IDENTIFICATION OF CYTOSOLIC SIALIDASE NEU2 ASSOCIATED PROTEINS BY MASS SPECTROMETRIC ANALYSIS

A Thesis Submitted to
the Graduate School of Engineering and Sciences of
Izmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

in Molecular Biology and Genetics

by
Seçil AKYILDIZ DEMİR

December 2013
İZMİR

We approve the thesis of **Seçil AKYILDIZ DEMİR**

Examining Committee Members:

_____

Assoc. Prof. Dr. Volkan SEYRANTEPE

Department of Molecular Biology and Genetics**,** Izmir Institute of Technology

_____

Prof. Dr. Talat YALÇIN

Department of Chemistry, Izmir Institute of Technology

_____

Assist. Prof. Dr. Bünyamin AKGÜL

Department of Molecular Biology and Genetics, Izmir Institute of Technology

20 December 2013

_____

Assoc. Prof. Dr. Volkan SEYRANTEPE

Supervisor, Department of Molecular Biology and Genetics

Izmir Institute of Technology

_____

Assoc. Prof. Dr. Ahmet KOÇ

Head of the Department of Molecular Biology and Genetics

_____

Prof. Dr. R. Tuğrul SENGER

Dean of the Graduate School of Engineering and Sciences

ACKNOWLEDGMENTS

I would like to thank all the people who contributed in some way to the work in this thesis. First and foremost, I thank to my supervisor Assoc. Prof. Dr. Volkan SEYRANTEPE for accepting me into her group, and for his patience, motivation, enthusiasm, and immense knowledge. During my thesis he contributed to a rewarding graduate school experience by giving me intellectual freedom in my work, engaging me in new ideas. His guidance helped me in all the time of research and writing of this thesis

Besides my advisor, I would like to thank my committee members Assist. Prof. Dr. Bünyamin AKGÜL and Prof. Dr. Talat TALÇIN for their suggestions, contributions and hard questions.

I would like to thank to Dr. Ahmet Tarık BAYKAL for Mass Spectrometry Analysis and Assoc. Prof. Dr. Yusuf BARAN and all Cancer Laboratory Members for their help during cell culture experiments.

I would like to thank to my supportive friend, Zehra Kevser TIMUR, and others who made the lab a friendly environment for working.

I would like to thank to my friends Murat DELMAN for his support and help during my thesis project and my co-worker Süleyman Can ÖZTÜRK for his helps and supports in dealing with experiments.

I am also thankful to all members of Molecular Biology and Genetics Department and Biotechnology and Bioengineering Central Research specialists for their sincere help and kindness during studies.

I would like to thank my family: my parents Nazlı AKYILDIZ and Sabahattin AKYILDIZ, and my brother, his wife Sercan AKYILDIZ, Deniz AKYILDIZ, and my niece Anıl Can AKYILDIZ for always believing in me, for their continuous love and their supports in my decisions. Without whom I could not have made it here.

Lastly, I would like to express my special thanks to my love Ismail DEMİR, for unending love.

ABSTRACT

IDENTIFICATION OF CYTOSOLIC SIALIDASE NEU2 ASSOCIATED PROTEINS BY MASS SPECTROMETRIC ANALYSIS

Sialidases (Neuraminidases) are the enzymes which remove sialic acids from glycoproteins, oligosacharides and glycolipids. Four mammalian sialidases have been identified which are lysosomal sialidase (Neu1), cytosolic sialidase (Neu2), plasma membrane sialidase (Neu3), and mitochondrial/lysosomal sialidase (Neu4). These enzymes differ in their subcellular localizations, expression levels in different cells and tissues, substrate specificities and optimum pH levels. Cytosolic Neu2 enzyme has an active role on a wide range of subtances including oligosaccharides, glycopeptides and gangliosides. Studies on the Neu2 enzyme also showed that this enzyme is involved in different cellular events including cancer metabolism, neuronal differentiation and myoblast differentiation, proliferation and hypertrophy. However, it has not been shown whether Neu2 interacts with other proteins within the cell. Therefore, in this study we aimed to identify Neu2 associated proteins by using InterPlay Mammalian TAP System and ESI-LC-MS/MS analysis. Proteins in the Neu2 protein complex were identified by three different database search software such as PGLS, Mascot and X!Tandem. As a result of experiment Actin proteins (Alpha Actin, Gamma Actin and Beta Actin), and Calsyntenin-2 protein were found as a candidate protein for Neu2 association. The interaction between Neu2 and β-Actin proteins was confirmed by western blot analysis.

ÖZET

KÜTLE SPEKTROMETRESI ANALIZI ILE SITOZOLIK SIALIDAZ NEU2 ILE ILIŞKILI PROTEINLERIN TANIMLANMASI

Sialidazlar (nörominidazlar) glikoprotein, oligosakkarit ve glikolipitlerden sialik asit adlı şekerli uzaklaştıran enzimlerdir. Lizozomal sialidaz (Neu1), sitoplazmik sialidaz (Neu2), hücre zar sialidazı (Neu3) ve lizozomal/mitokondrial sialidazdır (Neu4) olarak dört tane memeli sialidaz enzimi tanımlanmıştır. Bu enzimlerin hücre içindeki yerleri, hücrede ve dokuda ifade ediliş miktarları, substratları ve aktivite gösterdikleri pH aralıkları farklılık göstermektedir. Sitozolik NEU2 enzimi oligosakkaritler, glikopeptidler ve gangliosidleri içeren geniş bir yüzey yelpazesi üzerinde aktif olarak rol oynamaktadır. Ayrıca yapılan çalışmalarda Neu2 enziminin kanser metabolizması, nöron farklılaşması ve myoblast çoğalması, farklılaşması ve hipertropisi gibi farklı hücresel olaylarda görev yaptığı tespit edilmiştir. Fakat Neu2 enzimi hücre içerisindeki hangi proteinlerle etkileşime girdiği tam olarak tanımlanmamıştır. Biz bu çalışmamızda InterPlay Mammalian TAP System ve ESI-LC-MS/MS analizi kullanılarak Neu2 enziminin hücre içinde ilişkilide olduğu proteinler tespit edilmek amaçlanmıştır. Neu2 protein kompleksi içerisindeki proteinler PGLS, Mascot ve X!Tandem olmak üzere üç farklı veritabanı araştırma yazılımı ile tanımlanmıştır. Deneyin sonucu olarak Aktin proteinleri (Alfa Aktin, Gama Aktin ve Beta Aktin) ve Calsyntenin-2 proteini aday protein olarak bulunmuştur. Neu2 ile β-Aktin proteinleri arasındaki ilişki western blot analizi ile doğrulanmıştır.

To my wonderful family*...*

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

1.1. Sialidases

Sialidases, also known as Neuraminidases, (EC 3.2.1.18) belong to a family of exoglycosidases which are responsible for catalization of hydrolytic cleavage of nonreducing sialic acid residues from glycoconjugates such as glycoproteins and glycolipids (Fanzani et al. 2004; Monti et al. 2002). These enzymes cleave terminal α2→3, α2→6 and α2→8 sialyl linkages (Fanzani et al. 2004). They are widely distributed in nature from viruses and microorganisms to avian and mammalian species (Monti et al. 2002). Comparison of amino acid sequence of bacterial and viral sialidases shows that there is about 35% sequence identity. They all share F(Y)RIP domain in the amino terminal site of the protein and three to five times repeating "Asp boxes"(S-X-D-X-G-X-X-T/W) depending on the protein. Mammalian sialidases likely to microbial sialidases include F(Y)RIP domain and Asp boxes. Multiple alignment between mammalian and microbial sialidase amino acid sequences points out that there are highly conserved site in the catalytic region of the protein. This results in similar topology among bacterial, viral and mammalian sialidases (Monti et al. 1999).

In mammals four different sialidases have been purified, cloned and characterized so far. They all classified based on their subcellular localization; lysosomal sialidase (Neu1), cytoplasmic sialidase (Neu2), membrane bound sialidase (Neu3), lysosomal/-mitochondrial/-intracellular membranes sialidase (Neu4). In addition to differences in their subcellular localizations, their expression level in different cell and tissue, substrate specificities are also different. Their major subcellular localization , optimum pH level, total amino acids and chromosome location is shown in the Table1.1. These enzymes have involved in different cellular events as cell proliferation, differentiation, gangliosides and glycoproteins metabolism, clearance of plasma proteins, cell adhesion control, receptor modification, immunocyte function, and modeling of myelin (Monti et al. 2010). Involvement of sialidase in these biological

networks could be either direct or secondary to desialylation of various substrate(s) (Monti et al. 2002).

Table1.1. Comparison of mammalian sialidases Neu1, Neu2, Neu3, and Neu4 in terms of major subcellular localization, optimal pH, total amino acid and chromosome location (Adapted from: Miyagi et al. 2004; Monti et al. 2010)

Abbreviation	**Major Subcellular Localization**	**Optimum pH**	**Total Amino Acids (human)**	**Chromosome Location (human)**
Neu1	Lysosome	4.4-4.6	415	6p 21.3
Neu2	Cytosol	6.0-6.5	380	2q 37
Neu3	Plasma membrane	4.6-4.8	428	11q13.5
Neu4	Mitochondrial/Lysosomal/-intracellular membrane	3.2-4.8	496	2q37.3

1.1.1. Cytoplasmic Sialidase (Neu2)

Cytosolic sialidase Neu2 is an exoglycosidase removing terminal sialic acids from gangliosides and glycoproteins. Targets of Neu2 are thought to be as precursor sphingolipids and O-linked glycosylated proteins in cytosol because N-linked glycosylated proteins present within the endoplasmic reticulum and Golgi compartment (Rossi et al. 2009).

Cytosolic sialidases had been widely characterized after purification from rat liver, rat skeletal muscle and Chinese hamster ovary (CHO) cells cDNAs from diffent organism includes highly homologous polypeptides, with a conserved YRIP domain and two Asp boxes and potential N-linked glycosylation sites one for hamster and three for rat (Monti et al. 1999).

Human cytoplasmic sialidase (Neu2) gene was identified in 1999 by Monti et al using a sequence homology based approach. Chromosomal localization of Neu2 is 2q37. Its 2.4 kb ORF includes 1 intron about 1.25 kb and encodes a protein consisting

of 380 amino acids - 42 kDa with a two-Asp block consensus and the YRIP sequence. Its putative promoter sequence contains classical TATAA box and four E boxes which are binding regions for muscle- specific transcription factors (Monti et al. 1999).

Although cytosolic sialidase Neu2 enzyme is mostly expressed in the skeletal muscle (Miyagi et al. 1993), it is also expressed in other tissues such as liver (Miyagi and Tsuiki 1985), thymus (Kotani et al. 2001) and brain (Hasegawa et al. 2001).

1.1.2. Functions of Cytosolic Sialidase Neu2

1.1.2.1. Muscle Differentiation

Neu2 mRNA and its enzymatic activity are not detected during cell proliferation whereas they are gradually increase in post-mitotic myoblasts and reach maximum level in fully matured hypertrophic myotubes (Rossi et al. 2009).

Cytosolic sialidase gene in the rat skeletal muscle is highly expressed. It includes a TATA box and four E-box pairs having consensus binding sites for muscle-specific transcription factors in enhancer/promotor region of the gene (Monti et al. 2002). Transcriptional activity in this promoter region increases during myotube formation. Cytosolic sialidase have an important role in myoblast differentiation by desialylating some glycoconjugate(s) involved in this process. Function of cytosolic sialidase in myoblast differentiation was firstly demostrated *in vitro* studies with rat L6 myogenic cells. While Neu2 treatment induces myotube formation, treatment with Neu2 antisense oligonucleotides prevent the myotube formation (Sato and Miyagi 1996).

Over expression of rat cytosolic sialidase Neu2 induces myoblast differentiation in murine C2C12 myoblast cells. Following the stable transfection with rat Neu2 gene, myoblast cells resulted in spontaneous differentiation of myoblast cells under standard condition which is marked with formation of myogenin-positive myotubes. In addition, in these cells there is decrease in cyclin D1 protein showing the decrease in the proliferation rate of the myoblast cells (Fanzani et al. 2003).

IGF-1 stimulates proliferation, differentiation and hypertrophy of myoblasts by its specific binding to the IGF-I receptor (IGF-IR). This ligand-receptor interaction stimulates the activation of two major intracellular signaling pathways; the mitogen-

activated protein kinases (MAPKs) and the phosphatidylinositol 3 kinase (PI3K)/ serine–threonine protein kinase (AKT) (Fanzani et al. 2006).

IGF-1 promoting muscle proliferation is activated by the MAPK (serine/threonine kinases (RAF)/mitogen-activated protein kinase (MEK)/Extracellular signal-regulated kinase (ERK)) signaling pathway. Especially, ERK is a central and main component in this pathway (Jones et al. 2001; Samuel 1997).

Skeletal muscle hypertrophy is the increase in muscle size. During growth of mammalian increase in the muscle weight of pre-existing muscle fibers occurs in response to physical exercise instead of increase in the cell number. Hypertrophy has also important role during postnatal development (Fanzani et al. 2006; Glass 2005). Insulin-like growth factor 1 (IGF-1) has been shown to be sufficient to induce skeletal muscle hypertrophy stimulating the phosphatidylinositol-3 kinase (PI3K)/serine–threonine protein kinase (AKT)/mammalian target of rapamycin (mTOR) pathway (Figure 1.1). This pathway results in activation of translation initiation and elongation needed for synthesis of protein (Bodine et al. 2001). PI3K/AKT pathway is also important in activation of myoblast differentiation (Samuel 1997).

A link between IGF1 signaling pathway and Neu2 expression was shown in C2C12 cells. Activation of PI3K/AKT1/mTOR pathway causes an increase in Neu2 levels, both in terms of transcript level and enzyme activity. Transfection of an activated form of AKT stimulated hypertrophy by inducing high Neu2 activity. However, transfection of a kinase-inactive form of AKT prevented myotube formation by downregulation Neu2. Under standard condition Neu2-transfected myoblasts showed stronger differentiation but availability of PI3 kinase and mTOR inhibitors in the condition prevent the myotube formation in Neu2-transfected myoblasts. These results point out that increase of Neu2 level is a downstream event in myoblast differentiation and hypertrophy induced by IGF1 (Fanzani et al. 2006).

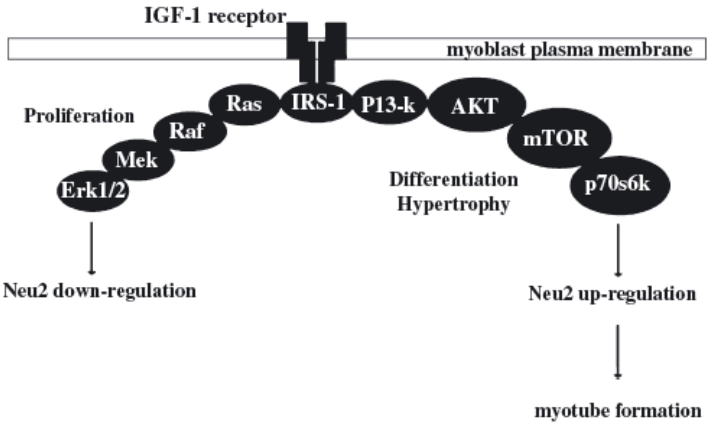

Figure1.1. Signaling pathways in the regulation of Neu2 expression in myoblasts cells. Autophosphorylation of IGF-1 receptor resulted in distinct downstream signals such as myoblast proliferation and differentiation / hypertrophy. Activation of the Ras–Raf–Mek–Erk pathway causes proliferation and Neu2 down regulation. Activation of the PI3K–AKT– mTOR–P70S6 pathway result in myoblast differentiation and hypertrophy and also up regulation of Neu2 which have an important role during myotube formation (Source: Fanzani et al. 2006)

When PI3K/AKT/mTOR pathway is down regulated, it leads to skeletal muscle atrophy (Bodine et al. 2001). Skeletal muscle atrophy resultes from decreased use of muscle under physiological and pathological conditions like ageing, muscle unloading, cancer, AIDS, denervation and dystrophies. Under these conditions, increased protein breakdown and decreased protein synthesis cause unbalanced protein levels and starting a reduction in myofiber size (Fanzani et al. 2008). Atrophied muscles can be reloaded and hypertrophied again (Bodine et al. 2001).

During myofiber atrophy there is down regulation of Neu2 expression in myotubes exposed to starvation or glucocorticoid treatment (Fanzani et al. 2008). In addition Neu2 mRNA and protein levels are downregulated when a macroautophagic process occurs in myotubes. Macroautophagy permit cell survival by the bulk degradation of proteins and organelles by lysosomal proteases rather than proteasome activation (Rossi et al. 2009).

1.1.2.2. Neuronal Differentiation

PC12 cells, a favored model for neural differentiation, can be stimulated to differentiate into neural phenotype by nerve growth factor (NGF) or human fibroblast growth factor 2 (FGF-2) treatments, and to proliferate by epidermal growth factor (EGF) treatment. Under normal condition cytosolic sialidase expression is very rare in PC12 cells whereas under differentiating and proliferating conditions the Neu2 expression is increased. These results indicate Neu2's potential role in these processes. The activity of Neu2 enzyme is detectable in PC12 cells at day 1–2 of NGF differentiation. This shows its potential role of Neu2 in the early steps of neuronal differentiation. In the 1.4 kb upstream of the Neu2 promoter there are binding regions such as the nuclear factors SP1 and AP1 and several E-box sequences for differentiating cells not for proliferating cells. EGF- dependent nuclear factors are found in the 5'upstream of this 1.4 kb fragment promoter. Presence of different E boxes on the same promoter indicates the transcriptional regulation of tissue specific genes (Fanzani et al. 2004).

1.1.2.3. Cancer Metabolism

Chronic myeloid leukemia (CML) is hematopoietic stem cell cancer resulted from a reciprocal translocation between chromosome 9 and chromosome 22. The formation of Bcr/Abl fusion protein with an uncontrolled tyrosine kinase activity results uncontrolled proliferation and insensitivity to apoptotic stimuli (Weisberg et al. 2007).

In the Bcr-Abl positive cells Bcr-Abl kinase induce a variety of downstream pathways such as mitogen-activated protein kinase cascade, Akt, Src kinases, the signal transducers and activators of transcription, and the nuclear factor kB. Activation of these pathways leads to increase in the expression of several anti-apoptotic proteins, such as Bcl-XL and Bcl-2. Anti-apoptotic proteins inhibit cytochrome c release from mytochondria and result in inactivation of caspases (Figure 1.2) (Tringali et al. 2007).

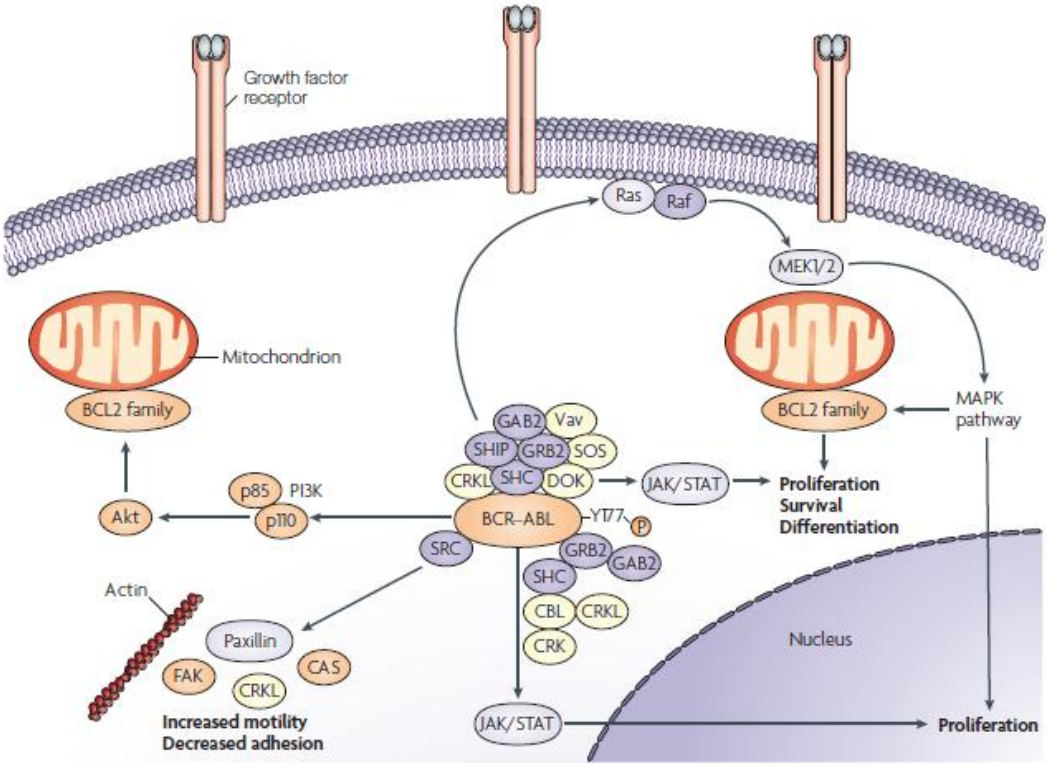

Figure 1.2. Signaling pathway of BCR-ABL kinase in Chronic Myeloid Leukaemia (Source: Weisberg et al. 2007)

K562 cells include the genetic background of chromic myeloid leukemia. Under the normal conditions cytosolic sialidase Neu2 is not expressed in K562 cells. Involvement of Neu2 sialidase in the regulation of cell proliferation and apoptosis was investigated. First of all, it has been shown that Neu2 transfection in K562 cells resulted in a marked 55 % decrease in the proliferation rate. Bcr-Abl activity was also decreased by modifying its downstream signaling pathway such as expression of mitochondrial anti-apoptotic regulatory proteins. Additionally, Neu2 expression leads to decrease 30% and 80% in mRNA of the antiapoptotic factor Bcl-XL and Bcl-2 respectively. Decrease in the expression levels of Bcl-2 and Bcl-XL genes makes the cells more sensitive to apoptotic stimuli (Tringali et al. 2007).

Neu2 expression in K562 cells cause 35% inhibition of tyrosine kinase activity of Bcr-Abl protein. Its inactivation affected activation and expression of Src kinase family members which is found in the downstream signaling pathway of Bcr-Abl protein. It causes also reduction in mRNA and protein levels of Src protein family. When all of the results come together, it was thought that the interaction between Neu2

and Bcr/Abl signaling pathways should be mediated by the desialylation of specific glycoproteins, promoted by the Neu2 enzyme (Tringali et al. 2007).

1.2. Identification of Proteins by Mass Spectrometry

Proteins are basic constructional and functional elements of living organisms. Monitoring all proteins within an organism is a discipline called as proteomics. Proteomics targets to identify proteins and find protein expression in different cell types of the organism, protein distribution in subcellular compartments of the organelles, post-translational modifications of the proteins, protein-protein interactions, relation between protein structure and function in the target organism (Blackstock and Weir 1999).

Peptide and protein identification is done commonly by mass spectrometry based proteomic (Elias and Gygi 2010). Simple principle of the mass spectrometer is the sorting charged particles according to their masses (m/z ratio) and producing spectrum including peaks and valleys which are important to identify target molecule. Generally, ‘‘bottom-up’’ work-flow is preferred in which proteins are digested into a mixture of peptides with an enzyme such as trypsin. Digestion is important because sensitivity of mass spectrometer is greater for peptides compared to proteins. Digested peptides are ionized and separated depending on their mass to charge ratio (m/z) in a mass spectrum (MS) (Vitek 2009).

Although peaks in the first run refer to peptide ions, the sequence of amino acids underlying each peak is not known. In the second run the mass spectrometer isolates the biological material from a peak and subjects it to high collision energy to identify amino acids. This energy breaks the peptide at different amide bonds, and the resulting fragments which are separated according to their m/z in a secondary spectrum (called MS2, MS/MS). Distances between peaks in the MS/MS spectrum are used to identify the peptide sequence (Vitek 2009).

In the mass spectrometry based proteomics firstly a set of spectra are obtained by ms/ms spectrometer and store peak information then each spectrum are examined against a list of proteins sequences by search engines such as Mascot (Wright et al. 2012), SEQUEST (Diament and Noble 2011), Open Mass Spectrometry Search Algorithm (OMSSA) (Vaudel et al. 2011), X!Tandem (Vaudel et al. 2011),

ProteinLynx Global Server (PGLS) (Prieto et al. 2012) and pFind (Wang et al. 2007) …etc.

The MS - based proteomic approach is commonly divided into two major steps such as the isolation, fractionation and purification of proteins, and the MS analyses of proteins. Protein complexes can be isolated by use of the ultracentrifugation, the sucrose density - gradient centrifugation, affinity chromatography, coimmunoprecipitation, or epitope-tag affinity purification. Following the isolation of protein complexes, gel based or gel-free separation techniques can be preferred for MS analysis. After the fractionation of protein complex on 1D or 2D gel system, proteins are digested in the gel by the protease (i.e., trypsin) followed by an MS analysis. For gel-free sepation the protein complex is digested in-solution protease (*i.e.*, trypsin) digestion followed by a 2-D-LC separation. Then digested peptides are analyzed by a database search for protein identification (Chang 2006).

For the analysis of gel based separation, first of all, proteins on SDS-PAGE gel were stained and bands of interest were proteolyticly digested and analyzed by matrix-assisted laser desorption / ionization – Mass Spectrometry (MALDI-MS) (Gavin et al. 2002). Ionization and generation of gas-phase molecules in MALDI is done by a matrix which include a high ratio protein sample to be analyzed (analyte). Matrix is generally composed of small organic molecules. In order to obtain protonated gas-phase molecules the matrix is mixed with the analyte at a high ratio and fixed onto a metal substrate. Laser beam is used to ionize analyte on the matrix. Ionization of the analyte occurs by transferring part of matrix's charge to the analyte. Matrix also protect the analyte from the disruptive energy of the laser beam (Mann, Hendrickson, and Pandey 2001). Figure 1.3 shows summary of protein identification by affinity purification coupled to MALDI ionization.

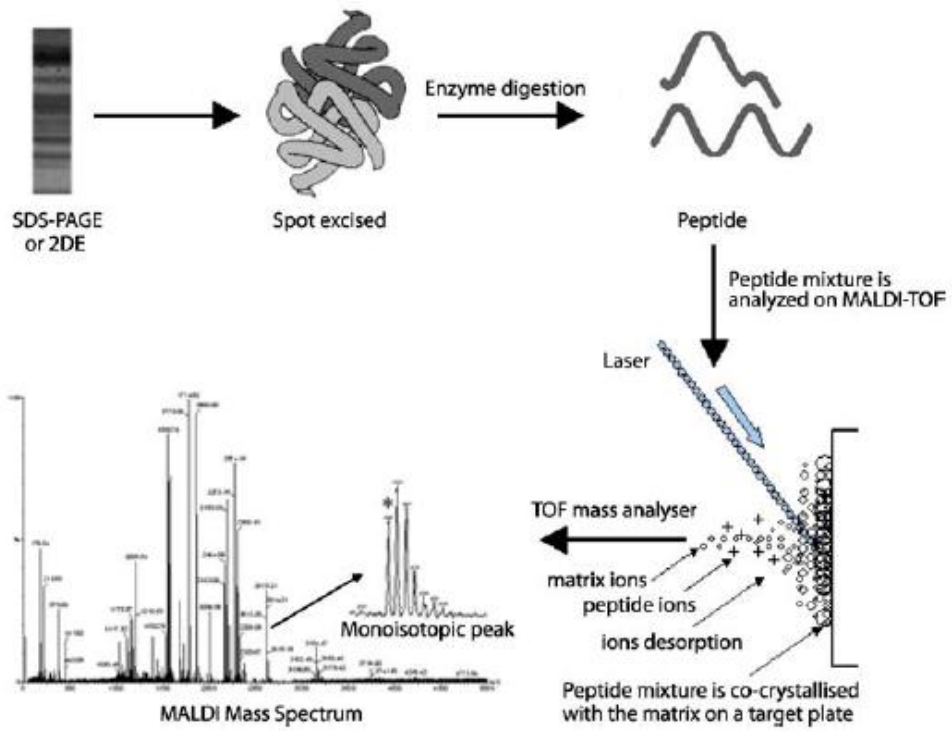

Figure 1.3. Protein identification by MALDI ionization coupled to MS
(Source: Abu-Farha, Elisma, and Figeys 2008)

For the analaysis of gel based separation, secondly, proteins on SDS-PAGE gel were stained with Coomassie stain and bands of interest were proteolyticly digested with trypsin. Then digested peptides were analyzed by electrospray ionization (ESI) coupled to LC-MS/MS analyzed. ESI, another ionization method, is used to produce gas-phase protonated molecules. Differently from MALDI, in ESI method analyte is dissolved at low concentration in a volatile solvent. Analyte in the solvent containing is pumped through a hypodermic needle a high voltage and a low flow rate to electrostatically scatter ions. Micrometer-sized droplets are obtained. These droplets rapidly evaporate while giving their charge onto the analyte molecules. Electrospray ionization helps preserving the structure of the sample being analyzed under atmospheric pressure (Abu-Farha et al. 2008; Loo et al. 1992; Wilm and Mann 1996). Figure 1.4. shows summary of protein identification by affinity purification coupled to electrospray ionization.

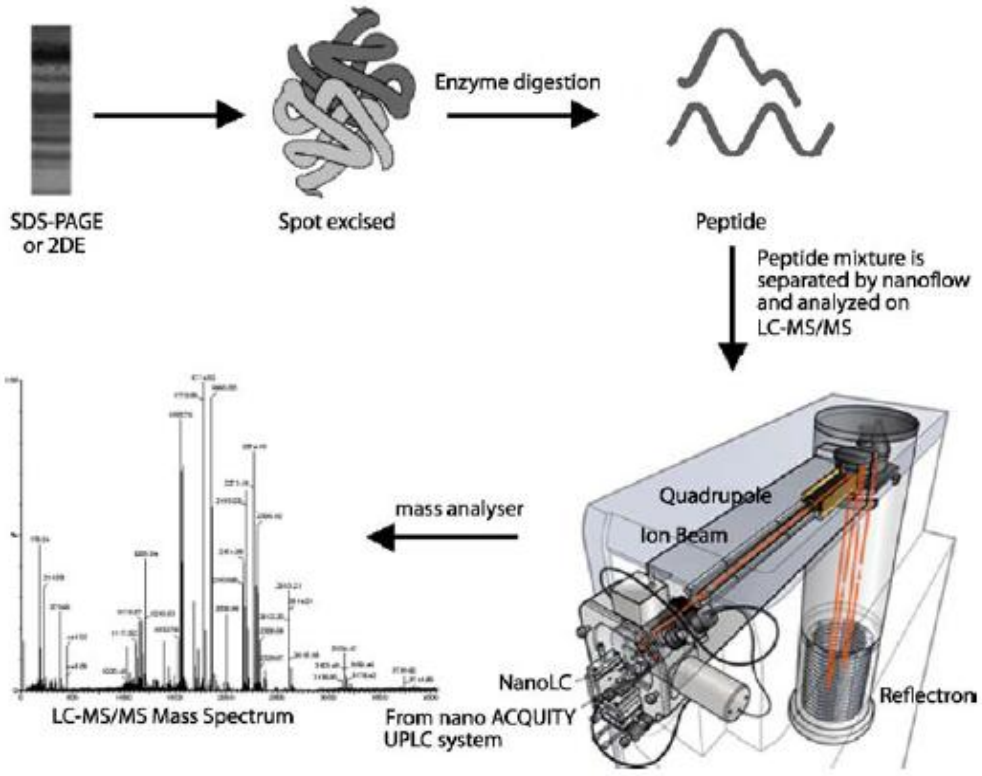

Figure 1.4. Identification of proteins with ESI-LC-MS/MS
(Source: Abu-Farha et al. 2008)

For best sensitivity, MS analysis is done in solution without separation of the purified protein complexes by SDS-PAGE. The "in-gel" strategy reduces the complexity of the samples by simplifies MS analyses, but decreases sensitivity due to poor recovery of low-abundant proteins from the gel matrix. Therefore "in-gel" method is more suitable for identification of major subunits of protein complexes Gel-free analysis is required when comprehensive identification of protein complex components is important (Kaiser et al. 2008).

1.3. Tandem-Affinity Purification Tag (TAP-Tag) Purification Systems

Most biological processes, DNA replication, RNA transcription, protein synthesis, protein degradation, molecular signaling etc., are regulated in response to intracellular or intercellular signals by multiprotein complexes rather than individual proteins. Since there are hundreds of protein complexes in the cells, their detailed

composition is not fully understood (Chang 2006). Therefore, identification of new protein-protein interactions is important to understand molecular function of proteins in cells and organisms. Mass spectrometry based proteomics combined with affinity-tag-based protein purification is one of the most effective methods to determine new protein-protein interaction (Kaiser et al. 2008).

Mass spectrometry based proteomics combined with tandem affinity purification tag (TAP-tag) is good method to identify sequences of proteins found in the protein complexes (Kaiser et al. 2008). There are several tandem-affinity tags as shown in the table 1.2. These epitope tags are hexahistidine (His6), strepII, FLAG, calmodulin-binding protein (CBP), maltose-binding protein (MBP), or protein A. In the one step purification system they are fused into either the N- or C-terminus of the coding region of the target protein in an expression vector for protein overexpression in the cells. If they are used in two step purification system, 2 tags fused together into either the N- or C-terminus of the target protein. This is called tandem affinity tags (Chang 2006).

Table 1.2. Generally prefered epitope-tags for MS-based proteomics analysis (Adapted from: Chang 2006)

Epitope tag	**Amino acid length**	**Purification method**	**Binding agent**	**Elution agent**
His	6 aa	Affinity	Ni-NTA	Imidazole
Strep II	8 aa (WSHPQFEK)	Affinity	Strep-Tactin	Desthiobiotin
FLAG	8 aa (DYKDDDDK)	Immunoaffinity	Anti-FLAG Ab	FLAG
Protein A	14 kDa	Affinity	IgG	TEV cleavage
MBP	40 kDa	Affinity	Amylose	Maltose
CBP	4 kDa	Affinity	Calmodulin	EGTA

Basically, protein of interest fused to TAP-tag is expressed in the cells and proteins that bind to target protein are purified. One or two-step purification depending on affinity interactions of the TAP-tag with its tag-specific binding agents helps to hold protein complexes on specific binding agent. TAP specific elution reagent provides isolation without disruption of target complex. Finally, obtained purified protein complexes are ready for mass spectrometer based analysis. If the isolation is done by

two step purification system, binding to specific binding reagent and elution with its specific elution reagent were done in 2 times in order. (Chang 2006; Kaiser et al. 2008)

1.3.1. Interplay Mammalian Tap Purification System

Interplay TAP systems (Agilent Technologies) is a kind of two-step purification. Two peptide tags as Streptavidin Binding Peptide (SBP) and Calmodulin Binding Peptide (CBP) allow for isolation of associated proteins without disrupting the targeted complex (Figure 1.5)**.** CBP and SBP can be fused into either the N- or C-terminus of the coding region of protein of interest (Figure1.6) (Manual of Interplay TAP system). The SBP tag (4.91 kDa), a synthetic sequence, has a high affinity for the streptavidin resin. It is effectively eluted by using biotin (Keefe et al. 2001). The CBP tag (2.96 kDa), part of a C-terminal fragment of muscle myocin light-chain kinase, has a high affinity for the calmodulin resin in the presence of calcium (Zhang, Yuan, and Vogel 1993). When calcium is removed from the environment with a chelating agent, CBP unbinds with calmodulin resin.

SBP tag	MDEKTTGWRGGHVVEGLAGELEQLRARLEHHPQGQREPSGGCKLG
CBP tag	KRRWKKNFIAVSAANRFKKISSSGAL

Figure 1.5. Amino acid composition of SBP tag and CBP tag
(Source: Manual of Interplay TAP system)

The SBP tag and the CBP tag can be eluted from their respective resins with its specific elution reagents to obtain purified protein complex (Manual of Interplay TAP system).

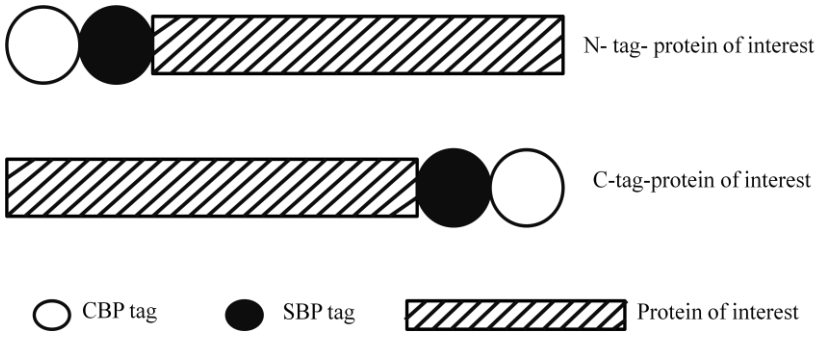

Figure 1.6. Schematic representation of N terminal and C terminal tagged protein of interest

1.4. Aim of the Project

The aim of the project is to identify cytosolic sialidase Neu2 associated proteins by mass spectrometric analysis. Neu2 enzyme has a role in different cellular events such as cancer metabolism (Tringali et al. 2007), neuronal differentiation (Fanzani et al. 2004) and myoblast differentiation (Fanzani et al. 2003), proliferation and hypertrophy (Fanzani et al. 2006). However, effects of Neu2 in these processes are not known. Therefore, in this project I intend to determine the candidate proteins working with Neu2 to carry out its cellular function.

CHAPTER 2

MATERIALS AND METHODS

2.1. Preparation of pCTAP Neu2 Expression Vector

The pCTAP expression vector in the InterPlay C-terminal Mammalian TAP System (Agilent Technologies - 240104) was used to clone and express human Neu2 gene with the SBP and CBP affinity tags.

2.1.1. Isolation of Total mRNA

Total RNA was isolated from human fibroblast cells by using trizol reagent (Life Technologies - 15596-026). Growth media was removed from 35 mm culture dish. 1 ml Trizol Reagent was added into culture dish to lyse the cells directly. Cells were lysed by pipetting several times. After incubating 5 minutes at room temperature, 0.2 ml of chloroform was added. Then it was incubated for 3 minutes at room temperature. After centrifugation at 12000 xg for 15 minutes at 4 °C, aqueous phase of the sample was removed. 0.5 ml 100 % isopropanol was added to aqueous phase placed into new tube. After the incubation at room temperature for 10 minutes, it was centrifuged at 12,000 xg for 10 minutes at 4 ^{o}C. Supernatant was removed from RNA pellet. The pellet was washed with 1 mL of 75% ethanol. After vortexing and centrifugation at 7500 xg for 5 minutes at 4 °C, supernatant was removed. RNA pellet was dried on the air for ~10 minutes. RNA pellet was resuspended in RNase-free water and incubated in water bath 55 °C for 10–15 minutes. It was stored at – 86 °C until use.

2.1.2. Preparation of Total Human cDNA

Total RNA, isolated from human fibroblast cells, was used to synthesize cDNA by random primers on RT-PCR. cDNA synthesis was carried out using RevertAid First Strand cDNA Synthesis Kit (Thermoscientific-K1621). After adding 1µl oligo $(dT)_{18}$ primer and 12 µl nuclease free water to 1 µg total RNA, the mixture was mixed gently, spin down and incubated at 65 °C for 5 min. Then, 4 µl 5X Reaction Buffer, 1 µl RiboLock RNase Inhibitor (20 u/µl), 2 µl 10 mM dNTP Mix and 1 µl RevertAid M-MuLV Reverse Transcriptase (200 u/µl) were added to the mixture. After mixing and centrifuging briefly, it was incubated for 60 min at 42 °C. The reaction was terminated by heating at 70 °C for 5 min. The prepared cDNA was stored at -86 °C until used in the PCR reaction.

2.1.3. PCR Amplification of Neu2 cDNA

Cytoplasmic sialidase Neu2 was amplified with forward primer including BamHI restriction enzyme (5'-AAAGGATCCATGGCGTCCCTTCCTGTCCTG-3') and reverse primer including HindIII restriction enzyme (5'-AAATTCGAACTGAGGCAGGTACTCAGCTGGG-3') by using total human cDNA as template and LongAmp Taq DNA Polymerase (New England Biolab -M0323). 1143 bp NEU2 (NM_005383) cDNA, obtained by using these primers, didn't include stop codon. Addition of BamHI and HindIII restriction enzyme sequences to primers resulted in 1158bp PCR product.

The PCR for Neu2 cDNA was performed with 100 ng total human cDNA in the 50 µl reaction mix including 10 µl 5X LongAmp *Taq* Reaction Buffer, 2 µl 10 µM of each primer, 1.5 µl 10 mM dNTPs and 2 µl LongAmp *Taq* DNA Polymerase. Conditions for PCR was; 1 cycle 30 seconds at 94 °C; 30 cycles 15 seconds at 94 °C, 20 seconds at 64 °C, 60 seconds at 65 °C; and 1 cycle 10 minutes at 65 °C.

2.1.4. Propagation of pCTAP Expression Vector

Interplay Mammalian TAP Kit (Agilent) includes 3 kinds of expression vectors named as pCTAP-A, pCTAP-B and pCTAP-C. pCTAP-A vector (Agilent) was suitable for Neu2 cDNA to prevent frame-shift mutations.

1 µl of pCTAP-A plasmid was transformed into chemically competent DH5α *E.coli* cells (New England Biolab - C2989K) using heat shock method. Cells were separated on 50 mg/ml kanamycin LB-Agar plates and incubated at 37 °C for 16 hours. Next day, transformant cells were selected and inoculated in 50 ml 50 mg/ml kanamycin LB-Broth for incubation at 37 °C for 16 hours. Following the overnight incubation, plasmid purification was performed using PureLink Plasmid DNA Purification Kit (Invitrogen - K2100-04).

50 ml LB culture having PCTAP A including bacteria was harvested by centrifuging at 4000 xg for 10 minutes. 4 ml Resuspention Buffer was added onto bacterial pellet. It was resuspended until obtain homogeneous mixture. 4 ml Lysis buffer was added into homogenous mixture and it was incubated for 5 minutes. After adding 4 ml precipitation buffer, it was centrifuged at 16000 xg for 10 minutes.

10 mL Equilibration Buffer was put onto the column to equilibrate the column. The solution was flowed by gravity.

Supernatant was load onto equilibrated column and flowed by gravity. Column including pCTAP-A plasmid washed twice with 10 ml wash buffer. Plasmid was eluted with 5 ml elution buffer. After addition of 3.5 ml isopropanol, it was centrifuged at 16000 xg for 30 min at 4 °C. Plasmid pellet was resuspended in 3 ml 70 % ethanol and it was centrifuged at 16000 xg for 5 minutes at 4 °C. After plasmid pellet was dried in air, pCTAP-A plasmid was resuspeded in 150 µl TE buffer.

pCTAP-A plasmid was confirmed using HindIII-HF enzyme (New England Biolabs - R3136). pCTAP vector was digested in a 50 µl reaction mix containing 1 ug pCTAP vector, 1 µl BamHI-HF (New England Biolabs - R3136) enzyme, 5 µl 10X NEBuffer by incubation at 37 °C for 3 hour. Then it was loaded on 1% agarose gel.

2.1.5. Ligation of Human Neu2 cDNA into pCTAP Expression Vector

cDNA of Neu2 was cloned into PCTAP vector from BamHI (5' of cDNA) and HindIII (3' of cDNA) restriction sites with direct cloning method (Figure 2.1). The reason of that they are noncutter to Neu2 cDNA according to RestrictionMapper digestion tool (http://www.restrictionmapper.org). BamHI and HindIII, sticky end restriction enzymes, were chosen to increase the cloning efficiency.

PCR product and pCTAP vector were digested in a 50µl reaction mix containing 15 µl of 50 µl PCR product or 1 µg pCTAP vector, 1 µl BamHI-HF (New England Biolabs - R3136) and 1µl HindIII-HF (New England Biolabs - R3104) enzyme, 5 µl 10X NEBuffer by incubation overnight at 37 °C.

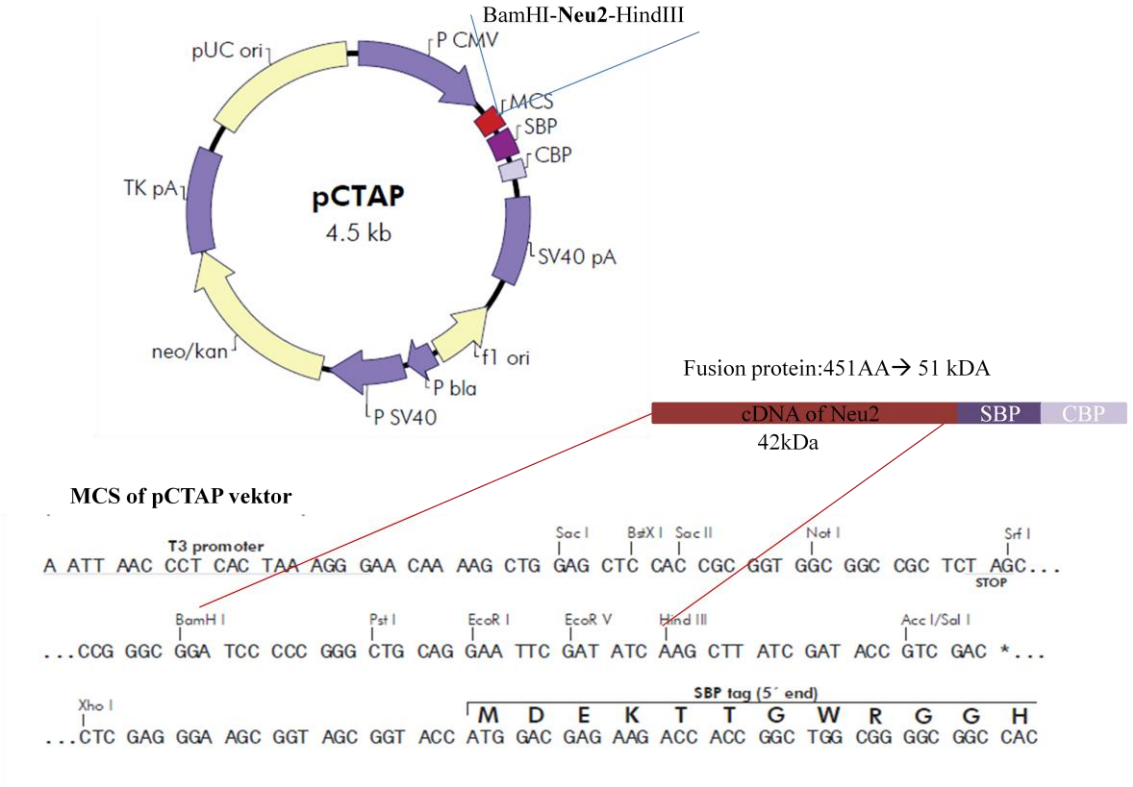

Figure 2.1. Cloning strategy of Neu2 cDNA into pCTAP vector

After digestion, PCR product and pCTAP vector were run on 0.8% agarose gel. After the visualization of PCR product under ultraviolet (UV) light, DNA fragment was cut from gel and isolated by using PureLink Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen - K2200-01). 3 volumes of Gel Solubilization Buffer was added

for every 1 volume of gel and placed into a 50 °C water bath until all gel dissolved. 1 gel volume of 100% isopropanol was added into dissolved gel slice.

After dissolved gel piece containing Neu2 cDNA and pCTAP vector were put into the center of a Spin Column inside a wash tube, it was centrifuged the tube at 16000 xg for 1 minute. The flow-through was discarded and 500 µl wash buffer was added. After centrifugation at 16000g two times, Neu2 cDNA was obtained with 50 µL Elution Buffer.

Concentrations of DNA samples were measured by using nanodrop (NanoDrop Technologies, Inc. ND-1000 spectrophotometer). Neu2 cDNA and pCTAP-A digested with BamHI and HindIII were ligated with T4 DNA Ligase (New England Biolabs - M0202). Vector:insert ratio in the ligation mixture was 1:3. Ligation mixture was including 20 ng pCTAP vector and 26 ng Neu2 cDNA, 2 µl T4 DNA ligase buffer, 1 µl T4DNA ligase. Ligation mixture was incubated at 16 °C overnight.

After ligation reaction completed 3 µl of ligation mixture were transformed into chemically competent DH5α E.coli cells (New England Biolab - C2989K) using heat shock method. Cells were separated on 50 mg/ml kanamycin LB-Agar plates and incubated at 37 °C for 16 hours. Next day, transformant cells were selected and inoculated in 5 ml 50 mg/ml kanamycin LB-Broth for incubation at 37 °C for 16 hours. Following the overnight incubation, plasmid purification was performed using alkaline lysis plasmid purification method.

Table 2.1. Solutions of alkaline lysis plasmid purification method

Solution I	**Solution II**	**Solution III**
50 mM Glucose	500 µl 0.4 N NaOH	60 ml 5M Potassium Acetate
25 mM Tris-HCl pH=8.0	100 µl 10% SDS	11.5 ml Glacial Acetic Acid
10 mM EDTA pH=8.0	400 µl water	28.5 ml water

1.5 ml of 5 ml LB culture was poured into micro centrifuge tube and centrifuged at 12000 xg for 30 second at 4 °C. After drying of bacterial pellet, it was resuspeded in 100 µl of ice-cold Solution I (Table 2.1) and 200 µl freshly prepared Solution II was added on it. It was mixed without vortex and 150 µl ice-cold Solution III was put on it. Mixture was vortexed and stored at ice for 5 minutes and centrifuged at 12000 xg for 5 minutes at 4 °C. 2 volumes of 100% ethanol were added on supernatant. Then, it was

vortexed and waited for 2 minutes at room temperature. After centrifugation at 12000 xg for 5 minutes at 4 °C, pellet was rinsed with 1 ml of 70% ethanol at 4 °C. Following the centrifugation at 12000 xg for 5 minutes at 4 °C, air dried pellet was dissolved in 20 µl water including 1 µl 10 mg/ml RNase.

Plasmid was confirmed by double digestion with NotI–HF (New England Biolabs - R3189S) and XhoI (New England Biolabs - R0146S) restriction enzymes. In 50 µl digestion mixture 2 µl plasmid DNA, 1 µl NotI-HF, 1 µl XhoI, 5 µl 10X NEBuffer, 0.5 µl 100X BSA and 40.5 µl ultrapure water was included. Secondly, plasmid was digested in a 50 µl reaction mix containing 1 ug pCTAP vector, 1 µl BamHI-HF (New England Biolabs - R3136) and 1 µl HindIII-HF(New England Biolabs - R3104) enzyme, 5 µl 10X NEBuffer and 41 µl ultrapure water by incubation overnight at 37 °C for 4 hours. Finally, digestion mixture was run on 1% agarose gel. Plasmid including human Neu2 cDNA was selected.

2.1.6. Sequence Analysis of pCTAP Neu2 Plasmid

After cloning of Neu2 cDNA into pCTAP plasmid, it was sequenced with T3 primer in Izmir Institute of Technology, Biotechnology and Bioengineering Research and Application Center in order to see whether there are errors in DNA sequence or stop codon removal. T3 primer binding site is 5′ AATTAACCCTCACTAAAGGG 3′.

2.1.7. Propagation of pCTAP Neu2 Expression Vector

Large scale inoculation for pCTAP Neu2 was prepared in 500 ml LB-Brotth containing 50 mg/ml kanamycin at 37 °C for 16 hours. After incubation, plasmids were purified with PureLink HiPure Plasmid Filter MaxiPrep Kit (Invitrogen - K210017).

Column provided by the kit was equilibrated with 30 mL Equilibration Buffer by gravity flow.

Bacteria in 500 mL LB culture were harvested by at 4000 xg for 10 minutes. After addition of 10 mL Resuspension Buffer to pellet, cells were resuspended until homogeneous. By adding 10 ml Lysis Buffer to homogeneous samples, it was incubated at room temperature for 5 minutes. After addition of 10 ml Precipitation Buffer, it was put onto equilibrated filter column. Lysate was passed through filter by gravity flow.

Filter cartridge was removed and colomn including plasmid DNA was wash with 50 mL Wash Buffer by gravity flow.

Plasmid DNA was eluted with 15 ml elution buffer in to 30 ml ultracentrifuge tube. After addition of 10.5 ml isopropanol, it was centrifuged at 16000 xg for 30 minutes at 4 °C. 5 ml 70% ethanol was added onto DNA pellet. It was centrifuged again at 16000 xg for 5 minutes. DNA pellet was dried in air. DNA pellet was resuspended in 300 µl TE Buffer. DNA concentration was measured by using nanodrop.

2.2. Transfection of pCTAP Neu2 to Mammalian Cell Lines for Optimization

pCTAP NEU2 does not include GFP or similar sequence which provides visual detection. Therefore, pEGFP-N2, a Green Fluorescent Protein (GFP) expressing plasmid, was used to determine optimum transfection reagent and vector ratio in our laboratory conditions. pEGFP-N2 was used as experimental control because after the transfection of pEGFP-N2 to mammalian cell lines such as Cos-7 cells, HeLa cells and Hek 293 cells, transfection efficiency could be observed under the fluorescence microscopy.

2.2.1. Subculturing of Mammalian Cell Lines

Frozen mammalian cell lines in different vial, stored at liquid nitrogen, were thawed in 37 °C water bath approximately 2 minutes. Then the thawed vial was decontaminated by spraying with 70% ethanol. After transferring vial contents into 9 ml full growth medium (DMEM (Dulbecco's Modified Eagle's Medium - Lonza - BE12 - 604F), 10% FBS (Fetal Bovine Serum - Lonza – DE14 - 801F), and 100 Units/ml Penicilin and 100 µg/ml Streptomysin (Gibco – 15140 - 122)), it was centrifuged at 800 rpm for 5 minutes. Cell pellet was resuspended with 5 ml full medium and dispensed into 25 cm^2 culture flask. It was incubated at 37 °C and 5% CO_2 incubator condition.

Medium of the cells was renewed every 2 or 3 days. When they come to 90%-100% confluence, it was subcultured at required amount. Culture medium was discarded. Cell layer was rinsed with 1XPBS (Phosphate Buffered Saline - Thermo

Scientific Hyclone - SH30258.02). 1 ml 0.25% (w/v) Trypsin - EDTA solution (Gibco-25200-056) was added to flask until cell layer was dispersed. 2 ml full medium was added onto dispersed cells and centrifuged at 800 rpm for 5 minutes. Cell pellet was resuspended with full medium and dispensed into new culture flask with 1:8 subculturing ratio. It was incubated at 37 °C and 5% CO_2 incubator. This subculturing procedure was continued throughout the transfection experiment in order to provide required amount of cells.

2.2.2. Transfection of pEGFP-N2 Vector into Mammalian Cell Lines by Polyethylenimine (PEI)

Transfection optimization was done separately for three types of mammalian cell lines such as Cos-7, HeLa and Hek 293 cell lines by using 6 well plates. On the day of transfection, cells were at 60% to 80% confluence. 2 ml full medium was changed with 1 ml FBS-free medium and put into 37 °C in 5% CO2 incubator. In order to obtain optimum PEI (Sigma-764647-1G):DNA ratio DNA and PEI was mixed at different concentrations (Table 2.2). 1 µg/ml PEI was put into 100 µl 150 mM NaCl in the first tube. Plasmid DNA was put into 100 µl 150 mM NaCl in the second tube. After tubes were vortexed and spinned, 15 minutes waited. Then, first tube was mixed with second tubes. After vortex and spin, 15 minutes waited again. PEI: DNA mixture was added to each well containing 1 ml FBS-free medium. After 6 h incubation at 37 °C in 5% CO2, 1 ml of medium containing 20% FBS was added to each well. After the 48 hour post transfection, transfection efficiency was determined under the fluorescence microscopy.

Table 2.2. DNA and PEI amount used for transfection optimization with pEGFP-N2

	1. well	**2. well**	**3.well**	**4. well**	**5. well**	**6. well**
DNA(µg)	0 µg	1 µg	2 µg	2 µg	3 µg	3 µg
PEI(1µg/ml)	9 µl	3 µl	4 µl	6 µl	6 µl	9 µl
DNA:PEI	0	1:3	1:2	1:3	1:2	1:3

2.2.3. Transfection of pCTAP Neu2 Expression Vector into Cos-7 Cells

pCTAP NEU2 expression vector, including NEU2 ORF tagged at the C-terminus with SBP and CBP tags, was transfected with the best DNA:PEI ratio obtained from pEGFP-N2 transfection. Same transfection steps were used as explained in part 2.2.2.

2.3. Identification of Recombinant NEU2 Protein by Western Blotting

2.3.1. Total Protein Isolation

After 48 hour post transfection with pCTAP NEU2, each well of 6 well-plate were washed two times with 1 ml 1X PBS. After harvesting by using steril scraper, cells were centrifuged at 800 rpm for 5 minutes. 25 µl lysis buffer for 1 well of 6 well plate was added onto pellet. Following the 1 hour on ice, it was centrifuged at 4 °C and 16000 rpm for 10 min. Obtained supernatant consisted of total protein isolated from pCTAP NEU2 transfected Cos-7 cells.

In the 10 ml lysis buffer there were 1% Triton X-100, 50 mM HEPES, 88 mg 150 mM NaCl, 10% Gycerol, 50 mM Tris. For 1 ml Lysis buffer 10 µl of a protease inhibitor cocktail (Sigma - P8340) and 10 µl of 100 mM of PMSF (Sigma - P7626) was added prior to using.

2.3.2. Western Blotting

2.3.2.1. Calculation of Protein Concentration

Protein concentration was determined with Bradford protein assay by using Coomassie Brilliant Blue G-250. Firstly, Bovine serum albumin (BSA) was prepared at different concentration by serial dilution 200 µg/ml, 150 µg/ml, 100 µg/ml, 50 µg/ml, 25 µg/ml, 10 µg/ml from 400 µg/ml BSA stock (Table 2.3). 10 µL of each

standard or unknown sample were pipetted into the appropriate 96 well-plate. 90 µL Coomassie Reagent was added to each well. After waiting 5 minutes, absorbance was measured at 595 nm wavelength and concentrations of isolated protein were determined using BSA standard graphic.

Table 2.3. Preparation of diluted Bovine Serum Albumin (BSA) standards

Vial	**Volume of Water**	**Volume and Source of BSA**	**Final BSA Concentration**
1	500 µl	500 µl of Stock 400 µg/ml	200 µg/ml
2	500 µl	500 µl of Vial 1 200 µg/ml	100 µg/ml
3	500 µl	500 µl of Vial 2 100 µg/ml	50 µg/ml
4	500 µl	500 µl of Vial 3 50 µg/ml	25 µg/ml
5	600 µl	400 µl of Vial 4 25 µg/ml	10 µg/ml

Coomassie Reagent was prepared by dissolving 50 mg of Commassie Brilliant Blue G-250 in 50 ml methanol and then adding 100 ml 85% (w/v) phosphoric acid (H_3PO_4) and 850 ml H_2O and filtering by using Whatman #1 paper. It was stored in a dark bottle at 4 °C.

2.3.2.2. Western Blotting

Western blot protocol was optimized firstly in our laboratory by using β-Actin antibody (Santa Cruz Biotechnology - sc-130657) to detect housekeeping gene β-Actin. Then the expresion of Neu2 protein on the mammalian cell line was detected by using Anti-Calmodulin Binding Protein Epitope Tag Antibody (Millipore - 07-482).

SDS-PAGE gel was prepared 5% stacking gel (3.5 ml dH_2O, 1.5 ml upper buffer, 1 ml %30 Acrylamide (Sigma), 60 µl 10% SDS (Applichem), 60 µl 10% APS (Applichem), 6 µl TEMED (Sigma)) and 10% resolving gel (5 ml dH_2O, 3 ml lower buffer, 4 ml %30 Acrylamide (Sigma), 60 µl 10% SDS (Applichem), 60 µl 10% APS (Applichem), 6 µl TEMED (Sigma))

20 µg protein was mixed with protein loading dye as protein:loading dye ratio at 4:1 and heated at 100 °C at 5 minutes. Mock transfected and pCTAP Neu2 transfected proteins were loaded at the same concentration to SDS page. It was migrated for 6 hours

at 60 V as vertical electrophoresis in running buffer. All of the solutions required during western blotting were shown in the Table 2.4.

Table 2.4. Preparation of western blotting solutions

Western Blotting Solutions	**Ingredient**
Running Buffer	0.25M Tris, 1.92 M Glycine, 1% SDS (w/v). Stored at 4 °C.
Transfer Buffer	48mM Tris, 39mM Glycine, 20% Ethanol (v/v), 1L dH_2O pH 9.2. Stored at 4 °C.
Washing Buffer	1X PBS, 0.05% Tween20. Stored at 4 °C.
Blocking Buffer	0.5% non-fat dry milk in washing buffer. Prepared freshly before use.
PBS	0.2 M NaH_2PO_4 was added to 250 ml 0.2 M Na_2HPO_4 until pH 7.4. 250 ml of this mixture was mixed with 250 ml 45 g including NaCl solution. Stored at room temperature.
Loading Dye	4% SDS, 10% 2-Mercaptoethanol, 20% Glycerol, 0.004% Bromophenol Blue, 0.125M Tris-HCl, pH 6.8.
Ponceau S solution	0.1% Ponceau S (w/v) in 5% Acetic acid

After electrophoresis, nitrocellulose membrane was activated by incubating in transfer buffer ~10 minutes at room temperature. SDS-PAGE gel including proteins was placed between filter paper and nitrocellulose membrane (Roche) in order to transfer proteins from gel to nitrocellulose membrane. After transfer, proteins were transferred to from nitrocellulose membrane at 60 V +4 °C for 3 hours.

Membrane was dyed Ponceau S (Sigma) dye to test whether all proteins were transfred corectly and washed with washing buffer. Membrane was blocked at 55 rpm shaker and room temperature for 1 hour using 10 ml blocking buffer.

After blocking, membrane was incubated with 10 ml of 10% blocking buffer with 2 µl β-Actin primary antibody or 2 µl Anti-Calmodulin Binding Protein Epitope Tag primary antibody at 55 rpm shaker and room temperature for 1 hour. Membrane was washed 5 times with 10 ml washing buffer for 5 minutes at 55 rpm and room

temperature. Membrane was incubated in 10 ml of 10% blocking buffer with 2 µl goat anti-rabbit IgG-HRP (Horseradish peroxide) secondary antibody (Santa Cruz - sc-2004) at 55 rpm shaker and room temperature for 1 hour. Membrane was washed 5 times with 10 ml washing buffer for 5 minutes at 55 rpm and room temperature.

Finally, 500 µl enhanced chemiluminescent substrate for HRP (Thermo) and 500 µl Enhancer Solution (Thermo) were spread on the membrane and incubated for 2 minutes at dark. Membranes releasing chemiluminescence were visualized using Gel Imaging System (Bio-Rad, Versadoc 4000MP) in Izmir Institute of Technology, Biotechnology and Bioengineering Research and Application Center.

2.4. Recombinant Neu2 Purification with TAP Purification System

Following 48 hour post transfection with pCTAP NEU2, cells was harvested and proteins were purified using InterPlay Mammalian TAP System (Agilent Technologies). In this system purification was done with Streptavidin resin followed by Calmodulin resin (Figure 2.2). Gentle washing and elution conditions allowed the protein–protein interactions to remain intact.

2.4.1. Transfection with pCTAP Neu2 Expression Vector into Cos-7 Cells

One day before the transfection, trypsinized Cos-7 cells were spread into 10X150 mm cell culture dishes. On the day of transfection, cell layer was 60% to 80% confluence and 20 ml full medium was replaced with 10 ml FBS-free medium and put into 37°C in 5% CO2 incubator for 30 min. Transfection of pCTAP Neu2 was obtained at the optimum PEI : DNA ratio. 126 µl 1µg/ml PEI was mixed 1400 µl 150 mM NaCl in the first tube. 42 µg plasmid DNA was mixed 1400 µl 150 mM NaCl in the second tube. After tubes were vortexed and spinned, 15 minutes waited. Then, PEI mixture in the first tube was mixed with DNA mixture in the second tubes. After vortex and spin, 15 minutes waited again. PEI-DNA mixture was added to each 150 mm cell culture dish including 10 ml FBS-free medium. After 6 h incubation at 37 °C in 5% CO2, 10 ml of medium containing 20% FBS was added to each well.

2.4.2. Purification of Neu2 and Neu2 Associated Proteins with TAP Purification System

Following 48 h post transfection with pCTAP Neu2, medium was removed from the culture dish. Cells were harvested by using steril scraper, washed with 1X PBS three times and collected to 30 ml polypropylene ultracentrifuge tubes.

Cells, resuspended in 10 ml lysis buffer, were subjected to three times freeze-thawing by incubating the cells in a –80 °C freezer for 20 minutes to freeze the cells, followed by incubating the cells in cold water to thaw the cells. Following the centrifugation at 16000 xg for 10 minutes, 40 µl 0.5 M EDTA and 7 µl 14.4 M β-Mercaptoethanol were put into supernatant. Then, 500 µl washed Streptavidin resin was added and tube was rotated at 4 °C for 2 hours to allow the tagged proteins to bind to the Streptavidin resin. Resin was obtained by centrifugation at 1500 xg for 5 minutes. It was washed two times in 1 ml of SBB by rotating the tube at 4 °C for 5 minutes. After washing, resin was collected by centrifugation at 1500 xg for 5 minutes. Following the addition of 1 ml SEB on to resin, it was rotated at 4 °C for 30 minutes to elute the protein complexes. Resin was collected by centrifugation at 1500 ×g for 5 minutes. Supernatant, including first step purified proteins, was transferred carefully to a fresh tube.

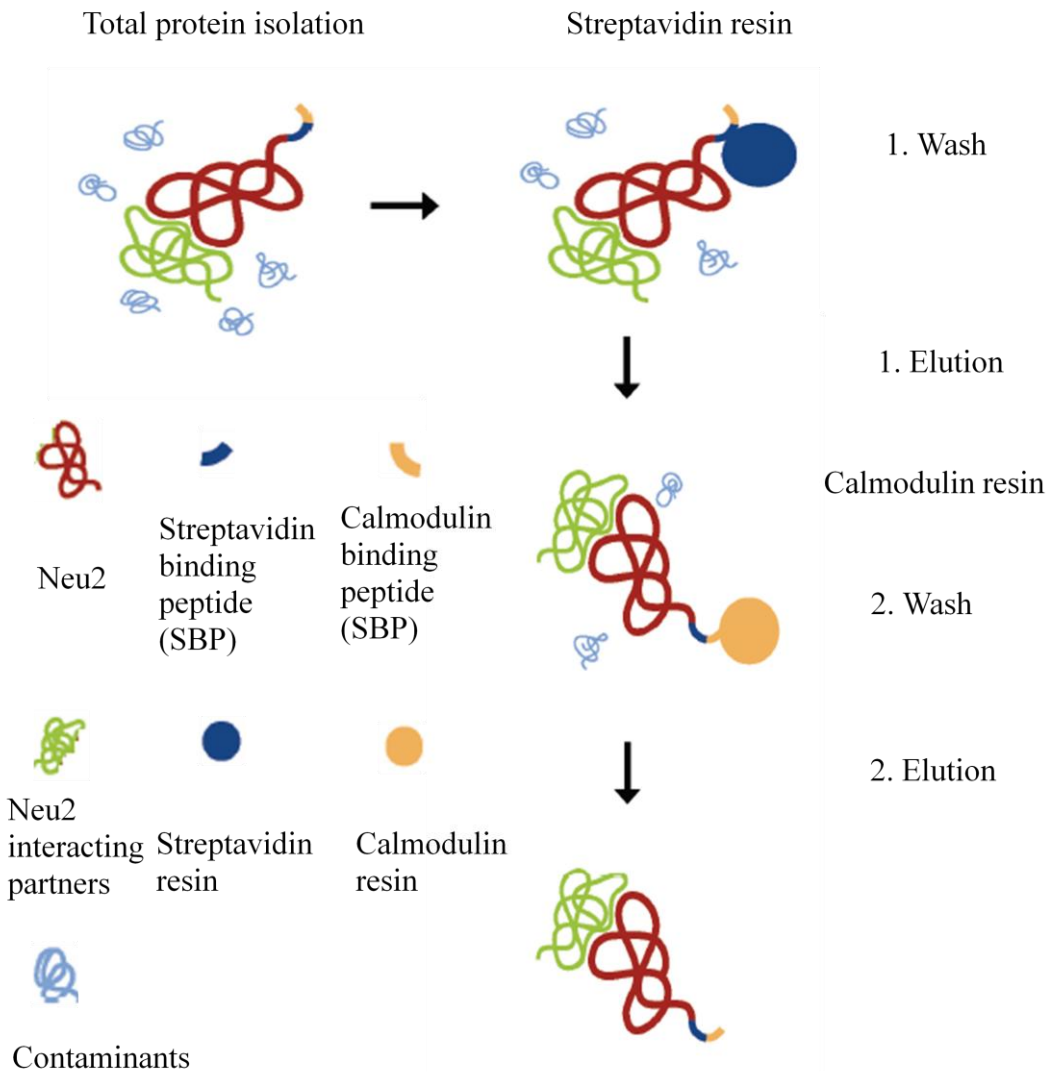

Figure 2.2. Shematic representation for purification of Neu2 associated proteins by InterPlay Mammalian TAP System

20 µl Streptavidin supernatant supplement and 4 ml CBB were added to the supernatant sequentially. After the addition of 250 µl washed Calmodulin resin, it was rotated at 4 °C for 2 hours to allow the protein complexes to bind to Calmodulin resin. Resin was recovered by centrifugation at 1500 xg for 5 minutes. It was washed two times with 1 ml of CBB by rotating the tube at 4 °C for 5 minutes. Following the centrifugation at 1500 xg for 5 minutes, protein complexes were eluted in 500 µl CEB by rotating tube at 4°C for 30 minutes. Recombinant Neu2 and its candidate associated protein complexes were obtained following the centrifugation at 1500 ×g for 5 minutes. Protein complexes were stored at -20 °C until using.

Table 2.5. Preparation of the reagents for isolation protocol

Reagent	**Procedure**
Lysis buffer	10 ml lysis buffer was prepared for isolation of transfected cell in 10X150 mm cell culture dish. 100 µl of a protease inhibitor cocktail (Sigma - P8340) and 100 µl of 100 mM of PMSF (Sigma - P7626) was added.
Streptavidin binding buffer (SBB)	SBB was prepared by adding 3.15 µl of 14.4 M β-Mercaptoethanol to 4.5 ml of SBB. In order to prevent protein degradation, 45 µl of protease inhibitor cocktail and 45 µl of 100 mM of PMSF was added.
Streptavidin elution buffer (SEB)	SEB must be prepared by adding 0.7 µl of 14.4 M β-Mercaptoethanol to 1 ml SEB. To prevent protein degradation, 10 µl of protease inhibitor cocktail and 10 µl of 100 mM was added to the mixture.
Calmodulin binding buffer (CBB)	CBB was prepared by adding 5.7 µl of 14.4 M β-Mercaptoethanol to 8.125 µl CBB. To prevent protein degradation, 81.25 µl of protease inhibitor cocktail and 81.25 µl of 100 mM of PMSF was added.
Calmodulin elution buffer (CEB)	CEB was prepared by adding 0.35 µl of 14.4 M β-Mercaptoethanol to 500 µl of CEB.
Streptavidin Resin	500 µl 50% streptavidin resin slurry was centrifuged at 1500 xg for 5 minutes. Resin was washed in 1 ml of SBB 2 times. After centrifugation at 1500 xg for 5 minutes, the resin was resuspended in 250 µl SBB.
Calmodulin Resin	250 µl 50% streptavidin resin slurry was centrifuged at 1500 xg for 5 minutes. Resin was washed in 1 ml of CBB 2 times. After centrifugation at 1500 xg for 5 minutes, the resin was resuspended in 125 µl CBB.

2.5. Identification of NEU2 Associated Proteins

2.5.1. Silver Staining of Tandem Affinity Purified Protein Complexes

After the purification of Neu2 and its associated proteins, they were observed on SDS gel by silver staining procedure. SDS gel (5% stacking phase and 10% resolving phase) was prepared and was loaded at this following order Marker / Total proteins / Streptavidin Elution proteins / Calmodulin Elution proteins. They were migrated at 60 V for 4 hours.

Gel was incubated in fixer solution for at least 1 hour. It was washed with 50% Ethanol 3 times for 20 minutes. After incubation in pretreatment solution for 1 min, it was washed in dH_2O 3 times for 20 seconds. After the incubation in developing solution until all purified proteins were visiable, developing solution was changed with stop solution. All of the solutions required for silver staining was shown in the table 2.6.

Table 2.6. Solutions required for Silver Staining of the gels

Solution	**Procedure**
Fixer Solution	150 ml Methanol, 36 ml Acetic Acid and 150 µl 37% Formaldehyde were mixed and completed to 300 ml with dH_2O
Pretreatment Solution	0.08 g $Na_2S_2O_35H_2O$ was dissolved in dH_2O
Silver Nitrate Solution	0.8 g Silver Nitrate and 300 µl 37% Formaldehyde was dissolved in dH_2O
Devoloping Solution	9 g potassium carbonate, 300 µl Formaldehyde and 8 ml from pretreatment solution completed to 400 ml with dH_2O
Stop Solution	200 ml Methanol and 48 ml Acetic Acid competed to 400 ml with dH_2O

2.5.2. Determination of Presence of Neu2-Tag Protein in the Purified Protein Sample by Western Blotting

After the purification of Neu2 and its associated proteins, presence of Neu2-tag protein through Streptavidin elution and Calmodulin elution step was confirmed with western blotting method with Anti-Calmodulin Binding Protein Epitope Tag Antibody. SDS gel (5% stacking phase and 10% resolving phase) was prepared and was loaded at this following order Marker / Total proteins / Streptavidin Elution proteins / Calmodulin Elution proteins. They were migrated at 60 V until the all marker separated.

Then proteins were transferred onto a nitrocellulose membrane at 60 V 4 °C for 3 hours. The membrane was blocked at 55 rpm shaker and room temperature for 1 hour using 10 ml blocking buffer. After blocking, the membrane was incubated with 10 ml 10% blocking buffer with 2 µl Anti-Calmodulin Binding Protein Epitope Tag Antibody at 55 rpm shaker and room temperature for 1 hour. At the next step, the membrane was washed 5 times with 10 ml washing buffer for 5 minutes at 55 rpm and room temperature. After that, membrane was incubated in 10 ml 10% blocking buffer with 2 µl Goat Anti-Rabbit IgG-HRP secondary antibody at 55 rpm shaker and room temperature for 1 hour. Membrane was washed 5 times with 10 ml washing buffer for 5 minutes at 55 rpm and room temperature.

At the last step, Neu2-tag protein was visualized using Gel Imaging System by using 500 µl enhanced chemiluminescent substrate for HRP and 500 ul Enhancer Solution.

2.5.3. Mass Spectroscopic Analysis

In order to perform mass spectrometric analysis of Neu2 protein complexes purified with InterPlay Mammalian TAP System, we made collaboration with Dr. Ahmet Tarık Baykal from Gene Engineering and Biotechnology Institute, TUBITAK.

Analysis was done in TUBITAK-MAM by using ESI-LC-MS/MS analysis. Firstly, overnight dialysis method was used to concentrate proteins with 3-7 IDa cutoff value. Next day, it was centrifuged at 15000 xg for 10 minutes. After the measurements of protein concentration with bradford reagent, 50 µg protein was added into a test tube and the total volume was fixed to 50 µl with water. The resulting protein mixture was

incubated at 80 °C for 15 minutes. After the addition of 100 mM DTT (Dithiothreitol) in 50 mM ammonium bicarbonate to protein mixture, it was incubated at 60 °C for 15 minutes. 200 mM IAA (Iodoacetamide) in 50 mM ammonium bicarbonate was put into protein mixture and it was incubated at room temperature in dark. As a positive control, enolase yeast protein was added to mixture. Finally, 1 μg of trypsin in 50 mM ammonium bicarbonate was added and incubated at 37 °C overnight.

50 μg trypsinized proteins were used to analyze in ESI-LC-MS/MS. After addition of 2 ul TFA (Trifluoroacetic acid) and 2 μl ACN (Acetonitril) to 50 μg trypsinized proteins, these peptides were separated using acetonitril gradient (5-40%) for 90 minutes by using nano Acquity UPLC (Ultra-High Performance Liquid Chromatography) system. Finally, mixture was analyzed in HDMS (High Definition Mass Spectrometry) to determine the proteins in Neu2 protein complex.

2.5.4. Bioinformatic Analysis

In the mass spectrometry based proteomics firstly a set of spectra was obtained by ms/ms spectrometer and store peak information then each spectrum was examined against a list of proteins sequences by database search software. During my thesis ProteinLynx Global Server (PGLS), Mascot and X!Tandem were used to determine the protein in the Neu2 protein complexes.

2.5.5. Conformation of Neu2 Interacting Protein by Western Blotting

After the purification of Neu2 protein complex and mass spectrometric analysis of these proteins, β-Actin protein was showed as candidate protein to interact with Neu2. In order to check the results of MS/MS analysis, SDS gel (5% stacking phase and 10% resolving phase) was prepared and was loaded at this following order Marker / Total proteins / Streptavidin Elution proteins / Calmodulin Elution proteins. They were migrated at 60 V until the all marker separated. Then proteins was transferred to nitrocellulose membrane at 60 V 4 °C for 3 hours. Then, membrane was blocked at 55 rpm shaker and room temperature for 1 hour using 10 ml blocking buffer. After blocking, membrane was incubated with 10 ml 10% blocking buffer with 2 μl β-Actin primary antibody at 55 rpm shaker and room temperature for 1 hour. At the next step,

membrane was washed 5 times with 10 ml washing buffer for 5 minutes at 55 rpm and room temperature. After that, membrane was incubated in 10 ml 10% blocking buffer with 2 µl Goat Anti-Rabbit IgG-HRP secondary antibody (Santa Cruz - sc-2004) at 55 rpm shaker and room temperature for 1 hour. Membrane was washed 5 times with 10 ml washing buffer for 5 minutes at 55 rpm and room temperature.

At the last step, 500 µl enhanced chemiluminescent substrate for HRP and 500 ul Enhancer Solution were spread on the membrane and incubated for 2 minutes at dark. Membranes were visualized using Gel Imaging .

CHAPTER 3

RESULTS

3.1. Cloning of cDNA of Neu2 into pCTAP Expression Vector

Cytoplasmic sialidase Neu2 was amplified with forward primer (5'-AAAGGATCCATGGCGTCCCTTCCTGTCCTG-3') including BamHI restriction enzyme and reverse primer (5'- AAATTCGAACTGAGGCAGGTACTCAGCTGGG-3') including HindIII restriction enzyme. 1143 bp NEU2 (NM_005383) cDNA, obtained by using these primers, didn't include stop codon. By the addition of BamHI and HindIII restriction enzyme sequences to primers resulted in 1158 bp PCR product which included 1143 bp NEU2 cDNA and didn't include stop codon (Figure 3.1).

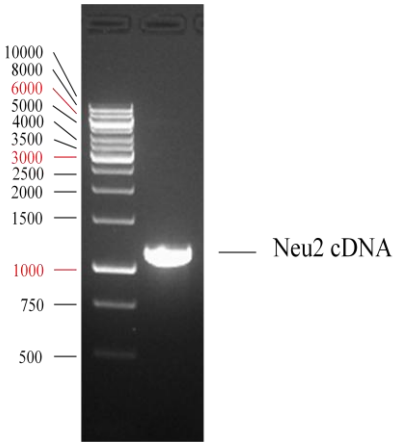

Figure 3.1. Agarose gel electrophoresis of PCR amplification of 1158 bp Neu2 cDNA

PCR amplification of 1158 bp Neu2 cDNA was obtained. Then, it was cloned into 4500 bp pCTAP expression vector. Prepared pCTAP Neu2 expression vector was conformed with NotI/XhoI and BamHI/HindIII double digestions. As a result of double digestion ~1600 bp Neu2 and ~4500 bp pCTAP expression vector was obtained. When the Neu2 cDNA is placed into pCTAP expression vector, NotI and BamHI is at the upstream site of the cDNA and XhoI and HindIII is at the downstream region of cDNA (Figure 3.2)(Figure 3.3). All the enzymes are noncutter for cDNA of Neu2.

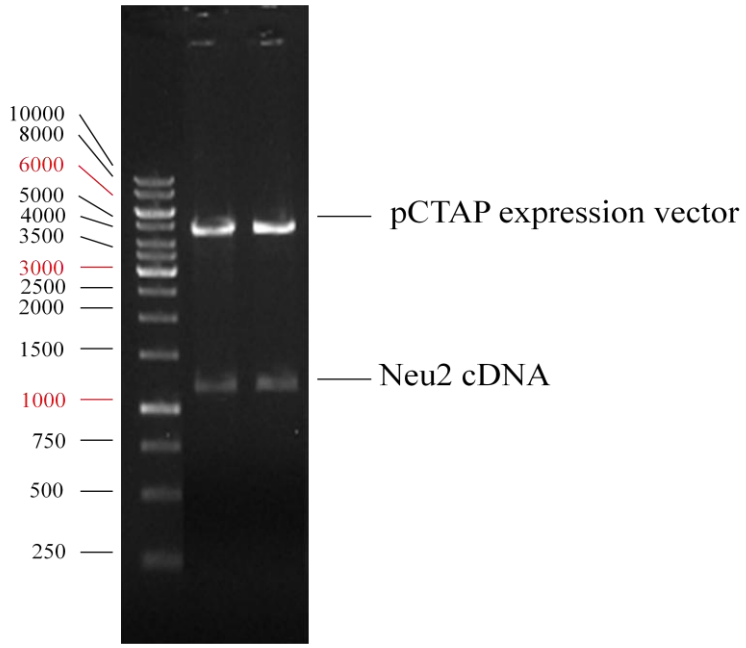

Figure 3.2. Agarose gel electrophoresis of XhoI/Not and BamHI/HindIII double digestions of pCTAP Neu2 expression vector. Gel images shows ~ 4500 bp fragment of pCTAP vector and ~1160 bp Neu2 cDNA

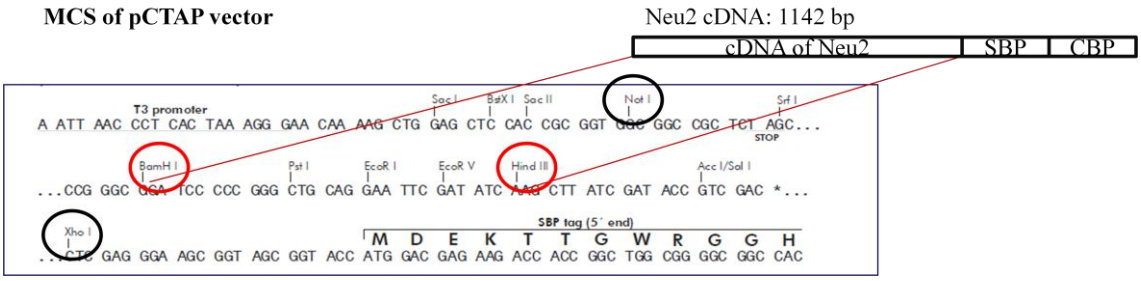

Figure 3.3. Schematic diagram show the position of Neu2 cDNA in pCTAP vector

After producing pCTAP-Neu2 plasmid, it was sequenced in Izmir Institute of Technology, Biotechnology and Bioengineering Research and Application Center in order to see whether there are errors in DNA sequence or stop codon removal. It was observed that there was no error in the DNA sequence. Removal of stop codon was figured out (Figure 3.4). Glutamine, the last amino acid found on the Neu2 cDNA sequence, was followed by HindIII restriction enzyme coming from reverse primer. Methionine is the start codon of Streptavidin Binding Peptide (SBP). The sequence between SBP start codon and HindIII restriction enzyme was the expected sequences coming from multiple cloning site of the original pCTAP vector.

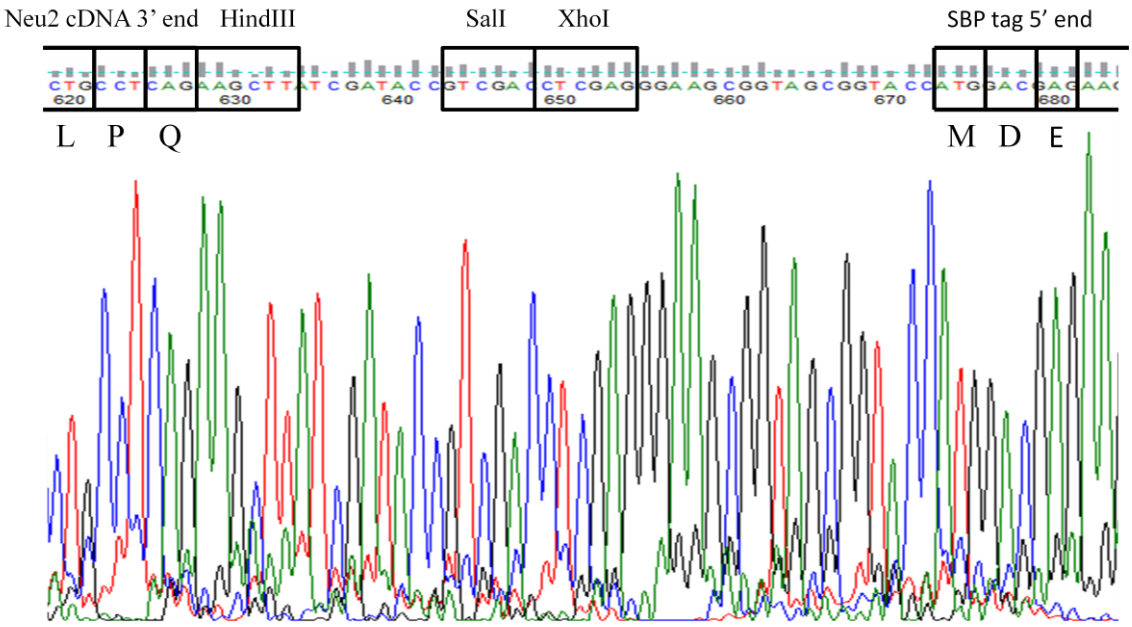

Figure 3.4. Sequence analysis of pCTAP Neu2. L: Leucine, P: Proline, Q: Glutamine, M: Methionine, D: Aspartic acid, E: Arginine. HindIII, SalI and XhoI are the restriction region between Neu2 3'end and Streptavidin Binding Peptide (SBP) tag 5'end

3.2. Transfection Optimization with pEGFP-N2 Vector on Mammalian Cell Lines

pCTAP Neu2 does not have green fluorescent protein (GFP) or similar sequence which provides visual detection. Therefore, pEGFP-N2, GFP expressing plasmids, was used as control to obtain optimum plasmid and transfection ratio. pEGFP-N2 was the control of experiment because after the transfection of pEGFP-N2 to mammalian cell lines such as Cos-7 cells, HeLa cells and Hek 293 cells, transfection efficiency could be observed visually under the fluorescence microscopy (Figure 3.5).

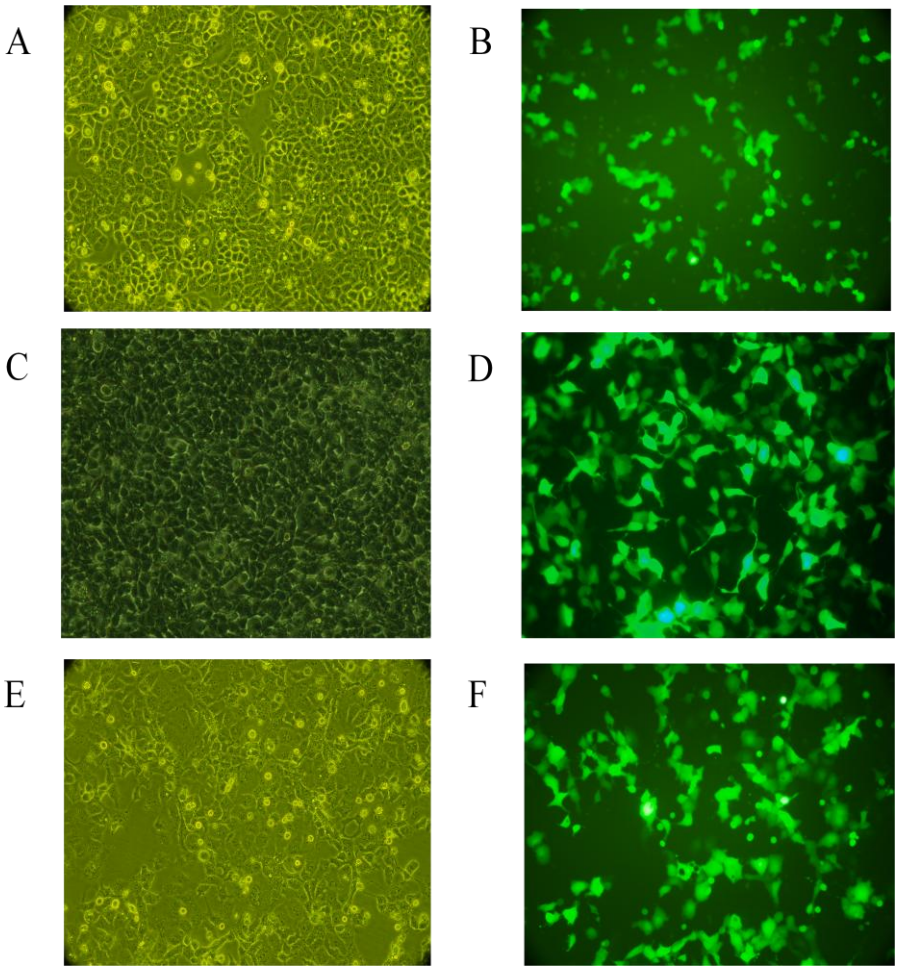

Figure 3.5. Following the 48 h post transfection with pEGFP-N2 plasmid A) Hela Cells C) Hek-293 Cells E) Cos 7 Cells under 10X magnification Inverted Light Microscope. GFP expression in the same area of B) Hela Cells D) Hek 293 Cells F) Cos 7 Cells Under 10X Magification Fluorescent Microscope

The best transfection efficiency was observed with 1:3 ratio of DNA:PEI. There was three different wells having 1:3 transfection ratio such as 1 µg DNA:3 µl PEI (1 µg/ml), 2 µg DNA:6 µl PEI (1µg/ml), 3 µg DNA:9 µl PEI (1 µg/ml). 3µg DNA with 9 µl PEI (1µg/ml) provided us highest transfection efficiency. The other ones were having inadequate amount of DNA to transfect the cells. When 1:2 ratio of DNA:PEI was used, it was observed that it is not efficient for transfection. 1:2 transfection ratio was obtained by using 2 µg DNA:4 µl PEI (1µg/ml) and 3 µg DNA:6 µl PEI (1µg/ml). Although 3 µg DNA provides the best transfection efficiency when used with 9 µl PEI, 3µg DNA was inadequate for 4 µl PEI. It should be that some of the DNA cannot bind to efficient transfection reagent and this excess amount of DNA should be found in the

cell culture medium. Cells expressing GFP was visualized under fluorescent microscope following the 48 h post transfection (Figure 3.5).

After the visual detection under fluorescent microscope, it was observed that the highest transfection efficiency in Cos-7 and Hek 293 cells. Therefore, in the following part of the experiment we used the Cos-7 cell line to transfect pCTAP Neu2.

3.3. Identification of NEU2-Tag Protein by Western Blotting

pCTAP Neu2 does not have green fluorescent protein (GFP) or similar sequence which provides visual detection. Therefore, during the experiment we transfected pCTAP Neu2 into Cos-7 cell line and determined whether recombinant Neu2 (Neu2-SBP-CBP) was expressed in the Cos-7 cell line by western blotting with Anti-Calmodulin Binding Peptide epitope tag primary antibody.

In order to do western blotting concentration of the isolated protein must be known. Therefore, Bradford assay was done by Coomassie Brilliant Blue G-250. Firstly, Bovine serum albumin (BSA) standart curve was prepared by using 200 µg/ml BSA, 150 µg/ml BSA, 100 µg/ml BSA, 50 µg/ml BSA, 25 µg/ml BSA, 10 µg/ml BSA (Figure 3.6). Then the concentration of isolated protein was determined according to this BSA standart curve.

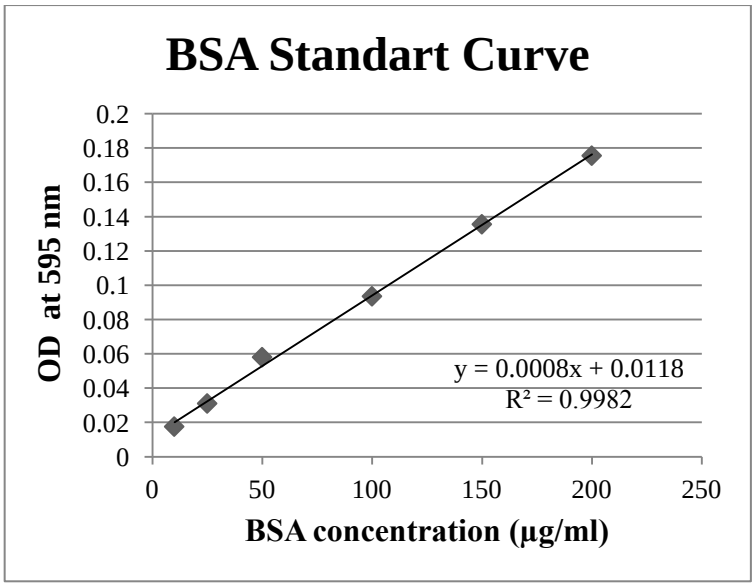

Figure 3.6. BSA standart curve

After the 48 hour post transfection of pCTAP Neu2 into Cos-7 cell line, expression of Neu2-tag (Neu2-SBP-CBP) protein was showed in the Cos-7 cell line by western blotting with Anti-Calmodulin Binding Protein Epitope Tag primary antibody. At the same time β-Actin, housekeeping gene, was determined as an internal control by using β-Actin primary antibody (Figure 3.7).

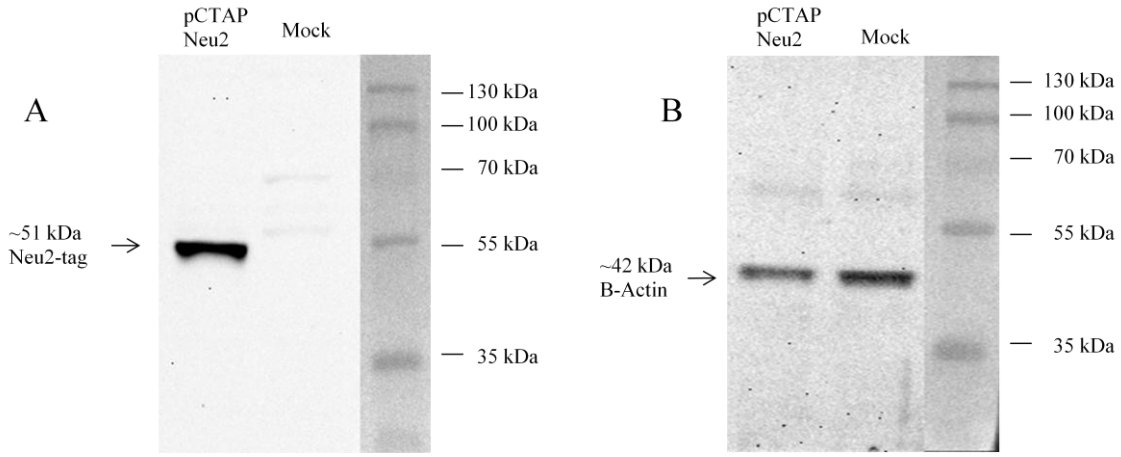

Figure 3.7. Detection of A) ~51 kDa Neu2-tag protein by Anti-Calmodulin Binding Protein Epitope Tag primary antibody and B) ~42 kDa β-Actin by β-Actin primary antibody by western blot analysis after the 48 h post transfection with pCTAP Neu2 plasmid

3.4. Identification of NEU2 Associated Proteins

3.4.1. Determination Neu2-tag protein in the purified Neu2 protein complex

Conservation of Neu2-tag proteins through two step of purification according to SBP and CBP was showed with Anti-Calmodulin Binding Protein Epitope Tag primary antibody (Figure 3.9).

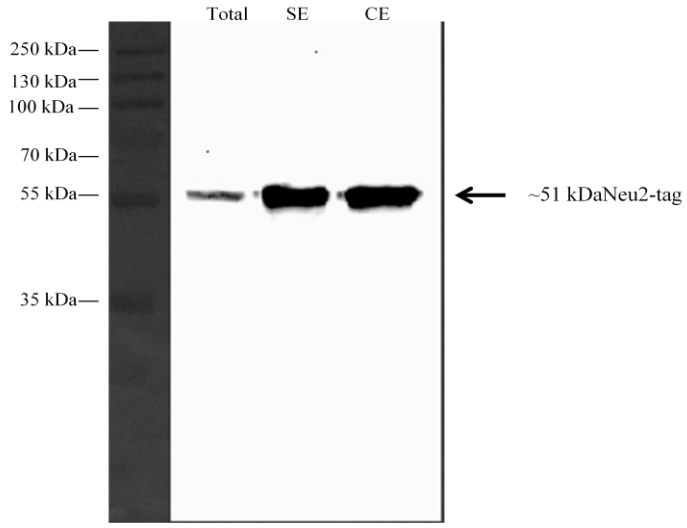

Figure 3.8. Detection of Neu2 tag protein by Anti-Calmodulin Binding Protein Epitope Tag primary antibody. Total: total proteins isolated from Cos-7 cells, SE: proteins obtained at the Streptavidin elution step, CE: proteins obtained at the Calmodulin elution step

3.4.2. Identification of Neu2 and Its Associated Proteins by Mass Spectrometric Analysis

Following the ESI-LC-MS/MS analysis, mass spectrometry raw data was obtained in the pkl file format. This file was analyzed by using 3 different database search softwares such as ProteinLynx Global Server (PGLS), Mascot and X!Tandem. 11 different protein was identified according to analysis with PGLS server (Table 3.1.) such as Yeast Enolase 1 (ENO1), and Human Calmodulin (CALM), Sialidase 2 (NEU2), Tyripsin 1 (TRY1), Histone H 1.3 (HIST1H1D), Actin Cytoplasmic 2 (ACTG1), Keratin type II Cytoskeletal 1 (KRT1), Heterogeneous nuclear ribonucleoproteins A2/B1(HNRNPA2B1**)**, DNA polymerase alpha subunit B (POLA2), Calsyntenin-2 (CLSTN2**)**, Keratin type I cytoskeletal 10 (KRT10).

Table 3.1. Results of ESI-LC-MS/MS analysis by using ProteinLynx Global Server (PGLS). mW: Molecular mass of protein in Dalton, pI: Isoelectric focusing point of the protein. PLGS Score: Probability of the match being random, Peptides: The number of matched peptide sequences, Theoretical Peptides: The number of the theoretical peptide sequences, Coverage: Protein sequence coverage in percentage

	Description	**mW (Da)**	**pI pH**	**PLGS Score**	**Peptides**	**Theoretical Peptides**	**Coverage (%)**
1	CALM HUMAN Calmodulin OS Homo sapiens GN CALM1 PE 1 SV 2	16826	3.9	6208	24	23	89
2	ENO1 YEAST Enolase 1 OS Saccharomyces Cerevisiae Strain ATCC 204508 S288c GN ENO1 PE 1 SV 3	46787	6.2	1762	20	28	31
3	NEUR2 HUMAN Sialidase 2 OS Homo sapiens GN NEU2 PE 1 SV 2	42227	6.4	1667	22	28	39
4	TRY1 HUMAN Trypsin 1 OS Homo sapiens GN PRSS1 PE 1 SV 1	26541	6.1	395	3	14	8
5	H13 HUMAN Histone H1 3 OS Homo sapiens GN HIST1H1D PE 1 SV 2	22336	11.5	349	2	21	10
6	ACTG HUMAN Actin cytoplasmic 2 OS Homo sapiens GN ACTG1 PE 1 SV 1	41765	5.2	280	13	34	24
7	K2C1 HUMAN Keratin type II cytoskeletal 1 OS Homo sapiens GN KRT1 PE 1 SV 6	65998	8.3	111	7	45	11
8	ROA2 HUMAN Heterogeneous nuclear ribonucleoproteins A2 B1 OS Homo sapiens GN HNRNPA2B1 PE 1 SV 2	37406	9.2	111	4	29	8
9	DPOA2 HUMAN DNA polymerase alpha subunit B OS Homo sapiens GN POLA2 PE 1 SV 2	65906	5	94	4	42	11
10	CSTN2 HUMAN Calsyntenin 2 OS Homo sapiens GN CLSTN2 PE 1 SV 2	106937	5.1	71	3	60	4
11	K1C10 HUMAN Keratin type I cytoskeletal 10 OS Homo sapiens GN KRT10 PE 1 SV 6	58791	5	62	4	35	9

10 different protein was identified according to analysis with Mascot software (Table 3.2.) such as Human Calmodulin (CALM), Sialidase 2 (NEU2), Keratin type II Cytoskeletal 1 (KRT1) Actin aortic smooth muscle (ACTA2), Actin cytoplasmic 1 (ACTB), Histone H 1.3 (HIST1H1D), Keratin type I cytoskeletal 10 (KRT10), Ubiquitin carboxyl-terminal hydrolase 25 (USP25), E3 ubiquitin-protein ligase (HERC2) and F box/LRR-repeat protein 19 (FBXL19).

Table 3.2. Results of ESI-LC-MS/MS analysis by using Mascot. Score: Ions score (-10log(P) where P is the probability that observed match is a random event) Mass: Molecular mas of the protein in Dalton, Matches: Number of peptide matches, Pep(sig): Number of significant peptide matches Sequences: Number of distinct sequences, Seq(sig): Number of significant distinct sequences. Ions scores **>36** show **identity** or **extensive homology** ($p<0.05$)

	Description	**Score**	**Mass**	**Matches**	**Pep(sig)**	**Sequences**	**Seq(sig)**
1	Calmodulin OS=Homo saphiens GN=CALM1 PE=1 SV=2	261	16827	12	9	9	7
2	Sialidase-2 OS=Homo sapiens GN=NEU2 PE=1 SV=6	174	42227	7	6	6	5
3	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6	117	65999	9	5	9	5
4	Actin, aortic smooth muscle OS=Homo sapiens GN=ACTA2 PE=1 SV=1	73	41982	6	3	6	3
5	Actin, cytoplasmic 1 OS=Homo sapiens GN=ACTB PE=1 SV=1	64	41710	8	5	7	4
6	Histone H1.3 OS=Homo sapiens GN=HIST1H1D PE=1 SV=2	41	22336	3	2	3	2
7	Keratin, type 1 cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6	35	58792	4	1	4	1
8	Ubiquitin carboxyl-terminal hydrolase 25 OS=Homo sapiens GN=USP25 PE=1 SV=4	25	122142	1	1	1	1
9	E3 ubiquitin-protein ligase OS=Homo sapiens GN=HERC2 PE=1 SV=2	24	526895	1	1	1	1
10	F-box/LRR-repeat protein 19 OS=Homo sapiens GN=FBXL19 PE=1 SV=3	13	75659	2	1	1	1

5 different protein was identified according to analysis with X!Tandem software (Table 3.3.) such as Tyripsin 1 (TRY1), Yeast Enolase 1 (ENO1), Sialidase 2 (NEU2), Human Calmodulin (CALM), and Actin alpha 1 skeletal muscle (ACTA1).

Table 3.3. Results of ESI-LC-MS/MS analysis by using X!Tandem. Mr: molecular mass of protein in kiloDaltons, Total: total number of tandem mass spectra that can be assingned to this protein, #: the number of unique peptide sequences associated with this protein sequences, %/%: the amino acid coverage of the protein in this assignment / the coverage corrected for peptide sequences, log(e): the base -10 log of the expectation that any particular protein assignment was made at random (E-value), log(I): the base -10 log of the sum of the fragment ion intensities in the tandem mass spectra used to make this assignment

	Description	**Mr**	**Total**	**#**	**%/%**	**log(e)**	**log(I)**
1	Tyripsin	24.4	$19_{1/2}$	6	29/36	-76.3	5.71
2	Enolase I	46.8	5	$5_{1/2}$	19/23	-37.3	4.57
3	Neuraminidase 2	42.2	4	3	15/20	-25.6	4.50
4	Calmodulin 2	16.8	2	1	11/16	-8.5	4.32
5	Actin, alpha1, skeletal muscle	42	1	1	4/5	-3.1	3.30

Table 3.4. Comparison of identified proteins by using 3 different database search programs as PGLS, Mascot, X!Tandem

Software	**PGLS**	**Mascot**	**X!Tandem**
1	Neu2	Neu2	Neu2
2	Calmodulin	Calmodulin	Calmodulin
3	Gamma Actin	Alpha Actin Beta Actin	Alpha Actin
4	Calsyntein 2	Ubiquitin Carboxyl-Terminal Hydrolase	
5	DNA Polymerase Alpha Subunit	E3 Ubiquitin-protein ligase	
6	Heterogeneous nuclear ribonucleoproteins	F-box/LRR-repeat protein	
7	Histone 1.3	Histone 1.3	
8	Trypsin		Trypsin
9	Enolase		Enolase
10	Kreatin Type II Kreatin Type I	Kreatin type II Kreatin Type I	

Each of the 3 software (PGLS, Mascot, X!Tandem) has their different algorithms to identify the protein samples. Therefore, some of them identify the same proteins as shown in the Table 3.4. Yeast Enolase 1 was the positive control of the ESI-LC-MS/MS analysis. It was added by us during overnight trypsin digestion. So as a result of experiment we both observe Typsin and Yeast Enolase 1 proteins. Neu2 cDNA was fussed to Streptavidin Binding Peptide and Calmodulin Binding Peptide. Therefore as a result of MS analysis, Human Calmodulin protein was observed and Neu2 sialidase was observed as expected. Endogeneously expressed Calmodulin protein was bind to Calmodulin Binding Peptide. Keratin type I cytoskeletal 10 (KRT10) and Keratin type II Cytoskeletal 1 (KRT1) are the proteins having key structure of human outlayer skin such as hair. Therefore, presences of them are the experimental contamination. Histone H 1.3 (HIST1H1D), Heterogeneous nuclear ribonucleoproteins A2/B1 (HNRNPA2B1) and DNA polymerase alpha subunit B (POLA2) are the nuclear proteins. When the cell lyses occur all the proteins both nuclear, cytoplasmic and plasma membrane proteins are isolated. Neu2 may have affinity to bind these nuclear proteins under *in vitro* experimental conditions but it is not easy that occurrence of this binding in the cell. Finally, Gamma Actin, Alpha Actin, Beta Actin, and Calsyntenin-2 **(**CLSTN2) proteins were found as a result of ESI-LC-MS/MS analysis. These proteins are our candidate proteins to have interacted in the cell in order to carry out its functions.

3.4.3. Identification of Neu2 and Its Associated Proteins by Silver Staining

Neu2 protein complexes were obtained with Interplay Mammalian Tap purification system. This is a kind of two step purification system. There are two peptide tags as Streptavidin Binding Peptide (SBP) and Calmodulin Binding Peptide (CBP). Elution was done firstly according to SBP tag then to CBP tag from their respective resins with its specific elution reagents to obtain purified Neu2 protein complex without disrupting. Then, total proteins, proteins coming from Streptavidin elution and proteins obtained by Calmodulin elution was used in order to show step base purification by silver staining (Figure 3.8). So identified proteins by different database search programs were estimated on the gel according to their molecular weights.

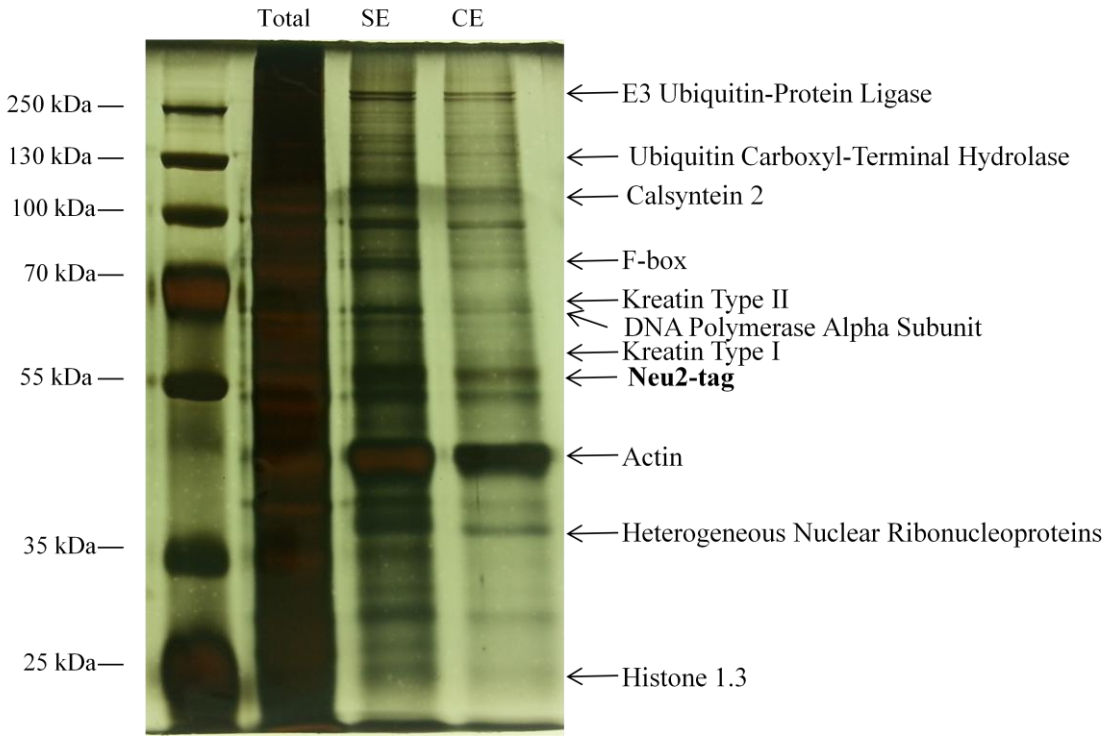

Figure 3.9. Detection of Neu2 and Neu2 associated proteins by silver staining. Total: total proteins isolated from Cos-7 cells, SE: proteins obtained at the Streptavidin elution step, CE: proteins obtained at the Calmodulin elution step

3.4.4. Neu2 Interacts with β-Actin Protein

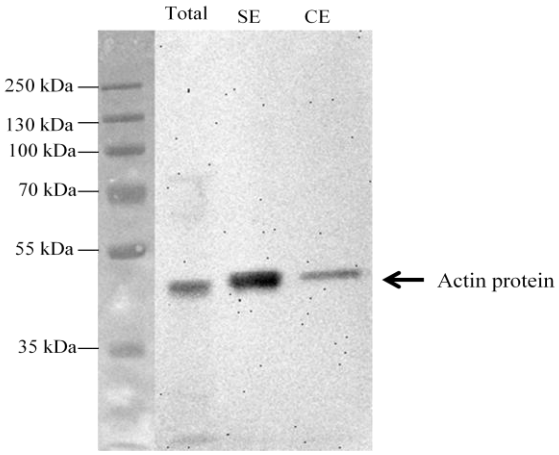

Figure 3.10. Detection of β-Actin protein by β-Actin primary antibody in the purified Neu2 protein complex by western blotting. Total: total proteins isolated from Cos-7 cells, SE: proteins obtained at the Streptavidin elution step, CE: proteins obtained at the Calmodulin elution step

Interaction between Neu2 and β-Actin protein was shown by western blotting with Neu2 protein complex purified by Interplay Mammalian Tap Purification System (Figure 3.10).

CHAPTER 4

DISCUSSION

Sialidases (Neuraminidases) removes sialic acids from glycoproteins, oligosacharides and glycolipids. Cytosolic sialidase enzyme (Neu2), the one of the four sialidase enzyme, has an active role on a wide range of subtances including oligosaccharides, glycopeptides and gangliosides. Neu2 enzyme has a function in different areas such as cancer metabolism, neuronal differentiation and myoblast differentiation, proliferation and hypertrophy. However, it has not been shown that Neu2 enzyme whether interacts with proteins within the cell to carry out its functions. During our research we identified candidate proteins such as Actin proteins (Alpha Actin, Gamma Actin and Beta Actin), and Calsyntenin-2 **(**CLSTN2) proteins 2. These proteins are important in order to carry out its functions in the cell.

Actin proteins are the important part of cytoskeletal. According to requirements of the cell polymerization and depolymerization of the actins take place (Bunnell et al. 2011). This is resulted from the globular (G) to fibrous (F) Actin transition. Actin amount is found in a dynamic equilibrium between monomeric G-actin and polymerized F-actin (Oda et al. 2009). They have a function in controlling cell shape, adhesion, division and migration (Bunnell et al. 2011). This process is the under control in the normal cells. However, cellular transformation and tumorogenesis cause the dysregulation of this process (Li et al. 2007). There are six highly conserved Actin isoforms in vertebrates. Four of them are expressed in striated muscle cells (αskeletal actin and αcardiac actin) and smooth muscle cells (αsmooth actin and γsmooth actin). The other two cytoplasmic β-Actin and γ-Actin isoforms are continuously expressed. Each isoform is produced by two different genes as Actb and Actg1. Actb encodes for β-Actin and Actg1 encodes for γ-Actin. β-Actin and γ-Actin includes the same amino acids sequence except for only four amino acid residues. The reason of the differences is result from posttranslational modifications (Belyantseva et al. 2009). Muscle actins are tissue specific and organized in contractile units while cytoplasmic β- and γ-actin are important for cell survival (Dugina et al. 2009). Alpha actin is found primarily in muscle, and Beta and Gamma Actin in other tissues. The amount of Beta and Gamma

Actin in different tissues are at different ratio (Erba et al. 1988). Cytoplasmic γ-Actin is necessary to maintain cytoskeletal integrity and function. However, it is not necessary building elements of developing cytoskeletal (Belyantseva et al. 2009).

Chronic myeloid leukemia (CML), the one type of the cancer, is resulted by a translocation between Abl tyrosine kinase and Bcr gene. The resulting fusion protein, Bcr–Abl, includes constitutive tyrosine kinase activity. BCR/ABL binds to monomeric and filamentous actin (F- actin) by an actin binding domain in the C terminus of the protein (McWhirter and Wang 1993). This interaction is necessary for Bcr–Abl localization to the plasma membrane and induction of cytoskeletal changes and alterations of cell adhesion (Wertheim et al. 2003). Abl protein normally has low binding affinity to F-actin. Addition of Bcr to Abl increases the binding affinity for F-actin. This is required for the transformation of cells. Deletion of the actin-binding domain of Abl in Bcr/Abl decreased its transformation ability (McWhirter and Wang 1993). At least 70% of the Bcr/Abl protein is localized at the cytoskeleton. This localization is likely to be involved in the binding to extracellular matrix proteins, cell motility, and cell morphology (Salgia et al. 1997).

Neu2 transfection in K562 (CML) cells resulted decrease 55% in the proliferation rate, 30% mRNA in mRNA of the antiapoptotic factor Bcl-XL and 80% of the mRNA of the antiapoptotic factor Bcl-2. Decrease in the expression levels of Bcl-2 and Bcl-XL genes resulted in cells that are more sensitive to apoptotic stimuli (Tringali et al. 2007). What is more, according to our results we found Gamma Actin as a candidate protein which interacts with Neu2 enzyme. Gamma Actin and Beta Actin are the part of F-actin (polymerazied actin). It was also known that Bcr/Abl protein interacts with F-actin via the C-terminus of the Bcr/Abl protein. K562 cells normally not expresses Neu2 enzyme and at the transfected cells cells starts to go to apaptosis. The reason of that might be high amount of Neu2 inteacts with the Actin filaments and cause the decrease in the interaction amount Bcr/Abl protein with the cytoskelatal. This might affect the enzymatic activity of Bcr/Abl protein and resulted with the apoptotic stimuli.

Calsyntein protein family localized in the postsynaptic membrane of excitatory central nervous system (CNS) synapses. There are three types of Calsyntein protein; Calsyntein-1, Calsyntein-2 and Calsyntein-3. Calsynteins are the transmembrane proteins which includes a large extracellular part with two cadherin-like repeats and a relatively small cytoplasmic segment. Calsyntein- 1 is the firstly identified postsynaptic

membrane protein with a highly acidic cytoplasmic segment with Ca_2-binding capacity. Calsyntein-2 is the calsyntenin-1-like protein. It is expressed in the cerebral cortex, the cerebellum, and some brain-stem nuclei. The highest expressions of calsyntenin-2 are found in putative GABAergic neurons throughout the cerebral and the cerebellar cortex (Hintsch 2002).

When PC12 cells were be stimulated to differentiate into neural phenotype by nerve growth factor (NGF) or human fibroblast growth factor 2 (FGF-2) treatments, the amount of Neu2 transcripts was increased although under normal condition Neu2 is a rare transcript in these cells. During the neuronal differentiation, Neu2 sialidase is important product. It is known that during neuronal differentiation transient Ca_2 intake to the cells are required in the early period of differentiation (Carey and Matsumoto 1999). The Calsyntein 2 is the candidate protein for Neu2 interaction. It is also the Calcium transporter protein localized in the brain. Effects of the Neu2 during neuronal differentiation can be with Calsyntein-2 protein by transferring Calcium from outside to inside of the cell or vice versa.

CHAPTER 5

CONCLUSION

In this study, cytosolic sialidase Neu2 associated proteins were analyzed by using InterPlay Mammalian TAP System and ESI-LC-MS/MS analysis. ESI-LC-MS/MS analysis was done in TUBITAK-MAM proteomic analysis research center by Dr. Ahmet Tarık BAYKAL. Proteins in the Neu2 protein complex were identified by three different database search software such as PGLS, Mascot and X!Tandem. As a result of experiment Actin proteins (Alpha Actin, Gamma Actin and Beta Actin), and Calsyntenin-2 (CLSTN2) proteins were found as a candidate protein. The interaction between Neu2 and β-Actin protein was confirmed by western blot analysis (Figure3.10).

Cytosolic sialidase enzyme (Neu2) has an active role on a wide range of substances including oligosaccharides, glycopeptides and gangliosides. What is more, Neu2 enzyme is functional in different areas such as cancer metabolism, neuronal differentiation and myoblast differentiation, proliferation and hypertrophy. However, it has not been shown that Neu2 enzyme interacts with which proteins within the cell to carry out its functions. In this study, we firstly found that Neu2 can interact with Actin proteins. K562 cell line, chromic myeloid leukemia cells, includes Bcr/Abl fusion protein. Bcr/Abl protein interacts with Actin filaments via the C-terminus of the Bcr/Abl protein. While K562 cells don't express Neu2 enzyme, Neu2 transfected K562 cells go to apaptosis. The reason of that might be high amount of Neu2 inteacts with the Actin filaments and cause the decrease in the interaction of Bcr/Abl protein with the cytoskelatal. This might affect the enzymatic activity of Bcr/Abl protein and resulted with the apoptotic stimuli.

The Calsyntein 2 is our candidate protein for Neu2 interaction. It is a Calcium transporter protein localized in the brain. During neuronal differentiation transient Ca_2 intake to the cells are required in the early period of differentiation. Neu2 can be effective during neuronal differentiation with Calsyntein-2 transporter protein.

REFERENCES

Abu-Farha, Mohamed, Fred Elisma, and Daniel Figeys. 2008. "Identification of Protein-Protein Interactions by Mass Spectrometry Coupled Techniques." *Advances in Biochemical Engineering/Biotechnology* 110(January):67–80.

Belyantseva, Inna a et al. 2009. "γ-Actin is required for cytoskeletal maintenance but not development." *Proceedings of the National Academy of Sciences of the United States of America* 106(24):9703–8.

Blackstock, W. P., and M. P. Weir. 1999. "Proteomics: quantitative and physical mapping of cellular proteins." *Trends in Biotechnology* 17(3):121–27.

Bodine, S. C. et al. 2001. "Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo." *Nature Cell Biology* 3(11):1014–19.

Bunnell, Tina M., Brandon J. Burbach, Yoji Shimizu, and James M. Ervasti. 2011. "β-Actin specifically controls cell growth, migration, and the G-actin pool." *Molecular Biology of the Cell* 22(21):4047–58.

Carey, M. B., and S. G. Matsumoto. 1999. "Spontaneous calcium transients are required for neuronal differentiation of murine neural crest." *Developmental Biology* 215(2):298–313.

Chang, Ing-Feng. 2006. "Mass spectrometry-based proteomic analysis of the epitope-tag affinity purified protein complexes in eukaryotes." *Proteomics* 6(23):6158–66.

Diament, Benjamin J., and William Stafford Noble. 2011. "Faster SEQUEST searching for peptide identification from tandem mass spectra." *Journal of Proteome Research* 10(9):3871–79.

Dugina, Vera, Ingrid Zwaenepoel, Giulio Gabbiani, Sophie Clément, and Christine Chaponnier. 2009. "Beta and gamma-cytoplasmic actins display distinct distribution and functional diversity." *Journal of Cell Science* 122(16):2980–88.

Elias, Joshua E., and Steven P. Gygi. 2010. "Target-Decoy Search Strategy for Mass Spectrometry-Bassed Proteomics" *Methods in Molecular Biology* 604:55–71.

Erba, Harry P., Roger Eddy, Thomas Shows, Larry Kedes, and Peter Gunning. 1988. "Structure, Chromosome Location, and Expression of the Human γ-Actin Gene: Differential Evolution, Location, and Expression of the Cytoskeletal β- and γ-Actin Genes. " *Molecular and Celular Biology* 8(4):1775-89

Fanzani, a, F. Colombo, R. Giuliani, a Preti, and S. Marchesini. 2004. "Cytosolic sialidase Neu2 upregulation during PC12 cells differentiation." *FEBS Letters* 566(1-3):178–82.

Fanzani, a. et al. 2003. “Overexpression of cytosolic sialidase Neu2 induces myoblast differentiation in C2C12 cells.” *FEBS Letters* 547(1-3):183–88.

Fanzani, Alessandro et al. 2008. “The enzymatic activity of sialidase Neu2 is inversely regulated during in vitro myoblast hypertrophy and atrophy.” *Biochemical and Biophysical Research Communications* 370(2):376–81.

Fanzani, Alessandro, Francesca Colombo, Roberta Giuliani, Augusto Preti, and Sergio Marchesini. 2006. “Insulin-like growth factor 1 signaling regulates cytosolic sialidase Neu2 expression during myoblast differentiation and hypertrophy.” *The FEBS Journal* 273(16):3709–21.

Gavin, Anne-Claude et al. 2002. “Functional organization of the yeast proteome by systematic analysis of protein complexes.” *Nature* 415(6868):141–47.

Glass, David J. 2005. “Skeletal muscle hypertrophy and atrophy signaling pathways.” *The International Journal of Biochemistry & Cell Biology* 37(10):1974–84.

Hasegawa, T., C. Feijoo Carnero, T. Wada, Y. Itoyama, and T. Miyagi. 2001. “Differential expression of three sialidase genes in rat development.” *Biochemical and Biophysical Research Communications* 280(3):726–32.

Hintsch, G. 2002. “The Calsyntenins—A Family of Postsynaptic Membrane Proteins with Distinct Neuronal Expression Patterns.” *Molecular and Cellular Neuroscience* 21(3):393–409.

Jones, N. C., Y. V Fedorov, R. S. Rosenthal, and B. B. Olwin. 2001. “ERK1/2 is required for myoblast proliferation but is dispensable for muscle gene expression and cell fusion.” *Journal of Cellular Physiology* 186(1):104–15.

Kaiser, Peter, David Meierhofer, Xiaorong Wang, and Lan Huang. 2008. “Tandem Affinity Purification Combined with Mass Spectrometry to Identify Components of Protein Complexes” *Methods in Molecular Biology* 439:1–16.

Keefe, a D., D. S. Wilson, B. Seelig, and J. W. Szostak. 2001. “One-step purification of recombinant proteins using a nanomolar-affinity streptavidin-binding peptide, the SBP-Tag.” *Protein Expression and Purification* 23(3):440–46.

Kotani, K. et al. 2001. “Cloning, chromosomal mapping, and characteristic 5’-UTR sequence of murine cytosolic sialidase.” *Biochemical and Biophysical Research Communications* 286(2):250–58.

Li, Yingzhu et al. 2007. “Bcr-Abl induces abnormal cytoskeleton remodeling, β1 integrin clustering and increased cell adhesion to fibronectin through the Abl interactor 1 pathway.” *Journal of Cell Science* 120(8):1436–46.

Loo, J. a et al. 1992. “High-resolution tandem mass spectrometry of large biomolecules.” *Proceedings of the National Academy of Sciences of the United States of America* 89(1):286–89.

Mann, Matthias, Ronald C. Hendrickson, and Akhilesh Pandey. 2001. "ANALYSIS OF PROTEINS AND PROTEOMES BY MASS SPECTROMETRY" *Annual Review of Biochemistry* 70:437-73

Manual, Instruction. "InterPlay Mammalian TAP System."

McWhirter, J. R., and J. Y. Wang. 1993. "An actin-binding function contributes to transformation by the Bcr-Abl oncoprotein of Philadelphia chromosome-positive human leukemias." *The EMBO Journal* 12(4):1533–46.

Miyagi, T. et al. 1993. "Molecular cloning and expression of cDNA encoding rat skeletal muscle cytosolic sialidase." *The Journal of Biological Chemistry* 268(35):26435–40.

Miyagi, T., and S. Tsuiki. 1985. "Purification and characterization of cytosolic sialidase from rat liver." *The Journal of Biological Chemistry* 260(11):6710–16.

Miyagi, Taeko, Tadashi Wada, Kazunori Yamaguchi, and Keiko Hata. 2004. "Sialidase and malignancy : A minireview." *Glycocongugate Journal* 20:189–98.

Monti, E., a Preti, E. Rossi, a Ballabio, and G. Borsani. 1999. "Cloning and characterization of NEU2, a human gene homologous to rodent soluble sialidases." *Genomics* 57(1):137–43.

Monti, Eugenio et al. 2010. "Sialidases in vertebrates: a family of enzymes tailored for several cell functions". *Advances in Carbohydrate Chemistry and Biochemistry* 64:403-79.

Monti, Eugenio, Augusto Preti, Bruno Venerando, and Giuseppe Borsani. 2002. "Recent development in mammalian sialidase molecular biology." *Neurochemical Research* 27(7-8):649–63.

Oda, Toshiro, Mitsusada Iwasa, Tomoki Aihara, Yuichiro Maéda, and Akihiro Narita. 2009. "The nature of the globular- to fibrous-actin transition." *Nature* 457(7228):441–45.

Prieto, Gorka et al. 2012. "PAnalyzer: a software tool for protein inference in shotgun proteomics." *BMC Bioinformatics* 13:288.

Rossi, Stefania et al. 2009. "The cytosolic sialidase Neu2 is degraded by autophagy during myoblast atrophy." *Biochimica et Biophysica Acta* 1790(8):817–28.

Salgia, R. et al. 1997. "BCR/ABL induces multiple abnormalities of cytoskeletal function." *The Journal of Clinical Investigation* 100(1):46–57.

Samuel, D. S. 1997. "The Mitogenic and Myogenic Actions of Insulin-like Growth Factors Utilize Distinct Signaling Pathways." *Journal of Biological Chemistry* 272(10):6653–62.

Sato, K., and T. Miyagi. 1996. “Involvement of an endogenous sialidase in skeletal muscle cell differentiation.” *Biochemical and Biophysical Research Communications* 221(3):826–30.

Tringali, Cristina et al. 2007. “Expression of sialidase Neu2 in leukemic K562 cells induces apoptosis by impairing Bcr-Abl/Src kinases signaling.” *The Journal of Biological Chemistry* 282(19):14364–72.

Vaudel, Marc, Harald Barsnes, Frode S. Berven, Albert Sickmann, and Lennart Martens. 2011. “SearchGUI: An open-source graphical user interface for simultaneous OMSSA and X!Tandem searches.” *Proteomics* 11(5):996–99.

Vitek, Olga. 2009. “Getting started in computational mass spectrometry-based proteomics.” *PLOS Computational Biology* 5(5):e1000366.

Wang, Le-heng et al. 2007. “pFind 2 . 0: a software package for peptide and protein identification via tandem mass spectrometry.” *Rapid Communucations in Mass Spectrometry* (973):2985–91.

Weisberg, Ellen, Paul W. Manley, Sandra W. Cowan-Jacob, Andreas Hochhaus, and James D. Griffin. 2007. “Second generation inhibitors of BCR-ABL for the treatment of imatinib-resistant chronic myeloid leukaemia.” *Nature Reviews. Cancer* 7(5):345–56.

Wertheim, Jason a et al. 2003. “Localization of BCR-ABL to F-actin regulates cell adhesion but does not attenuate CML development.” *Blood* 102(6):2220–28.

Wilm, M., and M. Mann. 1996. “Analytical properties of the nanoelectrospray ion source.” *Analytical chemistry* 68(1):1–8.

Wright, James C. et al. 2012. “Enhanced peptide identification by electron transfer dissociation using an improved Mascot Percolator.” *Molecular & Cellular Proteomics: MCP* 11(8):478–91.

Zhang, M., T. Yuan, and H. J. Vogel. 1993. “A peptide analog of the calmodulin-binding domain of myosin light chain kinase adopts an alpha-helical structure in aqueous trifluoroethanol.” *Protein Science: A Publication of the Protein Society* 2(11):1931–37.