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CHARACTERIZATION OF A NOVEL INTERACTOR/SUBSTRATE FOR THE
PRO-APOPTOTIC SERINE PROTEASE
OMI/HTRA2

by

VALERIE ANNE STRATICO
B.S. University of Central Florida, 2002

A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science
in the Department of Molecular Biology and Microbiology
in the College of Health and Public Affairs
at the University of Central Florida
Orlando, Florida

Fall Term
2004

ABSTRACT

OmiHtrA2 is a highly conserved mammalian serine protease that belongs to the HtrA family of proteins. Omi shares homology with the bacterially expressed heat shock protease *HtrA*, which functions as a protease at higher temperatures and a chaperone at lower temperatures. Additionally, Omi shares sequence similarity with the mammalian homologs L56/HtrA1 and PRSP/HtrA3. Omi was first isolated as an interacting protein of Mxi2, an alternatively spliced form of the p38 stress-activated kinase, using a modified yeast two-hybrid system. Omi localizes in the mitochondria and in response to apoptotic stimuli the mature form of this protein translocates to the cytoplasm. In the cytoplasm Omi participates in both the caspase-dependent as well as caspase-independent apoptosis. Additionally, recent studies suggest that Omi may have another unique function, maintaining mitochondrial homeostasis within the mitochondria. In an effort to further elucidate the function of Omi, a yeast two-hybrid screening was performed to isolate novel interacting proteins. This screening identified a novel protein (HOPS), as a specific interactor of Omi. The predicted amino acid sequence of this protein does not provide any information about its potential function in mammalian cells. However, experiments show that HOPS is cleaved *in vitro* by Omi. Furthermore, in response to apoptotic stimuli, HOPS is also degraded *in vivo*. This study suggests that HOPS could be a physiological substrate of Omi that is cleaved and removed during apoptosis.

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LIST OF ABBREVIATIONS

AIF.....	Apoptosis-Inducing Factor
Apaf-1.....	Apoptotic-Protease Activating Factor-1
ATP.....	Adenosine Triphosphate
BIR2/BIR3.....	Baculoviral IAP Repeat
CARD.....	Caspase Activation and Recruitment Domain
Caspases.....	Cysteine Aspartyl-Specific Proteases
DED.....	Death Effector Domain
DIABLO.....	Direct IAP Binding Protein with Low pI
DISC.....	Death Inducing Signaling Complex
DR3/DR6.....	Death Receptor
GFP.....	Green Fluorescence Protein
HtrA.....	High Temperature Requirement
HIS.....	Histidine
HOPS.....	HtrA2/Omi protein substrate
IAP.....	Inhibitor of Apoptosis Protein
IB.....	Insulin Like Growth Factor Binding Domain
IBM.....	IAP Binding Motif
LEU.....	Leucine
Mnd2.....	Motor Neuron Degeneration 2
MTS.....	Mitochondrial Targeting Sequence

PCR.....Polymerase Chain Reaction
PSORTPrediction of Protein Sorting Signals and Localization Sites
PRSP.....Pregnancy Related Serine Protease
SDS-PAGE.....Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SH3.....Src-homology 3
Smac.....Second mitochondria-derived activator of caspases
SP.....Signal Peptide
tBID.....truncated Bid
TNF-R1.....Tumor Necrosis Factor- Receptor 1
TRAIL.....TNF-Related Apoptosis-Inducing Ligand
X-gal.....5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside
XIAP.....X-linked Inhibitor of Apoptosis Protein

CHAPTER ONE: INTRODUCTION AND REVIEW OF LITERATURE

1.1 Cell Death-Apoptosis and Necrosis

Cell death is imperative in the development of a healthy organism, as well as in the maintenance of homeostasis that is necessary for life. One major type of cell death, apoptosis, plays an essential role in this delicate equilibrium. The average adult sheds 10 billion cells each day to preserve this balance (1). Apoptosis, or “programmed cell death”, allows an organism to control the number of cells within a tissue, while guarding from the harmful effects of unwanted cells. It is this form of programmed cell death that aids in regulating portions of the immune system, contouring the shape of fingers from webbing during fetal development, as well as eliminating cells that are superfluous, damaged, or infected (2, 3).

John Kerr and colleagues first defined apoptosis, in 1972, as a distinct process of cell death, differing from necrosis (4). This was followed by an important observation in the nematode, *Caenorhabditis elegans*, that of 1,090 cells exactly 131 are lost through the process of programmed cell death to form the adult worm (5). Pro-apoptotic proteins were later identified as major players in this pathway leading to cell death in the nematode. Examples of such proteins include the products of the nematode genes *ced-3* and *ced-4*. Each of these gene products share homology with genes in other species, suggesting that the mechanism of apoptosis is highly conserved (6, 7).

Cell death by apoptosis is most frequently characterized by morphological changes within the cell. There are four basic phases of apoptosis that include initiation, execution, disintegration, and elimination (6.). Some noted features are shrinkage of the cell, DNA fragmentation to approximately 200 base pairs, blebbing of the cell membrane, condensation of chromatin, and the formation of apoptotic bodies (Figure 1) (8). Since the cells are ultimately phagocytized, the lytic contents of the cells are not released into the surrounding environment; consequently this form of cell death is devoid of inflammation (6).

Due to the important role of apoptosis as a regulator of cellular homeostasis, modification of this process can have deleterious effects on an organism. Insufficient apoptosis correlates with the accumulation of unwanted or damaged cells, which may ultimately result in malignancy or autoimmune diseases (1, 9, 10). In contrast, excessive apoptosis has been associated with a variety of maladies, for example, Alzheimer's and Parkinson's diseases (8).

In the past a clear line could be drawn between apoptosis and the other major type of cell death, necrosis. These parameters allowed necrosis to be characterized by swelling of the cell, rupturing of the plasma membrane, and release of cellular contents into the surrounding environment leading to inflammation (Figure 1). However, current research suggests that although morphologically different, at times the two routes leading to cell death can overlap and may even share common pathways (11). In various diseases including cancer, autoimmune diseases, and neurodegeneration, apoptosis can occur in association with necrosis (12). This overlap is illustrated in another type of cell death, "aponecrosis", that is considered an intermediate between apoptosis and necrosis (13).

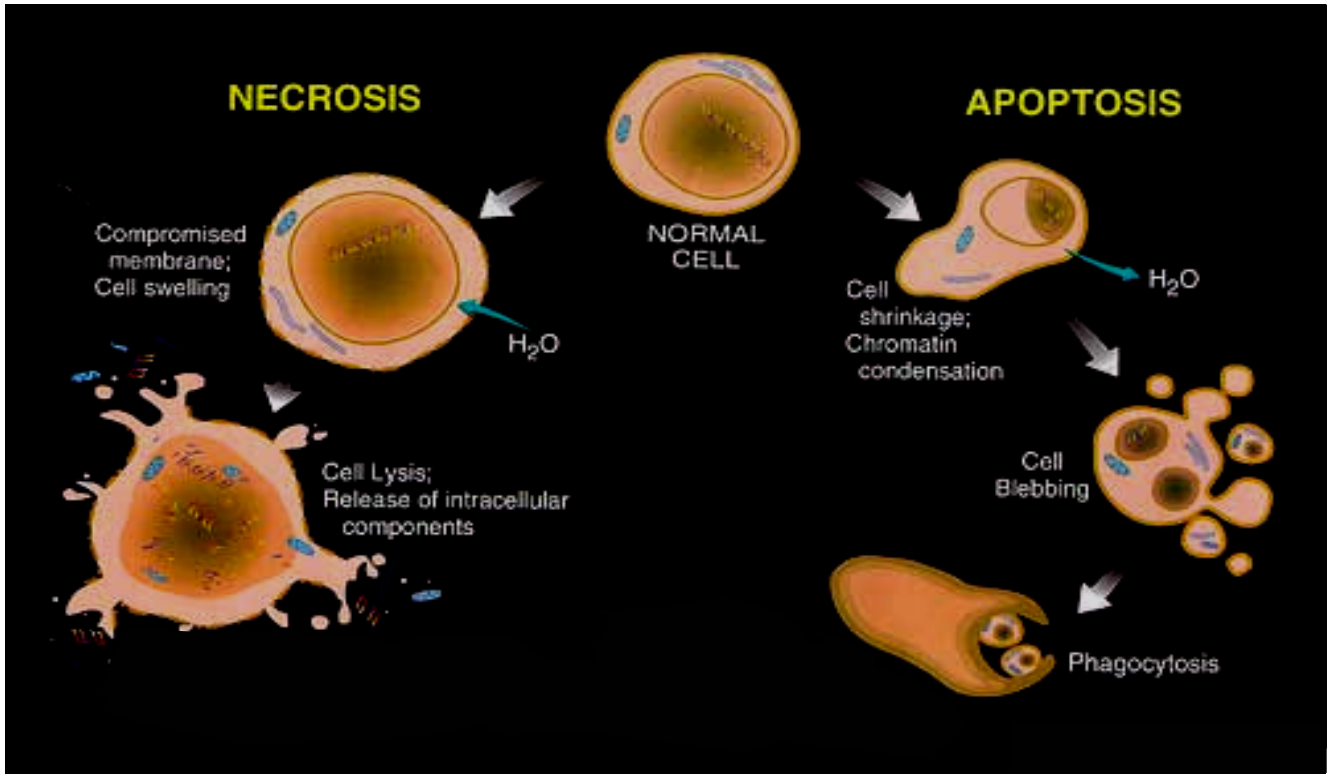


Figure 1: Morphological Changes Observed in Cell Death

On the right, the figure depicts the changes seen during apoptosis. Upon apoptotic stimuli, the cell begins to condense; this is followed by DNA fragmentation. The cell breaks off into smaller membrane-bound “apoptotic bodies”, that will eventually be phagocytized. In contrast, the left portion of the figure represents the morphological changes seen during necrosis. These include cell swelling that leads to the lysis of the cell, and the release of intracellular components into the surrounding tissue (picture taken from: <http://www.bioweb.uncc.edu/Faculty/Hughes/>).

1.2 Caspases-Mediators of Apoptosis

The role of caspases in programmed cell death was elucidated when homology was observed between the cell death genes of the nematode, *C. elegans*, and mammals (14). Much of the changes that can be observed within an apoptotic cell are the result of these homologous gene products, known as caspases, which cleave substrates after an aspartate residue. Currently, fourteen distinct members of this family of cysteine proteases have been identified (15).

Within this family of caspases, those involved in the apoptotic pathway are subdivided into two groups, the initiator and the effector caspases. The initiator caspases, also termed “upstream caspases”, include caspase-8, caspase-9, and caspase-10. Effector or “downstream” caspases are comprised of caspases -3, -6, -7. In response to an apoptotic signal, initiator caspases function to launch the apoptotic cascade by activating downstream effector caspases which break down the cell (16).

The specificity of each caspase for a substrate is determined by a sequence of four amino acids that is upstream to the cleavage site of the substrate. This cleavage may either activate the substrate or render it inactive so that it may no longer properly function. Some substrates of downstream caspases include cytoskeletal proteins, inhibitors of endonucleases, or proteins involved in signaling or repair. Ultimately resulting in the biological and morphological changes seen in cell death (17).

Caspases are made up of three domains: a small subunit, a large subunit, and an N-terminal pro-domain. Initially, caspases are expressed within the cell as zymogens, or pro-caspases, and remain inactive until each domain is separated by proteolytic cleavage. A

heterodimeric association of the large and small subunits, which later join to form a tetramer, follows this cleavage (16). Initiator caspases have a longer pro-domain that includes a site for protein-protein interaction. For caspase-8 and caspase-10 this domain is labeled DED, or death effector domain. The remaining initiator caspases including caspase-9 contain a caspase activation and recruitment domain (CARD). Both domains, through their interaction with other signaling proteins, take part in controlling this complex cascade leading to programmed cell death (7, 18).

1.3 Pathways of Cell Death

At present, two major mechanisms of apoptosis regulation have been defined. They are the caspase dependent and the caspase-independent pathways (Figure 2). The caspase-dependent pathway is composed of two independent sub-pathways (19). One, the extrinsic pathway, involves the initiation of death receptor molecules on the surface of the cell. Second, the intrinsic pathway, is mediated primarily through the mitochondria by various apoptotic signals (10). Although each pathway originates from a different location within the cell, the two converge with the activation of downstream effector caspases that result in the morphological changes seen in an apoptotic cell. The caspase independent pathway does not involve the activation of caspases, but instead executes cell death through other proteins. For example, apoptosis inducing factor (AIF), is released from the mitochondria, translocates to the nucleus

and induces apoptosis. Proteins such as Endonuclease G, granzymes, and calpains have also been shown to behave as proteases (7, 18, 20, 21).

The extrinsic pathway propagates the death signal following the binding of a ligand to a member of the tumor necrosis factor (TNF) superfamily of death receptors, located on the surface of the cell. Six various death receptors have been identified and include: CD95, TNF-R1, DR3, TRAIL-R1, TRAIL-R2, and DR6 (22). Each of these transmembrane death receptors contain a death domain located on the intracellular surface of the cell. This domain functions in binding to signaling molecules involved in the apoptotic pathway. Once a ligand binds to its respective receptor, the receptor trimerizes and then recruits various adaptor molecules that bind to the death domain. This complex is referred to as the death inducing signaling complex (DISC) (23). The adaptor molecules contain a death effector domain at their amino terminus that intimately interacts with initiator procaspase-8 leading to its activation. This signal is then transmitted to downstream caspases, resulting in apoptosis of the cell (6).

The intrinsic pathway is activated in response to various stimuli often referred to as “cellular stress”. These stressors, which include among others: ionizing radiation, cytotoxic drugs, and free radicals, are believed to cause a change in the mitochondrial membrane potential. This alteration in the membrane integrity leads to the release of a variety of pro-apoptotic proteins into the cytosol. One such protein, cytochrome c, forms a complex with the adaptor protein, Apaf-1, and ATP (24). This complex referred to as the apoptosome, then recruits and activates procaspase-9 (19, 20). Once active, caspase-9 proteolytically activates effector caspases, including procaspase-3, generating the morphological and physical changes in an apoptotic cell (16, 20).

Both the intrinsic and extrinsic pathway interconnects with the pro-apoptotic protein Bid. Bid is a member of the BH3 only, Bcl-2 family of proteins (6). This protein shares homology with the BH3 domain of the founding molecule of this family, Bcl-2 (25). Bid is processed into tBid by caspase-8 of the extrinsic pathway. Once truncated, tBid translocates to the mitochondria where it may interact with other proapoptotic proteins, such as Bax and Bak (26). Though the mechanism is not yet completely clear it is known that truncated Bid ultimately promotes the release of cytochrome c from the mitochondria (22).

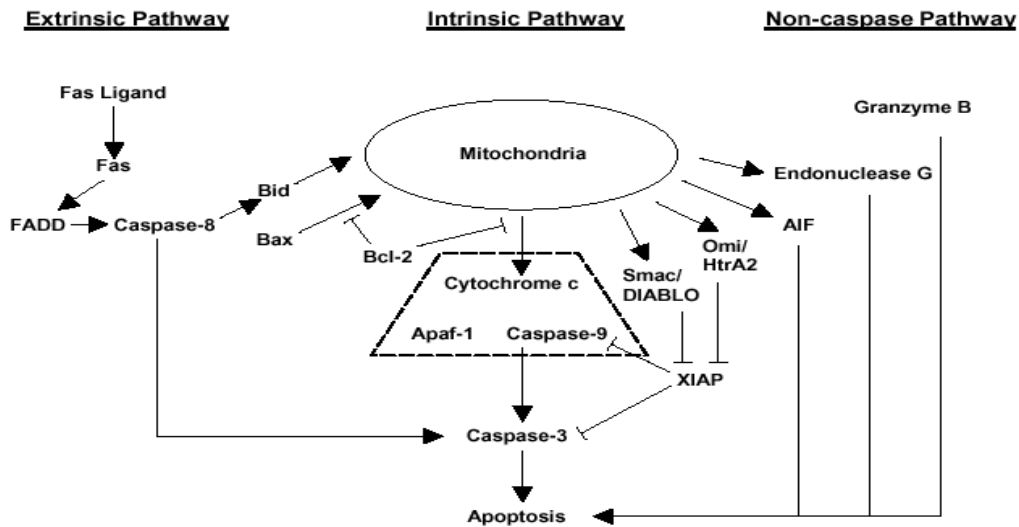


Figure 2: Pathways of Apoptosis

This figure is a schematic representation of the extrinsic, intrinsic, and caspase-independent cell death pathways. The extrinsic pathway is activated through the stimulation of death receptors on the surface of the cell. While, the intrinsic pathway is activated through various cellular “stressors” (20). Both pathways converge at the mitochondria, where the release of a variety of apoptotic proteins occurs. Among these is cytochrome c, which binds to Apaf-1, and caspase-9 forming the apoptosome. Additionally, Omi/HtrA2 and Smac//DIABLO are able to bind to XIAP, an endogenous inhibitor of caspases, thereby alleviating the inhibition of caspases and promoting apoptosis (7).

1.4 Preventing Apoptosis-Inhibitors of Caspases

The proteolytic cleavage of proteins by caspases is an irreversible event, therefore, it is essential that not only the activation but also the activity of these proteases be tightly regulated within the cell. Inhibitors of apoptosis (IAPs) are a class of proteins that function by binding to and inhibiting caspases so that healthy cells may survive (20).

IAPs were originally identified in baculoviruses where they functioned to inhibit apoptosis in host cells during viral infection. Since then the evolutionarily conserved IAPs have been found in viruses, yeast, flies, and mammals (8). Two motifs have been recognized as part of the structure of IAPs. All IAPs contain anywhere from one to three baculovirus IAP repeat domains (BIR), which are made up of approximately 70 amino acid residues. The BIR domain seems to be mandatory for the anti-apoptotic property of these proteins, and in some cases has been found to directly interact with caspases. Additionally, a RING finger domain can be found at the carboxy terminus of some inhibitors of apoptosis. Proteins that contain this RING domain are able to ubiquitinate other proteins in addition to initiating their own degradation (18, 27).

Currently, there are eight IAPs that have been identified: XIAP, c-IAP1, c-IAP2, NAIP, Survivin, Bruce, Livin, and ILP-2. These inhibitors of apoptosis are able to bind to either the pro-caspase or active form of caspases to inhibit their activity thereby preventing caspase-dependent programmed cell death (7).

1.5 Inhibitors of IAPs: Smac and Omi

In order for a cell to undergo the process of programmed cell death, the inhibition of caspases by IAPs must be terminated. Three proteins, Reaper, Hid, and Grim, originally identified in the insect *Drosophila melanogaster* perform this function (28). Recently, two other proteins, Smac/Diablo and Omi/HtrA2, were identified as mammalian homologs of these proteins. Omi is the focus of this study.

Both Smac/DIABLO and Omi/HtrA2 are nuclear encoded mitochondrial proteins that share an amino terminal sequence homology with the Reaper, Hid, and Grim proteins (29). This homologous sequence is referred to as the IAP binding motif (IBM). This sequence allows these proteins to bind to IAPs, permitting their release from caspases consequently enabling apoptosis to take place (7).

Smac (Second Mitochondrial Activator of Caspases) or DIABLO (Direct IAP Binding Protein with Low pI) is initially made as a 29 kDa precursor protein. This precursor translocates to the mitochondria where its mitochondrial localization signal is removed, generating the mature protein of 23 kDa. Upon apoptotic stimuli mature Smac/DIABLO is then released from the intermembrane space of the mitochondria into the cytosol, where it is able to bind to and sequester IAPs. Smac/DIABLO has been shown to bind to XIAP, c-IAP1 and c-IAP2, and Survivin. This binding to XIAP is primarily mediated through the BIR2 and BIR3 domains (30).

1.6 Characterization of Omi

Omi/HtrA2, was originally isolated as an interactor of Mxi2, the alternatively spliced form of the p38 stress activated kinase. Omi is a serine protease containing a high homology to the bacterial HtrA heat shock protease (31). The HtrA protein functions at higher temperatures as a protease and at lower temperatures as a chaperone. The Htr, or high temperature requirement, family of serine proteases is highly conserved from bacteria to humans (9). Omi is ubiquitously expressed, while the alternatively spliced form, D-Omi, is expressed only in the kidneys, thyroid, and colon (32). In healthy human cells Omi is restricted to the intermembrane space of the mitochondria, however, upon apoptotic stimuli it is released into the cytosol. Furthermore, the protease seems to be up-regulated during heat shock, ischemia or reperfusion, and endoplasmic reticulum stress (31).

Omi shares some characteristics with Smac/DIABLO but still maintains separate functions. Omi/HtrA2, like Smac/DIABLO, is known for its ability to inhibit IAPs, however, in this case it is through irreversible cleavage. In a similar fashion to Smac/DIABLO, Omi/HtrA2 is translocated to the mitochondria, where the protein is processed from the 50 kDa precursor to the 36 kDa mature protein. Omi/HtrA2 has been found to interact with and inhibit XIAP, thereby initiating apoptosis in a caspase-dependent fashion. Upon apoptotic stimuli Omi/HtrA2 is released into the cytosol where it binds to XIAP, however unlike Smac/DIABLO, Omi/HtrA2 binds preferentially to the BIR3 domain of XIAP. This interaction prevents the inhibitor from binding to caspase-9 thereby averting the activation of downstream effector caspases -3, -6, and -7 (29). Additionally, this protein has been shown to interact with cIAP1 and cIAP2. In contrast

to Smac/DIABLO, Omi/HtrA2 does not bind to Survivin. Furthermore, Omi has also been found to induce cell death through a caspase-independent mechanism, observed only when Omi is overexpressed in cells (30, 31).

Omi/HtrA2 shares homology with two other known proteases, HtrA1/L56 and HtrA3/PRSP (pregnancy related serine protease). HtrA1/L56 has been shown to be in higher concentration in osteoarthritic tissue, and at lower levels in fibroblasts transformed with SV40 virus. Both HtrA1 and HtrA3 are significantly expressed in placental tissue (33). While HtrA1 and HtrA3 share high functional homology, Omi is limited to having homology with these proteins only at the carboxy terminus of the protein. The amino terminal end of Omi contains a regulatory domain that is not present in the other serine proteases. Interestingly, each of the three proteases contains a highly conserved proteolytic domain. Although the function of HtrA1 and HtrA3 is not yet elucidated, the similarity between these two proteins suggests that they may in fact have similar functions (34).

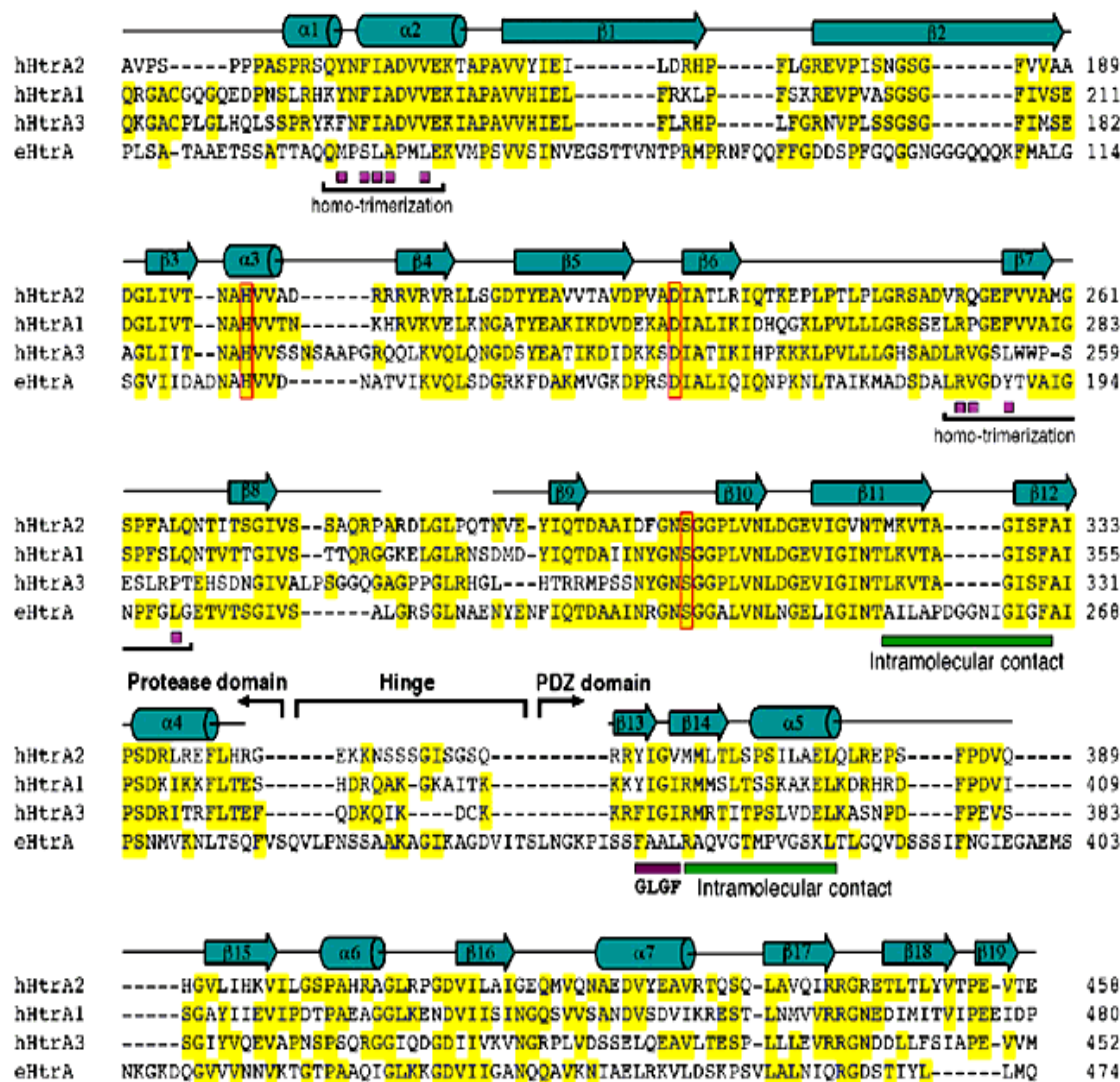


Figure 3: Sequence Comparison of Human HtrA Proteins and Homolog in *E. coli*

The alignment shows the similarities and differences between the three known HtrA family proteins as well as that of *E. coli*. Catalytic triad residues are boxed in red and the secondary structures are indicated above the sequences (35).

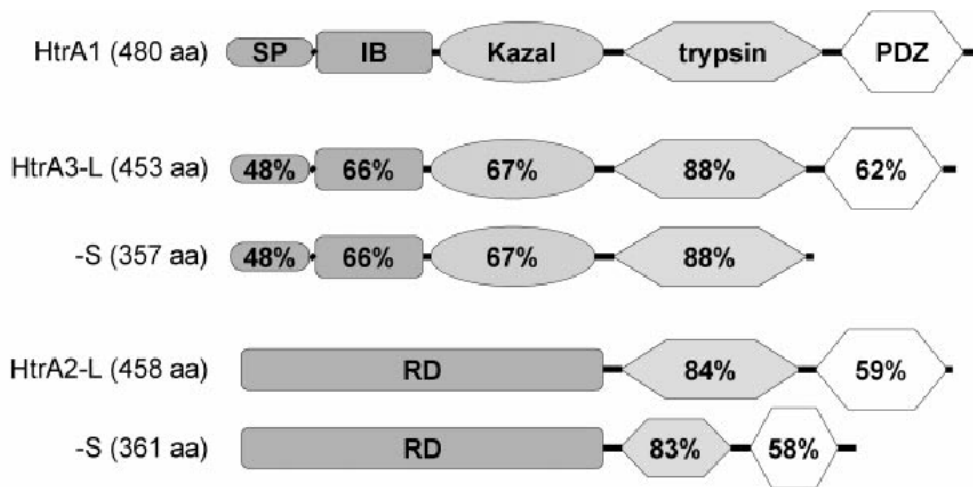


Figure 4: Domain Comparison of the Human HtrA Family

The diagram shows the differences between members of the HtrA family. HtrA1 and HtrA3 show a high degree of similarity, both having a signaling peptide (SP), IGF-binding domain (IB), a Kazal domain (Kazal), a trypsin like domain (trypsin), in addition to a PDZ domain (PDZ). In contrast, Omi contains an N-terminal regulatory domain instead of the SP, IB, and Kazal. For each domain the percent homology to HtrA1/L56 is shown (34).

1.7 Structure of Omi

Omi/HtrA2 contains three major domains including a regulatory domain at the amino terminus, a centrally located catalytic domain, and a single PDZ domain at the carboxy terminus. Within the regulatory domain exists a mitochondrial targeting sequence (MTS), a predicted transmembrane region, a phosphorylation site, and a SH3 binding domain. In addition, upstream to these features there is PRAXXTXXTP sequence that is repeated three times (31, 36). The serine protease's catalytic domain is comprised of three amino acids His 198, Asp 228, and Ser 306. This catalytic triad is folded with high similarity to that of trypsin, and is necessary for Omi's protease activity. Lastly, the PDZ domain is involved in interactions with other proteins, through its ability to recognize hydrophobic residues (35).

Omi is synthesized as a 458 amino acid precursor. Upon translocation to the mitochondria, 133 amino acids at the N terminus of the precursor are removed by cleavage. This exposes the conserved AVPS motif present in mature Omi that recognizes and binds IAPs (29). The mature form of Omi is composed of 7 α helices and 1 β strands. Together these structures form the serine protease domain as well as the PDZ domain (35).

When crystallized, the mature form of Omi/HtrA2 forms a homotrimer. This trimerization generates a pyramid structure housing the IBM sequences at the top and the PDZ domain at the bottom. The serine protease domain in addition to the N terminal sequences controls formation of this pyramid. Additionally, the formation of this structure seems to be imperative in the proteolytic pro-apoptotic function of Omi. A working model, proposed by Li

et al, suggests that perhaps the binding of a protein to the PDZ domain, leads to a conformational change in the trimer, which allows access to the catalytic site of the protease (35).

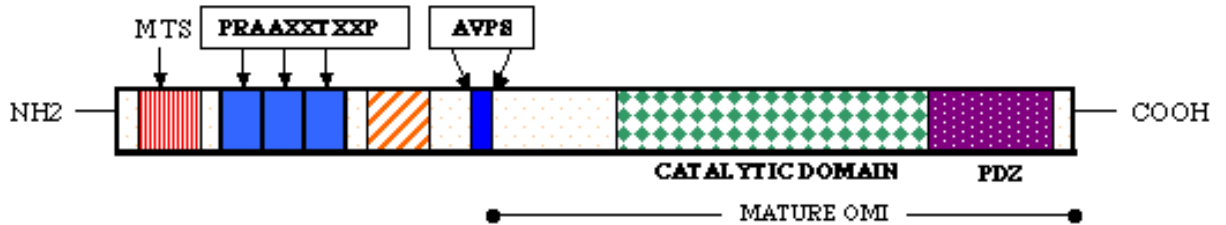


Figure 5: Functional Domains of Omi

Omi contains an N-terminal regulatory domain, a catalytic domain, and a C-terminal PDZ domain. The regulatory domain contains a mitochondrial targeting sequence (MTS) and three PRAAXXTXXP motif of unknown function. The mature form of Omi lacks the first 133 amino acids and has an exposed AVPS motif (31).

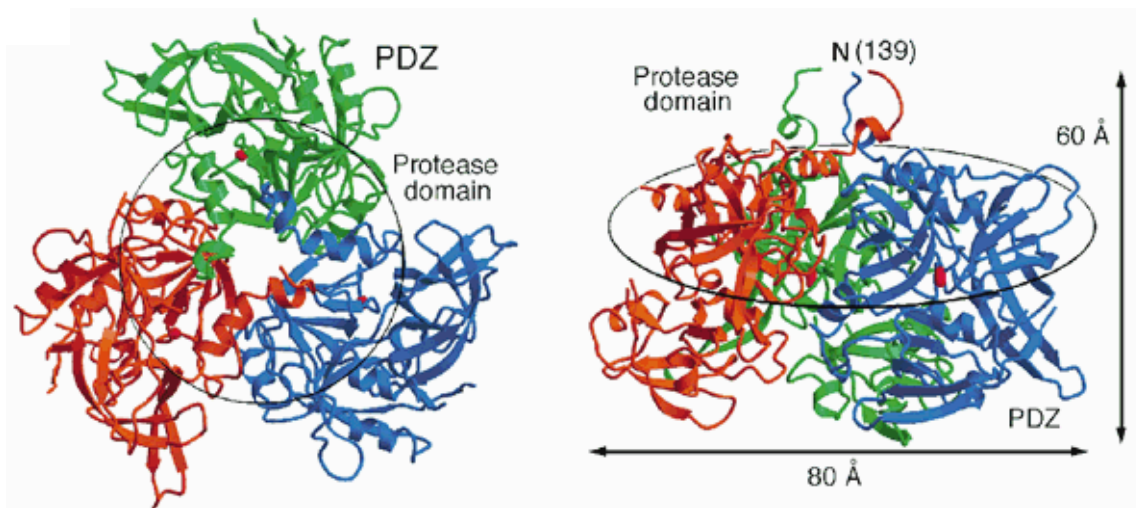


Figure 6: Trimeric Organization of Omi

Crystal structure analysis of Omi revealed that the active protease forms a trimer. Each subunits catalytic domain mediates the arrangement of this structure. The PDZ domain is at the base of the structure, while the IAP binding motif is situated at the top (35).

1.8 PDZ Domain of Omi

Recognized over 10 years ago, PDZ domains were originally identified in signaling proteins, as regions of high sequence homology. These domains have recently been identified as important protein-protein interacting regions necessary in a wide variety of proteins and diverse organisms. The PDZ domain is named after the first three proteins that contained a similar amino acid sequence: the post-synaptic density protein PSD-95, the *Drosophila melanogaster* Discs Large protein, and Zonula occludens 1 protein Zo-1 (37, 38). Presently, as many as 259 proteins within the human genome, have been suggested to contain PDZ domains (39).

Approximately 90 amino acids in length, PDZ domains specialize in binding to specific C-terminal motifs. These motifs are within 6 amino acids in length and require a free carboxylate group. Crystallographic data suggest that the binding of these C-terminal residues interact directly with the peptide binding groove (40). Hydrophobic in character, most PDZ domains select for a hydrophobic C-terminal residue (38, 41).

PDZ domains can be divided into three main groups for classification purposes. The distinction between these classes can be determined by the interaction at P₀ and P₋₂. In class I, the PDZ recognizes a serine or threonine at the P₋₂ position, as is the case with PSD-95. For the Class II PDZ domain, a hydrophobic residue occupies the P₀ and P₋₂ positions. The third class of PDZ favors a negatively charged amino acid at P₋₂. A fourth class of PDZ has been identified that prefers a negatively charged amino acid at the P₀ position (42).

In contrast to this classification scheme, Omi does not fall into any of these categories of PDZ domains. The binding selectivity of Omi/HtrA2 PDZ specificity has been shown to favor a valine or isoleucine at P₀. This amino acid is preceded by residues rich in either tyrosine, phenylalanine, or large aliphatic groups at positions P₋₁, P₋₂, and P₋₃ (43).

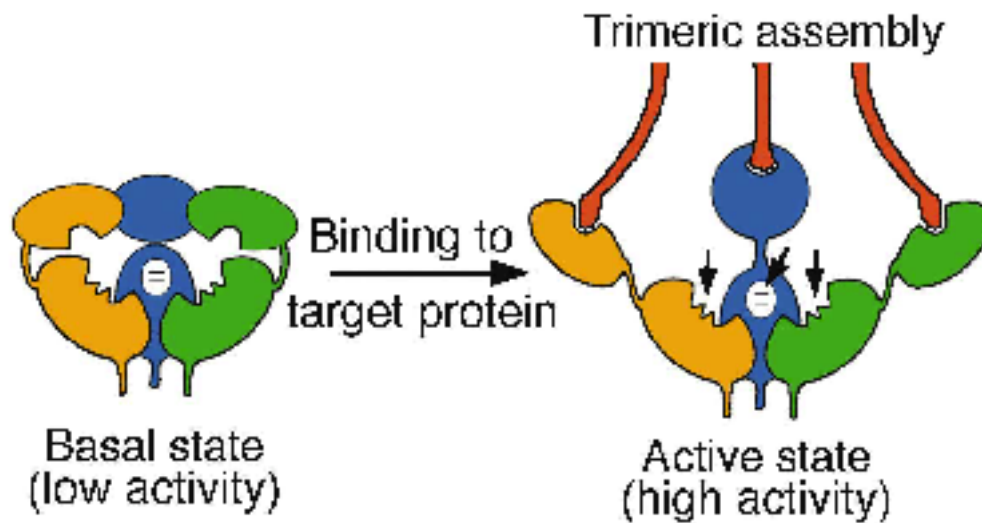


Figure 7: The PDZ Domain of Omi

The PDZ domain of Omi mediates the activity of the protease. After binding to a substrate the PDZ domain opens exposing the proteolytic domain (35).

1.9 Multiple Functions of Omi

In order to understand the various functions of Omi it might be necessary to identify all the physiological substrates of this protease as well as any other interacting proteins that could regulate its function. To date, Omi has been identified as an active participant in both caspase-dependent and caspase-independent cell death (44). Additionally, Omi has been suggested to have a unique housekeeping role in mitochondria (45).

Most of the studies published on Omi deal with its role in the caspase-dependent pathway of apoptosis. Omi is now known to activate caspase-dependent cell death by blocking and removing inhibitors of apoptosis proteins (IAPs). This function of Omi is carried out via two distinct mechanisms in the cytoplasm. One mechanism involves direct binding of Omi to IAPs which disrupts their association with active caspases (46). In the second mechanism, Omi is able to degrade and irreversibly remove IAPs. Omi does this by using its proteolytic activity after binding to the inhibitors(29, 36, 44, 47, 48). Inactivation of IAPs, in turn, leads to caspase-9 activation and eventually to apoptosis. The interaction of Omi with IAPs is mediated through the N-terminal AVPS of the mature Omi protein (28, 44). This AVPS motif is also found in all known IAP interacting proteins including *Drosophila* homologs.

Furthermore, Omi is involved in the caspase-independent pathway of cell death and is able to induce an “atypical form” of apoptosis (28, 44). This is independent of its AVPS motif, and instead relies entirely on its ability to act as a protease. This form of cell death is observed in Omi proteins lacking the exposed AVPS motif at their N-terminus but it is not seen in serine-protease inactive mutants (28, 44).

Lastly, Omi has been identified as a mediator of homeostasis within the mitochondria under normal physiological conditions. In *Mnd2* mutant mice, a missense mutation resulting in a Ser276Cys greatly reduces the protease activity of Omi/HtrA2 without affecting its protein levels or sub-cellular localization. This suggests that the function of Omi in mitochondria may involve the cleavage of specific substrates (45). These mice display signs of muscle wasting, neurodegeneration, and death by 40 days of age. The fact that these knockout mice show a neurodegenerative phenotype, suggests that perhaps a primary function of Omi, at least in mice, may lie within the mitochondria (45). This feature would concur with other pro-apoptotic proteins, such as cytochrome c and endonuclease G, that also have unique mundane functions in the mitochondria(49).

CHAPTER TWO: MATERIALS AND METHODS

2.1 Construction of LexA- Omi_{CAT} and LexA-HtrA1/L56 Baits

2.1a Polymerase Chain Reaction

The following primers were used in the PCR reaction for the cloning of the catalytic domain of Omi (amino acids 134-340) and HtrA1/L56 into pGILDA.

Primer A 5' Omi_{CAT} (MfeI):

5' CCC AAT TGG CCG TCC CTA GCC CGC CGC CC-3'

Primer B 3' Omi_{CAT} (XhoI):

5' GGC CTC GAG TCA TCC GGA GGA GGA ATT CTT CTT-3'

Primer C 5' HtrA1/L56 (MfeI):

5' CGC AAT TGG GGC AGG AAG ATC CCA ACA GTT TGC GC-3'

Primer D 3' HtrA1/L56(XhoI):

5'- GGC TCG AGC TAT GGG TCA ATT TCT TCG-3'

The restriction sites incorporated into each primer, MfeI or XhoI, are underlined. Each PCR reaction was performed in 50µl volume using 20ng of template DNA, 10µM of each primer, 5µl of 10X PCR buffer + Mg (Roche Diagnostics Corp.), 1.5 units of Taq polymerase (Roche Diagnostics Corp.), 100 µM dNTP (BD Biosciences). The reaction is placed in a thermal cycler and after an initial denaturing at 95°C is passed through the following cycle 25 times: 95°C denatur for 30s, variable annealing temperature (based on primer) for 40s, 72°C elongation

for a variable length of time based on size of PCR product. This followed by a final 7 minute elongation step at 72°C. Each PCR reaction is performed twice, the products of the reactions are then combined and precipitated.

2.1b Agarose Gel Electrophoresis

1.0 g of dried agarose (FMC BioProducts) was added to 100ml 1X TAE (0.04M Tris-acetate, 0.001M EDTA). This mixture was then microwaved until all agarose had completely dissolved (\approx 2 minutes). The volume was then brought up to 100ml again with water and the solution was allowed to cool. Ethidium Bromide was then added to a final concentration of 0.5 μ g/ml before pouring the solution into a gel tray. After the gel solidified, the combs were removed. 1X TAE electrophoresis buffer was added to the electrophoresis apparatus until the buffer just covered the gel.

2.1c Preparation of Samples for Agarose Gel Electrophoresis

1 μ l of 10X gel-loading buffer made of 30% glycerol (Fisher), and 2% Orange-G (Sigma) in 1X TAE, was added to DNA. The final volume was brought up to a minimum of 10 μ l, and the mixture was then loaded in a well. DNA markers (Bioline- 40ng/ μ l HyperLadder or 50ng/ μ l Lambda DNA BstEII Digest) were used to estimate the size of the DNA sample. Electrophoresis was performed at 50-120V, until the dye migrated the appropriate distance.

2.1d DNA Precipitation

3M Sodium Acetate, pH 5.5 was added to a DNA solution at 1/10 the volume of the sample. Additionally, 2X the sample volume of 100% ethanol -20°C was also added. The sample was mixed well and stored at -20°C for a minimum of 20 minutes. The sample was then centrifuged for 20 minutes at 4°C and 14000 rpm. The supernatant was then discarded. The pellet was washed in 300 μl of 70% ethanol -20°C , and centrifuged again for 5 minutes. The supernatant was then discarded and the pellet was dried for 10 minutes in the speed vacuum. The pellet was then resuspended in the appropriate volume of dH₂O or Tris EDTA, pH 8.0.

2.1e Restriction Enzyme Digestion

Double stranded DNA was incubated with the appropriate amount of restriction enzyme and its respective buffer as suggested by the supplier. Reactions were then incubated at 37°C for 1-4 hrs depending on the enzyme efficiency. For two separate digestions, DNA was precipitated after the first digestion and resuspended in the appropriate buffer needed for the second digestion. A general protocol for a 50 μl reaction is as follows:

5 μl Restriction Enzyme 10X buffer

0.5 μl BSA, 10 $\mu\text{g}/\mu\text{l}$

1 μl DNA, 1 $\mu\text{g}/\mu\text{l}$

42 μl sterile, dH₂O

1.5µl Restriction Enzyme,10U/µl

2.1f Ligation

DNA concentration of 1µl of insert and 1µl of vector were estimated using gel electrophoresis (2.1b). The ligation was performed using insert and plasmid DNA from gel purified RE digestion, an the Fast-Link DNA ligation Kit (EPICENTRE TECHNOLOGIES). In general a molar concentration of 2:1, insert:vector was used. A general protocol for a 15µl reaction is as follows:

1.5µl Fast-Link Ligation Buffer

1.5µl 10mM ATP

1 µl Fast-Link DNA Ligase

x µl insert

x µl vector

xµl dH₂O

The mixture is then incubated at room temperature for 5 minutes. This is followed by ligase deactivation at 70°C, for 15 minutes.

To precipitate the reaction, the ligation reaction is brought to a final volume of 50µl using dH₂O. 500µl of room temperature N-butanol is added and the solution is mixed very well. The sample was then centrifuged for 10 minutes at 4°C and 14000 rpm. The supernatant was then

discarded. The pellet was washed in 300 μ l of 70% ethanol -20°C , and centrifuged again for 5 minutes. The supernatant was then discarded and the pellet was dried for 10 minutes in the speed vacuum. The pellet was then resuspended in the appropriate volume, $\approx 4\mu\text{l}$, of dH_2O .

2.1g Bacterial Transformation

The DNA to be transformed was added to 100 μ l of a competent bacterial cell suspension (either DH5 α , BL21, or KC-8), mixed gently, and transferred to an electroporation cuvette (0.2 cm electrode gap, BIO-RAD) that had been cooled to 4°C . The cuvette was placed in the Gene Pulser (BioRad) that delivers a single exponential decay pulse of 2.5V. 1ml of room temperature LB without antibiotics was then added immediately. The entire mixture was then transferred to a sterile 15ml centrifuge tube and incubated at 37°C with shaking for 45 minutes. 50 μ l -150 μ l of the solution was then plated on agar plates containing the appropriate antibiotic, and incubated overnight at 37°C .

2.1h Isolation of Plasmid DNA from Bacteria: DNA Qiaprep Spin Miniprep

Plasmid DNA was isolated using QIAprep Spin Miniprep Kit according to the manufacturers instructions (QIAGEN). A single bacterial colony is chosen and grown overnight with shaking in 4ml LB broth with the appropriate antibiotic. 1.5ml of the bacterial culture is transferred to a microcentrifuge tube and centrifuged at 14000 rpm for 2 minutes. The supernatant is then aspirated. The pelleted bacteria is resuspended in 250 μ l of Resuspension Buffer P1, stored at 4°C , containing RNase (100mg/ml). 250 μ l of Lysis Buffer P2 is then added,

and the tube is gently inverted 4-6 times. The lysate is neutralized and adjusted to high-salt binding conditions by adding 350µl of neutralization Buffer N3. The tube is immediately and gently inverted 4-6 times and then centrifuged at 14000 rpm for 10 minutes at room temperature. The supernatant is then transferred to a QIAprep spin column resting in a 2ml collection tube. The column is centrifuged again for 1 minute and the flow-through is discarded. The QIAprep spin column is then washed using 500µl binding buffer (PB) and centrifugation at 14000 rpm, for 1 minute at room temperature. The flow-through is discarded. An additional wash is performed using 750µl of wash buffer (PE) and centrifugation at 14000 rpm, for 1 minute at room temperature. The flow-through is discarded. The residual wash buffer is then removed with an additional centrifugation for 1 minute. The QIAprep spin column is then placed in a clean 1.5ml microcentrifuge tube. By adding 50µl of Elution Buffer, EB, (10mM Tris-HCL, pH8.5) to the center of the column, letting stand for 1 minute, and centrifuging at 14000 rpm, for 1 minute at room temperature, the DNA is eluted.

2.1i Isolation of Plasmid DNA from Bacteria: DNA-Boiling Method Miniprep

A single bacterial colony is grown overnight with shaking in 4ml LB broth with the appropriate antibiotic. The culture is then placed on ice. 1.5ml of LB containing bacteria is transferred to a microcentrifuge tube and centrifuged at 14000 rpm for 2 minutes. The supernatant is then aspirated. The pellet is resuspended in 300µl of filtered STET (8% Sucrose, 5% 100X Triton, 50mM Tris-HCl pH8, and 50 mM EDTA). 25µl of 10mg/ml lysozyme is then added to each sample. The sample is vortexed briefly and placed in boiling water for 45

seconds. Then the sample is centrifuged at 4°C and 14000 rpm for 15 minutes. 200µl of the supernatant is transferred to a new 1.5ml microcentrifuge. To this, 230µl of -20°C isopropanol is added and the samples are mixed well. The tubes are placed at -20°C for 10 min and the centrifuged again at 4°C and 14000 rpm for 5 minutes. The supernatant is discarded and the pellet is washed with 300 µl of 70 % ethanol (-20°C). The pellet is then dried for 10 minutes in the speed vacuum. The pellet is then resuspended in dH₂O or TE.

2.1j DNA Sequence Analysis

CEQ 2000 Dye Terminator Cycle Sequencing was performed using the Quick Start Kit (Beckman). The reactions were performed in 0.2 ml thin walled tubes. All reagents were kept on ice while the sequencing reactions were prepared. Each reaction was performed in 10 µl volume using the appropriate amount of template DNA (based on the size of the insert), 3pM, and dH₂O (to adjust the volume to 10 µl). The components were mixed and then denatured for 2 minutes at 95°C. Next, 3µl of DTCS Quick Start Master Mix was added. The reaction is placed in a thermal cycler and is passed through the following cycle 30 times: 96°C denature for 20s, 50°C for 20s, 60°C for 4 minutes. This is followed by holding at 4°C. To this reaction 2.5µl of Stop Solution (1.5M NaOAc, 50mM EDTA prepared fresh by mixing equal volumes of 3M NaOAc and 100mM EDTA) and 1µl of 20mg/ml glycogen (Beckman Coulter) is added. The reaction is then transferred to a 1.5µl microcentrifuge tube and mixed thoroughly. To this 60µl of -20°C 100% ethanol is added. This is centrifuged immediately at 4°C and 14000 rpm for at least 15 minutes. This supernatant is carefully removed with a pipette and discarded. The pellet

is washed with 200µl of 70% ethanol (-20°C). The supernatant is again carefully removed with a pipette and discarded. The pellet is dried in the speed vacuum for 15 minutes and resuspended in 20 µl of Sample Loading Solution (Beckman Coulter).

2.2 Yeast Two Hybrid Interactions

2.2a Yeast Transformation

50ml of appropriate medium is inoculated with a single yeast colony to be transformed, and grown overnight with shaking at 30°C. The following day in a sterile 50ml Falcon tube the cells are harvested by centrifugation at 3800 rpm for 5 minutes. The supernatant is then discarded. The pellet is washed by resuspension in 10mL TE pH 7.5, and then centrifugation at 3800 rpm for 5 minutes. The supernatant is discarded. The pellet is resuspended in 5 ml LA (0.1M LiOAc in TE) and transferred to a 15 ml Falcon tube. The solution is then incubated for two hours at 30°C with shaking. The cells are harvested by centrifugation at 3800 rpm for 5 minutes. The supernatant is discarded and the pellet is resuspended in a variable amount, usually ≈5ml, of LA (depending on the size of the pellet). The yeast is then separated into 300µl aliquots in 1.5ml microcentrifuge tubes. Next, 1µg of DNA to be transformed is added along with 100µg of denatured salmon sperm DNA. To this, 700µl of 50% PEG4000 (diluted in 100ml of dH₂O) and the solution is mixed until homogeneous. Then the yeast is incubated in a 30°C water bath for 30 minutes. The yeast are then moved to a 42°C water bath for 15 minutes.

The cells are then centrifuged for 3 minutes at 14000 rpm and the supernatant is aspirated. The pellet is resuspended in 300µl of TE pH 7.5. 50µl-150µl of the solution is then plated on agar plates containing the appropriate selective medium, and incubated for 2 days at 30°C.

2.2b Yeast Drop-Out Medium

The yeast strain used in all experiments was auxotrophic, which allowed for selection by using specially prepared drop out medium. A liter of drop out medium contains the following: 2g appropriate “drop out” powder (lacking the appropriate amino acids) (50), 6.7g Yeast Nitrogen Base without amino acids (Sigma) and 900 ml of sterile water (for agar plates 20g of agar (USB) was added as well). The bottle was autoclaved and 2% weight-by-volume carbon source (Glucose or Galactose) was added.

2.2c Yeast Western Blot

3ml of yeast medium was inoculated with the yeast to be tested, the positive control, or the negative control, and incubated overnight at 37°C with shaking until an optical density of 0.4-0.7 at 600nm. The yeast was transferred to a 1.5ml microcentrifuge tube and centrifuged at 14000 rpm for 3 minutes. The supernatant was then aspirated and the pellet was resuspended in 50µl of 1X SDS-sample loading buffer (10% β-mercaptoethanol, 6% SDS, 20% glycerol and 1/40 X stacking buffer and 0.2mg/ml bromophenol blue). The samples were vortexed, placed on ice and then boiled for 5 min, along with a pre-stained protein marker (BioLabs). A short spin was performed to remove large cell debris. 20µl of sample and 15µl of marker were loaded on a

pre-cast gradient 4-20% Tris-Glycine gel (Invitrogen). The proteins were transferred to a PVDF membrane by semi-dry electrophoresis using a BioRad transfer blot. For proper transfer PVDF membrane (PALL) was first activated in 50 ml methanol (CH₃OH). Gel, membrane and 2 filter papers were equilibrated in 250 ml transfer buffer (9.6mM Tris, 7.8mM glycine and 20 % CH₃OH to a final pH ~9) for 5 min. The semi-dry transfer was arranged having the soaked filter paper on the bottom, followed by the membrane, gel and a second sheet of filter paper on top. The transfer machine was run for 1 hour at 15V. In order to block unspecific binding, the PVDF membrane was incubated overnight at 4° C in 10 ml 2% nonfat milk powder in TBST (250mM Tris pH 8, 1.25M NaCl and 0.1% Tween 20). The following day, either primary antibody α -LexA-HRP (used for “bait” proteins)(Santa Cruz) or α -HA (used for “prey” proteins)(Sigma) was diluted 1:1000 or 1:1500, respectively, in 5 ml 2% milk and used to incubate the membrane for 4 hours at room temperature. Before incubating with the secondary AB the membrane was washed in TBST for 5 minutes, and this was repeated 5 times. Secondary antibody Goat- α -rabbit-HRP (Cappel) was used when analyzing prey proteins at a 1:15000 dilution. When analyzing bait proteins, no secondary AB was used because the primary AB already contained the HRP tag. Secondary AB, when appropriate, was diluted in 2% milk and incubated with membrane for 1 hour. The membrane was washed in TBST for 5 minutes, and this was repeated 5 times. This was followed by two 5 minute washes in TBS (250mM Tris pH 8, 1.25M NaCl). Proteins were detected by a chemiluminescence ECL kit (PIERCE).

2.3 Screening Melanocyte Library

2.3a Yeast Two Hybrid: Interaction of Melanocyte Library

The yeast strain EGY48 was used as the host yeast strain for all two-hybrid interaction experiments. This yeast strain has an integrated LEU reporter gene with an upstream LexA operator, a pSH18-34 plasmid with an upstream LexA operator, and a LacZ reporter that directs the synthesis of β -galactosidase (50). Special shuttle vectors are used to clone the bait and prey. The melanocyte library was previously cloned in pJG4-5 (Zervos lab), which contains a galactose inducible promoter. While the bait proteins Omi (Zervos lab), Omi_{PDZ} (Zervos lab), Omi_{CAT}, and L56₁₅₆₋₄₈₀ were cloned into pGILDA, which also contains a galactose inducible promoter. The pGILDA vector is used for any bait, in this case Omi, whose continuous presence is toxic to the yeast (50). Positive interactions between bait and prey were detected by the presence of blue colonies when grown on GAL-Ura-Trp-His- selective medium plates containing X-Gal.

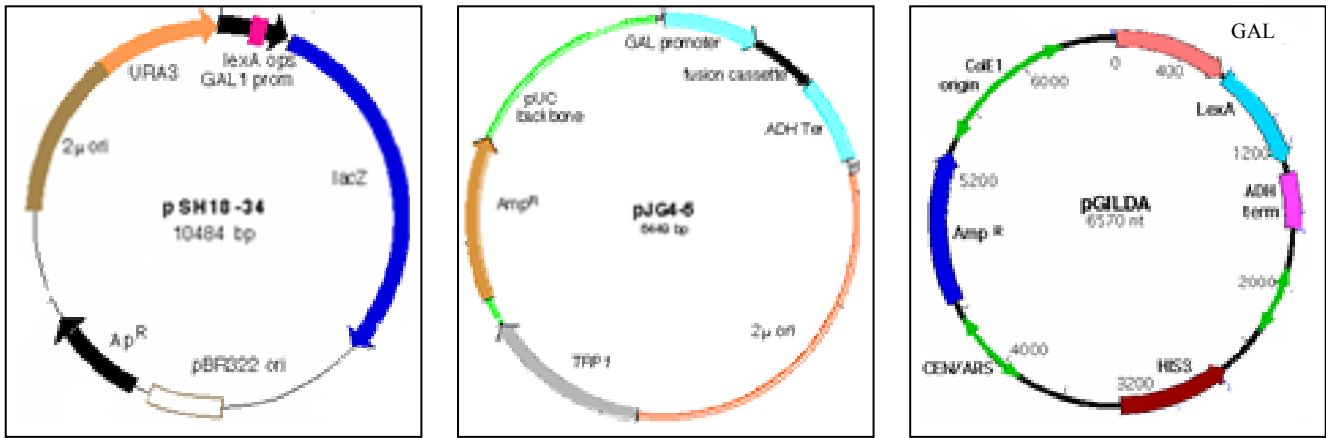


Figure 8: Yeast Plasmids: Map of pSH18-34, pJG4-5, and pGILDA Vectors

All vectors have a 2μ origin of replication in addition to Ampicillin resistance (Amp^R). A selectable marker is present to select for each plasmid when grown in yeast: URA3, TRP1, and HIS3 in pSH18-34, pJG4-5, and pGILDA, respectively. (Picture taken from: <http://www.fccc.edu/research/labs/golemis/InteractionTrapInWork.html>)

2.3b High Efficiency Yeast Transformation

A single yeast colony, EGY48 pSH18-34 pGilda-Omi₁₃₄₋₄₅₈, was picked and grown overnight at 30°C, with shaking, in 50 ml of U⁻ H⁻ Glu drop out medium. The next day the culture was diluted to 300ml with the same medium and grown to an OD_{600nm} of 0.9. Cells were harvested by centrifugation at 3800 rpm for 5 min. Supernatant was discarded and the cells were resuspended in 20ml of sterile H₂O. Spin was repeated for 5 min 3800 rpm, and supernatant discarded. Cells were resuspended in 20ml LA (0.1 M LiAc in TE) and spun again for 5 minutes. Lastly, the cells were resuspended in 5ml of LA. 100μl of competent yeast was aliquoted into sterile eppendorf tubes and the following was added: 10μg DNA melanocyte

library, 100µg of denatured salmon sperm DNA (carrier DNA), and 600µl of freshly prepared 40% PEG solution (50% PEG 4000 prepared in water then freshly diluted to 40% in LA solution). The mixture was resuspended, making sure the solution was homogeneous. The transformation reaction was incubated in a 30°C water bath for 30 min, and then heat-shocked at 42°C in water bath for 15 min. Transformed yeast were centrifuged for 30 seconds, the supernatant removed, and pellet resuspended in 500µl of sterile H₂O. 300µl of resuspended yeast was plated on 24 x 24 cm (Nalge Nunc) UHW Glu selective plates using glass beads (Fisher) and incubate at 30°C for two days.

2.3c Aliquoting Library

The number of colonies in a 2 x 2cm square was counted on 3 sample plates. An average for the squares is obtained and multiplied with 126.6 (the number of 2x2 cm squares in one large plate). This value is the average number of colonies per large plate. The plates are then placed at 4°C so that the agar will harden. Using autoclaved glass slides and aseptic technique, the colonies were scraped from the 10 plates and placed in a Falcon tube. Each plate was washed two times with 10ml of sterile autoclaved water. The yeast were resuspended and distributed evenly into 50ml Falcon tubes. Next, the yeast were centrifuged at 3800 rpm for 5 minutes. The supernatant was discarded and the pellet was washed with 50ml sterile H₂O, and centrifuged again for 5 minutes. This wash was repeated again. Lastly, the pellet was resuspended, in a volume equal to the size of the pellet, in filtered yeast freezing medium (65%glycerol, 0.1M

MgSO₄, 25 mM Tris, pH 7.4). The mixture was then aliquoted into small centrifuge tubes at 300µl each and placed in -80°C.

2.3d Titration

100µl of thawed “frozen melanocyte library” was grown in 5ml U^HW^L Gal/Raf for 5 hours at 30°C with shaking. Serial dilutions of this yeast culture are then prepared as follows:

Tube 1=100 µl from yeast solution in 900µl of sterile H ₂ O	-->1:500
Tube 2=100 µl from Tube 1 in 900µl of sterile H ₂ O	-->1:5,000
Tube 3=100 µl from Tube 2 in 900µl of sterile H ₂ O	-->1:50,000
Tube 4=100 µl from Tube 3 in 900µl of sterile H ₂ O	-->1:500,000
Tube 5=100 µl from Tube 4 in 900µl of sterile H ₂ O	-->1:5,000,000

100µl from each tube was then plated on U^HW^L Gal/Raf plates, and grown for two days at 30°C. Then the colonies from plates 3-5 were counted and multiplied by the dilution factor. This is done to determine the amount needed to be plated on a large U^HW^L Gal/Raf plate in order to yield the correct number of colonies that will represent the entire library. This extrapolated amount is plated on 24 x 24cm (Nalge Nunc) U^HW^L Gal/Raf plates for growth selection.

2.3e Screening

After growth selection was performed on U^HW^L Gal/Raf, the positive colonies were then streaked onto U^HW^L Glu plates, and grown for two days at 37°C. Next, the colonies were streaked onto U^HW^L Glu/X-Gal and U^HW^L Gal/Raf/X-Gal plates. For color selection, positive interactions between bait and prey were detected by the presence of blue colonies on the U^HW^L Gal/Raf/X-Gal plates and these were inoculated in U^HW^L Glu medium to select for the pJG4-5 melanocyte library plasmid. These were grown overnight at 30°C with shaking to release the plasmid from yeast.

2.3f Release plasmid from Yeast

A single yeast colony was picked with a sterile toothpick and grown in 1ml U^HW^L Glu medium overnight. Then, the culture was transferred to a screw cap microcentrifuge tube. The yeast was centrifuged for 1 minute at 14,000 rpm. The pellet was resuspended in 200µl of yeast lysis solution (2% TritonX-100, 1% SDS, 100mM NaCl, 10mM Tris pH 8.0, 1mM EDTA). Approximately 0.3 g of glass beads (Sigma) was added to assist in the lysis along with 200µl of Phenol-chloroform-isoamyl alcohol (25:24:1). The tube was capped and vortexed vigorously for 2 minutes or until the mixture became homogenous. The tube was centrifuged for 2 minutes to separate the phases, and the upper aqueous phase was transferred to a new microcentrifuge tube. DNA was precipitated as described in section 2.1d.

2.3g Transformation of Plasmid into KC8

The plasmids extracted from the yeast were transformed into KC8 bacterial cells using the transformation protocol in section 2.1g.

2.3h Sequencing of Melanocyte Library Positive Interactors

Positive clones were extracted from KC8 bacterial cells using the miniprep protocol in section 2.1h. These plasmids were then sequenced to identify the clone using the protocol in section 2.1j.

2.3i Retransformation in Yeast

The positive interactors for Omi₁₃₄₋₄₅₈ were retransformed into yeast containing pGILDA-Omi₁₃₄₋₄₅₈, pGILDA-Omi_{PDZ}, pGILDA-Omi_{CAT}, pGILDA-L56 using the yeast transformation protocol in section 2.2a.

2.4 Construction of pJG4-5-HOPS and GFP-HOPS

2.4a Polymerase Chain Reaction

The following primers were used in the PCR reaction for the cloning of pJG4-5-HOPS and pGFP-HOPS.

Primer E 5' pJG4-5-HOPS (EcoRI)

5' - CGG AAT TCA TGT TAA CTC TTA ATC TAG TTA AAC – 3'

Primer F 5' pJG4-5-HOPS (EcoRI)

5' – CGG AAT TCT ATG TTA ACT CTT AAT CTA GTT AAA C – 3'

Primer G 3' pGFP-HOPS and pJG4-5-HOPS (Sall)

5' – GCC GAC GTC GAC CTA TAT CAT AGT GAC TTC ATA TG-3'

The restriction sites incorporated into each primer XhoI and Sall are underlined. The PCR products were then digested with the appropriate enzyme and cloned into either pEGFP-C1 (Clontech), pJG4-5, or pET28A (Novagen).

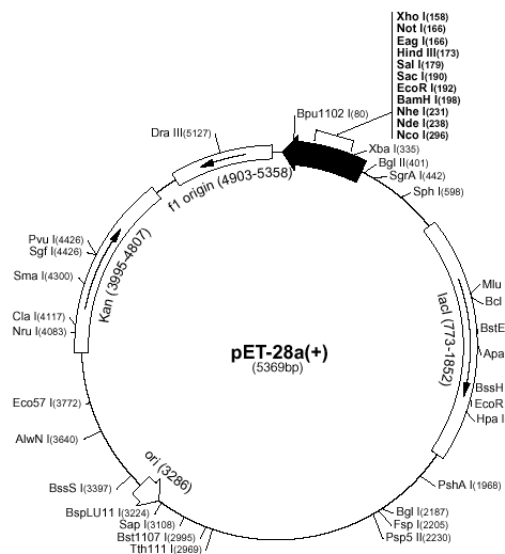
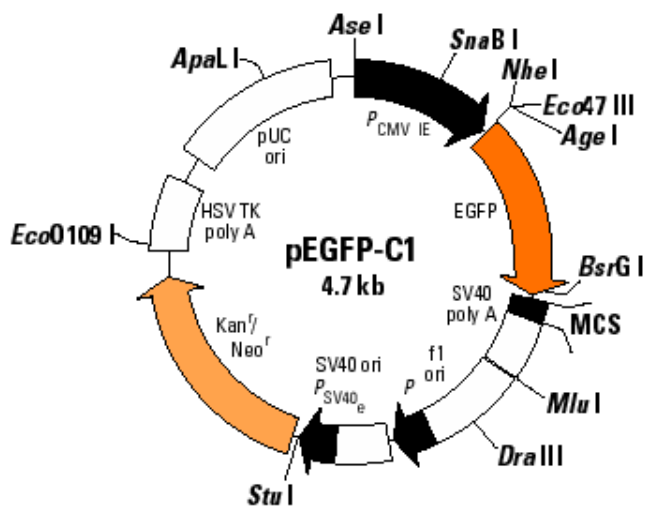


Figure 9: Maps of pEGFP-C1 and pET-28a Vectors

(A) Map of pEGFP-C1 vector used in localization experiments (figure taken from: www.clontech.com). (B) Map of pET-28a vector used in protein expression experiment (figure taken from: <http://www.emdbiosciences.com/html/NVG/AllTables.html>).

2.5 Cleavage Assay

2.5a TNT

The following primer was used in the PCR reaction for the TNT reaction of pJG4-5-HOPS:

Primer K 5' pJG4-5 with T7 promoter and Kozak sequence

5'-TAA TAC GAC TCA CTA TAG GGG ACG ACC ATG GTG CCA GAT TAT GCC
TCT CCC-3'

Primer L 3' pJG4-5

The PCR reaction was performed following the protocol in section 2.1a.

The general protocol for a 50 μ l TNT reaction is as follows:

TNT T7 Quick Master Mix (Promega)	40 μ l
[³⁵ S] methionine/cysteine (1,000Ci/mmol at 10mCi/ml) (Promega)	2 μ l
PCR generated DNA template	2.5-5 μ l
H ₂ O	3-5.5 μ l

The reaction is incubated at 30°C for 1.5 hours

2.5b Degradation Assay

The product of the TNT reaction was incubated with mature his-tag purified Omi₁₃₄₋₄₅₈ as shown in Table 1. The reaction was performed in a final volume of 20 μ l.

Table 1: Description of Cleavage Assay

+ve	-ve
[S ³⁵]-HOPS 5μl	[S ³⁵]-HOPS 5μl
Omi ₁₃₄₋₄₅₈ 5μl	Omi ₁₃₄₋₄₅₈ 0μl
Omi Assay Buffer 10μl	Omi Assay Buffer 15μl

The mixture was incubated at 37°C for 1 hour. 10μl of 2X sample loading buffer was then added to the reaction and the proteins were resolved on a 12% SDS gel under denaturing conditions.

2.6 Transfection in HeLa Cells

2.6a Growing HeLa Cells

HeLa cells, human cervical carcinoma cell line, were grown using F12-Nutrient Mixture (HAM) media (Life Technologies) supplemented with 10% FCS (Sigma), 2mM L-glutamine, 100U/ml penicillin and 100μg/ml streptomycin (Life technologies).

2.6b Transfections and Immunofluorescence Microscopy

For immunofluorescence, the cells were grown on cover-slips. Approximately 70% confluent cells were transfected with 2 μ g of pEGFP-HOPS using Lipofectamine Plus reagent according to the instructions provided by the manufacturer (Life Technologies). After 24 hours, the transfected cells were washed 3 times with PBS and fixed with 4% paraformaldehyde. The cover-slips were then placed on glass slides using Fluoromount-G solution (Southern Biochemical Association). The subcellular localization of GFP fusion proteins was monitored using a LSM510 confocal laser-scanning microscope (Carl Zeiss).

2.7 Transfection in 293T Cells

2.7a Growing 293T Cells

293T cells, human fibroblast cells, were grown using DMEM media (Life Technologies) supplemented with 10% FCS (Sigma), 2mM L-glutamine, 100U/ml penicillin and 100 μ g/ml streptomycin (Life technologies), 1.5g/L sodium bicarbonate (Fisher), 1mM sodium pyruvate (Sigma).

2.7b Induction of Apoptosis

HEK-293T cells were transfected with either GFP-HOPS or DsRED-Omi₁₃₄₋₄₅₈ using Lipofectamin Plus reagent according to the manufacturers directions (Life Technologies). The following morning the medium was removed. Fresh 293T medium was treated with either 2mmol H₂O₂ or 2mmol Cisplatin. The new medium was added to the cells and 12 hours after treatment the cells were analyzed.

2.7c Western Blot of Mammalian Cells

Twelve hours after treatment the cells were recovered by pipetting, and placed in a 2ml microcentrifuge tube. The wells were washed with 500µl of PBS. The tubes are spun at 3500-4000 rpm for 3 minutes. The supernatant is aspirated. The pellet is resuspended in 150µl of lysis buffer (1%Triton-X-100, 10%glycerol, 150mM NaCl, 20mM Tris pH7.5, 2mM EDTA, protease inhibitor cocktail SIGMA). The mixture is then placed in ice for 10 minutes. The cells are then lysed using a combination of freezing with dry ice for 2 minutes and then thawing the cells at 37°C for 1 minute. This process is repeated five times with a 30 second vortexing between each repeat. The cells are spun at 4°C and 14000rpm for 10 minutes. The supernatant is then transferred to a new tube and the protein concentration is measured for equal loading using a Bradford assay.

2.8 Purification of HOPS₁₃₃₋₂₃₃

2.8a Transformation of BL21

BL21 expression bacteria are used to for protein purification. The bacteria are transformed using 1µl of DNA from a 1:10 dilution of clean miniprep DNA, following the protocol in section 2.1g.

2.8b Protein Purification

BL21 bacteria transformed with pET28a-HOPS₁₃₃₋₂₃₃ were grown O/N in the appropriate LB containing Kanamycin. The following morning the bacteria were placed in the appropriate volume of medium with 50µg/ml. The culture was grown at 250rpm and 37°C to an OD₆₀₀ of 0.8. 1ml of bacteria was obtained before induction for analysis. The temperature was then dropped to 25°C and induction was performed using 1mM IPTG. Induction was done O/N. The following morning 1ml of bacteria is obtained for analysis, and the remaining culture is spun at 6000rpm and 4°C for 10 minutes. The supernatant is discarded and the pellet is placed on ice. The pellet was resuspended in 25-40ml of Lysis Buffer which is composed of Binding Buffer + 4X Protease Inhibitor Cocktail (SIGMA) diluted to a concentration of 1:100. This mixture was placed in a 50ml Falcon tube and lysozyme (10mg/ml) is added 1:100; the mixture was placed at 30°C for 15 minutes. 1% Triton X-100 was added and the solution was spun at room temperature for one hour. The cells are then sonicated 3 times, each time for one minute and then they were placed on ice for 1 minute. The mixture was then centrifuged at 4°C 13000rpm

for 30 minutes. The supernatant was filtered with .45micron filter and 4ml of 50% Ni-NTA beads were added. This was mixed O/N at 4°C. The next morning everything was placed in a column and the flow through was collected for analysis. The column was washed 2 times with 50ml of 1X Binding Buffer (8X=400mM NaH₂PO₄, 2.4M NaCl, 80mM Imidazole, 0.4% NaN₃). Then the column was washed 2 times with 50ml of 1X Wash Buffer (8X=400mM NaH₂PO₄, 2.4M NaCl, 160mM Imidazole, 0.4% NaN₃). The column is then eluted 2 times with 2ml of Elution Buffer (4X=200mM NaH₂PO₄, 1.2M NaCl, 1M Imidazole, 0.2% NaN₃). The eluant was then dialysed in Dialysis Buffer (40mM Hepes and 200mM NaCl). The purified protein was then subjected to SDS-PAGE to analyze purity.

2.9 Regulation Assay

2.9a Regulation Assay

Purified HIS-HOPS₁₃₃₋₂₃₃ was incubated with either his-tag purified mature Omi₁₃₄₋₄₅₈ or his-tag purified inactive OmiS-C₂₇₆ protein to see if HOPS₁₃₃₋₂₃₃ has a regulatory role on the protease activity of Omi. HOPS₁₃₃₋₂₃₃ was incubated together with either Omi₁₃₄₋₄₅₈ or OmiS-C₂₇₆ for 10 minutes. 2µl of 2.5mg/ml β-casein was then added to the mixture. The final reaction volume of 20µl was reached using Omi Assay Buffer (20mM NaH₂PO₄/Na₂HPO₄, pH 7.4, 200mM NaCl, 5% Glycerol). The samples were loaded side by side on a 12% SDS-PAGE and

the gel was stained with Coomassie for 3 hours and destained with Destaining Solution (20% Methanol, 10% Acetic Acid) overnight.

CHAPTER THREE: RESULTS

3.1 Cloning and Yeast Expression of LexA-Omi₁₃₄₋₃₄₀ and LexA-L56₁₅₇₋₄₈₅

DNA corresponding to the mature form of Omi₁₃₄₋₄₅₈ protein, the catalytic domain (amino acids 134-340) or the PDZ domain (amino acids 340-458) was cloned into the pGILDA yeast expression vector. Using these three different baits, one could assess which domain of Omi a potential interactor was binding.

In addition, as a control, DNA for the HtrA1/L56₁₅₇₋₄₈₀ protein was also cloned into the pGILDA vector. Since Omi and L56 share high sequence homology, this protein will be used to determine the specificity of any interactors.

The catalytic domains of Omi and L56₁₅₇₋₄₈₀ were expressed as Lex-A (DNA binding domain) fusion proteins using the pGILDA vector as described in Materials and Methods. Transcription of these bait proteins is under the control of a GAL1 promoter, and therefore are only expressed in yeast in the presence of galactose. These constructs were transformed into yeast cells and expression of the recombinant LexA-fusion proteins was monitored by Western Blot analysis of yeast whole cell extracts grown either in glucose or galactose.

LexA- Omi₁₃₄₋₃₄₀ has an estimated molecular weight of \approx 43 kDa, which corresponds to the estimated molecular weight of the catalytic domain, 23kDa fused with the LexA DNA-

binding domain, $\approx 20\text{kDa}$ (Figure 10). LexA- L56₁₅₇₋₄₈₀ has an estimated molecular weight of 56 kDa, which corresponds to the estimated molecular weight of L56₁₅₇₋₄₈₀, $\approx 36\text{kDa}$, fused with LexA $\approx 20\text{kDa}$ (Figure 11).

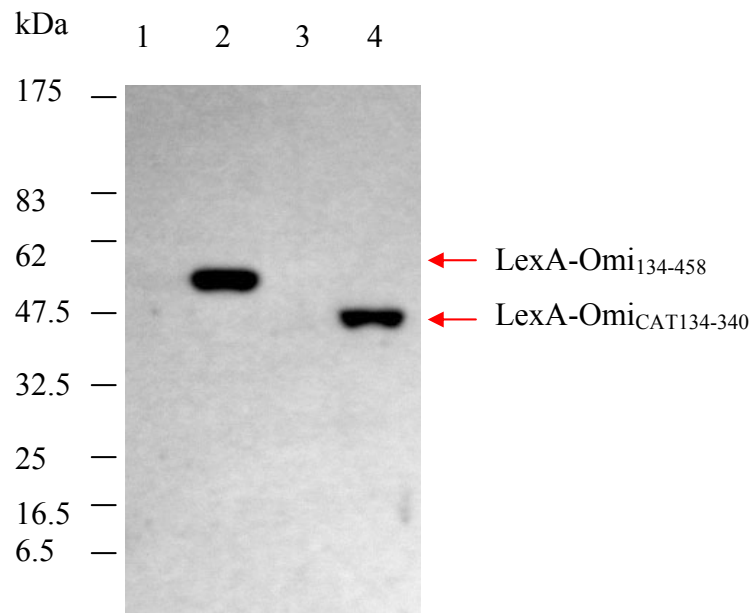


Figure 10: Cloning and Yeast Expression of pGILDA_{Omi134-340}

Yeast-expression of various baits in the presence of glucose or galactose. Lane 1: LexA-Omi₁₃₄₋₄₅₈ glucose; Lane 2: LexA-Omi₁₃₄₋₄₅₈ galactose; Lane 3: LexA-Omi₁₃₄₋₃₄₀ glucose; Lane 4: LexA-Omi₁₃₄₋₃₄₀ galactose.

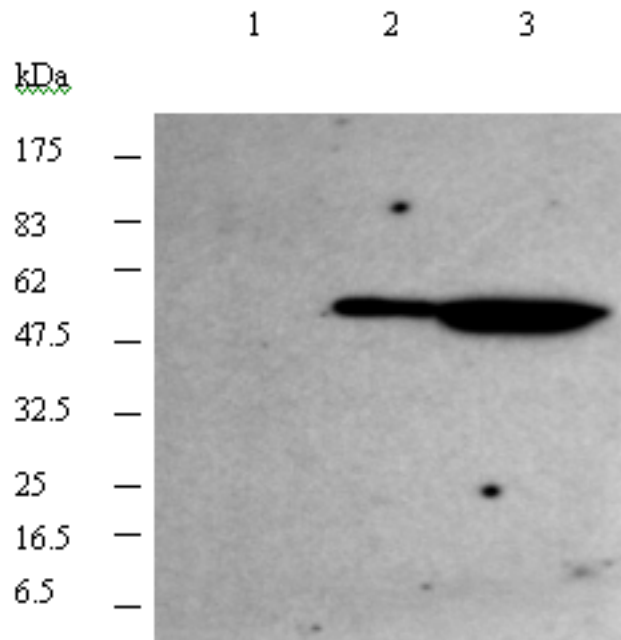


Figure 11: Cloning and Yeast Expression of pGilda-L56₁₅₇₋₄₈₀

Expression of LexA-L56₁₅₇₋₄₈₀ in the presence of glucose or galactose.

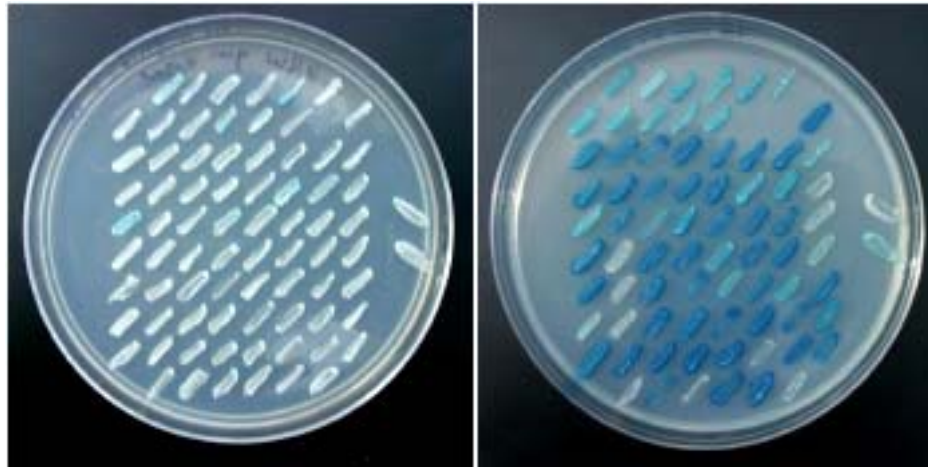
Lane 1: LexA-L56₁₅₇₋₄₈₀ glucose; Lane 2: LexA-L56₁₅₇₋₄₈₀ galactose; Lane 3: LexA-Omi₁₃₄₋₄₅₈ galactose. The size of both recombinant proteins is very similar.

3.2 Screening the Melanocyte cDNA Library

RNA was obtained from cultured, actively growing, human melanocyte cells. This RNA was used to make cDNAs, which was then cloned unidirectionally as an EcoR1/Xho1 fragment into the corresponding sites of the pJG4-5 vector (Zervos Lab). This melanocyte cDNA library was used in the yeast two hybrid screening.

The mature form of Omi₁₃₄₋₄₅₈ fused to LexA was used as the bait in this screening. Additionally, two reporter genes, the LexAop-LEU2 gene allowing the growth of the colonies in the absence of leucine and the LexAop-lacZ gene that directs the synthesis of β -galactosidase, were included. The number of clones per plate was estimated by counting the number of colonies in a 2x2 cm squares. Two squares were counted on each of the ten plates, averaged, and then extrapolated to estimate the number of clones per plate. A total of 7×10^5 colonies were present.

7 million primary yeast colonies were screened for cDNAs encoding proteins that potentially interact with the mature form of Omi. Of these seven million colonies 100 colonies were initially identified by their ability to grow on U⁻H⁻W⁻L⁻ Galactose selective plates. These were then tested on U⁻H⁻W⁻ Glucose/X-Gal and U⁻H⁻W⁻ Galactose/X-Gal plates. Fifty-one of them were found to have a galactose dependent phenotype. Twenty-five of the colonies were identified as false positives, correlating to either a blue colony on U⁻H⁻W⁻ Glucose/X-Gal that could not be further examined, or a white colony on U⁻H⁻W⁻ Galactose/X-Gal that suggests no protein-protein interaction (Figure 12).



U⁻H⁻W⁻ Glucose/X-Gal

U⁻H⁻W⁻ Galactose/X-Gal

Figure 12: Melanocyte cDNA Library Screening

A positive interaction between the bait protein and the prey protein, leads to blue colonies on U⁻H⁻W⁻ Galactose/X-Gal. White colonies suggest a negative protein-protein interaction. Blue colonies on the U⁻H⁻W⁻ Glucose/X-Gal plate correspond to false positives.

3.3 Characterization of Omi Interactors

The cDNA library plasmids, were rescued from each of the positive yeast clones, and introduced into KC8 bacteria by electroporation. The prey plasmids were then isolated using a Miniprep Kit (QIAGEN). The cDNAs were sequenced, and homology searches were performed using the BLAST program provided by on the NCBI server (www.ncbi.nlm.nih.gov/BLAST/). Screening in yeast was repeated using these 51 purified cDNA library plasmid as “prey” and either LexA-Omi₁₃₄₋₄₅₈, LexA-Omi_{CAT134-340}, LexA-Omi_{PDZ340-458}, or LexA-L56₁₅₇₋₄₈₀ as the “bait”.

The results of the yeast transformation revealed that of the 51 positive clones, 32 were true positives. A positive interaction, resulting in a blue colony on U⁺H⁺W⁻ Galactose/ X-Gal, between one of the bait proteins (LexA- Omi₁₃₄₋₄₅₈, LexA- Omi_{CAT134-340}, LexA- Omi_{PDZ340-458}, and LexA- L56₁₅₇₋₄₈₀) and the prey (interactors identified in melanocyte library cDNA screening) corresponds to a + on the chart. However, a negative interaction yielding no color change, corresponds to a – on the table. These 32 interactors were sequenced and 17 distinct family proteins were identified. Each isolated cDNA is a true interactor of Omi, however, these interactors may be localized in a different location then Omi under normal physiological conditions. This localization must be taken into consideration when choosing a particular interactor to characterize. There must be a possibility that Omi is able to encounter the interactor under normal conditions. One of these interactors we call HOPS, for HtrA/Omi protein substrate, was selected for further characterization.

Table 2: List of Omi Interactors Isolated From a Human Melanocyte cDNA Library

NAME	Omi ₁₃₄₋₄₅₈	Omi _{CAT134-340}	Omi _{PDZ340-458}	L56 ₁₅₇₋₄₈₀
Thyroid receptor interacting protein 15	+	-	-	+
Complement component 1, q subcomponent/ p32	+	-	+	-
Macrophage migration inhibitory factor	+	-	+	+
D-dopachrome tautomerase	+	+	+	+
Nucleostemin	+	+	+	+
HOPS	+	-	+	+
Protein kinase c like 2	+	-	+	-
Protein kinase c substrate 80K-H	+	+	+	-
Homo sapiens glutathione-s-transferase omega 2	+	+	+	-
SCAP2	+	+	+	+
Collagen type 11	+	+	+	-
Glutamate dehydrogenase 1	+	+	+	-
Homo sapiens calcium binding protein Cab 45 precursor	+	+	+	+
Scaffold Attachment Factor B	+	-	+	-
Crystallin, lambda 1	+	+	+	+
Supressor of Ty 4 homolog	+	+	+	-
Laminin receptor 1	+	-	+	-

A positive protein-protein interaction is represented by a +, while no protein-protein interaction corresponds to a -.

3.4 Characterization of HOPS

The HOPS protein was isolated twice of the 32 positive clones as a true positive interactor of LexA-Omi₁₃₄₋₄₅₈ in the melanocyte library screening. This protein is a novel protein of unknown function. HOPS is located at chromosome 7q22.3. Using the PSORT II program, HOPS, the protein is predicted to localize in either the nucleus or mitochondria.

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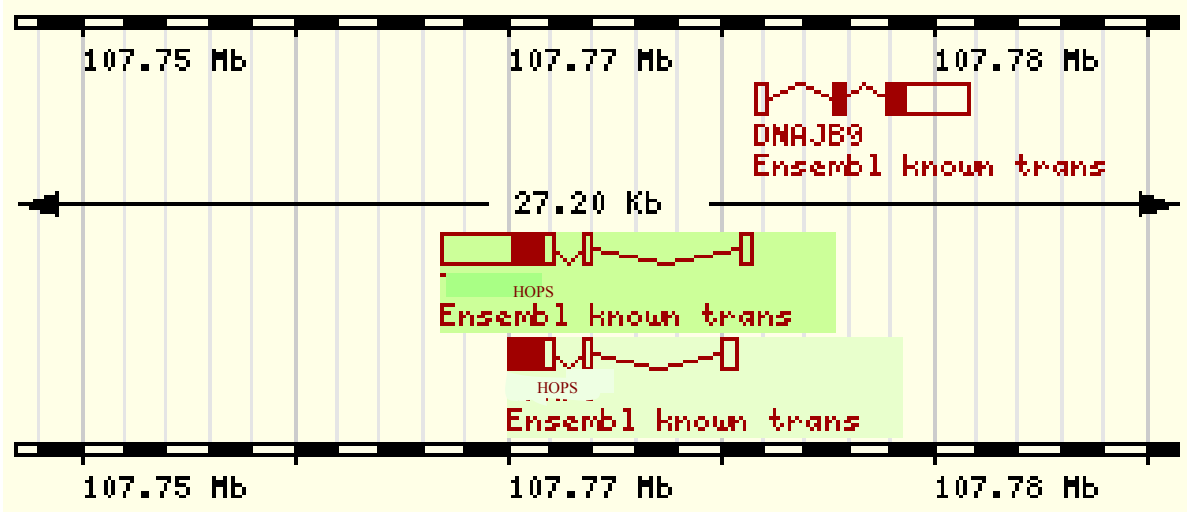
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421  att gaa gac tcc ttg tat aag gat gta gac tat ggg aca gaa gtt tta caa atc gaa cat 480
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   E A L I R Q L K Q E N W L S E E N V K I
661  ata gaa aac cat ttt aca aca tat gaa gtc act atg ata tag 702
   I E N H F T T V E V T M I *

```

Figure 13: Sequence of HOPS

Nucleotide and amino acid sequence of HOPS. The C-terminal portion underlined in red represents the clone obtained in the melanocyte library screening.

A. Genomic structure



B. Transcript Structure

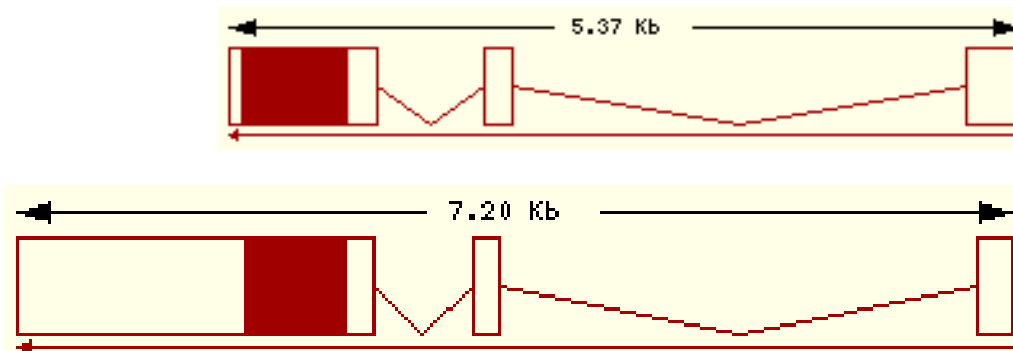


Figure 14: Genomic Structure of HOPS

(A) The gene is mapped to chromosome 7q22.3. (B) The transcript structure of HOPS. The gene contains three exons and two introns. Coding sequence is exclusively derived from Exon I. (Picture taken from:

http://www.ensembl.org/Homo_sapiens/transview?transcript=ENST00000313516&db=core)

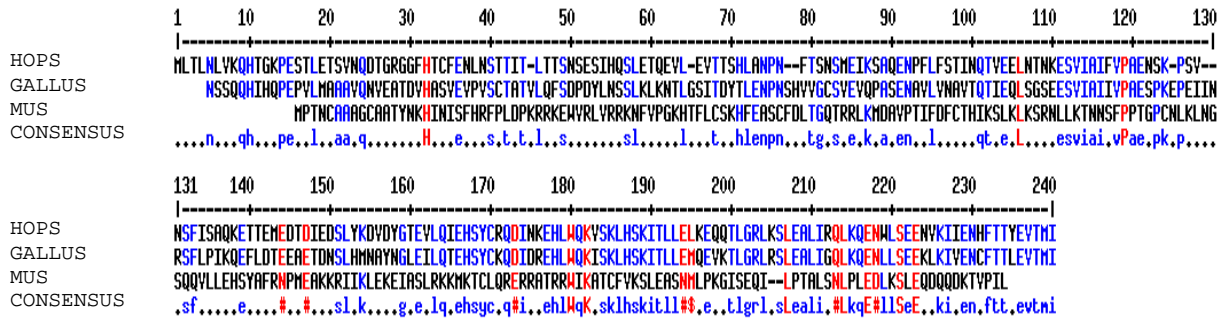


Figure 15: Alignment of Human HOPS with Mouse and Chicken Orthologues

Gallus: *Gallus gallus* (novel protein obtained from: www.ensembl.org), Mus: *Mus musculus* (NP_080056)

Table 3: HOPS Tissue Distribution

Testis	10.60
Prostate	1.51
Thyroid	1.51
Placenta	6.06
Colon	1.51
Stomach	1.51
Aorta	1.51
Liver	3.03
Cartilage	1.51
Head normal	1.51
Kidney	3.03
Lymph	1.51
Muscle (skeletal)	1.51
Hippocampus	1.51
Medulla	3.03
Frontal lobe	3.03
White matter	1.51
Total fetus	3.03
Fetal brain	4.54
Embryonic stem cell DMSO treated H9 cell line	1.51
Embryonic stem cell treated H1, H7, H9 retina	3.03
Embryonic stem cell retinoic acid and mitogenic hES cell line H7	1.51
Colon tumor RER	1.51
Embryonal carcinoma	3.03
Nervous tumor	3.03
Parathyroid tumor	1.51
Melanotic melanoma	3.03
Retinoblastoma	1.51
Acute myelogenous leukemia	1.51
Chronic myelogenous leukemia	1.51
Moderately differentiated adenocarcinoma	1.51
Adenocarcinoma	1.51
Hypernephroma cell line	1.51
Duodenal adenocarcinoma cell line	1.51
Transitional cell papilloma cell line	1.51
Mammary adenocarcinoma cell line	1.51
Human chondrosarcoma cell line	1.51
Mixed	1.51
Pooled	3.03
2 pooled wilms tumors: one primary and one metastatic to brain	1.51
Pooled lung and spleen	1.51
2 pooled tumors clear type	1.51
Three pooled meningiomas	1.51

The various tissue distributions of known ESTs for HOPS suggest a wide expression of this gene. The highest expression is seen in the brain and ectodermally derived organs, as well as cancer cells.

3.5 Expression of full-length HOPS protein in Yeast

The full-length cDNA clone of HOPS in the pCMV-SPORT6 vector was obtained (Open Biosystems I.M.A.G.E. Consortium). PCR was used to prepare DNA corresponding to the complete coding sequence using the primers described in Materials and Methods. HOPS full-length cDNA was cloned in frame with the B42 activation domain and HA-tag in the pJG4-5 vector. This prey protein, under the control of a GAL1 promoter, is expressed in yeast only in the presence of galactose. Whole cell extracts of the transformed yeast grown in either the presence of glucose or galactose, were subjected to SDS-PAGE. The expression of this B42 fusion protein was monitored by Western Blot analysis using HA-antibodies (ROCHE). The HOPS protein can be observed as a band at ≈ 36 kDa, this corresponds to the estimated molecular weight of HOPS, ≈ 26 kDa, in fusion with B42 and HA-tag, ≈ 10 kDa.

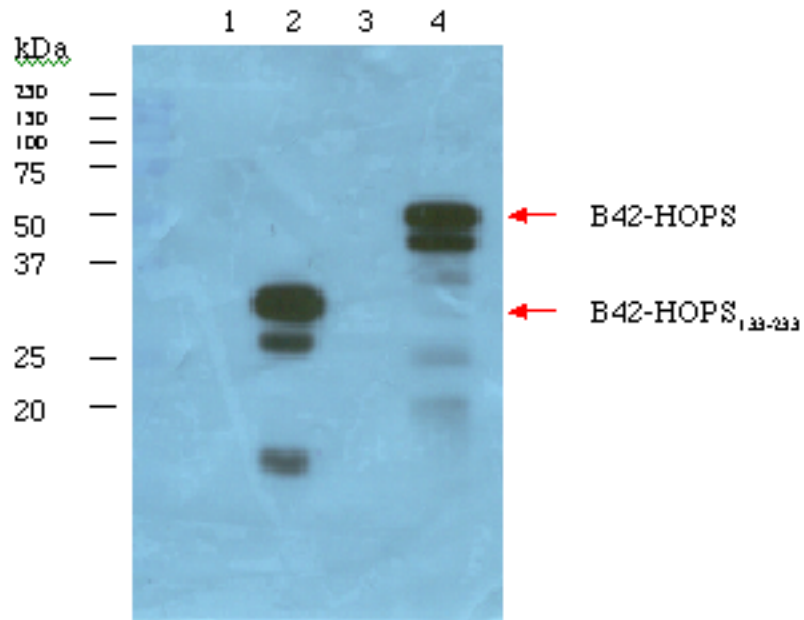


Figure 17: HOPS Expression in Yeast

Expression of HOPS₁₃₃₋₂₃₃ and full length HOPS protein in the presence of glucose or galactose. Equal amounts of yeast lysates were subjected to SDS-PAGE followed by Western Blot analysis using anti-HA primary antibody followed by goat-anti-mouse secondary antibody. Lane 1: HOPS glucose; Lane 2: HOPS galactose; Lane 3: HOPS₁₃₃₋₂₃₃ glucose; Lane 4: HOPS₁₃₃₋₂₃₃ galactose.

3.6 Interaction of Full-length HOPS Protein With Various Baits in Yeast

The full-length HOPS protein was expressed as a prey in a yeast strain EGY48 along with one of the following bait proteins: LexA-Omi₁₃₄₋₄₅₈, LexA-Omi_{CAT134-340}, LexA-Omi_{PDZ340-458}, LexA-L56₁₅₇₋₄₈₀. The full-length protein interacted with both the mature form of Omi₁₃₄₋₄₅₈ as well as the Omi_{PDZ340-458}. However, unlike the partial HOPS clone originally isolated from the melanocyte cDNA library (HOPS₁₃₇₋₂₃₃) the full length HOPS protein did not interact with L56₁₅₇₋₄₈₀.

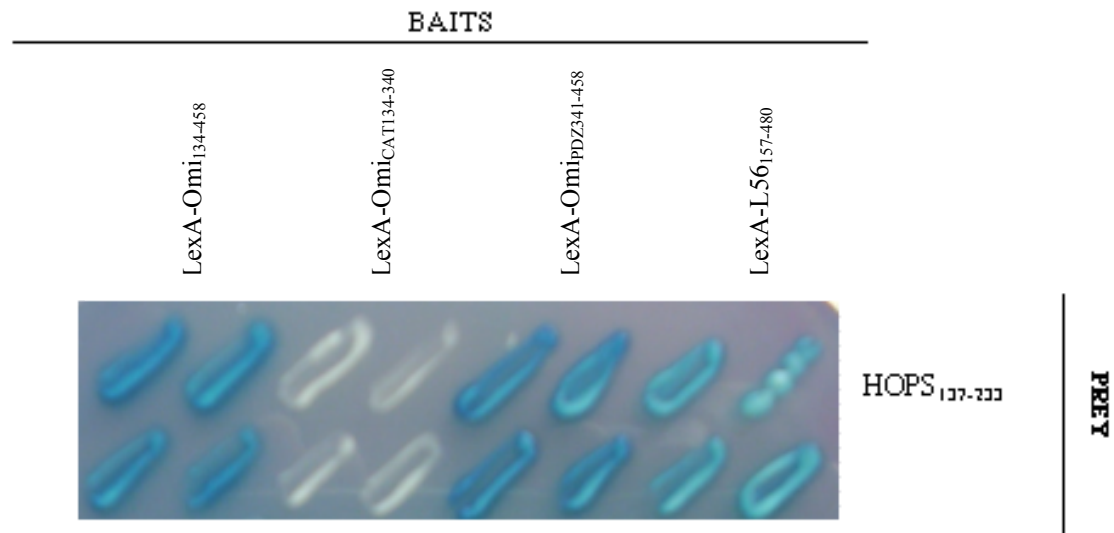


Figure 18: Specificity of the Partial HOPS Clone in Yeast

HOPS₁₃₇₋₂₃₃ was used as a prey against the following baits: LexA-Omi₁₃₄₋₄₅₈, LexA-Omi_{CAT134-340}, LexA-Omi_{PDZ340-458}, LexA-L56₁₅₇₋₄₈₀. HOPS₁₃₇₋₂₃₃ is shown to interact with L56 as well as Omi, and the PDZ domain of Omi.

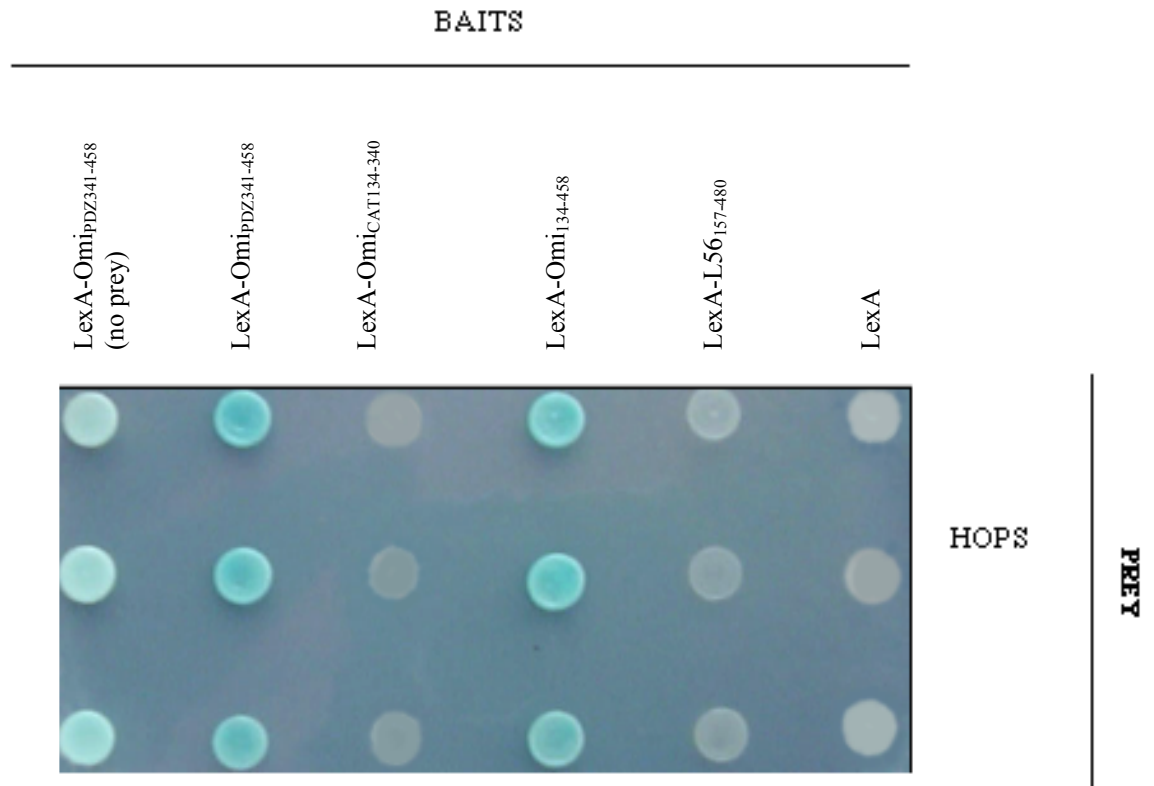


Figure 19: Specificity of the HOPS Clone in Yeast

Yeast colonies were transformed with the indicated bait and the full-length HOPS prey. A blue colony represents a positive protein-protein interaction, while white colonies indicate no interaction between bait and prey. HOPS is able to interact with Omi as well as the PDZ domain of Omi.

3.7 Degradation Assay

I investigated the possibility that Omi, a serine protease, might be able to degrade HOPS protein *in vitro*. [S^{35}]-labeled HOPS was used as a potential substrate against bacterially made Omi₁₃₄₋₄₅₈ in an *in vitro* assay as described (Materials and Methods) Briefly, His-Omi₁₃₄₋₄₅₈ was incubated with the [S^{35}]-HOPS in 20 μ l final reaction volume at 37°C for 1 hour. The products were subjected to SDS-PAGE and then observed by autoradiography.

In the presence of Omi₁₃₄₋₄₅₈, HOPS was cleaved. After degradation, HOPS can be seen as a band of \approx 36kDa and a new peptide band appears between 25kDa and 32kDa (Figure 20).

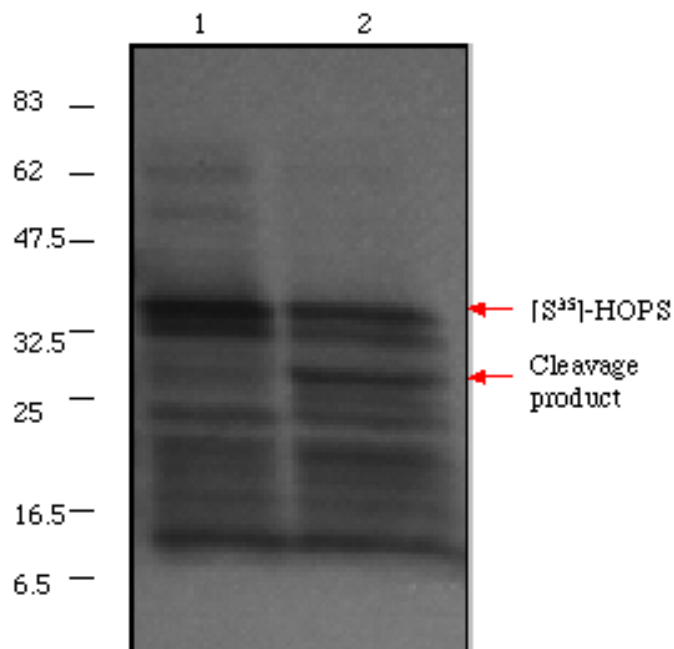


Figure 20: HOPS is Cleaved by Omi *In Vitro*

Lane 1: [S^{35}]-HOPS; Lane 2: [S^{35}]-HOPS + His-Omi₁₃₄₋₄₅₈.

3.8 Subcellular Localization of HOPS

The full-length HOPS protein was expressed as a fusion to green fluorescent protein using the pEGFP-C1 (Clontech) mammalian expression vector as described (Materials and Methods). This construct was co-transfected into HeLa cells with DsRed-Omi₁₃₄₋₄₅₈. The pDsRed1-C1 vector (Clontech) fuses a red fluorescence protein to the C-terminus of Omi. The HeLa cells were grown on a cover slip and 24 hours after transfection were fixed on the slide so that the fluorescent proteins could be observed using a confocal microscope. The subcellular localization of the fluorescent fusion proteins was observed (Figure 21).

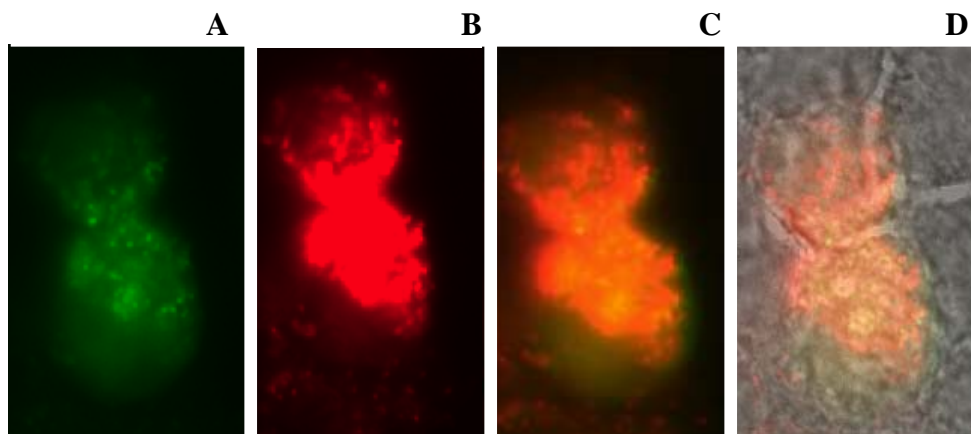


Figure 21: Co-localization of GFP-HOPS₁₃₇₋₂₃₃ and DsRED-Omi₁₃₄₋₄₅₈

HeLa cells were transiently co-transfected with both GFP-HOPS₁₃₇₋₂₃₃ (A) and DsRED-Omi₁₃₄₋₄₅₈ (B). After transfection the cells were fixed and examined by confocal microscopy. Merged fluorescent images show co-localization of GFP-HOPS and DsRED-Omi (C, D).

3.9 GFP-HOPS is Cleaved in Mammalian Cells in Response Cisplatin Treatment

293T cells were transfected with pEGFP-HOPS as described in Materials and Methods. The cells were treated with either H₂O₂ or Cisplatin for twelve hours. Equal amounts of whole cell extracts were subjected to SDS-PAGE and the protein level of GFP-HOPS was monitored by Western blot analysis using anti-GFP monoclonal antibodies, followed by goat-anti-mouse secondary antibody. GFP-HOPS can be seen as a band at ≈54 kDa, which corresponds to the estimated molecular weight of HOPS, ≈25kDa, and GFP, ≈29 kDa. In Figure 22, it can be seen that in the presence of Cisplatin the concentration of HOPS decreases. Equal loading was monitored using anti-βactin antibodies.

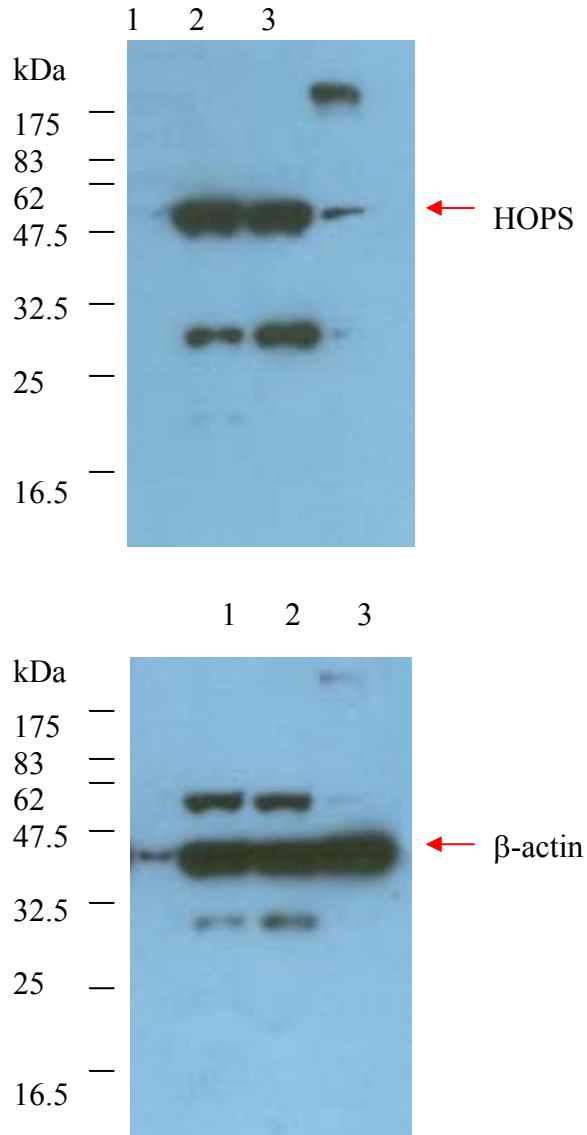


Figure 22: GFP-HOPS is Degraded in Mammalian Cells after Cisplatin Treatment.

(A) Degradation of GFP-HOPS proteins was monitored by Western Blot analysis. Equal amounts of whole cell lysates prepared from cells treated with H_2O_2 or Cisplatin were subjected to SDS-PAGE followed by Western Blot analysis using anti-GFP primary antibody followed by anti-mouse secondary antibody. Lane 1: GFP-HOPS; Lane 2: GFP-HOPS + H_2O_2 ; Lane 3: GFP-HOPS + Cisplatin. (B) To verify equal amounts of protein was used in each lane the blot from A was probed with β -actin antibodies.

3.10 Expression and Purification of HOPS₁₃₃₋₂₃₃ in Bacteria

PCR was used to amplify DNA corresponding to HOPS C-terminal (amino acids 137-233) domain and cloned into the pET-28A vector as described (Materials and Methods). The pET28A vector allows proteins to be expressed as fusions with a 6-His-tag. The construct was then transformed by electroporation into BL21 bacteria. Recombinant protein was purified using native-state conditions and Ni-NTA beads. The purified recombinant HOPS₁₃₃₋₂₃₃ protein was analyzed by SDS-PAGE stained with Coomassie Blue dye. C-terminal HOPS₁₃₇₋₂₃₃ can be seen as a band at ≈ 10 kDa, which corresponds to the estimated size (Figure 23).

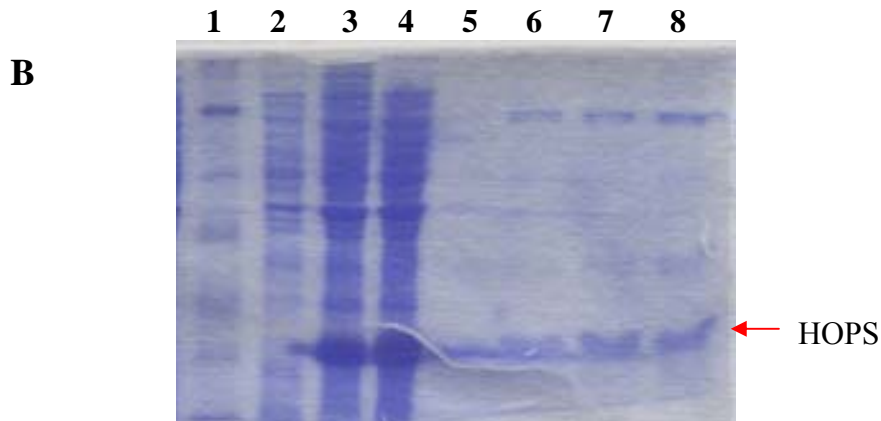
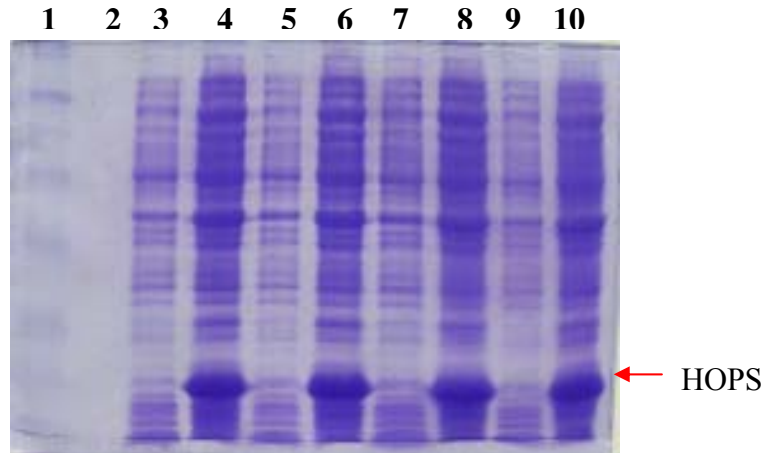


Figure 23: Expression and Purification of Recombinant His-HOPS₁₃₃₋₂₃₃ Protein

His-HOPS₁₃₃₋₂₃₃ was expressed in BL21 expression strain bacteria. Lane 1: Marker; Lane 2: empty; Lane 3,5, 7, 9: whole cell lysates of BL21 containing His-HOPS₁₃₃₋₂₃₃ not induced; Lane 4, 6, 8, 10: whole cell lysates of BL21 containing His-HOPS₁₃₃₋₂₃₃ induced with IPTG.

(B) His-HOPS₁₃₃₋₂₃₃ was purified from BL21 using Ni-NTA beads. Lane 1: Marker; Lane 2 and Lane 3: BL21 whole cell lysate not induced; Lane 4: BL21 whole cell lysate induced with IPTG; Lane 5: 5µl purified His-HOPS₁₃₃₋₂₃₃ elution 2; Lane 6: 10µl purified HOPS elution 2; Lane 7: 15µl purified His-HOPS₁₃₃₋₂₃₃ elution 2; Lane 8: 15µl purified His-HOPS₁₃₃₋₂₃₃ elution 1

3.11 Potential Regulation of Omi's Proteolytic Activity by Recombinant HOPS₁₃₃₋₂₃₃ Protein

The purified His-HOPS₁₃₃₋₂₃₃ protein was incubated with bacterially made Omi₁₃₄₋₄₅₈ in the presence of β -casein, a generic substrate for Omi. The results shown in Figure 24 suggest that the C-terminus of HOPS₁₃₃₋₂₃₃ has no regulatory control over Omi's proteolytic activity when tested against β -casein.

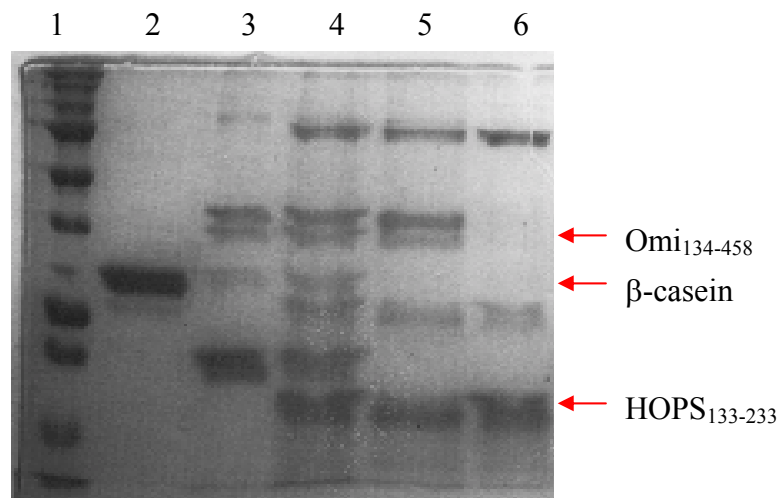


Figure 24: HOPS₁₃₃₋₂₃₃ Effect on the Proteolytic Activity of Omi *In Vitro*

HOPS₁₃₃₋₂₃₃ does not affect the proteolytic activity of Omi *in vitro*. His-HOPS₁₃₃₋₂₃₃ was incubated with His-Omi₁₃₄₋₄₅₈ in the presence of β -casein. Lane 1: Marker; Lane 2 β -casein; Lane 3: β -casein + His-Omi₁₃₄₋₄₅₈; Lane 4: β -casein + His-Omi₁₃₄₋₄₅₈ + His-HOPS₁₃₃₋₂₃₃; Lane 5: His-Omi₁₃₄₋₄₅₈ + His-HOPS₁₃₃₋₂₃₃; Lane 6: His-HOPS₁₃₃₋₂₃₃.

3.12 Bacterially Expressed HOPS₁₃₃₋₂₃₃ is Degraded *In Vitro* by Omi₁₃₄₋₄₅₈

The purified His-HOPS₁₃₃₋₂₃₃ protein was incubated with bacterially made Omi₁₃₄₋₄₅₈ over a time course of 24 hours. Figure 24 shows that even at 4 hours of incubation HOPS₁₃₃₋₂₃₃ is degraded.

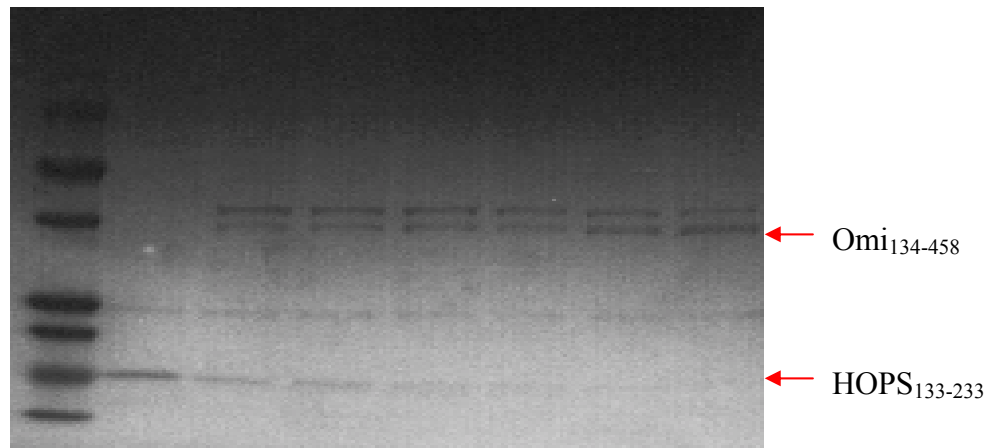


Figure 25: HOPS₁₃₃₋₂₃₃ is Degraded by Omi₁₃₄₋₄₅₈ *In Vitro*

Lane 1: Molecular Weight marker; Lane 2: His-HOPS₁₃₃₋₂₃₃ alone; Lane 3: His-Omi₁₃₄₋₄₅₈+His-Omi₁₃₄₋₄₅₈ 1 hour incubation; Lane 4: His-Omi₁₃₄₋₄₅₈+His-Omi₁₃₄₋₄₅₈ 2 hour incubation; Lane 5: His-Omi₁₃₄₋₄₅₈+His-Omi₁₃₄₋₄₅₈ 3 hour incubation; Lane 6: His-Omi₁₃₄₋₄₅₈+His-Omi₁₃₄₋₄₅₈ 4 hour incubation; Lane 7: His-Omi₁₃₄₋₄₅₈+His-Omi₁₃₄₋₄₅₈ 8 hour incubation; Lane 8: His-Omi₁₃₄₋₄₅₈+His-Omi₁₃₄₋₄₅₈ 24 hour incubation.

CHAPTER FOUR: DISCUSSION

Omi/HtrA2 is a serine protease belonging to the HtrA family of proteins and is highly conserved throughout different species. Omi protein shares homology with the bacterially expressed heat shock protease *HtrA*, that functions as a protease at higher temperatures and a chaperone at lower temperatures (31). Additionally, Omi shares sequence similarity with the mammalian homologs L56/HtrA1 and PRSP/HtrA3 (31, 34). However, this homology with Omi lies largely at the carboxy-terminus. Omi possesses a different amino-terminus which contains a regulatory domain not seen in the other two human homologs (34).

Omi has a series of functional domains including an amino-terminal regulatory domain, a catalytic domain, and a PDZ domain. The regulatory domain contains a mitochondrial targeting sequence (MTS), a predicted transmembrane region, a phosphorylation site, and an SH3-binding domain (28, 33). In the mature form of Omi, the MTS is removed, revealing a conserved IAP-binding motif, AVPS, at the new amino-terminus (29). The conserved catalytic domain has extensive similarity to serine proteases, such as trypsin, and is involved in the cleavage and degradation of substrates such as XIAP (35). In accord with other PDZ domains, including those of the bacterial HtrA, the PDZ domain of Omi is assumed to be involved in protein-protein interactions (44).

Omi has been identified as an active participant in both the caspase-dependent as well as caspase-independent pathways of cell death (28, 29, 44). In the caspase-dependent pathway, Omi binds and degrades IAPs, thus allowing apoptosis to proceed. This is accomplished by two mechanisms. First, by binding to IAPs through its N-terminal AVPS motif, hindering their

ability to bind to caspases (46). Secondly, Omi cleaves and irreversibly removes IAPs through its proteolytic activity (29, 36, 44, 47, 48). Omi is also involved in the caspase-independent pathway, but the mechanism remains unclear. The proteolytic activity of Omi is necessary and essential for this “atypical” cell death, and does not involve interaction with IAPs (28, 44).

Recent studies have revealed Omi has yet another unique function in mitochondria (12). A missense mutation of Omi was identified in *Mnd2* mice, which reduces the protease activity of the protein. This mutation results in a neurodegenerative phenotype rather than the excessive cell proliferation one would expect if Omi’s sole function were to induce apoptosis. This suggests that perhaps the primary function of Omi in mammalian cells deals with the maintenance of mitochondrial homeostasis, and resembling its bacterial homolog, functions as a chaperone (45).

Omi was first isolated as an interacting protein of Mxi2, an alternatively spliced form of the p38 stress-activated kinase, using a modified yeast two-hybrid system. The PDZ domain of Omi specifically recognizes the carboxy-terminus of Mxi2 (31). To date, there are several known interactors of Omi including the Mxi2, XIAP, cIAP1, the newly identified protein HAX1, and Presenilin1 (29, 31, 46). XIAP, cIAP and HAX 1 proteins are Omi substrates whose activation augments cell death. Presenilin1 has recently been shown to boast a different function; upon interacting with Omi, Presenilin 1 regulates its serine protease activity. A synthetic peptide encompassing the last 15 carboxy-terminal amino acids of Presenilin 1 is also able to substantially increase the proteolytic activity of Omi both *in vitro* as well as *in vivo* (51).

A screening of a primary melanocyte cDNA library was performed to isolate novel interacting proteins that may further elucidate the function of Omi. This cDNA library was

selected under the assumption that, unlike a cancer cell line, primary cells, such as the melanocytes used here, would have their apoptotic pathway intact and express a range of pro-apoptotic and anti-apoptotic proteins. Upon screening the cDNA library, a novel protein HOPS was identified. The isolated partial cDNA of HOPS contained the last 100 amino acids of the protein. This partial protein was shown to interact with the mature form of Omi, the PDZ domain of Omi, as well as the mature form of L56 in yeast two hybrid experiments. However, when the full length HOPS protein was tested in the same assay the results were even more promising showing that full length HOPS is specific for only Omi and its PDZ domain, and no longer binds to L56.

Though extensive sequence analysis of HOPS was performed, not much information was obtained that would suggest a potential function for this protein. HOPS shares sequence similarity with only two other proteins. The first is a hypothetical protein from *Homo sapiens*, DKFZp564I0422, and second is the protein RIKEN of *Mus musculus*; each contains only 31% and 48% sequence similarity, respectively. Analysis using PSORTII computer software suggested that the localization of HOPS was either nuclear (39%) or mitochondrial (30%). However, when transfected in HeLa cells the GFP-HOPS fusion proteins was found predominantly in the cytoplasm.

HOPS could be involved in the normal function of Omi under two different scenarios. In the first, HOPS could be a physiological substrate of Omi as is the case with XIAP, cIAP, HAX1. In the second, like the Presenilin 1, HOPS may regulate the serine protease activity of Omi. A degradation assay was performed to test the possibility that HOPS might be a substrate

for the Omi. Mature Omi was incubated with [S^{35}]-HOPS giving rise to one major cleavage product. This suggested the possibility that HOPS could indeed be a substrate of Omi.

Since the *in vitro* study revealed the possibility for cleavage, an *in vivo* experiment was designed to further confirm these results. GFP-HOPS was transfected in 293T cells and the cells were then treated with apoptosis-inducing agents including H_2O_2 and cisplatin. In accord with the *in vitro* degradation assay, a decrease in the amount of HOPS protein was observed during cisplatin-induced apoptosis. These results suggest that HOPS could be a physiological substrate of Omi that is cleaved and removed during the apoptotic process.

Both Mxi2 and Presenilin 1 have been shown to interact specifically with the PDZ domain of Omi. PDZ domains are known for their ability to recognize the carboxy-terminus of proteins. Mxi2 ends in the amino acids LTIYPHLMDIELVMI while Presenilin 1 ends with VQPFMDQLAFHQFYI. HOPS has sequence homology with both of these proteins as it ends in the following sequence KIIENHFTTYEVTMI. To ascertain whether or not HOPS alters the proteolytic activity of Omi, recombinant carboxy-terminal HOPS was purified from bacteria and used in an enzymatic activity assay. This recombinant HOPS protein encompassed only the last 100 amino acids. When expressed in bacteria, full length HOPS was insoluble, and therefore could not be purified using non-denaturing conditions. When the C-terminal HOPS was incubated with mature Omi in an *in vitro* assay, no change in the proteolytic activity of the enzyme was observed. A generic substrate, β -casein, was used in these experiments.

In conclusion, Omi has been shown to interact with a variety of proteins in mammalian cells. These proteins can be substrates or regulators of Omi's proteolytic activity. The isolation of HOPS brings us closer to understanding the mechanism of Omi's normal

function. Since the amino acid sequence of HOPS suggests little about its role in the cells, it is hard to speculate how its degradation and removal by Omi assists the apoptotic process. Further experiments currently under way in our lab will be used to provide further details on HOPS and its regulation during cell death.

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