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# CALPAIN 5: A NON-CLASSICAL CALPAIN HIGHLY EXPRESSED IN THE CNS AND LOCALIZED TO MITOCHONDRIA AND NUCLEAR PML BODIES

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# CALPAIN 5: A NON-CLASSICAL CALPAIN HIGHLY EXPRESSED IN THE CNS AND LOCALIZED TO MITOCHONDRIA AND NUCLEAR PML BODIES

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DISSERTATION

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A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine
at the University of Kentucky

By
Ranjana Singh

Lexington, Kentucky

Director: Dr. James W. Geddes, Professor, Department of Anatomy and Neurobiology

Lexington, Kentucky

2014

## ABSTRACT OF DISSERTATION

Calpain 5 (CAPN5) is a non-classical member of the calpain family. It lacks the EF-hand motif characteristic of the classical calpains, calpain 1 and 2, but retains catalytic and $Ca^{2+}$ binding non EF domains. *Tra-3*, an ortholog of CAPN5, is involved in necrotic cell death in *C.elegans*; although specific role of CAPN5 has not been investigated in the mammalian CNS. I compared relative mRNA levels of calpains in rat CNS, which revealed that CAPN5 is the second most highly expressed calpain. We examined relative levels of CAPN5 from late embryonic day 18 to postnatal day 90 and found lower mRNA but higher protein levels during CNS development. Using X –gal staining in *Capn5 +/-* mice, immunostaining of rat brain sections and SH-SY5Y cells, and subcellular fractionation of rat brain cortex, we found that CAPN5 is a non-cytoplasmic calpain localized in the nucleus and enriched in synaptic mitochondria. Proteinase K treatment of mitochondria and mitoplasts from B35 rat neuroblastoma cells and rat synaptic mitochondria revealed CAPN5 was localized on the inner mitochondrial membrane and released from mitochondria on membrane permeabilization with alamethicin. We used immunolabelling, confocal imaging, nuclear subfractionation and transient transfections to evaluate the subnuclear localization of CAPN5. CAPN5 was detected in punctate domains and associated with promyelocytic leukemia (PML) protein, a tumor suppressor protein. We further demonstrated that CAPN5 carries a nonconventional bipartite nuclear localization signal. Together, these findings demonstrate that CAPN5 is a non-cytosolic calpain, abundant in the CNS and localized to the mitochondria inner membrane and nuclear PML bodies.

Keywords: Sumoylation, Apoptosis, Cell death, Nuclear localization signal, Mitochondria

Ranjana Singh

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By

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# List of Abbreviations

AIF: Apoptosis Inducing Factor

ANT: Adenine Nucleotide Translocate

APAF1: Apoptotic Protease-Activating Factor1

ASP: Aspartyl protease

BAK: B Cell Lymphoma 2 (BCL-2) Antagonist or Killer

BAX: B Cell Lymphoma 2 (BCL-2) associated X protein

BID: BH3- Interacting Domain death agonist

C2: C2 domain

C2L: C2-like domain

CaMK: Ca2+/Calmodulin- dependent protein Kinase

CAPN5: Calpain 5

CASP: Caspase

CAST: Calpastatin

CYP-D: Cyclophilin D

CYT-C Cytochrome C

DEK1: Defective kernel 1

E18: Embryonic day 18

ESCRT: Endosomal sorting complex required for transport

GluR1: Glutamate Receptor 1

GR: Glycine-rich hydrophobic domain

IP3R : Inositol Triphosphate Receptor

IS1: CAPN3 [p94]-characteristic sequences

IS2: CAPN3 [p94]-characteristic sequences

IQ: a motif interactive with calmodulin

L domain: N-terminal domain of calpastatin

MIT: Microtubule interacting and transport motif

MOMP: Mitochondrial outer membrane permeabilization

NCX: $Na^{+}/Ca^{2+}$ exchanger

NS: CAPN3 [p94]-characteristic sequences

PC1: Protease core domain 1

PC2: Protease core domain 2

PEF(L): Penta-EF-hand domain in the catalytic large subunit

PEF(S): Penta-EF-hand domain in the regulatory small subunit

PKC: Protein Kinase C

PLEIAD : Platform element for inhibition of autolytic degradation; originally called SIMC1/C5orf25 (SUMO-interacting motif containing protein 1/chromosome 5 open reading frame 25)

PML: Promyelocytic leukemia protein

SNAP-25: Synaptosomal-associated protein of 25 kDa)

SNARE: SNAP Receptor

SOH: SOL-homology domain

tBID: Truncated BID

TDP-43: Transactive response DNA binding protein 43 kDa

VDAC: Voltage Dependent Anion Channel

XL: N-terminal extended domain of calpastatin

Zn: Zn-finger motif

# Chapter 1: Aims and background

## Preface

Calpains are $Ca^{2+}$ activated proteases. About 50 years ago, Dr. Guroff in a seminal work described $Ca^{2+}$ activated proteinases (Guroff, G. 1964.), now called calpain. Since then, calpains have been considered to be mainly cytoplasmic, although a small fraction is also thought to be associated with the cell membrane. Sixteen isoforms of calpains have been discovered thus far, and of these, 1 and 2 are the most investigated. Many studies show that calpains 1 and 2 contribute to the neurodegeneration following traumatic insult. In the CNS, apart from calpains 1 and 2, calpains 5, 7, and 10 are also present but they have domain architectures different than calpains 1 and 2. An ortholog of calpain 5 contributes to necrotic cell death in neurons of *C.elegans*. Hence, I became curious to investigate calpain 5 in the CNS. This dissertation presents the investigation of cellular biology of calpain 5.

## 1.1 Aims of the study

Calpain 5 (CAPN5) is a non-classical member of the calpain family (Barnes and Hodgkin, 1996, Dear et al., 1997, Matena et al., 1998, Ono and Sorimachi, 2012). The calpain family has 15 catalytic isoforms and two regulatory isoforms. Calpain 1 and 2, the first reported calpains (Guroff, 1964, Goll et al., 2003, Sorimachi et al., 2010, 2011a), are $Ca^{2+}$-activated proteases. They have a large catalytic subunit and a small regulatory subunit, each possessing $Ca^{2+}$ binding EF-hand motifs (Rizo and Sudhof, 1998, Hosfield et al., 1999, Strobl et al., 2000, Sorimachi and Suzuki, 2001, Khorchid and Ikura, 2002, Moldoveanu et al., 2002). CAPN5 has only one subunit containing three domains (N, CysPc, and C2L) similar to calpain1 and 2, but it has a unique domain T (also called C2) at the C-terminus. It also lacks $Ca^{2+}$ binding EF-hand domains (Barnes and Hodgkin, 1996, Dear et al., 1997, Matena et al., 1998); yet, it may be $Ca^{2+}$ activated (Waghray et al., 2004). *Tra-3*, an ortholog of CAPN5 plays a role in female sex determination in XX hermaphrodites and also contributes to neuronal necrotic cell death while knocking down *Tra-3* protects against necrotic death (Hodgkin, 1986, Barnes and Hodgkin, 1996, Syntichaki et al., 2002). CAPN5 has been associated with pathologic conditions such as polycystic ovary syndrome (Gonzalez et al., 2006), endometriosis (Penna et al., 2008), diabetes (Saez et al., 2007), Huntington's disease (Gafni et al., 2004) and autoimmune retinopathy (Mahajan et al., 2012).

Calpains 1,2,5,7 and 10 are also present in the CNS (Guroff, 1964, Zimmerman and Schlaepfer, 1984, Dear et al., 1997, Dear and Boehm, 1999, Ma et al., 2001, Waghray et al., 2004). We examined the relative expression of these calpains in the CNS, using a real-time comparative $C_T$ method ($\Delta\Delta C_T$) (Schmittgen and Livak, 2008). Calpain 2 had the highest levels of mRNA expression, exceeding CAPN 5 by 2.7 fold. CAPN 5 was the second most highly expressed calpain in the brain, followed in descending order by

calpains 7, 10, and 1 (Fig 1.8). Despite the higher mRNA expression and the importance of Tra-3 in neuronal necrotic death, CAPN5 has been minimally investigated in the CNS. Also, the role of CAPN5 in early development is controversial. CAPN 5 null mice are embryonically lethal in *Capn5*$^{tm1Dgen}$/*Capn5*$^{tm1Dgen}$ line (MGI accession no.: 3604529); however, *Capn5*$^{tm1Nde}$ /*Capn5*$^{tm1Nde}$ null progenies are normal and healthy (Franz et al., 2004). The specific aims of my work are to characterize CAPN5 5 in the CNS:

### 1.1.1 Specific aim 1: To examine the developmental regulation of calpain 5 in the CNS

Rat brain and spinal cord will be collected from rats at age E18 (embryonic day 18), P0 (post natal day 0), P5, P10, P15, P20, P30 and P90, N=4 per time point. Relative mRNA expression of CAPN5 using $\Delta\Delta C_T$ method will be examined at each time point. Similarly, Western blot against CAPN5 will be performed at each time point.

### 1.1.2 Specific aim 2: To examine the subcellular localization of calpain 5

Different subcellular fractions will be prepared from rat brain cortex. Nuclear, synaptic and non-synaptic mitochondria and cytosolic fractions will be probed through Western blot to find subcellular localization of CAPN5. Nuclear fraction will also be purified to nucleic acid binding and integrated nuclear protein fractions, and probed through Western blot. Immunohistocytochemistry will be performed on SH-SY5Y cells and on rat brain sections to confirm the subcellular localization of CAPN5.

### 1.1.3 Specific aim 3: To investigate a putative organellar targeting sequence on calpain 5

Once CAPN5 is localized, the study will aim to determine a putative organellar targeting sequence (for example, nuclear localization signal). Experimentally, the predicted organellar targeting sequence will be ligated into the pN1-ZsGreen1 vector such that

ZsGreen1 is expressed at the C-terminal of the peptide sequence. SHSY-5Y cells will be transfected with the vector to localize expression of the ZSGreen1.

## 1.2 Background

### 1.2.1 Classical calpains

#### 1.2.1.1 Calpain 1 and calpain 2

##### 1.2.1.1a Structure and nomenclature

Calpains 1 and 2 have large subunits with four domains (I, II, III and IV), and a small subunit with two domains (V and VI). A new nomenclature for calpains was proposed at the 2013 FASEB-sponsored conference 'The Biology of Calpains in Health and Diseases'. In this new nomenclature, the word 'calpain', such as calpain1 designates the active protein, while the abbreviation CAPN1 refers to the large subunit. The full composition of the calpain should be presented as CAPN1+CAPNS1 (large subunit + small subunit) or CAPN1/S1 for calpain 1 and CAPN2+CAPNS1 or CAPN2/S1 for calpain 2. Domain I is now named N-terminal domain (Fig 1.2). Domain II is divided into PC1 (protease core 1) and PC2 domains, and together PC1 and PC2 comprise the CysPc domain. Because domain III possesses β sandwich structure, it is referred to as the BS domain. At the level of the tertiary structure, domain III resembles the C2 like (C2L) domain similar to that described for protein kinase C and a phospholipase (Rizo and Sudhof, 1998, Corbalan-Garcia and Gomez-Fernandez, 2010). Therefore, it is also called C2L domain. This dissertation will mention domain III as C2L domain. Domains V and VI are designated as PEF (L) and PEF(S) (Penta-EF-hands) for the large and the small subunits, respectively. Domain V has been termed the GR (glycine rich) domain.

The N-terminal domain is presumed to contain a putative propeptide region (Strobl et al., 2000). The anchor helix of N-terminal domain is cleaved during enzyme activation. The CysPc domain performs the catalytic activity. PC1 possesses 'C' residue, PC2 contains residues 'H' and 'N' residues (Strobl et al., 2000, Reverter et al., 2001). C2L domain targets the protein to the cell membrane during activation (Rizo and Sudhof, 1998, Corbalan-Garcia and Gomez-Fernandez, 2010). CysPc and C2L domains also have $Ca^{2+}$ binding non EF hand sites (Strobl et al., 2000, Hata et al., 2001, Reverter et al., 2001). 1-4 EF hands in each of PEF (L) and PEF(S) domain binds to $Ca^{2+}$, which induces a conformational change and enzyme activation (Strobl et al., 2000). EF-5 motifs of PEF (L) and PEF (S) are involved in the heterodimerization of the two subunits (Lin et al., 1997).

#### 1.2.1.1b Activation of calpain 1 and calpain 2

Activation of calpain requires binding of $Ca^{2+}$ at three sites: EF hands regions of domains PEF (L) and PEF (S), an acidic loop region of domain III, and catalytic sub-domains PC1 and PC2. Activation occurs in two stages: conformational change and activation. In the conformational stage, inactive calpain exists as a heterodimer of large and small subunits. Binding of $Ca^{2+}$ to each of EF 1-4 motifs of PEF (L) and PEF (S) releases the contact between N-terminal anchor helix and EF-2 motif of PEF (S). $Ca^{2+}$ binding to the acidic loop of C2L domain releases the association between C2L and catalytic domain. These changes provide appropriate conformational change for the activation (Strobl et al., 2000, Reverter et al., 2001, Moldoveanu et al., 2002). $Ca^{2+}$ bound C2L is presumed to be responsible for membrane translocation of the calpains during the process of activation (Rizo and Sudhof, 1998, Corbalan-Garcia and Gomez-Fernandez, 2010). In the activation stage, $Ca^{2+}$ also binds one each to PC1 and PC2 domains and provides

rearrangement of the active cleft (Hata et al., 2001). This results in the repositioning of 'C' and 'H' residues of the catalytic triad, consequently making a functional cleft. 'C' and 'H' would otherwise be too far apart (>8.5 Å apart in calpain 1) to form an active enzyme. The functional distance is ~3.5 Å (Hosfield et al., 1999). The disturbance in the structure upon $Ca^{2+}$ binding leads to general autolysis of N terminal and C2L domains, leaving behind activated N terminal and CysPc domains together, and a heterodimer of PEF(L) and PEF(S) domains, Fig 1.3 (Chou et al., 2011). Previously it was thought that activation results into an active large subunit and a homodimer of small subunits (Nakagawa et al., 2001, Suzuki et al., 2004).

#### 1.2.1.1c Endogenous inhibition of calpain activation

Activities of CAPN1/S2 and CAPN2/S2 are inhibited by an endogenous inhibitor calpastatin (CAST). CAST has four calpain inhibitory domains (CIDs). Each CID has conserved 'A', 'B' and 'C' sub-domains (Fig 1.4). The 'L' domain at the N terminus helps CAST to anchor to the membrane. During calpain inhibition, subdomains 'A' bind to PEF domains of the large subunits while 'C' binds to small subunits. However, subdomain 'B' wraps around the remaining subunits such that it blocks the active cleft. It loops out and around the active cleft to avoid cleavage (Hanna et al., 2008, Mellgren, 2008, Moldoveanu et al., 2008). CAST possesses 34 exons: 1xa, 1xb, 1y, 1z, 1u, 2-29. Exons 1xa-1y-1z-2-29 are retained by CAST N-terminal spliced variants type I, whereas type II retains 1xb-1y-1z-2-29, type III retains 1u-2-29, and type IV retains 14t-29. 1u is only expressed in type III, and 14t is an exon present between exon 14 and 15, which is uniquely present in type IV (Goll et al., 2003).

#### 1.2.1.1d Physiological roles of calpain 1 and calpain 2

CAPN1/S1 and CAPN2/S1 are ubiquitous calpains, however CAPN2/S1 is not present in erythrocytes. These calpains have been implicated in many physiological roles such as cell motility, cell cycle regulation, regulation of gene expression, apoptosis and signal transduction pathway (Kishimoto et al., 1989, Hirai et al., 1991, Glading et al., 2002, Lu et al., 2002, Goll et al., 2003). Transient activation of calpains is required to perform its basic physiological role. To perform these functions, calpain cleaves a wide array of proteins from structural proteins (tau, β-tubulin), receptors and channels ( GluR1, IP3R ), signaling enzyme ( PKC, CaMK), apoptosis proteins (caspase -3, 7, 8, 9, AIF) to transcription and translational factor (c-fos, c-jun, TDP-43, eLF4G) (Saatman et al., 2010, Yamashita et al., 2012). Although calpains have been considered mainly cytosolic, some are now also considered mitochondrial. Additionally calpains may have a role in the nucleus. Purified CAPN2/S1 and CAPN1/S1 also proteolyse and release integrated nuclear H1 kinase, which otherwise is abolished in the presence of CAST (Mellgren, 1991). Also, during cell division, CAPN2/S1 relocates to the nucleus, associates with the chromosomes, and promotes precocious disassembly of the mitotic spindle and progression of mitosis (Schollmeyer, 1988). Calpains also appear important for early development. Knocking out CAPNS1 (*CAPN4* $^{-/-}$), results in the ablation of the both CAPN1/S1 and CAPN2/S1 activities and is embryonically lethal in mice (Arthur et al., 2000). This suggests that calpains 1 or 2 or both are required for early development. Selective knock out of calpain 2 (*CAPN2*$^{-/-}$) is also embryonically lethal (Dutt et al., 2006) and causes pre-implantation lethality between the morula and blastocyst stage. *CAPN* 1$^{-/-}$ mice are viable, although they exhibit a reduction in platelet aggregation and clot retraction (Azam et al., 2001). Li et al 2009 showed that levels of CAPN2/S1 were present throughout the brain during development; however CAPN1/S1 mainly appeared postnatally (Li et al., 2009).

#### 1.2.1.1e Pathological roles of calpain1 and calpain 2

Sustained activation of calpain triggers the pathological cleavage of its substrates. Calpains are involved in many pathological conditions such as cancer, stroke, Alzheimer's disease, amyotrophic lateral sclerosis (ALS), and traumatic brain and spinal cord injuries (Saatman et al., 1996, Trinchese et al., 2008, Storr et al., 2011, Yamashita et al., 2012, Yu et al., 2013). The pathology pertaining to calpain activation is well characterized in traumatic brain injury (TBI). Following TBI, in the injured cortex, calpain activation occurs as early as 15 min (Kampfl et al., 1996) to 4 hrs (Saatman et al., 1996) and has been reported to last up to 48 hours (Deng et al., 2007). Late activation at 3 and 7 days post injury has also been reported in the thalamus of the rat brain (Saatman et al., 1996). Activated calpains cleave αII spectrin, an intracellular cytoskeleton protein (Siman et al., 1984). Cleavage results in a 150 KDa and a calpain-specific 145 KDa breakdown fragment.

Calpain also contributes to cell death, apoptosis, and necrosis. Cell death is a consequence of a series of events. During apoptosis, in response to death ligands, DNA damage or endoplasmic reticulum (ER) stress, pro-apoptotic proteins (BH3-only protein, tBID) antagonize anti-apoptotic BCL-2 protein, which activates BAK or BAX. Dimerization of BAK or BAX forms high order oligomers at the mitochondrial surface, which permeabilizes the mitochondrial outer membrane. Meanwhile, increased $Ca^{2+}$ level in the matrix also triggers inner membrane permeabilization. Together, these events destabilize lipid membrane or form a transition pore consisting of VDAC, ANT and CYP-D. CYT-C and cleaved AIF are released into the cytosol. Cleaved AIF translocates to the nucleus to induce cell death, while CYT-C forms apoptosome to activate caspase 3 (CASP3) and CASP7 in the cytosol. CASP3 cleaves ICAD that translocates to the nucleus to induce DNA fragmentation (Yuan et al., 2003, Tait and

Green, 2010). Calpains contribute to many of these events (Fig 1.5). Cytosolic calpain1 cleaves BID to tBID, which activates proapoptotic protein BAK or BAX. Mitochondrial CAPN1 located in the inner membranous space cleaves Na+/$Ca^{2+}$ exchanger and leads to $Ca^{2+}$ overload in the mitochondrial matrix promoting inner membrane permeabilization (Garcia et al., 2005, Kar et al., 2009). Calpain 1 is also suggested to cleave AIF, however this hypothesis has yet to gain consensus (Polster et al., 2005, Cao et al., 2007, Joshi et al., 2009). Mitochondrial calpain 2 is proposed to cleave VDAC, which facilitates the release of truncated AIF (Ozaki et al., 2009). In necrosis, the high influx of $Ca^{2+}$ primarily through NMDA receptors activates calpains that activate cathepsins. Subsequently, activated cathepsins degrade various substrates (Yuan et al., 2003, Tait and Green, 2010). In *C.elegans*, CLP-1 (calpain like protein-1) acts upstream of aspartyl proteases (cathepsin), and ASP3 and 4 to induce necrosis (Syntichaki et al., 2002).

#### 1.2.1.2 Calpain 3

Calpain 3 is a muscle specific calpain. A splice variant of calpain 3, Lp82, is mainly present in rat/mouse lens and retina (Ma et al., 1999). Calpain 3 is similar to CAPN1/S1 and CAPN2/S1 but has 'NS' at the N terminal, 'IS1' at the N terminal end of PC2 domain, and 'IS2' between domains C2L and PEF(L) (Fig 1.1). Mutating calpain 3 causes abnormal sarcomere formation and limb-girdle muscular dystrophy type 2A (Richard et al., 1995, Kramerova et al., 2004). Calpain 3 is most rapidly autodegraded via a $Na^{+}$ dependent process in the absence of $Ca^{2+}$ (Ono et al., 2010) The regions IS1 and IS2 may be responsible for the faster autodegradation. (Sorimachi et al., 1989, Ono et al., 2010). The N-terminal region of IS2 binds specifically to N2A and M-line regions of muscle protein connectin/titin; hence muscle-associated calpain 3 is more stable (Sorimachi et al., 1995). Cytosolic calpain 3 interacts with PLEIAD [platform element for

inhibition of autolytic degradation; originally called SIMC1/C5orf25 (SUMO-interacting motif containing protein 1/chromosome 5 open reading frame 25)], which provides the regulatory scaffolding and suppresses the protease activity of calpain 3 in the cytosol (Ono et al., 2013). Using a knock-in mutation to abolish catalytic function (C129S), calpain 3 can perform a non proteolytic role by regulating $Ca^{2+}$ efflux in the sarcoplasmic reticulum (Ojima et al., 2011).

#### 1.2.1.3 Calpains 8, 9, 11, 12, 13 and 14

Calpains 8, 9, 11, 12, 13 and 14 are structurally similar to CAPN1/S1 and CAPN2/S1 (Fig 1.1). Calpains 8 and 9 are present specifically in the gastrointestinal tract. Mutations in calpain 8 and calpain 9 cause stress-induced ulcers. These two calpain isoforms exist as a heterodimer complex, also known as G-CAPN, and are denoted as CAPN8+CAPN9 or CAPN8/9. Calpain 8 performs the proteolytic function; however, calpain 9 provides stability to G-CAPN (Hata et al., 2010). Calpain 11 is present in testes; while calpain 12 is present in hair follicle cells. Calpains 13 and 14 are ubiquitous.

### 1.2.2 Non-classical calpains

#### 1.2.2.1 Calpain 5

##### 1.2.2.1a Characteristics features

Calpain 5 has a different structure than classical calpain; hence, it is called non classical calpain. It has only one subunit that possesses N-terminal, CysPc and C2L domains similar to the large subunit of the classical calpains. Instead of PEF (L), it has domain T, now called C2 domain (Fig 1.1).

Calpain 5 is an ortholog of the proteinTra-3 in *C.elegans*. Tra-3 is important for the processing of Tra-2A for female development in the XX hermaphrodite. However, it is not required in males; Tra-3-/- males are viable and normal (Hodgkin, 1986, Barnes and Hodgkin, 1996). Tra-3 acts upstream of aspartyl proteases ASP-3 and ASP-4 to participate in necrotic cell death. Knocking down Tra-3 provides protection from necrotic neuronal cell death in *C.elegans* (Syntichaki et al., 2002). The predicted protein sequence of the *TRA-3* gene has sequence homology with rat CAPN1 and CAPN2 in the regions of domains N, PC1/PC2 and C2L. Tra-3 does not have a PEF domain. Instead, it has a non –EF hand domain T, with little homology at the C-terminus of PEF(L) domain of classical calpains. The catalytic triad (C, H, N) in domain II is also conserved. (Barnes and Hodgkin, 1996). A homologous protein found in vertebrates was named calpain5, also referred as hTra3 (Dear et al., 1997).

Alignment of the predicted amino acid sequence of human and mouse CAPN 5 with the representative members of the vertebrate calpain family resulted in significant sequence homology over the entire coding region. Also, an unrooted phylogenetic tree generated from bootstrap analysis placed CAPN 5 with calpain 6 in a common group divergent from the group consisting of calpains 1, 2, 3 and 8 (Dear et al., 1997). Genomic

organization of mouse CAPN 5 and calpain 6 identified them as a new calpain subfamily with 11 introns at identical locations, 6 of which are in similar locations as those known vertebrate calpain members (Matena et al., 1998).

#### 1.2.2.1b Activation and inhibition of calpain 5

Calpain 5 can be activated in a manner similar to calpain 1 and 2 (Fig 1.3). Although CAPN 5 lacks EF hand motifs, $Ca^{2+}$ could bind to PC1, PC2 and C2 domains, providing an appropriate conformational change to make an active cleft. Activated calpains are presumed to be processed from the N-terminal. Using an antibody against the $1^{st}$ 30 aa of domain N, CAPN 5 was processed when SH-SY5Y cells were treated with maitotoxin (a potent calcium channel opener) and with A23187 (calcium ionophore), indicating that CAPN 5 level was regulated at higher calcium levels (Fig 1.6) (Waghray et al., 2004). Calpain 5 is believed to be activated at higher $Ca^{2+}$ concentration (personal communication with Dr Hiroyuki Sorimachi, Tokyo Metropolitan Institute of Medical Science, conclusion drawn based on his own lab work).

Endogenous inhibitors of CAPN 5 are unknown. Calpain 5 inhibition by CAST is mainly speculative at this point. CAST 'A' and 'C' subdomains bind to the PEF domains of large and small subunits of calpain 1 and 2, while CAST B subdomain performs inhibitory regulation of the catalytic cleft (Yang et al., 1994, Takano et al., 1995). Deletion of CAST binding domains does not affect the inhibition potency of CAST B subdomains. For example, a synthetic oligopeptide containing only inhibitory B domain loses the binding ability but retains moderate inhibitory activity (Ma et al., 1993, Ma et al., 1994). Since CAPN 5 lacks EF regions, CAST might not bind to it. However, CAST-mediated CAPN5 inhibition might be possible in the presence of appropriate conformation providing

chaperone(s). Calpain 5 is inhibited by small molecule calpain inhibitors. Treating SHSY5Y cells with MDL-28170, a calpain 1 & 2 inhibitor, protected processing of CAPN 5 in the presence of calcium channel opener maitotoxin (Waghray et al., 2004).

#### 1.2.2.1c Localization of calpain 5

Calpain 5 is a ubiquitous protein (Fig 1.7) (Waghray et al., 2004). Using $^{32}$P labeled DNA probes specific to human (Dear et al., 1997) and mouse (Dear and Boehm, 1999) RNA, CAPN5 was expressed in various tissues including brain. In mouse embryo, using in situ hybridization with an antisense probe, CAPN 5 was detected in thymus, and sympathetic and dorsal root ganglia (Dear and Boehm, 1999). Reverse transcription (RT) transcripts of CAPN 5 were detected in various human tissues including the CNS (Waghray et al., 2004). Relatively little is known about the subcellular localization of CAPN 5 except that when using RP3- CAPN 5 antibody (Triple Point Biologics), inactive and active forms were localized to both cytosol and nucleus (Gafni et al., 2004).

#### 1.2.2.1d Physiological role of calpain 5

The physiological role of CAPN5 is unknown. *Tra-3*, an ortholog of CAPN 5 is involved in female development in nematode XX hermaphrodites. Tra-3 is also involved upstream of aspartyl proteases in necrotic cell death pathways in *C. elegans*. The role of CAPN 5 is mainly speculative based on Tra-3 information. The viability of CAPN 5-null mice (CAPN5$^{tm1Nde}$ /CAPN5$^{tm1Nde}$) indicates that it may be dispensable during early development. CAPN5$^{tm1Nde}$ / CAPN5$^{tm1Nde}$ (129X1/SvJ * C57BL/6J, http://www.informatics.jax.org/allele/key/26357 ) mice created by inserting LacZ cassette in exon 4 are viable and fertile, although some are severely runted at birth and die by 2 months

of age (Franz et al., 2004). However, another CAPN 5- null mice (CAPN5$^{tm1Dgen}$ /CAPN5$^{tm1Dgen}$ , 129P2/OlaHsd * C57BL/6, http://www.informatics.jax.org/allele/key/40097 ) are embryonically lethal (MGI ID 3604529, ). Position of LacZ insertion in this line is unknown. Mutation in the same gene resulting in two different phenotypes could be due to the difference in the genetic background (personal communication with Dr. Peter Greer, Queen's university). These mice produce rather confusing information regarding the role CAPN 5 during early development.

### 1.2. 2.1e Pathological role of calpain 5

Calpain 5 has been associated with polycystic ovary syndrome (Gonzalez et al., 2006), obesity (Saez et al., 2008), risk factors for diabetes (Saez et al., 2007) and Huntington's disease (HD) (Gafni et al., 2004). In the striatum of the huntingtin knock-in mouse, a higher level of CAPN 5 was present compared to wildtype, indicating that CAPN 5 may contribute to HD pathogenicity (Gafni et al., 2004). Missense mutations in the CAPN 5 catalytic region cause autoimmune retinal neurodegeneration, an autosomal dominant neovascular inflammatory vitreoretinopathy (ADNIV). Further study is required to understand the role of CAPN 5 signaling pathway in the pathology of these diseases.

### 1.2.2.2 Calpain 6

Calpain 6 is also an ortholog of Tra-3. It is similar to CAPN 5 in that it possesses an N-terminal, PC1/PC2, C2L and C2 domains but does not contain EF hand regions (Fig 1.1). Unlike CAPN 5, it carries a natural mutation in the catalytic domain: the 'C' of PC1 is mutated to 'K' (K81 in human and mouse), making it a non proteolytic calpain (Matena et al., 1998). It is predominantly present in the placenta, embryonic muscle and cartilage.

Calpain 6 acts as a suppressor of muscle differentiation and development. It is also expressed in regenerating muscle and can suppress regeneration (Tonami et al., 2013)

#### 1.2.2.3 Calpain 7

Calpain 7 is an ortholog of PalB required by the fungus *E.nidulans* to adapt to alkaline conditions (Denison et al., 1995, Futai et al., 2001). Calpain 7 is a ubiquitous protein, and has a conserved microtubule interacting (MIT) domain at the N-terminus followed by CysPc and two C2L domains in tandem (Fig 1.1). Calpain 7 is proteolytically active and its MIT domains interact with a subset of endosomal sorting complex required for transport (ESCRT)-III-related proteins. Calpain 7 may act as a protease in the ESCRT pathway (Yorikawa et al., 2008, Osako et al., 2010, Maemoto et al., 2013).

#### 1.2.2.4 Calpain 10

Calpain 10 is similar to calpain 7, except that it does not possesses N-terminal MIT domains (Fig 1.1). A mutation in calpain 10 is linked to Type 2 or non-insulin-dependent diabetes mellitus (NIDDM) (Horikawa et al., 2000). Calpain 10 partially cleaves SNAP-25 (synaptosomal-associated protein of 25 kDa) of the SNARE (SNAP Receptor) complex to trigger insulin release through exocytosis (Marshall et al., 2005, Evans and Turner, 2007). Calpain 10 is present in the cytosol and mitochondria, as well as in the nucleus (Ma et al., 2001, Arrington et al., 2006). Degradation of calpain 10 is associated with the development of nuclear selenite cataract (Ma et al., 2001). In mitochondria, calpain 10 has been associated with the respiratory dysfunction, mPTP activation, and proteolysis of complex I subunits of the electron transport chain, which were protected when inhibited with calpeptin (fig 1.5) (Arrington et al., 2006).

#### 1.2.2.5 Calpain 15

Calpain 15 is a ubiquitous protein. It has homology with *Drosophila* small optic lobes (sol), a protein important for the growth of columnar neurons of the optic lobes (Delaney et al., 1991). Calpain 15 has N-terminal Zn-finger motifs followed by CysPc domain with a SOL homology domain at the C-terminal (Fig 1.1). Little is known about the function of calpain 15.

#### 1.2.2.6 Calpain 16

Calpain 16 is also called demi-calpain. It possesses only one part, PC1 of the CysPc core, and has a C-terminal IQ motif interactive with calmodulin (Fig 1.1) (Sorimachi et al., 2011b).

#### 1.2.2.7 Phytocalpain and other calpains

DEK1, a phytocalpain has a transmembranous N -terminal 'TM' domain followed by a CysPc protease core with a C2L C-terminal domain. DEK 1 is required for the aleurone cell development in the *Zea mays* endosperm (Lid et al., 2002, Lid et al., 2005).

Drosophila, nematode, fungus and yeast have calpain-like proteins, which have a conserved cysteine protease domain. In terms of similarity, these have common characteristic features with non-classical calpain compared to classical calpains. Non-classical calpains seems to be evolutionarily conserved across the species (Sorimachi et al., 2011b).

**Figure1.1** Classification of the calpain family

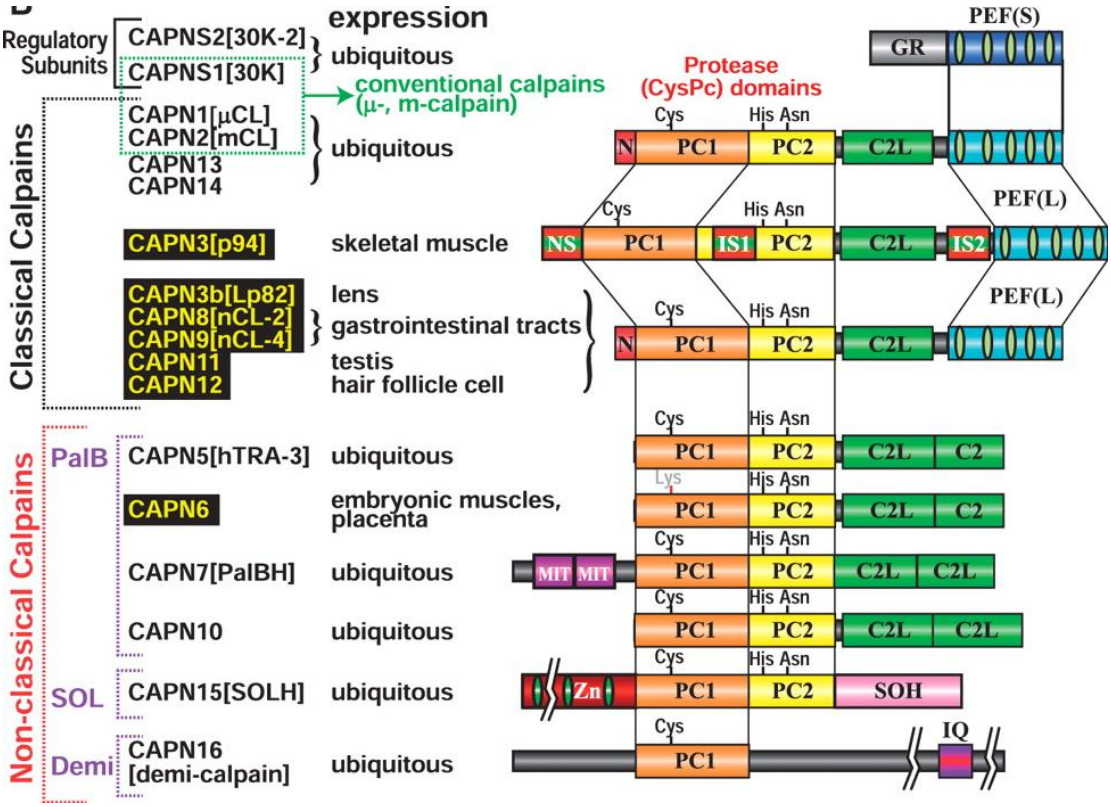

**Figure 1.1**: Classification of the calpain family. Reproduced from Sorimachi et al., 2011b with permission. Calpains are divided into classical and non-classical calpains. CAPNs 1and 2 are the best characterized calpains, and are referred to classical calpains. Other isoforms, which have domain architectures similar to CAPNs 1 and 2, are also classified as classical. Non classical calpains have conserved cysteine protease core with additional domains different than classical calpains. N: N-terminal anchor helix domain, PC1: Protease Core 1, PC2: Protease Core 2, C2: C2 Protein Kinase C (PKC ) conserved region 2 (Calcium binding, CalB), C2L: C2 Like domain, GR: Glycine rich, PEF: Penta EF hands Large (L) subunit or Small subunit(S), NS/IS1/IS2: CAPN3-characteristics sequences, MIT: Microtubule interacting and transport motif, Zn: Zn-finger motif, IQ: A motif interacting with calmodulin, and SOH: SOL homology domain.

**Figure 1.2** Newly proposed nomenclature of calpain domains

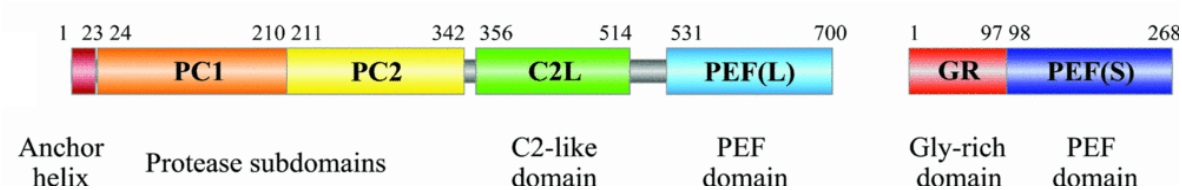

**Figure 1.2**: Newly proposed nomenclature of calpain domains. Domains of rat CAPN2 are shown. Reproduced from (Campbell and Davies, 2012) with permission. The newly proposed domains are N, anchor helix (initially domain I), PC1/PC2 (domain II), C2L (domain III) PEF-(L) (domain IV), GR (domain V), and PEF-(S) (domain VI). Please see Fig 1.1 legend for the abbreviations.

**Figure 1.3** A proposed mechanism of calpain activation

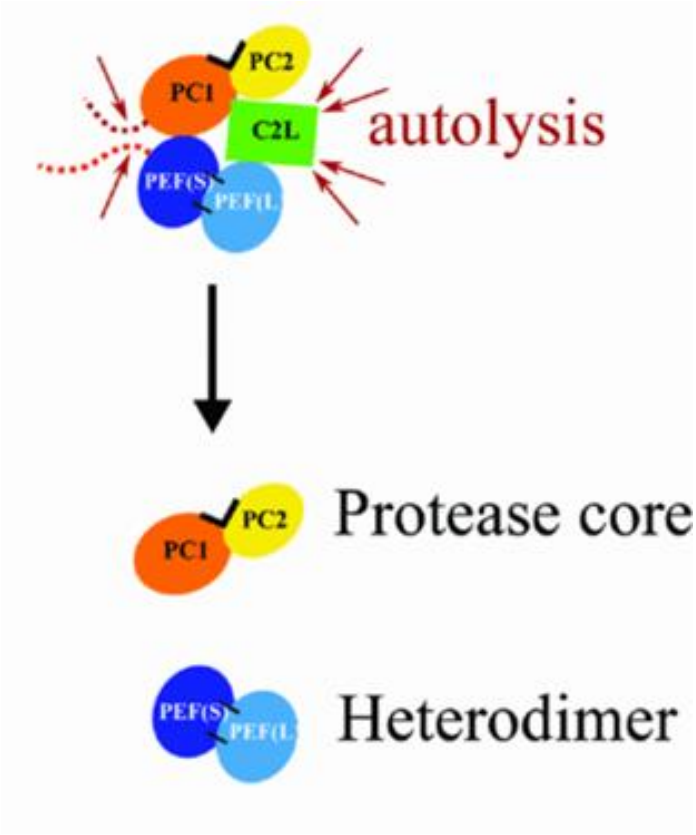

**Figure 1.3**: A proposed mechanism of calpain activation. Reproduced from Campbell and Davies, 2012 following the instructions at http://www.biochemj.org/bj/rights.htm . During the process of activation, $Ca^{2+}$ binds to 1-4 EF motifs of domains PEF (L) and PEF(S), and non EF-regions in PC1 and PC2 and C2L domains. Upon $Ca^{2+}$ binding, a general autolysis of N-terminal and C2L leaves behind a protease core and a heterodimer, PEF (L) + PEF (S).

**Figure 1.4** Classical calpains are inhibited by calpastatin

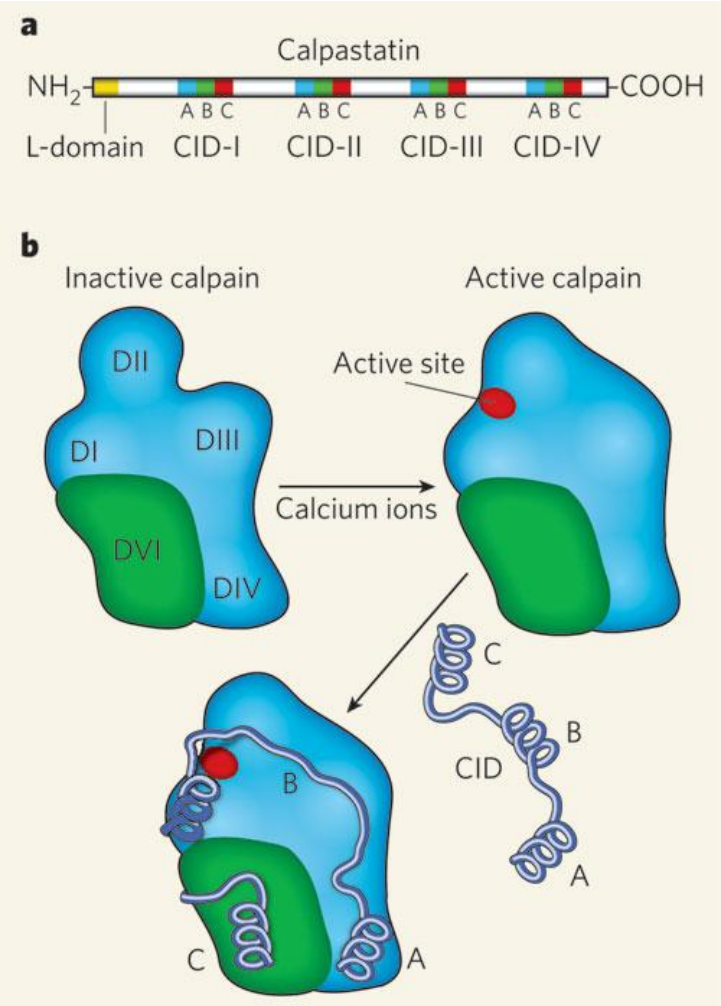

**Figure 1.4**: Classical calpains are inhibited by calpastatin. The image is reproduced from Mellgren, 2008 with permission. CAST subdomains A and C bind to PEF (L) and PFF (S), while subdomain B performs the inhibitory activity by blocking the active cleft. L-domain: anchor domain and CID: inhibitory domain

**Figure 1.5** Mitochondrial calpains and their possible role

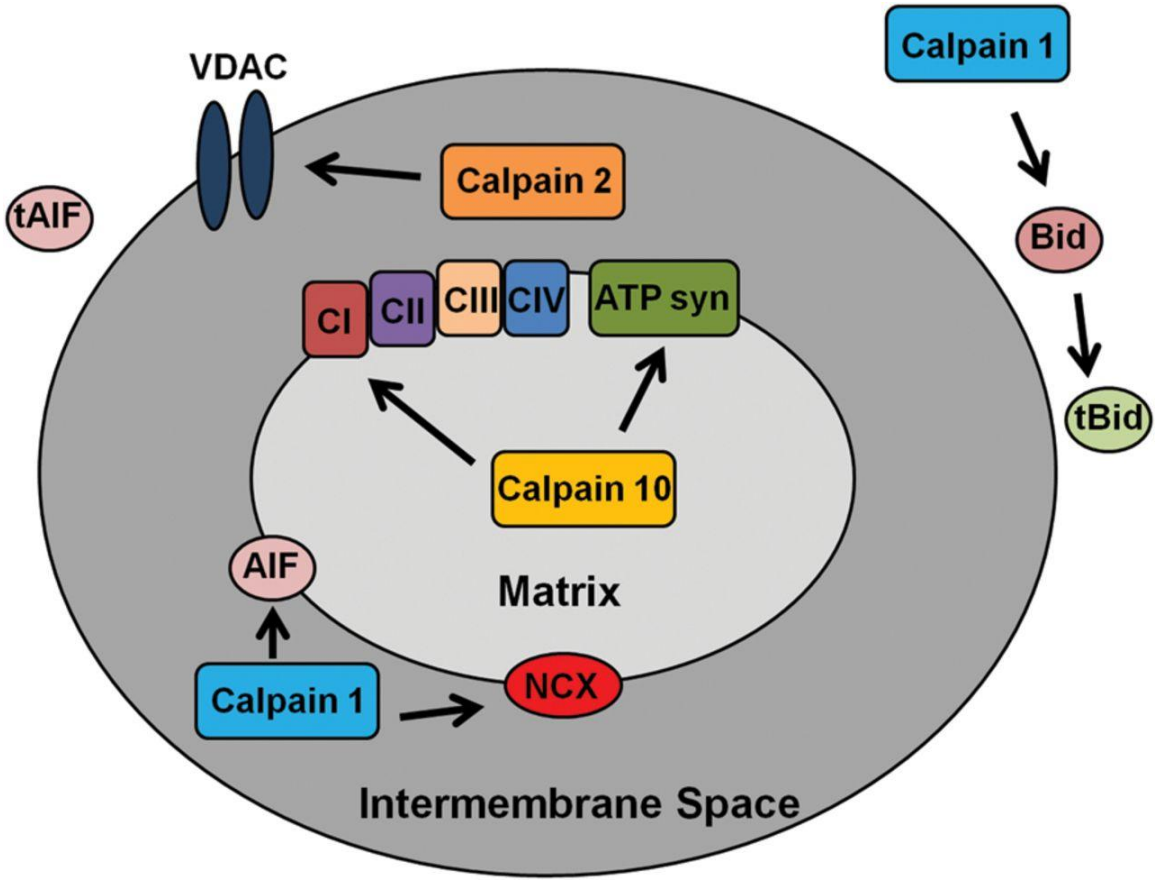

**Figure 1.5**: Mitochondrial calpains and their possible role. Reproduced from Smith and Schnellmann, 2012 with permission. Arrow indicates catalytic activity of the given calpain. Bid, tBid and tAIF are shown in the cytosol.

**Figure 1.6** Calpain 5 is calcium regulated

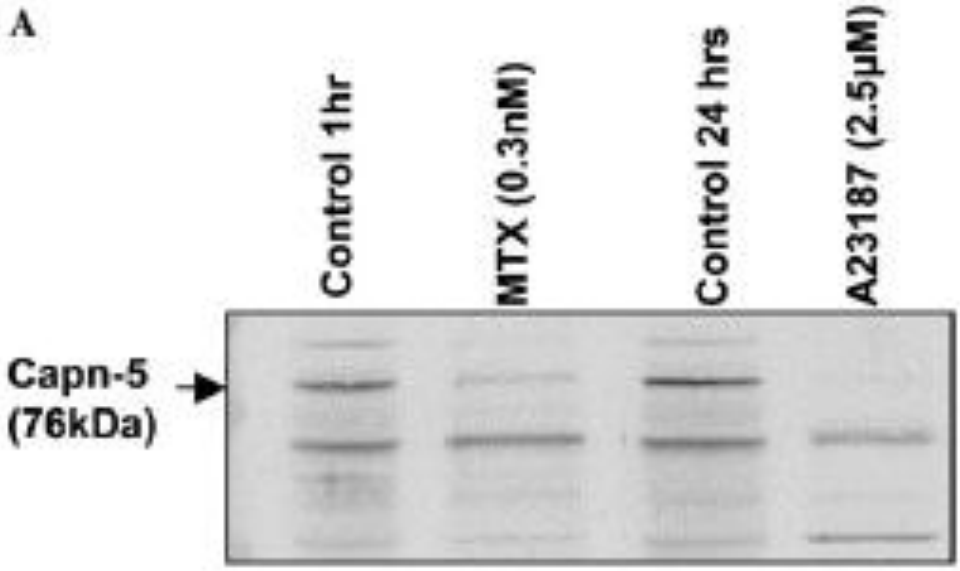

**Figure 1.6**: Calpain 5 is calcium regulated. Reproduced from Waghray et al., 2004 with permission. Using an antibody against N-terminal, CAPN5 was not detected in cells treated with $Ca^{2+}$ channel opener (Maitotoxin, MTX) and $Ca^{2+}$ ionophore (A23187) suggesting that the N-terminal of CAPN5 is processed in presence of $Ca^{2+}$.

**Figure 1.7** Calpain 5 RT transcript detected in various human tissues

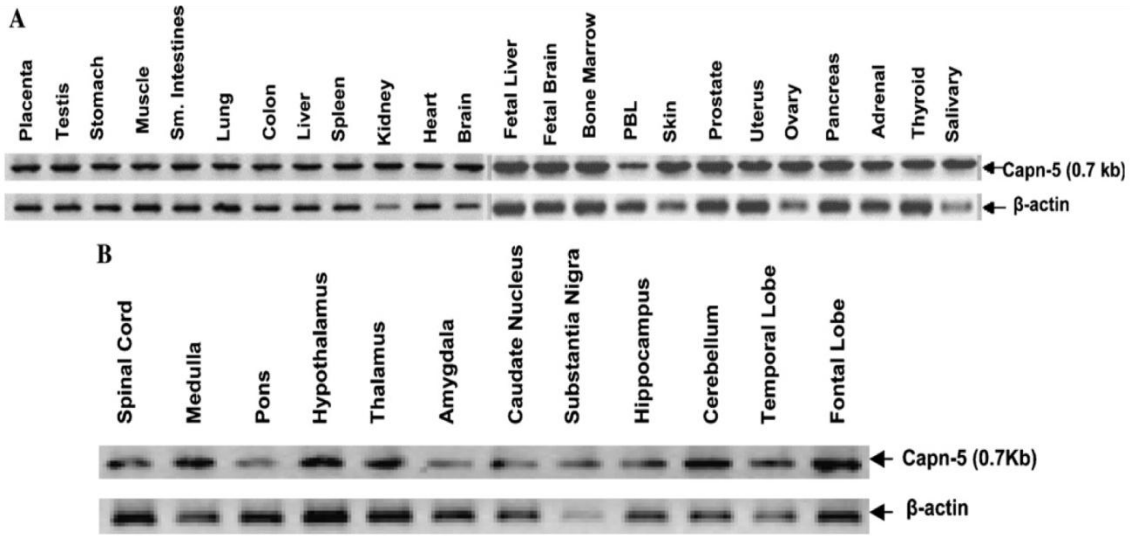

**Figure 1.7**: Calpain 5 RT transcript detected in various human tissues. Reproduced from Waghray et al., 2004 with permission.

**Figure 1.8** Calpain 5 is highly expressed in rat brain

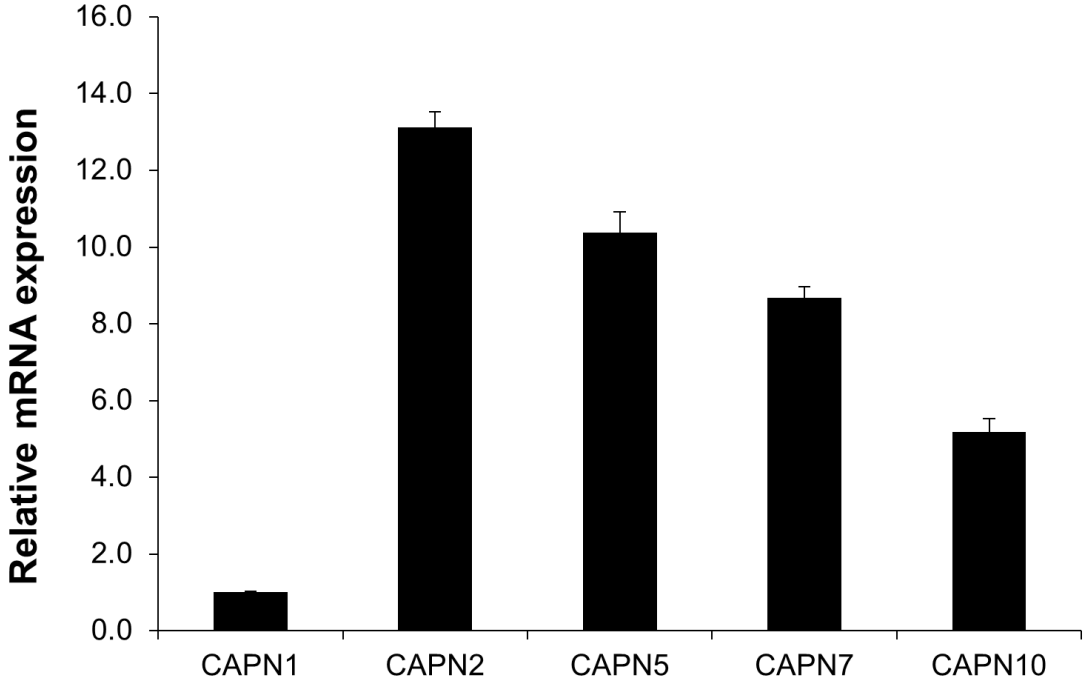

**Figure 1.8:**.Calpain 5 is highly expressed in rat brain. Relative mRNA expression of calpains 1, 2, 5, 7, and 10 was calculated using the comparative $C_T$ ( $\Delta\Delta C_T$) method in 3-month old male Sprague Dawley (SD) rat brain homogenate, N=4. $\Delta C_T$ of the each calpain isoform was obtained as a difference in the $C_T$ value from an endogenous control GAPDH. $\Delta\Delta C_T$ of the target gene was calculated by subtracting $\Delta C_T$ of the target gene from the $\Delta C_T$ value of reference gene, calpain 1. Relative mRNA expression of the target gene was then reported as $2^{-\Delta\Delta C}{}_{T.}$ The results show that after calpain 2, calpain 5 is the highest expressing calpain in the brain followed in descending order by calpains 7, 10 and 1. The results are expressed as the group means ± S.D, N=4.

# Chapter 2: Developmental regulation of Calpain 5

## Preface

The work shown in this chapter was performed as a group by Ranjana Singh, Ms.Vimala Bondada and Mr. Dingyuan Lou. Ranjana Singh collected various rat tissues: brain, spinal cord and liver at different developmental time points. Dingyuan Lou's work contributed to Fig 2.2. Vimala Bondada's work contributed to Figs 2.3.E and F. Ranjana Singh contributed to Figs 2.3. A, B C and D. Ranjana Singh also performed the statistical analysis of all the results. Fig 2.1 was reproduced with permission.

## 2.1 Introduction

The classical calpains, calpain 1 and 2, have a distinct large subunit and a common small subunit. Both the subunits have calcium binding EF hand motif. The small subunit is called calpain S1 and is also referred to as a regulatory subunit (Ono and Sorimachi, 2012). Non-classical CAPN5 contains only one subunit, similar to the large subunit of classical calpains; however, it does not contain EF hand motifs and a regulatory small subunit despite being calcium activated (Dear et al., 1997, Matena et al., 1998, Waghray et al., 2004). Calpains participate in many physiological functions such as cell motility, cell cycle, gene regulation, and cell death (Goll et al., 2003). They also appear to be involved in pre- and postnatal brain development (Li et al., 2009). The large subunit performs catalytic function whereas the small subunit regulates the process of activation. Therefore, both the subunits are required by typical calpains to perform a function. Knocking down CAPNS1 (CAPN4 $^{-/-}$) results in ablation of the both CAPN1 and 2 activities and is embryonically lethal in mice (Arthur et al., 2000). Selective knock out of calpain 2 (CAPN2$^{-/-}$) is also embryonically lethal (Dutt et al., 2006). CAPN 1$^{-/-}$ mice are viable, although they exhibit a reduction in platelet aggregation and clot retraction (Azam et al., 2001). This suggests that calpain 2, not calpain 1 is required for early growth. Along the similar hypothesis, Li et al 2009 detected a constant level of calpain 2 throughout rat brain development, whereas calpain 1 level were detected postnatally (Fig 2.1.).

Calpains or calpain-like proteins have been shown to play roles in the development of invertebrates and plant embryos. For example, in *Drosophila melanogaster* (dynamic changes in embryo cytoskeleton, dorsal ventral pattering and optic lobe development), *Plasmodium falciparum* ( transition to ring stage and progression of cell cycle.), *Danio rerio* (tissue specific expression during development), and in endosperm and embyo

development of plants such as *Arabidopsis thaliana and Zea mays* (Emori and Saigo, 1994, Lid et al., 2002, Friedrich et al., 2004, Lid et al., 2005, Lepage and Bruce, 2008, Russo et al., 2009) (Delaney et al., 1991, Friedrich et al., 2004).

Tra-3, an ortholog of calpain5, participates in nematode sex determination during development, and when mutated it results in partial masculinization of XX nematodes. Calpain 5 and 6 are orthologs of Tra-3 and have similar domain structures. Unlike calpain 5, calpain 6 carries a natural mutation in the catalytic domain: 'C' of PC1 is mutated to 'K' (K81 in human and mouse), making it a non proteolytic calpain (Matena et al., 1998). Calpain 6 is predominantly present during embryonic stage and suppresses muscle development and differentiation. It is also re-expressed in adult regenerating muscle and suppresses regeneration (Tonami et al., 2013). However, the role of calpain 5 is not known in pre- and postnatal development. Calpain 5 mRNA is present in the neurons of sympathetic and dorsal root ganglia in post coital day 16.5 mouse embryos (Dear and Boehm, 1999), implying that CAPN5 may be required for embryonic development. Additional evidence for its involvement in embryonic development is that CAPN5 null mice (CAPN5$^{-/-}$, *Capn5*$^{tm1Dgen}$/*Capn5*$^{tm1Dgen}$, MGI accession no.3604529) are embryonically lethal. However, CAPN5$^{-/-}$ in other line, *Capn5*$^{tm1Nde}$ /*Capn5*$^{tm1Nde}$, survive and are healthy barring some those are severely runted at birth (Franz et al., 2004). Hence, the role of CAPN5 in embryonic development as well as in the development of the CNS or other organs is not clear. Therefore, we pursued this study to understand the developmental regulation of CAPN5.

**Figure 2.1** Developmental regulation of calpain 1 and 2 in the rat brain

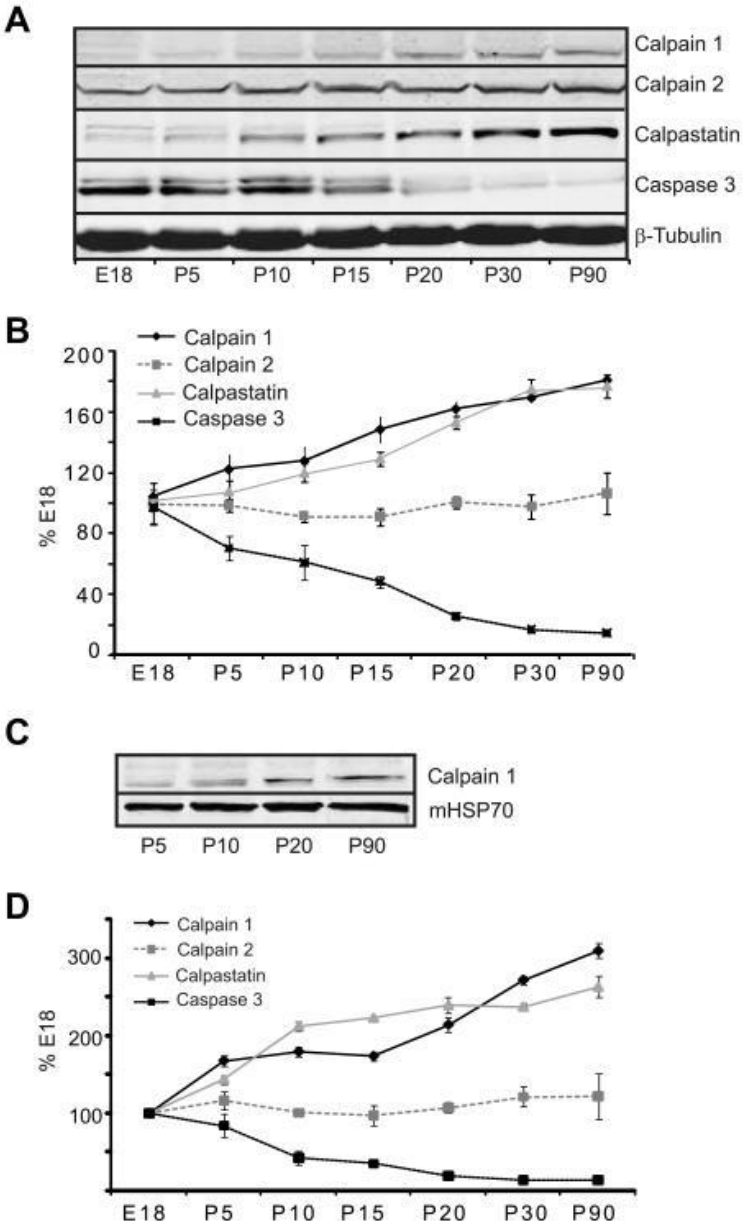

**Figure 2.1**. Western blots (**A,C**) represent the quantitative values of the immunoreactivity bands and mRNA expression detected using real time RT-PCR. Calpain1 progressively increased from E18 to P90; while, calpain 2 remained the same. Reproduced from (Li et al., 2009) with permission.

## 2.2 Materials and Methods

### 2.2.1 Experimental material

An Institutional Animal Care and Use Committee (IACUC) approved protocol was used to harvest brains from the rats and mice. SD rats of 18 days in utero (E18), postnatal day 0 (P0), 5, 10, 15, 20, 30 and 90 were used in this study. A timed pregnant female rat was euthanized with $CO_2$ to dissect out E18 from uterus. Postnatal rats up to 10 days of age were decapitated after desensitization in ice. Older rats were exsanguinated using $CO_2$ inhalation, followed by decapitation. Brain, spinal cord, and liver samples were removed and homogenized in the appropriate buffer as described below for qPCR and Western blot.

### 2.2.2 Antibodies and reagent

Antibodies against CAPN5 (ab28280, polyclonal), calpain1 (ab28257, polyclonal), and CAPN5 amino-terminal peptide (ab41310) were purchased from Abcam, Cambridge, MA. An antibody against calpain 2 (208729) was purchased from Calbiochem, Billerica, MA. An antibody against GAPDH (G8795), TRI reagent (T9424), and β-mercaptoethanol (M6250) were purchased from Sigma, St. Louis, MO. Protease inhibitor tablets (11873580001) were obtained from Roche, Indianapolis, IN. Secondary antibodies IRDye 800CW Anti-rabbit IgG (611-131-132) and IRDye 800 CW Anti-mouse IgG (610-131-121) were purchased from Rockland, Gilbertsville, PA. Additional reagents were purchased from Sigma (St. Louis, MO) or Thermo Fisher Scientific (Ashville, NC).

### 2.2.3 Quantitative PCR (qPCR)

Brain, spinal cord, and liver samples were homogenized in TRI reagent to extract total RNA. cDNA was prepared using an Applied Biosystems high capacity reverse transcription kit (AB # 4368814). Equal amounts of cDNA (100 ng) were used to perform qPCR using TaqMan gene expression master mix (AB 4369016). Reactions were performed in triplicate, plus a negative control without cDNA. The qPCR was programmed as an initial denaturation at 50°C for 2 min, followed by 95 °C for 10 min, 40 cycles @ 95 °C for 15 s, and 60 °C for 1 min, on a StepOne real-time PCR system (Applied Biosystems). The following rat gene transcripts were examined: CAPN1 (NCBI Reference Sequence NM_019152.2, TaqMan gene expression assay Rn00569689_m1); CAPN2 (NM_017116.2, Rn00567422_m1), CAPN5 (NM_134461.1, Rn00593213_m1) and GAPDH (NP_058704, Rn99999916-s1). Relative gene expression was determined using Comparative $C_T$ values. $\Delta C_T$ of the target gene at a particular developmental time point was obtained as a difference in the $C_T$ value from endogenous control GAPDH at the same point. $\Delta\Delta C_T$ value of the target gene was calculated by subtracting $\Delta C_T$ value of the target gene from the $\Delta C_T$ of a reference gene, calpain 1 at E18. The relative expression of target gene was then reported as $2^{-\Delta\Delta C}{}_T$.

### 2.2.4 Protein extraction and Western Blot

Brain, spinal cord, and liver samples were homogenized separately in twice the volume of 1xTBS supplemented with protease inhibitor mix (w/v). Homogenized mixture was ultracentrifuged at 124,000 X g for 20 min at 4$^{o}$C, and supernatant was stored at -80°C to use for Western blotting. Protein content of samples were assayed using Thermo Scientific Pierce® BCA protein assay reagent A (23228) and reagent B (1859078).

Protein (50 µg) was mixed in sample buffer supplemented with 5% β-mercaptoethanol, and boiled for 5 min then run on a 10% polyacrylamide gel (Biorad # 161-1155) or 4-12% Bis-Tris HCl gels (NuPAGE NP0335). Completely run gels were sandwiched in a Trans-Blot transfer pack (Biorad # 170-4158), and transferred to a nitrocellulose membrane using a Biorad semi dry Trans-Blot® Turbo TM transfer system. Following blocking in 5% skim milk in 0.05% Tween 20 in Tris-buffered saline, pH 7.6 (T-TBS) for 1 hr, the membrane was incubated with primary antibody (1:5000, CAPN5; 1:1000, CAPN1; 1:5000, CAPN2; 1:10,000, and 1:5000, GAPDH) in 5% skim milk in1X T-TBS (0.05% tween-20 ) overnight at 4ºC. The membrane was washed in T-TBS three times (3x) for 20 min each, followed by incubation with an appropriate secondary antibody (Anti-rabbit IgG, 1:5000; or anti-mouse IgG, 1:5000) at room temperature for an hr in dark conditions. The membrane was washed again 3x for 20 min each, and scanned using an Odyssey Infrared Imager (*LI-COR* Biosciences).

### 2.2.5 Statistical analysis

Quantified values of the immunoreactivity bands and qRT-PCR RQ data were analyzed using one-way ANOVA, followed by Tukey's multiple comparison tests.

## 2.3 Results

### 2.3.1 Ab28280 is specific to Calpain 5

On Western blots of rat brain homogenate, the antibody detected a prominent band at ~75 kDa. Pre-incubation of the antibody with the immunogenic peptide abolished the 75 kDa band on Western blots and the antibody did not detect purified rat calpain 2 (Calbiochem # 208718) (Fig 2.2 B &C). The antibody also detected a similar prominent band at ~75 KDa from SH-SY5Y cells expressing full length human CAPN5 fused with a FLAG tag on the C-terminal (Fig 2.2 D).

### 2.3.2 Calpain 5 is detected later in the post natal development in the CNS and liver

CAPN5 mRNA and protein levels were examined in the rat brain, spinal cord, and liver tissues, obtained from embryonic day 18 (E18), post natal day 0 (P0), P5, P10, P15, P20, P30 and P90 SD rats . CAPN5 mRNA levels were downregulated during CNS as well as liver development. However in the brain, the levels increased by 0.32 fold from E18 to P0, then decreased by a 0.67 fold until P90 (Fig 2.3 A). A similar mRNA developmental profile was obtained in spinal cord where the levels were increased 0.66 fold from E18 to P0, followed by a progressive decline up 0.92 fold by P90 (Fig 2.3 B). In liver, mRNA levels progressively decreased as much as 0.9 fold from E18 to P0 (Fig 2.3 C, table 2.1). In contrast to mRNA, CAPN5 protein levels were not detected at E18 and early post natal developmental points before P20. However, they were detected later in the post natal development of rat brain, spinal cord and liver (Fig 2.4, table 2.2).

### 2.3.3 Calpains 1 and 2 are differentially regulated in CNS and liver during development

Increasing calpain 1 and a constant calpain 2 protein levels during brain development have been previously reported (Li et al., 2009). Our results agree with Li et al. in that we found calpain 1 mRNA and protein levels were increased in the spinal cord during development. However in liver, CAPN1 mRNA declined while CAPN1 protein levels were unchanged. CAPN2 mRNA levels decreased from E18 to P90 in spinal cord, whereas, the protein levels increased. In liver, CAPN2 mRNA peaked at P10 and P15 with unchanged protein levels during the development.

In summary, calpains 5, 1 and 2 have dissimilar patterns of mRNA and protein expression because their mRNA and protein levels during development do not correlate. Calpain 5 protein was detected later in the development of the brain (P20), spinal cord (P10) and liver (P30).

## 2.4 Discussion

Calpain 5 mRNA and protein levels were evaluated in rat brain, spinal cord, and liver obtained from late embryonic to late postnatal developmental time points. The results revealed that mRNA level was downregulated from E18 to P90 but the protein level was upregulated during the postnatal brain, spinal cord, and liver development. Calpain 1 and 2 mRNA also did not correlate well with the protein levels. CAPN5 protein was detected mainly in the brain, spinal cord, and liver of adult rats as compared. The protein levels of CAPNs 1 and 2 varied in these tissues at different developmental time points.

The discrepancy between mRNA and protein levels can be explained in couple of ways. CAPN5 was detected using an antibody against amino terminal of domain I. Autolysis of

N-terminus has been inconsistently used as a marker for the activation of typical calpains (Cong et al., 1989, Baki et al., 1996, Neumar et al., 1996), including CAPN5 in two instances (Franz et al., 2004, Waghray et al., 2004). If N-terminus autolysis is also true for CAPN5, the protein level reported in this study represents mainly unprocessed CAPN5 and this could explain some of the discrepancies between mRNA and protein levels. The hypothesis that the activated calpains are processed from N-terminus is not universally accepted (Cong et al., 1989, Molinari et al., 1994). Such an inverse relation between mRNA and protein levels can also be described because calpains are long-lived proteins. Typical calpains have metabolic half-lives of as long as 5 days (Zhang et al., 1996). That means a relatively lower mRNA level may be enough to translate a large amount of the protein. Post translation modification at early developmental time point could also explain this discrepancy up to some extent. Modifications such as sumoylation may bar protein from being detected. RNA silencing could be another possibility. For example, *Rattus norvegicus* is predicted to have two microRNA (mir), mir-146a and mir-146b), which targets calpain 5 (http://www.microrna.org/microrna/getGeneForm.do ). This may result into silencing mRNA expression that may cause into the discrepancy between mRNA and protein level. A definitive explanation for unrelated calpain 5 mRNA and protein levels remain unknown.

Increase in mRNA level from E18 to P0 in brain and spinal cord, followed by a continuous decline postnatally, is not easily explained. At P0 (birth), the CNS needs to adapt quickly to a new temperature and environment, which would be underpinned by cellular, molecular and biochemical changes. Calpains are involved in many signal transduction pathways and in regulation of cell cycle and gene expression (Goll et al., 2003). Hence, an increase in CAPN5 mRNA at P0 would be needed to execute the above mentioned processes. But, at the same time CAPN5 protein was not detected at

P0 in our study (it was detected at P0 in spinal cord of only one rat). Therefore, if this hypothesis were true, the level of CAPN5 mRNA would be expected to change in liver, since the onset of independent functioning of liver may require alterations in many pathways that involve CAPN5. However in liver, CAPN5 mRNA did not increase at P0, it progressively declined from E18 to P90. The reason for an increase in CAPN5 mRNA in the CNS from E18 to P0 followed by a decline postnatally is unknown.

Our results show that CAPN5 protein appears earlier in spinal cord (P10) compared to brain (P15 - P20). In liver, it appears around P30. The CNS, especially brain, development is an ongoing process which lasts until adolescence (Stiles and Jernigan, 2010). As various functions are learned, brain and spinal cord continue to develop. Spinal cord develops more rapidly postnatally compared to brain. Hence, CAPN5, which may underpin some the cellular and physiological processes in spinal cord would be needed at an earlier stage compared to brain. Postnatal appearance of CAPN5 in brain coincides with the time when most of the characteristic features of an adult brain are achieved. Some of these features are larger perikarya, higher synaptic junction/$mm^3$ and axon density. (Eayrs and Goodhead, 1959, Aghajanian and Bloom, 1967). Since CAPN5 is predominantly present in nucleus and enriched in synaptic mitochondria (chapters 3 & 4), it suggests a role of CAPN5 in adult brain development and synaptic functions. Liver develops in three parts: embryonic liver development, morphogenesis, and organogenesis and is the shape of an adult liver by P30 and P90 with differentially matured hepatocytes (Apte et al., 2006). The late onset of CAPN5 in liver (P30) implies it has a role in differentiation of hepatocytes.

Calpain 5 is the $2^{nd}$ most highly expressed calpain in the adult rat CNS. It appears postnatally during brain (P20) and spinal cord (P10) development. The protein levels of calpain1 and CAST increase postnatally. Calpain 2 protein level remains unchanged

during development (Fig 2.1) (Li et al., 2009). Also, CAPN2$^{-/-}$ mice are embryonically lethal, whereas CAPN1$^{-/-}$ mice are viable. This indicates that calpain 2 is required for embryonic development. However, calpain 1 is required for postnatal adult CNS development. In adult rat brain, CAPN5 mRNA levels were 10-fold higher than calpain1 mRNA levels (Fig 1.8), which suggests that CAPN5 could be one of the major calpains in the adult brain. Calpains 5 and 6 are mammalian orthologs of Tra-3 and have similar domain structures. Unlike CAPN5, calpain 6 is non proteolytic as it lacks 'C' of the catalytic triad (Matena et al., 1998). It is present during embryonic development and suppresses muscle growth and differentiation (Tonami et al., 2013). Calpain 6 contributes to embryonic developments, whereas CAPN5 seems important in adult CNS development.

Proteases are required for programmed cell death or apoptosis during prenatal development.While caspases are involved in apoptosis and embryonic development, calpains are mainly involved in necrotic cell death following insult and injury (Kuan et al., 2000, Geddes and Saatman, 2010). Tra-3, an ortholog of CAPN5 in nematode is involved in necrotic cell death (Syntichaki et al., 2002). Caspase 3 levels decline postnatally; however, the levels of calpain1 and CAPN5 5 increase postnatally (Figs 1.8 & 2.1) (Li et al., 2009). A shift from apoptosis during embryonic development to necrosis in the adult CNS can be explained by the declining levels of caspase and rising levels of calpain1 and 5 during post natal CNS development. Since, CAPN5 mRNA was 10-fold higher than calpain 1 mRNA in the adult rat CNS, CAPN5 can be considered a major calpain involved in necrosis in the adult CNS.

In conclusion, CAPN5 appears during postnatal spinal cord (P10) and brain development (P20) along with calpain 1. Since CAPN5 mRNA levels are second to

calpain 2 and 10-fold higher than calpain 1 in the adult CNS, CAPN5 could be one of the major calpains in the adult CNS.

**Table 2.1 Statistical analysis of mRNA expression, One - way ANOVA followed by tukey's multiple comparison test**

| **Tukey's multiple comparison test** | BRAIN | LIVER | | |
|---|---|---|---|---|
| | CAPN5 | CAPN1 | CAPN2 | CAPN5 |
| | p values, One way ANOVA | | | |
| | <0.0001 | <0.0001 | <0.0001 | <0.0001 |
| | | | | |
| E18 vs P0 | P < 0.001 | *** | ns | ns |
| E18 vs P5 | P < 0.05 | **** | ns | ** |
| E18 vs P10 | P < 0.05 | **** | * | ns |
| E18 vs P15 | P < 0.001 | **** | ns | ** |
| E18 vs P20 | P < 0.001 | **** | ns | ** |
| E18 vs P30 | P < 0.001 | **** | ns | *** |
| E18 vs P90 | P < 0.001 | **** | ns | **** |
| P0 vs P5 | P < 0.05 | ns | ns | ns |
| P0 vs P10 | P < 0.001 | ** | ns | ns |
| P0 vs P15 | P < 0.001 | ** | ns | ns |
| P0 vs P20 | P < 0.001 | ** | ns | ns |
| P0 vs P30 | P < 0.001 | *** | ns | * |
| P0 vs P90 | P < 0.001 | ** | ns | * |
| P5 vs P10 | P < 0.001 | ns | ns | ns |
| P5 vs P15 | P < 0.001 | ns | ns | ns |
| P5 vs P20 | P < 0.001 | ns | ns | ns |
| P5 vs P30 | P < 0.001 | ns | ns | ns |
| P5 vs P90 | P < 0.001 | ns | ns | ns |
| P10 vs P15 | P > 0.05 | ns | ns | ns |
| P10 vs P20 | P > 0.05 | ns | * | ns |
| P10 vs P30 | P < 0.01 | ns | *** | ns |
| P10 vs P90 | P < 0.01 | ns | *** | ns |
| P15 vs P20 | P > 0.05 | ns | * | ns |
| P15 vs P30 | P > 0.05 | ns | ** | ns |
| P15 vs P90 | P > 0.05 | ns | ** | ns |
| P20 vs P30 | P > 0.05 | ns | ns | ns |
| P20 vs P90 | P > 0.05 | ns | ns | ns |
| P30 vs P90 | P > 0.05 | ns | ns | ns |

**Table 2.2 Statistical analysis of protein expression, One - way ANOVA followed by tukey's multiple comparison test**

| Tukey's multiple comparison test | BRAIN | LIVER | | |
|---|---|---|---|---|
| | CAPN5 | CAPN1 | CAPN2 | CAPN5 |
| | p values, One way ANOVA | | | |
| | <0.0001 | 0.02 | 0.79 | <0.0001 |
| | | | | |
| E18 vs P0 | P > 0.05 | P > 0.05 | - | P > 0.05 |
| E18 vs P5 | P > 0.05 | P > 0.05 | - | P > 0.05 |
| E18 vs P10 | P > 0.05 | P > 0.05 | - | P > 0.05 |
| E18 vs P15 | P > 0.05 | P > 0.05 | - | P > 0.05 |
| E18 vs P20 | P > 0.05 | P > 0.05 | - | P > 0.05 |
| E18 vs P30 | P < 0.01 | P > 0.05 | - | P < 0.01 |
| E18 vs P90 | P < 0.001 | P > 0.05 | - | P < 0.001 |
| P0 vs P5 | P > 0.05 | P > 0.05 | - | P > 0.05 |
| P0 vs P10 | P > 0.05 | P > 0.05 | - | P > 0.05 |
| P0 vs P15 | P > 0.05 | P < 0.05 | - | P > 0.05 |
| P0 vs P20 | P > 0.05 | P > 0.05 | - | P > 0.05 |
| P0 vs P30 | P < 0.01 | P > 0.05 | - | P < 0.01 |
| P0 vs P90 | P < 0.001 | P > 0.05 | - | P < 0.001 |
| P5 vs P10 | P > 0.05 | P > 0.05 | - | P > 0.05 |
| P5 vs P15 | P > 0.05 | P < 0.05 | - | P > 0.05 |
| P5 vs P20 | P > 0.05 | P > 0.05 | - | P > 0.05 |
| P5 vs P30 | P < 0.01 | P > 0.05 | - | P < 0.01 |
| P5 vs P90 | P < 0.001 | P > 0.05 | - | P < 0.001 |
| P10 vs P15 | P > 0.05 | P > 0.05 | - | P > 0.05 |
| P10 vs P20 | P > 0.05 | P > 0.05 | - | P > 0.05 |
| P10 vs P30 | P < 0.01 | P > 0.05 | - | P < 0.01 |
| P10 vs P90 | P < 0.001 | P > 0.05 | - | P < 0.001 |
| P15 vs P20 | P > 0.05 | P > 0.05 | - | P > 0.05 |
| P15 vs P30 | P > 0.05 | P > 0.05 | - | P < 0.01 |
| P15 vs P90 | P < 0.01 | P > 0.05 | - | P < 0.001 |
| P20 vs P30 | P > 0.05 | P > 0.05 | - | P < 0.05 |
| P20 vs P90 | P < 0.05 | P > 0.05 | - | P < 0.001 |
| P30 vs P90 | P > 0.05 | P > 0.05 | - | P > 0.05 |

**Figure 2.2** Ab28280 is specific to Calpain5

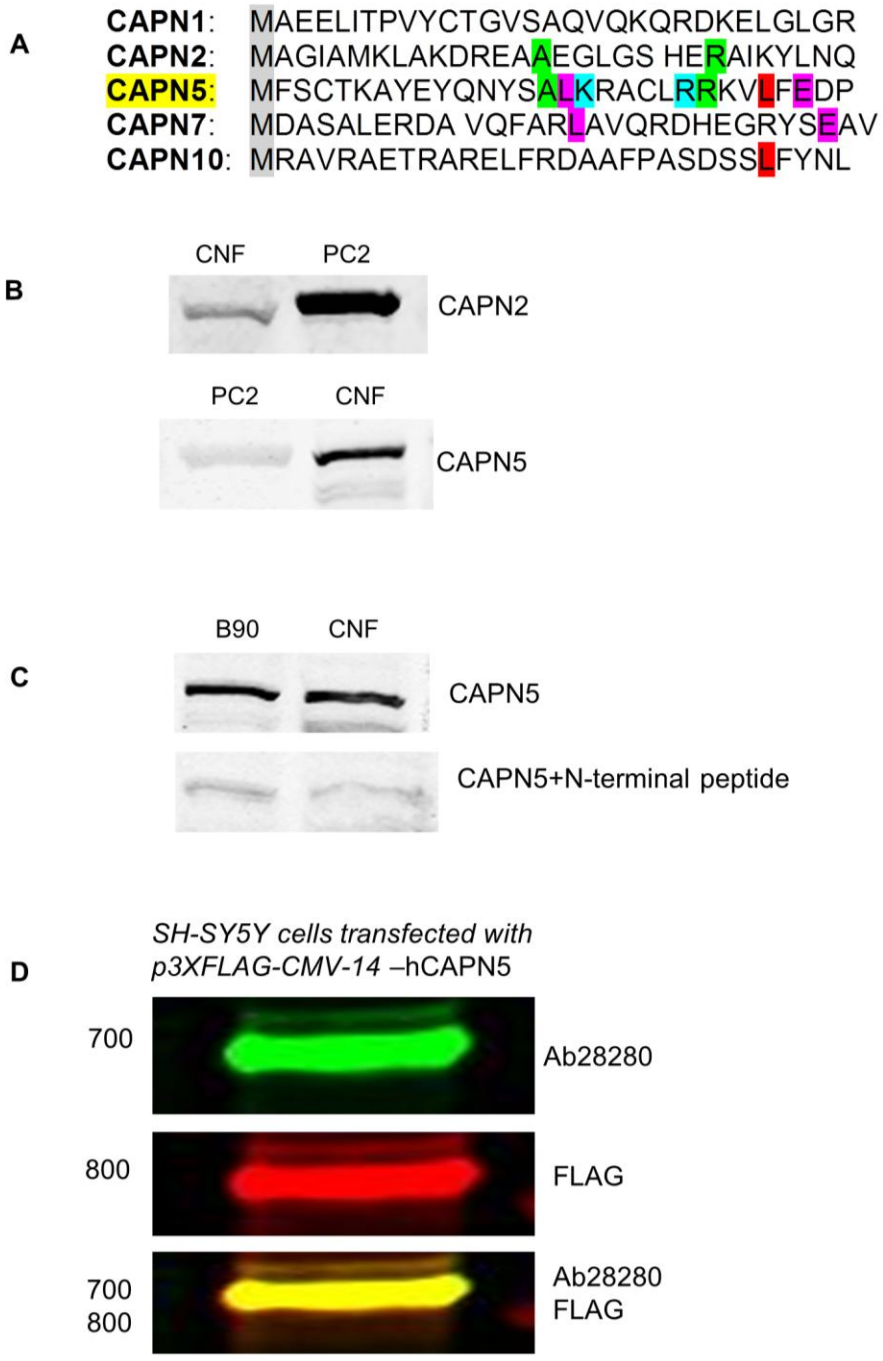

**Figure 2.2** Ab28280 is specific to Calpain5. Antibody ab28280 is raised against N-terminal end (aa 1-30) of CAPN5. The first 30 aa of the calpains 1, 2, 5, 7 and 10 present in the brain are fairly different. They have occasional matches (highlighted with the similar color), suggesting ab28280 may not cross react with another calpain isoform (**A**). CAPN5 does not detect purified rat calpain 2, CNF: Crude nuclear fraction, PC2: Purified calpain 2 (**B**). The antibody detected a prominent band at ~75 kDa. Pre-incubation of the antibody with the immunogen peptide abolished the 75 kDa band on Western blots, B90: Brain homogenate from a 90 day old rat (**C**). SH-SY5Y cells were transfected with a vector encoding the full length of human CAPN5 (hCAPN5) fused with a FLAG tag at the C-terminal. Cell lysate was prepared 24 h post transfection, and probed through Western blot. Ab28280 was linked with the secondary antibody IRDye 800CW anti-rabbit IgG. FLAG was detected using a FLAG-M2 antibody (Agilent technologies 200472) and was linked with the secondary antibody, IRDye 680 CW anti-mouse IgG (610-131-121). The blot was scanned with channel intensities 700 and 800 separately, and together gave a yellow fusion color. The antibody also detected a similar prominent band of ~75 KDa from SH-SY5Y cells expressing full length human CAPN5 fused with a FLAG tag on the C-terminal (**D**).

**Figure 2.3** The mRNA level of calpain 5 is downregulated in rat CNS and liver during development

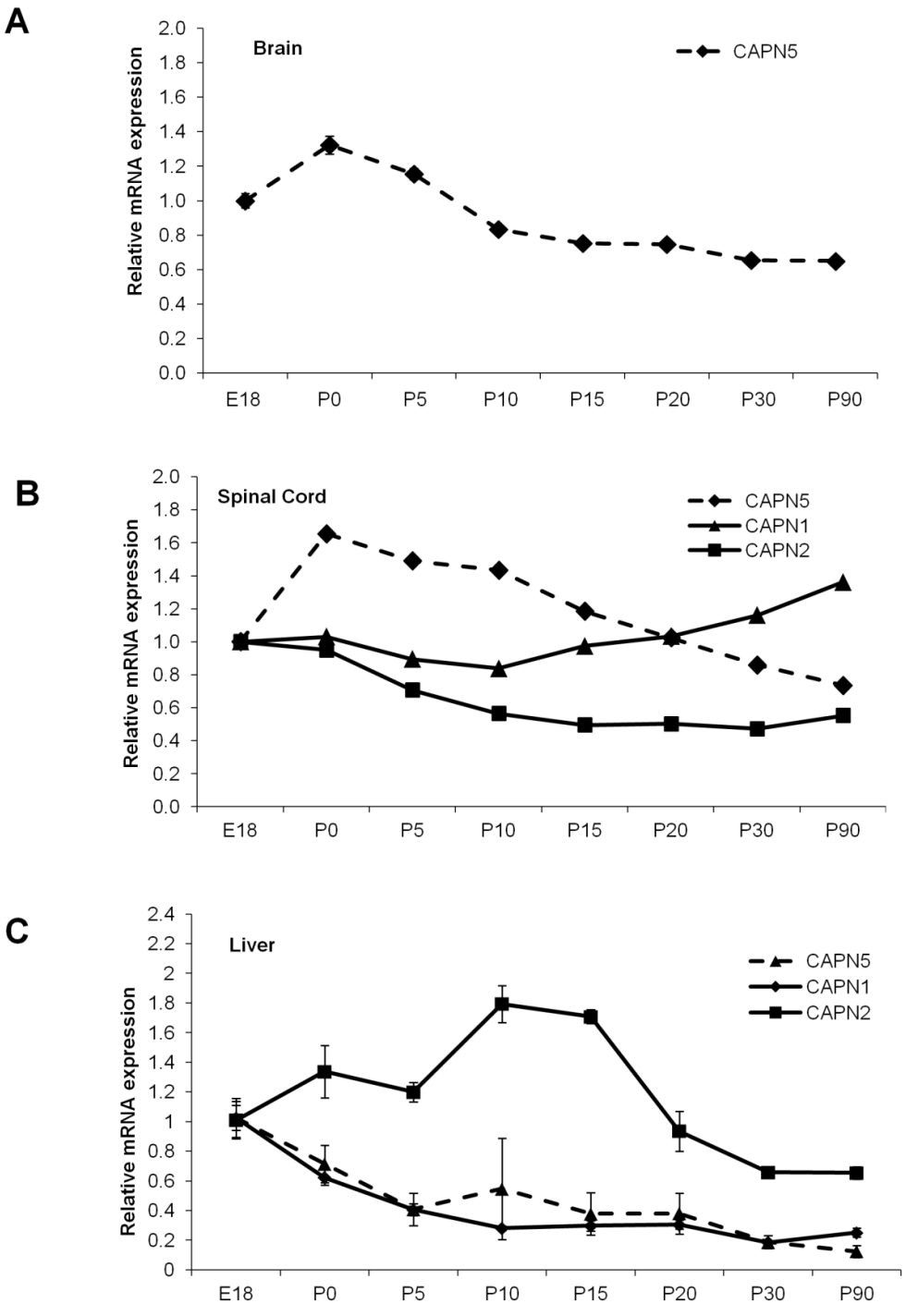

**Figure 2.3** The mRNA level of Calpain 5 is downregulated in rat CNS and liver during development. Comparative $C_T$ values ($\Delta\Delta C_T$) based mRNA expression were measured in SD rat brain, spinal cord and liver homogenates prepared from embryonic day 18 (E18), postnatal day zero (P0), P5, P10, P15, P20, P30 and P90 rats. $\Delta C_T$ at a particular developmental point was obtained as a difference in the $C_T$ value from endogenous control GAPDH at the same developmental point. $\Delta\Delta C_T$ value at each developmental time point was calculated by subtracting $\Delta C_T$ at that point from the $\Delta C_T$ of the reference point, E18. Relative expression was then reported as $2^{-\Delta\Delta C}{}_T$.

Compared to E18, calpain 5 mRNA levels increased until P0, then decreased gradually postnatally in brain (N=4 per time point) and spinal cord (N= 2 per time point). In liver, calpain 5 decreased gradually from E18 to P90 (N=4 per time point. The mRNA levels of calpain1 were elevated in spinal cord, however they declined in the liver during development. Calpain 2 mRNA levels declined in spinal cord but peaked around P10 and P15 during liver development. The data were analyzed using one-way ANOVA followed by Tukey's multiple comparison tests (see table 2.1), and reported as the group mean (N=4) ± S.D (**A** and **C**). The data were plotted as the group mean (N=2) (**B**) with no statistical analysis.

**Figure 2.4** CAPN5 is detected later in CNS development

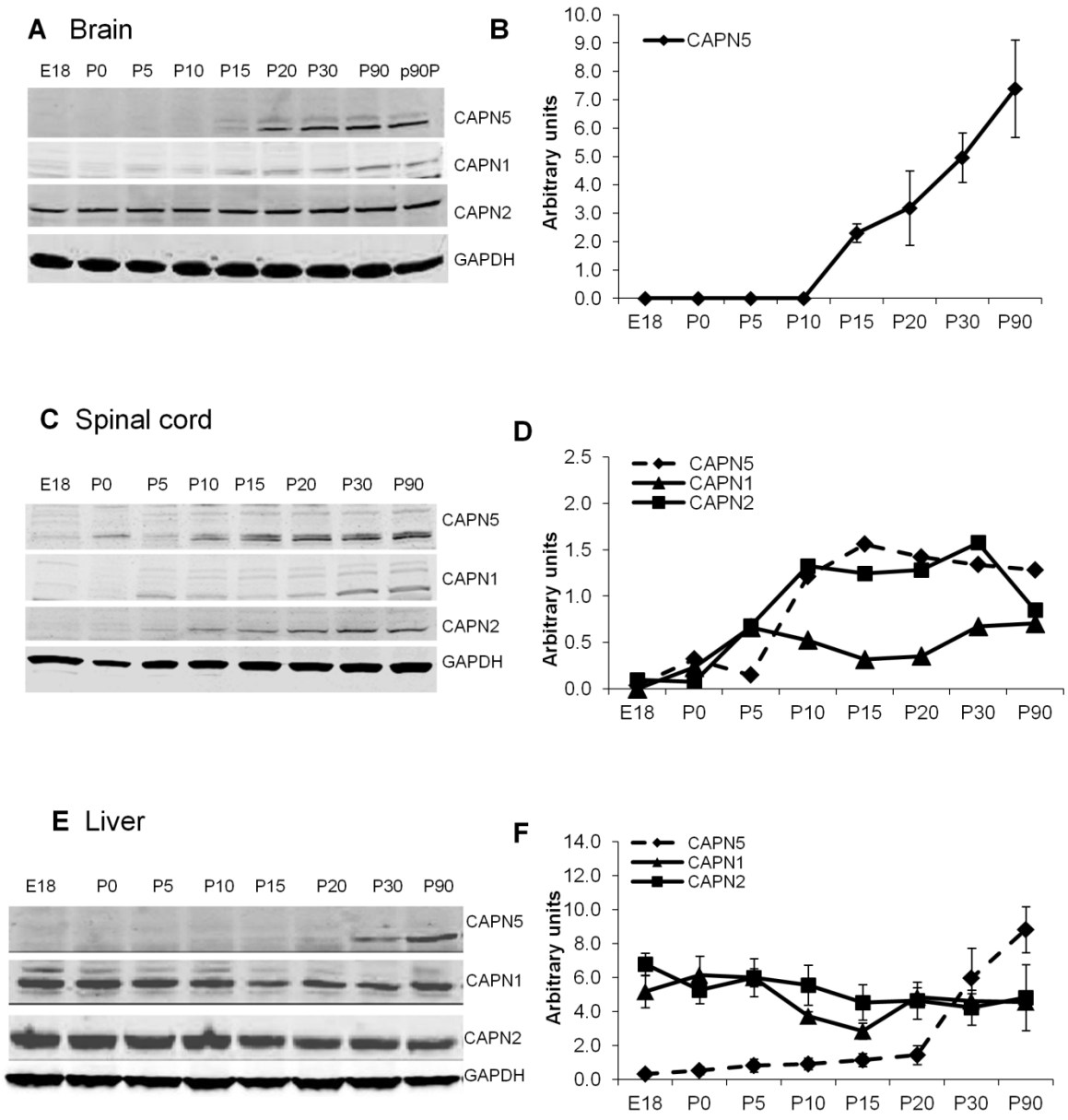

**Figure 2.4** CAPN5 protein is detected later in CNS development**.** To compare the protein levels, Western blot analyses were performed on SD rat brain, spinal cord, and liver homogenates for developmental time points, starting from E18 to P90. P90p is a pooled sample prepared by mixing equal amounts of protein from each of the four p90 homogenates, to use as a control for each of the four sets of the Western blot. In contrast to mRNA expression, CAPN5 levels progressively increased during the postnatal development of the brain, spinal cord, and liver. On the other hand, CAPN1 and CAPN2 remained almost constant during liver development. The level of CAPN2 is unchanged also during brain development. CAPN1 levels rose during post natal development of brain and spinal cord. Level of CAPN2 also increased postnatally in spinal cord. The quantitative values of the immunoreactivity bands were measured in Odyssey *LI-COR* software. Data were statistically analyzed as one-way ANOVA followed by Tukey's multiple comparison tests (see table 2.1). The results are expressed as the group means (N=4) ± SEM for **B** and **F**. The data are plotted as the group mean (N=2) for **D**, no statistical analysis was performed.

# Chapter 3: Nuclear localization of Calpain 5

## Preface

Work shown in this chapter is done by Ranjana Singh and Mr. Charles Mashburn. Mr. Charles Mashburn prepared the plasmids used in this study.

## 3.1 Introduction

The calpain family of $Ca^{2+}$-dependent cysteine proteases (Clan CA, family C2, EC 3.4.22.52-54) has 15 members in mammals, including the small regulatory subunit CAPNS1 (Ono and Sorimachi, 2012). These are subdivided into classical (CAPN1-3,8,9,11-14) and non-classical calpains (CAPN5-7,10,15,16) based on primary sequence, and into ubiquitous (CAPN1, 2,5,7,10,13-15,S1) and tissue-specific calpains (CAPN3,6,8,9,11,12) based on localization (Goll et al., 2003, Ono and Sorimachi, 2012). CAPN2 is not present in erythrocytes, but is present in other cells. The ubiquitous isoforms are presumed to play important roles in all cells, as knockout or mutations are often lethal, while the tissue-specific isoforms are required for more specialized functions. Most investigations have focused on the classical calpains CAPN1 (also referred to as μ-calpain, μCL) and CAPN2 (also referred to as m-calpain or mCL), which contain a C2-like domain, a penta-EF hand domain, and the cysteine protease domain. They are heterodimers, composed of the large CAPN1 or CAPN2 subunit and the CAPNS1 (CAPN4) small subunit. Their roles include apoptosis, cell migration, cytoskeletal remodeling, cell differentiation, necrosis/oncosis, platelet aggregation, and wound healing (Wang, 2000, Azam et al., 2001, Liu et al., 2004, Franco and Huttenlocher, 2005, Mellgren et al., 2007, Santos et al., 2012, Amini et al., 2013). Atypical calpains, which lack the EF hand motif characteristic of classical calpains but retain catalytic and $Ca^{2+}$binding domains, are less well understood.

Tra-3, the *C.elegans* ortholog of CAPN5, is essential for necrotic neuron death and is also involved in sex determination in nematodes (Barnes and Hodgkin, 1996, Syntichaki et al., 2002). CAPN6 is also an ortholog of Tra-3, but substitution of Cys with Lys at the active site results in a loss of proteolytic activity in eutherians (Matena et al., 1998). CAPN5, also referred to as hTra-3, lacks the penta-EF hand domain of classical calpains and has a C2 domain at the C-terminus (previously referred to as domain-T) (Sorimachi

et al., 2011a). CAPN5 is expressed in all rat and human tissues examined, including various regions of the CNS (Dear et al., 1997, Waghray et al., 2004). The subcellular localization of CAPN5 has not been examined previously.

Incubation of SH-SY5Y cell lysates with maitotoxin or the $Ca^{2+}$-ionophore A23187 results in CAPN5 proteolysis, presumably indicating activation (Waghray et al., 2004). *Capn5*$^{-/-}$ (*Capn5*$^{tm1Nde}$) mice are viable and fertile, although some are severely runted at birth and die by 2 months of age (Franz et al., 2004). However, another *Capn5* null mutant allele (*Capn5*$^{tm1Dgen}$) is embryonically lethal (MGI ID 3604529). CAPN5 polymorphisms have been associated with autoimmune retinal neurodegeneration (Mahajan et al., 2012), polycystic ovary syndrome (Gonzalez et al., 2006), endometriosis (Penna et al., 2008), diabetes (Saez et al., 2007) and Huntington's disease (Gafni et al., 2004). Based on importance of Tra-3 in neuron death in *C. elegans* and the relatively high expression of Capn5 mRNA in brain, we sought to further explore CAPN5 in the mammalian CNS.

## 3.2 Materials and Methods

### 3.2.1 Experimental animals.

The University of Kentucky Institutional Animal Care and Use Committee approved all procedures involving experimental animals. Animals included male Sprague-Dawley (SD) rats and *Capn5* heterozygous mice (C57BL/6J-*Capn5*$^{tm1Dgen}$/J; *Capn5*$^{+/LacZ}$). These were obtained as B6.29P2-*Capn5*$^{tm1Dgen}$/J mice from The Jackson Laboratory (Bar Harbor, ME) and were backcrossed 10 generations. For Western blot and fractionation studies, rats were exsanguinated using $CO_2$ inhalation, followed by decapitation. Brains were rapidly removed and homogenized in appropriate buffer for Western blot,

fractionation, or RNA isolation as described below. For immunohistochemical and X-gal staining studies, rats or mice were first perfused with PBS followed by 4% paraformaldehyde in PBS, pH 7.4.

### 3.2.2 Antibodies and reagent

Antibodies against CAPN5 (ab28280), APC (CC-1 clone, ab16749), PML (ab96051), β-tubulin (ab6046-100) and histone H3 (ab1791) were purchased from Abcam, Cambridge, MA. Anti NeuN (MAB377) and Anti-GFAP (MAB360) were obtained from EMD Millipore, Billerica, MA. An antibody against SMN (610647) was purchased from BD Biosciences, San Jose, CA. IRDye 800CW Anti-rabbit IgG (611-131-132) and IRDye 800 CW Anti-mouse IgG (610-131-121) were purchased from Rockland, Gilbertsville, PA. Hoechst 33258 ( H-3569 ) and conjugated secondary antibodies Alexa Fluor-488 anti-rabbit IgG ( A11005 ) and Alexa Fluor-594 anti-mouse IgG ( A11034 ) were purchased from Molecular Probes, Life technologies, Grand Island, NY. TRI reagent ( T9424 ), X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactoside, B4252), Pepstatin A ( P4265 ) and β-mercaptoethanol ( M6250 ) were purchased from Sigma, St. Louis, MO. *pN1-ZsGreen1* vector ( 632448) was purchased from Clontech Laboratories, Inc. Mountain View, CA. *p3XFLAG-CMV-14* (E7908) was purchased from Sigma, St. Louis, MO. Human *Capn5* cDNA (MHS1010-58128) was purchased from Thermo Scientific, Open Biosystems, Huntsville, AL. All oligonucleotides were ordered from Integrated DNA Technologies, Coralville, IA. Lipofectamine 2000 CD reagent (12566-014) was obtained from Invitrogen, Grand Island, NY. Pfu DNA polymerase (600135) was ordered from Agilent Technologies, Stratagene Division, La Jolla, CA. EcoRI-HF and BamHI-HF were purchased from New England Biolabs, Ipswich, MA. Rapid DNA Ligation kit (11 635 379 001) was obtained from Roche, Indianapolis, IN. One Shot® Stbl3™ competent bacteria

(C737303) was purchased from Invitrogen, Grand Island, NY. Additional reagents were purchased from Sigma (St. Louis, MO) or Thermo Fisher Scientific (Ashville, NC).

### 3.2.3 Quantitative PCR (qPCR)

Brain samples were homogenized in TRI reagent to extract total RNA. cDNA was prepared using an Applied Biosystems high capacity reverse transcription kit (AB # 4368814). Equal amounts of cDNA (100 ng) were used to perform qPCR using Taqman gene expression master mix (AB 4369016). Reactions were performed in triplicate, plus a negative control without cDNA. The qPCR was programmed as an initial denaturation at 50°C for 2 min, followed by 95 °C for 10 min, 40 cycles @ 95 °C for 15 s, and 60 °C for 1 min, on a StepOne real-time PCR system (Applied Biosystems). The following rat gene transcripts were examined: CAPN1 (NCBI Reference Sequence NM_019152.2, TaqMan gene expression assay Rn00569689_m1); CAPN2 (NM_017116.2, Rn00567422_m1), CAPN5 (NM_134461.1, Rn00593213_m1); CAPN7 (NM_001030037.1, Rn01453530_m1), CAPN10 (NM_031673.2, and GAPDH (NP_058704, Rn99999916-s1). Relative gene expression was determined using Comparative $C_T$ values. $\Delta C_T$ of the target gene was obtained as a difference in the $C_T$ value from endogenous control GAPDH. $\Delta\Delta C_T$ value of the target gene was calculated by subtracting $\Delta C_T$ value of the target gene from the $\Delta C_T$ of a reference gene, calpain 1. Relative expression of target gene was then reported as $2^{-\Delta\Delta C}{}_T$.

### 3.2.4 Cytosol and crude nuclear fractionation

Brain cortices were homogenized in a dounce homogenizer in isolation buffer containing 215 mM mannitol, 75 mM sucrose, 1mM EGTA, 20 mM HEPES and 1uM of pepstatin A. The homogenate was centrifuged at 1,300 X g for 3 min to obtain the crude nuclear

fraction as a pellet. The supernatant was spun again at 13,000 X g for 10 min to obtain cytosol as supernatant.

### 3.2.5 Nuclear subfractionation

Rat brain cortex was lysed and incubated with buffers provided in Qproteome nuclear protein kit (Qiagen, catalogue # 37582). The manufacturer's protocol was followed to obtain cytosol, nucleic acid binding proteins (NABP), and insoluble nuclear proteins (INP). Briefly, 50 mg of tissue was disrupted in dounce homogenizer in 1ml of lysis buffer NL supplemented with protease inhibitor solution and 0.1M DTT, followed by incubation on ice for 15 min. 50 µl of detergent solution NP was added to the solution and vortexed for 10 sec. Lysate was then centrifuged at 10,000 X g for 5 min to obtain cytosol as supernatant and pellet for nuclear subfractionation. The pellet was resuspended in 100 µl of extraction buffer NX1 supplemented with protease inhibitor solution, and incubated for 30 min on rotamix at 4°C. The suspension was spun down for 10 min at 12,000 X g to separate NABP fraction as supernatant and INP in pellet. The pellet was resuspended in extraction buffer NX2 supplemented with protease inhibitor, 0.1 M DTT and benzonase® nuclease, followed by incubation for an hour on rotamix at 4°C. The suspension was centrifuged for 10 min at 12,000 x g to obtain INP in supernatant.

### 3.2.6 Western blot

Protein content of samples were assayed using Thermo Scientific Pierce® BCA protein assay reagent A (23228) and reagent B (1859078). Protein (50 µg of each) was mixed in NuPAGE® LDS sample buffer (NP007) supplemented with 5% β-mercaptoethanol, and boiled for 5 min. Boiled samples were separated on 4-12% Bis-Tris HCl gels (NuPAGE NP0335) in MES SDS running buffer ( NuPAGE NP0002) and transferred to a 0.2 µm

nitrocellulose membrane. After blocking in 5% skim milk in 0.05% Tween 20 in Tris-buffered saline, pH 7.6 (T-TBS), for 1 hr, the membrane was incubated with primary antibody (CAPN5, 1:5000; SMN, 1:5000 BD; Histone H3, 1:5000; and β-tubulin, 1:10,000) in 5% skim milk in T-TBS overnight at 4ºC. The membrane was washed in T-TBS three times (3x) for 20 min each, followed by incubation with an appropriate secondary antibody (Anti-rabbit IgG, 1:5000; or anti-mouse IgG, 1:5000) at room temperature for 1 hr in dark conditions. The membrane was washed again 3x for 20 min each, and scanned using an Odyssey Infrared Imager (*LI-COR* Biosciences).

### 3.2.7 Identifying Capn5+/- mice and β-Galactosidase staining:

*Capn5*$^{tm1Dgen}$/J (*Capn5*$^{+/-}$) mice were used for this staining. To identify the genotype, DNA was extracted from tail tissue. The tissue was digested in 75 ul alkaline lysis solution (25 mM NaOH, 0.2 mM EDTA) at 95$^{o}$C 30 min/4$^{o}$C 10 min, followed by treatment with 2 ul of proteinase K (10mg/ml) at 55$^{o}$C 30 min/95$^{o}$C 10 min/4$^{o}$C 5 min. The resulting DNA solution was neutralized by adding 75 ul of 50mM Tris. PCR was set on Bio-RAD MJ mini Personnel Thermo Cycler or MJ Resaerch-PTC-200 Peltier thermo cycler, using primers and protocol provided by the Jackson Laboratory. Wild types were identified as PCR amplification of a single band of 209 bp and heterozygotes as two distinct bands of 209 and 452 bp.

*Capn5*$^{tm1Dgen}$/J (*Capn5*$^{+/-}$) male mice, approximately 3-months old, were perfused with PBS followed by 4% paraformaldehyde in PBS, pH 7.4. The brains were removed and postfixed overnight, then cryoprotected in 30% sucrose in PBS. The brains were frozen in powdered dry ice and coronally sectioned at 40 µm and stored in cryoprotectant (30 % ethylene glycol, v/v and 30% glycerol, v/v in 1xTBS) at -20°C until use. Sections were rinsed 3x in PBS then incubated with 1 mg/ml X-gal in 10 mM potassium ferricyanide, 5

mM potassium ferrocyanide, 2mM $MgCl_2$ at 37°C overnight or until the dark blue staining appeared. Following dehydration, clearing, and coverslipping, sections were viewed and photographed using brightfield microscopy.

### 3.2.8 Immunohistochemistry

Perfusion, fixation, and preparation of male rat brain sections were performed as discussed for β-galactosidase staining. Free floating brains sections were washed in TBS 3x followed by blocking in 5% natural goat serum (NGS) in T-TBS (0.1% Triton-X-100) for 30 min at room temperature. Brain sections were incubated with primary antibodies (CAPN5, 1:100; NeuN, 1:200; GFAP, 1:1000; and APC, 1:100) in 5% NGS-T-TBS overnight at 4°C. Primary antibody was omitted from negative controls. Sections were washed 3x in 1x TBS, followed by 1 hr incubation with appropriate 2°antibodies at 1:1000 dilution (Alexa Fluor-488 anti-rabbit IgG or Alexa Fluor -594 anti-mouse IgG), then washed 3x in TBS. Nuclei were stained with Hoechst 33258 at 10µg/ml. Brain sections were mounted on glass slides with Vectashield (H-1000, Vector Labs) fluorescence mounting medium and examined under a Leica AOBS TCS SP5 inverted laser scanning confocal microscope.

### 3.2.9 Cell Culture and Immunocytochemistry

*SHSY-5Y (ATCC # CRL-2266)* cells were cultured in complete growth medium (ATCC-formulated Eagle's Minimum Essential Medium (Cat# 30-2003 + 1% penstrap + 10% FBS) at 37ºC in an incubator maintained with 95% air and 5% $CO_2$. The cells were plated on 35mm glass bottom culture dishes. The following day, adherent cells were fixed in 4% paraformaldehyde in PBS, pH 7.4 for 15 min at room temperature, followed by permeabilization for 10 min with PBS containing 0.25% Triton X-100 (PBS/T). After

washing 3x with PBS, cells were incubated with 5% NGS-PBS/T for 30 min, then incubated with primary antibody (PML, 1:100 or CAPN5, 1:100) in 5% NGS-PBS/T overnight at 4°C. The next day, sections were washed 3x in PBS and incubated for 1 hr in the dark with 1:1000, Alexa Fluor-488 anti-rabbit IgG or Alexa Fluor -594 anti-mouse IgG, followed by 3x wash of 5 min each. Nuclei were stained with Hoechst 33258 at 10 µg/ml for 5 min. Cells were viewed under a Nikon Ti-E C2plus confocal microscope.

### 3.2.10 Plasmid preparation, transient transfection and confocal microscopy

Human *Capn5* cDNA (accession number BC018123.1) and oligonucleotides were purchased as described under *antibodies and reagents*. PCR oligonucleotide primers for *Capn5* were designed to allow cloning into *pN1-ZsGreen1* or *p3XFLAG-CMV-14* vector such that ZsGreen1or a FLAG tag was encoded at the C-terminal of the fusion product. PCR was carried out using Pfu DNA polymerase kit. The resulting DNA product was digested with EcoRI-HF and BamHI-HF. The product was then ligated into the vector using a Rapid DNA Ligation kit and transformed into One Shot® Stbl3™ competent bacteria. Plasmid DNA was isolated using Qiagen Maxi Prep Kit # 12263.

For transient transfections, SH-SY5Y cells were grown in 35 mm glass bottom culture dishes. At approximately 70% confluency, cells were transfected with 0.2µg of plasmid vector using Lipofectamine 2000 CD reagent. Transfected cells were imaged at 24 h post transfection. Prior to imaging, cells were stained for 1 hr with 10 µg/ml Hoechst 33258. Images were acquired on an Olympus IX81FV1000 confocal microscope.

### 3.2.11 Statistical Analysis

The quantified values of the relative intensity of the CAPN5 immunoreactivity band and qRT-PCR RQ data were analyzed using one-way ANOVA, followed by Tukey's multiple comparison tests.

## 3.3 Results

### 3.3.1 Calpain 5 is highly expressed in rat brain

CAPN5 is present in the CNS (Waghray et al., 2004), but its expression relative to other calpains is unknown. Using the real-time comparative $C_T$ method ($\Delta\Delta C_T$) (Schmittgen and Livak, 2008), we analyzed the average relative mRNA expression of several ubiquitous calpains (1, 2, 5, 7, 10) in adult rat brain (n=4) (Fig.1.8). CAPN2 had the highest levels of mRNA expression exceeding CAPN5 by 2.7 fold. CAPN5 was the second most highly expressed calpain in the brain, followed by CAPNs7, 10, and 1, in descending order.

To examine the cellular localization of CAPN5 mRNA expression, we utilized *Capn5*$^{tm1Dgen}$/J mice in which a LacZ-Neo555G cassette was inserted into the CAPN5 gene. These mice were created by DeltaGen (Moore, 2005) and obtained from Jackson Laboratories on a B6.129P2 background, then backcrossed onto C57BL/6J for 10 generations. *Capn5*$^{-/-}$ mice were embryonically lethal, while *Capn5*$^{+/-}$ mice were viable. Despite the lower gene dosage, CAPN5 protein levels were unchanged in brains of *Capn5*$^{+/-}$ mice compared to wild-type (results not shown). Another Calpain 5 mutant line, CAPN5$^{tm1Nde}$ /CAPN5$^{tm1Nde}$ is shown to be viable (see section 1.2.2.1d for the explanation on the difference in the phenotype) and supposedly have zero level of

calpain 5 protein. But, the data on the calpain 5 protein expression is unavailable (Franz et al., 2004). Representatives at European mouse mutant archive (EMMA), Germany, where these mice are housed were also unable to provide information on calpain 5 protein levels in these mice. These mice were being made available to us as frozen embryo, which would have taken a long time for us to breed the colonies. Since we already had maintained colonies of *Capn5*$^{tm1Dgen}$/J mice, I decided to choose *Capn5*$^{tm1Dgen}$/J mice to examine cellular localization of calpain5 based on lacZ expression.

In *Capn5*$^{+/-}$ mouse brain, X-gal staining was prevalent in the pyramidal neurons in the hippocampal formation, as well as in dentate granule and hilar neurons (Fig.3.1 A). X-gal staining was also observed in molecular layers and white matter indicative of expression in non-neuronal cells including astrocytes and oligodendrocytes. These results are consistent with CAPN5 being ubiquitously expressed in all cells.

### 3.3.2 Nuclear localization of Calpain 5

Using double-label immunocytochemistry, co-localization of CAPN5 immunoreactivity with anti-NeuN confirmed the neuronal localization of CAPN5, and also indicated that expression was predominantly nuclear (Fig. 3.1 B). Double-labeling of anti-CAPN5 with anti-glial fibrillary acidic protein (GFAP) and anti-adenomatous polyposis coli tumor suppressor protein (APC) demonstrated nuclear CAPN5 expression in astrocytes and oligodendrocytes (Figs. 3.1 C and D). However, localization was not exclusively nuclear as faint immunoreactivity was observed in the neuropil and a band of CAPN5 immunoreactivity was present in stratum lacunosum-moleculare in the hippocampal formation (not shown). This is a terminal zone of the perforant path and is rich in mitochondria (Kageyama and Wong-Riley, 1982), suggesting a possible presynaptic,

postsynaptic, or mitochondrial localization of CAPN5 in addition to the nuclear localization.

To further evaluate the subcellular localization of CAPN5, rat brain cortices were homogenized and separated into cytosolic and crude nuclear fractions. CAPN5 immunoreactivity was abundant in the crude nuclear fraction, but was not detected in the cytosolic fraction (Fig. 3.2 A). Additional subfractionation demonstrated that CAPN5 was enriched in the nucleic acid binding protein (NABP) fraction (Figs. 3.2 B and C). Unlike typical calpains, which are characterized as being mainly cytosolic (Yoshimura et al., 1984, Goll et al., 2003, Suzuki et al., 2004); these results illustrate that CAPN5 is predominantly a non-cytosolic calpain, present in the nucleus in the nucleic acid binding protein fraction.

### 3.3.3 Calpain 5 resides in punctate nuclear domains associated with PML bodies

The nuclear staining observed with CAPN5 appeared punctate in each of the cell types examined in rat brain (Fig. 3.2 D). Faint CAPN5 immunoreactivity was also detected in the nucleus outside of PML bodies. Extranuclear immunoreactivity was also observed. Punctate nuclear localization was also observed in SH-SY5Y neuroblastoma cells. The punctate nuclear localization suggests that CAPN5 is associated with one or more nuclear bodies (Dundr, 2012). In SH-SY5Y cells transfected with a plasmid expressing full length human CAPN5 fused with ZsGreen1 (*pN1-hCapn5-$_{1-640}$-ZsGreen1)*, similar punctate nuclear localization was observed with the CAPN5-ZsGreen1 fusion protein. A large amount of CAPN5 was expressed in cytoplasmic aggregates in the perinuclear region. Dot-like nuclear expression of CAPN5 was also observed following transient transfection of SH-SY5Y cells with a vector encoding human CAPN5 with a C-terminal FLAG tag, when using an anti-FLAG antibody (Agilent Technologies 200472). To

examine the nuclear domain, calpain 5 was colabeled with PML bodies ( promyelocytic leukemia protein). In the CNS, PML expression is biphasic—initially being expressed in immature neural progenitor cells, downregulated during differentiation, then re-expressed in mature neurons (Yu et al., 2003, Salomoni and Betts-Henderson, 2011). Calpain 5 was detected post natally around day $15^{th}$ / $20^{th}$ in rat brain. PML and Calpain 5 may interact via sumoylation (See below). Therefore, it could be hypothesized that CAPN5 could localize in PML bodies to regulate changes in gene expression or nuclear events for differentiation. Endogenous as well as the transiently expressed CAPN5 nuclear dots were colocalized or closely associated with PML bodies, using an antibody against PML protein (Figs. 3.3 A and B). PML independent CAPN5 dots were also observed.

Partner proteins associated with PML bodies are typically sumoylated or contain a SUMO interaction motif (Shen et al., 2006, Lallemand-Breitenbach and de The, 2010). Using the SUMOplot™ program, several high probability sumoylation motifs (where Ψ = a hydrophobic residue, K = the lysine conjugated to SUMO, x = any amino acid, and E = an acidic residue) are present on human CAPN5 (Table 3.1). CAPN5 contains a single probable sumo-interacting motif (KPEDEVLICI, aas 396-405), predicted using GPS-SBM 1.0 at medium threshold (http://sbm.biocuckoo.org/software.php).

### 3.3.4 Calpain 5 Nuclear Localization Signal

Transient transfection with *pN1-hCapn5*$_{1-640}$*-ZsGreen1* resulted in nuclear CAPN5-ZsGreen1 expression, while transfection with the *pN1-ZsGreen1* vector alone resulted in largely cytosolic ZsGreen1 expression (Figs. 3.4 A, B and Table 3.2). This suggests the possibility of a nuclear localization signal (NLS) in CAPN5. Analysis of the primary sequence of CAPN5 with PSORTII (Reinhardt and Hubbard, 1998) revealed a putative

bipartite NLS, KKPEDEVLICIQQRPKR (aa 395-411, two basics clusters of amino acids interspaced by 10-12 amino acids), in domain III of human CAPN5, along with possible monopartite NLS sequences (not shown). However, this sequence was not sufficient for the nuclear localization. The basic amino acids of the NLS interact with importin-α for the nuclear import (Gorlich et al., 1995, Lange et al., 2007) (Marfori et al., 2011). In studies to identify the NLS (summarized in Table 3.2), basic residues (K/R) of sequence 395-411 were mutated to 'N (to abort interaction with importin and hence nuclear localization) in full length *hCapn5* (*pN1-mhCapn5*$^{N}_{1-640}$*-ZsGreen1*); which did not abolish the nuclear localization. The PSORTII predicted bipartite NLS by itself was not sufficient since EVKKPEDEVLICIQQRPKRST, aa 393-413 (extended by 2 aa at each end), fused with ZsGreen1 (*pN1-hCapn5*$_{393-413}$*-ZsGreen1*) was unable to target the nucleus. The putative NLS was further extended at the C-terminus to include additional basic residues, KKPEDEVLICIQQRPKRSTRREG, aa 395-417, (*pN1-hCapn5*$_{395-417}$*-ZsGreen1*) to examine if additional basic residues at C-terminus may be required to drive the nuclear localization. However the resultant expression was mainly cytosolic (Fig 3.4 C and Table 3.2).

Then, we added few peptides proximal to aa 395-417. Fig 3.2 shows that calpain 5 was detected in the nucleic acid binding fraction. A thorough survey and in silico analyses show that 20% of NLS motifs co-localizes with the DNA binding region of the proteins (Cokol et al., 2000). Also, for 67% – 90% of the DNA binding proteins, the DNA binding region overlaps or is proximal to the NLS (LaCasse and Lefebvre, 1995, Cokol et al., 2000, Nair et al., 2003). Therefore, for the DNA binding protein the NLS could be longer. To start with, proximal upstream sequence YIFEV (aa 390-394) was added that resulted in the peptide sequence YIFEVKKPEDEVLICIQQRPKRSTRREG, (*pN1-hCapn5*$_{390-417}$*-ZsGreen1*). Transient transfection with this construct resulted in nuclear localization in distinct punctate or dot like domains (Fig. 3.4 D). This upstream sequence was not

sufficient, however, as PQYIFEVKKPEDEVLICIQQ, (*pN1-hCapn5*$_{388-407}$*-ZsGreen1*) was expressed in both the nucleus and cytosol (Table 2, figure not shown). Mutagenesis of basic residues (K/R) to A (*pN1-mhCapn5*$^{A}_{388-417}$*-ZsGreen1*) did not abolish the nuclear localization of the peptide sequence, but resulted in a more diffuse nuclear localization (Fig. 3.4 E). Together, the results suggest that YIFEVKKPEDEVLICIQQRPKRSTRREG is a NLS for CAPN5 and also contributes to the punctate localization.

A BLAST protein search (Altschul et al., 2005) against the putative NLS YIFEVKKPEDEVLICIQQRPKRSTRREG revealed that the sequence was unique to CAPN5 and highly conserved in mammals, with 100% identity in human, rat, and two amino acid differences in mouse. With occasional mismatch, the putative NLS aligned with CAPN5 protein of mammals, reptiles, amphibians and fish (Table 3.3). This sequence was unique to CAPN5 because other calpains do not carry a homologous sequence. These results indicate that domain III of CAPN5 contains a novel NLS, consisting of a bipartite region and upstream sequence.

## 3.4 Discussion

Most previous investigations of calpains in the CNS have focused on typical calpains 1 and 2, although several other calpains have been detected in the CNS including CAPNs 3, 5,10, and 12 (Ma et al., 2001, Konig et al., 2003, Shin et al., 2004, Waghray et al., 2004). In this study, we found that CAPN5 mRNA levels are second only to CAPN2 in relative abundance in the CNS. The much greater expression of CAPN2 vs. CAPN1 is consistent with previous findings (Li et al., 1996). Calpains are largely cytosolic proteases, although CAPNs 1, 2, and 10 have also been localized to mitochondria, with CAPNs 2 and 10 additionally being detected in the nucleus (Yoshimura et al., 1984, Ma et al., 2001, Goll et al., 2003, Suzuki et al., 2004, Garcia et al., 2005, Arrington et al.,

2006, Raynaud et al., 2008, Ozaki et al., 2009). In contrast, CAPN5 localization is predominantly nuclear where it is associated with PML nuclear bodies.

PML nuclear bodies are a collection of proteins arranged in spheres of 0.1-1.0 µm in diameter, localized to the nuclear matrix in most tissues and cell lines (Stuurman et al., 1992). They are organized by promyelocytic leukemia (PML) protein, which was discovered because of its involvement in acute promyelocytic leukemia (for review see de The et al., 2012). The PML protein forms the outer shell of the nuclear bodies, with partner proteins on the interior (Guiochon-Mantel et al., 1995). There are several PML isoforms with specific intracellular locations, with PML IV being the most extensively studied (Beech et al., 2005, Condemine et al., 2006). PML partner proteins are either sumoylated or contain a SUMO interaction motif (SIM) (Shen et al., 2006). PML nuclear bodies are not static structures, with partner proteins exchanging between the nuclear body and the nucleoplasm (Weidtkamp-Peters et al., 2008). Moreover, PML nuclear bodies release partners during mitosis as a result of desumoylation, and reform during the transition to G1 (Dellaire et al., 2006a, Dellaire et al., 2006b). PML nuclear bodies are also sensitive to cellular stress, which can result in either greater aggregation and increased size of the nuclear bodies or dispersion into microspeckles, depending on the nature of the insult (for review see [Lallemand-Breitenbach and de The, 2010]).

The localization of CAPN5 to PML nuclear bodies suggests that CAPN5 should contain a nuclear localization signal and one or more SUMOylation or SIM sites, similar to other nuclear body proteins such as SP100 and Daxx (Sternsdorf et al., 1999, Yeung et al., 2008, Santiago et al., 2009). Analysis of the human CAPN5 primary sequence using PSORTII (Horton and Nakai, 1997) revealed a putative classic 17 amino acid bipartite NLS KK[PEDEVLICIQQ]RPKR (395-411). Bipartite NLS signals consist of two clusters of basic amino acids, separated by approximately 10-12 amino acids with the prototype being the NLS for nucleoplasmin The basic amino acids interact with importin-

α for nuclear import via the classic import pathway (Gorlich et al., 1995, Lange et al., 2007) (Marfori et al., 2011). The putative NLS for CAPN5 contains two clusters of basic amino acids separated by 11 amino acids. However, this sequence was not sufficient for localization to the nucleus. The identified sequence sufficient for nuclear targeting (YIFEVKKPEDEVLICIQQRPKRSTRREG, 390-417) is consistent with critical residues found in other bipartite NLSs (Marfori et al., 2011). Surprisingly, mutagenesis of the basic residues did not prevent nuclear import of the 388-417 sequence.

The single putative CAPN5 SUMO-interacting motif (KPEDEVLICI, aa 396-405) in CAPN 5 is contained within the putative NLS. The prediction of SUMO-interacting motifs is not precise, but general features include an acidic domain adjacent to a string of three of V, I, and/or L amino acids. The putative CAPN5 SUMO- interacting motif contains each of these domains. Other PML proteins which contain sumo-interacting motifs include Daxx and SP100, in addition to PML itself (Lin et al., 2006, Kim et al., 2009).

The extranuclear aggregates of CAPN5-ZsGreen1 are similar to those observed following overexpression of another chimeric protein consisting of green fluorescent protein fused to an internal fragment of the Golgi complex protein (Fu et al., 2005). This raises the possibility that the observed association of CAPN5-ZsGreen1 is an artifact of overexpression. Arguing against this is that while extranuclear aggregates and aggresomes have been observed following expression of other chimeric green fluorescent fusion proteins (Johnston et al., 1998, Garcia-Mata et al., 1999), the presence of nuclear aggregates is largely restricted to expressed polyQ proteins (Fu et al., 2005). Nuclear aggregates of CAPN5-FLAG were also observed, demonstrating that the nuclear localization is not driven by ZsGreen1. Proteasomes are present in the nucleus where they can associate with PML bodies (Wojcik and DeMartino, 2003). Similar to cytosolic aggresomes, nucleolar aggresomes can also result from nuclear proteasome inhibition (Latonen, 2011). PML body proteins including PML and p53 may

translocate to the nucleoli under these conditions of proteotoxic stress, however many non-PML body proteins are also associated with nucleolar aggresomes, which are distinct from PML bodies (Latonen, 2011). The association of CAPN5 with nuclear bodies was observed with native CAPN5 in rat neurons in vivo and in cultured SH-SY5Y cells, further suggesting that the localization of CAPN5-ZsGreen1 to PML nuclear bodies is not an artifact.

Proteins associated with PML nuclear bodies are linked by their ability to be sumoylated (Bernardi and Pandolfi, 2007). Analysis of the CAPN5 sequence using sumoylation prediction algorithms SUMOsp (Xue et al., 2006) and SUMOplot (www.abgetn.com/sumoplot/) reveals several high probability sumoylation sites, including one at K395 within the NLS. Using proteomic analysis of the anti-HA affinity purified fraction from His6-HA-SUMO 1 K1 mouse brain, CAPN5 was identified as a candidate SUMO1-conjugated protein (Tirard et al., 2012). Thus, the putative NLS and sumoylation sites of CAPN5 are consistent with its localization to PML nuclear bodies.

PML nuclear bodies are implicated in the cellular response to stress, viral defense, transcriptional regulation, apoptosis, and cell senescence (Borden, 2002, Bernardi and Pandolfi, 2007, Lallemand-Breitenbach and de The, 2010, Dundr, 2012). PML bodies recruit a large number of partner proteins which are then sequestered, modified, or degraded (Lallemand-Breitenbach and de The, 2010). In the CNS, PML expression is biphasic—initially being expressed in immature neural progenitor cells, downregulated during differentiation, then re-expressed in mature neurons (Yu et al., 2003, Salomoni and Betts-Henderson, 2011). Differentiation of neuroblastoma cells with retinoic acid results in the upregulation of PML and increased prominence of PML nuclear bodies (Yu et al., 2003). In *Pml*$^{-/-}$ mice, there is decreased proliferation of neural progenitor cells, impaired differentiation, and reduced cortical thickness (Regad et al., 2009).

The functions of CAPN5 are largely unexplored and its role in PML nuclear bodies is not yet known. Small molecule calpain inhibitors inhibit differentiation of various cells (Kumar et al., 1992, Ueda et al., 1998, Patel and Lane, 1999, Yajima and Kawashima, 2002, Yajima et al., 2006). This was previously interpreted as involving calpains 1 and 2, but might also involve CAPN5 and is consistent with the role of PML bodies in differentiation. Both PML bodies and calpains are involved in regulating p53 following DNA damage (Sedarous et al., 2003, Alsheich-Bartok et al., 2008, Hetman et al., 2010) (Gostissa et al., 2003). A truncated isoform of βIV spectrin associates with PML nuclear bodies and may represent a scaffold to which other proteins bind (Tse et al., 2001). Spectrins are sensitive substrates of cytosolic calpains (Czogalla and Sikorski, 2005) and the presence of both CAPN5 and truncated βIV spectrin in PML nuclear bodies suggests that CAPN5 might regulate the association of various proteins with PML bodies.

Other proteases associated with PML include deSUMOylases. The best characterized are the sentrin-specific proteases (SENPs) (Drag and Salvesen, 2008). PML is a substrate of SENP1, also known as SUMO protease 2 (Best et al., 2002). The SENPs are cysteine proteases with a catalytic triad of His-Asp-Cys, as compared to His-Asn-Cys in CAPNs. Whether CAPN5 might also function as a SUMO protease is unknown. Of additional interest is that PML associates with nuclear aggregates in several neurodegenerative disorders, particularly polyglutamine disorders including spinocerebellar ataxia, Huntington's disease, dentatorubral-pallidoluysian atrophy, as well as amyotrophic lateral sclerosis (Skinner et al., 1997, Kaytor et al., 1999, Yamada et al., 2001a, Yamada et al., 2001b, Takahashi et al., 2002, Takahashi et al., 2003, Seilhean et al., 2004, Fu et al., 2005). Several of the aggregated proteins have been demonstrated to be calpain substrates (Kim et al., 2003, Gafni et al., 2004, Schilling et

al., 2006, Simoes et al., 2012, Hubener et al., 2013), with calpain inhibition attenuating the nuclear aggregation (Gafni et al., 2004, Haacke et al., 2007, Hubener et al., 2013).

In summary, the results of the present study demonstrate that CAPN5 is expressed at relatively high levels in the CNS, and is a non-cytosolic calpain localized to predominantly to the nucleus where it associates with PML nuclear bodies. CAPN5 contains a unique NLS as well as several potential sumoylation sites. The functions of CAPN5 remain to be determined. However, based on the roles of calpains and PML nuclear bodies, CAPN5 may be involved with neuronal differentiation, response to stress including DNA damage, transcriptional regulation, and regulation of cell death pathways.

**Table 3.1 Prediction of sumoylation motifs on human CAPN5 using SUMOplot™ program, Abgent, San Diego, CA**

| Human CAPN5 | | | |
|---|---|---|---|
| **No.** | **Position** | **Group** | **Score** |
| 1 | K543 | NSYVI **IKCE** GDKVR | 0.94 |
| 2 | K598 | LGQVH **LKAD** PDNLQ | 0.91 |
| 3 | K588 | WNHRV **LKDE** FLGQV | 0.91 |
| 4 | K273 | GLLAF **FKSE** KLDMI | 0.85 |
| 5 | K395 | QYIFE **VKKP** EDEVL | 0.82 |
| 6 | K418 | STRRE **GKGE** NLAIG | 0.67 |
| 7 | K52 | GPAVR **WKRP** KGICE | 0.54 |
| 8 | K276 | AFFKS **EKLD** MIRLR | 0.50 |
| 9 | K396 | YIFEV **KKPE** DEVLI | 0.48 |
| 10 | K312 | SKSER **EKMG** VTVQD | 0.33 |

SUMOplot™ analysis predicted high probability sumoylation motifs on human CAPN5. Sumoylation motifs are shown in bold with the predicted site of sumoylation, residues 'K' are underlined. The position of 'K' residue on the protein is also shown. The SUMOplot™ score system predicts probability based on comparison to known sumoylation sequences, with higher score indicating a greater probability of a sumoylation motif.

**Table 3.2 Summary of CAPN5-ZsGreen1 constructs and their subcellular distribution at 24h post-transfection.**

| Construct | Amino acid sequence | Cellular localization 24 h post transfection |
|---|---|---|
| *pN1-hCapn5$_{1-640}$-ZsGreen1* | Full length of CAPN5, aa 1-640 | Nuclear |
| *pN1-mhCapn5$^{N}_{1-640}$-ZsGreen1* | Full length of CAPN5 with K/R of PSORTII predicted bipartite NLS mutated to asparagine 'N' | Nuclear |
| *pN1-hCapn5$_{393-413}$-ZsGreen1* | EVKKPEDEVLICIQQRPKRST, PSORTII predicted bipartite NLS extended by 2 amino acids on either sides | Cytosolic |
| *pN1-hCapn5$_{395-417}$-ZsGreen1* | KKPEDEVLICIQQRPKRSTRREG, expanded bipartite NLS to include 2 additional basic residues on C-terminal | Cytosolic |
| *pN1-hCapn5$_{390-417}$-ZsGreen1* | YIFEVKKPEDEVLICIQQRPKRSTRREG, addition of upstream sequence to the extended bipartite NLS | Nuclear |
| *pN1-hCapn5$_{388-407}$-ZsGreen1* | PQYIFEVKKPEDEVLICIQQ, upstream sequence plus a portion of the bipartite NLS | Cytosolic and nuclear |
| *pN1-mhCapn5$^{A}_{388-417}$-ZsGreen1* | PQYIFEVAAPEDEVLICIQQAPAASTAAEG, basic residues mutated to alanine 'A' | Nuclear |

**Table 3.3 Putative NLS (YIFEVKKPEDEVLICIQQRPKRSTRREG) is unique to CAPN5 and is conserved across species.**

| Alignment following protein BLAST | Protein | Species |
|---|---|---|
| Sequence query (Putative NLS): YIFEVKKPEDEVLICIQQRPKRSTRREG | CAPN5 | *Homo sapiens* |
| YIFEVKKPEDEVLICIQQRPKRSTRREG | CAPN5 | *Rattus norvegicus* |
| YVFEVKKPEDEVLISIQQRPKRSTRREG | CAPN5 | *Mus musculus* |
| YIFEVKKPEDEILICIQQRPKRSTRVEG | CAPN5 | *Cricetulus griseus* |
| YIFDVKKPEDEVLICIQQRPKQSTRRDG | CAPN5 | *Mustela putorius furo* |
| YIFDVKKPEDEVLISIQQRPKQSTRRDG | CAPN5 | *Bos taurus* |
| YVFDVKKKPEDEVLICIQQKPKRTSRREG | CAPN5 | *Ornithorhynchus anatinus* |
| YVFNVKKAEDEVLVCIQQKPKRTSQKEG | CAPN5 | *Crotalus adamanteus* |
| FVFDVKKPEDEVLVCLQQKTKRTTRQEG | CAPN5 | *Xenopus tropicalis* |
| FVFDVKKPEDEVLVCLQQKTKRTTRKDG | CAPN5 | *Xenopus laevis* |
| YVFDVTKAEDEVLICLQQQDKR | CAPN5 | *Danio rerio* |
| YIFDVKKPEDEVLISIQQRPKQSTRRDG | Cysteine protease | *Desmodus rotundus* |

A homology search for putative NLS using BLAST (92) revealed that this sequence was conserved among CAPN5 protein of various organisms belonging to mammal, reptile, amphibia and fish. None of the other calpains carries a similar sequence. The upstream sequence (PQYIFEV) followed by a classical bipartite NLS (KKPEDEVLICIQQRPKRSTRREG) is a putative NLS of CAPN5.

**Figure 3.1** Calpain 5 is present in nuclei of neurons and glia

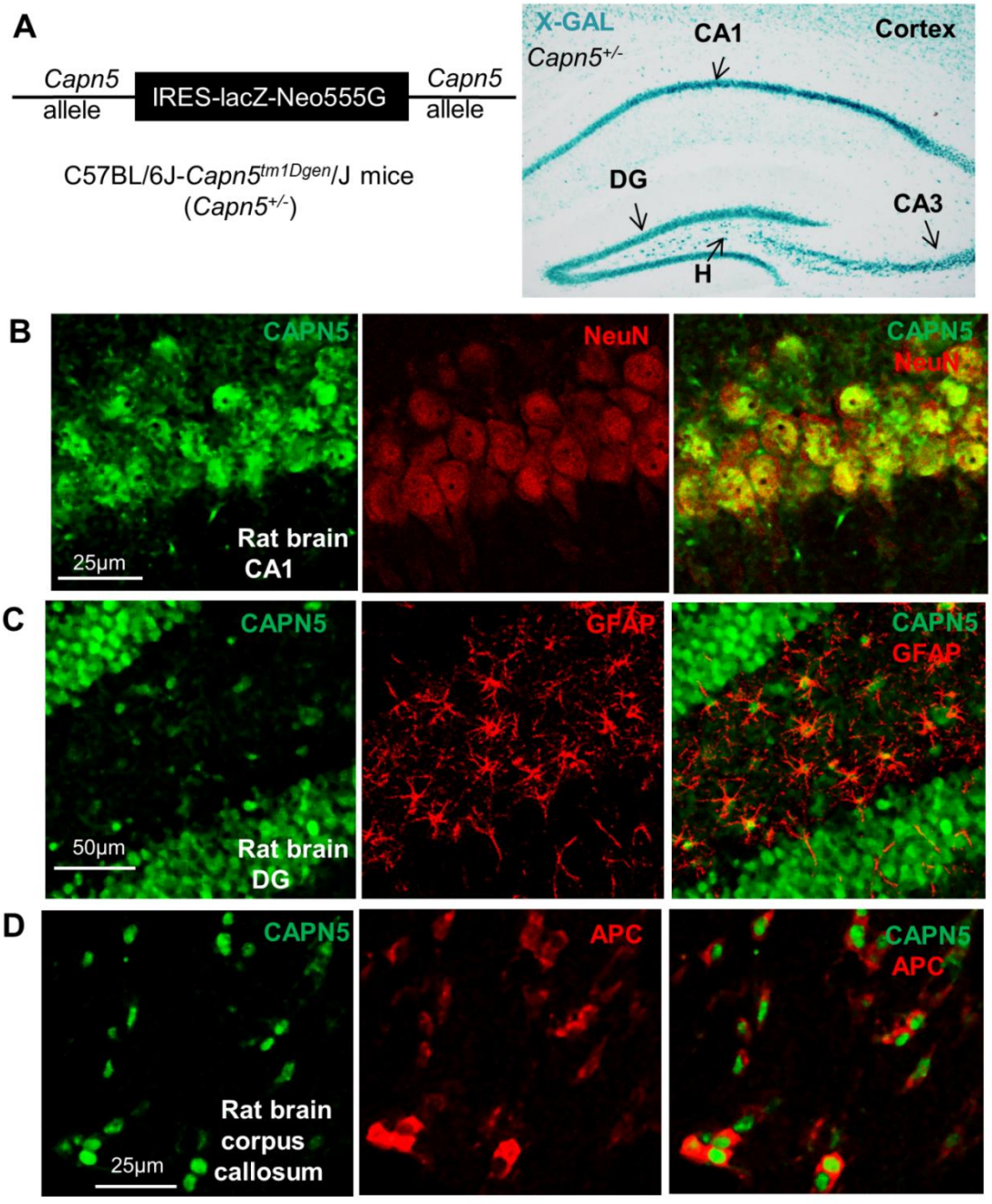

**Figure 3.1.** Calpain 5 is present in nuclei of neurons and glia. X-gal staining was performed on 40 µm coronal brain sections of ~ 3 month old male *Capn5*$^{+/LacZ}$ mice (N=6). In the hippocampal formation, X-gal staining was prominent in all neurons including CA1 and CA3 pyramidal neurons, granule cells of the dentate gyrus (DG) and hilar neurons (H). X-gal staining was also present in molecular layers, suggesting expression in glial cells and consistent with the ubiquitous expression of Capn5 mRNA (**A**).

Immunohistochemical localization of CAPN5 (***B-D)***. Confocal images were obtained following double immunolabelling of 40 µm coronal ~ 3 month old male SD rat brain sections. Co-localization of CAPN5 and NeuN (**B**), a neuronal nuclear protein (87) indicates that CAPN5 is predominantly localized to neuronal nuclei although faint extranuclear staining was also observed. CAPN5 immunoreactivity was evident in the nucleus of cells positive for glial fibrillary acidic protein (GFAP, an intermediate filament protein in astrocytes (88)) (**C**). In cells positive for the adenomatous polyposis coli (APC) protein, a marker of mature oligodendrocytes (89), CAPN5 immunoreactivity was also localized to the nucleus (**D**).

**Figure 3.2** Calpain 5 is enriched in the nuclear nucleic acid binding fraction

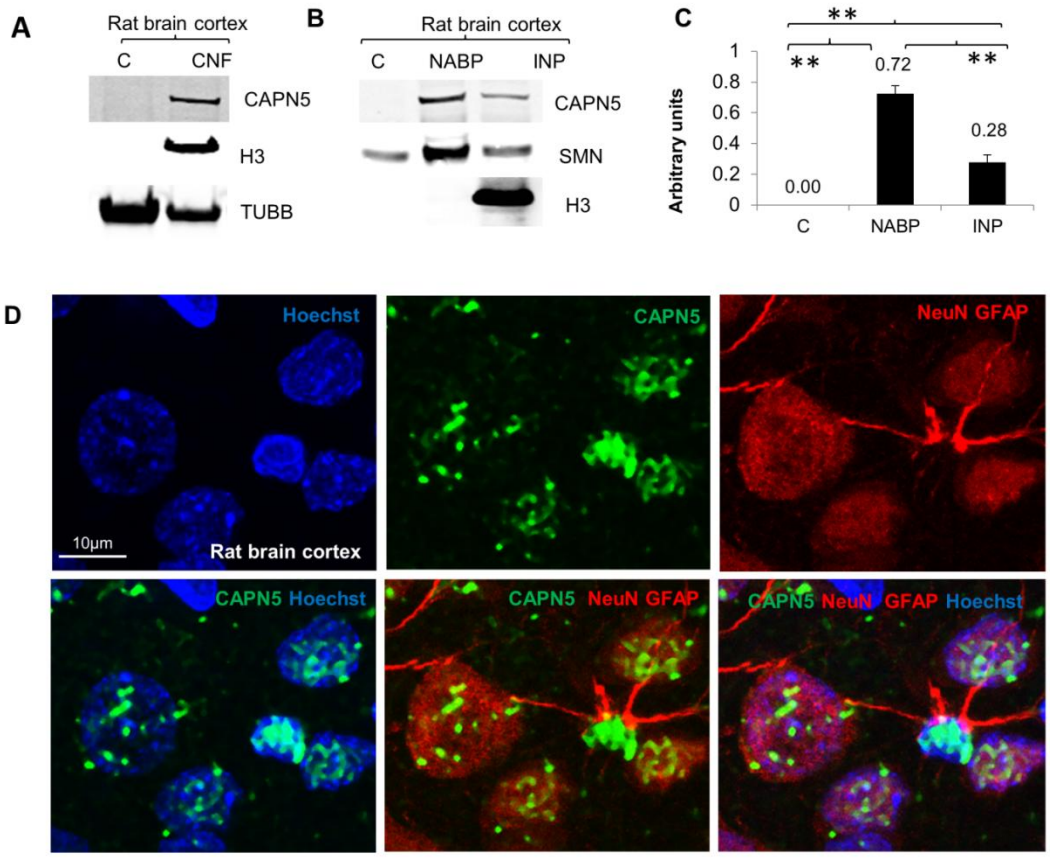

**Figure 3.2** Calpain 5 is enriched in the nuclear nucleic acid binding fraction. Using the Qiagen Nuclear Protein Kit, SD rat brain cortex was subfractionated into the cytosol (C), nucleic acid binding protein fraction(NABP) and insoluble nuclear protein fraction (INP), followed by probing for CAPN5 and marker proteins by Western blot. Following differential centrifugation, CAPN5 immunoreactivity was prominent in the crude nuclear fraction and was not detected in the cytosolic fraction (**A**). Marker proteins included Histone H3 (nuclear), and β-tubulin (TUBB) as a cytosolic marker. Following further nuclear subfractionation, CAPN5 was enriched in the NABP fraction but also detected in the INP fraction (**B**). Survival of motor neuron (SMN) protein resides both in the nucleus and cytosol (90,91), and was used as a marker for the NABP fraction. Histone H3 is a marker for INP fraction. Quantitation of the relative intensity of the CAPN5 immunoreactive band in (B) is shown in (C). The results, reported as group means ± S.D, N=3, were analyzed as one-way ANOVA followed by Tukey's multiple comparison test, ** $p<0.001$ (**C**). Confocal images of 40 µm male SD rat brain sections co-immunostained with CAPN5, NeuN, GFAP and Hoechst (a nuclear marker) show punctate nuclear localization of CAPN5 in each of the cell types (**D**).

**Figure 3.3** Calpain 5 is associated with PML bodies

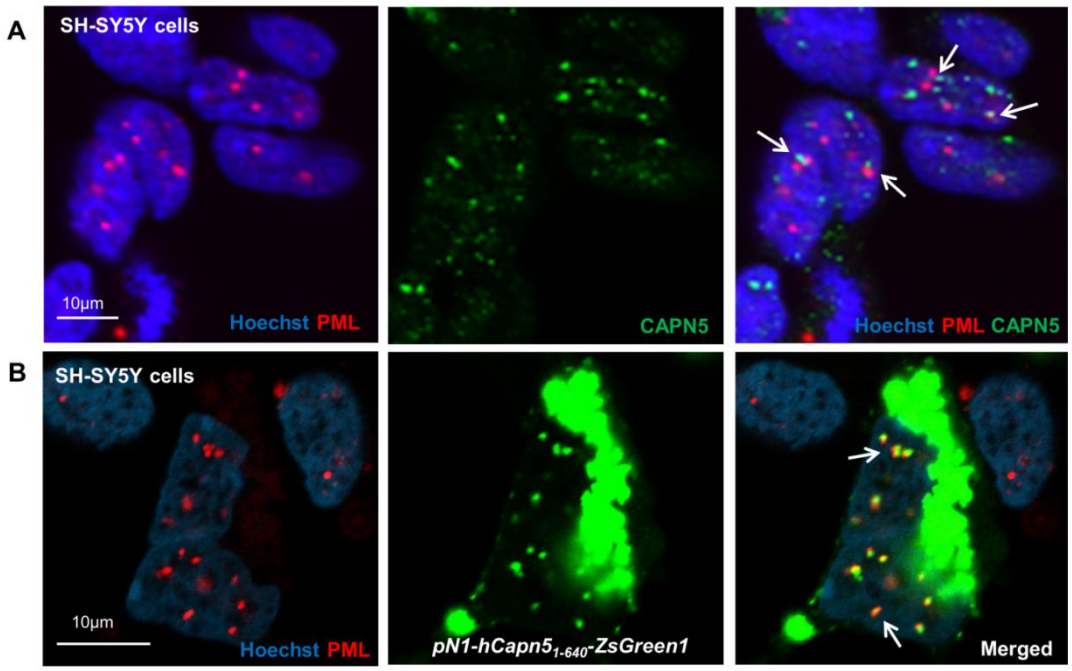

**Figure 3.3** Calpain 5 is associated with PML bodies. Co-immunolabeling of CAPN5 and PML in SH-SY5Y cells indicated that CAPN5 is localized with PML bodies. Independent CAPN5 domains were also observed. 'Arrow' indicates colocalization or close association of CAPN5 and PML (**A**). SH-SY5Y cells were transiently transfected with full length human calpain 5 cDNA (*hCapn5*) fused with *ZsGreen1* (pN1-*hCapn5*$_{1-640}$-*ZsGreen1*) using Lipofectamine 2000 CD reagent. At 24h post transfections, CAPN5-ZsGreen1 expression was detected in intranuclear punctate domains and as extranuclear aggregates. Immunocytochemistry against PML protein indicates that nuclear CAPN5 co-localizes with PML protein (**B**). PML protein undergoes sumoylation and is known to partner with sumoylated protein (23)**.**

**Figure 3.4** Calpain 5 nuclear localization signal YIFEVKKPEDEVLICIQQRPKRSTRREG (390-417)

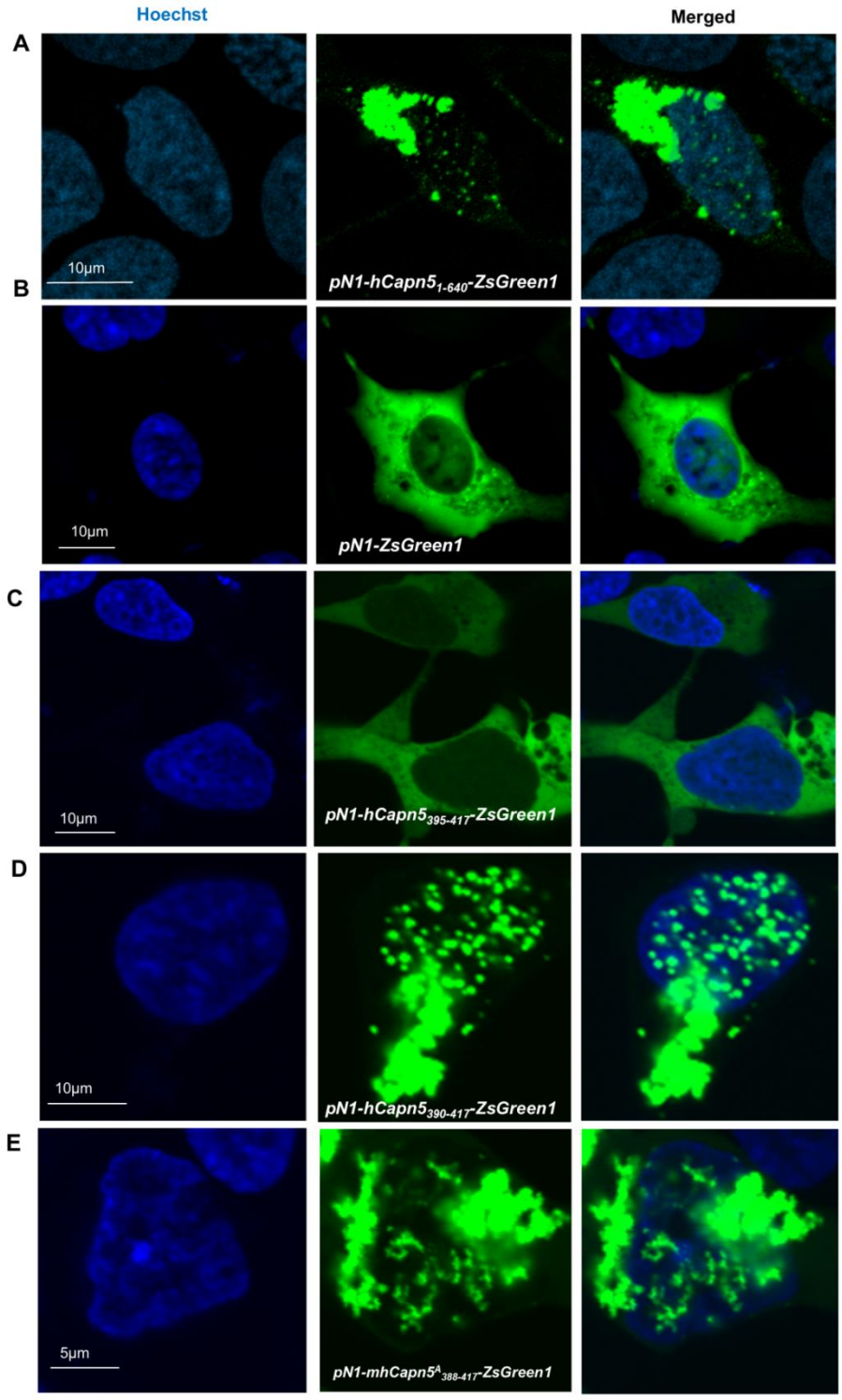

**Figure 3.4**. Calpain 5 nuclear localization signal YIFEVKKPEDEVLICIQQRPKRSTRREG (390-417). SH-SY5Y cells were transiently transfected with *pN1-hCapn5$_{1-640}$-ZsGreen1* (vector encoding full length of human CAPN5 fused with ZsGreen1 at C-terminal. The CAPN5-ZsGreen1 fusion protein was expressed in the nucleus in dot like domains 24h post transfection. (**A**). Following transfection with empty vector, *pN1-ZsGreen1,* the expressed ZsGreen1 protein was mainly cytosolic (**B**). Transient transfection with *pN1-hCapn5$_{395-417}$-ZsGreen1* (KKPEDEVLICIQQRPKRSTRREG, peptide sequence enclosing putative bipartite NLS) resulted in cytosolic localization of the fusion protein (**C**). Expanding this sequence to include the upstream amino acids PQYIFEV (*pN1-hCapn5$_{388-417}$-ZsGreen1,* YIFEVKKPEDEVLICIQQRPKRSTRREG ) resulted in nuclear localization of the fusion protein (**D**). Mutagenesis of the basic residues to alanine (*pN1-mhCapn5$^{A}_{388-417}$-ZsGreen1,* PQYIFEVAAPEDEVLICIQQAPAASTAAEG ) maintains nuclear localization but not the punctate appearance (**E**).

# Chapter 4: Mitochondrial localization of Calpain 5

## Preface

Work shown in this chapter is done by Ranjana Singh with one exception. Ms. Vimala Bondada performed proteinase K treatment of mitochondria and mitoplast from B35 rat neuroblastoma cells (Fig 4.3 D).

## 4.1 Introduction

Calpain 5 is a non-classical member of the calpain family (Barnes and Hodgkin, 1996, Dear et al., 1997, Matena et al., 1998, Ono and Sorimachi, 2012). The calpain family has 15 catalytic isoforms and two regulatory isoforms. CAPN1 and CAPN2 are the oldest known calpains (Guroff, 1964, Goll et al., 2003, Sorimachi et al., 2010, 2011a). Unlike classical calpains, CAPN5 has only one subunit containing three domains (N, CysPc and C2L) similar to classical calpains, and a unique domain T (now called C2) at the C-terminus. It also lacks $Ca^{2+}$ binding EF-hand domains (Barnes and Hodgkin, 1996, Dear et al., 1997, Matena et al., 1998), yet, it may be $Ca^{2+}$ activated (Waghray et al., 2004). We immunolabelled CAPN5 in the mouse brain section and found CAPN5 localized to the hippocampal stratum lacunosum moleculare (SLM) including other regions such as CA1, CA3 and dentate gyrus. SLM is a terminal zone of the perforant path and is rich in mitochondria (Kageyama and Wong-Riley, 1982).

Calpains 1, 2, and 10 are detected in the mitochondria (Garcia et al., 2005, Arrington et al., 2006, Ozaki et al., 2009) and are thought to participate in the events contributing to mitochondrial dysfunction and cell death, such as mitochondrial membrane destabilization, cleaving BID, Na+/$Ca^{2+}$ exchanger, AIF and VDAC (Mandic et al., 2002, Garcia et al., 2005, Kar et al., 2009, Ozaki et al., 2009, although whether calpain 1 cleaves AIF has been questioned (Polster et al., 2005, Cao et al., 2007, Joshi et al., 2009). In case of necrosis, high influx of $Ca^{2+}$ primarily through NMDA receptor activates calpains (Yamashima et al., 1994, Yamashima et al., 1996). An activated calpain such as calpain 1, can disrupt lysosomal membranes, resulting in the release of cathepsin B (Yamashima et al., 1998). Activated cathepsins degrade various substrates (Yuan et al., 2003, Tait and Green, 2010). In *C.elegans*, CLP-1 (calpain like protein-1) and calpain5 ortholog Tra-3 act upstream of aspartyl proteases (cathepsin) ASP3 and 4

to induce necrosis (Syntichaki et al., 2002). Additionally, calpain 10 has been associated with the respiratory dysfunction, mPTP activation and proteolysis of complex I subunits of electron transport chain (Arrington et al., 2006).

Based on the significant involvement of calpains in mitochondrial dysfunction and cell death, together with the localization of CAPN5 in the hippocampal stratum lacunosum molecular that is rich in mitochondria, we investigated the possibility of localization and role of CAPN5 in the mitochondria.

## 4.2 Materials and Methods

Male Sprague-Dawley (SD) rats, C57B/6J mice, SH-SY5Y human neuroblastoma cells and B35 rat neuroblastoma were used in this study.

### 4.2.1 Experimental animals

The University of Kentucky Institutional Animal Care and Use Committee approved all procedures involving experimental animals. Animals included Male Sprague-Dawley (SD) rats. For western blot and fractionation studies, rats were exsanguinated using $CO_2$ inhalation, followed by decapitation. Brains were rapidly removed and homogenized in appropriate buffer for western blot or fractionation as described below. For immunohistochemical study, rats were first perfused with PBS followed by 4% paraformaldehyde in PBS, pH 7.4.

### 4.2.2 Antibodies and reagents

Antibodies against CAPN5 (ab28280), β-tubulin (ab6046-100) and TOMM20 (ab56783) were purchased from Abcam, Cambridge, MA. . Anti NeuN (MAB377) and Anti-GFAP (MAB360) were obtained from EMD Millipore, Billerica, MA. Another CAPN5 antibody (GTX 103264) was purchased Gene Tex Inc., Irvine, CA. Calpain 2 specific antibody (208729) was purchased from Calbiochem, Billerica, MA. An antibody against CYT-C (556433) was ordered from BD Pharmingen, San Jose, CA. Antibodies against VDAC (PA1-954A) and mHSP70 (MA3-028) were purchased from Thermo Scientific, Ashville, NC. An antibody against AIF (SC-13116) was bought from Santa Cruz, Dallas, Texas. IRDye 800CW Anti-rabbit IgG (611-131-132) and IRDye 800 CW Anti-mouse IgG (610-131-121) were purchased from Rockland, Gilbertsville, PA. Hoechst 33258 ( H-3569 ), MitoTracker® Red 580 (M22425), and conjugated secondary antibodies Alexa Fluor-488 anti-rabbit IgG ( A11005 ) and Alexa Fluor-594 anti-mouse IgG ( A11034 ) were purchased from Molecular Probes, Life technologies, Grand Island, NY. Pepstatin A (P4265) and β-mercaptoethanol (M6250) were purchased from Sigma, St. Louis, MO. Alamethicin (A4665), Proteinase K (P4850) and Ficoll (F5415) were purchased from Sigma (St. Louis, MO). Additional reagents were purchased from Sigma (St. Louis, MO) or Thermo Fisher Scientific (Ashville, NC).

### 4.2.3 Western blot

Protein content of samples was assayed using Thermo Scientific Pierce® BCA protein assay reagent A (23228) and reagent B (1859078). Protein (50 µg of each) was mixed in NuPAGE® LDS sample buffer (NP007) supplemented with 5% β-mercaptoethanol, and boiled for 5 min. Boiled samples were separated on 4-12% Bis-Tris HCl gels (NuPAGE NP0335) in MES SDS running buffer ( NuPAGE NP0002) and transferred to a 0.2 µm

nitrocellulose membrane. After blocking in 5% skim milk in 0.05% Tween 20 in Tris-buffered saline, pH 7.6 (T-TBS), for 1 hour, the membrane was incubated with primary antibody (1:5000 of CAPN5, CAPN2, CYT-C, VDAC, TOMM20 or mHSP70, and β-tubulin, 1:10,000) in 5% skim milk in T-TBS overnight at 4ºC. The membrane was washed in T-TBS three times (3x) for 20 min each, followed by incubation with an appropriate secondary antibody (Anti-rabbit IgG, 1:5000; or anti-mouse IgG, 1:5000) at room temperature for 1 hr in dark conditions. The membrane was washed again 3x for 20 min each, and scanned using an Odyssey Infrared Imager (*LI-COR* Biosciences).

### 4.2.4 Immunocytochemistry

*SHSY-5Y (ATCC # CRL-2266)* cells were cultured in complete growth medium (ATCC-formulated Eagle's Minimum Essential Medium (Cat# 30-2003 + 1% Penicillin Streptomycin + 10% FBS) at 37ºC in an incubator maintained with 95% air and 5% $CO_2$. The cells were plated on 35mm glass bottom culture dishes. The following day, adherent cells were fixed in 4% paraformaldehyde in PBS, pH 7.4 for 15 min at room temperature, followed by permeabilization for 10 min with PBS containing 0.25% Triton X-100 (PBS/T). After washing 3x with PBS, cells were incubated with 5% NGS-PBS/T for 30 min, then incubated with primary antibody (1:100, CAPN5 and 1:500, mHSP70) in 5% NGS-PBS/T overnight at 4°C. The next day, sections were washed 3x in PBS and incubated for 1 hr in the dark with 1:1000, Alexa Fluor-488 anti-rabbit IgG or Alexa Fluor -594 anti-mouse IgG, followed by 3x wash of 5 min each. Nuclei were stained with Hoechst 33258 at 10µg/ml for 5 min. Cells were viewed under a Nikon Ti-E C2plus confocal microscope.

### 4.2.5 Immunohistochemistry

Rats were perfused with PBS followed by 4% paraformaldehyde in PBS, pH 7.4. The brains were removed and post fixed overnight, then cryoprotected in 30% sucrose in PBS. The brains were frozen in powdered dry ice and sectioned at 40 µm in the coronal plane. Brain sections were stored in cryoprotectant (30 % ethylene glycol, v/v and 30% glycerol, v/v in 1xTBS) at -20°C until use. Free floating brains sections were washed in TBS 3x followed by blocking in 5% natural goat serum (NGS) in T-TBS (0.1% Triton-X-100) for 30 min at room temperature. Brain sections were incubated with primary antibodies (CAPN5, 1:100 and NeuN, 1:200) in 5% NGS-T-TBS overnight at 4°C. Primary antibody was omitted from negative controls. Sections were washed 3x in 1x TBS, followed by 1 hour incubation with appropriate secondary antibodies at 1:1000 dilution (Alexa Fluor-488 anti-rabbit IgG or Alexa Fluor -594 anti-mouse IgG), then washed 3x in TBS. Nuclei were stained with Hoechst 33258 at 10µg/ml. Brain sections were mounted on glass slides with Vectashield (H-1000, Vector Labs) fluorescence mounting medium and examined under a Leica AOBS TCS SP5 inverted Laser Scanning Confocal Microscope.

### 4.2.6 Mitochondria Isolation from rat brain cortex

We used a method slightly modified from Naga et al., 2007. Rat brain cortical tissue was homogenized in dounce homogenizer in mitochondrial isolation buffer (MIB) containing 215 mM mannitol, 75 mM sucrose, 1mM EGTA, 20 mM HEPES and 1uM of Pepstatin A. Homogenate was centrifuged at 1,300 X g for 3 min to obtain crude nuclear fraction as pellet. The supernatant was spun again at 13,000 X g for 10 min to obtain cytosol as supernatant and crude mitochondria as a pellet. Crude mitochondria were purified through Ficoll gradient and layered on the top of 10% and 7.5 % Ficoll discontinuous

gradient ( appropriate amount of MIB was added to 20% Ficoll solution, 1M sucrose, 0.1M Tris- HCl and 0.5 M EGTA), then ultracentrifuged in a SW 55 Ti rotor at 124,000 X g at 4ºC for 30 min. The resulting pellet of non-synaptic mitochondria was dissolved in MIB and stored at -80º C until use. Synaptosomes were collected and placed in a nitrogen cell disruption bomb (Parr Instrument Company, model 4369, Moline, IL) at 1200 psi for 10 min (Brown et al., 2004), to break the synaptosomal membrane and liberate synaptic mitochondria. The synaptic mitochondria were purified and pelleted through ultracentrifugation on Ficoll gradient as mentioned above and stored in -80ºC until use.

### 4.2.7 B35 (ATCC # CRL-2754™) cell culture and mitochondria isolation

Cells were cultured in complete growth medium (ATCC-formulated Dulbecco's Modified Eagle's Medium, Cat # 30-2002 + 1% Penicillin Streptomycin $_{+}$10% fetal bovine serum) at 37$^{o}$C in an incubator maintained with air, 95% and $CO_2$, 5%. For subculture, adherent cells were rinsed with warm 0.05% trypsin followed by incubating the cells with 5 ml of 0.05% trypsin at room temperature (or at 37$^{o}$C) for 5 min or until cells detach. The detached cells were homogeneously mixed in 8 ml of fresh growth media, and equally dispensed to 4-6 subculturing flasks.

To isolate total mitochondria from B35 rat neuroblastoma cells, cells were detached with 0.05% trypsin and pelleted. The cell pellet was homogeneously mixed and incubated in MIB for 10 min and dounce homogenized. The homogenate was ultracentrifuged through 12, 26 and 40% of Percoll gradient at 27,000 X g for 10 min. After the spin, a mitochondrial fraction was obtained at the junction of 40 and 26% percoll gradient. Two consecutive washes of 5 min each were done in MIB at 14,000 X g and 11,500 X g.

### 4.2.8 Mitoplast isolation from rat brain cortex and B35 cells

Mitochondria (100 µg) was pelleted and resuspended in 10mM HEPES, pH 7.4, at 1mg/ml. The suspension was incubated on ice for 20 min on a slow rocker to rupture the outer membrane by hypotonic swelling. At the end of the incubation, an equal amount of 2x MIB was added to re-establish the osmolarity, and vortexed. The mix was centrifuged at 1,900 X g for 15 min to pellet mitoplast.

### 4.2.9 Proteinase K treatment of mitochondria and mitoplast

Mitochondria (50 µg) or mitoplast (obtained from 100 µg of mitochondria) was incubated with various concentrations of Proteinase K in MIB, pH 8.00 for 30 min at 37ºC. The reaction was stopped by adding 2mM PMSF for 10 min at 37ºC. Mitochondria/mitoplast was pelleted at 13,000 X g for 10 min/ 1,900 X g for 15 min, boiled with sample buffer, and separated through SDS-PAGE on a 4-12 % Bis-Tris gel.

### 4.2.10 Treatment of mitochondria with alamethicin

We used a method slightly modified from Joshi et al., 2009. Intact synaptic mitochondria (50 µg) was resuspended at 1mg/ml in respiration buffer (125 mM KCl, 2mM $MgCl_2$, 20mM HEPES, 2.5 mM $KH_2P0_4$, pH 7.2) containing 5 mM pyruvate, 2.5 mM malate and 150 µM ADP, and incubated on ice for 15 min. At the end of the incubation, 15 µM alamethicin was added and reincubated at 37°C for 20 min. The suspension was centrifuged with 13,000 X g for 10 min to collect pellet and supernatant separately to probe through Western blot.

### 4.2.11 SH-SY5Y cell culture, plasmid preparation, transient transfection and microscopy

Human *Capn5* cDNA (accession number BC018123.1 was purchased as described under *antibodies and reagents*. PCR oligonucleotide primers for *Capn5* were designed to allow cloning into *p3XFLAG-CMV-14* vector or *pN1-ZsGreen1* such that a FLAG tag or ZsGreen1 was encoded at the C-terminal of the fusion product. PCR was carried out using Pfu DNA polymerase kit. The resulting DNA product was digested with EcoRI-HF and BamHI-HF. The product was then ligated into the vector using a Rapid DNA Ligation kit and transformed into One Shot® Stbl3™ competent bacteria. Plasmid DNA was isolated using Qiagen Maxi Prep Kit # 12263.

SH-SY5Y cells were transfected with a vector encoding human CAPN5 fused with ZsGreen1 at C-terminus (*pN1-hCapn5$_{1-640}$-ZsGreen1*). Transfection was performed using lipofectamine 2000CD reagent. Transfected cells were labeled with Mito Tracker® red 580 at the concentration of 100 nM for 45 min. Cells were then imaged on Olympus DSU microscope.

### 4.2.12 Statistical analysis

The data were analyzed using one-way ANOVA, followed by Tukey's multiple comparison tests.

## 4.3 Results

### 4.3.1 Calpain 5 is localized to the nucleus and mitochondria.

Using anti-CAPN5 (ab28280), double labeling of CAPN5 and NeuN ( a marker for neuronal nuclei) in mouse brain cortex, we showed that calpain5 was not exclusively nuclear. Faint immunoreactivity was observed in the neuropil and a band of CAPN5 immunoreactivity was present in stratum lacunosum-moleculare in the hippocampal formation (Fig 4.1A). This is a terminal zone of the perforant path and is rich in mitochondria (Kageyama and Wong-Riley, 1982), suggesting a possible presynaptic, postsynaptic, or mitochondrial localization of CAPN5 in addition to the nuclear localization. Colabeling CAPN5 with mHSP70 in SH-SY5Y cells confirmed the nuclear as well as mitochondrial localization of calpain5 (Fig 4.1B).

### 4.3.2 Calpain 5 is enriched in synaptic mitochondria.

Cytosol and non-synaptic and synaptic mitochondria were probed against CAPN5 through western blot. CAPN5 was detected in the mitochondrial fraction, but not in the cytosolic fraction. Calpain 2 was used as a positive control for cytosol. CAPN5 was enriched in synaptic mitochondria compared to non- synaptic mitochondria (Figs 4.2A and 4.2B).

### 4.3.3 Calpain 5 is present on the inner mitochondrial membrane.

A broad spectrum protease, proteinase K (PK) is commonly used to check if a protein is localized on the surface of an organelle (Brdiczka and Krebs, 1973, Ebeling et al., 1974). Intact synaptic mitochondria were treated with 0, 50, 75 and 100 µg/ml PK. CAPN5 was

not digested at these concentrations, indicating that CAPN5 is not present on the outer surface of mitochondria. TOMM20, a translocase on the outer mitochondrial membrane (Pfanner and Wiedemann, 2002), was however digested at these treatments (Figs 4.3A and 4.3B).

Mitoplasts isolated from synaptic mitochondria were also treated with PK. Mitoplasts contain a matrix and intact inner membrane but no outer membrane, therefore AIF is exposed on mitoplasts since it is present on the inner membrane (Fig 4.3A). PK treatment of mitoplast resulted in digestion of AIF, as well as CAPN5. This suggests that CAPN5 is present on the inner mitochondrial membrane projecting into the inner membrane space (Fig 4.3C and 4.3D).

### 4.3.4 Calpain 5 is released from mitochondria on treatment with alamethicin

Alamethicin, a peptide antibiotic isolated from the fungus *Trichoderma Viride* forms anartificial channel in the membrane (Gostimskaya et al., 2003, Joshi et al., 2009). On treatment of intact synaptic mitochondria with alamethicin, CAPN5 was largely detected in the supernatant. CYT-C was used as a positive control for the release following membrane permeabilization (Liu et al., 1996, Otera et al., 2005). Following alamethicin treatment, CYT-C was detected mainly in the supernatant. These results indicate that similar to CYT-C, CAPN5 is released from mitochondria on membrane permeabilization (Fig 4.4).

### 4.3.5 1$^{st}$ 30 aa of Calpain 5 is insufficient to localize to the mitochondria

iPSORT (http://ipsort.hgc.jp/) predicted a putative N-terminal (1-30 aa) mitochondrial targeting sequence in human CAPN5 (hCAPN5) (Fig 4.5 A). SH-SY5Y cells were

transfected with a vector encoding 1-30 aa of hCAPN5 fused with ZsGreen1 at the C-terminal (*pN1-hCapn5*$_{1-30}$*-ZsGreen1*). When colabelled with MitoTracker®580, ZsGreen1 expression was not localized to the mitochondria, suggesting that 1-30 aa may not be sufficient to target to the mitochondria (Fig 4.5 B). Quite frequently ZsGreen1 expression was detected adjacent to the mitochondria, but not in the mitochondria.

## 4.4 Discussion

In chapter 3, we showed that CAPN5 was mainly present in nucleus, although extranuclear localization was also observed. In this study, by probing synaptic and non-synaptic mitochondrial fractions, and cytosol through Western blot, we found CAPN5 is also present in mitochondria with an enriched presence in the synaptic mitochondria. CAPN5 was not detected in the cytosol. Calpains 1, 2 and 10 have been detected in cytosol and in mitochondria (Garcia et al., 2005, Arrington et al., 2006, Badugu et al., 2008).

The inability to detect CAPN5 in the cytosol is unusual as this it not the case for other calpains. There are some non-convincing indications in the literature about cytosolic localization of CAPN5. In 293T cells transfected with human CAPN5 construct, CAPN5 was detected in the nucleus as well as in the cytosol. However, in untransfected cells, CAPN5 was mainly detected in the nucleus, which suggests that transfection with *Capn5* construct may have led to non-specific localization of CAPN5 in the cytosol (Gafni et al., 2004). Another study also briefly mentions cytosolic localization of CAPN5 in the stromal cells and endometrial glandular epithelial cells, although the immunostaining is not very evident without the images of higher magnifications (Penna et al., 2008). Undetectable levels of CAPN5 in cytosol together with its localization in nucleus and

mitochondria suggests that CAPN5 may have acquired specific roles in these cellular compartments. Recently, Mahanjan et al 2012 showed that a mutation in CAPN5 (R243L) causes mislocalization of CAPN5 to the cytosol (Mahajan et al., 2012).

Calpains are implicated in various mitochondrial functions. Mitochondrial CAPN10 has been associated with respiratory dysfunction, mPTP activation, and proteolysis of complex I subunits of electron transport chain, which were protected when calpain activity was inhibited with calpeptin (Arrington et al., 2006). At least four calpains, 1, 2, 5 and 10, are detected in mitochondria. So, mitochondrial respiratory dysfunction and complex I degradation may actually be attributed to specific or cumulative activation of all mitochondrial calpains. CAPN5 could also contribute to the process of cell death initiation in mitochondria because after its release from mitochondria, it might contribute to the cytosolic component of the cell death pathways. Calpain cleaves BID (Mandic et al., 2002). Truncated BID (tBID) oligomerizes BAK or BAX, which leads to permeabilization of mitochondrial membranes, followed by release of cytochrome C (CYT-C) and truncated AIF (tAIF) in the cytosol. White CYT-C engages in the pathway of caspase 3 activation, tAIF translocates to the nucleus to induce caspase independent cell death (Otera et al., 2005). The mechanism of AIF cleavage is still not clearly delineated. CAPN1 is present in the IMS of mitochondria and is thought to be involved in AIF cleavage (Polster et al., 2005, Cao et al., 2007), but a follow up study did not support this (Joshi et al., 2009). Once AIF is truncated, mitochondrial CAPN2 assists the release of tAIF by cleaving VDAC (Ozaki et al., 2009). $Ca^{2+}$ overload is continuously maintained during the cell death as calpain1 cleaves the mitochondrial inner membrane located $Na^{2+}/Ca^{2+}$ exchanger ( NCX) (Kar et al., 2009). CAPN5, being in spatial vicinity with AIF, could be involved in cleaving AIF. It could also participate in cleaving VDAC and $Na^{2+}/Ca^{2+}$ exchanger.

The mechanism of release of CAPN5 from the mitochondria is not clear. We showed that CAPN5 is present on the inner membrane but whether is anchored or loosely associated is also not clear. An anchored protein would need to be cleaved before release. However, if CAPN5 is loosely associated on the inner membrane, such as through electrostatic interaction, destabilization of mitochondrial membrane during cell death could result in the loss of association and release of CAPN5 from the mitochondria. After release, CAPN5 could contribute to the cytosolic events of the cell death. In cytosol, CYT-C binds to APAF1 forming apoptosome that activates CASP9, which in turn activates CASP7 and CASP3. CASP3 can also be directly activated by CAPN2 (Blomgren et al., 2001, Orrenius et al., 2003, Harwood et al., 2005). Once released, CAPN5 may participate with other protein complexes to cleave BID, CASP9, CASP3 and/or CASP7 to augment the cell death mechanics. Released CAPN5 could also cleave cathepsin D or E (aspartyl protease) to mediate lysosomal cell death pathway. Tra-3, an ortholog of CAPN5 in *C.elegans* contributes to necrotic cell death through engaging lysosomal and cytoplasmic aspartyl proteases (Syntichaki et al., 2002).

After release, CAPN5 could also translocate to ER-mitochondria associated membrane (MAM), where it may act as SUMO protease. $Ca^{2+}$ regulation at MAM requires interaction between mitochondria and ER (such as ATP is released from mitochondria in to the cytosol and released ATP activates $Ca^{2+}$ -ATPase pump at ER membrane), and reorganization of various proteins at MAM (Hayashi et al., 2009). One of these complexes at MAM is the PML complex with IP3, protein kinase Akt and protein phosphatase (PP2a). PML seems important to bind PP2a to IP3R, which favors dephosphorylation of IP3R over phosphorylation by AKT, hence leading to $Ca^{2+}$ influx in the mitochondria from ER (Wang et al., 1998a, Giorgi et al., 2010, Giorgi et al., 2011,

Pinton et al., 2011). SUMOylation could possibly be a way by which PML interacts with IP3R, AKT and PP2a and forms a complex. GPSsp2.0 predicted SUMOylation motifs at high threshold for all the three proteins, IP3R (Uniprot # Q14573; 7, SUMOylation motifs), PP2a (Q15173; 2) and AKT1 (B7Z5R1; 4). Triggered by a legitimate signal, released CAPN5 may translocate to PML-IP3-AKT-PP2a complex at MAM to deSUMOYlate the assembly to regulate $Ca^{2+}$ signaling.

Enriched levels of CAPN5 in synaptic mitochondria suggest a role of CAPN5 at presynaptic terminal. At the presynaptic terminal, calpains have been associated with glutamate release. SNARE [(soluble *N*-ethylmaleimide-sensitive fusion protein attachment receptor protein complex of synaptosomal-associated protein of 25 kDa (SNAP) Receptor] complex mediates the interaction and fusion of the synaptic vesicle to the presynaptic membrane (Popoli et al., 2012). In cultured rat cerebellar granule cells, ionomycin-induced calpain activation suppresses synaptic vesicle exocytosis and glutamate release (Ando et al., 2005). In pancreatic β-cell secretary granule exocytosis, CAPN10 triggers insulin release. CAPN10 binds to SNARE complex and partially cleaves SNAP-25 leading to remodeling of SNARE complex; hence triggering exocytosis and insulin release (Marshall et al., 2005, Evans and Turner, 2007). CAPN5 could be involved in exocytosis and glutamate release at presynaptic terminal. As shown in the results, CAPN5 is localized to the hippocampal stratum lacunosum moleculare, a terminal zone of the perforant pathways, which additionally supports synaptic localization of CAPN5.

The mechanism of CAPN5 localization to mitochondria is also unknown. N-termini of CAPN1 and CAPN10 possess mitochondrial targeting sequences (MTS) (Arrington et al., 2006, Badugu et al., 2008). Mitochondrial targeting proteins generally carry a cleavable N-terminus that is cleaved after import. Hydrophobic and positive residues of

the N-terminus make an amphipathic helix, which interacts with the transporter outer membrane (TOM) and transporter inner membrane (TIM) complexes for mitochondrial import. After import, the N-terminus is cleaved; however there are a few exceptions. For example, the N- terminal of calpain 1 ($1^{st}$ 30 amino acids that carry MTS) is retained on completion of the import (Badugu et al., 2008). Amino acids 1-30 of CAPN5 (MFSCVKPYEDQNYSALRRDCRRRKVLFEDP – the hydrophobic and positive residues are underlined) were not sufficient to target to mitochondria. SH-SY5Y cells were transiently transfected with a vector encoding amino acids 1-30 of CAPN5 fused with ZsGeen1 at C-terminal. The ZsGreen1 expression remained mainly cytosolic, suggesting that $1^{st}$ 30 amino acids of CAPN5 may not be enough to target to mitochondria. A considerable variation in the N-terminus length or/and amino acids composition through TOM/TIM import system has been reported (Pfanner, 2000, Truscott et al., 2003). Calpain 5 may require a longer sequence or have an internal MTS, which remains to be investigated. A variety of import sequences have been described (Chacinska et al., 2009). In many instances, ZsGreen1 expression was observed contiguous to the mitochondria, which suggests that the process of entry into the mitochondria may have been stalled. ZsGreen1, a large reporter protein (~38kDa), may hinder with the transport of the 1-30 aa peptide in to the mitochondria. Fusing this peptide with a Myc-tag, a small peptide (~1.2kDa) may be used to check if aa 1-30 is sufficient to enter the mitochondria.

In summary, CAPN5 is a non cytosolic calpain present in the nucleus and in mitochondria, where it is especially enriched in synaptic mitochondria. Calpain 5 is localized on the inner mitochondrial membrane and released following membrane permeabilization. It could be speculated that CAPN5 is involved in various functions

where other calpains are also involved. Specifically, it may be invloved in $Ca^{2+}$ regulation at ER-mitochondrial associated membrane that remains to be investigated.

**Figure 4.1** Mitochondria are one of the subcellular pools of CAPN5

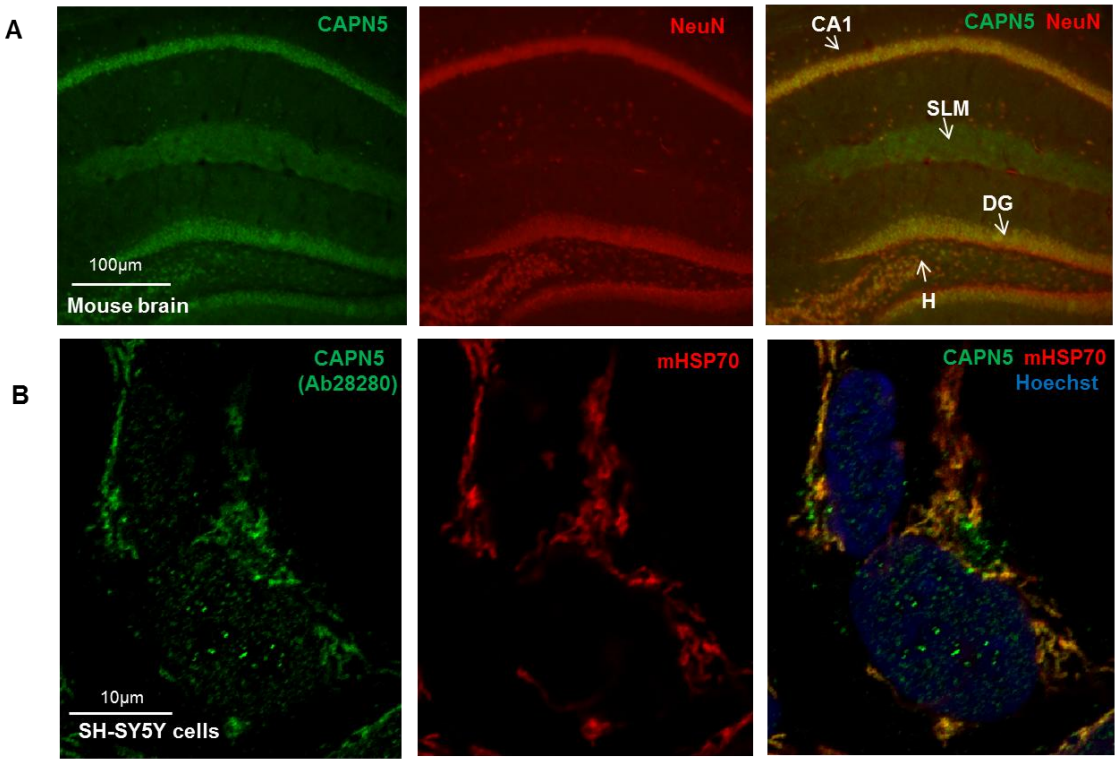

**Figure 4.1** Mitochondria are one of the subcellular pools of CAPN5. CAPN5 was not exclusively nuclear (colocalization with NeuN, in the regions of CA1, CA3, dentate gyrus/DG and hilar/H neurons) because a band of immunoreactivity was observed in the stratum lacunosum-moleculare (SLM) of the dentate gyrus. This pathway contains Schaffer collateral fibers from CA3 neurons as well as perforant path axons from entorhinal cortex. This is a terminal zone of the perforant path and is rich in mitochondria (Kageyama and Wong-Riley, 1982) (**A**). Through immunocolabelling and confocal microscopy, CAPN5 was detected in the nucleus as well mitochondria of human SH-SY5Y neuroblastoma cells (N= 5, culture dishes) (**B**).

**Figure 4.2** Calpain 5 is a non-cytosolic protein enriched in synaptic mitochondria

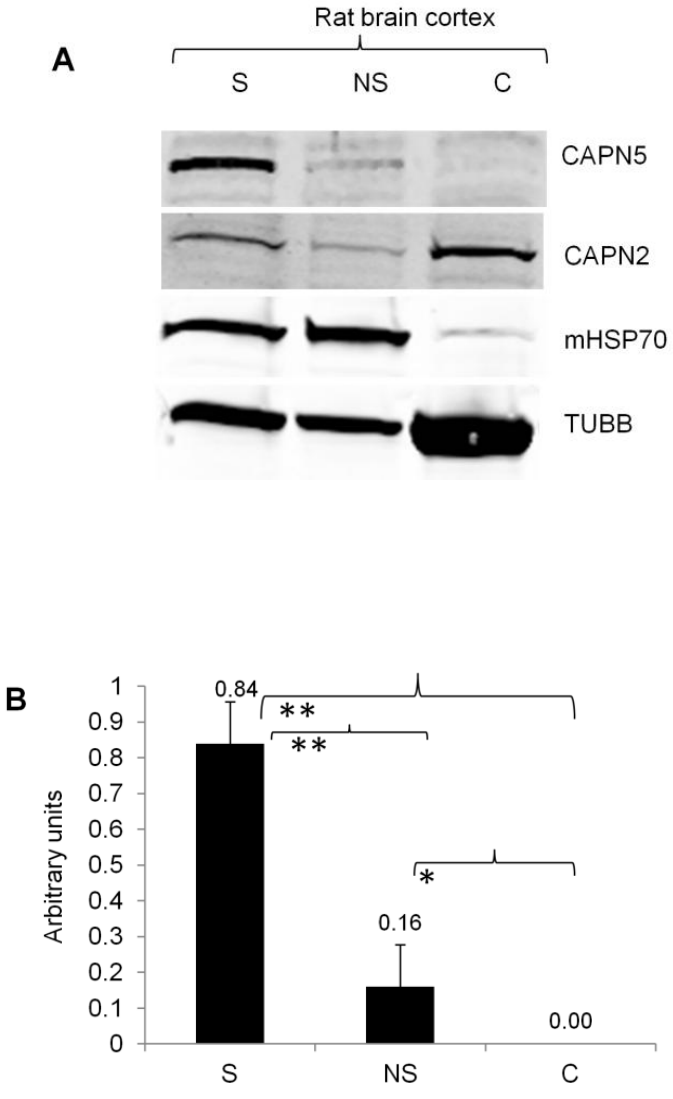

**Figure 4.2** Calpain 5 is a non-cytosolic protein enriched in synaptic mitochondria. Cytosolic fraction (C), non-synaptic (NS) and synaptic (S) mitochondria were obtained through differential centrifugation of ~ 3 month old rat brain cortex. The fractions were probed for CAPN5 by Western blot. Unlike CAPN2, CAPN5 was not detected in the cytosol but was enriched in the synaptic mitochondria compared to nonsynaptic mitochondria. mHSP70 and β-tubulin (TUBB) are mitochondrial and the cytosolic markers, respectively (**A**). The quantitative values of immunoreactivity band in (A) are plotted in (B). The data were statistically analyzed as one-way ANOVA followed by Tukey's multiple comparison test,* $p<0.01$, ** $p<0.001$. Results are expressed as the group means ± S.D, N=6 (**B**).

**Figure 4.3** Calpain 5 is present on the inner mitochondrial membrane

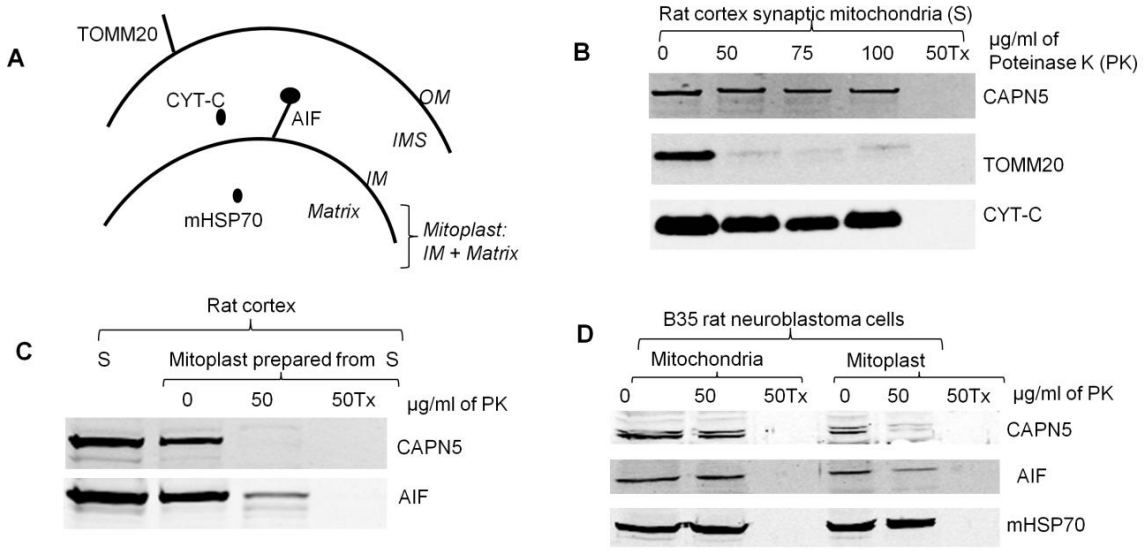

**Figure 4.3** Calpain 5 is present on the inner mitochondrial membrane. A schematic diagram describes various mitochondrial markers: TOMM20 is located on the cytoplasmic side of the outer mitochondrial membrane (*OM*). Treating isolated intact mitochondria with a board spectrum protease like proteinase K (PK) completely digests TOMM20. It is used as a positive control for PK treatment. AIF is a membranous protein anchored on the inner membrane (*IN*), and projects into inter membrane space (*IMS*). CYT-C and mHSP70 are soluble proteins present in the *IMS* and *matrix* of the mitochondria, respectively. AIF, CYT-C and mHSP70 are protected from PK digestion of the intact mitochondria. However, permeabilizing mitochondrial membranes with Triton-X-100 (Tx) results in the PK digestion of these proteins. *Mitoplast* can be fractionated from mitochondria as an intact *IN* covering *matrix*. PK treatment of the mitoplast is sensitive to the proteins like AIF, which is present on the outer surface of the inner membrane (**A**). 'S' represents intact synaptic mitochondria without any treatment. Treatment of intact rat synaptic mitochondria with varying concentrations of PK did not digest CAPN5 band, indicating that CAPN5 is present inside the mitochondria. TOMM20 was however digested. CYT-C, an *IMS* protein, was not digested on PK treatment (**B**). Mitoplasts fractionated from both rat synaptic mitochondria and mitochondria of B35 rat neuroblastoma cells were incubated with 50 ug/ml of PK. CAPN5 was digested similar to AIF suggesting it is present on the inner membrane (**C, D**).

**Figure 4.4** Calpain 5 releases from the mitochondria on membrane permeabilization

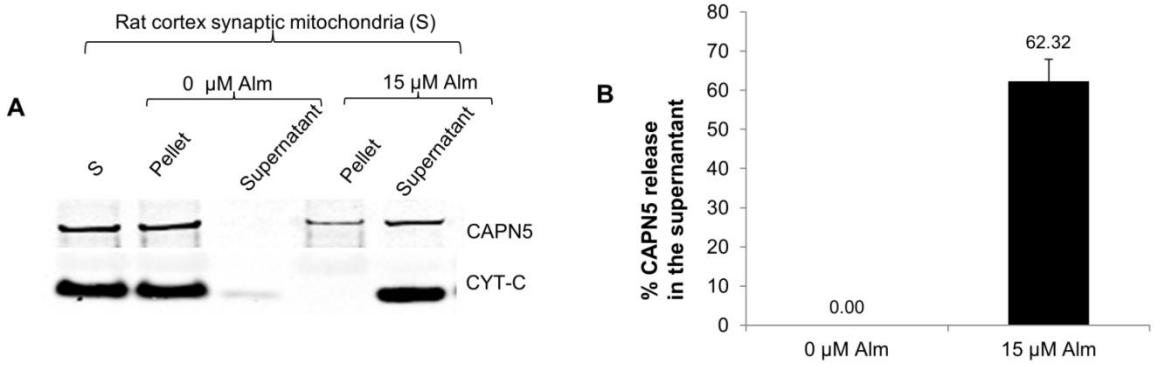

**Figure 4.4** Calpain 5 releases from the mitochondria on membrane permeabilization. Alamethicin forms artificial channels in the membrane. Swollen synaptic mitochondria (incubation of intact synaptic mitochondria in respiration buffer for 15 min, see method section for the details) were treated with 15μM alamethicin. CYT-C was released from mitochondria, and detected in the supernatant. CAPN5 was also largely detected in the supernatant, suggesting it is released from the mitochondria. 'S' represents intact synaptic mitochondria without any treatment (**A**). The fraction of CAPN5 release was calculated as a ratio of calpain 5 detected in the supernatant to the total calpain 5 present the pellet of untreated mitochondria. The result is reported as percentage release. Data were statistically analyzed as paired t test, ** p =0.0026, and reported as group means ± S.D, N=3. Arbitrary values of CAPN5 band were measured in Odyssey *LI-COR* software (**B**).

**Figure 4.5 1-30** aa of human Calpain 5 is insufficient to target to mitochondria

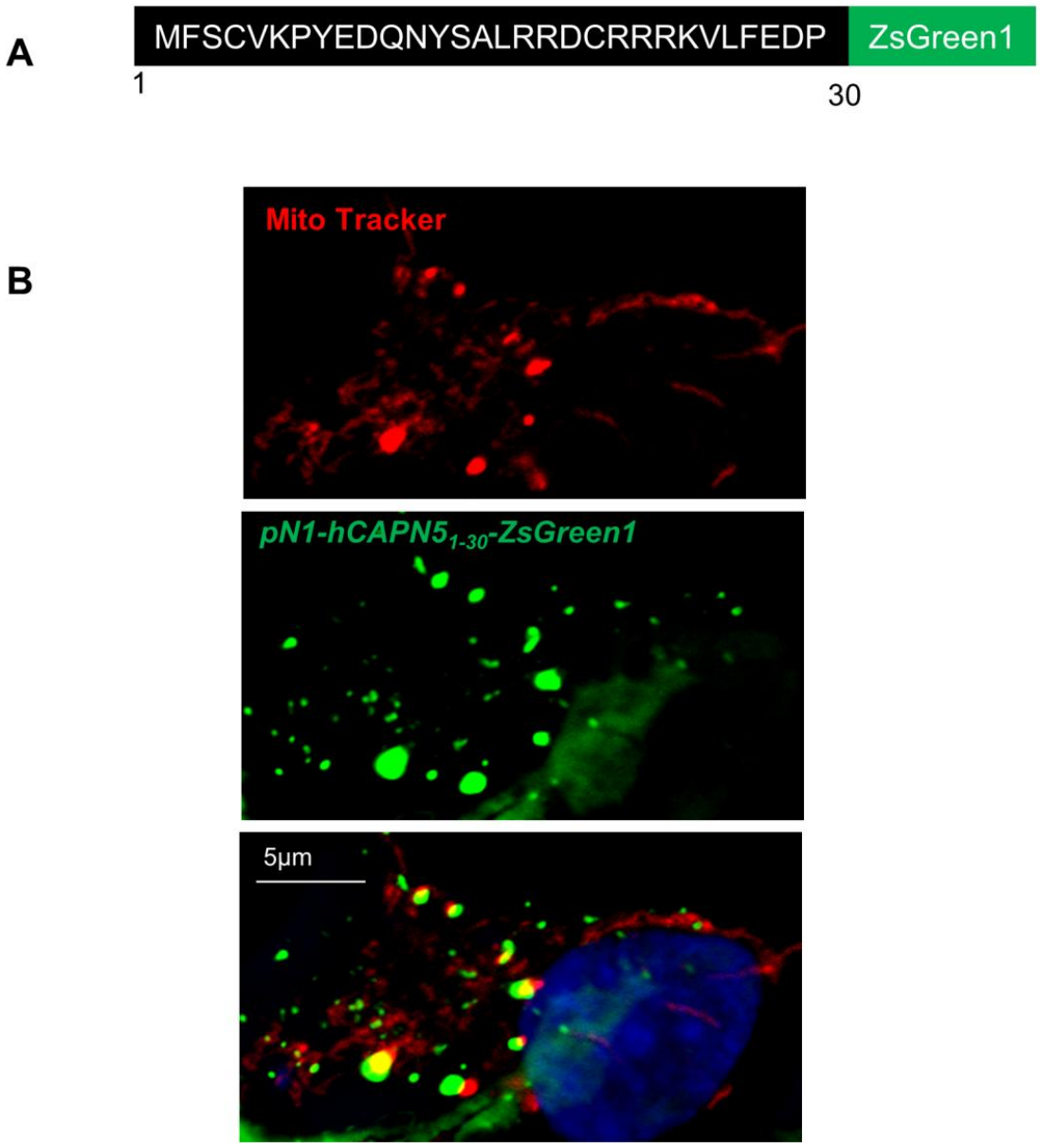

**Figure 4.5** 1 – 30 aa of human Calpain 5 is insufficient to target to mitochondria. SH-SY5Y cells were transfected with pN1-hCAPN5$_{1-30}$-ZsGreen1 encoding 1-30 aa of hCAPN5 fused with ZsGreen1 at the C-terminal (**A**). Cells were labeled with MitoTracker® Red 580 at the concentration of 100nM 15 mins prior to imaging. ZsGreen1 expression did not colocalize with mitochondria, suggesting that the 1-30 aa of CAPN5 may not be sufficient for targeting mitochondria. Zsgreen1 expression was detected adjacent to the mitochondria in many instances (**B**).

# Chapter 5: Discussion, summary and conclusion

Calpains are $Ca^{2+}$ activated proteases. Sixteen isoforms of calpains have been reported thus far. Calpains 1 and 2 are called the classical calpains. These are the most investigated calpains in the CNS. In the first report, calpain was describes as a soluble $Ca^{2+}$ proteinase, obtained in the soluble fraction of the brain (Guroff, 1964). Since then, calpains have been considered as cytoplasmic calpains, although a small fraction is now thought to associate with the cell membrane. Calpain 5 is a non-classical calpain because it has only one subunit possessing N-terminal, CysPc and C2L domains similar to the large subunit of the classical calpains. However, instead of PEF(L), CAPN5 CAPN5 has domain T, also called C2 domain (Fig 1.1). An ortholog of CAPN5, Tra-3, contributes to the necrotic neuron death *in C.elegans* (Syntichaki et al., 2002).

Calpain 5 is the second most highly expressed calpain in the CNS, after calpain 2. It appears postnatally during brain and spinal cord development. The protein levels of calpain1 and CAST also increase postnatally. Calpain 2 protein levels remain unchanged during development (Li et al., 2009). $CAPN2^{-/-}$ mice are embryonically lethal; however, $CAPN1^{-/-}$ mice are viable thus indicating that calpain 2 contributes prenatally and calpain1 postnatally to development. Calpain 5 mRNA levels were 10-fold higher than calpain1 mRNA levels in adult rat brain (Fig 1.8), suggesting that CAPN5 could be the major calpain in the adult brain. Calpain 5 is a non-cytosolic calpain, predominantly present in the nucleus, and enriched in synaptic mitochondria compared to non-synaptic mitochondria. In mitochondria, CAPN5 was present on the inner membrane and was released following outer membrane permeabilization. In the nucleus, CAPN5 was mainly detected in the nucleic acid binding protein fractions. Calpain 5 carries a nuclear localization signal (NLS) (aa 390-417), which encloses a classical bipartite NLS with a unique immediate upstream sequence. Calpain 5 colocalizes with PML bodies.

Classical and non-classical calpain protein structures in vertebrates have some similarity with the calpain domain architectures of lower organisms. Comparative genome and phylogenetic analyses of 34 unicellular eukaryotes have identified four ancient eukaryotic calpain domain architectures: CysPc, CysPc-C2L, MIT-CysPc-C2L and TML-CysPc-C2L (TML- transmembrane motif) (Zhao et al., 2012). Calpains in higher organisms may have evolved by shuffling ancient domains, adding novel domains, and/or modifying existing domains. Calpain 5 or Calpain 5-like protein is evolutionarily conserved across species such as mammal, amphibian, reptiles, fish and nematodes. Calpain5 is an ortholog of Tra-3, a cysteine protease in nematode. In nematodes, Tra-3 is important for the processing of Tra-2A for female development in hermaphrodites. (Hodgkin, 1986, Barnes and Hodgkin, 1996). Tra-3 has a sequence homology with the large subunits of rat calpain 1 and calpain 2 in the regions of domains N, PC1/PC2 and C2L. However, unlike calpains 1 and 2, Tra-3 lacks a PEF domain and instead has a non –EF hand domain T, with little homology at the C-terminus of PEF(L) domain of CAPNs 1 and 2. (Barnes and Hodgkin, 1996). A homologous protein found in vertebrates was named calpain 5 (Dear et al., 1997). Alignment of the predicted amino acid sequence of human and mouse CAPN5 with representative members of the vertebrate calpain family resulted in significant sequence homology over the entire coding region. Also, an unrooted phylogenetic tree generated from bootstrap analysis placed CAPN5 with calpain 6 in a common group divergent from the group consisting of calpains 1, 2, 3 and 8 (Dear et al., 1997). Genomic organization of mouse CAPN5 and calpain 6 identified them as a new calpain subfamily with 11 introns at identical locations, with 6 of them being in a similar location to those of the known vertebrate calpain members. (Matena et al., 1998).

Although CAPN5 and 6 have similar domain architecture (N, CysPc, C2L and C2 domains), CAPN6 carries a natural mutation in the catalytic domain, 'C' of PC1 is mutated to 'K' (K81 in human and mouse), making it a non proteolytic calpain (Matena et al., 1998). CAPN6 is predominantly present in the placenta, embryonic muscle, and cartilage. Loss of CAPN6 promotes embryonic skeletal muscle differentiation. CAPN6 is also expressed during muscle regeneration and suppresses regeneration (Tonami et al., 2013). On the other hand, CAPN5 is ubiquitous. CAPN5 was not detected at embryonic and early post natal developmental time points however it was detected in adult brain around 15 to 20 days postnatally. The function of CAPN5 is unknown, except that its ortholog Tra-3 is involved in sex determination and necrotic cell death (Syntichaki et al., 2002).

CAPN5 knockout mice present rather confusing information about the role of CAPN5 during development. CAPN5$^{-/-}$ progenies (*Capn5*$^{tm1Nde/\ tm1Nde}$) survive and are normal and healthy (Franz et al., 2004). However, a small portion these null mice were severely runted, and did not survive to adulthood. An IRES-LacZ-Neo cassette was inserted in the exon 4 of the CAPN5 alleles of *Capn5*$^{tm1Nde/\ tm1Nde}$ mice, which disrupted the catalytic domain. The difference in the CAPN5 protein levels in *Capn5*$^{tm1Nde/\ tm1Nde}$ and *Capn5*$^{+/+}$ has not been examined though. Another line of CAPN5 null mice (*Capn5*$^{tm1Dgen/tm1Dgen}$, MGI accession no.3604529) are available. These are embryonically lethal and die in utero before E3.5. However, the position of insertion of IRES-LacZ-Neo-555G in *Capn5*$^{tm1Dgen/tm1Dgen}$ mice is unknown.

The role of CAPN5 in the PML bodies remains to be investigated. A specific role of PML bodies is also unknown thus far. But, PML nuclear bodies are implicated in the cellular response to stress, viral defense, transcriptional regulation, apoptosis, and cell senescence (Borden, 2002, Bernardi and Pandolfi, 2007, Lallemand-Breitenbach and de

The, 2010, Dundr, 2012). For execution of these functions, SUMOylation, a reversible modification, is one key event for PML to recognize and recruit other proteins in the PML bodies. PML, a well-known SUMOylated protein together with partner proteins, e.g., DAXX and sp100 undergo SUMOylation (Duprez et al., 1999, Ishov et al., 1999, Zhong et al., 2000). CAPN5 contains the consensus SUMOylation motif (Ψ-K-x-D/E, hydrophobic residue- K, SUMOylation site- any residue- acidic residue), suggesting a possible mechanism of CAPN5 and PML partnering and the recruitment of the CAPN5 in the PML bodies. Several high and low probability SUMOylation sites (K residues) on CAPN5 are $K_{543}$, $K_{598}$, $K_{588}$, $K_{273}$, $K_{395}$, $K_{418}$, $K_{52}$, $K_{276}$, $K_{396}$ and $K_{312}$ (GPSsp 2.0: prediction of SUMOylation motif ). PML and partner proteins also interact through SUMO interaction motif (SIM). SIM consists of short stretch of hydrophobic residues, mainly 'V', 'L' or 'I' with flanking N or C –terminal serine and/or acidic residues. 'K' residue of SUMO interacts electrostatically with flanking acidic residues of SIM (Song et al., 2004, Song et al., 2005, Hecker et al., 2006). In addition to consensus SUMO motifs, CAPN5 also possesses two SIMs, aa 401-404 and aa 402-405 ($_{397}$PEDEVLICIQQ), flanked with acidic residues at N-terminal (GPS-SBM 1.0: prediction of SIM).

The role of CAPN5 in the PML body is mainly speculative at this point. Although CAPN5 could participate in many functions in which PML bodies participate in, the most appealing hypothesis seems to be the involvement of CAPN5 in the deSUMOylation or deconjugation of the SUMO modifications. SUMO protease enzymes such as yeast Ulp1 and Ulp2, and human SENPs are cysteine proteases, as is CAPN5 (Li and Hochstrasser, 1999, Mossessova and Lima, 2000, Bylebyl et al., 2003, Hickey et al., 2012). SUMO proteases belong to CE clan of the cysteine protease, and CAPN5 belongs to CA clan (http://merops.sanger.ac.uk). Proteases from CA and CE clan differ in the protein sequence, but have similar geometrical folds. In standard orientation, both

the proteases have upper helical fold and lower α/β folds. The relative positions of the 'C' and 'H' of the catalytic cleft provides a conserved catalytic geometry among CA and CE clan (Drag and Salvesen, 2008). SUMO proteases are involved in variety of functions. SENP5 is required for cell division. Knocking down SENP5 in HeLa cells causes growth inhibition, aberrant nuclear morphology and binucleate cells (Di Bacco et al., 2006). In absence of Ulp1 cell division is inhibited (Li and Hochstrasser, 1999). These findings suggest that deSUMOylation is required for the proper functioning of the cells. CAPN5 may be involved in deSUMOylation of the PML and/or the other partner proteins in the PML bodies, leading to dissolution of the bodies or removal of a particular partner protein from the PML bodies after the function is executed.

SUMO protease activity of CAPN5 may not be limited only to the nucleus. DRP1 (dynamin related protein-1), a substrate of SUMO protease is present in the mitochondria and involved in mitochondrial fission. During cell division, SENP5 translocates from the nucleus to the mitochondrial membrane, where it reverses the SUMO modification of DRP1 and leads to formation of an assembly of DRP1 polymers that promote mitochondrial fission (Zunino et al., 2007, Zunino et al., 2009). CAPN5 could also perform deSUMOylation roles in the mitochondria, one of which could be regulation of $Ca^{2+}$ release at the ER-mitochondria interface. At mitochondrial associated membrane (MAM) of ER, PML complexes with IP3, protein kinase Akt and protein phosphatase (PP2a). PML may be important for binding of PP2a to IP3R, which favors dephosphorylation of IP3 over phosphorylation by AKT; hence leading to $Ca^{2+}$ release in the mitochondria and induction of apoptosis. PML induces apoptosis in response to various stimuli such FAS, DNA damage and TNFα. Knocking down PML results in deregulation of $[Ca^{2+}]$ both in the ER lumen and mitochondria, i.e. higher in ER lumen and lower in mitochondria. $PML^{-/-}$ mice and mouse embryonic fibroblasts are resistant to

cell death (Wang et al., 1998b, Giorgi et al., 2010, Pinton et al., 2011). SUMOylation could possibly be a way through which PML interacts with IP3R, AKT and PP2a to form a complex. GPSsp2.0 predicted SUMOylation motifs at high precision threshold for all the three proteins, IP3R (Uniprot # Q14573; 7, SUMOylation motifs), PP2a (Q15173; 2) and AKT1 (B7Z5R1; 4). In response to a legitimate signal, CAPN5 may travel from inside mitochondria to the PML-IP3-AKT-PP2a complex at MAM to deSUMOYlate the assembly to regulate $Ca^{2+}$ signaling.

Calpain 5 could also shuttle between nucleus and mitochondria, where it could perform a SUMO specific and/or a general protease activity. In the nucleus, other than deSUMOylation, CAPN5 could be involved in rendering defense mechanisms, progression of mitosis and cleavage of transcription factors such as c-Fos, c-Jun, and p53, similar to other calpains. During cell division, calpain 2 is suggested to relocate to the nucleus, associate with the chromosomes, promote precocious disassembly of the mitotic spindle and progression of mitosis (Schollmeyer, 1988). Purified calpain 2 along with calpain 1 proteolyse nuclear proteins and release integrated H1 kinase, which otherwise was abolished in presence of calpastatin (Mellgren, 1991). Calpain 2 and 10 in the nucleus have been associated with selenite cataract formation (Hightower et al., 1987, Ma et al., 2001). Also, an Arabidopsis cysteine protease, RD19, translocates to the nucleus and complexes with pathogenic PopP2 effector to activate RRS1-R-mediated resistance response against bacterial wilt (Bernoux et al., 2008). In the mitochondria, other than SUMO specific proteolysis, CAPN5 could possibly be involved in cleaving VDAC, NCX, AIF or apoptotic-related proteins in mitochondria (as discussed in chapter 4),

Levels of CAPN5 increase postnatally and its presence ~15 – 20 days post birth may reflect the CAPN5 localization in synaptic mitochondria and PML bodies. PML bodies

are involved in neuron differentiation. In the CNS, PML expression is biphasic—initially being expressed in immature neural progenitor cells, downregulated during differentiation, then re-expressed in mature neurons (Yu et al., 2003, Salomoni and Betts-Henderson, 2011). Rat brain undergoes massive differentiation and synaptic formation at P15-P20 (Eayrs and Goodhead, 1959, Aghajanian and Bloom, 1967). Recruitment of CAPN5 into PML bodies could regulate changes in gene expression or nuclear events for differentiation. CAPN5 localization in the synaptic mitochondria could augment the processes required at the synaptic terminal to form new synaptic connections. One of those could be the release of neurotransmitters. Calpain 10 enhances insulin exocytosis by partially cleaving SNAP-25 of SNARE complex (Marshall et al., 2005, Evans and Turner, 2007). To perform the exocytosis of neurotransmitter, mitochondrial CAPN5 may be needed compared to calpains 1, 2 and 10, which are present in the cytosol, and possibly could play a role in exocytosis. Making new synaptic connections, more importantly 'correct' synaptic connections, is a highly regulated process. Hence, exocytosis of neurotransmitters would add another level of regulation if the mitochondrial membrane has to be permeabilized in a highly regulated manner for release of CAPN5.

A unique CAPN5 NLS and a similar subnuclear localization of full length CAPN5 as well as the bipartite NLS (395-417, KKPEDEVLICIQQRPKRSTRREG) need to be addressed. This sequence by itself does not contain any NLS activity but together with sequence YIFEV (390-394), YIFEVKKPEDEVLICIQQRP KRSTRREG (388-417) constitutes a novel putative NLS. Nonconventional NLSs have been reported, such as hydrophobic NLS of adenovirus E1A transcription factor lacking charged residues (Standiford and Richter, 1992), NLS of 38 amino acids rich in glycine and aromatic residues (termed M9) in human ribonucleoprotein A1 (Pollard et al., 1996) and leucine zippers in human

cytomegaloma virus UL84 (Lischka et al., 2003). While M9 import to the nucleus is mediated by transportin, UL84 utilizes interaction between leucine zipper (not the basic residues) and importin for nuclear import through importin α/β pathway. Furthermore, the basic clusters of UL84 do not carry NLS activity but are important for nuclear localization. Similarly, we found a nonconventional NLS in CAPN5 that possesses a classic bipartite NLS and a unique upstream sequence YIFEV. Neither the bipartite NLS nor YIFEV have any independent NLS activity. The mechanism of CAPN5 import to the nucleus remains to be investigated.

A similar subnuclear punctate localization of CAPN5 full length protein and CAPN5 NLS peptide is very interesting. In other words, NLS does not only enter the nucleus, but carries the information for specific subnuclear localization. A thorough survey and in silico analyses show that 20% of NLS motifs co-localizes with the DNA binding region of the proteins (Cokol et al., 2000). Also, for 67% – 90% of the DNA binding proteins, the DNA binding region overlaps or is proximal to the NLS (LaCasse and Lefebvre, 1995, Cokol et al., 2000, Nair et al., 2003). NLS of c-Fos and c-Jun overlaps with the DNA binding domains with the same stretch of residues carrying es both the NLS and DNA binding motifs . Examples are human PARP and androgen receptors and RNA binding domain of Human U1-70K, a U1 snRNA specific binding protein (LaCasse and Lefebvre, 1995). CAPN5 NLS could possibly also carry nucleic acid binding (NAB) motif. We showed that CAPN5 was enriched in NAB fraction. PML body-localized CAPN5 could directly bind to nucleic acid to regulate splicing and transcription since PML bodies complex with nascent RNA and CREB binding proteins (LaMorte et al., 1998, Wang et al., 1998a). The linkage between NLS and NAB motif may be necessary for efficient functioning. Continued occupancy of a NAB protein will require both import and nucleic acid binding. Basic residues of NLS may also enhance binding to the nucleic acid.

In conclusion this study elucidates that calpain 5 is the major calpain in the CNS, after calpain 2. Unlike typical calpains, it is not present in the cytosol but is mainly present in the nucleus and enriched in the synaptic mitochondria. In mitochondria, CAPN5 is present on the inner membrane, and is released when the outer membrane is permeabilized. Calpain 5 carries a unique NLS (YIFEVKKPEDEVLICIQQRPKRSTRREG, 390-417), which comprises a classical bipartite NLS and an immediate unique upstream sequence. Calpain 5 is enriched in the nucleic acid binding protein and I speculate that CAPN5's putative NLS also carries a nucleic acid bind domain. In the nucleus, CAPN5 is localized to the PML bodies and based on the role of the PML bodies, CAPN5 may be involved in transcriptional regulation, cell differentiation, cellular response to stress, viral defense, apoptosis, cell senescence, as well as protein sequestration, modification, and degradation.

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**Abstract publications**:

**1. Singh, R.**, Mashburn, C., Lou, D., Ghoshal, S., Graham, B., and Geddes, J. 2013**.** *Calpain 5 is highly expressed in the CNS, carries a unique NLS and localized to the nuclear promyelocytic leukemia protein.* (Abstracts submitted). To be presented at:

43rd annual meeting of SfN Nov 2013, San Diego, CA.

**2. Singh, R.**, Lou, D., Bondada, V., Ghoshal, S., Benson, A., Saatman, K., and Geddes, J. 2012. *Calpain5 is localized to neuronal mitochondria and nuclei, and is involved in caspase dependent cell death pathway. Journal of Neurotrauma* 29/10: A65-66. This was also presented at:

BGSFN symposium, March 2012, Lexington, KY
UL-SfN symposium, Apr 2012, Louisville, KY

**3**. Crowdus, C., Chen-Guang, Y., **Singh, R.**, Power, R., Geddes, J. 2012. *Enhancing endogenous protective mechanisms following spinal cord injury*. *Journal of Neurotrauma* 29/10: A82-83. Also presented at:

Physical Medicine and Rehabilitation Annual Resident Research Day, Cardinal Hill Rehabilitation Hospital, UK, KY, May 2012
NNS symposium, July 2012, Phoenix, AZ

**4. Singh, R.**, Benson, A., Saatman, K., and Geddes, J. 2011. *Calpain5 is expressed in neurons and may contribute to neurodegeneration following Traumatic Brain Injury*. *Journal of Neurotrauma* 28/6: A86. This was also presented at:

BGSFN symposium, Mar 2011, Lexington, KY
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**5**. Yu, C.G., **Singh, R[$].**, Li, Y., Raza, K., and Geddes J.W. 2010. *Calpain1 knockdown improves tissue sparing and locomotor function following spinal cord contusive injury*. *Annals of Neurosciences* 17/ supplement: 73.

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**1. Singh, R.,** Mashburn, C., Lou, D., Graham, B., and Geddes, J. 2013. *Calpain 5 is highly expressed in the CNS, carries a unique nuclear localization signal and is associated with promyelocytic leukemia protein.* Resubmitted after revision

**2. Singh, R.**, Bondada, V., Mashburn, C and Geddes, J. 2013. *Calpain 5 is enriched in synaptic mitochondria, localizes to the inner mitochondrial membrane and releases on outer membrane permeabilization.* Drafted.

**3.** Yu, C. G., Crowdus, C., Raza, K., **Singh, R.,** and Geddes J. 2013**.** *Fenbendazole improves pathological and functional recovery following traumatic spinal cord injury.* Resubmitted after revision

**4**. Dhillon, N. P. S., [#]**Ranjana, Ranjana**., Singh, K., Eduardo, I., Monforte, A. J., Pitrat, M., Dhillon, N. K. and Singh, P. P. 2007. *Diversity among landraces of Indian snapmelon (Cucumis melo var. momordica)*. *Genet Resour Crop Evol* 54: 1267-1283. SpringerLink
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**5.** Dhillon, N.P.S., **Ranjana, Ranjana**., Singh, K., Eduardo, I., Monforte, A.J., Dhillon, N.K. and Singh, P.P. 2007. *Horticultural and molecular characterization of landraces of Indian snapmelon* (*Cucumis melo* var. *momordica*). *Acta Hort* 752: 401-404. ISHS