INVESTIGATION OF THE CELLULAR MECHANISIMS UNDERLYING THE CARBOXYPEPTIDASE E MUTATION

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ABSTRACT

INVESTIGATION OF THE CELLULAR MECHANISIMS UNDERLYING THE CARBOXYPEPTIDASE E MUTATION

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Carboxypeptidase E (CPE) is an enzyme expressed in both endocrine and neuroendocrine cells functioning as both an exopeptidase and a sorting receptor. Recently, it has been reported that CPE plays a role in preventing neuronal cell death in the CA3 hippocampus so as to maintain normal cognitive function in the adult brain. Studies on CPE-knockout mice showed total degeneration of neurons in the CA3 region of the hippocampus in adult mice 4 weeks of age and older. Additionally, increased CPE expression was involved in protecting hippocampal neurons from oxidative stress-induced apoptosis. The neuroprotective role of CPE prompted us to search for possible mutations in the human CPE gene that might be linked to human neurodegenerative diseases. A non-redundant nucleotide sequence database search with the human CPE nucleotide sequence as queries identified an EST sequence entry from Alzheimer cortex tissue that had three adenosine inserts. This introduces 9 amino acids in the first beta-pleated sheet after the pro-domain of the mutant CPE protein, herein called QQ CPE due to the presence of two glutamine residues in the new sequence. The studies demonstrate that mutant CPE cannot

perform its enzymatic functions and at the same time it interferes with the wild type CPE activity. Expression studies in neuroblastoma cells (N2A) demonstrated that QQ CPE was made but failed to be secreted and instead degraded in the both proteosomes and lysosomes. The percent rescue of QQ CPE was higher in proteosomes compared to lysosomes, hence, QQ CPE was primarily degraded in proteosomes. Co-expression of WT and QQ CPE in N2A cell showed additive effects of degradation both in proteosomes and lysosomes.

Key words: Carboxypeptidase E, QQ CPE, Neurodegeneration

ÖZ

KARBOKSİPEPTİDAZ E MUTASYONUNUN HÜCRESEL MEKANİZMALARININ İNCELENMESİ

Kaşıkçı, Feride
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Karboksipeptidaz E (CPE) endokrin ve nöroendokrin hücrelerde ifade edilen hem ekzopeptidaz hem de hücre içi reseptör olarak görev yapan bir enzimdir. Yakın zamanda yapılan bir araştırmaya göre; CPE'nin yetişkin fare beyinlerinde hipokampüsün CA3 nöronlarında ölümü engellediği ve böylece kognitif fonksiyonlara normal bir şekilde devam edildiği gösterilmiştir. CPE nakavt fare modelinde 4 hafta ve üzeri yetişkinlerde hipokampüsün CA3 nöronlarında total yozlaşma gözlemlenmiştir. Ayrıca, CPE ifadesindeki artış hipokampal nöronları oksidatif strese bağlı apoptozdan korumaktadır. CPE'nin nöroprotektif rolü bizi insan CPE geninde nörodejenerasyona bağlı olası bir mutasyon aramaya yöneltti. Yinelenmeyen nükleotit dizini veritabanıyla insan CPE nükleotit dizini araştırılarak Alzheimer hastasının korteks dokusundan elde edilen ve 3 adenozin eklenmesiyle oluşmuş bir EST dizini bulunmuştur. Bu eklenme mutant CPE'nin pro-domain sonrasındaki ilk beta katmanına 9 amino asit eklenmesine sebep olmuş ve eklenen gruplardan 2'si glutamin olduğu için yeni dizin QQ CPE olarak adlandırılmıştır. Çalışmalarımıza göre, mutant CPE enzimatik görevlerini yerine getirememekte ve

aynı zamanda WT CPE aktivitelerine engel olmaktadır. Nöroblastoma hücrelerindeki (N2A) ifade çalışmalarımız QQ CPE'nin, üretildiği ama salgılanamadığı ve proteozom ve lizozomlarda yıkıldığını ispatlamıştır. QQ CPE'nin kurtarılma yüzde oranları lizozomlara kıyasla proteozomlarda daha yüksek olduğu için QQ öncelikle proteozomlarda yıkıma uğramıştır. N2A hücrelerinde QQ ve WT CPE birlikte ifade edildiğinde proteozom ve lizozomlara bağlı hücresel yıkım mekanizmalarını arttırdığı da görülmüştür.

Key words: Karboksipeptidaz E, QQ CPE, Nörodejenerasyon

To My Hopes,

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LIST OF ABBREVIATIONS

3D	Three Dimensional
ACTH	Adrenocorticotropin Hormone
AD	Alzheimer's Disease
ATF6	Activating Transcription Factor 6
BDNF	Brain Derived Neurotrophic Factor
BiP	Binding Immunoglobulin Protein
BSA	Bovine Serum Albumin
CART	Cocaine and Amphetamine Regulated Transcript
COPI	Coat Protein Complex I
COPII	Coat Protein Complex II
CP	Core Particle
CPA	Carboxypeptidase A
CPB	Carboxypeptidase B
CPD	Carboxypeptidase D
CPE	Carboxypeptidase E
CPM	Carboxypeptidase M
CPN1	Carboxypeptidase N1
CPO	Carboxypeptidase O
CSP	Constitutive Secretory Pathway
DMEM	Dulbecco's Modified Eagle Medium
ERAD	ER Associated Degradation
ER	Endoplasmic Reticulum
EV	Empty Vector
FBS	Fatal Bovine Serum
GA	Golgi Apparatus
GGA	Golgi-Localized, γ-Ear-Containing, ADP-Ribosylation Factor-Binding Proteins
IRE1α	Inositol-Requiring Protein-1α

ISG	Immature Secretory Granule
KO	Knock Out
LB	Luria Bertani
LSD	Lysosomal Storage Disorders
M6P	Mannose-6-Phosphate
ManI	Mannosidase I
Man II	Mannosidase II
MEF	Mouse Embryonic Fibroblast
MG101	Calpain Inhibitor I
MG132	Z-Leu-Leu-Ala-CHO
MPR	Mannose-6-Phosphate Receptor
N2A, Neuro2A	Neuroblastoma Cell Line
ODC	Omithine Decarboxylase
PBS	Phosphate Buffered Saline
PC	Prehormone Convertases
PERK	Protein Kinase RNA (PKR)-Like ER Kinase
POMC	Proopiomelanocortin
PSS	Prohormone Sorting Signal
RER	Rough Endoplasmic Reticulum
RP	Regulatory Particle
RSP	Regulated Secretory Pathway
RT	Room Temperature
SEM	Standard Error of Mean
SER	Smooth Endoplasmic Reticulum
SRP	Signal Recognition Particle
STD	Standard Deviation
SV	Synaptic Vesicles
T2DM	Type II Diabetes Mellitus
TAE	Tris-Acetate-EDTA
TBS	Tris Buffered Saline
TBS-T	Tris Buffered Saline-Tween-20

TE	Tris-EDTA
tER	Transitional Endoplasmic Reticulum
TGN	*Trans*-Golgi Network
TM	Transmembrane
TS	Thymidine Synthase
UPR	Unfolded Protein Response
WT	Wild Type
ZLLF	Z-Leu-Leu-Phe-CHO

CHAPTER 1

INTRODUCTION

1.1. Protein Sorting

The process by which the newly synthesized proteins are delivered to their correct destination is known as the protein sorting. It is a crucial process for not only communication between cells but also integrity and maintenance of the tissues (von Zastrow *et al.*, 1993; Kelly *et al.*, 1983; Green *et al.*, 1984) because most of the proteins are either signaling molecules or matrix proteins. Therefore the abnormalities in the protein sorting cause various types of diseases like neurological diseases (Chen *et al.*, 2001), diabetes (Harding *et al.*, 2001), cancer (Welsh *et al.*, 2003) or autoimmune diseases (Antonelli *et al.*, 2011).

In eukaryotic systems the protein sorting network involves mainly two pathways that are the secretory pathway (Bonnemaison *et al.*, 2013) and the non-secretory pathway (Tang *et al.*, 2014). The proteins targeted to the rough endoplasmic reticulum (RER) follow the secretory pathway and the remaining proteins follow the non-secretory pathway. The ribosomes carrying the translated proteins with an ER specific signal sequence are targeted to the RER and the rest of the translation completed there (Bonnemaison *et al.*, 2013). Other proteins are translated on the ribosomes that are free in the cytosol and targeted to the different types of organelles like mitochondria (Aich and Shaha, 2013), chloroplasts (Khan *et al.*, 2013), peroxisomes (Hasan *et al.*, 2013), and the nucleus (Sekimoto and Yoneda, 2012), depending on the specific sequences that they carry on. The proteins that lack any specific sequence are released to the cytosol. In the secretory pathway, the organelles; ER and the Golgi apparatus (GA) play the major role where proteins that need to be secreted are transported from ER within the budding vesicles that dock and fuse with the GA. Then proteins move along the GA and they are secreted out of the cell within the vesicles (Kienzele and von Blume, 2014).

1.1.1. The Role of Endoplasmic Reticulum in Protein Sorting

Endoplasmic reticulum (ER) is a member of the membrane network which extends from the nuclear membrane (Cooper, 2000). It can be classified with respect to its structure and functions (Fig.1). Structurally it has two distinct divisions that are nuclear and peripheral ER. The nuclear ER also known as nuclear envelope (Shibata *et al.*, 2010) surrounds the nucleus and the peripheral ER is a network of tubules (Terasaki and Jaffe, 1991) and cisternea (membrane sheets) that spread throughout cytoplasm (English *et al.*, 2009). The peripheral ER maintains the interaction between other organelles in the cell like GA, lysosomes, mitochondria, peroxisomes, vacuoles and late endosomes (Voeltz *et al.*, 2002). Functionally there are two types of ER that are smooth endoplasmic reticulum (SER) and RER (Baumann and Walz, 2001). The SER is devoid of membrane-bound ribosomes and responsible for phospholipid and steroid synthesis (Mc Master, 2001) and their delivery to the other organelles. It also functions in the detoxification and Ca^{++} metabolisms (Shibata *et al.*, 2006; Csordas *et al.*, 2006). The RER on the other hand has membrane-bound ribosomes and this way contributes to the protein sorting. It also involves in proper folding of proteins and their modifications. The protein compositions of RER and SER are also different. The proteins related to the translocation are all present in RER but not in SER (Vogel *et al.*, 1990; Voeltz *et al.*, 2002).

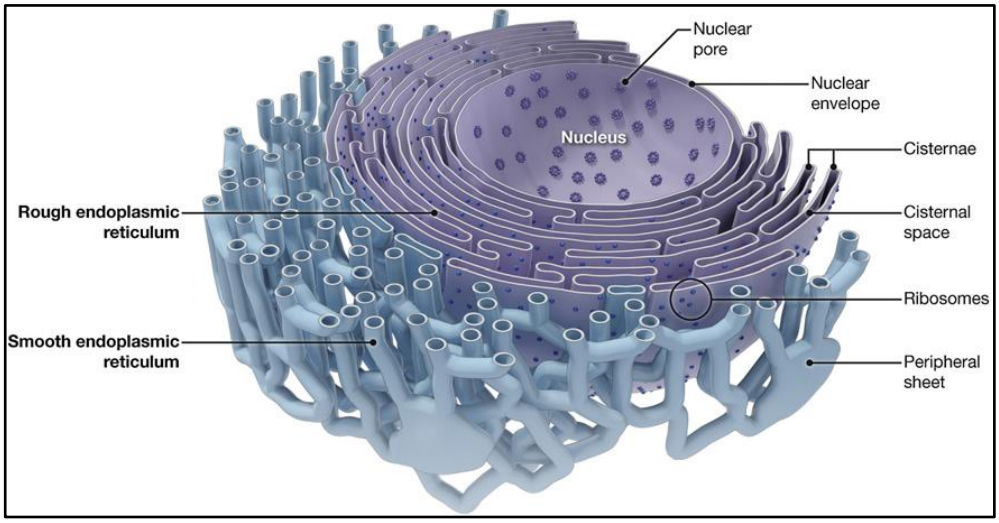

Figure 1. The ER network in the animal cells (Goyal and Blackstone, 2013).

The one-third of the all synthesized proteins is targeted to the secretory pathway (Kanapin *et al.*, 2003) carrying the ER specific N-terminal signal sequence (Walter and Johnson, 1994). This sequence is recognized by the signal recognition particle (SRP) and directed to the ER together with the ribosome (van Viliet, 2003) and bind to the cytosolic site of it (Matlack *et al.*, 1998). Then, the nascent protein is cotranslationally translocated to the ER lumen as the N-terminal signal sequence cleaved (Cross *et al.*, 2009; Zimmermann *et al.*, 2011). Within the ER lumen, with the assistance of different chaperones and enzymes, proteins reach their correct conformational structure (Lord *et al.*, 2000) and undergo some of post-translational modifications (Aebi, 2013). Proteins which fail to be modified are exported out through the transitional ER (tER) where the transportation vesicles formed (Glick, 2014).

The unfolded or misfolded proteins may also leave the ER within the vesicles (Fig.2); however, they are retrieved from the following station that is GA (D'Arcangelo *et al.*, 2013 and Gillion *et al.*, 2012). These proteins together with the ones that reside in the ER degraded with the ER-associated degradation (ERAD) system (Yoshida, 2003). This system requires the retrograde transport of the proteins from ER to the cytosol so that they can be degraded in the 26S proteasome through ubiquitination (Vembar and Brodsky, 2008). The activation of the ERAD system triggered by the unfolded protein response (UPR) modulators that are inositol-requiring protein-1α (IRE1α), activating transcription factor 6 (ATF6) and protein kinase RNA (PKR)-like ER kinase (PERK) through the binding immunoglobulin protein (BiP), a chaperone (Gardner and Walter, 2011). If the UPR mediated ERAD system fails to prevent the stress caused by the unfolded/misfolded proteins in the ER then apoptotic pathways get activated (Sano and Reed, 2013). The accumulation of the ER stress involves in many neurodegenerative diseases like Alzheimer's, Parkinson's or Huntington's diseases (Wang and Kaufman, 2012).

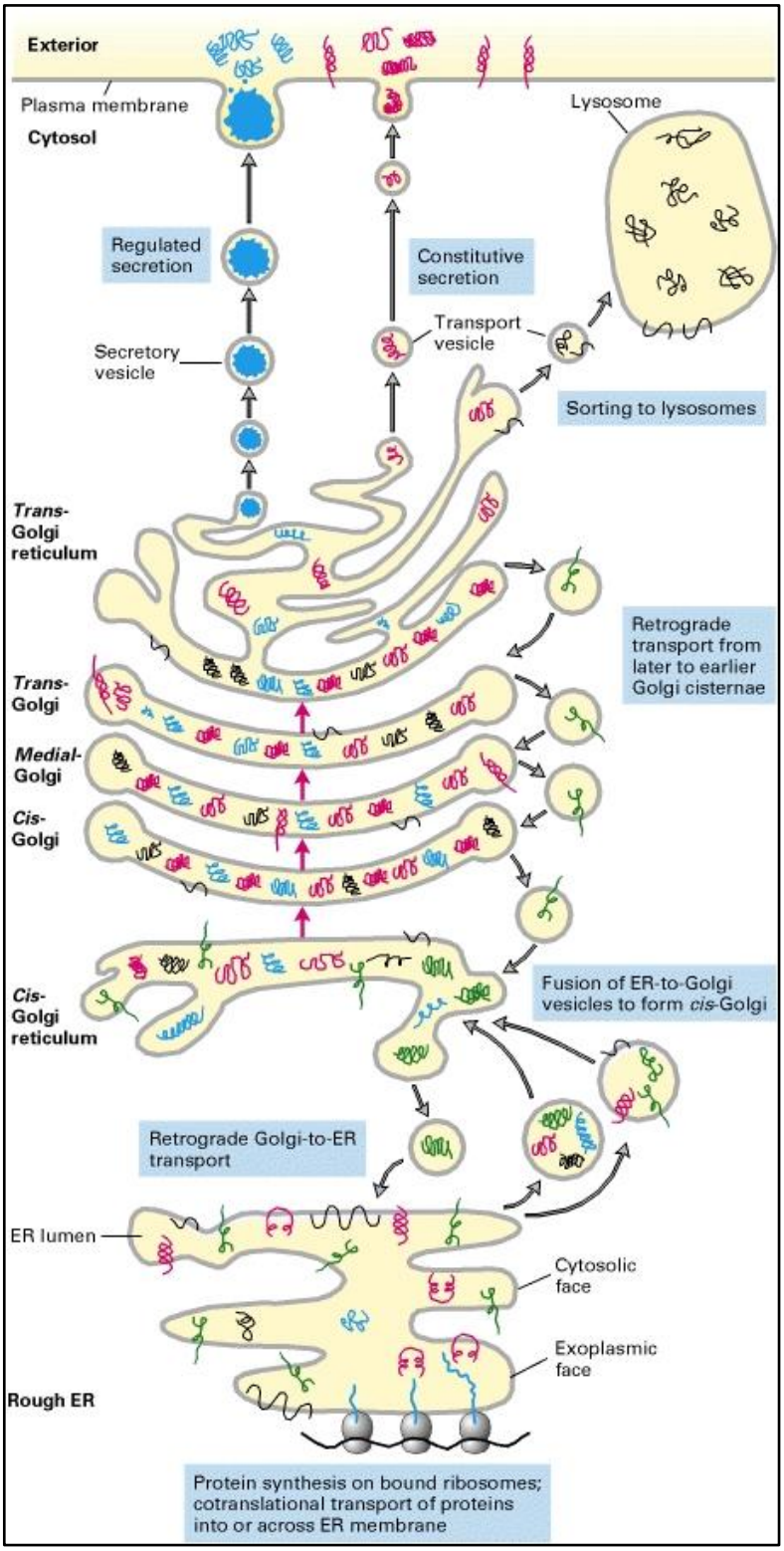

Figure 2. Endoplasmic reticulum and golgi in secretory pathway (Lodish *et al.*, 2000).

1.1.2. The Role of Golgi Apparatus in Protein Sorting

The GA was first discovered by the Camillo Golgi and this discovery confirmed decades after with the electron microscopy studies (Berger, 1988). Its structure composed of the flattened cisternal membranes (Short *et al.*, 2005) that shows both structurally and functionally distinct compartmentalization and divided into three parts as: *cis*-, *medial*- and *trans*-Golgi (Fig.2). Each compartment has different composition of enzymes that contributes to the glycosylation process that starts in the ER (Cylwik *et al.*, 2013). The *cis*-Golgi contains Mannosidase I (ManI) enzyme that functions in trimming the mannoses that is essential for the targeting the misfolded proteins to the ERAD system (Groisman *et al.*, 2011). It also contains phosphotransferases that tag the proteins with mannose-6-phosphate (M6P) that will target them to the lysosomes (Sun *et al.*, 2008). Apart from that the *cis*-Golgi contains the KDEL receptors that help ER to retrieve its chaperones (Cancino and Luini, 2013). The *medial*-Golgi with the Mannosidase II (Man II) further modifies the proteins (Cheong, 2010). The *trans*-Golgi compartment has different processing functions like tyrosine sulphation, proteolytic processing or sialylation (van Vliet *et al.*, 2003) and it also regulates the vesicular transport from the GA to the outside of the cell (Banfield, 2011).

1.1.3. Vesicular Transport From Golgi Apparatus

The proteins reached to the *trans*-Golgi transported to the different locations within the vesicles from the *trans*-Golgi reticulum or *trans*-Golgi network (TGN) (Fig.2). The vesicles are specialized depending on their destinations like lysosomes, cell surfaces, early/late endosomes, secretory storage granules, Golgi stacks or the ER (Kienzele and von Blume, 2014). Transport of the M6P receptors mediated by the Golgi-localized, γ-ear-containing, ADP-ribosylation factor-binding proteins (GGA) to the lysosomes in the clathrin coated vesicles via endosomes (Ghosh *et al.*, 2003). Early and late endosomes that function in the receptor recycling, receptor targeting to the lysosomes or endosomes- endosomes interaction mediated through

the small GTPases (Pfeffer, 2003). The retrograde transport between Golgi stacks and the ER are regulated by the COPI (coat protein complex I) and COPII (coat protein complex II) proteins (Barlowe and Miller, 2013). The release of the secretory storage granules are regulated with the pH and Ca^{++} concentration (Kienzele and von Blume, 2014) and their transport mediated through the microtubules (Park and Loh, 2008). At the low pH and in the presence of the Ca^{++} ions, the proteins, destined to be secreted, aggregate and condense and then form the immature granules. These immature granules together with proteins mature while they transported to the cell surface (Kögel *et al.*, 2013).

There are two main pathways for protein secretion to the cell surface through the secretory granules: constitutive (CSP) and regulated secretory pathway (RSP) (Fig.2). The secretion with the CSP is continuous and independent of the external stimulus. The proteins in vertebrates that follow this pathway are the serum and extracellular matrix proteins (Table 1). The secretion with the RSP is on the other hand dependent on the stimulus. The vesicle size is larger compared to the ones in the CSP because secretion is not continuous hence proteins released in high concentrations in a condensed form. Therefore these vesicles also called as dense core vesicles (Brion *et al.*, 1992). The proteins in vertebrates that follow this pathway are the peptide hormones, digestive enzymes and milk proteins (Table 1). The CSP is found in all cells however, the RSP is unique for neuroendocrine and endo/exocrine cells that functions in the generation of neuropeptides and peptide hormones (Thonen, 2000 and Egan *et al.*, 2003).

Table 1. Classes of secretory proteins in vertebrates (Lodish *et al.*, 2000).

Protein Type	Example	Site of Synthesis
Constitutive Secretory Proteins		
Serum proteins	Albumin	Liver (hepatocyte)
	Transferrin (Fe transporter)	Liver
	Lipoproteins	Liver, intestine
	Immunoglobulins	Lymphocytes
Extracellular matrix proteins	Collagen	Fibroblasts, others
	Fibronectin	Fibroblasts, liver
	Proteoglycans	Fibroblasts, others
Regulated Secretory Proteins		
Peptide hormones	Insulin	Pancreatic β-islet cells
	Glucagon	Pancreatic α-islet cells
	Endorphins	Neurosecretory cells
	Enkephalins	Neurosecretory cells
	ACTH	Anterior pituitary lobe
Digestive enzymes	Trypsin	Pancreatic acini
	Chymotrypsin	Pancreatic acini
	Amylase	Pancreatic acini, salivary glands
	Ribonuclease	Pancreatic acini
	Deoxyribonuclease	Pancreatic acini
Milk proteins	Casein	Mammary gland
	Lactalbumin	Mammary gland

The proteins in the immature granules are precursor proteins in the inactive state and they undergo modifications to become biologically active. The maturation

of the proteins is regulated by the processing enzymes that are mainly: prohormone convertases (PC) 1/3 and 2, carboxypeptidase E (CPE) (Steiner, 1998). PC cleaves the propeptides and produce C-terminal lysine and/or arginine extended intermediates. Then, these flanking dibasic aminoacids cleaved by CPE (Beinfeld, 2003). Further modifications may contribute to peptide hormone bioactivation such as amidation or acetylation (Takahashi and Mizusawa, 2013).

1.2. Carboxypeptidase E

Proteases are divided into two groups depending on the site of cleavage; endopeptidases that cleave within the molecule and exopeptidases that cleaves the end of the molecule. Carboxypeptidases are proteases cleaving the end proteins at the C-terminal. They function in catabolic reactions (Turk, 1999), protein maturation and other processes (Lou *et al.*, 2005). They grouped as metallo-, serine- and cysteine-carboxypeptidases based on their active site groups. Carboxypeptidase E (CPE), discovered in 1982 and named as enkephalin convertase (Fricker and Synder, 1982; Fricker, 1988), is an exopeptidase that functions in protein maturation by cleaving basic (Lysine, Arginine) aminoacids at the C terminal and also acts as a sorting signal in the RSP (Cool *et al.*, 1995) with many other functions (Cawley *et al.*, 2012). The activity of CPE requires Zinc (Zn^{++}) or Cobalt (Co^{++}) therefore it's a metallo-carboxypeptidase (Turk, 1999). The zinc-binding domain of metallo-carboxypeptidases is highly conserved (Rawlings and Barret, 1995). CPE differs from other carboxypeptidases with its acidic range of activity and localization (Manser *et al.*, 1990). Sequence comparison of CPE with other carboxypeptidases shows that it has sequence coverage of 55% with CPA, 37% with CPB, 32% with CPO, 82% with CPM, 86% with both CPN1 and CPD (Rawlings and Barret, 1995; Osterman *et al.*, 1992).

1.2.1. Synthesis, Structure and Distribution of CPE

CPE synthesized as a precursor protein that consists of 476 amino acids (Fig.3A). The 25 amino acids from the N terminal is the signal peptide that directs

proCPE to the RER. In the secretory vesicles, proCPE is further processed and 17 amino acid pro region is cleaved (Song and Fricker, 1995). Since proCPE is already enzymatically active, cleavage of the pro-region is not necessary for enzymatic activity or intracellular trafficking (Song and Fricker, 1997). The last 25 amino acids of CPE form the transmembrane (TM) region. CPE has two forms that are soluble and membrane associated one (Fricker, 1988). The soluble form of CPE aggregates at acidic conditions with the increase of Ca^{++} ion concentration (Song and Fricker, 1995) together with the cargo proteins which is important for protein sorting (Rindler, 1998). The three dimensional (3D) structure of membrane associated CPE (Fig.3B) modeled from the crystal structure of CPD due to high homology. On the 3D model enzymatically active site (Zinc binding site), prohormone sorting signal (PSS) binding site, α-helical transmembrane domain and cytoplasmic tail are shown. The C terminal cytoplasmic tail interacts with microtubules and involves in vesicle transport (Mitra *et al.*, 1994). The TM domain is amphipathic and required for CPE association with lipid rafts of TGN and granule membranes of RSP (Zhang *et al.*, 2003). It also mediates the CPE retrieval (Fricker *et al.*, 1990). In the granules soluble form of CPE generated with the cleavage of TM domain (Fricker and Devi, 1993). The PSS domain is unique to CPE and contains two basic aminoacids Arg_{255} and Lys_{260}. These residues are required for CPE to interact with acidic residues of prohormones and neuropeptides like proopiomelanocortin (POMC) (Cool *et al.*, 1995), proinsulin (Dhanvantari *et al.*, 2003) and brain derived neurotrophic factor (BDNF) (Lou, 2005). The enzymatically active site is responsible for zinc binding where zinc acts as a cofactor for CPE activity (Fricker, 2004).

CPE is localized at various tissues but the most distinct ones are neuropeptide rich regions of brain and endocrine tissues like hypothalamus (Lynch *et al.*, 1984), pituitary (Strittmatter, 1985), hippocampus and bovine adrenal medulla (Lynch, 1986). It is also present in Langerhans islets of pancreas, heart (Lynch *et al.*, 1988) and gut tissues and epithelial cells of stomach, colon and oviduct (Lynch, 1987). During development CPE occurs before the PCs at embryonic day (E) 10 at the

spinal cord and diencephalon and at E12 and E13 its expression increases at the developing nervous system (Zhang *et al.*, 1994).

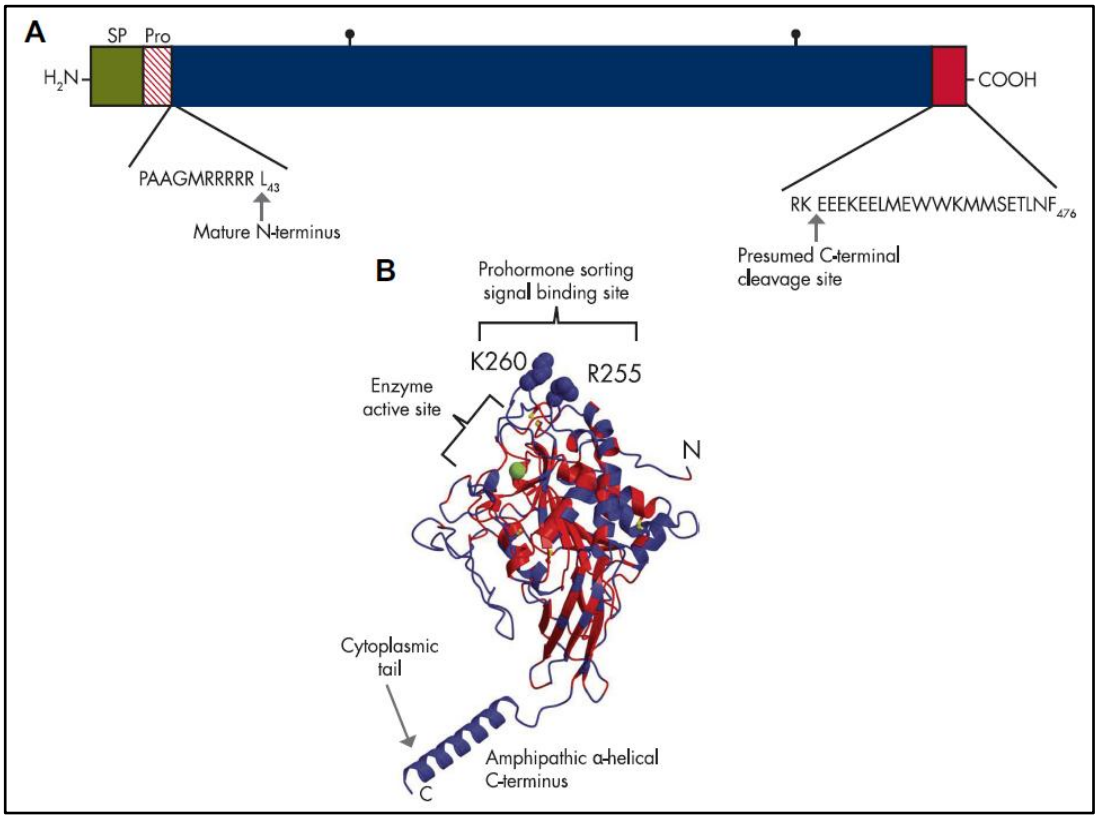

Figure 3. Structure of CPE. Schematic representation (A) shows the processing sites and 3D model (B) indicates the functional regions of CPE (Cawley *et al.*, 2012).

1.2.2. Functions of CPE

The peptides destined for delivery undergoes different modifications before secretion. When they reach to the TGN they are selectively packaged within the

vesicles and transported to the membrane through RSP. From TGN to their secretion site, CPE has mainly three known functions where they function as a prohormone sorting receptor, processing enzyme and in hypothalamus mediator for synaptic vesicle retention. At slightly acidic pH (5.8) and increased Ca^{++} concentrations peptide-CPE interaction occur and then they aggregate and condense together (Rindler, 1998). In addition CPE forms the amphipathic α-helix at acidic pH which generates the TM domain associating with the lipid rafts of TGN and then immature secretory granule (ISG) formed (Fig.4). After leaving the TGN both ISG and peptides maturation co-occurs. ISG mature into dense core vesicles when its content keeps condensing. Peptides on the other hand processes by PCs and CPE which is the second function. CPE as well processed in the vesicles and TM domain cleaved to increase the enzymatically more active soluble CPE number (Hook, 1985). As a processing enzyme CPE cleaves the Arg, Lys and poorly His residues at the C terminal (Fricker and Synder, 1982) which leads to activation of the peptide. The third function of CPE is to localize the synaptic vesicles (SV). There are three types of vesicles groups that are reserve pool (80% of whole vesicles), slow response pool (10% of whole vesicles) and readily releasable pool (1% of whole vesicles). These pools release their content in accordance with the strength of stimuli (Hou *et al.*, 2009). The membrane anchored CPE interacts with the surrounding proteins in the cytosol through its cytoplasmic tail and mediates the retention of SVs within the 0-100nm range of presynaptic terminal. It is seen that in CPE knock out (KO) mice, SVs located 300nm away from the presynaptic terminal and vesicles within the rage release their content more slowly compared to the wild type (WT) (Lou *et al.*, 2010).

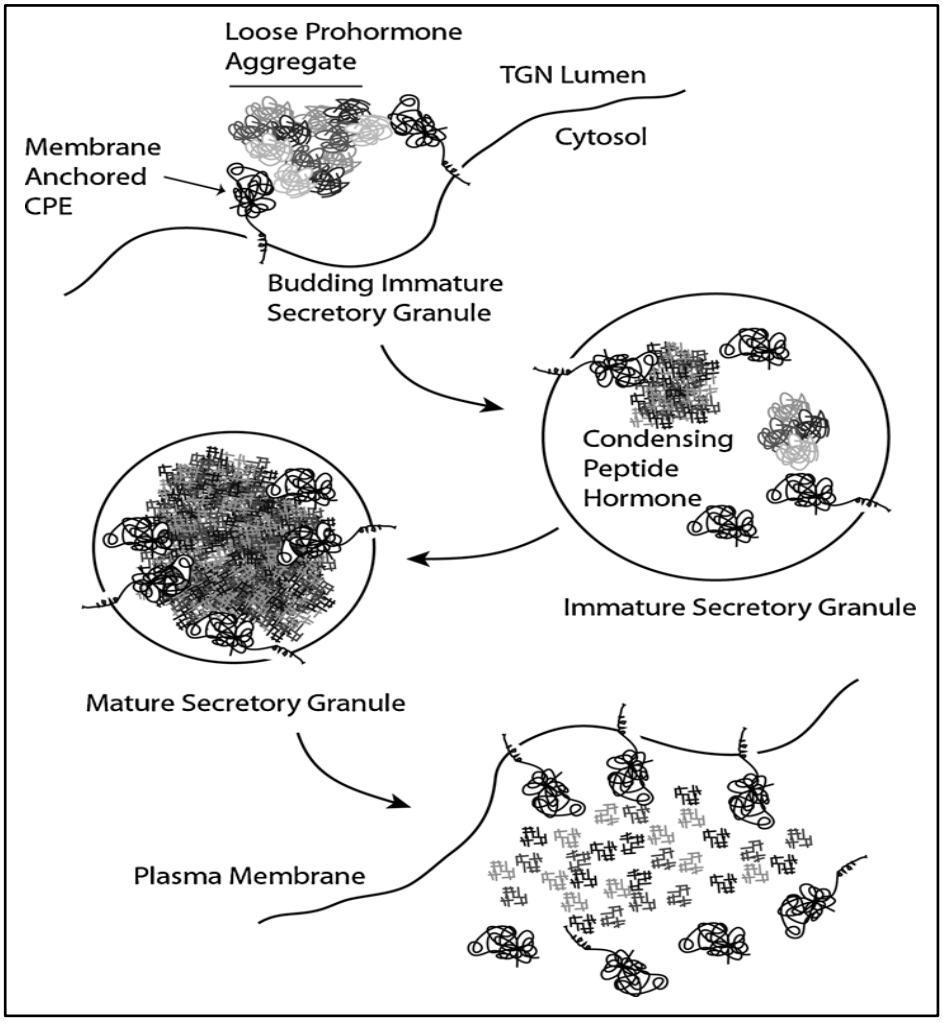

Figure 4. CPE in protein processing and sorting (Kim *et al.*, 2007).

CPE mediates different functions in various tissues. The solid information related to CPE functions comes from the studies on CPE mutations and CPE KOs. There are different mutations in CPE caused by single nucleotide polymorphism like S202P (Berman *et al.*, 2001) or R283W (Chen *et al.*, 2001). To study functions of CPE in different tissues, CPE KO was generated by deleting the exon 4 and 5 encoding PSS binding site and enzymatically active site (Cawley *et al.*, 2004).

1.2.2.1. CPE in Endocrine System

The R283W mutation is a missense polymorphism found in Type 2 diabetes mellitus (T2DM) patients causing Arg to Trp change. R283W mutation impairs the enzymatic activity of CPE as well as its stability at elevated temperatures. The patients with only one of this variant shows the early onset of T2DM. The homozygous state of R283W mutation carries the high risk of diabetes and hyperproinsulinism (Chen *et al.*, 2001).

The S202P is another substitution mutation with Ser to Pro change at position 202. It is termed as Cpe^{fat}/Cpe^{fat} mutant since one of the results of mutation is obesity. S202P causes loss of enzymatic activity of CPE and impairs the trafficking by RSP. It also prevents CPE processing and leads to degradation. Mutant CPE in the pituitary degraded in ER but in pancreas it is degraded partially and sorted through the CSP. These defects in CPE result in obesity, infertility and hyperproinsulinemia (Berman *et al.*, 2001).

The CPE KO mice were generated by deleting the 4^{th} and 5^{th} exons. The absence of CPE results in increase in food consumption and body weight with decrease in physical activity and rate of metabolism each of which part of the obesity phenotype. In addition body fat content with three fold increase, plasma leptin levels with four fold decrease, high glucose levels, and failure in processing insulin are the other defects compared to WT. The failure in gonadotropin releasing hormone (GnRH) processing also impairs the reproduction (Cawley *et al.*, 2004). Another problem caused by lack of CPE is increased osteoclast activity and precursor cocaine and amphetamine regulated transcript (CART) levels that cause low bone mineral density and ultimately impairments in bone regulation (Cawley *et al.*, 2010). It is also reported that in pancreatic β cells palmitate treatment decreases the CPE expression which leads to increased ER stress and apoptosis. This shows that CPE has protective functions against ER stress (Jeffrey *et al.*, 2008).

1.2.2.2. CPE in Nervous System

The studies with mutant and CPE KO support the idea that CPE activity highly required for the nervous system functioning. The absence of CPE alters the learning and memory (Woronowicz *et al.*, 2008) and SV locations and consequently neurotransmission (Lou, 2010). In the hippocampus neural degeneration with deformed dendrite morphology and also defects in spine formation were found (Woronowicz *et al.*, 2010). The increase in CPE levels in the CA3 and CA1 regions of hippocampus following transient global ischemia or *ex vivo* oxidative stress correspondingly increase in neuronal survival shows the neuroprotective role of CPE (Jin *et al.*, 2001; Woronowicz *et al.*, 2008). The neurodegeneration in the absence of CPE suggests the presence of a relationship between neurodegenerative diseases and CPE. The discovery of the new SNP in CPE gene in an AD patient brain confirms this suggestion and the new mutant CPE termed as QQ CPE (Cawley *et al.*, 2012). There are also spliced variants of CPE and one of them is called as CPE-ΔN has its first exon partially spliced from the N terminal. It induces proliferation and invasion in metastatic neuroendocrine tumors and localizes to the nucleus of these tumor cells. Therefore it is considered as a candidate marker for diagnosis of metastatic cancers (Lee *et al.*, 2011).

1.2.3. Mutation in CPE / QQ CPE

QQ CPE is the first mutation discovered to understand the CPE and neurodegeneration relation. It was found through manual screening of human CPE nucleotide sequence by BLASTing human full length CPE cDNA against GeneBank EST database. It was an undocumented mutation in EST database from Helix Research Institute, Japan. The mutation found in 3 different tissues in 2 patients out of 500. One of the mutations found in AD patient's cortex and others found in kidney tumor and thalamus tissues of the other patient (Utsunomiya *et al.*, 1998). It contains three adenosine insertions (Fig.5, BoxA) consequently nine amino acids substitutions (Fig.5, BoxB) in the first exon after the prodomain where two of these amino acids

are glutamine residues (Fig.5, BoxB). Therefore, this new mutation termed as QQ mutant, QQ CPE (Cawley *et al.*, 2012).

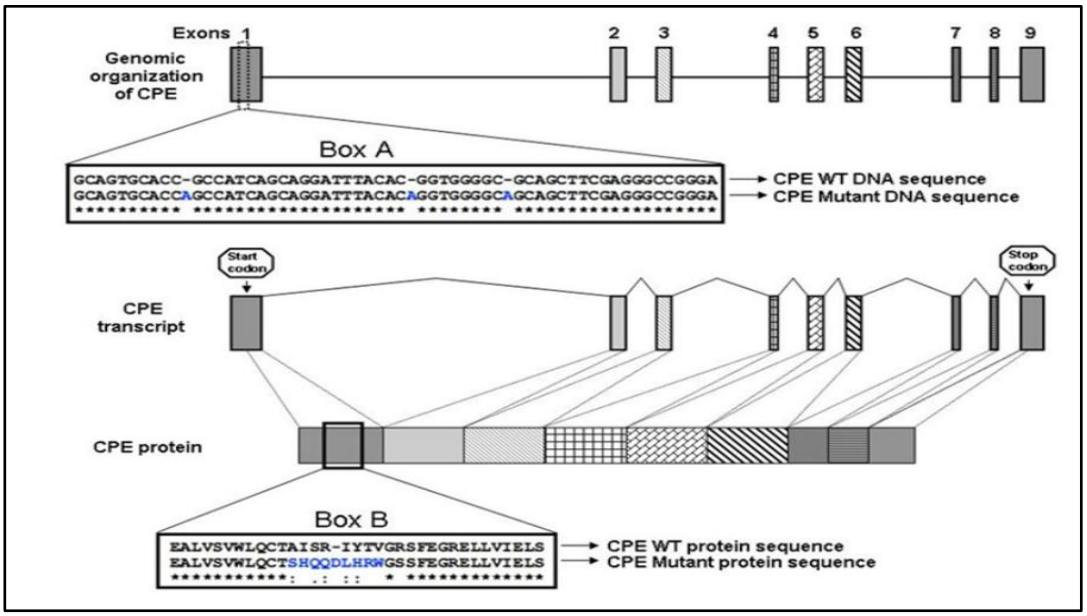

Figure 5. Genomic organization of QQ CPE. A non-redundant nucleotide sequence database search with the human CPE nucleotide sequence as queries identified an EST sequence entry from Alzheimer cortex tissue that had three adenosine inserts (indicated as blue, Box A). This introduces 9 amino acids in the first beta-pleated sheet after the pro-domain of the mutant CPE protein (indicated as blue, Box B), herein called QQ CPE due to the presence of two glutamine residues in the new sequence.

In the preliminary studies enzymatic activity of QQ CPE was analyzed with HPLC analysis (Fig.6). In the study COS7 cell line was used. This cell line lacks both RSP (Taylor et al., 1998) and CPE (Lee et al., 2011). COS7 cells transfected with both WT (Fig.6B) and QQ CPE (Fig.12C) cDNA constructs and untransfected

cells (Fig.6A) were used as negative control. One of the substrates of CPE, adrenocorticotropin hormone (ACTH) added to the media in the presence and absence of $CoCl_2$ (Cofactor of CPE) and GEMSA (inhibitor of CPE). ACTH contains one Arg and two Lys residues at the C terminal that are the targets of CPE. The analysis results that QQ CPE was enzymatically inactive.

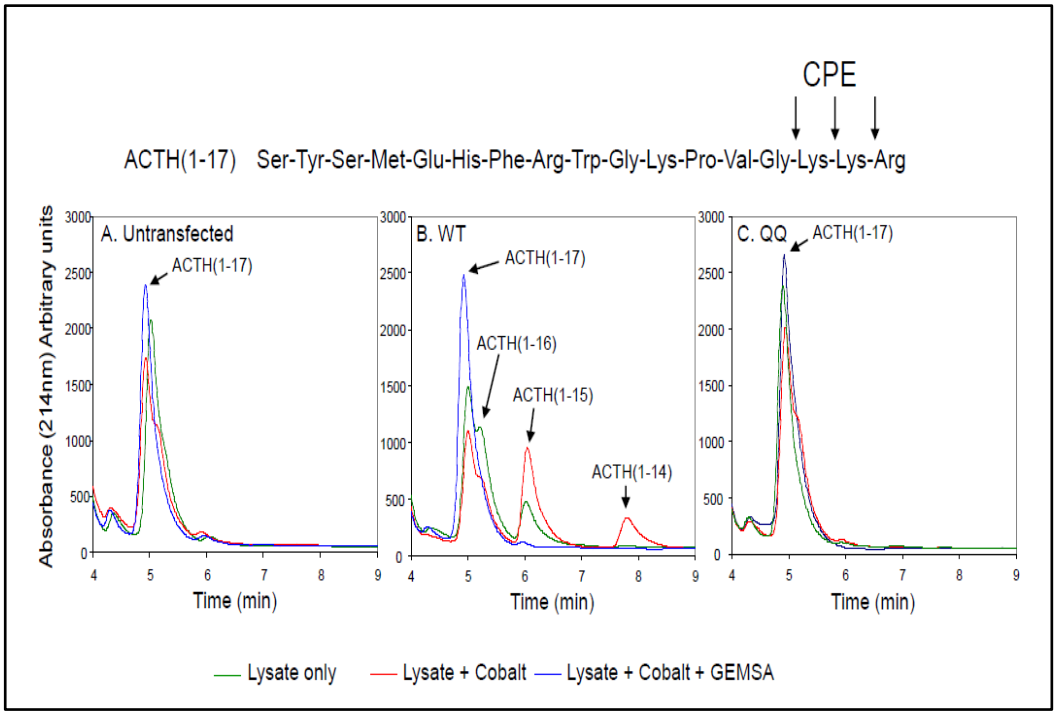

Figure 6. HPLC analysis of QQ CPE. Activity of QQ and WT CPE were tested under three conditions: lysate only (green), lysate+cobalt (red) and lysate+cobalt+gemsa (blue). Untransfected COS7 cells were used as control (A) and no cleaved ACTH was observed. WT CPE is enzymatically active so cleavage of ATCH occurs with lysate only and lysate+cobalt conditions. However with addition of CPE inhibitor GEMSA (blue), cleavage fails (B). QQ CPE however, fails to cleave the ACTH under three conditions (C) indicating that QQ CPE is enzymatically inactive (Cheng *et al.*, 2011).

In another study, the secretion of QQ CPE was analyzed with Western blot. The neuroblastoma cell line (Neuro2A, N2A) which has RSP but not CPE was used in the study. N2A cells were transfected separately with WT and QQ CPE cDNA constructs and also co-transfected. The empty vector (M, mock) transfected N2A cells were used as negative control. Then cell media was collected for secretion analysis and lysate collected to confirm the synthesis of the proteins (Fig.7). It showed that both WT (lane 2) and QQ (lane 3) CPE were present in the cell lysate. The media data showed that QQ CPE (lane 7) was not secreted outside of the cell as WT CPE (lane 6). In addition, QQ CPE hijacked the WT secretion when they were cotransfected (lane 8) as opposite to the cell lysate (lane 4). As expected in negative controls no expression of CPE was observed (lane 1, and 5, M: mock, transfection/empty vector).

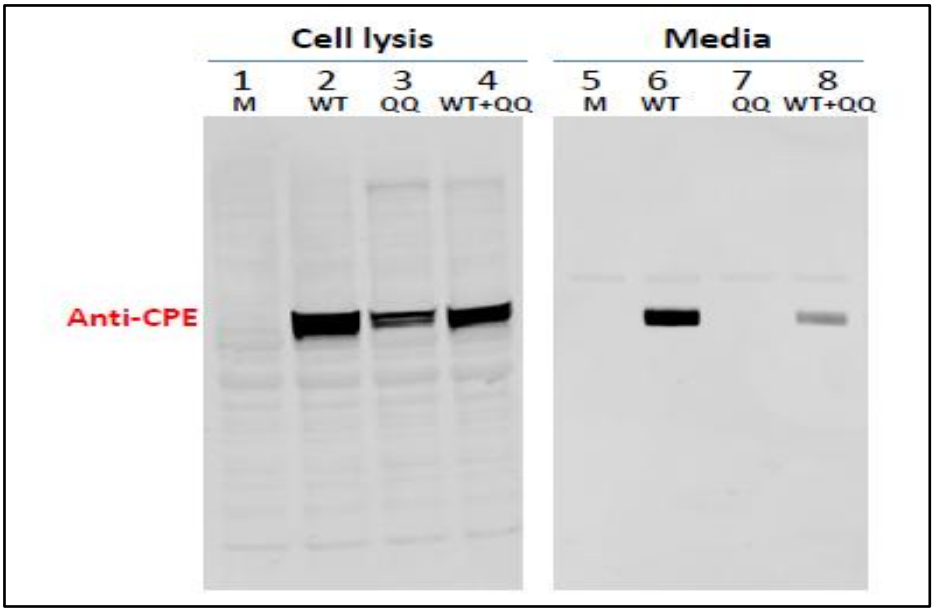

Figure 7. Secretion analysis of QQ CPE. The cell lysate and media from the transfected N2A cells were collected. Untransfected cells were used as negative control (lane 1,5). WT CPE is present in both cell lysate (lane 2) and media (lane 6). QQ CPE however is present in cell lysate (lane 3) but not in media (lane 7) indicating that unlike WT CPE, QQ CPE fails to be secreted. The co-transfection of WT and QQ CPE shows decreased expression of WT CPE (Cheng *et al.*, 2011).

From synthesis to secretion proteins undergo many modification and most of the proteins lost on the processes due to mis-modifications or-folding. Some of them produced excessively and together with the defective ones they cause stress in the cell and needs to be eliminated. The preliminary studies with QQ CPE was clarified that QQ CPE was not enzymatically active and secreted out. Therefore as other aberrant proteins QQ CPE also needs to be eliminated. This requirement of the cell is provided by the certain proteases and degradation machineries within the cell which were given in detail in the following section.

1.3. Protein Degradation Pathways

There are different pathways in the cell for aberrant proteins follow to be degraded. The pathways that were investigated in this study for the mutated proteins are the Ca^{++} regulated calpain, proteasomal and lysosomal pathways hence they were explained in the next sections.

1.3.1. Ca^{++} Dependent Calpain Pathway

Calpains are Ca^{++} dependent cysteine proteases that function as effector in the Ca^{++} signaling (Mellgren, 1987) and provide limited proteolysis of the target proteins (Ono *et al.*, 2010). They categorized into small and large calpains based on the size of the catalytic subunits. The large calpains divided into two groups that are typical and atypical. The atypical calpains lack the EF-hand motif at the C-terminal Ca^{++} binding domain but instead has additional domain (Kovács and Su, 2014). Calpain1 and 2 are the most characterized family members encoded by the *Capn1* and *Capn2* genes. They are also known as μ- and m-calpain, respectively. Since their activity depends on the micro- and milli-molar concentration of calcium (Suzuki, 1991). It was found that the ubiquitous calpains localized within the ER and Golgi (Hood *et al.*, 2004) where the autophagy regulatory complex (PI3K-Beclin) localizes as well (Kihara *et al.*, 2001). The studies with *Capn1*$^{-/-}$ mouse embryonic fibroblast (MEF)

cells also confirm that calpain deficiency impairs the autophagy and cause increase in apoptosis (Demarchi *et al*., 2006; Demarchi and Schneider 2007).

The catalytic subunit of μ- and m-calpains consists of four domains (I-IV) and regulatory subunit has two domains (V, VI). Domain I and VI interact with each other through the EF-hand motifs which may be crucial for the stability. Both domain IV and VI has five consecutive EF-hand motifs. The Cys105, His262 and Asn286 residues in the domain II regulates the catalytic activity of the calpain. There are at least three different regions for the Ca^{++} binding that are domain III, IV, and VI. The domain V functions as a membrane anchor (Fig.8). In the absence of Ca^{++} the subdomains IIa and IIb separated from each other preventing the hydrolysis of the substrates. When Ca^{++} binds to the three domains (III, IV, VI), then calpain activation occurs in two steps. In the first step, upon Ca^{++} binding, domain I auto-cleavage occurs resulting in separation of the subunits. This separation lets the subdomains IIa and IIb interact with each other. Then in the second step, Ca^{++} directly binds to cysteine residues on the subdomain IIa which allows the hydrolytic activity (Wu *et al.*, 2007).

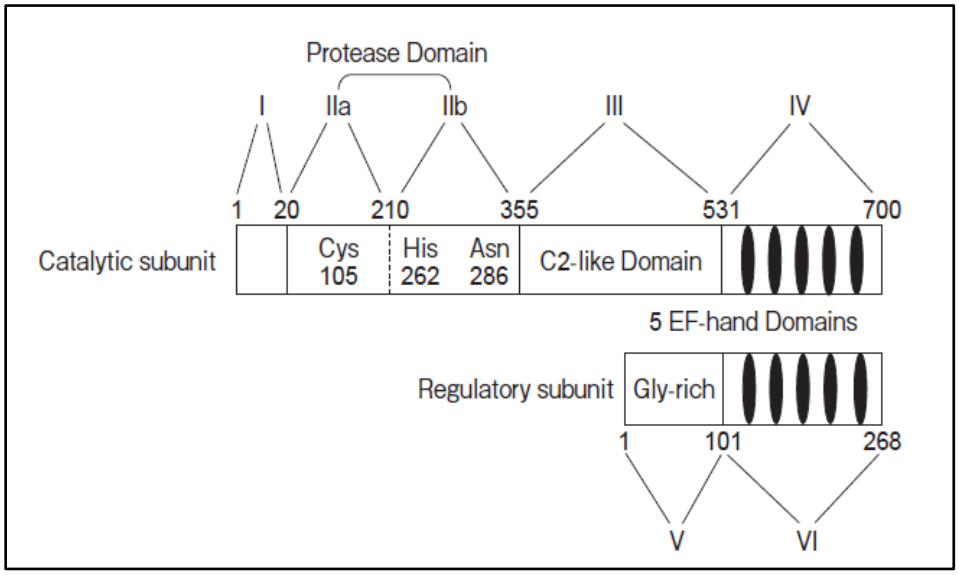

Figure 8. Schematic representation of classical calpain domains (Wu *et al.*, 2007).

Calpains play a role in embryonic and cytoskeletal remodeling, cell differentiation, apoptosis, necrosis and also learning and memory in the central nervous system due to their wide downstream targets. The defects of the pathway may cause to the different types of diseases like Alzheimer's disease, diabetes, muscular dystrophy, cardiac and cerebral ischemia, hypertension, traumatic brain injury, platelet aggregation, cataracts, restenosis and rheumatoid arthritis (Vanderklish and Bahr, 2000; Wu *et al.*, 2007).

1.3.2. Proteasomal Pathway

Proteasomes are another major protein degradation system in cells (Finley, 2009). They have both proteolytic and non-proteolytic functions. The proteolytic ones are degradation of short-lived proteins that mediate cell cycle, apoptosis, transcription and DNA repair and also degradation of misfolded aberrant proteins and breakdown of the intracellular pathogen proteins. They posses non-proteolytic activity in transcription and DNA repair (Rock and Goldberg, 1999).

After its discovery proteasomes are named as 26S proteasome based on its sedimentation coefficient. The 26S proteasome with its 2 subdomains has a complex structure and consists of more than 66 proteins. The 20S subcomplex is the catalytic core particle (CP) and the 19S subcomplex is the regulatory particle (RP). The 19S RP attaches on one or both end of the 20S CP. It is composed of two structures lid and base each of which consists of many ATPases (Rpt1-6) and non-ATPases (Rpn1-3, Rpn5-9, Rpn11-12, Rpn15/Sem1). The 20S RP composed of stacks of 4 rings each of which consists of seven subunits (Fig.9).

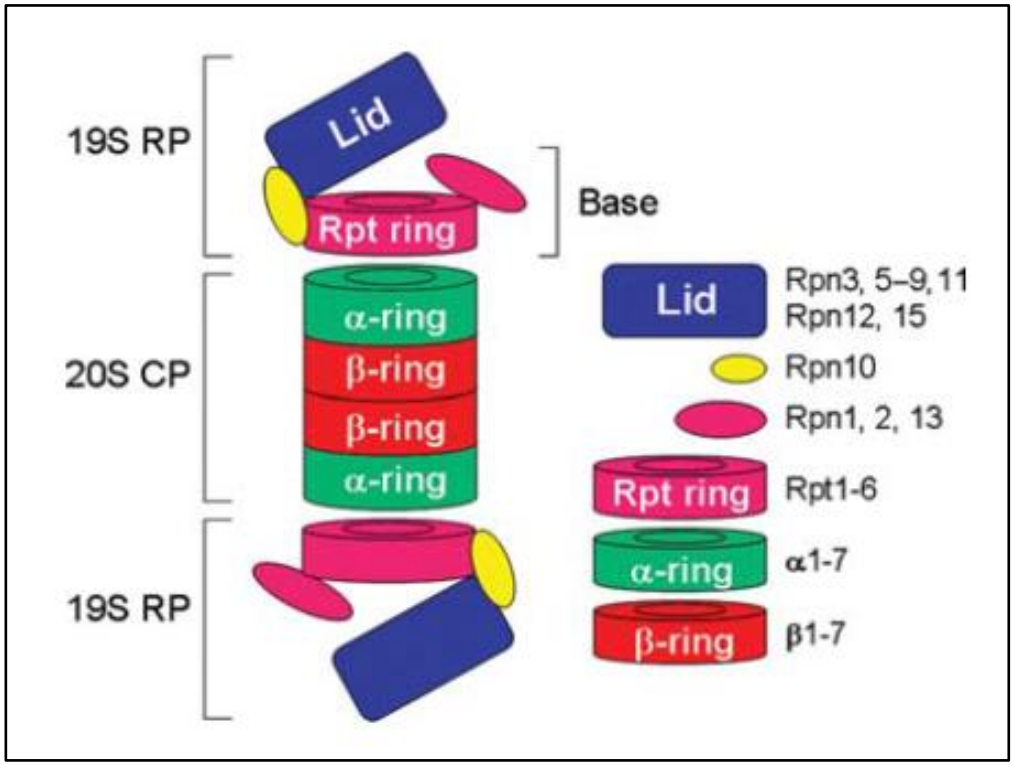

Figure 9. Structure of 26S proteasome (Xie, 2010).

The activity of the proteasome could be ubiquitin dependent (Pagan *et al.*, 2013) and independent (Erales and Coffino, 2014). Ubiquitin is a highly conserved small protein composed of 76 amino acids and found in all eukaryotic tissues (Hershko and Ciechanover, 1998). Ubiquitin tags the proteins by binding them covalently and this process called as ubiquitination (Fig.10). Ubiquitin can be tagged as a single molecule (monoubiquitination) or as a chain (polyubiquitination). Ubiquitination requires E1, E2 and E3 enzymatic activities for activation, conjugation and ligation of the ubiquitin (Jung *et al.*, 2009). Ubiquitin tagged target proteins were then targeted to the 26S proteasomes where ubiquitin(s) recognized by the lid of 19S RP and then target protein was unfolded and translocated and degraded within the rings of 20S CP (Schmidt *et al.*, 2005). After degradation of the target protein, polyubiquitin chain was released and deubiquitinated (Li and Li, 2011).

Proteasomal degradation can be independent of ubiquitin however there is no specific pathway. There are certain proteins studied for the ubiquitin independent proteasomal degradation like Rpn4, thymidylate synthase (TS) and omithine decarboxylase (ODC). The protein Rpn4 functions as an activator for transcription in yeast. It is degraded both ubiquitin dependent and independent manner because it has binding sites for both pathways. The recognition sites for each pathway different but still present in the 19S RP (Erales and Coffino, 2014). The case in the thymidylate synthase is different. It functions in methylation of the deoxyuridylate in mammalian cells. Degradation of TS happens only through ubiquitin independent pathway with a specific lysine independent sequence recognized at the N-terminal. The recognition of this signal by the proteasome is still not clear (Hoyt and Coffino, 2004). The ODC participates in polyamine biosynthesis in eukaryotes. It uses only ubiquitin independent pathway with a signal at the C-terminal. However, it also requires another enzyme activity that binds to the N-terminal (Jariel-Encontre, 2008 and Verma and Deshaies, 2000).

Defects in the proteasomal degradation systems were associated with cancer, aging, protein misfolding disorders, and proteinopathies (Suh et al., 2013). Also spinocerebellar ataxia and spinobulbar muscle atrophy were related to proteasomal disfunction (Orr and Zoghbi, 2007).

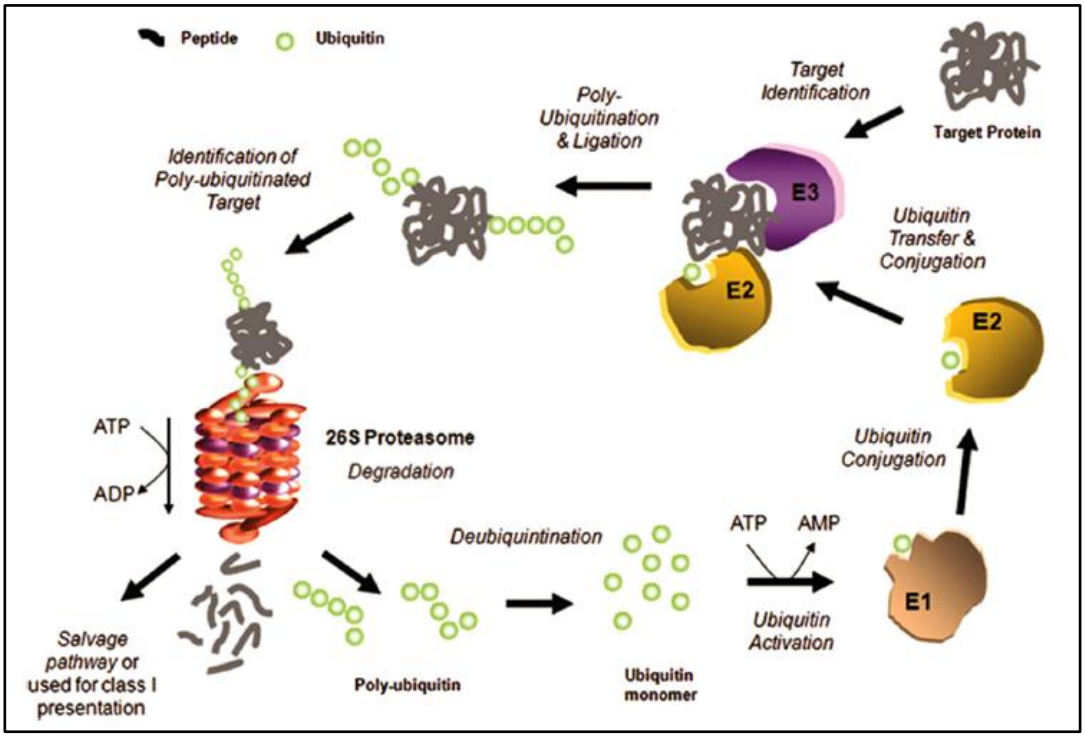

Figure 10. Protein degradation through the ubiquitin-dependent proteasome system (Suh *et al.*, 2013).

1.3.3. Lysosomal Pathway

Lysosomes are hydrolytic, membrane-enclosed organelles with an acidic environment and a dynamic structure which allows them to fuse with each other or certain other organelles (Luzio *et al.*, 2007). Being hydrolytic lysosomes contains many different enzymes like peptidases, proteases, phosphatases, lipases, nucleases and glycosidases and with this diverse content they function in the degradation of not only proteins but also carbohydrates, nucleic acids and lipids (Platt et al., 2012). The proteases and peptidases function in the protein degradation in lysosomes are cathepsins (B, D, H and L), dipeptidyl peptidase I and II, tripeptidyl peptidase,

arginyl aminopeptidase, carboxypeptidase A and B, prolyl- and tyrosyl-carboxypeptidases, and dipeptidase I and II (Dice, 2000).

The lysosomal enzymes delivered to the lysosomes after they are tagged with M6P in the *cis*-Golgi. In the TGN they bind to M6P receptors (MPRs) and then delivered to the endosomes where they dissociate from the MPRs and then transported to the lysosomes through the endosomes-lysosome fusion (Seaman, 2004). The enzymes without the M6P tag reach the lysosomes either by interacting with adaptor proteins (Peden *et al.*, 2004) or through the association with the ubiquitinated proteins (Pak *et al.*, 2006).

Lysosomes divided into two groups based on internalized substrates that are early lysosomes (contain only enzymes) and late lysosomes (contain enzymes and substrates). Lysosomes get their substrates either from outside (heterophagy) or from inside (autophagy) (Holtzman, 1989). Heterophagy occurs via endocytosis and endosomes fuse with lysosomes to leave the substrates (Fig 11.3). Autophagy occurs via autophagosome formation around the proteins that are destined to be degradation and then this autophagosome fuse with the lysosome (Fig 11.1-2).

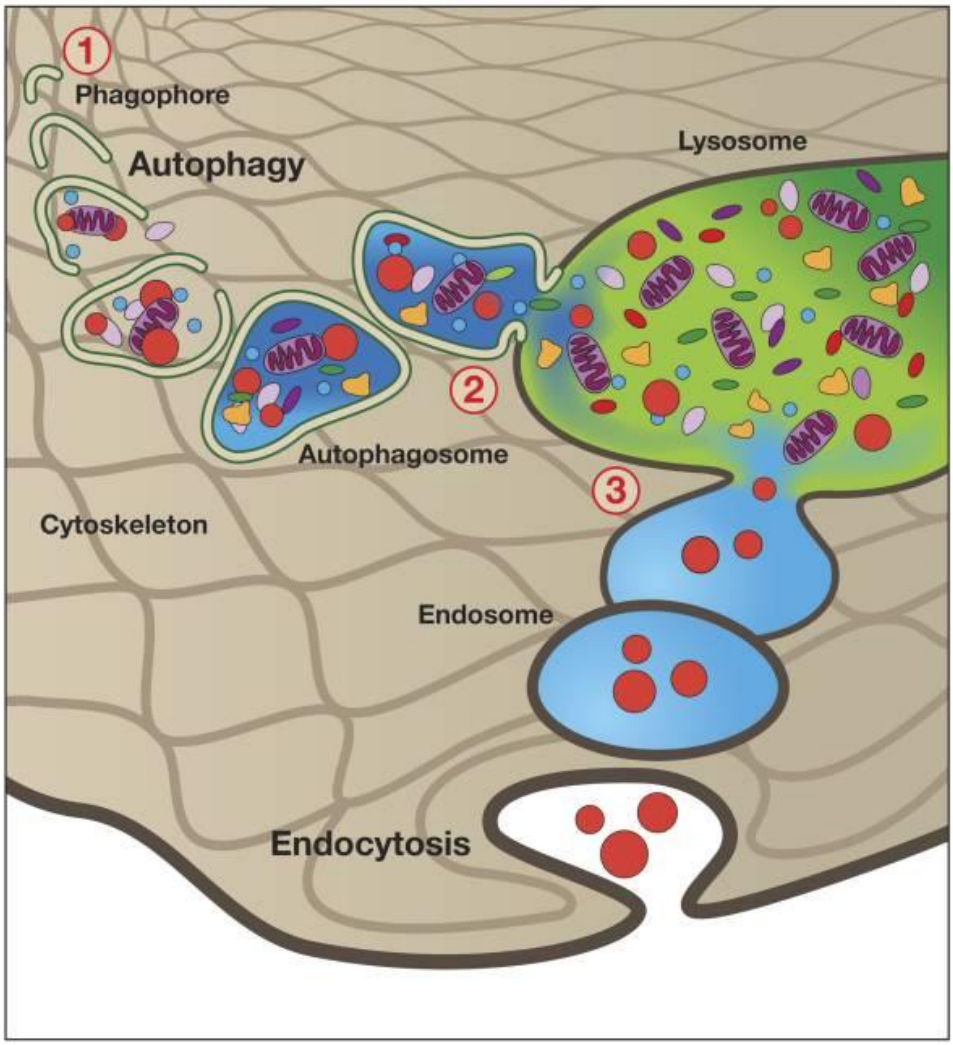

Figure 11. Proteins entering the lysosome (Stern *et al.*, 2012) indicated as 1: autophagy, 2: autophagosome fusion and 3: endosomal fusion with lysosome.

The delivery through endocytosis requires endosomes formation from the plasma membrane (Fig.12). The newly formed endosomes called as early endosomes and they mature into the late endosomes. On the process content of the endosomes recycles and late endosomes are formed. The recycling endosomes contains the proteins that need to be retrieved. The late endosomes contains the proteins to be

degraded. There are different mechanisms for the transfer of the late endosomes content to lysosomes. First, late endosomes directly matures into lysosomes. Second, vesicles employed to transfer endosomes' content to lysosomes. Third, lysosomes contacts with the late endosomes transiently and separates (kiss-and-run) and finally fourth, lysosomes and late endosomes fuse and form a hybrid (Fig.12).

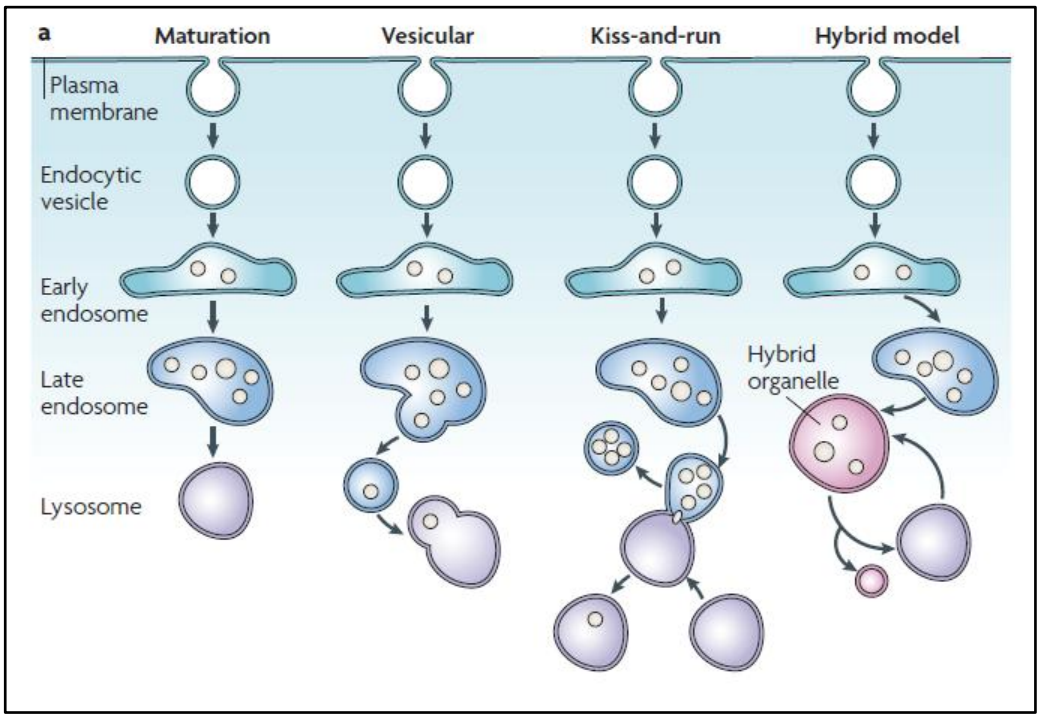

Figure 12. Mechanisms of endosomal delivery to lysosomes and lysosomal fusion (Luzio *et al.*, 2007).

Autophagy is the other fusion mechanism used by the lysosome to clean up the cytoplasmic content. Unlike endosomes, membrane origin of the phagophores is not clear but may have multiple sources like plasma membrane, ER, Golgi, or outer mitochondrial membranes (Falk *et al.*, 2012). The process of autophagy has mainly four steps: cargo selection, sequestration, fusion with lysosome and

degradation/recycling of the cargo (Lyon *et al.*, 2013). The selection of cargo can be specific or non-specific. The specific selection requires the association of the adapter proteins like p62 and NBR1. These adapter proteins bind to the ubiquitinated proteins and deliver them to the phagophore (Johansen and Lamark, 2011). The involvement of these adapter proteins in the selective autophagy suggests the presence of cross-talk between the ubiquitin-proteasome system and autophagy (Johansen and Lamark, 2011).

Defects in the lysosome functioning associated with different types of diseases like lysosomal storage disorders (LSD) or neurodegenerative diseases (Zhang *et al.*, 2009).

1.4. Aim Of The Study

The QQ mutation is a recently discovered novel mutation with a great potential to understand both new functions of CPE and also its connection to the neurodegenerative diseases. Preliminary studies shows that QQ CPE enzymatically inactive. It also fails to be secreted out of the cell and besides, it hijacks the WT CPE secretion. It was reported that another CPE mutation, S202P also could not be sorted instead showed tissue specific degradation. In pituitary it was totally degraded in the ER and in pancreas partial degradation occurred (Berman et al., 2001). In another study under ER stress driven conditions degradation of CPE lysosomes was shown (Jeffrey et al., 2008).

Based on the preliminary studies, we aimed to study degradation of QQ CPE and deduce cellular mechanisms of its degradation in N2A cells.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

All materials were provided in the Appendices A-N.

2.2. Methods

2.2.1. Molecular Biology

QQ CPE and WT CPE cDNA constructs kindly provided by the Loh lab (National Institutes of Health (NIH), USA) and shipped on the Whatman paper.

2.2.1.1. Plasmid elution From Whatman Paper

Plasmids were dropped on the Whatman paper within the circular regions (the Loh Lab). The circular regions on the whatman paper were cut and put into the 1.5 mL microcentrifuge tubes. The paper was wet with 50 µL of 1X TE (Tris-EDTA) buffer and incubated for 5 min at RT. To elute plasmids, the piece of Whatman paper spun down at 16000x*g* for 10 min. Then supernatant was taken and stored at -20°C as the first elute. Another 100 µL of 1X TE buffer was added to the paper and with the same protocol the supernatant was collected such that 70 µL as the second elute stored at -20°C and remaining 30 µL was kept at 4°C for further experiments. The 2 µL of the samples, were used for concentration measurements with NanoDrop2000 and the 5 µL was used for agarose gel electrophoresis to analyze the integrity of the samples. The rest was used for transformation experiments.

2.2.1.2. Transformation of Competent Bacteria

All experiments were done at 4°C unless indicated. Both QQ and WT CPE plasmids were kept on ice before starting the experiment. The main stock (at -80°C) of SOC medium was aliquoted and stored at -20°C and prewarmed to the RT before use. Then a vial of One Shot DH5α competent cells, 50 µL, was thawed and 3 µL of the plasmid sample, after vortexing, was added to the cells and mixed by tapping. Each vial was incubated on ice for 30 min. After securing the lids with parafilm the vials were kept in 42°C water bath for 30 sec and then put back on ice. By using aseptic techniques to avoid contamination, 250 µL of prewarmed SOC medium was added to each vial and put on shaker for 1h at 225 rpm, 37°C. Next, the transformation reaction solution was plated on agar plates containing 50 µg/mL selective antibiotics (ampicillin) with streak plate technique and incubated at 37°C for 16h. The transformation reaction solution was plated with different volumes: 20 µL and 50 µL to obtain single colonies. The remaining transformation reaction solution was stored in case of re-plating. The next day colonies were selected and plates were stored at the fridge for a week.

2.2.1.3. Colony Selection

The next day of plating colonies were selected with a sterile loop and inoculated into the LB medium, containing 100 µg/mL selective antibiotics (ampicillin). The inoculated LB medium was put on shaker for 16h at 37°C and 225 rpm. After incubation plasmid DNAs prepared.

2.2.1.4. Plasmid DNA Preparation

The plasmid DNA was prepared from small-scale (5 mL, miniprep) bacterial cultures grown in LB medium containing selective antibiotics at 37°C for 16 h from either a single colony of transformed bacteria, picked from the agar plate, or a glycerol stock. Using Thermo Scientific GeneJet Plasmid Miniprep Kit and applying aseptic techniques plasmids were isolated. The grown bacterial cultures (5mL) were first centrifuged at 6800x*g* for 2 min at RT and supernatant discarded. Then the pellet

was dissolved in 250 µL of resuspension buffer (RNAse A added) to protect the DNA from degradation and also to get rid of the RNA and 250 µL of lysis buffer added to reach the cytoplasmic content. To stop the over-lysation which may cause the denaturation of DNA due to NaOH content of the buffer, 350 µL of neutralization buffer was added and mixed by inversion. The mixtures were centrifuged for 6 min at 13.500x*g* and supernatants were transferred to the GeneJet spin columns. The columns then were centrifuged for 1 min at 13000x*g* and flow-through discarded. To purify the plasmid DNA, the washing step was performed by adding 500 µL of wash solution and centrifuged for 1 min at 13000x*g* which was repeated to remove the remaining wash solution. Then spin columns were transferred to fresh microcentrifuge tubes to elute the plasmid DNA. Fifty µL of elution buffer was added and incubated for 2 min then centrifuged for 2 min at 13000x*g* at RT. The concentration of the DNA measured with NanoDrop2000 and the integrity of DNA controlled with agarose gel electrophoresis.

2.2.1.5. Glycerol Stock Preparation

To obtain 15% glycerol stocks (% v/v) from the transformed bacterial cultures, 60% glycerol (% v/v) was prepared with dH_2O and 125 µL of 60% glycerol was added to the cryovials. Then 375 µL of bacterial culture grown from the single colony of transformed bacteria put into the glycerol added cryovials. As soon as the bacterial culture was added to the vials, they were frozen in the liquid nitrogen to and stored at -80°C.

2.2.1.6. Gel Electrophoresis

The integrity of the plasmid DNAs isolated from bacteria was controlled with agarose gel electrophoresis. First 0.8 % agarose gel was prepared (Appendix G). It was placed into the tank which was filled with the 1X TAE buffer. DNA samples (5 µL) mixed with 6X loading buffer (1 µL) and were loaded into the wells and run at

100 V for 1.5 h. As a control 3 µL of DNA ladder was also loaded. The image of the gel was taken with the Vilber Lourmat UV transilluminator (Appendix L).

2.2.2. Cell Culture

Mouse neuroblastoma (Neuro2A, N2A) cell line was selected for this study. The main reason for that it has got the RSP and also lack of CPE.

To prevent the contamination all cell culture works were performed in the laminar flow hood. The solutions and media (Appendix G, I) used were prewarmed before use by incubating at 37°C water bath for 10 min.

Table 2. Commonly used cell culture vessels.

Culture Vessel	***Growth Area (cm^2)***	***Medium Volume (mL)***	***Trypsin/EDTA Volume***
Culture Flask	75	12	3 mL
Culture Flask	25	5	1 mL
Culture Dish 10 cm	55	10	2 mL
6 well plate	6x9.5	3	600 µL
12 well plate	12x3.8	1.5	300 µL
24 well plate	24x1.9	1	200 µL

2.2.2.1. Propagation

The N2As were cultured in the DMEM-FBS growth medium at 37°C with 5 % CO_2. N2As have been thawed from the passage 3 (P3) cryogenic stock cultures. To split the N2As, first cells were washed with PBS and treated with trypsin/EDTA solution at 37°C for 5 min. The cells were collected in DMEM-FBS growth medium and centrifuged for 5 min at 150x*g*. The supernatant was discarded and cells were

resuspended in growth medium and plated in fresh culture vessels with desired dilutions such as1/3 to 1/10 ratios.

2.2.2.2. Freezing and Thawing

After cells were harvested as described earlier, they were centrifuged and then resuspended in freezing medium which contained 5% DMSO in cryovials. The cells were kept at -20°C for 1h and then stored at -80°C overnight (o/n). For the long term storage, the cells have been kept in liquid nitrogen vapor. During thawing the cells were, placed in 37°C water bath quickly to decrease the toxic effect of DMSO on cells to the minimum and they were suspended in prewarmed growth medium rapidly and centrifuged for 5 min at 150x*g*.

2.2.2.3. Transfection of Cells

The cultured cells were transiently transfected with cDNA constructs when they reached around 70% confluency. The cells were plated to the 6 well-plates (1/5 ratio), for each well, separately, 1 µg of cDNA was gently mixed into 100 µL of Opti-MEM and 2 µL of Lipofectamine2000 was gently mixed into 100 µL of Opti-MEM. After 5min incubation at RT, they were mixed gently and incubated for another 20 min at RT. Following the incubation period, the mix was applied onto the cells. Cells were incubated with mix for 24h and then lysed for protein analyses.

2.2.2.4. Inhibition Assays

The transfected cells were treated with different inhibitors to block the degradation pathways. The Calpain I regulated degradation was inhibited with Calpain Inhibitor I (MG101), a neutral cysteine protease inhibitor. It was reported that the E64, another inhibitor of cysteine proteases that was used in lysosomal inhibition experiments in this study, also functions as a calpain inhibitor (Moldoveanu *et al.*, 2004). Therefore MG101 inhibition was used to compare the

lysosomal activity with Calpain I activity. The N2A were cultivated on 6 well plates and when they reach 70-80% confluency they were transfected and after 24h, transfected cells were treated with MG101 (5μM) for 24h. Then proteins were isolated under denatured conditions and relative expression of CPE was detected with Western blot analysis. The primary antibodies were used against CPE and β-actin. The β-actin was used as a loading control to normalize the protein levels detected. The cells that are given the MG101 diluents were used as the control for MG101 treated cells. The empty vector (EV) transfected cells were used as negative control for both QQ and WT transfected cells.

The assessment of CPE rescue through the proteosomes was achieved by preventing the activity of proteosomes using different inhibitors like Z-Leu-Leu-Phe-CHO (ZLLF) and Z-Leu-Leu-Ala-CHO (MG132). At the beginning the ZLLF was used then changed to MG132. The MG132 acts by binding to the 20S CP of proteosome (Sutovsky *et al.*, 2005) and is more potent than ZLLF inhibition (Jamaluddin *et al.*, 1998). As described previously the N2A cells were cultivated on 6 well plates and grown to 70-80% confluency for transfection. After 24h, transfected cells were treated with ZLLF (15μM) for 24h.

Different lysosome specific inhibitors were used to understand the involvement of lysosomes in QQ CPE degradation. The first inhibitor used for this purpose was E64 (10μM) and then E64d (20μM) which is the membrane permeable form. The E64 and E64d inhibit the cathepsins A, B, H and L and some other cysteine proteases including calpains (Matsumoto *et al.*, 1999; Hook *et al.*, 2014). Pepstatin A (PepA) is another lysosomal inhibitor that acts on D and E type cathepsins (Yoshida *et al.*, 2006) was also tested for lysosomal inhibition.

The % rescue of CPE was calculated by dividing the treated quantifications over their controls.

2.2.3. Biochemistry

2.2.3.1. Cell Lysis and Protein Concentration

Transfected cells were washed with prewarmed PBS. The cells were lysed with M-PER (200 µL per 9.5cm^2 surface) plus 1Xprotease inhibitor cocktail at RT for 5 min with gentle shaking. The lysates were collected and centrifuged at 6,800x*g* for 5 min. The supernatant was taken into fresh tubes. The protein concentrations were measured by Bradford assay using Thermo Scientific Coomassie Protein Assay kit before stored at -20°C.

As standard different BSA concentrations (Appendix G) were used and assayed together with samples. Protein amount in each sample was detected based on the standard concentrations so that equal amounts were used for further analysis.

2.2.3.2. His-Tagged Protein Isolation

The cells, transfected with His-tagged WT and QQ CPE constructs, were first washed with PBS and then collected through trypsinization. The cells were resuspended in 500µL (per 50cm^2 surface area) of His-lysis buffer and lysed with sonication. For sonication, 6 cycles of 20s bursts at 50W with a sequential 20s cooling period were used. The sonicator was equipped with the microtip. The lysed cells were centrifuged at 6,800x*g* for 5 min. The supernatants were collected into new microcentrifuge tubes and a small volume of the supernatant was aliquoted for the control of isolation steps. Then 100 µL of Dynabeads His-tag isolation and pull down mixture taken into a fresh tube and beads were separated from the solution using neodymium magnetic rings. The collected supernatant was added onto the beads mixing gently and incubated for 7h on a shaker with slow and constant shaking. Following incubation beads were collected using neodymium magnetic rings. The beads were expected to bind with the His-tagged proteins and the nonspecifically bound proteins beads were washed 3 times with 100µL of His-washing buffer. The eluted samples from each step were stored in a fresh tube. The

His-tagged proteins beads were washed 3 times with 100µL of His-Elution buffer by incubating for 5 min on the shaker. The beads were incubated for 10 min at RT with reducing sample buffer. The protein concentrations from each step were determined using the Thermo Scientific Coomassie Protein Assay kit. The protein samples stored at -20°C. The isolated proteins were analyzed with SDS- and Native-PAGE gels (Fig.13).

2.2.3.3. Native-PAGE and Coomassie Staining

The His-tagged protein samples were isolated under native conditions and diluted with the 2X non-reducing sample. The proteins were separated by 10% native-PAGE on ice. After running the gels, they were stained with Coomassie blue for 1h at RT and destained until the protein bands were visible.

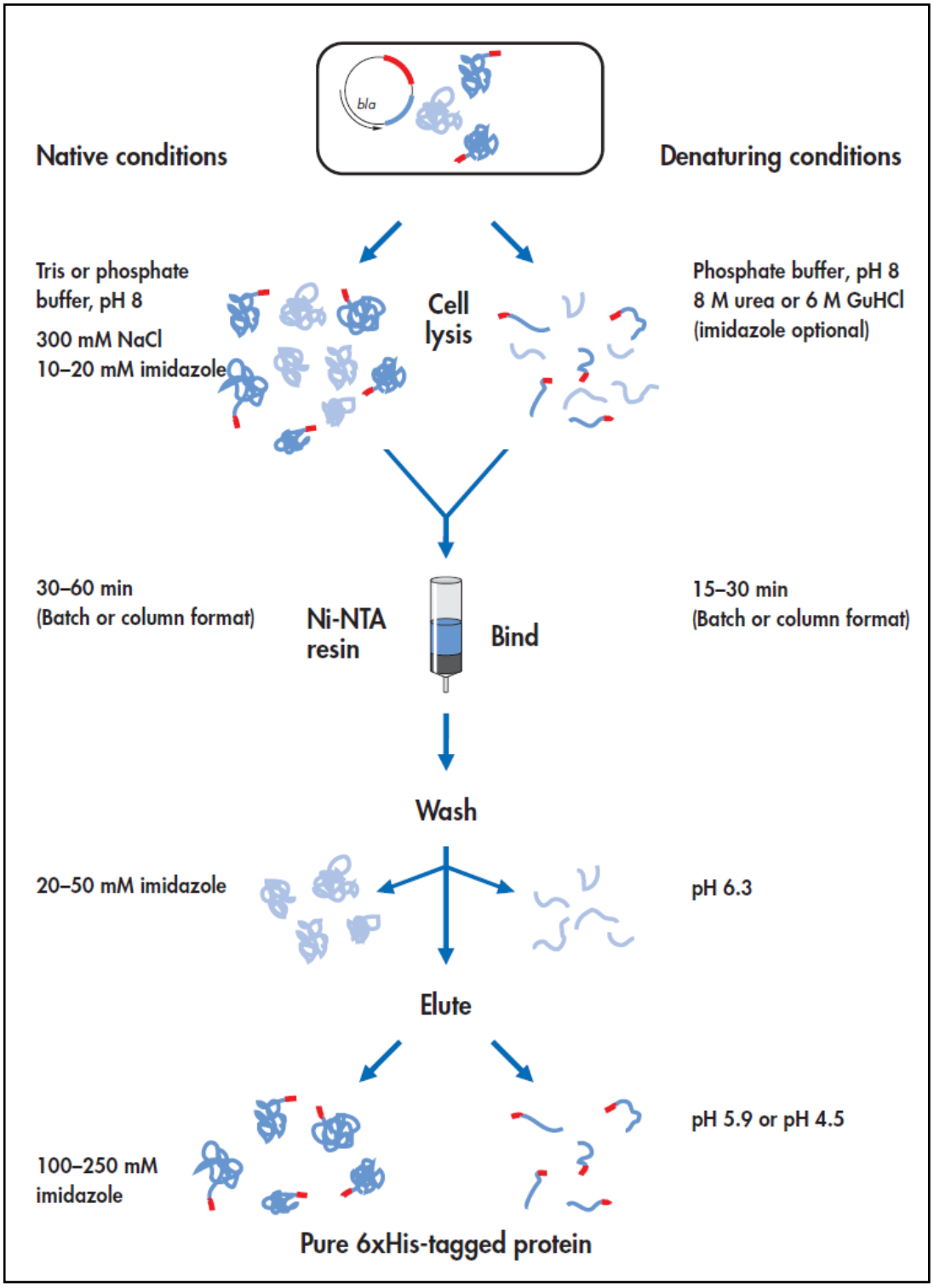

Figure 13. 6x His-tagged protein isolation. (Qiagen)

2.2.3.4. Immunoblotting

The samples were diluted in 4X loading buffer and separated by 10 % SDS–PAGE at 100V for 1.5h. They were transferred to 0.45-mm nitrocellulose membranes using the Bio-Rad wet blotting system at 400mA for 1 to 1.5 h using 1X transfer buffer. Next, the membranes were blocked with 5 % blocking solution for 1 h at RT and washed 3X with 1X TBS-T for 7 min to remove the unbound proteins. Subsequently the membranes were probed with the primary antibody o/n. After washing 3X with 1X TBS-T for 7 min, they were probed with horseradish peroxidase linked secondary antibody for 1h at RT. To remove excess secondary antibody membranes washed 1X with 1X TBS-T and then 1X with TBS to remove the Tween-20 for 7 min. For visualization, 1:1 mixture of luminal: peroxide ECL substrate was applied onto the probed membranes for 1 min at RT. The substantial chemiluminescent reaction was detected by exposure of the wrapped membranes to CL-XPosure Film in a dark room. Kodak X-Ray processor was used for fixing and developing the signal on the X-ray films.

2.2.3.5. Cell Death Detection

To detect the apoptotic effect of QQ CPE on N2A cells Roche Cell death detection Elisa^{Plus} kit was used based on the manufacturers' instructions. The microplates were blocked with freshly prepared 5% blocking solution. The cells were collected with trypsinization when they reach the 80-90% confluency. All procedures were performed at RT unless indicated.

After collection, the cells were resuspended in the lysis buffer and incubated for 30 min. Then lysate centrifuged at 200x*g* for 10 min. The 20 µL of the supernatant were transferred into the streptavidin coated microplates for analysis. Next, 80 µL of immunoreagent was added to each well and incubated on a shaker for 2h. Solutions were removed by tapping and washed for 3X with 300 µL incubation buffer. After washing microplates were incubated with 100 µL of ABTS solution on a shaker for 15 min until color development was sufficient for photometric analysis.

Then 100 µL of ABTS stop solution was added and color development measured at 405 nm against blank solution (100 µL ABTS Solution + 100 µL ABTS Stop Solution). Specific enrichment factor calculated as given below where mU corresponds for absorbance [10^{-3}]:

$$enrichment\ factor = \frac{mU\ of\ sample\ (dying - dead\ cells)}{mU\ of\ the\ negative\ control}$$

Apoptotic levels compared with the negative (< 100 mU) and positive control (> 600 mU) ranges.

2.2.4. Data Analysis

Western blot were quantified with the Fiji program and statistical analysis was performed with GraphPad Prism 6 program. For all data sets, the arithmetic average, standard deviation (STD) and standard error of the mean (SEM) were computed by the GraphPad Prism 6 program. Error bars depict the SEM. To examine the significance of the data sets the Student's t-test was used. Data were considered as significant with $^{*}p<0.05$ and as highly significant with $^{**}p<0.01$.

CHAPTER 3

RESULTS

3.1 Degradation of QQ CPE

The preliminary studies showed that QQ CPE is enzymatically inactive and fails to be secreted outside of the cell. Therefore it was assumed that QQ CPE was either degraded through one of the degradation pathways or aggregated somewhere inside the cell.

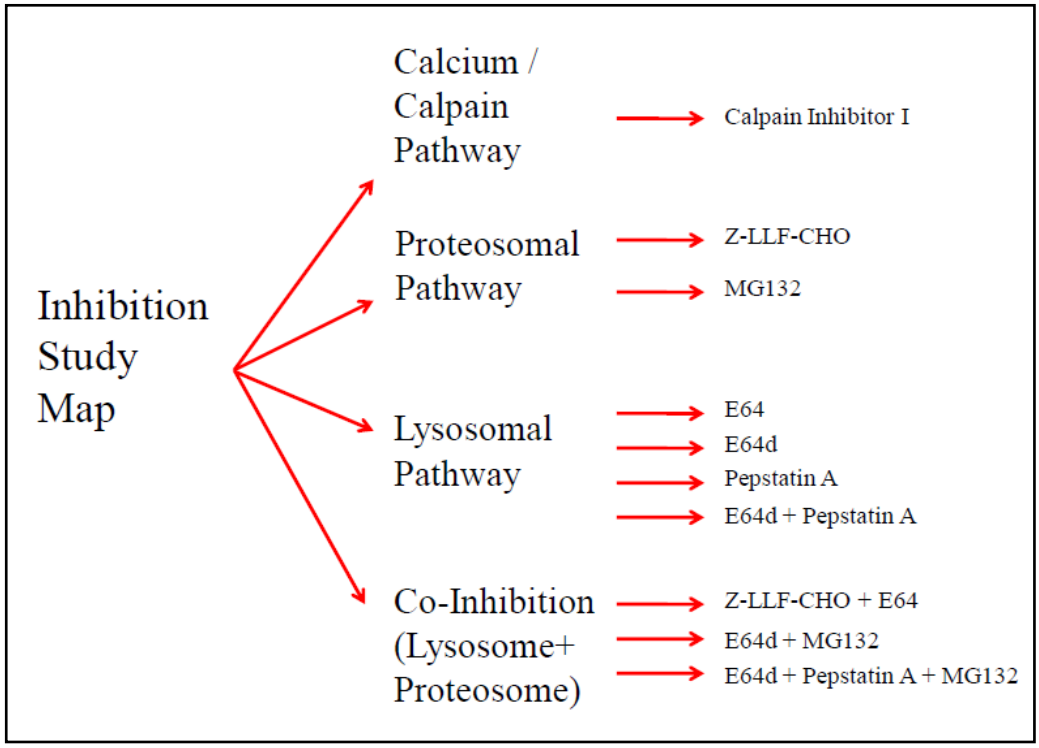

Figure 14. The map of inhibition study. The three different pathways studied on N2A cells that were treated with pathway specific inhibitors.

3.1.1. Proteosomal Inhibition Studies

Proteosomes are one of the major sites for proteolytic activity. Unlike calpains proteosomes degrade the proteins completely. The assessment of CPE rescue was achieved by preventing the activity of proteosomes using Z-Leu-Leu-Phe-CHO (ZLLF) and Z-Leu-Leu-Ala-CHO (MG132) inhibitors. Treatment with ZLLF resulted in inconsistent results where both QQ and WT CPE expressions were either increased (n=2) (Fig.15) or decreased (n=2).

Figure 15. CPE expression in ZLLF (10μM) treated N2A cells. Both QQ and WT CPE expression were increased with the treatment. β- actin used as loading control. Each lane contains 30 μg protein.

The cells treated with MG132 (20μM) shows different result compared to ZLLF. The relative CPE expression increases in both QQ and WT CPE transfected cells (Fig.16A, n=3). The loading control levels were also affected negatively in treated groups. Despite that, β-actin levels were consistent within the control and treated groups. The Western blot quantification results were provided in Table 3. The folded responses with the mean band densities (n=3) represented on the graph (Fig.16B). It was indicated that there was 4 fold difference between QQ treated and QQ control

(p=0.34) and two folds difference between WT treated and WT control (p=035). Due to high variance and low sample number results were statistically insignificant.

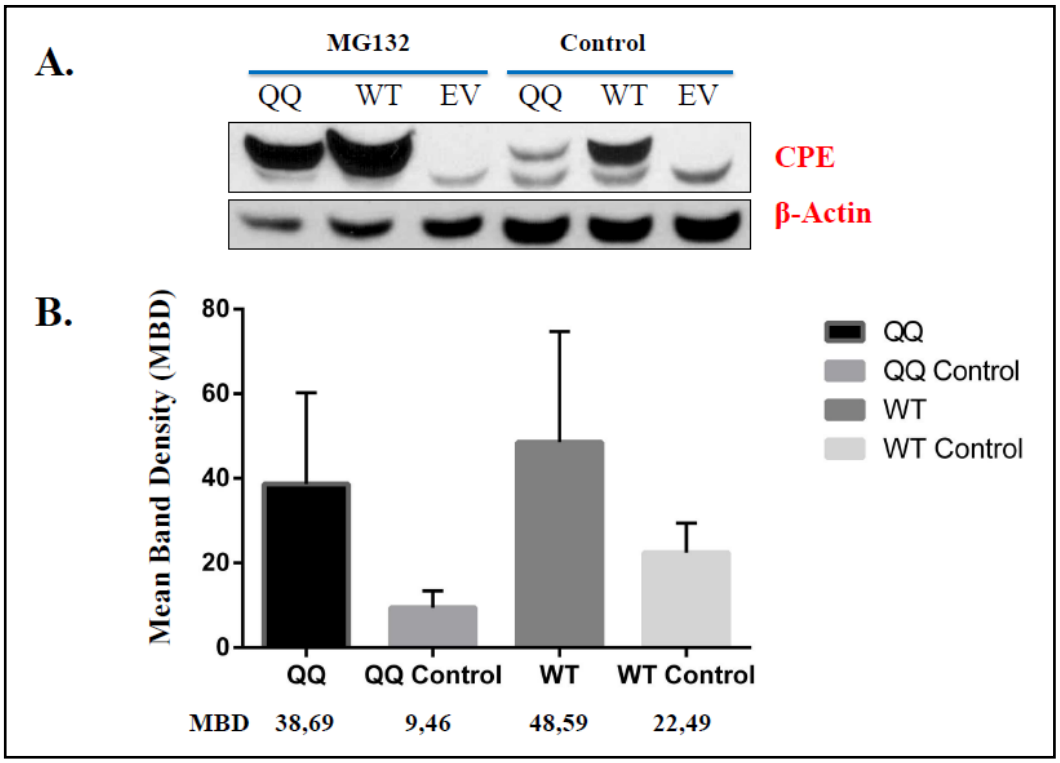

Figure 16. CPE expression in MG132 (20µM) treated cells. Both QQ and WT CPE expressions increased in MG132 treated cells compared to their controls (A). In the bar graph quantification of the Western blot is given as the fold responses optimized to EV controls (B). Both WT and QQ CPE got degraded in proteosomes.

Table 3. Quantification of MG132 treated cells.

	QQ	QQ Cont	WT	WT Cont	EV	EV Cont
CPE Folded Response	232	30	282	132	3	4
Beta Actin Folded Respone	94	270	148	256	188	272

3.1.2. Lysosomal Inhibition Studies

The lysosomes that lead to complete degradation of proteins studied by using E64 and E64d inhibitors. Treatment of N2A cells with E64 (10µM) showed conflicting results which were both relative increase (n=1) and decrease (n=2) in QQ CPE expression. Fig.17 represents the decreased expression of QQ CPE when WT CPE expression increases. This experiment was repeated for 3 times.

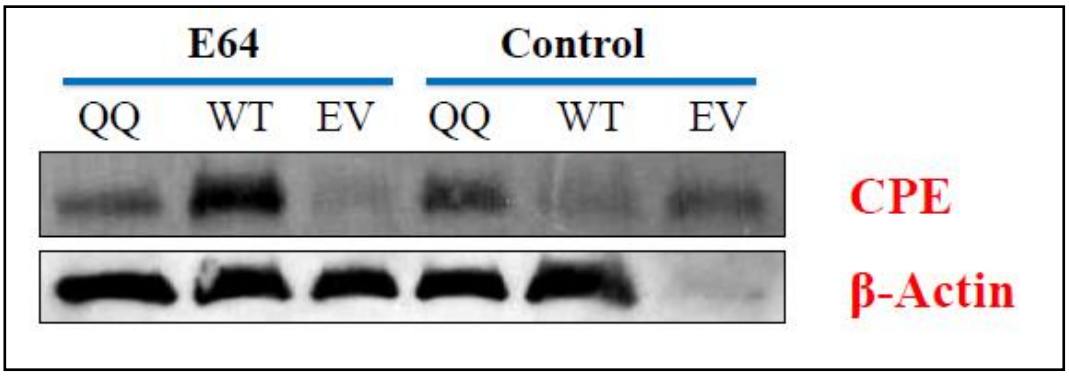

Figure 17. CPE expression in E64 (10µM) treated N2A cells. The QQ CPE expression decreases with E64 inhibition but WT CPE expression increases compared to their controls that shows E64 dependent lysosomal activity was not involved in QQ CPE degradation. 20µg protein loaded for each lane.

The treatment with E64d (20µM) however was rescued the QQ CPE and relative expression of WT CPE was not affected (Fig.18, n=3). PepA (1µM) treatment did not affect the both QQ and WT expressions (n=1) (Fig.18). At the high concentrations (2µM) increase in QQ expression was observed (n=1); however, loading control levels were inconsistently unbalanced (data not shown). Therefore PepA effect was concluded as inefficient but still used to achieve the complete inhibition of lysosome. The cells treated with both E64d and PepA (n=4) showed higher rescue for QQ CPE.

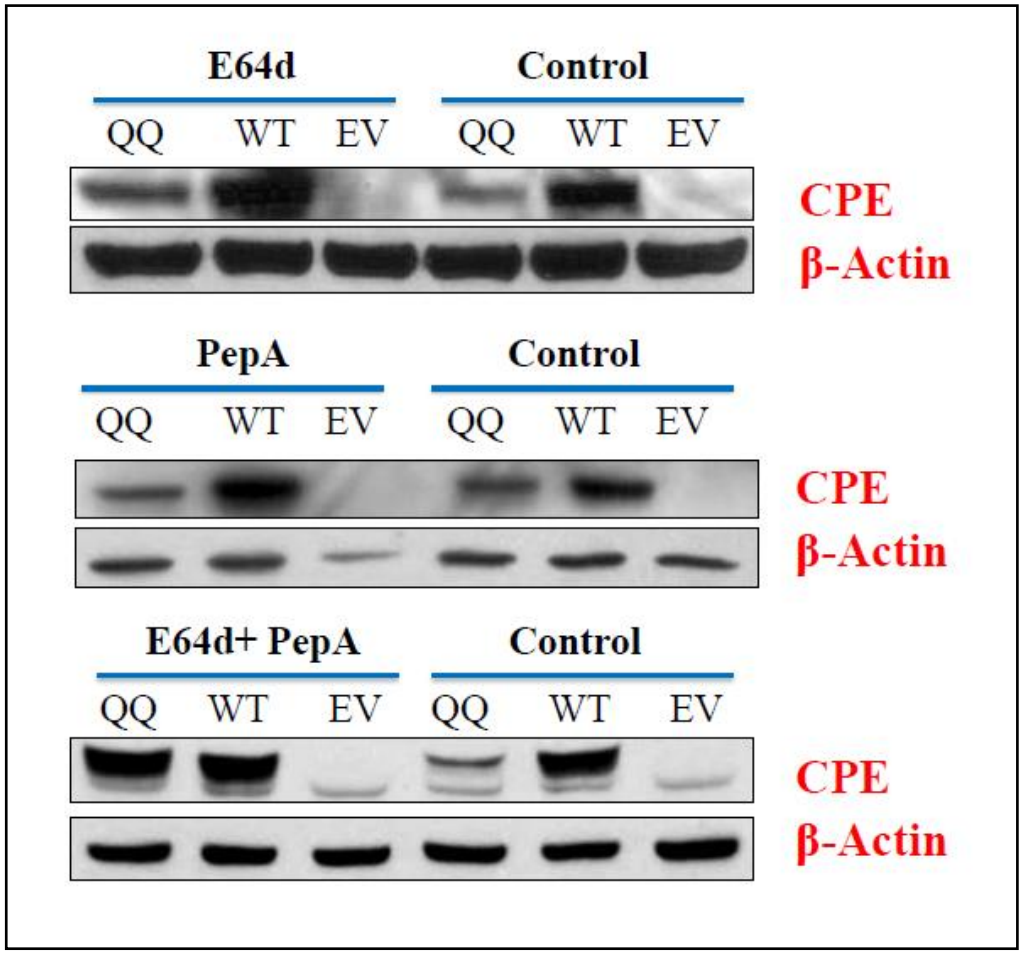

Figure 18. CPE expression in lysosomal inhibition. The N2A cells treated with E64d (20µM) and PepA (1µM) for 24h. The relative QQ CPE expression increases with

E64d treatment but was not affected from PepA treatment. The relative WT CPE expression was indifferent to the either treatments. Treatment with both E64d and PepA increased the QQ CPE rescue. 20µg protein loaded for each lane.

The optimized data for E64d and co-treatment with both inhibitors were shown in Table 4 and 5, respectively. The fold responses that normalized to the β-actin levels with mean band densities (n=6) represented on the graph for overall lysosomal inhibition studies (Fig.19). It was indicated that there was 3 fold difference between QQ treated and QQ control (*$p<0.01$) and 2 fold difference between WT treated and WT control ($p = 0.07$).

Table 4. Quantification of E64d treated cells.

	QQ	QQ Cont	WT	WT Cont	EV	EV Cont
CPE Folded Response	247	101	227	123	6	8
Beta Actin Folded Respone	95	88	88	89	88	93

Table 5. Quantification of E64d and Pepstatin A treated cells.

	QQ	QQ Cont	WT	WT Cont	EV	EV Cont
CPE Folded Response	146	39	137	117	12	11
Beta Actin Folded Respone	94	95	95	84	89	113

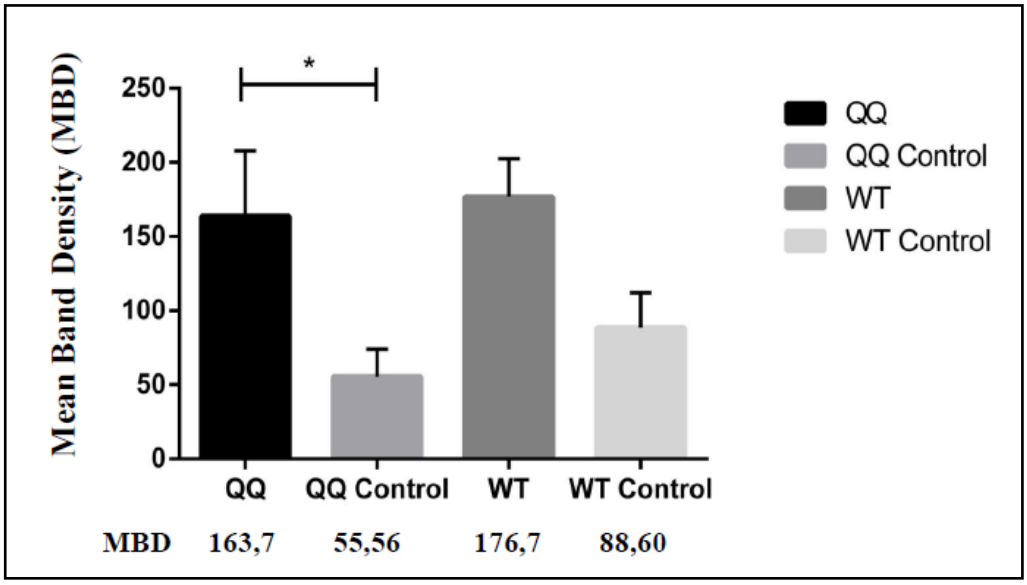

Figure 19. The fold response for the lysosomal inhibition. Western blots from the overall lysosomal inhibition studies were normalized to the β-actin levels. QQ CPE expression significantly increased (*p<0.01).

3.1.3. Calpain I Inhibition Studies

The Ca^{++} dependent Calpain pathway despite its limited proteolytic activity (Ono et al., 2010) in the absence of Calpain I (μ-Calpain) impairs the autophagy and increases the apoptosis (Demarchi et al, 2006; Demarchi and Schneider 2007). Therefore, the Ca^{++} dependent Calpain pathway was studies for QQ CPE degradation.

The expression studies with calpain Inhibitor I (MG101) demonstrated that calpain pathway was not involved in QQ degradation (Fig.20). The Fig.20 shows that despite inhibition of Calpain I proteases both QQ and WT CPE expressions were decreased slightly indicating that they were not degraded through the Calpain I proteolytic activity but some other way. The β-actin was used as a loading control to normalize the protein levels detected. The cells that are given the MG101 diluents were used as the control for MG101 treated cells. The EV transfected cells were used

as negative control for both QQ and WT transfected cells. This experiment was repeated two times.

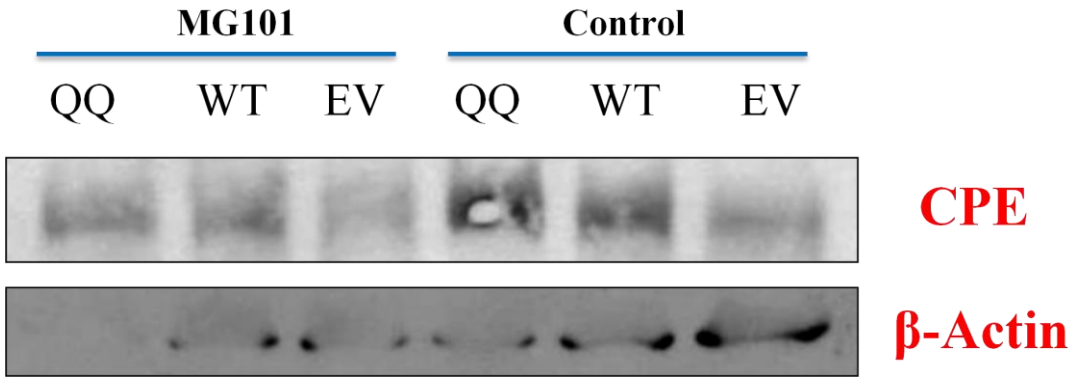

Figure 20. Calpain I inhibition: CPE expression in MG101 (5µM) treated N2A cells. Both QQ and WT expression slightly decreases in MG101 treated cells compared to their controls (EV: empty vector; negative control) which shows calpain pathway was not involved in QQ CPE degradation.

3.1.4. Co-Inhibition Studies

To observe the additive effect of both lysosomal and proteosomal inhibition N2A cells were co-treated with location specific inhibitors. First E64 and ZLLF (n=3) then E64d, Pepstatin A and MG132 (n=3) additive effects were studied. As it was shown in the Fig.21 ZLLF and E64 had had no effect separately, they showed additive effect together and rescued the QQ CPE. Since it was shown that both E64d and MG132 rescued the QQ CPE, when they used together (Fig.22) the increased rescue was as expected. The % rescue for each treatment was given in Table 7.

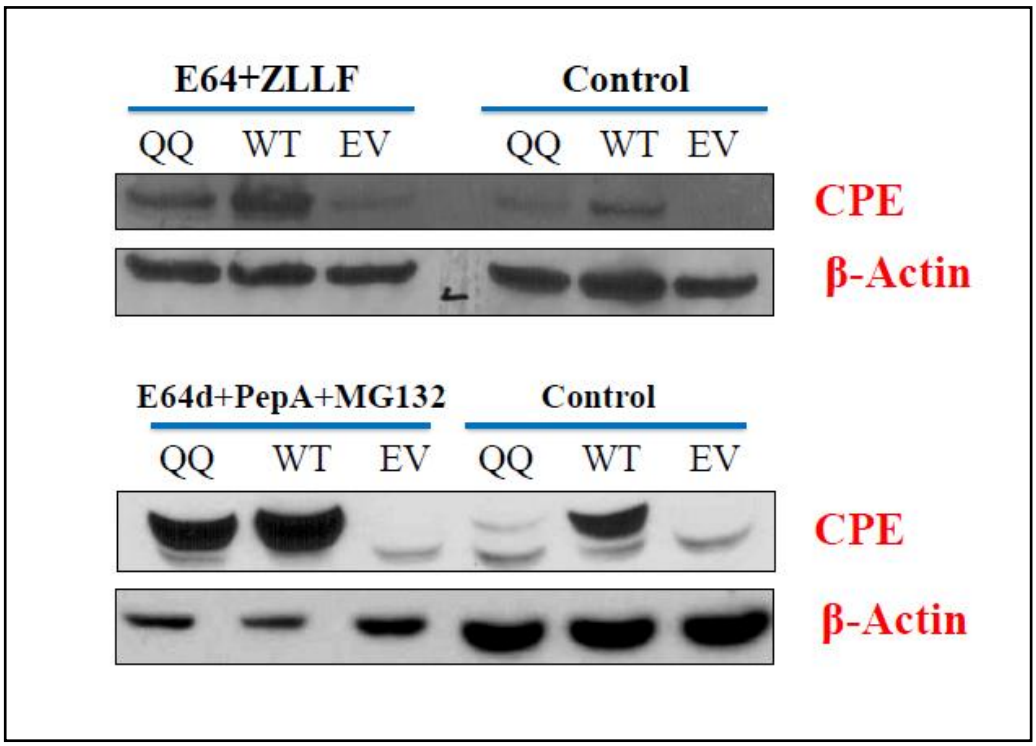

Figure 21. CPE expression in co-inhibited cells. The N2A cells treated with E64 (10µM), ZLLF (15µM), E64d (20µM), PepA (1µM) and MG132 (20µM) for 24h. The relative expression of both QQ and WT CPE increased with co-inhibition of proteosomes and lysosomes. 20µg protein loaded for each lane.

The optimized data for co-inhibition studies was shown in Table 6. The fold responses that normalized to the EV levels with mean band densities (n=3) represented on the graph for co- inhibition studies with E64d, PepA and MG132 (Fig.22). It was indicated that that there was a 1.9 fold difference between QQ treated and QQ control (p=0.64) and nearly no difference between WT treated and WT control (p = 0.91). Due to high variance and low sample number results were statistically insignificant.

Table 6. Quantification of co-inhibited cells.

	QQ	QQ Cont	WT	WT Cont	EV	EV Cont
CPE Folded Response	150	11	193	132	5	4
Beta Actin Folded Respone	1	118	1	92	1	118

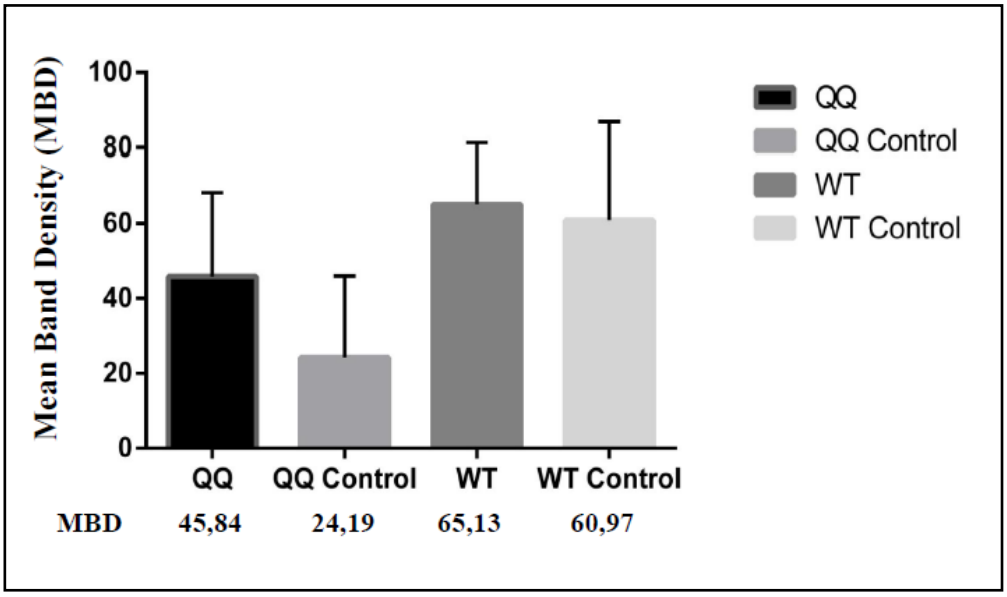

Figure 22. The fold response for co-inhibition studies. Western blots from the co-inhibition studies with E64d, PepA and MG132 were normalized to the EV levels. QQ CPE rescue was higher compared to the WT with co-inhibition of proteosomes and lysosomes.

The results of the inhibition of degradation pathways conclude that the Ca^{++} dependent calpain pathway was not used for QQ CPE degradation. Instead both lysosomes and proteosomes were involved in process. The % rescue, calculated based on treated over control ratio, suggesting proteosomal pathway preferred over

lysosomal pathway (Table 7). The WT CPE on the other hand was mainly degraded in proteosomes since lysosomal inhibition was not changed the relative expression of WT CPE.

Table 7. Percent rescue of CPE in inhibition studies

	QQ CPE Rescue %	WT CPE Rescue %	EV Rescue %
Lysosomal Inhibition	265	166	115
Proteosomal Inhibition	477	178	201
Co-Inhibition	1417	152	158

3.2. Aggregation Studies

To understand if the QQ CPE, failed to be secreted out, accumulates in the cell, proteins (QQ and WT CPE) were aimed to be studied in the native state. For this purpose His-tagged QQ and WT cDNA constructs were used. The N2A cells were transfected with His-tagged constructs and lysed with sonication on ice. Then His-tagged proteins were isolated using the Dynabeads, magnetic beads and run on native-PAGE. However the optimization of native-PAGE could not be concluded. Therefore proteins were run on the SDS-PAGE and analyzed with Western blot just to confirm the isolation process was successful. The primary antibodies were used against His, CPE and β-actin. The anti-His antibody is not monoclonal so non-specific bindings were observed. In the Fig.23 Western blot images of samples from each isolation steps were shown. The whole lysate (Fig.23.1) incubated with the magnetic beads for 7h and after incubation leftover sample collected (Fig.23.2). The

beads with attached His-tagged proteins were washed for 2 times (Fig.23.3-4). Then His-tagged proteins were eluted for 2 times (Fig.23.5-6). Then beads were incubated with reducing sample buffer (Fig.23.7). Samples were loaded at the equal volume so loading controls were not shown in the figure. In the process certain levels of proteins were lost (Fig.23.2-3). During the elution process proteins also were not eluted totally. The experiment was repeated for 6 times.

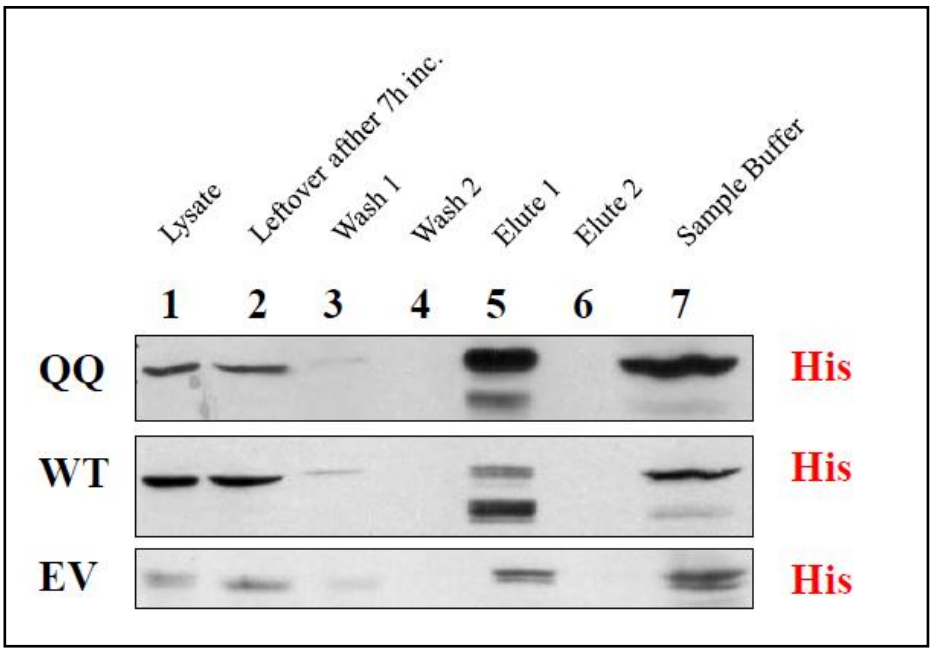

Figure 23. His-tagged protein expression. N2A cells transfected with his-tagged QQ, WT and EV constructs and proteins were isolated after 24h. The samples from each step subjected to the Western blot.

3.3. Cell Death Detection

The accumulation of misfolded or unfolded proteins in the cell causes the ER stress (Vembar and Brodsky, 2008). If these proteins fail to be degraded then the apoptotic pathways will be activated (Sano and Reed, 2013). Therefore it was of interest whether the presence of QQ CPE also triggers the apoptotic pathways and causes the cell death. To detect the apoptotic effect of QQ CPE on N2A cell, Roche Cell death detection Elisa^{Plus} kit was used (n=1). In the experiment 3 copies of the same cell batch were used. The apoptotic level detected based on the specific enrichment factor: positive (> 600 mU), negative (< 100 mU).

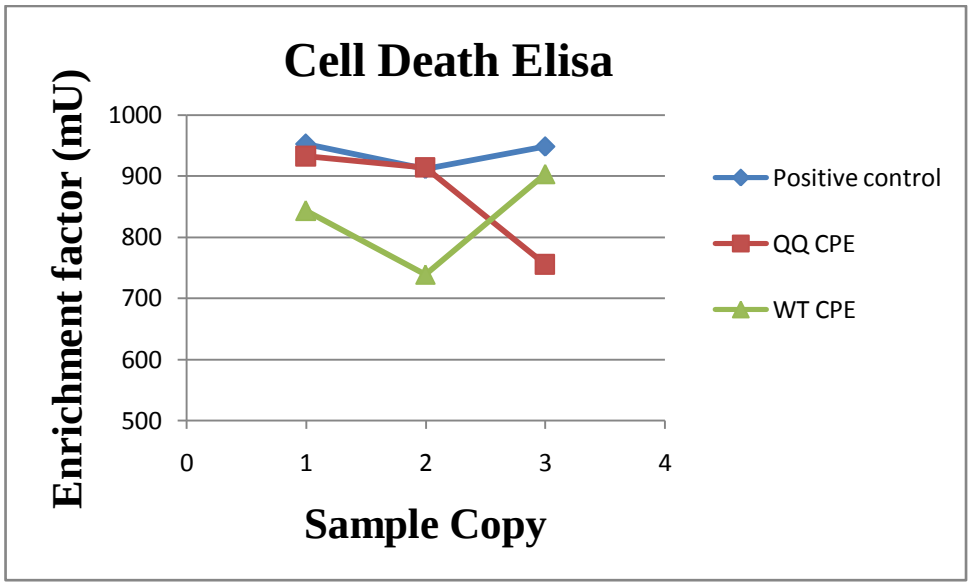

Figure 24. Cell death and QQ CPE. The cell death levels detected in the N2A cells transfected with QQ and WT CPE. Each sample contains three copies whose enrichment factor (mU) were calculated.

CHAPTER 4

DISCUSSION

The results of the inhibition of degradation pathways conclude that the Ca^{++} dependent calpain pathway was not used for QQ CPE degradation. Instead both lysosomes and proteosomes were involved in process. In the proteosomal inhibition, MG132 was more effective that the ZLLF. However MG132 treatment affected the β-actin levels negatively. This may be the result of both over-expression of CPEs and/or –inhibition of degradation pathways. Since the major proteolytic site of the cell was shut down it could be trying to overcome this by decreasing the production of certain the proteins. Another reason could be the accumulation of proteins that needs to be degraded that caused stress and increased the proapoptotic protein levels. Since β-actin was a substrate of proapoptotic enzymes (Kayalar *et al.*, 1996) its level was also influenced negatively. In the lysosomal inhibition, E64d was more effective than E64. E64 also inhibits the calpain I and with E64 treatments it was also confirmed that calpain pathway was not involved in degradation. In E64d treatments, QQ CPE was rescued with 265% ratio compared to its control. The PepA treatment did not affect the QQ and WT rescue. At high concentrations; the QQ expression was increased (n=1), however, loading control levels were inconsistently unbalanced (data not shown). Therefore PepA effect was concluded as inefficient but still used to achieve the complete inhibition of lysosome. In the co-inhibition studies shutting the both systems down increased the % rescue of QQ CPE but not affected the WT CPE rescue. It was concluded from the % rescue results that proteosomal pathway preferred over lysosomal pathway for QQ CPE degradation (Table 7). The WT CPE on the other hand was mainly degraded in proteosomes. In previous studies it was reported that mutant CPE (S202P) was also not sorted instead showed tissue specific degradation. In pituitary they all degraded in the ER and in pancreas partial

degradation occurred (Berman et al., 2001). In another study under ER stress driven conditions degradation of CPE in lysosomes was shown (Jeffrey et al., 2008). This study showed that the new mutant CPE (QQ) used proteosomal and lysosomal pathways for degradation.

The QQ levels at the controls were lower than the WT however it increased significantly with the proteosome and lysosome inhibition. The lower levels of QQ CPE at the controls may be the result of either low translation rate. This can be controlled by measuring the mRNA levels. This also be a result of rapid degradation of QQ CPE in the cell. This means that the presence of QQ CPE activates the degradation mechanisms rapidly.

The aggregation study indicated that His-tagged proteins were successfully isolated. Instead of native gel, aggregation of QQ CPE and its location could be shown with immunocytochemistry analysis where double staining of CPE and target region performed.

The cell death study was also not conclusive due to the lack of repeat. However, from the result we may speculate that the cells with QQ CPE were more prone to apoptosis than the cells with WT CPE.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

This study aimed to understand where the QQ CPE was degraded in the cell since QQ CPE cannot be sorted and enzymatically inactive and it impairs the secretion of WT CPE. For this purpose, three pathways were studied. These were Ca^{++} dependent calpain, proteosomal and lysosomal pathway. The Ca^{++} dependent calpain pathway was not involved in both QQ and WT degradation instead both proteosomal and lysosomal pathways were used. It was concluded from the % rescue that proteosomal pathway preferred over lysosomal pathway for QQ CPE degradation (Table 7). The WT CPE on the other hand was mainly degraded in proteosomes since lysosomal inhibition was not changed the relative expression of WT CPE.

Since the absence of CPE related to both neuroendocrine and neurodegenerative diseases; and also one of the QQ CPE found in the brain of Alzheimer patient, the relation of CPE with other neurodegenerative diseases could also be studied. In addition it could further studied whether the QQ CPE accumulate and aggregate in the cells. It was previously shown that the decrease of CPE leads to increase in ER stress mediated apoptosis therefore involvement of QQ CPE with ER stress and apoptosis could also be further investigated.

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APPENDIX A

CHEMICALS, REAGENTS, COMMERCIAL KITS & ENZYMES

Table A-1. Chemicals, reagents, commercial kits & enzymes

Chemicals	***Ordering information***	***Supplier***
30 % (w/v) Acrylamide/Bis solution 29:1 (3.3 % C)	10687.01	Serva
Acetic acid ($C_2H_4O_2$, 100%)	A3686-1L	AppliChem
Acetone deluxe Nntural	-	Etiler Kozmetik
Acrylamide (C_3H_5NO)	A3553-100G	Sigma
Agar	92144-500G	Fluka/Sigma
Agarose	A5093-500G	Sigma
Albumin standard	23209	Thermo Scientific
Ammonium peroxodisulfate($(NH_4)_2S_2O_8$)	1.01200.1000	Merck
Blotting grade non-fat milk powder	-	ConAgra Foods
Brilliant blue R ($C_{45}H_{44}N_3NaO_7S_2$)	B0149-25G	Sigma
Bromophenol blue ($C_{19}H_{19}Br_4O_5S$)	A2331,5G	AppliChem
Calpain inhibitor I ($C_{140}H_{227}N_{35}O_{44}S$)	C9181	Sigma
Cell death detection Elisa^{Plus}	11774425001	Roche
Chloroform ($CHCl_3$)	24216-2,5L	Sigma
Coomassie protein assay kit	23200	Thermo Scientific
Complete EDTA-free, proteinase inhibitor cocktail tablets	#1873580	Roche
di-Natrium hydrogen phosphate (HNa_2O_4P)	A2943-500G	AppliChem
DMSO	N182-5X10ML	Amresco
DNA ladder dye (6X)	#R0611	Thermo Scientific
Dynabeads His-Tag isolation and pulldown	101.04D	Invitrogen
E-64 ($C_{15}H_{27}N_5O_5$)	E3132	Sigma
E-64d ($C_{17}H_{30}N_2O_5$)	E8640	Sigma

Table A-1 (continued)

ECL Western blot detection reagent	#S RPN2134	GE Healthcare
EDTA ($C_{10}H_{16}N_2O_8$)	A5097,100G	AppliChem
Ethanol (C_2H_6O)	32221-2,5L	Sigma
Ethidium bromide	A1151,5G	AppliChem
Fetal bovine serum (FBS)	04-007-1B	Biological Industries
Glycerol ($C_3H_8O_3$)	GLY001.1	Bioshop
Glycine ($C_2H_5NO_2$)	33226-1KG	Sigma
Hydrochloric acid fuming 37%	1.00317.2500	Merck
Isopropanol (C_3H_8O)	24137-2,5L	Sigma
L-Glutamine solution	03-020-1B	Biological Industries
LB-Broth, Miller	71753-5-500G	Merck
Lipofectamine 2000	11668019	Invitrogen
Methanol (CH_4O)	24229-2,5L	Sigma
MG132(Z-Leu-Leu-Leu-Al, $C_{26}H_{41}N_3O_5$)	C2211	Sigma
M-PER mammalian protein extraction reagent	PHS007.500	Thermo Scientific
N,N'-Methylene bisacrylamide ($C_7H_{10}N_2O_2$)	M7279-100G	Sigma
Natriumdihydrogenphosphat 74onohydrate ($H_2NaO_4P.H_2O$)	A4229,1000G	AppliChem
Nitric acid (HNO_3, 65%)	84381-1L	Sigma
Opti-MEM	31985054	Invitrogen
Penicilin/Streptomycin	A2213	BiochromAG
Pepstatin A ($C_{34}H_{63}N_5O_9$)	P4265	Sigma
Phosphate buffered saline tablet	P4417-100Tab	Sigma
Ponceau S ($C_{22}H_{12}N_4Na_4O_{13}S_4$)	P3504-10G	Sigma
Potassium bicarbonate ($CHKO_3$)	60339-500G	Sigma
Potassium chloride (KCl)	1.04936.1000	Emsure
SOC medium	15544-034	Invitrogen
Sodium Chloride (NaCl)	A2942-500G	AppliChem
Sodium dedocyl sulfate ($C_{12}H_{25}NaO_4S$)	L4390-500G	Sigma
Sodium hydroxide pellets (NaOH)	06203	Sigma

Table A-1 (continued)

TEMED	0761-100ML	Amresco
Tris ($C_4H_{11}NO_3$)	A2264-1KG	AppliChem
Tryphan Blue ($C_{34}H_{24}N_5Na_4O_{14}S_4$)	A0668-10G	AppliChem
Trypsin-EDTA solution C	03-053-1B	Biological Industries
Tween 20	TWN508.1	Bioshop
Zefiran	-	Biomed
Z-Leu-Leu-Phe-CHO ($C_{29}H_{39}N_3O_5$)	C0358	Sigma
β-Mercaptoethanol	M3148-500ML	Sigma

APPENDIX B

PRIMARY AND SECONDARY ANTIBODIES

Table B-1. Primary antibodies

Primary Antibodies	***Size (kDa)***	***Ordering information***	***Supplier***
Anti-CPE, mouse	54	610759	BD Biosciences
Anti-CPE, mouse	54	MAB3587	R&D Systems
Anti-β Actin, rabbit	45	4970S	Cell Signaling
Anti-His, rabbit		ab125262	Abcam

Table B-2. Secondary antibodies

Secondary Antibodies	***Ordering information***	***Supplier***
Goat anti-mouse	ab97023	Abcam
Goat anti-rabbit	ab97051	Abcam

APPENDIX C

CONSUMABLE MATERIALS

Table C-1. Consumable materials

Consumable Materials	***Type***	***Supplier***
Assay plate, Costar	96 well	Corning
Cell culture dish	50cm^2	Corning
Cell culture flask, vent cap	25cm^2, 75cm^2	Corning
CL-XPosure Film	34089	Thermo Scientific
Cryogenic vial	2ml	Corning
Filtered pipette tips	10µl, 20µl, 200µl, 1000µl	Axygen
Filter System	500ml	Corning
Gel loading tips, Costar	200µl	Corning
Hyperfilm ECL 8x10 in	28-9068-37	GE Healthcare
Latex gloves, powder-free	Size S, M, L	ISOLAB
Microcentrifuge tubes	1.5 ml, 2 ml	Neptune
Multiwell cell culture plates	6, 12, 24 well	Corning
Nitril gloves, powder-free	Size S, M, L	ISOLAB
Nitrocellulose transfer membrane, Hybond ECL	RPN303D	GE Healthcare
Parafilm®	"M" laboratory film	Pechiney plastic packaging
Pasteur pipettes, sterile	084.22.001-3 ml	ISOLAB
Pipette tips	10µl, 20µl, 200µl,1000µl	Axygen
Plastic pipettes, Cellstar	5 ml, 10 ml, 50 ml	Greiner
Polystyrene, Polypropylene conical tube	15 ml, 50 ml	Corning
Protean extra thick blot paper	1703969	BioRad
Sertolab, bottle top filter	1 L (0.22 µm)	LLG

Table C-1 (continued)

Whatman paper	580x580mm	GmBH

APPENDIX D

EQUIPMENTS

Table D-1. Equipments

Equipment	***Model***	***Supplier***
4°C fridge	5092 NFY	Arçelik
-20°C freezer	-	Arçelik
-80°C freezer	Ultra low freezer	Thermo Scientific
Cell culture hood	Chemo cell-LRCX-UV	Teknomar
Centrifuge	Centrofriger-BL II	J.P. Selecta
CO_2 incubator	HERAcell 150	Heraeus
Electrophoresis apparatus	MQWL Easycast BI	Thermo Scientific
Gel documentation system	UV-Transillluminator	Vilber Lormat
Heating block	DMB-2	Q1 Topo
Incubator shaker	ZHWY-200B	Zhicheng
Liquid nitrogen tank	LS750	Taylor Wharton
Magnetic stirrer-AGE	F20320164	Velp Scientifica
Microplate absorbance reader	MultiscanGO	Thermo Scientific
Microwave	Intellowave MD 554	Arçelik
pH meter	WTW-pH 7110	Inolab
Pipette boy	Levo plus	Dragon Lab
Pipettes	P2, P20, P200, P1000	Thermo Scientific
Power supply	041BR83936	BioRad
Rocker	MR-1	Boeco
Shaker	PSU-2T	Boeco
Small microscope	CKX41	Olympus
Vacuum system	F110741	Gilson
Vortex, Wise mix	VM-10	Wisd Lab Instruments
Water bath	NB9	Nuve Bath
Ultrasonic processor	CP70T	Cole Palmer

Table D-1 (continued)

Western Blot analysis:		
Wet-blotting apparatus	Mini Trans-blot cell	BioRad
BioRad gel system	Mini PROTEAN tetra cell	BioRad
X-ray Film Processor	102	Kodak

APPENDIX E

PLASMIDS

pcDNA3.1 plasmids (Invitrogen, V790-20), QQ-CPE, WT-CPE, His-tagged QQ-CPE and His-tagged WT-CPE cDNA constructs, were kindly provided by Loh Lab (NIH, USA).

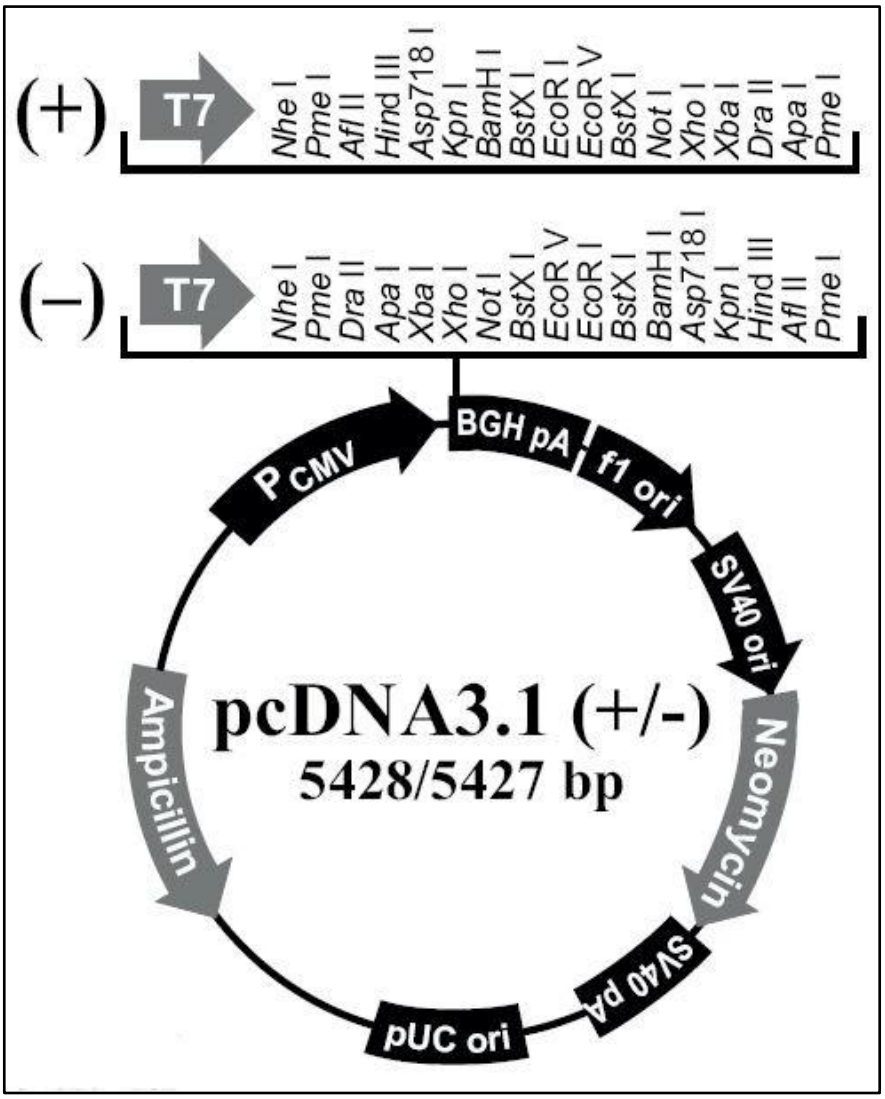

Figure E-1. pcDNA3.1 vector map. Retrieved from the Invitrogen user manual.

APPENDIX F

CELL LINES AND BACTERIA

Table F-1. Cell line(s)

Cell Line	***Origin***	***Culture Medium***
Neuroblastoma (Neuro2A)	Mouse	DMEM (High glucose), FBS

Table F-2. Bacteria

Bacteria Strain	***Genotype***
DH5α	F- φ80lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(rk-, mk+) phoA supE44 thi-1 gyrA96 relA1 tonA (confers resistance to phage T1

APPENDIX G

MEDIA AND STANDARD SOLUTIONS

1X Phosphate Buffered Saline Solution

PBS tablet	1
dH_2O	to 200 mL

Sterilized by autoclaving and stored at RT. One dissolved tablet yields 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4, at 25°C.

1M Tris-HCl, pH 7.4 or 8.0

Tris base	121.1 g
HCl	70 mL
HCl	42 mL
dH_2O	to 1L

Tris base was dissolved in 800 ml of dH_2O. Solution was adjusted to the desired pH with concentrated HCl, filled up to 1L total volume, sterilized by autoclaving and stored at RT.

50x Tris-acetate-EDTA (TAE) electrophoresis buffer

Tris base	242 g
Glacial acetic acid	57.1 mL
0.5 M EDTA (pH 8.0)	100 mL

dH_2O was added to 1L of total volume, the pH should be ~8.3. Stored at RT. Stock solution diluted to 1X with dH_2O to prepare both agarose gels and the electrophoresis buffer.

BSA Standards

Vials	Volume of Diluent	Volume and Source of BSA	Final BSA Concentration
A	0 µL	30 µL of stock	2 mg/mL
B	12.5 µL	37.5 µL of stock	1.5 mg/mL
C	32.5 µL	32.5 µL of stock	1.0 mg/mL
D	17.5 µL	17.5 µL of vial B dilution	0.75 mg/mL
E	32.5 µL	32.5 µL of vial C dilution	0.50 mg/mL
F	32.5 µL	32.5 µL of vial E dilution	0.25 mg/mL
G	32.5 µL	32.5 µL of vial F dilution	0.125 mg/mL
H	40 µL	10 µL of vial G dilution	0.025 mg/mL
I	40 µL	0 µL	0 mg/mL

BSA standards were prepared with respect to the diluents and stored at -20°C.

Tris-EDTA (TE) buffer, pH 8.0

Tris-HCl	10 mM
EDTA	1 mM

pH was adjusted to 8.0 by adding concentrated HCl and filled up to 1L volume. Sterilized by autoclaving and stored at RT.

Agarose gel (0.8%)

Agarose powder	0.4 g
1X TAE buffer	50 mL
EtBr	1 µL

The agarose powder mixed with the 1X TAE buffer and boiled in microwave for 2 min. When the agarose powder dissolved completely it was taken out of the microwave to cool down to the RT then EtBr was added and mixture was poured to the gel holder. As soon as the agarose mixture poured the combs were placed.

Blocking Solution for Elisa Microplates (5 %)

5 % (w/v) BSA	75 µL of stock solution
1X PBS	3 mL
0.025 % (v/v) Tween-20	3 µL

Prepared freshly and stored at 4°C.

APPENDIX H

MEDIA FOR BACTERIAL CULTURE

Luria-Bertani (LB) medium

LB powder	25 g
dH_2O	to 1L

Sterilized by autoclaving and stored at 4°C. Used with ampicillin with a concentration of 50µg/mL.

LB plates

LB agar	20 g
dH_2O	to 1L

Sterilized by autoclaving then antibiotics (50µg/mL ampicillin) were added and poured into petri dishes. The agar plates were stored at 4°C up to 2 weeks.

SOC Medium (Invitrogen, 15544-034)

Composition: 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose.

Aliquoted and stored at -20°C. Main stock was kept at -80°C. Before use prewarmed to the RT before use.

Antibiotics 1000x stock solution

Ampicillin	100 mg/mL

Aliquoted and stored at -20°C.

APPENDIX I

MEDIA AND SUPPLEMENTS FOR CELL CULTURE

DMEM-FBS growth medium

DMEM, High glucose (Biological Industries)	500 mL
FBS, European Grade(Biological Industries)	50 mL
Penicillin/Streptomycin	
L-Glutamine 29.2mg/ml in Saline (Biological Industries)	5 mL

Medium was kept at 4°C. Before use medium was incubated in water bath at 37°C.

DMEM-FBS freezing medium

DMEM-FBS growth medium	95 mL
DMSO	5 mL

Medium was kept at 4°C.

DMEM-FBS Transfection medium

DMEM, High glucose (Biological Industries)	500 mL
FBS, European Grade(Biological Industries)	50 mL
L-Glutamine 29.2mg/ml in Saline (Biological Industries)	5 mL

Medium was kept at 4°C. Before use medium was incubated in water bath at 37°C.

APPENDIX J

SOLUTIONS AND BUFFERS FOR WESTERN BLOT ANALYSIS

4x Tris-Cl/SDS (pH 8.8)

Tris base	18.2 g
SDS	0.4 g
H_2O	to 100 mL

pH adjusted with HCl or NaOH and stored at 4^{o}C.

4x Tris-Cl/SDS (pH 6.8)

Tris base	3.02 g
SDS	0.2 g
H_2O	to 50 mL

pH adjusted with HCl or NaOH and stored at 4^{o}C.

30% / 8% (w/v) Acrylamide Bisacrylamide

Acrylamide	30g
Bisacrylamide	0.8g
H_2O	to 100 mL

Stored at 4^{o}C and protected from the light.

10 % APS

APS	1.0 g
H_2O	to 10 mL

Dissolved and aliquoted then stored at -20^{o}C.

Coomassie Staining Buffer

Coomassie Brilliant Blue Reagent	2.5 g
Methanol (40%)	400 mL
H_2O	530 mL
Acetic acid (7% at last)	70 mL

Filtered and stored at RT, usable up to 2 months. First methanol and Coomassie BBR, and acetic acid with water mixed separately than mixed all together and filtered.

Coomassie Destaining Buffer

Methanol	200 mL
Acetic acid	200 mL
Isopropanol	200 mL
H_2O	to 2000 mL

Stored at RT.

4x sample buffer for reducing conditions

125m M Tris-Cl, pH 6.8	3.2 mL or	500mM Tris pH 6.8	2.5 mL
Glycerol 40%	4 mL or	50% Glycerol	8 mL
SDS 8%	0.8 g		
2-Mercaptoethanol 20%	2.0 mL		
Bromophenol blue 0.02%	2 mg		
H_2O	to 10 mL		

Aliquoted and stored at -20°C.

2x sample buffer for non-reducing conditions

1M Tris HCl pH 6.8	1.25 mL
Glycerol	2.5 mL
Bromophenol Blue	4 mg
H_2O	To 10 mL

Aliquoted and stored at -20°C.

Laemmli separating gel (10%)

H_2O	4.05 mL
1.5M Tris pH 8.8, 0.4% SDS	2.6 mL
30/8 % A/B	3.3 mL
10%APS	50µL
TEMED	5µL

Always prepared freshly, APS and TEMED were added just before pouring the gel.

Laemmli stacking gel (4%)

H_2O	6.1 mL
0.5M Tris pH6.8, 0.4% SDS	2.6 mL
30/8 % A/B	1.3 mL
10%APS	100 µL
TEMED	10 µL

Always prepared freshly, APS and TEMED were added just before pouring the gel.

Laemmli separating gel for non-reducing conditions (10%)

0.375M Tris pH 8.8	6.49 mL
30/8 % A/B	3.4 mL
10%APS	100 µL
TEMED	10 µL

Always prepared freshly, APS and TEMED were added just before pouring the gel.

Laemmli stacking gel for non-reducing conditions (4%)

0.375M Tris pH 8.8	4.275 mL
30/8 % A/B	0.67 mL
10%APS	50 µL
TEMED	5 µL

Always prepared freshly, APS and TEMED were added just before pouring the gel.

1x Electrophoresis Running buffer for non-reducing conditions (pH 8.3)

Tris base	6.057 g
Glycine	28.83 g
H_2O	to 2L

Buffer was stored at 4°C to be ready for use because Native-PAGE system was worked on ice to preserve the native structure of the sample protein.

10x Electrophoresis Running buffer (pH 8.3)

Tris base	30 g
SDS	10 g
Glycine	144 g
H_2O	to 1L

pH was adjusted with HCl to 8.3 and stored at RT, before use 1X buffer was prepared.

10x Transfer buffer

Tris base	30 g
Glycine	144 g
H_2O	to 1L

Mixed and stored at RT.

1x Transfer buffer

10X Transfer Buffer	100 mL
Methanol	200 mL
H_2O	to 1L

Prepared freshly before use.

10X TBS (pH 7.4)

Tris base	30 g
NaCl	80 g
KCl	2 g
H_2O	to 1L

pH was adjusted with HCl/NaOH to 7.4 and stored at RT, before use 1X buffer was prepared.

TBS Tween (TBS-T)

10X TBS	100 mL
Tween-20 (1%)	10 mL
H_2O	to 1L

Mixed and stored at RT.

5% Blocking solution

Milk powder	5 g
1X TBS-T	to 100 mL

Mixed and stored at 4^{o}C, prepared freshly, also1% blocking solution was prepared from 5% one freshly.

Stripping buffer

SDS 20%	2 mL or	SDS 10%	4 mL
β-Mercaptoethanol	160 µL		
1M Tris-Cl pH 6.7	1.25 mL or	0.5M Tris-Cl pH 6.8	2.5 mL
dH_2O	to 20 mL		

Mixed and stored at RT.

APPENDIX K

SOLUTIONS AND BUFFERS FOR HIS-TAGGED PROTEIN ISOLATION

His-Lysis Buffer (pH 8.0)

NaH_2PO_4	50 mM
NaCl	300 mM
Imidazole	2 mM
Tween 20	0.05%

pH was adjusted to 8.0 the buffer was filter sterilized and stored at 4°C.

His-Washing Buffer (pH 8.0)

NaH_2PO_4	50 mM
NaCl	300 mM
Imidazole	20 mM
Tween 20	0.05%

pH was adjusted to 8.0 then buffer was filter sterilized and stored at 4°C.

His-Elution Buffer (pH 8.0)

NaH_2PO_4	50 mM
NaCl	300 mM
Imidazole	500 mM
Tween 20	0.05%

pH was adjusted to 8.0 then buffer was filter sterilized and stored at 4°C.

APPENDIX L

AGAROSE GEL ELECTROPHORESIS RESULT

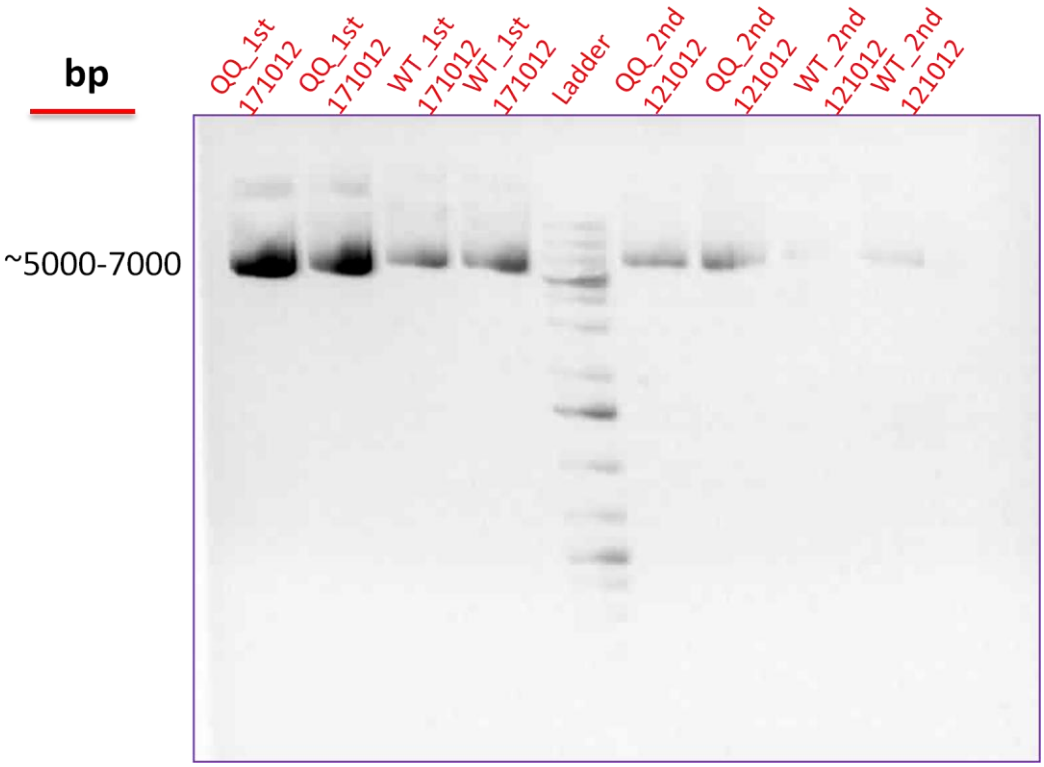

Figure L-1. Agarose gel electrophoresis for the isolated plasmid DNAs. First and second copy of the same WT and QQ CPE samples were shown. pcDNA3.1 was used for the constructs and molecular weight of the DNA was determined as expected.

APPENDIX M

GENERULER 1KB PLUS DNA LADDER

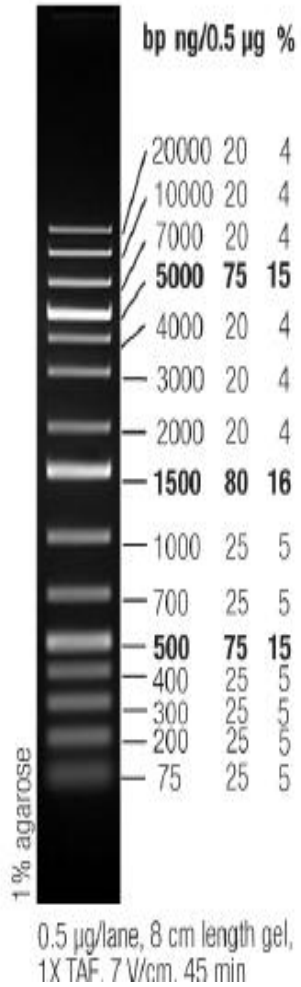

Figure M-1. Band profile of the Thermo Scientific GeneRuler 1kb Plus DNA Ladder 75 to 20.000 bp

APPENDIX N

PRESTAINED PROTEIN LADDER

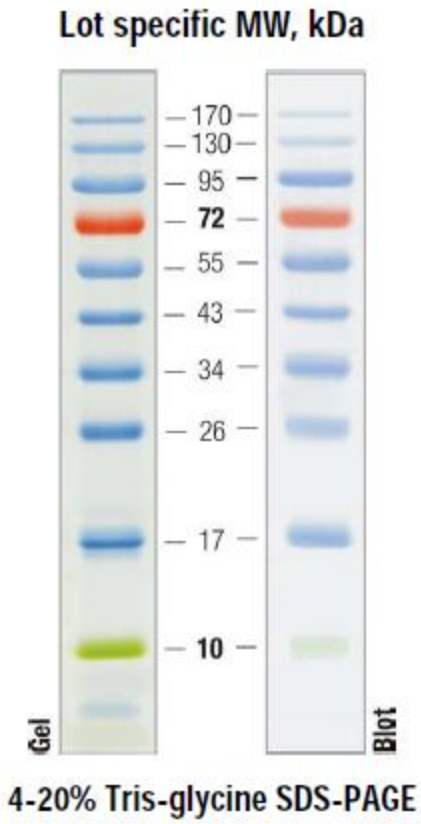

Figure N-1. Band profile of the Thermo Scientific PageRuler Prestained Protein Ladder