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Am Institute of Molecular Biology

in Mainz

Arbeitsgruppe um Dr. Falk Butter

Betreuerin: M. Sc. Patricia Schupp

Thema: RNA-binding Proteins of ANRIL

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Contents

1. Introduction

1.1 Personal Information

I am a 19-year-old high school graduate from Bavaria. My interest in biology started when I first participated in the German Biology Olympiad in 2020 and was in a laboratory for the first time during the Bavarian Landesseminar. In the third round of the Olympiad in 2022, I got the internship from Förderverein der BiologieOlympiade. After the internship, I want to study biochemistry in Leipzig.

1.2 Institute of Molecular Biology

The Institute of Molecular Biology is a modern and well-equipped research institute in Mainz. It is funded by the Boehringer Ingelheim Foundation and the State of Rhineland-Palatine. The scientists at IMB perform basic research in human development, aging and diseases on a cellular and molecular level (Institute of Molecular Biology, 2020). The IMB provides an informal and welcoming atmosphere and is very international, as many PhD students and postdocs come from abroad.

Figure 1: IMB from above
Institute of Molecular Biology. (n.d.). *Bird's eye view of IMB.* [Photograph]. https://www.imb.de/fileadmin/imb/IMB_Building/BirdsEyeViewfromCala.jpg

During my internship, I was in the group for Quantitative Proteomics led by Falk Butter. The Butter Group focusses on telomere biology, RNA biology, epigenetics and systems biology (Institute of Molecular Biology, 2021). I was supervised by PhD-student Patricia Schupp wo let me work on one of her projects about the lncRNA ANRIL and showed me another project of her.

1.3 Abstract

The long non-coding RNA (lncRNA) antisense non-coding RNA in the INK4 locus (ANRIL) at the human CFKN2A/B locus is associated with cancers and metabolic diseases. ANRIL improves metastasis and the growth of cancerous cells, whereas its knockdown decreases tumor size and increases apoptosis (Kong et al., 2018). As it has been shown that RBPs (RNA-binding proteins) play an essential role in regulating lncRNAs, analyzing the nuclear interaction proteins of ANRIL could explain the regulation of an oncogenic lncRNA and even lead to new therapeutical approaches. The aim of the project was to find interaction partners of ANRIL by performing quantitative proteomics. The experiments have already been conducted with randomly chosen fragments of ANRIL. To respect the influence of secondary structures of ANRIL, the experiment described in this report was specifically performed with exon 3 and 4. In our experiment, we could identify nine proteins binding exclusively to Exon 3 and 4 of

ANRIL. I want to thank the Förderverein der BiologieOlympiade, the Butter Group and especially my supervisor Patricia Schupp for enabling me this great internship.

Die lncRNA ANRIL auf dem menschlichen CFKN2A/B locus ist mit verschiedenen Krebs- und Stoffwechselerkrankungen assoziiert. ANRIL verstärkt Metastasierung und das Wachstum von Krebszellen, während ein Funktionsverlust hingegen die Tumorgröße reduziert und Apoptose verstärkt (Kong et al., 2018). Da bereits gezeigt wurde, dass RPBs eine essenzielle Rolle bei der Regulation von lncRNAs spielen, könnte eine Analyse der nuklearen Interaktionspartner von ANRIL zu einem besseren Verständnis der Regulation von ANRIL und neuen Therapieansätzen führen. Das Projekt, zu dem ich beigetragen habe, zielt darauf ab, RNA-bindende Proteine zu finden und zu quantifizieren. Das Experiment wurde schon einmal ähnlich durchgeführt, allerdings an kleineren ausgewählten Fragmenten. Um den Einfluss der Sekundärstruktur von ANRIL zu berücksichtigen, habe ich speziell die RBPs von Exon 3 und 4 untersucht. Letztendlich konnten wir neun Proteine finden, die speziell an Exon 3 und 4 binden. Ich möchte mich herzlich beim Förderverein, der Butter Group und vor allem meiner Betreuerin Patricia Schupp bedanken, die mir dieses großartige Praktikum ermöglicht haben!

2. Background Information

The human CDKN2A/B locus at 9p21.3 is associated with cancers and metabolic diseases. But the reason for this are probably not the proteins encoded by CDKN2A/B, which are cyclin-dependent kinase (CDK) inhibitors and therefore tumor suppressors, but a lncRNA antisense to these proteins called ANRIL. An indicator for this is that ANRIL regulates gene expression and chromatin modifications. Apart from that, SNPs in the ANRIL gene correlate to different cancers and different metabolic diseases such as type 2 diabetes, myocardial infarction, and stroke. ANRIL increases metastasis and growth of cancer cells, whereas its loss of function decreases tumor size and increases apoptosis (Kong et al., 2018).

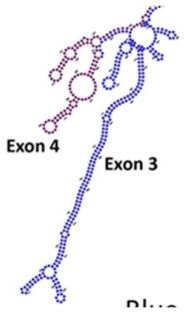

Figure 2: Secondary structure prediction of Exon 3 and 4 of ANRIL based on minimum free energy

The aim of the project is to find interaction partners of ANRIL. Therefore, quantitative interactomics on fourteen non-overlapping spanning the entire length of ANRIL NR_003529 were performed. The project has already been performed with smaler fragments of ANRIL, but as one can see in figure 2, ANRIL has characteristic secondary structures that might influence the results. Therefore, the experiment was repeated with ANRIL cut at the borders of its twenty-one exons.

3. Methods

3.1 Amplification with T7-Promotor and S1-Aptamer using PCR

Polymerase chain reaction (PCR) is used to make millions of copies of DNA segments. To select the DNA segment, two primers, short artificial DNA fragments that are complementary to the starting and ending point of the DNA fragment, are used. The forward primer contains the T7 promotor, which enables T7 RNA Polymerase to start transcription at +1. The reverse primer contains a streptavidin aptamer, which makes the DNA piece bind to streptavidin beads later (Rehm & Letzel, 2010)

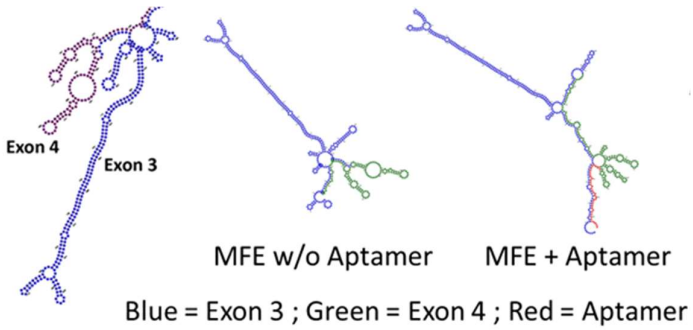

Figure 3: Secondary structure prediction of exons 3 and 4 in ANRIL, exons 3 and 4, exons 3 and 4 with streptavidin aptamer according to minimum free energy

We amplified Exon 3 and 4 of ANRIL. The sequence of ANRIL has therefore already been inserted into a plasmid. To be sure, that all our steps were successful and to find proteins, that are specific for ANRIL, we used pDEST plasmid as a control sequence.

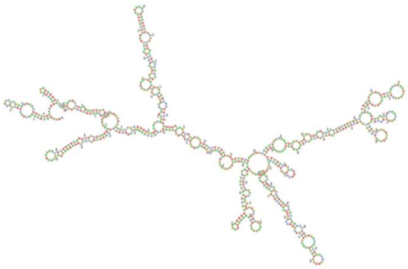

Figure 4: Secondary structure prediction of pDEST 17 according to minimum free energy

Apart from the primers, nNTPs, a buffer and a DNA Polymerase are needed for the PCR. These are mixed in a reaction tube and incubated in the thermocycler for 30 cycles. A cycle begins at 98°C which denatures the DNA helix, continues with annealing at 50°C, during which the primers hybridyze specifically to their complementary sequence and ends with elongation at 72°C which is the optimum temperature of the HF polymerase.

3.2 Agarose Gel Electrophoresis to check amplification

Agarose gel electrophoresis was used to check the amplification of our DNA in the PCR. The gel consists of an agarose matrix filled with water. It allows to separate DNA or RNA by size as the negatively charged nucleic acids move towards the positive pole in the gel camber. As the polymer matrix inhibits the flow of the molecules, smaller fragments can move faster. The molecules are therefore separated by size.

The smaller the gaps between the agarose chains are, the slower the DNA/RNA-fragments move. As we have relatively small pieces of DNA (500-800bp), a 2% agarose gel was used to separate the DNA molecules by size within short time.

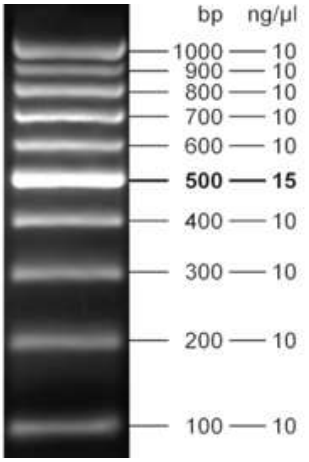

Figure 5: 100 bp DNA Ladder by Jena Bioscience
Jena Bioscience. (2021). 100 bp DNA Ladder: Data Sheet. , 1. https://www.jenabioscience.com/images/PDF/M-214.0003.pdf

To make the DNA pieces visible under UV-light, we added SYBR Safe to our gel. SYBR safe intercalates into the DNA helix, but is safer than ethidium bromide (ThermoFisher, 2016). We added Orange G to our samples, which visualizes the process during the electrophoresis, containing sodium dodecyl sulfate (SDS) for band sharpness and EDTA to stop enzymatic reactions. Then we loaded our samples as well as a DNA marker on the gel and let it run for 75 minutes at 100V. The DNA ladder consists of DNA pieces of defined length. By comparing the DNA ladder (figure 5) to the bands in our gel (figure 6), we could prove, that both our sample (528bp) and the control (802bp) have been amplified correctly.

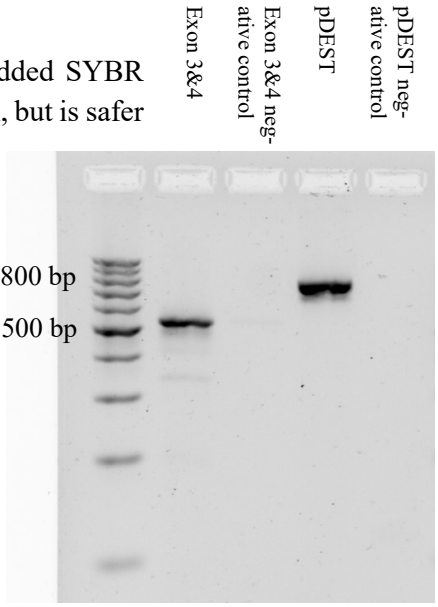

Figure 6: Bands in our 2% Agarose Gel after 75 min at 100V

3.3 T7-in-vitro Transcription of ANRIL "Arms"

We prepared the transcription of the exons by making a Master Mix containing Transcription Buffer, NTPs, T7Polymerase and RNase free water. The transcription buffer keeps the pH-value stable and contains Mg2+ ions which are cofactors for the DNA polymerase. As we have used a forward-primer containing the T7 promotor, we can now use the T7 Polymerase. To keep everything RNase free, we disinfected our working space and equipment with ethanol and worked with RNase free water. Before we added the master mix to the sample, we heated our sample for 3 min at 70°C to get rid of secondary structures. After that we mixed the master mix with each sample in a reaction tube and 2µL stored as a before-transcription control in the freezer. Then we incubated our transcription sample for 5 hours at 37°C. We used a special transcription buffer from the lab that turned murky when transcription is running so we could already see after 1h that our transcription was successful.

To check if our transcription was successful, we made a 2% agarose gel with SYBR Safe. We added 3x Orange G to our post-transcription samples and our pre-transcription controls. Moreover, we made one sample with RiboRuler DNA-Ladder. Before we let them run on the gel, we heated the samples to 70° for 5 minutes to get rid of secondary structures. We let the RNA samples, the RNA ruler and a DNA ladder run for 30 minutes at 100V.

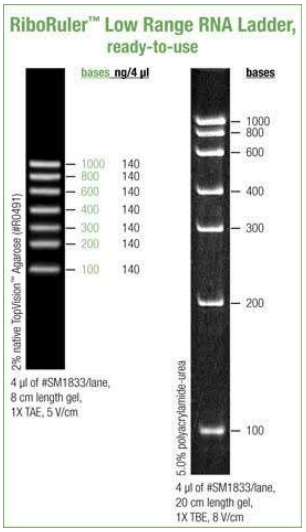

Figure 7: RNA RiboRuler ThermoFisher Scientific. (2019). *RiboRuler Low Range RNA Ladder*. www.thermofisher.com. Retrieved December 9, 2022, from https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0013098_RiboRuler_LowRange_RNA_Ladder_UG.pdf

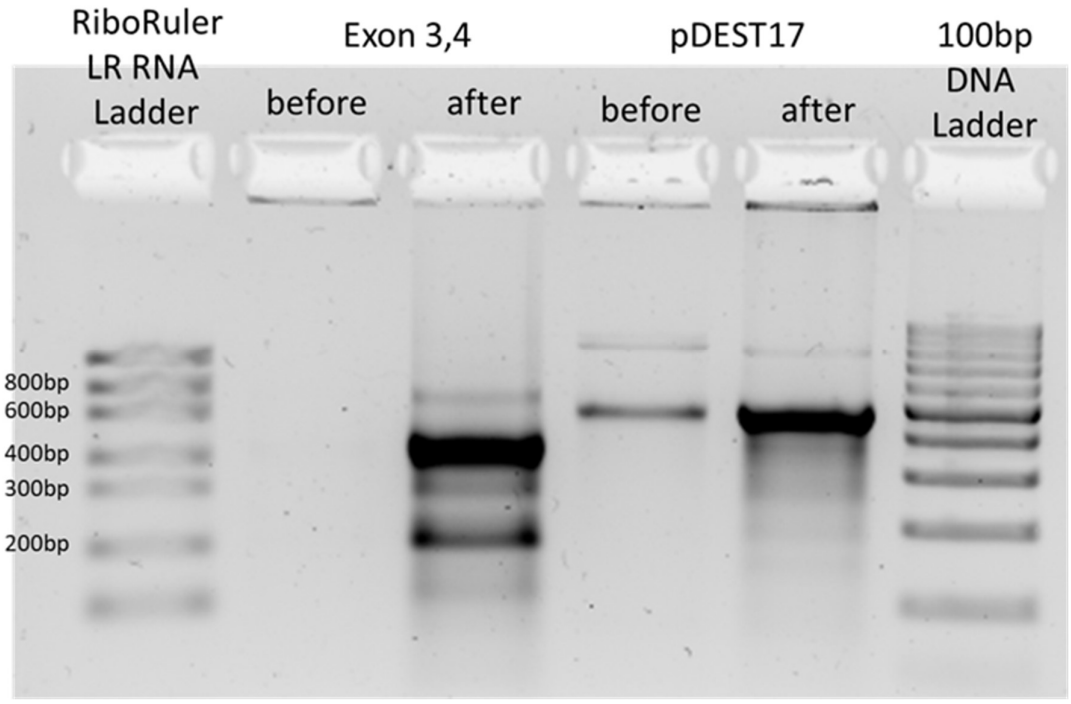

Figure 8: 2% Agarose Gel after 30 min at 100V

The bands at the after-transcription samples were larger than the bands of the before-transcription sample. As shown in figure 8, we could see the band of the pDEST17 control at the expected 800bp, but there were 2 bands for our Exon 3&4 sample. We were not sure if the RNA was degraded but apart from that, our transcription seemed to be successful.

3.4 Phenol/ Chloroform Extraction of RNA

Phenol is non-polar and has a higher density than water. Therefore, proteins and other cell components will dissolve in phenol, and nucleic acids stay dissolved in water. Chloroform supports this effect and ensures the separation of organic and aqueous phase.

First, RNase free water and phenol/ chloroform solution are added to the RNA pellet. Then, the solution is vortexed and centrifuged. Afterwards, the aqueous phase containing RNA is transferred to a new reaction tube.

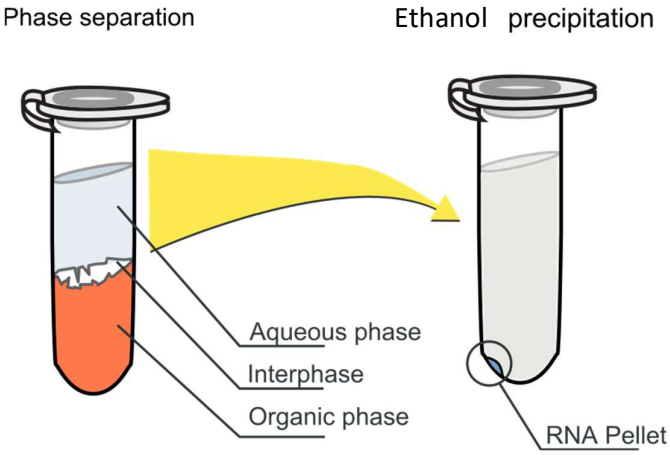

Figure 9: RNA extraction with phenol/ chloroform and ethanol
Difference Between DNA and RNA Extraction. (2018, June 1). [Illustration]. Pediaa.Com. https://pediaa.com/difference-between-dna-and-rna-extraction/

Ethanol has a higher dielectric constant than water and will leave the RNA pellet dehydrated. Afterwards, ions can form salts with the nucleic acids. Ethanol is added to the reaction tube as well as GlycoBlue for better visibility. The RNA-ethanol-solution is cooled at -80°C and then centrifuged. Afterwards, ethanol is carefully removed and water is added. The concentration of the solution and the purity of the RNA is measured by Nanodrop spectrometry.

3.5 Measure concentration with Nanodrop

The Nanodrop allows to quantify and analyze the purity of nucleic acids or protein samples easily and quickly. Only 1.0 to 2.0 µL of the sample that are directly pipetted onto the pedestal, but no cuvettes are needed. Nucleic acids have an absorbance maximum at 260nm (see figure 10) (Thermo Fisher, n.d.). The ratio of absorbance at 260 nm to the absorbance at 280 nm is used as a measure of purity in extractions of nucleic acids. A 260/280 ratio of ~1.8 is considered to be pure RNA, a ratio of ~2.0 is accepted as pure DNA. A ratio smaller than 1.8 means protein contamination (Thermo Fisher, 2012). The ratio of absorbance at 260 nm to absorbance to 230nm should be above 2.0. A lower ratio would indicate contaminations with wash solution, salts, phenol or proteins.

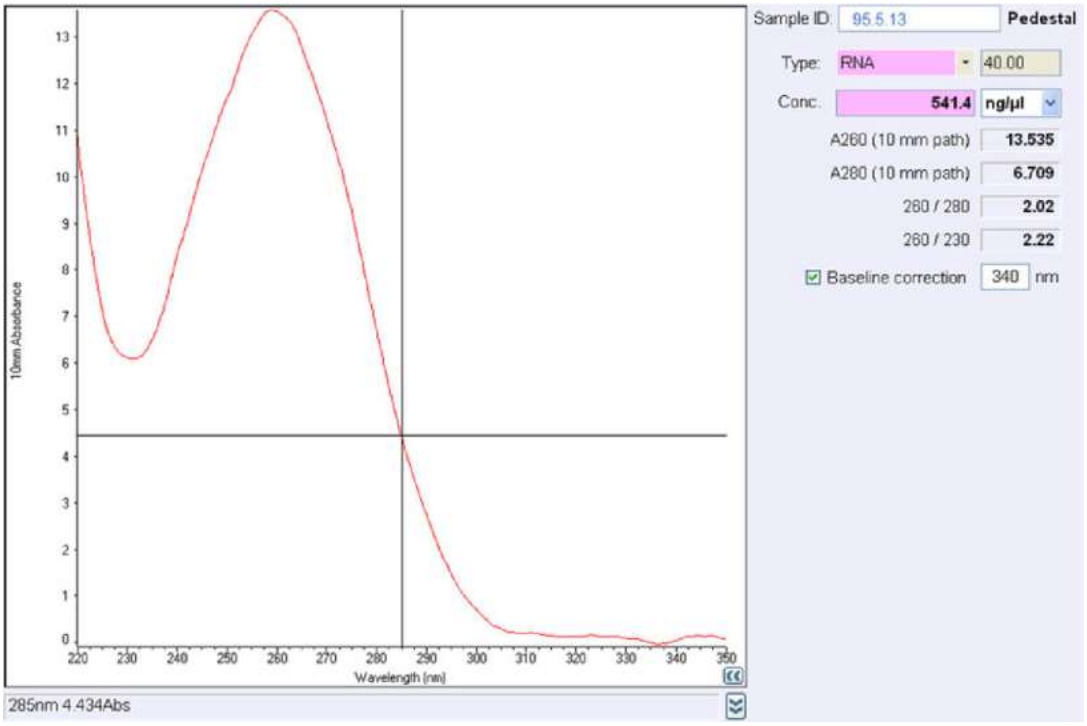

Figure 10: Nanodrop: RNA having absorbance maximum at 260 nm
Thermo Fisher. (2012, January). *Interpretation of Nucleic Acid 260/280 Ratios*.

Sample ID	User name	Date and Time	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230
Exon 34RNA	Schupp, Patricia	7/28/2022 1:13:33 PM	1553.5	ng/µl	38.837	20.919	1.86	2.17
pDEST 17 RNA	Schupp, Patricia	7/28/2022 1:15:11 PM	1481.6	ng/µl	37.040	19.131	1.94	2.25

Table 1 Nanodrop results of RNA samples

As one can see from our results (table 1), we can assure that our samples are pure RNA as the A260/A280 rations are between 1.8 and 2.1 and the A260/A230 ratios are above 2.0.

3.6 RNA binding test

The purpose of the RNA binding test is to check the binding capacity of the prepared RNA to magnetic Streptavidin C1 beads before performing the pull-down experiment. As we added the S1-aptamer in our amplification, the RNA samples should bind to the beads.

We prepared an RNA binding buffer and an RNA wash buffer. With this binding buffer, we washed the paramagnetic streptavidin beads, added our RNA samples and collected the supernatant in separate tubes. After incubating the samples at 4°C, we added Orange G master mix, which made the solutions turn brownish. We prepared the RNA RiboRuler with the same MM (Master Mix). Afterwards, we heated the samples in the Thermomix while shaking and loaded everything except the beads onto a 2% agarose gel.

The aim of this was to see that there were bands at the samples that had contained beads, which meant that our RNA samples were able to bind to the magnetic beads because of the S1 aptamers. The RNA that did not bind to the beads was washed away and was left in the supernatant. If there was also a band at the supernatant samples, this means, that the beads were saturated with RNA. As you can see in figure 12, the beads were saturated with RNA and our RNAs are able to bind to the beads.

As our gel was more concentrated than we had planned, we let it run for another 40 minutes. Unfortunately, one RNA sample was already digested, so we could not see it anymore.

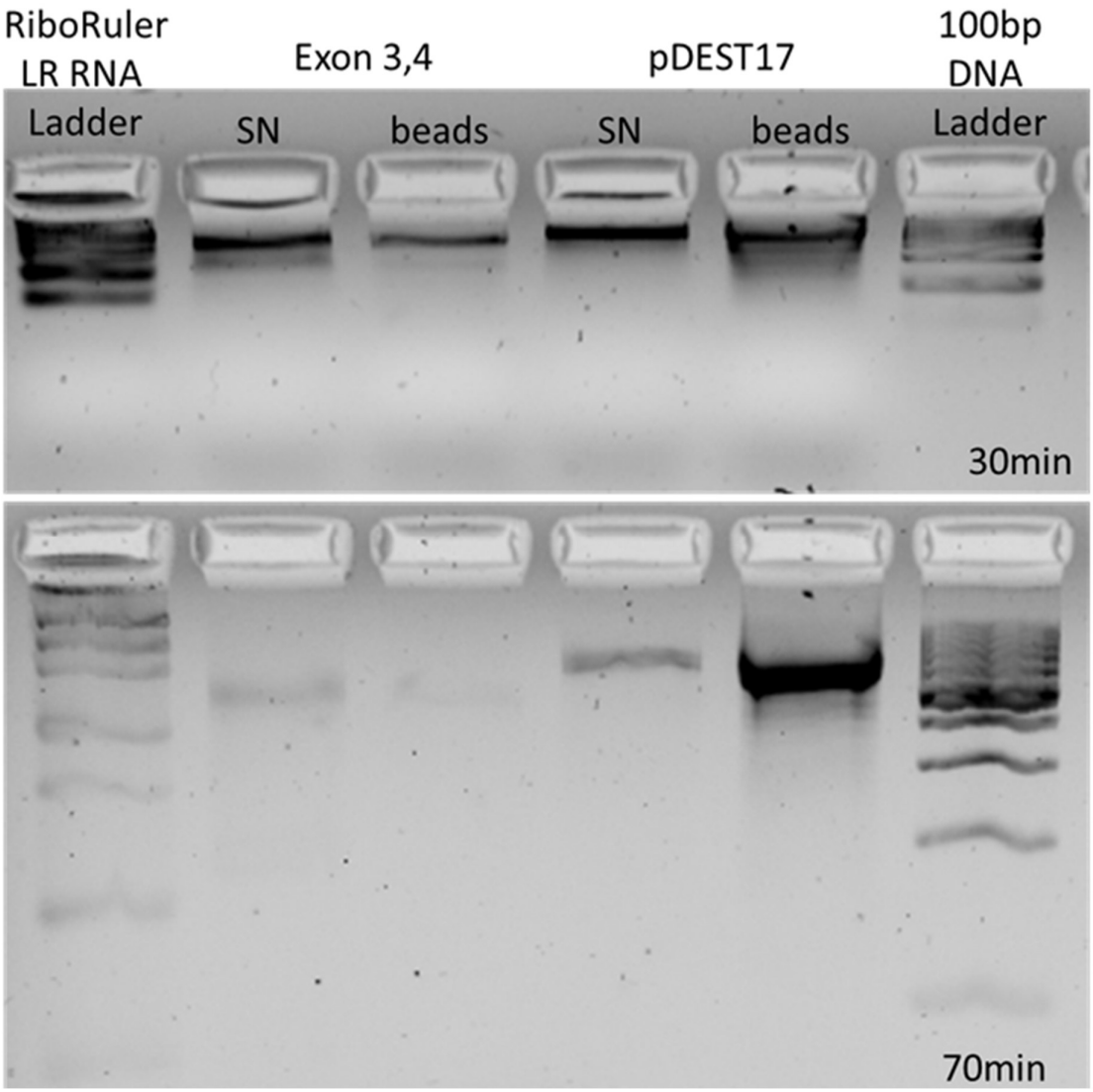

Figure 11: 4% agarose gel after 30 and 70 minutes at 100V

3.7 RNA Pull-down

An RNA pulldown is used to extract the RBP which are later identified by mass spectrometry (MS). Therefore the in vitro synthesized RNA is usually labeled (Cao et al., 2019), but in this experiment we choose a label-free approach (LFQ). The RNA sample is incubated with cellular lysates or nuclear extracts (Cao et al., 2019), in this case we added nuclear Hela extract to streptavidin S1 beads and the RNA samples. The extract leads to the formation of ribonucleoproteins (a complex of RNA and RBP). Apart from that, we added yeast tRNA that competes with the RNA during the pulldown to displace unspecifically binding RNA. Afterwards, the beads are washed again and the supernatant containing the RNA binding proteins is separated by SDS- PAGE. (Popova et al., 2015)

3.8 PAGE-Electrophoresis to separate pulled down proteins

We performed polyacrylamide gel electrophoresis (PAGE) to separate our pulled down proteins. During the SDS-PAGE, proteins are separated by size or molecular weight. Proteins migrate through a polyacrylamide gel containing SDS. SDS masks the intrinsic charge of the proteins by coating their surfaces, forming anionic complexes that have an overall charge proportional to the MW of the protein (Curreem et al., 2012). We prepared a NuPAGE LDS buffer containing DTT (Dithiothreitol). DTT is a reducing agent and cleaves the disulfide bonds of the proteins to get rid of secondary structures. We added the buffer to our sample and loaded this and a marker into the wells. After the gel was run, the gel was incubated with fixation buffer and then the proteins are detected by staining with Coomassie Brilliant Blue.

Then we let the gel destain overnight. As one can see in the picture, there were proteins in all samples.

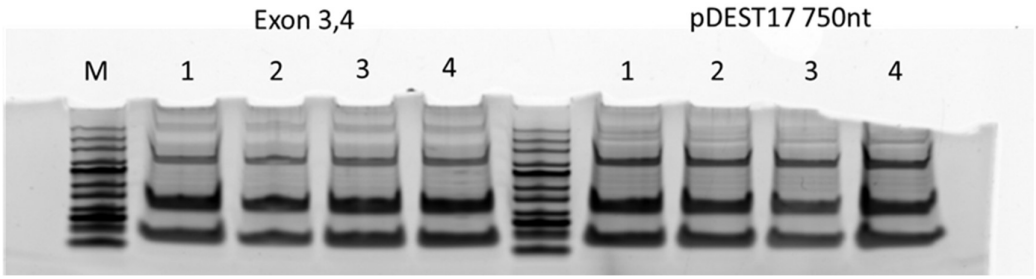

Figure 12: Bands after PAGE-Electrophoresis, stained in Coomassie

3.9 In gel digest

3.9.1 Destaining, linearization and dehydration

For the in-gel digest, the stained proteins of each sample are cut out of the gel into small pieces and transferred into wells of a 96 well plate. Then the proteins are destained with destaining buffer in multiple cycles to get rid of the Coomassie. Afterwards, the samples are incubated with digestion buffer containing ammonium bicarbonate. Then we used reduction buffer which contains dithiothreitol that breaks disulfide bonds in the proteins and linearizes them.

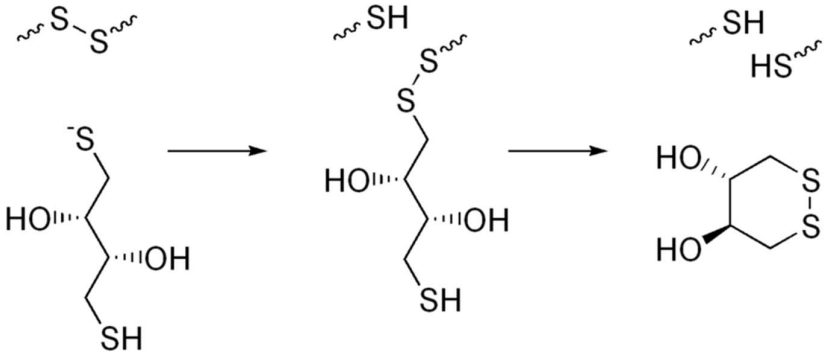

Figure 13: Thiol-desulfide exchanges proceeded by dithiothreitol
Edgar181. (2007, June 13). *Disulfide_reduction_by_DTT* [Illustration]. Commons.Wikipedia.Org. https://commons.wikimedia.org/wiki/File:Disulfide_reduction_by_DTT-2.png

Then we washed the gels with an alkylation buffer containing iodoacetamide that adds iodoacetamide groups to sulfhydryl groups and therefore prevents the formation of disulfide bonds.

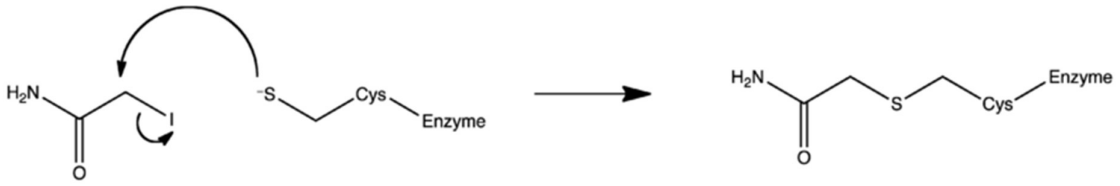

Figure 14: Mechanism of iodoacetamide modification of Cysteine
Mechanism of iodoacetamide modification of Cysteine. (2010, September 30). [Illustration]. Commons.Wikipedia.Org. https://commons.wikimedia.org/wiki/File:Iodoacetamide_mech_wiki.png

Then we used the digestion buffer again because the NH_4HCO_3 solutions provide a pH of 8 which is optimal for trypsin activity. Afterwards, we dried our gel pieces. The last step was to incubate our gel pieces with trypsin solution. Trypsin cleaves specifically at peptide bonds to the C-terminus of lysine and arginine residues, but not at the N-terminus of proline residues yielding peptides with protonable groups at their C-terminus (Rehm & Letzel, 2010). This step generates smaller peptides that can then be loaded into the MS and are easier to analyze.

The next day, we centrifuged our samples at 300 g multiple times to get rid of all the buffer and liquid. We added extraction buffer that contains acetonitrile which is hydrophobic, shrinks the gel pieces and squeezes the peptides out of the gel. The acetonitrile can later be removed from the samples in the speed-vac.

3.9.2 Stage tipping and elution

After the peptides have been isolated, salts and buffers must be removed from the sample. We used a C18 matrix to capture hydrophobic proteins. The peptides bind to the octyldecylsilane matrix whereas salts and buffers are washed off. Later the peptides are eluted from the tips by using acetonitrile buffer and centrifugation.

3.10 MS run

In the final step, the peptides are loaded into the MS, which allows the mass of an analyte to be determined very accurately. First, the samples run through the reversed-phase high performance liquid chromatography (HPLC) that separates the peptides by differentia elution from the column. The peptides are (together with buffer and increasing percentage of ACN) pumped through a non-polar stationary phase. Non-polar molecules and molecules with larger surfaces stay longer in the HPLC column, whereas polar molecules are eluted earlier. The buffer in the liquid phase is used because a changing pH would influence the solubility of an analyte in water. The RP-HPLC columns are washed before and after use to remove residual analytes and buffers (Rehm & Letzel, 2010).

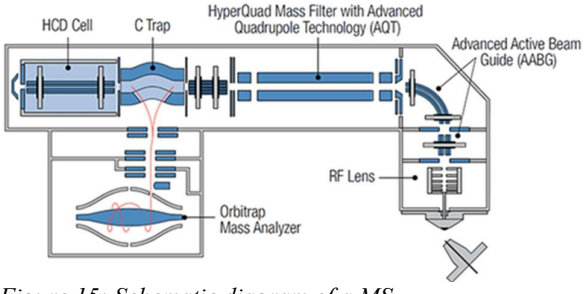

Figure 15: Schematic diagram of a MS
Researchgate.Net https://www.creative-proteomics.com/support/q-exactive-plus-hybrid-quadrupole-orbitrap-mass-spectrometer.html

The molecules eluted are then ionized by electrospray ionization. During the ESI process, a solution of the analyte is sprayed through a nozzle into a vacuum chamber. The solvent evaporates during the spray and the gaseous analyte is left. The ions are then transferred to a mass analyzer that measures m/z values of all peptides in the samples. Apart from that, peptides are separated into even smaller peptides by shooting them with inert gases (Berg et al., 2017). The MS also measures the M/Z values of the fragments of selected peptides. In the end, fragments can be identified by their mass, time and fragment pattern which is enough to identify their amino acid sequence (Rehm & Letzel, 2010). The data obtained is then analyzed with MaxQuant and an R script.

4. Results

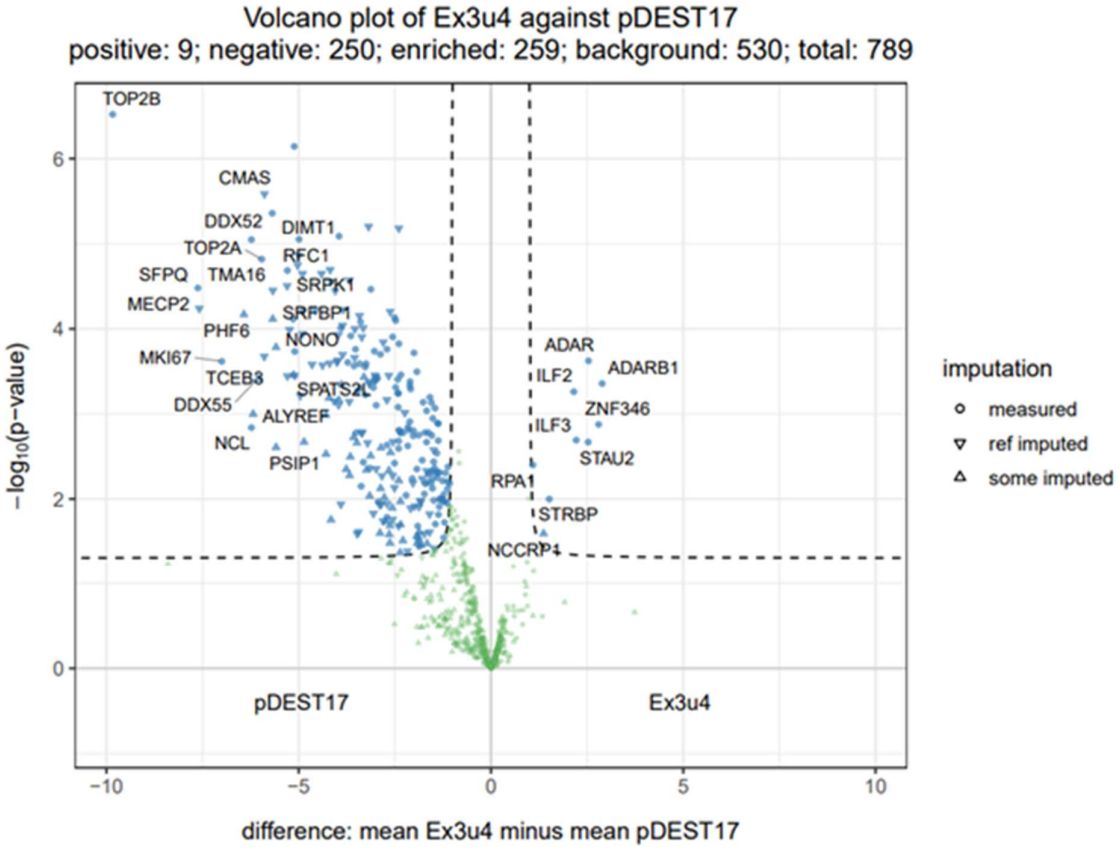

Figure 16: Volcano blot of pDEST 17 against Exon 3 and 4

On the x-axis of the volcano plot, the difference between the log2 of the means of the protein enrichment of the control pDEST17 and the ANRIL exon 3 and 4 is plotted. The y-axis shows -log10 p-value (Welch t-test) of enrichment across replicates, which is the confidence that the proteins are enriched. The background proteins are annotated as green dots. As one can see, there are nine proteins, that are specific for ANRIL exon 3 and 4. As they have already been found in a former experiment (not published yet), this data supports these results.

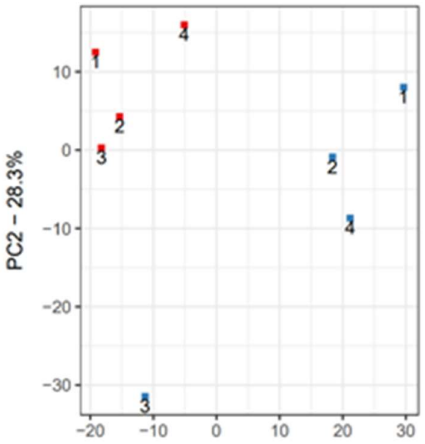

Figure 17: PCA of pDEST17 (blue) and Exon 3&4 (red)

Figure 18 shows the principal component analysis (PCA) of our experiment. PCA is a technique to reduce the dimensions and only uses the most important elements in the data (David & Jacobs, 2013).

In our experiment we could show that there are nine proteins that bind with high confidence to exon 3 and 4 of ANRIL. We assume that they mostly bind to exon 3 because of its greater length and its wide secondary structure that could possibly provide many opportunities to bind proteins.

5. Discussion

Further research needs to be conducted to find all the RBPs or ANRIL. Afterwards, proteins that bind specifically to ANRIL or do not have any other essential function need to be investigated. Finding regulatory mechanisms for these proteins could lead to a strategy to regulate the abundance of ANRIL and a therapy against certain kinds of cancer.

6. Conclusion and Acknowledgement

I want to thank the Butter Group and especially my supervisor Patricia Schupp for welcoming me at the IMB and providing me an insight into their work and research projects. During my internship, I learned a lot of techniques, improved my practical skills was also able to work partly independently on the project. I want to thank Patricia for showing me all the methods and spending so much time preparing and performing the experiments with me. I also want to thank Förderverein der BiologieOlympiade and Falk Butter for enabling the internship.

7. References

Berg, J. M., Tymoczko, J. L., Jr., G. G. J., Stryer, L., Held, A., Maxam, G., Seidler, L., Häcker, B., & Jarosch, B. (2017). *Stryer Biochemie (German Edition)* (8., vollst. überarb. Aufl. 2018 ed.). Springer Spektrum.

Cao, M., Zhao, J., & Hu, G. (2019). Genome-wide methods for investigating long noncoding RNAs. *Biomedicine & Pharmacotherapy*, *111*, 395–401. https://doi.org/10.1016/j.biopha.2018.12.078

Clark BA (honors)Christ's College Cambridge 1973PhD University of Brsitol (England) 1977, David P., Pazdernik, N., & McGehee, M. (2018). *Molecular Biology* (3rd ed.). Academic Cell.

Curreem, S. O. T., Watt, R. M., Lau, S. K. P., & Woo, P. C. Y. (2012). Two-dimensional gel electrophoresis in bacterial proteomics. *Protein & Cell*, *3*(5), 346–363. https://doi.org/10.1007/s13238-012-2034-5

David, C. C., & Jacobs, D. J. (2013). Principal Component Analysis: A Method for Determining the Essential Dynamics of Proteins. *Protein Dynamics*, *1084*, 193–226. https://doi.org/10.1007/978-1-62703-658-0_11

Difference Between DNA and RNA Extraction. (2018, June 1). [Illustration]. Pediaa.Com. https://pediaa.com/difference-between-dna-and-rna-extraction/

Edgar181. (2007, June 13). *Disulfide_reduction_by_DTT* [Illustration]. Commons.Wikipedia.Org. https://commons.wikimedia.org/wiki/File:Disulfide_reduction_by_DTT-2.png

Institute of Molecular Biology. (n.d.). *Bird's eye view of IMB.* [Photograph]. https://www.imb.de/fileadmin/imb/IMB_Building/BirdsEyeViewfromCala.jpg

Institute of Molecular Biology. (2020, December 10). *About the Institute*. IMB Mainz. Retrieved July 26, 2022, from https://www.imb.de/about-imb/aboutintroduction

Institute of Molecular Biology. (2021, February 8). *Quantitative Proteomics*. IMB Mainz. Retrieved July 26, 2022, from https://www.imb.de/research/butter/research

Jena Bioscience. (2021). 100 bp DNA Ladder: Data Sheet. , 1. https://www.jenabioscience.com/images/PDF/M-214.0003.pdf

Kong, Y., Hsieh, C. H. & Alonso, L. C. (2018). ANRIL: A lncRNA at the CDKN2A/B Locus With Roles in Cancer and Metabolic Disease. *Frontiers in Endocrinology*, *9*. https://doi.org/10.3389/fendo.2018.00405

Mechanism of iodoacetamide modification of Cysteine. (2010, September 30). [Illustration]. Commons.Wikipedia.Org. https://commons.wikimedia.org/wiki/File:Iodoacetamide_mech_wiki.png

Popova, V. V., Kurshakova, M. M., & Kopytova, D. V. (2015). Methods to study the RNA-protein interactions. *Molecular Biology*, *49*(3), 418–426. https://doi.org/10.1134/s0026893315020107

Rehm, H., & Letzel, T. (2010). *Der Experimentator: Proteinbiochemie/Proteomics (German Edition)* (6. Aufl. 2010 ed., Vol. 6). Spektrum Akademischer Verlag.

Schematic of the Q Exactive Plus mass spectrometer. (n.d.). Researchgate.Net. https://www.creative-proteomics.com/support/q-exactive-plus-hybrid-quadrupole-orbitrap-mass-spectrometer.html

Schupp, P. (2022, July). *Internship - RPBs*. P. Schupp. Protocols and explanations for my internship

The-concentration-and-purity-of-the-total-RNA-The-22-A260-A280-ratio-indicates-a-high. (2020). [Graph]. Researchgate.Net. https://www.researchgate.net/publication/342584281/figure/download/fig3/AS:90831

7253701632@1593571117089/The-concentration-and-purity-of-the-total-RNA-The-22-A260-A280-ratio-indicates-a-high.jpg

ThermoFisher Scientific. (2019). *RiboRuler Low Range RNA Ladder*. www.thermofisher.com. Retrieved December 9, 2022, from https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0013098_RiboRuler_LowRange_RNA_Ladder_UG.pdf

Thermo Fisher. (n.d.). *NanoDrop: How it works | Thermo Fisher Scientific - NL*. Retrieved July 28, 2022, from https://www.thermofisher.com/nl/en/home/industrial/spectroscopy-elemental-isotope-analysis/molecular-spectroscopy/ultraviolet-visible-visible-spectrophotometry-uv-vis-vis/uv-vis-vis-instruments/nanodrop-microvolume-spectrophotometers/nanodrop-products-guide/nanodrop-how-it-works.html

Thermo Fisher. (2012, January). *Interpretation of Nucleic Acid 260/280 Ratios*.

ThermoFisher. (2016). *SYBR Safe DNA Gel Stain*. https://www.thermofisher.com/content/dam/LifeTech/Documents/PDFs/PG1290-PJ8342-CO128458-SYBRsafe-Green-Fact-Sheets-Corp-FHR.pdf

All other pictures were taken by Patricia Schupp in the lab, by using RNAfold WebServer or by using an R script of Mario Dejung

8. Table of Figures