

## ABSTRACT

Title of Document:                       PROTEOMIC CHARACTERIZATION OF  
EXOSOMES SHED BY MYELOID-DERIVED  
SUPPRESSOR CELLS.

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Exosomes are a class of extracellular vesicles that have been shown to contribute to metastasis when derived from tumor cells. Myeloid-derived suppressor cells (MDSC) are an immature population of myeloid cells that accumulate in the tumor microenvironment and inhibit anti-tumor immunity. Given the role of the source cells, it is our hypothesis that MDSC-derived exosomes may contribute to or mediate the effects of MDSC in the tumor microenvironment. The goal of this work is to use mass-spectrometry based proteomics to characterize exosomes produced by MDSC that are induced by 4T1 mammary carcinoma. The protein content of the exosomes will be analyzed to determine if the exosomal proteome is representative of the parental cells or if it reflects active protein sorting.

Increased inflammation in the tumor microenvironment is associated with an increased population of MDSC, which further increases the level of immune suppression. Here, the relative change in abundance of exosomal proteins under a

heightened level of inflammation in the tumor microenvironment will be performed using the spectral count method.

While it is known that exosomes first form through invagination at the plasma membrane, the mechanism(s) through which the protein cargo is sorted into exosomes remains poorly understood. Given the role of ubiquitination in protein localization and trafficking, immunoaffinity enrichment coupled to mass spectrometry has been employed to identify exosomal proteins that carry this modification. Identification of the substrate proteins in MDSC-derived exosomes may provide insight into exosome formation and/or function.

PROTEOMIC CHARACTERIZATION OF EXOSOMES SHED BY MYELOID  
DERIVED SUPPRESSOR CELLS.

By

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## Dedication

This work is dedicated to my parents, Catherine and Michael Burke, who have made this possible through their unconditional love and support. To my brother, James Burke, who has taught me the true meaning of sacrifice through his military service. And to Marshall Harris for being a constant source of motivation and encouragement in everything I do. I am truly grateful.

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## List of Abbreviations

Alix	ALG-2 (apoptosis-linked gene-2)-interacting protein X
ARF1	ADP-ribosylation factor 1
DUB	Deubiquitinase
ESCRT	Endosomal-sorting complex required for transport
GO	Gene Ontology
GRAVY	Grand average of hydropathicity
HMGB1	High mobility group protein B1
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-1 $\beta$	Interleukin-1 $\beta$
IL-6	Interleukin-6
LC-MS/MS	Liquid chromatography/Tandem mass spectrometry
LETM1	Leucine zipper EF hand-containing transmembrane protein 1
MDSC	Myeloid-derived suppressor cells
PEC	Peritoneal exudate cells
PVDF	Polyvinylidene fluoride
R <sub>sc</sub>	Spectral count ratio
SDS	Sodium dodecyl sulfate
Tsg101	Tumor suppressor gene 101

# Chapter 1: Introduction

## Research Significance and Objectives

The role of a class of extracellular vesicles, termed exosomes, in the tumor microenvironment is currently an active area of research. Multiple reports have demonstrated that tumor cell-derived exosomes are composed of soluble factors and genetic material that contribute to metastasis.<sup>1</sup> However, the composition and function of exosomes is expected to reflect that of the parent cell.<sup>2</sup> The tumor microenvironment is composed of many cells that contribute to tumor-induced immune suppression, in addition to tumor cells; therefore, it is our interest to investigate the role of exosomes derived from myeloid derived suppressor cells (MDSC) in intercellular communication in the tumor microenvironment.

MDSC are immature myeloid cells that accumulate in the tumor microenvironment and are present in tumor bearing mice as well as patients with advanced cancer.<sup>3-5</sup> MDSC inhibit anti-tumor immunity through suppression of T cell activation and polarization of macrophages towards a tumor promoting phenotype.<sup>6, 7</sup> Furthermore, increased inflammation *in vivo* is associated with increased accumulation of MDSC in the tumor microenvironment, which propagates immune suppression.<sup>8, 9</sup> Several mechanisms of MDSC activity have been associated with suppression of T cell activation, some of which require cell-to-cell contact.<sup>8-10</sup> It is our hypothesis that extracellular vesicles shed by MDSC may mediate or contribute to

the immune suppressive activity and accumulation of MDSC in the tumor microenvironment.

MDSC produce extracellular vesicles that have all the characteristics<sup>11-13</sup> of exosomes. Electron microscopy experiments by Johnstone and coworkers, who contributed to the discovery of exosomes, have shown that exosomes are formed via invagination of the limiting membrane to form endosomes, which invaginate further to form multivesicular bodies. The multivesicular body fuses with the plasma membrane to release its contents into the extracellular milieu, which are then termed exosomes.<sup>14, 15</sup> MDSC-derived exosomes used in this work have been provided by Dr. Suzanne Ostrand-Rosenberg.<sup>16</sup> Here, mass spectrometry-based proteomics is used to identify the protein cargo of exosomes from MDSC induced by 4T1 mammary carcinoma (termed “conventional”) and 4T1 cells transfected with and expressing interleukin-1 $\beta$  (termed “inflammatory”). Due to the increased suppressive potency of MDSC under heightened inflammatory conditions, quantitative mass spectrometry is also employed to compare protein abundances in exosomes from conventional and inflammatory MDSC.

Ubiquitination has been shown to signal both the internalization of surface proteins and the sorting of proteins into endosomal proteins; both have been proposed as possible mechanisms of exosome biogenesis.<sup>17-20</sup> We utilize immunoaffinity enrichment coupled to tandem mass spectrometry to identify ubiquitinated proteins present in exosomes shed by inflammatory MDSC.<sup>21</sup> Biological assays have demonstrated that MDSC-derived exosomes carry proinflammatory proteins that contribute to the chemotaxis of MDSC and polarization of macrophages towards a

tumor promoting phenotype.<sup>16</sup> Therefore, it is of interest to the community to investigate the nature of the protein cargos of exosomes. The presence of ubiquitinated proteins in exosomes has been previously reported using western blot analyses<sup>22</sup>; however, the conjugated proteins were not identified.

Ubiquitin is an 8.5 kDa protein that forms an isopeptide bond through its terminal Gly76 and an  $\epsilon$ -amino group of a lysine in a substrate protein or another ubiquitin, which contains seven lysine residues (Lys6, 11, 27, 29, 33, 48, and 63). One of the multiple tryptic cleavage sites in ubiquitin is Arg74, which leaves Gly75 and Gly76 on the modified lysine of a substrate protein or an additional ubiquitin moiety.<sup>23-25</sup> This ubiquitin remnant can be identified through tandem mass spectrometry and bioinformatics analysis. Here, bottom up proteomic techniques are used to identify exosomal peptides that contain a glycinyglycine-modified lysine residue. Moreover, Gene Ontology annotations and the UniProt database will be used to look for trends in the cellular location (relative to the parental cell), protein function, size, and pI values of the ubiquitinated proteins. This may provide insight into the role of ubiquitination in exosome formation and/or function.

### Myeloid-Derived Suppressor Cells

Chronic inflammation and cancer are associated with an increase in a population of immature myeloid cells.<sup>4</sup> Activation by pro-inflammatory factors, such as interleukin-6 (IL-6) and interleukin-1 (IL-1 $\beta$ ), inhibit the differentiation of the immature myeloid cell population, termed myeloid-derived suppressor cells (MDSC).

<sup>7</sup> Factors produced by tumor cells, such as IL-6, activate MDSC in the tumor

microenvironment.<sup>7</sup> Under healthy conditions, myeloid cells from the bone marrow would differentiate into dendritic cells, macrophages, and/or granulocytes; however, the immature MDSC population that accumulates upon activation inhibits both innate and adaptive immunity and promotes tumor progression in the tumor microenvironment.<sup>6, 26</sup>

Under normal conditions, immature myeloid cells make up approximately 0.5% of mononuclear cells in the blood. However, a ten-fold increase in MDSC has been observed in cancer patients.<sup>4, 27-29</sup> The role of MDSC in promoting tumor progression inhibits successful immunotherapy in cancer patients.<sup>30</sup> The causal relationship between chronic inflammation and tumor progression was first proposed by Virchow in the 1800's; however, the mechanism(s) that mediate this relationship are not completely understood.<sup>8</sup> Therefore, a reduction in inflammation prior to immunotherapy has been proposed to improve the outcome of immunotherapy.<sup>9</sup>

MDSC skew the immune response and promote tumor progression through multiple activities including the induction of T regulatory cells and polarizing macrophages towards a tumor promoting phenotype.<sup>31-33</sup> MDSC production of interleukin-10 (IL-10) down-regulates macrophage production of interleukin-12 (IL-12) and skews macrophages from a tumoricidal phenotype to that of a tumor promoting M2-like phenotype.<sup>32,33</sup> Additional mechanisms of MDSC activity include production of arginase<sup>6,8,10,32,34,35</sup> and inducible nitric oxide synthase<sup>6, 36,37</sup>.

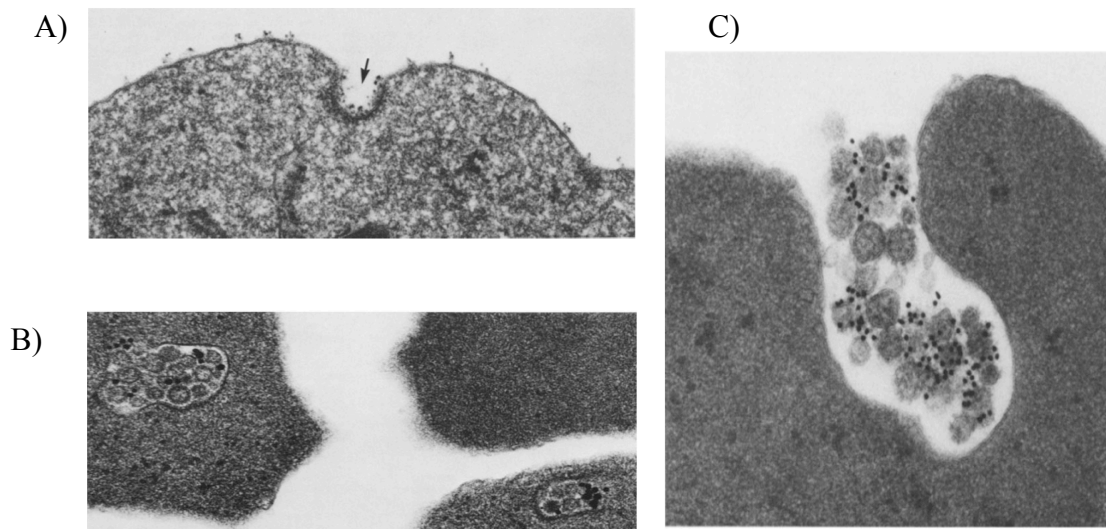
In addition to the pro-inflammatory factors produced by tumor cells, MDSC produce S100 A8 and A9, which are calcium-binding proteins that belong to the S100 protein family.<sup>4</sup> S100 A8 and A9 are secreted by MDSC and thought to form a

heterodimer that promotes MDSC accumulation in the tumor microenvironment.<sup>4</sup> In addition to producing the dimer, MDSC contain the N-glycan receptors for S100A8/A9 on their surface, which creates an autocrine feedback loop that ensures accumulation of MDSC in the tumor microenvironment and increased immune suppression.<sup>38</sup>

### Exosomes

Extracellular vesicles produced by cells include shedding microvesicles, apoptotic bodies and exosomes.<sup>1, 39</sup> Exosomes differ from microvesicles and apoptotic bodies in their size, sucrose density and composition (protein, lipid, and nucleic acids).<sup>1</sup> Extracellular vesicles were originally observed from cartilage in 1969.<sup>40</sup> However, in 1983 the exosomal biogenesis pathway was described (Figure 1).<sup>14, 41</sup> Exosomes are membrane-bound vesicles that are first formed through invagination at the plasma membrane to form early endosomes and mature to form intraluminal vesicles (Figure 1 a and b, respectively).<sup>15</sup> Further budding of the endosomal membrane generates multivesicular bodies that can either fuse with lysosomes for degradation or fuse with the plasma membrane to exocytose their contents into the extracellular space (Figure 1 c).<sup>15</sup> Exosomes are reported as 30-100 nm in diameter and contain marker proteins including heat shock protein 70, Alix [ALG-2 (apoptosis-linked gene 2)-interacting protein X], Tsg101 (tumor suppressor gene 101), and tetraspanins (CD63, CD9, and CD81).<sup>1, 42</sup>





**Figure 1.** Exosome biogenesis.<sup>15</sup>

More recently, a report demonstrated that exosomes derived from Epstein-Barr virus transformed B cells contain major histocompatibility complex (MHC) class II molecules<sup>43</sup> and suggested that exosomes may participate in intercellular communication.<sup>44</sup> Additionally, exosomes have been shown to promote angiogenesis, induce T regulatory cells, suppress natural killer cells, and inhibit DC maturation.<sup>45, 46</sup> Together, these functions suggest that exosomes may promote metastasis via the formation of a pre-metastatic niche.<sup>46, 47, 48</sup>

Exosomes can be purified from the parent cells and other contaminating vesicles present in cell culture through differential centrifugation.<sup>43, 49, 50</sup> Relatively low speed centrifugations at 300, 200, and 10,000 x g are first performed to eliminate cells, cellular debris, and large extracellular vesicles (greater than 100 nm in diameter).<sup>50</sup> Ultracentrifugation at 100,000 x g is then performed on the supernatant to obtain an exosome pellet, which may contain extracellular protein. A sucrose

density gradient can be used to further purify the exosomes, since the vesicles are expected to band in the gradient and protein aggregates are expected to sediment.<sup>43, 50,</sup>  
<sup>51</sup> Together, differential centrifugation and transmission electron microscopy, used to characterize vesicle size and morphology, are considered the gold-standard of exosome purification.<sup>43, 44</sup> More recently, the use of filtration and purification of exosomes based on classical exosome marker proteins have been increasingly used to obtain exosomes; however, the possibility of variability in the exosomes obtained from these methods has not been verified.<sup>12</sup>

While the biogenesis of exosomes has been reported,<sup>14, 41</sup> the exact mechanism of endosomal sorting of protein, lipid, and nucleic acids into exosomes remains unclear. Proteins belonging to the endosomal-sorting complex required for transport (ESCRT) have been identified in exosomes (Alix and Tsg101). Moreover, the exosomal marker proteins CD63 and CD81 have been found to co-purify with the ESCRT protein vacuolar sorting protein 4B (VPS4B) following exosome purification.<sup>52</sup> The ESCRT pathway is known to participate in intraluminal vesicle and multivesicular body formation.<sup>53</sup> It comprises 20 proteins, which make up four complexes. ESCRT-0 protein complex recognizes and traffics ubiquitinated proteins in the endosomal membrane, which is followed by membrane deformation by the ESCRT-1 and -II complexes. The ESCRT-III complex participates in membrane scission, releasing the intraluminal vesicle.<sup>54, 55</sup> Due to the overlap in endosomal and exosomal formation, including intraluminal vesicles and multivesicular bodies, it has been highly speculated that the ESCRT may participate in protein trafficking into exosomes. However, gene silencing of four ESCRT proteins (HRS, STAM, Tsg101,

and VPS4B) does not completely abolish multivesicular body formation.<sup>56</sup> This suggests that perhaps only part of the ESCRT pathway participates in exosome formation and/or other mechanisms may also participate in the protein sorting and vesicle formation.<sup>53</sup>

Additional mechanisms that have been reported to affect exosome formation and/or release include high luminal concentration of calcium and higher-order oligomerization of plasma membrane proteins.<sup>57, 44</sup> An increase in luminal calcium concentration has been shown to increase exosome production in erythroleukemia cell line K562,<sup>58</sup> oligodendroglial cells,<sup>59</sup> dendritic cells<sup>60</sup> and mast cells.<sup>61</sup> Additionally, higher-order oligomerization has been shown to target plasma membrane proteins to exosomes.<sup>57</sup> This result is consistent with the original work on exosome formation in maturing reticulocytes, in which aggregation of the transferrin receptor corresponds to sites of exosome formation.<sup>57, 62</sup> Interestingly, unlike an ESCRT-dependent formation, higher order oligomerization is independent of protein sequence and function.<sup>57</sup> Collectively, in addition to the potential role of the ESCRT pathway in exosome formation, evidence suggests that the luminal calcium concentration and higher-order oligomerization may also participate in the formation of intraluminal vesicles and multivesicular bodies that exocytose their contents into the extracellular space.

In addition to studying the mechanisms of protein trafficking into exosomes, another active area of research includes the RNA that is sorted into exosomes. In 2007, Valadi and coworkers reported the presence of messenger RNA (mRNA) and microRNA (miRNA) in exosomes.<sup>63</sup> Even more interesting is that exosomes obtained

from mice were able to transfer functional mRNA to human cells.<sup>44</sup> Since 2007, additional reports have demonstrated the presence of ribosomal RNA, mRNA, and noncoding RNAs including miRNA.<sup>64</sup> Due to previous observations that exosomes may mediate communication in the tumor microenvironment through chemokines and growth factors,<sup>64</sup> a new question is raised whether exchange of genetic material (miRNA) may also contribute to metastasis.<sup>65-67</sup>

Due to the promising role of exosomes in intercellular communication, another area of active research is the use of exosomes in clinical research. Exosomes are considered a promising candidate for clinical use due to their stability in circulation<sup>64, 68</sup> and a protein cargo that impedes exosome degradation.<sup>69</sup> Recently, mesenchymal stem cell-derived exosomes have been shown to reduce infarct size in mice.<sup>64</sup> Additionally, extracellular vesicle-based cancer vaccines are currently being evaluated in Phase I clinical trials and dendritic cell-derived extracellular vesicles will be evaluated for non-small-cell lung cancer in Phase II clinical trials.<sup>64, 70</sup> While the application of exosomes as cancer vaccines is promising, much still remains to be learned regarding the mechanisms of exosome formation and function.

## **Chapter 2: Comparative Exploration of Exosomes from Tumor-Induced Immune Suppressive Myeloid-Derived Suppressor Cells (MDSC) (Adapted from reference 16)**

### Introduction

In order for tumors to establish metastasis, tumor cells must evade the immune system.<sup>46,47</sup> Myeloid-derived suppressor cells have previously been shown to promote tumor growth through suppression of T cell activation and polarization of macrophages towards a tumor promoting phenotype.<sup>6,7</sup> However, exosomes have also demonstrated potential to contribute to metastasis through their ability to transfer proteins and RNA to recipient cells.<sup>46, 71</sup> Moreover, the ability of tumor cells to thrive is dependent upon communication between the tumor cells and the neighboring tissue and extracellular matrix.<sup>71</sup> It is possible that, in addition to tumor cell-derived exosomes, exosomes produced by cells present in the tumor microenvironment, such as MDSC, may contribute to tumor growth and metastasis. Tumor cell-derived exosomes in a mouse glioblastoma xenograft model have been shown to promote tumor growth.<sup>72</sup> Therefore, it is our goal to determine how exosomes derived from MDSC may also contribute to tumor growth in BALB/c mice carrying 4T1 mammary carcinoma using mass spectrometry-based proteomics.

As discussed earlier, increased inflammation is associated with an increase in MDSC accumulation and thereby increased immune suppression.<sup>8,9</sup> However, the mechanisms through which MDSC promote tumor progression are not completely

understood.<sup>8</sup> It has been shown that MDSC activity requires cell-to-cell contact and/or soluble mediators.<sup>8-10</sup> It is our hypothesis that MDSC-derived exosomes may contribute to MDSC accumulation and immune suppression in the tumor microenvironment through mediating intercellular communication. Therefore, the identification of MDSC-derived exosomal proteins whose abundance is significantly altered due to heightened inflammation is expected to provide insight into the role of exosomes in the tumor microenvironment.

Mass spectrometry-based proteomics is ideal for investigating the role of exosomes in the tumor microenvironment as proteins participate in most clinical conditions.<sup>73</sup> Additionally, changes in protein abundance do not correlate with mRNA abundance.<sup>74,75</sup> In order to compare the effect of heightened inflammation on protein abundance, available quantitation methods include stable isotope labeling (either metabolic or chemical tags) and label-free quantitation, which utilizes peptide precursor ion intensity or spectral counts.<sup>74</sup> It is believed that isotopic-labeling methods provide more accuracy, while label-free methods offer greater proteome coverage.<sup>74</sup> In this study, the spectral count method has been used to identify proteins with altered abundance under heightened inflammatory conditions.

The spectral count method is based on the assumption that the frequency at which a precursor peptide is selected for fragmentation correlates to the peptide, and therefore protein, abundance. The spectral count method requires that care is taken to avoid assigning identified peptides to multiple proteins, which would significantly alter peptide and protein quantitation.<sup>74</sup> Therefore, we have chosen to calculate spectral count ratios in this work with the requirement that each protein identification

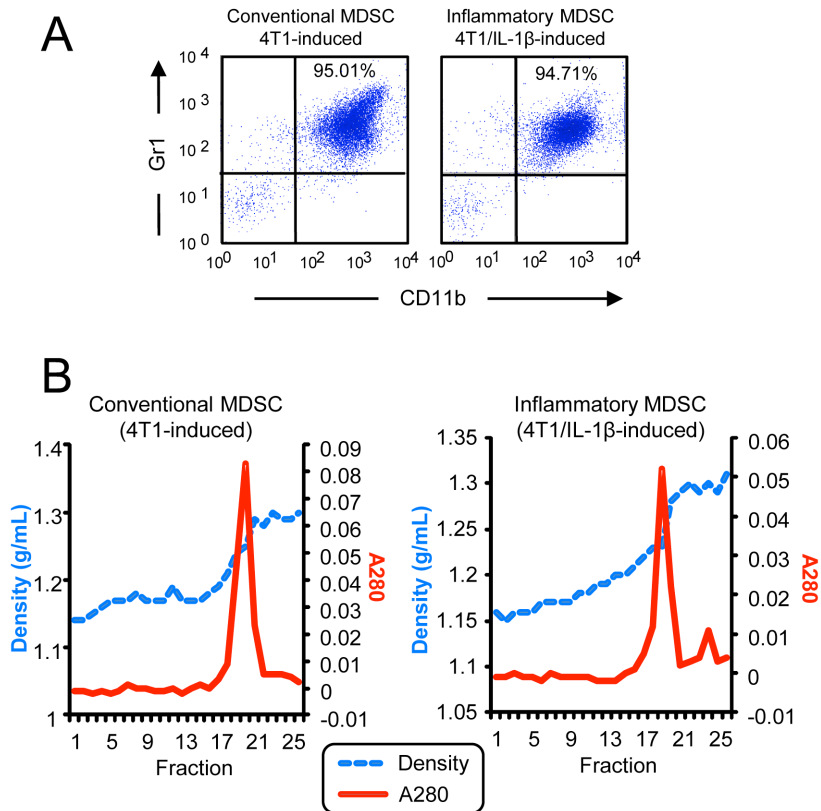
contain at least two distinct, unshared peptides and each unique peptide is assigned to only one protein.

Here, mass spectrometry-based proteomics followed by spectral count quantitation is used to identify differentially abundant exosomal proteins due to heightened inflammation in the tumor microenvironment. The results from this analysis will allow us to ask if the exosomal proteins identified reflect the known immunosuppressive function of the source cells, MDSC. Moreover, is it possible that the MDSC-derived exosomes can contribute to this activity in the tumor microenvironment? If so, this would provide evidence that the role of exosomes in the tumor microenvironment should be considered as potential obstacles to immunotherapy.

### Materials and Methods

#### **Myeloid-Derived Suppressor Cells**

BALB/c mice were injected in the mammary fat pad with 7000 wild type 4T1 mammary carcinoma cells or 4T1 cells stably transfected and expressing IL-1 $\beta$  as described. When tumors were greater than ~8mm in diameter (~3-4 weeks after initial inoculation), MDSC were harvested from the blood and monitored by immunofluorescence and flow cytometry for purity by expression of the MDSC markers Gr1 and CD11b (**Figure 2**).<sup>8</sup> MDSC used in experiments were >90% Gr1<sup>+</sup>CD11b<sup>+</sup>. MDSC induced by wild type 4T1 and 4T1/IL-1 $\beta$  tumor cells are termed “conventional” and “inflammatory” MDSC, respectively. All procedures with animals and animal-derived materials were approved by the UMBC and UMCP Institutional Animal Care and Use Committees.



**Figure 2:** A: Flow cytometry profile of MDSC expression for Gr1 and CD11b. B: Sucrose density (g/mL) and optical density (OD 280) plots of fractions from sucrose density gradients containing exosomes from conventional (left) and inflammatory (right) MDSC.

### Exosomes

Purified MDSC obtained from 2 or 3 mice ( $\sim 1 \times 10^8$  MDSC for each experiment) were plated at  $4 \times 10^6$  cells/mL in serum-free HL-1 medium (BioWhittaker, Walkersville, MD) and maintained at 37 °C with 5% CO<sub>2</sub>. After 16 hours the cultures were centrifuged at 805 x g for 5 min (Eppendorf 5810R centrifuge), the pellets discarded, and the supernatants centrifuged at 2090 x g for 30 min (Sorvall RC5C, SS34 rotor). The supernatants were then ultracentrifuged (Beckman L8 ultracentrifuge) at 100,000 x g for 20 hours at 10 °C using an SW40Ti



rotor. Supernatants were discarded, the pellets containing the exosomes were resuspended in PBS, and absorbances were measured at 260 and 280 nm. Protein content was assayed by Bradford Quick Start according to the manufacturer's directions (BioRad). Exosomes were stored at -80 °C until used. For migration experiments, exosomes were resuspended to the original volume of the conditioned medium from which they were obtained so a direct comparison of the effects of exosomes versus conditioned medium could be made. For the MDSC-macrophage cross-talk experiments, exosomes were used at 1x, 2.5x, and 5x concentrations. On average, 1 mL of conditioned medium contained 714 µg of exosomal protein.

#### **Sucrose Density Gradient Fractionation of Exosomes**

Freshly prepared ultracentrifuged exosomes were resuspended in 1.8-2.0 mL of 2.5 M sucrose/0.020 M Hepes and layered on the bottom of 9/16 in. x 3 ¾ in. polyallomer ultracentrifuge tubes (Beckman). A 10 mL gradient of 0.25 to 2.0 M sucrose in 0.020 M Hepes was then layered over the 2 mL containing exosomes for a total volume of 11.8-12 mL. Gradients were ultracentrifuged (Beckman L8 ultracentrifuge) at 10 °C for 16 hours at 100,000 x g in an SW40Ti rotor. Then, 0.5 mL fractions were collected and assessed by optical density (**Figure 2**). Density of the fractions was confirmed by refractometry.

#### **Transmission Electron Micrographs**

An aliquot of exosomes containing 0.03-0.3 pg total protein was suspended in 2% glutaraldehyde and was applied to a Formvar-coated grid and negatively stained with uranyl acetate. Electron micrographs were acquired using a Zeiss EM10 transmission electron microscope at an accelerating voltage of 80 keV.

### **Protein Analysis for In-solution Tryptic Digestion**

Aliquots of conventional and inflammatory exosomes containing 25 µg of total protein were lysed in 8 M urea in 50 mM ammonium bicarbonate and 1% of a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The exosomes in lysis buffer were centrifuged at 14,000 x g for 30 minutes with a 3 kDa molecular weight cutoff filter, and the supernatants were discarded. The process was repeated three times with the retentate from the previous wash step. After lysis, fifty millimolar ammonium bicarbonate was added to each sample to dilute the final urea concentration to >0.8 M, which is compatible with tryptic digestion. Each sample was then reduced in 20 mM dithiothreitol at 56 °C for 30 minutes followed by alkylation in 40 mM iodoacetamide at room temperature in the dark for 30 minutes. Three technical replicates of 7 µg total protein were analyzed by LC-MS/MS (using HPLC-MS/MS parameters outlined below) for three biological replicates each for conventional and inflammatory exosomes.

### **HPLC-MS/MS Analysis**

LC-MS/MS analyses were performed on a Shimadzu Prominent nanoHPLC (Shimadzu BioSciences, Columbia, MD) in-line with an LTQ Orbitrap XL (Thermo Fisher Scientific, San Jose, CA). Peptides prepared via tryptic digestion were injected onto an Acclaim PepMap 300 C18 precolumn (Dionex, Sunnyvale, CA) followed by desalting by 10% Solvent A (97.5% H<sub>2</sub>O, 2.5% ACN, and 0.1% formic acid) for 20 minutes. Peptides were fractionated on a C-18 analytical column (Grace Vydak, Deerfield, IL) with a linear gradient increasing from 0 to 40% solvent B (97.5% ACN, 2.5% H<sub>2</sub>O, and 0.1% formic acid) in 170 minutes, followed by an increase

from 40% to 85% solvent B in 40 minutes. Flow rate was 500 nL/min. Precursor scans were acquired in the orbitrap with a resolution of 30,000 at  $m/z$  400. In each cycle the nine most abundant ions were selected for fragmentation by collisional induced dissociation (CID), and product ion scans were acquired in the LTQ. A dynamic exclusion of 1 repeat count over 180 seconds was used.

### **Bioinformatics**

Peptides and proteins were identified by the PepArML meta-search engine<sup>76</sup> using the mouse reference proteome of the UniProtKnowledgeBase (June 2013) containing 50,807 sequences. Carbamidomethylation of cysteine was selected as a fixed modification, and oxidation of methionine and deamidation of asparagine and glutamine residues were selected as variable modifications. Peptide identifications from technical and biological replicates were pooled and filtered at 1% spectral FDR, as computed by PepArML using the method of Elias and Gygi,<sup>77</sup> and identified proteins were required to contain at least two distinct, unshared peptides, ensuring protein FDR of at most 0.01%. Spectral counts for identified proteins, spectral count ratios ( $R_{sc}$ ) as described in Old et al.,<sup>78</sup> and statistical significance for differential spectral counts were determined using in house software. Differential spectral count p-values were computed using the Fisher exact-test and corrected for multiple testing by transformation to false-discovery-rate (FDR) using the method of Benjamini and Hochberg.<sup>79</sup> Identified proteins are assigned to cellular components according to their Gene Ontology annotations and the PIR GO Slim.

### **MDSC Chemotaxis**

Cells used in the chemotaxis assay were >90% Gr1<sup>+</sup>CD11b<sup>+</sup> conventional MDSC as assessed by flow cytometry.<sup>35</sup> Five hundred microliters of fresh media, media from MDSC cultures (conditioned media), or MDSC-derived exosomes from the equivalent amount of conditioned medium in fresh media were placed in individual wells of 24-well plates (lower compartment). Monoclonal antibodies to S100A8, S100A9, or irrelevant control isotype matched antibodies (10 µg/500 µL; Santa Cruz Biotech) were included in some wells. Transwells with an 8 µm polycarbonate semipermeable membrane were then inserted in each well, and 1x10<sup>6</sup> MDSC in 100 µL of serum-free IMDM medium were placed in the transwells (upper compartment). Assembled transwells were incubated at 37 °C in 5% CO<sub>2</sub> for 3 hours, and the MDSC in the bottom chamber were then quantified by hemocytometer. Values for each sample are the average results of duplicate samples and three independent hemocytometer counts per well.<sup>38</sup>

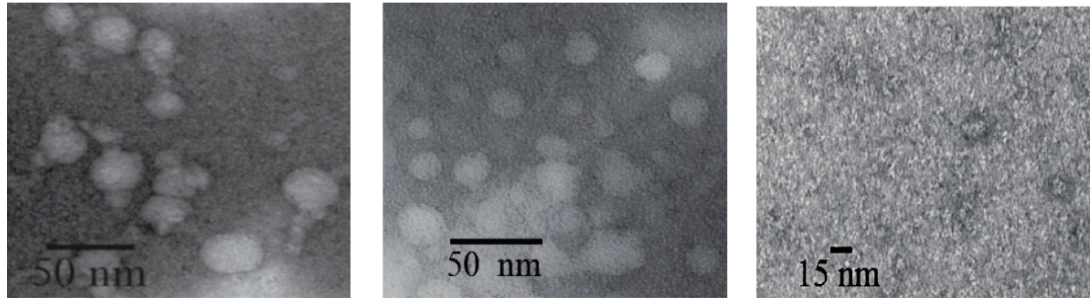
#### **MDSC-Macrophage Cross-Talk**

BALB/c mice were injected intraperitoneally with 1 mL of 3% thioglycolate, and peritoneal exudate cells (PEC) were harvested 4 days later. The percent of macrophages in the exudate was determined by flow cytometry analysis of the macrophage markers F4/80 and CD11b. PEC were plated in 24-well plates at 7.5 x 10<sup>5</sup> F4/80<sup>+</sup>CD11b<sup>+</sup> cells/well/500 µL DMEM medium supplemented with 10% fetal bovine serum and incubated at 37 °C in 5% CO<sub>2</sub> for 3 hours. Non-adherent cells (non-macrophages) were then removed, and the attached macrophages were washed with macrophage medium (DMEM/5% serum). Five hundred microliters of macrophage medium containing 7.5 x 10<sup>5</sup> conventional MDSC (>90% Gr1<sup>+</sup>CD11b<sup>+</sup>

cells) or MDSC-derived exosomes from  $7.5 \times 10^5$  (1X),  $18.7 \times 10^5$  (2.5X), or  $37.5 \times 10^5$  (5X) MDSC were then added to each well. MDSC and macrophages were activated with IFN $\gamma$  and LPS and co-cultured at a ratio of 1:1 ( $5 \times 10^5$  cells of each type/200  $\mu$ L/well) for 18 hours. Supernatants were harvested and assayed by ELISA for interleukin-12 (IL-12).<sup>9</sup>

### Results and Discussion

Cells produce several types of extracellular vesicles including exosomes, shedding microvesicles, and apoptotic bodies. Differential centrifugation is used to separate exosomes from their parental cells as well as larger extracellular vesicles (greater than 100 nm in diameter). However, further characterization is required in order to determine if the purified extracellular vesicles meet the criteria of exosomes.<sup>43</sup> After sucrose density gradient purification, MDSC-derived exosomes, prepared by Dr. Suzanne Ostrand-Rosenberg's laboratory at the University of Maryland Baltimore County, were further characterized using transmission electron microscopy. The purified MDSC-derived vesicles from both conventional and inflammatory MDSC equilibrate at a sucrose density of 1.2-1.3 g/mL (**Figure 2**) and are approximately 25-30 nm in diameter (**Figure 3**). The transmission electron micrograph images of both conventional and inflammatory exosomes reveal vesicles of similar diameter and morphology. The control grid shown in Figure 3 prevents the 15 nm artifacts from the negative staining from being considered as vesicles. Together, the sucrose density and diameter of the vesicles is consistent with the published characteristics of exosomes.<sup>11-13, 80</sup>



**Figure 3:** Transmission electron microscope images of (left) exosomes shed by conventional MDSC; (middle) exosomes shed by inflammatory MDSC; the TEM stain itself (right).

In order to compare the amount of exosomes produced by conventional and inflammatory MDSC, the total amount of exosomal protein was normalized for the number of MDSC cells used in each preparation. The amount of exosomes produced per cell, expressed as the amount of exosomal protein ( $\mu\text{g}$ ) per MDSC cell, was  $[1.1 \pm 0.1] \times 10^{-6}$  ( $n=4$ ) and  $[1.2 \pm 0.2] \times 10^{-6}$  ( $n=4$ ) for conventional and inflammatory MDSC, respectively. The similar ratios demonstrate that conventional and inflammatory MDSC produce the same amount of exosomes on a per cell basis.

Next, mass spectrometry-based proteomics was used to identify peptides, and therefore proteins, present in conventional and inflammatory exosomes from three biological replicates, each with three technical replicates. Three hundred and eighty seven proteins were identified (from 2528 peptides) in exosomes from conventional MDSC (Appendix Table 1), and 374 proteins were identified (from 2280 peptides) in exosomes from inflammatory MDSC (**Appendix Table 1**). Together, a total of 412 unique proteins were identified in both conventional and inflammatory exosomes.

This inventory was compared with several databases of exosomal proteins in order to evaluate the number of proteins in MDSC-derived exosomes that have been previously reported in exosomes from other cell types. ExoCarta was the first exosomal database established by Mathivanan and Simpson.<sup>81</sup> Approximately 83% of the proteins identified in MDSC-derived exosomes are present in the ExoCarta compendium (August 2013). Another resource is EVpedia, which was developed by Kim et al.<sup>82</sup> Approximately 87% of the proteins identified in MDSC-derived exosomes have been reported in EVpedia (August 2013). Lastly, Kalra and Mathivanan et al. have also developed a compendium for proteins from all types of extracellular vesicles, called Vesiclepedia.<sup>83</sup> When compared to the general database of vesicular proteins, about 93% of the proteins identified in MDSC-derived exosomes are also present in Vesiclepedia. Proteins identified in MDSC-derived exosomes that have not previously been reported in ExoCarta, EVpedia, or Vesiclepedia are listed in Appendix Table 2.

Among the inventory of 412 exosomal proteins identified in this study, several proteins considered to be characteristic of exosomes were observed. These proteins include annexins (A1, A2, A3, A6, A7, A11) and tetraspanins, including CD177 as well as GTPases, NCK microfibrils (NCK associated protein 1 like), heat shock proteins (heat shock cognate 71 kDa protein, heat shock 70 kDa protein 4, and HSP90 alpha and beta) and cytoskeletal proteins. Another protein of interest includes vacuolar-sorting protein 35 (VPS35), which is a member of the endosomal sorting complex required for transport. This pathway has been proposed to play a role in exosome formation.

Additional proteins of interest identified in MDSC-derived exosomes include several proteins belonging to the 26S proteasome. These proteins include 26S protease regulatory subunit 6A, 26S proteasome non-ATPase regulatory subunits 1, 2, 5, 6, 7, 11, and 13, proteasome subunit beta type-2, and proteasome subunit alpha type-6. Proteasomal subunits have also been observed in extracellular vesicles produced by T lymphocytes and have been shown to be proteolytically active.<sup>84</sup>

Interestingly, more than a dozen histones were identified in MDSC-derived exosomes as well as several elongation factors (1-gamma, 1-alpha 1 and 2), DNA topoisomerase, RNA helicase (ATP-dependent RNA helicase DDX39A) and a zinc finger protein (DBF-type zinc finger-containing protein 2 homologue). Recently, the high abundance of histones and other transcription-related proteins has gained attention from the exosome community.<sup>85</sup> It has been proposed that the transcription factors present in exosomes may alter signaling pathways in a way that enables disease progression or tumor growth or that perhaps the presence of nucleic acid binding proteins is required to allow the nucleic acids to fit within the limited space of exosomes.<sup>85</sup> The high abundance of histones and nucleic acid binding proteins in exosomes from various cell types highlights the need for further investigation of nucleic acid binding proteins in exosomes.

Several metabolic proteins have also been identified in MDSC-derived exosomes belonging to the pentose phosphate pathway. These proteins include glucose-6-phosphate 1-dehydrogenase X, 6-phosphogluconate dehydrogenase, and transketolase. Additionally, several proteins belonging to the glycolysis and gluconeogenesis pathways were identified (pyruvate kinase PKM, glucose-6-

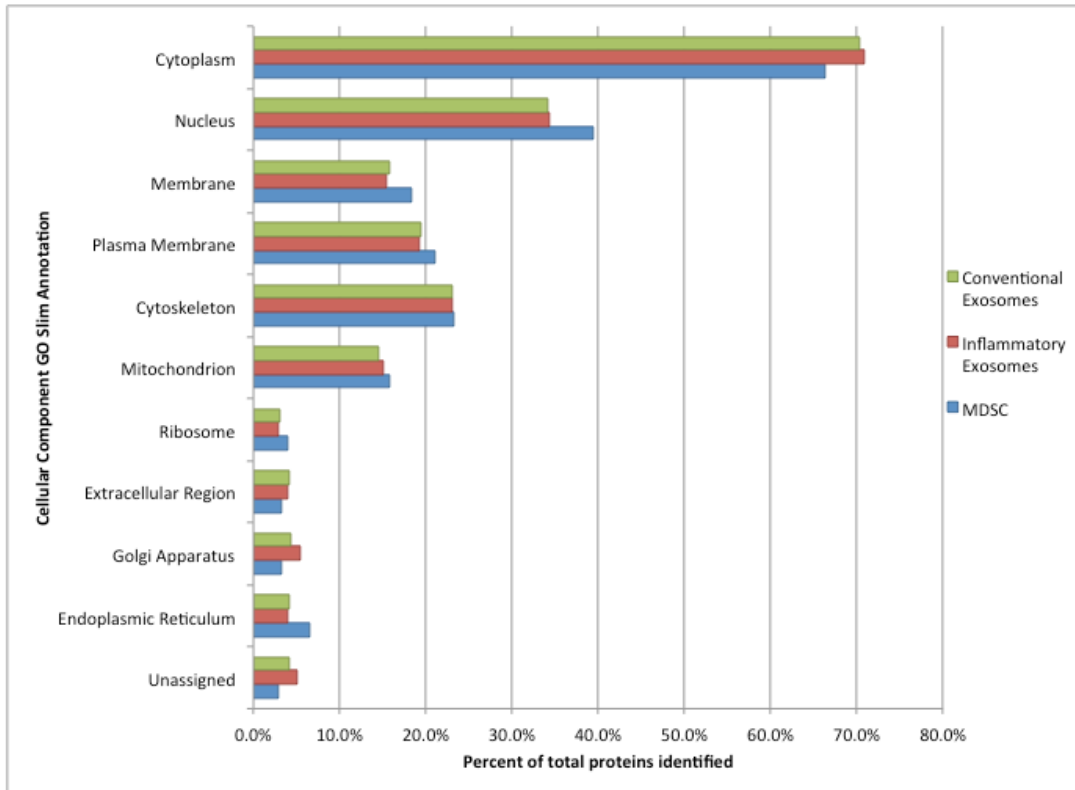


phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, alpha-enolase, L-lactate dehydrogenase A chain, fructose-bisphosphate aldolase, phosphoglycerate kinase 1, glycogen phosphorylase liver form, and glycogenin-1). These metabolic proteins have also been reported in exosomes derived from other cell types and are present in the exosomal databases.<sup>81-83</sup>

In addition to proteins previously observed in exosomes, proteins were identified in MDSC-derived exosomes that are critical for MDSC function and the proinflammatory response. Proteins S100A8 and S100A9 have been identified in MDSC-derived exosomes and are known to drive MDSC accumulation and immune suppression in the tumor microenvironment.<sup>38, 86</sup> Additionally, myeloid bacterenecin was identified, which has previously been reported to interact with immune cells and participate in the inflammatory response.<sup>87</sup>

In order to determine if the exosomal cargo of exosomes is representative of the parent cell or enriched for a subset of proteins, the cellular component GO annotations for the protein identifications for the conventional and inflammatory exosomes were compared to those belonging to 305 proteins identified in whole cell lysate analysis of conventional MDSC.<sup>88</sup> The cellular component GO annotations ascribed to exosomal proteins are relative to the parent cell, MDSC, as the exosomes do not contain organelles. The distribution of cellular component GO annotations for conventional and inflammatory exosomes shown in **Figure 4** illustrates that the protein content of the exosomes is similar. When the conventional and inflammatory exosomes are compared to the conventional MDSC, the distribution is similar with exceptions including nuclear and endoplasmic reticulum proteins. The exosomes

contain a greater abundance of nuclear proteins, including the histones and transcription factors described earlier.



**Figure 4:** Intracellular protein locations assigned by Gene Ontology annotations and the PIR GO Slim. Green: from exosomes from conventional MDSC; Red: from exosomes from inflammatory MDSC; Blue: from lysate<sup>88</sup> of conventional MDSC.

While the protein content of the conventional and inflammatory exosomes is similar, the spectral count method was used to determine how heightened inflammation in the tumor microenvironment alters protein abundance in the exosomes. Sixty-three of the 412 proteins (**Appendix Table 1**) identified in

conventional and inflammatory exosomes were found to differ in abundance by two fold or more (FDR  $\leq 0.05$ ). From the cohort of 63 proteins with altered protein abundance, 33 were found to decrease by at least two fold under heightened inflammatory conditions (**Table 1**). Several of the proteins with decreased abundance participate in the innate immune response including ficolin-1, C4b-binding protein, Chitinase-3-like protein 3, complement C3, and CD5 antigen-like proteins. Additional proteins of interest include cytoskeletal proteins spectrin beta 1, ankyrin-1, tubulin beta-1 chain, and nesprin-1. Ankyrins anchor transmembrane proteins and link them to the spectrin-actin cytoskeleton and participate in cell motility and intercellular communication.<sup>89,90</sup> Chemotactic proteins with decreased abundance include myeloid cysteine-rich protein and platelet factor 4. Together, the decrease in cytoskeletal and chemotactic proteins make it tempting to speculate that inflammation may alter proteins that participate in intercellular communication.

**Table 1:** Proteins with significantly greater abundance in conventional exosomes ( $R_{sc} \geq 1$  and FDR  $\leq 0.05$ ).  $R_{sc}$  is reported as the  $\log_2$  ratio of conventional versus inflammatory exosomes.

Accession	Protein	$R_{sc}$	FDR
P70390	Short stature homeobox protein 2	8.6	6.26E-157
P04919	Band 3 anion transport protein	6.4	5.05E-32
P08032	Spectrin alpha chain, erythrocytic 1	5.6	7.16E-19
Q8CIZ8	von Willebrand factor	4.8	3.56E-10
P08226	Apolipoprotein E	4.6	5.86E-09
Q61171	Peroxiredoxin-2	4.4	1.53E-07

Q9QUM0	Integrin alpha-IIb	4.3	6.14E-07
P01837	Ig kappa chain C region	3.7	1.28E-04
P29788	Vitronectin	3.3	1.50E-03
P49722	Proteasome subunit alpha type-2	3.3	1.50E-03
Q02357	Ankyrin-1	3.3	1.50E-03
Q3UGX2	Spectrin beta 1	3.3	1.50E-03
Q8K482	EMILIN-2	3.3	1.50E-03
Q9QWK4	CD5 antigen-like	3.0	9.63E-03
Q07797	Galectin-3-binding protein	2.8	1.74E-02
P11276	Fibronectin	2.7	3.08E-124
O70165	Ficolin-1	2.7	2.95E-02
P08607	C4b-binding protein	2.6	4.68E-03
P10605	Cathepsin B	2.5	8.26E-03
P07724	Serum albumin	2.5	7.87E-22
Q61646	Haptoglobin	2.1	1.08E-03
P01942	Hemoglobin subunit alpha	2.1	9.53E-47
P01872	Ig mu chain C region secreted form	2.1	7.19E-13
O35744	Chitinase-3-like protein 3	2.0	8.90E-05
P35441	Thrombospondin-1	1.9	4.55E-14
Q6ZWR6	Nesprin-1	1.9	1.54E-02
P62259	14-3-3 protein epsilon	1.5	6.12E-03
P82198	Transforming growth factor-beta-induced protein ig-h3	1.5	1.04E-02
Q8C2Q7	Heterogeneous nuclear ribonucleoprotein H	1.5	4.89E-02
A2AQ07	Tubulin beta-1 chain	1.5	1.88E-03
P02089	Hemoglobin subunit beta-2	1.4	2.67E-10
P01027	Complement C3	1.3	4.62E-08

P02088	Hemoglobin subunit beta-1	1.3	5.26E-15
Q8K426	Myeloid cysteine-rich protein	1.3	2.59E-02
Q9Z126	Platelet factor 4	1.1	4.71E-03
Q9R1P3	Proteasome subunit beta type-2	1.0	2.41E-02

Thirty exosomal proteins were found to increase in abundance (**Table 2**) due to heightened inflammation. Several GTP and ATP binding proteins were found to increase by at least two fold, which includes ATP-citrate synthase, ADP-ribosylation factor 1 (ARF1), and phosphatidylinositol 4-phosphate 3-kinase C2 domain containing subunit gamma. Interestingly, ADP-ribosylation factors, including ARF1, have been shown to participate in membrane curvature and budding to form extracellular vesicles.<sup>91,92</sup> Three proteins that participate in chemokine signaling that were identified include mitogen-activated protein kinase 3, cell division control protein 42 homolog and signal transducer and activator of transcription. Additional proteins of interest that were found to increase in abundance due to heightened inflammatory conditions are the biosynthetic proteins serine-tRNA ligase cytoplasmic, valine-tRNA ligase, aminopeptidase B, fatty acid synthase, ATP-citrate synthase, and elongation factor 1-gamma. Lastly, the vacuolar sorting-associated protein 35 was also found to increase in abundance, which is of interest because this protein participates in protein sorting.<sup>93</sup>

**Table 2:** Proteins with significantly greater abundance in inflammatory exosomes ( $R_{sc} \geq 1$  and  $FDR \leq 0.05$ ).  $R_{sc}$  is reported as the  $\log_2$  ratio of inflammatory versus conventional exosomes.

<b>Accession</b>	<b>Protein</b>	<b><math>R_{sc}</math></b>	<b>FDR</b>
Q5SS00	DBF4-type zinc finger-containing protein 2 homolog	3.6	1.48E-03
Q8VDP4	DBIRD complex subunit KIAA1967 homolog	3.1	1.39E-02
E9QQ35	Phosphatidylinositol 4-phosphate 3-kinase C2 domain-containing subunit gamma	3.1	1.10E-03
P12970	60S ribosomal protein L7a	3.0	2.29E-02
P84078	ADP-ribosylation factor 1	3.0	2.29E-02
P62908	40S ribosomal protein S3	2.8	4.49E-03
P08730	Keratin, type I cytoskeletal 13	2.8	3.76E-02
Q6ZQA0	Neurobeachin-like protein 2	2.8	3.76E-02
P26638	Serine--tRNA ligase, cytoplasmic	2.4	3.10E-02
P42227	Signal transducer and activator of transcription 3	2.4	3.10E-02
Q9D154	Leukocyte elastase inhibitor A	2.4	2.75E-05
Q9Z1Q9	Valine--tRNA ligase	2.2	7.61E-05
Q8VCT3	Aminopeptidase B	2.0	1.48E-03
P84096	Rho-related GTP-binding protein RhoG	1.9	1.91E-03
P42932	T-complex protein 1 subunit theta	1.8	3.28E-02
O88593	Peptidoglycan recognition protein 1	1.8	4.31E-03

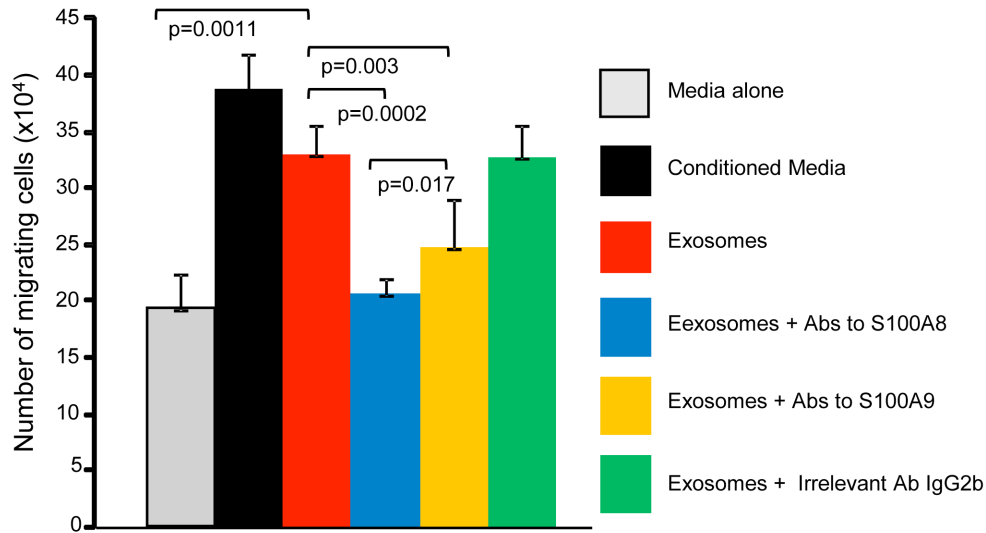
Q99KE1	NAD-dependent malic enzyme, mitochondrial	1.8	2.29E-02
O55029	Coatomer subunit beta'	1.7	1.49E-02
Q9EQH3	Vacuolar protein sorting-associated protein 35	1.7	9.63E-03
Q9CWJ9	Bifunctional purine biosynthesis protein PURH	1.6	1.48E-03
P80315	T-complex protein 1 subunit delta	1.5	2.40E-02
P19096	Fatty acid synthase	1.4	4.22E-03
P28293	Cathepsin G	1.3	1.72E-04
Q63844	Mitogen-activated protein kinase 3	1.2	3.10E-02
P60766	Cell division control protein 42 homolog	1.2	1.49E-02
Q8CCK0	Core histone macro-H2A.2	1.2	1.91E-03
Q9Z1Q5	Chloride intracellular channel protein 1	1.2	1.01E-02
Q9D8N0	Elongation factor 1-gamma	1.2	6.62E-03
Q91V92	ATP-citrate synthase	1.1	2.40E-02
P62827	GTP-binding nuclear protein Ran	1.0	1.15E-03

While the abundance of approximately 15% of the 412 proteins identified was found to differ by at least two-fold or more, two proteins of interest whose abundances were not found to differ due to heightened inflammation include proteins S100A8 and S100A9. These proteins are secreted by MDSC and are believed to form a heterodimer and drive the accumulation of MDSC, thereby increasing the immune suppression, at sites of inflammation including the tumor microenvironment.<sup>4,86</sup>

Proteins S100A8 and S100A9 have also been shown to contribute to polarization of tumoricidal macrophages to that of a tumor promoting phenotype.<sup>7</sup>

Because proteins S100A8 and S100A9 play a significant role in MDSC function and have been shown to be secreted extracellularly by MDSC, Dr. Suzanne Ostrand-Rosenberg's laboratory sought to determine if the extracellular S100A8 and S100A9 were actually active in the form of exosomes. The activity of S100A8 and S100A9 in MDSC-derived exosomes was assayed for MDSC chemotaxis and polarization of macrophages. The chemotaxis of MDSC by MDSC-derived exosomes was determined by measuring the number of MDSC migrating from the upper chamber of a transwell containing a semipermeable membrane to the lower chamber containing MDSC-derived exosomes (**Figure 5**). The MDSC-derived exosomes co-cultured with MDSC (upper chamber) were chemotactic for MDSC, approximately greater than 90% as chemotactic as the MDSC conditioned medium. When antibodies to S100A8 and S100A9 were added to MDSC-derived exosomes, a decrease in MDSC chemotaxis was observed. The decrease in chemotaxis due to the presence of the antibody for S100A8 is consistent with that for media alone. Together, the results indicate that MDSC-derived exosomes contain biologically active S100A8 and S100A9 that are chemotactic for MDSC. When the chemotaxis due to the MDSC-derived exosomes is compared to that of the conditioned media, the results suggest that most of the extracellular S100A8 and S100A9 is in the form of exosomes.

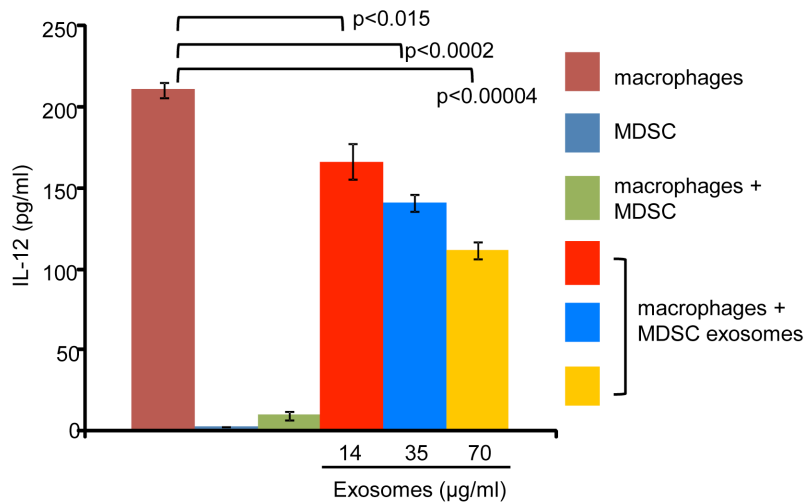




**Figure 5:** Exosomes shed by MDSC contain S100A8 and S100A9 proteins that are chemotactic for MDSC. MDSC were placed in the upper compartment of transwells and either tumor-conditioned medium or MDSC shed exosomes  $\pm$  antibodies to S100A8 or S100A9 were placed in the lower compartment. The number of MDSC migrating to the lower compartment was determined after 3 hours of incubation. Values are the average  $\pm$  SD of 3 independent cell counts of duplicate samples.

In addition to chemotaxis of MDSC, S100A8 and S100A9 contribute to the polarization of macrophages towards a tumor-promoting phenotype. This polarization is attributed to cross-talk between MDSC and tumoricidal (or M1) macrophages. MDSC produce IL-10, which decreases macrophage production of IL-12, resulting polarization of M1 macrophages towards a tumor promoting (or M2-like) phenotype.<sup>7</sup> Here, the production of IL-12 by macrophages co-cultured with  $7.5 \times 10^5$  conventional MDSC or MDSC-derived exosomes from 1x, 2x, and 5x conventional MDSC was measured using ELISA by the laboratory of Dr. Suzanne

Ostrand-Rosenberg. The results of the IL-12 assay indicate that MDSC-derived exosome contribute to the polarization of macrophages towards an M2-like phenotype, and this polarization correlates positively with the concentration of exosomes. However, the MDSC cells are more effective, on a per cell basis, than the exosomes.



**Figure 6:** Exosomes shed by MDSC polarize macrophages from a tumoricidal M1 phenotype to a tumor-promoting M2 phenotype by inhibiting macrophage production of IL-12. Type 1 macrophages were co-cultured alone, or in the presence of intact MDSC or exosomes shed by MDSC. IL-12 production by macrophages was measured by ELISA.

### Summary

Mass spectrometry-based proteomics has been used to analyze the protein content of exosomes produced by MDSC and how conditions in the tumor microenvironment alter the relative protein abundance. A total of 412 proteins have

been identified in exosomes from both conventional and inflammatory MDSC, which is similar to the protein content of the source cells. Highly abundant proteins were found to include histones, proteins that participate in metabolism, as well as the proinflammatory proteins S100 A8 and S100A9. While the total number of vesicles was not statistically different due to heightened inflammation in the tumor microenvironment, approximately 15% of the proteins identified were differentially abundant by 2-fold or more. Proteins that were found to increase in abundance include coatamer subunit beta and vacuolar protein sorting-associated protein 35, which are reported to participate in membrane budding and protein sorting, respectively.

Though highly abundant in the exosomes, the relative abundances of proteins S100A8 and S100A9 were not found to change by at least 2-fold due to heightened inflammation. However, biological assay results demonstrate that the exosomes contain biologically active S100A8 and S100A9 that accounts for approximately 90% of the activity that is observed in conditioned medium. Collectively, the biological assays illustrate the immunosuppressive activity associated with the MDSC is also mediated by the exosomes they produce. While MDSC are reported to inhibit successful immunotherapy in cancer patients,<sup>30</sup> the ability of exosomes to contribute to immune suppression suggests that extracellular vesicles may also need to be considered as obstacles to successful treatment.

# **Chapter 3: Ubiquitinated Proteins in Exosomes Exocytosed by Myeloid-derived Suppressor Cells (Adapted from reference 21)**

## *Introduction*

Exosomes are formed through the invagination of the plasma membrane to form early endosomes that mature into late endosomes, which are multivesicular bodies that contain intraluminal vesicles.<sup>44, 94</sup> While the overall pathway of exosome formation has previously been reported, the mechanism(s) through which protein, lipid, and RNA are sorted into exosomes remains poorly understood.<sup>1, 95</sup> Interestingly, exosomes from multiple parental cell types are found to be enriched, relative to their parental cells, in Alix [ALG-2 (apoptosis-linked gene 2)-interacting protein X] and Tsg101 (tumor suppressor protein 101) proteins, which are known to participate in the endosomal sorting complex required for transport (ESCRT).<sup>1</sup> ESCRT protein complexes participate in the sorting proteins at the endosomal limiting membrane,<sup>52</sup> which requires recognition of ubiquitinated proteins. The identification of ESCRT-related proteins in exosomes<sup>1, 22, 53</sup> and the overlapping pathways that participate in endosome formation between the ESCRT-dependent recycling of cell surface receptors and exosome formation has led to the speculation that exosome formation may be, at least in part, dependent on the ESCRT pathway. Moreover, gene silencing

of four proteins from ESCRT-0 and ESCRT-I protein complexes has been shown to alter exosome secretion.<sup>53</sup>

As mentioned earlier, the ESCRT pathway is dependent on the recognition of ubiquitinated proteins through ubiquitin interaction motifs.<sup>20</sup> Ubiquitin is a protein of approximately 8.5 kDa and forms an isopeptide bond through its C-terminal Gly76 and an  $\epsilon$ -amino group of a lysine belonging to another ubiquitin moiety or a substrate protein.<sup>23-25</sup> Conjugation of a ubiquitin molecule requires the activity of E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases.<sup>94</sup> Ubiquitin itself contains seven lysine residues (K6, K11, K27, K29, K33, K48, and K63), through which ubiquitin can form chains of various length and linkage types including linear or branched chains.<sup>95-97</sup> Recently, there have also been reports of ubiquitination at residues other than lysine, including serine, threonine and cysteine.<sup>98-100</sup>

Ubiquitination serves as a post-translational modification that alters protein interactions, subcellular localization and/or promotes protein degradation.<sup>96, 101,102</sup> The most well studied role of ubiquitination is the activity associated with K48-linked ubiquitin, which targets proteins to the 26S proteasome for subsequent deubiquitination and degradation.<sup>103-104</sup> It has been reported that the most efficient signal for proteasomal degradation is K48-linked ubiquitin chains longer than four ubiquitin moieties.<sup>95</sup> Also worth noting is that substitution of K48 to arginine causes cell death.<sup>105</sup> Endocytosis of cell surface receptors and protein trafficking are associated with K63-linked polyubiquitin and monoubiquitination.<sup>95,106</sup> Additional activities associated with ubiquitinated proteins include transcriptional regulation<sup>107</sup>

and DNA repair.<sup>108</sup> Among polyubiquitin chains identified from yeast cells, K48- and K63-linked ubiquitin are the most abundant linkages identified.<sup>20,24,95</sup> However, it is important to note that many ubiquitin conjugates are believed to be short-lived and therefore care must be taken during sample preparation.<sup>24</sup>

Proteomic characterization of ubiquitinated proteins has been much slower than other post-translational modifications, including phosphorylation and methylation,<sup>109</sup> which is in part due to the challenges associated with localizing the modification site in substrate proteins. Recent progress has been made using His-tagged ubiquitin conjugates<sup>110</sup> and anti-ubiquitin antibodies.<sup>111</sup> Following enrichment or purification of ubiquitinated proteins, the conjugates are typically digested with trypsin which cleaves C-terminally to Arg and Lys, including Arg74 of ubiquitin, which generates a ubiquitin remnant of Gly75 and Gly76 on the ubiquitinated lysine of a substrate protein. The fragment ion mass spectrum of a tryptic peptide containing the glycinyglycine ubiquitin remnant will contain two fragment ions with a mass difference of 242.12 Da corresponding to a glycinyglycine-modified lysine. This modification can be identified using available bioinformatic tools by allowing for a variable modification of glycinyglycine of lysine.<sup>24</sup> However, if a substrate protein contains one ubiquitination site, there would only be one lysine in the entire sequence carrying the modification.

The stoichiometry of unmodified peptides to peptides containing a ubiquitin remnant is not favorable for mass spectrometry analysis. This has led to the development of an antibody that recognizes glycinyglycine-modified lysine residues. Commercially available antibodies have been generated by preparing a histone H2A

antigen, which is lysine-rich, containing glycylglycine-modified lysines.<sup>109</sup> Peptide-level enrichment of glycylglycine-modified lysines, from whole cell extract, has been shown to greatly improve the identifications of tryptic peptides containing the ubiquitin remnant.<sup>98</sup> In fact, over 10,000 glycylglycine-modified lysines have been identified in over 4,000 proteins.<sup>112,113</sup>

Our goal is to identify ubiquitinated proteins in MDSC-derived exosomes in order to provide insight into exosome formation and/or function. It is important to note that most reports that demonstrate the identification of up to 4,000 ubiquitinated proteins start from milligram quantities of whole cell extract followed by peptide level enrichment for glycylglycine-modified lysines<sup>114</sup> or His-tagged ubiquitin.<sup>24</sup> In order to identify endogenous ubiquitinated proteins from limited starting material of MDSC-derived exosomes, an optimized immunoaffinity strategy was developed which included (1) protein level enrichment for ubiquitinated proteins followed by (2) peptide level enrichment for tryptic peptides containing glycylglycine-modified lysines.

### Materials and Methods

#### **Myeloid-derived Suppressor Cells**

BALB/c mice were injected in the mammary fat pad with approximately 7000 4T1 mammary carcinoma cells stably transfected to express IL-1 $\beta$ . When tumors were greater than approximately 8 mm in diameter (about 3-4 weeks after initial inoculation), MDSC were harvested from the blood, stained with fluorescently labeled monoclonal antibodies against markers of MDSC (Gr1 and CD11b), and analyzed by flow cytometry. Cell populations that were greater than 90%

Gr1<sup>+</sup>CD11b<sup>+</sup> were used in all experiments.<sup>8</sup> For each experiment, a total of about 1 x 10<sup>8</sup> MDSC were pooled from 2 to 3 mice. The UMBC and UMCP Institutional Animal Care and Use Committees approved all procedures with animals and animal-derived materials.

### **Exosomes**

MDSC were plated in serum-free HL-1 medium (Bio-Whittaker, Walkersville, MD) and maintained at 37 °C with 5% CO<sub>2</sub>. After 18 hours, the cultures were centrifuged at 805 x g for 5 min (Eppendorf 5810 rotor, Eppendorf, Hamburg), the pellets were discarded, and the supernatants were centrifuged at 2090 x g for 30 minutes (Sorvall RC5C, SS34 rotor, DuPont, Wilmington, DE). The supernatants were then ultracentrifuged at 100,000 x g for 20 hours at 10 °C (Beckman L8, SW40Ti rotor, Beckman, Pasadena, CA). The supernatants were discarded, and the pellets containing the exosomes were resuspended in PBS. Absorbances were measured at 260 and 280 nm. Exosomes were stored at -80 °C until use.

Exosomes were lysed in an optimized lysis buffer of 8 M urea in 50 mM ammonium bicarbonate with 50 μM of deubiquitinase inhibitor PR-619 (LifeSensors, Malvern, PA) and 1% of a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The exosomes in lysis buffer were centrifuged at 14,000 x g for 30 minutes with a 3 kDa molecular weight cutoff filter, and the supernatants were discarded. The process was repeated three times with the retentate from the previous wash step. After lysis, the buffer was diluted to <0.8 M urea in 50 mM ammonium bicarbonate. Protein content before the immunoprecipitation was measured by the Quick Start Bradford Assay (Bio-Rad, Hercules, CA).



### **Immunoprecipitation of Ubiquitinated Proteins**

Ubiquitinated proteins were enriched using Protein A-Sepharose 4B beads (Invitrogen, Carlsbad, CA) that had been incubated with anti-ubiquitin antibody 3933 (Cell Signaling Technology, Danvers, MA) in a 1:600 dilution with rotation for 4 hours at 4 °C. Excess antibody was removed from the beads by washing with 0.8 M urea in 50 mM ammonium bicarbonate and centrifuging three times at 3,000 x g for 2 minutes. One-hundred micrograms of exosome lysate was added to the Sepharose bead slurry and incubated with rotation overnight at 4 °C. The unbound fraction was collected via centrifugation at 500 x g for 5 min. The Sepharose bead slurry was washed with 50 mM ammonium bicarbonate and centrifuged at 1,000 x g for 5 minutes to remove non-specifically bound proteins. Bound proteins were eluted by incubating the Sepharose bead slurry in 0.2 M glycine, pH 2.6, for 1 hour at 4 °C and collected via centrifugation at 13,000 x g for 5 minutes. The elution was repeated, and the two elution fractions were combined.<sup>115</sup> Enriched fractions of ubiquitinated exosomal proteins were subsequently processed either by tryptic digestion in gel or in solution and immunoprecipitation of peptides containing glycylglycine-modified lysine residues.

### **In-Gel Tryptic Digestion of Ubiquitinated Proteins**

Proteomic studies were conducted on exosomal proteins enriched for ubiquitin conjugates by immunoprecipitation. Three biological replicates were resuspended in 2% SDS, 5% β-mercaptoethanol, and 62.5 mM Tris HCl and reduced at 90 °C for 5 minutes. The samples were then loaded onto 8-16% polyacrylamide gels (Bio-Rad) and subjected to electrophoresis for approximately 50 minutes at 200 V, 15 mA, and

50 W. The gels were stained using Coomassie blue (40% methanol, 20% acetic acid, 0.1% w/v Coomassie blue reagent 250; Thermo Scientific, San Jose, CA) stain and then excised into 13 slices. After destaining, proteins were reduced in 10 mM dithiothreitol at 56 °C for 30 minutes and alkylated in the presence of 10 mM methylmethanethiosulfonate at room temperature for 45 minutes. Tryptic digestion was performed on each gel slice overnight at 37 °C.<sup>116</sup> The extracted tryptic peptides were resuspended in 0.1% formic acid for injection into the LC-MS/MS. (See below for instrumental conditions)

### **Tryptic Digestion and Immunoprecipitation of Glycinyglycine-Modified Peptides**

Enriched fractions of ubiquitinated exosomal proteins from five biological replicates were frozen, lyophilized and resuspended in 50 mM ammonium bicarbonate. Proteins were reduced with 20 mM dithiothreitol for 30 minutes at 56 °C and alkylated with 10 mM methylmethanethiosulfonate for 45 minutes. One microgram of trypsin was added to each fraction, and digestion was performed overnight at 37 °C. As a positive control, a ubiquitin dimer linked with an isopeptide bond at K48 (LifeSensors, Malvern, PA) was also digested with trypsin under these conditions.

Peptides with glycinyglycine-modified lysine residues were enriched using Protein A-Sepharose 4B beads coupled to anti-diglycyl-lysine antibody GX41 (Millipore, Billerica, MA) using the same procedure as that with the anti-ubiquitin antibody, except that anti-diglycyl-lysine antibody was prepared at a 1:1000 dilution. The fractions of immunoprecipitated ubiquitinated proteins were added to the

Sepharose bead slurry and incubated with rotation overnight at 4 °C. The unbound fraction was removed via centrifugation at 500g for 5 minutes. The Sepharose bead slurry was washed with 50 mM ammonium bicarbonate and centrifuged at 1,000g for 10 seconds to remove non-specifically bound peptides. Bound peptides were eluted by incubating the Sepharose bead slurry in 0.2 M glycine, pH 2.6, for 1 hr at 4 °C and collected via centrifugation at 13,000g for 5 minutes. The elution was repeated, and the two elution fractions were combined. Prior to LC-MS/MS analysis, all fractions were desalted with C18 TopTip spin columns (Glygen, Columbia, MD) and resuspended in 0.1% formic acid.

### **Western Blotting**

All fractions were subjected to one-dimensional gel electrophoresis on an 8-16% Criterion precast gel (Bio-Rad) at 200 V, 50 mA, and 15 W for 56 minutes, followed by transfer to a PVDF membrane (EMD Millipore, Billerica, MA) at 100 V, 350 mA, and 35 W for 1 hr. Free ubiquitin, monoubiquitinated and polyubiquitinated proteins were detected by blotting with anti-ubiquitin antibody 3933 (Cell Signaling Technology) followed by anti-mouse IgG-HRP (Cell Signaling Technology). Protein bands were visualized with an Image Lab System (Bio-Rad, Hercules, CA) using the Gel-Doc program (Kodak Molecular Imaging Systems) and the SuperSignal West Dura chemiluminescent substrate (Thermo Fisher Scientific, Waltham, MA).

### **Extraction of Histones**

Exosomal histones were extracted using the EpiQuick Total Histone Extraction Kit (Epigentek, Farmingdale, NY) according to the manufacturer's

instructions and analyzed for ubiquitination via western blotting with anti-ubiquitin antibody 3933 as previously described.

### **LC-MS/MS and Bioinformatic Analysis**

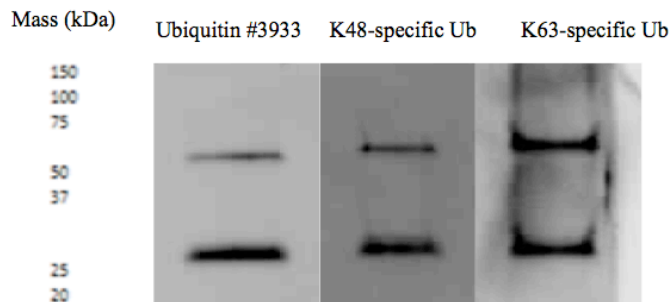
LC-MS/MS analyses were performed on a Shimadzu Prominence nano HPLC (Shimadzu Scientific Instruments, Columbia, MD) in-line with an LTQ-Orbitrap XL (Thermo Fisher Scientific). A 10  $\mu$ L aliquot of tryptic peptides was injected onto an Acclaim PepMap 300 C18 pre-column (Dionex, Sunnyvale, CA) followed by desalting with 10% solvent A (97.5% H<sub>2</sub>O, 2.5% acetonitrile, and 0.1% formic acid) in 85 minutes, followed by an increase from 40 to 85% solvent B (97.5% acetonitrile, 2.5% H<sub>2</sub>O, and 0.1% formic acid) in 85 minutes, followed by an increase from 40 to 85% solvent B in 20 minutes. The flow rate was 500 nL/min. Precursor scans were acquired in the orbitrap with a resolution of 30,000 at m/z 400. In each cycle, the nine most abundant ions were selected for fragmentation by collisional induced dissociation, and product ion scans were acquired in the LTQ. A dynamic exclusion of 1 repeat count over 180 seconds was used.

Peptide and protein identifications were made by the PepArML<sup>76, 117</sup> meta-search engine against the UniProt mouse database (July 2014). For the in-gel digestion, all peptide identifications were filtered at 10% spectral FDR, and proteins were required to be supported by at least 2 unshared peptides, bounding a protein FDR at 1%. For the two-step immunoaffinity enrichment of glycinyglycine-modified lysine-containing peptides, all peptides were filtered at 5% spectral FDR. A fixed modification included methylthio of cysteine residues and variable modifications of oxidation of methionine and glycinyglycine modification of lysine were allowed.

Proteins with at least one peptide containing a glycinyglycine-modified lysine were considered to be ubiquitinated. For all protein identifications based on a single peptide from the double immunoaffinity workflow, spectra are included in Appendix Figure 1. Subcellular location and function assignments for all protein identifications were made using the Protein Information Resource GO Slim (<http://pir.georgetown.edu>) using UniProt Gene Ontology annotations (July 2014).

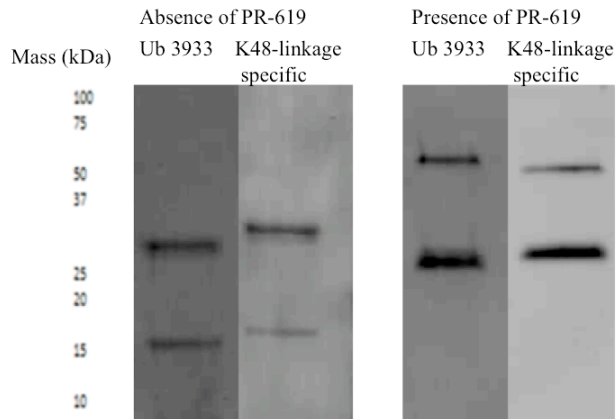
### *Results and Discussion*

Western blotting was first used to determine if MDSC-derived exosomes do in fact contain ubiquitinated proteins using a general ubiquitin antibody that recognizes free ubiquitin as well as monoubiquitinated and polyubiquitinated proteins. Additionally, antibodies specific to K48- and K63-linked polyubiquitin were also used. The Western blots shown in **Figure 7** confirm the presence of ubiquitinated proteins in MDSC-derived exosomes as well as the presence of polyubiquitin chains containing K48- and K63-linkages.



**Figure 7:** Western blots of lysates from MDSC-derived exosomes. Antibodies used are (left) anti-ubiquitin 3933, (middle) anti-K48-linked polyubiquitin, and (right) anti-K63-linked polyubiquitin.

In order to identify the exosomal substrate proteins carrying the ubiquitin modification(s) an immunoaffinity enrichment strategy was optimized. As noted earlier, most of the previous reports in which whole cell extracts were lysed and enriched at the peptide level for glycinyglycine-modified lysine-containing peptides started with milligram quantities of starting material; however, the amount of material that can be obtained from BALB/c mice carrying 4T1 mammary carcinoma is limited to approximately 100 µg per biological replicate. Moreover, it has been reported that deubiquitinating enzymes may be present in biological samples, which would significantly underestimate the number of ubiquitinated proteins identified. In order to address the second issue regarding deubiquitinating enzymes, exosome lysis and enrichment of ubiquitinated proteins was performed in the presence of a general deubiquitinase (DUB) inhibitor PR-619. The Western blot in **Figure 8** demonstrates the presence of DUB inhibitor PR-619 is required in order to maintain isopeptide linkage(s) between ubiquitin and substrate proteins due to the increase in mass observed in ubiquitinated proteins.



**Figure 8:** Western blot of lysates from MDSC-derived exosomes in the (left) absence and (right) presence of DUB inhibitor PR-619. Antibodies used are anti-ubiquitin 3933 and anti-K48-linked polyubiquitin.

Next, in-gel digestion of enriched ubiquitinated proteins, following immunoaffinity enrichment at the protein level using Sepharose beads coupled to anti-ubiquitin 3933 antibody, was evaluated by LC-MS/MS analysis. Mass spectrometry-based proteomic analysis resulted in 16 protein identifications from three biological replicates (**Table 3**). In order to report an exosomal protein as ubiquitinated, at least one tryptic peptide containing a glycylglycine-modified lysine must be observed. Initial peptide identifications and protein assignments were made by the PepArML meta search engine followed by manual curation of all tandem mass spectra assigned to confirm localization of the glycylglycine-modified lysine. Results from the in-gel digestion of exosomal proteins enriched for ubiquitinated proteins suggested that unmodified tryptic peptides were suppressing the identification of tryptic peptides containing the ubiquitin remnant, glycylglycine-modified lysine residues, which we require in order to claim that a protein is

ubiquitinated. In order to overcome this challenge, a second immunoaffinity enrichment was evaluated following the protein-level enrichment for ubiquitinated proteins and in-solution tryptic digestion. LC-MS/MS analysis of tryptic peptides obtained from a second enrichment using anti-diglycyl lysine antibody GX41 coupled to Sepharose beads resulted in 38 protein identifications from five biological replicates (**Table 4**). It is important to note that while the enrichment for glycinyglycine-modified lysine-containing peptides improved the identification of ubiquitinated exosomal proteins, the experimental design leads to several proteins based on single peptide identifications. These peptides are less reliable than the peptides listed in **Table 3**, and annotated spectra for single peptide identifications have been included in **Appendix Figure 1**.

**Table 3:** Ubiquitinated proteins and peptides identified from in-gel digestion of exosomal proteins with glycinyglycine-modified lysine residues.

Protein Accession	Protein Name	Number of non-overlapping peptides identified	Protein FDR	Peptide Sequence
F6XI62	60S ribosomal protein L7 (Fragment) <sup>‡</sup>	2	2.450E-05	REKKKKVATVPGTL KKKVPAGPKTLK(G G)K
P61161	Actin-related protein 2	3	6.486E-04	VVVC DNGTGFVK(G G)
P26040	Ezrin	3	6.490E-04	EELMLRLQDYEQK( GG)TKR
P17156	Heat shock-related 70 kDa protein 2	5	4.860E-06	HWPFRVVSEGK(G G)PK(GG)
P63158	High mobility group protein B1 <sup>‡</sup>	4	5.614E-04	WK(GG)TMSAK(GG)
P10922	Histone H1.0	4	5.610E-05	AAKPKKAASK(GG) APSK
				K(GG)KPAATPK(GG) K



				KAKPK(GG)VVK
				ASK(GG)PKKAKTVK PK
P15864	Histone H1.2 <sup>Ψ</sup>	11	3.920E-14	K(GG)ATGAATPKK AAK
				AKKPAAAAVTK(GG) )K
				K(GG)VAKSPK
				KAK(GG)VTKPKK
				AAK(GG)PKVAK
P43277	Histone H1.3 <sup>Ψ</sup>	9	2.738E-10	TPVKK(GG)KAK(GG) )
				SPKKVKAAG(GG)PK
				KAAKSPAKAK(GG)
				AKASK(GG)PKASKP K
P43274	Histone H1.4 <sup>Ψ</sup>	4	1.250E-10	AKKPAGAAK(GG)
				TVKPKAAKPK(GG)T SK(GG)
P43276	Histone H1.5 <sup>Ψ</sup>	10	3.040E-18	AKK(GG)TGAAKAK
				AKKPAGATPKKPKK (GG)
				K(GG)PAAAGVK
				VTKPKTAKPK(GG)A AKAK
Q07133	Histone H1t	3	6.486E-04	GKGK(GG)KSASAK( GG)
				TK(GG)AVKKPKATP TK(GG)
P27661	Histone H2A.x <sup>Ψ</sup>	5	4.860E-06	K(GG)SSATVGPK(G G)APAVGKK
P62806	Histone H4 <sup>Ψ</sup>	6	4.207E-07	GKGGK(GG)GLGK(G G)GGAK
Q6IFX2	Keratin, type I cytoskeletal 42 <sup>Ψ</sup>	9	2.729E-10	NK(GG)ILAATIDNAS IVLQIDNAR
P08071	Lactotransferrin	29	1.522E-31	GDADAMSLDGGYIY TAGK(GG)
P52480	Pyruvate kinase isozymes M1 <sup>Ψ</sup>	7	3.640E-08	GPEIRTGLIKGSGTA EVELK(GG)K

<sup>Ψ</sup> Indicates that the protein is reported previously to be ubiquitinated.

**Table 4:** Ubiquitinated proteins and peptides identified following immunoaffinity enrichment of tryptic peptides with glycylglycine-modified lysine residues.

Protein Accession	Protein Name	Number of peptides identified	Number of non-overlapping K(GG) containing peptides	Peptide FDR	Peptide Sequence
F6XI62	60S ribosomal protein L7 (Fragment)* <sup>Ψ</sup>	2	1	7.83E-02	REKKKKVATV PGTLKKKVP GPKTLK(GG)K
Q5SWU9	Acetyl-CoA carboxylase 1	1	1	4.48E-02	FGGNKVIEKVL IANNGIAAVK(GG) CMRSIR
E0CYH9	Carboxyl-terminal PDZ ligand of neuronal nitric oxide synthase protein	1	1	5.10E-02	KKKVSIMVSV DGVKVLK(GG) )KKKKLLLLQK
Q6P925	Cysteine-rich perinuclear theca 4	1	1	5.10E-02	AK(GG)RSKLLK KKRNPRSKLP K(GG)RSRHSLI R
Q9CQJ6	Density-regulated protein	2	1	5.10E-02	QKK(GG)K(GG) TVPQKVTIAKI PRAKKKYVTR
P08113	Endoplasmin	2	1	3.63E-02	LLKVIRK(GG) KLVR
P43275	Histone H1.1 <sup>Ψ</sup>	10	2	5.10E-02	KTVK(GG)TPK KPKKPAVSKK TSKSPKPKVV K
				5.10E-02	AKKVAKSPAK AKAVKPKASK AKVTKPK(GG) TPAKPK
P15864	Histone H1.2* <sup>Ψ</sup>	11	5	8.66E-02	K(GG)ATGAAT PKKAAK
				8.66E-02	AKKPAAAAVT K(GG)K
				8.66E-02	K(GG)VAKSPK
				8.66E-02	KAK(GG)VTKP KK

				8.66E-02	AAK(GG)PKVA K
P43274	Histone H1.4* <sup>ψ</sup>	4	2	8.66E-02	AKKPAGAAK( GG)
				8.66E-02	TVKPAAKPK( GG)TSK(GG)
P43276	Histone H1.5* <sup>ψ</sup>	10	7	8.66E-02	AKK(GG)TGAA KAK
				8.66E-02	AKKPAGATPK KPKK(GG)
				8.66E-02	K(GG)PAAAGV K
				8.66E-02	VTKPKTAKPK( GG)AAKAK
P15975	Inactive ubiquitin carboxyl- terminal hydrolase 53	1	1	4.48E-02	MAWVK(GG)F LRKPSGNL GK
B2RXC2	Inositol 1,4,5- trisphosphate 3- kinase B	1	1	2.15E-02	GTPASPRCGSP TPMETDK(GG) RVAPSLER
Q61781	Keratin type I cytoskeletal 14 <sup>ψ</sup>	2	1	0.00E+0 0	TIEDLKSK(GG) ILAATVDNAN VLLQIDNAR
Q6IFX2	Keratin, type I cytoskeletal 42* <sup>ψ</sup>	9	1	8.66E-02	NK(GG)ILAATI DNASIVLQIDN AR
Q924L1	LETM1 domain- containing protein 1	1	1	8.52E-03	MKGIQMLWA DGKK(GG)AR
Q0P5X1	Leucine-rich repeat and IQ domain- containing protein 1	1	1	5.10E-02	KLRKKLEPSVR LALFKKAK(GG) )NK(GG)VSVT K
P51960	Myb-related protein A	1	1	8.52E-03	WSLIAK(GG)H LK(GG)GR
E9Q5F6	Polyubiquitin-C (Fragment) <sup>ψ</sup>	4	2	1.87E-03	TLSDYNIQK(G G)ESTLHLVLR
				5.10E-02	MQIFVK(GG)T LTGK
Q9Z100	Probable carboxypeptidas e X1	2	1	2.60E-03	LRVIKKKIVV KKRK(GG)KLR
H3BKN5	Probable global transcription activator	1	1	9.13E-03	VLGRK(GG)LP KKRVRKKA MK(GG)KR

	SNF2L2				
H3BL88	Protein 9930021J03Rik	2	1	5.10E-02	K(GG)LKLTKM RAKKKKKK
E9Q6J5	Protein Bod11	2	1	3.99E-03	IKEVLKERKVL EKKV(GG)ALS KRRRK
J3QQ16	Protein Col6a3	1	1	3.63E-02	DLK(GG)IMVL MLTGDMQR
A2AU83	Protein GM14124	1	1	1.02E-02	AFSSPSGFLYH K(GG)R
E9PZM7	Protein Scaf11	1	1	5.10E-02	RK(GG)SVRRG RK(GG)PPLLK KKLRR
G3UWJ2	Protein Zfp69	1	1	2.93E-02	GEGPCMAESQ GPEDPILDVKN KLETK(GG)
F6SB18	RNA-binding protein 28	2	1	5.10E-02	KVLALPSHRGP KIRRLKERLRR IRQK(GG)
Q8C4U3	Secreted frizzled-related protein 1	2	1	1.29E-05	IVPKKKKPLKL GPIKKK(GG)EL KRLVFLK
Q9CZ91	Serum response factor-binding protein 1	2	1	4.05E-04	KEVKRIRVLVI RK(GG)LVRSV GRLKSKK
Q9CXH7	Shugoshin-like 1 <sup>Ψ</sup>	2	1	0.00E+0 0	EKRKNLAGI GK(GG)
G5E861	Sodium channel and clathrin linker 1 <sup>Ψ</sup>	1	1	3.77E-02	LQQENEQLQK ETEDLRKVAL EAQK(GG)
Q6PHS6	Sorting nexin-13	1	1	5.28E-03	DDQVK(GG)GT AEDLVETFFE EVEMEK
D3Z1Z3	Sphingosine-1-phosphate lyase 1	1	1	5.10E-02	KKLFKLIRKMP FIGRKVSKAK( GG)KDLVK(GG )
Q9CSP9	Tetratricopeptide repeat protein 14	1	1	5.10E-02	TK(GG)K(GG)I ETRAEKLRKLL KEEKRLKKK
P40630	Transcription factor A, mitochondrial	2	1	2.45E-05	QRRLKKKALV KRRELILGKP K(GG)R
Q5HZG4	Transcription initiation factor TFIID subunit 3	2	1	5.10E-02	LPSSVDVKKK LKKELKTKLK K(GG)KEKQR

Q6ZPJ3	Ubiquitin-conjugating enzyme E2 O <sup>Ψ</sup>	1	1	5.10E-02	KKSIPLSIKNLK (GG)RK(GG)HK RKKNKVTR
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\* Indicates that the protein was also identified in the in-gel digestion.

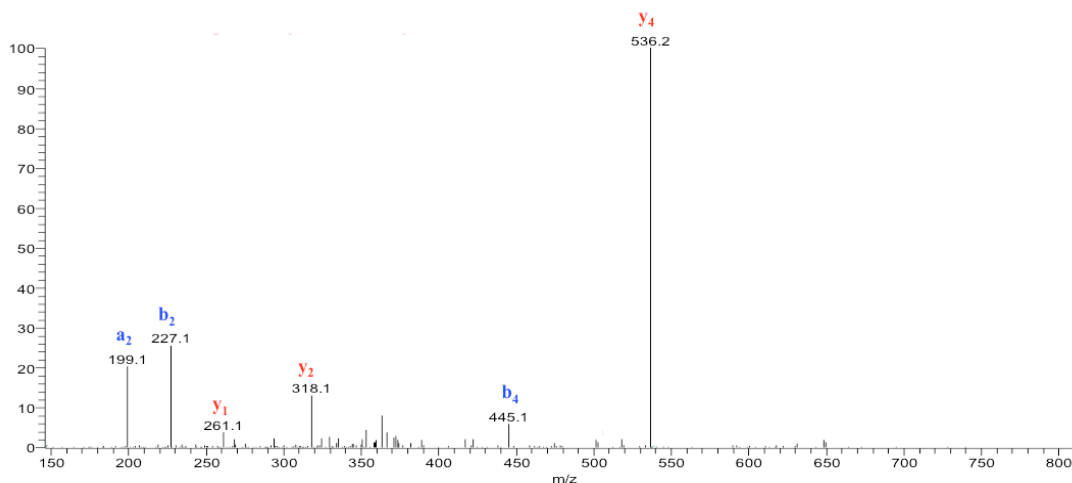
Ψ Indicates that the protein is reported previously to be ubiquitinated.

Together, a combined total of 65 tryptic peptides were identified containing glycylglycine-modified lysines corresponding to 50 ubiquitinated proteins (**Appendix Table 3**). From the combined cohort of 50 proteins, only ten of the proteins were previously identified in a proteomic survey of MDSC-derived exosomes, which highlights that enrichment of ubiquitinated proteins enables the identification of low abundance proteins that may not be identified in a complex sample. Consistent with the Western blots in Figures 7 and 8, polyubiquitin peptides containing glycylglycine-modifications at K6 and K63 were identified following enrichment for glycylglycine-containing peptides (**Table 4**). The identification of peptides assigned to polyubiquitin serves as an additional confirmation that the enrichment of ubiquitinated proteins was successful. The experimentally observed glycylglycine-modified lysine residues were compared to ubiquitination sites predicted *in silico* by a ubiquitination prediction tool, UbiProber.<sup>118</sup> Of the 65 ubiquitin remnant-containing peptides identified, the modification site of 42 peptides were also predicted with probabilities > 0.7, meeting UbiProber's confidence level.

Histones were identified as ubiquitinated with multiple conjugation sites and multiple unique peptides in both the in-gel digestion and two-step immunoaffinity enrichment of ubiquitinated exosomal proteins. Another protein of interest identified as ubiquitinated in exosomes includes high mobility group protein B1 (HMG B1). Parker *et al.* have recently reported that HMGB1 is secreted by MDSC cells in the

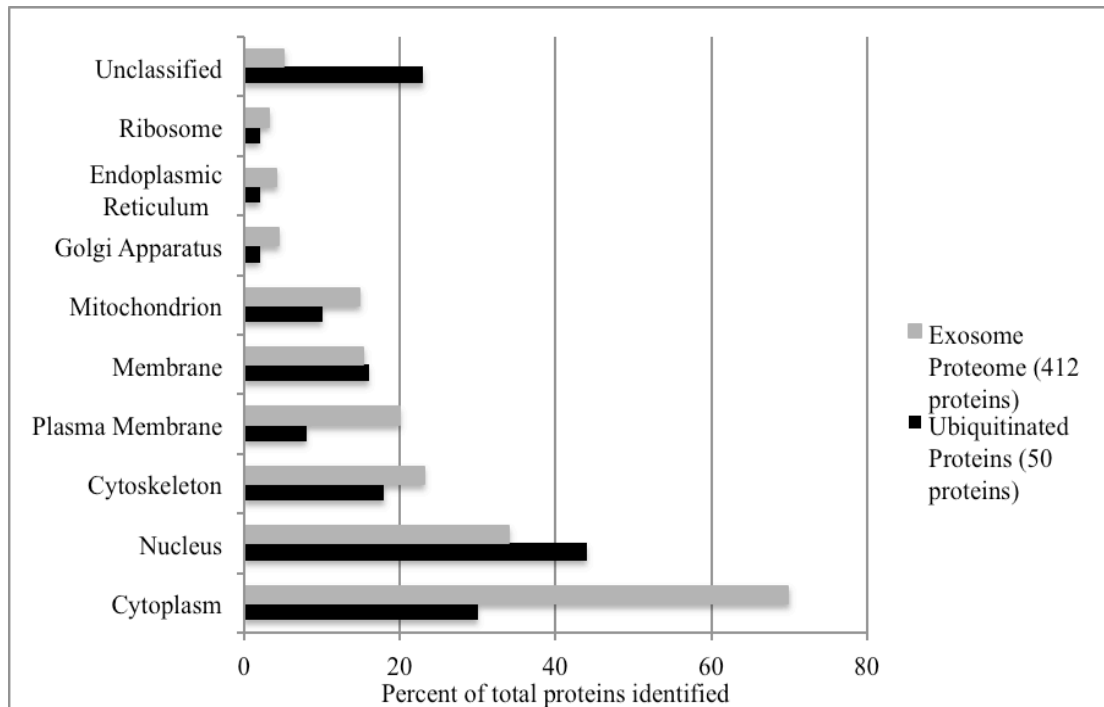
tumor microenvironment and contributes to the differentiation of MDSC and their associated immune suppressive activity.<sup>119</sup> However, the effect of ubiquitin on HMGB1 is not yet known.

From the combined list of 65 ubiquitin remnant-containing peptides, 15 peptides correspond to tryptic cleavage at glycinyglycine-modified lysine residues. This aberrant tryptic cleavage has been previously observed by others.<sup>25,109</sup> Tryptic digestion of K48-linked ubiquitin dimer was performed to confirm the identification of aberrant tryptic cleavage. In addition to the expected peptide containing a missed cleavage at the glycinyglycine-modified lysine (LIFAGK<sub>GG</sub>QLEDGR), the aberrant peptide corresponding to enzymatic cleavage of the glycinyglycine-modified lysine (LIFAGK<sub>GG</sub>) was also identified by tandem mass spectrometry (**Figure 9**). The ratio of the expected glycinyglyciny-product to the novel peptide is approximately 5:1, estimated by peak areas in selected ion chromatograms.



**Figure 9:** Tandem mass spectrum of the aberrant tryptic peptide (LIFAGK<sub>GG</sub>) produced following tryptic digestion of K48-linked ubiquitin dimer

Following identification of ubiquitinated exosomal proteins, the ubiquitinated cohort of 50 ubiquitinated proteins was compared to 412 proteins identified from a proteomic survey of MDSC exosome lysate<sup>16</sup> to determine if the ubiquitinated cohort exhibits unique characteristics. **Figure 10** presents a comparison of the UniProt subcellular localizations of exosomal proteins (referenced to the location in the parental cell), which demonstrates that the ubiquitinated cohort contains an increased percentage of nuclear proteins and a significantly lower percentage of cytosolic and plasma membrane proteins. The nuclear proteins identified in the ubiquitinated cohort include nine histones as well as other nucleic acid binding proteins (transcription factor A; density regulated protein; transcription initiation factor TFIID subunit 3, and protein Bod11) (Tables 3 and 4). It is important to note that histones, especially linker histones such as the histone H1 family, have been reported to be located in the cytoplasm and cell surface in addition to the nucleus<sup>120</sup> and several histones have been previously reported to be ubiquitinated.<sup>121-123</sup> The observation of ubiquitinated histones in MDSC-derived exosomes was confirmed by Western blot using anti-ubiquitin 3933 antibody following histone isolation from a total histone extract kit. Interestingly, 12 of the ubiquitinated proteins identified in MDSC-derived exosomes have no assigned cellular location. This is consistent with a previous report of the ubiquitinated proteins present in yeast expressing His-tagged ubiquitin,<sup>24</sup> where most of the proteins identified did not have assigned subcellular locations present in the available databases.



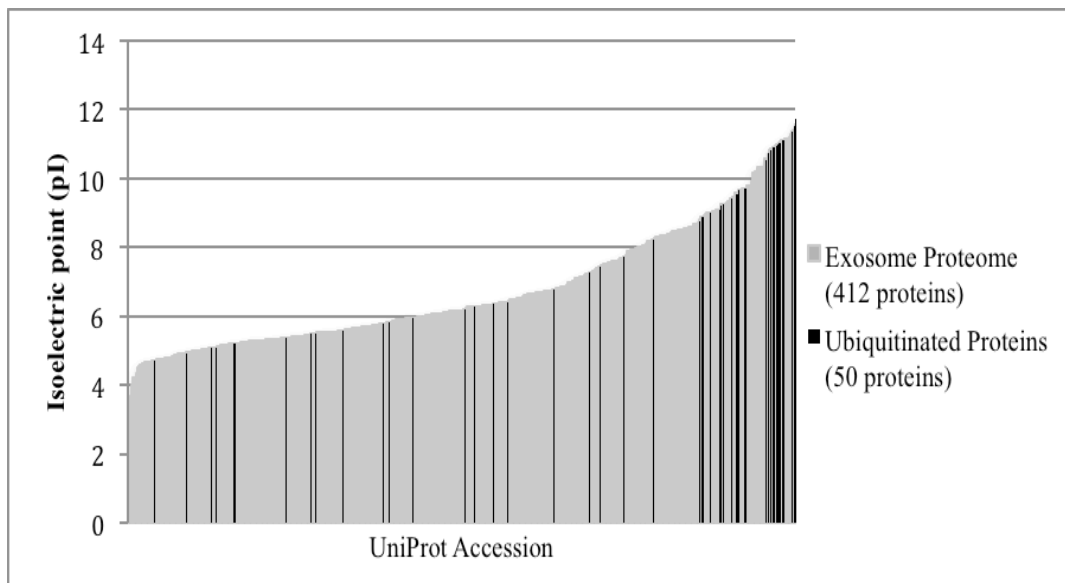
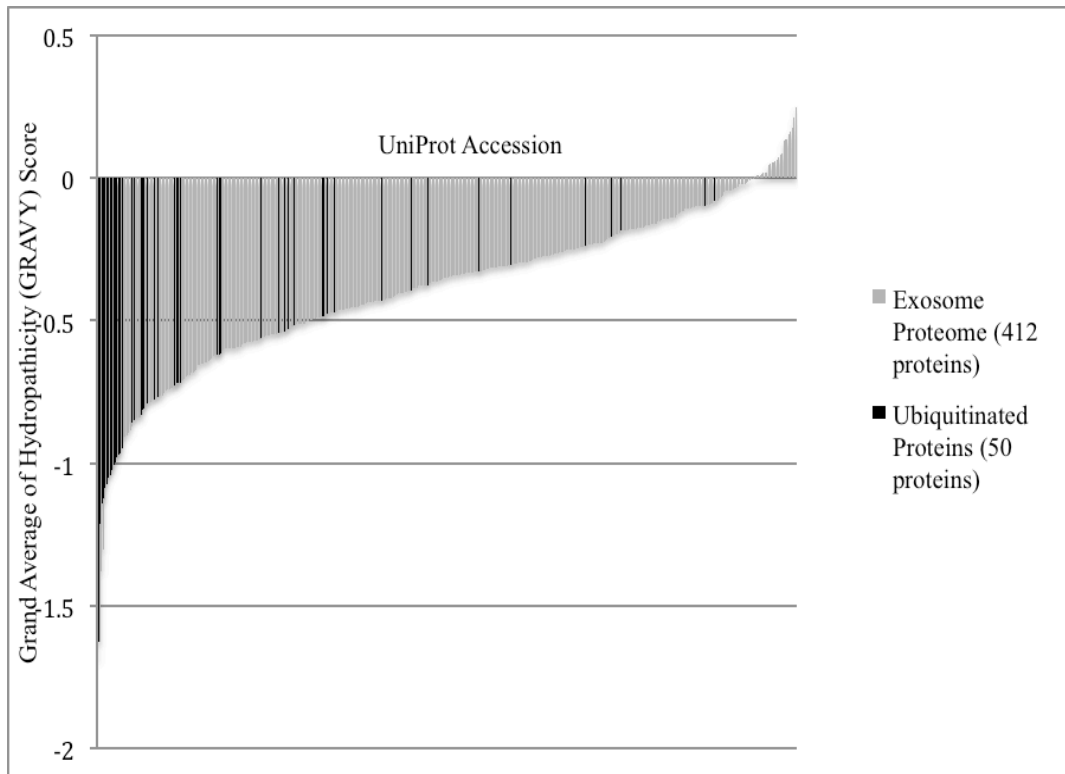
**Figure 10:** Protein locations assigned to MDSC-derived exosomal lysate (412 proteins) in grey and the ubiquitinated cohort (50+ proteins) in black. Some proteins have multiple locations.

Next, gene ontology annotations and the UniProt database were used to compare the distribution of protein sizes (sans ubiquitin), grand average of hydropathicity (GRAVY) scores, and isoelectric points of the ubiquitinated cohort to the 412 proteins identified in proteomic survey of MDSC exosomes. The intact masses of the proteins identified in the ubiquitinated cohort are evenly distributed between 11 and 327 kDa (sans ubiquitin). The GRAVY score and pI distributions of the ubiquitinated cohort are shown in **Figure 11**. Although both the ubiquitinated cohort and proteomic survey of MDSC exosomes illustrate a wide range of GRAVY scores (-0.081 to -1.627) and pI values (4.72 to 11.71), ubiquitinated proteins tend to



cluster as hydrophilic and basic proteins. Seventy-two percent of the proteins identified in the ubiquitinated cohort have a GRAVY score less than -0.5, and 50% of the ubiquitinated proteins have pI values greater than 9.00. Ubiquitin was not included in the calculation of GRAVY scores and pI values; however, monomeric ubiquitin has a GRAVY score of -0.489 and a pI value of 6.56. The observed bias of ubiquitinated proteins towards a high pH is consistent with observations reported by Chen and coworkers,<sup>118</sup> who reports that ubiquitinated proteins contain a greater abundance of positively charged amino acids.

Interestingly, 34 of identified proteins in the ubiquitinated cohort have not previously been reported to be ubiquitinated (**Tables 3 and 4**). Among these, sorting nexin 13 has been observed to participate in endosomal trafficking of ubiquitinated proteins.<sup>124</sup> Identification of two ubiquitinated keratins is consistent with the proposed role of protein aggregation in invagination, which is the initial step in exosome formation.<sup>62,125</sup> Other ubiquitinated proteins that are thought to play important roles in endosome and exosome formation include leucine zipper EF hand-containing transmembrane protein 1 (LETM1) and endoplasmic reticulum chaperone protein. Although the functions of the ubiquitinated proteins are not known, these two unconjugated proteins participate in transporting and maintaining high luminal concentrations of  $\text{Ca}^{2+}$  required for optimum exocytosis of exosomes.<sup>126</sup>



**Figure 11:** Distribution of (top) grand average of hydropathicity score (GRAVY) and (bottom) isoelectric point of MDSC-derived exosomal lysate (412 proteins) in grey and the ubiquitinated cohort (50+ proteins) in black.

## Summary

Due to the role of ubiquitination in protein localization and degradation, as well as its effects on protein-protein interactions,<sup>96,101,102</sup> it was the aim of this study to determine if MDSC-derived exosomes contain ubiquitinated proteins and to identify the substrate proteins that carry the modification(s). Here tandem mass spectrometry and immunoaffinity enrichment were used successfully to identify 65 exosomal peptides that contain the glycylglycine ubiquitin remnant, which corresponds to 50 exosomal proteins. Thirty-four of the proteins identified have not previously been reported to be ubiquitinated. Moreover, both Western blots and peptides identified as ubiquitin indicate that MDSC-derived exosomes contain polyubiquitinated proteins.

The cohort of 50 ubiquitinated proteins includes five proteins associated with exosome formation. This includes the two proteins known to participate in ion uptake and transfer, LETM1 and endoplasmic reticulum chaperone. Compared to a proteomic survey of exosome protein content (412 proteins), the ubiquitinated cohort has a greater abundance of nuclear proteins that includes ubiquitinated histones. Given the recent report<sup>44</sup> of exosomes transferring function mRNA to recipient cells, the presence of histones in exosomes is of great interest.

## Chapter 4: Conclusions and Prospectus

Mass spectrometry-based proteomics has been used in this work to characterize exosomes produced by the immunosuppressive cells, MDSC. MDSC promote tumor progression through suppression of T cell activation and polarization of macrophages towards a tumor-promoting phenotype.<sup>31-33</sup> This activity inhibits successful immunotherapy in cancer patients.<sup>30</sup> MDSC have also been shown to produce extracellular vesicles that meet the criteria of exosomes. The main objective of this work was to analyze the protein cargo of MDSC-derived exosomes in order to learn how these vesicles may contribute to immune suppression in the tumor microenvironment.

MDSC-derived exosomes were found to contain proteins that are considered characteristic of exosomes including annexins, tetraspanins and heat shock proteins. The spectral count method has identified 63 proteins whose abundance was found to differ by at least 2-fold or more due to heightened inflammation in the tumor microenvironment. Proteins that were found to decrease in abundance participate in the innate immune response (C4b-binding protein and complement C3) and chemotaxis (myeloid cysteine-rich protein and platelet factor 4). Additionally, proteins that increased in abundance due to heightened inflammation include proteins that participate in chemokine signaling (mitogen-activated protein kinase 3, cell division control protein 42 homolog and signal transducer and activator of transcription 3). Collectively, the effect of heightened inflammation on proteins that participate in innate immune response, chemotaxis, and chemokine signaling suggests that changes in the tumor microenvironment may alter intercellular communication.

Together, the proteomic analysis and biological assays indicate that MDSC-derived exosomes contain proteins that are representative of the parent cell and are biologically active. The exosomes have been shown to be chemotactic for MDSC and contribute to the polarization of macrophages towards a tumor promoting phenotype. Therefore, the exosomes contribute to the immune suppressive activities associated with MDSC in the tumor microenvironment. Given the role of MDSC in inhibiting successful immunotherapy, these results suggest that exosomes produced by MDSC should also be considered as potential obstacles in successful treatment.

In this work, mass spectrometry-based proteomics was also used with immunoaffinity enrichment to characterize ubiquitinated exosomal proteins. Some previous reports have demonstrated successful enrichment at the peptide level by enriching in one step for the glycinyglycine ubiquitin remnant. However, most reports use His-tagged ubiquitin and/or start from milligram quantities of total protein.<sup>24,114</sup> The method optimized in this study for the identification of endogenous ubiquitinated exosomal proteins from limited quantities of starting material is based on enrichment at the protein level for ubiquitinated proteins, followed by a peptide-level enrichment for the glycinyglycine ubiquitin remnant. This technique has been used to identify 50 ubiquitinated proteins in MDSC-derived exosomes, of which 34 have not previously been reported to be ubiquitinated.

Among the cohort of 50 ubiquitinated proteins are five proteins that are associated with exosome formation as well as ubiquitinated histones. Interestingly, histones located outside of the nucleus, such as the cytoplasm and plasma membrane, have been shown to possess proinflammatory activity.<sup>120</sup> Therefore, it is of great

interest to the exosome community to determine the location of the histones in the MDSC-derived exosomes.

As mentioned earlier, ubiquitin may have different effects on the conjugated protein depending on the ubiquitin linkage present as well as the degree of ubiquitination. While the identification of the exosomal substrate proteins carrying this modification has provided new insight on the role of this post-translational modification in exosome formation and/or function, the effect of this post-translational modification on the biological activity of the exosomal proteins is not yet known. However, more information can be gleaned from identifying the linkage-types present on the exosomal proteins as well as the degree of ubiquitination. For example, it would be of great importance to learn if the majority of ubiquitin modifications comprise monoubiquitin or polyubiquitin chains.

## Appendices

Appendix Table 1: Protein identifications and corresponding spectral count ratio ( $R_{sc}$ ) in exosomes from conventional and inflammatory MDSC.

Protein	Conv.: Distinct Peptides	Conv.: % Covera ge	Conv.: Est. FDR	Infl.: Distinct Peptides	Infl.: % Covera ge	Infl.: Est. FDR	Total: Spectra l Count	Rsc
A1BN54	12	19.6	1.29E-17	10	15.1	1.27E-12	125	- 0.301 17772 4
A2AF47	2	1.8	5.40E-06	0	0.0	1.00E+00	5	- 2.418 86030 7
A2AN08	0	0.0	1.00E+00	2	0.7	9.00E-06	3	1.668 83494 9
A2AQ07	7	25.7	2.05E-17	7	24.6	1.80E-14	45	- 1.622 33123 7
A6ZI44	11	34.0	1.09E-32	11	35.9	4.20E-37	160	- 0.636 66988 1
D3Z6Q9	2	7.8	1.12E-05	2	7.8	1.54E-05	5	- 0.483 84009 3
D3Z7R7	2	3.0	9.34E-06	1	2.9	4.01E-04	6	- 0.096 78912 6
E9PV24	7	10.1	2.65E-21	6	8.9	3.18E-20	98	- 0.800 18427 6
E9Q0F0	1	1.1	1.15E-03	2	1.3	7.94E-07	31	- 0.010 62782 2

E9Q0K6	11	26.4	1.47E-31	13	32.1	1.78 E-41	146	- 0.528 19365
E9Q133	7	28.4	1.60E-20	7	21.1	4.74 E-24	72	0.136 20484 4
E9Q604	8	11.4	3.67E-24	4	6.5	3.10 E-13	61	- 0.987 97878 1
E9QQ35	1	2.1	7.84E-03	2	2.1	1.01 E-06	18	2.923 55986 2
F6W687	5	43.8	1.13E-13	3	43.8	5.65 E-10	136	- 0.138 64175 5
F6YXS3	1	11.6	1.15E-03	2	19.6	1.61 E-07	7	0.846 71281 3
F8WGL3	3	20.7	3.57E-09	3	26.0	1.48 E-09	43	- 0.033 37068 2
F8WGM5	3	8.9	3.57E-09	2	6.4	3.20 E-07	10	- 1.605 97950 9
F8WH69	5	17.3	5.89E-15	8	28.0	6.72 E-26	61	- 0.416 41646 6
F8WI35	28	65.2	1.06E-08	17	59.3	7.50 E-10	1586	- 0.268 33147 7
G3UZJ4	1	13.9	1.15E-03	2	23.5	1.61 E-07	21	0.789 74130 4
G5E8N7	7	29.3	1.16E-20	7	29.3	8.24 E-24	107	0.141 06431 7
H3BKH6	7	45.1	4.44E-20	4	25.1	3.00 E-14	63	- 0.496 09322 7
O08692	15	58.7	7.65E-42	9	44.3	1.57 E-30	457	0.251 39631 3



O08756	3	22.2	2.20E-09	1	7.7	4.01E-04	14	- 0.810 60792 2
O08810	2	4.5	2.80E-06	2	4.5	3.40E-07	7	- 1.040 29299 7
O08992	3	20.7	1.10E-08	1	7.7	4.01E-04	40	- 0.646 73957 4
O09061	3	18.8	1.52E-09	2	12.9	1.61E-07	18	- 0.667 23170 2
O35286	2	3.5	1.32E-06	3	3.9	2.53E-10	7	0.208 09330 1
O35350	2	4.8	1.32E-06	5	11.5	1.84E-16	21	1.368 18161 3
O35598	2	4.1	1.32E-06	1	1.9	4.01E-04	7	- 1.040 29299 7
O35639	3	12.7	1.52E-09	3	12.4	2.07E-10	23	0.016 43387 4
O35643	5	11.2	5.30E-06	2	2.9	1.00E+00	17	- 1.181 06571 6
O35744	6	23.9	5.28E-18	3	11.3	2.80E-10	41	- 2.192 75835 3
O54890	2	5.5	1.32E-06	0	0.0	1.00E+00	5	- 2.418 86030 7
O55029	2	3.2	1.32E-06	3	5.7	6.45E-11	28	1.584 82850 3
O55222	0	0.0	1.00E+00	2	7.7	2.04E-06	2	1.281 78398 1
O70133	3	5.2	6.09E-09	3	4.6	2.88E-10	21	- 0.720 38434

								3
								-
O70138	3	9.2	1.52E-09	2	7.1	1.61E-07	34	0.414 41364 9
O70145	7	25.9	2.65E-21	8	25.7	6.05E-27	162	0.472 52025 8
O70165	3	16.2	1.52E-09	0	0.0	1.00E+00	7	- 2.819 45779 4
O88342	5	11.2	8.08E-15	6	19.1	5.11E-20	53	0.483 03151 8
O88593	1	13.2	1.15E-03	2	25.8	1.61E-07	37	1.613 19599 7
O88685	2	7.0	1.32E-06	3	9.3	6.45E-11	36	- 0.864 12914
O88844	3	9.4	1.32E-06	5	13.3	5.15E-14	60	0.561 03724 5
O89053	17	58.6	3.03E-49	19	60.7	1.46E-57	601	- 0.034 82676 5
P01027	19	55.9	1.34E-55	10	32.0	4.22E-34	153	- 1.474 86566 9
P01325	2	14.8	1.32E-06	2	14.8	1.61E-07	24	- 0.096 80651 8
P01794	2	23.6	9.58E-06	0	0.0	1.00E+00	4	- 2.167 29176 2
P01837	2	45.3	1.32E-06	0	0.0	1.00E+00	16	- 3.883 85617
P01872	10	29.1	4.03E-30	4	13.4	3.00E-14	123	- 2.235 97928 7
P01942	15	84.5	2.88E-44	8	83.8	4.08E-27	455	- 2.274 12756

								9
P02088	15	79.6	1.08E-26	10	73.5	1.08E-19	310	- 1.446 45389 8
P02089	10	78.2	4.90E-12	6	53.7	1.41E-06	174	- 1.593 13404 5
P02301	29	65.4	6.07E-09	18	59.6	3.69E-09	2372	- 0.236 13926 6
P04104	3	3.8	7.98E-09	2	3.8	1.61E-07	39	- 0.305 85436 8
P04919	11	24.5	3.19E-32	0	0.0	1.00E+00	107	- 6.536 26789 6
P06151	9	36.1	2.07E-26	8	32.8	4.20E-27	106	- 0.043 63521 2
P06745	9	23.5	7.29E-27	12	28.3	1.26E-39	176	0.000 24397 4
P06800	2	2.6	7.37E-06	2	4.3	4.84E-06	5	- 0.483 84009 3
P07356	6	32.2	6.21E-18	5	27.4	4.94E-17	41	- 0.565 41162
P07724	16	35.4	9.31E-48	5	13.3	2.57E-16	165	- 2.662 85554
P07742	0	0.0	1.00E+00	2	4.8	2.95E-06	3	1.668 83494 9
P07901	3	6.5	1.32E-06	4	7.5	6.45E-11	20	0.421 77969 5
P08030	5	38.3	2.01E-15	5	38.3	1.04E-17	109	0.616 75268
P08032	17	14.2	1.00E-47	0	0.0	1.00E+00	64	- 5.804 66809
P08071	48	69.7	9.30E-	45	64.1	5.56	2141	-

			139			E-151		0.059 00329 7
P08226	5	24.8	2.01E-15	0	0.0	1.00 E+00	31	- 4.787 00581
P08249	4	16.3	1.75E-12	3	13.9	6.45 E-11	26	- 0.713 65271 1
P08607	3	10.0	1.52E-09	1	3.0	1.29 E-03	14	- 2.760 10762 6
P08730	0	0.0	1.00E+00	2	6.8	6.30 E-07	7	2.625 87761
P08905	3	31.1	1.52E-09	2	27.0	1.61 E-07	36	- 0.705 47014 6
P09411	9	36.9	1.18E-26	12	47.0	7.87 E-40	265	0.759 89712 5
P09528	4	28.0	1.65E-11	2	20.3	4.49 E-07	27	- 1.227 52304 1
P0C0S6	11	56.3	3.05E-24	12	53.9	5.35 E-31	1102	0.029 01252 9
P10107	14	46.5	8.48E-42	11	36.1	1.23 E-37	278	- 0.682 56150 7
P10126	10	36.4	3.78E-28	12	38.5	4.96 E-37	120	0.476 49789 2
P10605	2	9.1	1.32E-06	1	3.8	5.86 E-03	13	- 2.655 10828 3
P10854	24	74.6	5.76E-12	17	67.5	3.40 E-07	2222	- 0.205 14799 8
P11247	21	33.7	4.29E-61	26	38.7	2.90 E-87	510	0.558 75722 3
P11276	63	39.5	1.22E-182	27	18.2	3.07 E-86	866	- 2.868

								49432 9
P11352	4	33.8	7.64E- 12	4	30.3	4.98 E-13	44	0.533 81676 9
P11499	4	8.7	1.83E- 09	3	5.7	1.61 E-07	24	- 0.535 80602 3
P11672	2	17.0	1.32E- 06	1	10.5	4.01 E-04	48	- 0.558 03408 5
P11835	13	23.2	1.78E- 37	6	10.6	3.25 E-18	60	- 1.054 34913
P11983	4	10.8	1.75E- 12	7	17.9	2.30 E-23	73	0.645 75733 3
P12815	1	14.1	1.15E- 03	3	35.1	1.41 E-08	10	1.412 39352 8
P12970	0	0.0	1.00E+ 00	2	7.9	6.30 E-07	8	2.790 96470 3
P13020	2	3.3	3.11E- 06	1	1.5	4.01 E-04	5	- 0.483 84009 3
P14069	3	32.6	1.52E- 09	3	32.6	6.45 E-11	66	- 0.265 64427 2
P14131	3	21.9	1.52E- 09	1	6.8	4.01 E-04	12	- 1.367 05696 1
P14152	2	11.1	4.43E- 06	0	0.0	1.00 E+00	2	- 1.475 35450 4
P14206	3	20.0	3.26E- 08	3	20.3	1.14 E-09	41	- 0.296 21822 1
P14685	2	4.5	4.43E- 06	3	12.6	1.56 E-09	14	1.002 91169 7
P14824	2	5.1	2.56E- 05	1	1.3	4.01 E-04	4	- 1.014

								38265 4
P14869	2	10.1	3.50E-06	3	20.5	1.38 E-09	17	- 0.244 92720 7
P15864	17	37.3	1.75E-12	17	28.8	2.83 E-20	973	0.344 68571 6
P16546	2	1.8	1.53E-05	1	0.9	6.96 E-03	4	- 1.014 38265 4
P16858	14	63.4	4.79E-41	18	71.5	1.35 E-59	598	0.203 40652 6
P17182	15	44.5	1.09E-42	17	46.5	3.25 E-57	407	0.151 05204 1
P17225	2	8.3	4.17E-05	0	0.0	1.00 E+00	2	- 1.475 35450 4
P17427	5	9.1	4.38E-14	2	3.4	1.61 E-07	17	- 1.181 06571 6
P17742	6	52.4	1.48E-17	4	40.2	2.59 E-14	56	- 0.907 07624 3
P17751	2	14.4	4.62E-06	2	15.4	1.76 E-06	5	- 0.483 84009 3
P19096	4	2.8	2.10E-12	10	7.8	1.59 E-33	64	1.199 41785 4
P20029	9	17.6	1.07E-20	3	6.7	4.01 E-04	36	- 0.096 81811 7
P20152	4	12.2	1.75E-12	2	7.1	1.96 E-06	15	- 0.596 45527 1
P21107	1	7.3	1.15E-03	1	5.2	5.39 E-04	12	0.303 80063 2
P22752	46	70.8	3.48E-	45	66.2	6.41	8165	0.083

			53			E-51		69747 9
P24270	6	15.2	6.11E- 18	4	11.6	2.59 E-14	80	- 0.026 84044 6
P25911	3	10.0	6.39E- 09	5	14.7	1.04 E-17	24	1.048 91208
P26039	36	27.0	2.01E- 104	31	24.4	1.32 E-96	390	- 1.044 59424 1
P26040	7	11.4	1.32E- 06	6	9.7	1.67 E-06	95	- 0.067 25156 5
P26041	10	18.5	8.79E- 15	9	16.6	2.62 E-16	132	- 0.096 91100 5
P26443	2	5.9	3.11E- 06	2	6.6	6.98 E-07	13	0.868 58275 7
P26516	1	8.7	1.15E- 03	1	5.3	4.01 E-04	8	0.459 65991 3
P26638	1	3.1	1.15E- 03	3	11.5	2.07 E-10	11	2.225 39344 8
P27005	5	74.2	1.01E- 14	7	92.1	1.97 E-22	510	0.727 75280 4
P27773	2	5.0	1.32E- 06	2	4.2	3.20 E-07	13	0.468 88772 5
P27870	1	2.0	5.14E- 03	1	1.7	4.01 E-04	3	0.433 75729 9
P28293	7	31.0	1.85E- 20	13	50.2	9.40 E-42	121	1.115 79700 7
P28352	1	6.0	4.61E- 03	1	2.2	7.10 E-03	3	0.433 75729 9
P28650	5	15.4	4.72E- 15	5	14.8	1.04 E-17	54	0.419 57635
P28798	2	4.8	1.32E- 06	3	7.8	1.30 E-09	15	- 0.261 88588 1

P29351	13	39.2	6.13E-39	12	34.2	9.26 E-40	236	0.367 76272 6
P29391	5	32.8	1.52E-14	4	41.0	1.62 E-13	37	- 0.613 81182
P29788	4	10.9	4.11E-12	0	0.0	1.00 E+00	12	- 3.503 13303 3
P30681	8	27.6	2.26E-17	5	25.7	7.51 E-13	55	- 0.761 02913 3
P31230	1	3.9	8.66E-03	1	5.5	4.66 E-04	2	- 0.096 78526 1
P31725	20	79.6	2.81E-59	18	77.9	7.76 E-59	2233	0.292 69634 1
P34884	1	18.3	4.61E-03	2	26.1	5.16 E-07	6	1.377 25730 6
P35441	19	23.2	2.21E-55	11	16.9	3.99 E-34	155	- 2.057 05630 5
P35700	1	5.5	1.15E-03	1	11.1	7.36 E-03	3	- 0.627 32975 4
P39054	5	9.5	2.32E-14	8	15.4	1.53 E-24	45	0.713 88664 7
P39654	2	5.7	2.62E-05	0	0.0	1.00 E+00	2	- 1.475 35450 4
P39655	2	5.4	2.46E-05	1	1.8	1.57 E-03	9	0.673 81256 5
P40124	10	36.5	8.53E-30	10	34.4	7.56 E-34	231	- 0.332 86136 5
P40142	15	31.1	1.05E-42	18	40.0	1.55 E-58	372	0.042 16706 4



P40201	1	0.7	7.84E-03	1	0.8	2.61E-03	2	- 0.096 78526 1
P41245	16	32.5	7.70E-45	8	19.0	1.55E-27	179	- 1.143 72221 3
P42227	1	3.4	1.15E-03	3	10.4	8.64E-10	11	2.225 39344 8
P42932	2	5.3	1.32E-05	6	14.6	3.06E-20	20	1.619 75015 7
P43274	17	37.4	1.32E-06	16	32.9	2.59E-14	945	0.295 07886
P43275	7	22.1	1.75E-12	9	22.1	2.80E-13	223	0.381 82198 1
P43276	10	26.5	6.18E-15	7	27.4	1.21E-16	108	- 0.789 51532 2
P45376	1	4.4	1.15E-03	2	10.1	6.30E-07	27	0.812 68446 9
P47738	13	43.9	1.04E-36	13	40.1	1.32E-42	249	0.052 62945 2
P47753	5	28.0	4.62E-12	3	15.0	1.61E-07	37	- 0.766 25396 6
P47754	4	21.3	1.52E-09	2	8.7	4.01E-04	20	- 0.354 01812 2
P47757	4	22.9	1.75E-12	5	29.6	1.21E-17	91	- 0.189 56896 8
P47791	3	10.1	1.52E-09	0	0.0	1.00E+00	6	- 2.633 01489 1
P47911	2	10.8	1.32E-06	2	7.8	1.87E-07	17	- 1.537 63167 1
P47955	2	51.8	1.07E-	0	0.0	1.00	6	-

			05			E+00		2.633 01489 1
P47962	2	8.1	1.59E- 06	1	3.4	4.01 E-04	6	- 0.096 78912 6
P48025	2	5.4	8.22E- 06	1	1.7	4.01 E-04	3	- 0.627 32975 4
P49722	4	23.5	1.75E- 12	0	0.0	1.00 E+00	12	- 3.503 13303 3
P50247	3	10.0	4.46E- 09	4	14.1	4.23 E-13	19	1.190 33879 1
P50516	2	8.1	3.11E- 06	2	8.1	1.61 E-07	37	0.270 53398 9
P50543	2	27.6	1.86E- 06	1	11.2	4.01 E-04	39	0.394 76911
P51150	1	6.3	1.15E- 03	1	4.8	3.29 E-03	3	0.433 75729 9
P51174	1	2.6	1.15E- 03	1	4.7	6.55 E-03	2	- 0.096 78526 1
P51437	3	35.8	8.39E- 09	2	19.7	1.61 E-07	47	- 0.038 50128 8
P52480	32	56.9	2.95E- 91	34	56.3	1.82 E- 111	1152	0.732 65808 9
P54071	2	4.9	1.15E- 03	6	16.2	5.44 E-16	21	0.789 74130 4
P54775	2	15.8	3.11E- 06	2	15.8	1.45 E-06	22	- 0.332 92957 4
P56480	1	3.6	1.15E- 03	3	9.1	6.33 E-10	5	0.290 26377 4
P57780	8	11.5	1.32E- 06	7	9.9	5.39 E-04	92	0.025 44899 4

P58252	12	25.4	4.53E-35	17	31.8	1.21 E-56	280	0.526 97745 2
P59999	2	13.1	1.32E-06	3	17.9	7.49 E-11	37	0.122 83375 3
P60122	2	6.6	1.32E-06	2	6.6	1.61 E-07	15	1.125 79629 3
P60229	0	0.0	1.00E+00	2	5.6	3.26 E-05	2	1.281 78398 1
P60335	3	12.1	1.52E-09	3	12.1	6.45 E-11	32	0.584 88677 7
P60710	54	90.1	6.93E-68	59	85.9	4.66 E-72	7298	0.324 41018 5
P60766	1	8.9	1.15E-03	5	42.9	1.72 E-13	57	1.080 32221 5
P60843	5	19.5	1.65E-14	5	16.0	6.03 E-17	71	0.666 89293 1
P61161	8	31.2	5.01E-23	10	35.0	8.44 E-33	110	0.005 86057 2
P61164	4	19.4	1.75E-12	5	22.3	1.04 E-17	49	- 0.491 66373 6
P61979	2	10.9	1.03E-05	2	6.6	7.94 E-07	22	0.378 64868 5
P62137	4	21.8	1.74E-11	4	20.6	3.47 E-13	91	- 0.065 98151 3
P62196	2	8.6	3.50E-06	0	0.0	1.00 E+00	4	- 2.167 29176 2
P62242	3	18.8	5.04E-08	2	12.5	1.41 E-06	8	- 0.653 24202 9
P62259	3	20.8	1.19E-08	2	16.1	1.61 E-07	34	- 1.656 06588 4

P62281	2	17.7	4.62E-06	2	17.7	3.40 E-07	13	0.468 88772 5
P62315	2	27.7	1.32E-06	2	27.7	1.61 E-07	59	- 0.049 89048 2
P62317	2	16.1	5.32E-06	1	15.3	4.01 E-04	25	0.433 85131 3
P62320	3	31.7	3.57E-09	2	15.1	3.40 E-07	21	- 0.720 38434 3
P62334	1	3.9	6.58E-03	3	10.5	6.45 E-11	8	1.777 85092 8
P62702	2	4.6	1.32E-06	2	8.0	1.61 E-07	15	0.751 34315 2
P62806	38	65.0	5.56E-109	33	62.1	2.74 E-109	8009	0.109 24666 5
P62814	2	6.7	1.32E-06	3	9.2	6.45 E-11	16	- 0.410 0143
P62827	9	40.7	3.93E-26	8	37.0	5.43 E-26	144	0.886 03670 1
P62855	2	23.5	9.18E-06	0	0.0	1.00 E+00	4	- 2.167 29176 2
P62880	2	9.1	1.59E-06	3	12.6	5.30 E-10	24	0.121 43128 4
P62889	2	30.4	3.11E-06	3	40.9	6.45 E-11	16	1.239 03475 3
P62908	1	5.3	2.70E-03	4	21.0	9.95 E-14	15	2.664 38908 7
P62962	5	37.1	1.92E-13	5	37.1	6.90 E-15	99	- 0.125 33630 1
P63005	0	0.0	1.00E+00	2	8.2	6.30 E-07	4	1.973 71737 5
P63017	16	33.7	9.74E-	18	38.1	3.15	309	0.135

			45			E-51		71108
P63085	2	6.7	3.23E-03	6	15.9	1.97E-10	9	1.247 30643 5
P63101	5	26.5	1.12E-14	4	29.0	2.59E-14	71	- 0.136 14022 2
P63158	3	14.9	1.99E-03	3	17.7	2.07E-06	25	- 0.201 80586
P63330	4	24.3	1.75E-12	4	24.3	1.01E-13	42	0.033 0016
P68033	34	67.9	4.97E-22	41	63.9	1.17E-32	1649	0.102 64196 5
P68040	6	34.4	6.78E-18	5	25.9	1.39E-17	46	- 0.215 93810 1
P68254	2	21.8	9.12E-06	1	11.9	7.51E-03	4	- 1.014 38265 4
P68368	11	34.2	4.11E-12	10	31.5	4.65E-10	196	0.195 31348 3
P68372	11	40.9	1.32E-06	11	39.1	4.01E-04	153	- 0.264 37794 7
P68373	12	36.3	2.01E-15	9	29.6	1.61E-07	186	- 0.250 46689 4
P68433	27	65.4	1.32E-06	17	59.6	8.73E-10	3787	0.018 97076 9
P68510	2	17.9	1.32E-06	2	17.9	1.61E-07	32	- 0.432 97872 6
P70168	7	15.5	1.53E-19	6	13.4	1.40E-18	41	- 0.163 19426 6
P70335	0	0.0	1.00E+00	2	2.7	6.30E-07	3	1.668 83494 9

P70390	40	13.8	3.17E-109	0	0.0	1.00 E+00	508	- 8.782 29104 5
P70460	3	10.1	1.83E-09	5	21.9	5.68 E-16	25	0.883 82305 4
P80313	2	7.5	1.32E-06	4	11.8	2.19 E-12	16	1.239 03475 3
P80314	2	7.1	1.32E-06	0	0.0	1.00 E+00	4	- 2.167 29176 2
P80315	4	13.2	1.75E-12	6	17.8	3.65 E-20	32	1.352 40495 6
P80316	4	16.5	2.53E-12	5	19.0	6.65 E-17	59	0.044 10865 9
P80317	2	7.0	1.32E-06	2	7.0	1.61 E-07	39	- 0.166 39077
P82198	6	17.6	4.06E-17	2	8.6	2.71 E-06	30	- 1.652 79212 5
P84078	0	0.0	1.00E+00	3	21.5	3.55 E-10	8	2.790 96470 3
P84091	1	6.4	1.15E-03	2	9.9	3.40 E-07	7	0.208 09330 1
P84096	5	30.9	5.93E-15	7	49.2	5.80 E-18	39	1.705 61573 4
P84228	33	65.4	1.52E-18	21	59.6	2.83 E-19	5337	- 0.271 92880 6
P97369	5	23.6	2.01E-15	5	23.6	1.04 E-17	54	0.633 72843 9
P97384	6	17.5	5.43E-18	7	17.5	1.44 E-23	66	- 1.058 16842 3
P99024	12	44.8	1.52E-09	12	43.0	1.61 E-07	156	- 0.279 50845

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Q00519	7	10.4	2.49E-20	5	7.5	1.01E-16	41	0.163 19426 6
Q00612	17	44.3	7.49E-50	21	50.7	2.76E-68	516	0.778 54123 6
Q01853	20	38.3	3.12E-54	13	24.9	8.08E-45	344	- 0.603 57152 1
Q02053	17	26.8	4.93E-50	16	25.0	5.73E-53	209	0.301 99199 4
Q02357	5	62.8	2.00E-14	0	0.0	1.00E+00	12	- 3.503 13303 3
Q04750	4	9.3	1.38E-11	1	2.1	4.01E-04	33	- 0.506 07197 5
Q05144	6	39.1	1.60E-17	10	66.7	8.16E-31	198	0.250 76507 4
Q07076	2	5.4	1.32E-06	3	8.9	7.49E-11	18	1.441 90733 6
Q07797	2	6.8	3.50E-06	0	0.0	1.00E+00	8	- 2.984 54682
Q3TCJ1	0	0.0	1.00E+00	2	7.5	2.50E-06	2	1.281 78398 1
Q3TEA8	2	4.7	1.32E-06	0	0.0	1.00E+00	4	- 2.167 29176 2
Q3THE2	1	5.8	1.15E-03	3	23.8	1.32E-09	29	0.955 34457 1
Q3TRM8	11	18.0	1.89E-32	10	16.6	5.46E-34	87	0.129 46922
Q3TXS7	2	3.8	1.94E-05	0	0.0	1.00E+00	2	- 1.475 35450 4

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Q3UGX2	5	3.5	8.95E-15	0	0.0	1.00 E+00	12	3.503 13303 3
Q3UKW 2	4	42.1	2.29E-11	2	16.8	1.61 E-07	72	- 0.891 48762 8
Q3UP87	4	20.8	1.75E-12	6	28.7	7.26 E-20	91	0.244 48279 2
Q3UW53	3	6.3	1.79E-08	3	4.2	1.28 E-10	14	- 0.096 79685 5
Q3UZZ4	5	12.7	2.01E-15	2	5.0	1.61 E-07	38	- 0.978 52139 5
Q3V1G4	1	2.5	8.34E-03	1	2.5	4.01 E-04	4	0.820 80826 7
Q571I9	4	9.0	1.75E-12	3	7.1	6.45 E-11	49	0.412 90102 4
Q5SQX6	3	1.7	1.01E-07	1	0.9	4.01 E-04	6	- 0.096 78912 6
Q5SS00	0	0.0	1.00E+00	2	0.3	2.45 E-07	13	3.414 54059 5
Q5SXR6	26	25.0	3.53E-75	24	22.9	3.71 E-75	252	0.130 76849 1
Q60605	5	45.7	1.32E-14	7	51.0	6.27 E-23	61	- 0.051 37049 2
Q60692	2	10.9	1.32E-06	1	4.2	4.01 E-04	10	- 1.053 83951 6
Q61081	1	5.5	1.15E-03	2	10.0	1.61 E-07	6	0.595 14620 1
Q61096	1	5.1	1.15E-03	1	5.1	4.01 E-04	4	0.820 80826 7
Q61171	5	34.8	8.20E-	0	0.0	1.00	26	-



			15			E+00		4.543 81391 7
Q61210	4	9.0	1.08E- 11	4	6.3	2.59 E-14	38	0.480 95553 8
Q61233	16	35.9	1.16E- 46	20	46.1	1.55 E-65	250	- 0.051 19692 7
Q61316	1	1.7	1.38E- 03	1	1.9	6.12 E-04	2	- 0.096 78526 1
Q61508	2	6.5	2.95E- 06	1	3.2	1.57 E-03	13	- 1.062 17453 5
Q61598	2	7.3	1.32E- 06	4	18.1	2.77 E-13	50	0.123 54976 2
Q61599	2	23.5	1.32E- 06	2	23.5	1.61 E-07	58	- 0.578 46751 6
Q61646	7	33.1	4.24E- 20	2	10.4	3.42 E-06	27	- 2.304 95236
Q61656	2	4.4	1.32E- 06	2	4.1	4.73 E-07	10	- 0.562 51418 6
Q61753	0	0.0	1.00E+ 00	2	6.4	1.12 E-06	3	1.668 83494 9
Q61990	2	8.3	1.32E- 06	2	8.3	3.20 E-07	11	- 1.218 92854 2
Q62465	3	6.9	6.39E- 09	1	4.7	4.01 E-04	6	- 0.788 72445 2
Q63844	6	23.4	2.01E- 15	8	24.5	5.72 E-17	44	1.082 03017 8
Q64514	2	4.1	2.96E- 05	1	0.9	4.01 E-04	4	- 1.014 38265 4

Q64522	40	60.8	2.65E-21	36	53.8	1.33E-27	6914	0.169 15369
Q64727	5	9.3	9.16E-15	4	7.1	2.59E-14	38	- 0.382 84420 2
Q69ZK0	1	5.0	4.69E-03	1	6.7	7.10E-03	4	- 1.014 38265 4
Q6GSS7	38	70.8	1.32E-06	34	63.8	6.45E-11	7436	0.130 51815
Q6IRU2	2	10.9	1.25E-05	0	0.0	1.00E+00	5	- 2.418 86030 7
Q6P069	2	16.4	1.32E-06	4	27.9	4.91E-13	32	0.070 70013 2
Q6P4T2	2	2.1	1.32E-06	0	0.0	1.00E+00	4	- 2.167 29176 2
Q6P5F9	3	4.9	1.83E-09	5	7.7	2.73E-15	54	- 0.508 38053
Q6P9Q4	1	0.9	9.05E-03	1	1.6	4.01E-04	3	0.433 75729 9
Q6PDI5	2	2.1	9.41E-06	3	2.5	6.54E-09	6	- 0.096 78912 6
Q6PDQ2	1	0.9	1.15E-03	2	1.5	1.32E-06	5	1.125 69069 3
Q6PHN9	1	5.5	1.15E-03	2	12.4	1.61E-07	11	- 0.310 94757 4
Q6ZQ38	2	4.5	1.59E-06	4	6.3	9.53E-12	10	0.368 92820 6
Q6ZQA0	0	0.0	1.00E+00	4	2.8	7.92E-14	7	2.625 87761
Q6ZWR6	6	8.9	4.51E-17	2	1.8	2.61E-06	18	- 2.032 05146 4

Q76MZ3	5	16.6	4.93E-15	4	10.7	2.59E-14	28	0.283 90888 6
Q78PY7	2	4.0	1.32E-06	2	4.4	3.72E-06	9	- 0.348 35960 3
Q80TE4	0	0.0	1.00E+00	2	1.9	4.71E-05	2	1.281 78398 1
Q80X41	2	11.4	1.86E-06	2	11.4	1.02E-05	11	- 1.218 92854 2
Q8BFY9	0	0.0	1.00E+00	2	3.8	2.65E-06	3	1.668 83494 9
Q8BFZ3	20	43.4	1.05E-08	27	47.9	1.54E-16	1790	0.526 85112 3
Q8BG32	3	8.5	1.52E-09	6	18.2	1.35E-18	33	0.827 95075 3
Q8BJY1	0	0.0	1.00E+00	2	6.3	1.61E-07	3	1.668 83494 9
Q8BT60	3	9.0	1.52E-09	3	9.0	7.49E-11	44	- 0.221 06290 5
Q8BTM8	34	242.6	4.86E-98	24	173.1	3.38E-80	318	- 0.664 17884 5
Q8BVQ9	3	12.0	1.52E-09	2	8.8	1.61E-07	20	- 0.615 38500 1
Q8BWT1	1	2.5	1.15E-03	2	6.0	3.66E-07	21	1.368 18161 3
Q8C147	4	10.8	1.96E-11	3	8.8	2.53E-10	13	0.468 88772 5
Q8C2Q7	3	10.6	1.52E-09	1	3.6	4.01E-04	18	- 1.635 50877 8
Q8CC86	0	0.0	1.00E+00	2	7.4	7.74E-07	3	1.668 83494

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Q8CCK0	4	12.4	1.38E-03	5	12.4	1.02E-05	92	1.061707064
Q8CG29	3	4.4	1.52E-09	3	4.4	6.45E-11	55	0.35879253
Q8CGP1	24	74.6	1.75E-12	16	54.0	4.66E-04	2185	-0.162329528
Q8CGP4	21	56.6	1.52E-09	22	58.9	3.27E-10	3428	0.039234262
Q8CIE6	4	3.9	1.75E-12	5	5.1	2.07E-17	30	0.628558382
Q8CIH5	6	8.8	3.34E-18	10	11.4	2.04E-31	48	0.364374648
Q8CIZ8	9	36.6	1.41E-26	0	0.0	1.00E+00	35	-4.955806826
Q8JZQ9	1	3.0	3.04E-03	1	2.1	4.01E-04	2	-0.096785261
Q8K0E8	12	36.4	4.20E-35	11	30.1	7.33E-37	164	-0.516463644
Q8K1B8	14	33.4	2.15E-39	16	34.7	6.66E-53	140	0.43624996
Q8K1X4	2	4.0	5.32E-06	1	1.4	4.01E-04	9	-0.867396613
Q8K426	2	23.1	1.32E-06	2	23.1	1.61E-07	30	-1.426921212
Q8K482	4	5.8	8.86E-12	0	0.0	1.00E+00	12	-3.503133033
Q8QZY1	3	9.4	1.52E-09	3	8.7	6.45E-11	11	0.117359661

Q8R010	2	12.8	4.43E-06	1	5.0	7.98E-04	5	0.290 26377 4
Q8R081	1	4.9	1.15E-03	2	7.7	1.61E-07	13	- 0.662 47950 3
Q8R1B4	3	2.9	2.52E-08	2	2.6	1.61E-07	10	0.860 25353 6
Q8R1Q8	2	5.9	1.32E-06	2	9.0	2.37E-06	5	- 0.483 84009 3
Q8R2S8	10	18.4	4.85E-30	9	19.8	5.35E-31	103	- 0.794 59301 9
Q8VCI0	0	0.0	1.00E+00	2	6.0	2.89E-05	3	1.668 83494 9
Q8VCM7	10	34.9	9.48E-30	9	31.4	1.72E-30	216	- 0.309 12038 8
Q8VCT3	4	11.7	3.91E-12	9	19.1	7.55E-30	37	1.825 23393
Q8VDD5	44	29.8	5.27E-123	36	24.6	4.18E-116	595	0.043 63309 9
Q8VDM4	3	6.2	1.52E-09	1	2.1	4.01E-04	14	- 0.448 32685 2
Q8VDP4	0	0.0	1.00E+00	2	5.1	2.65E-06	9	2.939 09119
Q8VDW0	2	11.0	1.32E-06	0	0.0	1.00E+00	4	- 2.167 29176 2
Q8VEK3	2	4.6	1.32E-06	1	2.8	4.01E-04	22	- 0.572 25785 7
Q8VHP7	2	11.3	3.24E-06	2	10.2	4.01E-04	20	- 0.885 47143 6

								- 2.418 86030 7
Q8VIJ6	2	7.9	3.50E-06	0	0.0	1.00 E+00	5	
Q91V92	3	4.6	1.52E-09	8	11.4	6.70 E-28	65	0.927 92933 1
Q91VI7	2	7.5	2.29E-05	3	12.3	7.44 E-09	6	0.595 14620 1
Q91YP3	1	3.8	1.15E-03	3	15.4	2.01 E-09	19	0.878 30903 4
Q91Z50	1	5.3	1.15E-03	1	2.9	4.01 E-04	4	- 1.014 38265 4
Q921G6	0	0.0	1.00E+00	3	7.8	1.36 E-10	6	2.439 43663 9
Q921I1	8	17.5	7.17E-24	4	9.0	2.59 E-14	141	- 0.689 00513 5
Q921M3	3	5.8	1.83E-09	0	0.0	1.00 E+00	6	- 2.633 01489 1
Q922B2	2	6.0	1.59E-06	4	10.4	6.92 E-14	9	1.247 30643 5
Q922D8	1	1.6	1.15E-03	3	4.3	6.45 E-11	6	1.377 25730 6
Q922U2	2	3.8	6.49E-06	1	2.1	4.01 E-04	47	0.555 56499 2
Q93092	2	7.7	5.28E-06	4	14.5	1.01 E-13	59	0.621 82106 5
Q99J77	1	8.9	5.03E-03	2	12.8	1.61 E-07	17	0.660 07316 5
Q99J14	1	4.4	1.15E-03	3	10.3	2.71 E-10	18	- 0.096 80072
Q99J16	2	12.5	1.32E-06	1	6.5	4.01 E-04	19	- 0.231 13159 2

Q99JY9	12	50.0	1.14E-34	10	42.8	1.45E-34	365	0.147 73236 4
Q99K48	3	25.1	3.57E-09	3	25.1	1.51E-09	47	- 0.749 22249 7
Q99KE1	3	12.4	1.25E-08	5	12.7	3.39E-16	24	1.599 59071 6
Q99KI0	0	0.0	1.00E+00	2	4.1	4.92E-07	4	1.973 71737 5
Q99KK2	2	14.5	9.58E-06	1	6.0	2.57E-03	5	- 1.319 26701 2
Q99KP6	2	8.1	4.62E-06	1	3.3	4.01E-04	18	0.185 65663 9
Q99LB4	0	0.0	1.00E+00	2	7.7	8.96E-05	2	1.281 78398 1
Q99LC5	0	0.0	1.00E+00	2	12.9	1.61E-07	2	1.281 78398 1
Q99MK8	2	5.7	5.34E-06	3	5.4	1.28E-10	8	1.060 86546 4
Q99NB9	2	2.8	2.74E-05	0	0.0	1.00E+00	5	- 2.418 86030 7
Q99PV0	4	3.6	7.84E-11	1	1.0	1.62E-03	8	- 1.971 43304 4
Q9CQV8	5	32.8	1.35E-14	4	32.0	2.14E-13	35	- 1.140 69343 9
Q9CVB6	3	17.7	8.96E-09	4	24.3	1.89E-12	91	0.181 98886 7
Q9CW03	2	2.5	1.07E-05	0	0.0	1.00E+00	2	- 1.475 35450 4
Q9CWJ9	3	11.0	6.21E-09	5	14.9	1.49E-15	54	1.471 96098

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Q9CZN7	1	7.5	4.59E-03	2	6.0	3.72E-06	6	0.595 14620 1
Q9CZU6	3	18.3	2.13E-09	4	16.6	2.59E-14	42	- 0.621 47544 5
Q9D0I9	1	2.4	1.15E-03	3	7.7	2.32E-09	4	0.820 80826 7
Q9D154	3	16.1	1.52E-09	8	33.5	1.20E-23	46	2.190 61727 7
Q9D2V7	7	19.8	3.99E-20	10	21.4	3.79E-30	92	0.460 04010 5
Q9D8N0	5	18.5	2.01E-15	8	27.0	9.15E-26	80	1.003 53995 9
Q9D906	1	4.3	1.15E-03	2	3.7	1.61E-07	13	0.868 58275 7
Q9DBG3	5	9.0	1.59E-06	4	7.8	2.16E-07	26	- 0.299 68296 7
Q9DBJ1	5	32.7	5.31E-15	6	28.3	1.19E-18	93	- 0.127 11669 7
Q9DCD0	8	23.8	7.17E-24	10	32.3	9.11E-33	219	0.151 66821 5
Q9DCH4	2	10.0	1.32E-06	1	5.3	4.01E-04	33	- 0.341 3204
Q9EPU0	0	0.0	1.00E+00	3	4.6	3.22E-09	4	1.973 71737 5
Q9EQH3	5	12.8	6.03E-14	7	12.7	3.67E-23	32	1.573 55587 3
Q9EQK5	12	25.2	2.65E-34	11	22.1	6.58E-33	113	0.028 23837 8
Q9ERK4	3	5.7	2.49E-08	2	3.0	1.61E-07	15	0.751 34315 2



Q9ESX5	1	3.9	2.70E-03	1	3.1	4.01E-04	5	- 0.483 84009 3
Q9ET01	25	38.6	2.02E-72	30	44.9	1.11E-94	602	0.172 32162 5
Q9JHK5	2	8.0	1.32E-06	1	4.0	4.01E-04	16	- 1.067 82532 4
Q9JHU4	13	5.5	1.73E-37	12	5.0	1.89E-38	79	- 0.634 46678 2
Q9JIF0	1	3.5	1.15E-03	2	8.7	1.61E-07	6	1.377 25730 6
Q9JIF7	2	4.2	3.11E-06	3	5.6	2.28E-10	12	0.303 80063 2
Q9JJ28	3	5.4	4.02E-09	3	4.0	6.45E-11	11	0.117 35966 1
Q9JKF1	21	20.5	4.14E-61	16	16.5	2.40E-53	164	- 0.623 43606 8
Q9JKR6	2	4.0	3.11E-06	2	4.0	6.98E-07	8	- 0.096 79105 8
Q9JL26	2	3.0	1.32E-06	0	0.0	1.00E+00	3	- 1.862 40740 4
Q9JM76	5	34.3	3.01E-14	3	24.7	6.45E-11	37	- 0.023 72680 2
Q9QUI0	3	24.9	2.59E-08	3	24.9	3.38E-09	20	0.421 77969 5
Q9QUM0	7	12.6	7.51E-21	0	0.0	1.00E+00	24	- 4.433 78149 4
Q9QUM9	4	17.9	1.75E-12	4	17.9	2.59E-14	41	0.102 57232

Q9QWK 4	2	6.5	1.04E- 05	0	0.0	1.00 E+00	9	- 3.132 67523 9
Q9QXK3	4	9.3	1.18E- 11	3	6.0	8.56 E-09	8	- 0.653 24202 9
Q9QZD9	2	5.5	1.32E- 06	0	0.0	1.00 E+00	2	- 1.475 35450 4
Q9QZQ8	20	38.5	1.92E- 20	21	48.2	1.14 E-29	4493	0.191 86854 5
Q9R062	4	24.6	1.03E- 11	6	23.1	3.38 E-19	43	0.615 14665 6
Q9R0N0	2	12.0	1.59E- 06	2	9.5	1.61 E-07	11	0.555 36917 7
Q9R0P5	3	26.1	8.48E- 09	3	29.1	6.45 E-11	18	0.773 31162 4
Q9R111	5	20.9	8.50E- 15	2	5.3	1.61 E-07	22	0.378 64868 5
Q9R1P0	3	21.5	2.63E- 09	1	8.4	4.01 E-04	40	- 0.646 73957 4
Q9R1P3	3	21.4	1.52E- 09	3	21.4	8.32 E-10	45	- 1.177 80671 2
Q9R1P4	2	8.7	1.32E- 06	2	9.9	1.61 E-07	8	- 0.653 24202 9
Q9WU78	10	22.2	8.19E- 29	9	17.2	1.42 E-28	68	- 0.763 75422 3
Q9WUA 2	1	2.2	9.94E- 03	2	4.2	3.20 E-07	3	0.433 75729 9
Q9WUM 3	2	10.7	2.01E- 05	1	2.3	7.98 E-04	8	- 1.971 43304 4

								- 0.078 10296 3
Q9WV32	8	31.7	3.05E- 24	9	36.3	5.35 E-31	151	
								- 1.014 38265 4
Q9WVJ2	3	12.2	2.09E- 08	1	3.5	5.17 E-03	4	
								- 0.278 71283 8
Q9WVK 4	7	26.6	1.63E- 19	6	18.7	8.28 E-21	77	
								1.973 71737 5
Q9Z0N1	0	0.0	1.00E+ 00	2	6.8	1.91 E-06	4	
								0.820 80826 7
Q9Z0P5	1	4.9	4.69E- 03	2	9.8	5.67 E-07	4	
								- 1.219 55738 2
Q9Z126	4	46.7	1.75E- 12	3	38.1	6.45 E-11	65	
								- 1.971 43304 4
Q9Z183	4	11.7	5.07E- 12	1	2.6	4.01 E-04	8	
								0.420 17365 2
Q9Z1E4	3	9.5	1.52E- 09	4	11.4	2.59 E-14	37	
								1.056 75388 5
Q9Z1Q5	5	39.0	1.30E- 14	8	36.1	1.55 E-27	66	
								2.025 52825 2
Q9Z1Q9	4	7.1	1.19E- 10	10	16.5	6.13 E-32	47	
								0.595 14620 1
Q9Z2L7	1	7.0	1.38E- 03	2	11.0	4.49 E-07	6	
								- 1.154 42979 2
Q9Z2U0	5	30.6	5.31E- 15	3	19.0	1.90 E-09	26	
								- 0.448 32685 2
Q9Z2U1	2	15.8	1.32E- 06	3	24.9	1.14 E-09	14	

Protein	Total: Distinct Peptides	Total: % Coverage	Total: Estimated FDR	Description
A1BN54	15	24.6	6.43E-24	Alpha actinin 1a
A2AF47	2	1.8	5.40E-06	Dedicator of cytokinesis protein 11
A2AN08	2	0.7	9.00E-06	E3 ubiquitin-protein ligase UBR4
A2AQ07	9	32.4	2.82E-20	Tubulin beta-1 chain
A6ZI44	12	39.2	1.14E-39	Fructose-bisphosphate aldolase
D3Z6Q9	3	12.5	1.77E-08	Bridging integrator 2
D3Z7R7	2	3.0	3.26E-06	Minor histocompatibility protein HA-1
E9PV24	7	10.1	1.37E-23	Protein Fga
E9Q0F0	2	1.3	7.94E-07	Protein Krt78
E9Q0K6	15	36.7	2.35E-47	Deoxynucleoside triphosphate triphosphohydrolase SAMHD1
E9Q133	9	32.9	1.08E-29	T-complex protein 1 subunit gamma
E9Q604	8	11.4	3.96E-25	Integrin alpha-M
E9QQ35	2	2.1	1.01E-06	Phosphatidylinositol 4-phosphate 3-kinase C2 domain-containing subunit gamma
F6W687	5	43.8	6.12E-15	Non-histone chromosomal protein HMG-17 (Fragment)
F6YXS3	2	19.6	1.61E-07	NEDD8-conjugating enzyme Ubc12 (Fragment)
F8WGL3	4	28.2	3.99E-12	Cofilin-1
F8WGM5	3	8.9	3.68E-10	Syntaxin-binding protein 2 (Fragment)
F8WH69	9	28.2	2.27E-28	Neutrophil cytosol factor 1
F8WI35	29	65.2	6.02E-12	Histone H3
G3UZJ4	2	23.5	1.61E-	Peroxiredoxin-6

			07	
G5E8N7	7	29.3	8.24E-24	Bifunctional polynucleotide phosphatase/kinase
H3BKH6	7	45.1	1.12E-22	S-formylglutathione hydrolase
O08692	17	68.9	4.41E-51	Myeloid bactenecin (F1)
O08756	3	22.2	7.68E-10	3-hydroxyacyl-CoA dehydrogenase type-2
O08810	2	4.5	3.40E-07	116 kDa U5 small nuclear ribonucleoprotein component
O08992	3	20.7	3.84E-09	Syntenin-1
O09061	3	18.8	1.85E-10	Proteasome subunit beta type-1
O35286	4	5.9	2.13E-13	Putative pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15
O35350	5	11.5	1.84E-16	Calpain-1 catalytic subunit
O35598	2	4.1	4.61E-07	Disintegrin and metalloproteinase domain-containing protein 10
O35639	4	17.3	2.13E-13	Annexin A3
O35643	5	11.2	5.30E-06	AP-1 complex subunit beta-1
O35744	7	26.1	1.12E-21	Chitinase-3-like protein 3
O54890	2	5.5	1.32E-06	Integrin beta-3
O55029	3	5.7	6.45E-11	Coatomer subunit beta'
O55222	2	7.7	2.04E-06	Integrin-linked protein kinase
O70133	4	5.9	3.31E-13	ATP-dependent RNA helicase A
O70138	3	9.2	1.85E-10	Neutrophil collagenase
O70145	10	32.2	7.99E-33	Neutrophil cytosol factor 2
O70165	3	16.2	1.52E-09	Ficolin-1
O88342	8	22.3	2.72E-25	WD repeat-containing protein 1
O88593	2	25.8	1.61E-	Peptidoglycan recognition protein

			07	1
O88685	3	9.3	6.45E-11	26S protease regulatory subunit 6A
O88844	5	13.3	5.15E-14	Isocitrate dehydrogenase [NADP] cytoplasmic
O89053	20	60.7	1.74E-62	Coronin-1A
P01027	19	55.9	1.03E-59	Complement C3
P01325	2	14.8	1.61E-07	Insulin-1
P01794	2	23.6	9.58E-06	Ig heavy chain V region HPCG14
P01837	2	45.3	1.32E-06	Ig kappa chain C region
P01872	10	29.1	6.94E-32	Ig mu chain C region secreted form
P01942	15	84.5	2.99E-47	Hemoglobin subunit alpha
P02088	15	79.6	1.11E-28	Hemoglobin subunit beta-1
P02089	10	78.2	1.71E-12	Hemoglobin subunit beta-2
P02301	29	65.4	1.62E-09	Histone H3.3C
P04104	3	3.8	4.35E-10	Keratin, type II cytoskeletal 1
P04919	11	24.5	3.19E-32	Band 3 anion transport protein
P06151	10	38.6	3.27E-32	L-lactate dehydrogenase A chain
P06745	12	28.3	1.26E-39	Glucose-6-phosphate isomerase
P06800	4	6.9	3.57E-11	Receptor-type tyrosine-protein phosphatase C
P07356	7	34.8	6.38E-23	Annexin A2
P07724	16	35.4	2.74E-49	Serum albumin
P07742	2	4.8	2.95E-06	Ribonucleoside-diphosphate reductase large subunit
P07901	5	9.4	6.45E-11	Heat shock protein HSP 90-alpha
P08030	5	38.3	1.04E-17	Adenine phosphoribosyltransferase

P08032	17	14.2	1.00E-47	Spectrin alpha chain, erythrocytic 1
P08071	54	72.0	3.78E-175	Lactotransferrin
P08226	5	24.8	2.01E-15	Apolipoprotein E
P08249	5	20.7	8.53E-17	Malate dehydrogenase, mitochondrial
P08607	3	10.0	1.52E-09	C4b-binding protein
P08730	2	6.8	6.30E-07	Keratin, type I cytoskeletal 13
P08905	3	31.1	1.85E-10	Lysozyme C-2
P09411	12	47.0	2.01E-40	Phosphoglycerate kinase 1
P09528	4	28.0	1.39E-12	Ferritin heavy chain
P0C0S6	14	56.3	6.15E-34	Histone H2A.Z
P10107	15	46.5	2.58E-49	Annexin A1
P10126	13	40.7	1.51E-39	Elongation factor 1-alpha 1
P10605	2	9.1	1.32E-06	Cathepsin B
P10854	25	74.6	1.48E-12	Histone H2B type 1-M
P11247	28	40.4	3.59E-93	Myeloperoxidase
P11276	66	40.7	3.81E-200	Fibronectin
P11352	5	39.3	5.73E-16	Glutathione peroxidase 1
P11499	5	10.2	2.23E-10	Heat shock protein HSP 90-beta
P11672	2	17.0	4.61E-07	Neutrophil gelatinase-associated lipocalin
P11835	14	25.7	3.32E-41	Integrin beta-2
P11983	8	20.3	2.64E-26	T-complex protein 1 subunit alpha
P12815	3	35.1	1.41E-08	Programmed cell death protein 6
P12970	2	7.9	6.30E-	60S ribosomal protein L7a

			07	
P13020	3	4.8	1.25E-09	Gelsolin
P14069	3	32.6	6.45E-11	Protein S100-A6
P14131	3	21.9	5.30E-10	40S ribosomal protein S16
P14152	2	11.1	4.43E-06	Malate dehydrogenase, cytoplasmic
P14206	4	25.4	3.47E-12	40S ribosomal protein SA
P14685	4	14.3	6.03E-12	26S proteasome non-ATPase regulatory subunit 3
P14824	3	6.4	1.03E-08	Annexin A6
P14869	3	20.5	1.38E-09	60S acidic ribosomal protein P0
P15864	23	37.7	3.25E-23	Histone H1.2
P16546	3	2.7	1.07E-07	Spectrin alpha chain, non-erythrocytic 1
P16858	20	74.5	1.21E-64	Glyceraldehyde-3-phosphate dehydrogenase
P17182	20	50.7	1.64E-64	Alpha-enolase
P17225	2	8.3	4.17E-05	Polypyrimidine tract-binding protein 1
P17427	6	11.2	8.15E-19	AP-2 complex subunit alpha-2
P17742	6	52.4	3.42E-20	Peptidyl-prolyl cis-trans isomerase A
P17751	4	25.4	8.12E-12	Triosephosphate isomerase
P19096	12	8.9	2.52E-39	Fatty acid synthase
P20029	10	19.2	3.75E-21	78 kDa glucose-regulated protein
P20152	4	12.2	6.09E-13	Vimentin
P21107	2	12.5	6.19E-07	Tropomyosin alpha-3 chain
P22752	50	70.8	9.03E-62	Histone H2A type 1
P24270	6	15.2	3.42E-20	Catalase



P25911	5	14.7	1.04E-17	Tyrosine-protein kinase Lyn
P26039	43	30.9	1.02E-131	Talin-1
P26040	8	11.4	5.30E-10	Ezrin
P26041	12	20.1	3.42E-22	Moesin
P26443	3	8.8	1.89E-09	Glutamate dehydrogenase 1, mitochondrial
P26516	2	14.0	4.61E-07	26S proteasome non-ATPase regulatory subunit 7
P26638	3	11.5	2.07E-10	Serine--tRNA ligase, cytoplasmic
P27005	7	92.1	1.97E-22	Protein S100-A8
P27773	3	6.9	3.68E-10	Protein disulfide-isomerase A3
P27870	2	3.7	2.06E-06	Proto-oncogene vav
P28293	13	50.2	3.48E-42	Cathepsin G
P28352	2	6.3	3.27E-05	DNA-(apurinic or apyrimidinic site) lyase
P28650	7	20.6	1.37E-23	Adenylosuccinate synthetase isozyme 1
P28798	5	12.6	1.72E-15	Granulins
P29351	14	41.0	7.11E-46	Tyrosine-protein phosphatase non-receptor type 6
P29391	6	48.6	7.11E-19	Ferritin light chain 1
P29788	4	10.9	4.11E-12	Vitronectin
P30681	10	38.1	3.68E-24	High mobility group protein B2
P31230	2	9.4	4.03E-06	Aminoacyl tRNA synthase complex-interacting multifunctional protein 1
P31725	23	79.6	1.40E-73	Protein S100-A9
P34884	2	26.1	5.16E-07	Macrophage migration inhibitory factor
P35441	20	24.8	1.21E-60	Thrombospondin-1

P35700	2	16.6	8.46E-06	Peroxiredoxin-1
P39054	9	16.4	4.13E-27	Dynamin-2
P39654	2	5.7	2.62E-05	Arachidonate 12-lipoxygenase, leukocyte-type
P39655	3	7.2	3.87E-08	Arachidonate 12-lipoxygenase, 12S-type
P40124	12	39.0	1.72E-39	Adenylyl cyclase-associated protein 1
P40142	20	40.0	8.69E-63	Transketolase
P40201	2	1.5	2.05E-05	Chromodomain-helicase-DNA-binding protein 1
P41245	16	32.5	1.48E-48	Matrix metalloproteinase-9
P42227	3	10.4	8.64E-10	Signal transducer and activator of transcription 3
P42932	6	14.6	2.81E-20	T-complex protein 1 subunit theta
P43274	22	38.8	2.59E-14	Histone H1.4
P43275	9	22.1	7.42E-14	Histone H1.1
P43276	12	33.2	4.27E-19	Histone H1.5
P45376	2	10.1	6.30E-07	Aldose reductase
P47738	16	46.4	1.67E-50	Aldehyde dehydrogenase, mitochondrial
P47753	5	28.0	5.63E-13	F-actin-capping protein subunit alpha-1
P47754	4	21.3	5.30E-10	F-actin-capping protein subunit alpha-2
P47757	6	34.2	1.39E-20	F-actin-capping protein subunit beta
P47791	3	10.1	1.52E-09	Glutathione reductase, mitochondrial
P47911	3	13.5	2.15E-10	60S ribosomal protein L6
P47955	2	51.8	1.07E-05	60S acidic ribosomal protein P1
P47962	2	8.1	5.55E-07	60S ribosomal protein L5
P48025	2	5.4	1.08E-	Tyrosine-protein kinase SYK

			06	
P49722	4	23.5	1.75E-12	Proteasome subunit alpha type-2
P50247	5	16.2	2.50E-16	Adenosylhomocysteinase
P50516	2	8.1	1.61E-07	V-type proton ATPase catalytic subunit A
P50543	2	27.6	6.47E-07	Protein S100-A11
P51150	2	11.1	3.79E-06	Ras-related protein Rab-7a
P51174	2	7.2	7.53E-06	Long-chain specific acyl-CoA dehydrogenase, mitochondrial
P51437	3	35.8	4.35E-10	Cathelin-related antimicrobial peptide
P52480	39	60.3	9.43E-126	Pyruvate kinase PKM
P54071	6	16.2	5.44E-16	Isocitrate dehydrogenase [NADP], mitochondrial
P54775	2	15.8	1.08E-06	26S protease regulatory subunit 6B
P56480	3	9.1	6.33E-10	ATP synthase subunit beta, mitochondrial
P57780	9	13.0	6.19E-07	Alpha-actinin-4
P58252	17	31.8	1.21E-56	Elongation factor 2
P59999	3	17.9	7.49E-11	Actin-related protein 2/3 complex subunit 4
P60122	2	6.6	1.61E-07	RuvB-like 1
P60229	2	5.6	3.26E-05	Eukaryotic translation initiation factor 3 subunit E
P60335	3	12.1	6.45E-11	Poly(rC)-binding protein 1
P60710	66	90.1	1.99E-80	Actin, cytoplasmic 1
P60766	5	42.9	1.72E-13	Cell division control protein 42 homolog
P60843	6	21.9	2.81E-19	Eukaryotic initiation factor 4A-I
P61161	12	38.6	7.79E-38	Actin-related protein 2
P61164	5	22.3	1.04E-	Alpha-centractin

			17	
P61979	3	14.8	7.13E-09	Heterogeneous nuclear ribonucleoprotein K
P62137	5	26.1	1.17E-15	Serine/threonine-protein phosphatase PP1-alpha catalytic subunit
P62196	2	8.6	3.50E-06	26S protease regulatory subunit 8
P62242	4	24.5	1.46E-11	40S ribosomal protein S8
P62259	3	20.8	1.44E-09	14-3-3 protein epsilon
P62281	2	17.7	3.40E-07	40S ribosomal protein S11
P62315	2	27.7	1.61E-07	Small nuclear ribonucleoprotein Sm D1
P62317	2	16.1	1.86E-06	Small nuclear ribonucleoprotein Sm D2
P62320	3	31.7	3.90E-10	Small nuclear ribonucleoprotein Sm D3
P62334	3	10.5	6.45E-11	26S protease regulatory subunit 10B
P62702	3	8.7	1.85E-10	40S ribosomal protein S4, X isoform
P62806	40	65.0	1.85E-127	Histone H4
P62814	4	11.9	7.42E-14	V-type proton ATPase subunit B, brain isoform
P62827	11	41.7	9.23E-34	GTP-binding nuclear protein Ran
P62855	2	23.5	9.18E-06	40S ribosomal protein S26
P62880	3	12.6	1.85E-10	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2
P62889	3	40.9	6.45E-11	60S ribosomal protein L30
P62908	4	21.0	9.95E-14	40S ribosomal protein S3
P62962	5	37.1	4.76E-15	Profilin-1
P63005	2	8.2	6.30E-07	Platelet-activating factor acetylhydrolase IB subunit alpha
P63017	19	40.7	2.11E-54	Heat shock cognate 71 kDa protein
P63085	6	15.9	1.97E-	Mitogen-activated protein kinase 1

			10	
P63101	6	31.4	1.91E-19	14-3-3 protein zeta/delta
P63158	4	21.9	7.97E-07	High mobility group protein B1
P63330	4	24.3	7.42E-14	Serine/threonine-protein phosphatase 2A catalytic subunit alpha isoform
P68033	46	68.2	2.06E-35	Actin, alpha cardiac muscle 1
P68040	7	37.2	1.84E-23	Guanine nucleotide-binding protein subunit beta-2-like 1
P68254	2	21.8	9.12E-06	14-3-3 protein theta
P68368	12	36.2	5.00E-13	Tubulin alpha-4A chain
P68372	13	45.2	4.61E-07	Tubulin beta-4B chain
P68373	13	38.3	2.44E-16	Tubulin alpha-1C chain
P68433	28	65.4	8.73E-10	Histone H3.1
P68510	2	17.9	1.61E-07	14-3-3 protein eta
P70168	7	15.5	9.37E-22	Importin subunit beta-1
P70335	2	2.7	6.30E-07	Rho-associated protein kinase 1
P70390	40	13.8	3.17E-109	Short stature homeobox protein 2
P70460	6	21.9	7.86E-19	Vasodilator-stimulated phosphoprotein
P80313	4	11.8	5.04E-13	T-complex protein 1 subunit eta
P80314	2	7.1	1.32E-06	T-complex protein 1 subunit beta
P80315	6	17.8	1.19E-20	T-complex protein 1 subunit delta
P80316	5	19.0	3.58E-17	T-complex protein 1 subunit epsilon
P80317	2	7.0	1.61E-07	T-complex protein 1 subunit zeta
P82198	6	17.6	2.82E-17	Transforming growth factor-beta-induced protein ig-h3
P84078	3	21.5	3.55E-	ADP-ribosylation factor 1

			10	
P84091	2	9.9	3.40E-07	AP-2 complex subunit mu
P84096	8	53.4	2.78E-21	Rho-related GTP-binding protein RhoG
P84228	33	65.4	2.08E-21	Histone H3.2
P97369	5	23.6	1.04E-17	Neutrophil cytosol factor 4
P97384	7	17.5	1.44E-23	Annexin A11
P99024	14	49.1	1.85E-10	Tubulin beta-5 chain
Q00519	7	10.4	9.24E-23	Xanthine dehydrogenase/oxidase
Q00612	22	51.1	1.12E-71	Glucose-6-phosphate 1-dehydrogenase X
Q01853	22	41.8	7.21E-67	Transitional endoplasmic reticulum ATPase
Q02053	20	28.6	7.55E-65	Ubiquitin-like modifier-activating enzyme 1
Q02357	5	62.8	2.00E-14	Ankyrin-1
Q04750	4	9.3	4.80E-12	DNA topoisomerase 1
Q05144	10	66.7	8.16E-31	Ras-related C3 botulinum toxin substrate 2
Q07076	3	8.9	7.49E-11	Annexin A7
Q07797	2	6.8	3.50E-06	Galectin-3-binding protein
Q3TCJ1	2	7.5	2.50E-06	BRISC complex subunit Abro1
Q3TEA8	2	4.7	1.32E-06	Heterochromatin protein 1-binding protein 3
Q3THE2	3	23.8	1.32E-09	Myosin regulatory light chain 12B
Q3TRM8	14	20.9	2.20E-45	Hexokinase-3
Q3TXS7	2	3.8	1.94E-05	26S proteasome non-ATPase regulatory subunit 1
Q3UGX2	5	3.5	8.95E-15	Spectrin beta 1

Q3UKW 2	4	42.1	2.79E- 12	Calmodulin
Q3UP87	7	29.1	8.34E- 23	Neutrophil elastase
Q3UW5 3	4	7.3	1.20E- 12	Protein Niban
Q3UZZ 4	5	12.7	2.44E- 16	Olfactomedin-4
Q3V1G 4	2	5.1	3.34E- 06	Olfactomedin-like protein 2B
Q571I9	5	10.3	8.53E- 17	Aldehyde dehydrogenase family 16 member A1
Q5SQX 6	4	2.6	4.04E- 11	Cytoplasmic FMR1-interacting protein 2
Q5SS00	2	0.3	2.45E- 07	DBF4-type zinc finger-containing protein 2 homolog
Q5SXR 6	32	29.7	1.08E- 98	Clathrin heavy chain 1
Q60605	7	51.0	5.50E- 23	Myosin light polypeptide 6
Q60692	2	10.9	4.61E- 07	Proteasome subunit beta type-6
Q61081	2	10.0	1.61E- 07	Hsp90 co-chaperone Cdc37
Q61096	2	10.2	4.61E- 07	Myeloblastin
Q61171	5	34.8	8.20E- 15	Peroxiredoxin-2
Q61210	7	12.9	2.44E- 22	Rho guanine nucleotide exchange factor 1
Q61233	22	49.6	1.41E- 72	Plastin-2
Q61316	2	3.6	8.46E- 07	Heat shock 70 kDa protein 4
Q61508	2	6.5	1.81E- 06	Extracellular matrix protein 1
Q61598	4	18.1	2.77E- 13	Rab GDP dissociation inhibitor beta
Q61599	2	23.5	1.61E- 07	Rho GDP-dissociation inhibitor 2
Q61646	7	33.1	2.94E- 20	Haptoglobin
Q61656	3	6.4	5.30E- 10	Probable ATP-dependent RNA helicase DDX5

Q61753	2	6.4	1.12E-06	D-3-phosphoglycerate dehydrogenase
Q61990	2	8.3	3.20E-07	Poly(rC)-binding protein 2
Q62465	3	6.9	2.23E-09	Synaptic vesicle membrane protein VAT-1 homolog
Q63844	9	29.2	6.58E-20	Mitogen-activated protein kinase 3
Q64514	3	5.0	1.19E-08	Tripeptidyl-peptidase 2
Q64522	43	60.8	1.33E-27	Histone H2A type 2-B
Q64727	5	9.3	1.36E-16	Vinculin
Q69ZK0	2	11.7	3.33E-05	Phosphatidylinositol 3,4,5-trisphosphate-dependent Rac exchanger 1 protein
Q6GSS7	41	70.8	6.45E-11	Histone H2A type 2-A
Q6IRU2	2	10.9	1.25E-05	Tropomyosin alpha-4 chain
Q6P069	4	27.9	4.91E-13	Sorcin
Q6P4T2	2	2.1	1.32E-06	U5 small nuclear ribonucleoprotein 200 kDa helicase
Q6P5F9	5	7.7	6.21E-16	Exportin-1
Q6P9Q4	2	2.5	3.63E-06	FH1/FH2 domain-containing protein 1
Q6PDI5	4	3.8	2.18E-11	Proteasome-associated protein ECM29 homolog
Q6PDQ2	3	2.4	1.52E-09	Chromodomain-helicase-DNA-binding protein 4
Q6PHN9	2	12.4	1.61E-07	Ras-related protein Rab-35
Q6ZQ38	5	8.2	4.25E-15	Cullin-associated NEDD8-dissociated protein 1
Q6ZQA0	4	2.8	7.92E-14	Neurobeachin-like protein 2
Q6ZWR6	7	9.9	2.29E-20	Nesprin-1
Q76MZ3	6	17.0	3.42E-20	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform



Q78PY7	3	6.0	4.27E-09	Staphylococcal nuclease domain-containing protein 1
Q80TE4	2	1.9	4.71E-05	Signal-induced proliferation-associated 1-like protein 2
Q80X41	2	11.4	1.86E-06	Serine/threonine-protein kinase VRK1
Q8BFY9	2	3.8	2.65E-06	Transportin-1
Q8BFZ3	29	49.2	1.54E-16	Beta-actin-like protein 2
Q8BG32	6	18.2	1.35E-18	26S proteasome non-ATPase regulatory subunit 11
Q8BJY1	2	6.3	1.61E-07	26S proteasome non-ATPase regulatory subunit 5
Q8BT60	3	9.0	7.49E-11	Copine-3
Q8BTM8	39	274.7	1.21E-122	Filamin-A
Q8BVQ9	3	12.0	1.85E-10	26S protease regulatory subunit 7
Q8BWT1	2	6.0	3.66E-07	3-ketoacyl-CoA thiolase, mitochondrial
Q8C147	5	13.7	2.74E-15	Dedicator of cytokinesis protein 8
Q8C2Q7	3	10.6	5.30E-10	Heterogeneous nuclear ribonucleoprotein H
Q8CC86	2	7.4	7.74E-07	Nicotinate phosphoribosyltransferase
Q8CCK0	5	12.4	1.02E-05	Core histone macro-H2A.2
Q8CG29	3	4.4	6.45E-11	Myosin IF
Q8CGP1	25	74.6	7.07E-13	Histone H2B type 1-K
Q8CGP4	25	58.9	3.76E-13	Histone H2A
Q8CIE6	5	5.1	2.07E-17	Coatomer subunit alpha
Q8CIH5	11	13.4	2.35E-34	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma-2
Q8CIZ8	9	36.6	1.41E-26	von Willebrand factor

Q8JZQ9	2	5.1	1.22E-06	Eukaryotic translation initiation factor 3 subunit B
Q8K0E8	14	39.1	2.62E-45	Fibrinogen beta chain
Q8K1B8	19	40.6	3.07E-60	Fermitin family homolog 3
Q8K1X4	3	5.4	2.13E-09	NCK associated protein 1 like
Q8K426	2	23.1	1.61E-07	Myeloid cysteine-rich protein
Q8K482	4	5.8	8.86E-12	EMILIN-2
Q8QZY1	4	11.7	7.42E-14	Eukaryotic translation initiation factor 3 subunit L
Q8R010	2	12.8	9.17E-07	Aminoacyl tRNA synthase complex-interacting multifunctional protein 2
Q8R081	2	7.7	1.61E-07	Heterogeneous nuclear ribonucleoprotein L
Q8R1B4	3	2.9	1.06E-09	Eukaryotic translation initiation factor 3 subunit C
Q8R1Q8	3	12.6	2.73E-09	Cytoplasmic dynein 1 light intermediate chain 1
Q8R2S8	11	22.4	7.06E-37	CD177 antigen
Q8VCI0	2	6.0	2.89E-05	Phospholipase B-like 1
Q8VCM7	12	40.8	6.15E-39	Fibrinogen gamma chain
Q8VCT3	10	20.6	1.94E-32	Aminopeptidase B
Q8VDD5	55	35.2	2.68E-168	Myosin-9
Q8VDM4	3	6.2	5.30E-10	26S proteasome non-ATPase regulatory subunit 2
Q8VDP4	2	5.1	2.65E-06	DBIRD complex subunit KIAA1967 homolog
Q8VDW0	2	11.0	1.32E-06	ATP-dependent RNA helicase DDX39A
Q8VEK3	2	4.6	4.61E-07	Heterogeneous nuclear ribonucleoprotein U
Q8VHP7	3	13.9	1.13E-06	Leukocyte elastase inhibitor B

Q8VIJ6	2	7.9	3.50E-06	Splicing factor, proline- and glutamine-rich
Q91V92	8	11.4	6.70E-28	ATP-citrate synthase
Q91VI7	3	12.3	5.10E-09	Ribonuclease inhibitor
Q91YP3	3	15.4	2.01E-09	Putative deoxyribose-phosphate aldolase
Q91Z50	2	8.2	4.61E-07	Flap endonuclease 1
Q921G6	3	7.8	1.36E-10	Leucine-rich repeat and calponin homology domain-containing protein 4
Q921I1	9	20.1	1.22E-28	Serotransferrin
Q921M3	3	5.8	1.83E-09	Splicing factor 3B subunit 3
Q922B2	4	10.4	6.92E-14	Aspartate--tRNA ligase, cytoplasmic
Q922D8	4	5.9	7.42E-14	C-1-tetrahydrofolate synthase, cytoplasmic
Q922U2	2	3.8	2.27E-06	Keratin, type II cytoskeletal 5
Q93092	4	14.5	1.01E-13	Transaldolase
Q99J77	2	12.8	1.61E-07	N-acetylneuraminic acid synthase (Sialic acid synthase)
Q99JI4	3	10.3	2.71E-10	26S proteasome non-ATPase regulatory subunit 6
Q99JI6	2	12.5	4.61E-07	Ras-related protein Rap-1b
Q99JY9	13	51.9	1.66E-42	Actin-related protein 3
Q99K48	3	25.1	5.05E-10	Non-POU domain-containing octamer-binding protein
Q99KE1	7	20.7	2.61E-21	NAD-dependent malic enzyme, mitochondrial
Q99KI0	2	4.1	4.92E-07	Aconitate hydratase, mitochondrial
Q99KK2	2	14.5	9.58E-06	N-acylneuraminic acid cytidyltransferase
Q99KP6	2	8.1	1.61E-06	Pre-mRNA-processing factor 19

Q99LB4	2	7.7	8.96E-05	Capping protein (Actin filament), gelsolin-like
Q99LC5	2	12.9	1.61E-07	Electron transfer flavoprotein subunit alpha, mitochondrial
Q99MK8	5	11.0	6.85E-16	Beta-adrenergic receptor kinase 1
Q99NB9	2	2.8	2.74E-05	Splicing factor 3B subunit 1
Q99PV0	4	3.6	2.76E-11	Pre-mRNA-processing-splicing factor 8
Q9CQV8	5	32.8	2.17E-16	14-3-3 protein beta/alpha
Q9CVB6	4	24.3	1.46E-12	Actin-related protein 2/3 complex subunit 2
Q9CW03	2	2.5	1.07E-05	Structural maintenance of chromosomes protein 3
Q9CWJ9	5	14.9	1.49E-15	Bifunctional purine biosynthesis protein PURH
Q9CZN7	3	13.5	1.71E-08	Serine hydroxymethyltransferase
Q9CZU6	5	23.1	2.98E-17	Citrate synthase, mitochondrial
Q9D0I9	3	7.7	1.21E-09	Arginine--tRNA ligase, cytoplasmic
Q9D154	9	36.9	1.38E-26	Leukocyte elastase inhibitor A
Q9D2V7	11	24.3	7.19E-34	Coronin-7
Q9D8N0	9	30.2	8.55E-29	Elongation factor 1-gamma
Q9D906	3	8.0	1.85E-10	Ubiquitin-like modifier-activating enzyme ATG7
Q9DBG3	5	9.0	2.16E-07	AP-2 complex subunit beta
Q9DBJ1	7	32.7	6.66E-22	Phosphoglycerate mutase 1
Q9DCD0	13	38.9	3.25E-41	6-phosphogluconate dehydrogenase, decarboxylating
Q9DCH4	2	10.0	4.61E-07	Eukaryotic translation initiation factor 3 subunit F
Q9EPU0	3	4.6	3.22E-09	Regulator of nonsense transcripts 1
Q9EQH3	8	16.5	7.97E-26	Vacuolar protein sorting-associated protein 35

Q9EQK5	15	28.3	1.36E-44	Major vault protein
Q9ERK4	3	5.7	4.35E-10	Exportin-2
Q9ESX5	2	7.1	1.08E-06	H/ACA ribonucleoprotein complex subunit 4
Q9ET01	32	46.4	2.49E-101	Glycogen phosphorylase, liver form
Q9JHK5	2	8.0	4.61E-07	Pleckstrin
Q9JHU4	22	8.4	1.53E-66	Cytoplasmic dynein 1 heavy chain 1
Q9JIF0	3	12.2	1.85E-10	Protein arginine N-methyltransferase 1
Q9JIF7	4	7.6	5.00E-13	Coatomer subunit beta
Q9JJ28	5	8.2	2.26E-16	Protein flightless-1 homolog
Q9JKF1	24	22.9	1.92E-77	Ras GTPase-activating-like protein IQGAP1
Q9JKR6	2	4.0	6.98E-07	Hypoxia up-regulated protein 1
Q9JL26	2	3.0	1.32E-06	Formin-like protein 1
Q9JM76	5	34.3	5.03E-16	Actin-related protein 2/3 complex subunit 3
Q9QUI0	3	24.9	1.07E-09	Transforming protein RhoA
Q9QUM0	7	12.6	7.51E-21	Integrin alpha-IIb
Q9QUM9	4	17.9	2.59E-14	Proteasome subunit alpha type-6
Q9QWK4	2	6.5	1.04E-05	CD5 antigen-like
Q9QXK3	4	9.3	1.03E-12	Coatomer subunit gamma-2
Q9QZD9	2	5.5	1.32E-06	Eukaryotic translation initiation factor 3 subunit I
Q9QZQ8	23	48.8	9.49E-32	Core histone macro-H2A.1
Q9R062	7	34.8	2.29E-21	Glycogenin-1
Q9R0N0	3	15.3	2.23E-10	Galactokinase

Q9R0P5	4	32.7	7.42E-14	Dextrin
Q9R111	5	20.9	1.03E-15	Guanine deaminase
Q9R1P0	3	21.5	9.16E-10	Proteasome subunit alpha type-4
Q9R1P3	3	21.4	1.85E-10	Proteasome subunit beta type-2
Q9R1P4	3	14.4	1.85E-10	Proteasome subunit alpha type-1
Q9WU78	11	23.1	1.14E-34	Programmed cell death 6-interacting protein
Q9WUA2	2	4.2	3.20E-07	Phenylalanine--tRNA ligase beta subunit
Q9WUM3	3	13.0	1.61E-08	Coronin-1B
Q9WV32	9	36.3	5.35E-31	Actin-related protein 2/3 complex subunit 1B
Q9WVJ2	3	12.2	2.09E-08	26S proteasome non-ATPase regulatory subunit 13
Q9WVK4	9	28.7	6.41E-28	EH domain-containing protein 1
Q9Z0N1	2	6.8	1.91E-06	Eukaryotic translation initiation factor 2 subunit 3, X-linked
Q9Z0P5	2	9.8	5.67E-07	Twinfilin-2
Q9Z126	4	46.7	7.42E-14	Platelet factor 4
Q9Z183	4	11.7	1.77E-12	Protein-arginine deiminase type-4
Q9Z1E4	4	11.4	2.59E-14	Glycogen [starch] synthase, muscle
Q9Z1Q5	10	47.7	2.04E-33	Chloride intracellular channel protein 1
Q9Z1Q9	12	20.0	1.38E-36	Valine--tRNA ligase
Q9Z2L7	2	11.0	4.49E-07	Cytokine receptor-like factor 3
Q9Z2U0	5	30.6	1.24E-15	Proteasome subunit alpha type-7
Q9Z2U1	3	24.9	1.14E-09	Proteasome subunit alpha type-5

**Appendix Table 2:** Proteins identified in MDSC-derived exosomes that have not previously been reported in ExoCarta, EVpedia, or Vesiclepedia.

Protein	Protein Name
D3Z6Q9	Bridging integrator 2
E9Q0F0	Protein Krt78
E9QQ35	Phosphatidylinositol 4-phosphate 3-kinase C2 domain-containing subunit gamma
O70138	Neutrophil collagenase
P01794	Ig heavy chain V region HPCG14
P01872	Ig mu chain C region secreted form
P08032	Spectrin alpha chain, erythrocytic 1
Q00612	Glucose-6-phosphate 1-dehydrogenase X
Q69ZK0	Phosphatidylinositol 3,4,5-trisphosphate-dependent Rac exchanger 1 protein
Q8K426	Myeloid cysteine-rich protein
Q99MK8	Beta-adrenergic receptor kinase 1

**Appendix Table 3:** Peptide and protein identifications of ubiquitinated proteins in MDSC-derived exosomes.

Accession	Precursor m/z	Charge	Precursor or neutral mass	Mass Diff. (Da)	Peptide	Modifications	Estfdr
A2AU83	805.88 73291	2	1609.75 9008	0.0411 85869	AFSSPSGFLY HKR	K12:+1 14.043	0.0214 87603
B2RXC2	973.45	3	2917.32 6525	1.9572 93152	GTPASPRCGS PTPMETDKR VAPSLER	C8:+45. 988,M1 4:+15.9 95,K18: +114.04 3	0.0214 87603
D3Z1Z3	1111.3 8	3	3331.11 6525	4.0582 98912	KKLFKLIRK MPFIGRKVS KAKKDLVK	K21:+1 14.043, K26:+1 14.043	0.0509 75293
E0CYH9	1104.3 95264	3	3310.16 2316	5.0170 78584	KKKVSIMVS VDGVKVLK KKKKLLLLQ K	K18:+1 14.043	0.0509 75293

E9PZM7	869.24	3	2604.69 6525	5.0388 55904	RKSVRRGRK PPLLKKKLR R	K2:+11 4.043,K 9:+114. 043	0.0509 75293
E9PZM7	1105.0 38208	3	3312.09 1149	4.0528 39383	RKSVRRGRK PPLLKKKLR RSVPPAEK	K2:+11 4.043,K 15:+114 .043	0.0820 93135
E9Q5F6	894.47 29614	2	1786.93 0273	0.0102 50948	TITLEVEPSD TIENVK	-	0
E9Q5F6	894.47 15576	2	1786.92 7465	0.0074 4333	TITLEVEPSD TIENVK	-	0
E9Q5F6	748.74 05396	3	2243.19 8144	0.0070 78604	TLSDYNIQKE STLHLVLR	K9:+11 4.043	0.0018 65672
E9Q5F6	534.31 73218	2	1066.61 8993	0.0055 03843	ESTLHLVLR	-	0.0052 58545
E9Q5F6	534.31 75049	2	1066.61 936	0.0058 70054	ESTLHLVLR	-	0.0018 65672
E9Q5F6	534.31 7627	2	1066.61 9604	0.0061 14194	ESTLHLVLR	-	0.0018 65672
E9Q5F6	534.31 78101	2	1066.61 997	0.0064 80405	ESTLHLVLR	-	0.0068 61064
E9Q5F6	534.31 70166	2	1066.61 8383	0.0048 93491	ESTLHLVLR	-	0.0250 19547
E9Q5F6	534.31 70166	2	1066.61 8383	0.0048 93491	ESTLHLVLR	-	0.0052 58545
E9Q5F6	460.59 66187	3	1378.76 6381	0.0020 54045	MQIFVKTLT GK	K6:+11 4.043	0.0509 75293
E9Q5F6	894.47 29614	2	1786.93 0273	0.0102 50948	TITLEVEPSD TIENVK	-	0.0509 75293
E9Q5F6	541.28 10669	2	1080.54 6484	0.0013 48413	TLSDYNIQK	-	0.0509 75293
E9Q5F6	748.74 05396	3	2243.19 8144	0.0070 78604	TLSDYNIQK ESTLHLVLR	K9:+11 4.043	0.0509 75293
E9Q5F6	534.31 73218	2	1066.61 8993	0.0055 03843	ESTLHLVLR	-	0.0509 75293
E9Q6J5	984.97	3	2951.88 6525	4.0253 57984	IKEVLKERK VLEKKVALS KRRRK	K14:+1 14.043	0.0509 75293
E9Q6J5	1136.3 8	3	3406.11 6525	6.0394 91568	TLEPKAPRIK EVLKERKVL EKKVALSKR	K5:+11 4.043	0.0782 98695
F6SB18	1108.3 8208	3	3322.12 2765	4.0525 59242	KVLALPSHR GPKIRRLKER LRRIRQK	K26:+1 14.043	0.0509 75293



F6XI62	1026.3 4	3	3075.99 6525	4.0577 7624	REKKKKVAT VPGTLKKKV PAGPKTLKK	K26:+1 14.043	0.0782 98695
F6XI62	940.61 51733	3	2818.82 2045	5.0512 22244	NFAELKVKR LRKKFALKT LRKAR	-	0.0782 98695
F7DCP6	820.47 96753	2	1638.94 3701	1.9448 06506	KPVASGVKK VAKSPK	K9:+11 4.043	0.0865 61747
F7DCP6	436.77 82898	2	871.540 9295	1.0121 5983	KVAKSPK	K1:+11 4.043	0.0865 61747
G3UWJ2	983.12 69531	3	2946.35 7384	1.9953 88887	GEGPCMAES QGPEDPILDV KNKLETK	C5:+45. 988,K26 :+114.0 43	0.0293 20987
G5E861	746.64 12354	4	2982.53 3641	0.9917 79278	LQQENEQLQ KETEDLRKV ALEAQK	K24:+1 14.043	0.0423 28041
H3BKN5	894.59 52759	3	2680.76 2353	5.0448 59277	VLGRKLPKK KRVKAM KKR	K5:+11 4.043,K 18:+114 .043	0.0913 32711
H3BL88	1096.7 1	3	3287.10 6525	4.0243 93416	LLIKKKGKH KKHKSGKKS ISKKAITK	K22:+1 14.043, K27:+1 14.043	0.0782 98695
H3BL88	702.15 69824	3	2103.44 7472	5.0497 63234	KLKLTKMRA KKKKKKK	K1:+11 4.043	0.0509 75293
J3QQ16	631.98 60229	3	1892.93 4594	- 0.0177 74552	DLKIMVLML TGDMQR	K3:+11 4.043,M 13:+15. 995	0.0376 78976
P08071	931.44 83032	4	3721.76 1913	1.0246 40011	SSTRQCIQAI VTNRADAM TLDGGTMFD AGKPPYK	C6:+45. 988,M1 8:+15.9 95,M25: +15.995	0.0865 61747
P08071	596.30 59692	2	1190.59 6288	0.0060 14229	QCIQAIVTNR	C2:+45. 988	0.0865 61747
P08071	652.37 87231	2	1302.74 1796	0.0122 14001	LRPVAAEVY GTK	-	0.0865 61747
P08071	454.25 57373	4	1812.99 1649	0.0070 20947	LRPVAAEVY GTKEQPR	-	0.0865 61747
P08071	575.82 31201	2	1149.63 059	0.0123 4949	THYYAVAV VK	-	0.0865 61747

P08071	908.16 40015	3	2721.46 8529	0.0195 30859	IPIGTLRPYL NWNPPASL EEAVSK	-	0.0865 61747
P08071	781.31 89087	2	1560.62 2167	0.0022 84999	FPNLCSSCAG TGANK	C5:+45. 988,C8: +45.988	0.0865 61747
P08071	902.39 39819	2	1802.77 2314	0.0116 66787	CASSPEEPYS GYAGALR	C1:+45. 988	0.0865 61747
P08071	495.56 94885	3	1483.68 499	1.0139 46576	CLRDNAGDV AFTR	C1:+45. 988	0.0865 61747
P08071	610.81 50635	2	1219.61 4477	0.0060 12929	GSTVFEELPN K	-	0.0865 61747
P08071	618.30 57861	3	1851.89 3883	0.0036 78606	LLCPDNTWK PVTEYK	C3:+45. 988	0.0865 61747
P08071	560.28 26538	3	1677.82 4486	0.0162 80082	ECHLAQVPS HAVVSR	C2:+45. 988	0.0865 61747
P08071	776.89 64233	2	1551.77 7197	0.0090 43888	QASGFQLFA SPSGQK	-	0.0865 61747
P08071	439.74 28589	2	877.470 0677	0.0043 05125	ESAIGFVR	-	0.0865 61747
P08071	1039.9 77051	2	2077.93 8451	0.0111 58651	VTCISFPTTE DCIVAIMK	C3:+45. 988,C12 :+45.98 8,M17:+ 15.995	0.0865 61747
P08071	658.84 19189	2	1315.66 8188	0.0050 82931	CGLVPVLAE NQK	C1:+45. 988	0.0865 61747
P08071	750.38 23853	3	2248.12 3681	0.0141 4693	SNGLDCVNR PVEGYLAVA AVR	C6:+45. 988	0.0865 61747
P08071	634.80 24902	2	1267.58 933	0.0060 18589	EDAGFTWSS LR	-	0.0865 61747
P08071	895.88 31177	2	1789.75 0585	0.0063 1668	FNEFFSQSCA PGADPK	C9:+45. 988	0.0865 61747
P08071	595.92 91992	3	1784.76 4123	0.0070 18216	SNLALCIGD EKGENK	C4:+45. 988,C7: +45.988	0.0865 61747
P08071	813.90 18555	2	1625.78 8061	0.0084 69401	LKDFELLCL DDTR	C8:+45. 988	0.0865 61747
P08071	713.69 04297	3	2138.04 7814	0.0087 59879	DFELLCLDD TRKPVTEAK	C6:+45. 988	0.0865 61747

P08071	781.76 51367	3	2342.27 1935	0.0125 28564	TDKVEVLQQ VLLDQQVQF GR	-	0.0865 61747
P08071	667.03 92456	3	1998.09 4262	0.0044 40056	VEVLQQVLL DQQVQFGR	-	0.0865 61747
P08071	675.77 52686	2	1349.53 4887	0.0066 73485	CPGEFCLFQS K	C1:+45. 988,C6: +45.988	0.0865 61747
P08071	590.96 31348	3	1769.86 5929	1.0168 61057	TKNLLFNDN TECLAK	C12:+45 .988	0.0865 61747
P08071	770.86 34644	2	1539.71 1279	0.0048 52215	NLLFNDNTE CLAK	C10:+45 .988	0.0865 61747
P08071	895.39 18457	2	1788.76 8041	0.0119 99662	QCSSSPLEA CAFLTQ	C2:+45. 988,C11 :+45.98 8	0.0865 61747
P08071	480.47 64099	4	1917.87 4339	0.0328 12016	GDADAMSL DGGYIYTAG K	K18:+1 14.043	0.0865 61747
P08113	740.52 96631	2	1479.04 3676	0.0299 20012	LLKVIRKKL VR	K7:+11 4.043	0.0362 53776
P10922	749.49 24316	2	1496.96 9213	1.0856 83449	AAKPKKAAS KAPSK	K10:+1 14.043	0.0865 61747
P10922	599.38 39722	2	1196.75 2294	1.0484 47592	KKPAATPKK	K1:+11 4.043,K 9:+114. 043	0.0865 61747
P10922	571.43 13965	2	1140.84 7143	2.0920 61417	KAKKPKVVK	K6:+11 4.043	0.0865 61747
P10922	763.49 63379	2	1524.97 7026	1.0258 10269	ASKPKKAKT VKPK	K3:+11 4.043	0.0865 61747
P15864	599.83 92334	2	1197.66 2817	0.0023 17173	ASGPPVSELI TK	-	0.0865 61747
P15864	554.29 05273	2	1106.56 5405	0.0046 19232	ALAAAGYD VEK	-	0.0865 61747
P15864	593.44 51294	2	1184.87 4609	0.0889 68197	IKLGLKSLVS K	-	0.0865 61747
P15864	532.00 45776	3	1592.99 0258	1.0016 66646	AGAAKAKKP AGAAKPK	-	0.0865 61747
P15864	663.90 01465	2	1325.78 4643	0.9655 76905	KPKKATGAA TPKK	-	0.0865 61747

P15864	452.95 07446	3	1355.82 8759	0.0401 92151	KATGAATPK KAAK	K1:+11 4.043	0.0865 61747
P15864	649.95 21484	2	1297.88 8647	1.1008 08507	AKKPAAAAV TKK	K11:+1 14.043	0.0865 61747
P15864	436.77 82898	2	871.540 9295	1.0121 5983	KVAKSPK	K1:+11 4.043	0.0865 61747
P15864	571.86 41968	2	1141.71 2743	0.9783 97443	KAKVTKPKK	K3:+11 4.043	0.0865 61747
P15864	548.06 33545	3	1641.16 6588	4.1313 81301	VKSASKAVK PKAAKPK	-	0.0865 61747
P15864	464.78	2	927.544 3499	1.9733 81032	AAKPKVAK	K3:+11 4.043	0.0865 61747
P15975	973.53 73535	2	1945.05 9057	0.0130 20001	MAWVKFLR KPSGNLGK	K5:+11 4.043	0.0485 65123
P17156	744.35 69946	2	1486.69 8339	0.0043 5449	TTPSYVAFT DTER	-	0.0865 61747
P17156	927.49 26147	2	1852.96 9579	2.0154 37876	HWPFRVVSE GGKPK	K12:+1 14.043, K14:+1 14.043	0.0865 61747
P17156	830.46 05103	2	1658.90 537	0.0174 36764	IINEPTAAAI AYGLDK	-	0.0865 61747
P17156	596.67 14478	3	1786.99 0868	0.0079 71342	IINEPTAAAI AYGLDKK	-	0.0865 61747
P17156	971.85 60791	3	2912.54 4762	2.0181 22353	NVLIFDLGG GTFDVSILTI EDGIFEVK	-	0.0865 61747
P26040	591.80 3833	2	1181.59 2016	0.0050 75432	APDFVIFYAP R	-	0.0865 61747
P26040	736.91 05835	2	1471.80 5517	0.0060 6404	QLLTLSNELS QAR	-	0.0865 61747
P26040	1098.5 46631	2	2195.07 7612	1.9563 39791	EELMLRLQD YEQKTKR	K13:+1 14.043	0.0865 61747
P27661	498.78 42102	2	995.552 7703	2.0501 2497	MSGRGKTGG K	M1:+15. 995	0.0865 61747
P27661	472.77 1759	2	943.527 868	0.0039 21802	AGLQFPVGR	-	0.0865 61747
P27661	972.87 54272	3	2915.60 2807	1.0223 99874	VGAGAPVYL AAVLEYLTA EILELAGNA AR	-	0.0865 61747

P27661	564.97 64404	3	1691.90 5846	0.0103 67505	HLQLAIRND EELNK	-	0.0865 61747
P27661	439.45 77332	4	1753.79 9632	0.8148 59417	KSSATVGP K APAVGKK	K1:+11 4.043, 9:+114. 043	0.0865 61747
P40630	983.32 35474	3	2946.94 7167	6.0329 6149	QRRLKKKAL VKRRELILLG KPKR	K22:+1 14.043	0.0782 98695
P40630	1101.7 2	3	3302.13 6525	5.0415 01192	EARQRRLKK KALVKRREL ILLGKPKR	K25:+1 14.043	0.0782 98695
P43274	614.84 32617	2	1227.67 0873	- 0.0001 90906	TSGPPVSELI TK	-	0.0865 61747
P43274	554.29 05273	2	1106.56 5405	0.0046 19232	ALAAAGYD VEK	-	0.0865 61747
P43274	593.44 51294	2	1184.87 4609	0.0889 68197	IKLGLKSLVS K	-	0.0865 61747
P43274	532.00 45776	3	1592.99 0258	1.0016 66646	AGAAKAKKP AGAAKPK	-	0.0865 61747
P43274	478.79 53491	2	955.575 0482	1.0139 15666	AKKPAGAAK	K9:+11 4.043	0.0865 61747
P43274	539.00 63477	3	1613.99 5568	3.0486 36985	TVKPKAAKP KTSK	K10:+1 14.043, K13:+1 14.043	0.0865 61747
P43275	1107.3 9	3	3319.14 6525	5.0447 33424	KTVKTPKKP KKPAVSKKT SKSPKPKV VK	K4:+11 4.043	0.0509 75293
P43275	1176.7 56958	3	3527.24 7399	3.0339 31575	AKKVAKSPA KAKAVKPKA SKAKVTKPK TPAKPK	K27:+1 14.043	0.0509 75293
P43275	946.96	3	2837.85 6525	6.0400 89296	TSKSPKKPK VVKAKKVA KSPAKAKAV K	-	0.0509 75293
P43275	671.38 7085	3	2011.13 778	0.0064 27571	KKPAGPSVS ELIVQAVSSS K	-	0.0509 75293

P43275	628.68 76831	3	1883.03 9574	0.0031 8514	KPAGPSVSEL IVQAVSSSK	-	0.0509 75293
P43275	1131.0 74463	3	3390.19 9914	5.0610 08248	TVKTPKKPK KPAVSKKTS KSPKKPKVV KAK	K3:+11 4.043	0.0782 98695
P43275	1064.3 6	3	3190.05 6525	4.0496 9656	TVKTPKKPK KPAVSKKTS KSPKKPKVV K	K23:+1 14.043	0.0782 98695
P43275	1140.7 40723	3	3419.19 8693	6.0535 67065	KTSKSPKKP KVVKAKKV AKSPAKAKA VKPK	K15:+1 14.043, K16:+1 14.043	0.0926 5694
P43276	606.84 71069	2	1211.67 8564	0.0024 14163	ATGPPVSELI TK	-	0.0865 61747
P43276	547.28 42407	2	1092.55 2831	0.0076 96069	ALAAGGYD VEK	-	0.0865 61747
P43276	593.44 51294	2	1184.87 4609	0.0889 68197	IKLGLKSLVS K	-	0.0865 61747
P43276	545.31 70166	2	1088.61 8383	1.9673 72851	AKKTGAAK AK	K3:+11 4.043	0.0865 61747
P43276	521.98 88916	3	1562.94 32	0.0189 14795	AKKPAGATP KKPKK	K14:+1 14.043	0.0865 61747
P43276	429.30 40161	2	856.592 3821	2.0949 12627	KPAAAGVK	K1:+11 4.043	0.0865 61747
P43276	613.39 33105	2	1224.77 0971	0.9995 83598	SPKKAKAAA KPK	-	0.0865 61747
P43276	464.31	2	926.604 3499	2.0287 02464	SPAKPKAVK	-	0.0865 61747
P43276	799.51 29395	2	1597.01 0229	1.0015 70898	AVKSKASKP KVTKPK	-	0.0865 61747
P43276	841.52 7832	2	1681.04 0014	0.9989 20775	VTKPKTAKP KAAKAK	K10:+1 14.043	0.0865 61747
P43277	564.89 86206	2	1127.78 1591	1.0992 08235	TPVKKKAK	K5:+11 4.043,K 8:+114. 043	0.0865 61747
P43277	599.83 92334	2	1197.66 2817	0.0023 17173	ASGPPVSELI TK	-	0.0865 61747
P43277	554.29 05273	2	1106.56 5405	0.0046 19232	ALAAAGYD VEK	-	0.0865 61747

P43277	593.44 51294	2	1184.87 4609	0.0889 68197	IKLGLKSLVS K	-	0.0865 61747
P43277	532.00 45776	3	1592.99 0258	1.0016 66646	AGAAKAKKP AGAAKKPK	-	0.0865 61747
P43277	663.90 01465	2	1325.78 4643	0.9655 76905	KPKKATGAA TPKK	-	0.0865 61747
P43277	648.39 55078	2	1294.77 5366	- 0.0332 08183	SPKKVKA AK PK	K9:+11 4.043	0.0865 61747
P43277	557.32	2	1112.62 435	- 0.0423 10432	KA AKSPAKA K	K10:+1 14.043	0.0865 61747
P43277	677.91	2	1353.80 435	- 0.0049 52136	AKASKPKAS KPK	K5:+11 4.043	0.0865 61747
P51960	768.91 80908	2	1535.82 0532	- 0.0480 89231	WSLIAKHLK GR	K6:+11 4.043,K 9:+114. 043	0.0085 17887
P52480	586.32 23877	2	1170.62 9125	0.0046 76959	LDIDSAPITA R	-	0.0865 61747
P52480	832.10 68115	3	2493.29 6959	1.0170 92194	EATESFASDP ILYRPVAVA LDTK	-	0.0865 61747
P52480	607.29 62646	2	1212.57 6879	0.0071 57161	ITLDNAYME K	M8:+15. 995	0.0865 61747
P52480	571.31 25	2	1140.60 935	0.0066 99664	GDLGIEIPAE K	-	0.0865 61747
P52480	1073.5 66528	2	2145.11 7407	1.0172 94857	LAPITSDPTE AAAVGAVE ASFK	-	0.0865 61747
P52480	793.90 08179	2	1585.78 5986	0.0123 5359	DAVLNAWA EDVDLR	-	0.0865 61747
P52480	766.40 93628	3	2296.20 4613	- 0.0705 15757	GPEIRTGLIK GSGTAEVEL KK	K20:+1 14.043	0.0865 61747
P61161	700.35 49194	2	1398.69 4189	2.0459 33195	VVVC DNGTG FVK	C4:+45. 988,K12 :+114.0 43	0.0865 61747
P61161	667.82 22046	2	1333.62 8759	0.0103 8118	DLMVGDEAS ELR	-	0.0865 61747
P61161	677.38 13477	2	1352.74 7045	0.0096 74361	ILLTEPPMNP TK	-	0.0865 61747

P62806	672.33 78906	2	1342.66 0131	0.9122 94314	GKGGKGLG KGGAK	K5:+11 4.043,K 9:+114. 043	0.0865 61747
P62806	442.59 33228	3	1324.75 6493	0.0101 98198	DNIQGITKPA IR	-	0.0865 61747
P62806	789.45 12329	2	1576.88 6816	0.0043 61436	ISGLIYEETR GVLK	-	0.0865 61747
P62806	495.29 55627	2	988.575 4754	0.0049 13312	VFLENVIR	-	0.0865 61747
P62806	567.77 771	2	1133.53 977	0.0044 70938	DAVTYTEHA K	-	0.0865 61747
P62806	663.85 78491	2	1325.70 0048	0.0098 76498	TVTAMDVV YALK	M5:+15. 995	0.0865 61747
P63158	751.32	2	1500.62 435	0.0040 09632	MSSYAFFVQ TCR	M1:+15. 995,C11 :+45.98 8	0.0865 61747
P63158	548.23 93799	2	1094.46 311	- 0.0549 96354	WKTMSAK	K2:+11 4.043,M 4:+15.9 95,K7:+ 114.043	0.0865 61747
P63158	760.92 60254	2	1519.83 6401	0.0005 62309	IKGEHPGLSI GDVAK	-	0.0865 61747
P63158	564.52 01416	4	2254.04 9266	1.0119 85918	LGEMWNNT AADDKQPYE KK	M4:+15. 995	0.0865 61747
Q07133	554.29 05273	2	1106.56 5405	0.0046 19232	ALAAAGYD VEK	-	0.0865 61747
Q07133	595.85 30273	2	1189.69 0405	1.0327 79856	GKGKKSASA K	K4:+11 4.043,K 10:+114 .043	0.0865 61747
Q07133	543.32	3	1626.93 6525	1.9739 43936	TKAVKKPKA TPTK	K2:+11 4.043,K 13:+114 .043	0.0865 61747
Q0P5X1	1081.3 57422	3	3241.04 8791	4.0561 42817	KLRKKLEPS VRLALFKKA KNKVSVTK	K19:+1 14.043, K21:+1 14.043	0.0509 75293
Q5HZG4	1076.0 26001	3	3225.05 4528	4.0469 71162	LPSSVDVKK KLKELKTK LKKKEKQR	K21:+1 14.043	0.0509 75293



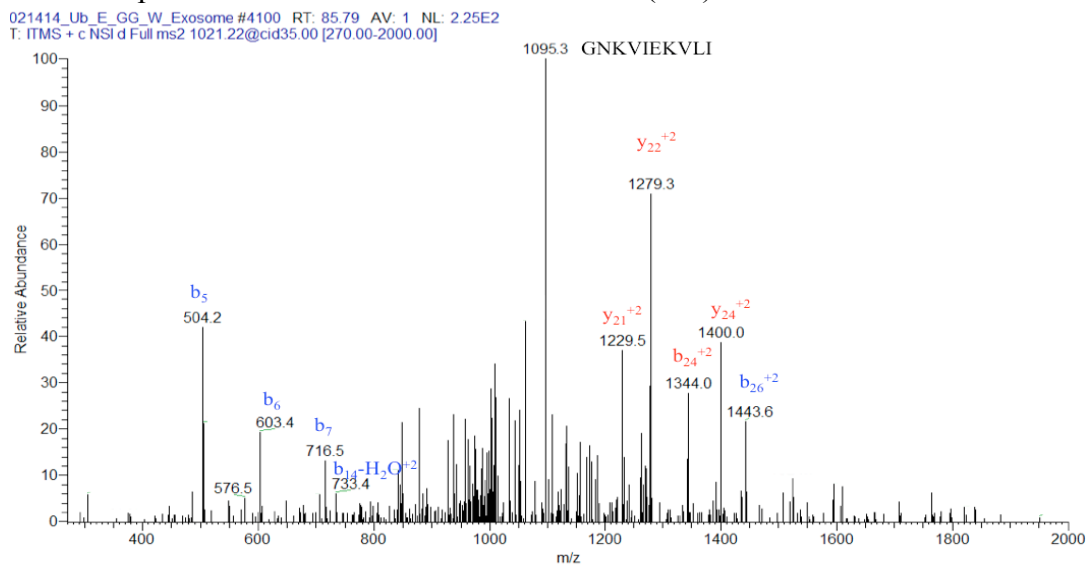
Q5HZG4	961.64	3	2881.89 6525	3.0541 3636	AKLPSSVDV KKKLLKKELK TKLKKK	K22:+1 14.043	0.0782 98695
Q5SWU9	1021.2 15698	3	3060.62 362	- 0.0278 37185	FGGNKVIEK VLIANNGIAA VKCMRSIR	K21:+1 14.043, C22:+45 .988	0.0448 4
Q61781	1018.8 94592	3	3053.66 0302	2.0038 03847	TIEDLKSKIL AATVDNAN VLLQIDNAR	K8:+11 4.043	0.0693 6416
Q61781	690.36 89575	2	1378.72 2265	0.0017 90815	TRLEQEIATY R	-	0.0509 75293
Q6IFX2	789.76 2207	3	2366.26 3146	- 0.0286 95778	NKILAATIDN ASIVLQIDNA R	K2:+11 4.043	0.0865 61747
Q6IFX2	633.83 83789	2	1265.66 1108	0.0004 54467	TKYETELNL R	-	0.0865 61747
Q6IFX2	459.57 22351	3	1375.69 323	0.0053 4993	MSVEADING LRR	M1:+15. 995	0.0865 61747
Q6IFX2	644.29 79736	3	1929.87 0446	0.9730 78162	ILNEMRDQY EKMAEK	M5:+15. 995,M1 2:+15.9 95	0.0865 61747
Q6IFX2	439.25 57983	2	876.495 9466	1.0312 28776	MAEKNRK	-	0.0865 61747
Q6IFX2	524.93	3	1571.76 6525	2.0089 36888	NRKDAEEWF FTK	-	0.0865 61747
Q6IFX2	586.76 96533	2	1171.52 3657	0.0050 70393	DAEEWFFTK	-	0.0865 61747
Q6IFX2	958.94 99512	2	1915.88 4252	2.0046 98336	DAEEWFFTK TEELNR	-	0.0865 61747
Q6IFX2	690.37 07886	2	1378.72 5927	0.0054 52924	TRLEQEIATY R	-	0.0865 61747
Q6P925	1134.0 5	3	3399.12 6525	3.0497 04528	AKRSKLKKK RNPRSKLPK RSRHSLIR	K2:+11 4.043,K 18:+114 .043	0.0509 75293
Q6PHS6	973.46	3	2917.35 6525	1.0341 03664	DDQVKGTAE DLVETFFEVE VEMEK	K5:+11 4.043,M 22:+15. 995	0.0052 77045
Q6ZPJ3	1054.0 1	3	3159.00 6525	3.0380 26352	KKSIPLSIKN LKRKHKRKK NKVTR	K12:+1 14.043, K14:+1 14.043	0.0509 75293

Q8C4U3	1101.0 7	3	3300.18 6525	3.9821 1888	IVPKKKKPL KLGPIKKKEL KRLVFLK	K17:+1 14.043	0.0509 75293
Q8C4U3	943.63	3	2827.86 6525	3.0081 3916	KIVPKKKKP LKLGPICKKE LKR	K5:+11 4.043,K 21:+114 .043	0.0509 75293
Q8C4U3	1105.7 1	3	3314.10 6525	3.0519 45528	IKEVKKENG DKKIVPKKK KPLKLGPIK	K11:+1 14.043, K27:+1 14.043	0.0509 75293
Q8C4U3	1368.8 8	3	4103.61 6525	5.0629 4988	MKIKEVKKE NGDKKIVPK KKKPLKLGPI KKKELK	M1:+15. 995,K24 :+114.0 43	0.0509 75293
Q8C4U3	683.13 81836	3	2046.39 1076	4.0453 47797	KIVPKKKKP LKLGPICK	K7:+11 4.043,K 8:+114. 043	0.0913 32711
Q924L1	924.00 56763	2	1845.99 5702	0.0250 53075	MKGIQMLW ADGKK(GG) AR	K13:+1 14.043	0.0085 17887
Q9CQJ6	1057.3 4	3	3168.99 6525	4.0613 92152	QKKKTVPQK VTIAKIPRAK KKYVTR	K3:+11 4.043,K 4:+114. 043	0.0509 75293
Q9CSP9	1076.3 50952	3	3226.02 9381	4.0516 55389	TKKIETRAEK LRKLLKEEK RLKKK	K2:+11 4.043,K 3:+114. 043	0.0509 75293
Q9CXH7	721.40 89355	2	1440.80 2221	- 0.0139 57386	EKRKNLAG IGK	K12:+1 14.043	0
Q9CZ91	1036.3 6	3	3106.05 6525	4.0371 25872	KEVKRIRVL VIRKLVRSV GRLKSKK	K13:+1 14.043	0.0509 75293
Q9Z100	793.89	3	2378.64 6525	3.9884 2796	LRVIKKKKIV VKRKKLR	K15:+1 14.043	0.0509 75293
Q9Z100	835.25 85449	3	2502.75 216	5.0508 00862	HVRLRVIKK KKIVVKKRK K	K16:+1 14.043	0.0509 75293
Q61781	1018.2 11426	3	3051.61 0802	3051.6 56498	TIEDLKSKIL AATVDNAN VLLQIDNAR	K6:+11 4.043	0

Q9CQJ6	814.51 87988	3	2440.53 2921	2434.4 72147	KQKRGGRG QIKQKKKT PQK	K20:+1 14.043	0.0782 98695
P08113	638.32 58667	2	1274.63 6083	1274.6 35407	ELISNASDAL DK	-	0.0509 75293
Q9Z100	961.64	3	2881.89 6525	2877.9 07257	VIKKKKIVV KKRKKLRHP GPLGTAR	-	0.0820 93135
Q9Z100	835.25 85449	3	2502.75 216	2497.7 01359	HVRLRVIKK KKIVVKKRK K	K16:+1 14.043	0.0509 75293
E9Q6J5	1136.3 8	3	3406.11 6525	3400.0 77033	TLEPKAPRIK EVLKERKVL EKKVALSKR	K5:+11 4.043	0.0782 98695
F6SB18	1037.0 2	3	3108.03 6525	3103.9 74776	RKVLALPSH RGPKIRRLKE RLRRIR	-	0.0509 75293
Q8C4U3	944.64	3	2830.89 6525	2824.8 58386	KIVPKKKKP LKLGPICKKE LKR	K1:+11 4.043,K 11:+114 .043	0.0509 75293
Q9CZ91	1057.0 31494	3	3168.07 1007	3163.0 18033	MRKEVKRIR VLVIRKLVRS VGRLKSK	M1:+15. 995	0.0782 98695
P40630	1105.3 92212	3	3313.15 3161	3308.1 11081	QRRLKKKAL VKRRELILLG KPKRPR	K7:+11 4.043,K 22:+114 .043	0.0782 98695

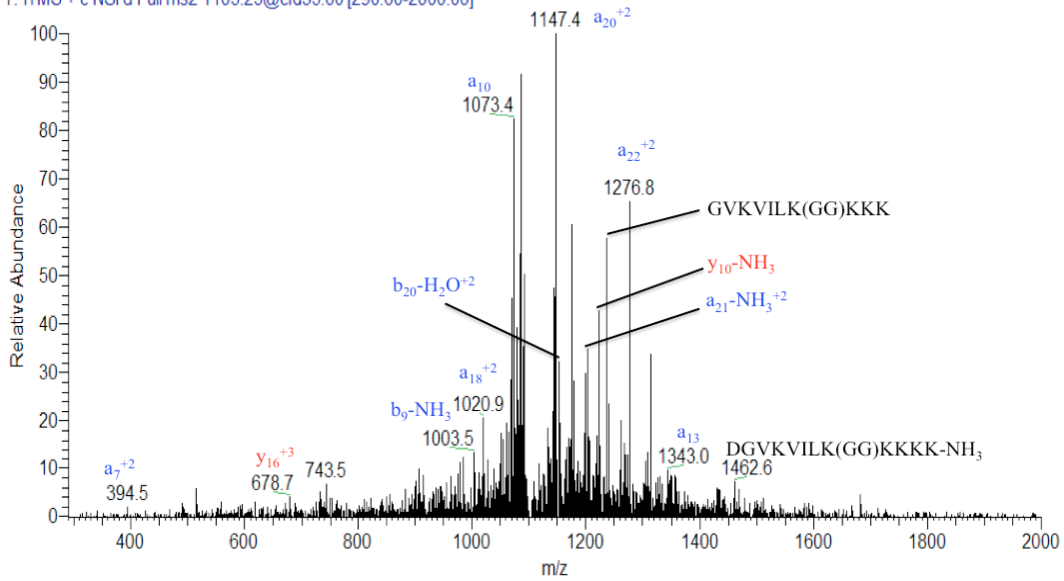
Appendix Figure 1: Annotated tandem mass spectra for all single peptide identifications containing a glycylglycine ubiquitin remnant. B-type (blue) and y-type ions (red) are labeled to the corresponding peaks in the tandem mass spectra shown.

a. Q5SWU9 Acetyl-CoA carboxylase 1 FDR 4.230E-2  
Peptide FGGNKVIEKVLIANNGIAAVK(GG)CMRSIR



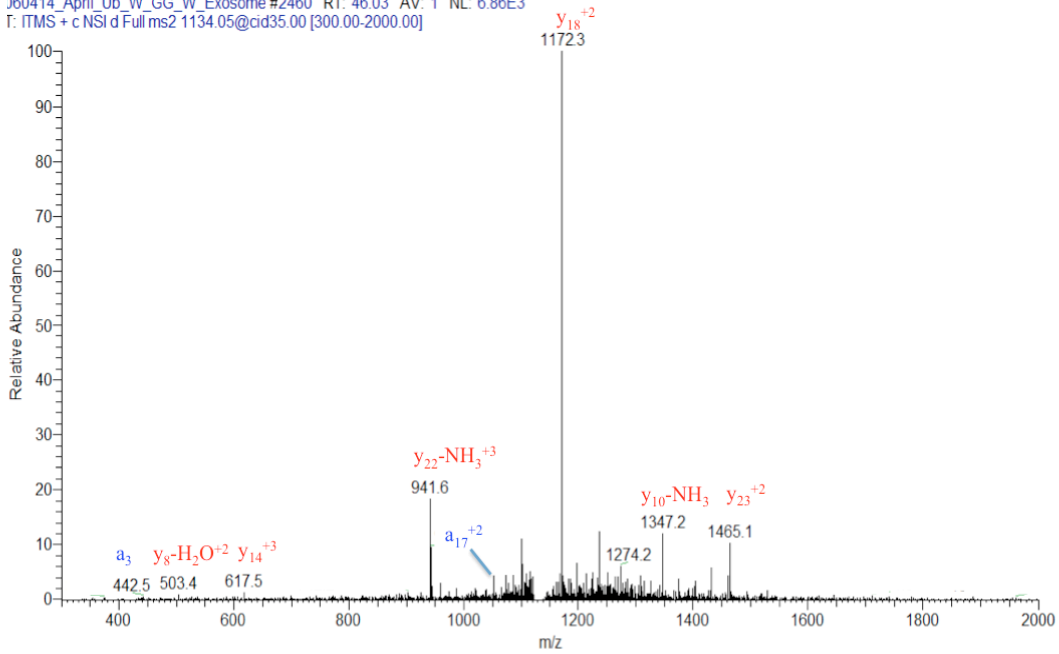
b. E0CYH9 Carboxyl-terminal PDZ ligand of neuronal nitric oxide synthase protein FDR 2.370E-4  
Peptide KKKVSIMVSDGVKVLK(GG)KKKLLLLQK

021414\_Ub\_E\_GG\_E\_Exosome#3363 RT: 65.22 AV: 1 NL: 9.23E2  
 T: ITMS + c NSI d Full ms2 1105.23@cid35.00 [290.00-2000.00]



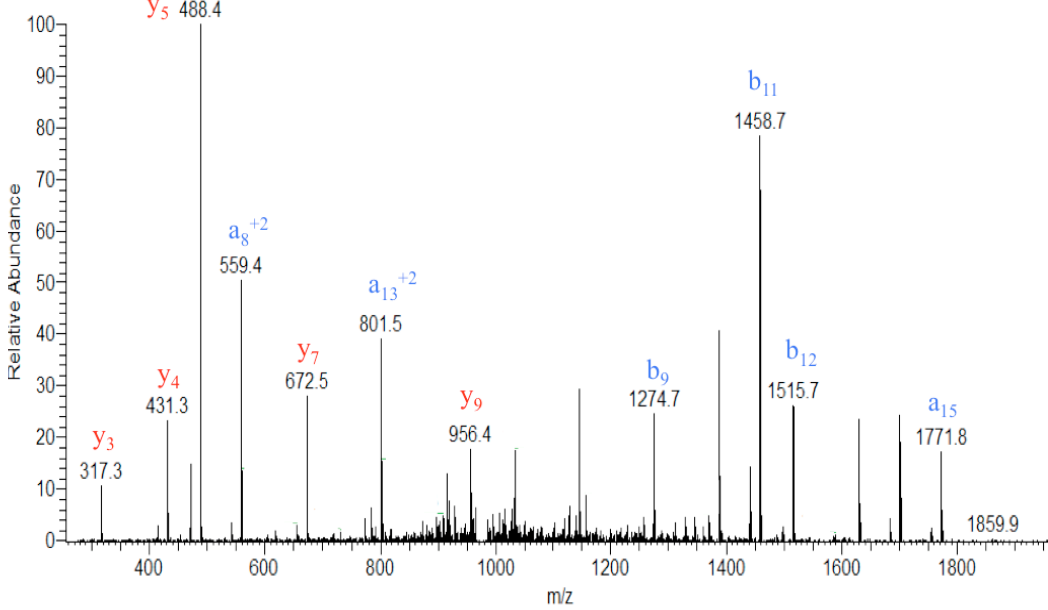
c. Q6P925 Cysteine-rich perinuclear theca 4 FDR 5.100E-2  
 Peptide AK(GG)RSKLLKKRNPRSKLPK(GG)RSRHSLIR

060414\_April\_Ub\_W\_GG\_W\_Exosome#2460 RT: 46.03 AV: 1 NL: 6.86E3  
 T: ITMS + c NSI d Full ms2 1134.05@cid35.00 [300.00-2000.00]



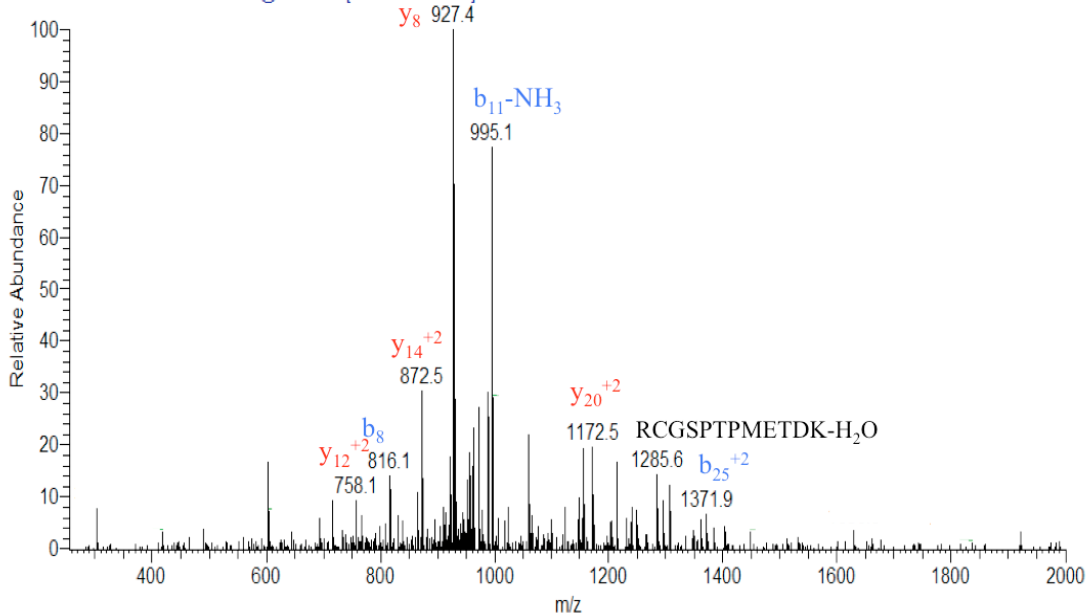
d. P15975 Inactive ubiquitin carboxyl-terminal hydrolase 53 FDR 4.484E-2  
Peptide MAWVK(GG)FLRKPSGNL GK

132514\_Ub\_E\_GG\_FT\_Exosome #3933 RT: 71.13 AV: 1 NL: 7.05E3  
ITMS + c NSI d Full ms2 975.04@cid35.00 [255.00-1965.00]



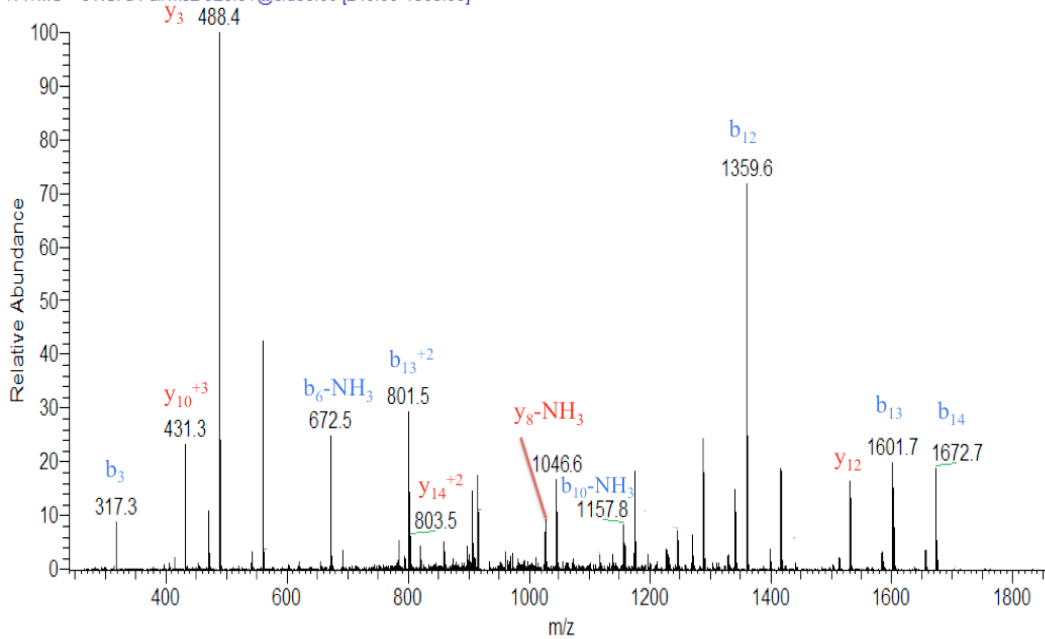
e. B2RXC2 Inositol 1,4,5-trisphosphate 3-kinase B FDR 2.15E-2  
Peptide GTPASPRCGSPTMETDK(GG)RVAPSLER

112614\_Exo\_Ub3933\_E\_GG\_E\_7\_3 #1928 RT: 50.35 AV: 1 NL: 4.76E2  
ITMS + c NSI d Full ms2 973.45@cid35.00 [255.00-2000.00]



f. Q924L1 LETM1 domain-containing protein 1 FDR 8.52E-2  
Peptide MKGIQMLWADGKK(GG)AR

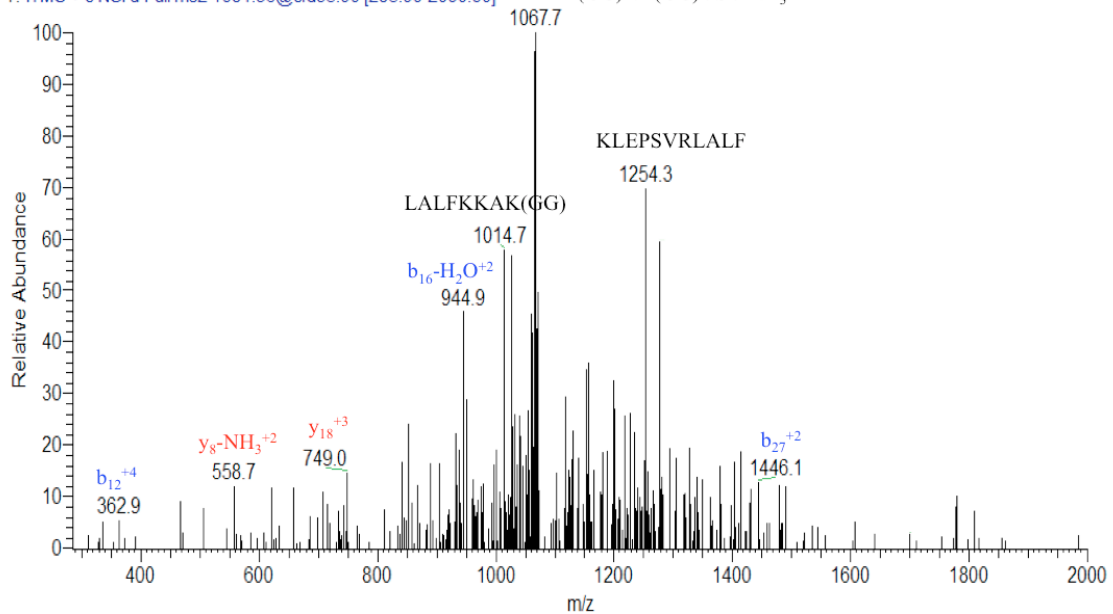
032514\_Ub\_W\_GG\_W\_Exosome #3486 RT: 62.81 AV: 1 NL: 2.74E4  
T: ITMS + c NSI d Full ms2 925.51@cid35.00 [240.00-1865.00]



g. Q0P5X1 Leucine-rich repeat and IQ domain-containing protein 1 FDR 5.09E-2

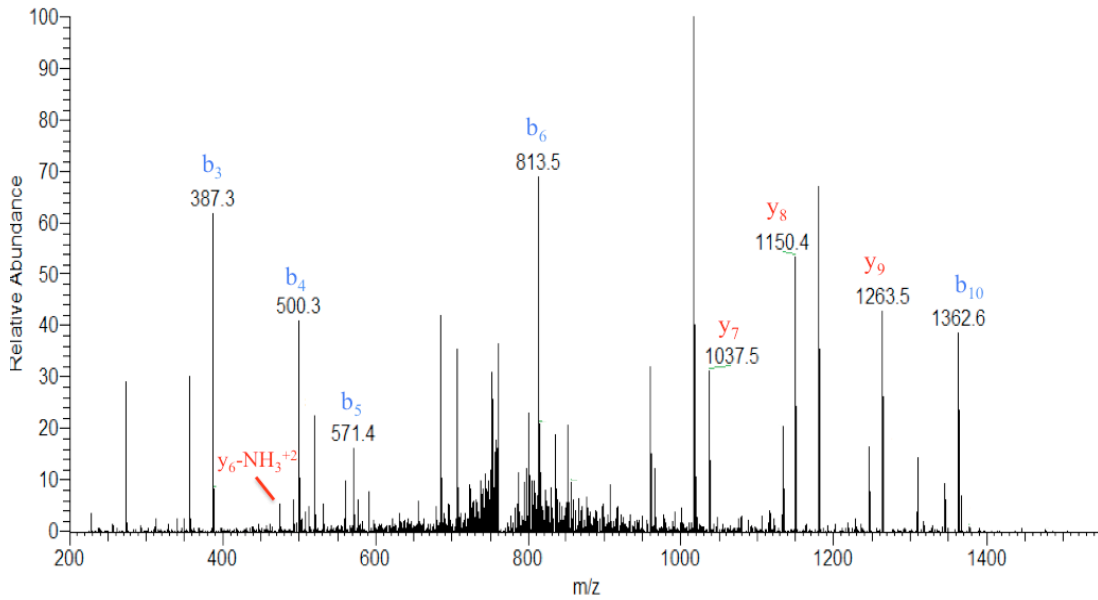
Peptide KLRKKLEPSVRLALFKKAK(GG)NK(GG)VSVTK

021414\_Ub\_W\_GG\_FT\_Exosome #867 RT: 26.72 AV: 1 NL: 1.08E2  
T: ITMS + c NSI d Full ms2 1081.36@cid35.00 [285.00-2000.00] KAK(GG)NK(GG)VSV-NH<sub>3</sub>



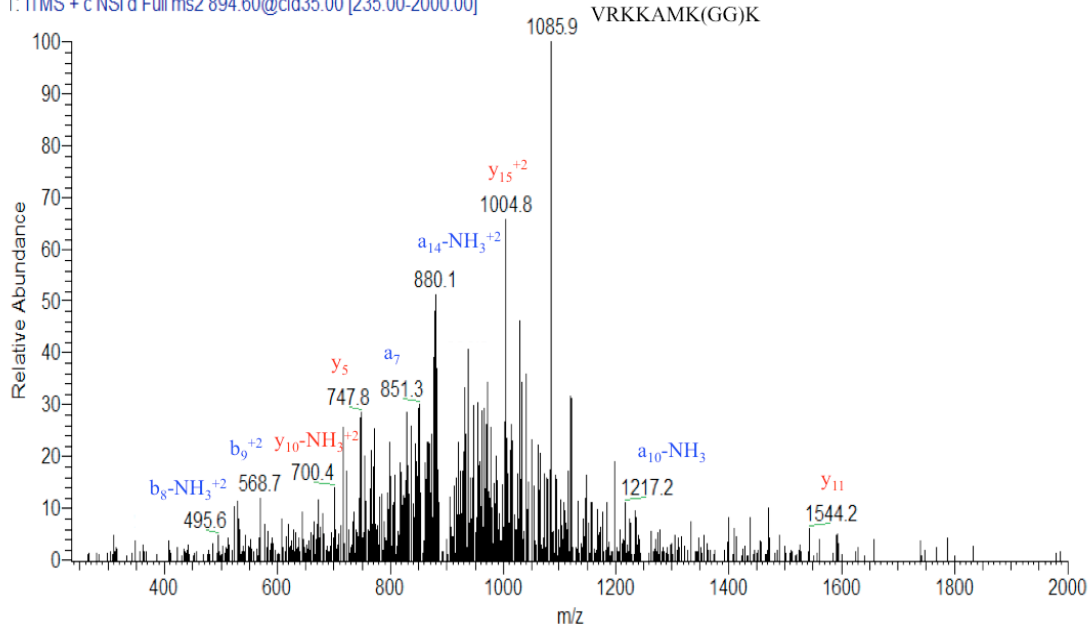
h. P51960 Myb-related protein A FDR 8.52E-3  
 Peptide WSLIAK(GG)HLK(GG)GR

21414\_Ub\_W\_GG\_FT\_Exosome #1470 RT: 37.58 AV: 1 NL: 2.46E3  
 : ITMS + c NSI d Full ms2 768.92@cid35.00 [200.00-1550.00]



i. H3BKN5 Probable global transcription activator SNF2L2 FDR 9.13E-3  
 Peptide VLGRK(GG)LPKKRVRKKAMK(GG)KR

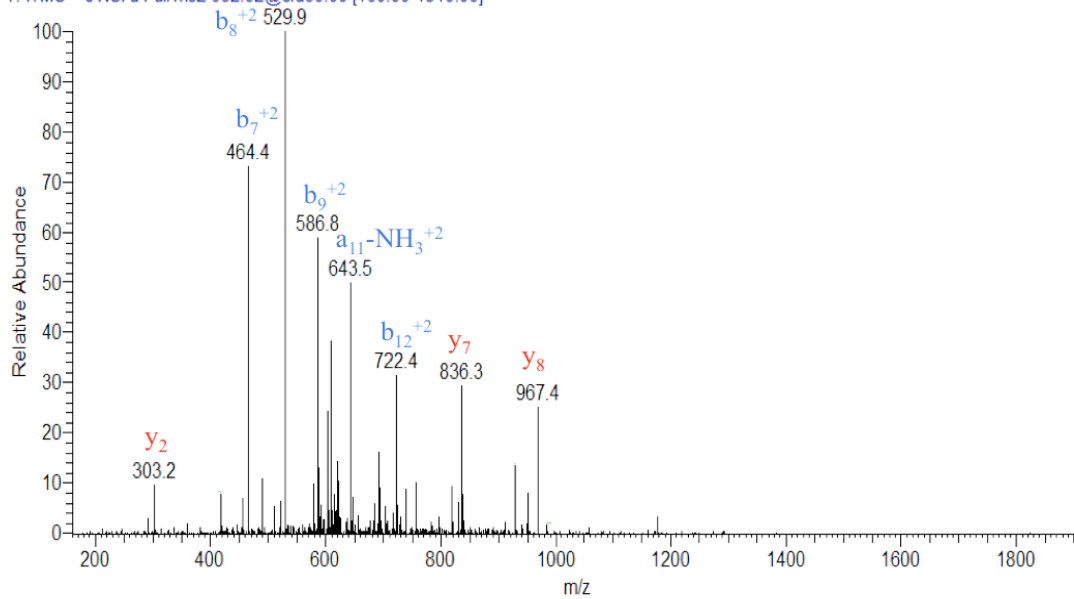
360414\_April\_Ub\_E\_GG\_W\_Exosome\_140608233604 #2038 RT: 39.65 AV: 1 NL: 4.11E2  
 : ITMS + c NSI d Full ms2 894.60@cid35.00 [235.00-2000.00]





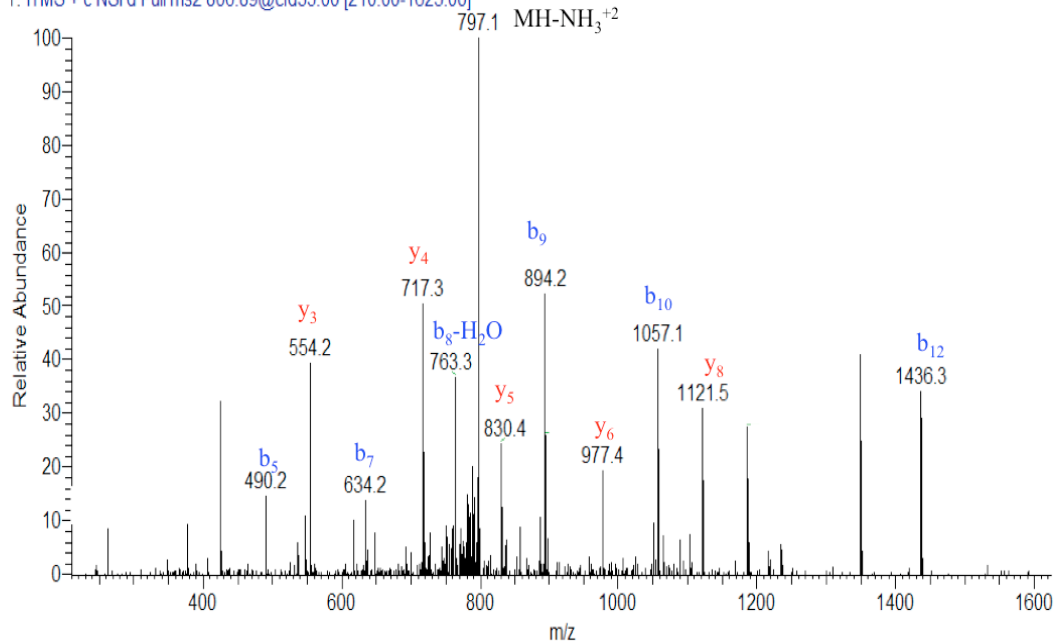
j. J3QQ16 Protein Col6a3 FDR 3.625E-2  
Peptide DLK(GG)IMVLMLTGDMQR

021414\_Ub\_E\_GG\_W\_Exosome #1821 RT: 41.33 AV: 1 NL: 5.29E3  
T: ITMS + c NSI d Full ms2 632.32@cid35.00 [160.00-1910.00]



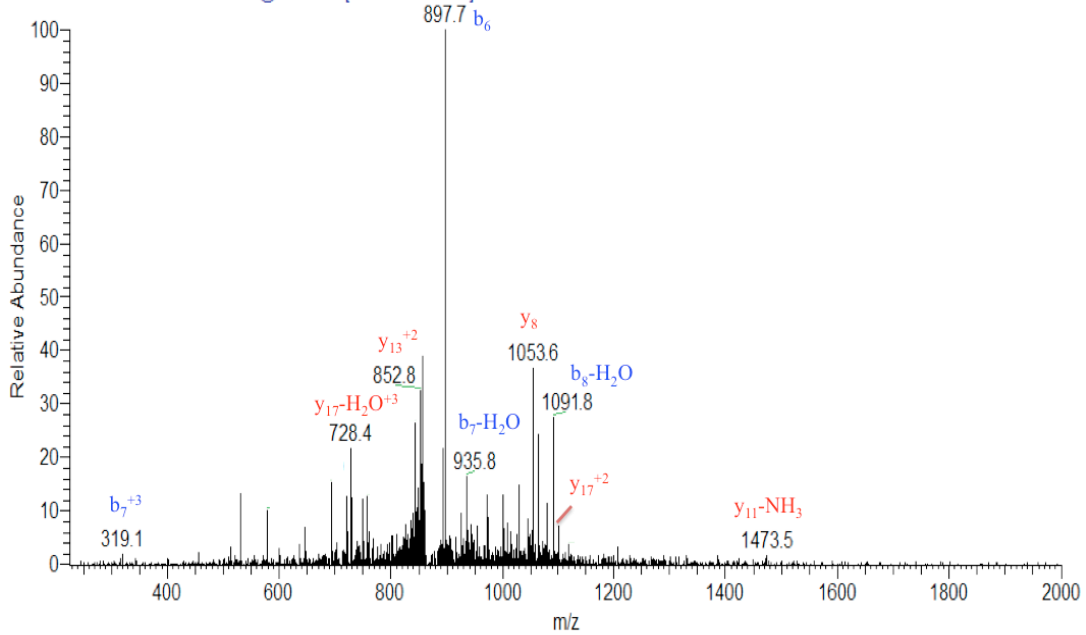
k. A2AU83 Protein GM14124 FDR 2.149E-2  
Peptide AFSSPSGFLYHK(GG)R

021414\_Ub\_E\_GG\_W\_Exosome #2894 RT: 60.68 AV: 1 NL: 6.91E2  
T: ITMS + c NSI d Full ms2 806.89@cid35.00 [210.00-1625.00]



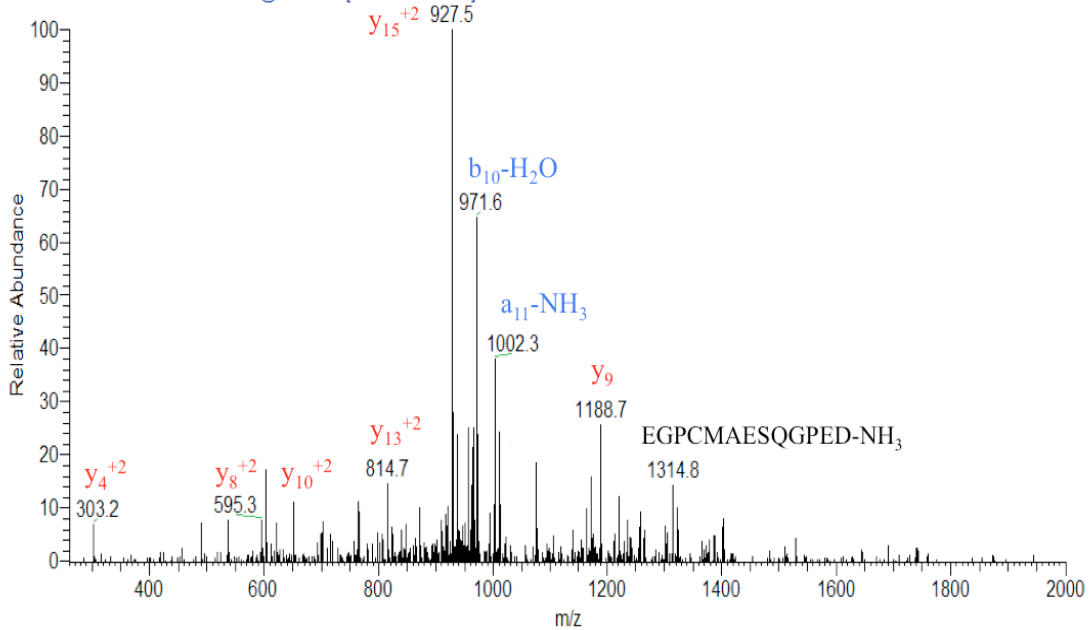
l. E9PZM7 Protein Scaf11 FDR 5.09E-2  
Peptide RK(GG)SVRRGRK(GG)PPLLKKKLRR

021414\_Ub\_W\_GG\_W\_Exosome #1629 RT: 38.55 AV: 1 NL: 1.67E3  
T: ITMS + c NSI d Full ms2 869.24@cid35.00 [225.00-2000.00]



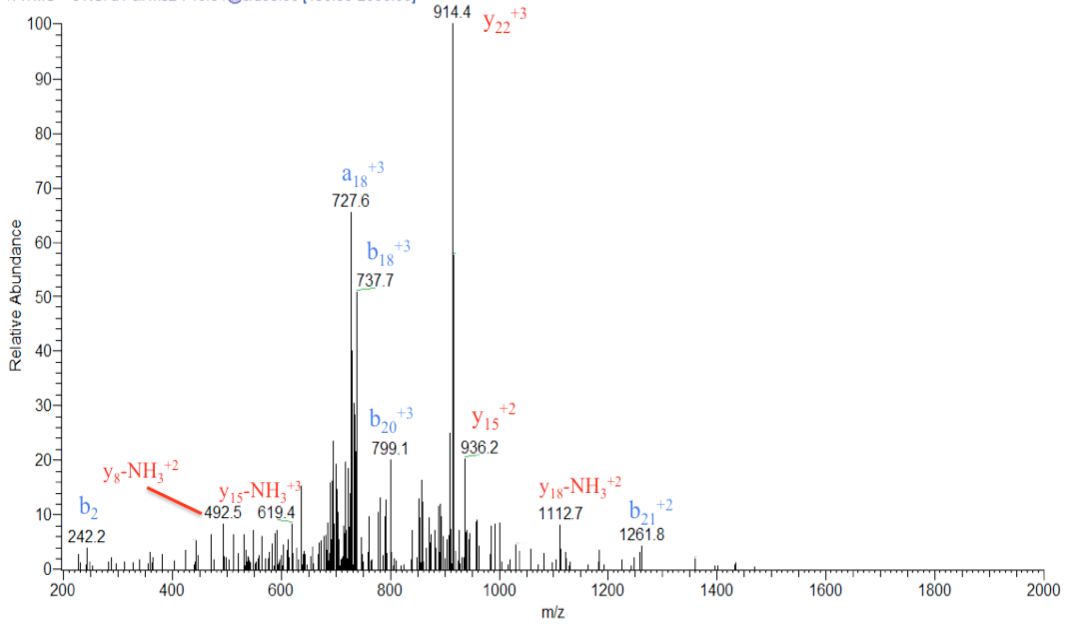
m. G3UWJ2 Protein Zfp69 FDR 2.932E-2  
Peptide GEGPCMAESQGPEDPILDVKNKLETK(GG)

021414\_Ub\_W\_GG\_E\_Exosome\_140217000458 #1575 RT: 39.40 AV: 1 NL: 5.02E2  
T: ITMS + c NSI d Full ms2 983.46@cid35.00 [260.00-2000.00]



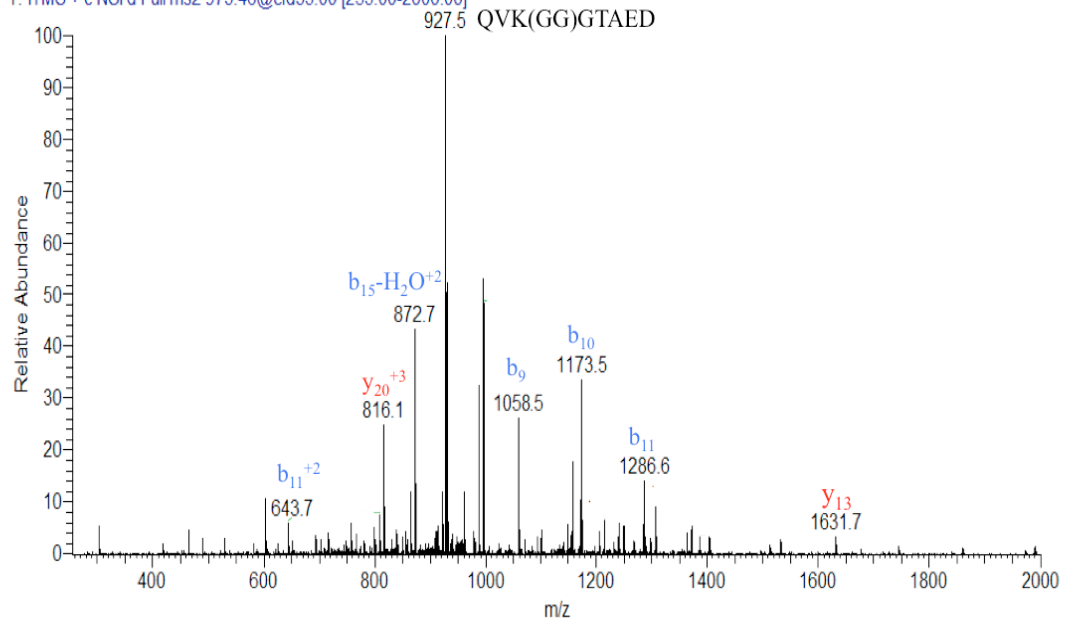
n G5E861 Sodium channel and clathrin linker 1 FDR 3.768E-2  
Peptide LQQENEQLQKETEDLRKVALEAQK(GG)

J21414\_Ub\_E\_GG\_FT\_Exosome\_140217031056 #726 RT: 25.80 AV: 1 NL: 1.95E2  
T: ITMS + c NSI d Full ms2 746.64@cid35.00 [195.00-2000.00]



o. Q6PHS6 Sorting nexin-13 FDR 5.28E-3  
Peptide DDQVK(GG)GTAEDLVETFFEVEMEKEK

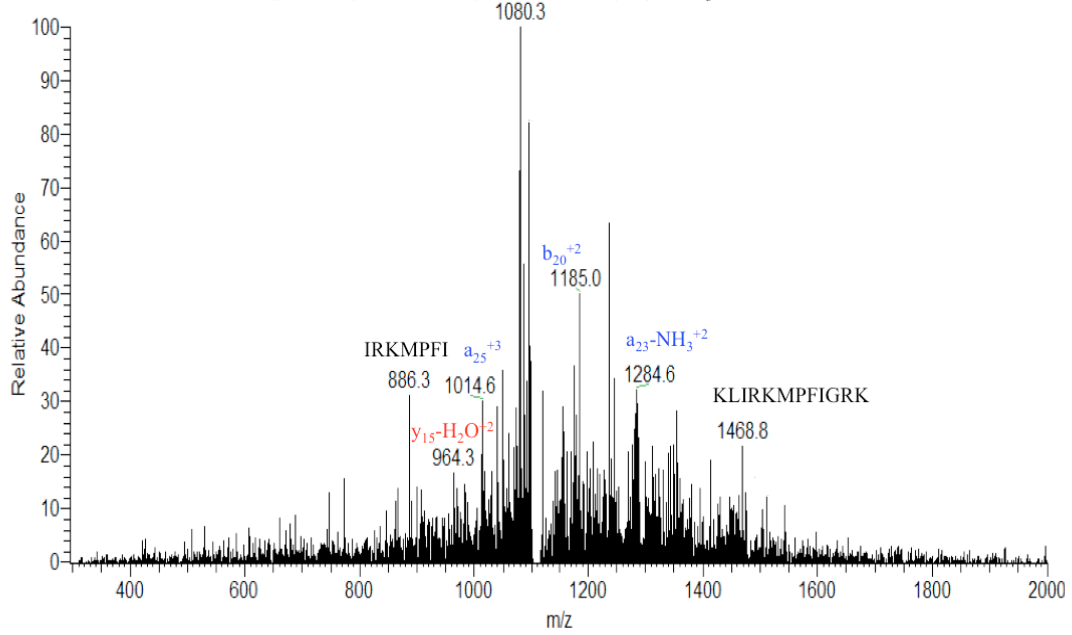
021414\_Ub\_W\_GG\_W\_Exosome\_140216205906 #1714 RT: 39.61 AV: 1 NL: 5.96E3  
T: ITMS + c NSI d Full ms2 973.46@cid35.00 [255.00-2000.00]



p. D3Z1Z3 Sphingosine-1-phosphate lyase 1 FDR 5.09E-2  
 Peptide KKLFLIRKMPFIGRKVSKAK(GG)KDLVK(GG)

J32514\_Ub\_W\_GG\_E2\_Exosome\_2#2341 RT: 46.26 AV: 1 NL: 2.15E3

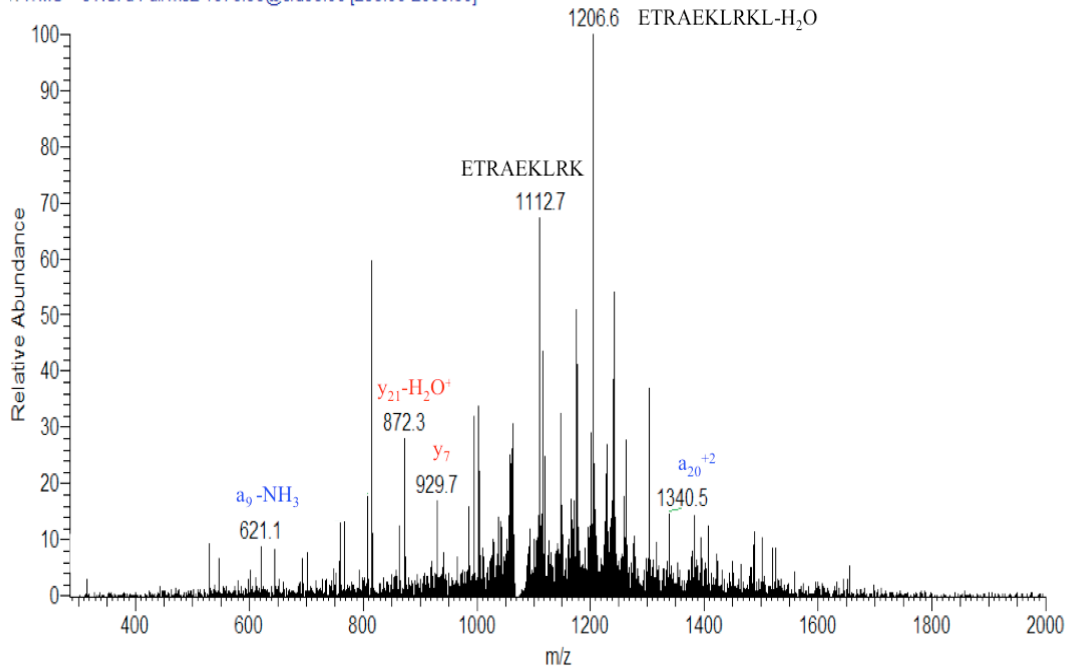
F: ITMS + c NSI d Full ms2 1111.38@cid35.00 [295.00-2000.00] GRKVS KAK(GG)K-NH<sub>3</sub>



q. Q9CSP9 Tetratricopeptide repeat protein 14 FDR 5.09E-2  
 Peptide TK(GG)K(GG)IETRAEKLRLKLLKEEKRLKKK

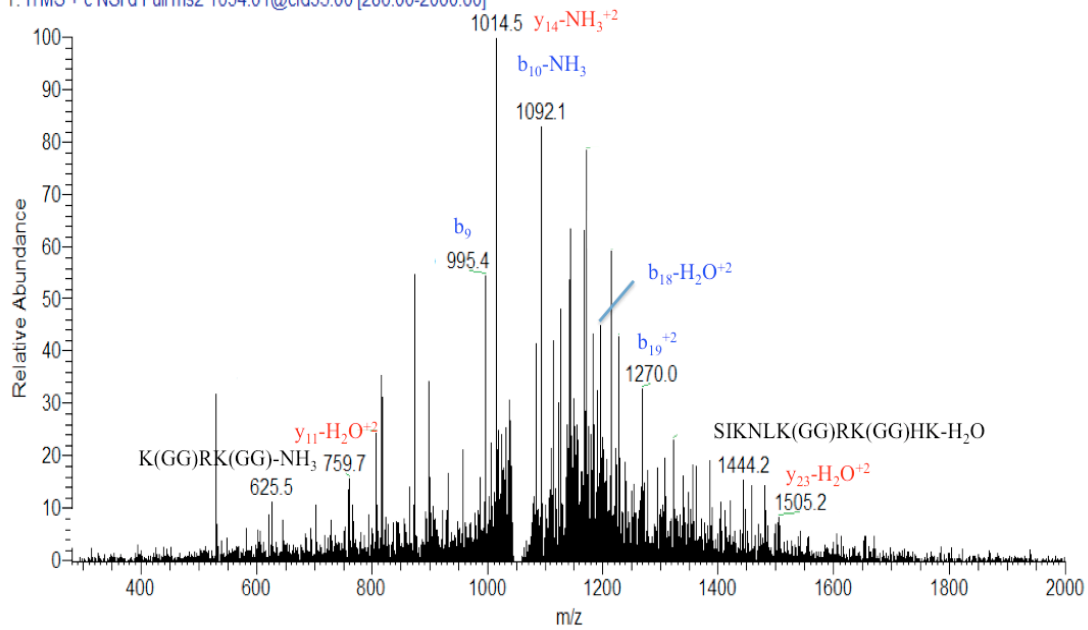
J21414\_Ub\_E\_GG\_FT\_Exosome\_140217031056#1858 RT: 46.75 AV: 1 NL: 2.27E3

F: ITMS + c NSI d Full ms2 1076.35@cid35.00 [285.00-2000.00]



r. Q6ZPJ3 Ubiquitin-conjugating enzyme E2 O FDR 5.09E-2  
Peptide K(SI)PLSIK(NLK)(GG)RK(GG)HK(RK)K(NK)VTR

060414\_April\_Ub\_W\_GG\_FT\_Exosome#2158 RT: 43.44 AV: 1 NL: 1.66E3  
T: ITMS + c NSI d Full ms2 1054.01@cid35.00 [280.00-2000.00]



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