

# Calpains, mitochondria, and apoptosis

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## Abstract

Mitochondrial activity is critical for efficient function of the cardiovascular system. In response to cardiovascular injury, mitochondrial dysfunction occurs and can lead to apoptosis and necrosis. Calpains are a 15-member family of  $\text{Ca}^{2+}$ -activated cysteine proteases localized to the cytosol and mitochondria, and several have been shown to regulate apoptosis and necrosis. For example, in endothelial cells,  $\text{Ca}^{2+}$  overload causes mitochondrial calpain 1 cleavage of the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger leading to mitochondrial  $\text{Ca}^{2+}$  accumulation. Also, activated calpain 1 cleaves Bid, inducing cytochrome c release and apoptosis. In renal cells, calpains 1 and 2 promote apoptosis and necrosis by cleaving cytoskeletal proteins, which increases plasma membrane permeability and cleavage of caspases. Calpain 10 cleaves electron transport chain proteins, causing decreased mitochondrial respiration and excessive activation, or inhibition of calpain 10 activity induces mitochondrial dysfunction and apoptosis. In cardiomyocytes, calpain 1 activates caspase 3 and poly-ADP ribose polymerase during tumour necrosis factor- $\alpha$ -induced apoptosis, and calpain 1 cleaves apoptosis-inducing factor after  $\text{Ca}^{2+}$  overload. Many of these observations have been elucidated with calpain inhibitors, but most calpain inhibitors are not specific for calpains or a specific calpain family member, creating more questions. The following review will discuss how calpains affect mitochondrial function and apoptosis within the cardiovascular system.

## Keywords

Calpains • Apoptosis • Mitochondria • Cardiovascular system

This article is part of the Review Focus on: **The Calpain Family in the Cardiovascular System**

## 1. Calpain family

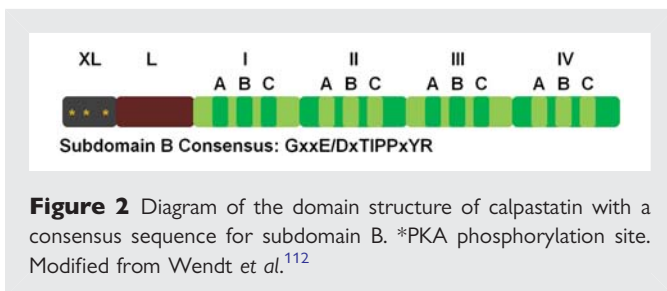
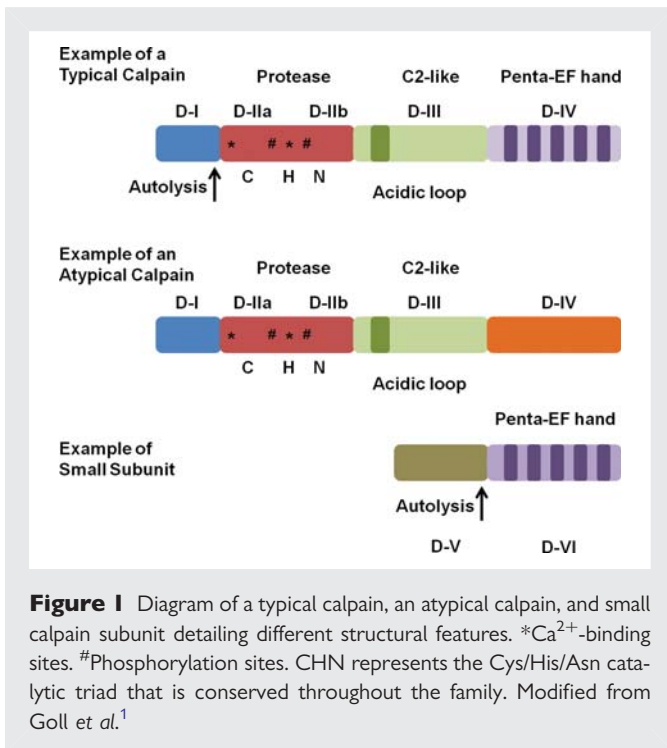
Calpains are  $\text{Ca}^{2+}$ -activated non-lysosomal cysteine proteases<sup>1</sup> and the first calpain discovered and purified by Dayton et al.<sup>2,3</sup> in 1976 was calpain 2. The calpain family is conserved in many different species, from fungi to humans.<sup>1</sup> In mammals, there are 14 large subunit members, one small subunit member, and one endogenous inhibitor. Some calpains are ubiquitously expressed—calpains 1, 2, 4, 5, 7, and 10<sup>1,4–9</sup>—whereas others are found in specific tissues: calpain 3 (skeletal muscle),<sup>9</sup> calpain 6 (placenta),<sup>10</sup> calpain 8 (smooth muscle),<sup>11</sup> calpain 9 (stomach),<sup>11</sup> calpain 11 (testes),<sup>12,13</sup> calpain 12 (skin after birth),<sup>14</sup> and calpain 13 (testes and lung).<sup>12</sup> Additionally, calpains are divided into two groups based on domain IV structure (Figure 1).<sup>1</sup> Typical calpains (1, 2, 3, 8, 9, 11, 12, and 14) contain a penta-EF hand in domain IV that can bind  $\text{Ca}^{2+}$ , the calpain small subunit (only calpains 1, 2, and 9 have been shown to dimerize), or calpastatin. Atypical calpains (5, 6, 7, 10, 13, and 15) lack a penta-EF hand in domain IV and are unable to bind the calpain small subunit or calpastatin.<sup>15–17</sup>

Most calpains contain four structural domains. In calpains 1, 2, and 9, domain I is cleaved after  $\text{Ca}^{2+}$  activation (autolysis) (Figure 1),<sup>1</sup> but

whether other typical calpains undergo autolysis of domain I is not clear. In atypical calpains, domain I is not cleaved and the function of domain 1 is unknown for many of these calpains, except for calpain 10 which contains a mitochondrial targeting sequence.<sup>18</sup> Domain II contains the active site, with the catalytic triad of cysteine, asparagine, and histidine, and this catalytic triad is conserved throughout the entire family, except for calpain 6 which lacks proteolytic activity.<sup>19</sup> In addition to containing the catalytic triad, crystallographic studies revealed that domain II can bind two atoms of  $\text{Ca}^{2+}$  and assist in calpain activation.<sup>16,20</sup> Domain III contains two  $\text{Ca}^{2+}$ -binding sites and a phospholipid-binding motif in the C2-like area.<sup>21</sup> These  $\text{Ca}^{2+}$  and phospholipid-binding residues are conserved throughout the family, except for calpain 10.<sup>16</sup> Also, domain III is believed to regulate calpain activity through specific electrostatic interactions and be involved in substrate recognition.<sup>16,22,23</sup> Domain IV contains the penta-EF hand that can bind  $\text{Ca}^{2+}$ , calpastatin, or the small subunit (calpain 4).<sup>1</sup> These penta-EF hands are thought to be one of the most important features in calpain activation.<sup>24</sup>

Calpain 4 (small calpain subunit) is a 28 kDa protein that dimerizes with typical calpains.<sup>1</sup> It only contains two domains, V and VI, and the

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only known function for domain V is to bind to the C-terminus region of domain IV in large calpain subunits.<sup>25</sup> Domain VI is identical to domain IV, in that it has a penta-EF hand that is available for Ca<sup>2+</sup>-binding and heterodimer formation.<sup>26</sup> Recently, calpain small subunit 2 (CSS2) was discovered and it can dimerize with calpain 2,<sup>27</sup> but CSS2 is not completely redundant with calpain 4 because knockdown of calpain 4 is embryonic lethal.<sup>28</sup>

Atypical calpains 5, 6, and 10 have the same general structure as typical calpains for the first three domains, but lack the penta-EF hand in domain IV.<sup>16,29</sup> Instead, atypical calpains contain a divergent domain IV, which presently has an unknown function. Since atypical calpains do not contain a penta-EF hand there are only several Ca<sup>2+</sup>-binding residues available, suggesting that large concentrations of Ca<sup>2+</sup> are not necessary for activation and that Ca<sup>2+</sup> modulates the activity.<sup>18</sup> Such differences in domain IV suggest different activation and inhibition patterns for atypical calpains.

Calpastatin, a protein that specifically inhibits calpains, has eight splice variants, ranging from 18.7 to 85 kDa.<sup>30–32</sup> The full-length calpastatin has six domains (XL, L, I, II, III, and IV) and domains I–IV contain subdomains A–C that are essential for calpain inhibition (Figure 2).<sup>1</sup> Of the four domains, the order of inhibition effectiveness is: I > IV > III > II.<sup>33</sup> Little is known about the XL domain other than the three protein kinase A phosphorylation sites,<sup>34</sup> and the function of

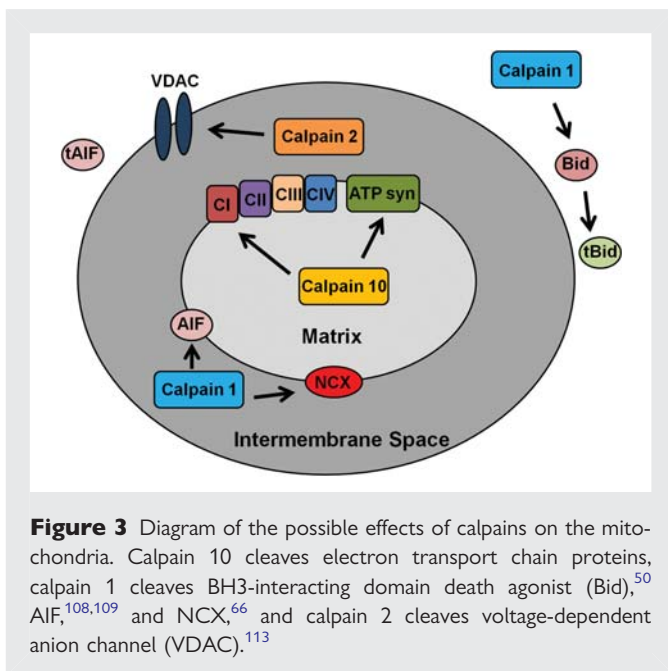
the L domain is still unclear. Because calpastatin must bind domain II and domain IV or VI to inhibit calpains, it seems unlikely that atypical calpains are inhibited. Therefore, atypical calpains must have other regulatory mechanisms. Further reading on the calpain family can be found by reviewing articles by Goll *et al.*<sup>1</sup> and Suzuki *et al.*<sup>16</sup>

## 2. Endothelial and smooth muscle cells

Endothelial cells are an essential part of the cardiovascular system through their functions in blood vessel formation, cell barrier, coagulation, vascular tone, inflammation and angiogenesis.<sup>35</sup> Many of these processes require cytoskeletal rearrangements, and numerous cytoskeletal proteins are known calpain substrates.<sup>1,36</sup> Indeed, calpains have been discovered in rabbit, bovine, and human endothelium.<sup>37</sup> More recently, Fujitani *et al.* purified calpains 1 and 2 and calpastatin from human umbilical vein endothelial cells and detected active calpains 1 and 2 by measuring the cleavage of talin and filamin.<sup>38</sup> Calpains 1 and 2 have been shown to be important in cell migration and proliferation in many mammalian cell types,<sup>1,39–41</sup> including endothelial cells.<sup>42–49</sup> A review focusing on the regulation and physiological roles of calpains can be reviewed in this same journal issue.

Recent research revealed that calpains can affect mitochondrial function and regulate apoptosis, but there has been limited research involving endothelial cells. Vindis *et al.*<sup>50</sup> showed that calpains are essential for apoptosis in human microvascular endothelial cells. Specifically, during oxidized-LDL-stimulated apoptosis, intracellular Ca<sup>2+</sup> increases and induces calpain activation, leading to BH3-interacting domain death agonist (Bid) cleavage, cytochrome c release, apoptosome formation, and caspase 3 activation (Figure 3). Using EGTA and calpeptin, Vindis' group was able to prevent apoptosis. Calpain cleavage of Bid can lead to several outcomes: (i) tBid-mediated Bak and Bax oligomerization, (ii) formation of tBid homodimers, and (iii) tBid-induced mitochondrial permeability transition pore opening and remodelling of the inner mitochondrial membrane,<sup>51–56</sup> which all lead to apoptosis. These data are in agreement with Walter *et al.*<sup>57</sup> and Pörn-Ares *et al.*<sup>58</sup> Under oxidized-LDL-stimulated apoptosis and calpain cleavage of Bid, calpain-independent apoptosis-inducing factor (AIF) release occurred.<sup>59</sup> From a pathological perspective, the increase in oxidized-LDL activation of calpain and induction of apoptosis in atherosclerotic areas<sup>60</sup> suggests that this type of apoptosis is important in atherothrombotic events.<sup>60,61</sup> Also, in lysophosphatidylcholine-induced apoptosis of endothelial cells, luteolin treatment reduced Ca<sup>2+</sup> influx which decreased calpain activation and prevented cytochrome c release.<sup>62</sup> This further implicates calpains as pro-apoptotic proteases.

Several researchers have reported roles for mitochondrial calpain 1 in endothelial cells. In rat heart microvascular endothelial cells, Moshal *et al.* reported that hyperhomocysteinemia (Hcy)-induced extracellular matrix remodelling by matrix metalloproteinase 9 (MMP-9) is caused by calpain 1 activation.<sup>63</sup> Hcy is defined as excess homocysteine in the blood, which leads to increased reactive oxygen species (ROS) production and vascular damage.<sup>64</sup> In this model, Hcy increased Ca<sup>2+</sup> flux, thereby activating calpain 1, which translocates from the cytosol to the mitochondria. Once in the mitochondria, calpain 1 increased ROS production, which activates extracellular-signal-regulated kinases 1/2 and MMP-9.<sup>63</sup> MMP-9 activation leads to the cleavage of the extracellular matrix causing vascular dysfunction.



Kar *et al.*<sup>65</sup> reported that in bovine pulmonary artery smooth muscle mitochondria, calpain 1 and calpastatin are bound to the mitochondrial inner membrane and treatment with the  $\text{Ca}^{2+}$  ionophore, A23187, which increases mitochondrial  $\text{Ca}^{2+}$  concentration, induced calpain 1 cleavage of the mitochondrial  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (NCX) (Figure 3).<sup>66</sup> Kar's group suggested that this is the main cause of  $\text{Ca}^{2+}$  accumulation in the mitochondria after injury.

Another important aspect of calpain biology is its role in ischaemia/reperfusion injury. Hoang *et al.* used calpain inhibitors (MDL28170, PD150606, and ALLN) in an *in vivo* model of ischaemic retinopathy to demonstrate that inhibition of calpain activity improves neovascular structure and function.<sup>67</sup> In this model, hypoxia causes calpain activation leading to new dysfunctional vessels that have a disrupted actin cytoskeleton. Interestingly, calpain inhibition increased stability and function in new vessels reducing retinal damage. However, several articles discussing ischaemia/reperfusion damage in endothelial cells report that cathepsin B is more important than calpains in apoptotic cell death.<sup>68–70</sup> Additional information about ischaemia/reperfusion injury and calpains can be learned from other review articles in this issue.

### 3. Kidney

The kidney plays an important role in the cardiovascular system by maintaining ion, water, and metabolic substrate homeostasis. Our laboratory has studied calpains in renal proximal tubular cell (RPTC) function and viability. We showed in various RPTC injury models that calpains 1 and 2 degrade cytoskeletal proteins and contribute to increased plasma membrane permeability, with  $\text{Ca}^{2+}$ ,  $\text{Cl}^-$ , and water influx leading to necrosis.<sup>71–75</sup>

Calpains 1 and 2 have a more direct role in apoptosis. Some research has shown that calpains are important for the inactivation of caspases,<sup>76,77</sup> whereas other reports suggest that calpains are necessary for caspase activation.<sup>78,79</sup> Specifically, Lee *et al.*<sup>78</sup> showed that calpains activate caspases in cadmium-induced apoptosis

in RPTC because calpain inhibitors reduced caspase 3 activity and cell death.<sup>79</sup>

In 2006, Arrington *et al.*<sup>18</sup> were the first to discover calpain 10 residing inside the mitochondria. Using rabbit mitochondria, calpain 10 was found in all of the mitochondrial compartments, with the majority of the activity in the mitochondrial matrix. Further analysis revealed that the first 15 amino acids on the N-terminus act as a mitochondrial targeting sequence. Calpain 10 has eight splice variants, ranging from 15 to 75 kDa in size, and it was shown using zymography that the 75, 56, and 50 kDa variants are in the mitochondria. Immunoblots for calpains 1 and 2 on mitochondrial fractions revealed calpain 10 to be the only calpain in the mitochondria. This was confirmed in rat and mouse renal mitochondria.<sup>80</sup> Interestingly, even though mouse, rat, and rabbit all contain the same splice variants, they did not migrate similarly during zymography, suggesting that there are different binding partners in each species. Additionally, mitochondrial calpain 10 did not require  $\text{Ca}^{2+}$  for activity, but  $\text{Ca}^{2+}$  increased its activity moderately.

We used PEST sequence analysis to elucidate mitochondrial calpain 10 substrates, proteins that contain a large number of proline, glutamate, serine, and threonine residues signal rapid degradation by either calpains or the proteasome.<sup>81–83</sup> NDUFV2, NDUFB8, ORP150, and ATP synthase- $\beta$  were confirmed to be calpain 10 substrates (Figure 3). After  $\text{Ca}^{2+}$  overload, mitochondrial calpain 10 cleaves these proteins (and possibly other unknown substrates), causing a decrease in state 3 respiration. Thus, mitochondrial calpain 10 plays an important role in degradation of electron transport chain proteins.

Recently, calpain 10 was shown to be important in renal ageing, and kidneys regress in size and function with ageing by an unknown mechanism.<sup>84</sup> Covington *et al.*<sup>85</sup> discovered that calpain 10 protein and mRNA levels are reduced in aged rat, mouse, and human kidneys and caloric restriction prevented the decrease. Caloric restriction also prevented degeneration of the rat proximal tubules with age.<sup>86</sup> Immunoblot analysis of the kidney and liver revealed that age did not affect calpain 1 and 2 protein or mRNA levels.<sup>85</sup> Additionally, liver calpain 10 was unaffected at any age. Using our primary RPTC model, we explored the effect of acute reduction in calpain 10 by administration of adenoviral-delivered calpain 10 shRNA.<sup>85</sup> Three days after adenovirus treatment when mitochondrial calpain 10 was  $\sim 80\%$  decreased with no effect on cytosolic calpain 10, we detected increased apoptosis. This suggests that renal mitochondrial calpain 10 is important in maintaining cellular viability and provides a possible reason why renal function decreases with age. Interestingly, when calpain 10 is overexpressed, there is mitochondrial swelling and cell death.<sup>18</sup> Thus, calpain 10 protein must be maintained at a specific level or cell death will occur.

Because calpain 10 has been implicated in type 2 diabetes,<sup>87</sup> we explored the effects of high glucose (17 mM) on primary RPTC.<sup>88</sup> Interestingly, mitochondrial calpain 10 protein levels increased from 3 to 12 h after incubation in high glucose and then returned to normal until 48 h and decreased until at least 120 h. Cytosolic calpain 10 was unaffected until 120 h. We detected decreased basal and uncoupled respiration, an accumulation of mitochondrial calpain 10 substrates (NDUFB8 and ATP synthase- $\beta$ ), and increased apoptosis 96 h after high-glucose incubation. Using the streptozotocin-induced rat diabetic nephropathy model, rats had decreased renal calpain 10 protein and mRNA, with no effects on renal calpains 1 and 2 after 10 weeks.<sup>88</sup> Accumulations of mitochondrial calpain 10 substrates and apoptosis were detected. Finally, calpain 10 siRNA knockdown of rat renal calpain 10 resulted in apoptosis and kidney

dysfunction.<sup>88</sup> These data demonstrate that diabetes results in decreased renal calpain 10 mRNA and protein, apoptosis, and decreased renal function and that a direct knockdown of renal calpain 10 in non-diabetic rats results in similar renal pathology, providing evidence that loss of renal calpain 10 may be a critical component of diabetic nephropathy.

Calpain inhibitors are promiscuous among calpain isoforms and other protease families. Thus, the use of calpain inhibitors to determine the role of calpains in physiology and pathology is difficult. Calpastatin is specific for typical calpains and the development of PD150606 provided a tool to inhibit typical calpains, because it binds the penta-EF hand in domain IV, but PD150606 can still inhibit multiple typical calpains.<sup>89,90</sup> Our laboratory developed a calpain 10-specific peptide inhibitor with an IC<sub>50</sub> of ~100 nM that prevented Ca<sup>2+</sup>-induced reduction in state 3 respiration and mitochondrial calpain 10 substrate cleavage.<sup>91</sup> While this inhibitor was not efficacious in cells, current research is focused on optimizing CYGAK to improve efficacy in cellular models.

## 4. Heart

Myocardial infarction can result in mitochondrial damage and apoptosis of cardiomyocytes.<sup>92</sup> Numerous studies have documented that calpains are important in ischaemia/reperfusion injury in the heart,<sup>93–96</sup> mitochondrial permeability transition,<sup>97,98</sup> and necrotic/apoptotic cell death.<sup>99,100</sup> Additional information about this type of injury can be found in another review article in this journal issue.

Trumbeckaite et al.<sup>101</sup> used isolated rabbit hearts and performed 45 min of ischaemia followed by 60 min of reperfusion with and without pretreatment of BSF 409425, a calpain inhibitor, and showed that BSF 409425 blunted the decrease in state 3 and the increase in state 4 respiration. Several other studies have demonstrated that calpain inhibition during ischaemia/reperfusion reduces apoptosis and infarct size.<sup>102–104</sup> These results suggest that excessive calpain activity plays a role in damaging mitochondria and oxidative phosphorylation during cardiac ischaemia/reperfusion injury.

One of the cytokines released after ischaemia/reperfusion injury is tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ).<sup>105</sup> TNF- $\alpha$  is known to induce apoptosis and inflammation.<sup>106</sup> Bajaj and Sharma showed the importance of calpains in TNF- $\alpha$ -induced apoptosis in the cardiac muscle cell line from the AT-1 mouse atrial cardiomyocyte tumor lineage cardiomyocyte cell line by pretreating cells with Z-LLY-fmk, a calpain inhibitor, for 30 min prior to TNF- $\alpha$  exposure for up to 12 h.<sup>107</sup> Over the time course, they demonstrated that Z-LLY-fmk decreased cleaved pro-caspase 3 and poly-ADP ribose polymerase (PARP), suggesting that calpains are important in the activation of PARP and caspase 3, and ultimately apoptosis.

Calpains have been reported to be pro-apoptotic proteases by mediating the cleavage of AIF after Ca<sup>2+</sup> overload (Figure 3).<sup>108,109</sup> AIF is bound to the inner mitochondrial membrane, and when there is mitochondrial damage, AIF can be cleaved allowing it to translocate to the nucleus and induce DNA degradation.<sup>110</sup> Chen et al.<sup>108</sup> showed in isolated heart mitochondria that the addition of exogenous Ca<sup>2+</sup> causes AIF release and that pretreatment with MDL28170, a calpain inhibitor, prevents this release. Therefore, it appears that the inhibition of mitochondrial calpain 1 prevents the release of AIF resulting in less cardiac cell death. However, there is some controversy because Ozaki et al. reported that liver mitochondrial calpain 1 mediated the release of AIF after Ca<sup>2+</sup> overload and pretreatment with MDL28170 prevented

this release.<sup>109</sup> Joshi et al.<sup>111</sup> reported that calpastatin and PD150606 pretreatment prior to Ca<sup>2+</sup> overload in liver mitochondria did not prevent AIF release, suggesting that calpain 1 or 2 is not involved in this process. Furthermore, these researchers confirmed that AIF release was calpain-independent in isolated brain mitochondria. Much research has been performed in non-cardiac tissue mitochondria regarding calpain 1 cleavage of AIF. Further research is needed to determine whether mitochondrial calpain 1 cleaves AIF in cardiac tissue.

## 5. Summary and conclusions

Calpains have been discovered in the mitochondria. Much of the mitochondrial calpain research performed to date has focused on its pro-apoptotic role after Ca<sup>2+</sup> overload, in particular, the cleavage of caspase 3 and/or AIF. There remains limited research on the physiological functions of mitochondrial calpains. However, calpain 10 regulates electron transport chain proteins and overexpression or knockdown of calpain 10 leads to mitochondrial dysfunction and cell death. Thus, mitochondrial calpain 10 protein must be tightly regulated for mitochondrial and renal cell function, but the underlying mechanisms need to be determined. Throughout the literature, many discrepancies exist concerning the role of calpains in physiology and different pathologies. Unfortunately, because the calpain active site is conserved throughout the entire family, creation of calpain isoform-specific inhibitors is difficult. Additionally, many popular calpain inhibitors also inhibit cathepsins, proteasome, and Lon protease (a mitochondrial matrix protease). Thus, many results must be confirmed with a knockdown model to ensure that calpain is the target and to identify the specific calpain isoform.

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