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Calponin Isoforms *CNN1*, *CNN2* and *CNN3*: Regulators for Actin Cytoskeleton Functions in Smooth Muscle and Non-Muscle Cells

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Abstract

Calponin is an actin filament-associated regulatory protein expressed in smooth muscle and multiple types of non-muscle cells. Three homologous genes, *CNN1*, *CNN2* and *CNN3*, encoding calponin isoforms 1, 2, and 3, respectively, are present in vertebrate species. All three calponin isoforms are actin-binding proteins with functions in inhibiting actin-activated myosin ATPase and stabilizing the actin cytoskeleton, while each isoform executes different physiological roles based on their cell type-specific expressions. Calponin 1 is specifically expressed in smooth muscle cells and plays a role in fine-tuning smooth muscle contractility. Calponin 2 is expressed in both smooth muscle and non-muscle cells and regulates multiple actin cytoskeleton-based functions. Calponin 3 participates in actin cytoskeleton-based activities in embryonic development and myogenesis. Phosphorylation has been extensively studied for the regulation of calponin functions. Cytoskeleton tension regulates the transcription of *CNN2* gene and the degradation of calponin 2 protein. This review summarizes our knowledge learned from studies over the past three decades, focusing on the evolutionary lineage of calponin isoform genes, their tissue- and cell type-specific expressions, structure-function relationships, and mechanoregulation.

Keywords

calponin isoform genes; actin cytoskeleton; smooth muscle; non-muscle cell motility; mechanoregulation

1. Introduction

Calponin was first identified in chicken gizzard smooth muscle, with a proposed function as a striated muscle troponin T-like protein that binds actin thin filaments for the regulation of smooth muscle contraction (Takahashi et al., 1986; Takahashi et al., 1988b). Extensive research followed and found that calponin is an actin filament-associated regulatory protein

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of 34–37 kDa (292–330 amino acids) expressed in both smooth muscle and many non-muscle cell types and functions as an inhibitor of actin-activated myosin ATPase.

In vitro protein binding studies demonstrated that calponin binds actin (Takahashi et al., 1986; Winder and Walsh, 1990) and cross-links actin filaments (Leinweber et al., 1999). Calponin also binds or interacts with many other cytoskeleton and related proteins, including tropomyosin (Takahashi et al., 1988a; Childs et al., 1992), myosin (Szymanski and Tao, 1997), tubulin (Fujii et al., 1999), desmin (Wang and Gusev, 1996; Mabuchi et al., 1997), gelsolin (Ferjani et al., 2006), Ca^{2+} -calmodulin (Takahashi et al., 1986), Ca^{2+} -S100 (Fujii et al., 1994), and phospholipids (Bogatcheva and Gusev, 1995). Calponin was also reported to interact with caldesmon (Graceffa et al., 1996) and α -actinin (North et al., 1994), although these observations may reflect a co-localization on actin filaments (Czurylo et al., 1997; Leinweber et al., 1999a). Functional significance of these biochemically detected bindings of calponin to cytoskeleton proteins or regulatory molecules is largely unknown and merits further investigation.

Besides modulating the function of smooth muscle myofilaments and contractility, calponin also regulates the actin cytoskeleton of non-muscle cells to effect on many cellular activities, such as proliferation, adhesion, migration, differentiation, phagocytosis and fusion. To summarize our current knowledge of the calponin isoform genes and proteins, this review focuses on the evolution, tissue and cell type-specific expression, structure-function relationships, transcriptional regulation and posttranslational modifications.

2. Three calponin isoform genes

Three isoforms of calponin encoded by three homologous genes have been found in vertebrate species: A basic calponin (calponin 1, isoelectric point (pI) = 9.4) encoded by *CNN1* (Gao et al., 1996), a neutral calponin (calponin 2, pI = 7.5) encoded by *CNN2* (Strasser et al., 1993) (Masuda et al., 1996) and an acidic calponin (calponin 3, pI = 5.2) encoded by *CNN3* (Applegate et al., 1994). In the human genome, *CNN1* is located on chromosome 19p13.2-p13.1 (Miano et al., 1997), *CNN2* on 19p13.3 (Masuda et al., 1996) and *CNN3* on 1p22-p21 (Maguchi et al., 1995) (Table 1).

A nomenclature of “*h*-calponins” was created based on an early observation of a low molecular weight form of calponin in human urogenital smooth muscle protein extracts (Draeger et al., 1991), which was named “*l*-calponin”. However, such low molecular weight isoform of calponin has not been confirmed with corresponding mRNA or any evidence of genomic structure. Therefore, the “*l*-calponin” band observed might be a product of partial proteolysis. Based on this fact, recent literature often used Calponin 1, Calponin 2, and Calponin 3, respectively, for the protein products of *CNN1*, *CNN2* and *CNN3* genes, which are also used in the present review.

Comparisons of cDNA sequences and the deduced protein primary structures demonstrated that calponin 1, 2 and 3 have largely conserved structures. The phylogenetic tree produced by alignment of amino acid sequences of the three calponin isoform genes in vertebrate species investigated showed that each of the calponin isoforms is conserved in the vertebrate

phylum whereas the three isoforms have significantly diverged during evolution (Fig. 1). This relationship likely reflects adaptations of the calponin isoforms to their potentially differentiated cellular functions.

Gene targeting studies showed that mice with the knockout of either *Cnn1* or *Cnn2* gene are viable and fertile (Takahashi et al., 2000; Huang et al., 2008). In contrast, *Cnn3* knockout resulted in embryonic and postnatal lethality due to defective development of the central nervous system (Flemming et al., 2015). The apparently critical role of calponin 3 in embryonic development appears to be consistent with a more ancient emergence as suggested by the higher degree of divergence among vertebrate *CNN3* genes than that of *CNN1* and *CNN2* (Fig. 1). The possibility that calponin 3 represents the prototype calponin that evolved into the three present-day isoforms is worth further investigation.

3. Tissue and cell type-specific expression of calponin isoform genes

The three calponin isoform genes exhibit different patterns of tissue and cell type-specific expressions, indicating functions corresponding to specific cellular environment and/or activity in different tissue and cell types.

The expression of *CNN1* encoding calponin 1 is highly specific to smooth muscle cells. The majority of previous studies on structure-function relationship of calponin were obtained from experiments using chicken gizzard calponin. Sequence similarity, physical properties, and immunological reactivity indicate that chicken gizzard calponin is an ortholog of the mammalian calponin 1. The contents of calponin 1 vary among different types of smooth muscle. For example, a high level of expression is seen in phasic smooth muscle of the digestive tract and a very low level of expression is found in avian trachea (Jin et al., 1996). The expression of calponin 1 in smooth muscle is up-regulated during development (Hossain et al., 2003). Calponin 1 is rapidly down-regulated when growth-arrested smooth muscle cells re-enter the G1 phase of the cell cycle to start proliferating (Samaha et al., 1996). The mature smooth muscle cell-specific high level expression of calponin 1 suggests a role in smooth muscle differentiation and contractile functions.

More recent studies reported the expression of calponin 1 in non-muscle cells, such as cultured human glomerular mesangial cells (Sugenoya et al., 2002), cultured pancreatic precursor cells (Morioka et al., 2003), periglomerular myofibroblasts in rat kidney (Lee et al., 2010) and sertoli cells in rat testis (Zhu et al., 2004). While it is potentially interesting to explore the function of calponin 1 in these mesenchymal types of cells, these observations were primarily based on antibody reactivity and require further investigation.

Calponin 2 is found in a broader range of tissue and cell types, including developing and remodeling smooth muscles and adult mature smooth muscles (Hossain et al., 2003), epidermal keratinocytes (Fukui et al., 1997), fibroblasts (Hossain et al., 2005), lung alveolar cells (Hossain et al., 2006), endothelial cells (Tang et al., 2006), myeloid white blood cells (Huang et al., 2008), myoblasts (Jiang et al., 2014), prostate cancer cells (Hossain et al., 2014) and platelets (Hines et al., 2014). These cell types can be paced in three groups: a) cells that are physiologically under high mechanical tension, e.g., smooth muscle cells in the

wall of hollow organs, epithelial and endothelial cells; b) cells that have high rates of proliferation, e.g., myoblasts; and c) cells that are actively migrating, e.g., fibroblasts and macrophages. The tissue distributions of calponin 2 may imply a role in regulating tension in the cytoskeleton and cell motility-based functions, especially cellular interactions with the extracellular mechanical environment (Liu and Jin, 2015).

Calponin 3 is found in the brain with expressions in neurons (Represa et al., 1995; Trabelsi-Terzidis et al., 1995), astrocytes (Agassandian et al., 2000), and glial cells (Ferhat et al., 1996), where it may function in regulating the actin cytoskeleton with a proposed role in the plasticity of neural tissues (Plantier et al., 1999; Ferhat et al., 2003). Calponin 3 is also found in embryonic trophoblasts and myoblasts with a function in cell fusion during embryonic development and myogenesis (Shibukawa et al., 2010; Shibukawa et al., 2013). A recent study reported calponin 3 in B lymphocytes whereas the physiological significance requires further investigation (Flemming et al., 2015).

4. Protein structure-function relationship of calponin isoforms

Primary structures of the three calponin isoforms have been determined in multiple species by cDNA cloning and sequencing. Current knowledge of the structure-function relationship of calponin was largely learned from biochemical studies of calponin 1 in smooth muscles. Summarized in Fig. 2, primary structure of the three calponin isoforms consists of a conserved N-terminal calponin homology (CH) domain, a conserved middle region containing two actin-binding sites, and a C-terminal variable region that constitutes the main differences among the isoforms.

4.1 The N-terminal CH domain

Outlined in Fig. 2, a sequence motif of ~100 amino acids in the N-terminal region of calponin (residues 29–129) is identified as the “calponin homology (CH) domain”. This nomenclature was based on the finding of its potentially homologous sequence motifs in a number of actin-binding proteins, including α -actinin, spectrin, filamin (Gimona et al., 2002) and plectin (Fontao et al., 2001). CH domain structure may also present as tandem repeats in some proteins. The CH domain in many proteins has been identified with functions of either a part of actin-binding site or serving as a regulatory structure for the actin binding activity.

In contrast to its observed functions in various other proteins, no definitive function has been demonstrated for the CH domain in calponin. The CH domain is not the binding site between calponin and F-actin nor regulates the modes of calponin-F-actin binding (Galkin et al., 2006). The CH domain in calponin was found to bind to extra-cellular regulated kinase (ERK) (Leinweber et al., 1999b) and calponin was co-immunoprecipitated with mitogen-activated protein kinase (MAPK) (Menice et al., 1997), suggesting that calponin may serve as adaptor protein in the ERK signaling cascades of smooth muscle and non-muscle cells.

4.2 Actin-binding sites in the middle region

A large collection of evidence demonstrated the role of calponin as an actin-binding protein that inhibits the MgATPase activity of smooth muscle myosin (Winder and Walsh, 1990;

Mezgueldi et al., 1992) (Abe et al., 1990) (Winder et al., 1993b) and hinders the movement of actin filaments over immobilized myosin heads (Shirinsky et al., 1992) (Haeberle, 1994). Calponin binds to F-actin through two binding sites in the middle region (residues 144–162 and 171–188 in chicken calponin 1). These two actin-binding sites bind F-actin in comparable manner and are conserved in the three calponin isoforms.

The effect on inhibiting actomyosin MgATPase was only detected for site 144–162, whereas site 171–188 was able to reverse the inhibition (Mino et al., 1998) suggesting a potentially regulatory function. Calponin induces actin polymerization and inhibits depolymerization of actin filaments. Addition of calponin greatly retarded the actin depolymerization process (Kake et al., 1995). The two actin binding sites of calponin share their binding sites on actin, *i.e.*, residues 18–28 and 360–372. Only binding to the 18–28 site results in a reduction in F-actin depolymerization, correlative to the fact that this site of actin determines the release of ATP from actin (Ferjani et al., 2010). These *in vitro* findings are consistent with the effect of calponin 2 on stabilizing actin cytoskeleton in living cells (Hossain et al., 2005).

Other than binding to F-actin, the region of residues 144–188 of calponin is also essential for the binding to tropomyosin and Ca^{2+} -calmodulin (Mezgueldi et al., 1995). Recent high-resolution structural studies of this region indicated that the sequentially positioned segments 140–146, 159–165, 189–195, and 199–205 tend to form four transient α -helices, probably providing the conformational malleability needed for the functionally promiscuous nature of this region of calponin (Pfuhl et al., 2011).

There are three repeating sequence motifs in calponin next to the C-terminal region. This repeating structure is conserved in all three isoforms and across species. Illustrated in Fig. 2, the first repeating motif overlaps with the second actin-binding site (residues 171–188) and contains the protein kinase C (PKC) phosphorylation residues Ser₁₇₅ and Thr₁₈₄ that have no counterparts in the first actin-binding site (residues 144–162). This structural feature is consistent with the hypothesis that the second actin-binding site plays a regulatory role in the binding of calponin to the actin filament. Similar sequences as well as potential phosphorylation sites are present in repeats 2 and 3 although their functions and regulation have not been determined. Therefore, the biological significance of these repeating motifs in calponin remains to be established.

4.3 The C-terminal variable region

The C-terminal segment of calponin is a variable region that has diverged significantly among the three isoforms. The variable lengths and amino acid compositions of the C-terminal segment produce the size and charge differences among the three calponin isoforms (Table 1). The corresponding charge features rendered calponin 1, 2 and 3 the name of “basic”, “neutral” and “acidic” calponins, respectively (Jin et al., 2008; Wu and Jin, 2008; Liu and Jin 2015).

The C-terminal segments of calponin have been shown to have differentiated effects on weakening the binding of calponin to F-actin. Deletion of the C-terminal tail segment of calponin strongly enhanced the actin-binding and bundling activities of all three isoforms, indicating a regulatory inhibition effect of the C-terminal variable region (Bartegi et al.,

1999) (Danninger and Gimona 2000). Domain-swap experiments demonstrated that the C-terminal segment of calponin 2 decreased the association all three isoforms to actin cytoskeleton whereas the C-terminal segment of calponin 1 had little effect. Accordingly, calponin 2 may have the lowest binding affinity for F-actin among three isoforms. The C-terminal tail regulates the interaction with F-actin by altering the function of the second actin-binding site of calponin (Burgstaller et al., 2002). These findings suggest that C-terminal variable region may determine the cell type-specific functions and/or subcellular distributions of the calponin isoforms.

The binding and interaction of calponin with its protein partners are summarized in Fig. 3.

5. Biological functions of calponin

Although the three isoforms of calponin have structural variations in the C-terminal region and exhibit distinct tissue and cell type-specific expressions, the two actin-binding sites are conserved among the isoforms. This conserved function forms a basis for the role of calponin in regulating various actin filament-based cellular activities.

5.1 Calponin 1 in the regulation of smooth muscle contractility

Calponin 1 was the first calponin isoform identified in differentiated smooth muscle cells (Takahashi et al., 1986; Takahashi et al., 1988b) and its function in regulating smooth muscle contractility has been extensively investigated. Data from numerous *in vitro* experiments suggest that calponin 1 functions as an inhibitory regulator of smooth muscle contractility through inhibiting actomyosin interactions (Takahashi et al., 1986; Allen and Walsh, 1994) (Takahashi et al., 1988a). In this regulation, binding of Ca^{2+} -calmodulin and PKC phosphorylation dissociates calponin 1 from the actin filament and facilitate smooth muscle contraction (Naka et al., 1990).

In vivo experimental data also support the role of calponin 1 as a regulator of smooth muscle contractility. Aortic smooth muscle of adult Wistar Kyoto rats, which naturally lacks calponin 1, is fully contractile, but showed decreased sensitivity to norepinephrine activation (Nigam et al., 1998) (Facemire et al., 2000). Proteolysis of calponin 1 by matrix metalloproteinase-2 in endotoxemic rats resulted in vascular hypocontractility when stimulated with phenylephrine (Castro et al., 2012). Vas deferens smooth muscle from calponin 1 knockout mice showed significantly faster maximum shortening velocity (Takahashi et al., 2000). Calponin 1 knockout mice exhibited the same rest mean arterial pressure (MAP) as that of WT mice, but blunted MAP response to phenylephrine administration (Masuki et al., 2003). These data suggest that although calponin 1 is not an essential element of smooth muscle contraction, it is a regulator of smooth muscle contractility and responsiveness to contractile activation.

More recent studies showed that calponin 1 co-localizes with and stabilizes actin stress fiber in smooth muscle cells, but translocates from the central contractile bundles consisting of smooth muscle α -actin and myosin to peripheral podosomes consisting of β -actin and other adhesion proteins in response to PKC agonist (Parker et al., 1998) (Dykes and Wright,

2007), suggesting that calponin 1 regulates contractile actin-myosin filaments as well as the non-contractile actin cytoskeleton in smooth muscle cells.

CARG box is a representative transcriptional factor binding element in genes encoding smooth muscle contractile proteins. The activation of CARG box is linked to the contractile phenotype of vascular smooth muscle cells. *CNN1* gene contains conserved CARG-rich regions, which is necessary for the smooth muscle-specific expression of calponin 1 *in vivo* (Long et al., 2011).

The expression of *CNN1* gene is markedly upregulated in postnatal and adult smooth muscle cells and may serve as a marker of smooth muscle differentiation and maturation (Duband et al., 1993). It was reported that *CNN1* gene was activated by angiotensin II-induced G-protein signaling in a time and dose-dependent manner. ERK1/2 might be partially involved in this signaling pathway (Dulin et al., 2001). *In vivo* experimental data also showed that angiotensin II could up regulate *Cnn1* gene expression in rat aorta (Castoldi et al., 2001).

5.2 Calponin 2 in the regulation of actin cytoskeleton in non-muscle cells

Calponin 2 is expressed in smooth muscle and multiple types of non-muscle cells, such as epidermal keratinocytes (Hossain et al., 2005), lung alveolar cells (Hossain et al., 2006), endothelial cells (Tang et al., 2006), fibroblasts (Hossain et al., 2005), blood cells of myeloid lineages (Huang et al., 2008), lymphocytes (Flemming et al., 2015), epithelial cells, myoblasts (Jiang et al., 2014), prostate gland epithelial cells (Hossain et al., 2014) and platelets (Hines et al., 2014). Like calponin 1 in smooth muscle cells, calponin 2 is an actin-binding protein associated with the actin cytoskeleton of smooth muscle and a broad range of non-muscle cells. Transfective expression of calponin 2 in cultured cells that lack endogenous calponin increased the resistance of actin stress fibers to cytochalasins B, indicating increased stability of the actin cytoskeleton (Hossain et al., 2005).

Various cellular functions that are regulated by calponin 2 have been identified in different cell types. Calponin 2 was first found to regulate the rate of cell proliferation. Significant amounts of calponin 2 are found in growing smooth muscle tissues such as embryonic stomach and urinary bladder as well as the uterus during early pregnancy (Hossain et al., 2003). The expression of calponin 2 decreases to lower levels in quiescent adult smooth muscle cells while the expression of calponin 1 is up-regulated (Hossain et al., 2003). These data suggest that although calponin 2 is an inhibitory regulator, it is required in rapidly proliferating cells, possibly as an equilibrium factor for the regulated dynamic changes of the actin cytoskeleton during cell proliferation and cytokinesis.

Supporting this notion, prostate cancer cells with decreased expression of calponin 2 and fibroblasts isolated from *Cnn2* gene knockout mice showed significantly increased rates of proliferation in culture (Hossain et al., 2014). On the other hand, over expression of calponin 2 in a rabbit smooth muscle cell line hindered cell proliferation with increased number of binuclear cells indicating a blocking of cytokinesis (Hossain et al., 2003). Consistently, boosting the level of calponin 2 in prostate cancer cells via transfective expression reduced the rate of cell proliferation (Hossain et al., 2014).

Calponin 2 regulates cell motility, presumably through inhibiting the actin activated myosin motor functions. An *in vivo* study found that calponin 2 mRNA is present in the protrusions at the leading edge of migrating neural crest cells. As the actin-driven formation of polarized cell protrusions is important for the migration of neural crest cells, knockdown of *Cnn2* expression in neural crest cells resulted in randomized outgrowth of protrusions and migration defects, accompanied by central stress fiber formation and reduced peripheral actin network (Ulmer et al., 2013). To demonstrate a common role of calponin 2 in regulating cell migration, primary fibroblasts and peritoneal macrophages isolated from calponin 2 knockout mice migrate faster than that of wild type control cells (Huang et al., 2008).

Calponin 2 may have effect on cell migration differently in different cell types and in different biological processes. A study showed that forced expression of calponin 2 in endothelial cells enhanced angiogenic cell migration *in vivo* and anti-sense calponin 2 RNA reduced chemotaxis of human umbilical vein endothelial cells in culture (Tang et al., 2006). This observation was opposite to the inhibitory effects on cell motility seen in many other cell types so far studied. A hypothesis is that a proper level of calponin 2 might be critical to maintain the physiological equilibrium of cell motility. Different biological processes in different cell types may require different internal set points for this physiological balance, in which the requirement for the level and activity of calponin could be different. This hypothesis is worth investigation.

Significant amounts of calponin 2 are found in human and mouse platelets (Hines et al., 2014). Platelet adhesion is a critical initial step in blood coagulation and thrombosis. In a microfluidic flow-based thrombosis assay, the time to the initiation of rapid platelet accumulation and thrombosis was significantly longer for blood samples from *Cnn2* knockout versus wild-type control mice (Hines et al., 2014). The effect of calponin 2 on facilitating the velocity of cell adhesion was also shown when prostate cancer cells expression high or low calponin 2 were compared on substrates of high or low stiffness (Hossain et al., 2014).

One of the interesting findings is that calponin 2 modulates immune cell function *in vitro* and *in vivo*. Significant amounts of calponin 2 are found in blood cells of myeloid lineage. Calponin 2-null bone marrow-differentiated monocytes from *Cnn2* gene knockout mice proliferated faster than wild type control cells. Calponin 2-null macrophages migrated faster and exhibit an enhanced phagocytotic activity (Huang et al., 2008). In global as well as in myeloid cell-specific *Cnn2* knockout mice, the development of inflammatory arthritis induced by anti-glucose-6-phosphate isomerase serum was significantly attenuated as compared with that in control mice (Hossain et al., 2011). Deletion of calponin 2 in macrophages also significantly attenuated the development of atherosclerosis lesions in apolipoprotein E knockout (*ApoE*^{-/-}) mice (Liu and Jin, 2015). These data demonstrate that calponin 2 regulates macrophage activities and controlling calponin 2 expression and function in macrophages may be explored for applications in the treatment and prevention of inflammatory diseases.

The mechanism by which calponin 2 regulates the functions of actin cytoskeleton remains to be investigated. In addition to stabilization of actin filaments (Hossain et al., 2005), our recent study demonstrated that calponin 2-null fibroblasts isolated from *Cnn2* knockout mice have increased cell traction force that is generated by myosin II motor activity (Hossain et al., 2015). This finding is consistent with the established role of calponin 1 as an inhibitory regulator of smooth muscle actomyosin ATPase and contractility (Walsh, 1991).

5.3 Role of calponin 3 in development, neuronal plasticity and cell fusion

Calponin 3 was found in stress fibers in skin fibroblasts and myofibroblasts in the proliferation phase of wound healing. *CNN3* knockdown in primary fibroblasts impaired stress fiber formation, resulting in decreased cell motility and contractile ability (Daimon et al., 2013).

Calponin 3 was detected in the brain with a potential function in regulating actin filaments during neuronal remodeling (Rami et al., 2006). Calponin 3 was also found in dendritic spines of adult hippocampal neurons to regulate dendritic spine plasticity (Ferhat et al., 2003). While mice with systemic knockout of *Cnn1* (Takahashi et al., 2000) or *Cnn2* (Huang et al., 2008), or both *Cnn1* and *Cnn2* (Feng et al., 2016) survive to adulthood and fertile, systemic knockout of calponin 3 in mice results in embryonic and neonatal lethality due to defect in the development of central nervous system (Flemming et al., 2015). This observation is consistent with the critical role of calponin 3 in the nervous system. Orthodenticle homeobox 2 (*Otx2*) is a group of homeobox gene that plays a role in embryonic brain and systemic development. It has been shown that *XclpH3*, the *Xenopus* homologue of *CNN3*, was directly stimulated by *Otx2* and involved in preventing cells convergence extension movements (Morgan et al., 1999).

CNN3 was found in the trophoblasts of human placenta and plays a role as a negative regulator of trophoblast fusion. Knockdown or dissociation of calponin 3 from cytoskeleton in response to PKC phosphorylation promoted fusion of trophoblasts (Shibukawa et al., 2010). Consistently, calponin 3 was also present in myoblasts as an inhibitory regulator of cell fusion. Overexpression of calponin 3 in C₂C₁₂ myoblasts inhibited cell fusion during *in vitro* differentiation, whereas *Cnn3* gene knockdown promoted cell fusion and the expression of skeletal muscle myosin. The inhibitory effect of calponin 3 was reversed upon phosphorylation by Rho-associated kinase 1/2 (ROCK1/2) (Shibukawa et al., 2013). These experimental data implicate a critical role of calponin 3 in embryonic development, neural plasticity and myogenesis, which is worth detailed investigation.

6. Regulation of calponin functions

Actin thin filaments are essential elements in the contractile machinery of smooth muscle cells. The non-muscle actin cytoskeleton plays multiple functions in various cellular activities. Therefore, the role of calponin in regulating these actin filament-based cellular functions is critical to many physiological and pathological processes. Extensive studies have investigated the regulation of calponin gene expression and function at the transcriptional and posttranslational levels.

6.1 Phosphorylation regulation of calponin

There is a large collection of *in vitro* experimental evidences demonstrating the phosphorylation regulation of calponin. Studies of calponin 1 have determined primary phosphorylation sites at residues Ser₁₇₅ and Thr₁₈₄ in the segment containing the second actin-binding site (Fig. 2). These residues and flanking sequences are highly conserved in all three isoforms and calponin 2 has an additional serine at position 177. However, phosphorylation of calponin 2 and calponin 3 remains to be directly demonstrated. Shown in Fig. 2, these phosphorylation sites are in the first of the three repeating motifs in the middle region of calponin. The downstream repeats 2 and 3 contain sequences similar to that of the second actin-binding site, including the potentially phosphorylatable serine and threonine residues. The phosphorylation status and physiological function of these potential phosphorylation sites remain to be investigated.

In vitro experimental data showed that the residues Ser₁₇₅ and Thr₁₈₄ of calponin 1 are phosphorylated by PKC (Naka et al., 1990). Direct association was found between calponin 1 and PKC α (Somara and Bitar, 2008) and PKC ϵ (Leinweber et al., 1999b). Calmodulin-dependent kinase II and Rho-kinase are also found to phosphorylate calponin in *vitro* at Ser₁₇₅ and Thr₁₈₄ (Walsh, 1991) (Kaneko et al., 2000). Of these two residues, the main site of the regulatory phosphorylation by calmodulin-dependent kinase II and Rho-kinase is Ser₁₇₅.

Unphosphorylated calponin binds to actin and inhibits actomyosin MgATPase. Ser₁₇₅ phosphorylation alters the molecular conformation of calponin and dissociates calponin from F-actin (Jin et al., 2000). The consequence is a release of the inhibition of actomyosin MgATPase and increase the production of force (Tang et al., 1996; Winder et al., 1993a; Winder and Walsh, 1993; Gerthoffer and Pohl, 1994).

Despite the overwhelming evidence for the phosphorylation regulation of calponin obtained from *in vitro* studies, phosphorylated calponin is not readily detectable *in vivo* or in living cells under physiological conditions (Barany and Barany, 1993; Gimona et al., 1992). Based on the observation that PKC phosphorylation of calponin 1 weakens the binding affinity for actin filaments (Jin et al., 2000), the phosphorylated calponin may dissociate from the actin cytoskeleton and be degraded. This potentially regulatory mechanism is worth testing.

PKC signaling is also proposed to regulate calponin redistribution in cells. It is observed that calponin 1 undergoes a PKC agonist-induced translocation from the central contractile filaments to the submembranous cortex (Parker et al., 1994). In addition to being a contractile regulatory protein, calponin may also serve as an adaptor protein connecting the PKC cascade (through phosphorylation of Ser₁₇₅) to the ERK cascade (through the CH domain), providing a scaffold for the ERK signaling complex (Leinweber et al., 2000). This hypothesis and the physiological significance of this function of calponin remain to be investigated.

An interesting finding was that calponin could directly activate PKC autophosphorylation in a lipid-independent manner (Leinweber et al., 2000). Dephosphorylation of calponin was catalyzed by type 2B protein phosphatase (Fraser and Walsh, 1995; Ichikawa et al., 1993).

6.2 Cytoskeleton tension regulates *CNN2* gene expression

A major finding toward fully understanding the physiological function and regulation of calponin is that the expression of *Cnn2* gene is regulated by mechanical tension in the cytoskeleton. Experimental studies have demonstrated that the expression of calponin 2 was significantly higher in cells cultured on hard versus soft gel substrates that produce high or low traction force and cytoskeleton tension in the cells (Hossain et al., 2005). The expression of calponin 2 in NIH/3T3 cells was decreased when cytoskeleton tension was reduced after inhibition of myosin II motor with blebbistatin (Hossain et al., 2006). The level of *Cnn2* mRNA was decreased along with the protein level, indicating a transcriptional regulation (Hossain et al., 2006). Supporting this notion, transfective expression of calponin 2 using a cytomegalovirus promoter behaved independently of the stiffness of culture substrate (Hossain et al., 2005).

A recent study has mapped the *cis*-regulatory elements responsible for the mechanical regulation of *Cnn2* gene promoter. Deletion and site-specific mutagenesis experiments identified the role of a binding site for transcriptional factor HES-1 (hairly and enhancer of split 1) in the 5'-upstream region of mouse *Cnn2* promoter (Jiang et al., 2014). Deletion or mutation of the HES-1 site in mouse *Cnn2* promoter abolished the mechanical regulation and resulted in a substrate stiffness independent high level of transcriptional activity. Therefore, the regulatory mechanism is via a low tension-induced repression of transcription. Corresponding to the down-regulation of *Cnn2* gene expression, the level of HES-1 increased in cells cultured on soft gel substrates in comparison with that in cells cultured on hard gels (Jiang et al., 2014). HES-1 is known to function downstream of the Notch-RBP J signaling pathway (Kageyama et al., 1997), which has been suggested to mediate cellular mechanoregulations (Morrow et al., 2005; Morrow et al., 2007).

It has been long established that chemical energy can readily be converted to mechanical force by ATPase- or GTPase-based motor proteins that hydrolyze the high energy bond in triphosphate. It is also widely observed that mechanical force have effects on cellular functions and gene expression. However, little is known about how the mechanical force signal and physical energy is converted to chemical signals that trigger biological responses in a living cell. The characterization of mechanical regulated *Cnn2* gene promoter provides not only an intriguing lead of such signal transduction but also a powerful experimental system to search for the upstream cellular sensor of mechanical signals.

6.3 Cytoskeleton tension regulates the degradation of calponin 2

Calponin 2 is also regulated by mechanical tension at the protein level. A rapid and selective degradation of calponin 2 occurs in lung tissues after a short period of deflation and loss of alveolar tension (Hossain et al., 2006). This low cytoskeleton tension-induced degradation of calponin 2 in collapsed lung was very effectively prevented in post mortem mouse lung simply by inflation with air to maintain the alveolar tension (Hossain et al., 2006). The tension-dependent stability of calponin 2 was confirmed in monolayer cells cultured on expanded elastic membrane by its rapid degradation after a reduction in the dimension of the cultural substrate to acutely reduce cytoskeleton tension (Hossain et al., 2006). In the meantime, transcriptional regulation is the primary determinant for the regulation of

calponin 2 in the cellular response to mechanical signals, based on the facts that calponin 2 expression became independent of substrate stiffness after deletion of the HES-1 repressor site from the *Cnn2* promoter (Jiang et al., 2014).

7. Calponin and cancer

Tumor cells derived from various calponin-positive cell types display a common feature of decreased calponin than that in their parental tissue. For example, calponin expression is significantly lower in leiomyosarcoma cells than that in normal smooth muscle cells (Horiuchi et al., 1998). Reduced calponin expression were also found in hepatocellular carcinoma (Sasaki et al., 2002), renal angiomyolipoma (Islam et al., 2004), mammary simple carcinomas (Martin de las Mulas et al., 2004), papillary carcinomas (Mosunjac et al., 2000), basal cell-like breast carcinoma (Hasegawa et al., 2008), metastatic basal cell carcinomas (Uzquiano et al., 2008), and prostate cancer (Meehan et al., 2002; Tuxhorn et al., 2002; Verone et al., 2013).

Reduced levels of calponin expression in tumor cells corresponded to alterations in the stability of actin cytoskeleton and dynamics with a correlation that the remaining calponin level positively related to the prognosis of the disease. A comparison between a metastatic prostate cancer cell line PC3-M and its parental cell line PC3 showed that PC3-M expresses significantly less calponin 2 and demonstrates faster rates of proliferation and migration (Hossain et al., 2014). Boosting the level of calponin 2 in PC3-M cells by transfective expression corrected this phenotype (Hossain et al., 2014). Consistently, transfective expression of calponin 1 in human fibrosarcoma, leiomyosarcoma, synovial sarcoma and osteosarcoma cells significantly reduced anchorage-independent growth and *in vivo* tumorigenicity, supporting calponin's function as a tumor suppressor (Yamamura et al., 2001; Horiuchi et al., 1999; Takeoka et al., 2002).

The decrease in calponin 2 results in weakened substrate adhesion in PC3-M prostate cancer cells as compared with that of PC3 cells. Interestingly, the low calponin 2-produced weaker adhesion of PC3-M cells is accompanied by increased dependence on substrate-stiffness (Hossain et al., 2014). This consequence may contribute to prostate cancer's high tendency of metastasis to the bone, a unique high stiffness tissue environment that facilitates the adhesion of arriving cancer cell to initiate metastasis.

A recent study reported the use of calponin 2 as a blood-based biomarker for the early diagnosis of breast cancer. Using monoclonal antibodies against calponin 2, sandwich ELISA determined the serum levels of calponin 2. Comparing samples from patients with breast cancer, benign diseases of the breast, and samples from healthy females showed increased serum levels of calponin 2 in 10.5% of patients with breast cancer in contrast to the all negative finding in the control groups (Debald et al., 2014).

8. Summary and remaining questions

Three decades since its discovery, calponin has been extensively studied and characterized to be an actin-filament associated regulatory protein with diverged functions in modulating smooth muscle contractility and non-muscle cell motility. The three isoforms of calponin

encoded by three homologous genes, *CNN1*, *CNN2* and *CNN3*, are of conserved structures in the N-terminal and middle regions, whereas diverged in the C-terminal segment corresponding to significant size and charge differences. The three calponin isoform genes are expressed under tissue- and cell type-specific regulation, reflecting diverged biological functions. Calponin 1 is specifically expressed at abundance in smooth muscle cells and plays a role in modulating contractility. Calponin 2 is expressed in both smooth muscle and many types of non-muscle cells and functions as a regulator of the actin cytoskeleton in fundamental activities such as cell proliferation, migration, adhesion, phagocytosis, angiogenesis, immune responses and cancer metastasis. Calponin 3 is expressed in smooth muscle and several types of non-muscle cells with demonstrated functions in regulating neuronal plasticity, and cell fusion with a critical role in embryonic development. The finding that the gene regulation and function of calponin 2 are both under mechanoregulation opened a new direction in calponin research leading to better understanding of cellular interactions with the mechanical environment and responses to force signals.

Based on our current knowledge, many interesting questions are ready to be addressed in order to further understand the regulation and structure-function relationship of calponin. For example, how does calponin regulate the actin filaments in smooth muscle contraction and the actin cytoskeleton in non-muscle cell motility? While phosphorylation clearly regulates calponin function in test tubes, why phosphorylated calponin is not accumulated in living cells? Are the regulatory functions of different isoforms of calponin exchangeable or distinct? How does the expression and function of calponin respond to mechanical tension signals? Are there any genetic diseases caused by calponin mutations? Would calponin blood test be widely applicable in cancer diagnosis? Future work along these directions would produce exciting information for the physiological and pathological significances of calponin in human health and diseases.

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https://en.wikipedia.org/wiki/Calponin_1,_basic,_smooth_muscle

https://en.wikipedia.org/wiki/Calponin_2

https://en.wikipedia.org/wiki/Calponin_3,_acidic

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Figure 1. Evolutionary lineage of vertebrate calponin isoforms

The phylogenetic tree of vertebrate calponin isoform genes is derived from protein sequence alignment performed with the Clustal V method using the MegAlign computer program (Lasergene; DNASTAR, Inc, Madison, WI). The degrees of evolutionary divergence are indicated by the lengths of lineage lines. Calponin isoforms 1, 2 and 3 are marked in green, blue and red, respectively. The NCBI database accession numbers of the sequences analyzed are: African clawed frog calponin 1, NP_001080753.1; African clawed frog calponin 2, NP_001085014.1; African clawed frog calponin 3, NP_001080482.1; Black flying fox calponin 1, ELK17809.1; Black flying fox calponin 2, ELK19295.1; Black flying fox calponin 3, ELK05808.1; Brandt's bat calponin 1, EPQ04681.1; Brandt's bat calponin 2, EPQ08061.1; Brown tree snake calponin 2, JAG68493.1; Cattle calponin 1, NP_001039844.1; Cattle calponin 2, AAI03381.1; Cattle calponin 3, NP_001033268.1; Channel catfish, AHH43034.1; Chicken calponin 1, NP_990847.1; Chicken calponin 2, NP_001135728.1; Chimpanzee calponin 1, NP_001267033.1; Chimpanzee calponin 2, JAA13388.1; Chimpanzee calponin 3, JAA44470.1; Chinese hamster calponin 1, EGV96164.1; Chinese hamster calponin 2, EGV99480.1; Chinese hamster calponin 3, EGW11625.1; Croaker calponin 1, KKF32470.1; Croaker calponin 2, KKF29575.1; Croaker calponin 3, KKF23334.1; Damara mole calponin 1, KFO28782.1; Damara mole calponin 2,

KFO28912.1; Damara mole calponin 3, KFO26533.1; Eastern diamondback rattlesnake calponin 2, AFJ49586.1; Ferret calponin 1, NP_001297140.1; Green turtle calponin 1, EMP37252.1; Green turtle calponin 3, EMP30806.1; Human calponin 1, NP_001290.2; Human calponin 2, AAI48265.1; Human calponin 3, AAB35752.1; King cobra calponin 3, ETE62831.1; Marmoset calponin 3, JAB48100.1; Mouse calponin 1, AAI38864.1; Mouse calponin 2, EDL31614.1; Mouse calponin 3, AAH85268.1; Mouse-eared bat calponin 1, ELK28732.1; Mouse-eared bat calponin 2, ELK29427.1; Mouse-eared bat calponin 3, ELK30701.1; Naked mole calponin 1, EHA97296.1; Naked mole calponin 2, EHB16944.1; Naked mole calponin 3, EHB05382.1; Orangutan calponin 2, NP_001124601.1; Rat calponin 1, NP_113935.1; Rat calponin 3, NP_062232.1; Rhesus macaque calponin 3, NP_001248738.1; Salmon calponin 1, NP_001139857.1; Salmon calponin 2, NP_001133873.1; Salmon calponin 3, NP_001133337.1; Sheep, calponin 1, NP_001009456.1; Snakehead calponin 2, AFJ79963.1; Timber rattlesnake calponin 2, JAG45504.1; Western clawed frog calponin 1, NP_001015796.1; NP_998841.1; Western clawed frog calponin 2, Western clawed frog calponin 3, NP_989257.1; Wild swine calponin 1, NP_999043.1; Yak calponin 1, ELR59973.1; Yak calponin 2, ELR60222.1; Yak calponin 3, ELR46902.1; Zebrafish calponin 2, NP_998514.1; Zebrafish calponin 3, NP_956047.1.

*The aliases of Africa clawed frog *Cnn1* and *Cnn2* genes deposited in NCBI database are annotated as *Cnn2* and *Cnn1*, respectively, which is inconsistent with the sequence homology analysis and requires further validation.

Figure 2. Linear structure and comparison of calponin isoforms

The illustrations summarize the primary structures and comparison of the three calponin isoforms. (A) The linear structural map primarily summarized from studies of chicken calponin 1 outlines the structural and functional domains of calponin. The N-terminal CH domain, the two actin-binding sites, the three repeating sequence motifs, and the C-terminal variable region are shown. The CH domain overlaps with the ERK binding region. Amino acid sequences of the two actin-binding sites in the three isoforms and the three repeating motifs of calponin 1 are shown in the insets. The regulatory phosphorylation sites Ser₁₇₅ and Thr₁₈₄ are located in the second actin-binding site that overlaps with the first repeating motif. Potentially phosphorylatable serine residues corresponding to Ser₁₇₅ are conserved in both repeats 2 and 3, whereas a Thr₁₈₄ equivalent is conserved in repeat 2. Different from calponin 1 and calponin 3, calponin 2 has an additional, potentially phosphorylatable, serine at position 177. (B) Each of the three calponin isoform genes contains seven exons. While the three isoforms are largely conserved in the N-terminal and middle regions, they have a C-terminal variable region encoded by exon 7 that is significantly diverged in length and amino acid sequences.

Figure 3. Proteins that interact with calponin

Multiple cytoskeleton and regulatory proteins have been reported to bind or interact with calponin. Summarized in this figure, calponin binds actin filaments through two actin-binding sites (ABS1 and ABS2) and other actin filaments-associated proteins tropomyosin via the N-terminal CH domain and gelsolin via the actin-binding sites. Calponin interacts with microtubules through the region including the actin-binding sites and the repeating motifs and with desmin through the region spanning from the CH domain to the actin-binding sites. The residues 144–182 of calponin interact with myosin. In the presence of Ca^{2+} , calmodulin and S100 bind the region of the actin-binding sites of calponin and reverse the calponin inhibition of the myosin MgATPase. An N-terminal 22-kDa fragment of calponin was reported to interact with phosphatidylserine and phosphatidylinositol.

*Caldesmon was reported to possibly interact with calponin but no definitive site was identified (Graceffa et al., 1996; Czurylo et al., 1997).

Table 1

Calponin isoform genes and their tissue-specific expression

Isoform Genes	<i>CNN1</i>	<i>CNN2</i>	<i>CNN3</i>
Protein product	Calponin 1	Calponin 2	Calponin 3
Location in human chromosome genome	19p13.2-p13.1	19p13.3	1p22-p21
Number of exons	7	7	7
Number of amino acids encoded	297	309	329
Molecular weight of encoded protein	33.2-kDa	33.7-kDa	36.4-kDa
Isoelectric point of protein product	9.10	7.23	5.84
Tissue and cell type of expression	Smooth muscle	Smooth muscle Neuronal tissue Epithelial cells Skin keratinocyte Fibroblast Myoblast Endothelial cell Myeloid blood cells B lymphocyte Carcinoma cells	Smooth muscle Neuronal tissue Fibroblast Myoblast Trophoblast B lymphocyte

The data presented are based on human calponin isoforms and genes, except for the tissue- and cell-specific expressions that are from studies of multiple vertebrate species.