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Enhanced retention of protein damage during ageing

Degree Project in Medicine

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ABSTRACT 3

BACKGROUND 4

AGING 4
AGING AND THE LIFE SPAN OF THE YEAST CELL 4
PROTEIN STRUCTURE AND AGING 5
PROTEIN QUALITY CONTROL SYSTEM MAINTAIN PROTEIN HOMEOSTASIS IN THE CELL 6
TEMPORAL QUALITY CONTROL SYSTEM 6
SPATIAL QUALITY CONTROL SYSTEM 6
LOSS OF PROTEOSTASIS IS AN EVOLUTIONARILY CONSERVED HALLMARK OF AGING 7
GENOME-WIDE IMAGING SCREEN FOR YEAST AGG IDENTIFIED YMR074C 8
YMR074C – A SUPPRESSOR OF DEGENERATIVE DEATH 8
SPINDLE POLE BODY FUNCTION IN PROTEIN QUALITY CONTROL 9
SPC110P IS PART OF THE SPB 10
AIM OF STUDY 10

METHOD 11

YEAST STRAINS 11
PLASMIDS 12
PLASMID SEQUENCE VERIFICATION 12
PLASMID PURIFICATION 12
DNA EXTRACTION FROM GEL 12
COMPETENT YEAST CELLS 12
YEAST CELL TRANSFORMATION 13
CLONING 13
POLYMERASE CHAIN REACTION 14
REACTION MIX 14
PRIMERS 14
REACTION CONDITIONS 15
GEL ELECTROPHORESIS 16
HEAT-SHOCK AND FORMALDEHYDE CELL SAMPLE FIXATION 16
FLOURESCENCE MICROSCOPY 16
MEDIA 17

RESULTS 17

THE UNCHARACTERIZED ORF, YMR074C, IS INVOLVED IN ASYMMETRIC SEGREGATION OF PROTEIN AGGREGATES DURING CELL DIVISION 17
COMPLEMENTATION SOUGHT TO RESCUE CELLS TO WT 19
OVEREXPRESSION OF YMR074C DISPLAYED ENHANCED AGGREGATE RETENTION 20
KAN-MARKERS LINKED TO THE GENE X WERE IDENTIFIED THROUGH PCR AMPLIFICATION. 22
INVERSE PCR. A STRATEGY TO DETERMINE THE GENOTYPE OF SPC110-221. 22

DISCUSSION 24

YMR074C REMAIN A VIABLE LOCUS FOR CONTINUED INVESTIGATION 24
ABANDONING SPC110-221 IN FAVOR OF MORE PROMISING RESEARCH CANDIDATES? 25

POPULÄRVETENSKAPLIG SAMMANFATTNING 26

ACKNOWLEDGEMENTS 28

REFERENCE LIST 29

ABSTRACT

Age can be reset in many different cell types through asymmetric cell division. During such a division, retention of aging factors in one cell allows the rejuvenation of the other. In unicellular organisms, it can provide a competitive advantage for the rejuvenated cell. In multicellular organisms, it is critical for maintenance of a stem cell lineage. Segregation of damage to the cell committed to differentiation can promote survival of the stem cell lineage, thereby allowing for long-term regenerative capacity of tissues that are critical to the organism's survival.

Asymmetric segregation of damaged proteins requires function of several cellular systems, many of which have been identified and characterized in the asymmetrically dividing (budding) yeast, *S. cerevisiae*, including the components of the actin cytoskeleton, protein quality control machinery, and intracellular trafficking. Each gene studied in singularity adds another jigsaw-piece of the big picture that is a complex cellular machinery of intertwining pathways. Mutations in these cellular components generally result in reduced asymmetry and often decreased cellular lifespan. In this study, we focused on one uncharacterized asymmetry generating gene, YMR074C, and the results indicated a decreased asymmetric segregation phenotype when knocked-out. Protein aggregates were quantified after exposure to heat-shock and Hsp104-GFP was used to localize these aggregates. Results confirmed its involvement in asymmetric protein segregation during mitosis.

Moreover, mutants with enhanced asymmetry have been less characterized, and the additional purpose of this study was to identify an unknown mutation in a yeast strain that displayed such enhanced capabilities. To accomplish this, a reverse PCR strategy was implemented, intending to amplify the nucleotide sequence up- and downstream from a known resistance marker thought to be linked to the mutation, thus revealing the affected locus. However, this strategy was unsuccessful and we could not locate the mutations responsible for the enhanced segregation phenotype.

BACKGROUND

Aging

There are several theories that try to explain the underlying cause(s) of aging. Aging has been suggested to be a programmed alteration in gene expression. In contrast, it has also been argued to be an accumulation of damage to the cell resulting in a steady decline in function. These are the two most prominent lines of thought that attempt to explain the process of aging, yet neither one fully provides a satisfying answer to the phenomenon.(Jin, 2010)

Several defining features of aging have been identified that are consistently seen in a wide range of organisms. Lopez-Otin et al. describe nine hallmarks of aging that all contribute to the accumulation of cellular damage and, therefore, the aging phenotype. They are: cellular senescense, loss of proteostasis, mitochondrial dysfunction, genomic instability, telomere attrition, epigenetic alterations, deregulated nutrient sensing, stem cell exhaustion and altered intercellular communication. (Lopez-Otin et al., 2013) While the features of aging are relatively straightforward to describe and define, the challange has been to determine how these features are linked and to attampt to work out what are the main driving forces of the aging process.

Aging and the life span of the yeast cell

The budding yeast, Saccharomyces cerevisiae, has proven to be a powerful model of the process of aging and has helped to define the features of aging common to many organisms. Who different approaches to studying aging in yeast have been used: chronological life span (CLS) and replicative life span (RLS). CLS defines as how long the cell can live when growth-arrested. RLS on the other hand, is defined to how many times the cell can divide and generate a daughter cell.(Longo, Shadel, Kaeberlein, & Kennedy, 2012; Sontag, Samant, & Frydman, 2017) Yeast culture might seem immortal as they continue budding for as long as there are sufficient nutrients. This was until 1959, when Johnston and Mortimer found a way to separate mother cell from its budded progenies. They discovered that the mother cell, which

represents the aging lineage, carries a finite replicative capacity.(Mortimer & Johnston, 1959) Since then, much work has gone into characterizing the decline seen in the aging yeast mother cell.

Protein structure and aging

A prominent feature in cells of any aging lineage is a gradual loss of protein function. Proteins are comprised of amino acids forming a chain, conjoined by covalent peptide bonds. The amino acid sequences are determined during translation, a process in which the ribosome is reading nucleotide triplets from RNA like a blueprint. The so-called polypeptide chain then fold into a three-dimensional structure that minimizes its free energy. Some proteins fold spontaneously into its three-dimensional shape. However, most require chaperone proteins to steer them along a productive folding pathway and prevents them from misfolding and aggregating inside the cell. Even when properly folded, proteins are constantly subjected to stress such as permanent oxidative or heat stress and are prone to misfold. A system of chaperones, called a protein quality control (PQC), has therefore evolved to counteract this damage and maintain protein homeostasis. (Alberts et al., 2019; Glover & Lindquist, 1998)

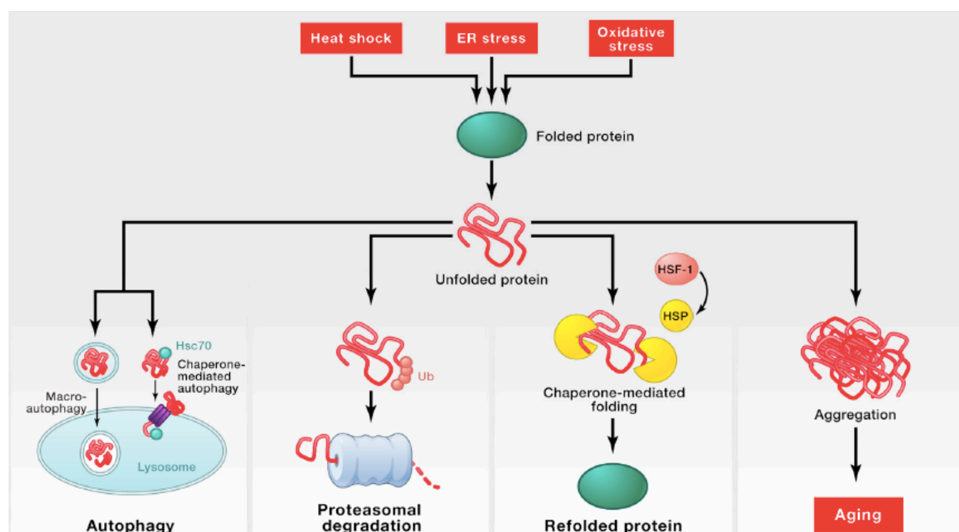

Figure 1. The different fates of synthesized proteins. (Lopez-Otin, Blasco, Partridge, Serrano, & Kroemer, 2013)

Protein quality control system maintain protein homeostasis in the cell

The PQC is of outmost importance for the cell to maintain protein homeostasis, otherwise called proteostasis. This PQC can be divided into two functional categories, namely temporal and spatial PQC.

Temporal quality control system

Temporal control system is aimed at refolding, removing or repairing proteins that cannot fulfill their intended function or that may otherwise cause damage to the cell. Damaged or misfolded proteins may interact in abberant fashion with other cellular components.(Josefson, Andersson, & Nyström, 2017) The chaperone network, eg Hsp104 that may refold proteins in assistance of Hsp 40 and Hsp70, is one crucial aspect of the temporal control system. Overexpressing chaperones have even been shown to extend the life span of some organisms (*Caenorhabditis elegans*)(Walker & Lithgow, 2003).

Spatial quality control system

One major purpose of the spatial control system is to isolate damaged, potentially toxic proteins, in concentrated inclusions to maintain a functional proteome. Upon increased proteostatic stress, protein aggregates scattered in the cell fuse under influence of an energy-dependant process to form inclusions. They are stored at specific sites in the cell. There may be more to identify but the two main ones are called JUNQ (juxtanuclear quality control) in tight association with INQ (intranuclear quality control) and IPOD (insoluable protein deposit). Some of these inclusions are destined for ubiquitinine-proteasome system (UPS) for degradation and some are cleared out by autophagy. Additionally, there are inclusion compartments containing aggregation-prone proteins that are retained in the cell.(Kaganovich, Kopito, & Frydman, 2008)

Additionally, the spatial quality control also facilitates the ability of the mother cell to asymmetrically divide accumulated protein aggregates between itself

and the daughter cell during mitosis. It has been established that carbonylated and oxidatively damaged proteins accumulate to a greater extent in the progenitor cell in relation to a greater replicative age. Such damaged proteins are associated with protein aggregates containing Hsp104p. (Erjavec, Larsson, Grantham, & Nyström, 2007) Protein remodeling factor Hsp104p, a disaggregase in the yeast chaperone machinery, is required to recognise and sequester protein aggregates to remain in the mother cell during mitosis.(Hill et al., 2016). It keeps the inclusion bodies in order to allow the progeny a rejuvenated proteome.(Nyström & Liu, 2014) The subcellular compartments of insoluble inclusions comprized of sequestered misfolded proteins, though mainly studied in yeast, have been proven to be clearly evolutionarily conserved in mammalian cells.(Gidalevitz, Prahlad, & Morimoto, 2011; Johnston, Ward, & Kopito, 1998)

Loss of proteostasis is an evolutionarily conserved hallmark of aging

Age-related neurological disease such as SCA (spinocerebellar ataxia), Mb Alzheimer, Mb Huntington and amyotrophic lateral sclerosis are becoming increasingly prevalent and the treatment of these diseases remain a challenge to modern healthcare. In these diseases, proteins of aberrant structure cause damage to the cell and thus interfere with normal cellular function.(Balch, Morimoto, Dillin, & Kelly, 2008; Hipp, Park, & Hartl, 2014) The presence of protein inclusions, formed from smaller protein aggregates is a hallmark of these age-related neurodegenerative diseases, even though they are etiologically diverse.(Kaytor & Warren, 1999) Furthermore, such inclusion bodies have been identified in aged neuronal cells in healthy animals, suggesting it is a normal process of the cellular protein quality control system, or PQC.(Schneider, Nystrom, & Widlund, 2018)

Several processes involved in protein aggregate retention and asymmetric sequestration during cytokinesis in yeast have been proven to be conserved even in higher eukaryotes. The inheritance of damaged proteins and inclusion bodies are linked with cells destined for longevity. For example, germ line stem cells divide into fully rejuvenated germ cells intended for reproduction. These cells will outlive the progenitor cell that retain damaged proteins. On the contrary, intestinal stem cells,

known to be long-lived, give rise to differentiating progenies that inherits aging factors as they in turn are destined for a high turn-over and have a shorter life span.(Bufalino, DeVeale, & van der Kooy, 2013)

Genome-wide imaging screen for yeast AGG identified YMR074C

Since asymmetric distribution of damaged protein represents a key strategy to maintain a rejuvenated lineage during cell division, a screen was performed to identify factors that are necessary for efficient retention of damaged proteins in the mother cell. A mutant library was generated using genetic synthetic array technology. The disaggregase Hsp104 was tagged with GFP for the entire yeast knockout collection (*S. cerevisiae*). Hsp104 binds protein aggregates.(Glover & Lindquist, 1998) The tagged collection, contained in 14 96-well plates, were heat-shocked at 42°C for 30 minutes to induce protein misfolding. Cells were allowed to recover briefly and Hsp104 labeled aggregates were subsequently counted. Asymmetry generating genes were defined as strains that had a decreased asymmetry in protein aggregates and cut-off was set to >25% deviation from WT. A total of 111 AGG were identified. A top hit in this screen was a relatively uncharacterized gene: YMR074C. Fold change in aggregate asymmetry for YMR074C was 1.47 compared to WT.(Hill et al., 2016)

YMR074C – A suppressor of degenerative death

An earlier study from 2015 had described a mitochondrial precursor over-accumulation stress pathway (mPOS), a type of mitochondria-mediated cell death in yeast.(Wang & Chen, 2015). Mitochondrial dysfunction is one of the hallmarks of aging. The efficacy of ATP-generation, largely dependant on the respiratory chain, tend to decrease over time, simultanously increasing electron leakage from the mitochondrial membrane. (Green, Galluzzi, & Kroemer, 2011)

The aforementioned study used a AAC2^{A128P}, a protein that causes protein misfolding and mitochondrial damage(Table 1) to understand how mitochondrial damage can cause cell death. By screening for suppressors of AAC2-induced cell death, they identified, among genes involved in TOR or chaperone function, several

uncharacterized genes including YMR074C. They therefore named this gene SDD-2 (suppressor of degenerative death-2).

Gene name	Species	Gene function	Organism response to knock-out mutation
ANT1 (SLC25A4)	Human	Encodes adenine nucleotide translocase. Involved in ATP/ADP exchange in the mitochondia.	Autosomal dominant progressive external ophtalmoplegia, cardiomyopathy, myopathy(Kaukonen et al., 2000; Palmieri et al., 2005)
AAC2^{A128P} (PET9)	Yeast	Orthologous to the human ANT1. ADP/ATP exchange.(Wang, Zuo, Kucejova, & Chen, 2008)	Protein misfolding, aging-dependant degenerative cell death. (Liu, Wang, & Chen, 2015)

Table 1. ”We modelled adPEO, an adult- or later-onset degenerative disease, by introducing the A128P mutation into the adenine nucleotide translocase Aac2p of *Saccharomyces cerevisiae*.”(Wang & Chen, 2015)

YMR074C is likely involved in some aspect of protein quality control. Disruption of YMR074C function had a negative impact on protein quality control resulting in reduced retention of damaged protein in the yeast mother cell during cell division. Overproduction of YMR074C was shown to boost the ability of the cell to handle stress in the form of mitochondrial precursor accumulation.(Hill et al., 2016)

Spindle pole body function in protein quality control

The spindle pole body (SPB) is the microtubule organizing center of the yeast cell. Its main function is to serve as an anchor for microtubules of the mitotic spindle which is used to segregate replicated chromosomes.(Seybold & Schiebel, 2013) (Holly A. Sundberg & Davis, 1997)

The SPB is the equivalent of the mammalian cell centrosome. The centrosome is found in many other higher eukaryotes which means multicellular lifeforms with differentiated cells forming tissues and organs. In a typical animal cell, microtubules originate from the centrosome and create a track-system in the cell cytoskeleton, widely incorporated in cellular transport. The centrosome and its microtubules are responsible for the transporting and positioning of membrane-enclosed organelles.(Alberts et al., 2019)

SPC110p is part of the SPB

JUNQ is an inclusion body, formed from smaller protein aggregates. It has been widely studied in S. cerevisiae. The mammalian equivalent of JUNQ, the aggresome, is dependent on microtubuli, whereas JUNQ is dependent on actin cables.(Hill et al., 2016) SPC110 has proven important for normal asymmetric inheritance of protein aggregates and necessary for the clearance of aggregated proteins. (Song et al., 2014).

From the yeast deletion library, two generated strains with temperature sensitive mutatant alleles of the SPC110 were available for further study performed in the Nystrom Lab. The first one is SPC110-220, which had a decreased asymmetric segregation of aggregated proteins and a confirmed mutation in the spc110 locus. The other one, SPC110-221, indicated to have an increased asymmetry. This contradictory phenotype attracted interest as it indicated a different mutation as was initially thought to be. However, the actual mutated locus was unknown.(H. A. Sundberg, Goetsch, Byers, & Davis, 1996; Tjeerdsma, 2019)

Aim of study

The purpose of this study is to characterize pathway involved in the rejuvenation of the daughter cell lineage, specifically how they promote the retention of protein aggregates in the mother cell during cell division.

Aim 1: Determine the function of the uncharacterized gene, YMR074C, in asymmetric segregation of damaged proteins during cell division.
Aim 2: Determine the location of the mutation in the genome that causes the enhanced segregation phenotype in the spindle pole body component mutant SPC110-221.

METHOD

Yeast Strains

A list of all the yeast strains used and created in this study (Table 2).

Strain name	Genotype	Plasmid	Source
BY4741 (wildtype, WT)	MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0	-	(Brachmann et al., 1998)
JBY001	MATa, his3Δ1, Hsp104GFP-HIS3MX, leu2Δ0, met15Δ0, ura3Δ0, ymr074cΔ:KanMX	-	This study
JBY002	MATa, his3Δ1, Hsp104GFP-HIS3MX, leu2Δ0, met15Δ0, ura3Δ0, ymr074cΔ:KanMX	pjb001	This study
JBY003	MATa, his3Δ1, Hsp104GFP-HIS3MX, leu2Δ0, met15Δ0, ura3Δ0, ymr074cΔ:KanMX	ppw411	This study
JBY006	MATa, his3Δ1, Hsp104-GFP, leu2Δ0, met15Δ0, ura3Δ0,	pjb002	This study
JBY007	MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0	ppw417	This study
Spc110-220(SGA)	MATa spc110-220::kanR can1Δ::STE2pr-Sp_his5 lyp1Δ leu2Δ0, ura3Δ0 met15Δ0	-	Synthetic Genetic Array Collection. Boone laboratory.
Spc110-221(SGA)	MATa spc110-221::kanR can1Δ::STE2pr-Sp_his5 lyp1Δ leu2Δ0, ura3Δ0 met15Δ0	-	Synthetic Genetic Array Collection. Boone laboratory.
PWY1104	MATa, his3Δ1, Hsp104GFP-HIS3MX, leu2Δ0, met15Δ0, ura3Δ0,	-	P. Widlund

Table 2.

Plasmids

Plasmids used in this study were integrated into the genomes of the strains studied (Table 3).

Plasmid name	Description	Bacterial markers	Yeast markers
pJB001	YMR074C_in pPW411_met15d0_NAT 8261bp	Ampicillin R (BLA)	ClonNAT
pJB002	GPD_YMR074CmCherry_lysfrg_prs405 8336bp	Ampicillin R (BLA)	LEU2
pPW411	met15d0_NAT	Ampicillin R (BLA)	ClonNAT
pPW417	GPD_mCherry_lysfrg_prs405	Ampicillin R (BLA)	LEU2

Table 3.

Plasmid sequence verification

All plasmids generated during this experiment were submitted for sequencing at Eurofins Genomics. Sequencing runs were analyzed by pairwise BLAST.

Plasmid purification

Plasmids were purified using QIAprep. Spin Miniprep Kit (QIAGEN, Hilden, Germany) according to manufacturer's protocol.

DNA extraction from gel

DNA fragments were gel extracted using a Thermo Scientific GeneJET Gel Extraction Kit (Thermo Fisher Scientific Baltics, Vilnius, Lithuania) according to manufacturer's protocol.

Competent yeast cells

The intended yeast strain was collected and inoculated in YPD overnight (200rpm, 30C). 1mL overnight culture was diluted into 50mL YPD. Flasks were incubated

(30C) until OD_{600}=1. The cells were centrifuged at 4,000rpm for 5 minutes. The pelleted cells were resuspended in 1mL FCC (5% glycerol, 10% high quality DMSO) and centrifuged (13,000rpm, 1min). The supernatant was discarded and the cells were resuspended in 500µL FCC and aliquoted into 5 tubes of 100µL each.

Yeast cell transformation

Competent yeast cells were thawed in a water bath (37°C, 15-30s) and centrifuged at 13,000rpm for 1 minute. The liquid was discarded and the cell pellet was resuspended in the following listed order (see Table 4).

PEG 3350 (50%w/v)	260µL	
ssDNA	10µL	
LiAc (1.0M)	36µL	
DNA	10µL	
Sterile H_2O	34µL	Total volume: 360µL

Table 4. Preparation of yeast competent cells.

The sample tubes were put in a water bath (42°C) for approx. 20 minutes and then centrifuged (13,000rpm, 1min). Supernatant was removed and replaced with a small volume YPD and vortex mixed to ensure that the cells were resuspended. The cells were plated on the appropriate selective medium agar plate by adding 10-50µL of the transformed cell solution.

Cloning

Plasmid pJB001 was created as follows: The YMR074C open reading frame with upstream and downstream sequences was amplified using primers PW1195 and PW1196 and purified using a GeneJet PCR purification kit (Thermo Scientific). The PCR fragment and plasmid pPW411 was digested with enzymes Asc-I and Pac-I with the recommended reaction conditions and purified.
The plasmid 5’ ends were dephosphorylated using a thermosensitive alkaline phosphatase. Dephosphorylated destination vector and YMR074C fragment were ligated using a T4 DNA ligase (Thermo Scientific) and transformed into DH5-α compentent cells.

Polymerase chain reaction

PCR was used to amplify a fragment containing KANMX-markers and neighboring sequence linked to locus of SPC110-220/221. For the YMR074C experiments, it was used for amplifying all genetic material intended for yeast cell transformation.

All components were added into PCR tubes to an end volume of 50µL. Phusion DNA polymerase, primers and dNTP were kept frozen and allowed to slowly and completely thaw before adding the components (Table ’Reaction mix’). The reaction programs are as listed in ’Reaction conditions’.

Reaction mix

Components added for all PCR reactions:

Phusion DNA Polymerase	-	1 µL
Forward primer	-	0.5µL
Reverse primer	-	0.5µL
dNTP	-	0.5µL
5x Phusion HF buffer	-	10µL
H2O (sterile)	-	36.5µL
Template	-	1µL
Total volume	-	50µL

Primers

List of primers used in PCR and their nucleotide sequence. Source: Per Widlund.

PW1195_YMR074C_locus_fw

gcattaattaaTGTATTATTATTAGCAGATGGTAATGGA

PW1196_YMR074C_locus_rev

gcattaattaaTGTATTATTATTAGCAGATGGTAATGGA

PW1203_newKanMx_ts_fw

GGTAAGGAAAAGACTCACGTTTC

PW1204_newKanMx_ts_rv

CATCGAGCATCAAATGAAACTG

PW1205_ YMR074C_N-term_S1

AATCTTCTTGCTCTCTGGATTACAATTCAGGAGGAAGTGTGTGCTCAGATATGCGTACGCT GCAGGTCGAC

PW1206_ YMR074C_N-term_S4

TTTTTCAACTGAGCCAATCTGGCTTCCCTAATAGCTTGTAACTCTGGGTCCATCGATGAATT CTCTGTCG

PW1207_YMR074C_ChkN-fw

CCTTGCAACAGAGAGCAATAG

PW1208_YMR074C_ChkN-rv

GACGTTGTTTGTAGCAATCAGT

PW1209_YMR074C_GPD_fw

gcaGGATCCATGGACCCAGAGTTACAAGC

PW1210_YMR074C_GPD_rv

cgttctagaATCAAAGAAGTCGTCATCATCTTC

PW1211_InverseKanMX_fw

GAAACGTGAGTCTTTTCCTTACC

PW1212_InverseKanMX_rv

CAGTTTCATTTGATGCTCGATG

Reaction conditions

PCR settings used in this study:

phus 5			
Cycle step	Temperature(°C)	Time	Amount cycles
Initial denaturation	98°	2:00min	1x
Denaturation	98°	30s	*
Annealing	60°	30s	*
Extension	72°	5:00min	* 32x
Final Extension	72°	7:00min	1x

phus 2			
Cycle step	Temperature(°C)	Time	Amount cycles
Initial denaturation	98°	2:00min	1x
Denaturation	98°	30s	*
Annealing	60°	30s	*
Extension	72°	2:00min	* 30x
Final Extension	72°	7:00min	1x

Table 5. Phus 5 program was used for the SPC110-220/221 reverse PCR amplification.
Phus 2 program is standard protocol for Phusion polymerase. It was used for all plasmid generation and for identifying KanMX in SPC110-220/211.

Gel electrophoresis

The gel was prepared by adding 2µL Midori Green in a 50mL centrifuge tube and adding 25mL agarose solution (0.5x TAE, 0.8% agarose). 5µL of the PCR product was mixed with 1µL 6x Loading Dye and put into the wells of the gel. The leftmost well of the gel was loaded with 5-6µL GeneRuler® 1 Kb DNA ladder. The gel was run for 20 minutes at 100V.

Heat-shock and formaldehyde cell sample fixation

Yeast colonies from selective medium agar plates were inoculated in 10mL YPD medium at 30°C overnight.
Using spectrophotometry (OD_{600}), cell density was determined and diluted in glass flasks to 1/10 in YPD, to a total volume of 20mL. The flasks were placed in a shaking incubator at 30°C, 180rpm. The flasks were removed at $OD_{600} = 0.5$ (approx.) where they are in mid-log phase. The flasks were put in a water-bath shaker at 38°C, 170rpm. At time points 0 and 90 minutes, 900µL samples were put into 1mL microcentrifuge plastic tubes containing 100µL 37% formaldehyde. The tubes were allowed to incubate for 30min-1 hour and then centrifuged at 13,000rpm for 1min. For each respective tube, the supernatant was removed and the cell pellet was washed in 1mL 1x PBS. The tubes were centrifuged and washed in a similar manner two more times and finally resuspended in 50µL PBS. The samples were stored at 4°C.

Flourescence Microscopy

Samples were examined under microscopy by pipetting approximately 0,4µL of the cell solution onto an objective glass and then covered with a glass cover slip. Using a Zeiss Axio Observer Z1 (Carl Zeiss AS, Oslo, Norge) flourescense microscope with computer software ZEN 2.3 Pro to create images of the cells. DIC filter was used to visualize the cells and a GFP-filter to localize intracellular protein aggregates. FIJI was used for image analysis frames (Schindelin, J.; Arganda-Carreras, I. & Frise, E. et al. 2012, https://imagej.net/Citing).

Media

Media:	Components:
YPD (yeast peptone dextrose), rich medium	1% Yeast extract 2% Glucose 2% Agar (for plates) 2% Peptone
Antibiotic selective LB, (for bacteria)	1% Typtone 1% NaCl 0,5% Yeast extract 50ug/mL Kanamycin

Table 6. Media used in this study.

RESULTS

The uncharacterized ORF, YMR074C, is involved in asymmetric segregation of protein aggregates during cell division

An uncharacterized ORF, YMR074C, was identified in a screen for genes defective in asymmetric segregation of misfolded proteins. (Hill et al., 2016) To confirm the asymmetric segregation phenotype, Hsp104GFP was transformed into the ymr074cD knockout strain from the yeast deletion collection. This strain, along with a wild type YMR074C/Hsp104-GFP control was heat-shocked continuously for 90 minutes and cells were sampled and fixed in formaldehyde solution at 0 and 90 minutes.
Three individual replicates each were examined for each strain respectively. The results from each replicate indicated a decreased asymmetric inheritance of protein aggregates. Hence, a larger amount of protein aggregates was inherited by the daughter cell in JBY001 compared to the WT-control. This confirmed the phenotype observed in the SGA screening background as can be seen in Figure 2. The mean value of aggregates found in the progeny during mitosis in the control was 31.4% (stdev 8.4%, se 5.9%), whereas the YMR074CΔ mean was 43.8% (stdev 12.3, se 8.7%). Paired t-test 0.172.

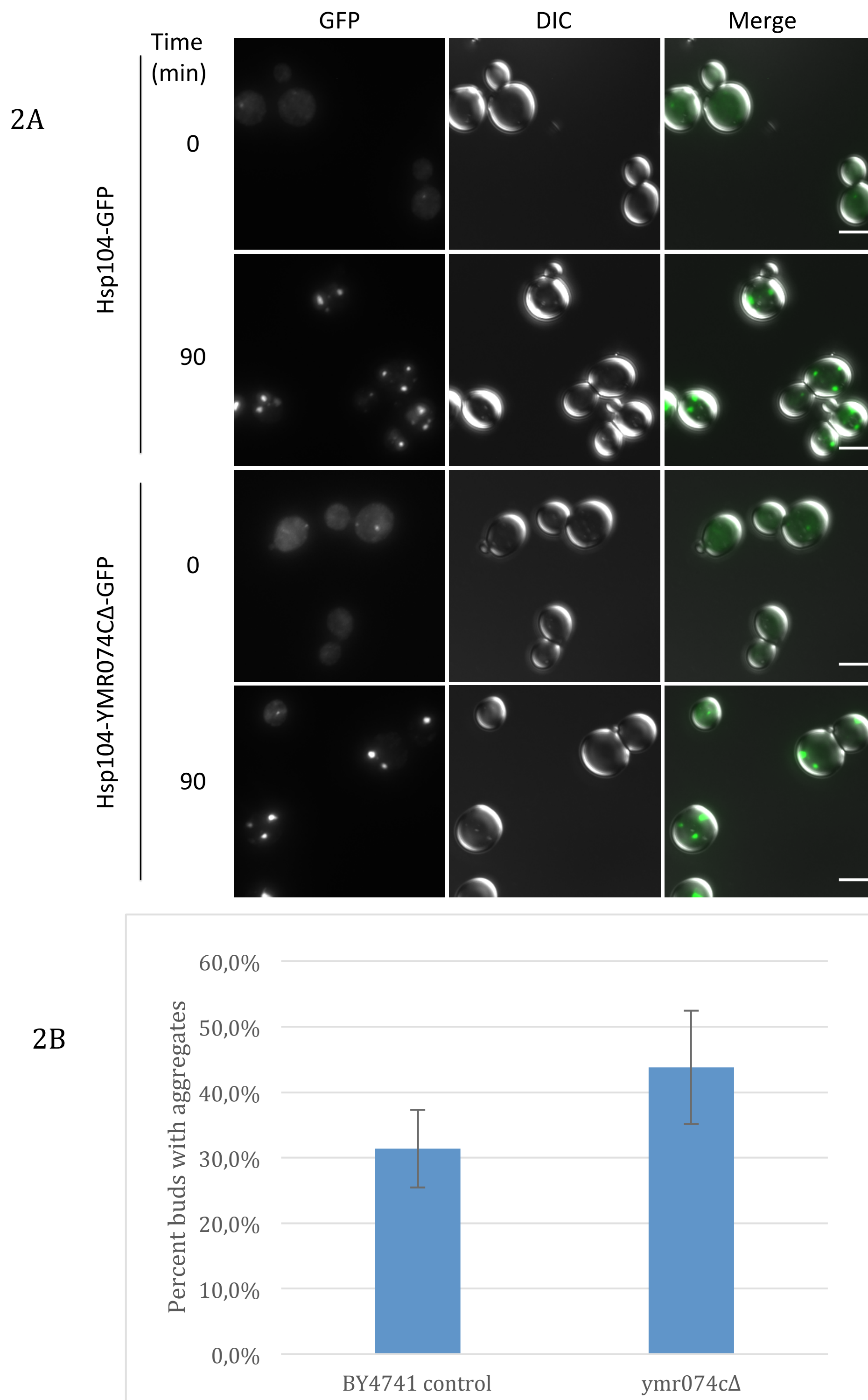

Figure 2. Results for YMR074C knock-out strains. The cells were observed under fluorescent light microscopy and examined for total cell count, total bud count and the amount of inclusion aggregates found in the buds during mitosis forming new daughter cells. 2A shows representative images for the visualized cells and their aggregates, shown in three modalities; DIC, GFP and Merge. YMR074CΔ displayed an abnormal count aggregates in the budding daughter cells. The control strain is WT with a functional GFP tag on Hsp104 (B). For the control, 290, 74 and 81 budded cells were counted respectively, wheras for the YMR074CΔ strain, 218, 91 and 51 budded cells were counted.

Complementation sought to rescue cells to WT

Having confirmed the AGG-phenotype, it was important to verify that YMR074C was indeed the gene responsible for this phenotype by doing a complementation experiment. Normally, a clone from the Molecular Bar-coded Yeast (MoBY) library can be used for complementation. Unfortunately, no YMR074C clone existed. The YMR074C open reading frame plus upstream and downstream sequences was therefore PCR amplified and cloned into integrating plasmid pPW411 to generate a complementation plasmid, pJB001. The knock-out strain ymr074cΔ (JBY001) was transformed with exogenous plasmid pJB001 and the control pPW411. Two isolates (iso 1 and iso 2) containing pJB001 were picked, as well as an empty vector (pPW411) control. As was done with the first experiments, three individual replicates for each respective isolate were run. Samples were taken at 0 and 90 minutes of continuous heat-shock at 38C (Figure 4).

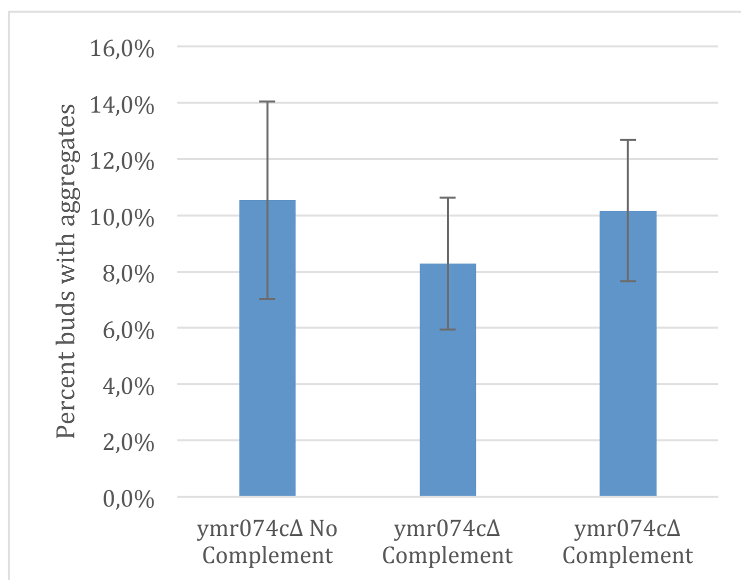

Figure 3. Aggregate count graph. Two plasmid complementation strains were counted. "No complement" strain served as an empty vector control. Paired t-test: 0.207(iso1), 0.861 (iso2). Paired t-test: 0.207, 0.861, respectively. <125 buds were counted from all the samples taken during heat-stress. Though, two samples from control strains (pPW411) at 0 minutes did not.

Partial rescue of the WT-phenotype could be observed through complementation (Figure 3). Compared to the control in which 10.5% (stdev 5.0%, se 3.5%) of buds had inherited Hsp104-GFP inclusion bodies, isolate 1 and 2 had 8.3% (stdev 3.3%, se 2.3%) and 10.2% (stdev 3.5%, se 2.5%), respectively. The overall percentage of aggregates in buds for this set of strains was much lower than expected, the expected average amount of inclusion body inheritance for wild-type cells is 30%. Therefore, all strains showed far fewer aggregates in their buds than expected.

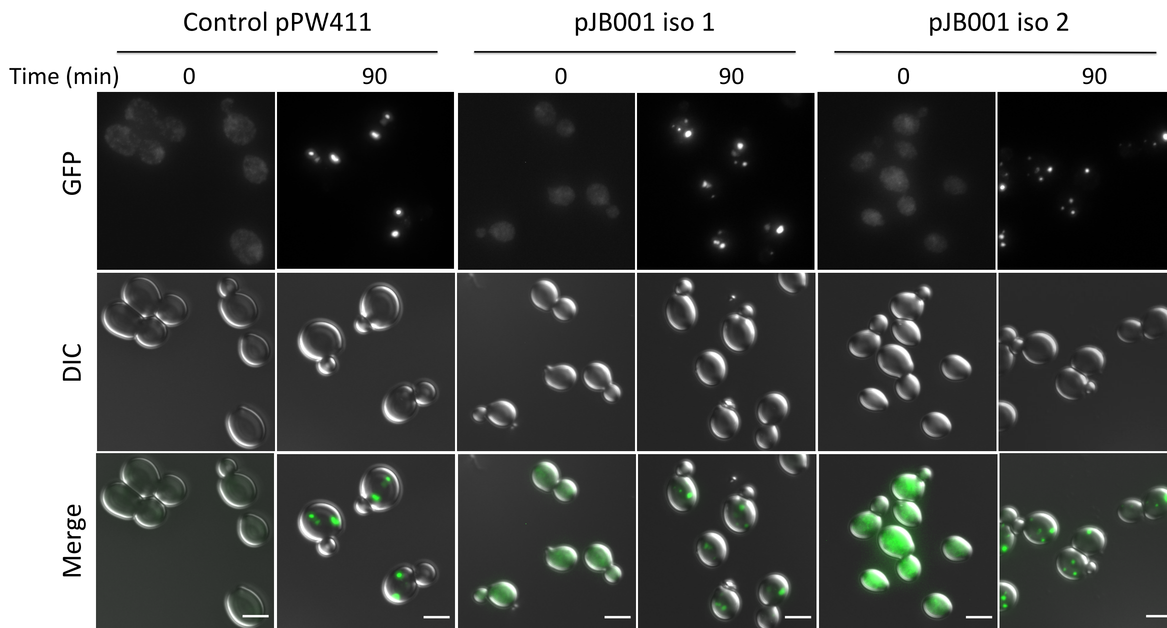

Figure 4. Representative images for the complementation. Aggregate flourescent signal was poor, therefore GFP and Merge pictures have been edited to better exemplify morphology. Cells were heat-shocked for 0 and 90 minutes as previosly. Cells shown in DIC, GFP and two channels merge. Strain names displayed at the top dividers.

Overexpression of YMR074C displayed enhanced aggregate retention

Since YMR074C was identified in a screen for high copy suppressors of mitochondrial damage, it was relevant to investigate the effects of overexpression of YMR074C. The YMR074C ORF was therefore cloned into plasmid pPW417 containing a GPD-promoter for overexpression. This plasmid was named pJB002. pJB002 and the pPW417 vector control were transformed into separate WT-strains containing Hsp104-GFP. Three replicates of inclusion body formation assay were run and samples were taken after 0 and 90 minutes of continuous heat-shock at 38°C as done previously.

For three overexpression experiments, the average amount of daughter cells inheriting aggregates was 5.8% (stdev 3.9%, se 2.7%). For the control, we expected a result similar to WT asymmetric inheritance. The wild-type control showed an average of 10.9% (stdev 3.1%, se 2.2%) of buds with aggregates. In comparison with the control, the over-expression strain increased asymmetry by 53.2%. The overall lever of inheritance was similar to the complementation experiments, but still far lower than

the original strains without plasmids. Nevertheless, overexpression of YMR074C has a positive effect on protein quality control as was predicted by experiments on mitochondrial damage.

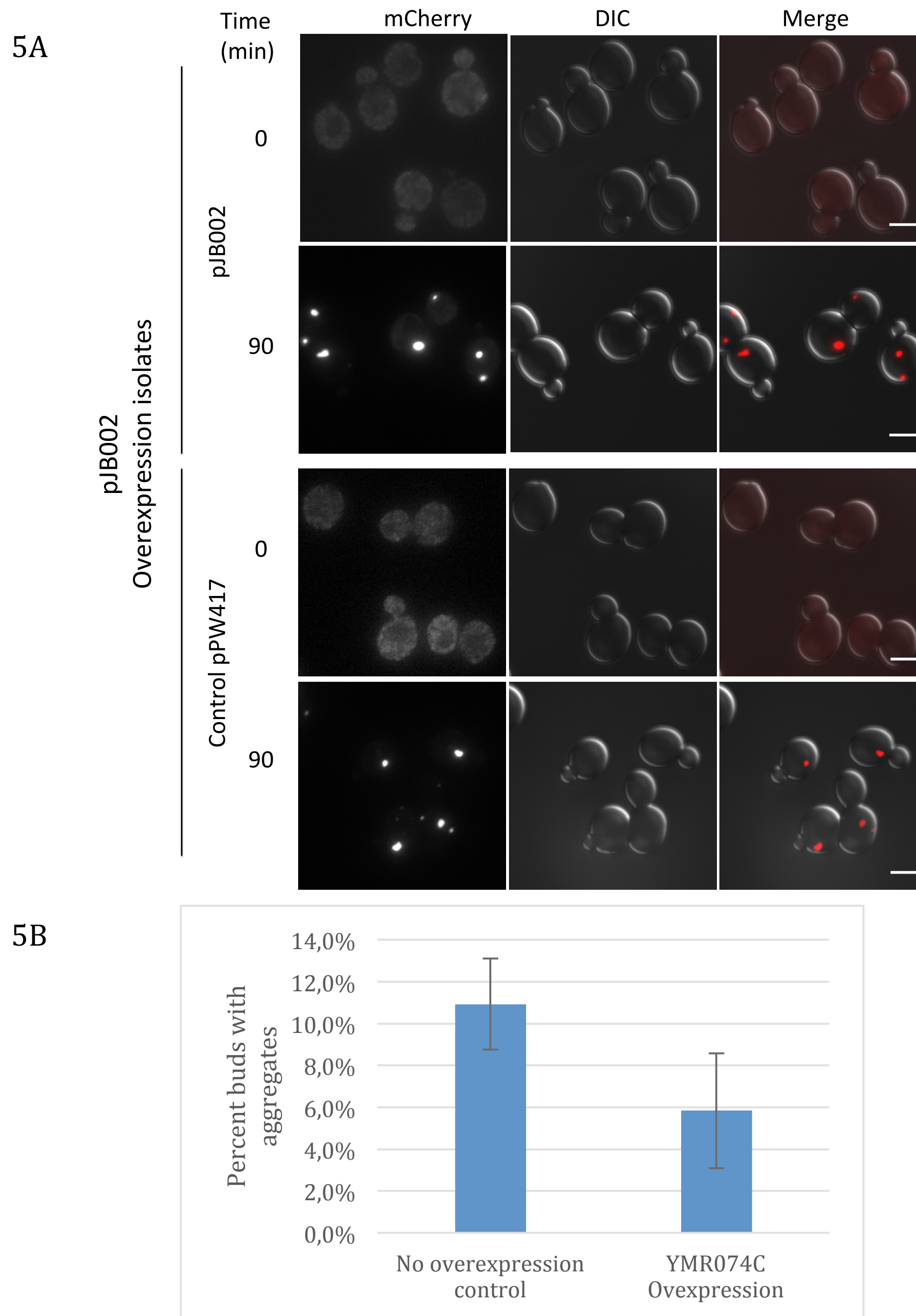

Figure 5. Results for YMR074C overexpression strains. Representative images. Cells shown in DIC, mRuby and merged channels. mRuby-aggregates are represented in red in Merge.(A)
>150 buds were counted for all replicates. The control contains an empty vector. The daughter cells showed a low overall aggregate-count, though significantly lower in the overexpression strains. Paired t-test: 0.027.(B)

KAN-markers linked to the gene X were identified through PCR amplification.

The spindle pole body component SPC110 was identified as a factor important for asymmetric segregation of damaged proteins in a screen for genes synthetic lethal with a sir2Δ deletion. (Song et al., 2014). In a recent study by the Nyström Lab(Tjeerdsma, 2019) it was shown that mutations in SPC110 had differing effect on the ability of the mother cell to retain flourenscent tagged protein aggregates in the mother cell during cell division. Interestingly, a ts mutant, spc110-221, displayed a dramatically enhanced retention of aggregates in the mother cell during mitosis. It was later shown, however, that the mutation responsible for this enhanced segregation phenotype was not found at the SPC110 locus. Due to the dramatic retention effect, it was important to determine the responsible mutation(s) and it was noted that the enhanced segregation phenotype was shown to have be linked to the KanMX resistance marker, indicating that a different ts mutant strain was likely mixed up with the spc110-221 strain.

An inverse PCR strategy was devised to identify the mutation(s) linked to the KanMX resistance marker. It was first necessary to identify sites in the KanMX marker of the spc110-221 mutant strain that could efficiently be used for amplification. A test amplification was performed using two primers designed to amplify a small fragment of the KanMX marker. Gel electrophoresis of these reactions showed flourescent bands at the expected kb-size, thus confirming the presence of the KanMX marker in the genome. In short, a preserved linked selection marker could be identified through PCR.

Inverse PCR. A strategy to determine the genotype of SPC110-221.

Having confirmed these two sites in the KanMX gene as good annealing sites, the reverse complement of the test primers were used in the inverse PCR strategy. The genome was extracted from cells of the spc110-220 and spc110-221 strains, i.e. genome preparation. Two restriction enzymes were selected to cut randomly in the genome that were not present in the KanMX marker. The resulting DNA fragments

were diluted, allowed to self-anneal, and ligated into circles using T4 DNA ligase. A fraction of this reaction was used as template in the final reverse PCR amplification.

Figure 7. Gel imaging. The gel (left) contains the first attempt at reverse PCR. Spc110-221 showed bands with little to no migration, indicating massive nucleotide chains that were inconclusive. The other gel (right) shows the last attempt. An initial PCR product was used as template for a second PCR amplification, and the result showed in the image. Bands of differing size along the gel did not provide us with material to continue the experiment.

Using two different digestion enzymes for both spc110-220 and spc110-221 did not provide any differing results on the gel as can be seen in Figure 7. The PCR product was run through gel electrophoresis twice. The initial PCR product was run in a second PCR reaction using the same primers and consequently run two times through gel electrophoresis with the same conditions. No consistent detectable product could be generated through reverse PCR for SPC110-221. For SPC110-221 a faint band with minimal migration could be seen, indicating a very large nucleotide chain that exceeded our expected result and could not be used to locate the unknown mutation. This meant that, using this method, we could not determine the locus or sequence of the mutated gene in the spc110-221 strain.

DISCUSSION

Proper segregation of damaged proteins in the progenitor during mitosis is an intricate feat and 10-15% of endogenous genes are important for this to function efficiently.(Sontag et al., 2017) A poorly functioning PQC system would increase the inheritance of damaged proteins to the daughter cells and these so-called aging-factors would accelerate aging of the cell. Understanding why this occurs is of outmost importance. If we are to understand how aging progresses and how the PQC system affects it.

YMR074C remain a viable locus for continued investigation

We were able to reproduce the asymmetric segregation phenotype observed for YMR074C knockout in a previous screen for asymmetry generating genes (AGG:s).(Hill et al., 2016) We could conclude from our results that knockout of YMR074C resulted in a diminished ability to segregate aggregates from the daughter cell. 39% more aggregates were found in the buds of ymr074CΔ cells compared to the wild type controls. This result is in accordance with the previous AGG screen from Nyström Lab, where ymr074CΔ had a 49% deviation from WT.

The AGG phenotype of ymr074CΔ could partially be complemented in relation to the plasmid control. However, paired t-test came out 0.207 and 0.861, respectively, which does not provide statistical significance for the complementation. This needs to be repeated to determine whether the partial complementation is real. Additional replicates would likely yield a result that is statistically significant.

pPW411 was used as a plasmid background for the YMR074C locus that we used to complement the knock-out strain. The control used for this experiment was BY4741 with a Hsp104-GFP tag with a pPW411 (empty vector control). This carries the advantage that both strains stay as similar as possible, save for the key point of the experiment. There might have been need for a non-plasmid control since the cells overall displayed a scarce amount of aggregates, both for this experiment as well as the latter induced over-expression. The complementation sought to rescue the cells

and revert to WT. We expected to see flourescent inclusions in roughly 30% of the buds, instead the results was approximately 10% for both complementation and control replicates. One possible explanation for this difference could be that the pictures from the microscope was noticably darker, meaning that the discrepancy of aggregate quantity would be because of poor visualisation of the aggregates.

Based on these figures, it would certainly be interesting to see how ymr074CΔ would affect life-span. Future research could also include a time-lapse experiment, fully microscopy based, to monitor inclusion formation. This would also account for more variables of aggregate inheritance, as this study only considered daughter cells as positive or negative. The location of the aggregates is also an important factor when studiyng inclusion formation and inheritance. An additional flourescent tag on a mitochondrial component (Tom70-mRuby) was, in fact, meant to be included in this study as a complement to the strains created for this study. The goal wa sto determine whether ymr074CΔ affected the documented association of aggregates with mitochondria as has been documented previously. Mitochondrial dysfunction is also a hallmark of aging (Lopez-Otin et al., 2013) and recent research indicates that apart from cytoplasmatic PQC there is also a mitochondrial PQC that might be relevant for the aggregate inheritance phenotype. However, this had to be excluded due to time-shortage.

Abandoning SPC110-221 in favor of more promising research candidates?

A recently identified mutant showed dramatically enhanced retention of damaged proteins in the mother cell during asymmetric cell division in *S. cerevisiae*.(Tjeerdsma, 2019) spc110-221 is not responsible for the observed phenotype, as it initially was thought to be. However, unveiling the mutation locus may provide interesting new questions as to why it happened, and to what extent this would affect the life-span of the strain. We know that knocking out AGG:s accelerate aging, but would it be wrongly assumed that enhanced function of segregation of damaged proteins in cell division would deccelerate aging? Does mutation X enhance the function of the gene in an unforseen way or does it simply knock-out the function, as induced deletion mutations in experimentation are intended to do. Such an instance

would be a rare event, as function impairment of endogeonus genes usually deteriorates the overall survival of the cell.

The results from the reverse PCR strategy used in this study could not provide any clues as to the location of the mutations responsible for the spc110-221 AGG phenotype (see Fig. 7). We thought that the KanMX marker was in the vicinity of the mutation locus in the genome. This proved to be the case for spc110-220, but not necessarily for spc110-221. If not, it would prove a bigger challange to figure out the actual mutation. Therefore, further investigative experiments are required to reveal the actual genotype of spc110-221. The same stategy could be implemented again, with a change of digestion enzymes or primers. One could also implement a genome library to perform a suppressor-screen. This would aim at restoring the ts phenotype of SPC110-221 by adding plasmids containing ORF:s of the yeast genome. However, this would be time consuming.

In spite of the possible interesting novel insights that the enhanced phenotype of spc110-221 may provide, so far the mutation appears to be out of our reach. To unravel the factual mutation would demand resources that does not stand in proportion to the plausible merits of contiued experimentation.

Populärvetenskaplig sammanfattning

Varför åldras vi? Om man kan bygga ett hus från marken och upp, borde det inte då vara en barnlek att därefter förhindra husets förfall? Varför skulle man inte kunna laga det man byggt?

Men orsaken bakom åldrande har aldrig varit närmre att bli upptäckt. På Nyström Lab arbetar man med en jästart, *Saccharomyces cerevisiae*, för att förstå mer om hur celler åldras. Det finns nämligen många likheter och flera paralleller att dras mellan den mänskliga cellen och jäst. Processer som är evolutionärt bevarade mellan arterna. Genom att studera jästcellen kan man således lära sig mycket om just åldrande, men även om åldersrelaterade sjukdomar såsom Alzheimer's, Parkinson's m.fl och bakgrunden till deras uppkomst i människan.

Denna studie berör proteiner i cellen. Proteiner bygger upp cellen, dess skelett, olika beståndsdelar och dess komplexa maskineri, alltid i arbete. Proteiner tillverkas i cellen, där DNA:t som finns i alla cellkärnor kan betraktas som ritningar. En enskild ritning, alltså ett enskilt segment av DNA, kallas för en gen. I jästcellen finns ca 8,000 gener, en bråkdel av vad som finns i mänskliga celler. Jag har studerat två olika gener och jag har valt att kalla dem SPC och YMR i denna text.

Proteiner har en invecklad struktur och en finkänslig sammansättning. Detta innebär att proteiner kan tillverkas felaktigt, skadas och förstöras. Detta sker kontinuerligt i cellen och hela tiden omsätter cellen proteiner. Hur skadade proteiner hanteras är avgörande för dess fortsatta överlevnad och funktionsgrad och en höggradig uppdamning av skadade proteinaggregat är direkt kopplat till ålderstecken. Särskilt intressant blir det när jästcellen celldelas, d.v.s knoppar av en bit av sig själv för att skapa en dottercell. Modercellen kan skapa en asymmetrisk delning av sig själv, behålla skadliga proteinsamlingar till dottercellens fördel.

YMR är ett protein som vi ännu inte vet mycket om förutom att den är involverad i den asymmetriska fördelningen av skadade protein mellan moder- och dottercell. Genom att behandla cellen i ett laboratorium har vi kunnat se att en högre produktion av YMR förbättrar modercellens förmåga att behålla skadliga proteiner medan utebliven YMR-produktion markant har försämrat samma aspekt.

SPC upptäktes i en tidigare studie och man uppmärksammade den jäststammens effektiva asymmetriska nedärvning mellan moder- och dottercell. En okänd mutation X var orsaken till detta och i denna studie har vi försökt hitta den mutationen. Projektet var dock fruktlöst trots flertalet försök och frågan kvarstår huruvida mutation X ligger till grund för cellens särskilda uppförande.

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