ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

CHARACTERIZATION OF BAG-1S/C-RAF INTERACTION TARGETING PEPTIDE

M.Sc. THESIS

Ecenur ÇEBİ

Department of Molecular Biology-Genetics and Biotechnology

Molecular Biology-Genetics and Biotechnology Programme

JULY 2022

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

CHARACTERIZATION OF BAG-1S/C-RAF INTERACTION TARGETING PEPTIDE

M.Sc. THESIS

Ecenur ÇEBİ
(521201110)

Department of Molecular Biology-Genetics and Biotechnology

Molecular Biology-Genetics and Biotechnology Programme

Thesis Advisor: Prof. Dr. Gizem DİNLER DOĞANAY

JULY 2022

ISTANBUL TEKNİK ÜNİVERSİTESİ ★ LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

BAG-1S/C-RAF ETKİLEŞİMİNİ HEDEFLEYEN PEPTİTİN KARAKTERİZASYONU

YÜKSEK LİSANS TEZİ

Ecenur ÇEBİ
(521201110)

Moleküler Biyoloji-Genetik ve Biyoteknoloji Anabilim Dalı

Moleküler Biyoloji-Genetik ve Biyoteknoloji Programı

Tez Danışmanı: Prof. Dr. Gizem DİNLER DOĞANAY

TEMMUZ 2022

Ecenur Çebi, a M.Sc.. student of ITU Graduate School student ID 521201110, successfully defended the thesis/dissertation entitled "CHARACTERIZATION OF BAG-1S/C-RAF INTERACTION TARGETING PEPTIDE", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor :	**Prof. Dr. Gizem DİNLER DOĞANAY** İstanbul Technical University	.....
Jury Members :	**Assoc. Prof. Ceren ÇIRACI** Istanbul Technical University	.....
	Asst. Prof. Hasan DEMİRCİ Koç University	.....

Date of Submission : 8 July 2022
Date of Defense : 25 July 2022

To my beloved grandparents,

FOREWORD

I would like to thank sincerely to people, who make this thesis possible, for their help, support and patience. First of all, I am grateful to my supervisor Prof. Dr. Gizem DINLER DOGANAY for giving me the great oppurtunity that being a member of her research team.

I would like to thank Ozge TATLI whom was not only a supervisor for four years but also an older sister and gracious friend for me I always admire. I also want to thank all GD Lab members for their help and friendship.

I would like to thank Betül Zehra Karakuş and Prof. Dr. Özge Can for peptide synthesis and Alp Ertunga Eyüpoğlu and Prof. Dr. Batu Erman for SPR experiments.

There is no such a word to express my feelings and gratitude to my family, my father Ismail CEBI, my mother Aysegul CEBI, and my sister Sude CEBI who have to put up me during my studies. With their unconditional love and support, I will always find the courage to move on and follow my dreams.

Last but not least I would like to thank myself for not giving up during any challenges and always pursing my dreams.

I would like to acknowledge TUBITAK for financial support (Project number: 117Z848 and 119Z261).

July 2022 Ecenur ÇEBİ

TABLE OF CONTENTS

Page

ABBREVIATIONS

BAG -1 **:** Bcl-2-associated athanogene 1
BAG -1L **:** Bcl-2-associated athanogene 1 Large
BAG -1M **:** Bcl-2-associated athanogene 1 Medium
BAG -1S **:** Bcl-2-associated athanogene 1 Small
Bcl-2 **:** B-cell lymphoma 2
BSA **:** Bovine Serum Albumin
DMEM **:** Dulbecco's Modified Eagle Medium
EDTA **:** Ethylenediaminetetraacetic acid
ERK **:** Extracellular signal-regulated kinase
Hsp70 **:** Heat shock protein 70
kDa **:** Kilodalton
LB **:** Luria-Bertani medium
MAP **:** Mitogen-activated Protein
MAPK **:** Mitogen-activated Protein Kinase
MEK **:** Mitogen-activated protein kinase
mRNA **:** Messenger Ribonucleic Acid
nm **:** Nanometer
pH **:** Power of Hydrogen
RAF **:** Rapidly Accelerated Fibrosarcoma
RAS **:** Rat sarcoma
SDS : Sodium Dodecyl Sulfate
SDS-PAGE **:** Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TBS **:** Tris-Buffer Saline
TBS-T **:** Tris Buffer Saline-Tween 20
TEMED **:** Tetramethylethylenediamine
µg **:** Microgram
µL **:** Microliter
µM **:** Micromolar

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHARACTERIZATION OF BAG-1S/C-RAF INERACTION TARGETING PEPTIDE

SUMMARY

ERK/MAPK cascade is one of the most important cellular pathways, which regulates many distinct physiological functions such as cell proliferation, migration, differentiation, and apoptosis. Since it has vital roles in many cellular functions, dysregulation of its members usually results in uncontrolled proliferation and development of cancer cells such as breast cancer. Ras and Raf serine/threonine protein kinases are mostly deregulated components of this pathway and mutations in both proteins have been identified approximately one-third of all human cancer types. Although there are several inhibitors against oncoproteins, because of the improving drug resistance, there is a need for the development of new drugs. One of the emerging interest is to target protein-protein interactions (PPIs) related with cancers.

Human Raf kinase family has three isoforms: A-Raf, B-Raf, and C-Raf. C-Raf and B-Raf are the important members of ERK pathway. Although B-Raf is dysregulated mostly, C-Raf has ability to suppress apoptosis and hence, both of the Raf kinase is vital for the cancer progression. The Raf kinases are regulated by several phosphorylation events and also, interactions with some proteins like BAG-1. Bcl-2 associated athanogene 1 (BAG-1) which is an anti-apoptotic co-chaperone protein is usually overexpressed in several cancer types making it possible oncoprotein. Moreover, its known that the interaction between BAG-1 and C-Raf promotes the C-Raf activation and stabilization. Therefore, targeting this interaction with small molecules and/or peptides would be effective therapeutics against different cancer types.

In our previous studies, the interaction surface between BAG-1S and C-Raf was determined and a peptidomimetic which coded as Pep3 was designed against the BAG-1S from natural sequence of C-Raf. The aim of this study was to characterize the interaction between BAG-1S and Pep3 and also, the interaction between BAG-1S and TPep3 which is cell penetrating form of Pep3. Firstly, His-tagged BAG-1S proteins were produced in mammalian and bacterial cells. Then, BAG-1S proteins were purified by Ni-NTA affinity purification in two steps. After first step, His-tag was cleaved with TEV protease and tagless proteins were eluted as flow-through in second step while the impurities and TEV proteases were remained bound to resin. Then, the purities of both proteins (produced in mammalian and bacterial) were calculated as >80% by SDS-PAGE. Secondary structure analysis of proteins was performed with circular dichroism and both of the proteins has been folded and showed primarily α-helix characteristics.

After BAG-1S proteins were characterized. Pep3 and TPep3 characterization were performed by mass spectrometry and circular dichroism analysis. Pep3 and TPep3 has been synthesized by Fmoc-based solid phase peptide synthesis and they were dissolved in 20 mM AMBIC and water respectively since the Pep3 shows hydrophobic characteristics. Molecular weights of peptides were measured as 2.066 kDa for Pep3

and 3.653 kDa for TPep3 which were compatible with the theoretical calculations. Both of the peptides showed β-sheet characteristics approximately 30% combined with random coils.

The interaction between BAG-1S and peptides were confirmed by crosslinking reaction with medium length crosslinker DSS (disuccinimidyl suberate). BAG-1S proteins were incubated with peptides separately to form interaction. After they formed interaction, the crosslink reaction was performed with 50-fold molar excess of DSS. The reactions were analyzed by immunoblotting. BAG-1S was shifted 2 kDa when interacts with Pep3 and 4 kDa with TPep3 comparing to the BAG-1S only reaction. These results were confirmed the interaction of BAG-1S with two peptides.

The binding kinetics of BAG-1S and Pep3 was measured by Surface Plasmon Resonance (SPR) with multi-cycle kinetics method. BAG-1S protein was captured on Protein G chip with Anti-BAG-1 and different concentrations of Pep3 was injected. Binding kinetics was calculated as 68.56 nM by 1:1 binding model showing that Pep3 binds BAG-1S with high affinity.

The effects of TPep3 on MAPK pathway and cell viability of MCF-7 breast cancer cells were analysed. MCF-7 cells were treated with different concentrations (0 uM to 50 uM) of TPep3 and total proteins were analysed with immunoblotting. C-Raf and p-C-RAf (S338) levels were decreased with the increasing concentration of TPep3. As a result of p-C-Raf inactivation, p-MEK levels were also decreased which showed the decrease in ERK pathway. Moreover, B-Raf and p-B-Raf (S446) levels were decreased. So, it can be said that peptide does not only affect the activitation of C-Raf but also B-Raf. To see the effects of peptide on other cell survival pathways, Akt and p-Akt (S473) levels were analysed and it was seen that their levels remained unchanged even with the highest peptide concentration. Lastly, the IC50 value was calculated as approximately 18 uM from the cell viability MTT assay. Together with all the results, TPep3 and Pep3 peptides have potential to be a promising therapeutics for cancer types relying on ERK pathway since the activity of pathway was decreased with the peptide treatment.

BAG-1S/C-RAF ETKİLEŞİMİNİ HEDEFLEYEN PEPTİTİN KARAKTERİZASYONU

ÖZET

Kanser dünya çapında ölümlerin en büyük sebeplerinden birisidir. Kanser hücreleri, sürekli çoğalma, vücudun başka bölümlerine metastaz yapma, hücre ölümüne karşı direnç gibi değişik özelliklere sahiptir. Bu özellikler hücresel yolakların düzensizliği yüzünden kaynaklanır ve devam ettirilir. Ras/Raf/MEK/ERK yolağı bu yolaklarlardan bir tanesidir. Ras/Raf/MEK/ERK yolağı (ERK yolağı), hücre çoğalması, migrasyonu, farklılaşması ve apoptoz gibi birçok farklı fizyolojik işlevi düzenleyen en önemli hücresel yolaklardan birisidir. Birçok hücresel fonksiyonda hayati rolleri olduğundan, üyelerinin düzensizliği genellikle meme kanseri gibi kanser hücrelerinin kontrolsüz çoğalmasına ve gelişmesine neden olur. Ras ve Raf serin/treonin protein kinazlar, bu yolağın çoğunlukla mutasyona uğrayan bileşenleridir ve her iki proteindeki mutasyonlar, tüm insan kanser türlerinin yaklaşık üçte birinde tanımlanmıştır. Raf kinazlara karşı geliştirilen birçok terapötik olmasına rağmen zaman içinde hastalar bu ilaçlara karşı direnç geliştirmektedir. İlaç direncinin artması nedeniyle de, yeni kanser ilaçlarının geliştirilmesine ihtiyaç vardır. Ortaya çıkan ilgi alanlarından biri, kanserlerle ilgili protein-protein etkileşimlerini (PPI'ler) hedeflemektir. Bu etkileşimleri hedeflemek için genelde yapısal biyoloji çalışmalarından yararlanılır ve küçük moleküller ya da peptidler tasarlanır.

Raf kinazlar (C-Raf ve B-Raf) ERK yolağının en önemli üyeleri olarak düşünülmektedir. İnsan Raf kinaz ailesinin üç izoformu vardır: A-Raf, B-Raf ve C-Raf. Kanser hücrelerinde çoğunlukla B-Raf mutasyona uğramasına ve düzensiz olmasına rağmen, C-Raf apoptozu baskılama yeteneğine sahiptir ve Ras-onkogen kanserlerde kanser oluşumu C-Raf üzerinden gerçekleşmektedir. Bu nedenle her iki Raf kinaz da kanser gelişmesi ve ilerlemesi için hayati öneme sahiptir. Raf kinazların stabilizasyonu ve aktivasyonu, domenlerindeki farklı fosforilasyon olayları tarafından ve ayrıca BAG-1 gibi bazı proteinlerle etkileşimleri tarafından düzenlenirler. Anti-apoptotik bir ko-şaperon proteini olan Bcl-2 ile ilişkili athanogen 1 (BAG-1), genellikle onkoprotein olarak çeşitli kanser türlerinde aşırı ifade edilir. İnsan BAG-1 proteinin ana 3 izoformu (BAG-1L, BAG-1M ve BAG-1S) bulunmaktadır ve bu 3 BAG-1 izoformu alternatif başlangıç tanslasyonu yoluyla tek bir mRNA'dan üretilmektedir. BAG-1 ve C-Raf arasındaki etkileşimin, C-Raf proteininin aktivasyonunu ve stabilizasyonunu desteklediği bilinmektedir. Ayrıca bu etkileşimin kanser hücrelerine uzun süreli sağkalımda da yarar sağladığı bilinmektedir. Bu nedenle, bu etkileşimin küçük moleküller ve/veya peptitler ile hedeflenmesi, BAG-1 proteinin aşırı ifade edildiği ve/veya Ras'ın düzensiz olduğu ve kanser hücrelerinin C-Raf üzerinden sağkalımı sağladığı kanser türlerine karşı etkili terapötikler geliştirilmesine olanak sağlayacaktır.

Daha önceki çalışmalarımızda BAG-1S ve C-Raf arasındaki etkileşim yüzeyi belirlenmiş ve C-Raf'ın doğal dizisinden BAG-1S'ye karşı Pep3 kodlu bir

peptidomimetik tasarlanmıştır. Pep3 peptidinin *in vitro* çalışmalarda BAG-1S ve C-Raf arasındaki etkileşimi bozduğu görülmüştür. Bu çalışmanın amacı, BAG-1S ile Pep3 arasındaki etkileşimi ve ayrıca BAG-1S ile Pep3'ün hücreye girebilen formu olan TPep3 arasındaki etkileşimi karakterize etmektir. Pep3 hidrofobik ve negatif yüklü bir peptid olduğu için hücre içine girebilen bir form değildir. Bu yüzden Pep3'ün N ucuna amino asitler eklenmiş ve hücreye girebilen formu (TPep3) tasarlanmıştır. İlk olarak, memeli ve bakteri hücrelerinde His-etiketli BAG-1S proteinleri üretilmiş ve daha sonra, BAG-1S proteinleri, iki aşamada Ni-NTA afinite saflaştırması ile saflaştırılmıştır. İlk adımdan sonra His-tag, TEV proteaz ile kesilmiş ve etiketsiz proteinler, ikinci adımda resine bağlanmamış olarak toplanırken, safsızlıklar ve TEV proteazlar resine bağlı kalmıştır. Daha sonra her iki saflaştırılmış proteinin (memeli ve bakteride üretilen) saflıkları SDS-PAGE ile >%80 olarak hesaplanmıştır. Proteinlerin ikincil yapı analizi sirküler dikroizm ile yapılmış ve 208 nm ve 222 nm'de minimum değerler gözlenmiştir. Bu sonuçlar, saflaştırılmış iki BAG-1S proteininin de katlı olduğunu ve α-heliks özellikleri gösterdiğini belirtmektedir.

BAG-1S proteinleri karakterize edildikten sonra. Pep3 ve TPep3 karakterizasyonu, kütle spektrometrisi ve sirküler dikroizm analizi ile gerçekleştirilmiştir. Pep3 ve TPep3, Fmoc bazlı katı faz peptit sentezi ile sentezlenmiştir. Pep3 hidrofobik olduğu için su yerine 20 mM AMBIC içinde çözülmektedir. TPep3 peptidi ise hidrofilik özellikler gösterdiği için su içinde çözülmüştür. Peptitlerin moleküler ağırlıkları Pep3 için 2.066 kDa ve TPep3 için 3.653 kDa olarak hesaplanmıştır. Bu değerler teorik olarak hesaplanan değerlerle uyum içinde olduğu içi peptitlerin doğru bir şekilde sentezlendiği kontrol edilmiştir. Peptitlerin ayrıca ikincil yapıları sirküler dikroizm yöntemi ile de kontrol edilmiştir. Pep3 %30 oranında β-tabaka özellikleri ve düzensiz sargı özellikleri göstermektediri. TPep3 peptiti de aynı şekilde çoğunlukla düzensiz sargı özellikleriyle birlikte β-tabaka özellikleri de göstermektedir.

BAG-1S ve peptitler arasındaki etkileşim, orta uzunlukta çapraz bağlayıcı DSS (disüksinimidil suberat) kullanılarak çapraz bağlama reaksiyonu ile doğrulanmıştır. Çapraz bağlama reaksiyonu kimyasal bir şekilde iki bağın kovalent olarak birbirine bağlanmasıdır. BAG-1S proteinleri, etkileşim oluşturmak için peptitler ile ayrı ayrı inkübe edilmiş ve etkileşim oluşturduktan sonra çapraz bağ reaksiyonu, 50 kat molar fazla DSS ile kurulmuştur. Reaksiyonlar immünoblotlama ile analiz edilmiştir. Sadece BAG-1S reaksiyonuna kıyasla Pep3 ile etkileşime girdiğinde BAG-1S proteini SDS-PAGE analizinde 2 kDa yukarıda yürümüş ve TPep3 ile etkileşime girdiğinde 4 kDa yukarıda yürümüştür. Bu yukarıda yürümeler peptitlerin ağırlıklarıyla uyumlu olduğu için BAG-1S proteininin peptitler ile etkileşimini doğrulamıştır.

BAG-1S proteininin Pep3 ve TPep3 peptitleri varlığındaki stabilitesi kısıtlamalı tripsinoliz yöntemi ile ölçülmüştür. BAG-1S proteini peptitler ile birlikte etkileşim kurması için inkübe edilmiştir. Daha sonra peptitlerin varlığında ve yokluğunda tripsin enzimi ile kesilmiştir. Tripsin enzimi ile kesilirken belli zaman sürelerinde tripsinli tüplerin içinden örnekler alınmış ve Laemmli ile reaksiyon susturulmuştur. Daha sonra immünblotlama yöntemi ile analiz edilmişlerdir. BAG-1S proteinlerinin her iki peptitin varlığında da tripsin ile tamamen kesilme süresinin uzadığı görülmüştür. Bu da BAG-1S proteininin her iki peptitle de etkileşimde olduğunu doğrulamış ve peptitlerin varlığında BAG-1S proteininin stabilitesinin arttığını söylemiştir. İki peptidin sadece BAG-1S protein örnekleri analiz edildiğine Pep3 proteininin kontrol örneğinin daha hızlı tripsinle kesildiği gözlenmiştir. Pep3 peptitli örneğin kontrolü olan sadece BAG-1S proteinine Pep3 20 mM AMBIC ile çözüldüğü için diğer örneğe eklenen peptid miktarı kadar 20 mM AMBIC eklenmiştir. Tripsin enziminin de

AMBIC varlığında aktivitesinin arttığı bilinmektedir. Bu yüzden iki BAG-1S arasındaki farkın AMBIC varlığında artan tripsin aktivitesi yüzünden olduğu düşünülmektedir.

BAG-1S ve Pep3'ün bağlanma kinetiği, çok döngülü kinetik yöntemiyle Yüzey Plazmon Rezonansı (SPR) ile ölçülmüştür. SPR iki molekül arasındaki bağlanma kinetiğini ve kinetik parametreleri ölçebilmeye yarayan bir yöntemdir. Bu deney için Protein G çipi kullanılmıştır. Öncelikler Anti-BAG-1 çipin üzerine kaplanmıştır. BAG-1S proteinleri Anti-BAG-1 yardımı ile çipin üzerine tutturulmuş ve daha sonra 500 nM'Dan 4000 nM'a kadar değişen konsantrasyonlarda Pep3 çipin üzerine enjekte edilmiştir. Belli bir süreden sonra enjeksiyon kesilmiş ve sonuçlar üzerinden bağlanma kinetiği hesaplanmıştır. Ayrıca artan Pep3 konsantrasyonlarında cihazdan alınan 'Response' değerlerinin de arttığı görülmüştür. Bu sonuç BAG-1S ve Pep3 arasındaki etkileşimin ikincil doğrulaması yerine de geçmektedir. Pep3 ve BAG-1S arasındaki bağlanma kinetiği 1:1 bağlanma modeli ile 68.56 nM olarak hesaplanmıştır. Bu değer nM seviyesinde olduğu ve 100 nM'ın da altında olduğu için Pep3 ve BAG-1S arasındaki etkileşimin yüksek afinite ile gerçekleştiği söylenebilmektedir.

TPep3 peptidi daha önce de söylendiği gibi Pep3 peptidinin N ucuna amino asit eklenmesiyle yeniden tasarlanmıştır ve hücre membranından geçebilme özelliğine sahiptir. TPep3'ün MAPK yolu üzerindeki etkileri ve MCF-7 meme kanseri hücrelerinin hücre canlılığı analiz edilmiş ve IC_{50} değeri hesaplanmıştır. MCF-7 hücrelerinde BAG-1 proteininin aşırı ifade edildiği bilinmektedir ve bu yüzden bu çalışma için MCF-7 hücre hattı kullanılmıştır. TPep3 peptitinin IC_{50} değeri MTT analizi ile 18 uM olarak hesaplanmıştır. Sonrasında MCF-7 hücreleri, farklı konsantrasyonlarda (0 uM ila 50 uM) TPep3 ile muamele edilmiş ve peptitle birlikte 24, 48 ve 72 saat inkübe edilmiştir. Daha sonra hücre proteinleri, immünoblotlama yöntemi ile analiz edilmiştir. TPep3 ile muamele edildikten 24 ve 48 saat sonrasında, artan TPep3 konsantrasyonu ile C-Raf ve p-C-Raf (S338) seviyelerinin azaldığı gözlenmiştir. Azalan p-C-Raf aktivitesinin sonucunda, p-MEK seviyelerinin de azaldığı gözlenmiş ve bu da ERK yolağındaki aktivitenin azaldığını göstermiştir. Ayrıca B-Raf ve p-B-Raf (S446) seviyelerinde de düşüş görülmüştür. Dolayısıyla peptidin sadece C-Raf aktivasyonunu etkilemediği, aynı zamanda B-Raf'ın aktivasyonunu da etkilediği söylenebilmektedir. 24 ve 48 saat analizlerinin aksine, TPep3 peptitdi ile inkübasyondan 72 saat sonra, p-C-Raf, C-Raf ve p-MEK seviyelerinin normale dönmeye başladığı ve peptitin etkisini kaybetmeye başladığı gözlenmiştir. Bunun sebebi olarak da iki olasılık düşünülmüştür: peptitin yarı ömrü kısa olduğu için peptit 48. saatten sonra etkisini kaybetmeye başlamıştır ya da C-Raf proteininin stabilizasyonu ve aktivitesi sadece BAG-1 proteini üzerinden düzenlenmediği için hücre 48. saatten sonra kendisini düzenlemek için diğer proteinleri ve yolakları aktive etmeyye başlamış olabilir. Son olarak TPep3'ün diğer hücre yaşam yolakları üzerindeki etkilerini görmek için Akt ve p-Akt (S473) seviyeleri analiz edilmiş ve en yüksek peptit konsantrasyonunda bile seviyelerinin neredeyse değişmediği görülmüştür. Son olarak, hücre canlılığı MTT deneyiyle ölçülmüş ve artan peptit konsantrasyonlarında hücre canlılığının azaldığı görülmüştür. Sonuç olarak Pep3 ve BAG-1S arasındaki etkileşimin yüksek afinitede gerçekleştiği gözlenmiş ve BAG-1S proteinin stabilitesinin TPep3 ve Pep3 varlığında stabilitesinin arttığı görülmüştür. Ayrıca TPep3 peptiti MCF-7 hücrelerinde 48 saate kadar ERK yolağının aktivitesinin azalmasına neden olmuştur. Tüm bu sonuçlar TPep3 peptitinin terapötik amaçlarla kullanabilme olasılığı olduğunu göstermiştir. Sonraki çalışmalar için farklı kanser hücreleri ve kombine terapiler kullanılabilir.

1. INTRODUCTION

1.1 Ras-Raf-MEK-ERK Pathway

Cells are able to respond extracellular signals and they can turn them into signals for cellular processes. In general, specific transmembrane receptors are activated by binding of extracellular ligands and this binding leads to transmission of signals via cellular signaling pathways (Shaul & Seger, 2007). Mitogen-activated protein kinase (MAPK) pathways are well-conserved pathways and they are said as one of the essential building blocks for intracellular signaling network since they regulate cell proliferation, survival, cell cycle progression, differentiation and development (Barbosa et al., 2021; Santini et al., 2019). There are four different defined MAPK pathways: ERK (extracellular signal-regulated kinase) pathway, JNK (c-Jun N-terminal kinase) pathway, p38 pathway and BMK-1 (Big MAP kinase-1) pathway (Burotto et al., 2014). ERK pathway (Figure 1.1) is one of the most studied MAPK pathway since it has role in proliferation, survival, differentiation and also, its members are upregulated in some of the cancer types (Dhillon et al., 2007). Initiation of the ERK cascade is usually occured by the activation of Ras, a small G protein, then, Ras protein activates Raf kinases . Raf kinases phosphorylate dual specificity MEK1 and MEK2 protein kinases which results in their activation. Then, activated MEK1 and MEK2 lead to dual phosphorylation of ERK1 and ERK2 kinases and activate them. Afterward, activated ERK proteins phosphorylate diverse cytoplasmic and nuclear substrates inducing changes in gene expression and/ or protein function (Burotto et al., 2014).

ERK pathway has a crucial role in the development and survival of cancer cells. Moreover, it is dysregulated nearly one-third of all human cancers. The dysregulation of ERK cascade is usually caused by the mutations on Ras and Raf kinases (Dhillon et al., 2007; Guo et al., 2019). Raf kinases could be accepted as key members of the ERK signaling pathway and they are used as targets for personalized cancer treatments (Jambrina et al., 2016). However, finding new therapeutics is of utmost importance,

since patients might develop drug resistance to Raf kinase inhibitors, which is a phenomenon known as “acquired drug resistance” (Haarberg and Smalley, 2015).

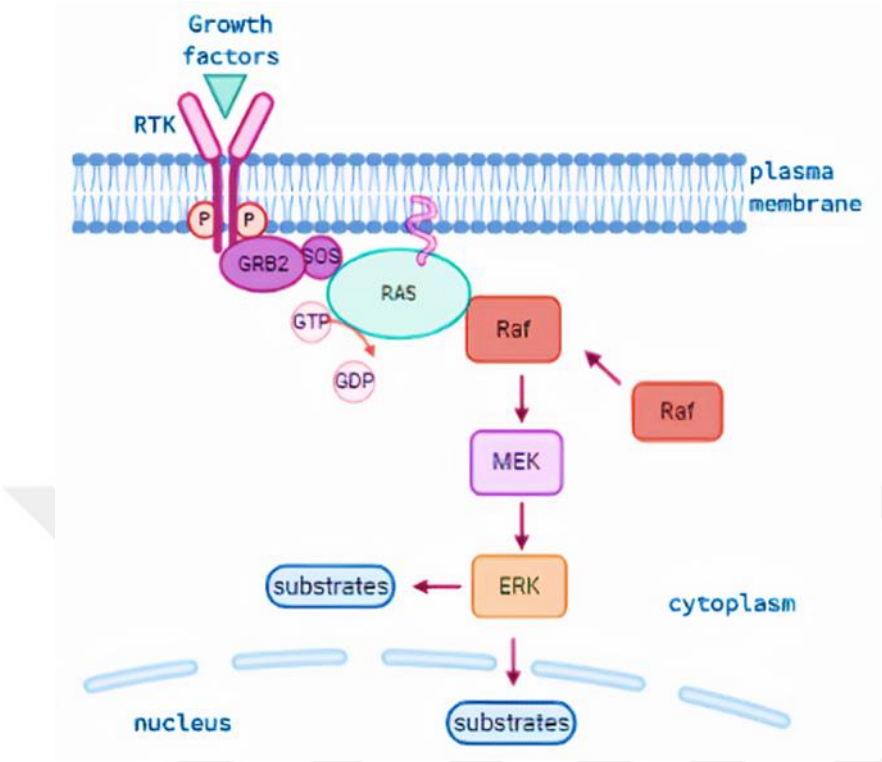


Figure 1.1 : The schematic representation of the Ras-Raf-MEK-ERK pathway. The pathway is initiated by the activation of Ras. Ras activates Raf kinases which activate MEKs. MEKs activate ERKs and activated ERKs phosphorylate several nuclear and cytoplasmic substrates (Adapted from Emuss and Marais, 2008).

1.2 Raf Kinases

1.2.1 General structure and functions of Raf kinases

Raf serine/threonine kinases are highly conserved protein kinase members of three-tiered ERK pathway. There are three mammalian isoforms of Raf kinase family: A-Raf, B-Raf and C-Raf/Raf-1 (Zhao & Luo, 2022). These three isoforms are common in terms of structural elements. They consist of three well conserved regions (CR) which have particular functions, CR1 and CR2 in the N terminus while CR3 in the C terminus (Figure 1.2). Conserved region 1 (CR1) contain a Ras-binding domain (RBD) that provide interaction with Ras and membrane phospholipids, leading to membrane recruitment. CR1 also contains a cysteine-rich domain (CRD) which function as secondary Ras binding site. Moreover, CR1 interacts with kinase domain via CRD for Raf autoinhibition regulation. Conserved region 2 (CR2) has important inhibitory phosphorylation sites. These sites have a role in negative regulation of Ras binding

and Raf activation. Conserved region 3 (CR3) contains kinase domain with activation segment. (Lavoie & Therrien, 2015; Matallanas et al., 2011). B-Raf also has a B-Raf specific region (BRS) in addition to three conserved regions. This region plays a role in heterodimerization with C-Raf and homodimerization (Terai & Matsuda, 2006).

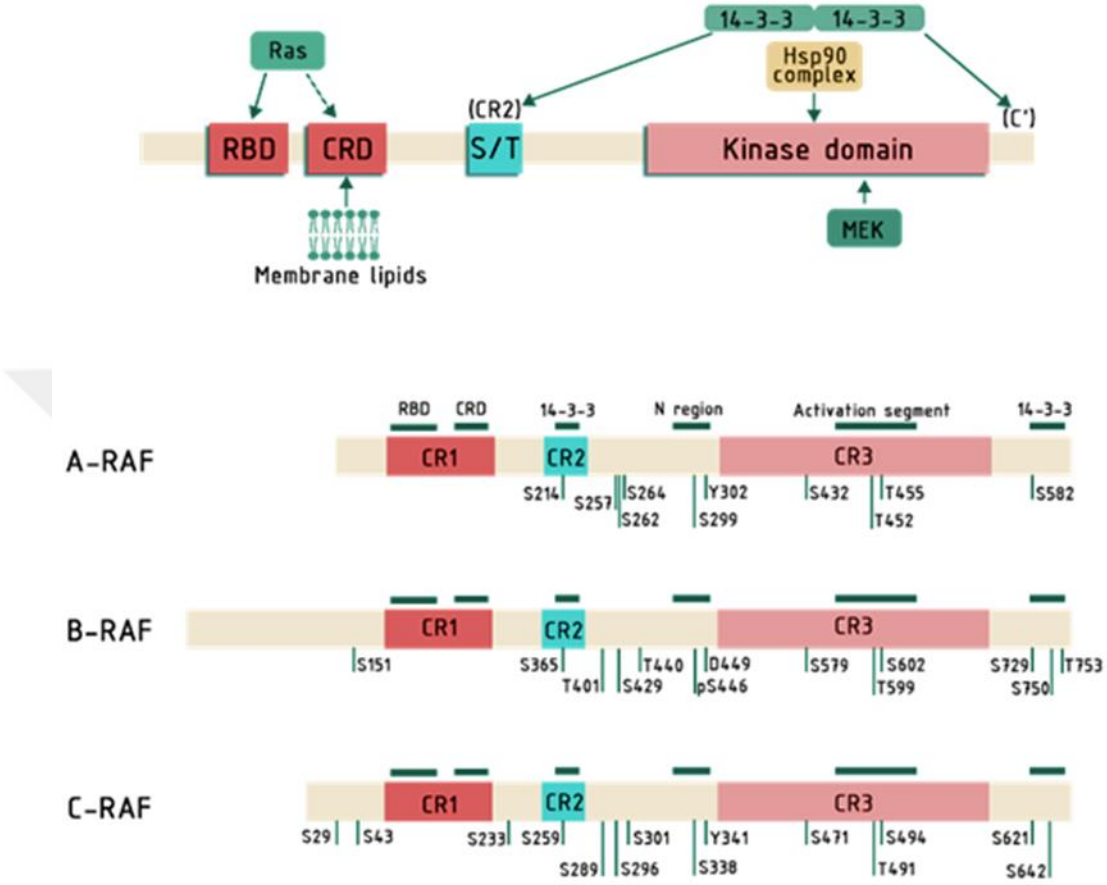


Figure 1.2 : Domain structure of Raf kinases and protein interactions. (Adapted from Roskoski Jr., 2010 and Terrell and Morrison, 2019).

In the absence of cellular stimuli, Raf kinases reside in the cytoplasm as auto-inhibited. Auto-inhibited Raf kinases are stabilized by 14-3-3 proteins that are bound to the phosphorylated serine residue at the amino terminus. After mitogen stimulation, Ras-GTP interacts with the Raf kinases through their RBDs and it initiates the recruitment of Raf kinases to the plasma membrane. Interaction with Ras also leads to dephosphorylation of a serine residue at amino terminus inducing the release of inhibitory 14-3-3. Translocation of Raf kinases to the plasma membrane provides contact with kinases that phosphorylate several activating sites in the negatively charged (N-) region. Ras clustering and membrane binding increase the Raf kinase concentration contributing to Raf dimerization which induces catalytic activity. The induced catalytic activity provides MEK phosphorylation and downstream signaling

which also implements negative feedback. Negative feedback is regulated by phosphorylation of inhibitory sites in distinct regions by ERK and also by dephosphorylation of Raf activatory phosphorylation sites (Matallanas et al., 2011; Lavoie and Therrien, 2015). The Raf activation cycle is shown in Figure 1.3.

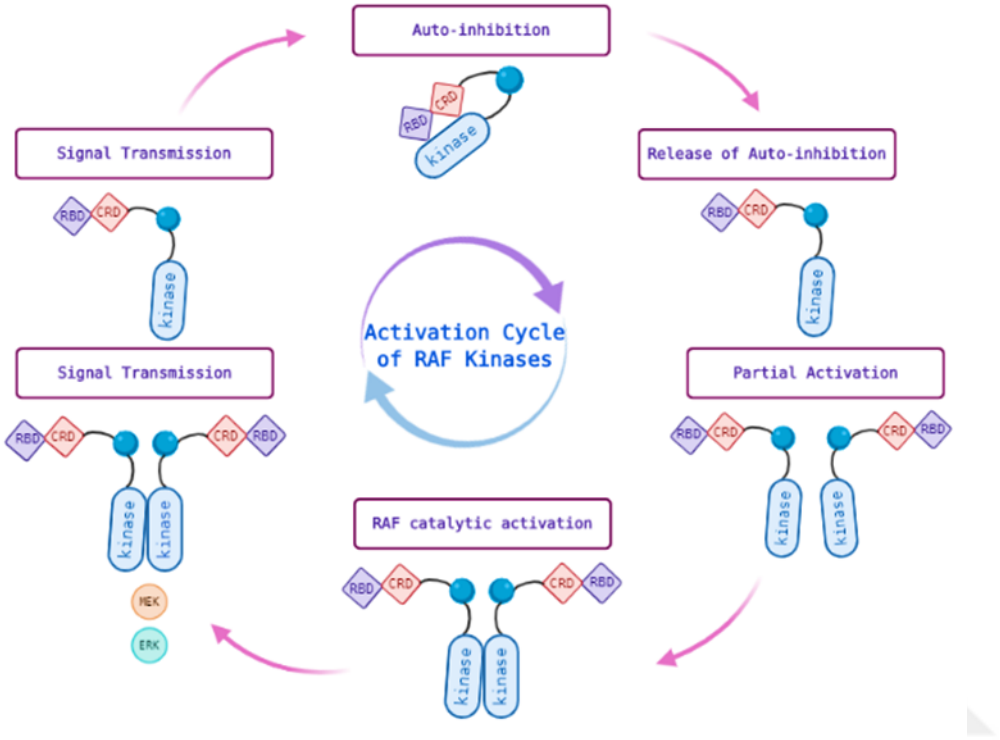


Figure 1.3 : Raf activation cycle. (Adapted from Lavoie and Therrien, 2015).

Raf kinases are core members of the ERK pathway and it has been shown that Raf kinases play important roles in many cellular processes such as cell cycle progression, cellular metabolism, and cell death (Leicht et al., 2007). Since they play significant roles in many physiological processes, dysregulation of Raf kinases results in the development and prolonged survival of cancer cells. Several gain of function mutations of Raf kinases have been previously described (Maurer et al., 2011).

1.2.2 C-Raf

C-Raf and B-Raf are key members of Ras/Raf/MEK/ERK pathway and their dysregulations mostly cause cancer cell development and proliferation. Apart from its kinase activity, C-Raf has also ability to hinder cell death with different ways. It has been shown that mouse embryos with *c-raf* did not live even though the ERK pathway was still activated showing that C-Raf is not only important for ERK pathway but also other cellular pathways (Galabova-Kovacs et al., 2006; Hu et al., 2013). Although the

mutations of C-Raf are rare than the B-Raf kinase, C-Raf is still associated with cancer cells and also, drug resistance (Zebisch et al., 2006; Zhao & Luo, 2022). The activation and stabilization of C-Raf has mediated by several factors and proteins such as Hsp-70 and BAG-1 (Song et al., 2001).

1.3 Bcl-2 Associated Athanogene (BAG-1)

BAG-1, a member of BAG family, is an anti-apoptotic and versatile protein which has a role in many cellular processes varying from apoptosis to proliferation and also, has interaction with many proteins like Bcl-2, Hsp70/Hsc70 (70 kDa heat shock proteins) and Raf-1 serine/threonine kinase (Liman et al., 2008; Tang, 2002; Townsend et al. 2003). Some major interaction partners of BAG-1 and cellular processes in which they have a role are shown in Figure 1.4.

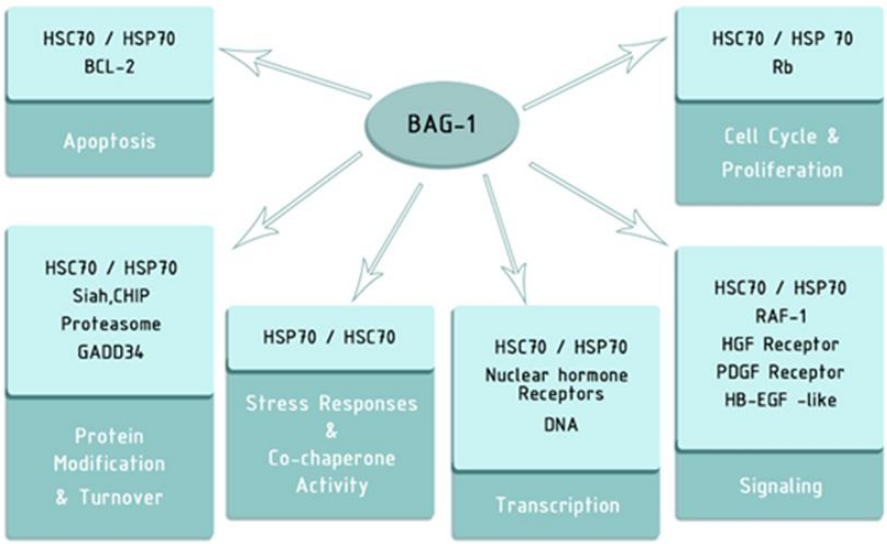


Figure 1.4 : BAG-1 interacting molecules and their function in cell (Adapted from Townsend et al., 2002).

Human BAG-1 gene is located on the 9th chromosome and it is comprised of seven exons. Human cells express three major isoforms of BAG-1 protein from single mRNA by alternative translation initiation mechanism: BAG-1L (52 kDa), BAG-1M (46 kDa), and BAG-1S (33 kDa) (Figure 1.5). Translation initiation codon "CUG" of BAG-1L is located at the upstream of the start site while BAG-1M and BAG-1S translation initiate at the first and second in-frame AUG codons, respectively (Townsend et al., 2003). The expression of BAG-1S is mediated by Internal Ribosome Entry Sequence (IRES)- dependent translation (Townsend et al., 2005). All BAG-1 isoforms have unique N-terminus and they share a common carboxy-terminus. Yang

et al. (1998) have also demonstrated fourth isoform weighing 29 kDa which is thought to be created by post-translational mechanisms in human cells. However, it cannot be detected all the time since its expression level is quite low (Aveic et al., 2011; Mariotto et al., 2020).

BAG-1 isoforms vary depending on the length of N-terminus which may cause functional diversity. BAG-1L has a nuclear localization signal at amino-terminus, while BAG-1M and BAG-1S do not have. Thereby, BAG-1L is located in the nucleus whereas others are predominantly found in the cytosol. Moreover, DNA- binding motif is found in BAG-1L and BAG-1M but it is absent in BAG-1S (Alberti et al., 2003; Townsend et al., 2003). Ubiquitin-like domain (UBL) and BAG domain (the conserved domain of approximately 100 amino acid), which are at C-terminus, are common for all isoforms (Figure 1.6). UBL of BAG-1 may have a role in the ubiquitin system as a regulatory, while the BAG domain is the part in which interaction with the Hsc70 and Hsp70 chaperones occur (Tang, 2002; Townsend et al., 2003).

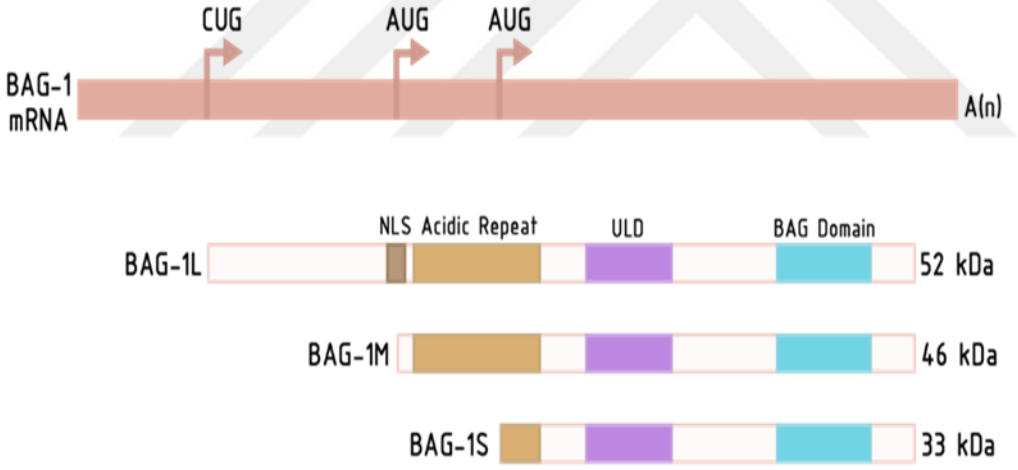


Figure 1.5 : Representative diagram of BAG-1 mRNA with alternative translation initiation sites and BAG-1 isoforms with their domains. (NLS, nuclear localization signal; UBL, ubiquitin- like domain). (Adapted from Cutress et al., 2002).

BAG domain, which forms three antiparallel helices is responsible for the interaction with ATPase domain of Hsp70/Hsc70, functioning as a nucleotide exchange factor (NEF). Binding of BAG-1 to ATPase domain leads to opening the nucleotide-binding cleft resulting in stimulation of release of bound ADP and polypeptide substrate from Hsc70 (Alberti et al.,2003; Mariotto et al., 2020).

The expression level of BAG-1 is increased in several human cancer types (Cutress et al., 2002). It was shown that BAG-1 overexpression may inhibit apoptosis induced by several inducers, such as heat shock, in distinct cell types leading to cell survival (Townsend et al., 2003).

1.4 Aim of the Study

MAPK/ERK pathway draw attention due to its functions on cell differentiation, growth, survival, and development. It has been shown that any dysregulation in ERK pathway components might lead to cancer development by causing one-third of all human cancers. Therefore, many inhibitors were designed to target this pathway's member. However, due to occuring drug resistance and paradoxical activation of other pathways, there is still need for the improvement of new cancer therapeutics. Targeting the protein-protein interactions to develop cancer drugs is becoming one of the emerging methods recently.

C-Raf is at the hub of ERK pathway and although its dysregulation is relatively less than Ras and B-Raf, it still promotes cancer cell survival especially, by becoming active when cells are treated with inhibitors targeting different oncoprotein. Therefore, developing a cancer therapeutic to target C-Raf would be efficient for treatments. BAG-1S is one of the interaction partners of C-Raf and it has been demonstrated that BAG-1S stabilizes and activate C-Raf. In previous studies done in our lab, a peptide has been designed to target C-Raf and BAG-1S interaction to decrease the kinase activity of C-Raf, hence, cell survival. The peptides (coded as Pep3 and TPep3) were designed to bind BAG-1S and it has been shown that Pep3 is able to disrupt interaction between BAG-1S and C-Raf. Thus, the aim of this study was to characterize the peptides and interaction of BAG-1S with peptides using several methods such as secondary structure analysis, crosslinking experiments and SPR analysis.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals, antibodies and commercial Kits

Chemicals, antibodies and commercial kits used in this study are represented in Appendix A.

2.1.2 Laboratory Equipment

The laboratory equipment used in this study are represented in Appendix A.

2.1.3 Buffers and Solutions

Separating (%12) and stacking (%5) gels for SDS-PAGE were prepared according to Table 2.1. Also, the recipe of 4X Laemmli buffer can be seen in Table 2.2.

Table 2.1 : Contents of separating (12%) and stacking (5%) gels.

Separating Gel (12%)	Volume	Stacking Gel (5%)	Volume
dH_2O	3.86 ml	dH_2O	2.92 ml
40% Acrylamide/Bisacrylamide	3.05 ml	40% Acrylamide/Bisacrylamide	540 µl
1.5M Tris-HCl pH: 8.8	3 ml	1.5M Tris-HCl pH: 6.8	500 µl
10% SDS	120 µl	10% SDS	40 µl
10% APS	120 µl	10% APS	40 µl
TEMED	12 µl	TEMED	4 µl
dH_2O	3.86 ml	dH_2O	2.92 ml

Table 2.2 : Contents of 4X Laemmli buffer.

4X Laemmli	Amount
Tris-HCl (pH 6.8)	0.2 M
SDS	8%
Glycerol	40%
EDTA	50 mM
β-Mercaptoethanol	4%
Bromophenol blue	0.08%

The recipes of 10X TBS, 10X Running Buffer (RB) and 10X Transfer Buffer are seen in Table 2.3.

Table 2.3 : Contents of 10X TBS, 10X Transfer Buffer (TB) and 10X Running Buffer (RB).

10X TBS (pH 8.0)	Amount	10X RB	Amount	10X TB	Amount
Tris Base	12.11 g	Tris Base	30.3 g	Tris Base	30.3 g
NaCl	87.66 g	Glycine	144 g	Glycine	144 g
dH_2O	to 1 L	SDS	10 g	dH_2O	to 1 L
		dH_2O	to 1 L		

2.2 Methods

2.2.1 Cell culture and protein isolation

HEK 293T (ATCC® HTB-22™) human embryonic kidney cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Thermo, USA) and 100 U/100 mg.ml-1 penicillin/streptomycin (Gibco, USA) in CO2 incubator (5% CO2 humidified air) at 37ºC. Since HEK 293T cells are known as their high capacity to produce recombinant protein production, they were used to produce recombinant BAG-1S production (Aricescu et al., 2006). After cells reached 90% confluency. media was removed and 1X 0.25% Trypsin-EDTA (Gibco, USA) was added to detach the cells for seeding. Detached cells were collected by centrifugation (300 xg, 5 minutes). Then, to each 100 mm cell culture dish, 3 x 10^6 cells were seeded and after they reached 90% of confluency, transient transfection was performed with cationic polymer, Polyethylenimine (PEI). For His_6TEVBAG-1S production, plasmid DNA (µg): transfection reagent (µl) ratio was 1:3. Appropriate amount of PEI (prewarmed at 37ºC) was added to 300 µl DMEM (each dish) and appropriate amount of DNA was added to another 300 µl DMEM, incubated for 5 minutes. Then, PEI mix was added onto the DNA mix and incubated for another 30 minutes. Mixture was added into each dish and proteins were collected after 48 hours.

In order to isolate proteins from transfected cells, firstly, media was discarded and cells were scraped with 1X TBS. They were centrifuged for 5 minutes at 5,000 xg, 4°C and supernatants were discarded. Pellets were lysed with RIPA lysis buffer (1% Triton X-100, 0.1% Sodium deoxycholate, 0.1% SDS, , 50 mM Tris-HCl , 150 mM NaCl, pH 7.4) supplemented with 5 mM PMSF and protease inhibitor cocktail (Roche, Switzerland) and incubated in ice for 20 minutes. Lysates were centrifuged for 20 minutes at maximum rate, at 4°C to remove cell debris after incubation. Supernatants were collected and stored in -80°C for further purification assays.

2.2.2 Purification of His_6TEVBAG-1S

His_6TEVBAG-1S proteins were purified by using Ni-NTA agarose resins (Qiagen, USA) in two step affinity purification since the protein is His-tagged. Briefly, cell lysates containing overexpressed His-tagged BAG-1S were incubated with Ni-NTA resin for 3 hours at 4°C by rotating. After incubation, supernatant was discarded and resin was washed with Wash Buffer (50 mM HEPES, 20 mM imidazole, 10% glycerol, 500 mM NaCl, 0.5% Triton X-100, pH 8.0) three times. Then, resin was washed with ATP Wash Buffer (20 mM HEPES, 20 mM MgCl2, 10% glycerol, 150 mM KCl, 10 mM ATP, pH 7.4) twice by incubating at 37°C for 10 minutes in order to remove chaperones that bound to the BAG-1S. Resin was washed with Wash Buffer once more and then, proteins were eluted with Elution Buffer (100 mM sodium phosphate, 10% glycerol, 500 mM NaCl, 500 mM imidazole, pH 7.8). To remove the His-tag, proteins were treated with TEV Protease (Invitrogen, USA) overnight at 4°C according to the manufacturer's protocol. After overnight incubation, proteins were incubated with Ni-NTA resin again and flow thhrough was collected because His-tag, TEV protease and nonspecific contaminants remain bound to resin. Lastly, buffer exchange was performed with Equilibration Buffer (20 mM HEPES, 150 mM NaCl, pH 7.4) and protein was concentrated. The concentration of protein was calculated with SDS-PAGE and protein was stored at -20°C for further experiments.

2.2.3 Production and purification of His_6TEVBAG-1S in *E. coli*

Full length HisTEVBAG-1S was produced in *E.coli* cells at 37°C. Since it has His-tag, they were purified with affinity chromatography in two steps like mammalian protocol. His-tag was cleaved with TEV protease and then, size exclusion chromatography was performed to eliminate remaining TEV proteins. Lastly, buffer exchange was performed with Equilibration Buffer 2 (20 mM HEPES, 150 mM KCl, 1 mM $MgCl_2$, pH 7.4) and protein was concentrated. Purified protein concentration and purity was measured with SDS-PAGE. Purified proteins were stored at -80°C for further experiments.

2.2.4 Mass spectrometry analysis of peptides

Molecular weight analysis of peptides was performed by using Acquity UPLC coupled with Waters Synapt G2-Si HDMS (Waters Corp). Pep3 and TPep3 samples were loaded on BEH C18 RP analytical column (temperature: 65°C) and peptide separation

was done with a gradient of 1-40% mobile phase B (acetonitrile) over 10 min followed by a quick ramp from 40% to 80% in 1 min, and isocratic elution at 80% for 2.5 min. Re-equilibration of the column was carried out over 10 min. Mobile phase C (water with 1% formic acid) and flow rate were kept constant at 10% and 200 µL/min, respectively.

All analyses were performed in positive electrospray ionization and resolution mode. Glu-fibrinopeptide B was infused to calibrate the time-of-flight analyzer from 50 to 2000 m/z. It was also used as an internal calibrant in reference spray with a frequency of 30 s-1. MassLynx Software was used for the data analysis (Waters Corp).

2.2.5 Crosslinking

In order to confirm the interaction between BAG-1S and Pep3, crosslinking reaction was performed with DSS crosslinker (Thermo Scientific, USA). Briefly, purified BAG-1S protein was incubated with 50-fold molar excess of Pep3 or TPep3 for 45 minutes at 37ºC to form interaction. DSS crosslinker was freshly prepared by dissolving appropriate amount of DSS in DMSO. After interaction is formed, 50-fold molar excess of DSS crosslinker was added into the mix and mixture was incubated for 30 minutes at room temperature. (BAG-1S only condition was used as control.) Then, reaction was quenched with addition of 50 mM Tris. The crosslinked proteins were analyzed with SDS-PAGE.

2.2.6 Secondary structure analysis

The secondary structures of BAG-1S, Pep3, TPep3 and BAG-1S together with Pep3 was analyzed by using circular dichroism spectrometry (Jasco J-1500-150). 2 µM BAG-1S samples and 50-fold molar excess of peptides were used. In order to analyze samples, far-UV CD spectra which was set to 195-250 nm were measured at 25°C. The samples were scanned as three replicas.

2.2.7 Binding kinetics analysis of Pep3 with BAG-1S

Surface Plasmon Resonance (SPR) was performed using a BIAcore T200 system (Cytiva, Sweden) with commercially available Series S sensor chip Protein G. Anti-Bag1 (Mouse mAb, CST) was captured on flow cell 1 and 2 to ~15 RU at flow rate, 10 ul/min for 60 s. Following, in-house expressed and purified Bag1 protein was captured on only flow cell 2 at flow rate, 10 ul/min for 60 s. For kinetic measurements,

HBS-EP+ (0.1 M HEPES with 1.5 M NaCl, 0.03 M EDTA and 0.5% v/v P20 Surfactant) was used as running buffer and all peptide dilutions were prepared with this buffer. Binding reactions were performed at 25°C and the samples were kept at 12°C. To assess binding kinetics and to avoid bulk effects, the multi-cycle kinetics method was used with the following dilutions, 0 nM, 500 nM, 1000 nM, 2000 nM, 4000 nM. Peptides were injected with 60 s association and 30 s dissociation at a flow rate of 30 µl/min through flow cell 1 and 2. Binding kinetics were calculated using Biacore T200 Evaluation Software (version 3.2.1, Cytiva) with the 1:1 binding model.

2.2.8 Limited trypsinolysis

In order to confirm interaction and also, stability, limited proteolysis was performed with trypsin digestion. Firstly, 2 uM BAG-1S proteins were incubated with 50-fold molar excess of Pep3 and TPep3 separately at 37^{o}C for 45 minutes to form interaction. Then, BAG-1S proteins with or without peptides were digested with Trypsin (Promega) at 37^{o}C. Samples were taken at certain time points (0 min, 1.5 min, 5 min, 10 min, 15 min, 45 min, 90 min). Reaction was quenched with 4X Laemmli buffer and samples were analyzed by immunoblotting. Datas were analyzed by normalizing values to 0 min. (20 uM AMBIC was also added to the BAG-1S only protein used for Pep3 analysis to eliminate the effect of AMBIC since Pep3 was dissolved in AMBIC.)

2.2.9 Treatment of MCF-7 cells with TPep3

To evaluate the effects of TPep3 on MAPK pathway and especially C-Raf, MCF-7 cells ($7x10^5$ cells) were seeded to each well of 6-well as duplicates for 3 different time points (24 h, 48 h, 72 h). After 24 hours, cells were treated with varying concentrations of TPep3 (1 µM, 5 µM, 10 µM, 20 µM and 50 µM) and one well was treated with water as negative control since the peptide was dissolved in water. Then, total proteins were isolated after 24 hours, 48 hours and 72 hours with NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris, pH: 7.4) supplemented with PMSF, Na_3VO_4, phosstop (Roche) and protease inhibitor cocktail (Roche) . Concentrations of total proteins were measured with Brafdord Assay by using BSA standards. The samples were analyzed by immunoblotting.

2.2.10 MTT cell viability assay

Cell viability of MCF-7 cells after treated with TPep3 was calculated by MTT assay. For this purpose, $4x10^3$ MCF-7 cells were seeded to each well of 96 well. After 24 hours, cells were treated with different concentrations (0 μM, 1 μM, 10 μM, 50 μM, 100 μM, 200 μM, 500 μM, and 1000 μM) of TPep3 as duplicates. After 48 hours, cells were incubated with MTT reagent for 3 hours and then formed crystals were dissolved with DMSO. Then, the absorbance values were measured at 570 nm and using 630 nm as reference. Absorbance values were normalized to control (0 uM) and represented as percentage. From the plot, IC50 value was calculated.

2.2.11 Statistical analysis

All of the datas of replicates were analyzed by using GraphPad Prism with Student's t test.

3. RESULTS

3.1 Characterization of BAG-1S

BAG-1S is a cochaperone protein which has shown to have a role in different cellular pathways and found as overexpressed in several cancer types. Moreover, it has been shown that it has a role in the activation and stabilization of C-Raf (Song et al., 2001; Wang et al., 1996). In our previous studies, interaction site between BAG-1S and C-Raf was discovered and a peptidomimetic inhibitor against BAG-1S was designed by using the natural sequence of C-Raf. To characterize interaction between BAG-1S and peptide, firstly, His-tagged BAG-1S protein was produced in mammalian and bacterial cells. Then, produced proteins were purified by two step affinity purification as described in Methods. The purity of proteins from mammalian (Figure 3.1a) and bacterial (Figure 3.1b) cells were calculated as >90% and >80% respectively by SDS-PAGE analysis.

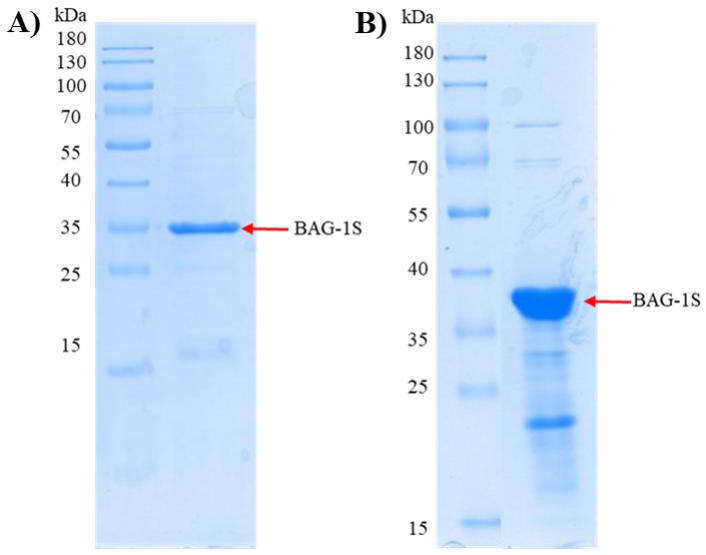


Figure 3.1 : SDS-PAGE analysis of purified BAG-1S. The thick band around 33 kDa (indicated with red arrow) represents the purified tagless full-length BAG-1S a) mammalian expression b) bacterial expression.

The secondary structure analysis of purified BAG-1S proteins were performed by using circular dichroism (Figure 3.2). Circular dichroism is a method which able to

analyze the secondary structure of proteins and peptides and also, it is a rapid method to determine if the purified proteins are folded. 2 μM of each purified protein was used to analyze and it was seen that both of the proteins were folded and as it was expected they were mainly found as α-helix combined with β-sheet (Briknarová et al., 2001). The percentage of secondary structure was quantified by using BeStSel webserver and it was calculated that mammalian BAG-1S had 73% α-helix while bacterial BAG-1S had 41% α-helix (Wien et al., 2022).

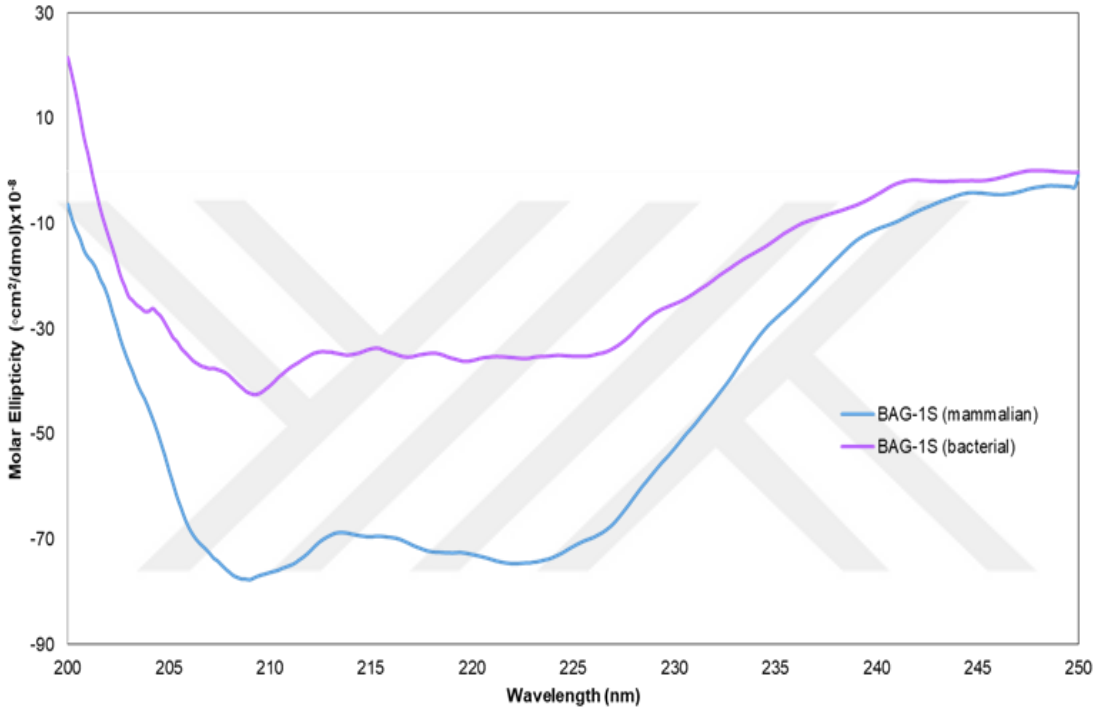
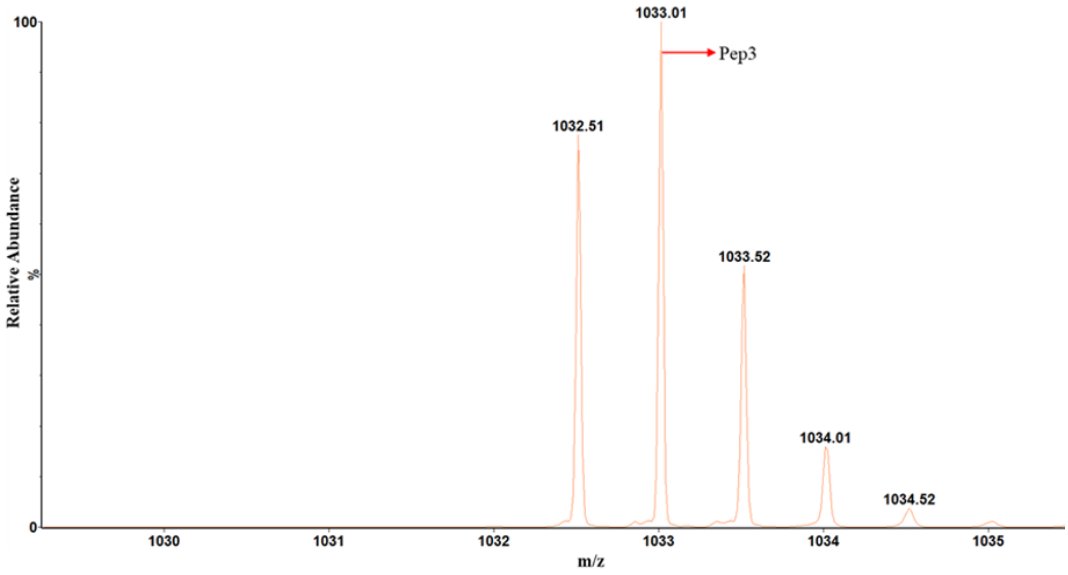


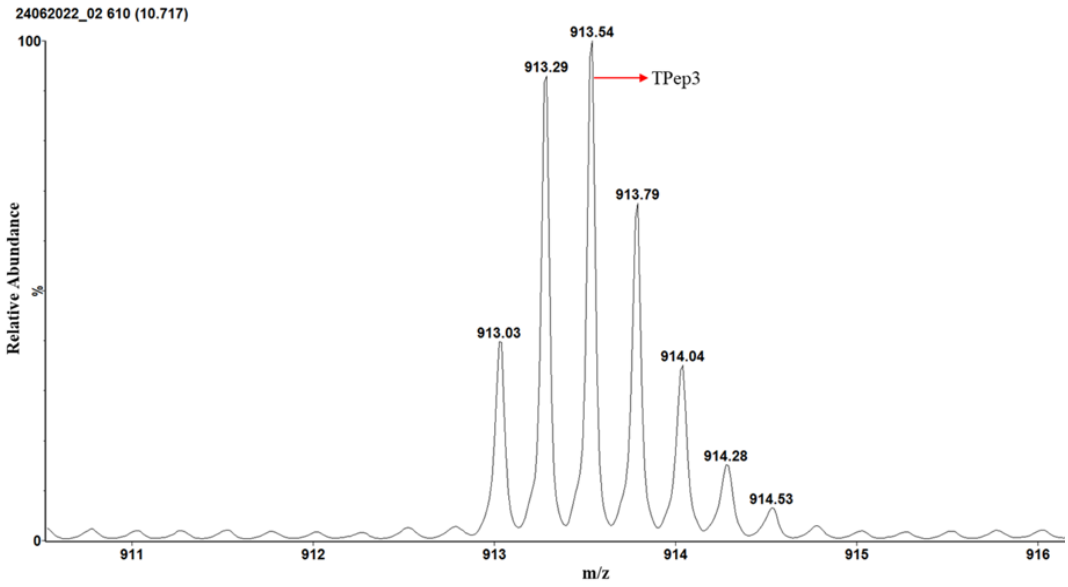
Figure 3.2 : Secondary structure analysis of mammalian (blue) and bacterial (purple) BAG-1S. Both of the proteins has folded and showed primarily α-helix characteristics together with β-sheet structure.

3.2 Characterization of Pep3 and TPep3

The peptidomimetic designed against BAG-1S and its cell penetrating version which were coded as Pep3 and TPep3 respectively, has been synthesized by Fmoc-based solid phase peptide synthesis. Since Pep3 shows hydrophobic characteristics, it was dissolved with 20 mM AMBIC while TPep3 was dissolved in water. Their molecular weight were analyzed by mass spectrometry (Figure 3.3). The molecular weight of peptides were calculated as 2.066 kDa and 3.653 kDa for Pep3 and TPep3 respectively. These results were compatible with the theoretical values calculated by ExPASy server (Gasteiger et al., 2005).



a) Pep3



b) TPep3

Figure 3.3 : The mass analysis of **a)** Pep3 and **b)** TPep3 by mass spectrometry. The highest peaks represent Pep3 and TPep3 as indicated with red arrow. Since the increase of Pep3 and TPep3 peaks was 0.5 (showing the charge as 2) and 0.25 (showing the charge as 4), the values were multiplied with 2 and 4 and their molecular weights were calculated as 2.066 kDa and 3.653 kDa respectively.

After the molecular weight of peptides were confirmed, their secondary structures were analyzed with circular dichroism (Figure 3.4). 100 µM of each peptide was used to analyze and then, the data were quantified with BeStSel webserver. According to analyze, Pep3 shows 36.3% β-sheet characteristics while TPep3 shows 34% β-sheet and main random coil characteristics.

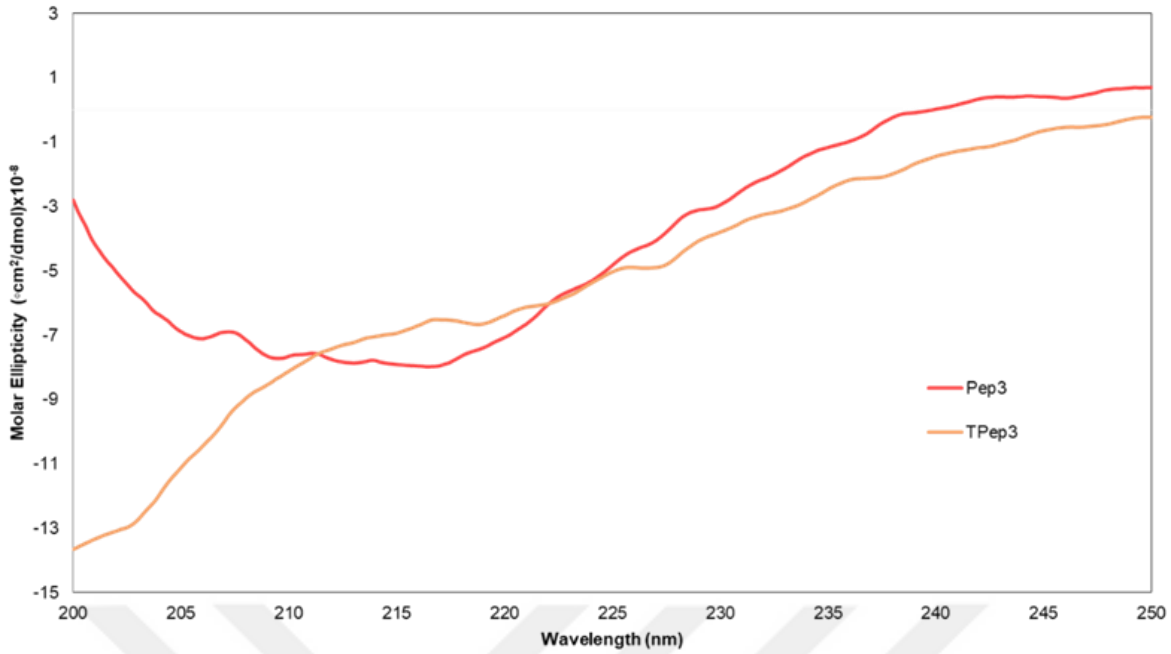


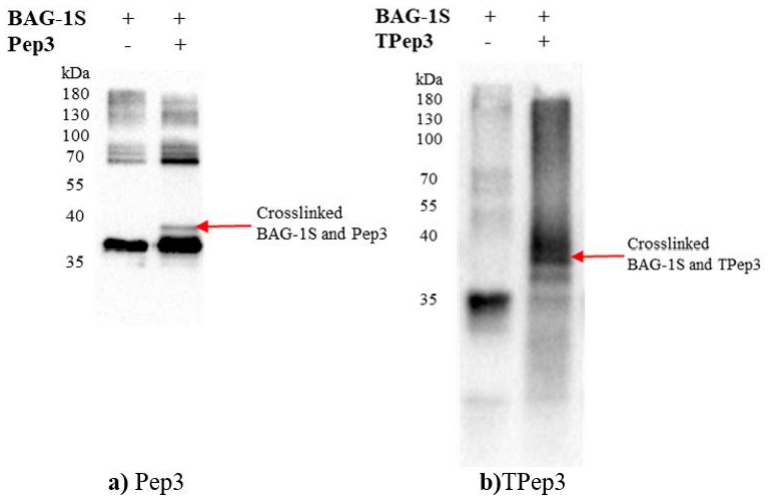
Figure 3.4 : Secondary structure analysis of Pep3 (red) and TPep3 (orange). Both of the peptides showed β-sheet characteristics while TPep3 showed also main random coil structure according to the analysis by BeStSel.

3.3 BAG-1S Interacts with Pep3 and TPep3

The interaction of BAG-1S with Pep3 and TPep3 was confirmed with crosslinking analysis. For crosslink reactions, mid-length (11.4 Å) crosslinker DSS was used. DSS is a NHS-ester crosslink reagent and form amide bonds with primary amino groups of proteins and peptides (Zybailov, 2013). After interaction was formed between BAG-1S and peptides by incubation at 37oC separately, the complexes were incubated with 50-fold molar excess of DSS crosslinker for 30 minutes at room temperature and reaction was quenched with Tris. The crosslinked proteins were analyzed by immunoblotting (Figure 3.5). The approximately 2 kDa and 4 kDa shifts were observed for BAG-1S protein in the presence of Pep3 (Figure 3.5a) and TPep3 (Figure 3.5b) respectively when compared with BAG-1S only sample. These protein shifts implied the interaction of BAG-1S with peptides.

After their interactions were confirmed, secondary structure analysis of BAG-1S with peptides were performed to see if there are major changes in protein structure upon binding peptides (Figure 3.6). Pep3 + BAG-1S interaction was formed with mammalian BAG-1S while for TPep3 interaction, bacterial BAG-1S was used. As seen in Figure 3.6, both of the proteins still had mainly α-helix characteristics. The

data were quantified by using BeStSel webserver and analyses also showed that the proteins were still found as folded and in α-helix structure mainly.



a) Pep3 **b)**TPep3

Figure 3.5 : Immunoblotting analysis of crosslinked BAG-1S in the presence and absence of a) Pep3 and b) TPep3. In the presence of Pep3 and TPep3, 2 kDa and 4 kDa shifts (red arrows) which confirms the interaction between protein and peptides were observed respectively.

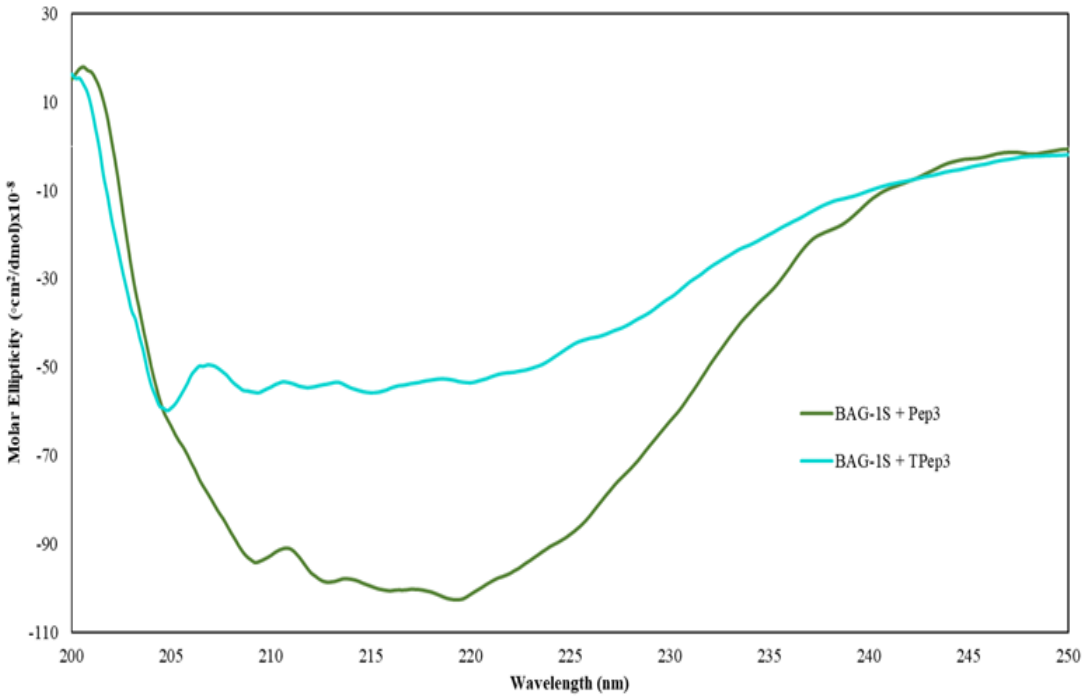


Figure 3.6 : Secondary structure analysis of BAG-1S proteins with Pep3 (green) and TPep3 (cyan). Pep3 interaction was formed with mammalian BAG-1S while TPep3 interaction was formed with bacterial BAG-1S. Even after the interactions were formed, poteins still showed primarily α-helix structure by BeStSel webserver analysis.

The stability of BAG-1S in the presence and absence of Pep3 and Tpep3 were analyzed with limited trypsinolysis. BAG-1S proteins were incubated with peptides and then,

tryptic digestion was performed at 37ºC. Samples were taken at specific time points and the amount of full-length BAG-1S protein was analyzed by immunoblotting (Figure 3.7). The analyses showed that BAG-1S bind to both peptides and moreover, in the presence of the peptides, the time required for the fully digestion of BAG-1S has been increased.

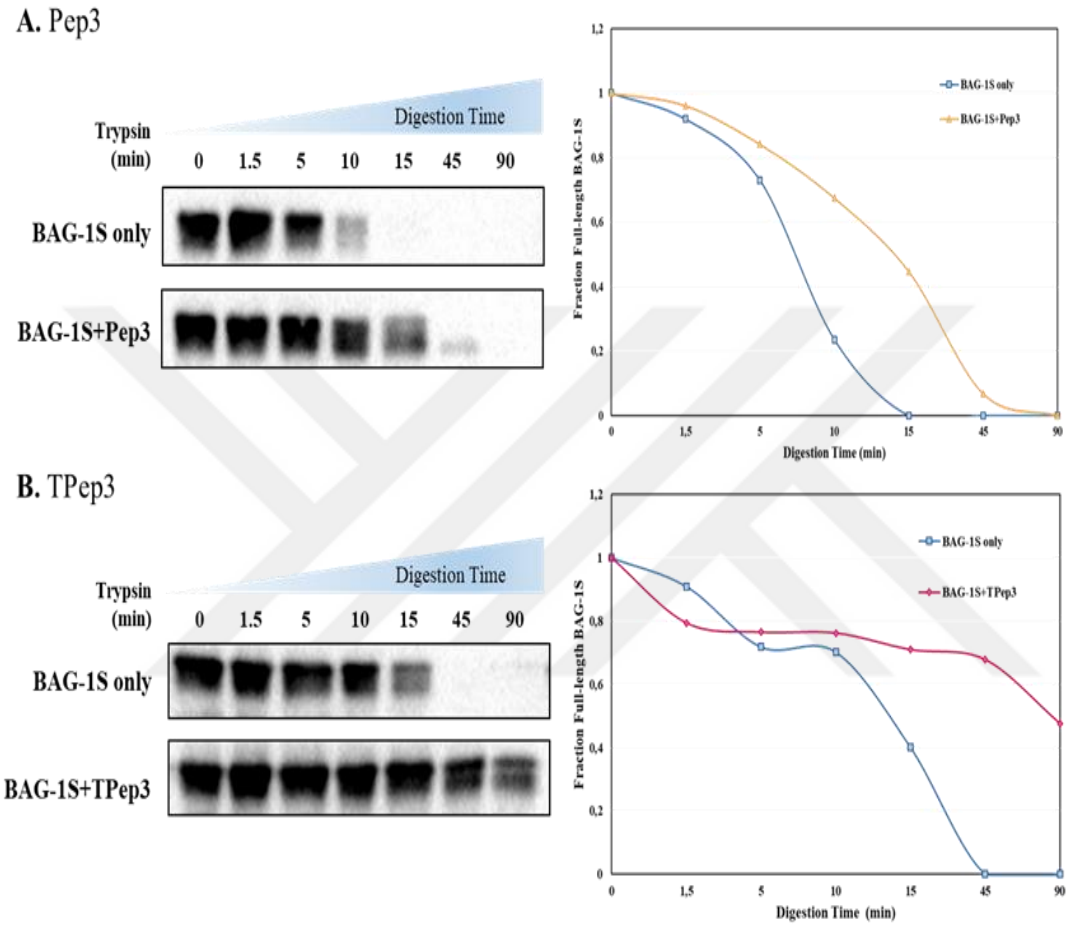


Figure 3.7 : Limited trypsinolysis of BAG-1S protein in the absence and presence of Pep3 and TPep3. The interaction with both peptides has increased the stability of full-length BAG-1S.

3.4 Pep3 Binds BAG-1S with High Affinity

After the interaction between BAG-1S and Pep3 was confirmed in vitro, the binding constant was measured by Surface Plasmon Resonance (SPR) analysis. Firstly, BAG-1 antibody was captured on Protein G chip and then, purified BAG-1S proteins were captured with antibody. Varying concentrations of Pep3 (0 nm to 4000 nm) were used to determine binding kinetics (Figure 3.8). The binding constant was measured as 68.56 nM.

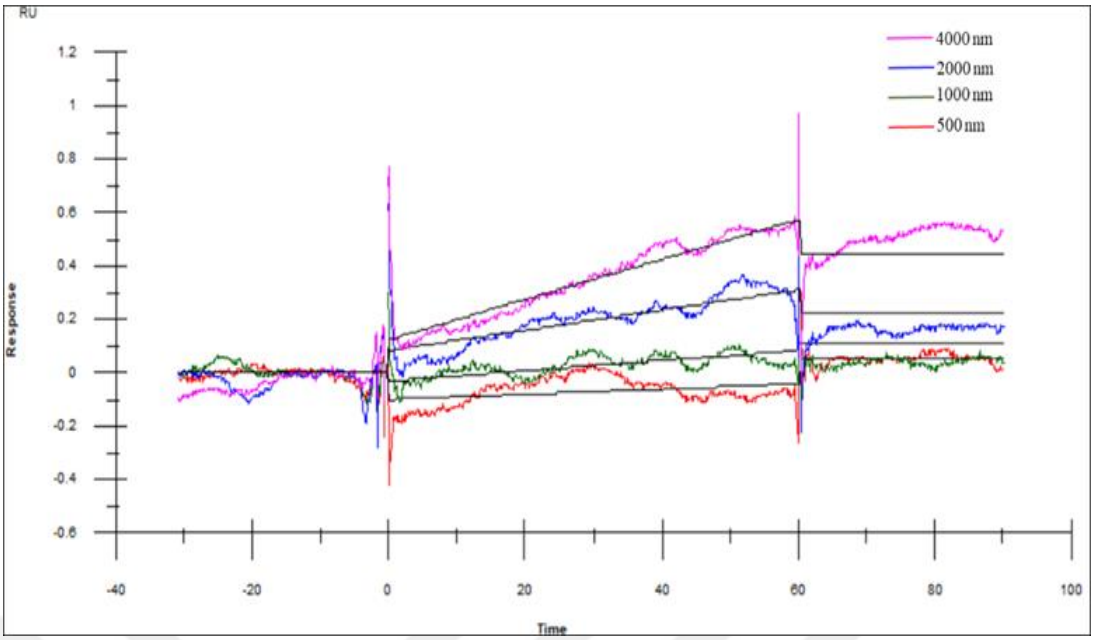


Figure 3.8 : Binding kinetics analysis of Pep3 to BAG-1S. Different concentrations of Pep3 were used to determine and it was seen that with the increasing concentration of Pep3, the response was also increased. Binding constant of Pep3 was measured as 68.56 nM.

3.5 TPep3 Decreases the Activity of MAPK Pathway in MCF-7 Cells

To determine the effects of TPep3, which was designed to penetrate cell membrane, on ERK pathway, MCF-7 cells were treated with varying concentrations (1 uM to 50 uM) of TPep3 for 24 h, 48 h and 72 h. MCF-7 cells (breast cancer) were used for this study since it is known that BAG-1S is overexpressed in this cancer cell line (Yang et al., 1998). After treatment with TPep3, members of MAPK pathways were analzyed by immunoblotting. As seen in Figure 3.9, C-Raf and p-C-Raf (S338) levels were decreased with the increasing concentrations of TPep3. Moreover, the levels of B-Raf and p-B-Raf were decreased when the concentration of peptide was increased. MEK and p-MEK (S218/222) levels were also analyzed . It was seen that although the level of MEK mostly remained same, the level of p-MEK was decreased with TPep3 treatment showing the decrease in the activity of MAPK pathway. In addition, it has been shown that the effect of TPep3 has been maintained only up to 48 hours and its effect has been started to decrease after 48 hours and nearly lost at 72 hours. Also, to see the effect of TPep3 on other cell survival pathways, Akt and p-Akt (S473) levels were analyzed. It was seen that their levels were remained same after treatment (Figure 3.9). Lastly, IC50 value of TPep3 was calculated by MTT analysis after 48 hours of TPep3 treatment. The cells were treated with the varying concentrations of TPep3 and

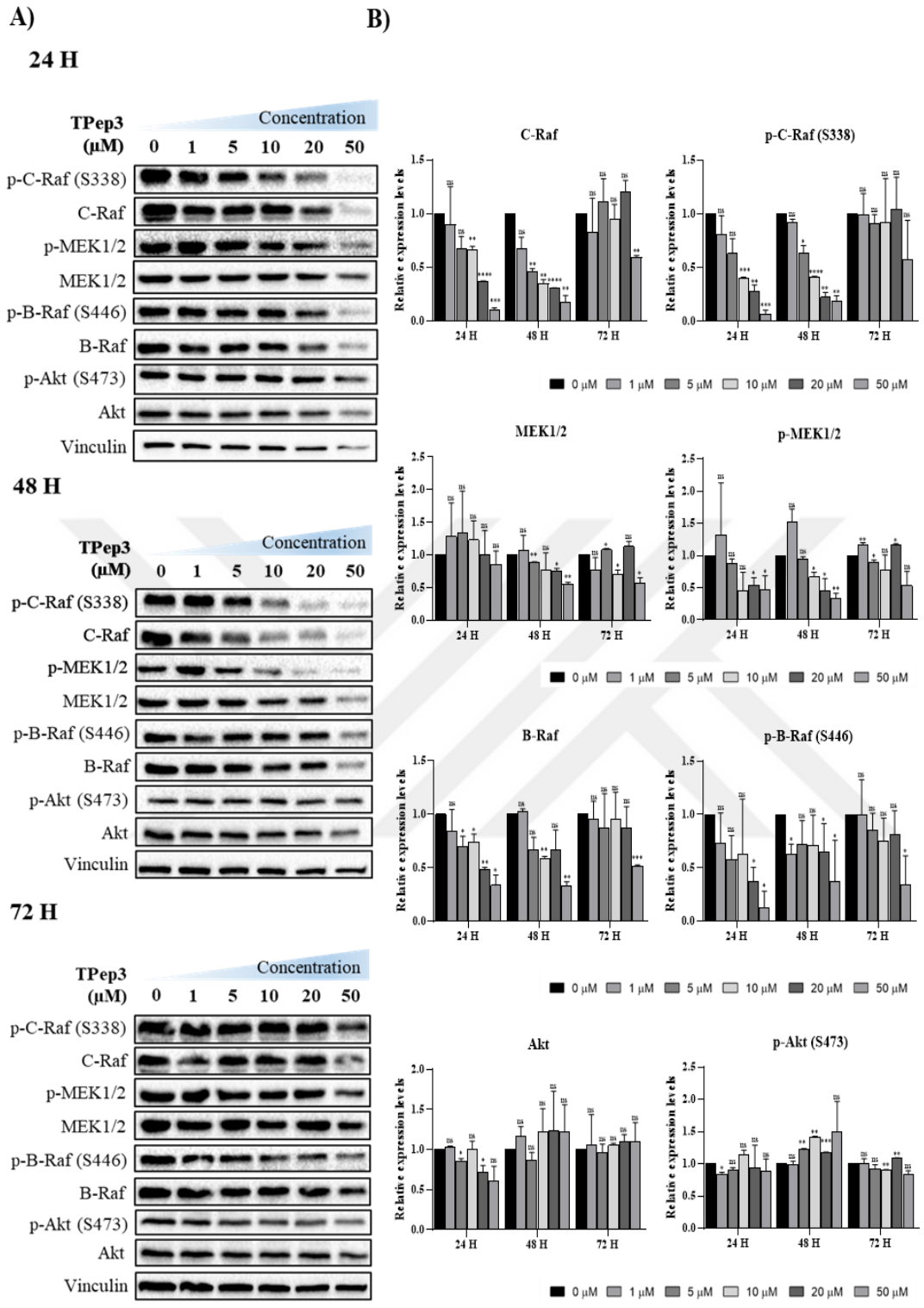


Figure 3.9 : Immunoblotting analysis of the effect of TPep3 on MCF-7 cell line after 24 h, 48 h, 72h. Increasing concentrations of TPep3 was lead to decrease of C-Raf and p-C-Raf levels which resulted the lower p-MEK levels. TPep3 also caused the decrease of B-Raf and p-B-Raf levels while p-Akt levels were remained same. Also, it has shown that the effect of TPep3 has decreased after 48 hours and almost lost at 72 hours.

after 48 hours, MTT analysis was performed by measuring the absorbance values. Absorbance values were normalized to the control value and cell viability was shown as percentage (Figure 3.10). IC50 value was calculated as approximately 18 uM.

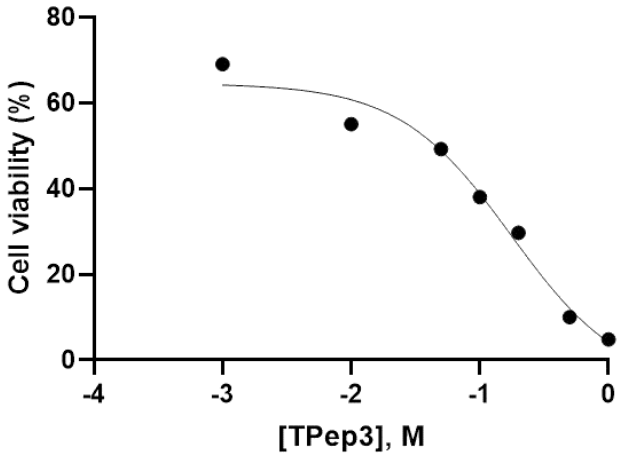


Figure 3.10 : Cell viability analysis of MCF-7 cells with MTT assay. IC50 value, which represents the inhibitor concentration needed to kill half of the cells, was calculated as 18 uM.

4. DISCUSSION

Drug resistance has become one of the limiting factor for cancer therapeutics. Because of the rising resistance to drugs, there is a need for the development of new therapeutics (Vasan et al., 2019). Protein-protein interactions (PPIs) play important roles in the regulation of cellular pathways and proteins' stability and activity (Bruzzoni-Giovanelli et al., 2018). Therefore, PPIs are considered as promising targets for new therapeutics and peptide inhibitors are getting more attention for this targets (Philippe et al., 2021). As mentioned above, cellular pathways, such as survival and proliferation, are regulated by many distinct factors whose dysregulations result in the development of cancer. One of the pathways that promote cancer progression is Ras/Raf/MEK/ERK cascade which regulates vital cellular functions (Mccubrey et al., 2007). C-Raf and B-Raf kinases are important players of ERK pathway and play roles in the upstream signalling of MEK and ERK. Anti-apoptotic BAG-1 interacts with B-Raf and C-Raf and BAG-1 has been shown that to have a role in stabilization and activation of C-Raf (Götz et al., 2005; Song et al., 2001). It has been shown that BAG-1 is overexpressed in several cancer types such as breast cancer, leukemia, and prostate cancer (Aveic et al., 2015; Cutress et al., 2002). Moreover, it has been demonstrated that C-Raf is able to be activated by BAG-1 in BAG-1 overexpressing cells indepent from Ras activation (Song et al., 2001) benefiting the cancer cells for prolonged survival and proliferation. Hence, targeting the interaction between BAG-1 and C-Raf complex would be a potential drug target for cancer progression and development.

The interaction between BAG-1S and C-Raf has been known over a decade however interaction surface was not determined up to now. In our previous study, we identified the interaction surface of this complex on both C-Raf and BAG-1S. Then, several peptides were designed against BAG-1S and C-Raf. One of the peptides which coded as Pep3 and designed against BAG-1S has shown to disrupt this complex. In this study, it was aimed to characterize the interaction between peptide and BAG-1S. Together with Pep3, its cell penetrating form which coded as TPep3 has been characterized.

Firstly, full-length His-tagged BAG-1S proteins were overexpressed in mammalian cells. Since the amount of purified protein obtained from mammalian cells was low, BAG-1S was also produced in bacterial cells. Both of the produced proteins were purified by Ni-NTA affinity purification in two steps since they have His-tag (Turk et al., 2021). After proteins were purified, they were analyzed and it was shown that both of the proteins were purified with >80% purity. According to the secondary structure analyses, both of the purified proteins were folded and they showed primarily α-helix characteristics as expected (Marzullo et al., 2022). After proteins were purified and characterized, Pep3 and TPep3 has been dissolved in 20 mM AMBIC and water respectively. Pep3 shows hydrophobic characteristics while TPep3 is mostly hydrophilic due to amino acid addition to make it cell penetrating peptide (Gräslund et al., 2011). Molecular weights of Pep3 and TPep3 were measured as 2.066 kDa and 3.653 kDa by mass spectrometry (Nielsen et al., 2005). These results were consistent with the theoretical molecular weights of Pep3 and TPep3 calculated from ExPASy server (Gasteiger et al., 2005). Secondary structure analysis of peptides were also performed and it was calculated that both of the peptides contained β-sheets combined with random coils by BeStSel webserver (Wien et al., 2022). Limited trypsinolysis was also performed to confirm interaction between BAG-1S and peptides and stability of BAG-1S. It has been clearly seen that in the presence of peptides, full-length BAG-1S was observed even after 90 minutes of tryptic digestion which confirms the interaction. Also, it can be seen that there is difference between BAG-1S only proteins which were used as controls of peptides. Since Pep3 was dissolved in 20 mM AMBIC, to eliminate the effect of AMBIC, 20 mM AMBIC was also added to the BAG-1S only sample. It has been known that the activity of trypsin is increased in the presence of AMBIC so the required time to digest all BAG-1S was decreased ((Heissel et al., 2018).

All of the proteins and peptides were characterized, the interaction of BAG-1S with Pep3 and TPep3 has been confirmed by crosslinking reaction with DSS. Crosslinking is a method to utilize the stabilization of interaction between proteins and/or protein-peptides. Therefore, it ables to analyze even the transient interactions of biomolecules. DSS is one of the most common NHS-ester crosslinker which reacts with primary amines and make interaction covalent (Belsom & Rappsilber, 2021). When BAG-1S proteins were crosslinked with peptides, approximately 2 kDa and 4 kDa shifts were

observed for Pep3 and TPep3 respectively. These protein shifts confirmed the interaction of BAG-1S with peptides. After binding was confirmed, the binding affinity of Pep3 to BAG-1S was measured with SPR. The binding constant (KD) was calculated as 68.53 nM which is considered as high affinity (Saw et al., 2021). This result increase the potential of Pep3 for using it as therapeutic target.

As mentioned before, C-Raf is in the center of ERK pathway and upstream signaling of MEK kinases. To see the effect of TPep3 on ERK pathway of breast cancer cells, MCF-7 cell line was treated with the varying concentrations of TPep3. MCF-7 cell line was used for this study since they are known with overexpression of BAG-1 protein (Yang et al., 1998). When the peptide concentration was increased, it was observed that the level of C-Raf and p-C-Raf (S338) proteins were decreased In correlation with this result, p-MEK levels were also shown as decreased as expected since the activation of MEK is provided by active C-Raf (Guo et al., 2020). Interestingly, it was seen that the levels of B-Raf and p-B-Raf (S446) were also decreased. Although, the interaction between B-Raf and BAG-1 is known, it was not determined if they interact directly or indirectly (Götz et al., 2005). These results suggest that either B-Raf may interacts with BAG-1S in complex with C-Raf and its phosphorylation could be affected by disruption of this complex or B-Raf may interact with the BAG-1 through the same interaction surface of C-Raf. Therefore, its activation was also decreased by the treatment of TPep3. In addition, Akt and p-Akt (S473) levels were analyzed to see the effects of peptide on other cell survival pathways. PI3K pathway, in which Akt is crucial member, is also dysregulated in several cancer types and a promising target for cancer therapeutics (Noorolyai et al., 2019). It was seen that the levels of Akt and p-Akt remained unchanged even with the highest concentration of TPep3. Lastly, cytotoxicity of TPep3 on MCF-7 cells was measured by MTT assay and IC50 value was calculated as approximately 18 uM.

In conclusion, designed peptides Pep3 and its cell penetrating version of TPep3 interacts with the BAG-1S. Pep3 binds the BAG-1S with high affinity and cellular assays showed that TPep3 has decreased the activity of C-Raf and makes it more unstable. Therefore, this peptide could be a promising target for BAG-1 overexpressing breast cancer cells. For further studies, the cytotoxicity of TPep3 could be analyzed on healthy cells and also, other BAG-1 overexpressed cancer cells and cancer cells that depend ERK pathway for prolonged survival could be analyzed.

REFERENCES

Alla, J. A., & Quitterer, U. (2022). The RAF Kinase Inhibitor Protein (RKIP): Good as Tumour Suppressor, Bad for the Heart. *Cells*, *11*(4). https://doi.org/10.3390/cells11040654

Aricescu, A. R., Lu, W., & Jones, E. Y. (2006). A time- and cost-efficient system for high-level protein production in mammalian cells. *Acta Crystallographica Section D: Biological Crystallography*, *62*(10), 1243–1250. https://doi.org/10.1107/S0907444906029799

Aveic, S., Pigazzi, M., & Basso, G. (2011). BAG1: the guardian of anti-apoptotic proteins in acute myeloid leukemia. *PloS one*, *6*(10), e26097.

Aveic, S., Viola, G., Accordi, B., Micalizzi, C., Santoro, N., Masetti, R., ... & Pigazzi, M. (2015). Targeting BAG-1: a novel strategy to increase drug efficacy in acute myeloid leukemia. *Experimental hematology*, *43*(3), 180-190.

Barbosa, R., Acevedo, L. A., & Marmorstein, R. (2021). The MEK/ERK Network as a Therapeutic Target in Human Cancer HHS Public Access Visual Overview. *Mol Cancer Res*, *19*(3), 361–374. https://doi.org/10.1158/1541-7786.MCR-20-0687.The

Belsom, A., & Rappsilber, J. (2021). Anatomy of a crosslinker. *Current opinion in chemical biology*, *60*, 39-46.

Briknarová, K., Takayama, S., Brive, L., Havert, M. L., Knee, D. A., Velasco, J., Homma, S., Cabezas, E., Stuart, J., Hoyt, D. W., Satterthwait, A. C., Llinás, M., Reed, J. C., & Ely, K. R. (2001). Structural analysis of BAG1 cochaperone and its interactions with Hsc70 heat shock protein. *Nature Structural Biology*, *8*(4), 349–352. https://doi.org/10.1038/86236

Bruzzoni-Giovanelli, H., Alezra, V., Wolff, N., Dong, C. Z., Tuffery, P., & Rebollo, A. (2018). Interfering peptides targeting protein–protein interactions: the next generation of drugs?. *Drug Discovery Today*, *23*(2), 272-285.

Burotto, M., Chiou, V. L., Lee, J. M., & Kohn, E. C. (2014). The MAPK pathway across different malignancies: A new perspective. *Cancer*, *120*(22), 3446–3456. https://doi.org/10.1002/cncr.28864

Cook, F. A., & Cook, S. J. (2021). Inhibition of RAF dimers: It takes two to tango. *Biochemical Society Transactions*, *49*(1), 237–251. https://doi.org/10.1042/BST20200485

Cutress, R. I., Townsend, P. A., Brimmell, M., Bateman, A. C., Hague, A., & Packham, G. (2002). BAG-1 expression and function in human cancer. *British journal of cancer*, *87*(8), 834-839.

Degirmenci, U., Wang, M., & Hu, J. (2020). Targeting aberrant RAS/RAF/MEK/ERK signaling for cancer therapy. *Cells*, *9*(1), 198.

Dhillon, A. S., Hagan, S., Rath, O., & Kolch, W. (2007). MAP kinase signalling pathways in cancer. *Oncogene*, *26*(22), 3279–3290. https://doi.org/10.1038/sj.onc.1210421

Galabova-Kovacs, G., Kolbus, A., Matzen, D., Meiss, K., Piazzolla, D., Rubiolo, C., ... & Baccarini, M. (2006). ERK and beyond: insights from B-Raf and Raf-1 conditional knockouts. *Cell cycle*, *5*(14), 1514-1518.

García-Gómez, R., Bustelo, X. R., & Crespo, P. (2018). Protein–Protein Interactions: Emerging Oncotargets in the RAS-ERK Pathway. *Trends in Cancer*, *4*(9), 616–633. https://doi.org/10.1016/j.trecan.2018.07.002

Gasteiger, E., Hoogland, C., Gattiker, A., Wilkins, M. R., Appel, R. D., & Bairoch, A. (2005). Protein identification and analysis tools on the ExPASy server. *The proteomics protocols handbook*, 571-607.

Godfrey, W. L., Rudd, C. J., Iyer, S., & Recktenwald, D. (2005). Purification of Cellular and Organelle Populations by Fluorescence-Activated Cell Sorting for Proteome Analysis. In *The Proteomics Protocols Handbook*. https://doi.org/10.1385/1-59259-890-0:067

Gräslund, A., Madani, F., Lindberg, S., Langel, Ü., & Futaki, S. (2011). Mechanisms of cellular uptake of cell-penetrating peptides. *Journal of Biophysics*, 2011. https://doi.org/10.1155/2011/414729

Götz, R., Wiese, S., Takayama, S., Camarero, G. C., Rossoll, W., Schweizer, U., Troppmair, J., Jablonka, S., Holtmann, B., Reed, J. C., Rapp, U. R., & Sendtner, M. (2005). Bag1 is essential for differentiation and survival of hematopoietic and neuronal cells. *Nature Neuroscience*, *8*(9), 1169–1178. https://doi.org/10.1038/nn1524

Guo, Y., Pan, W., Liu, S., Shen, Z., Xu, Y., & Hu, L. (2020). ERK/MAPK signalling pathway and tumorigenesis (Review). *Experimental and Therapeutic Medicine*, 1997–2007. https://doi.org/10.3892/etm.2020.8454

Harrison, C. (2003). GrpE, a nucleotide exchange factor for DnaK. *Cell stress & chaperones*, *8*(3), 218.

Heissel, S., Frederiksen, S. J., Bunkenborg, J., & Højrup, P. (2018). Enhanced trypsin on a budget: Stabilization, purification and high-temperature application of inexpensive commercial trypsin for proteomics applications. *PLoS ONE*, *14*(6), 1–16. https://doi.org/10.1371/journal.pone.0218374

Hu, J., Stites, E. C., Yu, H., Germino, E. A., Meharena, H. S., Stork, P. J. S., Kornev, A. P., Taylor, S. S., & Shaw, A. S. (2013). XAllosteric activation of functionally asymmetric RAF kinase dimers. *Cell*, *154*(5), 1036–1046. https://doi.org/10.1016/j.cell.2013.07.046

Huang, Y., Zhang, X. Y., An, S., Yang, Y., Liu, Y., Hao, Q., Guo, X. X., & Xu, T. R. (2018). C-RAF function at the genome-wide transcriptome level: A systematic view. *Gene*, *656*(January), 53–59. https://doi.org/10.1016/j.gene.2018.02.034

Lavoie, H., & Therrien, M. (2015). Regulation of RAF protein kinases in ERK signalling. *Nature Reviews Molecular Cell Biology*, *16*(5), 281–298. https://doi.org/10.1038/nrm3979

Jambrina, P. G., Rauch, N., Pilkington, R., Rybakova, K., Nguyen, L. K., Kholodenko, B. N., Buchete, N. V., Kolch, W., & Rosta, E. (2016). Phosphorylation of RAF kinase dimers drives conformational changes that facilitate transactivation. *Angewandte Chemie - International Edition*, *55*(3), 983–986. https://doi.org/10.1002/anie.201509272

Lavoie, H., & Therrien, M. (2015). Regulation of RAF protein kinases in ERK signalling. *Nature Reviews Molecular Cell Biology*, *16*(5), 281–298. https://doi.org/10.1038/nrm3979

Leicht, D. T., Balan, V., Kaplun, A., Singh-Gupta, V., Kaplun, L., Dobson, M., & Tzivion, G. (2007). Raf kinases: Function, regulation and role in human cancer. *Biochimica et Biophysica Acta - Molecular Cell Research*, *1773*(8), 1196–1212. https://doi.org/10.1016/j.bbamcr.2007.05.001

Matallanas, D., Birtwistle, M., Romano, D., Zebisch, A., Rauch, J., von Kriegsheim, A., & Kolch, W. (2011). Raf family kinases: Old dogs have learned new tricks. *Genes and Cancer*, *2*(3), 232–260. https://doi.org/10.1177/1947601911407323

Liman, J., Faida, L., Dohm, C. P., Reed, J. C., Bähr, M., & Kermer, P. (2008). Subcellular distribution affects BAG1 function. *Brain Research*, *1198*, 21–26. https://doi.org/10.1016/j.brainres.2008.01.010

McCubrey, J. A., Steelman, L. S., Chappell, W. H., Abrams, S. L., Wong, E. W., Chang, F., ... & Franklin, R. A. (2007). Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, *1773*(8), 1263-1284.

Marzullo, L., Turco, M. C., & Uversky, V. N. (2022). What's in the BAGs? Intrinsic disorder angle of the multifunctionality of the members of a family of chaperone regulators. *Journal of Cellular Biochemistry*, *123*(1), 22–42. https://doi.org/10.1002/jcb.30123

Minde, D. P., Maurice, M. M., & Rüdiger, S. G. D. (2012). Determining Biophysical Protein Stability in Lysates by a Fast Proteolysis Assay, FASTpp. *PLoS ONE*, *7*(10), 1–9. https://doi.org/10.1371/journal.pone.0046147

Nolan, A. A., Aboud, N. K., Kolch, W., & Matallanas, D. (2021). Hidden targets in raf signalling pathways to block oncogenic ras signalling. *Genes*, *12*(4). https://doi.org/10.3390/genes12040553

Noorolyai, S., Shajari, N., Baghbani, E., Sadreddini, S., & Baradaran, B. (2019). The relation between PI3K/AKT signalling pathway and cancer. *Gene*, *698*, 120-128.

Roskoski, R. (2018). Targeting oncogenic Raf protein-serine/threonine kinases in human cancers. *Pharmacological Research*, *135*(August), 239–258. https://doi.org/10.1016/j.phrs.2018.08.013

Santini, C. C., Longden, J., Schoof, E. M., Simpson, C. D., Jeschke, G. R., Creixell, P., Kim, J., Wu, X., Turk, B. E., Rosen, N., Poulikakos, P. I., & Linding, R. (2019). Global view of the RAF-MEK-ERK module and its immediate downstream effectors. *Scientific Reports*, *9*(1), 1–11. https://doi.org/10.1038/s41598-019-47245-x

Santos, E., & Crespo, P. (2018). The RAS-ERK pathway: A route for couples. *Science Signaling*, *11*(554), 1–3. https://doi.org/10.1126/scisignal.aav0917

Philippe, G. J. B., Craik, D. J., & Henriques, S. T. (2021). Converting peptides into drugs targeting intracellular protein–protein interactions. *Drug Discovery Today*, *26*(6), 1521–1531. https://doi.org/10.1016/j.drudis.2021.01.022

Saw, P. E., Xu, X., Kim, S., & Jon, S. (2021). Biomedical Applications of a Novel Class of High-Affinity Peptides. *Accounts of Chemical Research*, *54*(18), 3576–3592. https://doi.org/10.1021/acs.accounts.1c00239

Shaul, Y. D., & Seger, R. (2007). The MEK/ERK cascade: From signaling specificity to diverse functions. *Biochimica et Biophysica Acta - Molecular Cell Research*, *1773*(8), 1213–1226. https://doi.org/10.1016/j.bbamcr.2006.10.005

Song, J., Takeda, M., & Morimoto, R. I. (2001). Bag1-Hsp 70 mediates a physiological stress signalling pathway that regulates Raf-1/ERK and cell growth. *Nature Cell Biology*, *3*(3), 276–282. https://doi.org/10.1038/35060068

Terai, K., & Matsuda, M. (2006). The amino-terminal B-Raf-specific region mediates calcium-dependent homo- and hetero-dimerization of Raf. *EMBO Journal*, *25*(15), 3556–3564. https://doi.org/10.1038/sj.emboj.7601241

Nielsen, J. L., & Nielsen, P. H. (2005). Advances in microscopy: microautoradiography of single cells. *Methods in enzymology*, *397*, 237-256.

Townsend, P. A., Cutress, R. I., Sharp, A., Brimmell, M., & Packham, G. (2003). BAG-1: A multifunctional regulator of cell growth and survival. *Biochimica et Biophysica Acta - Reviews on Cancer*, *1603*(2), 83–98. https://doi.org/10.1016/S0304-419X(03)00002-7

Townsend, P. A., Stephanou, A., Packham, G., & Latchman, D. S. (2005). BAG-1: A multi-functional pro-survival molecule. International Journal of Biochemistry and *Cell Biology*, *37*(2), 251–259. https://doi.org/10.1016/j.biocel.2004.03.016

Turk, M., Tatli, O., Alkan, H. F., Ozfiliz Kilbas, P., Alkurt, G., & Dinler Doganay, G. (2021). Co-chaperone bag-1 plays a role in the autophagy-dependent cell survival through Beclin 1 interaction. *Molecules*, *26*(4), 854.

Ueda, Y., Hirai, S. I., Osada, S. I., Suzuki, A., Mizuno, K., & Ohno, S. (1996). Protein kinase C δ activates the MEK-ERK pathway in a manner independent of Ras and dependent on Raf. *Journal of Biological Chemistry*, *271*(38), 23512–23519. https://doi.org/10.1074/jbc.271.38.23512

Wang, H. G., Takayama, S., Rapp, U. R., & Reed, J. C. (1996). Bcl-2 interacting protein, BAG-1, binds to and activates the kinase Raf-1. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(14), 7063–7068. https://doi.org/10.1073/pnas.93.14.7063

Wien, F., Boros, E., Vad, H., Matthieu, R., Murvai, N., Lee, Y., & Goto, Y. (2022). *BeStSel : webserver for secondary structure and fold Andr as ´ Moln ar*. *50*(May), 90–98.

Vasan, N., Baselga, J., & Hyman, D. M. (2019). A view on drug resistance in cancer. *Nature*, *575*(7782), 299–309. https://doi.org/10.1038/s41586-019-1730-1

Yang, X., Chernenko, G., Hao, Y., Ding, Z., Pater, M. M., Pater, A., & Tang, S. C. (1998). Human BAG-1/RAP46 protein is generated as four isoforms by alternative translation initiation and overexpressed in cancer cells. *Oncogene*, *17*(8), 981–989. https://doi.org/10.1038/sj.onc.1202032

Yesilkanal, A. E., & Rosner, M. R. (2018). Targeting raf kinase inhibitory protein regulation and function. *Cancers*, *10*(9). https://doi.org/10.3390/cancers10090306

Zhao, J., & Luo, Z. (2022). Discovery of Raf Family Is a Milestone in Deciphering the Ras-Mediated Intracellular Signaling Pathway. *International Journal of Molecular Sciences*, *23*(9). https://doi.org/10.3390/ijms23095158

Zybailov, B. L. (2013). Large Scale Chemical Cross-linking Mass Spectrometry Perspectives. *Journal of Proteomics & Bioinformatics*, *01*(S2), 1–25. https://doi.org/10.4172/jpb.s2-001

Zebisch, A., Staber, P. B., Delavar, A., Bodner, C., Hiden, K., Fischereder, K., Janakiraman, M., Linkesch, W., Auner, H. W., Emberger, W., Windpassinger, C., Schimek, M. G., Hoefler, G., Troppmair, J., & Sill, H. (2006). Two transforming C-RAF germ-line mutations identified in patients with therapy-related acute myeloid leukemia. *Cancer Research*, *66*(7), 3401–3408. https://doi.org/10.1158/0008-5472.CAN-05-0115

APPENDICES

APPENDIX A: Materials

APPENDIX A

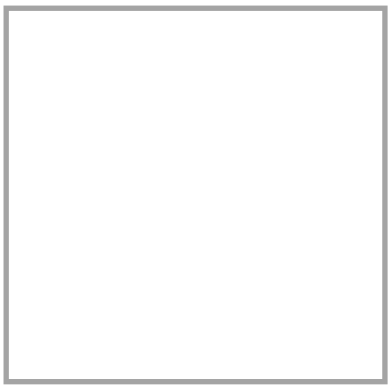
Table A.1 : Chemicals and antibodies used in this study

Chemicals & Antibodies	Brand
40% Acrylamide/Bisacrylamide	Bio-Rad
Ammonium persulfate	Sigma
Ampicillin	Sigma
Bovine serum albumin	Sigma
Bradford reagent	Bio-Rad
Methanol	Sigma
Non-fat dry milk	Santa Cruz
Peptone	Multicell
Phosphate buffer saline	EcoTech
Sodium chloride	Sigma
Sodium dodecyl sulfate	Sigma
TEMED	Sigma
Yeast Extract	Sigma
DMEM	Gibco
BAG-1 antibody (Mouse mAb)	Cell Signalling Technology
B-Raf antibody (Rabbit mAb)	Cell Signalling Technology
Vinculin antibody (Rabbit mAb)	Cell Signalling Technology
p-C-Raf (S338) (Rabbit mAb)	Cell Signalling Technology
C-Raf antibody (Rabbit mAb)	Cell Signalling Technology
p-B-Raf (S446) antibody (Rabbit mAb)	Cell Signalling Technology
Akt antibody (Rabbit mAb)	Cell Signalling Technology
MEK 1/2 antibody (Rabbit mAb)	Cell Signalling Technology
p-MEK 1/2 (S218/222)	Cell Signalling Technology
Anti-mouse IgG, HRP-linked	Cell Signalling Technology
Anti-rabbit IgG, HRP-linked	Cell Signalling Technology
p-Akt (S473) antibody (Rabbit mAb)	Cell Signalling Technology
Nonidet P40 (NP-40)	Biomatik
DMSO	GENAXXON

Table A.2 : Equipments and commercial kits used in this study.

Equipments & Commercial Kits	Brand
BIO-RAD WB System	Bio-Rad
Centrifuge	Eppendorf
Incubator with CO_2	ESCO
Laminar Air Flow Cabinets	Faster BH-EN 2003
Light Microscope	Olympus CH30 (USA)
Microplate Spectrophotometer	Bio-Rad Benchmark Plus
pH meter	Mettler Toledo Five-Easy
Pipettes	2.5, 10, 100, 200, 1000 µl
Power Supply	Bio-Rad Power Pac Basic
SDS-PAGE Gel Electrophoresis System	Bio-Rad Mini Protean
Thermal Cycler	Bio-Rad T100
NucleoSpin Plasmid Purification Kit	Macherey-Nagel
Water Distillation Unit	Millipore
Heat Block	Biosan
MiniSpin	Inovia Technology
Rotator	Stuart
ChemiDoc MP System	Bio-Rad
Circular Dichroism Spectrometer	Jasco J-1500-150
Mass Spectrometer	WATERS SYNAPT G2-Si
ECL substrate	Bio-Rad
Biosafety Cabinet	Hedlab

CURRICULUM VITAE



Name Surname **: Ecenur Çebi**

EDUCATION

- **B.Sc.** **:**2020, Istanbul Technical University, Faculty of Science and Letters, Department of Molecular Biology and Genetics
  2019 Summer, Exchange Student, University of Seoul, Seoul International Summer School
- **M.Sc.** **:**2022, Istanbul Technical University, Graduate School, Molecular Biology-Genetics and Biotechnology

PROFESSIONAL EXPERIENCE AND REWARDS:

- October 2018-July 2022. Istanbul Technical University, Department of Molecular Biology and Genetics, GDLab
- August 2019, Aziz Sancar Institute of Experimental Medicine, Department of Molecular Medicine
- August-September 2018, Aziz Sancar Institute of Experimental Medicine, Department of Molecular Medicine

PUBLICATIONS, PRESENTATIONS AND PATENTS ON THE THESIS:

- **Tatlı, Ö., Cebi, E., Dingiloglu, B., Turk, M., & Dinler Doğanay, G., (2021).** *Mapping the interaction surface of pro-survival BAG-1S with proto-oncogene C-Raf.* BioTurkiye International Biotechnology Congress. Turkey

OTHER PUBLICATIONS, PRESENTATIONS AND PATENTS:

- **Erdogdu, N. D., Can, N. D., Gül, C. M., Cebi, E., Akçay, İ. M., Ağaoğlu, N. B., Doğanay, H. L., Dinler Doğanay, G., (2022).** *Detection and Functional Analysis of CHEK2 Variants of Unknown Significance Found by Genetic Screening of Breast and Colorectal Cancer Patients*. 8th International Congress of the Molecular Biology Association of Turkey. İstanbul, Turkey

- **Tatlı, Ö., Türk, M., Çebi, E., Şahin, Z., & Dinler Doğanay, G., (2020).** *Anti-Apoptotik Bag-1 Isoformlarının Raf Kinazlarla Fosforilasyon Bağımlı Etkileşim.* 3. Uluusal Proteomik Kongresi (pp.27). İstanbul, Türkiye
- **Tatlı, Ö., Türk, M., Çebi, E., Şahin, Z., & Dinler Doğanay, G., (2020).** *Phosphorylation dependent interaction of pro-survival Bag-1 isoforms with MAPkinases*. 8. International Drug Chemistry Conference (pp.247). Antalya, Turkey
- **Türk, M., Tatlı, Ö., Çebi, E., Şahin, Z., & Dinler Doğanay, G. (2019).** *In vitro investigation of the interactions between Raf kinases and pro-survival BAG-1 in breast cancer cells* . 7th International Congress of the Molecular Biology Association of Turkey (pp.51). İstanbul, Turkey