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STUDIES ON THE POTENTIAL REGULATION OF USP30 BY OMI/HTRA2 PROTEASE

by

SUNMI JIN

A thesis submitted in partial fulfillment of the requirements
for the Honors in the Major Program in Biomedical Sciences
in the College of Medicine
and in the Burnett School Honors College
at the University of Central Florida
Orlando, Florida

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Thesis Chair: Dr. Antonis S. Zervos

ABSTRACT

This thesis intends to determine whether the deubiquitinating enzyme ubiquitin-specific protease 30 (USP30) is cleaved by Omi/HtrA2 (hereafter referred to as Omi) serine protease during mitochondrial stress. USP30 is a mitochondrial protein that is anchored in the outer mitochondrial membrane and has components in the intermembrane space (IMS) as well as in the cytoplasm. USP30's IMS component has a six-amino-acid sequence that is very similar to Omi's consensus cleavage sites. Under normal conditions, Omi resides exclusively within the IMS; therefore, if Omi were to cleave USP30, it would target the part of the protein located in the IMS component. Omi is known to play a crucial role in a variety of diseases states including cancers, neurodegenerative diseases, and metabolic disorders. Since Omi is a serine protease, it is assumed to carry out its normal function through the direct cleavage and degradation of specific substrates. If USP30 deubiquitinase is a *bona fide* substrate of Omi, this will provide new and essential information on the mechanism by which Omi regulates the polyubiquitination process during mitochondrial stress.

ACKNOWLEDGMENTS

I would like to express my gratitude to my thesis committee members who have played an indispensable role in the success of this project. First and foremost, I am indebted to my thesis chair, Dr. Antonis S. Zervos, whose encouragement and support has helped make this project a reality. The opportunity to be a part of his lab under his guidance for the past two years has allowed me to gain a more thorough understanding of molecular biology and develop my critical thinking skills as a scientist and student. I am incredibly grateful to Dr. Lucia Cilenti whose expertise, patience, and mentorship have been vital to the completion of this project. Throughout the process, Dr. Cilenti served not only as a fantastic mentor and researcher but also as an incredible role model and without her, this project could never have been realized. I would also like to thank Dr. Robert Borgon and Dr. Qun Huo whose critiques and constructive criticism have allowed me to improve my thesis. One other great scientist who played a crucial role in the completion of this journey is Dr. Camilla Ambivero. Finally, I want to extend my thanks to the members of Zervos' lab – Shannon Manuel, Stephany Bustamante, and Jomar Carrasquillo – whose encouragement and friendship have provided me with invaluable support during my time within the lab.

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LIST OF ABBREVIATIONS

ALS…… Amyotrophic Lateral Sclerosis

ATP …….. Adenosine Triphosphate

dH_2O…… Deionized water

Drp1…….. Dynamin-Related Protein 1

DUB…….. Deubiquitinase

FIS1……... Mitochondrial Fission Protein 1

GTP……... Guanosine Triphosphate

HECT…… Homologous to E6A carboxy-terminus

IMM…….. Inner mitochondrial membrane

IMS…… Intermembrane space

JAMM……... JAB1/MPN/MOV34

KAN…….. Kanamycin

LB…….. Lysogeny broth

MFF……... Mitochondrial Fission Factor

Mfn…… Mitofusin

MINDY……. MIU-containing novel DUB family

MJD……... Machado-Joseph disease

MPP …….. Mitochondrial Processing Peptide

OD……. Optical density

OMM……. Outer mitochondrial membrane

OPA1……. Optic Atrophy Protein 1

OTP……... Ovarian Tumor Proteases

PARL……. Presenilins-associated Rhomboid-like Protein

PCR……... Polymerase Chain Reaction

PD…….. Parkinson's Disease

PINK1…… PTEN-Induced Putative Kinase Protein 1

RBR……... RING-Between-RING

RING……. Really Interesting New Gene

ROS……... Reactive oxygen species

RPM …….. Rotations per minute

SDS……..... Sodium dodecyl sulfate

SDS-PAGE…… Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TAE……Tris-Acetate-EDTA

TE ……..Tris-EDTA

TEMED …….Tetramethylethylenediamine

TIM ……... Translocase of the Inner Mitochondrial Membrane

TOM ……..... Translocase of the Outer Mitochondrial Membrane

UPS …….....Ubiquitin-Proteasome System

USP ……... Ubiquitin Specific Protease

UCTH ……... Ubiquitin Carboxy-Terminal Hydrolases

CHAPTER ONE: INTRODUCTION AND REVIEW OF LITERATURE

1.1 The Ubiquitin-Proteasome System

The controlled and selective method that cells use to degrade proteins is known as the ubiquitin-proteasome system (UPS). The system involves a small, 76 residue (8 kDa) post-translational modifier known as ubiquitin and a trio of enzymes: the ubiquitin-activating factor (E1), ubiquitin-conjugating factor (E2), and ubiquitin-ligating factor (E3) [1, 2]. In human cells, there are two E1 enzymes, more than 30 E2s, and over 1000 E3s [3]. With the assistance of the three factors, the Gly76 residue in the ubiquitin molecule is covalently linked to the ε-amino group of an accepting lysine residue in the target substrate [2]. The ubiquitin molecule may sometimes also be linked to the amino terminus of a target substrate, albeit less frequently [4].

To ubiquitinate a substrate, the ubiquitin molecule is first adenylated in an energy-dependent manner and is subsequently attached to a cysteine residue within an E1's active site via a thioester bond. The activated ubiquitin is then transferred to the cysteine residue in an E2's active site. An E2 forms a high-energy thioester bond to produce ubiquitin-charged intermediates that will eventually interact with E3s. In the final step, the small ubiquitin molecule is transferred to an E3 and is ligated to the epsilon amino group of a lysine residue in the substrate in one of two ways, depending on the family of enzymes that the E3 belongs to [1, 2, 4]. The over 1000 E3s can be separated into three families and two categories according to how ubiquitin is transferred to the substrate. The homologous to E6AP carboxy-terminus (HECT) and RING-between-RING (RBR) families utilize the indirect method whereby ubiquitin is transferred to the cysteine residue in the enzymes' active sites and subsequently ligated to the substrate. The really interesting new gene (RING) family of E3 enzymes does not have an active site and thus simultaneously binds to the

ubiquitin-charged E2 and substrate in order to facilitate ligating the molecule to the target directly [5].

Ubiquitination may take the form of monoubiquitination, in which the modifier is added only once, or polyubiquitination, in which multiple ubiquitin molecules form chains on a substrate by linking to one another [4, 6]. Polyubiquitin chains form by linking ubiquitin moieties to one another using seven possible lysine residues within the molecule. These lysine residues are now known to be Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63 [4]. There is evidence that polyubiquitin chains with different structures may fulfill different functions depending on where the ubiquitin molecules form linkages to one another [7]. The most well-understood instance of polyubiquitination results from chains that form Lys48 linkages; polyubiquitin chains longer than four moieties resulting from Lys48 linkages are targeted for degradation via the 26S proteasome for destruction [6, 7] (Figure 1).

Proteasomes are complexes of proteins which are responsible for the recycling and degradation of proteins within cells. The 26S proteasome is the specific complex of proteolytic proteins that is associated with ubiquitin-dependent protein degradation, and it is composed of one 20S and two 19S subunits [1, 6]. The 20S core complex is a stack of four heptameric rings, while the 19S caps (also known as the lid and base) are groups of 15 to 20 enzymes that are thought to include at least 6 ATPases and a deubiquitinating enzyme. The 19S subunits are responsible for recognizing polyubiquitinated sequences and unfolding proteins so that they may enter and access active sites of the core complex in what is thought to be an energy-dependent process [6].

Although the UPS is responsible for protein degradation and turnover, it plays many other essential roles as well. Some other important functions fulfilled by ubiquitination include sequestering proteins to prevent their activity and targeting specific cellular contents to certain

multivesicular bodies within the cell [8-10]. Given the large number of substrates that it can interact with, the UPS is imperative to the proper functioning of the cell and has been implicated in a number of important processes such as signal transduction, transcriptional regulation, the immune response, apoptosis, and receptor downregulation [1, 6]. Malfunction of the UPS has been demonstrated to be linked to a number of disease states, particularly those that are neurodegenerative, including Huntington's disease, Alzheimer's disease, and amyotrophic lateral sclerosis (ALS) [11]. It has also been associated with other pathological conditions such as cystic fibrosis, some genetic autoimmune disorders, and in a number of cancers such as colorectal cancer, breast cancer, and prostate cancer [1, 11].

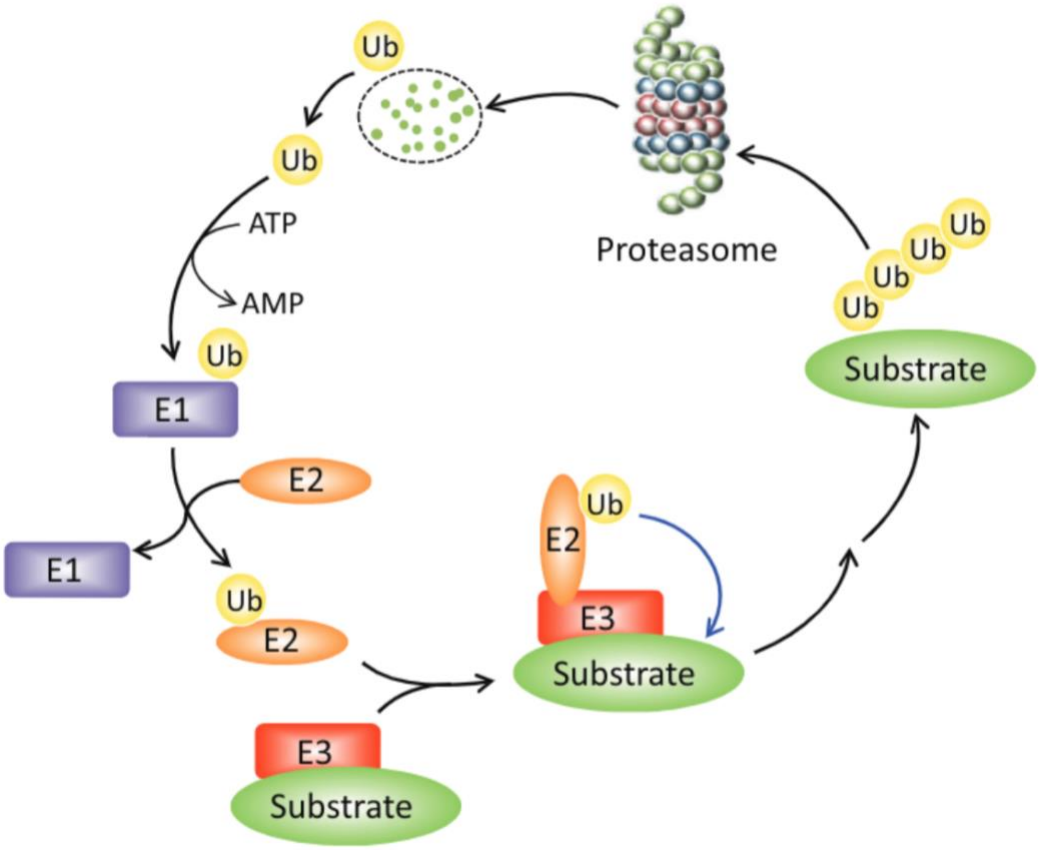

Figure 1: The Ubiquitination Process

Ubiquitination is a process that utilizes a three-enzyme cascade in order to transfer a ubiquitin molecule to the desired substrate. The three different enzymes are E1 (ubiquitin-activating factor), E2 (ubiquitin-conjugating factor), and E3 (ubiquitin-ligating factor). The two primary mechanisms that E3s utilize to ligate the ubiquitin moiety to the substrate are shown. In the above mechanism, ubiquitin is ligated indirectly by transferring it to the E3 active site (as in the case of the HECT and RBR families). In the below mechanism, ubiquitin is ligated directly without transfer to the E3 (as in the case of the RING family). [12]

1.2 Deubiquitination

In a process directly antagonistic to ubiquitination, deubiquitination is the process of removing ubiquitin from modified substrates. Deubiquitinases (DUBs) are responsible for removing or editing already-ligated ubiquitin molecules and therefore are involved in regulating the outcomes of the UPS [13]. Deubiquitinases are able to cleave ubiquitin molecules in polyubiquitin chains or directly from the surface of the protein substrate [14, 15].

The human genome codes for approximately 100 different deubiquitinating enzymes which fall into six different categories [16]. The five most well-characterized of these enzyme families are the ubiquitin carboxy-terminal hydrolases (UCTHs), the ubiquitin-specific proteases (USPs), the ovarian tumor proteases (OTPs), the Josephins, also known as Machado–Joseph disease proteases (MJDs), and the metalloproteases known as JAB1/MPN/MOV34 (JAMMs) [14]. The sixth and most recently discovered family of deubiquitinases are the MIU-containing novel DUB family (MINDYs) proteases [15, 16].

Deubiquitinating enzymes play an important role in regulating levels of free ubiquitin in the cell and in controlling the fate of ubiquitinated substrates, both through remodeling and through recycling [14]. Deubiquitinases recycle ubiquitin moieties at the proteasome and at the lysosome where they are responsible for cleaving ubiquitin before substrates are degraded [13, 14, 17]. They are important in remodeling ubiquitin chains on substrates as well, playing integral roles in determining the fates of protein substrates by potentially reversing the effects of ubiquitination and the UPS; this could take the form of changing the protein's activity and location or rescuing the originally ubiquitinated substrate from degradation [14]. Additionally, because ubiquitin is synthesized as a fusion protein in humans, deubiquitinases also have the responsibility of cleaving the fusion protein in order to generate free ubiquitin molecules (Figure 2). Some deubiquitinases

have also been found to be able to interact with E2s in order to prevent and interfere with the activity of E3 ligases [15]. Proper functioning of deubiquitinating enzymes depends on regulation within cells. Levels and activities of deubiquitinating enzymes are kept under control through various methods such as subcellular localization and post-translational modification. Subcellular localization also plays an important role in regulating deubiquitinase activity by determining which substrates the enzymes have access to, while post-translational modification such as phosphorylation and ubiquitination of deubiquitinating enzymes have been demonstrated to be crucial regulators in deubiquitinase activity [13, 14, 18].

Malfunction of deubiquitinating proteins can cause pathological states in many of the areas that the UPS regulates. The UPS and deubiquitination have been implicated in cell cycle regulation, intracellular signaling, transcriptional control, and their malfunctions have been linked to many neurodegenerative pathologies such as Machado-Joseph Disease, Parkinson's disease, and Alzheimer's disease [13, 14]. For this reason, understanding the significance of deubiquitination and its involved machinery is as important as deconstructing the impact of ubiquitination.

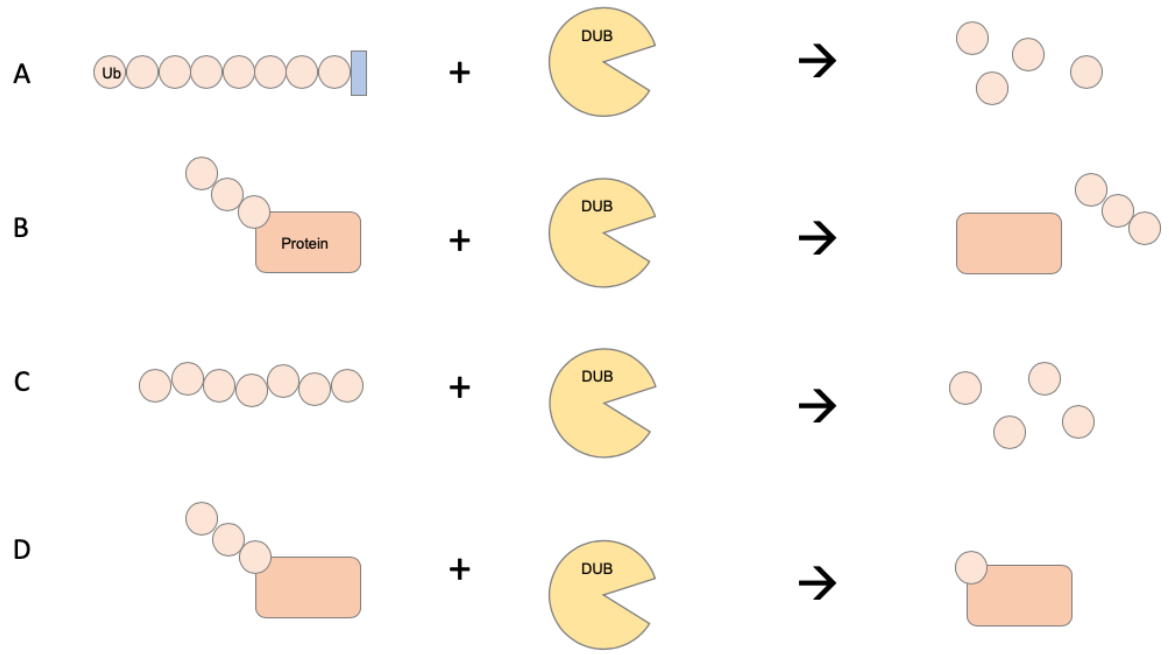

Figure 2: Functions and Processes of Deubiquitinating Enzymes

Deubiquitinases are crucial players in maintaining proper cell functioning. [3]

A. Ubiquitin in human cells is translated as a fusion protein. Deubiquitinases are responsible for processing and cleaving this linear fusion protein into useable free ubiquitin molecules
B. Deubiquitinases can rescue a protein targeted for degradation by removing polyubiquitin chains.
C. Chains that have been removed from substrates are cleaved deubiquitinating enzymes and recycled by processing them back into free ubiquitin for the cell to utilize.
D. Deubiquitinating proteins may change the fates of proteins that were originally tagged for destruction.

Different ubiquitin chains have been demonstrated to fulfill different functions. Deubiquitinases can edit polyubiquitin chains and change the fate of the ubiquitinated protein.

1.3 Mitochondrial Dynamics and Mitophagy

Mitochondria within cells exist in large, coordinated networks that are dynamic in nature; they are constantly shifting and changing in size, shape, and location [19]. These characteristics change because mitochondria in cells are constantly undergoing fusion (where two mitochondria come together to combine into one) and fission (where one mitochondrion splits into two) in order to discard damaged/dysfunctional portions of organelles and conduct quality control [19]. Increased oxidative stress may induce higher levels of mitochondrial fission as the organelles attempt to compensate for and discard damaged portions by targeting them for destruction and removal via cytochrome c release which may eventually lead to mitophagy [19]. Regulation of mitochondrial fusion and fission is thus essential to the survival of the cell.

Mitochondrial fusion is controlled mainly by mitofusin 1 (Mfn1), mitofusin 2 (Mfn2), and optic atrophy protein 1 (OPA1) [20] (Figure 3). Both Mfn1 and Mfn2 are anchored in the outer mitochondrial membrane (OMM) by their carboxy-terminus; their amino-terminus contains their catalytic activity. The cytosolic amino-terminus has GTPase activity that can facilitate binding to nearby mitochondria by providing required energy to fuse lipid bilayers [19]. They are essential for maintenance of the mitochondrial network within cells, and without them, mitochondrial fusion is shown to be impaired. OPA1 is a 120 kDa dynamin-like protein that is embedded within the inner mitochondrial membrane (IMM) that plays an integral role in cristae remodeling and mitochondrial fusion [21].

On the other hand, fission of mitochondria is regulated by a variety of proteins including dynamin-related protein 1 (Drp1), mitochondrial fission 1 protein (FIS1), mitochondrial fission factor (MFF), and mitochondrial dynamic proteins 49/51 kDa (MiD49/51) [19]. Drp1 is a GTPase that functions to mechanically constrict around the mitochondria and facilitate the early stages of

fission. FIS1 is anchored in OMM and serves to aid in forming the fission complex. In mammalian cells, MFF behaves as a mitochondrial receptor for Drp1. MiD49/51 are components of the fission complex that forms and without these proteins, Drp1 translocation to the mitochondrial decreases and mitochondria divide less frequently [22].

Mitophagy is a specialized form of autophagy that the cell uses in order to degrade and dispose of mitochondria that have been damaged. It is selective and non-random [23]. Its main function is mitochondrial quality control whereby it facilitates maintenance of a healthy mitochondrial network within the cell [24]. Mitochondrial damage may occur due to a number of factors or events. One of the main components in mitochondrial damage includes the production of reactive oxygen species (ROS) which can damage the cell. Some of the most prevalent ROS found in cells are superoxide anion, hydrogen peroxide, and hydroxyl radical, all of which are toxic byproducts of oxidative phosphorylation [25]. Mitochondria that sustain damage from an over-accumulation of ROS may experience IMM depolarization, which signals cues to begin mobilizing autophagy machinery [23]. Therefore, the cell protects itself from apoptosis by ensuring that damaged mitochondria are removed [19].

The most well-characterized pathway by which mitophagy initiates involves the functions of the serine-threonine kinase known as PTEN-induced putative kinase protein 1 (PINK1) and an E3 ligase known as Parkin [26]. Under normal conditions, mitochondria have high membrane potentials and PINK1 is able to pass through the translocase of the outer and inner membrane complex (TOM/TIM complex) and be cleaved by two proteins mitochondrial processing peptidase (MPP) and presenilins-associated rhomboid-like protein (PARL). When the membrane depolarizes in unhealthy mitochondria, the import of PINK1 is prevented and it accumulates on the surface of

the mitochondria, recruiting E3 ligase Parkin which ubiquitinates the mitofusin proteins and the outer membrane of the organelle [25, 26].

While mitophagy is essential to maintaining a healthy mitochondrial population and proper cellular function, its regulation is as well. One method through which this regulation is accomplished is the utilization of previously discussed deubiquitinating enzymes, which can act as negative regulators of mitophagy [26].

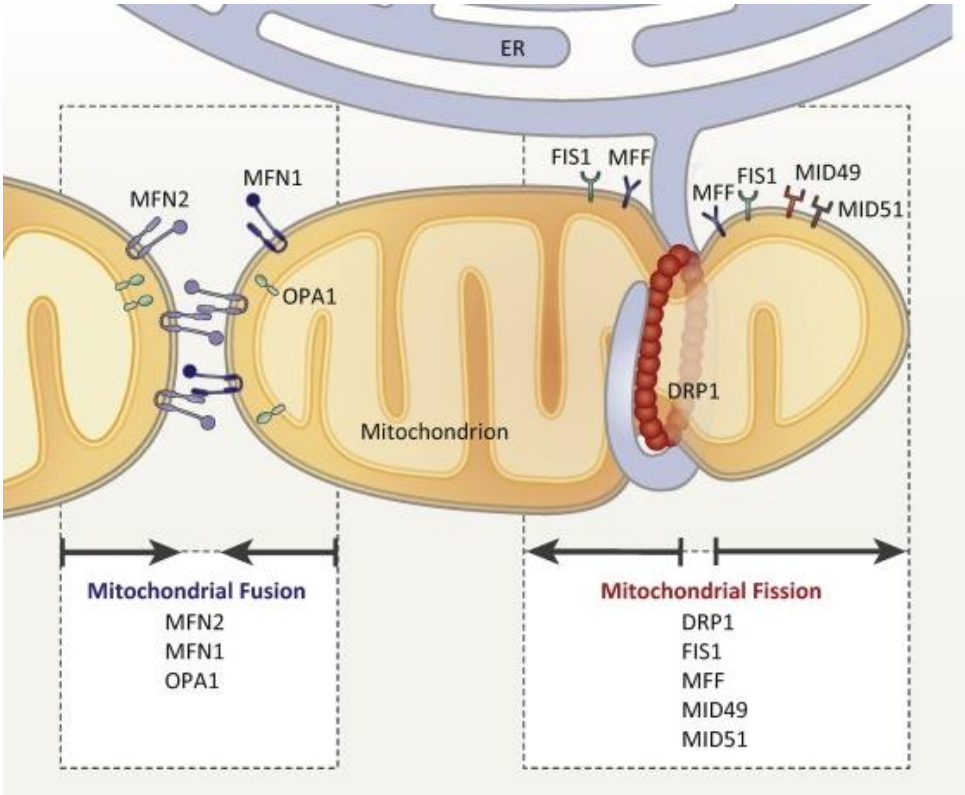

Figure 3: Mitochondrial Fusion and Fission

Mitochondrial fusion is mediated by Mfn1, Mfn2, and OPA1 proteins. Mfn1 and Mfn2 are responsible for outer mitochondrial membrane fusion while OPA1 mediates inner mitochondrial membrane fusion and cristae remodeling. Mitochondrial fission is facilitated by Drp1, FIS1, MFF, and MiD49/51. Drp1 functions as a mechanoenzyme that wraps around the circumference of a mitochondrion in order to physically constrict the organelle. FIS1 and MFF help Drp1 form the fission complex and MiD49/51 assists in Drp1 translocation to the mitochondria. The endoplasmic reticulum of the cell also facilitates physical constriction around the mitochondria during fission. [22]

1.4 Omi/HtrA2

Omi, also known as high temperature requirement protein A2 (HtrA2) is a 458 amino acid protein with molecular weight 49 kDa. It is an ATP-independent serine protease that resides within intermembrane space (IMS) of the mitochondria under normal conditions and maintains a healthy mitochondrial population [27, 28]. Under conditions of stress, Omi translocates to the cytosol of the cell where it facilitates cell death [29]. Mature and functional Omi, composed of amino acids 134-458, is a pyramidal, homotrimeric protein; at the top of this pyramid is an inhibitors of apoptosis protein (IAP) binding motif, also known as an IBM, which is responsible for Omi's interactions with IAPs. At the base of this pyramidal protein are three PDZ domains that function in substrate access to the active site within [30].

The function of Omi depends upon its location within the cell. When Omi translocates to the cytosol in response to apoptotic stimuli, it mediates both caspase-dependent and caspase-independent cell death via its serine-protease domain [31]. After proteolytic activation, Omi facilitates caspase-dependent cell death by binding to IAPs, thus deterring their function and triggering activation of caspases. For this reason, Omi was originally thought to be a proapoptotic protein [32]. However, when it is present within the IMS, Omi has been demonstrated to play integral roles in eliminating misfolded proteins. In cells lacking functional Omi, increased levels of an E3 ubiquitin ligase known as Mulan causes a decrease in Mfn2, resulting in higher levels of mitophagy [31]. In mice with nonfunctional Omi due to a Ser276Cys mutation, a progressive neuromuscular disease state known as motor neuron degeneration 2 (mnd2) develops [33]. Overexpression of Omi in transgenic mice led to a normal phenotype rather than showing an increase in apoptosis; this observation supports Omi function in neuroprotection, rather than apoptosis [32, 34].

Omi has been implicated in certain neurodegenerative disorders. Mnd2 mice exhibit many of the distinctive features of Parkinson's disease (PD) including reduced body weight, reduced organ size, muscle atrophy, loss of neurons in the basal ganglia's striatum, and early death [33]. Some studies have also linked Omi to Alzheimer's disease, as Omi has been demonstrated to interact with presenilin-1. The presenilin-1 gene has been identified as a pathogenic locus involved in Alzheimer's disease [35].

1.5 Ubiquitin Specific Protease 30

Ubiquitin specific protease 30 (USP30)–also known as deubiquitinating enzyme 30, ubiquitin thioesterase 30, ubiquitin-specific-processing protease 30, and ubiquitin carboxy-terminal hydrolase 30–is a 517 amino acid mitochondrial deubiquitinase protein that is anchored in the OMM within the cell (Figure 4). It is the only deubiquitination enzyme that is directly tethered to the OMM [36]. The enzyme has a domain in the mitochondrial IMS (1-35), a single transmembrane helix domain (36-56), and a catalytic domain in the cytoplasm (57-517) [37].

The catalytic activity of USP30 is responsible for negatively regulating Parkin-mediated mitophagy. When USP30 is mutated (Cys77Ser) to a catalytically inactive form, the enzyme is no longer effective at preventing mitophagy, insinuating that USP30's deubiquitinating activity is integral in stopping it [36, 38]. USP30 functions in an antagonistic manner to Parkin by removing ubiquitin molecules from substrates which were added by Parkin and is thus able to oppose Parkin-mediated mitophagy [24]. *In vitro*, USP30 preferentially cleaves Lys6, Lys48, Lys11, and Lys63 residues in order of most efficiently to least [24, 26, 39, 40]. Underexpression of USP30 with concomitant overexpression of Parkin promotes mitophagy when mitochondria are induced to depolarize; overexpression of USP30 reverses ubiquitination completed by Parkin and inhibits mitophagy. This demonstrates that the deubiquitinase is a key inhibitor of mitophagy and that it works in opposition to Parkin [24].

USP30 is additionally thought to regulate mitochondrial morphology and dynamics by facilitating the deubiquitination of Mfn1 and Mfn2; USP30's depletion using RNA interference has been demonstrated to result in heightened levels of mitochondrial fusion and longer, more interconnected mitochondrial networks [39].

USP30 has recently been the target of inhibition in treatments regarding certain neurodegenerative disorders, especially PD. Mutations in Parkin, which have been implicated in certain types of PD, are thought to be able to be compensated for by inhibition of USP30's activity. Removal of damaged or dysfunctional mitochondria is crucial in controlling excessive oxidative stress [26]. For this reason, it is thought that inhibition of USP30 as a negative mediator of mitophagy promotes mitochondrial quality control in cells by promoting clearance of damaged mitochondria. It has been demonstrated that by essentially removing the brakes on mitophagy in this way, clearance of damaged mitochondria was restored in Parkin-deficient cells [24].

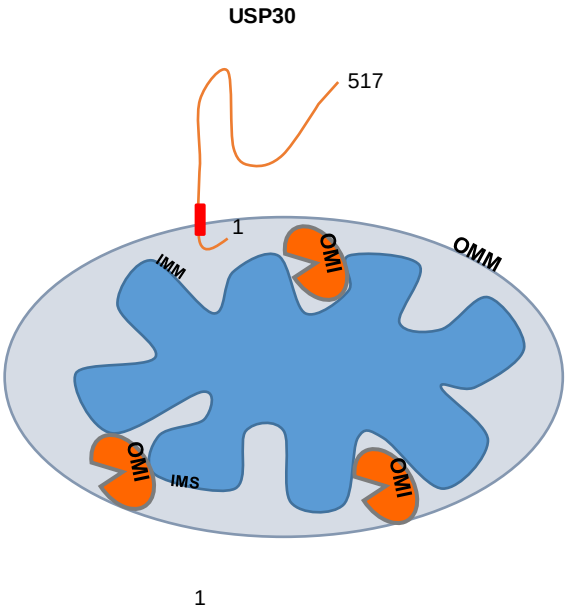

Figure 4: USP30 in the Outer Mitochondrial Membrane

USP30 is anchored in the outer mitochondrial membrane. It is a 517 amino acid protein with amino acids 1-35 in the intermembrane space, amino acids 36-56 spanning the OMM, and amino acids 57-517 in the cytoplasm of the cell. It is one of four proteins that are anchored to the OMM but is the only one that possesses deubiquitination activity. Omi is a serine protease that resides within the IMS of the mitochondria.

CHAPTER TWO: MATERIALS AND METHODS

2.1 Construction of cDNA Corresponding to E2E3-USP30-E2D1 Recombinant Protein

2.1a Construction of Fusion Protein Using Polymerase Chain Reaction

The cDNA corresponding to the fusion protein consisting of ubiquitin-conjugating enzyme E2E3 (E2E3), the IMS component of USP30, and ubiquitin-conjugating enzyme E2D1 (E2D1) (hereafter referred to as the fusion or recombinant protein of interest E3-USP30-D1) was created using a series of five different polymerase chain reactions (PCRs) (Figure 5). Table 1 lists the utilized primers for each reaction, the cDNA sequence each primer amplified, and the restriction site each primer contains. In reaction 1, the cDNA corresponding to E2E3 was amplified using forward primer TZ456 with an MfeI cut site and reverse primer TZ577 (Figure 5a).

Although the multiple cloning site of the pET-28a+ vector has restriction sites for EcoRI and XhoI within it (Figure 6), a primer with a cut site for EcoRI could not be utilized for this experiment because the cDNA sequence of E2E3 contains an EcoRI restriction site (Figure 7). If EcoRI and XhoI were used to digest the cDNA corresponding to the fusion protein, EcoRI would cleave it and render protein expression impossible. A primer with a restriction site for MfeI was utilized instead because the MfeI restriction endonuclease produces overhangs identical to the overhangs of EcoRI's restriction site but does not cleave the restriction site of EcoRI in the cDNA of E2E3 (Figures 7 and 8). Therefore, while EcoRI and XhoI were used to cleave the vector, MfeI and XhoI were used to cleave the insert so EcoRI would not cut the cDNA corresponding to the fusion protein.

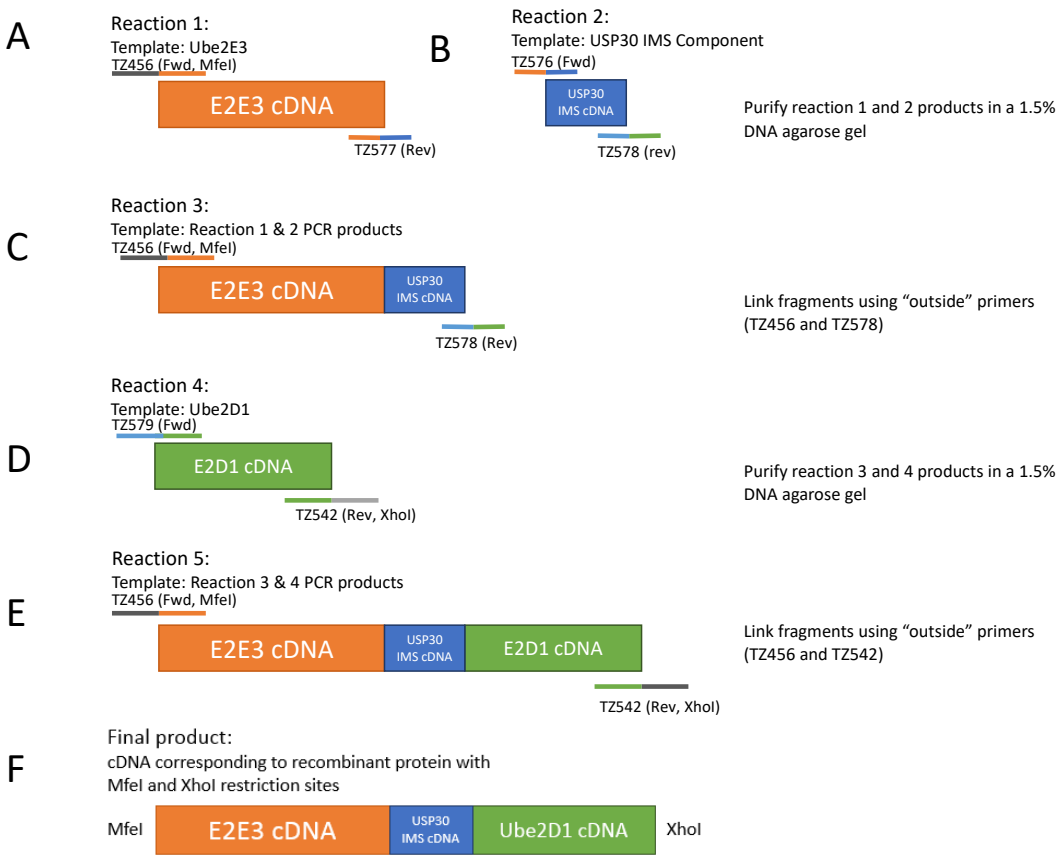

Figure 5: Overview of Fusion Protein Synthesis

An E3-USP30-D1 fusion protein was constructed to facilitate determination of whether Omi cleaves USP30's IMS component. Colors in the primers denote homology to the cDNA sequences with their same color (e.g., orange primer denotes homology to cDNA associated with E2E3).

A. In reaction one, forward primer TZ456 with an MfeI restriction site and reverse primer TZ577 were used to amplify E2E3 cDNA.
B. In reaction two, forward primer TZ567 and reverse primer TZ578 were used to amplify the USP30 IMS fragment's cDNA. The products of reactions one and two were purified in a 1.5% agarose gel.
C. In reaction three, the purified products of one and two were used as the PCR reaction template. The outside primers – forward primer TZ456 with an MfeI cut site and reverse primer TZ578 – were used to link the products from reaction one and two.
D. In reaction four, forward primer TZ579 and reverse primer TZ542 with an XhoI restriction site were used to amplify the E2D1 cDNA.
E. In reaction five, the purified products from reactions three and four were used as the PCR reaction template. The outside primers – forward primer TZ456 and reverse primer TZ542 – were used to link the products and create the fusion protein cDNA.
F. Pictured is the cDNA corresponding to the E3-USP30-D1 fusion protein with restriction sites for MfeI and XhoI that will be ligated into the pET-28a+ vector.

Table 1: Primer Sequences for Fusion Protein

Primer	cDNA Amplified	Sequence (5' → 3')	Restriction Site
TZ456 (Forward)	E2E3	GCGCAATTGATGTCCAGTGATAGGCAAAGGTC	MfeI
TZ577 (Reverse)	E2E3	CGGCCCGGGAGCTCAGTGTTGCGTACTTGGTCC	-
TZ576 (Forward)	USP30 (IMS)	GGACCAAGTACGCAACACTGAGCTCCCGGGCCG	-
TZ578 (Reverse)	USP30 (IMS)	TCCTCTTCAGCGCCAGTTCTTCATGACTTT	-
TZ579 (Forward)	E2D1	AAAGTCATGAAGAACTGGCGCTGAAGAGGA	-
TZ542 (Reverse)	E2D1	CCGCTCGAGTTACATTGCATATTTCTGAGTCCA	XhoI

Forward and reverse primers used to amplify the cDNA sequence of the E3-USP30-D1 fusion protein.

pET-28a(+) sequence landmarks	
T7 promoter	370-386
T7 transcription start	369
His•Tag coding sequence	270-287
T7•Tag coding sequence	207-239
Multiple cloning sites	
(*Bam*H I - *Xho* I)	158-203
His•Tag coding sequence	140-157
T7 terminator	26-72
lacI coding sequence	773-1852
pBR322 origin	3286
Kan coding sequence	3995-4807
f1 origin	4903-5358

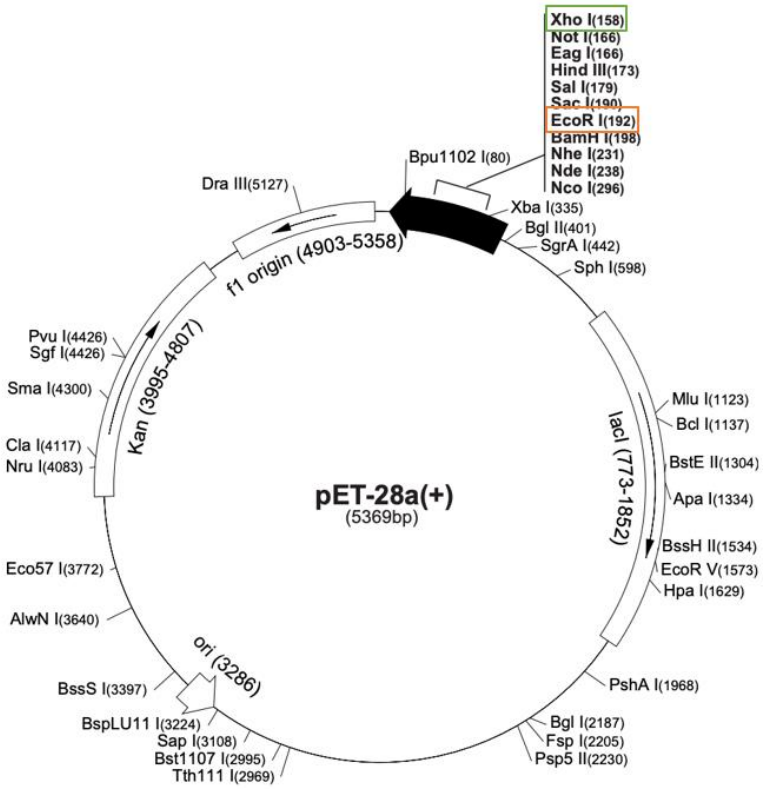

Figure 6: Overview of pET-28a+ vector

Overview of pET-28a+ bacterial expression vector including T7 lac promoter, addition of an amino-terminus His-tag, kanamycin resistance, multiple cloning sites, etc. The restriction sites of XhoI (green) and EcoRI (orange) are present.

E2E3 Nucleotide Sequence (624 nt)

ATGTCCAGTGATAGGCAAAGGTCCGATGATGAGAG
CCCCAGCACCAGCAGTGGCAGTTCAGATGCGGACC
AGCGAGACCCAGCCGCTCCAGAGCCTGAAGAACAA
GAGGAAAGAAAACCTTCTGCCACCCAGCAGAAGAA
AAACACCAAACTCTCTAGCAAAACCACTGCTAAGT
TATCCACTAGTGCTAAAAGAATTCAGAAGGAGCTA
GCTGAAATAACCCTTGATCCTCCTCCTAATTGCAG
TGCTGGGCCTAAAGGAGATAACATTTATGAATGGA
GATCAACTATACTTGGTCCACCGGGTTCTGTATAT
GAAGGTGGTGTGTTTTTTCTGGATATCACATTTTC
ATCAGATTATCCATTTAAGCCACCAAAGGTTACTT
TCCGCACCAGAATCTATCACTGCAACATCAACAGT
CAGGGAGTCATCTGTCTGGACATCCTTAAAGACAA
CTGGAGTCCCGCTTTGACTATTTCAAAGGTTTTGC
TGTCTATTTGTTCCCTTTTGACAGACTGCAACCCT
GCGGATCCTCTGGTTGGAAGCATAGCCACTCAGTA
TTTGACCAACAGAGCAGAACACGACAGGATAGCCA
GACAGTGGACCAAGAGATACGCAACATAA

Figure 7: Nucleotide Sequence for E2E3

Figure 7 shows the 624-nucleotide sequence of the DNA sequence corresponding to the E2E3 protein, which was one of the three parts of the cDNA for the fusion protein (Figure 5a). Outlined in orange is the restriction site for the restriction endonuclease EcoRI. If EcoRI was used to digest the cDNA insert, it would cut in the middle of the sequence. For this reason, EcoRI could not be utilized to prepare the cDNA for ligation, and MfeI was used instead.

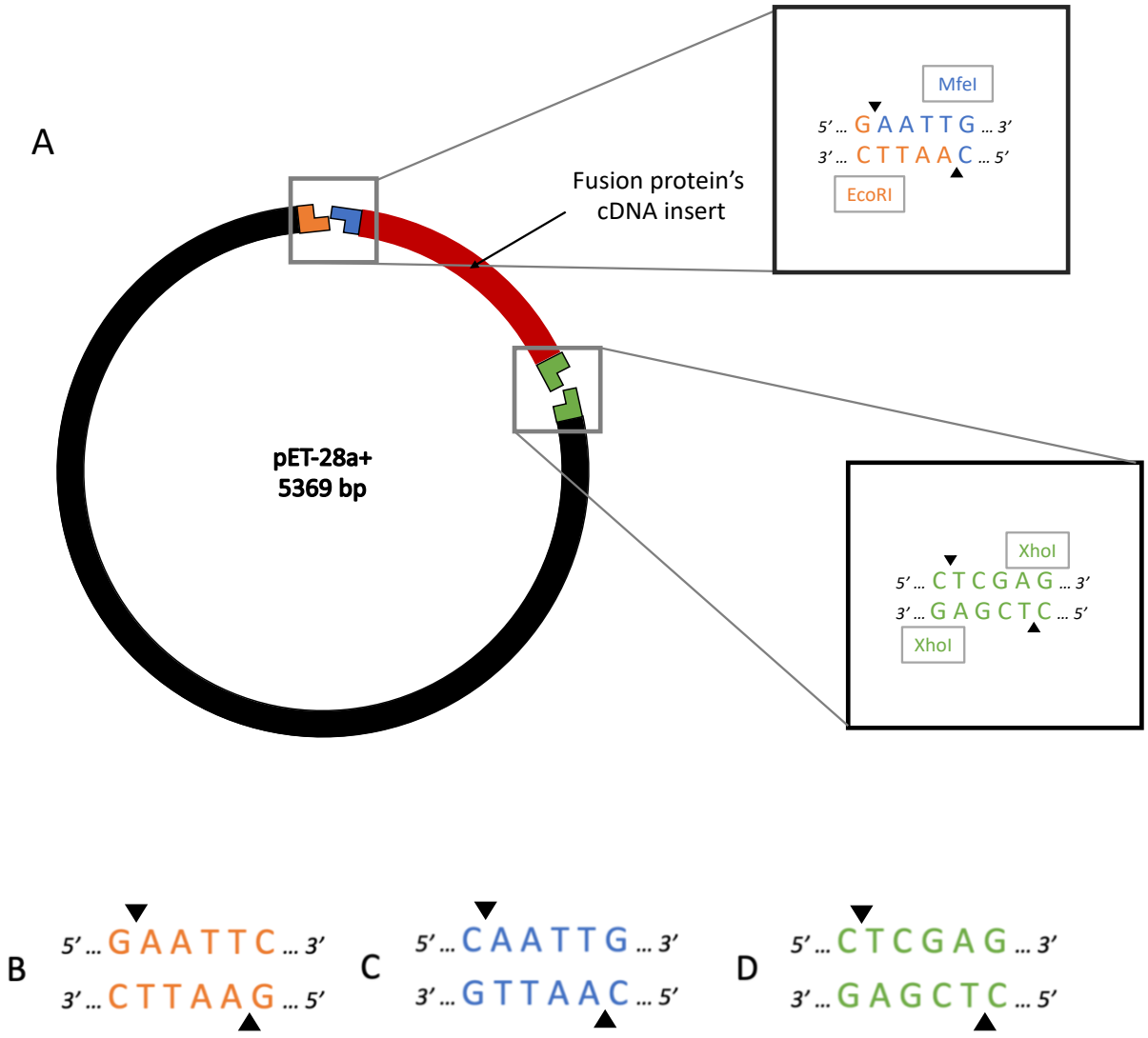

Figure 8: Justification for the use of MfeI Restriction Endonuclease

Represented in red is the cDNA corresponding to the recombinant E3-USP30-D1 protein and represented in black is the pET-28a+ vector. Orange denotes the restriction site for EcoRI, blue the site for MfeI, and green the site for XhoI.

A. Black boxes show the ligated overhangs of the respective endonuclease restriction sites. The overhangs for the restriction sites of EcoRI and MfeI are complimentary and thus can anneal.
B. The restriction site for EcoRI
C. The restriction site for MfeI
D. The restriction site for XhoI

As seen in Figure 5b, the cDNA corresponding to the IMS component of USP30 was amplified using forward primer TZ576 and reverse primer TZ578 for reaction 2. After the products from reactions 1 and 2 were purified using a 1.5% DNA agarose gel using the protocol outlined in section 2.1b, they were used as the template for reaction 3 and amplified using the outside primers (forward primer TZ456 with an MfeI cut site and reverse primer TZ578); this subsequently produced the fusion of the cDNA corresponding to E2E3 and the cDNA corresponding to USP30's IMS component (Figure 5c). In reaction 4, cDNA corresponding to E2D1 was used as the template and amplified using forward primer RZ579 and reverse primer TZ542 containing an XhoI cut site (Figure 5d). Once the products from reactions 3 and 4 were purified using a 1.5% DNA agarose gel, reaction 5 produced the cDNA corresponding to the fusion E3-USP30-D1 protein by linking the two products resulting from reaction 3 and 4. Thus, the final product for reaction 5 was made by using the respective outside primers (forward primer TZ456 with a restriction site for MfeI and reverse primer TZ542 with a restriction site for XhoI) as seen in Figures 5e and 5f.

The reaction mixtures and PCR programs utilized for each of the five PCR reactions can be found in tables 2-11.

Table 2: PCR Reaction Mixture for Reaction 1

Reagent	Amount
Template DNA (E2E3)	1 µL / 10ng
Forward primer (TZ456)	1 µL
Reverse primer (TZ577)	1 µL
10x PCR buffer (with Mg2+)	5 µL
dNTPs	1 µL
TAQ polymerase	0.3 µL
Sterile water	40.7 µL
Total	**50 µL**

Table 3: PCR Reaction Mixture for Reaction 2

Reagent	Amount
Template DNA (USP30)	1 µL / 10ng
Forward primer (TZ576)	1 µL
Reverse primer (TZ578)	1 µL
10x PCR buffer (with Mg2+)	5 µL
dNTPs	1 µL
TAQ polymerase	0.3 µL
Sterile water	40.7 µL
Total	**50 µL**

Table 4: PCR Reaction Mixture for Reaction 3

Reagent	Amount
Template DNA (USP30)	2.5 µL / 25ng
Template DNA (E2E3)	1 µL
Forward primer (TZ456)	1 µL
Reverse primer (TZ578)	1 µL
10x PCR buffer (with Mg2+)	5 µL
dNTPs	1 µL
TAQ polymerase	0.3 µL
Sterile water	38.2 µL
Total	**50 µL**

Table 5: PCR Reaction Mixture for Reaction 4

Reagent	Amount
Template DNA (E2D1)	1 µL / 10ng
Forward primer (TZ479)	1 µL
Reverse primer (542)	1 µL
10x PCR buffer (with Mg2+)	5 µL
dNTPs	1 µL
TAQ polymerase	0.3 µL
Sterile water	40.7 µL
Total	**50 µL**

Table 6: PCR Reaction Mixture for Reaction 5

Reagent	Amount
Template DNA (E2D1)	1 µL / 10ng
Template DNA (USP30+E2E3)	1 µL
Forward primer	1 µL
Reverse primer	1 µL
10x PCR buffer (with Mg2+)	5 µL
dNTPs	1 µL
TAQ polymerase	0.3 µL
Sterile water	39.7 µL
Total	**50 µL**

Table 7: PCR Program for Reaction 1

Cycles	Step	Temperature	Duration
1	Initial Denaturation	96°C	120s
25	Denaturation	96°C	30s
	Annealing	55°C	30s
	Elongation	72°C	45s
1	Final elongation	72°C	7min
1	Sample stored	6°C	Indefinite

Table 8: PCR Program for Reaction 2

Cycles	Step	Temperature	Duration
1	Initial Denaturation	96°C	120s
25	Denaturation	96°C	30s
	Annealing	55°C	30s
	Elongation	72°C	60s
1	Final elongation	72°C	7min
1	Sample stored	6°C	Indefinite

Table 9: PCR Program for Reaction 3

Cycles	Step	Temperature	Duration
1	Initial Denaturation	96°C	120s
25	Denaturation	96°C	30s
	Annealing	55°C	30s
	Elongation	72°C	60s
1	Final elongation	72°C	7min
1	Sample stored	6°C	Indefinite

Table 10: PCR Program for Reaction 4

Cycles	Step	Temperature	Duration
1	Initial Denaturation	96°C	120s
25	Denaturation	96°C	30s
	Annealing	55°C	30s
	Elongation	72°C	45s
1	Final elongation	72°C	7min
1	Sample stored	6°C	Indefinite

Table 11: PCR Program for Reaction 5

Cycles	Step	Temperature	Duration
1	Initial Denaturation	96°C	120s
25	Denaturation	96°C	30s
	Annealing	55°C	30s
	Elongation	72°C	45s
1	Final elongation	72°C	7min
1	Sample stored	6°C	Indefinite

2.1b Zymoclean Gel DNA Recovery Kit

To purify the products of PCR reactions 1, 2, 3, and 4, the suggested protocol from Zymoclean Gel DNA Recovery kit was followed. Necessary lanes of DNA from the agarose gel were excised using a razor blade and transferred into 1.5 mL Eppendorf tubes. If "V" was the volume of the agarose that was excised, 3V of the provided buffer ABD was added to the samples. They were allowed to incubate at 55°C for 5-10 minutes until the agarose gel was observed to have been completely dissolved.

The melted contents of the samples were transferred to a Zymo-Spin Column placed into the provided collection tubes and centrifuged from one minute at 13,300 RPM; flowthrough was discarded. Then, 200 µL of DNA Wash buffer was added and the columns were centrifuged for 30 seconds at 13,300 RPM. Flowthrough was again discarded, and the previous step was repeated once more. In the final step, 6 µL of DNA Elution Buffer was placed into the columns. The columns were placed into new 1.5 mL Eppendorf tubes and centrifuged for 30-60 seconds in order to elute the DNA.

2.1c Restriction Enzyme Digestion of cDNA

To prepare for the ligation, the insert and vector were both digested with EcoRI, MfeI and XhoI in three separate reactions. The reaction mixture for the restriction enzyme digestions included: 50 µL DNA, 37 µL sterile dH_2O, 10 µL 10x Restriction enzyme buffer, 1.5 µL BSA, and 1.5 µL *EcoRI* at 10 units/µL. This reaction mixture was placed in a 37°C water bath for one hour and allowed to react. After the reaction mixture had incubated for one hour, an additional 0.5 µL of *EcoRI* at 10 units/µL were added and allowed to react for an additional hour. Following the

two hours for digestion with EcoRI, DNA was precipitated from the solution to separate it from the buffer and enzyme and to repeat the digestions for MfeI and XhoI in their corresponding buffers. DNA agarose gel electrophoresis was used to determine the success of the restriction enzyme digestion and to estimate the amount of DNA in vector and insert solutions.

2.1d DNA Precipitation

The volumes of the samples in which the DNA was suspended were estimated and assigned a value of "V." 10% of V of 3M sodium acetate (pH 5.5), and 2V of pure ethanol at -20°C were added to the solution. The sample was mixed thoroughly and stored at -20°C for approximately 20 minutes before being centrifuged for 20 minutes at 4°C and discarding the supernatant. This centrifugation was repeated once more before 250 µL 70% ethanol was added to the solution. The sample was centrifuged for five minutes at 4°C, and then the supernatant was discarded. The pellet was allowed to dry for five to ten minutes in a speed vacuum before being resuspended in 50 µL sterile dH_2O.

2.1e DNA Agarose Gel Electrophoresis

A DNA agarose gel was used to ascertain the amount and size of DNA in each sample. To make a 1.5% gel, 100 mL of 1x TAE (0.04 M Tris-Acetate, 0.001 M EDTA) was combined with 1.5 g of agarose powder. This solution was poured into an Erlenmeyer flask and microwaved for approximately two minutes until all of the agarose powder was sufficiently dissolved. Afterward, the flask was run under cold water to cool the solution, and 5 µL of 0.5 µg/µL ethidium bromide was added. Upon swirling the flask to mix it, the solution was poured into a gel tray, which had

had combs placed into it. After ensuring that any bubbles were removed, the gel was then allowed to set, further cool, and solidify.

Once the gel had solidified, the combs were carefully removed. To prepare the samples for loading, 1-5 µL of the samples were combined with 5µL of 10x loading buffer (30% glycerol and 2% Orange-G, both dissolved in 1x TAE) before loading them into the wells. In the first well, 2-3 µL of GeneRuler 1kb DNA ladder was loaded to run alongside the samples. The gel was then completely immersed in 1x TAE and run at 150 V until the dye had migrated a sufficient distance. The DNA was visualized using UV light in a BioRad Gel-Doc machine.

2.1f DNA Ligation

To ligate the vector and insert together, the suggested protocol for Thermo Scientific Rapid DNA Ligation Kit was followed. This protocol called for the insert DNA to be at 3:1 molar excess over the vector and recommended approximately 0.1 ng of supercoiled vector DNA. The reaction mixture also included 3 µL 5x rapid ligation buffer and 1 µL of T4 DNA ligase (1 unit/µL). Sterile, nuclease-free water was added to bring the total volume to 15 µL. The mixture was vortexed and briefly spun down to collect to liquid and then was allowed to incubate at 22°C for five minutes. The ligation was then stored at 4°C.

2.2 Expression of E2E3-USP30-E2D1 Recombinant Protein

2.2a Bacterial Transformation into E. coli DH5α

Electro-competent *E. coli* DH5α cells were taken from a -80°C freezer and allowed to thaw on ice. Approximately 3 µL of the DNA ligation was added to the 200 µL aliquot of the electro-competent DH5α bacterial cells in a pre-chilled electroporation cuvette. The cuvette was placed into the Bio-Rad Gene Pulser, and a single exponential decay pulse of 2.5 kV at 400 Ω was delivered. Immediately following the pulse, 800 µL of antibiotic-free LB was added to the cuvette and gently mixed. The cells were transferred to 1.5 mL Eppendorf tubes and incubated for one hour at 37°C. Afterward, 50 µL, 150 µL, and 250 µL of cells were plated on Kanamycin-containing lysogeny broth (LB KAN) plates; glass beads were used to spread the bacteria, and the plates were incubated overnight at 37°C.

2.2b Isolation of Plasmid DNA from Bacteria: Boiling Method Miniprep

Colonies for miniprep were selected from LB KAN plates and grown overnight in 1.5 ml of LB KAN media at 37°C. Samples were then poured into 1.5 mL Eppendorf tubes and centrifuged for two minutes at 13,300 RPM. The supernatant was aspirated, and the pellet was subsequently resuspended in 300 µL of STET/lysozyme solution (8% sucrose, 5% 100X Triton, 50 mM of Tris-HCl pH 8, 50 mM of EDTA, 10 mg/mL of lysozyme). The samples were then boiled in water for one minute and centrifuged for 10 minutes at 13,300 RPM. After centrifugation, cellular debris was removed from the samples using a toothpick and discarded. 200 µL of isopropanol was added and the tubes were mixed gently but thoroughly via inversion. The samples were then centrifuged once more for 10 minutes at 13,300 RPM. The isopropanol was poured off,

and 300 µL of pre-chilled 70% ethanol was added to the tubes. Care was taken not to mix or disturb the tubes. The samples were centrifuged one last time for five minutes at 13,300 RPM before the supernatant was discarded and samples were allowed to dry in a speed vacuum for 10-15 minutes. Finally, samples were resuspended in 50 µL of TE.

2.2c Restriction Enzyme Digestion of Insert and Vector

In order to verify that the cDNA insert was properly cloned into the pET-28a vector, the vector and insert were digested once again using EcoRI and XhoI in a single double-digestion reaction. The reaction mixture for the restriction enzyme digestion included 5 µL DNA and 1.5 µL EcoRI-XhoI in the total reaction volume of 20 µL. The buffer used for this reaction was EcoRI buffer. This reaction mixture was placed in a 37°C water bath for one hour and allowed to react. Following the digestion, the product of the digestion was run on a DNA agarose gel. This was done in order to confirm that the insert was ligated successfully into the plasmid and to estimate the amount of DNA in vector and insert solutions.

2.2d Isolation of Plasmid DNA from Bacteria: NucleoSpin Plasmid Miniprep Kit

A clean miniprep was performed, and the suggested protocol in the NucleoSpin Plasmid miniprep kit was followed in order to sequence the cDNA for the E3-USP30-D1 fusion protein. Bacterial colonies were incubated overnight at 37°C in 1.5 mL LB KAN media. The next day, the bacterial suspension was poured into 1.5 Eppendorf tubes and centrifuged for two minutes at 13,300 RPM. The supernatant was aspirated out, and the pellet was resuspended in 200 µL of buffer A1 by pipetting up and down, taking care to ensure no bubbles were formed. Once it was

confirmed that there were no cell clumps remaining, buffer A2 was added, and the tubes were gently inverted six to eight times. The tubes were then allowed to incubate at room temperature for five minutes. Next, buffer A3 was added, and the tubes were mixed by inversion six to eight times once again. The samples were centrifuged at 13,300 RPM for 10 minutes at room temperature.

After the centrifugation was complete, the supernatant from the samples was decanted into NucleoSpin Plasmid (NoLid) Columns placed into 2 mL Eppendorf tubes. These columns were centrifuged for one minute at 13,300 RPM; flowthrough was discarded, and 600 µL of buffer A4/AQ, which was supplemented with ethanol, was added. Samples were then centrifuged for one minute. Flowthrough was discarded, and tubes were spun once again at 13,300 RPM for two minutes. The columns were then placed into new 1.5 mL Eppendorf tubes, and 50 µL of buffer AE was added to elute the DNA. The tube was allowed to incubate at room temperature for one minute before it was spun for one minute at 13,300 RPM to allow DNA elution.

2.2e Bacterial Transformation into E. coli BL21 (DE3)

Electro-competent *E. coli* BL21 (DE3) cells were taken from a -80°C freezer and allowed to thaw on ice 0.5µL of the vector DNA was added to the 50 µL aliquot of the electro-competent DH5α bacterial cells in a pre-chilled electroporation cuvette. The cuvette was placed into the Bio-Rad Gene Pulser, and a single exponential decay pulse of 2.5 kV at 400 Ω was delivered. Immediately following the pulse, 800 µL of antibiotic-free LB was added to the cuvette and gently mixed. The cells were transferred to 1.5 mL Eppendorf tubes and incubated for one hour at 37°C.

Afterward, 50 µL, 150 µL, and 250 µL of cells were plated on LB KAN plates; glass beads were used in order to spread the bacteria, and the plates were incubated overnight at 37°C.

2.2f Induction of Recombinant Protein Using IPTG

An isolated colony from the previously grown LB KAN plates was picked and grown in 3-4 mL of LB KAN media overnight to procure the starter culture. The following day, this starter culture was inoculated at a 1:100 dilution into 1 L of LB KAN media. The media was incubated at 37°C on a shaker moving at 250 RPM until the optical density (OD) at 600 nm was between 0.8 and 1.2 OD. When the OD reached a sufficient level, 2mM of isopropyl β-D-1 thiogalactopyranoside (IPTG) was added to induce the culture to express the E3-USP30-D1 fusion protein. The culture was then allowed to grow at 25°C for 4 hours. After induction, bacteria were centrifuged in 250 mL bottles at 6,000 RPM for 10 minutes until all of the bacteria had been spun down. The supernatant was discarded, and paste was kept and frozen at -80°C.

2.2g Lysis, Sonication, and Protein Purification

In order to lyse the collected cells, lysis buffer was made from 1X binding buffer (200 mM NaCl, 20 mM Tris pH 8, 10 mM imidazole), protease inhibitors, and 100 µg/mL total volume of lysozyme. The cell paste from the induction of the *E. coli* BL21 bacteria was removed from -80°C and resuspended in the lysis buffer using a 10 mL pipet. The suspension was then allowed to incubate for 20 minutes at 30°C. To eliminate the extreme turbidity of the suspension, it was sonicated and the DNA was sheared until the turbidity was similar to that of a normal protein solution. The suspension was then centrifuged at 15,000 RPM for 40 minutes at 4°C and the supernatant was collected.

The following protein purification was carried out at 4°C on ice. If the volume of the sample was designated "V", then 0.1V of Ni-NTA agarose resin beads were allowed to rotate with the supernatant in a 50 mL falcon tube for two to four hours in order to prepare to purify the His-tagged protein. The mixture of protein and Ni-NTA agarose resin beads was placed into a column and washed with 20V of 1X binding buffer. Next, the column was washed with 20V of 1X washing buffer (200 mM NaCl, 20 mM Tris pH 8, 50 mM imidazole). The column was then washed with 5-10V of 1X elution (200 mM NaCl, 20 mM Tris pH 8, 200 mM imidazole). The fractions were dialyzed in a 2 L beaker with dialysis buffer (20 mM Tris-HCl pH 8, 400 mM NaCl) overnight at 4°C. The buffer was changed two to three times throughout the night, and it was confirmed that the purified protein was the E3-USP30-D1 protein of interest through an SDS-PAGE and Western blot.

2.3 Assay

2.3a Omi/HtrA2 Digestion

To determine whether the IMS component of USP30 was a substrate of Omi, the fusion protein was digested in the presence of the serine protease. Approximately 3 µL (400 ng) Omi and two aliquots of 5 and 10 µL (400-800 ng) of recombinant protein were incubated in separate reactions. The reaction had a total volume of 15 µL containing Omi Assay Phosphate Buffer (20 mM Na_2HPO_4 pH 8, 10% glycerol, 200 mM NaCl) for one hour at 37°C. Following this assay, the reaction products were analyzed using SDS-PAGE and Coomassie Blue staining.

2.3b SDS-PAGE and Coomassie Blue Staining

BioRad glass plates were cleaned thoroughly using soap and water, sprayed with 70% ethanol, and allowed to air-dry completely. Once the glass plates were dried and assembled, the 12% resolving gel (10.7 mL of water, 7.5 mL of 40% acrylamide, 6.3 mL of 1.5 M Tris pH 8.8, 250 µL of 10% SDS, 250 µL of 10% APS, and 10 µL of TEMED) was dispensed into the glass plates and covered with isopropanol to ensure an even surface where the resolving gel would meet the stacking gel. The resolving gels were allowed to polymerize for at least 20 minutes. After the resolving gels had solidified, the isopropanol was rinsed out with water and the 5% stacking gel (7.3 mL of water, 1.25 mL of 40% acrylamide, 1.25 mL of 1.5 M Tris pH 6.8, 100 µL of 10% SDS, 100µL 10% APS, and 10 µL of TEMED) was dispensed into the glass plates. Combs were placed into the stacking gel and it was given another 20 minutes to polymerize. The completed gels were then wrapped with dampened paper towels, placed into Ziploc bags, and were stored in Tupperware containers where they were kept at 4°C for several days. When needed, the gels were

removed from 4°C and allowed to reach room temperature. The plastic combs were removed and the glass plates were placed into the BioRad Mini-PROTEAN Tetra cell gel electrophoresis apparatus.

In order to prepare the samples for SDS-PAGE, 5 µl of 4X sample buffer (10% β-mercaptoethanol, 6% SDS, 20% glycerol, 1/40 X stacking buffer, and 0.2mg/ml bromophenol blue) was added. The samples were boiled for 4 minutes and subsequently spun down. Once added to their corresponding lanes, the apparatus was filled with protein running buffer (0.02 M Tris, 0.003 M SDS, 0.19 M glycine, pH 8.3) and the samples were run at 20A until the dye had migrated a sufficient distance.

The gel was stained with Coomassie blue stain (1:1 methanol:dH_2O, 0.2% Coomassie powder, 0.1% acetic acid) for approximately 40 minutes until the gel was no longer able to be seen in the dye. The gel was then placed in destaining solution for at least 2 hours until the background was clear. This allowed for confirmation that the fusion protein was expressed and analysis of results and cleavage.

2.3c Denaturation of USP30 to Assist Potential Degradation by Omi/HtrA2 Protease

In order to confirm that any lack of cleavage was not attributable to an inability of Omi to access the potential cleavage site in the IMS component of USP30, the expressed recombinant protein was denatured through boiling and digested with Omi once more.

The fusion protein was boiled for 10 minutes and then re-digested according to the protocol outlined in section 2.3a. The denatured protein digestion was then analyzed and compared to the normal condition protein using SDS-PAGE and Coomassie blue staining.

CHAPTER THREE: RESULTS

3.1 Previous Data on USP30 and Omi

Prior research conducted by the on Omi and USP30 stipulated the potential of the IMS component of USP30 to be a substrate of the serine protease. The mature form of Omi is a 324 residue protein (amino acids 133-458) that contains a conserved AVPS motif, a catalytic domain, and a PDZ domain. The AVPS motif is what enables Omi to bind to and interact with IAPs; the efficiency of substrate cleavage is therefore determined by the substrate's IAP-binding motif, AVPS [41]. USP30's IMS component has a six-amino-acid sequence that is very similar to the consensus cleavage sites where Omi has been demonstrated to cleave (Figure 9). This provided the preliminary data to ask the hypothesis: is USP30 a substrate cleaved by Omi? Under normal conditions, Omi is a serine protease that resides exclusively within the IMS. For this reason, if USP30 is a substrate of Omi, USP30 must be cleaved within its IMS domain where Omi is able to physically access it *in vivo.*

In order to determine whether or not Omi/HtrA2 has the capability to cleave USP30, the portion of the USP30 DUB that resides within the IMS needed to be digested with Omi. However, due to the small size of USP30's IMS component, visualizing the possible results of Omi digestion of the 35 residue IMS fragment on a polyacrylamide gel would be difficult, as bands would be of molecular weights that are too small to decisively differentiate. In order to facilitate visualization and determine whether or not Omi/HtrA2 cleaves within this small region of USP30, a fusion protein was created.

The creation of the fusion protein, which was made by flanking USP30's IMS component with E2E3 and D2D1, fulfilled three requirements. Firstly, it allowed for better visual confirmation

USP30 Amino Acid Sequence (502aa)

MLSSRAEAAM TAADRAIQRF LRTGAAVRYK VMKNWGVIGG IAAALAAGIY
VIWGPITERK KRRKGLVPGL VNLGNTCFMN SLLQGLSACP AFIRWLEEFT
SQYSRDQKEP PSHQYLSLTL LHLLKALSCQ EVTDDEVLDA SCLLDVLRMY
RWQISSFEEQ DAHELFHVIT SSLEDERDRQ PRVTHLFDVH SLEQQSEITP
KQITCRTRGS PHPTSNHWKS QHPFHGRLTS NMVCKHCEHQ SPVRFDTFDS
LSLSIPAATW GHPLTLDHCL HHFISSESVR DVVCDNCTKI EAKGTLNGEK
VEHQRTTFVK QLKLGKLPQC LCIHLQRLSW SSHGTPLKRH EHVQFNEFLM
MDIYKYHLLG HKPSQHNPKL NKNPGPTLEL QDGPGAPTPV LNQPGAPKTQ
IFMNGACSPS LLPTLSAPMP FPLPVVPDYS SSTYLFRLMA VVVHHGDMHS
GHFVTYRRSP PSARNPLSTS NQWLWVSDDT VRKASLQEVL SSSAYLLFYE
RVLSRMQHQS QECKSEE

Figure 9: Amino Acid Sequence of USP30

The full-length amino acid sequence of USP30 is shown above. In blue are amino acids 1-35 which compose the IMS component. The underlined letters in blue compose the sequence where Omi is predicted to cleave. In red is the transmembrane domain, and in black is the component in the cytoplasm of the cell.

of cleavage, if it were to occur. It would be difficult, if not impossible to determine if cleavage was successful because the IMS component of USP30 is only 35 residues; by fusing two proteins to either side of IMS fragment, determining cleavage would be more straightforward because the fusion protein, now cleaved into two, would be at the approximate molecular weights of E2E3 and E2D1. Secondly, E2E3 and E2D1 were proved in previous experiments in Zervos lab not to be cleaved by Omi's protease activity, making them ideal candidates for this recombinant protein (Figure 10). Thirdly, noting that E2E3 is 23 kDa (207 amino acids) and E2D1 is 17 kDa (147 amino acids), the two proteins were of different enough molecular weights that their bands could easily be differentiated from each other on an SDS-PAGE analysis.

These previous data provided the primary justification for this project. It has been suggested that by interacting with USP30, Omi plays a function in regulating the ubiquitination process within the cell. Identifying whether this interaction occurs could shed light on the mechanisms by which Omi accomplishes this task.

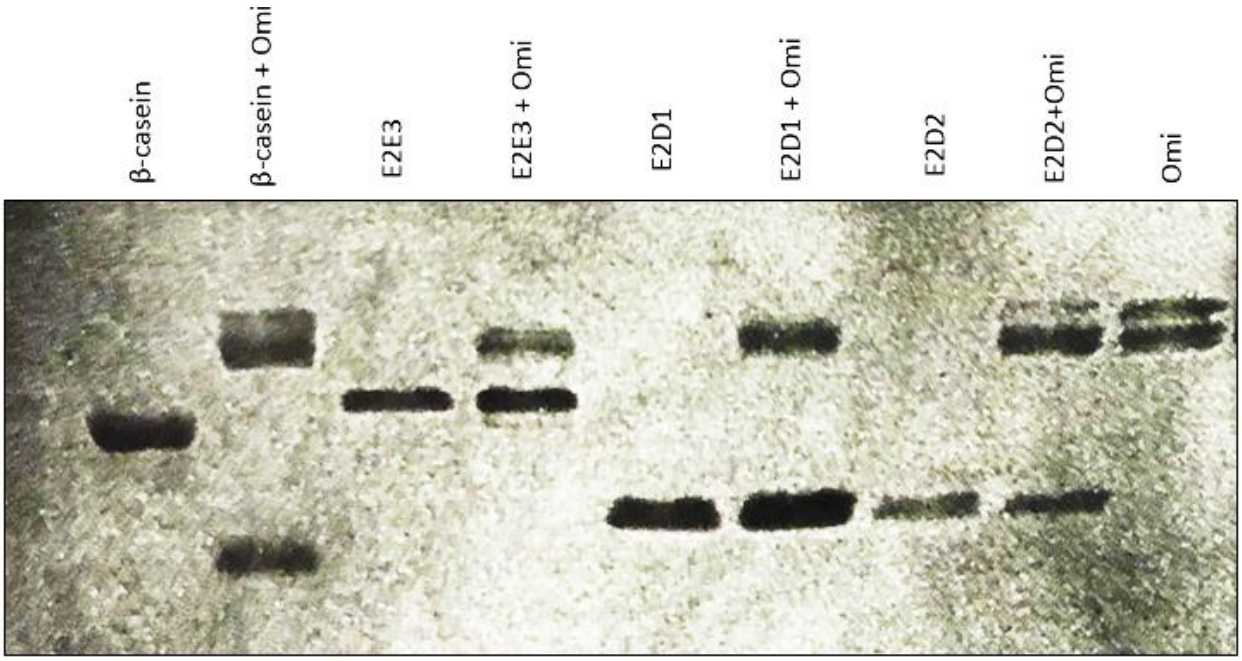

Figure 10: Interaction between Omi and other Well-Characterized Proteins within the Cell

The proteins β-casein, E2E3, E2D1, and E2D2 were all digested in the presence of Omi prior to the creation of the E3-USP30-D1 fusion protein's cDNA in order to ensure that the proteins flanking USP30's IMS component would not be cleaved by Omi.

3.2 Omi/HtrA2 Digestion Coomassie Blue Staining Analysis

The cDNA of the E2E3-USP30-E2D1 recombinant protein was cloned into the pET-28-a+ vector, cloned in *E. coli* BL21 cells, and expressed *E. coli* DH5α cells as described in chapter two. Then the protein was purified and digested in the presence of Omi in order to determine whether USP30 is a *bona fide* substrate of Omi. SDS-PAGE and Coomassie Blue staining was used to verify and analyze the results.

The fusion protein was expected to be approximately 45 kDa (389 amino acids), since E2E3 is 23 kDa (207 amino acids), E2D1 is 17 kDa (147 amino acids), and the IMS component of USP30 is 550 Da (35 amino acids). Differing amounts of fusion protein were run in lanes 1 and 3. The products of the initial digestion with Omi were run in lanes 2 and 4. Mature Omi is 37 kDa and was run alone in lane 5 for comparison. Because β-casein had been shown to be cleaved by Omi in previous experiments, it was used as a positive control. Conversely, since E2E3 had been tested previously to ensure that it was not a substrate cleaved by Omi, it was used as a negative control in this experiment (Figure 11).

As can be seen in lanes 2 and 4, only one band of interest can be identified at the molecular weight corresponding to the whole fusion protein (45 kDa). Had the cleavage been successful, one might have expected to see two bands present in both lanes: one band just above 23 kDa and the other at just above 17 kDa to demonstrate that the IMS component of USP30 had been cleaved. Therefore, in this experiment, USP30 was not shown to be a substrate that is cleaved by Omi.

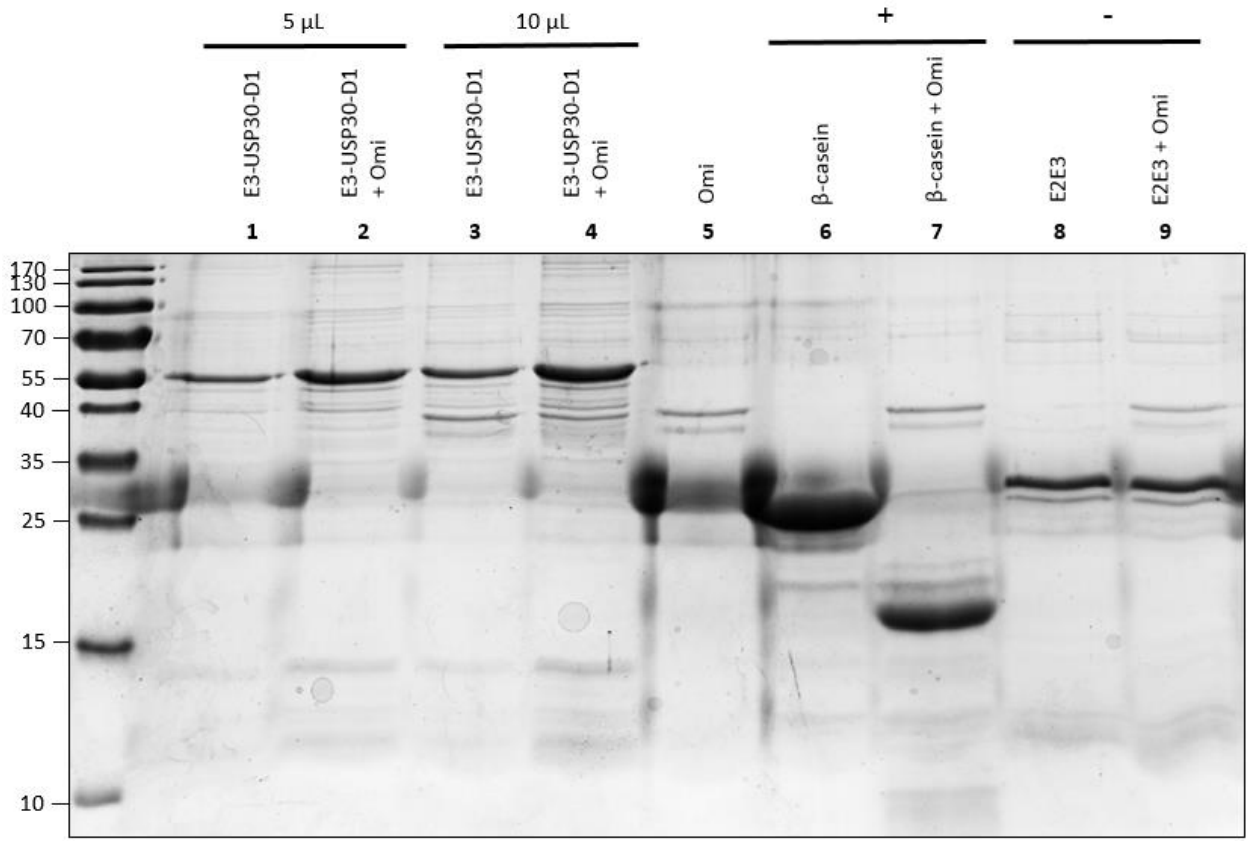

Figure 11: Coomassie Blue Analysis of Fusion Protein Omi Digestion

Omi is 37 kDa and the fusion protein is approximately 45 kDa.
Lane 1: 5 µl of E3-USP30-D1 protein
Lane 2: 5 µl of E3-USP30-D1 protein + Omi
Lane 3: 10 µl of E3-USP30-D1 protein
Lane 4: 10 µl of E3-USP30-D1 protein + Omi
Lane 5: Omi
Lane 6: β-casein (positive control)
Lane 7: β-casein + Omi (positive control)
Lane 8: E2E3 (negative control)
Lane 9: E2E3 + Omi (negative control)

3.3 USP30 Denaturation Coomassie Blue Staining Analysis

Following the failure of Omi to cleave the fusion protein within USP30's IMS component, the recombinant protein was boiled in order to denature it. This was done in order to determine if the lack of observable cleavage could be attributed to an accessibility issue. If Omi cleavage had not been observed due to the fusion protein structure, denaturation of the fusion protein would have allowed Omi to access and cleave the IMS component of USP30 within the fusion protein.

In lanes 1 and 2, the fusion protein and the fusion protein that had been digested by Omi were loaded, respectively. In lane 3, the denatured fusion protein was loaded and in lane 4, the denatured fusion protein which had been digested by Omi was loaded. E2E3 and β-casein were used once again as negative and positive controls, respectively (Figure 12).

The results of this experiment were similar to the results shown in Figure 11. The lanes which had the fusion protein digested with Omi, lanes 2 and 4, clearly show the 45 kDa band which represents the full length of the uncut fusion protein. Below those are the 37 kDa bands that denote the serine protease Omi, definitively confirming that USP30 is not a substrate of Omi/HtrA2.

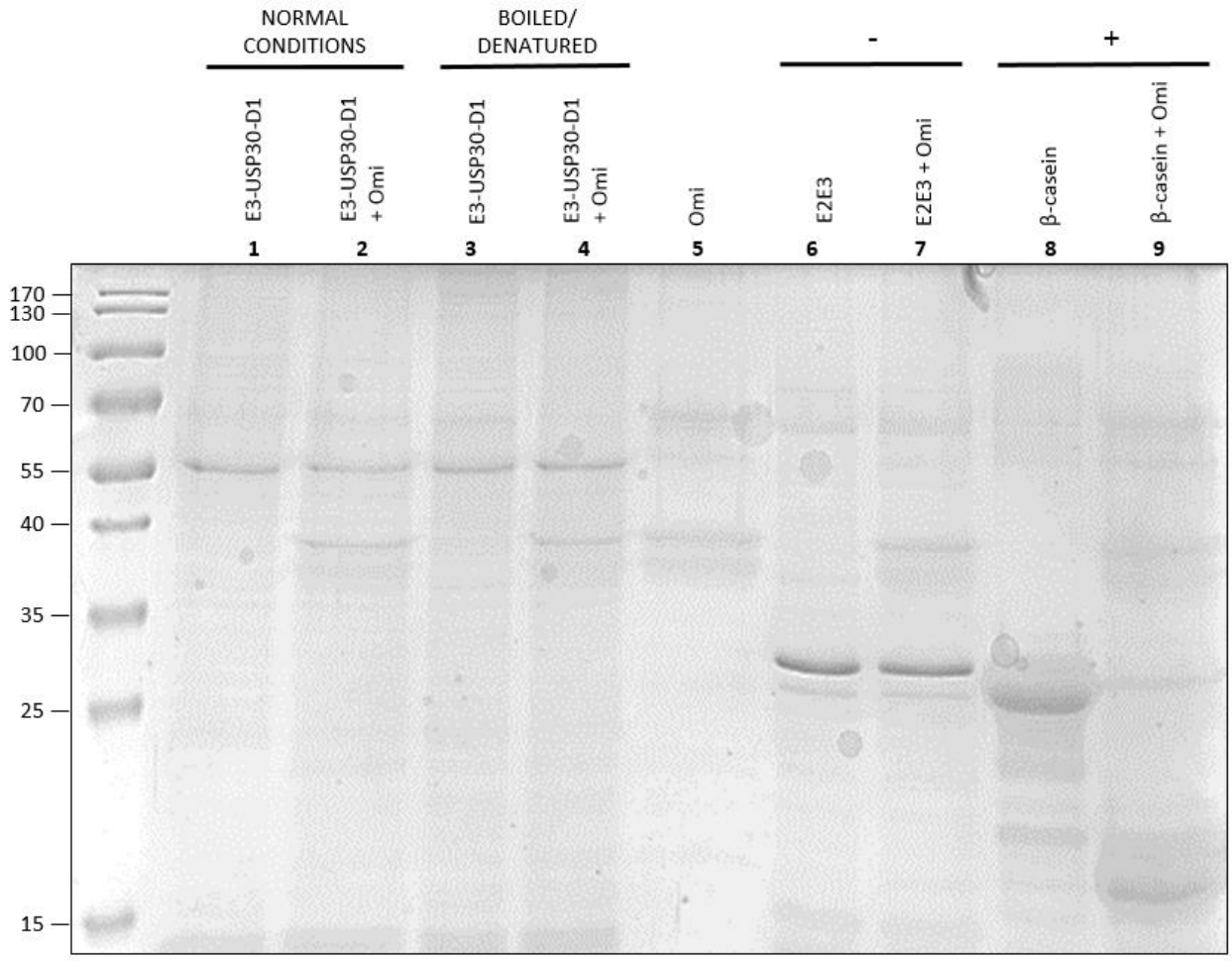

Figure 12: Coomassie Blue Analysis of Fusion Protein Denaturation and Omi Digestion

Omi is 37 kDa and the fusion protein is approximately 45 kDa.
Lane 1: E3-USP30-D1 protein
Lane 2: E3-USP30-D1 protein + Omi
Lane 3: Boiled E3-USP30-D1 protein
Lane 4: Boiled E3-USP30-D1 protein + Omi
Lane 5: Omi
Lane 6: E2E3 (negative control)
Lane 7: E2E3 + Omi (negative control)
Lane 8: β-casein (positive control)
Lane 9: β-casein + Omi (positive control)

CHAPTER 4: DISCUSSION

Omi is a serine protease that belongs to the eukaryotic HtrA family of proteins; the HtrA family is known to function in removing damaged/denatured proteins at very high temperatures. Omi, in particular, seems to serve a dual function depending on its intracellular location. When it is localized within the IMS, it helps to maintain mitochondrial homeostasis by playing a key role in mitochondrial protein quality control and facilitating fission/fusion dynamics [42, 43]; however, when it is released into the cytosol, it fulfills a pro-apoptotic function [33]. A lack of functional Omi causes a buildup of Mulan E3 ubiquitin ligase and increases levels of mitophagy within the cell [31].

USP30 is an important OMM-embedded DUB that is part of the peptidase C19 family and has been demonstrated to be a negative regulator mitochondrial fusion through deubiquitination of Mfn1 and 2 [36, 37]. It was also recently discovered in 2014 that overexpression of USP30 is capable of reversing mitophagy driven by Parkin and PINK1 [24]. Because malfunctioning mitophagy has been linked to Parkinson's disease, the potential for therapeutic uses was exciting and promising but has thus been inconclusive.

Further understanding of the mechanisms behind the action of Omi and USP30 may reveal important information about how Omi regulates protein quality control and polyubiquitination under conditions of stress. Additionally, notwithstanding the interconnected functions of the two proteins – such as regulating mitochondrial fusion/fusion and accelerating or deterring the path to mitophagy – USP30 and Omi also reside close to each another within the mitochondria. It is known that Omi and USP30 share many similar functions in terms mitochondrial dynamics and maintenance, but whether Omi interacts with USP30 to fulfill some of those functions and whether USP30 is a substrate of Omi is unknown. Combined with the presence of the six hydrophobic

amino acid residues within the IMS component of USP that bear resemblance to Omi's consensus cleavage sequence (Figure 9), these parameters provided the foundation for the hypothesis of our project.

We hypothesized that USP30 would be cleaved within its IMS component as a *bona fide* substrate of Omi. In this work, we tried to determine whether or not USP30 is a substrate of Omi by digesting the E3-USP30-D2 fusion protein in the presence of the serine protease. To elucidate the interplay between the two closely-situated mitochondrial proteins USP30 and Omi, the E3-USP30-D1 fusion protein was created (using the 35 residue IMS component of USP30 and the proteins E2E3 and E2D1) and was digested in the presence of Omi. Amino acids 35-502 of USP30 belong to the transmembrane and cytosolic domains of the DUB and were not expressed in the final fusion protein because cleavage could only have occurred where Omi and USP30 could have physically interacted with each other in the IMS. We created the E3-USP30-D1 recombinant protein that was suitable for the digestion and digested it in the presence of Omi in two different conditions. In the first the fusion protein was simply purified and digested. In the second, it was boiled and denatured before being digested in the presence of Omi to ensure that cleavage was not being impeded by inaccessibility due to the fusion protein's structure.

Analysis of the Coomassie Blue staining suggested that cleavage did not occur in either the normal or the denatured conditions. Given that the conditions in which USP30 resides were not perfectly mimicked by this project, further testing should be conducted in order to conclusively determine that USP30 is not a substrate of Omi. The *in vitro* system that was tested in this project contained only the E3-USP30-D2 substrate and Omi; it is possible that other proteins may participate in the cleavage *in vivo*. However, these proteins were not included in the *in vitro* system, which may explain the observed lack of cleavage. Additionally, the fusion protein that was created

is a chimeric protein that is not synthesized naturally within the cell; it contained only USP30's IMS component, which was flanked by two other proteins, E2E3 and E2D1. Thus, the free peptide should also be digested in the presence of Omi to test for cleavage. Although SDS-PAGE and Coomassie Blue staining cannot be used to analyze the results, other methods such as mass spectrometry may be used in order to determine if cleavage has occurred. Future work should focus on testing cleavage in the different conditions outlined above.

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