

NOVEL PROTEIN-PROTEIN INTERACTIONS REGULATE THE  
PROTEOLYTIC ACTIVITY OF THE PRO- APOPTOTIC SERINE  
PROTEASE, OMI/HTRA2

by

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## ABSTRACT

Omi/HtrA2 is a mitochondrial serine protease with high homology to the bacterial HtrA proteins. Omi promotes caspase-dependent apoptosis by binding and degrading IAPs-inhibitor of apoptosis proteins. Omi can also induce caspase-independent apoptosis but the actual mechanism is still unknown.

IAP's are not the only substrates cleaved by Omi. There are at least two more known substrates of Omi, the HAX-1 and the ped/pea-15 proteins. HS1-associated protein X-1 (HAX-1) is a mitochondrial protein, degraded by Omi after induction of caspase-dependent apoptosis. Ped/pea-15 is also an anti-apoptotic protein and is cleaved by Omi after induction of caspase-independent apoptosis.

The proteolytic activity of Omi is necessary and essential for its pro-apoptotic function. Recent studies suggest the proteolytic activity of Omi is regulated by specific protein-protein interactions. Presenilin was identified to be such a regulator of Omi. It binds to the PDZ domain of Omi via its carboxy-terminus and this interaction significantly increases the proteolytic activity of the enzyme.

My project was aimed to investigate the normal function of Omi in cell death and the mechanism of its regulation by isolating and characterizing novel Omi interactors. I screened a human melanocyte cDNA library using the yeast-two-hybrid system and Omi as the "bait" protein. Human Rad21 protein was isolated as a specific novel interactor of Omi. Human Rad21 interacted with the PDZ domain of Omi, the part of the protein

known to be involved in protein-protein interactions. Human Rad21 is a nuclear protein that plays a role in DNA double-strand break repair and sister chromatid cohesion during metaphase. Several reports suggest hRad21 has also a role in apoptosis; it is cleaved by caspase-3 and part of the protein becomes cytoplasmic. Human Rad21 was not cleaved by Omi in vitro and therefore it is unlikely to be a substrate. When tested in a proteolytic assay Rad21 was able to increase the proteolytic activity of Omi.

My work suggests a new mechanism whereby Omi and hRad21 can co-operate to induce cell death. This mechanism necessitates direct interaction of hRad21 with the PDZ domain of Omi resulting in increased proteolytic activity of the enzyme.

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## LIST OF ABBREVIATIONS

|                |  |
|----------------|--|
| AIF.....       | Apoptosis-Inducing Factor                                  |
| Apaf-1.....    | Apoptotic-Protease Activating Factor-1                     |
| BIR2/BIR3..... | Baculovirus 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                     |
| GFP.....       | Green Fluorescent Protein                                  |
| HtrA.....      | High Temperature Requirement A                             |
| HIS.....       | Histidine  |
| IAP.....       | Inhibitor of Apoptosis Protein                             |
| IBM.....       | IAP Binding Motif  |
| LEU.....       | Leucine  |
| Mnd2.....      | Motor Neuron Degeneration 2                                |
| MTS.....       | Mitochondrial Targeting Sequence                           |
| SDS-PAGE.....  | Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis |
| SH3.....       | Src-homology 3   |
| Smac.....      | Second mitochondria-derived activator of caspases          |
| TNF.....       | Tumor Necrosis Factor                                      |
| X-Gal.....     | 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside   |
| XIAP.....      | X-linked Inhibitor of Apoptosis Protein                    |

# CHAPTER ONE: INTRODUCTION AND REVIEW OF LITERATURE

## 1.1 Cell Death-Apoptosis, Necrosis and Autophagy

Cell death is an important mechanism used by multicellular organisms to replenish old cells, as well as remove damaged or transformed cells. It is also known as "Apoptosis" or programmed cell death where a cell has control over its demise to guard against the harmful effects of unwanted cells on the organisms, therefore keeping up the healthy state. There are many factors able to induce cell death including external factors like oxidative stress, excitotoxins, internal factors like accumulation of mutated proteins and byproducts of cell metabolism, cell signals like increased mitochondrial permeability that causes release of various pro-apoptotic proteins, and many other ligand-receptors mediated signals (1-5).

John Kerr and colleagues first defined "apoptosis", in 1972, as a different process of cell death apart from necrosis (4). This was shown in nematode, *Ceanorhabditis elegans*, that 131 of 1090 cells are lost through the mechanism of programmed cell death to form an adult worm (6). The specific proteins were later identified and called pro-apoptotic proteins, which play a major role in cell death of the nematode. Examples of such pro-apoptotic proteins in nematode include ced-3 and ced-4. These gene products share homology with other genes in different species, suggesting the mechanism of cell death (apoptosis) is highly conserved (7, 8).

There are four main steps in cell death: initiation, execution, disintegration and elimination. Apoptotic cells have characteristic features like cell shrinkage, chromatin

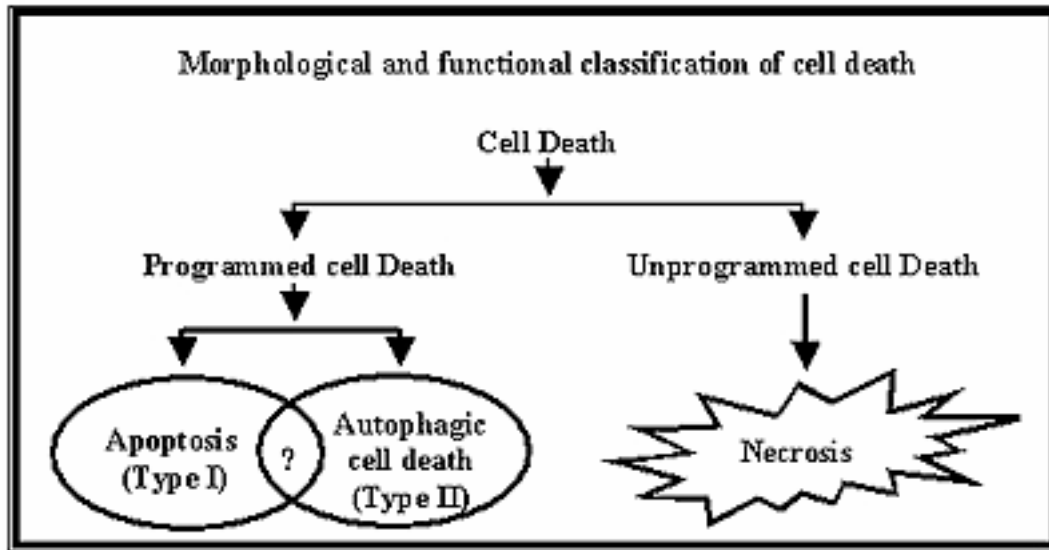
condensation, DNA fragmentation and blebbing of the cell membrane. The cell finally breaks into smaller pieces called apoptotic bodies that macrophages and neighboring cells recognize and phagocytose them. There is no spill of any cytoplasmic content of dying cell, which may cause inflammatory response (3, 7).

**Necrosis**, on the other hand is caused by physical disruption of the cell through injury, bacterial toxins, or nutritional deprivation. The cell loses functional control and osmotic pressure causing all of the organelles to swell, condensation of chromatin and finally cell bursts that release cytosolic components to the surroundings therefore mounting an inflammatory response that causes damage to surrounding tissues (9).

There is another type of cell death, which has gained a lot of attention recently and has not been characterized fully but is thought to play a major role in maintaining homeostasis of cells. This type of cell death is known as "**Autophagy**" or programmed cell death type-2. Autophagy is independent of caspase activation and does not lead to an inflammatory reaction (10-12).

Deregulation of cell death (excessive or too little cell death) can be harmful to an organism. When cell death occurs in non-dividing cells like neurons it can cause various diseases of nervous system including Parkinson's and Alzheimer's disease. Failure of cells to die can lead to autoimmune diseases, and resistance of some tumors to radiation or chemotherapy (13). There is evidence suggesting that these different types of cell death, despite being morphologically different, they overlap and merge into the same

pathway (Figure 1). This overlap can be clearly seen in neurodegenerative diseases and in virally infected cells (14).



**Figure 1: General Classification of Cell Death**

The cell can undergo demise either through programmed or unprogrammed way. Programmed cell death is subdivided in two types: Apoptosis and Autophagy with undistinguishable overlap in between them (12). Necrosis is another type of cell death where cells get badly injured and burst.

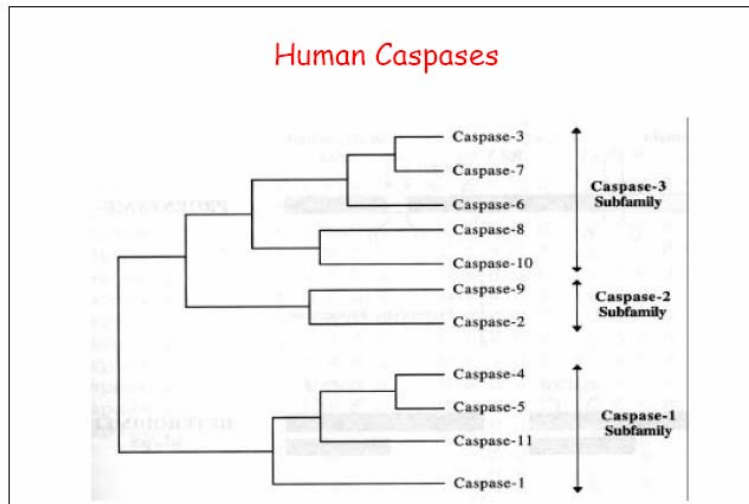
## 1.2 Caspases-Mediators of Apoptosis

Apoptosis, most of the time, is mediated through mitochondria that release different proteins upon induction of cell death. The most common proteins released are members of the Bcl-2 family of proapoptotic and anti-apoptotic proteins, and is the balance between these proteins that decides whether a cell dies or survives. The actual execution of a cell occurs through activation of proteolytic enzymes commonly known as caspases (15). Caspases are cell death proteases and they are conserved in all animals (16, 17). The name caspases itself denotes their function: Cystein-dependent ASpartyl-specific proteASE. Caspases are present in every cell of the body as inactive precursors called zymogens (18). After the cell receives a death signal either from extrinsic or intrinsic stress, caspases become activated and execute the cell in a highly regulated fashion. Caspases act on a variety of cellular proteins including: signal transduction proteins, cytoskeleton and nuclear proteins, chromatin-modifying proteins, DNA repair proteins and endonucleases to direct the cell for its demise by destroying cell-supporting proteins (19). Caspases also have other functions apart from apoptosis, such as caspases-1 (interleukin-1 beta convertase) which is involved in inflammation.

There are two types of caspases: initiator caspases and executioner (effector) caspases (20). The upstream caspase (initiator caspase) are activated by a death signal. Then interaction with adaptor proteins, activates the effector caspases (executioner caspase), which actually act as proteases to destroy cellular proteins. Initiator caspases include caspase -8, -9, -10 and effector caspases are comprised of caspase-3, -6, and -7. They are



also divided into subfamilies: caspase-3 subfamily, caspase-2 subfamily and caspase-1 subfamily (Figure 2), (6, 21).



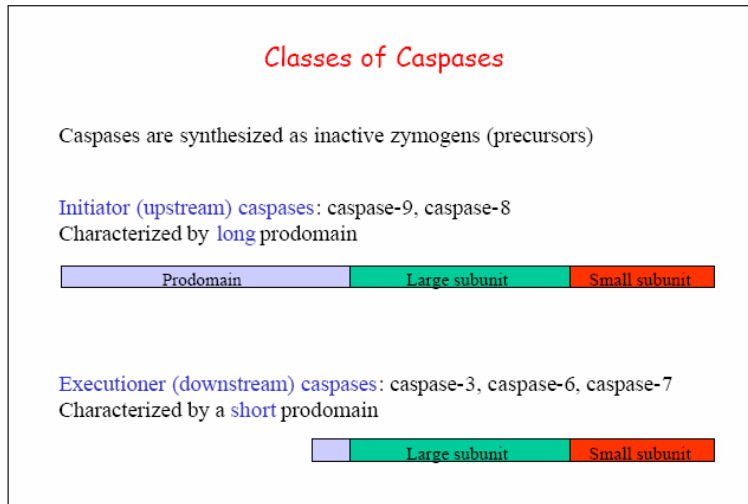
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**Figure 2: Major subfamilies of Caspases**

Caspases are subdivided into three subfamilies; caspase-1 subfamily, caspase-2 subfamily (include initiator caspases) and caspase-3 subfamily (include effector caspases).

[http://gsbs.uth.tmc.edu/courses/eukaryotic\\_gene\\_expression/Bergmann%20Lecture%201.pdf](http://gsbs.uth.tmc.edu/courses/eukaryotic_gene_expression/Bergmann%20Lecture%201.pdf)

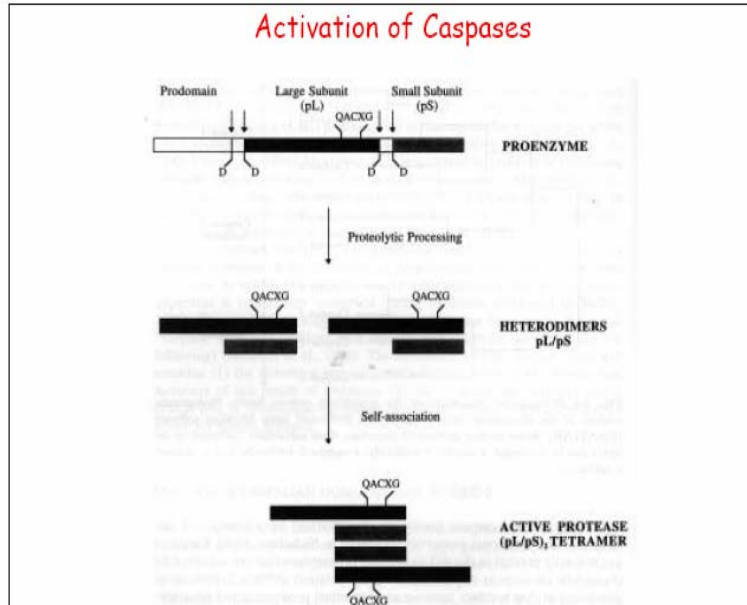
Structurally all zymogens/pro-caspases has a similar protease domain, which is a typical motif of this family of proteases. This domain is divided into three subunits: large subunit of 20kDa, small subunit of 10kDa and N- terminal peptide or a pro-domain of variable length. Initiator pro-caspases and inflammatory caspases contain a bigger pro-domain of more than 100 amino acids, while effector caspases have a shorter pro-domain of less than 30 amino acids (Figure 3), (22). These zymogens or pro-caspases remain inactive in a cell until they are separated by proteolytic cleavage. Following this cleavage, heterodimeric association of the large and small subunits occurs to form a tetramer, thus an active caspase (Figure 4), (18, 22). The long pro-domains contain a distinct motif called death effector domain (DED) and caspase activation and requirement domain (CARD). Procaspase-8, -10 contain two copies of DED, whereas the CARD domain is found in caspase-2, -4, -5, -9 and also seen in ced-3. These DED and CARD are similar to the motif DD (death domain) found in cell death adapter proteins that participates in protein-protein interaction. Association of CARD-CARD and DD-DD domain is mediated through charge-charge interactions while DED-DED is through hydrophobic interactions (22).



**Figure 3: Different domains of Caspases**

Initiator caspases are characterized by long prodomains and effector (executioner) caspases are characterized by shorter prodomains. Caspases with the prodomains are inactive in the cell. These prodomains need to be cleaved out for the caspases in order to become active.

[http://gsbs.uth.tmc.edu/courses/eukaryotic\\_gene\\_expression/Bergmann%20Lecture%201.pdf](http://gsbs.uth.tmc.edu/courses/eukaryotic_gene_expression/Bergmann%20Lecture%201.pdf)



**Figure 4: Active state of caspases**

After the prodomain is removed, the large and small subunits form the dimer. Finally, by self-associating the dimer forms the tetramer or active caspase.

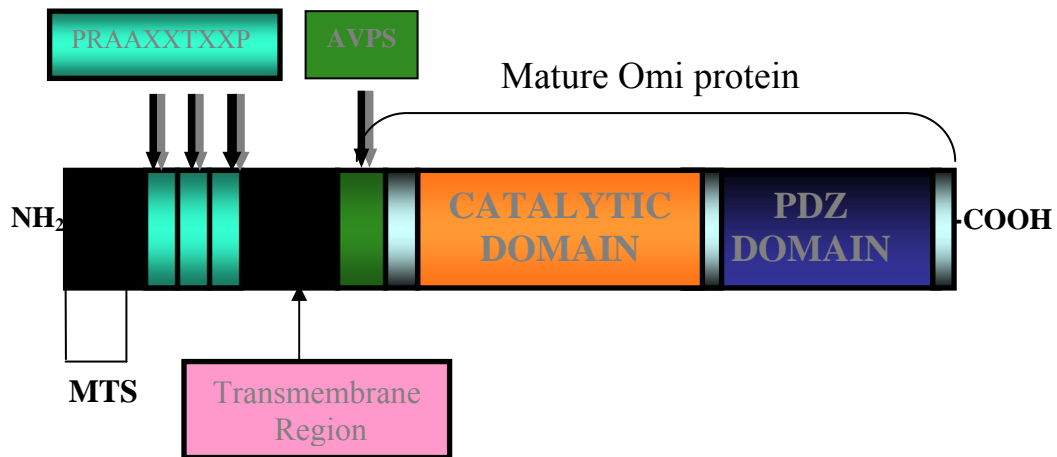
[http://gsbs.uth.tmc.edu/courses/eukaryotic\\_gene\\_expression/Bergmann%20Lecture%201.pdf](http://gsbs.uth.tmc.edu/courses/eukaryotic_gene_expression/Bergmann%20Lecture%201.pdf)

### 1.3 Isolation and Characterization of Omi protein

Omi was originally isolated as an interactor of Mxi2, an alternatively spliced form of p38 stress activated kinase using the yeast-two-hybrid system (23, 24). Omi is a serine protease with high homology to the bacterial HtrA proteins. HtrAs or high temperature requirement A is family of serine proteases, which are conserved from bacteria to humans (25). Their major function is to act as chaperons at lower temperature, but at higher temperatures they act as proteases to remove denatured or damaged proteins (25). The Omi protein also has homology to L56, a human homolog of bacterial HtrA1 (26).

#### 1.3a Sequence, Domains, Localization and Processing

Omi is expressed ubiquitously and the gene is localized on human chromosome 2p12. The protein consists of 458 amino acids has a molecular weight of 50kD and is present in the inter-membrane space of mitochondria (Figure 7). The first 60 amino acids represent a mitochondrial targeting sequence (MTS), which gets cleaved soon after the protein enters mitochondria. Omi has two other major domains: a catalytic domain and PDZ domain (Figure 5).

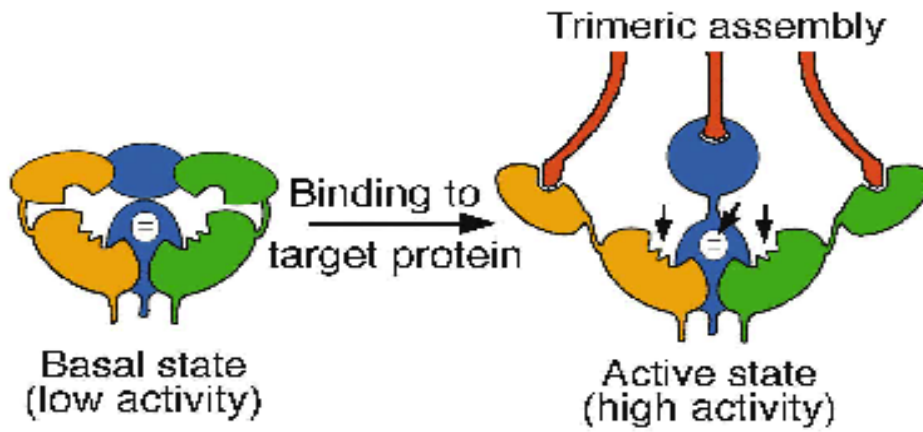


**Figure 5: Different Domains of Omi Protein**

The N-terminus of Omi protein has mitochondrial targeting sequence, which is removed as soon as it enters mitochondria. This is followed by three PRAAX repeats of unknown function and then the transmembrane region. The mature form of Omi protein (cytosolic form) starts from the "AVPS" motif (binds to IAPs) followed by catalytic and PDZ domains (26, 27).

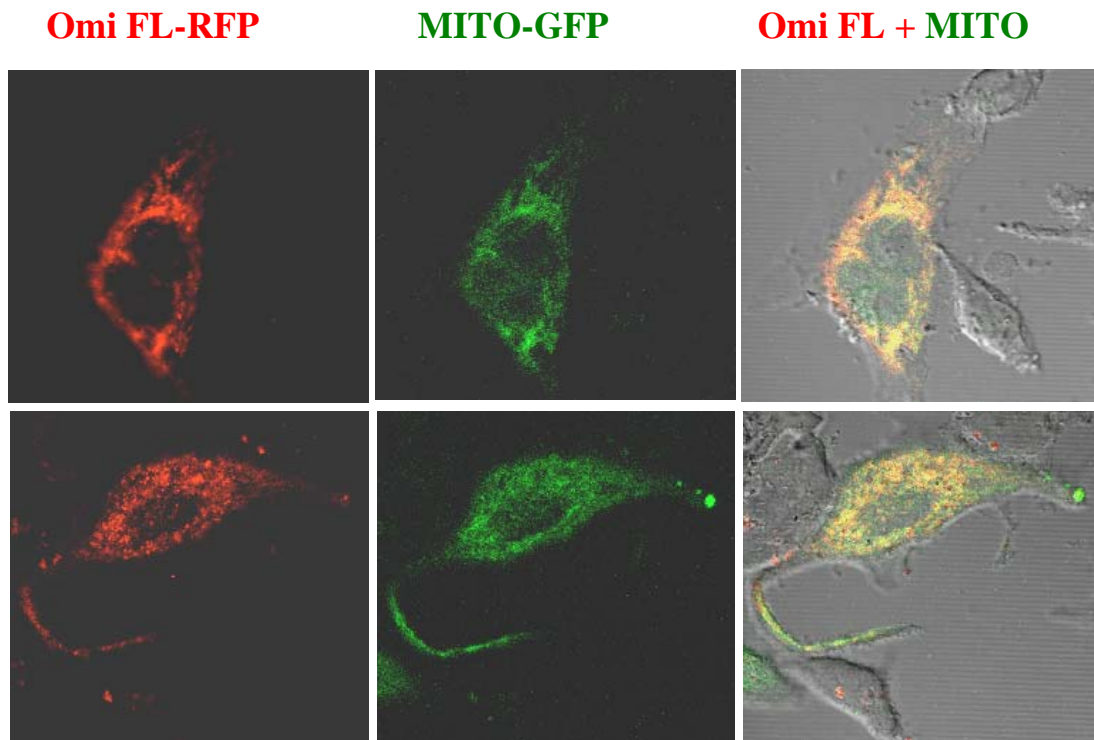
The N-terminus of the protein has three PRAAXTXXTP sequence repeats, whose function is unknown, a transmembrane region (105-125), a phosphorylation site (SPRS) and a SH3-binding domain (PPPASPR) (26, 28). The serine protease's catalytic domain (182-330) consist of the catalytic triad of His (198), Asp (228) and Ser (306), similar to the catalytic triad found in trypsin protease. Lastly, the PDZ domain (approx. 90 amino acids), which plays a major role in protein-protein interactions and has high affinity for the proteins with hydrophobic C-terminus (29). Trimerization of the Omi/HtrA2 is necessary for its protease/ pro-apoptotic activity. This trimerization generates a pyramid structure housing the IBM sequence at the top and the PDZ domain at the bottom. A model, proposed by Li et al (2002), suggest that perhaps binding of a protein or peptide to the PDZ domain may cause conformational changes in the trimer pyramidal structure and open up the catalytic site to act as protease (Figure 6), (29).





**Figure 6: Trimeric Structure of Omi.**

The PDZ domains in the trimeric form of the Omi remain in closed confirmation and open up after binding of the substrate or any interacting protein/peptide which will expose the catalytic site (29).

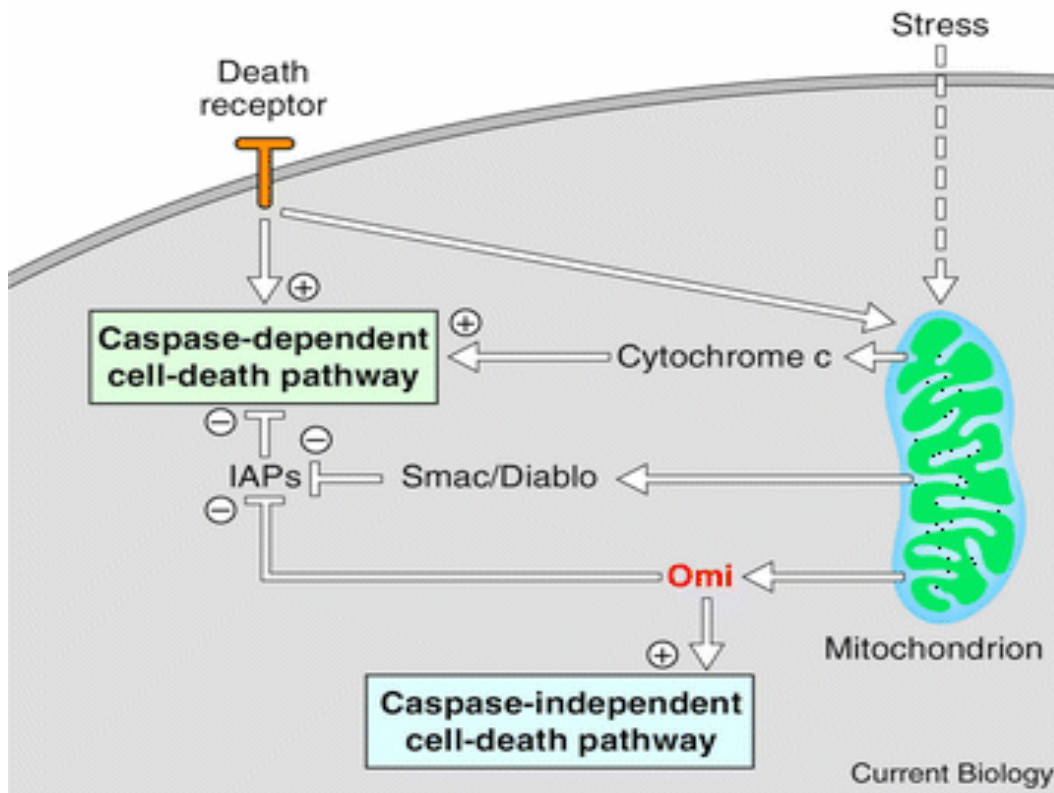


**Figure 7: Mitochondrial localization of Omi protein.**

Omi protein is localized in mitochondria and the signal peptide is removed as soon as it enters mitochondria. The picture shows the co-localization of Omi-RFP and Mitochondrial targeting sequence-GFP in the HeLa cells (L. Cilenti unpublished).

#### 1.4 Omi in Apoptosis

Omi resides in the intermembrane space of mitochondria, but it has been shown that upon apoptotic stimuli it is released in the cytoplasm. The first 133 amino acids of the N-terminus are removed or autocatalytically processed to form the mature Omi of 36kD. Omi promotes apoptosis by binding to the IAPs, the inhibitor of apoptosis proteins (Figure 8), (27). IAPs directly bind and inhibit the activity of the caspases through their baculovirus IAP repeat (BIR) domains (30). This binding to the IAPs is similar to Smac/DIABLO binding that occurs in mammalian cells or Reaper-like proteins (Reaper, Grim and Hid) in *Drosophila melanogaster* (31). The binding to X-IAP (mammalian X linked IAP) is mediated specifically through "AVPS" motif seen in all of these pro-apoptotic proteins (27, 32, 33). Thus, Omi activates the caspases by cleaving the IAPs resulting in the caspase-dependent pathway of cell death. Omi can also induce caspase-independent pathway of cell death through its serine protease's activity, but the actual mechanism is still unknown (Figure 8), (34).



**Figure 8: Omi in Caspase-dependent and Caspase-independent Cell Death.**

Omi is released in the cytoplasm after mitochondrial stress and induces apoptosis in two ways: caspase-dependent and caspase-independent. Omi is known to inhibit and degrade IAPs (the inhibitor of apoptosis proteins), which release caspases and induce caspase-dependent cell death (27, 31). Omi is also known to act through its protease activity to induce caspase-independent cell death, but the actual mechanism is still unknown (32, 33, 35).

### 1.5 Substrates and Regulators of Omi

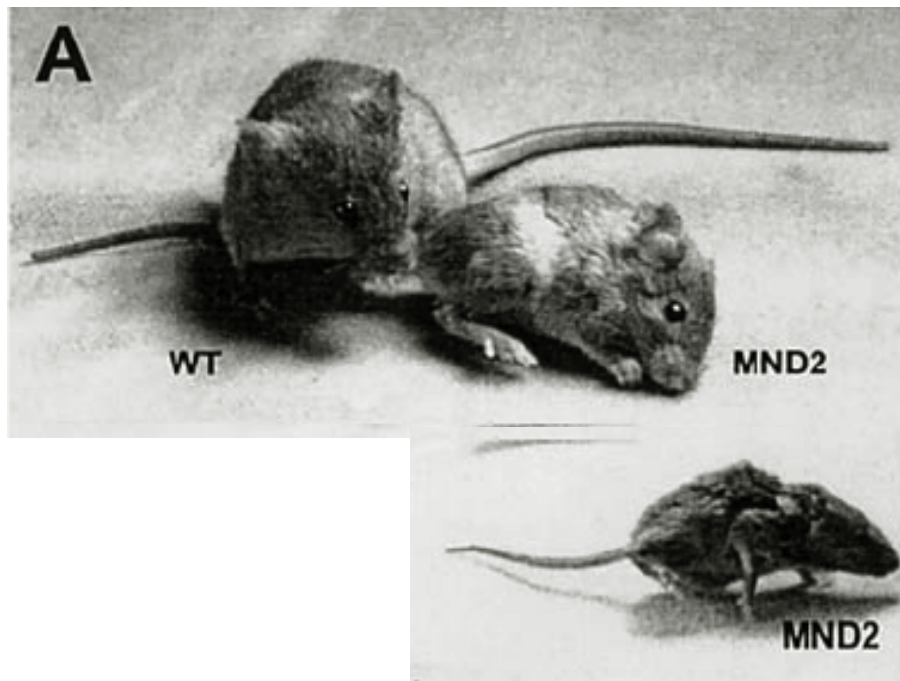
IAPs the inhibitor of apoptosis proteins, are the major substrates for Omi (27). After mitochondrial stress the mature form of Omi is released in the cytoplasm and through its N-terminus AVPS motif binds to X-IAP and degrades it. Similar to IAPs, there are other anti-apoptotic proteins degraded by Omi upon induction of apoptosis (27, 28, 31, 33). These proteins are both mitochondrial and cytoplasmic. A mitochondrial anti-apoptotic protein found to be degraded by Omi before it comes out in the cytoplasm is HS1 associated protein X-1 (HAX-1). HAX-1 belongs to Bcl-2 family of proteins. This protein binds both to PDZ and catalytic domains of Omi. Levels of HAX-1 decrease as the cell dies. Overexpression of HAX-1 protects cells against apoptosis (36).

A cytoplasmic anti-apoptotic protein ped/pea-15 also interacts specifically through its DED domain with mature form of Omi after induction of apoptosis. There is an inverse correlation between the ped/pea-15 level of protein and cell death. This mechanism may suggest a role of Omi in caspase-independent cell death (37).

Some proteins or peptide ligands regulate the protease activity of Omi by binding to the PDZ domain and not to the catalytic domain of Omi. One such peptide ligand is the C-terminus of Presenilin or PS1 protein that is implicated in Alzheimer's disease. The last 15 amino acids (a synthetic peptide) of PS1 bind the PDZ domain of Omi and open up the protease pocket of the trimeric form of Omi allowing access to the substrates (38).

### 1.6 Omi as a potential mitochondrial chaperon

It is now well established that Omi is involved in apoptosis following cellular stress leading to its release from mitochondria (27, 28, 39). In the cytosol Omi binds and degrades IAPs to activate caspases that in turn induce cell death. There is also evidence suggesting yet another role for Omi as a potential mitochondria chaperone (40, 41). This information is derived from studies on a mouse mutant *mnd2* (motor neuron degeneration 2). The *mnd2* mouse was described in 1990 as a spontaneous, recessively inherited mutation that came up on the C57BL/6J inbred background (40). The mutation is a missense mutation of Ser276Cys in the protease domain of Omi and very close to serine 306, the active serine. This mutation greatly reduces the proteolytic activity of Omi in the tissues of *mnd2* mice. The earliest symptoms seen in *mnd2* mice are altered gait and cessation of normal weight gain, followed by ataxia, repetitive movements and akinesia when compared to the normal mice with normal growth (Figure 9). Striatal neurons are damaged including the neurons in the central nervous system, brain stem, spinal cord and motor neurons are damaged at later stages. Early degeneration of mitochondria, apoptosis and necrosis are the noted features of the neuronal degeneration. The *mnd2* mice die within 40 days. The size and steady state of Omi mRNA is the same in the tissue of these mice when compare to wild type. Even though the molecular weight and protein concentration remains the same. Only the protease activity of Omi is affected. Thus, loss of protease activity of Omi seen in *mnd2* mice could possibly lead to accumulation of misfolded and damaged proteins in the mitochondria, increased sensitivity to induction of permeability transition and finally mitochondrial dysfunction leading to cell death (40).



**Figure 9: Phenotypic Comparison of WT and Mnd2 Mice**

In comparison to the wild type, the Mnd2 mice have severe symptoms of weight loss, organ size reduction, loss of striatal neurons and other neurons of the central nervous system. The Mnd2 phenotype is caused by the mutation of S267C in the catalytic domain. This mutation leads to the loss of protease activity of Omi thus accumulation of many misfolded and mutated proteins in the mitochondria. This suggests the role of Omi as an important mitochondrial chaperon (40).

The importance of Omi in maintaining the mitochondrial homeostasis is further demonstrated in mice where the Omi gene is knocked-out (KO) using homologous recombination. These mice show similar phenotype like the *mnd2* mice. Major symptoms in *Omi*<sup>-/-</sup> include loss of striatal neurons, loss in weight, reduction in organ size, movement disorder and several neurological abnormalities when compared to their wild or heterozygous littermates (42). Thus the data could be explained to show two different functions of Omi: under normal physiological conditions it is needed to maintain the homeostasis within the mitochondria and other during apoptosis it is released from the mitochondria to the cytoplasm and act as pro-apoptotic protein (41).



## CHAPTER TWO: MATERIALS AND METHODS

### 2.1 General cloning techniques

#### 2.1a Polymerase Chain Reaction

The required restriction sites are incorporated into forward and reverse primers. Each PCR reaction is performed in 50  $\mu$ l volume using 20ng of template DNA, 10 picoM of each primer, 5  $\mu$ l of 10X PCR buffer +  $Mg^{2+}$  (Roche Diagnostics Corp.), 1.5 units of Taq polymerase (Roche Diagnostics Corp.), 100 mM dNTPs (BD Biosciences). The reaction is placed in a thermal cycler and after an initial denaturing at 95 °C for two minutes is passed through the following cycle 30 times: 95 °C denature for 30s, variable annealing temperature (based on primer) for 30 sec, 72 °C elongation for a variable length of time based on size of PCR product. This followed by a final 7 minutes elongation step at 72 °C. Each PCR reaction is performed in duplicates, the products of the reactions are then combined and ethanol precipitated.

#### 2.1b 1% Agarose gel electrophoresis

1.0 g of dried agarose (FMC BioProducts) is added to 100 ml of 1X TAE (0.04M Tris-acetate, 0.001M EDTA). The mixture is then microwaved for approximately 2 minutes or until the agarose is completely dissolved. The volume is then brought up to 100 ml using water and the solution is left to cool down. Ethidium Bromide is then added to a final concentration of 0.5  $\mu$ g/ml and the solution is poured into a gel tray. When the gel is solidified, the combs are removed. Gel is run in a 1X TAE electrophoresis buffer into

the tray. To prepare the samples for electrophoresis, 5  $\mu$ l-10  $\mu$ l of 10X gel loading buffer made of 30% glycerol (Fisher), 2% Orange-G (Sigma) dissolved in 1X TAE, is added to DNA. Samples are loaded in the wells and electrophoresis is performed at 50-140 V until the dye reached the appropriate distance. DNA markers like Bioline-40 ng/ $\mu$ l, Lambda DNA BstEII digest or pEMBL DNA aTaqI digest are used to determine the size of the sample DNA.

### 2.1c Restriction enzyme digestion

DNA is incubated with the required restriction enzyme and its respective buffer as suggested by the supplier. The reaction is incubated at 37 °C (or else specified for the enzyme) for 1-4 hours depending on the enzyme efficiency. For two sequential digestions, DNA is precipitated after first digestion and then resuspended in the second digestion mixture. A general protocol for a 50  $\mu$ l reaction volume is as follows:

5  $\mu$ l     Restriction enzyme 10X Buffer

0.5  $\mu$ l   BSA, 10  $\mu$ g/ $\mu$ l

1  $\mu$ l     DNA, 1  $\mu$ g/ $\mu$ l

42  $\mu$ l    dH<sub>2</sub>O, sterile

1.5  $\mu$ l   Restriction Enzyme, 10 U/ $\mu$ l

### 2.1d Ligation

DNA concentration of insert and vector is estimated using DNA gel electrophoresis. Ligation is done using the gel purified insert and vector DNA after the RE digestion by the Fast-Link DNA Ligation Kit (Epicentre Technologies)).

In general, the molar concentration of insert to vector should be around 3:1. A protocol for a typical ligation reaction is as follows:

1.5  $\mu$ l Fast-Link Ligation Buffer

1.5  $\mu$ l 10 mM ATP

1  $\mu$ l Fast-Link DNA Ligase

x  $\mu$ l insert

x  $\mu$ l vector

x  $\mu$ l dH<sub>2</sub>O

---

15  $\mu$ l Final volume

The mix is then incubated at room temperature for 5 minutes, followed by deactivation of ligase for 15 minutes at 70 °C. It is precipitated by making the volume to 50  $\mu$ l using dH<sub>2</sub>O and 500  $\mu$ l of N-butanol. Samples are then centrifuged for 10 minutes at 4 °C at 14000rpm. The supernatant is discarded and samples are dried in a speed vacuum for 10-12 minutes. The pellet is resuspended in 4-6  $\mu$ l of dH<sub>2</sub>O.

### 2.1e Bacterial transformation

The DNA to be transformed is added to 100  $\mu$ l aliquot of competent bacterial cells (DH5 $\alpha$ , BL21 or KC8 cells), mixed gently, and transferred to an electroporation cuvette (0.2cm electrode gap, BIO-RAD). The cuvette is placed in a Gebe Pulser (BioRad) that delivers a single exponential decay pulse of 2.5 V. After the shock, 900  $\mu$ l of room temperature LB medium without any antibiotic is added to it. The entire solution is collected in a 15 ml falcon tube and incubated at 37 °C with shaking for 45 minutes.

150-300  $\mu$ l of solution is then plated on agar plates with appropriate antibiotic and incubated overnight at 37 °C.

#### 2.1f Isolation of Plasmid DNA from Bacteria: DNA Qiaprep Spin Miniprep

Plasmid DNA is isolated using QIAprep Spin Miniprep Kit according to the manufacturers' instructions (QIAGEN). A single bacterial colony is grown overnight with shaking in 3-4 ml of LB medium with antibiotic. The bacterial culture is collected in eppendorf tube and spun down for 2 minutes at 14000 rpm. The supernatant is aspirated out. The pellet is resuspended in 200  $\mu$ l of Resuspension Buffer P1, containing RNase (100 mg/ml). Then 200  $\mu$ l of Lysis Buffer P2 is added and the tube is inverted 5-6 times gently until the solution becomes transparent. The lysate is neutralized and adjusted to high-salt binding conditions by adding 300  $\mu$ l of Neutralization Buffer N3. The tube is immediately and gently inverted 4-6 times, until a white cloudy precipitation is formed. Centrifuge for another 10 minutes at 14000 rpm. The supernatant is then transferred to a QIAprep spin column, which is resting on a 2 ml collection tube. The column is centrifuged for 1 minute and the flow-through is discarded. The column is washed with 500  $\mu$ l of Buffer PB, spun for 1 minute, and the flow-through is discarded. The column is again washed with 750  $\mu$ l of Buffer PE, spun for 1 minute and discard the flow-through. The residual wash buffer is removed by an additional spin for 1 minute. The QIAprep spin column is then placed in a clean 1.5 ml microcentrifuge tube. 50  $\mu$ l of Elution buffer is added, allowed to bind for 1 min, centrifuge the tube for 1 minute to collect the DNA. The concentration of DNA is estimated using a spectrophotometer.

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

A single bacterial colony is grown overnight with shaking in 3 ml LB broth medium containing antibiotic. The culture is then collected in 1.5 ml eppendorf tube, spin down for 2 minutes. The supernatant is aspirated and the pellet is resuspended in 300  $\mu$ l of STET (8% Sucrose, 5% 100X Triton, 50 mM Tris-HCL pH 8.0 and 50 mM EDTA), Lysozyme and RNase. The sample is then boiled for 45 seconds and centrifuged for 15 minutes at 14000 rpm. The debris of cell wall and other cell contents are collected at the bottom and the supernatant contains the plasmid DNA. Using a toothpick, debris is removed and 230  $\mu$ l of room temperature Isopropanol is added to precipitate the plasmid DNA. The tube is spun for 10 minutes, the DNA forms pellet at the bottom, the supernatant is discarded and 70% room temperature ethanol is added to wash the pellet and spin for 5 minutes, discard the supernatant. The pellet is dried in a speed vacuum for 8-10 minutes. The pellet is resuspended in 30-50  $\mu$ l of water or TE.

## 2.1h DNA Sequence Analysis

CEQ 2000 Dye Terminator Cycle Sequencing was performed using the Quick Start Kit (Beckman). The reaction is performed in 0.2 ml thin-walled PCR tubes. All reagents are kept on ice while the sequencing reactions are prepared. Each reaction is performed in 10  $\mu$ l volume using the appropriate amount of template DNA (based on the size of the insert), required primer (3  $\mu$ M/ $\mu$ l) and dH<sub>2</sub>O (to adjust the volume to 10 $\mu$ l). The contents are mixed and then denatured for 2 minutes at 95 °C. Next, 3 $\mu$ l of DTCS Quick Start Master Mix is added. The reaction is placed in thermal cycler and the following cycle is

repeated 30 times: 96 °C denature for 30 seconds, annealing of primers for 30 seconds, elongation at 60 °C for 4 minutes. To stop the reaction 2.5 µl of Stop Solution (1.5 M NaOAc, 50 mM EDTA) is prepared fresh by mixing equal volumes of 3M NaOAc and 100mM EDTA) and 1 µl of 20 mg/ml glycogen (Beckman Coulter) is added. The reaction is then transferred to a 1.5 ml microcentrifuge tube and mixed thoroughly. Then 60 µl of 100% cold Ethanol is added. It is then centrifuged for 15 minutes at 14000 rpm in 4 °C. The supernatant is carefully removed using pipette. The pellet is washed with 400 µl of 70% cold Ethanol and spun for 5 minutes. The supernatant is removed and the pellet is dried for at least 20 minutes in a speed vacuum, and resuspended in 20 µl of Sample Loading Solution (Beckman Coulter). The reaction is now ready for sequencing.

## 2.2 Yeast Two-Hybrid Interaction

### 2.2a Standard Yeast Transformation

A single yeast colony is grown overnight in 50ml of appropriate medium at 30 °C. The cells are harvested by centrifugation at 3800 rpm for 5 minutes in a sterile 50 ml falcon tube. The supernatant is discarded and the pellet is washed with re-suspension in 10 ml of TE pH 7.5. Spin again at 3800 rpm for 5 minutes, discard the supernatant and re-suspend the pellet in 5ml LA (0.1M LiOAc in TE). The cells are collected in 15ml falcon tube and solution is incubated with shaking for 2 hrs at 30 °C. Cells are again harvested by centrifugation at 3800 rpm for 5 minutes. The supernatant is discarded and resuspend the cells in variable amount of LA ( $\cong$  5 ml or depending on the size of pellet). 300  $\mu$ l of Yeast cells are then aliquoted in 1.5 ml eppendorf tubes along with 100  $\mu$ g of denatured salmon sperm DNA. To this, 700  $\mu$ l of 50% PEG4000 (diluted in dH<sub>2</sub>O) is added and mixed until homogeneous. The yeast cells are incubated for 30 minutes at 30 °C water bath, then for 15 minutes at 42 °C water bath. The cells are collected by centrifugation for 3 minutes at 14000 rpm. The supernatant is aspirated and the pellet resuspended in 300  $\mu$ l of TE pH 7.5. 50-150  $\mu$ l of this solution is plated on appropriate selective medium plate and incubated for 2-3 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 specifically prepared drop-out media. A liter of drop-out medium includes: 2.0 g appropriate "drop-out" powder (lacking the appropriate amino acids), 6.7 g Yeast Nitrogen Base without amino acids (Sigma) and 900 ml of sterile water, for yeast plates add 20 g of Agar (USB). The solution is autoclaved and 2% weight-by-volume carbon source is added (Glucose or Galactose).

## 2.2c Yeast Western Blot

The yeast to be tested is grown in 3ml of appropriate medium overnight. Next day, yeast culture is divided into two eppendorf tubes. The culture is spun down and the supernatant is aspirated. Then yeast pellet is resuspended into different medium, one with glucose for negative control and one pellet with galactose for inducing the galactose promoter. Incubate yeast for 4-6 hrs. The yeast are collected by centrifugation for 3 minutes, and the supernatant is aspirated. To lyse the yeast cells, add 40  $\mu$ l of 1X SDS-sample loading buffer (10%  $\beta$ -mercaptoethanol, 6% SDS, 20% glycerol, 1/40X stacking buffer and 0.2 mg/ml bromophenol blue) and 40  $\mu$ l of TE. Samples are vortexed and placed on ice. The samples are boiled for 5 minutes along with pre-stained protein marker (BioRad) and then spun down to remove cell debris. 15-20  $\mu$ l of samples is loaded along with 10  $\mu$ l of marker on a pre-cast gradient (4-20% Tris-Glycine) gel (Invitrogen). The protein is then transferred to a PVDF membrane by semi-dry electrophoresis using a BioRad transfer blot. For proper transfer, PVDF membrane

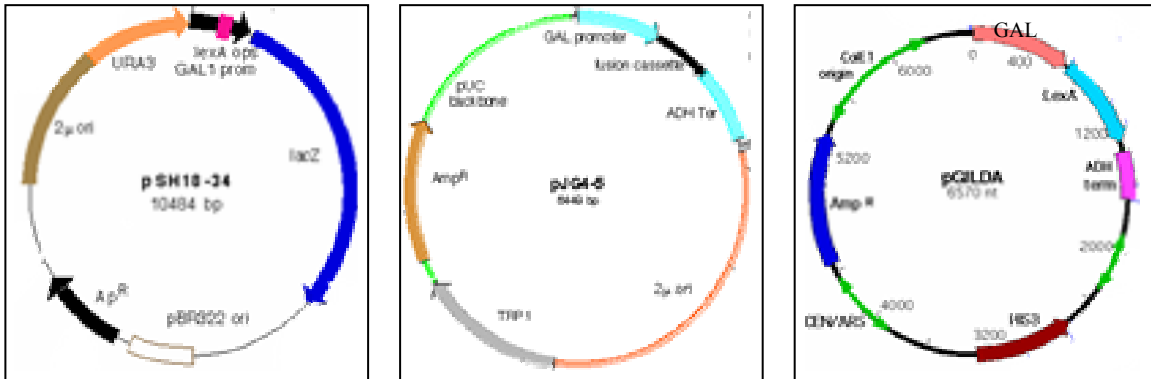


(PALL) is first activated in 20 ml of methanol (CH<sub>3</sub>OH). Then gel, membrane and two filter papers are equilibrated in 100ml transfer buffer (9.6 mM Tris, 7.8 mM Glycine and 20% CH<sub>3</sub>OH to a final pH 9.0) for 5 minutes. The semi-dry transfer is arranged by putting the filter paper on bottom, followed by gel then membrane and the filter paper on top. Push out any air that is trapped inside, and transfer the protein for atleast 45 minutes at 15 V. In order to block any non-specific binding, the PVDF membrane is incubated in 2% non fat milk solution dissolved in TBST (250 mM Tris pH8, 1.25 M NaCl and 0.1% Tween20) for at least 2 hrs. The membrane is incubated overnight with primary antibody  $\alpha$ -LexA-HRP (for "bait" proteins)(Santa Cruz) or  $\alpha$ -HA (for "prey" proteins)(Sigma), diluted 1:1000 or 1:5000 respectively in 2% milk. Then membrane is washed 5 times with TBST before putting it into secondary antibody. For "prey" proteins, secondary antibody used is Goat- $\alpha$ -Mouse diluted 1:2000 in 2% milk solution, and incubate the membrane for 1 hour. The membrane is washed 5 times with TBST and then 2-3 times with TBS (250 mM Tris pH8, 1.25 M NaCl). Proteins are detected using chemiluminescence ECL kit (PIERCE).

## 2.3 Screening Melanocyte cDNA Library

### 2.3a Yeast-two-hybrid screening

The yeast strain EGY48 is 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  $\beta$ -galactosidase. Two different shuttle vectors are used to clone the bait and prey. The melanocyte cDNA library is cloned in pJG4-5 (Zervos lab), which contains a galactose inducible promoter. All the bait proteins Omi (Zervos lab), Omi<sub>PDZ</sub> (Zervos lab), Omi<sub>CAT</sub>, and L56<sub>156-480</sub> were previously cloned into pGILDA, which also contains a galactose inducible promoter. Positive interactions between bait and prey proteins are detected by the presence of blue colonies when grown on U<sup>-</sup>H<sup>-</sup>W<sup>-</sup> GAL/Raf selective medium plates containing X-Gal.



**Figure 10: 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<sup>R</sup>).

A selectable marker is present, in order to select for each plasmid when grown in yeast.

URA3, TRP1, and HIS3 in pSH18-34, pJG4-5, and pGILDA.

<http://www.fccc.edu/research/labs/golemis/InteractionTrapInWork.html>.

### 2.3b High Efficiency Yeast Transformation

A single yeast colony of EGY48 pSH18-34 pGilda-Omi134-458, is picked and grown overnight at 30 °C with shaking in 50 ml of U<sup>H</sup> Glu drop out medium. The next day the culture is diluted to 300ml with the same medium and grown to an OD<sub>600 nm</sub> of 0.9. Cells are harvested by centrifugation at 3800 rpm for 5 minutes. The supernatant is discarded and the cells are washed with 20 ml of sterile water. The wash is repeated with water and cells are resuspended in 20 ml LA (0.1M LiOAc in TE), spun again for 5 minutes. Lastly cells are resuspended in 5ml of LA. 100 µl of competent yeast is aliquoted in eppendorf tube and following are added: 10 µg DNA (Melanocyte library in pJG4-5), 100 µg of denatured salmon sperm DNA (carrier DNA) and 600 µl of freshly prepared 40% PEG solution. The mix is resuspended until homogeneous. The transformation reaction is incubated at 30 °C for 30 minutes and then heat shocked at 42 °C for 15 minutes. Transformed yeast is centrifuged and the supernatant is aspirated. The pellet is resuspended in 500 µl of sterile water. Then 300 µl of resuspended yeast is plated on 22.5 x 22.5 cm (Nalge Nunc) U<sup>H</sup>W<sup>+</sup> Glucose selective plates and incubate at 30 °C for 2-3 days.

### 2.3c Aliquoting Library

The number of colonies in a 2 x 2cm square is counted on a sample plates. An average for the squares is obtained and multiplied with 126.6 (the number of 2 x 2cm squares in one large plate). This value is the average number of colonies per plate. The plates are then placed at 4 °C so that the agar will harden. Using autoclaved glass slides and aseptic

technique, the colonies from all the 10 big plates are scraped with the help of 20 ml of sterile water for each plate and collected into 50 ml falcon tubes. The yeast is centrifuged at 3800 rpm for 5 minutes. The supernatant is discarded and the cells are washed with 50 ml sterile water and spun again. The wash is repeated and the supernatant is discarded. Lastly, the pellet is resuspended in equal to the size of the pellet in filtered yeast freezing medium (65% glycerol, 0.1 M MgSO<sub>4</sub>, 25 mM Tris pH7.4). The mix is then aliquoted 300 µl into small centrifuge tubes and stored at -80 °C.

### 2.3d Titration

500 µl of thawed "frozen Melanocyte library" is grown in 4.5 ml (1:10 dilution) UHW 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 900 µl of sterile H<sub>2</sub>O → 1:100

Tube 2= 100 µl from Tube 1 in 900 µl of sterile H<sub>2</sub>O → 1:1,000

Tube 3= 100 µl from Tube 2 in 900 µl of sterile H<sub>2</sub>O → 1:10,000

Tube 4= 100 µl from Tube 3 in 900 µl of sterile H<sub>2</sub>O → 1:100,000

Tube 5= 100 µl from Tube 4 in 900 µl of sterile H<sub>2</sub>O → 1:1,000,000

100 µl from each dilution is then plated on UHW Gal/Raf plates and grown for 2-3 days at 30 °C. Then the colonies from plates 3-5 are counted and multiplied by the dilution factor. This is done to determine the amount needed to plate on a large UHW Gal/Raf plate in order to yield the approximate number of colonies that will represent the entire library. This extrapolated amount is plated on 24 x 24 cm (Nalge Nunc) UHW Gal/Raf plates for growth selection, and grows for 3-4 days at 30 °C.

### 2.3e Screening

After the selection is performed on U<sup>-</sup>H<sup>-</sup>W<sup>-</sup>L<sup>-</sup> Gal/Raf, the big colonies are picked with the help of toothpick and streaked on U<sup>-</sup>H<sup>-</sup>W<sup>-</sup>L<sup>-</sup> Gal/Raf, and let grow for another 2-3 days. The streaks are then transferred on U<sup>-</sup>H<sup>-</sup>W<sup>-</sup> Glu plates to have a master plate and grown for another 2 days. Next, the colonies are streaked onto U<sup>-</sup>H<sup>-</sup>W<sup>-</sup> Glu/X-Gal and U<sup>-</sup>H<sup>-</sup>W<sup>-</sup> Gal/Raf/X-Gal plates for color selection. The positive interaction between bait and prey is detected by blue colony on U<sup>-</sup>H<sup>-</sup>W<sup>-</sup> Gal/Raf/X-Gal plate and white colony on U<sup>-</sup>H<sup>-</sup>W<sup>-</sup> Glu/X-Gal plate. Corresponding blue colonies are picked from the U<sup>-</sup>H<sup>-</sup>W<sup>-</sup> Glu plate (master plate), and inoculated into W<sup>-</sup> Glucose medium to select for the pJG4-5 Melanocyte library plasmid and grown overnight at 30 °C with shaking.

### 2.3f Release of Plasmid from Yeast

The yeast culture grown in W<sup>-</sup> Glucose medium is transferred to a screw cap microcentrifuge tube. The yeast is centrifuged for 1min at 14000 rpm. The pellet is resuspended in 200 µl of yeast lysis solution (2% TritonX-100, 1% SDS, 100mM NaCl, 10mM Tris pH8.0, 1 mM EDTA). Approximately 0.3 g of glass beads (Sigma) is added to assist in the lysis along with 200 µl of Phenol-chloroform-isamyl alcohol (25:24:1). The tube is closed and vortexed vigorously for 2 minutes, or until the mixture become homogeneous. The tube is then centrifuged for 2 minutes to separate the phases, and the upper (where the actual plasmid will be) is collected to a new microcentrifuge tube. DNA is precipitated as described above.

### 2.3g Transformation of Plasmid into KC8

The plasmids extracted from the yeast are transformed into KC8 bacterial cells using the protocol for transformation in bacteria (section 2.1e).

### 2.3h Sequencing of Melanocyte Library Positive Interactors

Positive clones were extracted from KC8 bacterial cells using the miniprep protocol. These plasmids were then sequenced to identify the cDNAs (section 2.1h).

### 2.3i Retransformation and Specific Binding Assay in Yeast

The positive interactors for Omi<sub>134-458</sub> were retransformed into yeast containing pGILDA-Omi<sub>134-458</sub>, pGILDA-Omi<sub>PDZ</sub>, pGILDA-Omi<sub>CAT</sub>, pGILDA-L56 using the standard yeast transformation protocol (section 2.2a).

## 2.4 Cleavage Assay

### 2.4a TNT Reaction

The following primers were used in the PCR reaction for the TNT reaction of pJG4-5 Rad21 (partial clone)

Primer 5' pJG4-5 Fwd. 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 3' pJG4-5 Rev.

5' - GGA GAC TTG ACC AAA CCT CTG GCG - 3'

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

Then incubate the PCR reaction with the TNT Mix to label the Methionine/Cysteine.

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

|  |          |
|--|----------|
| TNT T7 Quick Master Mix (Promega)                            | 40 µl    |
| [ <sup>35</sup> S] Methionine/Cystein (1000 Ci/ml) (Promega) | 2 µl     |
| PCR generated DNA template                                   | 2.5-5 µl |
| H <sub>2</sub> O   | 3.5-5 µl |

The reaction was incubated at 30 °C for 1.5 hours.



## 2.4b Degradation Assay

The product of the TNT reaction is incubated with mature His-tag purified Omi<sub>134-458</sub> as shown below. The reaction is performed in 20  $\mu$ l final volume.

**Table 1: Description of Cleavage Assay**

| Positive                              | Negative                              |
|---------------------------------------|---------------------------------------|
| [ <sup>35</sup> S]-Rad21<br>5 $\mu$ l | [ <sup>35</sup> S]-Rad21<br>5 $\mu$ l |
| Omi <sub>134-458</sub><br>5 $\mu$ l   | Omi <sub>134-458</sub><br>5 $\mu$ l   |
| Omi Assay Buffer<br>10 $\mu$ l        | Omi Assay Buffer<br>10 $\mu$ l        |

The mixture is incubated at 37 °C for 1 hour. 10  $\mu$ l of 2X SDS sample loading buffer is then added to the reaction and the proteins are resolved on a 12% SDS gel under denaturing condition. Proteins are then transferred to a nitrocellulose membrane and samples are detected using autoradiography.

## 2.5 Construction of pJG4-5-hRad21 (416-631), hRad21 (416-627)

### 2.5a Polymerase chain reaction

The following primers were used in the PCR reaction for the cloning of pJG4-5-hRad21 (416-631) and (416-627)

Primer 5' pJG4-5-hRad21 (264) - MfeI

5' - CGG CCA ATT GAT GGA TGA GGA TGA TAA TGT ATC AAT G - 3'

Primer 3' pJG4-5-hRad21 (416-631) - XhoI

5' - CCG CTC GAG TTA TAT AAT ATG GAA CCT TGG TCC - 3'

Primer 3' pJG4-5-hRad21 (416-627) - XhoI

5' - CCG CTC GAG TTA CCT TGG TCC AGG TGT TGC G - 3'

After the PCR was performed, the reaction was digested with EcoRI (as the PCR has EcoRI site at 416th amino acid) and XhoI and cloned into pJG4-5.

## 2.6 Construction of pEGFP-hRad21 (279-631)

### 2.6a Polymerase Chain Reaction

The following primers were used to amplify the fragment of hRad21 (279-631) to clone into pEGFPC1

Primer 5' - pEGFP-hRad21 (279-631) - BglII

5' - GAA GAT CTT CAG TGG ATC CCG TTG AAC C - 3'

Primer 3' - pEGFP-hRad21 (279-631) - MfeI

5' - CCG CAA TTG TTA TAT AAT ATG GAA CCT TGG TC - 3'

The PCR was digested with BglII and MfeI and cloned into pEGFPC-1 vector.

**Figure 11: Map of pEGFP-C1**

Map of pGFP-C1 vector used in subcellular localization experiments.

[www.clontech.com](http://www.clontech.com).

## 2.7 Transfection of Mammalian Cells

### 2.7a Growing HeLa Cells

HeLa cells, human cervical carcinoma cell line, were grown using F-12 Nutrient Mixture (HAM) media (Life Technologies) supplemented with 10% FCS (Sigma), 2 mM L-Glutamine, 100 U/ml Penicillin and 100 µg/ml Streptomycin (Life Technologies).

### 2.7b Transfection and Immunofluorescence Microscopy

For immunofluorescence, the cells were grown on cover-slips. Approximately 70% confluent cells were transfected with 2 µg of pEGFP-Rad21 fusion protein using Lipofectamine Plus Reagent according to the instruction 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).

## 2.8 Protease Activity Assay

### 2.8a Protease Activity Assay

The protease activity assay is done in 15  $\mu$ l reaction volume. The enzyme His-Omi is pre-incubated with regulator; control peptide and hRad21 peptide on ice for 10 minutes. Then substrate  $\beta$ -casein was added in each reaction and incubated at 37 °C for 25 minutes. The reaction samples were loaded on SDS gel and stained with Coomassie Blue.

**Table 2: Description of Regulation Assay**

| Reaction | His-Omi | $\beta$ -casein | Control peptide | hRad21 peptide |
|----------|---------|-----------------|-----------------|----------------|
| <b>1</b> | -       | 4 $\mu$ g       | -               | -              |
| <b>2</b> | 400 ng  | 4 $\mu$ g       | -               | -              |
| <b>3</b> | 400 ng  | 4 $\mu$ g       | 200 $\mu$ M     | -              |
| <b>4</b> | 400 ng  | 4 $\mu$ g       | 400 $\mu$ M     | -              |
| <b>5</b> | 400 ng  | 4 $\mu$ g       | 600 $\mu$ M     | -              |
| <b>6</b> | 400 ng  | 4 $\mu$ g       | -               | 200 $\mu$ M    |
| <b>7</b> | 400 ng  | 4 $\mu$ g       | -               | 400 $\mu$ M    |
| <b>8</b> | 400 ng  | 4 $\mu$ g       | -               | 600 $\mu$ M    |
| <b>9</b> | 400 ng  | -               | -               | -              |

The reaction was incubated at 37 °C for 25 minutes and 10  $\mu$ l of 2X SDS sample loading buffer was added to each reaction and proteins are resolved on 12% SDS gel.

## 2.8 Co-Immunoprecipitation Assay

### 2.8a Co-Immunoprecipitation Assay

To investigate the interaction between endogenous Omi and hRad21 protein in mammalian cells, HEK293 cells were grown in 100 mm dishes. After subconfluent population, cultures were lysed using radioimmune precipitation assay buffer. Approximately 400 µg of total protein cell lysate was precleared using Protein G-agarose (Roche Applied Science) for 2 hours. The lysate was incubated with Omi rabbit polyclonal antiserum overnight at 4 °C. A control (preimmune) antiserum is also used. Protein G-agarose beads are collected by centrifugation, then washed four times with 1ml of radioimmune precipitation assay buffer. The samples were resuspended in 50 µl of SDS sample buffer. Proteins were subjected to SDS-PAGE and then transferred to nitrocellulose membrane. Western blot analysis was performed using hRad21 monoclonal antibody in 1:500 dilution (Novus Biologicals).

## 2.9 Construction of pEGFP-Autophagin-2

### 2.9a Polymerase Chain Reaction

Primer 5' - pEGFP-Autophagin2 - BglII

5' - GAA GAT CTA TGG AGT CAG TTT TAT CCA AG - 3'

Primer 3' - pEGFP-Autophagin2 - KpnI

5' - CGG GGT ACC CTA CAC ACT CAG AAT CTC AAA - 3'

Following the PCR, the reaction was digested with BglII and KpnI and cloned into pEGFPC1 (Clontech).



## 2.10 Construction of pET-28a-Autophagin-2 (309-398)

### 2.10a Polymerase Chain Reaction

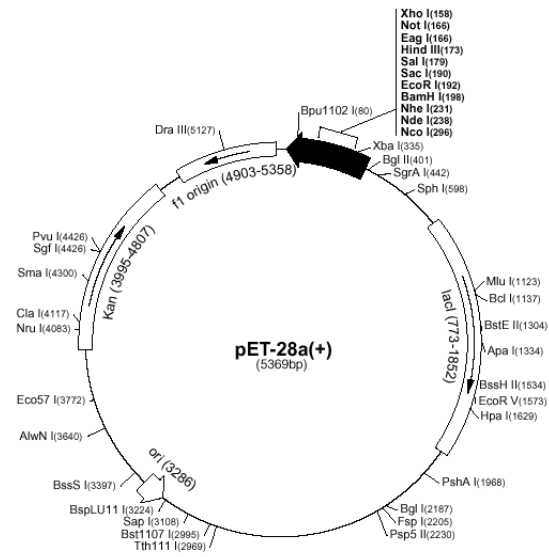
Primer 5' - pET-28a-Autophagin2 (309-398) - MfeI

5' - CCG CAA TTG ATG AAC ATC CTA AAC CTG GAT C - 3'

Primer 3' - pET-28a-Autophagin2 (309-398) - XhoI

5' - CCG CTC GAG CTA CAC ACT CAG AAT CTC AAA ATC - 3'

The PCR reaction was digested with MfeI and XhoI and cloned into pET-28a (NOVAGEN)



**Figure 12: Map of pET-28a vectors.**

Map of pET-28a vector used in protein expression experiment.

<http://www.emdbiosciences.com/html/NVG/AllTables.html>

## 2.11 Purification of Autophagin-2

### 2.11a Transformation of BL21

E.coli strain BL21 was used to transform the pET-28a vector for protein purification. The bacteria were transformed using few ng of DNA, following the protocol from section 2.1e.

### 2.11b Protein Purification under Denaturing Condition

Culture was grown in large volume such as 200 ml with shaking and induced overnight with IPTG (1 mM final concentration) at 25 °C. Next day, spun down the culture at 10,000 rpm for 10 minutes and throw away the supernatant. Freeze the cells in -80 °C for few minutes. Cells were thawed on ice and resuspended in 5ml Lysis Buffer (8M Urea, 0.1M NaH<sub>2</sub>PO<sub>4</sub>, 10mM Tris pH 8.0, 10 mM Imidazole + Protease Inhibitor (1mM final concentration). 70 µl of Triton X-100 (1% of volume) was added. The mixture was rotated at room temperature for 4-5 hrs (solution should become light and transparent). The mixture was sonicated 3 times for 1 minute each and 1 minute gap. The solution was spun for 30 minutes at 14000 rpm in 4 °C. The supernatant was filtered to get rid of any DNA in the solution. The Urea concentration was brought down to 6 M using Dilution Buffer (0.1M NaH<sub>2</sub>PO<sub>4</sub>, 10mM Tris pH 8.0). Approximately 2-3 ml of Ni-NTA beads was added and rotated overnight at 4 °C. Next day, the flow through was collected using a column. The column was washed with 100-140 ml of Wash Buffer (6M urea, 0.1M NaH<sub>2</sub>PO<sub>4</sub>, 0.01M Tris, 0.01M Imidazole, 10% Glycerol). The protein was eluted using Elution Buffer.

### 2.11c Incubation of Autophagin-2 (309-398) with His-Omi

The concentration of Autophagin-2 (309-398) protein was 2.5  $\mu\text{g}/\mu\text{l}$  and concentration of His-Omi is 0.5  $\mu\text{g}/\mu\text{l}$ . The reaction volume was 15  $\mu\text{l}$ . Total of 1  $\mu\text{g}$  of Autophagin2 (309-398) protein was loaded in the reaction. The reaction was incubated at 37 °C water bath.

**Table 3: Incubation of Autophagin2 (309-398) with His-Omi**

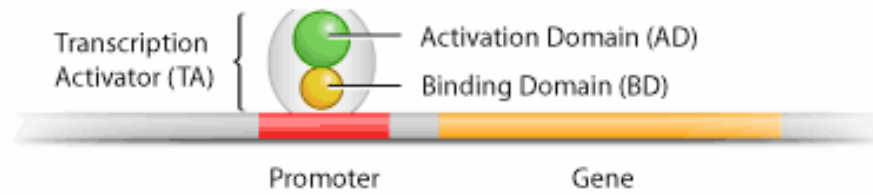
| Reagents                 | Reaction 1<br>(20 min) | Reaction 2<br>(20 min) | Reaction 3<br>(40min) | Reaction 4<br>(40min) |
|--------------------------|------------------------|------------------------|-----------------------|-----------------------|
| Autophagin2<br>(309-398) | 5 $\mu\text{l}$        | 5 $\mu\text{l}$        | 5 $\mu\text{l}$       | 5 $\mu\text{l}$       |
| His-Omi                  | -                      | 5 $\mu\text{l}$        | -                     | 5 $\mu\text{l}$       |
| Omi Assay<br>Buffer      | 10 $\mu\text{l}$       | 5 $\mu\text{l}$        | 10 $\mu\text{l}$      | 5 $\mu\text{l}$       |

Total of 15  $\mu\text{l}$  of reaction volume was prepared and loaded on SDS gel and stained with Coomassie Blue.

## CHAPTER THREE: RESULTS

### 3.1 Yeast Two Hybrid System

This system was used to identify genes encoding proteins that physically associate with a given protein in yeast cells. This method not only allows isolation of proteins that interact, but also can be used to identify particular domains/residues important for the interaction of two different proteins (43, 44). The basis of two-hybrid relies on the structure of particular transcription factors that have two physically separable domains: a DNA-binding domain and a transcription activation domain. When these two domains interact to form an initiation complex at the UAS (upstream activation sequence), it allows the transcription of a reporter gene (in this case the *LacZ* gene) (Figure 13), (45). These two domains are present in two different yeast vectors can be fused with different proteins to test for their interaction. The "bait" protein here contains the DNA-binding domain of LexA and the "prey" proteins contain a transcription activation domain (Figure 14), (45, 46).



**Figure 13: Normal Transcription:**

Normal transcription requires both the DNA-binding domain (BD) and the activation domain (AD) of a transcriptional activator (TA).

<http://www.biotech.ubc.ca/MolecularBiology/AYeastTwoHybridAssay/>

**Figure 14: Yeast two-hybrid system.**

The yeast two-hybrid technique measures protein-protein interactions by measuring transcription of a reporter gene. If protein X and protein Y interact, then their DNA-binding domain and activation domain will combine to form a functional transcriptional activator (TA). The TA will then proceed to transcribe the reporter gene that is paired with its promoter.

<http://www.bioteach.ubc.ca/MolecularBiology/AYeastTwoHybridAssay/>

### 3.2 Use of a human Melanocyte cDNA Library

This cDNA library was used because it was available in the desired vector (pJG4-5) to use straight away in the yeast-two-hybrid screening (Zervos Lab). The melanocyte cells used in preparing the mRNA for the cDNA construction were human primary cells.

Therefore, all the genes that are expressed in these cells would be comparable to the genes expressed in normal cells *in vivo*. This would eliminate the presence of any over expressed or under expressed genes that might be found in transformed cells. Since these were melanocytes, there could also be some tissue-specific genes being expressed. Above all, the cells were human and the Omi protein used as bait was also a human homolog.

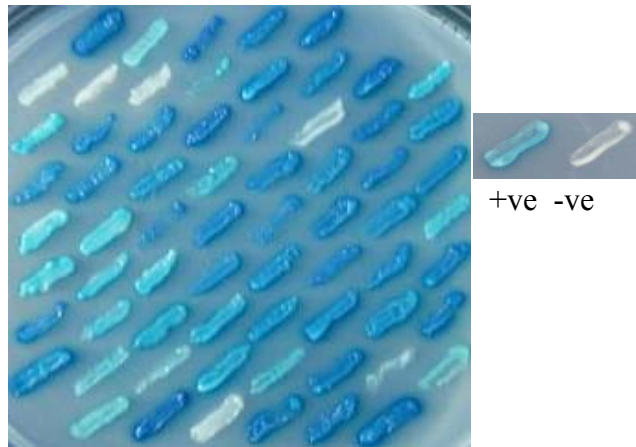


### 3.3 Screening the Melanocyte cDNA Library Using the Yeast-Two-Hybrid System

The melanocyte cDNA library was prepared from mRNA of actively growing human melanocyte cells. These cDNA were 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 this yeast-two-hybrid screening.

The mature form of Omi<sub>134-458</sub> fused to LexA was used as "bait" in the screening. Two reporter genes, the LexAop-LEU2 gene that allow the growth of colonies in the absence of leucine and the LexAop-lacZ gene that directs the synthesis of  $\beta$ -galactosidase, were used to make the selection of yeast colonies that may be interacting with Omi<sub>134-458</sub>. The number of clones per plate was estimated by counting the number of colonies in a 2x2 cm squares. Two squares were counted on a plate, averaged, and then extrapolated to estimate the number of clones per plate. A total of  $7 \times 10^5$  colonies were present.

These seven million colonies of yeast with the cDNA encoding the proteins might be the potential interactor of mature form of Omi. Out of these seven million, 67 colonies were picked to grow on U<sup>-</sup>H<sup>-</sup>W<sup>-</sup>L<sup>-</sup> Galactose selective medium plate. These same colonies were again tested on U<sup>-</sup>H<sup>-</sup>W<sup>-</sup> Glucose/X-Gal and U<sup>-</sup>H<sup>-</sup>W<sup>-</sup> Galactose/X-Gal plates. Forty-one of them were found to be true positive (blue color colony) on Galactose plate (Figure 15). Remaining twenty six were identified as false positives, correlating to either a blue colony on U<sup>-</sup>H<sup>-</sup>W<sup>-</sup> Glucose/X-Gal that could not be further examined, or a white colony on U<sup>-</sup>H<sup>-</sup>W<sup>-</sup> Galactose/X-Gal that shows no protein-protein interaction.



**Figure 15: Melanocyte cDNA Library Screening**

Out of 67 colonies screened on UH<sup>W</sup>Galactose/X-Gal, only 41 colonies were true positives. A positive interaction between the bait and prey proteins leads to blue colonies on UH<sup>W</sup>Galactose/X-Gal. A white colony represents no protein-protein interaction. Also, blue colonies on UH<sup>W</sup>Glucose/X-Gal correspond to false positive.

### 3.4 Characterization of Omi Interactors

The cDNA library plasmids were released from each of their positive yeast clones, and introduced into KC8 bacteria by electroporation. These 41 "prey" plasmids were then isolated and re-transformed into yeast for more specific screening using LexA-Omi<sub>134-458</sub>, LexA-Omi<sub>CAT134-340</sub>, LexA-Omi<sub>PDZ340-458</sub> and LexA-L56<sub>157-480</sub> as the "bait".

The result from yeast transformation revealed that out of the 41 positive clones, 32 were actually true positives. A positive interaction means a blue colony on UH<sup>W</sup> Galactose/X-Gal between one of the bait proteins (LexA-Omi<sub>134-458</sub>, LexA-Omi<sub>CAT134-340</sub>, LexA-Omi<sub>PDZ340-458</sub> and LexA-L56<sub>157-480</sub>) and the prey. These 32 positive plasmids were sequenced and analyzed using the BLAST program provided on the NCBI server ([www.ncbi.nlm.nih.gov/BLAST/](http://www.ncbi.nlm.nih.gov/BLAST/)). Finally 17 distinct proteins were identified and their specificity is shown on the chart below:

**Table 4: List of Omi Interactors Isolated from a Human Melanocyte cDNA Library**

| <b>NAME</b>  | <b>Omi<sub>134</sub>-<br/>458</b> | <b>Omi<sub>CAT134</sub>-<br/>340</b> | <b>Omi<sub>PDZ340</sub>-<br/>458</b> | <b>L56<sub>157</sub>-<br/>480</b> |
|--|-----------------------------------|--------------------------------------|--------------------------------------|-----------------------------------|
| Calcium binding protein Cab45 precursor                      | +                                 | +                                    | +                                    | -                                 |
| Scaffold attachment factor B (SAFB)                          | +                                 | -                                    | +                                    | -                                 |
| HS1 binding protein (HAX1)                                   | +                                 | +                                    | +                                    | -                                 |
| RAD21 homolog (S.pombe)(RAD21)                               | +                                 | -                                    | +                                    | +                                 |
| Chitobiase, di-N-acetyl-(CTBS)                               | +                                 | +                                    | +                                    | +                                 |
| 244Kb contig from human chromosome 11                        | +                                 | -                                    | +                                    | +                                 |
| Cleavage and polyadenylation specific factor 2,100kD (CPSF2) | +                                 | -                                    | +                                    | +                                 |
| PC326 protein  | +                                 | +                                    | -                                    | -                                 |
| Thyroid receptor interacting protein 15                      | +                                 | -                                    | +                                    | +                                 |
| Src family associated phosphoprotein 2                       | +                                 | +                                    | +                                    | +                                 |
| APG4 autophagy 4 homolog A (S. cerevisiae) (APG4A)           | +                                 | -                                    | +                                    | +                                 |
| Phosphoinositol 3-phosphate-binding protein-2                | +                                 | -                                    | +                                    | -                                 |
| Ubiquitin specific protease11                                | +                                 | +                                    | +                                    | -                                 |
| Crystallin,lambda 1  | +                                 | +                                    | +                                    | +                                 |
| Glutathione S-transferase omega 2                            | +                                 | +                                    | +                                    | -                                 |
| Squamous cell carcinoma antigen recognized by T cells        | +                                 | +                                    | +                                    | +                                 |
| MCM3 minichromosome maintenance deficient 3 (S.cerevisiea)   | +                                 | -                                    | +                                    | -                                 |

A positive protein-protein interaction is represented by +, no protein-protein interaction is represented by -.

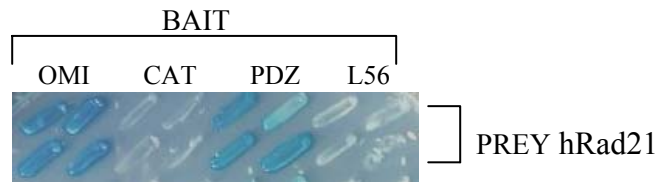
### 3.5 Characterization of hRad21

Normal development and homeostasis of the body requires regular cell cycle and cell apoptosis. Human Rad21 (hRad21) is a nuclear protein with an estimated molecular weight of 68-70kDa (47). It is located on chromosome 8q24 and encodes a protein containing 631 amino acids. Its main function is to act as a major cohesion subunit that keeps sister chromatids together until the transition from metaphase to anaphase stage in cell division cycle and in DNA double-strand-break repair damaged by  $\gamma$ -irradiation (48-50). The characterization of Rad21 was first recognized from its function in fission yeast, *Saccharomyces pombe*, and of Scc1/Mcd1 function in budding yeast, *Saccharomyces cerevisiae* (49). It is a hyper-phosphorylated protein, and phosphorylation occurs mainly on serine and some of threonine residues (50). Human Rad21 mRNA expression is highest at the end of the S phase and G2 phase of the cell cycle (48, 51). This protein has abnormal mobility on SDS-PAGE gels to 100-122kDa due to its hyper phosphorylation (50). Rad21 gene is conserved from yeast to humans and mouse (48). The proteolytic cleavage of the cohesion is done by a caspase like enzyme known as separase/separin, which is complexed with its inhibitor Pds1 (securin) before anaphase (52).

The N-terminal region of 329 amino acids is found to work in DNA repair and sister chromatids cohesion and only this N-terminal region is phosphorylated. The C-terminal part of Rad21 from amino acids 265-631 is less phosphorylated and the precise function of this part of the protein is unknown. This C-terminal is not necessary for cell cycle

check or DNA repair (50). Yeast Rad21-45 is a mutant, which is deficient in DNA double strand break repair and is hypophosphorylated (49, 50).

I isolated a partial cDNA clone for hRad21 protein from the melanocyte cDNA screening as the interactor of Omi through its PDZ domain (Figure 16). The partial clone represents 122-638 amino acid sequence of the protein.



**Figure 16: Interaction of partial clone of hRad21 in yeast**

The partial clone of hRad21<sup>122-631</sup> interaction with LexA-Omi<sup>134-458</sup>, LexA-Omi<sup>CAT134-340</sup>, LexA-Omi<sup>PDZ340-458</sup> and LexA-L56<sup>157-480</sup>. Human Rad21 is shown to interact with Omi and PDZ domain.

### 3.6 Human Rad21 in Apoptosis

It has already been shown that hRad21 is cleaved by separase during the separation of sister chromatids when cell is going from metaphase to anaphase (50). The hRad21 is also cleaved by caspase-3 and caspase-7 when a cell is undergoing apoptosis. Human Rad21 is cleaved at Asp279, the similar consensus sequence DXXD, which is cleaved by caspases-3 and -7 (Figure 17), (52, 53). This cleavage results in a 64kDa fragment seen only in cells undergoing apoptosis (52, 53). The same motif DSVD (DXXD) is conserved in hRad21 among different species like humans, mice and frogs. The C-terminus hRad21 is cytoplasmic as compared to the full-length protein that is nuclear (52, 53).

The specificity of proteolysis of hRad21 by caspase-3 during apoptosis is confirmed by using broad spectrum caspase inhibitor zVAD-fmk. Application of zVAD-fmk inhibited hRad21 proteolysis after inducing apoptosis using different stimuli (52, 53). Moreover, the C-terminal hRad21 is proapoptotic as its over-expression results in five to sevenfold increase in caspase-3 activity (52). Therefore, translocation of C-terminus 64kDa hRad21 fragment to the cytoplasm may provide a positive feedback to the cell in promoting apoptosis (52).



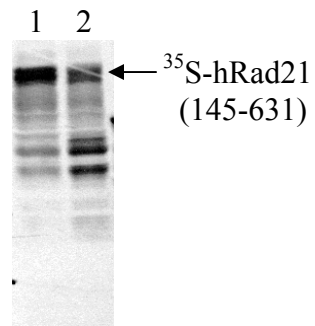
MFYAHFVLSKRGPLAKIWLAAHWDKCLKTAHVFEKNLESSVESIISPVKMALRTSGHL  
LLGVVRIYHRKAKYLLADCNEAFIKIKMAFRPGVVDLPEENREAAYNAITLPEEFHDFD  
QPLPDLDDIDVAQQFSLNQSRVEEITMREEVGNISILQENDFGDFGMDDREIMREGSAF  
EDDDMLVSTTTSNLLESEQSTSNLNEKINHLEYEDQYKDDNFGEGNDGGILDDKLI SN  
NDGGIFDDPPALSEAGVMLPEQPAHDDMDEDDNVSMGGPDS SPDSVDPVEPMPTMTDQTT  
LVPNEEEAFALEPIDITVKETKAKRKRKLI VDSVKELDSKTIRAQLSDYSDIVTTLDLA  
PPTKKLMMWKETGGVEKLFSLPAQPLWNNRLLKLFTRCLTPLVPEDLRKRRKGGEADNL  
DEFLKEFENPEVPREDQQQQHQORDVIDEPIIEEPSRLQESVMEASRTNIDESAMPPP  
PQGVKRKAGQIDPEPVMPPQQVEQMEIPPVELPPEEPPNICQLIPELELLPEKEKEKEK  
EKEDDEEEDEDEDASGGDQDQEERRWNKRTQQMLHGLQRALAKTGAESISLLELCRNTNR  
KQAAAKFYSLVVLKKQQAIELTQEEPYSDI IATPGPRFHII

**Figure 17: Sequence of hRad21**

Human Rad21 protein is encoded by 398 amino acids. The underlined sequence is where the caspase-3 cleaved the hRad21, which is after the Asp 279 residue. This cleaved sequence translocates to the cytoplasm where it increases the apoptosis pathway in an unknown manner (52, 53).

### 3.7 In-vitro Transcription and Translation

The partial clone of hRad21 protein that was isolated in melanocyte cDNA library screening is incubated with Omi to investigate if this protein is cleaved or degraded by Omi protease. [<sup>35</sup>S]-labeled hRad21 is incubated with His-Omi<sub>134-458</sub> in 20 µl of reaction volume at 37 °C for 1 hour. This partial protein was not degraded by Omi thus cannot be the substrate (Figure 18).



**Figure 18: TNT Reaction of Partial Clone of hRad21 with Omi**

Lane 1: [ $^{35}\text{S}$ ]-Rad21 (145-631); Lane 2: [ $^{35}\text{S}$ ]-Rad21 (145-631) + Omi<sub>134-458</sub>.

Partial hRad21 clone is not degraded by Omi in the reaction, thus, may not be the substrate of Omi.

### 3.8 Sequence similarity in different Omi's interactors

Omi was first isolated as an interactor of Mxi2 and bind with the C-terminus of Mxi2 (23). Recently, another protein Presenilin (PS1), a protein implicated in Alzheimer's disease was found to interact with Omi through its PDZ domain. The C-terminus of PS1 binds with PDZ domain and increases the proteolytic activity of Omi (38). Here also hRad21 interacts with the PDZ domain of Omi, and has a similar C-terminus sequence as PS1. Following is the last 20 amino acids of hRad21, PS1 and Mxi2 showing the specificity of amino acids that play a role in binding to PDZ domain of Omi.

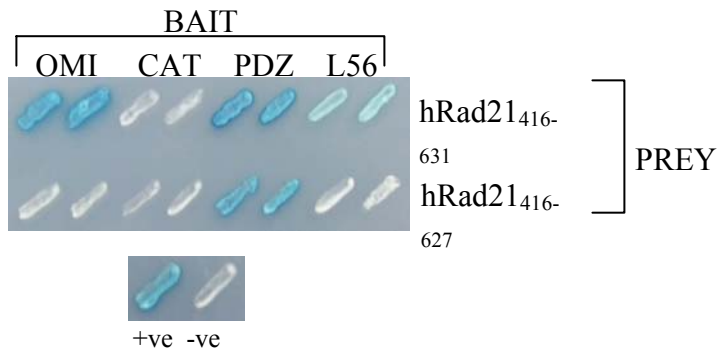
**Table 5: C-terminal alignment of Omi -PDZ Interacting Proteins**

| <b>Omi interacting proteins</b> | <b>Amino acids sequence</b> |
|---------------------------------|-----------------------------|
| hRad21, nm_006265               | TQEEPYSDI IATPGPRFHII *     |
| Presenilin 1, nm_000021         | ATDYLVQPFMDQLAFHQFYI *      |
| Mxi2, U19775                    | NPLGKLT IYPHLMDI ELVMI *    |

The proteins listed above interacted with Omi with their hydrophobic C-terminal amino acids residues. PS1 is found to be the regulator of Omi and has very high sequence homology to hRad21 in the last four amino acids.

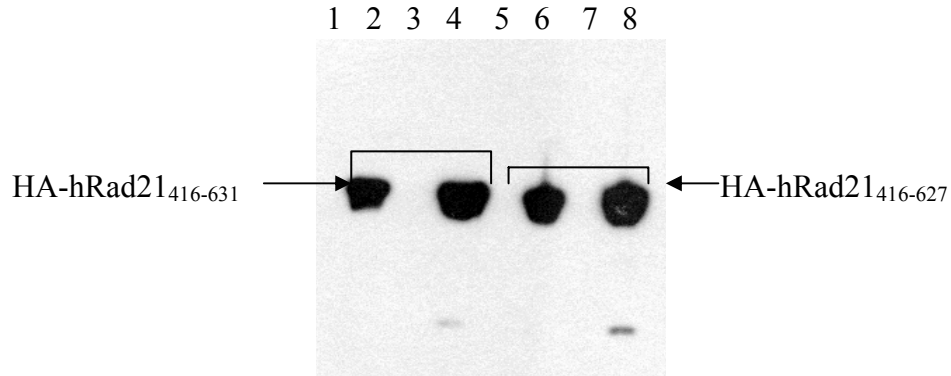
### 3.9 Specificity of hRad21 C-terminus with PDZ domain of Omi

In the Yeast-two-Hybrid isolation of partial length of hRad21 showed the interaction with only the PDZ domain of Omi. It has already been shown that PDZ domain has specificity in binding with the hydrophobic and aromatic amino acids like phenylalanine, isoleucine and tyrosine (29, 54). As hRad21 has all of the specific hydrophobic amino acids at its end, this may facilitate its binding with PDZ domain. Taking these facts into consideration, the C-terminal sequence of hRad21<sub>416-631</sub> and hRad21<sub>416-627</sub> was cloned into pJG4-5 yeast expression vector. Both constructs were transformed back into yeast to observe the interaction of hRad21 C-terminal with and without the last four amino acids with the PDZ domain of Omi. The construct hRad21<sub>416-627</sub> is not interacting with both Omi and the PDZ domain, as seen in the yeast transformation picture below (Figure 19). To confirm the proteins are made and are stable in yeast, the western blot using anti-HA antibody was performed (Figure 20).



**Figure 19: Specific Interaction of hRad21 C-terminus with Omi.**

Human Rad21<sub>416-631</sub> is interacting with Omi<sub>134-458</sub> and PDZ<sub>340-458</sub> in a similar manner as the partial clone that was isolated in the melanocyte cDNA library screening. But, the C-terminal clone of hRad21<sub>416-627</sub> is not interacting with both Omi<sub>134-458</sub> and PDZ<sub>340-458</sub> domain. The blue color of the hRad21<sub>416-627</sub> clone with the PDZ domain is due to self-activation of PDZ domain. Thus, confirming that the last four hydrophobic amino acids of hRad21 have the specificity of binding to PDZ domain of Omi.



### Figure 20: Protein Expression in Yeast

The prey proteins are under the Galactose inducible promoter. Transformed yeast were grown in the presence of glucose and galactose, whole cell extract are separated on a SDS-PAGE. The expression of fusion protein is monitored by Western Blot analysis using HA-antibody (ROCHE). Human Rad21<sub>416-631</sub> and hRad21<sub>416-627</sub> can be observed as a band at 54kDa.

Lane 1: hRad21<sub>416-631</sub> + Omi<sub>134-458</sub>-Glucose; Lane 2: hRad21<sub>416-631</sub> + Omi<sub>134-458</sub>-Galactose;

Lane 3: hRad21<sub>416-631</sub> + PDZ<sub>340-458</sub>-Glucose; Lane 4: hRad21<sub>416-631</sub> + PDZ<sub>340-458</sub>-

Galactose; Lane 5: hRad21<sub>416-627</sub> + Omi<sub>134-458</sub>-Glucose; Lane 6: hRad21<sub>416-627</sub> + Omi<sub>134-</sub>

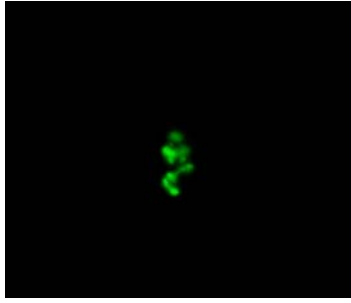
458-Galactose; Lane 7: hRad21<sub>416-627</sub> + PDZ<sub>340-458</sub>-Glucose; Lane 8: hRad21<sub>416-627</sub> +

PDZ<sub>340-458</sub> -Galactose.

### 3.10 Subcellular localization of hRad21

The partial clone of hRad21 (145-631) was fused to a green fluorescent protein (GFP) mammalian expression vector, to monitor its subcellular localization. This construct was then transfected into HeLa cells. The cells were grown on coverslips and then transfected with 2 $\mu$ g of DNA. Cells are fixed 24hrs after transfection and the fluorescent protein was observed using a confocal microscope. The partial clone of hRad21 protein seems to be localized in the nucleus consensus with the actual localization of hRad21 protein in the cell (Figure 21).



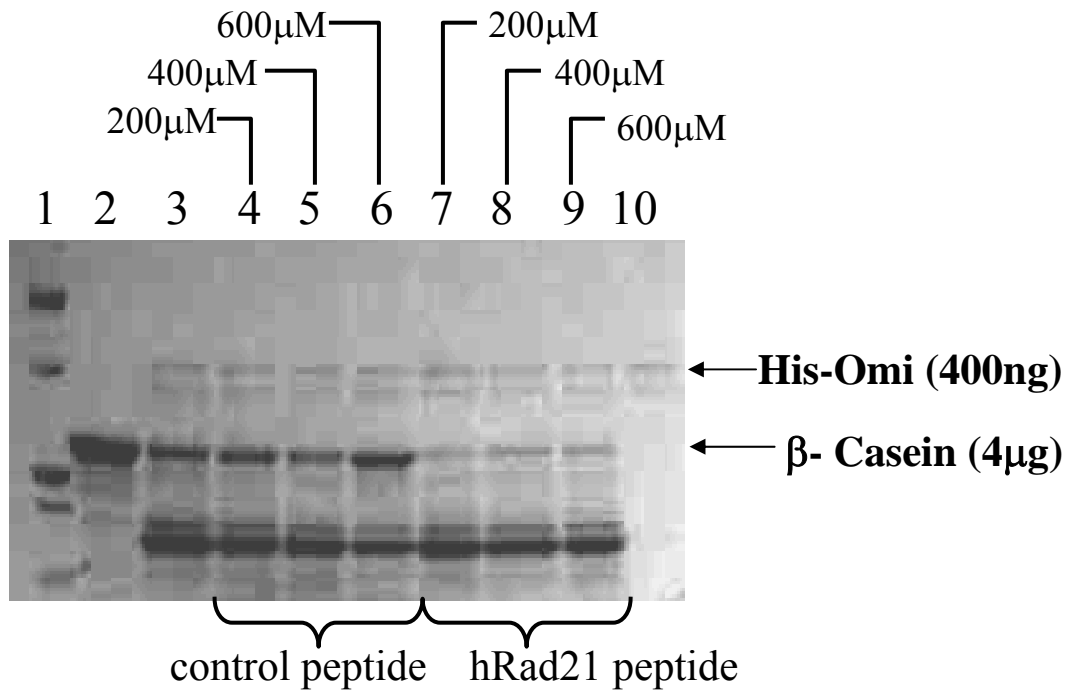


**Figure 21: Subcellular Localization of hRad21 in HeLa Cells**

HeLa cells are transfected with pEGFP-hRad21 (145-631). The fusion protein is localized in the nucleus mainly. Human Rad21 is a nuclear protein and plays an important role in DNA double strand break repair.

### 3.11 Potential Regulation of Omi's Proteolytic Activity by Synthetic peptide of hRad21

Using the TNT system I found that the hRad21 protein is not a potential substrate of Omi, at least, in vitro (Figure 18). The last few amino acids of hRad21 are necessary for specific binding to Omi<sub>134-458</sub> via its PDZ domain (Figure 19). The PDZ domain is important for specific protein-protein interaction. Any protein/peptide ligand that binds to PDZ domain of Omi is known to open up the trimeric structure of Omi, thus, exposing the catalytic site of Omi (29). Interaction of hRad21 with the PDZ domain of Omi may suggest that such a regulation exist. A synthetic peptide representing the last 15 C-terminal amino acids of hRad21 (YSDIATPGPRFHII) was made (BACHEM). The peptide is incubated with bacterially made His-Omi in the presence of  $\beta$ -casein, a generic substrate of Omi for 25 minutes at 37 °C. The picture below shows that, in comparison to the control, the presence of peptide increases the proteolytic activity of Omi against  $\beta$ -casein.



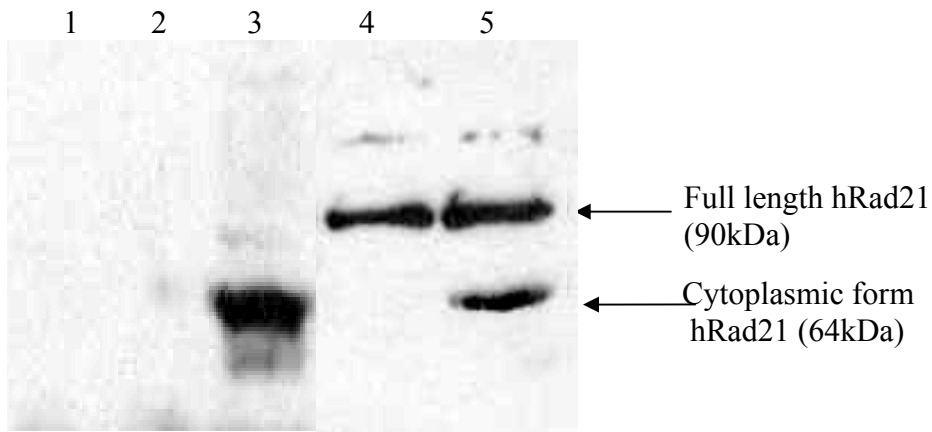
**Figure 22: Regulation of Proteolytic Activity of Omi**

Synthetic peptide representing the last 15 amino acids of hRad21 increases the proteolytic activity of His- Omi<sub>134-458</sub> towards β-casein.

Lane 1: Marker, Lane 2: β-casein (4 μg), Lane 3: β-casein + Omi (400 ng), Lane 4: β-casein + Omi + control peptide (200 μM), Lane 5: β-casein + Omi + control peptide (400 μM), Lane 6: β-casein + Omi + control peptide (600 μM), Lane 7: β-casein + Omi + hRad21 peptide (200 μM), Lane 8: β-casein + Omi + hRad21 peptide (400 μM), Lane 9: β-casein + Omi + hRad21 peptide (600 μM), Lane 10: His-Omi (400 ng).

### 3.12 Co-Immunoprecipitation of hRad21 with Omi

To investigate the interaction of endogenous Omi with hRad21 *in vivo*, HEK293 cells were used. Omi polyclonal antibody was employed followed by an anti-hRad21 monoclonal antibody. The results of this experiment clearly show a major polypeptide of approximately 100 kDa (full length hRad21) is present in actively growing cells. After induction of apoptosis another polypeptide appears of 64 kDa (processed cytoplasmic form of hRad21) in the cell extracts. After induction of apoptosis with etoposide (100 µg/ml), the 64 kDa specifically co-immunoprecipitates with Omi. In the absence of apoptosis this interaction is not observed (Figure 23). This is consistent with the fact that Omi is present in the mitochondria and only becomes cytoplasmic after induction of apoptosis.



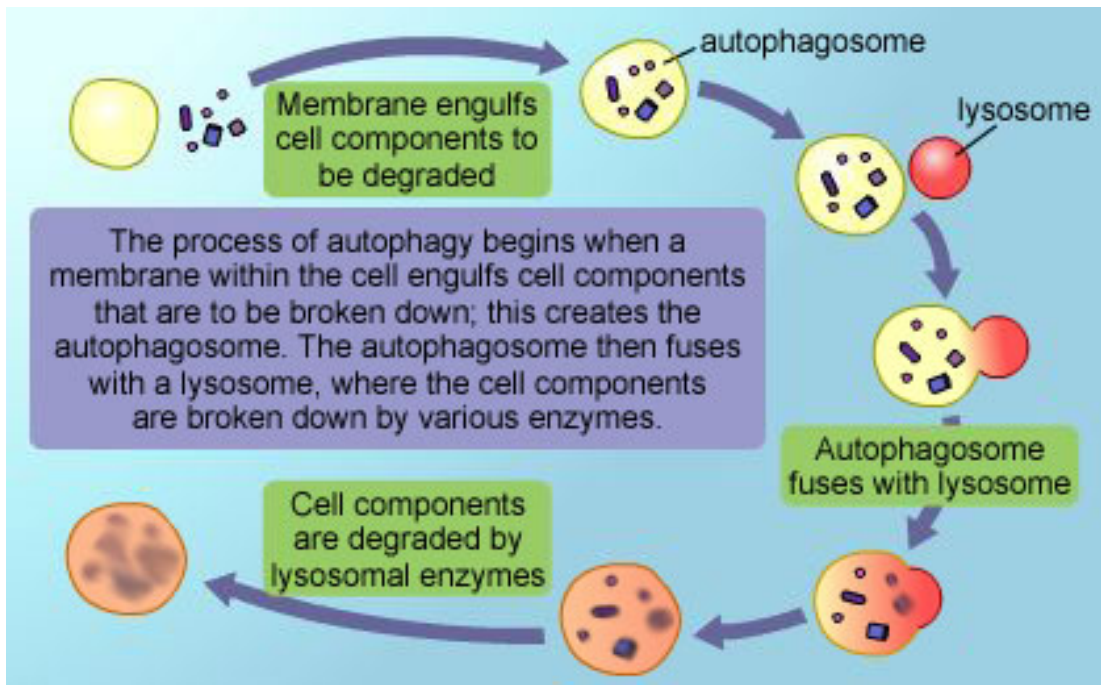
**Figure 23: Co-Immunoprecipitation of hRad21 with Omi**

The full length hRad21 is detected in HEK293 cell lysate on Western Blot. A 64 kDa appears only after induction of apoptosis. In the Co-IP assay the same 64 kDa band is precipitated along with Omi only when apoptosis is induced.

Lane 1: Co-IP with pre immune bleed, Lane 2: Co-IP without apoptosis, Lane 3: Co-IP after apoptosis, Lane 4: cell lysate, Lane 5: cell lysate after apoptosis.

### 3.13 Characterization of Autophagin-2

Autophagy is a biological process mainly involved in the destruction or removal of intracellular proteins and damaged organelles. Autophagy is done by means of forming an autophagosomal body to eat up the destined part of the cell. This process has been shown to be essential for cell homeostasis and for cell remodeling during differentiation, metamorphosis, and aging and above all non-apoptotic cell death (11). There are four different kinds of autophagy with related mechanisms: macroautophagy, microautophagy, crinophagy and chaperon mediated autophagy. Out of these macroautophagy is the one better studied (55). In a bigger sense macroautophagy can also be called autophagy (10, 14). Formation of a double membrane structure/body known as autophagosome that eats away the cytoplasm or unwanted organelle of the cell and finally fuses with the lysosome/vacuole to degrade its internal contents (Figure 24), (56).



**Figure 24: Basic Principal Involving Autophagy**

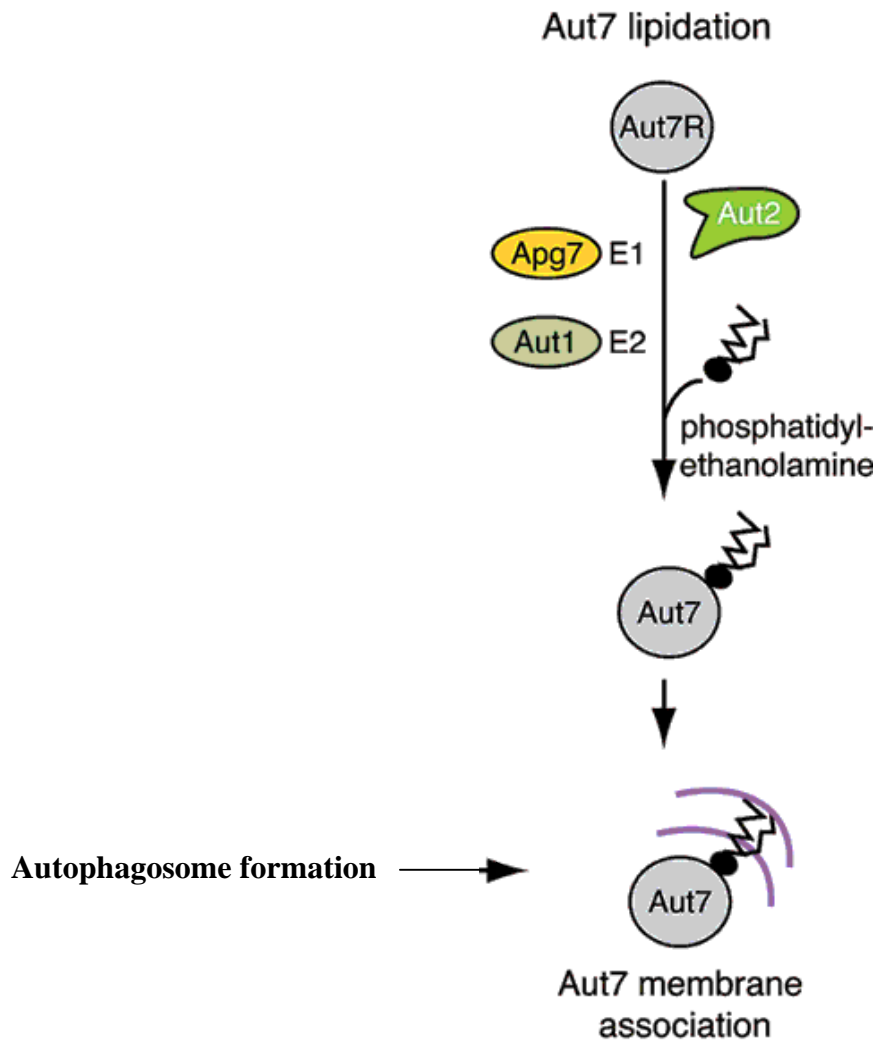
The unwanted components of the cell are degraded by Autophagy. This mechanism has recently gained attention as a caspase independent cell death and has been implicated in many neuronal diseases.

[http://www.stanford.edu/group/hopes/treatmts/pbuildup/f\\_h03autophagy.jpg](http://www.stanford.edu/group/hopes/treatmts/pbuildup/f_h03autophagy.jpg)

### 3.14 Autophagin-2

Human Autophagins are cysteine proteinases that have been discovered as homologs of yeast Apg4/Aut2 protein (57). Yeast Apg4/Aut2 is required for formation of autophagosome membrane structure. Apg4/Aut2 participates in the ubiquitin like system involving Apg8/Aut7. Apg8/Aut7 is cleaved by Apg4/Aut2 after Glycine residue and modify it to bind to PE (phosphotdylethanolamine) of the cell membrane (56, 57). Modification of Apg8/Aut7 after lipidation and then delipidation is required for the membrane rearrangement dynamics for formation of autophagosome (Figure 25), (56, 58). Four human cysteine proteinases closely related to yeast Apg4/Aut2 and with the similar enzymatic properties have been described (57).



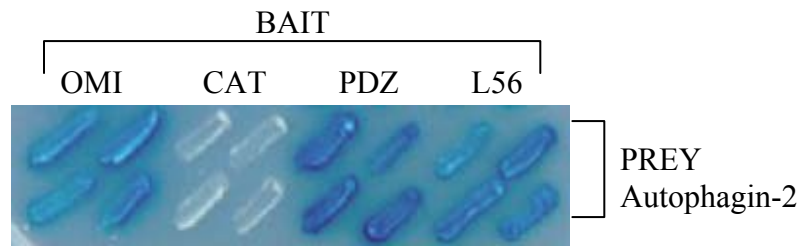


**Figure 25: Involvement of Aut2 in the Formation of Autophagosome**

The yeast Aut2 is required for the cleavage of Aut7 at Gly residue and help in the lipidation of Aut7. This modification of Aut7 is required for autophagosome formation. Autophagosome is a double membrane structure, which engulfs the cytoplasm or organelle and fuses with lysosome to degrade its internal contents.

<http://www.lifesciences.umich.edu/institute/labs/klionsky/fig4.gif>

I isolated a full length cDNA clone for Autophagin-2 as a novel interactor of Omi. Human Autophagin-2 interacts with Omi through its PDZ domain (Figure 26). The basic role of Autophagin-2 in the mechanism of autophagy has not been elucidated yet. Autophagin-2 is 1194bp long encoding 398 amino acids and located on chromosome Xq22. Northern blot analysis revealed that it expresses highly in fetal liver, skeletal muscles and to some extent in heart (57).

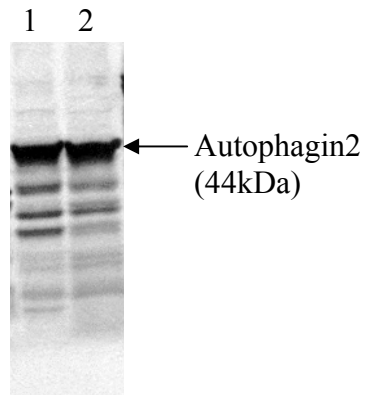


**Figure 26: Interaction of Autophagin-2 with Omi in Yeast**

Full length Autophagin-2 isolated in Melanocyte cDNA library screening is interacting with Omi<sub>134-458</sub>, PDZ<sub>340-458</sub> and L56<sub>157-480</sub>.

### 3.15 In-vitro Transcription and Translation of Autophagin-2 with Omi

To investigate the possibility that Omi protease can cleave Autophagin-2 protein in vitro. [<sup>35</sup>S]-labeled Autophagin2 was incubated with His-Omi<sub>134-458</sub> at 37 °C for 1 hour. The reaction was electrophoresed by SDS-PAGE and then observed with autoradiography (Figure 27). Autophagin-2 was not degraded by Omi<sub>134-458</sub> in vitro.

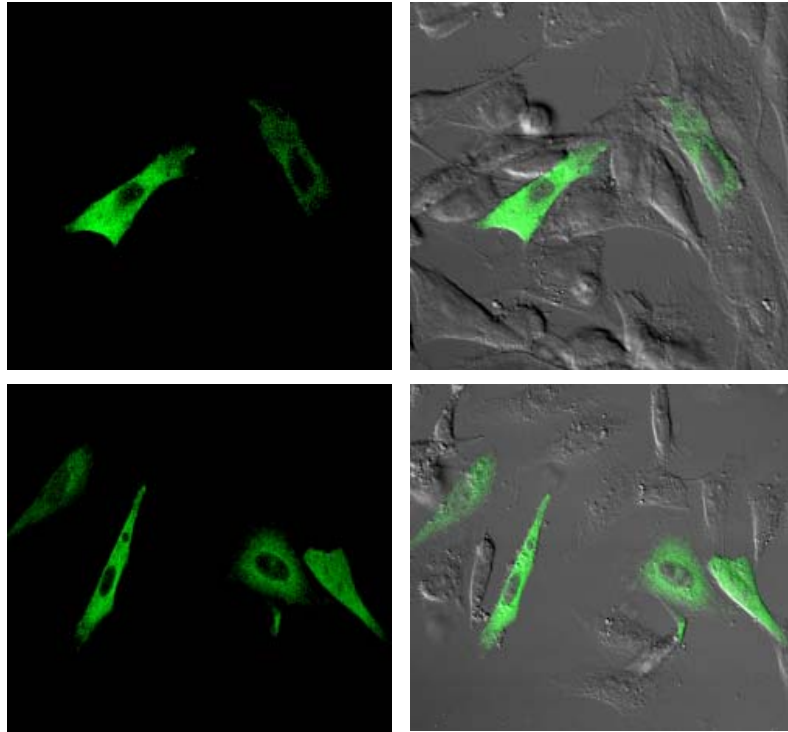


**Figure 27: Autophagin-2 with Omi in *In-vitro***

Lane 1: [<sup>35</sup>S]-Autophagin-2; Lane 2: [<sup>35</sup>S]-Autophagin-2+ His-Omi<sub>134-458</sub>

### 3.16 Subcellular localization of Autophagin-2 in HeLa cells

The full-length Autophagin-2 gene was sub-cloned into green-fluorescent protein (GFP) mammalian expression vector. The HeLa cells were grown on coverslips, and the next day transfected with 2 $\mu$ g of Autophagin-2 DNA. 24 hrs after transfection, cells were fixed on glass slides and observed under confocal microscope. The protein was found throughout the cytoplasm as previously reported for all Autophagin group of proteins (Figure 28).



**Figure 28: Subcellular Localization of Autophagin-2**

The fusion protein of pEGFP-Autophagin-2 is transfected into HeLa cells. Cover-slips were fixed and observed under the confocal microscope. The fusion protein was localized in the cytoplasm.

### 3.17 Bacterial expression and purification of C-terminus of Autophagin-2<sub>309-398</sub>

The C-terminus of Autophagi2 gene was subcloned into the bacterial expression vector pET-28A, which allows protein to be expressed as a fusion with a 6-His-tag. The vector is transformed into BL21 competent cells and protein expression is induced over night using IPTG (1 mM final concentration) at 25 °C. After checking the protein expression (Figure 29), the culture was grown in a large scale. The protein was purified using the native condition but could not get the proteins, may be it was insoluble. Then the recombinant protein was again purified using denaturing condition and Ni-NTA beads. Eluted protein was dialyzed for 3-4 days to get rid of urea. Different elutions were then analyzed by SDS-PAGE after staining with Coomassie Blue dye. C-terminal Autophagin-2 (309-398 aa.) can be seen as band at  $\cong$  13kDa, which corresponds to the estimated size in the protein (Figure 30).



1 2 3 4 5 6 7 8 9

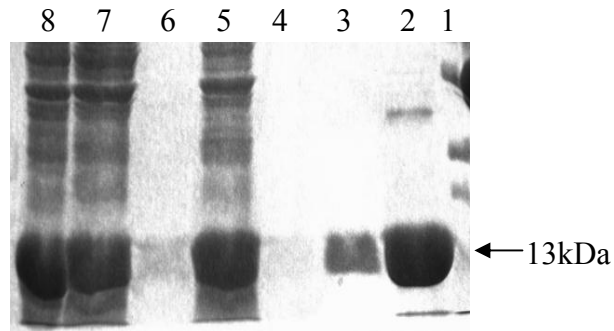
are needed to see this picture.

←13kDa

**Figure 29: Protein Expression and purification of Autophagin-2 (309-398).**

Four different clones were induced for 4 hrs and overnight to check for protein expression in pET-28a. Induction was done using IPTG (1 mM final concentration) overnight at 25 °C. Cell lysates were run on SDS-PAGE and stained with Coomassie Blue.

Lane 1: Marker, Lane 2: #4 (uninduced), Lane 3: #4 (induced 4 hrs), Lane 4: #4 (induced overnight), Lane 5: #15 (uninduced), Lane 6: #15 (induced 4 hrs), Lane 7: #15 (induced overnight), Lane 8: #7 (induced overnight), Lane 9: #16 (induced overnight).

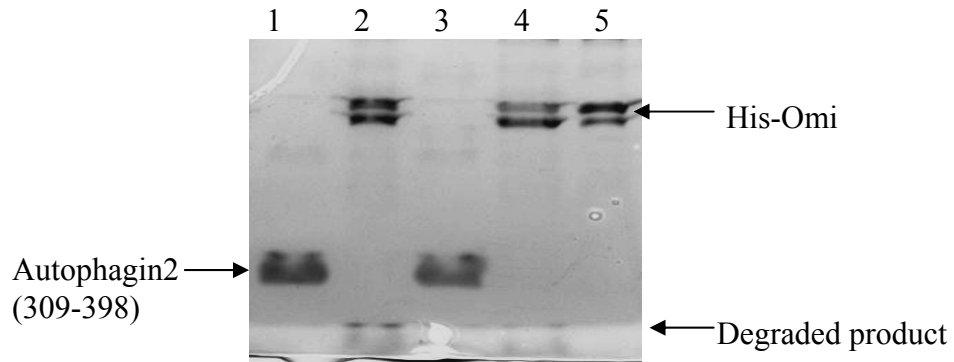


**Figure 30: Protein Purification under Denature Condition**

After the eluted fractions were dialyzed for 3-4 days, 10-15  $\mu$ l of each elution was run on a SDS-PAGE gel and stained with Coomassie Blue. In the picture from right to left; Lane 1: Marker, Lane 2: Elution 1, Lane 3: Elution 2, Lane 4: Elution 3, Lane 5: Flow through, Lane 6: Wash, Lane 7: Cell lysate (induced 4 hrs), Lane 8: Cell lysate (induced overnight).

### 3.18 Degradation of C-Terminus-Autophagin-2 (309-398) by His-Omi

The C-terminus-Autophagin-2 (309-398) protein was incubated with His-Omi to see if Omi degrades it. The incubation was done for different time points at 37 °C. The result shows that within 20 minutes for incubation with Omi, Autophagin-2 (309-398) is degraded completely (Figure 31). This degradation could possibly be because of the denaturing condition when the protein was purified. The protein could not get properly folded and the degradation by Omi is just non-specific towards the misfolded and hydrophobic residues of the protein. Since Autophagy is involved in caspase independent cell death, there could be some relation with Omi in triggering caspase-independent cell death. But still a lot of work needs to be done to show that this relationship exists in a physiological condition where a cell needs to undergo caspase independent cell death.



**Figure 31: Degradation of Autophagin-2 (309-398) by His-Omi**

The C-terminus-Autophagin-2 (309-398) protein is degraded completely by Omi within 20 minutes of incubation at 37 °C.

Lane 1: Autophagin-2 (309-398) (13kDa), Lane 2: Autophagin-2 (309-398) + His-Omi (20 minutes incubation), Lane 3: Autophagin-2 (309-398) (13kDa), Lane 4: Autophagin-2 (309-398) + His-Omi (40 minutes incubation), Lane 5: His-Omi (37kDa)

## CHAPTER FOUR: DISCUSSION

Apoptosis or programmed cell death is defined as the control of a cell over its own demise. There are various ways in which cells can die; apoptosis (programmed cell death type I), autophagy (programmed cell death type II) or necrosis. All of these pathways of cell death are different but still overlap with each other in undefined ways (10, 12).

Cell death can be triggered by external or internal stress signals. External signals include death receptor signals and internal signals include mainly mitochondrial stress. Upon induction of apoptosis various apoptotic proteins are released from mitochondria to the cytoplasm including: cytochrome-c, AIF and Omi/HtrA2 (25, 34). Omi 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 proteases HtrAs that function as proteases at elevated temperatures and as chaperones at lower temperatures (26). Omi is a nuclear encoded protein with a mitochondrial targeting sequence (MTS) at its N-terminus. This MTS is cleaved as soon as Omi enters mitochondria. Mature Omi protein lies in the mitochondrial inter membrane space where it may function as chaperone. Omi has two main functional domains; a catalytic domain and an amino terminal regulatory domain (29). Active Omi protein is present in a trimeric form where the catalytic domain is folded inside the PDZ domains. After the first 133N-terminal amino acids are removed they reveal a conserved IAP-binding motif, AVPS, at the N-terminus. Human IAPs like X-IAPs are the main substrates of Omi (27). IAPs are the Inhibitor of Apoptosis proteins that block the caspases. When Omi becomes cytoplasmic it binds and degrades IAPs, leading to activation of caspases to induce cell

death (28). This pathway of Omi is regarded as caspase dependent cell death (30, 33). Omi also plays role in caspase independent cell death where its proteolytic activity is essential, but the mechanism remains unclear. Apart from IAPs, Omi degrades ped/pea-15 an anti-apoptotic protein and led to caspase independent cell death (37). In the mitochondria Omi is also found to cleave another anti-apoptotic protein, the HS1 binding protein-X 1 (HAX-1). HAX-1 is degraded in the mitochondria on the onset of apoptosis before Omi is released to the cytoplasm (36).

A recent study suggests that some proteins/peptide ligands can regulate the activity of Omi by binding to the PDZ domain. This binding to the PDZ domain may open the protease domain and thus regulating its catalytic activity. Presenilin (PS1) is one of the regulators of Omi where its last 15 amino acids bind to the PDZ domain and increase its proteolytic activity (38).

Omi is also a potential chaperone when present in the mitochondria (41). The importance of its chaperon activity is emphasized after the discovery of mnd-2 mouse. The motor neuron deficient-2 mice have a single mutation that changes S276C in the catalytic domain of Omi. These mice show early symptoms like altered gait, cessation of normal weight gain, followed by ataxia, repetitive movements and akinesia when compared to the normal mice and die by 40 days of age. Destruction of striatal neurons and motor neurons is the most noted symptom in these mice. Neuronal degeneration is mainly due to degeneration of mitochondria, leading to apoptosis and necrosis (40). The activity of Omi can be restored in these mice by an artificial transgene provided from outside. This

evidence is further confirmed in the Omi knockout mice (KO) that show similar types of severe symptoms as the mnd-2 mice and die within 30 days (42). Thus, loss of protease activity of Omi seen in mnd-2 mice possibly results in accumulation of misfolded and damaged proteins in the mitochondria and finally mitochondrial dysfunction leading to cell death (40).

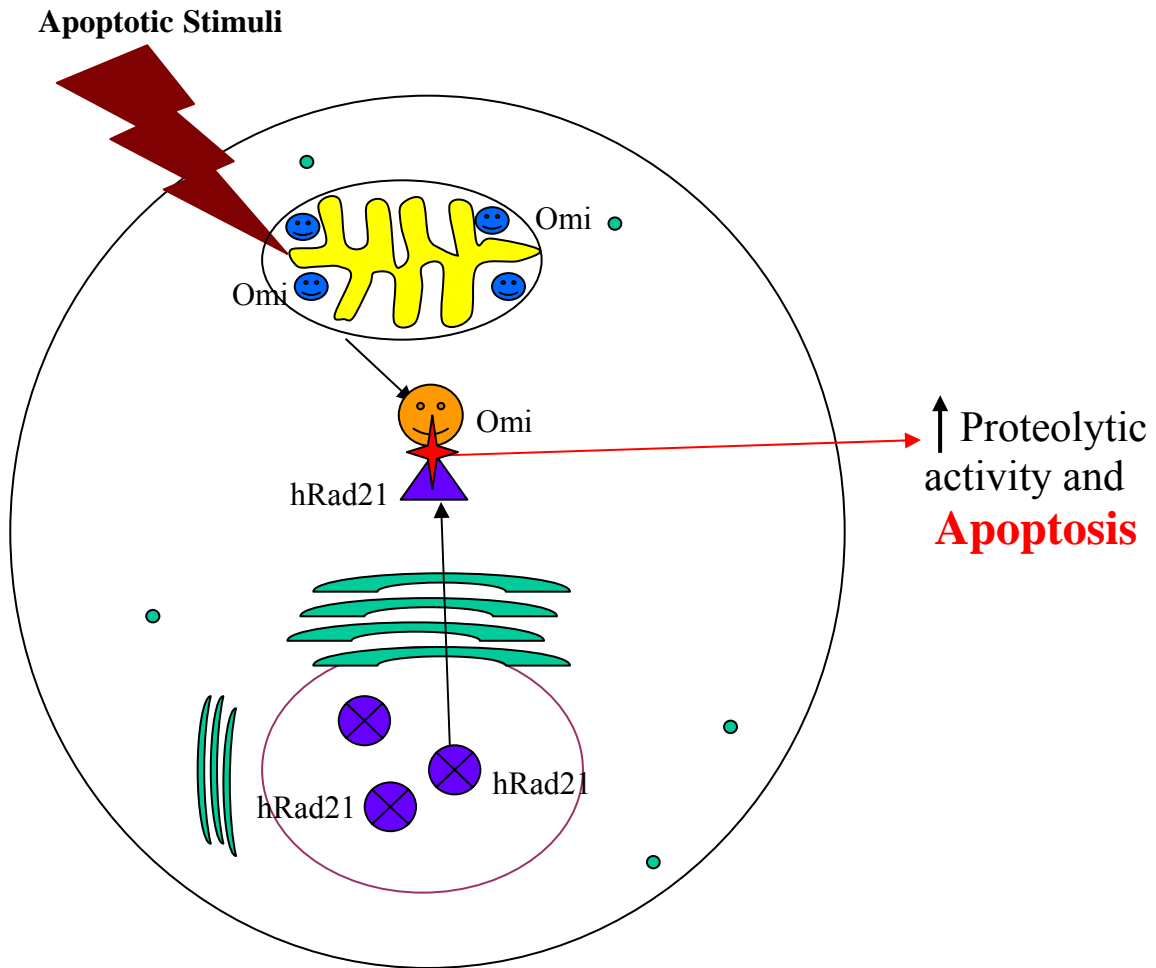
A screening of human melanocyte cDNA library was performed using a yeast-two-hybrid system to isolate novel Omi interacting proteins to further elucidate the function of Omi in the cell (Zervos Lab). This primary cDNA library was chosen for screening, with the assumption, that the primary melanocyte cells express physiological levels of various genes, unlike cancer cell lines that may have many deregulated pro- and anti-apoptotic genes.

Final selection was done on 32 true positive clones that were sequenced. Finally, 17 distinct genes are isolated and characterized using the NCBI Blast Search. Two different clones were chosen for further study: hRad21 (partial length) and full length Autophagin2. Human Rad21 is a nuclear protein and was first recognized in fission yeast, *Saccharomyces pombe*. The main function of hRad21 in yeast is DNA double-strand break repair after  $\gamma$ -irradiation (49). Human Rad21 is also involved in sister chromatids cohesion during the transition from metaphase to anaphase. Human Rad21 is the homolog of *S.pombe* Rad21 with a similar function. Like yeast Rad21, human homolog is a hyperphosphorylated protein. The estimated size of protein is 68-70kDa but has higher mobility of 110kDa on SDS-PAGE gels due to its hyperphosphorylation. The

N-terminus sequence of 267 amino acids is phosphorylated and has a role in DNA repair and sister chromatids cohesion. The C-terminus sequence is less phosphorylated and its basic function is still unknown (50). Some studies suggest that hRad21 is cleaved by caspase-3,-7 at Asp-279 after induction of apoptosis. The cleavage product of 64kDa then translocates to cytoplasm, where it enhances the activity of caspase-3 and apoptosis in an undefined manner (52, 53).

Human Rad21 was not cleaved by Omi but could still be a regulator of Omi by interacting with its PDZ domain. First, to show the specificity of interaction with the PDZ domain, the C-terminus of hRad21<sub>416-631</sub> and hRad21<sub>416-627</sub> were tested in yeast. After deletion of the extreme four carboxy-terminal amino acids of hRad21, it does not interact with the PDZ domain of Omi anymore. To show that, a synthetic peptide corresponding to the last 15 amino acids of hRad21 (YSDIATPGPRFHII) was used and it could specifically up-regulate the proteolytic activity of Omi in vitro. To investigate if these two proteins interact in mammalian cells before or after apoptosis, co-immunoprecipitation was performed. These experiments clearly showed that Omi and the truncated cytoplasmic form of hRad21 are indeed in a complex after induction of apoptosis. This is consistent with the fact that in normal cells these two proteins are in different subcellular compartments, Omi in the mitochondria and hRad21 in the nucleus. My studies suggest a model where hRad21 is co-operating with Omi after induction of apoptosis by directly regulating its proteolytic activity (Figure 32). Omi's proteolytic activity is necessary and essential for both caspase-dependent as well as caspase-independent cell death.





**Figure 32: Cartoon Showing the Interaction of Omi with hRad21**

Upon induction of apoptosis, the mitochondrial Omi and hRad21 both becomes cytoplasmic. Both of them interact in cytoplasm and enhance the apoptotic pathway.

Human Rad21 increases the proteolytic activity of Omi after they meet in cytoplasm.

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