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CHARACTERIZING THE FUNCTION OF THE N-TERMINAL DOMAIN OF
OMI/HTRA2

by

CHRISTINE NGUYEN

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

Fall Term, 2017

Thesis Chair: Dr. Antonis S. Zervos

ABSTRACT

The yeast two-hybrid system was used to isolate and characterize protein interactors of the N-terminal domain of the serine protease Omi/HtrA2 (high temperature requirement protein A2) encompassing amino acids 31-133. This large domain of Omi/HtrA2 is usually cleaved and removed through autoproteolysis to produce the mature form of the protein. Whether the N-terminal domain has any function after its removal is unknown. Omi/HtrA2 is involved in a variety of diseases including cancers, neurodegenerative disorders, and metabolic disorders, but thus far, it is assumed that its normal function is the degradation of specific substrates. To characterize any potential function of Omi/HtrA2's unique amino terminus, specific interactors were isolated. One such interactor was the small GTPase Rab2A protein. We discuss the implications of this interaction and its biological significance.

ACKNOWLEDGMENTS

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CHAPTER 1: INTRODUCTION AND REVIEW OF LITERATURE

Structure

Omi/HtrA2 (high temperature requirement protein A2) is an ATP-independent serine protease primarily localized in the mitochondrial intermembrane space, although it has also been identified in both the endoplasmic reticulum and the nucleus [1-4]. Within mitochondria, Omi plays key roles in maintenance of mitochondrial homeostasis and thus has been described as performing a pro-survival function [1, 5, 6]. However, in response to stress, Omi translocates to the cytosol, where it induces cellular apoptosis [2]. Omi was originally identified through a yeast two-hybrid screen for protein interactors of Mxi2, an alternatively spliced form of the p38 stress kinase [2]. It was later independently discovered through a yeast two-hybrid screen for protein interactors of presenilin-1, a protein linked to Alzheimer's disease [1].

Encoded by a gene found on human chromosome 2p12, Omi is a 458 amino acid protein of molecular weight 49 kDa that is expressed ubiquitously [2, 3]. Omi belongs to a family of proteins known as high temperature requirement A proteins (HtrA) and shows homology to bacterial HtrAs, a class of proteins with dual chaperone and peptidase function that varies according to the temperature [1]. Among mammals, the sequence for Omi has been found to be highly conserved and constitutes one of four HtrA proteases that have been identified, along with HtrA1, HtrA3, and HtrA4. Omi is unique within the mammalian HtrAs because it is mitochondrial while its paralogs are secreted [1].

The sequence of Omi consists of a mitochondrial targeting signal, transmembrane domain, a proteolytic trypsin like domain (including the catalytic triad of amino acids essential for its serine protease function), and a PDZ domain (Figure 1) [16]. X-ray crystallography has revealed that

Omi consists of seven α -helices and five β -sheets [16]. In its active form, Omi exhibits a homotrimeric structure with a pyramid shape: the IAP domain is located at the top of the pyramid while the PDZ domain forms the base of the pyramid. This PDZ domain restricts access to the active site and therefore regulates the activity Omi's serine protease domain [16]. Trimerization is crucial for Omi to function as mutation of a highly conserved phenylalanine 149 residue necessary for trimerization has been shown to abolish Omi's serine protease function [16, 29]. In its inactive form in the IMS of the mitochondria, Omi's transmembrane domain has been postulated to anchor the protein to the inner mitochondrial membrane. Omi is also found in its soluble active form within the IMS [18].

Alignment between the full length amino acid sequence of mouse and human Omi revealed that the mature form of the protein (amino acids 134-458) shows a high degree of similarity with about 95% of residues identical. However, the N-terminal domain has lower levels of similarity with only 61% of residues conserved (Figure 2). Within the N-terminal domain, the sequence for the transmembrane domain and mitochondrial targeting signal are similar, but the majority of the differences are located in the linker region between the targeting signal and transmembrane domain. Human Omi possesses a thrice repeated PRAXTXXTP motif within the linker region that is completely absent from mice. Alignment performed between the first 133 amino acids of Omi across various species including humans, mice, rats, cows, chimpanzees, and guinea pigs suggested that this PRAXTXXTP motif is only found in primates (Figure 3). The function of this PRAXTXXTP motif is unknown, but when Omi is expressed in *Escherichia coli* cells, the PRAXTXXTP motif was shown to cause human Omi to be much more susceptible to proteolytic degradation compared to mouse Omi [43].

Even though mitochondria contain their own genome, the proteins encoded by these genes are limited and mainly include subunits of the respiratory chain as well as ATP synthase. Therefore, the vast majority of mitochondrial proteins are nuclear encoded and after being synthesized on cytosolic ribosomes, must be targeted to mitochondria. This post-translational import usually occurs through an N-terminal signal sequence [32]. While the specific sequence of these targeting signals is not highly conserved among mitochondrial proteins, certain characteristics have been shown to be conserved including length (20-60 AA), charge (net positive), and the presence of stabilizing N-terminal residues in the mature protein [42]. Comparative analysis of the mitochondrial N-termini of various proteins from mice, humans, and yeast has revealed that Omi is unique in that its N-terminal pre-sequence is abnormally long at 133 amino acids [33]. This deviation serves as further impetus for investigating the potential fate of this N-terminal domain after its cleavage to form mature Omi.

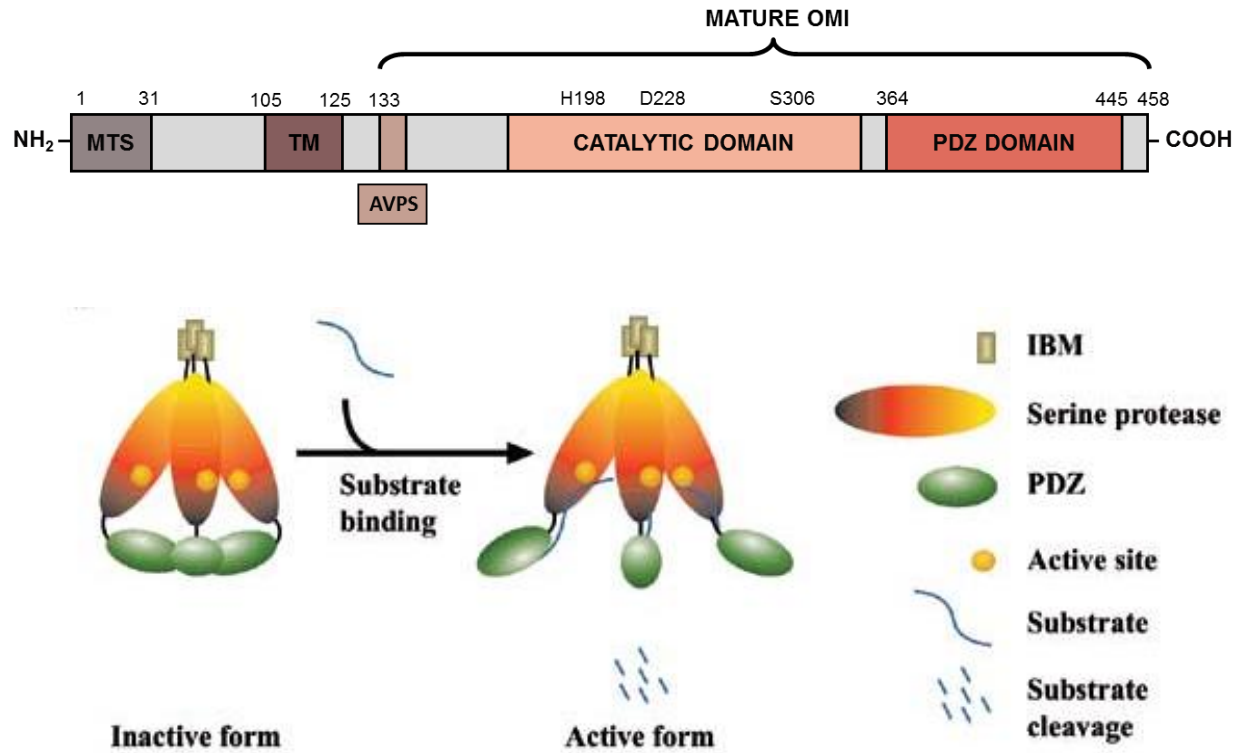


Figure 1. Structure of Omi

Omi is a 458 amino acid serine protease that consists of a large mitochondrial targeting signal, transmembrane domain, proteolytic trypsin-like domain, and a PDZ domain (including a conserved AVPS motif crucial for interactions with IAPs as well as the catalytic triad of amino acids). In its active form, Omi forms a homotrimeric pyramid structure with its IAP-binding domain (IBD) at the top and its PDZ domain at the base. The PDZ domain controls substrate access to the binding site [39].

	<i>Mitochondrial targeting signal</i>	
<i>Homo sapiens</i>	MAAPRAGRAGWSLRAWRALGGIRWGRRPRLT	70
<i>Mus musculus</i>	MAALKAGRGANWSLRAWRALGGIFWRKPELLA	70
<i>Rattus norvegicus</i>	MAALKAGRGANWSLRAWRALGGIFWRKPELLA	70
<i>Bos taurus</i>	MAALRAGRAGWSLRGWRALWGGRWGKGPLLT	70
<i>Cavia porcellus</i>	MAALRAGRGAAWILQGWRALGGILCGKGPLLT	70
<i>Pan troglodytes</i>	MAAPRAGRAGWSLRAWRALGGIRWGRRPRLT	70
	*** :***. * * :.*** * : * * :***** : **** * : : : * . * :	
	<i>Transmembrane domain</i>	
<i>Homo sapiens</i>	CLTSGTGPRAQLTAVT	133
<i>Mus musculus</i>	DLTSRTPDLWARLNVGTSGSSDQEARSPGSRREWLAVAVGAGGAVLLLLWGWGRGLSTVLA	133
<i>Rattus norvegicus</i>	NLTSGTDLWVRLTVGTPGSSDQEDCGSPGSRREWLAVAVGAGGAVLLLLWGWGRGPSTVLA	133
<i>Bos taurus</i>	CLRSRTSDLRRLIAGTPDPRTPEDSGTPGTRLRVWLAVALGAGGAVLLLFWGGGRGPVAVLA	133
<i>Cavia porcellus</i>	CLTSWTPTRR---TAENPDPPAREASGNPGALPHSWLVVTLGAGGALLLLWGRGRSPPAVLA	133
<i>Pan troglodytes</i>	CLTSGTGPRAQLTAVT	133
	* * . . * . * : : **.*:* **:* :** . ***	

Figure 3. Alignment of the N-terminal amino acid sequence of Omi/HtrA2 across numerous species

This alignment was performed using Clustal Omega and compares the first 133 amino acids of HtrA2 in *Homo sapiens* (human), *Mus musculus* (mouse), *Rattus norvegicus* (rat), *Bos taurus* (cow), *Cavia procellus* (guinea pig), and *Pan troglodytes* (chimpanzee). The numbers refer to the position of the amino acid residues. The functional domains present include: a mitochondrial targeting signal (aa 1-40) indicated in blue font and the transmembrane domain (aa 105-125) boxed in purple. The PRAAXTXXP motif is indicated in red. An asterisk (*) indicates identical residues, a colon (:) indicates residues with highly similar properties and a period (.) indicates residues with weakly similar properties.

Function

Omi plays dual roles in maintaining mitochondria homeostasis under normal conditions and promoting cell death under conditions of stress. Omi triggers cell death through two different pathways: caspase dependent cell death through interactions with inhibitors of apoptosis proteins (IAP) or caspase independent cell death through usage of its serine protease domain [17]. To induce caspase dependent cell death, Omi functions in a similar manner to proteins such as Smac/DIABLO, Grim, Reaper, and HID. These proteins are released from mitochondria to the cytosol following stress, and once in the cytosol, are able to bind to the BIR (baculovirus IAP repeat) domain of IAPs to deter their function and trigger activation of caspases that bring about cell death [19, 20]. In response to an apoptotic signal, Omi is processed into its active form through cleavage of its first 133 amino acids to produce the processed protein, which reveals the AVPS motif on its N-terminal domain essential for recognition and binding of the BIR or IAPs [19]. The importance of this motif has been verified in studies that observed that Omi's ability to induce cell death is drastically reduced by mutations within its AVPS motif or its serine protease domain, and its apoptotic function is altogether halted by mutations to both the AVPS motif and proteolytic domain [19]. Omi has also been observed to cause apoptosis through interactions with proteins outside of IAPs, such as ped/pea15 and HAX-1 [21, 22]. The serine protease activity of Omi, and hence its apoptotic function, has been shown to be effectively inhibited by UCF-101 [23].

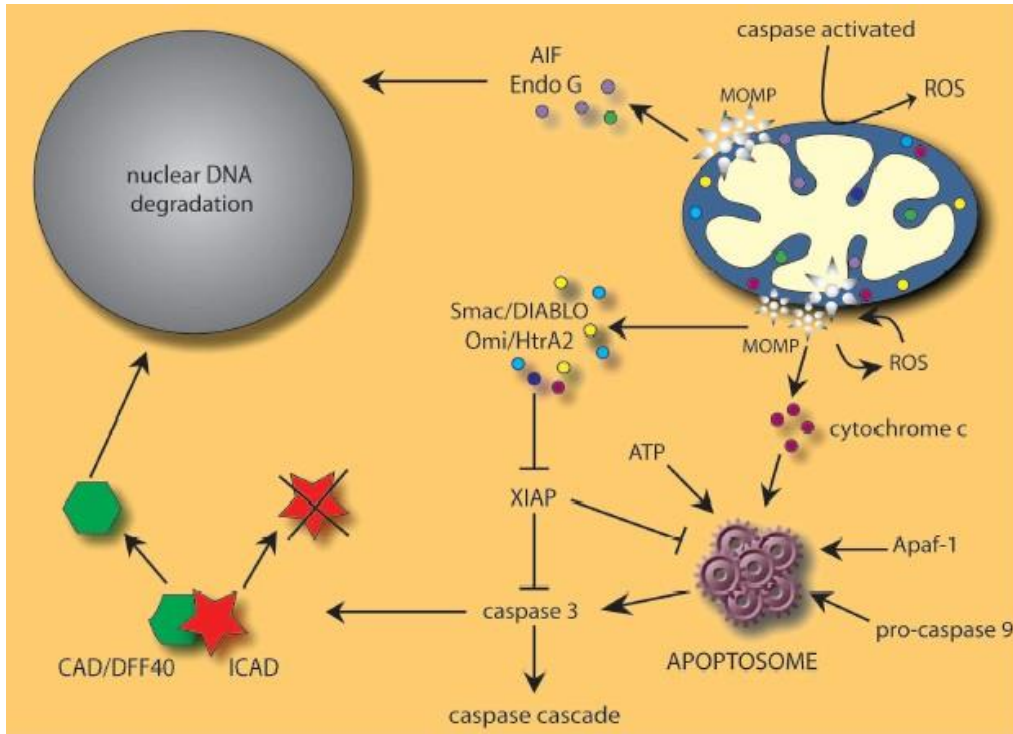


Figure 4. Programmed cell death via mitochondria

The release of pro-apoptotic proteins from the mitochondria into the cytosol induces cell death. One pathway through which this occurs involves the release of Smac/DIABLO and Omi/HtrA2, two mitochondrial intermembrane space proteins that antagonize IAPs. This leads to activation of caspase-3 and apoptosis. Omi/HtrA2 has also been postulated to induce caspase-independent cell death through its serine protease activity [40].

As mentioned previously, Omi also plays a key pro-survival function within the mitochondria. Increased mitophagy is observed for cells lacking functional Omi, due to Omi's role in regulating levels of the mitochondrial E3 ubiquitin ligase Mulan. Accumulation of the Mulan causes a concomitant decrease in mitofusion 2 (a protein which promotes mitochondrial fusion), leading to the observed mitophagy [19]. Furthermore, Omi has been suggested to play a key role in mitochondrial protein quality control and maintenance of mitochondrial homeostasis. Omi aids in the elimination of excessive or misfolded proteins within the mitochondrial intermembrane space [24]. The absence of Omi results in elevated levels of the mitochondrial proteins Lon protease 1 and prohibitin, leading to decreased mitochondrial membrane potential, the generation of reactive oxygen species, and a decrease in ATP production [28].

Omi's protective function is also clear through the Parkinsonian phenotype exhibited by *mnd2* mice that possess nonfunctional Omi through the homozygous mutation Ser276Cys. This mutation in Omi was postulated to limit substrate access to the active site of Omi, as deletion of the PDZ restores partial function [7]. Introduction of a human Omi transgene into the neuronal tissues of *mnd2* mice prevented development of the Parkinsonian phenotype, but the mice instead exhibited hallmark signs of premature aging including weight loss, hair loss, curved spine, and enlarged hearts [6]. Furthermore, overexpression of Omi in the neuronal cells of transgenic mice revealed a normal phenotype rather than increased signs of apoptosis, leading to the conclusion that Omi plays a neuroprotective role [25].

Implications for Disease

Non-functional Omi has been implicated in a variety of neurodegenerative disorders including Parkinson's disease (PD), Alzheimer's disease (AD), and Huntington's disease (HD). Mnd2 (motor neuron degeneration 2) mice possessing a homozygous mutation for Ser276Cys within Omi's proteolytic domain showed hallmark symptoms generally observed in PD including loss of neurons in the striatum of the basal ganglia, reduced body weight, reduced organ size, and muscle wasting before an early death (Figure 5) [7]. These mice exhibited increased vulnerability to stressed-induced permeabilization of the mitochondrial membrane that leads to cellular apoptosis [17]. Phosphorylation of Omi has also been suggested to be important to the functioning of its protease domain as mutations of the mitochondrial enzyme PTEN-induced putative kinase-1 (PINK1), which has been implicated in early onset AD, resulted in lower levels of phosphorylation of Omi near a site of mutation in human patients with PD [8].

This link between mutations affecting Omi's proteolytic activity and PD has been corroborated by screenings performed on German PD patients, which revealed high proportions of patients with the G399S (located in the PDZ domain) or A141S (located in the N-terminus of mature Omi) missense mutation compared to the general population [9]. Another study performed with Belgian PD patients showed that a R404W mutation (located in the PDZ domain) as well as variations in 5' and 3' regulatory regions that affect Omi expression increase susceptibility to PD [27]. However, the veracity of these findings is yet to be confirmed, as another group examining a cohort of North American patients found equal proportions of these mutations within a control group compared to a group afflicted with young and late onset PD [10].

Omi has also been suggested to be involved in various types of cancers. Gastric mucosal cells were found to have reduced expression of Omi compared to non-cancerous stomach cells, leading to the conclusion that perhaps a drop in Omi's apoptotic activity resulted in cancer development [13]. Researchers have also identified potential links between Omi expression and resistance to treatment with the alkylating agent cisplatin in ovarian cancer since cisplatin's mechanism of action involves increasing levels of Omi to promote apoptosis in cancerous cells and cisplatin resistant cancer cells had markedly lower levels of cytosolic Omi [14]. Conversely, Omi was found to be highly expressed in prostate cancer cells compared to both normal patients and those with benign prostate hyperplasia, leading researchers to conclude Omi contributes to prostate cancer development [15]. Because of the variability of gene expression observed among the various types of cancers, it is difficult to draw a definitive conclusion on the role of Omi in the development and progression of cancer. Nonetheless, Omi's vital role in balancing cell death and homeostasis suggests a high likelihood that aberrant expression of Omi could disturb this balance and trigger cancer.

Whole exome sequencing (WES) has led to identification of recessive mutations in Omi that cause 3-methylglutaconic aciduria (3-MGA-uria), a marker of metabolic dysfunction in which excessive amounts of 3-methylglutaconic acid are excreted. A study of four human patients lacking Omi due to a splicing mutation and a five base pair deletion observed increased susceptibility to apoptosis, abnormal cristae structure, and neurodegeneration in addition to 3-MGA-uria [30]. Similarly, another study using WES on five patients that presented with 3-MGA-uria noted recessive variants of Omi that caused seizures, hypotonia, neutropenia, and cardio-respiratory problems [31]. Both studies noted no abnormalities in mitochondrial network morphology or

deviations in the mitochondrial respiratory chain, although abnormal levels OPA1, a protein important in balancing fusion and fission in mitochondria, were observed [30, 31].

Thus, Omi has been implicated in a variety of diseases including cancers, neurodegenerative disorders, and metabolic disorders. Additional insight into Omi's mechanism of function through identification of protein interactors of its N-terminal domain could provide information about the pathways that result in disease phenotypes as well as possible methods to prevent or mitigate symptoms.

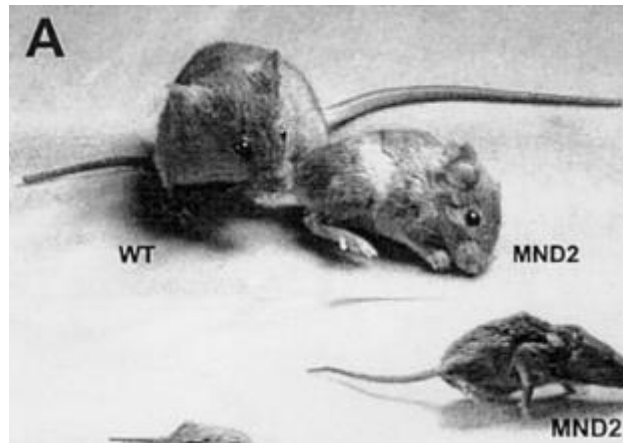


Figure 5. Phenotype of Mnd2 mice

Mnd2 mice with a homozygous mutation within Omi's catalytic domain exhibit neurological symptoms at 20 days postnatal that include involuntary movement and posture, akinesia, and an early death within 30-40 days postnatal [41].

CHAPTER 2: MATERIALS AND METHODS

Yeast Two-Hybrid Overview

The yeast two-hybrid screen was performed using the EGY48 yeast strain containing the pSH 18-34 reporter construct, which includes the LacZ gene for β -galactosidase under the control of LexA operators. These yeast also contain an endogenous leucine gene under the control of LexA operators. The protein of interest (Omi₃₁₋₁₃₃), termed the “bait,” was fused to the LexA DNA binding protein through the use of the pGilda vector. The pGilda vector contained a HIS3 selectable marker as well as a GAL1 promoter, which is repressed by glucose and induced by galactose. The potential interactors from the HeLa cDNA library, termed the “prey,” were fused to the B42 transcriptional activator through the use of the pJG4-5 vector, which contains a GAL1 promoter as well as a TRP1 selectable marker. Interaction between the bait and prey resulted in an active transcription factor that allowed for expression of two reporter genes. A positive interaction was indicated by yeast that was able to survive in media lacking leucine as well as produced a characteristic blue color in the presence of X-Gal.

Before performing the screen, a color and growth test were used to confirm that the two reporter genes could not be activated without interaction between the bait and prey. For the growth test, yeast that had been transformed with the pGilda vector containing Omi₃₁₋₁₃₃ was grown overnight in U⁻H⁻ glucose media and then transformed with the empty pJG4-5 vector. Varying dilutions were grown on U⁻H⁻W⁻L⁻ Galactose/Raffinose plates. The inability of the yeast to grow on medium lacking leucine confirmed that without interaction of the bait and prey, the gene for leucine was not activated. For the color test, yeast was streaked on U⁻H⁻W⁻ glucose plates and incubated for a few days. Colonies from this transformation were plated on U⁻H⁻W⁻

Galactose/Raffinose/X-Gal plates. The absence of blue colonies indicated that the bait protein did not self-activate to produce β -galactosidase.

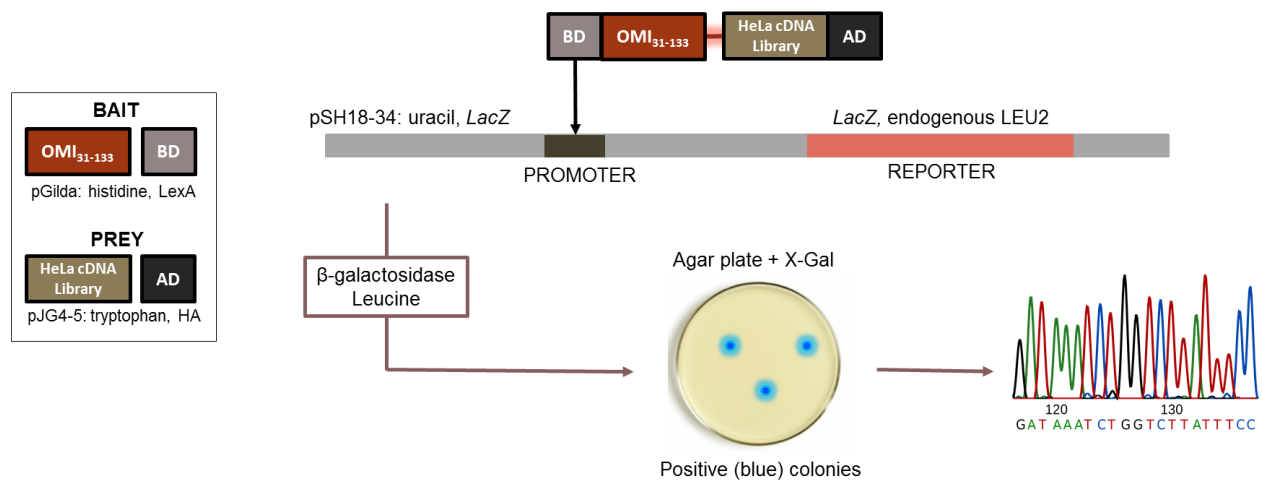


Figure 6. The Yeast Two-Hybrid System

Interaction between the LexA-*Omi₃₁₋₁₃₃* bait (fused to the binding domain) and the unknown prey protein from the HeLa cDNA library (fused to the activating domain) results in formation of an active transcription factor that binds to the LexA promoter. This results in the expression of the endogenous *LEU2* gene as well as the *LacZ* gene to form β -galactosidase and leucine. On a plate with X-Gal, positive interactors form blue colonies. DNA sequencing allows for identification of the specific interactor.

Polymerase Chain Reaction

The target DNA sequence corresponding to amino acids 31-133 of the N-terminal domain of Omi was amplified through PCR. The primers used in the PCR reaction and included restriction sites for EcoRI and BamHI (Table 1). For the PCR reaction, the following mixture was used: 20 ng of template DNA, 10 nM in 1 μ L of each of the primers, 5 μ L of 10X PCR Buffer containing Mg^{2+} , 1.5 units in 1 μ L of Taq polymerase, 10 μ M of dNTPs, and sterile water to bring the solution to a final volume of 50 μ L. The PCR program used was: denaturation for 2 minutes at 96°C followed by 25 cycles of denaturation for 30 seconds at 96°C, annealing for 30 seconds at 62°C, and elongation for 30 seconds at 72°C. The samples were then subjected to a final elongation for 7 minutes at 72°C and then kept at 4°C.

Table 1. Primer Sequences for Omi₃₁₋₁₃₃.

Primer	Sequence (5 → 3)	Restriction Site
Forward	GGCGAATTCTTGACCCCTGACCTCCGGG	EcoRI
Reverse	CGCGGATCCTTAGGCGAGGACGGCCGGAG	BamHI

These forward and reverse primers were used to amplify the cDNA sequence for Omi₃₁₋₁₃₃ with an EcoRI restriction site included the forward primer and a BamHI restriction site included in the reverse primer.

DNA Precipitation

1/10 sample volume of 3M sodium acetate pH 5.5 and 2 sample volumes of 100% ethanol kept at -20 °C were added to the DNA solution. This sample was mixed and then stored at -20 °C for at least 20 minutes. The sample were then centrifuged at 13,000RPM at 4 °C for 20 minutes. The supernatant was discarded. 250 µL of 70% ethanol was added to the pellet to wash away residual salt. The sample was centrifuged again at 13,000RPM for 5 minutes at 4 °C. The supernatant was discarded. The pellet was then dried for five minutes in a speed vacuum. The dried pellet was re-suspended in 50 µL of sterile water.

Restriction Enzyme Digestion

Both the vector and DNA insert were digested with BamHI and EcoRI in preparation for ligation. The restriction enzyme mixture included: 50 µL of DNA, 37 µL of sterile water, 10 µL of 10X Restriction Enzyme Buffer, 1.5 µL BSA, and 1.5 µL of the restriction enzyme EcoRI at 10 units/µL. This mixture was incubated at 37°C. At the end of an hour, an additional 0.5 µL of EcoRI was added. The mixture was left in the water bath for an additional hour for a total of two hours. Following the two hours, the DNA was precipitated from the solution and the digestion was repeated with the restriction enzyme BamHI. DNA gel electrophoresis was performed to determine whether the digestion was successful and to estimate the DNA concentration in the vector and insert solutions.

DNA Gel Electrophoresis

A 1.5% agarose gel was used to determine the size and concentration of the DNA present in each sample. This gel was created by mixing 100 µL of 1X TAE (0.04 M Tris-acetate, 0.001 M EDTA) with 1.5 g of agarose powder. This solution was microwaved for approximately 90-120 seconds until the agarose was observed to have completely dissolved. The solution was then cooled

under running cold water and 0.5 $\mu\text{g}/\mu\text{L}$ ethidium bromide was added and mixed by swirling. The solution was poured into a gel tray with well combs and left to solidify at room temperature. 2-5 μL of each sample of DNA was mixed with 5 μL of 10X Loading Buffer (30% glycerol, 2% Orange-G in 1X TAE) and loaded into the wells of the gel. A GeneRuler 1 kb DNA ladder was run along with the samples. The gel was covered with 1X TAE Buffer and run at 150V until the dye had migrated an appropriate distance in the gel. The gel was visualized using ultraviolet light in a Biorad Gel-Doc machine.

DNA Ligation

To determine the amount of vector and insert to use, gel electrophoresis was used to visualize and estimate the concentration of DNA in the insert and vector solutions. The amount of DNA used in the ligation should be roughly in a 3:1 insert to vector ratio. The reaction mixture included 1.5 μL of Ligation Buffer, 1.5 μL of ATP, 1 μL DNA ligase, 1.5 μL of 10X buffer, and varying amounts of insert and vector. A final volume of 15 μL was achieved by addition of sterile water to bring up the volume of the reaction mixture. The samples were incubated at room temperature for 20 minutes and at 72°C for 15 minutes in a heating block. The samples were stored at 4°C.

Bacterial Transformation

Approximately 3-4 μL of the DNA ligation was added into a 100 μL aliquot of competent DH5 α bacterial cells and transferred to an electroporation cuvette cooled to 4°C. The cuvette was placed in the Bio-Rad Gene Pulser to deliver a single exponential decay pulse of 2.5 kV at 400 Ω . 900 μl of room temperature LB medium was immediately added to the cuvette, which was gently mixed and transferred to a microcentrifuge tube. This tube was incubated at 37 °C for one hour

while spinning. Various volumes of the cells were plated on LB agar plates containing ampicillin and incubated overnight at 37 °C.

Isolation of Plasmid DNA from Bacteria (Boiling Method)

Individual colonies were isolated from the LB AMP plates and grown in 1.5 mL of LB AMP media overnight while shaking at 37 °C. The following day, the suspensions were poured into 1.5 mL microcentrifuge tubes and spun at 13,000 RPM for 2 minutes. The supernatant was aspirated and the pellet was resuspended in 300 µL of a STET/Lysozyme solution (8% sucrose, 5% 100X Triton, 50 mM of Tris-HCl pH 8, 50 mM of EDTA, 10 mg/mL of lysozyme). These samples were placed in a boiling water bath for 1 minute and centrifuged at 13,000RPM for 10 minutes. The pellet with cellular debris was removed with a toothpick and 200 µL isopropanol was added to the supernatant. The samples were inverted and mixed before centrifuging at 13000 RPM for 10 minutes. The supernatant was discarded and 300 µL of 70% ethanol was added to wash the pellet. This solution was centrifuged for 5 minutes at 13,000 RPM. The supernatant was discarded and the DNA pellet was dried in a speed vacuum for 10 minutes. The resulting dried pellet was resuspended in 50 µL of TE.

Isolation of Plasmid DNA from Bacteria (Clean Miniprep - QIAprep)

The QIAprep Spin Miniprep Kit was used to isolate plasmid DNA from bacteria that had been incubated with shaking overnight at 37°C in 1.5 mL of LB AMP media. These suspensions were transferred to microcentrifuge tubes and centrifuged at 13,000 RPM for 2 minutes at room temperature. The supernatant was aspirated and the pellet was resuspended in 200 µL of Resuspension Buffer P1 with RNase A (100 µg/mL). 200 µL of Lysis Buffer P2 was added, and the tubes were gently inverted several times until a homogeneously colored suspension was obtained. Next, 300 µL of Neutralization Buffer N3 was added and the tubes were once again

inverted several times until the solution appeared cloudy. The suspensions were centrifuged at 13,000 RPM for 10 minutes and the supernatant was transferred by pipette to a QIAprep spin column with a 2 mL collection tube. These columns were centrifuged for 1 minute at 13,000 RPM and the flow-through in the collection tube was discarded. The columns were then washed with 500 μ L of PB Buffer and centrifuged for an additional 1 minute at 13,000 RPM to remove any remaining wash buffer that might inhibit enzymatic reactions performed later on. The flow through was discarded once again followed by another centrifugation step for 1 minute at 1,3000 RPM. The spin columns were then transferred to a 1.5 mL clean microcentrifuge tube and 50 μ L of EB Buffer (10 mM Tris-HCl, pH 8.5) was added to the center of the column. This was allowed to sit for a minute before centrifuging for 1 minute at 13,000 RPM to elute the DNA.

Yeast Transformation

A single colony was inoculated in 50 mL of medium and grown overnight shaking at 30°C. The following day, the suspensions were centrifuged at 3800 RPM for 5 minutes and the supernatant was discarded. The cells were then washed with 10 ml TE pH 7.5 (10mM Tris pH 5, 1 mM EDTA pH 8) before being centrifuged once again at 3800 RPM for 5 minutes. The cells were resuspended in 5 mL LA (0.1 M LiAC in TE) and incubated in a shaker for 2 hours at 30°C. The cells were centrifuged at 3800 RPM for 5 minutes and the supernatant was discarded. The cells were once again resuspended in 5 mL of LA. 300 μ L of this solution was aliquoted into microcentrifuge tubes to which 1 μ L of denatured salmon sperm DNA and 1 μ g of DNA was added. 700 μ L of PEG (50% Peg 4000 in LA) was added and mixed thoroughly by micropipetting. The solution was then incubated in a water bath at 30°C for 30 minutes and then 42°C for 15 minutes. These cells were centrifuged at 13200 RPM for 3 minutes and the supernatant was

discarded. The pellet was resuspended in 300 μ L TE pH 7.5 and different volumes of the solution was plated on U⁻H⁻ Glucose plates. These plates were incubated for 2-3 days at 30°C.

Yeast SDS-PAGE and Western Blot

Single colonies of yeast were grown shaking overnight at 30°C in 2 mL of U⁻H⁻W glucose media. The following day, this suspension was centrifuged at 13200 RPM for 3 minutes at room temperature and the resulting supernatant was discarded. These cells were then washed with 1 mL of U⁻H⁻W⁻ galactose/raffinose media, and centrifuged at 13200 RPM for 3 minutes once again with the supernatant discarded. This pellet was resuspended in 500 μ L of U⁻H⁻W⁻ galactose/raffinose media. This solution was transferred to test tubes to which 500 μ L U⁻H⁻W⁻ galactose/raffinose media had already been added. These cells were incubated while rotating for 4 hours at 30°C. At the end of the incubation period, the cells were transferred to microcentrifuge tubes and boiled for 3 minutes. 0.1 g of 0.2 mm glass beads were added to the cells, which were vortexed for 2 minutes. 70 μ L of EBS (2% SDS, 80 mM Tris pH 6.8, 10% glycerol, 1.5% DTT, and 0.1 mg/ml bromophenol blue) was added to the cells and the samples were boiled for 1 minute and centrifuged for 1 minute at 13200 RPM. The supernatant was analyzed using SDS-PAGE with a 12% resolving gel and 5% stacking gel, and a Western transfer was performed with a PVDF membrane. α -LexA-HRP was used as the primary antibody for the bait fusion protein. This allowed for confirmation that the fusion protein was expressed and stable.

High Efficiency Yeast Transformation

An individual colony of EGY48 yeast containing pSH18-34 as well as LexA-Omi₃₁₋₁₃₃ in pGilda was grown overnight at 30 °C while shaking in 50 ml of U⁻H⁻ glucose medium. This culture was diluted the following day to 300 mL with U⁻H⁻ glucose medium and grown to an

OD_{600nm} of 0.9. The cells were then harvested by centrifugation at 3800 RPM for 5 minutes, and the resulting supernatant was discarded. The cells were washed with 20 mL of sterile water and centrifuged for another 5 minutes at 3800 RPM with the supernatant discarded. The solutions were resuspended in 20 mL LA (0.1M LiOAc in TE) and spun again for 5 minutes at 3800 RPM. This pellet was resuspended in 5 mL of LA. 100 µl of competent yeast was aliquoted into 1.5 mL microcentrifuge tubes. 10 µg DNA (HeLa cDNA library in pJG4-5), 100 µg of denatured salmon sperm DNA, and 600 µl of 40% PEG was added to the aliquots. The mixtures were resuspended until homogeneous. The transformation reaction was then incubated at 30°C for 30 minutes and then heat shocked at 42°C for 15 minutes. The samples were centrifuged and the supernatant was aspirated. The pellet was resuspended in 500 µL of sterile water. 300 µL of this solution of yeast was plated on 24 x 24 cm U⁻H⁻W⁻ galactose/raffinose plates and incubated at 30°C for 2-3 days.

Screening of Library

The yeast were grown on U⁻H⁻W⁻L⁻ galactose/raffinose plates for growth selection, and positive colonies were plated onto a U⁻H⁻W⁻ glucose plate. This plate was incubated at 30°C for 2-3 days. These colonies were then streaked onto U⁻H⁻W⁻ glucose/X-Gal and U⁻H⁻W⁻ galactose/raffinose/X-Gal plates. Blue colonies on these plates indicated a positive interaction.

Release of Plasmid from Yeast

Positive colonies were selected to perform a release the pJG4-5 plasmid with the HeLa cDNA library insert. These colonies were grown rotating at 30°C overnight in 1 mL of W⁻ glucose media. The following day, 800 µL of yeast was transferred to in a microcentrifuge screw-lid tubes and spun for 1 minute at 13200 RPM. The supernatant was aspirated and the pellet was resuspended in 200 µL of yeast lysis solution (2% TritonX-100, 1% SDS, 100 mM NaCl, 10 mM

Tris pH 8.0, 1 mM EDTA). 0.3 g of 150 μ m glass beads and 200 μ L of phenol-chloroform-isoamyl alcohol was added to the solution. The tubes were vortexed vigorously using a mini-beadbeater for 2 minutes. They were then centrifuged for 5 minutes at 13,200 RPM until three distinct layers were observed within the tube. The top layer contained the DNA of interest while the middle layer contained protein and the bottom layer contained cellular debris. Hence, the top layer was kept and a DNA precipitation procedure was performed.

KC8 Transformation

The plasmids were transformed into bacterial KC8 cells using electroporation. This solution was plated onto WM9 AMP plates and incubated overnight at 37°C. A single colony was selected from each plate and grown in LB AMP media overnight.

Retransformation

The boiling method was used to obtain the plasmid DNA from the KC8 cells. This was transformed back into yeast cells containing the pSH18-34 plasmid and the pGilda vector carrying the bait. These samples were plated onto U⁻H⁻W⁻ glucose plates. Colonies growing on these plates were subsequently grown on U⁻H⁻W⁻ Glucose/XGal plates and U⁻H⁻W⁻ Galactose/Raffinose/X-Gal plates for color selection. A colony that was blue on the U⁻H⁻W⁻ Galactose/Raffinose/X-Gal plates and white on the U⁻H⁻W⁻ Glucose/XGal was deemed a true positive interactor.

CHAPTER 3: RESULTS

Previous data on the N-terminal Domain of Omi

Previous experiments performed in the Zervos lab indicated the potential function of the N-terminal domain of Omi in promoting apoptosis under conditions of mitochondrial stress. Various constructs were created including a full length Omi and different portions of the N-terminal domain with amino acids 1-45 (includes the mitochondrial targeting signal and a few additional amino acids), amino acids 1-88 (includes the mitochondrial targeting signal and all three PRAAXXTXXP motifs), and amino acids 1-133 (includes the entire N-terminal domain that is cleaved off to form the mature protein, stopping just short of the AVPS motif) (Figure 7). The constructs were expressed as fusions to the green fluorescent protein (GFP).

These constructs were transfected into LLC-PK1 cells (pig proximal tubule epithelial cells) and treated with antimycin (1 μ M) for 24 hours. Antimycin acts as an inhibitor of cellular respiration by disrupting the electron transport chain and therefore prevents the production of ATP through oxidative phosphorylation. In addition, it causes the creation of toxic free radicals harmful to the cell. As a result, antimycin was used in this experiment to induce mitochondrial stress. Apoptosis was monitored using flow cytometry with AnnexinV staining. AnnexinV binds phosphatidylserine, and the presence of this molecule on the outer membrane of cells serves as an indicator of apoptosis. Apoptosis data was acquired for a population of 10,000 GFP transfected cells.

For all constructs, cells showed greater levels of apoptosis under conditions of stress compared to untreated cells. Cells possessing full length Omi showed the greatest level of apoptosis followed by the cells containing the full N-terminal domain. Cells containing Omi₁₋₁₃₃

showed significantly higher levels of apoptosis than cells transfected with a truncated N-terminal domain, suggesting that the N-terminal domain of Omi most likely plays a role in promoting apoptosis, although the mechanism through which this occurs is unclear (Figure 8).

These preliminary results served as the impetus for this project. It suggested that the N-terminal domain has a function within the cell, possibly related to apoptosis. Identifying interactors of this N-terminal domain could potentially give clues as to the specific function of the N-terminal domain of Omi.

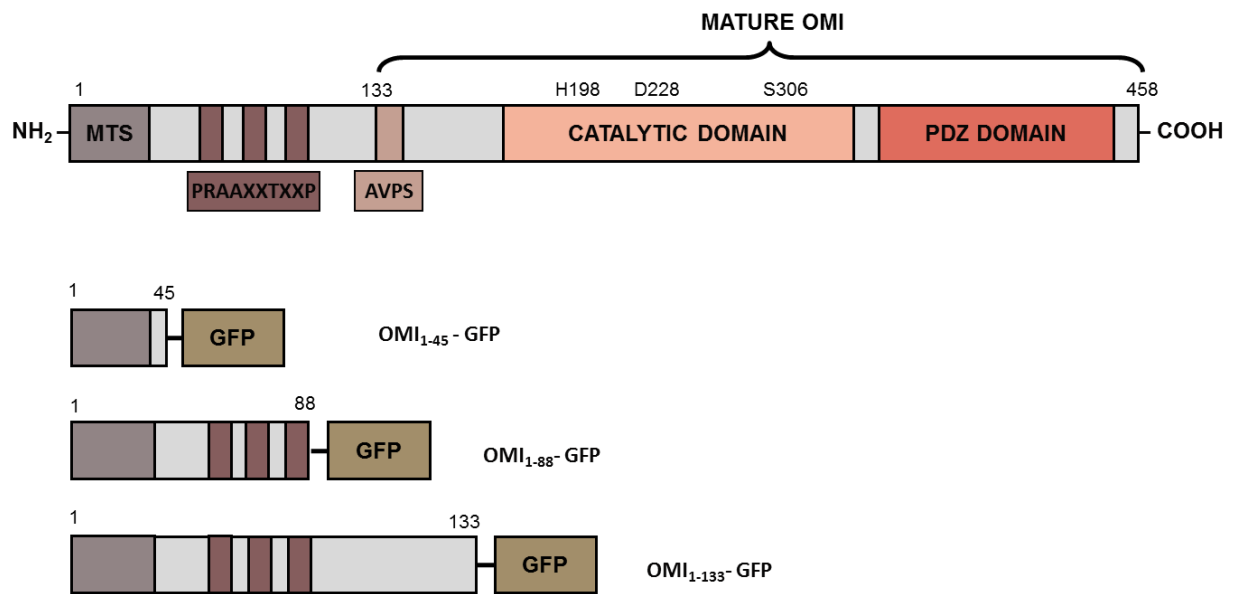


Figure 7. Omi constructs

A full length Omi includes a mitochondrial targeting signal, AVPS motif, catalytic domain containing the catalytic triad of residues, and a C-terminal PDZ domain. Various constructs containing different lengths of the N-terminal domain of Omi linked to GFP were created including amino acids 1-45, 1-88, and 1-33.

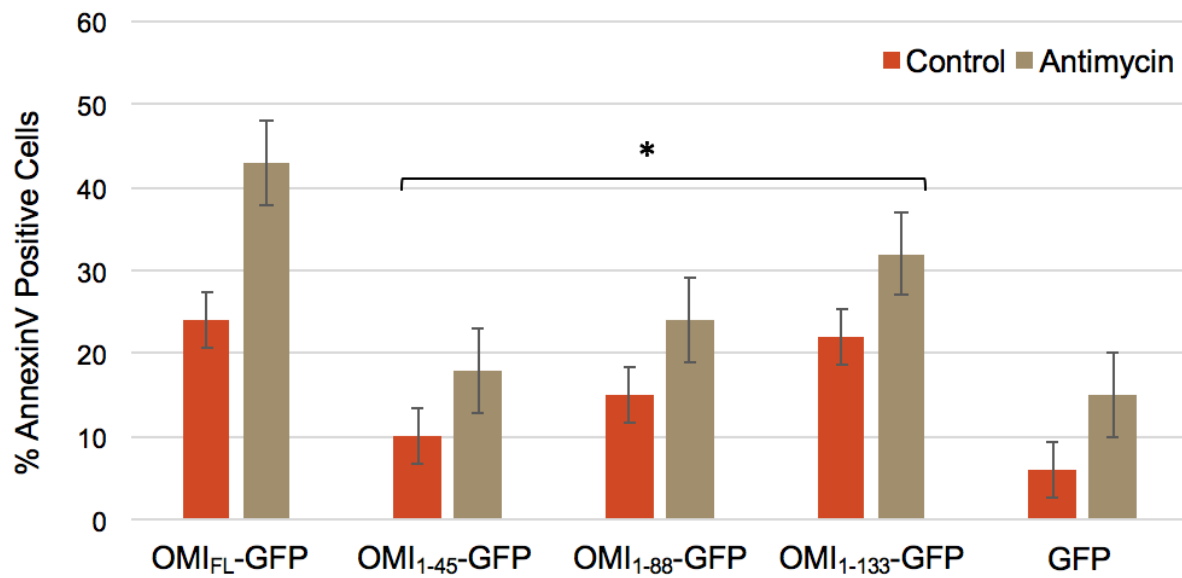


Figure 8. Flow cytometry to monitor apoptosis with different Omi constructs

LLC-PK1 cells (pig proximal tubule epithelial cells) were transfected with the various Omi constructs and treated with antimycin to induce mitochondrial stress (1 micromole for 24 hours). Flow cytometry with AnnexinV staining was used to measure apoptosis in the population of transfected cells. 10,000 GFP positive cells were acquired.

Expression and Stability of the Bait

The DNA fragment corresponding to Omi's N-terminal domain aa 31-133 was cloned into the pGilda vector, and the expression and stability of the bait in yeast was tested. Yeast were transformed with the LexA-Omi₃₁₋₁₃₃ plasmid and plated on U⁻H⁻ plates. These plates were allowed to incubate for several days at 30°C. Single colonies were picked from these plates and grown overnight in U⁻H⁻ media. These yeast were induced the following day with U⁻H⁻ galactose/raffinose media and lysed. Uninduced (control) yeast were grown in U⁻H⁻ glucose media. SDS PAGE and a Western blot analysis with LexA specific antibodies was used to verify that the LexA-Omi₃₁₋₁₃₃ bait was both expressed and stable within the yeast (Figure 9). Omi₃₁₋₁₃₃ is roughly 10.5 kDa and LexA is 24 kDa. Thus, it was expected that the LexA-Omi₃₁₋₁₃₃ would be 34.5 kDa. The Western blot shows no bands for the uninduced samples, since glucose represses the GAL1 promoter in pGilda. Conversely, galactose/raffinose promotes expression with a GAL1 promoter, so yeast in the induced condition expressed LexA or LexA-Omi₃₁₋₁₃₃, showing bands at 24 kDa and 34.5 kDa, respectively (Figure 9).

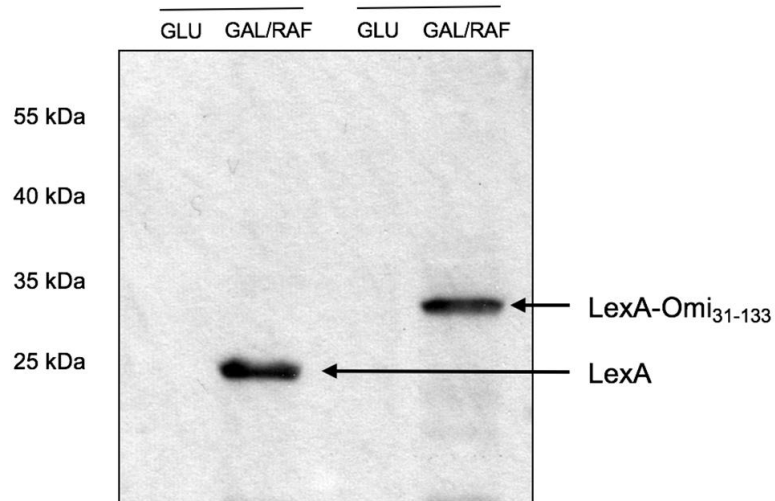


Figure 9. Expression and stability of LexA-Omi31-133 in yeast

The LexA-Omi₃₁₋₁₃₃ protein is 34.5 kDa, and the LexA protein alone is 24 kDa. Yeast transfected with the empty pGilda vector or the fusion bait LexA-Omi₁₋₁₃₃ were grown in U¹⁴C galactose/raffinose media (induced) or U¹⁴C glucose media (uninduced). Yeast extracts were separated by SDS-PAGE and analyzed by Western blotting using LexA specific antibodies.

Screen of HeLa cDNA library

The H2Y screen was used to isolate interactors against the LexA-Omi₃₁₋₁₃₃ bait as described in the Methods section. Several yeast colonies appeared that could potentially contain a specific interactor. The plasmid from these colonies was isolated and sequenced. DNA sequencing revealed the cDNA represents a partial length sequence for Rab2A, encoding amino acids 7-212 (Figure 10). PCR and specific primers were used to amplify a full length cDNA clone for the human Rab2A protein (Table 2). This was then cloned back to the pJG-4-5 vector and used to transform yeast. The full length Rab2A protein was also able to interact with the LexA-Omi₃₁₋₁₃₃ bait (Figure 11).

Table 2. Primer Sequences for Full Length Rab2A

Primer	Sequence (5 → 3)	Restriction Site
Forward	GGCGAATTCATGGCGTACGCCTATCTCTTC	EcoRI
Reverse	CCGCTCGAGTCAACAGCAGCCGCCCC	XhoI

These forward and reverse primers were used to create a full length clone for Rab2A with an EcoRI restriction site included the forward primer and an XhoI restriction site included in the reverse primer.


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ATGGCGTACGCCTAT CTCTTCAAGTACATC ATAATCGGCGACACA GGTGTTGGTAAATCA TGCTTATTGCTACAG
M A Y A Y L F K Y I I I G D T G V G K S C L L L Q
TTTACAGACAAGAGG TTTCAGCCAGTGCAT GACCTTACTATTGGT GTAGAGTTCGGTGCT CGAATGATAACTATT
F T D K R F Q P V H D L T I G V E F G A R M I T I
GATGGGAAACAGATA AAAC TTCAGATATGG GATACGGCAGGGCAA GAATCCTTTCGTTC ATCACAAGGTCGTAT
D G K Q I K L Q I W D T A G Q E S F R S I T R S Y
TACAGAGGTGCAGCA GGAGCTT TACTAGTT TACGATATTACACGG AGAGATACATTCAAC CACTTGACAACCTGG
Y R G A A G A L L V Y D I T R R D T F N H L T T W
TTAGAAGATGCCCGC CAGCATTCCAATTCC AACATGGTCATTATG CTTATTGGAAATAAA AGTGATTTAGAATCT
L E D A R Q H S N S N M V I M L I G N K S D L E S
AGAAGAGAAGTAAAA AAAGAAGAAGGTGAA GCTTTTGCACGAGAA CATGGACTCATCTTC ATGGAAACGTCTGCT
R R E V K K E E G E A F A R E H G L I F M E T S A
AAGACTGCTTCCAAT GTAGAAGAGGCATTT ATTAATACAGCAAAA GAAATTTATGAAAA ATTCAAGAAGGAGTC
K T A S N V E E A F I N T A K E I Y E K I Q E G V
TTTGACATTAATAAT GAGGCAAATGGCATT AAAATTGGCCCTCAG CATGCTGCTACCAAT GCAACACATGCAGGC
F D I N N E A N G I K I G P Q H A A T N A T H A G
AATCAGGGAGGACAG CAGGCTGGGGCGGC TGCTGTTGA
N Q G G Q Q A G G G C C *

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Figure 10. cDNA clone representing the Rab2A polypeptide is a specific interactor of LexA-Omi₁₃₁₋₁₃₃.

DNA and corresponding amino acid sequence of the partial clone representing part of the Rab2A protein that was isolated as a specific interactor in our yeast two-hybrid screen (blue). The DNA and amino acid sequence for the full length protein also includes the portion in black.

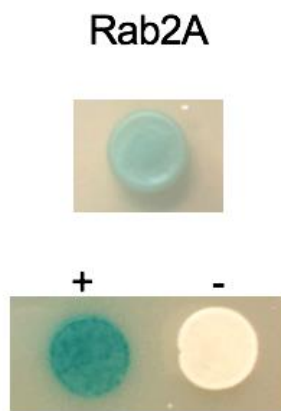


Figure 11. Full length Rab2A interacts specifically with LexA-Omi₃₁₋₁₃₃ in yeast

The cDNA library screen originally identified a partial length sequence of Rab2A as an interactor of Omi₂₁₋₁₃₃. A full length clone of Rab2A was produced, cloned back to the pJG-4-5 vector, and used to transform yeast. The characteristic blue color produced in the presence of X-Gal indicating expression of the reporter system confirms that full length Rab2A also interacts with Omi₂₁₋₁₃₃.

CHAPTER 4: DISCUSSION

Omi/HtrA2 was first identified as a mammalian homolog to the bacterial heat shock protein HtrA, and since its discovery, multiple studies have implicated this mitochondrial protease in the quality control of mitochondrial proteins, mitochondrial dynamics, autophagy, as well as apoptosis [2, 17, 24, 28, 38]. It has been assumed that all properties assigned to Omi are entirely mediated by its ability to function as a serine protease and degrade specific substrates. Some of these substrates have already been identified and represent a diverse group of both mitochondrial and cytoplasmic proteins. A paradox in the biological function of Omi is the presence of a very large N-terminal domain in the precursor Omi protein that is cleaved and removed to create the functional active protease. The normal function of this large N-terminal domain is unknown. It is assumed to be a mitochondrial targeting sequence, which does not help to explain its size or the various structural characteristics that it contains. We hypothesized that the amino terminal of Omi after it is removed from the precursor protein is stable and has a distinct function. In this work, we tried to identify the potential function of Omi's N-terminus by isolating and characterizing its specific interactors. This method has been used successfully in the past to define the function of a protein through its interactions. In addition, it provides information as to the potential stability of the N-terminus when expressed in yeast.

We performed a yeast two-hybrid screen, using the N-terminal domain of Omi (amino acids 31-133). Omi 1-30 amino acids represent the mitochondrial targeting sequence and were not included, as the success of the yeast two-hybrid relies on an interaction that occurs within the nucleus. The expression and stability of the LexA-Omi₃₁₋₁₃₃ suggested the Omi₃₁₋₁₃₃ polypeptide can be expressed and is stable in yeast cells. The yeast two-hybrid screen of a HeLa cDNA library

revealed a novel interactor, the Rab2A protein. Rab2A was able to specifically and strongly interact with the LexA-Omi₃₁₋₁₃₃.

The Rab (Ras-related in the brain) GTPases comprise a family of proteins within the Ras superfamily that are found within all eukaryotic organisms. Localized on the cytosolic side of intracellular membranes, Rab GTPases are essential for coordinating vesicle traffic within the cell through interactions with specific effector molecules such as tethering factors, kinases, and phosphatases [34, 36]. Rab has been shown to influence numerous aspects of trafficking including vesicle uncoating, budding, motility, fusion, and receptor signaling as well as play key roles in cellular signaling [34].

Rab GTPase activation is controlled by the antagonistic effects of GDP/GTP exchange factors (GEFs) and RAB GTPase activating proteins (GAPs). Rab alternates between two states: active Rab is bound to GTP while inactive Rab is bound to GDP. GEFs catalyze the conversion of GDP for GTP to form the active protein, and GAPs produce the opposite effect by promoting GTP hydrolysis to GDP [35]. With over 60 members of Rab proteins in the human genome, many Rab proteins have been shown to be the result of gene duplication events and have high sequence identity to one another. Even between eukaryotes, Rab function is highly conserved; yeast lacking a Rab enzyme can effectively use a mammalian homolog [34].

The Rab GTPase identified as an interactor of Omi, Rab2A, is suggested to be a key player in the process of autophagy and endocytosis. Rab2A was discovered to be a binding partner of a tethering complex known as HOPS (homotypic fusion and vacuole protein sorting), which is responsible for degradation of autophagosomes and endosomes. Combined with previous knowledge of Rab7's interaction with HOPS, a model was proposed through which Rab2A works

in conjunction with Rab7 in order to promote fusion of autophagosomes and endosomes with lysosomes. Rab2A knockdown or siRNA treatment resulted in accumulation of autophagic vesicles within the cell [37]. Previous research has revealed that Omi activates autophagy (more specifically mitophagy) by regulating the E3 ubiquitin ligase Mulan [38]. Thus, Rab2A's role in autophagy is of interest because it could describe another pathway through which Omi regulates autophagy within the cell. The potential link between Omi and Rab2A in autophagy has clinical significance since autophagy degrades pathogenic protein aggregates whose build up has been implicated in neurodegenerative diseases [38].

Further experimentation needs to be completed to confirm that this interaction between Rab2A and Omi occurs in mammalian cells under physiological conditions. If a specific antibody against the N-terminus of Omi can be created, experiments can be done to determine the subcellular localization of this domain (mitochondrial versus cytoplasmic). In addition, a specific antibody could be used to monitor the expression and stability of the amino terminal domain and its potential trafficking to the lysosomes during autophagy. Finally, whether the amino terminal domain of Omi is involved in the pathological conditions where the protease has been implicated needs further investigation. It is expected that the amino terminal domain of Omi is created through auto-degradation. As such, mutations that affect the protease activity of Omi will block the release of the amino terminal domain from the precursor protein. This could prove detrimental to cells by affecting several biological processes including autophagy. Thus, elucidation of the role of the N-terminal domain can result in better understanding of human disease and pathology.

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