ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

BAG-1 OVEREXPRESSION LEADS TO BAD PHOSPHORYLATION AT SERINE 136 REVEALING A ROUTE FOR SURVIVAL IN MCF-7 BREAST CANCER CELL LINE

M.Sc. THESIS

Salih DEMİR

Advanced Technologies

Molecular Biology-Genetics and Biotechnology

Thesis Advisor: Assist. Prof. Dr. Gizem DİNLER DOĞANAY

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

BAG-1'İN AŞIRI İFADESİ BAD SERİN 136 FOSFORLANMASINI SAĞLAYARAK MCF-7 MEME KANSERİ HÜCRE HATTINDA SAĞKALIMI İŞARET EDER

YÜKSEK LİSANS TEZİ

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Salih DEMİR, a **M.Sc.** student of ITU **Graduate School of Science, Engineering and Technology** student ID **521101111**, successfully defended the **thesis** entitled "**BAG-1 OVEREXPRESSION LEADS TO BAD PHOSPHORYLATION AT SERINE 136 REVEALING A ROUTE FOR SURVIVAL IN MCF-7 BREAST CANCER CELL LINE** which he/she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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FOREWORD

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December 2012

Salih DEMİR
(Biologist)

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ABBREVIATIONS

µg : Microgram
µL : Microliter
µm : Micrometer
µM : Micromolar
4-HRP : 4-hydroxy(phenyl)retinamide
aa : Amino acid
APS : Ammonium persulfate
ATP : Adenosine triphosphate
ATPase : Adenosine triphosphatease
ATRA : All-Trans Retinoic Acid
Bad : Bcl-2-associated death promoter
Bag-1 : Bcl-2-associated athanogene
Bax : Bcl-2 homologous antagonist/killer
Bcl-2 : B-cell lymphoma-2
bp : Base pair
CBB : Coomassie Brilliant Blue
DAPI : 4',6-diamidino-2-phenylindole
DiOC6 : 3,3′-dihexyloxacarbocyanine iodide
DMEM : Dulbecco's modified Eagle medium
DMSO : Dimethyl sulfoxide
DNA : Deoxyribonucleic acid
DTT : Dithiothreitol
E. coli : *Escherichia coli*
EDTA : Ethylenediaminetetraacetic acid
FBS : Fetal bovine berum
g : Gram
HRP : Horseradish peroxidise
Hsp70 : 70 kilodalton heat shock proteins
IPTG : Isopropyl β-D-1-thiogalactopyranoside
Kb : Kilobase
kDa : Kilodalton
L : Liter
LB : Luria-Bertani Broth
M : Molar
mA : Miliampere
MAP : Mitogen-activated protein
MAPK : Mitogen-activated protein kinase
mg : Miligram
min : Minute
mL : Milliliter
mm : Millimeter
mM : Milimolar

mRNA : Messenger ribonucleic acid
MT : Microtubule
MTs : Microtubules
NCBI : National Center for Biotechnology Information
ng : Nanogram
nM : Nanomolar
OD : Optical Density
PBS : Phosphate Buffered Saline
PCR : Polymerase chain reaction
pH : Power of hydrogen
PI : Propidium Iodide
pmol : picomole
RNA : Ribonucleic acid
rpm : Revolutions per minute
SDS : Sodium dodecyl sulphate
SDS-PAGE : Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec : Second
Ser : Serine
siRNA : Small interfering RNA
SOC : Super Optimal Broth with catabolite repression
TAP : Tandem Affinity Purification
TBS : Tris-Buffered Saline
TEMED : Tetramethylethylenediamine
UV : Ultraviolet
V : Volt

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BAG-1 OVEREXPRESSION LEADS TO BAD PHOSPHORYLATION AT SERINE 136 REVEALING A ROUTE FOR SURVIVAL IN MCF-7 BREAST CANCER CELL LINE

SUMMARY

Bcl-2 associated athanogene family (athano = against death in Greek) proteins are discovered by co-immunoprecipitation with Bcl-2 anti-apoptotic protein. Bag genes are evolutionally conserved and their homologes exist in yeasts, plants and animals. Bag family members control the cell homeostais in two different ways: (1) They contribute to maintain cell's vital activity under normal and stress conditions via regulating both positively and negatively the cycle of Hsp70/Hsc70 molecular chaperone system. For instance, the direction of the cell balance under normal and stress conditions are maintained by competitive binding of Hsp70 or Raf-1 to Bag-1. (2) They form complexes with transcription factors and nuclear hormone receptors, which are controlling various physiological processes such as apoptosis, tumorigenesis, neural differentiation and cell cycle independent from Hsp70/Hsc70 system.

Bag-1 isoforms are produced by the alternative translation initiation of single mRNA transcript; thereby they differ in their N-terminus related to translation initiation. Bag-1 isoforms appear to be differently localized in the cells. Main Bag-1 isoforms which are observed in humans are Bag-1S (36 kDa), Bag-1M (46 kDa) and Bag-1L (50 kDa). There is also another isoform of Bag-1, 29 kDa, however, it is very scarce in the cell, and thus is not consistently detected.

All of Bag-1 isoforms commonly contain both the ubiquitin-like domain (ULD) and Bag domain (BD) in their C-terminus. Even though ULD domain function is not clearly known, its evolutionary conservation and its relations with proteosome machinery indicate that ULD is necessary for stress tolerance. Bag-1L is localized in nucleus by means of its nuclear localization signal (NLS). NLS does not exist in Bag-1M, which is known to be cytosolic but some studies revealed Bag-1M translocation to nucleus via companion proteins, Bag-1S, on the other hand, always presents in cytosol. N-terminus of Bag-1L and Bag-1M consist of eight TXSEEX repeats. It is shown that these repeats have a role in DNA binding and transcription activation depending on their nuclear localizations. Having less repeat sequences, Bag-1S is the shortest isoform in the cells.

Bag-1 has many critical roles in the cell through its interactions with certain target molecules to regulate carcinogenesis, differentiation, motility, proliferation, cell survival, apoptosis and transcription. Nuclear hormone receptors, Siah, CHIP, HGF receptor, Hsp70/Hsc70 complex, Bcl-2, and C-Raf (Raf-1) are known interacting partners of Bag-1. Cell survival and apoptosis are the two major pathways that Bag-1 is involved in. Bag-1 interactions with C-Raf or Bcl-2 are main mechanism that determine cell fate. Through these interactions Bag-1 gets invoved in the regulation the balance between cell death and cell survival. Although Bag-1 involvement in

different mechanisms is known, the molecular details of these mechanisms and crosstalk between the pathways are not clear yet.

In this study, we aimed to elucidate the molecular mechanism of Bag-1-associated cell survival in MCF-7 breast cancer cell line. To understand Bag-1's influence on cell survival, we altered expression levels of Bag-1 in MCF-7 cells. TAP tagged-Bag-1 overexpressing plasmid was used for enhencement of Bag-1 level, and Bag-1 specific siRNA transfection was applied for down regulation of Bag-1. XTT cell proliferation assay and tryphan blue dye exclusion assay were performed to explain Bag-1's effect on cellular level. Western blot analysis was used for checking expression levels of proteins that take part in cell survival and signaling pathways to investigate changes in the expression after Bag-1 overexpression or silencing. To deduce molecular details of Bag-1-related cell survival we checked interaction partners of Bag-1 during cell survival using co-immunoprecipitation. We also did immunocytochemistry to demonstrate subcellular co-localization of Bag-1 with its interaction partners from the cell survival complex. Our results showed that C-Raf is involved in the complex that is formed by B-Raf, Hsp70, phospho-Akt and Bcl-2 in order to phosphorylate Bad at serine 136 residue for the prevention of apoptosis.

BAG-1'İN AŞIRI İFADESİ BAD SERİN 136 FOSFORLANMASINI SAĞLAYARAK MCF-7 MEME KANSERİ HÜCRE HATTINDA SAĞKALIMI İŞARET EDER

ÖZET

Bcl-2 asosiye athano gen (Bag) ailesi (Yunanca ölüm karşıtı athanostan) proteinleri anti-apoptotik Bcl-2 proteini ile ko-immunopresipitasyon sonucu bulunmuşlardır. Bag genleri evrimsel olarak korunmuştur ve mayalarda, bitkilerde ve hayvanlarda homologları mevcuttur. Bag ailesi üyeleri iki farklı yolla hücre homeostazını kontrol eder: (1) Hem pozitif hem negatif şekilde Hsp70/Hsc70 moleküler şaperon sisteminin çalışma döngüsünü regüle ederek hücrenin yaşamsal faaliyetlerini normal ve stres koşullarında sürdürmesine katkıda bulunur. Buna spesifik bir örnek, hücrenin normal ve stres halleri arasındaki dengesinin yönünün Bag-1'e Hsp70 ya da Raf-1'in yarışmalı bağlanması sonucu sağlanması ile verilebilir. (2) Apoptoz, tümör oluşumu, nöronal farklılaşma ve hücre döngüsü gibi birçok fizyolojik süreci kontrol eden transkripsiyon faktörleri ve reseptörler ile Hsp70/Hsc70 sisteminden bağımsız olarak kompleksler oluşturur.

Bag ailesi üyelerinin, insanlarda, kanser, AIDS ve Parkinson gibi kompleks hastalıklarda normal hücrelere kıyasla ekspresyon düzeylerinde ve hücre içi lokalizasyonlarında değişiklikler gözlenmektedir. Dolayısıyla, bu tip hastalıkları tahlil etmede Bag proteinlerinin hücre içi ekspresyonlarını ve lokalizasyonlarını incelemeye yönelik çalışmalardan yararlanılabilir. Örneğin, kanserli meme hücrelerinde yapılan çalışmalarla Bag-1 ekspresyonunun, hastalığın seyrinin izlenmesinde prognostik bir markör olarak kullanılabileceği gösterilmiştir.

Belirlenmiş Bag proteinleri Bag-1 (RAP46/HAP46), Bag-2, Bag-3 (CAIR-1/Bis), Bag-4 (SODD), Bag-5 ve Bag-6 (BAT3/Scythe) olup hepsi C-terminus yakınında BAG domen (BD) ifadesine sahiptir. Bag proteinlerinin genellikle N terminuslarında farklılık göstermesi her bir Bag ailesi üyesinin çeşitli proteinler, yolaklar ve/veya lokalizasyonlara göre spesifite göstermesini sağlar. Hücrelerde Bag-1 izoformlarinin ekspresyon düzeyleri farklılık göstermektedir. Bu izoformlar hücrelerde bulunma sıklıklarına göre Bag-1S, Bag-1L ve Bag-1M olarak sıralanabilir. Buna rağmen tümörlü hücrelerde ekspresyon düzeyi en yüksek olan Bag-1L izoformudur. Bunun nedeni ise Bag-1L'nin nüklear lokalizasyonuna bağlı olarak transkripsiyon faktörlerinin aktivitesini yönlendirmesi ve böylece hücre proliferasyonunu dolaylı yoldan tetiklemesi olabilir.

Bag-1 izoformlarının hücrelerde daha önce yapılmış çalışmalar sonucu belirlenmiş etkileşim partnerleri; Bcl-2, Raf-1, Hsc70/Hsp70 sistemi, nükleer hormon reseptörleri (NHR), ubikuitin/proteozom mekanizması ve DNA'dır. Bu etkileşimlerle Bag-1 izoformları, hücre büyümesi için kritik sinyal iletimi, proliferasyon, transkripsiyon gibi hücresel mekanizmalardan, hücre hareketi ve apoptoza varana kadar normal, malignan ve stres altında bulunan hücreler için önemli kontrol yolaklarında görev almaktadır. Bag-1 izoformlarinin belirtilmiş olan geniş

perspektifli bu hücresel aktiviteleri genel olarak Bag ailesi üyeleri için tanımlandığı gibi iki farklı şekilde kontrol ettiği literatürde yer almaktadır: (1) Hsp70/Hsc70 sistemi ile etkileşim yoluyla, (2) nüklear hormon reseptörlerine (NHR) bağlanmasıyla.

Bag-1 izoformlarinin Hsp70/Hsc70 moleküler şaperon sistemi ile etkileşiminde BAG domenleri Hsc70'in ATPaz domeninine bağlanır, konformasyonel değişiklikler yaratarak ADP'nin ATP ile değişimine etkide bulunur ve sonuçta bu şaperonların peptit bağlayan domenlerinin substrat proteinleri ile olan bağlanma/ayrılma kinetiklerini regüle eder. Bu şekilde bozunmuş hedef proteinin yeniden katlanıp katlanmayacağı kontrol edilmiş olur.

Bag-1 izoformları, Hsp70/Hsc70 sistemini hem pozitif hem de negatif yönde kontrol ederek sırasıyla hücrelerin efektif bir şekilde protein üretebilmesi sonucu hücre büyümesine ya da hücrelerin protein üretiminin yavaşlaması sonucu hücre ölümüne yol açabilir. Fakat bu kritik ayarlamada, henüz Bag-1'lerin Hsp70/Hsc70 sistemine tam olarak moleküler düzeyde nasıl bir kontrol mekanizması ile etkide bulunduğu net olarak bilinmemektedir. Şu ana kadar yapılmış bu konudaki çalışmalar tutarsız sonuçlar ortaya koymaktadır. Bazı çalışmalar Bag-1M'nin Hsp70/Hsc70 sistemine bağlanması sonucu oluşan konformasyonel değişikliğin ADP'nin ATP ile değişimine imkan vermeyerek protein katlanmasını engelleyici özellik gösterdiği yönündedir. Bunun yanı sıra, bir çalışmada Bag-1S'nin protein katlanmasına pozitif yönde bir etki göstermesine karşın daha sonra yapılan bir çalışmayla negatif yönde bir etkide bulunduğu ortaya çıkartılmıştır. Bu konudaki belirsizlikler, Bag-1 izoformlarının Hsp70/Hsc70 sistemi üzerinden hücre yaşamındaki etkilerinin anlaşılmasını güçleştirmektedir. Bu durumla ilgili çeşitli hipotezler ortaya atılmış ve bunları destekleyici bazı sonuçlara ulaşılmıştır. Kritik yolakların aydınlatılması yolunda literatürde yer alan örnekler aşağıda özetlenmektedir.

Bag-1'in Hsc70 ile etkileşimi sonucu Bcl-2 ile bir kompleks oluşturduğu gözlemlenmiştir. Anti-apoptotik Bcl-2 ile Bag-1'in etkileşimi, öncelikle Bag-1'in C-Raf (Raf-1) serin/treonin kinazı ile kompleks oluşturması sonucu gerçekleşir. Bunun sonucunda hücrenin yaşam ya da ölüm kaderini belirleyecek etkileşimlerin tetiklendiği ortaya çıkartılmıştır. Bag-1/C-Raf kompleksinin apoptoz öncüsü Bad (pro-apoptotik) proteinini fosforilasyonu sonucu, halihazırda hücrede bulunan Bcl-2'nin anti-apoptotik etkisini baskılayan Bcl-2/Bad etkileşimlerinin bozulabileceği ve böylelikle Bcl-2'lerin serbest kalarak apoptoz öncüsü Bax'in dimer oluşturmasını engellemesiyle hücrenin yaşamasının sağlandığı düşünülmektedir.

Bu çalışmayla amaçlanan, MCF-7 meme kanseri hücre hattında Bag-1-ilişkili sağkalım mekanizmasının moleküler detaylarının aydınlatılmasıdır. Bu amaç doğrultusunda Bag-1'in ekspresyon seviyeleri değiştirilmiş ve hücreye etkisi incelenmiştir. Bag-1'in aşırı ekspesyonunu sağlama amacı ile TAP-tag içeren Bag-1'in ekspresyonunu arttıran plazmit; Bag-1'i susturmak için ise Bag-1'e özgü siRNA kullanılmıştır. Bag-1'in ekspesyonundaki değişikler western blotlama ile doğrulanmıştır. Bag-1'in aşırı ifadesinin hücre canlılığına yaptığı pozitif etkiler XTT hücre canlılığı ve tripan mavisi sağkalım deneyleri ile gösterilmiştir. Bag-1'in aşırı ifadesinin MCF-7 meme kanseri hücrelerinde sağkalımı arttırdığı gösterildikten sonra sağkalım yolağında görev yapan proteinlerin ekspresyonunun Bag-1 ekspresynundaki değişimlerle birlikte nasıl etkilendiği western blotlama ile araştırılmıştır. Western blot analizi sonuçları önemli MAP (mitogen-activated protein) kinazlar olan B-Raf ve C-Raf ekpresyonlarının Bag-1 aşırı ifadesi ile

arrtığını göstermiştir. Ayrıca B-Raf'ın Serin 445, C-Raf'ın Serin 338 ve 289/296/301, Akt'nin Serin 473 bakiyelerinden fosfoslanma seviyelerinin ve Hsp70 expresyonunun da Bag-1 aşırı ekspesyonu ile arttığı, Bag-1'in susturulması ile azaldığı da western blotlama ile gözlemlenmiştir. Bag-1'ın hücre sağkalımını tetiklediği gösterildikten sonra Raf-Erk-MEK sinyal yolağının alt akışında bulunan proteinlerin de (Erk ve MEK) ekspresyonunu arttırdığı gösterilmiştir.

Östrojen bağımlı meme kanseri hücrelerinde Bag-1'in bu sağkalım kompleksindeki etkileşim partnerlerini incelemek için ko-immünopresipitasyon yapılmıştır. Bag-1, fosforlanmış Akt, B-Raf, C-Raf, Bcl-2 ve Hsp70'in oluşturduğu bir kompleksin hücrede sağkalım mekanizmasını tetiklediği gösterilmiştir. Bag-1'in hücre içinde forforlanmış Akt, B-Raf, Bcl-2, Hsp70 ve C-Raf ile birlikte lokalize olduğu immüno hücre boyaması ile ispatlanmıştır. Daha önce sinir hücreleri (birincil hücre hattı) ile yapılan bir çalışmada Bag-1-ilişkili sağkalım kompleksine C-Raf'ın dahil olmadığı bulunmuştur.

1. INTRODUCTION

1.1. Bag-1 gene and isoforms

Bag-1 (Bcl-2 associated athanogene gene) is located on human chromosome 9, band 12 and it encodes 3885 bp mRNA having 7 exons (Takayama et.al, 1995). The promoter has been dispersed along the positions -353 to -54 upstream of the first translational start codon and is a binding site for several transcription factors (Takayama et al., 2005).

There are multiple translation start sites on the nucleotide sequence of Bag-1 (Zheng et al., 2010). The first translation is started on mRNA sequence, at 66th codon (CUG) to encode Bag-1L (Zheng et al., 2010). Bag-1M is initiated from 279th codon (AUG), Bag-1S and Bag-1 are encoded from 411st and 504th amino acids, respectively (Zheng et al., 2010) (Figure 1.1). Even though alternative translation mechanism is not yet clear, there are hypothesis that might explain for the mechanism of translation (Townsend et al., 2005). While Bag-1L and Bag-1M isoforms are translated via of 5' cap structure of Bag-1 mRNA (Coldwell et al., 2001), Bag-1S isoform translation is dependent on an internal ribosome entry site (IRES). The ribosome binds to IRES element of the mRNA (Dobbyn et al., 2008).

According to these translation start sequences, ribosomes can settle on one of three certain sites diversely (Gehring et al., 2006). This selection of the site produces differential protein isoforms expression for Bag-1 (Gehring et al., 2006). The longest Bag-1 isoform is Bag-1L (50 kDa), and the others are Bag-1M/Rap46 (46 kDa), and Bag-1S (36 kDa) in human cells (Gehring et al., 2006). There is also another isoform of Bag-1, 29 kDa, however, it is very scarce in the cell and due to that is not consistently detected (Gehring et al., 2006).

Subcellular localization of Bag-1 isoforms is important for different biological mechanisms (Knee et al., 2001). The NLS (nuclear localisation signal or nuclear localisation sequence) domain of Bag-1 mRNA is responsible for nuclear localization of Bag-1L (Knee et al., 2001). Bag-1M has both nuclear and cytosolic

fractions of NLS according to the initiation site of translation of the mRNA (Brimmell et al., 1999). However, the localization of Bag-1 isoforms can be regulated by cellular stress conditions which can cause relocalization of Bag-1S and Bag-1M to the nucleus (Townsend et al., 2002).

These isoforms have different impacts on heat shock protein function, ubiqutination and different transcriptional activities (Liu et al., 2009). C- terminal "Bag domain" is found in all members of Bag protein family. Bag domain was first described as a conserved region which has about 50 amino acids (Takayama et al., 1999), but recent studies with advanced techniques like X-ray crystallograpghy, and multidimensional nuclear magnetic resonance (NMR) revealed that Bag-1 domain has 110-124 amino acids and it consists of three anti-parallel α-helices, each approximately 30-40 amino acids in length (Briknarova et al., 2001; Sondermann et al., 2001). These helices provide interaction sites for other proteins; the first and second helices (α-1 and α-2) interact with the serine/threonine kinase Raf-1 and the second and third helices (α-2 and α-3) are the sites of interaction with the ATPase domain of Hsc70/Hsp70 (Takayama at al., 2001; Sondermann et al., 2001; Song et al., 2001) and TNF-R1 (Antoku et al., 2001).

All three isoforms of Bag-1 has a ubiquitin- like domain (ULD) (Bricknarova et al., 2001). Ubiquitin is a ubiquitous 76 amino acid protein that is covalently attached to protein substrates to target proteins for degradation by proteosome, the major non-lysosomal proteolytic complex in cells (Pickard et al., 2001). Bag-1 has been shown to be ubiquitylated, but Bag-1 proteins have relatively long half-lifes (Luders et al., 2000). Therefore Bag-1 may play a role in coordinating ubiquitin system instead of participating in conjugation reactions (Townsend et al., 2003). Although the ULD of Bag-1 is structurally related to ubiquitin, Bag-1 is not covalently attached to protein substrates. The ULD is essential for some but not all biologic functions of Bag-1, and does appear to be significant for binding to the proteasome (Townsend et al., 2005).

Although Bag-1 predominantly functions to suppress apoptosis, most likely acting between the growth/survival receptors and the mitochondria, it also involves other regulatory points in the apoptotic cascade (Takayama et al., 2001). Bag-1 interacts with the ATPase domain of Hsc70, and Hsc70 may play a role intermediumte in the binding of Bag-1 to Bcl-2 (Cutress et al., 2003). Heat shock proteins such as Hsp70 and Hsc70, play an essantial role in assembly of multimeric apoptosis regulating

complex “apoptosome” via their charcteristic “chaperone” function (Townsend et al, 2003). Since Bag-1 directly binds to Hsp70/Hsc70, and regulates their activities by acting as a nucleotide exchange factor, it can be named as a “co-chaperone” (Nollen et al., 2000). It has been reported that in MCF-7 cells with a mutation of a single residue in the Bag domain of Bag-1S heat shock protein binding can be prevented, but this mutation did not affect C-Raf (Raf-1) interaction. Also, through this mutation, Bag-1’s protecting ability for heat shock-induced growth inhibition was prevented (Townsend et al., 2003). It has been demonstrated that at least in an artificial *in vitro* system, Bag-1M could interact with a wide range of proteins including c-Jun, c-Fos, c-Myc and Mos, which are important for the regulation of cell cycle (Zeiner et el., 1997).

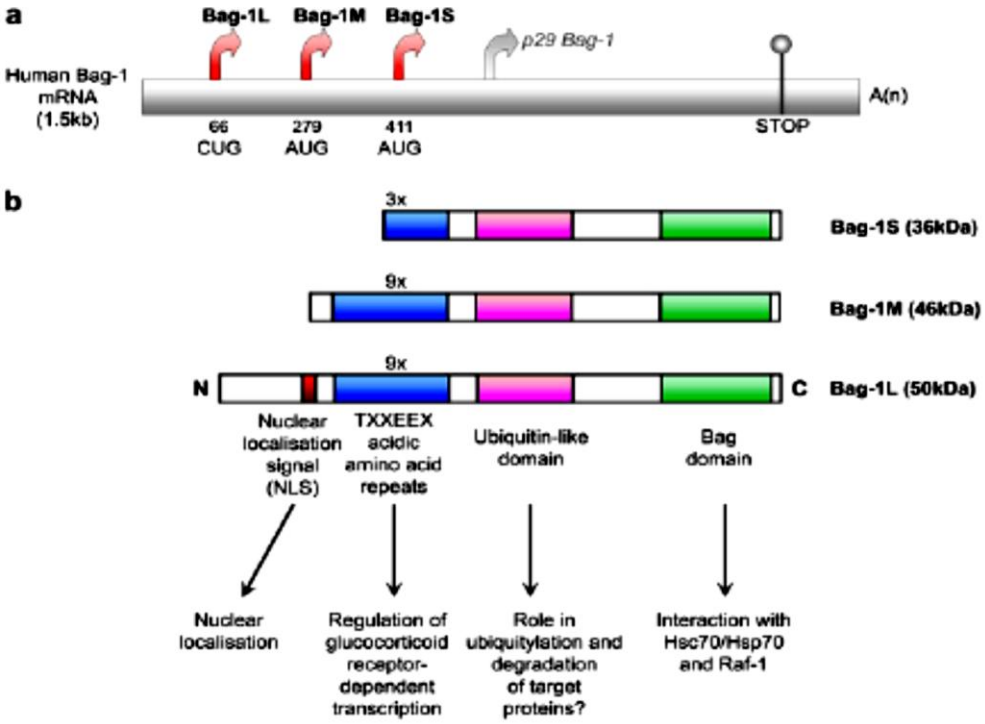

Figure 1.1 : Schematic presentation of Bag-1 mRNA. Alternative translation sites of Bag-1 mRNA (A). Bag-1 isoforms with the binding domains (B) (Zheng et al., 2010).

1.2 Apoptosis

Cell death is important to maintain cell population, especially during the developmental stages of both animals and plants (Alberts et al., 2008). Maintenance of the balance between cell division and tissue volume is provided by a process known as programmed cell death which usually occurs by apoptosis (“*falling of”* in

Greek) (Alberts et al., 2008). Apoptosis causes a wide range of morphological changes; cells shrink and condense, the cytoskeleton collapses, the nuclear envelope disassembles, and the chromatin condenses and breaks up into fragments (Alberts et al., 2008) (Figure 1.2).

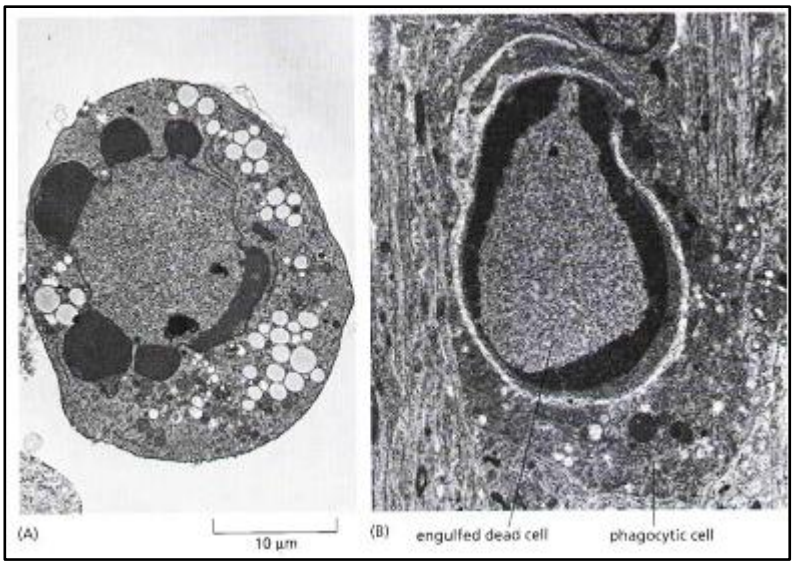

Figure 1.2 : The electron micrographs of apoptotic cells. The cell that died in a culture dish (A), died in developing tissue and engulfed by a phagocytic cell (B) (Alberts et al., 2008).

To understand the apoptotic morphology, including the early process of apoptosis, cell shrinkage and pyknosis can be monitored by light microscopy (Kerr et al., 1972). At the end of cell shrinkage, cells become smaller in size, the cytoplasm denses and the organelles become more tightly packed (Kerr et al., 1972). Pyknosis, also known as irreversible condensation of chromatin, is the most characteristic feature of apoptosis (Savill and Fadok, 2000). Extensive plasma membrane blebbing occurs after separation of cell fragments into apoptotic bodies, which is an indicator of late apoptosis, during a process called "budding" (Savill and Fadok, 2000). Apoptotic bodies, which include tightly packed organelles with or without nuclear fragment, are subsequently phagocytosed by macrophages, paranchymal cells, or neoplastic cells, and finally degraded within phagolysosome (Savill and Fadok, 2000). Tingible body macrophages which are responsible for the elimination of apoptotic cells engulf and digest apoptotic cells, and there is essentially no inflammatory reaction related neither with the process of apoptosis, nor with the removal of apoptotic cells (Savill and Fadok, 2000). Because: (1) apoptotic cells do not exudate their cellular components into surrounding interstitial tissue; (2) they are fastly phagocytosed by

surrounding cells; and (3) the engulfing cells do not produce anti-inflammatory cytokines (Savill and Fadok, 2000).

Apoptosis could be both a normal event or a response to a certain stimuli (Norbury and Hickson, 2001). Apoptosis takes place during development and aging and as a homeostatic mechanism to control cell population in tissues (Norbury and Hickson, 2001). Apoptosis also occurs as a defence mechanism in immune reactions (Norbury and Hickson, 2001).

1.2.1 Apoptotic pathways

The mechanisms of apoptosis are very complicated and sophisticated, involving an energy dependent cascade of caspases, however researchers were able to clasify the mechanisms of apoptosis into two major pathways (Elmore, 2007). They are (1) the extrinsic or death receptor pathway and (2) the intrinsic or mitochondrial pathway. Those two pathways are linked and that molecules in one pathway can affect the other (Igney and Krammer, 2002). There is an additional pathway, which can induce apoptosis via either granzyme A or granzyme B, and includes T-cell mediumted cytotoxicity, called as "perforin/granzyme pathway" (Igney and Krammer, 2002). All of these three pathways meet on the same termination point or in other words execution pathway (Igney and Krammer, 2002) (Figure 1.3).

Extrinsic pathways are related with the transmembrane receptor-mediumted interactions which involve death receptors that are members of the tumor necrosis factor (TNF) receptor gene superfamily, and they share similar cyteine-rich extracellular domains and have a cytoplasm domain of about 80 amino acids called the "death domain" (Locksley et al., 2001). Extrinsic phase of apoptosis is best characterized with the FasL/FasR and TNF-α/TNFR1 models, in which clustering of receptors and binding with the homologous trimeric ligand occur (Hsu et al., 1995). Cytoplasmic adaptor proteins, FADD (Fas-associated death domain) and TRADD are needed to transmit the death signal after getting activated by their receptors. FADD protein activation through Fas ligand binding to Fas receptor enables initiator caspase activation (Hsu et al., 1995). TRADD protein can also get activated and relay death signals with the recruiment of FADD and RIP by activation through binding of TNF ligand to TNF receptor (Hsu et al., 1995; Wajant, 2002). FADD then associates with procaspase-8 with the help of the dimerization of death effector

domain, and it results with the formation of death-inducing signalling complex (DISC), initiating the auto-catalytic activation of procaspase-8 (Kischkel et al., 1995).

Intrinsic pathways include a wide range of non-receptor mediumted stimuli (Garrido et al., 2006). Opening of the mitochondrial permeability transition (MPT) pore, loss of the mitochondrial transmembrane potential and release of cytocrome c, Smac/DIABLO, and the serine protease HtrA2/Omi into cytosol are the results of alterations in the inner mitochondrial membrane caused by stimuli such as radiation, hypoxia, viral infections, free radicals and loss of growth factors, hormones or cytokines that can lead to failure of cell death suppression thereby triggering the apoptosis (Garrido et al., 2006). Those proteins initiate the caspase dependent mitochondrial pathway. As a result of cytchrome c binding and activation Apaf-1 as well as procaspase-9, an "apoptosome" is formed (Chinnaiyan, 1999; Hill et al., 2004).

Cytotoxic T lymphocytes (CTLs) are able to kill target via extrinsic pathway, but they are also able to exert their cytotoxic effects on tumor cells or virus-infected cells via a novel pathway that involves secretion of the transmembrane pore-forming molecule perforin with a subsequent exophytic release of cytoplasmic granules through the pore and into target cell (Elmore, 2007). Granzyme A is one of these granules, and activates DNA nicking via DNAse NM23-H1(Elmore, 2007). Normally, the nucleosome assembly protein SET inhibits the NM23-H1 gene, but granzyme A protease cleaves the SET complex thus releasing inhibition of NM23-H1, resulting in apoptotic DNA degradation (Elmore, 2007). The other granule is granzyme B, which cleaves proteins at aspartates and activates procaspase-10 to cleave factors like ICAD (Inhibitor of caspase activated DNAse) (Elmore, 2007). Gryanzyme B can also directly activate caspase-3 by bypassing the upstream signaling pathway (Elmore, 2007).

Apoptotic mitochondrial events are controlled and maintained by the members of Bcl-2 family proteins, and tumor suppressor protein p53 has an important role in regulation of the Bcl-2 family members (Cory and Adams, 2002).

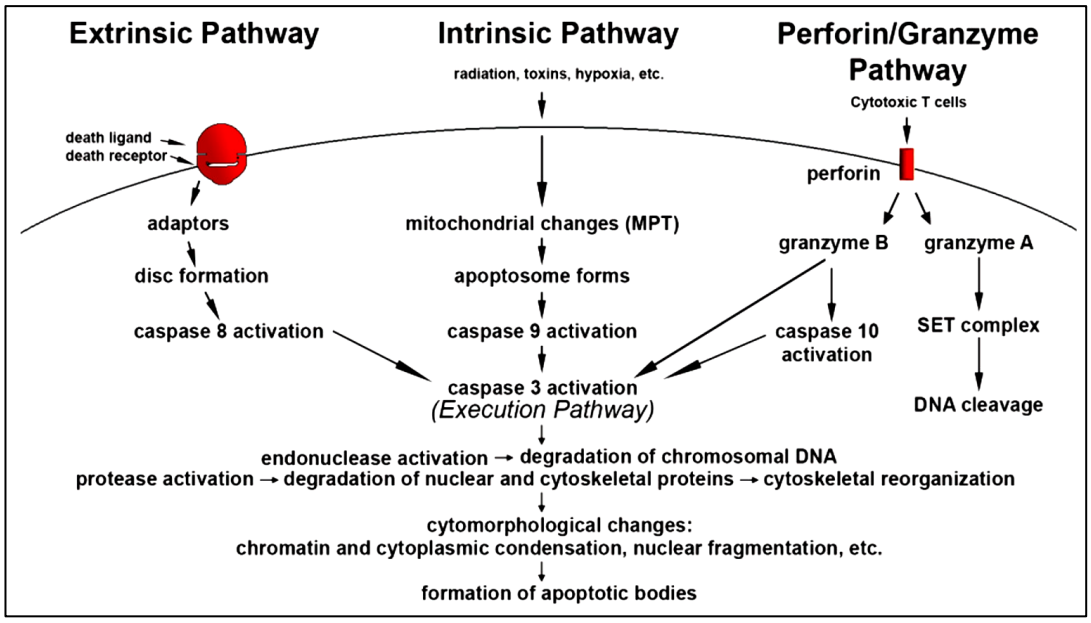

Figure 1.3 : Schematic demonstration of apoptotic pathways; in addition to two main pathways, extrinsic and intrinsic, perforin/granzyme pathway is shown (Elmore, 2007).

1.2.2 Bag-1 and down regulation of apoptosis

Some cells are resistant to stress, and they need higher levels of apoptotic stimuli for cell death (Hersey and Zhang, 2003). A wide variety of inducers including physiologycal alterations such as activation of mitogenic signaling pathways (i.e. Erk1/2, Akt), inactivation of certain apoptotic molecules (i.e. Fas receptor, Bax) and upregulation of anti-apoptotic genes such as Bcl-2 can influence the process of apoptosis (Hersey and Zhang, 2003; Duriano, 2008).

Apoptosis can be blocked in some conditions such as cancer, stress, hipometabolism, and aging (Hanahan and Winberg, 2000). Maintenance of homeostasis requires a balance between growth and cell death. Any type of parameter that affects the balance between growth and cell death can cause uncontrolled cell division and subsequently carcinogenesis (Hanahan and Winberg, 2000).

Bcl-2 protein family members are the most important molecules that regulate apoptosis (Youle and Strasser, 2008). Bcl-2 (B-cell lymphoma-2) gene was discovered at t(14;18) chromosome translocation breakpoint in B-cell follicular lymphomas (Tsujimoto et al. 1985). Bcl-2 family proteins take place in numerous biological processes such as prevention and induction of apoptosis (Zha et al., 1996). Bcl-2 family members share one or more of the four characteristic domains of

homology entitled the Bcl-2 homology (BH) domain (named BH1, BH2, BH3 and BH4) motives (Youle and Strasser, 2008) (Figure 1.4).

Bcl-2 family members have been grouped into three classes (Youle and Strasser, 2008). One class inhibits apoptosis (Bcl-2, Bcl-XL, Bcl-W, Mcl-1, Bcl-2L10 and Bcl-2A1), whereas a second class promotes apoptosis (Bax, Bak and Bok) (Youle and Strasser, 2008). The third class of BH3-only proteins (Bad, Bik, Bid, Hrk, Bim, Bmf, Noxa and Puma) have a conserved BH3 domain that can bind and regulate the anti-apoptotic Bcl-2 proteins to promote apoptosis (Youle and Strasser, 2008). It can be seen in Figure 1.4 that Bcl-2 family members have some functional domains such as α-helical segments (green bars), transmembrane (TM) domains (red lines). Sequence homologies of the BH1 (brown lines), BH2 (grey lines), BH3 (blue lines) and BH4 (orange lines) regions are also demonstrated. The three proteins in the shaded area are less well studied and cannot be categorized at this time (Youle and Strasser, 2008).

Bag-1 is a novel member of Bag family, which has been discovered via its interaction with Bcl-2 (Takayama et al., 1995). In the study of Takayama et al.,1995, it has been reported that co-transfection of Bcl-2 and Bag-1 expression plasmids rendered Jurkat T cells relatively more resistant to induction of cell death by staurosporine, anti-Fas antibody, and cytolytic T cells, whereas either Bcl-2 or Bag-1 alone was comperatively ineffective at providing protection from cell death (Takayama et al., 1995).

Bcl-2 is not the only interacting partner of Bag-1 during apoptosis. Bag-1 also has interactions with other anti- and pro-apoptotic molecules in the cell (Wood et al., 2009). Bax, Bcl-2, Bcl-XL and p53 have interactions with the Bag-1 isoforms, and these are crucially significant for tumorigenesis (Wood et al., 2009). Activation of the pro-apoptotic molecule Bax with an apoptotic inducer, involves subcellular translocation and dimerization (Gross et al., 1999). A substantial portion of Bax is monomeric and found in the cytosol or loosely attached to membranes in viable cells (Gross et al., 1999). However, following a death stimulus, cytosolic and monomeric Bax translocates to the mitochondria where it becomes an integral membrane protein and cross-linkable as a homodimer (Gross et al., 1999). In addition to Bax, Bak can also present conformational changes after induction of a death stimulus (Gross et al., 1999), but the presence of an anti-apoptotic molecule such as Bcl-2 or Bcl-XL can

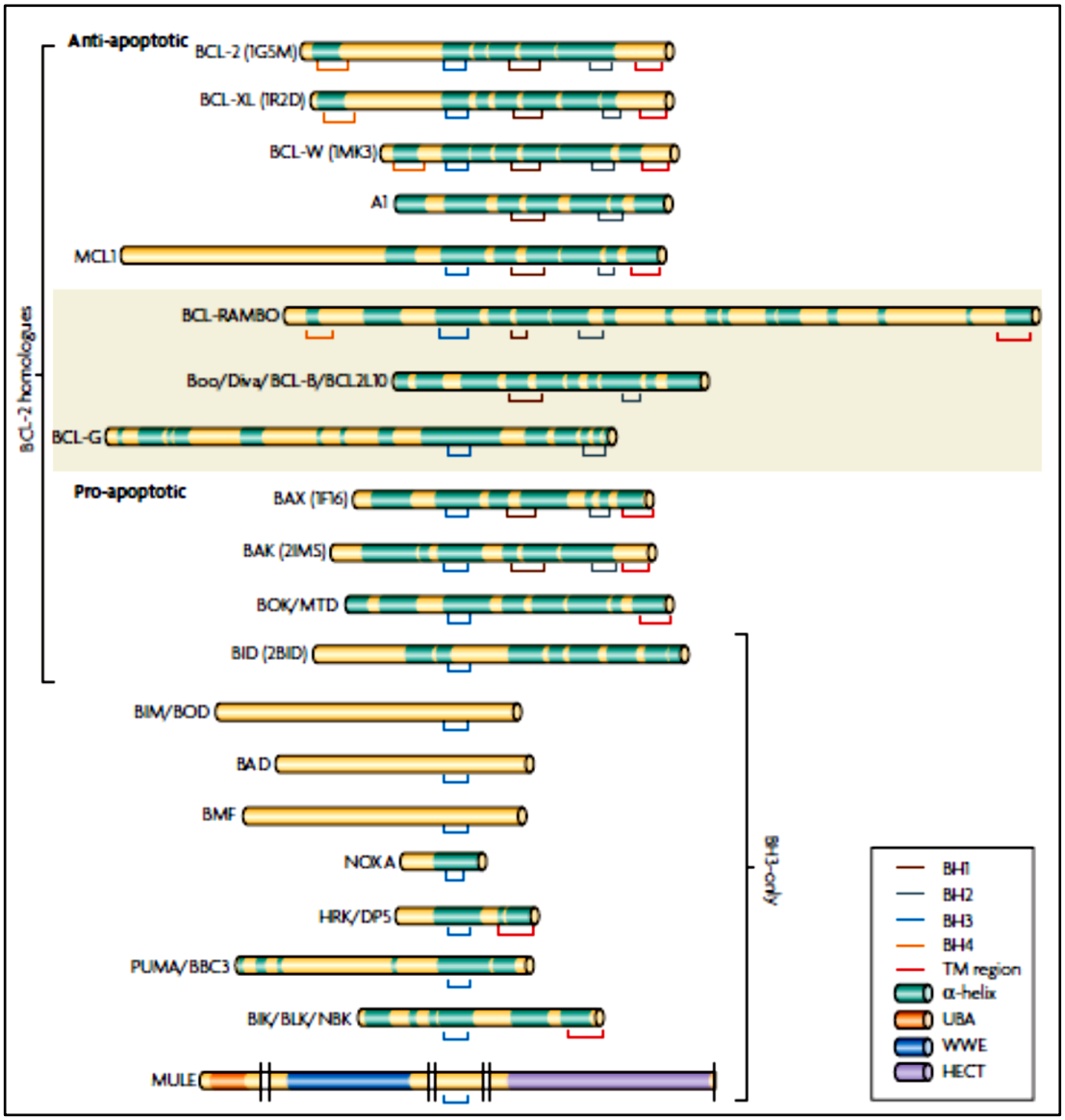

Figure 1.4 : Sequence alignment of Bcl-2 family proteins and BH3-only proteins (Youle and Strasser, 2008).

inhibit the activation of Bax folloving a death signal (Gross et al., 1998). Bcl-2 is an integral membrane protein heavily localized to mitochondria (Gross et al., 1998). It can be thought that Bcl-2 alone is not sufficent to inactivate Bax for the suppression of apoptosis. Therefore, Bag-1 is the found partner of Bcl-2 in the enhancement of its anti-apoptotic function (Takayama et al., 1995). It is explained that the reason for this might be the increased anti-apoptotic protein amounts on the mitochondria with the involvement of Bag-1. In a study, Bag-1 silenced HeLa-AS cells showed increased sensitivity to apoptosis incuced by chemotherapeutic drugs including staurosporine, paclitaxel, ATRA, and 4-HRP (Xiong et al., 2003). Underexpression of Bag-1 leads to decreased Bcl-2 levels and increased release amounts of

cytochrome c, leading to a increased sensitivity to apoptosis (Xiong et al., 2003). In Bag-1 silenced cells, Bax aggregation has been observed, which explains the Bax/Bcl-2 ratio change in mithochondria (an important parameter for apoptosis) (Xiong et al., 2003).

More importantly, a pro-apoptotic member of Bcl-2 family, Bad, inhibits Bcl-XL activity and thereby promotes Bak and Bax aggregation (Datta et al., 1999). This situation subsequently leads to 'cytochrome c' release to the cytosol, caspase activation and finally to apoptosis (Datta et al., 1999). Bad phosphorylation is important for the determination of cell fate. Phosphorylation of Bad from serines at the 112, 136 and 155 sites, rescue cells from apoptosis and modulates cell survival through interactions with 14-3-3 proteins (Peruzzi et al.,1999; Peso et al., 1997).

It has been ascertained that 14-3-3 has been found in the epigenetic mechanisms of cancer cells (Jelinek et al., 1994). As it is a histone kinase, it can bind to chromatin-modifying enzymes and transcriptional regulators such as histone deacetylases (HDACs), histone acetyltranferases (HATs), TATA-binding protein (TBP), and p53 (Jelinek et al., 1994). When it gets recruited with p53, they can regulate Bag-1 expression together. Once Raf-1 interacts with MEK, one of the MAP kinase modulators, it is targeted to nucleus to perform its epigenetic function (Jelinek et al., 1994). For this purpose, 14-3-3 may facilitate Raf-1 interaction with MEK in cytosol (Jelinek et al., 1994).

Phosphorylation of Akt is another important parameter for cell survival (Götz et al., 2005). Inhibition of apoptosis by Bad phosphorylation from serine 136 residue is maintained by activated Akt protein (Götz et al., 2005). In absence of Bag-1, Raf-1 activation and Akt phosphorylation are not sufficient to prevent apoptosis (Götz et al., 2005).

In a study with neuronal cells, Bag-1-related cell survival mechanism was investigated (Frabel et al., 2007). They create the model shown in Figure 1.5 to explain interactions during cell survival (Frabel et al., 2007). They claimed that Bag-1 forms a comlex with phosphotylated Akt, Hsp70, B-Raf and Bcl-2, and this complex phosphorylates Bad at Ser136. (Frabel et al., 2007). Phosphorylated Bad interacts with 14-3-3 scaffold protein instead of providing oligodimerization of pro

apoptotic proteins on mithochondrial membrane, and it leads cells to survival (Frabel et al., 2007).

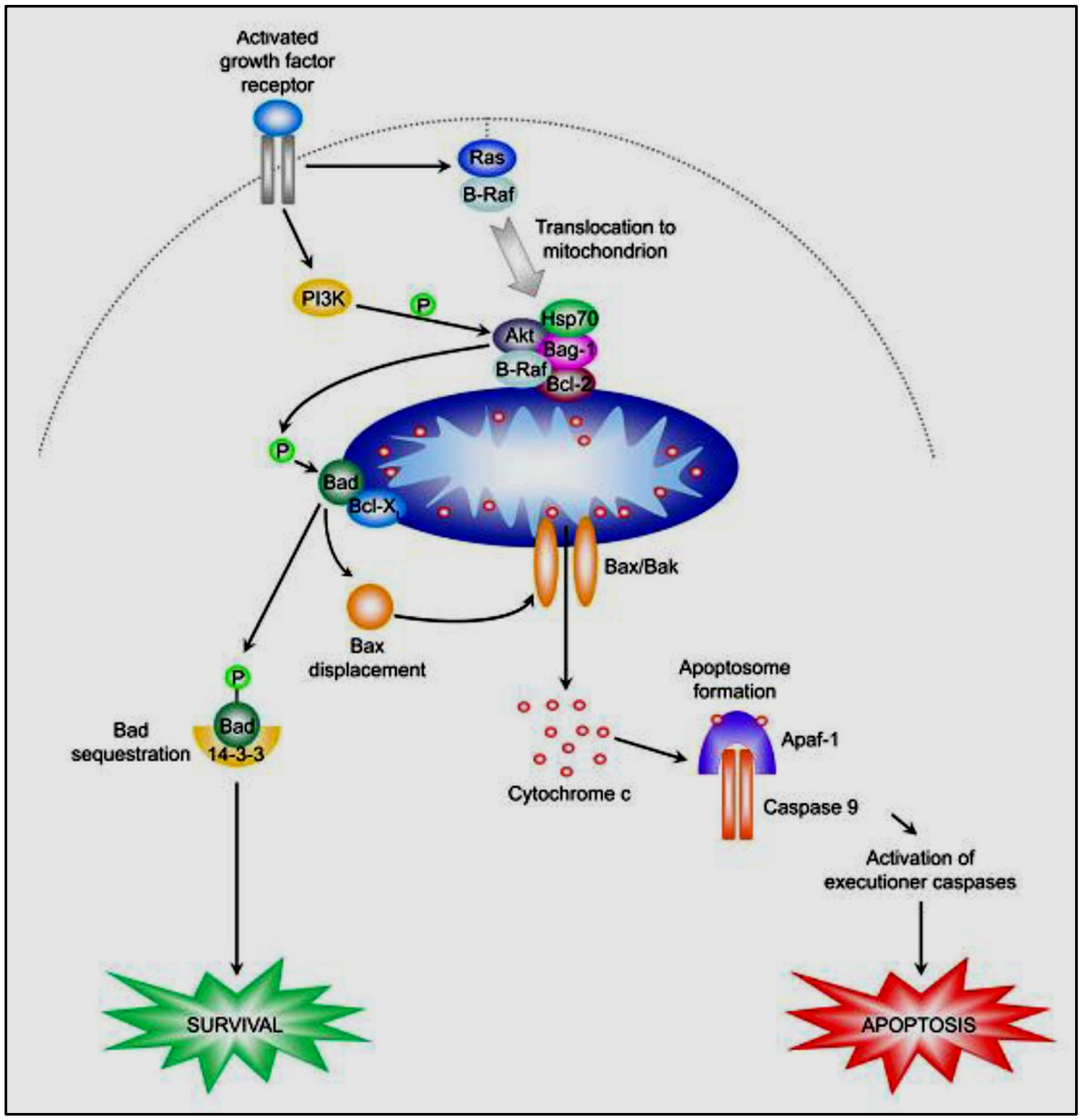

Figure 1.5 : A possible model of Raf-1 and Akt activation and regulation mechanism by Bag-1 (Frabel et al., 2007).

1.3. Bag-1's relationship with cancer and cell survival

Breast cancer is the most commonly occuring cancer in women, comprising 23% of all female cancers in the world, with an estimated 1.15 million cases diagnosed in 2002 (Ozmen, 2008). The lifetime risk of a woman developing invasive breast cancer is 12.6%, one out of 8 women in the United States will develop breast cancer at some point in her life (Richie et al., 2003). Distribution of breast cancer incidence in 2000s alters in different regions of Turkey due to the geographic, economic, social and cultural factors (Ozmen, 2008). Breast cancer incidence in western part of Turkey

(50/100.000) is more than two times in eastern part of Turkey (20/100.000) because of "Westernizing life" (early menarche, late menopause, first birth over 30 years, less breast feeding, etc.), and other related factors (Ozmen, 2008). It is known that Bag-1 is highly expressed in breast cancer cells (Sharp et al., 2004). Delineating the role of Bag-1 in breast cancer may provide new targeting strategies for both treatment and diagnosis of breast cancer. For this reason we chose MCF-7 human breast adenocarcinoma cell line which had been obtained from mammary gland of mammarial ephitelium. MCF-7 cells are estrogen receptor dependent cells isolated from a 69-year-old Caucasian female patient in 1970, and was established in 1973 (ATTC datasheet).

Bag-1 has many critical roles in the cell through its interactions with certain target molecules to regulate cancer, differentiation, motility and transcription (Cutress et al., 2002). Cell survival and apoptosis are the two major pathways that Bag-1 involves (Cutress et al., 2002). It is known that Bag-1 is overexpressed in cancer cells in comparison with the normal cells, thus Bag-1 can be used a prognostic marker for cancer (Cutress et al., 2002). As it is shown in Figure 1.6, Bag-1 interacts with a wide range of proteins to regulate different molecular mechanism in the cell (Wang et al., 1998). Bag-1 interactions with target proteins, and how they affect those mechanisms will be explained in following paragraphs.

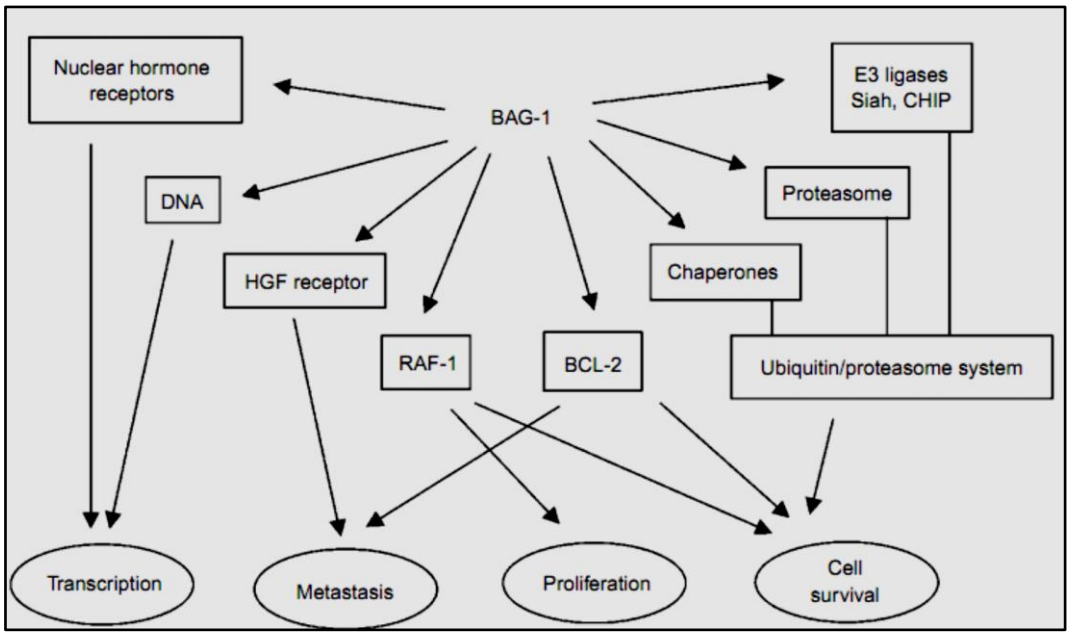

Figure 1.6 : Bag-1 interacting partners and their functions in the molecular pathways (adapted from Wang et al., 1998).

It has been previously reported that upregulation of Bag-1 protein expression is done by tumour-derived mutant p53. p53 is one of the molecules in cancer development, and the overexpression of Bag-1 in human cancer cells may arise from the transactivation of Bag-1 promoter by p53 mutants (Yang et al., 2008). Hence, Bag-1 overexpression in cancer cells may inhibit the apoptotic pathways using mutant p53 feedback loop (Yang et al., 2008). Also it has been demonstrated that Bag-1 inhibits p53-induced apoptosis, which has an impact on resistance for radiotherapy or chemotherapy (Oorschot et al., 1997).

Another important molecule in the cell is NF-κB which has been recently found to be regulated by Bag-1(Clemo et al., 2008). It is indicated that NF-κB inhibition is caused by Bag-1 gene knockdown and due to this relationship, Bag-1 can be used as an adjuvant for increasing the sensitivity of current therapeutic regimes (Clemo et al., 2008). siRNA-mediumted Bag-1gene silencing has also induced apoptosis in colorectal carcinoma cells via TRAIL and TNF-α that are found in extrinsic apoptotic pathway (Clemo et al., 2008). It has been showed that Bag-1 is significant for the regulation of NF-κB and IκBα, both involve activation of glucocorticoid receptors (Chen et al., 1995). In resting cells, most of NF-κB is bound to IκBα, and forms a very stable complex to protect IκBα degradation and inhibits NF-κB; both, nuclear localisation and transcriptional activation (Chen et al., 1995). Along with the stimulation, IκBα is phosphorylated by the IκB kinase (IKK) complex, and it targets IκBα for polyubiquitination and degradation in the 26S proteosome and causing the release of the NF-κB heterodimers (Chen et al., 1995). Further studies revealed that in the absence of Bag-1 phosphorylation and degradation of IκBα in response to pharbol complexes is elevated, and nuclear accumulation of NF-κB p65-p50 is significantly decreased (Maier et al., 2010). Furthermore, a recent study describes a novel role of Bag-1, sugesting Bag-1 as a co-regulator of gene expression via interaction with the p50-p50 NF-κB complexes (Southern et al., 2011). This study demonstrated that Bag-1 expression is elevated in the developing colorectal adenoma throught to metastatic lesions, which can be a sign for Bag-1's function as a selective regulator of p50-p50 NF-κB responsive genes in colorectal tumour cells (Southern et al., 2011).

It has been reported that Rb, mutifunctional protein with important roles in cell cycle regulation and apoptosis, interacts with Bag-1 (Arhel et al., 2003). Blocking of

Rb/Bag-1 interaction causes a change in the subcellular localisation of Bag-1 (Arhel et al., 2003). Since Rb/Bag-1 interaction has been detected in both adenoma- and carcinoma- derived cell lines, Rb-maintained nuclear localisation of Bag-1 is important for colorectal tumour progression (Arhel et al., 2003). Also it has been identified that following γ-radiation there is an alteration in subcellular localisation of Bag-1 (Barnes et al., 2005). They suggested that localisation change of Bag-1M (translocation) makes cells more sensitive to apoptosis (Barnes et al., 2005).

In a study about mouse liver progenitor cells, it has been shown that the effect of HGF (hepatocyte growth factor) on cell survival was increased by the overexpression of exogenous Bag-1 (Bardelli et al., 1996). Although it is known that C-terminal domain of Bag-1 binds to HGF receptor efficiently, overexpression of this region alone has no effect on the anti-apoptotic activity of HGF (Bardelli et al., 1996). These results indicate a possible interplay between Bag-1 and different growth factor receptors in the prevention of apoptosis and stimulation of cell survival (Bardelli et al., 1996).

Nuclear hormone receptors (NHRs) are another key targets for Bag-1 isoforms (Sharp et al., 2004). NHRs are ligand dependent transcription factors of which target genes take part in a wide range of cellular processes such as controlling cell growth, motility, cell division, apoptosis, differentiation and morphology (Cato and Mink, 2001). Bag-1 interacts with several NHRs inluding glucocorticoid receptors (GRs), estrogen receptors (ERs), androgen receptors (ARs), retinoic acid receptor (RAR), thyroid receptor, vitamin D3 receptor (VDR), progesterone receptor (PR) and mineralocorticoid receptor (MR). Bag-1 isoforms affect these receptors diversely. Interactions of Bag-1 isoforms with indicated receptors can be seen in Table 1.1.

Table 1.1 : Effects of Bag-1 isoforms on nuclear hormone receptors (adapted from Sharp et al., 2004).

Receptor	Effect of Bag-1 overexpression	Active Bag-1 isoforms	References
Vitamin D receptor	Activates/Inhibits	Bag-1L (not Bag-1S/M)	Guzey et al., 2000 Witcher et al., 2001
Androgen receptor	Activates	Bag-1L (not Bag-1S/M)	Froesch et al., 1998 Knee et al.,2001

Table 1.1 7 (continued) : Effects of Bag-1 isoforms on nuclear hormone receptors (adapted from Sharp et al., 2004).

Glucocorticoid receptor	Inhibits	Bag-1M/L	Schneikert et al., 2001
Retinoic acid receptor	Inhibits	Bag-1S	Liu et al., 1998
Thyroid hormone receptor	Inhibits	Bag-1S	Liu et al., 1998
Estrogen receptor	Activates	Bag-1L (not Bag-1S/M)	Cutress et al., 2003
Progesterone receptor	Inhibits	Bag-1M	Knapp et al., 2012
Mineralocorticoid receptor	Inhibits	Bag-1M	Knap et al., 2012

It has been reported that Hsp70 has a role as a direct negative regulator of Bag-1, and consequently as an indirect regulator of downstream effectors such as important serine/threonine kinase Raf-1 (C-Raf) (Song et al., 2001). They also have shown that function of Bag1 is to coordinate the cellular signals that determine the growth state of the cell with its environmental and physiological state; and they predicted that there may be a number of other points of regulatory crosstalk to ensure the various components of the cell growth biosynthetic apparatus are in communication with heat-shock proteins or other, unidentified, stress signaling molecules (Song et al., 2001). Bag-1 interacts with Raf-1 kinase from its C-terminus. Ras activates Raf-1 in order to tranduce a stimulation to mitogen-activated protein (MAP) kinase cascade. Also, it has been reported that Bag-1 uses a Ras-independent pathway for the Raf-1-dependent activation of the downstream MAP kinases (Song et al., 2001). They showed that Bag-1 mutants (deficient in binding to Hsp70) constitutively stimulated Raf-1/ERK activities, such that DNA synthesis was not repressed by heat shock (Song et al., 2001). They also showed that Hsp70 mutants (defective in binding to Bag-1) had no effect on Raf-1 activity and DNA synthesis when overexpressed (Song et al., 2001). These results suggest that down regulatory effects of Hsp70 on MAP kinase pathway and DNA synthesis require Bag-1interaction (Song et al., 2001). Also, Bag-1 interacts with E3 ligases CHIP and Siah-1 to stimulate CHIP-mediumted ubiquitylation of Raf-1 (Demand et al., 2001; Arndt et al., 2005).

Apart from its relationship with cancer, Bag-1 is an important molecule that gets involved in the regulation of cell survival and proliferation (Wang et al., 1996). In a

recent study it is indicated that the overexpression of Bag-1 in immortalized cell line Mcf-10A (also defined as normal breast epithelial cells) led to an attenuation of luminal apoptosis which is charecterized by an increased number of acini with filled lumens and enhanced caspase-3 activation (Anderson et al., 2010). In Bag-1-expressing acini, there is an enhanced activation of Raf-1 (C-Raf) protein which is known to influence both mitogenic and anti-apoptotic pathways (Anderson et al., 2010). Furthermore subsequent activation of a downstream signaling cascade through the phosphorylation of MEK and ERK1/2 might be the evidence of direct interaction of Bag-1 and Raf-1, though it is opposite to Bag-1's role in protecting against heat shock-induced apoptosis, in which its survival effects are dependent on Hsp70/Hsc70 binding and not on interaction with Raf-1 (Anderson et al., 2010) (Figure 1.7).

In another study on Bag-1 silenced mice with acute myeloid leukemia (AML) revealed that although Bag-1 does not have a direct anti-apoptotic role in AML, it is found to be important to control apoptosis in AML via its interactions (Aveic et al., 2011). Silencing Bag-1 affected the MAPK-pathway, specificially through down-regulation of ERK1/2 activity, and proliferative pathways (Aveic et al., 2011). However, it has been reported that in HeCaT epidermal keratinocytes, Bag-1 inhibits both proliferation and cell motility (Hinitt et al., 2011).

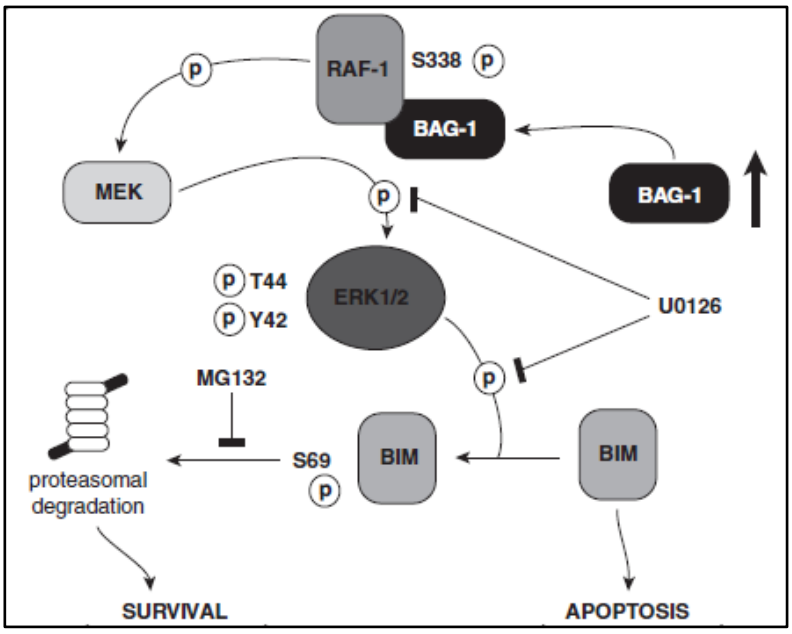

Figure 1.7 : A model of interaction between Raf-1 and Bag-1, and Raf-1 activation through phosporylation from serine 388 by Bag-1, resulting cell survival (Anderson et al., 2010).

1.4. Aim of the study

Bcl-2 associated athanogene family (athano = against death in Greek) proteins are discovered by co-immunoprecipitation with Bcl-2 anti-apoptotic protein. Bag genes are evolutionally conserved and their homologs exist in yeasts, plants and animals. Bag family members control the cell homeostasis in two different ways: (1) They contribute to maintain cell's vital activity under normal and stress conditions via regulating both positively and negatively the cycle of Hsp70/Hsc70 molecular chaperone system. For instance, the direction of the cell balance under normal and stress conditions are maintained by competitive binding of Hsp70 or Raf-1 to Bag-1. (2) They form complexes with transcription factors and nuclear hormone receptors which are controlling various physiological processes such as apoptosis, tumorigenesis, neural differentiation and cell cycle independent from Hsp70/Hsc70 system.

Even though Bag-1 involves a number of molecular pathways in the cell, none of them had been completely understood. Every cell type shows variations in the response of Bag-1 manipulations. In this study, we would like to understand the molecular mechanism of Bag-1-related cell survival in particularly estrogen receptor dependent breast cancer cell lines.

It is known that Bag-1 inhibits apoptosis and promotes cell survival, but molecular players or crosstalk between Bag-1 involved pathways are not clear yet. Once interacting partners of Bag-1 are elucitated in MCF-7 cells, Bag-1-related cell survival pathway will be understood. This will, in the long term, allow routes for the design of small molecules to manipulate the interactions of Bag-1, and, therefore offer solutions for estrogen receptor dependent cell cancer therapy.

2. MATERIALS and METHODS

2.1 Materials

2.1.1. Equipment

Equipment used in this study is shown in the table 2.1.

Table 2.1 : Laboratory equipment used in the study.

Equipment	Supplier Company
Laminar Air Flow Cabinets	FASTER BH-EN 2003
Pipettes	2.5 µL, 10 µL, 100 µL, 200 µL, 1000 µL, Gilson
Electronic Pipette	Finnpipette Thermo
Centrifuges	Biolab SIGMA 6K15, Beckman Coultier Microfuge®18, Beckman Coulter AvantiTM J-30 I, IECCL10 Centrifuge, Thermo Electron Corporation, Labnet, Labnet International C1301-230V
Quick Spin	Labnet International, C1301-230V
Magnetic stirrer	Dragon Lab, MS-H-S
pH Meter	Mettler Toledo, Five Easy
Light Microscope	Olympus CH30 (USA)
Hemacytometer	FisherLab Scientific, 0267110
High Pressure Steam Sterilizer	TOMY SX-700E
Precision Balance	Precisa 620C SCS
Balance	Precisa BJ 610 C
Ice Machine	Scotsman AF 10
SDS-PAGE Gel Electrophoresis System	BIO-RAD MiniProtean
Microwave	Arcelik MD582
Spectrophotometers	Shimadzu, UV-1601 Thermo Scientific NanodropTM 2000c BIO-RAD Benchmark Plus
Water Baths	Memmert, Elektro-mag M 96 KP
Vortex	Dragon Lab MX-F
Incubator with CO_2	Biolab SHEL LAB
Shaker	Forma Orbital Shaker, Thermo Electron Corporation
Freezers	Bosch (+4^{o}C), Bosch (-20^{o}C), New Brunswick Scientific (-80^{o}C)

Table 2.1 (continued) : Laboratory equipment used in the study.

TCS SP2 SE Confocal Microscope	Leica, Microsystems
Kodak Medical X-ray Processor	Kodak
Trans-Blot® Turbo™ Transfer System	BIO-RAD
DynaMag-2 Magnet	Invitrogen
Trans-Blot® SD Semi-Dry Transfer Cell	BIO-RAD
Power Supply	EC250-90 Apparatus Corporation BIO-RAD
Inverted Fluorescent Microscope	Olympus DC71

2.1.2 Commercial kits

Commercial kits used in this study are shown in the table below.

Table 2.2 : Commercial kits used in the study.

Kit	Supplier Company
QIAprep Spin Miniprep Plasmid Purification Kit	Qiagen, 27106
QIAGEN Plasmid Midi Kit	Qiagen, 12145
QIAGEN EndoFree Plasmid Maxi Kit	Qiagen, 12362
20X LumiGLO® Reagent and 20X Peroxide	Cell Signaling, 7003
SuperSignal® West Femto Maximum Sensitivity Substrate	Thermo, 34094
Restore Western Blot Stripping Buffer	Thermo, 21059
Lipofectamine™ 2000 Transfection Reagent	Invitrogen, 11668-019
Bag-1 siRNA (h)	Santa Cruz, sc-29211
Trans-Blot® Turbo™ Midi Nitrocellulose Transfer Packs	BIO-RAD, 170-4159
ProteoJET™ Mammalian Cell Lysis Reagent	Fermantas, K0301
XTT Cell Viability Kit	Cell Signaling, 9095

Table 2.2 (continued) : Commercial kits used in the study.

cOmplete Lysis-M Kit EDTA-free	Roche, 04719964001
TransPass™ D2 Transfection Reagent	BioLabs, M2554S
PhosSTOP	Roche, 04906845001
Quick Start Bradford Protein Assay Kit	BIO-RAD, 500-0203
X-tremeGENE HP DNA Transfection Reagent	Roche, 06366236001
Erase-It® Background Eliminator	Thermo, 21065
Dynabeads® Magnetic Beads	Invitrogen, 100-03D

2.1.3. Bacterial Assay

- Tryptone (BDH Laboratory)
- Yeast Extract (Merck)
- NaCl (Fluka)
- Agar (Merck)
- Ampicillin (Sigma-Aldrich)
- Neomycin (Sigma-Aldrich)
- Glycerol (Fluka)

2.1.4. Cell culture assay

- 25 cm^2, 75 cm^2 Tissue Culture Flask (TPP)
- 6 well, 24, 96 well Culture Plate (TPP)
- 5 mL, 10 mL, 25 mL Serological Pipettes (TPP)
- 250, 500 mL Vacuum Filtration System (TPP)
- Poly-L-Lysine (Sigma-Aldrich)
- 0,22 μm Syringe Filtre (TPP)

- 10 mL, 20 mL Syringe (Set Inject)
- DAPI (Invitrogen)
- Acridine orange (Applichem)
- PI (Applichem)
- Dioc6 (Fluka)
- Trypan blue (Sigma-Aldrich)
- Dulbecco's Modified Eagle Medium (DMEM) 1X (Gibco)
- Opti-MEM Reduced Serum Medium 1X (Gibco)
- Fetal Bovine Serum (FBS) (Gibco)
- RNAse-free water (Santa Cruz)
- Penicilin/Streptomycin Solution 100X (Biochrom)
- Trypsin-EDTA 0,25/0,02 Solution (Biochrom)
- Phosphate Buffered Saline (PBS) 10X pH=7,2 (Gibco)
- Dimethylsulphoxide (DMSO) (Fiedel-de Haën)
- Cell Scrapper (TPP)

2.1.5 Protein assay

- SDS (AppliChem)
- TEMED (AppliChem)
- Dithiothreitol (DTT) (Merck)
- β-Mercaptoethanol (Merck)
- Acrylamide (Sigma Aldrich)
- Bis-acrylamide (Sigma Aldrich)
- PMSF (AppliChem)
- Bromophenol Blue (Merck)
- Ammonium persulphate (APS) (Sigma-Aldrich)

- Biotinylated Protein Ladder (Cell Signaling)
- Prestained Protein Ladder (Fermantas)
- Nitrocellulose Paper (BIO-RAD)
- 3MM Whatman Filter Paper (Whatman)
- Tween-20 (AppliChem)
- Skimmed milk powder (%5) (OXOID)
- Bovine Serum Albumin (BSA) (Sigma-Aldrich)
- Alexa Flour 488 goat anti-rabbit (Invitrogen)
- Alexa Flour 647 goat anti-mouse (Invitrogen)
- DAPI (Sigma)
- Mounting Medium (Sigma)

2.1.6 Antibodies

All antibodies (both primary and secondary) were purchased from Cell Signaling Technologies of which properities are shown in the table below.

Table 2.3 : Primary antibodies used in this study.

Antibody	Type, Source	Dilution
α-Bag-1	Monoclonal, Mouse	1:500
α-β-actin	Monoclonal, Rabbit	1:500
α-Bcl-2	Monoclonal, Rabbit	1:250
α-Bax	Polyclonal, Rabbit	1:250
α-Bad	Polyclonal, Rabbit	1:250
α-Bim	Polyclonal, Rabbit	1:250
α-Phospho-Bad (Ser136)	Polyclonal, Rabbit	1:250
α-C-Raf	Polyclonal, Rabbit	1:500
α-Phospho-C-Raf (Ser338)	Monoclonal, Rabbit	1:500

Table 2.3 (continued) : Primary antibodies used in this study.

α-Phospho-C-Raf (Ser289/296/301)	Polyclonal, Rabbit	1:500
α-B-Raf	Monoclonal, Rabbit	1:500
α-Phospho-B-Raf (Ser445)	Polyclonal, Rabbit	1:500
α-A-Raf	Polyclonal, Rabbit	1:500
α-p44/42 MAPK (Erk1/2)	Monoclonal, Rabbit	1:1000
α-MEK1/2	Polyclonal, Rabbit	1:1000
α-Phospho-MEK1/2 (Ser217/221)	Polyclonal, Rabbit	1:1000
α-Phospho-Akt (Ser473)	Monoclonal, Rabbit	1:500
α-Hsp70	Polyclonal, Rabbit	1:500

Table 2.4 : Secondary antibodies used in this study.

Antibody	Epitope	Type	Dilution
Goat-α-Mouse	Mouse-IgG	Polyclonal goat; HRPconjugated	1:5000
Goat-α-Rabbit	Rabbit-IgG	Polyclonal goat; HRPconjugated	1:5000

2.1.7 General Chemicals

General chemicals which were used in this study are listed in the table below.

Table 2.5 : General chemicals used in this study.

Substance	Supplier Company
Ethanol	Riedel- de Haën
Methanol	Riedel- de Haën

Table 2.5 (continued) : General chemicals used in this study.

Isopropanol	Fluka
HCl	AppliChem
NaOH	AppliChem
Glicial acedic acid	Fluka
Glycerol	Fluka
Glycerol	Merck
Tricine	Merck
Glucose	Merck

2.1.8 Buffers and solutions

2.1.8.1 Separating acrylamide solution

Separating acrylamide solution was used for preparation separating gel. The amount and content of solution is given in Table 2.6.

Table 2.6 : Content of separating acrylamide.

Content	Amount
Acrylamide	46,5 g
Bis-acrylamide	1,5 g
dH_2O	up to 100 mL

2.1.8.2 Stacking acrylamide solution

Stacking acrylamide solution was used for the preparation of stacking gel. The amount and content of solution is given in Table 2.7.

Table 2.7 : Content of stacking acrylamide.

Content	Amount
Acrylamide	48 g
Bis-acrylamide	1,5 g

Table 2.7 (continued) : Content of stacking acrylamide.

dH_2O	up to 100 mL

2.1.8.3 6X sample buffer

6X sample buffer was used to denature protein samples which were loaded on SDS-polyacrylamide gel. Content of 6X sample buffer is shown in Table 2.8.

Table 2.8 : Content of 6X sample buffer.

Content	Amount
SDS	1,2 g
Bromophenol blue	6 mg
Glycerol	4,7 mL
0,5M Tris-HCl (pH: 6,8)	1,2 mL
dH_2O	2,1 mL

It was warmed and shaked until it dissolved, and 930 mg DTT or 5% β-Mercaptoethanol was added.

2.1.8.4 Running buffer for SDS-PAGE

Anode and the cathode buffers, both were used as SDS-PAGE running buffer, and their contents are given in Table 2.9 and Table 2.10, respectively.

Table 2.9 : Content of 1X anode buffer.

Content	Concentration	Amount
Tris-Base	0,2M	24,22 g
dH_2O		Up to 1 L (pH=8,9)

Table 2.10 : Content of 1X cathode buffer.

Content	Concentration	Amount
Tris-Base	0,1 M	182 g
Tricine	0,1 M	17,92 g

Table 2.10 (continued) : Content of 1X cathode buffer.

SDS	1 gr (1%)
dH_2O	Up to 1 L (pH=8,25)

2.1.8.5 10X TBS buffer

TBS (with HCl) was prepared and used as blocking and washing buffer during Western blot analysis. 10X stock solution was diluted to 1X concentration with dH2O to obtain working solution. Content of TBS buffer is shown in Table 2.11.

Table 2.11 : Content of 10X TBS buffer.

Content	Concentration	Amount
Tris-base	25 mM	3 g
Glycine	192 mM	14,4 g
Methanol	20 %	200 mL
SDS	0,05 %	0,05 g
dH2O		up to 1 L

2.1.8.6 TBS-T buffer

TBS-T solution was prepared for washing in Western blot analysis. Preparation of this buffer is shown in Table 2.12.

Table 2.12 : Content of TBS-T buffer.

Content	Concentration	Amount
TBS	1X	1 L
Tween-20	0,05 %	500 µL

2.1.8.7 Blocking and incubation buffer

Blocking and incubation buffer was used for blocking of membrane and incubation with primary antibody. Preparation of this buffer is shown in Table 2.13.

Table 2.13 : Content of blocking and incubation buffer.

Content	Concentration	Amount
TBS-T	1X	100 mL
Skimmed milk or BSA		5 g

2.1.8.8 PBS solution

PBS solution was prepared by dissolving 1 PBS tablet within 100 mL dH_2O.

2.1.8.9 PBS-T solution

To prepare PBS-T solution, 2 mL Tween-20 was added into 1 L PBS solution.

2.1.8.10 Elution buffer for immunoprecipitation

50 mM Glycine (pH 2,8) was prepared and used as elution buffer

2.1.9 Bacterial strain

Escherichia coli DH5α strain [F-, φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deo*R, *rec*A1, *end*A1, *hsd*R17(rk-, mk+), *pho*A, *sup*E44, λ-, *thi*-1, *gyr*A96, *rel*A1]

2.1.10 Bacterial culture medium and solution

2.1.10.1 LB medium

Luria Bertani (LB) Medium was prepared by dissolving 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 1L dH_2O. The medium was sterilized by autoclaving for 15 minutes (min) at 121°C. To prepare a selective medium, 50 µg/L ampicillin or neomycin was added after the medium was cooled down approximately to 55°C.

2.1.10.2 LB-agar medium

LB-Agar Medium was prepared by dissolving 10 g tryptone, 5 g yeast extract, 10 g NaCl and 20 g agar in 1L dH_2O. The medium was sterilized by autoclaving for 15 min at 121°C. To prepare a selective medium, 50 µg/L ampicillin was added after the medium was cooled down approximately to 55°C. After mixing the medium with ampicillin, the content was poured into 10 mm petri plates.

2.1.10.3 SOC medium

SOC medium was used to cultivate E. coli for 1 hour (h) after heat shock during transformation. 2 g tryptone, 5 g yeast extract, 0,058 g NaCl, 0,0186 g KCl, 0,095 g

$MgCl_2$, 0,23 g $MgSO_4$ and 0,36 g glucose was resolved in 100 mL dH_2O and sterilized at 121°C by autoclaving for 15 min.

2.1.10.4 $CaCl_2$ solution

$CaCl_2$ solution was used in the preparation chemically competent E. coli cells that were further used in transformation. The solution contains 60 mM $CaCl_2$, 10mM PIPES and 15% glycerol in dH_2O. pH was adjusted to 6,4 to dissolve PIPES and then was filter sterilized with 0,2 μm filter.

2.1.11 Plasmid

CS-L0198-M02 of OmicsLink Expression Clone was used as plasmid. Bag-1 human cDNA ORF Clone was inserted in pEZ-M02 vector after the removal of the stop codon and addition of a C-terminal TAP (tandem affinity purification)-tag containing sequentially CBP (calmodulin binding peptide) – TEV (tobacco etch virus protease)-Protein A from N to C terminus by Capital Biosciences. Whole plasmid size is 7285 bp with the 1038 bp ORF length. Ampicillin is the antibiotic for this plasmid, and neomycin is the stable selection marker. DH5α is the suitable bacterial strain for transformation.

2.1.12 Vector

pEZ-M02 vector information for Bag-1 overexpressing plasmid is demonstrated in Figure 2.1.

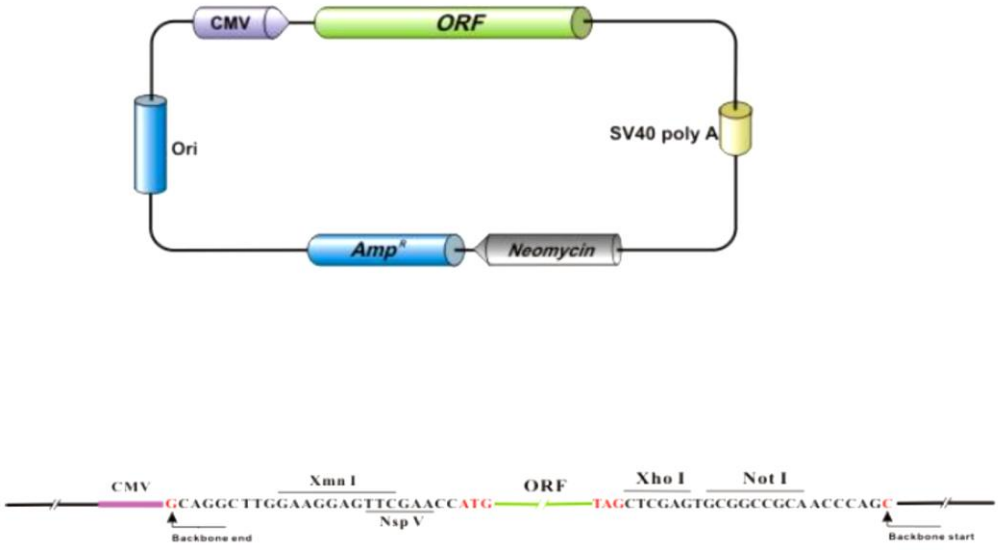

Figure 2.1 : Vector information for Bag-1 overexpressing plasmid.

2.1.13 Cell Line

MCF-7 cells are human breast adenocarcinoma cells which was derived from a metastatic site. Cell line was obtained from mammary gland of mammarial ephitelium. MCF-7 cells are adherent, estrogen receptor positive and expressed in blood type O Rh+. This cell line which has a doubling time of about 29 hours, was isolated from a 69-year-old Caucasian female patient in 1970, and was established in 1973.

2.1.14 Cell culture solutions

2.1.14.1 MCF-7 culture medium

MCF-7 cell culture medium is Gibco including containing 4500 mg/L D-Glucose, 110 mg/L Sodium Pyruvat. It was prepared with 10% FBS and 1% Penicillin/Streptomycin within DMEM (Dulbecco's Modified Eagle Medium) and then filter sterilized with a 0,2 μm filter.

2.1.14.2 MCF-7 freezing medium

For the preparation of MCF-7 freezing medium, 5% DMSO was supplemented to the serum free culture medium and sterilized with 0,2 μm filter

2.1.15 Bag-1 siRNA

Human Bag-1 siRNA was obtained from Santa Cruz and is a target-specific 19-25 nt siRNA designed to knock down gene expression. Each vial contains 3.3 nmol of lyophilized siRNA, sufficient for a 10 μM solution. Bag-1 siRNA was resuspended with 330 μL RNAse-free water and stored at -20^{o}C.

2.2. Methods

2.2.1 Competent cell preparation - $CaCl_2$ method

The bacterial competent cells have been chemically treated to allow the foreign DNA or plasmid to be taken in. *E.* coli-DH5α cells were taken from a glycerol stock culture by scraping with a tip, and it was put in 5 mL LB medium and incubated overnight at 37^{o}C in orbital shaker. The following day, 100 mL LB medium was inoculated with 5 mL culture solution and was incubated at 37^{o}C in orbital shaker. Cell density was measured by a spectrophotometer at optical density (OD) 600, and when it was reached to 0.6 the bacteria were transferred to 50 mL prechilled sterile

ultracentrifuge tubes and incubated on ice for 10 min. The cells were centrifuged at 1600xg for 7 min at 4°C, and then supernatant was discarded. Each bacterial pellet was resuspended in 10 mL ice-cold $CaCl_2$ and centrifuged for 5 min at 1600xg, and the supernatant was discarded. After centifugation, each bacterial pellet was resuspended in 10 mL ice-cold $CaCl_2$, and they were incubated on ice for 30 min. Then, centrifgation was performed again at 1600 x g for 5 min at 4°C, and each pellet was resuspended completely in 2 mL of $CaCl_2$. The competent cells were distributed into prechilled sterile microfuge tubes each containing 50 µL and they were stored at -80°C. For 50 mL $CaCl_2$ solution content is shown in Table 2.14.

Table 2.14 : $CaCl_2$ solution content.

Content	Concentration	Amount
$CaCl_2.2H_2O$	60 mM	0,442 g
PIPES	10 mM	0,15 g
Glycerol	15 %	7,5 mL (from 100 %)
dH_2O		Up to 50 mL

2.2.2 Transformation

For the transformation, competent cells were taken out from -80°C and thawed on ice. Purified plasmid DNA was mixed with competent cells and the tube was incubated on ice for 30 min. Then the tube was placed in 42°C for 45 sec and put back on ice for 2 min. 80 µL SOC medium was added and then culture was incubated at 37°C for 1 h in orbital shaker. Culture was plated on selective medium with appropriate antibiotic (LB-Amp). Plates were incubated overnight at 37°C.

2.2.3 Small scale plasmid DNA preparation (mini-prep)

Plasmid preparation was performed using QIAGEN, QIAPrep Spin Miniprep Kit for small scale (mini) preparation, following the instructions of the manufacturer. In this procedure, plasmid DNA was released from bacteria by alkaline lysis, and lysate was removed from the RNA by RNase. Then in the presence of chaotropic salt (guanidine HCl), DNA has an ability to bind selectively to glass fiber fleece in a centrifuge tube and remained bound. Washing steps was performed to remove contaminating bacterial components, and then low salt elution buffer was added to remove DNA

from the glass fiber fleece. After identification of the colonies containing the Bag-1L over-expressing plasmid, these colonies were taken into a 5 ml LB medium containing 5 µl suitable antibiotic (ampicilin) to prepare selection medium. Then, they were incubated overnight with vigorous shaking (200 rpm) at 37^{o}C. Plasmid preparation was performed using QIAGEN, QIAPrep Spin Miniprep Kit for small-scale (mini) preparations, following instructions of the manufacturer.

2.2.4 Medium scale plasmid DNA preparation (midi-prep)

Bag-1 constructs were transformed with *E. coli* DH5α strain. After the colonies rt containing our insert were reproduced in medium scale and purified with QIAprep Spin.

2.2.5 Determination of plasmid DNA concentration

The spectrophotometric analysis was used to determine recovery, purity and concentration of nucleic acid. The ratio of absorption at 260 nm vs 280 nm is commonly used to evaluate the purity of DNA with respect to protein contamination, since protein (in particular, the aromatic amino acids) absorbs at 280 nm. According to the literature, the ratio of absorbance (A260/A280) of a pure DNA solution is between 1,8 to 2,0. When protein contamination increases, the ratio decreases. 2 µL sample was placed onto a sensor and its concentration was determined with the help of NanoDrop 2000/2000c Operating Software.

2.2.6 Preparation of cell culture

2.2.6.1 Transferring the stock cell to culture flask

A frozen stock of MCF-7 cells were taken from -80^{o}C and after the cells were thawed in 37^{o}C water bath. They were diluted with MCF-7 culture medium, and centrifuged for 3 min at 1000 rpm. Cell pellet was dissolved with medium and transferred to the culture flasks.

2.2.6.2 Cell counting

Growing medium was removed and cells were detached by 1 mL Trypsin-EDTA treatment for 2 min at 37^{o}C. Then trypsin-EDTA solution was inhibited by 10 mL medium and cells were suspended. Then 10 µL suspension was taken and put onto the hemocytometer, cells were counted as the number of cells per 25 square (1 mm^{2}). The cell number calculation is as follows:

10^4 (constant number) x Amount of counted cell = Cell number/ml

Total cell number = Cell number/ mL x Total volume of cells (10 ml)

2.2.6.3 Cell passage

After cells were lifted and cell density was determined (80% and above), cells were put into the centrifuge tubes and centrifuged 1000 rpm for 3 min. The supernatant was discarded and pellet was resuspended with culture medium and $1x10^6$ cells were transferred to clean culture flask.

2.2.6.4 Cell freezing

After cell passage, cells resuspended in medium were transferred to freezing tube with freezing medium for future experiments. Estimated cell number per freezing tube was $1x10^6$ cell/mL.

2.2.7 Transient transfection of MCF-7 cells with Bag-1 plasmid

Transfection is the process to introduce a foreign DNA into the cell. In this study DNA was inserted into MCF-7 cells by lipofection. The synthetic cationic lipids that can form liposome are incubated with DNA. These liposomes interact with DNA through electrostatic interaction between the negatively charged nucleic acid and positively charged head group of synthetic lipid, and fuse with cultured cells. The liposome complex neutralizes the negatively charge of the nucleic acids, allowing association of the complex with the negative charged cell membrane. Entry of the liposome complex into the cell may occur by the processes of endocytosis or fusion with plasma membrane via the lipid moieties of the liposome. After cells were detached and cell number was calculated, 50.000 cells per well were seeded on 24 well tissue culture plate 2 day before the transfection, so that the cells were approximately 80% confluent on the day of transfection. 1 µg of plasmid DNAs are diluted in 200 µL serum-free DMEM (1X) and vortexed and quickly spun. Then, 3 µl FuGENE® HD Transfection Reagent (for western blot experiments) or 3 µl Transpass Transfection Reagent (for cellular experiments) was added to and vortexed and quickly spun for the formation of transfection complex, and later incubated for 15 min at room temperature. Transfection complex was shared to the wells with equal amounts, and cells were returned to the 37°C, 5% CO_2 incubator for 24 h or 48 h before analysis.

2.2.8 siRNA transfection

Santa Cruz human Bag-1 siRNA was used for the silencing of Bag-1 gene. Lyophilized siRNA stock was 330 μL RNAse-free water and diluted as a working solution for transfection. 40 pmol dilution of siRNA was optimum for MCF-7 cells for silencing. Opti-MEM® Reduced Serum Medium was used for dilution of siRNA to form transfection comlex. Since Opti-MEM® Reduced Serum Medium is an improved Minimal Essential Medium (MEM) that allows for a reduction of Fetal Bovine Serum supplementation by at least 50% with no change to growth rate or morphology and it is also recommended for use with cationic lipid transfection reagents, such as Lipofectamine reagent, it was chosen as a transfection medium. Lipofectamine 2000 of Invitrogen was used as lipid agent to increase permeability of the cell membrane, and the manufacture's instructions were followed.

2.2.9 Cell imaging

Visualization of MCF-7 cells is important for determination of cell morphology and alterations after Bag-1 plasmid transfection. $6x10^5$ cells were seeded into each well of 6-well plate. One day later cells were tansfected with the Bag-1 plasmid. Control and Bag-1 plasmid transfected cells were imaged using Olympus DC71 inverted microscope.

2.2.10 Fluorescent staining

Four different flurescent dyes were used as apoptotic markers for the determination of apoptotic cells under flurescent microscope.

Propidium iodide (PI) binds to DNA by intercalating between the bases with little or no sequence preference and with a stoichiometry of one dye per 4–5 base pairs of DNA. Once the dye is bound to nucleic acids, its fluorescence is enhanced 20- to 30-fold, the fluorescence excitation maximum is shifted ~30–40 nm to the red and the fluorescence emission maximum is shifted ~15 nm to the blue. Since PI is membrane impermeant and genarally excluded from viable cells, it is suitible for the identification of dead cells.

Acridine orange is a cell-permeant nucleic acid binding dye that emits green fluorescence when bound to dsDNA and red fluorescence when bound to ssDNA or RNA. By means of this unique property, acridine orange can be used for the detection of shreded DNA which is one of the indicators of apoptosis. After acridine

orange staining, healty cells gets dyed green while apoptotic cells are observed redish-orange.

The blue-fluorescent DAPI nucleic acid stain preferentially stains dsDNA; it appears to associate with AT clusters in the minor groove. Binding of DAPI to dsDNA produces a ~20-fold fluorescence enhancement, apparently due to the displacement of water molecules from both DAPI and the minor groove. DAPI dyes both healty and apoptotic cells to blue but the brightness of apoptotic cells are higher due to the nuclear fragmentation.

$DiOC_6$ is a fluorescent dye used for the staining of a cell's endoplasmic reticulum, vesicle membranes and mitochondria. Binding to these structures occurs via the dye's hydrophilic groups. $DiOC_6$ can be used to label living cells, however they are quickly damaged due to photodynamic toxicity, so cells stained with this dye can only be exposed to light for short periods of time. When exposed to blue light, the dye fluoresces green.

The usage of fluorescent dyes and colours of cells after staining is given in Table 2.15.

Table 2.15 : The apoptotic stains used in this study.

Dye	Dilution	Incubation time	Filter	Colour
Acridine orange	1:2000	5 min	Blue	Alive: Green Death: Orange
Propidium iodide	1:5000	30 min	Green	Alive: Not stained Death: Red
DAPI	1:1000	10 min	Green	Alive: Blue Death: Bright blue
$DiOC_6$	1:1000	15 min	Blue	Alive: Green Death: Yellow

2.2.11 Survival Assay (Trypan Blue Dye Exclusion Assay)

Trypan Blue is a vital dye of which reactivity is based on the fact that the chromopore is negatively charged and does not interact with the cell unless the

membrane is damaged. According to the time points, cells were counted each day for 4 days using dye exclusion assay. $0{,}5x10^5$ cells were seeded in each well of the 6-well plate one day before transfection. 24, 48, 72 and 96 h after transfection, growth medium was removed and cells were washed twice with PBS. Cells were trypsinized and gathered by centrifugation. Then, cells were exposed to 0.4% (w/v) Trypan blue dye (50µL) and DMEM (50µL) at 1:1 ratio. Dual-chamber FisherLab hemacytometer was filled with 10 µL of cells from trypan blue-DMEM mixture, and under a light microscope, non-viable cells were observed as stained while viable cells excluded the stain. Stained cells were counted and plotted.

2.2.12 XTT cell viability assay

Cell Signaling's XTT Cell Viability Kit was used for the detection of viable cells after different treatments (overexpression and silencing). The XTT Cell Viability Assay Kit is a colorimetric assay that detects the cellular metabolic activities. During the assay, the yellow tetrazolium salt, XTT, is reduced to a highly colored formazan dye by dehydrogenase enzymes in metabolically active cells. This conversion only occurs in viable cells and thus, the amount of the formazan produced is proportional to viable cells in the sample. The formazan dye formed in the assay is soluble in aqueous solution and can be quantified by measuring the absorbance at wavelength 450 nm using a spectrophotometer. An electron coupling reagent, such as PMS (N-Methylphenazonium methyl sulfate), can significantly improve the efficiency of XTT reduction in cells. $1x10^4$ cells were seeded in each well of 96-well plate one day before Bag-1 siRNA and Bag-1 plasmid transfection. Then, 24, 48 and 72 h after transfection XTT assay was applied, following the instructions of the manufacturer.

2.2.13 Immunostaining

Immunostaining is a biochemical technique used to visualize proteins in the cell. The primary antibodies which are specific for the intended protein and secondary antibodies which are specific for the IgG heavy and light chains of the primary antibody are used. Detection is usually performed by fluorescence. MCF-7 cells ($2x10^4$) were seeded on poly-L-lysine coated coverslips and transfected with Bag-1 plasmid DNA. After 48 h transfection, culture medium was removed, and cells were washed 2 times with PBS. Cells were fixed in prechilled methanol and incubated 15 min at -20^oC, and cells were washed 3 times with PBS. Blocking solution was

prepared 10% antibody specific serum, 10 mg/mL bovine serum albumin (BSA) and PBS, then, cells were blocked with blocking solution for1 h at room temperature. Primary antibodies were diluted in PBS (1:200 mouse monoclonal anti-Bag-1, 1:200 rabbit monoclonal anti-β-actin, 1:200 rabbit polyclonal anti-C-Raf, 1:200 rabbit monoclonal anti-B-Raf, 1:200 rabbit monoclonal anti-Bcl-2, 1:200 rabbit polyclonal anti-Hsp70, 1:200 rabbit monoclonal anti-Phospho-Akt (Ser473) antibodies). Cells were incubated with 300 µL antibody solution overnight at 4^{o}C. Following day primary antibodies were removed, and cells were washed 3 times with PBS. Cells were blocked with blocking solution 1 h at room temperature. Secondary antibodies were diluted with PBS (1:1000 both Alexa Flour® 647 goat anti-mouse, 488 goat anti-rabbit Alexa Flour®), and cells were treated with secondary antibody solution for 1 h at 37°C in dark. Cells were washed once for 5 min with PBS, then were incubated 1:1000 diluted DAPI for 5 min. After washing steps (3 times with PBS) 10 µL mounting medium was added into slides. Coverslips were put onto object slide and fixed by using nail polisher. Samples were visualized and analyzed by using Leica TCS SP2 SE Confocal Microscope with appropriate laser beams

and photos were taken under 63X magnification.

2.2.14 Total protein isolation

Total protein isolation from cells was performed by using ROCHE, cOmplete Lysis-M Kit EDTA-free. Each well of a 6 well-plate was seeded with $1X10^{6}$ cells, and transfected with Bag-1 plasmid DNAor Bag-1 siRNA. After 48 h transfection, one complete-M Mini tablet was dissolved in 1,5 mL ProteoJET™ Mammalian Cell Lysis Reagent Reagent to prepare working solution. Culture medium was removed, and cells were washed with PBS. Cells were gathered in to microcentrifuge tubes using cell scrapper and centrifuged at 13200 rpm for 5 min. 50 µL lysis buffer was added to each microcentrifuge tube and incubated for 15 min on ice on an orbital shaker. Microcentrifuge tubes were cemtrifuged at 20000 rpm for 15 min., and supernatants containing soluable proteins were transfered into clean tubes and stored -80°C.

2.2.15 Phospho-protein isolation

Phospho-protein isolation from cells was performed by using ROCHE, PhosSTOP; Phosphatase Inhibitor Cocktail Tablets supplied in EASYpacks. PhosSTOP provides

isolating or detecting phosphorylated proteins and benefits the ultimate in convenience to protect proteins against phosphatase activity. $1x10^6$ cells were seeded each well of 6 well plate and transfected with Bag-1 plasmid DNA or Bag-1 siRNA. 50 µL 10x PhosStop-Phosphatase Inhibitor Tablets, 50 µL cOmplete, EDTA-free Protease Inhibitor Cocktail Tablets and 500 µL ProteoJET™ Mammalian Cell Lysis Reagent were mixed, and cell lysis solution was prepared. Then instructions of the manufacturer were followed.

2.2.16 Bradford assay

The determination of proteins was performed using BIO-RAD's Quick Start Bradford protein assay. The principle of assay is rely on binding of Coommassie Brilliant Blue G-250 dye (also known as Bradford dye) to proteins. Under acidic conditions, the dye is predominantly in the doubly protonated red cationic form (A_{max} = 470 nm). However, when the dye binds to protein, it is converted to a stable unprotonated blue form (A_{max} = 595 nm). It is this blue protein-dye form that is detected at 595 nm in the assay using a spectrophotometer or microplate reader. The dye was diluted with distile water at 1:4 ratio. Bovine Serum Albumin Standard Set was chosen for microassay (the linear range of these assays for BSA is 1.25-10 µg/mL). 1X Bradford dye reagent was removed from the 4°C storage, and was warmed to ambient temperature. 200 µL of dye was shared each well in the plate, and all loading process was duplicated to confirm lineer range of standarts and to get more accurate results. 5 µL of BSA standarts were put into dye-loaded wells with the concentrations of 0,125; 0,25; 0,5; 0,75; 1; 1,5; 2 mg/mL, respectively. 5 µL of proteins of which concentrations were unkown were put into dye-loaded wells, and microplate was incubated at room temparature about 5 min. After incubation absorbance was measured at 595 nm on plate reader.

2.2.17 SDS-polyacrylamide gel electrophoresis of proteins (SDS-PAGE)

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis is a technique widely used to separate proteins according to electrophoretic mobility. In this method, tertiary structure of proteins is disrupted by SDS and reducing agents such as β-mercaptoethanol. The proteins are separated in gel according to their size. In this study, Fluka SDS Preparation Kit is used for preparation both 5% stacking gel and 10 % separating gel, contents of both were described in Table 2.16 and Table 2.17,

respectively. Prepared gels were poured between SDS-glasses and left for polymerization. After gel polymerized, samples, that were mixed 6X SDS-loading dye and boiled 95°C for 5 min to gain primary structure, were loaded in equal concentration into wells. The gel was placed into a tank and was run in a running buffer at 25 mA for 2 h.

Table 2.16 : Content of 5% stacking gel.

Contents	Volume
Stacking acrylamide	0,25 ml
Gel buffer	0,75 ml
dH_2O	2 ml
10% APS	20 µL
TEMED	2 µL

Table 2.17 : Content of 10% separating gel.

Contents	Volume
50% glycerol	2 ml
Separating acrylamide	1,22 ml
Gel buffer	2 ml
dH_2O	0,78 ml
10 % APS	75 µL
TEMED	7,5 µL

2.2.18 Western blotting

Western blot is used to detect specific proteins. After separation of denatured proteins according to size by SDS-PAGE, the proteins are transferred to nitrocellulose membrane where they are labeled using antibodies specific to the target protein. After SDS-PAGE, gel was taken from the tank and stripped off the glasses. Transfer of proteins from gel to the membrane was done in two different ways: (A) Semidry transfer; nitrocellulose membrane and 2 filter papers were put

into towbin electrotransfer buffer for 10 min for equilibration, then protein transfer was carried out in semi-dry blotting apparatus for 20 V for 30 min at 4 °C, (B) Trans-Blot Turbo transfer system; gel was put into Trans-Blot® Turbo Midi Nitrocellulose Transfer Packs, then protein transfer was carried out in Trans-Blot® Turbo tranfer system at 25 V for 8 min. After transfer, membrane was blocked with a blocking solution to prevent non-specific binding to empty regions on the membrane. In other words, efficient blocking simply reduces the background. After blocking, the membrane was incubated with 1:500 diluted with blocking solution primary antibodies, which are listed in Table 2.3, for overnight at 4 °C with gentle shaking. Following day, membrane was washed with TBS-T buffer for 5 min 6 times with gentle shaking, 1:5000 diluted secondary antibody was prepared in blocking solution, and was incubated with secondary antibody for 1 h at room temperature. After 5 times washing of membrane with PBS-T, 20X LumiGLO® Reagent or SuperSignal® West Femto Maximum Sensitivity Substrate was used for the detection of proteins according to manufacturer's instructions. The membrane was exposed to X-ray film for a certain time and then developed in Kodak Medical X-ray Processor according to manufacturer's instruction.

2.2.19 Protein stripping

Restore Western Blot Stripping Buffer was used to obtain different protein imaging from a single membrane. The reason that we chose this product was that since the product procedure does not include heating, protein loss due to the heat is prevented. Protein stripping was performed following instructions of the manufacturer.

2.2.20 Co-immunoprecipitation

Co-immunoprecipitation (CoIP) is a method used for isolation of intact protein complexes. In this study CoIP was applied for the investgation of Bag-1 protein's interacting partners in MCF-7 breast cancer cell lines. CoIP works by selecting an antibody that targets a known protein that is believed to be in an interaction with a large number of proteins. By targeting this known member with an antibody it may become possible to pull the entire protein complex out of solution and thereby identify unknown members of the complex, in this case unknown interacting partners. CoIP was performed for both Bag-1 and C-Raf proteins using monoclonal anti-Bag-1 and polyclonal anti-C-Raf antibodies, respectively. Invitrogen's

Dynabeads® Protein G was utilized as magnetic beads, following instructions of the manufacturer to attach selected antibodies. Bag-1 proteins bound with Bag-1 antibodies onto the beads were eluted using 20 µL elution buffer, and the tubes were incubated for 10 min at 70 °C in order to dissociate the complex. After dissociation of complex, proteins were mixed with 6X Laemmli buffer and loaded onto SDS gel for western blotting. Known and estimated interacting partners (Bcl-2, Hsp70, C-Raf, B-Raf, P-Akt) were checked using specific antibodies. Same procedure was introduced for immunoprecipitation of C-Raf.

3. RESULTS

3.1 Identification of Bag-1 isoforms

Immunodetection of Bag-1 with monoclonal α-Bag-1 antibody revealed three major isoforms of Bag-1 (first lane of the Figure 3.1). In second lane of Figure 3.2 MCF-7 cells were transfected with TAP-tag containing-Bag-1 overexpressing plasmid. Due to the alternative translation from N-terminus of Bag-1 mRNA, three major isoforms and their TAP-tag (~19 kDa) containing versions are visible (second lane of the Figure 3.1).

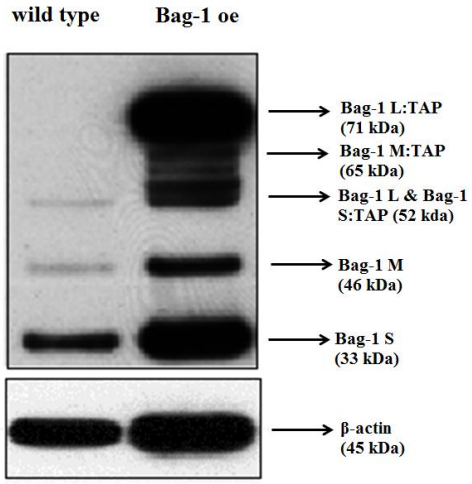

Figure 3.1 : Western blot analysis of TAP-tag containing Bag-1L transiently transfected MCF-7 cells. Immunodetection of Bag-1 reveals multiple band**s** due to the alternative translation of Bag-1 mRNA, three major isoforms and their 17 kDa TAP-tagged versions are visible (second lane of the Figure 3.1).

3.2. Imaging of cell morphology

Cell imaging was done to observe cell morphology at indicated time points. $6x10^5$ MCF-7 cells were seeded into 6-well plate, and cell pictures were taken with light microscopy. Wild type cells can be seen at the left side of Figure 3.2. MCF-7 cells were transfected with Bag-1 overexpressing plasmid, and cell pictures were taken (right side of Figure 3.2). Three different magnification was chosen to visualize cell

morphology. Results show that podia formation is clearer, and podia number is increased after 48 h from Bag-1 overexpression. Since MCF-7 cells are adherent cancer cells, these pictures might indicate that Bag-1 overexpression could be important for cell to cell communication and adhesion.

The black dots at the right side of Figure 3.2 are caused by transient transfection. These are lipid particles arised due to the application of lipid-based transfection reagent.

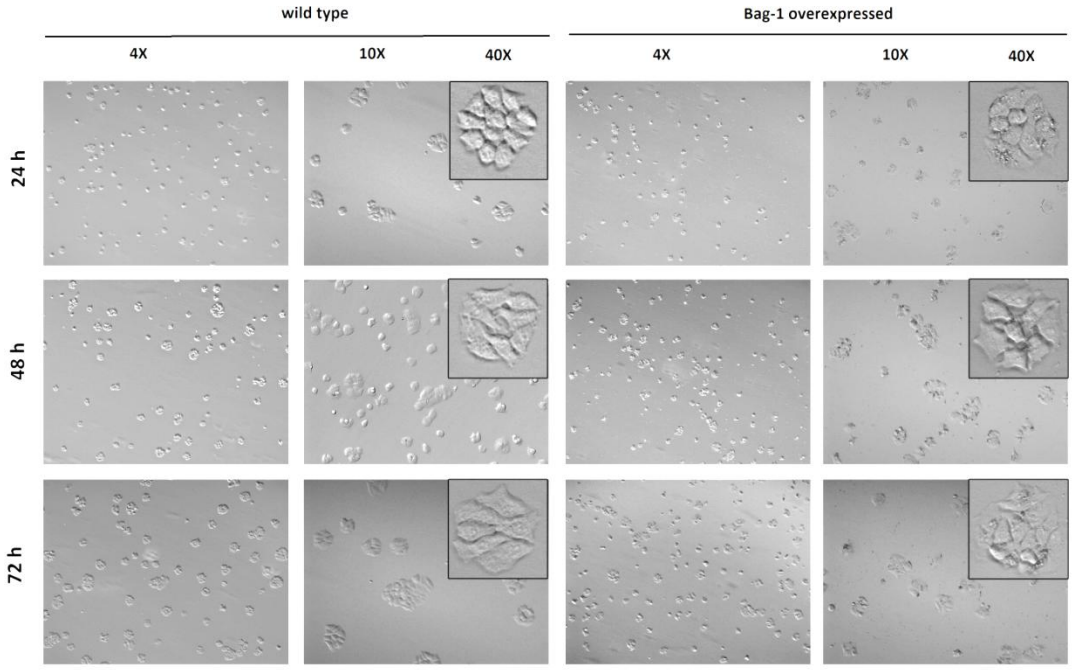

Figure 3.2 : Light microscopy images of cell morphologies**.** After 48 h from transfection with Bag-1 overexpressing plasmid podia number of cells were increased, and podia formation was become clearer.

3.3. Overexpression of Bag-1 promotes cell survival

3.3.1. MCF-7 cell viability alterations occur by Bag-1 amount change

To address the potential role of Bag-1 in control of cell survival and cell viability in MCF-7 breast cancer cells, we examined the expression and subcellular localization of Bag-1.

First, Bag-1 expression levels were detected with α-Bag-1 antibody. β-actin was used for normalization. Bag-1 overexpression and Bag-1 silencing were confirmed (Figure

3.3 -A) and densitometric analysis were reported with the usage of computer softwares (Figure 3.3 -B).

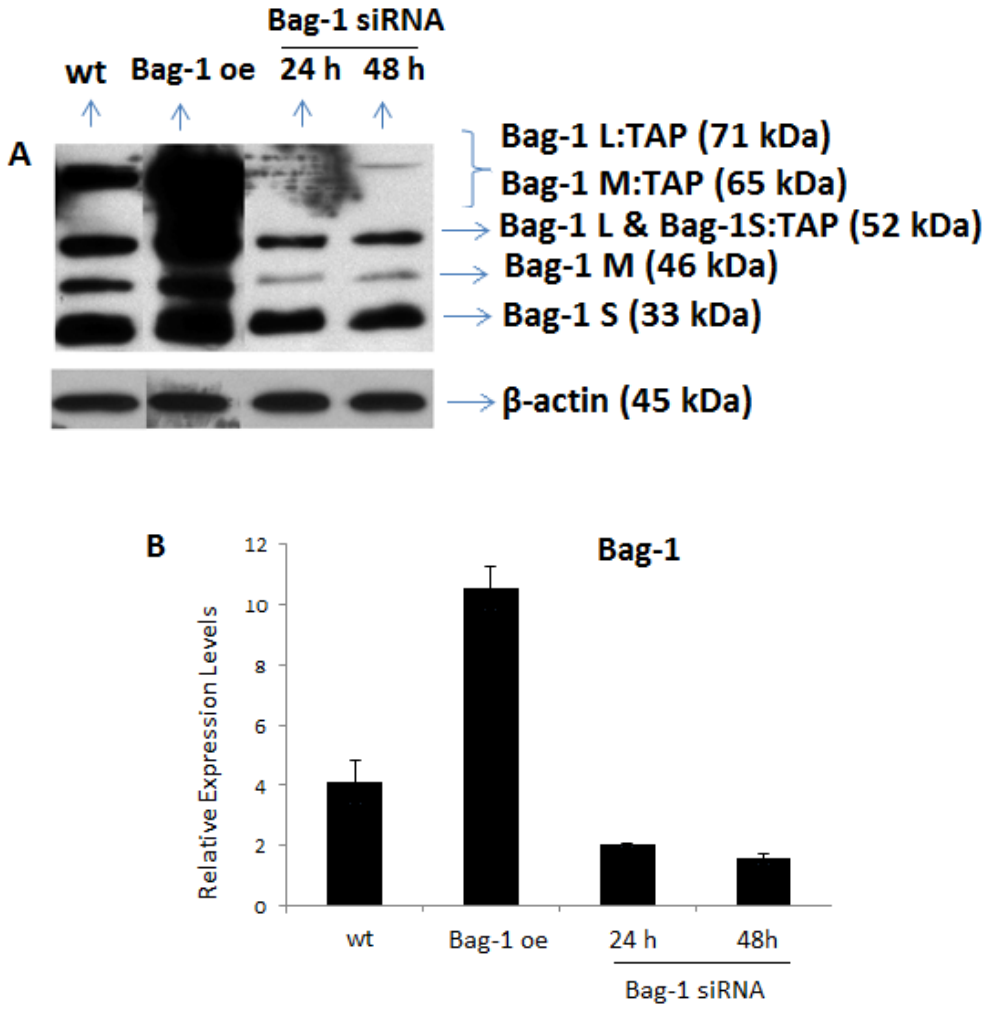

Figure 3.3 : Western blotting results of wild type, Bag-1 overexpressed and Bag-1 siRNA transfected MCF-7 cells. Bag-1 isoforms and alterations of expression levels of isoforms detected with α-Bag-1 antibody (A). Relative expression levels depend on desitometry of bands (B).

After verification of Bag-1 overexpression and silencing, we examined the level of change in cell viability upon Bag-1 alterations by cell survival (trypan blue dye exclusion assay) and XTT cell viability assays (Figure 3.4).

Wild type, Bag-1 overexpressed and Bag-1 siRNA treated MCF-7 cells were counted at indicated time points. Cells were collected, and trypan blue dye exclusion assay was performed (Figure 3.4-A). 10 µL of trypan blue/cell culture (1:1 ratio) mix was transfered into hemocytometer, and alive cells that dyed with trypan blue were

counted. Trypan blue assay results showed that Bag-1 overexpressed MCF-7 cell numbers are significantly higher than wild type (blue line) cells at the end of the 48 h and 72 h transfection (red line). On the other hand Bag-1 siRNA transfected cell numbers (green line) are fewer than wild type (Figure 3.4-A). This result might be an indication of Bag-1 silencing effect promotes apoptosis while with Bag-1 overexpression cell survival is modelated.

To ascertain percentage of viable cells after Bag-1 siRNA and Bag-1 overexpressing plasmid, we applied XTT cell viability assay (Figure 3.4-B). During the assay, the yellow tetrazolium salt, XTT, is reduced to a highly colored formazan dye by dehydrogenase enzymes in metabolically active cells. Thus, this conversion only occurs in viable cells. XTT cell viability assay showed that 48 h and 72 h Bag-1 overexpressed cells had the highest percentage of viability. However, in 48 h Bag-1 siRNA transfected cells, viability was decreased (Figure 3.4-B).

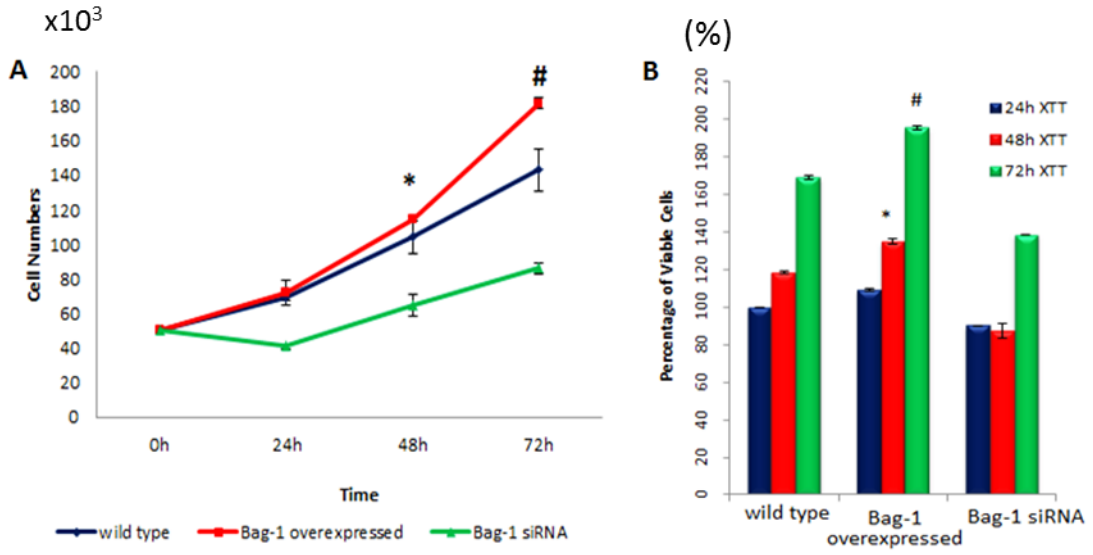

Figure 3.4 : MCF-7 cell viability gets altered due to the changes of Bag-1 expression level. Cell survival (trypan blue dye exclusion) assay revealed that viable cell numbers increased by Bag-1 overexpression (A). Viable cell numbers was lower in comparison to wild type MCF-7 cells (A). XTT cell viability test also revealed similar results (B). Bag-1 overexpression elevated percentage of viable MCF-7 cells while Bag-1 down regulation decreases the percentage of viable cells (B) in MCF-7 culture.

3.3.2 Analysis of Cell Survival Proteins Showed That Bag-1 Overexpression Promotes Cell Survival

3.3.2.1 In parallel to Bag-1 overexpression, B-Raf and C-Raf protein expressions are enhanced

B-Raf and C-Raf, they are most important members of Ser/Thr kinase Raf protein family. Both are involved in Raf-MEK-Erk pathway, which is mitogen-activated protein kinase cascade that controls many molecular mechanisms such as proliferation, differentiation and cell survival. Bag-1 interactions with C-Raf and B-Raf were previously suggested, therefore we first examined C-Raf/B-Raf signaling pathway that might potentially explain the route of Bag-1 involvement in cell survival. We performed western blot analysis with total proteins derived from wild type, Bag-1 overexpressed and Bag-1 siRNA transfected MCF-7 cells (Figure 3.5). Western blotting showed that overexpression of Bag-1 highly increases both B-Raf (Figure 3.5-A) and C-Raf (Figure 3.5-C) expression. Since overexpression of Bag-1 increased the expression levels of C-Raf and B-Raf, we checked the phosphorylation states of these. Western blot results showed that activatory site of C-Raf "Ser338 residue" (Figure 3.5-D) phosphorylation and phospho-C-Raf Ser289/296/301 (Figure 3.5-E) expressions were higher with Bag-1 overexpression. In addition, we observed that phospho-B-Raf Ser445 (Figure 3.5-B) level was elevated with Bag-1 overexpression. Akt kinase phosphorylation is essential for the prevention of cell death, and the initiation of survival signaling by means of interaction with B-Raf. Hence, we noticed that phospho-Akt at Ser473 (Figure 3.5-F) level was also increased with Bag-1 overexpression. Morever, we checked Hsp70, which is another main interacting partner of Bag-1, and western blotting revealed that Hsp70 expression elevated through Bag-1 overexpression and down regulated with Bag-1 silencing (Figure 3.5-G). All western blotting results were normalized with β-actin, and relative expression levels were reported via densitometric analyses (Figure 3.5-H).

3.3.2.2 Bag-1 overexpression stimulates MEK-Erk pathway

After confirmation of that Bag-1 promotes sell survival proteins, we decided to investigate the alterations on the downstream of the MAPK pathway. We applied

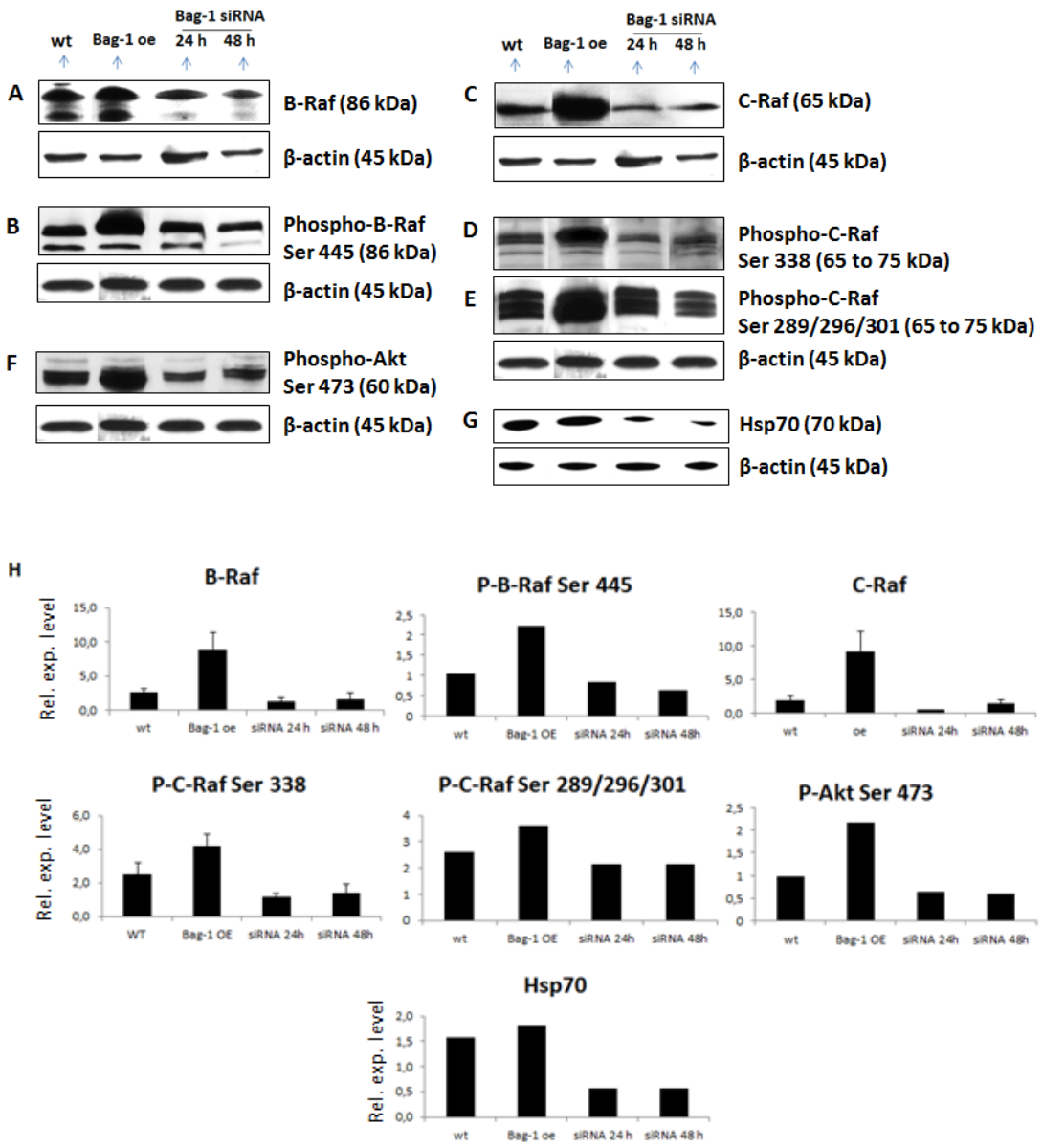

Figure 3.5 : Western blotting revealed that overexpression of Bag-1 upregulates B-Raf and C-Raf. Two of the known interacting partners of Bag-1 and also important players of cell survival signaling pathway, B-Raf and C-Raf expression was increased by Bag-1 overexpressed MCF-7 cells (A and C). Phosphorylation levels of survival pathway proteins were also examined. Phospho-B-Raf Ser445, phospho-C-Raf Ser338, phospho-C-Raf Ser289/291/301 (B,D and E). Expressions of phospho-Akt Ser473 and Hsp70, other known interacting partners of Bag-1 during cell survival, were checked, as well (F and G). Expression levels of indicated proteins were elevated with Bag-1 overexpression (H).

western blot analysis for Erk and MEK which are next proteins in MAPK dual cascade, and we observed that while Erk expression level did not change significantly, MEK expression level got higher with Bag-1 overexpression. We

noticed that phospho-MEK Ser221 expression level dramatically rose after Bag-1 overexpression (Figure 3.6).

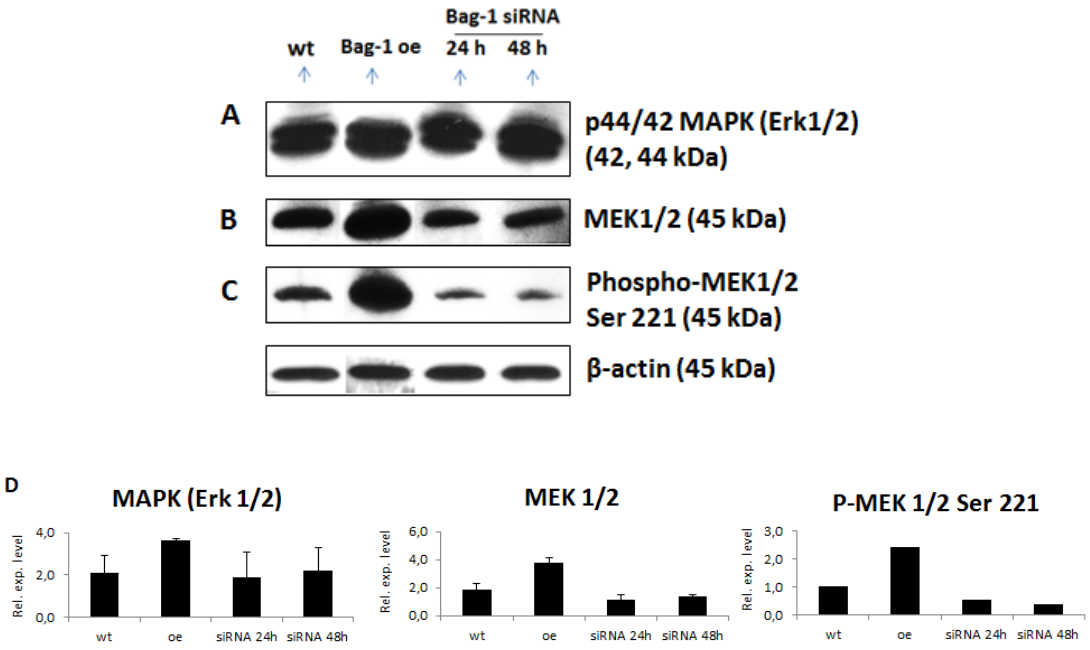

Figure 3.6 : Bag-1 overexpression modulates the activation the kinases in the downstream of MAPK pathway. Downstream players of MAP kinase dual cascade MEK1/2 and its phosphorylated form phospho-MEK1/2 Ser221 expressions changed in parallel with Bag-1 overexpression (B and C). However, we were not able to observe significant alteration in Erk1/2 expression level (A). All results were normalized with β-actin, and relative expression levels were calculated (D).

3.4. Identifying the interaction partners of Bag-1 during cell survival

Bag-1 interacts a group of proteins to form a complex to inhibit apoptosis, and direct cells to survival. We performed co-immunoprecipitation to identify interacting partners of Bag-1 during cell survival. We used magnetic beads which were loaded with α-Bag-1 antibody to pull down protein complex from total protein lysate. We first confirmed that Bag-1 immunoprecipitation was successful (Figure 3.7-A). Then, we checked interacting partners of Bag-1 with specific antibodies using western blotting (Figure 3.7). Co-immunoprecipitation revealed that Bag-1 interacts with B-Raf, Bcl-2, Phospho-Akt, Hsp70, and C-Raf (Figure 3.7- B to F). We used β-actin and A-Raf as the negative controls, as expected we did not observe any bands for these proteins (Figure 3.7- G and H).

A study about survival of neuronal cells revealed that Bag-1, B-Raf, Bcl-2, Phospho-Akt, and Hsp70 form a complex to provide Bad phosphorylation at Ser136. Phosphorylated Bad interacts with 14-3-3 scaffold protein, which leads cell to survival. Suprisingly, we discovered that C-Raf was also involved in this complex in MCF-7 breast cancer cells (Figure 3.7-F). Since MCF-7 cells are cancerous, C-Raf involvement with the detected complex may shed light to one of the paths of survival in breast cancer cells. It may indicate that Bag-1 overexpression in cancer cells stimulates C-Raf to direct cells to survival.

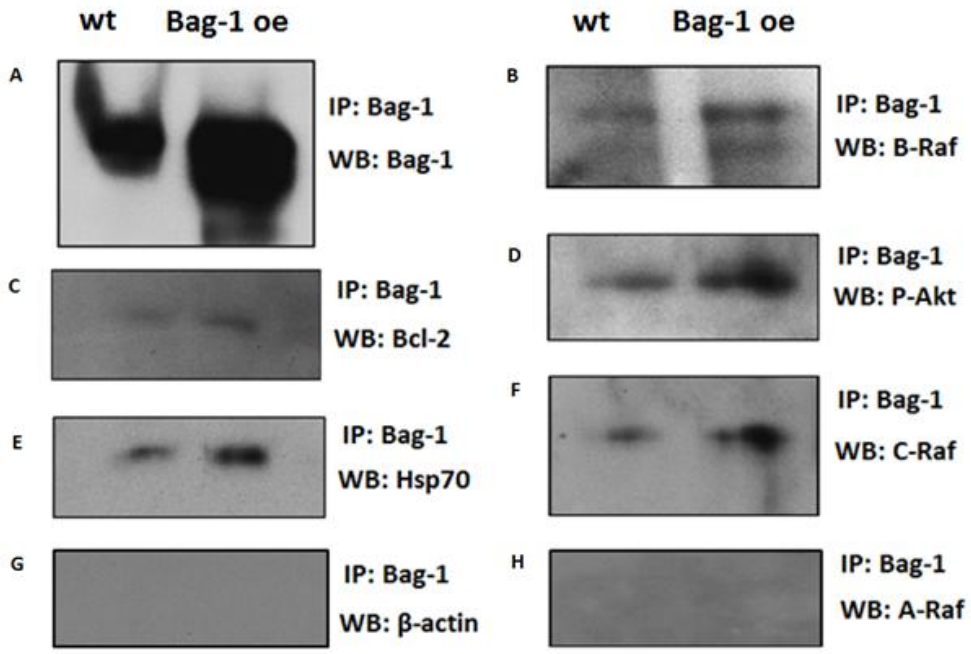

Figure 3.7 : Co-immunoprecipitation with α-Bag-1 antibody revealed the interacting partners of Bag-1 in the cell survival pathway. Verification of immunoprecipitation was done by western blotting . Results showed that Bag-1, B-Raf, Bcl-2, phosphorylated Akt, Hsp70, and C-Raf exist together (A, B, C, D, E, and F, respectively). β-actin and A-Raf was used as the negative controls, as expected any bands were observed for these proteins (G and H).

To verify the involvement of C-Raf in Bag-1-related cell survival complex, we applied co-immunoprecipitation for C-Raf. α-C-Raf antibody was used for the precipitation of C-Raf, and western blotting was performed to investigate indicated members of the survival complex (Figure 3.8). We confirmed that C-Raf exists in the complex along with B-Raf, Bag-1, Phospho-Akt, Bcl-2, and Hsp-70 (Figure 3.8-A to F). We used β-actin and A-Raf as the negative controls, as expected we did not observe any bands for these proteins (Figure 3.8-G and H).

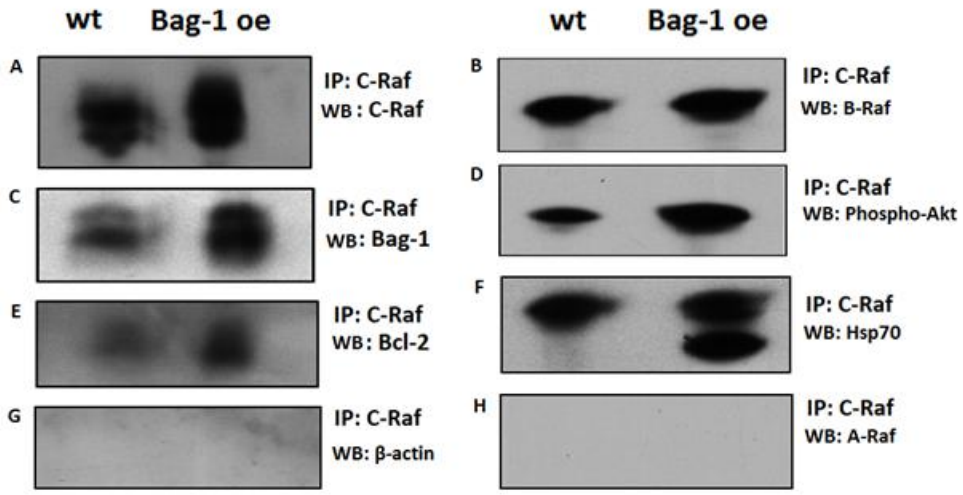

Figure 3.8 : Co-immunoprecipitation for α-C-Raf antibody confirmed that C-Raf's involvement in Bag-1-related survival complex. Western blotting for C-Raf, B-Raf, Bag-1, phosphorylated Akt, Bcl-2, and Hsp70 after C-Raf precipitation proved the co-existance of these proteins (A, B, C, D, E, and F, respectively). β-actin and A-Raf was used as the negative controls, as expected any bands were observed for these proteins (G and H).

3.5. Co-localisation of Bag-1 and its interacting partners

After detecting the co-existence of Bag-1 and its interacting partners, we used immunocytochemistry to display their co-localisation in the subcellular environment. We did double staining to Bag-1 overexpressed MCF-7 cells with Alexa Flour 647 goat anti-mouse for Bag-1 and Alexa Flour 488 goat anti-rabbit for the interacting partners. β-actin was used both as the negative control and reference protein. Since β-actin is a structural protein, it was useful to demonstrate the cell morphology (Figure 3.9-A). Co-localisation of Bag-1 with B-Raf, Bcl-2, Hsp70 and Phospho-Akt in cytoplasm can be seen at Figure 3.9- B, D, E and F, respectively. We also observed that C-Raf co-localisation with Bag-1 in the cytoplasm, but also C-raf was observed in the nucleus, revealing its secondary downstream role for the control of transcription in nucleus (Figure 3.9-C).

3.6. Alterations of Pro- and Anti-Apoptotic Protein Levels

As an anti-apoptotic protein Bag-1,it can get involved in the regulation of apoptosis. Thus, to investigate molecular mechanism of apoptosis in case of altered Bag-1 levels, we performed western blot analysis to determine expression levels of

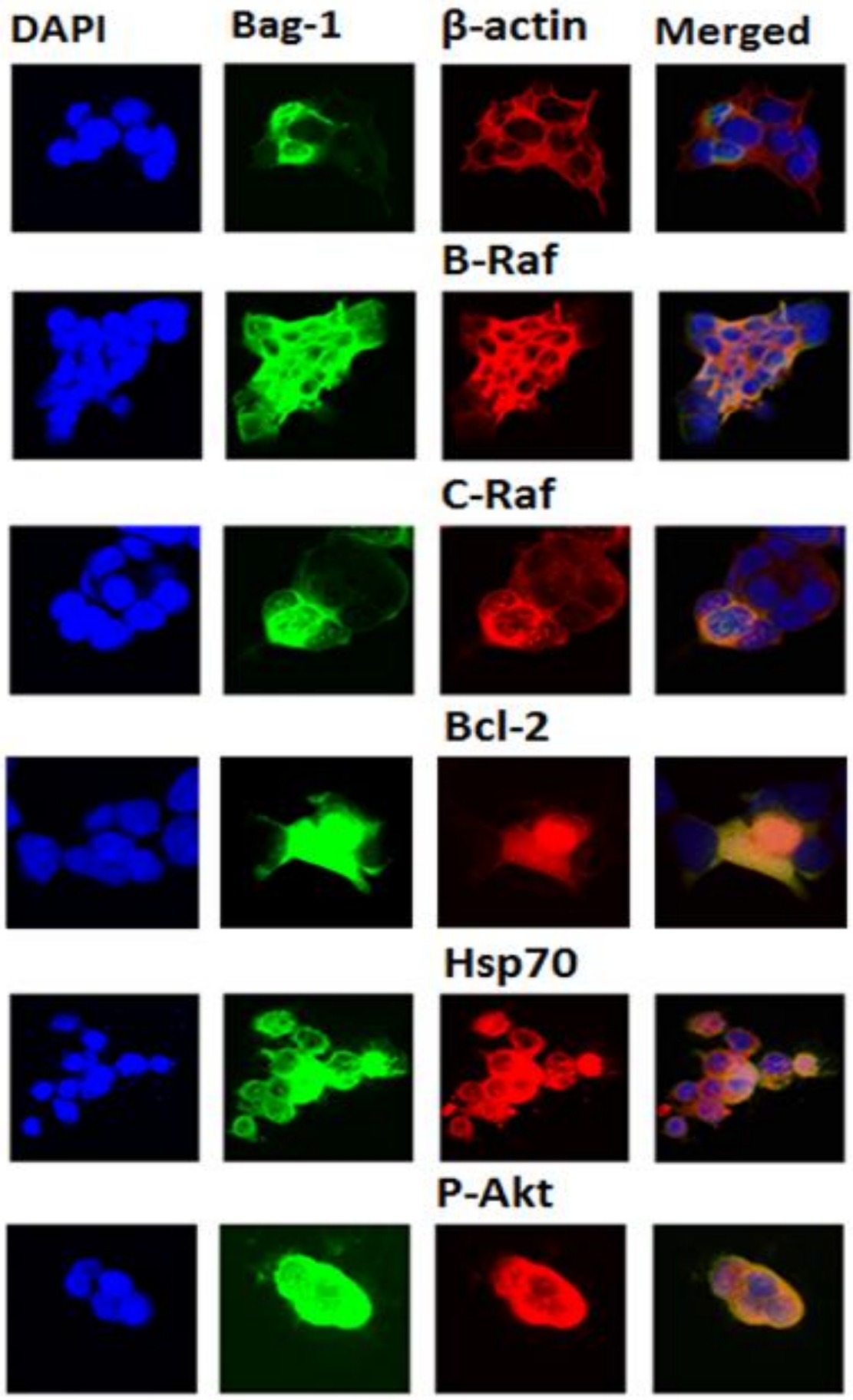
DAPI
Bag-1
β-actin
Merged
B-Raf
C-Raf
Bcl-2
Hsp70
P-Akt

Figure 3.9 : Subcellular co-localization of Bag-1 and its interacting partners in the survival complex. Since DAPI stains nucleus, it was used for detection of cells (Blue). Bag-1 dyed with Alexa Flour 647 goat anti-mouse (Green) while other indicated proteins dyed with Alexa Flour 488 goat anti-rabbit (Red). β-actin was utilized to demonstrate separate localisation with Bag-1 (A). Bag-1 co-localise with B-Raf, Bcl-2, Hsp70 and Phospho-Akt in cytosol (B, D, E, and F, respectively). We observed C-Raf co-localisation with Bag-1 in nucleus, but we also observed that C-Raf exists nucleus, independent from Bag-1 (C).

proteins and other anti-apoptotic proteins (Figure 3.10).

Bad and Bax are two members of Bcl-2 family. They both act as pro-apoptotic agents, but while Bad binds and regulates anti-apoptotic Bcl-2 proteins to promote apoptosis, Bax directly binds and inhibits Bcl-2 and other anti-apoptotic proteins. Bcl-2 is the most known anti-apoptotic protein which inhibits apoptosis, and promotes cell survival. When we altered the expression of Bag-1 protein, we did not observe any significant change on the Bad expression (Figure 3.10-A). However, we observed an increase on Bax expression with Bag-1 siRNA transfection, and Bax expression was decreased with Bag-1 overexpression (Figure 3.10-C). Bcl-2 expression level change was parallel to the Bag-1 expression level. There was an increase with Bag-1 overexpression, and a decrease with Bag-1 silencing (Figure 3.10-B). Ratio of pro-/anti-apoptotic proteins on mitochondrial membrane is used as a marker for apoptosis in cells. It is known that Bax/Bcl-2 ratio determines the susceptibility to apoptosis in melanoma cells by regulating mitochondrial function. Hence, we calculated the Bax/Bcl-2 ratio after both for Bag-1 overexpression and Bag-1 siRNA treatment of MCF-7 breast cancer cells. We could observe that Bax/Bcl-2 ratio decreased in Bag-1 overexpressed MCF-7 cells while dramatically increased in 48 h Bag-1 siRNA transfected MCF-7 cells. These results confirmed that Bag-1 downregulation increases sensitivity to apoptosis in MCF-7 cells (Figure 3.10-F)

We also checked Bim protein expression, which is an indicator of late apoptosis in certain carcinoma such as colon cancer and neuroblastoma. However our, results showed that expression of Bim isoforms was not altered by Bag-1 expression change (Figure 3.10-D).

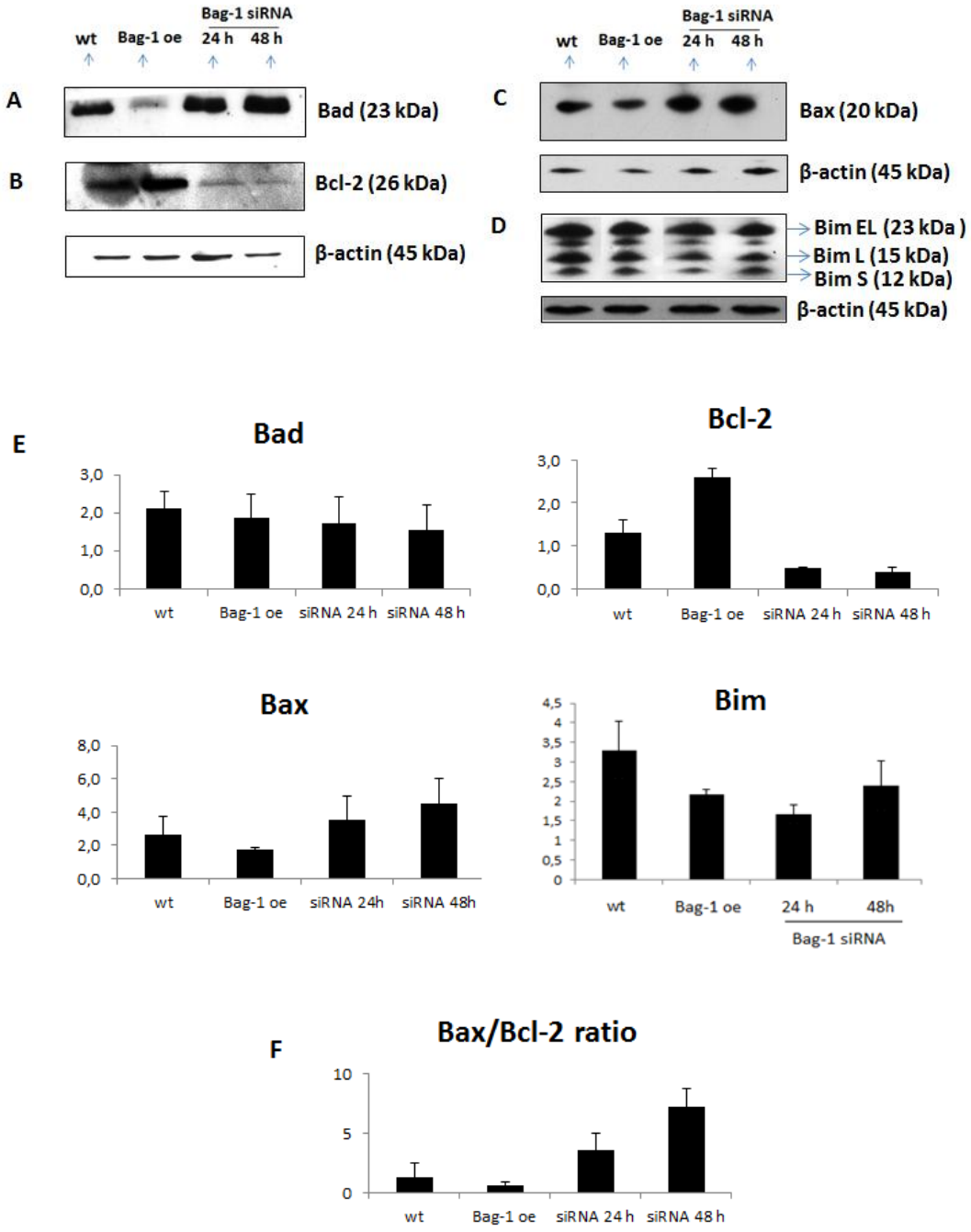

Figure 3.10 : Expressions of pro- and anti-apoptotic proteins on mitochondral membrane were altered by Bag-1 expression variations. Western blot analysis revealed that expression of pro-apoptotic proteins; Bad and Bim did not show any change by Bag-1 alterations (A and D). However expression of another important pro-apoptic, Bax, were upregulated by Bag-1 overexpression (C). Bcl-2, one of the most known anti-apoptotic proteins, expression change was parallel to the Bag-1 expression level (B). All results were normalized with β-actin, and densitometries of bands were calculated (E). Bax/Bcl-2 ratio was calculated to determine pro- and anti-apoptotic protein abundance on mithocondria (F).

3.7. Silencing of *bag-1* gene increases cell death in MCF-7 breast cancer cell line

To show the effect of Bag-1 protein level change on cell death, various fuorescent dyes were used (Figure 3.11).

Propidium iodide (PI) is an impermeant dye which stains dead cells and gets generally excluded from viable cells. PI intercalates into double stranded nucleic acids. Figure 3.11-A shows the PI-stained MCF-7 cells. First and second columnn were for wild type and Bag-1 overexpressed MCF-7 cells, where no PI staing were observed. With the transfection of Bag-1 siRNA to MCF-7 cells a significant increase in the amount of death cells were seen (third and fourth column of Figure 3.11-A).

Acridine orange is a cell-permeant nucleic acid binding dye which emits green fluorescence when bound to dsDNA and red fluorescence when bound to ssDNA or RNA. Wild type and Bag-1 overexpressed cells were dyed green because of their healty DNA. Since DNA is shreaded in apoptotic or death prone cells, they are dyed orange-red which can be seen in Bag-1 siRNA transfected cells at indicated time points (Figure 3.11-B).

DAPI is a nucleic acid stain that dye both healty and apoptotic cells. DAPI dyes healty cells blue (first and second column of Figure 3.11-C). However apoptotic cells were observed as bright dots because of the degradation of chromosomal DNA (third and fourth column of Figure 3.11-C).

$DiOC_6$ dyes endoplasmic reticulum of cells, vesicle membranes and mitochondria. Since healty cells have intact organelle membrane compartmentalisation, $DiOC_6$ stains healty cells green (first and second column of Figure 3.11-D). Apoptotic cells seem to be blurry-yellow because of the loss of membrane integrity of the organelles (third and fourth row of Figure 3.11-D).

3.8. Phosphorylation of Bad is upregulated by Bag-1 overexpression

Effects of Bag-1 overexpression on survival pathway proteins were demonstrated in Figure 3.5. We showed that increased amount of Bag-1 promotes cell survival by upregulating the expression levels of survival proteins such as C-Raf and B-Raf. To

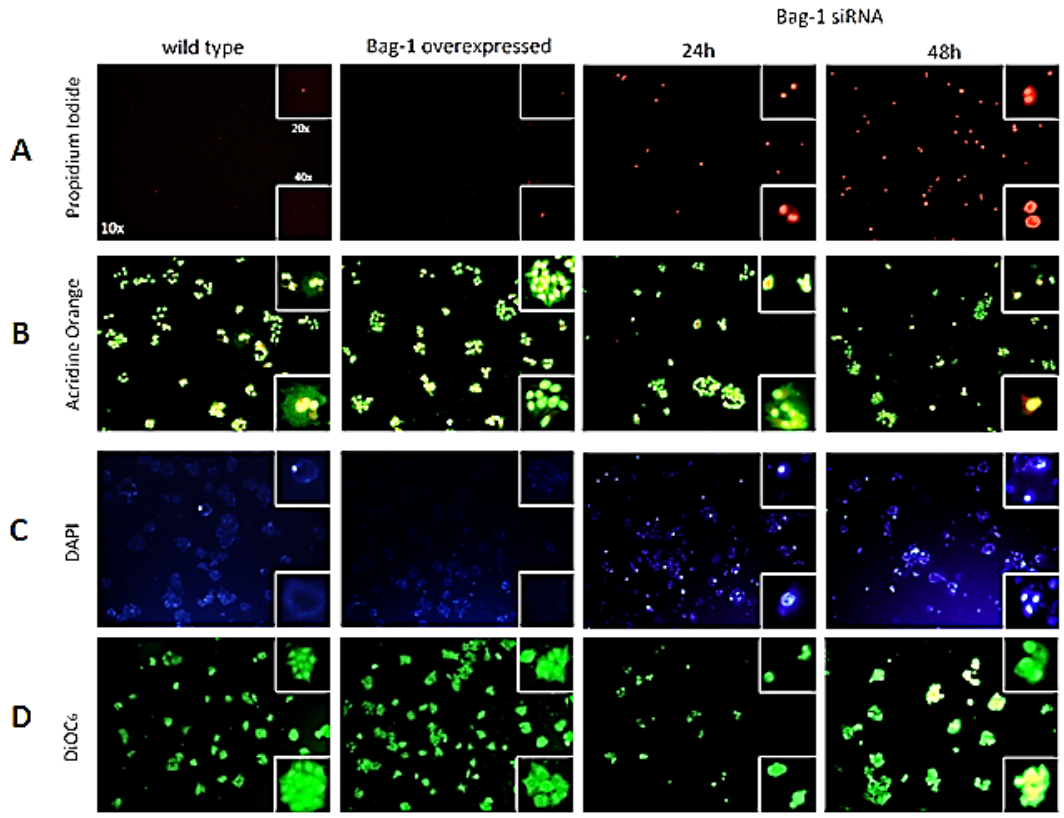

Figure 3.11 : Fluorescent staining showed that down regulation of Bag-1 elevated death cell population in MCF-7 culture. Propidium iodide, acridine orange, DAPI, and $DiOC_6$ stainings revealed that silencing Bag-1 increases cell death after 48 h Bag-1 siRNA transfection (A, B, C, and D, respectively).

investigate downstream mechanism of cell survival after Bag-1 overexpression, we investigated phosphorylation level of Bad protein. Although we did not see any significant change on Bad expression (Figure 3.12-A), we observed that phosphorylation level of Bad at Ser136 is dramatically upregulated by Bag-1 overexpression (Figure 3.12-B). Phosphorylation of Bad may lead to the prevention of cell death by a mechanism which includes selective association of the phosphorylated form of Bad with 14-3-3 scaffold protein isoforms. The interaction of Bad/14-3-3 occurs when Bad gets phosphorylated at serine 136, and triggered association of Bad with 14-3-3 appears to prevent Bad interactions with Bcl-XL and Bcl-2. Thus, interruption of Bad interactions with Bcl-XL or Bcl-2 inhibits pro-apoptotic oligomerization on mitochondrial membrane, which prevents cell death. Our results revealed that Bag-1 overexpression increases the phosphorylation level of Bad at Ser136, suggesting a route for cell survival through Bad/14-3-3 association.

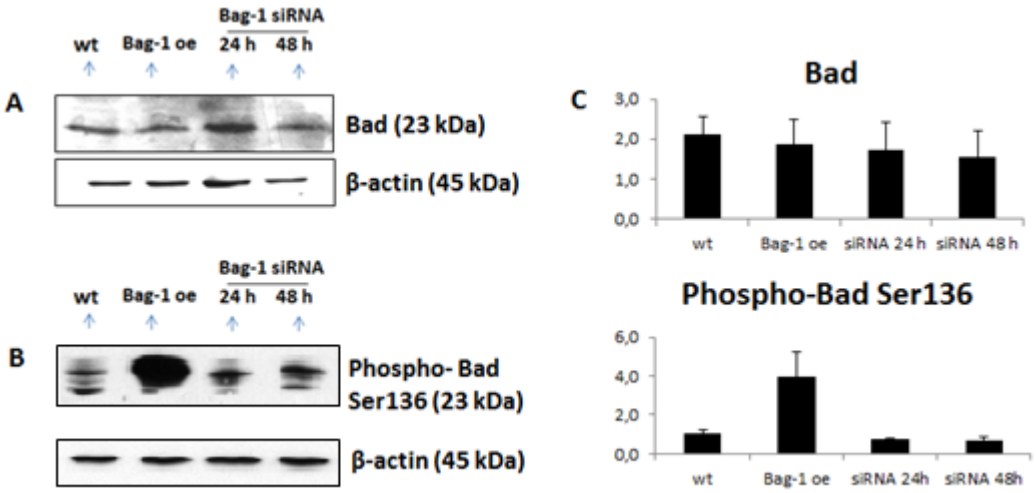

Figure 3.12 : Western blotting results showed that phosphorylation level of Bad at Ser 136 increases by Bag-1 overexpression. There was not any significant change in Bad expression by altered Bag-1 level (A). However, phoshorylation of Bad at Ser136 was significantly enhanced by the overexpression of Bag-1 (B).

4. DISCUSSION

Bag-1 (Bcl-2-associated athanogene) is an anti-apoptotic protein which involves a great number of molecular mechanisms such as ubiquitinilation, transcription, apoptotisis, cell survival, cell motility, proliferation, differantiation and tumorigenesis by means of its interactions with other proteins. Bag-1 protein has four major isoforms that are resulted from alternative translation; Bag-1L, Bag-1M, Bag-1S, and Bag-1 (Figure 3.1), and these isoforms have different impacts on heat shock protein function, ubiqutinilation machinery and different transcriptional activities. The subcellular localizations of Bag-1 isoforms differs between the isoforms; Bag-1S is located in cytosol while Bag-1L exists in the nucleus because of its nuclear localization signal. Bag-1M can usually be found in cytosol, but since it carries a part of the nuclear localization signal, it can be seen in the nucleus rarely (Sharp et al., 2004). Bag-1 is highly expressed in a variety of cancer types. The balance between apoptosis and cancer in the cell may be regulated by Bag-1 and its accompanying partners. Since Bag-1 is found near mitochondria and it has isoforms in both nucleus and cytosol, it can affect three central compartments of cells. Although it is known that Bag-1 interacts with Bcl-2 to inhibit apoptosis, Bag-1's role or molecular mechanism is not clear yet during the prevention of apoptosis or promotion of cell survival. However, it is important to consider Bag-1 as a prognostic marker or a therapeutic target in cancer (Townsend et al., 2003).

As it is shown in Figure 3.3, we studied altered levels of Bag-1 (overexpressed or silenced) to investigate effects of Bag-1 expression in Bag-1 involved molecular mechanisms. Preliminary data showed that overexpression of Bag-1 can be used as prognostic factor in several types of carcinomas (Tang et al., 1999). We demonstrated that forced-overexpression of Bag-1 increase resistance to cell death and promotes cell survival as shown with the trypan blue dye exclusion assay (Figure 3.4-A) and XTT cell proliferation assay (Figure 3.4-B).

Even though Bag-1 originally had been identified as a Bcl-2-interacting protein that potentiates its survival promoting activity, Bag-1 specifically interacts with C-Raf

and with B-Raf, but not with A-Raf. Binding of C-Raf to Bag-1 stimulates its kinase activity, and produces signals for cellular growth and differentiation. Hence it was hypothesized that Raf kinases which were activated by a Bag-1-dependent mechanism, acts as effector kinase of Bcl-2 phosphorylating targets such as Bad (Wang et al., 1996). We demonstrated that Raf kinases' protein levels, B-Raf and C-Raf, increased in Bag-1 overexpressed cells (Figure 3.5-A and C, respectively). Since phosphorylation stimulates the activities of Raf kinases and other survival pathway proteins, we checked their expressions via western blotting. We displayed that C-Raf phosphorylation at Serine 338 and Serine 289/296/301, which are activatory sites of C-Raf, residues increased in parallel with Bag-1 overexpression (Figure 3.5- D and E, respectively). Interestingly, we found that B-Raf phosphorylation at Serine 445 increases with Bag-1 overexpression as well (Figure 3.5-B). We also confirmed that expression of Hsp70 and phosphorylation levels of Akt at Serine 473 residue upregulated with Bag-1 overexpression (Figure 3.5-F and G, respectively). It is known that Bag-1 directly interacts with C-Raf to promote proliferation and cell survival (Song et al., 2001), but Götz et al. reported that activation of the MAPK pathway and FKHR phosphorylation is normal in *bag*-/- lack mice brain cells suggesting Bag-1 is not necessary for the activity of Raf kinases and Akt. However we investigate the downstream signaling pathway stimulated by C-Raf, and our results indicate that Erk1/2, MEK1/2, and phospho-MEK1/2 at Serine 221 expression levels elevated in parallel with increased C-Raf expression in Bag-1 overexpresssed MCF-7 cells (Figure 3.6).

It was denoted that expression of Bag-1 causes changes in MCF-10A cells (immortalized mammary ephitelial cells) grown in 3D cultures, namely a decrease in apoptotic cell number and prevention of apoptotic morphology. These effects are mediumted through the activation of the serine/threonine kinase C-Raf, with evidence of a direct interaction of Bag-1, then subsequent activation of a downstream signaling cascade by means of phosphorylation of MEK1/2 and Erk1/2 (Anderson et al., 2010).

In a study with neuronal cells, it was demonstrated that Akt, B-Raf, Bcl-2 and Hsp70 form a complex including Bag-1, which plays a crucial role on phosphorylation of Bad at Serine 136 residue, and leads cell to survival (Frabel et al., 2007). In light of our results and Anderson's studies, we thought that C-Raf might be involved in the

survival complex in cancer cells. To identify Bag-1 interacting partners during cell survival we performed co-immunoprecipitation with α-Bag-1 antibody and its interacting partners in survival complex. We precipitated Bag-1, then checked other proteins with western blotting using specific antibodies. The results presented that Bag-1 co-exists with B-Raf, phospho-Akt, Bcl-2, Hsp70 and C-Raf (Figure 3.7). We also did immunoprecipitation for α-C-Raf antibody to verify our findings. Western blot analysis for C-Raf precipitated proteins confirmed the co-existence of Bag-1, Hsp70, phospho-Akt, Bcl-2, B-Raf and C-Raf (Figure 3.8). Next step for the investigation of C-Raf as a part of survival complex was immunocytochemistry. Immunocytochemistry is suitable to show subcellular localisation of Bag-1 and its interacting partners by double staining. We observed that Bag-1 co-localise with phospho-Akt, Bcl-2, Hsp70, B-Raf and C-Raf in cytoplasm. However, we noticed that Bag-1 co-localise with C-Raf in nucleus, as well (Figure 3.9).

Since Bag-1 collaborates with Bcl-2, and promotes its anti-apoptotic function (Takayama et al., 1995), we wondered how Bag-1 effects other members of Bcl-2 family. Our results from western blot analysis confirmed that elevated expression of Bag-1 via forced overexpression increases Bcl-2 expression paralelly, and silencing Bag-1 decreases Bcl-2 expression in MCF-7 cells (Figure 3.10-B). We applied western blotting for pro-apoptotic agents of Bcl-2 family proteins including Bad, Bax and Bim. Bad is a BH3-only protein having conserved BH3 domain that can bind and regulate the anti-apoptotic Bcl-2 proteins to promote apoptosis while Bax directly binds and inhibits the Bcl2 and other anti-apoptotic family memebers (Youle et al, 2008). Our results for Bad showed any significant alteration on Bad expression either with Bag-1 overexpression or Bag-1 downregulation. However Bax expression increased in Bag-1 overexpressed MCF-7 cells, and elevated in 48 h Bag-1 siRNA treated MCF-7 cells (Figure 3.10-C). A study on melenoma cells showed that low Bax/Bcl-2 ratio was characteristic for death resistant cells and a high Bax/Bcl-2 ratio was characteristic for sensitive cells. Furthermore, they reported that Bcl-2 overexpression abolished apoptosis triggered by apoptotic stimuli like chemotherapeutic agents, confirming the importance of the Bax/Bcl-2 ratio as a rheostat that determines the susceptibility to apoptosis in melanoma cells by regulating mitochondrial function (Raisova et al, 2001). We calculated the Bax/Bcl-2 ratio after both for Bag-1 overexpression and Bag-1 siRNA treatment. We could

observe that Bax/Bcl-2 ratio decreased in Bag-1 overexpressed MCF-7 cells while dramatically increased in 48 h Bag-1 siRNA transfedted MCF-7 cells. These result confirmed that Bag-1 downregulation increases sensitivity to apoptosis in MCF-7 cells (Figure 3.10-F). It was noted in the paper by Anderson et al. (2010) that Bim expression increases in Bag-1 silenced MCF-10A cell line (also defined as normal cells). Nevertheless, our findings for Bim expression did not show any significant change in MCF-7 cells (Figure 3.10-D).

It was shown that forced overexpresssion of Bag-1 p50 (Bag-1L), p46 (Bag-1M), and p33 (Bag-1S) led to increased resistance to apoptosis (Chen et al., 2002). Also in a study on Hela cells revealed that a reduction of Bag-1 by antisense cDNA led to the sensitization to apoptosis when induced by several chemotheropeutic inducers such as staurosporine, paclitaxel, ATRA, and 4-HPR (Xiong et al., 2003). Furthermore, it was reported that decresing Bag-1 expression by means of RNA interference method incresed sensitivity to apoptosis (Takashi et al., 2003). To demonstrate effects of silenced Bag-1 we performed fluorescent staining using apoptotic markers including acridine orange, propidium iodide, DAPI, and $DiOC_{6.}$ Fluorescent stainings showed that apoptotic cell numbers increased in Bag-1 siRNA transfected MCF-7 cells (Figure 3.11). Bim expression can be affected through multiple signaling routes.

Since our results showed that Bag-1 overexpression increases expression levels of survival pathway proteins we would like to check phosphorylation levels of Bad, which prevent cell death and lead cells to survive. Altough expression of Bad, itself, did not show any significant changes in MCF-7 cells, our results diplayed that phosphorylated Bad at Serine 136 residue expression was significantly elevated in Bag-1 overexpressed MCF-7 cells (Figure 3.12). Phosphorylation of Bad mostly provided by Akt, but also B-Raf and C-Raf which are important kinases for neuronal survival (Wiesse et al., 2001). It was demonstrated that Bag-1, Akt, B-Raf, Bcl-2 and Hsp70 form a complex to phosphorylate Bad at Serine 136 residue, and leads cell to survival (Frabel et al., 2007). It is shown that Bag-1-related cell survival complex prevents Bad/Bcl-2 interaction by phosphorylatig Bad (Frabel et al., 2007). Phosphorylated Bad associates with 14-3-3 scaffold protein instead of activating cell death mechanism on mithochondria (Frabel et al., 2007). We found that C-Raf involves in Bag-1-related survival complex in MCF-7 breast cancer cells, which differs from neuronal cells. The reason for this might be that since we are using

cancerous cells, cell survival mechanism is triggered by the C-Raf activation in these cells.

In summary, Bag-1 overexpression decreases apoptosis, and promotes cell survival in MCF-7 breast cancer cells. During to the process of survival, expression of Raf kinases increases, and downstream signaling cascade MEK1/2 and Erk1/2 is become active. Bag-1 form a complex with its interacting partners to lead cells to survival. Our results suggest that C-Raf is involved in the complex that is formed by Bag-1, B-Raf, Hsp70, phospho-Akt and Bcl-2 in order to phosphorylate Bad at Serine 136 for the prevention of apoptosis. Thus, these results not only present a novel insight into deciphering the molecular mechanism of estrogen dependent breast cancer development, but also targeting Bag-1 or Bag-1/C-Raf interactions may provide an effective new strategy for therapeutic intervention.

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PUBLICATIONS/PRESENTATIONS ON THE THESIS

- **Demir S.,** Ahan R.E, Doganay G. D., 2012: "Identification of Bag-1 interacting partners from MCF-7 human breast cancer cell lines". *Keystone International congress* - Proteomics, Interactomes (F2), May 7-12, 2012, Stockholm, Sweden.

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