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CALPAINS FOR DUMMIES: WHAT YOU NEED TO KNOW ABOUT THE CALPAIN FAMILY.

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In memory of Dr Hiroyuki Sorimachi, a pivotal researcher in the calpain field

This review was written in memory of our late friend, Dr Hiroyuki Sorimachi, who, following the steps of his mentor Koichi Suzuki, a pioneer in calpain research, has made tremendous contributions to the field. During his career, Hiro also wrote several reviews on calpain, the last of which, published in 2016, was comprehensive [1]. In this manuscript, we decided to put together a review with the basic information a novice may need to know about calpains. We also tried to avoid similarities with previous reviews and reported the most significant new findings, at the same time highlighting Hiro's contributions to the field. The review will cover a short history of calpain discovery, the presentation of the family, the life of calpain from transcription to activity, the human diseases caused by calpain mutations and their therapeutic perspectives. In addition, the readers can find in **Table 1** the listing of **all the most relevant reviews in calpain research according to different subjects as well as the link of two** online resources created by Dr. Sorimachi.

Subject	Reference
Online resources	CaMPDB: a resource for calpain and modulatory proteolysis http://calpain.org/ [2] Calpain Research Portal, http://calpain.net/index.html
Whole calpain family	[1, 3-11]
Physiological system	[12] (Calpain-1 and -2 in the brain); [13] (Calpain-14 oesophagus)
Individual calpains	[14, 15]; [16] (Calpain-3)
Modulators of calpain activity	[1, 17]
Inhibitors	[18] (patent review); [17, 19] (cysteine protease inhibitors)
Crystal structures and domains/calpain activation	[20-23]
Pathological mutations in calpains	[24] (<i>CAPN3</i> , <i>CAPN10</i>); [25]

Table 1: Calpain review articles

THE HISTORY OF CALPAIN FAMILY DISCOVERY.

The calpain system (Clan CA, family C02; EC 3.4.22.17) is recognized as the third proteolytic system of the cell besides the lysosomal and proteasomal systems. In the following paragraph, we provide an historical perspective on calpain discovery. This would help a novice in the field

understand how the research on this class of proteins developed during the years, and what underlies the complexity of defining this superfamily. The first Ca^{2+} -dependent cysteine protease discovered is the one known today as calpain-2. In 1964, Guroff partially isolated calpain-2 from rat brain as a novel ubiquitous protease able to hydrolyse casein in a Ca^{2+} -dependent manner [26]. The same year, Meyer, Fisher and Krebs identified the same protein as a factor that activates phosphorylase kinase, and named it KAF (kinase activating factor). In 1968, KAF was then described as a Ca^{2+} -dependent proteolytic enzyme independently by Huston & Krebs and Drummond & Duncan [27-29]. In 1972, Goll's group re-identified (for the third time) a protein also corresponding to the current calpain-2 as Ca^{2+} -activated sarcoplasmic factor (CaSF) able to hydrolyse Z-lines [30]. Finally, in 1978, Imahori and colleagues purified this 80kD protease to homogeneity for the first time from chicken skeletal muscle and called it Ca^{2+} -activated neutral protease or CANP [31]. The same year, Murachi's group reported the occurrence of an inhibitor for CANP in rat liver known today as calpastatin, and partially purified it [32]. During this time, it was also demonstrated by protein purification and Ca^{2+} -activation assays that at least two different isoforms of Ca^{2+} -dependent proteases were present, with different sensitivities to Ca^{2+} concentrations [33]. In 1981, Murachi and colleagues reviewed all the literature of that time on this class of proteins and proposed that this new group of proteases, which requires an SH-reducing agent and the presence of Ca^{2+} for full activity, should be called calpain. "Cal" stands for calcium, like in calcitonin or calmodulin, whereas "-pain" stands for the resemblance with well-known thiol proteases such as papain and clostripain [34]. During this period, it was also discovered that both these proteases exist as the larger part of a heterodimer in association with a smaller subunit of 30kD (known today as calpain subunit 1 or CAPNS1) [35]. The determination that the active site of calpain contained a cysteine only arrived in 1983 through protein sequencing [36]. One year after, these authors also published the first nucleotide sequence of a calpain, following cloning of the cDNA of

what was named a calcium-dependent protease or CANP (probably the chicken CAPN11, that seems to be ubiquitous in birds) [37, 38]. This finding was the first to show that calpains contain domains sharing marked similarity to papain-like and calmodulin-like proteins [37].

As mentioned above, two different isoforms of these proteases were found, both ubiquitous, but with different sensitivities to Ca^{2+} [39, 40]. Interestingly, the two main research groups working on this new class of proteases kept using different names, creating confusion in the literature. Imahori's group named them μ -CANP and m-CANP based on the Ca^{2+} concentration necessary for their full activity (micro- and milli-molar, respectively) [40]. On the other side, Murachi's group used the names calpain-1 for the former and calpain-2 for the later [39].

The year 1989 changed the calpain field forever, when Sorimachi and colleagues, by screening human and rat muscle cDNA libraries, discovered and cloned a non-ubiquitous calpain for the first time, whose expression was mostly restricted to skeletal muscle, and called it p94 (known today as calpain-3) in reference to its molecular weight [41]. This finding led to the discovery of another tissue-specific calpain (known today as calpain-8) with expression restricted mainly in the stomach, suggesting the existence of a protease family with several members [42]. For this reason, Suzuki's group proposed a new nomenclature: μ -CL and m-CL for μ - and m-Calpain large subunits respectively, 30K for the small subunit and the analogical name nCL-x for the newly discovered members (where nCL stands for novel Calpain Large subunits and x for the associated number) [3, 42]. Calpain-3 was then named nCL-1 and the future calpain-8 nCL-2. However, this nomenclature was never adopted outside of their group and this again created confusion in the literature. Since these first findings, many other calpain members have been discovered. Their first appearance in the literature is listed in **Table 2**.

Today, the nomenclature uses “CAPN” for the genes and “calpain” for the protein products, followed by an analogical number to identify the specific member of the protein family (eg. CAPN1 and calpain-1) [7]. As is clear from **Table 2**, the analogical number in the gene/protein

name does not correspond to the order of discovery. This results from the fact that some members of the family (*CAPN8*, *9*, *15*) were named differently than “calpain” at the time of their discovery while the Human Genome Nomenclature Committee was including consecutive numbers for other calpain members (**Table 2 and Figure 1**). Another discrepancy is due to the fact that *CAPNS1* was incorrectly named *CAPN4*/Calpain-4 before the discovery of calpain-5. Consequently, the gene list of the 15 family members lacks a “*CAPN4*”, jumping from 3 to 5 (**Figure 1**). Of note, a second small subunit (*CAPNS2*) was identified in humans, homolog to *CAPNS1*, with a still unknown physiological role [43].

Gene	1st Discovery	Technique	Species	Original name
<i>CAPN1</i>	1980, Mellgren [33]	Protein purification	Dog	Calcium-dependent protease, peakI-type
<i>CAPN2</i>	1964, Guroff [26]	Protein purification	Rat	Neutral calcium-activated proteinase
<i>CAPN3</i>	1989, Sorimachi et al. [41]	cDNA library screening (by hybridization)	Human and Rat	P94 (nCL-1)
<i>CAPN5</i>	1997, Mugita et al. [44] and Dear et al.[45]	Blast search + cDNA cloning [44] PCR with redundant oligonucleotides[45]	Human and Mouse (C. elegans)	htra-3 (nCL-3) [44] <i>CAPN5</i> [45]
<i>CAPN6</i>	1997, Dear et al.[45]	PCR with redundant oligonucleotides	Human and Mouse	Capn6
<i>CAPN7</i>	1999, Franz et al. [46]	Blast search + hybridization Cloned in 2001[47]	Mouse and Human	<i>CAPN7</i> [46] PalBH[47]
<i>CAPN8</i>	1993, Sorimachi and Suzuki[42]	cDNA library screening (by hybridization)	Rat	nCL-2
<i>CAPN9</i>	1998, Lee et al. [48]	cDNA cloning	Mouse	nCL-4
<i>CAPN10</i>	2000, Horikawa et al. [49]	Genome-wide screen (for type 2 diabetes)	Human	<i>CAPN10</i> (approved by HGNC before publication)
<i>CAPN11</i>	1999, Dear et al. [50]	BlastN algorithm	Human (vertebrates)	<i>CAPN11</i>
<i>CAPN12</i>	2000, Dear et al. [51]	In situ hybridization and Northern blot analysis	Mouse and Human	<i>CAPN12</i>
<i>CAPN13</i>	2001, Dear and Boehm[52]	BlastN algorithm + Radiation hybrid mapping	Mouse and Human	<i>CAPN13</i>
<i>CAPN14</i>	2001, Dear and Boehm[52]	BlastN algorithm + Radiation hybrid mapping	Mouse and Human	<i>CAPN14</i>
<i>CAPN15</i>	1998, Kamei et al [53]	cDNA isolation	Human (Drosophila)	SOLH
<i>CAPN16</i>	2012, Hoogewijs et al. [54]	Comparative genomic studies	Human	<i>ADGB</i> , androglobin (these are also name and gene symbol approved by HGNC)

Table 2 – List of human calpain genes and their first appearance in the literature. CAPN1 and CAPN2 were purified as respective homodimers with the CAPNS1 subunit.

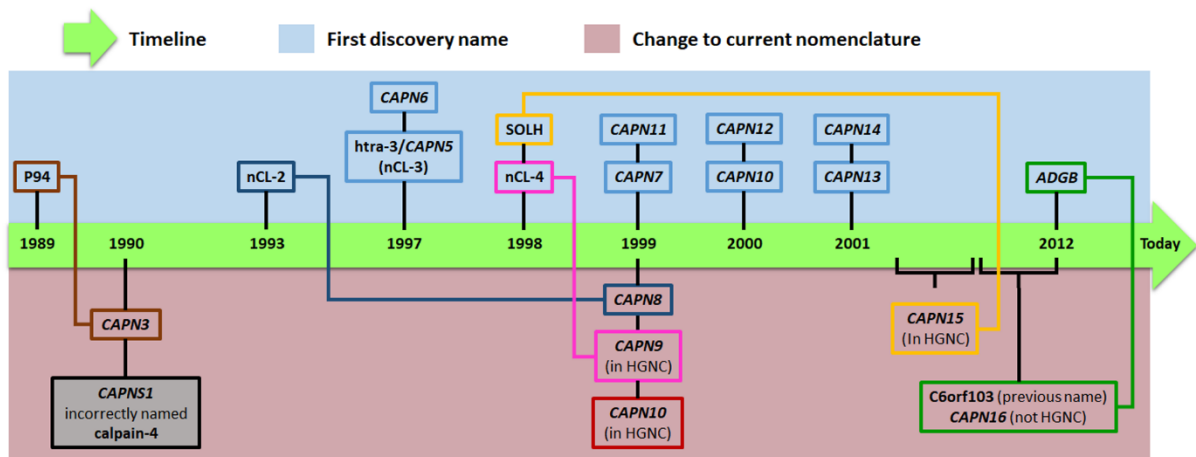


Figure1 – Nomenclature timeline of the vertebrate calpain family. In the upper panel, boxes indicate nomenclature proposed at the time of first appearance in the literature. In the lower panel, boxes indicate the name change to the current nomenclature for those calpains that were named differently at the time of discovery. The grey box with a black border indicates the incorrect inclusion of *CAPNS1* as a calpain gene. HGNC: Human Genome Nomenclature Committee. Although before the early 90's calpains were referred to by a variety of names, only the first discovery name and current nomenclature are provided, in order to simplify the figure.

A CURRENT DESCRIPTION OF CALPAIN FAMILY MEMBERS

The accepted definition of a calpain is a protein that has an amino-acid sequence significantly similar to the protease domain of conventional calpains, irrespective of proteolytic ability [6]. In mammals, the calpain family is composed of 15 gene products (**Figure 2, Table 2**) while additional calpains have been recognized in lower ontological classes [6, 7]. Because of their extensive sequence similarity, history and in the wake of protein kinase C nomenclature, the first two calpains discovered (-1 and -2) used to be called “conventional” [6]. The remaining family members are further divided into classical and non-classical calpains depending on the resemblance of their domain organisation to those of the conventional calpains (**Figure 2**). Moreover, based on their expression profile and physiological role, nine calpains are classified as ubiquitous and six as tissue-specific (**Figure 2**).

Over the years, even if complicated by the instability of these proteins, a number of crystal structures of different calpains and calpain domains have been solved. These structural studies have provided useful information into tertiary and quaternary structure organizations, as well as giving insight on the activation of both classical and non-classical calpains (**Table 3**). It is worth mentioning that the structure of calpain-2 was the first calpain crystal structure ever solved, in 1999, and to date the only one that has been solved in its entirety.

Protein	Domain solved	Species	Conditions	PDB* ID	Reference
Calpain-1	Calcium-bound protease core	<i>Rattus norvegicus</i>	Ca ²⁺ (C115S)	1KXR	[55]
	Calpain-1-like (First 48 and last 62 residues of calpain-2)	<i>Bos taurus</i> , <i>Rattus norvegicus</i>	Ca ²⁺ -free (C105S)	1QXP	[56]
	Protease core (PC1 & PC2)	<i>Rattus norvegicus</i>	Ca ²⁺ , leupeptin	1TL9	[57]
			Ca ²⁺ , E64	1TLO	
	Protease core	<i>Rattus norvegicus</i>	Ca ²⁺ , complexed with SNJ-1945, an alpha-ketoamide-type inhibitor.	2G8J	[58]
			Ca ²⁺ , complexed with SNJ-1715, a cyclic hemiacetal-type inhibitor	2G8E	
	Protease core	<i>Homo sapiens</i>	Ca ²⁺ , inhibited by ZLLYCH2F (G213A)	1ZCM	[59]
	Protease core	<i>Rattus norvegicus</i>	Ca ²⁺ , inactivated by WR13(R,R), an epoxysuccinyl-type inhibitor.	2NQI	[60]
			Ca ²⁺ , inactivated by WR18(S,S), an epoxysuccinyl-type inhibitor.	2NQG	
	Protease core	<i>Homo sapiens</i>	Ca ²⁺	2ARY	[61]
Protease core	<i>Rattus norvegicus</i>	Ca ²⁺ , inactivated by ZLAK-3001, an alpha-ketoamide	2R9F	[62]	
		Ca ²⁺ , inactivated by ZLAK-3002, an alpha-ketoamide	2R9C		
Calpain-2	Calpain-2	<i>Rattus norvegicus</i>	Ca ²⁺ -free (C105S)	1DF0	[63]
	Heterodimer	<i>Homo sapiens</i>	Ca ²⁺ -free	1KFX; 1KFU	[64]
	Calcium-bound protease core	<i>Rattus norvegicus</i>	Ca ²⁺ (C105S)	1MDW	[65]
	Heterodimer	<i>Rattus norvegicus</i>	K10T and C105S	1U5I	[66]
	Heterodimer	<i>Rattus norvegicus</i>	Ca ²⁺ , Calpastatin (C105S)	3DF0	[67]
	Heterodimer	<i>Rattus norvegicus</i>	Ca ²⁺ , Calpastatin (C105S)	3BOW	[68]
Calpain-3	Penta-EF-hand (PEF)	<i>Homo sapiens</i>	Ca ²⁺	4OKH	[69]
	Protease core	<i>Homo sapiens</i>	C129S with Ca ²⁺	6BDT	[70]
			C129A with Ca ²⁺ /CaCl ₂	6BGP	
			C129A Ca ²⁺ -free	-	
			E-64 added to a Ca ²⁺ - bound C129 core	6BJD	
Leupeptin added to a Ca ²⁺ - bound C129 core	6BKJ				
Calpain-5	Protease core	<i>Homo sapiens</i>	Ca ²⁺ -free	6P3Q	[71]
Calpain-7	Distal C2-like domain	<i>Homo sapiens</i>	Ca ²⁺ -free	2QFE	-
Calpain-8	Protease core	<i>Homo sapiens</i>	Ca ²⁺ , Leupeptin	2NQA	-
Calpain-9	Protease core	<i>Homo sapiens</i>	Ca ²⁺	1ZIV	[61]
	Protease core	<i>Homo sapiens</i>	Ca ²⁺ , Leupeptin	2POR	-
Calpain-13	Domain IV	<i>Homo sapiens</i>	Ca ²⁺	2I7A	-

Table 3: Crystal structures solved for the calpain family. (*) [72] Since the 2012 review by Campbell and Davies, inclusive of all calpain crystal structures known at that time [23], calpain-

3 (PDB ID: 6BDT and 4OKH) and calpain-5 (PDB ID: 6P3Q) crystal structures have been resolved.

Classical calpains

The classical group is comprised of nine calpains, of which calpain-1, -2, -13 and -14 are expressed ubiquitously while calpain-3, -8, -9, -11 and -12 expression is restricted to particular tissues (**Figure 2**). By definition, they all share the same structural composition. This organisation was clarified following determination of the tridimensional structure of calpain-2 in 1999 [63] as well as the subsequent calpains (**Table 3**). The classical calpains consist of: i) an N-terminal anchor helix region of variable length; ii) a catalytic domain named calpain-type cysteine protease conserved (CysPc), subdivided into two globular core domains called protease core 1 and 2 (PC1 and PC2 or DI and DII); iii) a calpain-type beta-sandwich (CBSW or DIII) domain and; iv) a penta-EF-hand domain (PEF or DIV). The N-terminal anchor is involved in the maintenance of the inactive state, as well as in Ca^{2+} -dependant activation. In CysPc, the catalytic activity is possible thanks to a triad of amino acids composed of a cysteine, a histidine and an asparagine, as determined via site directed mutagenesis [73]. From a tridimensional point of view, the active site resides in the catalytic cleft between the two PC domains, with the cysteine located in PC1 and the histidine and asparagine in PC2. The CBSW domain is also called C2-like (C2L) for the similarity with a common structural domain called C2, very often involved in Ca^{2+} -dependant membrane targeting [74]. The PEF domain is characterized by five repetitive EF-hand motifs and a helix-loop-helix structure involved in Ca^{2+} binding. The fifth EF-hand of the domain acts as a dimerization module to generate both homodimers and heterodimers [6].

Mammalian calpain-1 and -2 are the most studied and therefore well-known calpains, both are ubiquitously expressed with similarities in substrate specificities and mode of inhibition. As mentioned above, the functional form of calpain-1 and -2 exists as a heterodimer, where the gene product of CAPN1 or CAPN2 is the large catalytic subunit and the small 30 KDa protein acts as regulatory subunit (calpain subunit 1, or CAPNS1) [63, 64]. Interestingly, the crystal

structure of CAPNS1 was solved even before any calpain members. The subunit is composed of an N-terminal Glycine rich region (GR) and a PEF domain. The dimerization with the large subunit occurs through the fifth EF hands of their respective PEF domains [75]. CAPN1 and CAPN2, are the only calpains where association with CAPNS1 has been demonstrated to be required *in vivo*. In fact, disruption of CAPNS1 in mice results in destabilization of both calpains [76, 77].

Calpain-3 shares the same domains of the conventional calpains, but has additional specific sequences: NS at the N-terminal, the insertion sequence IS1 within the PC2 domain and the insertion sequence IS2 between the CBSW and PEF domains. The insertion sequence IS1 is primarily responsible for the uniquely rapid autolysis of calpain-3 that underlies enzymatic activation. The IS2 region contains a nuclear localization signal and calpain-3 has been observed in the nucleus and cytoplasm [78]. Interestingly, it was suggested that calpain-3 may have a non-proteolytic function with a role in Ca^{2+} release from the sarcoplasmic reticulum [79]. The resolution of the crystal structure of the PEF domain of calpain-3 demonstrated that this calpain is able to form a homodimer through its PEF domain [80]. In these homodimers, the calpain-3 monomers are oriented in such a way that allows the catalytic sites to be positioned at opposite ends [69]. Further investigation into the quaternary structure of calpain-3 revealed it can also form a homotrimer, the first demonstration of trimerisation of any calpain family member or PEF containing proteins [81].

Calpain-8 and -9 are mostly expressed in the gastrointestinal tract. Interestingly, it was shown that they are able to form a heterodimer with each other. The N-terminal anchor helix and C-terminal EF-hands of calpain-8 interact with the C-terminal EF-hands of calpain-9, forming a complex called gastric calpain (G-calpain) [82, 83]. The G-calpain is a protease complex where it has been shown that the catalytic activity is exerted by calpain-8, while calpain-9 functions

as a chaperone-like regulatory subunit [83]. Besides G-calpain, calpain-8 is also able to homodimerize and calpain-9 to form a complex with CAPNS1 [82, 83].

Little is known about the remaining four classical calpains, apart from their organ distribution. Calpain-13 is ubiquitous, while calpain-11 is expressed in testis and calpain-12 expression is restricted to hair follicle cells [7]. There is no evidence that they heterodimerise with CAPNS1, except for the avian form of *CAPN11* that was shown to be associated with the small subunit [84]. As for Calpain-14 initially thought to be ubiquitous, it is expressed at the highest level in the oesophagus where it constitutes a marker for tissue identity [85].

Non-classical calpains

Non-classical calpains present sequence homology with the classical calpains in the CysPc region but do not have complete similarity in domain organisation. In particular, they lack the PEF domain and for some the CBSW. According to the classification of Sorimachi [6], they are divided into three groups depending on their structure and additional domains: the PalB subfamily, which includes *CAPN5*, *CAPN6*, *CAPN7* and *CAPN10*, is the most evolutionarily conserved group, the Sol subfamily, which comprises only one calpain (*CAPN15*) and the single group of demi-calpain (*CAPN16*), a very divergent calpain. They are all ubiquitous apart from *CAPN6*, which was found to predominantly be expressed in embryonic muscles and placenta [45, 86].

The PalB subfamily takes its name from an alternative term for the human *CAPN7* (PALBH) through homology with the *Aspergillus* atypical calpain PalB [47]. The members of this family all lack a PEF domain making it implausible that they are able to bind the small subunit. The PEF domain is replaced either by a domain that is similar in structure to the classical C2 domain (for calpain-5 and -6), or by a second CBSW (for calpain-7 and -10). Calpain-5, historically called hTRA3 for its homology to the TRA-3 sex determination gene of *C. Elegans* [44], has

the structural feature of having 2 NLS that accounts for its dual localization in the nucleus and cytoplasm [87]. Furthermore, calpain-5 has a unique conformational feature of three extended flexible loops in each PC domain. These loops are proposed to account for the high Ca^{2+} concentrations required for optimal activity [71]. Calpain-6 is unique because it lacks a cysteine residue in the catalytic triad (substituted with Lys in humans) within the PC1 domain, suggesting that it may not possess proteolytic activity [45]. In this calpain, the C2-like domain mediates the interaction with microtubules rather than phospholipids [88, 89].

The SOL subfamily includes only *CAPN15*, also called SOLH in human, as the homologue of the small optic lobes (SOL) gene identified in *D. Melanogaster* [53]. Calpain-15 is a conserved ubiquitous protein that is highly expressed in the nervous system [90] and has up to 6 Zn-finger repeats at the N-terminus, recently shown to be able to bind polyubiquitin [91], a CysPC domain, and a SOL homology domain (SOH) at the C-terminal domain of unknown function [53].

The demi-calpain subfamily includes calpain-16, which consists of half the CysPC proteolytic domain and a calmodulin-binding motif (amino acid sequence motif IQ) in the C-terminal region [6]. The actual name of this protein is androglobin (ADGB) as registered in the HUGO database. Phylogenetic analyses indicate that this gene is probably not a descendant of an ancestral calpain, but rather a protein derived from the fusion of a calpain domain with a globulin in an exon-shuffling event [54]. Interestingly, the calpain domain of ADGB has retained only the cysteine of the typical triad but contains several His and Asn residues at non-standard positions. It is not yet defined whether calpain-16 has proteolytic activity [54].

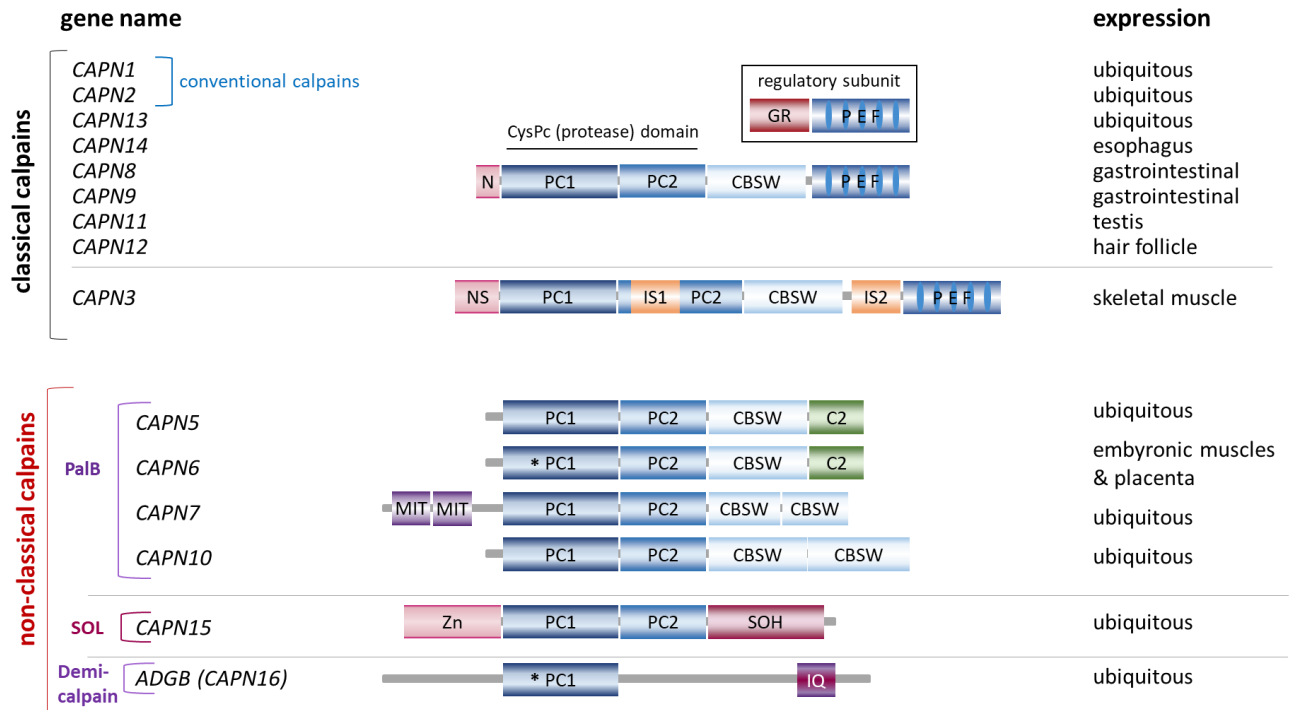


Figure 2: The domains of calpains. Abbreviations used for domain names: CysPc: calpain-type cysteine protease sequence motif defined in the conserved domain database at the National Center for Biotechnology Information, which is composed of two protease core domains 1 and 2 (PC1 and PC2). CBSW: calpain-type beta-sandwich domain (formerly called "C2-domain-like (C2L)" domain). PEF: penta-EF-hand domain. 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. IQ: calmodulin-interacting domain. Symbols: (*) change of one or more amino acids in the catalytic triad). This figure is an updated version of that found in Ono & Sorimachi (2012) [7]. Note - The tissue-specificity attributed to each calpain reflects the tissue in which it is predominately expressed. We refer the reader to the GTEx Portal (<https://gtexportal.org/home/>) for further details of tissue expression for each calpain.

FROM TRANSCRIPTION TO ACTIVITY

Proteolytic events are irreversible post translation modifications. Therefore, they require strict control to occur at the right time, the right place and on the right targets in order to keep homeostasis and avoid cell self-destruction. Calpains cumulate all existing possibilities for controlling proteolysis, from expression to conformational changes. In this part of the review, we will present these mechanisms using the most relevant examples to illustrate how such control can be achieved.

Expression and alternative splicing

The strategies that the cell uses to regulate calpain function start by spatio-temporal modulation of the level or the form of transcription. Restricting the expression of certain calpains only to the required cells and time with the goal to avoid abnormal functions and deleterious effects is a simple but extremely efficient way of activity modulation. The most exquisite example of this is given by calpain-3. In adults, this calpain is expressed solely in the skeletal muscle. Interestingly, gene transfer experiments showed that ectopic expression of calpain-3 in the heart leads to cardiotoxicity, demonstrating the absolute requirement to restrict the expression of the protein to the tissues that specifically require its activity [92]. The timing of expression is also an important regulation checkpoint. For example, during human and murine myogenesis, calpain-3 expression occurs only after innervation, a time when most of the other muscle-specific proteins are already expressed [93]. Calpain-3 also provides a perfect example for alternative splicing as a control mechanism [94]. Alternative splicing of exons coding IS1 and IS2 were observed across tissues such as brain, smooth muscle, and embryonic lens or during development [78, 95]. These domains are important for full proteolytic capacity and their absence results in calpain-3 isoforms with reduced autolysis and/or lack of proteolytic activity, while potentially keeping the non-proteolytic function [93].

Immature conformation of the protein

During protein formation, the association with a molecular chaperone to achieve the correct conformation appears to be necessary for at least some calpains. Such a requirement for chaperone intervention represents a means to prevent immediate activation of the protease, providing an additional point of modulation. A clear example for such a mechanism is obviously the conventional calpain small regulatory subunit (CAPNS1), which has a chaperone-like function on conventional calpains and is required for the stability of both calpain catalytic subunits. Only the formation of this heterodimeric structure allows the subsequent activation of conventional calpains [76]. Another example is G-calpain, the heterodimeric structure formed by calpain-8 and -9. The resulting protease complex functions with calpain-8 as the active proteolytic subunit and calpain-9 probably acting like a molecular chaperone, stabilizing the structure similarly to the action of CAPNS1 with calpain-2 [82, 83].

Interaction with inhibitors

One of the most intuitive regulatory mechanisms is the expression of modulatory proteins able to directly inhibit calpain by inducing conformational changes, which could impair proteolytic activity or substrate accessibility. Most of the data on modulators for these proteins have been generated from studies on conventional calpains and calpain-3, whereas there are very few examples of direct endogenous regulation of the other calpains. Calpastatin is the elite inhibitor of conventional calpains. It binds directly to all domains of the calpain molecule by occupying both sides of the active cleft, at the same time looping some of the adjacent amino acid residues to protect itself from proteolysis [67, 68, 96-98]. It was also shown that G-calpain can be inhibited by calpastatin like for the conventional calpains [83]. For the calpains that do not bind calpastatin, other safeguard mechanisms exist. For example, calpain-3 activity in skeletal muscle is heavily controlled through interaction with a giant protein of the sarcomere, titin. This protein has calpain-3 binding sites at the N2A region (I-band) and in the C terminus (M-line). The N2A titin is able to inhibit calpain-3 activity by protecting IS2 from autolysis [99-101]. In

addition, the association of calpain-3 with titin also forces the protease to a sarcomeric localization, providing another way to prevent aberrant activity via subcellular compartmentalization [92, 102].

From an inactive state to an active state

The activation of calpain is a multi-step process starting with a protein locked in an inactive conformation. The question of how a calpain is maintained in an inactive state was answered when the crystal structure of Ca^{2+} -free calpain-2 was solved. The structure showed that the geometric proximity of each residue of the catalytic triad would not allow the biochemical reactions to result in a nucleophilic and therefore active cysteine [63, 64]. As the crystal structures were solved of other calpain family members in their inactive states, in the absence of Ca^{2+} , the same arrangement of an unassembled catalytic triad was observed in calpain-1 and calpain-5 [56, 71]. For calpain-9, an unassembled catalytic triad was observed even in the calcium-bound state [61]. These observations suggests a general mechanism in accordance with the remarkable similarity in the 3D structure of the proteolytic cores of the different calpains.

The Ca^{2+} -induced activation mechanism of the proteases was described thanks to the observation of active versus inactive calpain-1 crystal structures [55, 103]. It appears that after binding of two Ca^{2+} ions, one to PC1 and one to PC2, an unstructured loop region within PC2 undergoes a conformational change becoming a β -sheet. As a result of the new conformation, a side chain in PC2 is displaced from the active cleft, permitting the proper formation of the catalytic triad [22]. Assembly of the protease core to an active state through the cooperative binding of two Ca^{2+} ions to the equivalent sites used in calpain-1 and -2 was also demonstrated for calpain-3 and -5 [70, 71]. As for calpain 9, the distance between the catalytic residues is still too great for the formation of the salt-bridge linking the two PC domains even after Ca^{2+} -induced conformational changes. This prevents formation of the active triad and indicates additional and currently unknown auto-inhibition and activation mechanisms for calpain-9 [61].

Conventional calpains require high concentrations of Ca^{2+} to be activated *in vitro* [104, 105]. A burning question within the calpain field is, how are calpains activated within the cell where it is difficult to reach such high Ca^{2+} levels? Evidences that the Ca^{2+} concentration required for calpain-1 activation is considerably lowered by the presence of particular phospholipids [106, 107] and that vicinity to the membrane and binding to phospholipids regulate calpain-2 activation support the hypothesis of subcellular compartmentalization as a mechanism of calpain regulation [108, 109]. Calpain-5 is another calpain requiring millimolar concentration of Ca^{2+} for its activation like calpain-2. The reason for this threshold is the presence of additional flexible loops leading to a particular protease core conformation associated with the lack of the Ca^{2+} -regulated PEF domain typical of the non-classical calpains [71]. Of interest, the cells where *CAPN5* is expressed have high resting Ca^{2+} levels. This necessity for extreme Ca^{2+} levels is likely an evolutionary adaptation providing either a specific cell defense mechanism to cells expressing high basal Ca^{2+} levels such as retinal cells [110] or a means to avoid mislocalized and/or accidental activation when associated with spatial compartmentalization.

Ca^{2+} -induced conformational changes to align the catalytic triad is only one layer of calpain activation. Proteolytic removal of N-terminal or internal peptides can be required for enzyme activation. Such cleavage can lead to additional structural changes to assemble the catalytic site, to free the active site sterically blocked by a peptide, or else change the interactions with other proteins including inhibitors. In particular, it has been demonstrated that the conventional calpains autolyse their first N-terminal domain rapidly after Ca^{2+} binding [111]. Resolution of the crystal structure of calpain-2 showed that the N-terminal anchor of calpain-2 binds to CAPNS1, tethering together the two molecules [63]. The internal autolysis of the N-terminal segment is an intermolecular event. Release of the tether between the large and small calpain subunits allows more conformational flexibility between PC1 and PC2 and therefore may aid

alignment of the catalytic triad. It is important to note, that while this autolysis occurs rapidly after Ca^{2+} -induced activation it is not essential for enzyme activation [112]. N-terminal autolysis has been shown to reduce the Ca^{2+} concentration required for activation [112]. Rapid autolysis of the N-terminal anchor has also been observed in calpain-1 [55], calpain-3 [113] and calpain-9 [61]. Calpain-3 is distinctive within the calpain family in that autolysis is so rapid that the full-length protein can barely be detected *in vitro* [14]. The underlying molecular mechanism is autoproteolysis of a further internal peptide, IS1, a sequence present at the interface of PC1 and PC2. The key event for calpain-3 activation is the intramolecular cleavage of IS1 that in turn allows for the opening of the cleft to calpain-3 substrates [70]. IS1 was found to be disordered and to act as an internal inhibitory polypeptide whose cleavage is required for full activation [113, 114]. This is supported by the crystal structure showing the N-terminus of IS1 residing and binding to residues within the active cleft, therefore blocking access to the catalytic cleft. In addition, autolysis leads to instability, regulating the duration of enzyme activity within the cell.

Additional post translation modifications can modulate calpain activity. Phosphorylation is one of the most common protein modifications and calpains are no exception. Several findings indicate that phosphorylation plays a major role in the regulation of calpain activation [115-117]. Tridimensional modeling revealed that phosphorylation at serine 369 in calpain-2 by protein kinase A generates constraints in domain movement, contributing to keep the protease in an inactive state [115]. In contrast, the phosphorylation of calpain-1 promotes its activity, by conformational changes yet to be characterized [116]. Interestingly, another reversible mechanism for control of proteolytic activity, acting directly through modification of the catalytic triad, has been proposed. Indeed, nitric oxide seems to reduce calpain-mediated proteolysis in cultured myotubes through nitrosylation of the cysteine of the active site [118, 119].

Finally, another indirect way to modulate calpain activity is to modify its interactors or substrates. For example, phosphorylation of calpastatin has been shown to decrease its inhibitory ability on conventional calpains [117]. It has recently also been found that different human cells express an alternatively spliced calpastatin (named hcast 3-25) that lacks all the exons coding for the inhibitory domains and that this form of calpastatin operates as a positive modulator of calpain-1 [120].

Substrate recognition

Those calpains that possess enzymatic activity exert an intracellular limited proteolytic activity on their target that is modulatory rather than degradative. Calpains do not “erase” but instead “modulate” their target by generating new fragments of the protein and consequently alter its original function with changes to protein-protein interactions or subcellular distribution [7, 121-123].

The analysis of crystal structures and kinetics has elucidated the positions surrounding the cleavage site that are most influential for calpain cleavage [57, 58, 60, 62, 67, 68, 124]. Peptide libraries, machine learning and position-specific scoring matrix analysis have been used to reveal amino acid sequence preferences and develop calpain cleavage site predictors [60, 124-131]. Structural data showed the active cleft is flanked by two flexible loops that may confer interaction specificity to calpain substrates through variability in sequences [57]. Calpain substrates identified thus far are involved in a range of cellular processes including signal transduction, membrane repair and structural sarcomeric proteins, many of which still need to be validated under physiological conditions [132-142].

ABNORMAL CALPAINS AND THERAPIES

What has been learnt from mutations in calpain genes?

The necessity for the various systems adopted to control calpain activity, described in the previous paragraphs, is also stressed by the large number of publications describing the deleterious consequences of calpain overexpression and/or exacerbated activity. At the same time, the biological importance of calpain is highlighted by the several pathological conditions associated with calpain inactivation or absence. An extensive number of studies are present in the literature regarding this topic, but this part of the review will focus on the genetic disorders caused by mutations in human calpain genes (**Table 2**), and on what we have learnt from the most important findings on mammalian animal models.

The identification of inactivating mutations in *CAPN3* as the genetic cause of limb girdle muscular dystrophy (LGMD) is a milestone that marked both the calpain and the muscular dystrophy fields [143]. The association of calpain deficiency with a pathology was in plain contrast with the initial concept of calpain hyperactivation as an aggravating agent in myopathies [144]. The discovery totally changed the perspective on the involvement of calpains in diseases, opening an entire new line of research. However, besides the calpain-3-related LGMD (LGMD-R1), so far only three additional monogenic disorders have been identified as being caused by mutations in calpain family members, one for the classical calpains and 2 for the non-classical calpains, while associations of pathological conditions with high-risk genetic variations is more frequent (**Table 4**).

LGMD-R1 is the most frequent form of LGMD, caused by recessive mutations in *CAPN3* and characterized by progressive skeletal muscle degeneration with no involvement of other tissues [143, 145]. The disease onset occurs in the first or second decade of life, leading to decreased ambulation and difficulties in performing daily life activities [145-148]. Recapitulation of the pathological phenotype in different calpain-3 murine models, although with reduced severity, confirmed mutations in *CAPN3* are disease causing. While there is a lack of understanding of the molecular mechanism leading to the disease, analyses of these models, as well as of human

biopsies, suggests an abnormal adaptation of the muscle fibers as a plausible pathological mechanism [15]. Dominant variants in *CAPN3* were also increasingly being associated with LGMD, creating a new subtype of the disorder: LGMD-D4, with similar but more mild symptoms and a delayed onset compared to LGMD-R1 [149, 150]. Further analyses showed a loss of proteolytic activity in the mutated proteins and a dominant negative effect on wild-type calpain-3 [150].

Pathological mutations in additional calpain family members were identified some seventeen years after the initial genetic diagnosis for calpain-3 was made. In 2012, mutations in *CAPN5* were reported to be responsible for autosomal dominant neovascular inflammatory vitreoretinopathy (ADNIV). This genetic disorder is characterized by a progressive retinal degeneration with presence of inflammatory T-cells and severe neovascularization, that leads to bleeding, formation of scar tissue and ultimately blindness [151]. ADNIV-causative mutations in *CAPN5* were demonstrated to lead to a gain-of function representing the first example, and the only one to date, of a dominantly inherited disease caused by an hyperactive calpain [152, 153]. These gain of function mutations are located in a region encoding a flexible loop whose function is to limit substrate access to the catalytic site instead, the mutations lead to increased proteolytic activity and lower Ca^{2+} -activation threshold [152, 153]. A mouse model heterozygous for one of these mutations (R243L) recapitulated the ADNIV phenotype, even if with milder symptoms, confirming the hypothesis of a dominant gain of function effect [152]. Interestingly, despite high expression of calpain-5 in other tissues, hyperactive mutation-related pathological effects were only observed in the ocular system [152]. This observation may be related to the high Ca^{2+} threshold required for activation and the high Ca^{2+} concentrations present in the retinal cells [154].

Despite the large number of studies, the first disease-causative human mutation in conventional calpains was identified more than 50 years after the initial discovery of calpain (**Tables 2 and 4**). Recessive mutations in *CAPN1* were identified in 2016 as the cause of neurological disorders such as spastic paraplegia, spinocerebellar ataxia or as a combination of both. Spastic paraplegia is characterized by progressive muscle stiffness and hyperreflexia in the lower limbs due to corticospinal tract degeneration [155]. Spinocerebellar ataxia is characterized by progressive degeneration of the cerebellum leading to gait imbalance associated with limb incoordination [156]. Other studies correlated additional *CAPN1* mutations to these neurodegenerative disorders, or even a combination of both [157-160]. These discoveries encouraged a deeper analysis of *Capn1*-KO mice, which were initially reported as showing no pathological phenotype, except for defects in platelet aggregation [161]. A more careful characterization of this mouse model suggested an implication of calpain-1 in a pro-survival pathway in cells of the cerebellum as well as in synaptic plasticity in both the hippocampus and cerebellum [156, 162]. Surprisingly, there is an absence of reported *CAPN2* disease-causing mutations in humans (**Table 4**). Of note, this might be related to a deleterious effect of the loss of function of this protease during development as shown by the fact that calpain-2 knock-out mice are embryonic lethal, suggesting the possibility of non-detected embryonic lethal *CAPN2* mutations in humans [163, 164]. Recently, it has also been proposed that *CAPN1* and *CAPN2* play opposite role in the brain: a neuroprotective action from calpain-1 and a neurodegenerative effect from calpain-2 [12, 165, 166]

Finally, this year, several variants in *CAPN15* have been associated with developmental eye disorders [90]. Loss of function mutations were identified in patients from 4 different families, presenting with microphthalmia and/or coloboma (disruption of the optic fissure closure). Some of the affected patients also displayed developmental delays and hearing loss. In the same study, it has been shown that calpain-15 KO mice suffered a marked growth deficit and exhibit various

eye defects, including anophthalmia or, microphthalmia and cataract, confirming a critical role for calpain-15 in the development of the ocular and neuromotor systems and comforting the implication of calpain 15 as a bona fide causative gene for the disease [90].

In addition to these monogenic diseases, it has also been suggested that single nucleotide polymorphisms (SNPs) in *CAPN8/9* might be linked with gastric ulcer susceptibility [82], that SNPs in *CAPN10* are linked to gestational diabetes mellitus [167], that mutations in *CAPN12* may aggravate symptoms of congenital ichthyosis [168] and that SNPs in *CAPN14* are associated with eosinophilic oesophagitis [169, 170] (**Table 4**). It is also interestingly to note that mutations in calpastatin have been reported to lead to a peeling skin syndrome [171].

Gene	Phenotypes associated to gene variant	Type of variant
<i>CAPN1</i>	*Spastic Paraplegia [155, 159, 160] and spinocerebellar ataxia [156].	Causative loss-of-function mutations
<i>CAPN3</i>	LGMD-R1 [143] *LGMD-D4 [149]	Causative loss-of-function mutations
<i>CAPN5</i>	*Autosomal dominant neovascular inflammatory vitreoretinopathy [152, 153, 172, 173].	Causative gain-of-function mutations
<i>CAPN8</i>	Gastric ulcers susceptibility [82]	Suspect that SNPs may be responsible for the susceptibility
<i>CAPN9</i>	Gastric ulcers susceptibility [82]	Suspect that SNPs may be responsible for the susceptibility
<i>CAPN10</i>	Type 2 diabetes [49, 167]	SNPs associated to disease
<i>CAPN12</i>	*Congenital ichthyosis [168]	Mutations that aggravate the existing phenotype
<i>CAPN14</i>	Eosinophilic oesophagitis [169, 170]	SNPs associated to disease
<i>CAPN15</i>	*Microphthalmia and/or coloboma [90]	Causative loss-of-function mutations

Table 4 – List of genetic calpain variations associated with human pathologies. New associations since last review from Sorimachi and colleagues in 2016 (*) [1].

Promising therapeutic approaches to cure a problem in calpain

According to the 2016 classification by Ono, Saido and Sorimachi, calpain-related diseases can be divided in three main categories: type 1, caused by exacerbated calpain activity; type 2, caused by parasites that use their or the host's calpain for their own survival; type 3, those genetic disorders caused by loss or gain of function mutations in one of the calpain genes (described in the previous chapters) [1]. Accordingly, a therapeutic strategy would involve either the inhibition of calpain activity (type 1, type 2 and gain of function type 3), or the rescue of calpain function (for loss of function type 3).

A comprehensive list of calpain inhibitors has been provided in a previous review by Sorimachi in 2016 [1]. We also refer the readers to a review by Dókus and colleagues about the known modulators of calpain activity and their possible application as potential drugs [17]. For now, only one inhibitor has been used in clinical trials [174-176]. Unfortunately, the mere inhibition of calpain might not be sufficient to resolve the pathology. Sometimes it may even have a series of undesired effects because of the lack of specificity, or the need for more fine-tuned regulation. Hyperactive mutations were identified in *CAPN5*. Interestingly, it was shown that calpain-5 KO mice do not present an apparent phenotype, suggesting that inhibition of calpain-5 might be a good candidate for a safe therapeutic strategy [177]. In addition, the availability of the crystal structure of calpain-5 in the inactive conformation provides useful structural information that can potentially guide effective inhibitor design [71].

For calpain loss-of-function mutations, the most intuitive strategy would be replacing the necessary level of expression via gene therapy. This may not be straightforward for a congenital form with developmental defects like in calpain-15 related diseases, but it might be for recessive mutations in LGMD-R1. To date, a gene transfer strategy mediated by vectors derived from the adeno-associated virus (AAV) is under development at Genethon for LGMD-R1. A first

preclinical proof of concept was obtained in 2006, with a study showing the efficacy of an AAV1 vector expressing calpain-3 under control of a muscle-specific promoter to restore the phenotype of mice deficient in calpain-3 after local administration [15]. The data showed transgene expression with correct protein localization at the sarcomere level and restoration of proteolytic activity, with correction of muscle atrophy and improvement in strength. However, a systemic administration of the same vector caused cardiotoxicity related to the ectopic expression of calpain-3 in the heart. To overcome the cardiotoxic adverse effect without compromising the therapeutic effect in skeletal muscle, modified vectors were developed [178]. A second generation vector was designed by introducing target sequences of a cardiac muscle-specific microRNA (miR208a) in the transgene expression cassette, or by the use of promoter sequences derived from endogenous promoters. In order to confirm the tolerance of this new type of vector, a pilot biodistribution and safety study was conducted in non-human primates. Results showed a total absence of cardiotoxicity after systemic injection with the modified vector. A study focusing on understanding the toxicity identified a mechanism explaining how the activity of calpain-3 is regulated in skeletal muscle [92]. A particular region of titin, a protein involved in the elasticity of the muscle to which calpain-3 binds, is indeed playing a role in inhibiting enzyme activity. Interestingly, this regulatory mechanism seems to be different between human and mouse hearts. With these encouraging results, a clinical trial is now in preparation at Genethon.

An emerging strategy to cure calpain disease is the genetic correction of calpain-linked mutations in patients-derived induced pluripotent stem cells for their prospective use in an autologous cell-based therapy. Promisingly, gene editing of LGMD2A iPSC has been recently proven to be effective in restoring CAPN3 expression level and in rescuing muscle function upon transplantation of corrected iPSC-derived muscle progenitors in immunodeficient CAPN3-KO mice (iPSC) [179].

CONCLUSIONS AND CONTRIBUTIONS BY DOCTOR HIROYUKI SORIMACHI TO THE CALPAIN FIELD

Lastly, in the following paragraphs, we want to give the reader a notion of the extent of Hiro's legacy to the calpain field. Those who knew him remember his heartfelt laugh, his love of French food and most importantly French wine. Work in the calpain field will continue and his memory will live on through it, and in the heart of his friends and colleagues.

Dr. Hiroyuki Sorimachi was the first to demonstrate the existence of tissue-specific calpains. In 1989, he and his colleagues screened cDNA hybridization libraries from human and rat muscle with a probe for the chicken calpain-1 and obtained a cDNA clone encoding for a novel member of the calpain family with homology of more than 50% to the ubiquitous human calpain-1 and -2. With a series of exquisite experiments for the time, they gave the first description of the structure of calpain-3 and via northern blots analyses they demonstrated that the expression of this new calpain was restricted to skeletal muscle [41]. In 1993, by screening cDNA libraries from various rat tissues, he also identified a new calpain with specific expression in the stomach (calpain-8) and showed that two isoforms of the protein were formed via alternative splicing [42]. Later in the nineties, while performing the genomic cloning of *CAPN8*, Dr. Sorimachi and Dr. Koichi Suzuki's research group identified another novel member of the calpain family (calpain-9) and demonstrated that its mRNA expression was restricted to the digestive tract [48]. Hiroyuki and Koichi also had a huge impact on our understanding of the structure of calpain. In 1998, they purified for the first time a recombinant human *CAPN2* using a baculovirus system, and in the early 2000s, the crystal structure of calpain-2 was published in collaboration with Dr. Wolfram Bode [64, 180]. Together with Dr. Suzuki, Hiroyuki's research triggered investigations into evolutionary relationships among calpain family members and was pivotal for the characterization of this superfamily [181]. Following this pioneering work, the advent of High-Throughput Sequencing Technologies

allowed for the identification of all 15 members of the calpain family listed above, half of which are predominantly tissue-specific (**Table 2**).

Dr. Sorimachi also contributed tremendously in unravelling the functions of several calpain family members. His most studied calpain family member was calpain-3. He was the first to describe the unusual short half-life of the muscle-specific calpain-3 and since then he went deeper into the understanding of its autolysis mechanism [101, 182, 183]. Dr. Sorimachi discovered calpain-3 interacts with titin and also described the mechanistic implications of this binding [3, 100, 101, 184, 185]. His laboratory also described the interaction between calpain-3 and substrates other than titin and provided evidence for a possible protein synthesis regulation by calpain-3 [186, 187] and contributed in our understanding of calpain-3 proteolytic activity and unconventional functions [79, 121, 123, 188-190]. The Sorimachi lab also discovered and characterized a gastrointestinal calpain complex formed by isoforms of calpain-8 and -9 involved in gastric mucosal defense [82, 83]. They described the localization of calpain-8 expression in gastric and duodenal mucosal cells, its Golgi localization and its interaction with the coatamer complex suggesting functions in membrane trafficking [191]. In addition, using a mouse model deficient for calpain-6, they were able to demonstrate how the loss of this protein promotes skeletal muscle development and regeneration [89]. Another Sorimachi's contribution was to demonstrate that calpain has a weak substrate specificity [124, 187].

Finally, he and his research group made huge efforts to make calpain information accessible to all through online resources (**Table 1**). The readers will find in these websites many information on the history and many other aspects of the calpain field.

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