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Identifying C-mannosylatedproteins in RAW264.7 cells via in vitro HSC70 Co-immunoprecipitation strategies

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IDENTIFYING C-MANNOSYLATED PROTEINS IN RAW264.7 CELLS *VIA IN VITRO* HSC70 CO-IMMUNOPRECIPITATION STRATEGIES

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By
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Approved by
Dr. John F. Rakus, Committee Chairperson
Dr. Derrick R. J. Kolling
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APPROVAL OF THESIS

We, the faculty supervising the work of Nicholas Ryan Kegley, affirm that the thesis, *Identifying C-mannosylated Proteins in RAW264.7 Cells via In Vitro Hsc70 Co-Immunoprecipitation Strategies*, meets the high academic standards for original scholarship and creative work established by the Chemistry Program and the College of Science. This work also conforms to the editorial standards of our discipline and the Graduate College of Marshall University. With our signatures, we approve the manuscript for publication.

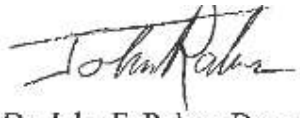

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TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT

C-mannosylation is a specific type of glycosylation in which α-mannose is covalently bound to the C_2 atom of a tryptophan via a carbon-carbon bond. Presently, it is known to occur on proteins that contain the W-x-x-W-x-x-W/C motif found within the Thrombospondin Type-1 Repeat (TSR), with 'x' denoting any amino acid. *C*-mannosylation has been implicated in a number of cellular functions, including protein secretion and type I cytokine receptor function. Previous research has shown that exogenous *C*-mannosylated peptides derived from TSR-containing proteins upregulate the production of the pro-inflammatory cytokine Tumor Necrosis Factor alpha (TNF-α) in RAW264.7 macrophage cells. This upregulation is substantially increased in the presence of Hsc70. When these peptides are internalized by RAW264.7 cells, a binding interaction occurs between the two proteins, which also correlates with increased TNF-α levels. With the knowledge that exogenous *C*-mannosylated peptides and Hsc70 can influence cytokine production, this study has focused on identifying endogenous *C*-mannosylated proteins in RAW264.7 cells that interact with Hsc70. The potential of a naturally occurring interaction between these two proteins could elucidate the importance of *C*-mannosylation in the innate immune response. Through Hsc70 co-immunoprecipitation, a potentially *C*-mannosylated protein has been identified in Junctophilin-1 (JPH1). JPH1 forms a junctional membrane complex (JMC) between the endoplasmic reticulum and plasma membrane of excitable cells, facilitating communication between calcium ion release channels. Tandem mass spectrometry data suggests that the hexose appears on a single tryptophan in a peptide bearing the sequence: E-G-E-W-A-N-N-K. This indicates that JPH1 may be an example of non-canonical *C*-mannosylation. To further explore the status of JPH1 as a *C*-mannosylated protein, the protein was overexpressed in HEK293T cells. This experiment showed that JPH1 was not *C*-

mannosylated in HEK293T cells. Noting the lack of modification, reverse transcriptase polymerase chain reaction (RT-PCR) was performed with both RAW264.7 and HEK293T RNA. These experiments showed that all known *C*-mannosyltransferase homologs (DPY19L1-4) were expressed in HEK293T cells, while only DPY19L1 and L4 were expressed in RAW cells. Further work is being conducted to overexpress JPH1 in RAW264.7 cells to determine why, or if, JPH1 is *C*-mannosylated in this particular cell line.

CHAPTER 1

GLYCOBIOLOGY AND THE INNATE IMMUNE RESPONSE

1.1 *N*- AND *O*-LINKED GLYCOSYLATION

Post-translational modifications compose a wide variety of reactions. As the name suggests, these modifications occur after the translation of a nucleotide sequence into a polypeptide. In some cases, a modification is necessary for a polypeptide to assume its final role as an active protein in a biological setting. This study focused on glycosylation, a post-translational modification that details the addition of a glycan (carbohydrate) to a specific amino acid within a motif of several amino acids in a protein.

Glycosylation is an enzymatically-driven process that occurs in the endoplasmic reticulum. One of the more thoroughly understood forms of glycosylation is known as *N*-linked glycosylation. In eukaryotes, this process begins at the surface of the ER membrane with dolichol phosphate (Dol-P), where mature *N*-linked glycans are built from sugar-nucleotide donors. Multiple transferases (enzymes that attach a carbohydrate to a substrate molecule) transfer carbohydrates from nucleotide donors such as UDP-GlcNAc and GDP-Mannose to build a 14-sugar glycan on Dol-P, forming $Glc_3Man_9GlcNAc_2$-P-P-Dol. During this process, a flippase moves the Dol-P-P glycan from the cytosolic side of the ER to the lumen side. The enzyme oligosaccharyltransferase takes this larger glycan and transfers it to an asparagine residue within the *N*-linked glycan motif of Asn-X-Ser/Thr to proteins that translocate across the ER membrane, providing proteins with a full length *N*-linked glycan that can be trimmed to serve other functions within the cell.[1] The function of these glycans is largely thought to be centered around proper folding and quality control of proteins.[2]

With *N*-linked glycosylation being one of the most well understood forms of glycosylation, other forms of glycosylation are generally thought to follow a similar pattern of formation. *O*-linked glycosylation provides many different glycans that are directly attached to proteins through a Ser/Thr residue; these include *O*-GlcNAc, *O*-GalNAc, *O*-glucose, and *O*-xylose. *O*-GlcNAcylation of proteins is catalyzed by the *O*-GlcNAc transferase (OGT) and the donor substrate UDP-galactose within intracellular areas like the nucleus and cytoplasm.[3] *O*-GalNAc is catalyzed by multiple different GalNAc transferases (GALNTs) that feature catalytic domains in the Golgi lumen to attach GalNAc (donated by UDP-GalNAc) to Ser/Thr.[4] Protein *O*-glucosyltransferase 1 (POGLUT1) adds both glucose and xylose to epidermal growth factor-like (EGF) repeats, and a deficiency in the enzyme results in muscular dystrophy in humans.[5] Another form of *O*-glycosylation adds to a different domain found across multiple proteins, like protein *O*-fucosyltransferase 1 and 2 (POFUT1, POFUT2). POFUT2 adds the monosaccharide fucose to thrombospondin type 1 repeats (TSRs) on Ser/Thr residues through an oxygen-carbon bond. This modification is essential to human development; a lack of *O*-fucosylation on TSRs results in the developmental disorder known as Peter's Plus Syndrome.[6] Fucosylated residues can also be elongated by other glycosyltransferases that are similarly important for healthy human development. Beta-1,3-glucosyltransferase adds glucose on top of a fucosylated Ser/Thr side chain, with further implications in the same developmental disorder for those lacking the modification.[7] **Figure 1** shows the linkages of amino acid side chains in both *N*- and *O*- glycans. All of these different protein modifications play roles in biological processes like protein folding/quality control and stages of healthy human development. A third form of glycosylation, *C*-mannosylation, has been implicated in the innate immune response through the upregulation of secreted, cell-signaling proteins known as cytokines.

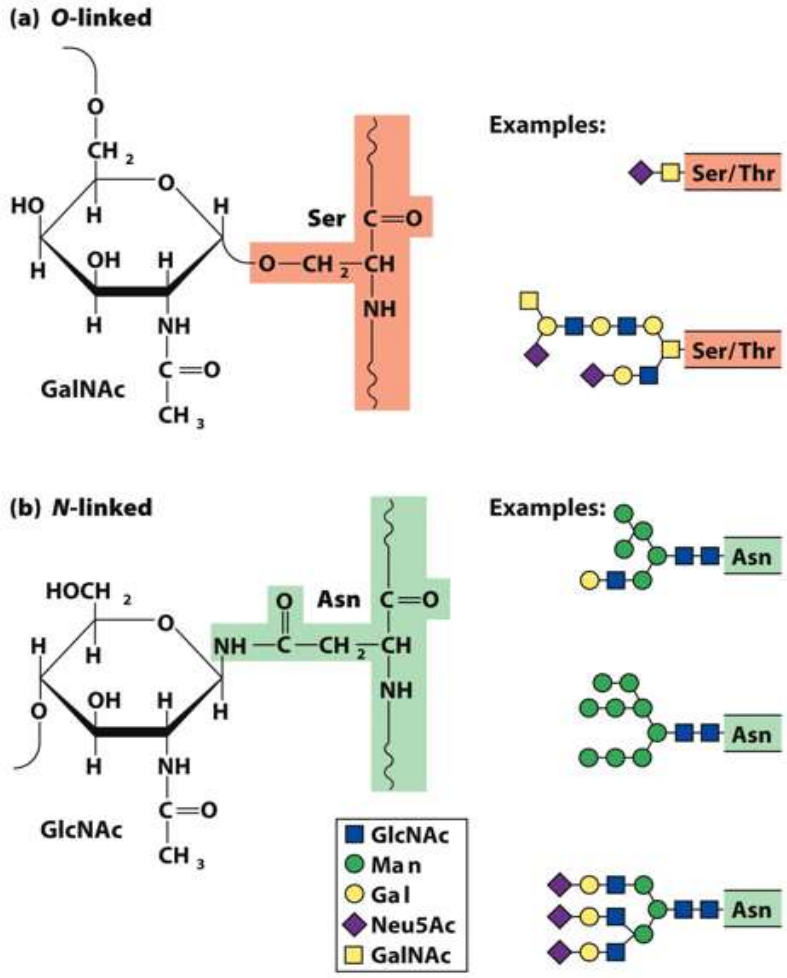


Figure 1: Examples of *O*- and *N*-linked glycosylation. Adapted from Lehninger, A. L., Nelson, D. L. 1., & Cox, M. M. (2008). *Lehninger Principles of Biochemistry* (5th ed.). New York; New Delhi: W.H. Freeman[8]

1.2 *C*-MANNOSYLATION

Both *N*- and *O*-linked glycosylation are implicated in important biological processes like protein stability, folding, and healthy human development. These modifications occur on specific amino acid side chains within consensus sequences. Like the two previously described glycosylation types, *C*-mannosylation features its own distinct modification site. *C*-mannosylation is the direct addition of a mannose monosaccharide by a *C*-mannosyltransferase

expressed in the endoplasmic reticulum. As opposed to a reaction occurring on a more reactive oxygen or nitrogen atom, the carbohydrate is added directly to the second carbon found on the indole ring of a tryptophan, as shown in **Figure 2**. For this study, this type of glycosylation was initially observed within the context of the thrombospondin type one repeat (TSR). TSRs feature more than one consensus sequence; upstream of the *O*-mannosylation site is a series of tryptophan residues. These are arranged as W-x-x-W-x-x-W/C, with 'x' denoting any other amino acid. This consensus sequence is the site of *C*-mannosylation and is thought to be required for the modification to occur.[9] **Figure 3** presents a TSR domain, with both sites of glycosylation highlighted.

Within the consensus sequence, the mannose can be found on all three tryptophan residues. No elongation occurs in this case of glycosylation; only the monosaccharide form has been observed, and four human homologs of the transferase have been identified.[10] *C*-mannosylation has been implicated in a few different functions. A lack of *C*-mannosylation in male sperm cells has been noted to result in the condition known as globozoospermia, an abnormal development of male sperm that results in infertility.[11] In another case, a lack of *C*-mannosylation on the protein ADAMTSL-1 saw a substantial increase in the intracellular levels of the protein. ADAMTSL-1 is known to be a protein that proceeds through the secretion pathway; this suggests that *C*-mannosylation is necessary for protein secretion.[11] These two cases underscore the importance of *C*-mannosylation in healthy cell development and secretory pathways. *C*-mannosylation has been noted to occur in cell lines like chinese hamster ovary (CHO) cells, and the murine RAW264.7 cell line.

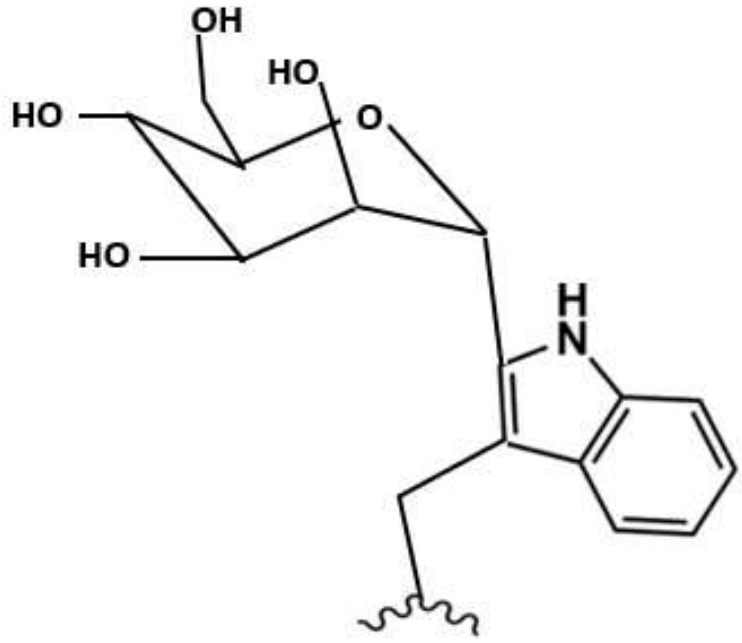


Figure 2: *C*-mannosylated tryptophan side chain. D-mannose is bound by a carbon to carbon on C2 of tryptophan's indole ring.

In murine macrophage-like cells (RAW264.7), expression of naturally *C*-mannosylated proteins was discovered by immunoblotting techniques. An antibody was built against synthetically mannosylated tryptophan (CMW) residues. Using this antibody with RAW264.7 cells, numerous protein bands were observed from immunostained cell lysates. Of the stained bands, one such mannosylated protein was Thrombospondin-1 (TSP-1). This corresponded to two of the most prominent bands observed by anti-CMW staining.[12]

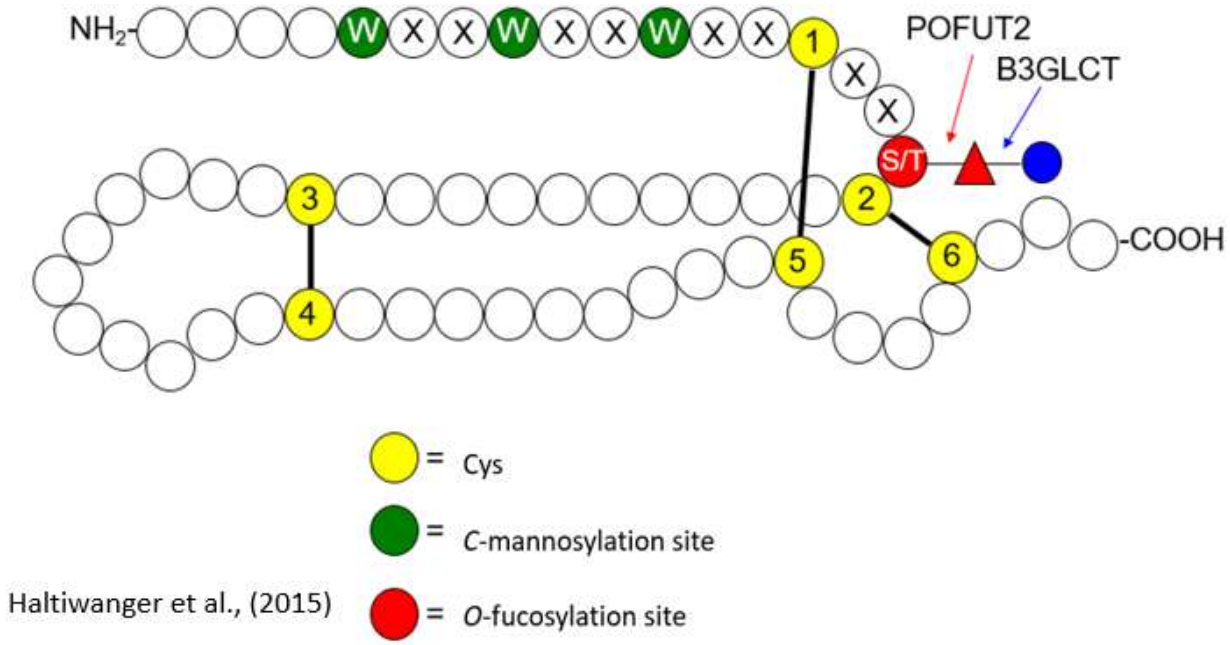


Figure 3: **Depiction of TSR with consensus sequences and glycosyltransferases**. Provided with consent by Dr. Robert S. Haltiwanger, University of Georgia.[13]

The identification of *C*-mannosylated proteins in RAW264.7 cells also showed that *C*-mannosylation was increased in hyperglycemic conditions. Noting these conditions as being able to produce cellular change, more work was conducted on observing the effects of *C*-mannose as an instigator of cellular response. RAW264.7 cells are typically used in immunology to studies with a focus on the innate immune response. Using chemically-derived *C*-mannosylated peptides, it was found that *C*-mannosylation upregulates the production of pro-inflammatory cytokines. Also, this upregulation was further increased when RAW264.7 cells were cultured with lipopolysaccharide infused media to simulate a bacterial infection, or in other words a period of cell stress and response.[14]

A separate study focused on the potential for *C*-mannosylated peptides to interact with other proteins that are in some way related to the innate immune response. When derived peptides were endocytosed by RAW264.7 cells and isolated after lysing the cells, other proteins were found to interact with the *C*-mannosylated peptide. Of these, Hsc70 was characterized by

blotting with anti-Hsc70 antibodies. This heat shock protein is an important molecular chaperone that facilitates proper protein folding during times of cellular stress, and was theorized to be important to the overall pro-inflammatory upregulation that LPS and *C*-Man-W were influencing.[15]

This same study showed that introduction of exogenous *C*-mannosylated peptides and Hsc70 to RAW264.7 cells could increase cytokine production. When paired together, the specific upregulation of TNF-α is higher than cases observed when these are introduced to the cells separately. Knowing that *C*-mannosylated peptides and Hsc70 can interact to cause an inflammatory response, and that LPS can also upregulate cytokine production, drove this study to focus on potential endogenous *C*-mannoyslated proteins in RAW264.7 cells.

1.3 DPY19

The gene encoding the primary *C*-mannosyltransferase was first detailed as an ER localized enzyme in *C. elegans.*[16] Dolichol-P-mannose is a known substrate for other *C*-mannosyltransferases like protein *O*-mannosyl transferases and oligosaccharyltransferases.[17,18] The Bakker group used this knowledge of the sugar donor to begin their study of the transferase. Using Drosophila cells, the group noted high levels of *in vitro* mannose transfer from radiolabeled Dol-P-Man to synthetic peptides bearing the consensus sequence. Noting that this activity was highest in ER-rich fractions of these cells, they concluded that this process occurred in a manner similar to other glycosyltransferases.[16]

There are four human homologs to DPY19, namely DPY19L1, 2, 3, and 4. DPY19L1 and L3 are known to *C*-mannosylate TSRs in mammalian cell lines, while DPY19L2 is important to the development of human sperm.[11,19] DPY19L4 is expressed in mammalian cell lines, but its function is unknown.[19] Though the enzyme shares homology with the other *C*-

mannosyltransferases, it doesn't exhibit *C*-mannosylation of TSRs. If the enzyme is indeed a *C*-mannosyltransferase, other targets or *C*-mannosylated tryptophans that are not part of the canonical motif have yet to be identified.[19]

1.4 HSC70

Heat shock cognate 71 kDa protein (Hsc70), is a constitutively expressed member of the larger Hsp70 family. As with other heat shock proteins, Hsc70 is a molecular chaperone; this class of proteins is expressed to facilitate the folding of other proteins. In the case of heat shock proteins, molecular chaperones facilitate protein folding during periods of intense cellular stress that includes sudden levels of high heat exposure. The inflammatory response to a bacterial infection causes a rapid increase in temperature that can halt protein expression in mouse cell lines, whereas heat shock proteins like Hsc70 are consistently expressed to prevent damage to other proteins.[20]

Previous studies have indicated that both Hsc70 and *C*-mannosylated peptides could be influential to the innate immune response. In humans, Hsc70 features three different substructures. These include an *N*-terminal ATPase domain, a peptide binding domain, and *C*-terminal domain.[21] The substrate binding domain would be the most likely point of interaction for a *C*-mannosylated protein and Hsc70. However, the manner in which this protein complex is formed has yet to be elucidated.

1.5 INNATE IMMUNE RESPONSE AND LIPOPOLYSACCHARIDE

The innate immune response is described as the non-specific reaction by proteins and cells to highly-conserved features of pathogens.[22] In macrophages, cell surface receptors recognize bacterial antigens to initiate the non-specific response. Lipopolysaccharide (LPS),

found on the surface of gram-negative bacteria, is the initiator of the macrophage inflammatory response to a bacterial infection.

Lipopolysaccharide is a component of the outer membrane of gram-negative bacteria. This molecule consists of a lipid linked to a repeating *O*-glycan (*O*-antigen) by another core glycan. This structure is recognized by the outer surface of the RAW264.7 cells *via* LPS-binding protein (LBP). The protein transports LPS to CD14, a coreceptor to toll-like receptor 4 (TLR4). TLR4 is an initiator of the innate immune response in macrophage cells, initiating a cascade of signaling events and ultimately phosphorylating nuclear factor-kappa B (NF-κB), a transcription factor that is incorporated in the nucleus once its subunits are phosphorylated.[22] This factor regulates the pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-α) at the transcription level.[23] This cytokine is responsible for inflammatory signal transduction that eventually leads to cellular apoptosis and necrosis.[25] **Figure 4** illustrates the signal transduction in the context of human monocytes, yet this pathway is conserved in the RAW264.7 cell line.

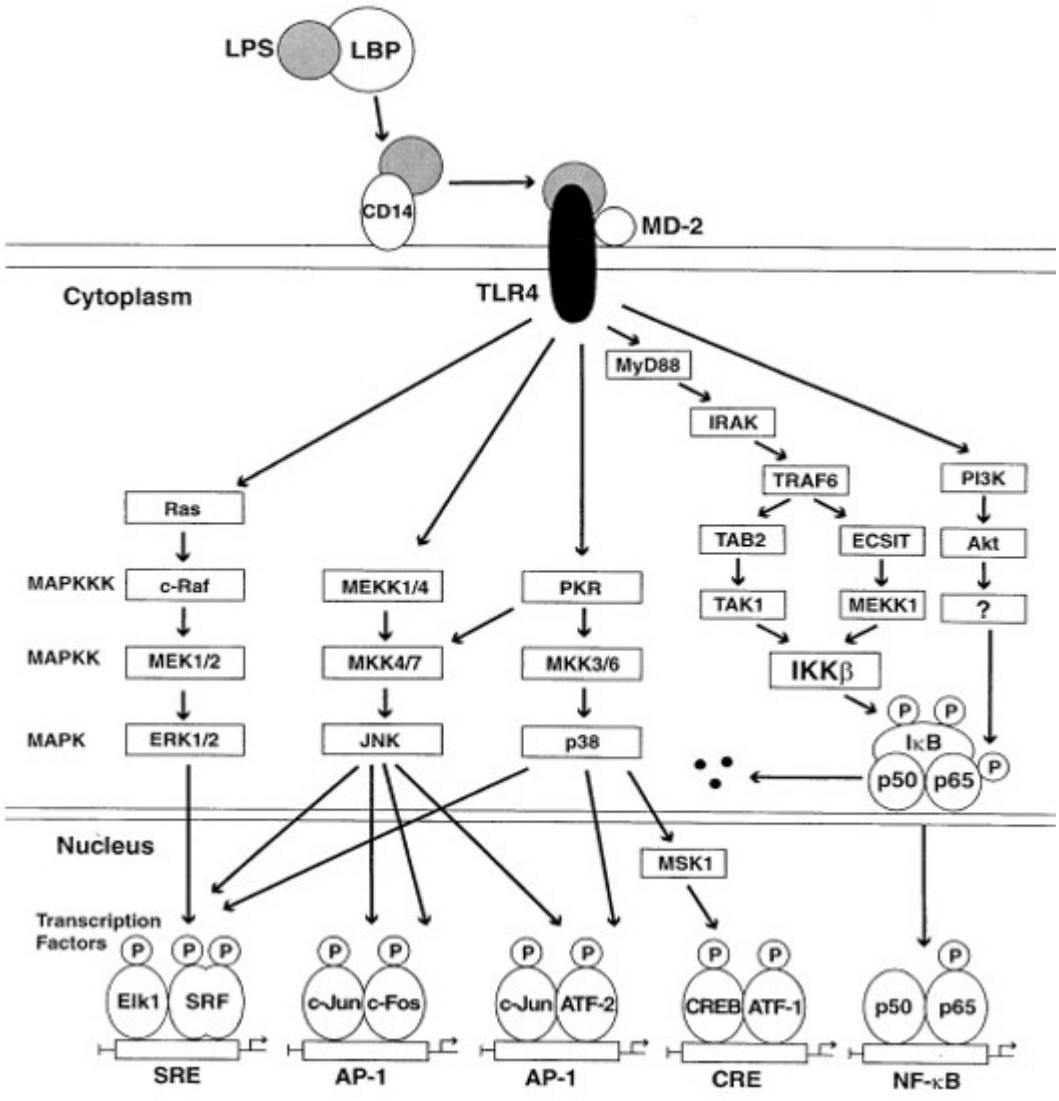


Figure 4: **Innate immunity pathways initiated by lipopolysaccharide**. Adapted from Guha, M., & Mackman, N. (2001). LPS induction of gene expression in human monocytes. *Cellular signaling*, 13(2), 85-94.[24]

Previous studies have indicated that Hsc70 and a *C*-mannosylated peptide may prove instrumental in the regulation of TNF-α expression in RAW264.7 cells, and could expand to human immune cells. Exploring the relationship between Hsc70 and its potential binding candidates could elucidate such a role for *C*-mannosylated candidates.

CHAPTER 2

METHODS

2.1 MAMMALIAN TISSUE CULTURE

RAW264.7 macrophage-like cells were the model system for this study. To simulate a bacterial infection to activate the macrophages, the cells were grown in culture media both with and without LPS. The LPS-treated media consisted of Dulbecco's Modified Eagle Medium (DMEM), 10% fetal bovine serum to supply vital growth nutrients, and penicillin/streptomycin to prevent bacterial infections. LPS-treated cells were serum-depleted 24 h prior to LPS stimulation. LPS was added at a concentration of 1 μg/mL as cell viability was previously determined to be greatest at this concentration.[26] Control cells received media without added LPS.

Timepoints of the RAW264.7 cells were collected throughout the study. Cells were collected at 0, 2, 4, 12, 24, and 48-h post-LPS stimulation. Stimulated cells were grown to roughly 70% confluence in either 10 cm^2 culture dishes in triplicate with non-LPS infused media, or in single 175 cm^2 flasks. Once a plate/flask reached this confluence level, the non-LPS media was aspirated from the container and replaced with LPS infused media, and the time point began from there. For example, a 0-h flask would have been collected immediately after LPS media was added, and a 12-h flask would have been collected 12 h after the media was added. Control cells were given fresh non-LPS media at the same time the LPS infused counterparts' media was swapped, then collected in parallel.

2.2 CO-IMMUNOPRECIPITATION

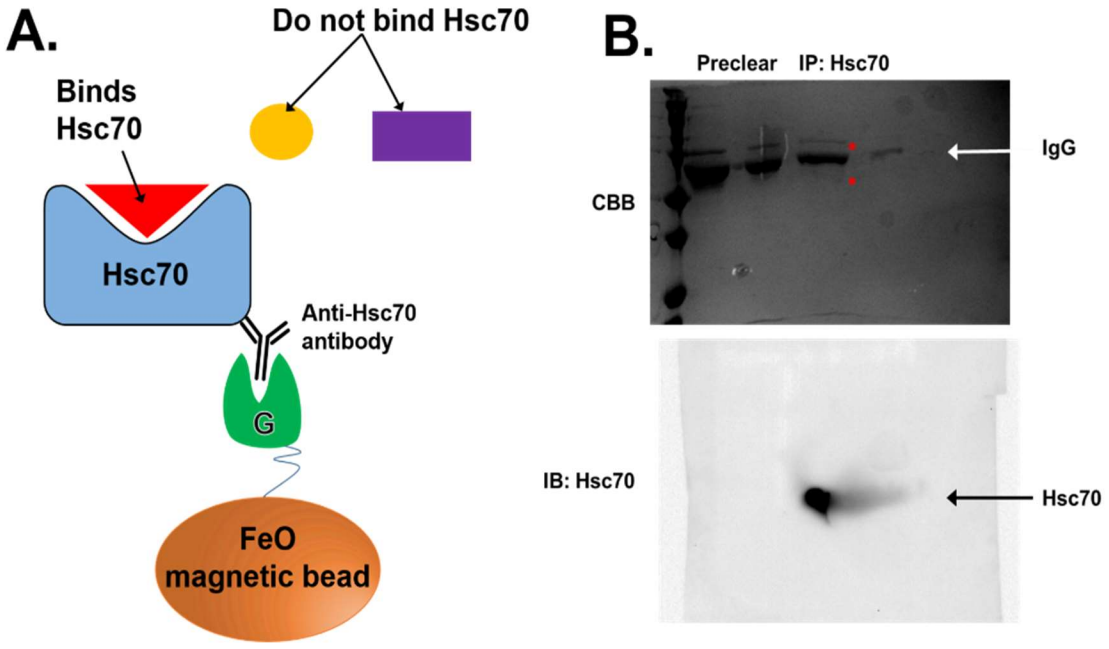


Figure 5: Hsc70 co-immunoprecipitation strategy
A) Schematic for Hsc70 co-immunoprecipitation. B) Top panel: separation of Hsc70 and IgG primary antibody *via* SDS-PAGE. Bottom panel: Hsc70 identification *via* western blot.

To isolate Hsc70 from RAW264.7 cells and characterize any interacting proteins, a co-immunoprecipitation strategy was utilized. A primary anti-Hsc70 antibody was incubated with magnetic beads conjugated to Protein G. The Protein G beads gave the antibody a stable, solid support to easily isolate the protein complex. **Figure 5** illustrates the binding schematic and preliminary results.

Noting that Hsc70 has been shown to interact and co-precipitate chemically derived *C*-mannosylated peptides, this strategy aimed to isolate Hsc70 and any other interacting protein.

The bead-antibody complex was incubated with crude RAW264.7 cell lysates to isolate Hsc70. Washing the complex with phosphate buffered saline (PBS) removed any proteins not interacting with Hsc70; applying heat eluted the Hsc70 protein complex from the antibody to visualize it *via* SDS-PAGE. **Figure 5** also illustrates this in panel B.

2.3 SDS-PAGE

Hsc70 has a known molecular weight of 70 kDa. The easiest way to identify this protein is thus to identify it by its weight. Electrophoresis was utilized heavily in this study to separate and characterize proteins based on their molecular weight. Various forms of electrophoresis allow for quick visualization of the large number of proteins in RAW264.7 cell lysates, as well as those present in immunoprecipitations and IP washes. The specific method of SDS-PAGE was utilized to separate proteins based solely on their weight without having to account for other features of proteins that could be part of the Hsc70 complex.

Pre-cast and poured BioRad minigels were utilized in SDS-PAGE experiments. Pre-cast gels and polyacrylamide were purchased from Bio-Rad (Hercules, CA, United States). These gels featured a 12% continuous layer of polyacrylamide to allow for uniform pore sizes, separating proteins based on their migration time across the gel. Larger proteins moved slowly towards the cathode, while smaller proteins moved more quickly. In the continuous gels, the polyacrylamide gel featured a stacking and resolving layer. The stacking layer allowed for proteins to migrate at the same pace and 'stack' into a single band. The second layer, or resolving layer, featured medium-sized pores based on the 12% polyacrylamide concentration in that layer. A higher percentage of polyacrylamide correlated with smaller pores, and lower percentages had larger pores. For poured gels, TEMED and ammonium persulfate from Thermo Fisher Scientific (Waltham, MA, United States) were used to promote polymerization of

acrylamide to form a gel matrix. Thermo Fisher SureCast Stacking (0.5 M Tris-HCl, pH 6.8) and Resolving (1.5 M Tris, pH 8.8) buffers were used alongside ammonium persulfate and TEMED to make the two gel layers. Gradient gels were also used, where gels had pores that decreased in size as proteins moved down the gel. The gradient gels eliminated the need for a stacking gel, as the changes in pore size prevented proteins of larger sizes from migrating past a certain point on the gel.

SDS-PAGE allowed for the migration of proteins based solely on their molecular weight due to the application of sodium dodecyl sulfate (SDS). A protein sample (crude cell lysate, immunoprecipitation elution, washes, etc.) was first reduced in a buffer containing beta-mercaptoethanol. Reducing buffers reduced disulfide bridges to unfold protein tertiary structure and provide a "linear" form of the proteins in the sample. The addition of SDS, a detergent, further unfolds proteins into a linear form.[27] The SDS molecule also conferred a negative charge to the proteins. Reduced protein samples were loaded into the top of an SDS-PAGE gel, where the anode is located. As an electric potential was applied to the gel, proteins migrated toward the cathode located at the bottom of the gel, being filtered by the porous resolving layer as they moved further down.[27]

To visualize the proteins trapped in the gel pores, two different staining methods were utilized. Typically, Coomassie blue staining was used to stain the gel. This stain binds to the trapped proteins, but doesn't bind as well to the gel components, meaning that it could easily be washed off using an acidic destaining solution. The manufacturer BioRad lists the sensitivity for this stain as between 30 – 50 ng of protein. However, sometimes even less protein was trapped in the gel pores, and still proved relevant to the study. To visualize such small concentrations of protein, silver staining was utilized. The Pierce Silver Stain for Mass Spectrometry kit, again

from Thermo Fisher, advertised a sensitivity for protein concentrations less than 0.25 ng, making it over a hundred times more sensitive than the Coomassie method. Similar to the Coomassie method, silver deposits bound the peptides to clearly visualize them. A destaining solution contained in the kit removed silver deposits so that peptides could be further characterized by mass spectrometry.

2.4 WESTERN BLOTTING

While gel electrophoresis allowed for detection of proteins based solely on mass, it did not reveal much information on the specific protein primary structure. Western blotting allowed for more specific identification of proteins isolated by gel electrophoresis using antibodies generated in host animals against the specific proteins of interest in this study. A common target of western blotting was Hsc70, allowing for proteins not detected by an Hsc70 antibody to be further examined as potential candidates in a *C*-mannosylated protein-Hsc70 complex.

Beginning with an SDS-PAGE gel, isolated proteins were transferred to a nitrocellulose membrane to allow for detection of proteins with a primary antibody. Nitrocellulose membranes feature a higher affinity for proteins than a gel; the porous nature of a gel would also present the issue of proteins diffusing through the gel matrix over time. Immobilizing the proteins on a membrane makes detection *via* antibodies possible. The primary antibodies were grown in a host animal different from the cell type that the proteins were isolated from. In this case, antibodies generated in hosts like goats and rabbits were selected as primary antibodies to avoid cross-reactivity with endogenous proteins from the same type of animal. Immunoglobin G antibodies (IgG) were the most common type of antibody used in this study.

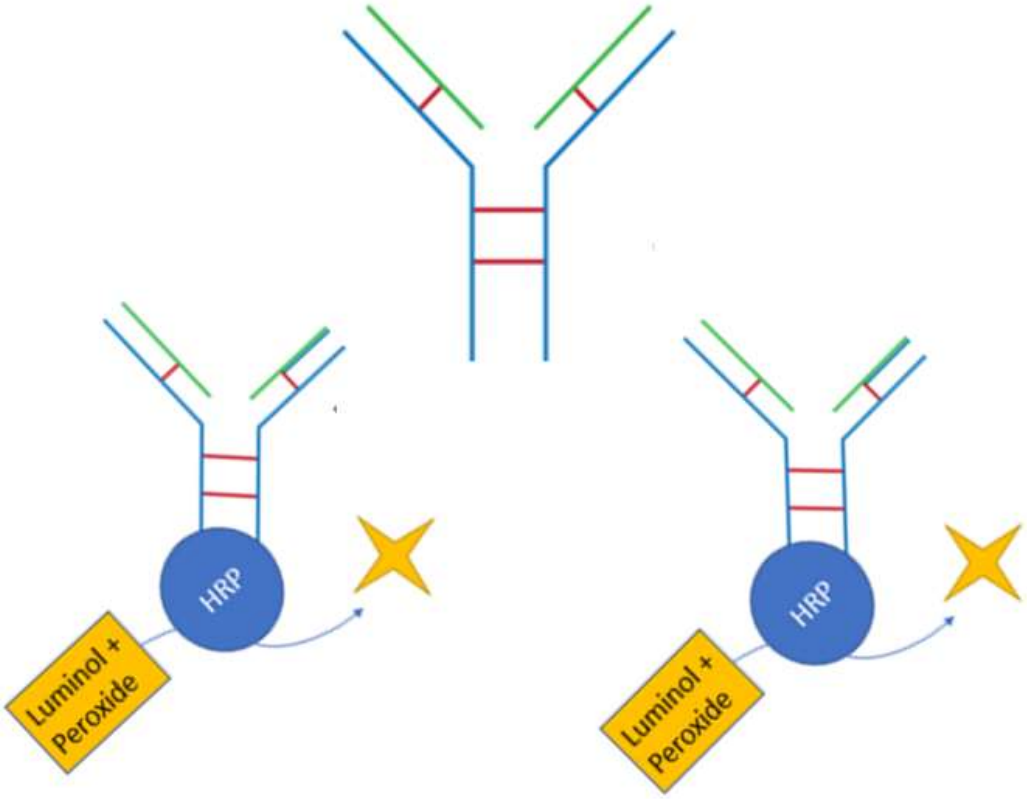


Figure 6: Secondary detection of IgG antibody via horse radish peroxidase-conjugated antibodies.

The IgG structure featured two heavy and two light chains; the heavy chains are linked together by disulfide bonds and linked to the separate light chains by separate disulfide bonds. The N-terminus featured the variable region where two antigen binding sites are located; this is where the primary antibody recognizes and binds to the antigen. The C-terminus featured a constant region, where the amino acids of the heavy chains are conserved across the IgG type. The constant region is where secondary detection of the primary antibody by an anti-IgG secondary antibody occurred.[28]

The choice of the secondary antibody was again determined by host reactivity and immunoglobin type. The variable region of the secondary antibody recognized the constant region of the primary. These goat/rabbit-generated secondary antibodies can recognize any IgG-

type antibody due to the conserved amino acid sequence across all IgG constant regions. To detect the secondary/primary antibody construct, two methods were used. Some secondary antibodies were conjugated with horseradish peroxidase (HRP), an enzyme that catalyzes the oxidation of a substrate to produce light. Luminol was frequently used as an HRP substrate, and its oxidation by peroxide causes a strong emission of light; this process is known as chemiluminescence, and allowed for visualization of the protein of interest by indicating where chemiluminescence occurred on the blotting membrane, as illustrated in **figure 6**. Other secondary antibodies were conjugated with IRDye, a product from LI-COR Biosciences (Lincoln, NE, United States). Instead of using an enzyme to catalyze a light-producing reaction, the IRDye simply absorbed light at one wavelength, then produced light of a particular wavelength. Using the LI-COR Odyssey imaging system, multiple blots were visualized with IRDye antibodies.[29]

2.5 MASS SPECTROMETRY SAMPLE PREPARATION

Preparation of SDS-PAGE samples for mass spectrometry is an essential process to acquire accurate data. Bands from the SDS-PAGE gel were physically excised using a razor blade and kept in microcentrifuge tubes. The bands in these gels contained proteins that were then reduced through the use of tris(2-carboxyethyl)phosphine (TCEP); this breaks disulfide bonds that maintain the tertiary structure of the protein. Dithiothreitol (DTT) was also used for this purpose. For preparation for the Orbitrap instrument, 100 µL of "reduction solution" (4 µL TCEP from 0.5 M stock and 96 µL 50 mM Tris-HCl pH 6.8) were added to excised gel pieces. Iodoacetamide is then used to alkylate the sulfhydryl (-S-H) groups in order to prevent reformation of the disulfide bonds.[30]

With the proteins alkylated, an enzymatic digestion by proteases such as trypsin and chymotrypsin was performed. Trypsin cleaved c-terminal to basic residues like lysine and arginine, whereas chymotrypsin cleaved after aromatic residues like tyrosine, phenylalanine, and tryptophan. Trypsin and chymotrypsin are both categorized as serine proteases; these enzymes utilized a serine sidechain within their active sites to perform a nucleophilic attack on the peptide bond which is cleaved.

Preparation of samples for MALDI-TOF characterization required some specific protocol changes. DTT reductions occurred in 25 mM $NH_4C_2H_3O_2$ at 56 °C for 1 h; 1.5 mg of DTT was dissolved in 1 mL 25 mM $NH_4C_2H_3O_2$, with 25 µL of the resulting solution added to each excised gel piece. Iodoacetamide alkylations utilized 25 µL of 55 mM iodoacetamide added to reduced samples. Gel pieces were then dehydrated with 25 mM NH_4HCO_3 in 50% acetonitrile prior to tryptic digests, which was performed in 25 mM $NH_4C_2H_3O_2$.

Following the enzymatic cleavages, peptides were extracted for analysis with a C18 ZipTip. The pipet tip is packed with a nonpolar, hydrophobic alkyl chain to do reverse-phase chromatography within the tip. The nonpolar stationary phase allowed for the weakly polar peptides to be trapped in the tip when extracted from solution. The tip was washed to eliminate any salts that would interfere with the ionization process; peptides were then eluted from the column using acetonitrile due to its high relative polarity. For the MALDI-TOF analysis, peptide solutions were mixed with matrix and spotted onto the sample plate. For the hybrid orbitrap-quadrupole analysis, the eluted peptide solutions were diluted to proper levels of acetonitrile concentration prior to injection onto a C_{18} column for HPLC separation.[31]

2.6 MATRIX ASSISTED LASER DESORPTION/IONIZATION – TIME OF FLIGHT (MALDI-TOF)

Western blot technologies essentially gave a positive/negative response as to whether a protein was expressed in a cell line. To know what specific protein was detected, mass spectrometry was necessary to identify proteins by the mass fingerprint technique. Mass spectrometry is an analytical technique in which ions are detected and masses determined. To accomplish this, ions are detected based on their relative kinetic energy when reaching a mass detector. The mass analyzer separates the peptide ions by their m/z values, their mass to charge ratio. The first type of mass spectrometry used in this study was matrix-assisted laser desorption ionization time-of-flight, or MALDI-TOF.

MALDI-TOF is a useful type of MS for analyzing biomolecules. Many other types of MS require volatile molecules that readily go into the gas phase; proteins are nonvolatile and require assistance to reach this phase. Applying enough heat to cause a protein to reach this phase would ultimately destroy or decompose the protein before a mass to charge ratio could be obtained. To assist a biomolecule in entering the gas phase, a crystalline matrix (organic acids with ring structures) is mixed with the biomolecule (analyte). The matrix absorbed photons from a high intensity N_2 laser, causing the matrix/analyte molecules to be desorbed into the gas phase at the same time. The matrix molecule transfers a proton to a basic group (like R, K, and N-terminus) in the peptide; the matrix α-cyano-4-hydroxycinnamic acid produced uniform $(M+H)^+$ peptide ions due to its acidity relative to the analyte. The peptide ions were accelerated into a flight tube, where each ion has the same kinetic energy based on the voltage on the accelerating lens. The time it takes an ion to reach a detector while passing through a flight tube is proportional to the mass-to-charge ratio. An equation for kinetic energy presents this information:

$$E = \frac{1}{2}mv^2$$

Kinetic energy is equal to the charge of an ion and the accelerating lens voltage, which rearranges the equation.

$$v = \sqrt{\frac{2V*e}{\frac{m}{z}}}$$

Factoring in the time delay (t) for the ions to reach the detector through a flight tube depends on the drift region found within the tube (L).

$$t = \frac{L}{\sqrt{2V*e}} * \left(\sqrt{\frac{m}{z}}\right)$$

The above equation gives the mass-to-charge (*m/z*) ratio as a function of time with respect to the path length and voltage (V) of the instrument.

2.7 ESI-LC MS ORBITRAP – Q EXACTIVE HYBRID QUADRUPOLE-ORBITRAP

Electrospray ionization (ESI) is also used to get nonvolatile biomolecules in the gas phase. ESI is considered a "soft" ionization technique in that it does not fragment the peptide into numerous smaller particles. The eluted peptide solution was passed through a charged nozzle at a high pressure to generate charged droplets. The solvent then transferred protons to the peptides to generate a range of ion species. Drying gases like nitrogen continually remove solvent around the now charged peptides; as the solvent volume was reduced, ions would be forced into smaller and smaller ion packets. The drying continued until only single ions were left in the gas phase. Unlike MALDI-TOF, where a vacuum co-crystalizes both the matrix and analyte, this method is purely solution based with no intermediate solid phase.[32]

The ion species sprayed from the ESI tip then travels to a quadrupole precursor ion selector, the first part of the tandem mass spectrometer. The quadrupole-filtered ions based on their m/z ratio by alternating radio frequencies. The quadrupole caused the ions to oscillate between the charged poles, where ions of a certain mass are spun out of the quadrupole; ions that filter through are then transferred to an HCD trap for fragmentation, which is later presented as MS/MS data by the mass analyzer located in the orbitrap. Fragmented ions were then released back to the curved linear trap.

The curved linear trap (C-trap) used a collision gas (nitrogen) to reduce the kinetic energy of ions that have been fragmented within the HCD trap. From the C-trap, reduced-energy ions were sent to the orbitrap for detection and analysis. The detection of the m/z values of the fragment ions was based on their characteristic oscillation frequencies as observed in the orbitrap.[31]

CHAPTER 3

HSC70, JUNCTOPHILIN-1, AND HEK293T TRANSFECTIONS

3.1 HSC70 CO-IMMUNOPRECIPITATION

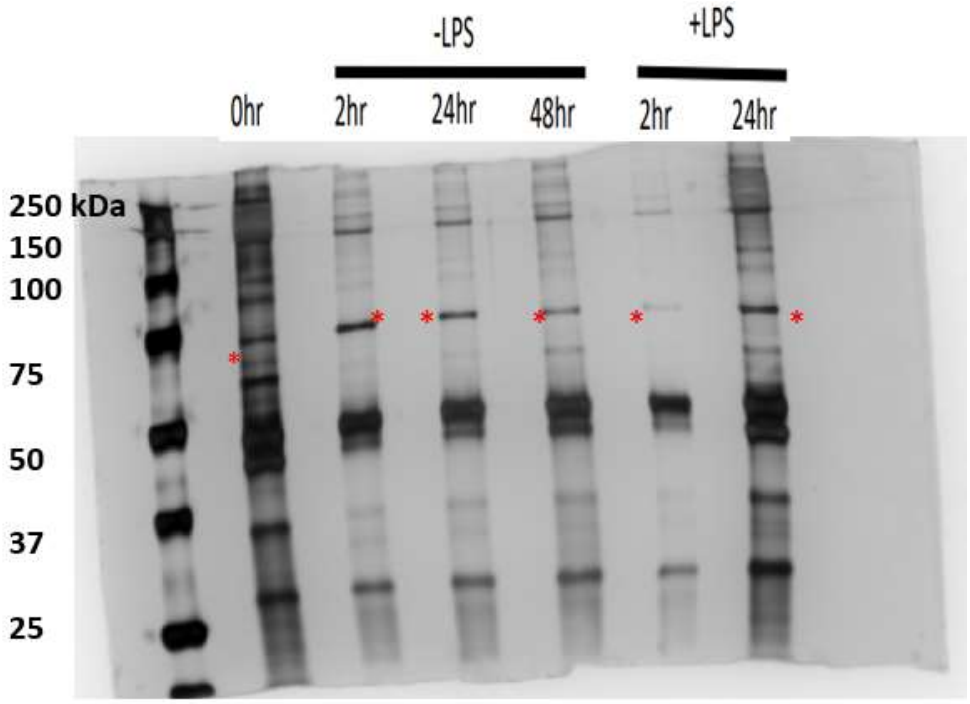


Figure 7: Silver stained SDS-PAGE gel of immunoprecipitated RAW264.7 lysates.

-LPS indicates non-stimulated samples, while +LPS indicates cells grown in LPS infused media. Red asterisks indicate protein bands roughly corresponding to 70 kDa when compared to the protein standard.

The outlined Hsc70 co-immunoprecipitation was performed across many different timepoints across the RAW264.7 cells. Hsc70 was precipitated from RAW cells with LPS infused media at 2 and 24 h post stimulation, and at 0, 2, 24, and 48 h without LPS. As **Figure 7** shows, numerous protein bands were visible alongside Hsc70 once the co-immunoprecipitations were visualized *via* silver-staining. As predicted with the co-immunoprecipitation strategy, any one of these bands were noted as potentially interacting proteins within an Hsc70-*C*-mannosylated protein complex. Each band in **Figure 7** not identified as Hsc70 was excised from the gel and prepared for identification *via* mass-spectrometry.

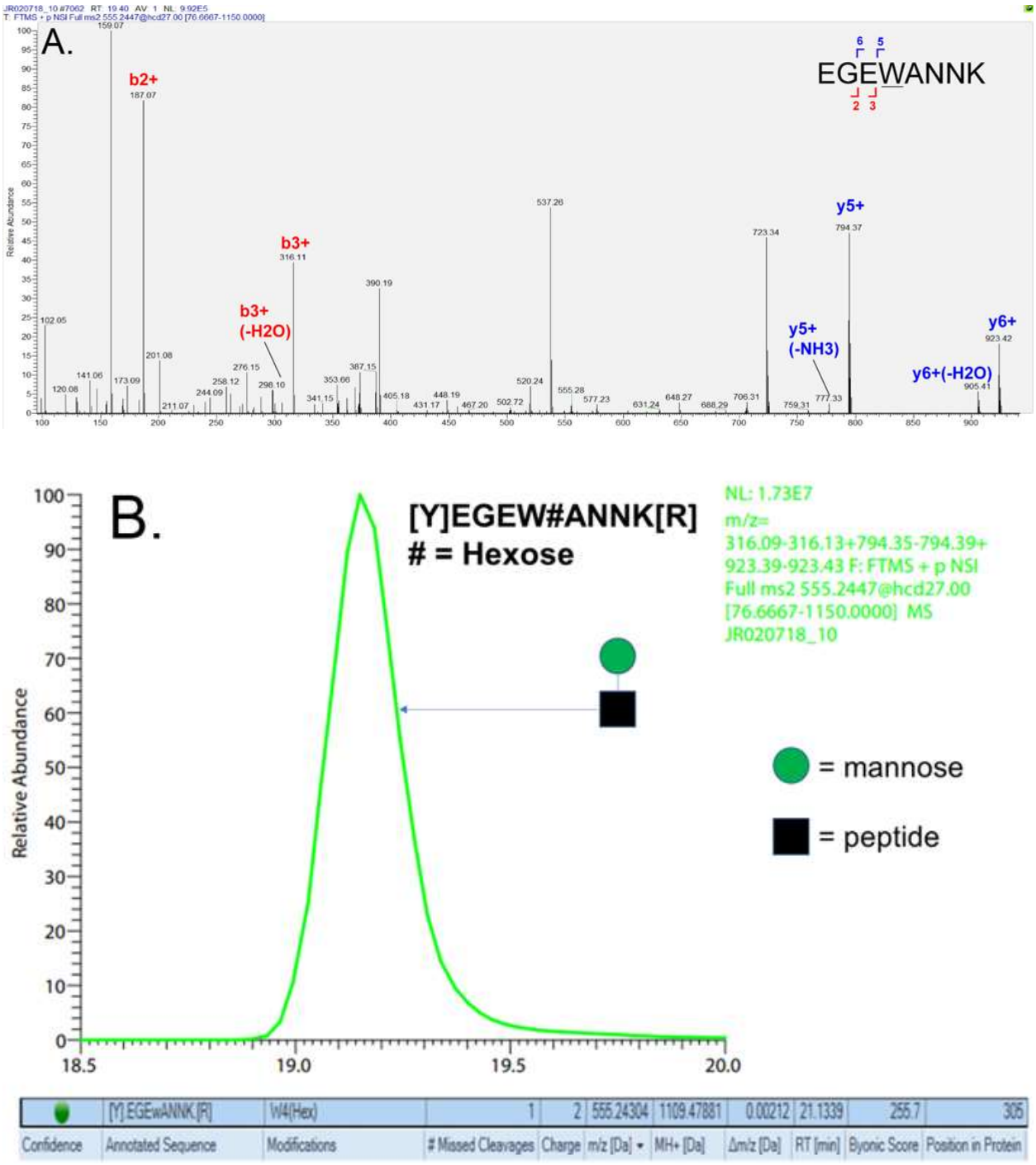


Figure 8: MS/MS spectra and extracted ion chromatogram of C-mannosylated JPH1 peptide. A) MS/MS collision induced dissociation spectra of JPH1 peptide. B) Extracted ion chromatogram of JPH1 peptide.

Many proteins were identified alongside Hsc70 from these co-immunoprecipitations.

Among these was Junctophilin-1 (JPH1), a protein typically found in skeletal muscle. A single

peptide bearing a hexose on a tryptophan residue was identified. Curiously, this peptide did not bear the W-x-x-W-x-x-W/C consensus sequence. Building an extracted ion chromatogram for the *C*-mannosylated peptide showed an almost 100% relative abundance of the modified peptide compared to an unmodified, or "naked" peptide, shown in **Figure 8** alongside its relevant MS/MS spectra. When researching the full primary sequence for JPH1, it was also noted that this protein did not contain a single TSR. To confirm the status of JPH1 as a putatively *C*-mannosylated protein, attention was turned to focus on overexpression of the protein in a cell line that would readily express the protein.

3.2 TRANSIENT JPH-1 TRANSFECTION - POLYETHYLENIMINE

A pCMV6 entry vector containing JPH1 (OriGene, Rockville, MD) was used to transiently transfect the full-length protein into HEK293T cells, a human embryonic kidney cell line used for their particularly robust ability to express transfected proteins. HEK293T cells were seeded at 850,000 cells per well in a 6-well culture plate. These cells were diluted in DMEM + 10% bovine calf serum, with no antibiotics present in the media. Cells were incubated overnight at 37 ºC and 5% CO_2. Afterward, a transfection mix containing 1.6 µg of JPH1 plasmid, 0.1 µg of human IgG construct as a positive transfection control, and 30.6 µL polyethylenimine (PEI) to facilitate the transfection. PEI condensed the plasmid into small charged particles to interact with the negatively charged surface of the HEK cells; the plasmid was subsequently endocytosed into the cell, where the cDNA was used to express the protein. A control master mix was also made in triplicate with pSec2Tag2 empty vector used in place of the JPH1 plasmid. Both master mixes were diluted in OPTI-MEM, a low-glucose growth medium.

Respective master mixes were added dropwise to the HEK293T growth media. Cells were incubated at 37 ºC and 5% CO_2 for 4 hours. After the incubation, the media was aspirated

from the cells, and the cells were washed with warm 1x PBS. Following the wash, 700 µL of OPTI-MEM was added to each well of cells and the plate was returned to the incubator. Cells were harvested and lysed roughly 48 hours post-transfection.

3.3 WESTERN-BLOT: CO-TRANSFECTED JPH1

Following the collection of transfected HEK293T cell lysates, crude lysates were boiled in a reducing buffer and run on a precast SDS-PAGE minigel. Protein bands trapped in the gel were transferred to a nitrocellulose membrane, and then blotted with an anti-Myc antibody at 1:1000 dilution in a 5% w/v buffer of powdered milk and TBS-T. The JPH1 vector also contained a coding region for a c-Myc tag and FLAG, two protein tags that were used as commonly recognizable antigens for primary antibodies. Secondary antibodies, goat anti-mouse and goat anti-human, were used at a dilution of 1:10,000 in the same buffer, binding the primary anti-myc antibody and human IgG construct respectively.

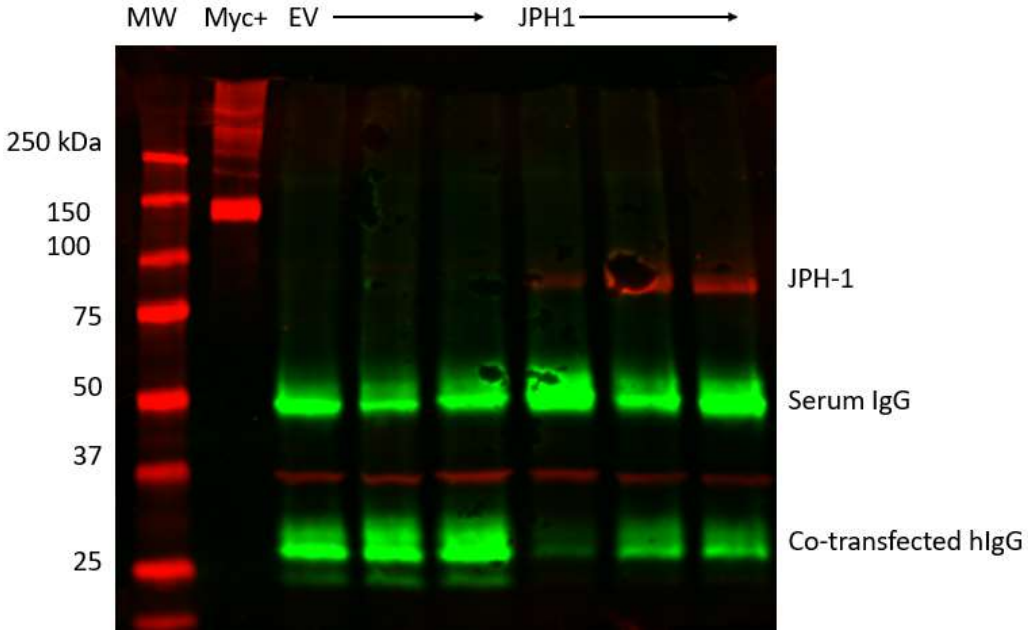


Figure 9: Western blot of JPH1-IgG co-transfected HEK293T cell lysates. Myc+ refers to myc-tagged ADAMTSL2 standard, EV refers to cells transfected with pSec empty vector, and JPH-1 refers to cells transfected with JPH-1 plasmid. All cells were transfected with a human IgG construct.

As expected, the primary anti-Myc antibody only bound the protein tag in the transfected crude cell lysates. Cells transfected with empty vector and hIgG showed no bands corresponding with c-Myc-tagged proteins. **Figure 9** shows this western blot, where the presence of Myc-tagged proteins was confirmed. To further confirm that the Myc-tagged bands were transfected Junctophilin-1, the same crude cell lysates were separated through another SDS-PAGE gel to be processed for mass spectrometry.

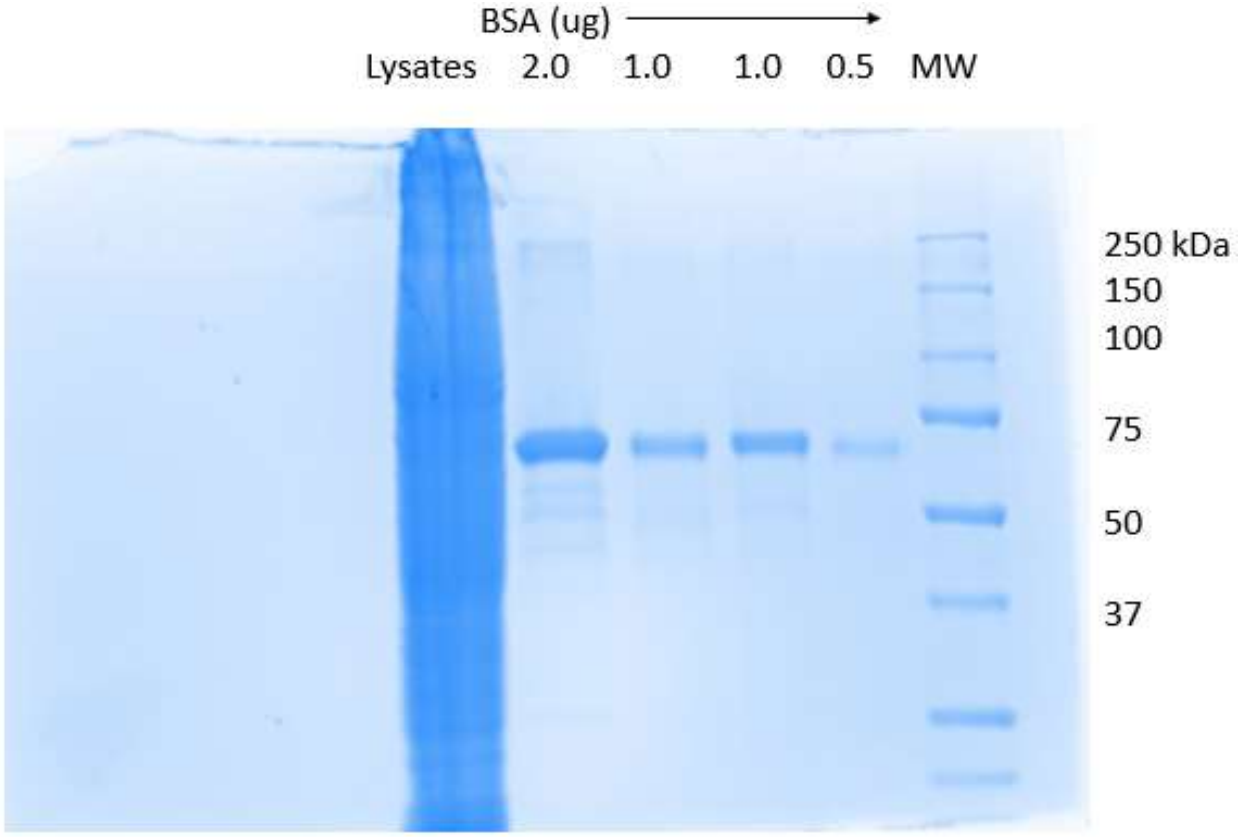


Figure 10: CBB stained SDS-PAGE gel of transfected HEK293T crude cell lysates and bovine serum albumin. Lysates are included in the leftmost lane and decreasing concentrations of BSA standard are included in the BSA labeled lanes. MW indicates the molecular weight standard.

Cell lysates were used in one lane, while bovine serum albumin (BSA) was used as a control to roughly estimate the concentration of protein observed in the lysate. As seen from the gel in **Figure 10**, the lysates far exceeded 2 µg of protein, so much so that the protein band of

interest was obfuscated. Comparing the gel to the previous western blot, a part of the lysate lane corresponding to roughly 75 kDa was excised from the gel to be reduced, alkylated, and cleaved with trypsin to be prepared for the mass spectrometer.

3.4 HYBRID QUADRUPOLE-ORBITRAP MS/MS

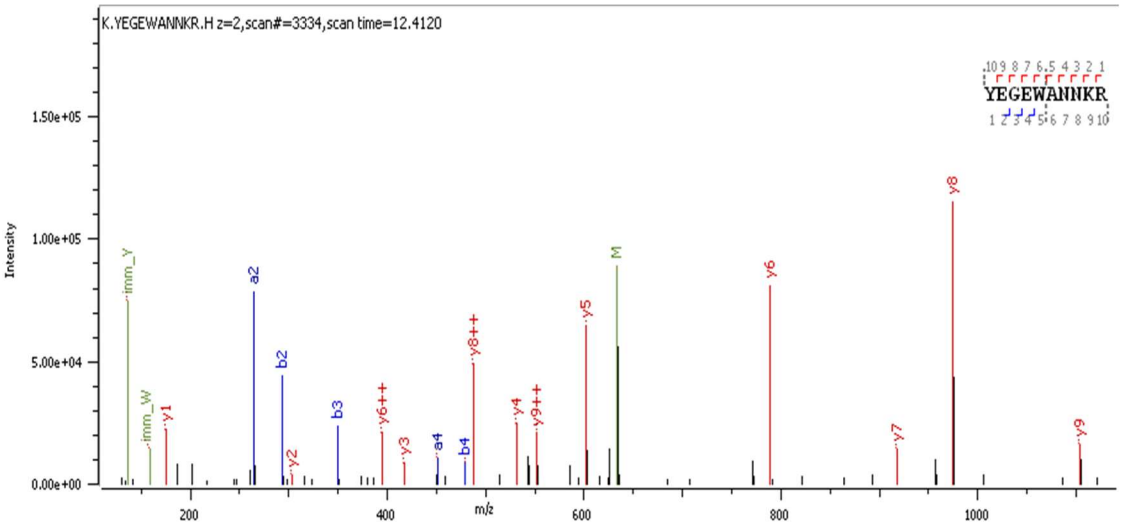
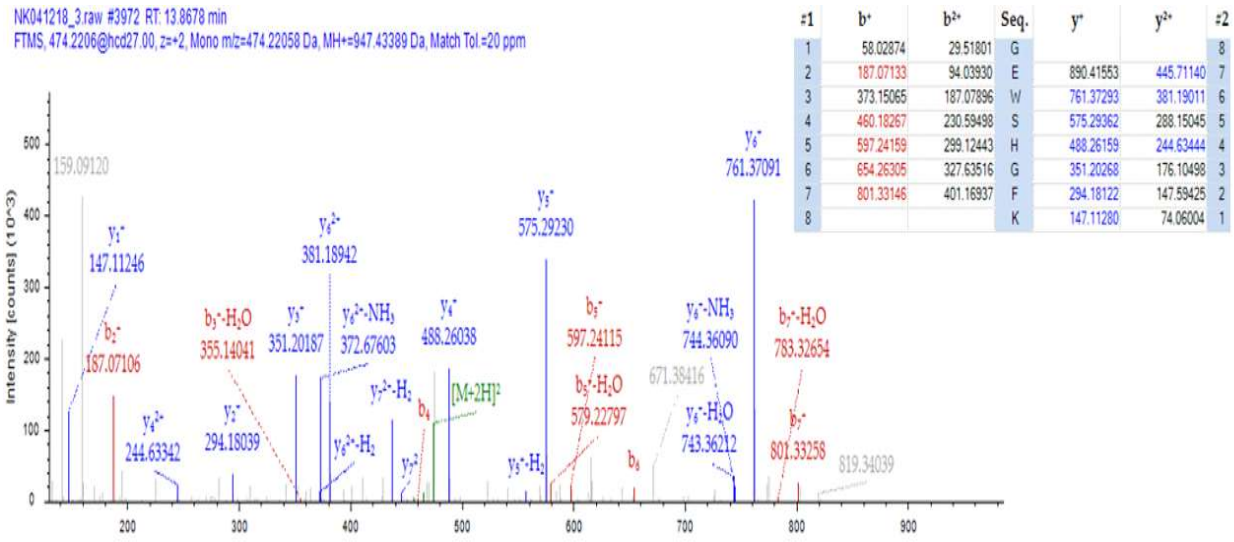


Figure 11: MS/MS spectrum of JPH1 peptide. Spectrum was taken from Byonic analysis software, with m/z ratio plotted on the x-axis and peak intensity plotted on the y-axis.

After the transfected proteins were proteolytically cleaved and injected into the mass spectrometer, tandem mass spectra were generated. The MS/MS data presented in **Figure 11** shows that a peptide nearly identical to the original endogenous JPH1 example was isolated from the transfected cells. Since only trypsin was used in this digest, an extra tyrosine was included on the peptide. When compared to the UniProt mouse protein database, this peptide was found to be unmodified in HEK293T cells. Other peptides featuring tryptophan were also identified by collision induced dissociation of peptides injected into the instrument. No TSR modules were noted in either peptide or motifs for *C*-mannosylation. A modified peptide different from the two presented here was detected by Byonic MS analysis software, but with a "low confidence" rating. The low confidence rating indicated that few ions were detected, and the peptide was constructed based on predictions made by the software.



#1	b+	b2+	Seq.	y+	y2+	#2
1	58.02874	29.51801	G			8
2	187.07133	94.03930	E	890.41553	445.71140	7
3	373.15065	187.07896	W	761.37293	381.19011	6
4	460.18267	230.59498	S	575.29362	288.15045	5
5	597.24159	299.12443	H	488.26159	244.63444	4
6	654.26305	327.63516	G	351.20268	176.10498	3
7	801.33146	401.16937	F	294.18122	147.59425	2
8			K	147.11280	74.06004	1

Figure 12: MS/MS spectrum of JPH1 peptide. Spectrum was taken from Byonic analysis software, with m/z ratio plotted on the x-axis and peak intensity plotted on the y-axis.

While JPH1 was successfully transfected into HEK293T cells, no *C*-mannosylated peptides were detected by the Orbitrap mass analyzer. **Figure 12** presents a second JPH1 peptide from the previously displayed gel, but this was also unmodified. As described previously, *C*-mannosylation has been found to occur in RAW264.7 cells to multiple proteins with the necessary consensus sequence. However, no data has been shown for this to be a naturally occurring process in HEK293T cells. With this knowledge, focus was turned back to the RAW264.7 system. Using multiple transfection methods, attempts were made to overexpress JPH1 in the cell line.

CHAPTER 4

RAW264.7 TRANSFECTIONS AND ENDOGENOUS IMMUNOPRECIPITATIONS

4.1 ELECTROPORATION OF RAW264.7 CELLS

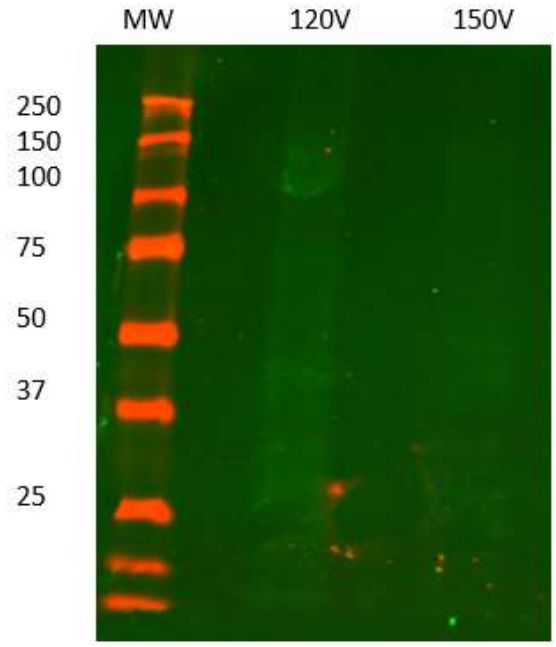


Figure 13: Western blots of electroporated RAW264.7 cells.
120V and 150V refer to potential difference settings at which cells underwent electroporation.

RAW264.7 cells proved to be difficult to transfect with plasmid DNA through conventional PEI based methods. One of the attempted methods at incorporating plasmid DNA in the cell line was electroporation. Cells were grown to a density of roughly 190,000 cells/mL, then placed in a cuvette along with roughly 18 µg JPH1 DNA. A brief pulse of electricity was passed through leads on opposite sides of the cuvette to open pores in the cell membrane, allowing the DNA to pass into the cytosol of the RAW264.7 cells.

Following the electroporation, cells were taken from the cuvette and resuspended in fresh DMEM growth media, then allowed to grow on a 10 $cm.^2$ plate. Cells were harvested once they had reached roughly 70% confluency, lysed, and subjected to the typical western blot protocol. Crude cell lysates that underwent electroporation at 120 and 150 V were blotted against using anti-Myc and IgG antibodies. No proteins were detected by the anti-Myc antibody, but some

bands appeared that corresponded with the IgG construct that was co-transfected with JPH1 DNA, as seen in **Figure 13**.

4.2 TRANSFECTION – LIPOFECTAMINE 2000 AND LTX

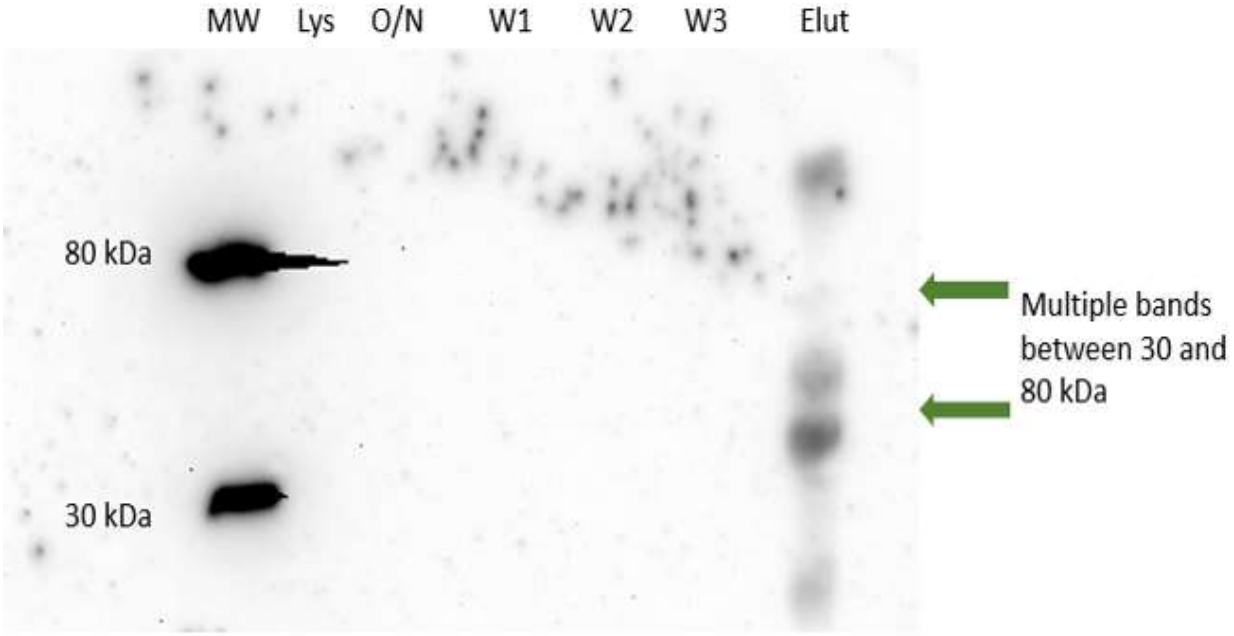


Figure 14: JPH1 immunoprecipitation and western blot Western blot of JPH1 transfected RAW264.7 cells that were also immunoprecipitated with an anti-JPH-1 antibody. MW indicates the molecular weight standard, with two chemiluminescent standard peptides. Lys is crude cellular lysate, O/N is the solution flowthrough after overnight immunoprecipitation. Washes 1-3 are indicated by W1, W2, W3, and eluted samples are included in the Elut lane. Primary antibody was used at 2 µg/mL buffer, secondary at 1:10,000 dilution.

Successive attempts at transfection of JPH1 into RAW264.7 cells using PEI proved to be unsuccessful. Lipofectamine is a class of reagents that form lipid-DNA complexes to facilitate transfections into mammalian cells by less stressful means. DNA is encased by a cationic liposome to overcome the repulsion between the plasmid and cell membrane, both of which are negatively charged. Two different Lipofectamine reagents were used, 2000 and LTX. Lipofectamine LTX is marketed as a reagent with a specific protocol outlined for RAW264.7 DNA transfections. Based on multiple western blots, Lipofectamine 2000 did not produce any viable JPH1 transfections for RAW264.7 cells. Lipofectamine LTX produced one western blot

that appeared to be a positive result for an anti-JPH1 blot of RAW264.7 cells that appeared to have been successfully transfected, as seen in **Figure 14**. Unfortunately, MALDI-TOF mass spectrometry of these protein bands did not return any results for JPH-1 peptides.

4.3 MALDI-TOF SPECTRA OF DIGESTED RAW264.7 PROTEINS

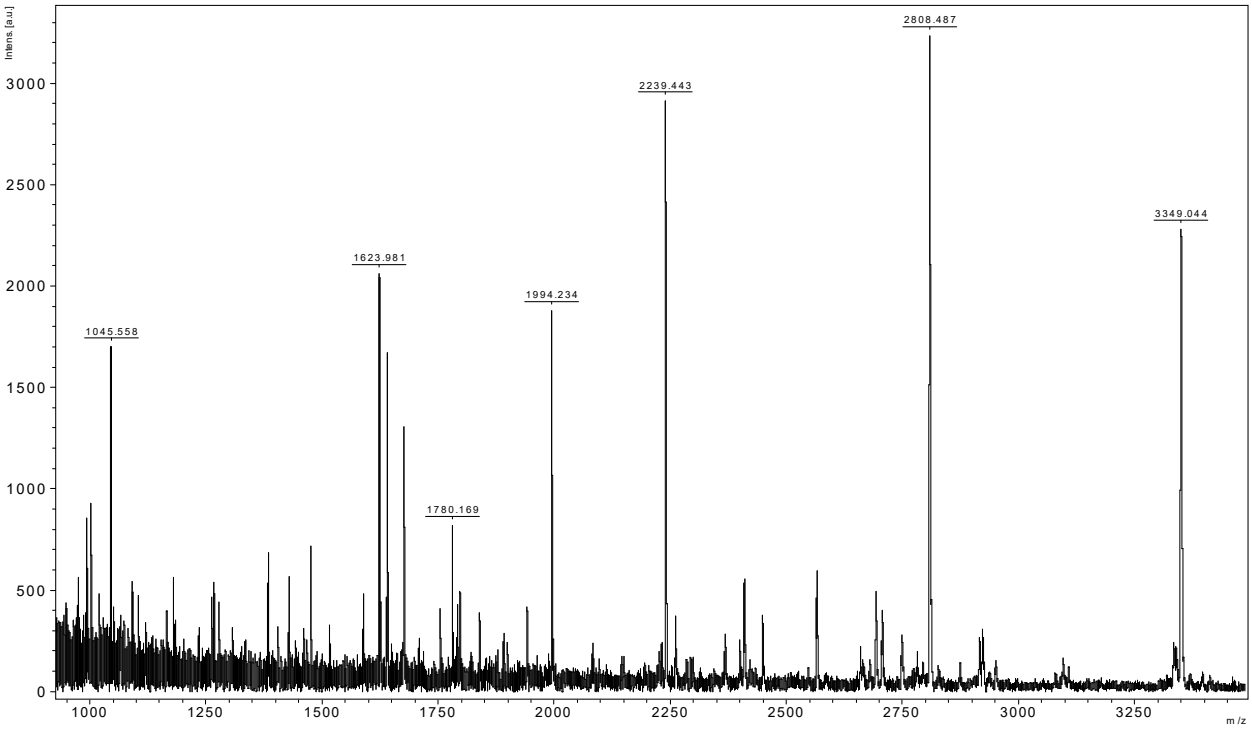


Figure 15: **MALDI-TOF MS spectrum of JPH1 transfected RAW264.7 cells.** X-axis represents m/z values, while y-axis represents relative intensity.

Further attempts at transfection of mouse JPH-1 into RAW264.7 cells were attempted after returning to Marshall University. Various concentrations of JPH-1 plasmid and lipofectamine reagents were used to facilitate the transfection. Crude cell lysates and immunoprecipitations of attempted RAW264.7 cell transfections were measured by Dr. Frost of the Marshall University Department of Chemistry to be characterized by MALDI-TOF mass spectrometry. Following the previously outlined mass spectrometry processing techniques, peptides were enzymatically digested for characterization *via* MALDI-TOF. **Figure 14** shows multiple peaks across a MS spectrum that correspond with the weight of various peptides.

However, these peaks did not correspond to previously identified peptides from JPH1, including the *C*-mannosylated peptide identified from the Hsc70 co-immunoprecipitation. Based on peptide fingerprinting and Mascot peptide database comparisons, 1110 m/z would correspond with the peptide YEGEWANNK, while 946 m/z would be EGEWANNK.

4.4 RyR1 IMMUNOPRECIPITATION

Facing difficulties with JPH1 transfections and immunoprecipitations, a new target for co-immunoprecipitation was chosen. The Ryanodine receptor type 1 (RyR1) is a protein that mediates the release of calcium ions stored within the sarcoplasmic reticulum, found within skeletal muscle. Using an anti-RyR antibody, an immunoprecipitation for endogenous RyR1 was performed, with the aim to co-precipitate JPH1 as part of an RyR1-JPH1 complex. **Figure 15** presents the RyR1 immunoprecipitation separated by and SDS-PAGE gel in the left panel, while the right panel presents a western blot of the same samples using an anti-JPH1 antibody.

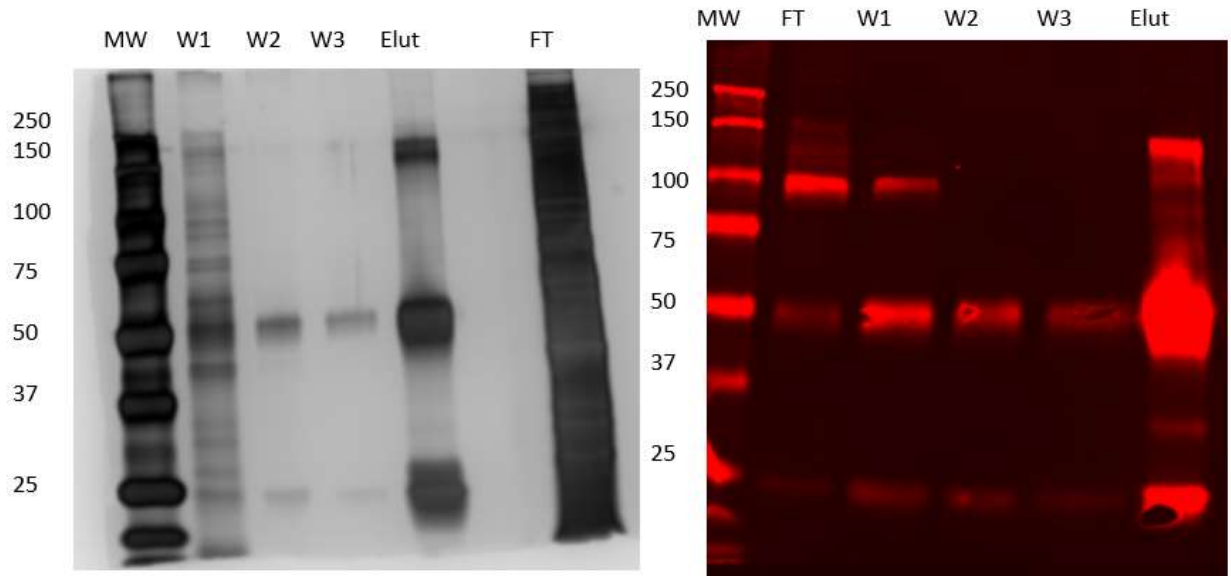


Figure 16: RyR1 immunoprecipitation and JPH1 western blot
Left panel: silver-stained SDS-PAGE gel of RyR1 IP from wildtype RAW264.7 cells. 'W' lanes indicate washes, 'Elut' indicates eluted proteins, and 'FT' indicates the overnight flowthrough solution. Right panel: anti-JPH1 western blot of RyR1-immunoprecipitated samples.

While the western blot in the right panel presents many positive instances of protein binding, these appear to have been false positives. Excising/digesting bands consistent across the SDS-PAGE gel and the western blot, no proteins were found to match JPH1. RyR1 is a large protein (over 250,000 Dalton) and could have aggregated at the top of the gel's stacking layer, preventing the RyR1-JPH1 complex from being separated by the gel. As for the false positives, the anti-JPH1 antibody could have recognized an epitope common between the JPH1 target and another non-specific protein.

4.5 JUNCTOPHILIN 1

Junctophilin 1 (JPH1) is a protein that features multiple integral membrane domains and is essential to the function of excitable cell types like muscle and neuronal cells. This protein is part of a larger family of proteins referred to as junctional membrane complex-associated proteins (JMC). The structure of JPH1 contains several amino terminal membrane occupation and recognition nexus (MORN) motifs; a motif of YxGxWxxGxRHGYG is conserved across JPH isoforms. These motifs constitute two different MORN domains that anchor the protein into the membrane of the ER, the organelle where *C*-mannosylation occurs.[33, 34] The initially identified *C*-mannosylated peptide featured a modification on W307 in its primary sequence. This would place the modification on MORN motif VII, the last MORN motif and one of the many domains that interact with the plasma membrane, as presented in **Figure 17**.

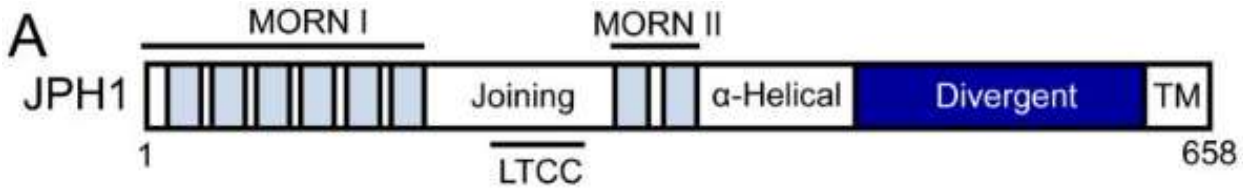


Figure 17: Junctophilin-1 diagram displaying MORN domains and connection to L-Type Calcium Ion Channels. Adapted from Landstrom, A.P. *et al* (2014).[35]

JPH1 is currently thought to function as a regulator for L-type Ca^{2+} channels (LTCC) through RyR1. These channels are essential to the initiation of skeletal muscle contraction. A joining region between MORN domains I and II interacts with the LTCC; JPH1 has also been shown to interact with RyR1 expressed in skeletal muscle *via* disulfide bonds, which **figure 18** presents. RyR1 is a mediator in the process of Ca^{2+} release from the sarcoplasmic reticulum (SR), the skeletal muscle equivalent of the ER. RyR1 and LTCC have been shown to colocalize in skeletal myoblasts *in vitro*; this colocalization is disrupted when JPH1 expression is silenced.[36, 37] All of this evidence points towards all three proteins operating in tandem with one another in the ER, but doesn't implicate them in the innate immune response.

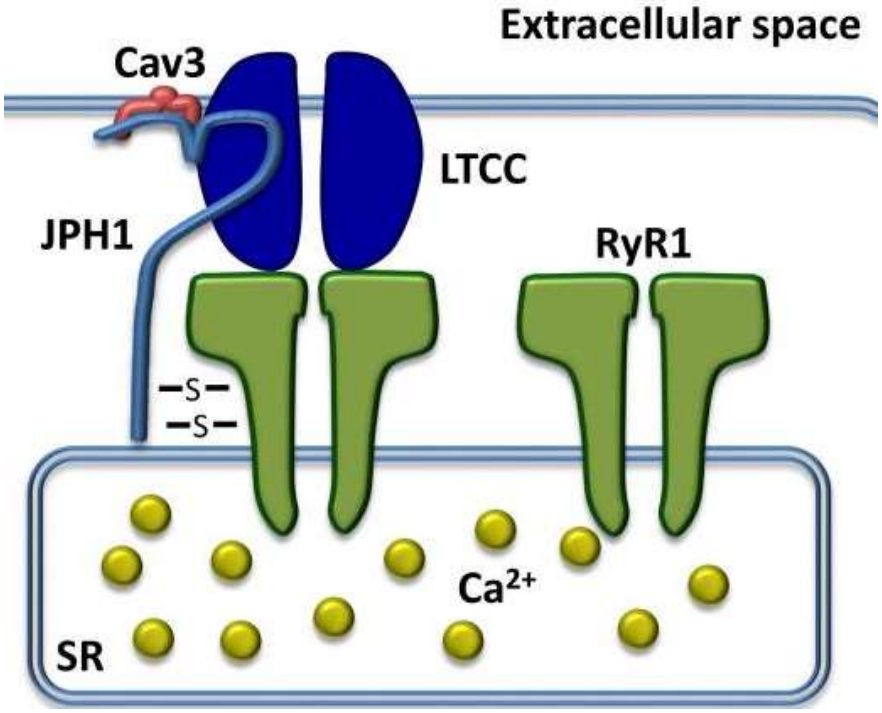


Figure 18: JPH1 and RyR1 interaction via disulfide bonds in skeletal muscle cells. Adapted from Landstrom, A.P. *et al* (2014).[35]

4.6 CONCLUSIONS

Hsc70 is usable as a powerful proteomics tool based on its easy-to-precipitate nature that has been demonstrated by this co-immunoprecipitation strategy. As a molecular chaperone protein, Hsc70 interacts with numerous proteins during the innate immune response to facilitate proper folding during a time of stress for all proteins. Being a constitutively expressed protein, Hsc70 is a prime target for co-immunoprecipitation even if the macrophage cell line hasn't been activated by LPS.

As for its utility as a tool for glycoproteomics, Hsc70 is still a viable option even if its full capabilities of isolating *C*-mannosylated proteins has yet to be discovered. This study has indicated at least two different proteins that were previously unknown to be *C*-mannosylated, with one of them being a non-canonical example of the modification. Currently, of the four DPY19 homologs believed to *C*-mannosylate proteins, the function of DPY19L4 is currently unknown. In knockout CHO cells, no difference in TSR *C*-mannosylation was noted when DPY19L4 was expressed or silenced.

Other work in the Rakus lab conducted by Karli Boober (unpublished) utilized RTPCR using RNA from both RAW264.7 and HEK293T cells. Junctophilin-1 was observed to be *C*-mannosylated on a specific peptide in RAW cells, but not HEK cells. Noting that DPY19L4 doesn't *C*-mannosylate TSRs, it was hypothesized that HEK cells did not express DPY19L4 and that perhaps RAW cells expressed this enzyme to *C*-mannosylate proteins that did not feature the canonical *C*-mannosylation sequence. However, Ms. Boober's data showed the opposite, with HEK293T cells expressing all four homologs.

Again, while only a handful of endogenous examples of *C*-mannosylated proteins were identified by a co-immunoprecipitation with Hsc70, this does not rule out its potential as a tool

for identifying modified proteins. The co-immunoprecipitation presented here has expanded our knowledge on *C*-mannosylation in that another as yet to be identified enzyme may be responsible for *C*-mannosylation in RAW264.7 cells. These non-canonical examples of *C*-mannosylation may also influence the innate immune response; deriving peptides like those identified in Junctophilin-1 and artificially *C*-mannosylating tryptophan side chains would provide peptides similar to those used in previous literature. Successfully permeabilizing the RAW cells would allow for these peptides to enter the cytosol. Downstream assays of TNFα production post-introduction of artificially modified peptides would provide evidence of their role in septic levels of pro-inflammatory cytokine production.

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APPENDIX A: APPROVAL LETTER

MARSHALL UNIVERSITY
www.marshall.edu

Office of Research Integrity

August 27, 2018

Nick Kegley
106 Overlook Drive
Hurricane, WV 25526

Dear Mr. Kegley:

This letter is in response to the submitted thesis abstract entitled *"Identifying Secreted and Cellular C-mannosylated Proteins in RAW264.7 Cells."* After assessing the abstract, it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Code of Federal Regulations (45CFR46) has set forth the criteria utilized in making this determination. Since the information in this study does not involve human subjects as defined in the above referenced instruction, it is not considered human subject research. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and a determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director