ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

IDENTIFICATION OF THE INTERACTION PARTNERS OF ANTI-APOPTOTIC BAG-1M ISOFORM IN BREAST CANCER AND BREAST EPITHELIAL CELLS

M.Sc. THESIS

Nisan Denizce CAN

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ISTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

ANTİ-APOPTOTİK BAG-1M İZOFORMUNUN ETKİLEŞİM PARTNERLERİNİN MEME KANSERİ VE MEME EPİTEL HÜCRELERİNDE TANIMLANMASI

YÜKSEK LİSANS TEZİ

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ARALIK 2017

Nisan Denizce CAN, a **M.Sc.** student of ITU **Institute of / Graduate School of Science Engineering and Technology** student ID **521151133**, successfully defended the thesis entitled "**IDENTIFICATION OF THE INTERACTION PARTNERS OFANTI-APOPTOTIC BAG-1M ISOFORM IN BREAST CANCER AND BREAST EPITHELIAL CELLS**", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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To my family,

FOREWORD

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TABLE OF CONTENTS

Page

ABBREVIATIONS

AR : Androgen Receptor
ACN : Acetonitrile
AMBIC : Ammonium bicarbonate
BAG : Bcl-2-associated athanogene
BAG-1 : Bcl-2-associated athanogene 1
BAG-1L : Bcl-2-associated athanogene 1 Large
BAG-1M : Bcl-2-associated athanogene 1 Medium
BAG-1S : Bcl-2-associated athanogene 1 Small
Bcl-2 : B-cell lymphoma 2
BN-PAGE : Blue native polyacrylamide gel electrophoresis
Bp : Base pair
BSA : Bovine serum albumin
CBP : Calmodulin binding peptide
DMEM : Dulbecco's modified eagle medium
DMSO : Dimethylsulfoxide
DNA : Deoxyribonucleic acid
EDTA : Ethylenediaminetetraacetic acid
ER : Endoplasmic reticulum
ERAD : Endoplasmic reticulum associated degradation
g : Gram
GR : Glucocorticoid receptor
HGF : Hepatocyte growth factor
HSP70 : Heat shock protein 70
IAD : Iodoacetamide
IgG : Immunoglobulin G
kDa : Kilodalton
L : Liter
LB : Luria-Bertani medium
LC-MS : Liquid chromatography - mass spectrometry
M : Molar
MAP : Mitogen-activated protein
MCF-7 : Michigan cancer foundation-7
mg : Miligram
min : Minute
ml : Mililiter
mM : Milimolar
mRNA : Messenger ribonucleic acid
NHR : Nuclear hormone receptor
OE : Overexpression
PBS : Phosphate buffered saline
PDIA3 : Protein disulfide isomerase family A member 3
pH : Power of hydrogen
PMSF : Phenylmethanesulfonylfuloride
RAR : Retinoic acid receptor

RAD23B : UV excision repair protein homolog B
RNA **:** Ribonucleic acid
rpm **:** Revolutions per minutes
SDS **:** Sodium dodecyl sulfate
SDS-PAGE **:** Sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA **:** Small interfering RNA
TAP **:** Tandem affinity purification
TBS **:** Tris buffer saline
TBS-T **:** Tris buffer saline - Tween 20
TCEP **:** Tris (2-carboxyethyl) phosphine
TEMED **:** Tetramethylethylenediamine
TEV **:** Tobacco etch virus
TR **:** Thyroid receptor
ULD **:** Ubiquitin-like domain
UV **:** Ultraviolet
VCP **:** Valosin containing protein
VDR **:** Vitamin D3 receptor
WT **:** Wild-type
µg **:** Microgram
µL **:** Microliter
µM **:** Micromolar

LIST OF TABLES

Page

LIST OF FIGURES

Page

IDENTIFICATION OF THE INTERACTION PARTNERS OF ANTI-APOPTOTIC BAG-1M ISOFORM IN BREAST CANCER AND BREAST EPITHELIAL CELLS

SUMMARY

BAG-1 (Bcl-2 associated athanogene-1) is an anti-apoptotic protein which is a member of BAG family that has been evolutionary conserved in many organisms. BAG-1 is involved in important mechanisms within the cell, as a multifunctional and an adapter protein. BAG-1 functions on the regulation of transcription, hormone activity, cell proliferation, cell motility, tumorigenesis, apoptosis and various cellular signaling pathways via its interactions with heat shock proteins, E3 ligases, nuclear hormone receptors, Raf-1 serine/threonine kinase family, and Bcl-2. When the functions in the cell are taken into account, BAG-1 allows the cell to survive and is overexpressed in many types of cancer. It is thought that BAG-1 has an important role in the development of breast cancer in particular.

There are three major isoforms of BAG-1 originated from alternative translation initiation sites on the same mRNA. Both of the three isoforms contain the BAG domain and ULD (ubiquitin like domain) at the C-terminus regions, the difference between the isoforms arise from the N-terminus sites. BAG-1L is the largest isoform, has the nuclear localization signal at the N-terminus and is found in the nucleus, the smallest isoform is the BAG-1S which is known as the most abundant isoform in cell and localized in the cytoplasm. BAG-1M can be found in both cytoplasm and nucleus with a variety of mediator proteins. It is known that BAG-1 isoforms can be specialized to the tissues and cells they are present in, but there is no detailed information in the literature on isoform-specific studies that lighten the proteins that each isoform interacts with and the mechanisms in which it is involved.

In this study, it was aimed to detect the interaction partners of BAG-1M isoform in breast cancer and breast epithelial cells.

In order to detect the interaction partners of BAG-1M, the vector plasmid that contains the N-terminus TAP-Tagged sequence of BAG-1M was used transfect cells and then BAG-1M was purified from the total protein isolated from the cells transfected. Proteins interacting with BAG-1M were first identified via LC-MS/MS (liquid chromatography-mass spectrometry) analyses for peptide mapping of the digested purified proteins. For verification of identified proteins, 2-DE performed on purified BAG-1M samples and spots of 2-dimensional gel were analyed on MALDI-TOF. For further confirmation, immunoblotting assays were applied on purified samples to check the existance of identified proteins.

Pathway analysis and protein-protein interaction networks of the detected proteins were analyzed using KEGG and STRING web servers. For the classification of the identified proteins, GO Enrichment assays were used.

In order to illuminate the cellular mechanisms of the complexes formed by BAG-1M and its interacting proteins, native forms of proteins were purified and complexes were

separated according to their size by blue native polyacrylamide gel electrophoresis (BN-PAGE). Each complex was cut from the gel, digested to peptides and analyzed on LC-MS/MS for peptide mapping to detect containing proteins.

The results obtained in all experimental studies showed that the interacting proteins of BAG-1M as heat shock protein 90 (HSP-90) , heat shock protein family member BiP (HSPA5), protein disulfide isomerase family member A3 (PDIA3), transitional endoplasmic reticulum ATPase (VCP) and UV excision repair protein RAD23 homolog B (RAD23B) which function in folding/refoldig of proteins and the ubiquitin-related proteasomal degradation.

Considering of the mechanisms that BAG-1 is involved, known partners and newly identified interacting proteins suggested that interacting with the proteins localized in endoplasmic reticulum, proteins functions in ubiquitination mechanism, proteasome subunits, and chaperons may be related to role of BAG-1M in endoplasmic reticulum associated degradation (ERAD).

Prediction of interaction surfaces of the identified proteins with the BAG domain and ubiquitin-like domain of BAG-1 were performed using the PRISM web server and evaluated according to the energy level between two proteins. Structural analyses showed that RAD23B, VCP and PDIA3 are capable to interact with BAG-1, while HSP-90 and HSPA5 interactions were not found as have possible physical interactions.

This study identified proteins that interacting with BAG-1M isoform in direct or indirect manner. More detailed studies that will be performed in the light of the experimental findings and structural analyses can contribute to the elucidation of isoform-specific functions of BAG-1, which is seen as a biomarker in breast cancer, and to the development of new therapeutic agents.

ANTİ-APOPTOTİK BAG-1M İZOFORMUNUN ETKİLEŞİM PARTNERLERİNİN MEME KANSERİ VE MEME EPİTEL HÜCRELERİNDE TANIMLANMASI

ÖZET

BAG-1 (Bcl-2 ilişkili athanogen-1) bir çok organizmada evrimsel süreçte korunmuş olan BAG ailesine ait anti-apoptotik bir proteindir. Hücre içerisinde önemli mekanizmada görev alan BAG-1, çok fonksiyonlu adaptör protein olarak bilinir. HSP70/HSC70 ısı şoku proteinleri, E3 ubikuitin protein ligazlar, nükleer hormon reseptörleri, Raf-1 serin/treonin kinaz ailesi ve Bcl-2 proteini gibi çeşitli moleküllerle olan etkileşimler ile BAG-1 transkripsiyon, hormon aktivitesi, hücre büyümesi, hücre hareketliliği ve proliferasyonu, tümörigenez, apoptoz mekanizması ve hücre içindeki önemli sinyal yolaklarını düzenlemektedir. BAG-1'in hücrenin yaşamını sürdürmesini sağlamakta olduğu düşünülmektedir. Yapılan çalışmalar meme kanseri, kolon kanseri ve prostat kanseri başta olmak üzere çeşitli kanser türlerinde BAG-1'in yüksek oranda ifade edildiğini göstermektedir. Hücre içerisindeki fonksiyonları göz önünde bulundurulduğu zaman BAG-1'in özellikle meme kanserinin oluşum ve gelişim sürecinde BAG-1'in önemli rolü olduğu düşünülmektedir.

BAG-1 proteini aynı mRNA üzerindeki farklı başlangıç bölgelerinden translasyon sonucu oluşan üç ana izoformdan oluşmaktadır. BAG-1'ın üç izoformu da C-ucunda BAG domaini ve ubikuitin benzer domaini bulundurmaktadır. İzoformlar arasındaki fark N-ucu bölgesinden kaynaklanmaktadır. En büyük izoform olan BAG-1L, N-ucunda nükleer lokalizasyon sinyaline sahiptir ve nükleusta bulunur. BAG-1S hücre içerisinde en baskın olarak bulunan ve sitoplazmada lokalize olmuş en küçük BAG-1 izoformudur. BAG-1M ise ekspresyon seviyesi BAG-1L ve BAG-1S'ye kıyasla daha az olan ve nükleer lokalizasyon sinyali bulunmamasına karşın çeşitli aracı proteinler ile hem sitoplazma hem de nükleusta lokalize olabilen BAG-1 izoformudur. İzoformların bulundukları doku ve hücreye göre özelleşebildiği bilinmekte ancak izoform spesifik çalışmalar ile her bir BAG-1 izoformun etkileştiği proteinler ve görev aldığı mekanizmalar ile ilgili detaylı bilgi bulunmamaktadır.

BAG-1M'nin hem sitoplazma hem de çekirdekte bulunabilmesi, bu izoformun hem DNA hem de sitoplazmik proteinler ile etkileşerek kanser sürecinde görev alabileceğini düşündürmüştür. Bu çalışmada BAG-1M izoformunun öncelikle BAG-1'in bilinen etkileşim partnerleri ile olan etkileşiminin incelenmesi ve hücre sağkalımı üzerindeki etkisinin gösterilmesi, sonrasında ise etkileşim partnerlerinin meme kanseri ve meme epitel hücrelerinde tespit edilmesi amaçlanmıştır. Yapılan bu çalışmanın BAG-1M'nin kanserli ve meme epitel hücrelerindeki farklı etkileşimleri göstererek bu izoformun meme kanseri oluşum ve gelişim sürecindeki rolünün aydınlatılmasına katkıda bulunması hedeflenmiştir.

BAG-1M'nin etkileşim partnerlerinin tespit edilebilmesi için öncelikle N-ucu TAP etiketi içeren BAG-1M dizisini içeren vektör plazmid hücrelere transfekte edilmiş, ardından TAP etiketi pürifikasyonu ile transfekte edilen hücrelerden elde edilen total protein içerisinden BAG-1M saflaştırılmıştır.

BAG-1M'nin hücre sağkalımındaki etkisi transfekte edilmemiş hücrelerle karşılaştırmalı olarak gösterilmiş, BAG-1M izoformunun yüksek ifadesinin hücre proliferasyonunu ve hücrelerin koloni oluşturma yatkınlığını arttırdığı hücre canlılığı testleri ile gösterilmiştir.

BAG-1'in bilinen etkileşim partnerleri olan HSP-70, Bcl-2, Raf-1, Akt, Raf-1 ve Akt'ın sırasi ile 338. ve 473'üncü serinlerinden fosforillenmiş formları ile BAG-1M etkileşiminin olduğu saflaştırılan BAG-1M örneklerinde immün blotlama ile gösterilmiştir.

Bilinen etkileşim partnerleri dışındaki etkileşen proteinlerin tespit edilmesi amacıyla saflaştırılan BAG-1M proteini tripsin ile peptitlerine parçalanmış ve peptit haritalama yöntemi için sıvı kromatografisi/kütle spektrometresi (LC-MS/MS) ile analiz edilmiştir. Tespit edilen peptitler PLGS (Protein Lynx Global Server) kullanılarak UNIPROT veritabanı ile eşleştirilerek kimliklendirilmiş, iki biyolojik tekrarın ikişer teknik tekrarı yapılarak ortak bulunan proteinler BAG-1M etkileşim partnerleri olarak kabul edilmiştir.

Sıvı kromatografisi/kütle spektrometresi analizleri sonucunda tespit edilen BAG-1M etkileşim partnerlerinin hücre içerisinde dahil oldukları mekanizmalar ve protein-protein etkişim ağları sırası ile KEGG ve STRING internet ağ sunucuları kullanılarak analiz edilmiş, proteinler GO Enrichment analizi kullanılarak moleküler fonksiyonlarına göre karbon metabolizması, hücre iskeleti organizasyonu, stres yanıtı ve endoplazmik retikulumdaki protein işlenmesi olmak üzere dört kategori altında incelenmiştir. BAG-1'in endoplazmik retikulum ilişkili degradasyonda görev alabileceğinin düşünülmesinden dolayı bu çalışmada endoplazmik retikulumda protein işlenmesi kategorisine odaklanılmış, etkileşimlerin kanserli ve epitel meme hücreleri arasındaki farkları incelenmiştir.

LC-MS/MS analizleri sonucunda kimliklendirilen proteinlerin doğrulanması amacı ile iki boyutlu jel elektroforezi (2-DE) yöntemi kullanılarak proteinler izoelektrik noktaları ve moleküler ağırlıklarına göre ayrılmış, jelden kesilen protein spotları MALDI-TOF analizleri ile kimliklendirilmiştir. Kütle spektrometresi ve iki boyutlu jel elektroforezi sonuçlarında ortak gelen proteinlerin BAG-1M izoformu ile etkileşimi meme kanseri ve meme epitel hücrelerinden saflaştırılan örneklerin immün blotlama analizleri ile de doğrulanmıştır.

BAG-1M izoformunun etkileştiği proteinlerle oluşturduğu komplekslerin görev aldığı hücresel mekanizmaları aydınlatması amacıyla doğal formları korunarak transfekte edilen hücrelerden izole edilen ve saflaştırılan proteinlerin oluşturduğu kompleksler mavi poliakrilamid jel elektroforezi ile moleküler ağırlıklarına göre ayrılmıştır. Her bir kompleksin jelden kesilmesi, peptitlerine parçalanması ve LC-MS/MS peptit haritalaması ile kompleksteki proteinler tespit edilmiştir. Komplekslerin incelenmesi ile tespit edilen proteinlerin proteazom alt birimlerini, ubikuitinasyonda görev alan proteinleri, endoplazmik retikulumda yerleşik olan proteinleri içerdiği görülmüş, bu proteinlerin aynı komplekslerde bulunması da BAG-1M'nin endoplazmik retikulum ilişkili degradasyonda görev alabileceği düşüncesini desteklemiştir.

Bu çalışmada yapılmış olan tüm deneysel çalışmalarda elde edilen sonuçlar, ısı şok proteini 90 (HSP-90), ısı şoku proteini 70 ailesi üyesi BiP (HSPA5), protein disülfid izomeraz A3 (PDIA3), geçişli endoplazmik retikulum ATPaz (VCP) ve UV eksizyon tamir proteini RAD23 homologu (RAD23B) gibi katlanma/yeniden katlanma mekanizmasında görevli ve ubikuitin ile ilişkili proteazomal bozunmada işlevi olan proteinlerle BAG-1M izoformunun etkileştiğini göstermiştir. Meme kanseri ve meme

epitel hücrelerinde BAG-1M ile VCP ve BAG-1M ile RAD23B etkileşimlerinin farklılık gösterdiği immün blotlama yöntemi ile gösterilmiştir. BAG-1M izoformunun VCP ve RAD23B ile kurduğu etkileşimin sadece meme kanseri hücrelerinde görülmesi bu etkileşimlerin kanser sürecinde önemli rolü olabileceğini düşündümüştür.

Yeni tespit edilen etkileşim partnerlerinin PDB veritabanında bulunan yapıları BAG-1'in BAG domaini ve ubikitin benzeri domaini ile PRIŞM web sunucusu kullanılarak incelenmiş, kurulabilecek olası kompleksler yapıları ve iki protein arasındaki enerji seviyesine göre değerlendirilmiştir. Yapısal analizler, RAD23B, VCP ve PDIA3'ün BAG-1 ile etkileşime geçebileceklerini, ancak HSP-90 ve BiP ile BAG-1 etkileşiminin BAG-1'in her iki domaininden de pek olası bulunmadığını göstermiştir.

Mavi-nativ jel elektroforezi ile kimliklendirilen kompleksler incelendiğinde, HSP-70 ile HSP-90 ve BiP'in bilinen etkileşimleri, BAG-1 ile HSP70 etkileşiminin biliniyor olması ve yapısal analizlerde BAG-1M ile HSP-90 ve BAG-1M ile BiP'in etkileşiminin olası bulunmaması, bu iki protein ile BAG-1M'nin birlikteliğine HSP-70 veya başka aracı proteinlerin sebebiyet verebileceğini düşündürmüştür.

Bu çalışma ile BAG-1M izoformunun doğrudan (fiziksel) veya dolaylı (fonksiyonel) olarak etkileştiği proteinler kimliklendirilmiştir. BAG-1'in rol aldığı mekanizmalar, bilinen etkileşim partnerleri ve yeni tanımlanan etkileşen proteinler göz önünde bulundurulduğunda, endoplazmik retikulumdaki yerleşik proteinlerle, ubikuitinasyon mekanizmasında görev alan proteinlerle, proteazom alt üniteleri ve şaperonlarla olan etkileşimleri BAG-1M izoformunun endoplazmik retikulum ilişkili degradasyon (ERAD) mekanizmasında fonksiyonu olabileceğini önerisini desteklemektedir.

Elde edilen deneysel bulgular ve gerçekleştirilen yapısal analizler ışığında gelecekte yapılacak olan bölgesel yönlendirilmiş mutasyonlar ile tespit edilen protein etkileşimlerinin bozulmasının hücre üzerindeki etkilerinin incelenmesi, degradasyon mekanizmasında hedef alınan substratların fonksiyonel analizler ile tespit edilmesi ve in vitro yüzey analizi çalışmaları ile etkileşimlerin analiz edilmesi gibi daha detaylı çalışmaların meme kanserinde bir biyobelirteç olarak görülen BAG-1'in BAG-1M izoformuna özgü fonksiyonlarının aydınlatılmasına, yeni tespit edilen etkileşimlerin kristal yapı çalışmalarına ve kanser tedavisinde kullanılabilecek küçük molekül sentezlenmesi ile yeni terapötik ajanlar geliştirilmesine katkıda bulunabileceği düşünülmektedir.

1. INTRODUCTION

1.1 BAG-1

Bcl-2 associated athanogene (BAG) family is evolutionarily protected between various organism such as yeast, plants and animals. BAG family members are known to be able to control their homeostasis in the cell by forming complexes with other proteins under normal and stress conditions by positively or negatively regulating certain processes. Six members of BAG family proteins have been found in human as BAG-1, BAG-2, BAG-3, BAG-4, BAG-5 and BAG-6. All family members have the BAG domain (BD) near the C-terminus and the differences in BAG family members are generally at their N-terminus which allow each member to work specific to divergent proteins, pathways or localizations. BAG proteins are thought to have the roles in HIV infection, Parkinson's disease and carcinogenesis in humans and so they may function as therapeutic targets (Kabbage and Dickman, 2008).

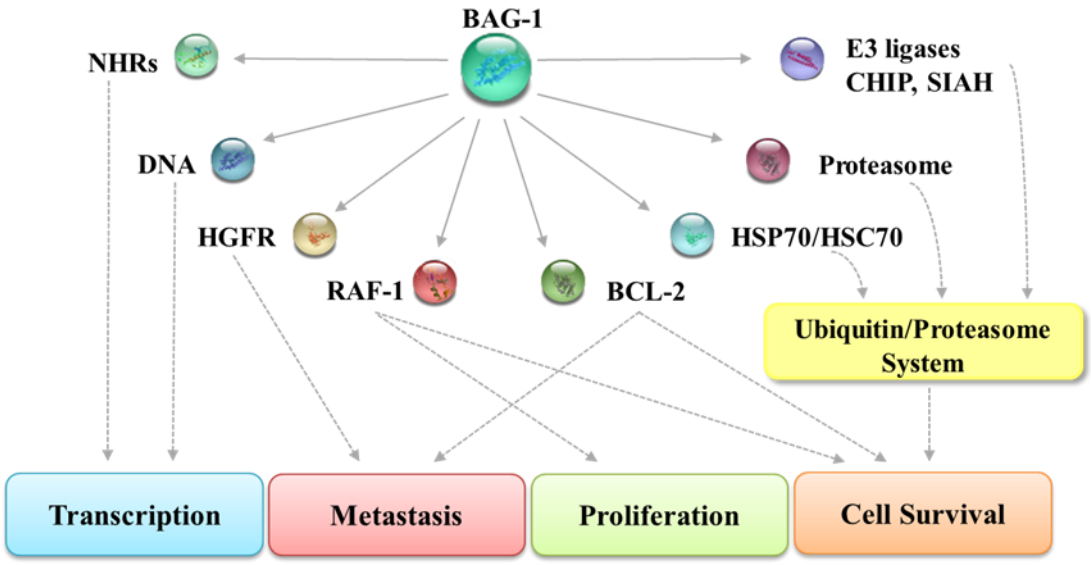


Figure 1.1 : BAG-1 interaction partners and tasks on the molecular pathways

BAG-1 (Bcl-2 associated athanogene 1) has been discovered as an interacting partner of Bcl-2 (Takayama et al., 1995) which has role in transcription, cell migration, proliferation, hormone activity, metastasis and apoptosis via interacting with Bcl-2, HSP70/HSC70, Raf-1 kinase, nuclear hormone receptors, hepatocyte growth factor receptor, DNA and ubiquitination/proteasome system (Townsend et al., 2003).

1.1.1 BAG-1 gene and isoforms

BAG1 gene which is located on the human chromosome 9, band 12, encodes 3885 base pair 7 exon mRNA (Gotz et al., 2005). The promoter that has been dispersed along the positions -353 to -54 upstream of the first translational start codon serves as binding site for several transcription factors. Alternative translation initiation of single mRNA transcript produces one minor and three major BAG-1 isoforms in human as BAG-1L, BAG-1M, BAG-1S and BAG-1 minor (Packham et al., 1997). BAG-1L is encoded by starting of translation at 66^{th} codon (CUG) on mRNA sequence. Initiation starting from 279^{th} codon (AUG) encodes BAG-1M. The initiation from the AUG codon at 411^{th} and 504^{th} amino acids encodes BAG-1S and BAG-1 minor respectively. However the mechanism of alternative translation has not been understood yet, hypothesis to explain the mechanism exists (Towsend et al., 2005). Translation of BAG-1L and BAG-1M isoforms occurs via 5' cap structure of mRNA (Coldwell et al., 2001), while the translation of BAG-1S depends on the binding of ribosome to an internal ribosome entry site (IRES) of mRNA (Dobbyn et al., 2008). Ribosomes can differ according to the initiation sites of translation, which can cause the generation of isoforms from a single BAG-1 mRNA.

The expression level of the shortest isoform BAG-1 minor (29 kDa) is significantly lower than the other isoforms (Gehring, 2006), whereas generally the most abundant isoform in the normal and tumor cells is known as BAG-1S (Packham et al., 1997). Isoforms differ in the N-terminus regions; BAG-1L (52 kDa), the largest isoform has nuclear localization site (NLS), so localizes in nucleus. BAG-1S (33 kDa) and BAG-1M (46 kDa) does not contain NLS, localizes in cytoplasm.BAG-1M can also be translocated in nucleus via accompanying proteins. Ten copies of TRSEEX repeats in the N-terminus of BAG-1L and BAG-1M bring those isoforms role in DNA binding and transcription activation (Zeiner et al., 1999). Repeats that rich in acidic amino acids are also exist in BAG-1S as four copies (Luders et al., 2000).

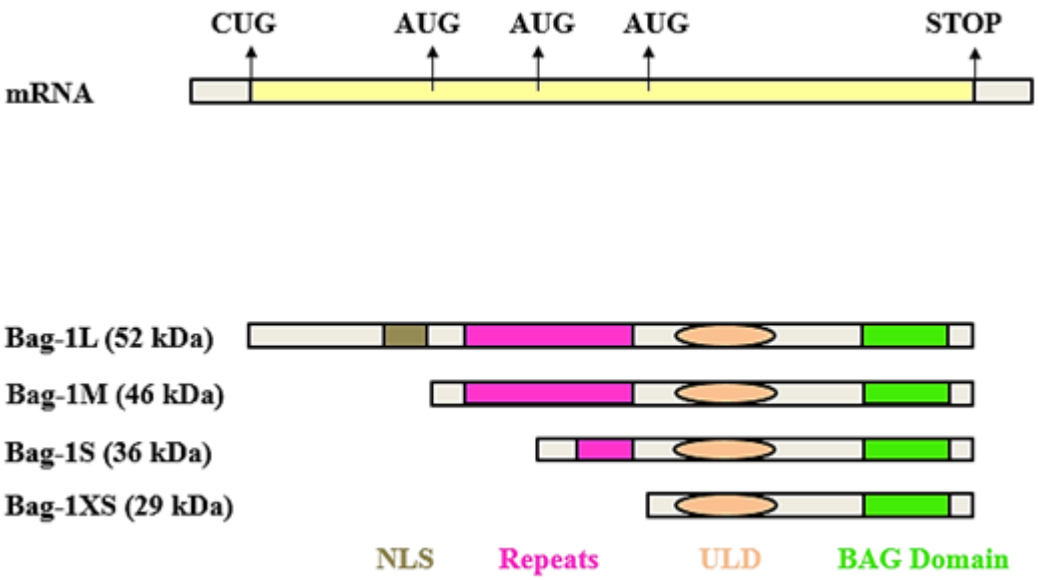


Figure 1.2 : Human BAG-1 isoforms. Four major BAG-1 isoforms obtained from alternative translation initiation of a single mRNA sequence are shown (NLS: Nuclear localization signal, ULD: Ubiquitin-like domain).

All BAG-1 isoforms include ubiquitin like domain (ULD) which has 81 amino acids near to their C-terminus regions. The function of ULD has not been clearly understood yet but suggested to be important for proteasome binding in an ATP-dependent manner (Takayama and Reed, 2001; Luders et al., 2000). Because BAG-1 proteins are stable, rather than being target of ubiquitin/proteasome machinery for degradation (Hohfeld et al., 2001), protection of ULD in evolutionary period and connection with the proteasome machinery is thought to be related to its role under the stress conditions (Alberti et al., 2002).

BAG domain, which exist in all isoforms C-terminus regions, functions as adapter protein that interacts with signaling molecules and molecular chaperones (Briknarova et al., 2001). HSC70/HSP70 heat shock protein family interaction of BAG-1 occurs from the two antiparallel alpha helices on BAG domain (Sondermann et al., 2001) which interfere the ATPase domain of Hsp70 and so modulates its function (Hohfeld, 1998). Interaction of BAG-1 with the kinase region of Raf-1 and B-Raf had been showed by the previous immunoprecipitation experiments (Gotz et al., 2005). It has been known that the interaction of BAG-1 protein with C-Raf activates C-Raf independently of Ras (Wang et al., 1996; Gotz et al., 2005). Ras-independent Raf activationis observed in cancerous cells that overexpressing BAG-1 (Song et al., 2001).

1.2 BAG-1 Interacting Proteins

1.2.1 Bcl-2

Bcl-2 had been identified as a gene against cell death in human follicular lymphoma of B cell origin (Tsujimoto et al. 1985) which blocks the apoptosis caused by stress conditions such as heat shock and nutrient deprivation. Bcl-2 binds Bax, which is a pro-apoptotic member of Bcl-2 family and by inactivation of Bax, releasing of cytochrome c and apoptosome formation is prevented. Interactions of BAG-1 with Bcl-2, increases Bcl-2 activation and localization in the mitochondria. It is thought that BAG-1 is needed to promote the anti-apoptotic activity of Bcl-2 and also to help inactivation of Bax. Additionally, it is suggested that BAG-1 increases the expression of proteins located in mitochondria that inhibit apoptosis (Takayama et al., 1995).

1.2.2 HSP70/HSC70

HSP-70s are ATP-dependent molecules that participate in a wide range of protein folding processes (Mayer, 2013). These proteins protect proteins under stress, prevent the grouping of unfolded proteins and provide a balance between folded and unfolded proteins. Heat shock protein 70 in the cytosol (HSC70) binds to immature polypeptides before leaving from the ribosome. HSP-70s have great importance in the cellular translocation and protein disaggregation (Alberts, 2002; Rios, 2005). Members of the HSP-70 family includes N-terminal ATPase domain and a hydrophobic peptide-binding domain. (Kiang and Tsokos, 1998). BAG-1 is one of the nucleotide exchange factors (NEFs) which are a distinct class of cochaperones that used to complement the ATPase cycle of HSP-70 and so control its substrate binding (Bukau B, 2006). The interaction between BAG-1 and HSP-70 plays a role in the folding of denatured substrate proteins for contributing the cell to maintain the vital activities in normal and stress conditions.

1.2.3 Raf-1

Serine/threonine protein kinase Raf-1 functions in the transmitting cell growth control signals from the surface of the cell to the nucleus. Graded phosphorylation facts occur and control the function of significant transcription factors by the activation of mitogen-activated protein (MAP) kinases by Raf-1 (Sharp et al., 2004). Translocation of Raf-1 to the mitochondria results with phosphorylation of BAD, which inhibits anti-

apoptotic activity of Bcl-2 when it is not phosphorylated. BAG-1 has been known to stimulate the activity of Raf-1 independently from Ras, which resulted in the regulation of cell growth signals under stress conditions and forward cell to survival pathway (Townsend et al., 2004)

1.2.4 CHIP

CHIP (C-terminus of HSC70 Interacting Protein) is an E3 ubiquitin-protein ligase that targets misfolding substrates to proteasomal degradation. BAG-1 and CHIP partnership may occur during the delivery of substrate that to be degraded. Although these two proteins are known as the cochaperones of HSC70 and thought to be related through HSP70, the direct interaction was indicated via co-immunoprecipitation assays (Demand et al., 2001). Interaction of BAG-1 and CHIP regulates the folding/refolding mechanism in degradation pathway by using multiubiquitination of CHIP as the rate-limiting step and forwards cell to survival (McDonough and Patterson, 2003).

1.2.5 Nuclear Hormone Receptors (NHRs)

NHRs are ligand-dependent transcription factors and are also second key targets of BAG-1. NHRs regulates the transcription of target genes via binding the DNA sequence in their promoter regions. Target genes of NHRs play important roles in cellular processes such as growth, division, motility, and death mechanism (Cato and Mink, 2001). BAG-1 modulates activity of NHRs including GR (glucocorticoid receptor), AR (androgen receptor), ER (oestrogen receptor), RAR (retionoic acid receptor), TR (thyroid receptor), PR (progesterone receptor) and VDR (vitamin D3 receptor). Overexpression of BAG-1 activates ER, AR and PR, which are known to be related to the development and progression of cancer (Sharp, 2004).

1.3 Newly Identified Interacting Proteins

1.3.1 HSP-90

Eukaryotic cytosolic HSP-90s have a broad class of proteins, including protein kinases and transcription factors, and regulate a variety of cellular processes with these interactions (Taipale, 2011). The HSP-90 chaperones are found in dimers and work in conjunction with auxiliary chaperones that support protein folding. The members of

the HSP90 family in the cytoplasm are HSP-90α and HSP-90β, whereas in the endoplasmic reticulum is Grp94 (Koyasu et al., 1986). HSP-90 have some common functions with HSP-70, such as interacting with misfolded proteins to prevent aggression, but HSP-90 cannot fold back the accidentally folded proteins into their natural state, unlike HSP-70. With the help of co-chaperones of HSP-90, it determines the stability and activity of proteins that control many cellular processes such as growth, proliferation, differentiation and cell death. This valuable feature makes HSP-90 a candidate for the development of new drug therapies (Mayer, 2015, Trepel, 2010, Johnson, 2012).

1.3.2 VCP

VCP (transitional endoplasmic reticulum ATPase) belongs to the type II AAA+ (ATPases Associated with diverse cellular Activities) protein family which is involved in the transitional endoplasmic reticulum (tER) formation and has a hexameric structure. VCP is also called p97 in mammalians and interacts with a wide variety of partners and cofactors. VCP functions in the ubiquitin-proteasome system by directing the critical steps in ubiquitin-dependent protein quality control (Yeo and Yu, 2016). VCP binds the ubiquitinated proteins and exports misfolded proteins to proteasome from ER for degradation in ATP dependent manner. It is also suggested that p97 protects cells from stress conditions by providing genome stability during proliferation (Meyer et al., 2012).

1.3.3 RAD23B

RAD23B is UV excision repair protein RAD23 homolog B that acts as polyubiquitin chain receptor and plays role in modulation of proteasomal degradation. RAD23B is able to simultaneously bind 26S proteasome and ubiquitinated substrates and carry them to the proteasome. Role of RAD23B in endoplasmic reticulum-associated degradation (ERAD) of misfolded proteins is also suggested (Wang et al., 2000). Shuttling of polyubiquitinated proteins to the 26S proteasome is thought to be related with the interaction of RAD23B with ribophorins that present at the base of 19S regulatory subunit of 26S proteasome (Rosenzweig et al., 2012). Function of RAD23B in breast cancer progression is suggested as negatively regulating of the degradation of the DNA repair factors, thus subsequently enhancement of DNA repair mechanism which leads growth advantage to tumor cells (Linge et al., 2014).

1.3.4 PDIA3

PDI family members are enzymatic chaperones that function in the correct refolding of misfolded proteins. PDIA3, one of the members of PDI family activates the disulfide bond isomerization or oxidation/reduction to maintain the correct protein folding in the endoplasmic reticulum (Freedman et al., 1994). PDIA3, an endoplasmic reticulum resident protein is known to have an effect on protecting cells from apoptosis by preventing the aggregation of misfolded proteins and modulating ER stress (Zhao et al., 2015).

1.3.5 BiP

BiP/GRP78 (78-kDa Glucose-Related Protein) is the best-characterized chaperone protein of endoplasmic reticulum that belongs to HSP70 family. 78 kDa glucose regulated protein has importance in ER protein quality control mechanism and controlling the transmembrane signaling molecules of endoplasmic reticulum due to its functions in translocating newly synthesized polypeptides across the ER membrane and role on protein folding and targeting misfolded proteins to endoplasmic reticulum associated degradation (ERAD) mechanism (Halperin et al., 2014). BIP has known to be the master regulator of unfolded protein stress by serving as an endoplasmic reticulum stress sensor with anti-apoptotic properties (Turano et al., 2011).

1.4 BAG-1 and Breast Cancer

BAG-1's role in apoptosis, cell proliferation, cell migration and autophagy, which are key players in progression in cancer development, has been showed by several studies, so BAG-1 is considered as significant prognostic molecule (Cutress et al., 2002). High expression of BAG-1 pushes down the caspase activity, protects cells from many apoptotic stimulates, increases the cell proliferation and metastasis, regulates various nuclear hormone receptors transcriptional activities (Takayama and Reed, 2001) and so suggested to have an important role in various cancer types according to the correlation with clinical parameters (Townsend et al., 2003). It has been showed that interactions with stress factors in the case of overexpression of BAG-1 modulates the survival pathways of breast cancer cells.

Breast cancer is an important health problem all over the world and one of the most common causes of cancer death in developed countries. Increased frequency of breast

cancer especially in women and covering 23% of all cancer types have increased the need for new approaches to treatment (Schuur & DeAndrade, 2015).

The identification of Bag-1 interacting molecules is critical for the development of new treatment strategies for cancer. Therefore, clarification of the role of Bag-1 isoforms may be a determining factor in the efficiency of the treatment.

1.5 Aim of The Study

The discovery of protein complexes in signal pathways that play a role in the formation and development of breast cancer helps to identify new target molecules that can be used therapeutically. In this regard, it is thought that the detection of the interaction partners of anti-apoptotic, adapter protein BAG-1 isoforms that are overexpressed in breast cancer may be an important step for lightening the apoptosis and survival behavior of cells in cell signaling. In this study, according to the lowest expression level than the other major isoforms and co-localization ability between nucleus and cytoplasm we aimed to detect the the interaction partners of the BAG-1M isoform. Detection of interacting proteins of BAG-1M may provide important contributions for the identification of novel target molecules that may be important for apoptosis or cell survival behavior of the breast cancer and breast epithelial cells.

2. MATERIALS and METHODS

2.1 Materials

2.1.1 Equipments

Equipments that were used in this study are listed in Table 2.1.

Table 2.1 : Laboratory equipments used in this study

Name of Equipment	Catalog Number	Supplier Company
Autoclave	GR110DF	Zealway
Balance	BJ 610C	Precisa
Balance	XB 220A	Precisa
Centrifuge	Mega Star 3.0R	VWR
Centrifuge	IEC CL10	Thermo Scientific
CO_2 Incubator	CB 150	Binder
Confocal Microscope	DM IRE2	Leica
Deepfreeze (-20^{o}C)	-	Bosch
Dry Blotting System	iBlot 2	Thermo Scientific
Dry Block Thermostat	Bio TDB-100	Biosan
Electronic Pipet Controller	ExactaCruz	Santa Cruz
Electrophoresis Chamber	Mini-PROTEAN Tetra Cell	Bio-Rad
Electrophoresis Chamber	XCell SureLock Mini-Cell	Thermo Scientific
Electrophoresis Power Supply	EPS 301	GE Healthcare
Freezer (-80°C)	DW-86L630	Aucma
Hemacytometer	New	Thoma
Ice Flaker	AF80	Scotsman
Imaging System	ChemiDoc XRS+	Bio-Rad
IEF System	Protean i12	Bio-Rad
Inverted Microscope	CK40	Olympus
Laminar Flow	BH-EN 2003	Faster
Magnetic Stirrer	MS-H-S	DragonLab
Mass Spectrometry	Synapt G2-Si	Waters

Tablo 2.1 (continued) : Laboratory equipments used in this study

Microcentrifuge	1730R	ScanSpeed
Micropipette (0.2□l-2□l)	NU02626	Thermo Scientific
Micropipette (2µl-20µl)	NU02766	Thermo Scientific
Micropipette (20µl-200µl)	NU08613	Thermo Scientific
Micropipette(100µl-1000µl)	NU03002	Thermo Scientific
Microplate Spectrophotometer	Benchmark Plus	Bio-Rad
Microwave Oven	ALMD 17 B	Altus
Mini Centrifuge	MINO-10K	Inovia
Nanodrop	NanoDrop 2000	Thermo Scientific
pH Meter	Seven Compact	Mettler Toledo
Platform Shaker	Duomax 1030	Heidolph
Rotator Mixer	HAG	FinePCR
Shaker	BBraun Certomat S II	Sartorius
Spectrophotometer	PharmaSpec UV-1700	Schimadzu
Refrigerator (+4oC)	A+	Bosch
Ultrapure Water System	Milli-Q Type 1	Merck
Vacuum Concentrator	Concentrator Plus	Eppendorf
Vortex	MX-F	DragonLab

2.1.2 Molecular Biology Kits

Molecular biology kits that were used in this study are listed in Table 2.2.

Table 2.2 : Molecular biology kits used in this study

Name of Kit	**Catalog Number**	**Supplier Company**
AcTEV Protease	12575-015	Thermo Scientific
Bradford Protein Assay Kit	5000006	Bio-Rad
Clarity Western ECL Substrate	1705061	Bio-Rad
DNA-midi Plasmid DNA Purification Kit	17252	Intron
iBlot 2 Nitrocellulose Regular Stacks	IB23001	Thermo Scientific
iN-fect DNA Transfection Reagent	15081	Intron
Mammalian Cell & Tissue Extraction Kit	K269-500	BioVision
ReadyPrep 2-D Starter Kit	1632105	Bio-Rad

ReadyStrip IPG Strips	1632000	Bio-Rad

2.1.3 Chemicals

Chemicals that were used in this study are shown in Table 2.3.

Table 2.3 : Chemicals used in this study

Name of Chemical	Catalog Number	Supplier Company
Acetic Acid	27225-2.5L-R	Sigma-Aldrich
Acetonitrile	1000302500	Merck
Acrylamide/Bis-Acrylamide (40%)	1610148	Bio-Rad
Agar Bacteriological Grade	800-010-LG	MultiCell
Ammonium Bicarbonate	1011315000	Merck
Ammonium Persulfate (APS)	A3678	Sigma-Aldrich
Ammonium Sulfate	A4418-1KG	Sigma-Aldrich
Ampicillin	K029.2	Roth
Bis-Tris	B9754	Sigma-Aldrich
Blotto, Non-Fat Dry Milk	sc-2325	Santa Cruz
Bromophenol Blue	B0126	Sigma-Aldrich
Bradford Reagent (1X)	500-0205	Bio-Rad
Bovine Serum Albumin (BSA)	A9418	Sigma-Aldrich
Choleratoxin	C8052	Sigma-Aldrich
Coomassie Brilliant Blue G-250	A2329-5G	Biomatik
Coomassie Brilliant Blue R-250	27816	Fluka
Digitonin	D141-100MG	Merck
Dithiothreitol (DTT)	K0311	Fermentas
DMEM (1X)	41966-029	Thermo Scientific
DMEM/F-12 (1:1) (1X)	11320-033	Thermo Scientific
DMSO	A2424	BioMatik
EDTA	E6758	Sigma-Aldrich
Epidermal Growth Factor (EGF)	E9644	Sigma-Aldrich
Ethanol Absolute	1009832500	Merck
Fetal Bovine Serum (FBS)	10270-106	Thermo Scientific
Formic Acid	1002632500	Merck
Glycine	sc-29096	Chem Cruz
Glycerol	800-040-LL	MultiCell

Table 2.3 (continued) : Chemicals used in this study

Horse Serum	16050-122	Thermo Scientific
Hydrochloric Acid (HCl)	07102	Sigma-Aldrich
Hydrochortisone	H0888	Sigma-Aldrich
IgG Sepharose 6 Fast Flow	17-0969-01	GE Healthcare
Insulin	I0516	Sigma-Aldrich
Iodoacetamide	163-2019	Bio-Rad
Isopropanol	1096342511	Merck
2-Mercaptoethanol	M6250	Sigma-Aldrich
Methanol	1060092511	Merck
Phosphoric Acid	04102	Sigma-Aldrich
PhosSTOP	04906845001	Roche
Sodium Chloride (NaCl)	sc-203274	Chem Cruz
NuPAGE LDS Sample Buffer (4X)	NP0007	Thermo Scientific
Phosphate-Buffered Saline (PBS) Tablets	003002	Thermo Scientific
Penicillin/Streptomycine	15140122	Thermo Scientific
Peptone from Casein Pancreatically Digested	1022390500	Merck
Prestained Protein Ladder (PageRuler)	26616	Thermo Scientific
Sodium Dodecyl Sulfate (SDS)	L3771	Sigma-Aldrich
TEMED	T9281	Sigma-Aldrich
Tricine	T0377	Sigma-Aldrich
Tris Base	600-127LG	MultiCell
Tris(2-carboxyethyl)phosphine (TCEP)	C4706	Sigma-Aldrich
Trypsin-EDTA (1X)	25200-056	Thermo Scientific
Trypsin from Porcine Pancreas	T6567-20UG	Sigma-Aldrich
Tween-20	TWN508.500	BioShop
Urea	C4706	Sigma-Aldrich
Yeast Extract	170200	Pronadisa

2.1.4 Antibodies

Antibodies that were used in this study are shown in Table 2.4

Name of Antibody	Type	Dilution
Anti-Rabbit IgG (CST, 7074S)	Polyclonal HRP-linked	1:5000 (5% Milk)
Anti-Mouse IgG (CST, 7076S)	Polyclonal HRP-linked	1:5000 (5% Milk)
14-3-3 (pan) (CST, 8312S)	Polyclonal Rabbit	1:500 (5% BSA)
Akt (CST, 9272S)	Polyclonal Rabbit	1:500 (5% BSA)
Bag-1 (CST, 3920S)	Monoclonal Mouse	1:500 (5% BSA)
Bag-3 (Sigma-Aldrich, SAB2103926)	Polyclonal Rabbit	1:500 (5% BSA)
Bcl-2 (CST, 4223S)	Monoclonal Rabbit	1:500 (5% BSA)
beta-Actin (CST, 4970S)	Monoclonal Rabbit	1:500 (5% BSA)
BiP (CST, 3177S)	Monoclonal Rabbit	1:500 (5% BSA)
B-Raf (CST, 9433S)	Monoclonal Rabbit	1:500 (5% BSA)
CHIP (CST, 2080S)	Monoclonal Rabbit	1:500 (5% BSA)
C-Raf (CST, 9422S)	Polyclonal Rabbit	1:500 (5% BSA)
Hsp27 (CST, 2402S)	Monoclonal Mouse	1:500 (5% BSA)
Hsp70 (CST, 4872S)	Polyclonal Rabbit	1:500 (5% BSA)
Hsp90 (CST, 4874S)	Polyclonal Rabbit	1:500 (5% BSA)
p-Akt (S473) (CST, 4060S)	Monoclonal Rabbit	1:500 (5% BSA)
p-B-Raf (S445) (CST, 2696S)	Polyclonal Rabbit	1:500 (5% BSA)
p-C-Raf (S/289-296-301) (CST, 9431S)	Polyclonal Rabbit	1:500 (5% BSA)
p-C-Raf (S338) (CST, 9427S)	Monoclonal Rabbit	1:500 (5% BSA)
PDIA3 (Sigma-Aldrich, SAB1410582)	Polyclonal Rabbit	1:500 (5% BSA)
RAD23B (CST, 13525S)	Monoclonal Rabbit	1:500 (5% BSA)
RNF34 (Abcam, ab169047)	Polyclonal Mouse	1:500 (5% BSA)
VCP (CST, 2648S)	Polyclonal Rabbit	1:500 (5% BSA)

Table 2.4 : Antibodies used in this study

2.1.5 Buffers and solutions

2.1.5.1 10X TBS solution

10X TBS was prepared with 87.66 g NaCl, and 24.22 g Tris Base in 1 L dH_2O and adjusted pH 7.6 with HCl.

2.1.5.2 10X Running buffer

10X Running Buffer was prepared with 144 g Glycine, 30.3 g Tris Base, and 10 g SDS in 1 L dH_2O.

2.1.5.3 20X Anode buffer

20X Anode Buffer was prepared with 209.2 g 50 mM Bis-Tris and 179.2 g Tricine (pH 6.8) in 1 L dH_2O.

2.1.5.4 SDS Polyacrylamide separating and stacking gels

SDS polyacrylamide separating gel (12%) and SDS polyacrylamide stacking gel (5%) were prepared as it is indicated in Table 2.5.

Table 2.5 : Content of 12% separating gel and 5% stacking gel

Content	Amount	
	Separating (12%)	Stacking (5%)
dH_2O	1.40 ml	1.93 ml
40% Acrylamide/Bis-acrylamide	270 µl	1.52 ml
1.5 M Tris-HCl	250 µl	1.5 ml
10% SDS	20 µl	60 µl
APS (10%)	20 µl	60 µl
TEMED (100%)	2 µl	6 µl

2.2 Methods

2.2.1 Vector cloning

The vector, pEZ-M02 was selected for cloning and TAP (Tandem Affinity Purification) tag which contains Protein A – TEV (tobacco etch virus) – CBP (calmodulin binding peptide) sequences was added to the N- terminus of Bag-1M sequence in order to purify Bag-1M isoform.

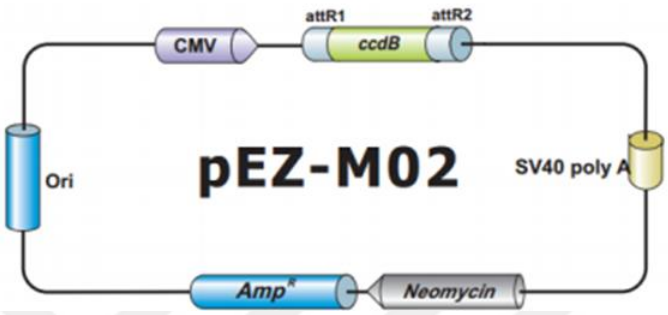


Figure 2.1 : pEZ-M02 clonning vector

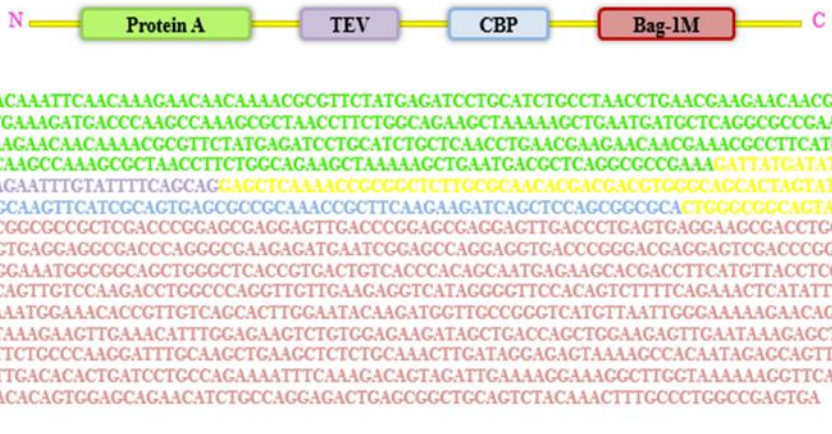


Figure 2.2 : Sequence of N-terminus TAP-Tagged Bag-1M isoform (TEV: Tobacco Etch Virus, CBP: Calmodulin Binding Peptide)

2.2.2 Competent cell preparation

E. coli-DH5α cells were incubated in 5 mL LB medium overnight at 37°C in orbital shaker, then 100 mL LB was inoculated with culture solution and incubated at 37°C untll the optical density (OD) reached 600. Culture solution were then transferred into 50 mL untracentrifuge tubes and incubated on ice for 10 min. Centrifugation was performed at 1600 xg for 7 min at 4°C. 10mL of ice-cold $CaCl_2$ was used to resuspend the bacterial pellet and centrifuged at 1600 xg for 5 min. After supernatant was

discarded, pellet was resuspended in 10 mL ice-cold $CaCl_2$ again and incubated on ice for 30 min. Bacterial culture was centrifuged at 1600 xg for 5 min at 4°C and pellet was completely resuspended in 2 mL of $CaCl_2$. Competent cells then were aliquated in microcentrifuge tubes and stored at -80°C.

2.2.3 Transformation

Component cells were taken from -80°C were thawed on ice and then were incubated with purified plasmid DNA for 30 min on ice. Plasmid DNA taken by cells was maintained with heat shock as incubation on 42°C for 45 sec then incubation on ice for 2 min. Then 80 µL SOC medium was added and cells were placed in orbital shaker at 37°C for 1 h. Culture then plated on LB-Amp and incubated overnight at 37°C.

2.2.4 Plasmid DNA preparation

Plasmid isolation was performed in conformity with manufacturer's protocol (DNA-midi SV plasmid DNA Purification Kit, iNtRON Biotechnology). Isolated plasmid's concentrations and were determined by using Nanodrop.

2.2.5 Cell culture

MCF-7 (ATCC-HTB-22) human breast epithelial cancer cell line was cultured in DMEM (Dulbecco's modified Eagle's medium) with 10% fetal bovine serum, 10 units/ml penicillin-G and 10 mg/ml streptomycin. MCF-12A (ATCC-CRL-10782) human breast non-tumorigenic epithelial cell line was cultured in 1:1 mixture of DMEM and Ham's F12 medium which supplemented with 5% horse serum, 20 ng/ml epidermal growth factor, 100 ng/ml cholera toxin, 0.01 mg/ml insulin, 500 ng/ml hydrocortisone 10 units/ml penicillin-G and 10mg/ml streptomycin. Cells were incubated in 5% CO_2 moisturized air at 37°C. Cells were split up while reaching 90% confluency by PBS (phosphate buffered saline) and 0.25% Trypsin-EDTA.

2.2.6 Plasmid transfection

MCF-7 and MCF-12A cells were seeded into 6-well plates as $0.5x10^6$ cell/well. After 24h from seeding, transient transfection was performed for both pEZ-M02 which includes N-terminus TAP tagged Bag-1M sequence and pEZ-M02 vector without any insertion. After the determination of effective dose for the plasmid(Figure 3.1), 100 µl serum free medium, 1 µg plasmid and 3 µl transfection reagent according to the 1:3

ratio of the manufacturer's instruction (In-fect in vitro Transfection Reagent, iNtRON Biotechnology) per well was incubated for 15 minutes at room temperature. Equal amounts of transfected complex were transferred into the wells and cells were maintained at 37°C, in a 5% CO_2 incubator for 48h before protein isolation.

2.2.7 Trypan Blue cell survival assay

Cells were seeded in 12-well plate as $0{,}5x10^5$ cells per well one day before transfection. Growth medium was removed, cells were washed with PBS, trypsinized, centrifuged and resolved in 50 µL of 0.4% Trypan blue dye and 50 µL medium 24, 48 and 72 h after transfection. Stained cells were counted under a light microscope, because trypan blue is a vital dye and does not interact the cells with damaged membrane, results with staining of only living cells.

2.2.8 MTT cell viability assay

Cells were seeded in 96-well plate as $1x10^4$ cell per well. 24, 48 and 72 h after transfection, 5mg/mL of 3-4,5-Dimethyl-2-thiaxolyl-2,5-diphenyl-2H-tetrazolium bromide (MTT) was added and incubated at 37° C for 4h. Then media containing MTT reagent was removed, 100 µL of DMSO added each well and after 5 min incubation in dark at room temperature absorbance was determined between 570 – 655nm using microplate reader.

2.2.9 Colony formation assay

Cells were seeded in 6-well plate as $0{,}3x10^4$ cell per well, transfected and incubated in 5% CO_2 moisturized air at 37°C for 10 days. Fixation was performed with methanol/acetone and cells were stained with 0.5% Crystal Violet. Obtaining colonies were examined and photographed under inverted microscope.

2.2.10 Total protein isolation

Transfected cells were scraped from the culture plates and lysed on ice using Mammalian Cell Extraction Kit (BioVision, Maryland, USA). Cell lysates were centrifuged at 10,000xg for 10 minutes at 4°C, concentration of isolated proteins were measured by Bradford Assay using bovine serum albumin (BSA; 2 mg/ml) as a standard.

2.2.11 Immunoblotting analysis

Denatured proteins were separated according to their size by SDS-PAGE, then iBlot Turbo transfer system (Invitrogen, CA, USA) was used to transfer the proteins on gel to the nitrocellulose membranes at 20 V for 7 minutes. Blocking of the membranes was carried out with 5% milk/TBS-T solution (Tris Buffered Saline containing 0.1% Tween-20) for 1 hour at room temperature. Blocked membranes were incubated with primary antibodies overnight at 4°C and then anti-mouse or anti-rabbit IgG-HRP conjugated secondary antibodies for 2 hours at room temperature. Washing membranes was maintained with TBS-T after the incubation with both primary and secondary antibodies. Image Lab software program and ChemiDoc imaging system (Bio-Rad, CA, USA) was used for the visualization of proteins which were entreated with Clarity ECL Western Blotting Substrate (Bio-Rad, CA, USA).

2.2.12 TAP-Tag purification

Tandem affinity purification was applied for the isolation of TAP-tagged Bag-1M protein and its binding partners from the total protein that isolated from transfected cells. GE Healthcare IgG Sepharose 6 Fast Flow were used to purification by following the manufacturer's instructions. Beads were washed two times with TST solution and then incubated with protein lysate overnight at 4°C, latter unbound proteins were collected and 20X Tev Buffer, 0.1 M DTT and AcTEV Protease (Invitrogen Novex AcTEV Protease) were supplemented on the precipitated beads to maintain the elution. After overnight incubation at 4°C, beads were centrifuged at 1500 RPM for 3 minutes and supernatant contains eluted proteins was collected. Beads were washed with TST solution, centrifuged and supernatant collected as washing control. Bradford Assay was applied to evaluate the concentration of quantified protein samples.

2.2.13 2-Dimensional gel electrophoresis (2-DE)

Purified proteins were desalted using ReadyPrep 2D Cleanup Kit (Bio-Rad, CA, USA) according to the manufacturer's protocol. Desalted proteins were suspended in rehydration buffer (8 M urea, 50mM DTT, 2% CHAPS, 0.2% Bio-Lyte, 0.001% Bromophenol Blue) and loaded onto 7 cm pH 3-10 nonlinear immobilized pH strip (Bio-Rad, CA, USA) and incubated for half an hour to saturate to strip. PROTEAN IEF Cell (Bio-Rad, CA, USA) was used to carry out the isoelectric focusing. Rehydration was performed at 30 V for 12 hours pursued by 300 V for 1 hour, 500 V

for 1 hour, 1000V for 1 hour, 3000 V for 1 hour and then focusing was carried out at 8000 V until 80,000 Vh at 20°C. After focusing have completed, strips were equilibrated using equilibration buffer (50 mM Tris-HCl pH 8.8, 6M urea, 30% glycerol, 2% SDS, 0.2% Bromophenol Blue) with additional 1% DTT for 15 minutes at room temperature. For further equilibration strips were incubated with equilibration buffer with the addition of 2.5% iodoacetamide for 15 minutes at room temperature. Equilibrated IPG strips were placed on the top of 12% SDS-polyacrylamide gel and sealed with 0.5% overlay agarose. Protean Mini Cell System (Bio-Rad, CA, USA) was used to SDS-PAGE step at 125 V until Bromophenol Blue dye reach the end of the gel. Staining of the gel performed by 20 min incubation with 5 g of Ammonium sulphate, 1 g of phosphoric acid and 62.5 mg Commasie G-250 dye in 50mL dH_2O and latter 12.5 mL methanol was added. After overnight incubation gel was stored in dH2O at 4°C. ChemiDoc Imaging System (Bio-Rad, CA, USA) was use for visualization. Molecular weights of proteins was estimated according to the protein marker and isoelectric point of proteins was forecasted according to their movement on the pH gradient strip.

2.2.14 In solution tryptic digestion

Purified proteins (20 µg) were exposed to 80 µL 10 M urea for 15min at RT then 25 µL 25 mM DTT, 120 µL 50 mM iodoacetamide and 60µL 25 mM DTT respectively added into the tube, every step has 1 hour incubation at RT. Digestion was provided by adding of 1 µl Trypsin (1 mg/ml) and overnight incubation at 37°C. Formic acid was used to stop trypsin activity and peptide mixture was stored at -20°C until LC/MS analysis.

2.2.15 Blue native polyacrylamide gel electrophoresis (BN-PAGE)

Protein isolation from transfected cells was performed using 4X Native Page Sample Buffer and 5% digitonin instead of Mammalian Cell Extraction Kit and centrifuged at 20,000xg for 30 min to ensure preserving of the protein complexes. Purification procedure was applied and eluted proteins were concentrated using Ultra-0.5 mL Centrifugal Filters (Amicon, EMD Millipore) according to manufacturer's instructions. XCell SureLock Mini-Cell Electrophoresis System (TermoFisher) was used for performing of BN-PAGE. Concentrated-purified proteins samples were prepared with 4X Native Page sample Buffer, Commasie G-250 and 5% digitonin,

loaded into 4-16% gradient NativePAGE Bis-Tris Protein Gel (GE Healthcare). Electrophoresis was started with using anode buffer (50 mM Bis-Tris, 50 mM Tricine pH 6.8) and cathode buffer dark blue (50 mM Bis-Tris, 50 mM Tricine pH 6.8, 0.02% Coomassie Brilliant Blue G-250) at 150 V for 30 min at 4°C. After proteins passed through one - third of gel cathode buffer dark blue was replaced with light blue (50 mM Bis-Tris, 50 mM Tricine pH 6.8, 0.002% Coomassie Brilliant Blue G-250) and applied power was increased to 250 V until the proteins reach end of the gel. After the run has completed, gels were fixed with fixative solution (40% Methanol, 10% Acetic Acid) for 30 min at room temperature.

2.2.16 In gel tryptic digestion

Spots of the complexes on BN-PAGE gel were cut and incubated with 100 µL ACN for an hour by shaking at 800 rpm, at RT. After discarding the ACN, 200 µL 2mM TCEP (in 50 mM AMBIC) were added into the tubes containing gel pieces and incubated by shaking for 30 min. Then 100 µL 12 mM IAD (in 50 mM AMBIC) were used to treat gel spots at 37°C, in the dark for 30 min. Supernatant was discarded, gel pieces were incubated with a solution that contains 150 µL 50 mM AMBIC and 50 µL ACN for 30 min, this step were repeated until the gel pieces completely clarify from the blue color. Then supernatant was discarded and gels were dried via using vacuum concentrator. 200 µL ACN was added on the gels, shaken at RT for 20 min and completely white and opaque gel pieces were incubated with 20µL of 20 ng/µL trypsin at 37°C overnight. Supernatant that collected after trypsin incubation was dried and peptides were dissolved in 32 µL 0.5% formic acid and 8 µL 400 mM AMBIC. Peptide mixtures were stored at -80°C until the LC/MS analysis.

2.2.17 Protein identification by mass spectrometry

Peptide mixture were analyzed in MSE mode by LC-MS (Synapt G2 Si, Waters) with Acquity UPLC BEH C-18 reverse phase column (Waters Corporation, Milford, Mass., USA) for peptide mapping analysis. The sample were injected in the system in proportions such that acetonitrile: water: 1% formic acid (1:89:10) was at a volume of 10 µL with a flow rate of 0.2 ml/min. Glu-Fib measurement was taken at 30 seconds as a reference mass to eliminate possible deviations and the detection of MSE data, screening was performed at 50-2000 m/z.

The protein list of Homo sapiens from UniProt database was uploaded in the FASTA format. Spectral processing, peak listing and database searches were performed by Protein Lynx Global Server 3.0.3 (Waters Corporation, Milford, Mass, USA) and resulting spectra was identified by scanning in the system-loaded UniProt Homo sapiens protein list. The accuracy of peptide identification was selected to be greater than %95. The method parameters were set as minimum ion fragment per peptide 3, minimum number of ions per protein 7, minimum number of peptides per protein 3, protein modification carbidomethylation and monoisotopic and peptide load +1.

2.2.18 Statistical analysis

Band density determination and densitometric analysis were performed on PhotoShop CS5. GraphPad-PRISM 6 software computed standard deviations with biological replicates using statistical p values ANOVA and t-test.

2.2.19 Interactome analysis

Interactome analyses of identified proteins were maintained by using of two available webservers as STRING (Protein-protein interactions network) and KEGG (Kyoto Encyclopedia of Genes and Genomes).STRING (http://string-db.org/.) database which aims to show known or predicted interactions between proteins was used to analyze protein-protein interactions of the identified proteins from purified samples. KEGG PATHWAY (http://www.genome.jp/kegg/tool/map_pathway1.html) server which presents the higher order functional information of proteins was used to analyze the pathways that found proteins were commonly involved.

2.2.20 Structural analysis

Protein Interactions by Structural Matching (PRISM) Protocol server was used to perform the prediction of protein-protein interactions and modeling structural complex of these proteins (URL http://cosbi.ku.edu.tr/prism). Structures of the proteins to be predicted are obtained from Protein Data Bank (PDB). Predicted interaction sites were compared according to the fiberdock energy of surfaces. Protein-protein interaction predictions which have lower fiberdock energy than -10 were accepted as favorable results.PyMOL software (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC) was used to analyze the predicted interactions obtained from PRISM.

3. RESULTS

3.1 Confirmation of Transfections by Immunoblotting

Confirmation of transfections with N-terminal TAP-tagged BAG-1M plasmid and mock (empty vector) plasmid was maintained by immunoblotting analysis. BAG-1M has been overexpressed in both MCF-7 breast cancer cells and MCF-12A breast epithelial cells. Because the BAG-1M sequence has a TAP-Tag (21 kDa) at its N-terminus region, BAG-1M isoforms have additional molecular weight according to TAP and seen at 67 kDa. The mock plasmid was used to control the effect of the vector on the cell and Bactin was used as a loading control and each result was normalized to Bactin.

The effective dose of BAG-1M plasmid was determined by transfection of different amounts of plasmid to MCF-7 breast cancer cell line. After immunoblotting assay for dose determination and relative fold increase analyses of band densities 1µg plasmid transfection per well was approved (Figure 3.1).

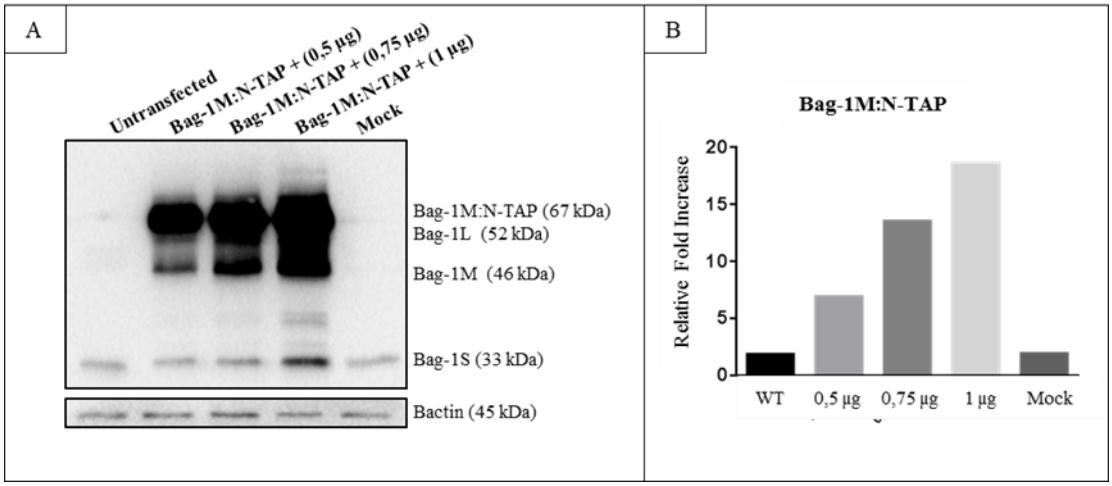


Figure 3.1 : BAG-1 overexpression controls of different amount of N-terminal TAP-tagged BAG-1-M plasmid transfected MCF-7 breast cancer cells A) Immunoblotting result of BAG-1M expression for three different concentration of plasmid (10 µg protein) B) Relative fold change of BAG-1M expressions in comparison to proteins isolated from untransfected cells and mock plasmid transfected cells.

According to the overexpression levels and relative fold changes between different amounts of plasmid transfected cells, 1 µg plasmid per well was seen as most effective dose and further experiments were done with the selected amount for both MCF-7 breast cancer cells (Figure 3.2) and MCF-12A non-tumorigenic breast cells (Figure 3.3).

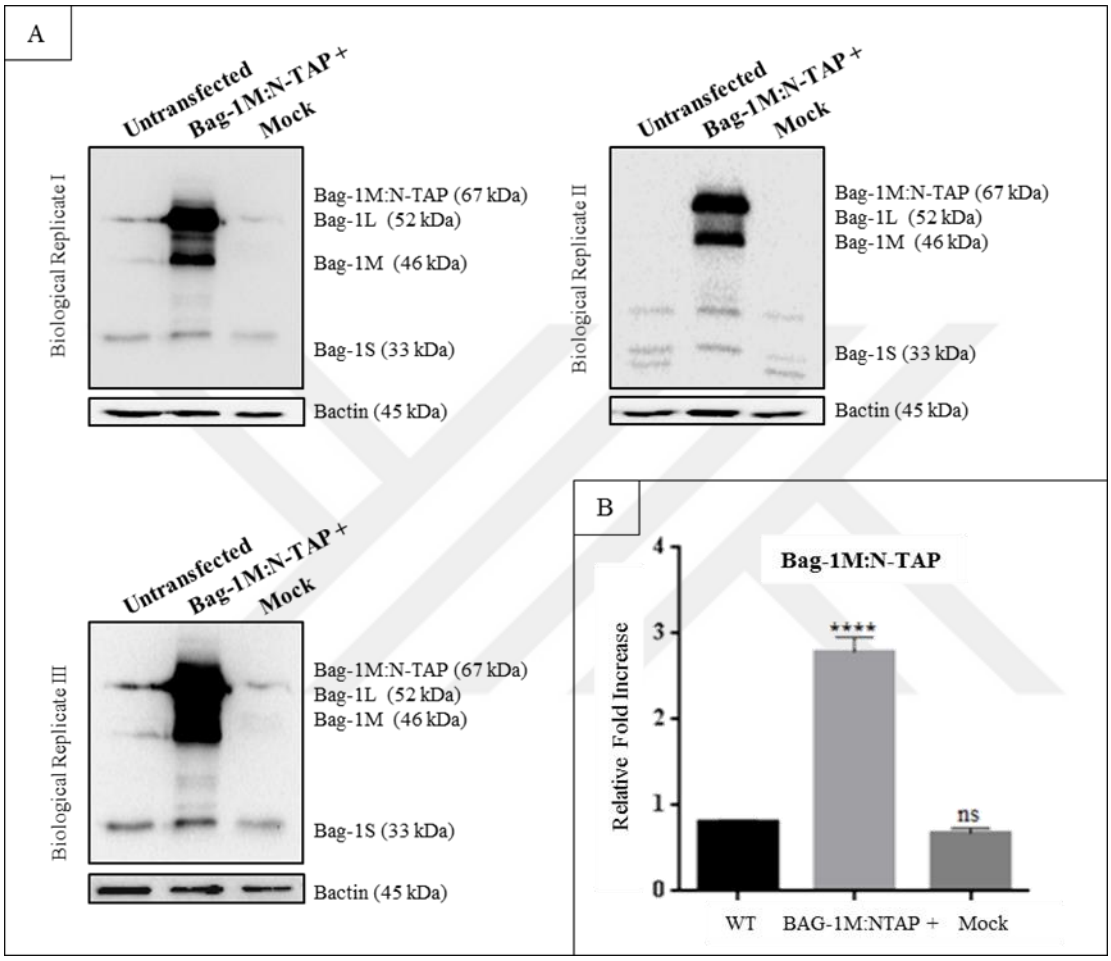


Figure 3.2 : BAG-1M overexpression in MCF-7 breast cancer cells A) Immunoblotting results of BAG-1M expression for three biological replicates (10 µg protein) B) Relative fold change of BAG-1M expression in comparison to untransfected and empty plasmid vector transfected.

For the statistical evaluation of the obtained results, densitometric analysis was performed and analyzed by Tukey's method in GraphPad Prism6 program. When the results are analyzed, it is observed that BAG-1M expression in MCF-7 and MCF-12A cell lines transfected with the N-terminus TAP-tagged BAG-1M plasmid is increased in comparison tountransfected cells and BAG-1 expressions of empty plasmid transfected cells are as expected almost identical with the untransfected cells.

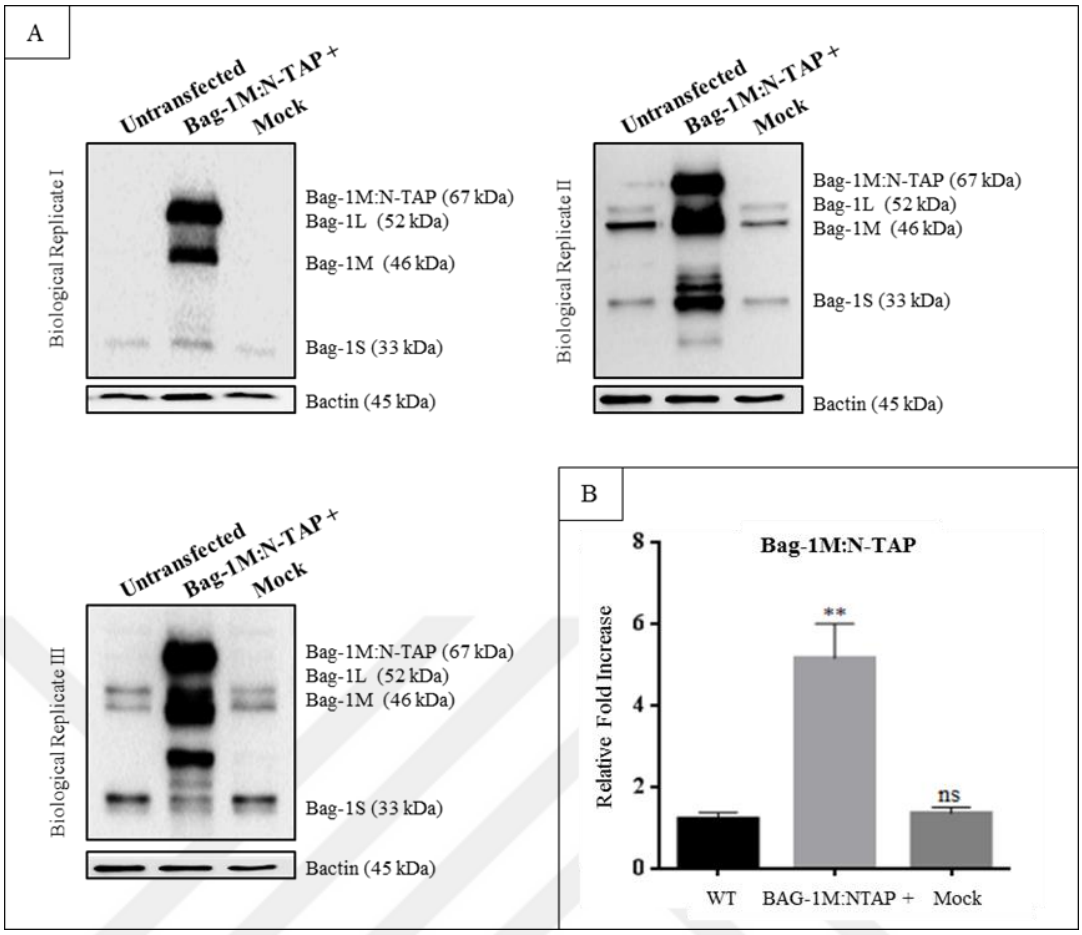


Figure 3.3 : BAG-1-M overexpression in MCF-12A breast non-tumorigenic cells A) Immunoblotting results of BAG-1M expression for three biological replicates (10 µg protein) B) Relative fold change of BAG-1M expression in comparison to untransfected and empty plasmid vector transfected.

3.2 Effects of BAG-1M Expression on Cell Survival

Cell viability assays were used to demonstrate the effects of BAG-1M overexpression on cell survival and cytotoxicity in MCF-7 breast canscer and MCF-12A non-tumorigenic breast cells. Absorbance measurements for MTT assay and cell counting for Trypan Blue assay were performed three times at the time intervals of 24 h and effect of BAG-1M expressionwas evaluated by comparing the relative fold increase for MTT and cell numbers for trypan blue with untransfected cells.

MTT and trypan blue results showed that, BAG-1M overexpression significantly increased cell survival in both MCF-7 and MCF-12A cell lines compared to the untransfected and mock transfected cells. (Figure 3.4 and Figure 3.5).

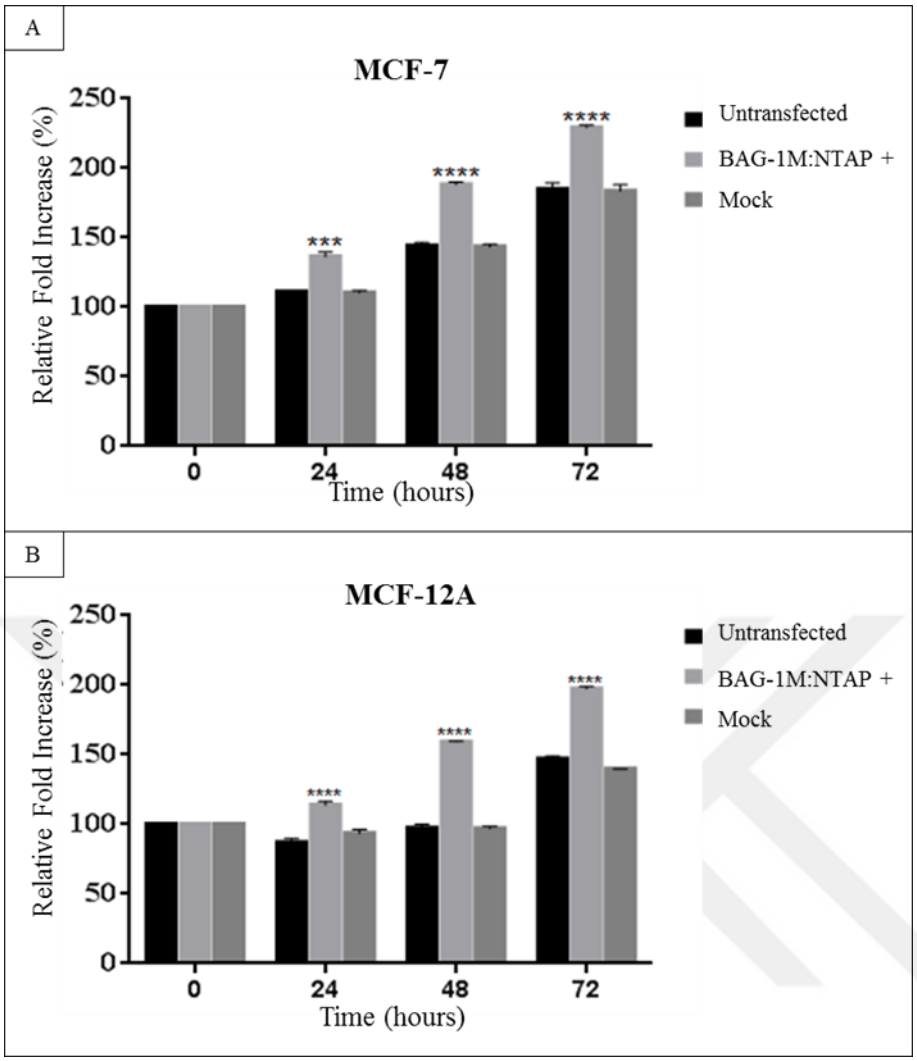


Figure 3.4 : MTT results of A) MCF-7 breast cancer cells, B) MCF-12A non-tumorigenic breast cells in the time intervals of 24, 48 and 72 hours after transfection. Analyses were performed thrice for verification of obtained results.

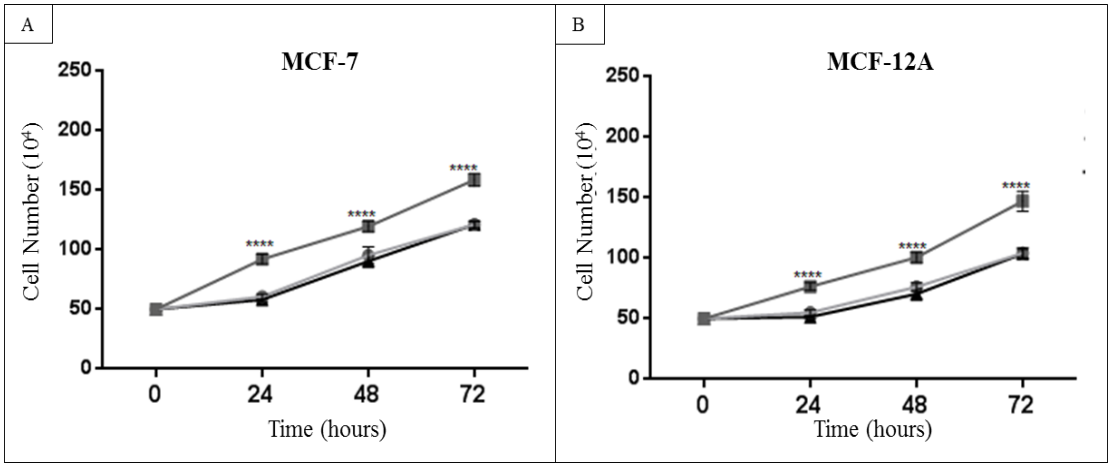


Figure 3.5 : Trypan blue cell viability assay results of A) MCF-7 cells, B) MCF-12A cells in the time intervals of 24, 48 and 72 hours after transfection. Obtained data were verified by repeating cell viability assay three times.

Collony Formation Assay was performed to test the effects of BAG-1M and the empty plasmid in MCF-7 and MCF-12A cell lines. Transfection of cells with N-terminus TAP-tagged BAG-1M plasmid showed to increase the cell proliferation rate and enhance colony formation. Mock plasmid transfected cells were observed to have equal viability with untransfected cells and formed colonies just as untransfected cells (Figure 3.6).

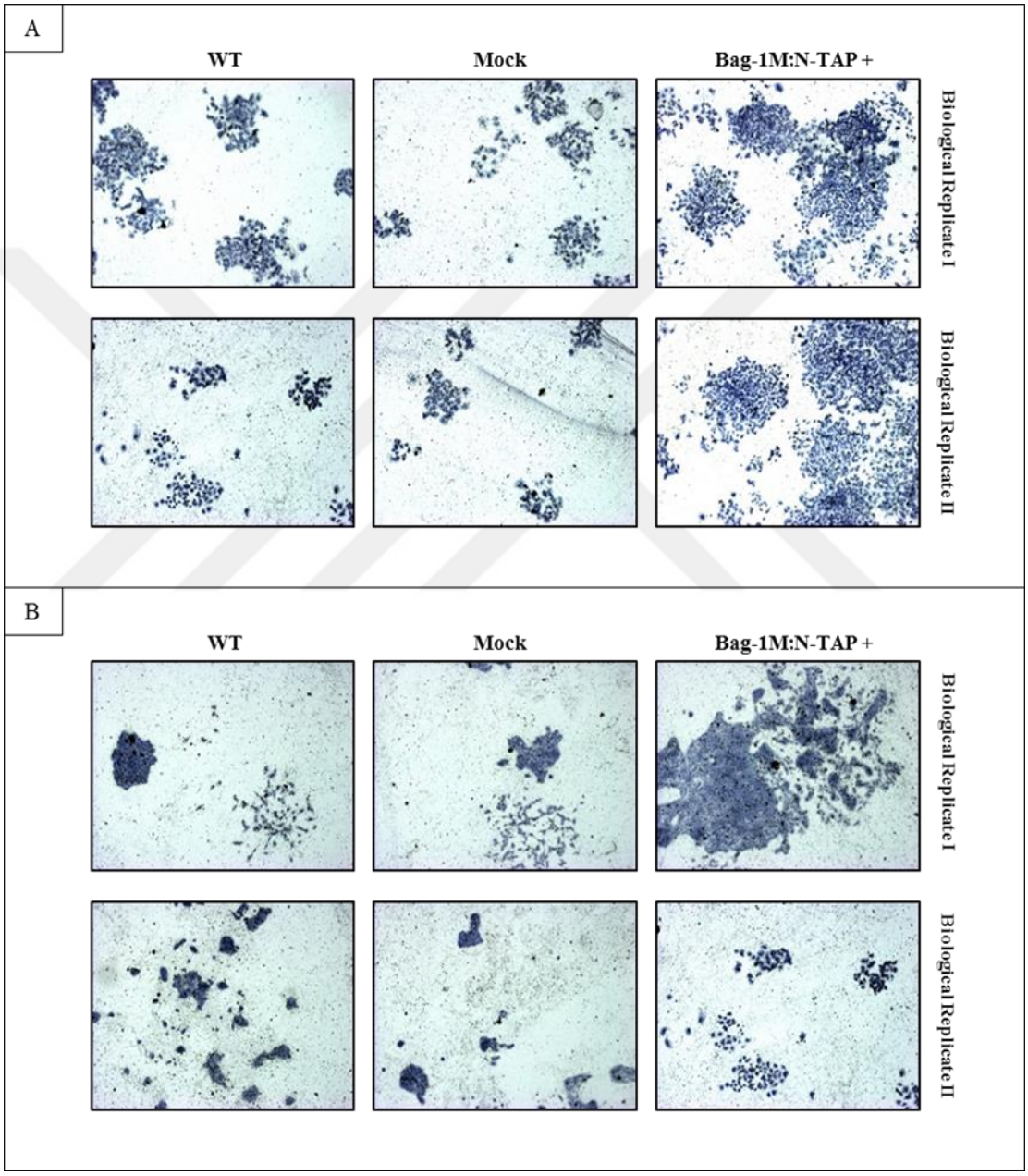


Figure 3.6 : Colony Formation assay results of untransfected, empty vector transfected and N-terminus TAP-tagged Bag-1M plasmid transfected A) MCF-7 breast cancer cell lines and B) MCF-12A nontumorigenic breast cells. All results were performed twice to confirmation on two biological replicates.

3.3 TAP-Tag Purification of BAG-1M

BAG-1M purification was performed via TAP-Tag, IgG Sepharose beads were incubated with total proteins that isolated from MCF-7 and MCF-12A cells transfected with BAG-1M plasmid. After the incubation of beads with total proteins, supernatant was collected as unbound proteins, second incubation was used to maintain the cleavage of BAG-1M by TEV protease; then supernatant collected as eluted proteins which include BAG-1M and its direct or indirect interacting proteins. Because the TEV cleavage site of the sequence is between protein A and CBP; purification process ended up with N-terminus CBP-added BAG-1M, which causes additional 3 kDa molecular weight.Last step was aimed to control the efficiency of protease, beads were washed in harsh conditions and supernatant was stored as wash.

Immunoblotting analysis were performed to compare total protein that isolated from the cells that transfected with N-terminus TAP-tagged BAG-1M plasmid, unbound proteins, eluted protein complex and proteins that obtained after the wash step. BAG-1 immunoblotting results showed that in both MCF-7and MCF-12A, purification of BAG-1M was succeed for three biological replicates of each cell line (Figure 3.7).

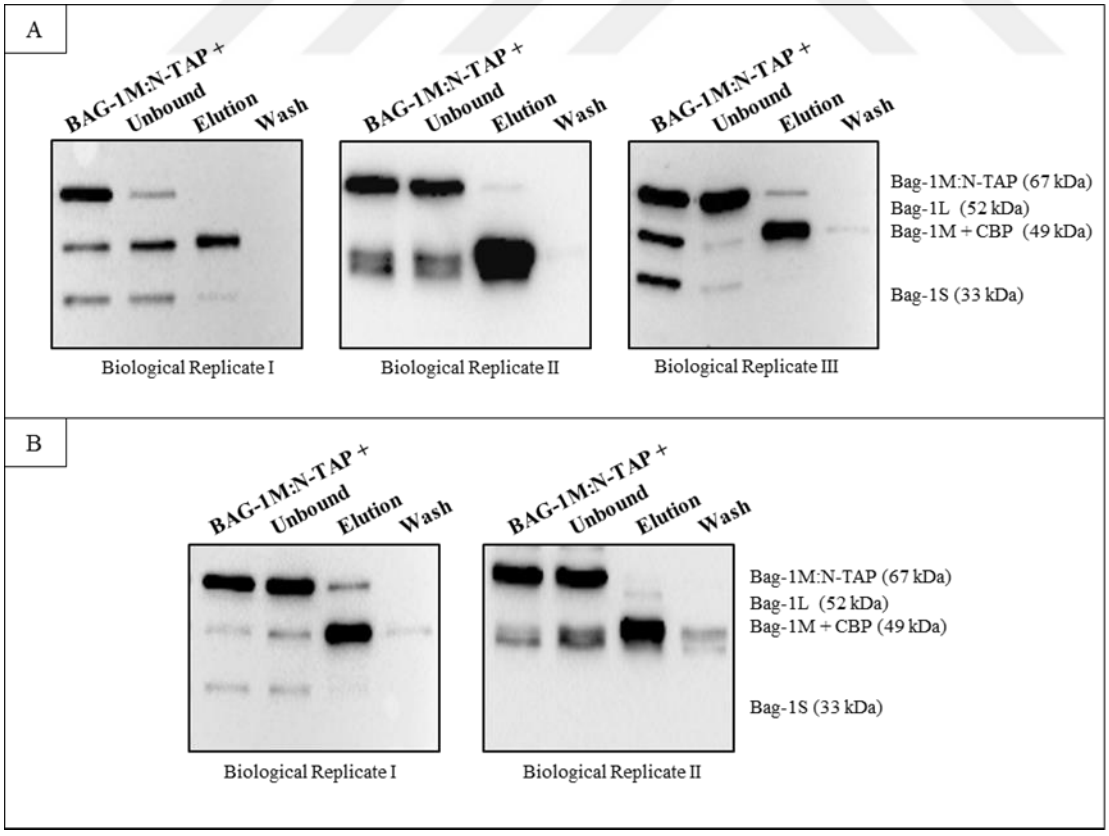


Figure 3.7 : TAP-Tag purification immunoblotting results of A) MCF-7 breast cancer cell lines and B) MCF-12A non-tumorigenic breast cells.

3.4 Immunoblotting Analysis of BAG-1M Interactions with Known Interacting Proteins of BAG-1

BAG-1 interacting proteins that known according to the previous studies on the literature are Bcl-2, HSP-70, CHIP, B-Raf, C-Raf and Akt. Because known interactions are not isoform specific, to see whether BAG-1M is forming a complex with these proteins, eluted proteins from purification were analyzed via immunoblotting assays.

Total proteins that isolated from N-terminal TAP-tagged BAG-1M transfected cells were expected to include all interaction proteins of BAG-1; because of the expressions of all BAG-1 isoforms in the cell and so TAP-Tagged BAG-1M is not the only BAG-1 isoform in the total protein complex. Unbound proteins were also anticipated to contain known partners, too. Interaction of BAG-1M with the known interacting partners of BAG-1 were shown in the elution of the TAP-Tag purification results.

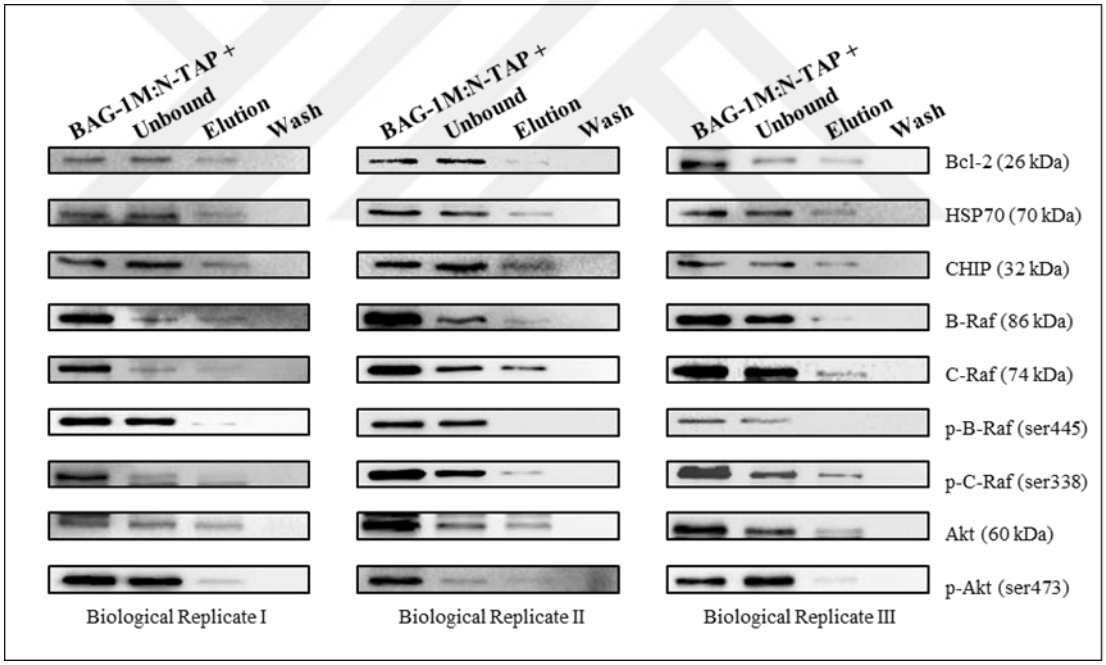


Figure 3.8 : Immunoblotting results of known interacting proteins of BAG-1 in purified samples from BAG-1M transfected MCF-7 cell line breast cancer.

Immunoblotting results of purified BAG-1M from MCF-7 breast cells that transfected with N-terminal TAP-tagged BAG-1M plasmid showed that Bcl-2, HSP-70, CHIP, C-Raf, Akt, phosphorylated forms of C-Raf at serine 338, B-Raf at serine 445, and Akt at serine 473 are interacting with BAG-1M. Confirmation of analyses were maintained

by verifying the results with biological replicates. B-Raf interaction of BAG-1M was not observed in MCF-7 cells (Figure 3.8).

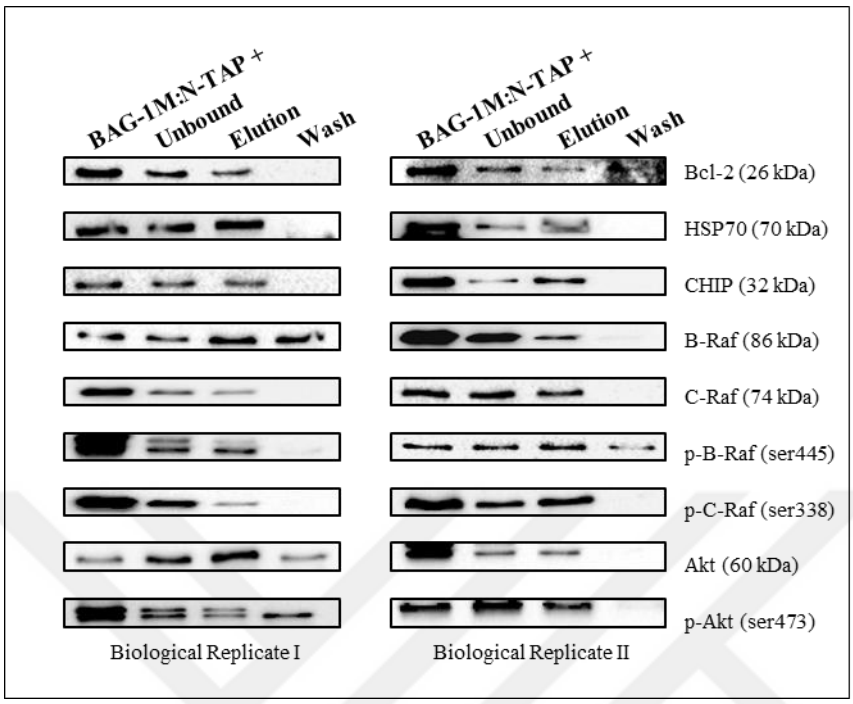


Figure 3.9 : Immunoblotting results of known interacting proteins of BAG-1 in purified samples from BAG-1M transfected MCF-12A breast epithelial cell line.

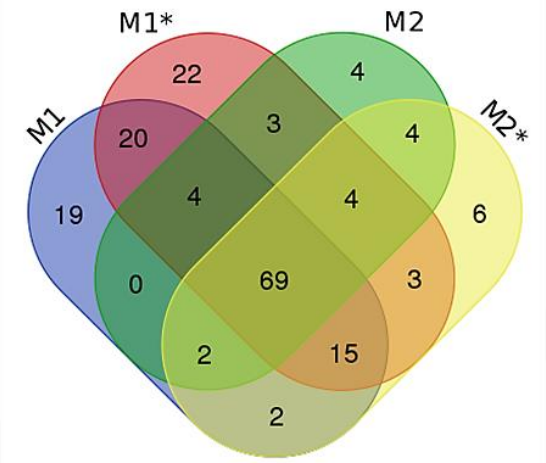
Immunoblotting analysis of purified BAG-1M from MCF-12A cells that transfected with N-terminus TAP-tagged BAG-1M plasmid performed twice for confirmation of the data observed. Results showed that BAG-1M isoform is interacting with Bcl-2, HSP-70, CHIP, C-Raf, B-Raf, Akt and phosphorylated forms of C-Raf at serine 338 f B-Raf at serine 445, and Akt at serine 473 in MCF-12A non-tumorigenic breast cell line.

3.5 Identification of the Interaction Partners of BAG-1M Isoform

Purified BAG-1M samples from the total protein isolated from the N-terminus TAP-tagged BAG-1M plasmid transfected MCF-7 breast cancer cells were digested into peptides by using trypsin. Peptide mixture was analyzed to identify the interaction partners of BAG-1M by the MS^E mode on LC-MS/MS for peptide mapping. LC-MS/MS analyses were performed four times as two biological replicates and two technical replicates for each biological replicates to deal with the possible experimental errors. Venn Scheme was used to specify the common proteins that found

in all replicates which were accepted as the interacting proteins of BAG-1M in MCF-7 breast cancer cell line and used for further analyses.

According to the Venn Scheme of LC-MS/MS results, 68 proteins were found in BAG-1M purified samples as physical or functional interacting proteins of BAG-1Misoform (Figure 3.10).



REPLICATES	PROTEINS
M1 M1* M2 M2*	ACTN4, HSPD1, ANXA2, ENO1, HSPA5, TUBB, TKT, CFL1, HSPB1, YWHAQ, SFN, PPIA, HSD17B10, EEF2, GAPDH, ATP5B, MDH2, NPM1, CTSD, G6PD, TUBB3, GPI, PGAM1, PCBP1, GSN, ATP5A1, YWHAG, PKM, CALR, UBA1, PRDX1, SET, CA2, PGD, NME1, PCNA, HSP90AB1, VCP, ALDOA, LDHA, NME2, NCL, APRT, UGDH, BAG1, FBP1, HSPA8, PDIA3, TUBB4B, TRIM28, MIF, PFN1, HNRNPK, RAD23B, GDI2, TUBA1B, EIF4A1, ISG15, AGR2, HSP90AA1, P4HB, TPI1, YWHAB, PGK1, YWHAZ, YWHAE, EEF1A2, HSPA1A
M1 and M2 are the biological replicates, group names with * are the technical replicates.	

Figure 3.10 : Venn Scheme of the proteins that identified by the LC-MS/MS analyses of BAG-1M purified samples from N-terminus TAP-tagged BAG-1M transfected MCF-7 cells and the list od the common proteins that found at all replicates.

Protein-protein interaction networkanalysis of 68 proteins that found as BAG-1M interacting proteins was performed by using STRING web server (Figure3.11).

To further overcome with the complex network profile of the interaction partners of BAG-1M, proteins were catagorised according to GO (Gene Onthology) Enrichment Aanlysis under four classes that they involved in as carbon metabolism, cytoskeleton organisation, cellular response to stress and protein processing in endoplasmic reticulum (Figure 3.12).

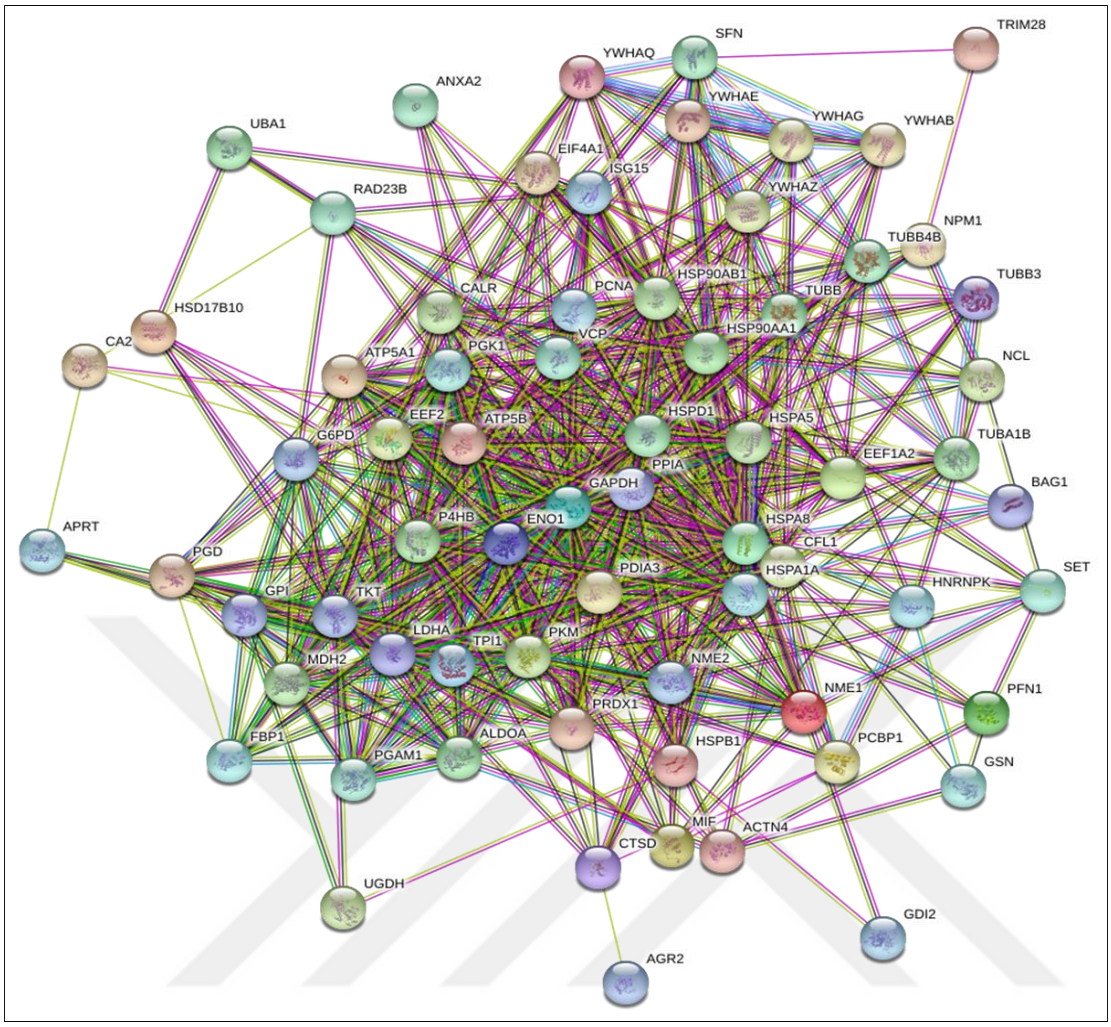


Figure 3.11 : Protein-protein interaction network of 69 proteins that found as common in all biological replicates.

Proteins that signed under carbon metabolism were Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), alpha-enolase (ENO1), posphoglycerate kinase 1 (PGK1), L-lactate dehydrogenase A (LDHA), triosephosphate isomerase (TPI1), Pyruvate kinase PKM (PKM), phosphoglycerate mutase 1 (PGAM1), Fructose-bisphosphate aldolase A (ALDOA), Fructose-1,6-bisphosphatase 1 (FBP1), Glucose-6-phosphate isomerase (GPI), 6-phosphogluconate dehydrogenase (PGD), glucose-6-phosphate 1-dehydrogenase (G6PD), transketolase (TKT) and malate dehydrogenase (MDH2). Interaction of BAG-1M with the proteins that plays role in carbon metabolism is thought to be related with the requirement of the specific interactions of cellular metabolism to support cell growth and survival in the cancer cells.

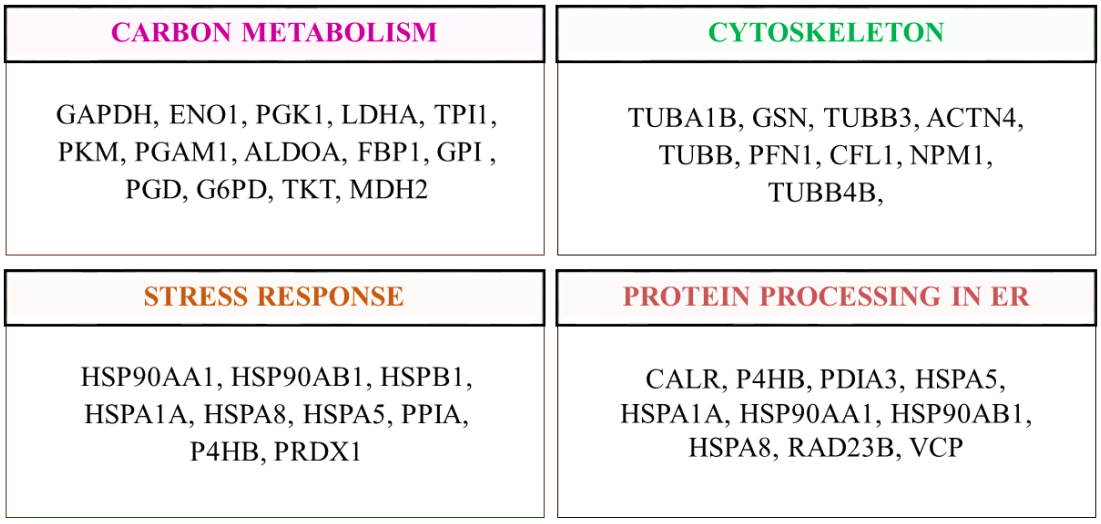


Figure 3.12 : Four enriched class of proteins according to GO Enrichment Analysis as carbon metabolism, cytoskeleton organisation, stress response and protein processing in endoplasmic reticulum.

Functioning proteins in cytoskeleton organisation were alpha actinin-4 (ACTN4), tubulin alpha-1B (TUBA1B), tubulin beta (TUBB), tubulin beta-3 (TUBB3), tubulin beta-4B (TUBB4B), nucleophosmin (NPM1), gelsolin (GSN), profilin-1 (PFN1) and cofilin-1 (CFL1).Reorganization of the cytoskeleton and migratory capabilities are required for malignant cells which can explain the interaction of BAG-1M with the proteins that have role in cytoskeleton organization.

Stress response classification comprised heat shock protein 90-alpha (HSP90AA1), heat shock protein 90-beta (HSP90AB1), heat shock protein beta-1 (HSPB1), heat shock 70 kDa protein 1A (HSPA1A), heat shock cognate 71 kDa protein (HSPA8), 78 kDa glucose-regulated protein (HSPA5), peptidyl-prolyl cis-trans isomerase A(PPIA), protein disulfide-isomerase (P4HB) and peroxiredoxin-1 (PRDX1).BAG-1M interaction with these proteins probably can maintain the protection of cells from apoptosis by overcoming the stress conditions that capable to induce apoptotic pathways.

The class that had been focused on was protein processing in endoplasmic reticulum which includes calreticulin (CALR), protein disulfide-isomerase (P4HB), protein-disulfide isomerase A3 (PDIA3), heat shock 70 kDa protein 1A (HSPA1A), 78 kDa glucose-regulated protein (HSPA5), heat shock protein 90-alpha (HSP90AA1), heat shock protein 90-beta (HSP90AB1), heat shock cognate 71 kDa protein (HSPA8), UV excision repair protein RAD23 homolog B (RAD23B) and transitional endoplasmic

reticulum ATPase (VCP). BAG-1 also has function in the protein processing in endoplasmic reticulum and it is thought that with the interaction partners that involved in this category, it plays role in the refolding or the degradation of the unfolded or misfolded proteins that cause endoplasmic reticulum stress, thus protect cell to undergo the cell death pathways.

Protein-protein interaction network analysis of the proteins that involved in protein processing in endoplasmic reticulum was performed by using STRING web server which showed the interactions of BAG-1 with HSPA8 and HSPA1A which are already known interacting proteins, HSPA5 according to the interaction of their putative homologs in other species (Figure 3.13).

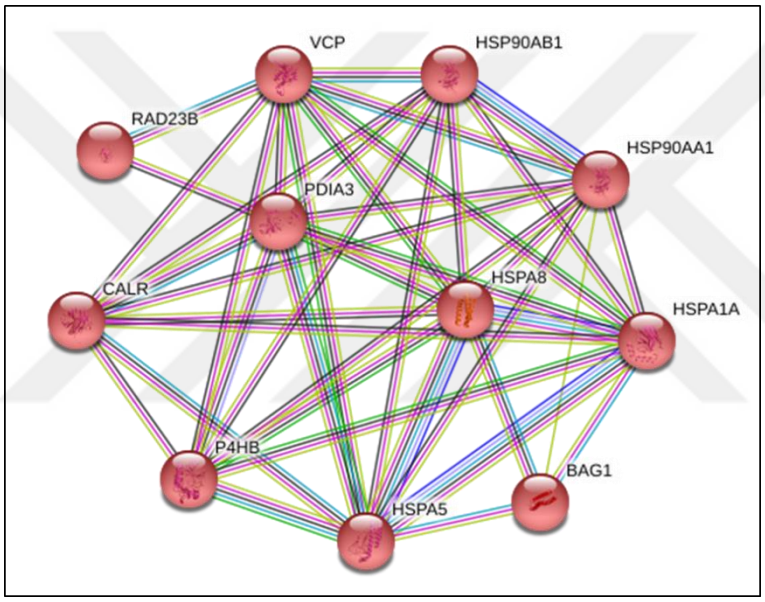


Figure 3.13 : Protein-protein interaction network between identified interaction partners of BAG-1M that involved in protein processing in endoplasmic reticulum.

KEGG (Kyoto Encyclopedia of Genes and Genomes) mapping pathway was used to show the layout of proteins that are the members of protein processing in endoplasmic reticulum.

To verify the results obtained from LC-MS/MS analysis 2-DE was performed.Purified BAG-1Msamples from MCF-7 and MCF-12A cells that transfected with N-terminal TAP-tagged BAG-1M plasmid were loaded onto strips with range of pH 3-10 and seperated according to the isoelectric points, then SDS-PAGE was performed for further seperation with regard to their molecular weights. After staining with colloidal commassie and imaging of the gel, spots were cutted and analyzed via MALDI-TOF at Kocaeli University, Faculty of Medicine, Department of Medical Biology for the

identification of the proteins. Spots cut from the 2D gels of BAG-1M purified samples from MCF-7 cells that transfected with N-terminus TAP-tagged BAG-1M plasmid were identified as heat shock 70 kDa protein (HSPA1A), calreticulin (CALR), protein-disulfide isomerase (P4HB), protein-disulfide isomerase A3 (PDIA3), transitional endoplasmic reticulum ATPase (VCP), UV excision repair protein RAD23 homolog B (RAD23B), 78 kDa glucose-regulated protein (HSPA5), heat shock protein beta-1 (HSPB1), 14-3-3 protein zeta/delta (YWHAZ), peroxiredoxin-1 (PRDX1), profilin-1 (PFN1), cofilin-1 (CFL1), fructose-bisphosphate aldolase A (ALDOA) and alpha-enolase (ENO1) (Figure 3.14).

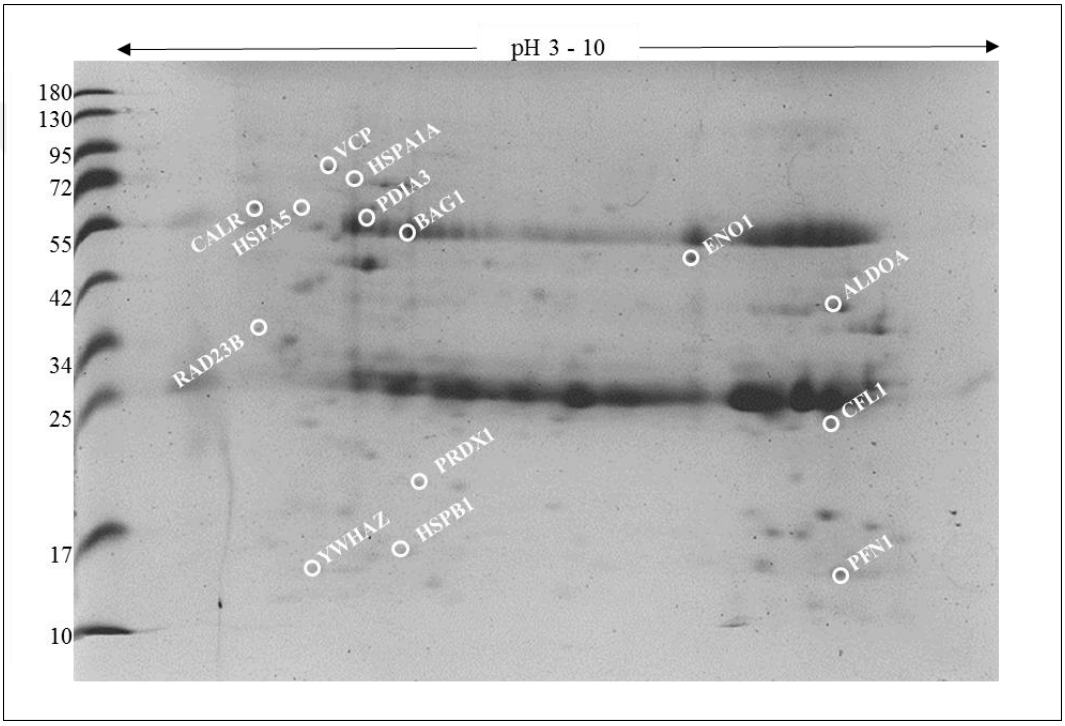


Figure 3.14 : 2-Dimensional gel image of BAG-1M purified proteins from MCF-7 breast cancer cells and the identified protein spots by MALDI-TOF analysis.

Protein spots obtained from the 2-DE of the MCF-12A cells were analyzed by using PDQuest software and identified according to the overlapping spots of MCF-7 gels. Heat shock 70 kDa protein (HSPA1A), calreticulin (CALR), protein-disulfide isomerase A3 (PDIA3), and 78 kDa glucose-regulated protein (HSPA5) were identified in 2D gels of MCF-12A cells (Figure 3.15).

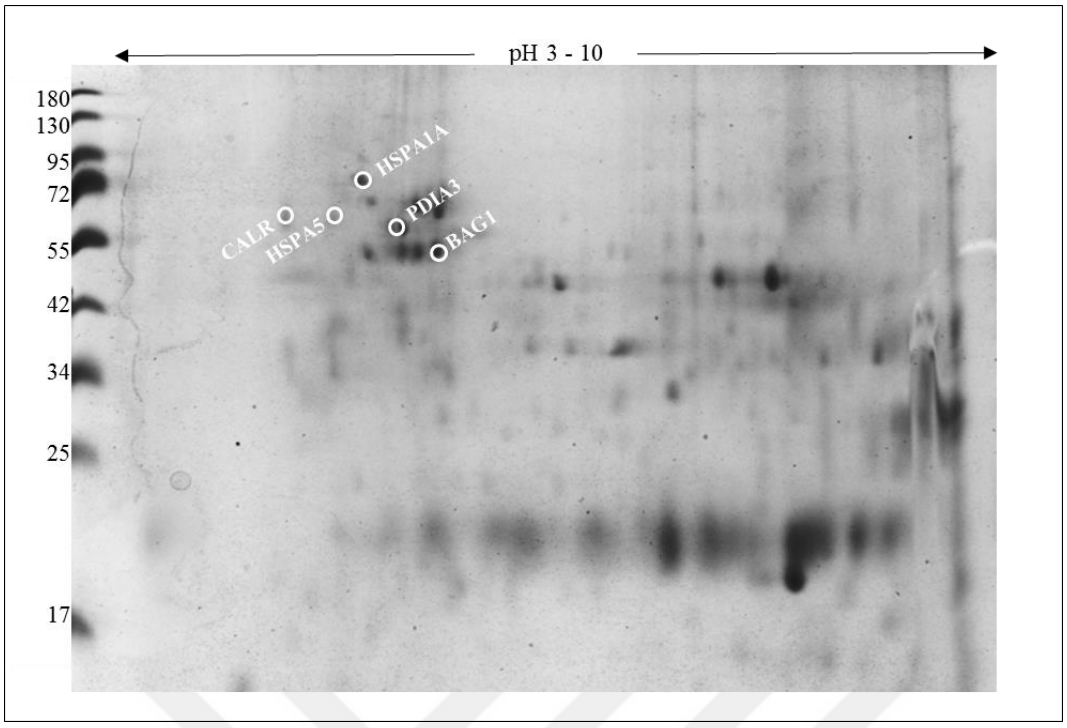


Figure 3.15 : 2-Dimensional gel image of BAG-1M purified proteins from MCF-12A non-tumorigenic breast cells and the identified protein spots by PDQuest software.

For further verification, immunoblotting assays were performed for purified samples from both MCF-7 and MCF-12A cells transfected with N-terminus TAP-tagged BAG-1M plasmid to see wheter the identified proteins exist on elution.

Immunoblotting analyses of MCF-7 breast cancer cells showed that 78 kDa glucose-regulated protein (HSPA5), protein-disulfide isomerase A3 (PDIA3), heat shock protein 90 (HSP-90), transitional endoplasmic reticulum ATPase (VCP) and UV excision repair protein RAD23 homolog B (RAD23B) were pulled from the total protein complex while BAG-1M purification. To insure from the obtaining data, immunoblotting assays were performed thrice. (Figure 3.15)

The same set of immunoblotting assays were performed twice for MCF-12A non-tumorigenic breast cells. When the results were analyzed, it was shown that 78 kDa glucose-regulated protein (HSPA5), protein-disulfide isomerase A3 (PDIA3) and heat shock protein 90 (HSP-90) were interacting with BAG-1M, however the interaction of transitional endoplasmic reticulum ATPase (VCP) and UV excision repair protein RAD23 homolog B (RAD23B) does not exist in MCF-12A cell line (Figure 3.16).

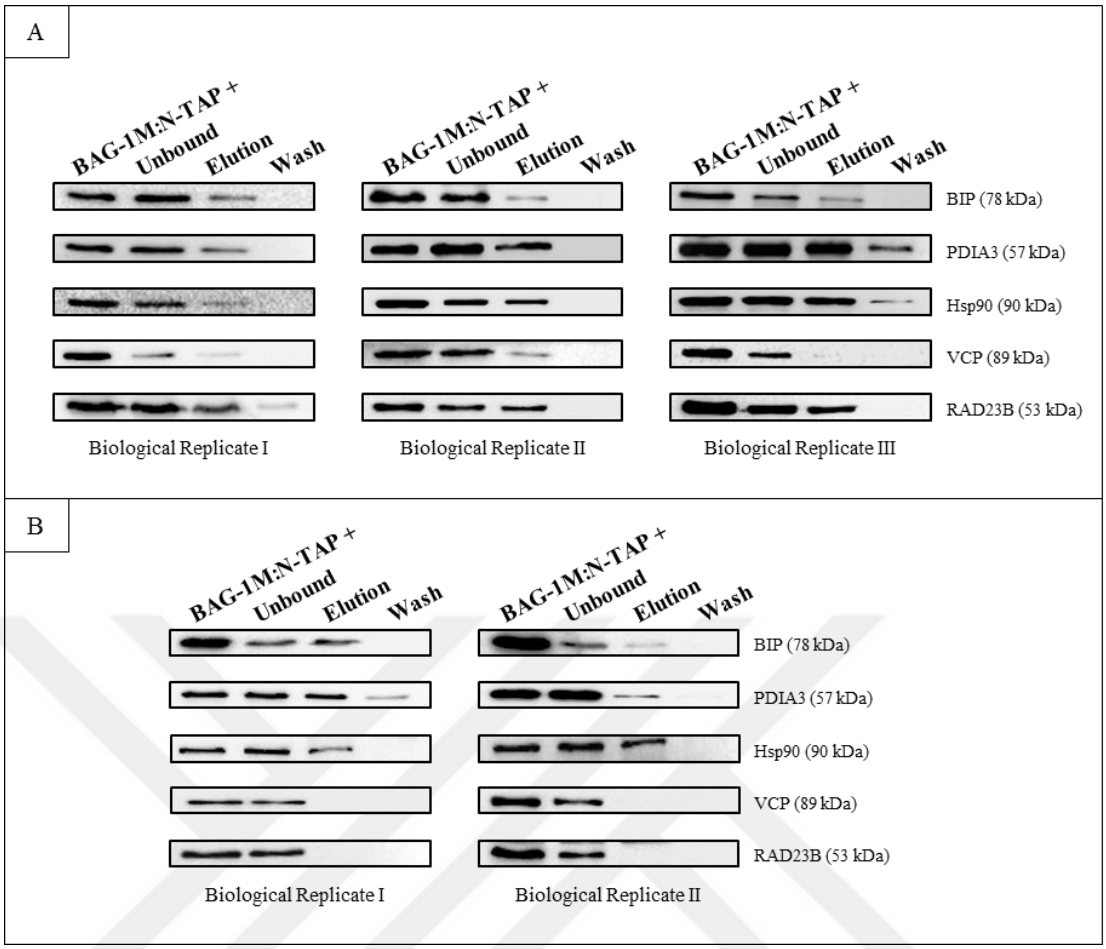


Figure 3.16 : Immunoblotting results of newly identified interaction partners of BAG-1Mthat belongs to protein processing in endoplasmic reticulum category in A) MCF-7 breast cancer cells B)MCF-12A breast non-tumorigenic cells.

3.6 Identification of BAG-1M Involved Complexes by PAGE Analyses

Total protein isolation from MCF-7 breat cancer cells transfected with N-terminal TAP-tagged BAG-1Mplasmid were maintained by protecting the native forms of proteins. Purification of BAG-1M performed from native protein samples whose interactions were not destroyed. Protein complexes were separatedaccording to their molecular weights by performing the BN-PAGE protocol (Figure 3.17).

Stained bands which represents a protein complex were cut from BN-gel, digested with trypsin into peptides and analyzed on MS^E mode of LC-MS/MS for peptide mapping. Because the protein purification was performed by pulling BAG-1M, each complex were expected to involve BAG-1. (Figure 3.18)

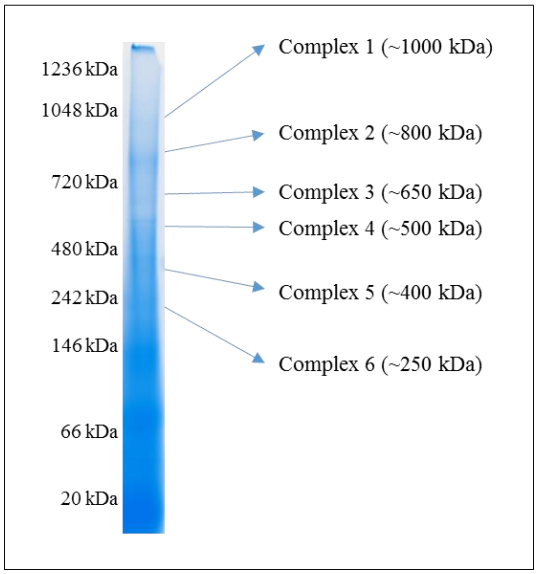


Figure 3.17 : Gel image of BAG-1M purified samples isolated fron N-terminal TAP-tagged BAG-1M transfected cells in their native forms.

1st complex which has approximate 1000 kDa molecular weight were found to conrain BAG-1, heat shock 70 kDa protein 1A (HSPA1A), heat shock cognate 71 kDa protein (HSPA8), 26S proteasome regulatory subunits, tubulins and actin.

Anaysis of 2nd complex that has approximate 800 kDa molecular weight showed that BAG-1, transitional endoplasmic reticulum ATPase (VCP),HSPA1A, HSPA8, proteasome subunits, tubulins and ATP synthase subunit beta (ATPB).

Complex 3 was including BAG1, VCP, HSPA1A, HSPA8, 26S proteasome non-ATPase regulatory subunits, tubulins, peroxiredoxin-1 (PRDX1) and 60 kDa heat shock protein (HSPD1).

BAG-1, heat shock protein 90 alpha and beta (HSP90AB1, HSP90AA1), 78 kDa glucose-regulated protein (HSPA5), protein-disulfide isomerase A3 (PDIA3), UV excision repar protein RAD23 homolog B (RAD23B), protein-disulfide isomerase (P4HB), HSPA1A, HSPA8, calreticulin (CALR), E3 ubiquitin-protein ligase CHIP (STUB1), proteasome subunits, tubulins, heterogeneous nuclear ribonucleoprotein K (HNRNPK), proteins incvolved in carbon metabolism andstress response also found in the fourth complex analysis.

400 kDa-weighted fifth complex were comprisied BAG-1, heat shock protein 90 alpha and beta (HSP90AA1, HSP90AB1), 78 kDa glucose-regulated protein (HSPA5), heat

shock cognate 71 kDa protein (HSPA8), heat shock protein 70kDa (HSPA1A), proteasome subunits and tubulins.

Proteins in the last complex which has approximately 250 kDa, were indentified as BAG-1, heat shock protein 90 alpha and beta (HSP90AA1, HSP90AB1), 78 kDa glucose-regulated protein (HSPA5), UV excision repair protein RAD23 homolog B (RAD23B), HSPA1A, HSPA8, tubulins, glycolytic enzymes.

BAG-1M BN-PAGE

Complex 1 (~1000 kDa)		**Complex 2 (~800 kDa)**	
BAG1, HSPA8, HSPA1A, PSMC6, PSMC3, TUBB, TUBA1A, TUBA1B, TUBB4B, ACTB		BAG1, **VCP**, HSPA8, HSPA1A, PSMA1, PSMB1, TUBA1B, TUBB4B, TUBB, ATP5B	
Complex 3 (~650 kDa)		**Complex 4 (~500 kDa)**	
BAG1, **VCP**, HSPA1A, HSPA8, PSMD14, PSMD3, PSMD7, PSMD13, PSMD12, PSMD11, PSMD6, TUBA1A, TUBB, TUBB4B, TUBB3, PRDX1, HSPD1		BAG1, **HSP90AB1**, **HSP90AA1**, **HSPA5**, **PDIA3**, **RAD23B**, **P4HB,** HSPA1A**,** HSPA8, **CALR**, STUB1, PSMC4, PSMD1, TUBA1B, TUBB, GAPDH, ALDOA, LDHA, MDH2, HNRNPK, HSPB1, UBA1, HSPD1, ENO1, TPI1, MIF, PKM, PRDX1, EEF2, TRIM28	
Complex 1 (~400 kDa)		**Complex 1 (~250 kDa)**	
BAG1, **HSP90AA1**, **HSP90AB1**, **HSPA5**, HSPA8, HSPA1A, PSMD2, PSMC1, TUBB, TUBB4B, PRDX1, TPI1, LDHA, EEF1A1, MDH2		BAG1, **HSP90AA1**, **HSP90AB1**, **HSPA5**, **RAD23B**, HSPA1A, HSPA8, TUBA1B, TUBB, TPI1, LDHA, GAPDH, ALDOA, EEF1A1, MDH2, NME1, PKM, ENO1	

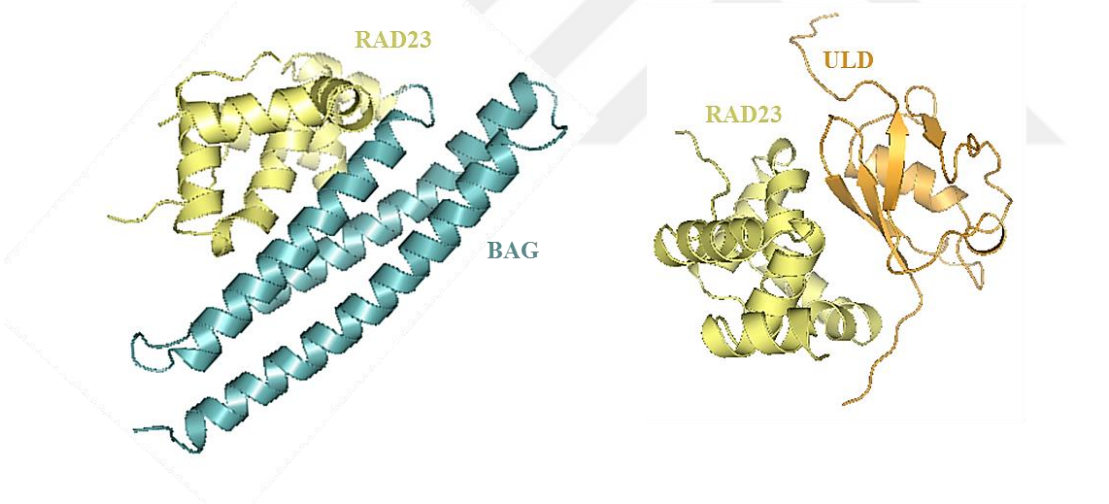
Figure 3.18 : List of the identified proteins and their complexes which were cut from the gel, digested and analyzed on LC/MS-MS.

3.7 Structural Analyses of BAG-1 and Newly Identified Interacting Proteins

Interaction partners of BAG-1M that identified by previous experiments were structurally analyzed via PRISM protein-protein interaction prediction tool which is served by Koc University, Computational Systems Biology Lab. Proper structures of the proteins were selected from PDB (Protein Data Bank) and were run on PRISM with both ULD and BAG domain of BAG-1. PDB ID's and detailes of all proteins were given in Appendix C.

Favorability of interactions were eliminated according to the fiberdock energy that is estimated by web server, results whose energy is below -10 were considered as the interactions that have possibilty tooccur.

First, RAD23 and BAG-1 interaction was analyzed, according to the PRISM results interaction of RAD23 and BAG-1 from BAG domain was not seem possible while ULD of BAG-1 and RAD23 interaction was observed to have favorable energy to interact (Figure 3.19).

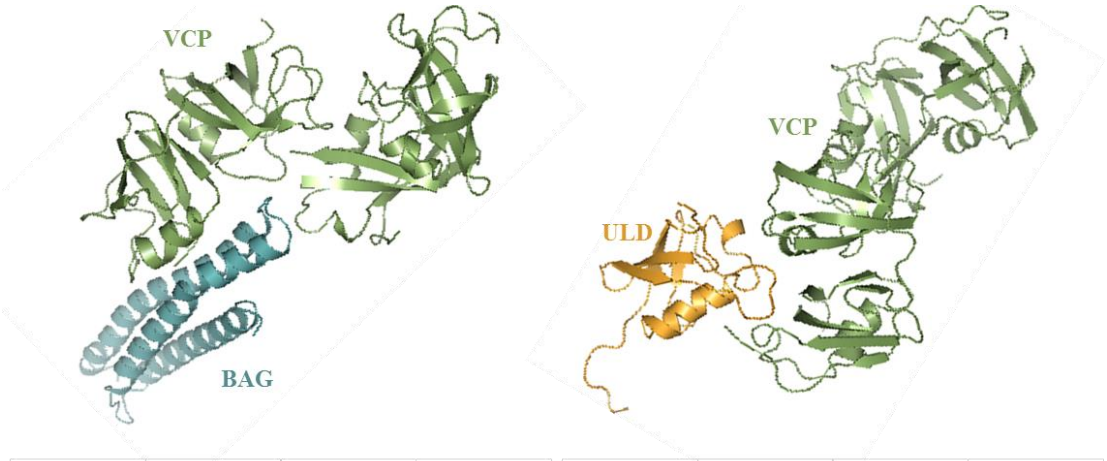


RAD23	Bag Domain	Interface	Energy
2qsfX	1hx1B	3urrAB	-4.37

ULD	RAD23	Interface	Energy
1wxwA	2qsfX	3hs2GH	-26.26

Figure 3.19 : Predicted interaction interface of UV excision repair protein RAD23 and BAG domain (left), and ubiquitin like domain (ULD)interaction interface (right).

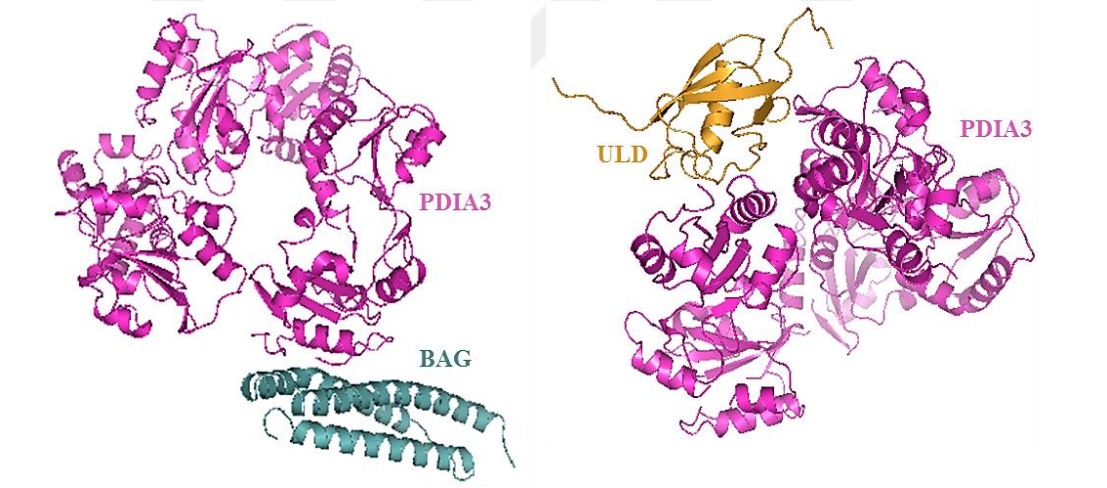
Transitional endoplasmic reticulum ATPase (VCP) and BAG-1 interaction predictions ended up with the fiberdock energy levels as -16.95 from BAG domain of BAG-1 and -18.51 from ULD of BAG-1 which can be accepted as significant (Figure 3.20).



VCP	Bag Domain	Interface	Energy	ULD	VCP	Interface	Energy
5x4lAB	1hx1B	1l7cBC	-16.95	1wxwA	5x4lAB	1mo1AB	-18.51

Figure 3.20 : Predicted interfaces of transitional endoplasmic reticulum ATPase (VCP) and BAG domain (left), and Ub-like domain of BAG-1 (right).

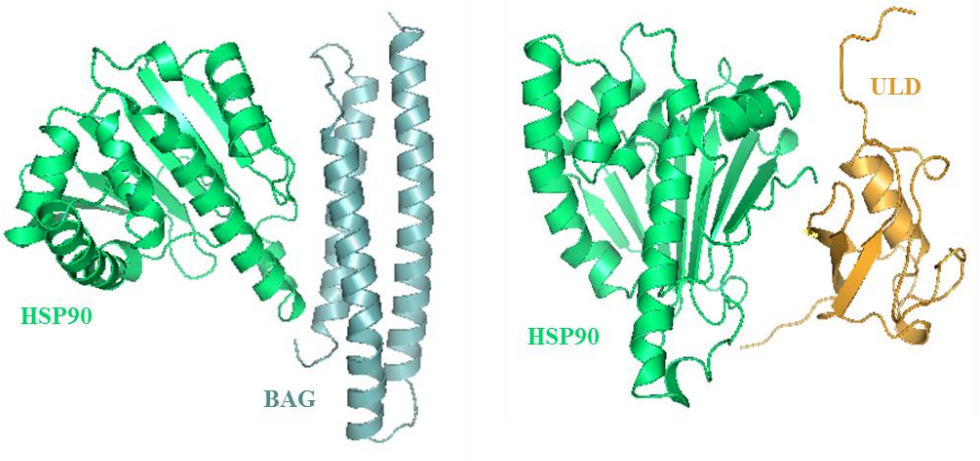
Structural analysis of PDIA3 and BAG-1 complex results showed that, as VCP interaction of BAG-1, these proteins are able to interact from both domains of BAG-1 according to favorable fiberdock energy estimations (Figure 3.21).



Bag Domain	PDIA3	Interface	Energy	ULD	PDIA3	Interface	Energy
1hx1B	2h8l	2wlrAB	-25.63	1wxwA	2h8l	1ffvAC	-30.02

Figure 3.21 : Predicted interaction interface of protein-disulfide isomerase A3 (PDIA3) and BAG domain (left), and Ub-like domain of BAG-1 (right).

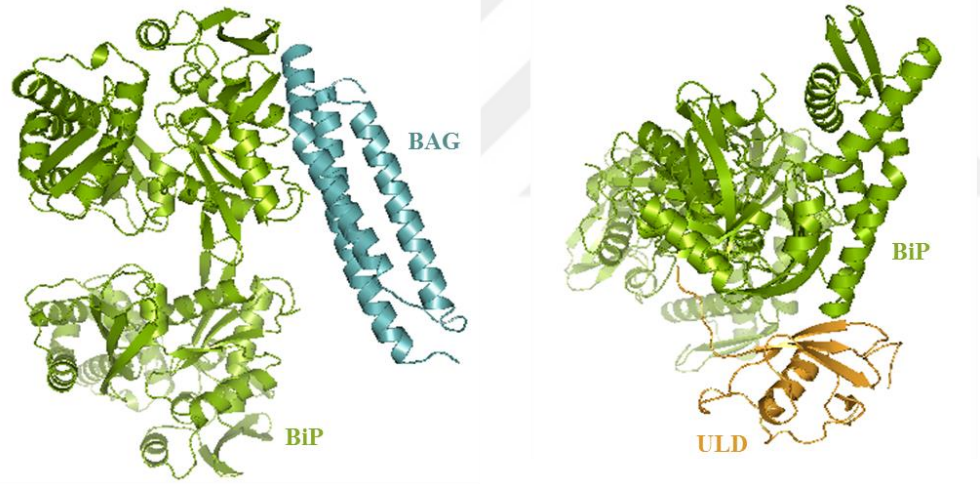
.PRISM protein-protein interaction predictions of HSP-90 and BAG-1 complex and BiP and BAG-1 complex were ended up with not significant fiberdock energies (Figure 3.22 and Figure 3.23).



Bag Domain	HSP90	Interface	Energy
1hx1B	5lrzA	1xsvAB	-8.06

HSP90	ULD	Interface	Energy
5lrzA	1wxvA	3r4oAB	-10.83

Figure 3.22 : Predicted interfaces of heat shock protein 90 (HSP90) and BAG domain (left), and Ub-like domain of BAG-1 (right).



HSPA5	Bag Domain	Interface	Energy
5evz	1hx1B	1ylmAB	-9.62

ULD	HSPA5	Interface	Energy
1wxvA	5evz	3hs2GH	-0.98

Figure 3.23 : Predicted interfaces of 78 kDa glucose-regulated protein (HSPA5) and BAG domain (left), and Ub-like domain (right).

Structural analyses that maintained by using PRISM web server showed that, BAG-1 and RAD23 interaction may occur via ULD domain of BAG-1. VCP and PDIA3 were seem to be able to interact with BAG-1 from both ULD and BAG domains. However, according to the fiberdock energies estimated by PRISM, HSP90 and BiP interaction of BAG-1 were thought to occur by intermediary proteins that interacting simultaneosly with BAG-1 and HSP90 or BiP.

4. DISCUSSION

BAG-1 (Bcl-2-associated athanogene), a member of BAG family is an adapter protein which has been evolutionary protected between several organisms. Four isoforms of BAG-1 are present in human cells as BAG-1L, BAG-1M, BAG-1S and BAG-1 minor that are translated from different initiation sites on the same mRNA (Townsend et al., 2005). BAG-1 has known to be involved in various molecular mechanisms such as cell motility, proliferation, transcription, ubiquitination and apoptosis under the favor of its interaction partners Bcl-2, serine/threonine kinase Raf-1, heat shock proteins HSP70/HSC70, E3 ligase CHIP, and nuclear hormone receptors (Wang et al., 1996). It is known that BAG-1 modulates HSP-70 chaperone activity and also leads cell survival as a result of independent interactions with Bcl-2 or Raf-1 (Song et al, 2001).

Several studies interpreted that BAG-1 can be considered as one of the key players in carcinoma, especially in breast cancer (Sharp et al., 2004). Because three major isoforms of BAG-1 is present in human cells and their specific functions in detail has not been described yet, to find out the role of BAG-1M, the isoform that localize in cytoplasm with the ability of translocation to nucleus, this study aimed to determine its interacting proteins in breast cancer and breast non-tumorigenic epithelial cell lines which may be important for lightening of BAG-1M function in cancer progression.

BAG-1 has discovered as an anti-apoptotic protein associated to Bcl-2, whose overexpression has been showed the relation with raised cell viability in breast cancer (Takayama and Reed, 2001). In this study cell survival assays showed that overexpression of BAG-1M isoform in MCF-7 human breast cancer and MCF-12A non-tumorigenic breast cells increases the cell proliferation over time. Moreover, N-terminal TAP-tagged BAG-1M transfected cells enhanced colony formation in contrast to untransfected cells. The function of BAG-1M on cell proliferation was in tune with the idea of overexpressed BAG-1 is enhancing growth rate. In order to understand if BAG-1M is interacting with known partners of BAG-1 and thus increasing cell survival, TAP-Tag purified samples were analyzed to check the interactions with Bcl-2, HSP70, Raf-1, Akt and B-Raf with the phosphorylated forms

of these proteins which are considered as active forms that forward cells to survive. Results of immunoblotting assays showed that BAG-1M is interacting with all partners of BAG-1 in both cell lines which means it can take place in cell survival mechanism via its Bcl-2 and Raf kinases interactions. Also the role of BAG-1M in ubiquitination and protein folding mechanism was confirmed by CHIP and HSP-70 interactions.

Identidication of BAG-1M interacting proteins was maintained by performing LC-MS/MS analysis of purified proteins. Four repeats of peptide mapping ended up with common 69 proteins with aproximately 60% correlation. Proteins were classified according to GO enrichment analysis as carbon metabolism, cytoskeleton organization, stress response and protein processing in endoplasmic reticulum. BAG-1M interaction with the proteins that involved in carbon metabolism was thought to related with the requirement of energy in the cancer development and progression mechanism. Identification of proteins takes place in cytoskeleton organization was interpreted as the provision of migratory capabilities of cells which have importance on metastasis. Interaction with the stress response protein were thought to be related to anti-apoptotic activity of BAG-1, which may regulates stress-induced cell death by its interactions with stress response proteins.

The catogory focused on this study was protein processing in endoplasmic reticulum which was already thought as a mechanism that BAG-1 is involved (Amm et al., 2014), was containing identified proteins as heat shock proteins HSP90 and HSPA5, ER stress response protein PDIA3, proteins that have role in proteasomal degradation such as VCP and RAD23B.

Confirmation of the interaction of BAG-1M with identified proteins via LC-MS/MS was maintained by MALDI-TOF analysis of 2-Dimensional gel spots and immunoblotting assays of BAG-1M purified protein samples from both MCF-7 breast cancer and MCF-12A non-tumorigenic breast cells.

LC-MS/MS analyses, 2-DE gel spots and immunoblotting assay results pointed the new interacting proteins involved in protein processing in ER as HSP-90, BiP, PDIA3, RAD23B, and VCP in MCF-7 cells.Immunoblotting assays and 2D gel spots analysis of MCF-12A cells ended up with existance of BAG-1M interaction with HSP-90, BiP and PDIA3, while RAD23 and VCP interactions were not be observed in non-tumorigenic cells which may indicate the role of these complexes in carcinogenesis.

According to the observed experimental data and previous knowledge about BAG-1, interaction with ER resisdent proteins PDIA3 and BiP, also interactions with the proteins take place in ubiquitin/proteasomal mechanism such as VCP and RAD23B suggested that BAG-1 may regulates the endoplasmic reticulum associated degradation (ERAD) of pro-apoptotic molecules or stress factors to protect cells from apoptosis.

In order to find out formed complex BAG-1M involved in, Blue Native gel electrophoresis (BN PAGE) was performed on purified samples and each complex were analyzed on MS^E mode of LC-MS/MS. Altough the limitation of the peptide mapping from the native gel was the possibility of overlapping the complexes with the same or near molecular weights, as they identified by previous experiments, HSPA5, HSP90, VCP, RAD23B and PDIA3 were found in complexes with BAG-1.

Structural analysis were performed to see the possibilities of BAG-1M interaction with newly identified prtoteins by PRISM protein-protein interaction prediction tool. Because full length structure of BAG-1 does not exist, BAG domain and ubiquitin like domain (ULD) of BAG-1 were separately run on PRISM with target proteins. Structural analyses showed that VCP and PDIA3 interaction of BAG-1 can occur via both domains of BAG-1, whereas RAD23 interaction was seem to be arised from only ULD. HSP-90 and BiP interactions were not found to have favorable fiberdock eneriges to considered as physical interacting proteins of BAG-1.

Examination of proteins that identified by LC-MS/MS analysis of protein complexes cut from BN-PAGE gels showed that all complexes were containing HSPA1A and HSPA8 which are the known interacting proteins of BAG-1 and may be the mediator proteins to cause the functional interactions of BAG-1M with newly identified proteins.

Altough verification of newly identified proteins were maintained by performing different methods, experimenting with biological replicates and structural analyses, further studies including site-directed mutagenesis would eliminate the indirect interaction partners, thus physical interactions would be made certain.

According to the potential of BAG-1 as a marker for diagnosis and cancer therapy, isoform specific mechanisms and interacting partners are important to developing of new therapeutic agents in cancer treatment. Besides, this study with the structurally

confirmed potential interacting proteins, may lead the crystal structure studies on complexes of BAG-1 and its binding partners. Detailed analyses of new complexes would contribute significant knowledge in literature.

5. CONCLUSION

BAG-1 has three isoforms in human cells as BAG-1S, BAG-1M and BAG-1L which differ according to localizations and expression levels. BAG-1M is the isoform that has lowest expression and interestingly has the ability of translocating between nucleus and cytoplasm altough it has not nuclear localization signal.

BAG-1M was found to have an affect that enhances the cell survival and colony formation behavior of both breast cancer and breast epithelial cells.

Molecular pathways that BAG-1M is involved in was found as carbon metabolism, cytoskeleton organisation, stress response and protein processing in endoplasmic reticulum. While the interaction of BAG-1M wirh the proteins included in the protein processing in endoplasmic reticulum were analyzed in breast cancer and breast non-tumorigenic cells. RAD23B and VCP interaction of BAG-1M was shown to occur in cancer cells, but not in breast epithelial cells, which is thought to suggest a role of these complexes in cancer development or progression. Structural analyses were confirmed the possibility of RAD23B and VCP interaction of BAG-1.

Interactions with the endoplasmic reticulum resident proteins, proteins involved in ubiquitin/proteasome system, and chaperones suggest that BAG-1M has a function in endoplasmic reticulum associated degradation (ERAD) mechanism. In the light of the information from the results of this study, model of proteins complexes were created for breast non-tumorigenic cells (Figure 5.1) under normal conditions and breast cancer cells (Figure 5.2) under unfolded protein response.

Hereafter, determination of the substrates of BAG-1M mediated endoplasmic reticulum associated degradation mechanism via functional analyses, eliminating the indirect interaction partners of BAG-1M, and performing the structural experiments for the revealing the interacting surfaces would provide more information about the mechanism that BAG-1M is functioning which may lead the design of the small molecules that target the specific sites of BAG-1, where the interactions with the

proteins involved in endoplasmic reticulum associated degradation mechanism occur from and cause the progression of breast cancer.

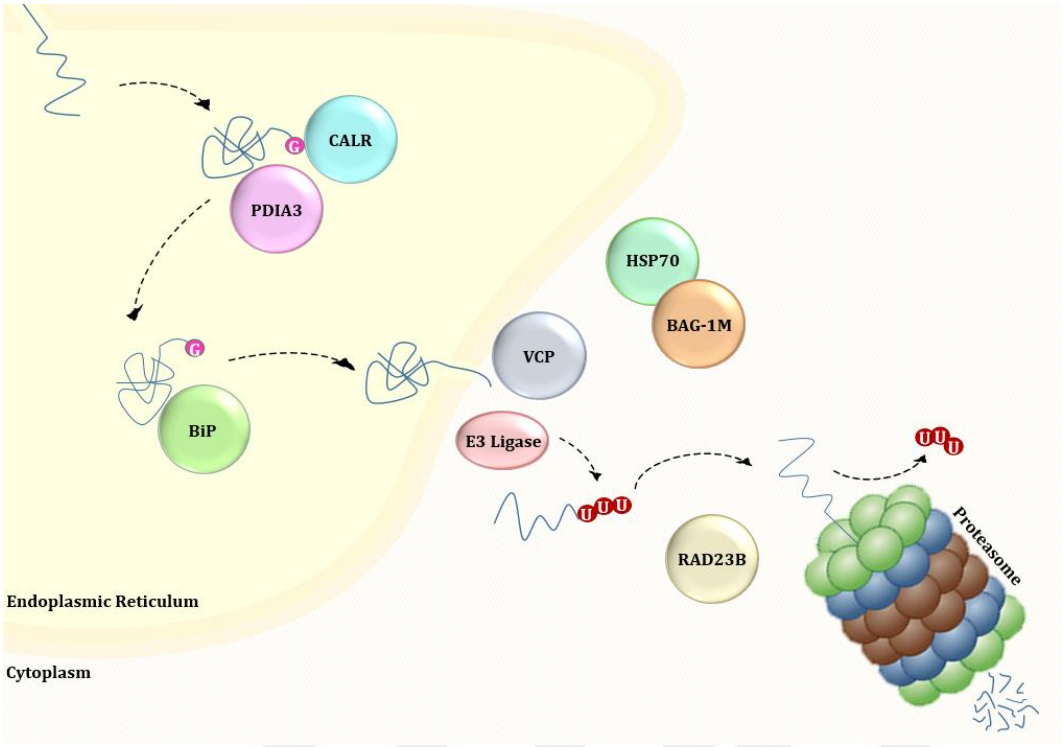


Figure 5.1 : Model of ERAD mechanism in MCF-12A breast non-tumorigenic cells

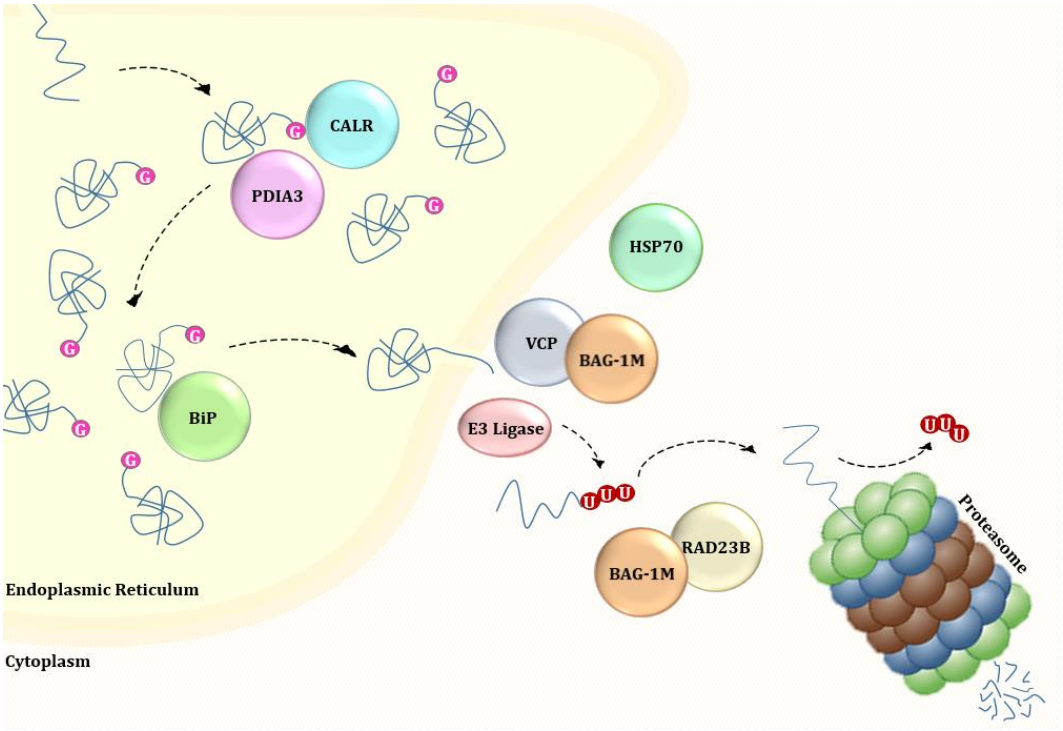


Figure 5.2 : Model of ERAD mechanism in MCF-7 breast cancer cell line

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APPENDICES

APPENDIX A : Raw Data of LC-MS/MS Peptide Mapping Analyses

APPENDIX B : PDB Details

APPENDIX C : BAG-1 mRNA Sequence

APPENDIX A

Table A.1 : First LC-MS/MS resullts of MCF-7 BAG-1M:N-TAP purified proteins

Accession Number	Gene ID	PLGS Score	Peptides Number	Theoretical Peptides	Coverage (%)
P60709	ACTB	36915.82	29	37	80.2667
P04406	GAPDH	30969.69	55	26	82.3881
P04792	HSPB1	29853.39	32	15	89.7561
P63104	YWHAZ	28025.88	17	30	63.2653
P68363	TUBA1B	23503.3	24	35	78.4922
Q9BQE3	TUBA1C	22326.44	22	35	70.6013
Q71U36	TUBA1A	22285.27	28	35	75.6098
Q13748-2	TUBA3C	18161.94	15	30	59.0909
P07437	TUBB	17277.95	25	32	87.6126
Q6PEY2	TUBA3E	16767.38	16	34	61.1111
P06733	ENO1	16734.48	48	39	65.8986
P68371	TUBB4B	15931.58	32	32	76.4045
O95994	AGR2	15409.43	16	18	73.1429
P68104	EEF1A1	13862.74	17	36	50.4329
P04075	ALDOA	11965.7	42	34	81.8681
P10809	HSPD1	11789.98	24	57	54.1012
P62736	ACTA2	10745.08	42	37	42.4403
P23528	CFL1	10671.59	19	21	64.4578
P07737	PFN1	10506.94	15	16	58.5714
Q05639	EEF1A2	10430.88	14	36	53.9957
O60701	UGDH	9637.472	29	54	70.4453
P31949	S100A11	9254.305	10	10	68.5714
P62937	PPIA	9147.298	24	16	83.0303
P08238	HSP90AB1	9126.486	46	75	62.2928
P31946	YWHAB	8165.852	10	25	59.7561
P07900	HSP90AA1	7784.658	48	101	66.1202
P62258	YWHAE	7617.677	13	31	52.549
P11142	HSPA8	7330.61	37	55	64.8607
P14174	MIF	7125.437	6	6	83.4783
P13929	ENO3	6577.247	9	37	37.0968

P00338	LDHA	6219.497	10	38	54.8193
P22392	NME2	5970.845	7	15	46.7105
P0DMV8	HSPA1A	5696.101	39	54	66.3027
P14618	PKM	5299.798	41	49	69.3032
P09972	ALDOC	4534.658	6	35	17.8571
P60174	TPI1	4045.361	16	26	58.7413
Q99933	BAG1	3908.928	15	34	42.6087
P13639	EEF2	3856.636	27	80	48.4848
Q13509	TUBB3	3807.414	30	32	45.5556
P40926	MDH2	3775.83	12	32	64.2012
P27348	YWHAQ	3670.006	7	28	47.3469
P61981	YWHAG	3654.117	8	27	46.1538
Q01105	SET	3590.306	5	19	23.1034
P52565	ARHGDIA	2992.765	4	23	52.451
P32119	PRDX2	2634.888	10	19	62.6263
P07339	CTSD	2584.166	17	31	43.932
Q06830	PRDX1	2558.815	20	20	69.3467
O75747	PIK3C2G	2483.727	6	116	7.5433
P07355	ANXA2	2345.836	8	34	33.6283
P06576	ATP5B	2336.936	14	41	53.4972
P61978	HNRNPK	2273.187	10	41	36.933
P00558	PGK1	2267.95	13	38	57.3142
P61204	ARF3	2253.741	6	17	53.5912
P15531	NME1	2252.355	5	16	38.1579
P00918	CA2	2206.71	7	21	43.8462
P05161	ISG15	2175.889	4	12	48.4848
P18669	PGAM1	2070.495	9	22	49.6063
P09467	FBP1	2026.074	12	33	62.426
P06748	NPM1	1987.14	7	26	27.8912
Q04917	YWHAH	1957.697	7	29	26.0163
P52209	PGD	1881.45	13	43	52.588
P29401	TKT	1839.359	23	48	60.0321
P31947-2	SFN	1788.615	5	23	36.1111
Q99497	PARK7	1689.04	7	29	58.7302
P60842	EIF4A1	1586.707	14	47	51.4778

P60981-2	DSTN	1546.238	4	18	41.8919
P11021	HSPA5	1513.164	16	60	34.2508
P34931	HSPA1L	1462.281	17	54	29.0172
P06744	GPI	1439.936	16	50	58.6021
Q58FF8	HSP90AB2P	1398.56	10	49	28.8714
P12004	PCNA	1386.635	6	25	55.9387
P17066	HSPA6	1307.991	10	59	20.5288
P30041	PRDX6	1231.481	6	29	33.0357
P30050	RPL12	1187.839	3	18	28.4848
P27824	CANX	1111.681	9	50	32.0946
P62820	RAB1A	1086.013	4	22	28.7805
P27797	CALR	1082.034	11	52	46.7626
Q14240	EIF4A2	1051.272	8	51	28.7469
Q15257	PTPA	1050.281	5	30	26.5363
Q96CN7	ISOC1	1034.641	8	21	41.6107
P26641	EEF1G	997.3229	7	40	36.1556
Q9H0U4	RAB1B	988.2962	4	21	29.3532
P30084	ECHS1	969.8068	4	28	24.4828
P30043	BLVRB	941.913	5	13	58.2524
Q15365	PCBP1	913.8634	7	28	44.1011
P62829	RPL23	840.0098	3	14	32.8571
P06396	GSN	828.4485	15	55	40.5371
P07741	APRT	812.6904	4	20	41.1111
P22314	UBA1	797.7346	15	72	32.7977
P50395	GDI2	784.5074	5	44	19.1011
O43707	ACTN4	760.626	14	93	30.0768
P30086	PEBP1	733.6564	4	16	45.4545
Q99714	HSD17B10	733.2983	5	20	39.4636
P17931	LGALS3	726.5529	4	13	10.4
P31943	HNRNPH1	721.6763	5	33	19.5991
P52597	HNRNPF	704.6179	5	32	28.1928
Q13263	TRIM28	703.9753	5	62	17.9641
P31939	ATIC	691.4221	8	56	30.9122
P19338	NCL	689.6178	10	74	17.7465
P11413	G6PD	689.0336	8	57	31.2621

P37802	TAGLN2	688.1257	4	26	40.7035
P54727	RAD23B	674.8271	4	22	21.874
Q02790	FKBP4	653.938	7	44	24.183
Q9NR45	NANS	639.0416	5	27	28.9694
P07237	P4HB	636.5197	10	55	26.378
P50454	SERPINH1	631.2811	3	36	12.6794
P22626	HNRNPA2B1	621.6864	3	32	19.2635
P55060	CSE1L	604.4983	4	74	11.2255
Q9BR76	CORO1B	588.1726	3	36	15.1329
P05388	RPLP0	563.2357	3	27	20.8202
P08865	RPSA	555.3992	4	27	24.7458
Q14974	KPNB1	540.2103	6	65	17.8082
P08758	ANXA5	533.6252	3	33	19.0625
Q14697	GANAB	506.976	7	77	15.7839
P55072	VCP	464.9026	7	72	18.2382
P49588	AARS	462.1758	6	91	13.843
Q07021	C1QBP	460.2771	3	20	34.0426
P30101	PDIA3	445.8206	3	54	6.8192
Q15366	PCBP2	438.3573	3	27	19.726
Q13200	PSMD2	430.7621	4	95	9.6916
P25705	ATP5A1	420.2182	6	51	24.0506
Q00610	CLTC	409.3557	12	142	17.1343
O75369-4	FLNB	354.2019	7	166	8.6977
P50991	CCT4	336.46	3	48	11.5028
P78371	CCT2	335.65	3	51	10.6542
P26599	PTBP1	335.0751	3	39	11.1111
Q86VP6	CAND1	295.6497	3	104	7.6423
P14625	HSP90B1	264.0286	3	92	4.9813
Q96K30	RITA1	151.4264	4	20	2.6022
P02768	ALB	131.3089	4	61	3.6125

Table A.2 : LC-MS/MS results of the technical replicate of firstly analyzed MCF-7 BAG-1M:N-TAP purified proteins

Accession Number	Gene ID	PLGS Score	Peptides Number	Theoretical Peptides	Coverage (%)
P04792	HSPB1	38180.61	27	15	84.3902
P60709	ACTB	36034.22	32	37	83.2
P68363	TUBA1B	29264.77	25	35	82.7051
P04406	GAPDH	28745.05	59	26	84.4776
P63104	YWHAZ	27409.67	13	30	52.2449
Q13748-2	TUBA3C	24197.29	14	30	56.6986
Q6PEY2	TUBA3E	23350.44	15	34	58.8889
P68366-2	TUBA4A	22463.67	18	35	66.5127
P06733	ENO1	21739.36	39	39	65.2074
P68104	EEF1A1	15816.16	21	36	63.6364
O95994	AGR2	14203.93	18	18	73.1429
P23528	CFL1	12707.24	20	21	65.6627
P07437	TUBB	11871.59	26	32	74.5496
P68371	TUBB4B	11599.84	36	32	71.0112
P68032	ACTC1	11502.82	21	37	56.7639
P10809	HSPD1	11416.07	24	57	60.0349
Q6S8J3	POTEE	11123.63	18	114	19.1628
P04075	ALDOA	10832.7	38	34	81.8681
P62937	PPIA	10084.33	25	16	79.3939
Q05639	EEF1A2	9734.75	18	36	57.0194
P11142	HSPA8	9613.66	36	55	66.8731
P07737	PFN1	9310.15	14	16	57.1429
P22392	NME2	9008.123	8	15	55.9211
P31946-2	YWHAB	8739.17	7	25	37.2951
P14174	MIF	8521.247	4	6	75.6522
O60701	UGDH	8502.382	33	54	71.4575
P31949	S100A11	8438.954	13	10	68.5714
P62258	YWHAE	8262.521	16	31	67.0588
P08238	HSP90AB1	7276.936	40	75	56.2155
P15531	NME1	7248.786	6	16	49.3421
P07900	HSP90AA1	6891.735	40	101	51.2295

P14618	PKM	6564.379	42	49	67.42
P0DMV8	HSPA1A	5500.015	36	54	64.7426
Q01105	SET	4726.743	8	19	34.1379
P27348	YWHAQ	4656.4	8	28	49.3878
P00338	LDHA	4530.82	15	38	69.8795
P61981	YWHAG	4035.559	10	27	56.2753
P07339	CTSD	3537.326	18	31	59.9515
Q99933	BAG1	3359.526	12	34	42.029
P05161	ISG15	3237.177	3	12	58.1818
P13639	EEF2	3121.416	26	80	47.4359
P40926	MDH2	3105.588	9	32	51.7751
Q06830	PRDX1	2976.375	20	20	81.407
P60174	TPI1	2892.024	12	26	53.8462
Q04917	YWHAH	2806.137	7	29	28.0488
P31947-2	SFN	2712.018	5	23	25.0
P05386	RPLP1	2539.123	3	8	51.7544
P60842	EIF4A1	2501.694	13	47	48.7685
P52565	ARHGDIA	2370.047	5	23	55.8824
Q13509	TUBB3	2334.96	29	32	48.8889
P00918	CA2	2307.829	7	21	41.9231
P06748	NPM1	2295.456	5	26	21.0884
P32119	PRDX2	2264.126	9	19	48.4848
P09467	FBP1	2082.625	8	33	50.2959
P06576	ATP5B	2033.688	14	41	53.4972
P63241	EIF5A	1996.455	4	14	33.1169
P00558	PGK1	1965.91	17	38	56.5947
P18669	PGAM1	1908.029	9	22	51.9685
P11021	HSPA5	1860.426	19	60	43.1193
Q58FF7	HSP90AB3P	1846.247	15	62	21.943
Q99497	PARK7	1791.194	8	29	52.381
P29401	TKT	1743.107	23	48	56.3403
P34931	HSPA1L	1736.401	17	54	25.897
P61978	HNRNPK	1566.505	9	41	30.4536
P60981-2	DSTN	1506.972	4	18	41.8919
P39687	ANP32A	1480.256	4	18	27.3092

P17066	HSPA6	1468.741	11	59	22.8616
P27797	CALR	1408.255	15	52	52.518
Q15843	NEDD8	1406.595	3	9	34.5679
Q92688-2	ANP32B	1373.556	4	16	40.5128
P26641	EEF1G	1354.617	8	40	32.9519
Q15365	PCBP1	1351.169	7	28	42.4157
Q14240	EIF4A2	1337.782	6	51	25.5528
P30050	RPL12	1309.237	3	18	27.8788
Q9H0U4	RAB1B	1281.417	7	21	45.2736
Q8N0Y7	PGAM4	1270.882	4	21	27.1654
P40925	MDH1	1212.391	4	43	28.7425
Q13263	TRIM28	1191.835	9	62	29.9401
O00299	CLIC1	1147.373	3	22	26.1411
P18085	ARF4	1130.685	5	17	49.4444
O75747	PIK3C2G	1129.677	5	116	5.8824
P52209	PGD	1113.498	5	43	28.3644
P84077	ARF1	1104.539	7	17	69.6133
P13693	TPT1	1096.598	3	16	43.0233
P05062	ALDOB	1089.848	3	33	12.9121
P11413	G6PD	1089.528	18	57	54.1748
P06744	GPI	1078.043	13	50	34.2294
Q99714	HSD17B10	1037.771	6	20	47.8927
P84085	ARF5	1026.553	5	17	48.3333
Q58FF8	HSP90AB2P	1005.074	10	49	33.8583
P12004	PCNA	1005.01	6	25	60.1533
P09651	HNRNPA1	992.9473	4	32	20.6989
P30084	ECHS1	988.4885	4	28	23.7931
P07355	ANXA2	984.9102	5	34	25.0737
Q58FF6	HSP90AB4P	981.6727	4	58	13.2673
Q15366-6	PCBP2	863.3679	3	27	19.9446
P07741	APRT	860.179	3	20	31.1111
O43707	ACTN4	854.764	12	93	25.1372
P30043	BLVRB	806.8812	4	13	27.6699
P22314	UBA1	785.9836	19	72	38.3743
P37802	TAGLN2	763.952	5	26	47.7387

P08865	RPSA	761.3416	3	27	24.7458
P22626	HNRNPA2B1	759.1482	4	32	21.5297
Q9NR45	NANS	756.2419	5	27	37.6045
P07237	P4HB	750.4034	7	55	20.8661
P52597	HNRNPF	724.588	4	32	19.2771
P27824	CANX	696.6346	7	50	27.7027
P26599	PTBP1	675.6244	6	39	28.6252
P50395	GDI2	665.1646	5	44	25.618
P30086	PEBP1	652.5181	4	16	49.1979
O75390	CS	634.8287	6	33	25.9657
P06396	GSN	614.1158	9	55	30.179
P25705	ATP5A1	595.5125	9	51	36.8897
P63244	RACK1	585.1907	4	29	22.7129
P13804	ETFA	579.3265	4	27	22.8228
P54727	RAD23B	575.1616	3	22	16.6259
P31943	HNRNPH1	570.5313	4	33	18.7082
Q12931-2	TRAP1	559.8965	3	72	6.298
P08758	ANXA5	546.285	3	33	19.0625
P55072	VCP	545.7092	10	72	23.8213
P49588	AARS	530.1185	5	91	10.7438
P19338	NCL	499.5539	12	74	20.5634
P20618	PSMB1	484.9835	3	27	25.3112
P30101	PDIA3	575.5306	6	54	19.2079
Q92538	GBF1	459.6753	4	151	2.3131
P31939	ATIC	458.2007	8	56	27.5338
Q96CN7	ISOC1	457.0365	5	21	30.8725
Q00610	CLTC	452.5719	19	142	22.6269
Q14697	GANAB	432.1828	8	77	23.8347
P38646	HSPA9	387.9535	5	75	10.6038
P31150	GDI1	381.3416	6	37	31.5436
Q9Y365	STARD10	364.4425	3	19	17.5258
Q02790	FKBP4	363.8271	4	44	12.854
Q01518	CAP1	351.3481	3	49	12.4211
O75369-4	FLNB	324.2772	7	166	5.4419
P48735	IDH2	288.1072	3	43	5.7522

Q00839	HNRNPU	274.1102	3	69	8.9697
P14625	HSP90B1	152.196	3	92	1.7435
P53675-2	CLTCL1	145.6055	4	130	4.9905
Q96K30	RITA1	140.0281	3	20	2.6022

Table A.3 : LC-MS/MS resullts of biological replicate of MCF-7 BAG-1M:N-TAP purified proteins

Accession Number	Gene ID	PLGS Score	Peptides Number	Theoretical Peptides	Coverage (%)
P60709	ACTB	30230.5	29	37	80.8
P04792	HSPB1	23558.67	23	15	74.1463
P68363	TUBA1B	15518.71	21	35	76.4967
Q71U36	TUBA1A	14829.99	23	35	73.3925
P10809	HSPD1	14702.21	20	57	58.9878
Q9BQE3	TUBA1C	14466.26	20	35	73.0512
P06733	ENO1	14022.78	45	39	64.977
P04406	GAPDH	13995.87	45	26	86.2687
P04075	ALDOA	12234.65	37	34	83.5165
P68366-2	TUBA4A	11493.23	15	35	54.7344
P63104	YWHAZ	11473.76	13	30	48.5714
P68032	ACTC1	11280.11	17	37	54.3767
Q5VTE0	EEF1A1P5	10964.65	41	36	60.6061
P07437	TUBB	10420.88	24	32	72.5225
P68371	TUBB4B	9690.265	38	32	73.4831
P62937	PPIA	9106.142	24	16	83.0303
P07737	PFN1	9045.995	14	16	79.2857
O95994	AGR2	8201.557	20	18	80.0
P15531	NME1	8143.328	10	16	55.2632
P14174	MIF	8114.893	7	6	80.0
O60701	UGDH	7878.803	26	54	69.4332
P22392	NME2	7710.118	6	15	55.2632
P11142	HSPA8	7076.502	29	55	61.6099
P23528	CFL1	6956.317	12	21	63.8554
Q13885	TUBB2A	6745.564	18	32	62.9214
Q05639	EEF1A2	6487.584	19	36	60.0432

P62258	YWHAE	6282.763	11	31	53.3333
P00338	LDHA	5337.543	6	38	34.6386
P0DMV8	HSPA1A	5287.784	33	54	59.7504
P08238	HSP90AB1	5012.043	38	75	52.6243
P07900-2	HSP90AA1	4791.381	33	116	55.5035
P07900	HSP90AA1	4743.639	41	101	60.3825
P31946-2	YWHAB	4485.815	9	25	58.1967
P31949	S100A11	3441.525	9	10	56.1905
P05161	ISG15	3200.434	4	12	44.8485
P14618	PKM	2985.471	34	49	75.1412
P06748	NPM1	2641.773	5	26	23.4694
P07339	CTSD	2586.799	18	31	69.9029
P40926	MDH2	2476.517	8	32	51.7751
P61981	YWHAG	2464.61	4	27	28.7449
P05386	RPLP1	2449.37	4	8	51.7544
Q9Y281	CFL2	2356.174	8	20	48.7952
P13639	EEF2	2192.274	25	80	45.5711
Q13509	TUBB3	2188.887	28	32	38.8889
P07355	ANXA2	1762.698	13	34	50.4425
Q9UI54	PRO0628	1692.362	3	5	27.2727
P61978	HNRNPK	1663.916	16	41	50.7559
P27348	YWHAQ	1661.258	8	28	44.4898
P60842	EIF4A1	1477.913	15	47	55.9113
P18669	PGAM1	1451.456	5	22	41.3386
O00299	CLIC1	1413.46	4	22	36.5145
P06576	ATP5B	1398.391	14	41	48.0151
P32119	PRDX2	1339.837	8	19	61.1111
P11021	HSPA5	1317.619	24	60	42.8135
P00558	PGK1	1289.827	15	38	57.0743
Q15365	PCBP1	1177.014	6	28	41.8539
Q99497	PARK7	1141.982	5	29	30.6878
P31947-2	SFN	1105.775	4	23	32.8704
P12004	PCNA	1041.422	6	25	60.1533
Q99933	BAG1	977.8799	10	34	36.2319
P29401	TKT	931.991	11	48	31.4607

Q01105	SET	919.2538	3	19	18.2759
Q06830	PRDX1	905.6166	10	20	68.8442
P11413	G6PD	893.2961	6	57	26.7961
P07741	APRT	852.4309	3	20	31.1111
P60174	TPI1	825.8353	7	26	43.007
P40925	MDH1	821.4749	3	43	25.4491
P09467	FBP1	735.1293	6	33	28.4024
P47756	CAPZB	701.6404	4	31	22.0217
Q15084	PDIA6	615.0433	6	44	38.8636
P00918	CA2	609.7629	4	21	44.2308
Q13263	TRIM28	593.9703	6	62	18.6826
P10599	TXN	566.35	3	9	60.9524
P06744	GPI	543.5633	7	50	30.4659
O43707	ACTN4	542.9114	6	93	13.7212
P25705	ATP5A1	540.7919	4	51	16.094
P07237	P4HB	511.7315	8	55	25.3937
P22314	UBA1	509.501	8	72	17.9584
P52209	PGD	502.4141	3	43	9.1097
P54727	RAD23B	487.4856	3	22	12.7382
P62826	RAN	474.5939	3	20	29.6296
Q99714	HSD17B10	460.3398	3	20	30.6513
Q96CN7	ISOC1	438.7895	4	21	28.5235
P27797	CALR	411.6411	3	52	23.9808
P55209	NAP1L1	406.1511	3	28	16.1125
P38646	HSPA9	396.8154	4	75	14.2857
P06396	GSN	383.1104	4	55	15.0895
P55072	VCP	373.4989	5	72	13.2754
P48735	IDH2	371.9536	3	43	10.8407
Q96K30	RITA1	346.118	4	20	2.6022
P50395	GDI2	331.6561	3	44	17.7528
P26599	PTBP1	313.569	5	39	17.5141
P19338	NCL	310.1627	4	74	9.7183
P30101	PDIA3	251.9147	3	54	8.1188

Table A.4 : LC-MS/MS results of the technical replicate of the biological replicate of the analyzed MCF-7 BAG-1M:N-TAP purified proteins

Accession Number	Gene ID	PLGS Score	Peptides Number	Theoretical Peptides	Coverage (%)
P63261	ACTG1	30597.61	51	37	89.0667
P04792	HSPB1	17531.36	25	15	79.0244
P06733	ENO1	16778.67	42	39	66.8203
P68363	TUBA1B	16179.99	21	35	89.8004
Q71U36	TUBA1A	14755.68	23	35	89.8004
Q9BQE3	TUBA1C	14488.25	18	35	79.2873
P04406	GAPDH	13019.34	37	26	90.1493
P68032	ACTC1	12868.44	18	37	51.9894
P10809	HSPD1	12022.54	22	57	52.007
P63104	YWHAZ	12000.78	11	30	63.6735
O95994	AGR2	11239.88	13	18	60.0
P04075	ALDOA	10713.7	40	34	85.4396
P07437	TUBB	10346.19	23	32	70.9459
P07737	PFN1	9950.927	15	16	80.7143
P68104	EEF1A1	9271.47	21	36	60.1732
P68371	TUBB4B	9209.581	34	32	75.5056
P62937	PPIA	7585.132	22	16	76.3636
P23528	CFL1	7053.714	11	21	50.0
O60701	UGDH	6535.595	20	54	59.5142
P11142	HSPA8	6235.024	27	55	55.5728
P15531	NME1	5931.227	12	16	73.0263
P14174	MIF	5650.786	8	6	58.2609
P14618	PKM	5046.713	35	49	70.2448
P00338	LDHA	5003.368	9	38	50.9036
P09972	ALDOC	4772.021	8	35	32.967
P31946-2	YWHAB	4407.234	7	25	40.9836
P0DMV8	HSPA1A	4142.563	35	54	70.2028
P07900	HSP90AA1	4026.479	36	101	49.0437
Q05639	EEF1A2	3905.937	16	36	55.2916
P08238	HSP90AB1	3696.044	35	75	50.0
P62258	YWHAE	3556.033	10	31	52.549

Q9BUF5	TUBB6	3393.772	8	33	23.5426
P07339	CTSD	3200.792	14	31	58.7379
P31949	S100A11	3088.996	9	10	74.2857
P05386	RPLP1	3077.141	4	8	51.7544
P05161	ISG15	2719.064	7	12	59.3939
P13639	EEF2	2600.116	29	80	52.7972
P52565	ARHGDIA	2505.57	3	23	41.6667
Q3ZCM7	TUBB8	2444.287	12	32	40.0901
Q13509	TUBB3	2330.156	34	32	50.6667
P07355	ANXA2	2275.239	14	34	52.2124
P06748	NPM1	2266.707	4	26	23.4694
P11021	HSPA5	2058.026	22	60	50.6116
P40926	MDH2	1955.524	14	32	66.568
Q9Y281	CFL2	1661.288	8	20	24.0964
P06576	ATP5B	1631.67	15	41	58.7902
Q01105	SET	1452.242	5	19	26.5517
Q99933	BAG1	1416.642	7	34	26.087
Q06830	PRDX1	1359.282	14	20	71.8593
P07741	APRT	1356.071	4	20	41.1111
O00299	CLIC1	1340.182	4	22	37.7593
P00558	PGK1	1334.065	18	38	61.3909
Q15365	PCBP1	1269.145	7	28	49.7191
P60174	TPI1	1262.985	12	26	54.5455
P18669	PGAM1	1196.044	6	22	51.5748
P00918	CA2	1168.418	3	21	34.6154
Q58FF7	HSP90AB3P	1085.916	8	62	14.9079
P61981	YWHAG	1082.99	6	27	37.247
P61978	HNRNPK	1042.388	14	41	57.6674
P34931	HSPA1L	1016.179	14	54	24.805
P31943	HNRNPH1	997.1948	4	33	15.1448
P31947	SFN	994.2861	5	27	30.6452
Q9UI54	PRO0628	989.6329	3	5	27.2727
P27348	YWHAQ	983.0424	7	28	46.5306
P12004	PCNA	976.1941	5	25	48.659
P17066	HSPA6	917.3652	7	59	11.1975

P11413	G6PD	867.7175	13	57	42.1359
P60842	EIF4A1	843.9269	11	47	41.8719
P13693	TPT1	809.3802	3	16	43.0233
Q14240	EIF4A2	798.1409	8	51	34.6437
P06744	GPI	785.1648	9	50	30.4659
P09467	FBP1	778.4921	6	33	28.6982
P29401	TKT	745.6019	12	48	39.8074
P62826	RAN	742.7097	4	20	48.6111
Q9H0U4	RAB1B	731.5067	3	21	29.8507
Q04917	YWHAH	702.9286	4	29	8.5366
P07237	P4HB	670.0535	10	55	31.1024
Q15084	PDIA6	636.2172	4	44	30.0
P50395	GDI2	573.8947	5	44	24.4944
Q13263	TRIM28	568.5576	7	62	21.9162
P63241	EIF5A	559.4131	3	14	17.5325
O43707	ACTN4	558.9625	6	93	11.3063
Q99714	HSD17B10	545.0331	5	20	39.4636
P22314	UBA1	541.7365	13	72	26.3705
Q15366-8	PCBP2	499.7659	3	25	22.3602
P08758	ANXA5	497.5329	3	33	20.625
P52209	PGD	455.4922	3	43	11.1801
Q9NR45	NANS	438.7314	4	27	25.6267
P25705	ATP5A1	432.5443	7	51	19.1682
P38646	HSPA9	431.6451	3	75	12.813
P54727	RAD23B	487.4856	5	22	32.0388
P55072	VCP	399.9561	4	72	11.1663
P05388	RPLP0	396.9088	3	27	20.8202
Q14697	GANAB	327.3035	3	77	8.1568
P27797	CALR	278.9723	3	52	16.5468
P19338	NCL	275.0543	3	74	7.8873
P06396	GSN	272.3326	4	55	15.2174
P30101	PDIA3	462.8283	4	54	8.73762
P12814	ACTN1	221.4334	3	85	4.148
Q08752	PPID	218.9689	3	42	1.6216

APPENDIX B

*BAG-1 BAG Domain PDB Details

PDB ID: 1HX1 Crystal structure of BAG domain in complex with the HSC70 ATPase domain

1HX1:B|PDBID|CHAIN|SEQUENCE

GNSPQEEVELKKLKHLEKSVEKIADQLEELNKELTGIQQGFLPKDLQAEALC
KLDRRVKATIEQFMKILEEIDTLILPENFKDSRLKRKGLVKKVQAFLAECDTV
EQNICQET

*BAG-1 ULD PDB Details

PDB ID: 1WXV Solution structure of ubiquitin-like domain of Bcl-2 binding athanogene-1

1WXV:A|PDBID|CHAIN|SEQUENCE

GSSGSSGLTVTVTHSNEKHDLHVTSQQGSSEPVVQDLAQVVEEVIGVPQSFQ
KLIFKGKSLKEMETPLSALGIQDGCRVMLIGKKNSGPSSG

*RAD23 PDB Details

PDB ID: 2QSF Crystal structure of the RAD4-RAD23 complex

2QSF:X|PDBID|CHAIN|SEQUENCE

GSGNASSGALGTTGGATDAAQGGPPGSIGLTVEDLLSLRQVVSGNPEALAPL
LENISARYPQLREHIMANPEVFVSMLLEAVGDNMQDVMEGADDMVEGEDIE
VTGEAAAAGLGQGEGEGSFQVDYTPEDDQAISRLCELGFERDLVIQVYFACD
KNEEAAANILFSDHAD

*VCP PDB Details

PDB ID: 5X4L Crystal structure of the UBX domain of human UBXD7 in complex with p97 N domain

5X4L:A|PDBID|CHAIN|SEQUENCE

GGSPNRLIVDEAINEDNSVVSLSQPKMDELQLFRGDTVLLKGKKRREAVCIV
LSDDTCSDEKIRMNRVVRNNLRVRLGDVISIQPCPDVKYGKRIHVLPIDDTVE

GITGNLFEVYLKPYFLEAYRPIRKGDIFLVRGGMRAVEFKVVETDPSPYCIVA
PDTVIHCEGEPIKREDEEE

***PDIA3 PDB Details**

PDB ID: 2H8L Crystal structure of the bb' fragment of ERp57

2H8L:A|PDBID|CHAIN|SEQUENCE

GPLGSPASVPLRTEEEFKKFISDKDASIVGFFDDSFSEAHSEFLKAASNLRDNY
RFAHTNVESLVNEYDDNGEGIILFRPSHLTNKFEDKTVAYTEQKMTSGKIKK
FIQENIFGICPHMTEDNKDLIQGKDLLIAYYDVDYEKNAKGSNYWRNRVMM
VAKKFLDAGHKLNFAVASRKTFSHELSDFGLESTAGEIPVVAIRTAKGEKFV
MQEEFSRDGKALERFLQDYFDGNLKRYLKSEPIPESNDGAAAS

***HSP90 PDB Details**

PDB ID: 5LRZ Crystal structure of HSP90 in complex with A003643501

5LRZ:A|PDBID|CHAIN|SEQUENCE

HMETFAFQAEIAQLMSLIINTFYSNKEIFLRELISNSSDALDKIRYESLTDPSKL
DSGKELHINLIPNKQDRTLTIVDTGIGMTKADLINNLGTIAKSGTKAFMEALQ
AGADISMIGQFGVGFYSAYLVAEKVTVITKHNDDEQYAWESSAGGSFTVRT
DTGEPMGRGTKVILHLKEDQTEYLEERRIKEIVKKHSQFIGYPITLFVE

***BiP PDB Details**

PDB ID: 5EVZ Crystal structure of human GRP78 (70kDa heat shock protein 5 / BiP) ATPase domain in complex with ADP and inorganic phosphate

5EVZ:A|PDBID|CHAIN|SEQUENCE

MHHHHHHSSGRENLYFQGDVGTVVGIDLGTTYSCVGVFKNGRVEIIANDQG
NRITPSYVAFTPEGERLIGDAAKNQLTSNPENTVFDAKRLIGRTWNDPSVQQ
DIKFLPFKVVEKKTKPYIQVDIGGGQTKTFAPEEISAMVLTKMKETAEAYLG
KKVTHAVVTVPAYFNDAQRQATKDAGTIAGLNVMRIINEPTAAAIAYGLDK
REGEKNILVFDLGGGTFDVSLLTIDNGVFEVVATNGDTHLGGEDFDQRVME
HFIKLYKKKTGKDVRKDNRAVQKLRREVEKAKRALSSQHQARIEIESFYEGE
DFSETLTRAKFEELNMDLFRSTMKPVQKVLEDSDLKKSDIDEIVLVGGSTRIP
KIQQLVKEFFNGKEPSRGINPDEAVAYGAAVQAGVLSG

APPENDIX C

GenBank: AF022224.1, Homo sapiens Bcl-2-binding protein (BAG-1) mRNA, complete cds

ACGCCGCGCTCAGCTTCCATCGCTGGGCGGTCAACAAGTGCGGGCCTGGC
TCAGCGCGGGGGGGCGCGGAGACCGCGAGGCGACCGGGAGCGGCTGGG
TTCCCGGCTGCGCGCCCTTCGGCCAGGCCGGGAGCCGCGCCAGTCGGAG
CCCCCGGCCCAGCGTGGTCCGCCTCCCTCTCGGCGTCCACCTGCCCGGAG
TACTGCCAGCGGGCATGACCGACCCACCAGGGGCGCCGCCGCCGGCGCT
CGCAGGCCGCGGATGAAGAAGAAAACCCGGCGCCGCTCGACCCGGAGCG
AGGAGTTGACCCGGAGCGAGGAGTTGACCCTGAGTGAGGAAGCGACCTG
GAGTGAAGAGGCGACCCAGAGTGAGGAGGCGACCCAGGGCGAAGAGAT
GAATCGGAGCCAGGAGGTGACCCGGGACGAGGAGTCGACCCGGAGCGA
GGAGGTGACCAGGGAGGAAATGGCGGCAGCTGGGCTCACCGTGACTGTC
ACCCACAGCAATGAGAAGCACGACCTTCATGTTACCTCCCAGCAGGGCA
GCAGTGAACCAGTTGTCCAAGACCTGGCCCAGGTTGTTGAAGAGGTCAT
AGGGGTTCCACAGTCTTTTCAGAAACTCATATTTAAGGGAAAATCTCTGA
AGGAAATGGAAACACCGTTGTCAGCACTTGGAATACAAGATGGTTGCCG
GGTCATGTTAATTGGGAAAAAGAACAGTCCACAGGAAGAGGTTGAACTA
AAGAAGTTGAAACATTTGGAGAAGTCTGTGGAGAAGATAGCTGACCAGC
TGGAAGAGTTGAATAAAGAGCTTACTGGAATCCAGCAGGGTTTTCTGCCC
AAGGATTTGCAAGCTGAAGCTCTCTGCAAACTTGATAGGAGAGTAAAAG
CCACAATAGAGCAGTTTATGAAGATCTTGGAGGAGATTGACACACTGAT
CCTGCCAGAAAATTTCAAAGACAGTAGATTGAAAAGGAAAGGCTTGGTA
AAAAAGGTTCAGGCATTCCTAGCCGAGTGTGACACAGTGGAGCAGAACA
TCTGCCAGGAGACTGAGCGGCTGCAGTCTACAAACTTTGCCCTGGCCGAG
TGAGGTGTAGCAGAAAAAGGCTGTGCTGCCCTGAAGAATGGCGCCACCA
GCTCTGCCGTCTCTGGATCGGAATTTACCTGATTTCTTCAGGGCTGCTGGG
GGCAACTGGCCATTTGCCAATTTTCCTACTCTCACACTGGTTCTCAATGAA
AAATAGTGTCTTTGTGATTTGAGTAAAGCTCCTATTCTGTTTTTCACAAAA
AAAAAAAA

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