INVESTIGATION OF THE EFFECTS OF GTF2A1-ANTISENSE LONG NON-CODING RNA ON CELL FATE

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

MASTER OF SCIENCE

in Molecular Biology and Genetics

by
Yusuf Cem ÇİFTÇİ

December 2022
İZMİR

ACKNOWLEDGEMENTS

Foremost, I would like to express my sincere gratitude to my supervisor Prof. Dr. Bünyamin AKGÜL. His guidance, advice and immense knowledge carried me through all the stages of my Master Thesis. I want to express my thanks and regards to TUBITAK (Scientific and Technological Research Council of Turkey) due to their support and funding (Project No: 122Z759).

I would like to express my thanks to my committee members Assoc. Prof. Dr. Özden YALÇIN ÖZUYSAL and Assist. Prof. Dr. Yavuz OKTAY for their valuable time and insightful comments to support my thesis.

I would like to give my warmest thanks to Dr. İpek ERDOĞAN VATANSEVER and Dr. Özge TÜNCEL for their technical support, friendship, and guidance. They have a heart of gold. I am also deeply thankful to Dilek Cansu GÜRER, Ayşe Bengisu GELMEZ and Bilge YAYLAK for their great support and friendship. I extend my gratitude to my other colleagues, Vahide İlayda KAÇAR, Şirin Elife CEREN, Melis ATBİNEK, Merve KARA, Azime AKÇAÖZ and Buket SAĞLAM for their help. I am also thankful to Biotechnology and Bioengineering Central Research specialists Özgür OKUR for her kindness and help during my studies.

Last but not the least, I would like to extend my gratitude to my family for endless support and Nil Hazal EKENEL who is my bluebird singing in my heart.

ABSTRACT

INVESTIGATION OF THE EFFECTS OF GTF2A1-ANTISENSE LONG NON-CODING RNA ON CELL FATE

Apoptosis is a distinct mode of programmed cell death whereby cellular contents are broken down and accumulated in the apoptotic bodies. The vast majority of the genome consists of non-coding RNAs (ncRNA). NcRNAs can be divided into groups depending on their length, for example, long non-coding RNA (lncRNA) longer than 200 nucleotides. It has been demonstrated that these have important roles in the development, and treatment of cancer and in other diseases that critically affect human life. Considering the lncRNAs' mechanisms of action on apoptosis, they modulate activity of transcription factors, regulate miRNAs, and interact with proteins related to histone mechanisms such as chromatin modifier. In this perspective, GTF2A1-AS which is an uncharacterized and novel lncRNA was found as one of highly expressed lncRNA in transcriptomic data obtained from HeLa cells treated with cisplatin. The potential role of GTF2A1-AS within the cell was investigated through the transcriptomic data provided by GTF2A1-AS knockdown. It has been found that specific gene clusters mainly enriched in the pathway which is Defective Homology directed Repair through Homologous Recombination. In this process, double-strand breaks are repaired with the help of BRCA1/2, RAD50, RAD51, PALB2 proteins which are known as DNA damage response proteins. Thus, the genes related with DNA damage response were selected to validate the transcriptomic data. In light of this information, GTF2A1-AS knockdown has resulted in an increase in the early apoptosis in HeLa cells. Additionally, when GTF2A1-AS knockdown was combined with cisplatin, it sensitized HeLa cells against cisplatin by affecting late apoptosis, specifically. Consequently, GTF2A1-AS as a cisplatin inducible lncRNA modulates apoptosis and chemosensitivity in HeLa cells.

Keywords: *Apoptosis, DNA Damage Response, Long Non-Coding RNA, Antisense, GTF2A1-AS,*

ÖZET

GTF2A1-ANTİSENS UZUN KODLAMAYAN RNA'SININ HÜCRE KADERİ ÜZERİNDEKİ ETKİLERİNİN ARAŞTIRILMASI

Apoptoz, hücresel içeriklerin parçalandığı ve apoptotik cisimlerde biriktiği, programlanmış hücre ölümünün farklı bir modudur. Genomun büyük çoğunluğu kodlayıcı olmayan RNA'lardan oluşur. Kodlamayan RNA'lar, uzunluklarına bağlı olarak gruplara ayrılabilir, örneğin, 200 nükleotidden uzun olan, uzun kodlamayan RNA (ukmRNA). Bunların kanser ve insan hayatını kritik derecede etkileyen diğer hastalıkların gelişimi ve tedavisinde önemli rolleri olduğu kanıtlanmıştır. UkmRNA'ların apoptoz üzerindeki etki mekanizmaları göz önüne alındığında, transkripsiyon faktörlerinin aktivitesini modüle ederler, miRNA'ları düzenlerler ve kromatin değiştirici gibi histon mekanizmalarıyla ilgili proteinlerle etkileşime girerler. Bu açıdan, sisplatin ile muamele edilmiş HeLa hücrelerinden elde edilen transkriptomik verilerde, karakterize edilmemiş ve yeni bir uzun kodlamayan RNA olan GTF2A1-AS, yüksek oranda eksprese edilen ukmRNA'lardan biri olarak bulundu. GTF2A1-AS'nin hücre içerisindeki potansiyel rolü, GTF2A1-AS susturulması yoluyla sağlanan transkriptomik veriler aracılığıyla araştırıldı. Spesifik gen kümelerinin, "Homolog Rekombinasyon Yoluyla Kusurlu Homolojiye Yönelik Onarım" yolağında ağırlıklı olarak yoğunlaştığı bulunmuştur. Bu süreçte DNA hasar yanıt proteinleri olarak bilinen BRCA1/2, RAD50, RAD51, PALB2 proteinleri yardımıyla çift zincir kırıkları onarılır. Böylece, bu transkriptomik verilerini doğrulamak için DNA hasar yanıtı ile ilgili genler seçildi. Bu bilgiler ışığında GTF2A1-AS susturulması, HeLa hücrelerinde erken apoptozda artış ile sonuçlanmıştır. Ayrıca sisplatin ile birleştiğinde özellikle geç apoptozu etkileyerek HeLa hücrelerinin sisplatine karşı hassasiyetine yol açmıştır. Sonuç olarak, bir sisplatin indüklenebilir ukmRNA olarak GTF2A1-AS, HeLa hücrelerinde apoptozu ve kemosensitiviteyi düzenler.

Anahtar Kelimeler: *Apoptoz, DNA Hasar Yanıtı, Uzun Kodlamayan RNA, Antisens, GTF2A1-AS*

TABLE OF CONTENTS

LIST OF FIGURES…….....vii

LIST OF TABLE…….....……ix

CHAPTER 1. INTRODUCTION……..1

1.1. Overview of Apoptosis. . ……1

1.1.1. Intrinsic Pathway……... ……..2

1.1.2. Extrinsic Pathway…….. ……..4

1.2. Long Non-Coding RNAs……4

1.2.1. Classification of Long Non-Coding RNAs……..5

1.2.2. Mode of Actions of Long Non-Coding RNAs…….7

1.2.3. The Role of Long Non-Coding RNA in Apoptosis……..8

1.3. GTF2A1-AS Long Non-Coding RNA …….11

1.4. Aim……...12

CHAPTER 2. MATERIALS AND METHODS……..13

2.1. Cell Culture, Transfection, Drug Treatment…. … ……...……13

2.2. Apoptosis Rate Measurement…… …… ..……14

2.3. RNA Isolation……...14

2.4. cDNA Synthesis and Quantitative PCR……15

2.5. Bioinformatic Analysis…….16

2.6. Protein Isolation and Western Blotting…….17

CHAPTER 3. RESULTS…….19

3.1. Expression Level of GTF2A1-AS In The Presence of Cisplatin..…19

3.2. Revealing The Potential Role of GTF2A1-AS……..……19

3.3. Bioinformatic Analysis of Transcriptomic Data……22

3.4. Time-Kinetic Experiments…… …….. ……..…26

3.5. Effect of GTF2A1-AS Knockdown on Apoptosis…...……..28

3.6. Effect of GTF2A1-AS Knockdown on Chemosensitivity of HeLa Cells Against Cisplatin……..30
3.7. Effect of GTF2A1-AS Overexpression on Apoptosis……..32
3.8. Effect of GTF2A1-AS Knockdown on Chemosensitivitv of HeLa Cells Against Cisplatin……..34
3.9. Effect of GTF2A1-AS on Its Coding Gene……..37

CHAPTER 4. DISCUSSION……...38

CHAPTER 5. CONCLUSION……..…43

REFERENCES……44

LIST OF FIGURES

Figures **Page**

Figure 1.1. Schematic Illustration of Intrinsic and Extrinsic Pathways of Apoptosis. ..…3
Figure 1.2. Schematic Illustration of Classification of LncRNAs With Respect to Genomic Arrangements……6
Figure 1.3. Schematic Illustration of Classification of LncRNAs With Respect to Mode of Actions……. ……..8
Figure 1.4. Schematic Representation of Chromosomal Location GTF2A1-AS……..…….11
Figure 3.1. Expression Levels of GTF2A1-AS In Between HeLa Cells Treated With Cisplatin and DMSO……...20
Figure 3.2. The Potential Phenotypic Profiles of GTF2A1-AS Gene …….……..21
Figure 3.3. qPCR Result of GapmeR mediated GTF2A1-AS Gene ……..……..23
Figure 3.4. The Most Significant Enriched Pathways For Common Differentially Expressed Genes…… . ……...24
Figure 3.5. Gene Set Enrichment Analysis (GSEA) Results of Differentially Expressed Genes From GTF2A1-AS Knockdown RNA-seq Data.……..……25
Figure 3.6. qPCR Results of Phenotype Related Candidate Genes ……..26
Figure 3.7. Apoptotic Rates of GTF2A1-AS-Gapmer mediated Hela cells With Respect To Time-Kinetic Manner……..…….…….28
Figure 3.8. Representation of Apoptotic Rates of GTF2A1-AS-GapmeR Mediated Hela Cells…… ..……..... 29
Figure 3.9. Representation of Apoptotic Rates of GTF2A1-AS-GapmeR Mediated Hela Cells In Combination With Cisplatin Treatment…….31
Figure 3.10. qPCR Results of Overexpression of GTF2A1-AS Gene….……32
Figure 3.11. Representation of Apoptotic Rates of pcDNA3.1-GTF2A1-AS Mediated Hela Cells…...……...33
Figure 3.12. Representation of Apoptotic Rates of pcDNA3.1-GTF2A1-AS Mediated Hela Cells In Combination With Cisplatin Treatment…..……35

Figures **Page**

Figure 3.13. Representation of cPARP Expression Levels of pcDNA3.1-GTF2A1-AS Mediated Hela Cells In Combination With Cisplatin Treatment……....36
Figure 3.14. qPCR results of GTF2A1 mRNA Expression Under Different Conditions…….….37
Figure 4.1. Putative Protein Interactions of GTF2A1-AS With Respect To POSTAR3 Tool……..42

LIST OF TABLE

Table **Page**

Table 2.1. Representation of The Primer Sequences Used In This Study……....16

CHAPTER 1

INTRODUCTION

1.1. Overview of Apoptosis

The word apoptosis was first defined by Kerr, Wyllie, and Currie in 1972 to describe a type of cell death that differs from others in terms of its physical properties (Kerr et al. 1972). Apoptosis is a process in which a cell stops its growth and division in a controlled manner, thus resulting in death before the cell contents are released into the environment. Apoptosis is sometimes referred to as cell suicide or more generally programmed cell death. There are proteases, defined as caspases, which are necessary for the initiation of apoptosis in the cell, and the process is initiated depending on their activation. These caspases are divided into two as initiator and executioner caspases, depending on their intracellular function (Elmore 2007). Due to their role as initiator caspases, caspases 8 and 9 are induced when a disorder is detected in the cell and cause activation of caspases 3, 6, and 7 as executioner caspases. With the activation of the executory caspases, several events are initiated in the cell, such as breaking the DNA into small pieces, degrading the proteins in the nucleus and cytoskeleton, and forming the apoptotic body (Martinvalet et al. 2005; Poon et al. 2014). Generally speaking, apoptosis can be distinguished from necrosis, a type of unprogrammed cell death, both morphologically when viewed under the microscope and with various molecular biology applications. Apoptotic bodies that occur after the formation of apoptosis prevent the cell contents from spreading to the surrounding environment, and this reduces the risk of negative effects on the surrounding tissues. These cells are then destroyed by macrophages, an immune system cell that is permanently present in the tissues (Elmore 2007). Apoptosis is conserved in complex organisms from an evolutionary perspective and is also governed by certain genes throughout the process (Lockshin and Zakeri 2004).

Apoptosis can be initiated by a mechanism called the intrinsic pathway, by detecting damage in the cell with various sensors. On the other hand, apoptosis can also

be initiated by the immune system contacting a damaged cell by a mechanism called the extrinsic pathway (Sica et al 1990; Oppenheim et al 2001) (Figure 1.1). Considering that apoptosis occurs in approximately $1x10^9$ cells per day in the human body, it seems that apoptosis works as a protection mechanism in complex organisms and eliminates damaged cells (Elliott and Ravichandran 2010). The fact that the apoptosis mechanism is mutated, as in cancer cells, or if it works less, causes the cells to divide uncontrollably. In other case, apoptotic enzymes are highly activated in Alzheimer disease (Dickson 2004).

1.1.1. Intrinsic Pathway

The intrinsic pathway, also known as the mitochondrial pathway of apoptosis, involves stimuli that can have a wide variety of effects within the cell (Igney and Krammer 2002). This subtype of apoptosis requires various factors released from the mitochondria to occur, and this process can originate in two ways, called positive or negative. These signals, known as negative signals, occur due to the lack of hormones required for cell growth, cytokines, and growth factors required for intercellular interaction in the outer periphery of the cell. Prosurvival proteins such as Puma, Noxa, and Bax, which are normally inactive in a healthy cell, are induced due to the lack of prosurvival signals and prepare the ground for the initiation of apoptosis. On the other hand, intrinsic apoptosis can also be initiated by hypoxia, which is active due to oxygen deficiency, also known as the positive pathway, toxins produced by bacteria or various pathogens, radiation, or reactive oxygen species with increased levels in the cell (Brenner and Mak 2009). However, for some cells, such as neutrophils, this provides an advantage for the survival mechanism (Walmsley et al. 2005). Caspase-9, which plays a role as an initiator caspase in the intrinsic pathway of apoptosis, binds to the caspase recruitment (CARD) domain of APAF-1, known as apoptotic protease activating factor 1, which has an important effect on the intrinsic pathway. In a normal cell, APAF1 is mostly found with CARD domain I blocked, preventing Caspase-9 from binding and the initiation of apoptosis. Apoptosis initiated by positive or negative stimuli causes changes in the membrane of the mitochondria inside the cell, thus opening the pores in the outer membrane of the mitochondria, and mitochondrial permeability transition is provided. Proteins known as proapoptotic proteins such as cytochrome c and Smac/Diablo are

released from mitochondria to the cytoplasm and irreversibly maintain apoptosis (Cain et al. 2002). Cytochrome c released from mitochondria binds to the WD domain of APAF-1 monomers, causing a conformational change. As a result, APAF-1 causes both oligomerization and the emergence of the CARD domain. These conformational changes allow several APAF1 to come together to form a complex known as an apoptosome (Acehan et al. 2002). CARD domains in the middle of the apoptosome complex are positioned to allow Caspase-9 assembly. Then, the apoptosome bound to the activated Caspase-9 enzymes becomes capable of converting the executioner procaspase-3 into its activated form, which will cause complete cell death (Cain et al. 2002). However, Smac/Diablo proteins released from mitochondria contribute to the occurrence of apoptosis by inactivating IAPs, which are apoptosis inhibitor proteins. However, IAP inactivation alone cannot induce apoptosis without the release of cytochrome c (Ekert and Vaux 2005).

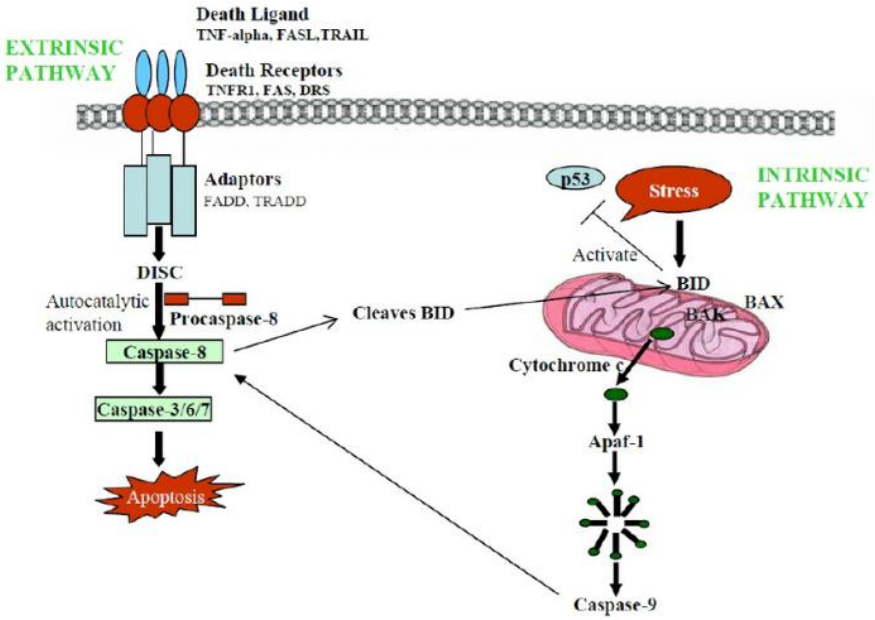


Figure 1.1. Schematic illustration of intrinsic and extrinsic pathways of apoptosis. Depending on type of stimuli, apoptosis is triggered by two distinct pathways which are intrinsic and extrinsic pathways. In intrinsic pathway, the stimuli occurred within the cell cause mitochondrial pore formation, apoptosome formation and eventually caspase-9 activation. In extrinsic pathway, death ligands are sensed by death receptors and which in turn, causes Death inducing signaling complex (DISC) and eventually, caspase-8 activation (Source: D'Arcy, 2019).

1.1.2. Extrinsic Pathway

The extrinsic pathway, which can also be defined as the death receptor pathway of apoptosis, is induced by death ligands produced by natural killer cells and macrophages that bind to death receptors in the cell membrane (Igney and Krammer 2002). These death ligands trigger the conversion of procaspase-8 to its active form, caspase-8 (Kim et al. 2004). Death receptors belong to a superfamily, also known as tumor necrosis factor, and these receptors are activated specifically by death ligands (Bossen et al. 2006). When the death ligand binds to the death receptor, it causes procaspase-8 to recruit through the death-inducing (DED) domain to the DISC domain located in the cytoplasmic part of the death receptor. However, the death-inducing signal complex (DISC) domain contains several proteins identified as the FAS-associated death (FADD) domain and the TNF-receptor (TNFR)-associated death (TRADD) domain to enable interaction with procaspase-8 (Kim et al. 2004). Dimerization and induction of procaspase-8 occur by recruitment of procaspase-8 to the DISC domain, and as a result, activated caspase-8 can trigger apoptosis in two different ways. The pathway chosen to induce apoptosis depends on the status of cells classified as type 1 or type 2 (Samraj et al. 2006). In type 1 cells, the Caspase-8 executor interacts directly with caspases, enabling them to be activated and directly causing apoptosis. In the absence of proteins released from the outer membrane of mitochondria in type 2 cells, IAPs directly stop the activation of executioner caspases (Spencer et al. 2009).

1.2. Long Non-Coding RNAs

Only 2% of the human genome is composed of protein-coding genes, and the remaining part is not functionally defined. Research over the past 20 years has triggered an interest in the remaining 98%, and it has been revealed that the vast majority of this portion consists of ncRNAs (Birney et al 2007). ncRNA can be divided into groups depending on their length, for example, microRNAs are 22 nucleotides in length on average or lncRNA are longer than 200 nucleotides. It has been demonstrated that these RNAs have important roles in the development, diagnosis, and treatment of cancer and in

other diseases that critically affect human life, such as cardiovascular diseases, and neuronal disorders. (Peng et al. 2017; Korostowki et al 2012; Faravelli et al. 2017).

1.2.1. Classification of Long Non-Coding RNAs

In general, lncRNAs are a type of ncRNAs, apart from previously defined ribosomal RNAs longer than 200 nucleotides (Kapranov et al. 2007). However, Amaral's group brought a new perspective to this definition. According to this definition, lncRNAs are ncRNAs that can act as primary transcripts, unlike RNAs that are smaller in length. Another distinguishing feature is that, according to this side, the length of lncRNAs is not defined as an important principle (Amaral et al. 2011). Therefore, there are ncRNAs that are normally quite short but functionally included in the lncRNA class, for example, BC1 that has been shown to have a function in neurons (Tiedge et al. 1991)

Although possessing open reading frame and protein-coding are unique to protein-coding genes, there are many similarities between lncRNAs and protein-coding genes. Most lncRNAs have a 5' cap, polyadenylation at the 3' end and they have many exon regions. Also, transcription of both is carried out by RNA polymerase II or III (Ahanda and Ruby 2009; Erdmann et al. 1999). Another feature is that lncRNAs regulate gene expression in a transcriptional and posttranscriptional manner by interacting with various transcriptional factors and adaptor proteins (Dykes and Emanueli 2017). One difference between the two, from an evolutionary point of view, is less conservation compared to protein-coding genes (Johnsson et al. 2014). As shown in the studies, the function of lncRNAs is determined by the tertiary structure formed by folding within itself after transcription. However, studies continue to classify lncRNAs and identify new functional aspects, and there are specific methods for their classifications (Yan et al. 2016).

LncRNAs are classified in two ways, based on their functional properties and their location in the genome. In the classification according to their functional properties, they are divided according to a) their effects on the DNA sequence, b) the ways of action, such as taking a role at the transcriptional and posttranscriptional level, c) their target mechanisms, and d) their genomic locations (Dykes and Emanueli 2017). They are divided into 5 sub-groups based on genomic locations: 1) sense lncRNAs that create transcripts with protein-coding genes from the same DNA chain and in the same direction, but are likely to contain many exons or introns; 2) antisense lncRNAs that generate

transcripts from the opposite DNA strand of protein-coding genes, but are likely to contain many exons and introns; 3) intronic lncRNAs that generate transcripts only from the intronic region of a protein-coding gene; 4) intergenic lncRNAs located between two protein-coding genes and generate transcript in the same direction; 5) bidirectional lncRNAs that are located only within the 1 kb portion of the promoter region of the protein-coding gene and generate transcripts from the opposite DNA strand (Kopp et al. 2019) (Figure 1.2).

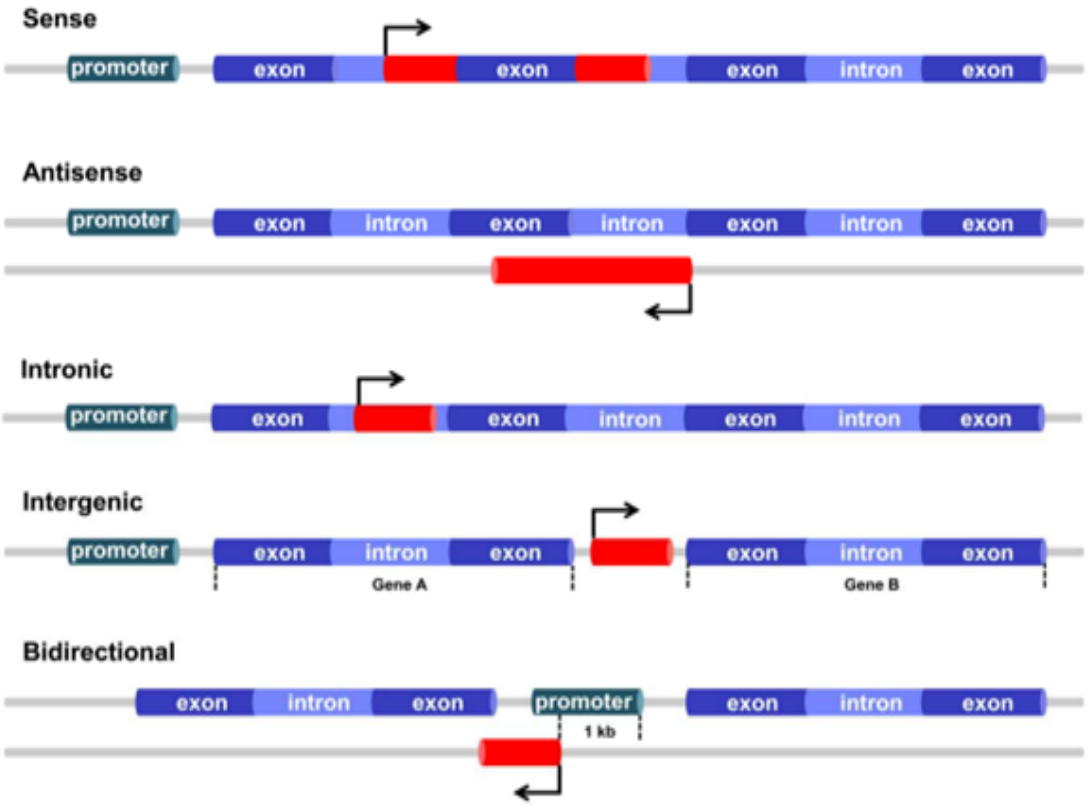


Figure 1.2. Schematic illustration of classification of lncRNAs with respect to their genomic context. In this scope, lncRNAs are classified as sense, anti-sense, intronic, intergenic and bidirectional (Source: Hermans-Beijnsberger et al. 2018).

1.2.2. Mode of Actions of Long Non-Coding RNAs

Recent functional analyses of lncRNAs have begun to reveal the mode of actions of lncRNAs. Therefore, based on this information, lncRNAs are classified as a signal, decoy, guide, scaffold, enhancer, and circular lncRNA according to their mechanism of action (Ma et al. 2013) (Figure 1.3).

Signal lncRNAs are expressed at specific times temporally, for example during early development. Its effects vary depending on the type of cell it is in, and changes in expression can be observed depending on the stimuli (Wang and Chang 2011). For example, KCNQ1OT1 lncRNA contributes to cell differentiation during early development by interacting with chromatin-modifying enzymes that alter chromatin structure to affect gene expression (Pandey et al. 2008). Decoy lncRNAs change their activities by interacting with transcription factors, chromatin modifiers, and RNA binding proteins, which can significantly affect gene expression in the cell (Wang and Chang 2011). For example, TERRA interacts directly with the enzyme telomerase, a member of the lncRNA telomerase complex, thereby affecting its biological function (Redon et al. 2010). As another example, MALAT1 as a decoy lncRNA directly interacts with metastasis-triggering factors, causing an increase in the expression of genes that increase cancer progression (Tripathi et al. 2010). Guide lncRNAs combine with ribonucleoproteins to form a complex, which directs these complexes to target genes, regulating the activation or inhibition of related genes (Wang and Chang 2011). Guide lncRNAs and scaffold lncRNAs, which can be compared due to their mechanism of action, differ from each other in the types of protein complexes with which they interact, as several studies have shown. Scaffold lncRNA has also been shown to regulate chromatin and histone modifiers. However, there is also disagreement as to whether these lncRNAs are guide or scaffold (Gutmann 2011; Tsai et al. 2010). Enhancer lncRNAs formed from the enhancer region of the gene have a significant effect on the activation of the relevant gene (Long et al. 2017). For example, some enhancer lncRNAs have been shown to regulate the transcription of genes activated in the presence of estrogen (Li et al. 2013).

Classification of lncRNAs remains contradictory and needs strong proof. For example, it has been observed that some lncRNAs can be both decoy and signal lncRNA

as their mechanism of action, while at the same time they can be both intergenic and intronic in genomic locations. It is thought that this contradictory situation will be clearly understood as researches on the tertiary structure of lncRNAs give clearer results. Thus, it will be easier to understand the protein complexes or transcription factors with which they interact in the cell and in which pathways they play a role. In addition, understanding these tertiary structures will further increase the activity of molecular tools that can be developed to silence lncRNAs and reveal their role in various diseases. One of the classification methods developed to understand the effect of proteins in signaling pathways is intracellular location. Likewise, this classification for lncRNAs will be an important step in understanding both their interaction with proteins and their functions (Shukla et al. 2018; Kashi et al. 2016).

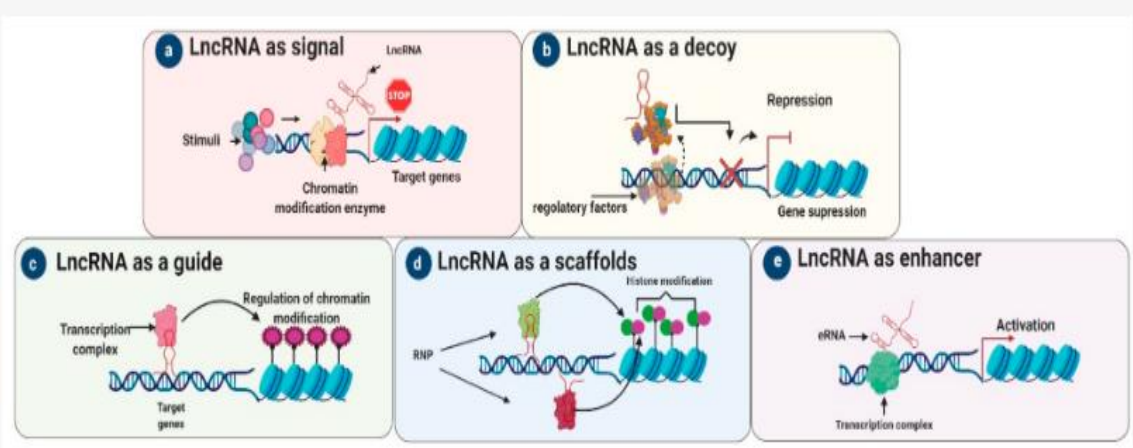


Figure 1.3. Schematic illustration of classification of long non-coding RNAs with respect to their modes of action. They are categorized into groups which are signal, decoy, guide, scaffold and enhancer (Source: Bridges et al. 2021).

1.2.3. The Role of Long Non-Coding RNA in Apoptosis

Considering the effect of lncRNAs on apoptosis and the mechanism of action it uses to interact with other components in the cell, it has been seen that they mostly change the activity of transcription factors, regulate miRNAs, and interact with proteins related to histone mechanisms such as chromatin modifier (Takeiwa et al. 2021). Early work on this topic was clarified by the identification of lncRNAs involved in apoptosis and cell division, which are important for the progression of cancer cells. For example, linc-p21

regulates apoptosis by modulating p53 and affecting DNA damage susceptibility (Huarte et al. 2021). lnc-CCNL1-3:1 lncRNA is overexpressed in patients with polycystic ovary syndrome. Accordingly, using the RPIseq method, it was determined that FOXO1 and lncCCNL1-3:1 could interact and play an important pathway in the cell. Rescue experiments to demonstrate this mechanism showed that decreasing the expression of FOXO1 in the cell caused CCNL overexpression to cause a low level of apoptosis. Thus, the relationship of this lncRNA with FOXO1 has critical importance in terms of the presence of FOXO1 in the nucleus and thus the occurrence of apoptosis (Huang et al. 2021). In another study, up to 10,214 different expression levels of lncRNA were detected in HeLa cells treated with cisplatin to observe the lncRNA profile in apoptotic conditions (Gurer et al. 2021). From this point of view, it can be said that lncRNA can directly or indirectly regulate cell death depending on its expression levels in the cell.

Considering the mechanisms required for triggering apoptosis, lncRNAs may act by regulating the intrinsic pathway proteins from the Bcl-2 protein family or p53 (Shi et al. 2018). For example, lncRNA TUG1 can affect apoptosis in different ways in terms of regulation of the intrinsic pathway. In a study by Liu et al. it was shown that a decrease in the expression of a proapoptotic protein BAX occurred as a result of direct interaction between lncRNA TUG1 and EZH2 in lung cancer cells (Liu et al. 2017). In another study, lncRNA caused an increase in AEG1 expression by interacting with miR-129-5p with the sponge mechanism, which is one of the TUG1 action mechanisms. Thus, this lncRNA has been shown to have an oncogenic role in human malignant melanoma. Mechanistically, melanoma cells with suppressed lncRNA TUG1 level showed decreased expression of Bcl-2 and increased expression level of Caspase-3, which ultimately led to apoptosis (Long et al. 2018). Another lncRNA that contributes to cancer progression by increasing its expression level in cancer cells is lncRNA UCA1. It has been revealed that Ets-2, which is mainly found in bladder cancer, increases UCA1's expression by binding to its own promoter. In this way, Ets-2 regulates the transcription of UCA1 and activates the Akt signaling pathway, and as a result, the triggering of apoptosis in the cell is prevented (Wu et al. 2013). Wu et al. carried out the following study, while studies continued to elucidate how cancer cells develop mechanisms to suppress apoptosis. Accordingly, they provided an explanation for the inability to trigger apoptosis in cancer cells despite the presence of endoplasmic reticulum-induced stress, by revealing the effect of lncRNA GOLGA2P10 on the intrinsic pathway in hepatocellular carcinoma cancer

cells. As a result of ER-induced stress, an increase in lncRNA GOLGA2P10 expression level occurs, which triggers the PERK/ATF4/CHOP signaling pathway. The effect of this pathway in suppressing apoptosis is made possible by regulating BCL-xL and phosphorylated BAD proteins (Wu et al. 2020).

Studies have shown that lncRNAs can also play a role in the extrinsic pathway of apoptosis (Jiang et al. 2021). For example, the alternatively spliced soluble Fas receptor, which does not contain Exon 6, interacts with the Fas ligand, preventing the occurrence of extrinsic apoptosis. The regulation of this Fas receptor-specific posttranscriptional mechanism is mediated by the lncRNA FAS-AS1 expressed in lymphoma cells. During alternative splicing, the lncRNA FAS-AS1 binds to RBM5, and the splicing of exon 6 is skipped. In other studies, it has been reported that as a result of excessive methylation of the lncRNA FAS-AS1 by EZH2, it causes an increase in the production of soluble Fas, and as a result, it leads to apoptosis inhibition (Sehgal et al. 2014). It has been reported that there is an increase in the expression of lincRNA HOXA-AS2 in NB4 cells due to Tretinoin treatment, also known as ATRA. However, after silencing the lincHOXA-AS2 gene, an increase in caspase-3, 8, and 9 activations and BAX protein was observed in ATRA-treated cells. Based on these studies, in order to reveal the role of lincHOXA-AS2 in suppressing apoptosis, an increase was observed in TRAIL expression levels and the application of neutralizing antibodies to stop TRAIL activation caused the suppression of Caspase-8 and 9 in cells treated with ATRA after silencing HOXA-AS2. Considering all these studies, it has been suggested that HOXA-AS2 has the task of suppressing apoptosis in NB4 cells treated with ATRA and may be associated with the apoptosis pathway originating from TRAIL (Zhao et al. 2013). Recent studies have shown that anticancer drugs targeting TRAIL are not sufficient to stop the development of cancer due to apoptosis in pancreatic cancer. As the reason for this, Yang et al. stated that the sensitivity of pancreas cells to anticancer drugs varies according to the expression level of lncRNA HOTAIR. The increase in HOTAIR expression causes methylation in the promoter of the DR5 gene via EZH2, and DR5 cannot be activated (Yang et al. 2017).

1.3. GTF2A1-AS Long Non-Coding RNA

GTF2A1-AS is one of the significant candidate lncRNAs differentially expressed under apoptotic conditions induced by cisplatin in HeLa cells (TUBİTAK Project 113Z371) (Ahmadov 2015).

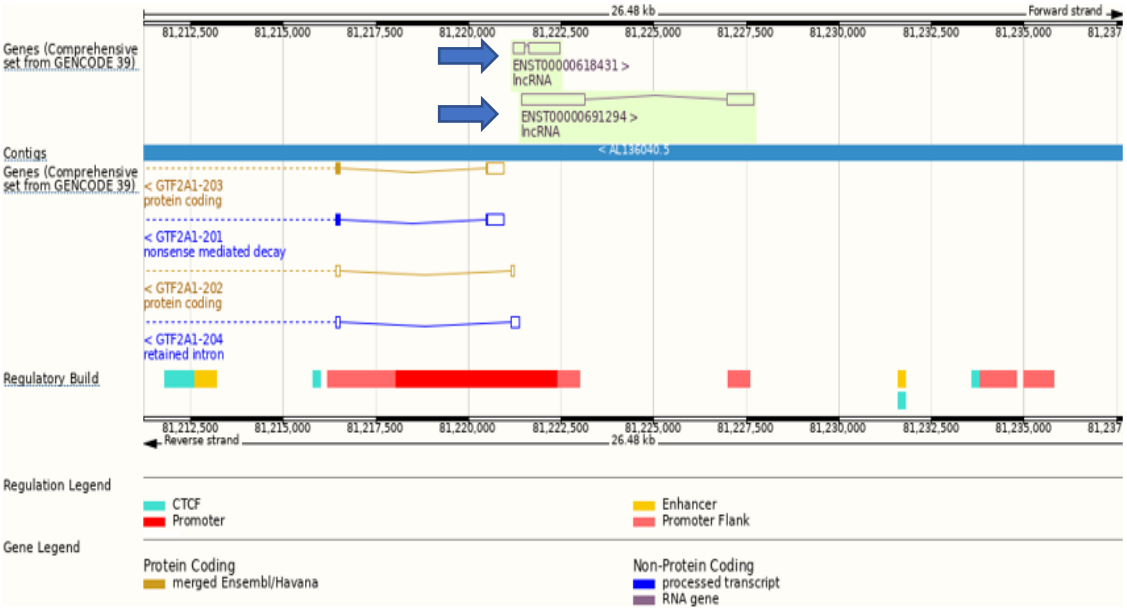


Figure 1.4. Schematic representation of chromosomal location GTF2A1-AS. The lncRNA located at coordinates 81,219,474 and 81,223,420 on human chromosome 14 has two transcripts which are ENST00000691294.1 with a length of 2406 bp and ENST00000618431.1 with a length of 1130 bp. They also overlap with coding transcripts of GTF2A1 gene.

GTF2A1-AS is an antisense lncRNA located at coordinates 81,219,474 and 81,223,420 on human chromosome 14. This lncRNA, also known as CTD-2506P8.6, is localized between GTF2A1 and STON2. This non-protein-coding transcript localized in the antisense direction overlaps with the 5' prime of the protein-coding GTF2A1 gene and possesses also two variants of this gene, ENST00000691294.1 with a length of 2406 bp and ENST00000618431.1 with a length of 1130 bp (Figure 1.4).

There is no data on GTF2A1-AS lncRNA in the literature yet. The overlap of this lncRNA with GTF2A1 is significant, especially given their potential to regulate genes located near antisense lncRNAs (Bonasio and Shikhattar, 2014). As a member of general transcription factors, RNA polymerase II stabilizes its binding to DNA for transcription

initiation. A malfunction that will occur here creates an obstacle to the continuation of the transcription event and creates transcription stress. However, the increase in the genes expressed to reduce this stress, especially in cancer cells, has an important place in the progression and proliferation of cancer. The importance of general transcription factors and their binding proteins draws attention to research aimed at stopping cancer progression, which has increased frequently in recent years. The feedback obtained from these studies, which led to dramatic results on cancer cells, is that the resulting transcriptional stress causes a high rate of apoptosis (Choi et al. 2019; Chakraborty et al. 2021). In a study carried out by Guo's group, the effects of IFN subtypes on chronical mucosal HIV-1 pathogenesis were demonstrated. The RNA-seq data showed that GTF2A1-AS lncRNA was one of the genes that was upregulated as interferon-stimulated genes against HIV infection in $CD4^+$ T cells in the gut (Guo et al. 2020). Therefore, this may encompass the role of GTF2A1-AS which is cisplatin inducible lncRNA in HeLa cells in our study.

Considering all this information, it can be concluded that there is a gap in the knowledge of whether GTF2A1-AS lncRNA has a contribution to regulation of GTF2A1 gene, or about the regulatory manner of GTF2A1-AS lncRNA on the expression of the GTF2A1 gene either transcriptionally or post-transcriptionally and more importantly, the effect of GTF2A1-AS lncRNA on cell apoptosis/proliferation. Revealing its effect on cell fate would provide to widen our knowledge about RNA Pol II-driven transcription or DNA damage response.

1.4. Aim

The aim of this study is to understand the function of GTF2A1-AS lncRNA by using the reverse genetic method.

CHAPTER 2

MATERIALS AND METHODS

2.1. Cell Culture, Transfection, and Drug Treatment

HeLa cells purchased from DSMZ GmbH were cultured in humidified air containing 5% CO2 at 37°C in RPMI 1640 (Gibco) medium supplemented with 10% fetal bovine serum (FBS) (Gibco). In every two days, the cells were passaged as the cells reached the number of 2.0 – 2.5 x 10^6 cells in 75 cm^2 cell culture flask. Treatment of HeLa cells with Cisplatin was carried out to the culture of 0.3 x 10^6 cells on a 6 well-plate (Sarstedt) and cells were grown overnight. To reach fifty percent apoptosis level in HeLa cells as performed previously (TUBITAK Project 113Z371), dose and time dependent kinetic experiments regarding to Cisplatin were carried out. To preserve chemical stability of Cisplatin (TRC Canada) it was dissolved in DMSO (Applichem) in order to have 83.2 mM fresh stock. In the experiments, Cisplatin treatment was optimized as 80 μM for 16 hours and as a control, DMSO was used. 6-well plates (Sarstedt) were used to carry out the experiments which aim to silence the gene of interest. 7.5 x 10^4 cells were cultured overnight in the media containing FBS without antibiotics before the experiment had been conducted. Then, the mixture used in the transfection process was prepared by the following stages; initially, 40 nM GTF2A1-AS GapmeR (Qiagen, Cat. No: LG00191600) probes were added to RPMI 1640 (with L-Glutamine, Gibco) media without antibiotic and FBS followed by subsequent addition of 4.5 μl of FuGENE HD Transfection Reagent (Promega). After incubating the transfection mixture of final volume of 150 μl for 15 minutes at the room temperature, it was added delicately to the media to distribute evenly. After transfection process was done, the cells were incubated for 24, 48 and 72 hours, and in the rest of the experiments, incubation time has been set to 48 hours. As a control negative antisense LNA GapmeR which has no target within the cell was used. In the experiments for overexpression of the gene of interest, gene sequence of GTF2A1-AS was synthesized commercially (Epoch) and it was cloned into pcDNA3.1(+) as a mammalian expression vector (Invitrogen) in order to construct

pcDNA3.1-GTF2A1-AS. As in the silencing experiments, general procedure of transfection was applied for the overexpression process. Then to avoid from toxicity resulting from plasmid DNA, media of cells which were transfected with pcDNA3.1-GTF2A1-AS plasmid replaced with fresh RPMI 1640 (with L-Glutamine, Gibco) containing FBS. After transfection process was done, the cells were incubated for 24, 48 and 72 hours, and in the rest of the experiments, incubation time has been set to 48 hours.

2.2. Apoptosis Rate Measurement

The levels of apoptotic cells were measured by FACSCanto Flow Cytometry (BD) by using Annexin V-FITC and 7AAD-PerCP (BD) stains. After cells were harvested with Trypsin-EDTA (Gibco, 0.25%), washed with ice-cold PBS (Gibco) and centrifuged at 1500 rpm for 5 minutes. Then supernatant containing PBS was removed completely and the pellet was resuspended with 50 µL of 1X annexin binding buffer (BD). After addition of both 10 µL of Annexin V and 7AAD into the cell suspension, cells were incubated for 15 minutes in the dark at room temperature. After that, the cells stained with Annexin V and 7AAD were suspended into 200 ml of PBS before flow cytometric analysis to detect apoptosis. Living cells were counted as both Annexin V- and 7AAD-negative. The cells in the early apoptotic stage were counted as Annexin V-positive and 7AAD-negative. The cells in the late apoptotic stage were counted as Annexin V- and 7AAD-positive. Necrotic and dead cells were counted as Annexin V- negative and 7AAD-positive.

.

2.3. RNA Isolation

After harvesting cells with Trypsin-EDTA (Gibco, 0.25%), they were washed twice with ice-cold PBS. Then, 1 mL of cold TRIzol (Invitrogen) were added into cells for lysis. After cells were dissolved, resulting lysate was incubated at the room temperature to dissociate the nucleoproteins completely. After that 0.2 µL of chloroform (Sigma) was transferred into tubes containing 1 mL of TRIzol and the tubes were shaken vigorously for 20 seconds. Tubes were allowed to incubate 2-3 minutes at room temperature and centrifugation was conducted at 12,000 x g at 4°C for 15 minutes. Then

the upper aqueous phase was collected from the divided three phases which formed in the tube as RNA content in the upper phase, DNA content in the middle phase, and organic content of the cell in the bottom phase. The upper aqueous phase was transferred delicately into a new tube by considering the 45° angle of the tube in order to prevent the mixing of the middle and bottom phases. Moreover, 0.5 mL of 100% RNase-free isopropanol (Sigma) was added to the tube containing the upper aqueous phase together with 1 µL of RNase free glycogen (Sigma). The tubes were incubated at -20°C for 2 hours and then they were centrifuged for 10 minutes at 12,000 x g at 4°C. After removing the supernatant delicately, 1 mL of 75% ice-cold ethanol was added into tubes to wash the pellet, and tubes were then centrifuged at 7,500 x g at 4°C for 5 minutes. This process was repeated twice for each wash. After removing the supernatant delicately again, the tubes were left to air-dry for 5 minutes. RNA concentrations of the samples were detected in the NanoDrop Spectrophotometer (Thermo Fisher Scientific) after dissolving pellets in 20-50 µL of RNase free water. Quality control of RNA samples obtained from cells was made by the criterion regarding to 260/280 and 260/230 absorbance ratios. To ensure that RNA samples were not contaminated by DNA or organic solvent, samples were run on 1% agarose gel for 30 minutes at 100V. Finally, the visualization of gel was carried out under AlphaImager (Model IS-2200, AlphaImager High Performance Gel Documentation, and Image Analysis System)

2.4. cDNA Synthesis and Quantitative PCR

Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) was used to synthesize cDNA. 1 µg total RNA was used in combination with 1 µl of Random Hexamer primer, 4 µl of 5X Reaction Buffer, 1 µl of RiboLock RNase Inhibitor, 2 µl of 10 mM dNTP mix and 1 µl of RevertAid M-MuLV RT, respectively. After centrifuging the tubes for short time, the reaction was incubated 5 minutes at 25°C, 60 minutes at 42°C and 5 minutes at 70°C incubation. Then, 20 µl of cDNA mixture was diluted into 5 ng/µl concentration by adding 180 µl of nuclease free water into it.

qPCR reactions were set up with 6.25 µl of GoTaq® qPCR Master Mix (Promega), 4.25 µl of nuclease-free water, 1 µl of 5 µM forward and reverse primer mix and 1 µl of cDNA. Standard two-step PCR amplification was conducted with incubation

of the samples at 95°C for 2 minutes as initial denaturation, 45 cycles of denaturation at 95°C for 15 seconds and annealing at 60°C for 1 minute as a melting step. The genes used in the experiments were normalized with respect to GAPDH which is a housekeeping gene. (Table 2.1)

Table 2.1. The representation of primer sequences used in this study.

Genes	Forward 5'-3'	Reverse 5'-3'
GTF2A1-AS	Qiagen Cat. No. LPH00619A	
GAPDH	ACTCCTCCACCTTTGACGC	GCTGTAGCCAAATTCGTTGTC
FOS	GCCTCTCTTACTACCACTCACC	AGATGGCAGTGACCGTGGGAAT
JUN	CCTTGAAAGCTCAGAACTCGGAG	TGCTGCGTTAGCATGAGTTGGC
CDK12	CGAACTCAGCAAAATGGCTCCTC	TTGGATGGAGGTGGCTCTTCGA
RMI2	GGCAGGGTAGTGATGGCGGAC	CCTGAACCACTCCCATCACCAT
NUF2	TGGAGACTCAGTTGACTGCCTG	ATTTGGTCCTCCAAGTTCAGGCT
P21	AGGTGGACCTGGAGACTCTCAG	TCCTCTTGGAGAAGATCAGCCG
EGR1	AGCAGCACCTTCAACCCTCAGG	GAGTGGTTTGGCTGGGGTAACT
DKK1	GGTATTCCAGAAGAACCACCTTG	CTTGGACCAGAAGTGTCTAGCAC
FGF21	CTGCAGCTGAAAGCCTTGAAGC	GTATCCGTCCTCAAGAAGCAGC

2.5. Bioinformatic Analyses

A Venn Diagram was the commonly shared differentially expressed genes from two different RNA-seq data which are obtained from Hela cells treated with 80 μM Cisplatin and Gapmer mediated GTF2A1-AS silencing (Gurer et al. 2021). These genes were selected for the criteria which are Fold Change ≥ 1.5 and p-value ≤0.05. Then, these genes were analyzed in the Reactome database which provides bioinformatic tool in order to interpret the data and represents 30 statistically significant pathways enriched with genes of interest (Jassal et al. 2020).

Gene Set Enrichment Analysis (GSEA) were used to analyze the differentially expressed genes in the RNA-seq obtained from HeLa cells mediated GTF2A1-AS silencing, specifically. The approach represents whether a gene set between different biological states was enriched in a phenotype as statistically significant (Subramanian et al. 2005). These genes were selected for the criteria which are Fold Change ≥ 1.5 and p-value ≤0.05.

2.6. Protein Isolation and Western Blotting

HeLa cells which were treated with Cisplatin or GTF2A1-AS GapmeR were harvested and centrifuged at 1500 rpm for 5 minutes with addition of 1X PBS. After dissolving the cell pellets in the tubes by adding 2 µl of 100x protease inhibitor (CST) and 48 µl of RIPA solution which is composed of 25 mM Tris•HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS (CST), the tubes were vortexed for 1 minute and held on ice for 10 minutes. To lyse the cells completely, this procedure was performed four times, repetitively. Moreover, centrifugation of the samples was conducted at 14.000xg for 10 minutes to get rid of cell debris. After transferring the supernatant into a new tube, the protein sample was diluted as 1:4 ratio in order to determine its protein concentration. 200 µl of Bradford solution was mixed with the protein sample and its concentration was measured at 595 nm. Isolated protein was kept at -20°C for a day to preserve its stability. To initiate Western Blotting, firstly, 10% separating gel which consisted of 40% acrylamide mix, preparations of separating buffer pH 8.8, APS and TEMED, and 5% stacking gel which consists of 40% acrylamide mix, stacking buffer pH 6.8, APS and TEMED were made. After loading 20 µg of protein into wells separately, colored protein ladder (New England Biolabs) was loaded to determine molecular weight of protein of interest. Then, electrophoresis was conducted for 3-4 hours at 80-100V via PowerPac Basic Power Supply (Bio-Rad) for separation of proteins with respect to their molecular weights in the tank containing 1X Running Buffer which consists of 25 mM Tris and 190 mM glycine. Two Whatman filter papers, protein gel and PVDF membrane (Thermo Scientific) were placed via Sandwich method in order to conduct wet transfer of proteins on gel. Subsequently, the cassette was placed into the

tank containing 1X Transfer Buffer which consists of 25 mM Tris, 192 mM glycine, pH 8.3 with 20% methanol. The transfer was conducted at 20V overnight. After the transfer has been ended, the membrane was blocked with 5% non-fat dry milk (CST) containing TBS-T Buffer which consists of 1x Tris Buffered Saline and 1% Tween 20 (FISHER) at room temperature for 1 hour. After washing the membrane with TBS-T Buffer for 10 minutes three times repetitively, PARP1 and Caspase-3 primary antibodies (CST) as 1:1000 dilution were incubated with the membrane overnight at 4°C in order to increase its efficiency. IgG-HRP conjugate secondary antibody (CST) was incubated with the membrane for 1 hour at room temperature. After the washing steps of the membrane with TBS-T, the membrane was incubated with the chemiluminescent ECL substrate (Bio-Rad) to visualize the proteins on the membrane for 3 minutes and was visualized under ChemiDoc XRS+ (Bio-Rad). ß-actin was used as a housekeeping gene.

CHAPTER 3

RESULTS

3.1. Expression Level of GTF2A1-AS In The Presence of Cisplatin

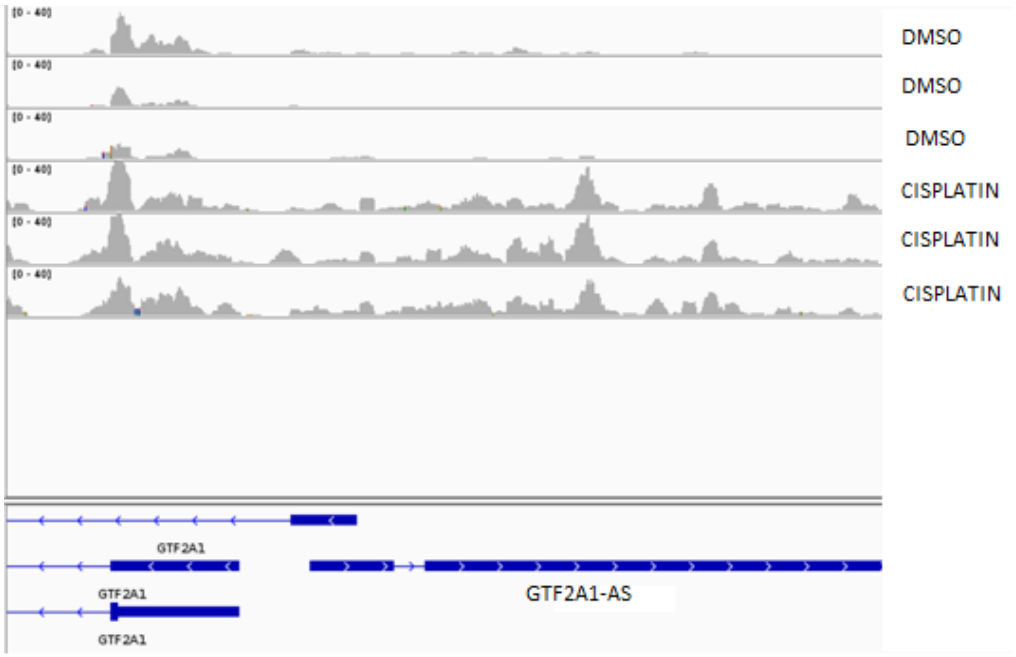
Since GTF2A1-AS is one of the highly upregulated LncRNA in the transcriptomic data obtained from HeLa cells treated with cisplatin, experimental validation was carried out to show its expression level. In Integrated Genome Viewer (IGV), transcriptomic profiles of GTF2A1-AS and three GTF2A1 transcripts were analyzed for three replicates of both DMSO and cisplatin-induced HeLa cells (Figure 3.1A). The peaks showing counts of transcripts of gene of interest demonstrated differences of expression levels of GTF2A1-AS and GTF2A1 genes between control and treatment groups. Quantitative PCR experiment was conducted in order to find out what the peaks refer to in the manner of the gene level of GTF2A1-AS. The gene expression of GTF2A1-AS was found as 3,94 Log_2 fold change, compared to its level which is 4,92 Log_2 fold change in the cisplatin-induced RNA-seq data. DMSO was used as a control group (Figure 3.1B).

3.2. Revealing The Potential Role of GTF2A1-AS

In light of information obtained from transcriptomic data showing differentially expressed genes in the presence of cisplatin, GTF2A1-AS was found as the highly expressed candidate by confirming it experimentally. Considering that evolutionary conservation is one of the signs of the functionality of a gene, Multiple Sequence Analysis (MSA) was carried out for the GTF2A1-AS among 38 species, based on EPO gene sequence. It has been found that GTF2A1-AS was conserved among 7 species that are relatively close to human, evolutionarily by showing high conservation. Other species have shown low conservation (Figure 3.2A). This result raised the probability that GTF2A1-AS would be responsible for a role within a cell, functionally. To test this, the expression level of GTF2A1-AS was analyzed throughout different normal tissues and cancer cell lines, based on FPKM level which is fragments per kilobase of transcript per

million fragments mapped (Gibb et al. 2011). It has been found that GTF2A1-AS is expressed in testis at most and in embryonic stem cells, secondly for normal tissues (Figure 3.2B). In addition to this, GTF2A1-AS is also expressed in Breast Invasive Carcinoma cell line (T-47D) at most and in Prostate Adenocarcinoma cell line (DU-145) secondly, based on TCGA database. (Figure 3.2C).

A



B

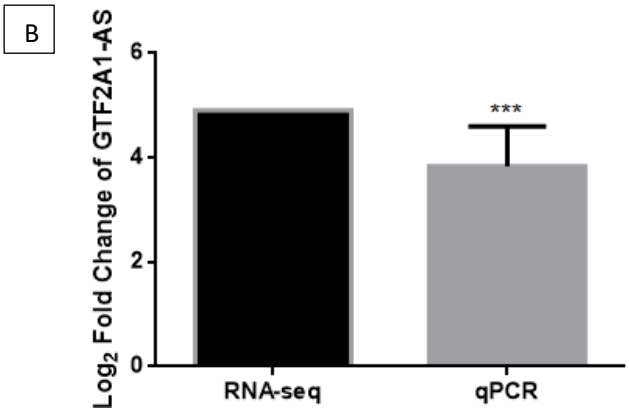


Figure 3.1. Expression levels of GTF2A1-AS in HeLa cells treated with Cisplatin and DMSO. (A) The expression levels of GTF2A1-AS were demonstrated in RNA-seq data (Gürer et al. 2021). (B) Expression level of GTF2A1-AS gene was validated by quantitative PCR experiment. ΔΔCt method was used to calculate $\log_2$ fold change by normalizing to GAPDH. The experiments were conducted in triplicates. Statistical analyses were performed by using Student's t-test, $p<0.001$ (***).

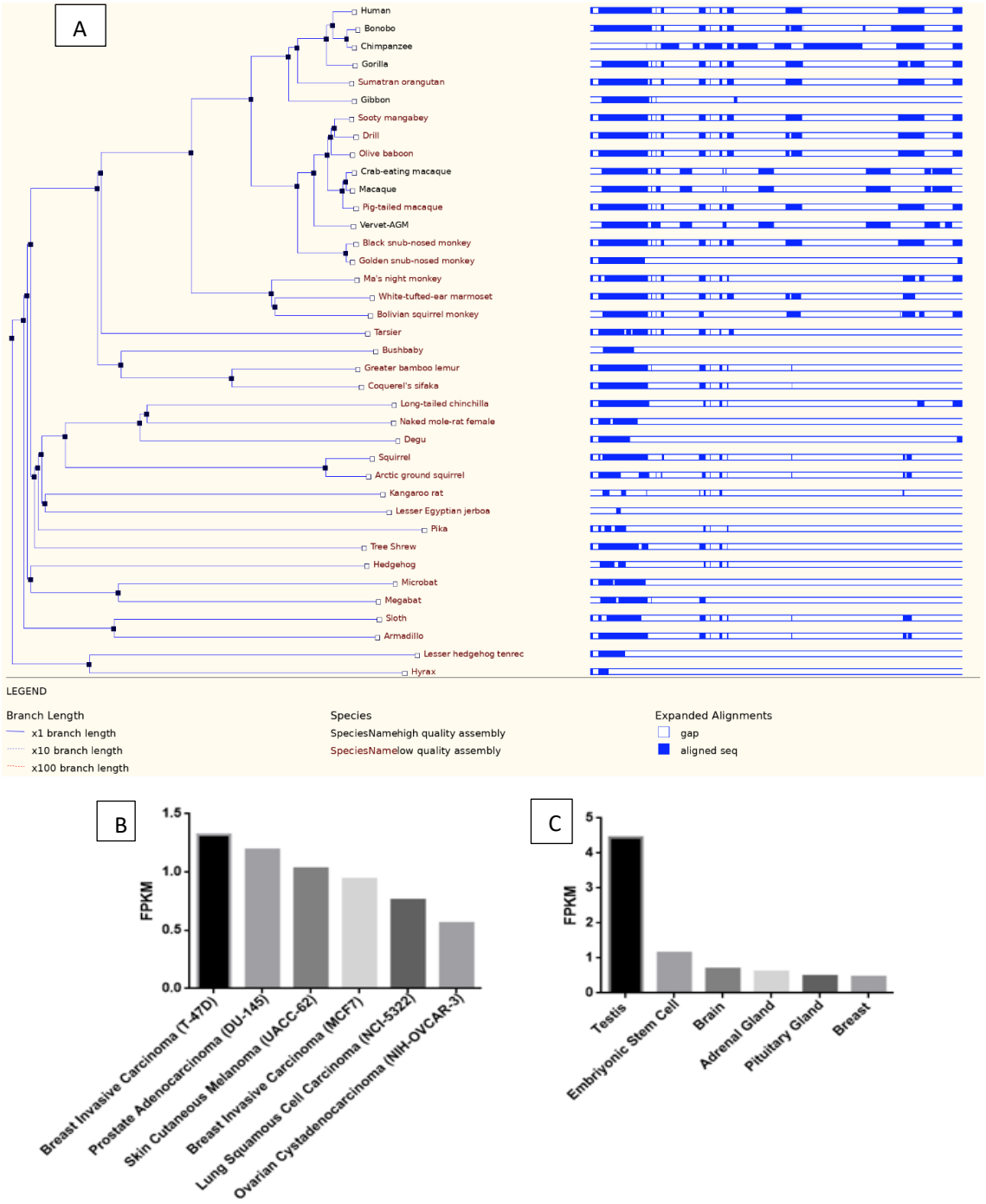


Figure 3.2. The potential phenotypic profiles of GTF2A1-AS gene. (A) Evolutionary conservation of GTF2A1-AS among 38 different species by using Multiple Sequence Alignment (MSA) analysis, based EPO gene sequence. The expression profile of GTF2A1-AS in different (B) normal and (C) cancer cells, based on FPKM level (fragments per kilobase of transcript per million fragments mapped).

3.3. Bioinformatic Analysis of Transcriptomic Data

Tissue-specific expression and evolutionary conservation in higher mammals of GTF2A1-AS gene suggest that it would serve as a function within the cell. To examine whether the change in the expression level of GTF2A1-AS would affect phenotype of the cell, GTF2A1-AS gene was silenced via GTF2A1-AS-GapmeR. GapmeRs which are short DNA antisense oligomers interact with RNA of interest and mediate its degradation via RNase H. Quantitative PCR was carried out to show the change in its expression level and it was found that the expression level of GTF2A1-AS was decreased as 2.3 Log_2 fold change. Negative GapmeR was used as a control (Figure 3.3).

The transcriptomic data were obtained from HeLa cells under apoptotic conditions in HeLa cells induced by cisplatin. In this transcriptomic data, GTF2A1-AS was a lncRNA that is highly expressed. Thus, it has been hypothesized that GTF2A1-AS knockdown would lead to apoptosis. Another transcriptomic data were obtained from HeLa cells mediated by GTF2A-1-AS-GapmeR to reveal the mechanism behind its apoptotic process. Since GTF2A1-AS was a cisplatin-inducible lncRNA, it would share with one of the cisplatin's mechanisms of action. Therefore, the Venn Diagram was plotted for commonly shared differentially expressed genes between Cisplatin-induced RNA-seq and GTF2A1-AS knockdown RNA-seq which has already been performed. The threshold of the genes for both two RNA-seq was determined as $p<0.05$ and ± 1.5 fold change. There were 1981 common differentially expressed genes found (Figure 3.4A). These genes were then analyzed in Reactome Pathway Database which showed the most significant 25 enriched pathways. DNA damage response-related pathways were found as the most significant ones (Figure 3.4B).

To validate these pathways, Gene Set Enrichment Analysis (GSEA) was carried out for 2072 genes obtained from GTF2A1-AS knockdown RNA-seq (Figure 3.5). Finally, DNA damage response-related candidate genes were selected to validate GTF2A1-AS knockdown RNA-seq data (Figure 3.6).

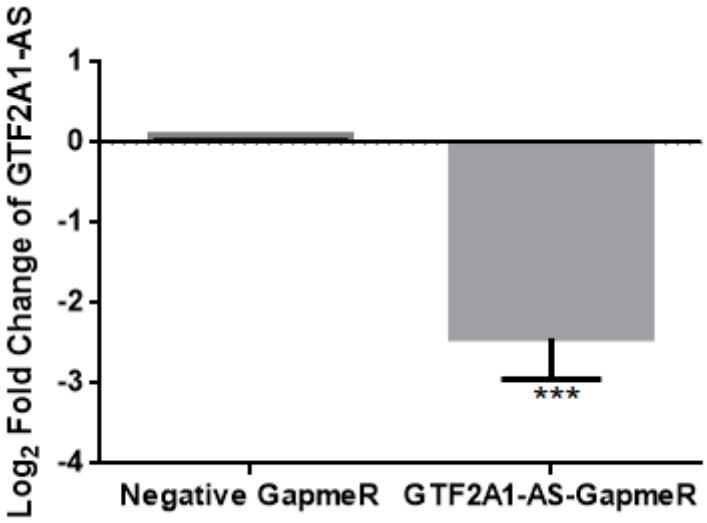
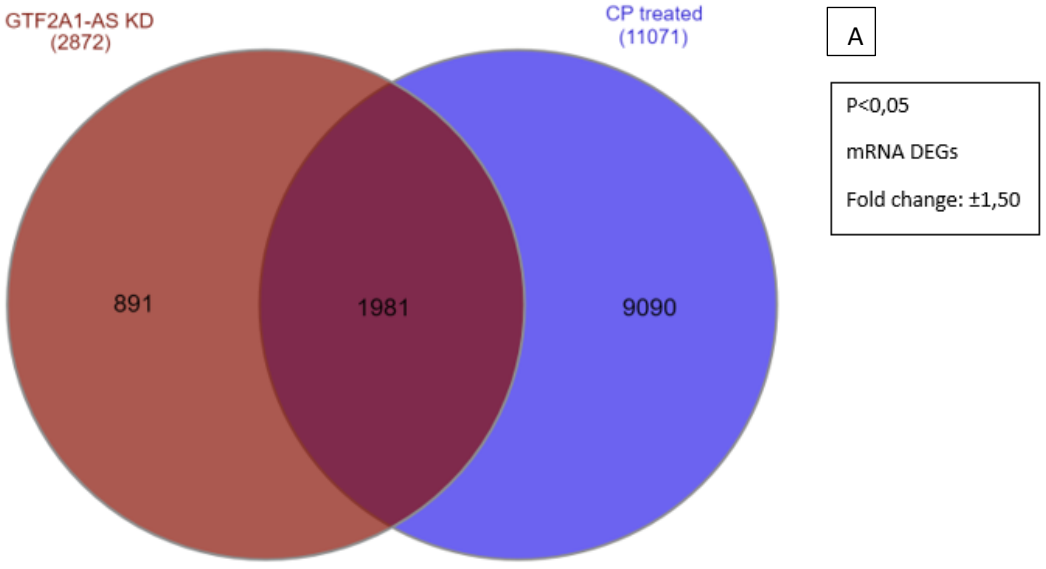


Figure 3.3. qPCR results of GapmeR mediated GTF2A1-AS gene. (A) GTF2A1-AS-GapmeR was used for silencing experiment and Negative GapmeR was used as a negative control. ΔΔCt method was used to calculate $\log_2$ fold change of the gene by normalizing to GAPDH. The experiments were conducted in triplicates. Statistical analyses were performed by using Student's t-test, $p<0.001$ (***).



B

Pathway name	Entities				Reactions	
	found	ratio	p-value	FDR*	found	ratio
Impaired BRCA2 binding to PALB2	11 / 24	0.002	8.81e-04	0.475	1 / 1	7.18e-05
Defective HDR through Homologous Recombination (HRR) due to PALB2 loss of function	11 / 25	0.002	0.001	0.475	2 / 2	1.44e-04
Defective HDR through Homologous Recombination (HRR) due to BRCA1 loss-of-function	11 / 25	0.002	0.001	0.475	1 / 1	7.18e-05
Defective HDR through Homologous Recombination Repair (HRR) due to PALB2 loss of BRCA1 binding function	11 / 25	0.002	0.001	0.475	1 / 1	7.18e-05
Defective HDR through Homologous Recombination Repair (HRR) due to PALB2 loss of BRCA2/RAD51/RAD51C binding function	11 / 25	0.002	0.001	0.475	1 / 1	7.18e-05
FOXO-mediated transcription of cell cycle genes	11 / 27	0.002	0.002	0.706	15 / 22	0.002
RUNX3 regulates NOTCH signaling	8 / 16	0.001	0.003	0.706	7 / 7	5.02e-04
NOTCH4 Intracellular Domain Regulates Transcription	10 / 26	0.002	0.005	0.762	8 / 9	6.46e-04
Resolution of D-loop Structures through Synthesis-Dependent Strand Annealing (SDSA)	11 / 31	0.002	0.006	0.762	1 / 1	7.18e-05
Interleukin-37 signaling	12 / 36	0.002	0.007	0.762	1 / 14	0.001
Estrogen-dependent nuclear events downstream of ESR-membrane signaling	10 / 29	0.002	0.01	0.762	7 / 12	8.61e-04
G1/S-Specific Transcription	13 / 43	0.003	0.011	0.762	28 / 28	0.002
Transcription of E2F targets under negative control by DREAM complex	9 / 25	0.002	0.011	0.762	12 / 12	8.61e-04
TP53 Regulates Transcription of Cell Cycle Genes	17 / 65	0.004	0.015	0.762	20 / 42	0.003
Signaling by ERBB2 in Cancer	11 / 36	0.002	0.017	0.762	53 / 62	0.004
NGF-stimulated transcription	15 / 56	0.004	0.018	0.762	20 / 37	0.003
DEx/H-box helicases activate type I IFN and inflammatory cytokines production	4 / 7	4.62e-04	0.019	0.762	3 / 5	3.59e-04
Resolution of D-loop Structures through Holliday Junction Intermediates	11 / 37	0.002	0.02	0.762	7 / 9	6.46e-04
Attenuation phase	13 / 47	0.003	0.021	0.762	5 / 5	3.59e-04

Figure 3.4. The most significantly enriched pathways for common differentially expressed genes. (A) Venn Diagram of common differentially expressed genes for both GTF2A1-AS knockdown RNA-seq and Cisplatin-induced RNA-seq. (B) Commonly shared differentially expressed genes were analyzed by using Reactome Pathway Database. The pathways of interest in the red rectangular were chosen according to the criterion whose p-value is less than 0.005.

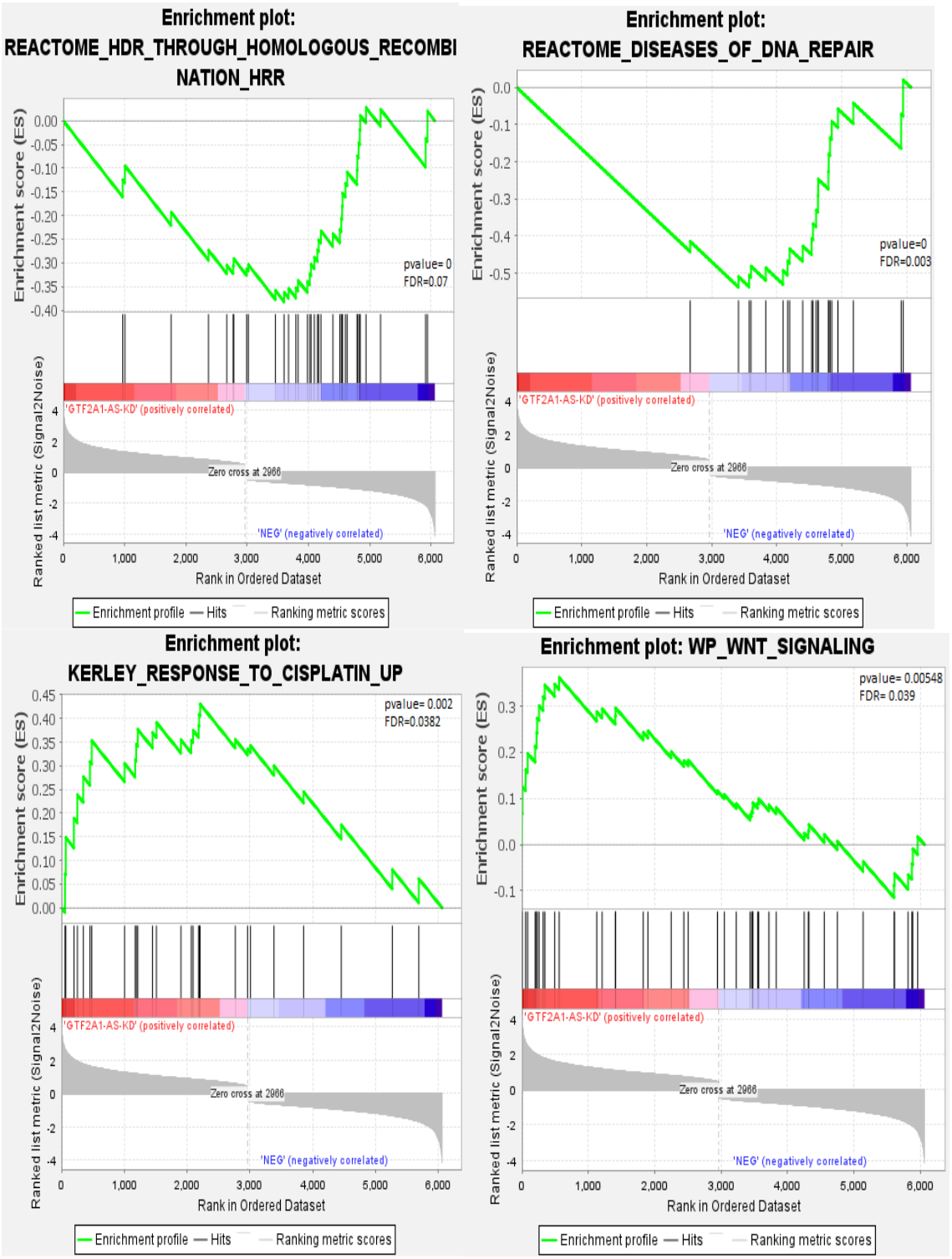


Figure 3.5. Gene Set Enrichment Analysis (GSEA) results of differentially expressed genes from GTF2A1-AS knockdown RNA-seq. (A) The genes used for the analysis were chosen for the following criteria: Fold Change > 1.5, p value< 0.05. The pathways of interest were chosen for the following criteria: FDR< 0.25, p value< 0.05.

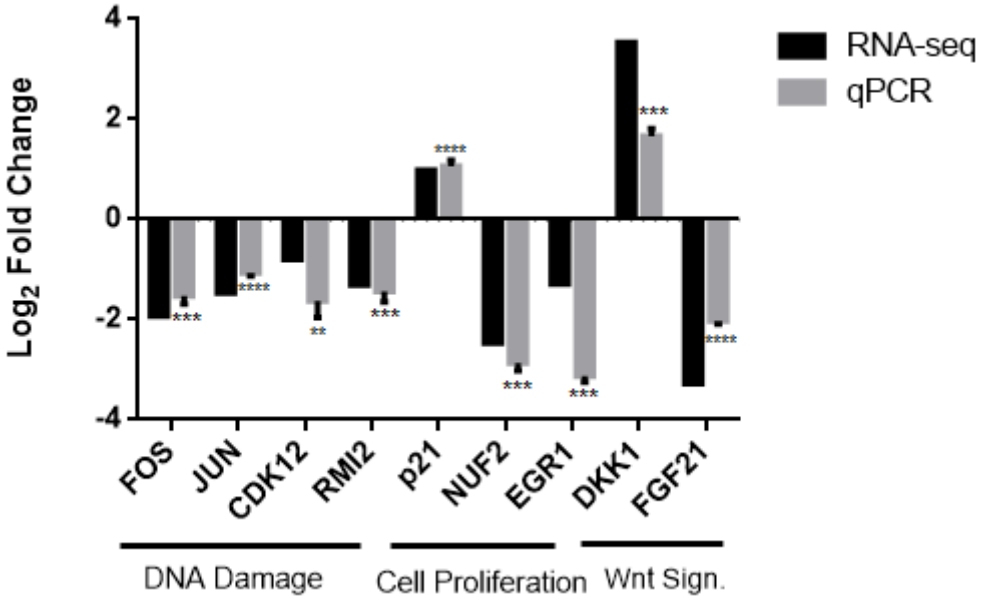
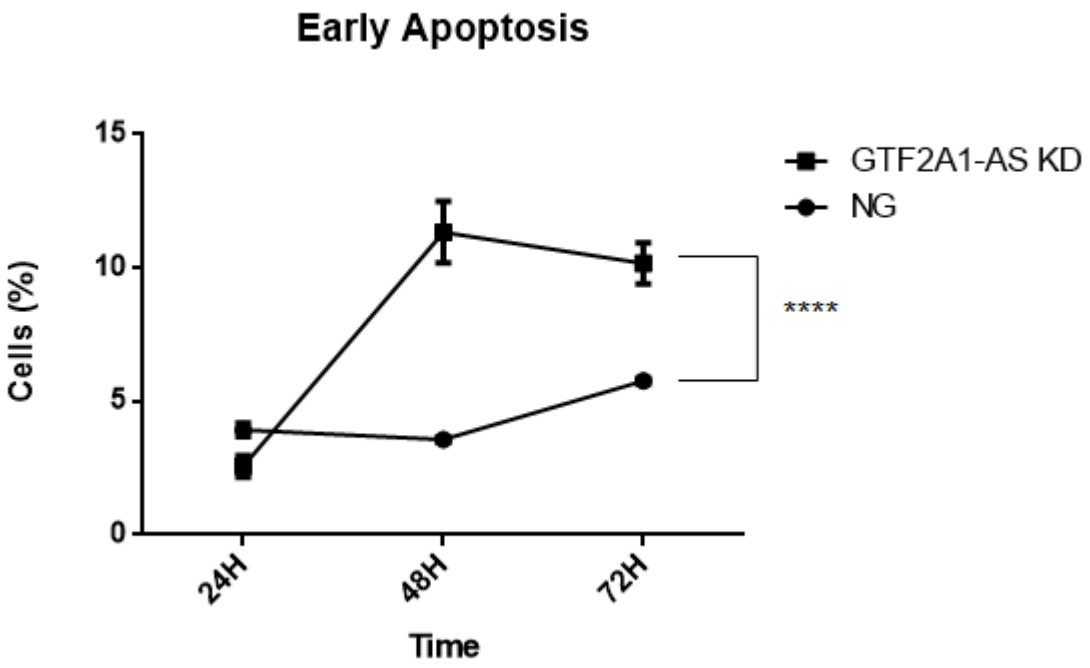


Figure 3.6. qPCR results of phenotype-related candidate genes. The gene levels of FOS, JUN, CDK12, RMI2, p21, NUF2, EGR1, DKK1 and FGF21 were compared to their levels in GTF2A1-AS knockdown RNA-seq. ΔΔCt method was used to calculate log_2 fold change of the gene by normalizing to GAPDH. The experiments were conducted in triplicates. Statistical analyses were performed by using Student's t-test, $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****).

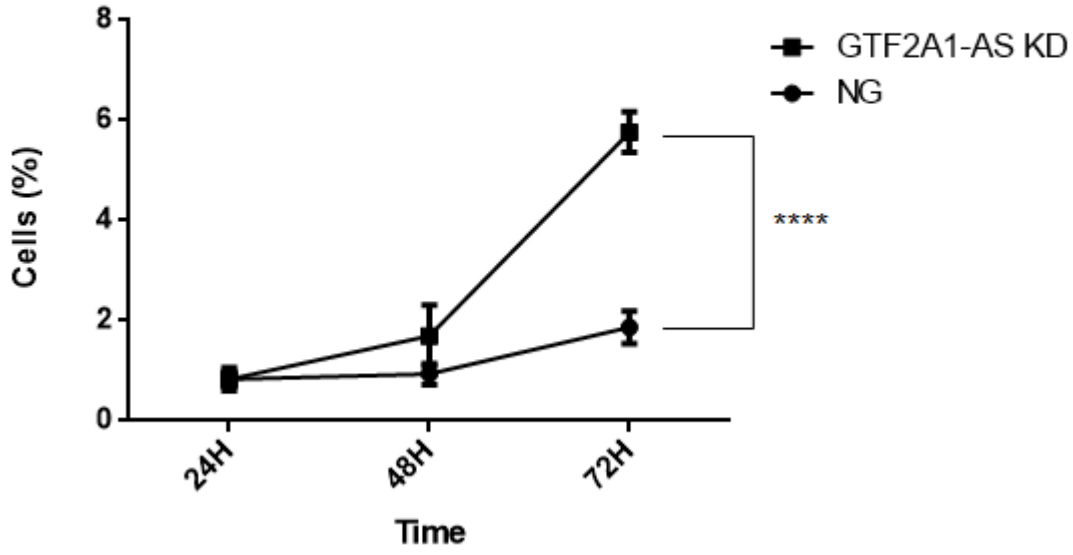
3.4. Time-Kinetic Experiments

Considering that DNA damage leads to apoptosis, eventually, it was tested at which time point apoptotic cell death reached the highest level. For this, silencing experiments of GTF2A1-AS were performed in three different time intervals which are 24, 48, and 72 hours. It has been found that early apoptosis rates reached the highest level at the end of GTF2A1-AS-Gapmer-mediated silencing experiment for 48 hours (Figure 3.7A). Late apoptosis and dead cells was at the lowest level at 48 hours. (Figure 3.7B and C).

A


Early Apoptosis
Cells (%)
15
10
5
0
24H
48H
72H
Time
GTF2A1-AS KD
NG
****

B


Late Apoptosis
Cells (%)
8
6
4
2
0
24H
48H
72H
Time
GTF2A1-AS KD
NG
****

C

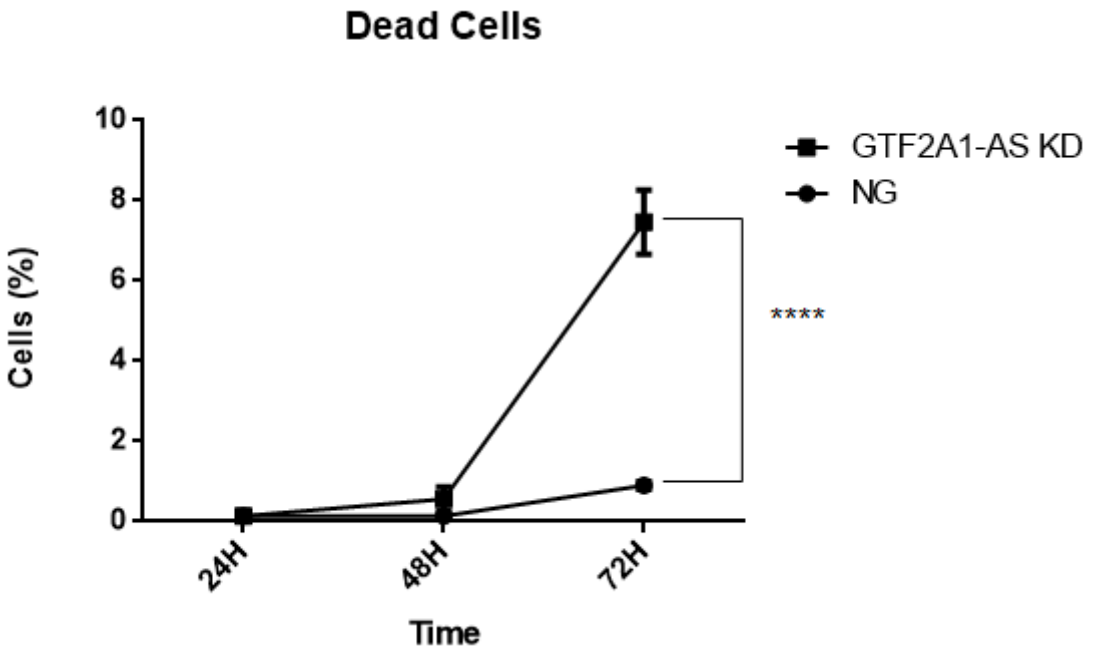


Figure 3.7. Apoptotic rates of GTF2A1-AS-Gapmer mediated Hela cells with respect to time-kinetic manner. Apoptosis rates were measured by using flow cytometry in three different time intervals which are 24, 48 and 72 hours. The cells were stained with Annexin V-FITC and 7AAD-PerCP. AnnV+/7AAD-, AnnV+/7AAD+, AnnV-/7AAD+ cells were considered as (A) early apoptotic, (B) late apoptotic and (C) dead cells, respectively. The experiments were conducted in triplicates. Statistical analyses were performed by using Student's t-test, $p<0.0001$ (****).

3.5. Effect of GTF2A1-AS Knockdown on Apoptosis

Apoptotic rates of HeLa cells mediated by GTF2A1-AS-GapmeR for 48 hours were measured by flow cytometry. Negative GapmeR was used as a negative control. It has been found that GTF2A1-AS knockdown increased early apoptosis rate by 16,6 percent in HeLa cells (Figure 3.8).

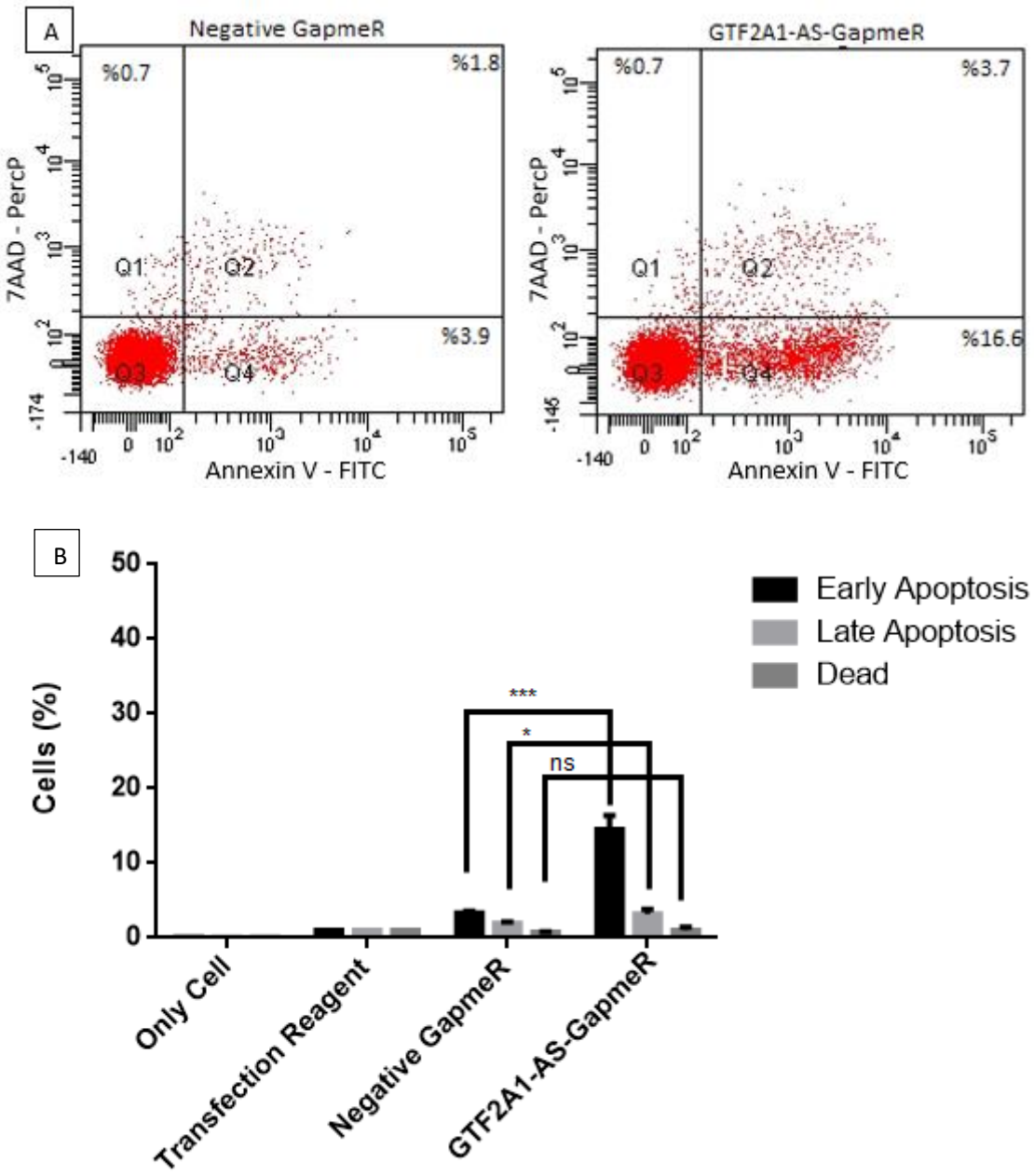
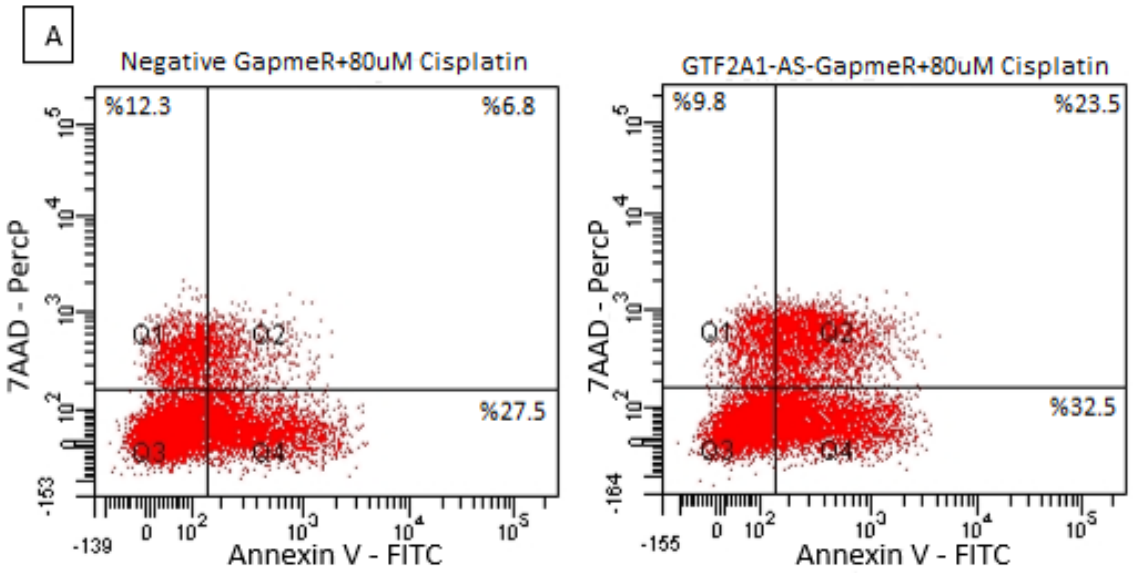


Figure 3.8. Representation of apoptotic rates of GTF2A1-AS-GapmeR mediated Hela cells. (A) Dot plots were demonstrated for Negative and GTF2A1-AS-GapmeR mediated Hela cells. Q1, Q2, Q3 and Q4 indicate dead, late apoptotic, live and early apoptotic cells, respectively. Each dot represents one cell. Apoptotic rates were measured by flow cytometry. (B) The bar graph shows apoptotic rates of HeLa cells mediated by GTF2A1-AS-GapmeR for 48 hours. The experiments were conducted in triplicates. Statistical analyses were performed by using Student's t-test, $p>0.05$ (ns), $p<0.05$ (*), $p<0.001$ (***).

3.6. Effect of GTF2A1-AS Knockdown on Chemosensitivity of HeLa Cells Against Cisplatin

Given that GTF2A1-AS would share with one of the cisplatin's mechanisms of action, based on the bioinformatic analysis above, it was hypothesized that the combined effects of GTF2A1-AS knockdown with cisplatin treatment would increase chemosensitivity of HeLa cells against cisplatin. For this, HeLa cells were treated with 80 µM concentration of cisplatin after GTF2A1-AS knockdown for 48 hours as control group was comprised of HeLa cells treated with 80 µM cisplatin. As a result, the rate of late apoptosis was increased from 6,8 to 23,5 percent whereas there was no significant change in early apoptotic and dead cells in the combination treatment (Figure 3.9).



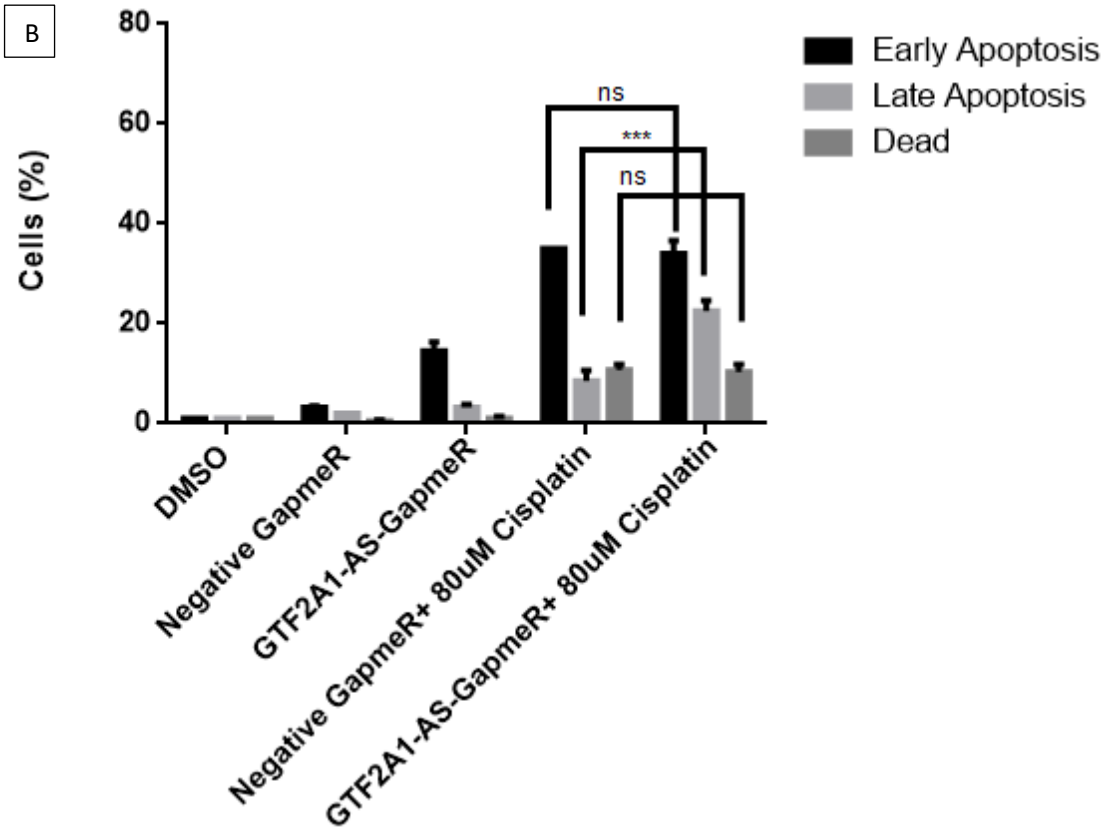


Figure 3.9. Representation of apoptotic rates of GTF2A1-AS-GapmeR mediated Hela cells in combination with cisplatin treatment. (A) Dot plots were demonstrated for Negative and GTF2A1-AS-GapmeR mediated Hela cells in combination with 80 µM concentration of cisplatin treatment. Apoptotic rates were measured by flow cytometry. (B) The bar graph shows apoptotic rates of HeLa cells mediated by GTF2A1-AS-GapmeR for 48 hours in combination with 80 µM concentration of cisplatin treatment for 16 hours. The experiments were conducted in triplicates. Statistical analyses were performed by using Student's t-test, $p>0.05$ (ns), $p<0.001$ (***).

3.7. Effect of GTF2A1-AS Overexpression on Apoptosis

Since GTF2A1-AS-GapmeR mediated silencing increased the apoptotic level in HeLa cells, it was assumed that overexpression of GTF2A1-AS would reverse its effect on HeLa. For this, pcDNA3.1-GTF2A1-AS construct was used to overexpress the GTF2A1-AS gene and pcDNA3.1 was used as an empty vector and control. After overexpressing experiments for 48 hours, expression level of GTF2A1-AS was found as 10 $\log_2$ fold change (Figure 3.10). When its apoptotic measurements were carried out, it was found that there was no change in the apoptotic cell death, compared to control group (Figure 3.11).

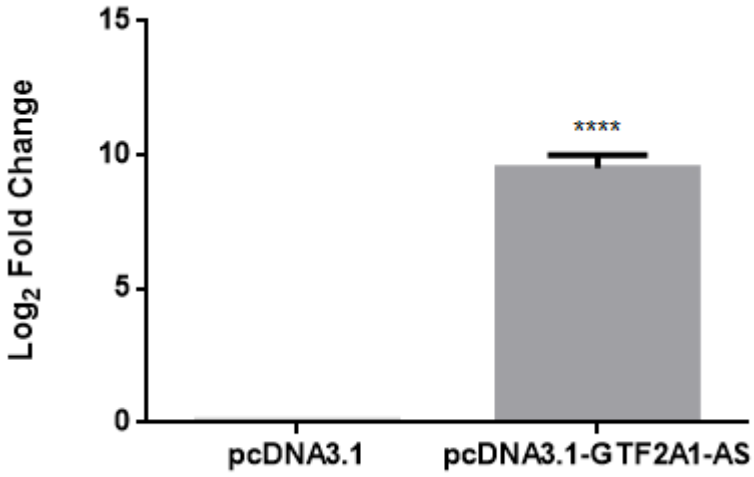


Figure 3.10. qPCR results of overexpression of GTF2A1-AS gene. pcDNA3.1-GTF2A1-AS was used for overexpressing experiment and pcDNA3.1 was used as a negative control. ΔΔCt method was used to calculate $\log_2$ fold change of the gene by normalizing to GAPDH. The experiments were conducted in triplicates. Statistical analyses were performed by using Student's t-test, $p<0.0001$ (****).

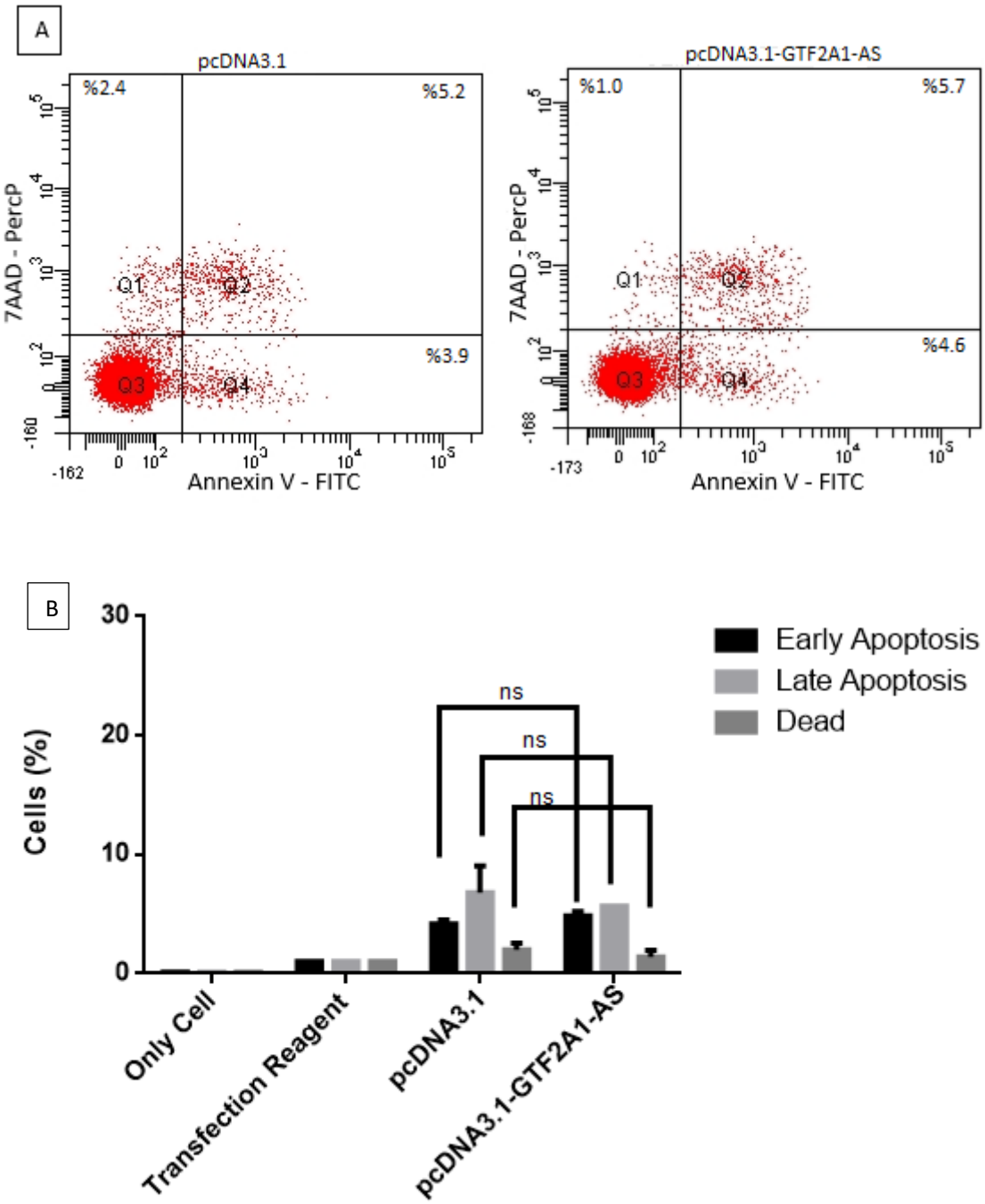
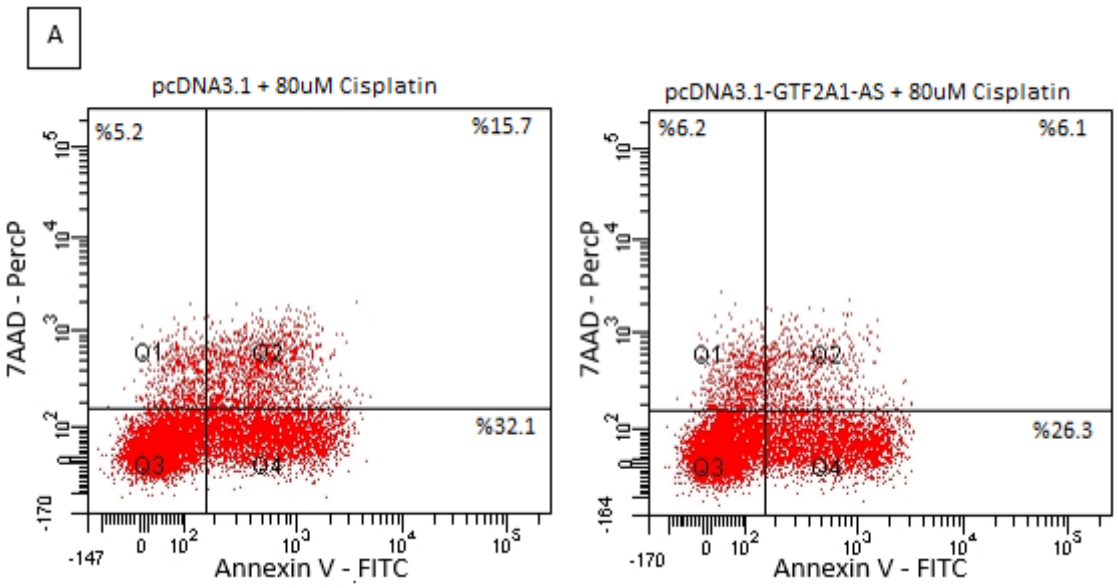


Figure 3.11. Representation of apoptotic rates of pcDNA3.1-GTF2A1-AS mediated Hela cells. (A) Dot plots were demonstrated for pcDNA3.1 and pcDNA3.1-GTF2A1-AS mediated Hela cells. Apoptotic rates were measured by flow cytometry. (B) The bar graph shows apoptotic rates of HeLa cells mediated by pcDNA3.1-GTF2A1-AS for 48 hours. The experiments were conducted in triplicates. Statistical analyses were performed by using Student's t-test, $p>0.05$ (ns).

3.8. Effect of GTF2A1-AS Overexpression on Chemosensitivity of HeLa Cells Against Cisplatin

Considering that GTF2A1-AS knockdown increased the chemosensitivity of HeLa cells against cisplatin treatment, it was assumed that GTF2A1-AS overexpression would drive a protective effect on HeLa cells against cisplatin by increasing its chemoresistance. For this, HeLa cells were treated with 80 µM concentration of cisplatin after overexpression experiment for 48 hours while they were only treated with same concentration of cisplatin as a control. As a result, whereas the rate of early apoptosis was slightly decreased from 32,1 to 26,3 percent, a major decreasing change was observed in late apoptotic cells from 15,7 to 6,1 percent in the combination treatment, compared to control group. There was no change in dead cells between the two groups (Figure 3.12). Additionally, PARP levels were analyzed between groups to show this change in protein level. As cPARP level was found as 32.9 fold in combination treatment, it was 28,9 fold in the control group. GTF2A1-AS overexpression showed no significant effect on cPARP level, compared to empty vector (Figure 3.13).



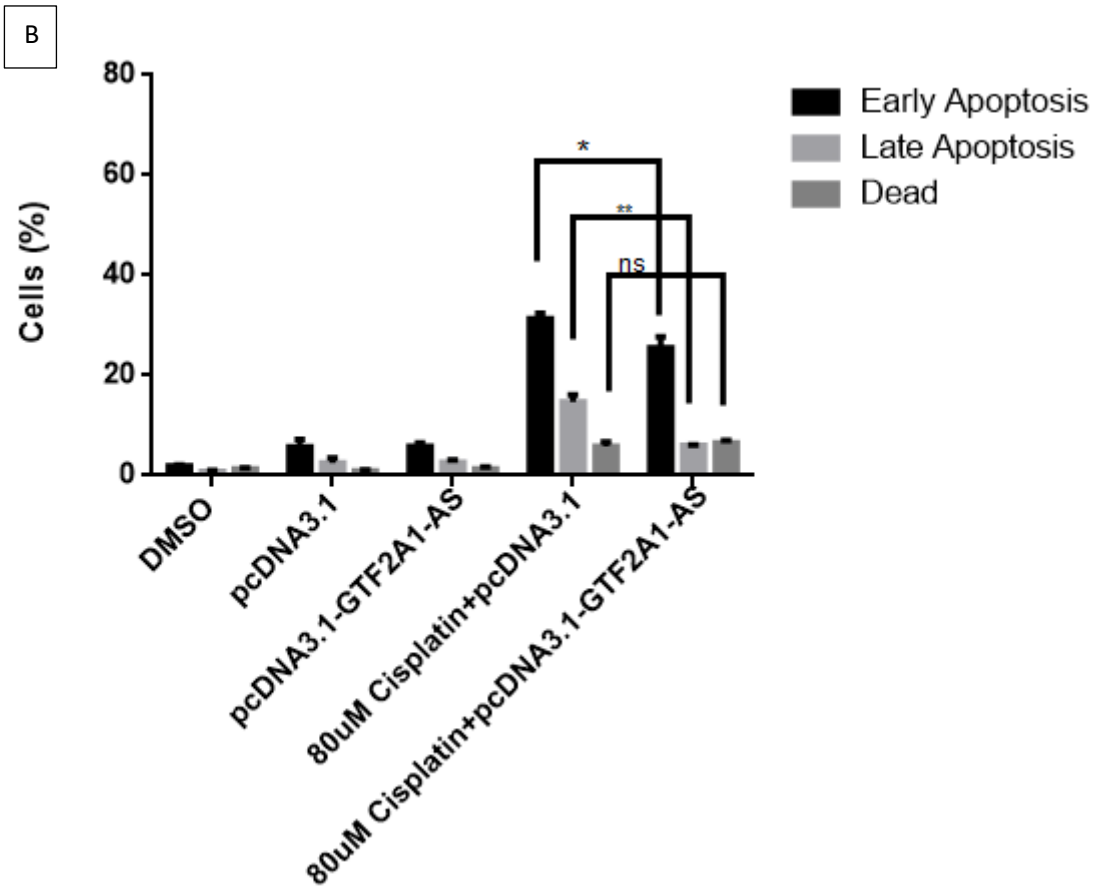


Figure 3.12. Representation of apoptotic rates of pcDNA3.1-GTF2A1-AS mediated Hela cells in combination with cisplatin treatment. (A) Dot plots were demonstrated for pcDNA3.1 and pcDNA3.1-GTF2A1-AS mediated Hela cells in combination with 80 μM concentration of cisplatin treatment. Apoptotic rates were measured by flow cytometry. (B) The bar graph shows apoptotic rates of HeLa cells mediated by pcDNA3.1-GTF2A1-AS for 48 hours in combination with 80 μM concentration of cisplatin treatment for 16 hours. The experiments were conducted in triplicates. Statistical analyses were performed by using Student's t-test, $p>0.05$ (ns), $p<0.05$ (*), $p<0.01$ (**)

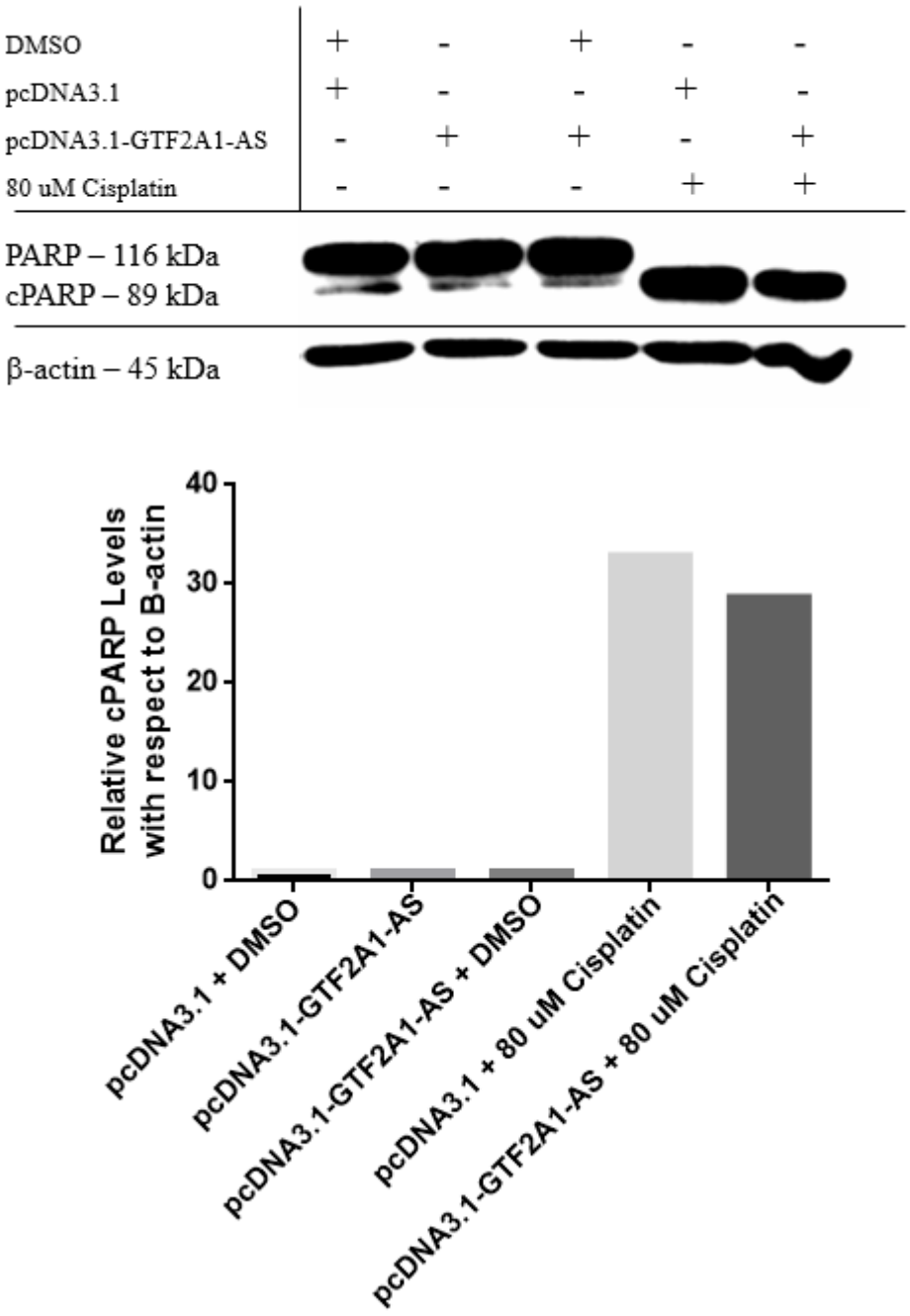


Figure 3.13. Representation of cPARP expression levels of pcDNA3.1-GTF2A1-AS mediated Hela cells in combination with cisplatin treatment. Western Blotting analysis of cPARP was demonstrated for pcDNA3.1 and pcDNA3.1-GTF2A1-AS mediated Hela cells in combination with 80 μM concentration of cisplatin treatment. The analysis were measured by ImageJ software. β-actin was used as a control. The experiments were conducted as once.

3.9. Effect of GTF2A1-AS in Its Coding Gene

To reveal the effects of GTF2A1-AS on apoptosis, mechanistically, quantitative PCR experiments were carried out to show expression levels of GTF2A1 gene under the conditions of silencing and overexpression of GTF2A1-AS. As a result, it was found that there was no significant change in the expression levels of GTF2A1 mRNA (Figure 3.14).

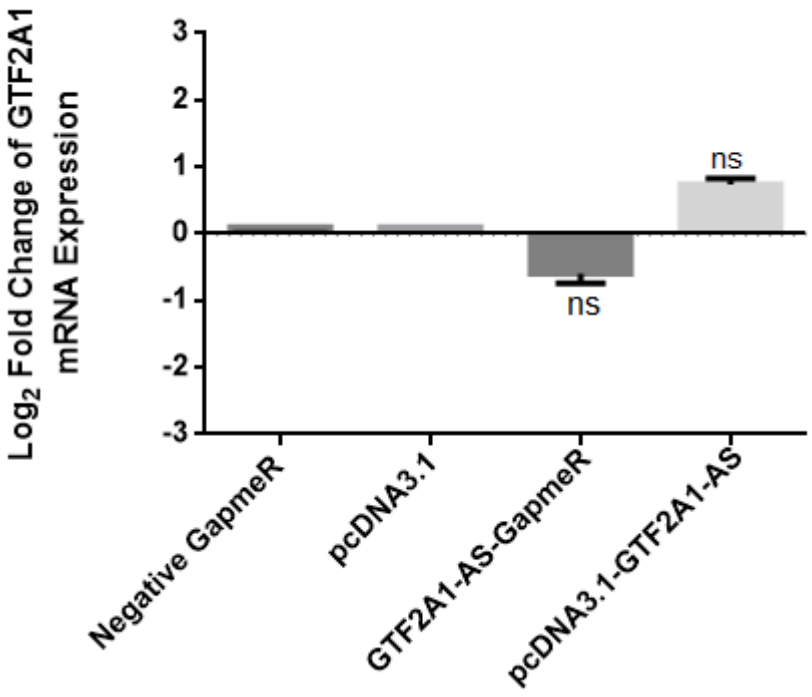


Figure 3.14. qPCR results of GTF2A1 mRNA expression under different conditions. The expression level of GTF2A1 gene was measured with respect to HeLa cells mediated by GTF2A1-AS-GapmeR and pcDNA3.1-GTF2A1-AS construct. ΔΔCt method was used to calculate $\log_2$ fold change of the gene by normalizing to GAPDH. The experiments were conducted in triplicates. Statistical analyses were performed by using Student's t-test.

CHAPTER 4

DISCUSSION

GTF2A1-AS which is an uncharacterized and novel lncRNA was found as one of the highly expressed lncRNA in transcriptomic data obtained from HeLa cells treated with cisplatin. In fact, there was no study about its functionality and role within the cells. Thus, this gap in the literature has drawn our attention to work on it. Additionally, there is also another reason sparked our interest most that GTF2A1-AS lncRNA overlaps to 5' portion of its coding gene which is GTF2A1. This transcription factor takes up a vital place in the formation of pre-initiation complex at the beginning of transcription process. By interacting with other general transcription factors, it allows RNA Polymerase II to be recruited into the promoter sequence of a gene. It is inevitably necessary for the cell itself (Merle and Cramer 2017). Therefore, genomic arrangement between GTF2A1-AS and GTF2A1 gene indicates that there would be a reasonable interaction with each other. All these strong reasons tempted us to choose this promising lncRNA. The transcriptomic data were then analyzed IGV to reveal the difference between expression levels of GTF2A1-AS gene provided by DMSO and cisplatin-treated HeLa cells, visually (Figure 3.1A). This difference was further validated by performing qPCR experiment. It was found that expression level of GTF2A1-AS was 3.92 $\log_2$ fold change as almost similar to its level in Cisplatin-induced RNA-seq data (Figure 3.1B).

Determining whether a gene is functional or not would be challenging in the case which the gene is uncharacterized and newly identified. For that reason, evolutionary conservation and expression of the gene in normal and cancerous tissues encompass us to understand its association with the phenotype. Therefore, the expression level of GTF2A1-AS was screened among the normal tissues and cancer cell lines based on TCGA. Interestingly, it has been found that GTF2A1-AS was expressed mostly in the testis among normal tissues and in Breast Invasive Carcinoma cell line (T-47D) among cancer cell lines (Figure 3.2B and C). The fact that lies behind these findings would be that GTF2A1 has particular significance for developmental process requiring high and secure proliferative ability. The cell proliferates in the case in which genomic stability is

under control (Veenstra and Wolffe 2001). On the other hand, breast cancer cells possess high metastatic capacity to invade distant parts of the body among other cancer types. Thus, they require a higher proliferative rate and tightening control of DNA repair (Wang et al. 2019). Together with this information, it would be possible that GTF2A1-AS may play a role in genome stability. Additionally, Multiple Sequence Analysis (MSA) indicates evolutionary conservation among species. As a result of this, 7 species which are particularly higher mammals showed high conservation of GTF2A1-AS gene sequence (Figure 3.2A).

Evolutionary conservation and tissue-specific expression of GTF2A1-AS has urged us to interrogate the putative role of GTF2A1-AS within the cell. To find out this, GapmeR-mediated silencing has been performed for GTF2A1-AS in HeLa cells in order to have transcriptomic data. Since GTF2A1-AS is a cisplatin inducible lncRNA (Gürer et al. 2021), it has been put forward that GTF2A1-AS would contribute to one of cisplatin's mechanisms of action within the cells. Thus, a Venn Diagram has been plotted for commonly shared differentially expressed genes for the genes provided by both Cisplatin-induced RNA-seq and GTF2A1-AS knockdown RNA-seq data (Figure 3.4A). Those 1981 common genes were then analyzed in Reactome Pathway Database to understand in which pathways those genes were enriched. It has been found that specific gene clusters were mainly enriched in the pathway which is Defective Homology directed Repair through Homologous Recombination (HRR) (Figure 3.4B). In this process, double-strand breaks are repaired with the help of BRCA1/2, RAD50, RAD51, PALB2 proteins which are known as DNA damage response proteins (Ledermann et al. 2016). It was the expected result for the genes from Cisplatin-induced RNA-seq because it is a platin-based drug that has higher genotoxicity, leading to double-strand breaks in the genome. Strikingly, these were also found in GTF2A1-AS knockdown RNA-seq data. It means that GTF2A1-AS may be responsible for DNA damage response, somehow. To strengthen this finding, for only the genes provided by GTF2A1-AS knockdown RNA-seq data, GSEA has been performed to compare the genes with the desired gene set in the GSEA database (Figure 3.5). As Reactome pathway analysis only shows most significant 25 pathways in the list regardless of what has been desired, GSEA provides us to choose pathway of interest. Therefore, DNA damage-related pathways have been also found by confirming the pathways found in Reactome database.

To validate the GTF2A1-AS knockdown RNA-seq data, DNA damage response-related genes which are FOS, JUN, CDK12, RMI2, NUF2, p21, EGR1, DKK1 and FGF21 were selected (Figure 3.6). The rationale behind this selection was that firstly, FOS and JUN come together to form the AP-1 transcription factor that modulates mismatch repair specific proteins (Humbert et al. 2003). After silencing the GTF2A1-AS, expression levels of both FOS and JUN were decreased. Then, RMI2 is a RecQ helicase which plays role in homologous recombination and double-strand break repair (Wei et al. 2022). The expression level of RMI2 was decreased as well. The last gene chosen for DNA damage response is CDK12. It is a kinase which interacts with RNA Pol II and directs it to express the genes specific to DNA damage response, such as BRCA1/2, FOS, JUN, RAD50, RAD51, ATR, FANCD (Manavalan et al. 2019). CDK12 would be thought as the central hub for DNA damage response because it involves in 3' end processing of the transcripts during transcription and recognizes poly-adenylation signals (PAS) by phosphorylating it (Pilarova et al. 2020). Surprisingly, most DNA damage response genes have longer PAS compared to other genes so that CDK12 shows selectivity for those genes. Due to this fact, any defect in CDK12 protein or gene leads to higher genome instability and also sensitizes the cells to genotoxic agents, resulting in nascent transcripts for those genes (Blazek et al. 2011). The expression level of CDK12 was also decreased by validating the transcriptomic data. Although fundamental roles of NUF2, p21 and EGR1 are related to cell proliferation, they contribute to DNA repair. As NUF2 involves in chromosomal segregation, EGR1 upregulates p53 in the case that DNA damage occurs (Li et al. 2014; Baron 2006). P21 is only activated when defects in the genome, result the cell cycle arrest in G1/S (Ticli et al. 2022). While expression levels of EGR1 and NUF2 decreased, the expression level of p21 increased. DKK1 is a strong inhibitor of Wnt signaling and has pro-apoptotic features. In most of cancer types, aberrant Wnt signaling occurs by promoting survival of cancer cell. In the literature, in response to alkylating agent, as DKK1 expression shows a significant increase in brain tumor cells, FGF21 shows a decrease (Shou and Multani 2002). In our case, after silencing GTF2A1-AS, they confirmed the literature. These results obtained from bioinformatic analysis have addressed the role of GTF2A1-AS in the DNA damage response, eventually leading to apoptosis.

Considering that putative role of GTF2A1-AS in DNA damage response leads to apoptosis, GTF2A1-AS was silenced via GapmeR to measure its effect on apoptosis. To

determine at which time point apoptosis reaches highest level regarding to silencing of GTF2A1-AS, time-kinetic experiments were performed as 24, 48, and 72 hours (Figure 3.7). It has been found that in the experiment for 48 hours, early apoptosis has reached highest level among other time intervals. In addition to this, late apoptosis and dead cells have found as lower levels comparing others. Since GTF2A1-AS is a cisplatin inducible lncRNA, it has been hypothesized that GTF2A1-AS knockdown sensitizes HeLa cells against cisplatin treatment. To test this hypothesis, chemosensitivity of HeLa cells treated with GTF2A1-AS-Gapmer was examined to interrogate the combined effect with cisplatin (Figure 3.8). It has been found that only late apoptosis has made a difference compared with early apoptosis and dead cells. It would mean that GTF2A1-AS knockdown combined with cisplatin treatment has shifted the population found in early apoptosis to late apoptotic stage by increasing cell death response.

Apoptosis measurements were performed to examine whether overexpression affects apoptotic rate or not and there appears to be no effect on apoptosis. Furthermore, it was hypothesized that it would reverse the effects of GTF2A1-AS knockdown combined with cisplatin on chemosensitivity of HeLa cells (Figure 3.12). It has been found that GTF2A1-AS overexpression combined with cisplatin treatment showed protective effect on HeLa cells. The major difference was seen again in late apoptotic population. Another explanation for this difference in the late apoptotic stage would be made that this population in late apoptotic stage would owe to different type of cell death modality. Although it requires further investigation, it would be reasonable explanation for this observation when our bioinformatic results were considered. To show this change, Western blotting experiment has been performed for PARP expression. That’s why PARP1 is one of the major sensors in the case that DNA damage occurs. In apoptosis process, it is cleaved by Caspase-3. Thus, although it requires for further experiments to confirm the result, it has been found that GTF2A1-AS overexpression combined with cisplatin treatment showed decrement in level of cPARP, regarding to only cisplatin treatment (Figure 3.13).

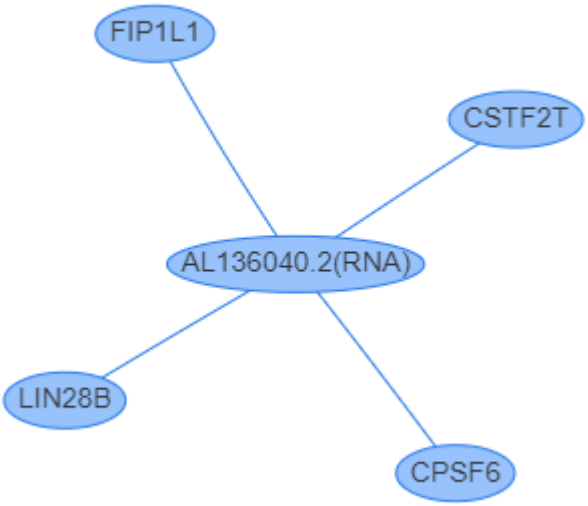


Figure 4.1. Putative protein interactions of GTF2A1-AS with respect to POSTAR3 tool.

To understand how GTF2A1-AS affects cell fate mechanistically, expression level of GTF2A1 was measured under different conditions of GTF2A1-AS by considering cis-acting mechanism of lncRNAs. It has been observed that there was no change in its expression level (Figure 3.14). It would be hypothesized that GTF2A1-AS acts via a different mechanism by interacting with distal proteins as trans-acting mechanism for a possible explanation of this situation. Using POSTAR3 tool hypothetical protein interaction was analyzed based on PAR-CLIP experiments (Figure 5.1). According to the results, two proteins which are CPSF6 and CSTF2T sparked our interest because both Cleavage and Polyadenylation Specific Factor 6 (CPSF6) and Cleavage Stimulation Factor Subunit 2 Tau Subunit (CSTF2T) are involved in 3' end processing (Hollerer et al. 2014). It is worth mentioning that interaction of GTF2A1-AS with one of these proteins may affect activity of CDK12, leading to a defect in DNA damage response (Pilarova et al. 2020). Therefore, further studies are required to demonstrate this relationship.

CHAPTER 5

CONCLUSION

In this study, the aim was to investigate the effect of a novel and uncharacterized GTF2A1-AS on the cell fate. Based on the transcriptomic data, the role of GTF2A1-AS in DNA damage response has potentially led to apoptosis in the case which GTF2A1-AS was silenced. In conjunction with this GTF2A1-AS deficient HeLa cells have shown increased chemosensitivity against cisplatin. This phenotypic association was further supported by the expression levels of specific DNA damage response genes.

Evolutionary conservation and tissue-specific distribution of GTF2A1-AS has shed light on its potential function within the cell. By taking advantage of bioinformatic analysis, DNA damage-related pathways have been enriched in the presence of the genes provided by GTF2A1-AS knockdown RNA-seq. Thus, GTF2A1-AS knockdown has resulted in apoptosis in HeLa cells, as expected. Additionally, whereas GTF2A1-AS knockdown has increased the chemosensitivity of HeLa cells, GTF2A1-AS overexpression has shown protective effects on HeLa cells. Mechanistically, in the manner of determining the upstream target of GTF2A1-AS, any changes in the expression levels of GTF2A1 which is its coding gene have been observed. However, although it remained to be clarified, POSTAR3 tool has pointed out the putative proteins which have the probability to interact with GTF2A1-AS. They would widen our knowledge about understanding GTF2A1-AS’s mechanism of action within the cell, particularly on DNA damage response.

REFERENCES

Acehan, Devrim, Xuejun Jiang, David Gene Morgan, John E Heuser, Xiaodong Wang, and Christopher W Akey. 2002. "Three-Dimensional Structure of the Apoptosome." *Molecular Cell* 9, no. 2: 423–32. https://doi.org/10.1016/s1097-2765(02)00442-2.

Ahanda, Marie-Laure Endale, Thomas Ruby, Håkan Wittzell, Bertrand Bed'Hom, Anne-Marie Chaussé, Veronique Morin, Anne Oudin, Catherine Chevalier, John R. Young, and Rima Zoorob. 2008. "Non-Coding RNAS Revealed during Identification of Genes Involved in Chicken Immune Responses." *Immunogenetics* 61, no. 1: 55–70. https://doi.org/10.1007/s00251-008-0337-8.

Ahmadov, Ulvi. 2015. "Identification of Long Non-Coding Rnas That Regulate Apoptosis in Human (MSc Thesis, Izmir Institute of Technology, 2015)."

Amaral, Paulo P., Michael B. Clark, Dennis K. Gascoigne, Marcel E. Dinger, and John S. Mattick. 2010. "LncRNAdb: A Reference Database for Long Noncoding RNAS." *Nucleic Acids Research* 39, no. suppl_1. https://doi.org/10.1093/nar/gkq1138.

Baron, V, E D Adamson, A Calogero, G Ragona, and D Mercola. 2005. "The Transcription Factor EGR1 Is a Direct Regulator of Multiple Tumor Suppressors Including TGFB1, PTEN, p53, and Fibronectin." *Cancer Gene Therapy* 13, no. 2: 115–24. https://doi.org/10.1038/sj.cgt.7700896.

Birney E., Stamatoyannopoulos J.A., Dutta A. 2007. "Identification and Analysis of Functional Elements in 1% of the Human Genome by the Encode Pilot Project." *Nature* 447, no. 7146: 799–816. https://doi.org/10.1038/nature05874.

Blazek, Dalibor, Jiri Kohoutek, Koen Bartholomeeusen, Eric Johansen, Petra Hulinkova, Zeping Luo, Peter Cimermancic, Jernej Ule, and B. Matija Peterlin. 2011. "The Cyclin K/CDK12 Complex Maintains Genomic Stability via

Regulation of Expression of DNA Damage Response Genes." *Genes & Development* 25, no. 20: 2158–72. https://doi.org/10.1101/gad.16962311.

Bonasio, Roberto, and Ramin Shiekhattar. 2014. "Regulation of Transcription by Long Noncoding RNAS." *Annual Review of Genetics* 48, no. 1: 433–55. https://doi.org/10.1146/annurev-genet-120213-092323.

Bossen, Claudia, Karine Ingold, Aubry Tardivel, Jean-Luc Bodmer, Olivier Gaide, Sylvie Hertig, Christine Ambrose, Jürg Tschopp, and Pascal Schneider. 2006. "Interactions of Tumor Necrosis Factor (TNF) and TNF Receptor Family Members in the Mouse and Human." *Journal of Biological Chemistry* 281, no. 20: 13964–71. https://doi.org/10.1074/jbc.m601553200.

Brenner, Dirk, and Tak W Mak. 2009. "Mitochondrial Cell Death Effectors." *Current Opinion in Cell Biology* 21, no. 6: 871–77. https://doi.org/10.1016/j.ceb.2009.09.004.

Bridges, Mary Catherine, Amanda C. Daulagala, and Antonis Kourtidis. 2021. "LNCcation: Lncrna Localization and Function." *Journal of Cell Biology* 220, no. 2. https://doi.org/10.1083/jcb.202009045.

Cain, Kelvin, Shawn B. Bratton, and Gerald M. Cohen. 2002. "The Apaf-1 Apoptosome: A Large Caspase-Activating Complex." *Biochimie* 84, no. 2-3: 203–14. https://doi.org/10.1016/s0300-9084(02)01376-7.

Chakraborty, Prasun, and Kevin Hiom. 2021. "Dhx9-Dependent Recruitment of BRCA1 to RNA Promotes DNA End Resection in Homologous Recombination." *Nature Communications* 12, no. 1. https://doi.org/10.1038/s41467-021-24341-z.

Chirackal Manavalan, Anil Paul, Kveta Pilarova, Michael Kluge, Koen Bartholomeeusen, Michal Rajecky, Jan Oppelt, Prashant Khirsariya, et al. 2019. "CDK12 Controls G1/S Progression by Regulating RNAPII Processivity at Core DNA Replication Genes." *EMBO reports* 20, no. 9. https://doi.org/10.15252/embr.201847592.

Choi, Hee-Joo, Sora Jin, Hani Cho, Hee-Young Won, Hee Woon An, Ga-Young Jeong, Young-Un Park, et al. 2019. “Cdk12 Drives Breast Tumor Initiation and Trastuzumab Resistance viaWntandIrs1-Erbb-Pi3K Signaling.” *EMBO reports* 20, no. 10. https://doi.org/10.15252/embr.201948058.

D’Arcy, Mark S. 2019. “Cell Death: A Review of the Major Forms of Apoptosis, Necrosis and Autophagy.” *Cell Biology International* 43, no. 6: 582–92. https://doi.org/10.1002/cbin.11137.

Dickson, Dennis W. 2004. “Apoptotic Mechanisms in Alzheimer Neurofibrillary Degeneration: Cause or Effect?” *Journal of Clinical Investigation* 114, no. 1: 23–27. https://doi.org/10.1172/jci22317.

Dykes, Iain M., and Costanza Emanueli. 2017. “Transcriptional and Post-Transcriptional Gene Regulation by Long Non-Coding RNA.” *Genomics, Proteomics & Bioinformatics* 15, no. 3: 177–86. https://doi.org/10.1016/j.gpb.2016.12.005.

Ekert, Paul G., and David L. Vaux. 2005. “The Mitochondrial Death Squad: Hardened Killers or Innocent Bystanders?” *Current Opinion in Cell Biology* 17, no. 6: 626–30. https://doi.org/10.1016/j.ceb.2005.09.001.

Elliott, Michael R., and Kodi S. Ravichandran. 2010. “Clearance of Apoptotic Cells: Implications in Health and Disease.” *Journal of Cell Biology* 189, no. 7: 1059–70. https://doi.org/10.1083/jcb.201004096.

Elmore, Susan. 2007.“Apoptosis: A Review of Programmed Cell Death.” *Toxicologic Pathology* 35, no. 4: 495–516. https://doi.org/10.1080/01926230701320337.

Erdmann, V. A., M. Szymanski, A. Hochberg, N. de Groot, and J. Barciszewski. 1999. “Collection of Mrna-like Non-Coding RNAS.” *Nucleic Acids Research* 27, no. 1: 192–95. https://doi.org/10.1093/nar/27.1.192.

Faravelli, Irene, and Stefania Corti. 2017. “MicroRNA-Directed Neuronal Reprogramming as a Therapeutic Strategy for Neurological Diseases.” *Molecular Neurobiology*. https://doi.org/10.1007/s12035-017-0671-7.

Guo, Kejun, Guannan Shen, Jon Kibbie, Tania Gonzalez, Stephanie M. Dillon, Harry A. Smith, Emily H. Cooper, et al. 2020. “Qualitative Differences between the IFNA Subtypes and Ifnβ Influence Chronic Mucosal HIV-1 Pathogenesis.” *PLOS Pathogens* 16, no. 10. https://doi.org/10.1371/journal.ppat.1008986.

Gurer, Dilek Cansu, İpek Erdogan, Ulvi Ahmadov, Merve Basol, Osama Sweef, Gulcin Cakan-Akdogan, and Bünyamin Akgül. 2021. “Transcriptomics Profiling Identifies Cisplatin-Inducible Death Receptor 5 Antisense Long Non-Coding RNA as a Modulator of Proliferation and Metastasis in Hela Cells.” *Frontiers in Cell and Developmental Biology* 9. https://doi.org/10.3389/fcell.2021.688855.

Guttman, Mitchell, Julie Donaghey, Bryce W. Carey, Manuel Garber, Jennifer K. Grenier, Glen Munson, Geneva Young, et al. 2011. “LincRNAs Act in the Circuitry Controlling Pluripotency and Differentiation.” *Nature* 477, no. 7364: 295–300. https://doi.org/10.1038/nature10398.

Hantsche, Merle, and Patrick Cramer. 2017. “Conserved RNA Polymerase II Initiation Complex Structure.” *Current Opinion in Structural Biology* 47: 17–22. https://doi.org/10.1016/j.sbi.2017.03.013.

Hermans-Beijnsberger, Steffie, Marc van Bilsen, and Blanche Schroen. 2018. “Long Non-Coding RNAS in the Failing Heart and Vasculature.” *Non-coding RNA Research* 3, no. 3: 118–30. https://doi.org/10.1016/j.ncrna.2018.04.002.

Hollerer, Ina, Kerstin Grund, Matthias W. Hentze, and Andreas E Kulozik. 2013. “Mrna 3′End Processing: A Tale of the Tail Reaches the Clinic.” *EMBO Molecular Medicine* 6, no. 1: 16–26. https://doi.org/10.1002/emmm.201303300.

Huang, Jiayu, Jun Zhao, Xueying Geng, Weiwei Chu, Shang Li, Zi-Jiang Chen, and Yanzhi Du. 2021. “Long Non-Coding RNA LNC-CCNL1-3:1 Promotes Granulosa Cell Apoptosis and Suppresses Glucose Uptake in Women with Polycystic Ovary Syndrome.” *Molecular Therapy - Nucleic Acids* 23: 614–28. https://doi.org/10.1016/j.omtn.2020.12.008.

Humbert, O. 2003. “HMSH2 Expression Is Driven by AP1-Dependent Regulation through Phorbol-Ester Exposure.” *Nucleic Acids Research* 31, no. 19: 5627–34. https://doi.org/10.1093/nar/gkg781.

Jassal, Bijay, Lisa Matthews, Guilherme Viteri, Chuqiao Gong, Pascual Lorente, Antonio Fabregat, Konstantinos Sidiropoulos, et al. 2020. “The Reactome Pathway Knowledgebase.” Nucleic Acids Research 48 (D1): D498–503. https://doi.org/10.1093/nar/gkz1031.

Jiang, Na, Xiaoyu Zhang, Xuejun Gu, Xiaozhuang Li, and Lei Shang. 2021. “Progress in Understanding the Role of Lncrna in Programmed Cell Death.” *Cell Death Discovery* 7, no. 1. https://doi.org/10.1038/s41420-021-00407-1.

Johnsson, Per, Leonard Lipovich, Dan Grandér, and Kevin V. Morris. 2014. “Evolutionary Conservation of Long Non-Coding RNAS; Sequence, Structure, Function.” *Biochimica et Biophysica Acta (BBA) - General Subjects* 1840, no. 3: 1063–71. https://doi.org/10.1016/j.bbagen.2013.10.035.

Kashi, Kaori, Lindsey Henderson, Alessandro Bonetti, and Piero Carninci. 2016. “Discovery and Functional Analysis of Lncrnas: Methodologies to Investigate an Uncharacterized Transcriptome.” *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms* 1859, no. 1: 3–15. https://doi.org/10.1016/j.bbagrm.2015.10.010.

Kerr, J F, A H Wyllie, and A R Currie. 1972. “Apoptosis: A Basic Biological Phenomenon with Wideranging Implications in Tissue Kinetics.” *British Journal of Cancer* 26, no. 4: 239–57. https://doi.org/10.1038/bjc.1972.33.

Kim, Ji, Su Lee, Su Oh, Song Han, Hye Park, Mi-Ae Yoo, and Ho Kang. 2004. “Methyl Jasmonate Induces Apoptosis through Induction of BAX/Bcl-XS and Activation of Caspase-3 via ROS Production in A549 Cells.” *Oncology Reports*. https://doi.org/10.3892/or.12.6.1233.

Kopp, Florian, and Joshua T. Mendell. 2018. “Functional Classification and Experimental Dissection of Long Noncoding RNAS.” *Cell* 172, no. 3: 393–407. https://doi.org/10.1016/j.cell.2018.01.011.

Korostowski, Lisa, Natalie Sedlak, and Nora Engel. 2012. “The KCNQ1OT1 Long Non-Coding RNA Affects Chromatin Conformation and Expression of KCNQ1, but Does Not Regulate Its Imprinting in the Developing Heart.” *PLoS Genetics* 8, no. 9. https://doi.org/10.1371/journal.pgen.1002956.

Ledermann, Jonathan A., Yvette Drew, and Rebecca S. Kristeleit. 2016. “Homologous Recombination Deficiency and Ovarian Cancer.” *European Journal of Cancer* 60: 49–58. https://doi.org/10.1016/j.ejca.2016.03.005.

Li, Wenbo, Dimple Notani, Qi Ma, Bogdan Tanasa, Esperanza Nunez, Aaron Yun Chen, Daria Merkurjev, et al. 2013. “Functional Roles of Enhancer RNAS for Oestrogen-Dependent Transcriptional Activation.” *Nature* 498, no. 7455: 516–20. https://doi.org/10.1038/nature12210.

Liu, Huan, Guizhi Zhou, Xin Fu, Haiyan Cui, Guangrui Pu, Yao Xiao, Wei Sun, et al. 2017. “Long Noncoding RNA TUG1 Is a Diagnostic Factor in Lung Adenocarcinoma and Suppresses Apoptosis via Epigenetic Silencing of Bax.” *Oncotarget* 8, no. 60: 101899–910. https://doi.org/10.18632/oncotarget.22058.

Liu, Qiang, She-Jiao Dai, Hong Li, Lei Dong, and Yu-Ping Peng. 2014. “Silencing of NUF2 Inhibits Tumor Growth and Induces Apoptosis in Human Hepatocellular Carcinomas.” *Asian Pacific Journal of Cancer Prevention* 15, no. 20: 8623–29. https://doi.org/10.7314/apjcp.2014.15.20.8623.

Lockshin, Richard A., and Zahra Zakeri. 2004. “Apoptosis, Autophagy, and More.” *The International Journal of Biochemistry & Cell Biology* 36, no. 12: 2405–19. https://doi.org/10.1016/j.biocel.2004.04.011.

Long, Jianwen, Qiqige Menggen, Qimige Wuren, Quan Shi, and Xianming Pi. 2018. “Long Noncoding RNA Taurine-Upregulated GENE1 (TUG1) Promotes Tumor Growth and Metastasis through tug1/Mir-129-5p/Astrocyte-Elevated Gene-1

(AEG-1) Axis in Malignant Melanoma." *Medical Science Monitor* 24: 1547–59. https://doi.org/10.12659/msm.906616.

Long, Yicheng, Xueyin Wang, Daniel T. Youmans, and Thomas R. Cech. 2017. "How Do Lncrnas Regulate Transcription?" *Science Advances* 3, no. 9. https://doi.org/10.1126/sciadv.aao2110.

Ma, Lina, Vladimir B. Bajic, and Zhang Zhang. 2013. "On the Classification of Long Non-Coding RNAS." *RNA Biology* 10, no. 6: 924–33. https://doi.org/10.4161/rna.24604.

Martinvalet, Denis, Pengcheng Zhu, and Judy Lieberman. 2005. "Granzyme a Induces Caspase-Independent Mitochondrial Damage, a Required First Step for Apoptosis." *Immunity* 22, no. 3: 355–70. https://doi.org/10.1016/j.immuni.2005.02.004.

Oppenheim, J. J., J. M. Wang, A. Hishinuma, and K. Matsushima. 1992. "Properties and Binding Characteristics of Monocyte Chemotactic and Activating Factor (MCAF)." *Mononuclear Phagocytes*, 389–96. https://doi.org/10.1007/978-94-015-8070-0_52.

Oppenheim, Ronald W., Richard A. Flavell, Sharon Vinsant, David Prevette, Chia-Y. Kuan, and Pasko Rakic. 2001."Programmed Cell Death of Developing Mammalian Neurons after Genetic Deletion of Caspases." *The Journal of Neuroscience* 21, no. 13: 4752–60. https://doi.org/10.1523/jneurosci.21-13-04752.2001.

Pandey, Radha Raman, Tanmoy Mondal, Faizaan Mohammad, Stefan Enroth, Lisa Redrup, Jan Komorowski, Takashi Nagano, Debora Mancini-DiNardo, and Chandrasekhar Kanduri. 2008. "KCNQ1OT1 Antisense Noncoding RNA Mediates Lineage-Specific Transcriptional Silencing through Chromatin-Level Regulation." *Molecular Cell* 32, no. 2: 232–46. https://doi.org/10.1016/j.molcel.2008.08.022.

Peng, W-X, P Koirala, and Y-Y Mo. 2017. “LncRNA-Mediated Regulation of Cell Signaling in Cancer.” *Oncogene* 36, no. 41: 5661–67. https://doi.org/10.1038/onc.2017.184.

Pilarova, Kveta, Jan Herudek, and Dalibor Blazek. 2020. “CDK12: Cellular Functions and Therapeutic Potential of Versatile Player in Cancer.” *NAR Cancer* 2, no. 1. https://doi.org/10.1093/narcan/zcaa003.

Poon, Ivan K., Christopher D. Lucas, Adriano G. Rossi, and Kodi S. Ravichandran. 2014. “Apoptotic Cell Clearance: Basic Biology and Therapeutic Potential.” *Nature Reviews Immunology* 14, no. 3: 166–80. https://doi.org/10.1038/nri3607.

Redon, Sophie, Patrick Reichenbach, and Joachim Lingner. 2010. “The Non-Coding RNA Terra Is a Natural Ligand and Direct Inhibitor of Human Telomerase.” *Nucleic Acids Research* 38, no. 17: 5797–5806. https://doi.org/10.1093/nar/gkq296.

Samraj, Ajoy K., Eric Keil, Nana Ueffing, Klaus Schulze-Osthoff, and Ingo Schmitz. 2006. “Loss of Caspase-9 Provides Genetic Evidence for the Type I/II Concept of CD95-Mediated Apoptosis.” *Journal of Biological Chemistry* 281, no. 40: 29652–59. https://doi.org/10.1074/jbc.m603487200.

Sehgal, L, R Mathur, F K Braun, J F Wise, Z Berkova, S Neelapu, L W Kwak, and F Samaniego. 2014. “Fas-Antisense 1 Lncrna and Production of Soluble versus Membrane FAS in B-Cell Lymphoma.” *Leukemia* 28, no. 12: 2376–87. https://doi.org/10.1038/leu.2014.126.

Shi, Yao, Chen Lv, Li Shi, and Guanjun Tu. 2017. “Meg3 Inhibits Proliferation and Invasion and Promotes Apoptosis of Human Osteosarcoma Cells.” *Oncology Letters*. https://doi.org/10.3892/ol.2017.7463.

Shou, Jiang, Francis Ali-Osman, Asha S Multani, Sen Pathak, Paolo Fedi, and Kalkunte S Srivenugopal. 2002. “Human DKK-1, a Gene Encoding a Wnt Antagonist, Responds to DNA Damage and Its Overexpression Sensitizes Brain Tumor Cells

to Apoptosis Following Alkylation Damage of DNA." *Oncogene* 21, no. 6: 878–89. https://doi.org/10.1038/sj.onc.1205138.

Shukla, Chinmay J, Alexandra L McCorkindale, Chiara Gerhardinger, Keegan D Korthauer, Moran N Cabili, David M Shechner, Rafael A Irizarry, Philipp G Maass, and John L Rinn. 2018. "High-Throughput Identification of Rna Nuclear Enrichment Sequences." *The EMBO Journal* 37, no. 6. https://doi.org/10.15252/embj.201798452.

Spencer, Sabrina L., Suzanne Gaudet, John G. Albeck, John M. Burke, and Peter K. Sorger. 2009. "Non-Genetic Origins of Cell-to-Cell Variability in Trail-Induced Apoptosis." *Nature* 459, no. 7245: 428–32. https://doi.org/10.1038/nature08012.

Statello, Luisa, Chun-Jie Guo, Ling-Ling Chen, and Maite Huarte. 2020. "Gene Regulation by Long Non-Coding RNAS and Its Biological Functions." *Nature Reviews Molecular Cell Biology* 22, no. 2: 96–118. https://doi.org/10.1038/s41580-020-00315-9.

Subramanian, Aravind, Pablo Tamayo, Vamsi K. Mootha, Sayan Mukherjee, Benjamin L. Ebert, Michael A. Gillette, Amanda Paulovich, et al. 2005. "Gene Set Enrichment Analysis: A Knowledge-Based Approach for Interpreting Genome-Wide Expression Profiles." Proceedings of the National Academy of Sciences 102, no. 43: 15545–50. https://doi.org/10.1073/pnas.0506580102.

Takeiwa, Toshihiko, Kazuhiro Ikeda, Kuniko Horie-Inoue, and Satoshi Inoue. 2021. "Mechanisms of Apoptosis-Related Long Non-Coding RNAS in Ovarian Cancer." *Frontiers in Cell and Developmental Biology* 9. https://doi.org/10.3389/fcell.2021.641963.

Ticli, Giulio, Ornella Cazzalini, Lucia A. Stivala, and Ennio Prosperi. 2022. "Revisiting the Function of p21cdkn1a in DNA Repair: The Influence of Protein Interactions and Stability." *International Journal of Molecular Sciences* 23, no. 13: 7058. https://doi.org/10.3390/ijms23137058.

Tiedge, H, R T Fremeau, P H Weinstock, O Arancio, and J Brosius. 1991. “Dendritic Location of Neural BC1 RNA.” *Proceedings of the National Academy of Sciences* 88, no. 6: 2093–97. https://doi.org/10.1073/pnas.88.6.2093.

Tripathi, Vidisha, Jonathan D. Ellis, Zhen Shen, David Y. Song, Qun Pan, Andrew T. Watt, Susan M. Freier, et al. 2010. “The Nuclear-Retained Noncoding RNA MALAT1 Regulates Alternative Splicing by Modulating SR Splicing Factor Phosphorylation.” *Molecular Cell* 39, no. 6: 925–38. https://doi.org/10.1016/j.molcel.2010.08.011.

Tsai, Miao-Chih, Ohad Manor, Yue Wan, Nima Mosammaparast, Jordon K. Wang, Fei Lan, Yang Shi, Eran Segal, and Howard Y. Chang. 2010. “Long Noncoding RNA as Modular Scaffold of Histone Modification Complexes.” *Science* 329, no. 5992: 689–93. https://doi.org/10.1126/science.1192002.

Veenstra, Gert Jan, and Alan P Wolffe. 2001. “Gene-Selective Developmental Roles of General Transcription Factors.” *Trends in Biochemical Sciences* 26, no. 11: 665–71. https://doi.org/10.1016/s0968-0004(01)01970-3.

Walmsley, Sarah R., Cristin Print, Neda Farahi, Carole Peyssonnaux, Randall S. Johnson, Thorsten Cramer, Anastasia Sobolewski, et al. 2005.“Hypoxia-Induced Neutrophil Survival Is Mediated by Hif-1α–Dependent NF-KB Activity.” *Journal of Experimental Medicine* 201, no. 1: 105–15. https://doi.org/10.1084/jem.20040624.

Wang, Hua, Dongxi Xiang, Ben Liu, Aina He, Helena J. Randle, Kelvin Xi Zhang, Anushka Dongre, et al. 2019. “Inadequate DNA Damage Repair Promotes Mammary Transdifferentiation, Leading to BRCA1 Breast Cancer.” *Cell* 178, no. 1. https://doi.org/10.1016/j.cell.2019.06.002.

Wang, Kevin C., and Howard Y. Chang. 2011. “Molecular Mechanisms of Long Noncoding RNAS.” *Molecular Cell* 43, no. 6: 904–14. https://doi.org/10.1016/j.molcel.2011.08.018.

Wei, Wei, Xiaomei Ying, Liang Chen, Qingmei Sun, Xiaohuan Lu, Yang Xia, Rubin Xu, et al. 2022. “Recq Mediated Genome Instability 2 (RMI2): A Potential Prognostic and Immunological Biomarker for Pan-Cancers.” *Aging* 14, no. 9: 4107–36. https://doi.org/10.18632/aging.204076.

Wu, Meng-Zhi, Tao Fu, Jin-Xi Chen, Ying-Ying Lin, Jin-E Yang, and Shi-Mei Zhuang. 2020. “LncRNA GOLGA2P10 Is Induced by PERK/ATF4/Chop Signaling and Protects Tumor Cells from ER Stress-Induced Apoptosis by Regulating BCL-2 Family Members.” *Cell Death & Disease* 11, no. 4. https://doi.org/10.1038/s41419-020-2469-1.

Wu, Wenjing, Shuwan Zhang, Xu Li, Mei Xue, Sancheng Cao, and Wei Chen. 2013. “ETS-2 Regulates Cell Apoptosis via the AKT Pathway, through the Regulation of Urothelial Cancer Associated 1, a Long Non-Coding RNA, in Bladder Cancer Cells.” *PLoS ONE* 8, no. 9. https://doi.org/10.1371/journal.pone.0073920.

Yan, Kun, Yasir Arfat, Dijie Li, Fan Zhao, Zhihao Chen, Chong Yin, Yulong Sun, Lifang Hu, Tuanmin Yang, and Airong Qian. 2016. “Structure Prediction: New Insights into Decrypting Long Noncoding RNAS.” *International Journal of Molecular Sciences* 17, no. 1: 132. https://doi.org/10.3390/ijms17010132.

Yang, Shan-zhong, Fei Xu, Tong Zhou, Xinyang Zhao, Jay M. McDonald, and Yabing Chen. 2017. “The Long Non-Coding RNA HOTAIR Enhances Pancreatic Cancer Resistance to TNF-Related Apoptosis-Inducing Ligand.” *Journal of Biological Chemistry* 292, no. 25: 10390–97. https://doi.org/10.1074/jbc.m117.786830.

Zhao, Hang, Xueqing Zhang, Josias Brito Frazão, Antonio Condino-Neto, and Peter E. Newburger. 2013. “Hox Antisense Lincrna HOXA-AS2 Is an Apoptosis Repressor in All*Trans*Retinoic Acid Treated NB4 Promyelocytic Leukemia Cells.” *Journal of Cellular Biochemistry* 114, no. 10: 2375–83. https://doi.org/10.1002/jcb.24586.