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Mapping Functional domains of ORP3 required for suppression of ER aggregation in ALS8

Sohail Syed

I. Introduction

Although Amyotrophic Lateral Sclerosis (ALS) is a relatively rare disease, it affects 1 per 100,000 people (Zarei et. al). ALS is a late-onset neurodegenerative disease, a condition where the cells in motor neurons die off resulting in patients having limited to no movement in the motor neurons where ALS is present (Zarei et. al). Within ALS, there are 27 subtypes with each one having its own associated gene (Nishimura et. al). Looking specifically at ALS8, the associated gene VAPB (Vesicle-associated membrane protein-associated protein B) causes the onset of the disease (Nishimura et. al).

VAPB is a gene found in humans, that when modified by the P56S (Proline 56 to Serine) mutation causes ALS8 (Prosser et. al). VAPB encode proteins containing a cytoplasmic-Major Sperm Protein (MSP) that aid in the Unfolded Protein Response (UPR) by helping with folding accumulations of unfolded/misfolded proteins (Yin Liu et. al). The P56S mutation lies within the MSP domain, which when expressed, inhibits Endoplasmic Reticulum (ER)-to-Golgi transport disrupting the natural flow of the cell, deactivating the UPR causing clusters of improperly folded proteins to aggregate illustrated in **Fig. 1**. If proteins are misfolded or unfolded the cell will undergo apoptosis, a form of programmed cell death, as the cell cannot properly function (Prosser et. al).

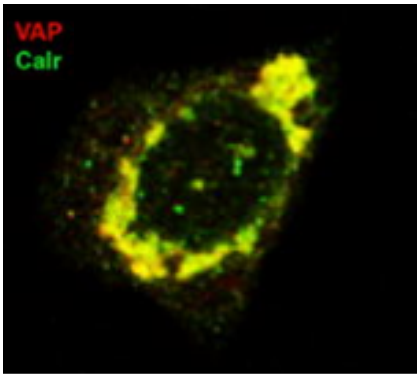

Figure 1: ER aggregation caused by VAPB-P56S due to the wildtype VAPB-P56S being insoluble. Red regions indicate where VAPB is colocalized. Green regions represent regions where Calreticulin, an ER marker protein for displaying movement of proteins in ER membrane, are located. Yellow regions indicate where VAPB and Calreticulin aggregates (adapted from Prosser et. Al, 2008).

Proteins with the FFAT (two phenylalanines in an acidic tract) motif rescues ER aggregation allowing the cell to properly fold and pass proteins through, seen in **Fig. 2**. The FFAT motif rescues the cell by binding to microtubules from the ER and nuclear membrane blocking the mutated VAPB from binding within the ER (Kaiser et. al). Genes within the oxysterol-binding protein (OSBP) family, a family of genes that shuttle lipids to different compartments in the cell, contain the FFAT motif and have been discovered to rescue cells infected by ALS8 (Lehto et. al).

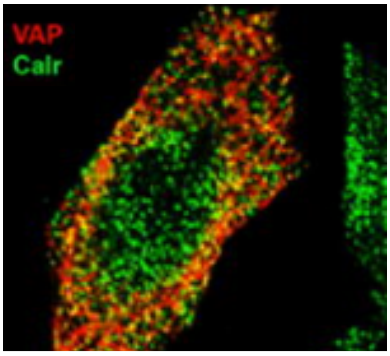

Figure 2: Normal ER functioning Cell Red and Green regions indicate VAPB and Calreticulin properly diffusing from the ER throughout the cell (adapted from Prosser et. Al, 2008).

According to the Darbyson et al paper, of the different OSBP-related proteins (ORP), ORP3 helps the best with rescuing of ER aggregation, best visualized in **Fig. 3**. Through their research, they found that ORP3 reduces the size and number of VAPB-induced expansions. VAPB is known to cause vacuole expansions of ER

related proteins that when overexpressed and insoluble due to the P56S mutation causes ER aggregation. ORP3 reducing the number of expansions allows for the cell to properly diffuse the ER proteins. Darbyson et. al also uncovered that ORP3 changes the solubility of VAPBs. In doing so, ORP3 restricts for immovable obstacles, created by the binding of VAPs to microtubules, to be steadily be created.

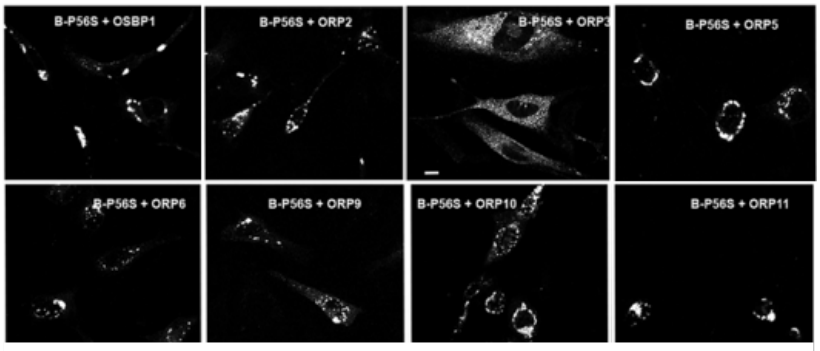

Figure 3: ORP3 Rescues ER Aggregation from Overexpression of VAPB-P56S ER aggregation caused by the P56S mutation is seen in the OSBP1, ORP2, ORP5, ORP6, ORP9, ORP10, and ORP11 cells. Within the cell with ORP3 and VAPB-P56S there is normal ER function. Images were taken of cells stained with FLAG antibodies for ORPs and VAPBs (adapted from Darbyson et. al).

ORP3 contains three major regions. The FFAT motif, OSBP-related domain (ORD), and the pleckstrin homology (PH) domain. The ORD domain is a hydrophobic region of ORPs that regulate the binding of sterols and other membrane binding surfaces (Raychaudhuri et. al). The PH domain bind phosphoinositides, which interact with proteins and guide their activity through the cell (Lemmon)(Falkenburger et. al). Of interest is to find which section of ORP3 specifically helps with rescuing ER aggregation.

To understand the effects of ORP3 in rescuing a cell from the VAPB-P56S mediated aggregation, it is important to map out the regions of ORP3 and submit them for testing. Understanding why certain regions rescue mutated cells gives insight as to why other ORPs fail in rescuing ALS8 cells.

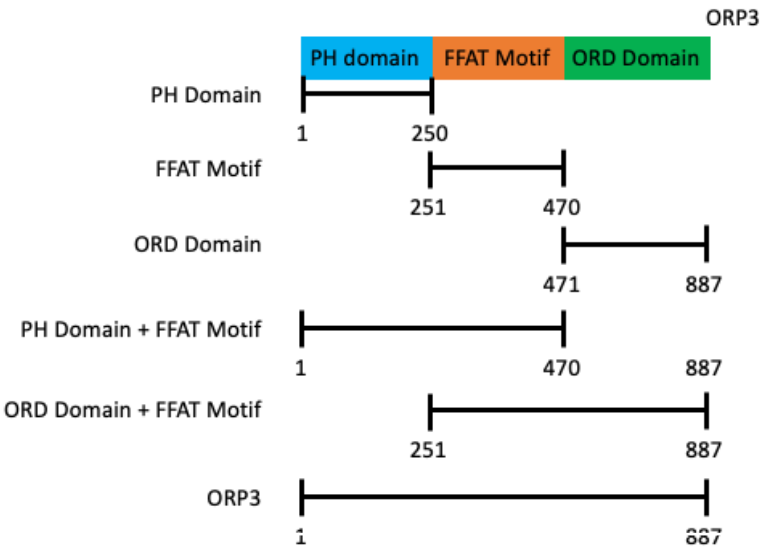

Figure 4: ORP3 Split into its Regions of Interest The above image displays the sections that will be tested for mapping out ORP3. The PH domain from 1-250 amino acids, FFAT Motif 251 – 470 amino acids, and the ORD domain 471-887 amino acids. The FFAT motif will also be the PH domain and ORD domain as well to test. ORP3 as a whole will also be tested.

II. Experiment

The aim of this experiment is to determine which defined region of ORP3, specifically the PH domain, FFAT motif, and ORD domain, helps in rescuing ER aggregation of ALS8 cells. Understanding which domain is the main component allows for further analysis and possible creation of chimeric proteins. The controls for this experiment will be ORP3 as a whole and the mutated AAAT motif as a positive and negative control respectively. Through use of the Polymerase Chain Reaction (PCR) protocol the different regions can be amplified and expressed. Through molecular cloning, the amplified

regions can be purified and digested into plasmids for cell use. Testing of aggregation rescuing will be tested using immunofluorescence (IF) of the cells.

II.A. PCR Protocol

In order to use certain regions in ORP3, these domains need to be amplified. The PCR protocol can specifically target certain regions of the ORP3 and amplify. The length of ORP3 is 887 amino acids (Gregorio-King et. al). According to Lehto et. al, they found that the PH domain exists in its entirety from 52-152, the FFAT motif to be within 449-453, and the ORD domain to be from 454-887. For the basis of this experiment the regions will be 1-250 for the PH domain, 251-470 for the FFAT motif, and 471-887 for the ORD domain illustrated in **Fig. 4**.

Using PCR, the regions of interest can be amplified. For the protocol to work, DNA polymerase, primers, a buffer, and nucleotides are needed in addition to the ORP3. For this experiment Taq polymerase will be used to read and create a complimentary copy of the regions. Taq Polymerase is used due to its convenience, high purity with little to no contamination, and cost effectiveness. Using the tactic of Collier et. al for their PCR experiment, primers will be designed using Primer Express software and chemically synthesized for the specific regions of interest illustrated in **Fig. 5**. These primers will also add a restriction site at the 5' end in order to stop PCR from replicating more than necessary. A buffer will be used in order to regulate the pH and salt concentration of the PCR process. And lastly the nucleotides, dATP, dTTP, dGTP, and dCTP, will be used in order to create the complimentary DNA. I

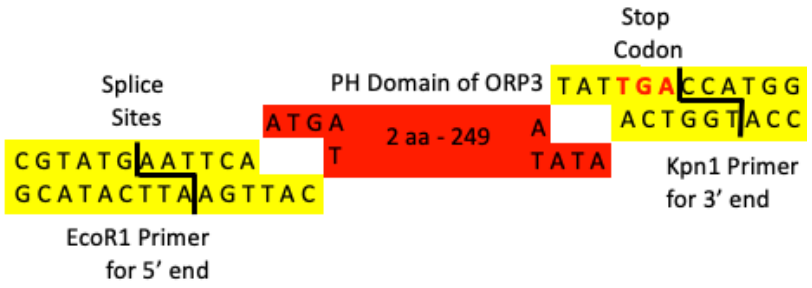

Figure 5: Attachment of EcoR1 and Kpn1 primers The above image displays the primers used for restricting ORP3 and for creation of attachment sites for the primer. EcoR1 is used at the 5' end, and Kpn1 is used at the 3' end, due to them not making cuts on ORP3. A stop codon is added before Kpn1 to stop tranlation(adapted from Collier et. al).

The PCR protocol will have the solution go through three main steps of denaturation, annealing, and extension. In the first step, the solution will be heated to 95C in order to disrupt the Hydrogen bonds in order to separate the ORP3 into its separate strands. From there, the solution is cooled to 54C to allow the designed primers to bind to their respective strands there by annealing the ORP3 region. In order for extension to happen, the solution is heated to 72C in order to have the Taq Polymerase bind to the primers and add nucleotides to the strands. This process will be repeated for 30-35 rounds in order to amplify the regions million-fold.

II B. Molecular Cloning

In order to have the amplified ORP3 regions enter the ALS8 cells, plasmids are needed. The molecular cloning process can cleave PCR products at the desired amino acid and digest them with empty plasmids in order to combine them. After combination of the regions and empty plasmids,

they can be sent into the cell using horizontal gene transfer done in the Lacroix et. al experiment where bacterial plasmids transferred DNA to human cells.

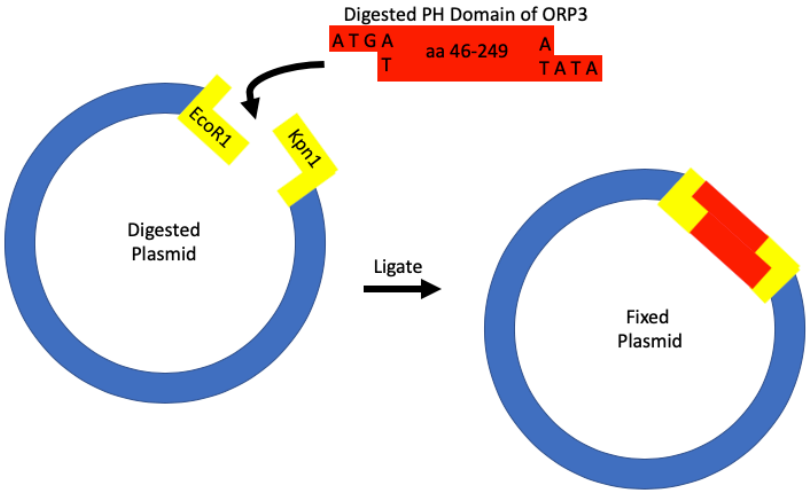

Figure 6: Molecular Cloning through DNA ligation of Inserts and Vectors The digested empty plasmid is fixed with the same restriction enzymes used on the primers from **Fig. 5**. Using the PCR products as inserts, they can be ligated and fixed into plasmid by digestion on agarose gel in order to isolate and purify insert. The plasmid used is pFlag-CMV2 in order to allow for flagging needed by IF (adapted from Topcu and Prosser et. al).

Molecular cloning is done through use of inserts and vectors. Inserts are the genes or fragments of interest, while the vectors are the empty plasmids that will be modified by the inserts for DNA transfer (Topcu). Using the PCR products as inserts, they can be by digested, breaking the DNA into small fragments, by inserting restriction enzymes that target the restriction sites on the primers to cleave the domain from the entire ORP3 gene. After creating the digested inserts, the empty vector plasmid will be digested with the same restriction enzymes in order to create the same overhanging illustrated in **Fig. 6**.

In order to combine the empty plasmid and inserts into the appropriate vector, DNA ligation must occur. Ligation starts by mixing in a 3:1 ratio of inserts and vectors in the presence of T_4 DNA ligase. T_4 DNA ligase is used because of its convenience and fast working qualities. Using the Froger et. al experiment's heat shock method where plasmid DNA is rapidly heated after being cooled in ice in order for it to be transformed into E. coli so that the fixed plasmids can grow and be used. Antibiotics, ampicillin, will be used in order to kill off the untransformed cells.

II C. Transfecting Plasmids and IF

To allow the ORP3 to interact with the ALS8 cells, the plasmids need to be transfected into the cell. This is done through incubation of the plasmids with the cells (Fechheimer et. al). In order to find whether or not the ORP3 region of interest is beneficial to the cell, IF is necessary. IF is the process of visualizing molecules through microscopy with colored tags. Illustrated in **Fig. 1, Fig. 2,** and **Fig 3** IF pictures how the molecules of interest move throughout the cell.

Using the same method used by Prosser et. al and Darbyson et. al, the cells will be fixed and imaged through use of flag-antibodies specific to VAPB-P56S and ORP3. Having antibodies specific to the genes of interest allows for staining to occur with the fluorescence dyes, so that the confocal microscope can pick up their movements and create an image.

III. Discussion

If the experiment goes according to plan, the positive control and FFAT motif are expected to rescue the ER from its aggregation. The mutated AAAT motif would be expected to have aggregates remain after being inputted into the cell. Results stemming from the PH+FFAT and ORD+FFAT tests are expected to return positive for rescuing aggregation due to the FFAT motif being present. Results from the PH and ORD domains is unknown as research hasn't been conducted on those specific regions in respect to ALS8 cells, so there are no expectations but rather hope that they work by themselves.

Which regions remove aggregation will affect my conclusion as to which specific region is necessary for rescuing the ER. If specific regions work chimeric proteins can be synthesized to help with other ORPs. ORP6 by itself doesn't help but would ORP6 with a region replaced by ORP3's working region help? **Fig. 1** shows the FFAT motif helping but there isn't any clear evidence to show that the other domains rescue.

The problem exists that if none of the regions work by themselves, the study brings forth minimal beneficial data for ALS8 patients. The reasons for them not working maybe because another region isn't expressed also, leading to further research. Another problem can stem from the FFAT motif by itself may return a false positive result due to its presence being very small in the plasmids.

It will be difficult to interpret the results as the plasmids entering the cells process isn't entirely known. A result could show aggregation due to the fact that the transfection of the plasmid's success is unknown. If there is an in-between for aggregates remaining and the proteins moving throughout the membrane, it will be difficult to interpret the success of the domain.

Despite these problems, mapping out the regions of ORP3 will allow research to uncover why certain proteins rescue aggregation over others. In addition, the working regions can be applied to other proteins in the form of chimeric proteins where regions of different proteins regions are replaced by ORP3's regions. And if failure is seen in each test the results offers insight for regions working together to combat ER aggregation in ALS8 cells.

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Supplemental:

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841 rvleenhveh qprffrksdd dswvsngtyl elrkdlgfsk ldhpvlw
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S.1: Amino Acid sequence of ORP3 (Gregorio-King et. al)