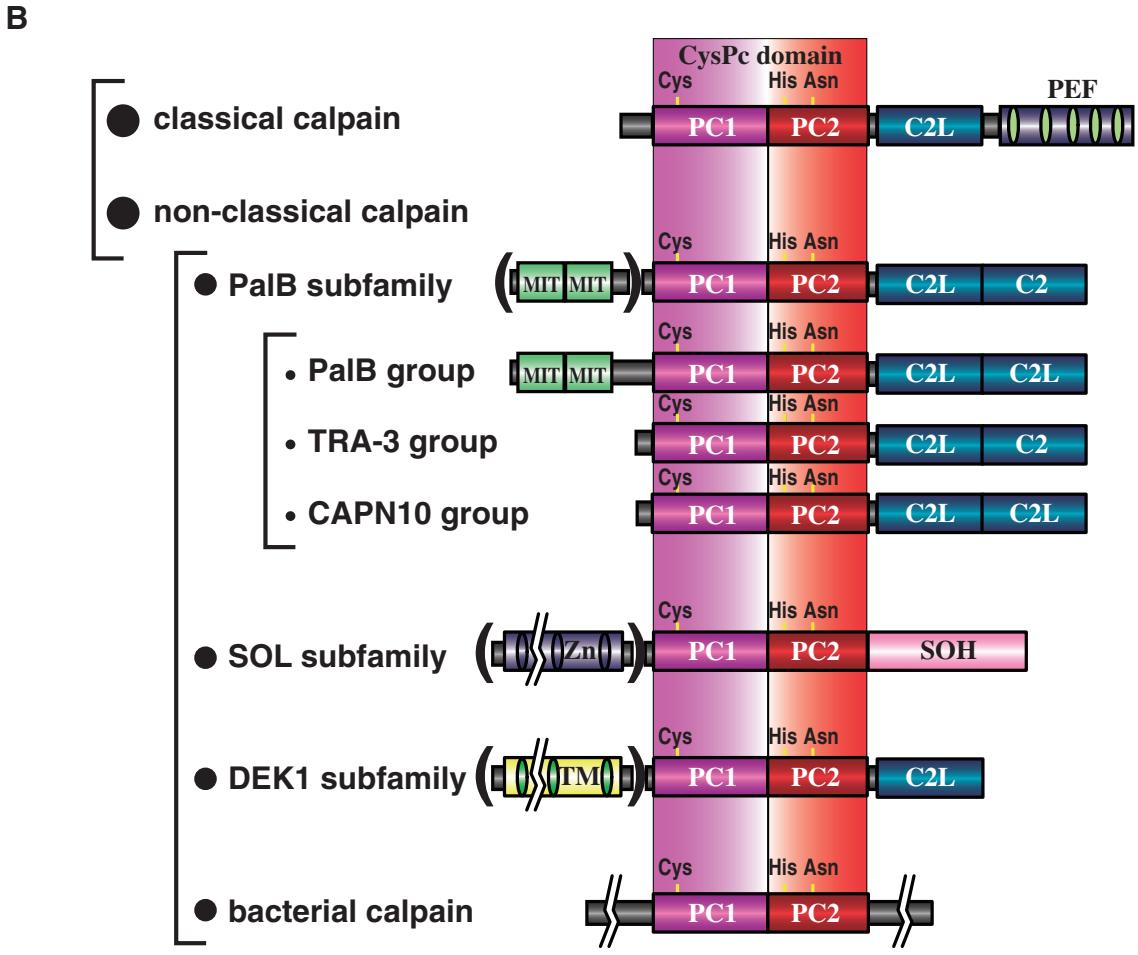


Fig. 14. Schematic structures of *T. thermophila* and *Z. mays* DEK1s.
A. The number of TM spanners is taken from prediction programs and may be corrected in the future. Domain names for *Z. mays* DEK1 are taken from ref. 62). B. Structural classification and consensus domain structures of calpain super family members. See Fig. 1 legend for symbols.

Many subsequent family analyses have been reported, identifying substantial numbers of both pathogenic and polymorphic mutations in *CAPN3*.[190),191)] However, no mutational hot spot has been found, so a genetic diagnosis of calpainopathy is not easy. A total of 2,530 mutations, including 456 unique ones, have been reported in the *CAPN3* gene so far (available at the Leiden Muscular Dystrophy Pages http://www.dmd.nl/). Among the 80 gene entries with mutations responsible for muscular dystrophy, *CAPN3* has the third most, coming only after dystrophin and lamin A/C. One characteristic of *CAPN3* gene mutations is that more than half (62.7%) of them are nucleotide substitutions, of which missense mutations are dominant (60.3% among all substitutions, and 37.8% among all pathogenic mutations) (Fig. 15A).

Although some missense mutations found in LGMD2A patients are reported relatively frequently, such as R490Q/W, R769Q, and A236T, most of them are distributed all over the protein, between the N-terminus and C-terminus, including 215 unique mutations at 176 independent loci (21.4%) among the 821 aa residues (Fig. 15B). A comparison of vertebrate CAPN3/p94s (including the two gene products from fishes) revealed 241 residues that are conserved in all species (Fig. 16), including 83 of the above 176 missense loci, indicating that conserved aa residues are important for CAPN3/p94 functions. Conservation in the CAPN3/p94-characterizing regions NS, IS1, and IS2 is relatively low; however, it should be noted that some missense mutations, such as R49C/H, P319L, and S606L, which are in the NS, IS1, and IS2 regions respectively, occur relatively frequently. These observations suggest that sequence conservation of the CAPN3/p94-characterizing region is not necessarily required, but its length and a few important aa, usually near the borders of these regions, must be conserved for proper function.

LGMD2A and *CAPN3* mutations are the first—and so far, only—example of a clear cause–effect relationship between human disease and calpain gene mutations.

4.2. Gastrointestinal tract-specific calpains. CAPN8/nCL-2 and CAPN9/nCL-4 are predominantly expressed in surface mucus cells, called pit cells, in the stomach. Smaller amounts are expressed in the goblet cells in the intestines.[179),192),193)] Among mammalian calpains, CAPN8/nCL-2 is most similar to CAPN2/mCL (62% identical). Unlike m-calpain, however, *E. coli*-expressed recombinant CAPN8/nCL-2 exhibits Ca^{2+}-dependent autolytic and caseinolytic activity without CAPNS1/30K, and it forms a homo-di∼oligomer *via* C2L domain *in vitro*.[146)] *Xenopus laevis* has a CAPN8/nCL-2 ortholog named xCL-2, which causes severe developmental defects when disrupted.[194)]

CAPN9/nCL-4 is another classical calpain homolog specific for gastrointestinal tracts. It is equally similar to all other classical calpains at the aa level, suggesting that CAPN9/nCL-4 is the molecule closest to the ancestral calpain species.[193)] CAPN9/nCL-4 is reported to be involved in tumorigenesis[195)] and in lumen formation in breast epithelial cells induced by an adhesion molecule.[196)] Recombinant human CAPN9/nCL-4 requires CAPNS1/30K for its activity *in vitro*, and such a complex has Ca^{2+}-dependent activity that is inhibited by calpastatin and other Cys protease inhibitors, similar to conventional calpains.[147)]

In contrast, the *in vivo* characteristics of these calpains are rather different. Experiments using *Capn8*$^{-/-}$ and *Capn9*$^{-/-}$ mice showed that neither CAPN8/nCL-2 nor CAPN9/nCL-4 forms a stable complex with CAPNS1/30K; rather, they form a hybrid complex with each other, now designated as gastric calpain (G-calpain).[57)] As is the case for *Capns1*$^{-/-}$, disruption of either *Capn8* or *Capn9* caused down-regulation of another molecule, suggesting that each species contributes to the stability and the functionality of the other. Both *Capn8*$^{-/-}$ and *Capn9*$^{-/-}$ mice seem healthy under normal conditions, but they are significantly more susceptible to ethanol-induced gastric ulcers.[57)] CAPN8/nCL-2 knock-in (*Capn8*$^{CS/CS}$) mice that express the protease-inactive CAPN8/nCL-2:C105S mutant instead of wild-type CAPN8/nCL-2 also show stress-induced gastropathy. This indicates that CAPN8/nCL-2 and CAPN9/nCL-4, in the form of G-calpain, mediate key events for gastric mucosal defense.[57)]

A single nucleotide polymorphism (SNP) database search revealed that human *CAPN8* and *CAPN9* have several aa-substituting SNPs.[57)] An *in vitro* expression study indicated that some of these SNPs compromised proteolytic activity of G-calpain. Thus, evaluation of the link between these SNPs and susceptibility to gastrointestinal disorders is on list of things to do.

4.3. The PalB subfamily. PalB was first identified in the ascomycete *E. nidulans* as a product of the gene responsible for the fungi's adaptation to alkaline conditions.[63)] Its orthologs were then widely identified, from *S. cerevisiae* to humans (Fig. 12).[64),158)] The structural consensus for PalB

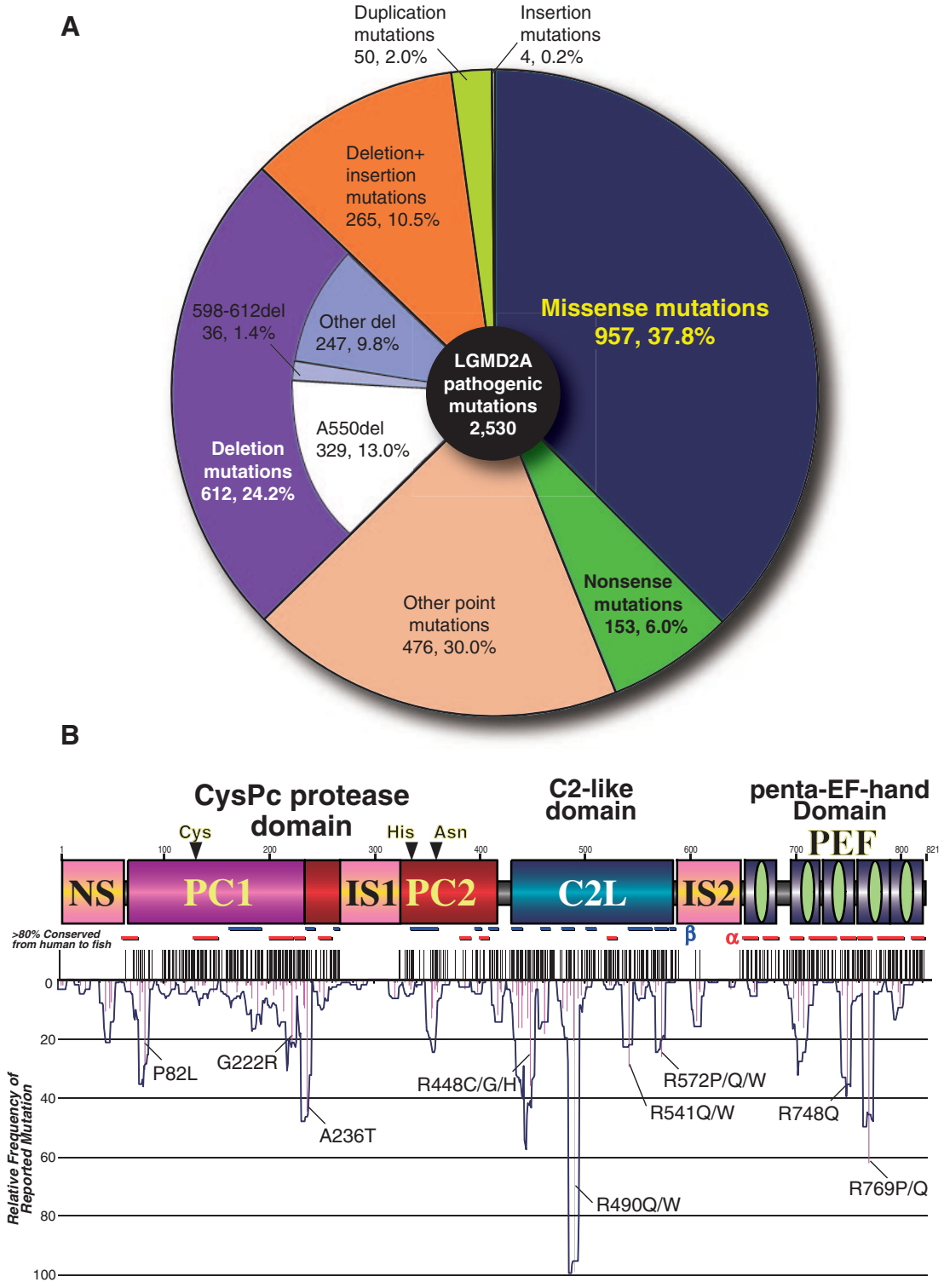


Fig. 15. Pathogenic CAPN3 mutations in calpainopathy patients.
A. Summary of calpainopathy mutation types. B. Calpainopathy missense mutation positions and frequencies (relative to the maximum value). Black vertical lines indicate aa residues that are conserved more than 80% of vertebrates as shown in Fig. 16B. α and β indicate α-helix and β-strand secondary structures of the corresponding m-calpain 3D structure. See Fig. 1 legend for other symbols.

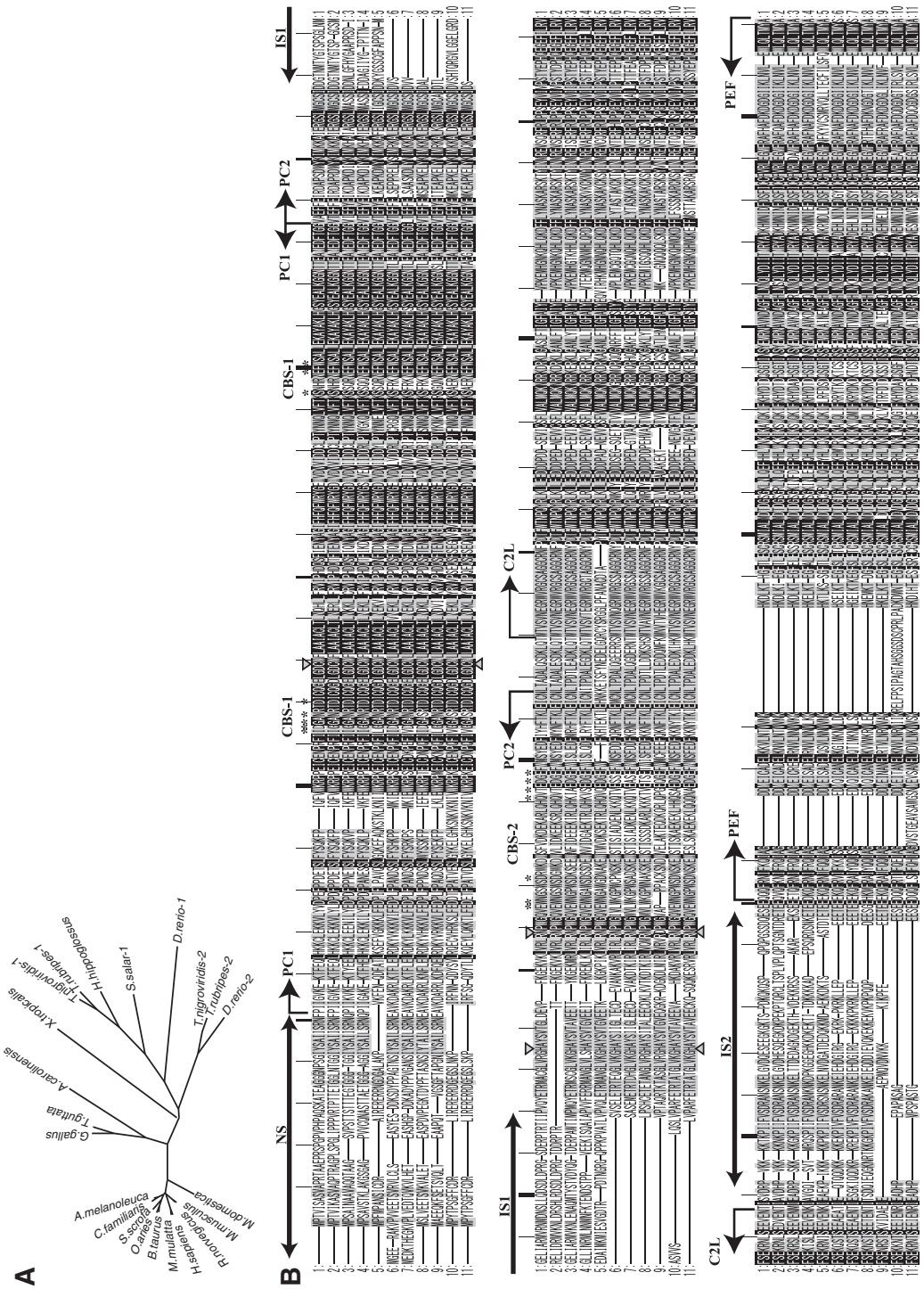


Fig. 16. Phylogenetic tree and sequence alignment of CAPN3 proteins.

A. Phylogenetic tree of vertebrate CAPN3. See Fig. 3A for methods. Explicit CAPN3 homologs are found only in vertebrates. B. Sequence alignment of CAPN3s. Fishes have two CAPN3 homologs (type-1 and -2); the latter does not have explicit IS1 or IS2 regions. Sequences: 1: *H. sapiens*, NP_000061; 2: *M. domestic*, ENSMODP00000022330; 3: *G. gallus*, NP_001004405; 4: *A. carolinensis*, ENSACAP00000015362; 5: *X. tropicalis*, ENSXETP00000026925; 6: *Takifugu rubripes* (type-1), ENSTRUP00000046251; 7: *Hippoglossus hippoglossus* (type-1), ACY78226; 8: *Salmo salar* (type-1), NP_001158880; 9: *D. rerio* (type-1), XP_001337065; 10: *T. rubripes* (type-2), ENSTRUP00000016935; 11: *D. rerio* (type-2), ENSDARP00000094244. For other explanation, see Fig. 12B.

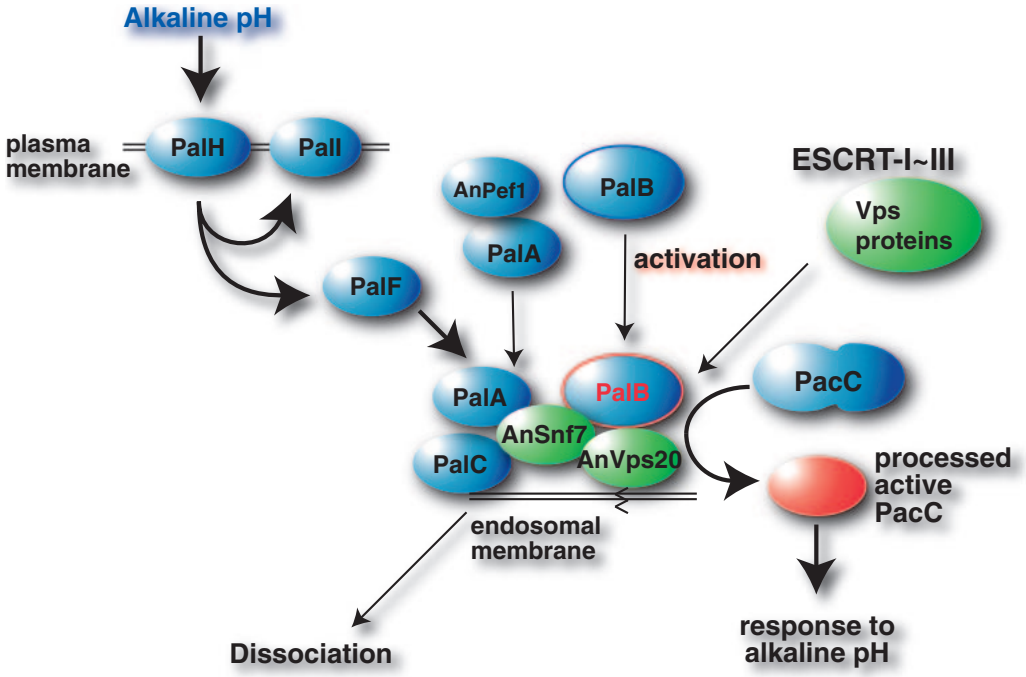


Fig. 17. Pal-PacC pathway and corresponding molecules in yeast and human.
Schematics of signaling pathways involving PalB of fungi. In fungi, a membrane protein (PalH) senses ambient pH and transduces the alkaline signal to PalI. An arrestin homolog, PalF, further transduces the signal to PalA, which forms a complex with AnPef1. At the endosomal membrane, PalA forms the PalB active complex to proteolyze PacC at the C-terminus, thus activating this key transcription factor. Activated PacC regulates gene expression under alkaline conditions. This pathway is highly conserved in yeast and possibly conserved in human; corresponding molecules: PalH (fungi)-Rim21 (yeast)-? (human), PalI-Rim9-?, PalF-Rim8-arrestin, PalA-Rim20-Alix/AIP1, PalC-Ygr122w-?, AnPef1-Pef1/Ygr058w-ALG-2, AnSnf7-Snf7/Vps32-CHMP4s, PalB-Rim13/Cpl1-CAPN7/PalBH, and PacC-Rim101-C2H2 Zn-finger proteins? See text for details.

subfamily members is $(MIT)_{0-2}$-CysPc-C2L-C2L/C2 (Fig. 14B). Strict PalB homologs have $(MIT)_2$-CysPc-C2L-C2L (the upstream C2L was once called PBH (PalB-homology) because of its highly divergent sequence[48]), whereas CAPN10 and TRA-3 homologs have CysPc-C2L-C2L and CysPc-C2L-C2, respectively.

4.3.1. Strict PalB homologs in fungi. *E. nidulans*, like many other microorganisms, grows over a wide pH range. The gene locus *palB* was identified from mutants that mimic the gene expression profiles for acid environments even under normal/neutral conditions, corresponding to *pal* genes (*pal* stands for "*p*hosphatase mutants: loss in activity at *al*kaline pH but normal or increased activity at acidic pH"). PalB is involved in the proteolytic activation of a key transcription factor named PacC, which governs the entire regulation of pH-dependent changes in gene expression, such as the expression of alkaline phosphatase under alkaline conditions. This pH adaptation system is here called the Pal-PacC pathway (Fig. 17).

Mutations in six genes, *palA*, *palB*, *palC*, *palF*, *palH* and *palI*, that mimic the effects of growth at acid pH, and one, *pacC*, that mimics those at alkaline pH, were identified.[197)–199)] These genes were characterized from 1995, starting with the identification of the *palB* gene product as a novel and divergent calpain homolog.[63),200)–203)] Primary structures of these *pal* gene products showed that PalB was a calpain homolog, and PalH and PalI had TM domains. Protein interaction analyses showed that PalF, a distant homolog of mammalian arrestin, binds to the large cytosolic domain of PalH,[204)] and that PalF's phosphorylation and ubiquitylation under alkaline conditions depend on PalH and PalI. These findings indicate that PalF–PalH interactions mediate pH signaling, similar to the arrestin–receptor interactions that regulate various processes in mammals, such as photo-signaling.[205)] These findings strongly imply the existence of an corresponding mammalian signaling pathway(s) involving arrestin and CAPN7/PalBH.

PacC (674 aa) usually forms an inactive, closed conformation, which protects it from activation by intramolecular interactions, and undergoes limited proteolysis to become active.[206),207)] First, PacC is processed between aa 479–502 by a primary processing protease, PalB, in a pH-regulated manner. This changes PacC to an open form susceptible to proteolysis by the proteasome in a pH-independent constitutive manner, simultaneously creating the processed active form (252–254 aa).[207),208)] It is thought that ubiquitylation is not involved in this process, which is a rare mode of action for proteasome.[209)–212)]

4.3.2. Strict PalB homologs in yeasts and mammals. The above-described fungus Pal-PacC pathway is well conserved in budding yeasts. The *S. cerevisiae* PalB ortholog is Rim13/Cpl1. It was first identified in 1993 as one of the *RIM* genes, which reduce the expression of sporulation-specific genes when mutated.[213),214)] Among the *RIM* gene products, Rim101 corresponds to fungus PacC, and plays a central role in this signaling system, as does PacC. The proteolytic regulation of Rim101 was first discovered in 1997,[215)] and the responsible protease was identified as Rim13/Cpl1 in 1999.[64)] Subsequently, *RIM8*, *9*, *20*, and *21*, which correspond to *palF*, *I*, *A*, and *H*, were identified.[200),201)]

Saccharomycetes' usual environment is rather acidic, and its alkaline adaptations function in near-neutral pH conditions. Therefore, this adaptive system, here called the Rim pathway, has practical importance for human health. Pathogenic and saprophytic yeasts like *Candida albicans* and *Yarrowia lipolytica* also have conserved Rim pathways. For example, *C. albicans* uses this pathway when it infects and invades mammalian skin in neutral pH environments, and disrupting the Rim pathway compromises its pathogenicity.[216),217)] *Y. lipolytica*[218),219)] and *Usitlago maydis*[220)] are aslo reported to use an identical system.

The Pal-PacC and Rim pathways relate to membrane trafficking, and involve ESCRT (endosomal sorting complex required for transport) and Vps (vacuolar protein sorting) proteins.[65),221)–229)] Based on a genomic study that showed Rim and Vps interactions, it is demonstrated that these two proteins had a functional relationship in *S. cerevisiae* and *C. albicans*.[223),228),230)] Screening of suppressor mutants for *rim21* and *rim9* revealed functional relationships between the Rim and Vps proteins.[65)] These studies clarified components of the Rim signaling cascade and the hierarchy: *RIM8*, *9*, and *21* act upstream of *RIM13/CPL1* and *RIM20*, and Rim13/Cpl1 proteolyzes Rim101 at the endosomal membranes with the aid of the ESCRT-III subcomplex as a scaffold (Fig. 17).

The Pal-PacC and Rim pathways are the first examples that genetics thoroughly uncovered the molecular components and mechanisms of calpain-mediated systems. These pathways also exemplify that calpains function as modulator proteases, affecting their substrates' functions by limited proteolysis. Theoretical extension of the knowledge of these pathways is anticipated to provide important keys for understanding other calpain systems, including those that involve CAPN7/PalBH.

There are few published reports on CAPN7/PalBH, a mammalian ortholog of PalB.[158),231)–233)] Many studies have examined other homologous components; for instance, arrestin and Alix/AIP1, which correspond to PalA and F, respectively. It should be noted that interactions among Rim20, Fef1/Ygr058w, and Snf7/Vps32 are conserved in mammals as Alix1/AIP1, ALG-2, and CHMP4, respectively.[234)–237)] Although multiple interactions of Alix/AIP1 have been reported,[236),238)] the physiological roles of these proteins and CAPN7/PalBH have not been clarified. Another message from these studies is that it is essential to take ubiquitous unconventional calpains such as CAPN7/PalBH into account along with the conventional calpains when analyzing various biological phenomena, but very few reports achieve this.[239)]

4.3.3. TRA-3 homologs in the PalB subfamily. TRA-3/CLP-5 is classified into the PalB subfamily in the broad sense, but TRA-3/CLP-5 has a distal C2 instead of C2L domain, and no N-terminal MIT domain (Fig. 14B). The distal C2 domain was once called a T domain (T from TRA-3), because its primary sequence rather diverges from that of other C2 domains. TRA-3/CLP-5 was first identified by genetics as the product of the gene, *tra-3* (transformer: XX animals transformed into males), which is involved in the sex determination cascade of *C. elegans*.[61)] Its action as an enzyme has not been characterized; however, its protease activity is necessary for female development in XX hermaphrodites, in the processing of the TRA-2A membrane protein.[240)] In contrast, TRA-3/CLP-5 and another *C. elegans* calpain, CLP-1, are components of a neuronal necrotic death cascade, upstream of the aspartic proteases ASP-3 and ASP-4, which may correspond to mammalian cathepsins D and E.[241)] An SNP in *tra-3* is also reported to be involved in nematode body-size determination.[242)]

Mammals have two TRA-3 orthologs, CAPN5/hTRA-3 and CAPN6; both share more than 30% aa identity with TRA-3 (Fig. 13).[243),244)] CAPN5/hTRA-3 has Ca^{2+}-dependent autolytic activity and is sensitive to several calpain inhibitors.[245)] CAPN5/hTRA-3 is expressed in almost all tissues in varying amounts. Analysis of *Capn5*$^{-/-}$ mice showed that CAPN5/hTRA-3 is expressed in a subset of T cells, but is dispensable for development.[246)] SNPs of human *CAPN5* were reported to be associated with polycystic ovary syndrome, diastolic blood pressure, and cholesterol levels,[247),248)] and CAPN5/hTRA-3 and CAPN15/SOLH are upregulated in chronic intermittent, but not constant, heart hypoxia.[249)]

On the other hand, CAPN6 in eutherians (placental mammals) naturally has aa substitutions in the most important residue in the active site triad (C→K in humans, see Fig. 13B), strongly suggesting that eutherian CAPN6 has no proteolytic activity. Interestingly, CAPN6 in marsupialia (*Monodelphis domestica*; gray, short-tailed opossum) and in chicken (*Gallus gallus*) have the active-site residues Cys-His-Asn; moreover, frog (*X. tropicalis*) and fish (*Danio rerio*) have three TRA-3 homologs, which also have the active-site residues. This is in contrast to CAPN11, which is a classical calpain expressed specifically in the testis in eutherians, whereas the *CAPN11* gene is ubiquitously expressed in chicken but was lost in marsupialia. Mouse CAPN6 is predominantly expressed in embryonic muscles, placenta, and in several cultured cells. It is intriguing to consider the physiological function of eutherian CAPN6 and CAPN11, which are expressed in the placenta and testis, respectively. CAPN6 was shown to be involved in regulating microtubule dynamics,[250)] and was subsequently shown to regulate cell motility in cultured cells,[251)] although the *in vivo* physiological functions of CAPN6 and CAPN11 are still unclear.

4.3.4. CAPN10 homologs in the PalB subfamily. CAPN10 also belongs to the PalB subfamily in the broad sense, but CAPN10 differs from PalB in that it has no N-terminal MIT domain (Fig. 14B). *CAPN10* was identified as a risk factor for non-insulin-dependent diabetes mellitus (NIDDM, type 2 diabetes) in a large-scale genetic association study.[162)] An association was found between G→A SNP (UCSNP-43, the G allele is the at-risk genotype) in intron 3 of *CAPN10* and susceptibility to NIDDM in Mexican Americans and in a Northern European population from the Botnia region of Finland. There is no molecular explanation why this SNP causes susceptibility to NIDDM, although G/G homozygotes in UCSNP-43 were reported to have reduced *CAPN10* mRNA expression in skeletal muscle.[252),253)]

The *Capn10* gene is also a candidate gene responsible for a phenotype of the Otsuka Long-Evans Tokushima Fatty rat, a NIDDM animal model.[254)] Quantitative trait locus (QTL) analyses using *Capn10*$^{-/-}$ mice and two strains, LG/J and SM/J, with low and high obesity phenotypes showed that *Capn10* is included in the obesity QTL, *Adip1*, indicating involvement of CAPN10 in obesity also in mice.[255)]

Studies using *Capn10*$^{-/-}$ or CAPN10-overexpressing Tg mice suggest that CAPN10 is involved in type 2 ryanodine receptor-mediated apoptosis.[256)] Although the phenotypes of these mice have not been described, they do not have embryonic lethality. Ubiquitous distribution and dynamic changes in CAPN10's cellular localization was shown by a CAPN10-specific antibody.[257)] CAPN10 was also demonstrated to be involved in GLUT4 vesicle translocation during insulin-stimulated glucose uptake in adipocytes.[258)] Arrington *et al.* showed that CAPN10, localized to mitochondria, mediates mitochondrial dysfunction by cleaving Complex I subunits and activating the mitochondrial permeability transition.[259)]

4.4. The SOL subfamily. The first genetic calpain study examined the *Drosophila* gene *small optic lobes* (*sol*).[60)] A mutation in *sol*, such as *sol*^{1078}, results in the absence of certain classes of columnar neurons in the optic lobes, leading to specific alterations in flight and walking maneuvers. The longest transcripts from this gene encodes a 1,597-aa protein belonging to the calpain SOL subfamily. *Drosophila* SOL is composed of the N-terminal six-Zn-finger motif-containing domain and the C-terminal calpain-like protease domain (Fig. 7B). Most of the protease domain is deleted in the *sol*^{1078} mutation, demonstrating that SOL as a protease is essential for forming proper optic lobes. Unfortunately, there have been no further reports dissecting the molecular mechanisms of neurodegeneration involving SOL.[34)] Mammals have one SOL ortholog, CAPN15/SOLH,[260),261)] but its physiological role is not clear. In parallel with PalB subfamily, this evolutionarily interesting molecules including their mammalian homologs beg further study.

4.5. Phytocalpain. A plant calpain, now called phytocalpain, was first identified in the sugarcane EST database by Correa *et al.*, and was named SC

cal1.[262] A sequence similarity search using SC cal1 as bait subsequently identified other phytocalpains: from the dicotyledons, *Vitis vinifera* (pinot noir grape), *A. thaliana*, and *Solanum melongena* (eggplant); from the monocotyledons, *Zea mays* (maize), *Oryza sativa* (rice plant), and *Hordeum vulgare* (barley); and from gymnospermae, *Pinus taeda* (loblolly pine).[163] A genetic study identified phytocalpain as the *defective kernel 1* (*dek1*) gene product, DEK1, required for aleurone cell development in the maize endosperm.[62] Since then, DEK1 homologs in *A. thaliana* and *Nicotiana benthamiana* (tobacco) have been found to have similar functions.[166),263]

A biochemical study using *E. coli*-expressed recombinant maize DEK1 CysPc-C2L domains showed that its Ca^{2+}-activated caseinolytic activity depends on the predicted active site Cys residue, although this activity was much lower than that of the conventional calpains under the study conditions used.[164] DEK1 is the only calpain homolog in the *Arabidopsis* genome,[263] and it is important for regulating growth in this plant. Although the full-length DEK1 protein localizes to membranes, intramolecular autolytic cleavage releases the calpain domain into the cytoplasm, and this domain is sufficient to fully complement *dek1* mutants.[263),264]

As shown in Fig. 14A, from the N-terminus of maize DEK1, a possible signal peptide is followed by the first TM region (A), eight-TM region (B1), large loop region (C), third TM domain with 14–15 spanners (B2), a domain of unknown function with no homology to other proteins (D), the calpain protease (CysPc) domain, and C2L domain.[62),164] The number of TM spanners of B2 region is predicted to be 15 or 14, respectively, by SOSUI or TMHMM Ver.2.0,[265),266] which makes the topology of C region and calpain domains on the same or opposite sides of cell membrane. This topology is very important for DEK1 function, and it should be examined biochemically.

Among the 15 human calpains, the protease domain of non-classical CAPN15/SOLH is the most similar to the DEK1 CysPc domain, with which it shows almost 40% aa sequence identity. Surprisingly, DEK1's C-terminal C2L domain is rather similar to that of classical mammalian calpains, such as CAPN1/μCL, CAPN3/p94, and CAPN8/nCL-2 (25–30% identity). Therefore, DEK1 is considered to consist of three evolutionarily different modules: a TM domain, a CysPc domain, and a C2L domain. In addition, it has been suggested that DEK1 homolog of the protista *T. thermophila* (Fig. 14A) resulted from a lateral gene-transfer event from a green alga-type endosymbiont of ciliates as an explanation for this inter-kingdom similarity.[46]

4.6. Other calpain members. *Leishmania* and *Trypanosoma* have around 20 calpain homologs each, which are likely to contribute to cell morphogenesis, drug resistance, and stress-response mechanisms.[267)–273] Some trypanosome calpains have N-terminal domains with weak similarity to calpastatin. As described above, some of the calpain homologs have substitutions in one or more of the well-conserved active-site triad residues, Cys-His-Asn. This non-proteolytic family of calpain homologs includes eutherian CAPN6 (Fig. 3), some of the schistosome (Fig. 5) and nematode (Fig. 6) calpains, insect CALPC (Fig. 7B) and all of the *Trypanosoma* homologs.[34),241),267] The generation of these calpain species is interesting from an evolutionary viewpoint, and elucidating their physiological functions will reveal additional roles played by the calpain superfamily, *e.g.*, possible non-proteolytic functions.

Concluding remarks

Gordon Guroff, Darrel E. Goll, Kazutomo Imahori, Koichi Suzuki, and Takashi Murachi established numerous milestones in the study of calpains, including its discovery,[1] finding its relationship to skeletal muscle,[5] purifying it to homogeneity,[7] labeling calpain and calpastatin,[12] cloning calpain[13] and calpastatin cDNA,[143] determining the 3D structure,[109] and many more. Their highly comprehensive and educational reviews[17),48),49),274),275] have been cited many times. These heroes will always live in the memory of many calpain researchers. They made great strides in the study of calpains, but much work remains to elucidate calpain's physiological roles. The divergence of its physiological functions and the lack of a good activation marker are major obstacles. Recent progress in genetics and structural biology has led to new and invaluable information about calpains. Considering calpain's divergent physiological functions, breakthroughs in the field of calpain studies will affect an enormously wide range of life science fields.

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Profile

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Profile

Yasuko Ono was born in 1971 and started her research career as a post-doctoral researcher of JSPS in 1999 at The University of Tokyo, after receiving her Ph.D. from The University of Tokyo. From 2000, she studied on muscle regulatory proteins for three years at University of Arizona, USA as a post-doctoral research fellow abroad of JSPS. In 2003, she became a CREST postdoctoral researcher of Japan Science and Technology Agency at The University of Tokyo. In 2004, she moved to Rinshoken joining to Calpain Project. In 2009, she was promoted to a chief researcher at Rinshoken. She was awarded Murachi Award at FASEB Summer Research Conference in 2001, Young Investigator's Award of the JSPP in 2008, Outstanding Presentation Award at RRW in 2010, and The Young Scientists' Prize of the Commendation for Science and Technology by the Minister of Education, Culture, Sports, Science and Technology in 2011. She has been trying to enjoy tackling to challenge questions casted by the calpains, especially those by numbers 3 and 7.