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Institute of Molecular Biology
Mainz
Group Leader: Dr. Falk Butter
Supervisor: Lara Perez

Study of Telomere-Binding Proteins in *Saccharomyces
Cerevisiae* by Quantative Proteomics

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1. Introduction

1.1 Personal

I am an 18-year-old secondary school graduate who completed a scientific internship at the Institute of Molecular Biology Mainz (IMB) from August 08 - 26, 2016. My interest in biology grew tremendously during tenth grade. In the course of an *American Secondary Schools for International Students and Teachers (ASSIST)* scholarship, I attended the private boarding school *Chatham Hall* that year. During my daily biology classes which were very hands-on, I started becoming particularly interested in molecular biology. For my last two years of school in Germany, I participated in a dual enrollment program (Juniorstudium) at the *University of Hamburg* in the field of Molecular Life Sciences and completed an internship for 1.5 weeks at the *Section for Translational Surgical Oncology* at the *University Medical Center Schleswig-Holstein* in Lübeck in summer 2015. Through these experiences, I started considering a biochemistry major in university. Up to this moment, however, I was never able to actually participate actively in a laboratory, so I was unsure whether I would enjoy the practical part. I am very grateful for this internship which was an award of the *Förderverein der Internationalen BiologieOlympiade e.V.* for my participation in the 3rd qualifying round of the German competition for the International Biology Olympiad in spring 2016.

1.2 About the IMB

The IMB is a 5-year-old institute, funded by the Boehringer Ingelheim Foundation, which is located on campus of the University Mainz. The research focuses on DNA repair, epigenetics, and developmental biology. Inside the building, there are Core Facilities which offer equipment for various fields. During my time at the IMB, the *International Summer School* for bachelor and master students took place. Many of the people working at the institute are international PhD students, so the



Figure 1.2.1: The Butter group in front of the IMB

atmosphere is very vivid and welcoming.

I spent my time with the group of Dr. Falk Butter who studies quantitative proteomics, mostly by using a mass spectrometer. The device is for instant used to identify proteins binding specific DNA or RNA. Lara Perez, a PhD student who studies telomeric binding proteins in several yeast species, was my supervisor during this project.

1.3 Abstract

I used mass spectrometry to study proteins that bind the telomeres of *S. cerevisiae*, the most well known species of yeast (baker's yeast/ brewer's yeast). From Lara's cultures, I chose a cell colony for cultivation, lysed the cells, and purified the proteins. Afterwards, I added the proteins to a DNA bait. This bait DNA had a structure similar to telomeric DNA because I synthesized it with primers of a repetitive sequence from *S. cerevisiae* telomeres. The proteins that bound specifically the target DNA were analyzed using a mass spectrometer. I found a total of 7 proteins significantly more enriched in the bait than in the control. They included RAP1, an essential DNA-binding transcription regulator that is also involved in telomere length maintenance and recruitment of other telomeric binding proteins. The presence of the two mitochondrial proteins ABF2 and MRPL31 shows that the proteins might also bind the streptavidin beads which were used to fix the DNA. According to the "*Yeast Genome Database*", the other proteins which are HMO1, PHR1, MIG2, and SDD4 have so far not been regarded telomere specific.

2. About the Project

2.1 Proteomics

My project can be classified as a proteomics project because the focus was protein profiling by means of mass spectrometry. The mass spectrometer measures the mass-to-charge (m/z) ratio of the peptides in order to detect the corresponding proteins.

2.2 *S. Cerevisiae*

The yeast species *S. cerevisiae*, budding yeast, is the most studied eukaryotic "model organism" (Botstein *et al.* 1997) [1]. Knowledge about proteomics of *S. cerevisiae* has been applied before to study the homologous proteins in humans. At least 71 human genes are mutations of *S. cerevisiae* genes. The "discovery in yeast of two close homologs (RAS1 and RAS2) of the mammalian ras proto-oncogene" (Botstein *et al.* 1997) [1] is an example of such homologs. "Given sufficient nutrients, yeast cells double in number every 100 minutes or so" (Herskowitz 1988), so it is relatively easy to calculate the amount of cells and the phase (lag, exponential growth, stationary, death) of a culture [2]. For the analysis of proteins, cultures in late exponential growth are used.

2.3 Telomeres

Telomeres are the ends of linear chromosomes in eukaryotes. The length of telomeres (the ends of chromosomes) is one of the factors that determine if a cell stops dividing or enters apoptosis (programmed cell death)[3]. Proteins binding to telomeres preserve their structure by preventing chromosome fusion, protecting telomeres from rapid shortening and preventing homologous recombination at chromosome ends [4].

[1] Botstein, David, Steven A. Chervitz, and J. Michael Cherry. "Yeast as a Model Organism." *Science* (New York, N.Y.). U.S. National Library of Medicine, 29 Aug. 1997. Web. 26 Aug. 2016. <<http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3039837/>>.

[2] Herskowitz, I. "Life Cycle of the Budding Yeast *Saccharomyces Cerevisiae*." *Microbiological Reviews*. U.S. National Library of Medicine, Dec. 1988. Web. 26 Aug. 2016. <<http://www.ncbi.nlm.nih.gov/pmc/articles/PMC373162/>>.

[3] De Magalhães, João Pedro. "Telomeres and Telomerase." *The Cellular Timekeepers and Human Aging*. N.p., n.d. Web. 18 Aug. 2016. <http://www.senescence.info/telomeres_telomerase.html>.

[4] Wellinger, Raymond J., and Zakian, Virginia A.. "Everything You Ever Wanted to Know About *Saccharomyces cerevisiae* Telomeres: Beginning to End." *Genetics*, Vol. 191, 1073-1105, August 2012

2.4 Telomere Functions [1]

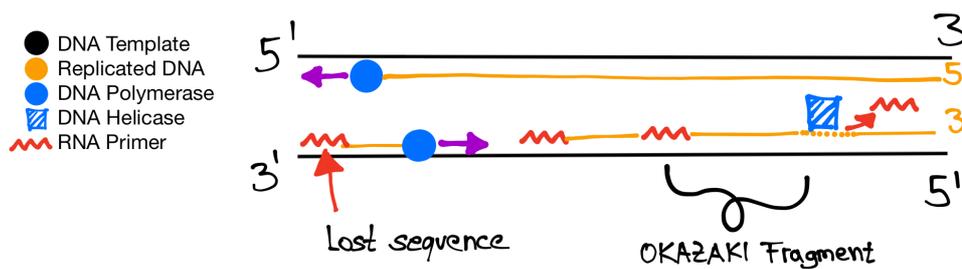


Figure 2.4.1: Scheme of *End-Replication-Problem*

When replicating the chromosomes, the DNA polymerase cannot continue replication all the way up to the 5' end of the lagging strand. The enzyme starts at new primers after replicating a short sequence to build so called *Okazaki* fragments in 5' - 3' direction on the lagging strand. After the final RNA primer is removed, there is a piece of DNA at the very end where the last primer attached that cannot be replicated by the DNA Ligase because there is no sequence on the other side of the primer: "Without DNA to serve as template for a new primer, the replication machinery is unable to synthesize the sequence complementary to the final primer event" (De Magalhães and João Pedro 2014). Thus, a short sequence is lost with each replication cycle. This phenomenon is called the *End-Replication-Problem*. Also, due to the overhang of the 3' end, telomeres from different chromosomes could fuse. The enzyme telomerase is one of the known mechanisms that elongate telomeres and prevent too rapid shortening. The forming of so called t-loops is another important function of telomeres because it protects from chromosome fusing and from being recognized as a double strand break (DSB). Without this capping, telomeres were very similar in structure to DSBs which are subjected to DNA repair machineries.

2.5 *S. Cerevisiae* Telomere Structure [2]

In vertebrates, the telomeric DNA consists of hundreds of tandem repeats of the short base sequence TTAGGG. The structure of the telomeres of *S. cerevisiae*, however, varies in each of the approx. 300bp of simple repeats. The sequence is therefore abbreviated as "C₁₋₃A/TG₁₋₃" (Wellinger 2012). Due to the abundance of Cs and Gs which form three hydrogen bonds, the bonding of the strands is very tight. The 3' strand is G-rich and longer than the C-rich 5' strand. There is also a subtelomeric region in *S. cerevisiae* between the telomere and the rest of the chromosome with a unique structure, consisting of X' and Y' strands. X' regions are repetitive sequences which have few histones and are present in all organisms, while Y' regions are superfluous, similar to euchromatin in nucleoside packing and can move like transposons. In these *Telomere Associated Sequences* (TAS elements), "reporter genes inserted into this region are silenced" (Grigliatti *et al.* 2008) which is known as the *Telomere Position Effect* (TPE) [3]. Due to its special structure, telomeres are bound by particular proteins. These proteins for example recruit telomerase, a ribonucleo protein that is able to elongate telomeres. Other already studied functions include gene silencing and telomere capping.

[1] De Magalhães, João Pedro. "Telomeres and Telomerase." : The Cellular Timekeepers and Human Aging. N.p., n.d. Web. 18 Aug. 2016. <http://www.senescence.info/telomeres_telomerase.html>.

[2] Wellinger, Raymund J., and Zakian, Virginia A.. "Everything You Ever Wanted to Know About *Saccharomyces cerevisiae* Telomeres: Beginning to End." Genetics, Vol. 191, 1073-1105, August 2012

[3] Grigliatti, Thomas A. *et al.* "Telomeric Position Effect-A Third Silencing Mechanism in Eukaryotes." PLOS ONE. N.p., Dec. 2008. Web. 17 Aug. 2016. <<http://journals.plos.org/plosone/article?id=10.1371%2Fjournal.pone.0003864>>.

3. Material and Methods

3.1 Preparation of Target DNA [1]

DAY 1

Preparation of the biotinylated target DNA

Annealing (30 min)

- 12.5 uL forward DNA-strand (100 pmol/uL)
- 12.5 uL reverse DNA-strand (100 pmol/uL) (total ca. 50 ug)
- 5 uL annealing buffer (200 mM Tris-HCl, pH 8.0; 100 mM MgCl₂;
- Fill up to 50 uL with DI water (20 uL ddH₂O)

Primers

forward sequence:

GTGGGTGTGGTGTGGGTGTGGGTGT
GTGTGGTGTGGTGTGGTGTGGGTGTGTGTGT

Reverse sequence:

ACACACCACCCACACCCACACACACCC
ACACACCCACACCACACACCCACACACC

Heat mixture for 10 min at 80 °C and let them cool down to RT. Switch off thermoblock, then 1 h to cool down to ca 38 °C). Take samples out and cool down in rack for ca 30 min @ RT).

Phosphorylation and Polymerisation (2 hours + overnight)

Prepare master mix

- 10 uL 10x T4 DNA ligase buffer (Fermentas)
- 5 uL PEG 6000 (24% (m/v) Polyethylenglycol)
- 1 uL 1M DTT
- 5 uL 100 mM ATP (self made)
- 27 uL H₂O
- 2.5 uL T4 Polynucleotide Kinase (10 U/uL) f.c. 50 units

add 50 uL annealed oligos (1:1 reaction oligos:mix)

Incubate 2 hours at 37 °C, cool to RT

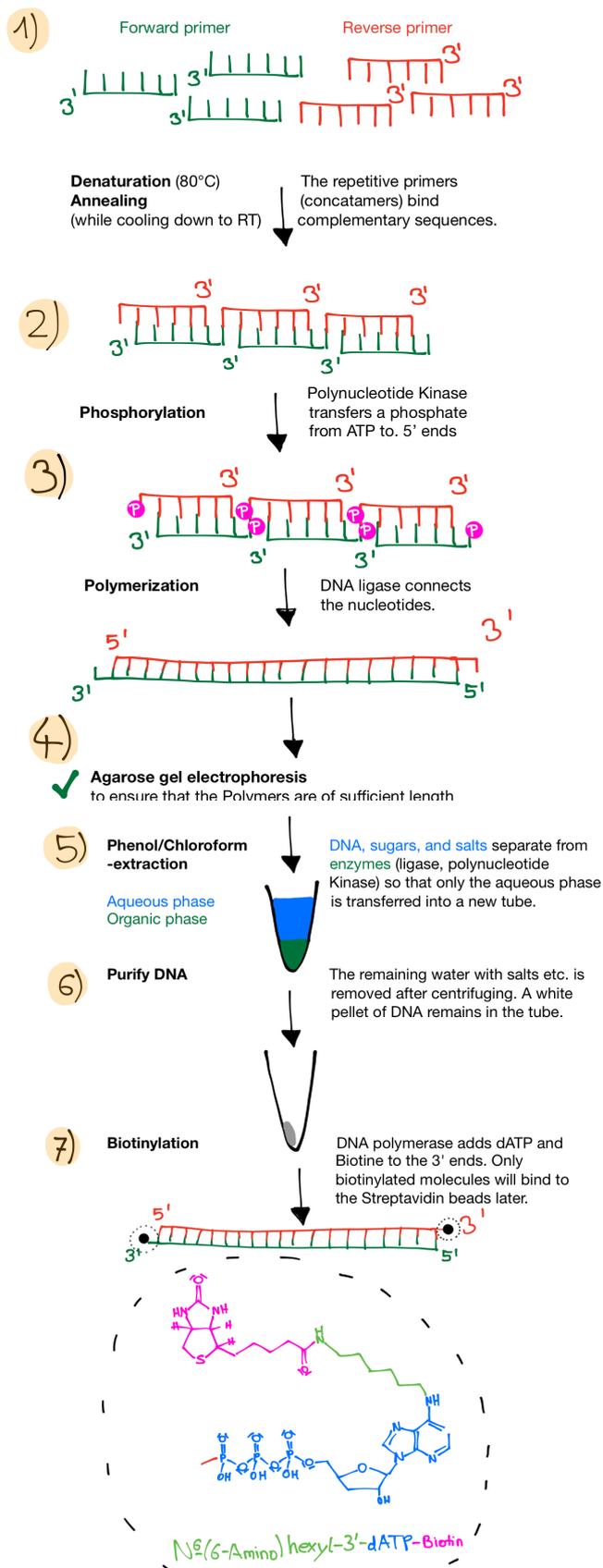
- add 4 uL T4 DNA ligase

Ligate/polymerize oligos at room temperature overnight.

Monitor the process of ligation by agarose gel electrophoresis:

Prepare agarose gel:

- 1.5 g Agarose powder
 - 100 mL TBE buffer (1 x concentrated)
- Heat in microwave and add 4 uL of SYBR DNA stain safe for 1.5% agarose gel.



Prepare mix for pockets:

- 1 uL loading dye
- 2 uL sample
- 3 uL DI water

Fill 5 uL of each mix into the pockets and do gel electrophoresis at 120 V for 30 min.

Average length should be at least 10-mers. (The initial primer length was 25 bp -> 2500 bp minimum bait length).

DAY2

Phenol/chloroform-extraction (2 hours)

- add 2 volume of DI water (250 uL)
- add 300 uL Phenol/Chloroform/IAA (25:24:1) pH 8.0, vortex centrifuge 2 min at 16.000 xg
- Transfer aqueous phase to new tube, mix with 1 ml EtOH 100% (undenatured) room temperature
- Precipitate for 10 min @ -80 C
- Centrifuge at highest power (14 800 x g) for 45 min at 4 °C
- Resuspend pellet in 37 ul DI water

Biotinylation (overnight)

Prepare master mix if possible

- 5 uL 10 x Polymerase buffer (Reaction buffer for Klenow fragment)
- 5 uL 0.4 mM Biotin-7-dATP (*Jena Bioscience, NU-835*)
- 3 uL DNA polymerase 30 Units (klenow fragment exo- 5 U/uL)

Add 13 ul of master mix directly to the pellet resuspended in water.

Incubate at 37 °C overnight

DAY3

Size-exclusion chromatography (5 min)

- Open Sephadex G-50 column and loose lid
- Centrifuge at 2000 rpm for 2 min to remove storage solution
- Add max. 50 uL of biotinylation reaction to the column and centrifuge at 2000 rpm for 1 min into 1.5 mL Eppi

Store the 4 samples at -20 °C.

8)

Size-exclusion chromatography

Sephadex matrix
Purified DNA



The sephadex G-50 column separates the DNA from the DNA polymerase.

9)

The product: Biotinylated target DNA



S. cerevisiae 1, S. cerevisiae 2, control 1, control 2

Figure 3.1.1: Scheme of Preparation of Target DNA

3.2 Cultivating *S. Cerevisiae*

Preparation of 0.5 L DSMZ 393 YPD medium [1]:

- 5.0 g Yeast extract
- 10.0 g peptone from soybean enzyme digest (2x)
- 10.0 g glucose
- 0,5 l Distilled Water (DI water)

Fill into 2 bottles of 0,5 l and autoclave.

Pick a colony derived from a single wt cell from Lara's dissection (*S. cerevisiae* wild type alpha) and spread on agar plate. Leave it in the incubator at 30 °C for 48 h.

Pick two colonies from the plate and give each into a falcon tube with 10 mL of liquid YPD medium. Store at 4 °C over night.

Measure the Optical Density (OD) at 600 nm after diluting the colonies in liquid medium to a 20% mix with water:

- 50 uL *S. cerevisiae* in YPD
- 950 uL DI water

Dilute *S. cerevisiae* in YPD to a OD₆₀₀ of 0.2 in 150 mL:



Figure 3.2.1: Colony after 48 h

[1] List of Recommended Media for Microorganisms." DSMZ: List of Media for Microorganisms. N.p., n.d. Web. 27 Aug. 2016. <<https://www.dsmz.de/catalogues/catalogue-microorganisms/culture-technology/list-of-media-for-microorganisms.html>>.

Use this formula: $V_{\text{final}} \times [C]_{\text{final}} = V_{\text{initial}} \times [C]_{\text{initial}}$

Leave the bottles in the incubator at 30 °C mildly shaking until the OD₆₀₀ is approx. 0.6-0.8. Measure absorbance to ensure the right cell density.

3.2 Cell Lysis and Protein Extraction [1]

Spin 100 mL culture of OD₆₀₀ 0.8-1.0 at 3000rpm. Resuspend pellet in 200 ul IP buffer (without NP40):

- 50 uM Tris pH7.5
- 150 uM NaCl
- 5 uM MgCL₂
- 8 uM PMSF
- 1 tablet Roche per 25mL

Put into fast prep tubes and do 2 x 30 s at full power in BeadMiller/ Fast Prep Instrument with one minute ice in between runs.

Add 800 uL IP buffer with NP40 (2 uL NP40 per 1 mL), vortex buffer quickly and transfer extract into a new Eppendorf tube.

Spin 13 000 rpm for 5 min and put supernatant (SN) into a new tube.

Spin 13 000 rpm again for 5 min and measure protein concentration with Bradford:

Dilute the SN 1:20

- 4 uL extract
- 76 uL DI water

Fill 35 uL of this diluted extract into cuvette and add 1 mL protein dye (1 x concentrated, diluted from 5 x concentrated stock)

For the control to adjust the photospectrometer use 35 uL DI water and 1 mL protein dye (1 x concentrated, diluted from 5 x concentrated stock).

Measure known protein concentration as well: 0.25, 0.5, 1.0, 10.0

Calculate the protein concentration of samples using Bradford method and standard curve with BSA. Freeze lysates at -20 °C until used for pull down.

3.3 Pull Down Yeast Lysates [2]

- 1) Prepare the beads: 50 ul beads/ eppi. Calculate 4 eppis/ sample if using dimethyl-labeling. **Wash the beads 3 times with 500 ul PBB buffer.**
- 2) Prepare biotinylated DNA. Dilute 50 ul DNA (from biotinylation protocol 12 uL) in PBB+DTT buffer. Each 50 uL biotinylated DNA is diluted with 200 uL PBB+DTT.
- 3) Mix beads (1) and biotinylated DNA (2). Incubate 30 min @RT in the rotating wheel.

TOTAL: 2 EPPIS/ SAMPLE. The beads will bind the biotinylated DNA.



Telomeric DNA

110 ul beads resuspended in
200 uL PBB+DTT buffer with 50 uL DNA



Control DNA

110 ul beads resuspended in
200 uL PBB+DTT buffer with 50 uL DNA



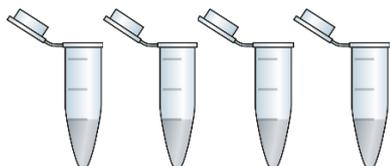
Figure 3.3.1: Washing of beads on magnetic rack

SAVE SUPERNATANT AFTER INCUBATION @ -20 °C!!!! IT CAN BE REUSED UP TO 3 TIMES.

[1] Institute of Molecular Biology Mainz: "CO-IP (Co-immunoprecipitation) all at 4°C"

[2] Institute of Molecular Biology Mainz: "Pull down for yeast lysates using di-methyl labeling afterwards"

- 4) Wash beads with telomere sequence bait and control sequence bait 3 x 200 uL PBB+DTT buffer.
- 5) Prepare a Master mix including:
 - a. 2 ul Salmon Sperm 10 mg/mL(DNA competitor)
 - b. Sample extract containing 500 ug of protein. (Calculate volume)
 - c. PBB+DTT buffer
 - d. Final: 200 uL per eppi. For each sample, 400 uL .Prepare master mix 4.5X per sample.
- 6) Divide the clean beads into the tubes. 50 uL beads/ eppi (resuspend each eppi in 100 uL PBB+DTT and divide in two)



Each eppi 50 uL beads resuspended.

- 7) Add 200 uL of mix to each of the eppis containing 50ul beads.
 - 8) Incubate 1h @4 °C in the rotating wheel (cold room).
 - 9) Wash beads 3 x 200 uL in PBB+DTT buffer (4 times up and down)
 - 10) Resuspend beads VORTEX in 25 uL loading buffer buffer and boil 10 min @70 C in agitation.
- May be freezed at -20 °C to later use. If so, reboil again the mix. Repeat steps 1-10 with supernatant from step 3).
- 11) Place them on rack to remove beads and take the supernatant. Load 25 uL in NuPAGE 4-12% gradient gel. Run 180 V 10 min in MOPS running buffer.
 - 12) Fix with MS-fixing solution 5 min lightly agitated @RT.
 - 13) Stain 15 min @RT with Coomassie Blue and de-stain solution overnight with water.
 - 14) Rinse the gel with purified (VE) water and leave with paper inside a box over night.

PBB+DTT buffer:

0,15 M NaCl + 0,05 M Tris H-Cl pH 7.5 + 0,5 % IQEPAC + 0,005 M MgCl₂ in DI water. Add 1 mM final DTT right before start.

#Loading buffer:

NuPAGE LDS (4X stock) 1X + 100 mM final DTT. 25 ul loading buffer/ well.

- 6.25 uL NuPAGE
- 2.5 uL DTT 1M
- 15.25 uL H₂O

3.4 In-Gel Protein Digestion [1]

DAY 1:

Take a picture of gel with BioRad.

In-gel protein digestion (6 hours + overnight + 5 hours):

1. Place gel on a clean glass plate and rinse with DI water.
2. Excise bands with a clean scalpel (cut as close to the edge of the band as possible).
3. Chop each band into pieces of approx. 1x1 mm and transfer to a fresh Eppendorf tube (8 tubes total).

4. Destain gel pieces with 1 mL **destaining buffer** (50n% Ethanol, 50b% ABC solution), 2 x 30 min at 25 °C on rotating wheel until they are destained or slightly blue. Discard liquid each time. (~1 h). ROTATION.
5. Dehydrate gel pieces in (400 μ L/ eppi) volume of **100% Acetonitrile (ACN)** for 10 min at 25 °C, shaking. Repeat twice until gel pieces are hard and white. Discard the liquid afterwards. ROTATION.
6. Dry the sample in a speed-vac (mode V-AQ) for 5 min or until the gel pieces are bouncing in the tube.
7. Cover the gel pieces in 200 μ L / eppi **reduction buffer** (for 1 mL: 10 μ L DTT, 990 μ L ABC solution) and incubate for 60 min at 56 °C to rehydrate and reduce them. Discard ALL the liquid afterwards. Reduction: 30 min-2 h.
8. Cover the gel pieces in 150 μ L **alkylation buffer** (for 0.5 mL: 50 μ L IAA (0.5 M), 450 μ L ABC solution) and incubate the gel pieces **IN THE DARK** for 45 min at RT (150 μ L). Discard the liquid afterwards.
9. Wash gel pieces with 300 μ L **ABC solution** for 20 min at 25°C. Discard the liquid afterwards.
10. Dehydrate gel pieces in volume (400 μ L) of **100 % ACN** for 10 min at 25 °C, 800 rpm shaking. Discard the liquid afterwards.
11. Repeat steps 9-10. Incubate with 100% ACN until gel pieces are hard and white. Discard liquid afterwards.
12. Dry the sample in a speed-vac (mode V-AQ) for 5 min or until the gel pieces are bouncing in the tube.
13. Rehydrate the gel pieces in the **LysC solution** (for 200 μ L: 158 μ L ABC, 2 μ L Lys C) at 37 °C. 160 μ L should be used to at least cover the dehydrated gel pieces. Incubate over night.

DAY 2

Extraction of peptides:

1. Recover supernatant of trypsin/ lysin digest and transfer into fresh Eppendorf tube.
2. Extract peptides **twice** with 200 μ L **extraction buffer** (30 % ACN, 70 % DI water) for 15 min at 25 °C, shaking at 1400 rpm. After each extraction cycle spin down gel pieces, recover supernatant and combine with step 1 in one eppi. **DO NOT MIX PHASES!!**
3. Dehydrate gel pieces **4 x** with 100 μ L **100% ACN** for 10 min at 25 °C, shaking at 1400 rpm. Spin down and combine supernatant with step 2.
4. Do **NOT** mix the samples this will prolong the drying process.
5. Dry sample in a speed-vac (mode V-AQ) until 10 - 20 % original volume (100 μ L) to remove acetonitrile @ RT. (1.5 hours, heat up to 60 °C if it takes longer)

3.5 Preparation for Mass Spectrometer and Analyzation [1]

Stage Tip Purification

- Methanol
- Buffer A (0.1 % formic acid)
- Buffer B (80 % ACN, 0.1% formic acid)

1. Prepare desalting tips using Empore C18 material 2 layers
2. Activate with 50 μ L methanol: shortly spin 30 sec @ 500 x g to see if material is dense



After 30 sec @ 500 x g liquid level should be slightly below the second line in the tip.



Figure 3.5.1: Stage Tip with C18 Matrix

3. spin for 5 min at 500 x g to elute methanol, **check if through!**

Otherwise repeat.

4. Wash with 50 μ L buffer B, 5 min at 500 x g, **check if through!** Otherwise repeat.
5. Wash with 50 μ L buffer A, 5 min at 500 x g, **check if through!** Otherwise repeat.
6. Add sample and centrifuge 5-10 min at 500 x g (until no liquid is left)
7. Wash column with 50 μ L buffer A, 2 min at 500 x g
8. Elute the peptide in 30 μ L buffer B using a syringe directly in 24-well plate fitting the auto sampler or centrifuge using the tip adapter
9. Speed-vac the plate for 10 min to evaporate the ACN (setting V-AQ)
10. Add 8 μ L buffer A to end up with 14 μ L total volume
11. Pipette half of the volume to another "back-up" plate and freeze at -20°C

Place each sample next to a control and insert plate with samples in the mass spectrometer to be analyzed.

Max Quant Analysis:

Open file from mass spectrometer in MaxQuant and select "Label Free Quantification (LFQ)", LysC" as enzyme. Do not select "fast mode" and start program.

RStudio analysis:

Open file from MaxQuant in RStudio and add to directory. Open library and run protocol from Mario Dejung (LFQ Analysis for *S. cerevisiae*). The program will create different graphs and a volcano blot, showing the enriched proteins. Research the proteins which were incubated with telomere bait significantly versus proteins binding to the shuffle control bait.

4. Results

4.1 Agarose Gel To Control Bait DNA Length

Loading from left to right:

1. 1 Kb marker
2. *S. cerevisiae* bait 1
3. *S. cerevisiae* bait 2
4. Control 1
5. Control 2
6. 100 bp marker

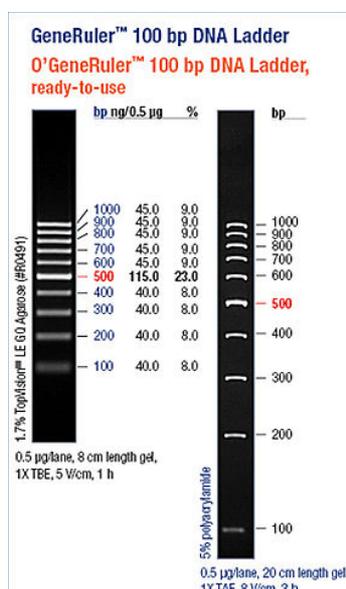


Figure 4.1.2: 100 bp DNA Ladder

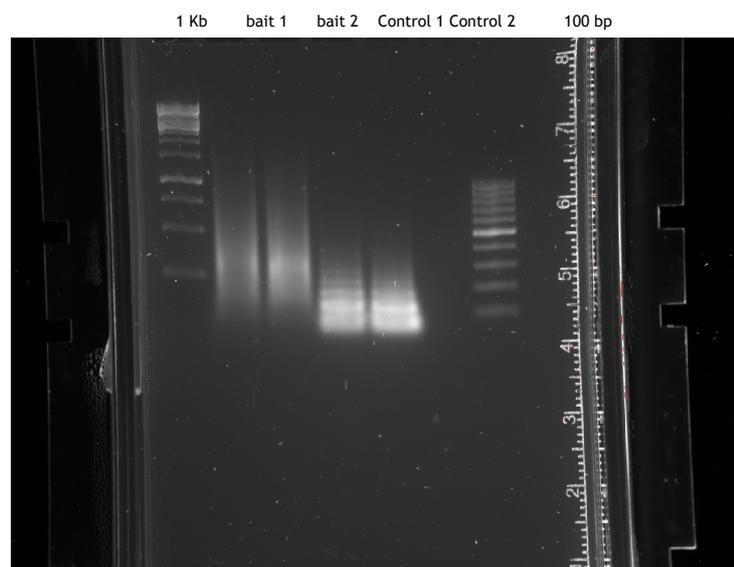


Figure 4.1.1: Agarose Gel

All lanes contain nucleotides and a typical ladder is visible in the control lanes. The length of the telomeric baits is approx. 100bps which can be inferred from comparing the ladder to the 100 bp marker in the lane at the right. The length of the

strands varies a lot, therefore the ladder appears blurry. Due to the repetitive structure of the primer DNA, the length varies more than in the control. The control DNA with a random sequence will be used later to contrast the proteins that bound only the bait to those that bind the random sequence as well.

4.2 Absorbance of Samples and Protein Concentration by Bradford Assay

The Absorbance is measured and due to the known protein concentrations which are measured as well, the X value can be calculated. $OD_{595} = \text{Absorbance}$

$$X = \text{Absorbance} : \text{concentration}$$

Calculate the protein concentration of samples by finding the mean of the x values and dividing the measured absorbance of the sample by this value.

After finding the X value, the protein concentration of the lysate can be calculated.

Actual protein concentration of sample without dilution: $0.6608 \times 20 = 13.216$

Sample	Measured OD	X value	Protein Concentration
Control 0.25	0.4655	1.862	0.25
Control 0.5	0.85	1.7	0.5
Control 1.0	1.3955	1.3955	1.0
Control 10.0	5.021	Not used due to the high OD value	10.0
<i>S. cerevisiae</i>	1.092	(mean of all above) 1.6525	0.6608

Figure 4.2.1: Measured Absorbance and Calculated Protein Concentration

4.3 Acrylamid Gel for Proteins

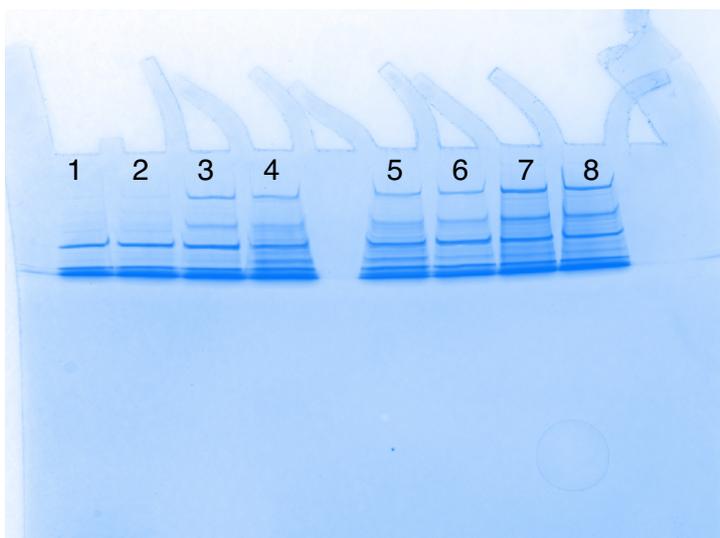


Figure 4.3.1: Acrylamid Gel

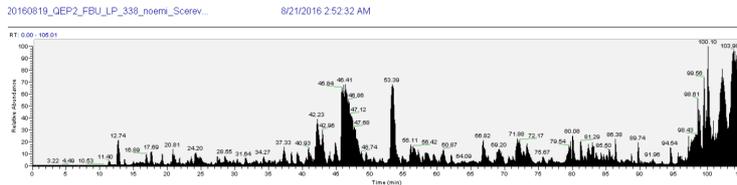
Loading from left to right:

1. *S. cerevisiae* Day 1 (Aug. 16)
2. *S. cerevisiae* Day 1 (Aug. 16)
3. Control Day 1 (Aug. 16)
4. Control Day 1 (Aug. 16)
5. *S. cerevisiae* Day 2 (Aug. 17)
6. *S. cerevisiae* Day 2 (Aug. 17)
7. Control Day 2 (Aug. 17)
8. Control Day 2 (Aug. 17)

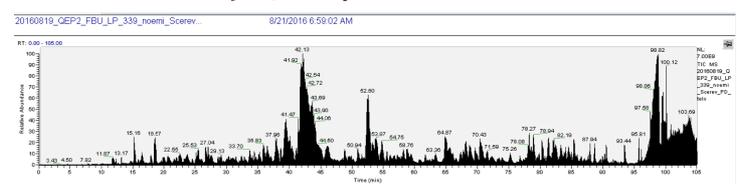
The coloring shows that all lanes contain many peptides, while the control lanes are slightly darker which means that more proteins bound the random DNA sequence. Since the lanes of the bait DNA and

the control DNA look similar, it's probable that many of the same proteins bound either sequence. The mass spectrometry will detect to which proteins the peptides belong and the "R Studio" analysis will show the significant differences between the telomeric sequence and the control.

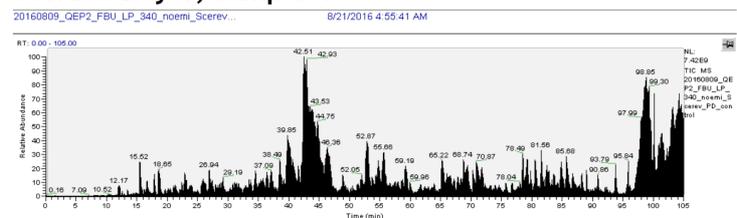
S. Cerevisiae Day 1, sample 1



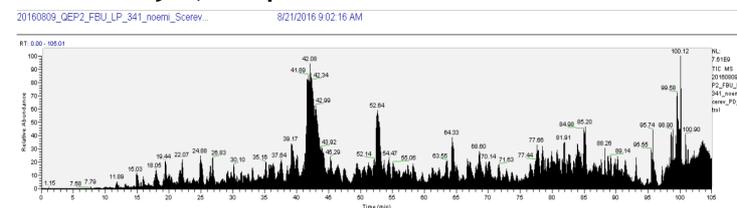
S. Cerevisiae Day 1, sample 2



Control Day 1, sample 1



Control Day 1, sample 2



4.4 Mass Spectrometer Data

Figure 4.4.1 shows the relative abundance of peptides over the time that the mass spectrometer runs. After being ionized, the positively charged peptides are accelerated through a magnetic field which causes them to deflect. Smaller peptides are deflected more than larger peptides and the more an ion is charged the more it is deflected as well. The mass spectrometer electrically detects this deflection and is thus able to calculate the mass.

In the graphs on the left, each peak represents a different peptide. Smaller and more charged peptides are detected before the larger peptides. The x axis represents the time that the peptides need to travel through the mass spectrometer. The y axis shows the abundance of each peptide.

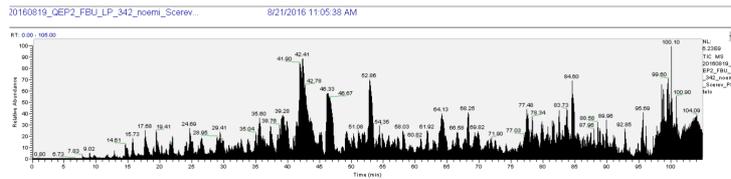
A certain peptide is always

accelerated and deflected the same way.

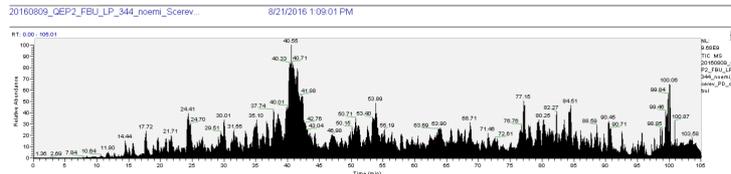
All graphs, except for the last one show a high peak, that is a high peptide abundance, after approx. 42 min. That means that they all contain a large amount of the exact same peptide.

In the next step, the program "MaxQuant" is used to identify the proteins. Many of the peptides could belong to several different proteins. Therefore filtering steps are necessary in order to only use those proteins that are certainly present in the probe for the analysis.

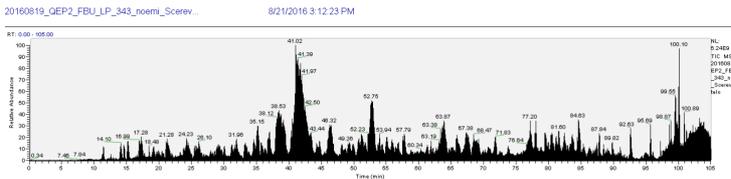
S. Cerevisiae Day 2, sample 1



S. Cerevisiae Day 2, sample 2



Control Day 2, sample 1



Control Day 2, sample 2

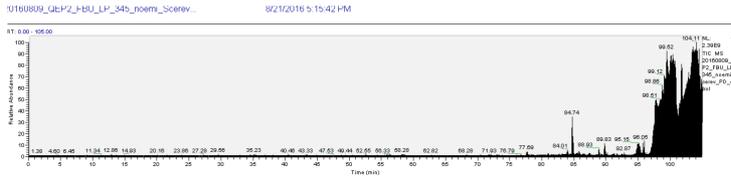


Figure 4.5.1: Protein Groups After Filtering Steps

The volcano plot shows the negative logarithm of the p value on the y axis and the logarithm of the fold change between the abundance of the proteins on the x axis. The p value indicates how likely an enrichment in the sample is if the proteins were in fact distributed equally among the sample and the control. It is a statistical value that is used to be more certain that the enrichment of a protein is not a coincidence. The fold change indicates how many times more a protein is enriched in the bait DNA compared to the random sequence. Proteins that are highly enriched in the sample DNA appear toward the top right of the plot.

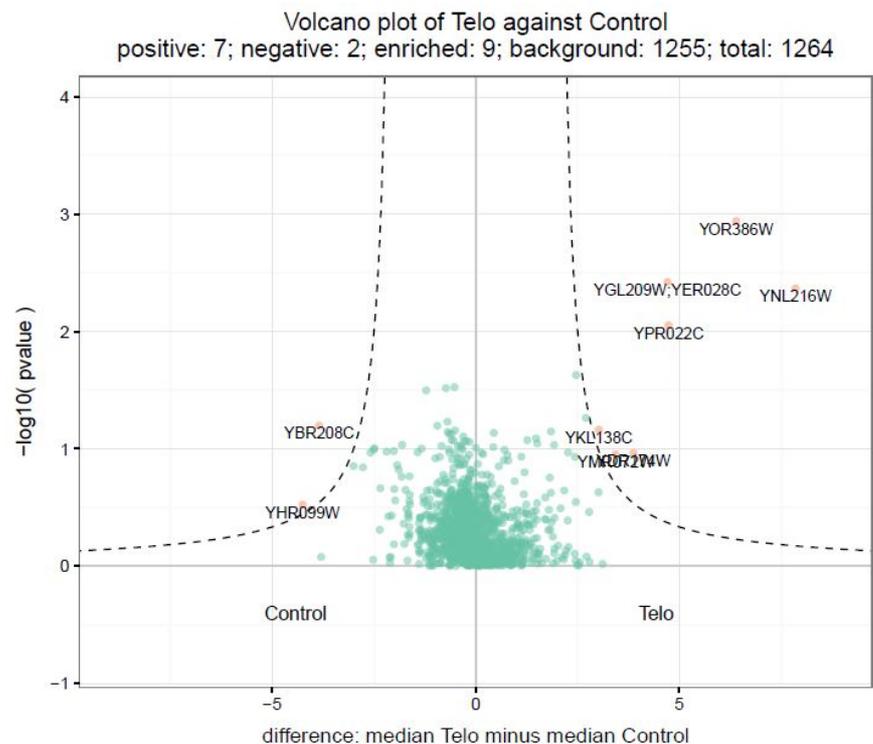


Figure 4.5.2: Volcano Plot of Telomeric Against Control Sequence

4.5 R Studio Data

Figure 4.5.1 shows that 532 of the 1985 bound proteins are actually used for the analysis. The other ones are sorted out during the following filtering steps:

1. Remove contaminants, reverse sequences, and proteins detected due to specific modifications.

2. Remove proteins with too few unique sequences and too many razor sequences (razors = peptides that could belong to different proteins)

Steps 3-5 remove proteins that are detected in less than 4 samples.

By means of these steps the number of detected proteins is reduced from 1 985 to 532 (Figure 4.5.1). These proteins are only proteins that have to be present in the samples because their unique sequences have been detected by the mass spectrometer.

Figure 4.5.2 compares the intensity of the detected peptides in the bait to those detected in the control. The green cloud in the center represents the proteins which were detected equally in telomeric and control sequences. Only the proteins whose abundance exceeds the negative logarithm of the p-value (dashed line), are regarded to be significant. The volcano plot shows the proteins which are significantly enriched in the telomeric bait:

- YOR386W
- YGL209W
- YNL216W
- YPR022C
- YKL138C
- YDR174W
- YMR072W

5. Discussion

5.1 Telomeric Binding Protein Analysis

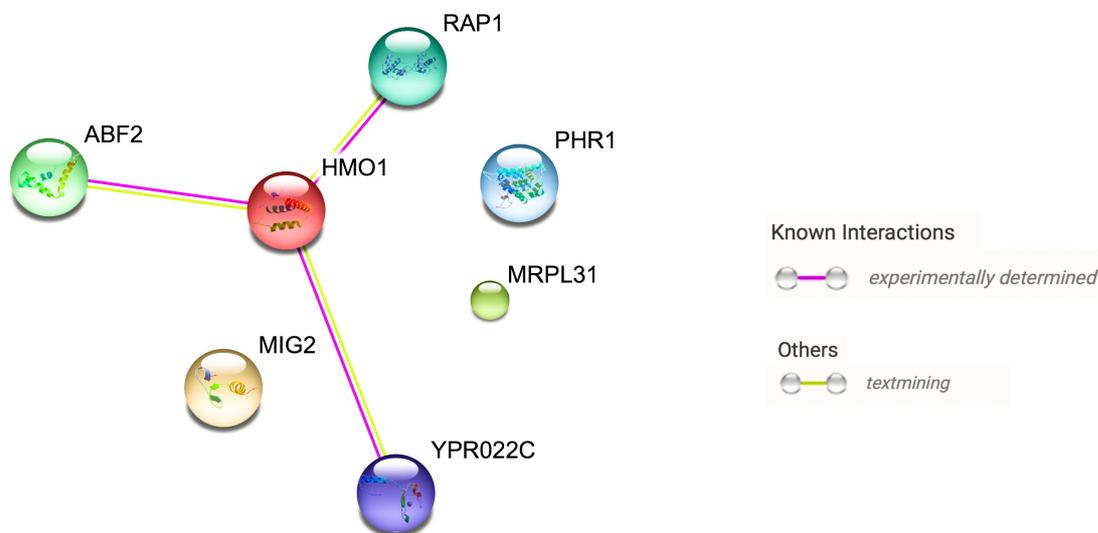


Figure 5.1.1: Interactions of Detected Proteins

• [RAP1 \(YNL216W\)](#) [1]:

As expected, the protein RAP1 which is essential to *S. cerevisiae* telomeres bound abundantly to the bait. This can be regarded as a positive control and shows that the bait simulates the telomeric region well. RAP1 is a DNA-binding transcription regulator that binds several loci in the genome. Its functions include transcription activation and repression, chromatin silencing, but also telomere length maintenance. At the telomeres of *S. cerevisiae*, it recruits the essential Sir proteins.

• [HMO1 \(YDR174W\)](#) [2]:

HMO1 is member of the chromatin-associated high mobility group (HMG) and has several functions in chromatin packing, looping and bending. It is also involved in RNA polymerase I promoter regulation. Since there is possibly an interaction of HMO1 and RAP1 as seen in figure 5.1.1 which has been detected by a tandem affinity purification assay, it does not necessarily bind telomeric sequences [3].

[1] "RAP1." Saccharomyces Genome Database. N.p., n.d. Web. 27 Aug. 2016. <<http://www.yeastgenome.org/locus/S000005160/overview>>.

[2] "HMO1." Saccharomyces Genome Database. N.p., n.d. Web. 27 Aug. 2016. <<http://www.yeastgenome.org/locus/S000002581/overview>>.

[3] Zheng, Jiashun. "Epistatic Relationships Reveal the Functional Organization of Yeast Transcription Factors." *Molecular Systems Biology*. Nature Publishing Group, 2010. Web. 28 Aug. 2016.

- [ABF2 \(YMR072Wr\)](#) [1]:

This is a mitochondrial protein which is involved in mDNA replication and recombination. Therefore, ABF2 probably bound HMO1 but not the bait itself. As seen in figure 5.1.1, an interaction of these protein has been experimentally determined by using a tandem affinity purification assay [2].

- [PHR1 \(YOR386W\)](#) [3]:

This protein is a photolyase which repairs DNA damage caused by UV light. UV light allows thymine or cytosine pairs on the same strand to form pyrimidine dimers which are broken by PHR1. Sequences which are abundant in neighboring thymines and cytosines are therefore often checked by photolyases. Since the bait was not exposed to sunlight, most likely the DNA was not dimerized but simply checked by the enzyme. This is reasonable because of the C-rich sequence of the reverse primer (see page 5).

- [MRPL31 \(YKL138C\)](#) [4]:

This protein is part of the large ribosomal subunit in mitochondria. It is probably either a "coincidence" that MRPL31 also bound the telomeric sequences or it in fact bound the straptavidin beads. It might also interact with one of the other proteins such as ABF2 or MIG2 because they are also a mitochondrial proteins.

- [MIG2 \(YGL209W\)](#):

MIG2 is a zink finger protein required for glucose repression [5]. It represses cellular respiration in mitochondria in case of a low glucose level inside a cell and is necessary for SUC2 expression [6]. SUC2 hydrolyses sucrose to glucose and fructose [7]. During high glucose concentrations, MIG2 relocates to the nucleus. There are so far no studies that show where MIG binds in the nucleus [5]. Due to the high affinity of a zink finger, it my either bind the bait or one of the other proteins.

- [SDD4 \(YPR022C\)](#):

Green fluorescent protein fusion has localized YPR022C in the nucleus and in the cytoplasm [8]. It is a putative transcription factor which is activated in response to MMS, a DNA-damaging agent [9]. It also plays a role in the suppression of the pathway mitochondrial precursor over-accumulation stress (mPoS) which leads to mitochondria-mediated cell death in yeast [10]. The pathway of the suppression takes probably place in the cytosol because all the suppressors were found in the cytosol. Up to now, there are no studies that show where SDD4 binds in the nucleus. As illustrated in figure 5.5.1 by the purple line connecting YPR022C to HMO1, experiments have shown that mutations or deletions in these two genes at once are far more dramatic for fitness than a dysfunction of only one of the proteins [11], [12]. It is possible that the proteins are both involved in DNA looping at telomeres.

[1] "ABF2." Saccharomyces Genome Database. N.p., n.d. Web. 27 Aug. 2016. <<http://www.yeastgenome.org/locus/S000004676/overview>>.

[2] "Lab Experiments." 7 Items (*Saccharomyces Cerevisiae*). N.p., n.d. Web. 28 Aug. 2016.

[3] "PHR1." Saccharomyces Genome Database. N.p., n.d. Web. 27 Aug. 2016. <<http://www.yeastgenome.org/locus/S000005913/overview>>.

[4] "MRPL31." Saccharomyces Genome Database. N.p., n.d. Web. 27 Aug. 2016. <<http://www.yeastgenome.org/locus/S000001621/overview>>.

[5] Lutfiyaya, L. L. *et al.* "WikiGenes - Collaborative Publishing." WikiGenes. N.p., n.d. Web. 27 Aug. 2016. <<https://www.wikigenes.org/e/gene/e/852663.html>>.

[6] Fernández-Cid, A. *et al.* "Glucose Levels Regulate the Nucleo-mitochondrial Distribution of Mig2." National Center for Biotechnology Information. U.S. National Library of Medicine, Feb. 2012. Web. 27 Aug. 2016. <<http://www.ncbi.nlm.nih.gov/pubmed/22353369>>.

[7] "SUC2." Saccharomyces Genome Database. N.p., n.d. Web. 27 Aug. 2016. <<http://www.yeastgenome.org/locus/S000001424/overview>>.

[8] "Wang X and Chen XJ (2015) | SGD." Wang X and Chen XJ (2015) | SGD. N.p., n.d. Web. 27 Aug. 2016. <<http://www.yeastgenome.org/reference/S000180950/overview>>.

[9] "SDD4." Saccharomyces Genome Database. N.p., n.d. Web. 27 Aug. 2016. <<http://www.yeastgenome.org/locus/S000006226/overview#reference>>.

[10] Wang, Xiaowen *et al.* "A Cytosolic Network Suppressing Mitochondria-mediated Proteostatic Stress and Cell Death." Nature International Weekly Journal of Science. N.p., 20 July 2015. Web. 27 Aug. 2016. <<http://www.nature.com/nature/journal/v524/n7566/full/nature14859.html>>.

[11] "BioGRID Interaction Database." HMO1 (YDR174W) Result Summary. N.p., n.d. Web. 28 Aug. 2016.

[12] Zheng, Jiashun *et al.* "Epistatic Relationships Reveal the Functional Organization of Yeast Transcription Factors." Molecular Systems Biology. Nature Publishing Group, 2010. Web. 28 Aug. 2016.

In order to substantiate the results, there are many possible follow up experiments. For instance, an experiment could show if ABF2, HMO1, and YPR022C also bind the telomeric DNA in the absence of RAP1. If they do not bind, this experiment could substantiate the protein interactions in figure 5.1.2.. If they do bind, the experiment could give evidence that the hypothesis that ABF2, HMO1, and YPR022C bind telomeric sequences is right. Furthermore, it should be tested whether they also bind in vivo.

It is unlikely that the mitochondrial proteins ABF2 and MRPL31 also bind in the living organism. This could be tested by protein tagging.

HMO1 and YPR022C, however, have been associated with the nucleus before. In fact, HMO1 could be involved in telomeric DNA looping and YPR022C could be a telomeric transcription factor. It is crucial to keep in mind that some telomeric sequences are transcribed so YPR022C could be either an activator or a repressor. Moreover, it should also be tested if any of the proteins bind the streptavidin beads. Washed streptavidin beads should be incubated with the same lysate as before. For example, the zinc finger of MIG2 might actually bind the beads. Another hypothesis is that this protein indeed binds the telomeres and can repress transcription in telomeric regions. After several follow up experiments, the function of those proteins that actually bind the telomeric sequence in vitro and in vivo could be analyzed with further studies.

As stated in the beginning of the report (page 4), many *S. cerevisiae* proteins have homologues in mammals. RAP1 is for example homologous to the human activator repressor protein hRAP1 which regulates telomere length in humans [1]. HMG1/2, the human high mobility group genes are similar in function and structure to the homologue HMO1 in *S. cerevisiae* [2]. The human homologue of ABF2 is TFAM, a mitochondrial transcription factor that is also involved in DNA replication and repair [3], [4]. Due to the homologues, new knowledge about proteins that protect telomeres in *S. cerevisiae* could be used to study humans. The proteins could maybe be detected in human cells. Studying telomeres is crucial for understanding aging and cancer because they are elongated in cancer cells, but often shortened in senescent cells [5]. Therefore, the study of telomeric binding proteins is relevant to medicine as well.

5.2 Evaluation of Results

It is important to question the reproducibility of this experiment because in several steps, errors influence the outcome. The first step portraying that an error had occurred was during the agarose gel electrophoresis to control the bait length. Inaccurate pipetting, the error of the pipet itself or other factors might be the reason for the surprising short bait DNA.

The length of approx. 100 bp corresponds to only 4 x the length of the primer, while the recommended length of 10 mers would be 2500 bp.

[1] O' Connor, Matthew S. "The Human Rap1 Protein Complex and Modulation of Telomere Length*." The Human Rap1 Protein Complex and Modulation of Telomere Length. N.p., n.d. Web. 28 Aug. 2016.

[2] Alekseev, S. Yu. "HSM2 (HMO1) Gene Participates in Mutagenesis Control in Yeast *Saccharomyces Cerevisiae*." HSM2 (HMO1) Gene Participates in Mutagenesis Control in Yeast *Saccharomyces Cerevisiae*. N.p., n.d. Web. 28 Aug. 2016.

[3] "MTF1 - Mtf1p." WikiGenes - Collaborative Publishing. N.p., n.d. Web. 28 Aug. 2016. <<https://www.wikigenes.org/e/gene/e/855268.html>>.

[4] "Genes and Mapped Phenotypes." National Center for Biotechnology Information. U.S. National Library of Medicine, n.d. Web. 28 Aug. 2016.

[5] De Magalhães, João Pedro. "Telomeres and Telomerase." : The Cellular Timekeepers and Human Aging. N.p., n.d. Web. 18 Aug. 2016. <http://www.senescence.info/telomeres_telomerase.html>.

Another point, where an error became obvious was after the Mass spectrometer analysis. The graph (figure 4.4.1) shows that control 2 Day 2 contained almost no proteins and not even the high peak at 42 min., representing LysC. Thus, the sample was lost for example in the stage tip or while diluting the proteins afterwards. Therefore, the final volcano blot contains 4 samples but only 3 controls. This number should still be significant but 4 controls would be even more accurate.

Figure 4.5.1 shows that remarkably few of the 1985 proteins are quantified 4 times. Thus, the variability of the proteins in the samples is very high which could mean that the circumstances for the samples varied more than expected. Filtering steps 1 and 2 also sorted out a significant portion of the proteins which is not surprising because the presence of various contaminants (e.g. human proteins) and peptides which are lacking sufficient unique sequences to be identified is basically inevitable.

In conclusion, the data is statistically relevant but prior to a follow up project, the experiment should be repeated to increase reproducibility.

6. Conclusion

Over the past three weeks, I got to know new techniques like the plating of a yeast culture, Size-exclusion chromatography, stage tip preparation or evaluating data using MaxQuant and RStudio. I enjoyed being able to actually implement "my own" project and doing much practical work. In addition to my own project, I gained insight of the working atmosphere for PhD students and I was able to watch and assist during DNA extraction, a PCR, and a western blot as well.

In the beginning even opening an Eppendorf tube with the left hand while pipetting with the right hand was challenging. I think I learned many practical skills but also much theoretical background. In the first week, I enjoyed attending the lectures of the *International Summer School*. That way, I got an impression of the work of some other groups at the IMB.

I am particularly thankful that Lara took much time to answer all of my questions thoroughly, that Sarbrina allowed me to assist during a western blot, and that Anja explained me in-depth how a mass spectrometer worked (even though I still do not understand all the details to be honest). The whole Butter group was extremely welcoming and happy to share their experiences. I enjoyed having lunch with the whole group and seeing that the PhD students helped each other a lot. This positive impression of collaboration and cohesion of the group encourages me a lot to do more internships like this one. Prior to this internship, I had not been sure whether I would enjoy working inside a laboratory for a whole day. Now I am certain that biochemistry is in fact the right major for me and I am looking forward to my studies even more.

Special thanks to

the *Förderverein der Internationalen BiologieOlympiade e.V.* for organizing this internship for me,
to Dr. Falk Butter for allowing me to join his group at the IMB,
and to my supervisor Lara Perez for preparing my project.

I can definitely picture myself coming to the IMB again.

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- Institute of Molecular Biology Mainz: "CO-IP (Co-immunoprecipitation) all at 4° C"
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7.2 Pictures

IMB Logo: https://pbs.twimg.com/profile_images/434290120196227072/HcUZtaf6_400x400.jpeg

Figure 4.1.2: <http://www.ri.com.my/#!gen-ts-nucleic-acid-electrophoresis/cwed>

Figure 5.1.1: <http://string-db.org/cgi/network.pl?taskId=qLNSwldxVJFS>

8. Appendix

8.1 Western Blot After Telo Pulldown with HIS-Tag

Blotting buffer:

22.5 g Glycin + 5.5 g Tris + 400 ml MeOH ad 2 l w DI water (stored at 4°C)

10x TBS buffer:

12.18g Tris(hydroxymethyl)-aminomethan + 87.5g NaCl pH adjust to 7.5 ad 1L w DI water (autoclave or sterile filter)

1x TBS buffer:

100 ml 10x TBS + 900 ml DI water

1x TBS-T buffer:

100 ml 10x TBS + 0.5 % Tween 20 + 0,02 % Triton X-100

Blocking Solution:

0.55 g Blocking Reagent (provided in Penta-His HRP Conjugate Kit) + 110 ml 1x Blocking Buffer + 0,1% Tween 20

Heat to 70°C until completely solved, keep at 4°C after cooled down

- Run a gel according to your samples (time and buffer depend on the protein you want to look at)
- Take gel out of the chamber & rinse it in DI water, afterwards incubate it in Blotting buffer for 5 min
- Wet the membrane with H₂O and also incubate it in Blotting buffer for 5 min
- Start building the blotting sandwich with the holder (black side facing down then gel has to come first), the wet sponges and two soaked filter papers:
 - o Roll the sandwich with a 15 ml Falcon tube to get rid of air bubbles
 - o Put the gel on the sandwich and afterwards the membrane
 - o Roll again but make sure nothing moves
 - o End with two more soaked filter papers, the second sponge and close the holder
- Fill the Blotting chamber with 1 l of blotting buffer and place the sandwich (black to black)
- Blot for 60 min at constant 300 mA (this time and power also depends on the proteins and the system that you are using : Wet Blot in this case)
 - o Blotting Buffer used for 2nd time
- After blotting remove membrane and stain gel with Coomassie for 15 min and destain with water to see what didn't get blot
- Wash the membrane with TBS buffer for 2 times 5 min
 - Removes the SDS from the proteins to help them re-nature and fold back to their natural secondary and/or tertiary structure
- Incubate the membrane with blocking solution for 60 min
 - o Fill 30 ml of Blocking solution into a 50 ml falcon tube and place the membrane inside (used 1st time)
 - The blocking reagent blocks the remaining free binding spots on the membrane and is undetectable by the antibody (to secure specific signal)
- Wash membrane 2x 5 min in TBS-T buffer
 - Contains detergent to remove excess blocking reagent
- Incubate with the anti His-HRP conjugate (Kit provided, 1:1000 in 5 ml Blocking Solution) for 1h (used for 1st time)

- The primary antibody is already coupled to the HRP enzyme (Horseradish peroxidase) so no secondary antibody is needed for detection
- Wash 2x 5 min in TBS-T buffer & additional 5 min in TBS buffer (RT)
 - Removes not bound antibody from the membrane to avoid unspecific signal and the last TBS step removes the detergent from the membrane which could interfere with the chemiluminescence reaction
- Take a clean glass plate and mix 500 µl of each ECL Western Blot Detection Reagent
 - Solution 1: Luminol and Enhancer containing buffer
 - Solution 2: Peroxide containing buffer
 - In the presence of Hydrogenperoxide HRP oxidises Luminol to 3-aminophthalate which emits light at 428 nm, with the help of the enhancers the light is amplified and detectable in our system
- Put membrane proteins facing down on the glass plate and onto the solution for 30 sec
- Transfer membrane onto black screen and detect with ChemiDoc XRS+ System
 - Program: Western Blot Chemi for Detection
 - Western Blot Colour for marker