INVESTIGATION OF THE INTERACTION BETWEEN DR5-AS LONG NONCODING RNA AND CAPRIN1 PROTEIN

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ABSTRACT

INVESTIGATION OF THE INTERACTION BETWEEN DR5-AS LONG NONCODING RNA AND CAPRIN1 PROTEIN

Cell proliferation is the crucial process for many physiological incidents such as tissue and organ development, wound healing, and immune system reactions. It is achieved by the growth and division of cells in a multicellular organism. Investigation of molecules involved in the regulation of cell cycle mechanism provides insight into reasons and treatments of the diseases such as cancer. In recent years, information that acquired from deep sequencing reveals that several proteins and non-coding RNAs have crucial role in the regulation of cell cycle and proliferation. Death receptor 5 antisense (DR5-AS) is a novel long non-coding RNA (lncRNA) transcript that is cisplatin inducible and is involved in modulation of cell proliferation and cell cycle in HeLa cells. When DR5-AS lncRNA was knocked down, the morphology of HeLa cells became spherical without inducing apoptosis. Although this lncRNA reduces cell proliferation via a cell cycle arrest at S and G2/M phases, mechanism behind this cell cycle arrest is not known. lncRNAs work in complexes with RNA, DNA, and protein interactions in the cell. There are several experimental and bioinformatical approaches to investigate RNA: protein interactions such as PAR-CLIP. In this approach, proximal protein and RNAs are covalently bonded with UV radiation. Then this complex is immunoprecipitated with specific antibodies. According to PAR-CLIP data of DR5-AS lncRNA, CAPRIN1 is a cell cycle associated protein that has the highest interaction score. The results suggest that CAPRIN1 and DR5-AS work reversely in cell proliferation although under the cisplatin treatment, CAPRIN1 enhances the expression of DR5-AS lncRNA. All these observations were confirmed by many quantitative experiments. Conclusively, this study provides a clue about how DR5-AS lncRNA might regulate cell cycle and proliferation through CAPRIN1 protein.

Keywords: *Cell Cycle, Proliferation, Long Non-Coding RNA, DR5-AS, CAPRIN1*

ÖZET

DR5-AS UZUN KODLAMAYAN RNA'SININ CAPRIN1 PROTEİNİ İLE ETKİLEŞİMİNİN ARAŞTIRILMASI

Hücre proliferasyonu, doku ve organ gelişimi, yaraların iyileşmesi ve bağışıklık sistemi reaksiyonları gibi birçok fizyolojik olay için çok önemli bir hücresel işlevdir. Çok hücreli bir organizmada hücrelerin büyümesi ve bölünmesi işlemidir. Hücre döngüsü mekanizmasının düzenlenmesinde görev alan moleküllerin araştırılması kanser gibi hastalıkların nedenlerinin ve tedavilerinin anlaşılmasını sağlar. Son yıllarda, derin sekanslamadan elde edilen bilgiler, birçok proteinin ve kodlamayan RNA'nın hücre döngüsü ve proliferasyonun düzenlenmesinde önemli bir role sahip olduğunu ortaya koymaktadır. DR5-AS, sisplatin ile indüklenebilir kodlamayan bir RNA transkripsiyonudur ve HeLa hücrelerinin hücre proliferasyonu ve hücre döngüsünde bir modülatör olarak yer alırken, DR5-AS lncRNA susturulduğunda, HeLa hücresinin morfolojisi apoptozu indüklemeden küresel hale gelir. Bu lncRNA, S ve G2/M fazlarında hücre döngüsü durdurma yoluyla hücre çoğalmasını azaltmasına rağmen, bu hücre döngüsü durdurmasının arkasındaki mekanizma bilinmemektedir. lncRNA'lar hücrede RNA, DNA ve protein etkileşimleri ile çalışır. RNA: protein etkileşimlerini araştırmak için PAR-CLIP gibi birkaç deneysel ve biyoinformatik yaklaşım vardır. Bu yaklaşımda, birbiriyle etkileşecek kadar yakın olan protein ve RNA'lar UV radyasyonu ile kovalent olarak bağlanır. Daha sonra bu kompleks, spesifik antikorlarla çöktürülür. DR5-AS lncRNA'nın PAR-CLIP verilerine göre CAPRIN1, en yüksek etkileşim skoruna sahip, hücre döngüsü ile ilişkili bir proteindir. Sonuçlar, sisplatin uygulaması altında CAPRIN1'in DR5-AS lncRNA ekspresyonunu arttırmasına rağmen, CAPRIN1 ve DR5-AS'nin hücre proliferasyonunda tersine çalıştığını göstermektedir. Tüm bu gözlemler qPCR ile doğrulanmıştır. Sonuç olarak, bu çalışmada, DR5-AS lncRNA'nın CAPRIN1 proteini aracılığıyla hücre döngüsünü ve proliferasyonu düzenlediğine dair oldukça önemli veriler sunulmaktadır.

Anahtar Kelimeler: *Hücre Döngüsü, Proliferasyon, Uzun Kodlamayan RNA, DR5-AS, CAPRIN1*

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CHAPTER 1

INTRODUCTION

Central dogma of molecular biology dictates that flow of the information can only pass-through RNA from DNA, then RNA, which was believed to be an intermediate molecule, generates a phenotype by translating into protein (Jarroux, Morillon, and Pinskaya 2017; Hüttenhofer, Schattner, and Polacek 2005). In the view of such information, scientists anticipated that a significant portion of the human genome must have a coding capacity. Completed genome projects have revealed that only 1% of the genome has the capacity to be translated into proteins and the rest is transcribed but does not produce proteins (Collins, Morgan, and Patrinos 2003; Eddy 2012). These large portions of the genome were initially considered as transcriptional noise. But progress in the sequencing technology has provided a new perspective to this transcriptional noise and it has led to the discovery of a novel class of non-translated RNAs. There are 20,000 protein-coding genes in mammalian genome and more than 100,000 transcripts (Fernandes et al. 2019; Ponting, Oliver, and Reik 2009). This knowledge gives rise to revision about the definition of junk DNA, so it is known that this junk DNA has essential roles in the cell (Jarroux, Morillon, and Pinskaya 2017).

1.1. Long Non-Coding RNAs

Non-coding RNAs can be classified based on their localization, length, or functions and these are identified as RNA molecules which do not have functional open reading frame (ORF). Long Non-Coding RNAs are a subclass of non-coding RNAs which are named according to their length because they contain more than 200 nucleotides (nt). Some lncRNAs may have a 5’ cap structure and/or polyA tail so they might be present in the cytosol. Primarily lncRNAs are transcribed by RNA Polymerase II and some lncRNAs can produce small peptides by translation (Hüttenhofer, Schattner, and Polacek 2005; Qian et al. 2019; Brannan et al. 1990; Li and Liu 2019). Although lncRNAs are not conserved evolutionary in comparison with mRNAs, their presence is very common in several organisms such as animals, plants, prokaryotes and even viruses (Brannan et al.

1990). Their functions that are barely known may show differences in each organism because they have specific characters in different organisms. Their abundance is not too much in the cell, however it is apparent that they have involved many crucial processes ranging from cell cycle, cell proliferation, invasion to tumorigenesis. They can be oncogenes and/or tumor suppressors or may involve in drug resistance in cancer cells (J. yan Wang, Lu, and Chen 2019).

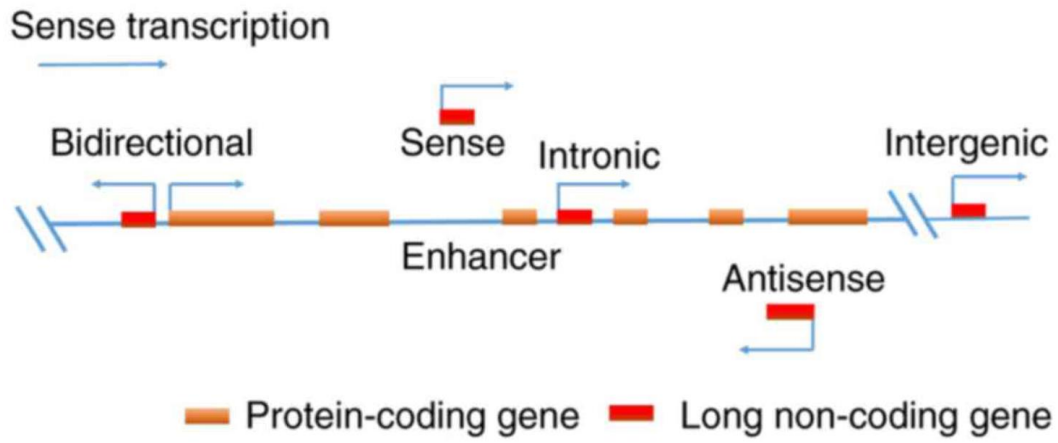

Figure 1.1. Long non-coding RNAs are classified based on their genomic context and location with respect to neighboring protein-coding genes as bidirectional, intronic, intergenic, sense and antisense.
(Source: Zhou et al. 2018)

1.1.1. Classification of Long Non-Coding RNAs

Long non-coding RNAs can be classified according to their different characteristics in the following way: genome location and context, exerted effect of DNA sequences, mechanism of functioning and targeting mechanism (Ma, Bajic, and Zang 2013). Long non-coding RNAs are placed and transcribed from different regions in the genome. There are five major classes based on genomic context and those are intergenic, intronic, bidirectional, sense and antisense lncRNA (Hombach and Kretz 2016; Chen, Feng, and Wang 2018; Esteller 2011). Intergenic lncRNAs are transcribed from intergenic region in both strands in contrast intronic lncRNAs are transcribed from entirely introns from protein-coding genes (Esteller 2011; Matsui and Corey 2017). Sense lncRNAs are transcribed from the sense strand that means it is in the same direction with protein-coding gene and they overlap with some part of protein-coding gene or include

all sequence of the protein-coding gene while antisense lncRNAs are transcribed from the antisense strand which means in the opposite strand of protein-coding gene and overlap with both exonic and intronic regions or include protein-coding gene sequence completely (Fang and Fullwood 2016; Jarroux, Morillon, and Pinskaya 2017). And the last group is bidirectional lncRNAs which are transcribed from the opposite strand of the protein-coding gene like antisense lncRNAs. However, there is a difference between them. Bidirectional lncRNAs do not overlap with protein-coding gene sequences (Meng et al. 2017). Long non-coding RNAs are located commonly in the nucleus thus they may have effect on DNA sequences and transcriptional regulation. Therefore, lncRNAs can be classified based on their effects exerted on DNA sequences: cis-lncRNAs and trans-lncRNAs. Cis-acting lncRNAs have role in the regulation of the expression of genes in proximity while trans-acting lncRNAs involve in the regulation of the expression of genes in distant (Ma, Bajic, and Zang 2013).

1.1.2. Functional Roles of Long Non-Coding RNAs

In recent years, many lncRNAs have been discovered in animals and plants owing to advances in transcriptome analyses such as microarrays, tiling arrays, expressed sequence tags (ESTs) and RNA-seq. The biological functions of several animal lncRNAs have been established; however, only a few plant lncRNAs have been examined (Zhang, Wang, and Zhu 2019). lncRNAs have been implicated in epigenetic, transcriptional regulation, post-transcriptional regulation, and translational regulation as well as post-transcriptional modification according to research (Dykes, and Emanueli 2017). Besides, lncRNAs play a variety of regulatory roles in both humans and animals. Dosage compensation effects, genomic imprinting, chromatin modification and remodeling are the key regulatory roles at the chromatin level. Also, lncRNAs are involved in the regulation of alternative splicing, cell differentiation, and cell cycle regulation. Differential expression of these lncRNAs provides clues about the incidence of a variety of illnesses including cancer (Jarroux, Morillon, and Pinskaya 2017; Gupta, Shah, and Wang 2010). Based on the current understanding of lncRNA function, these transcripts are classified into those mediating chromatin modifications and DNA methylation related with epigenetic regulation, proteins and DNA interactions related with regulation in transcriptional level, and mRNA processing at the post-transcriptional level, along with

direct interactions with proteins to control translation of proteins and post-transcriptional modification (Matzke, and Mosher 2014; Zang, Wang, and Zhu 2019).

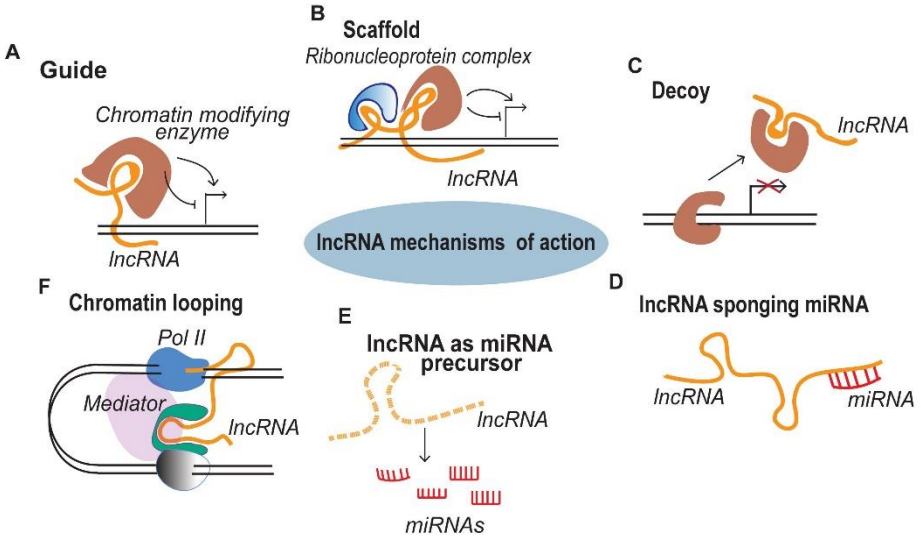

Figure 1.2. Long non-coding RNAs mechanisms of action
(Source: Sweta et al. 2019)

1.1.3. Working Mechanisms of Long Non-Coding RNAs

Long non-coding RNA expressions can be correlated with exceptional cell type and cell state specificity and, their localization within the cell exhibits their biological significance for their molecular functions. Based on the cellular locations, they can be classified into two major groups; nuclear lncRNAs and cytoplasmic lncRNAs (Miao et al. 2019).

Specified by the mechanism of action where they are in the cells, lncRNAs may work *in cis* on neighboring genes regulation while *in trans* on distantly located genes regulation or their molecular targets that are found in nucleus or cytoplasm (Wang and Chang 2011). Their actions are different based on proximity of their targets however, the basic working principle is the same for both situations. Mechanisms of lncRNA actions can be classified as chromatin regulators, transcriptional regulators, and post-transcriptional regulators. The number of lncRNAs are able to recruit chromatin-remodeling complex to mediate epigenetic modifications such as polycomb repressive complex 2 or chromatin-activating complexes. In addition, lncRNAs can mediate the

activity of transcriptional factors and they can act as a co-factor to enhance target gene transcription. For example, Evf2 ncRNA recruits the transcription factor DIX2 to induce expression of proximal protein-coding genes. Besides, lncRNAs are able to define complementary sequences so this provides capability of post-transcriptional regulation processes such as capping, splicing, transport, translation, stability and degradation. MALAT1 is an example for regulation of splicing, it has the ability of alternative splicing by interacting with splicing factors. Some nuclear lncRNAs may interact with DNA and attend formation or disruption of chromosomal loops so they can reorganize the chromosomal architecture. In the addition to these, lncRNAs may regulate the subnuclear structures such as Polycomb bodies (Bhat et al. 2016; Neguembor, Jothi, and Gabellini 2014; López-Urrutia et al. 2019).

On the other hand, cytoplasmic lncRNAs are transported to the cytosol after their process and some of these assist translocations of other proteins. Also, they play role in the translational regulation in various ways. They can act as a sponge and cause the sequestration of miRNAs thus they can alter the expression of mRNAs which are targeted by sequestered miRNAs. In another way for translational regulation, some lncRNAs can directly alter the translation rate of specific mRNAs by recruitment or inhibition of polysome loading. Lastly, lncRNAs can alter mRNA decay by increasing the mRNA stability such as methylation or stimulate mRNA decay by recruiting the degradation complex (Salmena et al. 2011; Tang et al. 2019; Carlevaro-Fita et al. 2016)

1.2. DR5-AS Long Non-Coding RNA and Its Function on Cell Proliferation

Death receptor 5 antisense (DR5-AS) lncRNA was one of the differentially expressed lncRNAs under cisplatin induction (Ahmadov 2015). The name of this lncRNA comes from its location as it overlaps (803 bp) with the DR5 gene, which is involved in cell proliferation and apoptosis. AC107959.2 gene (DR5-AS) is a natural antisense transcript (NAT) that is 2,636 bp long in the human reference genome GRCh38.p13 and it is located on chromosome 8p21.3. DR5 is a p53-dependent TNF-related apoptosis-inducing ligand (TRAIL)-induced receptor, which is involved in apoptosis, miRNA biogenesis, survival, and proliferation. This cell surface receptor, which is activated by TRAIL, trigger the cell transduction pathways such as caspases and cause induction of

apoptosis (Ke et al. 2018). In addition, DR5 is regulated by p53 and ATF3 in response to the DNA damage and it may enhance cell proliferation and apoptosis according to the interactions with different sets of signal transduction pathways. Besides that, DR5 can be translocated in nucleus by interacting with importin B1 and play role in the upregulation of LIN28B and HMGA2, promoting cell growth by interacting with miRNA processing machinery (Mert and Sanlioglu 2016).

The DR5-AS gene is annotated as a transcript with three exons (Gibb et al. 2011). Based on the previous data, DR5-AS gene is conserved among various species, and it is differentially expressed in different cancer cells. According to the previous data in this laboratory, DR5-AS 5' and 3' borders were defined, and it encodes a 2636-nt transcript without any tail. Also, it appears to be localized primarily in the nucleus (Gurer et al. 2021; Sweef 2020). DR5-AS lncRNA knockdown causes an issue in cell proliferation and cell cycle flow by triggering ae cell cycle arrest in the metaphase stage in HeLa cells regulating the expressions of key proteins which have role in cell cycle processes such as ANAPC2, ANAPC4, GADD45B and CENPP (Gurer et al. 2021).

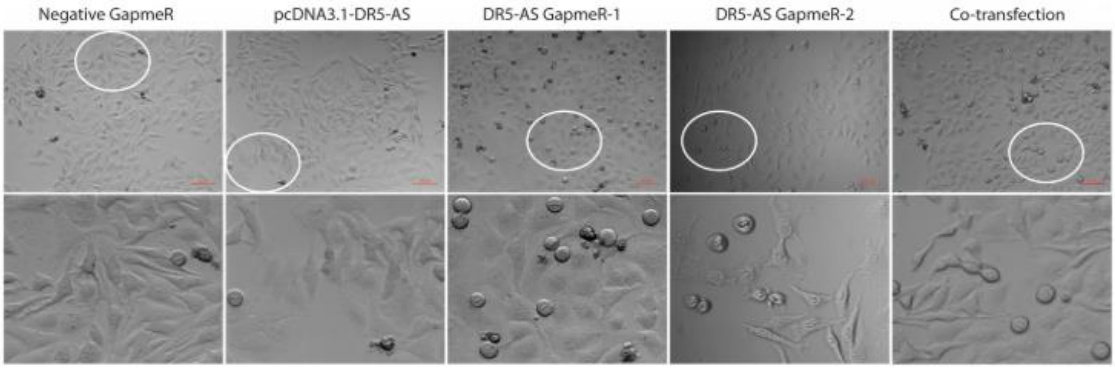

Figure 1.3. DR5-AS knockdown alters cell morphology in HeLa cells. Brightfield microscopy analysis of HeLa cell morphology under treatment of Negative GapmeR, overexpression vector, DR5-AS GapmeR, and co-transfection. (Source: Gurer et al. 2021)

1.3. Experimental and Bioinformatical Approaches to Study lncRNA and Protein Interaction

With the beginning of the discovery of first lncRNA, several approaches have been developed to understand whether lncRNA is a functional molecule and if it is

functional, then how it can act to this function. It is widely known that lncRNAs can act as a regulator in many biological processes like mRNAs although they are not translated into proteins (McDonel and Guttman 2019). So, how they can act their regulatory functions?

According to the literature, lncRNAs have the ability to interact with other nucleic acids (DNA or RNA) and proteins directly or indirectly in the cell to achieve their regulatory functions. With the direct interactions, they can trigger sequestration or releasing transcript by controlling gene expression and with the binding of DNA they can inhibit the transcription. Besides, with the indirect interaction, they can control recruitment of other molecules and formation of functional complexes, or they can work as a scaffold and attend the chromatin formation (Hombach and Kretz 2016). RNA and proteins are interrelated molecules that may interact directly to affect each other's functions and life cycles (Ramanathan, Porter, and Khavari 2019).

Beginning with demonstrated of lncRNAs functionality, the following step is the examination of the molecular mechanism behind that function. For this purpose, there are two reciprocal methods for determination of lncRNA and protein interaction *in vivo*. The first one is RNA immunoprecipitation, in which the lncRNA is tagged with probes and the protein which interacts with this lncRNA can be precipitated with the help of cross-linking. This precipitated protein or proteins can be defined by mass spectrometry. RNA immunoprecipitation can be a challenging method because lncRNA abundance in the cell is very limited. The second one is protein immunoprecipitation; this approach allows the identification of all RNAs which interact with precipitated protein of interest by isolation RNAs from precipitation. This method is useful for determination of target lncRNAs that interact with specific RNA-binding proteins (Selth, Gilbert, and Svejstrup 2009; Ramanathan, Porter, and Khavari 2019). Moreover, there is another newly developed approach to identify in-cell RNA-protein interactions called as incPRINT. This high-throughput method is based on quantification of luminescence. MS2 luciferase tagged lncRNA is co-transfected with flag tagged RNA binding protein library into MS2CP luciferase-expressing cell. With the luciferase assay, lncRNA-protein interactions can be defined, and protein of interest can be identified with ELISA. However, this approach is limited for determination of indirect RNA-protein bindings (Graindorge et al. 2019).

The following part after validation of lncRNA-protein interaction is examination of functional outcome of this specific interaction. For this purpose, some phenotypic observations can be done by targeted mutagenesis at the specific site of lncRNA-protein

interaction. Then, this observed phenotype should be confirmed by rescue trails. Consequently, there are several approaches to study functions of lncRNA-protein interactions. According to the scientific questions, different approaches can be chosen (McDonel and Guttman 2019).

Although there are several high-throughput experimental approaches to investigate lncRNA-protein interaction, computational methods are necessary to screen the potential interactions and choose candidates before experimental validation. Each of these computational predictors can follow different strategies that are dependent on some biological databases. Starbase. POSTAR, RAIN, RNAInter and NPInter are some of the examples of these databases and each of these databases have own advantages and limitations. These databases can contain curated lncRNA-protein interactions or contain RNA-binding motifs (Zhang et al. 2020).

PAR-CLIP (Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation) is one of the experimental and bioinformatical approaches employed to examine lncRNA-protein interactions. In this approach, proximal lncRNA and protein are covalently bound (crosslinked) with UV irradiation. After crosslink, the resultin complexes are immunoprecipitated with previously determined specific antibodies and RNA is isolated and deep sequenced. The result of this sequence gives a map thus lncRNAs that interact with protein of interest can be identified (Lin and Ouyang 2020; Spitzer et al. 2014). To investigate proteins that have a potential to interact with DR5-AS lncRNA, a PAR-CLIP data that was previously published was analyzed (Baltz et al. 2012). According to this data analysis, there are 6 candidates selected based on the highest interaction score (>0.5). These candidates are given in figure 1.4. and CAPRIN1 has the highest interaction score with 0.999317 and most related one in cell proliferation and cell cycle. However, this approach gives false positive results so these candidates must be validated by experimental approaches (Baltz et al. 2012; Lin and Ouyang 2020).

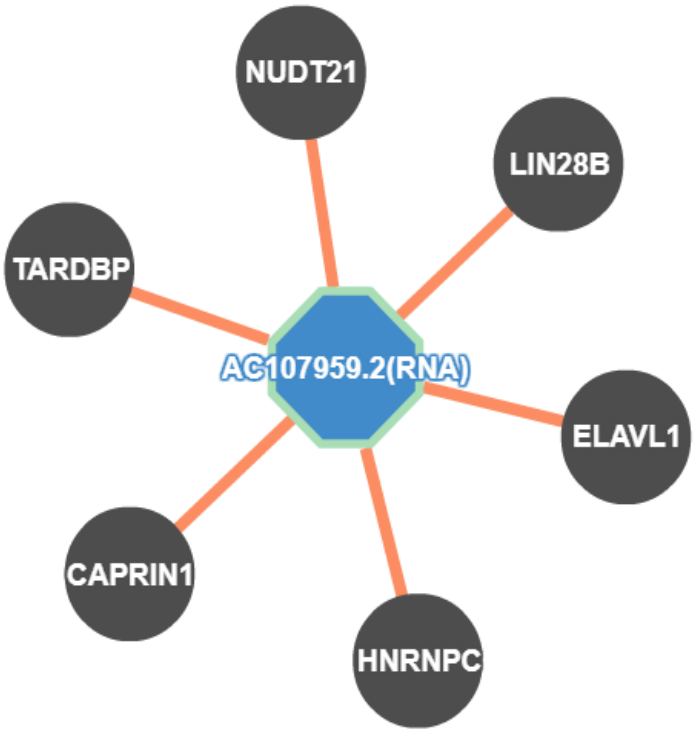

Figure 1.4. Protein interactions of DR5-AS lncRNA
(Source: Baltz et al. 2012).

1.4. CAPRIN1 Protein and DR5-AS Long Non-Coding RNA

Cell cycle associated protein 1 (CAPRIN1) is an RNA-binding protein that is encoded by CAPRIN1 gene. This gene is located on chr11:34,051,063-34,102,610 plus strand according to the GRCh38 and has 50,948 bases. Additionally, it encodes a 709-amino acid protein, and its molecular weight is 116 kDa.

Caprin1 is a part of caprin family which is highly conserved cytoplasmic phosphoprotein. It is ubiquitously expressed so initially high expression of caprin1 is defined in dividing cells of the thymus. Despite having low expression in slowly dividing cells such as kidney or muscles, expression of caprin1 has been reported to be high in the brain. Caprin1 has RNA binding characteristic such as RGG motif and RG enrichment region due to it is an RNA binding protein that is essential for cell proliferation. It is related with controlling of the cell cycle associated genes and alteration in caprin1 plays role in oncogenesis so it can affect cell survival and growth. It can act alone or interact with other RBPs such as fragile X mental retardation protein. According to the data in the

literature, caprin1 binding mRNA targets are more than 6,064 and these mRNAs have various biological functions in cell structure, RNA metabolism, RNA translation, signal transduction and ubiquitylation. Also, caprin1 binds selectively to c-Myc and cyclin-D2 mRNAs so it is involved in cell growth, migration, and differentiation. These two mRNAs are involved in acceleration of cell progression through the G1 to S phase transition (Wang, David, and Schrader 2022). Therefore, they promote cell growth and viability, and they may have important role in the tumorigenesis. On the other hand, caprin1 triggers upregulation of expression in immune checkpoint proteins and formation of the stress granules in the tumor cells so it contributes to adaptation of tumor cells to radiation and harsh conditions (Yang et al. 2019; Wang et al. 2005; Qiu et al. 2015).

Knock-down of DR5-AS lncRNA causes stacking of cells at S phases in the cell cycle and cells with low expression of caprin1 show delay in the G1-S transition in the cell cycle (Gurer et al. 2021; Wang et al. 2005). According to all this information and PAR-CLIP data, DR5-AS lncRNA may bind with CAPRIN1 protein and they can involve in cell cycle processes.

1.5. Aim

This study aims to uncover the molecular and phenotypic effects of DR5-AS lncRNA and CAPRIN1 protein interactions on cell proliferation and cell cycle.

CHAPTER 2

MATERIALS AND METHODS

2.1. Cell culture, Transfection, and Drug Treatment

HeLa cells were provided from DSMZ GmbH (Gibco). The culture conditions were RPMI 1640 medium (with L-Glutamine, Gibco), supplemented with 10% fetal bovine serum (FBS) (Gibco) with humidified air with 5% CO_2 at 37ºC. Cells were subcultured every other day in a 75 cm^2 cell culture flask with 2.0 – 2.5 x 10^6 cells.

For overexpression experiments, pHTN-HaloTag-CMV-neo vector (Promega) was linearized with *Eco*RI (NEB) restriction enzyme and isolated from 1% agarose gel after running 1 hour at 100 V. The coding sequence of CAPRIN1 gene was constructed by In-Fusion Cloning (5X In-Fusion HD Enzyme Premix, Takara). In this ligation-independent cloning, to amplify the coding sequence of CAPRIN1 gene, primers were designed with 18-25 nucleotides homologous to our insert and 15 nucleotides extensions homologous to the of the linearized pHTN-HaloTag-CMV-neo vector. (Forward primer sequence: 5^-GATCGCTTCCGAATTCATGCCCTCGGCCACC, reverse primer sequence: 5'-GTTGAGCTCTGAATTTTAATTCACTT GCTGAGTGTTCATTTGCG). As a result, CAPRIN1 construct was generated by performing gel isolation after PCR amplification. pHTN-HaloTag-CMV-neo – CAPRIN1 construct was generated by in-fusion reaction in a 1:2 molar ratio and this construct was transformed into DH5-α competent cells. Colonies were plated on selective media and checked for cloning accuracy by colony PCR. Plasmid isolation was performed by NucleoSpin Plasmid Mini Kits (Macherey Nagel) from successfully transformed colonies and construct was sequenced. After verification of sequence, plasmid was isolated with NucleoBond Xtra Midi EF (Macherey Nagel) as endotoxin free to transfect HeLa cells. One day before the experiment, 7.5 x 10^4 cells per plate were seeded on 6 well-plates (Sarstedt) and allowed to be grown overnight in FBS containing RPMI 1640 (with L-Glutamine, Gibco) without antibiotics. Following day, transfection mixture was prepared by following steps; 1500 ng of plasmid DNA was transferred in RPMI 1640 (with L-Glutamine, Gibco) without FBS and antibiotics followed by addition of 4.5 µl of FuGENE HD Transfection Reagent

(Promega) then mixture was vortexed for 3-5 seconds. Total reaction mixture was set 150 µl and this mixture was incubated at room temperature for 10 minutes. Until then, the media of cells were refreshed with RPMI 1640 (with L-Glutamine, Gibco) with FBS. After completion of incubation, mixture was spun shortly and added into the media drop by drop to ensure even distribution. The total volume of the each well was 2 mL and after 1h from transfection, medium of transfected cells was refreshed by RPMI 1640 (with L-Glutamine, Gibco) containing FBS due to toxicity of plasmid DNA transfection. After transfection, the cells were incubated for 12 to 72 hours. The subsequent experiments were set to 24 hours incubation for plasmid DNA and 72 hours for only vector. In this experiment set up, there were 4 sample groups. First one was control HeLa cells that placed RPMI 1640 (with L-Glutamine, Gibco) containing 10% FBS, second was control cells that only containing transfection reagent (FuGENE HD Transfection Reagent, Promega), third group was pHTN-HaloTag-CMV-neo vector transfected cells as a negative control and the last one was pHTN-HaloTag-CMV-neo – CAPRIN1 plasmid transfected cells. As a positive control to test transfection efficiency, green fluorescence protein (GFP) containing vector was transfected in the same way and after transfection, transfected cells were imaged by fluorescence microscope to confirm transfection. After that, proteins were isolated using TRIzol (Thermo) from plasmid DNA and only vector transfected HeLa cells. Transfection accuracy and protein expressions were confirmed by Western-Blot using Anti-HaloTag monoclonal antibody (Promega).

Silencing experiments were performed with si-CAPRIN1 (Dharmacon). Before transfection, 100 µM siRNA solution was prepared for si-CAPRIN1 and off target siRNA (Dharmacon) as a negative control in 1x siRNA buffer (60 mM KCl (Sigma), 6 mM HEPES-pH 7.5 (Gibco), and 0.2 mM $MgCl_2$ (Applichem)). All stock solutions were stored at -20 ºC.

1 x 10^5 cells were seeded on 6 well-plates (Sarstedt) and incubated overnight. Tube 1 included siRNA and tube 2 included DharmaFECT transfection reagent were diluted separately. Tube 1 was prepared by mixing 0.5 µl of 100 µM siRNA and 199.5 µl of RPMI 1640 (with L-Glutamine, Gibco) medium without serum. Tube 2 was prepared by adding 4 µl DharmaFECT transfection reagent in 196 µl of serum free RPMI 1640 (with L-Glutamine, Gibco). Both tubes were incubated for 5 minutes at room temperature separately and then tube 2 was added to tube 1 and total volume was fixed as 400 µl. Before adding 1600 µl antibiotic-free RPMI 1640 (with L-Glutamine, Gibco) with FBS

to this mixture, it was incubated for 20 minutes at room temperature. Up to that time, culture medium was refreshed with 2000 µl fresh medium. After completion of incubation, mixture was spun shortly and added into the media drop by drop to ensure even distribution. Cells were incubated at 37°C in 5% CO_2 for 72 hours.

After a total of 72-hour incubation, RNAs were isolated from negative and CAPRIN1 siRNAs transfected HeLa cells with TRIzol (Thermo) and then cDNAs were synthesized from these RNAs using RevertAid First Strand cDNA synthesis Kit (Thermo). Silencing was confirmed by qPCR by using GoTaq qPCR Master Mix (Promega). At the same time, proteins were isolated from these cells by TRIzol (Thermo) and silencing was shown in protein level by using Anti-CAPRIN1 (Sigma-Aldrich) Antibody by Western Blotting.

For cisplatin treatment, 0.3 x 10^6 of HeLa cells were seeded on a 6 well-plate (Sarstedt) and incubated overnight. According to the previous studies (TUBITAK Project 113Z371), dose and time of cisplatin experiments were performed to achieve 50% early apoptosis rate in HeLa cells. 83.2 µM fresh stock of cisplatin (SantaCruz) was prepared by using DMSO as a diluting agent. Following experiments in HeLa cell were fixed as 80 µM for 16 hours and DMSO 0.1% (v/v) was used as negative control.

For determination of drug sensitivity after transfection, 0.3 x 10^6 HeLa cells were seeded on a 6 well-plate (Sarstedt) and incubated overnight to treat the transfected cells with cisplatin. Transfection protocol was acquired as stated above. 40 µM cisplatin (SantaCruz) was applied to pHTN-HaloTag-CMV-neo – CAPRIN1 transfected cells after 8 hours of transfection and incubated for 16 hours. Cisplatin treated cells that transfected with pHTN-HaloTag-CMV-neo vector were used as a control groups.

2.2. CLIP and RNA Extraction

Interaction between DR5-AS lncRNA and CAPRIN1 protein was investigated by UV Crosslinking and Immunoprecipitation (CLIP) method after CAPRIN1 protein was overexpressed with HaloTag vector in HeLa cells. Medium was removed from empty vector (only pHTN-HaloTag-CMV-neo) and test vector (pHTN-HaloTag-CMV-neo – CAPRIN1) transfected HeLa cells and cells were triggered with UV irradiation at 254 nm 200 mj/cm^2 using UVP crosslinker. Then, cells were scraped off the plates and lyzed with lysis buffer and sonication for 5 minutes. A part of lysate was saved as an input for control

and rest of it was immunoprecipitated with Magne HaloTag Beads (Promega) by incubation with lysates and beads with rotation at 4ºC for 16 h. After denaturing washes of beads, DNAse I treatment was applied to immunoprecipitated sample and then HaloTag protein was cleaved from CAPRIN1 protein by HaloTEV Protease (Promega). Precipitated CAPRIN1 protein was treated with Proteinase K mix (5M NaCl, 1M Tris pH 7.0, 10% SDS, H_2O) at 37ºC for 30 min and RNA was extracted from this precipitation by phenol-$CHCl_3$ with centrifugation at max speed for 10 minutes at 4ºC. Then, RNA was precipitated by using ethanol incubation at -20ºC for 16 hours. RNA pellet was dissolved in 8 µl of H_2O and quality of the RNAs were confirmed by NanoDrop (Thermo) measurements and $A_{260/230}$ – $A_{260/280}$ ratios (1.8 - 2.2). cDNAs were synthesized using RevertAid First Strand cDNA Synthesis Kit (Thermo) and expression of DR5-AS lncRNA was investigated using DR5-AS specific primers (Qiagen) and GoTaq qPCR Master Mix (Promega) in RotorGene (Qiagen) equipment. As a positive control c-Myc was used which is known the interactions with CAPRIN1 protein and GAPDH was used as a negative control for qPCR analysis.

For RNA extraction with TRIzol (Invitrogen), cells were harvested with Trypsin-EDTA (Gibco, 0.25%) and were washed twice with ice-cold PBS. Then, 1 mL of TRIzol (Invitrogen) was added to the cell pellets and cells were incubated at room temperature for 5 minutes for complete dissociation of nucleoprotein complexes. After incubation, 0.2 ml of chloroform (5:1 ratio) (Sigma) was added and the following step was vigorous shaking for 15 seconds. After 2 minutes incubation, cells were centrifuged at 12,000 x g at 4ºC for 15 minutes. This step is important for phase separation and collection of RNA without any DNA or protein contamination. The upper phase that contains RNA was collected by 45º angling to ensure of disturbing the other two phases. After collection of RNA, 0.5 mL of 100% isopropanol (Sigma) and 2 µl of glycogen (10 mg/ml) (Sigma) were added onto the RNA and incubated for 2 hours at -20ºC. After that RNA was precipitated by centrifugation at 12,000 x g 4ºC for 10 minutes. After removal of supernatant, RNA pellet was washed with 1 mL of 75% ice-cold ethanol followed by centrifugation at 7,500 x g 4ºC for 5 minutes. After that, ethanol was removed completely by air-dry and RNA pellet was dissolved with 20 µl of nuclease-free water. RNA quality was checked as described above.

2.3. Protein Extraction and Western Blot

For protein extraction, cells were washed by 1x ice-cold PBS to remove residual media after removing of culture media. Then cells were collected by centrifugation at 1200 RPM for 10 minutes and supernatant was removed clearly. Then 23 µl RIPA lysis buffer (1 x 10^6 cells) was added to cell lysate with 2 µl protease inhibitor cocktail (100X) (CST). Lysates were vortexed 1 minute, followed by incubation on ice for 10 minutes. This period was repeated four times and at the end, samples were centrifuged at 15.000 x g for 10 minutes at 4°C. Supernatant was collected carefully into new collection tube and proteins were stored at -80°C.

Proteins were quantified by Bradford assay. Proteins were diluted 5-fold and 100 µl Bradford, 5 µl protein and 100 µl Bradford were added respectively into 96 well-plates. This was set as 3 technical replicates and nuclease free water was used for blank in the same amount with proteins. Proteins were measured at 495 nm with spectrometer. After determination of protein concentrations, the volume needed for 20 µg protein to load into SDS gel was calculated. After proteins were prepared, 4X Laemmli Loading Buffer was added to the protein mixtures and final volume was adjusted with dH_2O to 25 µl. Also 5% of ß-mercaptoethanol was added to final mixture to reduce disulphide bridges. Proteins were denaturized at 95°C for 5 minutes and then before loading into SDS gel, 10% separating gel (40% acrylamide mix, separating buffer pH 8.8, APS and TEMED), and 5% stacking gel (40% acrylamide mix, stacking buffer pH 6.8, APS and TEMED) were prepared. Proteins were run at 80 V till stacking and 100 V till separating enough. Color protein marker (New England Biolabs) was used as reference for proteins. After completion of running step, proteins were transferred to PVDF (Thermo Scientific) membrane at 20 V for 16 h in cold environment. In wet transfer, the SDS gel and membrane are sandwiched between sponge and paper. Before transfer, PVDF membrane was activated with methanol. When transfer was completed, membrane was blocked with 5% of non-fat dry milk (CST) prepared with 1x Tris Buffered Saline (TBS) and 1% Tween 20 (FISHER) with shaking 1 h at room temperature to prevent non-specific background binding. After blocking step, the membrane was washed with TBS-T solution for 3 times for 30 minutes in total, followed by primary antibody hybridization which were mouse HaloTag monoclonal antibody (Promega) diluted in PBS (1:1000) and goat antibody CAPRIN1 (Abcam) diluted in 5% non-fat dry milk (1:1000) for 1h with shaking

at room temperature. Then as a secondary antibody, anti-mouse IgG-HRP conjugate (1:10000) and anti-goat IgG-HRP conjugate antibody (CST) were used, and membrane was incubated at room temperature for 1 h. Membrane was washed with TBS-T after each step. For visualization of proteins, membrane was incubated for 1 min with ECL (Millipore) and image was taken from BIO-RAD, VERSADOC 4000 MP equipment. ß-actin (rabbit) was used as a loading control with 1:5000 dilution.

2.4. cDNA synthesis and Quantitative PCR

RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) was used for cDNA synthesis. Master mix was set up in following order: 2 µg of RNA, 1 µl of OligodT primer or Random Hexamer primer (Thermo) and Nuclease free water up to 12 µl were mixed, then 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 (Thermo) were added into previous mix. This reaction was set up at 42ºC for 60 minutes and then 70ºC for 5 minutes for termination of the reaction. To dilute the samples, 20 µl of nuclease free water was added into 20 µl of cDNA mixture and final concentration was fixed at 1000 ng/µl. Stocks were stored at -80ºC.

For qPCR reaction, GoTaq qPCR Master Mix (Promega) was used, and reaction was set up in this following way: 6.25 µl of master mix, 1 µl of cDNA, 4.75 µl of nuclease free water and 0.5 µl of primer mix (Forward and Reverse). Standard two-step PCR amplification was applied by Rotor-Gene Q 2plex Platform (Qiagen). Steps was followed this order: 2 min at 95ºC, 15 seconds at 95ºC and 1 min at 60ºC (45 cycle). All primer sequences which were used in qPCR analysis are presented in Table 2.1. GAPDH was used as a housekeeping gene for normalization in all experiments and all reactions were performed with 3 biological replicates.

GoTaq 1-Step RT-qPCR (Promega) system was used for CLIP RNAs. The reaction mix was prepared by combining the GoTaq qPCR master mix, GoScript RT mix, PCR primers and nuclease-free water as described below: 10 µl of GoTaq master mix (2x), 0.4 µl of GoScript RT mix (50x), 1 µl of forward and reverse primer mix and final volume was adjusted to 20 µl with nuclease-free water after 1 µl of RNA template was added. Cycling condition was followed in this way: 15 minutes at 42ºC for reverse transcription, 10 minutes at 95ºC for reverse transcriptase inactivation and DNA

polymerase activation, 40 cycles for 10 seconds denaturation at 95ºC, 30 seconds annealing at 60ºC and 30 seconds extension at 72ºC.

Table 2.1. The list of primer sequences that were used in this study

Genes	Forward 5'-3'	Reverse 5'-3'
DR5-AS	Qiagen Cat. No. PH15855A-200	
DR5	CAGGTGTGATTCAGGTGAAGTGG	CCCCACTGTGCTTTGTACCTG
CAPRIN1-In Fusion	GATCGCTTCCGAATTCATGCCCTCG GCCACC	GTTGAGCTCTGAATTTTAATTCACT TGCTGAGTGTTCATTTGCG
CAPRIN1	GTGCGGACTGACCTGAAACA	TGTCCCGTTCAGGGTCTACT
MYC	CATCAGCACAACTACGCAGC	GCTGGTGCATTTTCGGTTGT
GAPDH	ACTCCTCCACCTTTGACGC	GCTGTAGCCAAATTCGTTGTC

2.5. Measurement of Proliferation

WST-8 assay was used to measure proliferation of cells with several transfection conditions. Before addition of cell proliferation reagent, time zero absorbance measurement was performed. After addition of 10 µl of WST-8 (Abcam) to the cell media, cells were incubated at 37ºC with 5% CO_2 for 2 hours. Subsequent to the incubation, spectrometric measurement was performed at 460 nm wavelength. Media, which is used during process, RPMI 1640 with 10% FBS, was used as blank.

2.6. Measurement of Apoptosis

Cisplatin treated Hela cells were analyzed by flow cytometry (FACSCANTO, BD) to measure apoptosis rate using Annexin V-FITC and 7AAD (BD) staining. Before staining, cells were harvested by Trypsin- EDTA (Gibco, 0.25%) and washed with 1x PBS. After removal of residual PBS, both Annexin V-FITC and 7AAD were diluted 1:10 by using PBS and 50 ml of Annexin binding buffer (BD), 10 ml Annexin V-FITC and 10 ml 7AAD were added to cells respectively. Then cell suspension was incubated 15

minutes at room temperature in the dark place. Starting analysis, cells that were stained were diluted with 200 ml 1x PBS. For control of analysis, unstained cells and monochromatic controls consisted of only annexin V FITC- and only 7AAD-stained cells were used. According to the analysis, cells that were not stained with any of these dyes were considered as viable cells; cells that were stained only Annexin V-FITC were considered as early apoptotic, cells that were stained with both annexin V and 7AAD were considered as late apoptotic and cells stained only with 7AAD were determined as dead cells.

CHAPTER 3

RESULTS

3.1. Construction of HaloTag-CAPRIN1 Overexpression Plasmid

DR5-AS lncRNA modulates cell morphology and cell proliferation in HeLa cells (Gurer et al. 2021). According to PAR-CLIP data, DR5-AS potentially interacts with 6 proteins and CAPRIN1 is one of them with a score of 0.999317 (Baltz et al. 2012). To investigate this cell cycle mechanism, CAPRIN1 protein was cloned into pHTN HaloTag CMV-neo vector by in-fusion cloning (Figure 3.1). To this extent, the vector was restricted with *Eco*RI restriction enzyme and run on an agarose gel (1%) at 100 V for 30 minutes. The agarose gel electrophoresis result showed that the CAPRIN1 insert had the *Eco*RI restriction sequence. Therefore, this enzyme cuts from two different sites of this plasmid and size of this small part is smaller than the PCR product size (Figure 3.2A).

After validation of sequences of constructed endotoxin free plasmid, the construct was transfected into HeLa cells by transient transfection and incubated for 48 hours. 48-hour post-transfection, the expression level of DR5-AS was examined to find out the extent of expression. qPCR results showed the validation of overexpression, CAPRIN1 was upregulated almost 2^8-fold in HeLa cells (Figure 3.2B). pHTN HaloTag CMV-neo vector, empty vector, was used as a negative control.

3.2. CAPRIN1 Protein Expression Depending on Time

Besides, upregulation in the CAPRIN1 mRNA was promising to validate the overexpression of CAPRIN1 in the protein level, CAPRIN1 construct was transfected to HeLa cell with different incubation times. After 12-, 24-, 48- and 72-hours proteins were isolated and examined with western blotting by using HaloTag primary antibody.

Interestingly, the HaloTag-CAPRIN1 band intensity decreased in a time-dependent manner while the intensity of fractionated bands increased. Thus, CAPRIN1 protein expression was the highest in 24h incubation and then while protein expression

was downregulated, CAPRIN1 protein was degraded into small proteins within increase of incubation time (Figure 3.3). β-actin was used as a loading control and empty vector (EV) expressed only the halo tag with a MW of 35 kDa as expected. When proteins were treated with TEV protease, degradations in CAPRIN1 were not determined due to CAPRIN1 and HaloTag had been separated from each other. Thus, it was concluded that, these all degradations have a part of CAPRIN1 protein, and the reason of this result can be related with using of HaloTag antibody instead of CAPRIN1 antibody. The possible start site of this fractionation of CAPRIN1 protein is c-terminal because HaloTag binds to CAPRIN1 protein in N-terminus.

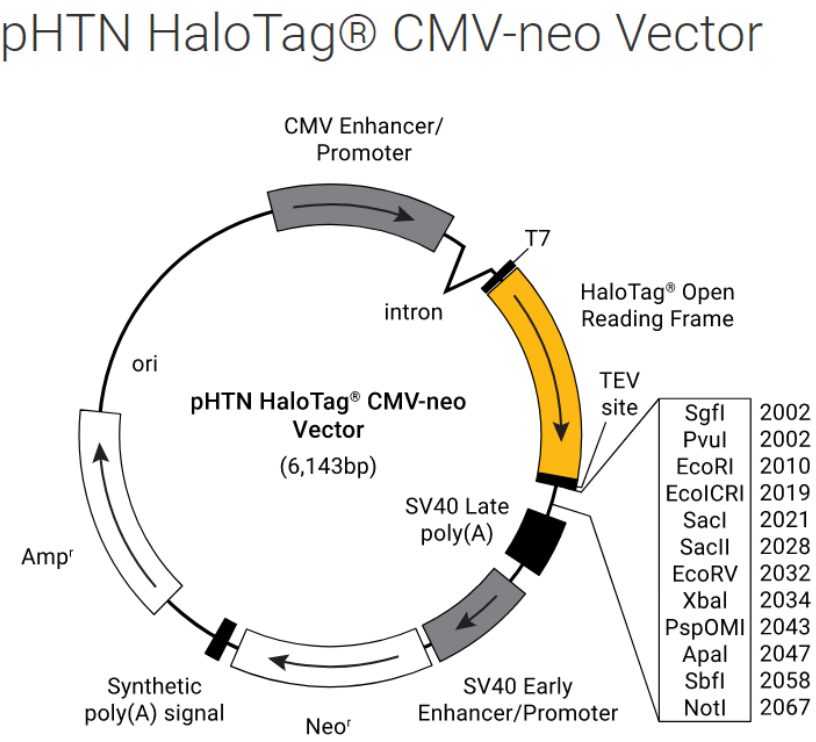

Figure 3.1. Empty vector (EV) that was used CAPRIN1 overexpression with restriction sites.
(Source: Promega)

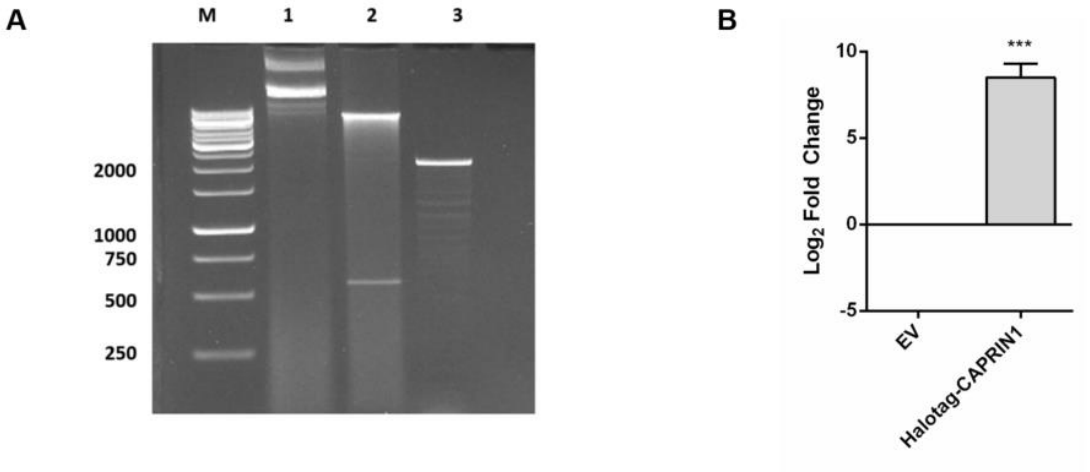

Figure 3.2. Construction of CAPRIN1 plasmid. (A) After cloning of CAPRIN1 into EV, cloning was confirmed with *Eco*RI restriction enzymes. Samples were run in agarose gel (1%) at 100V for 30 minutes. M; DNA marker, 1; uncut plasmid, 2; cut plasmid, 3; PCR product. (C) Log_2 fold change value in response to CAPRIN1 overexpression in 48h. Experiments were performed in triplicates. Student's unpaired t-test was used for statistical analysis, P>0.05. P<0.01 (**), P<0.001 (***), P>0.0001 (****).

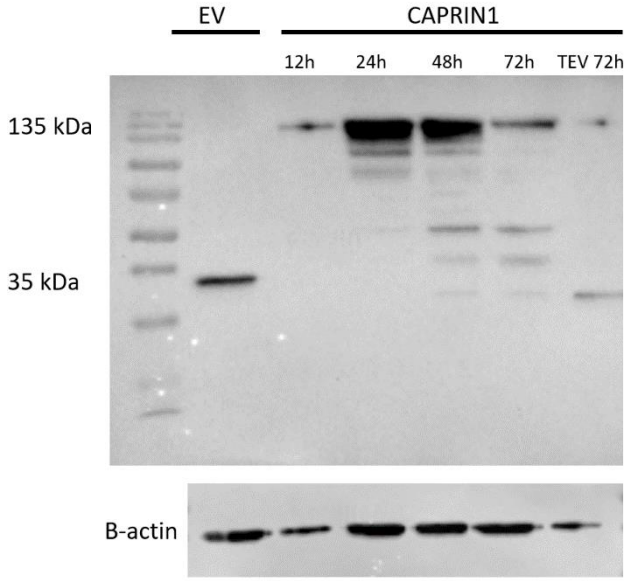

Figure 3.3. Time dependent change of CAPRIN1 protein expression in HeLa cells. EV; empty vector, TEV 72h; protein sample that digested with TEV protease. β-actin was used as a loading control.

3.3. UV Crosslinking and Immunoprecipitation (CLIP) of CAPRIN1

HaloTag technology is a powerful tool for protein analysis and labeling. It allows comprehensive analysis of the protein functions and interactions required only single construct. The CLIP method which has high specificity and affinity was used to validate PAR-CLIP data and to confirm DR5-AS lncRNA and CAPRIN1 interaction. CAPRIN1 and DR5-AS lncRNA was covalently bonded via UV crosslinking at 254 nm after CAPRIN1 overexpression (24h) in HeLa cells. After pull-down with magnetic beads which have Halo ligand, protein complex was separated from HaloTag by TEV digestion and RNA was isolated. The presence of DR5-AS in that complex was confirmed by quantitative PCR (Figure 3.4).

According to the CLIP result, it is exciting that both molecules are precipitated in the same complex. qPCR result validates that DR5-AS interacts with CAPRIN1 protein. Expected product size of DR5-AS primer was 91 bp. When samples were run on 1% agarose gel at 100V for 30 minutes, bands were observed for total RNA sample and CAPRIN1 sample as expected. However, for NT and EV samples only primer dimers were observed. In this experimental set up, NT was a control for genomic DNA contamination from outside and EV was a control for any contaminations inside the cell (Figure 3.5). In consideration of both DR5-AS and CAPRIN1 localize in the nucleus, effect of the interaction between them must be investigated on transcription or cellular phenotype.

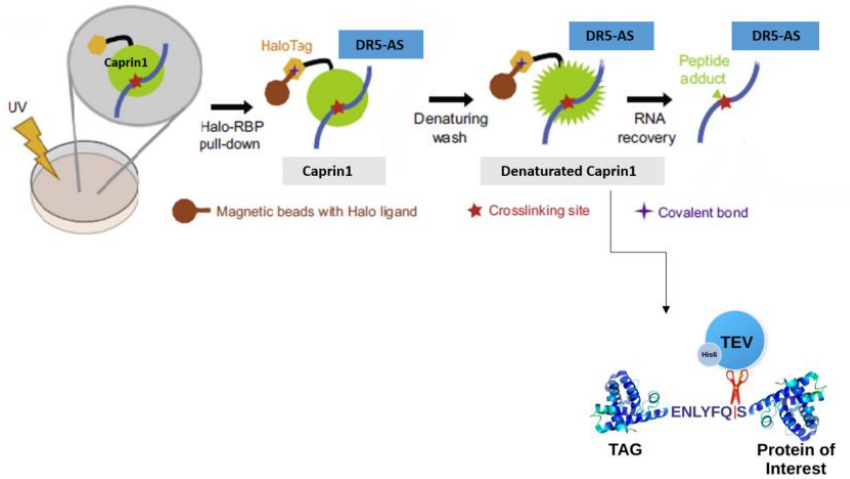

Figure 3.4. Schematic representation of CLIP method.

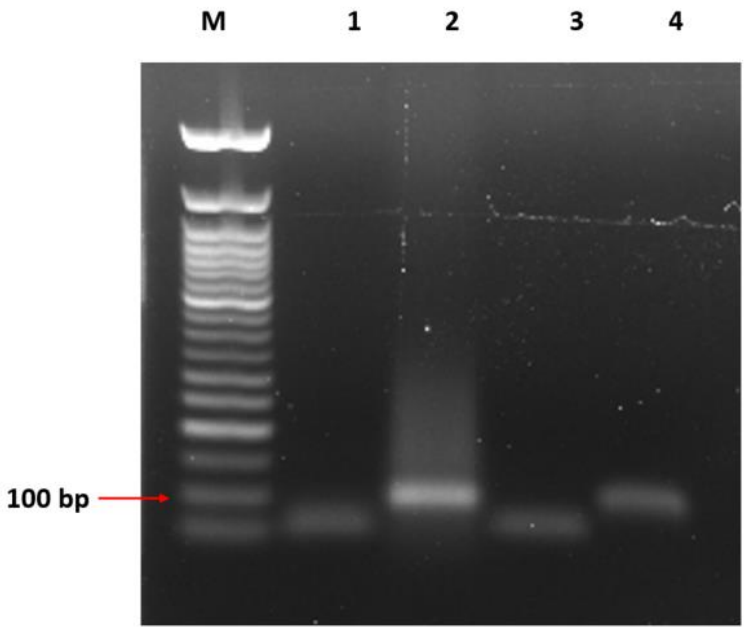

Figure 3.5. RNA immunoprecipitation of overexpressed CAPRIN1 and validation of DR-AS lncRNA CAPRIN1 interaction by qPCR. Samples were run 1% of agarose gel for 30 minutes at 100V. M; DNA marker, 1; No Template (NT) control, 2; Total RNA, 3; EV, 4; CAPRIN1.

3.4. Cisplatin Treatment and Apoptosis Measurement

DR5-AS is a cisplatin inducible lncRNA although its expression has no remarkable effect on apoptosis rate of HeLa cells (Gurer et al. 2021). However, this lncRNA binds to CAPRIN1 protein and it is possible that it can regulate apoptosis via this protein or CAPRIN1 protein may affects DR5-AS lncRNA on apoptosis rate regulation. To test these hypotheses, 40 µM cisplatin was applied to Hela cells after 8 hours of CAPRIN1 overexpression and apoptosis rates were measured. 0.05% (v/v) DMSO was used as a negative control and EV was used for transfection control. The results showed that cisplatin caused a decrease in the live cell population. However, there was no significant changes in the rate of early and late apoptotic cells transfected with empty or overexpression vector (Figure 3.6A). Besides, RNAs were isolated from CP-treated and CAPRIN1-overexpressed HeLa cells and gene expression levels of DR5, DR5-AS and CAPRIN1 were quantified by qPCR. Cisplatin treatment caused almost a 2^5-fold decrease in CAPRIN1 expressions in both situations although cisplatin treatment

in CAPRIN1 overexpressed cells caused almost a 2^3-fold increase in DR5-AS expressions in contrast to cisplatin treatment without CAPRIN1 overexpression cells (Figure 3.6B). Additionally, there was slight recovery effect of CAPRIN1 overexpression on HeLa cell, but it was not significant.

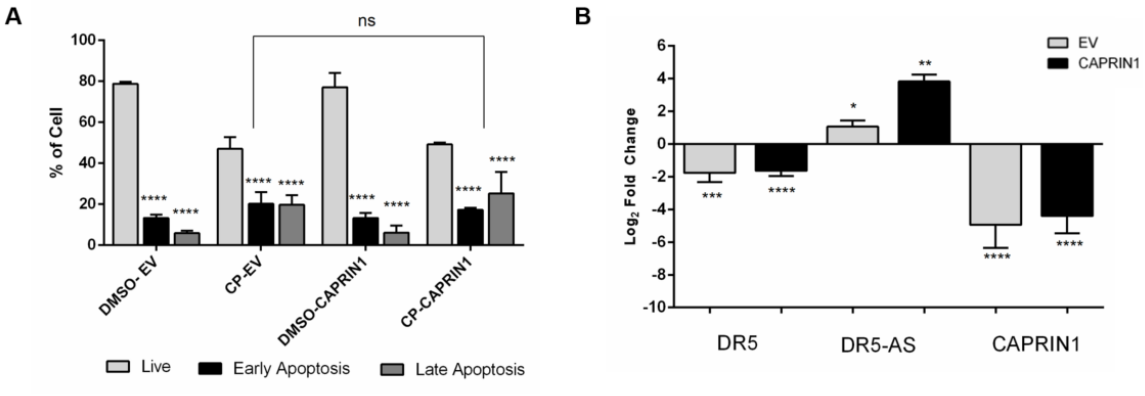

Figure 3.6. (A) CP-induced apoptosis rate measurements of CAPRIN1 overexpressed HeLa cells. Flow cytometry analysis was performed via Annexin V and 7AAD staining. Annexin V (+) and 7AAD (-) cells represent early apoptosis whereas Annexin V (+) and 7AAD (+) cells were considered as late apoptotic. Cells that were not stained neither Annexin V nor 7AAD represent live population. CP; cisplatin, EV; empty vector. (B) Log_2 fold changes of DR5, DR5-AS and CAPRIN1 expression under apoptotic conditions. GAPDH was used for normalization. Experiments were performed in triplicates. After 8 hours of CAPRIN1 overexpression', 40 µM CP and 0.05% (v/v) DMSO as a negative control were applied and incubation was carried until 24 hours. Student's t-test was performed for statistical analysis, $P>0.05$. $P<0.01$ (**), $P<0.001$ (***), $P>0.0001$ (****).

According to the previous results (Figure 3.3 and Figure 3.6B), it is known that CAPRIN1 protein degrades in a time-dependent manner and CAPRIN1 expression decreased under the cisplatin treatment. To test the potential effect of cisplatin on time-dependent degradation of CAPRIN1 protein, apoptosis rates were measured following different incubation times of cisplatin. Cell viability sharply decreased below 50% following 16- and 24-hours incubation of CP and the rate of early apoptotic cells increased to almost 50% in that time in comparison to 2-, 4- and 8-hours incubations (Figure 3.7A).

Log_2 fold changes of DR5, DR5-AS and CAPRIN1 were quantified by qPCR in a time kinetic analysis. CAPRIN1 expressions decreased while DR5-AS expression slightly

increased parallel to the CP incubation time. There was no correlation between the DR5 expression pattern and the CP incubation time (Figure 3.7C). Moreover, 40 µM CP treatment of HeLa cells for 16 hours caused a 2^5-fold decrease in CAPRIN1 and a $2^{2.5}$-fold decrease in DR5 while a $2^{2.5}$-fold increase in DR5-AS expressions (Figure 3.7B).

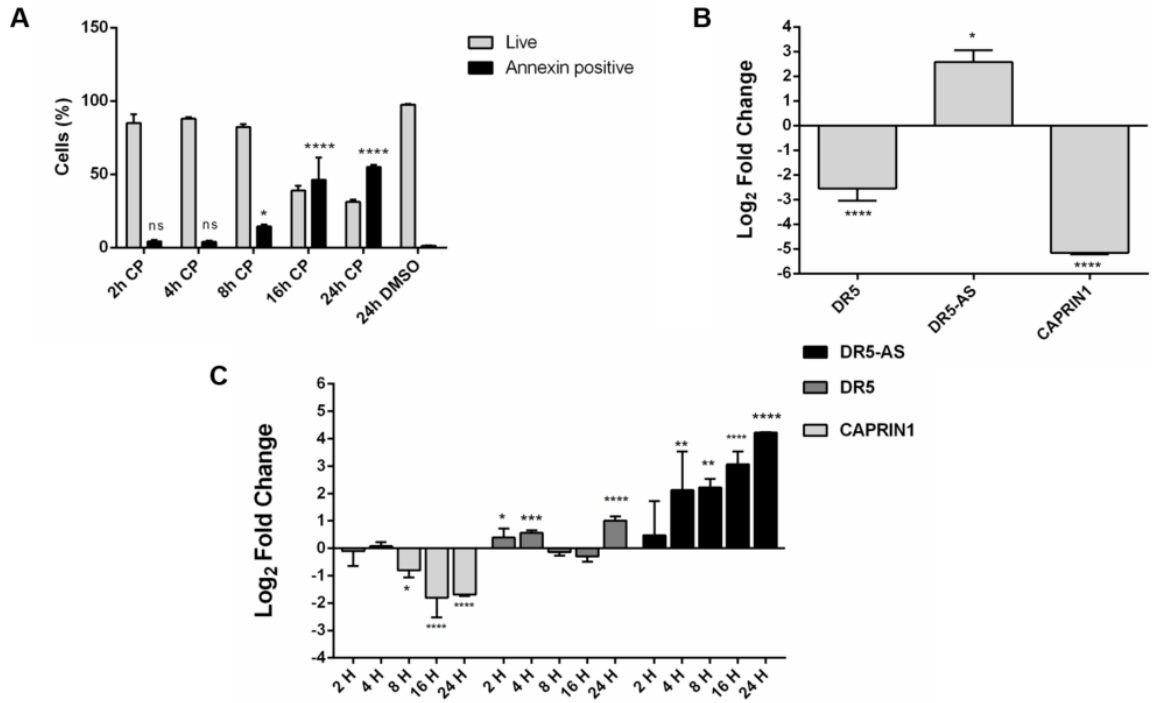

Figure 3.7. (A) Apoptosis rate measurement of HeLa cells depending on time under 80 µM CP treatment. (B) Quantitative PCR results of DR5, DR5-AS and CAPRIN1 expressions after 40 µM CP treatment with 16 hours incubation. (C) Quantitative PCR results of DR5, DR5-AS and CAPRIN1 expressions in 80 µM CP treated HeLa cells depending on incubation time. All experiments were performed in triplicates. GAPDH was used for normalization. Statistical analysis was performed by Student's t-test, P>0.05. P<0.01 (**), P<0.001 (***), P>0.0001 (****).

3.5. Effect of CAPRIN1 Expression on DR5-AS lncRNA

Under cisplatin treatment, CAPRIN1 overexpression caused a significant increase in DR5-AS lncRNA expressions. To test whether it is dependent on drug treatment or not, gene expression levels of DR5, DR5-AS and CAPRIN1 were quantified by qPCR after overexpression and silencing of CAPRIN1 and DR5-AS separately. When CAPRIN1 was overexpressed almost 2^8-fold in HeLa cells following 24-hour incubation, there was no

remarkable change in expressions of DR5-AS lncRNA while there was nearly 2^1-fold increase in DR5 expression. However, when DR5-AS was overexpressed almost 2^{17}-fold in HeLa cells with 72 hours incubation, there was a slight increase in CAPRIN1 expression while there was no change in DR5 expression (Figure 3.8).

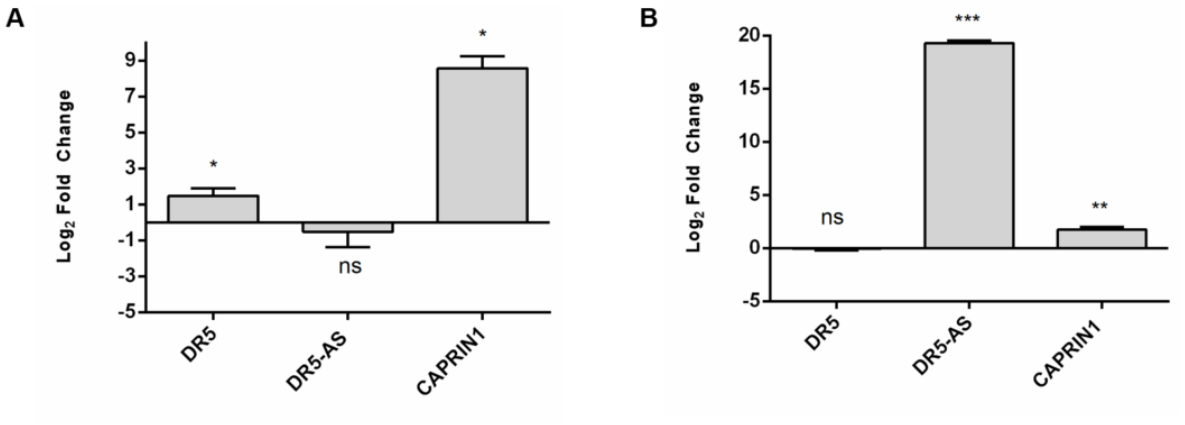

Figure 3.8. Quantitative PCR results of DR5, DR5-AS and CAPRIN1 expressions on HeLa cells (A) with CAPRIN overexpression, (B) with DR5-AS overexpression. All experiments were performed in triplicates. For normalization GAPDH was used. Student's t-test was performed, P>0.05. P<0.01 (**), P<0.001 (***), P>0.0001 (****).

On the other hand, when CAPRIN1 was silenced by using siRNA in 48- and 72-hours, CAPRIN1 expression was decreased nearly 2^4-fold in both while there was no significant change in DR5-AS expression with 48 hours incubation. However, in 72 hours silencing, DR5-AS expression was slightly increased (Figure 3.9).

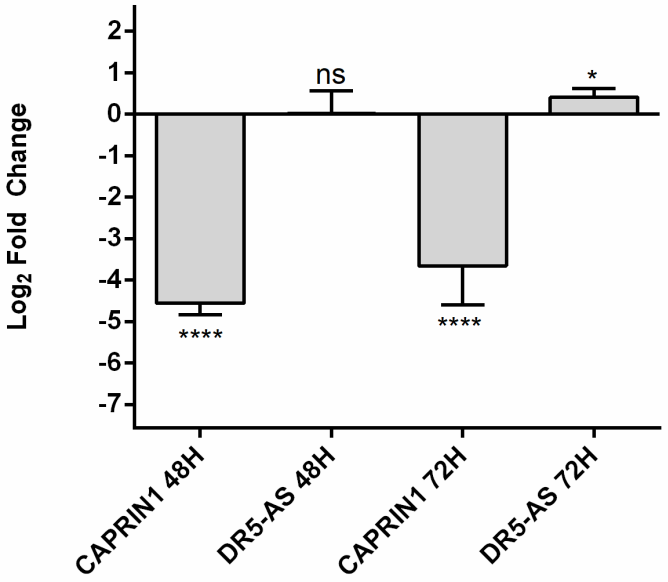

Figure 3.9. Gene expression levels of DR5-AS and CAPRIN1 on HeLa cells with CAPRIN1 silencing 48- and 72- hours. Experiments were performed in triplicates. Quantification was performed by GAPDH normalization. Student's t-test was applied all experiments to calculate p-values, P>0.05. P<0.01 (**), P<0.001 (***), P>0.0001 (****).

3.6. Cell Proliferation Measurement

CAPRIN1 is a cell cycle-associated protein (Wang et al. 2005), and it interacts with DR5-AS lncRNA which is involved in the regulation of proliferation. Knockdown of DR5-AS lncRNA caused morphological changes in HeLa cells via cell cycle arrest at mainly G2/M phases (Gurer et al. 2021). To examine the effect of CAPRIN1 expression on HeLa cell morphology and cell proliferation, proliferation rates were measured in CAPRIN1 overexpressed and silenced HeLa cells. It was known in the literature, CAPRIN1 absence causes a decrease in proliferation (Wang et al. 2005) and Figure 3.10B suggests this information. When CAPRIN1 was silenced with 48 hours cell proliferation rate was decreased to nearly 50% however in 72 hours there was no significant change in proliferation. Although CAPRIN1 was overexpressed with 24 hours incubation, cell proliferation rate was decreased to 75% (Figure 3.10A). This result suggests that DR5-AS and CAPRIN1 work in reversible manner.

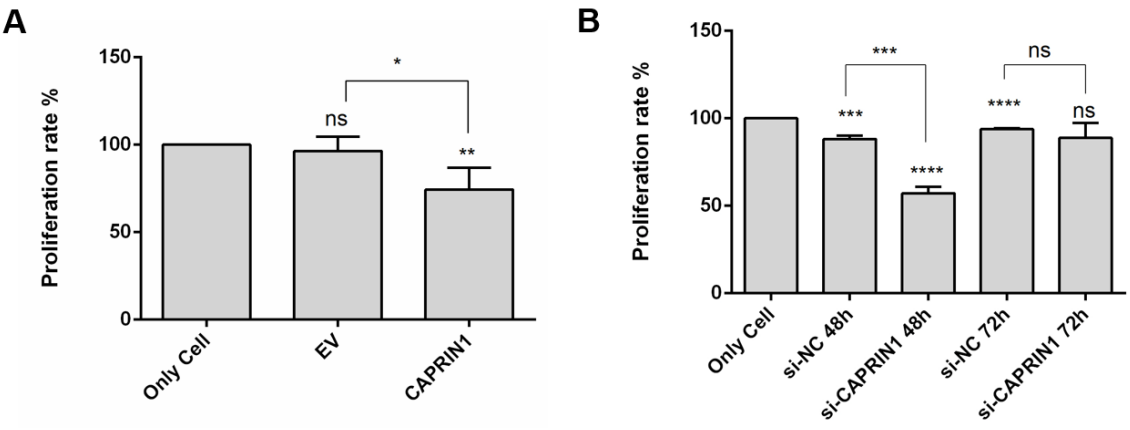

Figure 3.10. Proliferation rate measurement by spectrophotometric analysis using WST-8 staining at 460 nm (n=4). (A) Effect of CAPRIN1 overexpression on cell proliferation. (B) Effect of CAPRIN1 silencing on cell proliferation, si-NC; negative control. All experiments were repeated three times. Statistical analyses were performed using Student's t-test, P>0.05. P<0.01 (**), P<0.001 (***), P>0.0001 (****).

CHAPTER 4

DISCUSSION

DR5-AS is a cisplatin inducible nuclear natural antisense lncRNA that has a role in cell proliferation and metastasis in HeLa cells. According to the previously published data, DR5-AS knockdown cause morphological change in HeLa cells due to cell cycle arrest at mainly G2/M phases (Gurer et al. 2019). However, mechanism behind this regulation was an open question. It is known that lncRNAs are involved in many regulatory mechanisms via RNA, DNA, and protein interactions (Bhat et al. 2016). To understand how DR5-AS causes decrease in proliferation rate, several functional investigations were performed. In consideration of RNA-seq data, selected candidate genes expressions were analyzed by qPCR after DR5-AS silencing. Interestingly, this result indicates that ANAPC2, ANAPC4 and CENPP genes that are crucial for cell cycle, show a decrease in expression while an increase is observed in p21 protein expression (Gurer et al. 2019). In the light of this information, it is obvious that DR5-AS has a role in cell proliferation and cell cycle.

According to the PAR-CLIP data, there are 6 candidate proteins which have a high potential to interact with DR5-AS and among them, CAPRIN1 has the highest score. Additionally, it is known that CAPRIN1 is a cell cycle-associated protein, and it is crucial for cell proliferation. In the literature, suppression of CAPRIN1 expression resulted in slower rate of cell proliferation due to stacking at G1 phase of the cell cycle (Wang, David, Schrader 2022). Therefore, it was a promising protein candidate, and it was hypothesized that DR5-AS lncRNA regulates cell proliferation and cell cycle through interacting with CAPRIN1 protein. To investigate this potential interaction and its effects, CAPRIN1 protein should be cloned into the HaloTag plasmid. HaloTag was chosen due to its high affinity and specificity. Moreover, it is easy to study with HaloTag construct because only one construct allows the comprehensive analysis of many candidates. After selection of the technology, CAPRIN1 sequence was amplified by PCR while plasmid was linearized with EcoRI restriction enzyme then, cloned into HaloTag plasmid by in-fusion cloning. In-fusion cloning is a cloning technique based on homologous recombination. Thus, it allows directional cloning of any PCR fragment or multiple

fragments into any vector. Besides that, it is cheaper and faster than other cloning techniques. Figure 3.1 represents the HaloTag vector that was used in this study. After cloning, plasmid was restricted with EcoRI enzyme to confirm the product and run on the agarose gel (Figure 3.2A). When plasmid was restricted, backbone of vector and a small fragment were observed although only one restriction enzyme was used. Explanation of this result is that CAPRIN1 sequence has the EcoRI restriction site, so this enzyme cuts both HaloTag vector backbone and CAPRIN1 product. This confirmed HaloTag-CAPRIN plasmid that was endotoxin free plasmid was transfected into HeLa cells for 48 hours. After incubation time, the qPCR result reveals that overexpression of CAPRIN1 was achieved successfully (Figure 3.2B).

Besides, showing the CAPRIN1 overexpression in mRNA level, western blotting experiment was performed with different overexpression time of CAPRIN1 to investigate its protein expression. Prominently, CAPRIN1 protein was degraded in a time dependent manner from the c-terminal because HaloTag is bound to CAPRIN1 from n-terminal. The band intensity of HaloTag-CAPRIN1 decreases while the intensity of fractionated fragment band increase over the time (Figure 3.3). The band intensity of CAPRIN1 protein was the highest at 24 hours so incubation time was set up for 24 hours for further overexpression experiments. It was an interesting result because in the literature there is no information about CAPRIN1 protein degradation. After confirmation of CAPRIN1 overexpression in both mRNA and protein level, the next step was validation of a possible interaction between DR5-AS and CAPRIN1 in HeLa cells. UV crosslinking and immunoprecipitation (CLIP) method is one of the widely used techniques for detection of RNA-protein interactions (Figure 3.4). In this method, proximal RNAs and proteins were bonded covalently with UV irradiation. This covalent bond allows to protect this interaction during denaturing washes. This result reveals that DR5-AS lncRNA was present into the immunoprecipitated CAPRIN1 complex as expected (Figure 3.5). Total RNA was used as a positive control while EV and NT were negative controls. There were primer dimers in EV and NT agarose gel result around at 50 bp while the expected band size for DR5-AS was 91 bp.

It was known that DR5-AS is a cisplatin inducible lncRNA so after validation of their interaction, the next step to test the hypothesis was to investigate CAPRIN1 protein reaction under CP treatment and its effect on DR5-AS lncRNA and apoptosis. The flow cytometry analysis of CAPRIN1 overexpression under CP treatment showed that, there

was no remarkable change in the early and late apoptosis when compared with EV as a negative control (Figure 3.6A) Besides that, to understand the effect of CAPRIN1 overexpression with CP treatment on DR5 and DR5-AS expressions, qPCR experiment was performed. The qPCR result exhibits that CAPRIN1 expression was downregulated with CP induction (Figure 3.7B), however, DR5-AS expression was remarkably upregulated with CAPRIN1 overexpression under CP treatment in contrast with CP treatment without CAPRIN1 overexpression (Figure 3.6B). This result reveals that CAPRIN1 enhances the DR5-AS expression under CP treatment. On the other hand, CAPRIN1 protein degradation was shown in Figure 3.3 and to investigate effect of CP treatment on CAPRIN1 expression in a time dependent manner, apoptosis rates were measured. According to these results, CAPRIN1 expression was decreased over the time while DR5-AS expression was increased (Figure 3.7A). Additionally, qPCR result also validated flow cytometry analysis (Figure 3.7C).

It was shown that DR5-AS and CAPRIN1 work reversible under CP treatment. For further investigation of CAPRIN1 and DR5-AS interactions, CAPRIN1 and DR5-AS expressions were investigated separately with overexpression and silencing experiments. qPCR results reveal that DR5-AS overexpression causes a slight increase in CAPRIN1 (Figure 3.8B) while CAPRIN1 overexpression does not significantly change the expression of DR5-AS (Figure 3.8A). However, with the silencing of CAPRIN1, DR5-AS expression slightly increases in 72 hours (Figure 3.9). All experiments support that DR5-AS and CAPRIN1 work in a reversible manner without drug treatment. In consideration of results, CAPRIN1 may somehow regulates the DR5-AS lncRNA. The next step was further phenotypic characterization of CAPRIN1 protein by proliferation measurement. In the literature, it was known that CAPRIN1 is an essential protein for proper cell cycle, and the absence of CAPRIN1 leads to decrease in proliferation. The results of this study promote the literature information. When CAPRIN1 was silenced, proliferation rate was decreased significantly in 48 hours however there was a recovery in proliferation rate in 72 hours (Figure 3.10B). This recovery may result from transient transfection of CAPRIN1 or positive feedback mechanisms. However, the interesting thing was, CAPRIN1 overexpression in HeLa cells leads to decrease proliferation rate also (Figure 3.10A). In this case, this result makes sense due to DR5-AS knockdown leading to decrease in proliferation and DR5-AS might regulates CAPRIN1 protein. To understand this mechanism, CAPRIN1 expression should be examined in protein level.

CHAPTER 5

CONCLUSION

Under our experimental settings, we confirmed DR5-AS lncRNA and CAPRIN1 protein interactions based on PAR-CLIP data by performing CLIP and many phenotypic characterization experiments. CAPRIN1 is a cell cycle associated protein that was degraded in a time dependent manner in HeLa cells. Moreover, DR5-AS silencing and CAPRIN1 overexpression cause decrease in cell proliferation separately, and their expression levels were quantified as reversible in the HeLa cells. Under CP treatment, CAPRIN1 expression was decreased while DR5-AS was increased over the time. But the interesting result was CAPRIN1 overexpression enhanced the DR5-AS expression under cisplatin treatment. This data shows that DR5-AS and CAPRIN1 work in reverse without CP treatment.

CAPRIN1 was initially selected based on PAR-CLIP data and this data was validated by performing crosslinking and immunoprecipitation experiment. DR5-AS deficiency causes cell cycle arrest in metaphase stage and CAPRIN1 is crucial for proper cell cycle. While DR5-AS involves in the regulation of key proteins that involve in cell cycle like ANAPC2, ANAPC4, CENPP and p21, CAPRIN1 binds to MYC and cyclin D2 mRNAs and involve in the regulation of FOXM1 and CenpF directly or indirectly. To understand mechanism underlying the regulation of cell cycle progression, many other experiments must be performed. As a future direction, interaction between CAPRIN1 and key proteins which involve in cell cycle progression should be investigated. Also, DR5-AS lncRNA may regulate CAPRIN1 protein and this must be considered to test this hypothesis. If any relations can be revealed about CAPRIN1, DR5-AS and other protein(s), the exact mechanism of this cell cycle arrest at metaphase stage can be more understandable. Eventually, CAPRIN1 can be a possible diagnostic marker after further investigations in different cancer cells and *in vivo* studies and may have a role in chemotherapy resistance.

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