

# **A Functional Study of the IMPAS Family of Proteins**

A Major Qualifying Project Report

Submitted to the Faculty of the

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the

Degree of Bachelor of Science

in

Biology and Biotechnology

by

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April 26, 2012

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## ABSTRACT

The IMPAS family of proteases is not well characterized, but some members may be associated with neurodegenerative diseases, such as Alzheimer's disease. In this project, potential IMPAS substrates and pathways were investigated *in vitro* by transfecting plasmids encoding various IMPAS proteins and potential substrates into HEK293 cells and monitoring the cellular levels of predicted substrate proteins by Western blots. The results show that ODZ4, Syntaxin 5A, and truncated ST14 ( $\Delta$ ST14) likely do not serve as substrates. Protease hIMP1 was validated to cleave HCV core protein, as in a previous report. A putative connection between IMPAS and the autophagy pathway was revealed.

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## **ACKNOWLEDGEMENTS**

Thanks to Dr. Evgeny Rogaev for sponsoring my senior project in his lab at the UMass Medical School, and for all your help through the project. His care and efforts towards my success were extremely appreciated. I would also like to thank Anastasia Grigorenko for all her knowledgeable assistance in the lab with trouble shooting, and her teaching me unfamiliar protocols was critical in completing this project. A thank you to the lab of Dr. Matsuura at the Research Institute of Microbial Diseases for providing the Hepatitis C virus core protein plasmid used in this project. Lastly, I would like to sincerely thank Dr. David Adams from WPI for helping initiate this project and for helping me with all the paperwork, writing, and all the little details along the way that made my project experience a great success.

# BACKGROUND

## Alzheimer's disease

Alzheimer's disease (AD) is a neurodegenerative disorder primarily affecting older individuals worldwide. AD gets its name from Alois Alzheimer, a German physician, who first described a condition he observed in 1906 in an early onset case, Auguste Deter, whom he treated for almost 5 years. Auguste displayed progressive memory impairment, unpredictable behavior, various speech and motor issues, recognition problems, trouble processing questions, and even hallucinations. Postmortem examination of the brain showed some disturbing brain alterations: cerebral cortex atrophy, senile plaques in the cortex, and neurofibrillary abnormalities (Grigorenko & Rogae, 2006). These physical and behavioral symptoms have become the hallmark signs of Alzheimer's disease.

The common early symptoms of AD include a variety of mental complications such as confusion, short term memory disturbances, attention difficulty, language issues, and even personality changes. Although, there is a basic outline of the stages of disease progression individuals are effected in different ways. The first stage, defined as mild, can last for 2-4 years, and can be stressing to the individual because in most cases the patient can perceive the mental changes at some level. In this stage, getting lost and losing things are a common occurrence; this, if they are still working, can begin to negatively affect job performance. Stage two represents the loss of individuality; although the patient can perform simple tasks alone, the drop into a more disabled life leads them to require much more care. This often results in depression, irritability, restlessness, and withdrawal. At this point there may be loss of the ability to read, write, communicate, and they may become a danger to themselves when left alone. Stage three is the most severe, and is generally characterized by complete dependence. At this stage patients need

help with meals, and cannot recognize people they have known for their entire lives. Even more severely they may lose control of bodily functions such as swallowing or bowel movements. Constant care is required due to the almost non-existent memory and complete physical dependence (Alzheimer's Disease Research, 2012). This disease is highly debilitating to the patient, and proves to be a great challenge for their families. As of now there is no cure for AD, which only makes such a diagnosis harder to bear as a patient or a family member.

AD affected an estimated 5.4 million Americans in 2011, 5.2 million of which were over the age of 65 (Alzheimer's Association, 2011). Thirteen percent of American's over the age of 65, approximately 1 in 8 people, have AD; and 43 percent, nearly half of people over the age of 85. AD remains the 6<sup>th</sup> leading cause of death in American's of all ages, and the 5<sup>th</sup> leading cause of death in individuals over the age of 65 (Alzheimer's Association, 2011). A startling fact to consider is that while the other major causes of death (stroke, heart disease, HIV/AIDS, prostate and breast cancer) have declined in the past 10 years, AD deaths increased (Alzheimer's Association, 2011). This statistic is affected by the trend of longer lifetimes in modern countries; however, the ever rising numbers of affected individuals indicates that this debilitating disease, and others like it, should be more greatly researched to try to decrease the incidence.

Characteristic histopathological changes in the brain of AD patients are neurofibrillary tangles and amyloid senile plaques, much like those seen in the original patient. Neurofibrillary tangles (NFTs) are intracellular conglomerates of tau protein found in neurons. They consist primarily of the abnormally phosphorylated microtubule associated protein tau. This distorted structure eventually leads to a loss of cell shape and cell death. Senile plaques are an extracellular insoluble spherical structure with a dense central core made up of  $\beta$ -amyloids ( $A\beta$ -peptides) which aggregate and are deposited in the brain, often adjacent to capillaries and larger

blood vessels (Cras et al., 1991; Grigorenko & Rogaev, 2006). A $\beta$  peptides are a product of amyloidogenic proteolysis of the conjugated protein  $\beta$ -amyloid precursor protein (APP) (Grigorenko & Rogaev, 2006; Paolo & Kim, 2011). These A $\beta$  peptides are neurotoxic, and result in the dementia and cognitive impairment associated with AD, even before the fibrils and plaques form (Grigorenko & Rogaev, 2006). The core of the plaque is surrounded by abnormal (dystrophic) neurites. Other than multiple forms of A $\beta$  peptides, senile plaques also consist of glucosaminoglycan (GAG), heparan sulfate proteoglycan (HSPG), serum amyloid P (SAP), apolipoprotein E (ApoE),  $\alpha$ 2-macroglobin,  $\alpha$ 1-antichymotrysin, and other complement factors (Grigorenko & Rogaev, 2006). These other factors may also play a critical role in the formation of plaques and neurofibrillary tangles.

### **Amyloid Precursor Protein**

Amyloid precursor protein (APP), present on the surface of neurons and glial cells, can be inappropriately processed by beta and gamma secretase to produce the A $\beta$  peptide that forms senile plaques. APP is a type I transmembrane protein, and functions as a receptor on the outer cell membrane (Grigorenko & Rogaev, 2006). The APP gene maps to chromosome 21, and several families (Indiana, London, Swedish, Dutch) with early-onset AD show mutations in APP (Goate et al., 1991). Early onset familial cases also map to the presenilin genes (gamma secretase) (see below) located on chromosome-14. However, late-onset cases do not show a link to known gene mutations.

## Presenilins

Presenilin proteins 1 and 2 (PSEN1/PS1, and PSEN2/PS2) are polytopic integral membrane proteins that span the membrane six to eight times. They are involved in the processing of the APP protein and in the Notch-signaling pathway (Tomita et al., 2001; Grigorenko & Rogaev, 2006). Specifically, they alter the selectivity of cleavage by the  $\gamma$ -secretase complex of type I transmembrane proteins, such as APP (Grigorenko et al, 2002; Xia & Wolfe, 2003). This APP cleavage is site-specific, and cuts within a single transmembrane domain (Moliaka et al., 2004). Endoproteolysis occurs to these type I proteins, generating N- and C-terminal fragments (NTF and CTF) which form heterodimers which are incorporated into high molecular weight (HMW) protein complexes. These HMW complexes are tightly related to the functionality (Tomita et al., 2001).

Mutations in the APP processing mechanisms result in an increased production of the senile plaque forming A $\beta$  peptides (Grigorenko & Rogaev, 2006). Both of the PSEN proteins contain a proline-alanine-leucine-proline (PALP) motif which is critical to the function. The first proline is correlated with  $\gamma$ -secretase function and cell metabolism functions (Tomita et al., 2001). PSENs are highly conserved, and can be found in a wide variety of organisms including: protists, plants, invertebrates, and vertebrates. However, they are not found in bacteria or fungi (Tomita et al., 2001; Grigorenko & Rogaev, 2006). The highly conserved nature of these proteins indicates a level of high importance. The AD mutations in APP, PS1, and PS2 are primarily inherited in an autosomal dominant manner (Grigorenko & Rogaev, 2006), and are critical in early-onset AD.

## IMPAS

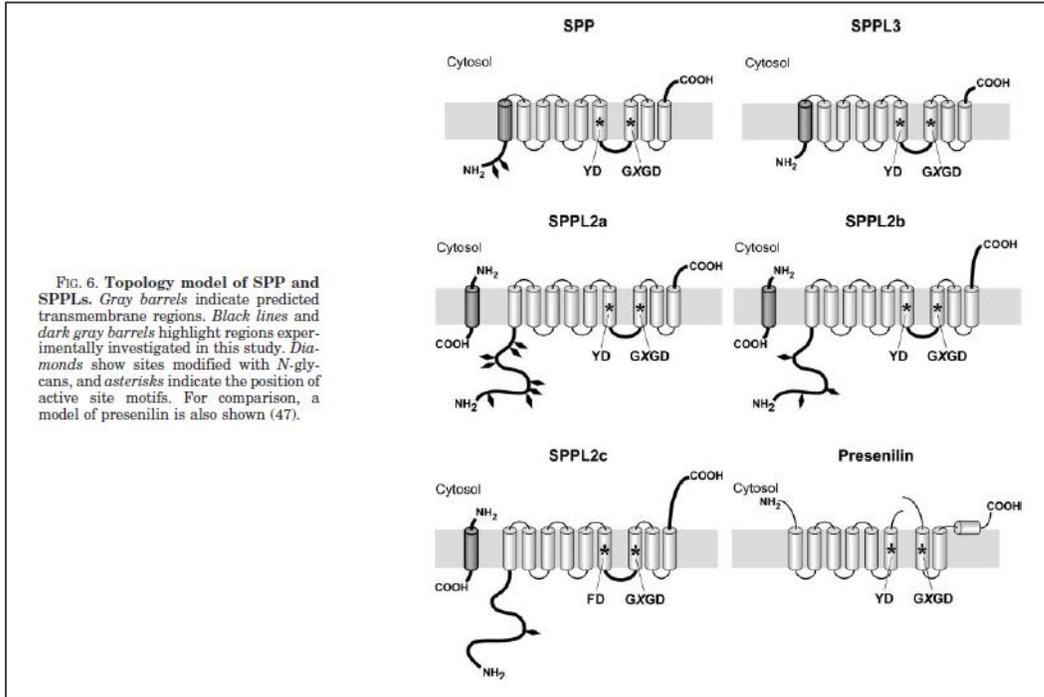
The IMPAS (intra-membrane-aspartic-protease) proteins are a group of multi-pass trans-membrane proteins, related in homology to the PSEN transmembrane domains (Xia & Wolfe, 2003; Moliaka et al., 2004). More specifically, these proteins belong to the GXGD-type aspartyl protease family, in which aspartate is included in the active site motif within the transmembrane domain-7 (Martin et al., 2009). IMPAS proteins, when named using the IMPAS system (**Table-1**) are ordered from the most to the least conserved in evolution; although the proteases are also known by other names.

**Table-1: Classification of Various IMPAS Proteins.**

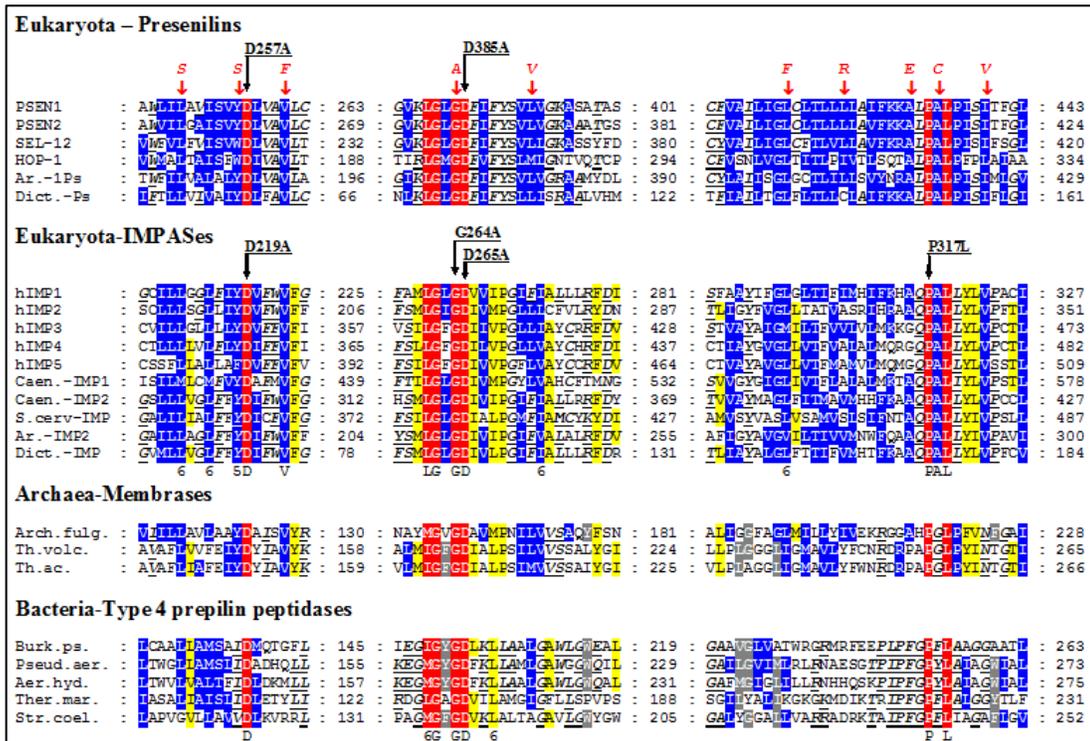
<b>Chromosome</b>	20	12	15	19	17
<b>Location</b>	20q11.21	12q24.31	15q21.2	19q13.3	17q21.31
Protein Group Classification Parallels					
<b>IMP</b>	IMP1	IMP2	IMP3	IMP4	IMP5
<b>SPP</b>	SPP	SPPL3	SPPL2A	SPPL2B	SPPL2C
<b>PSH</b>	PSH3	PSH1	PSH5	PSH4	PSH2
<b>PSL</b>	PSL3	PSL4	PSL2	PSL1	PSL5

The basic structure of different IMPAS proteins is shown in **Figure-1**. Three general structures are shown, including IMP1, IMP2 (upper row), IMP3, IMP4, and IMP5 (middle and lower rows) and Presenilin (lower row, right). The diagram especially shows the transmembrane regions and orientation within the membrane. The amino acid sequences of a few of the highly conserved regions are shown in **Figure-2**. The PAL motif in PS proteins is also conserved in the IMPAS proteins. Two other highly conserved regions are the first (DxxV) and second (GxGD) aspartate domains present in all IMPs, also found in presenilins (Grigorenko et al, 2002). It has been found that the catalytic domain is embedded in the cell membrane oriented opposite of the presenilins and has the capability to cleave type II oriented transmembrane proteins (Friedmann

et al, 2004; Grigorenko & Rogaev, 2006). Members in this group of intramembrane-cleaving proteases are predicted to cut type II oriented substrates (Friedmann et al, 2004).



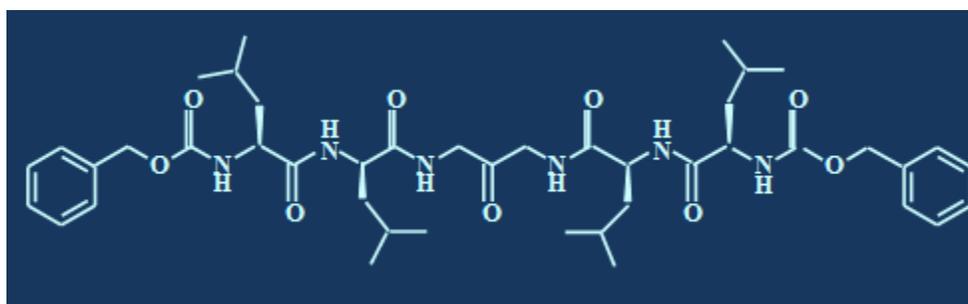
**Figure-1: Diagram of the Basic Structure of IMPAS Proteins.** Several topology models of SPP (IMP1), SPPL3 (IMP 2), SPPL2A (IMP3), SPPL2B (IMP4), SPPL2C (IMP5), Presenilin are shown. (Friedmann et al., 2004)



**Figure-2: Amino Acid Alignments of Three Main Conserved Regions in the IMPAS Proteins. (Gringorenko et al, 2002)**

Impas-1 (IMP1), a bi-aspartic polytopic protease, is capable of cleaving transmembrane precursor proteins and signal peptide peptidases, and may have other functions that are still unknown. In experimental cell culture conditions, induced IMP1 was found to have the capability to cleave the PS1 protein. IMP1 was discovered to be responsible for liberating hydrophobic signal peptides from the N-terminus of secreted or transmembrane proteins during co-translocation into the endoplasmic reticulum (Martin et al., 2009). IMP1 has also been found to be crucial in the generation of cell surface histocompatibility antigen-E epitopes, and the processing of hepatitis C virus polyproteins (Martin et al., 2009). However, the full role and substrates of endogenous IMP1 remain undetermined (Moliaka et al., 2004).

Ce-IMP-2 (a homolog of human IMP1) has been found to be critical to *Caenorhabditis elegans* development (Grigorenko et al., 2004). Inactivation of Ce-IMP2 results in embryonic lethality, and in molting defects arresting development in the larval stage. (Z-LL)<sub>2</sub>-Ketone (**Figure-3**) is a IMP1-specific inhibitor, used to help identify the function of this interesting protein. The highly conserved nature of IMP1 makes it of great interest because of its potential influences on neurodegeneration and normal development.



**Figure-3: Structure of (Z-LL)<sub>2</sub>-Ketone, An IMP1-Specific Inhibitor.**

Impas 2 (IMP2) is another of these presenilin-like proteases found in the endoplasmic reticulum. This locational similarity to IMP1 would imply that this protein may also have signal peptide removal functions as well (Martin et al., 2009). IMP2, combined with the known Site-1 protease (S1P) and site-2 protease (S2P), are required for the activation of membrane transcription factor ATF6 – a necessary mediator of the mammalian ER unfolded protein response (Kobialko, 2007). In *Danio rerio* (zebrafish) when the IMP2 homolog was knocked down, the result was cell death, indicating it is critical for development (Nyborg et al., 2006).

Impas-3 (IMP3) and Impas-4 (IMP4) are other homologs in this protease family which are 50% identical and 70% homologous to each other (Behnke et al., 2011). However, IMP3 is expressed in all major adult human tissues, while IMP4 is detectable chiefly in the adrenal cortex and mammary gland (Behnke et al., 2011). After an initial cleavage of the Bri2 fragment by furin

and related proteases, IMP3 and IMP4 execute proteolysis on the N-terminal transmembrane fragment of Bri2 (Martin et al., 2008). These two proteases have also been studied in relation to TNF $\alpha$  and have been found to perform an active role in processing this cytokine in dendritic cell cultures to release a reverse signal component TNF-ICD (Friedmann et al., 2006). Murine IMP3 has been found to contain a single tyrosine-based motif that mediates its transport to the lysosome; this motif is highly conserved as shown in a multispecies alignment (Behnke et al., 2011). IMP3 and IMP4 may play a critical role in the processing of transmembrane proteins and in signaling pathways involving the lysosome.

Impas-5 (IMP5) is the least conserved of the known proteases in this group, with no determined substrate or function. The substrate or substrates are most likely a type II oriented transmembrane protein. IMP5 has also been found only in humans and at low expression (Grigorenko & Rogaev, 2006). More research needs to be done to determine the function of this most specific member of the IMP family of proteins.

## IMPAS Substrates

### HCV

Hepatitis C Virus (HCV) is a blood born pathogen causing Hepatitis C, a potentially chronic liver disease which can result in long term health complications and death (CDC, 2009). The HCV genome consists of a single-stranded, positive sense RNA molecule encoding a polyprotein in its only open reading frame (McLauchlan et al., 2002). This protein is approximately 3000 amino acids long, which can be divided into two parts: The first is the N-terminal third which makes up the core and two envelope proteins (E1 and E2). The remainder consists of the non-structural virion proteins (McLauchlan et al., 2002). Viral products are

produced during maturation through a series of cleaving events to the polyprotein (McLauchlin et al., 2002). Hepatitis core protein, the substrate used in this project, is generated during maturation from the most N-terminal region and is 191 amino acids in length (McLaughlin et al., 2002; Boulant et al., 2005). It contains a 50 amino acid hydrophobic region, D2, which is responsible for core association with lipid droplets and also with the ER (Boulant et al., 2005). A 20 aa signal sequence targets the protein to the membrane of the endoplasmic reticulum (ER) and later serves as a signal to the downstream E1 protein (Boulant et al., 2005). A signal peptidase cleaves the signal sequence, leaving the Hepatitis core protein anchored in the ER membrane (McLaughlin et al., 2002). IMP1 has been found to cleave the hepatitis core protein within the ER membrane allowing for release from the membrane and further trafficking (Okamoto et al., 2008). However, if there is any mutation in the signaling sequence, then cleavage by IMP1 is eliminated and the HCV core protein remains stuck in the ER membrane (McLauchlin et al., 2002).

## ODZ4

ODZ4 is a type II transmembrane protein that is highly expressed in the brain and less in other tissues (Zhou et al., 2003). It belongs to the teneurin/odd oz (TEN-M/ODZ) transmembrane protein receptor family which is highly conserved, and can be found in *Caenorhabditis elegans* (roundworms), *Danio rerio* (zebrafish), mice, and humans (Oohashi et al. 1999; Lossie et al., 2005). Four main ODZ4 homologs have been identified in mice and humans (ODZ1-4); it was found that these four types show separate but sometimes overlapping expression in the brain during development of mouse pups (Zhou et al., 2003). These distribution patterns may mean that these transmembrane proteins relate to neuronal connectivity

(Kenzelmann et al., 2007). The ODZs may be a novel substrate for a transmembrane protease; extending the function from an extracellular adhesion molecule to also being a signaling molecule (Zhou et al., 2007). Multiple mutations in the *ten-m/odz* gene have been found to result in embryonic lethality in mice (Zhou et al., 2003). Other popular models for studying this family of proteins are *Caenorhabditis elegans* and *Drosophila melanogaster* (fruit fly).

## Syntaxin 5A

Members of the syntaxin and synaptobrevin families form complexes, and promote vesicle docking and fusion (Dascher et al., 1994). Proteins in the syntaxin, synaptobrevin and SNAP-25 families all comprise a group called SNARE proteins which form a stable complexes that act as receptors (Xu et al., 2002). Syntaxins are made up of a cytoplasmic domain, a single hydrophobic membrane anchor, and an extracellular carboxy-terminal tail (Dascher et al., 1994). Syntaxin 5A (STX5A) is a component of the transportation mechanisms involved in transfer of material from the endoplasmic reticulum to the golgi body in mammalian cells (Dascher et al., 1994). Experiments using nonviable STX5A without a transmembrane domain, or inhibition via antibodies, suggests that STX5A is required for the fusion of carrier vesicles at the cis-face of the Golgi body (Xu et al., 2002). It has also been shown that hypomorphic mutations in STX5A lead to sterility in male *Drosophila melanogaster* due to failure in cytokinesis as well as sperm maturation (Xu et al, 2002). After mitosis, STX5A has been found to be necessary to reassemble of the Golgi complex (Xu et al., 2002). STX5A is a critical protein in the function of the Golgi body, and in turn many signaling pathways, mitosis, and reproduction.

## ST-14

ST-14 encodes a large protein which functions as a type II transmembrane serine protease (Khang et al., 2005). It has been found co-localized in cell-to-cell contact sites in epithelium cells of the thymus with E-cadherin, and may have a role in cell adhesion (Khang et al., 2005). Knockout mouse pups lacking in this gene have severely abnormal skin development, and suffer fatal dehydration due to the malfunctioning epithelial barrier (Khang et al., 2005). ST-14 also plays a critical role in cell adhesion during pre-implantation development, as well as other stages of early development (Khang et al., 2005). There are two forms of this protein generally found within cells: the full length protein and the N-terminally truncated protein (referenced going forward as  $\Delta$ ST-14). Experiments using *in vivo* cell labeling indicate that in cell culture the long form is quickly processed into the truncated form.  $\Delta$ ST-14 has been found in cell culture media and on the cell surface (Cho et al., 2001). It has been found that this protein may functions in cell migration, as well as cancer invasion and metastasis (Cho et al, 2001). This protein is a type II-oriented protein which has critical roles in development; it is a potential substrate of one of the members of the IMPAS family of proteases.

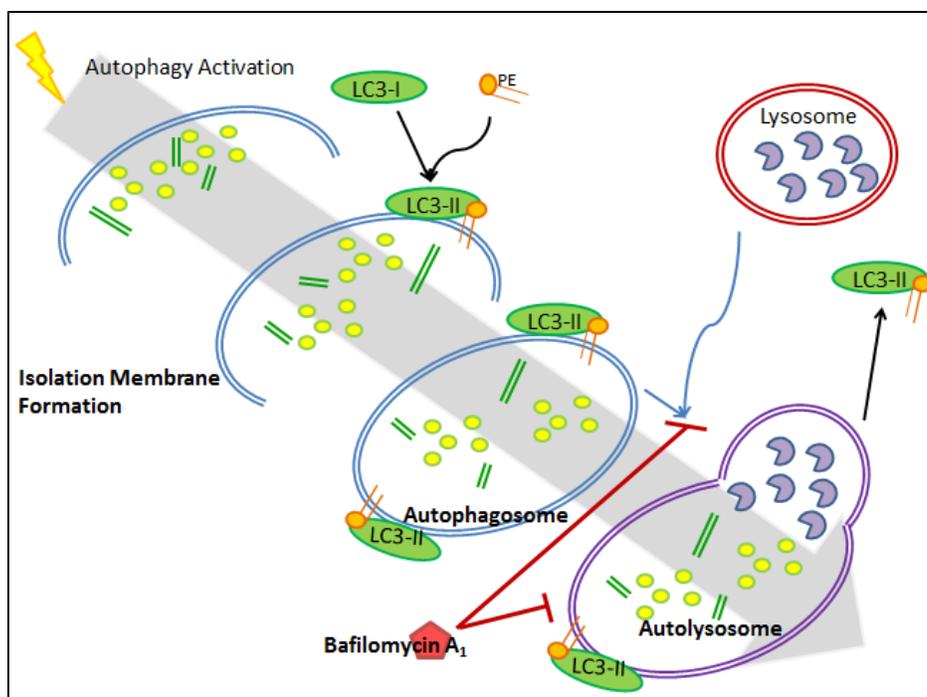
## Autophagy

The field of autophagy, the mechanism relating to cells undergoing prograded cell death during starvation, is a fast growing area of study, and there are many factors required that can still be explored. Autophagy is a cell process using a cell's lysosomal mechanisms to degrade its own components (Fader et al, 2009). In this process, autophagosomes are produced, which are vesicles that capture and deliver cytoplasmic material and bring them to the lysosome (Rabinowitz & White, 2010). The autophagosomes fuse with endosomes and/or lysosomes

forming amphisome and autolysosomes, respectively, as part of the maturation process and the autophagy pathway (Pankiv et al., 2007). Large cell structures including organelles and protein aggregates can be broken down by lysosomes when the cell reaches a state of macroautophagy- or a programmed cell death- resulting from a cellular stress. In a state of starvation, macroautophagy is activated to provide necessary nutrients for survival (Rabinowitz & White, 2010). Basal autophagy is a routine function in the cell which rids the cell of toxins and waste products (Rabinowitz & White, 2010). Bafilomycin A<sub>1</sub> is an inhibitor of autophagy which changes the lysosomal pH and blocks the ability for the autophagosome and the lysosome to bind (Klionsky et al., 2009).

The IMPAS proteases may play a role in the autophagy pathway by potentially activating components, releasing other signaling mechanisms, or cleaving protein compounds for degradation. Accumulation of autophagosomes, required in the function of the pathway, can be measured using various techniques including electron microscopy image analysis with fluorescent GFP-LC3 markers or using LC3 lipidation screening via Western blotting (Klionsky et al., 2009), results of which can lead to the discovery of more potential autophagy related factors.

Light chain 3 (LC3), a human homolog of Atg8, is a ubiquitin-like protein used as an autophagic marker due to its fluctuation during the autophagy response (Pankiv et al., 2007). The role of LC3 in the autophagy process is shown in **Figure-4**, notice how it binds to the membrane and remains in that location. The blocking role of Bafilomycin A<sub>1</sub> is also shown in the figure.



**Figure-4: Diagram of the Role of LC3 Protein in Autophagy.**

There are three main forms of LC3, the first of which is pro-LC3, the unprocessed form, which is cleaved post-synthesis by Atg4B exposing the C-terminal end resulting in LC3-I. The LC3-II form has phosphatidylethanolamine (PE) conjugated to the C-terminal end which is related with the formation of autophagosomal membranes (Pankiv et al., 2007). The lipidated LC3-II form remains on mature autophagosomes until after fusion with lysosomes and therefore makes LC3 a good marker for autophagy (Rabinowitz & White, 2010). An important point is that the overall level of LC3 protein may not change, but the variance in levels between the two different LC3 forms can indicate what is being affected in the pathway (Klionsky et al., 2009). Western blotting is an easy way to monitor the changes between these two forms in the autophagy pathway; when there is autophagosome production, there is an increase in LC3-II formation which is observed as a second lower band in Western blot analysis. LC3-I runs at 18kD during western blotting, while LC3-II run at 16kD. However, it was found to be

unsuccessful when using a *Drosophila melanogaster* model. Another concern is that LC3-I is more sensitive to freezing, thawing and degradation in SDS sample buffers; therefore, the samples should be processed as soon as possible (Klionsky et al, 2008).

## **PROJECT PURPOSE**

The cellular functions of the IMPAS family of proteases are not well known, but some family members may be associated with neurodegenerative diseases, such as Alzheimer's disease. This project will investigate the potential substrates of some IMPAS family members in HEK293 cells transfected with plasmids encoding various IMPAS family members and plasmids encoding potential substrates. Western blots are then used to monitor the molecular weights of potential substrate proteins to determine whether cleavage was induced in cells over-expressing the IMPAS protein. The project will focus on potential type II oriented transmembrane protein substrates for these proteases. And the potential effects on autophagy are also observed. Uncovering the function of the highly conserved and critical IMPAS proteases could promote discoveries the field of neurodegenerative diseases.

## METHODS

### Plasmid Constructs Used

Several plasmids were used throughout this project (see Tables below) that encoded a variety of proteins of interest. These plasmids were constructed by other personnel in our laboratory, except for HCV which was provided by the Dr. Matsuura's lab. Plasmids containing IMP2 and IMP3 WT were provided by Applied BioSystems. The vector containing PS22 was provided by Mayo Clinic (USA).

<b>IMPAS Constructs</b>		
<b>Inserted Gene</b>	<b>Plasmid Name</b>	<b>Expressed Protein</b>
IMP1 WT	pcDNA4 Myc HisB	Intramembrane Protease 1 Wild-type
IMP1 D219A	pcDNA4 Myc HisB	Intramembrane Protease 1 with mutation at 1st aspartic acid conserved region to an alanine.
IMP1 D265A	pcDNA4 Myc HisB	Intramembrane Protease 1 with mutation at 2nd aspartic acid conserved region to an alanine.
IMP1 P317A	pcDNA4 Myc HisB	Intramembrane Protease 1 mutation in conserved PALP region: 1st Proline to alanine
IMP1 P317L	pcDNA4 Myc HisB	Intramembrane Protease 1 mutation in conserved PALP region: 1st Proline to leucine (a chemically similar amino acid)
IMP1 A318D	pcDNA4 Myc HisB	Intramembrane Protease 1 mutation in conserved PALP region: alanine to aspartic acid
IMP2 WT	pCMV SPORT 6	Intramembrane Protease 2 Wild-type
IMP3 WT	pCMV SPORT 6	Intramembrane Protease 3 Wild-type
IMP4 WT	pcDNA4 Myc HisB	Intramembrane Protease 4 Wild-type
IMP4 D359A	pcDNA4 Myc HisB	Intramembrane Protease 4 with mutation at 1st aspartic acid conserved region to an alanine.

Substrate Constructs			
Inserted Gene	Plasmid Name	Expressed Protein	Chromosomal Location
HCV	pcDNA3 Flag tag	Hepatitis Core Virus	
STX 5A	pcDNA3 Flag tag	Syntaxin 5A	11q12.3
ODZ4	pcDNA3 Flag tag	odd Oz/ten-m homolog 4	11q14.1
$\Delta$ ST-14	pcDNA3 Flag tag	suppression of tumorigenicity 14-processed/shortened form	11q24-q25

### Plasmid Cloning and Isolation

Plasmids were transformed into DH5 $\alpha$  cells, a high efficiency competent cell line (Invitrogen). 2  $\mu$ l of plasmid DNA (approximately 400-600 ng) was transformed into 30  $\mu$ l of the cell mix using the provided Invitrogen protocol. The plasmid conferred ampicillin-resistance; therefore all selections were performed by plating onto ampicillin plates. Selected colonies were cultured overnight in 4-5mL of LB broth with ampicillin at 37°C with shaking. The plasmids were isolated using QIAprep Spin Miniprep kits (QIAGEN) following their Plasmid DNA Purification Procedure with a few changes to increase the efficiency, including changes to introducing the DNA to the column and in eluting. Before spinning down the sample in both steps, the liquid was allowed to incubate in the column for approximately 5 minutes, after which it was centrifuged for 2 minutes at 800 rpm, then for 1 minute at 13,200 rpm. The elution step was modified to be performed twice, each time with half the listed elution volume. For the second elution, the column was rotated 180 degrees from its original orientation in the centrifuge to ensure a more even elution. The final plasmid DNA concentration was measured using a Nanodrop procedure. After cleaning the machine's stage and blanking it, 1  $\mu$ l of the plasmid

DNA sample was placed onto the stage. A reading of the purity and concentration was then taken.

## Sequencing Reactions

Plasmids were sequenced to verify the gene inserts. All constructs were sequenced from a T7 primer is located upstream of the multiple cloning site containing the inserted DNA.

Plasmids IMP1 and IMP1D265A were sequenced additionally with a BGH primer, and IMP2 and IMP3 were also screened with M13 forward and M13 reverse primers. The reaction mixture contained 2  $\mu$ l of the purified plasmid DNA template, 2  $\mu$ l of 1  $\mu$ M primer, 4  $\mu$ l of DEPC-treated H<sub>2</sub>O, and 2  $\mu$ l of BigDye. The mixture was then run in a thermal cycler with the following program: 28 cycles of 1 minute at 95°C, 1 minute at 55°C, 4.5 minutes at 60°C; followed by a 4°C holding temperature. Samples were then brought to the Deep Sequencing Core at the UMass Medical School.

The obtained DNA sequences were then searched on NCBI to confirm the gene identity. The IMP1 and IMP1 D265A genes were identical except for an intended single nucleotide change which results in the protein structural change of D265A, the intended mutation at this second highly conserved aspartic acid site. Sequence analysis was done using the freeware program Chromas Version 2.33. The other IMPAS mutation genes had been previously verified.

## Cell Culture

This project used human embryonic kidney cells (HEK293). These cells were obtained from ATCC, and grown on 10mm plates in DMEM High Glucose 1X media (Invitrogen) supplemented with 10% Fetal Bovine Serum, 1% Antibiotic-Antimycotic liquid (Invitrogen), and 1% Nonessential amino acids (NEAA). The day before transfection, the cells were split onto 6-

well plates in 3 mL of media. The cells were adherent and were detached using TrypLE Express (Life Technologies) through a 5 minute incubation period at 37°C. The cells were then plated at approximately 60-70% confluence and allowed to adhere and grow overnight (to approximately 80% confluence).

## Transfections

All transfections were done using Lipofectamine 2000 (Invitrogen) following the provided protocol and recommendations. HEK293 cells were used for all experiments, and were cultured in 6-well plates to approximately 70-80% confluence at the time of transfection. 4 µg of plasmid DNA was used per well on a 6-well plate. Therefore, in dual transfections, 2 µg of each plasmid were transfected. 1.5 mL of media was removed from the plates before plasmid addition, therefore after plasmid addition there was a 2 mL total volume in the well. Lipofectamine is incubated first in Opti-Mem Low Serum Media (Invitrogen) for 5 minutes. Before addition to the cells, plasmids were incubated for 20 minutes in 0.5 mL Opti-Mem Low Serum Media (Invitrogen) with Lipofectamine. (Z-LL)<sub>2</sub>-ketone (CalBiochem), an IMP1 inhibitor, diluted in DMSO was added to a concentration of 40 µM. DMSO was also used as a control at the same concentration because it was used for dilution. The transfected cells were allowed to grow 24 hours before collection and cell lysing.

## Cell Lysates

Cell lysates were prepared in two different ways. The medium was first removed from the plate and the cells were detached using 5 mL of PBS buffer (Invitrogen) and collected into a 15mL Falcon tube. The cells were spun down for 5 minutes at 12,000 rpm. The PBS was then removed without disturbing the pellet.

For standard protein isolation, 200  $\mu$ l of RIPA buffer (Thermo Scientific) was added, and the cells incubated on ice for 20-30 minutes and sonicated using the Sonifier 150 (Branson).

For membrane-specific protein isolation, the cells were resuspended in HEPES buffer (Invitrogen) and immediately flash frozen in dry ice. The samples at this point were stored overnight at  $-80^{\circ}\text{C}$ . The following day, the cells were thawed slowly on ice for 1 hour. The samples were then mixed six times using a 22G syringe (Becton Dickinson) and centrifuged for 1 minute at  $1,000\times g$  ( $4^{\circ}\text{C}$ ), the resulting pellet was the nuclear fraction. The supernatant and pellet were stored at  $-80^{\circ}\text{C}$ . The supernatant was then centrifuged for 1 hour at  $16,000\times g$  ( $4^{\circ}\text{C}$ ), and the supernatant was then removed and stored at  $-80^{\circ}\text{C}$ . The pellet, containing the membrane fraction, was then resuspended in 100  $\mu$ l of RIPA buffer (Thermo Scientific) and allowed to incubate on ice for 20-30 minutes and sonicated.

## Western Blots

Each protein lysate sample was measured via spectrophotometry to determine total protein concentration. 1.0  $\mu$ l of protein lysate was placed in a cuvette with 999  $\mu$ l of QuickStart™ Bradford Dye Reagent 1x (Bio-Rad). After blanking the spectrophotometer, a standard curve was produced using QuickStart™ Bovine Serum Albumin (BSA) Stand Set. All samples were then measured and then approximately 100 to 500ng protein of each sample was prepared for Western Blotting using Lane Marker Sample Buffer (Thermo Scientific). The samples were boiled at  $95^{\circ}\text{C}$  for 5 minutes, and then allowed to cool for 5 minutes before being centrifuged for 1 minute at 13,200 rpm to remove insoluble debris. The samples, and the MagicMark XP Western Blot Protein Standard (Invitrogen), were then loaded onto the wells of a 15% acrylamide gel, and run at 100 V for approximately 90 minutes until the sample dye

reached the bottom of the gel. The gel was then set up for transfer onto a methanol-activated Immobilon Transfer Membrane (Millipore). Single membrane transfers were performed for approximately 2-3 hours at 35 volts, while double membrane transfers required 4 hours at 35 volts.

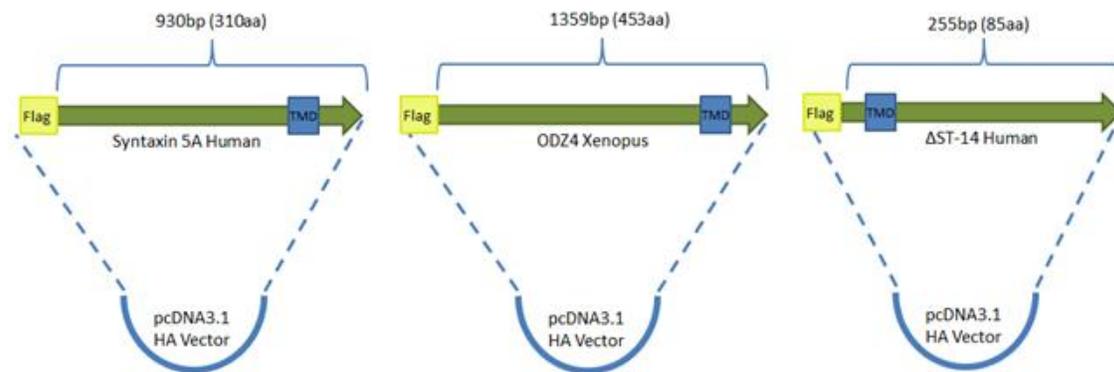
The membranes were blocked using 0.5% Blotting-Grade Blocker (nonfat dry milk) (BioRad) dissolved in TBST (TBS buffer supplemented with 0.1% Tween). Membranes were then stained with primary antibodies: Anti-flag antibodies (*Sigma* F1804) for screening of the substrates, or anti-LC3 antibodies (LC3B D11, XP Rabbit mAb (Cell Signaling) for autophagy studies. After washing thoroughly with TBST, the membranes were incubated with secondary antibodies: anti-mouse antibodies for the anti-flag antibodies, and anti-rabbit for the anti-LC3 antibodies. After a second wash cycle with TBST-Tween, the membranes were developed for imaging using SuperSignal West Dura Extended Duration Substrate kit (Thermo Scientific) following the standard protocol. Digital images were captured at various exposures using the VersaDoc Imaging System (BioRad). The membrane was stripped of antibodies by incubating for 30 minutes in Restore™ Western Blot Stripping Buffer (Thermo Scientific), and the membranes were again blocked in milk and stained with appropriate antibodies, if desired.

Membranes were also stained for GAPDH which is used to normalize the quantity of protein loading. This is important if the results are quantitatively examined. The antibody used is anti-GAPDH (Ambion). The secondary antibody is anti-mouse. All procedures were done as previously mentioned.

# RESULTS

## Substrates

In this project, the function of the IMPAS (IMP) family proteases was investigated to identify potential substrates. First, various type II transmembrane proteins were investigated as potential substrates by overexpressing various IMPs in HEK293 cells along with potential substrates in co-transfection experiments, and using western blots to monitor potential substrate cleavage. **Figure-5** shows the plasmid constructs encoding three example substrates to be assayed. The IMPAS protease and potential substrate were over-expressed in human embryonic kidney (HEK293) cells allowing for potential cleavage. The cells were then collected, lysed, and analyzed by Western blotting for substrate cleavage. The expected sizes of the uncleaved and cleaved substrate proteins are listed in **Table 2**.

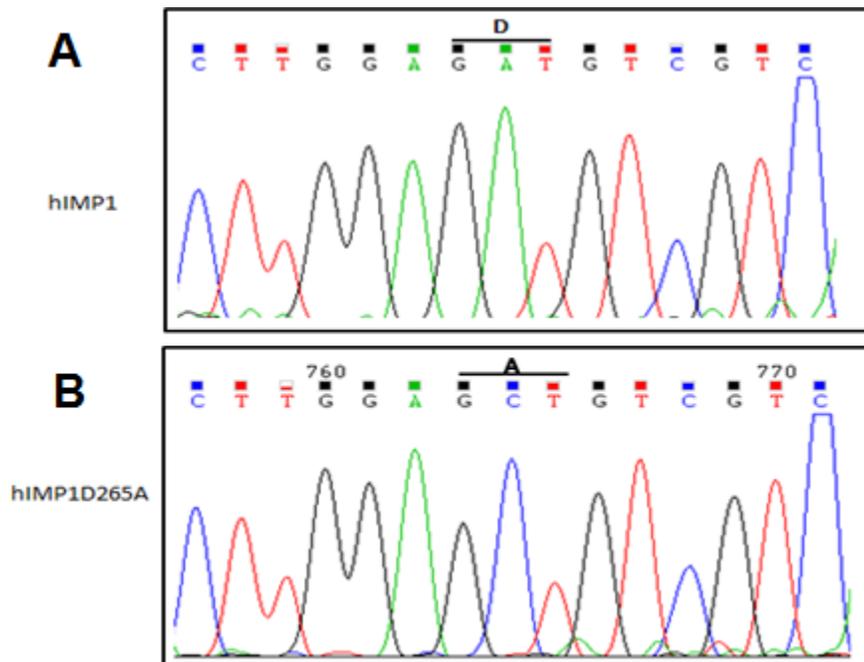


**Figure-5: Example of Three Plasmid Constructs Encoding Potential Substrate Proteins.** The substrate genes are cloned into pcDNA3.1 HA vector including a flag tag (yellow Flag) and a transmembrane domain (TMD, blue). The TMD is the expected cleavage region by the IMPAS proteases

**Table-2: Expected Substrate and LC3 Marker Protein Sizes, Uncleaved and Cleaved.**

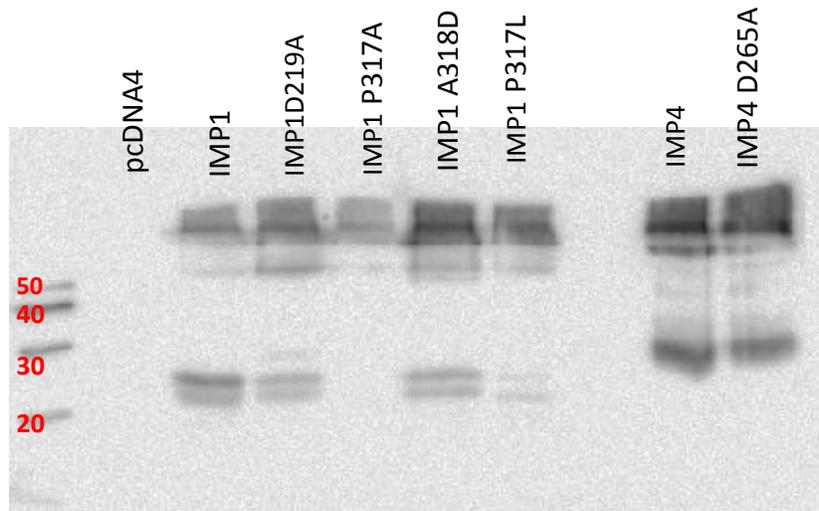
<b>Potential Substrate and LC3 Marker Proteins</b>	<b>Predicted Molecular Weight Uncleaved</b>	<b>Predicted Molecular Weight Cleaved in TMD</b>
Syntaxin 5A	35.09 kD	34.19 kD
ODZ4	50.57 kD	42.63 kD
$\Delta$ ST-14	15.02 kD	12.85 kD
LC3-I	18 kD	N/A
LC3-II	16 kD	N/A
HCV	25 kD	23 kD

The genes encoded in each of the plasmid constructs were sequenced to confirm identity, and the sequences were all confirmed relative to the NCBI database. The D265A mutant of IMP1 contains a single amino acid change, caused by a single base pair mutation, so the sequences were screened more closely, using a Chromas2 program (**Figure-6**). The correct IMP1 D265A mutation was confirmed (**Figure 6B**).



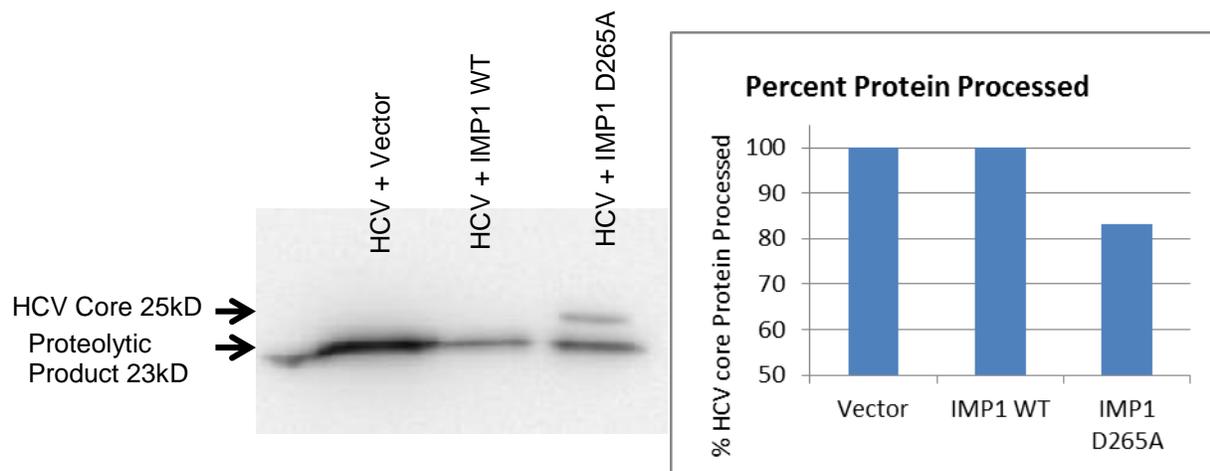
**Figure-6: Sequencing Data for WT hIMP1 and hIMP1D265A.** DNA sequence is color coded corresponding to the base pair. The triplet coding for the mutated amino acid region is labeled using a black bar lying above it, and the single letter code for the amino acid.

The expression of the IMP proteases in the HEK293 cells was confirmed by immunoblots. The plasmids containing the genes coding for IMP1, IMP4, and the mutants of each were transfected, and the myc-tagged proteins were identified by immunoblots (**Figure-7**). The vector (pcDNA4) was tested as a control as it does not express the myc tag. The expression of IMP1 and IMP4 was confirmed. As expected, the negative control, vector pcDNA4 had no expression. IMP1, IMP4, and all of their variants show expression in the model HEK293system.



**Figure-7: Verification of IMP1 and IMP4 Protease Expression in Transfected Hek293 cells.** The proteins are visualized with anti-myc primary antibodies which bind to the myc-tag on the proteins which is encoded by the plasmid vector. The first lane shows the MagicMarkXP (Invitrogen) size marker.

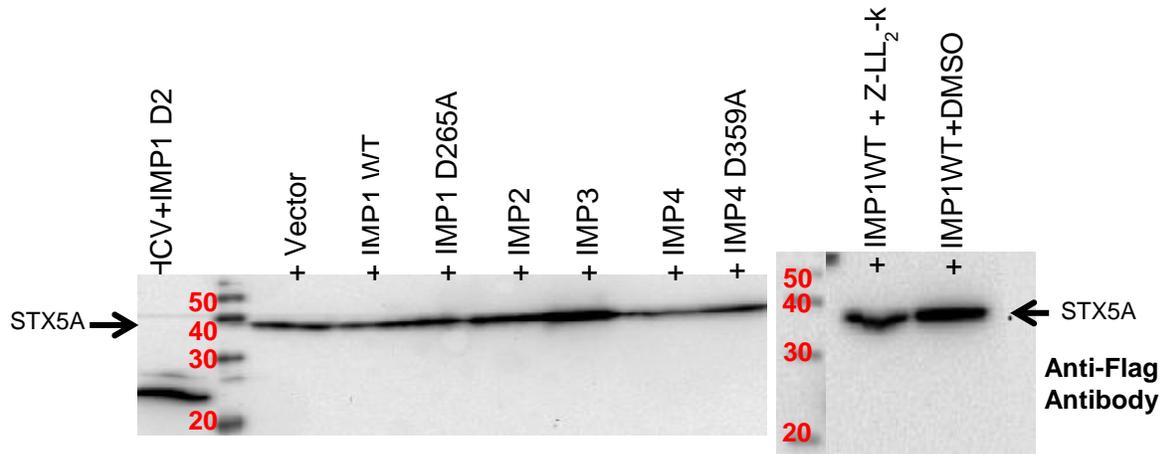
Next, the Hepatitis C virus (HCV) core protein was tested as a potential substrate for protease hIMP1, based on preliminary data indicating it might act as a substrate (Okamoto et al, 2008). The data (**Figure-8**) show that vector by itself (left lane) does not affect the ability of endogenous IMP1 to process the HCV protein, leaving only the proteolytic product. The addition of ectopically expressed wild-type hIMP1 (middle lane) still allows for the complete processing of the protein. However, the addition of hIMP1 mutated within the second conserved aspartate region (D265A) (right lane) inhibits the ability of the endogenous IMP1 to process the HCV core protein, shown in the higher molecular weight unprocessed HCV protein band. Approximately 20% of the HCV core protein remained unprocessed when the mutant IMP1 protein was expressed. Thus, protease hIMP1 may indeed act to cleave HCV core protein.



**Figure-8: Cleavage of HCV Core Protein by hIMP1 in HEK293 Cells.** Mutant IMP1 (D265A) (right lane) inhibits the proteolytic activity of the HCV core protein substrate in the endogenous and ectopically expressed IMP1 leaving upper unprocessed form. The membrane is treated with anti-flag antibodies to visualize the flag-tagged HCV core protein.

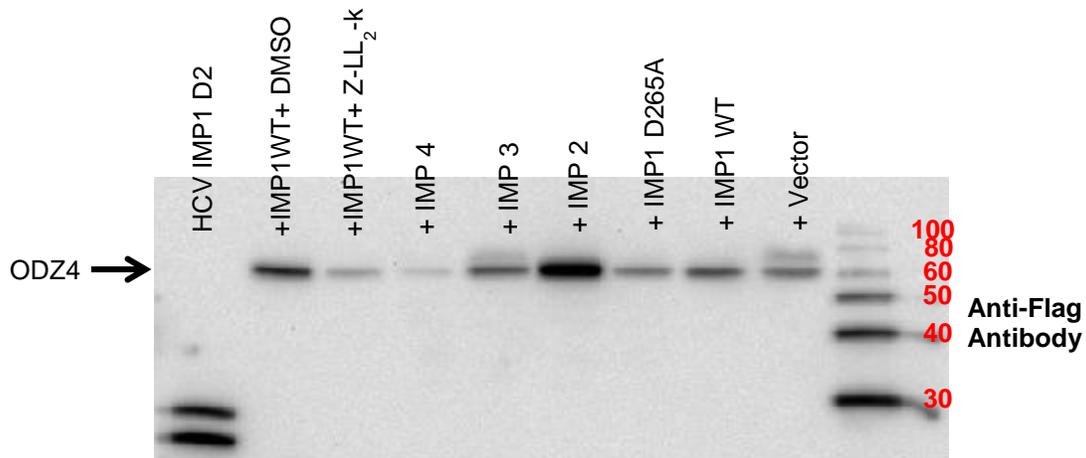
Substrates for the members of the IMP family of proteins remains primarily undetermined, so other putative substrates were assayed, including type II transmembrane proteins that function in neuronal connectivity, signaling, or development. The IMP proteases, and the substrate of interest were overexpressed in HEK293 cells via co-transfections, and proteins were assayed to investigate potential cleavage. The Syntaxin 5A (STX5A) substrate protein ran at approximately 35 kD (**Figure-9**), the expected size without cleavage, regardless of which IMP protease was also expressed. The tested mutants containing a single amino acid change within the highly conserved second aspartic region also reveal no effect on the STX5A substrate. The sample containing both IMP1 and Z-LL<sub>2</sub>-ketone (IMP1 inhibitor) also showed no

change in protein size. Neither, IMP1, IMP2, IMP3, nor IMP4 were found to cleave Syntaxin 5A in the HEK293 model system.



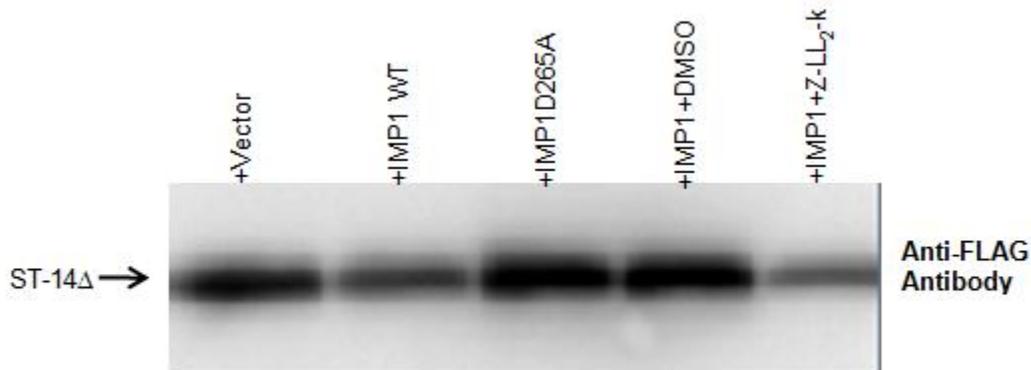
**Figure-9: Syntaxin 5A is Not Cleaved by Proteases IMP1, IMP2, IMP3, or IMP4.** The ladder used in each image is the Magic Mark XP Protein ladder (Invitrogen), and the sizes of those bands are indicated in the figure in red. Z-LL<sub>2</sub>-k is the abbreviation used in the figure for the (Z-LL)<sub>2</sub>-ketone inhibitor, which in this case had no effect on cleavage of STX5.

ODZ4 is another type II transmembrane protein identified as a putative substrate for the IMP proteins. The cleavage capabilities were again tested using transfection and Western blotting (**Figure-10**). The bands for ODZ4 ran at a molecular weight just above 60 kD, which is an unexpected size, seeing as uncleaved ODZ4 is expected to be approximately 50 kD, and cleaved protein is supposed to run at 42 kD. Cleavage by these proteases certainly would not result in the protein becoming larger. The IMP mutants also revealed no change in protein size. The sample containing (z-LL)<sub>2</sub>-k (IMP1 inhibitor) also shows no protein size alteration. Therefore, ODZ4 does not appear to be a cleavage substrate of proteases IMP1, IMP2, IMP3, or IMP4.



**Figure-10: ODZ4 is Not Cleaved by Proteases IMP1, IMP2, IMP3, or IMP4.** The ladder used in each image is the Magic Mark XP Protein ladder (Invitrogen) and the sizes of those bands are indicated in the figure in red. Z-LL<sub>2</sub>-k is the abbreviation used in the figure for the (Z-LL)<sub>2</sub>-ketone inhibitor.

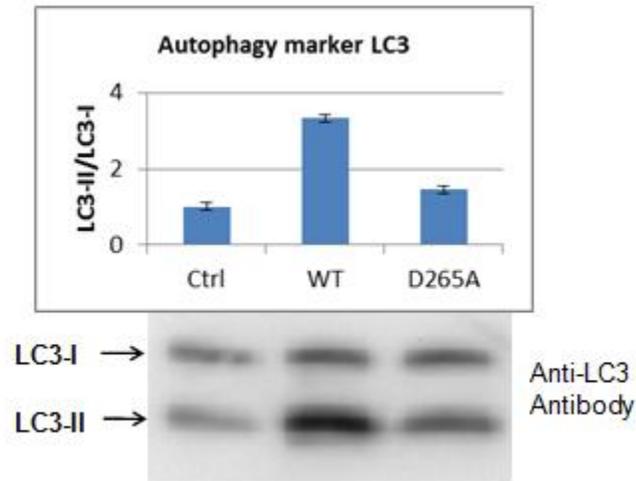
Substrate  $\Delta$ ST-14 was also screened for potential cleavage by IMP1 through transfection and Western blotting (**Figure-11**). All of the bands ran at the same level on the gel which is expected if the substrate is not cleaved. The bands ran just below the 20 kD band the appropriate size for the uncleaved protein. The cleaved size is estimated to be much smaller (~12 kD). The right lane on the membrane contains the  $\Delta$ ST-14, IMP1, and z-LL<sub>2</sub>-k (IMP1 inhibitor) and the bands runs in line with all the others. Thus,  $\Delta$ ST-14 does not appear to be a cleavage substrate of IMP1.



**Figure-11:  $\Delta$ ST-14 is Not a Cleavage Substrate of IMP1.** The ladder used in each image is the Magic Mark XP Protein ladder (Invitrogen) and the sizes of those bands are indicated in the figure in red. Z-LL<sub>2</sub>-k is the abbreviation used in the figure for the (Z-LL)<sub>2</sub>-ketone inhibitor.

## Autophagy Tests

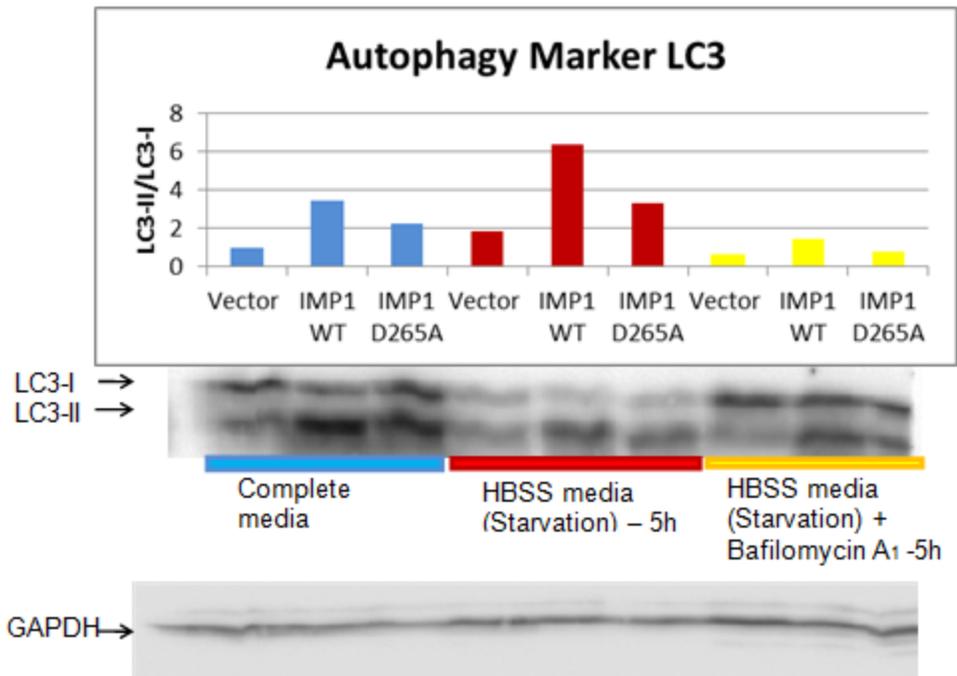
The effects of overexpression of the IMP1 protease on the autophagy pathway was also studied using transfection and Western blotting, using an endogenous LC3 protein (LC3-II is a known autophagy marker). The proportions of LC3-II, the processed form to the original LC3-I form, was used for comparison and is represented graphically along with each blot. First, the effects of the control vector, vector encoding IMP1, and vector encoding an IMP1 mutant (single base pair in the second conserved aspartic region-D265A) were transfected separately into HEK293 cells and investigated for autophagy levels (**Figure-12**). The lower 16 kD LC3-II band is of the expected size, and is increased with the addition of IMP1 WT to the cells [ratio ~3]. The control plasmid and D265A mutant have far lower LC3-II expression [ratio < 2].



**Figure-12: Autophagy Analysis of hIMP1 in HEK293.** The over-expression of wild-type hIMP shows upregulation of the autophagy marker LC3-II, while the D265A mutant shows no significant change.

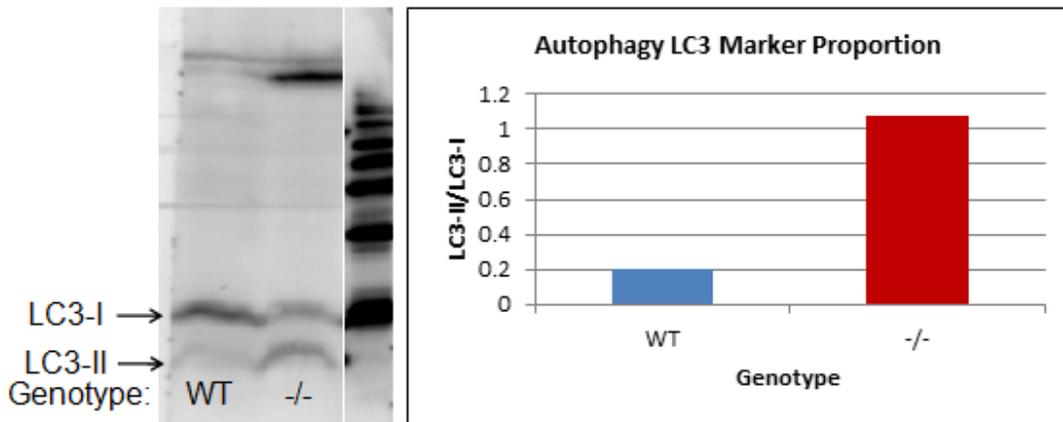
Autophagy is increased when cells are starved. The effect of the vector, wild-type IMP1, and the D265A mutant, are examined for their effects on autophagy with natural upregulation of the pathway. The HEK293 model system was used in three conditions after transfection: one being a control complete media, a five hour starvation condition, and a five hour starvation period with Bafilomycin A<sub>1</sub>- an autophagy pathway inhibitor (**Figure-13**). The vector samples were used as controls. The samples overexpressing WT IMP1 represent the effect of IMP1 on the autophagy pathway in the HEK293 model system. The D265A mutant (single amino acid mutation in the second conserved aspartic region) represents an overexpressed mutated IMP1 gene. The effects of autophagy by vector, wild-type IMP1, and IMP1 D265A mutant in complete media were confirmed in this second experiment, showing an increase in autophagy with the overexpression of wild-type IMP1. It can be seen that the wild-type IMP1 bands show a predominant LC3-II (lower band) than the other two in each set. This is even more apparent in the LC3-II to LC3-I ratio represented in the graph. The mutant does show an increase though not

nearly as high as the wild-type. Bafilomycin A<sub>1</sub> shows a marked decrease in all LC3-II expression as it is inhibiting that pathway, indicating that the observed increased signals actually represent autophagy not inhibition. At the bottom of the figure a stain for GAPDH reveals equal loading, and so the intensity of the bands can be compared.



**Figure-13: Starvation and Inhibited Autophagy Analysis in HEK293 Cells.** Sets are color coded to indicate the cell growth condition. Blue denotes control cells grown in complete media. Red indicates cells that were starved for 5 hours after transfection using Hank's Balanced Salt Solution (HBSS). Yellow represents cells treated with bafilomycin which are also starved for 5 hours in HBSS.

A knock out mouse model lacking the IMP1 gene was also investigated. Liver tissues of P0 pups were screened for autophagy via Western blotting using the anti-LC3 antibody (**Figure 14**). Pups from the same litters were compared. The wild-type sample shows the predominant form of LC3 to be LC3-I, and limited to no lower band. The knockout pup sample has both bands; the protein concentration is dominated in the lower molecular weight LC3-II band – indicating increased autophagy when compared to the control wild-type pup. The ratio of LC3-II to LC3-I is represented graphically, to more clearly indicate the levels of autophagy in each sample. This increased autophagy is even more apparent in the graphic representation where the ratio for the knockout is over 1, and the wild-type has a ratio of 0.2.



**Figure-14: Screening of IMP1 Knockout P0 Pups for Autophagy.** The IMP1 knockout pup is indicated by the (-/-) genotype and the red bar on the graph, which shows an increase in LC3-II relative to LC3-I.

## DISCUSSION

All of the protease and potential substrate sequences cloned into the plasmid vectors were verified by sequence analysis. The other mutants were confirmed previous to this experiment. The model system used was HEK293 cells and the expression of the proteases in this cell line was confirmed by immunoblots using the myc-tag fused to the expressed proteins. Samples were prepared without boiling and stained for the myc-epitope tag (Figure 7). HCV core protein a published IMP1 substrate was confirmed and shows IMP1 is able to cleave in the model system (Figure 8). Both the vector and wild-type IMP1 do not inhibit cell from processing the HCV core protein. However, the D265A mutant, single point mutation in the highly conserved second aspartic acid region, of IMP1 inhibits HCV core protein cleavage by endogenous IMP1. Therefore, the HEK model system is effective for experiments with this family of proteases.

Based on the data presented in this project, Syntaxin 5A (STX5A), ODZ4, and  $\Delta$ ST-14 are not cleavage substrates of IMPAS. Both STX 5A and  $\Delta$ ST-14 ran at the expected uncleaved molecular weights: 35kD and 15kD respectively (Figures 9 and 11). However, ODZ4 ran at just over 60 kD (Figure 10), approximately 10 kD higher than expected for the uncleaved protein. This indicates that there is some other cellular processing of this protein occurring in the model system. None of the considered substrates of IMPAS were actually cleaved and can be ruled out. However, pinpointing the exact substrates for the members of the IMPAS family of proteases will be critical in determining their function; it will also allow for more accurate research into the potential neurodegenerative diseases it may be related to.

The role of IMP1 in the autophagy pathway was investigated as another angle to narrowing down the function of this newly discovered family of proteases. In the HEK293

model, overexpression of wild-type IMP1 significantly increased autophagy (Figure 12). It is clear that with the addition of wild-type IMP1, the ratio of LC3-II to LC3-I is calculated to be at approximately three; whereas with the control vector and with the D265A mutant of IMP1 the ratio is well under two. This indicates that the functional IMP1 causes this increase. Starvation naturally induces the autophagy pathway, and the addition of wild-type IMP1 autophagy is still increased further (Figure 13). D265A mutant saw increase in autophagy when compared to the vector in both complete media and more noticeably when starved. The addition of Bafilomycin-A1, an autophagy inhibitor, shows a marked decrease in autophagy in all samples (Figure 13). This indicates that the increase in the lower molecular weight LC3-II band is due to an increase in autophagy not due to inhibition in the pathway. In the HEK293 model system it is demonstrated that wild-type IMP1 increases autophagy; this means that the protease may be integral in some part of the pathway.

An IMP1 loss of function knockout (KO) mouse model was also tested, and liver tissue cells were screened for autophagy. The KO P0 pup shows much higher autophagy than its wild-type litter mate (Figure 14). The ratios were dynamically different at approximately 1.1 and 0.2 respectively. In this model system, the results reveal that without IMP1 expression the autophagy is increased, while the wild-type litter mate which expresses IMP1 has limited autophagy. The complete lack of IMP1 also increases autophagy.

IMP1 is a protease that demonstrates a putative role in the autophagy pathway. The protein is most likely highly regulated due to the high levels of autophagy when it is overexpressed or when it is absent. The protease should also be studied in a variety of living tissues because of the differences shown between the model systems. HEK293 cells being kidney cells may have different outcomes than the liver tissues used in the KO mouse study. The

function of the IMP family of proteases, especially IMP1, in the autophagy pathway still requires more investigation.

In conclusion, the role and function of the IMP family of proteins still remain vastly unknown. Syntaxin 5A, ODZ4, and  $\Delta$ ST-14 have been ruled out as potential substrates. However, a putative connection has been uncovered between IMP1 and autophagy. Further investigation into the substrates and potential role in the autophagy pathway of IMPAS is still necessary.

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