UiT
THE ARCTIC
UNIVERSITY
OF NORWAY

Department of Medical Biology

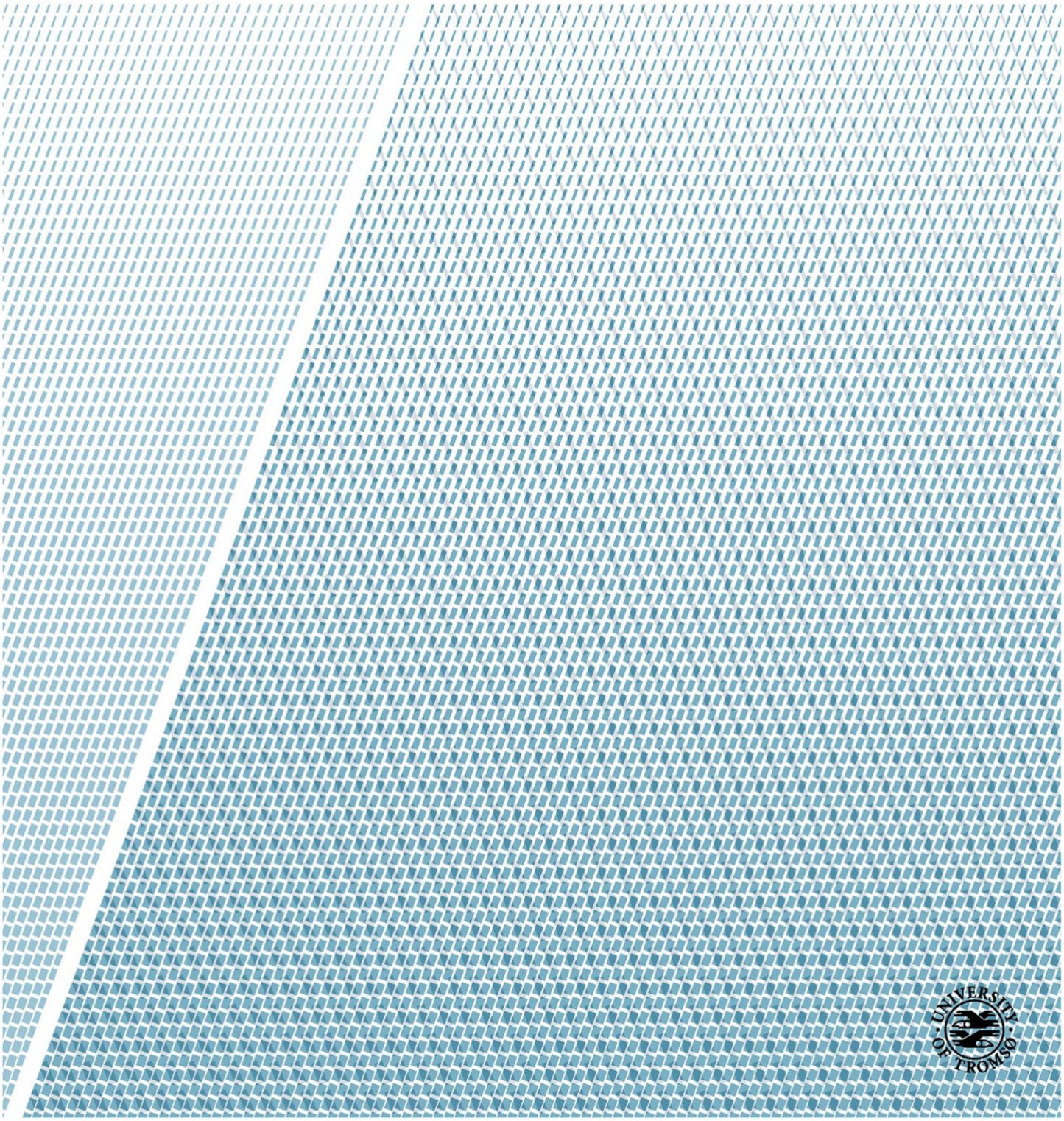
# Experimental gene expression modulation

*A practical and theoretical approach*

—

**Ingrid Arctander Rosenlund**

*Master thesis in MED 3950 June 2019*



# Preface

Originally, the aim of this thesis was to investigate the expression levels of the novel long non-coding RNA (lncRNA) *FAM83H-AS1* in formalin-fixed paraffin-embedded (FFPE) core needle biopsy samples of breast cancer by RNA fluorescence in-situ hybridisation. Due to technical and administrative issues with the Confocal Microscope at the Faculty of Health Sciences changing the objectives of this research project was considered necessary.

The purpose of this thesis is to create a stable knockout model with the CRISPR/Cas9 method for programmable genome editing, and comparing this technique to gene knockdown with small interfering RNAs (siRNAs) and short hairpin RNAs (shRNAs) via the RNA interference (RNAi) system endogenous to cells.

The work for this project was carried out in the RNA and Molecular Pathology Research Group (RAMP) at the Department of Medical Biology, UiT-The Arctic University of Norway. RAMP carries out research on lncRNAs and their cellular function in breast cancer. The research group possesses RNA sequencing data from several cell lines, and from these data numerous novel lncRNAs have been identified, including the lncRNA *FAM83H-AS1* that will be the focus of this thesis. The project is financed by the RNA and Molecular Pathology Research Group using internal and external funding. The work on this project has been carried out in close collaboration with my supervisor, postdoctoral fellow Erik Knutsen.

I would like to thank assistant professor Maria Perander for giving me the opportunity to complete my master thesis in her group. And I would like to express my utmost gratitude to my supervisor, Erik Knutsen, for his impeccable guidance and patience throughout the many peaks and valleys of this project, even when away on holiday. I am grateful for my colleagues in the research group that provide a stimulating and social work environment. A special thanks to postdoc Marta Tellez Gabriel, with whom I share an office, for always lifting my spirits.

Tromsø 02.06.19

Ingrid Arctander Rosenlund

# Table of Contents

## Summary

The aim of this project was to create a stable knockout cell model with the CRISPR/Cas9 method for programmable genome editing that would make it possible to run long term assays to further investigate the cellular function of the long non-coding RNA *FAM83H-AS1*. The CRISPR/Cas9 method is compared to gene knockdown with small interfering RNAs (siRNAs) and short hairpin RNAs (shRNAs) via the RNA interference (RNAi) system in cells.

The cell lines T-47D, MDA-MB-468, and BT474 were transfected with the CRISPR/Cas9 components by electroporation and single cell sorted by flow cytometry. Wild type T-47D cells were transfected with siRNAs towards the target gene. knockout status of the CRISPR clones was determined by PCR, and the expression levels of *FAM83H-AS1* and *FAM83H* was measured with RT-qPCR.

Our attempt at creating a CRISPR knockout cell line resulted in eight T-47D CRISPR clones; three heterozygous knockout clones, and five clones where the *FAM83H-AS1* gene remained intact in both alleles. We were not successful at making a homozygous knockout clone, or expanding CRISPR clones in MDA-MB-468 and BT474 cells. Analyses of the T-47D clones with RT-qPCR showed variable expression levels of *FAM83H-AS1* in the three heterozygous clones. The expression level of *FAM83H* seems to follow the expression of *FAM83H-AS1* in all clones. This was not seen with siRNA knockdown of *FAM83H-AS1*, and suggests a relationship between the sense, *FAM83H*, and antisense gene, *FAM83H-AS1*, at the transcriptional level.

Conclusively, there are several advantages and disadvantages with each knockdown strategy, and as our results show the CRISPR/Cas9 method is not the most suitable option for long term knockout of gene expression in our epithelial cell lines. Knockdown with shRNA might prove to be a feasible alternative as stable gene silencing is possible and some of the methodological issues with the CRISPR/Cas9 technique is overcome.

# 1. Introduction

## 1.1 Long non-coding RNAs and their involvement in cancer

The majority of the human genome consists of non-coding sequences that when transcribed are not translated to proteins. These sequences generate a large variety of non-coding RNA (ncRNA) molecules (1) that in the past were considered to be largely non-functional and therefore of little importance to the development of human diseases (2). Previous research has been mainly concerned with identifying our protein coding genes, and the machinery involved in protein synthesis, included the non-coding tRNAs and rRNAs vital to a cell's function, as proteins were perceived to be the functional units responsible for cellular activity (2).

However, in more recent years several ncRNAs with regulatory functions have been discovered (3); a large amount of research on micro RNAs (miRNAs) have been produced, and lately an interest in long non-coding RNAs (lncRNA) has emerged. The latter have gained much attention during the past decade and has become a particularly interesting addition to research on the molecular mechanisms of cancer (1).

LncRNAs are broadly defined as non-coding transcripts longer than 200 nucleotides (nt). They are mainly transcribed from RNA polymerase (pol) II promoters and undergo processing by 5' capping, polyadenylation, and splicing (4-6). LncRNAs are often transcribed from loci located in intronic and exonic regions of protein coding genes they overlap with, or from intergenic regions (6). They can be classified according to their location as long intergenic ncRNA (lincRNA) transcribed from genomic regions between genes; exonic sense lncRNA transcribed in the sense direction overlapping with exons of protein coding genes; exonic antisense lncRNA transcribed in the antisense direction overlapping with exons of protein coding genes; intronic lncRNA transcribed from introns in protein coding genes with no overlap with exons; and bidirectional transcripts that share a promoter with, and is transcribed in the opposite direction of, a neighbouring protein-coding gene (7, 8).

LncRNAs are thought to be under selection and strictly regulated, as the expression of these transcripts seem to be highly tissue specific, indicative of important functional properties and making them potential biomarkers (2, 9-11). LncRNAs are involved in a number of regulatory processes of gene expression, including the regulation of embryonic development (2, 11). The role of lncRNAs in cellular processes have been described in several papers, and lncRNAs can also be classified according to their function as either: guides for proteins to specific DNA

sequences or RNA molecules in nuclear transportation; enhancers stimulating transcription of particular genes; scaffolding RNAs that assemble ribonucleoprotein complexes to specific sites; or decoys that inhibits protein function by sequestering, allosteric modification or by blocking protein binding sites (10, 12, 13). Furthermore, cytoplasmic lncRNAs have been identified that regulate mRNA stability, protein synthesis, and the localization of specific proteins (2, 5).

The dysregulation of many lncRNAs, including abnormal levels of expression and changes to their primary and secondary structure, has been associated with the initiation and progression of cancer, as well as clinical variables and prognosis (2, 14-16). Such as the extensively studied lncRNA Hox transcript antisense intergenic RNA (HOTAIR) that promotes invasion and metastasis of breast cancer. High expression levels of HOTAIR is a powerful predictor of poor prognosis in breast cancer patients (2, 17, 18). The lncRNA X-inactive specific transcript (Xist) is involved in X-chromosome silencing in women (19), and loss of expression has been linked to breast, ovarian, and cervical cancer (20).

## 1.2 The long non-coding RNA *FAM83H-AS1*

The lncRNA *FAM83H-AS1* is transcribed from a 12.2 KB long gene, located on chromosome 8 (q24.3). It is situated in close proximity to the protein coding gene *FAM83H*, with their 5'ends <400 nt apart. For this reason, *FAM83H-AS1* is not a true antisense lncRNA, but a bidirectional lncRNA.

Initial analyses carried out by the research group on RNA-sequencing data from 23 matched breast cancer tumour samples and normal mammalian tissue samples show that *FAM83H-AS1* is more highly expressed in cancerous tissue than in normal tissue. In addition, high expression of *FAM83H-AS1* was also associated with worse prognosis in breast cancer by analysing the expression of *FAM83H-AS1* in the publically available gene expression database TCGA.

The lncRNA *FAM83H-AS1* is a novel non-coding transcript with only a few recent publications addressing its cellular function and role in various cancer types. High expression levels of the lncRNA have been found in several types of cancer, and studies have shown that knockdown of *FAM83H-AS1* significantly impairs cell proliferation, migration, and invasion (9, 21-26), possibly through G2 arrest in the cell cycle (21). High expression levels of *FAM83H-AS1* is also associated with poor prognosis in all sub types of breast cancer, and can be used as a novel independent prognostic marker for luminal subtype breast cancer (9). Knockdown of *FAM83H-AS1* in colorectal cancer (CRC) exerts an anti-proliferative effect in a Notch signal dependent manner, and is associated with worse overall survival in CRC patients (24). In cervical cancer, *FAM83H-*

*AS1* is regulated by HPV-16 E6 independent of the tumour suppressor p53, and it is associated with poor survival in cervical cancer patients (22). The lncRNA *FAM83H-AS1* has also been found to be an independent prognostic marker in ovarian cancer, and contributes to the radio-resistance and metastasis of the disease (25). In lung adeno carcinoma (LUAD), high expression levels of *FAM83H-AS1* is associated with poor patient survival, and it has been suggested that *FAM83H-AS1* contributes to the progression of LUAD by targeting MET/EGFR and their downstream signaling ERK1/2 and AKT (21).

Considering our preliminary data on *FAM83H-AS1* and recent publications, it is of particular interest to determine this lncRNA's cellular function and role in the initiation and development of cancer.

LncRNA *FAM83H-AS1* is located in close proximity to the protein coding gene *FAM83H*. FAM83H protein is located in the nucleus, and is predicted to play a role in the structural development and calcification of tooth enamel (27), and it is involved in the organisation of the keratin cytoskeleton and formation of desmosomes (28). Truncated mutations in the *FAM83H* gene is associated with a severe variety of the disease amelogenesis imperfecta, a disorder of tooth development where the quantity and/or quality of dental enamel is reduced (27). Further, recent reports suggest that FAM83H is involved in tumorigenesis; it has been associated with poorer survival of patients with clear cell renal cell carcinoma (29), and is involved in the progression of hepatocellular carcinoma (30).

## 1.3 Genome editing in experimental research

Gene knockdown or knockout is the process by which the expression of one or several genes in a cell or organism is eliminated or reduced for experimental or therapeutic purposes. Gene knockdown can be achieved both at a transcriptional and post- transcriptional level, and can be either transient or permanent.

With gene knockdown no permanent changes to the target gene is made, but the expression level is reduced by interfering with the mRNA transcribed from the gene loci and thereby diminishing its function. Gene knockout, on the other hand, involves the direct interference with the target gene by inducing alterations in its DNA sequence. This can be used to create a stable and permanent suppression of gene expression.

Gene silencing can be achieved with several techniques, such as knockdown via the endogenous RNA interference (RNAi) system using small interfering RNAs (siRNA) and short hairpin RNAs (shRNA), or knockout with the CRISPR/Cas9 method which is based on a bacterial defence mechanism.

## 1.4 Gene knockout with CRISPR/Cas9

The CRISPR/Cas9 technique is a genome editing technique that uses RNA molecules to guide nucleases to specific target sites in the DNA where it cuts the DNA strand. This allows researchers to create specific modifications to the target area of interest in the genome (31).
The method was established as a technique for programmable genome editing by scientists in 2012 after discovering that the adaptive bacterial immune system could be manipulated and used for gene editing in eukaryotic cells (32). Specifically, a family of endonucleases were described, the CRISPR associated proteins (Cas), characterised by their use of dual-RNAs for site-specific DNA cleavage, showing great potential for utilisation in RNA-programmable genome editing (32). The Cas endonuclease family is endogenous to bacteria and archaea, and the proteins are part of the defence mechanism against viruses and plasmids; the so called clustered regularly interspaced short palindromic repeats (CRISPR) (33). CRISPR/Cas is a system consisting of *cas* genes, organised in operons under the control of a single promoter, and CRISPR arrays, genomic regions where multiple incorporations of viral DNA and plasmid DNA sequences are located, originating from past invasions. The *cas* genes and the CRISPR arrays are interspersed with identical repeats (31-33).
In the case of new invasions, the adaptive immune system will recognise segments of the viral or plasmid DNA and initiate its defence mechanism by activating and guiding the Cas nuclease to the target DNA. Cas then binds to the DNA and introduces double-stranded (ds) breaks, in effect causing the destruction of viral or plasmid DNA which terminates the invasion (32, 34).
The CRISPR/Cas9 method has proven to be a simple and efficient method for genome editing, and has shown great potential in experimental research and gene therapy.
The mechanism is utilised in experimental research for genome editing by exploiting the endonuclease activity of Cas9 to remove or insert a specific DNA sequence of a target gene (34, 35). An RNA sequence complementary to the desired cleavage location on the target gene is designed, a so called guide RNA (gRNA). The gRNA transports Cas9 to the target location in the genome where it binds to a specific protospacer adjacent motif (PAM), located 3-4 nt

downstream of the intended cut site, the Cas9 then creates a ds break in the DNA (31, 35). The PAM motif for Cas9 is 5'-NGG-3', where N is any nucleotide. Different Cas nucleases recognises different PAM sequences, thus limitations for potential cut sites in the genome are few (31).

Following the ds cleavage, the DNA repair apparatus endogenous to the cell will attempt to mend the damaged DNA. The imperfect repair carried out by these mechanisms are then exploited to create specific changes to the cell's genome (31).

The CRISPR/Cas9 components can be transported in different forms, and with various methods into cells. The system can be delivered as DNA that enters the nucleus and is transcribed to mRNA coding for gRNA and/or Cas9; RNA where Cas9 is delivered as mRNA and translated in the cytoplasm; or delivered directly to the cytoplasm or nucleus as ribonucleoprotein complexes (RNP) without any further need for transcription or translation (31, 35). The components are delivered to the cells cytoplasm or nucleus by transfection; either physical, chemical, or viral-mediated transfection, depending on the cell line and the need for transient vs stable effects on the genome (31).

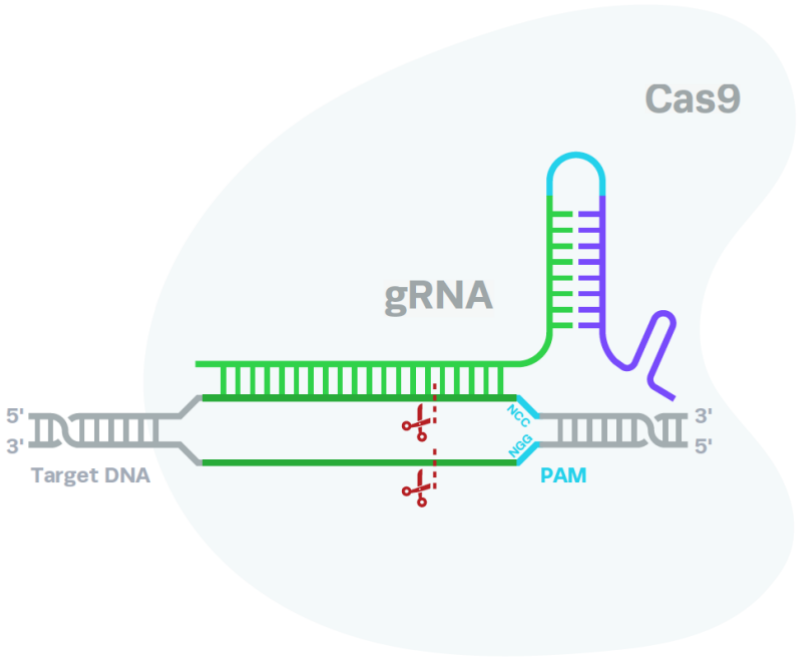



**Figure 1:** Illustration of the CRISPR/Cas9 system with guide RNA (gRNA) and Cas9 nuclease binding to the DNA target site and PAM motif, forming a ribonucleoprotein complex.
From the ebook: Cell Engineering 101: The Complete Guide to CRISPR Knockouts. Synthego. Available from: https://app.hubspot.com/documents/2418554/view/25560344?accessId=4f338d.

## 1.5 Gene knockdown with small interfering RNAs

One of the most important discoveries in biology is the endogenous RNAi mechanism in eukaryotic cells that regulate gene expression, and the possibility of utilizing this system for gene silencing in biological research and therapeutics (36, 37). In 2018 the first siRNA drug, Patisiran by Alnylam® Pharmaceuticals, was approved by the Food and Drug Administration in the U.S for the therapeutic silencing of the disease-associated gene expression in the hereditary disease transthyretin-mediated amyloidosis (38).

The RNAi system was first described in plants, and later in *Caenorhabditis elegans* and mammalian cells (36, 39). The system is activated when double stranded RNA (dsRNA) species are introduced into a cell's cytoplasm. The system consists of several key components that together regulate gene expression post-transcriptionally by the selective silencing of mRNA complementary to the target gene (37). In the nucleus of the cell, the double-strand-specific ribonuclease (RNase) III enzyme Drosha processes precursor micro RNAs (pri-miRNA) and shRNA. In the cytoplasm the RNA-induced silencing complex (RISC) processes and binds dsRNA before targeting mRNA for degradation. RISC consists of Dicer, Argonaute proteins, dsRNA-biding proteins, TAR-RNA-binding protein (TRBP), and Protein R (PKR)-activating protein (PACT) (40, 41). Dicer is an RNase III enzyme that processes dsRNA into shorter siRNA segments 19-27 bp in length. Argonaute proteins and dsRNA-biding proteins binds and loads the siRNA into the RISC. Argonaute-2 (AGO2), the only Argonaute protein with ribonuclease activity in humans, cleaves the target mRNA (40, 42, 43). TAR-RNA-binding protein (TRBP) is needed for dsRNA cleavage by Dicer and the following association between the siRNA and RISC. Protein R (PKR)-activating protein (PACT) associates with Dicer and TRBP for dsRNA cleavage (40).

The use of siRNAs has become the most commonly applied technique for gene silencing in biological research. SiRNAs are short dsRNA molecules that operate within the RNAi pathway, and induces post-transcriptional gene silencing by degrading mRNA and thereby preventing translation. The siRNAs are about 19-27 base pairs in length, have phosphorylated 5' ends, and hydroxylated 3´ends with a characteristic two nucleotide overhang (Figure 1) (36, 37, 44). They are homologous to the target gene and induces its effect by complete complementary base pairing with the mRNA. Synthetic siRNAs can be designed to complement any target gene, and can be introduced effectively into cells by transfection. They are useful in studying gene function, as well as drug sensitivity and targeting (36, 40, 45, 46).

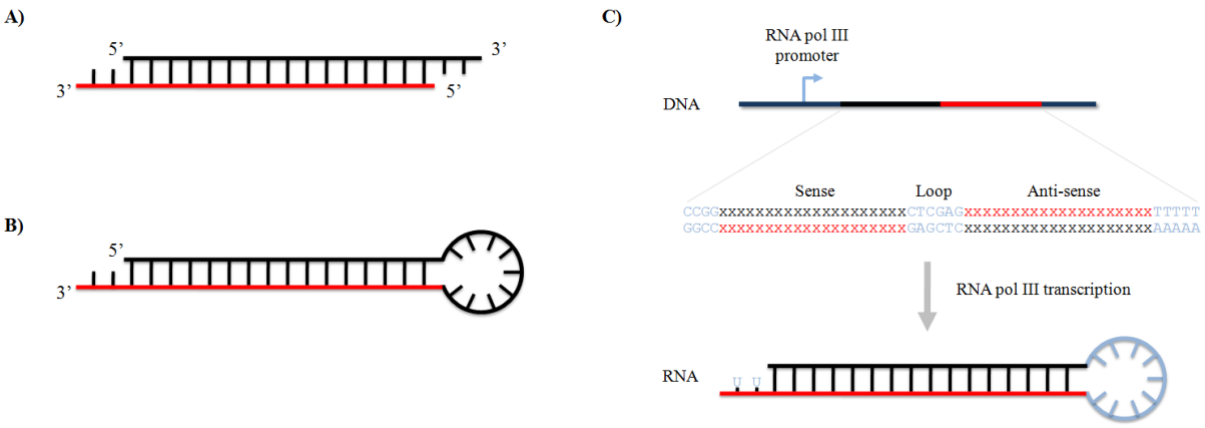



**Figure 2:** The characteristic structure of small interfering RNAs (siRNA) and short hairpin RNAs (shRNA).
A) Structure of siRNA with the characteristic 2nt 3' overhang.
B) Structure of shRNA with sense and antisense sequences separated by a loop sequence.
C) shRNA construct for insertion into expression vectors.
From: O'Keefe EP. siRNA and shRNAs: Tools for Protein Knockdown by Gene Silencing. Materials and Methods. 2013;197(3).

When introducing siRNAs into a cell by transfection they accumulate in the cytoplasm, although there are a few reports of siRNAs being translocated to the nucleus (40), where they are incorporated into RISC, which results in an ATP dependent activation of the complex (47). When in contact with RISC the dsRNA is unwound to form single stranded siRNA. The single stranded RNA with the most thermodynamically unstable 5' end remains attached to the RISC while the other strand is degraded (41). The complex is then guided by the siRNA to search for and bind to the complementary mRNA in a sequence-specific manner to form perfect base pairing. mRNA cleavage is then induced by the ribonuclease activity of AGO2 in the centre of the duplex region 10 nt from the 5' end of the siRNA (47). The mRNA is further cleaved and degraded by other endogenous nucleases (43). The result is inhibition of translation and thus reduced gene expression. Processed endogenous miRNAs, on the other hand, binds to target RNA imperfectly with mismatches that leads to repression of translation but not RNA degradation (42). Imperfect base pairing might also occur with exogenous introduced siRNAs and cause off-target effects by repressing translation without mRNA degradation (46).

## 1.6 Gene knockdown with short hairpin RNAs

Another way of utilising the RNAi system for gene silencing is by introducing shRNAs into cells by the means of a vector, either plasmid or viral. The DNA segment encoding the shRNA is integrated into the cell's genome via viral transfection (43). When successful this allows for more stable and long-term knockdown of gene expression.

ShRNAs are dsRNA molecules connected by a region of unpaired nucleotides forming the hair pin loop, making them structurally similar to endogenous miRNAs, as seen in figure 3. Although similar, the two molecules differ in that miRNAs contain internal mismatches causing bulging in their secondary structure, whereas shRNAs do not have these characteristics as the sense and antisense strand form perfect base pairing (47).

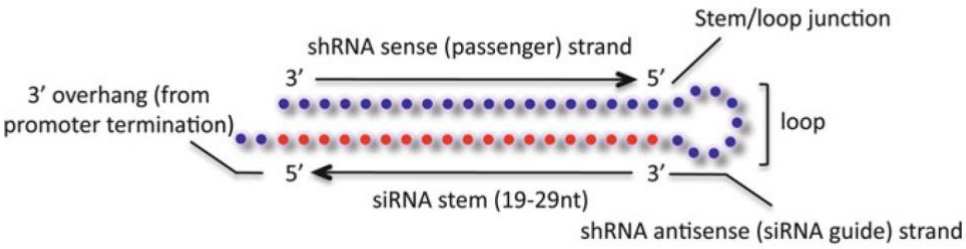



**Figure 3:** The structure of a typical shRNA. The loop connects the 3' end of the upper sense strand with the 5' end of the lower antisense strand. The antisense strand is complementary to the target mRNA sequence and becomes the siRNA guide strand.
From: Lambeth LS, Smith CA. Short Hairpin RNA-Mediated Gene Silencing. In: Taxman DJ, editor. siRNA Design: Methods and Protocols. Totowa, NJ: Humana Press; 2013. p. 205-32.

ShRNAs are introduced into cells by vectors, integrated into the genome, and transcribed by RNA pol III or pol II promoters (43). After transcription they are converted into siRNAs by the RNAi pathway in the same manner as endogenous miRNAs. Before being exported into the cytoplasm by Exportin-5 in a Ran-GTP-dependent manner (47), they are processed by Drosha and the dsRNA binding domain protein DGCR8 to form pre-shRNAs. Once in the cytoplasm the shRNA is recognised and cleaved by Dicer and TRBP/PACT in the RISC machinery. This removes the hairpin loop and creates 19-27 nt long siRNAs (40, 43, 47). The resulting siRNA is incorporated into RISC and causes gene silencing by the degradation of target mRNA in the same manner as synthetic exogenous siRNAs (47).

# 2. Material and Methods

## 2.1 Cell culturing

HCT116 (ATCC® CCL-247™), HeLa (ATCC® CCL-2™), A549 (ATCC® CCL-185™), MDA-MB-468 (ATCC® HTB-132™), and T-47D (ATCC® HTB-133™) cells were all purchased from the American Type Culture Collection (ATCC). HCT116, MDA-MB-468, and T-47D were cultured in RPMI 1640 (Sigma-Aldrich). T-47D were grown in the presence of 0.006 mg/ml insulin (Sigma-Aldrich). HeLa was cultured in Eagel's Minimum Essential Medium (Sigma-Aldrich). A549 was cultured in Nutrient Mixture F-12 Ham (Sigma-Aldrich).
All mediums were supplemented with 10% Fetal bovine serum (FBS) (Biochrom) and 1% penicillin-streptomycin (Sigma-Aldrich). All cell lines were incubated in a 5% CO2 humidified incubator at 37°C. Subculturing of all cell lines was done according to the recommendations by ATCC.

## 2.2 CRISPR/CAS9 knockout by electroporation

For the generation of *FAM83H-AS1* knockout cells, a two gRNAs strategy targeting upstream and downstream of the desired target sequence was utilized in order to generate two DNA breaks that could create a relegation of the two broken ends with the loss of the genomic sequence in between (Figure 4C). Synthetic single guide RNAs (sgRNAs) and Cas9 enzyme were purchased from Synthego (USA). Delivery of complexed sgRNA and Cas9 was done by electroporation using the Cell Line Nucleofector™ Kit V (Lonza).

A nucleofector mix was made combining 43ul of Nucleofector solution with 9.5ul Nucleofector supplement. SgRNA was diluted to 100uM in Low TE buffer. In a 1.5mL tube, 1.8ul sgRNA was added together with 1ul Cas9, and 22.2 ul Nucleofector Mix to a total volume of 25ul. The solution was then incubated at room temperature for a minimum of 10 min. Leading up to the experiment cells were cultured in growth medium under normal conditions. At the day of the experiment the cells were split and collected from the culture flask at 60% confluency. 100,000 cells were transferred to a 1.5ul tube, spun down at 100g for 10 min. The supernatant was removed and the cells suspended in 25ul Nucleofector mix. The cells in the Nucleofector mix were then transferred to the 1.5mL tube containing the sgRNA-Cas9 mix. The solution was transferred to an electroporation tube and electroporated with program P-020. After the

electroporation, 50ul of growth medium was added to the cell suspension and the cells were seeded out equally in two wells on a 12-well plate containing 450ul of warm growth medium. The growth medium was renewed after 24h. Cells from one of the two wells were harvested for DNA isolation 72 hours after the CRISPR/Cas9 knockout, to verify the efficiency of the knockout. The cells in the remaining well were left to recover for 3-5days, but not reaching more than 30% confluency. Conditioned growth medium was prepared by sterile filtering, with a 0,2 uM filter, growth medium used to culture the mother cell line for a minimum of 2 days. 10ml of the sterile filtered growth medium was mixed with 10mL sterile filtered foetal bovine serum (FBS). The solution was then added to 50mL of fresh growth medium. 200ul medium was then transferred to each well on 96-well plates. The cells in the remaining well on the 12-well plate were split, spun down at 100g for 10 min, the supernatant removed, and the cells suspended in 500ul of warm PBS. The cells were then transferred to a flow tube through a filter cap to separate clusters of cells into single cells. Single cell sorting was performed into the 96-well plates containing conditioned growth medium with flow cytometry, sorting for live and dead cells. After 1 week the 96-well plates were screened for colonies. After two weeks the growth medium was replaced with fresh conditioned growth medium in the wells containing colonies. When colonies reached a number of >200 cells, cells were transferred from the 96-well plates to 24-well plates to allow further expansion of the colonies. After cells reached a high confluency in 24-well plates, ¼ of the cells in each well was collected for DNA isolation. The DNA from each colony was screened to verify whether the cells were homozygous wild type (WT), heterozygous knockout or homozygous knockout. The colonies were then allowed to expand further.

## 2.3 siRNA knockdown by reverse transfection

For transient knockdown of the *FAM83H-AS1* transcript, cells were transfected with siRNAs towards the mRNA of the target gene using Lipofectamine® 2000, according to the reverse transfection protocol provided by the manufacturer, Thermo Fisher Scientific. With this approach siRNA is transported in micelles of the Lipofectamin® 2000 through the cell membrane into the cells cytoplasm. All transfections were done in triplicates, one triplicate of non-transfected cells and one triplicate with a scramble siRNA were used as negative controls.

In reverse transfection as opposed to conventional transfection, the cells are seeded out in the

transfection mix on the day of the experiment, followed by incubation between 24-48h before harvesting. Reverse transfection was carried out in 12-well plates. For each well transfected the following mixtures were prepared. Lipofectamin® 2000 was mixed gently before use, then 2,0 ul Lipofectamine® 2000 was diluted in 125 ul Opti-Mem® I Medium. The solution was mixed gently and incubated for 5 min at room temperature. In a separate tube, 1.5ul siRNA from a 20uM stock concentration was diluted in 125ul Opti-MEM® I medium and mixed gently. After the 5-min incubation, the diluted siRNA was added to the tube with Lipofectamin® 2000, mixed gently and incubated for 15 min at room temperature to allow complex formation to occur. 1000 ul of complete growth medium, with 200,000 cells/mL, was added to each tube containing RNAi molecule–Lipofectamine® 2000 complexes. Giving a final volume of 1250ul and a final siRNA concentration of 24nM. The solution was mix gently before cells were seeded in 12-wells. Cells were incubated at 37°C in humidified conditions with 5% $CO_2$ and harvested 24 hours after transfection. Knockdown was verified by RT-qPCR.

## 2.4 RNA isolation

RNA was isolated from cells either harvested during splitting of the cell cultures, or from 12-well plates after reverse transfection with siRNAs. Growth medium was removed from cells and washed with 1 mL PBS to completely remove growth medium and serum. Cells in suspension were first spun down for 2 min at 13000g. Further, cells were lysed with 300-600ul TRI Reagent® (Zymo Research), depending on the number of cells harvested. Total RNA was isolated using the Direct-zol RNA miniPrep by Zymo Research, according to the manufacturer's protocol. Total RNA was reconstituted in 25ul RNase free water. Concentrations and purity was measured by NanoDrop 2000 from Thermo Fisher Scientific. Isolated RNA was stored at -70°C until further use.

## 2.5 DNA isolation

DNA was isolated from the CRISPR clones when splitting the colonies. The cell suspension was spun down for 2 minutes at 13000g, and the growth medium removed. DNA was isolated using the Wizard® Genomic DNA Purification Kit by Promega. The DNA pellet was air dried after washing with 70% ethanol, and rehydrated in 25ul of DNA Rehydration Solution overnight at 4°C. DNA concentrations and purity was measured by NanoDrop 2000 from Thermo Fisher Scientific. Isolated DNA was stored at 4°C until further use.

## 2.6 cDNA synthesis and real time quantitative polymerase chain reaction (RT-qPCR)

cDNA synthesis for 10ul and 20ul reactions was carried out with equal RNA dilutions for all samples, and was performed with SuperScript™ IV Reverse Transcriptase from Thermo Fisher Scientific according to the manufacturer's suggestions. Total RNA was denatured at 65°C for 5 minutes. Time and temperature for each step are specified in the table 1 below.

**Table 1: Temperature and time specifications used for cDNA synthesis**

| Step | Time in minutes | Temperature in °C |
|---|---|---|
| Pre cDNA | 5 | 65 |
| cDNA synthesis | 10 | 23 |
| | 10 | 53 |
| | 10 | 80 |

Real time quantitative polymerase chain reaction (RT-qPCR) was used to amplify, detect, and quantify the target genes *FAM83H-AS1* and *FAM83H*. RT-qPCR was run on LightCycler 96 (Roche Life Science) with the SYBR green reaction mix FastStart Essential DNA Green Master from Roche Life Science, and 0.25uM forward and reverse primer. Thermal cycle conditions were 95°C 10 minutes and 40 cycles of 95°C 10 seconds, 60°C 10 seconds and 72°C for 10 seconds. Experiments were done in triplicates, and the ΔΔCq method was used for fold change calculations. GAPDH was used as reference gene.

## 2.7 Polymerase chain reaction (PCR)

The isolated DNA from the clones was screened using polymerase chain reaction (PCR) to verify the knockout status of each clone. DNA samples were amplified using LA Taq DNA polymerase from TaKaRa according to the manufacturer's suggestions, and run for 30 cycles. Time and temperature as specified in the table 2 below. Samples were run on a 1% agarose gel at 100V for 40 min.

**Table 2: Temperature and time specifications for polymerase chain reaction**

| Step | Time in seconds | Temperature in °C |
|---|---|---|
| Initial denaturing | 10 | 98 |
| Denaturation | 15 | 60 |
| Primer annealing | 120 | 68 |
| Extension | 300 | 68 |

# 3. Results

## 3.1 Genomic analysis of the *FAM83H-AS1* transcript

The *FAM83H-AS1* transcript still remains relatively undescribed as to its transcription start site, the true structure of the mRNA transcribed from its genomic loci, and its relationship with the adjacent sense transcript *FAM83H*. Figure 4 displays data from the UCSC gene browser on the *FAM83H-AS1* transcript and the H3K27ac mark in 7 cell lines from ENCODE. The H3K27ac mark is an acetylation modification on the 27$^{th}$ lysine residue in the DNA packaging protein histone 3 (48). This histone modification can be used to distinguish active enhancers from poised enhancer elements. Thus, H3K27ac is an important enhancer mark (49). It is found in close proximity to transcriptional start sites and is associated with the active transcription of many mammalian genes (49, 50).

The H3K27ac data from ENCODE suggest there are two promoter regions in the area between the sense transcript *FAM83H* and antisense *FAM83H-AS1* (Figure 4A). Further, based on RNA-Seq data mapped to the human genome previously generated by the RNA and Molecular Pathology group, the sequencing reads suggests that *FAM83H-AS1* does not have a transcriptional start site at the proposed start site at exon 1, but rather at exon 3 (Figure 4B). It is not known if *FAM83H* and *FAM83H-AS1* share the same promoter. Based on the observations of a more downstream transcriptional start site seen in the RNA-Seq data, and the presence of two promoter regions in the data from UCSC, we hypothesize that there might be separate promoters for *FAM83H* and *FAM83H-AS1*. By further analysis of the RNA-Seq data, exon 3 seems to be joined with exon 4, and exon 4 is connected to exon 5. According to the sequencing reads it does not appear to be an intron between exon 5 and 6, but rather that exon 5, the intron following, and exon 6 make up one large exon.

Based on the observation above, the strategy for knocking out *FAM83H-AS1* was to introduce double stranded DNA breaks upstream of exon 3, within the region that potentially act as a promoter for *FAM83H-AS1*, and within exon 5 (large new defined exon) (Figure 4C).

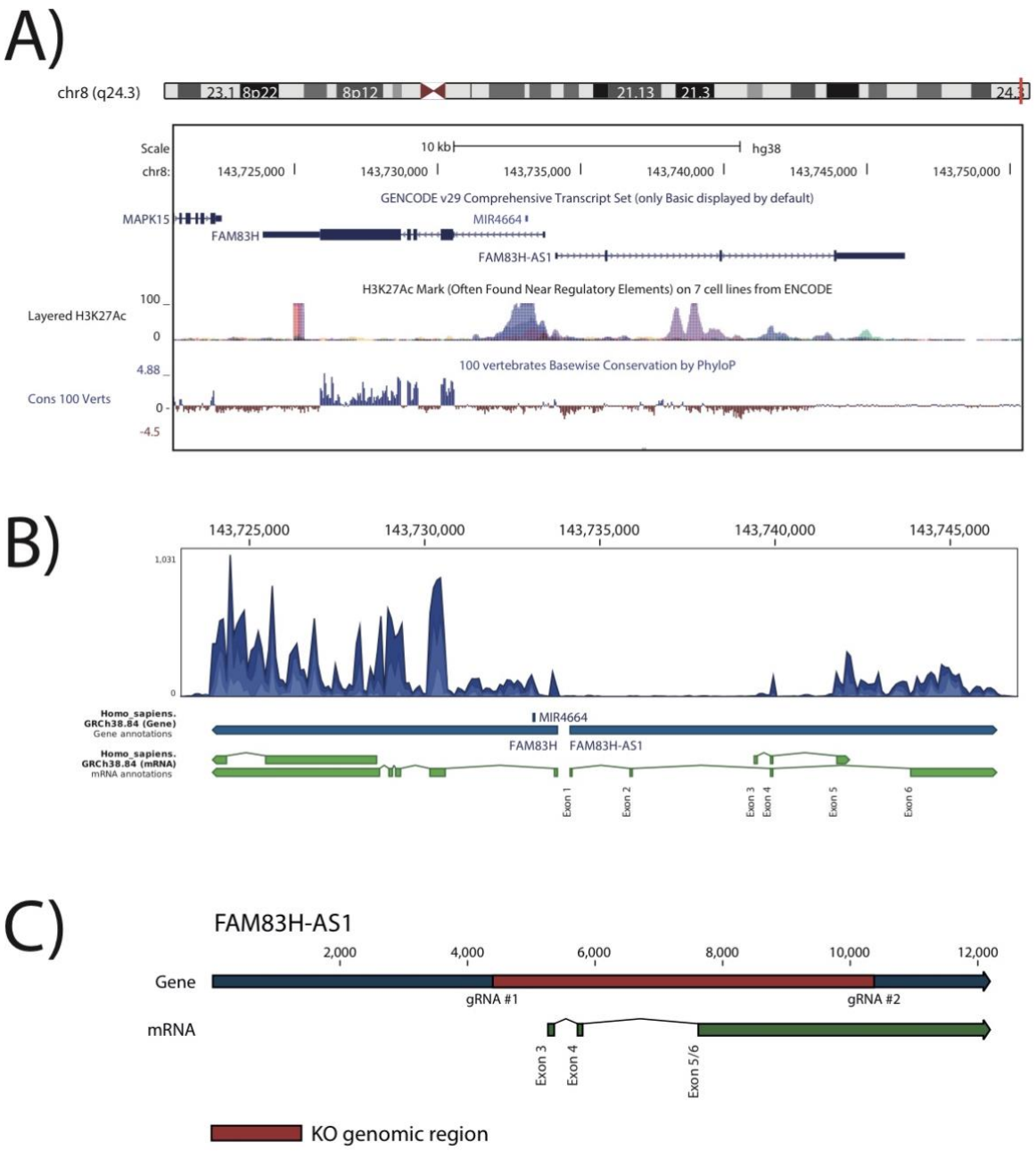



**Figure 4:** Genomic analysis of the *FAM83H-AS1* transcript.
A) Genomic loci of *FAM83H-AS1* extracted from the UCSC Genome browser (https://genome.ucsc.edu, Human Dec. 2013 GRCh38 Assembly). Included is the H3K27ac Mark in 7 cell lines from ENCODE and the 100 vertebrates Basewise Conservation by PhyloP track.
B) RNA-Seq data on *FAM83H-AS1* and the protein coding sense transcript *FAM83H* mapped to the human genome (GRCh38.84).
C) The proposed *FAM83H-AS1* gene with highlighted CRISPR/Cas9 genomic knockout (KO) region, showing the binding sites of the two guide RNAs (gRNA) at the target cut sites.

## 3.2 *FAM83H-AS1* is highest expressed in the breast cancer cell line T-47D

In the initial steps of the project *FAM83H-AS1* expression levels were investigated in cell lines of various types of cancer, to ascertain what cancer type and cell line would be best suited to create a knockout cell line. Recent literature has shown that increased expression of the *FAM83H-AS1* transcript is associated with poor prognosis in colorectal cancer (24), lung cancer (21), and cervical cancer (22), amongst others. Several cell lines were cultured and then screened using RT-qPCR with *FAM83H-AS1* specific primers, including one cervical cancer cell line (HeLa), one colorectal cancer (CRC) cell line (HCT116), one lung cancer cell line (A549) and the breast cancer cell lines MDA-MB-468 and T-47D. From previous investigations the group possesses data on the expression levels of *FAM83H-AS1* in the breast cancer cell lines MDA-MB-231, Hs 578T, BT549, HCC1569, SKBR3, MCF7, and BT474. As seen in figure 5 the highest expression level of *FAM83H-AS1* was found in the breast cancer cell line T-47D. The lung cancer cell line A549 has a relatively low expression of *FAM83H-AS1*, as does the cervical cancer cell line HeLa and the CRC cell line (HCT116). Thus, it was decided to carry out the CRISPR/Cas9 knockout in T-47D cells.

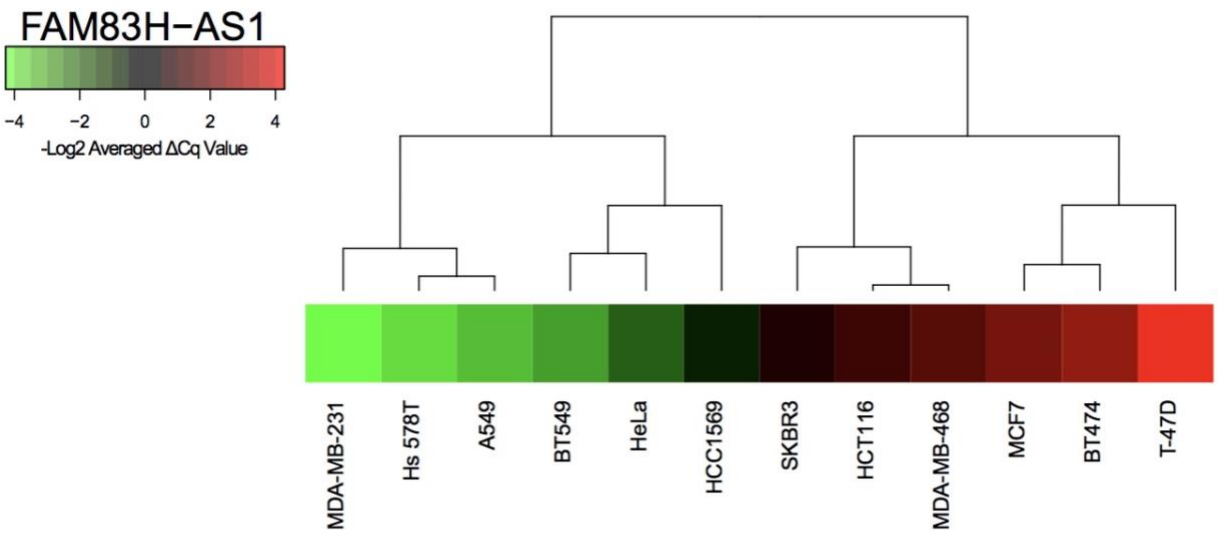



**Figure 5:** Heat map showing average ΔCq values of *FAM83H-AS1* in multiple cell lines. The highest and lowest expression levels are seen in the breast cancer cell lines T-47D and MDA-MB-231, respectively. The lung cancer cell line A549 and cervical cancer cell line HeLa show the lowest expression of the three other cancer types screened, followed by the CRC cell line HCT116 and breast cancer cell line MDA-MB-468.

## 3.3 Only heterogeneous knockout was achieved by CRISPR/Cas9

From the single cell sorted T-47D CRISPR pool we were able to expand 8 clones. The T-47D CRISPR clones are monoclonal populations of cells, where all cells in each clone population contain the same knockout inducing indel profile. The knockout clones can be either heterozygous with knockout of the target gene in only one allele, or homozygous with knockout in both alleles. PCR analysis of the clones was carried out to determine the knockout status of each clone, with DNA from wild type T-47D cells as the control sample.

All samples were analysed with two sets of primers. The first set of primers bind to a target sequence within the CRISPR/Cas9 knockout genomic region, and the second set of primers bind to sequences outside this region, as seen in figure 6A. For the wild type T-47D control sample with the target gene intact in both alleles, a product of 838 bp long is expected with primer set #1, but no product with primer set #2 (as the product would be around 6500 nt long and this is not feasible with the PCR settings used in this setup). For clones with the target gene still present in both alleles we expect the same result as in the wild type T-47D control sample. If a clone is a heterozygous knockout there will be a product with primer set #1, as with the wild type control, and a 508 bp long product with primer set #2. Whereas in homozygous knockout clones there will be no product with primer set #1, but a product 508 bp long with primer set #2.

The PCR results in figure 6B and C show that clone 4, 6, and 8 are heterozygous knockouts, with one intact *FAM83H-AS1* allele. In clones 1, 2, 3, 5 and 7 the target gene is still present in both alleles.

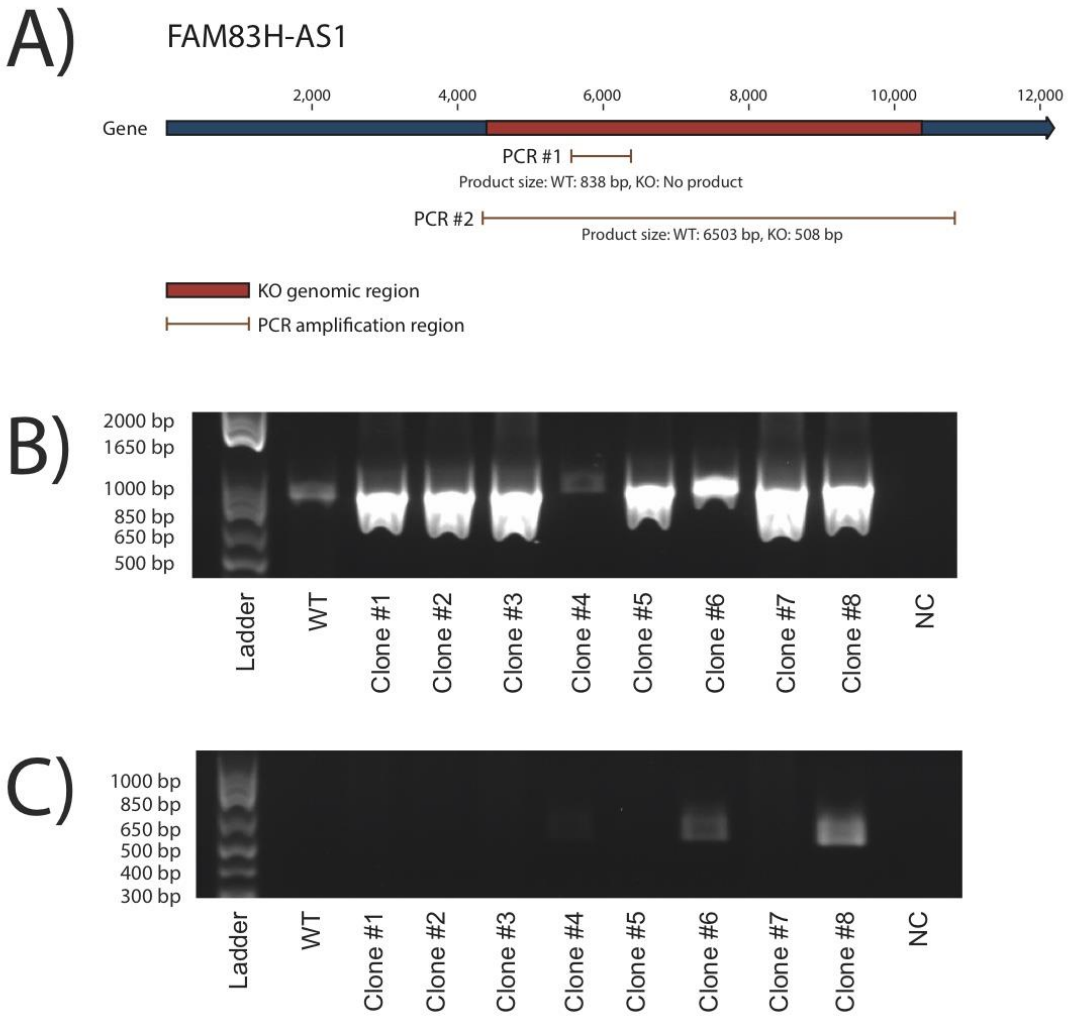



**Figure 6:** PCR results of the CRISPR/cas9 clones.

A) *FAM83H-AS1* gene with knockout (KO) genomic region highlighted in red. Amplification regions of PCR primer set #1 and #2 are shown.

B) PCR results with primer set #1 showing a PCR product in the WT control and in all CRISPR clones.

C) PCR results with primer set #2, showing a PCR product in CRISPR clones 4, 6 and 8. These are heterozygous knock out clones with one allele missing the *FAM83H-AS1* transcript.

## 3.4 *FAM83H-AS1* expression levels are changed in heterozygous knockout clones

The analysis of the clones with RT-qPCR was carried out with specific primer sets towards *FAM83H-AS1* to investigate the RNA expression levels. The first set of primers towards *FAM83H-AS1* is located within the knockout genomic region and set number two located outside this region, as shown in figure 7A. The RT-qPCR analysis showed variable expression levels of the transcript between the knockout clones (figure 7B and C). None of the clones showed complete loss of the transcript.

Knockout clones 1, 2, 3, 5, and 7 with intact *FAM83H-AS1* in both alleles showed similar expression levels to the wild type controls with both primer sets. In the heterozygous knockout clones 4, 6, and 8 a substantial variation in expression levels between the clones was observed. The expression level of clone 6 was downregulated compared to the wild type controls. In contrast, knockout clone 4 showed increased expression levels with both primer sets compared to wild type. Clone 8 showed similar expression levels to wild type controls.

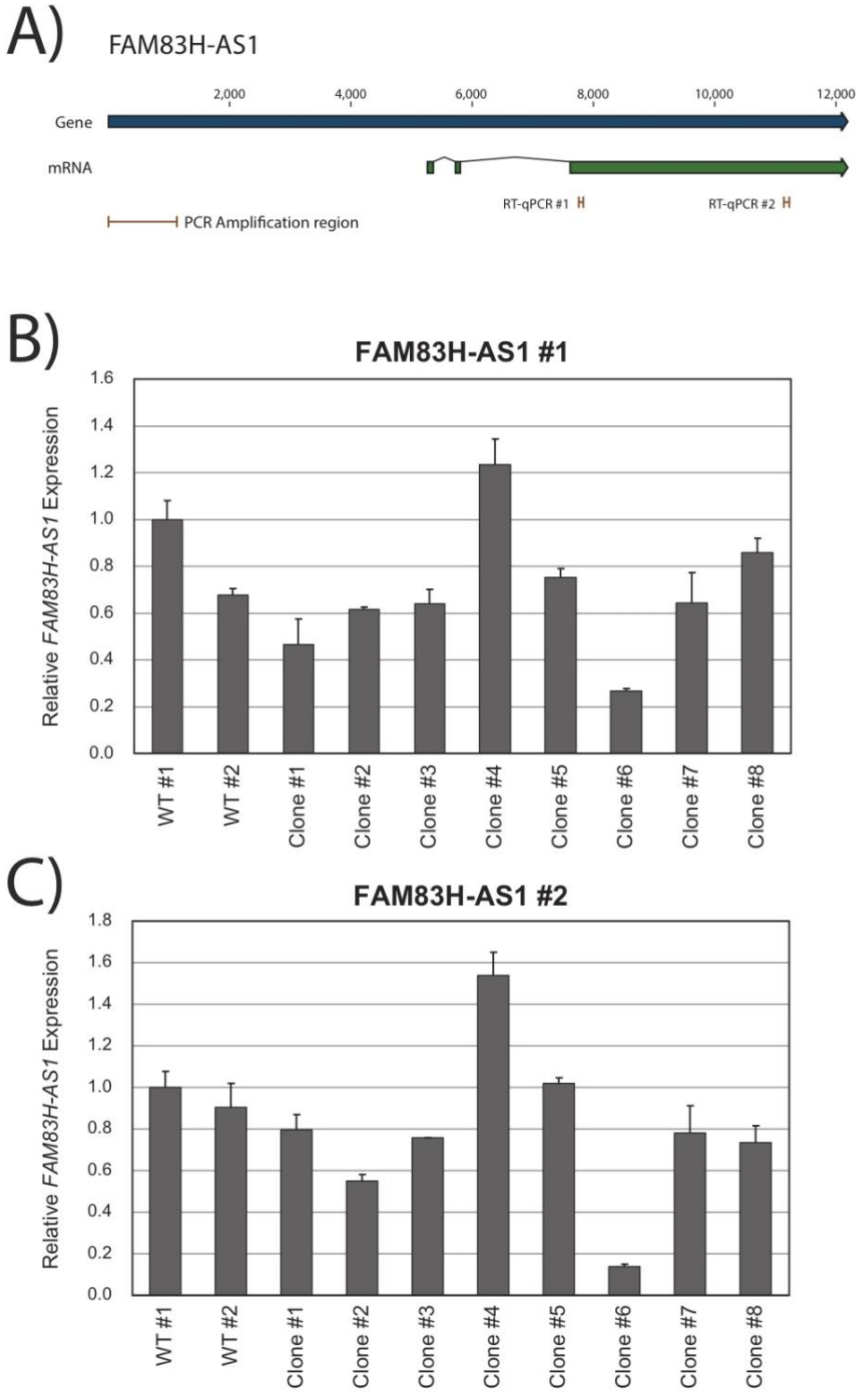



**Figure 7:** *FAM83H-AS1* expression levels analysed by RT-qPCR.
A) Showing the *FAM83H-AS1* gene and its transcribed mRNA, with the location of the RT-qPCR primers within exon 5, and the PCR amplification region.
B) *FAM83H-AS1* expression levels in two wild type (WT) and the eight CRISPR clones with RT-qPCR primer set #1
C) *FAM83H-AS1* Expression levels in two wild type (WT) and the eight CRISPR clones with RT-qPCR primer set #2

## 3.5 The expression level of *FAM83H* is affected by the knockout

Analysis of the clones with RT-qPCR was also carried out with a primer set towards the protein coding sense strand *FAM83H*. The analysis show that the transcription of the protein coding sense strand *FAM83H* and the antisense *FAM83H-AS1* seem to follow each other (r=0.685), as seen in figure 8.

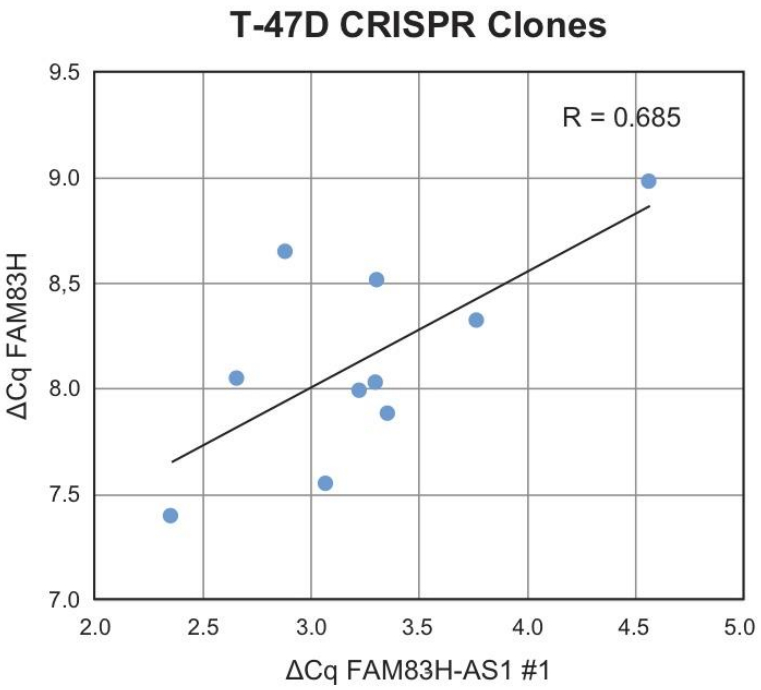



**Figure 8:** Scatter plot showing the relationship between the expression levels of lncRNA *FAM83H-AS1* and the protein coding transcript FAM83H. There is a moderate positive correlation between the two transcripts, R=0.685.

### 3.6 siRNA knockdown of *FAM83H-AS1* in T47D cells does not affect *FAM83H* expression

To further investigate the relationship between the *FAM83H-AS1* and *FAM83H* transcript, we carried out knockdown of *FAM83H-AS1* with three different siRNAs in wild type (WT) T-47D cells (Figure 9A). Expression levels of both transcripts were analysed 24h after the transfection with the siRNAs, and compared with a non-transfected control and two negative siRNA scramble controls (Figure 9B). With transient knockdown of *FAM83H-AS1* the protein coding *FAM83H* sequence was unaffected, as shown in figure 9C.

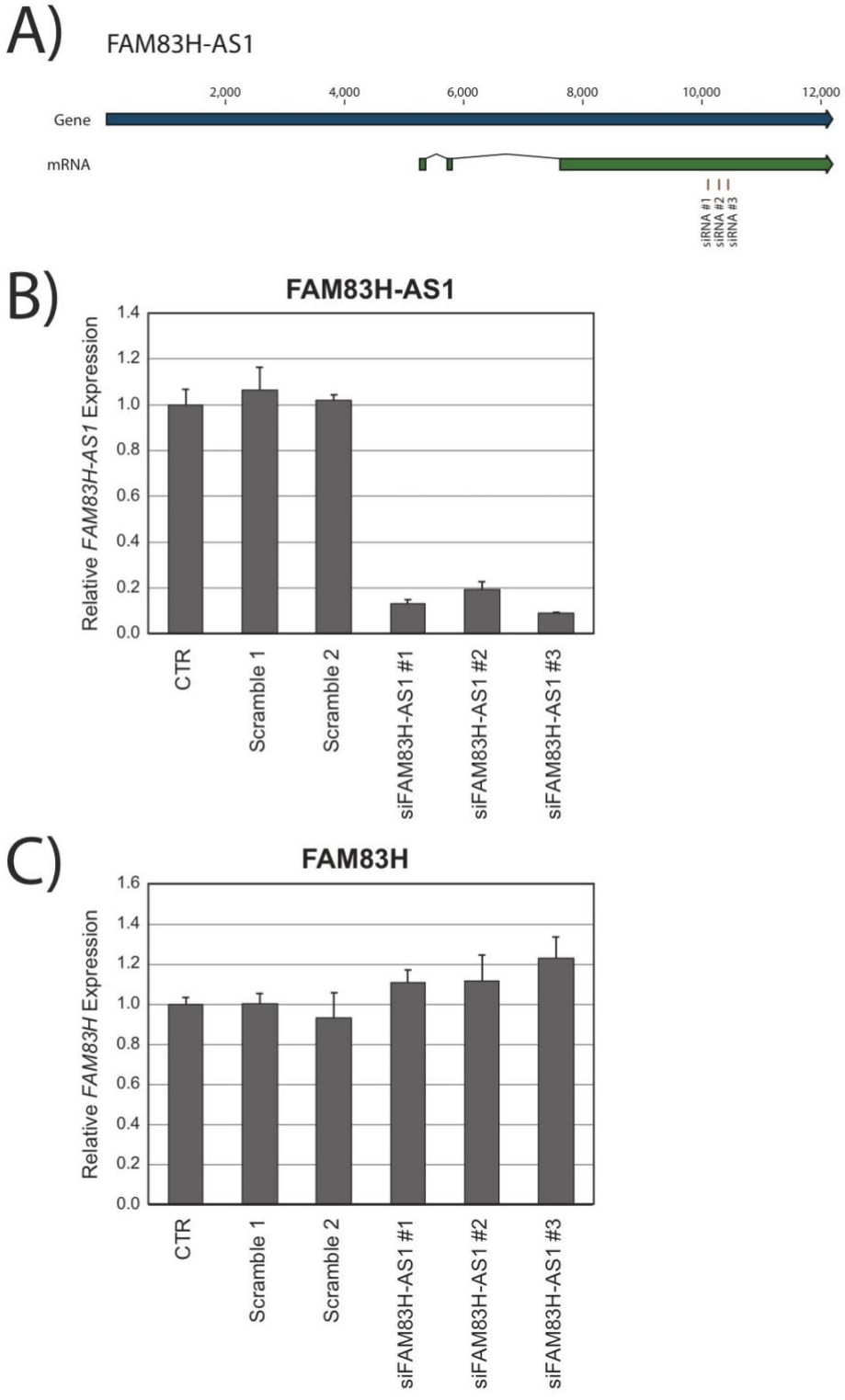



**Figure 9:** Knockdown of *FAM83H-AS1* with siRNAs.

A) Showing the *FAM83H-AS1* gene and mRNA with the location of the three siRNAs in exon 5.

B) Expression levels of *FAM83H-AS1* in wild type (WT) T-47D cells after 24h knockdown with three different siRNAs. Expression levels are compared with a non-transfected control sample (CTR) and two scramble control samples.

C) Expression levels of FAM83H after 24h knockdown of *FAM83H-AS1*.

## 3.7 CRISPR/Cas9 knockout was not successful in additional cell lines

An attempt was also made at creating *FAM83H-AS1* knock out cells in the two epithelial breast cancer cell lines MDA-MB-468 and BT474. However, this attempt was not successful. The BT474 cells did not recover from the electroporation, whereas the MDA-MB-468 CRISPR/Cas9 cells did not recover from single cell sorting with flow cytometry.

## Discussion

As shown in the results from the PCR analyses of the clones, our attempt at creating a CRISPR knockout cell line in T-47D cells resulted in eight clones, where three of the clones were heterozygous knockout cells. In the remaining five CRISPR clones the *FAM83H-AS1* transcript was still present in both alleles. We were therefore not successful in create a homozygous knockout clone. The three heterozygous knockout clones, number 4, 6 and 8, showed variable expression levels of *FAM83H-AS1* by RT-qPCR analysis. Interestingly, there were opposing results in CRISPR clones 4 and 6, with a significant increase in the expression level of *FAM83H-AS1* in clone 4, and a reduction in the expression level in clone 6. These variable expression levels may be due to differences in the location and type of mutations resulting from the CRISPR/Cas9 interference with the *FAM83H-AS1* transcript. The clones have yet to be sequenced, and thus a weakness with this present study is that the location of the cut sites and the resulting changes to the cells' genome remain unknown. Although the target gene is still present in one allele in the heterozygous clones, there might be alterations to this remaining loci of *FAM83H-AS1*, affecting its transcription, structure, and/or function. The increased expression of *FAM83H-AS1* observed in clone number 4 could in this way be a result of interference with regulatory elements at the DNA level causing the loss of negative feedback mechanisms, resulting in a lack of control of transcription. Similarly, the reduction in the expression level of *FAM83H-AS1* in clone 6 could be due to loss of the transcript from one allele, and significant damage to the second allele. The CRISPR/Cas9 technique is a very efficient method when working with protein coding genes, as a frame shift mutation will alter the reading frame and the amino acid sequence of the protein completely, resulting in a loss of function for that protein (31). However, when working with ncRNAs this is not the case, as single nucleotide changes might not be sufficient to alter the function of the ncRNA. Unless a complete removal of the gene is achieved it is difficult to know how point mutations will affect the ncRNAs function.

The results from RT-qPCR analyses of *FAM83H-AS1* and *FAM83H* expression levels of the CRISPR clones, show that there is a positive correlation in the expression of these two transcripts. This suggest a regulatory relationship between the two transcripts, and might imply they are sharing the same promoter or regulatory elements, resulting in coactivation of transcription of the genes. Our initial hypothesis was that the two transcripts do not share the same promoter, but the result from the expression analysis point towards that the second promoter that was

partially removed by the knockout might have some enhancer function towards *FAM83H*. This positive correlation of expression was not observed with knockdown of the target gene with siRNAs that exerts their effect through the RNAi system. Thus, knockdown of *FAM83H-AS1* by degradation of the RNA does not affect *FAM83H*. This suggest that the relationship between the expression of *FAM83H-AS1* and *FAM83H* is not through posttranscriptional mechanisms, again pointing toward important regulatory elements within the DNA sequence of the *FAM83H-AS1* gene with regulatory function on *FAM83H* expression. However, as previously mentioned, from these preliminary results the cut sites and resulting mutations within each clone are still uncharacterized.

We were not able to create a homozygous knockout clone in our chosen cell line and this might be partially due to methodological challenges with the CRISPR/Cas9 method. The electroporation step in the CRISPR/cas9 method is very damaging to the cells, and a large number of cells are unable to recover from this process. The T-47D breast cancer cell line is an epithelial cell line. Epithelial cells are adherent cells that grow in clusters and dependent on their neighbouring cells for growth factors and other signalling molecules. Thus, the optimal condition for epithelial cells in tissue culture is growing in close proximity to each other, as they do not thrive in single cell conditions, making clone expansion after single cell sorting of the CRISPR/Cas9 cell pool challenging. Some of these problems with the CRISPR/Cas9 method can be resolved by gene knockdown through the RNAi pathway. As shown in our results, knockdown of the *FAM83H-AS1* transcript with siRNA gives a high knockdown efficiency, as well as being a quick and uncomplicated technique compared to the time consuming CRISPR/Cas9 method. Further, knockdown with siRNAs by reverse transfection is less damaging to the cells than the electroporation and single cell sorting in the CRISPR/Cas9 method. Although all transfection reagents are toxic to cells, improvements to the chemical composition and structure of transfection reagents and the synthetic siRNAs have made it possible to achieve a high degree of gene knockdown with reduced cell death and off-target effects (46). Theoretically, siRNAs can be designed for any wanted target gene in any cell type, as all cells possess the RNAi system. There are also several commercial sources of siRNAs available that have been functionally validated, making them easily accessible (36, 45, 46, 51). However, siRNAs mainly accumulate in the cytoplasm (40); thus, the effect of siRNAs is limited to genes expressed in the cytoplasm as opposed to the nucleus of the cell. While gene knockdown with siRNAs solve some of the methodological issues with the CRISPR/Cas9 method, the effect of the knockdown is only transient. To overcome the problem of transient vs stable effect of knockdown, as well as the

methodological challenges with CRISPR/Cas9, the use of shRNAs could prove to be a feasible alternative. With the use of shRNAs, a stable integration of the expression construct into the cells genome is possible, resulting in long-term expression of the shRNA which is further processed into functional siRNAs (47). This gives a stable knockdown of the target gene. With the shRNA method it is also possible to create inducible promoters, allowing the transcript of the target gene to be switched on and off (43, 47). Moreover, the use of viral vectors for the delivery of the RNAi components is a good option in cell lines that are difficult to transfect, and might be a more suitable option for future work with our epithelial cell lines. Nonetheless, with shRNAs as with the CRISPR/Cas9 technique, the method is time consuming, taking into account the amount of work that goes into creating and preparing the construct and the following selection of the shRNA positive cells either by drug resistance or fluorescent markers (43).

The creation of homozygous knockout was also attempted in two additional breast cancer cell lines, but also here the attempts were not successful. Not succeeding at creating a homozygous knockout clone might not alone be due to a methodological problem with negative effects of electroporation and single cell sorting, or low efficiency of the CRISPR/Cas9 system, but rather that a homozygous knockout of the target gene is not a viable situation for the cell. This would be somewhat in line with recent literature on *FAM83H-AS1* in various cancer types demonstrating a profound reduction in proliferation with the knockdown of the transcript through the RNAi pathway with siRNAs and shRNAs (21, 22, 24-26), and a mechanism for this reduction with cell cycle arrest in the G2 phase has been proposed (24). Further, a recent study on the resistance to radiotherapy in ovarian cancer show that knocking down *FAM83H-AS1* expression reduces cell viability, leading to more cell death, and knockdown increases the effect of radiation treatment (25). Gene knockdown through the RNAi pathway which targets the mRNA of a chosen gene, will never result in a complete knockdown of the transcript, and the effect is transient. If we hypothesise that *FAM83H-AS1* is indispensable for the cells' survival, with transient and incomplete knockdown there will still be a functional transcript left, allowing the cell to survive and recover. Perhaps interfering with the transcript itself at the DNA level with CRISPR/Cas9 affects the cell in a more profound way with mutations rendering the transcript with diminished or complete loss of function resulting in the cell being unable to survive.

The CRISPR-Cas9 system for gene editing is a relatively new technique, and has been assumed to be fairly specific with little off-target effects. Research carried out to investigate the effects

of CRISPR/Cas9 on the genome has mostly looked at effects close to the target site, and has found that the most common on-target DNA repair associated damage are insertions and deletions (indels) of <20bp (34, 52). Additionally, the research on Cas9 induced lesions have been carried out on cancerous cell lines, who's genome and DNA repair mechanisms in general are abnormal compared with healthy cells and tissues (52).

However, a recent study on the off-target effects of CRISPR/Cas9 in non-cancerous cells and tissues has shown that Cas9 is not as specific as initially assumed. The use of CRISPR/Cas9 resulted in genomic rearrangements involving large indels far away from the intended target site, giving rise to novel mutations in the genome (52). Although this is particularly concerning when it comes to the therapeutical application of CRISPR/Cas9, it must also be taken into consideration in experimental research, as off target effects could be wrongly interpreted as effects of silencing the target gene of interest.

Although not interacting directly with the genome as in the CRISPR/Cas9 method, off-target effects also arises from using both siRNAs and shRNAs. Even though an siRNA might contain an RNA sequence that form perfect base pairing with its target mRNA, the siRNA is not necessarily specific to that mRNA and might cause unwanted off-target effects by degradation of non-target mRNA. In fact, most validated siRNAs have not been extensively tested for such unwanted effects (46). Consequently, there is a high probability of off-target effects occurring with unknown consequences for the functioning of the cell.

Furthermore, there are other unwanted effects with knockdown strategies utilising the RNAi system that must be taken into account. With the RNAi pathway and the introduction of exogenous dsRNA into a cell's cytoplasm the problem of immunostimulatory responses arise. The presence of foreign dsRNA in the cell's cytoplasm triggers the activation of Toll-like receptor 3 (TLR3) and retinoic acid-inducible gene I protein (RIG-I) (46, 51, 53), all part of the cell's endogenous protection mechanism against viral infections. The extent to which the immune response is generated depends on the length of the dsRNA, as well as the concentration of dsRNA in the cytoplasm (37). The introduction of foreign dsRNA to the cytoplasm of a cell and the resulting immune response are stressors that in an experimental setting might impact the results from knockdown and functional assays.

However, in an effort to reduce these unwanted off-target effects, methods are being developed to chemically modify siRNAs to increase their specificity to the target mRNA and making them less detectable by the cell's immune system (46). Such chemical modifications include the addition of 2'-O-methylation of the lead strand of the siRNA, in this way off-target

effects are to an extent avoided while the knockdown effect on the target gene remains intact (36, 51).

## Conclusion

In conclusion, in our attempt at creating a CRISPR knockout cell line we were not successful at making a homozygous clone. However, the effort resulted in three heterozygous knockout clones. Analyses of the clones with RT-qPCR with primers towards the *FAM83H-AS1* transcript as well as the sense *FAM83H* gene, showed variable expression levels of the target transcript in the three heterozygous clones, and the expression level of the sense transcript seems to follow the expression of the antisense transcript in all clones. This suggests a relationship between the sense and antisense gene at the transcriptional level, as this tendency was not seen with knockdown of *FAM83H-AS1* with siRNAs targeting the transcript at the mRNA level.

There are advantages and disadvantages with both the CRISPR/Cas9 method for genome editing and gene knockdown though the RNAi pathway with siRNAs and shRNAs. Knockdown with siRNA is a simple, quick, and efficient method for transient knockdown of the target gene, but is only suitable for cells capable of transfection and for running short term functional assays. Both the CRISPR/Cas9 and shRNA method are time consuming and has some methodological challenges. However, the use of viral transfection for transportation of the RNAi components into the cell in the shRNA method is less damaging to the cells than the electroporation and single cell sorting of the CRISPR/Cas9 cell pool, and might be better suited for knockdown of gene expression in epithelial cell lines that does not thrive in single cell conditions. Viral transfection is also a better option for difficult to transfect cell lines.

With all three methods, off-target effects are an issue. The CRISPR/Cas9 method is not as specific as previously believed, and can lead to large genomic rearrangements far away from the intended cut site. Likewise, siRNAs and shRNAs might be complementary to the target mRNA, but they are not necessary specific, and could bind to other mRNAs and cause degradation or partial loss of function. Further, the introduction of exogenous dsRNA into the cells cytoplasm in the RNAi pathway triggers an immune reaction in the cells by activating Toll-like receptors, as the RNAi pathway is a cellular defence mechanism against viral and bacterial attacks. Depending on the extent of this activation, it causes cellular stress that could affect the results of experimental assays.

The aim of this project was to create a stable knockout cell model that would make it possible to run long term assays to further investigate the cellular function of the *FAM83H-AS1* transcript. As our results show, the CRISPR/Cas9 method might not be the most suitable option for long term knockout of gene expression in the T47D epithelial cell line.

Future work with the T-47D CRISPR clones should include sequencing of the clones' genome to identify cut sites and the resulting mutations in the target transcript, as well as investigating potential downstream effects of knockout of the *FAM83H-AS1* transcript. Functional assays should also be carried out to closer examine the effects of partial knockout of the *FAM83H-AS1* transcript in our heterozygous clones.

# References


1. Pointing CP, Oliver PL, Reik W. Evolution and Functions of Long Noncoding RNAs Cell. 2009;136:629-41.
2. Wapinski O, Chang HY. Long noncoding RNAs and human disease. Trends in Cell Biology. 2011;21:354-61.
3. University Y. Giraldezlab: micro RNAs [Available from: http://www.yale.edu/giraldezlab/miRNA.html.
4. Morris KV, Mattick JS. The rise of regulatory RNA. Nature Reviews Genetics. 2014;15:423-47.
5. Ulitsky I, Bartel DP. Linc RNAs: genomics, evolution and mechanism. Cell. 2013(154):26-46.
6. Mattick JS, Rinn JL. Doscovery and annotation of long noncoding RNAs. Nature Structural Molecular Biology. 2015;22:5-7.
7. T D. The GENCODE v7 catalog of human long noncoding RNAs: Analysis of their gene structure, evolution and expression. Genome Research. 2012;22:1775-89.
8. M L, J K, J J. Long Noncoding RNAs in Metabolic Syndrome Related Disorders. Mediators Inflammation. 2016:1-12.
9. Yang F, Lv SX, Lv L, Liu YH, Dong SY, Yao ZH, et al. Identification of lncRNA FAM83H-AS1 as a novel prognostic marker in luminal subtype breast cancer. Onco Targets Ther. 2016;9:7039-45.
10. Wang K, Chang HY. Molecular mechanisms of long noncoding RNAs. Molecular Cell. 2011;43:904-14.
11. Batista PJ, Chang HY. Long noncoding RNAs: cellular address codes in development and disease. Cell. 2013(152):1298-307.
12. Clark MB, Mattick JS. Long noncoding RNAs in cell biology. Seminars in Cell and Developmental Biology. 2011;22:366-76.
13. Zong X, Tripathi V, Prasanth KV. RNA splicing control: yet another gene regulatory role for long nucleat noncoding RNAs. RNA Biology. 2011;8:968-77.
14. Gibb EA, Vucic EA, Enfield KSS, Stewart GL, Lonergan KM, Kennett JY, et al. Human Cancer Long Non-Coding RNA Transcriptomes. PLoS ONE. 2011;6(10):e25915.
15. Cheetham SW, Gruhl F, Mattick JS, Dinger ME. Long noncoding RNAs and the genetics of cancer. 2013;108(12):2419-25.
16. Yang G, Lu X, Yuan L. LncRNA: a link between RNA and cancer. Biochimica et Biophysica Acta- Gene Regulatory Mechanisms. 2014;1839(11):1097-109.
17. Zhao W, An Y, Liang Y, Xie X. Role of HOTAIR lon noncoding RNA in metastatic progression of lung cancer. European Review for Medical and Pharmacological Sciences. 2014:1930-6.
18. Gupta RA, Shah N, Wang KC, Kim J, Horlings HM, Wong DJ, et al. Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. Nature. 2010;464:1071-8.
19. West JA, Davis C, Sunwoo H, Simon MD, Sadreyev RI, Wang PI, et al. The long noncoding RNAs NEAT1 and MALAT1 bind active chromatin sites. Molecular Cell. 2014;55:791-802.
20. Weakly SM, Wang H, Yao Q, Chen C. Expression and function of a large non-coding RNA gene XIST in human cancer. World journal of surgery. 2011;35(8):1751-6.
21. Zhang J, Feng S, Su W, Bai S, Xiao L, Wang L, et al. Overexpression of FAM83H-AS1 indicates poor patient survival and knockdown impairs cell proliferation and invasion via MET/EGFR signaling in lung cancer. Scientific Reports. 2017;7:42819.

22. Barr JA, Hayes KE, Brownmiller T, Harold AD, Jagannathan R, Lockman PR, et al. Long non-coding RNA FAM83H-AS1 is regulated by human papillomavirus 16 E6 independently of p53 in cervical cancer cells. Scientific Reports. 2019;9(1).
23. Yang L, Xu L, Wang Q, Wang M, An G. Dysregulation of long non-coding RNA profiles in human colorectal cancer and its association with overall survival. Oncology Letters. 2016;12(5):4068-74.
24. Lu S, Dong W, Zhao P, Liu Z. lncRNA FAM83H‐AS1 is associated with the prognosis of colorectal carcinoma and promotes cell proliferation by targeting the Notch signaling pathway. Oncology Letters. 2017;15:1861-8.
25. Dou Q, Xu Y, Zhu Y, Hu Y, Yan Y, Yan H. LncRNA FAM83H-AS1 contributes to the radioresistance, proliferation, and metastasis in ovarian cancer through stabilizing HuR protein. European Journal of Pharmacology. 2019;852:134-41.
26. Bi YY, Shen G, Quan Y, Jiang W, Xu F. Long noncoding RNA FAM83H-AS1 exerts an oncogenic role in glioma through epigenetically silencing CDKN1A (p21). J Cell Physiol. 2018;233(11):8896-907.
27. Xin W, Wenjun W, Man Q, Yuming Z. Novel FAM83H mutations in patients with amelogenesis imperfecta. Scientific Reports. 2017;7(1):6075.
28. Kuga T, Sasaki M, Mikami T, Miake Y, Adachi J, Shimizu M, et al. FAM83H and casein kinase I regulate the organization of the keratin cytoskeleton and formation of desmosomes. Scientific Report. 2016;6.
29. Kim KM, Hussein UK, Bae JS, Park S-H, Kwon KS, Ha SH, et al. The Expression Patterns of FAM83H and PANX2 Are Associated With Shorter Survival of Clear Cell Renal Cell Carcinoma Patients. Frontiers in Oncology. 2019;9.
30. Kim KM, Park S-H, Bae JS, Noh SJ, Tao G-Z, Kim JR, et al. FAM83H is involved in the progression of hepatocellular carcinoma and is regulated by MYC. Scientific Reports. 2017;7.
31. Synthego. Cell Engineering 101: The Complete Guide to CRISPR Knockouts: Synthego. Available from: https://app.hubspot.com/documents/2418554/view/25560344?accessId=4f338d.
32. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity. Science. 2012;337(6096):816-21.
33. Wiedenheft B, Sternberg SH, Doudna JA. RNA-giuded genetic silencing systems in bacteria and archaea. Nature. 2012;482:331-8.
34. Halsne R. CRISPR mindre presis enn først antatt. Tidsskriftet for Den norske legeforening. 2019;139(2).
35. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, et al. Multiplex Genome Engineering Using CRISPR/Cas Systems. Science. 2013;339(6121):819-23.
36. Dana H, Chalabatani GM, Mahmoodzadeh H, Karimloo R, Rezaiean O, Moradzadeh A, et al. Molecular Mechanisms and Biological Functions of siRNA. International Journal of Biomedical Science. 2017;13(2).
37. siRNA Design: Methods and Protocols. Walker JM, editor. Hertfordshire: Humana Press; 2013.
38. Mullard A. FDA approves landmark RNAi drug. Nature Reviews Drug Discovery. 2018;17:613.
39. Bartlett D, Davis M. Insights into the kinetics of siRNA-mediated gene silencing from live-cell and live-animal bioluminescent imaging. Nucleic Acid Research. 2006;34(1):322-33.
40. O'Keefe EP. siRNA and shRNAs: Tools for Protein Knockdown by Gene Silencing. Materials and Methods. 2013;197(3).

41. Nakanishi K. Anatomy of RISC: how do small RNAs and chaperones activate Argonaute proteins? Wiley Interdiscip Rev RNA. 2016;7(5):637-60.
42. Höck J, Meister G. The Argonaute Protein Family. Genome Biology. 2008;9(2).
43. Moore CB, Guthrie EH, Huang MT-H, Taxman DJ. Short Hairpin RNA (shRNA): Design, Delivery, and Assessment of Gene Knockdown. Methods of Molecular Biology. 2010;629:141-58.
44. Elbashir SM, Lendeckel W, Tuschl T. RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes & Development. 2001;15(2):188-200.
45. Xia H, Mao Q, Paulson HL, Davidson BL. siRNA-mediated gene silencing in vitro and in vivo. Nature Biotechnology. 2002;20(10):1006-10.
46. Naito Y, Ui-Tei K. Designing Functional siRNA with Reduced Off-Target Effects. In: Taxman DJ, editor. siRNA Design; Methods and Protocols. Methods in Molecular Biology. Hertfordshire, UK: Humana Press; 2013. p. 57-68.
47. Lambeth LS, Smith CA. Short Hairpin RNA-Mediated Gene Silencing. In: Taxman DJ, editor. siRNA Design: Methods and Protocols. Totowa, NJ: Humana Press; 2013. p. 205-32.
48. Pradeepa MM. Causal role of histone acetylations in enhancer function. Transcription. 2016;8(1):40-7.
49. Creyghton MP, Cheng AW, Welstead GG, Kooistra T, Carey BW, Steine EJ, et al. Histone H3K27ac separates active from poised enhanvers and predicts developmental stage. Proceedings of the National Academy of Sciences of the United States of America. 2010;107(50):21931-6.
50. Tie F, Banerjee R, Stratton CA, Prasad-Sinha H, Stepanik V, Zlobin A, et al. CBP-mediated acetylation of histone H3 lysine 27 antagonizes Drosophila Polycomb silencing. Development. 2009;136:3131-41.
51. Wittrup A, Lieberman J. Knocking down disease: a progress report on siRNA therapeutics. Nature Reviews Genetics. 2015;16(9):543-52.
52. Kosicki M, Tomberg K, Bradley A. Repair of double-strand breaks induced by CRISPR–Cas9 leads to large deletions and complex rearrangements. Nature Biotechnology. 2018;36(8):765-72.
53. Yu M, Levine SJ. Toll-like receptor, RIG-I-like receptors and the NLRP3 inflammasome: key modulators of innate immune responses to double-stranded RNA viruses. Cytokine Growth Factor Rev. 2011;22(2):63-72.

## GRADE

The following pages contains GRADE evaluation of the five articles below:

- Yang F, Lv SX, Lv L, Liu YH, Dong SY, Yao ZH, et al. Identification of lncRNA FAM83H-AS1 as a novel prognostic marker in luminal subtype breast cancer. Onco Targets Ther. 2017;9:7039-45.

- Lu S, Dong W, Zhao P, Liu Z. lncRNA FAM83H-AS1 is associated with the prognosis of colorectal carcinoma and promotes cell proliferation by targeting the Notch signaling pathway. Oncology Letters. 2017;15:1861-8.

- Barr JA, Hayes KE, Brownmiller T, Harold AD, Jagannathan R, Lockman PR, et al. Long non-coding RNA FAM83H-AS1 is regulated by human papillomavirus 16 E6 independently of p53 in cervical cancer cells. Scientific Reports. 2019;9(1).

- Dou Q, Xu Y, Zhu Y, Hu Y, Yan Y, Yan H. LncRNA FAM83H-AS1 contributes to the radioresistance, proliferation, and metastasis in ovarian cancer through stabilizing HuR protein. European Journal of Pharmacology. 2019;852:134-41.

- Zhang J, Feng S, Su W, Bai S, Xiao L, Wang L, et al. Overexpression of FAM83H-AS1 indicates poor patient survival and knockdown impairs cell proliferation and invasion via MET/EGFR signaling in lung cancer. Scientific Reports. 2017;7:42819.

**Referanse:**
Yang F, Lv SX, Lv L, Liu YH, Dong SY, Yao ZH, et al. Identification of lncRNA FAM83H-AS1 as a novel prognostic marker in luminal subtype breast cancer. Onco Targets Ther. 2017;9:7039-45.

**Studiedesign: Ekperimentell studie**

**Grade – kvalitet**
Svak
2

| Formål | Materiale og metode | Resultater | Diskusjon/kommentarer/sjekkliste |
|---|---|---|---|
| To uncover the dysregulated lncRNAs in luminal subtype breast cancer, which might offer potential biomarkers for prognostic evaluation and gene therapy.<br><br>**Konklusjon**<br>796 significantly differentially expressed lncRNAs in luminal subtype breast cancer were identified. The lncRNA *FAM83H-AS1* was identified as a novel independent prognostic marker in luminal subtype breast cancer.<br><br>**Land**<br>China<br><br>**År data innsamling**<br>2016 | **Populasjon**<br><br>**-kasus**<br>Three tissue samples of primary breast cancer. RNA was isolated and then sequenced.<br>**-kontroller**<br>Three adjacent non-cancerous tissue samples. RNA was isolated and then sequenced.<br>**Inklusjons-/eksklusjonskrit.**<br>lncRNA expression in sequenced samples was quantified as Reads Per Kilobase per Million Mapped Reads (RPKM). lncRNAs with sum read counts <10 across all samples were abandoned.<br>RPKM values from sequencing of tissue samples were compared with RPKM expression values of lncRNAs in The Cancer Genome Atlas (TCGA) data set of 626 samples of luminal subtype breast cancer and 105 non-tumorous tissues, as well as corresponding clinical parameteres and follow-up information.<br><br>**Konfunderende faktorer**<br>Only data from TCGA used. Results not shown across different cohorts.<br><br>**Statistiske metoder**<br>EBseq algorithm was used to identify differentially expressed lncRNAs between cancer tissues and adjecent non-cancerous tissues.<br>Differences in RNA expression between | **Hovedfunn**<br><br>**Hazard ratio**<br><br>HR=2.440<br><br>High FAM83H-AS1 expression was the only independent prognostic factor in luminal subtype breast cancer after multivariate Cox proportional hazards regression analysis<br><br>**CI**<br><br>95% CI=1.238-4.807<br>*P*=0,010.<br><br>**Bifunn**<br>FAM83HAS1 was found to be a prognostic marker for all breast cancer subtypes, *P*=0.028. | **Sjekkliste:**<br>• **Er formålet klart formulert?**<br>Yes, the purpose of the study is clearly states.<br><br>• **Er studie design egnet for formålet?**<br>Yes<br><br>• **Valg av kasus og kontroller**<br>Breast cancer tissues and adjacent non-cancerous tissue samples were obtained after informed consent of all participants and with approval of ethical committee.<br><br>• **Diagnosen validert?**<br>The diagnosis of luminal type breast cancer comfirmed by postoperative immunohistorchemistry.<br><br>• **Kan det utelukkes at kontrollgr. fri for aktuelle sykdom?**<br>Yes<br><br>• **Kan resultatene overføres til praksis?**<br>Yes, *FAM83H-AS1* could be used as an independent prognostic marker in breast cancer.<br><br>• **Støtter litteratruen resultatene?**<br>Yes, srecent studies have shown that *FAM83H-AS1* is associated with poor prognosis in several types of cancer.<br><br>• **Styrke:**<br>○ Sequencing data from tissue samples compared with data from TCGA.<br>○ Large data set with 626 samples of luminal subtype breast cancer.<br><br>• **Svakhet:**<br>○ The data is descriptiv. There is not functional data or suggested hypoteses for the functional mechanism of *FAM83H-AS1.*<br>○ Few control samples |

**Referanse:**
Lu S, Dong W, Zhao P, Liu Z. lncRNA FAM83H-AS1 is associated with the prognosis of colorectal carcinoma and promotes cell proliferation by targeting the Notch signaling pathway. Oncology Letters. 2017;15:1861-8.

**Studiedesign: Eksperimentell studie**

**Grade – kvalitet**
Svak-Middels
2

| Formål | Materiale og metode | Resultater | Diskusjon/kommentarer/sjekkliste |
|---|---|---|---|
| The aim of the study is to reveal the dysregulation of FAM83H-AS1 in colorectal carcinoma (CRC) samples and elucidate its underlying associations with the Notch signaling pathway.<br><br>**Konklusjon**<br>*FAM83H-AS1* was found to be upregulated in both CRC tissues and cell lines, wherein acting as an oncogene via activation of the Notch signaling pathway.<br><br>**Land**<br>China<br><br>**År data innsamling**<br>2017 | **Populasjon -kasus og kontroll**<br>40 tissue samples of primary CRC tissues and their paired adjacent tissues<br><br>**Viktige konfunderende faktorer**<br>Not relevant<br><br>**Metoder**<br>Expression profiles of FAM83H-AS1 and two Notch signaling-associated molcules, Notch1 and Hes1, was measured by qPCR reaction and Western Blot analysis.<br><br>A specific shRNA was used to silence FAM83H-AS1 expression in cell lines.<br><br>MTT and colony formation assays were perforemd to measure the growth effect of sileced FAM83H-AS1.<br><br>Pearson $X^2$ test was used to evaluate the association between FAM83H-AS1 expression and clinical features.<br><br>Spearman's correlation analysis was used to analyse the association between FAM83H-AS1 and Notch1 and Hes1 expression levels in CRC tissues.<br><br>Kaplain-Meier method wa used to compare the overal survival curves between highly-expressed and low-expressed FAM83H-AS1 groups via log-rank test. | **Hovedfunn**<br><br>*FAM83H-AS1*, Notch1 and Hes1 were significantly upregulated in CRC tissues, p<0,001.<br><br>*FAM83H-AS1 show* significnt positiv correlation with Notch1 and Hes1. R=0.613<br><br>The expression level of FAM83H-AS1, Notch1 and Hes1 was increased in four CRC cell lines compared with the human colonic epithelial cell line.<br><br>High expression level of FAM83H-AS1 in CRC patients was associated with advanced tumor stage (p=0.004), large tumor size (p=0.002) and worse overall survival (p=0.006).<br><br>FAM83H-AS1 silencing suppressed the expression of Notch1 and Hes1, both in mRNA and protein levels. The effect was reversed by rescue with Jagged-1/Fc.<br><br>Silencing of FAM83H-AS1 significantly inhibits cell viability, proliferation, colony formation and migration.<br><br>**Bifunn** | **Sjekkliste:**<br>**Er formålet klart formulert?**<br>Yes<br><br>• **Er kasus-kontroll design egnet for formålet?**<br>Yes<br><br>• **Rekruttering av kasus og kontroller**<br>CRC tissues and adjacent non-cancerous tissue samples were obtained after informed consent of all participants and with approval of ethical committee.<br><br>• **Diangosen validert?**<br>Differentiation grade, pathological stage, grade and nodal status validated by pathologist<br><br>• **Kan det utelukkes at kontrollgr. fri for aktuelle sykdom?**<br>Yes<br><br>• **Tror du på resultatene?**<br>Yes<br><br>• **Kan resultatene overføres til praksis?**<br>FAM83H-AS1 could be used as a prognostic marker in CRC. The results of the functional mechanism of FAM83H-AS1 contributes to understanding the disease mechanism of CRC.<br><br>• **Støtter litteraturen resultatene?**<br>Yes, several studies have shown the same effect of FAM83H-AS1 on proliferation, migration and invasion.<br><br>• **Styrke:**<br>○ Relativeley large tissue sample size<br>○ Functional assays show that FAM83H-AS1 has a profound effect on proliferation, colony formation and migration.<br>○ The study suggests a functional mechanism of FAM83H- |

**Referanse:**
Barr JA, Hayes KE, Brownmiller T, Harold AD, Jagannathan R, Lockman PR, et al. Long non-coding RNA FAM83H-AS1 is regulated by human papillomavirus 16 E6 independently of p53 in cervical cancer cells. Scientific Reports. 2019;9(1).

**Studiedesign: Eksperimentell studie**

**Grade – kvalitet**
**Svak-Middels**
2

| Formål | Materiale og metode | Resultater | Diskusjon/kommentarer/sjekkliste |
|---|---|---|---|
| To identify host lncRNAs affected by HPV infection, and to identify an HPV-16 E6 regulated gene that is altered from the early stages of HPV infection until carcinogenesis.<br><br>**Konklusjon**<br>The lncRNA *FAM83H-AS1* is up-regulated in primary keratinocytes expressing HPV-16 E6, in HPV-16 positive human cervical cancer cell lines and cervical tumor samples.<br>FAM83H-AS1 is regulated by HPV-16 E6 by p300 and not p53. It is involved with cellular proliferation, migration and apoptosis, and associated with poor survival in cervical cancer patients.<br><br>**Land**<br>USA<br><br>**År data innsamling**<br>2018 | **Populasjon**<br>**-kasus og kontroller**<br>Cervical cancer cell lines (HPV-16,-17 and -18 positive), and cervial cancer tissue samples.<br><br>Non-cancerous cells and tumour samples, as well as and HPV-16 negative cervical cancer cells.<br><br>TCGA data of cervical cancer samples from different stages and normal cervix samples.<br>-196 cervical cancer tissue samples<br>-3 non-cancerous tissue samples<br><br>**Metoder**<br><br>Knock down of *FAM83H-AS1*, P53 and p300 with siRNAs in cell lines.<br>Expression levels were analysed using RT-qPCR analysis.<br><br>Data from the UCSC Genome Browser on potential binding sites within the F*AM83H-AS1* promoter was analysed<br><br>Cellular fractionation was carried out to determine the localization of *FAM83H-AS1*, with U6 as a nuclear RNA control and $\beta$-actin mature mRNA as a cytoplasmic RNA control | **Hovedfunn**<br><br>*FAM83H-AS1* was found to be upreglated in cervical cancer cell lines and tissue samples, and is associated withh worse overall survival (p=0.027).<br><br>Higher expression of *FAM83H-AS1* was found in all the HPV-16 positive cell lines compared with primary human cervical keratinocytes.<br>Lower expression levels were found in HPV-16 negative cell lines.<br><br>P53 is a major HPV E6 target, which is involved in cell proliferation, DNA repair and apoptosis. Knock down of p55 did not alter expression levels of *FAM83H-AS1*. This indicates a a regulation by HPV-16 E6 in a p53 independent manner.<br><br>Three predicted p300 binding sites in the promoter of FAM83H-AS1 was found using the UCSC Genome Browser. Knock down of p300 resulted in reduction of FAM83H-AS1 expression levels. This suggests direct or indirect regulation of FAM83H-AS1 by p300.<br><br>**Bifunn** | **Sjekkliste:**<br>• **Er formålet klart formulert?**<br>Yes, ain of study is clearly stated.<br><br>• **Tror du på resultatene?**<br>Yes<br><br>• **Kan resultatene overføres til praksis?**<br>Research is carried out in cell lines and results cannot automatically be transfered to humans and the clinical treatment of cervical cancer.<br><br>• **Støtter litteraturen resultatene?**<br>Yes, several recent studies have found FAM83H-AS1 to be upregulated in various types of cancer and this is associated with poor prognosis in cancer patient.<br><br>• **Styrke:**<br>○ Reproduced data from other recet studies<br>○ Some functional data with hypothesis of a mechanism of *FAM83H-AS1* involvement in the initiation and development of cervical cancer<br>○ Takes into concideration the nearby *FAM83H* gene, and finds that *FAM83H-AS1* does not regulate tanscription of *FAM83H*.<br><br>• **Svakhet:**<br>○ Few non-cancerous controls in data from TCGA<br>○ Few functional assays<br><br>• **Har resultatene plausible biologiske forklaringer?**<br>Yes |

**Referanse:**
Dou Q, Xu Y, Zhu Y, Hu Y, Yan Y, Yan H. LncRNA FAM83H-AS1 contributes to the radioresistance, proliferation, and metastasis in ovarian cancer through stabilizing HuR protein. European Journal of Pharmacology. 2019;852:134-41.

**Studiedesign: Kasus-kontroll**

**Grade – kvalitet**
**Svak-Middels**
2

| Formål | Materiale og metode | Resultater | Diskusjon/kommentarer/sjekkliste |
|---|---|---|---|
| The study aim to explore the role of lncRNA FAM83H-AS1 in radioresistance and metastasis in ovarian cancer (OC).<br><br>**Konklusjon**<br>FAM83H-AS1 contributes to the radioresistance and cell metastasis in ovarian cancer through stabilising Hu-Antigen R (HuR protein). FAM83H-AS1 could serve as an independent prognostic marker in OC.<br><br>**Land**<br>China<br><br>**År data innsamling**<br>2018 | **Populasjon**<br>**-kasus og kontroll**<br>• OC tissue samples<br>-80 matched human ovarian cancer tissues and corresponding non-cancerous tissues.<br><br>• 4 OC cell lines and 1 normal ovarian epithelial cell line.<br><br>**Hovedeksponering:**<br>Exposure of cells lines to radiation therapy<br><br>**Metoder**<br>Data expressed as the mean +- S.D.<br><br>Differences between groups analysed using a Students *t*-test when only 2 groups, or 1-way analysis of variance when more than 2 groups were compared.<br><br>RT-qPCR used to analyse expression levels.<br><br>Cell transfection with shRNA for gene knock down.<br><br>For patient survival analyses Kaplan-Meyer and log-rank test were performed.<br><br>P< 0.05 was considered statistically significant.<br><br>All experiments were run til triplicates. | **Hovedfunn**<br><br>*FAM83H-AS1* was significantly uregulated in tumour tissue compared to non-tumor tissue. There was a higher expression level in metastatic tissue compared with non-metastatic tissue.<br><br>FAM83H-AS1 expression level is associated with:<br>• Tumour size: P=0.001<br>• FIGO stage: P= 0.023<br>• Lymph node metastasis: P=0.026<br><br>High expression of FAM83H-AS1 was associated with poor prognosis in OC, p=0.015.<br><br>Knock down of FAM83H-AS1 significantly reduces cell proliferation, invasion and migration, and increases sensitivty to radiation.<br><br>**Bifunn**<br>FAM83H-As1 could serve as an independent prognostic marker in OC. | **Sjekkliste:**<br>• **Er formålet klart formulert?**<br>Yes<br><br>• **Er studie design egnet for formålet?**<br>Yes<br><br>• **Diangosen validert?**<br>Yes, by histological examination of tissue samples and commercially purchased OC cell lines.<br><br>**Kan det utelukkes at kontrollgr. fri for aktuelle sykdom?**<br>Yes, determined non-cancerous by histological examination.<br><br>• **Tror du på resultatene?**<br>Yes<br><br>• **Kan resultatene overføres til praksis?**<br>FAM83H-AS1 can be used as an independent prognostic marker in OC.<br><br>• **Støtter litteratruen resultatene?**<br>Yes, several recent studies show similar results.<br><br>• **Styrke**<br>○ Similar results as other recent studies on *FAM83H-AS1.*<br>○ Some functional data on proliferation, invasion and igration as well as shows that *FAM83H-AS1* is involved in resistance to radiotherapy in OC.<br>○ Relatively large tissue sample size and matching controls.<br><br>• **Svakhet**<br>• OC tissue samples only from one population<br>• No strong functional data on FAM83H-AS1.<br><br>• **Har resultatene plausible biologiske forklaringer?**<br>Yes |

**Referanse:**
Zhang J, Feng S, Su W, Bai S, Xiao L, Wang L, et al. Overexpression of FAM83H-AS1 indicates poor patient survival and knockdown impairs cell proliferation and invasion via MET/EGFR signaling in lung cancer. Scientific Reports. 2017;7:42819.

**Studiedesign: Kasus-kontroll**

**Grade – kvalitet**
**Middels** 3

| Formål | Materiale og metode | Resultater | Diskusjon/kommentarer/sjekkliste |
|---|---|---|---|

**Formål**

The study hypothesizes that FAM83H-AS1 may play an oncogenic role in lung cancer progression, with the aim of characterizing this lncRNA for its diagnostic and prognostic potential, and examine which cancer related pathways are affected by it.

**Konklusjon**

MET/EGFR and their downstreams signaling ERK1/2 and AKT may be the targets of FAM83H-AS1 in lung cancer.
FAM83H-AS1 expression was significantly increased in lung cancer, and correlated with worse patient survival.
Knockdown of FAM83H-AS1 impairs tumour cell proliferation, migration and invasion, possibly through cell cycle regulation inducing G2 arrest.

**Land**

USA

**År data innsamling**

2016

**Materiale og metode**

**Populasjon**
**-kasus (definisjon/validert?)**

RNA-Seq data sets from 3 independent tumor cohorts:

- The University of Michigan cohort with 67 lung adenocarcinoma (LUADs) and 6 matched normal tissue samples.
- The Korean cohort with 85 LUADs and 77 normal tissue samples.
- The Cancer Genome Atlas LUAD cohort with 309 LUADs and 73 normal tissue samples.

FAM83H-AS1 expression and its prognostic significance was validated in an independent cohort from the University of Michigan (UM) with 101 LUADs and 19 noraml tissue samples.

**Statistiske metoder**

Data was analyzed using GraphPad Prism 6 and R software.

The Receiver Operating Characteristic (ROC) curve analysis was used for diagnostic accuracy measured by the area under the curve (AUC).

Kaplan-Meyer and log-rank test were used for survival analysis.

Proliferation, migration and invasion data was evaluated by Student's t-test. A two-tailed p value <0.05 was considered significant.

Correlation of proliferation related genes

**Resultater**

**Hovedfunn**

FAM83H-AS1 expression was significantly increased in LUADs, with AUC >0.9 in all 3 cohorts.

Independent validation cohort:

- FAM83H-AS1 expression significantly higher in LUADs than controls: p=0.001 and AUC=0.87.
- FAM83H-AS1 was not associated with tumor stage, differentation and other clinical variables.

Significantly decrease in proliferation by >30% in 7 out of 12 cell lines after siRNA knockdown of FAM83H-AS1.

FAM83H-AS1 knockdown with siRNA induced cell cycle arrest at the $G_2$ phase determined by flow cytometry.

RT-qPCR was performed on 20 genes involved in pathways of MET/EGFR, cell cycle and EMT:

- MET mRNA was decreased by 40% after siRNA knockdown of FAM83H-AS1
- Other genes were not affected

**Bifunn**

**Diskusjon/kommentarer/sjekkliste**

**Sjekkliste:**

- **Er formålet klart formulert?**

Yes

- **Er studie design egnet for formålet?**

Yes

- **Diangosen validert?**

Yes, by histological examination of tissue samples and commercially purchased cell lines.

- **Kan det utelukkes at kontrollgr. fri for aktuelle sykdom?**

Yes, done by histological examination of tissue samples.

- **Var kasus-kontrollgruppene hentet fra sammenlignbare befolkningsgrupper?***

Yes

- **Tror du på resultatene?**

Yes

- **Kan resultatene overføres til praksis?**

It is possible that FAM83H-AS1 can become a prognostic and diagnostic marker, as well as a potential therapeutic target in lung cancer.

- **Støtter litteratruen resultatene?**

Yes

- **Styrke**
  - Analysis of a large set of data with several different cohorts from different populations showing the same results.
  - Suggests a mechanism for *FAM83H-AS1* involvement in lung cancer via MET/EGFR siganling.
  - Similar results to other recent studies on FAM83H-AS1

- **Svakhet:**