Function of a novel protein, Pah1, in the G1/S checkpoint in fission yeast

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A thesis for the Master's degree in Molecular Biology

Department of Biosciences
Faculty of Mathematics and Natural Sciences

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Department of Cell Biology
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60 Study points

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http://www.duo.uio.no/

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ABSTRACT

Our group discovered a novel G1/S checkpoint in fission yeast. This checkpoint is activated in response to ultraviolet (UV) irradiation leading to a delayed entry into S phase, and its activation is totally dependent on the Gcn2 kinase. The main substrate of Gcn2 is eIF2α and thus Gcn2 had not been seen as a cell-cycle regulator. One intriguing aspect of the checkpoint was the question of how a translational regulator, which is thought to reside in the cytoplasm, can achieve its effect on a nuclear event such as DNA replication.

In a recent publication it is shown that GCN2 in mammalian cells phosphorylates the methionine tRNA synthetase (MRS) in response to UV irradiation. The MRS is part of a bigger complex called the multisynthetase complex (MSC) which also contains additional proteins which are not synthetases. One of these is the aminoacyl tRNA synthetase-interacting multifunctional protein 3 (AIMP3) which binds to the MRS. UV irradiation causes GCN2 to phosphorylate the MRS leading to the release of AIMP3 which is then translocated to the nucleus. Interestingly, previous work identified AIMP3 as a tumor suppressor that can interact with cell-cycle regulators such as ATR. Here we identify a putative AIMP3 homologue termed Pah1 (for Pombe aimp homologue) and characterize its role with a special focus on its involvement in the G1/S checkpoint.

In this study we have deleted the gene encoding pah1 in fission yeast cells demonstrating that it is not essential for cell survival. We went on to show that the delayed entry into S phase after UV irradiation is shortened in the *pah1Δ* cells, suggesting that Pah1 is required, but not essential, for the full length delay. By tagging the Pah1 protein with a GFP tag we could investigate whether the protein translocated to the nucleus after UV and our preliminary data suggests that is does, indicating that its functions are conserved.

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Oslo, May 2014

Herman Sebastian Folkestad Evensen

ABBREVIATIONS

AIMP3	Amino-acyl-tRNA synthetase-interacting multifunctional protein 3
ARS	Aminoacyl-tRNA synthetase
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related protein
BSA	Bovine serum albumin
Bp	Base pair
CAK	Cyclin-dependent kinase activating kinase
CDI	Cyclin-dependent kinase inhibitor
CDK	Cyclin-dependent kinase
clonNAT	Trade name for antibiotic nourseothricin
DMSO	Dimethyl sulphoxide
DNA	Deoxyribo nucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetraacetic acid
eEF	Eukaryotic elongation factor
eIF	Eukaryotic initiation factor
eRF	Eukaryotic release factor
EMM	Edinburgh minimal media
EtOH	Ethanol
Fwd	Forward
g	Gram
G1	First gap phase
G2	Second gap phase
$G418^R$	G418 resistance
Gcn2	General control non-derepressible-2
GDP	Guanosine diphosphate
GFP	Green fluorescence protein
GTP	Guanosine triphosphate
Hri	Heme-regulated inhibitor
IgG	Immunoglobulin G
IgM	Immunoglobulin M
Kb	Kilo base
kDa	Kilo Dalton
LiAC	Lithium acetate
Log	Logarithmic
M	Mol/L
M phase	Mitosis
MCM	Mini-chromosome maintenance
Met	Methionine

MetOH	Methanol
Min	Minute
mL	Milliliter
MQ-H_2O	MilliQ- H_2O
mRNA	Messenger RNA
MRS	Methionyl-tRNA synthetase
MSC	Multisynthetase complex
Nat^R	ClonNAT resistance
NER	Nucleotide excision repair
Nm	Nanometer
Nt	Nucleotides
OD	Optical density
ORC	Origin recognition complex
PAGE	Polyacrylamide gelelectrophoresis
Pah1	Pombe aimp homologue 1
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PVDF	Polyvinylidine fluoride
RNA	Ribonucleic acid
RNase A	Ribonuclease A
Rev	Reverse
Rpm	Rounds per minute
SDS	Sodium dodecyl sulphate
S phase	DNA synthesis phase
S. cerevisiae	*Saccaromyces cerevisiae*, budding yeast
Ser	Serine
S. pombe	*Schizosaccaromyces pombe*, fission yeast
TAE	Tris-acetate-ethylenediaminetraacetic acid
TBS-T	Tris-buffered saline tween-20
TC	Ternary complex
TCA	Trichloroacetic acid
TE	Tris EDTA
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
tRNA	Transfer RNA
U	Units
UV	Ultraviolet
UVC	Ultraviolet C (280 nm – 100 nm)
UVER	UV-damaged DNA endonuclease-dependent excision repair
V	Volt
YE	Yeast extract
YFP	Yellow fluorescence protein
µL	Microliter

°C	Degree Celsius

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1 Introduction

1.1 *Schizosaccharomyces pombe* as a model organism

A model organism is a species that is used to study and better understand different biological phenomena. These could be processes such as cellular growth, division and DNA repair. Good model organisms are defined by their easy handling in the lab, ability to be genetically manipulated and their low cost. A few examples of commonly used model organisms are the small flowering plant Arabidopsis thaliana, the fruit fly *Drosophila melanogaster* and the mouse. All of these are multi-cellular, but uni-cellular organisms such as the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* are also much used in science. What model organism is chosen depends on the question asked. When studying cellular mechanisms such as cell-cycle regulation and division, *S. pombe*, also called fission yeast, is a popular organism to use.

Fission yeast was first described in 1893 by Paul Lindner who isolated it from East African millet beer. He gave it the name pombe which comes from the Swahili word for "beer". It wasn't until the 1960s that fission yeast was used as a model organism for studying growth control (Forsburg, 2005).

S. pombe is a unicellular, rod shaped eukaryote fungus, with a cell length of 12-15 µm and a diameter of 3-4 µm (figure 1.1). As its name suggests it divides by binary fission, in contrast to S. cerevisiae which divides by budding. Even though they are both a type of yeast, the two separated from a common ancestor 1 billion years ago (Forsburg, 2005). In 2002 *S. pombe* was the sixth eukaryote to have its entire genome sequenced. Its genome has a size of 13.8 Mb and includes approximately 5100 protein coding genes which are distributed on three chromosomes (Wood et al., 2002) (www.pombase.org).

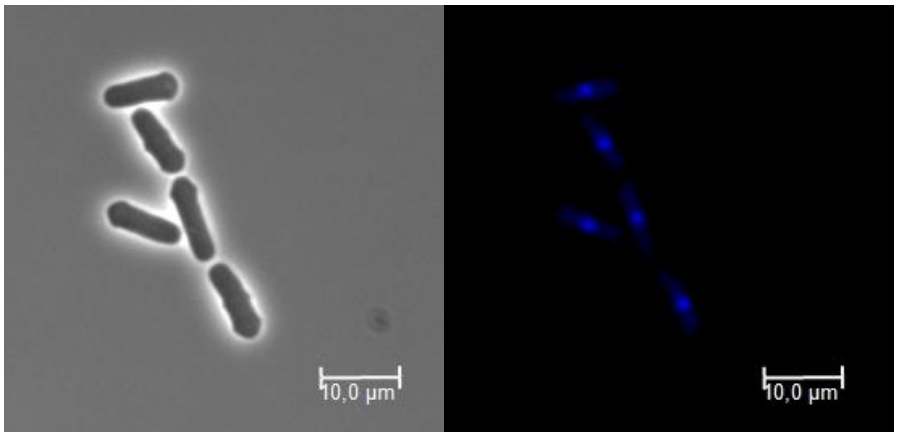


Figure 1.1: A microscopy picture of fission yeast cells. Phase contrast is used on the picture to the left, and on the picture to the right fluorescence is used to visualize the DAPI stained nuclei.

S. pombe has become a popular model organism for several reasons. It has a short generation time, can be maintained in a haploid or diploid state and classical methods in genetics can be applied. It is also non pathogenic, which makes it easy to handle in the lab. *S. pombe* is much used to study the cell-cycle and the response to DNA damage because homologues of genes involved in these mechanisms can be found in human cells (pombenet, appendix I). It has also been shown that about fifty genes in *S. pombe* are homologous to genes involved in disease in humans, half of them related to cancer (Wood et al., 2002). A deletion library which covers 98.4 % of the fission yeast genome has also been made (Kim D. U. et al., 2010), and the localization of 4431 fission yeast proteins has been determined (Matsuyama et al., 2006).

1.2 *S. pombe* cell-cycle

S. pombe has a cell-cycle which is quite similar to that of the general eukaryotic cell-cycle. It consists of four distinct phases termed G1 (gap 1), S (DNA synthesis), G2 (gap 2) and M (mitosis) phase.

G1 is an important phase, where the cell has to decide whether it should progress into a new cell-cycle or enter stationary phase. DNA replication occurs during S phase, and in M phase the DNA is distributed to the two daughter nuclei. As can be seen on figure 1.2, the G2 phase of fission yeast cells is longer than the other phases and the cells spend up to 70 % of their time in G2. This series of events is tightly regulated, and leads to cell division, producing two equally sized daughter cells. In 2001 Paul Nurse together with Leland Hartwell and Tim Hunt received the Nobel prize in Physiology or medicine for their research on regulators of the cell-cycle (appendix I).

Fission yeast cells are commonly found as haploid cells that multiply asexually, but the cells can also enter a meiotic cell-cycle, and multiply sexually by meiosis and sporulation. This is dependent on the growth condition. *S. pombe* will only mate when starved. When mating the cells will conjugate and form diploid zygotes. After a short time meiosis will occur and four haploid spores are made, producing zygotic asci.

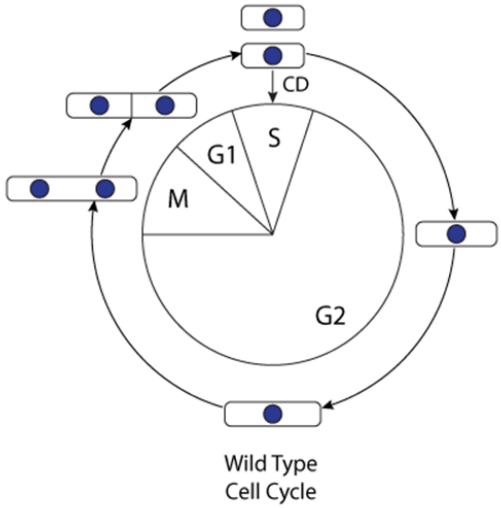


Figure 1.2: An illustration of the fission yeast cell cycle, showing how G2 is the longest phase and where the cells spend most of their time. It should be noted that the cytokinesis is completed during S phase. The picture is a modified form from Novak et al., 1988.

1.2.1 Cell-cycle regulation and checkpoints in *S. pombe*

To ensure that the cell-cycle events occur in the correct order and at the right time, the phases of the cell-cycle are strictly regulated. In eukaryotes this important task is performed by a family of proteins called cyclin-dependent kinases (CDK). The CDKs are constitutively expressed through the cell-cycle, and they regulate the transition from one phase to the next by substrate phosphorylation. They are regulated by several means: 1) interaction with cyclin partners, 2) inhibitory phosphorylation and 3) direct inhibition by proteins called cyclin-dependent kinase inhibitors (CDIs). Cyclins are regulatory proteins whose expression levels fluctuate through the cell-cycle, and the complexes they form with CDKs are specific for the different phases in the cell-cycle. CDK activation by phosphorylation can also take place and this is performed by CDK-activating kinases (CAKs).

In *S. pombe* one CDK has been identified, Cdc2, (Nurse, 1990) and it has been shown to interact with four different cyclins, Cig1, Cig2, Cdc13 and Puc1 (Moser and Russel, 2000). In addition a CDI named Rum1 has been identified. This protein targets Cdc13 for degradation and also inhibits the complexes Cdc2/Cdc13 and Cdc2/Cig2 during G1 (Benito et al., 1998). Cig2 is the main cyclin which promotes the onset of S phase (Mondesert et al., 1996), and when Rum1 is targeted to degradation at the end of G1 by Cdc2/Cig1 and Cdc2/Puc1 (Benito et al., 1998) the cells can progress into S phase. Cdc13 promotes the onset of mitosis (Booher et al., 1989) and the Cdc2/Cdc13 complex is inhibited by the kinases Mik1 and Wee1 during S and G2 phase (Baber-Furnari at al., 2000, Rhind and russel 2001). It should also be noted that CAKs have been identified in *S. pombe*. A CAK complex consisting of the proteins Mcs2, Mcs6 and Pmh1 have been found to activate Cdc2 by phosphorylation (Hermand et al., 2001).

Processes in the cell-cycle phases must be properly completed before the cell can progress further. Control mechanisms called checkpoints will stop or delay the cell-cycle in response to DNA damage or if some processes are not correctly finished (Hartwell and Weinert, 1989). The checkpoint machinery, when activated, halts the cell-cycle to ensure that the proper actions can be performed. If the cell has acquired DNA damage that is beyond repair the cell can be targeted for destruction. Three DNA-damage checkpoints have been identified in *S. pombe* (Rhind and Russel, 1998, Caspari and Carr, 1999), namely the intra-S checkpoint, the S/M checkpoint and the G2/M checkpoint. The intra-S checkpoint delays DNA replication, so that DNA repair can be carried out. The S/M checkpoint prevents the cell from entering mitosis until DNA replication is finished and the G2/M checkpoint will delay the onset of mitosis in response to DNA damage. These checkpoints target CDK activity through inhibitory phosphorylation, and they depend on checkpoint Rad proteins which can sense DNA damage. Our group has previously discovered a novel checkpoint, regulating the G1/S transition in response to some, but not all, DNA-damaging agents (Tvegård et al., 2007, Krohn et al., 2008). This checkpoint does not target CDK activity, but inhibits the onset of DNA replication.

If a checkpoint should lose its activity, this could cause aberrations in chromosome replication and distribution and also increased cell proliferation which can cause cancer. Understanding the cell-cycle regulation and the mechanism behind checkpoints is therefore very important in the fight against cancer.

1.2.2 G1/S phase transition

The G1 phase is a critical point in the cell-cycle. At this time the cell decides whether it is favorable to continue through another cell-cycle or if it should enter a quiescent phase. This decision is influenced by the extracellular surroundings of the cell. If the conditions are appropriate, the cell will start to prepare for DNA replication, and entry into S phase will take place. The decision of whether or not the cell should enter stationary phase is made at a point in the cell-cycle called START, and it is located in early G1 in yeast cells. In mammalian cells this same point is called the restriction point.

DNA replication in eukaryotes starts at regions of the DNA called origins of DNA replication. In *S. pombe* these origins are often found in intergenic regions which are very large and A-T rich (Dai et al., 2005). When the cells prepare for replication, a protein complex called origin recognition complex (ORC) binds to the origin of DNA replication. The ORC consist of six sub-units, Orc1-6, and this complex attracts replication factors which are needed to initiate DNA replication. The binding of ORC to chromatin attracts the proteins Cdc18 and Cdt1 which cooperatively promotes DNA replication (Nishitani et al., 2000). Cdc18 and Cdt1 will promote the assembly of the MCM (mini chromosome maintenance) complex on the origins of replication, forming the pre-replicative complex (pre-RC). The MCM protein complex is conserved in all eukaryotes (Forsburg, 2004) and it consists of six proteins, MCM2-7, which form a hexamer with helicase activity. When the pre-RC is formed, the cell is licensed for replication.

Controlling the expression of many genes specific for the G1/S transition, such as cig2, is done by the transcription factor Cdc10. To arrest cells in G1, temperature sensitive cdc10 mutant strains are often used. Cells containing this mutation will grow as normal when incubated at the permissive temperature of 25°C. When the temperature is raised to 36°C, which is called the restrictive temperature, Cdc10 will be inactivated and the cells will arrest in G1. Strains carrying this mutation were used to synchronize cells in G1 in this study.

1.3 DNA damage checkpoints

The checkpoints found throughout the cell-cycle are, as mentioned above, important for regulating the cell-cycle, and to allow the cells to repair damage to the DNA. If cells are exposed to damaging agents such as UV, ionizing radiation or oxidation, so-called DNA damage checkpoints are activated and halt the cell-cycle in order to repair the damage. These checkpoints can be found in G1, intra-S and G2. If the cell does not get enough time to repair the DNA, genome instability might occur.

The protein kinases ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related protein (ATR) plays a central role in the DNA damage responses, and they are highly conserved in eukaryotes (Abraham, 2001). These two proteins are members of the phosphatidylinositol-3-kinase-like kinase family (PIKKs) and in response to DNA damage, such as DNA strand breaks or lesions, they can promote DNA repair, cell-cycle arrest, apoptosis (as reviewed in Marechal and Zou, 2013).

In this study UVC light with a wavelength of 254 nm was used to irradiate the *S. pombe* cells (4.1.7). The result of such irradiation is the production of cyclobutan pyrimidine dimers (CPDs) and 6-4 photoproducts. In *S. pombe* a protein called Rad3, which is the fission yeast homologue of the mammalian ATR, is activated in response to DNA damage. Rad3 will then target the checkpoint kinases Chk1 and Cds1. Chk1 is activated by Rad3 in response to DNA damage in late S phase or in G2 (Martinho et al., 1998), while Cds1 is activated in S phase (Lindsay et al., 1998). These two kinases target CDK activity by causing the inhibitory phosphorylation of Cdc2 through mediator proteins. After contracting UV damages as the ones mentioned above, the fission yeast cells repair these through the nucleotide excision repair pathway (NER) and the UV-damaged DNA endonuclease-dependent excision repair pathway (UVER). The UVER pathway is not conserved like the NER pathway is. During the NER pathway, damage is recognized, followed by incision on both sides of the lesion by two endonucleases. Excision, resynthesis and ligation follows. The UVER pathway operates in quite a similar way, but only one endonuclease performs the incision step.

1.4 Translation

Translation is the cellular process where mRNA transcripts are read and translated into proteins by RNA-protein complexes called ribosomes. The ribosomes are made up of two sub-units, one large (60S) and one small (40S). The nucleotide sequence of mRNA is read as codons, triplets of nucleotides which encode amino acids. One codon encodes one specific amino acid. Translation can be divided into three stages; initiation, elongation and termination. During initiation the $tRNA^{Met}$ is placed at the start codon on the mRNA in the 40S ribosome sub-unit. It is the eukaryotic initiation factors (eIFs) that organize this action. $tRNA^{Met}$ is called the initiator tRNA, and it always contains methionine. In elongation aminoacids delivered by tRNAs are joined together by covalent bonding to create a polypeptide which can be hundreds and even thousands of peptides long. The joining of amino acids is catalyzed by the ribosomal RNA and eukaryotic elongation factors (eEFs). The termination of protein synthesis occurs when the ribosome reaches a stop codon. The stop codon is recognized by proteins called eukaryotic release factors (eRFs) which causes the release of the polypeptide, and the subsequent dissociation of the ribosome complex.

1.4.1 Regulation of translation initiation

To ensure that the correct protein is expressed at the appropriate time, the regulation of gene expression is very important. During exposure to certain stresses such as UV irradiation or nutritional starvation, it is important that genes involved in protecting the cell against these stresses are activated and the corresponding protein is translated. Other important instances where correct gene expression is imperative, is during the cell-cycle. As mentioned in 1.2.1, the expression of the correct cyclin at the right time is important to let the cell progress through the cell-cycle. The regulation of gene expression can take place on several different points in the DNA-to-protein process, and during translation it is most often the initiation event that is regulated. This is usually done through modification of initiation factors.

A detailed overview of cap-dependent initiation can be seen in figure 1.3. At the start of initiation, a 43S pre-initiation complex (PIC) containing eIFs and Met-tRNAi interacts with the m7G(5')pppN cap of mRNA. This interaction is facilitated by the cap-binding complex eIF4F. After attachment of mRNA the complex starts to scan for the AUG start codon. When AUG is recognized the eIF2- bound GTP is irreversibly hydrolyzed to GDP. After hydrolysis,

eIF2 and the other initiation factors are released from the PIC and the large 60S sub-unit joins to make the 80S ribosome complex. This form of initiation is called cap-dependent initiation of translation.

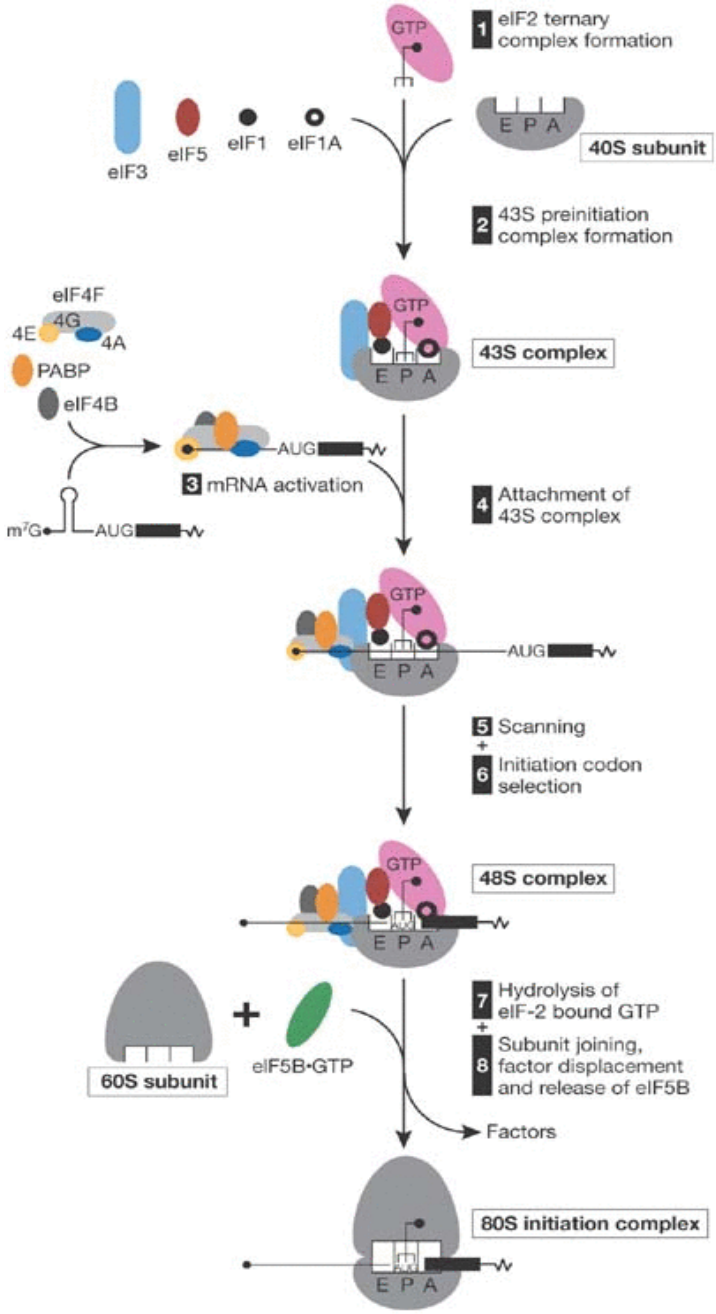


Figure 1.3: Overview of cap-dependent initiation. The ternary complex containing eIF2-GTP/Met-tRNAiMet, together with eIF3, eIF5, eIF1, eIF1A and the 40S sub-unit form the 43S pre-initiation complex. eIF4F consists of eIF4A, E and G, and activates mRNA in cooperation with poly(A) binding protein (PABP) and eIF2B by binding to the m7GpppN cap of the mRNA. This complex unwinds RNA structures and scans along the mRNA for the start codon site. When found the 48S complex is formed. The following hydrolysis of the eIF2 bound GTP, induced by eIF5B, mediates the binding of the 60S sub-unit and the dissociation of eIF2-GDP, eIF5B and other factors, which creates the 80S initiation complex. This marks the end of the initiation phase, and elongation follows (Holcik and Pestova, 2007).

Regulation of translation initiation can be carried out in one of two ways. Global translational control, in which most of the mRNA in the cell is affected, and mRNA-specific regulation where only certain sets of mRNA are regulated (Gebauer and Hentze, 2004). The targets of regulation are different in these two methods. Global control of translation will usually modify the eIFs or the proteins they interact with. The initiation factor eIF2 and the cap-binding complex eIF4F are targets that are frequently modified by global control. mRNA-specific control on the other hand targets elements on the mRNA, which can be found in the untranslated regions, through regulatory proteins or non-coding RNA (Gebauer and Hentze 2004, Sonenberg and Hinnebusch 2009).

1.4.2 The eukaryotic initiation factor-2α

eIF2 is a protein complex consisting of the three sub-units eIF2-α, eIF2-β and eIF2-γ, and together with GTP it mediates the binding of Met-tRNAiMet to the 40S ribosome sub-unit during initiation of translation (figure 1.3). The complex containing eIF2-GTP and Met-tRNAiMet is called the ternary complex. Met-tRNAiMet can only be transferred to the 40S sub-unit when eIF2 is bound to GTP, thus making it the rate-limiting step in the construction of the ternary complex. The substitution of GDP to GTP is catalyzed by the guanine nucleotide-exchange factor eIF2B, but phosphorylation of eIF2α at serine-51 (Ser52 in *S. pombe*) will inhibit this exchange and thus hinder the formation of the ternary complex. The decrease in the number of ternary complexes causes downregulation in translation of global mRNAs, but also upregulation of specific mRNAs. These mRNAs that are specifically translated after eIF2α phosphorylation are often associated with stress responses such as UV irradiation, heat shock and starvation (Holcik and Sonenberg, 2005).

1.4.3 Family of eIF2α kinases

In mammalian cells four kinases that phosphorylate eIF2α have been identified. These are general non-derepressible (GCN2), haem-regulated inhibitor kinase (HRI), protein kinase RNA (PKR) and PKR-like endoplasmic reticulum kinase (PERK). The kinases share a similarity in their kinase domain, but they have a distinctive regulatory domain which makes them respond to different kinds of stress. GCN2 has a regulatory domain which is related to histidyl-RNA synthetase (HisRS) which is activated when binding uncharged tRNAs during amino acid starvation (Dong et al., 2000). HRI is activated by stress responses such as heme

deficiency, oxidative stress and heat shock in erythroid cells (Lu et al., 2001). PKR is activated in an antiviral response (Barber, 2005) and PERK is activated by the presence of unfolded proteins in the endoplasmatic reticulum (Harding et al., 2000).

The protein kinase Gcn2 is highly conserved in eukaryotes, and it has been shown that human GCN2 can functionally substitute the Saccharomyces cerevisiae Gcn2 (Dever et al., 1993). A Gcn2 homologue in S. pombe has also been identified (Zhan et al., 2004). The most studied mechanism of Gcn2 activation is the binding of uncharged tRNAs during amino acid starvation and the subsequent phosphorylation of eIF2α which causes the selective translation of the transcription factor Gcn4 in budding yeast (ATF4 in mammals). Gcn4 activates genes involved in the biosynthesis of amino acids (as reviewed in Hinnebusch, 2005). A few other examples of stresses that activate Gcn2 is exposure to hydrogen peroxide (H_2O_2), treatment with DNA alkylating reagents such as methyl methanesulfonate (MMS) and UV irradiation (Krohn et al., 2008, Tvegård et al., 2007). Exactly how these stresses might cause the accumulation of uncharged tRNAs is not known for sure. Homologues of HRI can also be found in fission yeast, these are named Hri1 and Hri2 and they stimulate the phosphorylation of eIF2α in response to heat shock, arsenite and oxidative stress (Zhan et al., 2002, Zhan et al., 2004).

1.5 Background

Our group has previously identified a novel checkpoint regulating the G1/S transition in fission yeast (Nilssen et al., 2003, Nilssen et al., 2004, Tvegård et al., 2007). Cells exposed to UV irradiation (245 nm) in G1 delays the onset of S phase by a mechanism which is absolutely dependent on the protein kinase Gcn2. A delay in the loading of MCMs, and hence pre-RC formation, together with the expression of the Cdc2 inhibitor Rum1, which is only expressed in G1, were used to confirm that the UV induced delay occurs in G1 and not in S phase (Tvegård et al., 2007). In addition to the delay, downregulation of global translation was also observed.

Gcn2 is responsible for phosphorylating Ser52 of eIF2α in response to UV irradiation, and is required for both the delay and the reduced translational rate at least when translation is measured in vitro (Tvegård et al., 2007). eIF2α is known to be a target for translational regulation and it is phosphorylated in response to several different forms of stresses. To

further investigate its role in the G1/S delay, Ser52 was replaced with alanine which cannot be phosphorylated. After UV irradiation of the eIF2αS52A mutant, there was no significant delay in pre-RC formation, suggesting that eIF2α phosphorylation is necessary for delaying the assembly of the pre-RC complex. Surprisingly, translation was also reduced in the mutant, although not as much as in wild type cells (Tvegård et al., 2007) suggesting that Gcn2 has another way to regulate translation after UV. Gcn2 is known to be in the cytoplasm, and there is no indication or report on it being in the nucleus, but it has an effect on nuclear processes such as initiation of replication. Gcn2 has also been shown to have an effect on mitotic entry (Petersen and Nurse, 2007). When cells are put into medium with poor nitrogen resources, the cells accelerate entry into mitosis, entering at a smaller size. This is dependent on Gcn2, but independent of eIF2α. How Gcn2 achieves this is not known, but one possibility is through its role in translation, indirectly. This is currently being investigated by our group. However, in a recent publication a new substrate of Gcn2 in mammalian cells was identified. This substrate is methionine tRNA synthetase (MRS) and its function is to initiate translation by transferring methionine to the initiator tRNA (Kwon et al., 2011). MRS can be found in the multisynthetase complex (MSC) which consists of 9 aminoacyl tRNA synthetases, including MRS and also some proteins that are not tRNA synthetases. These are aminoacyl tRNA synthetase-interacting multifunctional protein 1 (AIMP1), AIMP2 and AIMP3 (Lee et al., 2004). AIMP3 is a tumor suppressor protein and it has been shown that when cells are exposed to UV irradiation, GCN2 phosphorylates MRS at serine 662 which causes a conformational change that releases AIMP3 (Kwon et al., 2011). AIMP3 is then translocated to the nucleus (figure 1.4). Interestingly AIMP3 was previously shown to interact with ATM/ATR which are components of the DNA-damage checkpoint system. This interaction has been shown to upregulate p53 in response to UV irradiation and oncogenic stress (Park et al., 2005, Park et al., 2006). Cross talk between MRS and eIF2α phosphorylation was also shown. The levels of phosphorylated MRS was increased when the levels of eIF2α was decreased, and vice versa, suggesting a cooperation between these two in controlling translational inhibition (Kwon et al., 2011).

We were wondering whether there is an AIMP3 homologue in fission yeast which has a corresponding role, and links the cytoplasmic Gnc2 to nuclear functions. Our investigations have shown that there is a MSC, containing a putative MRS homologue called Rar1, and a possible AIMP3 homologue in fission yeast. The purpose of this master thesis is to investigate the properties of this putative homologue termed Pombe aimp homologue 1 (Pah1).

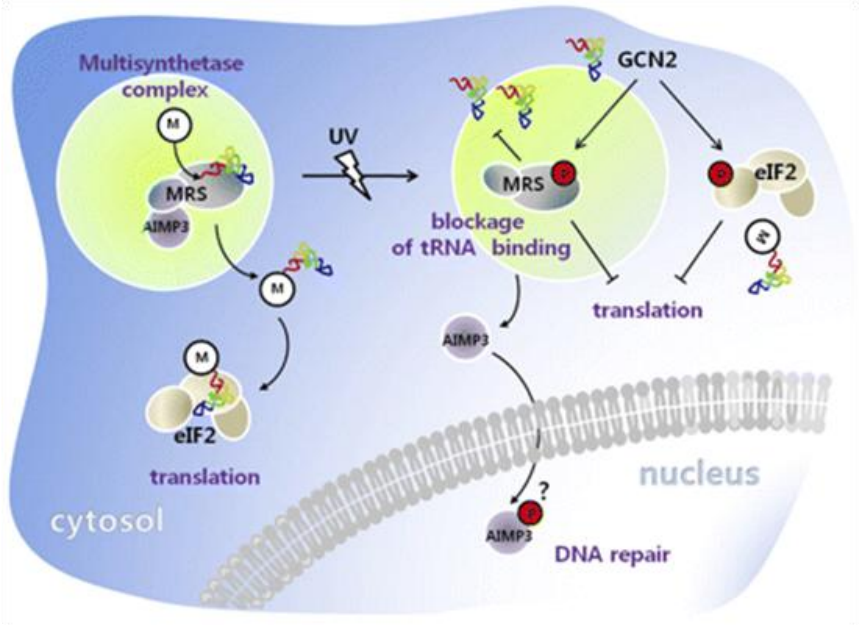


Figure 1.4: Dual role of MRS. Ordinarily MRS catalyses the attachment of methionine to the initiator tRNA. AIMP3 can be bound to the N-terminal of MRS, and upon UV irradiation, GCN2 phosphorylates Ser662 of MRS which blocks the binding of methinonine to initiator tRNA and causes the release of AIMP3. AIMP3 is then translocated to the nucleus where it can interact with the DNA damage repair system. It is believed that the translocation of AIMP3 to the nucleus is due to post translation modifications, possibly by phosphorylation.

2 AIM OF STUDY

The purpose of this master thesis is to characterize the putative AIMP3 homologue Pah1, with the main focus of studying its possible involvement in the G1/S checkpoint in *S. pombe*.

3 MATERIALS

3.1 *S. pombe* strains

The following strains (table 3.1) were used in this study.

Table 3.1 *Schizosaccharomyces pombe*

Strain	Genotype	Supplier
#489	cdc10-M17 h-	Lab collection
#1138	gcn2::ura4+ cdc10-M17 ura4-D18 h-	Lab collection
#1142	cdc10-M17/cdc10-M17 ade6-M210/ade6-M216 ura4-D18/ura4-D18 h-/h-	Lab collection
#1229	cdc10-M17 mcm6:GFP:kanR ade6-M210 ura4-D18 h+	Lab collection
#1312	pku80::ura4+ ura4-D18 leu1-32 ade6-704 h-	Lab collection
#1929	aimp::natMX6/ aimp+ cdc10-M17/cdc10-M17 ade6-M210/ade6-M216 ura4-D18/ura4-D18 h-/h+ mcm6:GFP:kanR / /mcm6+	This study
#1930	cdc10-M17/cdc10-M17 ade6-M210/ade6-M216 ura4-D18/ura4-D18 h-/h+ mcm6:GFP:kanR /mcm6+	This study
#1931	aimp::natMX6/ aimp+ cdc10-M17/cdc10-M17 ade6-M210/ade6-M216 ura4-D18/ura4-D18 h-/h+	This study
#2010	aimp::natMX6 cdc10-M17 ade6(M216 or-M210) ura4D18 h+	This study
#2011	aimp::natMX6 cdc10-M17 ade6(M216 or-M210) ura4D18 MCM2:YFP:kanMX6 h+	This study
#2012	cdc10-M17 aimp:GFP:natMX6 h-	This study

3.2 Primers and plasmids

3.2.1 Primers

Table 3.2: The following primers were used in this study.

Number	Primer name	Direction	Sequence	Supplier
#1	MCM2 tag fwd	Forward	5'-TGATTATTGATTCCTTTGTGAATGCCCAAAAAATGAGTGTTAAACGAAGTTTGTCAAGAACATTTGCTAAATATCTTATTCGGATCCCCGGGTTAATTAA-3'	DNA technology A/S
#2	MCM2 tag rev	Reverse	5'-ACAGATGCAGTAACAAAAGGAGGGTCAAAGAACGGGGAAATCAAAATCACTGGACTCCATAAACTGATTGTGTCAAAAAAGAATTCGAGCTCGTTAAAAC-3'	DNA technology A/S
#3	aimpD fwd	Forward	5'-ATCTCTTATAAAGAACCATACAACTTAACGTTTTCGTTTATTCGAATTGCATATCTTTTCATCTGTACTTCTACCGCAGACGGATCCCCGGGTTAATTAA-3'	DNA technology A/S
#4	aimpD rev	Reverse	5'-TTCATATATAAAAAGAACAAAAAGTATTTGGAATTCACTGTTATCTTCTTTTCTATACTTTAAGTACATCAACTTTAGAAGAATTCGAGCTCGTTTAAAC-3'	DNA technology A/S
#5	aimp tag fwd	Forward	5' -GGTTGCATAGGCTATTTGTCAAGGGAAAAAAGGACCTCGGATTCTGTAAAGCTCAAACTGTTGTCAATGGCACATTAAGTCGGATCCCCGGGTTAATTAA-3'	DNA technology A/S
#6	In aimp sense	Forward	5'-CCTGGATTTACAAGTGGAGAGG-3'	DNA technology A/S
#7	us aimp sense	Forward	5'-GATATGCAACGTCTTTGGTTGA-3'	DNA technology A/S
#8	ds aimp as	Reverse	5'-GGACTAAGACAAAGGTTGTGCC-3'	DNA technology A/S
#9	Us 5' aimp sense	Forward	5'-CGAAATGTAGAGTAAATTGG-3'	DNA technology A/S
#10	Rev5' homology + nat	Reverse	5'-CTTAATTAACCCGGGGATCCGTCTGCGGTAGAAGTACAGAT-3'	DNA technology A/S
#11	Fwd3' homology + nat	Forward	5'-GTTTAAACGAGCTCGAATTCTTCTAAAGTTGATGTACTTAAAG-3'	DNA technology A/S
#12	Ds3' aimp rev	Reverse	5'-CAGAAGCCACCAAATTTAAA-3'	DNA technology A/S
#13	Us 5' aimp sense2	Forward	5'-CGAAAGCTTGATTCTTAGTA-3'	Invitrogen
#14	Ds 3' aimp rev2	Reverse	5'-GGTTTACTGTCGAACTATTT-3'	Invitrogen
#15	NatMX6 sense	Forward	5'-ACATAAACAACCATGGGTAC-3'	DNA technology A/S
#16	In MCM2 Sense	Forward	5'-TACTGTGCGTCATCTGGAGTCT-3'	DNA technology A/S
#17	DS MCM2 as	Reverse	5'-CCCTTTAAATTTGGAAATGGAC-3'	DNA technology A/S

3.2.2 Plasmid templates

The following plasmids were used as templates when making transforming DNA for transformation in *S. pombe*.

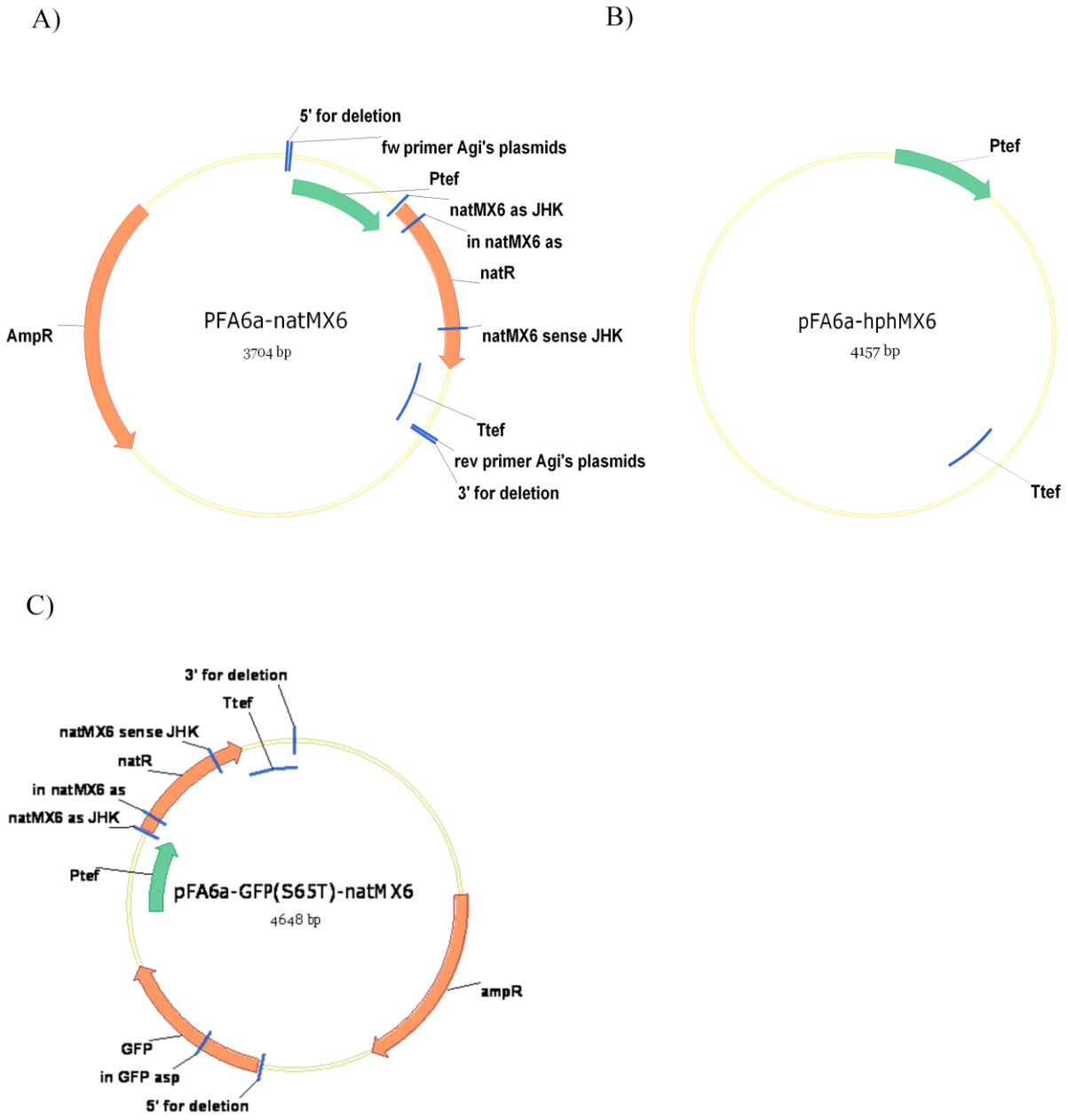


Figure 3.1: A) A map of plasmid #274 pFA6a-natMX6. B) A map of plasmid #275 pFA6-hphMX6. C) A map of plasmid #309 pFA6a-GFP(S65T)-natMX6. Plasmid #397 pFA6-YFP-kanMX6 was also used, but is not shown.

3.3 Enzymes

The enzymes used in this study is listed in table 3.3 except for the enzymes included in kits (3.6)

Table 3.3: enzymes

Enzyme	Supplier
NdeI	New England Biolabs
PmeI	New England Biolabs
Ribonuclease A	Sigma-Aldrich
SacI	New England Biolabs
S.H.P/H.PJ. Helix Pomatia Juice (helicase)	Life technologies
Zymolase 20T	Nacalai Tesque

3.4 Antibodies

Table 3.4: Antibodies used for immunodetection and visualization in this study.

Antibody	Antigen	Origin	Concentration	Supplier
Primary	GFP	Mouse	1:2000	abCam
	eIF2α-P	Rabbit	1:3000	Life Technologies
	Tubulin	Mouse	1:30 000	Sigma-Aldrich
Secondary	Anti-mouse, IgG+IgM alkaline phosphatase linked whole antibody	Goat	1:5000	GE Healthcare
	Anti-rabbit, IgG alkaline phosphatase linked whole antibody	Goat	1:10 000	GE Healthcare

3.5 Molecular weight standards

The molecular weight standards in table 3.5 were used in order to estimate the size and amount of DNA and protein in a sample. Pictures of the molecular weight standards can be seen in appendix 2.

Table 3.5: Molecular weight standards used in this study.

Molecular weight standards	Range	Supplier
O'generuler 1kb DNA ladder, ready-to-use	250–10 000 bp	Fermentas
Generuler 100bp pluss DNA ladder	100-3 000 bp	Fermentas
Dual Color Precision Plus Protein Standard	10-250 kDa	Bio-Rad

3.6 Kits

Table 3.6: Kits

Kit	Supplier
Nucleospin Extract II	Macherey-Nagel
Accuprime Pfx DNA Polymerase	Invitrogen
DreamTaq DNA polymerase (2X)	Thermo Scientific

3.7 Chemicals and reagents

Table 3.7: Chemicals and reagents

Supplier	Chemicals
Arcus kjemi	2-propanol
Biotium	GelRed
Fermentas	6X Orange loading dye
GE Healthcare	ECF substrate, ECF dilution buffer, Membrane blocking agent
Intergen	Sheared herring testes
Kemetyl Norge AS	Ethanol
Life Technologies	Sytox green
Merck	Acetone, Methanol
New England Biolabs	NEBuffer#4, BSA
Roche	Protease inhibitor cocktail tablets
Sigma-Aldrich	Acetic acid, adenin, agarose type 1, bromphenol blue, citric acid, DMSO, DTT, EDTA, G418, glucose, glycerol, glycin HCl, KAc, KHP, lithium acetate, NaF, NaCl, NaN_3, NH_4Cl, Peg 4000, phloxine B, PIPES, SDS, sodium sulphate, sorbitol, spermidin HCl, spermine HCl, TCA, Tris, Triton X-100, Tween-20, uracil
WERNER BioAgents	ClonNAT

3.8 Solution

3.8.1 Growth Media and agar plates

Growth Media	Ingredients
EMM medium	31.77 g/L EMM Supplemented with 80 µg/mL amino acids when needed
EMM agar plates	48 g/L EMM agar
EMM with Phloxine B	EMM supplemented with 20 µg/mL Phloxine B
MEA agar plates	30% malt extract, 20% g/L agarose
YE 50 medium	0.5% Yeast extract, 30 g/L glucose, 50 mg/mL histidine, 50 mg/mL leucine, 50 mg/mL adenine, 50 mg/mL uracil, 50 mg/mL lysine
YE 50 agar	Same YE medium as above and 17 g/L agar in addition
YE 50 agar with ClonNAt	YE 50 agar supplemented with 100 µg/mL ClonNat
YE 50 with G418	YE 50 agar supplemented with 100 µg/mL G418
YE 50 with phloxine B	YE 50 agar supplemented with 20 µg/mL Phloxine B

3.8.2 Buffers and other solutions

Solution	Ingredients
Agarose gel solution, 1 % / 0.8 %	1 % / 0.8 % agarose 1 x TAE buffer
Citrate/phosphate pH 5.6	7.1 g/L Na_2HPO_4 11.5 g/L citric acid

Citrate/phosphate/EDTA/sorbitol	50 mM citrate/phosphate pH 5.6 40 mM EDTA pH 8.0 1 M sorbitol
Denaturing sample buffer (2x)	1 M tris-HCL pH 6.8 50% Glycerol 10% SDS 0.2 M DTT 0.1% Bromphenol blue
EDTA pH 8.0 (0.5 M)	146.12 g/L EDTA NaOH to pH 8.0
EMM sorbitol pH 7.0	15 mM KH phtallate 15 mM Na_2HPO_4 90 mM NH_4Cl 1.2 mM Sorbitol NaOH to pH 7.0
Extraction buffer	20 mM Pipes-KOH pH 6.8 0.4 M sorbitol 1 mM EDTA 150 mM KAc To 50 mL extraction buffer: 1.0 M spermidine HCl 1.0 M spermin HCl
LiAC/TE (10X)	1 M lithium acetate 1X TE Acetic acid to pH 7.5
PEG/LiAc/TE	40% PEG 4000 1x LiAc/TE
Potassium acetate pH 5.0 (5M)	118 g KAc 46 mL acetic acid to pH 5.0
Running buffer (10x)	30.2 g/L Tris 144 g/L glycine 1% (w/v) SDS
STOP buffer (2x)	20 mM Tris-HCl pH 8.0 150 mM NaCl 50 mM NaF 10 mM EDTA 1 med mer NaN_3

TAE buffer (10x)	48.8 g Tris base 11.4 mL glacial acetic acid (17.4 M) 3.7 g EDTA Dilute buffer to 1x by adding MQ-H_2O
TBS-T	20 mM Tris-HCL pH 7.5 8 g/L NaCl 0.05% (v/v) Tween-20
TE pH 7.5 (10X)	0.1 M Tris-HCl pH 8.0 0.01 M EDTA pH 8.0 0.02 HCl to pH 7.5
Transfer buffer	50 mM Tris 380 mM glycine 0.1% (w/v) SDS 20% (v/v) methanol

4 METHODS

4.1 Cell biology methods

4.1.1 Growth and maintenance of *S.pombe*

Yeast media

Yeast extract medium (YE) is a growth media for *S. pombe* which is rich in nutrients and provides optimal growth conditions. *S. pombe* cells may be grown in liquid YE and also on YE agar plates. Wild type strains have a generation time of 3 hours when growing in YE at 25°C, while mutants may have longer generation times. Antibiotics such as clonNAT and G418 may be added to YE for selection purposes.

Edinburgh minimal medium (EMM) is a poor, defined growth medium, and the yeast cells will have a generation time of around 4 hours for wild type cells at 25°C. Auxotroph mutants will not grow in this medium unless the required supplement is added.

Starting a liquid culture

When starting a liquid culture, a loop of cells are inoculated in 10 mL YE over night at 25°C, without shaking. This is to grow the cells up to early stationary phase. The next day the pre-culture is used to make a main culture in either YE or EMM depending on the purpose of the experiment. The main culture is grown in a shaking water bath at 25°C over night.

Optical density is used to measure the cell density in a liquid culture. The density is measured at 595 nm using a spectrophotometer (Hitachi U-1900), and a OD_{595} of 0.1 corresponds to 2 x 10^6 cells/mL for wild-type cells. When doing experiments it is important that the cells are growing exponentially. To achieve a main culture with cells in log phase the following equation is used:

$$V_{preculture} = \frac{V_{total} * OD_{desired}}{OD_{preculture} * 2^n}$$

where $V_{preculture}$ is the volume of pre-culture that is to be inoculated, V_{total} is the total volume of the new main culture, $OD^{desired}$ is the desired OD_{595} at a specific timepoint, $OD_{preculture}$ is the OD_{595} of the pre-culture. The number of generations, n, is calculated by dividing the time the cells are grown in liquid on the generation time of the strain used.

$$n = \frac{time}{gen\ time}$$

Growth on malt extract agar

In this study, growth on malt extract agar was used to make new strains by crossing, and for tetrad analysis. Conjugation and sporulation in fission yeast can only take place during starvation (Moreno et al., 1991), and MEA is a very poor medium which quickly runs out of nutrients for the yeast cells after a couple of rounds of cell divisions.

Strain maintenance

S. pombe strains can be frozen down and stored at -80°C for several years. The cells are frozen down in a 1:1 ratio of YES medium and 50 % glycerol. When preparing a glycerol stock, a small culture is grown for 1 day at 25°C and 0.8 mL is transferred to a cryotube and mixed with 0.8 mL 50 % glycerol. The stock is stored in a -80°C freezer.

When re-isolating a frozen stock, a small amount of cell suspension is scraped off using a sterile lab spatula and patched out on YE agar (or appropriate selective medium). The plate is incubated at 25°C for 4-5 days before cells are streaked out for single colonies on a new YE agar plate. The cells are incubated at 25°C for 3 days. YE plates can be kept at 4°C for two months as short term storage. To prevent the agar from drying out the plates are sealed with parafilm. During experiments fresh colonies were used.

4.1.2 Transformation of *S. pombe*

Transformation of *S.pombe* was done using the "Bähler's method" (Bähler et al., 1998), but with some modifications. After heat shock the cells were diluted in ½YE and incubated over night at 25°C (instead of being washed once with YE then resuspended in 0.5 mL MQ-H_2O and plated onto YE).

1) Spin down 50 mL/transformation of OD =0,2 at 1700 x g for 3 min.
2) Wash once with equal volume of MQ-H_2O. Spin down at 1700 x g for 4 min.
3) Resuspend the cell pellet inn 1 mL of MQ-H_2O. Transfer to Eppendorf tube. Spin down at 16 000 x g for 1 min.
4) Wash once with 1ml of LiAc/TE. Spin down at 16 000 x g for 1 min.
5) Resuspend the cell pellet in 100 µL/transformation of LiAc/TE.
6) Mix 100 µL of the concentrated cells with 2 µL sheared herring testes DNA and 10 µL of transforming DNA [100 – 300 ng DNA]. Incubate at room temperature (RT) for ~10 min.
7) Mix gently with 260 µL of 40 % PEG/LiAc/TE and incubate for 30-60 min at 25 °C.
8) Add 43 µL of DMSO and heat shock the cell suspension for 5 min at 42 °C in water bath.
    a. Spin down at 16 000 x g for 1 min.
9) Dilute cells in ½YE and incubate overnight at 25°C with rotation.
10) Spin down at 1700 x g for 3 minutes and resuspend in 1 mL MQ-H_2O. Plate out 200 µL on 5 selective plates and incubate at 25°C for 5-6 days.
11) Streak out single colonies onto selective plates.

4.1.3 Genetic crossing and random spore analysis

A newly created *S. pombe* strain may be crossed with existing ones to change the genomic information, adding or removing genotypes. Only strains with different mating types, h+ and h-, may be crossed. The strains are crossed on MEA plates since conjugation and sporulation only occurs when the cells starve.

In this study a diploid strain heterozygous for the deletion was crossed to a haploid strain. Haploid and diploid spores from the cross were identified with the use of YE + phloxine B plates. Phloxine B is a red stain that can be used to differentiate between living and dead yeast cells. The stain will accumulate in dead cells and give the colony a dark pink color (Moreno et al., 1991). Because diploid colonies contain more dead cells than haploid ones, they will have a darker pink color making it is easy to distinguish between haploid and diploid colonies.

1) Inoculate 500 µL MQ-H_2O with a toothpickful of both strains that are to be crossed. Vortex.
2) Spot 20 µL on MEA plate and let it dry.
3) Incubate at 25°C for 2 days.
4) Check for zygotic asci under a microscope.
5) If there are enough asci, inoculate 500 µL MQ-H_2O with a toothpick full of cells from the cross. Streak across the whole spot!
6) Add 4 µL helicase to the suspension and incubate for 3 hours at 36°C, incubator (Techne Dri-Block DB-2D).

7) Look for spores under the microscope.
8) Add 1 mL MQ-H_2O and spin down, 16 000 x g for 2 minutes at 4°C. Do this twice.
9) Resuspend pellet in 1 mL MQ-H_2O and plate out 20 µL and streak out for single colonies on YE + phlox B plates. Incubate at 25°C until colonies appear.
10) Identify haploid and diploid colonies based on the intensity of their pink color and separate them on new YE plates.
11) Replica plate onto selective plates.

The helicase used in this method is an enzyme from the gut of the snail *Helix pomatia*. It is used to break down the cell wall of yeast cells.

4.1.4 Replica plating

Different selective markers can be used in *S. pombe* and replica plating is a way to screen for these markers and other phenotypes. Auxotrophs, nutritionally defective mutants, are common markers in fission yeast and the genes frequently affected are adenine, leucine, lysine and uracil. Resistance towards clonNAT and G418 are also very commonly utilized. In this study replica plating was used to screen for a haploid mutant and desired genotypes after transformation.

Place a sterile filter paper on top of a plastic replica-block. Press the colonies gently onto the filter paper from the master plate. Then press fresh selective plates as required onto the filter paper and thus the colonies are transferred preserving the original spatial pattern. EMM plates were used when screening for auxotrophs. The replica plates are incubated at 25°C for 2-3 days. Auxotrophs will not grow on EMM plates that do not contain the required supplement.

4.1.5 Tetrad dissection

By performing tetrad analysis, spores from a single conjugation event can be identified, separated and allowed to germinate to form single colonies. A row of four colonies will therefore derive from one mating and meiosis. This is a powerful technique which can be used to construct strains and to investigate genotypes and phenotypes.

Two strains are crossed on MEA and incubated at 25°C for two days. A light microscope is used to check for asci. A fine line with cells from the cross is streaked out on a new YE plate, and asci are pulled approximately 1-2 mm from the line using a light microscope and micromanipulator. The micromanipulator has a glass needle which is used to pull asci and

spores. After the asci are pulled the plate is incubated for 3-5 hours at room temperature until the ascus walls have broken down. The micromanipulator is then used to separate the spores in a line, having 3-5 mm of space between each spore. The plate is incubated at 25°C until the spores have started to make colonies.

4.1.6 Synchronization of *S. pombe* cells

Before being UV irradiated, the cells are synchronized in G1 by using *cdc10-M17*, temperature sensitive strains (1.2.2). A culture of cells is grown to log phase, as described above (4.1.1), and the culture is shifted up to 36°C for 4 hours in a water bath. When the cells are incubated at 36°C, they will progress until G1 phase and then arrest. The OD_{595} at shift up is between 0.1 -0.2.

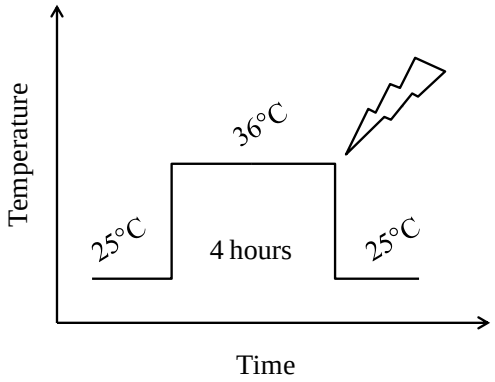


Figure 4.1: Synchronization and UV irradiation of *S. pombe* cells in G1 phase. The graph depicts how cells are shifted up to 36°C and subsequently UV irradiated after one generation time. When cells are growing in EMM the generation time is 4 hours.

4.1.7 UV irradiation of *S. pombe* cells

Cells are irradiated in EMM due to the fact that YE contains macromolecules and other factors which can absorb some of the UV light. The cell culture is poured into a petri dish of appropriate size, so the liquid layer has a height of 3 mm. The culture is under constant stirring when they are irradiated with 254 nm UVC light (Sylvania Fluorescence Lamp, UVC light). Cells are synchronized in G1 and irradiated immediately after release (Figure 4.1).The cells receive an irradiation dose of 1100 J/m^2. Before irradiating, the intensity of the UVC-light (W/m^2) is measured using a UVX radiometer (AH Diagnostic) and the following equation is used to calculate the correct dose:

$$seconds\ of\ irradiation = \frac{J/m^2}{W/m^2}$$

Because light with wavelength 254 nm don't go through water very efficiently, the dose of 1100 J/m^2 is not the exact amount of what the cells absorb. But this is countered by the constant conditions during irradiation, which makes sure that the given dose is proportional to the absorbed dose. After irradiation in G1 the cell survival is around 15 % (Nilssen et al., 2003) whereas the same dose delivered in G2 leads to >95 % survival (Christiane Rothe, personal communications).

4.1.8 Measuring generation time

Measuring the generation time in *S. pombe* is done by growing a culture in YE (as described in 4.1.1) to log phase, OD_{595}=0.2, and measuring the OD_{595} every 30 minutes for 6 hours. There is a linear relationship between the number of cells growing in log phase and the OD_{595} of the culture which means that a doubling in OD_{595} equals a doubling in cell number.

Using the following equation one can calculate the generation time:

$$G = \frac{t}{n}$$

Where t is the time t_2-t_1 and n is the number of generations.

4.1.9 Flow cytometry

By using the principles of light scattering and emission of fluorescence, flow cytometry can be used to count, sort and examine microscopical particles such as cells. This is done by suspending particles in a stream of fluid and exposing them to a beam of exciting light. Fluorescent tagged molecules are especially well suited for this technique. The obtained data can be analyzed using flow cytometry software. A method for interpreting *S. pombe* results has been developed and published by Knutsen et al., 2011.

Taking samples for flow cytometry:

1) Spin down 1 mL of cell suspension. Centrifuge at 16 000 x g for 1 minute. Remove supernatant.
2) Resuspend in 1 mL cold EtOH, -20 , while vortexing. Store samples in a fridge.

Preparing samples for flow cytometry:

1) Spin down 500 µL sample for 2 minutes at 16 000 x g. Remove supernatant.
2) Resuspend the pellet in 1.0 mL 20 mM EDTA. Spin down for 2 minutes at 16 000 x g. Discard supernatant.
3) Resuspend the pellet in 1.0 mL 20 mM EDTA. Spin down for 2 minutes at 16 000 x g. Discard supernatant.
4) Resuspend pellet in 500 µL 20 mM EDTA containing 0.1 mg/mL RNase A. Incubate 3 hours or overnight at 36°C
5) Add 0.5 mL 20 mM EDTA containing 2 µM Sytox Green, so that the concentration in the sample is 1.0 µM Sytox Green.
6) Run the LSR II flow cytometer (BD Bioscience) as described in the core facility manual.

4.2 DNA methods

4.2.1 Polymerase chain reaction (PCR)

PCR is a technique that can rapidly amplify a specific segment of DNA. The method consists of 20-40 cycles and each cycle is comprised of three steps called denaturation, annealing and elongation. During the first step, denaturation, the double-stranded DNA is denatured by heat, which creates single-stranded DNA. In the next step, annealing, the temperature is lowered so oligonucleotide primers can anneal to the single-stranded DNA. In the third and last step called elongation, a DNA polymerase will extend the primers in a 5' -3' direction. This happens in the presence of deoxyribonucleotides, and the temperature is set accordingly to the optimal temperature for the polymerase used. The result of 20-40 cycles of these three steps is a exponential amplification of the DNA sequence of interest.

General PCR

Accuprime pfx DNA polymerase (Invitrogen) was used for most of the PCRs in this study. This enzyme possesses a proofreading 5' to 3' exonuclease activity which increases the fidelity. The polymerase is bound to an antibody which makes it inactive at room temperature, but upon heating in the first step of denaturation, the polymerase is released, providing a "hot start" which enhances specificity, sensitivity and yield. Because of this antibody bound form,

the PCR reaction can be assembled at room temperature. For PCR reaction set ups and programs see appendix II.

S. pombe Colony PCR

Colony PCR is a technique that can be used to screen for successful insertions/deletions into the genome of *S. Pombe*. SDS is used to extract DNA from the cells, and Triton X-100 is added to prevent the SDS denaturing the polymerase by forming mixed micelles.

A fresh colony is picked and put in 50 µL 0.25 % SDS in TE. The suspension is boiled for 5 minutes before being centrifuged for 5 minutes at 16 000 x g. 30 µL of the supernatant is recovered and can be used as template for future PCRs.

Dreamtaq master mix from Fermentas was used when doing colony PCRs in this study. This ready-to-use mastermix contains dreamtaq buffer, $MgCl_2$, dNTPs and Taq polymerase. This polymerase comes from the thermophilic bacterium *Thermus aquaticus* and it can withstand high temperatures that would normally denature other proteins. It does not have a proofreading 5' to 3' exonuclease activity.

4.2.2 One-step gene replacement

One-step gene replacement is an efficient method to delete, tag or overexpress genes in S. pombe (Bähler et al., 1998). The principle of this approach is to transform the target strain with a cassette containing the desired modification to the target gene (deletion, mutation, tag etc), an appropriate marker and targeting homologous regions. The ends of the transforming DNA will target the integration of the transformed cassette at the homologous target genes by recombination. The execution of this method was done as described in the article by Bähler et al., 1998. In this technique the transforming DNA is generated by PCR using hybrid primers, which contain ca 80 nucleotides of targeting sequence homologous to the desired site of integration and ca 20 nucleotides that anneal to the template containing the modification to be introduced.

For gene deletion, the deletion cassette consists of a marker and the gene specific 80 nucleotides on either side. A plasmid containing a suitable marker is used as a template in the PCR reaction. The same principle is applied when tagging genes. A plasmid template containing, for instance, clonNAT resistance and GFP is used to create a tagging cassette. For

C-terminal tagging the gene-specific sequenceses in the primers are homologous to the target sequences just upstream and downstream of the STOP codon. The deletion/tagging cassette is integrated through homologous recombination, using the cells own machinery. The transforming DNA invades the homologous region, induces a strand break which is repaired by homologous recombination and can integrate the exogenous DNA into the targeted locus (figure 4.2). The cells are transformed using Bählers method (4.1.2).

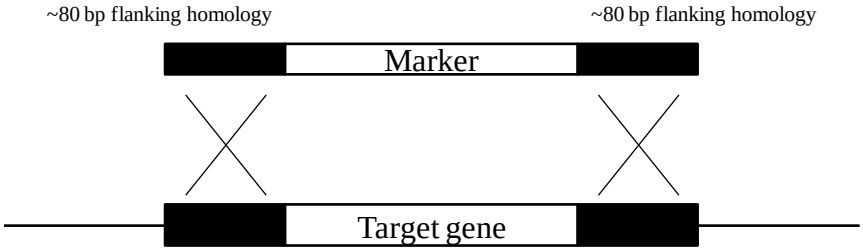


Figure 4.2 : Illustration showing how a deletion cassette with 80 bp of flanking homology is integrated into the target locus by homologous recombination. The drawing is not to scale.

4.2.3 Agarose Gel Electrophoresis

Agarose gel electrophoresis uses an electrical field to separate DNA fragments according to their size and charge. The negatively charged DNA fragments will migrate through a matrix of agarose toward a positive electrode, and this method can resolve fragments with differences down to approximately 50 nucleotides.

A low percentage of agarose will separate bigger fragments and a higher concentration will separate smaller fragments. Short fragments will migrate faster and further than the bigger fragments due to the pores in the agarose gel. This method can also be used to isolate and purify a DNA fragment of interest.

Different percentage gels were used in this study, mostly 0.8 % and 1 %. After electrophoresis the gel is stained with GelRed nucleid acid gel stain (Biotium). All gels in this study were visualized using 365 nm UV light ChemiGenius Bio Imaging System, Syngene.

1) Prepare and cast a gel with the wanted agarose percentage in a gel chamber.
2) Fill electrophoresis chamber with 1X TAE and put the agarose gel in the chamber.
3) Mix samples with 6X DNA loading buffer and load samples and molecular weight standard on the gel.
4) Run the gel on 120 V for 60-75 minutes.
5) Remove gel from chamber and put in GelRed (diluted 1:10000) for at least 30 minutes before visualization under UV light.

4.2.4 QIAxcel

PCR products were checked using either gel electrophoresis as described above or the QIAxcel system from QIAgen. This machine operates through the principle of capillary electrophoresis. Fragments migrate through small gel filled capillaries towards an anode, and are excited by light emitting diodes. The signal is picked up by detection fields and sent to the QIAxcel ScreenGel software for further interpretation.

The instruction manual from the manufacturer was followed when checking PCR products.

4.2.5 Genomic mini-prep

To isolate genomic DNA from *S. Pombe* the following protocol was used.

1) Grow a 10-15 mL culture to saturation in YE
2) Pellet cells at 1700 x g for 3 min at 4°C
3) Wash once with STOP buffer or ice cold water
4) If STOP buffer was used, wash once with 1.0 mL citrate/phosphate/EDTA/sorbitol
5) Resuspend cells in 1.0 mL citrate/phosphate/EDTA/sorbitol containing 2.5 mg Zymolase (20T). Transfer to an Eppendorf tube and incubate at 36°C for 30-60 minutes. Check for ghosts by adding a drop of 10% SDS on the microscope-slide (1 µL SDS to 9 µL of the digested cells).
6) Pellet cells at 16 000 x g at 4 °C for 1 minute.
7) Resuspend in 0.5 mL of TE
8) Add 25 µL 20% SDS, mix by inverting the tube several times. Incubate at 65°C for 1 hour.
9) Add 175 µL 5 M potassium acetate (pH 5), vortex well and keep on ice for 15 minutes.
10) Centrifuge at 16 000 x g for 10 minutes at 4°C.
11) Carefully transfer 0.5 mL of the supernatant to another Eppendorf tube.
12) Add 0.5 mL ice-cold isopropanol and mix.
13) Centrifuge at 16 000 x g for 10 minutes at 4°C. Remove all of the supernatant.
14) Wash pellet with 500 µL 70% ethanol.
15) Let the pellet air dry before resuspending in 500 µL dH_2O.
16) Run 10 µL on agarose gel to check the quality of your DNA.

4.2.6 DNA purification

After enzymatic reactions such as PCR and restriction cutting, the DNA needs to be purified to remove leftover primers, nucleotides and enzymes. Purification of DNA was done using the kit NucleosSpin Extract II, Macherey-Nagel. DNA purification was also done using ethanol. When using the kit, the manufacturer's instructions were followed with one exception; DNA was eluted in dH_2O instead of Buffer NE.

Protocol for DNA purification using ethanol:

1) Collect the DNA in a 2 mL tube and measure the volume
2) Add 1/10 volume of 3 M NaAc (pH 5.2)
3) Add 2.5 volume of 96% EtOH
4) Centrifuge at 16 000 x g for 20 minutes at 4°C. Remove supernatant
5) Add 500 µL of 70% EtOH and centrifuge at 16 000 x g for 5 minutes at 4°C. Remove supernatant
6) Let the pellet air dry before eluting it in MQ-H_2O
7) DNA should be stored at -20°C

4.2.7 Restriction analysis

Restriction analysis uses restriction enzymes, which are endonucleases that can cut double stranded DNA at specific recognition sequences. The resulting DNA fragment after digestion can be predicted by using known restriction enzymes. In this study restriction analysis was used to verify PCR products. Several restriction enzymes have been used as listed in table 3.3.

When performing a restriction digestion the following reaction mixture was used and incubated at 36°C for 1-2 hours:

1.0 µL DNA template (50-1000 ng)
1.0 µL 10X BSA (When needed)
0.5 µL Restriction enzyme (5-20 U)
1.0 µL NEBuffer #4
6.5 µL MQ-H_2O

Products from the restriction reaction were checked by agarose gel electrophoresis (4.2.3).

4.2.8 DNA quantification

The concentration of DNA samples were measured using the Nanodrop 1000 spectrophotometer (Thermo scientific). This instrument determines the concentration based

on the absorbance of 260 nm UV light in the DNA sample. The supplied productions manual was followed when measuring DNA concentration.

4.3 Protein methods

4.3.1 Protein isolation in *S. pombe*

The following protocol is used when taking samples for protein isolation:

1) Spin down 1 x 10^8 cells at 1700 x g for 4 minutes.
2) Resuspend in 1 mL fresh STOP buffer, transfer to tube with screw cap and spin at 16 000 x g for 1 minute.
3) Remove the supernatant and snap-freeze the pellet in liquid nitrogen.
4) Store cells at -80°C.

TCA extraction of total cell protein

Trichloroacetic acid extraction was used to precipitate total cell protein in this study.

1X sample buffer is diluted from 2X denaturing sample buffer which contains 10 %SDS and 0.2M Dithiothreitol (DTT).

1) Allow frozen samples to thaw on ice for a couple of minutes.
2) Resuspend pellet in 1 mL of 20 % TCA (no incubation needed).
3) Centrifuge at 16 000 x g for 1 minute at 4°C. Remove supernatant.
4) Wash the cells with 1 mL of 1 M Tris (pH 8.0) in order to neutralize the TCA.
5) Centrifuge at 16 000 x g for 1 minute at 4°C and remove supernatant.
6) Resuspend in 1X sample buffer.
7) Boil for 2 minutes at 95°C.
8) Add acid-washed glass beads to a little below the meniscus and boil for another 2 minutes.
9) Break the cells with Fast-Prep-24, MP Biomedicals (4 x 30 sec).
10) Boil for 2 more minutes and centrifuge at 16 000 x g 1 minute at 4°C.
11) Put a 1.5 mL screw cap tube in the bottom of a 15 mL falcon tube. With a hot needle, pierce the bottom of the ribolyzer tube and set it on top of the screw cap tube in the falcon tube.
12) Centrifuge at 1700 x g for 5 minutes.
13) The glass beads will now be in the top tube. Retrieve the screw cap tube from the bottom of the falcon tube with tweezers. Discard the tube containing the glass beads.
14) Store samples at -80°C or continue to SDS-PAGE

4.3.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is used to separate proteins according to their molecular weight. The protein samples are treated with SDS, an anionic detergent which binds to proteins and denature them. This is to prevent secondary and tertiary protein structure from interfering and to give the proteins a uniform negative charge. The samples are also treated with DDT which disrupts the disulfide bonds that stabilize the tertiary structure of proteins. The proteins will start migrating towards the anode when an electrical field is applied, and the migration is affected by the size of the proteins and also the size of the pores in the polyacrylamide gel. It is the concentration of the polyacrylamide gel and the ratio between acrylamide and bisacrylamide that decides the size of the pores so it is important to choose a gel with appropriate pore size for the proteins of interest. The top part of the gel has a lower concentration of polyacrylamide than the rest of the gel and is called the stacking gel. The reason for this is to concentrate the protein sample before it enters the other part of the gel called the separating gel.

In this study 10 % pre-cast gels were used. BioRad, LifeTechnologies

Running the SDS-PAGE

The samples are boiled at 95°C for 3-5 minutes before they are loaded into the gel. This is to ensure the total denaturation of the proteins. After boiling they are centrifuged at 16 000 x g for 1 minute, 4°C.

1) Assemble the gel chamber (BioRad or Invitrogen depending on the gel used). Fill the inner chamber with 1X running buffer and check if there is any leakage.
2) Remove comb from the gel and wash wells with 1x running buffer using a syringe.
3) Load molecular weight standard and the protein samples.
4) Fill chamber with 1X running buffer until it is a little above the anode.
5) Run at 120 V for 50-60 min or until the color front has run out of the gel.
6) Disassemble the gel chamber. Remove the gel from its cast and soak in 1x transfer buffer for 15 minutes before continuing to blotting.

4.3.3 Western blotting. Semi-dry protein blotting

Western blotting is a method that uses an electrical field to transfer proteins from a SDS-PAGE gel to a membrane before treatment with antibodies. The electrical current is applied at

a 90° angle to the gel (figure 4.3). Because of the earlier treatment with SDS, the proteins have a negative charge and will migrate towards the anode, and onto the membrane. The transfer buffer contains SDS, which elutes proteins from the gel, and methanol which improves the binding of proteins to the membrane.

Semi-dry blotting is used for proteins of up to 100 kDa in size.

The following protocol was used:

1) Soak the membrane in MetOH for 10 seconds. Wash the membrane with MQ-H_2O, then cover it with MQ-H_2O in a trey and shake it for 10 minutes.
2) Put the gel, filter paper and membrane in separate trays with transfer buffer and shake them for 15 minutes.
3) Place the filter paper, membrane and gel in a semi-dry transfer cell (BioRad Trans-Blot semi-Dry) at 15V for 40 minutes.

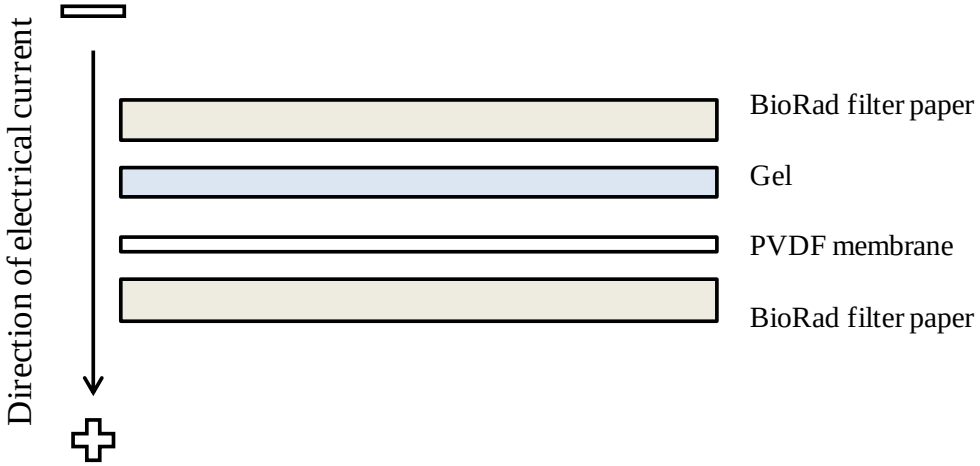


Figure 4.3: The figure illustrates how the different parts of the blot are put together in the transfer cell. When the electrical field is applied, the current will move from the positive electrode to the negative electrode.

Immunodetection

After blotting the proteins can be detected using specific antibodies. In this study visualization of proteins was done by indirect detection using a primary and secondary antibody. The membrane is first blocked in 5 % dry milk in TBS-Tween, this is to prevent nonspecific binding of antibodies which can give background. After blocking, the membrane is incubated with a primary antibody with specificity towards the protein of interest. Excess antibody is washed away in the next step with TBS-T before the membrane is incubated in secondary antibody. This secondary antibody is species specific and it must correspond with the primary

antibody. The secondary antibodies used in this study are conjugated to an alkaline phosphatase which will dephosphorylate it's substrate ECF which in turn produces a fluorescent product.

The following protocol for immunodetection was used.

1) Block the membrane in 5 mL of 5 % blocking agent, TBS-T dry-milk, for 1 hour.
2) Incubate the membrane with primary antibody. Over night with rotation at 4°C.
3) Wash the membrane with 5 mL TBS-T. 3 x 5 minutes.
4) Incubate the membrane in secondary antibody for 1 hour at room temperature.
5) Wash the membrane in 5 mL TBS-T, 3 x 5 minutes.
6) Put the membrane on a glass plate and remove excess liquid with a paper towel or a filter paper.
7) Add 1-2 mL ECF and incubate for 5 minutes. Remove excess ECF in the same way as described in the previous step.
8) Expose the membrane with transilliminator using EtBr filter, ChemiGenius Bio Imaging System, Syngene.
9) Cover the membrane in aluminum foil and store in a fridge.

4.3.4 MCM-chromatin extraction.

This *in situ* extraction technique is based on the protocol developed by Kearsey et al., 2005.

The cells are permeabilized with Zymolase and then treated with detergent (Triton X-100) in an appropriate buffer which will remove all soluble proteins in cell, leaving behind proteins bound to insoluble structures such as chromatin. The samples are split into two, cells treated with Triton X-100 and cells not treated with Triton X-100. Cells untreated with triton are used as a control to make sure that all non-chromatin bound proteins are extracted. The protein of interest can be tagged with a fluorescent protein and visualized using fluorescence microscopy.

1) Transfer around 15 mL cell culture to a falcon tube. Add 150 µL of 1M NaN_3.
   Spin tubes (1500 x g, 4 min, 4^oC)

2) Resuspend in 1.8 mL EMM sorb buffer and transfer to 2 mL eppendorf tubes.
3) Spin tubes (1500 x g, 1 min, 4^oC)
4) Resuspend in 450 µL EMM sorb buffer containing 10 mM DTT
5) Add 100 µL of 40 mg/mL zymolyase 20T
6) Digest at 36C for 8-15 min
7) Test for adequate digestion by adding 2 µL 10 % SDS to a 2 µL sample (cells should go dark under phase contrast microscope, aim for >95% phase dark)
8) Add 1.5 mL EMM sorb buffer

9) Spin tubes (1500 x g, 1 min, 4°C)
10) Resuspend in 2 mL EMM sorb buffer
11) Spin tubes (1500 x g, 1 min, 4°C)
12) Resuspend in 2 mL extraction buffer
13) Spin tubes (1500 x g, 1 min, 4°C)
14) Resuspend in 0.9 mL extraction buffer containing protease inhibitor (Roche Complete Protease inhibitor cocktail tablets # 11697498001)
15) Split into 2*450 µL
    a. To one 450 µL add 50 µL extraction buffer plus 10 % Triton X-100 and incubate 5 min at 25 °C inverting periodically (+T, extracted sample)
    b. The other 450 µL is kept on ice in the meantime (-T, control sample)
16) Spin tubes (1500 x g, 1 min, 4°C)
17) Take off supernatant, flick tubes so cells come off tube wall and add 1.5 mL COLD (-20C) methanol.
18) Leave on ice for at least 6 mins.
19) Spin tubes (1500 x g, 1 min, 4°C)
20) Resuspend in 500µl COLD (-20C) acetone
21) Store tubes at -20°C. Cells are okay for a few days.

4.4 Microscopy

During this study several different microscopes were used. Light microscopy (Zeiss, Germany) objective 20x, was used to look for asci, pulling tetrads and investigating cell growth. Phase contrast and fluorescence microscopy (Leica DM6000B, Wetzlar, Germany), 63x/1.40 Oil objective, was used when looking at GFP and YFP tagged cells with DAPI staining.

4.4.1 Fixation

When taking samples at different time points it is important that the cells are arrested at that exact time point so they can be investigated later. This can be done by fixing the cells after the sample is collected.

When fixing with ethanol, methanol or acetone, the cells are rapidly dehydrated and proteins are made less soluble. Before viewing fixed samples under the microscope, they are mounted on polysine slides (Thermo scientific), which uses an adhesive surface and electrostatic forces to increase attachment of cells to the slide.

Fixation in 70 % ethanol is performed as described in 4.1.9.

Methanol and acetone fixation

The following protocol is used when cells are first fixated in methanol then resuspended in acetone:

1) Filter 3-5 mL of cell suspension with a Durapore HVLP filter (Millipore). Place the filter in a 50 mL tube containing 10 mL methanol and swirl the tube a couple of times to rinse the membrane. Remove filter and spin cells at 1700 x g for 3 minutes.
2) Remove the methanol and resuspend the cells in 1 mL acetone. Tubes should be stored at -20°C.

Fixing in 1:1 Methanol acetone

The following protocol is used when fixing cells in 1:1 Methanol acetone:

1) Filter 3-5 mL of cell suspension a Durapore HVLP filter (Millipore). Place the filter in a 50 mL tube containing 10 mL 1:1 methanol and acetone. Swirl the tube a couple of times to rinse the membrane.
2) Remove the filter and store the cells at -20°C.

4.4.2 DAPI staining

DAPI, 4’,6-diamidino-2-phenylindole, is a nuclear stain which stains DNA in both living and fixed cells. It is used to visualize the nucleus when using a fluorescent microscope, and because of the strong affinity towards DNA there is very little background staining.

To DAPI stain cells, take 2 µL of DAPI (0.2 µg/mL) in mounting medium and apply to a cover slip. Place the cover slip on top of your sample. The sample can be viewed immediately after staining.

5 RESULTS

5.1 Preliminary BLAST search to find the AIMP3 homologue in *S. pombe*.

A BLAST search was performed to find the AIMP3 homologue in *S. pombe* termed *pah1*. The search identified a protein of 450 amino acids. Alignment of Aimp3 and Pah1 showed an overall similarity of 12.3 % (appendix IV). However, AIMP3 is much shorter than Pah1 and when we compared the N-terminal half of Pah1 the two proteins shared 32.7 % similar residues and 17.4 % identical residues over this region. Interestingly, a search of with the C-terminal part of the Pah1 identified AIMP1with a 34.2 % similarity and 25.1 % identity. Since Pah1 shares sequence homology with both AIMP3 and AIMP1, it might also share functions with both.

5.2 Constructing the *pah1* deletion mutant

In a genome-wide study of gene deletions in *S. pombe* the *pah1* gene was found to be nonessential (Kim et al., 2010). Therefore we first attempted to delete the gene in a haploid strain carrying the *cdc10-M17* mutation (#489). We chose a *cdc10-M17* strain, because this makes it possible to arrest the cells in G1, and we don't have to add this mutation after we have successfully deleted the *pah1* gene. A deletion cassette containing 80 bp homology on either side to direct integration to the desired locus, was constructed as described in chapter 4.2.2 using primers #3 and #4, see table 3.2. ClonNAT (nat^R) resistance was used as a marker for the deletion. The construct was transformed, as described in chapter 4.1.2, into the *cdc10* strain, and 48 transformants were analyzed by colony PCR (4.2.1) using primers #6 and #8. These primers anneal in and downstream of *pah1*, respectively. Transformants are taken from a selective plate containing 100 µg/ml clonNAT. The expected product from the strain carrying the wild type *pah1* is 513 bp and no band is expected in the transformants carrying the *pah1Δ*. None of the colonies showed the expected result for the deletion, but all had the band for the wild type gene. We concluded that the integration of the PCR product occurred at other sites in the genome and that the gene replacement was not successful.

To increase the chances of integration at the *pah1* site by homologous recombination, we decided to transform in a *pku80Δ* strain. The deletion of *pku80* causes the cells to be deficient in non-homologous end joining, and thus integration through homologous recombination is likely to occur at a higher frequency. The *pku80Δ* mutant (#1312) was transformed with the same deletion cassette as described above. 24 new transformants were checked using the same primers as in the previous colony PCR. No transformants showed the result expected for the *pah1* deletion (data not shown).

We suspected that the deletion might cause the cells to be very sick, or that it is not viable at all in a haploid. Therefore we decided to try to delete the gene in a diploid strain (#1142) instead. The strain was transformed with the same *pah1Δ* cassette as previously described, and transformants were checked by colony PCR using primers #7 and #8. Because the strain was diploid, the primers need to anneal outside the gene of interest. The expected wild-type band is 2089 bp and 1982 bp for the *pah1* deletion. If the transformant is carrying the *pah1Δ*, an extra band of 1982 bp should appear close to the wild type band. However, only wild type bands were observed (data not shown). To increase the chance of correct homologous recombination, we decided to increase the flanking homology from 80 bp to 300 bp. This was done by a two-step PCR approach as described by Krawchuck et al. (1999), see figure 5.1. During the first PCR the 5' and 3', ~300 bp long fragments were made. These two fragments have homology to just upstream and just downstream of *pah1* and were made using hybrid reverse and forward primers respectively. The hybrid primers also contained a sequence with homology towards the natMX6 cassette carrying the nat^R marker. The primers used were #9 and #10, and primers #11 and #12, respectively. In the second PCR the products from the previous reaction are annealed to the natMX6 cassette, extended, and the outermost primers #9 and #12 are added to amplify the deletion cassette. The PCR products were checked using the QIAxcel machine, but no bands could be seen.

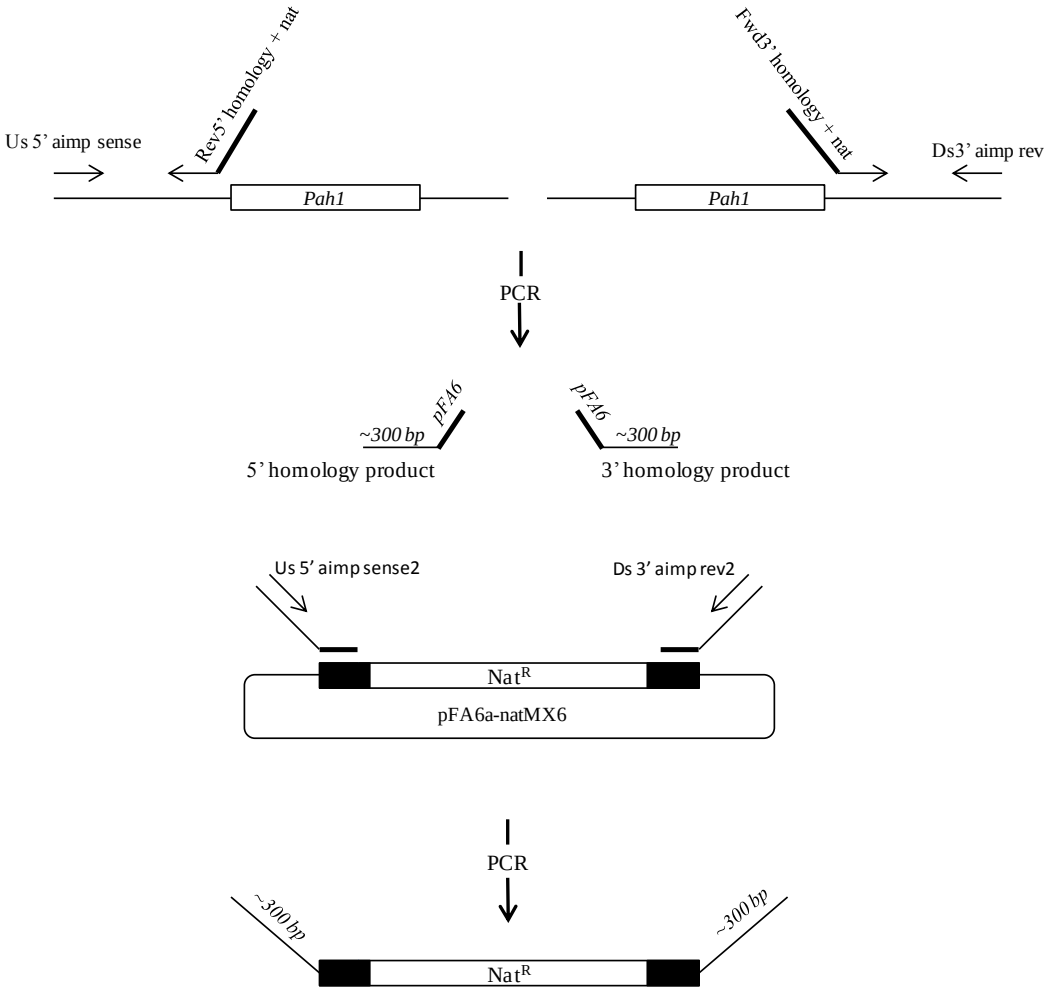


Figure 5.1: A scheme depicting how the deletion cassette with long, flanking tracts of homology is made by PCR. The first step is to produce the 300 bp flanking homology tracts upstream and downstream of the *pah1* gene. These two products are then mixed together with the pFA6a template and the two outermost primers from the previous PCR, to create the deletion cassette. The flanking homologies direct the cassette to the target locus and it is integrated by homologous recombination. The drawing is not to scale.

The primers used to create the deletion cassette, #9 and #10, were selected to be optimal in combination with their respective reverse and forward primers, but they had a low score in a primer-designing software when considered with regards to each other. The primers were tested on genomic DNA from wild-type cells to see if we could optimize the PCR reaction, but this approach did not work. We therefore designed new primers, #13 and #14, where we changed the annealing position of the primers 20-30 bp further downstream and upstream on the 5' and 3' homology fragments respectively. The deletion cassette was made using the new primers and transformed in the same diploid strain (#1142) as before. Transformants were analyzed by colony PCR using three different primer pairs (table 5.1).

Table 5.1:

Primer pair	Expected band size, WT	Expected band size, deletion mutant
15 and 8	No band	766 bp
6 and 8	513 bp	No band
7 and 8	2089 bp	1982 bp

The result from the colony PCR is shown on figure 5.2.

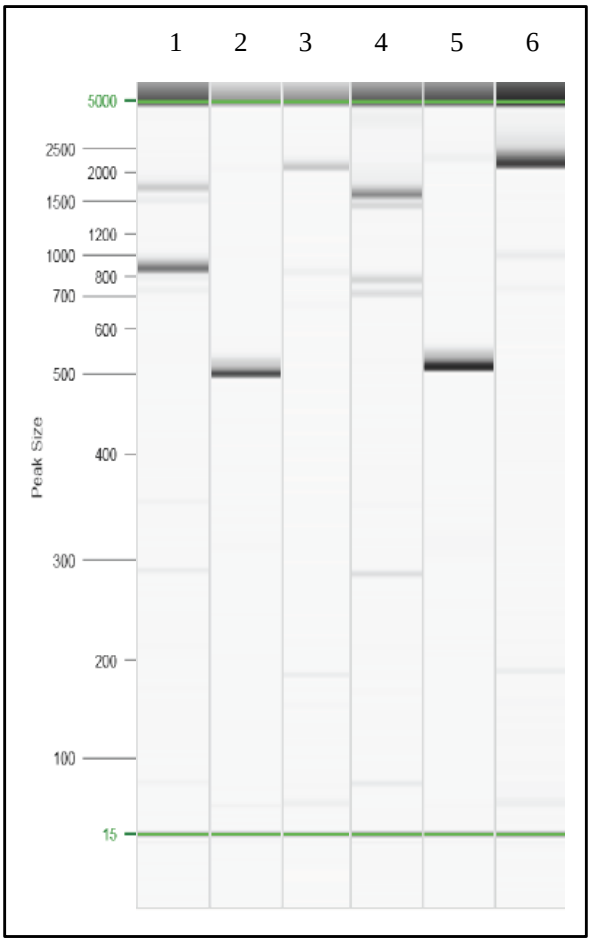


Figure 5.2: Colony PCR results, showing the pah1*Δ* mutant and wild-type side by side. PCR products were analysed using the QIAxcel machine. Lanes 1-3 contain the PCR products from the pah1*Δ* mutant made by the primer pairs #15 and #8, #6 and #8, #7 and #8 respectively. Lanes 4-6 contains the PCR product using wild type template, and the order of the primers is the same as for the deletion mutant. All lanes are from the same gel, but cut out and placed next to each other to show the difference. The separation is performed using the QIAxcel machine.

The first lane contains the primers #15 and #8. Primer #15 anneals inside the nat^R marker and primer #8 anneals a little downstream of the *pah1* gene. If the transformation has been successful a band size of 766 bp is expected with this primer pair while no band is expected in a wild type strain. The band that can be observed in this lane has a size of approximately 800

bp which is slightly bigger than expected. Lane 4 contains the PCR product made using the same primers and wild type DNA as template. No band is expected here. What we can see though are two weak bands with sizes a little above 700 bp. These might be non-specific bands created during the PCR. Bands such as these can be prevented by reducing annealing time, lowering the chances of primers annealing to wrong sequences. Unspecific bands can also be seen in lane 1 and 4 at around 1500 bp. Such unspecific bands can come from contamination, or simply that the primers have annealed at the wrong place in the genome.

Lane 2 contains primers #6 and #8. Primer #6 anneals inside of the *pah1* gene. Since the transformation was done in a diploid, it is to be expected that we get a wild-type band in a possible mutant. A band of the same size can be seen in lane 5 containing the wild-type.

In lane 3 the primer pair #7 and #8 was used. Primer #7 anneals a little upstream of the *pah1* gene. In a *pah1Δ* mutant two bands are expected to show up, one wild-type band and one mutant band. This should create a doublet, but no such doublet could be seen on the screen gel. This is probably because the Qiaxcel machine cannot separate fragments which are so similar in size. There is only 107 bp difference between the two bands. A band which is slightly bigger can be seen in lane 6 containing the same primers and wild-type DNA.

Due to the uncertainty of the outcome, a restriction reaction was set up to confirm that the correct mutant had been obtained. The PCR product in lane 1 was purified using the NucleosSpin Extract II kit from Macherey-Nagal (3.6) and cut with restriction endonucleases PmeI and SacI. Two fragments of size 365 bp and 401 bp is expected when the PCR fragment is cut with PmeI and two fragments of sizes 374 bp and 392 bp are expected when SacI is used (figure 5.3).

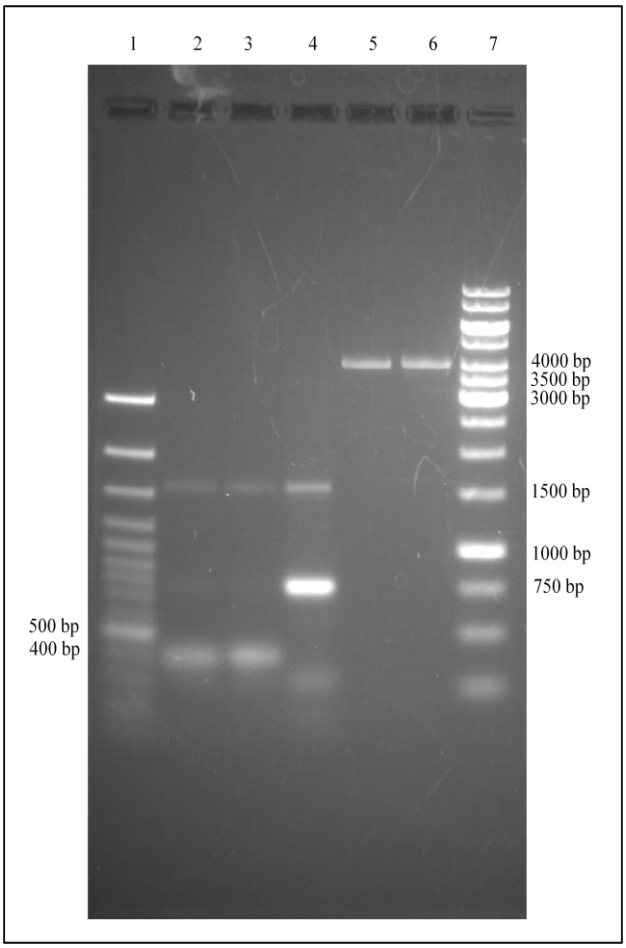


Figure 5.3: Analysis of PCR product made by primers #15 and #16. 1) Generuler 100bp pluss DNA ladder. 2) PCR fragment cut with PmeI. 3) PCR fragment cut with SacI. 4) Uncut PCR fragment. 5) Plasmid #275 cut with PmeI. 6) Plasmid #275 cut with SacI. 7) O'generuler 1kb DNA ladder, ready-to-use.

The products were run on a 1% gel and incubated in GelRed over night. In lane 2 a thick, diffuse band can be seen. This is the two bands expected after cutting the PCR product with PmeI. It is a bit difficult to separate the two bands since they are so close in size, but the thickness of the band suggests both bands are present. The same can be said for the bands in lane 3. A gel with a higher percent of agarose could solve this problem. In lane 4 the uncut PCR fragment can be seen just above the 750 bp marker band, which is close to the expected size of 766 bp. In lanes 5 and 6 plasmid #275 cut with PmeI and SacI respectively can be seen. The plasmid has a size of 4157 bp corresponding nicely to the observed bands in lanes 5 and 6.

The plasmid was added as a control to show that the restriction enzymes are functioning properly. Uncut plasmid will supercoil and migrate further in the gel, creating several bands in one lane. This is not the case when plasmids are linearized by a restriction reaction, which

is apparent in this gel. The same unspecific bands at around 1500 bp and 300 bp, which could be seen in the previous result (figure 5.2 Lane 1 and 4) can also be seen on this gel.

5.2.1 Generating a haploid *pah1Δ* strain by crossing

In order to investigate whether the *pah1Δ* is viable in haploid cells and to obtain progeny with desirable genotypes such as *cdc10-M17*, we performed a genetic cross between the *pah1Δ* strain and #1229 as described in 4.1.3. The cross was performed on a MEA plate, and after zygotic asci had formed the cells were treated with helicase to degrade the ascus walls and the vegetative cells so spores could be plated out on YE + Phloxine B plates. After colonies had formed, haploid colonies, having a light pink color, were identified and transferred to a new YE plate. Diploid colonies, with a dark pink color, were transferred to a different YE plate. The plates were incubated at 25°C over night before they were replica plated onto the following plates:

EMM + ade 25 ^{o}C
EMM + ura 25 ^{o}C
EMM + ade + ura 25^{o}C
YE + clonNAT 25 ^{o}C
YE 36 ^{o}C
EMM + phloxin B 36 ^{o}C

As mentioned above the marker used to select for the *pah1Δ* mutant is nat^{R} and after two days, growth could be observed on the plate containing clonNAT and diploid cells. Three new strains, 1929, 1930 and 1931 (table 3.1), were obtained and frozen down in cryo tubes, as described in 4.1.1. No growth was observed at the selective plate with haploid cells. We started to suspect that the deletion was only viable in diploids but after 5 days, growth could be seen on the plate containing clonNAT and haploid cells. A colony PCR was performed with primers #6 and #8 (figure 5.4). No band is expected if the cells have the deletion. As can be seen in lane 1, containing template from the haploid spore, no band can be seen proving the *pah1Δ* is viable in haploid cells. In lane 2 wild type template were added as a negative control. A band can be seen at 513 bp in lane 2 which is expected for cells not carrying the *pah1* deletion.

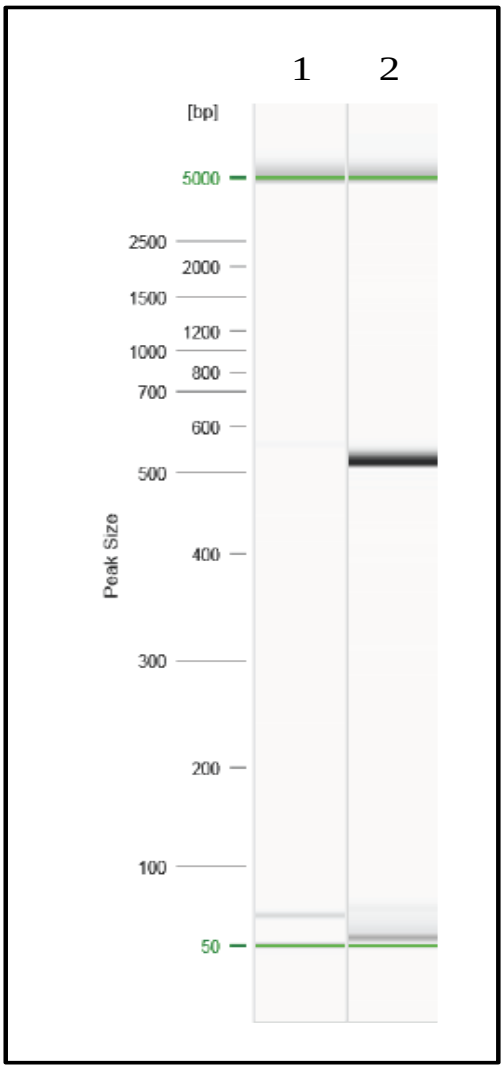


Figure 5.4: Analysis of PCR products made with primers #6 and #8. The QIAxcel system was used to analyze the products. Lane 1 contains the haploid *pah1Δ* mutant. Lane 2 contains wild type template.

5.2.2 Measuring the generation time

When the haploid *pah1Δ* mutant was streaked out for single colonies on fresh YE plates, it seemed to be growing at a rate comparable to that of wild type strains. Due to this change in growth rate, we decided to measure the generation time to investigate the possibility that deleting *pah1* somehow interfered with the growth of the cells. A culture of *pah1Δ* cells were grown to log phase (4.1.1) and the OD_{595} was measured as described in 4.1.8 for 6 hours.

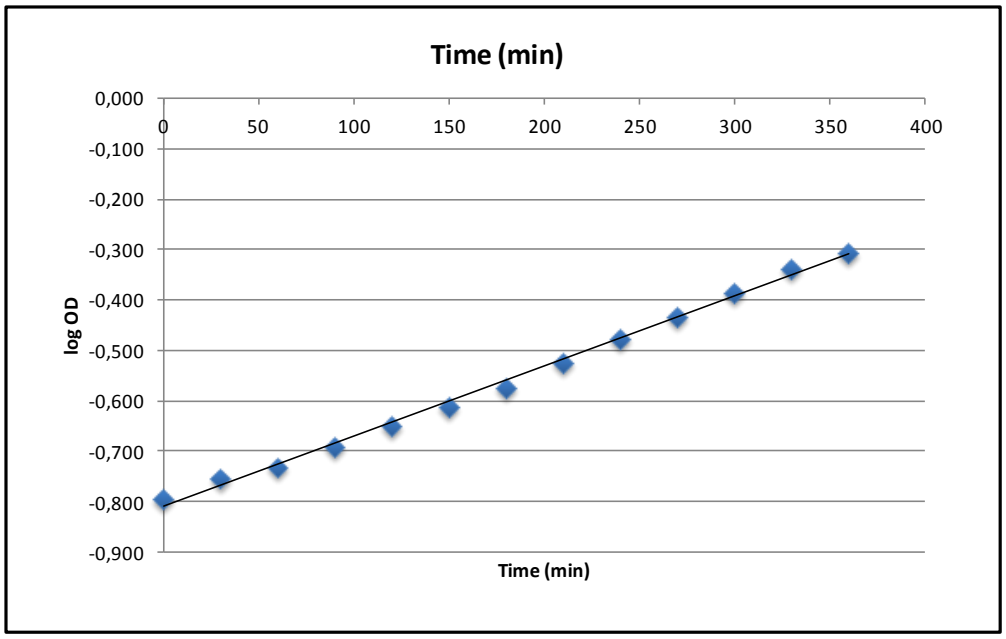


Figure 5.5: Graph depicting the generation time in the *pah1Δ* strain. Log OD_{595} is given on the X-axis, while time is given on the Y-axis. The X-axis is on a negative scale because log is taken of OD_{595}.

The generation time was calculated to be ~215 minutes, which is approximately 35 minutes longer than the generation time for wild type cells. This-close-to normal generation time in spite of the late appearance of the haploid colonies after plating the spores (above) made us wonder if the *pah1Δ* strain had picked up a suppressor mutation. To explore this possibility, we performed a tetrad dissection

5.2.3 Investigating a possible suppressor mutation by tetrad dissection

A suppressor mutation is an additional mutation which can alleviate the phenotype of a pre-existing mutation. Suppressor mutations can be either intragenic or extragenic, meaning they are located in the same gene as the pre-existing mutation or somewhere else in the genome respectively. To test whether there was a suppressor mutation conferring the wild type growth rate, strain #489 was crossed to the *pah1Δ*. Tetrad dissection was performed as described in 4.1.5. Table 5.2 shows the possible outcomes of the cross between the *pah1Δ* carrying the putative suppressor and the #489 wild type strain.

Tetrad type					
PD		**T**		**NPD**	
genotype	**growth**	**genotype**	**growth**	**genotype**	**growth**
pah1Δ/suppressor	Fast	*pah1Δ*/suppressor	Fast	*pah1Δ*	slow
pah1Δ/suppressor	Fast	*pah1Δ*	Slow	*pah1Δ*	slow
WT	Fast	WT/suppressor	Fast ?	WT/suppressor	Fast ?
WT	Fast	WT	Fast	WT/suppressor	Fast ?

Table 5.2: Possible outcomes after crossing the *pah1Δ* strain carrying the putative suppressor mutation with a wild type strain. PD: parental ditype, T: tetratype, NPD: non-parental ditype

We suspected that the *pah1Δ* might have made the strain slow growing and that the suppressor mutation alleviated this effect. If the suppressor mutation is segregated away from the *pah1Δ* during meiosis, we will see a difference in how fast the spores are growing compared to each other. Spores with the *pah1Δ* genotype would use several days to form colonies, while the *pah1Δ*/suppressor spores would grow at a normal rate. After having separated the spores from all zygotic asci, the plates were incubated at 25°C for two days. Small colonies had formed from almost all the spores indicating good spore viability. After a couple of more days the plates were inspected again. No new colonies had formed.

As a control for the tetrad analysis the colonies were replica plated onto several plates containing different supplements, such as adenine, uracil and clonNAT. The plates displayed a segregation of genotypes 2:2 as expected, confirming that four and four colonies identified derive from one ascus each. A total of 25 tetrads were pulled. We concluded that the presence of a suppressor mutation is highly unlikely.

5.3 The G1/S checkpoint in the *pah1Δ* strain

In this project we wished to investigate the putative role of Pah1 in the G1/S checkpoint after UV irradiation. And so, to examine whether the transient delay into S phase after UV irradiation is intact or abolished in the *pah1Δ* mutant, we first tried to measure it using flow cytometry.

Cells were grown to log phase and arrested in G1-phase by a *cdc10* block (4.1.6) and UV irradiated (254 nm, UVC) by a dose of 1100 J/m^2 (4.1.7). Samples for flow cytometry were taken at time points, 0, 30, 60, 90 and 120 minutes. The cells were stained with sytox green as described in 4.1.9. The results from the flow cytometry had a poor resolution and it was not possible to conclude whether there was a delay or not (data not shown). We decided to do a MCM-chromatin binding assay instead to determine if there was delay.

5.3.1 Tagging MCM2

The minichromosome maintenance protein complex (MCM2-7) is loaded onto chromatin in G1 phase and is needed to form the pre-RC. No replication can take place before this complex is made. MCM loading is therefore a convenient marker when investigating S phase entry.

In order to perform the MCM-chromatin extraction we needed to tag one of the MCMs, since there was no pre-existing MCM tag in the *pah1Δ* strain. Because of the already existing clonNAT resistance marker in the deletion strain, another marker had to be chosen for the MCM tagging. We chose to tag MCM2 and to use G418 resistance as the marker. The tagging cassette was made using primers #1 and #2, along with plasmid #397, containing YFP and $G418^R$, which was used as the template in the PCR to amplify the tagging cassette (figure 5.6). Integration of the tagging cassette is based on the same principles as for gene replacement by PCR (4.2.2). Cells are transformed with the linear tagging cassette carrying the desired construct and marker. Flanking the construct and marker are stretches that contains homology towards the desired DNA sequence. The tagging cassette was transformed into the *pah1Δ* strain using the slightly altered Bählers method described in 4.1.2.

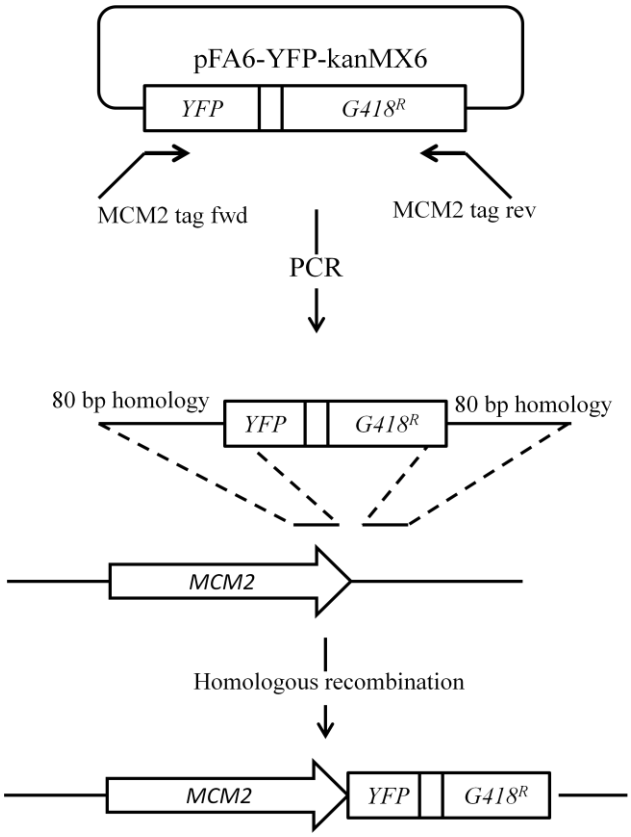


Figure 5.6: Homologous integration of the PCR-made tagging cassette. The pFA6-YFP-kanMX6 vector was used as template when making the tagging cassette containing the YFP sequence and the G418^R marker. The cassette was made by PCR, using 100 nt primers. The forward primer contains 80 nt of homology towards the 3' end of *MCM2* upstream of the STOP codon and 20 nt homology towards the 5' end of YFP. The reverse primer has similar 80 nt homology to a bit downstream of *MCM2* and 20 nt complementary to the 3' end of *G418^R*. The resulting PCR product has a size of 2565 bp, and is integrated into the *pah1Δ* strain by homologous recombination. It is worth noting that when designing primers for C-terminal tagging it is important to make sure the *YFP* sequence is in frame with the protein that is to be tagged so they are translated together, creating the correct fusion protein. The drawing is not to scale.

After the transformation procedure, cells were incubated at 25°C on selective YE plates containing G418 for several days. When colonies grew up several was transferred to a second selective plate and incubated further for two days. The presence of the MCM2-YFP tag was confirmed by colony PCR (figure 5.7) and by using phase contrast and fluorescent microscopy (63x/1.40 Oil objective, Leica DM6000B, Wetzlar, Germany). The primers used in the colony PCR anneals in and downstream of *MCM2*. A band can be seen at 571 bp in the lane containing wild type template which is expected for cells not carrying the MCM2:YFP tag. In lane 1 containing the template from MCM2:YFP tagged cells, no band can be observed. We know from previous experience that this result usually means that the protein of interest has been successfully tagged. Why a bigger band corresponding to the wild type PCR product plus the marker and tag is not made in the PCR we don't know. This band should

have a size of 3136 bp. Previous attempts have been made to try and optimize the PCR, but without any results. Colony PCR is thus used as a fast screening method to find possible transformants. To further confirm the presence of the tag, microscopy and/or western blotting is used.

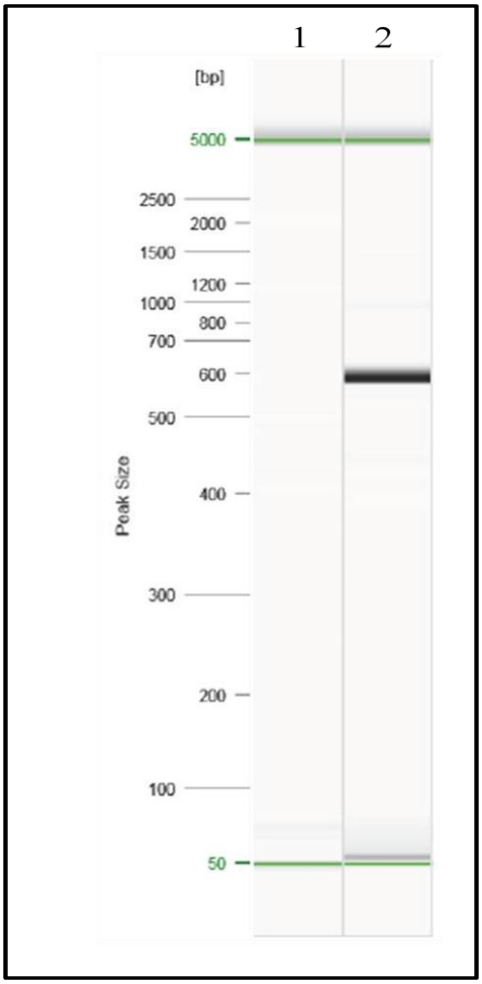


Figure 5.7: Analysis of PCR products made by the primers #16 and #17. Lane 1 contains template from cells carrying the MCM2:YFP tag. Lane 2 contains wild type template.

5.3.2 MCM-chromatin extraction

After having successfully tagged *MCM2* we went on to carry out the MCM-chromatin extraction. During this procedure all soluble proteins will be extracted, and only proteins bound to insoluble structures will be retained. This makes us able to see when MCM2, together with the rest of the MCM complex, is loaded onto chromatin in G1, forming the pre-RC which indicate that the cell is ready for replication in S phase.

pah1Δ cells were arrested in G1 by a *cdc10* block and subsequently exposed to UV irradiation by a dose of 1100 J/m^2. Samples were collected at timepoints 0, 30, 60, 90 and 120 minutes. After all samples had been collected the extraction was performed as described in 4.3.4. Before viewing the cells, they were mounted on polylysine slides and stained with DAPI. A Leica microscope was used to visualize the cells, applying fluorescence and phase contrast (63x/1.40 Oil objective, Leica DM6000B, Wetzlar, Germany). Several pictures were taken and at least 200 cells were counted for each time point.

After cells have been arrested in G1 and UV irradiated, most of the cells have entered S phase 60 minutes after release (Tvegård et al., 2007). Previous results from our lab show that there is a 30-40 minute delay in MCM loading after UV irradiation. In a wild type strain the MCM-chromatin binding reaches a peak 60 minutes after release from a *cdc10* block. After UV irradiation MCM-chromatin binding peaks approximately 30 minutes later and the loading is not as high as in the control cells (Figure 5.7 C)).

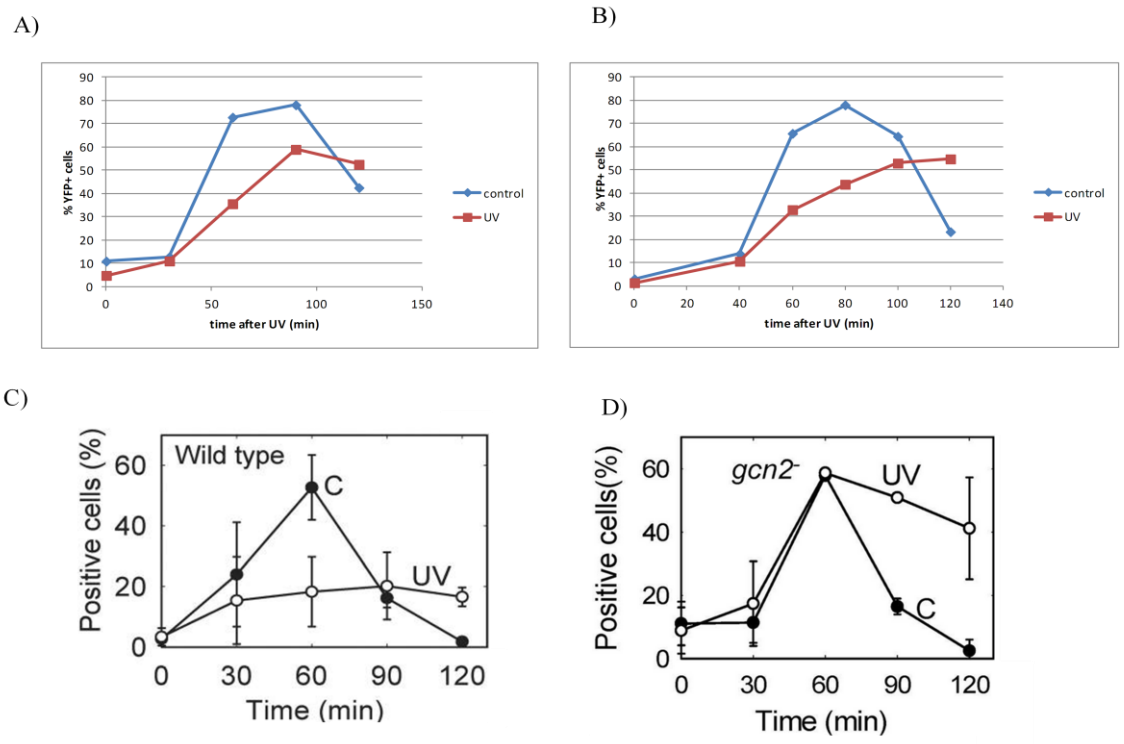


Figure 5.8: Plots depicting MCM-chromatin loading. Note that for technical reasons these experiments were not performed in parallel. Plot A) shows the first extraction results of the *pah1Δ* strain, with time points 0, 30, 60, 90 and 120. The Y-axis show the percentage of positive YFP signals in the cells and the X-axis shows the time after release from the block. A second extraction B) was performed in order to achieve better kinetics with the same deletion mutant. Samples were taken at time points 0, 40, 60, 80, 100 and 120 minutes during the second extraction. Both C) and D) are plots from the Tvegård et al., 2007 article and they are added for the purpose of comparison. They depict the MCM-chromatin loading in C) wild type cells and D) *gcn2Δ* cells. A 30 minute delay of MCM loading can be observed in the wild type strain after irradiation, while no delay is observed in the *gcn2Δ* cells. Note that the strains used in C) and D) have tagged MCM6 with GFP. Extractions were performed on the same strains with MCM2-GFP and gave similar results (data not shown).

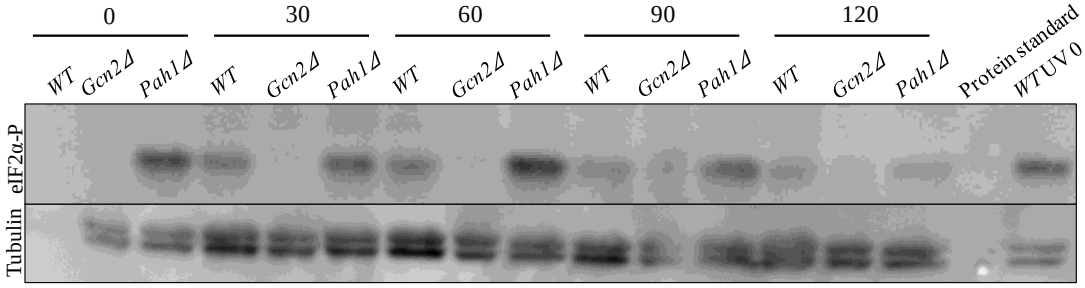
The control cells of the *pah1Δ* mutant seem to peek later than in the wild type and *gcn2Δ* cells (figure 5.8). The peak for the control cells can be seen around 80 minutes after release in both of the extractions which is 20 minutes later than seen in wild type cells.

The MCM loading in the UV irradiated cells seem to reach a plateau around 100 minutes after irradiation, which is 20 minutes later than the control cells. Additionally, there seems to be a higher amount of YFP positive cells in the *pah1Δ* strain after irradiation compared to the wild type, but not as high as the percentage of positive cells in the *gcn2Δ* strain. This difference in the amount of cells positive for MCM loading indicates that the delay is only partial in the *pah1Δ* strain. We conclude that there is a shortened and partial delay in the *pah1Δ* strain after UV irradiation.

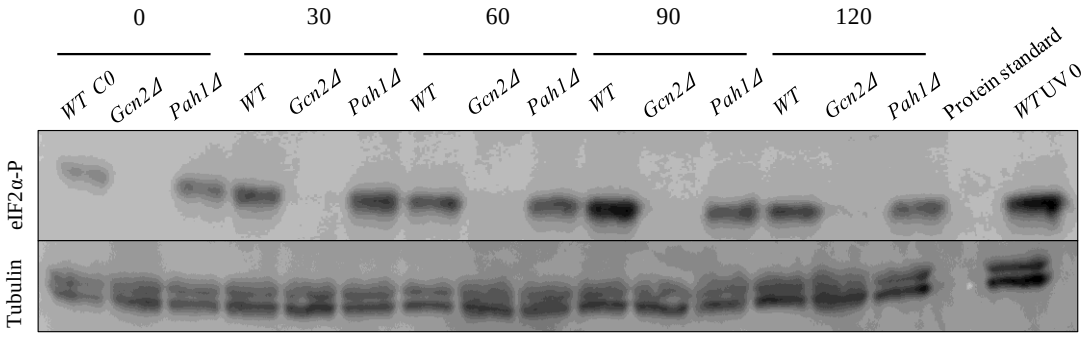
5.4 eIF2α phosphorylation with and without UVC.

Gcn2 phosphorylates eIF2α after UV irradiation (Tvegård et al., 2007), which correlates with delayed entry into S-phase and also a down regulation of translation (1.5). To investigate if the *pah1Δ* has any affect on the phosphorylation of eIF2α, cells were arrested in G1 and UV irradiated immediately after release. Protein samples for western blot were collected at time points 0, 30, 60, 90 and 120 minutes after release (as described in 1.5). Wild type, *pah1Δ* and *gcn2Δ* cells were analyzed.

A) Control



B) UV



C) Relative protein levels

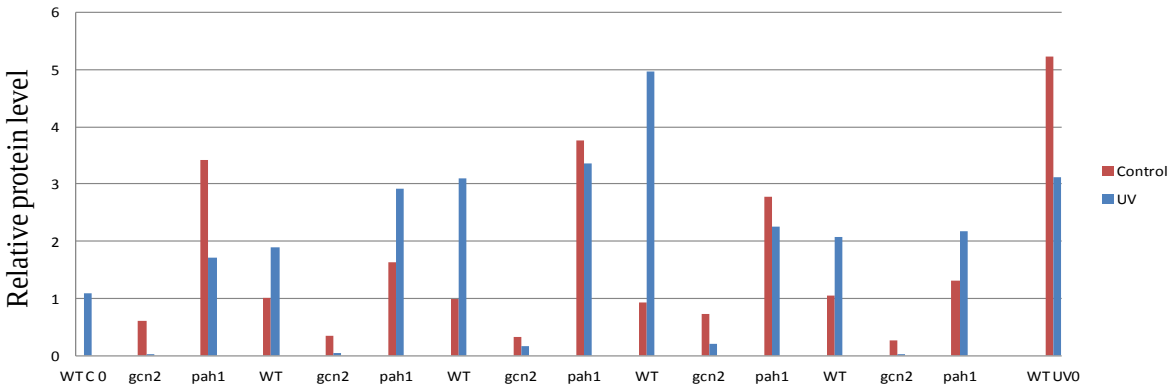


Figure 5.9: Investigating levels of phosphorylated eIF2α by western blotting. Western blot was performed as described in 4.3.3 using antibody against eIF2α phosphorylated on Ser52, which gives a band at around 34 kDa. Tubulin (50 kDa) was used as a loading control. The results in A) show phosphorylated eIF2α without UV. The WT UV 0 is loaded for the purpose of comparison between control and UV samples. B) This blot shows the same strains after UV. Here WT control 0 was loaded as a control to compare with the UV irradiated cells. Note that the WT C0 and WT UV0 have been loaded in the wrong order. C) A quantification was done using the program Genetools (Syngene) to look at the relative protein levels in the blots. The eIF2α phosphorylation is normalized to tubulin.

As can be seen in figure 5.9 A) the first lane containing sample wild type control cells at time point 0 was lost during the experiment, and no protein can be seen here. The rest of the lanes containing samples from wild type cells, show only a weak band corresponding to a small amount of eIF2α phosphorylation which is to be expected even without irradiating the cells. No bands can be seen in the lanes containing protein samples from *gcn2Δ* cells. This is as expected since Gcn2 is the kinase which phosphorylates eIF2α. Surprisingly, dark bands corresponding to eIF2α phosphorylation can be seen in most of the lanes containing protein samples from *pah1Δ* cells, indicating that the deletion might cause stress to the cells.

When we look at the irradiated cells (figure 5.9 B)) phosphorylation can be seen in the lanes containing samples from wild type cells. No phosphorylation can be seen in the *gcn2Δ* cells. Both of these results are as expected. We can also observe phosphorylation in the *pah1Δ* cells.

As can be seen from the tubulin blots the loading was not even, and we chose to perform a quantification of the relative protein levels in the blots (figure 5.9 C)). It appears that the *pah1Δ* strain shows a higher than normal eIF2α phosphorylation for control cells at all time points. After irradiation it is also possible to see an induction of eIF2α phosphorylation in the *pah1Δ* strain, but when we compare the relative protein levels of eIF2α phosphorylation with and without UV it is difficult to tell if there is a higher level of eIF2α phosphorylation or not. It is possible that *pah1Δ* cells are stressed because of the deletion, and this causes the phosphorylation, but we would then expect a consistently higher phosphorylation level in the irradiated cells. Due to this we cannot conclude whether the cells are stressed or not.

5.5 Localization of Pah1 after UVC irradiation.

AIMP3 has been shown to translocate to the nucleus after UV irradiation (Kwon et al., 2011) where it interacts with ATM/ATR (Park et al., 2005). This led us to investigate whether this mechanism is also found in *S. pombe* cells. We decided to tag Pah1 with a GFP tag and expose the cells to UV irradiation.

5.5.1 Tagging of Pah1

Pah1 was C-terminally tagged with a GFP tag using the same principles as depicted in figure 5.6. Primers #4 and #5 together with plasmid #309 was used to create the tagging cassette. Cells from strain #489 (wild type) were transformed using the same modified Bähler method as for the MCM2 tagging procedure. Nat^R was used as the selective marker. The GFP tag was confirmed by colony PCR and western blot using anti-GFP as primary antibody (figure 5.10). Pah1 has a molecular weight of 50.6 kDa and the GFP protein is 25 kDa. The fusion product should show up as a band at 75 kDa.

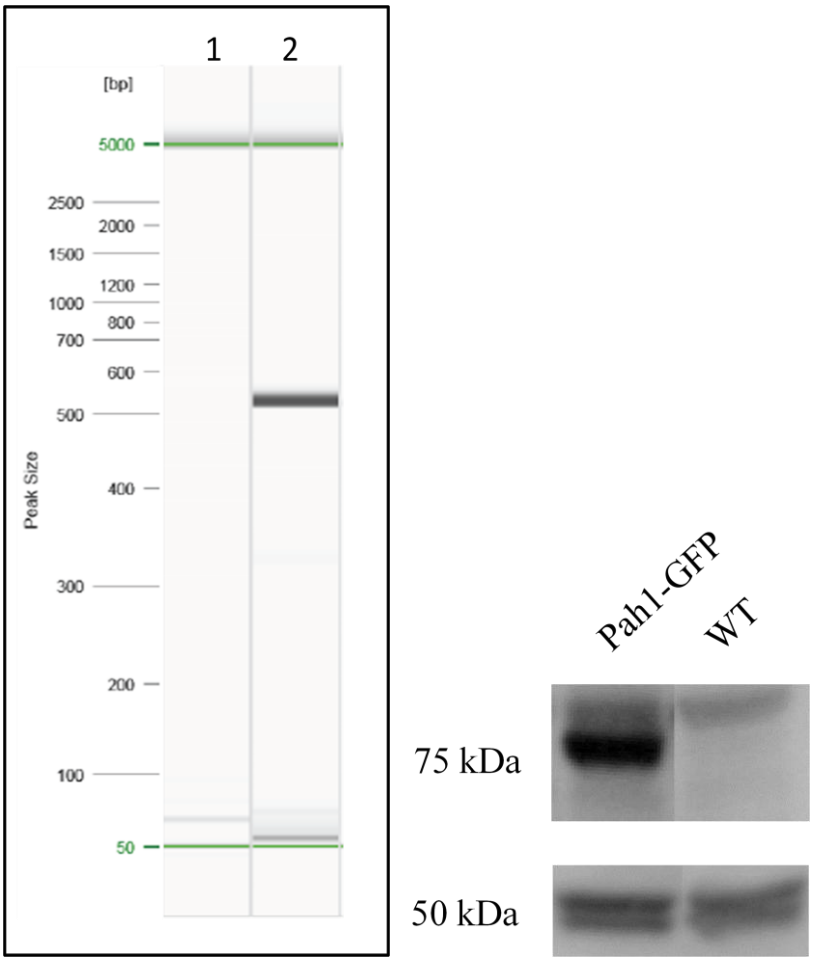


Figure 5.10: PCR and Western blot showing the presence of the Pah1:GFP tag. A PCR was run with the primers #6 and #8 which anneal in and downstream of *pah1*. The PCR products were analyzed by using the QIAxcel machine. The results can be seen to the left. Lane 1 contains the strain carrying the Pah1:GFP tag, and lane 2 contains wild type template. A blot was performed to further confirm the presence of the GFP tag. This blot can be seen to the right. It shows the Pah1:GFP fusion protein at 75 kDa and tubulin as loading control at 50 kDa. The wild type is added as a negative control. The lanes from the western blot have been cut and placed next to each other to give a better picture.

As can be seen in the PCR result in figure 5.10, lane 1 containing template from Pah1:GFP cells contains no band while in lane 2 containing wild type template a band can be seen at 513 bp which is the expected band for cells not carrying the tag. As mentioned in 5.3.1, for unknown reason there is no PCR product in the lane containing template from cells where the protein of interest have been successfully tagged. In this case the primers used should produce a band of size 2703 bp corresponding to the size of the wild type band and the size of the transforming DNA (2190 bp), but no such band can be seen. To further confirm the presence of the tag we performed a western blot which can also be seen in figure 5.10. A band can be observed at 75 kDa which corresponds to the Pah1:GFP fusion protein. No band can be seen in the negative control as expected.

The presence of the Pah1:GFP tag was also confirmed by visualizing the cells using the Leica microscope (63x/1.40 Oil objective, Leica DM6000B, Wetzlar, Germany). A strong GFP signal could be observed from the cytosol. We concluded that the transformation was successful and went on to investigate the localization of Pah1 after UVC irradiation. The *pah1:GFP* strain received the strain number #2012.

5.5.2 Pah1 localization after UVC irradiation

Before we started to irradiate the GFP tagged cells, we examined which fixation method gave the best GFP and nuclear signal. We tried to fix in 70 % ethanol, methanol followed by acetone and lastly a 1:1 mix of methanol and acetone (4.4.1). When fixing in alcohol, especially ethanol, the tertiary structure of proteins may be affected as a result of disrupted hydrogen bonds, which can affect the GFP signal. This is why several different fixatives were tried before performing the experiment. We found that cells fixed in a 1:1 mix of methanol and acetone gave the best GFP and nuclear signal when looking at the cells under the microscope.

The cells were arrested in G1 by *cdc10* block and release, and subsequently UV irradiated (1100 J/m^2). One sample was taken before irradiation as a control. The rest of the samples were taken and at the following time points: 0, 10, 20, 30 and 60 minutes after UV irradiation. When the cell samples were taken, they were fixed immediately. Before we viewed the samples under the microscope, they were first stained with DAPI. This staining is important as it confirms whether the Pah1 translocates to the nucleus after irradiation.

As can be seen on the pictures from the microscope it is difficult to decide whether there is an increased GFP signal in the nucleus after UV or not because of the strong cytoplasmic signal. In the control cells the GFP signal is clearly excluded from the nucleus. In the UV irradiated samples the nucleus appears less clearly identifiable and this is consistent with some of the Pah1:GFP translocating into the nucleus around 20 minutes after irradiation. Because it is hard to determine the GFP signal by eye we performed a quantification of the cells using a program called Image J to investigate whether the nuclear signal increased after irradiation. Pictures of some of the cells before and after UV can be seen in figure 5.11. The diagram showing the intensity of the nuclear signal can be seen in figure 5.12.

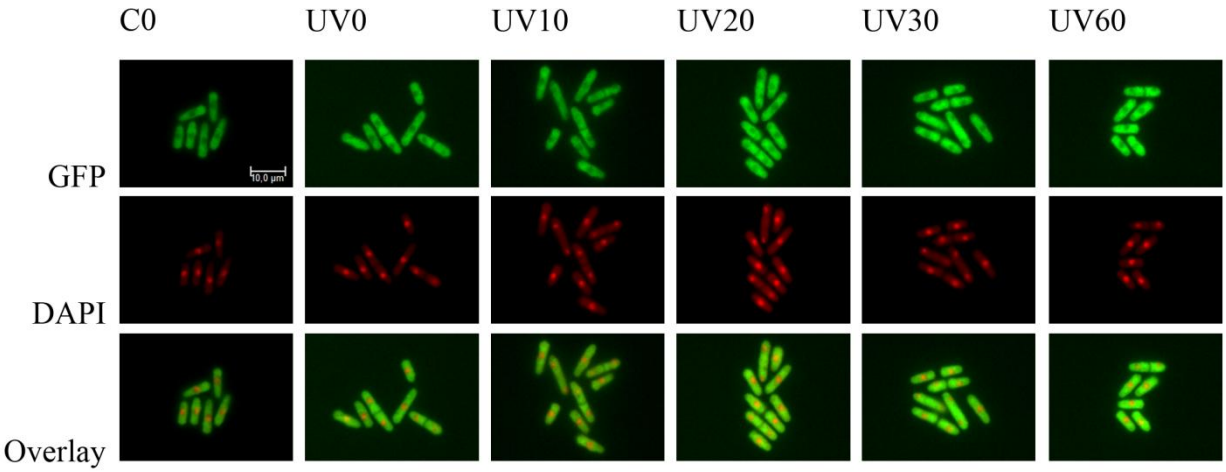


Fig 5.11: Localization of Pah1 after UV irradiation

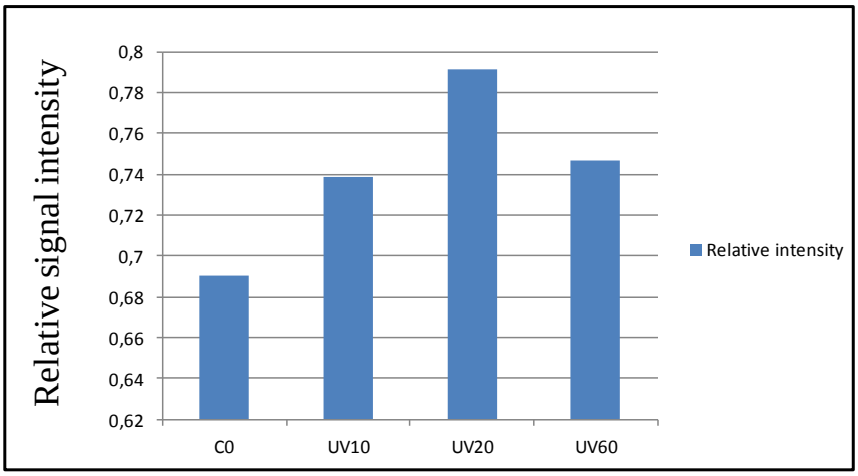


Figure 5.12: This plot shows the relative signal intensity, nucleus to cytosol. The intensity was measured at time points 0, and 10, 20 and 30 minutes after irradiation. The background intensity was measured for each cell and has been subtracted from both the cytoplasmic and nuclear signal. Note that the intensity was measured on a single plane, and not by using z-stacks.

As can be seen in figure 5.12, the intensity of the nuclear signal rises after irradiation, and 60 minutes later, the intensity is dropping. These preliminary results indicate that Pah1 enters the nucleus after UV irradiation.

6 DISCUSSION

The purpose of this master thesis was to delete the gene encoding the Pah1 protein and characterize the mutant, focusing on its putative role in the G1/S checkpoint in fission yeast. In this chapter I will first discuss the deletion of the *Pah1* gene, due to the fact that it was a big part of the project. I will then go on discussing how it affects the G1/S transition, eIF2α phosphorylation, and the proteins possible nuclear entry after UV irradiation, considering what is known in mammalian cells, and previous work done in our lab. In the end I will touch upon the role of AIMP3 in translational initiation in mammalian cells in the light of recent, unpublished results from our group.

6.1 Deletion of the pombe aimp homologue

Gene replacement is a powerful tool which can give us valuable information about the function of a protein by deleting the gene encoding it. *S. pombe* is often a favored organism to use, when doing gene replacement because of the relatively simple transforming protocol. In the method paper by Bähler et al. (1998) they report efficiencies of homologous integration between 6-63% using short, ~80 bp flanking sequences, when deleting genes. In *S. cerevisiae* they have even managed to target integration of DNA fragments by using what they call micro-homology, with 30 bp of flanking homology (Manivasakam et al., 1995). We therefore tried deleting the *Pah1* using short flanking sequences in a haploid *Cdc10*-M17 strain (#489) as described in the results (5.2), but without any luck. After a failed attempt in a *pku80Δ* strain (#1312) we thought that the gene might be essential for survival, so we tried transforming in a diploid strain (#1142), but also this transformation yielded no right transformants. In a paper by Krawchuk and Wahls (1999) they describe three, transcriptionally-silent genes, which only had a homologous integration efficiency of 4% using short flanking homology sequences. When they increase the homology of the flanking sequences to more than 250 bp they achieved integration efficiencies up to 100%. We decided to try this strategy, which finally made it possible to delete the *pah1* gene. Another possible approach which we did not try is transformation by electroporation. This method has been reported to give high efficiency transformation in *S. pombe* cells (Prentis 1991, Suga and Hatakeyama, 2001)

Why some genes are more difficult to delete than others is difficult to say. Non homologous recombination is a problem that can give integrants at the "wrong" sites, but even in a *pku80Δ* strain we obtained no transformants carrying the *pah1Δ*. The localization of the locus on chromatin might also impact the efficiency of the integration. As reported in the paper by Krawchuk and Wahls (1999) the genes they tried to delete were transctriptionally-silent, which means that they might be located in positions with tightly packed chromatin, called heterochromatin, and they are not expressed in mitotic cells. This would make them harder to target. *pah1* is shown to be constitutively expressed throughout the cell cycle (Rustici et al., 2004) and the level of its transcription can be comparable to some housekeeping genes (Marguerat et al., 2012). These findings suggest that the *pah1* loci is probably not located in a heterochromatin rich position on the chromosome, and doesn't explain why it was so difficult to delete.

In the end we managed to delete the gene by increasing the length of the flanking homology tracts. We made the deletion in a diploid strain, and to investigate if the deletion was viable in a haploid we crossed the diploid carrying the *pah1Δ* with a haploid strain (#1229) as described in 5.2.1.

6.2 A possible suppressor mutation in the haploid *pah1Δ* strain

We obtained a haploid *pah1Δ* mutant, but it was very slow growing. We suspected that the slow growth pace was caused by the deletion, but when the mutant was streaked out on new YE plates, its growth seemed comparable to that of wild type cells. Due to this change we measured the generation time of *pah1Δ* cells growing in log phase. The generation time was close to that of wild type cells which led us to suspect that the cells might have obtained a spontaneous suppressor mutation which alleviated the slow growing phenotype from the *pah1Δ* cells. To investigate this we performed a tetrad dissection. During meiotic recombination it is likely that the deleted *pah1* gene and the suppressor mutation will segregate away from each other giving progeny with different genotypes. In the presence of the suppressor mutation the spores should form colonies which have a wild type generation time and spores having the *pah1Δ* should form slower growing colonies. To our surprise all colonies came up at the same time which could mean (1) there is no suppressor mutation and the reason the haploid cells were slow growing was because they came out from triploid

meiosis, or (2) the suppressor mutation is situated in close proximity to the deleted *pah1* gene which causes them to co-segregate during recombination. It is not very likely that the deletion and suppressor mutation are in close proximity, but if it is we can calculate the approximate distance between them. To do this we use the following formula:

Recombination frequency = ((½ T + NPD) /total tetrads)*100

Where T is tetratype, a tetrad containing four different genotypes, and NPD is non-parental ditype, a tetrad containing two different genotypes which are both recombinant. We pulled 25 tetrads and all of the spores formed colonies in only a couple of days. If we imagine that tetrad number 26 should give a tetratype containing slow growing spores we would get the following equation:

((½ * 1 + 0) / 26)*100=1.9 %

A recombination frequency of 1 % is equal to 1 cM (centimorgan). Centimorgan is a unit used for measuring genetic linkage. In *S. pombe* 1.1cM is approximately 10kb (Young et al., 2002). If there should be a suppressor mutation present in our *pah1Δ* strain, it would have to be situated within ~17 kb of the deletion. This is not very likely, but we cannot exclude the possibility of it being present. We investigated which genes were in this region and they can be seen in appendix V. None of these genes seem to be involved in translation.

We tried making a conditional deletion mutant by fusing the hormone binding domain (HBD) of the estrogen receptor (ER) to the Pah1 protein, but we were not successful. By fusing the HBD to a protein of interest, one can easily control the activity of this protein depending on the media the cells are growing in (Bøe et al., 2008). If estradiol is added to the growth media, the tagged protein is activated. This strain would have been useful to back up results which we had gotten with the *pah1Δ* strain, and could also have proven that there is no suppressor mutation in this strain. Lastly, we suspected that the deletion might affect the sporulation because they came up late after the triploid cross, but the high spore viability observed during the tetrad dissection makes this an unlikely explanation.

6.3 Deletion of *pah1* leads to a partial delay in the G1/S transition

As mentioned in the Introduction (1.5) our group has previously shown a novel checkpoint mechanism regulating the G1/S transition in response to UV irradiation. After irradiation the cells delayed S phase entry for at least 30 minutes (Tvegård et al., 2007). In mammalian cells a delay in MCM loading can also be observed after UV irradiation in G1 which is partially dependent on GCN2 (Tine W. Håland, personal communication).

To investigate if there was a delay after UV irradiation in our *pah1Δ* strain, we looked at the pre-RC loading after irradiation. MCM loading in the unirradiated control cells peaked at around 80 minutes, which is 20 minutes later than in wild type cells. The irradiated cells reached their peak ~20 minutes after the control cells. The amount of YFP positive irradiated cells was also higher than that observed in irradiated wild type cells, which suggests a reduced and partial G1 delay in the *pah1Δ* strain. These results indicate that Pah1 is required, but not essential for the full length delay.

In both *Saccharomyces cerevisiae* and human cells there has been shown that UV irradiation induces a checkpoint mechanism which arrests the cells in G1 and activates the kinase ATR, whose homologue in *S. cerevisiae* is termed Mec1 (Siede et al., 1996, Tibbetts et al., 1999). No such mechanism involving the *S. pombe* ATR homologue Rad3 has been described, but our group has shown that the processing of UVC-induced lesions through the NER or UVER pathway is needed for the G1/S delay and that Rad3 can contribute to the G1 delay in a repair deficient mutant (Bøe et al., 2012). Further investigation into the role of Rad3 in the G1 delay was performed, but working with *rad3Δ* strains proved difficult. When looking at pre-RC loading after UV irradiation in several different *rad3* strains with differently tagged MCMs, the length of the G1 delay varied greatly (Cathrine Bøe, personal communication). This difference in delay suggests that there is a genetic interaction and therefore possibly a functional link between MCM loading and Rad3, but how this mechanism works we cannot say. This makes the MCM-chromatin loading assay inadequate when investigating the G1/S transition after UV irradiation.

In exponentially growing cells after UV irradiation a small increase of G1 cells and decrease of S phase cells can be detected by flow cytometry analysis. This small increase/decrease can not be observed in *rad3Δ* cells (Cathrine Bøe, Jon H. Knutsen, Beata Grallert, personal

communication). However, ATR was recently reported to be required for a step in early G1, and inhibiting ATR radiosenzitizes cells irradiated in G1 (Gamper et al., 2013). Therefore a role of Rad3 in early G1 related to MCM loading is important to explore. Furthermore, given the suggested interaction of AIMP3 and ATR in human cells (Kwon et al., 2011) and the observed delayed MCM loading in unirradiated *pah1Δ* cells (this work) it is tempting to speculate that the early-G1 role of ATR might possibly also involve AIMP3. However, as Pah1 is cytoplasmic in unirradiated cells, the observed delayed MCM loading might well be an indirect effect. It would be highly attractive to investigate if Pah1 and Rad3 are interacting after UV irradiation.

6.4 eIF2α phosphorylation in the *pah1Δ* strain

In unirradiated *pah1Δ* cells we can see an induction in eIF2α phosphorylation. This phosphorylation can also be seen in irradiated cells, but there seems to be a variation in the relative protein levels between irradiated and unirradiated cells. We would expect that the irradiated cells show further induction of eIF2α phosphorylation, but when looking at the quantification it is not possible to say if the levels of phosphorylation are higher or lower because the levels of phosphorylations are not consistently higher in irradiated cells. The quantification is only from one set of blots, and the experiment should be performed again to get more conclusive results, but due to a shortage of time we were not able to repeat this experiment. These preliminary results suggest that the *pah1Δ* somehow stresses the cells and induces eIF2α phosphorylation. If this is true and eIF2α phosphorylation leads to downregulation of translation we would expect this to affect the generation time, but the *pah1Δ* cells have a generation time close to that of wild type cells. Recent results from our group suggest that downregulation of global translation after UV can be mediated independently of Gcn2 and eIF2α phosphorylation (Jon Halvor J. Knutsen, personal communications). However, the exact contribution of eIF2α phosphorylation to the downregulation of global translation has been difficult to judge. If it should be shown that eIF2α is phosphorylated in *pah1Δ* cells without UV and no downregulation of global translation can be found then these results correspond nicely with the recent findings of our group.

6.5 Preliminary results suggests a nuclear translocation of Pah1 after UV irradiation

Kwon et al., (2011) showed that AIMP3 is released from the MRS complex and translocated into the nucleus after UV irradiation in mammalian cells. We therefore wanted to examine if this was the case in *S. pombe* by tagging Pah1 with a GFP tag. Unfortunately we were only able to do the localization experiment once, due to shortage of time. A clear GFP signal was observed in the cytosol, and the GFP signal in the nucleus seemed to be getting slightly stronger some time after UV, but it was difficult to see due to the strong fluorescent signal in the cytosol and the low resolution of the microscope. If Pah1 is moving into the nucleus, it might only be a small fraction of the cytosolic portion of the protein. This would make it hard to detect in the nucleus. We measured the intensity of the GFP signal in the nucleus relative to the cytosol and we could see an increase in signal intensity 10 and 20 minutes after UV. At 60 minutes the intensity had started to sink. These preliminary results indicate that Pah1 is translocated to the nucleus after UV irradiation, but further work need to be done in order to confirm this. In the article by Kwon et al., (2011) they reported that they had to use higher magnifications to be able to see UV-dependent AIMP3 foci formation in the nucleus and so our next plan was to use the confocal microscope, which has a much higher resolution and contrast. The cells should also be mounted on a thin layer of agar to prevent them from having different angles which makes it hard to get a good focus on all the cells in the field of view. A couple of other strategies we also considered was lectin staining the membrane of control or UV irradiated cells, so both unirradiated and irradiated cells can be examined at the same time in the same field which makes it easier to compare. One could also do a digitonin extraction which permeabilizes the cell membrane, leaving only the content of the nucleus intact.

We looked for a nuclear localization signal in the Pah1 sequence, but no such sequence could be found. It is possible that Pah1 is modified in some way after UV irradiation that signals nuclear entry. It could also be that Pah1 interacts with Rad3 in the cytosol, and that they enter the nucleus together. One article also suggests that the nuclear entry of AIMP3 might be controlled by post translational modification (Kim et al., 2008) and this theory is further strengthened in the Kwon et al. (2011) article where they found that phosphorylation of AIMP3 after UV might be a possible modification. This was found by performing a 2D-PAGE analysis of nuclear AIMP3.

Should it be shown that Pah1 translocates to the nucleus after UV in *S. pombe* it strengthens the possibility that Pah1 is involved in performing similar tasks in fission yeast as it is in mammalian cells. *S. pombe* would then prove to be a good model organism to further study the properties of Pah1.

6.6 AIMP3 in translational initiation

In mammalian cells, it is suggested that MRS is phosphorylated at Ser662 in a GCN2 dependent matter after UV irradiation. This phosphorylation causes a conformational change that releases AIMP3, and reduces the catalytic activity of MRS, making it unable to transfer Met to initiator tRNA (Kwon et al., 2011). As a result global translation is downregulated. In a recent article it is shown that AIMP3 recruits and mediates the binding of eIF2γ to the MRS-AIMP3 complex (Kang et al., 2012). The eIF2γ subunit is the main binding site for GTP/GDP. They also show that AIMP3 delivers Met-$tRNA_i^{Met}$ to eIF2 which is important for the TC formation. In $AIMP3^{+/-}$ MEF cells there was a 40% reduction in global translational rate suggesting that the transfer of Met-$tRNA_i^{Met}$ to eIF2 mediated by AIMP3 is important for global translation. This is interesting because recent results from our group indicate that downregulation of global translation after UV is mediated through a mechanism independent of Gcn2 and eIF2α phosphorylation as mentioned above (Jon Halvor J. Knutsen, personal communication). Perhaps there are different mechanisms working in fission yeast, but it would be interesting to investigate whether the MRS homologue Rar1 is a substrate of Gcn2, and if Rar1 and Pah1 are in some way interacting, contributing to the binding of Met-$tRNA_i^{Met}$ to eIF2 and thus affecting the global translational rate.

6.7 Conclusion

In this study we have constructed a *pah1Δ* mutant. The deletion is viable in haploid *S. pombe* cells and the generation time of the deletion mutant was close to that of a wild type strain. We then went on to show that Pah1 is required, but not essential, for the full G1 delay after UV irradiation. In addition, the *pah1* deletion might stress the cells and cause phosphorylation of eIF2α, but this need to be further confirmed. Our preliminary data also indicate that Pah1 translocates into the nucleus after UV, but we have not been able to confirm this. Taken into consideration that AIMP3 in mammalian cells have been shown to enter the nucleus and interact with ATM/ATR after UV, it is not unlikely that this mechanism exists in fission yeast

as well. A possible hypothesis could be that in response to UV Pah1 will enter the nucleus and interact with Rad3, affecting the MCM loading through this interaction, but further research is needed to confirm this hypothesis.

6.8 Further work

More research is needed to further characterize the AIMP3 homologue Pah1 in fission yeast. First of all the localization experiment needs to be repeated so we can confirm whether Pah1 enters the nucleus after UV irradiation or not.

The experiment regarding the phosphorylation status of eIF2α with and without UV should be repeated in order to further test whether eIF2α is phosphorylated in irradiated cells. In vivo translation rates should be measured in unirradiated and irradiated cells. These results together with recent work done in our lab could prove helpful to investigate to what extent eIF2α phosphorylation is important for regulating downregulation of translation or not.

The next logical step would be to see whether Pah1 and Rad3 interact after UV irradiation. By tagging both proteins and performing a co-immunoprecipitation (co-IP) one could see if the two proteins precipitate together. Antibody against tagged Pah1 can be used to capture the protein in a solution and precipitate it away from the other proteins. If Pah1 and Rad3 interact and bind tightly together, we should be able to confirm the presence of Rad3 by running the protein sample containing eluted Pah1 on a gel and by using antibodies against tagged Rad3 we can perform a western to see if Rad3 has precipitated together with Pah1. By carrying out this co-IP in a wild type and *gcn2Δ* strain we would be able to determine if the interaction is regulated by Gcn2. Co-IP could also be performed to investigate the interaction between Rar1 and Pah1.

It should also be investigated if Gcn2 phosphorylates Rar1 after UV irradiation, to see if this mechanism is conserved. This could be done by performing a 2D gel electrophoresis which seperates proteins based on their isoelectric point and protein mass. We could then investigate the phosphorylation status of Rar1 after UV irradiation, and whether this phosphorylation is performed by Gcn2.

Further research should focus on the translational rate, investigating whether Pah1 and Rar1 has similar functions in *S. pombe* as they have in mammalian cells. Translational rates can be

measured using a metabolic labeling approach by incorporation of the noncanonical amino acid L-Homopropargylglycin (HPG; LifeTechnologies) into proteins followed by chemoselective fluorescence tagging by means of “click chemistry” (Dieterich et al., 2010) using the Click-it Cell reaction buffer kit (LifeTechnologies). The rates of translation can be measured by flow cytometry or western blot.

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APPENDIX

Appendix I: Internet references

Appendix II: PCR set ups and programs

Appendix III: Molecular weight standards

Appendix IV: AIMP3 alignments

Appendix V: Genes next to *pah1*

Appendix I: Internet references

http://www.pombase.org

http://www.nobelprize.org/nobel_prizes/medicine/laureates/2001/

http://www-bcf.usc.edu/~forsburg

Appendix II : PCR set ups and programs

PCR reaction set ups.

Accu prime pfx polymerase PCR set up:

Solution	**For 50 µL**
10X buffer	5.0 µL
5M NaCl	0.5 µL
$MgSO_4$ (10 – 30 mM)	5.0 µL
Forward primer (10 µM)	1.0 µL
Reverse primer (10 µM)	1.0 µL
Template (10 pg – 200 ng)	1.0 µL
DMSO	1.25 µL
H_2O	34,85 µL
Accu Prime pfx polymerase	0,4 µL

This set up is used when making deletion/tagging cassette with short tracts of flanking homology. Note that the template concentration varies depending on what type of template is used. The concentration given above is for genomic DNA. When using plasmid as template a concentration of 10-20 ng is enough. The reason for this difference in concentration is due to the size of the template used.

The same set up was used when making the deletion cassette with long tracts of flanking homology, but then the 5' and 3' homologous sequences were added in addition. The template and flanking tracts were set up to be in a 1:3 molecular ratio.

Colony PCR set up:

Solution	**For 20 µl**
Template (10 pg – 200 ng)	1.0 µL
dH_2O	3.0 µL
Triton X-100 (10%)	4.0 µL
Forward primer (10 µM)	1.0 µL
Reverse primer (10 µM)	1.0 µL
DreamTaq Master Mix (Fermentas)	10.0 µL

PCR programs

Accu prime program:

	Cycles	**Time**	**Temp**
Initial denaturation	1	2 min	95 °C
Denaturation	30	45 sec	95°C
Annealing		45 sec	48°C
Elongation		140 sec	68°C
Final elongation	1	15 min	68°C
Hold	--	--	12°C

S. pombe colony PCR program:

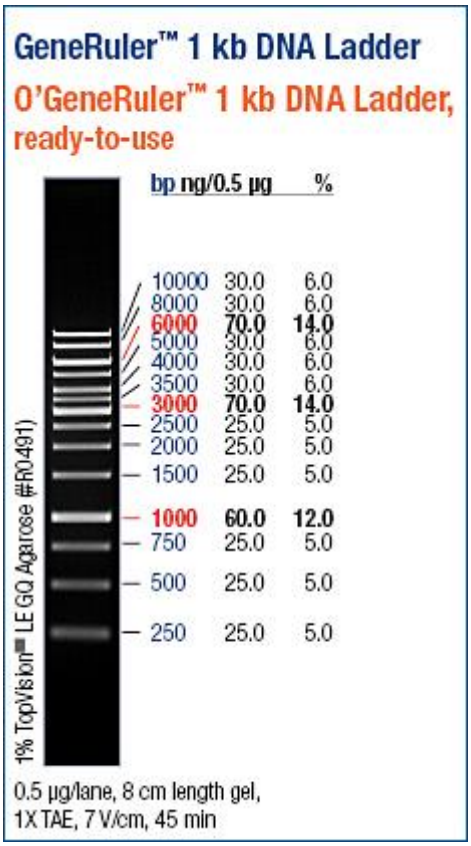
	Cycles	**Time**	**Temp**
Initial denaturation	1	2 min	94 °C
Denaturation	34	15 sec	94°C
Annealing		30 sec	42°C
Elongation		5 min	68°C
Final elongation	1	10 min	68°C
Hold	--	--	12°C

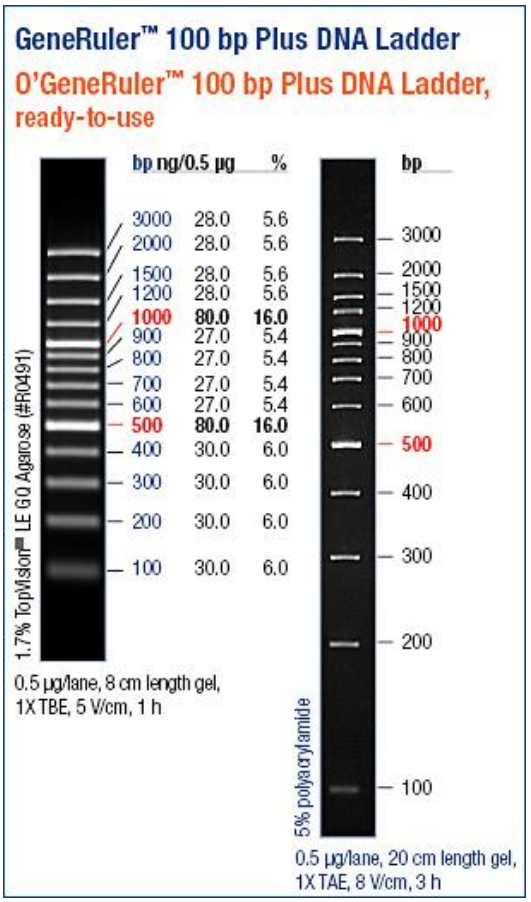
Deletion cassette with long tracts of flanking homology PCR program:

	Cycles	**Time**	**Temp**
Initial denaturation	1	2 min	95 °C
Denaturation	6X	45 sec	95°C
Annealing		45 sec	40°C
Elongation		180 sec	68°C
Secondary denaturation	1	120 sec	95°C
Denaturation	5X	45 sec	95°C
Annealing		45 sec	45°C
Elongation		150 sec	68°C
Denaturation	25X	45 sec	95°C
Annealing		45 sec	48°C
Elongation		150 sec	68°C
Final elongation	1	15 min	68°C
Hold	--	--	12°C

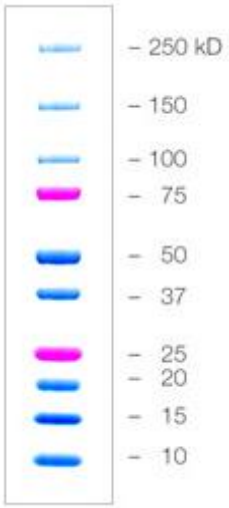
The first 6 rounds of this PCR program is added to amplify the template and the flanking sequences, and the primers are added after this step. The annealing temperature in the main step is raised to 48°C after the first 5 cycles of the main program.

Appendix III: Molecular weight standards





Precision Plus Protein Dual Color:



Appendix IV: AIMP3 alignments

	Identities	Similarities
Pombe aimp – AIMP3	8.4	12.3
Pombe aimp N term – AIMP3	17.4	32.7
Pombe aimp C term – AIMP1	25.1	34.2

Appendix V: Genes next to *pah1*

The following genes are located in a vicinity of 17 kb upstream and downstream of *pah1*.

Upstream:

Gene	Molecular function
SPAC30C2.03	Not known
mmd1	Oxireductase activity
dhc1	ATPase activity
SPAC1093.05	RNA binding

Downstream:

Gene	Molecular Function
erv14	oxidoreductase activity
dml1	GTPase activity
SPAC30C2.07	Not known
SPAC30C2.08	Not known
SPAC1635.01	transmembrane transporter activity
SPAC144.01	Not known
iec1	DNA binding
ade2	Ion binding