Studies on E2 Conjugation Enzyme Partners of Mulan E3 Ubiquitin Ligase

Rebekah J. Fitzpatrick

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STUDIES ON E2 CONJUGATION ENZYME PARTNERS OF MULAN E3 UBIQUITIN LIGASE

by

REBEKAH J. FITZPATRICK

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 Honors College at the University of Central Florida
Orlando, Florida

Spring Term, 2018

Thesis Chair: Antonis Zervos, PhD
ABSTRACT

Mulan is an E3 ubiquitin ligase embedded in the outer mitochondria membrane. Mulan’s participation in the ubiquitination process is conducted through its cytosol-exposed RING finger domain, and its ability to modulate protein ubiquitination makes it a key player in mitochondrial and cellular homeostasis. Mulan is known to be involved in mitochondrial fission, fusion, mitochondrial stress, apoptosis, and Parkin-independent mitophagy. Dysregulation of Mulan in mice has been shown to correlate with human neurodegenerative disorders and heart disease. Accumulation of Mulan is predicted to be responsible for the motor neuron degeneration 2 (mnd2) phenotype in mutant mice through the deregulation of the Mulan-dependent pathway of mitophagy. The purpose of this study was to utilize both a yeast two-hybrid screen as well as an in vitro profiling assay to characterize interactions between Mulan and potential E2 conjugating enzymes. Through these studies, Ube2D1, Ube2D2, and Ube2D3 were identified as strong interactors with the Mulan-RING domain. The tissue specific expression and protein levels of these E2 conjugating enzymes was further investigated in mouse tissues by SDS-PAGE and Western blot analysis. They all had similar patterns of expression and were present in brain, heart, kidney, and liver tissues, with the highest level seen in the brain. This data demonstrates that Mulan has a primary function in the brain and it suggests that Mulan’s deregulation might be involved in the development and progression of neurodegeneration.
ACKNOWLEDGMENTS

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LIST OF ABBREVIATIONS

AD  Activation Domain
Akt  Serine-Threonine Protein Kinase
AMP  Ampicillin
ampR  Ampicillin Resistance
APS  Ammonium Persulfate
BAM  Beside a Membrane
BD  Binding Domain
BSA  Bovine Serum Albumin
CCCP  Carbonyl Cyanide m-Chlorophenyl Hydrazine
Cd  Cadmium
cDNA  Complementary DNA
dH2O  Deionized Water
dNTP  Deoxyribonucleotide Triphosphate
Drp1  Dynamin Related Protein 1
DTT  Dithiothreitol
DUB  De-ubiquitination
ECL  Enhanced Chemiluminescence
EC109  Esophageal Squamous Carcinoma Cells
EDTA  Ethylenediaminetetraacetic Acid
ER  Endoplasmic Reticulum
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>G1</td>
<td>Gap phase 1</td>
</tr>
<tr>
<td>GABARAP</td>
<td>Gamma-Amino-Butyric Acid Receptor Associated Protein</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-Phosphate Dehydrogenase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine-5'-Triphosphate</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>HBAST</td>
<td>Human Brain Astrocytes</td>
</tr>
<tr>
<td>HECT</td>
<td>Homologous to the E6-AP Carboxyl Terminus</td>
</tr>
<tr>
<td>HeLa</td>
<td>Henrietta Lacks</td>
</tr>
<tr>
<td>HIS3</td>
<td>Histidine Gene 3</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse Radish Peroxidase</td>
</tr>
<tr>
<td>hTERT</td>
<td>Human Telomerase Reverse Transcriptase</td>
</tr>
<tr>
<td>IMM</td>
<td>Inner Mitochondrial Membrane</td>
</tr>
<tr>
<td>IMS</td>
<td>Intermembrane Space</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun N-terminal Kinase</td>
</tr>
<tr>
<td>KD</td>
<td>Knock Down</td>
</tr>
<tr>
<td>KO</td>
<td>Knock Out</td>
</tr>
<tr>
<td>LA</td>
<td>Lithium Acetate</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny Broth</td>
</tr>
<tr>
<td>LC3</td>
<td>Microtubule-Associated Protein 1, Light Chain 3</td>
</tr>
<tr>
<td>MAVS</td>
<td>Mitochondrial Antiviral-Signaling Protein</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Human Breast Cancer Cells</td>
</tr>
<tr>
<td>MDV</td>
<td>Mitochondrial Derived Vesicle</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>Mfn</td>
<td>Mitofusin</td>
</tr>
<tr>
<td>MHCII</td>
<td>Major Histocompatibility Complex II</td>
</tr>
<tr>
<td>mnd2</td>
<td>Motor Neuron Degeneration 2</td>
</tr>
<tr>
<td>MPP</td>
<td>Mitochondrial Processing Peptidase</td>
</tr>
<tr>
<td>MPT</td>
<td>Mitochondrial Permeability Transition</td>
</tr>
<tr>
<td>Mulan</td>
<td>Mitochondrial Ubiquitin Ligase Activator of NF-κB</td>
</tr>
<tr>
<td>NEB</td>
<td>New England BioLabs</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor Kappa-Light-Chain Enhancer of Activated B Cells</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear Localization Sequence</td>
</tr>
<tr>
<td>NRK-52E</td>
<td>Rat Renal Tubular Epithelial Cells</td>
</tr>
<tr>
<td>O/N</td>
<td>Over Night</td>
</tr>
<tr>
<td>OMM</td>
<td>Outer Mitochondrial Membrane</td>
</tr>
<tr>
<td>OPA1</td>
<td>Optic Atrophy 1</td>
</tr>
<tr>
<td>Ori</td>
<td>Origin of Replication</td>
</tr>
<tr>
<td>PARL</td>
<td>Presenilin Associated Rhomboid Like Protein</td>
</tr>
<tr>
<td>PCP</td>
<td>Phencyclidine</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson's Disease</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene Glycol</td>
</tr>
<tr>
<td>PINK1</td>
<td>PTEN Induced Putative Kinase 1</td>
</tr>
<tr>
<td>polyUb</td>
<td>Polyubiquitination</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene Fluoride</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>RIG-1</td>
<td>Retinoic Acid-Inducible Gene 1</td>
</tr>
<tr>
<td>RING</td>
<td>Really Interesting New Gene</td>
</tr>
<tr>
<td>RNF</td>
<td>Ring Finger Protein</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>rpm</td>
<td>Rotations Per Minute</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small Interfering RNA</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
</tr>
<tr>
<td>SS DNA</td>
<td>Salmon Sperm DNA</td>
</tr>
<tr>
<td>STET</td>
<td>Sucrose-Triton-EDTA-Tris solution</td>
</tr>
<tr>
<td>SUMO</td>
<td>Small Ubiquitin-like Modifier protein</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-Acetate EDTA</td>
</tr>
<tr>
<td>TAQ</td>
<td>Thermus Aquaticus</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered Saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered Saline with Tween</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TIM</td>
<td>Translocase of the Inner Membrane</td>
</tr>
<tr>
<td>TMD</td>
<td>Transmembrane Domain</td>
</tr>
<tr>
<td>TOM</td>
<td>Translocase of the Outer Membrane</td>
</tr>
<tr>
<td>TRP1</td>
<td>Tryptophan Gene 1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>UAS</td>
<td>Upstream Activation Sequence</td>
</tr>
<tr>
<td>Ub</td>
<td>Ubiquitin</td>
</tr>
<tr>
<td>U-H-W-</td>
<td>Uracil- Histidine- Tryptophan-</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>X-Gal</td>
<td>5-Bromo-4-Chloro-3-Indolyl-β-D-Galactopyranoside</td>
</tr>
</tbody>
</table>
CHAPTER 1: INTRODUCTION AND REVIEW OF LITERATURE

Mitochondria ubiquitin ligase activator of NF-κB (Mulan), also coined Mitochondria-anchored protein ligase (MAPL), growth inhibition death E3 ligase (GIDE), Hades, Mitochondrial ubiquitin protein ligase 1 (Mul1), C1orf166, and RNF218, is one of three E3 ubiquitin ligases located within the outer mitochondrial membrane (OMM) [1-5]. Mulan is a 352-amino acid protein with a molecular weight of 39.8 kDa. The protein includes two transmembrane domains (TMDs) and it is orientated in the OMM such that both the N- and C-termini face the cytosol, leaving a large BAM (beside a membrane) / GIDE domain within the intermembrane space (IMS) (Figure 1) [6]. The C-terminus includes a RING finger domain, the key component for Mulan’s E3 ligase activity and a major regulator of cellular and mitochondrial dynamics. An intact RING finger (amino acids 259-352) and proper localization to the mitochondria is required for Mulan’s participation in cell growth, mitophagy, mitochondrial fission/fusion, apoptosis and vesicle transport [1]. The BAM-RING regions are highly conserved, supported through phylogenetic analysis that determined Mulan to be present with the origin of holozoan and viridiplantae lineages [6]. Mulan is expressed ubiquitously with the highest expression in the heart, placenta, liver, kidney, and brain [1].
A. Schematic diagram indicating the three mitochondrial E3 ubiquitin ligases and their topology on the OMM.

B. Mulan is a 352-residue protein, and includes two TMDs, a BAM/GIDE domain within the IMS, and the RING finger domain facing the cytosol. The RING finger domain is responsible for the ligase activity of the protein.

Figure 1: Mulan E3 ubiquitin ligase location and domains
1.1 The Ubiquitin-Proteasome System

Mulan’s main molecular function is as an E3 ubiquitin ligase in the ubiquitination process. Ubiquitination is a cytosolic system of targeted protein degradation that uses ubiquitin (Ub) and a three-enzyme cascade. Ub is a small molecule (76 amino acids) that is covalently attached as a post-translational modification to a substrate [7]. Attachment occurs at the amino terminus or at internal lysine residues of the substrate by Ub’s C-terminal Gly76. Multiple rounds of ubiquitination result in a polyubiquitin (polyUb) tag, Ub molecules attaching to each other at one of seven internal lysine residues, with the Lys48 and Lys63 sites being the most frequently polyubiquitinated. Lys48 polyUb has been associated with degradation by the 26S proteasome [8]. PolyUb shuttles include a Ub-associating domain to bind to the Ub, a Ub-like domain to target the complex to the proteasome, and innate abilities to protect the polyUb tag from deubiquitinating enzymes (DUBs), the modulators for the reverse process [7].

Ubiquitination occurs in a series of three steps with enzymes E1, E2, and E3: (1) E1 activates ubiquitin, (2) E2 conjugates ubiquitin to a carrier, and (3) E3 ligates Ub to the substrate target (Figure 2). The human genome encodes 2 E1s, about 50 E2s, and approximately 700 E3s [9]. The two most common classes of E3 ubiquitin ligases include: RING finger proteins and HECT domain enzymes. For the RING finger E3 proteins, there is a complex formed between the E2, E3, and substrate for the Ub transfer, whereas HECT domains include a Ub-thiolester-intermediate before transfer [7]. Mulan, as an E3 ubiquitin ligase of the RING finger protein class, participates in the direct translocation of the activated Ub from an E2 conjugating enzyme to the substrate, which often targets the substrate for degradation by the 26S proteasome.
SUMOylation is a similar process to ubiquitination that adds SUMO molecules onto targets as a post-translation modification. SUMO is a ubiquitin-like modifier that, when added, aids in a variety of cellular processes including localization, signaling, and maintenance of other post-translational modifications. SUMOylation also involves a three-enzyme cascade: (1) E1 heterodimer, (2) E2 ligase Ubc9, and (3) and E3 ligase. Mulan has been reported to also function as an E3 SUMOylation ligase [10].
Ubiquitination is a process that involves three different steps with three different enzymes: an E1 ubiquitin activator, an E2 conjugating enzyme, and an E3 ligating enzyme. Mulan is an E3 ligating enzyme, responsible for tagging Ub molecules to substrates transferred from an E2. This transfer requires formation of a complex between E2, E3, and the substrate.

Figure 2: The Ubiquitination process and Mulan E3 ubiquitin ligase
1.2 Mitochondrial Dynamics

Mulan’s function has also been implicated in mitochondrial fusion, fission, apoptosis, and mitochondrial vesicle transport [1-3,10].

Mitochondria are constantly adjusting their size, shape, and location, and together they exist in a coordinated network. These changes result from mitochondrial fusion (the combining of two mitochondria into one) and fission (the splitting of one mitochondrion into two) (Figure 3). Fusion is controlled by mitofusin 1 and 2 (Mfn1 and Mfn2): the proteins are tethered to the OMM and their cytosolic component contains a GTPase responsible for binding an adjacent mitochondrion; OPA1, a dynamin-like GTPase located within the IMS or IMM, is responsible for IMM fusion and the regulation of cristae morphology [11]. Mulan ubiquitinates Mfn2 and targets it for degradation [5].

Mitochondrial fission is controlled by the GTPase Dynamin-related protein 1 (Drp1). An increase in Drp1 leads to an increase in fission. This protein is a substrate for Mulan’s E3 SUMOylation activity. During induction of apoptosis, Mulan SUMOylates Drp1, which results in stabilization of a mitochondria-endoplasmic reticulum complex that permits calcium flux. In turn, this leads to cristae remodeling, and cytochrome c release, which activates the caspase dependent pathway of apoptosis [12].

Overall, Mulan stabilization of Drp1 by SUMOylation and breakdown of Mfn2 by ubiquitination enhances fission and impedes fusion [13]. Mulan knockdown with siRNA has also been shown to result in mitochondrial perinuclear clustering, whereas Mulan accumulation has been shown to result in mitochondrial fragmentation [1,7].
Apoptosis is a process of cell death and it can occur due to many forms of cellular stress, including OMM depolarization. Mulan is involved in apoptosis by ubiquitinating the tumor suppressor p53 and p73 proteins [4,14], as well as Akt, an antiapoptotic protein [15,16]. In addition, Mulan activates the protein kinase JNK just before the apoptosis associated caspase cascade is initiated [3]. In contrast, activation of the NF-κB transcription factor by Mulan during conditions of ER stress leads to the production of several antiapoptotic factors [17]. Therefore, Mulan’s placement in apoptosis often appears contradictory and the dynamics are still not highly understood.

Lastly, one of Mulan’s initial functions was defined by its presence in mitochondrial-derived vesicles (MDV) that fuse with peroxisomes. However, most peroxisomes do not contain Mulan, and only about 5-15 MDVs contain Mulan per cell. Vesicles that possess Drp1 were also found to block Mulan’s ability to cause mitochondria fragmentation and resulted in increased interconnectedness of the endoplasmic reticulum [2]. An interactor of Mulan, Vps35, is involved in Mulan-MDV formation and transport of Mulan to the peroxisome [18]. Vps35 deficiency leads to an increase in Mulan expression and mitochondrial fragmentation. Vps35 mutation is linked to familial PD [19].
Mitochondria alternate their shape and size through the processes of fission and fusion in order to maintain health and manage damage. Fission is the splitting of one mitochondrion into two and involves the Drp1 protein. Fusion is the combining of two mitochondria to form one and involves the Mfn1, Mfn2, and OPA1 proteins. Damaged mitochondria exit the cycle through mitophagy.

Figure 3: Mitochondrial fission and fusion
1.3 Mitophagy

Autophagy is the process of cytoplasmic sequestering within an autophagosome and the subsequent degradation by the merging with a lysosome. Nutrient deprivation, starvation, or hormone release can result in autophagy for the salvaging of precursor metabolites, however, autophagy is a continuous process that requires no stress for baseline functioning [20]. Autophagosomes are double-membraned vesicles that work to isolate damaged or excess cellular material. The overall process of autophagy includes induction, formation of the autophagosome, fusion of the autophagosome with a lysosome, breakdown of the cellular components with the lysosome’s hydrolytic enzymes and release of the products into the cytosol [21].

Mitophagy is a form of autophagy that specifically targets mitochondria for degradation and is responsible for maintaining a healthy mitochondrial population. The term “mitophagy” was coined to emphasize the selective and non-random nature of mitochondrial destruction [20]. Mitochondria may become damaged or dysfunctional, for example, through dysregulation of fission/fusion, through age, through mitochondrial permeability transition (MPT), or through an accumulation of reactive oxygen species (ROS) such as hydrogen peroxide, superoxide, and hydroxyl radical, the byproducts of oxidative phosphorylation in aerobic metabolism that cause mutation [7,20,21]. The ROS result in a depolarization of the mitochondrial membrane potential, an event that forces the signaling for autophagosome engulfment and mitophagy. Even in non-proliferating tissues, mitochondria are still replaced every 10-25 days through the binary fission of pre-existing mitochondria to generate the new and mitophagy to destroy the old [20].
Mitophagy was first associated with PINK1 and Parkin, proteins whose gene mutations are linked to Parkinson’s Disease (PD). PINK1 is a mitochondrial targeted serine-threonine kinase and Parkin is an E3 ubiquitin ligase in the cytosol [5, 22, 23]. In healthy mitochondria with high membrane potential, PINK1 is imported into the mitochondria and degraded. When a mitochondrion is damaged, and the membrane potential is lowered, the import of PINK1 is inhibited and it accumulates on the OMM. PINK1 phosphorylates Ub and Mfn2, which then recruits Parkin. Binding of Parkin to the phospho-ubiquitin chain and further phosphorylation of Parkin by PINK1 directly leads to activation of Parkin’s ubiquitin ligase activity for OMM substrates. Extensive ubiquitination of OMM proteins leads to mitophagy. Parkin ubiquitinates p62, which binds LC3 and leads to autophagosome formation [24] (Figure 4).

Studies on Drosophila indicate that downregulation of Mfn2, mitochondrial fusion promoting, and upregulation of Drp1, mitochondrial fission promoting, results in the suppression of PD phenotype when PINK1 or Parkin are absent [22]. Mitophagy can be induced in vitro with the mitochondrial uncoupler carbonyl cyanide m-chlorophenyl hydrazine (CCCP) for study purposes [23].

The role of Parkin as a universal inducer of mitophagy has recently been challenged [5]. Parkin expression is restrictive and inactivation of the protein in mice produces no discernible phenotype [25]. Recent work from our lab as well as others suggest that Mulan is involved in a mitophagy pathway that is independent of Parkin [26]. Studies have also suggested that Mulan is capable of compensating for the loss of PINK1/Parkin and that expression of wild-type Mulan can suppress the PD phenotype when PINK1/Parkin are mutated or when Mfn2 is overexpressed. Additionally, fly mutants have shown that the removal of Mulan in PINK1/Parkin mutants
results in aggravated PD phenotype and double knockouts (PINK1/Mulan or Parkin/Mulan) result in further muscle degeneration and potential lethality. Mouse cortical neurons with Parkin KO or Mulan KD (knock down) have shown decreased mitochondrial membrane potential.

Interaction between Mulan and PINK1/Parkin is thought to not be direct, but exist through Mfn2: analysis of HeLa cells show stabilization of Mfn2 when Mulan is not present (Parkin is not expressed in HeLa cells and does not confound) [5].

Mulan has also been found to be directly regulated by the mitochondrial Omi/HtrA2. Omi is a serine protease that normally resides in the IMS, exhibiting a role in mitochondrial homeostasis with protein quality control. Additionally, Omi is released into the cytosol and participates in apoptosis during stress. Inactivation of Omi’s protease ability by a single mutation (S276C) leads to the mnd2 (motor neuron degeneration 2) phenotype in mutant mice, exhibiting neurodegeneration, premature aging, muscle wasting, and parkinsonian features [26].

We have previously found that Mulan is a substrate of Omi and that mnd2 mutant mice show an accumulation of Mulan in their tissues. It is thought that Mulan’s increase results in mitochondrial dysfunction that mirrors mitochondrial uncoupling and leads to mitophagy. Imbalance in mitochondria numbers may result in energy deficiency for high-energy consuming cells, including muscle cells, neurons, and cardiomyocytes, leading to the observed mnd2 phenotype. Omi interacts with a domain of Mulan that is similar in residue sequence to a known substrate of Omi, Inhibitor of Apoptosis Proteins [26]. Omi is thought to be phosphorylated by PINK1 before interacting with Mulan (24).

Further support of Mulan’s role in mitophagy comes from studies where Mulan was shown to ubiquitinate ULK1 in the regulation of selenite-induced mitophagy [27].
In healthy mitochondria, the kinase PINK1 is transported into the OMM and IMM by TOM and TIM, respectively. Normal degradation then occurs with PARL and MPP. In damaged mitochondria, PINK1 becomes trapped inside TOM, and phosphorylates Mfn2, which attracts Parkin E3 ubiquitin ligase to the mitochondria for phosphorylation. Parkin then ubiquitinates many mitochondrial proteins, including p62, which binds LC3 and initiates autophagosome formation.
1.4 Mulan E2 Interactors

Mulan is an E3 ubiquitin ligase and as such it will interact with specific E2 conjugating enzymes in order to carry out its function. Our lab recently identified four specific E2 conjugating enzymes as specific interactors of Mulan in a yeast two-hybrid screen of a HeLa cDNA library. These enzymes are: Ube2E2, Ube2E3, Ube2G2, and Ube2L3. In addition, a modified yeast two-hybrid screen was used where Mulan’s RING domain was fused to each of the E2s to isolate potential substrates for the Mulan\textsubscript{259-352}-E2 complex. GABARAP was isolated as a key interactor for the Mulan\textsubscript{259-352}-Ube2E3 fusion protein; this protein is a member of the Atg8 family and participates in mitophagy, a potential link in subsequently explaining Mulan’s participation in mitophagy [28].

An alternative approach was recently used in our lab where recombinant Mulan-RING protein was tested directly against most of the common E2s in an in vitro ubiquitination assay. This was done to verify our yeast two-hybrid system results and to overcome a potential problem that not all E2s might be present in the HeLa cDNA library. The profiling assay identified Ube2D1, Ube2D2, Ube2D3, and Ube2E3 as very likely partner conjugating enzymes for Mulan (Figure 9).

By studying Mulan’s E2 interactors, we hope to gain further insight into a pathway by which Mulan E3 ubiquitin ligase regulates mitophagy and cell death following mitochondrial stress, and how the inactivation of this pathway may be responsible for mnd2 phenotype in mice.
CHAPTER 2: MATERIALS AND METHODS

2.1 Overview of the Yeast Two-Hybrid System

The yeast two-hybrid system was used to verify and confirm protein-protein interactions between Mulan’s RING domain and its anticipated E2 interactors: Ube2D1, Ube2D2, and Ube2D3. Ube2E3 acted as a positive control. In the yeast two-hybrid (Figure 5), the protein of interest and its potential interactor are separately cloned into different plasmid vectors, the plasmids are transformed into yeast, expression is induced, and the produced proteins may interact with each other and with an already present yeast plasmid to produce a color change [29].

The protein of interest, Mulan259-352, is fused to a LexA DNA binding domain (BD) and cloned into a pGilda vector. This is known as the bait. The LexA DNA binding domain component oversees the locating and binding of the bait to the Upstream Activation Sequence (UAS) for the lacZ reporter gene located on the pSH18-34 yeast plasmid. A collection of potential protein interactors from a HeLa cDNA library are then fused with an activation domain (AD) and cloned into a pJG4-5 vector. These are known as the prey. If the bait interacts with an unknown prey, then the binding domain and the activation domain will be in close enough proximity to create the full, functional transcription factor at the UAS (the complex: UAS-BD-Bait-Prey-AD). This leads to the transcription of the lacZ reporter gene for the synthesis of beta-galactosidase and leucine (Figure 5). The gene for leucine is not located on the pSH18-34 plasmid, however, its transcription is managed at the same promoter, providing a check that the proper plasmid is present and functioning [29].
In the presence of X-gal and galactose, the beta-galactosidase cleaves X-gal and a blue color is produced. This positive interaction must be further tested to determine if it is a true interaction between the proteins.

This yeast two-hybrid was abridged since the Ube2D prey were already isolated. A complete scan of the library was unnecessary as we were looking specifically for interactions implied in the in vitro profiling assay for Mulan. Second, Mulan259-352 was previously cloned into pGilda and transformed into EGY48 yeast for past experiments, and this stock was used.
The bait consists of Mulan$_{259-352}$ fused to a LexA-DNA binding domain and it is expressed in yeast. The prey is the full length E2 protein fused to the Activation domain (AD). If the prey interacts with the bait, it will activate transcription of the reporter gene (usually β-galactosidase) and yeast will turn blue.

Figure 5: Schematic diagram of the yeast two-hybrid system
2.2 Construction of pJG4-5-Ube2D Prey

2.2.1 Polymerase Chain Reaction

Prey Primer Construction:

Ube2D1: 5’ (EcoR1) – 5’ GGC GAA TTC ATG GCG CTG AAG AGG ATT CAG 3’
3’ (Xho1) – 5’ CCG CTC GAG TTA CAT TGC ATA TTT CTG AGT CCA 3’

Ube2D2: 5’ (EcoR1) – 5’ GGC GAA TTC ATG GCT CTG AAG AGA ATC CAC 3’
3’ (Xho1) – 5’ CCG CTC GAG TTA CAT CGC ATA CTT CTG AGT 3’

Ube2D3: 5’ (EcoR1) – 5’ GGC GAA TTC ATG GCG CTG AAA CGG ATT AAT AAG G 3’
3’ (Xho1) – 5’ CCG CTC GAG TCA CAT GGC ATA CCT CTG AGT CCA 3’

DNA for Ube2D1, Ube2D2, and Ube2D3 was amplified with the preceding primers at EcoR1 and Xho1 restriction enzyme sites. The PCR mix included: 1 µl of DNA Template, 100 µM of Forward Primer (eurofins), 100 µM of Reverse Primer (eurofins), 1 µl of dNTPs (Clontech), 5 µl of (10x) PCR Running Buffer (NEB), 40.8 µl of sterile water, and 0.2 µl of TAQ Polymerase (Roche Diagnostics Corp). The thermocycler abided by the following: 2 minutes of template denaturing at 95 °C, 29 cycles, and a final elongation period of 7 minutes at 72 °C. Each cycle consisted of denaturing at 95 °C for 30 seconds, primer annealing at 56 °C for 30 seconds, and TAQ Elongation at 72 °C for 35 seconds. Samples were kept on ice. Template DNA for Ube2D2 was taken from a HeLa library.
2.2.2 Agarose Gel Electrophoresis

Gels were prepared by adding 1.5 g of agarose powder (Promega) to 100 ml of (1x) TAE DNA Running Buffer. Microwaving for 2 minutes with intermittent solution swirling permitted the agarose to dissolve. 5 µl of ethidium bromide, the UV-associated viewing agent, was added while the solution was warm. Solution was then poured into a gel casket with two combs. Gel was left to solidify during the cooling process. After cooling, the combs were removed, and the gel was cut to give the appropriate quantity of wells. TAE buffer was poured over the gel, filling the casket, until the gel was fully immersed. Sample preparation included combining 2 µl of PCR DNA with 5 µl of DNA dye. The GeneRuler 1 kb plus DNA ladder (ThermoFisher) of multiple known DNA sizes was pipetted into the first well, and then each of the samples were added. Viewed with a gel doc, ethidium bromide fluorescence indicated the location and comparative size of DNA samples.

2.2.3 Precipitation

PCR product was precipitated to remove residual salts from the PCR buffer solution and to further purify the amplified DNA. Sample volume was brought up to 100 µl with water. 10 µl of sodium acetate (10% of sample volume) and 200 µl of (100%) ethanol (2x the sample volume) were added. Tubes were inverted several times and stored at -20 °C for 30 minutes. Next, tubes were spun at 13,200 rpm for 20 minutes within a 4 °C fridge. Supernatant was removed, pellet was dried in a speed vacuum for 15 minutes, and re-suspended in 50 µl of water.
2.2.4 Restriction Enzyme Digestion

5 µl of precipitated DNA (Ube2D1, Ube2D2, and Ube2D3 inserts, and stock pJG4-5 vector), 5 µl of (10x) Restriction Enzyme Buffer (NEB), 0.5 µl of (100x) BSA (NEB), 41.5 µl of dH2O, and 1 µl of restriction enzyme were combined and incubated in a 37 °C water bath. After an hour, 0.5 µl more enzyme was added, and incubation continued for another hour. This was performed first using Xho1 restriction enzyme, and then separately conducted using EcoR1 restriction enzyme, each with their appropriate buffer. Next, the insert DNA was precipitated, and the 4-5 vector DNA was ran on a 1.5% agarose gel, extracted and purified according to the methods in the Gel DNA Recovery Kit (Zymoclean). 50 µl of dH2O was added to the samples to bring the volume up to 100 µl. Samples were precipitated.

2.2.5 Ligation

2 µL of digested DNA was run on a 1.5% agarose gel and the quantity of DNA within 1 µl of insert and 1 µl of 4-5 vector was estimated. DNA used for ligation should be 3:1 for the vector to insert ratio. Each sample received 4 µl of vector, varied insert DNA (7 µl Ube2D1, 7 µl Ube2D2, 5 µl Ube2D3, 0 µl (Negative control), 3 µl of (5x) buffer, 1 µl of ligase, and enough dH2O to bring each sample up to 15 µl. Samples were incubated at room temperature for 20 minutes. 35 µl of dH2O was added to bring the samples up to 50 µl. Desalting occurred with the addition of 500 µl of (10x) butanol. Tubes were inverted several times and spun at 13,200 rpm for 15 minutes. Butanol was removed by pipette and tubes were dried for 15 minutes in a speed vacuum. Pellet was resuspended in 10 µl of dH2O.
2.2.6 Bacterial Transformation

Electrocompetent bacteria cells (Dh5α) were retrieved from a -80 °C freezer and thawed slowly on ice. Electroporation cuvettes were also chilled. 3 µL of ligated DNA was added to the tube of thawed bacteria cells (50 µL) and the entire solution was transferred to its respective cuvette. The Gene Pulser (Bio Rad) was set to 2.5 kV at 400 Ω. After electroporation, 800 µL of LB was quickly added to each cuvette, mixed, and transferred to a fresh tube. Samples were incubated by rotator for 1 hour at 37 °C. 250 µL of each sample was then plated on LB + AMP plates, with plastic beads (Fisher) to spread, and left to incubate over-night (O/N). Grown colonies were selected the following day and added to 1.5 ml of LB + AMP broth. Tubes were also incubated O/N.

2.2.7 Isolation of Plasmid from Bacteria: DNA-Boiling Miniprep

Cells and media were transferred into 1.5 mL Eppendorf tubes and spun at 13,200 rpm for 2 minutes at room temperature (RT). The supernatant was removed by aspiration and the pellet was resuspended in 300 µL of STET-lysozyme solution (8% sucrose, 5% (100X) Triton, 50 mM Tris-HCl pH 8, 50 nM of EDTA, 10 mg/mL of lysozyme). Samples were then boiled for 1 minute and spun at 13,200 rpm for 10 minutes. Mucus pellets were removed with a toothpick. 200 µL of (70%) isopropanol was added, and tubes were mixed by inverting. Samples were spun again at 13,200 rpm for 10 minutes. Isopropanol was removed by pouring out the supernatant in one motion. 200µL of (70%) ethanol was added with no mixing. Samples were spun at 13,200 rpm for 5 minutes. Ethanol was also removed by pouring out the supernatant in one motion and
the tubes were dried in a speed vacuum for 15 minutes. Pellets were resuspended in 100 µL of Tris-EDTA (TE) and vortexed using a Fisher Vortex Genie 2 for 10 minutes.

2.2.8 Isolation of Plasmid from Bacteria: DNA QIAprep Spin Miniprep

The methods suggested within the QIAprep Spin Miniprep Kit (QIAGen) were used. Bacteria grown O/N was spun at 11,200 rpm for 2 minutes. Supernatant was aspirated out and the pellet was resuspended in 200 µL of Buffer P1, which contains lysozyme and RNAse. Then, 200 µL of Buffer P2 was added, which contains SDS, tubes were inverted to mix and left at RT for 5 minutes. 250 µL of Buffer N3 was added, the tubes were inverted to mix, and spun at 11,200 rpm for 10 minutes. Supernatant was loaded into a QIAprep spin column and centrifuged for 1 minute. The flow through was discarded, the column was washed with 600 µL of Buffer PB, and tubes were centrifuged for 3 minutes. A subsequent wash occurred. The column was spun for 1 more minute to dry. Finally, the QIAprep spin column was placed in a fresh 1.5mL Eppendorf tube and the column was eluted with 50µL of Buffer EB (10 mM Tris-Cl pH 8.5) before a final centrifugation for 1 minute.

2.2.9 Restriction Enzyme Double Digestion

To test which DNA samples contained a successfully transformed plasmid-insert combination, a double digestion with EcoRI and Xho1 was performed. A master mix was prepared and equally distributed to a fresh 50 µL Eppendorf tube per sample. Each sample contained: 11.4 µL of dH2O, 2 µL of (10X) Restriction Enzyme Buffer (NEB), 0.2 µL (100X) BSA (NEB), 0.2 µL of EcoR1, and 0.2 µL of Xho1. 6 µL of DNA brought each sample up to 20
µL. All samples were incubated at 37 °C for 2 hours. 2 µL of each digested sample was then run on an agarose gel with RNAse loading dye and tested by visualization to determine insert presence. Clean DNA samples from the mini prep that contained the desired insert were stored at 20 °C.
2.3 The Yeast Two-Hybrid

2.3.1 Yeast Drop-Out Medium

Yeast drop-out powder (Sigma) consists of all 20 amino acids except the amino acids desired for drop-out mediums. The yeast two-hybrid plasmids associated with this study have selectable markers that permit them the ability to self-produce certain amino acids; a colony’s presence in drop-out medium specific to an amino acid the plasmid can produce, therefore, indicates the plasmid’s presence within the colony. To distinguish yeast pSH18-34 plasmid, uracil (U⁻) is left out from the medium (Figure 6). To distinguish the presence of pGilda after transformation, histidine (H⁺) is left out (Figure 7), and to distinguish the presence of pJG4-5 after transformation, tryptophan (W⁻) is left out (Figure 8). Expression for all of the vectors are under the GAL1 promoter and can be induced in the presence of galactose and repressed in the presence of glucose.

Yeast drop-out medium consisted of the following: 1 g dropout powder (Sigma), 3.35 g Yeast Nitrogen Base (Sigma), 10 g glucose or 10 g galactose and 5 g raffinose dissolved in dH₂O to a volume of 500 ml, and 10 g agar (Apex) if the medium was for plates. Solution was autoclaved for 40 minutes and cooled before the addition of 50 ml (10X) BU salt and 1 ml X-Gal if the medium was for blue-white screening. Media with agar was poured into plates, left to solidify, and stored at 4 °C, while bottled non-agar media was left at room temperature.
A LacZ reporter plasmid present in the EGY48 yeast strain and replicated with the 2 µm origin. Expression of LacZ is controlled by 8 LexA operators with the GAL1 promoter. Additionally, URA3 serves as a selectable marker and the ampR enables ampicillin resistance.
A cloning vector that fuses the bait protein of interest with LexA, which acts as the DNA binding domain. The ARS/CEN ori is used to replicate in yeast using the GAL1 promoter. HIS3 serves as the selectable marker and amp<sup>R</sup> is present for ampicillin resistance. Cloning utilizes the EcoR1 and XhoI restriction sites.

Figure 7: pGilda vector used for Mulan<sub>259-352</sub> bait cloning
A cloning vector that fuses the prey proteins with B42, which acts as the DNA Activating Domain. The 2 µm ori is used to replicate in yeast using the GAL1 promoter. TRP1 serves as the selectable marker and ampR is present for ampicillin resistance. Cloning utilizes the EcoRI and XhoI restriction sites. Additionally, this vector includes a nuclear localization sequence (NLS) and a hemagglutinin (HA) epitope tag.
2.3.2 Yeast Transformation

50 ml U⁻H⁻ glucose medium was inoculated with a single EGY48 yeast colony and grown shaking O/N at 30 °C; this colony was stock from previously transformed yeast with LexA-Mulan259-352 and, therefore, possessed not only the normal yeast plasmid pSH18-34, but also pGilda. Cells were harvested at 38,000 rpm (Allegra 6R Centrifuge) for 5 minutes. The supernatant was poured out and the cells were washed with 10 ml of TE pH 7.5 (10 mM Tris pH 5, 1 mM EDTA pH 8). Cells were spun for 5 minutes and the TE was poured out. Cells were resuspended in 5 ml LA (0.1 M LiAC in TE) and incubated shaking at 30 °C for 2 hours. Cells were spun at 3,000 rpm for 5 minutes and the LA was poured out. Cells were resuspended in 5 ml of LA. Salmon sperm DNA (SS DNA) was boiled for 5 minutes. 300 µL aliquots of yeast + Mulan259-352 cells, 2 µL of denatured SS DNA, and 5 µl of clean (miniprep) prey DNA were combined for transformation. 700 µL PEG (50% Peg 4000 in LA) (Sigma) was added to the solution, and resuspended to mix. Incubation occurred in a 30 °C water bath for 30 minutes. Cells were heat shocked for 15 minutes at 42 °C in a second water bath and collected by centrifugation at 13,200 rpm for 3 minutes. Supernatant was aspirated, and the pellet was resuspended in 300 µL of TE pH 7.5. 100 µL of transformed yeast was plated on U⁻H⁻ W⁻ glucose plates and grown for 2 days. Grown colonies were selected the following day and added to 1.5 mL of U⁻H⁻W⁻ glucose media. Tubes were also incubated O/N.
2.3.3 Yeast Induction

Cells and media were transferred into 1.5 ml Eppendorf tubes and centrifuged at 32,000 rpm for 3 minutes. Supernatant was poured out and 1 ml of U′H′W′ galactose/raffinose media was added to wash. After centrifugation for 3 minutes, the supernatant was aspirated out and 500 µL of fresh U′H′W′ galactose/raffinose was added. Yeast was resuspended with vortexing. An additional 1 ml of U′H′W′ galactose/raffinose media was added to the Eppendorf tubes and 1 ml of U′H′W′ glucose media was added to the original incubation tubes. New and old tubes were incubated for 4 hours at 30 °C. Cells and media were then transferred to fresh tubes, spun, and the supernatant was removed. 100 µL of ESB (2% SDS, 80mM Tris pH 6.8, 10% glycerol, 1.5% DTT, and 0.1 mg/ml bromophenol blue) was added to the yeast samples and to Ube2D1 and Ube2E3 non-induced samples for control. Resuspension revealed blue coloring. Samples were boiled for 5 minutes, vortexed thoroughly, boiled for an additional minute, and spun down to collect. Samples were stored at -20 °C.

2.3.4 Yeast SDS PAGE and Western Blot

Induced and non-induced sample supernatant was analyzed using SDS PAGE with the 12% resolving and 5% stacking gels. Samples were prepared with the addition of 5 µL (4X) sample buffer (10% β-mercaptoethanol, 6% SDS, 20% glycerol, 1/40 stacking buffer, and 0.2 mg/ml bromophenol blue) to 20 µl of supernatant and 1 minute of boiling. A western transfer was performed using a PVDF membrane. The membrane was activated with 10 ml methanol, 10 ml (5X) TBS buffer and 30 ml dH2O before transfer. α-HA (Sigma) (binds to the prey-fusion protein) mouse monoclonal primary antibody was diluted 1: 5,000 in 2% milk-TBST (250 mM
Tris pH 8, 1.25 M NaCl, and 0.1% Tween20) and α-LexA-HRP (SC) (binds to the bait-fusion protein) mouse monoclonal primary antibody was diluted 1: 500. After blocking with milk and washing with TBST, the membrane was incubated O/N at 4 °C shaking with the appropriate primary antibody. The membrane was washed, rabbit α-mouse, HRP conjugated secondary antibody (Jackson ImmunoResearch) diluted 1: 10,000 was used for 1-hour incubation, and the membrane was washed again. Protein visualization occurred using the chemiluminescence ECL kit (PIERCE). Blue sensitive autoradiography film (MIDSCI) was used for development in a SRX-101A (Konica).

12% resolving gels consisted of the following: 8 ml 40% acrylamide mix (BioRad), 10 ml dH2O, 6.3 ml 1.5 M Tris pH 8.8, 250µL 10% SDS, 250µL 10% APS, and 10µL TEMED (Fisher). 5% stacking gels consisted of the following: 1.25 ml 40% acrylamide mix (BioRad), 7.3 ml of dH2O, 1.25 ml 1.0 M Tris pH 6.8, 100µL 10% SDS, 100µL 10% APS, and 10 µl TEMED (Fisher).

2.3.5 Yeast Streaking and Spotting

Colonies from the yeast transformation were selected and streaked onto a UH+W-glucose plate. Colonies that grew on this master plate were used to streak a UH+W-galactose / raffinose plate. Results were photographed 2 days later.

The remaining protein-induced yeast was vortexed and transferred to a fresh 2 ml tube. 5 µl was used to spot a UH+W-galactose / raffinose plate. Results were photographed the next day.
2.4 Mouse Tissue Analysis

2.4.1 Mouse Tissue Preparation

Tissue from the brain, heart, liver, and kidneys were extracted from wild-type mice. Homogenization was performed with an ULTRA TURRAX (IKA) for all samples besides the brain, which was done by hand. A Bradford assay was performed to determine protein concentration and quantity of each sample needed for the equalized loading of 20 µg. Sample buffer was added, the samples were boiled for 1 minute, and they were briefly spun down to collect. Samples were stored at -20°C [30].

2.4.2 Mouse Tissue SDS PAGE and Western Blot

Techniques were performed as previously described. Primary monoclonal antibodies included: rabbit α-Ube2D1 (Abcam), α-Ube2D2 (Abcam), α-Ube2D3 (Abcam), and α-Ube2E3 (Novus Biologicals) diluted 1: 5,000. Secondary antibodies included goat α-rabbit, HRP conjugated (Jackson ImmunoResearch) diluted 1: 10,000. GAPDH was used as a loading control in each gel.
CHAPTER 3: RESULTS

3.1 Transformation and Screening of Mulan259-352 Interactors

Our lab’s previous yeast two-hybrid screen isolated both Ube2E2 and Ube2E3 as bona fide interactors of Mulan259-352 [28]. The in vitro profiling assay identified these two, as well as three additional E2s: Ube2D1, Ube2D2, and Ube2D3 (Figure 9).

To verify the validity of interaction for these proteins, Ube2D1, Ube2D2, and Ube2D3 were separately cloned in-frame into pJG4-5 vectors as described in Chapter 2. This yeast two-hybrid was abridged since the Ube2D prey were already identified, making the HeLa library scan unnecessary. Yeast cells pre-transformed with Mulan259-352 were transformed with the synthesized prey and streaked onto U- H- W- glucose + X-gal plates and U- H- W- galactose / raffinose + X-gal plates following induction. All three of the Ube2D prey showed an intense blue color on the galactose / raffinose plates (Figure 10).

Ube2D1, Ube2D2, and Ube2D3 have approximate molecular weights of 11 kDa. The HA tag and other proteins associated with the pJG4-5 vector have an estimated molecular weight of about 14 kDa. Therefore, HA-Ube2D fusion proteins should have an approximate molecular weight of 25 kDa (Figure 11).
An in vitro ubiquitination assay that uses recombinant His-Mulan\textsubscript{259-352} with each of the indicated E2 enzymes. Ube2D3, Ube2E3, Ube2D2, Ube2D1 conjugating enzymes were able to work efficiently with Mulan\textsubscript{259-352} in this assay. E2 proteins previously identified in our lab through the yeast two-hybrid are boxed in red and new proteins are boxed in blue.

Figure 9: Results of the E2 selection and profiling assay for Mulan
**Figure 10**: Interaction of the various E2 enzymes with Mulan\textsuperscript{259-352} in yeast

Full length Ube2D1, Ube2D2, and Ube2D3 were cloned in-frame in the pJG4-5 vector and transformed in yeast expressing LexA-Mulan\textsuperscript{259-352} bait. Ube2E3 was previously cloned and characterized in our lab. Interaction between the bait and the different prey was tested on X-gal plates. Blue color indicates a positive protein-protein interaction.
Figure 11: Expression and stability of HA-E2 prey in yeast cells

Western blot analysis showing induced (i) and non-induced (ni) yeast expression for Ube2D family members and Ube2E3. Primary antibody used was α-HA (Sigma). Lane 1: Induced 4-5 vector. Lane 2: Non-induced Ube2D1. Lane 3: Induced Ube2D1. Lane 4: Induced Ube2D2. Lane 5: Induced Ube2D3 Clone A. Lane 6: Induced Ube2D3 Clone B. Lane 7: Non-induced Ube2E3. Lane 8: Induced Ube2E3.
3.2 Tissue Specific Expression of Mulan\textsubscript{259-352} Interactors

To investigate the expression of the various E2s in mouse tissues, brain, heart, kidney, and liver tissue homogenates were prepared as described in Chapter 2 and analyzed by SDS-PAGE and Western blotting. Antibodies used were monoclonal rabbit α-Ube2D1 (Abcam), α-Ube2D2 (Abcam), α-Ube2D3 (Abcam), and α-Ube2E3 (Novus Biologicals).

Figure 12 shows that the expression and protein levels are very similar between all four E2s in the mouse tissues tested. Highest expression is seen in the brain and lowest in the heart.
Figure 12: Tissue specific expression and protein levels of various E2 partners of Mulan

Western blot analysis showing the expression of Ube2D1, Ube2D2, Ube2D3, and Ube2E3, proteins in mouse tissues. GAPDH was used as a loading control.
3.3 Characterization of Ube2D Interactors

There are four members of the Ube2D family, including Ube2D1, Ube2D2, Ube2D3, and Ube2D4. Protein sequences among the three studied family members are high in homology. NCBI BLAST indicates that Ube2D1 shares 89% identity with Ube2D2 and 88% identity with Ube2D3. Ube2D2 and Ube2D3 share 97% identity (Figure 13). Since these proteins are all E2 conjugating enzymes, it follows that high homology should mark similar functioning in certain areas.

Not much is known about the Ube2D family and their definitive roles in the cell, however, the research has encompassed a variety of molecular processes. One of the first studies described cadmium (Cd) exposure, a cause of renal dysfunction, decreasing Ube2D family expression in rat renal tubular epithelial (NRK-52E) and human cells. It was hypothesized that the inhibition of the Ube2D proteins enabled p53 accumulation and p53-dependent apoptosis under Cd exposure [31]. Results varied for different cell types and pointed to an association of Cd to renal toxicity, but not hepatic toxicity in mice. Human brain astrocytes (HBASTs) uniquely showed Cd increasing Ube2D3 expression [32]. Inorganic arsenic also increased p53 expression, and it decreased expression in Ube2D1 and Ube2D2, but not in Ube2D3, within NRK-52E cells. Inorganic mercury did not affect p53 or Ube2D protein levels. It was suggested that arsenic, but not mercury, increased p53-dependent apoptosis through the suppression of Ube2D proteins like Cd [33].
Alignment for Ube2D1, Ube2D2, and Ube2D3 proteins shows high sequence homology.

Asterisks indicate sites of varied amino acid residues. Coloring corresponds to amino acid properties.

**Figure 13: Protein sequence alignment for Ube2D family members**
3.4 Characterization of Ube2D1

Ube2D1 has been shown to interact with the RING finger of two E3 ubiquitin ligases besides Mulan. The herpes simplex virus, for example, requires the E3 ubiquitin ligase ICP0 for proper initiation of the lytic cycle of infection and degradation of proteins involved in DNA repair, centromere assembly, and antiviral proteins. The study pointed to Ube2D1 being a key component to the system by which ICP0 initiates activation from viral quiescence [34]. Second, Ube2D1 has been shown to interact with the E3 ubiquitin ligase March-I. Normal function for March-I involves lowering the expression for MHC-II and CD86 proteins in antigen presenting cells, proteins that mediate CD4 T cell activation. Ube2D1 is thought to aid in the ubiquitination of March-I itself with an unknown E2, leading to March-I expression decrease and the upregulation of MHC-II [35].

Lastly, Ube2D1 has been questioned for its potential influence in neurodevelopmental processes that may be linked to schizophrenia; Ube2D1’s selection was based on its association with the p53 pathway and its location at the chromosome region 10q21.1, which is adjacent to the ANK3 gene, a likely schizophrenia relation found in previous single nucleotide polymorphism (SNP) studies. SNPs in Ube2D1 for a case-control study did not correlate with schizophrenia for this population, however, there was an observed reduction in the risk of cancer for the patients over the decade study, surmised to be related to potential polymorphisms in p53 [36]. Ube2D1 continued to be involved in study due to its relation to long-term neurodevelopmental effects and altered brain function upon dysfunction. In follow up research, Ube2D1 showed no difference in basal concentrations for post-mortem schizophrenia patients
within the dorsolateral prefrontal cortex; however, Ube2D1 was significantly increased in mice under phencyclidine (PCP) treatment, a model for schizophrenia, at the juvenile time point [37].
3.5 Characterization of Ube2D2

The least amount is known about Ube2D2 among the three Ube2D family members analyzed. Perhaps the most important relationship studied for this protein is Ube2D2’s supported regulation of Parkin in Parkin-dependent mitophagy.

The knockdown of Ube2D2 is correlated with a reduction in the clearing of depolarized mitochondria with autophagic mechanisms, though the process of PINK1 membrane-embedding and Parkin translocation remains constant; this may point to redundancy in E2 action. Knockdown of Ube2D2 with additional E2 knockdowns (Ube2N and Ube2L3) goes further to decrease Parkin polyubiquitination of the mitochondria and decrease p62 recruitment [38].

Parkin is now thought to exhibit mechanistic features similar to the HECT ubiquitin ligase family: the RING Ube2D-binding domain is covered and autoinhibited. PINK1 phosphorylation permits the domain release, Ube2D2 can form a thioester bond to Parkin, and ubiquitination follows. Knockdown of specific E2 partners, therefore, directly relates to decreased Parkin ubiquitination and subsequent mitophagy [38]. Specifically, Ube2D2 with Parkin can monoubiquitinate Mfn1 [39].

An identical relationship with Parkin was also linked to Ube2D3 [38].
3.6 Characterization of Ube2D3

Ube2D3 has been studied with high consideration for tumorigenesis and cancer progression; it was found to interact with human telomerase reverse transcriptase (hTERT). hTERT expression usually correlates to telomerase activity and activation, leading to radio resistance for cancer cell lines, which was countered with Ube2D3 presence. Downregulation of Ube2D3 resulted in hTERT and cyclin D1 accumulation, cell proliferation, and quicker transitions from G1 to S phase in human breast cancer cells (MCF-7) [40]. Ube2D3 was also found to be significantly lower in esophageal cancer lines. The negative correlation linkage between hTERT and Ube2D3 demonstrated a strong predicting factor for the progression of human esophageal cancer [41]. Additionally, further study with esophageal squamous carcinoma cells (EC109) indicated that Ube2D3 overexpression prolonged G1 phase, decreased telomere length, and enhanced radio sensitivity again through the degradation of hTERT, reiterating the proposed link found in MCF-7 lines [42]. These results were consistent in Eca-109 cells and it was further showed that downregulating Ube2D3 led to faster DNA repair after IR exposure [43]. This data suggests that Ube2D3 manipulation might provide benefits for advancing radio therapy due to its supported link in cell-cycle control, DNA damage repair, and tumorigenesis [41]. However, care should be taken after considering the Ube2D family’s abundant involvement in alternative pathways.

Second, Ube2D3 has been shown to aid in the ubiquitination of RIG-1, which induces the aggregation of MAVS on mitochondria. This leads to downstream activation of transcription
factors and initiation of the innate immune response for viral protection through type I interferon production [44].
CHAPTER 4: DISCUSSION

The natural mitochondrial cycle involves a continual flow between fission and fusion. Fission can result in unequal mitochondria sharing and the creation of a depolarized membrane for one of the daughters [11-13]. This shift from homeostasis creates an increase in ubiquitination and may lead to mitophagy, a form of autophagy that destroys damaged mitochondria [20-23]. If the cell cannot manage the excessive damage, the cell may engage in apoptosis. Mitophagy and apoptosis can lead to disease since an imbalance in mitochondria may starve cells of energy and lead to neurodegeneration, muscle atrophy and wasting, and parkinsonian phenotype [26].

Mulan is an E3 ubiquitin ligase that has been implicated in all of these steps towards disease through interactions with its RING finger domain [1] (Figure 14). Though much research has been done concerning Mulan’s RING domain, there has been less focus on the full classification of Mulan’s E2 interactors, their role in a Mulan-derived pathway for mitophagy, and the potential link to mnd2 mutant mice phenotype. Previously in our lab, a yeast two hybrid system was used that identified Mulan interactors. In that study, the RING domain of Mulan (amino acids 259-352) served as bait in a yeast two-hybrid screening and potential interactors were cloned into pJG4-5. Strong and comparable protein-protein interactions were confirmed to exist between Mulan and the full-length E2 conjugating enzymes, Ube2E2 and Ube2E3. Since the yeast two-hybrid system has limitations, an in vitro ubiquitination assay with a recombinant Mulan-RING domain was used to monitor potential activity with these E2s and any additional E2 interactors that were not shown in the yeast two-hybrid. This screen identified the same
interactors, Ube2E2 and Ube2E3, as well as three additional E2s: Ube2D1, Ube2D2, and Ube2D3. These family members showed considerable promise as strong interactors and potential partners (Figure 9). All E2’s were cloned into the pJG4-5 vector as full-length proteins and their interaction with Mulan’s RING domain was tested side by side. They all showed strong positive interaction with the Mulan bait (Figure 10).

Therefore, we have shown that Ube2D1, Ube2D2, and Ube2D3 are able to interact with Mulan and work with it in an in vitro ubiquitination assay. This data strongly supports the notion that these interactions are real, functional and can potentially exist within mammalian cells.

Ube2D1, Ube2D2, and Ube2D3 have been previously studied and are known to be involved in contending p53-directed apoptosis, neurodegeneration in juvenile mice, possessing anti-cancer properties, aiding in innate immunity, aiding in the viral lytic cycle, and regulating the Parkin-dependent mitophagy [31-44]. Though these proteins possess high homology and some similar roles, their independent functions have a wide variance. This indicates the potential for different interactions with Mulan.

Since Mulan as an E3 ubiquitin ligase can only interact with one E2 at a time, we investigated if the tissue distribution or protein level of the various E2s can determine the identity of the Mulan/E2 combination at any time. Ube2D1, Ube2D2, Ube2D3, and Ube2E3 proteins showed no major differences among each other in their expression levels within the tissue types. They also displayed great overlap between tissues, indicative of ubiquitous activity.

One result of significance was the high levels of E2 protein expression in the brain, as opposed to the expression in the heart, kidney, and liver (Figure 12). This directly points to Mulan having vital functioning with the brain. Dysregulation of Mulan, therefore, might be
responsible for neurodegeneration and a related loss of function within the brain – neurons are highly energy demanding, therefore, an increase in mitophagy involving Mulan might be responsible for phenotype. Since Mulan expression is elevated in mnd2 mutant mice, this data might also indicate that the dysregulation of Mulan is responsible for the neurodegeneration apparent in mnd2 mice. A second result showed Ube2D expression was lower in the heart compared to the liver and kidney samples (Figure 1). This is interesting since Mulan expression is typically raised in the heart [1,26].

This data does not preclude the option that Mulan initiates its own mitophagy through collected interactions with other, non-Ube2D interactors. The specific relationship that exists between Mulan and Parkin is also still unknown and should be studied further; Mulan is thought to be able to prevent PD phenotype in the absence of PINK1/Parkin, yet its accumulation leads to PD phenotype in the presence of non-mutated PINK1/Parkin in mice [5,26]. This still points to a Mulan-dependent mechanism for mitophagy.

The next phase of the project has already been initiated. Further in vitro assays were performed with Mulan259-352 – E2 protein combinations looking to isolate potential substrates that interact with each individual ubiquitination complex. Combinations studied include: Mulan + Ube2D1, Mulan + Ube2D3, and Mulan + Ube2E3. Several potential substrates were identified for each combination and are being further analyzed.

Future work should focus on quantifying data for E2 expression in varied tissue samples with qPCR since western blot visualization alone is qualitative. Also, assays should be performed that involve stressing the cells, isolating the mitochondria and cytoplasm, and observing if there were changes in E2 expression present between these locations. This would be
particularly important since the Ube2D proteins are localized within the cytoplasm and must migrate to interact with mitochondria-bound Mulan. Lastly, it would also be interesting to conduct temporal studies in accordance with mice development. This could reveal E2 characterization specific to time for growth patterns or Mulan-related disease progression.
Mulan E3 Ubiquitin Ligase is known to be involved in mitochondrial dynamics, mitophagy, and apoptosis. Mulan interacts with Mfn2 and Drp1 to affect fission and fusion; it also activates NF-κB, p53, and JNK pathways. Mulan itself is regulated by Omi protease during mitochondrial stress. Mulan also drives a Parkin-independent pathway to mitophagy. During mitophagy, Mulan interacts with GABARAP and can compensate for the absence of PINK1 and Parkin. As an E3 ligase, its activity and substrate specificity are dictated by specific E2 partners.
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