The calpain system as a potential target for pelvic muscle reinforcement

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ABSTRACT

The fascial and muscular components within the pelvic floor create a support mechanism that facilitates storage and voiding of urine. Their constituents are mainly fibrillar collagens I and III, which are responsible for maintaining tensile strength. Stretching and recoiling is enabled by the elastic fibers consisting of elastin on a scaffold of microfibrils, fibrillin-1 and -2. Calpains are intracellular Ca2+-dependent cysteine proteases found in almost all eukaryotes and some bacteria. Calpains display limited proteolytic activity at neutral pH, proteolyzing substrates to transform and modulate their structures and activities, and are therefore called "modulator proteases". By making selective limited proteolytic cleavages, they modulate the activity of enzymes, including key signaling molecules, and induce specific cytoskeletal rearrangements, accounting for their roles in signal transduction and structural stabilization. Understanding these mechanisms should provide avenues for novel therapeutic strategies to treat pathological processes such as urinary incontinence and pelvic prolapse.

INTRODUCTION

Urinary incontinence (UI) in women has been claimed as a social disease thus its frequency has exceeded 5%. It is a distressing disorder, which affects mostly the elderly. Incontinence may take various forms: urge incontinence, stress incontinence, and mixed incontinence are the three main types. UI risk factors have been classified as urogynecological, constitutional, neurological, and behavioral. The anatomic structures that prevent urinary incontinence during elevations in abdominal pressure, can be divided into 2 systems: a sphincteric system and a supportive system. The action of the vesical neck and urethral sphincteric mechanisms at rest constrict the urethral lumen and keep urethral closure pressure higher than bladder pressure. The striated urogenital sphincter, the smooth muscle sphincter in the vesical neck, and the circular and longitudinal smooth muscle of the urethra all contribute to closure pressure. The mucosal and vascular tissues that surround the lumen provide a hermetic seal, and the connective tissues in the urethral wall also aid coaptation. Decreases in striated muscle sphincter fibers occur with age and parity [1, 2].

Pregnancy and childbirth are considered to be the most important UI risk factors. The persistent increased pelvic pressure, which is a result of hormone changes and increased uterine during pregnancy, leads to the damage of pelvic tissue. During vaginal delivery, the extremely stretched pelvic tissue resulted in the structure changes of muscles and supporting tissue around urethra, and ultimately induces urinary incontinence. The fascial and muscular components within the pelvic floor create a support mechanism that facilitates storage and voiding of urine. Their constituents are mainly fibrillar collagens I and III responsible for maintaining tensile strength. Stretching and recoiling is enabled by the elastic fibers consisting of elastin on a scaffold of microfibrils, fibrillin-1 and-2. In the assembly of the elastic fibers different elastin associated proteins are involved, among them the fibulins [3-9].

Urinary incontinence is in part attributed to qualitative and quantitative changes in connective tissue of the urogenital tract. It is reported that some of the smooth muscles from women with stress urinary incontinence appeared to show atrophy, apomorphosis, or apoptosis [10]. It was discovered that women with pelvic organ prolapse had collagen degradation and elastic proteins reduction e.g. fibulin-5, elastin, smooth muscle actin, myosin heavy chain, and caldesmon [11-15].

The calpain family of proteases are widely expressed in almost all the organs. Calpains have been implicated in basic cellular processes including cytoskeletal rearrangements, different signal transduction pathways, and cell apoptosis. In pathological status, abnormal elevation of Ca^{2+} activates calpains, which accelerate the degradation of various cytoskeleton, and ultimately induces the tissue damages [16].

Although our current understanding of the mechanisms underlying these processes remains limited, recent studies have begun to shed light on this subject. Here, we discuss advances that have provided insight into where calpains fit into the elastic proteins structure, and how the activities of calpains are modulated.

Calpains

Calpains (calcium-activated non-lysosomal proteases; CAPN) were originally identified as a unique class of calcium activated proteolytic enzymes present in the cytosolic fraction of brain extracts. These proteases were later named calpains to reflect their calcium dependency and homology with the protease domain of the papain family of cysteine proteases (papain, caspases, and cathepsin B, L, and S) [16, 17].

Molecular biological studies have shown that calpains constitute a superfamily, which exists ubiquitously in organisms ranging from humans to microorganisms, and showing limited proteolytic activity at neutral pH. Calpains result in the proteolysis of a broad spectrum of cellular proteins and a distinguishing feature of their activity is their ability to confer limited cleavage of protein substrates into stable fragments rather than complete proteolytic digestion. Calpains are regarded as a bio-modulators, because properties of the substrate proteins are often modulated upon hydrolysis by calpain. Therefore, calpains are considered a representative of the intracellular modulator proteases that govern various cellular functions such as signal transduction and cell morphogenesis [18, 19].

Humans have 15 genes that encode a calpain-like protease domain, and they generate diverse kinds of calpain homologues with combinations of several functional domains such as Ca^{2+}-binding domains and transmembrane domains. Among the best studied calpains are mammalian µ-calpain (also called calpain-1 or µCAPN) and m-calpain (also called calpain-2 or mCAPN), which are called the "conventional" calpains. They are mainly localized in the cytosol, show ubiquitous expression, and exhibit Ca^{2+}-dependent proteolytic activity. They are both heterodimers and consist of a large (80K) distinct µ- and m-calpain catalytic subunits, and a common small (30K) calpain regulatory subunit. The 80K of µ- and m-calpains isolated by dissociation in urea and that obtained by expression of the cDNA possess a full protease activity if properly folded, indicating that 30K is not essential for protease activity [18,19]. 30K also plays a role as a chaperon and is essential for 80K to have correct conformation. µ and m-calpains dissociate into subunits in the presence of Ca^{2+}. Calpains are regulated by their interacting partner, the endogenous calpain inhibitor calpastatin, which is encoded by the *CAST* gene [16, 18, 20].

Calpain family members

Actually, 15 human calpain genes have been numbered, *CAPN1–3* and *5-16*. The other two genes encode smaller regulatory proteins that associate with some of the catalytic subunits to form heterodimeric proteases. Several calpain isoforms are ubiquitously expressed, whereas many demonstrate tissue-specific expression patterns. Within the cell, the localization patterns of calpains are complex and somewhat variable, which means that their subcellular localization might be dynamically regulated and constitutes an important factor in the modulation of their functions [18, 21, 22]. Table 1 shows the calpains expression patterns in both tissues, as well as in the cell.

Calpains structure

The catalytic (large) and regulatory (small) subunits of conventional calpains can be divided into four (I-IV) and two domains (V-VI), respectively (Fig. 1). Domain I is present at the N-terminus of some calpains, and interacts with domain VI of the noncatalytic (small) subunits and may be important for stability. N-terminus of this domain is autolysed upon its initial activation by Ca^{2+}. This results in a lower requirement for Ca^{2+}, and different substrate specificity. The protease domain II is composed of two subdomains (IIa and IIb) with its substrate binding cleft in-between. It contains the active site catalytic triad Cys105, His262, and Asn286. Domain III consists of eight ß-strands arranged in a ß-sandwich, a structure very similar to TNF-& and the C2-domains found in various Ca^{2+}-

Table 1. Human calpain related genes, expression patterns and localization of its protein products [16, 18, 23].

Gene	Chr.	Calpain protein	Protease activity/ association with 30K	Expression (highest)/ localization in the cell
Catalytic subunits				
CAPN1	11q13	Calpain – 1	+/+	Ubiquitous (ascending and descending colon; esophagus; placenta; thyroid; trachea)/ diffuse cytoplasmic; endoplasmic reticulum; extracellular; Golgi apparatus; nucleus; plasma membrane
CAPN2	1q41-q42	Calpain – 2	+/+	Ubiquitous except for mammalian erythrocytes (kidney; lung; stomach; transverse colon; trachea)/ adhesion complexes; caveolae; diffuse cytoplasmic; endoplasmic reticulum; extracellular; Golgi apparatus; lipid rafts; nucleus
CAPN3	15q15	Calpain – 3	+/-	Skeletal muscle/n.d.
CAPN5	11q14	Calpain – 5	+/-	Ubiquitous (brain; kidney; liver; lung; testis; trachea)/ diffuse cytoplasmic; nucleus
CAPN6	Xq23	Calpain – 6	-/-	Placenta, embryonic muscles/ n.d.
CAPN7	3p24	Calpain – 7	N.d./-	Ubiquitous/ diffuse cytoplasmic; nucleus
CAPN8	1q41	Calpain – 8	+/-	Brain; digestive tract; stomach; testis/ n.d.
CAPN9	1q42.1-43	Calpain – 9	+/+	Digestive tracts, heart; stomach/ n.d.
CAPN10	2q37.3	Calpain – 10	N.d./n.d.	Ubiquitous (heart)/ diffuse cytoplasmic; nucleus
CAPN11	6p12	Calpain – 11	N.d./n.d.	Stomach; testis/ n.d.
CAPN12	19q13.2	Calpain – 12	N.d./n.d.	Hair follicle/ n.d.
CAPN13	2p22-p21	Calpain – 13	N.d./n.d.	Ubiquitous (lung; testis)/ n.d.
CAPN14	2p21-22	Calpain – 14	N.d./n.d.	Not detected
SOLH CAPN15	16p13.3	Calpain – 15	N.d./n.d.	Ubiquitous (brain)/ n.d.
C6ORFI03/CAPN16	6q24.3	Calpain – 16	-/n.d.	Ubiquitous/ n.d.
Regulatory subunits				
CAPNS1	19q13	CAPNS1	No	Ubiquitous (heart; interventricular septum; kidney; pancreas; prostate; skeletal muscle; testis)/ diffuse cytoplasmic; endoplasmic reticulum; Golgi apparatus; nucleus; plasma membrane
CAPNS2	16q13	CAPNS2	No	Ubiquitous (bladder; esophagus; prostate; trachea)/ n.d.
Calpain inhibitor				
CAST	5q15	Calpastatin	No	Ubiquitous (interventricular septum)/-

Chr. – chromosome; N.d. – not yet detected

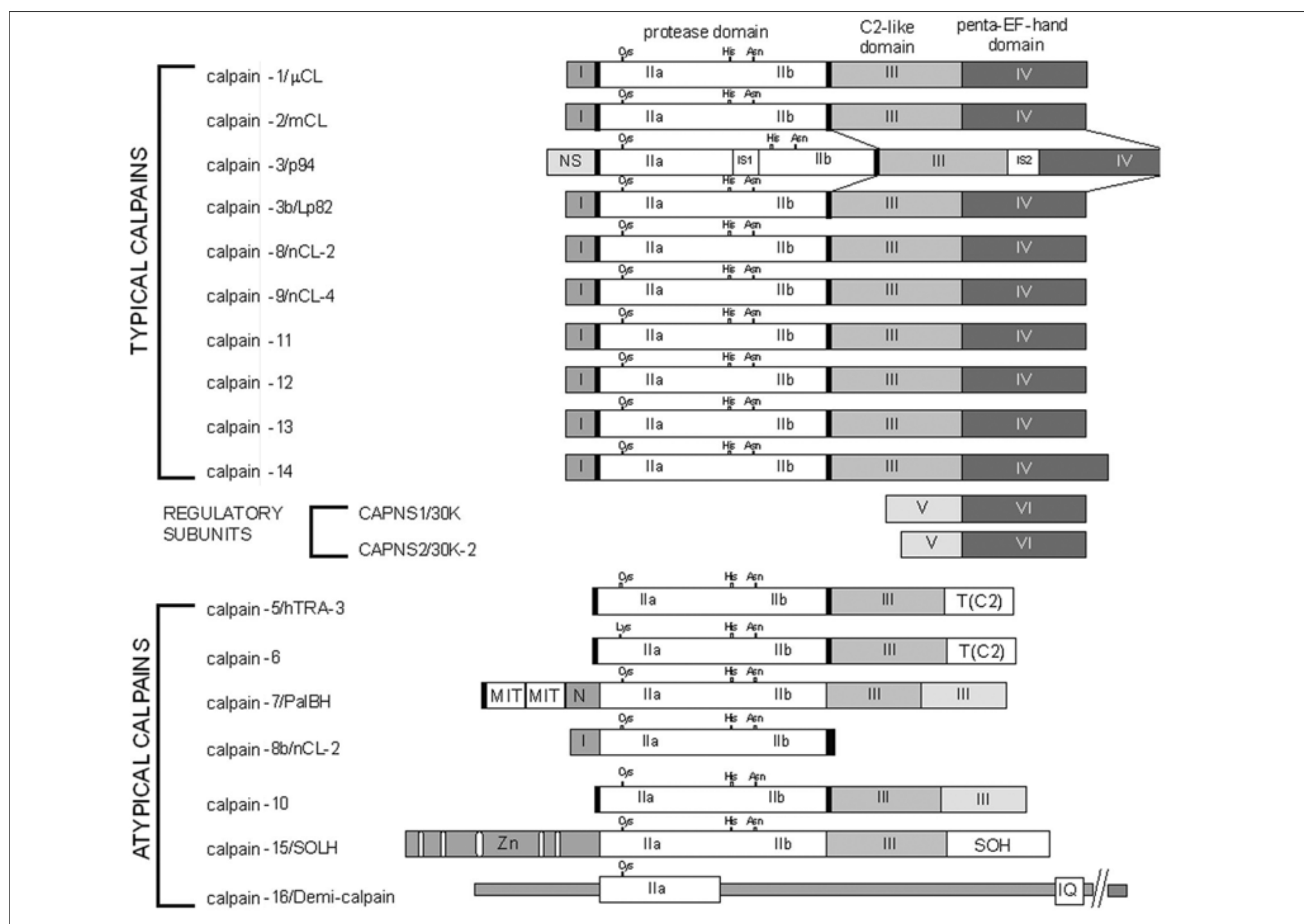

Fig. 1. Domain structures of human calpain family members. Typical calpains are composed of four domains (I–IV), whereas in the case of atypical calpains, certain domains of typical calpains have been deleted or replaced. The small subunit of calpain is composed of two domains (V and VI). Symbols used are: I – the N-terminal regulatory domain; IIa and IIb – the protease subdomains containing the active sites, Cys and His + Asn, respectively; III - the C2-!ike Ca^{2}+-binding domain; IV and VI – five EF-hand containing Ca^{2+}-binding PEF domain; V – glycine-rich hydrophobic domain; MIT – microtubule interacting and trafficking domain; NS, IS1, and IS2 – calpain-3-specific sequences; SOH – SOL subfamily homology domain; SOLH – small optic lobe homology; Zn- Zn-finger motif-containing domain.

regulated proteins such as protein kinase C isoforms and synaptotagmin. This domain binds Ca^{2+} and phospholipids. Domains IV and VI in the large and small subunits, respectively, contain five sets of EF-hand Ca^{2+} binding motifs similar to those found in calmodulin. The extreme COOH-terminal fifth EF-hand motif in IV and VI cannot bind Ca^{2+} but interacts with each other to assemble heterodimers. Domain V of the small subunits appears to have a very flexible structure as a consequence of being glycine rich. This domain is thought to interact with plasma membrane and/or membrane proteins through hydrophobic interactions. Most of this domain is autolysed during activation [16, 24].

Calpain activation and regulation

Calpain exists in the cytosol as an inactive enzyme and translocates to membranes in response to increases in the cellular Ca^{2+} level. The Ca^{2+} concentration required for proteolytic and other activities of the calpains are much higher than the 50-300 nM Ca^{2+} that exist in living cells [25]. Studies have also described molecules that seemed to reduce the Ca^{2+} requirements of the calpains in *in vitro* assays. For example, isovalerylcarnitine reduces the Ca^{2+} concentration required for maximal proteolytic activity of calpain-2 and increases its specific activity. At the membrane, calpain is activated in the presence of Ca^{2+} and phospholipids. Because calpains contain calcium-binding EF-hand motifs in domains IV and VI and because domain IV of calpain-1 and domain IV of calpain-2 are different, these were originally presumed to be responsible for the calcium dependent activation of calpains [24].

Furthermore, functional studies have demonstrated that domain II alone exhibits calcium-dependent protease activity and that non-EF-hand calcium-binding sites within the protease domain act as a calcium switch to align the catalytic triad [26-28].

Autocatalytic hydrolysis of domain I take place during activation, and dissociation of 30K from 80K occurs as a result. Activated calpain or 80K hydrolyzes substrate proteins at membranes or in cytosol after release from membranes, and has a lower requirement for calcium. In the absence of Ca^{2+}, two protease subdomains IIa and IIb are separated by structural constraints imposed by domain interaction. Ca^{2+}-induced structural changes that release the constraints are prerequisite for activation to form a functional catalytic site [18, 24].

Phosphorylation of calpain might be another important mechanism for activity regulation. Calpain-2 is activated by phosphorylation of Ser50 by the ERK (extracellular signal-regulated protein kinase), mitogen-activated protein (MAP) kinase and by EGF-induced pathway whereas calpain-1 does not contain a phosphorylatable site in this region. The MAP kinase kinase MEKK1 is required for normal calpain-2 activity [29]. MEKK1 associates with focal adhesion kinase (FAK) in adhesion complexes and appears to act upstream of ERK in the regulation of calpain-2 activation [30].

Calpain activity can also be inhibited by phosphorylation. Cyclic-AMP-mediated activation of protein kinase A (PKA) can block EGF-induced activation of calpain-2. This appears to occur through phosphorylation of calpain-2 by PKA, which probably restricts domain movement and freezes calpain-2 to an inactive conformation.

The residues in calpain-2 (Ser369 and Thr370) that appear to be the protein kinase A targets are conserved in other calpains, which suggests that phosphorylation of domain III represents yet another mechanism for regulating calpain activity [31, 32].

In addition, PKA reduces calpain-2 activity by blocking phosphatidylinositol-4,5-bisphosphate (PIP2) binding in the C2 domain of calpain. Recent observations indicate that PIP2 acts as a cofactor for calpain-2 and that phosphorylation by ERK or PKA alters the cellular distribution of the enzyme to modulate activity. Localization of calpain-2 at the plasma membrane, through PIP2 anchorage, is important for the activation of the protease [33, 34].

The binding of phospholipids also decreases the calcium requirement for calpains *in vitro* [35]. During the initial studies on purifying calpain-2, it was discovered that muscle extracts having calpain activity also contained a calpain inhibitor [36]. The name calpastatin was proposed for this inhibitor by Takashi Murachi in 1979. It consists of an N-terminal L domain that contains an N-terminal XL region, and four repetitive inhibitory domains (I–IV). The intrinsically unstructured nature of calpastatin allows it to reversibly inhibit up to four calpain heterodimers [20].

The equilibrium binding of calpains to calpastatin is extremely pH sensitive and decreases as pH decreases. Immunolocalization results suggest that the calpains and calpastatin are frequently localized in cells, so the cells must possess some mechanism to allow calpain activity in the presence of calpastatin. Otherwise, increased CA^{2+} concentrations would cause calpastatin binding before the calpains could initiate any proteolytic activity [28, 37].

Experiments of the binding of autolytic fragments of calpains to calpastatin indicated that calpastatin bound to both domains IV and VI of the calpain molecule. Subsequent studies using expressed subdomains of the calpastatin molecule showed that a 14-amino acid subdomain was conserved around the four domains of the calpastatin molecule and bound specifically to domain IV of calpain in a Ca^{2+} dependent manner [36].

Research showed that optimal conditions for *in vitro* calpain activity were pH 7.5 at 25°C. However, this condition does not naturally occur in a slaughtered animal. Minimal activity is seen at 5°C / pH 5.5. Factors such as the reduced solubility of calcium at low pH and temperatures or extraction buffer pH could affect calpain activity. Unless the homogenate is kept above pH 6.5, calpain activity reduces significantly because the enzyme is precipitated [38].

Calpains substrates and their catalytic action of protein structure

Dysfunction of smooth muscle and connective tissue appears to be directly related to the phenomenon of urinary incontinence in women. Particularly important are the proteolytic mechanisms that may change the structural and functional properties of proteins forming the urogenital system. While the sliding filament model adequately describes the basic mechanism of contraction in all muscle types, there are significant differences between skeletal and smooth muscle. An appreciation of these differences stems from the observation that although smooth muscle lacks troponin, its contractile activity is still regulated by cytoplasmic calcium levels [39, 40].

In addition to the major contractile proteins actin and myosin, smooth muscle cells contain numerous proteins involved in the regulation of contraction. Actin and myosin are the contractile proteins and form the actin and myosin filaments of the myofibril. Cross-bridges are formed between the actin and myosin filaments and conformational changes in myosin generate the contractile force during contraction. In the relaxed state, a muscle generates very little tension, and can easily be stretched by a force pulling on it. This means that there are no cross-bridges between actin and myosin filaments and the filaments of each sarcomere slide passively over one another. In contrast to actin and myosin, tropomyosin plays the role of regulatory protein and assists in turning the contractile process 'on' and 'off' [39, 40].

In addition to the major contractile proteins actin and myosin, smooth muscle cells contain numerous proteins involved in the regulation of contraction, such as such as caldesmon, calponin, and tropomyosin (strengthens microfilaments); in regulation of microfilament stability, such as gelsolin (fragments microfilaments); in organization of the microfilament network, such as filamin (crosslinks microfilaments), α-actinin, and fimbrin (bundle microfilaments); and a set of proteins involved in the attachment of microfilaments to plasma membrane, such as vinculin, talin, paxillin, and tensin [40]. The major proteins of the intermediate filament system in smooth muscle cells are desmin and vimentin [41, 42].

Many contractile and cytoskeletal proteins have muscle-specific variants that can be used as smooth muscle cell phenotypic markers: α- and -γ-smooth muscle actins, smooth muscle myosin heavy chains SM-1 and SM-2, *meta*- and γ-vinculin, desmin, heavy (h)-caldesmon, smooth muscle α-tropomyosin, and calponin. A significant portion of these proteins are substrates for calpains [40, 43].

So far, two experimental papers were published on the expression of calpains in material derived from women with urinary incontinence and uterovaginal prolapse. Wu et al. [44] found overexpression of calpain-2 and low expression of calpastatin in periurethral vaginal tissues that were taken at 1-1.5 cm below the urinary meatus. In the studied material Mucosa, submucosa, connective tissue, and smooth muscle were contained.

In turn, Chen and coworkers studied the expression of calpain-1 and calpain-2 in the vaginal walls of women with and without uterovaginal prolapse [45]. They conclude that calpain expression may be compromised in the anterior vaginal wall of women with uterovaginal prolapse who have abnormal histologic changes in the vaginal connective tissues or have anterior vaginal laxity.

Although the mechanism of calpain action in these tissues is not clear, it can be assumed that it is linked to proteolysis of structural proteins, both smooth muscle and connective tissue.

Croall et al., found that the calmodulin binding proteins, caldesmon and calponin, are cleaved by both major isoforms of calpain *in vitro* [17]. Qualitatively, the cleavage pattern of each substrate is unchanged by the presence or absence of calmodulin suggesting that the interaction between calmodulin and these calmodulin-binding proteins does not alter substrate recognition by calpain. Authors conclude that calmodulin-binding domains do not provide substrate recognition sites for calpains. It seems likely that the calmodulin-like regions of calpain function to bind calcium and to regulate enzyme conformation as required for activity and that they do not interact directly with most substrates [46]. Similar results were obtained for calponin by Tsunekawa and coworkers [47].

In turn, Yoshimoto et al., suggested that acidic calponin, an actin binding protein expressed in smooth muscle, is also cleaved by calpain, and this protein might be involved in the calpain-regulated actin cytoskeleton. Further proteins degraded by calpain-2 are actin and vimentin [48].

Yoshida et al., demonstrated proteolysis of two important cytoskeletal proteins, actin and vimentin, in the lens fiber cells by calpain [49].

Connective tissue is the most important part of pelvic supporting tissue, which maintains the flexibility and toughness of pelvic floor. Collagen secreted by fibroblast is the major compartment of connective tissue. It is reported that collagenolytic activity might be related with the occurrence of urinary incontinence [50-52].

Von Wnuck Lipinski and coworkers found that collagen I and III fragments proteolytically released from the extracellular matrix

by matrix metalloproteinases may propagate apoptosis of smooth muscle cells by calpain-mediated inactivation of anti-apoptotic proteins such as X-chromosome-linked inhibitor of apoptosis (xIAP) [53, 54]. They suggest that degraded collagen fragments simultaneously activated an apoptotic pathway triggered by calpain/caspase activation and a survival pathway triggered by NF-κB activation. The survival pathway was dominant over the apoptotic as substantial cell death took place only after inactivation of NF-κB.

CONCLUSIONS

Calpain activity contributes to remodeling of the actin cytoskeleton, cell migration, and oncogenic transformation. Structure-function relationships for various calpain family members is now providing valuable insights into the complex regulation of these proteases and should help to design therapies for disorders involving calpain activation in the future.

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