EXAMINATION OF STABLE INTRONIC SEQUENCE RNA PROFILE UNDER APOPTOTIC CONDITIONS

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ABSTRACT

EXAMINATION OF STABLE INTRONIC SEQUENCE RNA PROFILE UNDER APOPTOTIC CONDITIONS

Apoptosis is a process of programmed cell death. Cisplatin, a chemotherapeutic drug, activates intrinsic pathway of apoptosis while TNF-alpha, a death ligand, activates the extrinsic pathway of apoptosis. Noncoding RNAs involve in regulation of apoptotic pathways at post-transcriptional level. Stable intronic sequence RNAs (sisRNAs) are the novel class of non-coding RNAs which can be generated by splicing- dependent and independent mechanisms. sisRNAs transcribed from their intronic promoter may contain 5' cap and polyA tail. Despite the reports of several studies about sisRNAs in *Xenopus* and *Drosophila*, a genome-wide profile of sisRNAs in human is lacking. Therefore, we aimed to identify sisRNAs profile that are transcribed from their intronic promoter under cisplatin- and TNF-alpha- mediated apoptosis conditions. In this thesis study, the deep sequencing of total RNA, polyA + and polyA eliminated fractions from cisplatin-, TNF-alpha-, DMSO-treated cells were performed. Differentially expressed intronic transcripts were analysed by DE-kupl algorithm. The intronic transcripts both in total RNA and polyA + RNA fractions but not in polyA eliminated fractions were screened visually on Integrated Genome Viewer (IGV) and selected as sisRNA candidateS. 48 sisRNA candidates were detected in cisplatin-treated data while 33 sisRNA candidates were detected in TNF-alpha- treated data. 5' and 3' RACE PCRs were performed for determination of transcriptional units of sisRNA candidates. Overexpression of sisR-DOCK7-IT1 caused 8.09% increase in total apoptosis of HeLa cells in 48 hours. sisR-DOCK7-IT1 triggers the activation of apoptosis but the mechanism of its induction of apoptosis is still unknown.

Keywords: *Apoptosis, Stable intronic sequence RNA, Non-coding RNA, RNA-seq*

ÖZET

APOPTOTİK KOŞULLAR ALTINDA STABİL İNTRONİK SEKANS RNA PROFİLİNİN İNCELENMESİ

Apoptoz bir programlı hücre ölümü şeklidir. Kemoterapötik ilaç olan sisplatin apoptozun içşel yolağını aktive ederken, ölüm ligandı olan TNF-alfa ise apoptozun dışsal yolağını aktive eder. Apoptozun transkripsiyon sonrası düzenlenmesinde kodlamayan RNA rol alır. Kodlamayan RNA sınıfının yeni üyesi olan stabil intronik sekans RNA'lar (sisRNA) kırpılmaya bağlı ve bağımsız olmak üzere iki mekanizma ile üretilirler. Kendi intronik promotörüne sahip olan sisRNA'lar 5'şapka ve çokluA kuyruğa sahip olabilirler. *Xenopus* ve *Drosophila*'da yapılan çalışmalarda birçok sisRNA raporlarsa da henüz insanda genom düzeyinde bir çalışma bulunmamaktadır. Bu sebeple, sisplatin ve TNF-alfa uygulanması sonrası koşullarda ifade edilen intronik promotörlü sisRNA'ları belirlemeyi amaçladık. Bu çalışmada, sisplatin, DMSO ve TNF-alfa ile müdahale edilmiş hücrelerden elde edilen toplam, çokluA pozitif ve çokluA negatif RNA fraksiyonları için derin sekanslama gerçekleştirildi. Farklı ifade edilen intronik transkriptler DE-kupl algoritması kullanılarak analiz edildi. Toplam ve çokluA pozitif RNA fraksiyonlarında görülen fakat çokluA negatif RNA fraksiyonlarında görülmeyen intronik transkriptler manuel olarak Integrated Genome Viever üzerinden tarandı. Analiz sonucunda sisplatin uygulanmış koşullarda 48, TNF-alfa uygulanmış koşullarda ise 33 sisRNA adayı listelendi. Adayların 5' ve 3' sınırları 5'/3' RACE PCR ile belirlendi. Adayların fenotip üzerindeki etkilerinin taranması amacıyla, sisR-DOCK7-IT1 hücrede aşırı-ifade ettirildi. 48 saatlik aşırı-ifade sonrasında toplam hücre apoptozu %8,09 oranında arttı. sisR-DOCK7-IT1'in hücre apoptozunda rol aldığı bulundu fakat nasıl bir mekanizma ile etkili olduğu hala bilinmemektedir.

Anahtar Kelimeler: *Apoptoz, stabil intronik sekans RNA, Kodlamayan RNA, RNA-seq*

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CHAPTER 1

INTRODUCTION

1.1. Apoptosis

Apoptosis is a form of programmed cell death that has distinct morphological and biochemical characteristics (Elmore 2007). In the history, the apoptosis term was firstly used by Kerr, Wyllie, and Currie in 1972 (Kerr, Wyllie, and Currie 1972). The following studies in *Caenorhabditis elegans* showed the role of apoptosis in development of nematode (Ellis and Horvitz 1986). Apoptosis is a process that occurs in living organisms to maintain the homeostasis of cells in a population and as a defence mechanism in immune reactions for removal of damaged cells (Ellis and Horvitz 1986). In the early stages of apoptosis, the morphological changes start to be observable under light microscopy. Due to induction of the activation of apoptosis via various stimuli, firstly cells start to get smaller, demonstrating a dense cytoplasm and irreversible chromatin condensation, pyknosis, occurs. Following the pyknosis, nucleus condensation and its fragmentation become visible. The cell detachment and the protrusions of cell membrane cause the formation of apoptotic bodies. Apoptotic bodies are separated blebs from the apoptotic cells that contain cellular organelles and fragmented nucleus (Kerr, Wyllie, and Currie 1972; Elmore 2007). Finally, the apoptotic bodies are removed by the engulfment of macrophages without any inflammation occurring in the tissue (Savill and Fadok 2000).

1.2. Mechanism of apoptosis

Apoptosis mainly can be accomplished by intrinsic, extrinsic, and perforin/granzyme pathways based on their initiator signals. The initiator signals cause the activation of specific initiator cysteine proteases known as caspases for each pathway. While intrinsic and extrinsic pathways crosstalk with each other in the activation of caspases, finally all three pathways undergo the execution pathway due to activation of caspase 3 (Elmore 2007; Igney and Krammer 2002).

Extrinsic apoptosis pathway is triggered through external signals/death ligands that bind to transmembrane death receptors such as Fas receptors (FasR), tumour necrosis factor 1, 2 (TNFR-1, TNFR-2) receptors (Fulda and Debatin 2006; Nair et al. 2014). Upon the specific ligand-receptor interaction, pro-apoptotic proteins such as FADD bind to the docking sites of death receptor which are death domains. FADD is an adaptor protein that facilitates the formation of death-inducing signalling complex (DISC). The formation of DISC leads to the recruitment of initiator caspase, pro-caspase 8 and their binding causes the activation of caspase- 8. Activated caspase-8 involves in activation of the downstream executioner caspases (caspases-3, - 6 and - 7) (Elrod and Sun 2008).

The life-or-death decision of cells is executed by mitochondrial apoptotic proteins, Bcl-2 proteins, which are categorized into three subgroups, namely pro-apoptotic, anti-apoptotic and the BH3-only proteins (Czabotar et al. 2013). Intrinsic apoptosis pathway is induced through the non-receptor mediated extracellular or intracellular signals (DNA damage, hypoxia, ER stress etc.) within cells which causes changes in the inner mitochondrial membrane permeability (Czabotar et al. 2013; Norbury and Hickson 2001; Wu and Bratton 2013). These stimuli lead to activation of initiator BH3-only family proteins and therefore promote the activation of pro-apoptotic effectors BAX and BAK proteins while inhibiting the action of anti-apoptotic proteins (Youle and Strasser 2008). These alterations cause the opening of mitochondrial pores and release of pro-apoptotic proteins to cytosol. Firstly, release of cytochrome c, SMAC/DIABLO, and the serine protease HtrA2/Omi involve in activation of caspase-dependent pathway. Subsequent binding of cytochrome c to APAF-1, leads formation of apoptosome. Apoptosome recruits the pro-caspase -9 and induces its dimerization and autocatalysis (Bratton and Salvesen 2010). This causes the activation of caspase-9 and

-3, consecutively. On the other side, SMAC/DIABLO and the serine HtrA2/Omi exert their function in promotion of apoptosis by inhibiting IAP (inhibitor of apoptosis proteins) (Zhou et al. 2005; Du et al. 2000; Yang et al. 2003). Another group of pro-apoptotic proteins including apoptosis inducing factor (AIF), endonuclease G (EndoG) and CAD are released from mitochondria in late stages of apoptosis. AIF and EndoG translocate from mitochondria to nucleus. This results in DNA fragmentation in nucleus as independently from caspases, meanwhile CAD involves in oligonucleosomal DNA fragmentation as caspase-3 dependent (Nirmala and Lopus 2020).

Despite the fact that apoptosis is induced through extrinsic and intrinsic pathways separately, they may crosstalk by cleavage of pro-apoptotic protein BID. Caspase-8 induces the cleavage of BID into truncated BID (tBID). This cleavage results in increase in permeabilization of mitochondrial membrane and thus, it will cause the amplification of apoptotic signals (Billen, Shamas-Din, and Andrews 2008; Igney and Krammer 2002).

Although the cytotoxic T lymphocytes (CTLs) trigger the apoptosis to perform their cytotoxicity upon viral infection to kill target cells via the extrinsic pathway, they can also mediate the apoptosis via perforin/granzyme pathway (Igney and Krammer 2002). In this mechanism, CTLs secrete a pore forming cytolytic protein, perforin, and cytoplasmic granules that contains serine proteases granzyme A and B (Kist and Vucic 2021). Granzyme B exerts its function by cleaving the aspartate residues of proteins that will lead to activation of pro-caspase- 10. Additionally, Granzyme B can cause cleavage of ICAD (Inhibitor of Caspase Activated DNAse) (Enari et al. 1998). Through the activation of caspase-9 and caspase-3, cells undergo apoptosis upon Granzyme B secretion (Afonina, Cullen, and Martin 2010). Granzyme A involves in apoptosis by activating DNA nicking via DNAse NM23-H1. NM23-H1 results in DNA degradation in cells. Granzyme A induces the apoptosis via caspase-independent fashion (Elmore 2007).

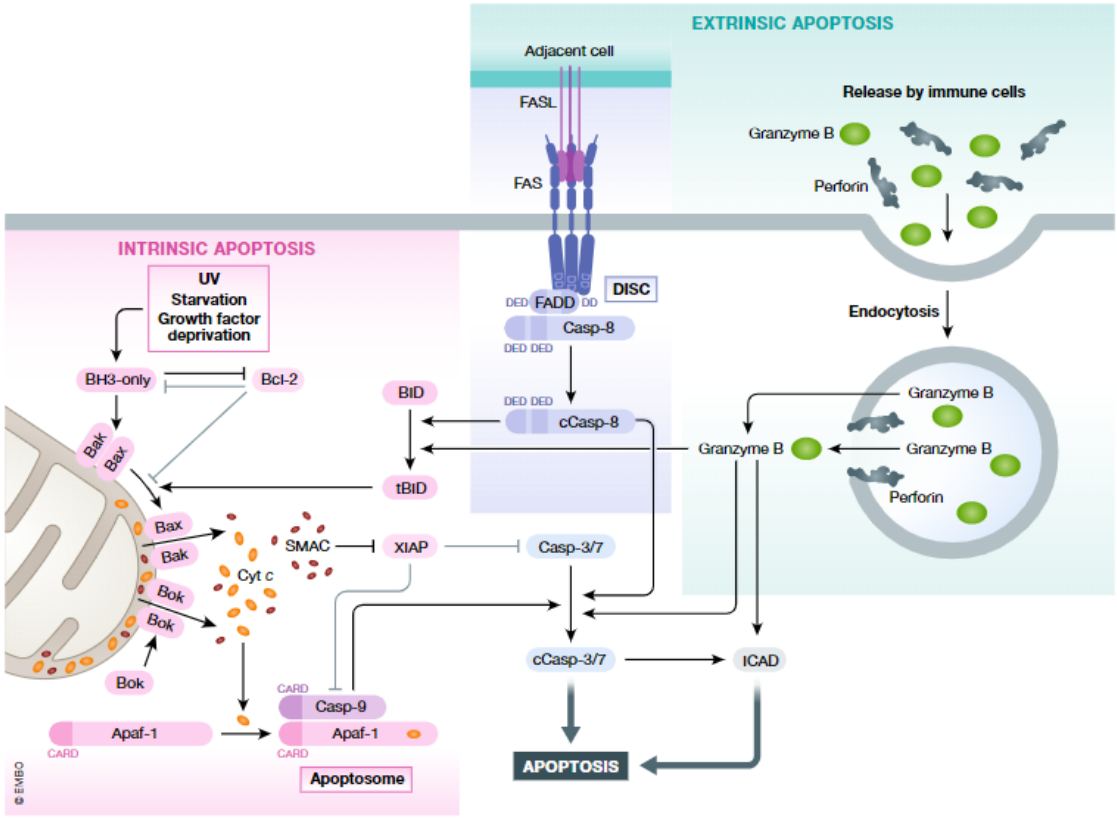

Figure 1.1. Caspase-dependent apoptosis pathways. (Source: Kist and Vucic 2021)

1.3. Regulation of apoptosis

The balance of death and life is crucial for living organisms. Therefore, apoptosis is tightly regulated at different levels. The regulation of apoptosis at protein level occurs through the interactions of Bcl-2 family proteins, caspase activation or post-translational modifications of pro-apoptotic or anti-apoptotic proteins (Singh, Letai, and Sarosiek 2019). Additionally, its regulation at the transcriptional level is mainly controlled through interaction of transcription factors with the regulatory regions of apoptosis-related genes. The regulation at post-transcriptional level is achieved by RNA binding proteins (RBP) and noncoding RNAs, mostly miRNAs and lncRNAs (Dudgeon et al. 2009).

1.4. Noncoding RNAs

The mystery of human genome began to unravel through the Human Genome Project in the beginning of 21st century. Initial sequencing data showed that ~ 98% of human genome consists of the non-coding part (Venter et al. 2001). Non-coding sequences are transcribed to the non-coding transcripts that are not able to encode a functional protein (Costa 2007). Noncoding RNAs (ncRNAs) can be classified, based on their length, as small and long noncoding RNAs. Small ncRNAs are the transcripts smaller than 200 nucleotides in length. Micro RNAs (miRNAs), small interfering RNAs (siRNAs), small nuclear RNAs (snRNA), small nucleolar RNAs (snoRNA), tRNA derived RNAs (tsRNA) and Piwi-interacting RNAs (piRNA) fall under the small ncRNAs (S. Yang et al. 2018). Long noncoding RNAs (lncRNAs) are the transcripts longer than 200 nucleotides. lncRNAs are categorized as antisense, sense, intronic, bidirectional, intergenic and enhancer lncRNAs based on the genomic position they are transcribed (Fernandes et al. 2019). Based on their structure lncRNAs are classified in linear and circular forms (Qin, Li, and Zhang 2020). The novel class of non-coding RNAs, stable intronic sequence RNAs, also can be categorized under lncRNAs.

ncRNAs act as gene expression regulators at various levels such as epigenetic, transcriptional, and post-transcriptional levels. The best characterized classes of ncRNAs are miRNAs and lncRNAs. miRNAs can bind to 3' untranslated region (UTR) of target mRNAs. Due to miRNA-mRNA complementarity they degrade target mRNA or repress their translation (O'Brien et al. 2018). LncRNAs act as molecular signals, decoys, guides, and scaffolds in gene regulation (Gao et al. 2020). They can recruit transcription factors or directly bind to transcription factors to inhibit their functioning (Wang and Chang 2011). Additionally, they compete with mRNAs as endogenous competitors of RNAs to attract miRNAs on lncRNAs. Therefore, they can regulate the target gene expression post-transcriptionally via lncRNA:miRNA sponge mechanism (Alkan and Akgül 2022). Lastly, lncRNAs can regulate the chromatin dynamics through the recruitment of chromatin modifiers and chromatin modellers at epigenetic level (Zhang et al. 2019)

ncRNAs play crucial role in physiological processes of development and diseases. Their roles as regulators of cancer gene network and biomarker in diseases have been demonstrated (Fernandes et al. 2019). LncRNAs play crucial role in regulation

of apoptosis by affecting the activity of transcription factors, histone modification complexes and miRNAs and by changing the stability apoptotic proteins in cancer cells (Takeiwa et al. 2021). LncRNA regulation is achieved in intrinsic pathway of apoptosis by targeting the p53 or Bcl-2 family genes (Liu et al. 2017; Zhou et al. 2007). For example, *Taurine up regulated 1 lncRNA (TUG1)* regulates apoptosis by downregulating pro-apoptotic BAX levels through its interaction with EZH2 in lung cancer cell (Liu et al. 2017). According to another study, *TUG1* can also regulate the apoptosis by competing for astrocyte elevated gene-1 (AEG1) with miR-129-5p in malignant melanoma (Long et al. 2018).

1.5. Stable intronic sequence RNAs (sisRNAs)

Introns are non-coding sequences located within coding sequences in particular genes. Introns are removed during processing of pre-mRNA in eukaryotic cells through splicing (Lee and Rio 2015). The spliced-out introns are usually unstable and immediately degraded only within seconds or minutes (Hesselberth 2013). The existing knowledge about stable intronic sequences is limited with few exceptions of small Cajal bodies, small nucleolar RNAs and microRNAs (Cech and Steitz 2014). Until recently the presence of intronic transcripts has been neglected by researchers. In 2012 Gall et al. reported that some intronic transcripts are deposited in the oocyte of the frog *Xenopus tropicalis* and existed till beginning of zygotic transcription via a high-throughput RNA-seq study. Actinomycin D treatment and U2 depletion were performed in the oocyte of *Xenopus tropicalis* to determine the stability of intronic transcripts. It is observed that even after the treatment, the abundance of intronic transcripts did not change. The deposited intronic transcripts transcribed from single long intron is called as stable intronic sequence RNAs (sisRNAs) (Gardner et al. 2012). Through the recent discoveries sisRNAs can be defined specifically as the unusually stable transcripts that may contain polyA tail and 5’ cap and sourced from the coding regions of protein coding host genes (Chan and Pek 2019).

Different forms of sisRNAs can be generated through various mechanisms. The biogenesis of sisRNAs mainly occurs via splicing-dependent and splicing independent

mechanism (Chan and Pek 2019). sisRNAs can be found in lariat, circular and linear forms as localized in the cytoplasm and nucleus of cells (Osman, Tay, and Pek 2016). In *Xenopus*, nuclear sisRNAs exist in linear or lariat forms while the cytoplasmic sisRNAs are only found in lariat form (Jin, He, and Silva 2020). The first proposed mechanism for sisRNA biogenesis is splicing-dependent mechanism. Since pre-mRNAs undergo splicing, the spliced-out introns are in a lariat form (Hesselberth 2013). Through the activity of lariat debranching enzyme, the transcripts in a lariat form transforms to linear sisRNAs or they are trimmed from their 3' end and transform to the circular sisRNA form (Gardner et al. 2012; Tay and Pek 2017; Talhouarne and Gall 2014; Pek et al. 2015) . In addition to these, debranched linear sisRNAs can also re-circularize to the circular sisRNA form (Talhouarne and Gall 2018). sisRNAs also can be generated through an exon-back-splicing-mechanism. Depending on the abundance of complementary elements such as Alu repeats in pre-mRNA, the ligation occurs between 3' end and 5' ends of two succeeding exons which have intron interspersed between them (Li et al. 2015a).

As distinct from the splicing-dependent mechanism, sisRNAs can be generated through the direct transcription from their intronic promoter or by using the cognate host gene promoter without a requirement for pre-mRNA splicing (Jia Ng et al. 2018). It is considered that they can be originated from intron-retained (IR) transcripts, or they can undergo intronic cleavage and have partially retained introns. Additionally, it is known that intron-retention of some detained nuclear transcripts is unusually stable and these nuclear transcript contain full-length unspliced introns within it (Chan and Pek 2019). These detained transcripts which are transcribed from cognate host gene promoter and are with polyA tail can be categorized as sisRNAs (Boutz, Bhutkar, and Sharp 2015). Alternatively, sisRNAs can be generated as unspliced forms of cognate-host genes with splice junctions. These transcripts were detected in Epstein–Barr virus (EBV) in different sizes and in different forms with or without polyA tail (Moss and Steitz 2013). Lastly, exposure to UV irradiation may lead to formation of shorter RNA isoforms because of early termination of transcription and polyadenylation (Williamson et al. 2017).

As far as we know, sisRNAs are identified in different organisms including *Xenopus tropicalis*, *Drosophila melanogaster*, *Arabidopsis thaliana*, human and mouse (Tomita et al. 2015; Wu et al. 2018; Laurent et al. 2012; Gardner et al. 2012; Pek et al. 2015). The forms of sisRNAs are circular intronic, exon-intron circRNA and linear

intronic RNAs in human (Zhang et al. 2013; Li et al. 2015; Tomita et al. 2015). These RNAs are firstly categorized as intronic RNAs in the year they are discovered, and after the discovery of sisRNAs they are classified as sisRNAs in human. *SMYD3-CLIP* is an example of intronic RNA identified in HCT-116 cells. The study focuses on the identification of guiding RNAs that recruit repressive polycomb complex PRC2 to specific chromatin regions for epigenetic regulation. The interaction of first intron of *SMYD3* gene (also called *SMYD3-CLIP*) with EZH2 domain identified. Through this interaction, expression of host gene, *SMYD3* which is a chromatin modifier enzyme is downregulated in human colorectal cells (Guil et al. 2012).

Recent studies reported that sisRNAs may regulate gene expression through various pathways. *sisR-1, sisR-2 and sisR-3* are the maternally deposited linear sisRNAs in *Drosophila* (Jia Ng et al. 2018; Pek et al. 2015; Osman and Pek 2018). When deep sequencing was performed from unfertilized egg of *Drosophila*, *sisR-1, sisR-2 and sisR-3* were interestingly detected in polyA-positive RNA fractions, not in polyA eliminated ones. The presence of polyadenylation signal near to the 3' end of intronic transcripts and having 5' ends close to 5' splice sites of introns arose the question of if they are transcribed by a splicing-independent mechanism. The cloning of intronic regions to UAS/Gal4 reporter system plasmids revealed that these sisRNAs have their own intronic promoters. They are transcribed in a splicing-independent manner, not as by-products of splicing (Jia Ng et al. 2018). The studies to decipher their biological significance showed that they can cause the gene repression of their targets, *lncRNA ASTR, dFAR1*, and the *lncRNA CR44148* respectively (Jia Ng et al. 2018; Pek et al. 2015; Osman and Pek 2018). The common feature of these three sisRNAs is having free 3' tail in their secondary structure. According to proposed mechanism for their function, the complementary base-pairing via 3' tail may cause the degradation of their targets (Jia Ng et al. 2018). *sisR-1* involves in negative feedback mechanism by downregulating lncRNA ASTR. lncRNA ASTR modulates the expression of pre-mRNA of *rga* gene, the host gene of sisR-1. Since the *rga* functions as stem cell differentiation factor, this feedback mechanism regulates germline stem cell differentiation (Pek et al. 2015). *sisR-2* plays role in the maintenance of germline stem cell during nutritional stress by downregulating *dFAR1* expression, a gene involves in fatty acid metabolism. Additionally, *sisR-2* is negatively regulated by *bantam* miRNA while *sisR-2* modulate the transcription of *bantam.* The action of regulation of *bantam* prevents decrease in germline stem cells by inhibiting the action of

sisR-2 during nutritional stress (Osman and Pek 2018). sisR-3 targets *lncRNA CR44148* via complementary base pairing with 3' tail. This interaction results in repression of *lncRNA CR44148.* While sisR-3 has high expression in larvae and pupae stages of development, *lncRNA CR44148* is highly expressed in embryos of *Drosophila*. Knockdown of *sisR-3* elicits that *sisR-3* is crucial for larvae and pupae development (Jia Ng et al. 2018).

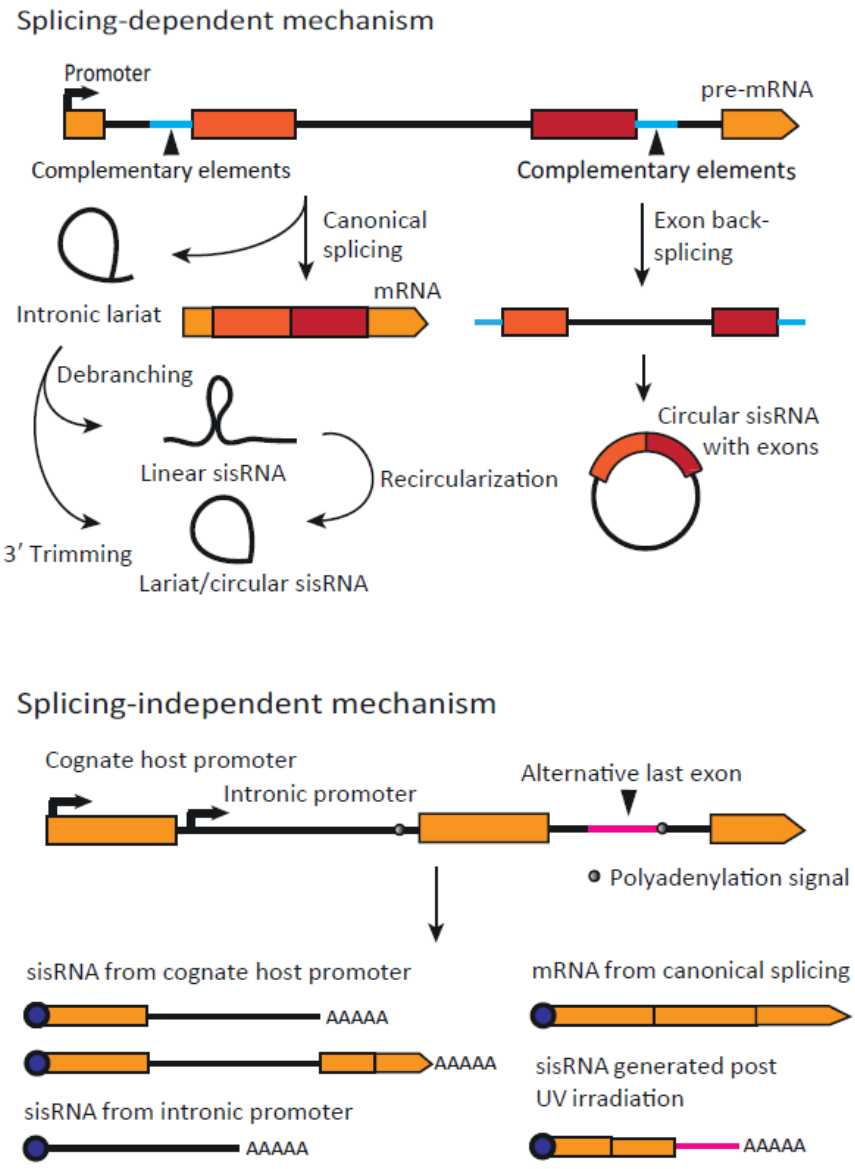

Figure 1.2. The biogenesis of stable intronic sequence RNAs. **A.** Splicing dependent mechanism **B**. Splicing-independent mechanism. (Source: Chan and Pek 2019)

1.6. sisRNAs and apoptosis

There is limited information about roles of sisRNAs in human. There are few established sisRNAs in human such as *ci-ankrd52*, *circEIF3J*, *circPAIP2*, *Eleanors* and *SMYD3- CLIP* (Tomita et al. 2015; Guil et al. 2012; Y. Zhang et al. 2013; Li et al. 2015). *Eleanors* and *SMYD3- CLIP* are the examples of linear sisRNAs (Guil et al. 2012; Tomita et al. 2015). At the time of their discovery, they were categorized as intronic RNAs however with the discovery of sisRNAs they were re-categorized as linear sisRNAs. *Eleanors* was firstly detected in the intron of *ESR1* after prolonged oestrogen-depletion in MCF7 cells. Further studies revealed that it plays role in breast cancer resistance adaptation by interacting with *Upstream-Eleanor* (Tomita et al. 2015). On the other side, *SMYD3-CLIP* was studied in HCT-116 cells. It caused repression in the gene expression of host gene by interacting with EZH2 (Guil et al. 2012). To sum up, there is not any genome-wide study in human that focuses on linear sisRNA profile under apoptotic conditions. Additionally, a sisRNA with an intronic promoter has not been reported in human yet.

1.7. Aim

The objective of this study to investigate stable intronic sequence RNA profile under cisplatin- and TNF-alpha-induced apoptotic conditions and to characterize differentially expressed sisRNA candidates.

CHAPTER 2

MATERIALS and METHODS

2.1. Cell culture, drug treatment and transfection

HeLa cells (DSMZ GmbH) were cultured in RPMI 1640 (with L-Glutamine, Gibco) medium with 10% fetal bovine serum (FBS) (Gibco) in a humidified atmosphere at 37°C and 5% CO_2.

For induction of apoptosis via drug (cisplatin) or ligand (TNF-alpha) treatment, $1x10^6$ HeLa cells were seeded on 10 cm dishes (Sarstedt) as three biological replicates. The cells were incubated overnight at 37°C, in 5% CO_2. Freshly prepared 80 μM cisplatin (CP) (Toronto Research Chemicals) dissolved in dimethyl sulfoxide (DMSO) was added on previously seeded HeLa cells and incubated for 16 hours at 37°C. DMSO was used as negative control of CP as 0.1 % (v/v). TNF-alpha at a concentration of 75 ng/mL (Merck Millipore) in combination with 10 μg/ml cycloheximide (CHX) (Applichem) was added on previously seeded HeLa cells and incubated for 24 hours at 37°C CHX without TNF-alpha was used as negative control. TNF-alpha is not sufficient to induce apoptosis alone so it requires cycloheximide for induction (Bhattacharya et al. 2003). The given concentrations and incubation duration of CP, DMSO, TNF-alpha and CHX-treatments were optimized previously as part of TUBITAK Project (113Z371). HeLa cells reached LD_{50} after CP and TNF-alpha treatment when the proper concentration and incubation duration were followed.

The overexpression of sisRNA candidates were performed by transfecting the pcDNA3.1(+) construct with sisRNA transcriptional unit. Prior to transfection, $7.5x10^4$ HeLa cells were grown overnight on 6-well plate. The media on the cells were changed and 1850 μl fresh RPMI was added on them. Then, 4.5 μl FuGENE® HD Transfection Reagent (Promega), 1500 ng plasmid and RPMI without FBS were added till the reaction volume reaches 150 μl. The transfection mixture was vortexed and incubated at room

temperature for 10 minutes. Then, the transfection mixture was added on the cells dropwise for even distribution. Transfection experimental set up consisted of pcDNA3.1(+) with candidate sisRNA, empty vector, only transfection reagent control and only cell control with three biological replicas. After 24-, 48- and 72-hours incubation at 37°C and 5% CO_2, transfected cells were harvested for further experiments.

2.2. Apoptosis measurement

The apoptosis percentage of CP, DMSO, TNF-alpha and CHX-treated cells were measured by flow cytometry (FACS CANTO, BD). Annexin V-FITC and 7AAD-PerCP (BD) were used for labelling purposes. Prior to staining, cells were trypsinized with Trypsin-EDTA (Gibco, 0.25%) and washed with 1X PBS (Gibco). Since dead cells are also important for total apoptosis profile, collected culture media and PBS were saved. After centrifugation at 1000 rpm for 5 min, 50 µl of 1X annexin binding buffer (BD) were used to dissolve the cell pellet. The cells in annexin binding buffer were stained with 10 µl of Annexin V-FITC and 10 µl of 7AAD-PerCP, diluted as 1:10. After 15 min incubation at room temperature in dark, cells were diluted with 200 µl 1X PBS for flow cytometric analysis. Three control groups were prepared for analysis as unstained cell, two monochromatic controls for Annexin V-FITC and 7AAD-PerCP. The cells which are not stained with both of dyes are accepted as live. Early apoptotic population was distinguished by Annexin V positive and 7AAD negative cell profile, while Annexin V positive and 7AAD positive cells are accepted as late apoptotic population. The cell population that is Annexin V negative and 7AAD positive are accepted as dead cells.

2.3. Total RNA isolation and DNase treatment

CP, DMSO, TNF-alpha, CHX-treated cells were treated with Trypsin-EDTA (Gibco, 0.25%) and washed with 1x PBS. The collected cells were centrifuged at 1200 rpm for 5 min. Cell pellets were used for total RNA isolation by using RNeasy Midi RNA

isolation kit (Qiagen) for deep sequencing. During total RNA isolation, manufacturer's instructions were followed as mentioned below. Cells were homogenized by passing them 5-10 times through a 20-gauge RNase-free needle with syringe in 2 mL Buffer RLT. Then, 2 mL 70% RNase-free ethanol was mixed with cell lysate. All cell lysate loaded on RNeasy midi column placed on 15 mL centrifuge tube, centrifuged for 5 min and flow-through was discarded. For DNase digestion on column, 2 mL Buffer RW1 was transferred into column, centrifuged for 5 min and flow-through was discarded. 20 µl of DNase I stock solution was mixed gently by flicking with 140 µl of Buffer RDD and transferred into the column. The column was incubated at room temperature for 15 min. Then, 2 mL Buffer RW1 was pipetted into column, incubated for 5 min, centrifuged and flow-through was discarded. 2.5 mL Buffer RPE was added on column to wash it, centrifuged for 2 min and flow-through was discarded. Another 2.5 mL of RPE was added on column, and centrifuged for 5 min. Then, the column was transferred to fresh 15 mL collection tube. For elution, first 100 µl RNase-free water transferred directly on column, left for 1 min to stand, and centrifuged for 3 min. The elution step was repeated once to obtain higher yield of RNA. All centrifugation steps were performed at room temperature and maximum speed (3200 x g) during protocol. Isolated total RNA concentrations were measured by NanoDrop Spectrophotometer (Thermo Fisher Scientific). Purity of total RNA was checked by controlling 260/280 and 260/230 ratios, which should be approximately 2. Then, the integrity of total RNA was checked by running 1% agarose gel in TBE buffer (1X Tris-borate-EDTA buffer consisting of 89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA.) for 30 min at 100 V.

Additionally, total RNA from drug and ligand treated cells were isolated to be used in RACE and qPCR experiments by using TRIzol™ reagent (Invitrogen). Cell pellets were prepared as mentioned above. Total RNA isolation was performed according to manufacturer's instructions. Cell pellets were resuspended with 0.5 mL TRIzol™ reagent and after incubation for 5 min at room temperature, 0.2 mL chloroform (Sigma) was added on the sample. The cells in TRIzol™ and chloroform were shaken for 15 seconds vigorously to obtain successful phase separation and incubated at room temperature for 3 minutes. Then, centrifugation was performed for 15 minutes 12.000 x g at 4°C to achieve phase separation. The upper phase containing the RNAs were transferred to a fresh tube. Followed by 0.5 mL of 100% RNase free isopropanol (Sigma) addition on the phase and incubation at room temperature for 10 minutes, sample was

centrifuged for 10 minutes at 12.000 x g at 4°C. After centrifugation, supernatant was removed carefully, and the RNA pellet was washed with 0.5 mL of 75% ice-cold ethanol. Centrifugation was performed for 5 minutes at 5000 x g at 4°C and all residual ethanol was removed from RNA pellet. The tube with RNA pellet bottom left as lid open for air dry. After the white RNA pellet turned to transparent colour, then RNA pellet was dissolved with 20 µl of RNase free water (Gibco). After the concentrations of RNAs were measured with NanoDrop Spectrophotometer (Thermo Fisher Scientific), they were stored at -80 °C. The integrity check on agarose gel was performed as mentioned above.

DNase treatment for the total RNA in the range of 20-40 µg was performed by mixing 2 µl Turbo DNase (Invitrogen), 10X reaction buffer and nuclease-free water and incubating for 20 minutes at 37°C. After incubation, by adding 10X inactivation buffer and incubating for 5 minutes, the activity of DNase was inactivated. After that, the mixture was centrifuged at 12000x g for 1.5 minutes and the supernatant that contained the DNase treated total RNA was transferred to fresh tube.

For RACE PCR experiments, polyA+ RNA was isolated by mRNA isolation kit (New England Biolabs). As starting material, $5x10^5$ DMSO-treated HeLa cells were used. 100 µl of oligod$(T)_{23}$ beads were resuspended for 30 minutes at room temperature before starting the experiment. Additionally, all buffers in kit were allowed to reach to room temperature before 1 hour. Oligod$(T)_{23}$ beads were mixed with 200 µl of lysis buffer and agitated for 2 minutes for bead equilibration. At the same time, cells were mixed with 500 µl of lysis buffer and swirled by hand. Cell lysate was mixed for 5 minutes at room temperature with agitation. The lysis buffer in beads were removed by magnetic rack and beads were resuspended by pipetting with addition of cell lysate into tube. Bead and cell lysate mixture were incubated at room temperature for 10 min with agitation. Later, the sample tube was placed on magnetic rack and the supernatant was discarded. The beads were washed by Wash1 buffer and fully resuspended by pipetting. Beads were incubated for 1 minute at room temperature with agitation. The washing step with Wash1 Buffer was repeated twice. The same steps for washing were performed with Wash2 Buffer twice as performed with Wash1. Removal of all residuals of wash buffers is crucial so quick spin of sample tubes can be performed during protocol. Then, the beads were resuspended with 500 µl of Low salt buffer by pipetting and incubated for 1 minute at room temperature with agitation. The beads were eluted for releasing of polyA + RNAs by adding 60 µl of elution buffer. The sample was incubated for 2 minutes at 50 °C with

agitation by using thermoshaker. Lastly, the sample tube was placed on magnetic rack and the eluent was transferred to fresh nuclease-free tube. After spectrophotometric concentration measurement with NanoDrop Spectrophotometer, polyA + RNAs were stored at -80 °C.

2.4. Deep sequencing and bioinformatic analysis

Three biological replicates of total RNAs from CP, DMSO and TNF-alpha treated of HeLa cells (in total 9 samples) were shipped Fasteris (Switzerland) for RNA sequencing. A total of 27 libraries were prepared from these samples as follows: 9x for total RNA plus rRNA depletion, 9x for stranded mRNA library purified with polyA selection and 9x stranded non polyA RNA libraries (eluated fraction recovered from polyA selection step). The deep sequencing was performed with 2x100 bp paired-end reads by NovaSeq 6000, SP-200. According to the sequencing data output, approximately 24-29 million of paired-end reads for each library was expected. When the FASTQ data was achieved, the bioinformatic analyses were performed in Non-Coding RNA/ Akgül Lab by Dilek Cansu Gürer. Bioinformatic analysis was performed by two different methods. In first method, DE-kupl algorithm which is a k-mer based and reference genome independent method was used. By DE-Kupl algorithm, the differentially expressed k-mers and k-mer contigs were found, the contigs were aligned to the reference genome (GRCh38.p13) and annotation of contigs (GENCODE v.39) were completed. DE-kupl algorithm was performed by comparing the expression k-mers of control groups to treatment group. For example, when DMSO total RNA k-mers were compared to CP or TNF-alpha treated total RNA k-mers, DMSO polyA+ RNA k-mers were compared CP or TNF-alpha treated polyA+ RNA k-mers. In the second method, RNA-seq reads were aligned to the reference genome (GRCh38.p13) by STAR Aligner to create BAM files (.bam). and indexed with SAMtools to create BAM index file (.bai). BAM files were required to observe RNA-seq reads on Integrative Genome Viewer (IGV).

As a result of bioinformatic analysis with DE-kupl algorithm, the intronic contig files (intron.tsv) of control vs treatment RNAs for total, polyA+ and polyA eliminated RNAs were obtained. The intronic contigs in polyA+ files were screened on IGV visually.

Possible sisRNA candidates with intronic promoters were expected as observed both in total RNA and polyA + RNA fractions but not in polyA eliminated fractions. Candidate sisRNAs were selected based on the following criteria: a clear intronic peak on IGV with distinct 5' and 3' ends, the intronic peak that is not dispersed through the whole intron and is longer than 200 nucleotides in size, having polyadenylation signal close to the 3' end of intronic transcripts (canonical poly(A) signals "AATAAA" and "ATTAAA" and non-canonical polyA signals "AGTAAA", "TATAAA", "CATAAA", "GATAAA", "AATATA", "AATACA", "AATAGA", and "ACTAAA"). All the intronic transcripts that suited the criteria were listed by visual screening of all intronic contigs for each treatment samples.

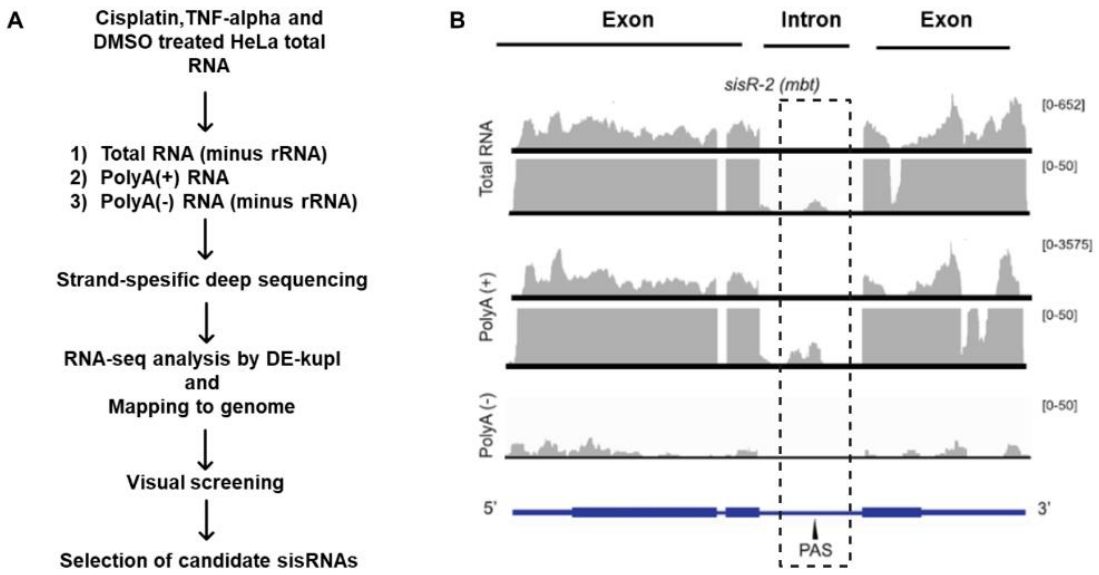

Figure 2.1. The experimental design of sisRNA identification A. The workflow of sisRNAs with intronic promoter identification strategy. B. An example of sisRNA (sisR-2) that is visualized on IGV. The B part figure was modified from Jia Ng et al. 2018.

2.5. cDNA synthesis and quantitative PCR

RevertAid First Strand cDNA Synthesis Kit (Thermofisher Scientific) was used for the complementary DNA conversion from both total RNA and polyA + RNAs. Total RNA of 4 µg or 300 ng was used to set up a 20 µl reaction in the presence of 1 µl oligodT 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 Reverse transcriptase. For proper cDNA conversion, reaction was incubated at 25 °C for 5 minutes, at 42°C for 60 minutes at 42°C and lastly at 70°C for 5 minutes. The cDNA which was converted from total RNA for qPCR applications were diluted with 20 µl of nuclease free water and stored at -80°C.

qPCR reaction was set up with 6.25 µl of RealQ Plus 2x Master Mix Green (Ampliqon), 0.5 µl of 10 µM reverse primer, 0.5 µl of 10 µM forward primer, 1 µl of cDNA and 4.25 µl of nuclease-free water. Two-step amplification program in Rotor-Gene Q 2plex Platform (Qiagen) was selected and following incubation times were set up on program. Reaction mixture was incubated for 15 minutes at 95°C, followed by 40 cycles of denaturation for 30 seconds at 95°C and completed with annealing for 60 seconds at 60 °C. GAPDH was used as a housekeeping gene for calculation of $2^{-\Delta\Delta CT}$. All experiments were conducted as three biological replicates with 2 technical replicates.

2.6. 5’ and 3’ Rapid Amplification of cDNA ends (RACE) PCR

5’ and 3’ RACE experiments were performed by using the 5’/3’ RACE Kit, 2nd generation (Roche). For 3’ RACE protocol, first-strand cDNA synthesis was set up by mixing 4 µl of cDNA synthesis buffer, 2 µl of deoxynucleotide mixture, 1 µl oligodT-Anchor primer, 12 µl polyA + RNA and 1 µl Transcriptor reverse transcriptase, reaching to 20 µl final reaction volume. The mixture was incubated for 60 minutes at 55°C and then, for 5 minutes at 85°C. The cDNA was used for PCR amplification with TEMPase HotStart DNA polymerase (Ampliqon). For 3’ RACE PCRs of candidates, an internal forward primer (GSFP1) for each candidate was designed and anchor primer was used as reverse primer. For 5’ RACE protocol, first-strand cDNA synthesis was performed by

mixing 4 µl of cDNA synthesis buffer, 2 µl deoxynucleotide mixture, 1.25 µl of 10 µM GSRP1 (Gene specific reverse primer 1), 11.75 µl of polyA+ RNA and 1 µl of Transcriptor reverse transcriptase, reaching to 20 µl final reaction volume. Before continuing to the next polyA-tailing reaction of 5' RACE PCR, the cDNA was cleaned up with Monarch PCR and DNA clean up kit (New England Biolabs) by following the oligonucleotide clean up protocol according to manufacturer's instructions. The cDNA volume was completed to 50 µl with nuclease-free water and mixed with 100 µl of DNA clean-up binding buffer. Then, 300 µl of absolute ethanol was mixed with previous mix of cDNA. Fresh silica column was placed on its collection tube and all the sample was loaded on column, and centrifuged. Flow-through was discarded and 500 µl of wash buffer was added on column, and centrifuged. This washing step was repeated twice. Then, the column was centrifuged without adding any solution to remove the residual ethanol. Lastly, the column placed into fresh tube, was eluted with 20 µl of DNA elution buffer, incubated for 1 minute at room temperature and centrifuged. All centrifugation steps in DNA clean-up protocol were for 1 minute at 16000 x g at room temperature. Pure cDNA elution was followed by then concentration measurement by NanoDrop Spectrophotometer.

For poly(A) tailing of first strand of cDNA, 19 µl of purified cDNA sample, 2.5 µl of reaction buffer and 2.5 µl of 2mM dATP were mixed in a microcentrifuge tube and incubated for 3 minutes at 94°C. Then, the sample tube was placed on ice immediately. 1 µl of Terminal transferase rec. (80U/ µl) was added into tube, pipetted well and the reaction mixture was incubated for 30 minutes at 37°C. At the last step of incubation, the reaction mixture was incubated for 10 minutes at 70°C to inactivate terminal transferase. In the next step, PCR amplification of poly(A) tailed cDNA was performed with TEMPase Hot Start DNA Polymerase. The following reagents were mixed for PCR reaction; 2.5 µl of 10X buffer, 1 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTP mix, 0.5 µl of 10 µM GSRP2 (Gene specific reverse primer 2) and 0.5 µl of oligod(T)- anchor primer, 0.3 µl of TEMPase DNA polymerase, 2.5 µl of poly(A) tailed cDNA and 17.2 µl of nuclease-free water. The PCR mixture was incubated at thermal cycler (Blue-Ray Biotech) according to following touchdown PCR conditions; 1 cycle of initial denaturation for 15 minutes at 95°C, 10 cycles of denaturation for 30 seconds at 95 °C, annealing for 40 seconds at 46-52°C (-0.6°C for each cycle) and extension for 1 minutes

per kb at 72°C, 25 cycles of denaturation for 30 seconds at 95°C, annealing for 40 seconds at 48°C and extension for 1 minutes per kb at 72°C, and 1 cycle of final extension for 5 minutes per kb at 72°C. After PCR amplification, PCR product can be run on 1% agarose gel, however if the gene with low expression levels that is tried to be amplified, it may not be able to be observable on agarose gel. In this case, even if there is no product on agarose gel, this cDNA product can be used for next step of 5' RACE experiment.

1 cycle	Initial denaturation	15 minutes at 95 °C
10 cycles	Denaturation	30 seconds at 95 °C
	Annealing	40 seconds at 46-52 °C (-0.6°C each cycle)
	Extension	1 minute/per kb at 72 °C
25 cycles	Denaturation	30 seconds at 95 °C
	Annealing	40 seconds at 48 °C
	Extension	1 minute/per kb at 72 °C
1 cycle	Final extension	5 minutes at 72 °C

Figure 2.2. Touchdown PCR conditions for amplification of poly(A)-tailed cDNA with TEMPase DNA polymerase.

The next step of 5' RACE PCR was amplification of touchdown PCR product with an internal GSRP3 (Gene Specific Reverse Primer 3) and Anchor primer. For this reaction, 2.5 µl of 10X buffer, 1 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTP mix, 0.5 µl of 10 µM GSRP3 and 0.5 µl of Anchor primer, 0.3 µl of TEMPase DNA polymerase, 1 µl of 1:10 diluted touchdown PCR product and 17.2 µl of nuclease-free water were mixed. Annealing temperature of GSRP3 and Anchor primer were screened in 5°C range by gradient PCR. The PCR mixture was incubated under following conditions; 1 cycle of initial denaturation for 15 minutes at 95°C, 35 cycles of denaturation for 30 seconds at 95 °C, annealing for 40 seconds at 50-65°C and extension for 1 minutes per kb at 72°C, and final extension for 5 minutes at 72°C. Gradient PCR products were run on 1% agarose gel for 50 min at 100V, and imaged by Chemidoc XRS+ Gel Imaging System (Bio-Rad).

Table 2.1. The list of primers used in this study.

Primer name	**Primer sequence (5'→ 3')**
Anchor primer	GACCACGCGTATCGATGTCGAC
Oligod(T)-anchor primer	GACCACGCGTATCGATGTCGACTTTTTTTTTT TTTTTTT
sisR-DOCK7-IT1 GSRP1	CCTCCACCTCAATCACTTGCT
sisR-DOCK7-IT1 GSRP2	ATCATCCATCTTCTCGGGTGC
sisR-DOCK7-IT1 GSRP3	TTGTTCTGAGTTCGCCTGTGA
sisR-DOCK7-IT1 GSFP1	TCACAGGCGAACTCAGAACAA
sisR-DOCK7-IT1 qPCR RP	ATCATCCATCTTCTCGGGTGC
sisR-DOCK7-IT1 qPCR FP	TTGTTCTGAGTTCGCCTGTGA
sisR-DOCK7-IT1 OV-NheI	CTAGCTAGCCCTCCTTCACGCGTCT
sisR-DOCK7-IT1 OV-XhoI	CCGCTCGAGCCTCTGTCATTTAATT
sisR-PLPBP-IT4 GSFP1	AACCAACCCTGCACTCCTAG
sisR-MXD4-IT3 GSRP1	ATGGTTTGCTGGTGGGAGAG
sisR-MXD4-IT3 GSRP2	CGGAAGTCCCTGTGTAAACCA
sisR-MXD4-IT3 GSRP3	CAGGCGGACAGGACTTACG
sisR-MXD4-IT3 GSFP1	TAGGTTAGGTTGGCCCTCGT
sisR-MXD4-IT3 qPCR RP	GCACACTCCTCCACCTTCTC
sisR-MXD4-IT3 qPCR FP	ATGGTTTGCTGGTGGGAGAG
sisR-SSH2-IT2 GSFP1	GACCATCATCTCCTCCCCATTC
sisR-C11orf24-GSFP1	CAGGATGGGGATCACGCAAT
GAPDH-forward	ACTCCTCCACCTTTGACGC
GAPDH-reverse	GCTGTAGCCAAATTCGTTGTC

2.7. DNA extraction from agarose gel and PCR clean up

The DNA fragments that need to be cloned were extracted from 1% agarose gel after running for 50 minutes at 100V. For gel extraction and PCR clean up steps, NucleoSpin Gel and PCR clean up (Macherey-Nagel) was used according to

manufacturer's instructions. For gel extraction, the extracted gel amount was weighed, the twice volume of Buffer NT1 (Binding Buffer) was added, and the gel was melted at 50°C till it becomes completely dissolved. For PCR clean up protocol, the minimum reaction volume was completed with nuclease-free water if it is less than 50 µl. The twice volume of Buffer NT1 was mixed with PCR product. The fresh column was placed into a collection tube and a maximum of 700 µl of melted gel product in Buffer NT1 or PCR product in NT1 buffer was transferred on column. The column was centrifuged for 30 seconds at 11000 x g and flow-through was discarded. To wash column, 700 µl of Buffer NT3 (Wash Buffer) was transferred to column, centrifuged for 30 seconds at 11000 x g and flow-through was discarded. Wash step was repeated twice. Then, to completely remove the ethanol in wash buffer, column was centrifuged for 3 minutes at 11000 x g without adding any component. For elution of DNA, the column was placed on fresh microcentrifuge tube and 16 µl of Buffer NE (Elution Buffer) was directly transferred into top of silica membrane of column and incubated at room temperature for 1 minutes. Lastly, the column was centrifuged for 1 minutes at 11000 x g, and the concentration of DNA fragment was measured by NanoDrop Spectrophotometer. The eluted DNA was stored at -20°C till use.

2.8. Molecular cloning and plasmid isolation

5' and 3' RACE PCR products were cloned into pMD20 (Takara Bio) T-vector to reveal the sequence information of products by TA cloning. Prior to TA cloning, A-tailing of PCR products that were isolated from gel was performed by mixing 15 µl of PCR product, 5 µl of 10X Standard *Taq* Reaction Buffer (New England Biolabs), 1 µl of 10 mM dATP (Thermo Fisher Scientific), 0.2 µl of *Taq* DNA Polymerase (New England Biolabs), and 28.8 µl of nuclease-free water till final volume 50 µl. Reaction mixture was incubated for 20 minutes at 72°C. Then, PCR clean up protocol with Nucleospin Gel and PCR clean up kit was performed. The ligation of A-tailed PCR product and pMD20 T-vector was set up by mixing the PCR product and vector with a 3:1 (3 unit insert : 1 unit vector) ligation ratio. For ligation reaction, the proper amounts of A-tailed PCR product, 0.5 µl pMD20 T-vector (25 ng), 1 µl T4 DNA ligase (New England Biolabs), 2 µl T4

DNA ligase buffer (New England Biolabs) and nuclease-free water till 20 µl were mixed in microcentrifuge tube. The reaction mixture was incubated at least 4 hours at room temperature.

Transformation of chemically competent DH5α cells was done by using 2 µl of previously prepared ligation mix. Firstly, DH5α cells were thawed on ice for 2-3 minutes, and then 2 µl of ligation mix was pipetted into cells. The transformation mixture was incubated on ice for 25 minutes. Then, for heat-shock step, DH5α cells were immersed into water bath (Witeg) which was at exactly 42°C for 50 seconds, and directly chilled on ice for 2 minutes. After that, the cells were transferred into 900 µl SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) and placed into orbital shaker (ThermoForma) for 1 hour at 37°C for recovery.

pMD20 T-vector contains *lacZ* gene therefore blue-white screening can be applied to distinguish the colonies with insert. Before 30 minutes of spreading the transformed cells onto LB agar plate with 100 mg/µl final concentration of ampicillin, 40 µL X-Gal Solution (20 mg/mL) (Thermo Fisher Scientific) and 40 µL of 100 mM IPTG solution (Bioshop) were mixed and spread to the surface of LB agar plate with ampicillin. After overnight incubation at 37°C in incubator, white colonies were selected as positive colonies with insert, while blue colonies did not have any insert in plasmid. For confirmation of white colonies whether it is false positive or not, colony PCR was performed. Colony PCR protocol is the same with the aforementioned protocol of TEMPase DNA polymerase. In colony PCR instead of 1 µl of cDNA as template, a white colony was picked with a sterile toothpick and mixed in the previously prepared PCR master mix. After running the samples on 1% agarose gel, the PCR products with correct product size were accepted as colonies with insert.

For Mini-Prep plasmid isolation, single colonies were inoculated into 4 mL of LB broth with inoculation loop and incubated at 37°C with 180 rpm shaking for overnight. During plasmid isolation, NucleoSpin Plasmid isolation kit (Macherey-Nagel) was used, and protocol was followed according to manufacturer's instructions for high-copy plasmid DNA isolation. Overnight bacterial cultures were transferred into fresh 1.5 mL tubes and centrifuged for 30 seconds. The supernatant was removed, and the remaining bacterial culture was transferred to the same tube and centrifuged again for 30 seconds. Then, the LB media as supernatant was fully removed from the bacterial pellet. The

bacterial pellet was resuspended with 250 µl of Buffer A1 (RNase A added) and then 250 µl of Buffer A2 was added to the resuspended cells for cell lysis, followed by incubation for 5 minutes. To neutralize the lysis reaction, 300 µl of Buffer A3 was added, and tube was inverted by hand gently till the blue cell lysate turned into white. Then, to clarify the cell lysate before loading to column, mixture was centrifuged for 8 minutes. After centrifugation, to enable plasmid binding to column, 700 µl of supernatant was loaded onto fresh column on collection tube and centrifuged for 1 minute. Flow-through was discarded. The column was washed with 500 µl of Buffer AW (recommended for sequencing reactions) and 600 µl of Buffer A4 subsequently. Each wash step was completed by 1 minute centrifugation and discarding of flow through. The washed column was centrifuged without adding any buffer to dry silica membrane of column for 3 minutes. Then, column was placed on to fresh microcentrifuge tube and 50 µl of Buffer AE was added onto the centre of silica column carefully to elute the plasmid DNA and incubated for 1 minute on benchtop. Then, the column was centrifuged for 1 minute. At the end, the plasmid DNA concentration was measured with NanoDrop Spectrophotometer, samples were stored at -20 °C. All centrifugation steps were followed at room temperature and at 11000 x g.

To be able to use the overexpression plasmid constructs in transfection, endotoxin-free plasmids were purified by using NucleoBond® Xtra Midi EF (Macherey-Nagel). As starter culture, 1 mL of overnight bacterial liquid culture from single colony were inoculated into 100 mL fresh LB broth and incbubated at 37°C with 180 rpm shaking for overnight. The bacterial culture was transferred into 50 mL tubes (BD Falcon) and centrifuged at 3200 xg for 30 minutes at 4°C. When the supernatant was removed, the bacterial pellet was dissolved in 8 mL Buffer RES (RNase A added). In case of having two 50 mL centrifuge tubes, it is possible to add 4 mL Buffer RES to each tube to resuspend cells and then transfer them into the same tube. Then, 8 mL of Buffer LYS was added into the resuspended cells for lysis and mixture was mixed by only inverting. The mixture was incubated at room temperature for 5 minutes. In this time, the equilibration of column was performed by adding 12 mL of Buffer EQU by swirl movements of serological pipette during transfer. After lysis incubation was completed, 8 mL of Buffer NEU was added to cell lysate to neutralize the lysis reaction and the sample tube was inverted gently till the blue colour changes to white. Then, the cell lysate was centrifuged at 3200 xg for 10 minutes at 4°C for clarification. After centrifugation, the supernatant

was transferred to the column by swirling for DNA binding to column. It is important to not to take white precipitate which may clog the column. Flow through was allowed to flow with gravitation. First washing of column filter and column was performed by addition of 5 mL of Buffer EQU by swirling the pipette. Then, the filter was removed, and second wash step was performed with addition of 8 mL Buffer WASH only to the column. In the next step to elute the plasmid DNA, 5 mL elution buffer was transferred onto column and the flow through was collected by placing 50 mL centrifuge tubes under the column. The eluted plasmid was precipitated by addition of 3.5 mL room-temperature isopropanol and mixed very well. Then, the mixture was centrifuged at 3200 x g for 30 minutes at 4°C. After centrifugation, the supernatant was completely removed, and the pellet was washed with 2 mL of room-temperature 70% ethanol, centrifuged at 3200 x g for 10 minutes at 4°C, and all ethanol was removed from pellet. The pellet was left for air-drying, but it is important to that not to have over-dried case. Following the complete drying of the pellet, the pellet was dissolved in nuclease-free water of 200 µl. The concentrations of isolated plasmids were checked by NanoDrop Spectrophotometer. Additionally, the integrity of purified plasmids was checked by running them 1% agarose gel for 1 hour at 100V.

2.9. Preparation of overexpression constructs

The PCR products for cloning of full-length cDNA sequence to the pcDNA3.1 (+) Mammalian Expression Vector (Invitrogen) were amplified by DMSO-treated polyA+ cDNA by Q5® High-Fidelity DNA Polymerase (New England Biolabs). The forward and reverse primers were used in PCR amplification were containing restriction sites for the enzymes that would be used for cloning in further steps. PCR reaction was set up by mixing 5 µl of 5X Q5 Reaction Buffer, 5 µl of 5X Q5 High GC Enhancer, 0.5 µl of 10 mM dNTPs, 1.25 µl of 10 µM forward primer, 1.25 µl of 10 µM reverse primer, 1 µl of polyA+ cDNA, 0.25 µl of Q5 High-Fidelity DNA Polymerase and 10.75 µl of nuclease-free water. PCR reactions were incubated according to following conditions: 1 cycle of initial denaturation at 98°C for 30 seconds, 35 cycles at 98°C for 10 seconds, at 50-72 °C (depends on primer properties) for 30 seconds, and at 72°C for 30 seconds per

kb and as last step 1 cycle of final extension at 72°C for 2 minutes. Amplified specific PCR products were isolated from 1% agarose gel after 50 min run by NucleoSpin Gel and PCR clean up kit. 4 μg of pcDNA3.1 (+) Mammalian Expression Vector was restricted with proper restriction enzymes for amplified PCR product, and Alkaline Phosphatase, Calf Intestinal (CIP) (New England Biolabs) treatment to the restricted plasmid was applied at the same reaction. According to protocol 4 μl of restriction enzyme 1, 4 μl of restriction enzyme 2, 0.3 μl of CIP, 4 μg of pcDNA3.(+), 5 μl of 10X CutSmart Buffer (New England Biolabs) and nuclease-free water till 50 μl were mixed in the tube and incubated at 37°C for 30 minutes. The double digested pcDNA3.1 (+) was run on 1% agarose gel for 1 hour with 100V and extracted from agarose gel by using by NucleoSpin Gel and PCR clean up kit. Prior to ligation reaction, amplified PCR fragment was restricted with restriction enzyme pair. For restriction reaction, 1 μl of restriction enzyme 1 per 1 μg, 1 μl of restriction enzyme 2 per 1 μg, 15 μl of PCR product, 5 μl of 10X CutSmart Buffer and nuclease free-water till 50 μl were mixed in the tube and incubated at 37°C for 30 minutes. Digested PCR product was run on agarose gel for 50 min under 100V and extracted from agarose gel by using by NucleoSpin Gel and PCR clean up kit. The final isolated concentrations of double digested pcDNA3.1(+) and PCR product were measured by NanoDrop Spectrophotometer.

Ligation reaction was set up according to 3:1 (insert:vector ratio). The calculated proper amounts of double digested vector and PCR product were ligated by mixing them with 2 μl 10X T4 Ligation Buffer (New England Biolabs), 1 μl of T4 DNA Ligase (New England Biolabs) and nuclease-free water till 20 μl. Then, the ligation reaction was incubated at room temperature for 10-15 minutes. The ligated product was transformed into the DH5α competent cells as mentioned in molecular cloning section.

CHAPTER 3

RESULTS

1.8. Induction of apoptosis by CP and TNF-alpha in HeLa cells

HeLa cells were treated with 80 μM CP to induce intrinsic apoptosis pathway. As negative control, HeLa cells were treated with 0.1 % (v/v) DMSO. The early apoptosis rate of cells was increased from 3.23% to 47.46% upon CP treatment. In parallel with the apoptosis rate, the viability of CP-treated cells reduced from 95.46% to 46.8 % (Figure 3.1B, $P \leq 0.0001$).

75 ng/mL of TNF-alpha in combination with 10 μg/ml cycloheximide (CHX) was used to induce the extrinsic apoptosis pathway in HeLa cells. TNF-alpha requires cycloheximide for induction of apoptosis since it is not able to trigger apoptosis by itself (Singh, Letai, and Sarosiek, 2003). As negative control group, 10 μg/ml CHX treated HeLa cells were used. After 24 hours incubation time, the apoptosis rate of early apoptotic TNF-alpha treated HeLa cells increased from 8.7% to 57.56%. The viability of HeLa cells upon TNF-alpha treatment decreased from 89.33% to 38.33 % (Figure 3.1C, $P \leq 0.0001$)

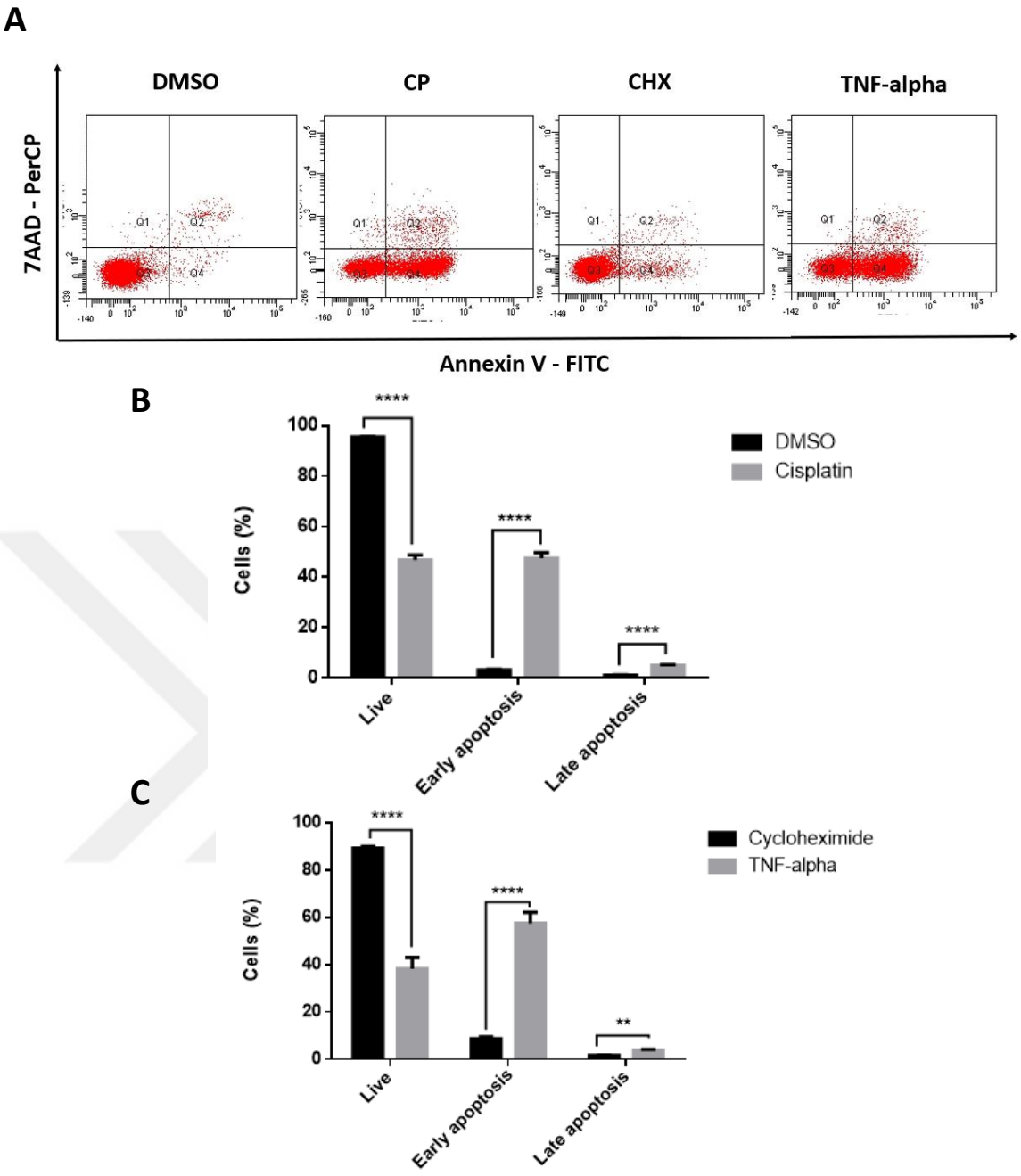

Figure 3.1. The flow cytometry analysis of DMSO, CP, CHX and TNF-alpha-treated cells. **A.** Representative FACS profiles of DMSO, CP, CHX and TNF-alpha-treated cells. AnnexinV-FITC/7AAD-PerCP staining was performed for detection of apoptosis rate. Q1 population (AnnexinV-/7AAD+) indicates dead cells, Q2 population (AnnexinV+/7AAD+) indicates late apoptotic cells, Q3 population (AnnexinV-/7AAD-) indicates live cells and Q4 population (AnnexinV+/7AAD-) indicates early apoptotic cells. **B-C.** The histogram of apoptosis rate of CP and TNF-alpha-treated HeLa cells with mean ± SD of three independent experiments. Statistical analyses by applying student's t-test were performed. (****P ≤ 0.0001, ** P ≤ 0.01)

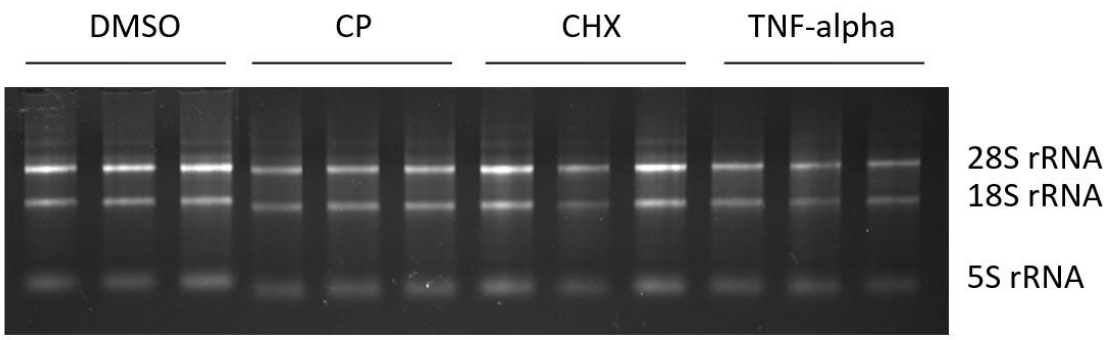

Figure 3.2. The total RNAs that are isolated from DMSO, CP, CHX and TNF-alpha treated HeLa cells, after DNase treatment.

The total RNAs from DMSO, CP, CHX and TNF-alpha treated HeLa cells were isolated by using TRIzol™ reagent. The application of Turbo DNase to the total RNAs was performed to eliminate any residual genomic DNA. After DNase treatment, the concentration of RNAs were measured by Nanodrop spectrophotometer and the 260/280 and 260/230 values were approximately 2 which was accepted as pure. Additionally, total RNAs were run on 1% agarose gel to check their integrity. The intact 28S, 18S and 5S rRNA bands showed the absence of degradation of total RNA (Figure 3.2).

1.9. Bioinformatic analyses and candidate selection

The isolated total RNAs of DMSO, CP and TNF-alpha treated HeLa cells with three independent biological replicas were subjected to deep sequencing company by Fasteris (Switzerland). Total RNAs were fractionated into polyA + and polyA eliminated fractions with the help of oligod(T) beads. Each RNA sample was sequenced as total RNA, polyA+ and polyA eliminated fractions. In total, deep sequencing of 27 samples was achieved by NovaSeq 6000 technology. The number of reads of RNA-seq data was varying between 27-38 million. It was higher than what is guaranteed by company as expected read depth.

The raw RNA-seq data were analysed by using two different methods. Firstly, a k-mer based analysis were performed by using DE-kupl algorithm. DE-kupl is a reference genome independent method that determines the differentially expressed 31-mers (k-mer)

and then creates larger contigs from these 31-mers. DE-kupl filters and masks the k-mers that show exact match with already annotated transcripts. Through this masking event, it enables to find novel transcripts within genome. In the next step of DE-kupl, differentially expressed contigs were aligned to reference genome to and classified based on their properties as intronic, SNV, antisense, lincRNA etc. In this study, DE-kupl algorithm was performed by comparing the DMSO and CP-treated conditions and comparing the DMSO and TNF-alpha treated conditions for each fraction separately. In the second analysis, classical RNA-seq analysis were performed. DE-kupl algorithm is not able to create the .bam files to observe the transcripts on Integrated Genome Viewer (IGV). Therefore, STAR alignment was performed and .bam and .bai files were created to visualize the transcripts on IGV.

Since the sisRNAs were expected to be observed on intronic regions the locations obtained in intron.tsv file by DE-kupl analysis were screened visually on IGV one by one. The intronic transcripts that have the following properties 1) not dispersed through whole intron of cognate host gene 2) having distinct 5' and 3' borders of intronic peak 3) having polyadenylation signal near to the 3' end 4) longer than 200 nucleotides in length 5) having the count number on IGV in the range of 0 to 200, were listed as candidate sisRNAs. The nomenclature of sisRNAs was done by containing sisR- at first part, the name of cognate host gene in the middle part and the intron number that the sisRNA is localized at last part. sisR-DOCK7-IT1 is one of the upregulated sisRNAs after CP treatment. This candidate sisRNA localized on first intron of DOCK7 gene. The intronic peak of sisR-DOCK7-IT1 has distinct 5' and 3' borders and contains ''AATAAA'' canonical PAS signal sequence near to the 3' end (Figure 3.3).

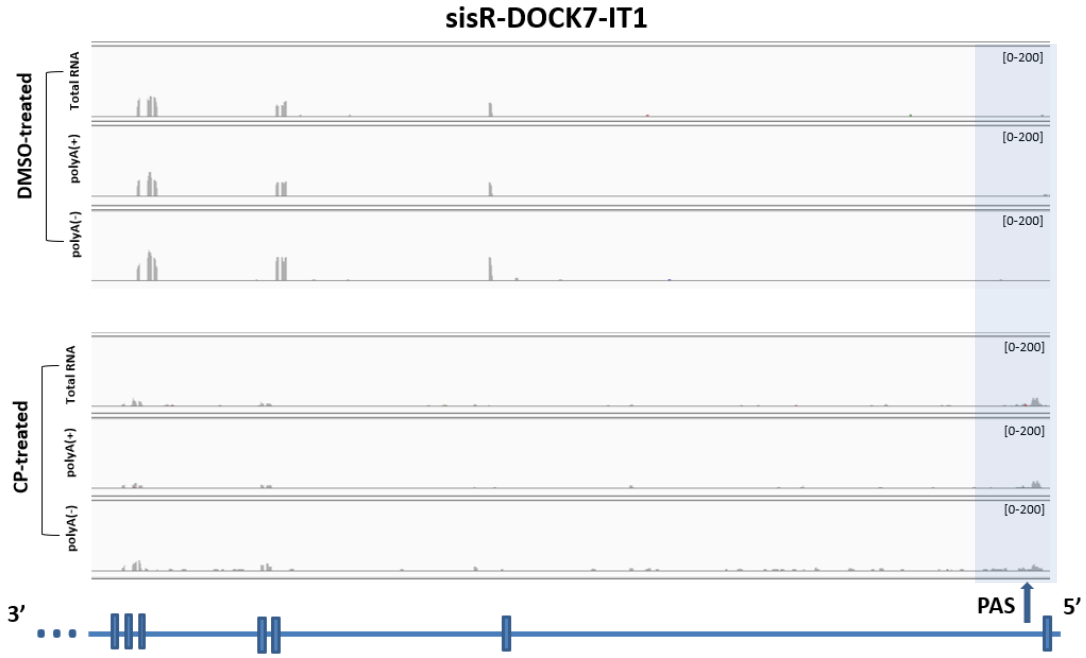

Figure 3.3. The IGV snapshot of sisR-DOCK7-IT1 (indicated by blue coloured region) from DMSO and CP-treated total, polyA+ and polyA eliminated RNA fractions. Scale 0-200.

According to DMSO vs CP polyA+ fraction DE-kupl analysis, 54701 contig locations that correspond to the 5578 genes were screened. In total, 48 candidate sisRNAs were listed based on CP-treatment RNA-seq data. The candidate sisRNAs were classified based on their differential expression level and the polyadenylation signal (PAS) that they have. The candidate sisRNAs were categorized based on differential expression levels; $log_2FC>1$ as upregulated sisRNA, $log_2FC<-1$ as downregulated sisRNA. In total, 37 (77% of total) sisRNA candidates were upregulated and 11 (23% of total) sisRNA candidates were downregulated. Candidate sisRNAs from CP-treatment data showed variety in distribution of PAS near to the end. The major group of sisRNAs harboured canonical PAS. In total, 32 of candidate sisRNAs (67% of total) possessed canonical PAS and 4 of total candidates (8% of total) possessed noncanonical PAS. Additionally, 12 of total candidates (25 % of total) carried sisRNA properties but did not contain PAS near to 3' end of them (Figure 3.4).

According to DMSO vs TNF-alpha polyA+ fraction DE-kupl analysis, 13254 contig locations that correspond to the 3219 genes were screened. Total 33 sisRNA candidates were listed from TNF-alpha treatment analysis. When the selected candidates

for TNF-alpha treatment were categorized based on their differential expression, 29 of them (76% of total) were upregulated and 4 of them (12% of total) were downregulated. Additionally, 22 of sisRNA candidates (67% of total) harboured canonical PAS while 8 of them (24% of total) had noncanonical PAS. 3 of total sisRNA candidates (9% of total) did not possess PAS while carrying sisRNA characteristics upon TNF-alpha treatment (Figure 3.4).

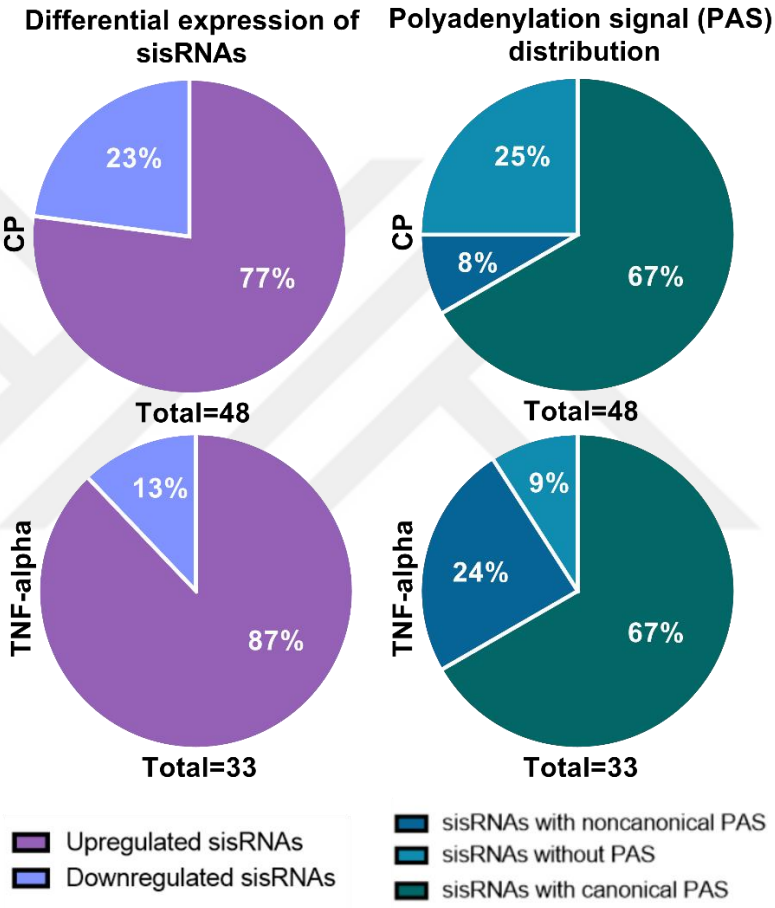

Figure 3.4. The categorization of selected candidate sisRNAs after visual screening **A.** The categorization of differentially expressed candidate sisRNAs that were selected visually after DE-kupl analysis. The candidates with $log_2FC>1$ were accepted as upregulated, the candidates with $log_2FC<-1$ were accepted as downregulated. Log_2FCs were calculated according to DMSO vs CP and DMSO vs TNF-alpha by DE-kupl algorithm. **B**. The categorization of candidate sisRNAs according to polyadenylation signal (PAS). sisRNAs with canonical PAS possessed ‘’AATAAA” and “ATTAAA’’ sequences near to 3’ end, sisRNAs with noncanonical PAS possessed “AGTAAA”, “TATAAA”, “CATAAA”, “GATAAA”, “AATATA”, “AATACA”, “AATAGA”, and “ACTAAA” near to 3’ end and sisRNAs without PAS did not contain any PAS sequence at their 3’

end. The total number of candidate sisRNAs selected from DMSO vs CP RNA-seq data was 48. The total number of candidate sisRNAs selected from DMSO vs TNF-alpha RNA-seq data was 33. (PAS: polyadenylation signal)

After completing the visual screening for CP and TNF-alpha datasets, 5 sisRNA candidates were selected for characterization of these intronic transcripts. sisR-MXD4-IT3 was one of the sisRNA candidates which is downregulated after TNF-alpha treatment, while sisR-SSH2-IT2 was upregulated upon TNF-alpha treatment. sisR-DOCK7-IT1, sisR-PLPBP-IT4 and sisR-C11orf24-IT2 were selected as sisRNA candidates which were upregulated upon CP treatment. Additionally, the PAS sequences of these candidates that were near to the 3' ends candidates on IGV, were indicated in Table 3.1.

Table 3.1. The list of candidate sisRNAs that were studied

Candidate	Drug/ligand inducible	Gene expression regulation	PAS sequence
sisR-MXD4-IT3	TNF-alpha	Downregulated	AATAAA
sisR-SSH2-IT2	TNF-alpha	Upregulated	AATAAA
sisR-DOCK7-IT1	Cisplatin	Upregulated	AATAAA
sisR-PLPBP-IT4	Cisplatin	Upregulated	AATAAA
sisR-C11orf24-IT2	Cisplatin	Upregulated	AATAAA

1.10. Determination of transcriptional units of candidates by 5'/ 3' RACE PCR

The 5' and 3' ends of candidate sisRNAs were established by performing RACE PCR experiments. In 3' RACE PCR experiments, cDNA conversion was performed by

using oligod(T)- Anchor primer that enables to amplify the 3’ end of transcripts in further PCR reactions easily. After sequencing of 3’ RACE PCR products, it was expected to observe anchor sequence (‘’GTCGACATCGATACGCGTGGTC’’) next to poly(A) sequence. According to Sanger sequencing results, 3’ ends of all candidates were determined successfully. The 3’ end sequences of candidates were given in Table 3.2. Candidate sisRNAs possessed poly(A) tail after PAS sequence in varying length of nucleotides from 14 to 31.

Table 3.2. The list of 3’ RACE PCR sequences of candidate sisRNAs.

Gene name	**3’ RACE PCR sequence**
sisR-MXD4-IT3	TGATTGTCACAGCAATGTCTGTGTGTGTTTTTTTTTA AGCGTAGAAAACCTGGAAAATACAGAAAAGCACAA AGCTAAAGAAATAAAATTGCCGGGTGCTTCGCCTGG AGACGCAAAAAAAAAAAAAAAAGTCGACATCGATA CGCGTGGTC
sisR-SSH2-IT2	TTGACCATCATCTCCTCCCCATTCTCCCACCAATCCC CAATCTGGTGTGAATTTTCTGAATTGATACCTATATT GATCCAGGGTTAAATAAACAAAGTATAATACTAAAA AAAAAAAAAAAAAAAGTCGACATCGATACGCGTGGT C
sisR-DOCK7-IT1	AGCAAGTGATTGAGGTGGAGGTTTCAAAGCCTTTCA CCTACTTGTGGTGGTTGGTTGCCACCTTTCTCCCACC CGCCAAATAAAAATTAAATGACAGAGGAAAAAAAA AAAAAAAAAAAAGTCGACATCGATACGCGTGGTC
sisR-PLPBP-IT4	TGTTATGGGATTTATAACTATGTAAAAGCAAAATGC ATGACAACAATAACAGAGGGGGATAAACAGAAGTA CACTATTGTAAAGTTCTTAAACTGTGTGAAGTAGTGT AACACACCTTGAAGGTGGACTGTTGTAAGTTAAACC CCAAAGCAACCAATAAAATAACACAATAAAGAGTT ATAGCTTATAAGTCAACAAAGGAGATAAAAAAAAA AAAAAAAAAAAAAGTCGACATCGATACGCGTGGTC
sisR-C11orf24-IT2	GTCTGTTCCAAGGCAAAGAACTGGACAAGAAGGAG GAGGTTCATTCAGTACATTGAGTCTGATCCTCCCTGC TGTAGCTGGAGCACGGTCCCTGAAGCTGGCTGAAGT GCTAAAGCAGGGGCTAGCAAACTGGGGCAGAATCC CACCTGCTGCCTGTTTGTATAAATAAAGTTTTATTGG GACACAAAAAAAAAAAAAAAAAAAAAAAGTCGAC ATCGATACGCGTGGTC

In 5’ RACE PCR experiments, cDNA was converted by using gene specific reverse primer and A-tail to the 3’ end of cDNA was added by terminal transferase. A-tail enabled the amplification of specific product with oligod(T)- Anchor primer and gene specific reverse primer. It is expected to observe the anchor sequence near to the A-tail at the 5’ end of candidate. Among the 5 candidates, only 5’ ends of sisR-MXD4-IT3 and sisR-DOCK7-IT1 were determined. According to the 5’/3’ RACE PCR results, sisR-MXD4-IT3 is 792 bp in length and sisR-DOCK7-IT1 is 427 bp in length.

Table 3.3. 5’ RACE PCR sequences of sisR-MXD4-IT3 and sisR-DOCK7-IT1

Gene name	**5’ RACE PCR sequence**
sisR-MXD4-IT3	AGGCGGACAGGACTTACGTACGTGGTGTCGGGTGAA AGATCAAGTGAGGAAATGGAGAACAAGGTCCGAAA AAAAAAAAAAAGTCGACATCGATACGCGTGGTC
sisR-DOCK7-IT1	GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTC CTCCTTCACGCGTCTCTCTCTTCCTGAGAAATCTGAC AGGAACCCTCTTTGCACCCTCGGGCCAGGTTCAAAG TGGACTTGTGCGTCCAGGATTGAAAACGTGTGGACA GAAT

sisR-DOCK7-IT1’s 5’ and 3’ RACE PCR experimental design were shown below (Figure 3.5). 3’ RACE PCR product were amplified at 60 ^{o}C which is approximately 300 bp in length (Figure 3.5B). 5’ RACE PCR final product were amplified which is approximately 250 bp in length at multiple temperatures specifically (Figure 3.5C). Sanger sequencing of RACE PCR products enabled to determination of transcriptional unit of sisR-DOCK7-IT1 (Figure 3.5D).

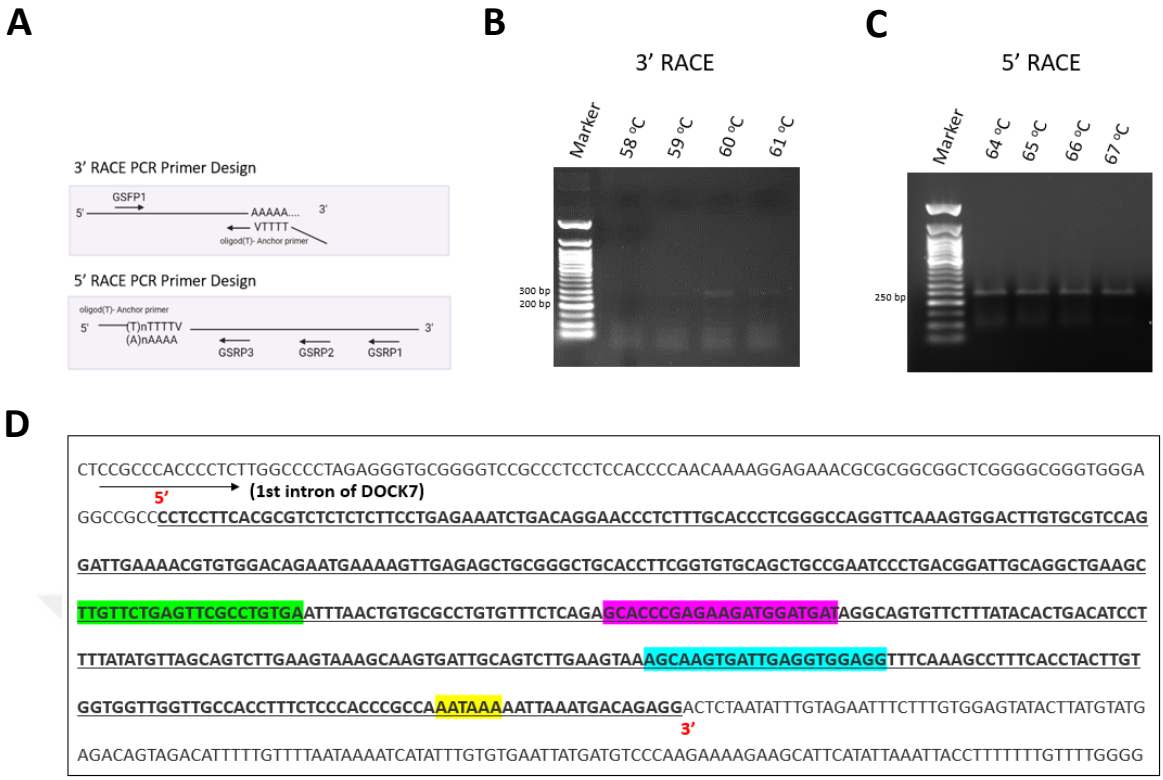

Figure 3.5. The overview of experimental design of RACE PCRs belong to sisR-DOCK7-IT1. **A.** The locations of primers used in 3' and 5' RACE experiments. B. -C. Gradient 3' RACE PCR reactions were set up to determine the specific annealing temperature of GSFP1 and oligod(T)-Anchor primer. **C.** The final nested 5' RACE PCR products. The reaction was performed by using Anchor primer and GSRP3. TEMPase Hot start DNA polymerase was used for every PCR reaction. **D.** The sequence of transcriptional unit of sisR-DOCK7-IT1 according to 5' and 3' RACE PCRs. The underlined sequence shows sisR-DOCK7-IT1. Different colours used to highlight the primer sequences. GSFP1 and GSPRP3: Green, GSRP1: Turquoise and GSRP2: Pink. PAS is also highlighted with yellow. Marker is 50 bp DNA ladder (New England Biolabs).

1.11. Determination of gene expression levels of candidates by qPCR

The differential expression levels of sisRNA candidates that had the full-length sequences determined, were measured by qPCR. Even if the candidates were selected from specific drug/ligand treatment, their expression levels were measured by using the cDNAs of CP and TNF-alpha treated HeLa cells. According to qPCR results, sisR-DOCK7-IT1 was upregulated 4.27-fold upon CP treatment while it was downregulated

4.16-fold downregulated upon TNF-alpha treatment. On the other side, sisR-MXD4-IT3 was downregulated in both treatment conditions. It was downregulated 2.77-fold upon CP treatment while 3.03-fold downregulation was observed upon TNF-alpha treatment (Figure 3.6).

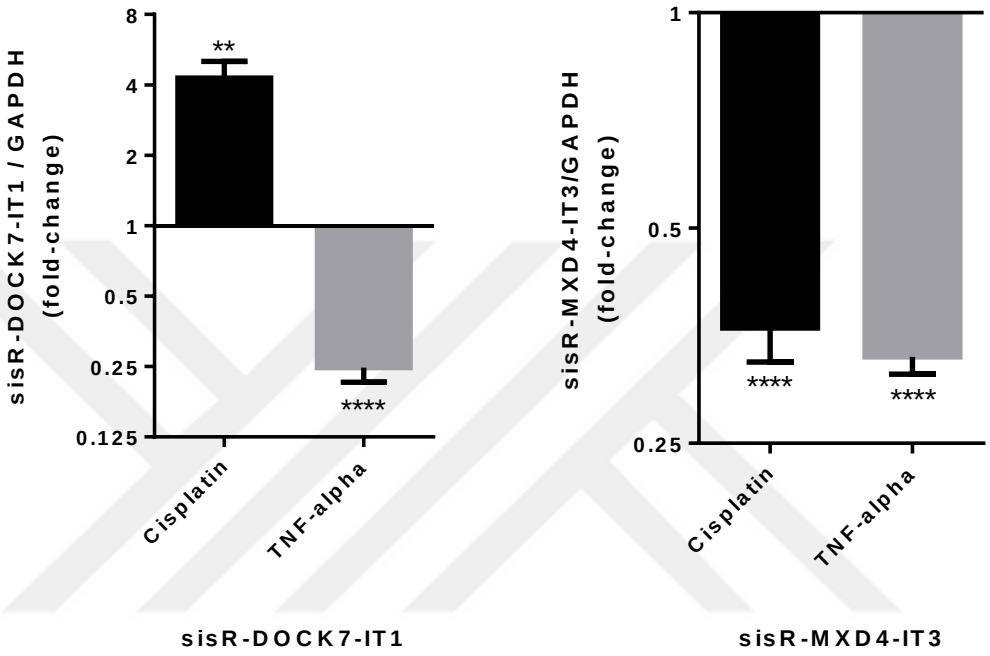

Figure 3.6. The expression levels of sisR-DOCK7-IT1 and sisR-MXD4-IT3 upon CP and TNF-alpha treatment. DMSO-treated cDNA was used as control of CP-treated condition while cycloheximide treated cDNA was used as control of TNF-alpha treated cells. GAPDH was used for normalization of results. Student's t-test was applied as statistical test by using GraphPad Prism 6. The experiment was performed with 3 independent biological and two technical replicas for each condition. (****$P \leq 0.0001$ and ** $P \leq 0.01$)

1.12. Construction of overexpression plasmid of sisR-DOCK7-IT1

The full-length sequence of sisR-DOCK7-IT1 that were determined via RACE PCR was amplified with primers designed from 5' and 3' end sequences of it with NheI and XhoI restriction sites. PCR reactions were incubated at varying temperatures from 65 to 69 ^{o}C. At each temperature except 69 ^{o}C, specific band at correct size of sisRNA-

DOCK7-IT1 with restriction enzyme sites was obtained (Figure 3.7A). The PCR product approximately at 500 bps in length was isolated from agarose gel for cloning into pcDNA3.1(+) mammalian expression vector. Prior to cloning, pcDNA3.1(+) restricted with NheI and XhoI restriction enzymes and isolated from agarose gel (Figure 3.7B).

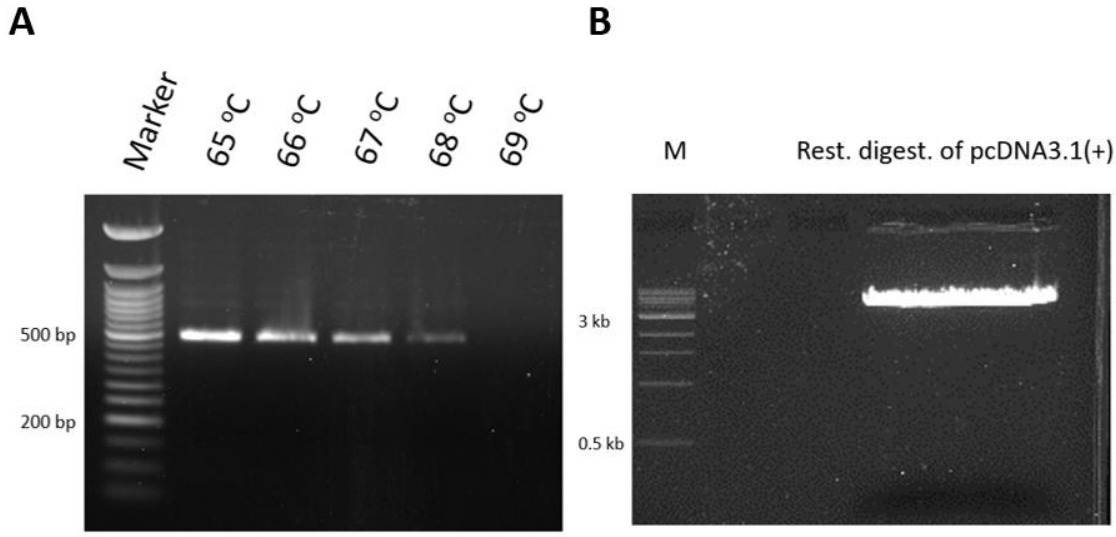

Figure 3.7. The PCR reaction of sisR-DOCK7-IT1 and preparation of plasmid backbone. **A.** The PCR products of full-length sequence of sisR-DOCK7-IT1 amplified by Q5 Taq Polymerase at different temperatures. 50 bp DNA ladder (New England Biolabs) was used as marker. **B.** The pcDNA3.1 (+) restricted by NheI and XhoI restriction enzymes prior to cloning. Marker is 1 kb DNA ladder (New England Biolabs). The products were run on 1% agarose gel for 50 min at 100V.

The ligation reaction was set up with sisR-DOCK7-IT1 product and pcDNA3.1 (+) restricted product and transformed into DH5α competent cells. The colonies with sisR-DOCK7-IT1 were confirmed with colony PCR. Positive colonies with insert were selected for plasmid isolation and isolated plasmids were confirmed with Sanger sequencing. When the pcDNA3.1(+) + sisR-DOCK7-IT1 were confirmed by Sanger sequencing, the large-scale endotoxin free plasmid isolation was performed. The isolated plasmids were run on agarose gel for quality control and restricted with NheI and XhoI restriction enzymes for double check (Figure 3.8A).

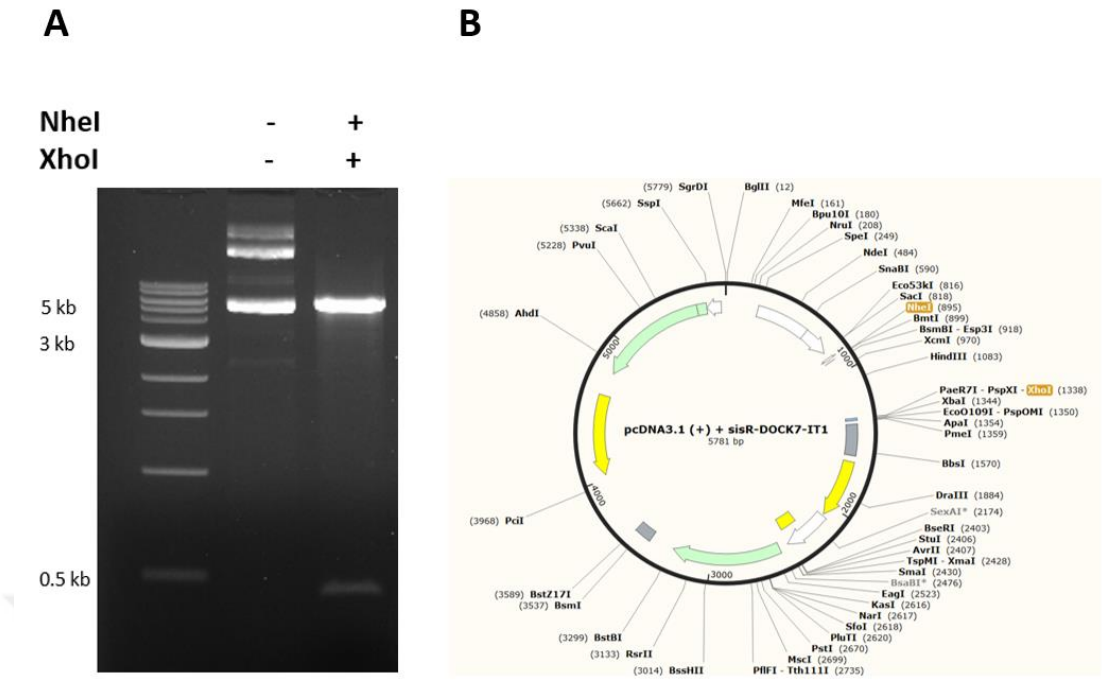

Figure 3.8. pcDNA3.1(+) + sisR-DOCK7- IT1 plasmid quality control and plasmid map **A.** The transfection quality pcDNA3.1(+) + sisR-DOCK7- IT1 plasmid as without and with restricted form with NheI and XhoI restriction enzymes. GeneRuler 1 kb DNA ladder (Thermo Scientific) was used as marker. **B.** Plasmid map of pcDNA3.1(+) + sisR-DOCK7- IT1 was created by using SnapGene 6.0.

1.13. Overexpression and phenotypic studies of sisR-DOCK7-IT1

The overexpression study of sisR-DOCK7-IT1 was performed by transfecting pcDNA3.1 (+) + sisR-DOCK7-IT1 into HeLa cells and incubating HeLa cells for 24, 48 and 72 hours. As control group, pcDNA3.1 (+) was transfected into cells. The transfected cells were harvested after 24-, 48- and 72-hours of incubation, and their total RNAs were isolated. After that, the overexpression level of sisR-DOCK7-IT1 for each incubation time were measured by qPCR. The fold changes of sisR-DOCK7-IT1 compared to only pcDNA3.1(+) transfection for 24-, 48- and 72-hours incubation were 10,523.1, 7116.94 and 6.72 respectively (Figure 3.9).

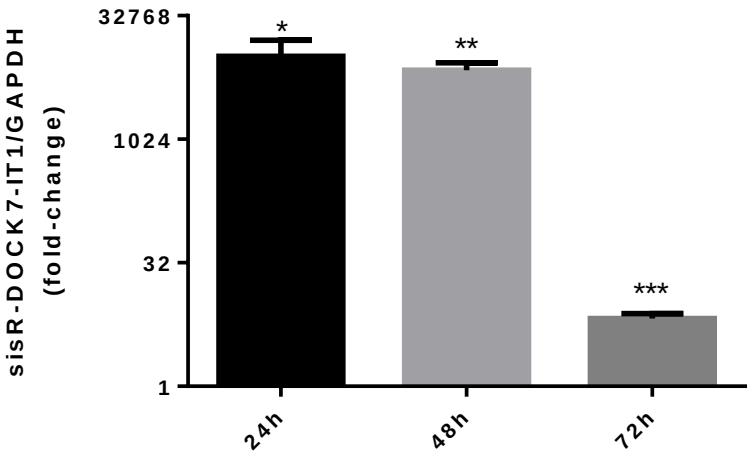

Figure 3.9. qPCR analysis for siR-DOCK7-IT1 expression levels. In qPCR experiment the cDNAs that were synthesized by using pcDNA3.1(+) + sisR-DOCK7-IT1 transfected cells for 24-, 48-, and 72-hours. As control group, only pcDNA3.1(+) transfected cells for 24-, 48-, and 72-hours were used. GAPDH was used for normalization. Student's t-test was applied as statistical test by using GraphPad Prism 6. The experiment was performed with 3 independent biological and two technical replicas for each condition. (***$P \leq 0.001$, ** $P \leq 0.01$ and * $P \leq 0.05$)

The effect of overexpression of sisR-DOCK7-IT1 on cell apoptosis, the apoptosis rates of pcDNA3.1(+) + sisR-DOCK7-IT1 transfected cells for 24-, 48-, and 72-hours were analysed after AnnexinV/7AAD staining by flow cytometry. After 24 hours of transfection, while 14% of cell population was early apoptotic cells in control group, it was 17.5% in pcDNA3.1(+) + sisR-DOCK7-IT1 transfected cells. After 48 hours of transfection of pcDNA3.1(+) + sisR-DOCK7-IT1, an increase in cell apoptosis rate compared to control group from 2.2% to 7.1% were measured. Lastly at 72 hours of transfection, early apoptotic cells were in 1.9% of population of control group, while early apoptotic cells were in 2.4% of cell population of pcDNA3.1(+) + sisR-DOCK7-IT1 transfected cells. Any significant change in cell apoptosis depending on the sisRNA transfection was not observed for 72 hours (Figure 3.10B).

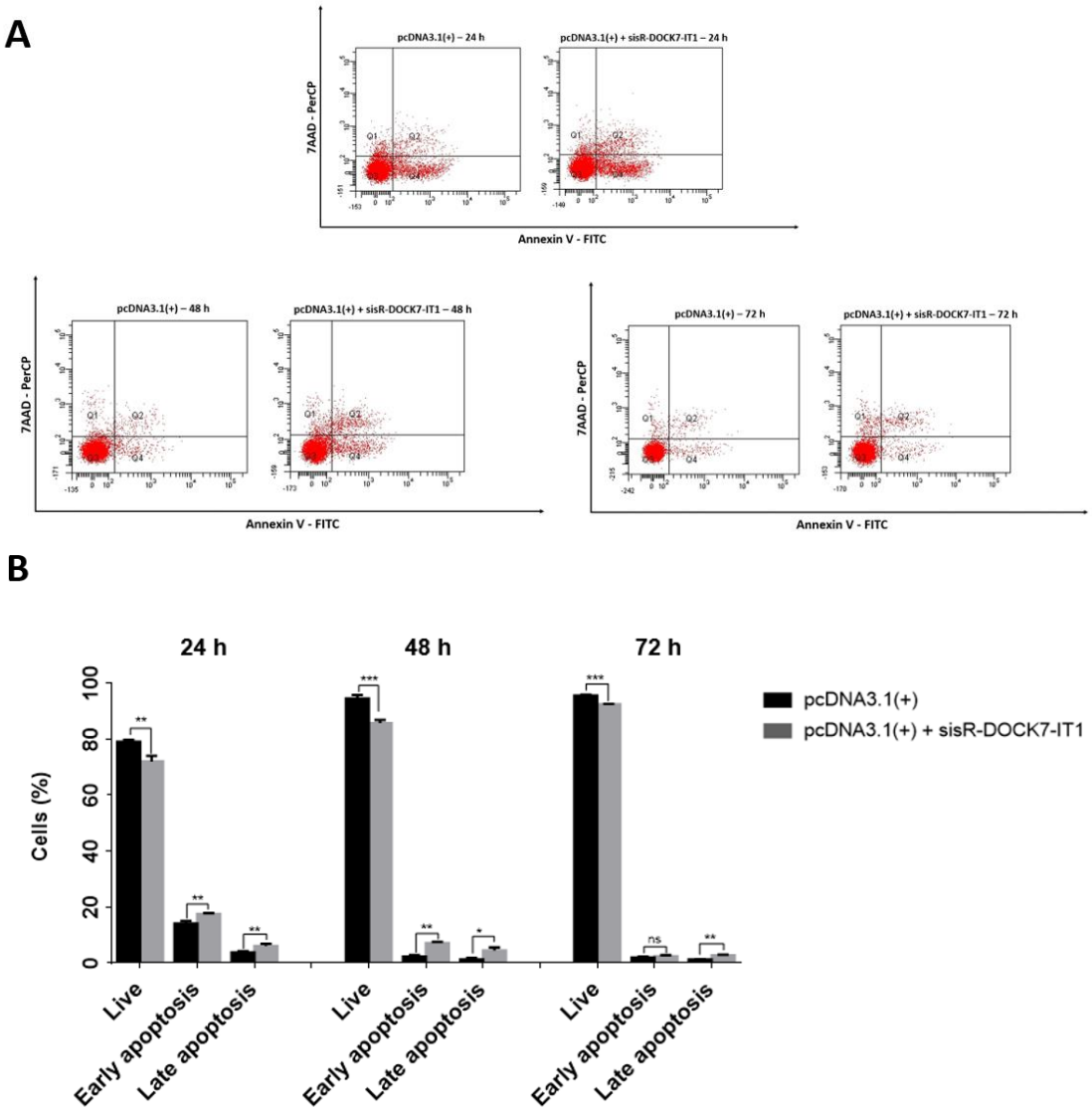

Figure 3.10. The apoptosis rate measurement of pcDNA3.1(+) + sisR-DOCK7-IT1 transfected cells for 24-, 48- and 72-hours of incubation by flow cytometry. A. Representative FACS profiles of pcDNA3.1(+) as control and pcDNA3.1(+) + sisR-DOCK7-IT1 transfected cells for 24-, 48- and 72-hours of incubation. AnnexinV-FITC/7AAD-PerCP staining was performed for detection of apoptosis rate. Q1 population (AnnexinV-/7AAD+) indicates dead cells, Q2 population (AnnexinV+/7AAD+) indicates late apoptotic cells, Q3 population (AnnexinV-/7AAD-) indicates live cells and Q4 population (AnnexinV+/7AAD-) indicates early apoptotic cells. B. The histogram of apoptosis rates of pcDNA3.1(+) + sisR-DOCK7-IT1 transfected cells for 24-, 48- and 72-hours of incubation. The histogram was drawn with mean ± SD of three independent experiments by GraphPad Prism 6.0. Statistical analyses were performed by applying student's t-test. (***P ≤ 0.001, ** P ≤ 0.01, * P≤ 0.05, ns: non-significant)

CHAPTER 4

DISCUSSION

sisRNAs are novel class of non-coding RNAs that were transcribed from introns (Chan and Pek 2019). Introns and intronic transcripts were neglected by many researchers for long years. It was believed that they were rapidly degraded after they were excised in pre-mRNA splicing. Some exceptions of intronic RNAs which are not degraded and are stable such as small nucleolar RNAs and microRNAs were reported in previous decades (Cech and Steitz 2014). sisRNAs were firstly recognized by the team of Joseph Gall in 2012. They realized that some intronic transcripts were unusually stable for 2 days and even when they perform Actinomycin D treatment or U2 depletion, their abundance was not affected (Gardner et al. 2012). Further studies in *Drosophila* revealed the existence of different forms of sisRNAs such as circular, lariat and linear. Circular and lariat forms of sisRNAs are generated by splicing-dependent mechanism. The interesting feature of linear sisRNAs, they can be transcribed through splicing-independent mechanism by using their own intronic promoter or using host gene's intronic promoter (Chan and Pek 2019; Jia Ng et al. 2018). Jun Wei Pek's team reported that some intronic transcripts were only detected in polyA+ fractions of total RNA and they had polyadenylation signal (PAS) near to 3' end of the transcript. To determine the intronic transcripts genome-wide in *Drosophila*, they performed deep sequencing of total RNA, polyA+ and polyA eliminated fractions. They screened all the intronic regions of genes visually on IGV after classic RNA-seq analysis. They listed the intronic regions that followed their sisRNA criteria, but it was important that all these transcripts do not overlap with the sequences of already annotated transcripts. Their findings and experiments showed that these RNAs detected in polyA+ fraction of total RNA with PAS are the linear sisRNAs which are transcribed by using their intronic promoter (Jia Ng et al. 2018). In our study, we aimed to identify the differentially expressed candidate linear sisRNAs under apoptotic conditions of HeLa cells. With this purpose, HeLa cells were treated with CP, TNF-alpha and DMSO and deep sequencing were performed by using their RNA in three fractions; total RNA, polyA+ RNA and polyA- eliminated RNA. Since human genome is larger

than *Drosophila*, the screening of whole intronic regions one by one visually is insurmountable method. In this study, we took the advantage of DE-Kupl algorithm. Classical RNA-seq analysis method is limited to give information about the transcripts that have already been annotated (Dobin et al. 2013). DE-kupl is a reference genome independent and a k-mer based method. DE-kupl creates 31 nt k-mers (31-mers) by partitioning 100 bp reads. Then, the 31-mers that show exact match with already annotated genes are masked, and sequencing errors are filtered. Differentially expressed k-mers based on control vs test conditions are listed and larger contigs that are extended by merging 31-mers were created. At the end of the analysis, the contigs are aligned to reference genome. Since DE-kupl masks the already annotated genes, it helps to identify new biologically meaningful transcripts such as sisRNAs (Audoux et al. 2017). The locations of contigs were searched on IGV instead of screening whole genome by eye. However, the key point is DE-kupl does not give the fold change of intronic transcript, it only gives the fold change of differentially expressed contigs. Contigs cover not whole intronic transcript but only a limited region to calculate a fold change. Even DE-kupl gives pre-liminary information about the expression levels of transcripts, their gene expression levels should be confirmed with qPCR. Usage of DE-kupl algorithm for identification of sisRNA is a new outstanding approach for widening our understanding. After completing the visual screening of locations indicated in DE-kupl analysis, total 48 and 33 candidates were selected from CP- and TNF-alpha treatment data, respectively. Most of the candidates were carrying the canonical PAS sequence and most of them were upregulated under drug/ligand treatment conditions. According to current knowledge about PAS sequence usage, 58.2% of mRNAs possess AAUAAA, 14.9 % of mRNAs possess AUUAAA hexamer as PAS and the rest of them have non-canonical signals (Beaudoing et al. 2000). Having a majority of sisRNA candidates with canonical PAS strengthens the evidence that they may have polyA tail.

Following the selection of candidates, firstly 3' RACE PCRs were performed to check whether they have polyA tail or not. 3' RACE PCR has simple logic. During preparation of cDNA, instead of usage of oligod$(T)_{18}$ or random hexamer, cDNA is converted with an oligodT primer that has an anchor sequence that helps to the label the transcripts with polyA tail. Following PCR reaction was performed with internal forward primer and anchor sequence primer. Even if it has simple logic, it was difficult to obtain a product at first and then obtaining a specific product. Since the sisRNAs are rare

transcripts, cDNA conversion by using total RNA was not an effective way. To provide enrichment of polyA+ transcripts, polyA+ RNA was used in cDNA conversion instead of total RNA. Usage of polyA+ RNA in cDNA conversion helped to get PCR product in PCR step but multiple products on agarose gel. As next optimization step, usage of Hot start DNA polymerase was preferred instead of standard DNA polymerase and this change improved the results to get specific product after PCR. By applying these changes, the 3' RACE PCRs of 5 candidate sisRNAs were completed and their 3' ends were established by this way.

5' RACE PCR was more complex than 3' RACE PCR. In cDNA conversion, it was not possible to use oligodT-Anchor primer since 5' end of transcript does not have polyA tail. Instead of oligodT-Anchor primer, cDNA conversion was performed by using gene specific reverse primer (GSRP1). After cDNA conversion, the clean-up of synthesized cDNA was required for next step of experiment. In next step, adenylation of 3' end of cDNA was performed by terminal transferase. However terminal transferase is an ultra-sensitive enzyme to any residual ethanol or salt remained during clean up. Trials to obtain the purest cDNA for successful adenylation reaction took a long time. Nevertheless, usage of new clean-up kit (Monarch PCR clean up, New England Biolabs) enabled us to get more efficient results. After that, adenylated cDNA was used for first PCR reaction with GSRP2 and oligodT-Anchor primer. In that step, the annealing temperatures of GSRP2 and oligodT-Anchor primer was quite different, and it was not possible to amplify specific product with classical PCR protocol. For this purpose, we improved touchdown PCR conditions (Figure 2.2). Touchdown PCR have different range of annealing temperature and cause the amplification region of interest. Since sisRNAs are not abundant, in first PCR conditions it was not possible to observe product on agarose gel because they were not amplified enough for visualization. Even no band of product is observed on agarose gel, touchdown PCR product can be used by diluting 1:10 in second PCR reaction by using Anchor primer and GSRP3. The PCR product obtained at last reaction was cloned into T-vector and according to the sequencing results 5' end of candidate can be established.

After 3' and 5' RACE PCRs, the transcriptional units of sisR-MXD4-IT3 and sisR-DOCK7-IT1 were determined. Since only the contig differential expression fold change was known from DE-kupl analysis, their gene expression levels were measured

by qPCR. Their pattern of regulation was proved, and fold changes were calculated by qPCR.

The full-length cDNA sequence of sisR-DOCK7-IT1 determined by RACE PCR was cloned into pcDNA3.1(+) mammalian expression vector. The overexpression study was conducted to determine the phenotypic effects of sisR-DOCK7-IT1 on HeLa cells. Since the optimal transfection time was unknown, the incubations times were adjusted as 24-, 48h-, and 72 hours after pcDNA3.1(+) + sisR-DOCK7-IT1 transfection. After transfection, replicates were split in two groups for total RNA isolation and apoptosis detection by AnnexinV/7AAD staining in flow cytometry. The overexpression levels of sisR-DOCK7-IT1 were 10,523.1, 7116.94 and 6.72 for 24-, 48- and 72-hours of incubation respectively. According to the apoptosis rate measurements by flow cytometry, the early apoptotic cells were increased by 3.5% in 24 hours of incubation. The early apoptosis cell percentage in 24 hours of incubation was higher when compared to 48 hours. Transfection of pcDNA3.1(+) may cause the cytotoxicity however the cytotoxic effect can be encountered by defence mechanism of cells and was overcome by time. Additionally, this may be caused by variation in seeded cell number. Because the population doubling time of HeLa cells is 42.6 h (Sato et al. 2016), they do not reach high confluency and low cell density may be the cause of the high percentage of early apoptotic cells in 24 hours. Therefore, the percentage of early apoptotic cells in 48 hours of incubation was quite low. The percentage of early apoptotic cells were increased 4.9% while late apoptotic cells were increased to 3.17% for 48 hours of incubation. In 72 hours of transfection, a dramatic decrease in gene expression was measured therefore any notable change in apoptosis rate was not measured, which may be attributed to transient transfection. Since the transient transfection has reduced potency at 72 hours, we may not be able to observe its effect. Taking into account of the qPCR results and phenotypic effects of transfection, 48 hours of incubation was selected as optimal time. To sum up, overexpression of sisR-DOCK7-IT1 in HeLa cells for 48 hours triggered apoptosis by 8.09% in total. The mechanism of induction of apoptosis via sisR-DOCK7-IT1 is still unknown but its overexpression caused significant change in apoptosis of HeLa cells.

CHAPTER 5

CONCLUSION

In this study 48 candidate sisRNAs upon CP-treatment and 33 candidate sisRNAs upon TNF-alpha treatment data were identified by taking advantage of DE-kupl algorithm. The majority of candidate sisRNAs were upregulated upon drug/ligand treatment. 5 sisRNA candidates were selected for 5'/ 3' RACE PCR. 3' ends of sisR-MXD4-IT3, sisR-SSH2-IT1, sisR-DOCK7-IT1, sisR-PLPBP-IT4, sisR-C11orf24-IT2 were established by 3' RACE PCR. The transcriptional units of sisR-MXD4-IT3 and sisR-DOCK7-IT1 were determined after completion of the 5' RACE PCR. The gene expression levels of sisR-MXD4-IT3 and sisR-DOCK7-IT1 were measured by qPCR. sisR-MXD4-IT3 had decreased gene regulation pattern in both drug/ligands treated cells (CP; 2.77-fold, TNF-alpha; 3.03-fold). On the other hand, sisR-DOCK7-IT1 had increased gene regulation pattern in CP-treated cells (4.27-fold), while it was downregulating in TNF-alpha-treated cells (4.16-fold). Overexpression studies were performed to elucidate the phenotypic effect of sisR-DOCK7-IT1 on HeLa cells. HeLa cells were incubated for 24-, 48- and 72-hours after transfection of pcDNA3.1(+) + sisR-DOCK7-IT1. According to the results the optimal time for overexpression of sisR-DOCK7-IT1 was 48-hours. After 48 hours of incubation, the sisR-DOCK7-IT1 levels increased 7116.94-fold and 8.09% increase in total apoptosis were measured by flow cytometry.

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