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Impact of genetic insights into calpain biology

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Calpain has long been an enigmatic enzyme, although it is involved in a variety of biological phenomena. Recent progress in calpain genetics has highlighted numerous physiological contexts in which the functions of calpain are of great significance. This review focuses on recent findings in the field of calpain genetics and the importance of calpain function. Calpain is an intracellular Ca^{2+}-dependent cysteine protease (EC 3.4.22.17; Clan CA, family C02) found in almost all eukaryotes. It is also present in a few bacteria, but not in archaebacteria. Calpain has limited proteolytic activity; rather, it transforms or modulates the structure and/or activity of its substrates. It is, therefore, referred to as a 'modulator protease'. Within the human genome, 15 genes (*CAPN1-3*, *CAPN5-16*) encode a calpain-like protease (CysPc) domain along with several different functional domains. Thus, calpains can be regarded as a distinct family of versatile enzymes that fulfil numerous tasks *in vivo*. Genetic studies show that a variety of defects in many different organisms, including lethality, muscular dystrophies and gastropathy, actually stem from calpain deficiencies. The cause-effect relationships identified by these studies form the basis for ongoing and future studies regarding the physiological role of calpains.

Keywords: calcium ion/calpain/intracellular proteolysis/muscular dystrophy/stomach ulcer.

Abbreviations: aa, amino acid(s); aar, amino acid residue(s); C2 domain, conserved domain 2 originally defined for protein kinase C; C2L domain, C2 domain-like domain; CANP, Ca^{2+}-activated neutral protease (calpain); *CAPN*, calpain gene; *CAPNS*, calpain regulatory small subunit gene; 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 at the National Center for Biotechnology Information (cd00044); GR domain, Gly-rich domain; HSP, heat-shock protein; KA, kainic acid; LGMD2A, limb-girdle muscular dystrophy type 2A (calpainopathy); MARP2, muscle-specific ankyrin-repeat protein-2 (MARP2); MIT motif, microtubule interacting and transport motif; NIDDM, noninsulin-dependent diabetes mellitus (type 2 diabetes); PC1 and PC2, 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; QTL, quantitative trait locus; Tg, transgenic.

An enzyme corresponding to calpain was first described in 1964 by Guroff (*1*). In 1978, calpain was purified to homogeneity for the first time and named CANP (Ca^{2+}-activated neutral protease) (*2*). Calpain was soon established as one of the most intriguing and enigmatic enzymes. In 1984, cDNA cloning led to the publication of the complete primary structure of the calpain catalytic subunit (*3*), and structure–function analyses became central to calpain studies. The number of cDNA and genomic cloning studies then exploded, and hundreds of new calpains and related molecules were identified. Among these were the 15 human calpain genes, now referred to as *CAPNn* (short for calpain; $n = 1$, 2, 3 and 5–16). There are two genes encoding smaller regulatory subunits, *CAPNS1* and *CAPNS2* (short for calpain regulatory small subunit) and one encoding calpastatin, *CAST*, the endogenous inhibitor protein specific for calpain.

Calpains constitute a distinct group of intracellular cysteine proteases that are found in almost all eukaryotes and a few bacteria. They were originally defined as cytosolic proteases, exhibiting Ca^{2+}-dependent proteolytic activity at a neutral pH. Calpain activity is strictly regulated, as is the case for other intracellular proteases such as proteasomes and caspases. However, unlike proteasomes and lysosomal proteases, calpains act for proteolytic processing, rather than for degradation, i.e. calpains proteolyse their substrates at one, or a few, sites. After calpain-mediated proteolysis, the substrate proteins are transformed into new functional states, thereby affecting various cellular functions, including signal transduction and cell morphogenesis. Therefore, calpain is referred to as an intracellular 'modulator protease'. Again, unlike proteasome and lysosomal proteases, which function in a co-ordinated step-wise fashion alongside proteins that mediate ubiquitination and the formation of intracellular compartments such as autophagosomes and endosomes, calpain is distinctive in that its functions depend only on one or two molecules.

The importance of the physiological role of calpains is demonstrated by a variety of defects caused by

compromised calpain function in different tissues and organisms. These include embryonic lethality, muscular dystrophies, gastropathy, lissencephaly, tumourigenesis, impaired neurogenesis and deficient sex determination, defects in aleurone cell development and alkaline stress susceptibility, all of which are described in detail in the following sections.

This review uses the general gene-product nomenclature for calpains (Table I), i.e. mammalian calpain gene products are referred to as CAPN1 for *CAPN1* gene product (μ-calpain catalytic large subunit, abbreviated as μCL), CAPN2 for *CAPN2* gene product (m-calpain catalytic large subunit, mCL), etc., and calpains previously known by other names have the 'old' name placed after the above mentioned name in square brackets, e.g. CAPN1[μCL] and CAPN2[mCL].

The calpain superfamily and its classification

Distribution of calpains between species

The most extensively studied calpains are the major ubiquitous mammalian μ- and m-calpains (in this review, for clarity, subunit composition of calpain enzymes is added after each enzyme name, e.g. μ-calpain [CAPN1/S1] (CAPN1/S1 is short for CAPN1/CAPNS1) and m-calpain [CAPN2/S1]). These are defined as 'conventional' calpains; all others are termed 'unconventional' calpains. The chicken μ/m-calpain [CAPN11/S1] was well studied during the early years of calpain research, and can be also defined as a conventional calpain (*2*). Intriguingly, CAPN11[μ/mCL] is ubiquitously expressed in chickens, but is specific to the testis in eutherians, and absent altogether from marsupialia (*4*).

The consensus sequence encoding the calpain protease domain is well established, and is referred to as 'CysPc' (cd00044) in the conserved domain database of the National Center for Biotechnology Information (NCBI), which contains the sequences of almost all the calpain homologues identified from different living organisms. Calpains belong to the papain superfamily of cysteine proteases, but their similarity to papains and cysteine cathepsins is weak and less significant than the similarities between different calpains. Therefore, it is reasonable to define calpains as 'proteins comprising aa sequences significantly similar to the protease domain of the human conventional calpain catalytic subunit', which is equivalent to those included in CysPc family above described.

The above definition encompasses 15 human genes that encode calpains (Table I). *Anopheles gambiae* (mosquito), *Drosophila melanogaster* (fruit fly), *Schistosoma mansoni* (blood fluke), *Caenorhabditis elegans* (nematode), *Arabidopsis thaliana* (thale-cress), *Emericella* (*Aspergillus*) *nidulans* (ascomycete), and *Saccharomyces cerevisiae* (budding yeast) have seven, four, seven, fourteen, one, two, and one calpain genes, respectively (*5*). Calpain genes are not present in *Encephalitozoon* or *Schizosaccharomyces pombe*. So far, in prokaryotes, 53 calpains have been identified in 42 eubacteria of the 1,006 fully-sequenced bacterial genomes in the database (*5*). These bacterial species have up to four calpain genes in their genome; however, 96% of bacteria, including *Escherichia coli* and all of archaebacteria, have no calpain genes.

Structure of conventional calpains

The mammalian conventional calpains, μ- and m-calpains are heterodimers comprising a common regulatory small subunit (CAPNS1[30K]; *ca.* 30 kDa) and a distinct catalytic large subunit (CAPN1[μCL] or CAPN2[mCL], respectively; *ca.* 80 kDa). Human CAPN1[μCL] and CAPN2[mCL] are 62.4% identical at the aa level. Accordingly, they have almost indistinguishable substrate and inhibitor specificities, and both are almost ubiquitously expressed. However, they differ in terms of the *in vitro* Ca^{2+} concentration (μM versus mM) required for proteolytic activity. It is assumed that their functions are fundamental and essential for the regulation of various biological phenomena, although their precise roles remain unknown.

The catalytic and regulatory subunits of conventional calpains can be divided into four and two domains/regions, respectively (Fig. 1). Historically, there are several different nomenclatures for the domains and their boundaries (Fig. 1A), which have caused confusion among calpain researchers. Therefore, this review proposes and uses the new coordinated nomenclature as shown in Fig. 1A. When activated by Ca^{2+}, conventional calpain displaces the N-terminal anchor α-helix of the catalytic subunit (previously called anchor or domain I) through autolysis, resulting in functionality at lower Ca^{2+} concentrations, different substrate specificity and, in some cases, further subunit dissociation. Thus, autolysis of this anchor helix is one of the intrinsic regulatory mechanisms critical for calpain activity.

In isolation, the protease (CysPc) domain of m-calpain has Ca^{2+}-dependent protease activity (*6*, *7*). The elaborate activation mechanism of calpain was revealed using 3D structural analysis. In the absence of Ca^{2+}, the CysPc domain comprises two separate protease core domains, PC1 (previously called protease D-I, or protease subdomain IIa) and PC2 (D-II or IIb) (Fig. 1A). These core domains form a functional protease domain when each Ca^{2+} binding site in PC1 and PC2 (CBS-1 and -2, respectively) binds Ca^{2+} (*8–10*) (Fig. 2). In other words, calpain remains structurally inactive in the absence of Ca^{2+}. This is reasonable because calpain resides in the cytosol, where it is in direct contact with a large number of proteins and its activity must be strictly regulated.

Ca^{2+} also binds to the C2 domain-like (C2L) domain (previously called domain III) and the penta-EF-hand (*11*) [PEF(L) and PEF(S)] domains (domains IV and VI of the catalytic large and regulatory small subunits, respectively), each of which is composed of five EF-hand motifs. Although not apparent from the primary sequence, the 3D-structures of the C2L and C2 domains show similar β-sandwich alignment (Fig. 2). The fifth EF-hand (EF-5) motif within each of the PEF domains contributes to heterodimer formation. Interestingly, the N-terminal anchor helix of m-calpain contacts the EF-2 motif of PEF(S). This interaction is broken either by Ca^{2+} binding to

Table I. Human genes for calpains and their regulatory subunits.

Gene	Chromosome location	Phenotype of gene deficiency in mice	Gene product name[a]	Aliases	Classical (typical) calpain	Expression: Ubiquitous	Expression: Tissue-specific calpain	Expression: Note	Homologs in non-vertebrates	Active site res.[b]: Cys	Active site res.[b]: His	Active site res.[b]: Asn	domains[c]: C2L	domains[c]: C2	domains[c]: PEF
CAPN1	11q13	Platelet dysfunction	CAPN1	μ-calpain large subunit (μCL), calpain-1, μCANP/calpain-I large subunit, μ80K	✓	✓				+	+	+	+	−	+
CAPN2	1q41-q42	Embryonic lethal	CAPN2	m-calpain large subunit (mCL), calpain-2, mCANP/calpain-II large subunit, m80K	✓	✓		Except erythrocytes		+	+	+	+	−	+
CAPN3	15q15.1- q21.1	Muscular dystrophy	CAPN3	p94, calpain-3, calpain-3a, nCL-1	✓		✓	Skeletal muscle		+	+	+	+	−	+
			CAPN3:ex1B|2-5|7-14|17-24, CAPN3:ex1B|2-5|7-14|17|18B|19-24, etc.)	Lp82, Lp85, etc.	✓		✓	Lens, retina (not in humans)		+	+	+	+	−	+
			CAPN3:ex1-14|16-24	p94:Δex15	✓		✓	Skeletal muscle		+	+	+	+	−	+
			CAPN3:ex1C|2-14|16-24	Up84	✓	✓				+	+	+	+	−	+
			CAPN3:ex1D|13-24	Tp36			✓	Testis		−	−	−	+/−	−	+
			CAPN3:ex1E|19-24	Mp18			✓	Melanoma		−	−	−	−	−	+/−
CAPN5	11q14	Sudden death[d]	CAPN5	calpain-5, hTRA-3, nCL-3		✓		Abundant in testis and brain	Nematode TRA-3	+	+	+	+	+	−
CAPN6	Xq23	n.r.[e]	CAPN6	calpain-6, calpamodulin, CANPX			✓	Embryonic muscles, placenta	Nematode TRA-3	−	+	+	+	+	−
CAPN7	3p24	n.r.	CAPN7	calpain-7, PalBH		✓			*Aspergillus* PalB, yeast Rim13 [CplI]	+	+	+	++	−	−
			CAPN7:ex1-8|9B							+	+	+	++	−	−
CAPN8	1q41	Stress-induced gastric ulcer	CAPN8	nCL-2, calpain-8, calpain-8a	✓		✓	Gastrointestinal tracts		+	+	+	+	−	+
			CAPN8:ex1-9|10B	nCL-2′, calpain-8b						+	+	+	−	−	−
			CAPN8:ex1-9|11-21	nCL-2:Δex10,17∼fs, calpain-8c						+	+	+	+	−	+/−
CAPN9	1q42.11- q42.3	Stress-induced gastric ulcer	CAPN9	nCL-4, calpain-9, calpain-9a	✓		✓	Gastrointestinal tracts		+	+	+	+	−	+
			CAPN9:ex1-7|9-21	nCL-4:Δex8, calpain-9b	✓					+	+	+	+	−	+
CAPN10	2q37.3	No significant phenotype	CAPN10	calpain-10, calpain-10a		✓				+	+	+	++	−	−
			CAPN10:ex1-8|9B|10	calpain-10b						+	+	+	+	−	−
			CAPN10:ex1-7|10-12	calpain-10c						+	+	+	+	−	−
			CAPN10:ex1-8|10	calpain-10d						+	+	+	+	−	−
			CAPN10:ex1-7|7B	calpain-10e						+	+	+	+/−	−	−
			CAPN10:ex1-2|3B|4-6	calpain-10f						+	−	−	−	−	−
			CAPN10:ex1-2|13-14	calpain-10g						−	−	−	−	−	−
			CAPN10:ex1|10-12	calpain-10h						−	−	−	+/−	−	−
CAPN11	6p12	n.r.	CAPN11	calpain-11, μ/mCL (chicken)	✓		✓	Testis		+	+	+	+	−	+
CAPN12	19q13.2	n.r.	CAPN12	calpain-12, calpain-12a, calpain-12A	✓		✓	Hair follicle		+	+	+	+	−	+
			CAPN12:ex1-11|12B	calpain-12b, calpain-12B						+	+	+	+/−	−	−
			CAPN12:ex1-11|13	calpain-12c, calpain-12C						+	+	+	+/−	−	−
			CAPN12:ex1-9|20-21	calpain-12d (mouse)						+	+	+	−	−	+/−
CAPN13	2p22-p21	n.r.	CAPN13	calpain-13	✓	✓				+	+	+	+	−	+
CAPN14	2p23.1-p21	n.r.	CAPN14	calpain-14	✓	✓				+	+	+	+	−	+
CAPN15 [SOLH]	16p13.3	n.r.	CAPN15	calpain-15, SOLH		✓			*Drosophila* SOL	+	+	+	−	−	−
CAPN16 [C6orf103]	6q24.3	n.r.	CAPN16	Demi-calpain, calpain-16, C6orf103		✓				+	−	−	−	−	−
CAPNS1	19q13.1	Embryonic lethal	CAPNS1	CANP/calpain small subunit, 30K, css1, calpain-s1, calpain-4, CAPN4	[f]	✓		Calpain regulatory subunit	PEF proteins				−	−	+
CAPNS2	16q12.2	n.r.	CAPNS2	calpain small subunit 2, 30K-2, css2, calpain-s2	[f]	✓		Possible calpain regulatory subunit	PEF proteins				−	−	+

[a]Splicing variant products are expressed as exon (ex) numbers used for translation. Exon numbers 1, 2, 3 etc. correspond to the representative transcripts that generate the longest polypeptide [thus, exons 8, and 9–15 of CAPN10 in the original report (*16*) are renumbered as exons 7B, and 8–14 here, respectively]. If alternative exons are used, they are shown as ex1B, 1C, 12B etc. (number 1 indicate initiation exon).
[b]+ indicates that the molecule has well-conserved aar consisting of the active site triad, and − means that it has other aar in that position.
[c]+ or − indicates that the molecule has, or does not have, a corresponding domain and +/- indicates that it contains a part, but not a full, of the corresponding domains. For acronyms, see Fig. 1.
[d]This phenotype disappeared after sufficient backcross.
[e]Not yet reported.
[f]CAPNS1 and CAPNS2 are not 'calpain' according to the definition adopted in this review.

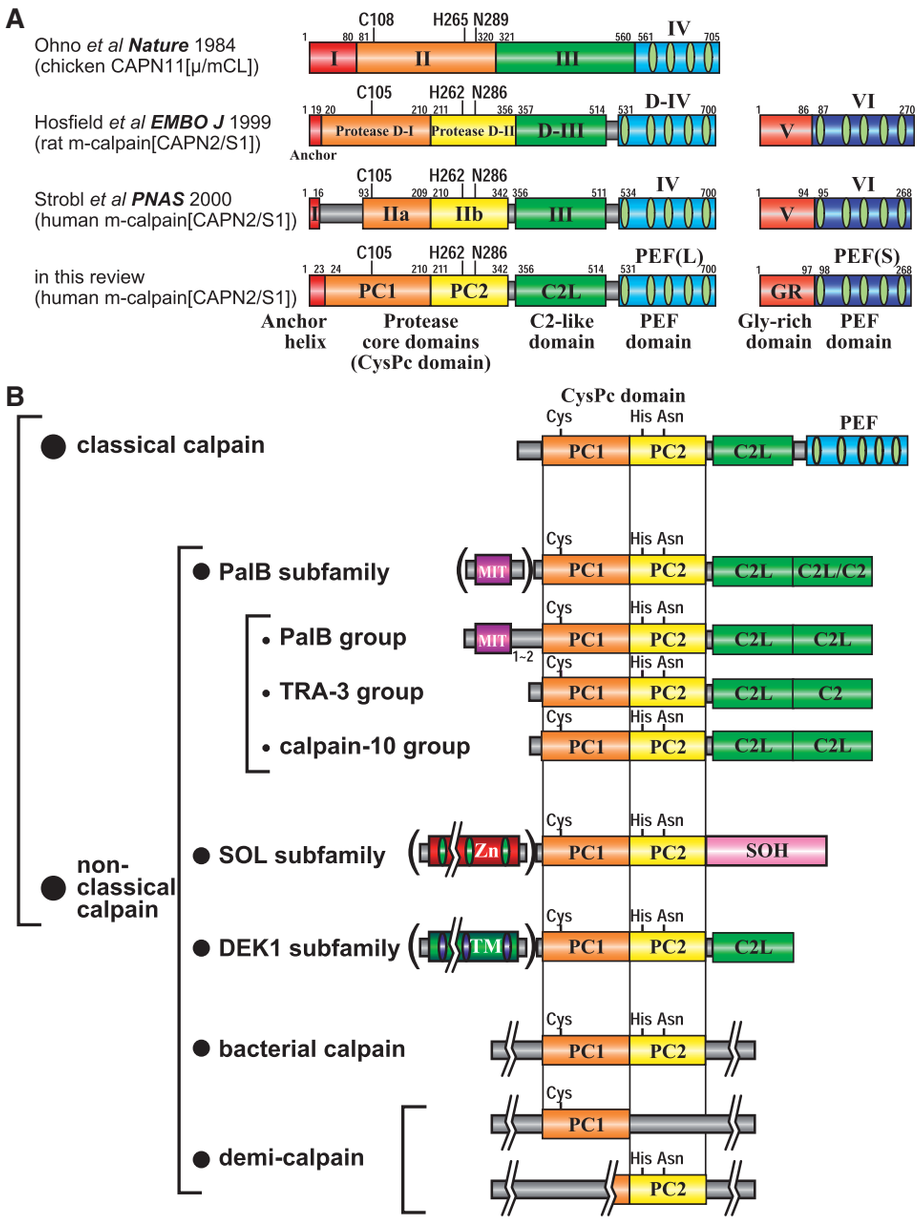

Fig. 1 Strutural classification and consensus domain structures of calpain super family members. (A) Domain nomenclature. Ohno *et al.* (*3*) first described the domain structure of chicken CAPN11[μ/mCL] from the primary structure. Hosfield *et al.* (*66*) and Strobl *et al.* (*67*) solved the 3D structure of m-calpain [CAPN2/S1], and defined the domains from the viewpoint of a 3D structure. These nomenclatures, however, are not necessarily consistent with one another, and this review proposes that domains be sectioned and named as indicated: the domain boundaries were mainly defined from the viewpoint of a 3D structure and evolutional conservation (Drs Robert L. Campbell and Peter L. Davies, personal communication), and, for the domain names, I, II, III etc. were eliminated for clarity and acronyms representing function and/or structure of each domain were used instead (see below). (B) Calpain homologues have been identified in almost all eukaryotes and in some bacteria. Their structures are classified as described in the main text. Consensus domain structures for classification are shown. Symbols: PC1 and PC2, protease core domains 1 and 2 in the calpain protease (CysPc) domain; C2L, C2-domain-like domain; PEF(L/S), penta-EF-hand domains in the large(L)/small(S) subunit; GR, glycine-rich hydrophobic domain; MIT, microtubule interacting and transport motif; C2, C2 domain; Zn, Zn-finger motif; SOH, SOL-homology domain; TM, transmembrane domain.

the EF-2 motif or by autolysis of the N-terminus upon activation (*12*).

The N-terminal Gly-rich (GR) domain (also called domain V) of CAPNS1[30K] contains hydrophobic Gly-clusters, most of which are autolysed during activation. This domain is almost invisible in the 3D structure, indicating a very 'soft' structure. In humans, an intron-less gene, *CAPNS2*, encodes a paralogue of CAPNS1[30K], the physiological role of which remains unclear.

Structural classification of calpains

The calpain superfamily is divided into several subfamilies according to domain structure. These divisions are supported from an evolutionary viewpoint (Figs 1B, 3 and Supplementary Fig. S1) (*5*). Calpains

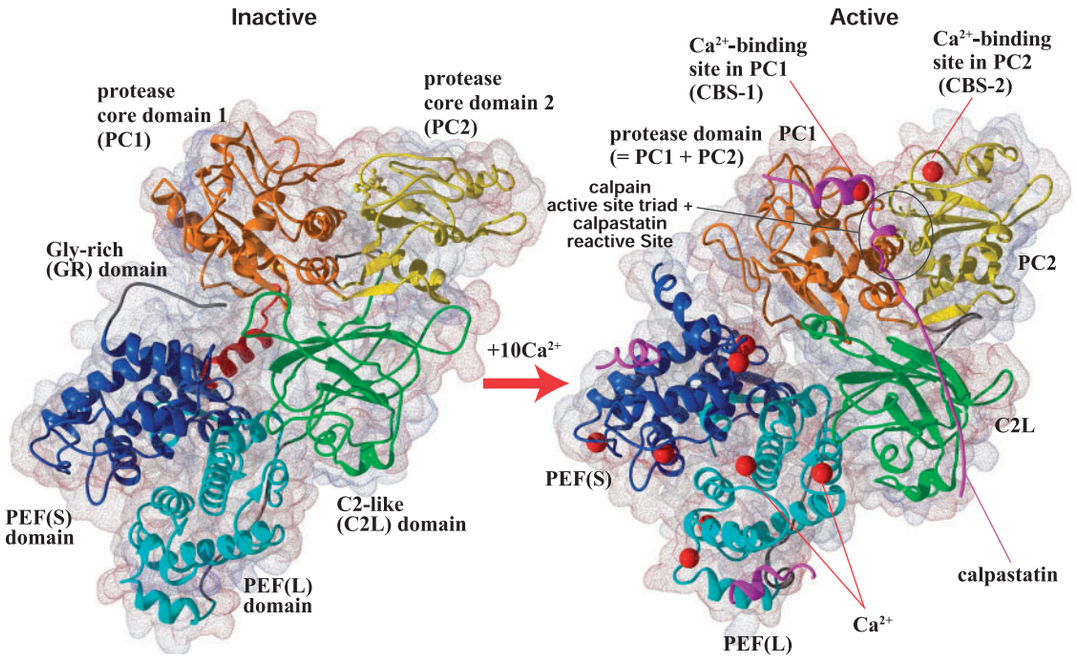

Fig. 2 Schematic showing the 3D structure of inactive and active m-calpain. Schematic 3D ribbon structures superimposed by the surface-type structures of inactive (Ca^{2+}-free) and active (Ca^{2+}- and calpastatin-bound) forms of m-calpain using PDB data, 1KFX and 3DF0 (*9*). The active protease (CysPc) domain is formed by the fusion of the PC1 and PC2 core domains after binding of a single Ca^{2+} by each of the Ca^{2+}-binding site in both core domains (CBS-1 and -2). The active site is circled in black. Red balls represent Ca^{2+}. Only a small part of GR domain is visible because of its 'soft' structure.

with a domain structure similar to that of CAPN1[μCL] and CAPN2[mCL] are termed 'classical' calpains (*13*), i.e. they contain C2L and PEF domains in addition to the CysPc domain (Fig. 1B) [although they were also called 'typical' calpains, 'typical' is not appropriate because of their non-typical distribution among all living organisms, i.e. 'classical' calpains are only found in vertebrates, insects, and schistosome (Table II)]. Accordingly, 'non-classical' calpains (also called 'atypical' calpains) do not contain both of C2L and PEF domains. Of the 15 representative products of human calpain genes, nine are classical and six are non-classical (Fig. 3).

Most human classical calpains are conserved in other vertebrates (Table II); fish have a duplicate set of most of these genes (*4*). Only a few classical calpains have been identified in invertebrates: *S. mansoni*, *D. melanogaster*, and *A. gambiae* have four, three, and three, respectively (*5*). No classical calpain homologues have been found in *C. elegans*, trypanosomes, plants, fungi, or *S. cerevisiae* (*5*).

Non-classical calpain CysPc domains are 30–75% identical to one another at the aa level and contain distinct domains (Figs 1 and 3). Some of these domains, such as the transmembrane (TM) domain, are not found in human calpains. 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, led to the hypothesis that calpain molecules were generated evolutionarily by combining an ancestral calpain-type cysteine protease gene with genes encoding other functions.

Subfamilies of non-classical calpains

Non-classical calpains can further be classified into several subfamilies according to their domain structures. Human CAPN7[PalBH] is the most evolutionarily conserved human calpain and its homologues are found in vertebrates, yeasts, fungi, protists, nematodes and insects (except *Drosophila*), but not in plants (Table II) (*14*, *15*). CAPN7[PalBH] homologues commonly contain two C2L domains in tandem, each of which diverge (greatly or moderately) from those of conventional calpains, and conserved microtubule interacting and transport (MIT) motifs at the N-terminus (Fig. 1B).

Human CAPN10, the longest product of the *CAPN10* gene (*16*), contains slightly divergent C2L domains in succession at the C-terminus. CAPN10 homologues are only found in vertebrates, and do not contain an MIT domain (*13*). TRA-3 is found in nematodes and vertebrates, but not in insects or lower organisms (Table II) (*5*). TRA-3 homologues, including human CAPN5[hTRA-3] and CAPN6, contain C2L and C2 domains in succession at the C-terminus (Figs 1B and 3B). Thus, CAPN10 and TRA-3 homologues can be grouped together with the CAPN7[PalBH] homologues within the PalB subfamily, all of which contain the structural consensus of two tandem C2L and/or C2 domains at the C-terminus (Fig. 1B).

Another evolutionarily-conserved subfamily is SOL, which occurs in vertebrates, insects, nematodes, and green algae, but not in fungi and yeasts. The structure of SOL homologues is characterized by a varying number of Zn-finger motifs within the N-terminal

Table II. Calpains in various living organisms and their correspondence to human calpains.

Classification	Subfamily	Subgroup	Consensus structure (Fig. 1)	Human	Mouse	Chicken	Frog	Fish	Mosquito	Fly	Schistosome	Nematode	Fungi	Yeast	Plant	Bacteria
Classical (typical) calpain		(w/o ISs)	CysPc-C2L-PEF	CAPN1[μCL]	✓	✓	✓	✓	✓[a]	✓	✓					
				CAPN2[mCL]	✓	✓	✓	✓								
				CAPN3[p94]	✓	✓	✓	✓								
				CAPN8[nCL-2]	✓	✓	✓	✓								
				CAPN9[nCL-4]	✓	✓	✓	✓								
				CAPN11	✓	✓										
				CAPN12	✓											
				CAPN13	✓	✓										
				CAPN14		✓		✓								
Non-classical (atypical) calpain	PalB	PalB	MIT-CysPc-C2L-C2L	CAPN7[PalBH]	✓	✓	✓	✓	✓		✓	✓	✓	✓[d]		
		TRA-3	CysPc-C2L-C2	CAPN5[hTRA-3]	✓	✓	✓	✓	✓		✓	✓				
				CAPN6	✓	✓	✓									
		CAPN10	CysPc-C2L-C2L	CAPN10	✓	✓	✓	✓	✓			✓	✓			
	SOL		Zn-CysPc-SOH	CAPN15[SOLH]	✓	✓	✓	✓	✓	✓		✓				
	demi		PC1 or PC2	CAPN16[demi-calpain]	✓	✓	✓	✓	✓		✓	✓				
	DEK1		(TM)-CysPc-C2L	CAPN10:ex1-7|9|10B[calpain-10b][b,c]		✓				✓[b,c]		✓[b]			✓	
	bacterial		CysPc	CAPN8:ex1-9|10B[nCL-2][c]	✓[c]											✓

[a]Insects and schistosomes have calpains in other subfamilies, which are not significantly similar to specific human classical calpains but show equal similarity.
[b]These do not contain transmembrane (TM) domain.
[c]These are alternatively splicing products of the genes that encode calpains in other subfamilies, and other species without ✓ may have such gene products.
[d]Yeast Rim13/Cpl1 does not contain a sequence that can be explicitly identified as MIT motif.

domain. They also share an SOL-homology (SOH) domain (Figs 1B and 3).

Plant calpain, called phytocalpain, was first identified in *Saccharum officinarum* (sugarcane) in 2001 (*17*). The maize calpain homologue, DEK1 (defective kernel 1), was shown to be involved in aleurone cell development in 2002 (*18*). DEK1 homologues are found in various plants, including rice plants and *Arabidopsis*. They contain TM and C2L domains at their N- and C-termini, respectively. A DEK1 homologue is also found in *Tetrahymena thermophila* (*5*). Human CAPN10:ex1-8|9B|10 [calpain-10 b] and *Drosophila* CALPA', alternative splicing gene products of *CAPN10* and *CalpA*, respectively, and some nematode calpains have a similar domain structure to that of DEK1 (CysPc-C2L$_{\text{-COOH}}$), but lack the TM domain (*5*). These calpains are grouped together to form the DEK1 subfamily (Fig. 1B).

Bacterial calpain was first identified in *Porphyromonas gingivalis* in 1992 and is known as tpr (thiol protease) (*19*). Subsequent bacterial genome projects identified several calpain homologues. These are the most divergent calpain species, sharing similarity only within the CysPc domain (*5*).

Tissue specificity of calpains

In addition to their structural features, calpains are also independently categorized according to their tissue and organ distribution. Some human calpains are ubiquitously expressed, whereas others are expressed only in specific tissues or organs (Table I). The widely accepted assumption is that ubiquitous calpains play a fundamental role in all cells, whereas tissue-specific calpains, as the name suggests, have tissue-specific roles. Defects in ubiquitous calpains may be lethal (one such example is *Capn2*$^{-/-}$ and *Capns1*$^{-/-}$ mice; see below) (*20–24*), whereas defects in tissue-specific calpains may cause tissue-specific symptoms, one example of which is muscular dystrophy caused by a defective *CAPN3* or *Capn3* gene (*25–27*).

At the same time, conventional calpains tend to be over-activated in muscular dystrophies, cardiomyopathies, traumatic ischemia, and lissencephaly, probably due to compromised intracellular Ca^{2+} homeostasis caused by these diseases. Since over-activity often exacerbates the disease state, conventional calpain inhibitors are currently used to prevent the progression of such diseases (*28*).

Genetics of conventional calpains

Genetic studies of conventional calpains

Calpain studies using genetically modified mice first published in 2000 revealed that disrupting the mouse *Capns1* gene (encoding calpain regulatory small subunit, CAPNS1[30K]) causes embryonic lethality before E11.5 (*23*, *24*). This showed that conventional calpains were necessary for mammalian life, encouraging further research into this enigmatic enzyme.

A deficiency in CAPNS1[30K] causes down-regulation of the CAPN1[μCL] and CAPN2[mCL] proteins, indicating that CAPNS1[30K] is required

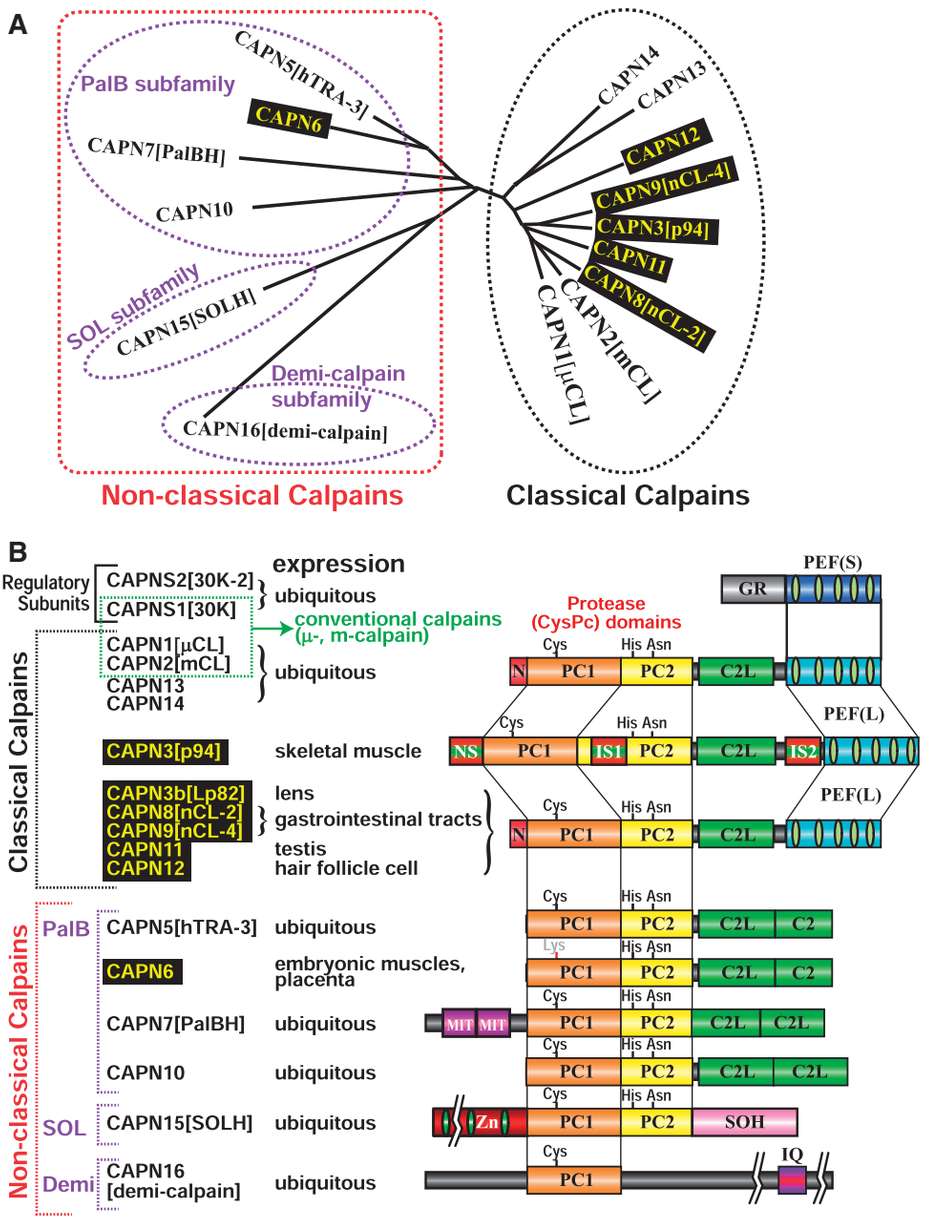

Fig. 3 Phylogenetic tree and schematic structures of human calpains. (A) Phylogenetic tree of human calpains. The tree was drawn using the neighbour-joining/bootstrap method after aligning all the sequences using MAFFT v6.240 (at http://align.genome.jp/mafft/, strategy: E-INS-i; see Supplementary Fig. S1 for alignment). Non-classical calpains further consist of three subfamilies, and one of which shows further divergence within the subfamily, i.e. the PalB subfamily is composed of the strict PalB, TRA-3, and CAPN10 groups. (B) Schematic structures. Names for calpains are by the general nomenclature of gene product (e.g. *CAPN1* → CAPN1) used in this review, and their previous names, if any, are shown in square brackets after the general names. Black and highlighted letters indicate ubiquitous and tissue/organ-specific calpains, respectively (Table I). Symbols: IQ, a motif interactive with calmodulin; NS/IS1/IS2, CAPN3[p94]-characteristic sequences. See the legend to Fig. 1 for an explanation of the other symbols.

for the stability 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 a long incubation; however, in cells, unfolded large calpain subunits are probably degraded by other proteases before they can form active conformations. Also, since *Capns1*$^{-/-}$ embryonic stem (ES) cell growth and adhesion are not noticeably different from those of the wild-type (WT) cells, it appears that activity of conventional calpains is not necessary at the cellular level under standard culture conditions.

Disruption of the mouse *Capn1* or *Capn2* genes produces contrasting results: *Capn1*$^{-/-}$ mice appear normal and are fertile (*29*), whereas *Capn2*$^{-/-}$ mice die before the blastocyst stage (*20*). This suggests that μ- and m-calpain differ in terms of their function and/or expression levels, at least at specific developmental stage(s). Cells from *Capns1*$^{-/-}$ mice are ideal tools for unequivocally demonstrating the role of

conventional calpains in specific cellular events. CAPNS1[30K] is required for the induction of senescence (*30*), Ca^{2+}-dependent repair of wounded plasma membranes (*31–33*), and macroautophagy (*34*).

Genetic studies of calpastatin

Disruption of mouse *Cast* does not result in a significant phenotype under normal unstressed conditions (*35*). This suggests that conventional calpains do not normally function dynamically, and that calpastatin is not required for calpain regulation. However, neuronal cells in $Cast^{-/-}$ mice are significantly more susceptible to intra-hippocampal injections of kainic acid (KA), which causes apoptosis by excitotoxicity, than those in WT mice. The effect of KA is reduced in transgenic (Tg) mice that overexpress calpastatin in neuronal cells, but not in Tg mice overexpressing caspase inhibitor p35 (*36*). These results suggest that KA-induced neuronal cell death is mediated by calpain, but not by caspases.

Tg mice overexpressing calpastatin in muscle tissue also appear healthy with no detectable defects. When these mice were crossed with *mdx* mice, which have a nonsense *Dmd* mutation that causes a mild muscular dystrophic phenotype due to a lack of dystrophin, the dystrophic phenotype was significantly ameliorated in the resultant calpastatin-overexpressing *mdx* mice (*37*).

Studies using genetically-modified mice have also had an impact on food science, e.g. the post-mortem tenderization of muscles, which is an important step in producing quality meat. Both calpastatin-overexpressing and $Capn1^{-/-}$ mice show reduced post-mortem proteolysis of muscle proteins (*38*, *39*). Thus, calpains have now become a target for controlling meat quality.

Genetic approaches to unveiling the functions of unconventional calpains

CAPN3[p94]

The first tissue-specific calpain, CAPN3[p94], was identified in 1989 and is predominantly expressed in skeletal muscle (*40*). Although this classical calpain is ~50% identical to CAPN1[μCL] and CAPN2[mCL], it contains three additional characteristic regions, NS, IS1 and IS2, which are located at the N-terminus, in the PC2 domain, and between the C2L and PEF domains, respectively (Fig. 3B). These regions endow CAPN3[p94] with some unique features.

The most characteristic property of CAPN3[p94] is its extremely rapid autodegradation (half-life *in vitro* <10 min) which depends on both IS1 and IS2. Conventional calpain inhibitors such as calpastatin and E64 have little effect on CAPN3[p94] activity. Surprisingly, autodegradation is Na^{+}-dependent in the absence of Ca^{2+}, establishing CAPN3[p94] as the first example of an intracellular Na^{+}-dependent enzyme (*41*). The physiological relevance of the Na^{+}-dependency of CAPN3[p94] is still unclear, but its *in vitro* substrate specificity differs depending on whether it is activated by Ca^{2+} or Na^{+} (*41*). CAPN3[p94] binds specifically to the N2A and M-line regions of the gigantic filamentous muscle protein connectin/titin and one of the binding sites is the N-terminal region of IS2. The autodegradative activity of CAPN3[p94] is almost completely suppressed *in vivo*, most probably because it binds to N2A connectin/titin. The N2A region is also involved in the alignment of important subcellular structures within muscle tissue, such as the sarcoplasmic reticulum (SR) and T-tubules.

In 1995, *CAPN3* mutations were shown to be responsible for limb-girdle muscular dystrophy type 2A (LGMD2A), also called calpainopathy (*25*). $Capn3^{-/-}$ mice emulate a human calpainopathy-like phenotype (although less severe), indicating that calpainopathy is caused by defects in *CAPN3* (*26*, *42*). Calpainopathy and *CAPN3* mutations are the first and only examples thus far of a clear cause-effect relationship between human disease and calpain gene mutations.

Calpainopathy appears to be primarily caused by compromised CAPN3[p94] protease activity, rather than by damaged structural properties. This was confirmed using CAPN3[p94] knock-in ($Capn3^{CS/CS}$) mice, which express a structurally intact, but inactive, CAPN3[p94]:C129S mutant resulting in a muscular dystrophy phenotype (*27*). Intriguingly, however, $Capn3^{CS/CS}$ mice show a less severe phenotype than $Capn3^{-/-}$ mice, indicating that proteolytically inactive CAPN3[p94] retains some function, possibly related to structural properties in the SR resulting from its association with ryanodine receptors (*27*, *43*).

Studies using $Capn3^{CS/CS}$ mice have provided some insights into the molecular mechanisms underlying the pathogenesis of calpainopathy. First, CAPN3[p94] shows a stretch-dependent distribution. The amount of CAPN3[p94] at the M-line relative to that in the N2A region of the myofibrils decreases as the sarcomere lengthens (Fig. 4A). Surprisingly, this change in localization is delayed when CAPN3[p94] is inactive, as in $Capn3^{CS/CS}$ mouse muscle; thus, identifying one of the molecular mechanisms by which calpainopathy develops. Second, $Capn3^{CS/CS}$ mice show impaired adaptation to physical stress, accompanied by compromised exercise-induced up-regulation of muscle ankyrin-repeat protein-2 (MARP2) and heat-shock proteins (HSPs). These findings suggest that the stretch-induced dynamic redistribution of CAPN3[p94], which is dependent on its protease activity, functions in surveillance of myofibrilar conditions, and that this system is essential for protecting muscle tissue from degeneration, particularly under physical stress conditions (Fig. 4B) (*43*).

Although a substantial number of pathogenic *CAPN3* mutations have been identified, no mutational hot spot has been found (*5*). In total, 2,530 mutations (456 of them unique) have been reported in human *CAPN3* (Table III). One characteristic of *CAPN3* mutations is that more than half of them are point mutations, and the majority of these are missense mutations (Table III). Missense mutations found in LGMD2A patients are also distributed throughout the protein, including 215 unique mutations at 176 independent loci among the 821 aa residues (aar) (*5*). A comparison between CAPN3[p94] proteins in different vertebrates

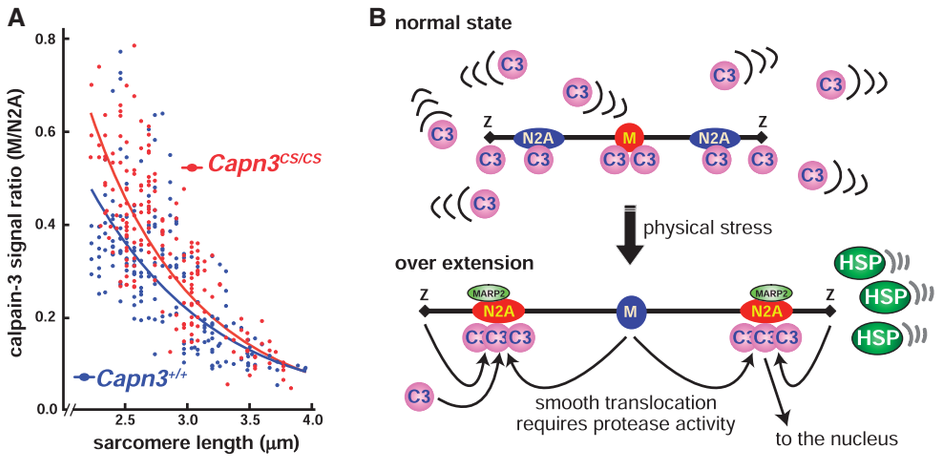

Fig. 4 Stress responses in skeletal muscle cells mediated by CAPN3[p94]. (A) Ratio of the CAPN3[p94] signal at the M-line relative to that in the N2A region plotted against sarcomere length. CAPN3[p94] localization in WT and *Capn3*$^{CS/CS}$ mouse muscle was analysed by confocal microscopy using specific antibodies. The results show the distribution of the M-lines, N2A regions, and/or Z-bands in both types of mice. The M/N2A ratio becomes smaller as the sarcomeres extend. Two fitted exponential curves (the blue and red lines represent WT and *Capn3*$^{CS/CS}$ mice, respectively) were statistically different between WT and *Capn3*$^{CS/CS}$ mice (*43*). (B) The physiological functions of CAPN3[p94]. Under normal conditions, CAPN3[p94] localizes in various parts of skeletal muscle cells, where it monitors their status. When muscle cells are under physical stress, such as over-excise, CAPN3[p94] (C3) accumulates at the N2A region of myofibrils and transduces stress signals to the nucleus *via* interactions with MARP2. At the same time, HSPs are induced for protection, a process that involves CAPN3[p94] activity (*43*).

Table III. Classification, frequency and ratio of calpainopathy mutations in *CAPN3*.

Classification	Mutation type	Frequent example	Frequency	Unique	Unique aar	Ratio (%)	Ratio within the same mutation type (%)
Point mutation			***1,586***[a]			***62.7***	
	Missense		**957**	**215**	**176**	**37.8**	
		R769Q(G2306A)	56			2.2	5.9[b]
		R490Q(G1469A)	48			1.9	5.0
		R490W(C1468T)	43			1.7	4.5
		A236T(G706A)	41			1.6	4.3
		R748Q(G2243A)	35			1.4	3.7
		P82L(C245T)	28			1.1	2.9
	No change (synonymous, in intron, or in regulatory regions)		**430**	**105**		**17.0**	
		(T96C)	44			1.7	10.2
		(G946-1A)	41			1.6	9.5
		(G1992+1T)	21			0.8	4.9
	nonsense		**153**	**24**	**24**	**6.0**	
		R748*(C2242T)	41			1.6	26.8
		R110*(C328T)	38			1.5	24.8
		Q738*(C2212T)	8			0.3	5.2
Deletion			***612***			***24.2***	
	Frameshift/termination		**484**	**42**		**19.1**	
		T184Rfs*36(550Adel)	329			13.0	68.0
		I661*(1981Adel)	20			0.8	4.1
		P22Qfs*35(60Adel)	9			0.4	1.9
		H690Rfs*9(2069-2970del)	9			0.4	1.9
	aa deletion		**111**	**24**		**4.4**	
		F200-L204del(598-612del)	36			1.4	32.4
		K254del(759-761del)	23			0.9	20.7
		I47del(140-142del)	12			0.5	10.8
Deletion+insertion			***265***			***10.5***	
		R788Sfs*14(2362-2363del+insTCATCT)	247			9.8	93.2
		D295Lfs*57(883-886del+insCTT)	14			0.6	5.3
Insertion			***54***			***2.1***	
Others/unknown			***13***			***0.5***	
Total			***2,530***	***456***		***100***	

[a]The value in the first row of each classification is taken from the Leiden Muscular Dystrophy Pages at http://www.dmd.nl/. Other values are calculated using the data in the Leiden database.
[b]The values in the right column are calculated from the 'frequency' of each specific mutation divided by the 'frequency' of the same 'mutation type' (missense or nonsense).

(including the two gene products from fish) showed that 424 aar are more than 80% conserved between species (*5*). These conserved aar include 128/176 (72.7%) of the above-mentioned missense loci. Conservation within the CAPN3[p94]-characterizing regions (NS, IS1, and IS2) is relatively low; however, it should be noted that some missense mutations, such as the R49C/H, P319L, and S606L mutations within the NS, IS1 and IS2 regions, respectively, occur relatively frequently (*5*). This suggests that strict sequence conservation within the CAPN3[p94]-characterizing regions is not necessarily required, but a few important aar, usually near the borders of these regions, must be conserved to maintain proper function.

CAPN8[nCL-2] and CAPN9[nCL-4]

These gastrointestinal tract-specific calpains are predominantly expressed by the surface mucus-secreting cells (pit cells) in the stomach. Smaller amounts are expressed by the goblet cells in the intestines. Of the human calpains, CAPN8[nCL-2] shows the highest degree of similarity to CAPN2[mCL] (61.4% identity). Moreover, *CAPN8* and *CAPN2* closely locate (head-to-head) on chromosome 1q41 (chromosome 1 in mice) and their exons 1 are only 36-kb distant (*cf. CAPN9* locates *ca.* 6.9-Mb downstream of *CAPN2* in the same direction). However, unlike m-calpain, recombinant CAPN8[nCL-2] expressed in *E. coli* exhibits Ca^{2+}-dependent activity without the need for CAPNS1[30K], and forms a homo-di/oligomer *via* C2L domain *in vitro* (*44*).

CAPN9[nCL-4] is another classical calpain homologue specific to the gastrointestinal tract and shows almost equal similarity to all other classical calpains at the aa level. CAPN9[nCL-4] is involved in tumourigenesis and in lumen formation by breast epithelial cells induced by carcinoembryonic antigen-related cell adhesion molecule 1 (*45*). Recombinant human CAPN9[nCL-4] requires CAPNS1[30K] for activity *in vitro*, and the activity of this complex is Ca^{2+}-dependent. The activity of both CAPN9[nCL-4] and CAPN8[nCL-2] is inhibited by calpastatin and other cysteine protease inhibitors in a manner similar to that seen for conventional calpains.

By contrast, these calpains show rather different characteristics *in vivo*. Endogenous CAPN8[nCL-2] and CAPN9[nCL-4] in mouse stomach form a hybrid heterodimer called G-calpain [CAPN8/9] (G for gastric), and neither CAPN8[nCL-2] nor CAPN9[nCL-4] form a stable complex with CAPNS1[30K] (*46*). Similar to the effect of *Capns1*$^{-/-}$ on the levels of CAPN1[μCL] and CAPN2[mCL], disruption of either mouse *Capn8* or *Capn9* causes down-regulation of the other gene product, suggesting co-dependency in terms of stability and functionality of both gene products. This point is supported by the finding that the residual CAPN9[nCL-4] in *Capn8*$^{-/-}$ mice does not display autolytic activity (*46*).

Capn8$^{-/-}$ and *Capn9*$^{-/-}$ mice appear healthy under normal conditions; however, they are significantly more susceptible to ethanol-induced gastric ulcers (*46*). CAPN8[nCL-2] knock-in (*Capn8*$^{CS/CS}$) mice expressing a protease-inactive CAPN8[nCL-2]:C105S mutant show stress-induced gastropathy, indicating that CAPN8[nCL-2] and CAPN9[nCL-4] (in the form of G-calpain [CAPN8/9]) mediate key components of gastric mucosal defence (*46*).

Gastric mucosal defence is a complex process involving mucus secretion and the migration of pit cells differentiated from stem cell progenitors. β-COP, a subunit of the COPI coatamer complex involved in ER-Golgi retrograde transportation, is a substrate for CAPN8[nCL-2]. This suggests the possibility that G-calpain is involved in the regulation of mucus secretion. However, expression of CAPN8[nCL-2] in cranial neural crest cells, where it is involved in cell motility, is regulated by ADAM13, a transmembrane metalloprotease containing a disintegrin domain (*47*). Taken together, the results of these studies indicate the involvement of G-calpain in pit cell migration.

As an alternative approach to studying the physiological functions of G-calpain, a single nucleotide polymorphism (SNP) database search showed that human *CAPN8* and *CAPN9* contain several SNPs that result in aa substitutions (*46*). An *in vitro* expression study showed that the G-calpain variants resulting from these SNPs have compromised proteolytic activity, suggesting that individuals expressing certain *CAPN8* and *CAPN9* variants may suffer gastrointestinal dysfunction.

Strict PalB homologues (the PalB group within the PalB subfamily)

PalB was first identified in *E. nidulans* as a product of the gene responsible for the fungus's adaptation to alkaline conditions (*15*). Subsequently, its orthologues were identified in *S. cerevisiae* (Rim13 [Cpl1]) and humans (CAPN7[PalBH]) (*5*, *14*). *E. nidulans*, like many other microorganisms, grows over a wide pH range. The *palB* gene locus was identified as one of the *pal* genes that show defective alkaline adaptation when disrupted. PalB is involved in the proteolytic activation of the PacC transcription factor, a key regulator of pH-dependent gene expression. Here, we refer to this pH adaptation system as the Pal-PacC pathway (Fig. 5).

The Pal-PacC pathway contains *palA*, *palB*, *palC*, *palF*, *palH*, *palI* and *pacC*. PalF is a distant homologue of mammalian arrestin and binds to the large cytosolic domain of PalH. The phosphorylation and ubiquitination of PalF under alkaline conditions depend on PalH and PalI, suggesting that pH-sensing is mediated *via* the interaction between PalF and PalH (similar to that observed for the arrestin receptor, which regulates various processes in mammals, such as photo-sensing). PacC usually adopts an inactive conformation during intramolecular interactions, and is activated by proteolytic removal of its C-terminus. PalB is primarily responsible for the processing of PacC in response to increased pH, which is followed by further proteolysis by the proteasome to yield the active form.

The Pal-PacC pathway is well conserved in yeasts and the orthologues are listed in Table IV. The yeast calpain, Rim13 [Cpl1], was identified as a product of the *RIM* genes. Like PacC and PalB, Rim101 is proteolytically regulated by Rim13 [Cpl1] (*14*),

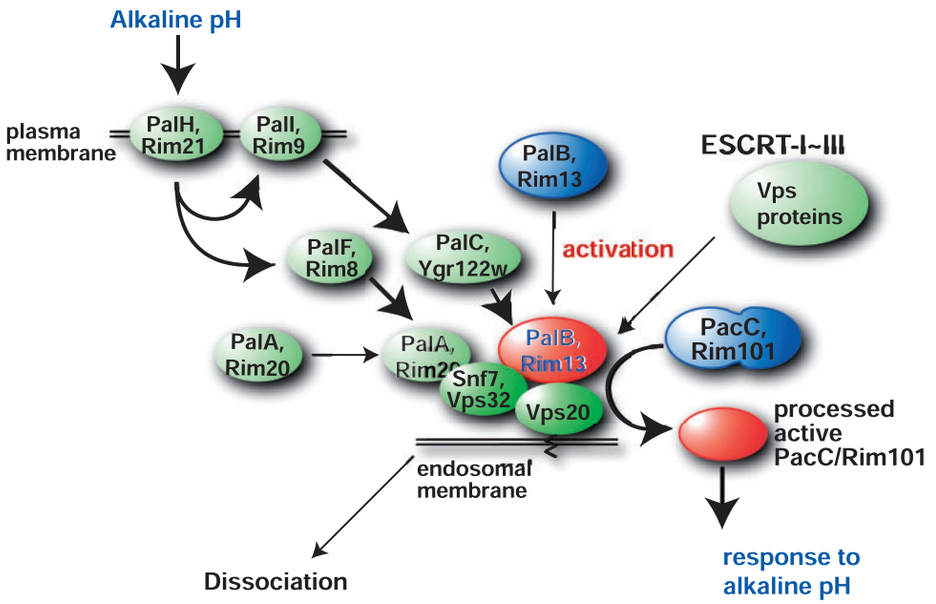

Fig. 5 Pal-PacC and Rim pathways. Schematic showing the alkaline-response signalling pathways in fungi and yeast. A membrane protein (PalH/Rim21) senses a shift from an ambient to an alkaline pH and transduces signals to PalI/Rim9. An arrestin homologue, PalF/Rim8, further transduces the signal to PalA/Rim20. At the endosomal membrane, PalA/Rim20 supports the formation of the PalB/Rim13 active complex to proteolyse PacC/Rim101 at the C-terminus, thereby activating this key transcription factor. Activated PacC/Rim101 orchestrates the gene expression required for survival under alkaline conditions (Table IV).

Table IV. Role of fungal, yeast and human Pal-Pac/Rim pathway members.

General role	*Emericella nidulans*	*Saccharomyces cerevisiae*	*Homo sapiens*
Sensor?	PalH	Rim21	?
Transducer?	PalI	Rim9	?
Arrestin	PalF	Rim8	arrestins
Adaptor	PalA	Rim20	Alix/AIP1
Regulator?	PalC	Ygr122w	?
Adaptor	AnSnf7	Snf7 [Vps32]	CHMP4 [VPS32]A, B, and C
Calpain	PalB	Rim13 [Cpl1]	CAPN7[PalBH]
Membrane anchoring	AnVps20	Vps20	CHMP6 [VPS20]
Transcription factor	PacC	Rim101	C2H2 ZNFs ?

? indicates that a role or a corresponding molecule is not experimentally shown or missing, respectively.

although in this case the proteasome is not involved. Under normal conditions, the environment for *Saccharomycetes* is rather acidic and this adaptive system [the Rim pathway (Fig. 5)] functions in near-neutral pH conditions. One example of a biological event in which the Rim pathway plays a role is the infection and invasion of mammalian skin by pathogenic and saprophytic yeasts such as *Candida albicans* and *Yarrowia lipolytica* at neutral pH. Disrupting the Rim pathway compromises the pathogenicity of these organisms (*48*).

The Pal-PacC and Rim pathways relate to membrane trafficking and involve the ESCRT (endosomal sorting complex required for transport) and Vps (vacuolar protein sorting) proteins. The Pal-PacC and Rim pathways are the first examples of genetic studies that are being used to thoroughly elucidate the molecular components and mechanisms underlying calpain-mediated systems (Fig. 5) (*49*, *50*). These pathways also exemplify the fact that calpains function as modulator proteases, affecting the function of their substrates through limited proteolysis. Theoretical extension of the knowledge of these pathways is anticipated to provide important keys to understanding other calpain systems, especially those that involve CAPN7[PalBH] (*51*).

The TRA-3 group within the PalB subfamily

TRA-3 [CLP-5] was first identified as the product of one of the genes involved in the sex determination cascade of *C. elegans*. The Ca^{2+}-dependent protease activity of TRA-3 [CLP-5] is necessary for the processing of TRA-2A, which is required for female development in XX hermaphrodites. On another front, TRA-3 [CLP-5] and CLP-1 are both components of a neuronal necrotic death cascade, which acts upstream of the aspartic proteases, ASP-3 and ASP-4. ASP-3 and ASP-4 correspond to mammalian cathepsins D and E (*52*). In addition, an SNP in *tra-3* is reported to be involved in nematode body-size determination (*53*).

Mammals express two TRA-3 orthologues, CAPN5[hTRA-3] and CAPN6 (*5*). CAPN5[hTRA-3]

has Ca^{2+}-dependent autolytic activity and is sensitive to several calpain inhibitors. *CAPN5* is expressed at varying levels in almost all tissues. Analysis of *Capn5*$^{-/-}$ mice shows that CAPN5[hTRA-3] is expressed by a subset of T cells, but is not required for development. SNPs within CAPN5[hTRA-3] are associated with polycystic ovary syndrome, diastolic blood pressure, and cholesterol levels (*54*), and expression of both *Capn5* and *Capn2* is up-regulated in caerulein-induced acute pancreatitis in mice (*55*).

CAPN6 proteins expressed in eutherians (placental mammals) and schistosomes have a naturally occurring aa substitution at the most important residue in the active site triad (C→K in humans, Fig. 3B); strongly suggesting that these CAPN6 proteins have no proteolytic activity. Interestingly, CAPN6 proteins expressed in marsupialia (e.g. *Monodelphis domestica*, opossum) and birds (e.g. *Gallus gallus*, chicken) retain all the active-site residues. Moreover, *X. tropicalis* and *Danio rerio* (zebrafish) express three TRA-3 homologues, all of which retain the active-site residues (*5*). Mammalian CAPN6 is predominantly expressed in embryonic muscles, placenta, and in several cultured cell lines. CAPN6 is involved in regulating microtubule dynamics (*56*) and motility in cultured cells (*57*), although the *in vivo* physiological functions of CAPN6 are still unclear.

The CAPN10 group within the PalB subfamily

A large-scale genetic association study identified an SNP within intron 3 of *CAPN10* that is linked to susceptibility to non-insulin-dependent diabetes mellitus (NIDDM, type 2 diabetes) (*16*), although there is no clear molecular explanation as to why this is so. *Capn10* is also a candidate gene responsible for the NIDDM phenotype in the Otsuka Long-Evans Tokushima Fatty (OLETF) rat. Quantitative trait locus (QTL) analyses using *Capn10*$^{-/-}$ mice and two other inbred strains with low and high obesity phenotypes (LG/J and SM/J) show that *Capn10* is a component of the obesity QTL, *Adip1*. This indicates that CAPN10 is involved in obesity in mice (*58*).

Studies using *Capn10*$^{-/-}$ or CAPN10-overexpressing mice suggest that CAPN10 is involved in type 2 ryanodine receptor-mediated apoptosis (*59*). Although phenotype of *Capn10*$^{-/-}$ mice was not described, they are not embryonic lethal. CAPN10 is ubiquitously distributed and its cellular localization is dynamic. When localized in the mitochondria, it mediates mitochondrial dysfunction by cleaving Complex I subunits and promotes mitochondrial permeability transition. CAPN10 is also involved in GLUT4 vesicle translocation during insulin-stimulated glucose uptake in adipocytes.

The SOL subfamily

The first genetic calpain study identified the *Drosophila* gene, *small optic lobes* (*sol*), in 1991 (*60*). A mutation in *sol* results in the absence of certain classes of columnar neurons from the optic lobes, the differentiation and/or survival of which is dependent on the protease activity of SOL. SOL comprises an N-terminal six Zn-finger motifs and C-terminal CysPc domain (*5*, *60*). Unfortunately, there is no other report analyzing the molecular mechanisms involved in this process. Mammals express a single SOL orthologue, CAPN15[SOLH], but its physiological role remains unclear. In parallel with the PalB subfamily, the SOL subfamily (including their mammalian homologues) is a group of evolutionarily interesting molecules that warrant further study.

Phytocalpain

A plant calpain, now called phytocalpain, was first identified from the sugarcane expressed sequence tag (EST) database (*17*). Subsequently, many other phytocalpains were identified from dicotyledons, monocotyledons, and gymnospermae. A genetic study identified phytocalpain as the *defective kernel 1* (*dek1*) gene product, DEK1, which is required for aleurone cell development in the maize endosperm (*18*).

Recombinant maize DEK1 CysPc-C2L domains show Ca^{2+}-activated caseinolytic activity, depending on the predicted active site Cys residue. DEK1 is the only calpain homologue in the *Arabidopsis* genome, and it is important for regulating growth in this plant. Although the full-length DEK1 protein localizes to membranes, intramolecular autolytic cleavage releases the CysPc-C2L domains into the cytoplasm. These domains are sufficient to fully complement *dek1* mutants (*61*).

DEK1 contains a potential signal peptide sequence followed by possible 3×7 TM regions, and CysPc and C2L domains (*5*, *18*). Although the CysPc domain of DEK1 is divergent from mammalian calpains, the C-terminal C2L domain is significantly similar to that of classical calpains such as CAPN1[μCL], suggesting that DEK1 consists of evolutionarily-different modules. One explanation for this inter-kingdom similarity is that the DEK1 homologue of the protista *T. thermophila* (*5*) resulted from a lateral gene-transfer event from a green alga-type endosymbiont of ciliates (*13*).

Other calpain members

Leishmania and *Trypanosoma* each express around 20 calpain homologues, which likely contribute to cell morphogenesis, drug resistance, and stress-response mechanisms (*62*, *63*). Some trypanosome calpains have N-terminal domains with weak similarity to calpastatin. As described above for TRA-3 homologues, some calpain homologues show substitutions in one or more of the well-conserved active-site triad residues. This non-proteolytic family of calpain homologues includes eutherian and schistosome CAPN6, several of the schistosome and nematode calpains, *Drosophila* CALPC, and all the *Trypanosoma* homologues (*5*, *13*). The evolutionary background for the occurrence of these calpain species is quite interesting, and elucidating their physiological functions will expand our knowledge regarding the functions of the calpain superfamily, e.g. possible non-proteolytic functions (*43*).

Conclusion

A major advantage of genetic studies is that several calpain members can be directly linked to various biological phenomena in terms of cause and effect. This connection is very hard to achieve with biochemical studies alone. Genetic studies have revealed entrances (defects in calpain genes) and exits (phenotypes) that should greatly encourage scientists working in the field of calpain research. Considering the different phenotypes of the various organisms whose calpain genes have been genetically manipulated, it can be said that calpains are involved in the fine-tuning of defence systems against a variety of stresses. However, uncovering the molecular mechanisms that connect the entrances and exits is another difficult path that needs to be negotiated, and biochemical studies will be essential in achieving this. Unfortunately, very little has been discovered regarding the precise molecular processes involving calpains, and much work is still needed to elucidate their physiological roles. Nevertheless, the authors believe that genetic comprehension of the context in which calpain functions will greatly accelerate its further study. Considering its diverse physiological functions, further breakthroughs in the study of calpain will have an enormous impact on many rapidly advancing fields in the life sciences.

Because space is limited, the references sited here are mainly recent genetics papers. For other references, see reviews 5, 13, 64, and 65.

Supplementary Data

Supplementary Data are available at *JB* online.

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Conflict of interest

None declared.

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