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The effect of TRAP150 on HPV16 E1 gene regulation

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Civilingenjörsprogrammet i molekylär bioteknik

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Abstract

Human papilloma virus (HPV) is a small DNA virus that is known as the causative agent in cervical cancer. The virus is found worldwide and causes more than 300 000 deaths yearly. Although there are many types of HPV, HPV16 is found in 99 % of cervical cancer cases. There are several vaccines available but as of now there is no antiviral treatment for the disease. Thus, it is further studies are required to find potential targets or biomarker which can be used to develop new treatments.

The viral genome is divided into early and late genes. Where the early genes are active during the early stage of infection and the late genes are upregulated at the late stage of infection. The early (E) genes E6 and E7 are oncogenic. E6 prevents the cells from going into apoptosis and E7 drives proliferation. The E1 and E2 genes are involved in the replication of the viral genome

The HPV16 genome is polycistronic, meaning that the mRNAs that the virus produces encodes for several proteins. Gene regulation is thus very important for the virus. The HPV only have two promoters which means that it has to utilize other methods to control its gene expression. It uses alternative splicing to control the expression of its genes. Using different splice sites the virus can produces many different pre-mRNAs. The splice sites are in turn controlled by regulatory elements called enhancers and silencers. These elements acts as binding places for cis-acting elements (RNA) and trans-acting factors (protein).

This report focuses on the regulation of the E1 gene. It investigates whether the trans-acting factor TRAP150 affects the regulation of the E1 gene. Using reporter plasmids to with a reporter Luciferase gene the expression of the gene can be studied.

The results indicate that TRAP150 does induce splicing of E1, although it is not clear how it does this. It also seems that the splicing variant E1C upregulated by the presence of TRAP150. Using deletion mutants to study the TRAP150 interaction, it was also found that the N-terminal is essential for splicing to happen. This also concurs with previously reported results.

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Populärvetenskaplig sammanfattning

Humant papillomvirus (HPV) är ett virus som kan infektera oss människor och vid långvarig infektion orsakar cancer. Viruset är mest känt som orsaken till livmoderhalscancer och ligger bakom upp till 99 procent av cancerfallen. Trots att det nu finns flera vaccin som skyddar mot viruset i hög grad, finns det ännu inte något läkemedel att använda i de fall som cancer uppstått. Den vanligaste behandlingsformen då är att operera och avlägsna cancercellerna (1177 2020). Att operera bort cellförändringarna är en effektiv behandling men en opraktisk procedur, speciellt i länder som inte har ett lika välutvecklat sjukvårdsystem som här i Sverige. Därför bedrivs forskning för att kunna framställa ett läkemedel som förhindrar att viruset förökar och på så sätt förhindrar cancerns framväxt. För att kunna göra detta måste forskarvärlden bättre förstå sig på hur viruset förökar sig och även vilka steg som är viktiga när viruset har infekterat en cell samt vilka komponenter som påverkar dessa processer.

Viruset är förhållandevis litet och har endast möjlighet att producera åtta stycken proteiner. Detta innebär att viruset inte har förmåga att föröka sig själv utan är beroende av cellens egna maskineri. Eftersom att viruset endast producerar åtta proteiner är generna som producerar dessa essentiella för att viruset ska överleva. Generna kan delas upp i två grupper så kallade tidiga och sena gener, dvs gener som är aktiva under det tidiga stadiet av infektionen och gener som är aktiva under den senare delen av infektionen. De tidiga generna tillverkar proteiner som utnyttjar cellens egna processer för att tillverka nya kopior av sin arvsmassa. De tidiga generna producerar även proteiner som gör att cellen inte dör, vilket annars är ett sätt som celler skyddar sig mot virusinfektioner, och ett protein som får cellen dela sig med en högre frekvens än vanligt. De sena generna producerar proteiner som går till det skyddande proteinhöljet (kapseln) som behövs för att viruset ska kunna infektera nya celler.

Det ovanliga med HPVs gener är att de överlappar, det vill säga att från samma DNA sträcka kan man få flera proteiner. Det betyder att från samma mall klipper och klistrar viruset så att det i slutändan kan producera olika proteiner. Forskare har viss insyn i hur detta fungerar men har ännu inte hittat ett sätt att blocka dessa processer så pass att det kan användas för att utveckla läkemedel som kan användas i kampen mot cancer. Detta projekt fokuserar på en av HPVs gener och om ett protein, kallat TRAP150, kan påverka virusets förmåga att klippa i genen.

Innehållsförteckning

Abbreviations

HPV	*Human papillomavirus*
TRAP150	*Thyroid Hormone Receptor Associated Protein 3*
SD	*Splice donor*
SA	*Splice acceptor*
nLuc	*Nano-Luciferase*
ORF	*Open reading frame*
wt	*Wild type*
Rb	*Retinoblastoma*
CDS	*Protein coding sequence*
ORF	*Open reading frame*
pAE	*Early polyadenylation site*
pAL	*Late polyadenylation site*
RBP	*RNA binding proteins*
hnRNPs	*Heterogeneous nuclear ribonucleoprotein*
EJC	*Exon junction complex*
CMV	*Cytomegalovirus*
DMEM	*Dulbeccos's Modified Eagle Medium*
Wb	*Western blot*
RIPA	*Radioimmunoprecipitation assay*
NMD	*Nonsense-mediated decay*

1 Introduction

Human papillomavirus (HPV) is a virus that is commonly known for causing cervical cancer. According to the World Health Organization (WHO), cervical cancer is the fourth most common cancer in women with over 600 000 new cases and 340 000 deaths yearly. HPV16 and HPV18 are so-called high-risk viruses and are responsible for about 70% of HPV-linked cancer cases (World Health Organization 2022). Of these two virus types, HPV16 is the most common (Orrù *et al.* 2012). There are several vaccines available that provide good protection against both HPV16 and HPV18 as well as several other types. In unvaccinated individuals, most HPV infections clear out spontaneously within a year, but in cases where the infection is sustained, there is a risk of cervical cancer development. These cases require continuous screenings to monitor the progression of the infection. This allows for the identification of pre-cancerous lesions. Most HPV-associated deaths occur in lower-income countries where there are no widespread vaccination campaigns and less access to preventative care (World Health Organization 2022). The most common treatment for HPV is to surgically remove the cancerous cells. Therefore there is a need for the development of an antiviral treatment that can be administered to patients non-invasively.

This project focuses on details of how HPV16 E1 gene expression is regulated. More precisely how does the Thyroid Hormone Receptor Associated Protein 3 (THRAP3, also known as TRAP150) controls post-transcriptional modifications of the E1 pre-mRNA. Previous studies in the Schwartz group have indicated that the TRAP150 protein has a regulatory effect on the splicing of HPV16 oncogenes E6 and E7 mRNA (unpublished data in Stefan Schwartz group). Overexpression of TRAP150 showed that splicing between splice donor (SD) 226 and splice acceptor (SA) 409 increased the generation of the E7 mRNA. As E7 is one of the oncogenic genes, it offers a potential target for therapy. TRAP150 also induces alternative splicing of HPV16 E2 pre-mRNA, referred to as E1C mRNA. The function of E1C is currently unknown. However, high levels of E1C mRNA and E6*I mRNA are used to diagnose cervical lesions with an elevated risk of progressing to cervical cancer (Höfler *et al.* 2015). Studies indicate that high levels of E1C mRNA and E6*I mRNA may be linked to elevated levels of TRAP150, therefore TRAP150 could be a potential biomarker or therapeutic target for pre-cancerous lesions and/or cervical cancer (Höfler *et al.* 2015).

1.1 HPV life cycle

HPV is a small DNA virus with a genome size of about 8kb and with a tropism for mucosal cells. It infects basal cells in the cervical epithelium, thereby gaining access to actively dividing cells. The virus binds to HPV receptors on the cell membrane of the basal keratinocyte, and after entry, the virus is transported into the nucleus (Kajitani & Schwartz 2020). HPV is strictly epitheliotropic which means that is fully dependent on the cell's

replication machinery to reproduce. HPVs life cycle is tied to the differentiation of the keratinocyte which it has infected. In basal cells, a small number (50-100) of copies are produced during the very early stage of infection. The virus then enters the maintenance stage, where the genome is amplified, and the early genes are expressed (Nilsson *et al.* 2018). The early stage of infection is identified by gene expression controlled by the early promoter and the early polyadenylation signal. This promotor has the ability to generate most of the early transcripts (Wu *et al.* 2017). As the infection progresses, the expression of some genes is downregulated so the late stage can progress (Figure 1).

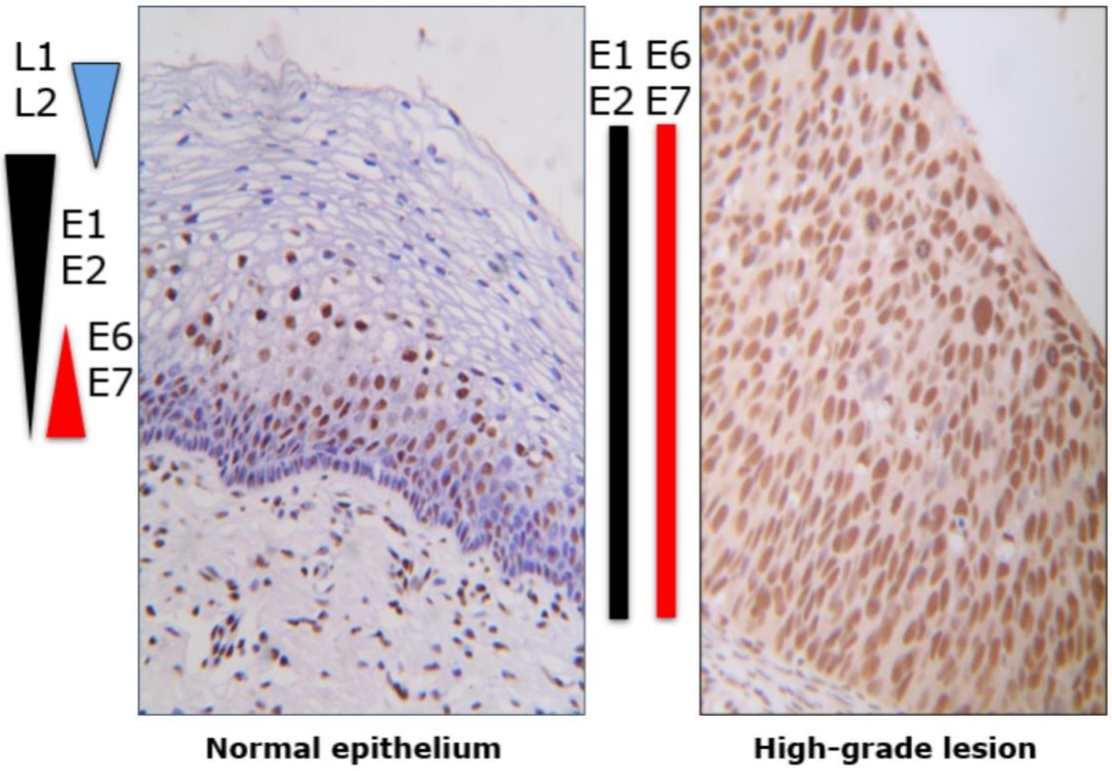

Figure 1. *Regulation of the HPV16 genes during the different stages of infection. A wider triangle indicates that the expression of the gene is high, and as the expression drops, the tringle thins out. Several of the HPV16 genes need to be expressed at the same level, which is also indicated by the triangles. The left picture shows normal uninfected basal epithelial cells, whilst the right picture shows epithelial cells which are infected by HPV16. Figure from Naoko Kajitani*

As the HPV16 genome encodes for a few genes (Figure 2), these proteins have distinct functions and are essential for the viral life cycle. The E8 gene has been linked to the control of the viral DNA copy number during the early stages of infection (Cerasuolo *et al.* 2020). The E1 and E2 proteins are essential in the initial replication and maintenance of the viral genome. E1 has helicase activity which opens up the DNA at the origin of replication (*ori*). E2 then tethers itself to E1 and positions it on the HPV genome and recruits the host's replication machinery. E2 also plays a part in partitioning, transcription, and polyadenylation (Wu *et al.* 2017, Nilsson *et al.* 2018).

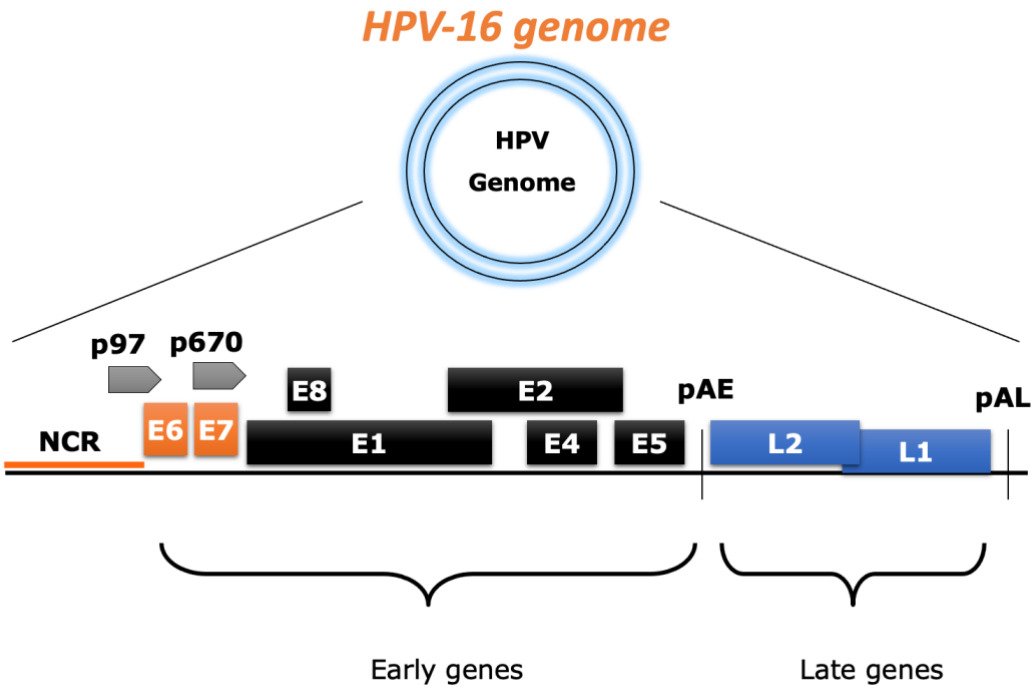

Figure 2. *A schematic representation of the HPV16 genome. Upstream of the early genes the none coding region (NCR) is located. There are two promoter, p97 and p670, that are located amongst the early genes. p97 regulates the transcription of the early genes and p670 regulates transcription of the E1, E2, E4, E5, E8 and the late genes. The two polyadenylation sites, pAE and pAL, are also included. The oncogenes E6 and E7 are represented in orange whilst the rest of the early genes are represented in black. The viral capsid genes, L1 and L2, are blue. Note that E8 is not represented here but is located within the E1 gene. Figure from Naoko Kajitani*

During cell division, the HPV DNA is distributed into both the daughter and mother cells. The daughter cell is not attached to the epithelial membrane but rather moves toward the upper layers of the epithelium. As this happens, DNA synthesis starts to shut down, affecting the viral replication (Kajitani & Schwartz 2020). In response to this, the HPV16 E7 protein binds to proteins of the retinoblastoma (Rb) family (Nilsson *et al.* 2018). The Rb family consists of regulatory proteins which regulate entry into the S-phase of the cell cycle. They do this by binding to the E2F transcription factor, and thus, when E7 binds to Rb proteins it allows free E2F to accumulate in the cell and pushing it into S-phase (Henley & Dick 2012, Nilsson *et al.* 2018). This allows the virus to regain access to cell's replication machinery. The natural response to the re-entry of the cell cycle is to induce apoptosis, but this is prevented by E6. The E6 protein binds to p53 and degrades it, and blocks the p53-mediated apoptosis (Nilsson *et al.* 2018).

During the late stage of infection, the amount of the E2 protein in the cells increases (Figure 1). This promotes E2 binding to the early promotor, p97, thereby effectively shutting down the production of E6 and E7 (Figure 2). This then allows the cells to resume normal differentiation processes, allowing them to determinably differentiate. The terminal differentiation is necessary as it induces the late promotor, p670, which allows for the

transcription of E1, E2, E4 and E5 (Figure 2) (Wu *et al.* 2017, Kajitani & Schwartz 2020). The function of E4 and E5 is not known, but it has been suggested that high levels of E4 are necessary for the production of L1 and L2 as well as for the release of novel virus (Johansson & Schwartz 2013, Wu *et al.* 2017). E5 has been linked to the virus ability to avoid the host immune system by affecting the endocytic trafficking and increasing the ability for reproduction (Kajitani & Schwartz 2020). As soon as the cell enters the final differentiation steps, the late promotor becomes active (Figure 1). L1 and L2 are structural proteins that make up the viral capsid. These proteins have a strong immunological effect which may be why it is essential that they are repressed until the host cell reaches the upper epithelium (Orrù *et al.* 2012).

1.2 HPV gene regulation

In order to produce a functional protein, introns need to be removed from the pre-mRNA and exons need to be joined. In HPV this also includes the removal of certain exons to generate alternatively spliced mature mRNAs. The HPV genome differs from the human genome as the exons are long and frequent. In most cells, exons are rare and about 160 bp in length, meaning cellular pre-mRNA consists mainly of introns (around 6938 bp on average) (Kajitani & Schwartz 2020). This makes identification of exons amongst the introns challenging. However, this is not the case with HPV, as up to 92 % of the genome consists of protein-coding sequences (CDSs). The HPV16 genome consists mainly of open reading frames (ORF) and CDSs, of which several are intersecting (Kajitani & Schwartz 2020).

HPV16 has two major promoters; the early promotor p96 and the late promotor p670. The virus contains also w two polyadenylation sites; pAE (early) and pAL (late) (Figure 2). As the name indicate the early promotor and polyadenylation sites are active during the early stages of infection. The late equivalents are active during the late stages of infection. The early promotor regulates the expression of the early genes, although E1, E2, and E4 can be expressed from mRNA generated by the pAL. The late promotor becomes active when the cell has undergone differentiation (Nilsson *et al.* 2018). A third promoter located in the open reading frame (ORF) of E8 was found recently and seems to regulate the production of E8 (Cerasuolo *et al.* 2020). Gene regulation is crucial for any organism and since HPV has relatively few promoters it is limited in how it controls it genes. HPV16 transcripts are polycistronic meaning that are several genes encoded in one mRNA. To produce the different mRNAs the virus uses alternative splicing (Kajitani & Schwartz 2020). The different splicing alternatives are shown in Figure 3.

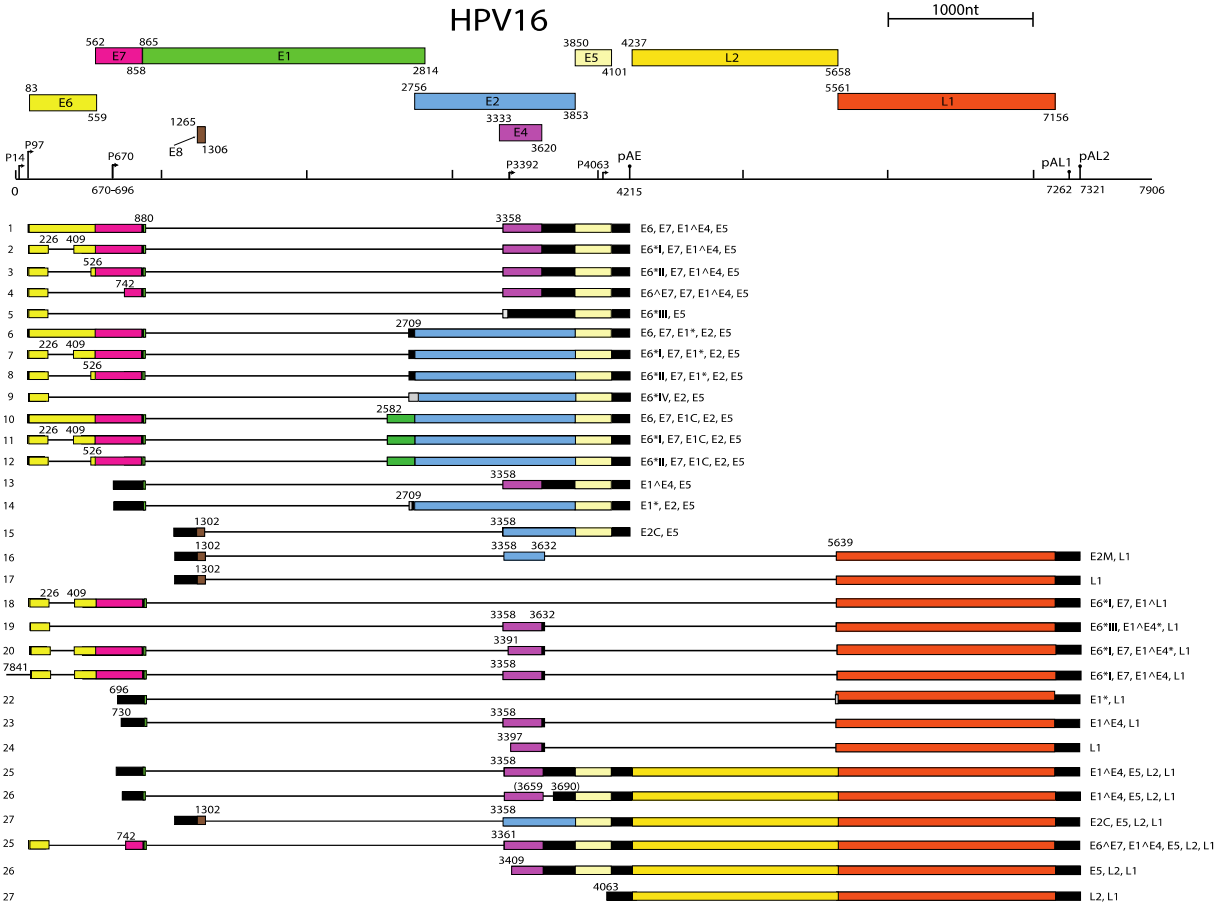

Figure 3. *A schematic drawing of the HPV16 genome and splicing alternates it can produce. At the top is the HPV16 genome with the promotors and polyadenylation sites indicated. Each gene is marked with its first and last nucleotide. Below, the different splicing alternatives are shown. The SD (splicing donor) and SA (splicing acceptor) for each mRNA are written at the end of each block. Note that the SD's and SA's are indicated only by their number. The straight lines are areas of the mRNA that have been cut out. To the right at each row which splice alternatives that have been produced are stated. Figure by Naoko Kajitani.*

Splicing regulation is of utmost importance for maintaining the viral life cycle. Several factors influence the splicing of an mRNA, like the strength of the splice site and polyA signal, the presence of cis-acting elements, and the amount of trans-acting factors (Kajitani & Schwartz 2020). A strong splice site is defined by a consensus sequences recognized by the spliceosome. The consensus sequence for a canonical 5'-splice site is (C/A)AG_**GU**(A/G)AGU and for a 3'-splice site (C/U)nX(C/U)**AG**_(A/G) (Kajitani & Schwartz 2020). There is no consensus sequence for weaker splice sites; hence, the splicing events depend on how well various RNA binding proteins (RBPs) bind to these weaker spice sites. The RBPs interact with enhancer elements located close to the splice site. In HPV16 none of the splice sites have consensus signals and are thus suboptimal (Kajitani & Schwartz 2020). However, most HPV16 splice sites have short sequences of C/U intersected by A/G, with SA2709 and SA5639 being the exception. This indicates that the strength of the splice acceptor does not affect the efficiency of the splice site and that enhancers and silencers are

greatly involved in the control HPV16 gene expression. Even if the splice sites have various efficiency, none are utilized to their maximum potential as they compete with each other (Kajitani & Schwartz 2020).

The splice sites in HPV16 are regulated to respond to the differentiation steps that the cell undergoes during the HPV infection cycle. Changes to the splice site efficiency will alter the usage of the other splice sites. This will result in changes in the levels of the produced mRNAs that need to be synchronized for the virus life cycle to progress. Therefore, the virus needs to control the activity of its various splice sites during the entire infection (Kajitani & Schwartz 2020).

There are at least four known splice donors (SD) in the HPV16 genome SD226, SD880, SD1302 and SD3632 and seven splice acceptors (SA) SA409, SA526, SA742, SA2582, SA2709, SA3358, and SA5639. For splicing of early mRNAs SD226, SA409, SA742, SD880, SD1302, SA2582, SA2709, and SA3358 are used. For splicing of late mRNAs SD880, SD1302, SA2582, SA2709, SA3358, SD3632, and SA5639 are used (Figure 3) (Kajitani & Schwartz 2020). Which splice sites (SD vs. SA) is used depends on regulatory elements within the pre-mRNA called splicing enhancers and silencers. These regulatory elements are recognized by the trans-acting factors (proteins), which can activate (splicing activators) or repress (splicing repressors) splicing reactions. Typical splicing activators are so-called SR proteins containing serine (S) and arginine (R) amino acid stretches (so-called RS-domain) (Shepard & Hertel 2009). The SR proteins bind to pre-mRNA and modulate splicing reactions by protein-protein contacts occurring via the RS domain. Similarly, the splicing repressors bind to pre-mRNA, but instead of activating, they form protein-protein interactions which block spliceosome function (Shepard & Hertel 2009). Most of the splicing repressors belong to the heterogeneous nuclear ribonucleoprotein (hnRNP) family of proteins. SA3358 and SA5639, and SD3632 are hot spots for regulatory protein, which supports the possibility that each splice site is regulated by several factors (Kajitani & Schwartz 2020).

The E1C mRNA is spliced using the SD880 and the SA2582. This spliced mature mRNA is believed to generate a high level of protein although its function is not well understood. However, the function of this protein has remained enigmatic. Notably, Höfler *et al.* have proposed that high levels of E1C mRNA relative to the levels of L1 mRNA could be used as a biomarker for high-grade lesions (Höfler *et al.* 2015).

1.3 TRAP150

Not much is known about the exact function of the TRAP150 protein, and there is also not solved structure for the protein. The C-terminal is about 50 % homologous to the transcriptional repressor BCLAF1, and it has an arginine-serine (RS) rich region at the N-terminal (Lee *et al.* 2010). Both TRAP150 and its homolog BCLAF1 have been found to interact with the splicing machinery, which indicates that they have an effect on pre-mRNA

splicing. However, it is unclear how it exactly functions during splicing reaction (Lee *et al.* 2010). Within the BCLAF1 homologous region, there is a smaller region (~ 90 amino acids) that is homologous to the exon junction complex (EJC) component MLN51 (Lee & Tarn 2012).

TRAP150 seems to be a multifunctional protein as it has been identified as a component of the spliceosome and the transcription mediator complex TRAP/Mediator. It has also been found to associate with the mRNA export factor TAP, suggesting it may have a role in post-splicing events (Lee *et al.* 2010). Lee *et al.* also found that the protein appears in nuclear speckles, assisting in pre-mRNA splicing. After splicing, TRAP150 remained associated with the mRNA and facilitated interactions with the EJC (Lee *et al.* 2010). It is possible that the TRAP150 RS domain could interact with other SR proteins or export factors and, via them, interact with the EJC and nuclear export complexes (Varia *et al.* 2013). Although the EJC is associated with spliced transcripts, many transport proteins do not, which seems to contradict this theory. Instead, it has been suggested that TRAP150 could affect the stability of the interaction between EJC and the mRNA as well as regulate the degradation of the mRNA (Varia *et al.* 2013). Lee *et al.* found that TRAP150 interacted with the EJC and factors involved nonsense-mediated decay (NMD), which further points to TRAP150 having a role in mRNA decay. They found that the MLN51 region at the C-terminal was involved in the degradational activity (Lee & Tarn 2014). By making truncated versions of the protein Lee & Tarn also found that the N-terminal, with the RS-domain, was necessary for pre-mRNA splicing. As TRAP150 is known to associate with the export factor TAP, TRAP150 could act as an adaptor to TAP and promote the recruitment of mRNA to the nuclear pore complex for export to the cytoplasm. Results from Lee *et al.* suggest that TAP and TRAP150 interact directly. When knocking down TRAP150, apparent differences in mRNA dispersal were noted, which further supports its participation in nuclear export and mRNA stability (Varia *et al.* 2013).

A previous study from another Lee & Tarn has shown that the N-terminal deletion of TRAP150, ΔN190, but not C-terminal deletion ΔC359 is critical for pre-mRNA splicing (Lee *et al.* 2010). To understand the molecular mechanism of how TRAP150 regulates HPV16 mRNA splicing, for example, through the interaction of RNA-protein or that protein-protein, we aim to determine functional differences of TRAP150 mutants on the HPV16 mRNA splicing enhancement. Therefore, TRAP150 deletion mutants, ΔN190 and ΔC359, encoding plasmids were constructed according to the previous study (Lee *et al.* 2010).

2 Material and methods

2.1 pE1nLuc

In this project, several plasmids are used, with the main reporter plasmid being pE1nLuc. This plasmid was constructed in-house, encoding the whole E1 protein, which is neighboring a Nano-Luciferase (nLuc) gene and controlled by the cytomegalovirus (CMV) promoter. It is used to study which of the splicing alternatives, E1 and E1C, is produced by the changes in luciferase expression (see Figure 4). The start codon for nLuc and the stop codon for E1 have been removed. E1 contains SD880, SA2582, and SA2709 with 880^2709 being the favored splice site. As a result, the splicing event leads to an out-of-frame CDS with the nLuc gene, consequently rendering the nLuc signal inactive under normal conditions. The other splicing alternatives are in frame with nLuc, meaning luciferase will be expressed. This can then be detected in several ways, including Nano-Luciferase assays and Western blot (wb).

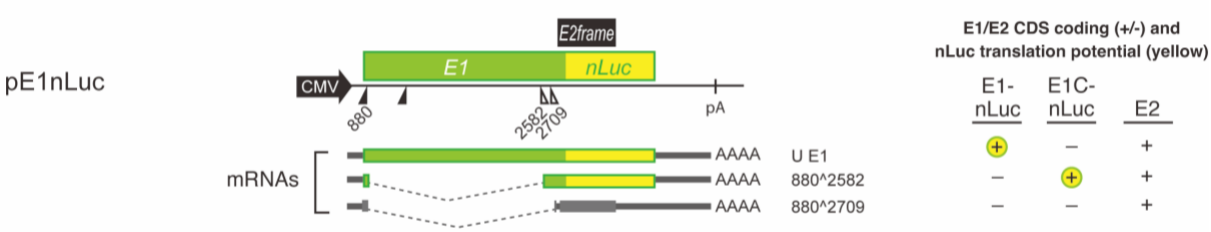

Figure 4. *A schematic illustration of the pE1nLuc plasmid shows the different splicing alternatives. The spliced mRNAs that produce Nano-Luciferase (nLuc) is shown on the right side. Figure from Naoko Kajitani*

My host, Prof. Schwartz group, constructed the pE1-nLuc plasmid. To construct the plasmid, the HPV16 E1 gene was amplified using PCR from pBR322-HPV16 (kindly provided by Dr. Ola Forslund) and the nLuc gene was amplified from pNL1.1 (purchased at Promega) was inserted between Cla I/BamH I or BamH I/Xho I sites, respectively, in CMV-promoter driven empty vector, pCL086 (Collier *et al.* 2002).

2.2 pE6E7nLuc

The E6E7nLuc plasmid encodes for the HPV16 genes E6 and E7 flanked by the nLuc reporter gene. The plasmid is driven by a CMV promoter, and the E7 stop codon and the nLuc start codon have been removed. The E7 gene is fused to the nLuc gene. The E6E7 region contains SD226, SA409, SA526, and SA742, which generates intron-retained E6-, 226^409-spliced, 226^526-spliced or 226^742-spliced mRNAs (see Figure 5). Among them, 226^409-spliced (also known as E6*I) mRNA has been believed to contribute to the E7 protein expression even though intron-retained E6 mRNAs also contain E7 CDS (Tang *et al.* 2006). The plasmid is used to determine the expression levels of E6 and E7 by measuring the nLuc signal.

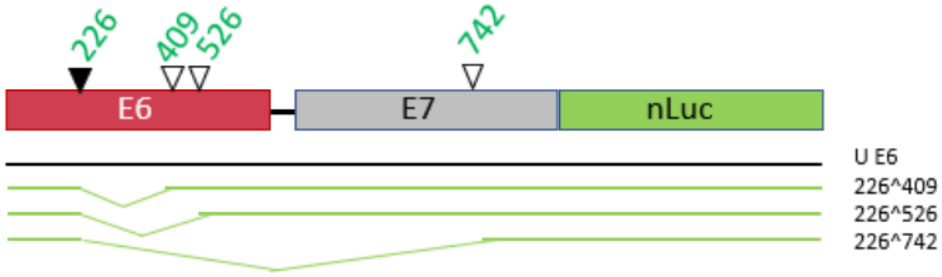

Figure 5. *A schematic illustration of the pE6E7nLuc plasmid shows the different splicing alternatives. The SD is indicated by a black triangle and the SA with white triangles. If the spliced mRNA is in frame with nLuc the mRNA is presented as green. Figure from Naoko Kajitani*

The plasmid was constructed in Schwartz group (unpublished). Briefly, the HPV16 E6E7 gene region that was PCR-amplified from pBR322-HPV16 (kindly provided by Dr. Ola Forslund) was replaced to the HPV16 E1 gene in pE1nLuc (described above) between Sal I/BamH I sites.

2.3 TRAP150 plasmids

The pcDNA3-TRAP150-Flag was kindly gifted from Dr. W.Y. Tarn (Lee & Tarn 2014). The plasmid contains the entire TRAP150 coding sequence fused to a Flag-tag. The latter enables TRAP150 detection by western blotting.

The pcDNA3-TRAP150-dN190-Flag and pcDNA3-TRAP150-dC359-Flag plasmids were constructed to express an N-terminal 190 amino acid or C-terminal 359 amino acid deletion mutants of TRAP150, respectively. They were created via Phusion site directed mutagenesis using the pcDNA3-TRAP150-Flag plasmid as a template (unpublished). Both of the deletion mutants are fused to the C-terminal Flag-tag (Figure 6).

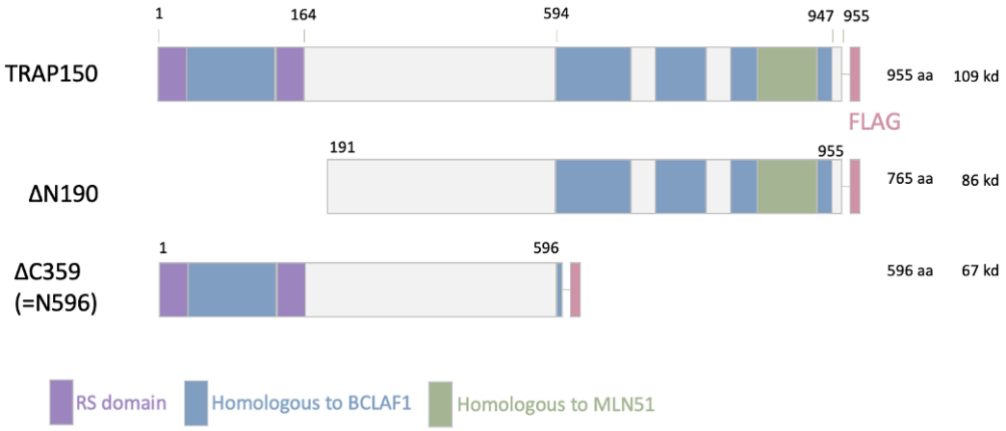

Figure 6. *A schematic illustration of the TRAP150 region for the plasmids pTRAP150-Flag, pTRAP150-dN190-Flag and pTRAP150-dC359-Flag. For the TRAP150-Flag plasmid the entire TRAP150 gene was conserved and*

fused to a Flag-tag at the C-terminal. For the pTRAP150-dN190-Flag the first 190 amino acids were removed and for pTRAP150-dC359-Flag the last 359 aa were deleted. The amino acid (aa) number and expected molecular weight (kd) for each protein is specified. Figure from Naoko Kajitani

2.4 Culturing and splitting HeLa cells

The HeLa cell culture was started from a culture provided in the lab. The cells were then split according to the protocol found in Appendix 8.1.1. The cells were split into different fractions depending on when the experiment was planned. Before each experiment, the condition of the cells was checked using the microscope. The cells were grown in Dulbeccos's Modified Eagle Medium (DMEM, Cytiva), if the cells were not use within two days, the media was changed. The cells were kept in an incubator at +37°C between experiments. At the start of each experiment, the cells were counted and diluted to the concentration required for each experiment. The cells were then seeded in an appropriate plate and incubated until the cells were 70-80 %confluent.

2.5 Purification of plasmids

The plasmids were purified using the NucleoBond™ Xtra Midi Plus from Macherey-Nagel™. The protocol can be found in Appendix 8.1.2. After purification, the plasmids were diluted with Ambion™ DEPC – Treated water by ThermoFisher™ and the concentration was determined using a NanoDrop™ One/One^{C} Microvolume UV-Vis Spectrophotometer from ThermoFisher™.

2.6 Transfection and Luciferase assay

Several transfections were carried out during the project. HeLa cells were transfected using Turbofect according to the manufacturer's protocol (Thermo Fisher Scientific). A detailed protocol and the exact experimental setups can be found in Appendix 8.1.3 . 4*10^5 HeLa cells per well were seeded and incubated until the cells were 70-80% confluent. In short, a mixture of Turbofect and DNA was incubated for 20 min at room temperature and then added dropwise to the HeLa cells. The total amount of DNA used for transfection was always 400 ng. Twenty-four hours after transfection the cells were lysed using 5x Lysis buffer. The samples were then collected, and a mixture of cell lysate and Nano-Glo were analysed. For each sample, three biological replicates were used, and two technical replicates per biological replicate were used during analysis.

2.7 RNA extraction and RT-PCR

RNA extraction was performed according to the protocol in Appendix (8.1.4). Briefly, HeLa cells were cotransfected using Turbofect (like above), amount of DNA used for transfection was 400 ng. Four hours after transfection the media (DMEM) was changed as the high

concentration of DNA can be toxic to the cells. After 24 hours, the cells were lysed using TRI reagent according to protocol. Reverse transcription as performed on the sample using random primers, the cDNA was then amplified using PCR. The PCR product was run on a electrophoresis gel and analysed.

2.8 Construction of new TRAP150 deletion plasmids construction

New deletion plasmids were constructed using Phusion Site-Directed Mutagenesis Kit from ThermoFisher™. The TRAP150-Flag plasmid as a template. Using phosphorylated primers different sizes of the template was amplified. Thus, the new fragments will lack some of the TRAP150-Flag plasmid. To confirm that the fragments were of the right size they were run on an electrophoresis gel. The fragment is then amplified using PCR and then ligated using T4 DNA ligase. The plasmids were subsequently transformed into competent DH5α cells. Following incubation, the cells were streaked on ampicillin agar plates and incubated at 37°C for 24 hours. To confirm that the colonies contained the right fragment, colony-PCR was performed. The colonies confirmed to contain the right fragment were incubated in LB with ampicillin overnight. To purify the plasmids, the NucleoBond™ Xtra Mini Plus from Macherey-Nagel™ was used. The concentration was determined using a NanoDrop™ One/One^{C} Microvolume UV-Vis Spectrophotometer from ThermoFisher™. The plasmids were then sent for sequencing at BioFisher. To confirm that the sequenced fragment matched the desired sequence, the sequence was examined using BLAST ®. The sequenced fragment was aligned against the whole TRAP150 gene. When the sequence was confirmed, the plasmids were then diluted to the desired concentration using Ambion™ DEPC–treated water by ThermoFisher™. The concentration was determined using a NanoDrop™ One/One^{C} Microvolume UV-Vis Spectrophotometer from ThermoFisher™. A complete protocol can be found in Appendix 8.1.5.

2.9 Western blot

Western blot was performed according to the protocol found in Appendix 8.1.6. HeLa cells were cotransfected with Turbofect according to the manufacturer's protocol (Thermo Fisher Scientific). Four hours after transfection the media (DMEM) was changed as the high concentration of DNA can be toxic to the cells. After 24 hours, cell lysate was collected by suspending HeLA in radioimmunoprecipitation assay (RIPA) buffer. After SDS-PAGE, the proteins were transferred nitrocellulose membranes, blocked in PBS containing 0.2% Tween 20 and stained with specific primary antibodies (Appendix 8.1.6).

3 Results

3.1 TRAP150 induces splicing in E1

To see if TRAP150 could induce splicing of E1, pE1nLuc was cotransfected with wild-type TRAP150 and two deletion mutants of TRAP150 (dC359 and dN190) into HeLa cells. If TRAP150 could induce splicing of the E1 pre-mRNA, the nLuc gene will be expressed. Thus, incubating cell lysates with Nano-Glo reagent would produce a luminescence signal, which can be recorded using luminometer.

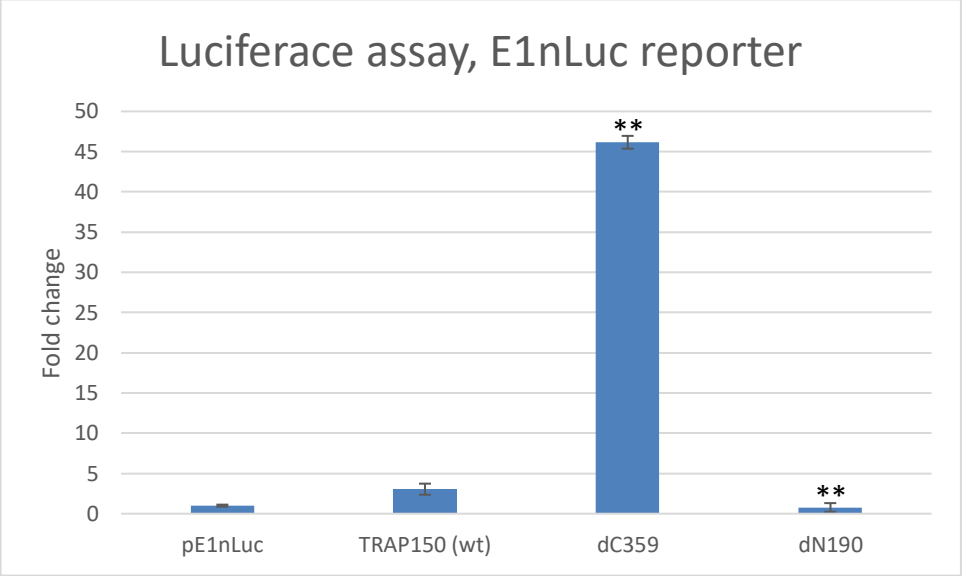

Figure 7. *Results from a Luciferase (nLuc) assay using pE1nLuc as a reporter gene. Data shown as fold change (pE1nLuc = 1), with standard deviation. *; P <0.05, **; P<0.01*

As shown in Figure 7, transfection of the TRAP150(dC359) plasmid induced high nLuc luminescence, while the dN190 mutant reduced the amount of luminescence. Unsurprisingly, the dN190 mutant was not very effective at inducing mRNA splicing (= high nLuc signal as it lacks the RNA-binding domains (RS domain, Figure 6) and hence cannot regulate splicing. However, the fact that the dC359 mutant had about 40 times stronger nLuc induction than the wild-type counterpart was unexpected.

The luciferase assay cannot reveal which HPV16 E1 alternative splicing isoform is induced by TRAP150 as both the unspliced mRNA and SD280^2582 can cause nLuc expression (Figure 4). Therefore qualitative studies like western blot and RNA extraction and analysis are required.

HeLa cells were cotransfected with pE1nLuc, TRAP150(wt) and the TRAP150 deletion mutants for the RNA extraction. As the deletion mutants and the TRAP150(wt) induce nLuc mRNA expression, bands representing unspliced E1 and E1C should be visible (see Figure 4).

The unspliced band should be visible around 2000 bp, E1C around 250 bp and E2 about 180 bp. As can be seen in Figure 8, this was not the case.

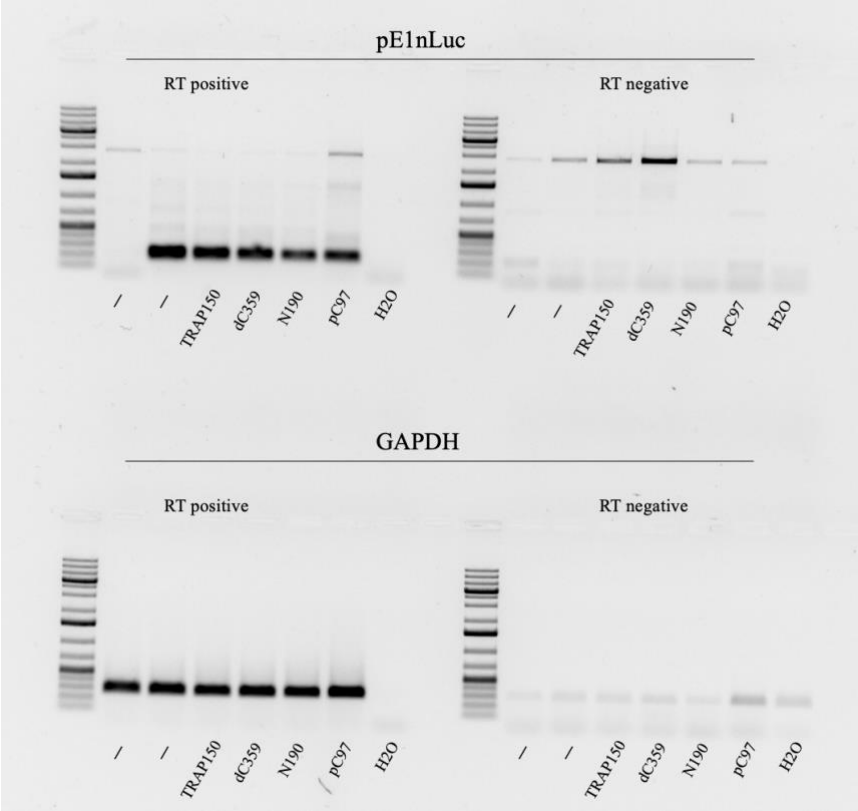

Figure 8. *RT-PCR results from HeLa cells transfected with the pE1nLuc reporter plasmid and various TRAP150 encoding plasmids. Agarose gel showing migration of the E1nLuc or GAPDH amplicons. RT negative; RT-PCR TRAP150(wt) reaction without reverse transcriptase. The RT negative is a specificity control for the reaction.*

Several tries to optimize the protocol were made, including changing primers and prolonging the amplification cycles. None of these changes resulted in bands of expected size, *i.e.* E1=2000 bp, E1C=250bp and E2180 bp. It was suggested that the E1 plasmid had low yield, at least compared to other HPV16 reporter plasmids used by the group. Alternatively, it could be that the transfection efficiency was low or perhaps that the induction using TRAP150 may not be strong enough to produce a significant amount of mRNA to be detected by RT-PCR. Because of this, it was decided to proceed with western blotting to determine which nLuc protein is produced.

3.2 Splicing of E6E7 is induced by TRAP150

TRAP150 was preliminarily shown to increase HPV16 E6/E7 mRNA splicing, i.e. the splicing from SD226 to SA409 (226^409) (unpublished data). To determine the effect of TRAP150 dC359 and dN190 on HPV16 E6/E7 226^409 mRNA splicing, another nLuc

reporter plasmid pE6E7nLuc was cotransfected with TRAP150 or deletions expressing plasmids, followed by nLuc assay as shown in Figure 7.

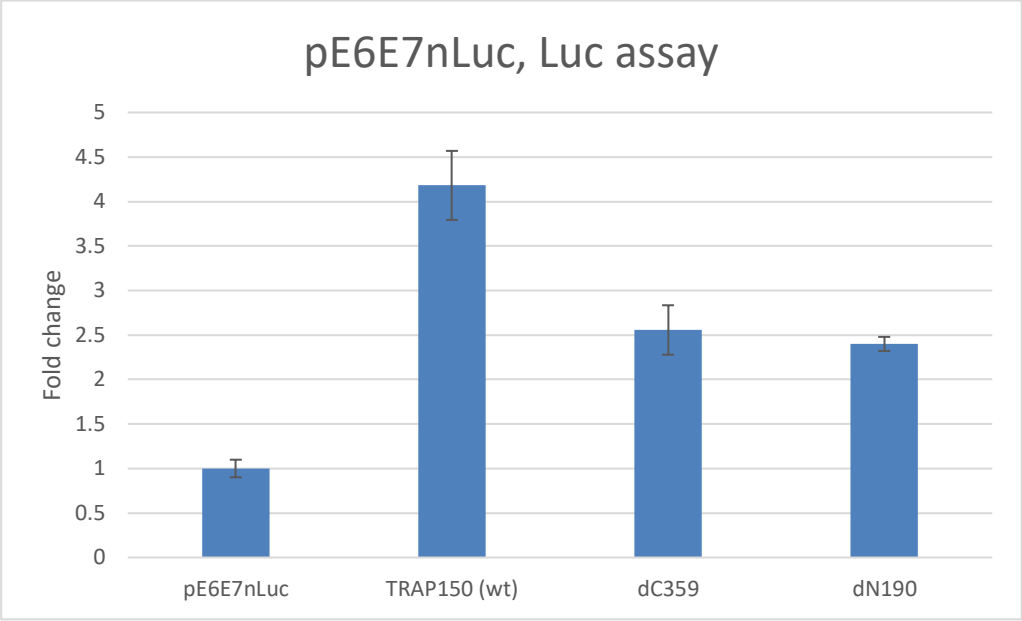

Figure 9. *Results from a nLuc luciferase assay using pE6E7nLuc as a reporter plasmid. HeLa cells were transfected with the indicated plasmids and analysed as in Figure 5. Data shown as fold change (pE6E7nLuc = 1), with standard deviation. *; P <0.05, **; P<0.01.*

As shown in Figure 9, transfected TRAP150(wt) plasmid caused E6 and E7 splicing shown as a read-out in nLuc assay. Notably, the raw nLuc values were lower when compared to pE1nLuc Surprisingly, the dC359 mutation showed much weaker effect than the TRAP150(wt) on pE6E7nLuc compared to pE1nLuc (compare Figures 7 and 9). The dN190 mutant behaved similarly as it did on the pE1nLuc reporter plasmid, i.e., expression was about half of the TRAP150(wt) effect.

3.3 Constructing new deletions plasmids to study TRAP150's function

As the result from the luciferase assay with pE1nLuc indicated that the TRAP150 mutant dC359 was better at inducing the production of nLuc compared to the wild-type protein (Figure 7), it we interested in which regions on TRAP150 were important for the function of protein as a splicing regulator. Since the dN190 mutant also reduce the production of nLuc (Figures 7 and 9), we decided to study if the nLuc production would be restored if less of the N-terminal part of TRAP150 is deleted. Therefore, five new plasmids were constructed.

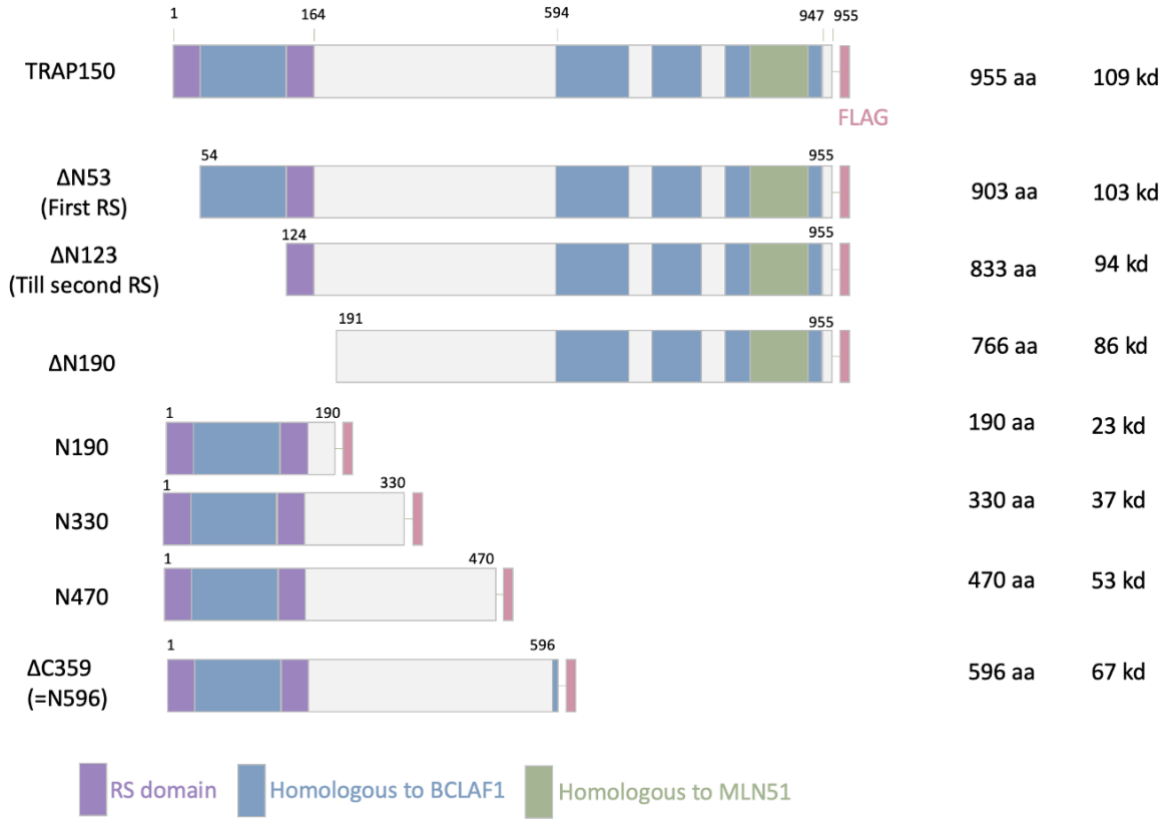

Figure 10. *A schematic illustration of the TRAP150 region for the plasmids pTRAP150-Flag, pTRAP150-dN190-Flag, pTRAP150-dN123-Flag, pTRAP150-dN53-Flag, pTRAP150-N190-Flag, pTRAP150-N330-Flag, pTRAP150-N470-Flag and pTRAP150-dC359-Flag. The pTRAP150-dN190-Flag, pTRAP150-dN123-Flag and pTRAP150-dN53-Flag have deletions in the N-terminal of various sizes with a Flag-tag fused at the C-terminal. pTRAP150-N190-Flag, pTRAP150-N330-Flag, pTRAP150-N470-Flag, and pTRAP150-dC359-Flag all have different deletions within the C-terminus with a Flag-tag fused at the C-terminus. The amino acid length (aa) and molecular weight (kd) of each protein are shown.*

Three of the plasmids had deletions in the C-terminal of TRAP150 (N190, N330 and N470), and two had deletions in the N-terminal (dN123 and dN53). dN53 only lacks the first 53 amino acids of the RS domain, while dN1223 lacks the first RS domain as well as the domain that is homologous to BCLAF1 (Figure 10). Like dC359 the N190, N330, and N470 all lack the domains homologous to MLN51 and several domains homologous to BCLAF1.

Using phosphorylated primers the pTRAP150-Flag was amplified according to Figure 10. The fragments were then amplified using PCR. To confirm the fragments were the right length they were run on an electrophoresis gel (Figure 11). The dN53 is the largest fragment followed by dN123. N470 is the third largest, N330 is the second smallest and N190 is the smallest fragment (see Figure 10). The bands in the gel corresponds to this order.

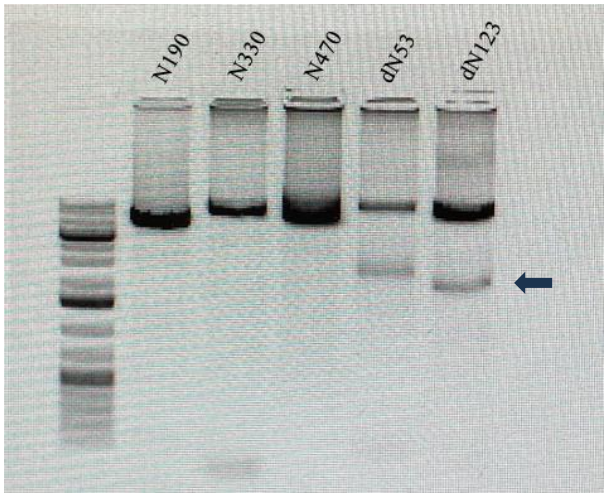

Figure 11. *PCR results confirming showing the sizes of the fragments of TRAP150. dN53 is 903 aa long and dN123 is 833, in these lanes unspecified bands can be seen, indicated by an arrow. The N190 is the smallest fragment with 190 aa, N330 is 330 aa long and N470 is 470 aa long.*

The fragments then underwent Dpnl digestion and ligation using T4 DNA ligase. All fragments were the right size, but some additional bands could be seen for the dN53 and dN123 mutants.

Ligated plasmids were then transformed into competent DH5α cells, and a colony PCR was done to confirm that the cells had taken up the correct plasmids. After this was confirmed, the plasmids were sent for sequencing. BLAST was used to examine if there were any mutations in the plasmids. There were no mutations in the N190, N330, N470, and dN53 plasmids, but the dN123 plasmid had mutations at the start codon. The mutation most likely occurred because the primers were located very close to the start codon. The mutation could also be an error at the sequencing level; it was decided to continue to use the plasmid and then send new samples for sequencing.

3.4 E1C production by TRAP150

HeLa cells were transfected with the aforementioned plasmids to test how the new TRAP150 mutants would affect the nLuc expression. After lysing the transfected cells the relative nLuc luminescence was measured. As shown in Figure 12, none of the TRAP150 N-terminal deletion mutants produced the same luciferase signal as the wild-type protein. However, as the TRAP150 N-terminal deletions got smaller, the nLuc expression did increase. All of the mutants with the C-terminal deletions were better at inducing nLuc expression (= read-out of splicing) when compared to the wild-type TRAP150 protein. dC359 had the biggest fold change followed by the dN190, N470 and N330 mutant proteins.

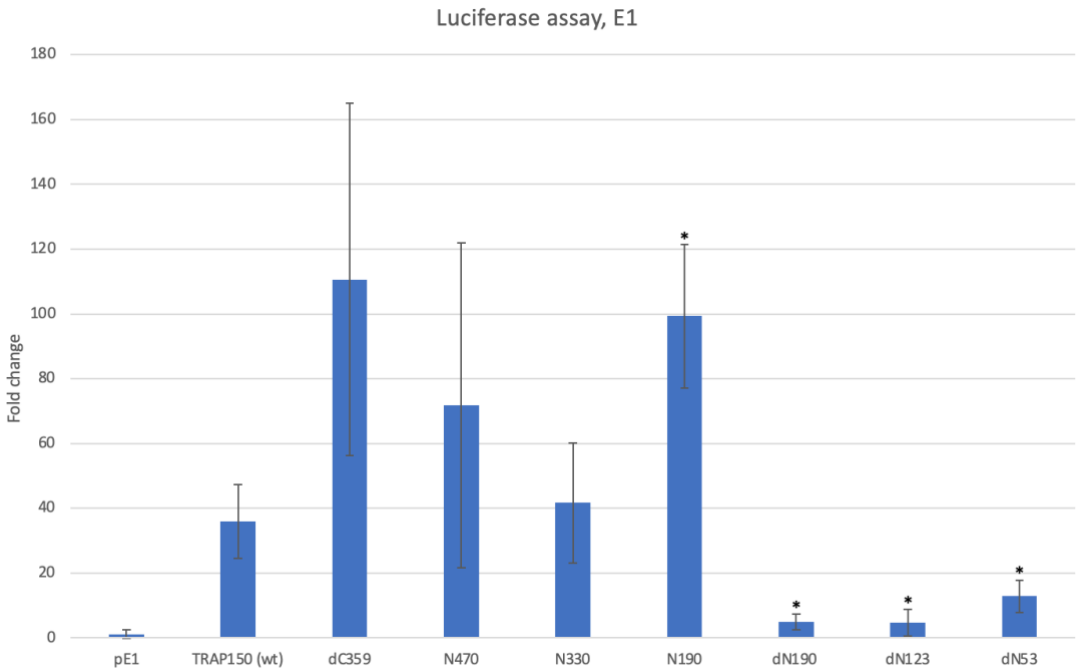

Figure 12. *Results from a nLuc luciferase assay using pE1nLuc as the reporter. HeLa cells were transfected with the indicated plasmids and analyzed as in Figure 5. Data shown as fold change (pE1nLuc = 1), with standard deviation. *; P <0.05, **; P<0.01.*

It is worth noting that there were quite large differences in luminesces between the replicates resulting in large error bars. Therefore, an assay with four biological replicates was done to see if certain values could be considered as outliers. However, this was hard to determine.

To see which splicing alternatives were induced by the plasmids western blot of the nLuc protein was performed. Since RNA extraction has proven unsuccessful with this plasmid, no attempt at RNA extraction was made using the new plasmids. Unfortunately, due to time restrictions there was no room for optimization of the plasmids.

There were three western blots performed: one anti-Flag, one anti-nLuc and one anti-Histone 3 (Figure 12). In the anti-nLuc western blot, detection of protein products from the spliced mRNA indicates that correct E1 splicing has occurred as the E1 mRNA is spliced to the nLuc cDNA (Figure 3, E1-nLuc or E1C-nLuc). In the anti-Flag western blot the TRAP150 mutations can be detected as the proteins contain a Flag tag epitope (Figure 10). The anti-Histone 3 western blot is used as a control to assure equal protein loading to the SDS-PAGE.

As can be seen in Figure 12, transfection of the C-terminal TRAP150 deletion mutants caused accumulation of the nLuc protein, which most likely corresponds to the E1C-nLuc protein. The expression levels also support the results from the Luciferase assay, with dC359 and N190 having the highest expression and N470 and N330 having the lowest.

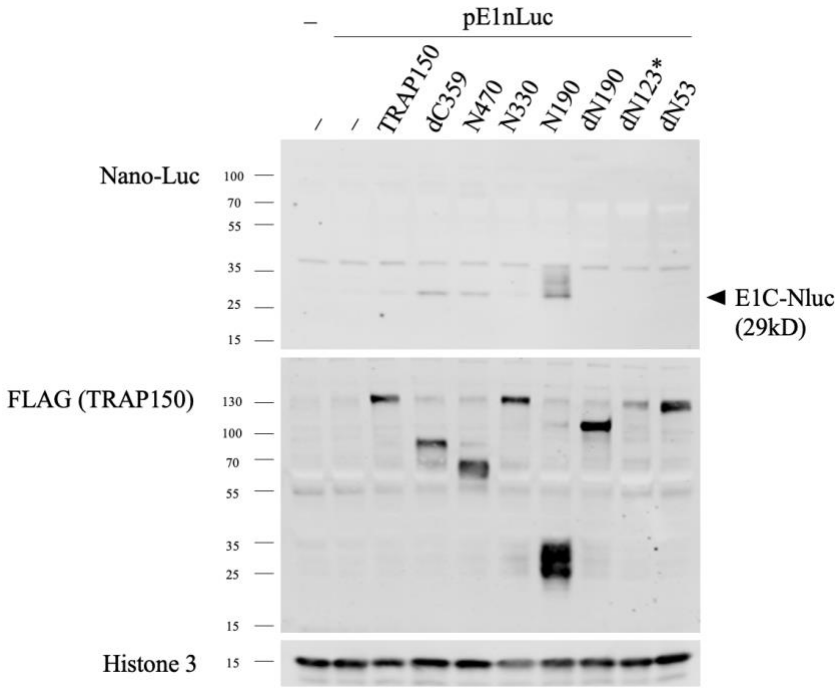

Figure 12. *Results from western blots detecting Flag-tagged TRAP150 mutant, nLuc and Histone 3 proteins. The black triangle indicates the bands that corresponds to the size of the E1C protein.*

Notably, N330 migrated the same size as the TRAP150(wt) protein when detected with the anti-Flag antibody. This could either be a technical issue or the expression from the plasmid may be low. Further studies are required to determine the cause of this unexpected migration. Also, as expected, the dN123 plasmid had a very faint band, most likely due to the mutation at the start codon.

4 Discussion

4.1 TRAP150 induces splicing of E1

The results indicate that TRAP150 does affect the splicing of E1 mRNA, as there was an obvious increase in the amount of nLuc produced when compared to the sample expressing only the pE1nLuc reporter plasmid (Figure 7). Unsurprisingly, TRAP150 N-terminal deletions resulted in a lower nLuc expression. The N-terminus of TRAP150 is where the annotated RS-domains are located which means that deletions in this region affects the proteins ability to bind to mRNA. However, none of these results provide any further knowledge of how TRAP150 exactly regulates the E1 pre-mRNA splicing. The luciferase assays, RT-RNA, and western blot experiments are indirect measures of TRAP150-mRNA interaction, which means that TRAP150 could affect any stage of mRNA splicing. Previous

studies from Lee *et al.* have shown that TRAP150 does affect the splicing of some pre-mRNA, but the exact details of how TRAP150 interferes with splicing reaction remain enigmatic. Therefore, further studies into the interaction are warranted before TRAP150 could be considered a therapeutic target for cervical cancer.

4.2 TRAP150s effect on E6E7 splicing

TRAP150 could induce splicing of the E6 and E7 pre-mRNA (Figure 7). This has been seen previously by the Schwartz lab (unpublished). However, the induction of the E6/E7 reporter gene was less effective than with the pE1nLuc. The TRAP150 mutant dN190 behaved similarly to the Luciferase assay results using E1 (see figure 7 and 9). This is not surprising as the dN190 lacks the RS domain that is necessary for RNA interactions. What is interesting is that the dC359 mutant did not produce higher amounts of nLuc than the wild type. In the Luciferase assay with E1, the dC359 was much more effective at inducing splicing than the wt. This could indicate that TRAP150 interacts with E6/E7 pre-mRNA differently than E1 pre-mRNA. However, these results were less convincing than the experiments performed with E1 as there were larger differences between measurements of the same biological replicate. Therefore, to be able to say something more conclusively, further studies are recommended. As the TRAP150 effect on E6/E7 differs from E1, I would like to do additional studies with the new TRAP150 deletion plasmids to see how the different deletions affect the splicing of E6/E7 pre-mRNA.

4.3 Induction with new deletion mutants

The examination done with the new TRAP150 mutants showed similar results to the previous luciferase assays using pE1nLuc. Namely, the mutants with deletions at the N-terminus had a lower nLuc protein induction than the wild-type TRAP150. This also concurs with the results from the western blotting. Once again, this is not surprising as they all have deletions at the RNA-binding domain. However, it is interesting that even with a small deletion of 53 amino acids (dN53), the efficiency was reduced by more than half. As expected, the C-terminal deletions all had a higher induction than the wild type. Although it is interesting that the level of expression went down as more of the C-terminal part of the protein was deleted. There are no domains known to affect TRAP150 binding to mRNA. Thus, this might indicate that this region has some important steric function for splicing. The expression level from N190 is almost as high as for dC359, which is interesting as dN190 lacks almost the whole region between the RS-domain and the BCLAF1 homologous domain. This could be an indication that deletions between aa 164 and 394 cause it to lose its function and, when doing so may act as a steric hindrance when interacting with other molecules.

However, Lee *et al* reported that deletions in the N-terminus do not affect the C-terminus's ability to participate in pre-mRNA degradation. This could explain why we could not see any

restoration of the induction levels by dN53 or dN123. Although dN123 had mutations in the start codon so it is not surprising that there was no change in induction levels there.

The results also showed that when mutating TRAP150 C-terminus (N190, N330, N470, and dC259) the splicing became even more efficient than the wild type. This is consistent with the result of dN190 that has a deletion of N-terminal RS domains and lost the production of E1C proteins. As TRAP150 is a very unstructured protein, the deletions may have allowed it to interact more efficiently with its interaction partners.

4.4 Problems with incubator

At the later stage of this project, there was a problem with the incubator, where the cells were kept between and during the project. This could affect the results as the cells may have been kept in a suboptimal milieu. It was noted that the cells were growing slowly and were, at one point, discarded for this reason. This did not affect the results of the luciferase assays or the western blot, as the issue had been dealt with by then, but it may have affected the cells during RNA extraction, which may be a reason for the low expression levels during the extraction. When there were issues with the incubator, the experiments using cells were stopped, which also affected the timeline of this project.

4.5 Problems with RNA extraction

One of the most challenging phases of this project was doing RNA extraction. Although several rounds of RNA extraction were performed, none produced reliable results. Most of the experiments produced no bands at all. This could be because the transfection efficiency was low and therefore, the amount of RNA was low. However, the transfection efficiency was not a problem for the luciferase assay.

Different ways of optimizing the extraction were also tried, like using other primer sets and more cycles during the PCR.

It could also be that the induced expression from the plasmid was relatively low. This idea was proposed after comparison with another luciferase assay (unpublished) where the expression from the pE6E7nLuc was almost 40-fold higher than the expression from the pE1nLuc.

There were also contamination issues, first believed to be in the buffers or solutions used during the experiment since water controls of PCR reaction showed PCR bands, indicating the contamination of cDNA in PCR reaction components. So they were discarded, but the problem remained. Finally, the primers used were determined as the cause of the contamination. At the end the experiment was abandoned, and it was decided to do western

blot instead to determine which alternatively spliced mRNA was produced via the expression of TRAP150.

5 Conclusion

As a conclusion, it seems that TRAP150 does have an effect on the splicing of the HPV16 E1 protein, resulting in the production of HPV16 E1C protein. Not only did the wt induce splicing but the C-terminal deletion plasmids seems to induce an even higher level of gene expression. The results of the western blot indicated that the mRNA splice variant produced was the E1C variant. Deletions to the N-terminal drastically reduced TRAP150s ability to induce splicing and this was still the case even with a small deletion of 53 amino acids. Taken together, these results indicates that N-terminal RS domains of TRAP150 are critical to induce HPV16 880^2582 splicing and to produce E1C.

The results also showed that TRAP150 could induce splicing of E6 and E7 but less efficiently than with E1. In E6 and E7 the deletion plasmids had a lower expression than the wt which suggests that TRAP150 might interact with E6 and E7 differently than with E1.

However, as none of these are experiments that shows an direct interaction it is hard to say how TRAP150 affects splicing. Therefore, further studies are required to determine how TRAP150 affects splicing.

6 Acknowledgements

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7 Referenser

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8 Appendix

8.1 Protocols

8.1.1 Culturing and splitting HeLa cells

1. Check that the cells are okay.
2. Remove media from the plate using a automatic pipette.
3. Wash with 5 ml PBS for 10 s, then remove.
4. Add 1 ml trypsin to the plate and incubate för 3 min at 37°C. After 3 min check that cells have started to come off the bottom of the plate. If not incubate for 0.5-1 min.
5. Add 1 ml of DMEM and wash the plate several times until all cells are loose.
6. Add approximately 10 ml of DMEM to a new plate
7. Take desired amount of cell culture from the first plate and add (drip) onto the new plate.
8. Shake and swirl the plate in order to spread out the cells on the plate.
9. Store in an incubator (37°C) until use.

8.1.2 Purification of plasmids

1. Culture cells in 1 ml of LB with proper resistance in 37°C shaker
2. Transfer 1 ml of culture into 100 ml LB with proper resistance. Keep in 37°C shaker overnight.
3. Perform plasmid mini prep according to Macherey-Nagel™ NucleoBond™ user guide.
4. Measure concentration of the plasmids and store in 20°C.

8.1.3 Transfection for luciferase assay

Day One: Cell preparation

1. Seed HeLa cells on 24 wells plate with $5x10^4$ cells/wells

Day Two: Transfection

1. Check cells after 24 hours if cells are 70-80 % confluent.
2. Prepare transfection mix with Turbofect
   - Total DNA amount/well: 500 ng
   - pCL086: negative control
   - pE1nLuc: reporter plasmid
   - pTRAP150-FLAG: TRAP150 Wild Type

- pTRAP150-aC359-FLAG: TRAP150 Deletion 1
- pTRAP150-dN190-FLAG: TRAP150 Deletion 2

Transfection mix

1 reaction	N	trap150 0ng	trap150 400ng	C359 400ng	N190 400ng
Serum free DMEM	92,5	92,5	92,5	92,5	92,5
Reporter plasmid (50ng/ul)	0	1	1	1	1
pTRAP150-FLAG (100ng/ul)	0	0	4	0	0
pTRAP150-FLAG-dC359 (100ng/ul)	0	0	0	4	0
pTRAP150-FLAG-dN190 (100ng/ul)	0	0	0	0	4
pCL0806 (100ng/ul)	5	4,5	0,5	0,5	0,5
H2O	0,5	0	0	0	0
Turbofect	2	2	2	2	2
Total	100	100	100	100	100

OBS: The total volume of plasmids added to the wells should be the same, otherwise add water to the ones have a smaller volume

3. After adding the TurboFect incubate at RT for 20 min
4. Add the transfection mix in the wells

Day Three: Data Collection

1. Check cells are fine
2. Lyse cells at 24 hrs after transfection.
3. Prepare passive lysis buffer:
   - 5xpassive lysis buffer: ddH_2O =1:4
4. Discard culture medium.
5. Wash cells with 200 rt PBS on each well, gently swirl it for 10-20 s. Discard PBS.
6. Add 200ul passive lysis buffer to each well.
7. Incubate for 15 min whilst shaking.
8. Pipette up and down and transfer lysate into a new 96 well plate.

- Store samples in -20 °C until use.

9. Measure luciferase in cell lysates.
10. Prepare nano-glo reagent
    - *Nano-Glo* reagent: *Nano-Glo* buffer =1:50
11. Fill 96 wells plates, add
    - PBS 44ul
    - Lysate 1ul
    - Nano-glo reagent 5ul
12. Incubate for 3 min
13. Data collection

8.1.4 RNA extraction

RNA extraction with TriReagent and Directzol RNA miniprep columns, spin at 12,000xg
1 mL of TriReagent/well of 6 wells-plate is best.
This protocol is good for HeLa

Day one:

1. Seed HeLa cells on a 6 well plate, $4x10^5$ cells/well

Day two:

1. Check that cells are fine
2. Prepare transfection mix with Turbofect

Transfection mix

1 reaction	N	trap150 0ng	TRAP150 400ng	C359 400ng	N190 400ng	(-)
Serum free DMEM	370	370	370	370	370	370
Reporter plasmid (50ng/ul)	0	4	4	4	4	0
pTRAP150-FLAG (100ng/ul)	0	0	4	0	0	0
pTRAP150-FLAG-dC359 (100ng/ul)	0	0	0	4	0	0
pTRAP150-FLAG-dN190 (100ng/ul)	0	0	0	0	4	0
pC97	0	0	0	0	0	4
pCL0806 (100ng/ul)	20	18	14	14	14	18
H_2O	2	0	0	0	0	0
Turbofect	8	8	8	8	8	8
Total	400	400	400	400	400	400

3. Add 400 ul of transfection mix to respective well
4. Incubate at 37 °C for 4 hrs
5. After 4 hrs replace media containing transfection mix with 2 ml DMEM
6. Incubate for 20 hrs at 37 °C

Day three:
For extraction of total RNA:

1. Remove media and wash with 200 uL of PBS
2. Add 1 mL TriReagent to plate (6 wells-plate, 60 mm dish: reagent per $1x10^5$- 10^7cells)
3. Pipet the lysate up and down several times (sometimes more than 20 times) to homogenize
4. Transfer to Epp tube (can store in -20 °C at this stage)
5. Incubate 5 min at r.t.
6. Add 0.2 mL of chloroform. Close lids and shake tubes vigorously by hands.
7. Incubate for 2-3 min
8. Centrifuge the sample for 15 min at 14,000xg at 4°C
9. Transfer the aqueous phase containing the RNA to a new tube
10. Add 0.5 mL isopropanol to the aqueous phase, per 1 mL of TRIReagent used for lysis.

11. Incubate for 10 min on ice
12. Centrifuge for 10 min at 14,000xg at 4 °C
13. Discard the supernatant with a micropipette
14. Add 0.5-1 mL of 75 % ethanol
15. Shake tube and then centrifuge for 5 min at 14,000xg at 4 °C
16. Discard the supernatant with a micropipette
17. Air dry the RNA pellet for 5-10 min
18. Resuspend the pellet in 80 uL of H_2O with 10 uL 10xDNase reaction buffer $MgCl_2$, 10 uL DNase I (1 U/uL, SIGMA, #AMPD1) and 1 uL Ribolock

For Clearing DNA:

19. Mix and incubate at 37 °C for 1 h
20. Add 200 uL of phenol/chloroform and 100 uL of H_2O, vortex 30 s, spin 5 min at r.t.
21. Transfer 200 uL aqueous phase into a new tube and add 20 uL 3 M NaOAc (pH5.2) and 500 uL EtOH, mix invert, keep it at -20 °C for 30 min
22. Spin 10 min at 14,000 rpm, 4 °C, remove supernatant
23. Add 300 uL 70 % EtOH
24. Spin 5 min at 4 °C and remove the supernatant
25. Lett pellet air-dry
26. Add 20 uL H_2O to resuspend
27. Check RNA concentration with NanoDrop
28. Per sample add 1 uL random primers, 1 uL dNTP (10 nM), 500 ng-1 ug RNA. Add ddH_2O until total volume is 12 uL
29. Incubate at 65 °C for 5 min. Place on ice, quickly.
30. To each sample add 4 uL Front strand buffer, 2 uL 0.1 M DTT and 1 uL RiboLock
31. Incubate at 37 °C for 2 min. Keep on ice
32. Add 1 uL M-MLV RT to each sample
33. Perform PCR
    a. 25 °C for 10 min
    b. 37 °C for 50 min
    c. 70 °C for 15 min
    d. 12 °C for ∞

8.1.5 Phusion-site directed mutagenesis kit (Thermo Scientific)

- According to protocol, this kit is available up to 10kb plasmid.
- Order 5’ phosphorylated primers. Otherwise ligate phosphate on primers.
- Follow the manufacture protocol.
    - PCR amplification
    - Digestion of parental methylated template plasmid with DpnI
    - Circularization of mutated PCR products with T4 ligase.

1. Prepare PCR reaction mix.

Component	50uL Rxn.	Final conc.
H2O	32.5 uL	
5x Phusion HF buf	10 uL	1x
10 mM dNTP	1 uL	200uM each
Fw primer (10uM)	2.5 uL	0.5uM
Rv primer (10uM)	2.5 uL	0.5uM
Template DNA (10ng/uL)	1 uL	10ng
Phusion hot start DNA pol	0.5 uL	0.02 U/uL

2. Run PCR

Step	Temp	Time	Number of cycles
Initial denaturation	98oC	30s	1
Dnaturation Annealing Extension	98oC 65-72oC* 72oC	5-10s 10-30s 15-30s/kb	25
Final extension	72oC 4oC	5-0 min	1

*58oC can work.

3. Run gel electrophoresis

Take 1-5 uL sample from the PCR reaction for agarose gel electrophoresis and verify PCR amplification.

4. Perform DpnI digestion

Add 1uL of FastDigest DpnI directly to the PCR reaction and incubate at 37oC for 15 min. There is no need to inactivate DpnI prior to ligation.

5. Prepare 10uL ligation reaction mix.

a. Take 10-20ng (1-5uL) of PCR product from the mutagenesis reaction after DpnI digestion.
b. Add 2uL of 5x Rapid Ligation Buffer.
c. Adjust the reaction vol to 9.5uL with H2O and mix.
d. Add 0.5uL of T4 DNA ligase and mix thoroughly.
e. Centrifuge briefly and incubate at r.t. for 5min.
f. Chill on ice, then transform or store at -20oC.
g. Transform 2.5uL of ligation for transformation: [option] prepare negative control: w/o ligase

6. Pick up 3-10 colonies for miniprep and sequencing.

a. Miniprep:
    a. Pick up 3-10 colonies and cultivate in 3-10 mL of LB (+Amp) for 16-20hrs
    - If needed, make a master LB plate for the colonies.
    b. Follow manufacture protocol (NucleoSpin Plasmid, Mni kit for plasmid DNA, Macherey-Nagel)
    c. Digest 100-500 ng of plasmid DNA with restriction enzyme(s) and evaluate if digested DNA fragments are predicted sizes.
b. Sequencing:
    a. Send miniprep plasmid DNA to sequencing (see different file).
c. Midi or Maxiprep
    a. After confirmation of sequencing, amplify plasmid DNA with midi/maxiprep.

CONFIRMATION of subcloning
Miniprep or colony PCR

8.1.6 Western blot

SDS page and Western Blot Nluc

1. Transfect cells in 6 wells (4*105cells/well) plate by TurboFect and change medium after 4 hours.
   Total DNA 2ug/well
   TurboFect 8ul/well

2. Collect cells after 24 hours
   RIPA + Proteinase inhibitor 200ul/well

Cells lysate can be stored at -80°C
Centrifuge 1 min at top speed

3. Sample denaturation
   Denaturation medium: Prepare more to filling up the empty wells
   100 ul 5x Laemmli buffer+ 60ul beta-Me (just before use) for 8 samples
   100ul sample + 20ul denaturation medium, store the rest of the sample at -80°C
   Incubate at 95°C for 5-10 min
   Centrifugation: top speed, 1 min (*can also be store at -20°C until use)

Load samples to precast gel*
Add running buffer to the indicated line
Run at 80V for 15 min
Run at 120V until samples reach the end of the gel

4. Transfer
   Assemble the sandwich in 1xtransfer buffer
   Run at180mA for 1.5h

5. Block in blocking buffer 1 hour

6. Primary antibody: anti-Nluc mouse (500ug/ml)
   Dilute anti-Nluc mouse to 1ug/ml (10ml blocking buffer (0.2% tween20)+20ul)
   Incubate at 4°C overnight

   Anti-tublin: 1:10000(original)

7. Wash with PBST for 5min x3 times

8. Secondary antibody IRDye 680 Goat anti mouse 1:10000 in blocking buffer for 1hour

9. Wash with PBST for 5min x3 times

10. Wash additionally with PBS for 5 minx1

11. Check results