

FUNCTIONAL EFFECTS OF ATAD2 GENE EXPRESSION IN BREAST CANCER

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By

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We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.



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ABSTRACT

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The ATAD2 gene is a newly investigated gene of which the expression levels are associated with the disease prognosis in many types of cancer and especially breast cancer and that is known to be overexpressed usually through gene amplification and E2F/RB pathway activation. ATAD2 (ATPase family, AAA domain-containing 2) can be overexpressed due to amplification or other regulatory mechanisms in many cancers such as lung, breast, prostate and liver. The fact that ATAD2 has an AAA+ ATPase and bromodomain indicates that it may be a good target for anti-cancer therapy. However, it is necessary to clarify the role of the ATAD2 gene in tumorigenesis before strategies that target ATAD2 are developed. We evaluated a regulator that consistently shows high expression in breast cancer in this study. ATAD2 (ATPase family AAA domain-containing protein 2) is a gene that regulates important cellular activities such as cell proliferation and invasion. This study aimed to clarify the mechanism of action of ATAD2 in breast cancer. The ATAD2 expression of the MCF7 and T47D cell lines with high ATAD2 gene expression was silenced with siRNA and the expression levels of all genes were screened. Gene chip expression analyses revealed that the suppression of ATAD2 in breast cancer cells indicated a role in the regulation of microtubule organization, cell growth, cell adhesion and important signal pathways such as EGFR, FGFR, MAPK, and PI3K. Functional studies with breast cancer cells have

supported the gene expression analysis results. Our study revealed that silencing of ATAD2 lead to suppression of ER(-) breast cancer cell migration but not ER(+) cancer cell migration. The same experiments causes a marked decrease in the colony formation capacity and proliferation potential of HCC1937 cells while there was no change in SKBR3 and ER(+) cells. ATAD2 silencing also lead to a senescence response in all breast cancer cells.

We investigated the molecular mechanisms of action of ATAD2 to determine the factors underlying the biological effect. The MCF7 and HCC1937 cells were used to clarify its action on the main cellular signal pathways. We found that ATAD2 silencing induced an apoptosis response in both cell types. Intrinsic pathways are activated with caspase-9 cleavage in MCF7 cells while high Bcl₂ and Bcl_{XL} expression prevents caspase-9 cleavage in HCC1937 cells. Decreased ATAD2 did not cause a difference in the p53 protein level in either cells but while p21 expression was increased in just MCF7 cells, RB phosphorylation was inhibited in both cell lines. The results indicate that dysregulation of proteins involved in intracellular control pathways triggers the senescence mechanism.

ER α gene expression has been shown to be suppressed as a result of siRNA suppression of ATAD2 gene expression in MCF7 cells. This result indicates that ATAD2 has a role in ER α regulation. ATAD2 gene expression has been found to decrease following Gefitinib suppression of EGFR signaling while EGF treatment of serum-starved MCF7 cells caused increased ATAD2 gene expression. These results indicate that EGFR could be a possible upstream activator of ATAD2. This gene expression pattern also points towards a positive feedback mechanism between ATAD2+ER α and EGFR.

Although it is known that EGFR is frequently overexpressed in breast cancer and cross-talk with the estrogen receptor, we do not have detailed information on the mechanism of their interactions. ‘Pathway Enrichment’ analysis of microarray studies have revealed EGFR signaling as one of pathways enriched in the genes downregulated with decreased ATAD2 expression.

The silencing of ATAD2 and ER α together prevents EGFR expression in MCF7 cells while silencing of ATAD2 by itself in HCC1937 cells does not cause a change in EGFR expression but prevents its phosphorylation in the Tyr1173 region of the receptor. The ATAD2-suppressed EGFR activity in HCC1937 cells did not lead any change in the Akt level or MEK/ERK activity. The down-stream signaling pathway analysis of the EGFR has revealed that Akt protein

expression is suppressed when ATAD2 is silenced in MCF7 cells. The increase in the MEK/ERK signaling activity with decreased ER α expression in the same cells was suppressed with decreased ATAD2 expression.

In conclusion, the high expression of the ATAD2 gene in breast cancer stimulates growth of cancer cells while its interaction with the EGFR signaling pathway could be one of the causes of the pro-oncogenic effects of the gene. Its suppression together with EGFR could provide an option for new therapeutic applications in breast cancer studies.

Key words: ATAD2, ER α , EGFR, breast cancer, senescence, migration, MAPK, Akt

ÖZET

ATAD2 GEN İFADESİNİN MEME KANSERİNDE İŞLEVSEL ETKİLERİ

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ATAD2 geni, meme kanseri başta olmak üzere bir çok kanser türünde, çoğunlukla gen amplifikasyonu ve E2F/Rb yolağı aktivasyonu ile aşırı ifade gösterdiği bilinen ve ifade seviyeleri hastalığın prognozu ile ilişkili olan, henüz yeni araştırılan bir gendir. ATAD2 (ATPaz ailesi, AAA domain içeren 2) akciğer, meme, prostat ve karaciğer gibi bir çok kanserde amplifikasyon veya diğer düzenleyici mekanizmalar nedeniyle fazlaca ifade edilmektedir. ATAD2'nin AAA+ ATPaz ve bromodomain'inin olması iyi bir anti-kanser ilaç hedefi olabileceğini düşündürmektedir. Ancak ATAD2'yi hedef alan stratejiler geliştirilmeden önce ATAD2 geninin tümörigenezdeki rolü aydınlatılmalıdır. Bu çalışmada, meme kanserinde sürekli olarak yüksek ifade gösteren regülatörlerden biri araştırılmıştır. ATAD2 (ATPase family AAA domain-containing protein 2), hücre çoğalması ve invazyonu gibi önemli hücresel faaliyetlerin düzenleyicisi olan bir gendir. Bu çalışma, ATAD2'nin meme kanserindeki çalışma prensibini aydınlatmayı hedeflemiştir. ATAD2 genini yüksek ifade eden hücre hatları MCF7 ve T47D'de ATAD2 ifadesi siRNA'lar ile susturularak Affymetrix mikroçipleri ile tüm genlerin ifade düzeyleri taranmıştır. Mikroçip gen ifade analizleri sonucu ATAD2'nin meme kanseri hücrelerinde ifadesi bastırıldığında; mikrotübül organizasyonu, hücre büyümesi, hücre adezyonu

ve EGFR, FGFR, MAPK, PI3K gibi önemli sinyal yollarının düzenlenmesinde görev aldığını gösterilmiştir. Meme kanseri hücreleriyle yapılan işlevsel çalışmalar gen ifade analizi sonuçlarını desteklemektedir. Çalışmalarımız ATAD2'nin susturulması ile ER(-) meme kanseri hücrelerinin göçünü baskılanırken ER(+) kanser hücrelerinin göçünü etkilemediği gösterildi.. Aynı deneyler HCC1937 hücrelerinin koloni oluşturma kapasitelerini ve çoğalma potansiyellerini önemli ölçüde düşürürken, SKBR3 ve ER(+) hücrelerinde bir değişim gözlenmedi. Ayrıca, ATAD2 susturulması, bütün meme kanseri hücrelerinde senesens yanıtına yol açmıştır.

Biyolojik etki mekanizmasının altında yatan sebepleri ortaya çıkarmak için ATAD2'nin moleküler çalışma prensibi araştırılmıştır. Temel hücresel sinyal yolları üzerindeki etkisini aydınlatmak için MCF7 ve HCC1937 hücreleri kullanılmıştır. Buna göre, ATAD2'nin susturulması her iki hücre tipinde de apoptozis cevabı indüklemiştir. MCF7 hücrelerinde, caspaz-9 yıkımı ile intrinsik yollar aktifleştirilirken, HCC1937 hücrelerinde yüksek Bcl₂ and Bcl_{XL} ifadesi caspaz-9 yıkımını engellemiştir. ATAD2'in düşüşü her iki hücrede de, p53 protein seviyesinde bir farklılığa yol açmadı ama sadece MCF7 hücrelerinde p21'in ifadesinde bir artış gözlenirken, RB fosforilasyonu her iki hücrede de engellendi. Sonuçlar gösteriyor ki hücre içi kontrol yollarında görev alan proteinlerin regülasyonunun bozulması senesens mekanizmasını tetikledi.

MCF7 hücrelerinde ATAD2 gen ifadesinin siRNA ile baskılanması sonucu ER α gen ifadesinin de baskılandığı gösterilmiştir. Bu sonuç, ATAD2'nin, ER α regülasyonunda görev aldığını işaret etmektedir. EGFR aktivitesinin Gefitinib ile baskılanması sonucu ATAD2 gen ifadesinin azaldığı ve ayrıca serum açılığındaki MCF7 hücrelerinin EGF ile muamelesi sonucu ATAD2 gen ifadesinin arttığı gözlenmiştir. Bu sonuçlar EGF sinyal yolağının ATAD2'nin akış üstü aktivatörü olabileceğini düşündürmektedir. Bu gen ifade paterni ATAD2+ER α ve EGFR arasında pozitif bir geri-bildirim ağı olduğunu işaret etmektedir.

EGFR'nın meme kanserinde sıklıkla yüksek ifade edildiği ve östrojen reseptörü ile etkileşim halinde olduğu bilinmekle beraber etkileşim mekanizmasına dair detaylı bir bilgi bulunmamaktadır. Mikroçip deney sonuçlarının 'Pathway Enrichment' analizi sonucu ATAD2 ifadesindeki azalma ile EGFR sinyal yolağında görev alan genlerin ifadesinde düşüş olduğu gözlenmiştir.

ATAD2 ve ER α 'nın birlikte susturulmasının MCF7 hücrelerinde EGFR ifadesini engellerken, ATAD2'nin HCC1937 hücrelerinde tek başına susturulması EGFR ifadesinde bir değişime yol açmadığı ancak reseptörün Tyr1173 bölgesindeki fosforilasyonunun engellendiği gösterilmiştir. HCC1937 hücrelerinde ATAD2 tarafından baskılanan EGFR aktivitesi, ne Akt seviyesinde ne de MEK/ERK aktivitesinde bir değişime yol açmıştır. EGFR reseptörünün akış aşağı sinyal yollarının analizi sonucu, MCF7 hücrelerinde ATAD2'nin susturulması ile Akt protein ifadesinin bastırıldığı gözlemlenmiştir. Aynı hücrelerde, ER α ifadesinin düşüşüyle görülen MEK/ERK sinyal yolağı aktivitesindeki artış ATAD2 gen ifadesinin düşüşü ile baskılanmıştır.

Kısaca, ATAD2 geninin meme kanserindeki yüksek ifadesi, kanser hücrelerinin büyümesini sağlarken, EGFR sinyal yolağı ile olan etkileşimi genin pro-onkogenik etkilerinin altında yatan sebeplerden biri olabilir. ATAD2'nin EGFR ile birlikte baskılanması meme kanseri çalışmalarına yeni bir tedaviye yönelik seçenek olabilir.

Anahtar kelimeler: ATAD2, ER α , EGFR, meme kanseri, yaşlanma, göç, MAPK, Akt



To my beloved family...

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ABBREVIATIONS

ATAD2/ANCCA/PRO2000: AAA+ nuclear coregulator cancer associated

BRD: Bromodomains

CASP9: Caspase 9

CCND1: Cyclin D1

CCNE1: Cyclin E1

CDH1: Cadherin 1

CDK: Cyclin-dependent kinases

CDKN1: Cyclin-Dependent Kinase Inhibitor 1A, P21

DEG: Differentially expressed genes

DSB : Double strand breaks

DCIS: Ductal carcinoma in situ

DMSO: Dimetil sülfoksit

EGFR: Epidermal growth factor receptor

EMT: Epithelial-mesenchymal transition

ER: Estrogen receptor

ESR1: Estrogen receptor α gene

E2: Estradiol

FDR: False Discovery Rate

GEO : Gene Expression Omnibus

H3K36: Histone 3 lysine 36

HCC: Hepatocellular carcinoma

HPRD: Human protein reference database

IC-NST: Invasive carcinoma of non-specific type

IARC: International Agency for Research on Cancer

LBC: Lobular breast carcinoma

SA- β -GAL: Senescence-associated beta-galactosidase

MOMP: Mitochondrial outer membrane permeabilization

LCIS: Lobular carcinoma in situ

IHC: Immunohistochemistry

MDM2: Mouse double minute 2 homolog

MAPK: Mitogen-activated protein kinases

PR: Progesterone receptor

RT: Room temperature

pRB: Retinoblastoma protein

RIN: RNA Integrity number

TNBC: Triple-negative breast cancer

TP53: Tumor Protein P53

RB1: Retinoblastoma

CHAPTER 1. INTRODUCTION

1.1 CANCER

Cancer is a generic disease that is not limited to any part of body. It can originate in any type of cells. Cancer cells are transformed from normal cell. The accumulated epigenetic and molecular alterations within the cells drive abnormal cell development. They show uncontrolled growth and indefinite proliferation capacities¹. It is a disease that has existed in human history from the beginning. However its recognition dates back to Greek physician Hippocrates time. He first used the carcinos and carcinoma terms to describe different tumor forms. The next contribution was by Roman physician Celsus with his translation of the original term to the Latin word, “cancer”². This terminology has been kept up-to-date. Today the name “cancer” bore the burden of 8.2 million deaths worldwide just in 2012. It is the leading cause of death. The statistical trends estimate 22 million new cancer cases within the next two decades³. We have been in a war against this affliction for over the quarter of century. We have witnessed many major discoveries during these years. They have revealed the key hallmarks of cancer cells. These cells' complexity has been the real challenge for disease management. It is not a straightforward process, as evidence indicates it is a multistep process⁴. The studies have revealed six biological abilities acquired by cancer cells during their development period⁵. Cancer cells are defective structures; however their abnormalities create a system in their complexity. Six hallmarks of cancer have been listed in figure 1.1. They are common for all cancer types, so in fact these acquired capabilities are the references to define cancer.

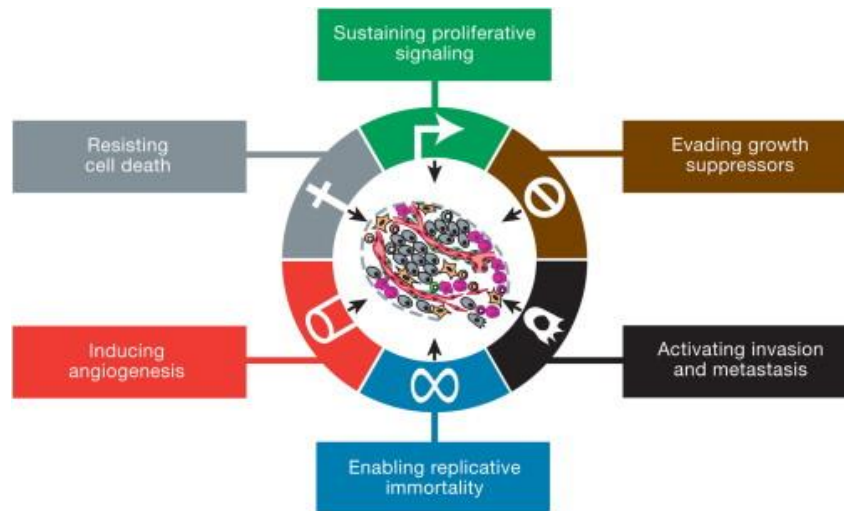


Figure 1. 1: The hallmarks of cancer: Six capabilities of cancer cells acquired during tumorigenesis

1.2 BREAST CANCER

Cancer has been categorized into subtypes to make it easier to manage. The age, sex, genetic background, environmental factors affect the tendency of a person to develop a specific cancer subtype. World cancer statistics revealed the most common cancers for each gender in the 2012 report. Accordingly, breast and prostate cancers are the most common types in females and males, respectively. While lung cancer appears to be most prevalent cancer worldwide in men, breast cancer is the leading cancer worldwide in women corresponding to more than 25% of new cases diagnosed in 2012⁶.

As the CDC (Centers for Disease Control and Prevention) report suggests, breast cancer is the major cause of cancer mortality for women worldwide⁷. Breast cancer is the malignant tumor that develops from breast tissue. It originates from the epithelial or stromal component of the normal mammary gland. The gland consist of ducts and lobes. They are structurally embedded in the stroma. The main function of breast tissue is milk production and its storage^{8,9} A normal breast is made up of 15-20 lobes. Lobes are formed of grouped lobules which are milk producing glands. They are connected by milk ducts. Stroma, composed of fatty and connective tissue, envelopes and protects the lobes, blood and lymphatic vessels and supports breast tissue as a whole¹⁰. Lymph is a fluid that contains waste products from tissue and immune cells. Lymphatic vessels

connected to lymph nodes located around the breast drain off lymph fluid from the tissue¹¹. During puberty, breast tissue responds to hormonal changes and begins to develop. Estrogen and progesterone hormones stimulate mammary gland growth. The secretion of prolactin and somatotropin from the pituitary gland is involved in the regulation of breast development. The stimulation increases the branching of the duct system and the mass of glandular tissue during puberty¹². Deregulated hormone stimulation is the driving force of breast cancer development¹³⁻¹⁵. It is a heterogeneous disease with a variety of subtypes¹⁶⁻¹⁹. To improve the diagnosis and prognosis of breast cancer, there are a few classification systems depending on the histological, molecular and functional characteristics of breast cancer cells^{9,20,21}.

1.2.1 SUBTYPES OF BREAST CANCER

1.2.1.1 Histological Classification of Breast Cancer

Histological classification refers to the grading of tumor growth pattern²². Breast cancer is predominantly classified into two histological subtypes which are in situ carcinoma and invasive carcinoma. They are further sub-classified into either ductal or lobular carcinomas^{20,23}.

Carcinoma in Situ refers to the limited malignant development of breast tissue in ducts and lobules by basement membrane. It is further divided into *Ductal carcinoma in situ (DCIS)* and *lobular carcinoma in situ (LCIS)*. *DCIS* is the most common non-invasive breast cancer subtype. It is further classified into five subtypes which are *comedo carcinoma*, *solid*, *cripriform*, *papillary* and *micro papillary*^{23,24}. The major risk factors of recurrence in *DCIS* are defined as grade, size and margins. The death risk is lower than 2% among patients diagnosed with *DCIS*²⁵. *LCIS* is generally observed in women aged between 40 and 50 years. It often cannot be detected by mammography. The patients diagnosed with *LCIS* have a 30-40 % of developing invasive carcinoma^{26,27}. Invasive breast carcinoma is also differentiated into further subtypes. *IC-NST* is the most common subtype of carcinoma contributing to 70-80% of patients diagnosed with invasive carcinoma²⁸. Irregular and enlarged nuclei which are the characteristics of poorly differentiated tumors, can be observed in *IC-NST*. These tumors are defective in terms of tubule formation²⁹. The last group of histologic classification is *metaplastic carcinoma*. This class is rarely observed among breast cancer patients. It comprises of less than 1 % of all cases. They are

divided into *matrix producing carcinomas*, *squamous cell carcinomas* and *carcinomas with prominent spindle cell components*. The prognosis of this subtype is poor^{30,31}.

1.2.1.2 Molecular Classification of Breast Cancer

The heterogeneous nature of cancer has created the need of alternative classifications of breast cancer. The discovery of the century, microarray technology, has made the molecular categorization of breast cancer possible. The microarray technique has enabled the identification of the molecular basis of cancer heterogeneity³²⁻³⁵. High throughput gene expression analysis has sub-classified breast cancer into five categories^{9,17,22}. They offer well-defined diagnostic and therapeutic tools since they are based on the intrinsic changes in cancer cells. Table 1.1 summarizes the molecular subtype signatures of breast cancer³⁶. The current model determines the classifications of cancer cells according to their cell specific markers such as hormone receptors, growth factor receptors, cell proliferation markers, over-activated signaling pathways, cell cycle related factors, metastasis related markers. The spectrum may be extended with addition of microenvironment factors or the expression of non-coding RNAs^{17,37-40}. The established categories today are luminal A and B, HER2 positive, Basal-like and normal breast-like classes^{40,41}.

Luminal types are recognized by the expression of ER, PR, Bcl-2, CK8/18+ are molecular signatures. Luminal type cells show good differentiation and good prognosis unlike the other three subtypes due to their ER- feature. CK8/18 is a luminal type specific epithelial marker^{17,39}. Further microarray analysis have revealed differences in gene expression pattern of two luminal types. Luminal B subtypes express HER2 receptor unlike luminal A, so type B may cause worse prognosis in breast cancer³⁸. The molecular background of cancer cells affect the overall survival predictions. The breast cancer statistics indicate patients with basal-like subtype have the shortest disease-free survival. Together luminal types are make up 65-70% of all breast cancer cases^{35,42,43}. Luminal subtype cells are all ER+, so considering the frequency of luminal type diagnosis among of breast cancer patients, estrogen receptor status becomes the most distinctive marker in breast cancer⁴³. Therefore, the regulation and functions of ER is one of most attractive topics of breast cancer researches. It is also the most studied target in drug development. In fact two inhibitory drugs, tamoxifen and fulvestran, have been developed to prevent its action in

estrogen responsive breast cancer cells. However, almost 30% of patients are reported to resist hormone therapies in long term⁴⁴⁻⁴⁶. Therefore, additional molecular signatures are required to develop more optimal treatment strategies in breast cancer.

1.2.2 SIGNALING PATHWAYS IN BREAST CANCER CELLS

In addition to the efforts to classify breast cancer cells, signaling pathways over stimulated or suppressed in cancer cells have been investigated. Depending on the activated signaling networks, cancer cells acquire distinct capabilities⁴⁷⁻⁴⁹. The hallmarks of cancer (figure 1.1) imply that cancer cells activate the signaling molecules involved in proliferation, cell growth, cell motility, anti-cell death, genomic instability, angiogenesis. Cancer is a gradual process resulting from the disruption of pathway regulation^{1,5}. Cross-communication of pathways allow cells to create a favorable environment for proliferation. Not only they can stimulate their self-proliferation, but they make neighbor cells to secrete favorable factors for their growth^{50,51}.

1.2.2.1 Apoptosis Pathways

Apoptosis is a tightly regulated cell death program. It is a critical process to eliminate unwanted cells from the population. For the integrity of the organism, apoptotic signaling should be regulated strictly to maintain the balance between survival and death⁵²⁻⁵⁴. Apoptotic pathways are frequently dysregulated in cancer and this protects cancer cells from death signals. It can be initiated by intrinsic or extrinsic signals^{5,55,56}.

Intrinsic pathway can be activated by cellular stress, UV radiation, heat, viral infection, serum deprivation, DNA damage^{57,58}. This internal changes have been recognized by intracellular molecules which are the signal messengers for the induction of mitochondrial outer membrane permeabilization (MOMP). The molecules responsible for the regulation of MOMPS are BCL-2 family proteins. Their activities decide the intrinsic apoptotic fate. BCL2 family has both pro- and anti-apoptotic proteins. Pro-apoptotic proteins include bax, bak, bid, bik, bad, DIVA and bok proteins while anti-apoptotic proteins include bcl2, bcl-xl, mcl1, bcl-w and bcl-b. This family additionally contains BH3-only proteins, a subgroup of pro-apoptotic proteins which are bim,

tbid (truncated form) and PUMA. Structural analysis has revealed the possible protein-protein interactions between BCL2 family proteins. The sub cellular localization of proteins varies in the cells. BAX proteins are predominantly localized in the cytosolic part of mitochondria while BAK is an inner membrane protein of mitochondria. The subcellular localization of these proteins is important for the maintenance of cellular integrity in the absence of a death signal because the changes in their location may initiate an apoptotic response. Intrinsic apoptotic pathway is also known as mitochondrial apoptosis because the factors initiating the process are released from apoptosis. The intrinsic signals relayed by intracellular sensor molecules induce BH3-only proteins. They in turn activate BAX proteins. Following death stimuli, BAX translocate to inner compartment of mitochondria. BAK proteins also translocate into the inner mitochondrial membrane upon a death signal as with BAX. There they become integral membrane proteins and show conformational change and form homodimers within the membrane. Homo-oligomerization induces MOMP. However there are two activation models of BAX/BAK proteins. The indirect model suggests that BCL2 proteins bind to BAX/BAK proteins physically and maintain their inactive state. Activated “sensitizer” BH3-only proteins bind to BCL2 proteins and this allows the release of BAX/BAK proteins for mitochondrial translocation and MOMP induction. According to the direct activation model, activated “activator” BH3-only proteins can directly bind to BAX/BAK proteins and activate them. As highlighted, there are two types of BH3-only proteins which can be classified as sensitizers and activators. Sensitizers are unable to interact with BAX/BAD proteins, so only activate them by binding to BCL2, while activators directly interact with BAX/BAD proteins. Studies have demonstrated direct interactions of tBid (activator BH3-only protein) with BAX on/in membrane and confirmed “tBid induced BAX mediated MOMP activation “. Studies have also revealed the presence of BCL2 or BCLXL, anti-apoptotic proteins, inhibited the activation of BAX even in the presence of a death signal. MOMP induction results in the release of cytochrome c and apoptotic factors from mitochondria to cytoplasm. Its release induces its oligomerization with Apaf-1 and caspase 9 which in turn results in the activation of caspase 9. The activation of caspase 9 results in the cleavage of its downstream caspases-3 and -7 and their subsequent activation^{54,59-67}.

The extrinsic pathway is initiated by the stimulation of death receptors such as TNFR1, FAS, TRAIL1 and TRAIL2. Upon stimulation, death receptor oligomerization recruits adaptor proteins, such as FADD or TNFR1-associated death domain protein, to the receptor domain.

These adaptor proteins activate caspase-8. Its activation induces the cleavage of its downstream caspases which are caspase-3,-6,-7. Caspases -8 and-9 are called initiator caspases while caspases-3,-6,-7 are executioner caspases. The activation of downstream executioner caspases may be insufficient to exert an apoptotic response by death receptor signaling. That is why the extrinsic apoptotic pathway requires the activation of the intrinsic pathway as well in some cases. The extrinsic pathway induces MOMP with Bid activation by caspase-8. Then tBid translocates to the mitochondria and induces the release of apoptotic factors into cytoplasm. These factors in turn activate both caspase-3 and-8, so enable an appositive feedback loop which enhances apoptotic response^{54,68-74}.

1.2.2.2 EGFR Signaling in Breast Cancer

EGFR is one of the cell-surface receptors of cells. Its overexpression is associated with poor differentiation of cells in breast cancer⁷⁵⁻⁷⁷. It is frequently observed in triple negative breast cancer subtypes, so it suggests it may stimulate the formation of aggressive breast cancer⁷⁸⁻⁸⁰. The receptor exerts its action with activation of the PI3K/Akt, Ras-Raf-MAPK, JNK pathways⁸¹⁻⁸⁴. The activation of signaling pathways shows differential biological effects depending on the nature of the stimuli or which pathways are activated. The stimulation of the receptor is reported to induce cell proliferation, cell motility, metastasis, anti-apoptotic response and angiogenesis^{75,85}. The activation of EGFR in cell culture has promoted loss of cell polarization and epithelial features of cells⁸⁶. The inter-EGFR family cross-talks have been often observed with HER2. The co-expression of EGFR and HER2 is indicated to be inversely correlated with estrogen receptor expression. The heterodimer interaction of EGFR with HER2 increases invasive abilities of breast cancer cells^{87,88}. The reports show that EGFR induces E-cadherin loss with the presence of mesenchymal markers such as vimentin. That is one way to promote EMT (epithelial-mesenchymal transition) in cancer cells. The inhibition of EGFR activity with erlotinib inhibitor has increased E-cadherin expression and inhibited cell motility in cancer cells. This supports the suggestions that EGFR may be involved in the EMT process^{89,90}. EGFR is one of molecular markers for basal-like breast cancer subtype⁴¹. Studies have demonstrated that the inhibition of

Table 1. 1: Molecular classification subtypes of breast cancer³⁶

Breast cancer population	Subtypes	Detection method	Current treatment	Markers of response	Markers of resistance	Exploitable markers
ER+ and/or PgR+	Luminal A	Gene expression profile	HT alone	High ER levels	ER levels <10%	ER-alpha aberrations
	Luminal B	Ki67 label index >14% Lack of expression of PgR	HT + mTOR inhibitors CT	High Ki67 levels	HER2 overexpression Low Ki67 levels	FGFR 1 PI3K/PTEN/CCND1 alterations
HER2+ ER+	HER2-enriched	Gene expression profile	CT + anti-HER2 (single or double block) ± mTOR inhibitors HT + anti-HER2	High Ki67 levels	p95 HER2	D16 HER2
	Luminal A	HER2 amplification			High ER levels	Immune metagene
	Luminal B	ER overexpression Ki67 label index >14%				ER-alpha aberrations FGFR 1 PI3K/PTEN/CCND1 alterations
HER2+ ER–	HER2-enriched	Gene expression profile HER2 amplification	CT + anti-HER2 (single or double block) ± mTOR inhibitors	High Ki67 levels	p95 HER2	D16 HER2 Immune metagene FGFR 1 PI3K/PTEN/CCND1 alterations IFNG STAT 1
Triple negative breast cancer	Basal-like	Gene expression profile	CT	High Ki67 levels		BRCA1 mutations
	Nonbasal-like	Lack of expression of ER and PgR Lack of expression of HER2				TOP 2A P53 mutations BCL2 TIL FGFR 2 NOTCH Caveolin 1

EGFR signaling prevents the expression of RAS and MYC signatures in TNBC cells⁹¹. Its involvement in important cellular functions made EGFR an attractive target for drug development. There have been a few quite effective inhibitors against its activity. Gefitinib is one of them. It was the first selective inhibitor for the receptor. It binds to ATP-binding site of enzyme and inhibit its kinase activity. This prevents its action in the RAS signaling cascade⁹²⁻⁹⁴.

1.3 ATAD2 AND CANCER

1.3.1 ACTIVATION OF ATAD2 GENE EXPRESSION IN CANCER

Cancer is the black death of the 21st century. A total of 19.3 million new cancer cases has been estimated for 28 cancer types till 2025 in the GLOBOCAN 2012 report of International Agency for Research on Cancer (IARC). According to the latest statistics on cancer incidence released by the World Health Organization, there have been 14 million new cases already worldwide in just 2012, 11.9 % of which were breast cancer⁶. The drastic increase in breast cancer incidence compared to previous reports are worrying and urge the scientific community to act to prevent and control breast cancer progression globally. Ineffective treatments have demonstrated the need for improvements in disease management. Cancer researchers have been searching of new approaches to cancer studies to develop better treatment strategies. Massive efforts have been made for the extensive analysis of comparative gene expressions profiling of cancer cells. Gene expression profiling determines the genetic constitution of individual cells and reveals the expression pattern of each gene at transcriptional level in cell type- specific manner⁹⁵⁻⁹⁷. Sequencing or DNA microarray methods are high throughput techniques used to detect differential expression of the genes that changes from cell to cell under varying conditions. They provide the characterization of thousands of gene expressions simultaneously. Genome-wide scale microarray analyses are quite useful to associate the biological relevance of altered gene expressions between cancer cells or between cancer cells and normal cells^{42,43,98,99}. The progress in molecular biology has turned this technology into a tool

enabling disease classification, the characterization of mechanisms involved in disease progression, the identification of diagnostic biomarkers, and correlating marker expressions with disease responses. This approach has opened the door to a better understanding of the molecular pathogenesis of cancer and to develop more effective targeted therapies that enable more accurate disease management. Since the first publications in 1995, gene expression studies have revised the perspective on genetics over the last 20 years. Following the introduction of cDNA hybridization technique by Schena et al. at that time, the first ever results on the application of microarray in expression-profiling has emerged in the next year with a study by the Stanford group^{100,101}. Afterwards, research on comparative gene expression patterns has gained momentum and the field took another direction to seek cancer-associated specific gene markers; that is what is currently recognized as the best prognostic and predictive tool and as the basis of future optimized cancer therapies. Nowadays the cDNA hybridization technique, known as microarray, has become a routine protocol in laboratories. Breast cancer studies have benefited from this scientific development in the field. There are studies that have adapted a genome wide approach to identify gene signatures from breast cancer cells. Quite a few groups have identified genes responsive to drug treatments or involved in their resistance and have revealed expression signatures of patients treated with them¹⁰²⁻¹⁰⁵. Several studies showed the association of expression patterns with deregulated signaling pathways and their oncogenic outcomes. These genes should be evaluated in the context of their-involved mechanisms and can be regarded as markers of specific cellular events in breast cancer such as hypoxia, p53, PTEN loss, wound healing response and epithelial-mesenchymal transitions¹⁰⁶⁻¹¹⁰. A great number of prognostic gene-expression signatures of breast cancer have been identified in the literature in recent years together with their relevant functions and clinical validations¹¹¹⁻¹¹⁴. Ectopic activation of these genes may cause the loss of normal cell functionality and make them gain oncogenic activity. The activated genes in turn may initiate epigenetic mis-regulation of other silent genes and this goes on and on with activation of others. This dysregulation loop drives into a malignant state in the end. Even though we, to a great extent, have mastered the oncogenic functions of these genes, we are rarely aware of their normal physiological status and their potential to drive malignancy. We do not know in which step of malignant transformation they may take a role. One example of such genes is

ATAD2 which is also known as ANCCA and PRO2000. It was identified with microarray studies¹¹⁵. The first reports on this gene have dated back to early 2000s when it was recognized as significant gene signature in breast cancer¹¹⁶. ATAD2 was identified as one of 70-gene signatures predicting the clinic outcome of distant metastasis in 2001 and also one of 76-gene signatures predicting the high risks of distant metastasis in primary tumors of 286 patients diagnosed with breast cancer in 2005^{35,116}. Its elevated expression level has been reported to be observed in more than 70 % of breast cancer patients¹¹⁷. Its high expressions have been supported in multiple studies¹¹⁷⁻¹²⁷. Among an independent cohort of 225 primary human breast tumors, Immunohistochemistry (IHC) results have demonstrated the overexpression of ATAD2 in 63% of estrogen positive cells, in 86% of estrogen negative cells and in 88% of TNBC cells¹²¹. This result implies the correlation of high ATAD2 expression with all three types of breast cancer. However, its overexpression is not limited to breast cancer. Its high expression has been reported in prostate cancer, lung cancer, B-cell lymphoma and hepatocellular carcinoma (HCC)¹²⁸⁻¹³¹. Figure 1.2 illustrates the expression levels of ATAD2 in various cancer types (GSE2109)¹¹⁸. It is expressed higher in almost all cancer types compared to their corresponding normal cells. The Kaplan-Meier graph demonstrates that its high expression is correlated with poor prognosis of lung cancer¹¹⁸. The studies support the strong association of prostate cancer progression and ATAD2 expression. Then, the question of how ATAD2 is upregulated in such a variety of cancer types has arisen. Independent studies have implied that it may be correlated with the gene number. One of them has reported that the integrated analysis of copy-number and their expression analysis has revealed 8q24 amplification in 72% of the cases in breast cancer^{117,119,124,132}. It is the site that ATAD2 is located in the genome and it is a region that frequently amplified in cancer^{133,134}. Another study has revealed its genomic amplification in HCC as well¹³⁵.

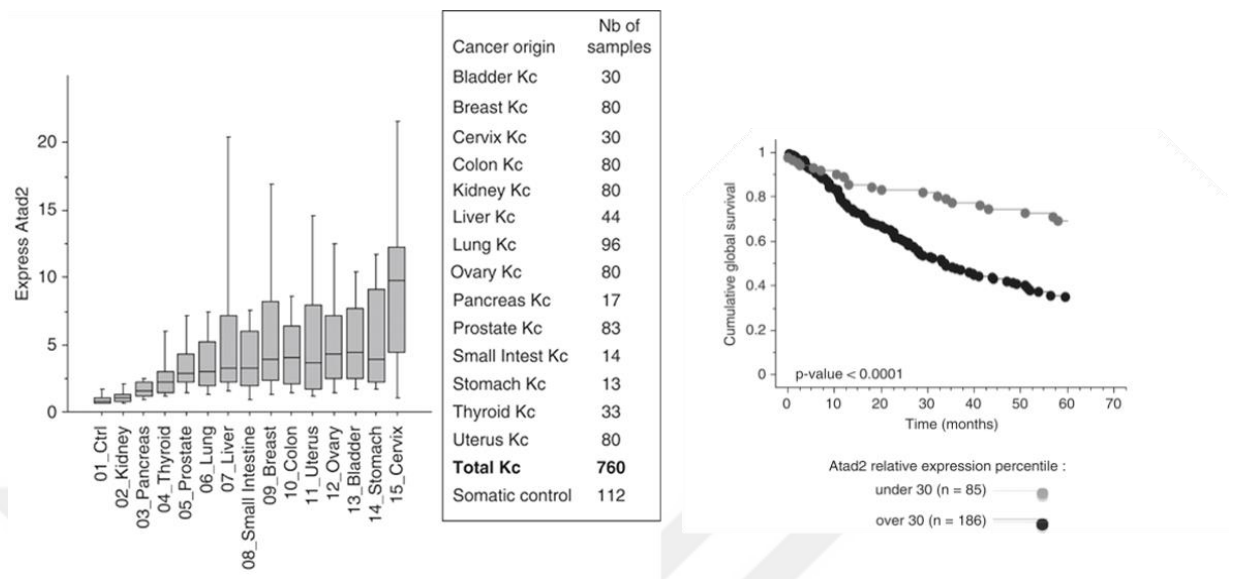


Figure 1. 2: Left graph: ATAD2 expression levels across various cancer types Right graph: Kaplan-Meier graph of cumulative global survival of lung cancer patients according to their ATAD2 expression levels.

1.3.2 THE FUNCTION AND REGULATION OF ATAD2 ACTIVITY IN CELLS

AAA+ nuclear coregulator cancer associated (ANCCA) is a recently identified nuclear protein which is a member of the AAA+ ATPase family. It possess a bromodomain that is responsible for its binding to acetylated histones¹¹⁵. Phylogenetic studies illustrate its highly conserved ATAD2 homologues in the eukaryotic kingdom. ATAD2-like proteins have been found to be conserved from yeast to plants to human. These proteins share the highest similarities within the sequence of two distinctive domains; AAA+ ATPase domain and the bromodomain. AAA+ ATPase domain present in all living organisms and sequence alignment studies have revealed AAA+ ATPase as the highest conserved region in ATAD2 protein across evolution. This domain may function in different cellular processes¹³⁶. The mutations that occur at this domain of ATAD2 appear to affect its ATP binding capacity and its binding to acetylated histones¹¹⁸. The overall conservation of amino-acid sequences in both bromodomain and ATPase regions suggest the necessity of its global structure. Yta7, ATAD2 homologue of *Saccharomyces cerevisiae*, is the most studied homologue in

literature. Experiments with Yta7 indicated a third conserved region located in the upstream region of the AAA+ ATPase domain in the N-terminal site of gene. The negatively-charged nature of this region suggests its involvement in histone binding. High similarity between Yta7 and ATAD architecture implies some shared functions. Therefore, the functional studies with this homolog have provided insight into the possible functions of ATAD2 in humans as well. They have revealed a possible function in chromatin regulation, such as the regulation of nucleosome density. They suggest a role as a histone chaperone altering histone density of chromatin. These findings imply the transcriptional activity of ATAD2 as a scaffold protein or as chromatin remodelers. Yeast and mammalian studies on ATAD2 functions showed complementary results¹³⁶. In humans, ATAD2 was found to interact with estrogen and androgen receptors and act as a co-activator for these nuclear receptors. The later reports showed its requirement for full transcriptional activity of receptors. It seems ATAD2 stimulates the activation of estrogen and androgen responsive genes in the presence of hormones^{115,127}. Work on transcriptional regulation of the ATAD2 gene has shown two spliced variants of the gene. The longer form functions in chromatin binding while the shorter form cannot act in the same way and it is found mainly in the cytosolic part. This difference has been attributed to the lack of 300 amino-acids at the N-terminal end of the gene at shorter variants compared to its corresponding functional variant. The studies revealed the importance of this missing region in the interaction of protein with the androgen receptor and E2F factor. Therefore, just the longer variant may exert its transcriptional activity^{118,123,127}. It was found that ATAD2 can directly induce the activation of H3K36 methyltransferase, NSD2/WHSC1 gene expression so that it may regulate post-translational histone modifications^{115,118}. This finding indicates that the transcriptional activity of ATAD2 in humans may be the result of its chromatin remodeling function. It is also an inference supporting yeast studies. Figure 1.3 represents a diagram of domains located within the ATAD2 protein. As we already know, it has 2 AAA+ ATPase domains and 1 bromodomain. While the ATPase domain may mediate cellular events such as the facilitation of protein folding and unfolding, assembly or disassembly of protein complexes, protein transport and its degradation, it regulates significant cellular functions such as replication, recombination, repair and transcription^{137,138}. Bromodomain binds to acetylated lysine residues and may be involved in protein-protein interactions and at the

same time may play a role in the assembly or activation of multi-component complexes involved in transcriptional activation^{139,140}. In addition to these two significant domains, the PhosphoMotifFinder feature of human protein reference database (HPRD) illustrates the presence of an experimentally verified tyrosine (T) phosphorylation site at 1084 in the ATAD2 protein. This can be an important observation since this phosphorylation motif is located in the bromodomain region and it can be a post-translation modification site for the regulation of ATAD2 activity. The regulation of its expression is still in the dark, However there is one study related its expression conversely miR-372 expression and there was a binding site for miRNA in 3'UTR of mRNA. Experiments confirmed that ATAD2 is suppressed with miR-372 expression in HCC cells and this is the first proof of ATAD2 as a miRNA target¹²⁵. Likewise, this can be the first clue regarding its regulation in the cell. However, there are findings related with its expression levels showing fluctuation throughout the cell cycle. There is a study from 2010 in which global scale proteome and phosphoproteome of the human cell cycle has been investigated¹⁴¹. Accordingly, the ATAD2 protein level increased in G1/S and S phase with a peak at the late S phase, and then declined in the G2 and M phase. Conversely, it showed the highest phosphorylation site occupancy in the M phase and stayed high in G1 and through the G1/S checkpoint, and then came a dramatic decrease in S and it was at the lowest level in the G2 phase. This suggests that ATAD2 may be activated by phosphorylation in mitotic cells.

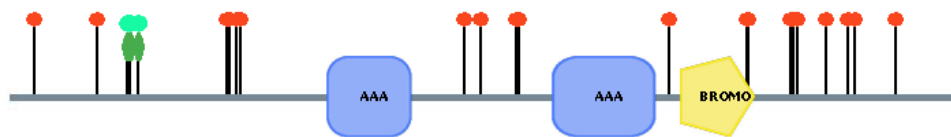


Figure 1. 3: The graphical representation of domains on ATAD2 protein with Human Protein Reference Database (HPRD)¹⁴².

1.3.3 ONCOGENIC FUNCTION OF ATAD2 IN CANCER

The conservation of its structure across living organisms indicates the functional significance of ATAD2 in the cell. The further evaluation of its transcriptional activity has

revealed more information on its cellular activity. It can interact with E2F transcription factor and play a role in RB/E2F downstream signaling as co-activator¹²³. It enhances the cell cycle control of their transcriptional activities. There is one study showing that ATAD2 recruits MLL methyltransferase to the promoter regions of E2F responsive genes^{115,122}. Another one showed it may activate the expressions of H3K36 and NSD2 (NF-Kb co-activator) methyltransferases^{115,118}. ATAD2 may increase the expression of RB/E2F downstream target genes and make the pathway to work at full efficiency in both ways. The Retinoblastoma protein (pRB) is a tumor suppressor gene. It plays a vital role in the regulation of cell proliferation. It is the responsible protein of the G1 checkpoint. Its interaction with the E2F transcription factor suppresses cell cycle progression if the conditions are not convincing to progress through the S phase. Its loss of function may induce a deregulated cell cycle which leads to tumor progression¹⁴³. That is why the RB/E2F pathway has been disrupted in a variety of cancer types, mostly through its genomic mutations¹⁴⁴. Considering this pathway is placed in the center of cell cycle control, without its control mechanisms E2F regulated genes show aberrant expressions. One of the E2F target genes is MYC oncogenes. It is frequently overexpressed in tumors^{145,146}. It is adjacent to ATAD2 in the genomic map, so it is often co-amplified with ATAD2 gene in cancer. However, it is known that amplification of MYC does not always reflect its true expression levels, and that ATAD2 expression mostly correlates with its genomic amplification^{135,147}. Besides, it has been shown ATAD2 is involved in the MYC complex and cooperates in the activation of MYC target genes. In fact, researchers showed that ATAD2 could be the limiting factor for the transcription of MYC-responsive genes¹¹⁷. Therefore, it is suggested that ATAD2 may be the driving force for MYC to exert its oncogenic activity. The transcriptional regulatory functions of ATAD2 seem to be down to its bromodomain. Bromodomains (BRDs) are protein domains binding to acetylated lysine residues of histones. Lysine acetylation is a type of histone modification. It is one of best known epigenetic processes. These modifications are the carriers of the epigenetic state of cells through divisions. Each modification reflects distinct gene activation status. Their effects are dependent on the specific residue and to what extent the residue is modified. A correlation has been found between specific posttranslational modifications of histones with transcriptional events; such that although all acetylations of lysine residues on H3 and H4

are associated with transcriptional activation, methylation of lysine residues may be either associated with transcriptional repression (H3K9, H3K27) or activation depending on amino acid modified and methylation degree. Genome-scale coordinated histone modifications are closely coupled to gene expression changes between cells¹⁴⁸. The nuclear macromolecular complexes recruit specific proteins such as bearing BRDs to genomic regulatory regions. These proteins in turn recognize specific histone acetylated sites and induce chromatin regulation by targeting chromatin modifying molecules¹⁴⁹. Therefore, there is a strong biological relevance of BRDs action in cells. Overexpression of these proteins have been reported in cancer cells and they show correlation with patient survival¹⁵⁰. ATAD2 is one of these proteins with BRD and its high correlation with poor prognosis and high disease recurrence has been stated among breast cancer patients^{117,118}. ATAD2 as a BRD containing protein has been found to be involved in the transcriptional activation of the cell cycle, differentiation and apoptosis related genes¹²³. ATAD2 selectively binds to acetylated residues of histones. H3K14ac is the most preferentially recognized mark by the ATAD2 bromodomain and this mark is quite abundant at the promoter regions of E2F responsive genes. . The requirement of a functional ATPase domain of ATAD2 for protein multimerization has been demonstrated and upon the loss of this activity the protein has lost its effective binding to acetylated histones as well, so this implies that the two domains intercommunicate to exert the transcriptional activity of the protein^{117,118}. When microarray data sets have been examined, the highest correlated genes with ATAD2 expression have been classified into cell proliferation and mitosis processes in breast cancer. Further examination has revealed that four of 45 kinesin family genes have been co-expressed with ATAD2 as well. These kinesins function in the assembly of mitotic spindles and chromatin segregation. The experimental validation of the microarray result, conducted by the Zou J. X. group in 2014, has supported the preliminary data. They confirmed ATAD2-mediated upregulation of mitotic kinesins in breast cancer. Its association with histone modifications and its evidenced elevated expression at both the transcriptional and protein level during the G1/S or S phase of the cell cycle show that it may be involved in activation, sustenance or termination of cellular events¹⁵¹. The association of its overexpressed state with cellular events has been revealed with down-regulation experiments. Accordingly, they found the absence of protein hindered prostate

and lung cancer cell proliferation. Independent studies have reported regression in breast cancer cell proliferation after ATAD2 suppression^{118,120,151}. One study showed decreased colony formation ability of HeLa cells after downregulation. However, there was no change in cell proliferation rate after downregulation in exponentially growing cells¹¹⁷. Normal fibroblasts showed decreased ATAD2 expression after serum starvation and blocked cell cycle progression, but then re-upregulated upon serum supplementation and resumed the cell cycle. However, the addition of serum inhibited cell cycle progression after ATAD2 downregulation under serum starvation conditions because DNA synthesis had been blocked¹¹⁷. Altogether, studies support its involvement in tumorigenesis and they indicate its cooperation with cellular signaling pathways to execute its oncogenic functions. So far they could not highlight the underlying interactions yet.

1.3.4 THE ROLE OF ATAD2 IN ESTROGEN SIGNALING

ATAD2 frequently shows high expression in breast cancer cells^{116,121}. It is a gene responsive to both estrogen and androgen hormones^{115,127}. Its expression is strongly upregulated with estrogen/androgen stimulation in breast cells. It is also recruited to the promoter regions of estrogen responsive genes. Molecular studies have revealed its cooperative work with estrogen receptor α (ER α). Its enhancer role in the transcriptional activation of these genes indicated its co-activator role for ER α in breast cancer cells. It supports the activity of ER α . Its presence is required in transcriptional regulation of some ER induced genes such as cell cycle progression, DNA synthesis and replication related CCND1, MYC, survivin, SGK1, ACTR. ATAD2 selectively controls the expression of ER α target genes¹²³. The same group later stated its similar transcriptional activities for androgen responsive genes as well and it is required for full activity of the receptor¹²⁷. One study specifically reported the correlation of stimulation of early prostate development mediated by testosterone with increased ATAD2 expression¹⁵². Altogether, they placed ATAD2 in the center of steroid hormone signaling. Investigations across primary endometrial cancers revealed that high expression of ATAD2 is strongly associated with increased MYC signaling but their co-expression is negatively associated with ESR1 expression in the tumors¹³². Similarly, ATAD2 expression is frequently found to be high in

triple negative breast cancer tumors and one microarray study on the identification of genes regulated by ER α in MCF7 cells has displayed its downregulation by estrogen 12 h after treatment in the culture. ATAD2 was found to be in direct physical interaction with ER α , but it is not involved in the recruitment of the receptor to its target genes. On the other hand, the results indicate ATAD2 acts on the recruitment of CBP to gene promoters¹¹⁵. CBP is a bromodomain bearing protein demonstrated to be involved in E2 induced hyperacetylation of histones¹⁵⁰. ATAD2 downregulation prevented CBP occupancy at the promoter regions of cyclinD1, c-myc and E2F1 genes. The mutation experiments emphasized that ATPase activity is necessary for ATAD2 to mediate E2 induced gene expressions as an ER α coactivator because the mutant form of the protein in this domain diminished ER α target gene expression¹¹⁵. Further functional studies of E2-induced ATAD2 activity revealed its association with kinesin expression in cancer cells. Kinesins are motor proteins and they are expressed in a cell-specific manner. They are responsible for the organelle transport on microtubules. They function in cell division. Expression of 26 kinesin genes is regulated by E2 in MCF7 cells¹⁵³. Among these genes, it was found that ATAD2 mediates the upregulation of mitotic kinesins and downregulation of non-mitotic kinesins via ER α following E2 induction¹⁵¹. Further expression analysis showed striking similarities between functions of ATAD2 and another nuclear protein, ACTR. This is a member of p160 coactivator family and acts as coactivator of nuclear receptors just like ATAD2¹⁵⁴. The protein, also recognized as a proto-oncogene, is overexpressed in 30%-40% of breast cancer cells and 10% of its overexpression is reported to be associated with gene amplification in breast tumors. The studies showed its requirement for both E2 dependent and independent breast cancer cell proliferation. The investigations indicated its role as E2F coactivator in cell cycle control mechanisms of breast cells. Its cooperation with ERBB2 is believed to be one of the reasons of antiestrogen resistance and its ectopic expression transforms human mammary epithelial cells¹⁵⁵⁻¹⁵⁷. Chromatin IP (ChIP) assays have demonstrated the direct interaction of ATAD2 with the ACTR and E2F proteins. ATAD2 is also known as E2F coactivator and both ACTR and ATAD2 induce E2F-responsive gene expression. ATAD2 binds to E2F1 on the promoter of the ACTR gene, so it may directly regulate its expression in breast cancer cells. It is assumed there is a positive feedback loop between ACTR and ATAD2 and ATAD2 is a direct target of the ACTR as

well. ACTR was previously shown to activate its own expression and now the underlying mechanism of this appears to be its transcriptional cooperation with the ATAD2 protein. The overexpression of both ACTR and ATAD2 synergistically increased the expression of E2F target genes 200%^{115,121}. Overall the results suggest it is required for both cell proliferation of both hormone-responsive and hormone-nonresponsive breast cancer cells and for the expression of cell cycle and DNA synthesis related E2F target genes such as cyclin E1, cyclin A2, cyclin 1, cdk2, cdc2, cdc6.

1.4 CELLULAR SENESCENCE

Cellular senescence has been described with the Hayflick discovery. He showed that normal fibroblast cells went under growth arrest after a limited number of divisions. This was an irreversible state since even though cells have been induced with growth factors, cell proliferation did not start again, so cells are said to have a definite replicative lifespan¹⁵⁸. Over four decades after Hayflick's finding, we now are aware that cancer cells do not have same control mechanism on cell growth as normal cells. The senescence process does not work for them. Therefore, they are defined as immortal cells. The accepted reason behind the protection of cancer cells from senescence is that 90% of them have overexpressed telomerase activity, so they keep stable telomere ends even with infinite divisions^{159,160}. The reports supporting this suggestion came from mutation experiments of the telomerase enzyme. The mutated form of telomerase could not sustain cancer cell proliferation¹⁶¹. Likewise, the senescence response was dependent on p53 and RB pathways as well¹⁶²⁻¹⁶⁵. However despite continuing research, it is still a complex topic. There are differing opinions on the beneficial effects of senescence in vivo. There are some studies that reported mutant p53 could induce cell growth in vivo and double mutant could inhibit early tumor development in mice. It is accepted as the indications of the natural senescence mechanism in vivo. On the other hand, there are studies proving senescent cells may induce cancer cell growth also in vivo. For instance, the mimic of an in vivo system in which TGF- β deficient fibroblasts stimulated adjacent epithelial cell growth^{164,165}. The molecular basis of these cellular interactions has been investigated in depth under this topic and revealed many aspects of senescent cells.

1.4.1 CHARACTERISTICS OF SENESCENT CELLS

To find a clear definition of senescence is still challenging. Scientists have therefore identified a few criteria to detect senescent cells. Multicellular organisms are the mixture of both mitotic and post-mitotic cells. While mitotic cells correspond to cells still having proliferating abilities, post-mitotic cells, as the name states, are no longer capable of proliferation. While G0 seems to be their common point, the difference between the two states is that mitotic cells are still responsive to external factors and can exit G0 and re-enter into the cell cycle. These cells are defined as quiescent cells at the G0 point. When growth factors in the medium have been withdrawn, cells stop growing and enter into G0 which is a reversible state. Post-mitotic cells exit the cell cycle indefinitely and cannot reinitiate cellular proliferation¹⁶⁶. However there should be more than a simple serum withdrawal to push cells into irreversible growth arrest. One way to arrest cells is the induction of CDK inhibitors. CDK inhibitors act as the sensors of cell cycle and let or prevent cell cycle progression depending on the cellular conditions. They may cause cell cycle arrest even in the presence of growth stimulation¹⁶⁷⁻¹⁶⁹. Therefore a strange situation occurs during the progress of senescence mechanism. CDK inhibitors such as p21, p16 ensure that the cell cycle does not reinitiate and it remains permanent in this case because growth factors cannot stimulate proliferation of senescent cells; however, they can promote the growth-pathways¹⁷⁰⁻¹⁷². That is why senescent cells display over activated growth associated signaling pathways such as MAPK and mTOR while their downstream cell cycle progression is blocked. This state is called cellular hypertrophy^{173,174}. As a result, the first sign of senescent cells is their cell cycle arrest coupled with increased intracellular activities of growth pathways. The senescence response differs depending on the genetic makeup of cells¹⁷⁵. Some cell types exhibit preferential specific growth arrest. For instance, senescent mouse fibroblasts are frequently arrested in the G1 phase; however defective kinase activity induces G2/M arrest in the same cells. The second known sign is hyper-secretion from senescent cells which is a natural result of their increased cellular functions. It is found that senescent cells secrete mitogenic factors to their microenvironments¹⁷⁶. It explains the previous observations of that senescent cells may promote neighbor cell growth. Another senescence indication is apoptosis resistance. Apoptosis is a natural

programmed cell death induced by intra- or extra-cellular signals. It is a significant tumor-suppressive mechanism. Reports show senescent cells remain mainly arrested and do not go down the cell death route¹⁷⁷⁻¹⁷⁹. This may be the explanation for the accumulation of post-mitotic cells with age in vivo instead of being wiped out entirely. However, one study showed both facets of apoptosis and senescence association. They reported that while senescent human fibroblast cells did not resist apoptosis in the presence of Fas death receptor signaling, they did not show any sign of apoptosis with growth factor depletion this time^{177,180}. There could be yet unknown causes behind this phenomenon. In parallel with that, subsequent studies revealed that deregulated apoptotic proteins may change the cell fate. This means senescent cells may undergo apoptosis or the other way around. They highlight the p53 protein activity as the common protein of both pathways, so it is believed it may be the link between the two mechanisms¹⁸¹. The senescent cells show striking change in their gene expression pattern. They have elevated expressions of CDK inhibitors; p21 and p16. They function in p53 and RB/E2F signaling pathways in the cell and we know these pathways are highly deregulated in cancer. These pathways control pRB phosphorylation during the cell cycle. p21 and p16 function as tumor suppressors. They keep pRB at hypophosphorylated state and maintain arrested cell cycle status. Senescent cells have elevated cyclins D and E, but repressed cell cycle stimulating gene expression such as cyclins A and B. It is suggested that some of them are repressed because E2F transcription factor activity is inhibited by pRB hypophosphorylation^{171,182-184}. On the other hand, as we already mentioned as the over-secretory feature of senescent cells, the genes associated with secreted growth signals is overexpressed in these cells. Last but not least, senescence reference is the activity of a lysosomal enzyme. Mammalian cells express a specific enzyme called β -galactosidase in this organelle. This enzyme exerts a senescence specific activity range and under senescence conditions it could be detectable at pH 6.0. This enzyme uses 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) as substrate. This activity is detected by histochemical staining in senescent cells. It is used as a biomarker of senescence and enables its quantitative determination¹⁸⁵⁻¹⁸⁷. Another senescent marker detected can be senescence-associated DNA—damage foci (SDFs). It preserves DNA damage related proteins such as phosphorylated H2AX (γ -H2AX) and

53BP1 protein. This focus occurs as a result of DNA damage due to dysfunctional telomeres.

1.4.2 REASONS OF CELLULAR SENESENCE

Senescence may be induced by various signals. It can be the result of dysfunctional telomeres. Telomeres are the repetitive ends of DNA. They are protected with cap proteins from degradation and damage because their integrity is important for proper DNA synthesis. These regions cannot be replicated during proliferation, so during each division, cells lose between 50-200 bp from the DNA end. After a limited number of cell divisions, cells stop dividing due to end-replication problems. Senescence is triggered when telomeres are shortened to a critical point¹⁸⁸⁻¹⁹⁰. The underlying cause is that dysfunctional telomeres induce DNA damage response (DDR). This process initiates subsequent cascades of protein activations involved in the DNA repair mechanism. Two of them are adaptor protein 53BP1 and chromatin modifier H2AX (γ -H2AX) proteins which are located in DNA damage loci, as mentioned before, which may be used to detect senescent cells^{188,191}. This end-replication problems may be overcome with telomerase enzyme activity. This end-replication problems may be overcome with telomerase enzyme activity. This enzyme has a catalytic subunit to add telomeric repeats to the DNA end; however in normal cells this protein is expressed at the basal level and insufficient to compensate the loss repeats. On the other hand, cancer cells and germ cells exhibit overexpression of this protein and they can escape telomere shortening with its activity¹⁹²⁻¹⁹⁵. Studies have shown severe DNA damage may initiate senescence response as well¹⁹⁴. Especially double strand breaks (DSBs) is the major causes of DNA damage- dependent senescence. It is suggested that the damage signal may activate p53 pathways and induce senescence-dependent growth arrest. In fact, the studies indicate both telomere-dependent and DNA-damage initiated senescence may activate p16/Rb or p53/p21 signaling pathways^{188,191,196-198}. The most striking cause of cellular senescence is oncogene-induced senescence. Oncogenes are activated cancerous genes. They can drive cells into transformation. This suggests senescence induction following oncogene activation is one of tumor suppressive mechanisms. This phenomenon has been supported with observations by RAS and its downstream targets RAF, MEK,

ERBB2, E2F induced cellular senescence. The studies on MEF cells showed the functional activity of both p53 and RB is required for cells to induce senescence when RAS is activated¹⁹⁹⁻²⁰³. However, human fibroblast cells could show senescence response in the absence of their activity. This result may be attributed to the activation of MDM2 and p19 proteins, which are inhibitors and activators of p53 respectively, by RAS and their expression levels decide the activity of p53 in the cell^{202,204-206}. Another protein appears to be in the center of the RAS induced senescence mechanism is p38-mitogen activated protein. It is activated after RAS expression. Moreover, the studies revealed the path from RAS activation to senescence induction. It indicates that RAS overexpression causes ROS accumulation in cells, which in turn increases p38 activity. Enhanced p38 signaling activates the p16 pathway and this induces the senescence response of the cells. There are some reports also suggesting direct activation of p16 with ROS induction for RAS-dependent senescence response²⁰⁷. Cell populations are heterogeneous structures with each of them having a distinct genetic background; therefore gene expression patterns of cells determine their senescence response.

1.4.3 SENESCENCE PATHWAYS

The senescence response is regulated with tumor suppressive signaling mechanisms; which are p53/p21 and p16/RB pathways¹⁶⁶. They can be activated together or individually. p53 is a tumor suppressive protein involved in the regulation of the cell cycle, differentiation and apoptosis. There are intracellular control mechanisms regulating the activity of p53. MDM2 protein facilitates its proteosomal degradation while p19 inactivates MDM2 activity inducing p53 action in turn. The activation of p53 means the transcription of cell cycle arrest and apoptosis related genes. One of the transcriptional target genes is p21. It is considered as the main downstream target of p53. It is a member of CDK inhibitors which prevent CDK2/cyclin E and CDK4/cyclin D activity. DNA damage responses induce the activation of the p53 pathway. Possibly the extent of damage decides the fate of cell and whether to enter into senescence^{172,208}. The dysregulation of p53 and p21 expression reduces senescence response in the cells^{209,210}. RB protein has a critical role in the regulation of cell cycle at the transition point from G1 to S phase. It is found in the

hypophosphorylated state at the early cell cycle stage to inhibit the transcriptional activity of E2F. Through progress to the S phase it is phosphorylated and this releases E2F factor. Its phosphorylation has been mediated by CDK4-6 and CDK2 bound to their partner cyclins; cyclin D and E respectively. The expression of cyclins has been induced with growth stimulation. Cyclin D expression is demonstrated to increase through RAS pathway activation²¹¹. P16 is another CDK inhibitor inhibiting RB phosphorylation and subsequent G1/S progression. p16 is one of the RAS downstream target genes. The activation of its expression induces cellular senescence through inhibition of E2F activity, and therefore G1 cell cycle arrest¹⁷². There is cross-talk between these two senescence inducing pathways. The loss of p16/pRB activity has been shown to upregulate p53/p21 expressions. It is suggested the E2F induced ARF expression may be the reason of this. The deregulation of any proteins involved in these pathways may prevent cells from triggering the senescence process¹⁶².

1.5 AIM OF THE RESEARCH

Epigenetic changes drive the initiation and progression of cancer with the stimulation of constitutive activity of cellular signaling mechanisms^{212,213}. There are numerous studies utilizing high throughput expression analyses linking upregulation of transcriptional regulatory genes to over-activated growth signaling in many cancer types. Dysregulation of regulators may lead to loss/gain of their functions in breast cells. Their regulation plays an important role in breast cancer progression⁵. The expression profiles in breast cancer have revealed one of these dysregulated transcriptional regulators known as ATAD2/ANCCA to be highly upregulated in breast carcinoma. Its overexpression is reported to be observed at more than 70% of breast cancer patients¹¹⁷, yet information on its functions was still missing in literature. Thus, a detailed analysis of this gene's function in breast cancer was aimed in this study.

The first aim of the study was to reveal altered genes following ATAD2 suppression in breast cancer cells with high throughput gene expression analysis. Next, enrichment analyses were applied to significantly downregulated genes with ATAD2.

The second aim was to carry out functional assays with ATAD2 downregulated breast cancer cells to reveal its biological effects.

The third aim emerged as the analysis of molecular functioning of ATAD2 in breast cancer and its involvement in EGFR signaling after revealing the inhibitory effect of its downregulation on EGFR signaling with microarray studies.

CHAPTER 2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. GENERAL LABORATORY MATERIALS

General laboratory materials are chemicals, reagents, solutions, kits, commercially supplied products and equipments used in the applications of molecular biology. We followed the manufacturers' recommendations for their storage. The lists at the bottom provide detailed information about the materials and the preparation of solutions used during the experiments.

2.1.1.1. Chemicals and Reagents

Table 2. 1: The list of Chemicals, reagents and enzymes used for general laboratory processes

Name	Catalog#	Company (Country)
2-mercaptoethanol	M3148	Sigma Aldrich (USA)
Agarose	BHE500	Prona (Spain)
Ampicillin	A0839	Applichem (Germany)
Agar	05039	Sigma Aldrich (USA)
Acrylamide	A9099	Sigma Aldrich (USA)
Ammonium persulfate	A3678	Sigma Aldrich (USA)
Bactotryptone	1612	Conda (Spain)
Bis-acrylamide	M7279	Sigma Aldrich (USA)
Bovine Serum Albumin Fraction V (BSA)	10735078001	Roche (USA)

Bromophenol blue	B5525	Sigma Aldrich (USA)
Brilliant Blue R	B0149	Sigma Aldrich (USA)
Crystal Violet	V5265	Sigma Aldrich (USA)
DEPC	A0881	Applichem (Germany)
dNTP	R0182	Thermo Scientific (USA)
Ethidium Bromide	17898	Thermo Scientific (USA)
EDTA	A3562	Applichem (Germany)
ECL Prime	RPN2232	Life Sciences (USA)
Gene Ruler DNA Ladder (1 kb)	SM0311	Thermo Scientific (USA)
Gene Ruler DNA Ladder (50 bp)	SM373	Thermo Scientific (USA)
Glacial acetic acid	27225	Sigma Aldrich (USA)
Glycine	G8898	Sigma Aldrich (USA)
Giemsa	453616	Carlo Erba (Spain)
IPTG	A4773	Applichem (Germany)
Kanamycin	60615	Sigma Aldrich (USA)
K-ferrocyanide	P3289	Sigma Aldrich (USA)
K-ferricyanide	P3667	Sigma Aldrich (USA)
Molecular Biology Grade Water	SH30538	Thermo Scientific (USA)
Nuclear fast red	N3020	Sigma Aldrich (USA)
Proteinase K	P2308	Sigma Aldrich (USA)
Proteinase inhibitor (PI) cocktail	P8340	Sigma Aldrich (USA)
PageRuler Prestained Protein Ladder, (170kDa)	26616	Thermo Scientific (USA)
PageRuler Plus Prestained Protein ladder, (250 kDa)	26619	Thermo Scientific (USA)
Propidium Iodide (PI)	421301	Biolegend (USA)

Rnase A	R6513	Sigma Aldrich (USA)
Roche PVDF Membranes (0.2uM)	3010040001	Roche (USA)
SDS	71725	Sigma Aldrich (USA)
Sulforhodamine B (SRB)	230162	Sigma Aldrich (USA)
Taq DNA Polymerase	EP0402	Thermo Scientific (USA)
Triton X-100	T8787	Sigma Aldrich (USA)
TEMED	1610801	Biorad (USA)
Trizma Base	T1503	Sigma Aldrich (USA)
Tween-20	822184	Merck /Germany)
Trichloroacetic acid (TCA)	33731	Sigma Aldrich (USA)
X-Gal	R0404	Thermo Scientific (USA)
Yeast extract	1702	Conda (Spain)

2.1.1.2. Routinely used solutions

Routinely used solutions were prepared freshly each time and they were used for western blot, gel-electrophoresis, colony formation assay, SA- β -Gal assay and transformation. They are listed in table 2.2.

Table 2. 2: The list of routinely used buffers/solutions

Buffer/solution	Recipe prepared for 100 ml
RIPA buffer (500 ul)	15 μ l 5M NaCl+ 25 μ l 1M Tris-HCL (pH = 8.0)+ 50 ul 10X Proteinase inhibitor+ 6.25 ul 10% SDS+ 0.625 μ l Triton X100+ 25 ul 10X DOC+378,125 ul ddH ₂ O
Bradford solution (1L)	100 mg Coomassie brilliant blue (G-250)+50 ml 95% ethanol+100 ml 85% phosphoric acid+ volume up to 1 L with ddH ₂ O Filtrate with Whatman paper.
6X Loading Dye	Mix 0.012 g of Bromophenol blue with 0.012 g of Xylene-cyanol in 8ml 0.5 M EDTA and add 80ml of glycerol
5x Protein Loading Buffer	Mix 0.001g of Bromophenol blue with 2g of SDS and 62.5 mM Tris-HCL (pH:6.8) and 15% glycerol. Add 5% β -mercaptoethanol just before loading.
5X Running Buffer	Mix 1.5 g of Tris-base with 7.2 g of glycine and with 0.5 g of SDS in dH ₂ O
Wet Transfer Buffer	Mix 0.6 g of Tris-Base with 2.88 g of glycine in 15% Methanol in dH ₂ O
10X TBS	Mix 1.22 g of Tris-base with 8.78g of NaCl in dH ₂ O. Set pH = 8.0
TBS-T (0.2%) (500ml)	Mix 1ml Tween-20 in 50 ml 10X TBS in ddH ₂ O
Mild stripping buffer	15 g glycine+1gr SDS+ 10ml Tween-20+ddH ₂ O
Ponceau S staining solution	1gr Ponceau S+50 ml acetic acid+ ddH ₂ O

Coomassie Staining Buffer	Mix 0.25 g of Coomassie Brilliant Blue in 45 ml MetOH in 10 ml glacial acetic acid
Destaining Solution	Mix 7% glacial acetic acid in 20% MetOH
50X TAE	Mix 24.2 g of Tris-base with 1.86 g of EDTA in 5.71 ml glacial acetic acid
PI staining solution	50 ug/ml PI + 0.1 mg/ml RNase-A + 0.05% Triton-x + PBS
Crystal Violet Solution	Dissolve 0.5% crystal violet in 25% MetOH
Na-P Buffer (40 ml)	Prepare with 10.2 µl 1M Na ₂ HPO ₄ + 29.8 µl NaH ₂ PO ₄
SA-B-Gal Solution (3 ml)	Dissolve 600 µl 200mM Citric acid + 600 µl Na-P buffer + 150 µl 100mM Kferrocyanide + 150 µl 100mM K-ferricyanide + 90 µl 5mM NaCl + 60 µl 100mM MgCl ₂ + 75 µl 40mg/ml X-gal in 1313 µl ddH ₂ O
LB	Dissolve 1 g of Tryptone with 0.5 g of yeast extract; with 1 g of NaCl; with 2.5 g of Bactoagar
Transformation Buffer	Mix 1.09 g MnCl ₂ .4H ₂ O + 0.22 g CaCl ₂ .2H ₂ O + 1.87 g KCl in 2 ml 0.5M Pipes and set pH:6.7

2.1.1.3. Kits

Kits were used to purify RNA from cell pellets and for its subsequent validation with RT-qPCR studies. They are listed in table 2.3.

Table 2. 3: The list of Kits used in laboratory processes.

Kit Name	Catalog#	Company Name(Country)
Nucleospin RNA extraction kit	740955	Macherey Nagel (Germany)
RevertAid First Strand cDNA Synthesis Kit	K1622	Thermo Scientific (USA)
DyNAmo HS SYBR Green qPCR Kit	F-410L	Thermo Scientific (USA)
PureLink Quick Plasmid Miniprep Kit	K210011	Thermo Scientific (USA)
BD Pharmingen FITC BrdU Flow Kit	559619	BD Biosciences (USA)

2.1.1.4. Primers

The listed primers were used for PCR and RT-qPCR studies. They were synthesized by the Iontek or Sentromer companies. The primers were handled in accordance with the manufacturer's guidance. They were dissolved in water and stored in a final concentration of 100 μ M.

Table 2. 4: The list of primers used in the studies

Primer Name	PCR	Primer Sequence	Tm (°C)	Size (bp)
GAPDH F GAPDH R	RT	GGCTGAGAACGGGAAGCTTGTCAT CAGCCTTCTCCATGGTGGTGAAGA	60	140
CASP9 F CASP9 R	RT	GTTGCGGCGTCGCTTCTCCT ACTGCAGGTGGCTGGCCTGA	60	170
ATAD2 F ATAD2 R	RT	TGAAAAGGCTTTGGCAATTC TTGCGATGCCGATAAATACA	60	167
ESR1 F ESR1 R	RT	AGACATGAGAGCTGCCAACC GCCAGGCACATTCTAGAAGG	60	299
EGFR F EGFR R	RT	GCAAGAGGCAGGCTCAGCAA GCGGACCTACCTAGGCAATG	60	227
EGR1 F EGR1 R	RT	AGCAGCACCTTCAACCCTCA CACCTTCTCGTTGTTTCAGAG	60	99

ADAM23 F ADAM23 R	RT	TCAGCGATCTCTGTGCAACT CACATTAAGGTTCCACCACG	60	95
ADAMTS1 F ADAMTS1 R	RT	GGCTGATGTTGGAAGTGTGT ACACGTGGCCTAATTCATGG	60	104
C-MYC F C-MYC R	RT	CAGCTGCTTAGACGCTGGATTTT ACCGAGTCGTAGTCGAGGTCAT	60	115
N-MYC F N-MYC R	RT	CCCTGAGCGATTCAGATGAT AATGTGGTGACAGCCTTGGT	60	115
CCNE1 F CCNE1 R	RT	CGGTATATGGCGACACAAGA CTGGTGCAACTTTGGAGGAT	60	114
CCND1 F CCND1 R	RT	CCCTCGGTGTCCTACTTCAA GAAGCGGTCCAGGTAGTTCA	60	147
CDH1 F CDH1 R	RT	GACTCGTAACGACGTTGCAC GGTCAGTATCAGCCGCTTTC	60	119
RB1 F RB1 R	RT	TGTAACAGCGACCGTGTG TTGGACTCTCCTGGGAGATG	60	131
TP53 F TP53 R	RT	ATTGGAGCCAGACTGCCTTC GCAGGCCAACTTGTTCAGTG	60	138
AKT2 F AKT2 R	RT	CCATGAATGAGGTGTCTGTC GGCCTCCAGGTCTTGATGTA	60	73
BRCA1 F BRCA1 R	RT	ACAGCTGTGTGGTGCTTCTGTG CATTGTCCTCTGTCCAGGCATC	60	107

2.1.1.5. Antibodies

The listed antibodies were used in western blot studies. Their working concentrations were optimized under the guidance of their manufacturers.

Table 2. 5: The list of Antibodies used in the studies

Name	Catalog#	Company (Country)
Rabbit anti α-ATAD2 polyclonal antibody *		

Mouse anti-E Cadherin [HECD-1]	ab1416	Abcam (UK)
Mouse anti-Vimentin [V9]	ab8069	Abcam (UK)
Rabbit anti-calnexin antibody	C4731	Sigma Aldrich (USA)
Anti-rabbit IgG-HRP	6154	Sigma-Aldrich (USA)
Anti-mouse IgG-HRP	M7001	Dako (Denmark)
ERα Antibody (F-10)	sc-8002	Santa Cruz Biotechnology
Monoclonal Anti-β-Actin antibody	A5441	SIGMA
EGFR (C74B9) anti-rabbit antibody	2646S	Cell signaling
p-Her2/ErbB2 anti-rabbit antibody	2244S	Cell signaling
Akt anti-rabbit antibody	9272S	Cell signaling
Phospho-Akt (Ser473) Antibody	9271	Cell signaling
RB1 antimouse antibody (4H1)	9309S	Cell signaling
Phospho-Rb (Ser780) Antibody	9307	Cell signaling
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Rabbit Antibody	4370	Cell signaling
Phospho-p38 MAPK (Thr180/Tyr182) Rabbit Antibody	4511	Cell signaling
Monoclonal Mouse Anti-Human p53 Protein Clone DO-7	M7001	Dako
Caspase-9 Antibody	9502	Cell signaling
BID Antibody	2002	Cell signaling

Bax Antibody	2774	Cell signaling
Bcl-2 Antibody (C-2)	sc-7382	Santa Cruz Biotechnology
Bcl-xL Antibody (H-5)	sc-8392	Santa Cruz Biotechnology

* Primary antibody was produced and provided by Dr. Mehmet Öztürk's lab.

2.1.1.6. Equipments

The equipment used for all experiments are listed in table 2.6.

Table 2. 6: The list of Equipment used in the studies

The utilized instrument	Company Name (Country)
AxioCam MRc5 image capture device	Carl Zeiss (Germany)
BD Accuri™ C6 Cytometer	BD Biosciences (USA)
Centrifuges 5810 and 5810 R	Eppendorf (Germany)
PCR Thermal cycler	Applied Biosystems (USA)
Stratagene Mx3005P Real-Time PCR System	Agilent (USA)

2.1.2. CELL CULTURE MATERIALS

Cell culture materials were used to carry out the experiments at the culture room. They were sterilized materials and handled under aseptic conditions. All work with these materials was performed under a laminar flow hood.

2.1.2.1. Cell culture reagents

Table 2. 7: The list of chemicals, reagents and kits used during cell culture studies

Product Name	Catalog#	Company (Country)
DMEM, Low Glucose	SH30021	GE Healthcare (UK)
DMEM:HAM's F-12 1:1	SH30023	GE Healthcare (UK)
RPMI 1640	SH30027	GE Healthcare (UK)
McCoy's 5A medium	F 1015	Biochrom (Germany)
Fetal Bovine Serum	CH30160	GE Healthcare (UK)
L-glutamine	SH30034	GE Healthcare (UK)
Non-Essential Amino Acids	SH30238	GE Healthcare (UK)
Penicillin/Streptomycin (Antibiotics)	SV30010	GE Healthcare (UK)
Sodium Pyruvate	11360	Thermo-Fischer Scientific (USA)
Hydrocortisone	H4001	Sigma Aldrich (USA)
Insulin	I9278	Sigma Aldrich (USA)
Epidermal Growth Factor	E9644	Sigma Aldrich (USA)
PBS	SH30256	GE Healthcare (UK)
Trypsin/EDTA	SV30031	GE Healthcare (UK)
Dimethyl sulfoxide (DMSO)	A1584	Applchem (Germany)
OptiMEM I	11058	Thermo-Fischer Scientific (USA)
Lipofectamine RNAiMAX transfection reagent	13778	Thermo-Fischer Scientific (USA)
Lipofectamine 2000 transfection reagent	11668	Thermo-Fischer Scientific (USA)

2.1.2.2. Cell lines and Media

Table 2. 8: The list of cells lines and their respective growth media used for our studies

Cell Line	Medium
BT20	Normal Medium
BT474	Normal Medium+10µg/ml insulin
CAMA-1	Normal Medium
HCC-1937	RPMI-1640 medium+10%FBS+1% Penicillin/Streptomycin+ 1% nonessential aminoacids+ 1mM sodium pyruvate
MCF10A	DMEM/Ham'sF12+ 10%FBS+1% Penicillin/Streptomycin+ 1% nonessential aminoacids+10µg/ml insulin+ 20 ng/ml EGF+0.5 mg/ml hydrocortisone
MCF12A	DMEM/Ham'sF12+ 10%FBS+1% Penicillin/Streptomycin+ 1% nonessential aminoacids+10µg/ml insulin+ 20 ng/ml EGF+0.5 mg/ml hydrocortisone
MCF7	Normal Medium
MDA-MB-157	Normal Medium+ 1mM Sodium pyruvate
MDA-MB-231	Normal Medium
MDA-MB-361	Normal Medium+1mM Sodium pyruvate
MDA-MB-453	Normal Medium
MDA-MB-468	Normal Medium
SKBR-3	McCoy's 5A Medium+10%FBS+1% Penicillin/Streptomycin+ 1% nonessential aminoacids+1mM sodium pyruvate
T47D	RPMI-1640 medium+10%FBS+1% Penicillin/Streptomycin+ 1% nonessential aminoacids+ 1mM sodium pyruvate
ZR-75-1	Basic RPMI-1640+10%FBS+1% Penicillin/Streptomycin+ 1% nonessential aminoacids+4.5g/L glucose
*Normal Medium: DMEM (low glucose)+10%FBS+1% Penicillin/Streptomycin+ 1% nonessential aminoacids	

2.1.2.3. Nucleic acids

Table 2. 9: The list of vectors and siRNA oligos used in transfection studies

Name	Catalog#	Company	Target sequence
pSuper-GFP/Neo	VEC-PRT-0006	OligoEngine	pSR-ER α -458: 5'-TTC AGA TAA TCG ACG CCA G-3' pSR-ER α -499: 5'-GTA CCA ATG ACA AGG GAA G-3'
ATAD2 SureSilencing shRNA Plasmid	KH16398N	Qiagen	Set of 4 shRNA plasmids targeting different section of ATAD2 gene
FlexiTube GeneSolution for ATAD2	GS29028	Qiagen	siRNA_2: CTGGTACTTAGGCACCATA AA siRNA_3: AAGGCATTATATAAAGATCG A siRNA_4: AAGAATAATTAGCAGCGTT A siRNA_5: CCGGATAAAGAAGAACGA CA

2.2. METHODS

2.2.1. LABORATORY TECHNIQUES

2.2.1.1. RNA isolation from cell pellets and its quality/quantity determination

For RNA purification, cells were trypsinized and detached from surface. Cell suspensions were collected into falcons and centrifuged at 15,000 g at +4 C for 5 min. Following aspiration of supernatant, cells re-centrifuged in cold PBS under same conditions. After discarding supernatant, cell pellets were quick frozen in liquid nitrogen to inhibit metabolic activities within cells. RNA was isolated from prepared cell pellets according to the instructions described in MN Nucleospin RNA kit's user protocol. After purification, RNA quantity and quality was determined with the NanoDrop ND-100 spectrophotometer. 2 ul from each RNA sample was used to measure its absorbance values at 260, 280 and 230 nm. The ratio of 260/280 represents the purity of the RNA. A value of around 2.0 is accepted as pure RNA. The 260/230 ratio is accepted as secondary measurement criteria of sample purity. The samples with values in the range of 2.0-2.2 are usable. After a quality check, RNA samples were stored in nuclease-free water at -80 C.

2.2.1.2. Complementary DNA (cDNA) preparation from isolated total RNA samples

Prepared RNA samples were used for further experiments. cDNA was prepared from 500ng-1ug of RNA samples depending on the total quantity from each samples. The RevertAid 1st strand cDNA synthesis kit from Thermo Scientific was used for cDNA preparation. We followed the protocol described in the kit. Oligo-dT primers were used for RNA samples isolated from cell pellets. The prepared cDNAs were been stored at -20C.

2.2.1.3. Polymerase chain reaction (PCR)

The quality of prepared cDNAs were controlled by PCR with *GAPDH* primers. For each sample, a negative control, not including cDNA, was prepared to check the presence of any contamination in PCR materials. The reaction conditions were as follows: 30 cycles of 15 sec at 90 C; 15 sec at T_m ; 15 sec at 72 C. The PCR products were run in 2.5% agarose gel and visualized under UV light.

2.2.1.4. Quantitative reverse transcription PCR (RT-qPCR)

The prepared cDNAs were used to quantify the expression of genes of interest. RT-qPCR experiments were performed with DyNAmoTM HS SYBR Green qPCR kit. The reaction conditions were as follows: initial denaturation at 95 C for 5 min; then continuing with 40 cycles of 15 sec denaturation at 95 C; 20 sec annealing at T_m ; 15 sec elongation at 72 C; at the end a final elongation for 5 min at 72 C. The dissociation curve was formed at 55 C for 10 sec. Each sample was studied in duplicate. Average C_t values of the duplicates were used for normalization. All expression results were normalized to their respective internal control gene which was *GAPDH*. Their relative gene expressions were determined using the comparative C_T method referred as the $2^{-\Delta\Delta C_t}$ method. and provided the fold change value due to treatment.

$$\Delta\Delta C_T: [(C_T\text{gene of interest}- C_T\text{internal control})_{\text{treated}} - (C_T\text{gene of interest}- C_T\text{internal control})_{\text{untreated}}]$$

2.2.1.5. Total protein isolation from cell pellets

Cell pellets were prepared as explained in the RNA isolation step. Pellets were incubated with RIPA-lysis buffer for 30 min on ice. The added buffer quantity varied in the range of 50ul-15ul depending on the pellet amount. They were then homogenized with sonication to extract nuclear proteins efficiently. Later, homogenized samples were centrifuged at 13,000

g, for 20-30 min at 4 C. The supernatant of the cell lysate was used for protein detection studies.

2.2.1.6. Determination of total protein concentrations

We used two methods to detect protein concentrations. The first one was Bradford measurement. The principle of the assay is binding of proteins to coomassie dye under an acidic environment. This causes a color change from brown to blue in the presence of protein and the intensity of the color is positively correlated with the protein amount. Before protein measurements, we prepared a standard curve. We added 0, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20 ul of BSA standards at 2 mg/ml concentration onto ddH₂O and 900 ul Bradford solution into each test tube for a final volume of 1 ml. After incubation at room temperature for 5-10 min, we measured absorbance at 595 nm with spectrophotometer. Then we plotted a standard curve and this was used to calculate protein concentrations with known absorbance values. 2 ul protein samples were used with 900 ul Bradford and 98 ul ddH₂O. The second method was the Pierce BCA Protein Assay. The working principle of the method depends on the formation of Cu⁺² complexes under alkaline conditions. This formed purple-blue complexes proportional with the presented protein amounts. This is a highly sensitive method. 200 ul of BCA working solution (prepared by 50:1 of A:B reagents) is added onto 25 ul of standard and unknown samples/well in the microwell plates. They are incubated at 37°C for 30 min in the dark and the absorbance is measured at 540-590 nm by a plate reader.

2.2.1.7. Western blot

Following measurement of their concentrations, proteins were used for western analysis. Initially we prepared SDS-polyacrylamide gels. We used 10% or 12% gels for proteins depending on their molecular weight. SDS-PAGE was run in freshly prepared 1X running buffer at 90 V till proteins passed the stacking gel and then the voltage was increased to 120V and they were run till the loading dye reached the end of the gel. Afterwards, the

proteins were transferred to PVDF membrane in wet transfer buffer. After the transfer, the membrane was incubated in blocking solutions which were 5% BSA or milk powder prepared in 1X TBS with 0.05 %-2% Tween-20 for 1 hour on a shaker working slowly at room temperature. After blocking, the membrane was incubated with primary antibody solutions overnight at +4°C on a shaker at moderate speed. Following primary antibody incubation, membrane was washed three times for 10 min each with the same TBS-T, used to prepare antibody solution, on a shaker working fast to get rid of excessive antibodies. Then the membrane was placed into 1:5000 diluted secondary antibody solution prepared in 5% milk or BSA solution prepared in same TBS-T used for washing. Then the membrane was washed again with TBS-T to discard excessive secondary antibody and in the end the membrane was used to visualize the proteins of interest by developing with ECL prime system in a dark room with X-ray films. The incubation conditions and the antibody dilutions along with the tween concentration in TBS was optimized according to each antibody.

2.2.1.8. Transformation of pSUPER.retro vector into competent DH5α cells

We took two ready-to-use knockdown products, pSR-ER-458 and pSR-ER-499, and an empty vector, pSR-Empty, from Dr. Elif Erson's lab from METU. They were prepared by cloning shRNA oligos into the BglII/XhoI sites of the pSuper-GFP/Neo vector. We transformed these recombinant products into competent *E. coli* DH5α strains. Competent cells were prepared as 50-500ul aliquots. We used 150-200ul for transformation for each vector. After dilution of plasmids with water, and then vortexing, 1ul of plasmid was added onto competent cells. They were incubated on ice for 20 min; for 45-50 sec at 42 C; and for 2 min on ice in this order. Following adding 950ul of SOC medium (richer than LB agar and should be kept at +4 C and warmed before using), cells were incubated with constant agitation at 150 rpm for 1.5 hours at 37 C. 100ul of transformed cells were then spread onto LB-agar plates containing 1X ampicillin (100 ug/ml). Cells were let to grow overnight at 37 C. The next day, colonies were picked and let to grow in kanamycin broth medium for an additional cycle. Ready colonies were used for the following isolation protocol.

2.2.1.9. Isolation of amplified plasmids from transformants

Following the transformation step, cells were lysed and the amplified vectors were purified. We used the PureLink Quick Miniprep Kit from Invitrogen protocol. The purified plasmids were quantified with a NanoDrop ND-1000 spectrophotometer. To confirm the presence of insert, plasmids were digested with EcoRI and HindIII and XhoI. We then ran the fragments in the gel and determined if positive clones had the correct insert within the recombinant vector.

2.2.2. CELL CULTURE TECHNIQUES

2.2.2.1. Culture conditions and maintenance of cultured cell lines

All cells were maintained in CO₂ incubators set to 5% at 37C and 95% humidity. Cells were grow in the medium optimized to their characteristics. They were passaged every 2-3 days and split 1:2-1:5 depending on their proliferation rates. For storage of cells, initially cells were washed with 1X PBS, then trypsinized and detached from flasks. Collected cells were centrifuged and the medium discarded. Cell pellets were dissolved in 1.5-2 ml of freshly prepared freezing medium containing 90% FBS and 10% DMSO and were preserved in sterile cryo-vials. After cells were kept at the -20°C freezer for 1 hour, they were transferred to -80°C freezer overnight and later stored in liquid nitrogen for longer periods. When the cells were to be thawed, they were taken out of liquid nitrogen and mixed with growth medium. Following centrifuge to remove DMSO, the cell pellets were dissolved in growth medium and moved into T-25 flasks. The following day, if cells reached sufficient confluence in the flask, they were transferred into a T-75 flask; otherwise the medium was changed and they were left to incubate. All cell culture studies were carried out under laminar flow hoods in a sterile environment and aseptic techniques were applied during handling of the cells.

2.2.2.2. Transient transfection

Various cell types were transfected to silence either *ATAD2* or *ESR1* genes or both. HCC1937 and SKBR3 cells were transfected with Hs_ATAD2_2 and Hs_ATAD2_5 FlexiTube siRNA (Qiagen) using the RNAiMAX transfection reagent. We used the reverse transfection protocol supplied by the manufacturer for siATAD2 transfection experiments. We decided the optimum concentrations of siRNAs for the most efficient transfection as 25uM, 25uM and 100uM final concentrations in the medium for MCF7, HCC1937 and SKBR3 cells respectively. Following incubation of 5ul transfection reagent with siRNAs in 500 ul of Opti-MEM I Reduced serum medium, we seeded 200.000 cells /well into 6-well tissue culture plate. They were left to 72 hours incubation. MCF7 cells were co-transfected with Hs_ATAD2_2 and Hs_ATAD2_5 siRNAs; and pSR-ER α -499 plasmid to silence both *ATAD2* and *ESR1* gene. MCF7 cells were first forward transfected with pSR-ER vectors. We did forward transfection using Lipofectamine 2000 and applied the protocol the manufacturer recommended for transient transfections with shRNAs. After 200.000 cells/well were seeded on 6-well plates, the transfection reagent-shRNA complex incubated in Opti-MEM I reduced medium for 20 min was added onto cells. They were left to incubate for 24 h. The next day, the medium was changed and the RNAiMAX- siRNA complex was added onto the cells and left for further 48 h incubation. At the end of treatments, the cells were collected and the cell pellets were divided as 1/3 and 2/3 to be used for RT-qPCR and western blot analysis respectively.

2.2.2.3. Stable transfection

To obtain stably transfected clones, cells should be selected in Geneticin supplemented medium. Therefore, we initially determined Geneticin concentrations to be used as the selective reagent for stable cells. For this purpose, we carried out kill curve assays at the concentrations of 0 ug/ml; 200 ug/ml; 400 ug/ml; 600 ug/ml; 800 ug/ml; and 1000ug/ml with MCF7, T47D, HCC1937 and SKBR3 cells. Following treatment of cells with given concentrations of antibiotic for two weeks, cell viability was measured with the SRB assay. After fixation of cells with ice-cold 10% TCA for 1 hr at 4C, cells were incubated in SRB

solution, prepared with 0.04 g SRB dye dissolved in 10 ml 1% acetic acid, for 10 min at RT in dark. After washing and drying, dye was extracted out of the cells with addition of 0.5X TBS and plates were read at the range of 495-525 nm to measure the absorbance. We determined the concentrations at which cells showed least viability for the selection of stable clones. SureSilencing shRNA plasmids (Qiagen) were used for stable downregulation of *ATAD2* gene in these cells. The kit had a set of 4 shRNA plasmids targeting different regions of the gene. 5 ul Lipofectamine 2000 reagent has been used per well for efficient transfection and forward transfection has been carried out. Cells were initially seeded as 250,000 cells/well in 6-well cell culture plates. After 24 hours, lipofectamine 2000 was incubated with 2 ug shRNA in 250 ul Opti-MEM medium for 20 min. Then these complexes were added onto cells and left to incubate for 72 hours. After three days, cells were transferred to 150 mm petri dishes including medium supplemented with Geneticin, the concentration of which had been pre-determined. Cells were grown in the dishes until colonies became visible. This may take 3-4 weeks. During this time, we constantly renewed the medium every-3-4 days. When colonies were formed, we picked them up with 1000 ul pipettes filled with 0.2 ml of medium and transferred them into 24 well-tissue culture plates. We placed each colony into separate wells. Once colonies expanded to high confluence in the wells, we first passaged them into 12-well plates and later into 6-well plates. At the end, they were placed into T-25 and T-75 flasks in order. During all these processes, cells were always grown in antibiotic supplemented medium. To assess the downregulation of the gene, some of the cells were collected and harvested to perform RT-qPCR and western blot experiments to determine transcription and protein expression levels of *ATAD2*.

2.2.2.4. 2D colony formation assay

For this assay, cells were cultured to be 1000 cells/well in 6-well culture plates. They were seeded in triplicate. Cells should be separated as single cells, so homogenization of cell suspension is crucial during seeding. After 2-3 weeks incubation, we proceeded with fixation of the cells. The incubation duration varied according to the proliferation rate of the cells. Cells were fixed with ice-cold MetOH for 15 min in the freezer. Then the cells

were stained with 0.5% crystal violet solution for 15 min. Following the washing step with ddH₂O, the cells were left to dry. Images of the wells were obtained and the colony numbers and their sizes were determined with the ImageJ tool.

2.2.2.5. *In vitro* scratch assay

Cells were seeded to be 250,000 cells/well in 6-well culture plates. They were seeded in triplicate. The next day when cells were expected to reach 90-95% confluence, we drew a straight line in the middle of wells with 10 ul tips. We aspirated the medium and washed cells with PBS to remove detached cells. The media was replaced with medium containing 0.01% FBS. We took pictures of the gap starting from time 0 up to 72 h every 24 hours with 10X amplification. The migration rate was determined with the TScratch tool²¹⁴.

2.2.2.6. Senescence-associated β -galactosidase (SA- β -Gal) staining

Cells were seeded as 1000 cells/well on cover-slip in each well of 6-well plates. The experiments were run in triplicate. They were left to grow for 3 weeks. At the end of 3 weeks, cells were washed with 1X PBS and fixed with 1ml of 3% formaldehyde for 15 min. Later they were washed twice with 1X PBS. Cells were incubated in freshly prepared SA- β -gal solution for 18-24 hours at 37C in dark. After incubation, cells were washed again with 1X PBS twice and fixed with ice-cold MetOH this time for 5 min. Then the fixed cells were stained with nuclear fast red as counter stain for 5 min at RT, and we washed the cover slips with ddH₂O to remove excessive dye. Cover slips were then taken off the plates. A little glycerol was placed on the slide and a cover slip mounted such that the bottom part was facing the slides. Colonies were counted under the light microscope at 10X and 20X amplification. The ratio of colored cells to all cells in a colony gave the percent of senescent cells.

2.2.2.7. Serum starvation studies

T47D, MCF7, HCC1937, SKBR3, BT20, MDA-MB-231 cells were seeded in 150 mm culture dishes as 3×10^6 cells and in 6-well plates as 1×10^5 cells/well for 24 hours in 4 sets as shown in figure 2.1. The next day, the media were exchanged with growth medium supplemented with either 10% FBS or 0.01% FBS(+ 100nM NaSel) and they were incubated for 48 hours and 7 days. At the end of incubation, the viability of cells in 6-well plates were analyzed with SBR assay and their cell cycle analysis were performed with FACS. Cells were cultured as 2.5×10^6 cells/well for FACS analysis. The cells seeded on 150 mm culture dishes were used for the quantification of ATAD2 protein levels in the cells. For ATAD2 quantification, cells were separated into 3 tubes for protein expression analysis and 2 tubes were prepared as negative controls. Cells were fixed in FACS lysis buffer for 10 min. After washing with 1 X PBS, they were centrifuged at 1600 rpm for 6 min. Cells were treated with permeabilization solution (500 ul) for 10 min at +4 C and after washing they were centrifuged at 1600 rpm for 6 min. The pellets were dissolved in 100 ul of primary ATAD2 antibody solution (1/200 diluted) and incubated for 1 hour on ice at a +4C room. After washing with 1 X PBS, they were centrifuged at 1600 rpm for 6 min. The supernatant was removed. The negative controls were not treated with primary antibody solution. All samples were dissolved in 100 ul of secondary antibody solution conjugated with FITC (1/250 diluted) and incubated for 1 hour at +4C at dark. Following washing with 1X PBS twice, cells were centrifuged at 1600 rpm for 6 min. The cell pellets were suspended in 1ml of 1X PBS and kept on ice at dark until flow cytometry analysis. The ATAD2 amount was quantified with the BD FACSCalibu machine. The geometric means of replicates were calculated.

2.2.2.8. Gefitinib treatment and MTT assay

MCF7 cells were seeded as 250.000/well in 6-well plates. To determine the molecular effects of different doses of Gefitinib, we treated cells with 0 ug/ml; 5 ug/ml; 10 ug/ml; 20 ug/ml; and 40 ug/ml final concentrations of drug. Since it was dissolved in DMSO, we made a parallel set-up in which cells were treated with corresponding DMSO

concentrations. Following 48 h incubation, the cell pellets were collected and used for RNA isolation as described previously. To determine the effect of different doses on cell proliferation, an MTT assay was carried out. Cells were seeded at a density of 2000 cells/100 ul per well in 96-well culture plates. They were incubated for 24 hours. The next day, their medium was changed with medium containing Gefitinib at predetermined concentrations. Each treatment was done in four replicates. After 24 and 48 hours incubations, we added 110ul/well solution, containing 100 ul medium+10ul MTT solution (0.5 mg/ml) onto the cells in each well (we did not aspirate the medium early on). Plate was incubated for 4 hours at 37 C. Then 100 ul/well SDS-HCl solution was added and incubated further for 4-18 hours. The next day, we measured the absorbance at 550-600 range.

2.2.2.9. Epidermal growth factor (EGF) treatment

MCF7 cells were seeded as 250.000 cells/well in 6-well plate. After 24 hours, the medium was changed with phenol-red free DMEM containing 0.1% charcoal-stripped FBS referred to as starvation medium. This eliminated the estrogenic effect of phenol red in the medium. Cells were cultured for 24 hours in this medium. Then EGF at 100 ng/ml final concentration was added to wells. Cells were stimulated with EGF for 24, 48 and 72 hours. Following incubation, cell pellets were collected and they were used for RNA isolation as described previously.

2.2.2.10. FACS analysis

250.000 cells/well were seeded on 6-well plates in triplicates. After 3 days incubation, to detect the cell death percentage as well, the medium in each well was transferred into separate tubes. Then the cells were washed with 1X PBS and the PBS was collected into the same tubes also, and cells were then trypsinized and the detached cells were transferred into the same tubes again. They were centrifuged at 1500g for 5 min at +4°C. After removing supernatant, cell pellets were resuspended in 1 ml of 1X PBS. Cell suspensions

were vortexed occasionally by adding 2,5 ml of ice-cold EtOH drop by drop to prevent cell clumps during fixation. Cell suspensions were incubated on ice for 30 min with again occasional vortex. Fixed cells could be stored up to 3 months at +4°C room. For flow cytometry analysis, cells were stained with propidium iodide (PI). Initially, cell suspensions were centrifuged and the supernatant was removed. Then, cell pellets were incubated in PI staining solution for 40 min at 37°C in dark with occasional vortex. Following incubation, 3 ml of 1X PBS was added onto stained cells. They were centrifuged at 1500g for 5 min at +4°C. The supernatant was removed and stained cells were resuspended in 500 ul-1ml of 1X PBS and finally they were transferred to FACS tubes for subsequent cell cycle analysis.

2.2.2.11. BrDU-7AAD cell cycle assay

To detect cells actively DNA synthesizing along with their cell cycle distribution, we used the BD Pharmingen FITC BrDU flow Kit. We followed the protocol provided in the manufacturer's user manual. The bottom image represents an example for the measurement of actively proliferating cells with anti-BrDU FITC and total DNA content with 7-AAD. The regions corresponding to each phase are defined as follows: R3:G0/G1; R4:S phase; R5: G2+M; R6:apoptotic

2.2.2.12. Statistical analysis

All experiments were performed in triplicate. The statistical significance of experiments were determined with GraphPad Prism 6.0 software. Comparative analysis of the scratch assay, colony formation assay, FACS, BrDU staining, and MTT assay was performed with the two-tailed t test. However, comparison test for all SA- β -Gal studies and for scratch assay of MCF7 and T47D shATAD2 clones was done with two-way analysis of variance. Threshold for significance (P value) was set to 0.05. The value smaller than P was accepted as significant.

2.2.2.13. Microarray data analysis

We used two independent datasets to analyze differential gene expressions. One of them was our own expression dataset obtained during a previous study (project no 111T434) in which the *ATAD2* gene was silenced in MCF7 and T47D cells. We downloaded the other dataset from GEO (GSE27473) in which the *ESR1* gene was silenced in MCF7 cells. Expression data were extracted as CEL files and the first adjustment was normalization. We used the BRB-Array tool 3.8²¹⁵ for all transformations applied to data. We performed normalization with the justRMA method. Quality control of the samples was performed with software. Scatter plot of replicates and the ratio of 3':5' ends of ACTB and GAPDH gene signals were used as control steps. Following confirmation of data quality, they were used for class comparison function of software to identify differentially expressed genes (DEGs). Genes were filtered by at least 1.5 fold change in expression at $p < 0.05$. Next, intersect gene lists utility tool was applied to obtain intersected genes. Functional annotation of selected genes was performed with DAVID Bioinformatics Resources 6.8 Beta tool²¹⁶. We selected Human Genome U133 Plus 2 Array as the background during annotation analysis. The determined DEGs were further analyzed for pathway enrichment analysis with the Reactome V56 tool²¹⁷ using $FDR < 0.05$ and $p < 0.05$ filtering criteria.

CHAPTER 3. RESULTS

The altered *ATAD2/ANCCA* gene expression levels in various breast carcinomas, regardless of their receptor status, compared with non-carcinomas led us to focus our attention on its yet unknown biological functions in breast cells. As a gene shown to be overexpressed in more than 70 % of breast cancer patients, *ATAD2* is suspected to be involved in carcinogenesis^{16,117-119,125}. Our aim was to shed light on the role of this specific gene in breast cancer. Because the extent of its contribution to cancer progression is still unclear, we based our studies on two topics. These were the study of *ATAD2* functions in breast carcinoma cells and the mechanism of action of *ATAD2* in breast carcinoma. ER positive (ER+) and ER negative (ER-) breast cancer cell lines with high *ATAD2* gene expression were used for our experiments. Experiments were conducted to evaluate the effect of its downregulation on cellular cytotoxicity, cellular senescence, cell proliferation and growth, colony formation and its invasive ability in these cells. One of the main experiments was the co-targeting of estrogen receptor and *ATAD2* genes to hinder their cellular activity in ER(+) cells, so that their mutual functions in these cells were analyzed at the molecular basis. Microarray analyses of *ATAD2* downregulated MCF7 and T47D breast carcinoma cells (project no 111T434, carried out by Gurbet Karahan) and ER α downregulated MCF7 cells (GSE27473) were carried out to gain further insight into the mechanism of *ATAD2* activity. Short-listed gene names were identified after determination of co-down/upregulated between two data sets and their functional annotations were analyzed. The results gave an idea of intracellular signaling mechanisms in which *ATAD2* may be involved.

3.1 ANALYSIS OF INDEPENDENT MICROARRAY DATASETS

Microarray experiments were carried out by Gurbet Karahan. During a previous study (project no 111T434, carried out by Gurbet Karahan), which was the starting point of this project as well, MCF7 and T47D cell lines were transiently transfected with siATAD2 (25nM) and siControl (25nM) for 72h. Transfections were run in triplicate. Their ATAD2 expressions were determined at both mRNA and protein levels by RT-qPCR and western blot experiments respectively. Protein expressions of the gene were downregulated to undetectable levels within siATAD2 transfected MCF7 cells (Figure 3.1.A) and downregulated more than 90% in T47D cells, yet one of the replicates (siATAD2 R3) displayed greater downregulation compared to the other two (Figure 3.2.A). The quality of RNA samples to be used in microarray analysis was detected by the Agilent 2100 Bioanalyzer (Figure 3.1-2.B). RNA quality is determined by RIN values assigned for each sample by the Agilent Bioanalyzer²¹⁸. It detects 18S and 28S rRNA fragments in total RNA and calculates their concentrations. The 18S/28S ribosomal ratio represents the RIN value. RNA Integrity number (RIN) is a tool to estimate the quality of total RNA samples. This ratio changes across species and distinct tissue types. It can be within the range of 1 to 10. A RIN value greater than 7 is a prerequisite for reliable microarray studies even though it is preferable to have a value higher than 8. RIN values lower than 7 indicate RNA degradation in the samples²¹⁹⁻²²¹. While all MCF7 samples have RIN values greater than 9, T47D siCtrl 3 has a RIN value of 7.60 but other T47D samples showed RIN values greater than 8. Hence, all samples had acceptable RIN values to be used for chip analysis.

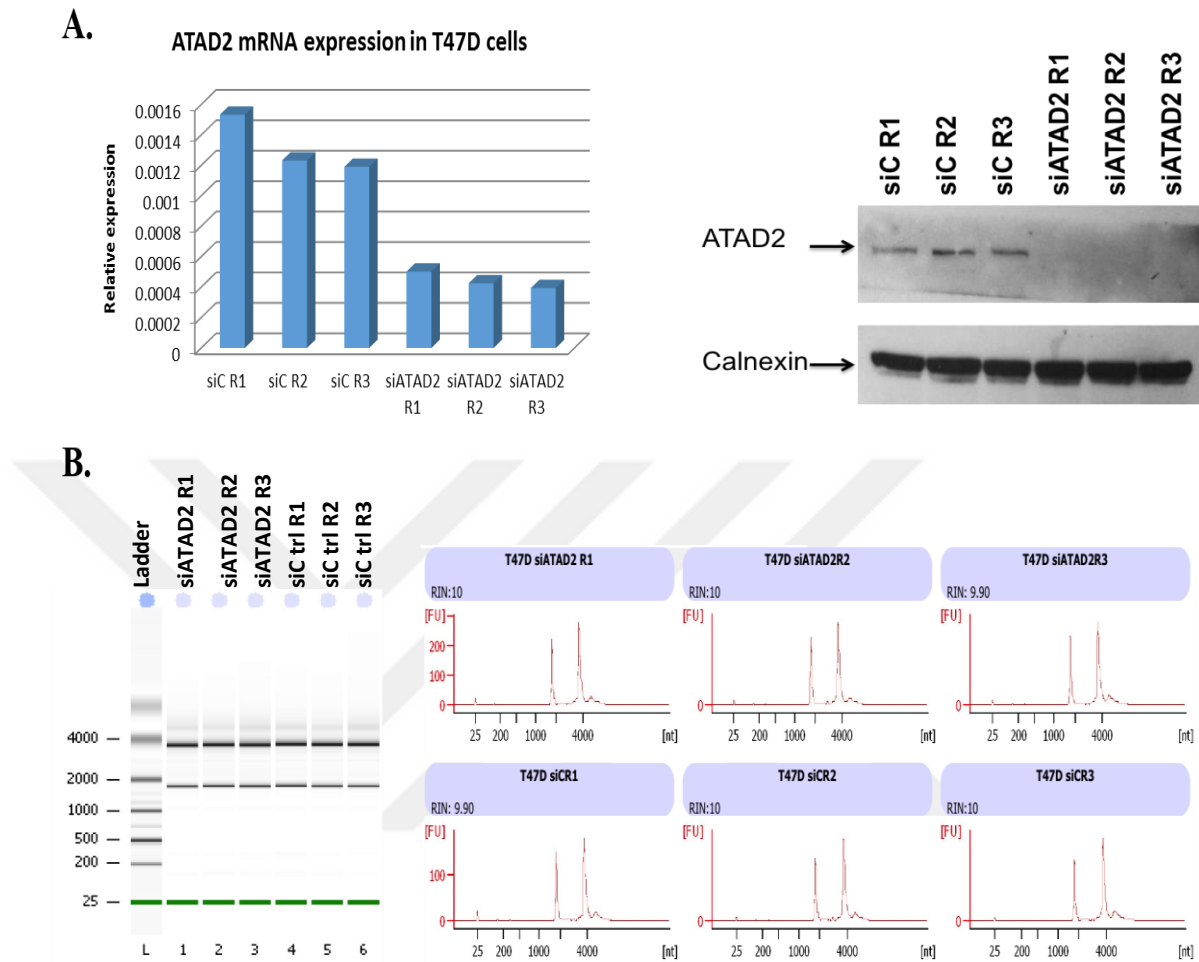


Figure 3. 1: Validation of effectiveness of siATAD2 treatment and Determination of initial total RNA quality by Agilent 2100 Bioanalyzer before the microarray experiments in T47D cells.

(A)Left cDNA was prepared from RNA samples used for microarray experiments and subsequent RT-qPCR was carried out for ATAD2 gene expression analysis. **Right** After protein isolation from siATAD2 (25nM) and siControl (25 nM) treated T47D cells, the western blot experiment was carried out with ATAD2 antibody. **(B) Left** The gel image shows ribosomal RNA band intensity. While the upper and lower bands correspond to 28S rRNA and 18S rRNA respectively, the bands at the bottom (green colored) indicate 5.8S rRNA baseline bands. **Right** The Bioanalyzer electropherogram of total RNA shows peaks of 18S and 28S ribosomes respectively. Peak ratios (18S/28S) were calculated and the RNA Integrity (RIN) values were assigned for each sample.

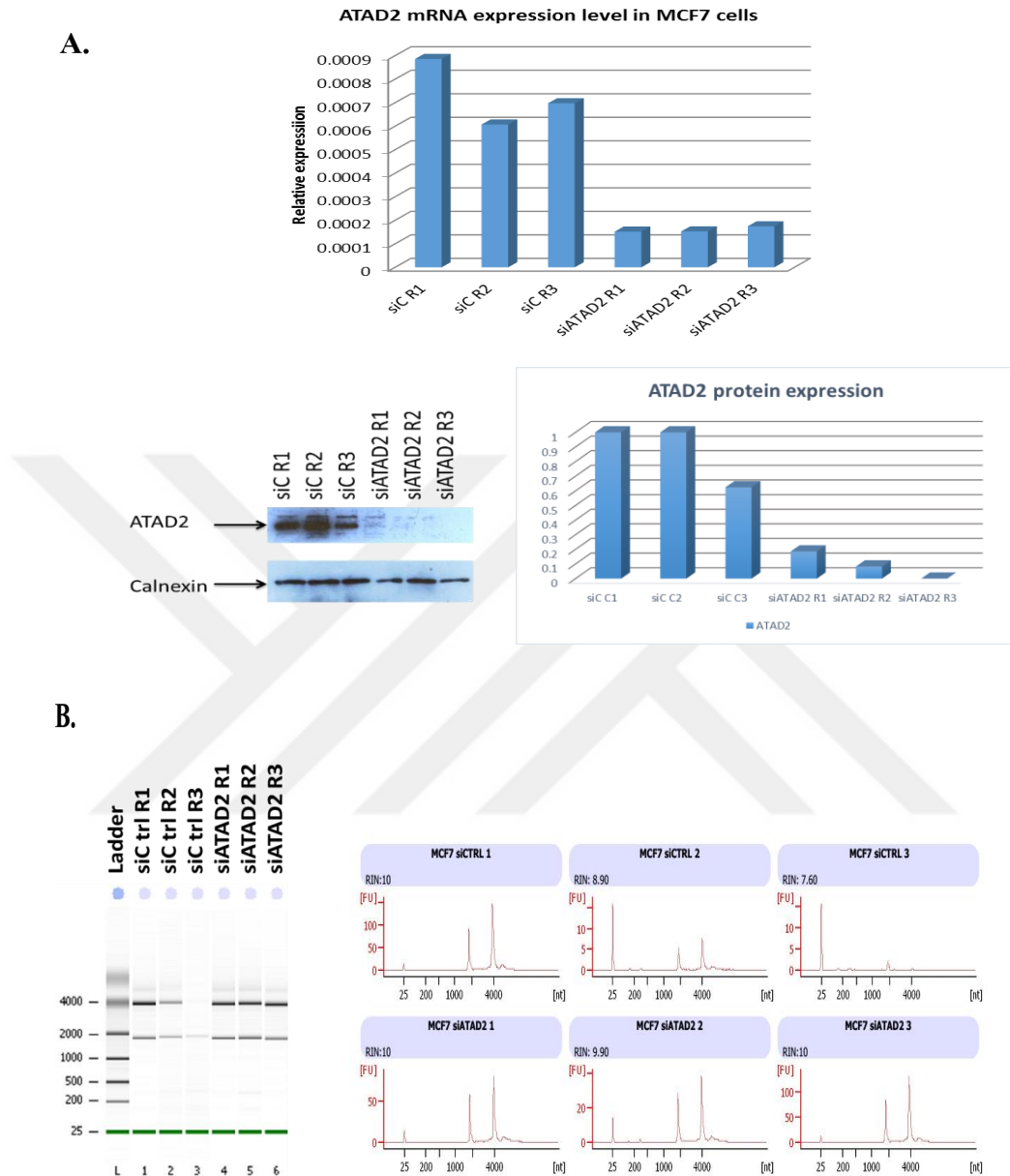


Figure 3. 2: Validation of effectiveness of siATAD2 treatment (A) and Determination of initial total RNA quality by Agilent 2100 Bioanalyzer (B) before the microarray experiments in MCF7 cells.

(A) Left cDNA was prepared from RNA samples used for microarray experiments and subsequent RT-qPCR was carried out for ATAD2 gene expression analysis. **Right** After protein isolation from siATAD2 (25nM) and siControl (25 nM) treated MCF7 cells, a western blot experiment was carried out with ATAD2 antibody. **(B) Left** Computerized gel image shows ribosomal RNA band intensity. While the upper and lower bands correspond to 28S rRNA and 18S rRNA respectively, the bands at the bottom (green colored) indicate 5.8S rRNA baseline bands. **Right** The Bioanalyzer electropherogram of total RNA shows peaks of 18S and 28S ribosomes respectively. Peak ratios (18S/28S) were calculated and the RNA Integrity (RIN) values were assigned for each sample.

Following the chip hybridization process, normalization (RMA method) and gene expression profiles of samples were performed using BRB-ArrayTools²¹⁵. RNA degradation plots, one of the functions of BRB-Array Tools, was performed to assess the RNA quality of samples. It is another tool to indicate potential RNA degradation of processed samples. The plot shows the average intensity of each probe across probe-sets. The slope indicates potential RNA degradation of hybridized cRNA to each array. Degraded RNA will have a very high 5' to 3' slope. Hence, the standardized slope is used as another indicator of RNA degradation. Any array different from others would stand out in a degradation plot^{222,223}. The parallel trend in degradation plots indicates the intact RNA in the samples. Hence, RNA degradation plots confirmed the integrity of processed RNA (Figure 3.3) and the data was ready to be used for further analysis.

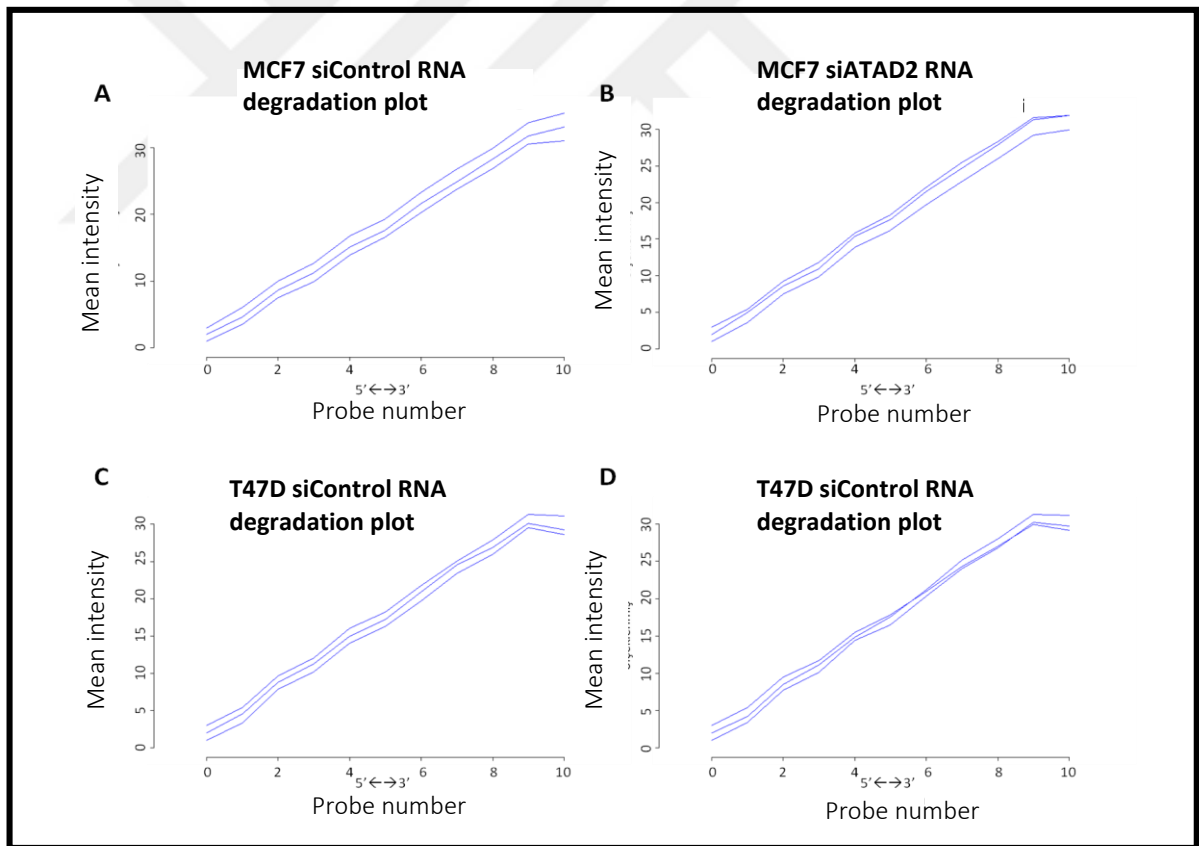


Figure 3. 3: Quality control analysis of the processed RNAs used for microarray experiments.

RNA degradation plots of MCF7 siControl (A), MCF7 siATAD2 (B), T47D siControl (C), T47D siATAD2 (D) showed average intensity of probes across each array, ordered from the 5' to the 3' end. Each curve corresponds to an array.

3.1.1 GENE EXPRESSION PROFILES OF TREATED BREAST CANCER CELLS

3.1.1.1 Altered gene expression by ATAD2 downregulation in MCF7 and T47D cells

Probe sets significantly changed ($p < 0.05$) (t-test) by at least 2 fold were detected, upon ATAD2 downregulation, with the class comparison function of BRB-ArrayTools. All ATAD2 probe sets displayed at least 3-fold decrease in expression. When we compared the treatment effect between MCF7 and T47D cells (Table 3.1), they seemed to respond differentially. While the upregulated and downregulated probe numbers were quite close in total for T47D cells, the downregulated probes were much larger in number compared to upregulated ones in MCF7 cells. In addition, there are many more affected probe sets in total for MCF7 cells. As things stand, MCF7 cells were more sensitive to ATAD2 downregulation compared to T47D cells.

Table 3. 1 Probe numbers with 2 or more fold change ($p < 0.05$)

Cell lines	Upregulated probes	Downregulated probes
MCF7	474	901
T47D	221	237
Both	126	263

*up/downregulated probe sets showing 2 or more fold change at $P < 0.05$ (t-test)

When the genes with at least 2 fold change ($p < 0.05$, t-test) after ATAD2 downregulation were examined, they were altered in the same direction in both MCF7 and T47D cells. For instance, 87% of downregulated genes in T47D were also downregulated in MCF7 cells (Figure 3.4). Table 3.2 represents the first 25 common genes in both MCF7 and T47D cells with most significant fold changes ($p < 0.05$, t-test) determined with the class comparison function of BRB-ArrayTools.

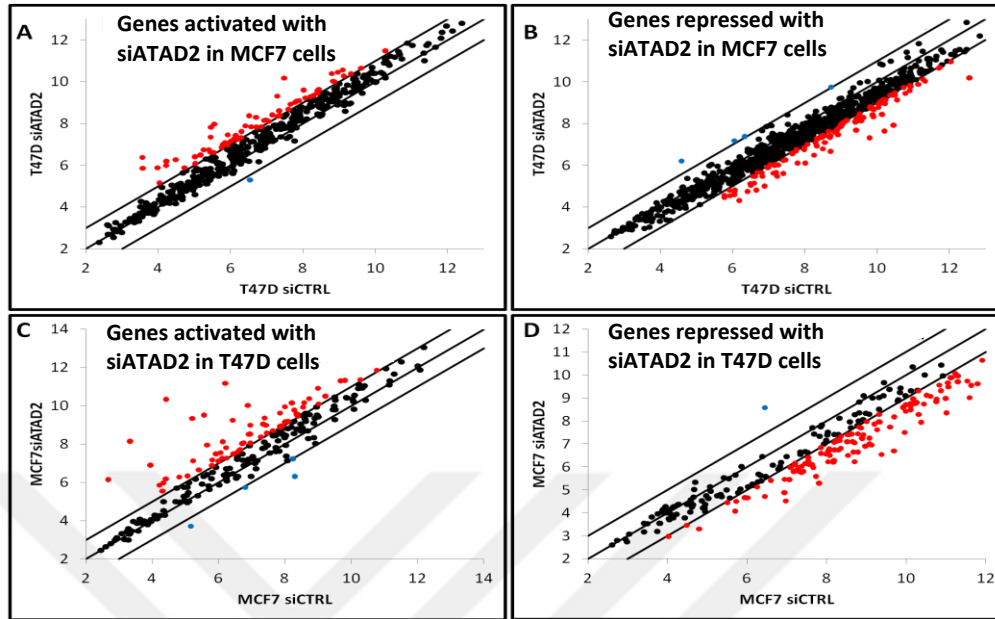


Figure 3. 4: 2-D interactive scatterplots of activated (A-C) and repressed (B-D) genes in siATAD2 treated MCF7 and T47D cells.

Genes with at least 2-fold change ($P < 0.05$, t-test) were selected for scatterplot analysis. While genes that were altered in the same direction by siATAD2 treatment in MCF7 and T47D cells were highlighted in red, genes altered in opposite directions were highlighted in blue. Two lines represent the 2-fold thresholds, so this means the genes between two lines showed less than 2-fold change.

Table 3. 2: The first 25 probes that were significantly changed in both MCF7 and T47D cells ($P < 0.05$)

Probes	Gene Name	P value (t-test)	Fold change
206488_s_at	CD36	0.0323	12.65
203665_at	HMOX1	0.0006	10.25
211138_s_at	KMO	0.0020	9.54
204137_at	GPR137B	0.0220	9.30
205306_x_at	KMO	0.0012	9.10
230278_at	NA	0.0198	8.91
209555_s_at	CD36	0.0578	8.24
229620_at	SEPP1	0.0112	5.32
1561817_at	NA	0.0012	4.91

202917_s_at	S100A8	0.0106	4.56
207802_at	CRISP3	0.0001	4.39
201667_at	GJA1	0.0121	4.12
226363_at	ABCC5	0.0000	3.91
1553226_at	NCRNA00052	0.0138	3.79
203767_s_at	STS	0.0000	3.78
239823_at	NA	0.0000	3.57
205352_at	SERPINI1	0.0004	3.38
233576_at	HMGCLL1	0.0001	3.27
214455_at	HIST1H2BC	0.0208	3.23
223503_at	TMEM163	0.0002	3.23
207430_s_at	MSMB	0.0059	3.22
228345_at	CHIC1	0.0010	3.16
208180_s_at	HIST1H4H	0.0011	3.04
232035_at	HIST1H4H	0.0012	3.00
1569454_a_at	LOC283352	0.0011	2.97
205239_at	AREG	0.0001	-6.41
222740_at	ATAD2	0.0000	-6.12
218782_s_at	ATAD2	0.0000	-5.90
228401_at	ATAD2	0.0000	-5.50
212636_at	QKI	0.0002	-4.49
218031_s_at	FOXN3	0.0005	-4.43
226633_at	RAB8B	0.0000	-4.16
222846_at	RAB8B	0.0001	-4.04
226085_at	CBX5	0.0124	-3.88
222494_at	FOXN3	0.0002	-3.87
209337_at	PSIP1	0.0001	-3.63
219294_at	CENPQ	0.0078	-3.43
208925_at	CLDND1	0.0007	-3.37
215030_at	GRSF1	0.0002	-3.33
225060_at	LRP11	0.0100	-3.31
239903_at	NA	0.0011	-3.26
206247_at	MICB	0.0110	-3.25

221935_s_at	C3orf64	0.0001	-3.25
209230_s_at	NUPR1	0.0023	-3.21
224129_s_at	DPY30	0.0037	-3.17
217980_s_at	MRPL16	0.0002	-3.08
224922_at	CSNK2A2	0.0005	-3.05
235266_at	ATAD2	0.0001	-3.00
238015_at	C4orf46	0.0032	-2.96
225479_at	LRRC58	0.0032	-2.94

3.1.1.2. Functional annotation of common genes altered by ATAD2 downregulation

To evaluate treatment effects on cells, functional annotation analysis was performed for common genes upregulated or downregulated in MCF7 and T47D cells upon ATAD2 downregulation. DAVID Bioinformatics Resources 6.8 Beta^{216,224}, an online functional annotation tool, was used. It is used to extract biological meanings from a list of genes. Hgu-133 plus 2.0 array was used as background during analysis. The tool gives an enrichment score for each cluster and it sorts them according to their scores. Clusters contain related terms (e.g. functions/pathways) of genes of interest. The tool assigns a P value for each term displaying its significance in the clusters. The smaller the P values, the more significant they are. The highest enrichment score and lower P values give the most interesting functions of the relevant genes. The first 5 most significant clusters of annotation analysis are shown. Table 3.3 and table 3.4 represent the clusters of upregulated and downregulated genes respectively. It seems ATAD2 downregulation has resulted in the upregulation of genes function in nucleosome organization and extracellular or intracellular cellular response. It induced the downregulation of genes involved in the immune response and protein metabolism related functions and genes involved in the regulation of cell proliferation and cancer associated intracellular signaling pathways.

Table 3. 3: Functional clustering of common upregulated genes in MCF7 and T47 cells upon ATAD2 downregulation

Category	Term	P value
Annotation Cluster 1	Enrichment Score: 3	
GOTERM_CC_DIRECT	nucleosome	0.000004
KEGG_PATHWAY	Systemic lupus erythematosus	0.000288
GOTERM_BP_DIRECT	chromatin organization	0.000346
KEGG_PATHWAY	Alcoholism	0.000757
GOTERM_CC_DIRECT	nuclear nucleosome	0.000819
GOTERM_MF_DIRECT	protein heterodimerization activity	0.001312
GOTERM_BP_DIRECT	nucleosome assembly	0.003889
GOTERM_MF_DIRECT	DNA binding	0.803452
Annotation Cluster 2	Enrichment Score: 1.95	
GOTERM_CC_DIRECT	nuclear nucleosome	0.000819
GOTERM_BP_DIRECT	nucleosome assembly	0.003889
GOTERM_BP_DIRECT	defense response to Gram-positive bacterium	0.017656
GOTERM_BP_DIRECT	innate immune response in mucosa	0.032416
GOTERM_BP_DIRECT	antibacterial humoral response	0.097237
Annotation Cluster 3	Enrichment Score: 1.44	
KEGG_PATHWAY	Valine, leucine and isoleucine degradation	0.007707
GOTERM_BP_DIRECT	branched-chain amino acid catabolic process	0.041984
GOTERM_CC_DIRECT	mitochondrial matrix	0.144221
Annotation Cluster 4	Enrichment Score: 1.37	
GOTERM_BP_DIRECT	cotranslational protein targeting to membrane	0.005343
GOTERM_BP_DIRECT	IRE1-mediated unfolded protein response	0.039661
GOTERM_BP_DIRECT	endoplasmic reticulum unfolded protein response	0.054578
KEGG_PATHWAY	Protein processing in endoplasmic reticulum	0.289342
Annotation Cluster 5	Enrichment Score: 0.81	
KEGG_PATHWAY	Viral carcinogenesis	0.001297
KEGG_PATHWAY	FoxO signaling pathway	0.022453
KEGG_PATHWAY	Glioma	0.082450
KEGG_PATHWAY	Proteoglycans in cancer	0.101448
KEGG_PATHWAY	Melanoma	0.109286
KEGG_PATHWAY	Chronic myeloid leukemia	0.116467
KEGG_PATHWAY	Small cell lung cancer	0.167019
KEGG_PATHWAY	Hepatitis B	0.212149
KEGG_PATHWAY	Non-small cell lung cancer	0.242204
KEGG_PATHWAY	Thyroid hormone signaling pathway	0.290768
KEGG_PATHWAY	Pancreatic cancer	0.292280
KEGG_PATHWAY	PI3K-Akt signaling pathway	0.334763
KEGG_PATHWAY	Cell cycle	0.341654
KEGG_PATHWAY	Epstein-Barr virus infection	0.373711
KEGG_PATHWAY	Prostate cancer	0.425932
KEGG_PATHWAY	Measles	0.634436
KEGG_PATHWAY	Pathways in cancer	0.640309

Table 3. 4: Functional clustering of common downregulated genes in MCF7 and T47 cells upon ATAD2 downregulation		
Category	Term	P value
Annotation Cluster 1 Enrichment Score: 2.3		
GOTERM_BP_DIRECT	response to cytokine	0.00030
GOTERM_MF_DIRECT	ciliary neurotrophic factor receptor activity	0.00340
GOTERM_BP_DIRECT	ciliary neurotrophic factor-mediated signaling pathway	0.00553
KEGG_PATHWAY	Jak-STAT signaling pathway	0.06038
Annotation Cluster 2 Enrichment Score: 2.1		
GOTERM_MF_DIRECT	histone deacetylase binding	0.00021
GOTERM_CC_DIRECT	histone deacetylase complex	0.04179
GOTERM_MF_DIRECT	repressing transcription factor binding	0.04496
Annotation Cluster 3 Enrichment Score: 1.8		
KEGG_PATHWAY	Spliceosome	0.00422
GOTERM_BP_DIRECT	RNA splicing	0.00692
GOTERM_BP_DIRECT	mRNA splicing, via spliceosome	0.02892
GOTERM_CC_DIRECT	catalytic step 2 spliceosome	0.05633
Annotation Cluster 4 Enrichment Score: 1.6		
GOTERM_BP_DIRECT	Notch signaling pathway	0.00576
KEGG_PATHWAY	Notch signaling pathway	0.02778
GOTERM_BP_DIRECT	positive regulation of Notch signaling pathway	0.05167
Annotation Cluster 5 Enrichment Score: 1.3		
GOTERM_BP_DIRECT	negative regulation of growth	0.01014
GOTERM_BP_DIRECT	cellular response to zinc ion	0.06222
GOTERM_BP_DIRECT	response to metal ion	0.09026
KEGG_PATHWAY	Mineral absorption	0.09837

3.1.2 DETERMINATION OF DIFFERENTIALLY EXPRESSED GENES (DEGs)

ATAD2 is a recently identified nuclear protein. It acts as a transcriptional co-activator of estrogen receptor α . Its elevated expression in breast cancer has been associated with its regulatory effect on estrogen target genes. It is recruited to estrogen responsive gene promoters by ER α ¹¹⁵. Decreased expression of some cell proliferation/survival related ER responsive genes, in the absence of ATAD2, confirms its regulatory activity in the transcription of these genes^{115,117,127}. What's revealed so far indicates that ATAD2 as an ER α co-regulator may play a significant role in the deregulation of cellular functions and maintenance of breast cancer cells when it is overexpressed. Its potential functions in main cellular events were analyzed initially with bioinformatics studies. Another microarray data set GSE27473) from Gene Expression Omnibus (GEO) along with our datasets was used for pathway analysis. This was an expression data set of MCF7 cells that were ER α downregulated. Data was RMA normalized by using BRB-ArrayTools. The genes that were significantly ($P<0.05$) downregulated by at least 1.5 fold were determined using the class comparison function of BRB-ArrayTools. To obtain ER responsive genes co-expressed with ATAD2 in breast carcinoma, probe sets downregulated in MCF7 cells with ER α silencing were subtracted from the list of significantly ($P<0.05$) and commonly downregulated genes by at least 1.5 fold of MCF7 and T47D cells with ATAD2 silenced. A total of 69 intersected genes were detected. Functional annotation of these genes was analyzed using DAVID Bioinformatics Resources 6.8 Beta. Gene ontology (GO) enrichment analysis was performed on DAVID. FDR <0.05 and EASE <0.1 cut-off²²⁵ was applied for the analysis and enriched genes were extracted from DAVID. A total of 30 genes passed through this filtration (Table 3.5). Pathway enrichment analysis was then conducted with these differentially expressed genes (DEGs).

Table 3. 5: The list of enriched genes passed through filtering (FDR <0.05 ; EASE <0.1)

ARNT	aryl hydrocarbon receptor nuclear translocator
C6orf120	chromosome 6 open reading frame 120
CRLF1	cytokine receptor-like factor 1
CXCL12	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)
CXCL16	chemokine (C-X-C motif) ligand 16
DLL1	delta-like 1 (Drosophila)
ENPP5	ectonucleotide pyrophosphatase/phosphodiesterase 5 (putative function)

ERBB3	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)
FGFR3	fibroblast growth factor receptor 3
GAL	galanin prepropeptide
GRIK3	glutamate receptor, ionotropic, kainate 3
IGFBP5	insulin-like growth factor binding protein 5
JAG2	jagged 2
JAK2	Janus kinase 2
LFNG	LFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase
MATN2	matrilin 2
MEGF6	multiple EGF-like-domains 6
NETO2	neuropilin (NRP) and tolloid (TLL)-like 2
NTN1	netrin 1
OLFM1	olfactomedin 1
PCDHB14	protocadherin beta 14
PGR	progesterone receptor
PLA2G12A	phospholipase A2, group XIIA
PRRG4	proline rich Gla (G-carboxyglutamic acid) 4 (transmembrane)
QPCT	glutamyl-peptide cyclotransferase
RAB3IP	RAB3A interacting protein (rabin3)
SEMA3C	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C
STMN3	stathmin-like 3
TP53I11	tumor protein p53 inducible protein 11
ULK2	unc-51-like kinase 2 (C. elegans)

3.1.3. PATHWAY ENRICHMENT ANALYSIS OF DEGs

Pathway enrichment analysis of DEGs (Table 3.5) was performed. The Reactome V56 tool^{217,226} was used to associate altered biological functions in breast carcinoma. FDR<0.05 and p<0.05 cut-off points were applied to identify differentially enriched pathways. A total of 108 pathways related with our DEGs were identified. DEGs represent the ER α responsive genes altered with ATAD2 downregulation, indicating they are the genes regulated by both ATAD2 and ER α in ER(+) breast carcinoma cells. Top 50 significantly (P<0.05) enriched pathways of ER α responsive genes downregulated upon ATAD2 silencing are listed in table 3.6. The altered genes function mainly in cellular proliferation, survival, cell cycle, migration, and adhesion signaling pathways. When enriched pathways were analyzed in depth, it revealed the potential participation of the ATAD2 gene in the regulation of some major signaling cascades such as MAPK, EGFR, and PI3K/Akt. Considering that these are commonly altered genes in both ATAD2 and ER α

downregulated breast carcinoma cell lines, ATAD2 may exert these effects as a partner of the estrogen receptor.

Table 3. 6: Top 50 significantly (P<0.05) enriched pathways of ER α responsive genes downregulated upon ATAD2 silencing

Pathway name	Entities p Value	Entities FDR
FGFR3 mutant receptor activation	2.41E-05	0.003152481
Signaling by activated point mutants of FGFR3	2.41E-05	0.003152481
Signaling by FGFR3 in disease	1.18E-04	0.010269154
Constitutive Signaling by NOTCH1 t(7;9)(NOTCH1:M1580_K2555) Translocation Mutant	3.57E-04	0.018570946
Signaling by NOTCH1 t(7;9)(NOTCH1:M1580_K2555) Translocation Mutant	3.57E-04	0.018570946
Nuclear signaling by ERBB4	5.24E-04	0.022514806
Diseases of signal transduction	8.62E-04	0.025325629
Signaling by NOTCH3	8.73E-04	0.025325629
Signaling by NOTCH4	8.73E-04	0.025325629
Signaling by FGFR in disease	0.001114108	0.027916694
FGFR3 ligand binding and activation	0.001213769	0.027916694
Signaling by ERBB4	0.001494545	0.028945298
Signaling by NOTCH1 HD Domain Mutants in Cancer	0.001608072	0.028945298
Constitutive Signaling by NOTCH1 HD Domain Mutants	0.001608072	0.028945298
Constitutive Signaling by Aberrant PI3K in Cancer	0.002590527	0.029410389
SHC-mediated cascade:FGFR3	0.002824042	0.029410389
IL-6-type cytokine receptor ligand interactions	0.002824042	0.029410389
NOTCH2 Activation and Transmission of Signal to the Nucleus	0.003105904	0.029410389
t(4;14) translocations of FGFR3	0.003899717	0.029410389
Interleukin-6 family signaling	0.006200806	0.029410389
Activated NOTCH1 Transmits Signal to the Nucleus	0.006200806	0.029410389
Negative regulation of FGFR3 signaling	0.006200806	0.029410389
PI3K/AKT Signaling in Cancer	0.006415741	0.029410389
Signaling by NOTCH2	0.007020814	0.029410389
PI-3K cascade:FGFR3	0.0088184	0.029410389
PI3K events in ERBB2 signaling	0.0088184	0.029410389
PIP3 activates AKT signaling	0.0088184	0.029410389
PI3K events in ERBB4 signaling	0.0088184	0.029410389
PI-3K cascade:FGFR2	0.0088184	0.029410389
PI-3K cascade:FGFR4	0.0088184	0.029410389
PI-3K cascade:FGFR1	0.0088184	0.029410389

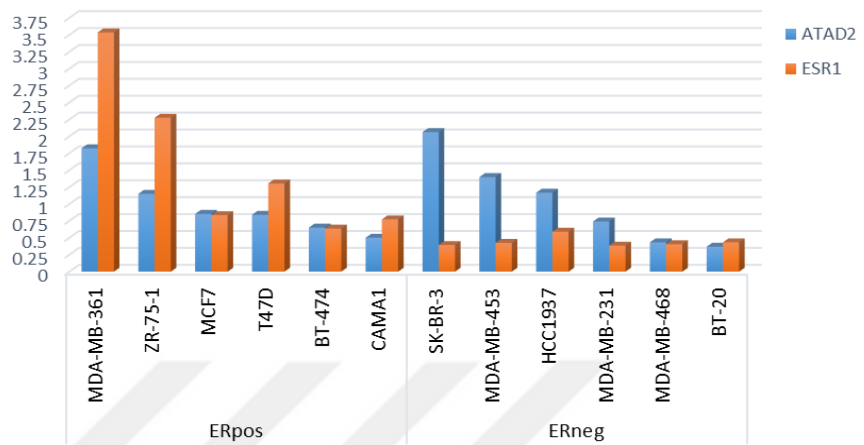
3.2. VALIDATION OF FUNCTIONAL SIGNIFICANCE OF ATAD2 GENE IN BREAST CANCER

3.2.1. EXPRESSION ANALYSIS OF ATAD2 GENE IN BREAST CELL LINES

There have been individual reports showing high ATAD2 expressions in different cancer types but none have specifically compared its expression in distinct breast cancer cell lines at the same time so far. Differential expression of ATAD2 was evaluated in breast carcinoma cells depending on their estrogen receptor status. ATAD2 expression levels were determined at both mRNA and protein levels (Figure 3.5-6). GSE44836 microarray data set from GEO was used to evaluate differential expressions of ATAD2 and ESR2 genes in both ER positive and ER negative breast cancer cells (Figure 3.5A). The data analysis showed that not only ER positive cells but also ER negative breast cancer cell lines had high ATAD2 expression levels. RT-qPCR experiments were performed to confirm microarray results (Figure 3.5 B). ATAD2 and ER α protein expressions were analyzed with western blot (Figure 3.6 A-B). The results demonstrate that ATAD2 expression is higher in breast carcinoma cells than non-carcinoma cells at both expression levels. The upregulated expression levels in ER(-) cells indicate that ATAD2 may have a function that is independent of the estrogen receptor in breast cells. While MDA-MB-361 cells among ER(+) cells showed the greatest ATAD2 expression at both mRNA and protein levels, SKBR3 and MDA-MB-453 cells had highest transcript and protein expressions of ATAD2 respectively among ER(-) cells. There seems to be no correlation between ATAD2 and ER α expression at protein levels; however, their expressions at the mRNA level are more comparable. MCF7 and T47D cells display the highest co-expression of these two genes at the protein level and that is why they were selected as ER+ cells to be used in future stable transfection experiments for functional assays.

A.

ATAD2 and ESR1 expression profiles of Breast cancer cells (GSE44836)



B.

ATAD2 mRNA expression levels in Breast Cells

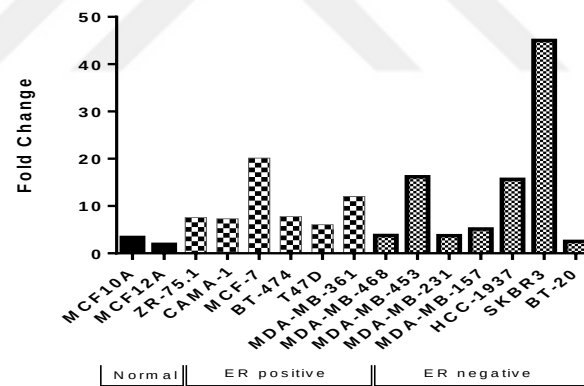


Figure 3. 5: Expression profiles of ATAD2 and ER α in Breast carcinoma and non-carcinoma cell lines.

(A) Differential expressions of *ATAD2* and *ESR1* genes were determined in 12 breast carcinoma cell lines (GSE44836). Gene expressions were normalized by justRMA method. Breast carcinoma cells were grouped according to their estrogen receptor (ER) status. The gene expressions were divided by the geometric mean of each gene across the group. A value of “1” indicates that the given breast carcinoma cell line shows average expression of the gene of interest. Greater than “1” indicates higher expression of the gene compared to other cell lines. (B) Relative ATAD2 expression levels were determined across selected cancerous and non-cancerous breast cell lines by RT-qPCR. The target gene expression was normalized to the housekeeping gene, GAPDH, expression level. The cells were grouped according to their estrogen receptor (ER) status.

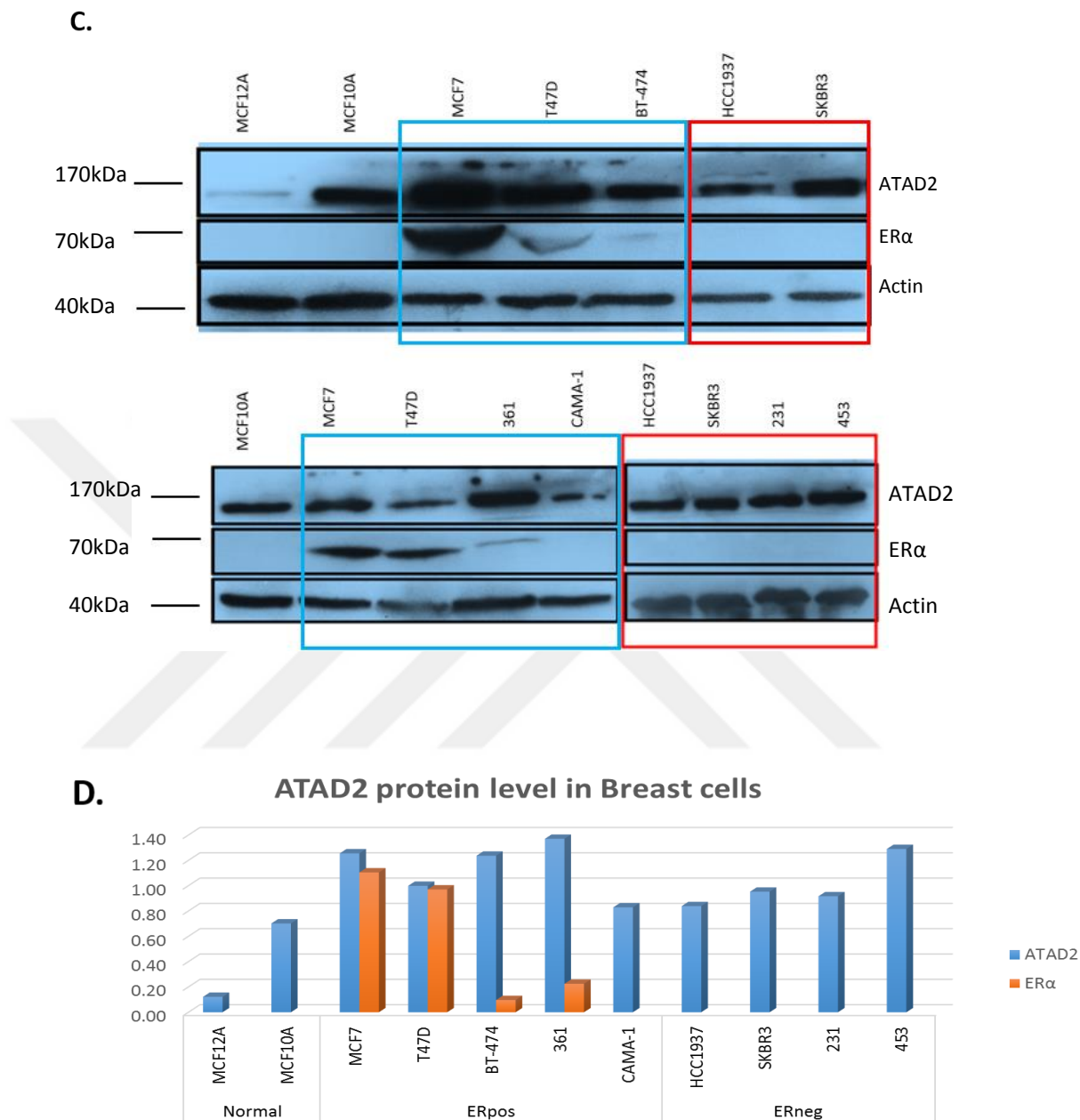


Figure 3. 6: Validation of protein expression levels of ATAD2 and ERα in Breast carcinoma and non-carcinoma cell lines.

(A) Western Blot was performed using ATAD2 (160 kDa) and ERα antibody (66 kDa) (santa cruz, sc-8002) after protein extraction from selected breast carcinoma and non-carcinoma cells. **(B)** Protein expressions were normalized to input (β-Actin, 45kDa) protein and their relative expression levels were calculated and demonstrated in the bar graph. The cells were grouped according to their estrogen receptor (ER) status.

3.2.2. EFFECT OF SERUM STARVATION ON ATAD2 EXPRESSION IN BREAST CARCINOMA CELLS

Serum is a growth supplement used in culture media to stimulate signaling pathways related with proliferation and cell survival. It contains growth factors and cytokines. It supplies the metabolic requirements of cells in the culture²²⁷. During some studies, the serum in the medium is removed to evaluate various procedures. This method allows the assessment of the direct effect of treatments on the cells in the absence of other interfering factors²²⁸⁻²³¹. Serum starvation experiment was carried out by Gurbet Karahan. MCF7, T47D, SKBR3, HCC1937, BT-20 and MDA-MB-231 cells were grown under serum-starvation conditions and then alterations in ATAD2 expressions in the cells were measured. After cells were seeded on 6-well plates for 48 h and 7 days in normal growth medium (10% FBS) and serum free medium (0.01 % FBS), the cell viability was measured with SRB assay. Another group was set for FACS analysis. The expression levels of ATAD2 as a response to serum starvation were evaluated. The results show that upon serum deprivation, ATAD2 expression decreased in MCF7 and T47D cells, increased in BT-20 and 231 cells, but did not show significant change in HCC1937 and SKBR3 cells (Table 3.7). Expression levels were evaluated by flow cytometry. MCF7 and T47D cells showed the highest decrease in cell viability after both 48-hour and 7-day starvation while proliferation of SKBR3 and BT20 cells did not change much after 48 hours. However, the proliferation rate of SKBR3 cells was inhibited more than 50% after 7 days of starvation. BT-20 cells maintained high viability even after 7 days of lack of growth stimulation (Figure 3.7). The cell cycle analysis following 48 hours of serum starvation showed that MCF7 cells had a slight increase in G2/M population whereas T47D cells displayed an increase in the apoptotic cell population. MDA-MD-231, SKBR3 and HCC1937 displayed the same pattern where cell cycle distribution decreased in the G2/M and S phase but increased in G1, while treatment-induced G1-arrest was seen in BT-20 cells (Table 3.8).

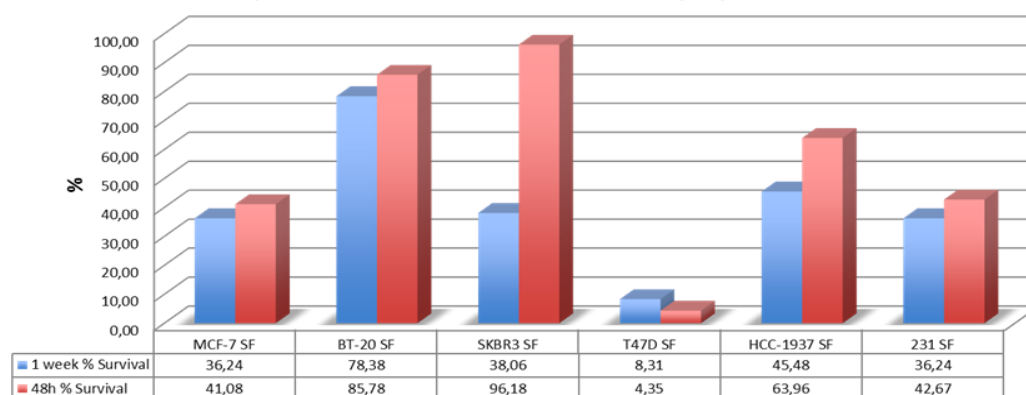


Figure 3. 7: Determination of cell viability after 48 hours and 7 days serum starvation.

MCF7, BT-20, SKBR3, T47D, HCC1937 and MDA-MB-231 breast carcinoma cell lines were cultured to be 1×10^5 cells/well on 6-well plates in the media supplemented with either 10% FBS or 0.01% FBS(+ 100nM NaSel). They were seeded in triplicates. The cells were grown for 48 hours and 7 days under these conditions. At the end of the incubation, the viability of the cells was determined by SRB assay. They were read at 515nm with the ELISA reader. The average of the measurements was represented in the bar-graph.

Table 3. 7: Quantification of ATAD2 protein in the cells following 48 hours of serum starvation; measured by average readings of fluorescent labelled antibodies.

	FBS Neg	SF Neg Av	ATAD2 FBS Av.	ATAD2 SF Av.	FBS/Neg	SF/Neg
MCF7	3.38	3.38	16.45	12.17	4.87	3.60
HCC-1937	3.09	3.15	12.87	13.63	4.16	4.33
T47D	3.30	5.40	20.15	14.56	6.12	2.70
SKBR3	2.95	3.12	9.50	10.77	3.23	3.45
BT20	3.05	3.06	5.47	10.01	2.79	3.08
MDA-MB-231	3.12	3.12	5.03	6.15	1.61	1.97

*MCF7, BT-20, SKBR3, T47D, HCC1937 and MDA-MB-231 breast carcinoma cell lines were cultured to be 3×10^6 cells/well on 150 mm culture dishes in the media supplemented with either 10% FBS or 0.01% FBS(+ 100nM NaSel). The cells were grown for 48 hours under these conditions. At the end of the incubation, cells in each dish were separated into 3 tubes for protein expression analysis and 2 tubes were prepared as negative controls. After permeabilization, cells were conjugated with primary ATAD2-antibodies and antibodies were stained with fluorescent labelled probes. Negative controls were not conjugated with ATAD2 antibody. Protein amounts were quantified by flow cytometric measurement. The geometric means of the measurements are listed in *table 3.7*.

Table 3. 8: Cell cycle analysis of cells following 48 hours of serum starvation				
	BT20		MDA-MB-231	
	FBS	SF	FBS	SF
Apoptotic	30.66	13.04	2.42	4.08
G0/G1	37.15	56.43	54.15	61.11
S	7.11	6.44	20.73	12.46
G2/M	23.71	24.09	22.3	20.57

	SKBR3		HCC1937	
	FBS	SF	FBS	SF
Apoptotic	19.72	2.57	3.79	5.01
G0/G1	50.52	58.1	41.08	47.23
S	7.48	6.44	15.96	15.57
G2/M	21.96	32.92	38.87	30.8

	MCF7		T47D	
	FBS	SF	FBS	SF
Apoptotic	0.31	0.12	0.54	9.22
G0/G1	56.63	53.64	62.36	52.01
S	7.98	4.92	10.89	15.12
G2/M	35.22	41.47	25.17	23.08

* MCF7, BT-20, SKBR3, T47D, HCC1937 and MDA-MB-231 breast carcinoma cell lines were cultured to be 2.5×10^5 cells/well on 6-well plates in the media supplemented with either 10% FBS or 0.01% FBS(+ 100nM NaSel). They were seeded in triplicate. The cells were grown for 48 hours under these conditions. At the end of the incubation, cells were fixed and stained with PI (propidium iodide) solution. The cell-cycle profile of the cells was analyzed with FACS. The geometric means of the measurements are listed in *table 3.8*.

3.2.3. ANALYSIS OF CELLULAR PROCESSES IN ATAD2 SILENCED BREAST CANCER CELL LINES

ATAD2 function in estrogen receptor positive (ER+) and (ER-) breast carcinoma cell lines was analyzed to reveal its effects on cellular functions as ER α co-activator. Breast carcinoma cell lines were selected looking at their ATAD2 and ER α gene expression profiles (Figure 3.5-6). Accordingly, MCF7 and T47D cells were selected as ER a (+), whereas SKB3 and 1937 were selected ER (-) cell lines. All displayed considerably high protein expressions. ATAD2 downregulated stable clones were generated as explained in the materials and method section. Clones were screened for their ATAD2 mRNA expressions, then two control and two shATAD2 clones for MCF7 and T47D were determined based on their expression levels (Figure 3.8A). Protein expressions of selected clones were detected (Figure 3.8-9 B). While shATAD2_1_C17 and shATAD2_1_C21 T47D clones displayed 75% and 85% downregulation in ATAD2 protein expression respectively, expression levels in shATAD2_1_C10, shATAD2_1_C15 MCF7 clones were reduced 97% and 89% respectively, compared to shCntrl clones. These selected 8 clones were used in all functional assays.

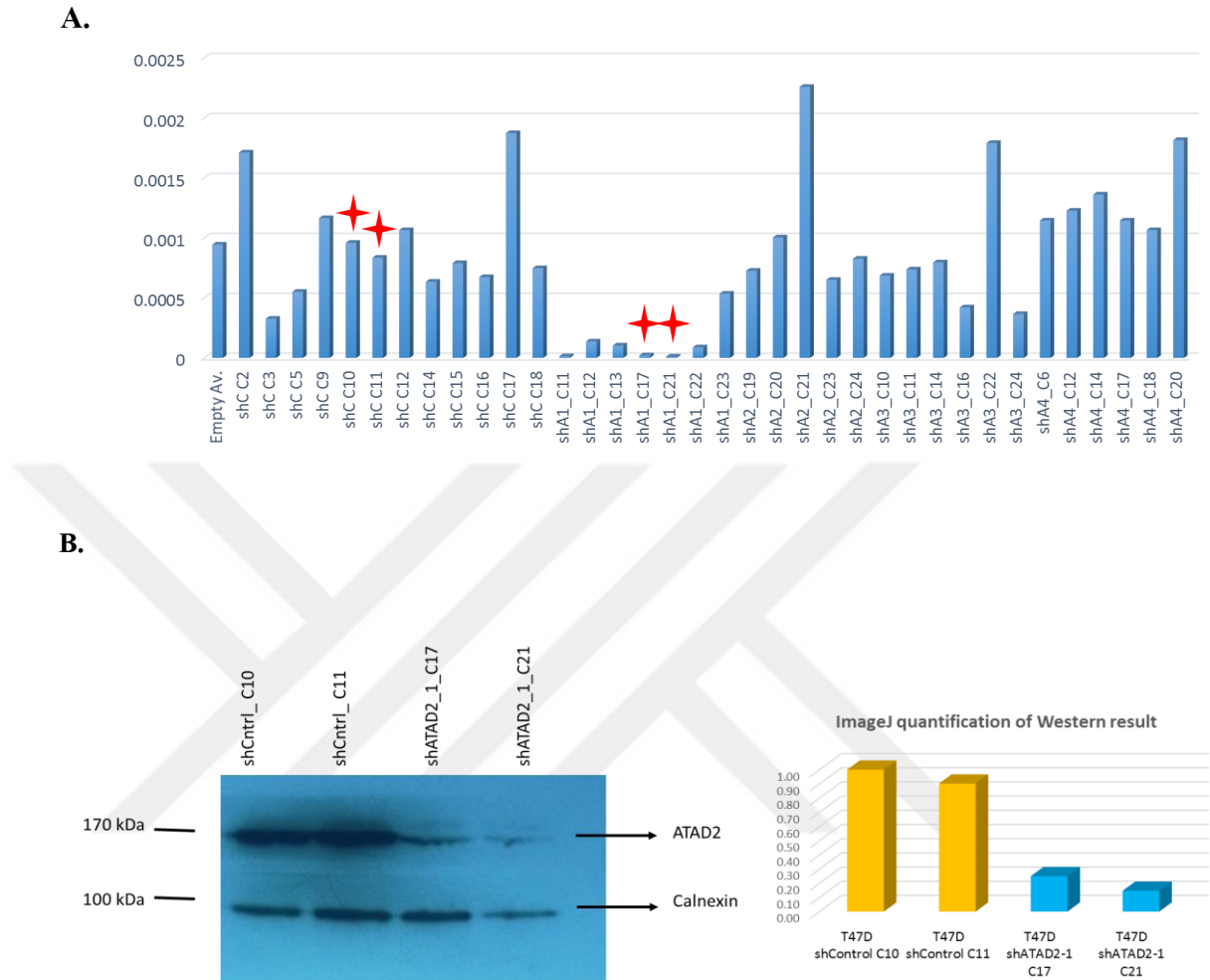


Figure 3. 8: Validation of ATAD2 expression in stably-transfected T47D cells.

(A) ATAD2 gene expression was screened across stable T47D clones. RT-qPCR was performed and ATAD2 expression levels were normalized to the housekeeping gene, GAPDH. Two sh-controls with expression levels close to average of all shCntrl and two sh-ATAD2 clones with the least gene expression levels were selected for western blot analysis. They were highlighted with red stars. **(B)** ATAD2 protein expression was determined in the selected clones that were shCntrl_C10, shCntrl_C11, shATAD2_1_C17, shATAD2_1_C21 with western blot analysis. ATAD2 primary antibody used during western blot was produced by Dr. Mehmet Oztürk's lab. Its expression levels were normalized to input Calnexin (90 kDa) (Sigma, C4731). The relative ATAD2 (170 kDa) expressions in the clones were illustrated in the graph **(B, left)**. Stable transfection of these cells with shATAD2_1 plasmid (SureSilencing, NM_014109) resulted in the 75% and 85% downregulation of the ATAD2 protein in shATAD2_1_C17 and shATAD2_1_C21 clones respectively, compared to shCntrl clones.

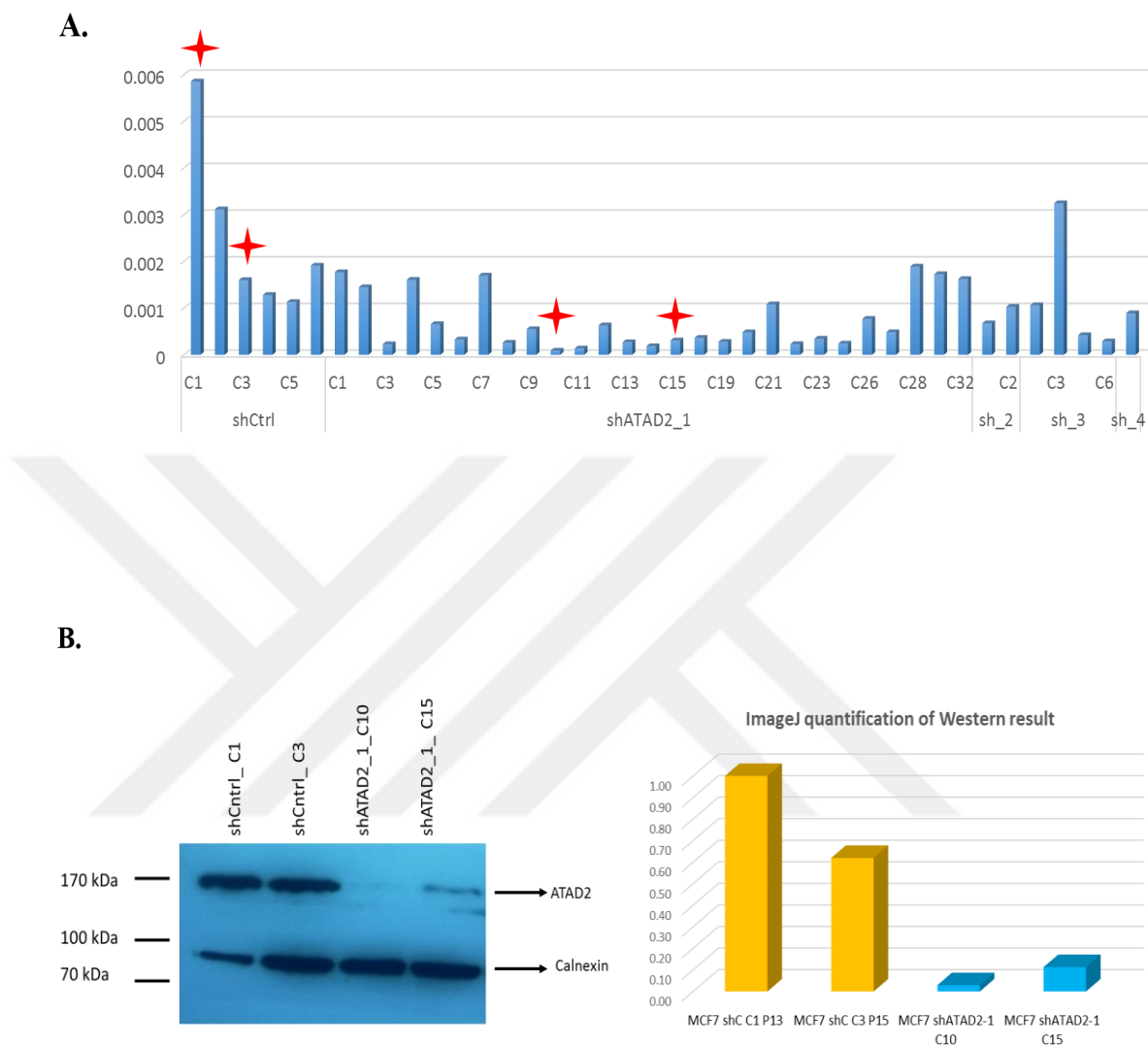


Figure 3. 9: Validation of ATAD2 expression in stably-transfected MCF7 cells.

(A) ATAD2 gene expression was screened across stable MCF7 clones. RT-qPCR was performed and ATAD2 expression levels were normalized to the housekeeping gene, GAPDH. Two sh-controls with expression levels close to average of all shCtrl and two sh-ATAD2 clones with the least gene expression levels were selected for western blot analysis. They were highlighted with red stars. **(B)** ATAD2 protein expression was determined in the selected clones that were shCtrl_C1, shCtrl_C3, shATAD2_1_C10, shATAD2_1_C15 with western blot analysis. ATAD2 primary antibody used during western blot was produced by Dr. Mehmet Oztürk's lab. Its expression levels were normalized to input Calnexin (90 kDa) (Sigma, C4731). The relative ATAD2 (170 kDa) expressions in the clones were illustrated in the graph **(B, left)**. Stable transfection of these cells with shATAD2_1 plasmid (SureSilencing, NM_014109) resulted in the 97% and 89% downregulation of the ATAD2 protein in shATAD2_1_C10, shATAD2_1_C15 clones respectively, compared to shCtrl clones.

ATAD2 expression is induced with estrogen (E2) stimulation. This means increased ATAD2 interaction with ER α in the presence of E2 and in turn the upregulation of ER responsive genes. However, as reported previously, ER(-) breast cancer cells were shown to have quite upregulated ATAD2 expressions (Figure 3.5-6). These aggressive cancer cell types do not respond to estrogen stimulation due to lack of its receptor. The question is how and why ATAD2 maintains its overexpression level in ER- cells. This implies an E2-independent regulation of ATAD2 expression and its function independent from ER activity. Therefore, ATAD2 function in ER(-) cells was investigated. For this purpose, ER(-) cells, namely SKBR3 and HCC1937, were transfected transiently with siATAD2. The transfection conditions have been explained in the material and methods section. 100% downregulation of the gene was confirmed by western blot analysis for both cell types upon transfection (Figure 3.10).

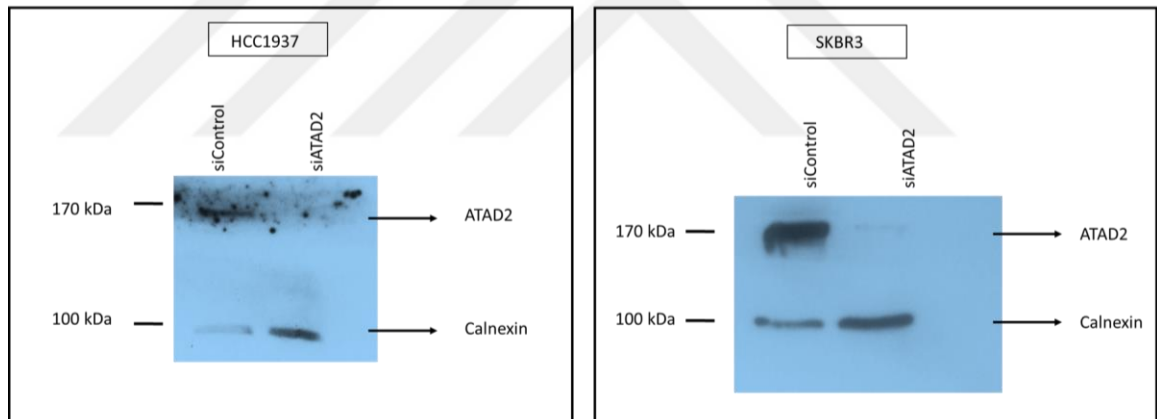


Figure 3. 10: Validation of ATAD2 expression in siATA2 treated ER(-) cells.

ATAD2 protein expression was determined in siATAD2 treated HCC1937 (**A**) and SKBR3 (**B**) cells with western blot analysis. They were treated with 25nM and 100nM final concentration of siATAD2 respectively. ATAD2 primary antibody used during western blot was produced by Dr. Mehmet Oztürk's lab. Its expression levels were normalized to input Calnexin (90 kDa) (Sigma, C4731). Transient transfection of these cells resulted in 100% downregulation of the ATAD2 protein in treated cells, compared to siContrl cells.

3.2.2.1 Analysis of cell migration in ATAD2 suppressed breast carcinoma cells

Migration is a normal cellular process required for development and maintenance of biological integrity. However it is deregulated in tumorigenesis and has evolved into one of the main features of malignancy^{232,233}. Therefore cell motility studies are one of easiest and useful tool to evaluate treatment effects. Scratch assay is one of these methods that mimic *in vivo* 2D migrations of cells²³⁴⁻²³⁶. Hence, it was performed to study the effect of ATAD2 downregulation on cell migration.

Following successful ATAD2 downregulation, the scratch assay was performed with both ER(+) and ER(-) cell types. Cell migration ability of cells was determined by measuring the distance they invaded into the scratch during a specific time period. Photos of cells were taken every 24 hours for 3 days. MCF7 shATAD2_1_C10 was the only clone showing significant decrease in cell motility within ER(+) cells. While two T47D clones were not affected significantly from the treatment, it induced invasive motility of cells for MCF7 shATAD2_1_C15 clone significantly. Due to the inconsistency in the results, whether ATAD2 downregulation had an inhibitory or inducer effect on cell migration of ER(+) breast carcinoma cells was not clear. Conversely, in the case of ER(-) cells, they had spot-on results because the results showed that ATAD2 depletion has significantly inhibited cell migration in both HCC1937 and SKBR3 cells. Not only did the treatment impair their cellular movement, it also reduced adhesion of cells. This indicates ATAD2 may be involved in cell-to-cell contact in ER(-) breast carcinoma cells. HCC1937 especially showed detachment from the surface during the 3 days of the scratch assay post-ATAD2 downregulation. This could be easily deduced from the increasing wound area of siATAD2 treated cells.

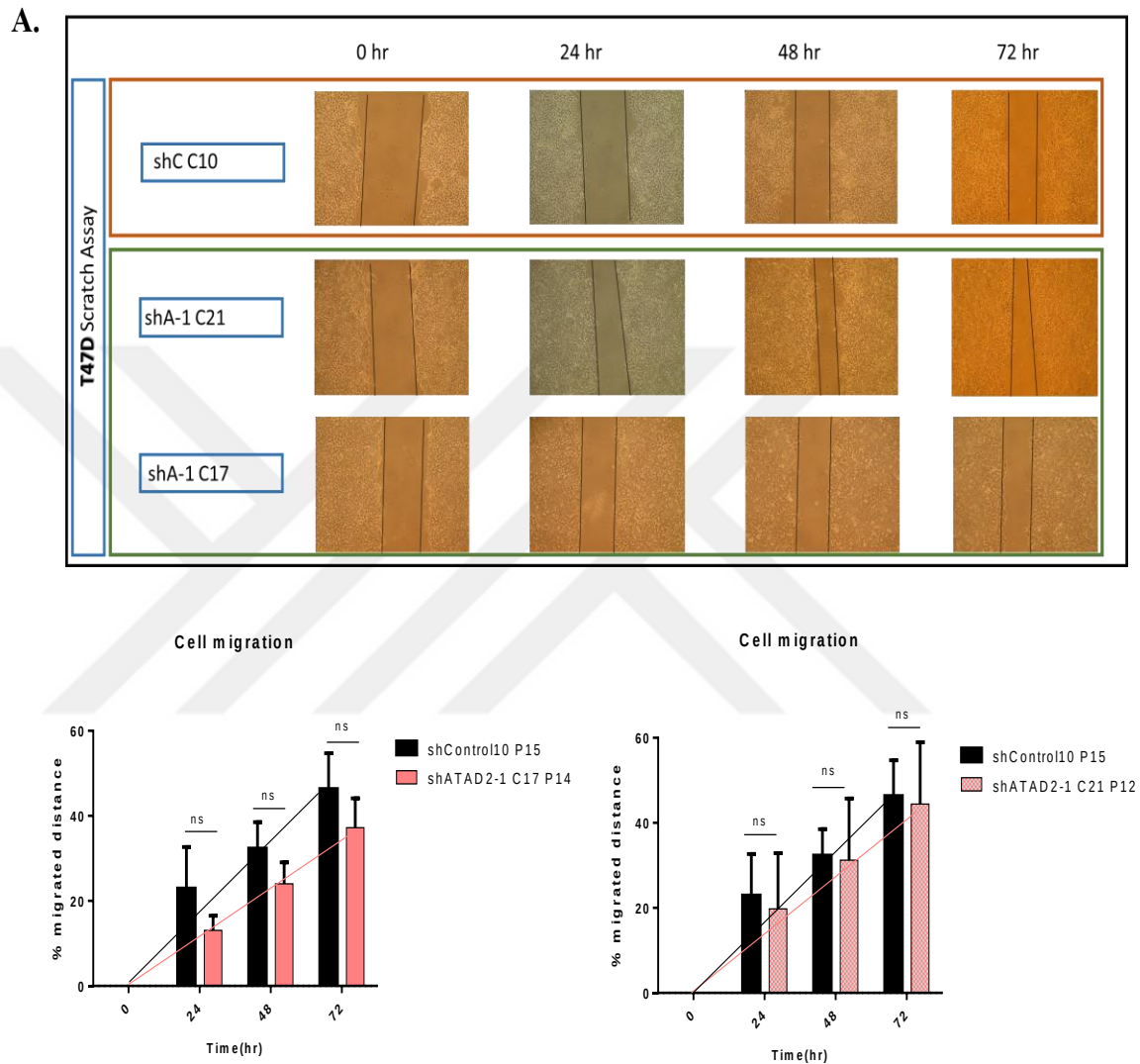


Figure 3. 11: The Effect of ATAD2 down-regulation on migration of breast carcinoma cells.

(A) While ATAD2 downregulation in T47D cells did not result in a significant difference in cell migration, (B) it affected migration of MCF7 cells significantly. Wound closure of shATAD2_1_C10 cell clones was reduced significantly, yet it promoted cell motility of shATAD2_1_C15 cell clones. (C-D) The migration of both siATAD2 treated HCC1937 and SKBR3 cells were inhibited significantly with ATAD2 downregulation. In treated SKBR3 cells, the wound area at the end of 3 days was bigger than that at the starting point (time 0). This implies that SKBR3 cells not only displayed inhibited cell motility but also reduced cell-to-cell attachment after ATAD2 silencing. The statistical significance of the results was calculated with the two-tailed t-test.

B.

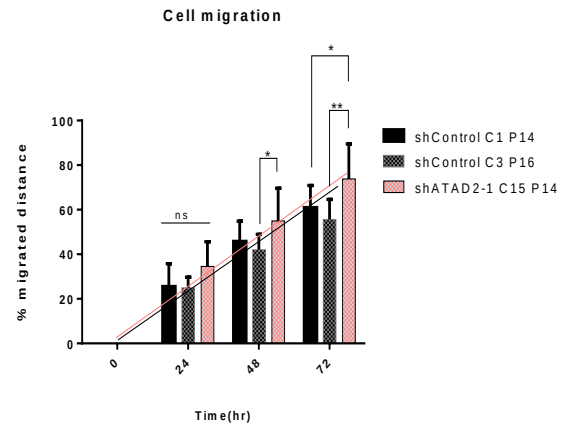
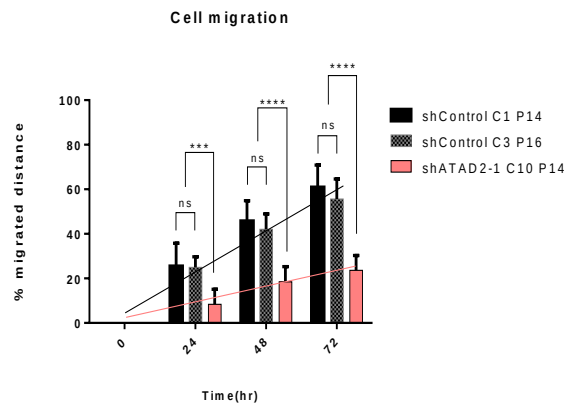
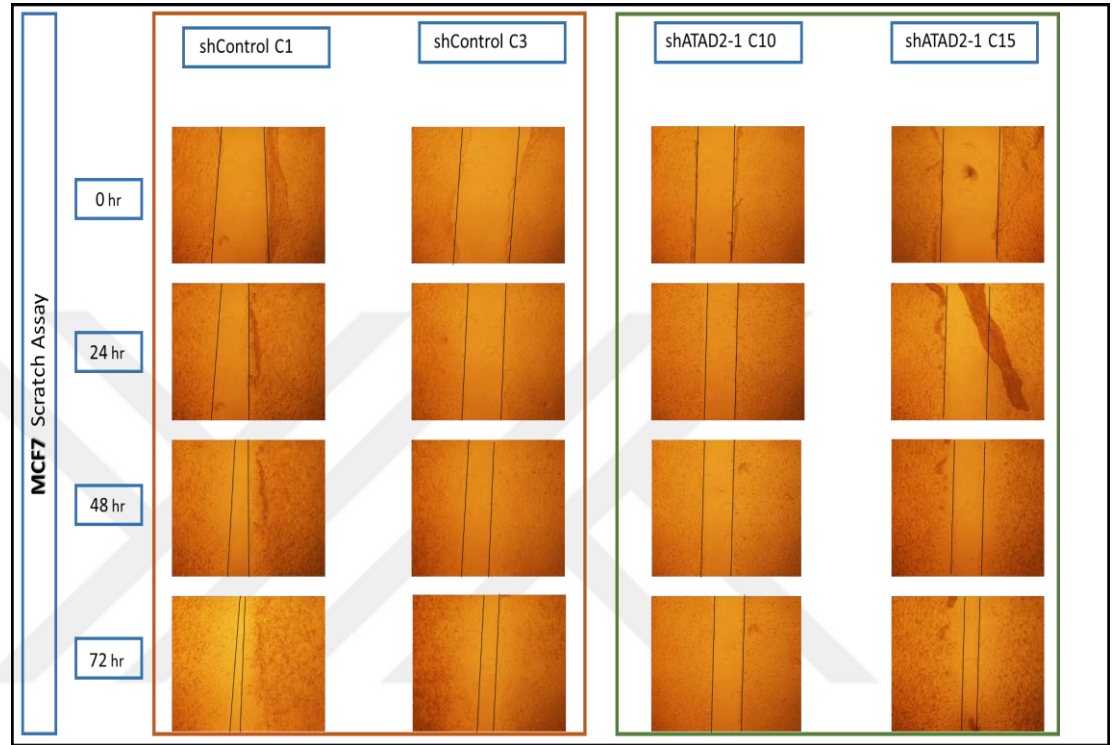


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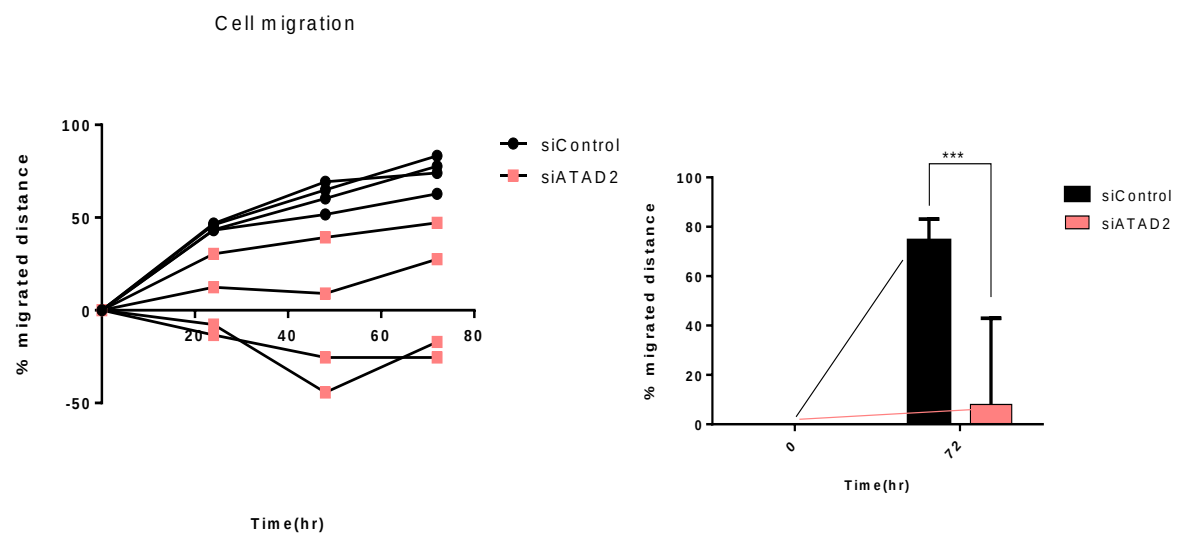
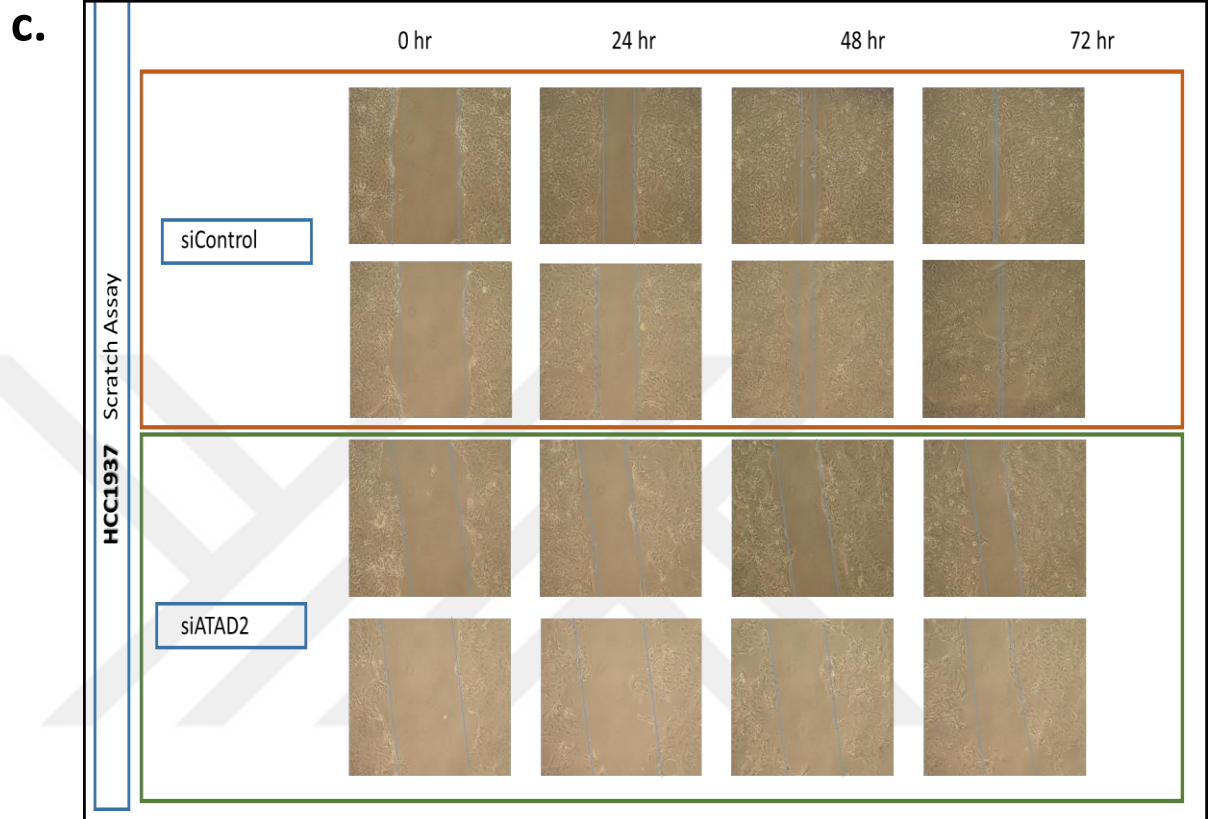
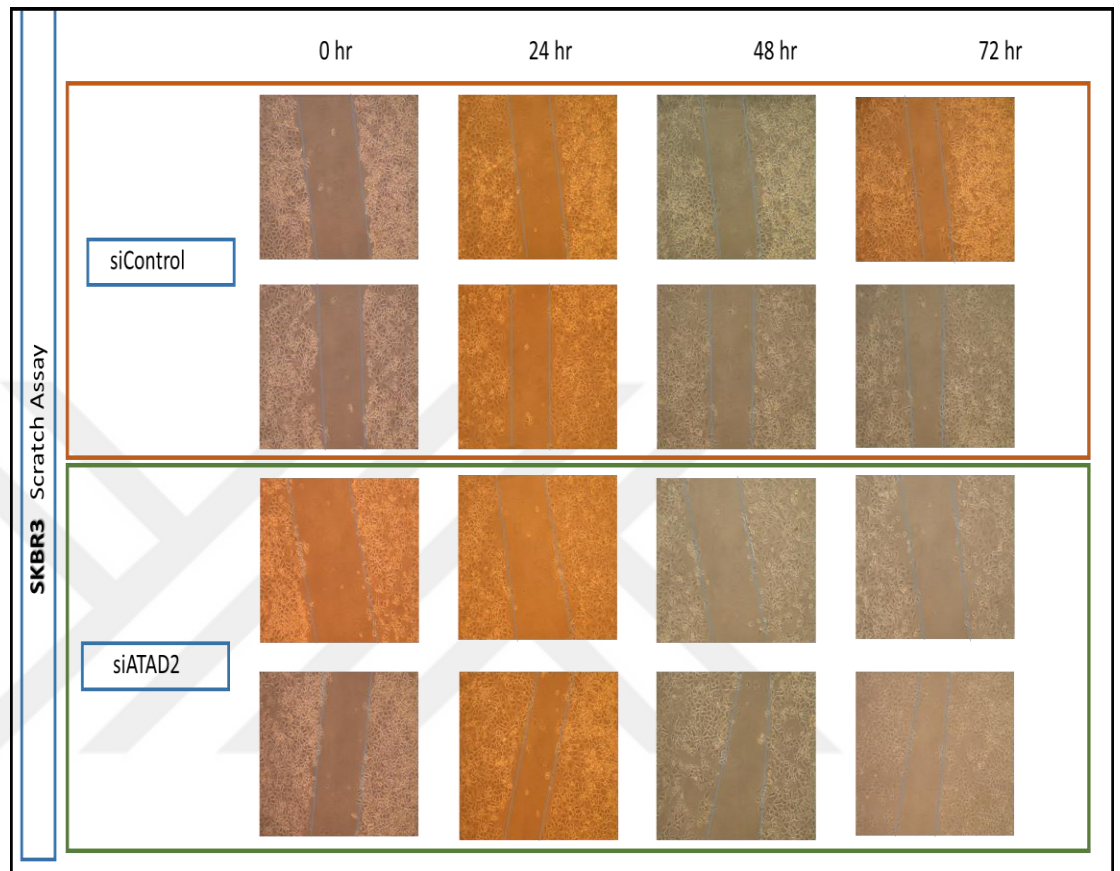


Figure 3.11 continued.

D.



Cell migration

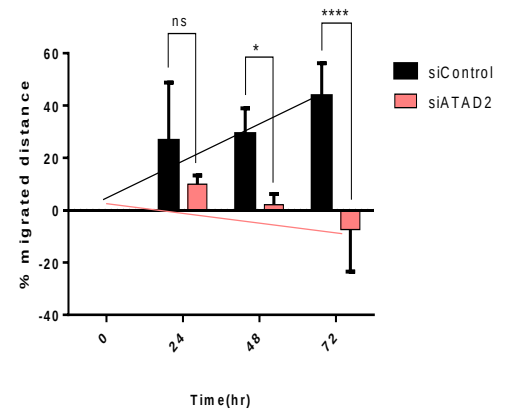
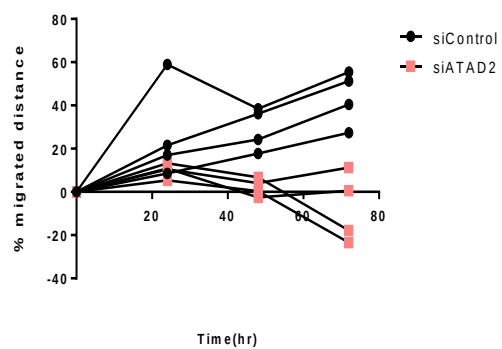


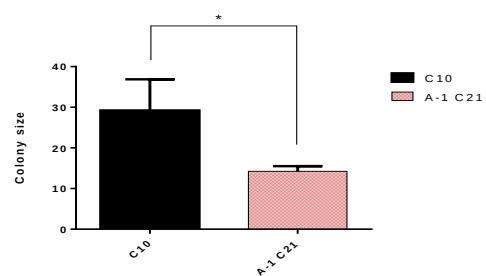
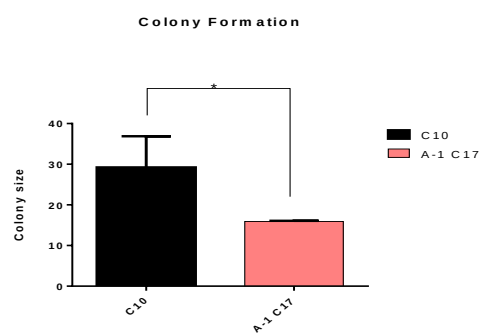
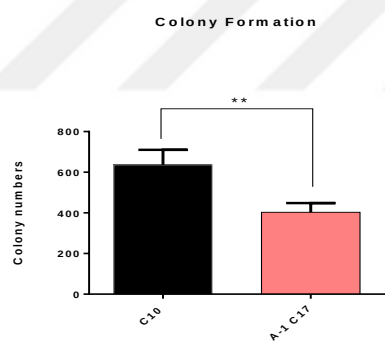
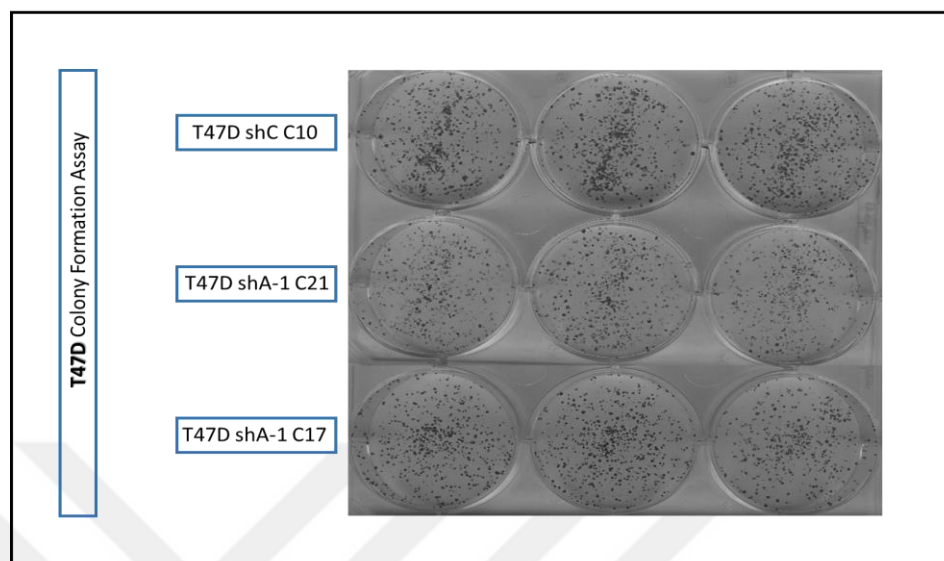
Figure 3.11 continued.

3.2.2.2 Analysis of colony formation in ATAD2 suppressed breast carcinoma cells

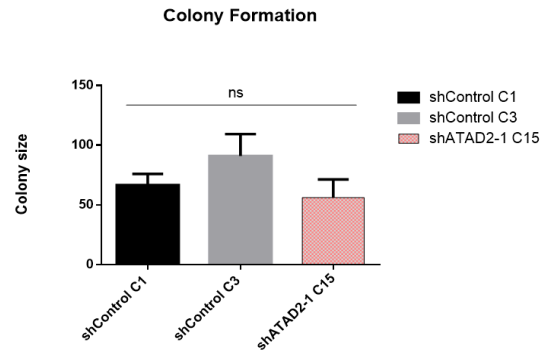
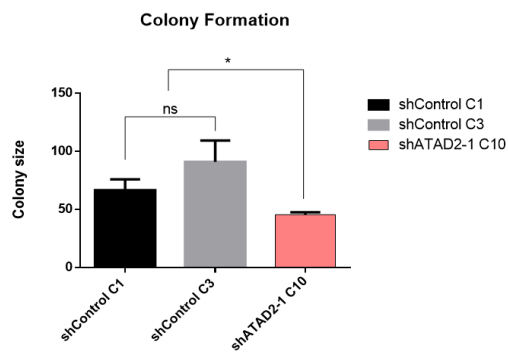
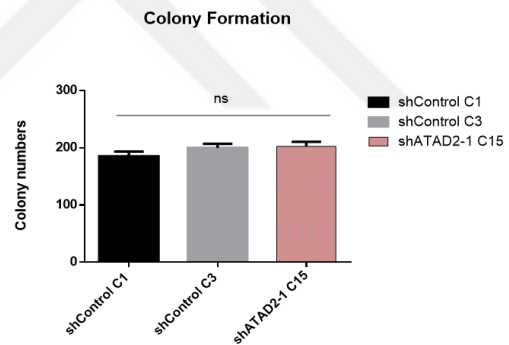
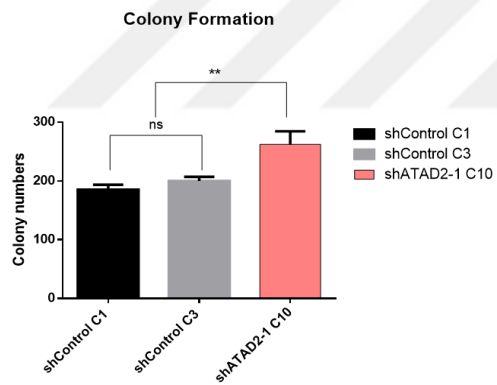
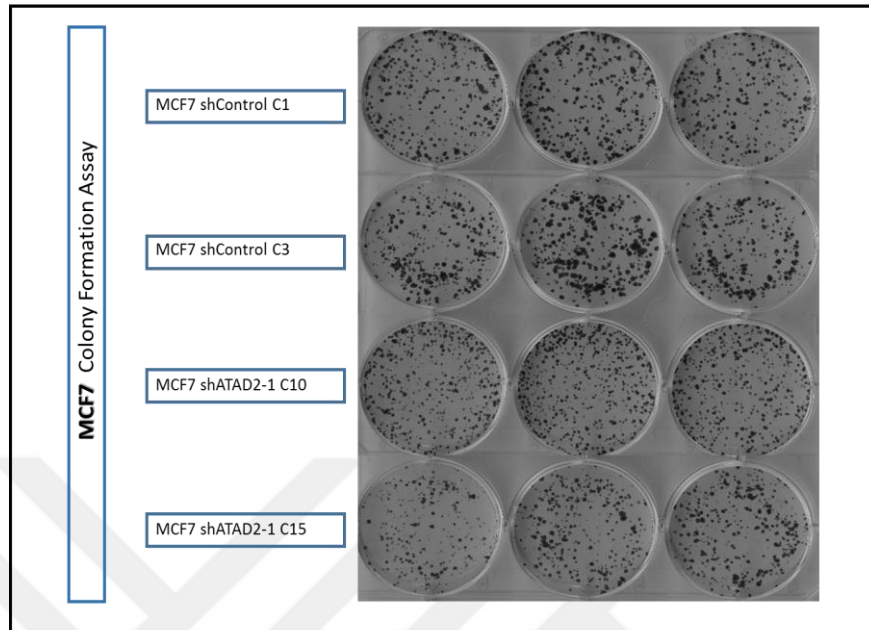
Clonogenic assay determines the ability of single cells to propagate into colonies; in other words, the “unlimited “ division potential of single cells. Uncontrolled division is one of the hallmarks of tumorigenesis. It is the most studied feature of cancer cells. Clonogenic assay is one of numerous methods to test this feature and probably the easiest²³⁷⁻²³⁹. Hence, this method was carried out for both ER(+) and ER(-) cell types. The effect of ATAD2 downregulation on both the colony forming capacity of breast cancer cells and on their established colony sizes was compared (Figure 3.12).

T47D shATAD2_1_C17 and MCF7 shATAD2_1_C10 clones showed significant reduction not only in colony numbers but also in colony sizes. However, while T47D shATAD2_1_C21 cells were not affected in colony numbers, there was a significant decrease in colony size. There was no difference in neither colony numbers nor colony sizes of MCF7 shATAD2_1_C15 cells. Colony size represents the division potency of cells such as cellular proliferation. Therefore, differences in ATAD2 downregulation levels between clones suggest that there may be a dose effect in the regulation of reproductive capacity of cells. Two ER(-) breast cancer cells did not show compatible results in clonogenic assay. ATAD2 silencing in HCC1937 cells resulted in a significant decrease in both colony numbers and colony sizes. However, SKBR3 cells did not show any significant change in colony number or in colony size upon treatment. Therefore, the results suggest that the effect of ATAD2 downregulation on cellular proliferation and colony formation potentials of cells could be dependent on other unknown factors apart from estrogen receptor status of breast carcinoma cell lines. Colony formation assay is reported to be an indication of the onset of replicative senescence²⁴⁰. Taking into account that the majority of breast carcinoma cells displayed difference in colony sizes in response to ATAD2 downregulation, suggesting its effect on the regulation of cellular proliferation, we next evaluated their replicative capacities.

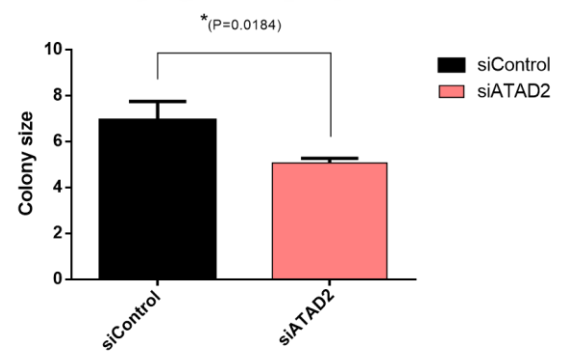
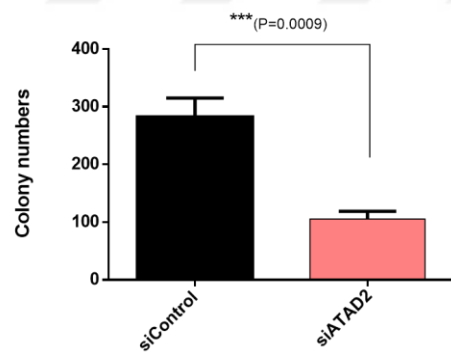
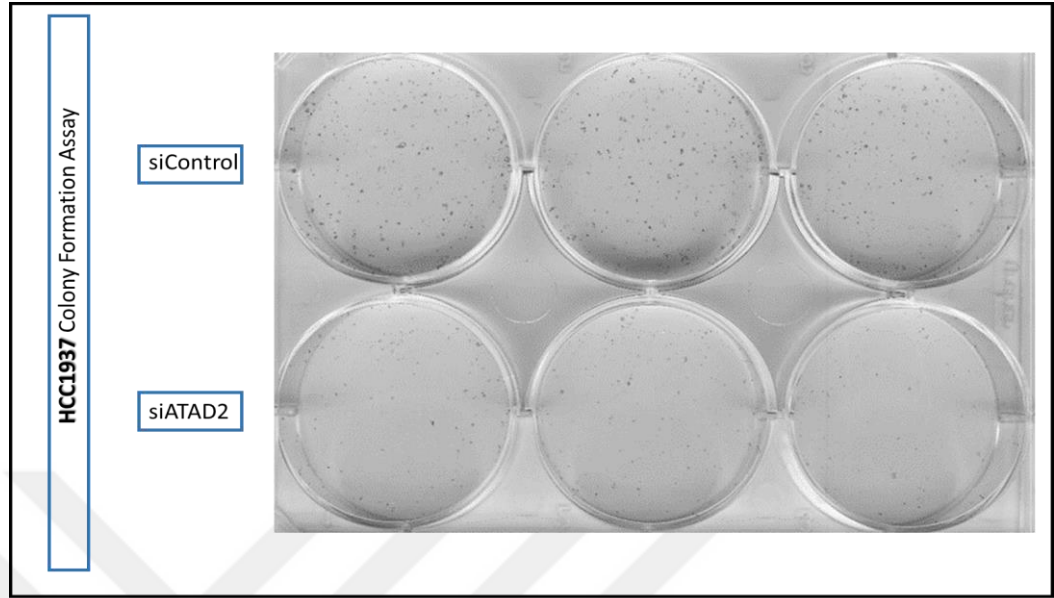
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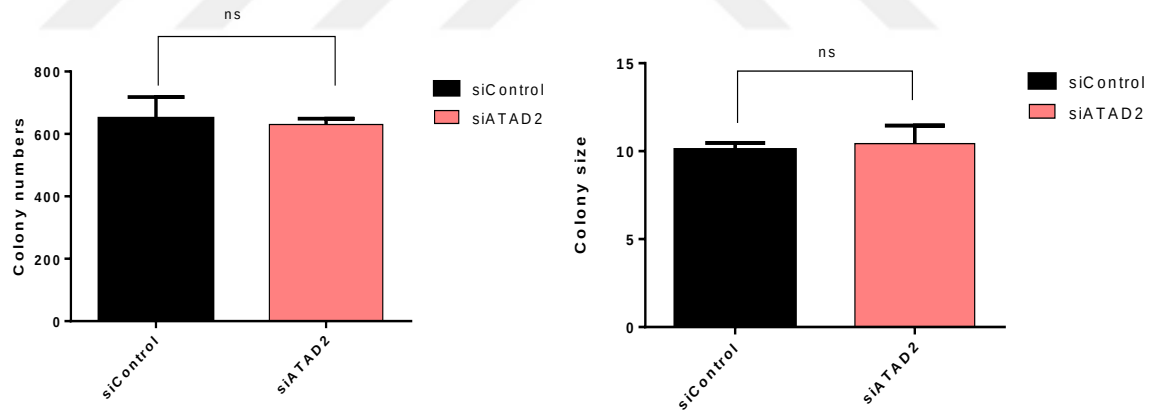
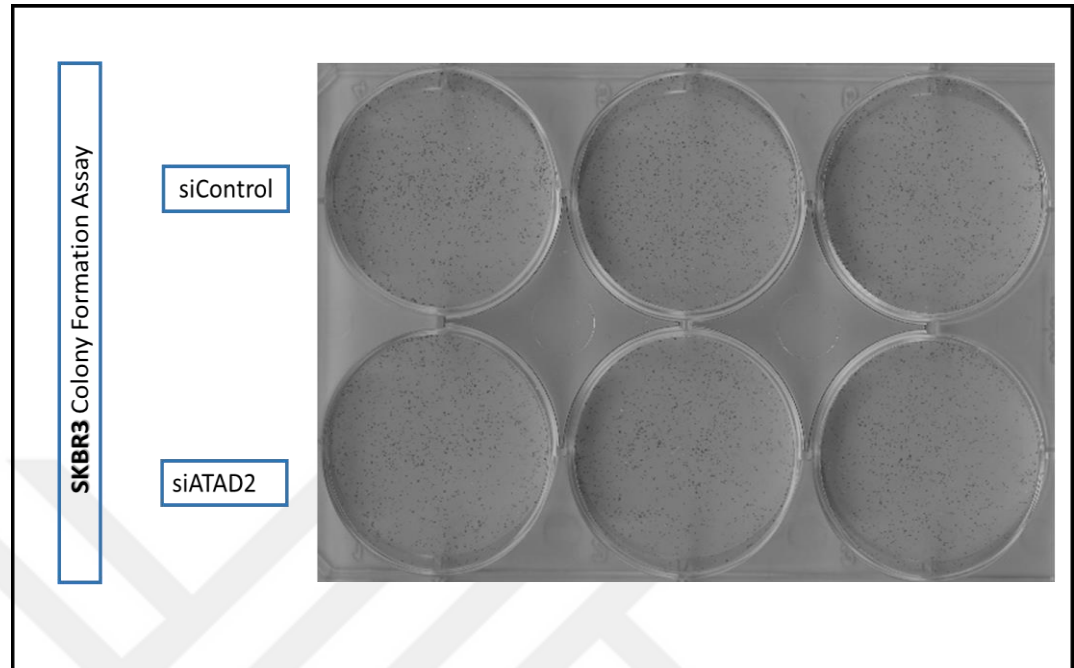


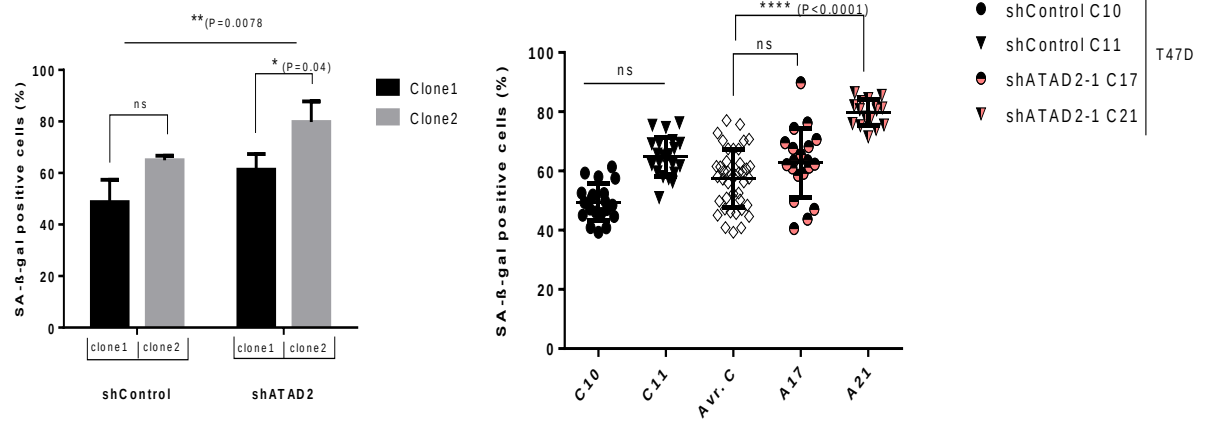
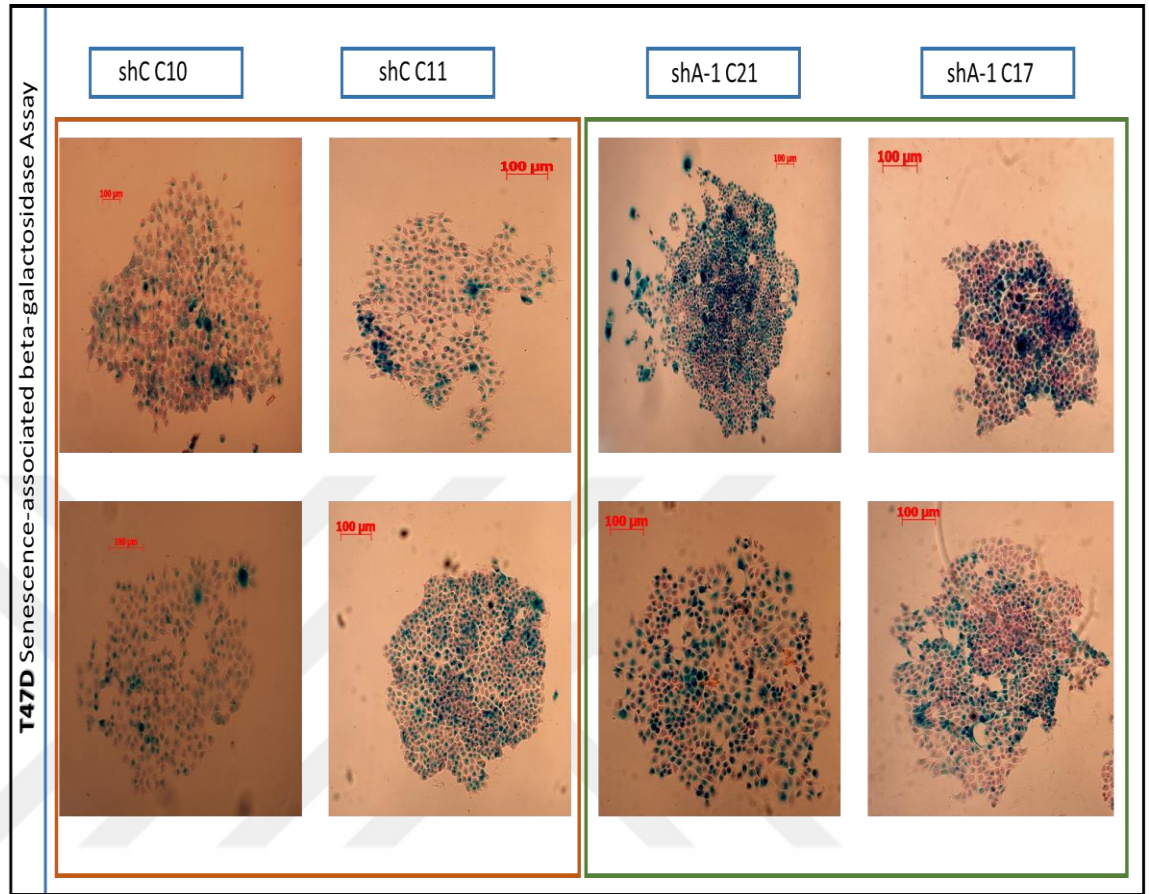
Figure 3. 12: The Effect of ATAD2 down-regulation on colony formation of breast carcinoma cells.

ATAD2 downregulation resulted in significant reduction in both colony numbers and colony size of shATAD2_1_C17 cell clones, yet it reduced only colony sizes of shATAD2_1_C21 cell clones. (B) While both MCF7 shATAD2_1_C10 and shATAD2_C15 clones displayed significant reduction in colony numbers, neither showed significant difference in colony sizes. (C-D) ATAD2 silencing in HCC1937 cells resulted in decreased numbers and sizes of colonies, while it affected neither colony numbers nor sizes in SKBR3 cells. The statistical significance of the results was calculated with the two-tailed t-test ($p < 0.05$) in comparison to their respective control cells.

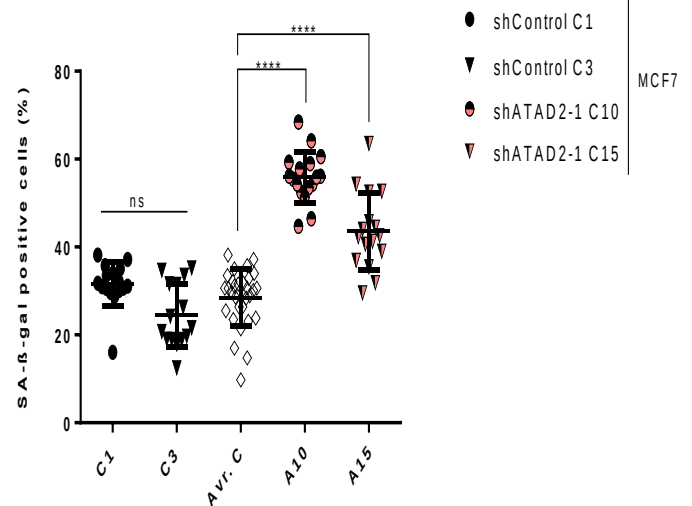
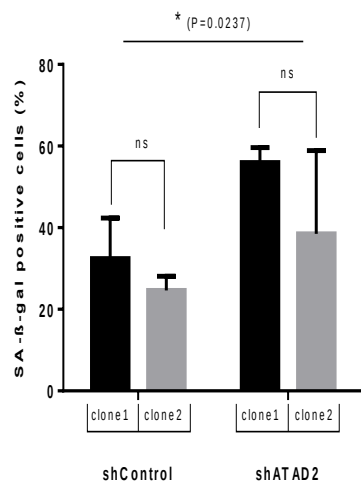
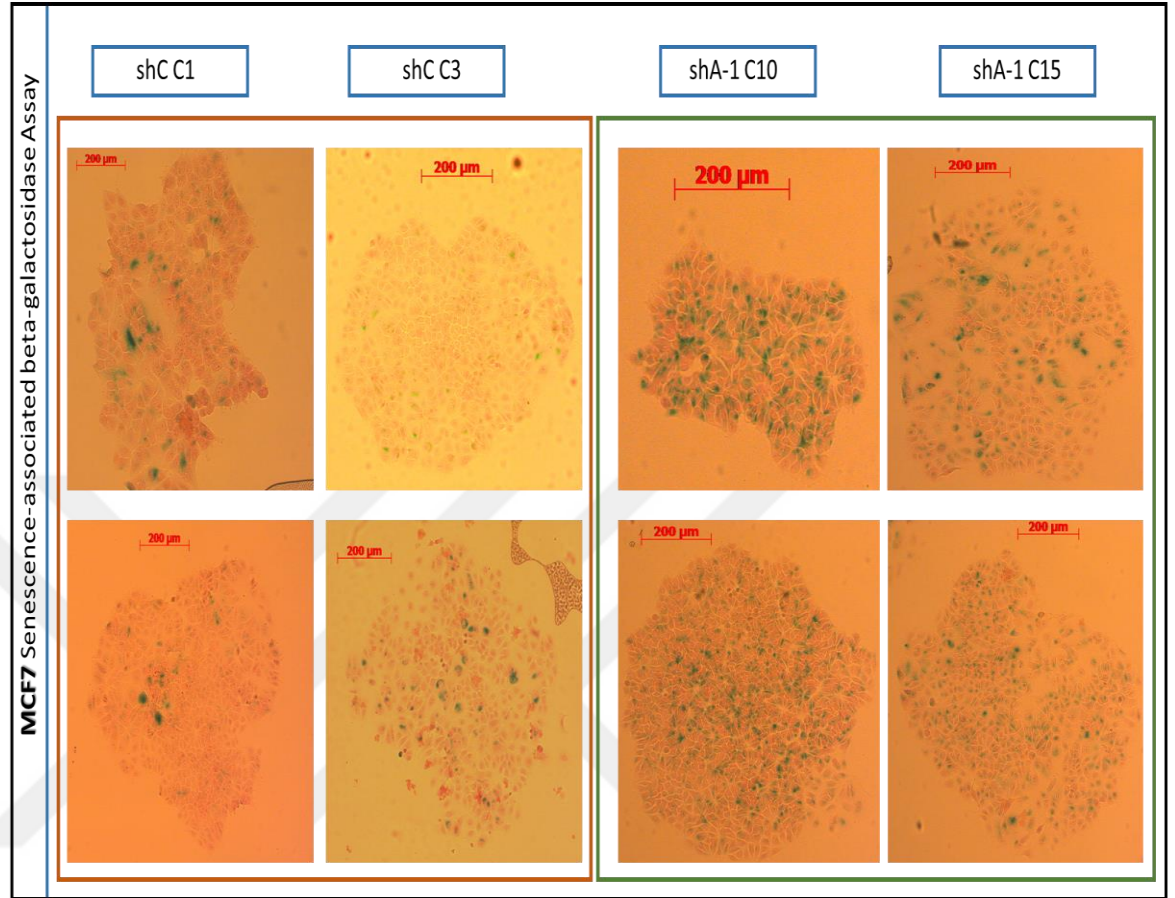
3.2.2.3 Analysis of senescence response in ATAD2 suppressed breast carcinoma cells

Limited replicative capacity is defined as one of the normal cell characteristics. When the control mechanisms of replication are disrupted, the cell loses its brakes and progresses through the cell cycle non-stop. Unlimited proliferation capacity is one of the main features of cancer⁵. Cancer cells acquire this ability when they are able to escape from the regulation barriers in the cell cycle. Senescence regulation, which is another tumor suppressive mechanism of normal cells, is one of these barriers for carcinogenesis. Senescence is an irreversible arrested state in which cells remain viable but cannot proliferate^{167,241,242}. There are a few main characteristic of senescent cells by which we may distinguish them from proliferating ones in a cell population as explained in detail in the introduction part. Two of them are important for the assay since we determine the senescent cells with them and they are enlarged cell size and expression of pH-dependent β -Gal activity^{185,186}. The latter is a biomarker for senescent cells. The senescence status of ER(+) and ER(-) cells were measured by using the activity of this biomarker. MCF7 and T47D shsATAD2 clones and siATAD2 treated SKBR3 and HCC1937 cells showed significant induction of cellular senescence upon treatment, compared to their respective controls (Figure 3.13). The clones with lower ATAD2 expression showed higher senescence response in ER(+) cells, suggesting a strong link between ATAD2 downregulation and senescence activation. Therefore, ATAD2 may function in the senescence regulation of breast carcinoma cells.

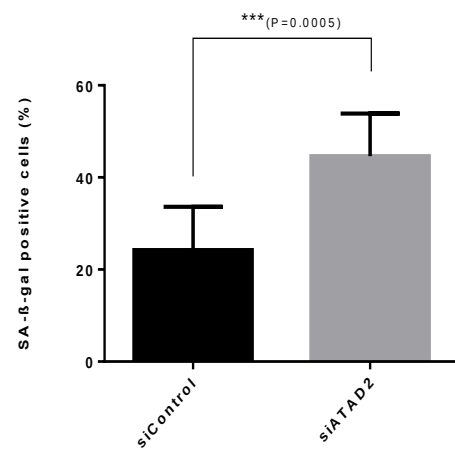
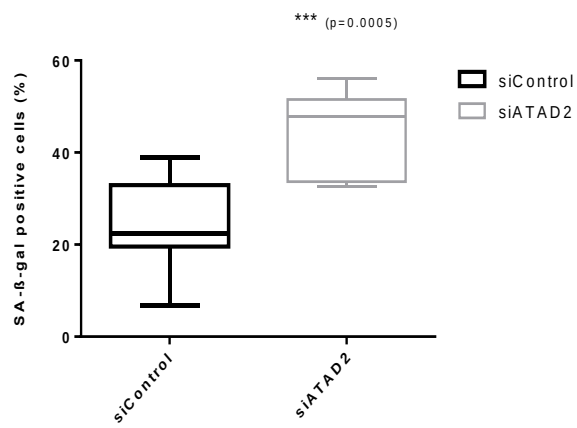
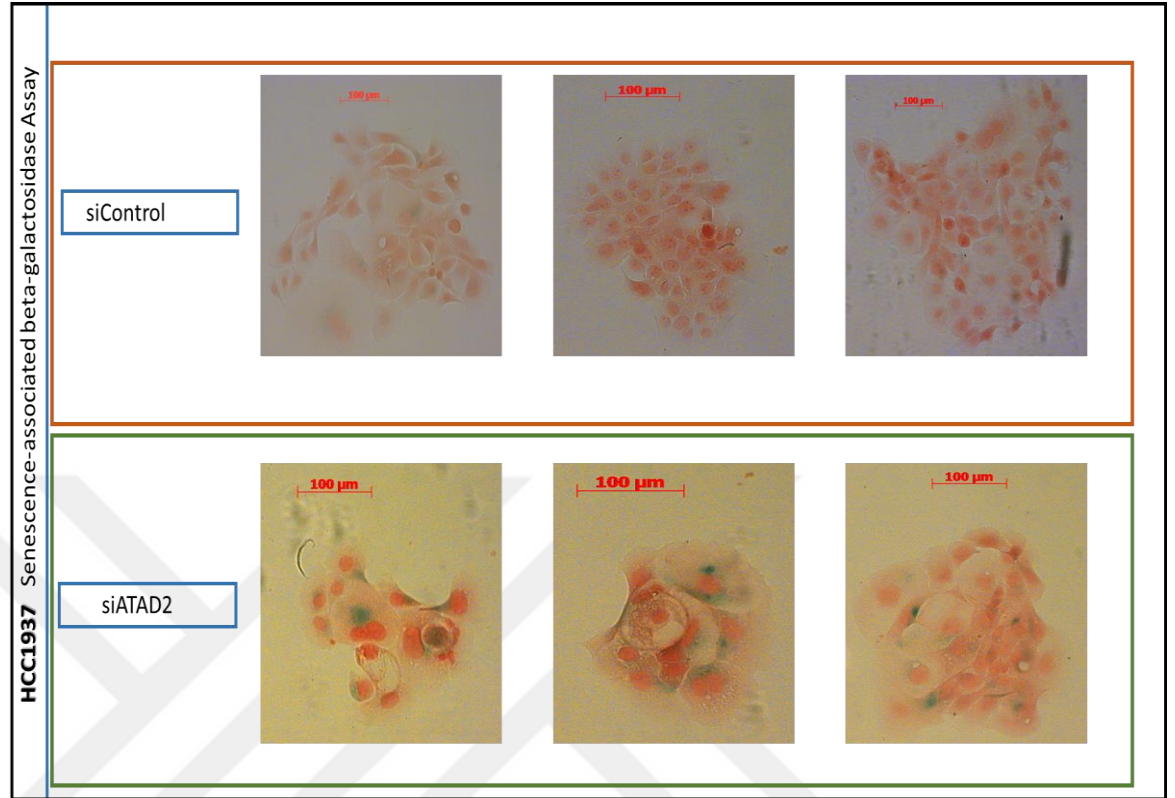
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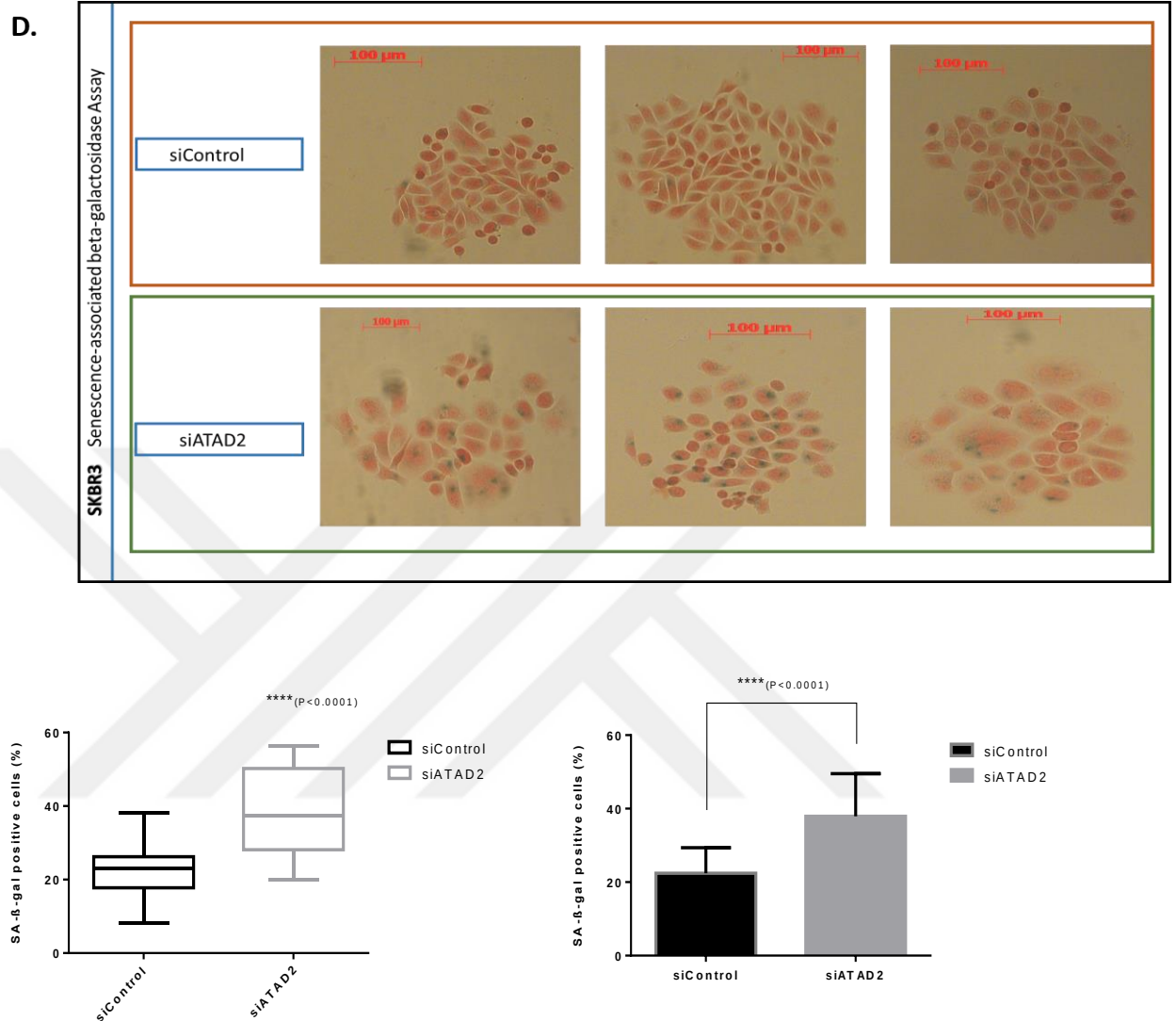


Figure 3. 13: The Effect of ATAD2 down-regulation on senescence response of breast carcinoma cells.

The figures represent senescence-associated β -Gal activity in breast carcinoma cells. The stained cells indicate a positive senescence response. The percent of stained cells (SA- β -Gal +) in each colony was calculated and the bars show average percent of total colonies for each clone. **(A-B)** Both T47D and MCF7 shATAD2 clones displayed significant increase in senescence response after ATAD2 downregulation. The response was negatively correlated with ATAD2 expression levels within the cells. There was no clone difference between either shATAD2 or shcontrols of MCF7 cells. However there was a significant difference between two T47D shATAD2_1_C17 and shATAD2_1_C21 clones. **(C-D)** Likewise, ATAD2 silencing induced senescence phenotype in HCC1937 and SKBR3 cells. There was a significant difference between controls and treated cells. The statistical comparison of all data was performed with the two-way ANOVA method (P<0.05).

3.2.2.4 Analysis of cell growth in ATAD2 suppressed breast carcinoma cells

Irreversible growth arrest is a prerequisite for a senescence program. To find out if cell cycle distributions of treated cells support senescence assay results, BrDU staining was carried out. BrDU is a thymidine analog and used to determine proliferation and cell growth status of cells. It is a flow cytometric analysis which enables detecting proliferating cells by incorporating BrDU into newly synthesized DNA and growth arrest by 7-AAD staining of total DNA²⁴³⁻²⁴⁶. With the combination of two dyes, we get a two-colored cytometric analysis which allows us to characterize cell cycle kinetics of cells. BrDU and 7-AAD staining were performed for both ER(+) and ER(-) cells. Figure 3.14 demonstrates the scatter plot of all readings. The results were collected in a table (Table 3.9). ATAD2 downregulated stable MCF7 and T47D clones did not show significant difference in their cell cycle distributions compared to their respective controls. However, the percent of proliferating cells decreased with ATAD2 deficiency in MCF7 cells. Treatment has increased G2/M arrested cells while decreasing proliferating cells and G1 phase cells in both SKBR3 and HCC1937 cells while HCC1937 cells displayed higher sensitivity to transfection treatment since the apoptotic cell population was observed up to 10% in both control and siATAD2 treated cells, though treated cells showed more increase in apoptotic cells amounts yet not significantly. Therefore, even though ATAD2 downregulation affected cellular growth of breast carcinoma cells, the differences were not statistically significant. Only adherent cells were analyzed by BrDU staining protocol. However, floating, detached cells in the medium may indicate the presence of apoptotic cells. To detect apoptotic cells, ATAD2 downregulated cells were analyzed with FACS as well. Cells were stained with PI staining following 3 days of treatment. While ATAD2 deficiency did not lead any apoptosis induction in MCF7, T47D and SKBR3 cells, it induced cell death in HCC1937 cells. FACS analysis results supported the notion that loss of ATAD2 activity may hinder normal cellular functions of cancer cells and inhibit proliferation in HCC1937 cells ($p < 0.05$, Figure 3.15).

	HCC1937		SKBR3			T47D				MCF7		
	siControl	siATAD2	siControl	siATAD2	C10	C11	A17	A21	C1	C3	A10	A15
Apoptotic	13.5	18.0										
G1	35.6	28.2	25.1	21.6	71.1	73.8	72.3	72.2	57.2	55.3	58.3	58.3
S	22.1	22.8	20.7	13.2	19.6	12.3	19.0	15.7	31.6	31.7	29.2	28.1
G2/M	13.8	15.4	41.6	51.5	4.9	8.8	4.5	6.6	3.8	6.5	7.1	6.0

Table 3. 9: The representative of average percent of cell populations in cell cycle phases determined with BrDU assay. The experiments were carried out in triplicate.

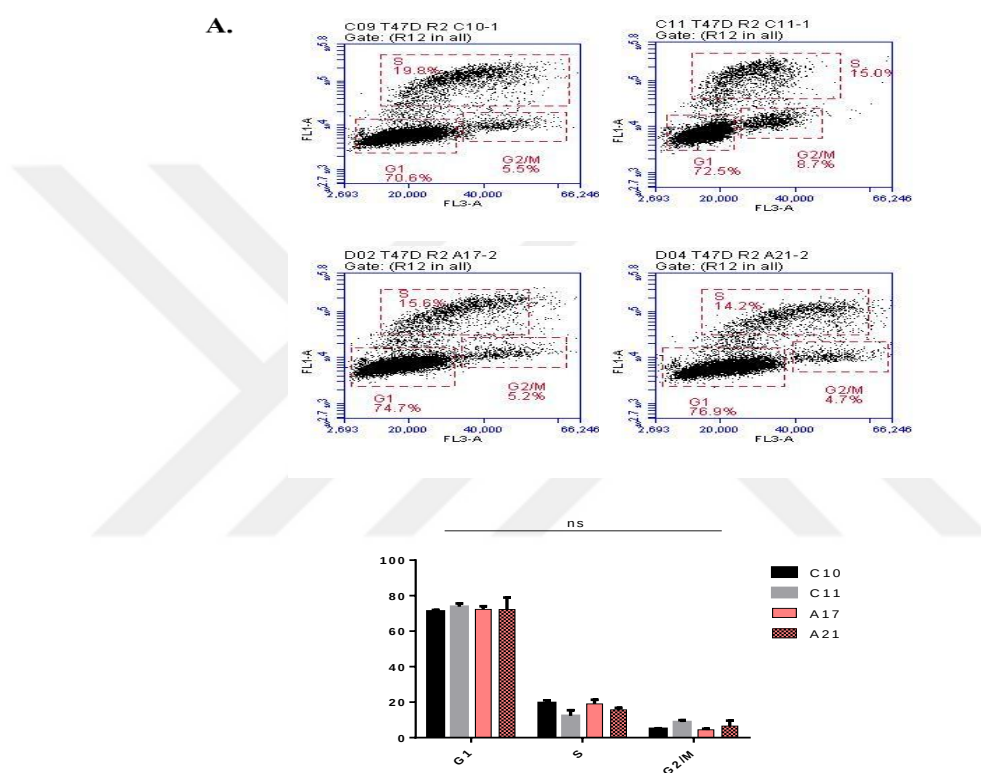


Figure 3. 14: Quantitative Cell cycle analysis of 7-AAD and BrDU stained ATAD2 downregulated breast carcinoma cells.

The percentage of cells in each phase of cell cycle was determined by flow cytometry. Actively synthesized DNA was labelled with BrDU and total DNA content was stained with 7-AAD. 2×10^5 cells/well of MCF7 and T47D shclones and siATAD2/siControl treated HCC1937 and SKBR3 cells were seeded on 6-well plates in triplicate. Cells were incubated for 3 days under normal growth conditions. Following harvesting, cells were stained using the BrDU staining protocol (BD Pharmingen BrDU flow kit, 559619). Table 3.9 represents the average of cell percent at G1, S and G2/M for the cell lines. **(A-C)** Differences between treated and control cells was non-significant across all cell types according to the t-test. There was no treatment effect. ATAD2 downregulation resulted in neither a decrease in the S phase nor an increase in the G1 or/and G2/M phases in breast carcinoma cells. The statistical validation was carried out with two-way ANOVA for stable clones and unpaired t-test for ATAD2 silenced HCC1937 and SKBR3 cells.

B.

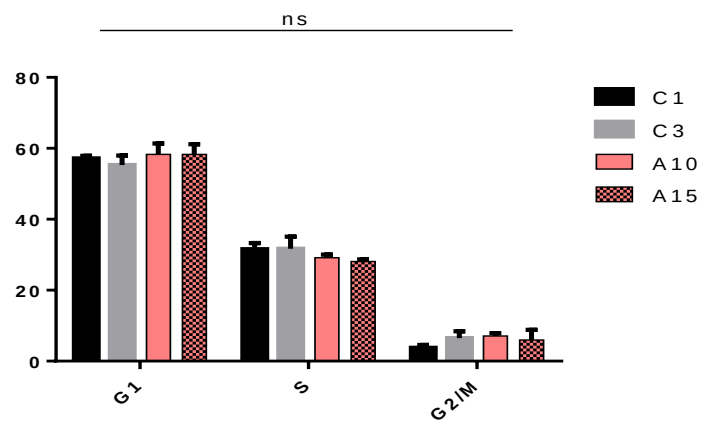
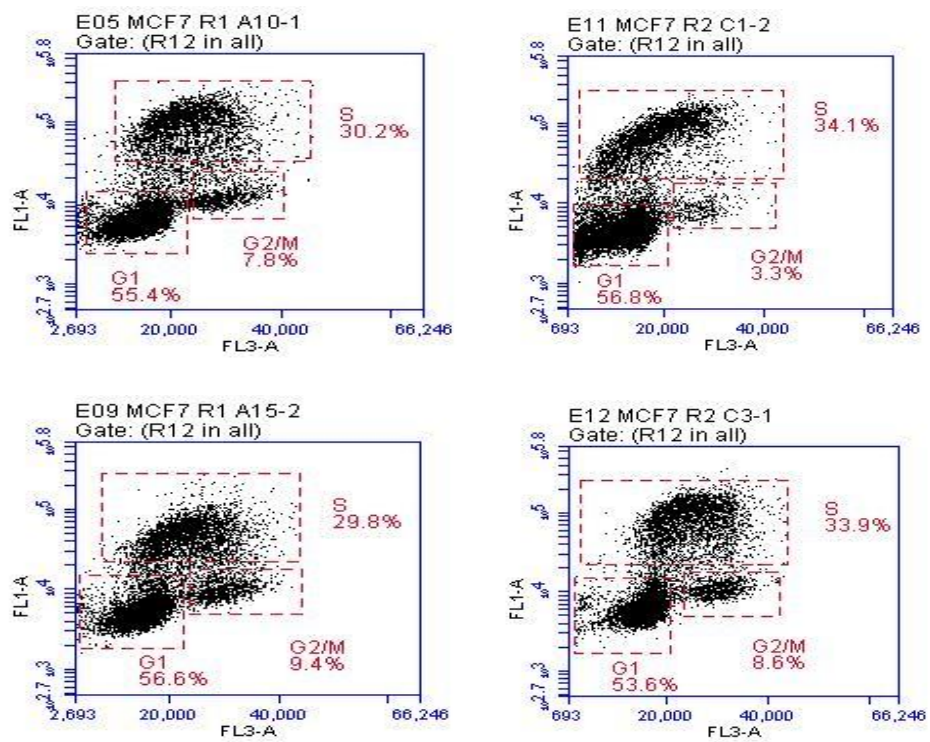
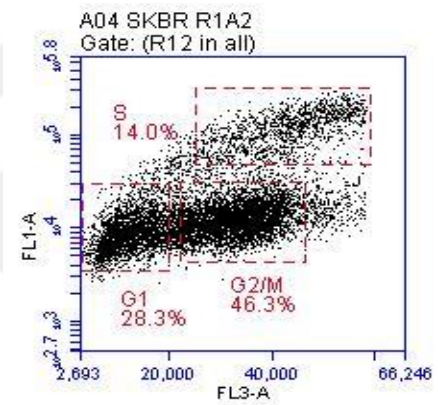
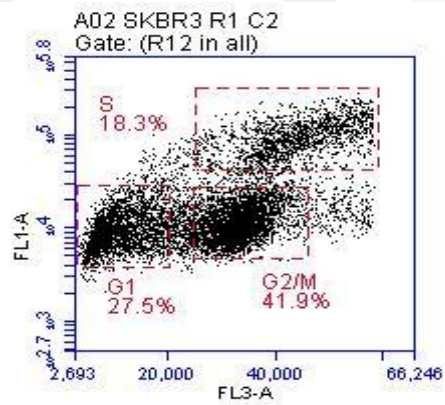
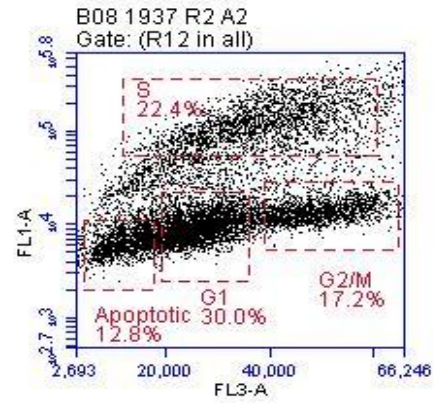
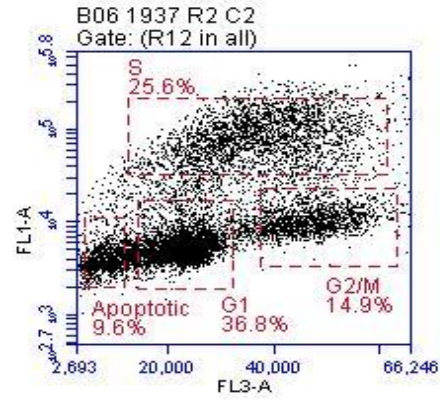
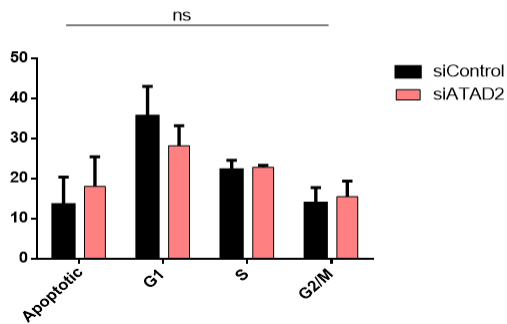


Figure 3.14 continued.

C.



HCC1937



SKBR3

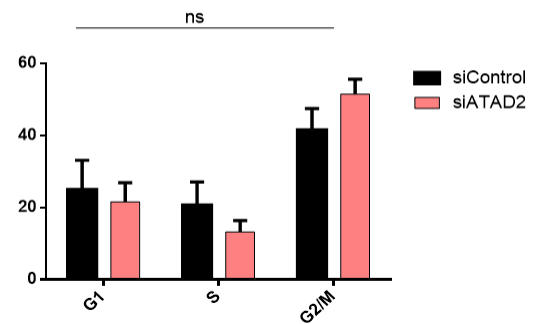


Figure 3.14 continued.

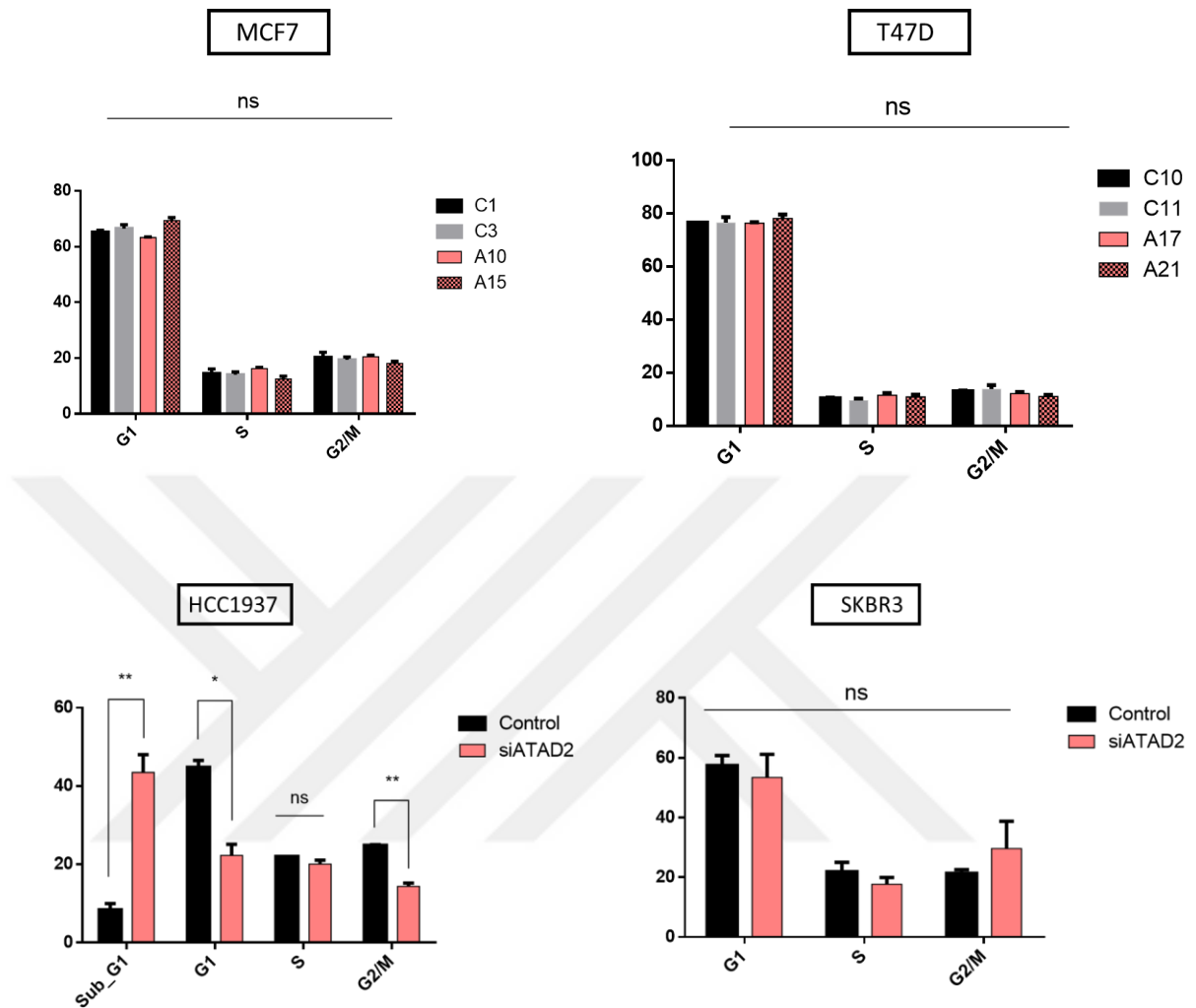


Figure 3. 15: Quantitative Cell cycle analysis using flow cytometry in ATAD2 downregulated breast carcinoma cells.

Flow cytometry analysis was performed with PI staining in ATAD2 downregulated stable MCF7 and T47D clones and siATAD2/siControl treated HCC1937 and SKBR3 cells. The percentage of cells in the G1, S and G2/M phases was determined. The experiments were conducted in triplicate. While ATAD2 silencing induced an increase in the sub-G0/G1 (Apoptotic) cell population in HCC1937 cells, it did not result in a significant difference for stable clones and SKBR3 cells. The statistical validation was carried out with two-way ANOVA for stable clones and unpaired t-test for ATAD2 silenced HCC1937 and SKBR3 cells.

3.3. VALIDATION OF UNDERLYING MOLECULAR FUNCTION OF ATAD2 GENE IN BREAST CANCER

The results of our functional studies suggest that ATAD2 expression is required for the maintenance of major cellular functions in ER(+)/ER(-) breast carcinoma cells. The lack of its expression has hindered malignant cell motility and induced cell type specific senescence state, and/or cell death in these cells. We then questioned how ATAD2 executes these responses. We ground the rest of the research on this question and proceed with elucidation of underlying molecular mechanisms leading to diverse cell fates upon ATAD2 downregulation in breast carcinomas.

3.3.1. ASSESSMENT OF ATAD2 FUNCTIONS IN BREAST CARCINOMA

In the literature ATAD2 has been defined in the context of estrogen signaling and its function is connected with E2 action because it acts primarily as ER α co-regulator in ER+ breast cancer cells. Reports have revealed its activator effect on selective control of ER α target genes¹¹⁵. The relevance of its expression with cell cycle progression has been detected previously and its involvement in cellular senescence response in breast cancer was revealed in this study. However, the underlying cause of its actions is still quite obscure. At this part of the research, we aimed to uncover the mechanism of action of ATAD2 gene as an estrogen receptor co-regulator in MCF7 cells.

ATAD2 and ER α genes were co-targeted in MCF7 cells and their protein expressions were downregulated more than 80% (Figure 3.17). pSR-ER α knockdown constructs were used to suppress estrogen receptor expression in these cells. Initially, the construct to be used for co-transfection studies was determined and its effectiveness at 2 ug concentration was shown with western blot analysis (Figure 3.16). The most effective concentration of siATAD2 was determined as a final concentration of 25nM for MCF7 cells during previous studies (project no 111T434). The transfection conditions have been explained in detail in the materials and methods section. 2×10^5 cells/well of MCF7 cells were seeded in 6-well

plates in triplicate. Three different transfection conditions were applied in parallel under normal growth conditions; siATAD2 (25 nM) transfection for 72 hours, shER α -499 (2 ug) transfection for 72 hours, and siATAD2 (25 nM) transfection for 48 hours followed by shER α (2 ug) transfection for 24 hours. After 3 days, the cells were collected. While 2/3 of the cells were used for protein analysis, 1/3 was used for RNA quantification. The mRNA and protein expressions of the ATAD2 and ER α genes upon transfection are demonstrated in figure 3.17. While siATAD2 treatment downregulated ATAD2 protein expression to undetectable levels, shER α treatment of cells resulted in more than 90% reduction of ER α protein expression. Accordingly, ATAD2 silenced cells showed greater than 90% reduction in ER α expression as well at both mRNA and protein levels, suggesting that ATAD2 regulates the transcriptional expression of ER α gene. However, ER α downregulation led to only 30% decrease in ATAD2 protein expression despite more than 80% reduction in its mRNA levels. Their co-targeting resulted in 95% and 85% downregulation of ATAD2 and ER α protein levels respectively. Their reported mutual function in carcinogenesis hints their co-expressions in breast carcinoma. Their altered expressions after transfections confirm these suggestions. To observe the effects of differential ER α expression on ATAD2 expression, MCF7 cells were transfected with different concentrations of shER α plasmid and the ATAD2 expression levels were analyzed (Figure 3.18). mRNA levels usually are not correlated with protein expression and do not indicate biological activity of the gene of interest, possibly due to complex post-transcriptional modifications involved in the translational process. Therefore, protein expression levels were more reliable for our study. We analyzed protein expression of ATAD2 with western blot. Receptor expression showed a gradual decrease with increasing concentrations of shER α -499 plasmids. The knockdown efficiency was about 95% repression with 3 ug of plasmid. ATAD2 protein expression was reduced by 40% in cells transfected with 1 ug of plasmid, yet its levels increased up to 70% in 2 ug-plasmid transfected cells and retained its high level in 3-ug transected cells as well. It looks as though ATAD2 expression was upregulated to compensate the loss receptor activity when receptor expression decreased to a certain threshold.

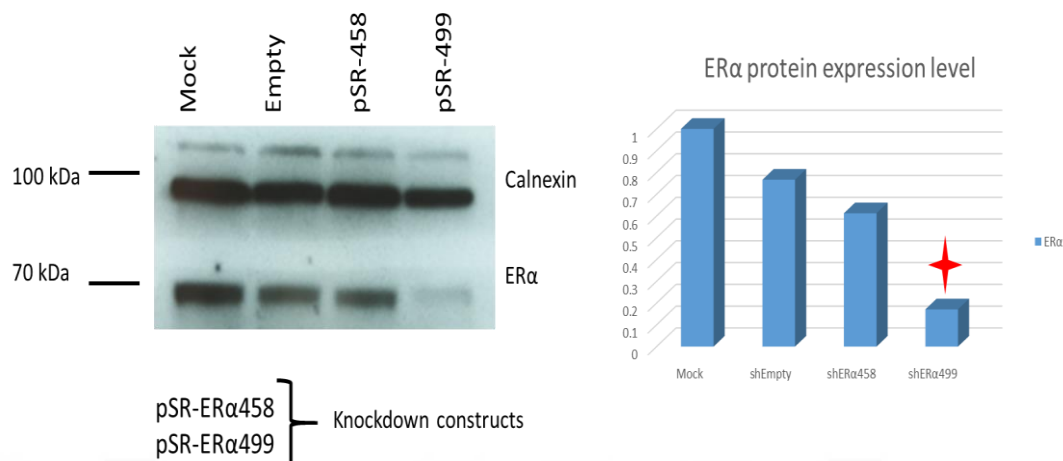


Figure 3. 16: Determination of an effective pSR-ERα knockdown construct for transient-transfection experiments in MCF7 cells.

MCF7 cells cultured as 2×10^5 cells/well were transfected with 2 ug of pSR-ERα knockdown constructs for 72 hours. Following protein extraction from transfected and control cells, western blot analysis was carried out with ERα (66 kDa) (santa cruz, sc-8002) and calnexin (90 kDa) (Sigma, C4731) antibodies. The bars show relative expressions of ERα protein over calnexin normalization. We detected transfection with the pSR-ER-499 construct resulted in better ERα downregulation. While pSR-458 transfection suppressed 40% of the protein expression in MCF7 cells, cells transfected with pSR-499 displayed only 17% of the ERα protein expression compared to control cells (highlighted with a red star).

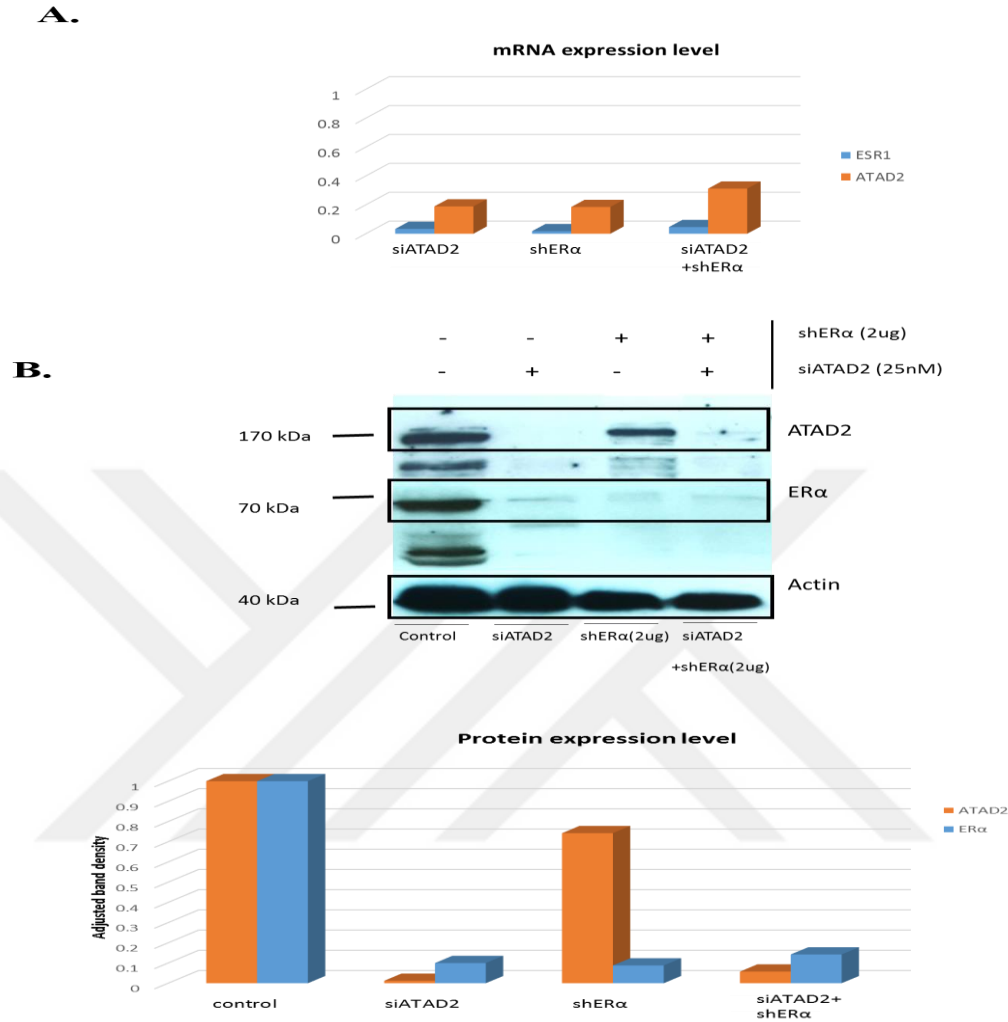


Figure 3. 17: Co-suppression of ATAD2 and ERα expression in MCF7 cells.

2×10^5 cells/well of MCF7 cells were seeded in 6-well plates in triplicates. Three different transfection conditions were applied to cultured cells. They were as follows: siATAD2 (25 nM) or siControl (25 nM) transfection alone for 72 hours, shERα-499 (2 ug) or shEmpty (2 ug) transfection alone for 72 hours, siATAD2 (25 nM) transfection for 48 hours followed by shERα (2 ug) transfection for 24 hours under normal growth conditions. Cells were collected and 2/3 were used for protein isolation, and 1/3 for RNA quantification. **(A)** RT-qPCR of *ATAD2* and *ESR1* in treated MCF7 cells. Transcripts were normalized to internal control, *GAPDH*. The experiment was run in duplicate reactions. **(B)** After protein extraction from treated cells and control cells, ATAD2 and ERα expressions were assessed by western blot analysis. ERα (66 kDa) (santa cruz, sc-8002), ATAD2 antibody (160 kDa) (produced by Dr. Mehmet Oztürk's lab) and β-Actin antibody (45 kDa) were used to detect proteins. Relative expressions of ERα and ATAD2 proteins over actin normalization were calculated (bar-graph). While ATAD2 silenced cells showed greater than 90% reduction of ERα protein levels, ERα downregulation led to a 30% decrease in ATAD2 protein expression. Their co-targeting resulted in 95% and 85% downregulation of ATAD2 and ERα protein levels respectively.

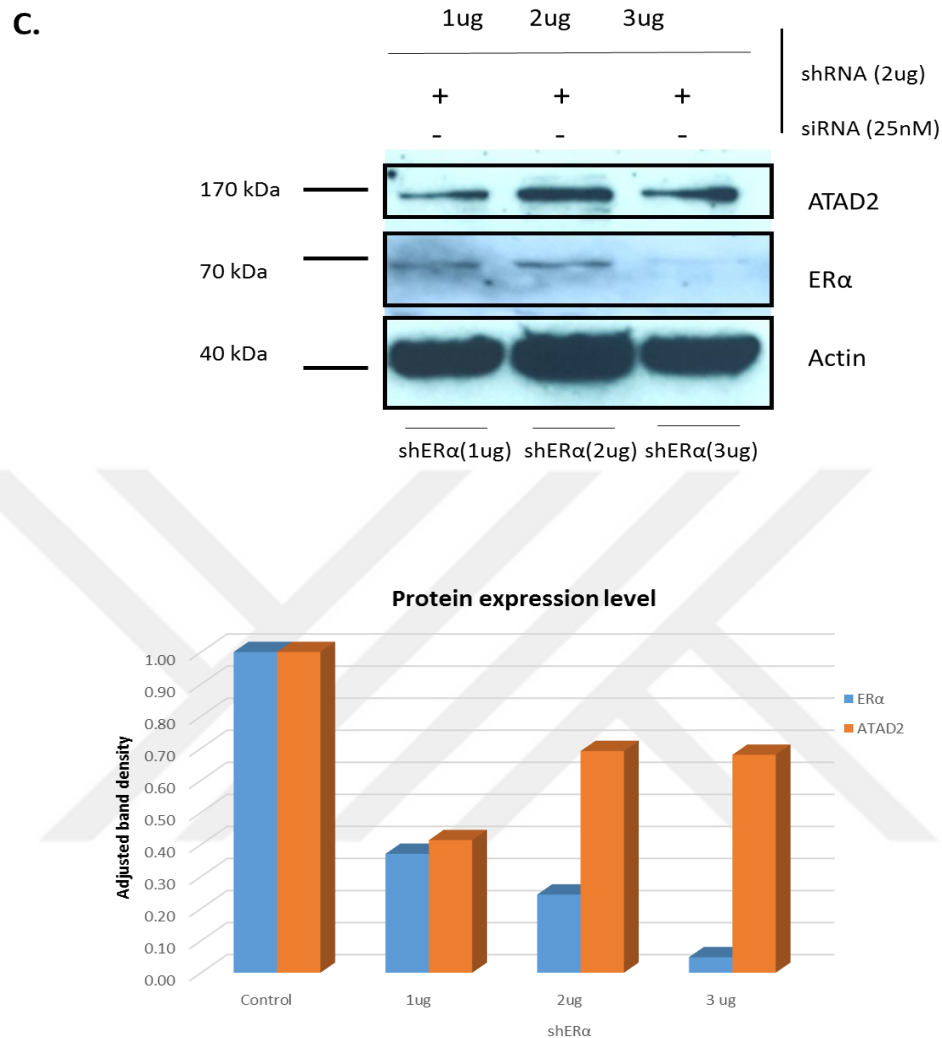


Figure 3. 18: Downregulation of ERα in MCF7 cells.

2×10^5 cells/well of MCF7 cells were seeded in 6-well plates in triplicate. The cultured cells were transfected with 1ug, 2ug or 3ug of either shERα-499 or shEmpty plasmids. Cells were incubated for 3 days under normal growth conditions. Following protein extraction from transfected cells, western blot experiment was carried out with ATAD2 antibody (160 kDa) (produced by Dr. Mehmet Oztürk's lab), ERα antibody (66 kDa) (Santa Cruz, sc-8002) and β-Actin antibody (45 kDa). Relative expressions of ERα and ATAD2 proteins over actin normalization were calculated (bar-graph). ERα protein levels were reduced with increasing concentrations of shERα-499 plasmids. While transfection with 1 ug of plasmid led to a greater than 60 % reduction of ERα protein levels, the knockdown efficiency was about 95% repression with 3 ug of plasmid. ATAD2 protein expression was reduced by 40% in 1 ug-plasmid transfected cells, yet its levels showed increase up to 70% in 2 ug plasmid transfected cells and stayed at that level in 3 ug transfected cells also. No positive correlation was detected between ATAD2 and ERα protein expressions.

Estrogen receptor negative breast cancer cells are not responsive to E2 induction and the expression of ATAD2 is known to be regulated by estrogen positively. Then, how does ATAD2 function in these cells and exert its effects? We think there may be a molecular basis of E2-independent actions of ATAD2 in ER- breast cells. To assess this assumption, ATAD2 downregulated stable clones of HCC1937 cells were generated. There was just 1 clone showing successful downregulation of ATAD2, so molecular characterization of ATAD2 studies in ER(-) breast carcinoma cells was carried out with 1 shATAD2 clone and 2 selected shcontrol clones (shCntrl_C2 and shCntrl_C5). Figure 3.19 show that ATAD2 expression was downregulated more than 80 and 75% at mRNA and protein levels respectively in the HCC1937 shATAD2_1 clone.

3.3.1.1. Validation of the selected genes by RT-qPCR in treated cells

Relative expressions of a group of selected genes in ATAD2&ER α co-suppressed MCF7 cells were determined with RT-qPCR. They were selected according to their functions. The microarray analysis revealed the potential functions of ATAD2 in major cellular signaling pathways. One of them was EGFR signaling. EGFR is suggested to be over-activated in the majority of breast carcinoma and in cross-talk with estrogen signaling. There are accumulating reports showing its cooperation with ER α during tumorigenesis^{247,248}. Therefore, its expression and some of its target genes were analyzed; these were EGR1, ADAM23, ADAMTS1. MYC genes are oncogenes reported to co-function with ATAD2 in the transcriptional regulation of cell survival genes. CCND1 and CCNE1 genes are cyclin genes controlling cell cycle progression while CDKN1 (p21), RB and TP53 genes are involved in senescence response. AKT2, CASP9, CDH1 and BRCA1 are cell proliferation related genes. Figure 3.20 represents expression levels of genes at 3 transfection conditions, which were siATAD2 treated, shER α treated or siATAD2+shER α co-treated. There is consistency in the expression of genes between 3 conditions. The most downregulation was observed in *EGFR* and *EGR1*, an EGF responsive gene, in addition to *ATAD2* and *ESR1* genes. Increase in the expression of *Myc* oncogenes and in senescence markers *p21* and *p53* and in other EGFR target genes *ADAM23* and *ADAMTS1* was observed.

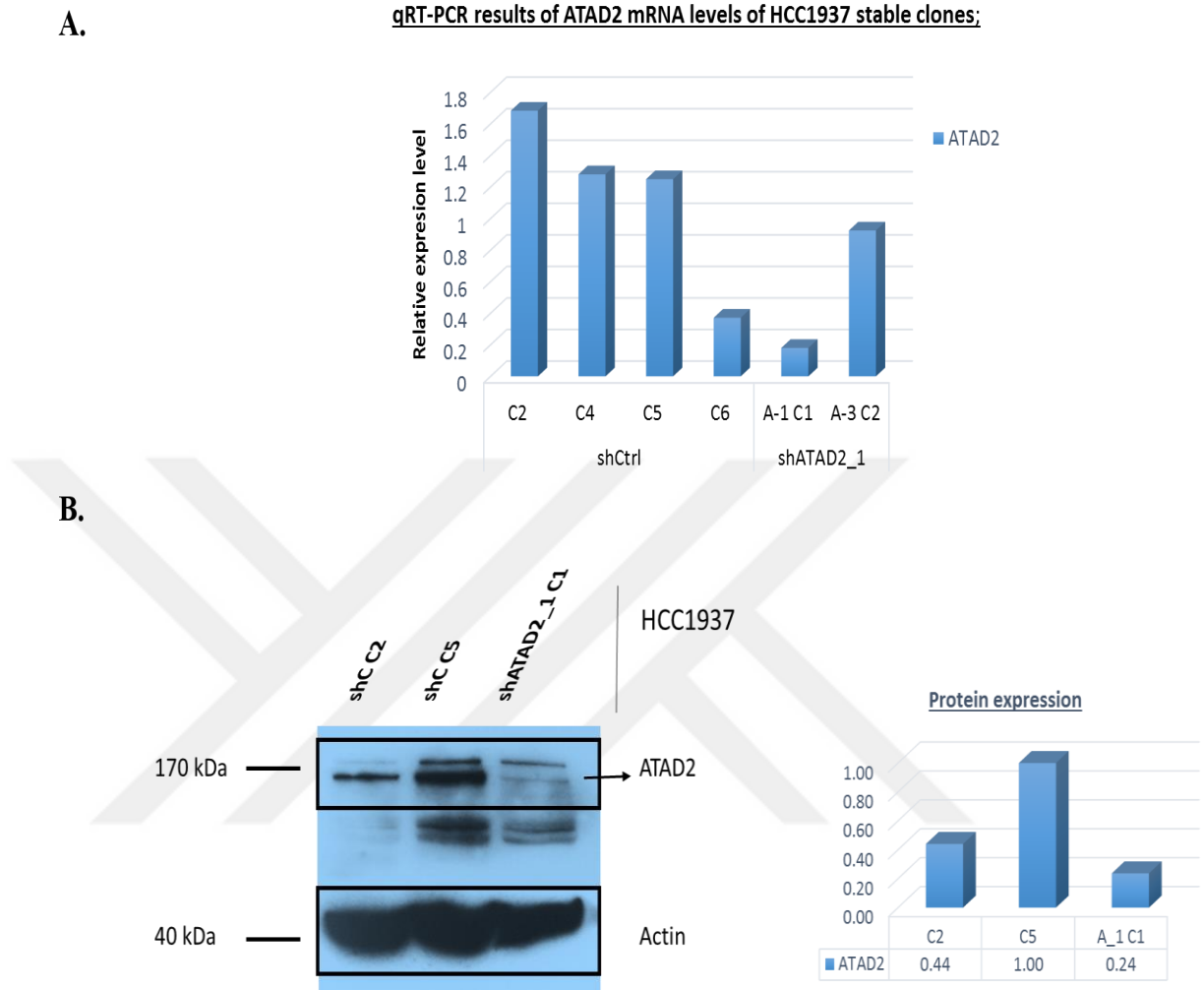


Figure 3. 19: Validation of ATAD2 expression in stably-transfected HCC1937 cells.

(A) ATAD2 gene expression was screened across stable HCC1937 clones. RT-qPCR of *ATAD2* was performed and transcripts were normalized to the housekeeping gene, *GAPDH*. The experiment was run in duplicate reactions. Two sh-controls and one sh-ATAD2 clone with the least gene expression level were selected for western blot analysis. They were highlighted with red stars. **(B)** ATAD2 protein expression was determined in the selected clones, which were shCntrl_C2, shCntrl_C5, shATAD2_1_C1 with western blot analysis. ATAD2 primary antibody used during western blot was produced by Dr. Mehmet Oztürk's lab. Its expression levels were normalized to input β -Actin (45 kDa). The relative ATAD2 (170 kDa) expressions in the clones were illustrated in the graph. **(B, left)**. Stable transfection of these cells with shATAD2_1 plasmid (SureSilencing, NM_014109) resulted in greater than 80% reduction in the ATAD2 protein level compared to shCntrl_C5. Control clones were generated with empty vectors. However, the shCntrl_C5 clone showed higher ATAD2 protein level compared to the shCntrl_C2 clone.

On the contrary, reduction in the expression of two important genes involved in cell growth, namely *CCND1* and *pRB*, was observed.

Relative mRNA expression levels of the same genes was analyzed in the ATAD2 downregulated stable HCC1937 clone (Figure 3.21). These cells also confirmed EGFR downregulation upon ATAD2 silencing. EGFR, EGR1, Myc oncogenes, ADAM23 and ADAMTS1, CCND1 and pRB genes all showed decreased expression levels. p21 and p53 gene expressions, involved in growth arrest, increased. ADAM genes especially seemed to be downregulated drastically. They are the genes functioning in the cell-cell and cell-matrix interactions. Remembering the observed loss of cellular motility and decreased cellular adhesion of HCC1937 cells upon ATAD2 downregulation in the scratch assay, this finding of decreased expressions of adhesion molecules complements what we observed in the functional assay.

	Fold change																
MCF7	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
siATAD2	-2.4	-4.9	-4.7	-2.3	1.4	-0.5	3.1	4.5	0.6	-1.4	3.0	-1.0	2.2	-1.0	0.5	-0.3	0.3
shER α	-2.4	-5.8	-5.1	-3.5	1.3	0.4	2.1	3.3	0.4	-2.6	2.5	-2.2	1.6	-0.9	0.3	0.4	0.3
siATAD2+shER α	-1.7	-4.4	-4.5	-4.1	1.5	0.6	2.6	3.5	1.1	-2.4	1.3	-2.0	1.2	-1.1	0.8	0.0	0.1

Figure 3. 20: RT-qPCR analysis of selected genes in siATAD2 (25nM) or/and shER α -499 (2ug) treated MCF7 cells.

1: ATAD2; 2: ESR1; 3:EGFR; 4: EGR1; 5: ADAM23; 6: ADAMTS1; 7: c-MYC; 8: N-MYC; 9: CCNE1; 10: CCND1; 11: CDKN1; 12: RB1; 13: TP53; 14: AKT2; 15: CASP9; 16: CDH1; 17: BRCA1. Fold changes greater than 2 have been highlighted in **bold**. All transcripts were normalized to internal control, GAPDH gene. The experiments were run in duplicate reactions.

	Fold Change													
HCC1937	1	2	3	4	5	6	7	8	9	10	11	12	13	14
shATAD2_1_C1	-3.0	-2.5	-0.2	-4.4	-2.1	-2.5	-0.7	-0.2	0.0	0.1	-0.6	-0.5	-0.4	-0.3

Figure 3. 21: RT-qPCR analysis of selected genes in HCC1937 shATAD2_1_C1 clone.

1: ATAD2; 2: EGFR; 3: EGR1; 4: ADAM23; 5: ADAMTS1; 6: C-MYC; 7: N-MYC; 8: CCNE1; 9: CCND1; 10: CDKN1; 11: RB1; 12: TP53; 13: AKT2; 14: BRCA1. Fold changes greater than 2 have been highlighted in **bold**. All transcripts were normalized to internal control, GAPDH gene. The experiments were run in duplicate reactions.

3.3.1.2. Effect of ATAD2 depletion on the regulation of apoptosis

Cell death may occur through apoptosis or necrosis processes. While apoptosis is a programmed cellular mechanism, necrosis is an unprogrammed premature cell death triggered by abnormal external factors such as infection or inflammation. There are different proteins involved in the regulation of these two forms of cell death and we may distinguish them with molecular characterization of proteins functioning in these processes^{249,250}. In cancer cells, the regulation mechanism of cell death is disrupted, and that is why they are resistant to cell death-inducing external signals. The main common point of anticancerous agents is to sensitize cancer cells to cell death stimuli²⁵¹. Whether ATAD2 downregulation might have triggered an inner apoptosis signal was investigated in breast cancer cells. The expression levels of proteins involved in the intrinsic apoptosis pathways were analyzed. Individual downregulations of ATAD2 and ER α or their combined-inhibition led to pRB hypophosphorylation in MCF7 cells. Decreased phosphorylation of this protein inhibits cell cycle progression through G1 to S phase and leads to growth arrest in G1. Bid, a pro-apoptotic protein, is localized in the cytosolic part of mitochondria in inactive form and its activated (cleaved) form is associated with release of cytochrome c from mitochondria, which results in caspase-9 activation. Decreased amount of full-length Bid proteins was observed, suggesting increased cleaved Bid fragments. The primary Bid antibody we used detected just full-length Bid protein. The cleavage of Bid has induced caspase 9 cleavage, meaning its activation. 37 kDa caspase-9 fragments were detected in the blot. This confirmed the activation of intrinsic apoptotic pathway through caspase-9 activation in MCF7 cells.

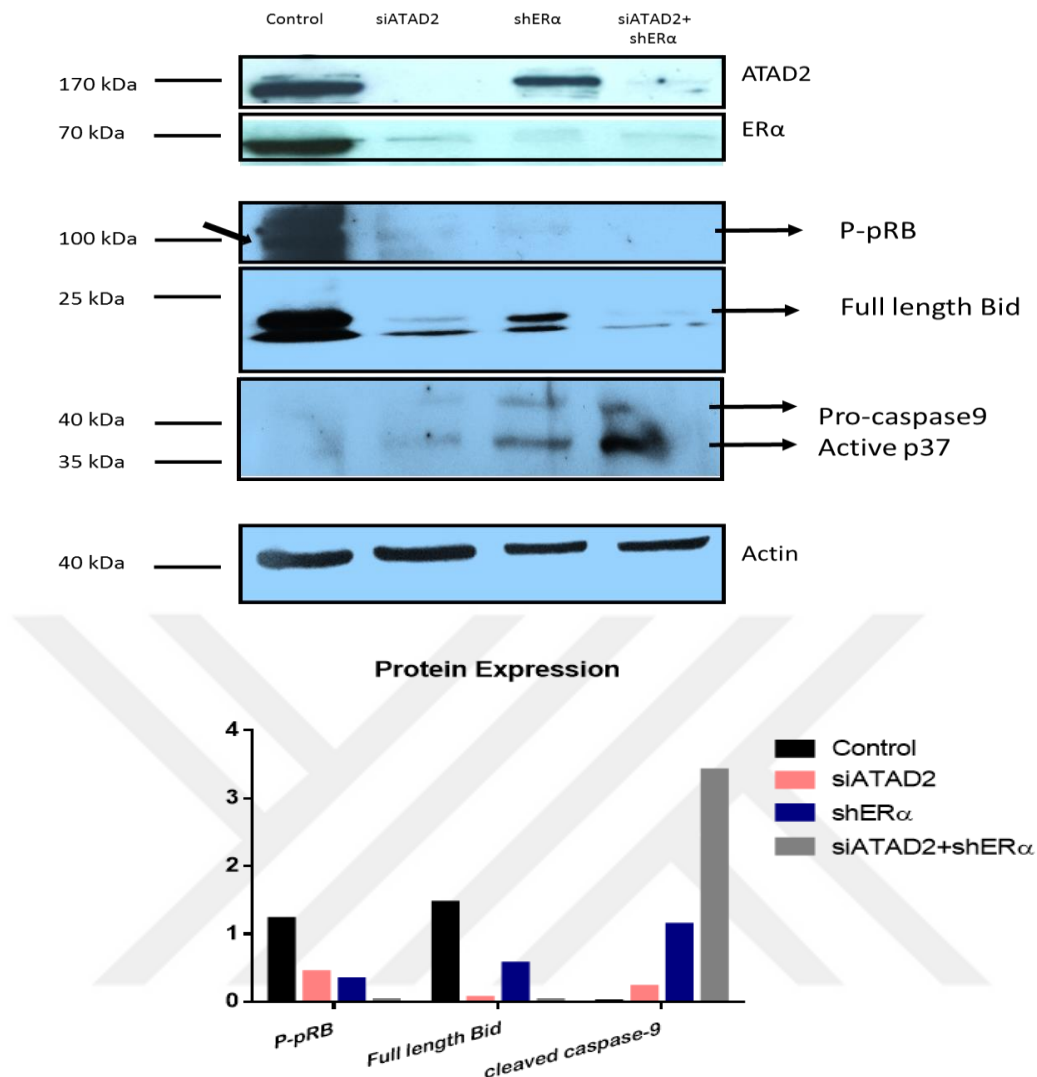


Figure 3. 22: Effect of ATAD2 or/and ERα knockdown on the expression of apoptotic proteins in MCF7 cells.

MCF7 cells were treated with either siATAD2 (25 nM) or shERα-499 (2 ug) for 72 hours; or co-targeted with 48 hours siATAD2 (25 nM) transfection following 24 hours shERα (2 ug) treatment. Then, whole-cell extracts were prepared and apoptotic proteins were analyzed with western blot. Phospho-RB (ser780) (cell signaling, #9307, 110 kDa), full length Bid (cell signaling, #2002, 22kDa) and cleaved-Caspase-9 (cell signaling, #9502, 37 and 47 kDa) protein levels were detected. B-Actin was used as input to show equal loading. ATAD2 proteins over actin normalization were calculated (bar-graph). RB phosphorylation was inhibited in treated MCF7 cells compared to control. Full-length Bid protein expressions were reduced to almost undetectable levels for siATAD2 (25 nM) and siATAD2 (25 nM)+ shERα-499 (2 ug) cells. Cleaved caspase-9 could not be detected in control cells and its levels showed a gradual increase from siATAD2 (25 nM), shERα-499 (2 ug) to siATAD2 (25 nM)+ shERα-499 (2 ug) treated cells. The highest cleaved-caspase-9 protein level was observed in co-transfected cells.

HCC1937 clones showed consistent results with MCF7 cells on the regulation of cell growth. ATAD2 downregulation decreased phosphorylation of RB proteins in these cells as well. Decreased full length of pro-apoptotic Bid proteins was observed, meaning its cleavage and activation. Bcl2 protein expression seemed to be decreased with ATAD2 downregulation. Not cleavage of caspase-9 in shATAD2_C1 cells was observed compared to shCntrl_C5. The result suggests the trigger of apoptotic response but not completed through intrinsic pathways following ATAD2 depletion in ER(-) cells.

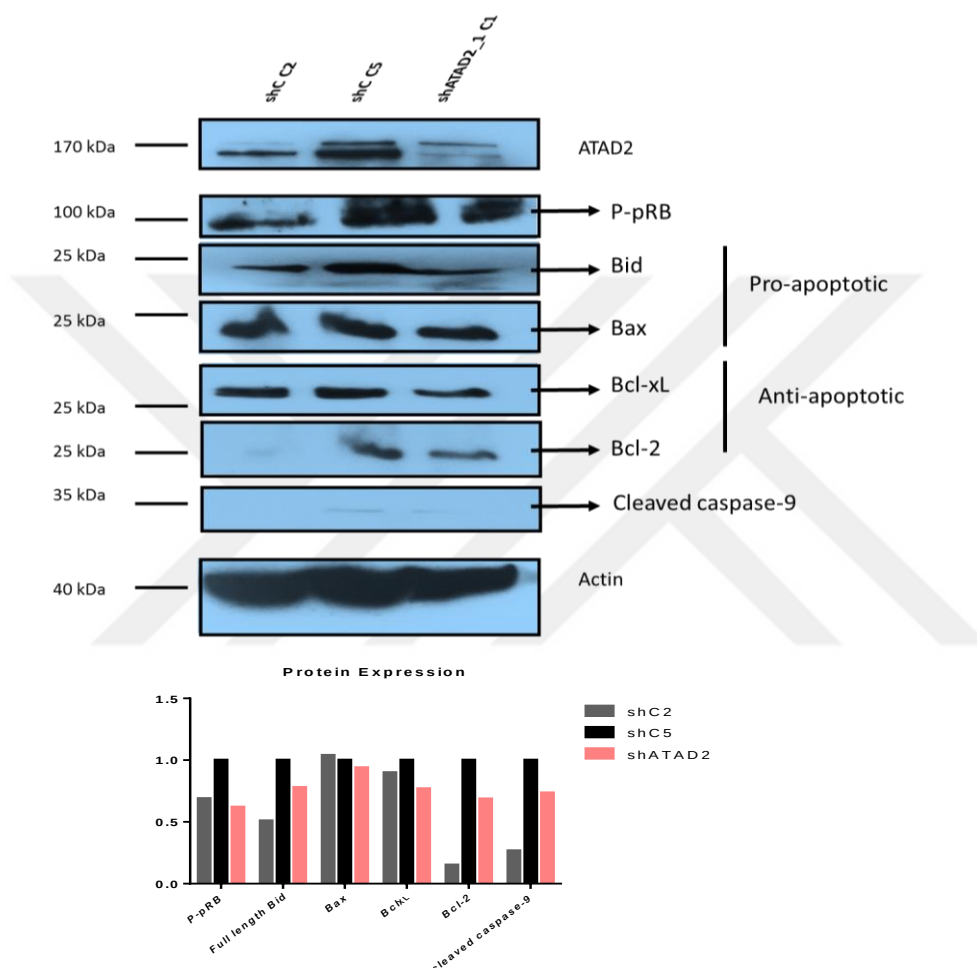


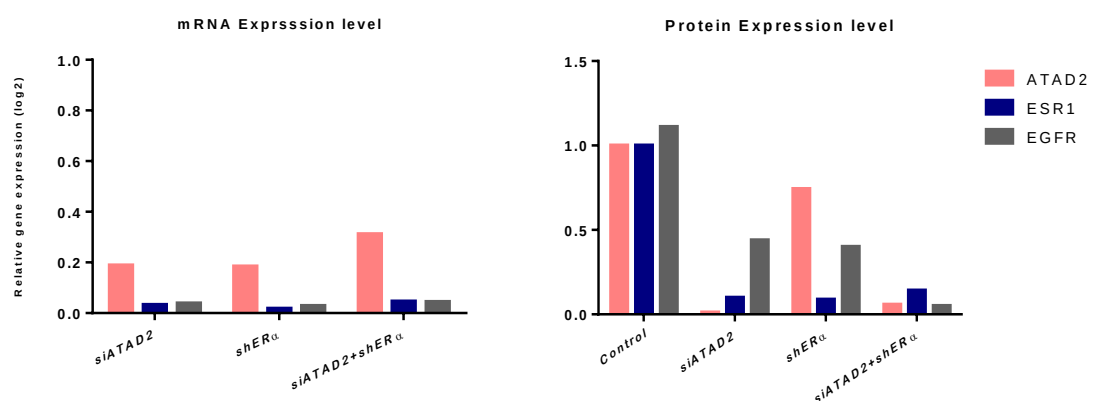
Figure 3. 23: Effect of ATAD2 knockdown on the expression of pro-apoptotic and anti-apoptotic proteins in ATAD2 down-regulated HCC1937 cells.

Whole-cell extracts were prepared from the ATAD2 downregulated-stable HCC1937 cell clone, shATAD2_1_C1 and shCntrl clones. Apoptotic proteins were analyzed with western blot. Phospho-RB (ser780) (cell signaling, #9307, 110 kDa), full-length Bid (cell signaling, #2002, 22kDa), Bax (cell signaling; #2772, 20 kDa), BCL-xL (santa cruz, sc-8392, 30 kDa) and cleaved-Caspase-9 (cell signaling, #9502, 37 and 47 kDa) protein levels were detected. B-Actin was used as input to show equal loading. ATAD2 proteins over actin normalization were calculated (bar-graph). With RB phosphorylation, the full length Bid protein and BCL-xL expression levels were reduced in ATAD2 downregulated HCC1937 cells, Bax expression did not show any difference compared to control cells.

3.3.1.3. Effect of ATAD2 depletion on EGFR signaling

Antitumor effects of ATAD2-downregulation on breast cancer have been evaluated with functional studies and its ability to protect cells from cellular senescence and cell death has been confirmed. To evaluate these effects, expressions of apoptosis and cell proliferation related genes at the mRNA and protein expression levels were analyzed. Pro-apoptotic signaling seemed to be induced after ATAD2-downregulation and cell cycle progression was arrested in G1 or G2/M. To highlight downstream signaling pathways interconnected with these effects, the candidate signaling networks in which ATAD2 may function has been investigated. EGFR signaling was included in the list of enriched signaling pathways of significantly downregulated genes upon ATAD2 silencing (Table 3.6). Besides, the RT-qPCR results support decreased EGFR expression in breast carcinoma cells after ATAD2 downregulation (Figure 3.20). Therefore, we thought the activity of ATAD2 in EGF signaling might be one of the underlying causes of its oncogenic effects. EGFR executes its effect through activation of multiple sequential signaling cascades in breast cancer cells, namely MAPK and PI3K/Akt. We identified key proteins of these signaling pathways and their protein expressions were analyzed, in addition to EGFR, in treated breast carcinoma cells. Co-inhibition of ATAD2 and ER α together resulted in the complete suppression of EGFR expression at the protein level in MCF7 cells. The co-inhibition seemed to decrease Akt protein expression as well in these cells. We could not detect any change in the phosphorylation status of p38 MAPK.

A.



B.

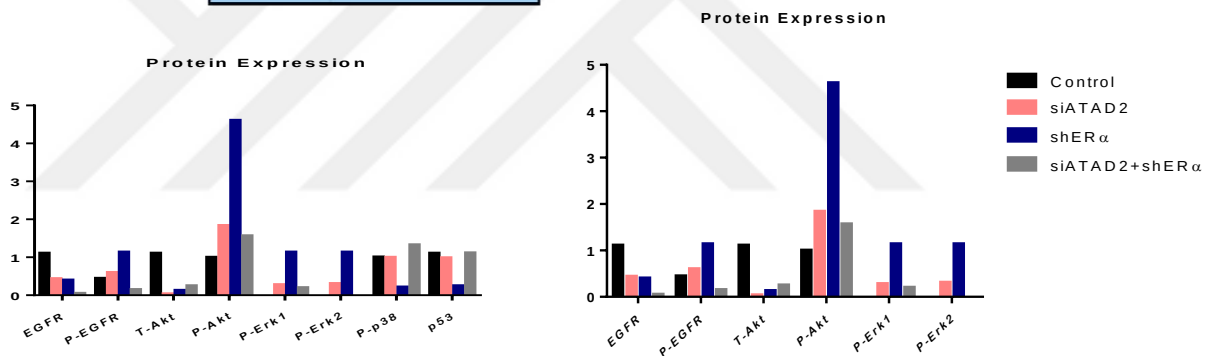
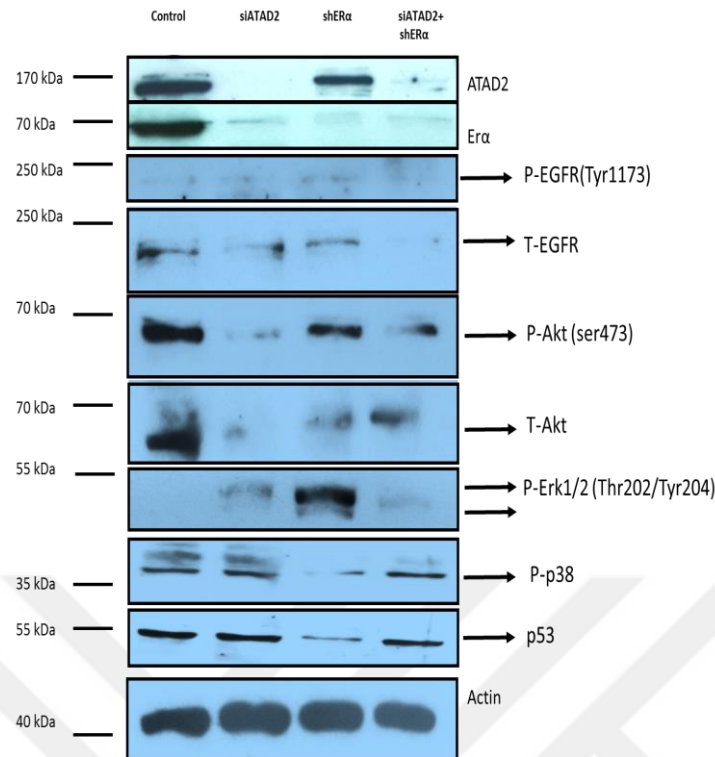


Figure 3. 24: Effect of ATAD2 or/and E α knockdown on the expression and on the activity of EGFR and EGFR stimulated signaling proteins in MCF7 cells.

Figure 3.17 showed the expressions of *ATAD2*, *ESR1* and *EGFR* genes determined by RT-qPCR in MCF7 cells. **(A)** Their relative expression levels are illustrated in the graph. Both *ESR1* and *EGFR* gene expressions were reduced to almost undetectable levels. **(B)** MCF7 cells were treated with either siATAD2 (25 nM) or shER α -499 (2 ug) for 72 hours; or co-targeted with 48 hours siATAD2 (25 nM) transfection following 24 hours shER α (2 ug) treatment. Then, protein was isolated from whole-cell extracts. EGFR and proteins involved in EGFR signaling were analyzed with western blot. While EGFR protein levels were reduced by half in siATAD2 (25 nM) or shER α -499 (2 ug) transfected cells compared to control, they were suppressed more than 90% in co-transfected (siATAD2+ shER α -499) cells. While Akt expression and P-Erk1/2 were decreased in siATAD2 or co-transfected cells, P-Erk1/2 increased in shER α -499 transfected cells. Phosphorylation of p38 and p53 expression levels showed more than 50% reduction in shER α -499 transfected cells.

Functional assays have revealed the inhibitory effect of ATAD2 depletion on cell migration in ER(-) cells. HCC1937 cells are of the basal-like breast cancer type with an aggressive molecular subtype limiting hormone-based therapeutic options. EMT markers such as vimentin have been shown to be highly expressed and epithelial markers such as E-cadherin may be lost in these cells (Table 1.1). We decided to look into epithelial and EMT marker expressions at the protein level. There was no difference in their expressions at all. However, there was a slight decrease in p53 expression in treated cells. It appears that a visible drop in EGFR gene expression at the mRNA level was not translated into its reduced protein expression in ATAD2 downregulated HCC1937 cells. However, its phosphorylation at Tyr1173 was inhibited. This is one of the autophosphorylation sites in the receptor. The receptor activates MAPK and PI3K/Akt signaling following EGF stimulation. The expression levels of key proteins involved in these signaling pathways and their activities were analyzed. Initially the effect of hypophosphorylated EGFR receptor on ERK1/2 activity was controlled. There was no difference in its phosphorylation status. Next, its effect on EGF-induced PI3K/Akt activation was controlled. ATAD2 knockdown has led to enhanced Akt phosphorylation. There was no change in total Akt expression, which means its increased phosphorylation levels is the direct result of ATAD2 downregulation on the Akt activity in the cell.

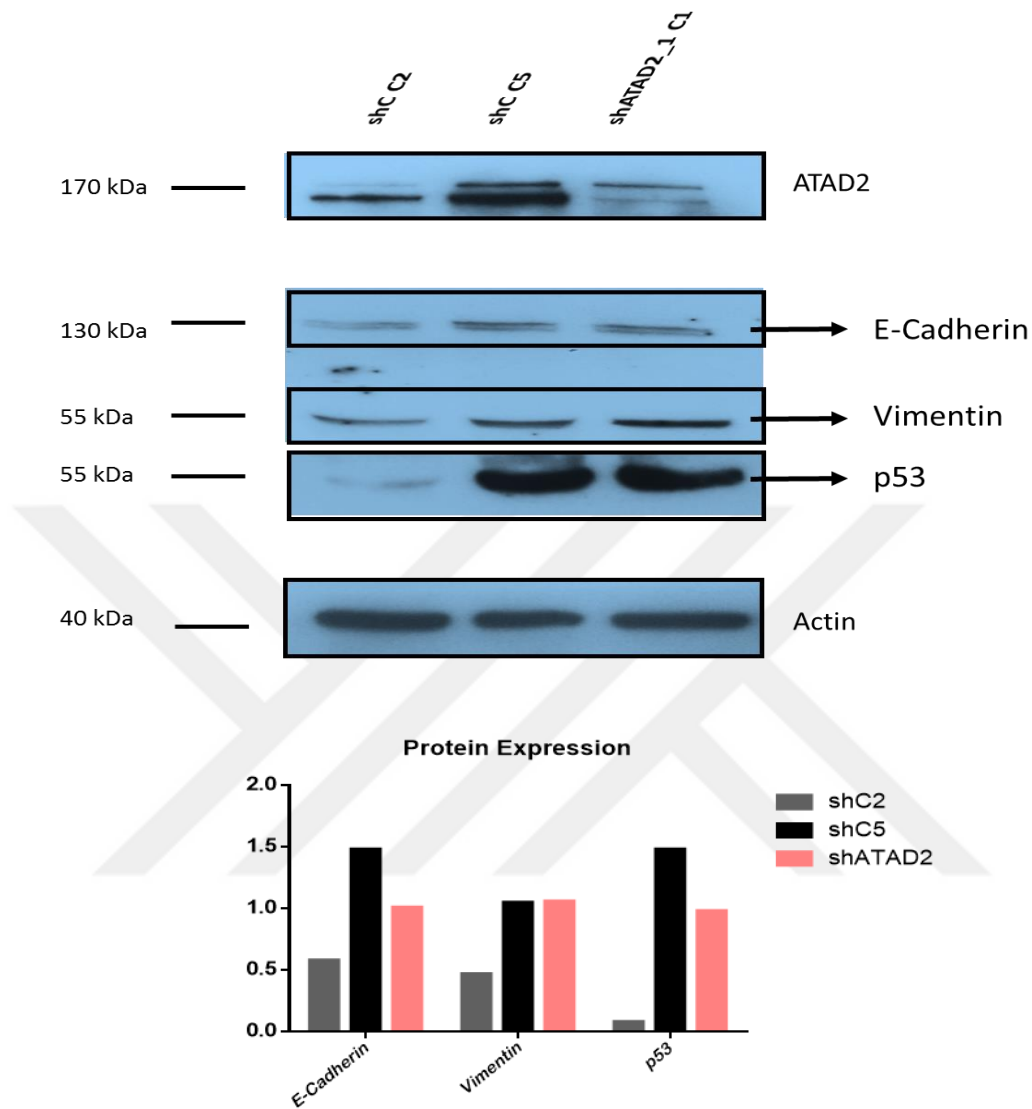


Figure 3. 25: Effect of ATAD2 knockdown on the expression of EMT markers in ATAD2 down-regulated HCC1937 cells.

Whole-cell extracts were prepared from ATAD2 downregulated-stable HCC1937 cell clone, shATAD2_1_C1 and shCntrl clones. Protein expressions of EMT markers were analyzed. E-cadherin protein (Abcam, ab1416) and α -Vimentin protein (Abcam, ab8069) were detected. The expression levels of p53 protein were controlled as well. B-Actin was used as an input to show equal loading. Protein levels over actin normalization were calculated (bar-graph). While Vimentin did not show any change in its expression level compared to shcntrl_C5, E-cadherin and p53 levels were reduced slightly in ATAD2 downregulated cells.

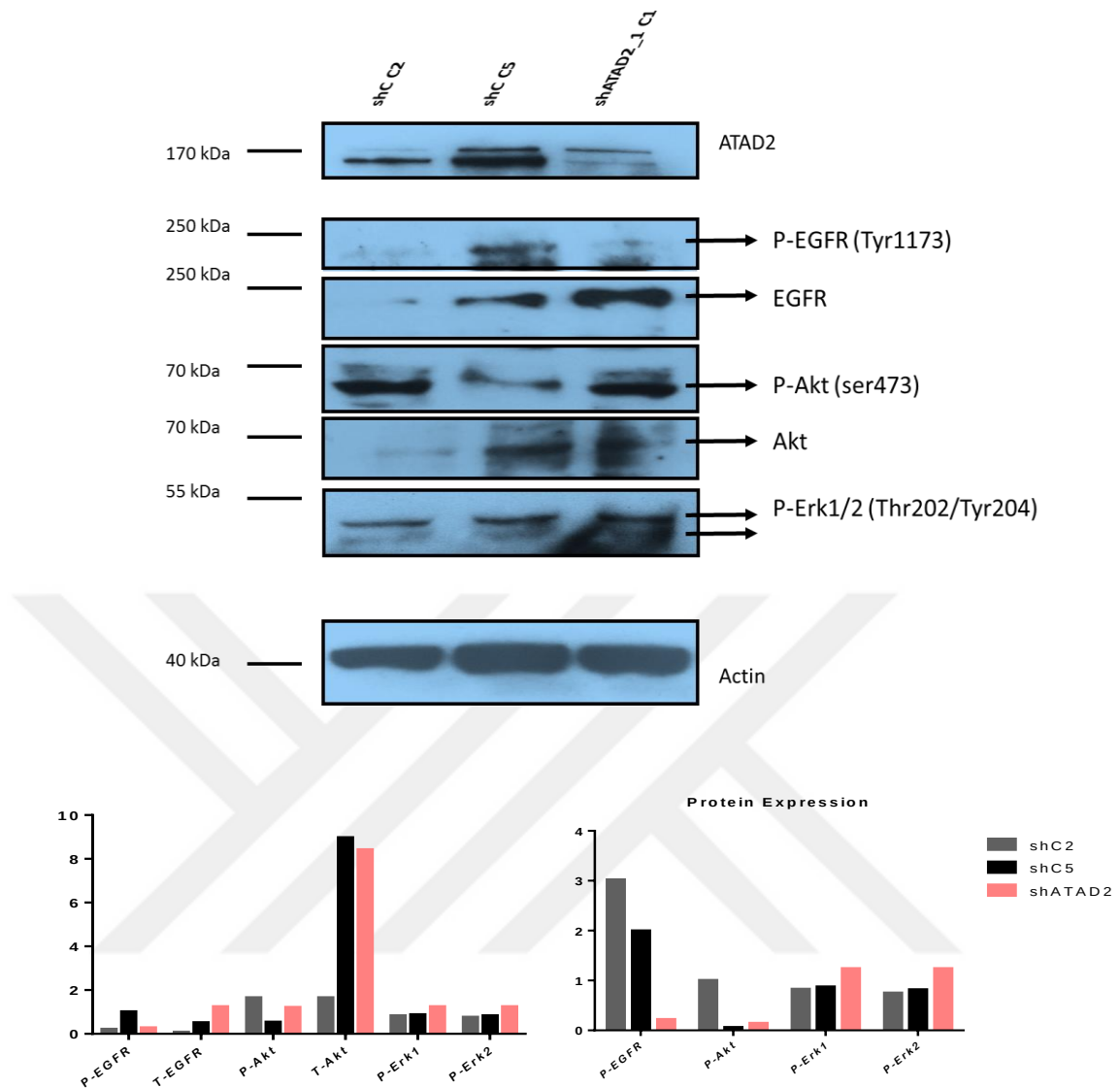


Figure 3. 26: Effect of ATAD2 knockdown on the expression and the activity of EGFR and EGFR stimulated signaling proteins in ATAD2 down-regulated HCC1937 cells.

Figure 3.18 showed the expressions of *ATAD2* and *EGFR* genes determined by RT-qPCR in stable HCC1937 cell clones. **(A)** Their relative expression levels were illustrated in the graph. *EGFR* gene expression was decreased 2-fold, **(B)** while its protein expression increased in ATAD2 downregulated cells. However, its phosphorylation was inhibited in these cells. Neither expression nor the phosphorylation levels of Akt showed any difference, yet a slight increase in Erk1/2 phosphorylation was observed.

3.3.2. ASSOCIATION OF ATAD2 GENE WITH EGFR SIGNALING IN MCF7 CELLS

Finding out EGFR was regulated by the mutual transcriptional activity of ATAD2 and ER α (Figure 3.24), we questioned the requirement of ER α activity for ATAD2 to exert its effect. In other words, was its control on EGFR expression due to its regulatory effect on ER α expression? As a reminder, ATAD2 downregulation alone has resulted in the inhibition of estrogen receptor expression of up to 90% in MCF7 cells (Figure 3.17). To clear this dilemma, mRNA expression of three genes (ATAD2, ER α and EGFR) was determined at different time points (48 and 72 hours) by RT-qPCR (Figure 3.27). We set two different conditions for each treatment. Cells were treated with the following:

- 1) siATAD2 (25nM) for **48** hours and **72** hours or
- 2) shER α -499(2ug) for **48** hours and **72** hours or
- 3) **co-trns 1:** siATAD2 (25Nm) for **48** hours + shER α -499(2ug) for **72** hours or
- 4) **co-trns 2:** siATAD2 (25Nm) for **72** hours + shER α -499(2ug) for **48** hours.

Transfection efficiency increased with longer incubation time. ATAD2 and ESR1 expressions showed greater downregulation after 72 hours transfection with siATAD2 and shER α -499 plasmid respectively. ESR1 expression was suppressed at both 48 and 72 hours post-siATAD2 treatment, supporting previous results (Figure 3.17). ATAD2 expression was lower at 48 hours compared to 72 hours post-transfection with shER α . EGFR expression was downregulated in all 4 transfection conditions. However, its expression showed positive correlation with ESR1 expression levels. Their mRNA expression patterns suggest that ATAD2 downregulation inhibits ER α expression dramatically as an early event. Subsequent decrease in EGFR expression is a secondary effect of downregulated ER α activity.

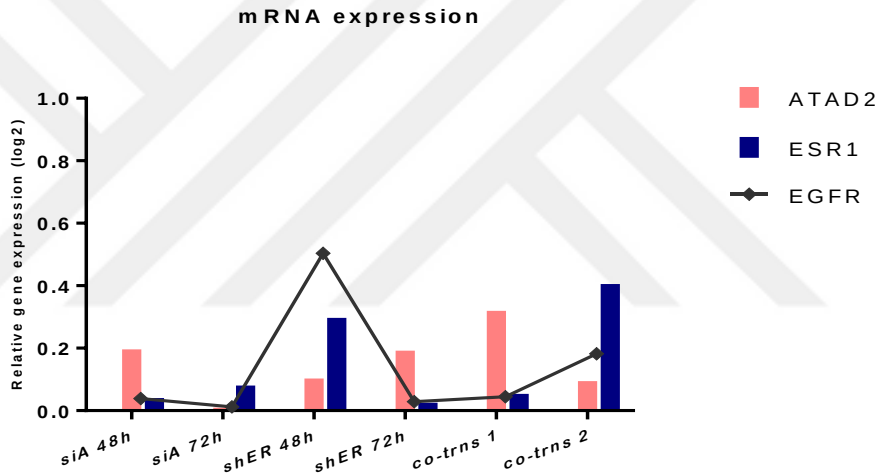
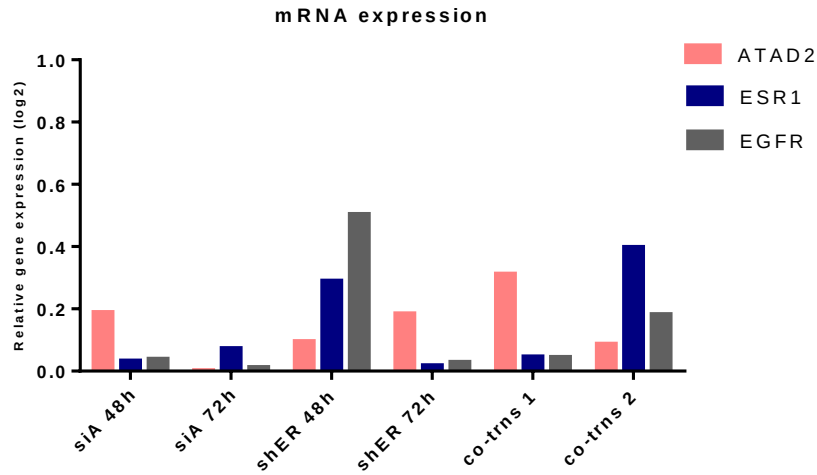


Figure 3. 27: RT-qPCR analysis of ATAD2, ESR1 and EGFR in transfected MCF7 cells.

2×10^5 cells/well seeded MCF7 cells were transfected with as follows: 1: siATAD2 (25nM) for 48 hours and 72 hours; 2: shER α -499(2ug) for 48 hours and 72 hours; 3: **co-trns 1:** siATAD2 (25Nm) for 48 hours + shER α -499(2ug) for 72 hours and **co-trns 2:** siATAD2 (25Nm) for 72 hours + shER α -499(2ug) for 48 hours. Total RNA was isolated from whole-cell extracts and EGFR transcript levels were analyzed with ESR1 and ATAD2 expressions. All the values were normalized with the GAPDH reference gene. Relative expression levels were represented in log₂ scales. ATAD2 and ESR1 expressions showed greater downregulation after 72 hours transfection with siATAD2 and shER α -499 plasmid respectively. At both occasions of siATAD2 treatment, ESR1 expression was suppressed as well. ATAD2 expression was lower at 48 hour compared to that at 72 hour post-transfection with shER α . EGFR expression showed positive correlations with ESR1 expression in all transfection conditions.

Results suggest ATAD2 is placed in the upstream of EGFR signaling and regulates its transcriptional expression. To further analyze the regulation mechanism between ATAD2 and EGF signaling, cellular response of MCF7 cells to Gefitinib (EGFR inhibitor) treatment and EGF stimulation were observed. We investigated whether EGF may also be able to modulate the expression of the ATAD2 gene as well.

3.3.2.1. Effect of Epidermal Growth Factor (EGF) stimulation on ATAD2 expression

EGF is reported to mimic E2 action²⁵² and enable activation of ER α to exert its activity²⁵³. Considering ATAD2 expression is upregulated with E2 induction and it acts as a transcriptional co-activator of ER α . Its expression may be induced by EGF as well. This assumption was tested with EGF stimulation. MCF7 cells were grown in starvation medium containing 0.1% charcoal-stripped FBS phenol-red free medium for 24h and then EGF at a final concentration of 100ng/ml was added in the medium and cells were incubated for 24h, 48h and 72h. Figure 3.28 shows decreasing levels of ATAD2 expression with prolonged time of serum starvation and the rapid and robust increase in its expression upon EGF stimulation. ER α expression also increased drastically with hormone stimulation and EGFR gene expression displayed the same expression trend with the ER α gene. This result also supports our previous assumption that ER α downregulation-mediated EGFR suppression is more likely a secondary effect of ATAD2 silencing in MCF7 cells.

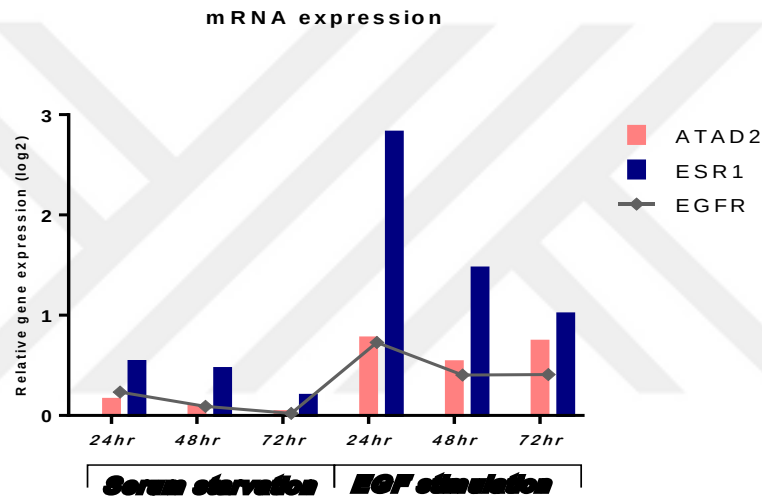
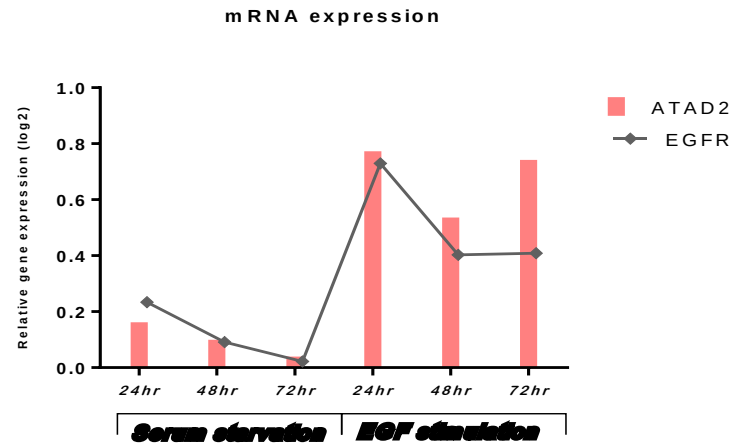


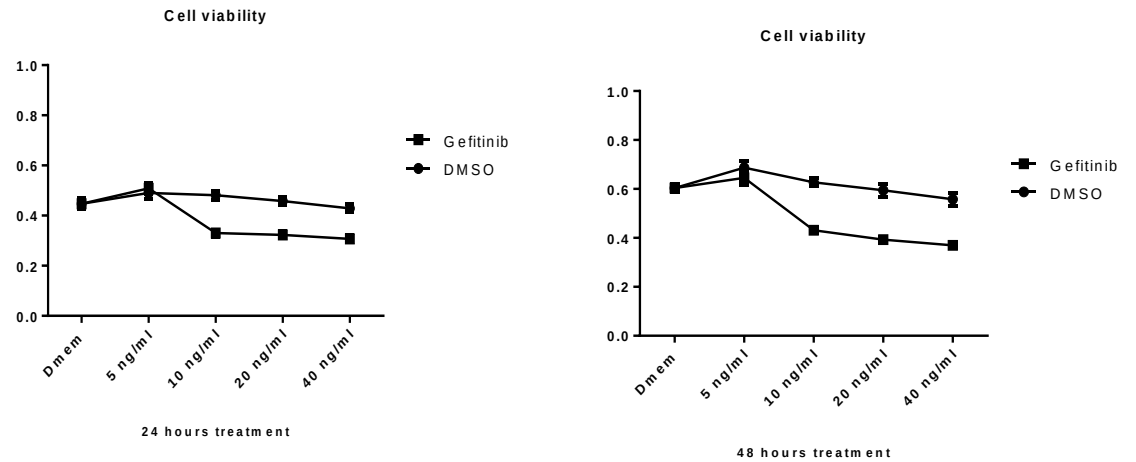
Figure 3. 28: RT-qPCR analysis of ATAD2, ESR1 and EGFR in Epidermal Growth Factor (EGF)-stimulated MCF7 cells.

Cells were grown in EGF (100 ng/ml) added medium after 24 hours serum starvation in phenol red free media containing 0.1% charcoal-stripped FBS. They were stimulated with EGF for 24, 48 and 72 hours. Cell lysates were prepared at indicated times in the presence or absence of EGF. ATAD2, ESR1 and EGFR expressions were analyzed by RT-qPCR. All values were normalized with the GAPDH reference gene. Relative expression levels were represented in log₂ scales. Genes showed greater reduction in the expression with extending incubation time under serum starvation conditions. They were upregulated in response to EGF stimulation. ESR1 was activated most at 24 hour post-treatment. However, its expression showed decline with increasing time period of EGF-stimulation. ATAD2 expression showed more or less steady levels at different time points post-EGF treatment, yet there was a slight increase in its expression at 72 hours compared to that at 24 hours. EGFR expression showed positive-correlations with ESR1 expression after EGF treatment.

3.3.2.2. Inhibitory effect of Gefitinib treatment on ATAD2 expression

EGFR activity in MCF7 cells was inhibited with Gefitinib treatment. This is an EGFR inhibitor targeting its autophosphorylation sites so that the receptor cannot be activated even in the presence of its ligand⁹³. Cells were treated with different concentrations of drug, which were 5 ng/ml, 10 ng/ml, 20 ng/ml and 40 ng/ml. MTT assay was performed to determine cell viability at 24 and 48 hours post-drug treatment. Results showed no difference between the viability of cells at these time points, so cells were incubated for 48 hours. After treatment, cells were collected and mRNA expression of ATAD2, ER α , EGFR genes were analyzed by RT-qPCR. The drug seems to be effective on cell proliferation. At 40 ng/ml concentration, it inhibited the proliferation of almost 40% of the cells. With increasing concentration of the drug, ATAD2 expression is further downregulated. At 40 ng/ml concentration, EGFR inhibition downregulated ATAD2 expression by 50%. Both EGF stimulation and EGFR inhibition have revealed the regulatory role of EGF signaling on ATAD2 expression, so results suggest that there are a possibly feedback regulation between EGFR and ATAD2.

A.



B.

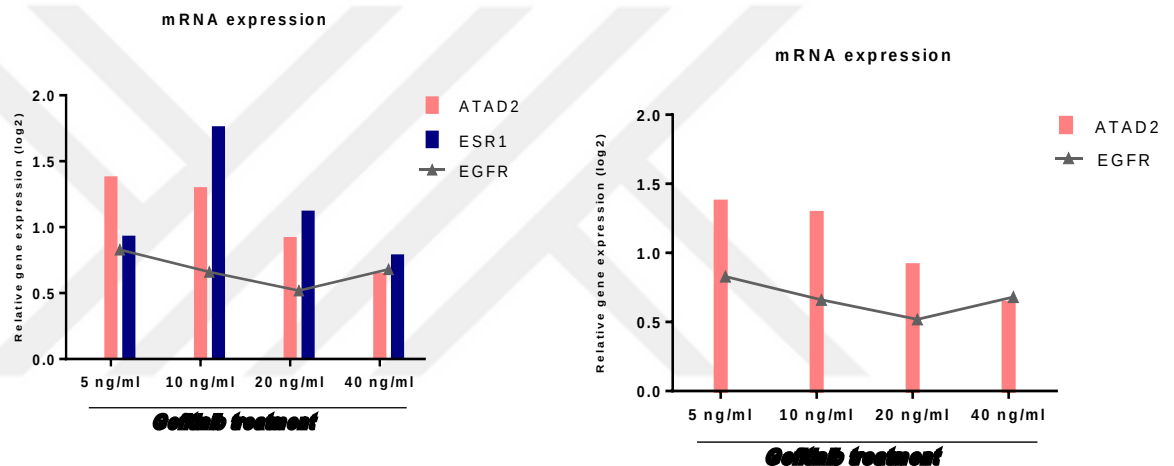


Figure 3. 29: Effect of different doses of Gefitinib on ATAD2, ESR1 and EGFR expression in MCF7 cells

(A) MCF7 cells were treated with different doses of Gefitinib (0 ng/ml; 5 ng/ml; 10 ng/ml; 20 ng/ml; 40 ng/ml) and with DMSO at the concentrations in which Gefitinib dissolved for 24 hours and 48 hours. Cell viability was determined with the MTT assay. **(B)** 24 hours after cells were seeded as 2×10^5 cells/well in 6-well plates, they were treated and incubated for 48 hours under these conditions. Cell lysates were prepared at indicated times in the presence or absence of EGF. ATAD2, ESR1 and EGFR expressions were analyzed by RT-qPCR. All values were normalized to the expression levels in MCF7 cells treated with 0 ng/ml, following their normalization with the GAPDH reference gene. Relative expression levels were represented in $\log_2$ scales. ATAD2 expression showed gradual decrease with increasing doses of Gefitinib. ESR1 expression resulted in an increase at 10 ng/ml, but later showed reduced expression at higher concentrations. Inhibition of EGFR activity down-regulated transcript expression of itself and higher doses led to lower EGFR levels in MCF7 cells. EGFR expression showed positive-correlations with ATAD2 expression after Gefitinib treatment.

CHAPTER 4. DISCUSSION

The intensive studies of molecular regulatory networks have highlighted more than 100 genes that act as executors of signal integration in the cell. They regulate signal transmission from top-to-down. They are transcriptional regulators of the genome. Their combinational actions control simultaneous tissue specific gene expression due to different sets of these regulatory elements²⁵⁴⁻²⁵⁸. They are classified under two groups as oncogenes and tumor suppressors which compete for the binding sites on DNA and exert their effects through these regions. Isolation of these protein-DNA complexes has revealed that many of them were actually transcription factors. They are building blocks of regulatory units and function in important cellular events. Their activities shape cellular responses²⁵⁸⁻²⁶⁰. Therefore, their expressions are strictly controlled in the cells. However, they are commonly deregulated in cancer cells. Control mechanisms for major regulators seem not to work and the transcriptional circuitry is quite disrupted in these cells²⁶¹⁻²⁶⁷. They are involved in a number of intricate molecular interactions and their oncogenic activations disturb cellular balance. Consequently, deregulated regulatory networks have resulted in over-activated cell growth signaling. Therefore, cancer development is partly the consequences of expression change of genes that regulate cell proliferation such as improper oncogene expression and disabled tumor-suppressor gene expression. Transcriptional profiling analysis of breast tumors has uncovered a list of molecular abnormalities associated with transcription factors. A number of them act as activators and their overexpression is common in breast cancer. Since deregulation of these factors have been implicated in disease development, to reveal more of them and the mechanisms by which they drive oncogenesis lies at the heart of breast cancer studies²⁵⁴.

ATAD2 is recognized as one of these activators. Its over-expression across many cancer types including breast cancer, relative to their corresponding normal cells, has been indicated and supported with independent studies^{16,115,117-119,121,122,127}. Moreover, a strong correlation has been found between its expression status and poor prognosis of the disease^{118,121,122,126}. More specifically, its co-activator function for ER α has been demonstrated in ER(+) positive breast cancer cells¹¹⁵. However, our knowledge regarding its involvement in transcriptional circuitry is limited mostly with repertoire of

the cell cycle genes. Its activation in concert with key nuclear regulatory proteins has been associated with abnormal cell growth, but the context of its molecular relationships has not been highlighted yet. Therefore, we studied functions of the ATAD2 gene using both morphological and molecular concept to bridge the gap between its biological effects and transcriptional cascades regulated with its nuclear activity.

Its expression level in a breast cancer cell panel at both the mRNA and protein levels were screened. Its expression at high levels were demonstrated in most of breast cancer cells, including both ER(+) and ER(-) cell types. Since it is defined as one of main ER α co-activators in breast cancer, its relation with estrogen receptor expression was examined. However, among ER(+) cells, its expressions does not seem to be correlated with ER α levels. While all of them showed elevated ATAD2 protein expression, ER α did not exhibit the same pattern. ATAD2 is an estrogen receptor-responsive gene, suggesting that its expression may be upregulated with receptor activity. However, what we observed is that despite the relatively reduced receptor expression, the ATAD2 gene can maintain its overexpression level, as seen in BT-474 cells for instance. Its expression was then compared between two distinct molecular subtype of breast cancer which were ER(+) and ER(-) cells and found to be comparable. It is an indication that its expression is partly independent from receptor status. Its generic overexpression in breast cancer may be down to its known regulatory control on cell growth, so its elevated expression may be a favorable acquisition during cancerogenesis. Cancer cells have an advanced adaptation abilities under stress conditions. They can rearrange cellular signaling and gene expressions to protect themselves from cell death^{5,268,269}. These acquired adaptive mechanisms may increase our understanding of cellular activities in cancer cells. Therefore, we removed serum from media and exposed breast cancer cells to metabolic stress. Variance in ATAD2 expression was observed under unfavorable conditions. Both cell growth and cellular proliferation of treated cells were examined along with their relative ATAD2 gene expressions. Serum starved MCF7, T47D, SKBR3, HCC1937, MDA-MB-231, BT-20 cells were used for simultaneous comparison of their cell viability and flow cytometric analysis after 48-hour and 7-day culture incubation. Our gene expression profiling studies revealed that while BT20 cells had normal endogenous levels of ATAD2 expression, the other five cell lines overexpressed it. In both MCF7 and T47D cells, ATAD2 expression has decreased following serum depletion and their subsequent cellular proliferation is significantly

halted. T47D cells became the most effected cell lines in all with more than 90% inhibition of cell proliferation for both 48h and 7-days. MCF7 cells were the closest with more than 60% inhibition. We noted MCF7 cells could show higher cell viability rate in spite of lesser gene expression compared to T47D cells. This may imply T47D cells are more sensitive to ATAD2 depletion. BT-20 and MDA-MB-231 cells displayed increased gene expressions. While BT20 could induce exactly 2 fold rise in ATAD2 expression, the increase in MDA-MD-231 was minimal. BT20 cell viability was not affected much from starvation but proliferation of MDA-MB-231 cells was inhibited by 50%. These two results are actually compatible with each other and we suppose the extent to which ATAD2 is upregulated or downregulated affects cell viability. BT20 cells showed adaptation to lower growth stimulation through increased ATAD2 activity in the cell, yet MDA-MB-231 cells did not respond in the same way and could not compensate for the lack of a growth signal with increased ATAD2 activity, suggesting the increase in ATAD2 expression alone was not sufficient for these cells. The protein level did not change in HCC1937 and SKBR3 cells after starvation, but their proliferation was inhibited. SKBR3 resisted more or less 48h starvation, but after 7-days its viability was cut down to 30% as well.

When we grouped these cells according to their estrogen receptor status, we realized a trend in their responses. ER(+) cells are the ones influenced most from serum starvation. They responded with both decreased ATAD2 expressions and decreased cellular proliferation. Considering that they are estrogen responsive cells, our study supports the suggestions that ATAD2 functions predominantly as transcriptional effector of estrogen and estrogen responsive gene in these cells. On the other hand, ER(-) cells are not hormone responsive due to lack of receptors, so unlike ER(+) cells, they either showed increased ATAD2 expression or did not change at all. The variety in their endogenous ATAD2 expression might have played a role in the difference of ER (-) cell response to cellular stress. BT20 cells, having low endogenous ATAD2 levels, recovered from anti-proliferative conditions by inducing its overexpression, while MDA-MD-231 cells, having high endogenous ATAD2 expression already, showed sensitivity to serum deprivation. ATAD2 may ensure metabolic stability under stress conditions in cancer cells and its overexpression can be one of adaptation tools to support self-sufficient growth, which is one of hallmarks of cancer.

We next investigated the cell cycle status after 48 h of serum starvation. While MCF7 cells did not show any change in their cell cycle distribution even after serum deprivation, cell cycle results for T47D cells supported decreased cellular viability with reduction in the percent of cells in the S phase and increase in G1/G0 and in apoptotic cell numbers. It is interesting not to see any shift in cell cycle status of MCF7 cells after treatment but decreased cell viability at the same time. We could not find a sensible reason for this yet. The results for T47D cells indicates that absence of growth signals deregulated ATAD2 levels in the cell which in turn altered expression of its cell cycle-associated target genes. For instance, cyclin D and E are among the target genes of ATAD2 and they are required for cell cycle progression through the S phase for DNA synthesis. Therefore, to observe G0/G1 arrest in response to decreased ATAD2 levels following serum starvation in these cells (ER+) was quite expected. Treatment increased cells accumulated in the G0/G1 phase in all ER(-) cells, suggesting serum deprivation induced G0 arrest in these cells. However, in two out of four ER negative cells, ATAD2 expression did not change and in others it was increased. Therefore, either ATAD2 may not be directly involved in cell cycle mechanisms in ER(-) cell types or its activity is inhibited somehow.

After detection of its generic overexpression in breast cancer cells, we decided to carry on our experiments to reveal biologic activity of ATAD2 in the malignant phenotype of cancer cells. Its high expression was shown in most of the breast carcinoma cells compared to normal non-carcinomas. This can be an implication of oncogenic function of this protein at the cellular level. For this purpose, two ER(+) and two ER(-) breast cancer cell lines were determined based on their ATAD2 expression profiles. MCF7 and T47D cells were selected for functional studies due to their highest co-expression of both ATAD2 and ER α at protein level. SKBR3 and HCC1937 cells were the selected ER(-) cell lines for studies. They were the cells used for all experiments in the rest of the thesis. They were transfected to downregulate ATAD2 expression and a number of functional assays were conducted to understand its effects on cellular events.

The relevance of ATAD2 gene expression with cell cycle progression has been reported previously and supported with a great number of studies. However, to what extent it functions within the cell is unknown yet. Therefore, its cellular activity was studied on a broader basis. Functional annotation analysis of our gene expression datasets of ER positive cells has revealed significantly deregulated genes upon ATAD2

downregulation, suggesting possible involvement of ATAD2 on the dynamic control of microtubule network organization. Knowing that microtubules participate as well in the events leading to cell migration, we tested the effects of ATAD2 silencing on cell motility. We carried out the scratch assay to study cell migration in vitro. This is a cellular process deregulated frequently in tumorigenesis. Therefore, it is the favorite target of cancer therapeutics in recent years after being recognized as one of main features of malignancy. However, the studies so far have not been very successful due to high plasticity of cancer cells⁵. They have the ability to adapt their migration mechanisms. After finding out that ATAD2 expression may enable cancer cells to adapt to cellular stress during serum starvation experiments, we thought ATAD2 may be involved in migration mechanisms of cancer cells as well. However, studies showed that ATAD2 activity is apparently not essential for cellular motility of ER(+) cells while it has a pivotal role in the regulation of cell migration in ER(-) breast carcinomas. The next studies have been interconnected with each other. The colony formation assay has tested their unlimited division capacity. There were two parameters for this study to evaluate the treatment effect, which were colony numbers and colony sizes. Assuming cells were equally seeded at the beginning of experiment, if treatment does not induce cell death after some time, we do not expect the fraction of seeded cells to change at the end. Looking at the results of the ER(+) clones, the colony numbers seem to stay constant but colony sizes become significantly smaller after treatment, suggesting reduced proliferation ability of the cells. Therefore, we believe the colony size may be a better reference for the reduced viability capacity of cancer cells. On this basis and accepting colony size as the reference point of limited reproductive capacity, loss of ATAD2 activity may suppress colony forming abilities of ER(+) breast cancer cells. While colony forming abilities of HCC1937 cells were suppressed in both colony number and colony size content, SKBR3 cells appear to be not affected. Colony formation capacity of cells is suggested to be a potential indicator for the senescence mechanism. Therefore, cells were examined regarding the association of ATAD2 with the senescence response. β -Gal activity was used as a marker senescence activation¹⁸⁶. In both breast cancer subtypes of ER(+) and ER(-)), ATAD2 downregulation induced significant cellular senescence. Moreover, the senescence response was getting stronger with less ATAD2 expression. Another criteria of senescence activity is to observe enlarged cell size. Even though it was not much clear in ER(+) cells, cell morphologies have transformed into flat, enlarged cells after ATAD2 suppression especially in the

ER(-) group. The senescence program is a tumor suppressive mechanism and an irreversible cell cycle arrest is a must for this mechanism. To further confirm the activation of the senescence response, cell cycle progression and proliferation of treated cells were studied. However, none of the cells could exhibit a significant difference in cell cycle distribution after treatment. In the case of ER(+) cells, the number of cells arrested in G1 was quite high even in controls for T47D cells. For functional studies, stable ATAD2 silenced ER positive cells were used. Therefore, we suggest antibiotic selection might have created a metabolic stress on these cells. G2/M population has increased slightly for MCF7 and SKBR3 cells. Despite the differences observed in the percentage of cells accumulated in cell cycle phases between treated and control cells, these were not statistically significant ($P > 0.05$, t-test). HCC1937 cells stimulated apoptosis following G1 arrest upon treatment ($P < 0.05$). So far our studies indicated ATAD2 downregulation stimulated senescence response in all cells; however ER(+) and SKBR3 cells did not show senescence-induced cell cycle arrest. FACS experiments were carried out with 3-days cultured cells, while cells were cultured for at least 2 weeks for the senescence assay. These incompatible results may be relevant with differences in the duration of studies. The lack of ATAD2 function may be showing its full effect on the phenotype in an extended time of period. Therefore, the results indicate that ATAD2 may take part in the senescence mechanism in breast cancer. Its downregulation inhibited large-sized colony formations from single cancer cells and hampered cell migration.

We observed a variety of cellular responses to intracellular ATAD2 protein depletion. This may be attributed to molecular differences between subtypes such that ER negative cells appeared more responsive to ATAD2 expression at cellular level. However, even its effects differed across them. The loss of ATAD2 activity seemingly disrupted cellular functions of HCC1937 dramatically; on the other hand, SKBR3 cells appear to be not affected in the cell cycle, yet it still showed senescent morphology. Considering each breast cancer cell has a distinctive intracellular molecular substructure, it is not surprising to find out they exhibit heterogeneity in their responses. They indicate ATAD2 expression is required for maintenance of cancer cells, but its functional competency may depend on the molecular background of the cells. To summarize, the lack of its expression has induced regression in some malignancy phenotypes and

induced a specific senescence state, and ultimately inhibited cell growth and caused subsequent cell death for some cell types.

The next question to answer then was which ATAD2 gene activities at the protein level allow cells to execute these responses. We based the rest of our research on this question and proceeded with elucidation of the underlying molecular mechanisms leading to diverse cell fates. ATAD2 gene function is connected with E2 action because it acts primarily as ER α co-regulator in ER positive breast cancer cells. Reports have revealed its activator effect on selective control of ER target genes. However, with the regulation of particular gene sets, what does ATAD2 activity aim for? Underlying causes behind its actions are still quite obscure. At this part of our research we tried to determine the mechanism of action of the ATAD2 gene as an ER α co-activator in MCF7 cells.

Transfection experiments were carried out with either siATAD2 or shER α or their co-transfection in MCF7 cells. It seems that ATAD2 downregulation has inhibited ER α expression as well at both the mRNA and protein level. On the other hand, downregulation of ER α receptor could only inhibit 30% of ATAD2 expression at the protein level. This suggests that ATAD2 regulates ER α expression.

The combinational analysis of gene expression data sets of ATAD2 downregulated MCF7+T47D cells and ER α downregulated MCF7 cells presented a short- list of significantly altered signaling mechanisms in the cell after treatments. Among the names, EGFR signaling caught our attention. As explained in introduction chapter, EGFR signaling is deregulated in various breast cancer types. It plays a pivotal role in the progression of breast cancer. Its overexpression or disrupted EGFR signaling pathway has been suggested to be correlated with abnormal cell growth and poor prognosis⁷⁵⁻⁸⁰. Therefore, we evaluated its expression in MCF7 cells. RT-qPCR supported microarray results and EGFR expression was reduced with ATAD2 downregulation. Then, to check whether its decreased expression is an obstacle to exert its function, the expression of one of its target genes was measured. EGR1 is a transcriptional factor. It is highly expressed in breast cancer despite its known function as a tumor suppressor in a group of other cancer types²⁷⁰⁻²⁷². RT-qPCR confirmed that its expression was downregulated in response to decreased EGFR levels as well. However, we know that mRNA levels are not always an indication of the cellular

activity of genes. Protein expression is the most reliable reference for this. Therefore, to confirm qPCR results protein expressions were analyzed with western blot. EGFR protein level was reduced by half with siATAD2 and shER α treatments separately, but its expression was inhibited completely with co-suppression of ATAD2 and ER α .

EGFR exerts its biological functions through activation of the MAPK and Akt pathways⁸¹⁻⁸⁴. We hypothesized the depletion of EGFR would inhibit the activation of these signaling cascades. For this reason, both the expression and activity of key signaling proteins involved in these two pathways were analyzed. Akt phosphorylation was reduced following ATAD2 downregulation and co-suppression in MCF7 cells. However, inhibited EGFR was not the reason of reduced Akt activity since individual ATAD2 downregulation or dual-inhibition of ATAD2 and ER α suppressed Akt expression (Figure 3.24). Individual ER α downregulation also decreased Akt expression but not as much as when ATAD2 activity is prevented. Results suggests ATAD2 is one of main transcriptional regulators of Akt gene, and the inhibition of both ATAD2 and ER α is required for complete downregulation of EGFR expression at the protein level.

Erk 1/2 and p38 phosphorylations were controlled for MAPK signaling activity. Erk1/2 phosphorylation was enhanced in treated cells and its highest phosphorylation levels were observed in ER α downregulated MCF7 cells (Figure 3.24). In ER positive cells, estrogen regulates a number of its target gene transcription through MAPK. Estrogen receptor deficient cells or estrogen receptor positive cells that were resistant to hormone therapy display over-activated MAPK signaling and the general assumption is that upregulation of MAPK and other signaling pathways such as NF-KB mediates reduced ER expression^{82,273-276}. However, this was not the case here. We showed that the other way around is also a possibility because downregulation of receptor induced up-regulated Erk1/2 activity. ER signaling and MAPK pathway is interconnected and they regulate the activity of each other. However in the case of ATAD2 downregulation alone or co-downregulation, we cannot see similar enhanced activity in Erk1/2. It seems that ATAD2's effect on the pathway is minimal. Moreover, downregulation of ATAD2 along with ER seems to block pathway activation. We believe ATAD2 mediated EGFR downregulation may inhibit the downstream MEK/ERK cascade. Another sequential signaling cascade activated by EGFR is p38 MAPK. Therefore, we next checked the phosphorylation status of the p38 protein. ATAD2 could not induce any change in protein activity, but ER loss alone reduced the phosphorylation level of protein. The p38

cascade is another downstream target of estrogen signaling. Activated ER α induces activity of p38 kinases. The relationship between p38 and p53 pathways has been indicated in different cancer types. The reduced p53 expression could be the result of cross talk between ER-induced p38 pathway and p53 signaling. We could not find any direct relationship between p53 expression and EGFR but cells that acquired resistance to EGFR inhibitors are mostly associated with p53 loss and a few studies have demonstrated that restoring p53 expression resensitized cells to EGFR inhibitors. In our study, there was no indication of a potent relation between them. All in all, ATAD2 seem to not be involved in p53 and p38 MAPK signaling directly or indirectly through EGFR in MCF7 cells.

The studies have hinted to a positive correlation between increased cellular proliferation with ATAD2 overexpression in breast carcinomas^{116,117,120,121}. Our functional analysis indicated alteration in cell cycle progression in the cells, although not significant, and increased senescence response following ATAD2 depletion. To reveal the detailed mechanism underlying these effects, we examined the expression and the activity of key proteins functioning in the cell cycle and apoptotic pathways.

The expression of two significant cyclins involved in the G1/S transition has been detected with qPCR. Cyclin D expression decreased with ATAD2 downregulation. It is the cyclin which associates with CDK 4/6 and forms active complexes responsible for the early phosphorylation of tumor suppressor RB protein in G1^{277,278}. pRB forms complexes with the E2F transcription factor in its inactive state. When RB is phosphorylated, it dissociates from E2F which allows transcriptional activation of E2F responsive genes required for S phase. Cyclin E is among target genes of E2F. Free E2F following RB phosphorylation activates cyclin E transcription, so early cyclin D synthesis induces subsequent cyclin E expression. The accumulation of cyclin E through G1 enables its interaction with CDK 2^{143,172,211}. This complex later allows G1/S progression through hyperphosphorylation of RB at late G1. Later, hyperphosphorylation activates even more cyclin E transcription and this leads to a positive feedback cycle allowing its accumulation through G1/S. Cyclin E increased slightly upon ATAD2 downregulation. However, as we understand with decreased phosphorylation level of RB in the western blot, decreased cyclin D prevented RB activation. Therefore cyclin E expression did not accumulate to its required level and could not enable RB hyperphosphorylation. This was the first detailed mechanism

underlying ATAD2 mediated G1 arrest in breast cancer cells. pRB hypophosphorylation inhibited cell cycle progression through the S phase.

Next we controlled protein expression levels of key apoptotic proteins in the intrinsic pathway. The pathway stimulation is initiated with BH3-only protein activation. Therefore we first checked Bid cleavage, a BH3-only protein. Loss of ATAD2 activity in the cell has induced Bid cleavage. tBid (truncated form) can interact with BAX and induce MOMP activation. Disruption of mitochondrial membrane integrity releases cytochrome c and pro-apoptotic proteins which results in the activation of caspase-9. We next looked at caspase -9 cleavage to confirm if tBid resulted in successful MOMP activation. It did; however the highest apoptotic response was observed following co-suppression of ATAD2 and ER. . We suggest their co-inhibition has sensitized MCF7 cells to apoptosis. We did not check but we believe, considering MCF7 cells are caspase-3 deficient, that the intrinsic apoptotic response took place with the sequential activation of caspase-7 and -6 following caspase-9 cleavage.

Inhibition of EGFR signaling may be the cause of the apoptotic response in MCF7 cells. If so, this leads to another question. How did ATAD2 regulate EGFR expression? It could be either by transcriptional regulation or by induction of lysosomal degradation. On the basis of the gene expression results, it seems the mechanism by which ATAD2 regulated EGFR gene expression was transcriptional. Phosphorylation of ER α at S118 is a marker of elevated ER signaling in breast cancer and its over-activation is frequently associated with drug resistance^{279,280}. The studies have revealed that EGFR mediated-ERK1/2 activates ER phosphorylation at this site in estrogen independent manner²⁸¹⁻²⁸⁴. Moreover, EGF can mimic estrogen action and it can stimulate cross-talk with ER α signaling pathways, leading to estrogen insensitivity in ER positive cells²⁸⁵. In depth studies have indicated that resistance to estrogen deprivation and to effective inhibitors, such as tamoxifen and fulvestran, is partly based on the association of ER α with EGFR signaling. This was due to the translocation of ER α to the cellular membrane and its direct or indirect interaction with EGFR on the membrane. The studies displayed that ER binding to EGFR increased with a response to tamoxifen²⁸⁶⁻²⁹⁰. Hence, ER signaling may merge into the downstream of EGFR pathways and synergize its effect. Therefore, considering the cross-talk between ER α and EGFR signaling pathways and that ATAD2 is a transcriptional activator of ER α , we asked if EGFR signaling might have modulated ATAD2 gene expression as well. Inhibition of EGFR activity with Gefitinib has

diminished ATAD2 expression. Both gefitinib treatment and EGF stimulation experiments have revealed that ATAD2 gene expression is mediated by EGFR signaling at the transcriptional level. Moreover, its mRNA expression level was positively correlated with EGFR expression. All in all, the results indicated that not only did ATAD2 act as downstream effector of EGFR signaling but it might also be an upstream initiator of EGFR gene expression. This implies a positive feedback regulation between EGFR and ATAD2.

The synergistic activity of ATAD2 and ER α suggests that they act together to regulate EGFR transcription. However so far we could not find an answer to whether transcriptional control of ATAD2 on EGFR expression was dependent on its regulatory effect on ER α expression. We set two different conditions to compare their expressions and clear this mystery. ATAD2 silencing apparently reduced ER α expression in the early event and subsequent inhibition of EGFR expression was a secondary effect. In other words, ER α down-regulation mediated EGFR suppression is a secondary effect of ATAD2 silencing in ER positive breast cancer cells.

ER(-) cells are not responsive to estrogen induction. However we still observed high ATAD2 levels in these cells, additionally its high expression levels remained unchanged during serum starvation experiments for HCC1937 and SKBR3 cells, and still they could respond to ATAD2 downregulation in functional assays. Then how did ATAD2 exert its effects in ER negative cells? What is the molecular basis of its E2-independent actions in these cells? To clear these questions we set experiments with stable ATAD2-downregulated HCC1937 cells. These cells are characterized as a basal-like subtype of breast cancer. One of their molecular signatures of them is an overexpressed EGF receptor. EGFR expression is correlated with ER loss. These cells have 5382insC mutated BRCA1 expression^{32,103}. Mutated BRCA1 is suggested to be associated with high EGFR expression in breast cancer¹⁰³. Due to their molecular constitution, EGFR activity seem to be predominant signaling mechanism in HCC1937 breast cancer cells.

Looking at the gene expression levels following ATAD2 downregulation in HCC1937 cells, a dramatic decrease in the expression of ADAM23 and ADAMTS1 genes along with EGFR and EGR1 was observed. ADAM proteins are disintegrin and metalloproteases. They function in cell-cell and cell-matrix interactions^{291,292}. Several

ADAM family proteins known to mediate EGFR transactivation are stimulated by GPCRs in a variety of cell types^{293,294}. ADAM23 is an adhesion molecule. ADAMTS1 is suggested to have tumor suppression function in breast cancer cells. Expression of both genes is frequently downregulated in tumor progression^{295,296}. HCC1937 cells belong to an aggressive cancer subtype. Considering the oncogenic functions of ATAD2, we would have expected the upregulation of ADAM proteins following ATAD2 suppression, but on the contrary both genes showed a decrease. We could not make any sense of their relation with ATAD2 in that aspect. However, there were a few studies suggesting that ADAM23 may interact with intracellular cytoskeletal systems^{297,298}, so their ATAD2-mediated overexpression may be attributed to this function. Further investigation is needed for a clear picture.

In parallel with MCF7 cells, ATAD2 downregulation in HCC1937 cells reduced cyclin D expression as well. Consistent with previous results, RB phosphorylation was inhibited. HCC1937 cells were the most sensitive cells to ATAD2 downregulation among all four cell lines used during our functional assays. They showed the most dramatic responses in cellular events. We observed that their colony forming abilities and cell migration had been inhibited. These effects may be associated with reduced expression of ATAD2-mediated adhesion molecules. These cells also displayed a shift in their cell cycle distribution more than other breast cancer cells and in parallel a significant increase in apoptotic cells upon inhibition of ATAD2 activity. Therefore, we examined expressions of apoptosis-related proteins. The apoptosis pathway seem to be initiated with Bid cleavage again. However, caspase-9 was not activated in the treated cells. There are studies indicating that Bcl₂ and Bcl_{XL}, the anti-apoptotic proteins, would prevent MOMP activation even in the presence of a death signal. Hence, we checked their expressions in treated cells and this was actually the reason. It appears that both Bcl₂ and Bcl_{XL} protein expressions were decreased slightly, yet the results indicates their insufficient downregulation inhibited the progress of the apoptosis pathway through caspase-9 (Figure 3.23). The proteosomal degradation of anti-apoptotic proteins such as Bcl₂ and Bcl_{XL} is required to trigger signaling cascades of the intrinsic apoptosis pathway because they block the activation of Bax activity upon death signal. It is known that the expression levels of pro- and anti-apoptotic molecules decide the cell fate. Their ratio allows or inhibits cell death.^{61,63,67} However, we observed an apoptotic response to ATAD2 downregulation in these cells in flow cytometry analysis, so how did they exert

this effect? Therefore, another possibility is that ATAD2-downregulation triggered the extrinsic pathway through activation of initiator caspase -8 and subsequent activation of executioner caspases 3-6-7 in HCC1937 cells. If that's the case, the question would be how ATAD2 loss can stimulate death receptors? Maybe the cells stimulated autocrine secretion of the death signal. This assumption should be tested cautiously first before drawing any conclusion.

Functional assays have revealed an inhibitory effect of ATAD2 depletion on cell migration in HCC1937 cells. As a basal-like subtype, they have aggressive molecular features limiting their therapeutic options. EMT markers are highly expressed while epithelial markers are generally lost in these cells¹⁰³. It is suggested that EGFR overactivation may play a role in EMT promotion and the invasive character of these cells²⁹⁹⁻³⁰¹, so we decided to look at the expression state of epithelial and EMT markers. However neither vimentin nor E-cadherin showed any change in their expressions compared to control. Hence, it is clear that ATAD2 did not show its effects on cell motility by means of these proteins. A study demonstrated high ATAD2 expression that in HCC tissues is negatively correlated with expression of CTNNA1, which is a catenin family protein playing a role in cell adhesion and in the same study the knock-down of ATAD2 led to down-regulation of protein¹²⁵. These results are consistent with what we observed in our qPCR result for the ADAM23 and ADAMTS1 genes. It appears that ATAD2 regulates cellular motility by the regulation of cellular adhesion molecules.

ATAD2 reduced EGFR expression at the mRNA level in ER negative cells as well. However, the visible drop in its transcription did not translate into its protein level. On the other hand, we realized its phosphorylation at Tyr1173 has been inhibited with ATAD2 downregulation. It is one of autophosphorylation sites in the receptor and it is known to be involved in MAPK and PI3K/Akt signaling activation following EGF stimulation^{302,303}. We analyzed the expression of key proteins of these pathways. ERK1/2 activity was not affected from loss of EGFR activity. However, it caused enhanced Akt phosphorylation. There wasn't any change in total Akt levels, so it is clear that ATAD2 loss increased Akt activity in the cells. How? HCC1937 cells are PTEN deficient. Studies have revealed cells lacking PTEN have a stable Akt activity at higher threshold and they do not respond to EGFR inhibitors alone³⁰⁴. Our result supports this observation as well and we confirm once more that Akt activity is independent of EGFR signaling in PTEN deficient cells. That is why Akt activity could

not be suppressed in these cells although ATAD2 downregulation inhibited EGFR activation. These emphasize the importance of molecular background on the variety of cellular response. The evaluation of results should therefore be done within the molecular context of the cells.

The most consistent results were observed in the senescence response of all cell types during functional studies of ATAD2. Without exception, independent from their receptor status, all demonstrated activated cellular senescence. Known molecular signatures of senescence response were analyzed, which were p21, p53 and pRB. Senescent cells undergo a chain of molecular changes including p16 and p21 upregulation. The activation of these Cdk allows accumulation of unphosphorylated retinoblastoma protein (pRB) in the cell. Unphosphorylated pRB provokes negative regulation of cell cycle progression^{305,306}. Even though the contribution of Cdk to the senescence mechanism is undisputed, the precise role of each Cdk is not fully understood yet. However, the consensus is that both induce senescent G1 arrest by preventing formation of cyclin-Cdk complexes^{307,308}. Antiproliferative signals stimulate p21 elevation in senescent cells^{168,198}, yet the precise mechanism is still obscure. In MCF7 cells, p21 mRNA expression was increased by 3 folds in response to ATAD2 suppression. It is the main transcriptional target of p53 factor in senescent mechanism; yet, considering that p53 protein expression did not change upon ATAD2 downregulation, increasing p21 may be the driving factor for the induction of senescence response in MCF7 cells. However, p21 mRNA and p53 protein expression levels were hardly changed upon ATAD2 suppression in HCC1937 cells. The western analysis showed ATAD2 downregulation inhibited pRB phosphorylation in both MCF7 and HCC1927 cells. The hypophosphorylation state of protein blocks cell cycle progression through the S phase. Therefore, while elevated p21 levels and hypophosphorylation of pRB are seemingly the underlying inducers of senescence response mediated by ATAD2 depletion in MCF7 cells, it is not clear yet for HCC1937 cells.

We provided a basis for understanding the molecular mechanisms behind cellular functions of the *ATAD2* gene. The relation of the gene with major cellular signaling pathways in breast cancer was studied. We discovered the cross-talk between EGFR signaling and ATAD2 gene through ER α signaling in ER(+) carcinoma cells. On the other hand, although we evidenced its regulatory effect on EGFR activity also in ER(-)

carcinoma cells, their convergent targets could not be highlighted yet in these cells. We described EGFR and ATAD2 as upstream regulators of each other, leading to a positive feedback loop in breast cancer, and propose that ATAD2 exerts its putative tumorigenic effects, such as proliferative, pro-apoptotic and pro-invasive, through EGFR signaling. Breast cancer cells probably favor gain-of-function mechanisms related with the ATAD2 gene, which in turn potentiates EGFR activity.



CHAPTER 5. FUTURE PERSPECTIVES

ATPase family AAA domain-containing 2 (ATAD2) has been identified as a pivotal regulator involved in cell proliferation. Its aberrant expression is reported to contribute to the progression of many different cancer types. ATAD2 was shown here to be highly expressed across breast carcinoma cells independent of their estrogen receptor status. Functional studies of ATAD2 have revealed that it mediates cellular senescence in breast carcinoma and migration of ER(-) breast cancer cells. It is a novel gene that acts in transcriptional circuitry of ER signaling as a co-activator. Further studies have revealed a part of the molecular mechanisms of ATAD2 underlying its effect on cellular processes. The observations have indicated its involvement in EGFR signaling in breast carcinoma. They have also highlighted ATAD2 as an important upstream of EGFR to enhance oncogenesis.

The microarray analysis of ATAD2 downregulated breast cancer cells has uncovered various genes differentially expressed between the treatment and control groups. Further annotation studies have enabled the identification of related cellular processes with selected significantly altered genes. It is required to mine the data for further candidate signaling mechanisms related to ATAD2 activity in breast cancer. The involvement of ATAD2 in EGFR signaling was revealed during those studies. The potential candidates would be used for functional analysis in line with their association with ATAD2. FGF signaling was one of the significantly enriched pathways of downregulated genes following ATAD2 depletion in breast cancer cells. Therefore, it is important to investigate the potential functions of ATAD2 in FGF signaling as well.

Senescence cells illustrate over-activation of growth promoting signaling pathways leading to cellular hypertrophy. Considering ATAD2 downregulation provoked senescence mechanism in breast cancer cells, growth pathways, such as mTOR, should be analyzed in these cells.

ATAD2 was identified as a common signature gene upregulated in expression profiles of breast cancer cells. The importance of this study is that it revealed the first implications regarding the regulation of ATAD2 gene expression. The potential positive-regulatory feedback between EGFR and ATAD2 was highlighted at the

transcription level, yet the results should be validated further with protein analysis. If the results are confirmed, ATAD2 with EGFR may serve as a therapeutic target for breast cancer.

Another important inference of this study was that the molecular background of cancer cells affects the treatment outcome. Breast cancer is a heterogeneous disease and there is a high variety within tumors. Differences among cell types should be considered during the management process. In our study, HCC1937 cells, with over-activated EGFR signaling, showed high sensitivity to ATAD2 suppression. However, SKBR3 cells with upregulated HER2 signaling were not affected to the same degree. This suggests that HER2 overexpression would compensate for the loss of ATAD2 activity in these cells. Therefore, their combined inhibition may offer a better option for disease management. In conclusion, the additional molecular signatures specific to each type along with ATAD2 may provide new approaches for optimal therapeutic strategies.

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APPENDICES

Appendix A- Gene lists

Appendix Table 1: Top 50 probe sets upregulated by ATAD2 silencing in T47D cells (P< 0.05)

#	Probe set	Gene symbol	Parametric p-value	Fold Change*
1	1561817_at	NA	< 1e-07	7.14
2	203665_at	HMOX1	< 1e-07	6.67
3	39248_at	AQP3	< 1e-07	5.88
4	205306_x_at	KMO	< 1e-07	5.26
5	203153_at	IFIT1	< 1e-07	5.26
6	211138_s_at	KMO	< 1e-07	5.26
7	207430_s_at	MSMB	< 1e-07	5.00
8	240272_at	NA	< 1e-07	4.55
9	213796_at	SPRR1A	< 1e-07	4.55
10	229450_at	IFIT3	< 1e-07	4.17
11	226363_at	ABCC5	< 1e-07	4.00
12	207802_at	CRISP3	< 1e-07	3.85
13	206263_at	FMO4	< 1e-07	3.70
14	223503_at	TMEM163	< 1e-07	3.70
15	1569454_a_at	LOC283352	< 1e-07	3.70
16	203767_s_at	STS	< 1e-07	3.57
17	233576_at	HMGCLL1	< 1e-07	3.45
18	205483_s_at	ISG15	< 1e-07	3.45
19	239823_at	NA	< 1e-07	3.45
20	226757_at	IFIT2	< 1e-07	3.33
21	202411_at	IFI27	< 1e-07	3.23
22	209969_s_at	STAT1	< 1e-07	3.23
23	220622_at	LRRC31	< 1e-07	3.23
24	237168_at	NA	< 1e-07	3.23
25	1553602_at	MUCL1	< 1e-07	3.23
26	218943_s_at	DDX58	< 1e-07	3.23
27	204415_at	IFI6	< 1e-07	3.13
28	206378_at	SCGB2A2	< 1e-07	3.13
29	201411_s_at	PLEKHB2	< 1e-07	3.03
30	226702_at	CMPK2	< 1e-07	3.03
31	212688_at	PIK3CB	< 1e-07	3.03
32	209368_at	EPHX2	< 1e-07	3.03
33	210297_s_at	MSMB	< 1e-07	3.03
34	229778_at	C12orf39	< 1e-07	2.94
35	231233_at	NA	< 1e-07	2.94

36	236125_at	NA	< 1e-07	2.94
37	207174_at	GPC5	< 1e-07	2.94
38	241310_at	NA	< 1e-07	2.94
39	216634_at	PLCH1	< 1e-07	2.86
40	240069_at	NA	< 1e-07	2.86
41	203820_s_at	IGF2BP3	< 1e-07	2.86
42	207018_s_at	RAB27B	< 1e-07	2.86
43	220115_s_at	CDH10	< 1e-07	2.86
44	201410_at	PLEKHB2	< 1e-07	2.78
45	202883_s_at	PPP2R1B	< 1e-07	2.78
46	204137_at	GPR137B	< 1e-07	2.78
47	205352_at	SERPINI1	< 1e-07	2.78
48	207437_at	NOVA1	< 1e-07	2.78
49	1557038_s_at	LOC100289373	< 1e-07	2.78
50	230278_at	NA	< 1e-08	2.78

**Fold change: siATAD2/siCntrl*

Appendix Table 2: Top 50 probe sets downregulated by ATAD2 silencing in T47D cells (P< 0.05)

#	Probe set	Gene symbol	Parametric p-value	Fold Change*
1	206502_s_at	INSM1	< 1e-07	7.25
2	222740_at	ATAD2	< 1e-07	5.92
3	218782_s_at	ATAD2	< 1e-07	5.78
4	228401_at	ATAD2	< 1e-07	5.56
5	205239_at	AREG	< 1e-07	5.15
6	210609_s_at	TP53I3	< 1e-07	5.12
7	204014_at	DUSP4	< 1e-07	4.22
8	226633_at	RAB8B	< 1e-07	4.12
9	231040_at	NA	< 1e-07	4
10	230356_at	NA	< 1e-07	3.95
11	242385_at	RORB	< 1e-07	3.94
12	224694_at	ANTXR1	< 1e-07	3.83
13	215030_at	GRSF1	< 1e-07	3.76
14	222846_at	RAB8B	< 1e-07	3.67
15	212636_at	QKI	< 1e-07	3.66
16	224215_s_at	DLL1	< 1e-07	3.58
17	227963_at	NA	< 1e-07	3.57
18	221935_s_at	C3orf64	< 1e-07	3.47
19	218031_s_at	FOXN3	< 1e-07	3.42
20	240633_at	DOK7	< 1e-07	3.41
21	222494_at	FOXN3	< 1e-07	3.32
22	209285_s_at	C3orf63	< 1e-07	3.28
23	209337_at	PSIP1	< 1e-07	3.24

24	214023_x_at	TUBB2B	< 1e-07	3.23
25	227654_at	FAM65C	< 1e-07	3.18
26	227361_at	HS3ST3B1	< 1e-07	3.18
27	202350_s_at	MATN2	< 1e-07	3.17
28	228455_at	RBM15	< 1e-07	3.12
29	206115_at	EGR3	< 1e-07	3.1
30	219115_s_at	IL20RA	< 1e-07	3.09
31	200709_at	FKBP1A	< 1e-07	2.99
32	235266_at	ATAD2	< 1e-07	2.94
33	230892_at	NA	< 1e-07	2.94
34	204619_s_at	VCAN	< 1e-07	2.93
35	238803_at	HECTD2	< 1e-07	2.84
36	206067_s_at	WT1	< 1e-07	2.78
37	203570_at	LOXL1	< 1e-07	2.77
38	232024_at	GIMAP2	< 1e-07	2.77
39	214119_s_at	FKBP1A	< 1e-07	2.76
40	1567014_s_at	NFE2L2	< 1e-07	2.75
41	212073_at	NA	< 1e-07	2.73
42	201508_at	IGFBP4	< 1e-07	2.73
43	215719_x_at	FAS	< 1e-07	2.73
44	1556054_at	NA	< 1e-07	2.71
45	208925_at	CLDND1	< 1e-07	2.7
46	202271_at	FBXO28	< 1e-07	2.69
47	217980_s_at	MRPL16	< 1e-07	2.69
48	202409_at	NA	< 1e-07	2.68
49	238719_at	NA	< 1e-07	2.64
50	239010_at	FLJ39632	< 1e-07	2.62

*Fold change: 1/(siATAD2/siCtrl)

Appendix Table 3: Top 50 probe sets upregulated by ATAD2 silencing in MCF7 cells (P<0.05)

#	Probe set	Gene symbol	Parametric p-value	Fold Change*
1	206488_s_at	CD36	< 1e-07	62.5
2	209555_s_at	CD36	< 1e-07	40.0
3	204137_at	GPR137B	< 1e-07	31.3
4	228766_at	CD36	< 1e-07	31.3
5	230278_at	NA	< 1e-07	28.6
6	204637_at	CGA	< 1e-07	23.8
7	211138_s_at	KMO	< 1e-07	17.5
8	203665_at	HMOX1	< 1e-07	16.4
9	205306_x_at	KMO	< 1e-07	15.4
10	201427_s_at	SEPP1	< 1e-07	15.2
11	210095_s_at	IGFBP3	< 1e-07	12.5

12	229620_at	SEPP1	< 1e-07	11.1
13	202291_s_at	MGP	< 1e-07	10.0
14	226535_at	ITGB6	< 1e-07	10.0
15	202917_s_at	S100A8	< 1e-07	9.1
16	241873_at	NA	< 1e-07	7.7
17	212143_s_at	IGFBP3	< 1e-07	7.7
18	201667_at	GJA1	< 1e-07	7.7
19	209392_at	ENPP2	< 1e-07	7.1
20	1553226_at	NCRNA00052	< 1e-07	7.1
21	205234_at	SLC16A4	< 1e-07	6.7
22	206280_at	CDH18	< 1e-07	6.7
23	216092_s_at	SLC7A8	< 1e-07	6.3
24	207245_at	UGT2B17	< 1e-07	5.9
25	214455_at	HIST1H2BC	< 1e-07	5.9
26	220448_at	KCNK12	< 1e-07	5.6
27	1553449_at	C16orf81	< 1e-07	5.6
28	1554190_s_at	C10orf81	< 1e-07	5.6
29	202752_x_at	SLC7A8	< 1e-07	5.6
30	219768_at	VTCN1	< 1e-07	5.6
31	227919_at	UCA1	< 1e-07	5.3
32	208083_s_at	ITGB6	< 1e-07	5.3
33	202748_at	GBP2	< 1e-07	5.3
34	212912_at	RPS6KA2	< 1e-07	5.3
35	214652_at	DRD1	< 1e-07	5.3
36	210141_s_at	INHA	< 1e-07	5.3
37	218559_s_at	MAFB	< 1e-07	5.3
38	207802_at	CRISP3	< 1e-07	5.0
39	201278_at	DAB2	< 1e-07	5.0
40	236193_at	HIST1H2BC	< 1e-07	5.0
41	211653_x_at	AKR1C2	< 1e-07	4.8
42	1553746_a_at	C12orf64	< 1e-07	4.8
43	213371_at	LDB3	< 1e-07	4.8
44	201998_at	ST6GAL1	< 1e-07	4.8
45	209310_s_at	CASP4	< 1e-07	4.8
46	225915_at	CAB39L	< 1e-07	4.8
47	231929_at	IKZF2	< 1e-07	4.8
48	209474_s_at	ENTPD1	< 1e-07	4.8
49	1560071_a_at	NA	< 1e-07	4.5
50	205357_s_at	AGTR1	5E-07	4.3

**Fold change: siATAD2/siCntrl*

Appendix Table 4: Top 50 probe sets downregulated by ATAD2 silencing in MCF7 cells (P< 0.05)

#	Probe set	Gene symbol	Parametric p-value	Fold Change*
1	205239_at	AREG	< 1e-07	7.97
2	226085_at	CBX5	< 1e-07	7.17
3	222740_at	ATAD2	< 1e-07	6.33
4	237435_at	NA	< 1e-07	6.14
5	205258_at	INHBB	< 1e-07	6.06
6	218782_s_at	ATAD2	< 1e-07	6.01
7	203913_s_at	HPGD	< 1e-07	6
8	211548_s_at	HPGD	< 1e-07	5.84
9	218031_s_at	FOXN3	< 1e-07	5.74
10	231145_at	NA	< 1e-07	5.67
11	219294_at	CENPQ	< 1e-07	5.55
12	212636_at	QKI	< 1e-07	5.5
13	225060_at	LRP11	< 1e-07	5.49
14	228401_at	ATAD2	< 1e-07	5.43
15	206247_at	MICB	1E-07	5.43
16	238506_at	LRRC58	< 1e-07	4.86
17	218729_at	LXN	< 1e-07	4.83
18	232197_x_at	ARSB	< 1e-07	4.81
19	203741_s_at	ADCY7	< 1e-07	4.75
20	224129_s_at	DPY30	< 1e-07	4.54
21	222494_at	FOXN3	< 1e-07	4.5
22	214156_at	MYRIP	< 1e-07	4.44
23	222846_at	RAB8B	< 1e-07	4.44
24	209230_s_at	NUPR1	< 1e-07	4.4
25	229459_at	FAM19A5	< 1e-07	4.36
26	242342_at	NA	< 1e-07	4.28
27	203432_at	TMPO	< 1e-07	4.25
28	226633_at	RAB8B	< 1e-07	4.21
29	208925_at	CLDND1	< 1e-07	4.2
30	239903_at	NA	< 1e-07	4.18
31	206074_s_at	HMGA1	< 1e-07	4.16
32	235088_at	C4orf46	< 1e-07	4.12
33	238015_at	C4orf46	< 1e-07	4.08
34	238623_at	NA	< 1e-07	4.08
35	209337_at	PSIP1	< 1e-07	4.06
36	209560_s_at	DLK1	< 1e-07	4.04
37	1562484_at	C17orf104	1E-07	4.04
38	225479_at	LRRC58	< 1e-07	4.03
39	236513_at	NA	2E-07	4.01
40	226702_at	CMPK2	1E-07	3.95
41	225438_at	NUDCD1	< 1e-07	3.92

42	235363_at	NA	< 1e-07	3.89
43	225681_at	CTHRC1	< 1e-07	3.89
44	226611_s_at	CENPV	< 1e-07	3.87
45	206091_at	MATN3	1.8E-06	3.86
46	224071_at	IL20	< 1e-07	3.85
47	211958_at	IGFBP5	< 1e-07	3.81
48	238029_s_at	SLC16A14	< 1e-07	3.79
49	212215_at	PREPL	< 1e-07	3.78
50	213397_x_at	NA	< 1e-07	3.73

**Fold change: 1/(siATAD2/siCntrl)*



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