

Thesis for the Master's degree in Molecular Biosciences
Main field of study in Molecular Biology

**Characterisation of eukaryotic initiation factor-2 α in
the fission yeast *Schizosaccharomyces pombe*.**

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60 study points

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Abstract

Our group has shown that ultraviolet (UV) irradiation of fission yeast cells in G1 phase induces a delayed entry into S phase (Nilssen et al., 2003). More recently, we found that the cell cycle delay is accompanied by a general depression of translation and phosphorylation of the eukaryotic translation initiation factor eIF2 α (Tvegard et al., 2007). Both the cell cycle delay and downregulation of translation are absolutely dependent on the Gcn2 kinase, which is known to phosphorylate eIF2 α at Ser52 and thereby reduce global translation. However, the checkpoint is only partially dependent on eIF2 α Ser52 phosphorylation, indicating that there must be more Gcn2 targets in fission yeast.

One attractive hypothesis is that other sites on eIF2 α might be phosphorylated by Gcn2. In this work, intention was to investigate additional phosphorylation sites on eIF2 α in response to UV irradiation. Using bioinformatics tools and comparisons with published observations on eIF2 α in *S. cerevisiae*, wheat and brine shrimp, a number of potential phosphorylation sites were predicted. Indeed, some of these sites were shown to be phosphorylated in fission yeast in the presence of thiabendazole in a recent paper (Wilson-Grady et al., 2008).

To confirm the predicted phosphorylation sites, eIF2 α was tagged by an epitope tag. Then protein extracts were prepared from cells treated with UV irradiation as well as from untreated control cells and analyzed MS and 2D-Western. Preliminary results suggest that eIF2 α is indeed phosphorylated on other sites, in addition to Ser52, after UV-irradiation. However, further studies are needed to fully understand eIF2 α 's involvement in the downregulation of translation and in the G1/S delay after UV irradiation, as well as the exact role of Gcn2 in the observed modifications.

Abbreviations

2D	Two dimensional	LC	Liquid chromatography
AA	Amino acid	LiAc	Lithium acetate
ABC	Ammonium bicarbonate	Log	Logarithmic
ACN	Acetonitrile	M phase	Mitosis
APS	Ammonium persulphate	Mb	Mega base
BCA	Bicinchoninic acid	MEA	Malt extract agar
bp	Base pair	min	Minutes
BSA	Bovine serum albumine	MQ-H ₂ O	MilliQ-H ₂ O
C	Copy	mRNA	Messenger RNA
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>	MS	Mass spectroscopy
CDK	Cyclin-dependent kinase	NEB	New England Biolabs
cDNA	Complementary DNA	OD	Optical density
CKI	Cyclin-dependent kinase inhibitor	PA	Polyacrylamide
CK-II	Casein kinase II	PABP	Poly(A) binding protein
DMSO	Dimethyl sulphoxide	PAGE	Polyacrylamide gel electrophoresis
DNA	Deoxyribo nucleic acid	PCR	Polymerase chain reaction
dsRNA	Double-stranded RNA	PEG	Polyethylene glycol
DTT	Dithiothreitol	PERK	PKR-like ER kinase
<i>E. coli</i>	<i>Escherichia coli</i>	pI	Isoelectric point
EDTA	Ethylenediaminetetraacetic acid	PKR	Protein kinase R
eEF	Eukaryotic elongation factor	Pre-RC	Pre-replication complex
EGTA	Ethylene glycol tetraacetic acid	PVDF	Polyvinylidene fluoride
eIF	Eukaryotic initiation factor	RNA	Ribonucleic acid
EMM	Edinburgh minimal media	rpm	Rounds per minute
ER	Endoplasmatic reticulum	rw	Reverse
eRF	Eukaryotic release factor	S phase	Synthesis phase
ESI	Electrospray ionisation	<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
EtBr	Ethidium bromeide	<i>S. pombe</i>	<i>Schizosaccharomyces pombe</i>
fw	Forward	SDS	Sodium dodecyl sulphate
G1	First gap phase	SOC	Super Optimal borth for Catabolite repression
G2	Second gap phase	TBE	Tris-borate-EDTA
Gcn2	General control non-derepressible-2	TBS-T	Tris-buffered saline Tween-20
GDP	Guanosine diphosphate	TBZ	Thiabendazole
GEF	Guanine nucleotide exchange factor	TCA	Trichloroacetic acid
GTP	Guanosine triphosphate	TE	Tris EDTA
HRI	Heme-regulated inhibitor	TEMED	N,N,N',N'-tetramethylethylenediamine
IEF	Isoelectric focusing	TFA	Trifluoroacetic acid
IgG	Immunoglobulin G	Tm	Melting temperature
IPG	Immobilised pH gradient	Tris	2-Amino-2-(hydroxymethyl)-1,3-propanediol
IPTG	Isopropyl β-D-1-thiogalactopyranoside	tRNA	Transfer RNA
IRES	Internal ribosome entry site	U	Units
ISR	Integrated stress response	UV	Ultraviolet
Kb	Kilo base	UVC	Ultraviolet C (280 nm – 100 nm)
kDa	Kilo Dalton	V	Volt
LB	Luria Bertani	YE	Yeast extract

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1. Introduction

1.1 Model organisms in biological science

Life is characterised by certain biological processes such as metabolism, homeostasis, adaptation, organization, and perhaps most important, growth and division. These processes are carried out by the cell. A cell is the smallest entity that has all the characteristics of life and all living organisms consist of one or more cells.

All living organisms on earth are ordered into the three domains of life; eukaryotes, bacteria, and archaea. Animals, plants, protists and fungi are all eukaryotes. Eukaryotic cells have a membrane-bound nucleus and compartmentalisation of organelles. The two other domains of life, archaea and bacteria, are also known as prokaryotes.

One of the most fundamental tasks in biology is to understand how cell growth and division are regulated. The use of model organisms in science has made it possible for the scientists to gain insight into the fundamental properties of life; how cells grow and divide, how they store and express their genetic information, how energy is made and used, and how homeostasis is achieved.

Model organisms are chosen for their advantages in the lab and should possess qualities such as small physical size, small genome, and a short life-cycle. A good model organism should also be easily grown in the lab and should be amenable to experimental manipulation, such as mutation and genetic crossing. Model organisms should also have characteristics that make them appropriate to serve as model systems. Model organisms used in basic science should for example have well conserved translational apparatus or checkpoint mechanisms.

Famous much-used model organisms for the study of multi-cellular organisms are the fruit fly *Drosophila melanogaster*, the nematode *C. elegans*, the zebrafish *Danio*

erio, the plants *Arabidopsis thaliana*, wheat, and the mouse. To study and answer more fundamental questions such as growth and division, single cells in culture are preferred. Human cell cultures are extensively used in basic research, but often are single-celled eukaryotic organisms, such as the budding yeast *Saccharomyces cerevisiae* or the fission yeast *Schizosaccharomyces pombe*, preferred. Human cells are diploid and their normal regulation of cell division has to be changed in order to maintain the cells in culture. Their redundant proteins and reaction pathways makes them also a much more complicated model than their single-celled counterparts. So useful are single-celled model system, that cell cycle analysis carried out in budding yeast and fission yeast by Leland H. Hartwell Paul Nurse, respectively, was honoured with the Nobel prize in Medical or Physiology in 2001 (Appendix I).

The budding yeast is probably the most used eukaryotic model organism. It has been a pioneer in both translational and cell cycle research. Thus, due to the fact that fission yeast's regulation of translation is closer to that of humans (reviewed in Hinnebusch, 2005), the fission yeast is used as a model organism in this study.

1.1.1 Fission yeast as a model organism

The fission yeast, *S. pombe*, was isolated from East African millet beer and systematically described in 1893 by P. Lindner, but limited genetic studies were first started in the late 1940s. It was further developed as an experimental model for studies of genetics by Urs Leupold, and for studying the cell cycle by Murdoch Mitchison (Forsburg, 2005).

S. pombe is a non-pathogenic single-celled free living archiascomycete fungus (Figure 1.1), with many common features of more complicated eukaryotes. The cells are cylindrically shaped, 12 to 15 μm in length and 3 to 4 μm in diameter (PombeNet at Forsburgs Lab, Appendix I), which, in contrast to the budding yeast, divides by fission. *S. pombe* is a unique fungus that is characterized by features of ascomycetes (cell wall, spore formation, fermentation capacity), but which is extremely divergent in terms of gene sequence. Phylogenetic analysis suggests that fission yeast diverged

from the budding yeast around 330-420 million years ago, and from metazoans from 1 000 -1 100 million years ago (reviewed in Sipiczki, 2000). *S. pombe* has a 13,8 Mb genome distributed between three chromosomes, and it contains around 4900 genes, which is the smallest number of protein-coding genes recorded for an eukaryotic organism (Wood et al., 2002).

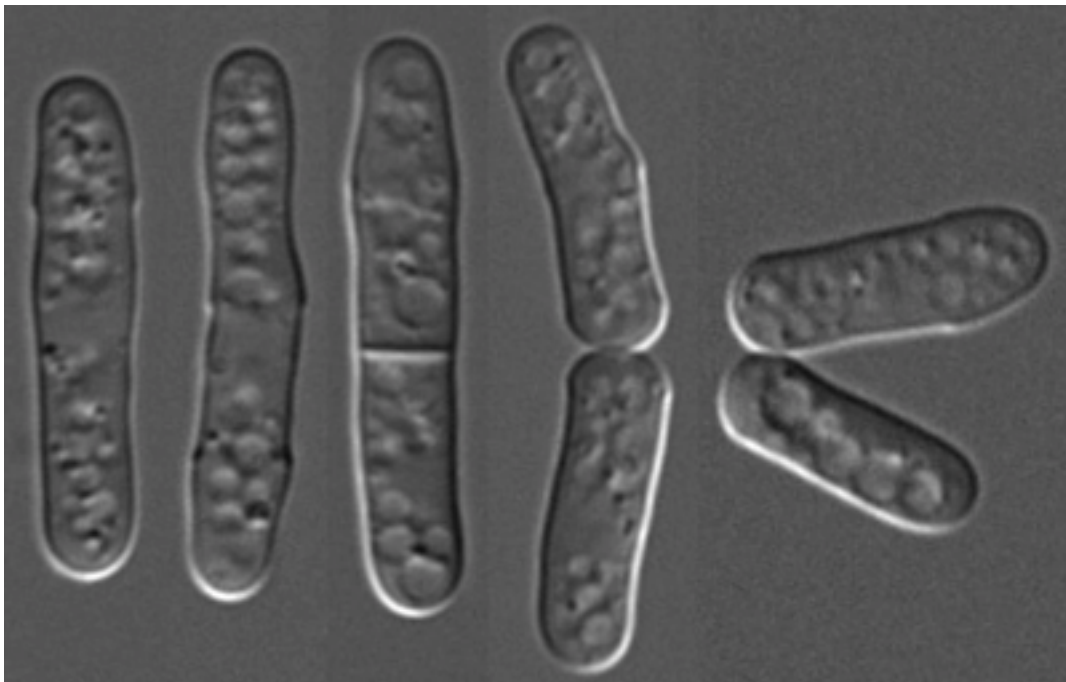


Figure 1.1 Microscopic picture of the model organism *Schizosaccharomyces pombe*. The fission yeast in different phases of the cell cycle (the picture is copied from Steve's place, Appendix I).

Fission yeast has, over a short period of time, grown to be a popular model organism to investigate basic biological processes, especially in the field of cell cycle control and differentiation. Fission yeast can easily be maintained in haploid or diploid state under normal laboratory conditions. Classical genetic methods can easily be done to identify new genes, or to do functional analysis of already identified genes. It is a well liked model organism since it is amenable to both classical and molecular genetic analysis, and to biochemical and cell biology studies (Forsburg, 1999; Forsburg, 2005).

Corresponding to the increased use of fission yeast as a model organism, the collection of available molecular tools has increased. The fission yeast genome was the sixth genome to be sequenced and fully annotated (Wood et al., 2002). Global

transcription responses have also been extensively characterized, including the periodic gene expression program of the cell cycle (Rustici et al., 2004; Wilhelm et al., 2008), and the global transcriptional responses to meiosis and sporulation (Mata et al., 2002), pheromone response (Mata and Bähler, 2006) and during environmental stress (Chen et al., 2003). A global analysis of protein localization in fission yeast has been carried out, determining localization of 4 431 proteins out of the total 4900 fission yeast genes (Matsuyama et al., 2006), and a genome-wide deletion mutant library covering more than 93 % of the genome (Bioneer, Appendix I) is available for use. All the existing data sets are merged in a curated database at the Sanger Institute (GeneDB, Appendix I).

The increased data gathered through genome-wide analyses mentioned above, including a large collection of temperature-sensitive mutants, a wide variety of plasmids, and various expression systems makes fission yeast a powerful experimental model.

1.2 The cell cycle

A proliferating cell undergoes a series of distinct stages, which involve the replication of the chromosomes and the division of one cell into two daughter cells. This process is known as the cell cycle and consists of four phases. S phase and M phase are the phases where DNA is replicated and distributed between the daughter cells, respectively. Between S and M, there are two gap phases, named first gap (G1) phase and second gap (G2) phase. The cell will continue to grow and carry out translation in all phases, except for M phase where very little if any growth is detected (Alberts et al., 2002).

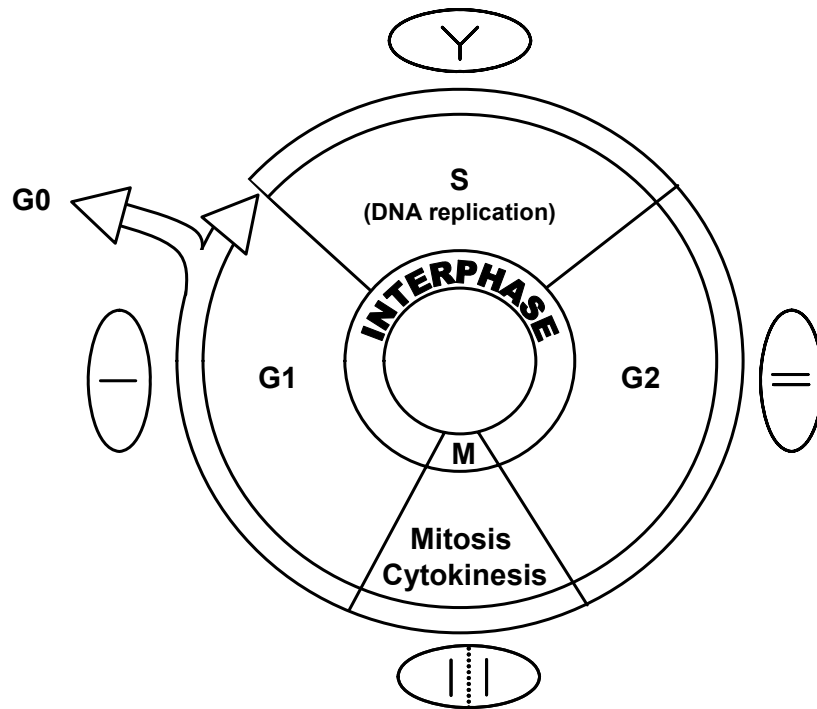


Figure 1.2 A schematic drawing of the cell cycle. The oval shapes represent cells. The lines inside the cells represent copies of DNA, while the Y-shape represents replicating DNA. The stippled line represents the actomyosin ring, which contracts and constricts the cell membrane to cleave the cell into two during cytokinesis.

1.2.1 Regulation of the cell cycle

The cell cycle is a dynamic process and can be regulated both positively and negatively by various extracellular and intracellular signals. Cyclin-dependent kinases (CDKs) are key components of the cell-cycle control system. CDKs are protein kinases which regulate their substrates by phosphorylation and thereby control cell cycle transitions. Their activity and probably substrate specificity depends on association of the kinase with cyclins. CDKs are regulated by three main mechanisms: (1) association with their cyclin partners, (2) suppression by inhibitory phosphorylation, and (3) inhibitory proteins called CKIs. CDKs are constitutively expressed during the cell cycle. Cyclins are regulatory subunits of the CDKs, and they are expressed and degraded in specific stages of the cell cycle. G1-cyclins bind CDK in G1 phase and are involved in preparation for DNA replication and commitment to proceed in the cell cycle. S-cyclins are active during S phase and required for initiation of DNA replication, and M-cyclins regulate events in M phase

(Alberts et al., 2002). CKIs bind and inhibit the activity of the CDKs. In the model organism fission yeast, Cdc2 is the only known CDK (Nurse, 1990), while Rum1 is the only known CKI (Benito et al., 1998).

Each phase of the cell cycle has to be completed before the cell can continue the cell cycle. Several control mechanisms, known as cell cycle checkpoints, ensure that the cell cycle phases progress correctly and that the DNA is not damaged. In the event of DNA damage or perturbed replication, the checkpoint mechanisms delay or arrest the cell cycle (Alberts et al., 2002). If the cell fails to respond to normal growth-inhibitory signals, cancer may be the result. The three major checkpoints identified in fission yeast are the intra-S checkpoint, the S/M checkpoint, and the G2/M checkpoint. The intra-S checkpoint slows down replication when DNA is damaged, the S/M checkpoint prevents mitosis until replication of DNA has finished, and the G2/M checkpoint prevents mitosis when DNA is damaged. To stop the cells progressing further in the cell cycle the checkpoints often target CDK activity either by inducing CKIs and/or by increasing the inhibitory phosphorylation on the CDK. In addition, a novel G1 checkpoint has been discovered in fission yeast (Nilssen et al., 2003; Tvegard et al., 2007). This checkpoint inhibits preparation for DNA replication, but unlike the checkpoints described above, does not inhibit CDK activity.

1.2.2 The G1/S transition in fission yeast

In G1 phase, before the beginning of DNA replication, the cell has to decide whether to enter the mitotic cell cycle or go into a non-proliferating state known as the G0 phase (see Figure 1.2). This is a crucial decision, since once started on S phase, the cell is committed to dividing. Entering the S phase without the cell being in an optimal condition may lead to mutations, chromosomal fragmentations and genomic instability. In a multicellular organism, such events can lead to cancer or inheritable diseases.

Preparation for S phase involves the assembly of the pre-replication (pre-RC) complex, which consists of the heterohexameric origin-recognition complex and the proteins Cdc18 and Cdt1. Replication origins bound by the pre-RC can then attract further replication factors, like the minichromosome maintenance protein complex (Mcm2-7). Initiation of replication also requires the activity of two kinases, where one is a CDK. In fission yeast Cdc2 associates with the Cig2-cyclin to perform the required functions for replication initiation (reviewed in Moser and Russell, 2000). Then, as S phase commences, Cdc2 is rapidly phosphorylated and thereby inactivated. Thus, Cdc2 phosphorylation coincides with entry into S phase and serves as a convenient molecular marker of S phase entry. In addition to the cell-cycle dependent accumulation of Cig2, the activity of Cig2-Cdc2 is also regulated by the CKI Rum1. Rum1 accumulates in M phase and persists through G1 phase (Benito et al., 1998). Thus, the presence of Rum1 serves as a molecular marker for G1.

Regulation of transcription is an important tool to ensure the timely expression of genes during the cell cycle. The expression of 80-90% of all fission yeast genes is cell-cycle regulated (Rustici et al., 2004). Cdc10 is the main transcription factor responsible for the G1-S-phase-specific gene expression, including transcriptional activation of several replication initiation factors and of Cig2. Therefore will a *cdc10* mutant arrest in G1 phase. In the following work is a temperature sensitive *cdc10* mutant used to synchronize the fission yeast cells in G1 phase.

1.3 Translational regulation in eukaryotes

An organism's DNA encodes all of the RNA and protein molecules required to construct its cells, regulate the cell cycle and repair damage to the DNA itself. All the important genes need to be expressed and by far the most need to be translated into a protein to carry out their functions. Gene expression is regulated at many of the steps in the pathway from DNA to RNA to protein (Alberts et al., 2002). Examples of steps where gene expression may be regulated are transcription, export of messenger RNA (mRNA) from the nucleus and translational control. Regulation of translation makes

it possible to have a direct and rapid response to nutrient deprivation, development, differentiation, and stress. Translational control of pre-existing mRNA allows faster reaction to these events, than for example transcriptional control, which has to be followed by processing, transport from nucleus to cytoplasm and then by translation. Other benefits of translational control are reversibility, fine control, spatial control, and flexibility (Mathews et al., 2007).

1.3.1 Overview of translation

Translation of the mRNA into protein is performed by the ribosome, a large nucleoprotein assembly found in the cytoplasm of all cells. The ribosome consists of a large 60S subunit and a small 40S subunit, which perform peptidyl transfer and decoding, respectively. The process of translation can be divided into four parts: (1) initiation, (2) elongation, (3) termination, and (4) ribosome recycling. The initiation phase places an initiator tRNA (Met-tRNA_i^{Met}) at the start codon of the mRNA in the 80S initiation complex. This phase is carried out by eukaryotic initiation factors (eIFs). The elongation phase of protein synthesis is a cycle of aminoacyl-tRNA delivery and peptide bond formation repeated hundreds of times. Elongation is carried out by the enzymatic activity of the ribosomal RNA, together with elongation factors (eEFs) to improve the accuracy. Termination of protein synthesis is carried out by release factors (eRFs) when the acceptor site on the ribosome meets a stop codon. After termination, the ribosome subunits need to be dissociated. This process is known as ribosome recycling and probably carried out together by eIFs and eRFs (Pisarev et al., 2007).

Cellular levels of protein often do not correlate with their corresponding mRNA levels. Several elements of the translational apparatus may be a target to regulate protein levels in the cell: (1) Modification of initiation factors and/or their interaction with other proteins might control recognition or binding of mRNA. This may in turn affect rate of translation or mRNA selectivity. Regulation of initiation is the main target of translational regulation, and is both a rapid and energy economical method

to regulate translation. (2) Post-translational regulation of elongation factors will mainly affect the elongation rate. (3) Modifications of the ribosome may affect translation initiation, elongation, termination or ribosome recycling (reviewed in Mathews et al., 2007). (4) Additionally, mRNA may contain structural elements which determine and modulate translational efficiency by *trans*-acting protein factors. The process of initiation and its regulation is described in more detail below.

1.3.2 Regulating translation initiation

There are at least two different types of translation initiation in eukaryotes. In most mRNAs, translation is initiated by interaction between the cap-binding protein complex eIF4F and the m⁷GpppN cap structure located at the 5' end of mRNA (Figure 1.3). This is known as cap-dependent initiation of translation. In contrast, some viral and cellular mRNAs initiate translation at internal ribosome entry sites (IRES), which is not dependent on the cap structure. Further discussion will focus on cap-dependent translation.

Initiation of translation consists of a series of steps; each step is promoted by one or more initiation factors. Each step of translation initiation is described in detail in Figure 1.3. Most of the known control mechanisms affect initiation of translation. There are two distinct types of initiation regulation: (1) global translational control and (2) mRNA-specific translational control (reviewed in Gebauer and Hentze, 2004). Global control of translation often results from changes in the phosphorylation state of eIFs or proteins that interact with them. Common targets of global control are the cap-binding complex, eIF4F, which is a major target of translational control by extracellular stimuli. Another target of global control is the ternary complex component eIF2, as described in Section 1.3.3. Common targets of mRNA-specific control are usually regulatory elements in untranslated regions upstream or downstream of the coding region of mRNA. Factors affecting regulatory elements in mRNA can either be protein complexes or microRNA (Gebauer and Hentze, 2004; Sonenberg and Hinnebusch, 2009).

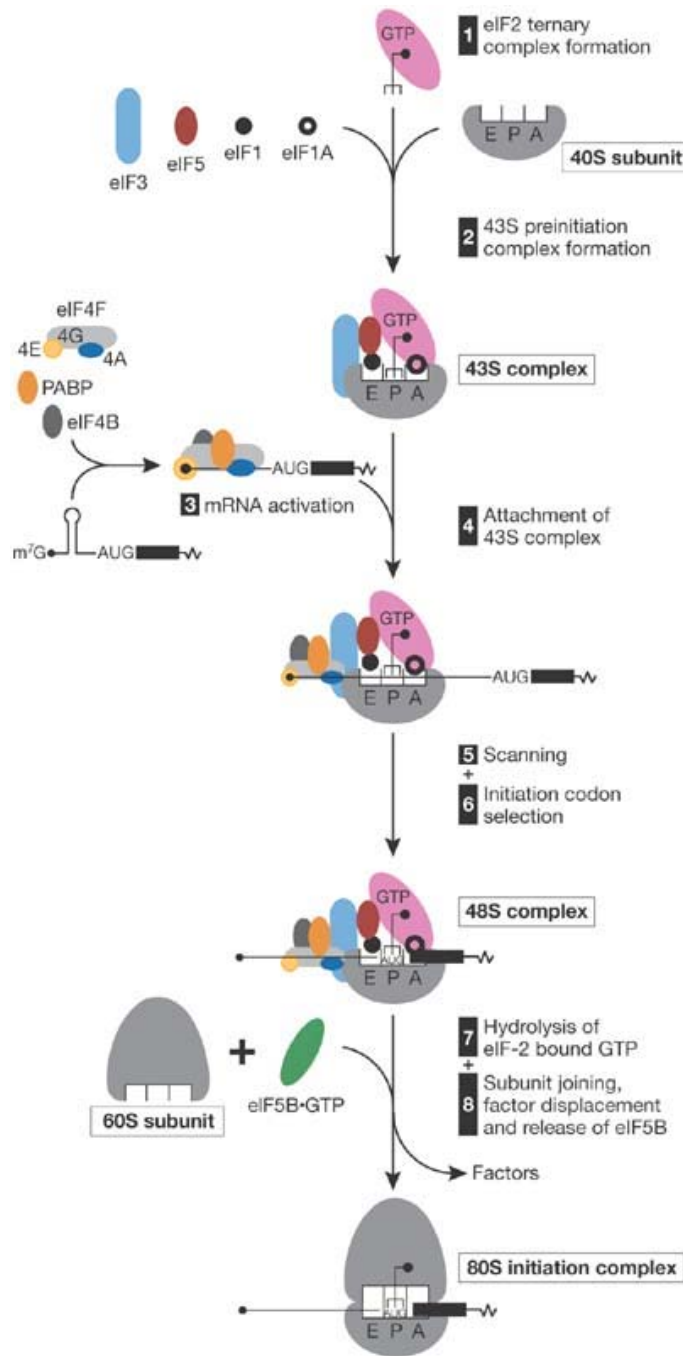


Figure 1.3 Cap-dependent initiation of translation in eukaryotes. eIF2-GTP-Met-tRNA^{iMet} ternary complex forms and joins with eIF3, eIF5, eIF1, eIF1A and the 40S ribosomal subunit to make the 43S preinitiation complex. mRNA is activated by binding of eIF4F, which consists of eIF4E, eIF4G and eIF4A, to the m⁷GpppN cap together with poly-A binding protein (PABP) and eIF4B. Activated mRNA is attached to the 43S complex making a 48S complex, where it unwinds RNA structures and scans for a start codon (most often AUG). When the initiation codon is selected, eIF-2 bound GTP is hydrolysed to GDP. This will join the 60S subunit and eIF5B-GTP to the 48S complex, displace the bound initiation factors and release eIF5B to make the 80S initiation complex. The initiator-tRNA is now bound to the P site of the ribosome and the elongation cycle can be started (Holcik and Pestova, 2007).

1.3.3 Eukaryotic initiation factor-2 α and its kinases

As described in figure 1.3 above, eIF2 mediates the binding of Met-tRNA_i^{Met} to the ribosome during initiation of translation and is one of the main targets for regulation of initiation. eIF2 consists of the three subunits; eIF2 α , eIF2 β , and eIF2 γ . In addition, eIF2 binds guanine nucleotides and in its GTP-bound state it interacts with Met-tRNA_i^{Met} (Figure 1.4). Phosphorylation of the serine-51 (Ser51) residue of the α -subunit in mammals is a highly conserved regulatory event and is a main player in the integrated stress response (ISR). Phosphorylation of this residue converts eIF2 from a substrate to an inhibitor of eIF2B, the guanine nucleotide exchange factor for eIF2. Thus, the level of phosphorylated eIF2 α regulates the rate at which eIF2 can be recycled to the GTP-bound, active form. As a consequence of the phosphorylation, concentration of the ternary complex is reduced, thereby preventing translation initiation. This results in inhibition of global mRNA translation. However, due to several specific mechanisms, stress response proteins are selectively translated (reviewed in Holcik and Sonenberg, 2005; Proud, 2005; Sonenberg and Hinnebusch, 2009).

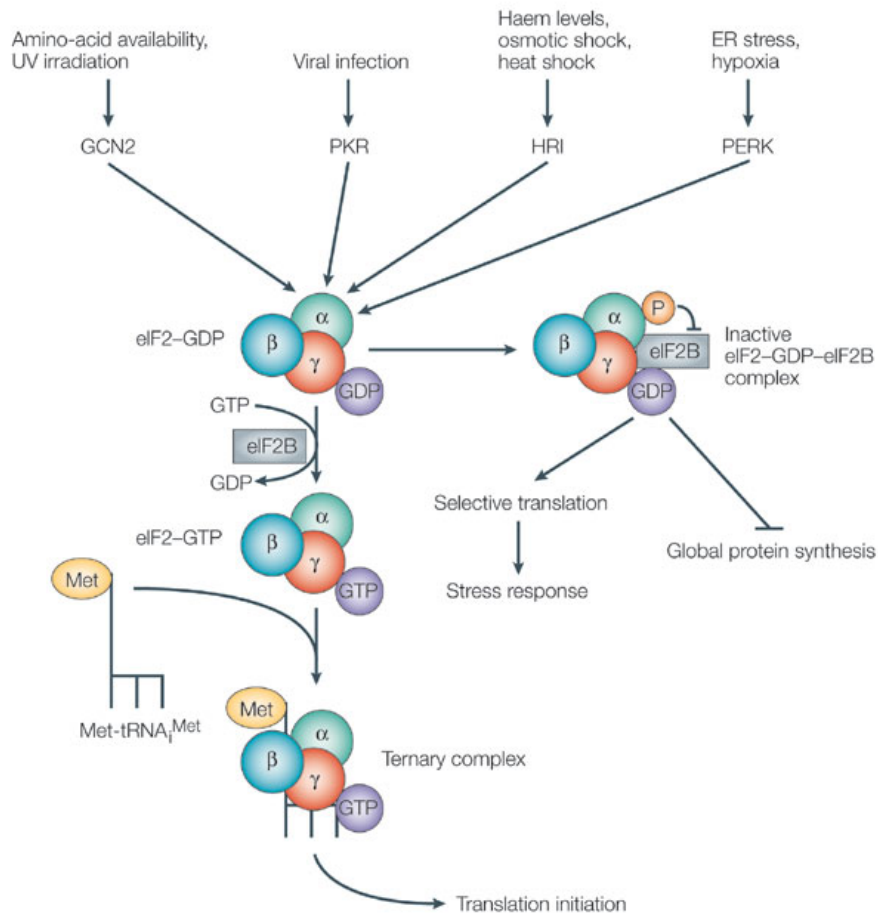


Figure 1.4 Regulation of translation initiation by eukaryotic initiation factor-2α. Many stress conditions result in eIF2α phosphorylation. In mammals, there are four different protein kinases; general control non-derepressible-2 (GCN2), protein kinase RNA (PKR), haem-regulated inhibitor kinase (HRI) and PKR-like ER kinase (PERK). eIF2-GTP, which consists of eIF2α, eIF2β, eIF2γ and guanosine triphosphate, binds Met-tRNA_i^{Met} to make the ternary complex necessary for cap-dependent translation in eukaryotes. During translation initiation, GTP is hydrolysed and eIF2 needs to be recharged following each round of initiation. This recharge is catalyzed by eIF2B, which is a guanine nucleotide exchange factor (GEF), exchanging GDP by GTP. Phosphorylation of eIF2α at the Ser51 residue (or Ser52 in fission yeast) produces an inactive eIF2-GDP-eIF2B complex. Inactivation of the GDP-GTP exchange ultimately results in global down-regulation of protein synthesis, but also in selective translation of a subset of mRNAs, which allows cells to adapt to the stress condition. (Holcik and Sonenberg, 2005)

Four kinases that phosphorylate the Ser51 residue of eIF2α have been identified in mammalian cells; general control non-derepressible-2 (GCN2), protein kinase RNA (PKR), haem-regulated inhibitor kinase (HRI) and PKR-like ER kinase (PERK). All of these kinases share a conserved eIF2α kinase domain in addition to an unique regulatory domain, which makes the protein able to respond to distinct stimuli (Dever et al., 2007). The regulatory domains are either found in the N-terminal region, C-terminal region, or within the conserved eIF2α kinase domain. GCN2 has a domain

resembling histidyl-tRNA synthetase, which is activated upon binding to uncharged tRNAs that accumulate in cells starved for any amino acid (Dong et al., 2000). Gcn2 is also activated upon UV irradiation by an unknown mechanism (Deng et al., 2002; Tvegard et al., 2007). PKR has a dsRNA-binding domain which is activated in response to viral RNAs and viral dsRNA genomes (Wu and Kaufman, 1996). HRI has a primary function in erythroid cells to coordinate globin synthesis with available iron by phosphorylating eIF2 α when heme-levels are low. In addition, HRI is known to respond to heavy metal exposure, heat shock, osmotic stress, and nitric oxide (Lu et al., 2001; McEwen et al., 2005). PERK is activated under endoplasmatic reticulum (ER) stress conditions and accumulation of unfolded proteins (Harding et al., 2000).

In fission yeast, there are three known proteins related to the eIF2 α kinase family: Gcn2, Hri1, and Hri2 (Zhan et al., 2002). Hri1 and Hri2 induce phosphorylation of eIF2 α in fission yeast by heat shock, arsenic and oxidative stress (Dunand-Sauthier et al., 2005; Zhan et al., 2004; Zhan et al., 2002). Gcn2 is known to phosphorylate eIF2 α after amino acid starvation (Tvegård, 2007), as well as UV irradiation (Tvegard et al., 2007) and other cellular stresses in fission yeast (Krohn et al., 2008).

1.4 Background

Previous work in our lab has led to the discovery and initial characterisation of checkpoint mechanisms regulating the G1/S transition in fission yeast (Nilssen et al., 2004; Nilssen et al., 2003; Tvegard et al., 2007). Fission yeast cells exposed to UV light (254 nm) in G1 phase delay DNA replication. Several lines of evidence support the notion that the cells delay in G1; for example inhibition of pre-RC formation, and expression of the Cdc2 inhibitor Rum1 (Tvegard et al., 2007), the lack of Cdc2 phosphorylation, and the absence of an active S-phase-specific checkpoint kinase Cds1 (Nilssen et al., 2003).

Coinciding with the observed cell cycle delay, cell growth slows down after UV irradiation. This is due to phosphorylation of the Ser52 residue of the initiation factor

eIF2 α , a standard pathway for regulation of translation (as discussed in Chapter 1.3.3) (Tvegard et al., 2007). Phosphorylation of the Ser52-residue of eIF2 α in response to UV is performed by the Gcn2 kinase. After UV irradiation in fission yeast, Gcn2 is required for both the translation downregulation and the cell cycle delay in G1, thus providing a link between the cell cycle and cell growth (Grallert and Boye, 2007).

It is currently unclear whether and how the two pathways are coupled beyond the activation of Gcn2 after UV irradiation. Phosphorylation of Ser52 of eIF2 α coincides with the cell cycle delay after UV and various other treatments (Krohn et al., 2008), suggesting that the phosphorylated form of eIF2 α might be a signal to delay G1/S progression. However, an alternative possibility is that it is the low translation activity per se that is directly responsible for the cell cycle delay. In an attempt to clarify the significance of eIF2 α phosphorylation, the Ser52 residue was exchanged for an alanine residue (an eIF2 α S52A mutant), which cannot be phosphorylated. Surprisingly, this mutant still down-regulated translation after UV irradiation, suggesting that Ser52 phosphorylation is not the only mechanism for translation downregulation. Since a *gcn2* mutant loses the ability to down-regulate translation after UV irradiation (Tvegard et al., 2007), the additional mechanism(s) must also be Gcn2-dependent. Analysis of the mutant did not reveal the significance of Ser52 phosphorylation for checkpoint activation. Pinpointing the additional pathway(s) for translation downregulation after UV might allow us to identify the signal for the cell cycle delay.

In the following work, I shall focus on the downregulation of translation. The unexpected downregulation of translation in the eIF2 α S52A mutant after UV irradiation makes the following predictions possible; (1) there are other phosphorylation sites on eIF2 α targeted by Gcn2 and/or (2) Gcn2 targets other proteins that affect translation (Figure 1.5). If the latter prediction is true, these proteins are probably not directly involved in the cell-cycle delay. One such target might be eIF4E isoform 2 in fission yeast, which is known to be a stress-response factor (Ptushkina et al., 2004).

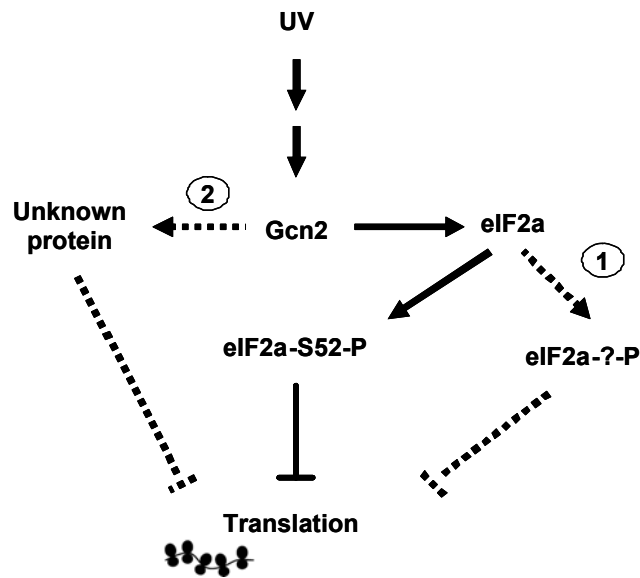


Figure 1.5 Downregulation of translation after UV irradiation in G1 phase. In addition to Ser52-phosphorylation of eIF2 α , the following or alternative events may also lead to a downregulation of translation: (1) Gcn2 phosphorylates additional residues on eIF2 α and/or (2) Gcn2 phosphorylates an unknown protein in addition to eIF2 α . Dashed lines indicate hypothetical pathways which are not proven experimentally.

Aim of study

The main goal of this work is to further characterise the molecular mechanisms involved in down-regulation of translation after UV irradiation. I will do the following to achieve this goal:

- 1) Generate methods to identify and purify eIF2 α from fission yeast.
- 2) Investigate whether there are other phosphorylation sites on eIF2 α in addition to Ser52.
- 3) Investigate whether there is a difference in the phosphorylation status of eIF2 α before and after UV irradiation.
- 4) Investigate whether these sites are phosphorylated in a Gcn2-dependent manner.

2. Materials

2.1 Cell strains

2.1.1 Bacterial strains

The following *E. coli* strains (Table 3.1) were used for transformation and/or expression of recombinant protein.

Table 3.1 Escherichia coli

Strain	Genotype	Supplier
TOP10	F- mcrA Δ (mrr-hsdRMS-mcrBC) Φ 80lacZ Δ M15 Δ lacX74 recA1 araD139 Δ (araleu) 7697 galU galK rpsL (StrR) endA1 nupG	Invitrogen
BL21 Star (DE3)	F- ompT hsdSB (rB-mB-) gal dcm rne131 (DE3)	Invitrogen
BL21 CodonPlus-RIPL (DE3)	F- ompT hsdS(rB- mB-) dcm+ Tetr gal λ (DE3) endA Hte [argU proL Camr] [argU ileY leuW Strep/Specr]	Stratagene

2.1.2 S. pombe strains

The following *S. pombe* strains (Table 3.2) were used in this study. All the strains are derivatives of the *Schizosaccharomyces pombe* L972 h⁻ strain.

Table 3.2 Schizosaccharomyces pombe

Strain	Genotype	
19	L972 h-	
20	h+	
148	his3-D1 leu1-32 ura4-D18 ade6-M210 h-	
489	cdc10-M17 h-	
1042	cdc10-M17 ura4-D18 leu1-32 h+	
1311	pku70::his3+ leu1-32 his3-D1 ade6-M216 ura4-D18 h+	
1471	tif211:13myc:kanMX6 his3-D1 ade6-M216 ura4-D18 leu1-32 pku70::his3+ h90	This study
1473	tif211:13myc:kanMX6 his3-D1 ade6-M210/ade6-M216 ura4-D18 leu1-32 h-	This study
1488	tif211:13myc:kanMX6 cdc10-M17 ura4-D18 leu1-32 [his3-D1 ade6-M216/ade-M210]? h-	This study

2.2 DNA

2.2.1 PCR and DNA sequencing primers

All the primers presented in Table 3.3 were used for PCR experiments except for sequencing primers. The DNA sequencing primers used were T7-fw-sequencing and ATG-fw-sequencing. Melting points (T_m) were determined by the suppliers.

Table 3.3 PCR and DNA sequencing primers

Primer name	Direction	Sequence 5' – 3'	Supplier
CACC-tif211-fw	Forward	CACCATGTTCGACGACAAGCTGCAG	Invitrogen
CACC-tif211B-fw	Forward	CACCATGTATACCACAATTGGATG	Invitrogen
tif211-rw	Reverse	TTACTCAGAACCGCTTTGGT	Invitrogen
tif211-UAA-rw	Reverse	CTCAGAACCGCTTTGGTCAT	Invitrogen
T7-fw-sequencing	Forward	TAATACGACTCACTATAGGG	GATC Biotech
ATG-fw-sequencing	Forward	CCGAAACAAGCGTCATGGAG	GATC Biotech
tif211_C-tag_fw	Forward	TTGCCGATTTGATGAAAAAATTTGA AAAGGAAAATGCTGAAATCTCTGG TGATGAAGAGGATGACCAAAGCGG TTCTGAGCGGATCCCCGGGTTAATT AA	Invitrogen
tif211_C-tag_rw	Reverse	AGAACCCAGATTATAATGGAACA CTAAAGTGCCAAAAATTCACAATG AAAAAGCGTATATTAGCTAGTGTCT AATCCATGAATTCGAGCTCGTTTAA AC	Invitrogen
in_tif211	Forward	GAAGATGTTTCATACCGAGGAGG	Invitrogen
us_tif211	Reverse	AATCCTCCCCAAAGCAGATATT	Invitrogen
in_kanR	Reverse	GTTATTCATTCGTGATTGCG	Invitrogen

2.2.2 Plasmids

The following plasmid (Figure 3.1) was used for cloning, transformation and recombinant expression in *E. coli*.

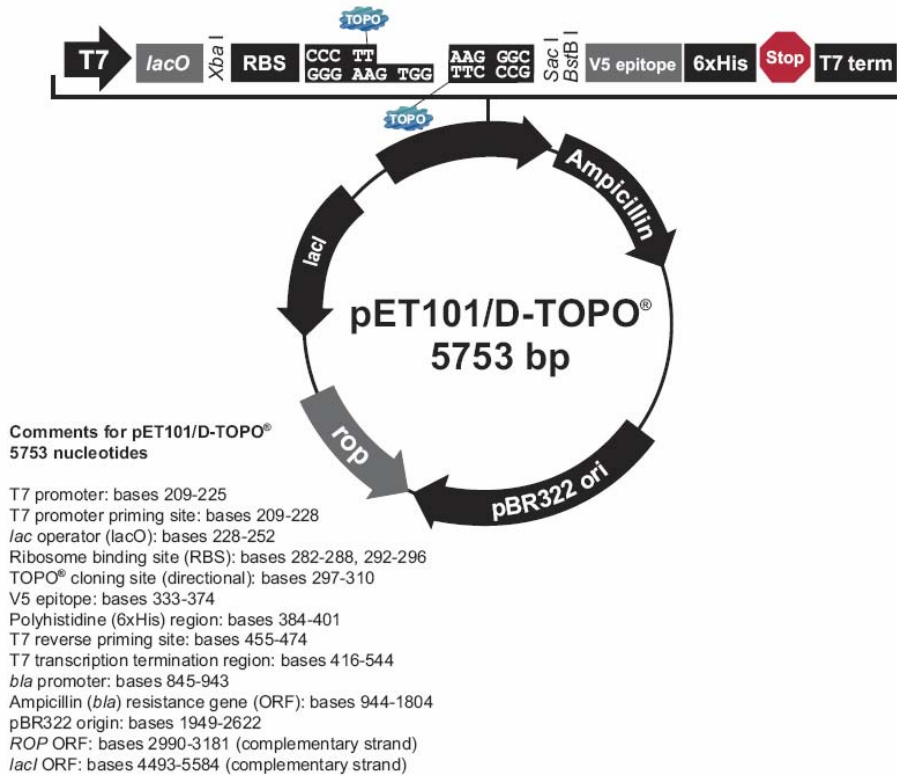


Figure 3.1 Map of pET101/D-TOPO. A PCR product is cloned into the TOPO cloning site between bases 297 and 310. pET101/D-TOPO was supplied by Invitrogen and is a high-level expression vector (Copied from Invitrogen, Appendix I).

The following plasmids (Figure 3.2) were used as a template for transformation of *S. pombe*.

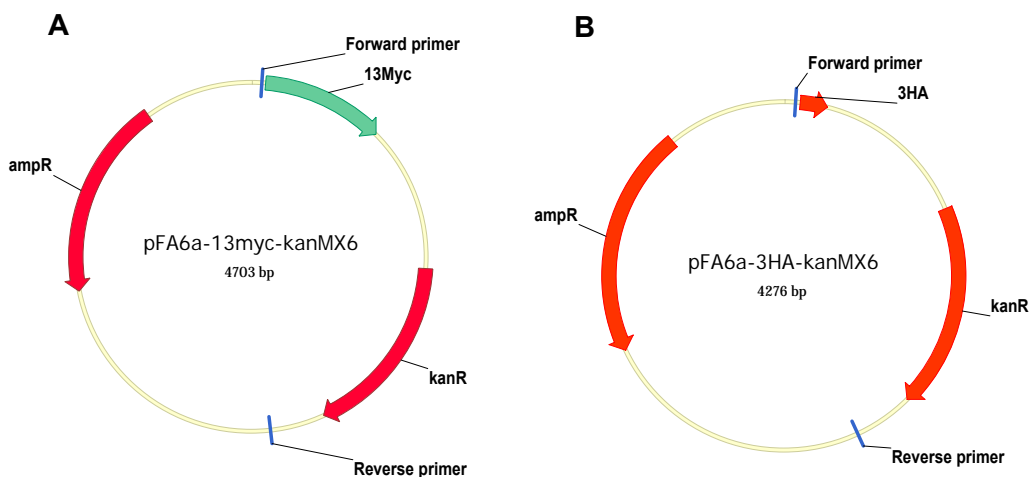


Figure 3.2 Map of pFA6a-13myc-kanMX6 and pFA6a-3HA-kanMX6. Plasmids used for tagging of a protein of interest with either (A) 13myc-tag or (B) 3HA-tag (Bähler et al., 1998).

2.3 Enzymes

Enzymes presented in Table 3.4 were used for PCR (AccuPrime Pfx DNA polymerase), restriction analysis (HindIII, MscI, PstI), crossing of fission yeast strains (Helikase), mass spectroscopy (trypsin), and for flow cytometry (Ribonuclease A).

Table 3.4 Enzymes

Supplier	Enzyme
BioSeptra	S.H.P/H.P.J Helix Promatia Juice (helikase)
Invitrogen	AccuPrime Pfx DNA Polymerase
New England Biolabs	Hind III, MscI
Fermentas	PstI
Promega	Trypsin
Sigma-Aldrich	Ribonuclease A

2.4 Cloning, DNA isolation and DNA purification kits

The kits presented in Table 3.5 were used for cloning (Champion pET Directional TOPO Expression Kit), DNA isolation and purification (QIAquick Gel Extraction Kit, QIAprep Spin Miniprep Kit), polymerase chain reaction (High Fidelity PCR Master), and for determining the concentration of proteins in a solution (BCA Protein Assay Kit).

Table 3.5 Kits used in cloning, DNA isolation and DNA purification

Supplier	Kit
Invitrogen	Champion pET101 Directional TOPO Expression Kit
Qiagen	QIAquick Gel Extraction Kit, QIAprep Spin Miniprep Kit
Roche	High Fidelity PCR Master
Thermo Scientific	BCA Protein Assay Kit

2.5 Antibodies

Antibodies used for protein immunoblots or immunoprecipitation are listed in Table 3.6.

Table 3.6 Antibodies

Antibody	Supplier
Primary antibody:	
Purified Mouse Anti-Human c-Myc	BD Pharmingen
Rabbit (polyclonal) Anti-eIF2 α [pS^{52}] Phosphospecific Antibody, Unconjugated	Biosource
Serum from rabbit SY0988 and SY0989 (immunization against peptide)	Eurogentec
Secondary antibody:	
Goat anti-mouse, IgG + IgM (H+L), alkaline phosphatase-linked antibody	GE Healthcare
Goat anti-rabbit, IgG + IgM (H+L), alkaline phosphatase-linked antibody	GE Healthcare

2.6 Molecular weight standards

The molecular weight standards (Table 3.7) were used to estimate size and amount of proteins or DNA in gels. A visual representation of the molecular weight standards used is shown in Appendix II.

Table 3.7 Molecular weight standards

Molecular weight standards	Range	Supplier
DNA molecular weight standards:		
2-Log DNA Ladder	0.1-10.0 kb	NEB
Protein molecular weight standards:		
Dual Color Precision Plus Protein Standard	10-250 kDa	Bio-Rad

2.7 Chemicals and reagents

The chemicals and reagents listed in Table 3.8 were used in this study.

Table 3.8 Chemicals and reagents

Supplier	Chemicals
Arcus	Ethanol
BD	Bacto Agar, Bacto Tryptone, Bacto Yeast Extract, methanol
Bio-Rad	Acrylamide/bis solution (37.5:1), Bio-safe Coomassie Brilliant Blue Stain, APS, Ponceau S stain solution, TEMED
Fermentas	Buffer O ⁺ with BSA
Formedium	EMM+N Broth
GE Healthcare	DeStreak Rehydration Solution, IPG buffer pH 3.5-5.0, Immobiline DryStrip Cover Fluid, Membrane Blocking Agent, ECF substrate, ECF dilution buffer,
Intergen	Sheared herring testes DNA
Invitrogen	10X AccuPrime Pfx Reaction Mix, Sytox Green
Merck	Acetonitrile, ammonium bicarbonate, iodoacetamide, malt extract, NH ₄ HCO ₃ , octylglucoside, TFA
New England Biolabs	NEBuffer #2, NEBuffer #4
Sigma-Aldrich	Acetic acid glacial, adenine, Agarose type 1, ampicillin, bromphenol blue, citric acid, DMSO, boric acid, chloramphenicol, DTT, EDTA, EGTA,

2.8 Solutions

2.8.1 Growth medium and agar plates

E. coli

LB-medium	1 % Tryptone 0.5 % Yeast Extract 0.5 % NaCl NaOH to pH 7.0 Supplemented with: 100 μ g/mL ampicillin or 50 μ g/mL chloramphenicol when needed
LB-plates	As above plus: 15 g/L agar
SOC-medium	2.5 mM KCl 10 mM MgCl ₂ 10 mM MgSO ₄ 20 mM glucose

S. pombe

EMM-medium	32.3 g/L EMM+N Broth Supplemented with: 225 μ g/mL amino acids when needed
MEA-plates	30 % Malt Extract 20 g/L agarose
YES-medium	0.5 % Yeast Extract 30 g/L glucose 250 mg/L histidine 250 mg/L leucine 250 mg/L adenine 250 mg/L uracil 250 mg/L lysine
YES-plates	As above plus: 20 g/L agar Supplemented with: 100 μ g/mL kanamycin when needed

2.8.2 Buffers and other solutions

Agarose gel solution, 0,8 %	0,8 % agarose
Binding Buffer	TBE buffer
DNA loading buffer (6 X)	20 mM sodium phosphate
	0.5 M EDTA, pH 8.0
	40 % (w/v) sucrose
	0,25 % bromphenol blue
EDTA, pH 8.0 (0,5 M)	146,12 g/L EDTA
	NaOH to pH 8.0
Electrophoresis buffer (10 X)	30,2 g/L Tris
	144 g/L glycine
	1 % (w/v) SDS
Elution Buffer	0.1 M citric acid
Equilibration buffer	6 M urea
	75 mM Tris-HCl, pH 8.8
	29.3 (v/v) % glycerol
	2 % (w/v) SDS
	0.002 % (w/v) bromphenol blue
HB buffer (2 X)	30 mM EGTA
	50 mM Hepes-KOH, pH 7.5
	120 mM β -glycerophosphate
	30 mM p-nitrophenylphosphate
	30 mM MgCl ₂
	2 mM DTT
	0.2 mM sodium vanadate
	KOH until EGTA is dissolved
LiAc/TE (10 X)	1 M lithium acetate
	1 X TE
	Acetic acid to pH 7.5
Na-acetate buffer, pH 5.2 (3 M)	408,24 g/L sodium acetate
	Glacial acetic acid to pH 5.2
PEG/LiAc/TE	40 % PEG 4000
	1 X LiAc/TE
Protein sample buffer (3 X)	0,1 M Tris-HCl, pH 6.8
	3.1 % (w/v) SDS
	25 % (v/v) glycerol
	300 mM DTT
	0,25 % bromphenol blue
Separation gel solution (10 %)	4 % (v/v) acrylamide/bis solution (37.5:1)
	0.125 M Tris-HCl, pH 6.8
	0.1 % (w/v) SDS
	0.025 (w/v) APS
	0.2 % (v/v) TEMED
Stacking gel solution (4 %)	10 % (v/v) acrylamide/bis solution (37.5:1)
	0.375 M Tris-HCl, pH 8.8
	0.2 % (w/v) SDS
	0.025 (w/v) APS
	0.2 % (v/v) TEMED

STOP buffer (2 X)	20 mM Tris-HCl, pH 8.0 150 mM NaCl 50 mM NaF 10 mM EDTA 1 mM NaN ₃
TBE (5 X)	54 g/L Tris 27.5 g/L boric acid 10 mM EDTA, pH 8.0
TBS-T	20 mM Tris-HCl, pH 7.5 8 g/L NaCl 0,05 % (v/v) Tween-20
TE (10 X)	12,11 g/L Tris 1,49 g/L EDTA NaOH to pH 7.5
Transfer buffer	5.8 g/L Tris 29 g/L glycine 0,1 % (w/v) SDS 20 % (v/v) methanol
Tris-HCl, pH 6.8 (0.5 M)	60,57 g/L Tris HCl to pH 6.8
Tris-HCl, pH 7.5 (1.0 M)	121.14 g/L Tris HCl to pH 7.5
Tris-HCl, pH 8.0 (1.0 M)	121.14 g/L Tris HCl to pH 8.0
Tris-HCl, pH 8.8 (1.5 M)	181.71 g/L Tris HCl to pH 8.8

3. Methods

3.1 DNA Methods

3.1.1 Polymerase chain reaction

Polymerase chain reaction, or PCR, is an automated and rapid method used to amplify a specific segment of single- or double-stranded DNA. This method consists of 20-30 cycles, and each cycle repeats three steps. In the first step, called denaturation, the DNA to be amplified is denatured by heat. In the second step, called annealing, oligonucleotide primers hybridize to opposite strands of DNA. In the third step, elongation, a DNA polymerase catalyzes, in the presence of excess dNTPs, the growth of a new strand between the primers in 5' to 3' direction. This sequence of events results in an exponential amplification of the product of interest.

In this work, PCR was used for cloning, analysis of transformants, and for sequencing. PCR programs are listed in Table 4.1 and 4.2. Primers are listed in Table 3.3.

General polymerase chain reactions

Polymerase chain reactions were generally done using AccuPrime Pfx DNA polymerase (Invitrogen), which possesses a proofreading 3' to 5' exonuclease activity to enhance fidelity. This polymerase is provided in an antibody-bound form, which is inactive. The antibody dissociates and thus the enzyme is activated upon heating to high temperature during the first denaturation step, which will automatically provide a "hot start" that increases specificity, sensitivity and yield, and enables us to assemble the reaction at room temperature.

5 μ L of AccuPrime Pfx Reaction mix (Invitrogen), 1,5 μ L of each primer (10 μ M stock), 0,5 μ L AccuPrime Pfx DNA polymerase (Invitrogen), 0,5 μ L template (10 pg

– 200 ng) and dH₂O were added to a total of 50 µL. The PCR reactions were run on the program “AccuPrime PCR” summarized in Table 4.1.

Table 4.1 AccuPrime PCR program

Step	Cycles	Time	Temperature
Initial denaturation	1	2 min	94 °C
Denaturation		15 s	94 °C
Annealing	35	30 s	50 °C
Elongation		2 min	68 °C
Final elongation	1	15 min	68 °C
Hold	---	---	4 °C

S. pombe colony PCR

This colony PCR method for *S. pombe* uses SDS to extract DNA from the cells. SDS would denature the polymerase used, so Triton X-100 is added which forms mixed micelles with SDS and thus “neutralizes” it. Triton X-100 has no adverse effects on the PCR reaction (Ken Sawin, personal communication).

A fresh colony was dispensed into 50 µL 0,25 % SDS in TE and the solution was boiled on a heating block for 5 minutes and centrifuged in a microcentrifuge for 1 min at 13 000 rpm. 1 µL of the supernatant was added to 4 µL of dH₂O and 5 µL 10 % Triton X-100. Then 1,25 µL of both forward and reverse primer (10 µM stock) and 12,5 µL High Fidelity PCR Master (Roche) were added to the mixture. The PCR reactions were run on the program “Colony PCR” (Table 4.2). Note the prolonged extension time.

Table 4.2 Colony PCR program

Step	Cycles	Time	Temperature
Initial denaturation	1	2 min	94 °C
Denaturation		15 s	94 °C
Annealing	35	30 s	50 °C
Elongation		5 min	68 °C
Final elongation	1	10 min	68 °C
Hold	---	---	4 °C

3.1.2 Cloning of PCR products into pET expression vectors

Directional cloning of blunt-end PCR products was done using the Champion pET101 Directional TOPO Expression Kit (Invitrogen). The pET101 expression vector have two features; TOPO cloning and directional cloning. TOPO cloning

makes use of the reaction carried out by Topoisomerase I from *Vaccinia* virus to clone PCR products into a pET101/D/TOPO vector (Section 3.2.2) without the use of a ligase. Correct orientation, or directional cloning, is ensured by adding the four bases CACC to the forward primer, which is engaged in base-pairing by the GTGG overhang in the cloning vector. Cloning was carried out according to the manufacturer's instructions.

3.1.3 Agarose gel electrophoresis

Agarose gel electrophoresis is a method for separating DNA fragments ranging in size from ~0,5 kb to ~25 kb and it can also be used to identify and purify a DNA fragment of interest. A higher percentage of the agarose gel, will separate smaller DNA fragments. The protocol can be divided into three stages:

- 1) Prepare and cast a gel with an agarose concentration of 0,8 % in 0,5X TBE buffer in an Owl B1 EasyCast Mini Gel System (Thermo Scientific).
- 2) Load the samples on gel in 1X DNA loading buffer, before running the gel in 0,5X TBE at constant voltage (120 V) for ~75 minutes. Samples were run together with a molecular weight standard to determine the size (and concentration) of the DNA sample. The DNA standard used was 2-Log DNA Ladder (see Appendix II).
- 3) After running, the gel was stained in 20 mL water with 10 μ L 1 mg/mL ethidium bromide to a final concentration of 0,5 μ g/mL for ~15 minutes. DNA was visualised under 365 nm UV light in ChemiGenius Bio Imaging System (Syngene Systems).

3.1.4 DNA purification

Purification of DNA from agarose gels was done using QIAquick Gel Extraction Kit (Qiagen). The manufacturer's instructions were followed with the following exception: To completely remove ethanol from the samples after the washing step,

the columns from QIAquick Gel Extraction Kit were placed in the 36°C incubator for 10 minutes before elution.

3.1.5 Isolation of plasmid from *E. coli*

Isolation of plasmids from *E. coli* was done using the QIAprep Spin Miniprep Kit (Qiagen). A single colony was used to inoculate 2 mL LB medium with the appropriate selective antibiotic. 1,5 mL of the culture was used to purify plasmid DNA and 0,5 mL of the culture stored at 4°C for further use. The manufacturer's instructions were followed, with the following exception; to completely remove ethanol from the samples, the columns had to be incubated in the 36°C incubator for 10 minutes before elution.

3.1.6 Restriction analysis

Digestion of DNA with restriction endonucleases, which recognise short DNA sequences and cleave double-stranded DNA at specific sites, was used to verify PCR products and plasmids. Several different restriction endonucleases, listed in Table 3.4, were used in the following reaction mixture: 1 µL DNA substrate (50-1000 ng), 1X reaction buffer, 5-20U restriction enzyme, 1X BSA when required, and MQ-H₂O to a final volume of 10 µL. The samples were incubated for 2-3 hours at 37°C and analyzed by agarose gel electrophoresis (section 4.1.3). When two different restriction enzymes were used together, the most compatible buffer, according to the manufacturer, was employed.

3.1.7 Quantification of DNA by spectrophotometry

Concentration of DNA samples were determined based on their absorbance of 260 nm UV light in GeneQuant Pro spectrophotometer (Amersham Bioscience) according to the supplied manual. DNA samples were diluted 1:100 in MQ-H₂O and added to 10 mm UV plastic cuvette (Biochrom).

3.1.8 DNA Sequencing

Sequencing of DNA was performed by GATC Biotech AG (Germany).

3.2 General methods in cell biology

3.2.1 Growth of *E. coli*

E. coli cultures were inoculated with a small number of cells, either from a toothpick or from a liquid culture, and grown in LB media with appropriate selective antibiotics for ~16 hours at 37°C with vigorous shaking.

3.2.2 Transformation of *E. coli*

Competent *E. coli* cells are able to take up external plasmid DNA, replicate the plasmid and keep the plasmids in the following cell divisions. This may be exploited to amplify plasmids for isolation from the cells or to express proteins encoded on the plasmid. Competent *E. coli* strains were transformed with plasmid DNA according to the manufacturer's instructions with the following exception: Selective plates were incubated at 36°C instead of the recommended 37°C.

3.2.3 Protein expression in *E. coli* using T4-based expression system

The Champion pET101 Directional TOPO Expression Kit (Invitrogen) was used for recombinant expression of proteins in *E. coli*. In the pET101/D/TOPO vector, expression of recombinant genes is controlled by endogenously transcribed bacteriophage T7 promoter with a *lac* operator sequence. The *lac* repressor, encoded in the bacterial genome by *lacI*, represses expression of T7 RNA polymerase without the inducer isopropyl β-D-thiogalactoside (IPTG). When T7 RNA polymerase is produced, it binds to the T7 promoter and transcribes the recombinant gene.

Pilot expression was done according to the manufacturer's manual, with different expression conditions summarized in Section 5.1.3 and 5.1.4.

3.2.4 Growth and handling of *S. pombe*

Storage and re-isolation of frozen cultures

For short time storage, *S. pombe* strains may be kept on YES-plates at 4°C for at least two months. The plates should be sealed with parafilm.

S. pombe can be stored at - 80°C and remain viable for several years. To make a glycerol stock for long time storage, cells were grown up in 0.8 mL YES medium for 1 day at 25°C and mixed with 0.8 mL 50 % glycerol in a cryotube. Cultures were placed at -80°C.

To re-isolate frozen cultures, a small amount of frozen culture was scraped off using a sterile spatula and patched onto YES-plate. The plate was then incubated at 25°C for 5 days. A patch of visible cells should be streaked out to single colonies on YES plate and incubated at 25°C for 3 days.

Liquid cultures

S. pombe cells in liquid culture grown at 25°C in YES medium have a generation time of 3 hours. Cells grown in Edinburgh minimal medium (EMM) or some mutant cell lines, have a prolonged generation time. Optical density at 595 nm (OD₅₉₅) may be used to measure cell density in a liquid culture. The generation time of an exponentially growing strain, the time it takes for a population to double its total mass, may be calculated by measuring OD₅₉₅ at different time points and plot this on a growth curve.

During physiological experiments, it's important to keep the cells in log phase, with an OD₅₉₅ of 0.1-0.3. OD₅₉₅ = 0,1 corresponds to 2×10^6 cells/mL. It is a linear relationship between OD₅₉₅ and cell number up to OD₅₉₅ = 0.4-0.5. EMM is normally used to grow the cells. To make a liquid culture:

Inoculate 10 mL YES medium with a loop of cells and incubate the pre-culture over night at 25°C without shaking to grow the cells up to early stationary phase. Use the pre-culture to inoculate a bigger culture. With a given generation time, one can use the following formula to determine the volume needed of the pre-culture to achieve a definite OD after n generations:

$$V_{pre} = \frac{V_{tot} \cdot OD_{desired}}{OD_{pre} \cdot 2^n},$$
 where V_{pre} is the volume of pre-culture to inoculate larger

culture, V_{tot} is to total volume of the culture, $OD_{desired}$ is to desired OD_{595} of the total culture, OD_{pre} is OD_{595} of the pre-culture and n is the number of expected generations.

Cells were grown for time = n number of generation * generation time in a shaking water bath at 25°C.

S. pombe cells seem to grow badly at low cell density, so OD_{595} of less than 0.05 should not be used to start a culture.

3.2.5 Transformation of *S. pombe*

The following protocol, a modified form of “Bählers method” (Bähler et al., 1998), were used to transform *S. pombe* cells with exogenic DNA:

- 1) Spin down 50 mL cell culture/transformation of $OD_{595} = 0.2$ at 3000 rpm for 4 min.
- 2) Wash once with equal volume of water. Spin down at 3000 rpm for 4 min.
- 3) Resuspend the cell pellet in 1 mL of water. Transfer to Eppendorf tube. Spin down at 10 000 rpm for 1 min.
- 4) Wash once with 1 mL of LiAc/TE. Spin down at 10 000 rpm for 1 min.
- 5) Resuspend the cell pellet in 100 μ L/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 for ~10 min.
- 7) Mix gently with 260 μL of 40 % PEG/LiAc/TE and incubate for 30-60 min at 25 $^{\circ}\text{C}$.
- 8) Add 43 μL of DMSO and heat shock the cell suspension for 5 min at 42 $^{\circ}\text{C}$ in water bath. Spin down at 10 000 rpm for 1 min.
- 9) Wash once with 1 mL YES. Spin down at 10 000 rpm for 1 min.
- 10) Resuspend in 0.5 mL water. Transfer the cell suspension to 40 mL YES and incubate at 25 $^{\circ}\text{C}$ in shaking water bath over night. Spin down at 3000 rpm for 4 min. Resuspend in 200 mL water. Plate out 200 μL onto YES+kanamycin plates or other appropriate selective plates.
- 11) Incubate the plates at 25 $^{\circ}\text{C}$ for 6 days.
- 12) Streak out single colonies onto selective plate.

3.2.6 Crossing and random spore analysis of *S. pombe* strains

Crossing and mating of various *S. pombe* strains with different mating type, h^{+} and h^{-} , is used to combine newly made mutations with different genetic backgrounds. To cross *S. pombe* strains:

- 1) Mix a loop of two strains of different mating types in 30 μL of MQ-H₂O and plate onto a MEA plate. Incubate for 2 days at 25 $^{\circ}\text{C}$. The cells will make zygotes and sporulate due to scarce nutrients in the MEA medium.
- 2) Inoculate a toothpick full of cells in 500 μL MQ-H₂O with 1 μL helicase. Incubate in room temperature for 1 day. This will kill vegetative cells.

- 3) Plate 30 μL of suspension out onto plates and streak out for single colonies. Incubate at 25 °C until visible colonies.

One can identify mating type by crossing the strain with an unknown mating type with known strains of both h^+ and h^- mating type. After two days of incubation at 25°C, the plates may be stained with iodine vapour for 1-5 minutes. A cross where spores have been made, where the unknown strain was crossed to the strain of the opposite mating type, will turn dark due to the starch in the spore walls.

3.2.7 Synchronization and UV irradiation of *S. pombe* cultures

Synchronization of the fission yeast culture was done using a *cdc10-M17* mutant as described in the Section 1.2.2. Cells were shifted up to 36°C for four hours to arrest the cells in G1 phase, then immediately UV irradiated (Figure 4.1).

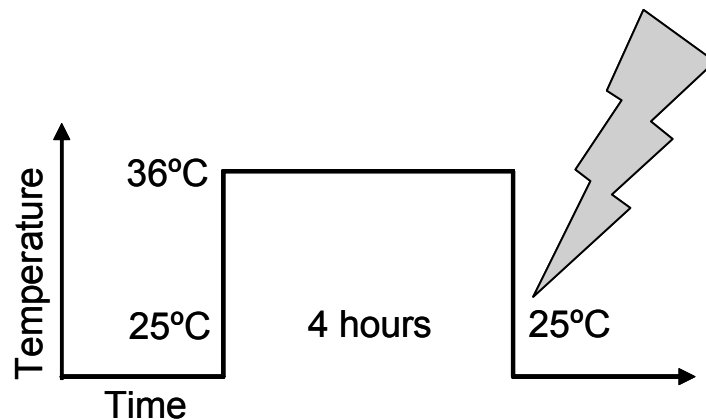


Figure 4.1 Synchronization and UV irradiation of fission yeast cells. The figure shows a graphical representation of the experimental setup.

UV irradiation of 254 nm (Sylvania Fluorescence Lamp, UVC light) was given to cells at $OD_{595} = 0,2$ in Petri dishes under constant stirring in a Klean hood. The UV dose was measured by a UVX radiometer (AH Diagnostic) in W/m^2 . To administrate the right dose (J/m^2), use the formula:

$$\text{Seconds of irradiation} = \frac{J / m^2}{W / m^2}.$$

Protein samples were taken shortly after the irradiation.

3.2.8 Flow cytometry

Flow cytometry is a method to analyse light scattering and fluorescence from single particles. With this method, one can count, study and sort microscopic particles suspended in a stream of fluid. This method is especially well-suited to study cells stained with fluorescence molecules. A flow cytometer may analyse thousands of cells each second and has a very high sensitivity. These qualities make flow cytometry an invaluable method to study cell cycle progression and make it possible to follow the yeast cell through the different phases of the cell cycle (Figure 4.2).

Taking cell samples for flow cytometry

2 mL cell suspension was spun down at 13 000 rpm for 2 minutes and the supernatant was removed. Cells were resuspended on vortexer with 1 mL ice cold 70 % EtOH. The samples were stored in a refrigerator.

Preparation of flow samples

The following protocol was used to prepare flow samples:

- 1) Spin down 500 μ L sample for 2 min at 13 000 rpm.
- 2) Discard supernatant and resuspend pellet in 1.0 mL 20 mM EDTA. Spin down for 2 min at 13 000 rpm.
- 3) Discard supernatant and resuspend pellet in 1.0 mL 20 mM EDTA. Spin down for 2 min at 13 000 rpm.
- 4) Discard supernatant and resuspend pellet in 500 μ L 20 mM EDTA containing 0.1 mg/mL Ribonuclease A.
- 5) Incubate over night at 36 °C.

- 6) Add 0.5 mL 20 mM EDTA containing 2 μ M Sytox Green, so that final concentration in the sample is 1.0 μ M Sytox Green.
- 7) Run the LSR II flow cytometer (BD Bioscience) as described in the core facility manual.

Interpretation of histograms

The transition from G1 to S phase may be measured by the flow cytometer as an increase in the amount of DNA. The amount of DNA is measured indirectly by the amount of Sytox Green binding to DNA (Figure 4.2). Fission yeast cells in log phase will have a peak at 2C DNA content.

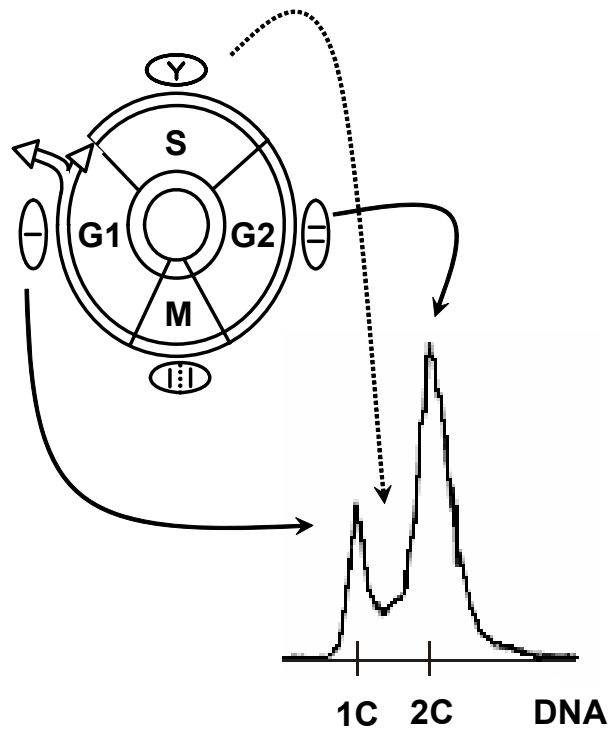


Figure 4.2 Interpretation of DNA histograms. The oval shapes represent cells, while the line inside represents 1C (one copy) DNA. The Y-shape in S-phase cells represents replicating DNA. The line drawn from the cell cycle figure indicate where in a DNA histogram different cells in different phases will show up. The first top at 1C will show cells that are in G1 phase of the cell cycle. The second top will at 2C will show cells in G2 phase. The area between the two tops will show cells in S phase. For more about the cell cycle, see Section 1.2.

3.3 Protein methods

3.3.1 Isolation of proteins from *S. pombe*

Preparation of cell samples for protein isolation:

- 1) Spin down 1×10^8 cells at 3000 rpm for 4 min.
- 2) Resuspend in 1 mL fresh, ice-cold STOP buffer, transfer to 2 mL Eppendorf tubes, and spin down at 13 000 rpm for 1 min.
- 3) Wash the cells in 1 mL STOP buffer. Keep the tubes cold. Spin down at 13 000 rpm for 1 min.
- 4) Remove the STOP buffer and snap-freeze the pellet in liquid nitrogen.
- 5) The cells can now be stored at $-80\text{ }^{\circ}\text{C}$.

Trichloroacetic acid (TCA) precipitation of total cell protein

Trichloroacetic acid (TCA) precipitation is one of the most widely used methods for precipitation of total protein in *S. pombe*. TCA precipitation done in *S. pombe* was based on Kelly's Super Method:

- 1) Add 300 μL of acid washed glass beads and 200 μL 20 % TCA.
- 2) Ribolyze the samples in the FastPrep FP120 ribolyser (Thermo Scientific) for 3 x 20 sec at speed 6.5. Cool down between each run.
- 3) Check cell breakage under the microscope.
- 4) Add 400 μL 5 % TCA to each sample.
- 5) Place on 1.5 mL Eppendorf tube at the bottom of a 15 mL Falcon tube. Make a hole in the lid for the samples and place on top of the 1.5 mL Eppendorf tube (lid against lid). Spin the tubes at 3000 rpm for 2 min. Remove the tubes. The tube at the bottom contains the sample.

- 6) Spin at 13 000 rpm for 5 min and remove ALL supernatant.
- 7) Resuspend the protein sample in 200 μ L 1 x protein sample buffer.
- 8) Boil the samples for 5 min.
- 9) Spin at 13 000 rpm for 3 min.
- 10) Run a SDS-PAGE or freeze the samples at -80°C .

HB buffer protein extraction

Some methods, such as immunoprecipitation (Section 4.3.6), are not compatible with TCA precipitation of proteins. For immunoprecipitation, total protein was extracted in HB buffer. The protocol is essential as described for TCA precipitation, except that 300 μ L of acid washed glass beads was added to the cell pellet together with 200 μ L HB buffer. After ribolysing the samples, 600 μ L of HB buffer was added to each sample. For a normal SDS-PAGE (Section 4.3.3), total protein extract in HB buffer are mixed with 2 x protein sample buffer and ran as described.

3.3.2 Quantification of proteins

Quantification of the protein concentration was done using BCA Protein Assay Kit (Thermo Scientific) according to the manufacturers' instruction. The BCA assay employs bicinchoninic acid to determine the total level of protein in a solution. This assay relies on a reaction where the peptide bonds in protein reduce Cu^{2+} ions from the cupric sulphate to Cu^{+} . Thereafter, two molecules of bicinchoninic acid chelates with each Cu^{+} , forming a purple-colored product that absorbs light at a wavelength of 562 nm. Since the reduced Cu is proportional to the amount of protein in the solution, the amount can be quantified using a spectrophotometer.

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

Electrophoresis is used to separate proteins according to their mass. However, migration in an electric field depends on the net charge of the proteins, which in turn depends on their sequence (the number of ionisable residues) and the pH. To avoid this problem, sodium dodecyl sulphate (SDS) is added in the protein sample buffer. This will cause the proteins to be covered with negative charge. On average, one SDS molecule binds strongly for every two amino acid residues and therefore all the proteins will then have the same charge-to-mass ratio as they do in the absence of SDS. This makes it possible to separate proteins based on their molecular mass by electrophoresis.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli et al. (1970), using a BioRad Gel Modular Mini-Protean II Electrophoresis System. Unless otherwise stated, a polyacrylamide concentration of 10 % was used.

- 1) Two glass plates were washed in water and 70 % ethanol and assembled in a cassette as described by the manufacturer.
- 2) The separation gel solution (Section 3.8.2) was prepared and poured into the gap between the two glass plates to a level about 1 cm below the top. The gel was overlaid with water to create an even surface of the gel.
- 3) After approximate 30 minutes of polymerisation, the water was removed from the gel.
- 4) The stacking gel solution (Section 3.8.2) was poured on top of the separation gel and the well forming comb was inserted between the glass plates.
- 5) The stacking gel was left for polymerisation for another 30 minutes.
- 6) The gel cassette was assembled with the electrode cassette and placed in the electrophoresis chamber. Electrophoresis buffer was filled to a level above the wells before the comb was removed.

- 7) The wells were washed with running buffer and protein samples were loaded to the wells using a small pipette tip.
- 8) The electrophoresis chamber was filled with electrode buffer and the lid was attached on the top of the chamber. The electrophoresis apparatus was connected to an electric power supply and the electrophoresis was carried out at room temperature at 180 V until the tracking dye reached the bottom of the gel.

3.3.4 Two-dimensional gel electrophoresis (2D-PAGE)

2D-PAGE separates protein according to their isoelectric point in the first dimension and by mass (SDS-PAGE) in the second dimension. This makes 2D-PAGE a powerful tool in separating each protein and their isoforms, making it easier to monitor differences in expression or modifications of most cellular proteins than conventional SDS-PAGE (Section 4.3.3). 2D-PAGE was performed as described by Angelika Görg (2004).

Sample preparation

Total protein extract from cell pellet was prepared as described below:

- 1) Add 200 μ L DeStreak Rehydration solution (GE Healthcare) to each pellet together with 200 μ L acid-washed glass beads (Sigma).
- 2) Break cells for 3 x 20 sec at max rpm (6.5) with 1 min rest between each cycle on ice in the FastPrep FP120 ribolyser (Thermo Scientific).
- 3) Remove glass beads as described in Section 4.3.1.
- 4) Centrifuge for 30 min at 4°C at 13 000 rpm.
- 5) Transfer supernatant to new tube.
- 6) Dilute 20 μ L sample in 320 μ L DeStreak Rehydration solution and add a final concentration of 0.5 % IPG Buffer pH 3.5-5.0.

Rehydration of immobilised pH-gradient strips and isoelectric focusing

Linear immobilised pH gradient (IPG) strip (Immobiline DryStrip, GE Healthcare) with a length of 18 cm and a pH interval between pH 4-5 were rehydrated using 340 μ L DeStreak Rehydration solution including the total protein sample (known as rehydration loading) in regular IPGphor Strip Holder. IPG-strips and sample were covered with Immobiline DryStrip Cover Fluid to minimize evaporation and urea precipitation. The strips were rehydrated for 10-20 hours at 20°C.

In the first dimension, the proteins are separated according to their pI. A proteins pI is determined by the number and identity of charged amino acids and by post-translationally modified amino acids. Isoelectric focusing (IEF) was performed with the Ettan IGPphor II Isoelectric Focusing System (GE Healthcare), connected to a PowerPac HV power supply (Bio-Rad). The IEF was carried out with the parameters given in Table 4.3. After the run, the IPG strips were stored in 10 mL plastic pipettes at -80°C.

Table 4.3 Parameters¹ for isoelectric focusing of pH 4-5 strips

Step	Step voltage mode	Voltage (V)	Time (h:min)	Volt-hours (kVh)
0	Rehydration	0	10:00	0,0
1	Step-and-hold	500	1:00	0,5
2	Gradient	1000	1:00	0,8
3	Gradient	8000	3:00	13,5
4	Step-and-hold	8000	5:30	44,0
Total			20:30	58,8

¹ 50 μ A/strip. Automatic cooling set at 20°C.

Equilibration of IPG-strips

The IPG strips were equilibrated twice, each time for 15 min in 10 mL equilibration buffer to saturate the IPG strips with the SDS-buffer required for second dimension separation. In the first step of the equilibration, 2,5 mg/mL DTT was added to preserve the fully reduced state of denatured proteins. In the second step, iodoacetamide at a concentration of 45 mg/mL were added to alkylate thiol-groups of the proteins. This prevents re-oxidation, which may cause vertical streaking during the running of the second dimension.

Second dimension: SDS-PAGE

The second dimension was performed using Protean II xi Electrophoresis system (Bio-Rad), essentially as described in Section 4.3.3. The gels were cast without stacking gel and wells, at an acrylamide concentration of 10 %. A small piece of Watman filter paper was soaked in 10 μ L DualColor Precision Plus Protein Standards (Bio-Rad) and placed at the left side of the gel. The equilibrated IPG gel strip were rinsed in running buffer and applied onto the SDS-PA gel. Care was taken to get close contact between the first and second dimension and the strip was sealed to the second dimension using 0.5 % agarose gel in running buffer with bromphenol blue. The Protean II xi system (Bio-Rad) was assembled as described by the manufacturer and connected to PowerPac HV power supply (Bio-Rad). Electrophoresis was performed over night as described in Table 4.4 without cooling. After electrophoresis, the gels were blotted onto PVDF membranes as described in Section 4.3.5.

Table 4.4 Parameters for running two PA-gels (2. dimension)

Step	Constant mA	Max voltage (V)	Max watt (W)	Time (h.min)
1	40	1000	250	00:15
2	14	1000	250	18:00
3	20	1000	250	01:00

3.3.5 Immunoblotting and immunodetection

Immunoblotting, also known as Western blotting, is a method to transfer proteins onto a porous membrane and thereafter probing this blot with antibodies. Antibody-antigen complexes are then detected using a labelled secondary anti-immunoglobulin antibody to identify specific proteins. Immunoblotting is a sensitive method and suitable for detection of low abundance proteins. Antibodies used in immunodetection in this work are listed in Table 3.5.

Immunoblotting

Proteins were separated using either SDS-PAGE (Section 4.3.3) or 2D-PAGE (Section 4.3.4) and transferred to a 0.45 μm Immobilon-P PVDF membrane (Millipore) using a Trans-Blot SD semi-dry cell (Bio-Rad) as described by the manufacturer. The following protocol was followed:

- 1) The gel were put in transfer buffer for 5 minutes
- 2) PVDF membranes were cut and put in 100 % MeOH and then soaked in MQ-H₂O before transferred to transfer buffer for 5 minutes.
- 3) Wet two Watman filter papers in Transfer buffer and put on the blot apparatus.
- 4) Put the membrane on top of the filter papers. Remember to mark the top of the membrane with a pencil or cut one corner.
- 5) Put the gel on top of the membrane.
- 6) Wet two Watman filter papers in Transfer buffer and put on top of the gel.
- 7) Remove the air so that no air-bubbles remain.
- 8) Allow the blotting to occur for 45 minutes at 15 V.

Monitoring the transfer of protein

The transfer of proteins from the gel to the membrane was monitored quantitatively by staining both the gel and membrane. The gel was stained with Coomassie Brilliant Blue as described below. The PVDF membrane was stained with Ponceau S. A good transfer was characterized by a weak and even staining of the gel and a strong and even staining of the membrane.

Immunodetection

The protocol used is described below.

- 1) Wash the membrane in 5 mL TBS-T for 5 min at RT.

- 2) Block the membrane in 5 mL 5 % membrane blocking agent/TBS-T for 1 hour at RT.
- 3) Incubate the membrane overnight at 4 °C with 5 mL primary antibody of appropriate concentration (as in the manufacturer's instructions) in TBS-T.
- 4) Rinse once, and then wash 1 x 10 min and 2 x 5 min with 5 mL TBS-T.
- 5) Incubate the membrane for 1 hour at RT with 5 mL secondary antibody at a appropriate concentration, as indicated by the manufacturer, in TBS-T.
- 6) Rinse once, and then wash 1 x 10 min and 2 x 5 min with 5 mL TBS-T.
- 7) Remove residual liquid from the membrane using a paper towel.
- 8) Cover the membrane with ECF substrate. ECF is a fluorescent substrate for alkaline phosphatase-based detection.
- 9) Stop the reaction when you see bands, and don't let it run for more than 5 min.
- 10) Dry the membrane in the dark.
- 11) Take picture. Use the transilluminator with EtBr filter.
- 12) Save the membrane covered in filter paper and silver paper in the fridge.

3.3.6 Immunoprecipitation of proteins

Immunoprecipitation is a technique in which a protein of interest is isolated by binding to a specific antibody attached to a sedimentable matrix. In this case, the sedimentable matrix is magnetic beads (Dynabeads, Invitrogen) coated with Protein G. Protein G binds with high affinity to IgG. Antibodies used in immunoprecipitation are listed in Table 3.5.

Washing Procedure

The washing is facilitated by the use of a magnet (DynaL MPC). Dynabeads are resuspended in an adjusted volume so that sample and Dynabeads volumes together are the same as the bead-volume originally pipetted from the vial.

- 1) Resuspend the Dynabeads Protein G, thoroughly in the vial (e.g. by vortexing 1-2 min or rotating on a roller) to obtain a homogeneous suspension.
- 2) Transfer 20 μL /500 mg IP Dynabeads Protein G to a test tube at room temperature.
- 3) Place the test tube on the magnet for one minute and pipette off the supernatant.
- 4) Remove the test tube from the magnet and add 0.5 mL 0.1 M Na-acetate buffer, pH 5.2.
- 5) Repeat step 3, 4 and 3.

Ig Capture Procedure

- 1) Resuspend the washed Dynabeads in 18/IP 0.1 M Na-acetate buffer, pH 5.2.
- 2) Add 2 μL antibody/IP to the solution containing Dynabeads. Take out a sample.
- 3) Incubate with slow tilt rotation mixing for 30 min at room temperature. Take out a sample of the supernatant.
- 4) Place the test tube on the magnet for 2 minutes and pipette of the supernatant.
- 5) Remove the test tube from the magnet and add 0.1 M NA-acetate buffer, pH 5.2.
- 6) Repeat step 4, 5, 4, 5 and 4. Take out a sample.
- 7) Resuspend in 20 μL /IP of 0.1 M Na-acetate buffer, pH 5.2.

Binding of antigen and target protein elution

- 1) Add 1 mg sample protein to 40 μL antibody-coated Dynabeads Protein G.

- 2) Incubate with gentle rotation in cold room for a 1 h.
- 3) Place the tube on the magnet for 2 min to collect the Dynabeads-Ig complex at the tube wall. Pipette off and save supernatant.
- 4) Rinse the beads once at room temperature in HB buffer and change buffer by the use of a magnet.
- 5) Wash the beads 3 x 10 min in cold room with rotation. Change the buffer by the use of a magnet. Save the buffer from the last wash.
- 6) Boil in 50 μ L SDS sample buffer for 5 min at 100 °C.

3.3.7 Affinity purification of IgG

Affinity purification of IgG uses the same basic principles as immunoprecipitation of proteins, but the goal is not to isolate a protein of interest, but the IgG itself. Protein A, which binds IgG from rabbit with high affinity, is coated to a matrix inside a HiTrap Protein A column (GE Healthcare).

Sample preparation

The sample should be adjusted to the composition of the binding buffer. This was done by diluting the sample with binding buffer (diluted 1:3 sample/binding buffer). The sample was then filtered and centrifuged immediately before it was applied to the column.

Purification

- 1) Prepare collection tubes by adding 60-200 μ L of 1 M Tris-HCl pH 8.0 per mL of fraction collected.
- 2) Fill the syringe with binding buffer; sodium phosphate pH 7.0. Remove stopper and connect the column to the syringe “drop to drop” to avoid introducing air into the column.

- 3) Remove the snap-off end at the column outlet.
- 4) Wash the column with 10 column volumes of binding buffer at 1 mL/min for 1 mL column.
- 5) Apply the sample, using a syringe.
- 6) Wash with 5-10 column volumes of binding buffer.
- 7) Elute with 2-5 column volumes of elution buffer; citric acid pH 3 – 6.

3.3.8 Staining of proteins

Coomassie staining of polyacrylamide-gels

Coomassie Brilliant Blue (R-250) is an organic dye that binds to almost all proteins, but has a lower detection limit of 8-29 ng protein/band in an SDS-PA gel. Staining was done using Bio-Safe Coomassie G-250 Stain (Bio-Rad). SDS-PA gels were washed 3 x 5 min in MQ-H₂O. The water was removed and enough stain was poured over to cover the gels and gently shaken for 1 hour, then the stain was removed and the gels were rinsed in water for at least 30 min.

Ponceau S staining of PVDF membranes

Following the transfer of proteins from gel to membrane, the membrane was placed in Ponceau S for 5 minute, destained for 2 minute in water, and visually inspected. Before immunodetection, the stained membrane was completely destained by soaking in water for an additional 10 minutes.

3.3.9 Mass spectroscopy

Mass spectroscopy (MS) is an analytical method to accurately determine the molecular mass of almost any ionisable chemical compound by separating molecular ions according to their mass to charge ratio (m/z) (reviewed in Aebersold and Mann, 2003). The sensitivity of mass spectroscopy is so high that even post-translational

modifications, like phosphorylation, might be detected (reviewed in Mann and Jensen, 2003).

Collection of proteins from SDS-PA gels

All equipment used was cleaned with 50 % ACN/ 0,1 % TFA before use. The SDS-PA gel was stained with Coomassie as described to visualise the proteins. The protein bands of interests were cut out using a sharp scalpel. The gel pieces were stored in 1.5 mL eppendorf tubes. Gloves were worn to avoid contamination of the samples with keratin.

In-gel trypsination

- 1) Cut SDS-PA gel pieces into small cubes (1 mm²). Wash 3 x 1 mL ddH₂O in 1 hour with shaking..
- 2) Add 50 µL 100 % acetonitrile (AcN) for 10 min and remove supernatant.
- 3) Vacuum dry for 15 min (5' RC, volatile, low temp, SpeedVac).
- 4) Add 50 µL 10 mM dithiothreitol (DTT) in 100 mM NH₄HCO₃ and incubate for 1 hour at 56°C. This will reduce cystein-disulfide bridges.
- 5) Cool down to room temperature, remove supernatant and add same volume iodoacetamide (55 mM in 100 mM NH₄HCO₃) and place in the dark for 45 min in RT (alkylation of free sulfhydryl groups to carboxyamidomethylcystein).
- 6) Remove supernatant and wash the gel pieces in 100 µL 100 mM NH₄HCO₃/ ammonium bicarbonate (ABC) for 10 min.
- 7) Dehydrate with 50 µL 100 % AcN for 5 min.
- 8) Repeat steps 6. and 7.
- 9) Vacuum dry for 15 min (5' RC, volatile, low temp, SpeedVac).

- 10) Inactive trypsin solution (Promega; 100 ng/ μ L dissolved in acetic acid pH ~4,5) from -70°C should be added to at least 2:1 (v/v) 25 mM NH_4HCO_3 (pH 7.8). Defrost in RT for activation. The ratio between trypsin:protein should be from 1:20 to 1:100 (w/w). Final amount of trypsin is 50 ng, which is enough for 1+ μ g of protein.
- 11) Rehydrate with trypsin solution, incubate for 30 min at 4 °C. The trypsin solution should be used as an external control of the MS.
- 12) Add 0.1 % (w/w) octylglucoside in ABC and 10 % ACN (final).
- 13) Incubate for 37°C over night.
- 14) Add 20 μ L 30 % ACN in 0.1 % TFA for 45 min to extract the peptides. Spin down and transmit the supernatant to a new tube. Redo with 50 % ACN in 0.1 % TFA and 70 % ACN in 0.1 % TFA.
- 15) Vacuum dry for 30 min (5' RC, volatile, low temp, SpeedVac) or until all the solvent has evaporated. This can now be stored at - 20°C for MS analysis.
- 16) For ESI/LC/MS: Solve in 5 μ L 5 % ACN in 0.1 % FA.

LC-MS/MS and data analysis

The LC-MS/MS was carried out on a XCT Ultra Ion Trap (Agilent) connected to a Nano-Chip LC 1100 (Agilent). The spectra analysis was done using Data Analysis and Spectrum Mill (Agilent).

Mass spectrometry and data analysis was carried out by Anders Øverbye, Section for Proteomics & Mammalian Cell Biology, Dept. of Cell Biology, Institute for Cancer Research.

3.4 Bioinformatical prediction of phosphorylation sites

Prediction of phosphorylated amino acids was done using NetPhosYeast (Appendix 1).

3.5 Production of antibodies against a peptides

Production of antibodies against a peptide was performed by MedProbe.

4. Results

To characterise and study a protein of interest (Section 5.4 and 5.5) one must be able to detect and isolate the protein. A commonly used approach to this end is immunochemistry. Immunochemistry utilises antibodies for detection (immunoblotting) and isolation (immunoprecipitation). Antibodies can be made against the whole protein (Section 5.1) or a short peptide derived from the protein (Section 5.3). One may also tag the gene of interest with an antigenic determinant, or epitope, which may be detected by commercially available antibodies (Section 5.2).

4.1 Expression of recombinant eIF2 α in *Escherichia coli*

To use a recombinant protein to immunise an animal, and thereby make antibodies, one must first overexpress the protein in an appropriate organism. *E. coli* was decided to be used to express the protein of interest, which is an organism often used to express recombinant proteins. The expression system used is based on the bacteriophage T7 RNA polymerase (Studier et al., 1990). The RNA polymerase of bacteriophage T7 uses both promoters and termination signals rarely encountered in DNA unrelated to T7 DNA which, in principle, makes the T7 expression system completely selective (Studier et al., 1990). This, in combination with fast elongation and earlier experience with this expression system in our lab, makes this system attractive to use.

4.1.1 Expression of full-length *tif211* from *S. pombe*

eIF2 α is encoded by the *tif211* gene in fission yeast (Aslett and Wood, 2006). The first step in the production of recombinant eIF2 α proteins was to amplify this gene by polymerase chain reaction (Section 4.1.1). A primer pair was chosen to amplify the full-length *tif211* gene (Figure 5.2). A cDNA library from fission yeast was used as template, because the *tif211* gene contains two introns, which *E. coli* can't splice

out. A cDNA library is a collection of cloned complementary DNA (cDNA) fragments, which is produced from transcribed and spliced mRNA and therefore lacks the introns. The PCR product was cloned into a pET101/D-TOPO vector, making the pET101/D/tif211 expression plasmid. The pET101/D-TOPO expression vector allows expression of exogenous proteins under the control of the T7 promoter in *E. coli*. Plasmids were isolated (Section 4.1.5) from bacterial cultures made from colonies growing on selective plates. Restriction analysis (Section 4.1.6) was carried out to verify correct insertion and orientation. In addition, the plasmid was sequenced to verify that the gene sequence was correct (data not shown).

A BL21 Star (DE3) *E. coli* cell carries the T7 DNA polymerase gene under control of the *lacUV5* promoter. The BL21 strain was transformed with the pET101/D/tif211 expression plasmid (Section 3.2.2). The transformants were grown for one hour at 37°C and used to inoculate LB-medium. This pre-culture was grown overnight at 37°C with shaking and inoculated in LB-medium the following day. When this main culture reached an $OD_{600} \sim 0,4$ the culture was split and induced with IPTG at different concentrations varying from 0,1 – 1,0 mM. Addition of IPTG, isopropyl β -D-thiogalactoside allows expression of T7 DNA polymerase from the *lacUV5* promoter, which in turn allows expression of the protein of interest under the control of T7 promoter on the expression plasmid. Samples were taken after three hours and analyzed using SDS-PAGE (Section 4.3.3). The plasmid pET101/D/lacZ-V5-6xHis was used as a positive control for the induction. Addition of IPTG to cells containing the expression control plasmid, will express lacZ-V5-6xHis protein. Induction of a prominent band after induction of IPTG was observed (Figure 5.1A). This suggests that transformation was successful, and that growth and induction conditions were appropriate. The expected size of Tif211 is 34,5 kDa. No prominent band of this size was observed in any of the samples (Figure 5.1B).

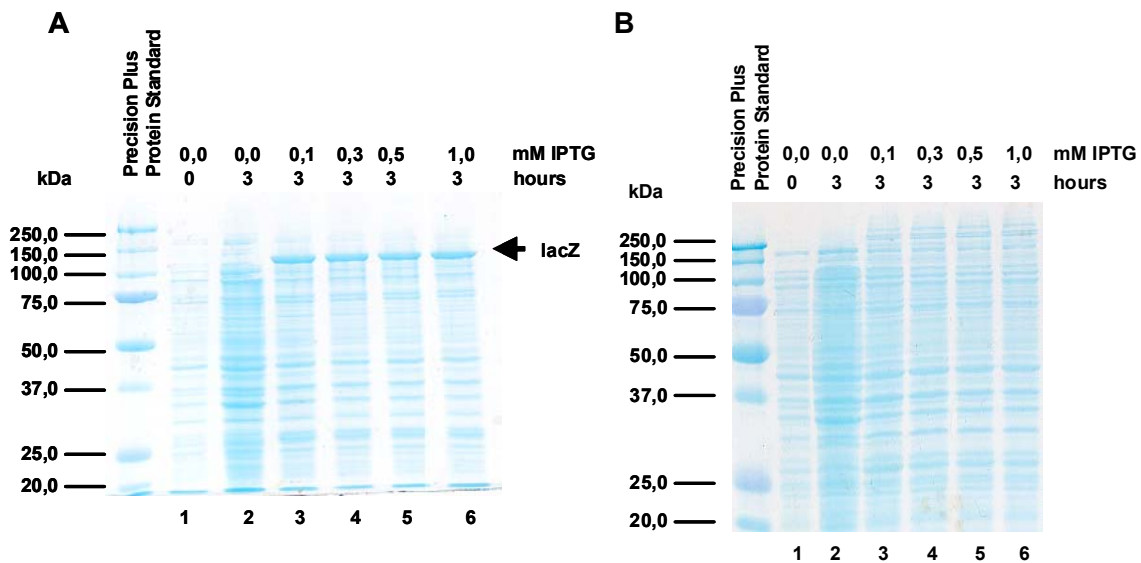


Figure 5.1 Expression of *tif211* and *lacZ* in *E. coli*. *E. coli* cultures containing either (A) pET101/D/*lacZ*-V5-6xHis or (B) pET101/D/*tif211* expression plasmid were induced with IPTG as described in the text. Samples were taken and separated on a 12 % SDS-PA gel at 190 V for ~1 hour and stained with Coomassie Blue. The big arrowhead indicates the position of LacZ, expressed after induction with IPTG.

4.1.2 Expression of truncated *tif211*

eIF2 α from fission yeast contains a S1 motif, which also is present in several *E. coli* proteins. The S1 motif is an RNA-binding motif found in proteins involved in initiation of translation and mRNA turnover (Bycroft et al., 1997). Therefore it is conceivable that the S1 motif present on Tif211 might interfere with the translational apparatus of *E. coli*. In order to avoid this potential problem, an expression plasmid was constructed to express a truncated Tif211 that lacks the S1 motif.

Primers were designed to amplify a product which lacked the S1 motif, removing the 375 first base pairs of the *tif211* gene, starting at an AUG codon. The primers were designed so that the truncated form of *tif211*, hereafter called *tif211B*, would be expressed both with and without a V5-6xHis tag at the C-terminal end of the protein (Figure 5.2).

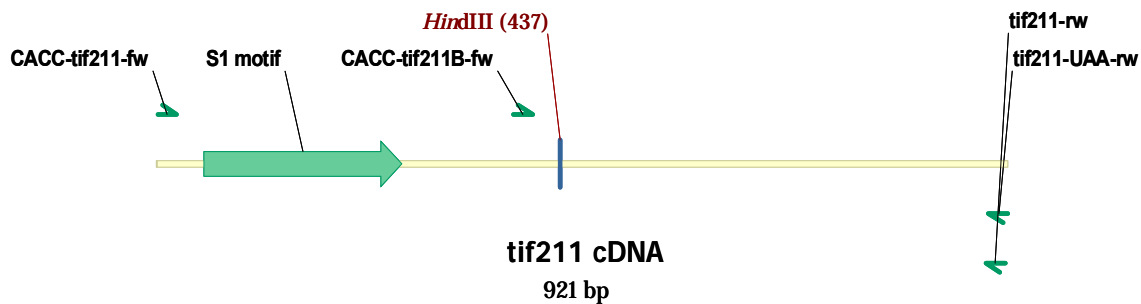


Figure 5.2 Primers used for amplification of *tif211* and *tif211B*. Primer sequences are given in Table 3.3. This figure was drawn in Vector NTI (Invitrogen).

The making of the expression plasmids pET101/D/*tif211B* and pET101/D/*tif211B*-V5-6xHis and the induction of protein expression were performed as described above, using the same positive control. However, the expression of neither *tif211B* nor *tif211B*-V5-6xHis was observed (data not shown).

4.1.3 Alternative strategies to overcome the expression problem

Highly toxic genes interfere with the physiology of *E. coli* during the growth phase (Saïda, 2007). Even though the pET101/D/TOPO vector is designed to reduce the low basal expression of T7 RNA polymerase and thereby the heterologous protein, a highly toxic heterologous gene may lead to plasmid instability or cell death. To solve this potential problem, 1 % glucose was added to the LB medium to further reduce the basal expression of T7 and thereby that of the toxic protein. The recombinant proteins were still not expressed after IPTG induction (data not shown). It is possible that both Tif211 and Tif211B proteins expressed in *E. coli* interfere with the bacterial translational apparatus or other indispensable parts of *E. coli*.

The expression problems were therefore thought to arise only after substantial quantities of the toxic protein had accumulated in the cells, probably minutes after gene expression was induced. Growth rates were measured before and after induction with IPTG and were found to be markedly reduced in the cells containing pET101/D/*tif211* or pET101/D/*tif211B* expression plasmid compared to the positive expression control plasmid pET101/D/*lacZ*-V5-6xHis after induction. There was also

no increase in the amount of total proteins content after three hours of induction in cells containing pET101/D/tif211 or pET101/D/tif211B expression plasmid.

The toxicity of the expression plasmids may impose selective pressure on the cells to lose or rearrange the plasmid. To examine if plasmid instability or cell death was the cause of the problem, I induced protein expression as described above and did a miniprep (Section 4.1.5) to re-isolate the expression plasmids. Miniprep, and the following restriction analysis, showed that the cells most likely still contained the original expression plasmids, so I concluded that plasmid instability or rearrangement of the plasmid in the growth and/or induction phase was not the problem.

There are several methods available to improve the expression of toxic genes. Expression in conditions which decrease the translation rate of recombinant protein may give the cells more metabolic control over the toxic protein. The toxic protein may even be competed-out and prevented from binding to its targets (Saïda, 2007). To express *tif211* and *tif211B*, several different expression conditions (proposed in Saïda, 2007) were attempted;

- 1) induction with a low IPTG concentration (10 μ M),
- 2) samples taken at different time points (1 – 4 hours), or
- 3) induction at 22°C for 3 and 22 hours.

Even induction of protein in stationary phase were tried (as described in Ou et al., 2004). Still, no production of Tif211, Tif211B or Tif211B-V5-6xHis was observed (data not shown).

4.1.4 Codon usage

Codon usage is probably the single most important factor in recombinant gene expression in prokaryotes (reviewed in Gustafsson et al., 2004). High-level expression of recombinant proteins from other organisms can deplete one organism's

pool of rare tRNAs and stall translation. As shown in Table 5.1, several rare codons are used in the *tif211* gene. This may hamper, or even stall, translation in *E. coli*.

Table 5.1 Rare codons in *tif211*^{1,2}

Amino acid	Rare codon ³	Times used	Rescued by BL21-CodonPlus-RIPL
Arginine (R)	AGA	2	Yes
Glycine (G)	GGA	4	No
Leucine (L)	CTA	1	Yes
	CTC	3	No
	TTA	4	No
	CTT	7	No
	TTG	11	No
Proline (P)	CCC	3	Yes
Serine (S)	AGT	2	No
	TCG	2	No
	TCA	3	No
Threonine (T)	ACA	4	No

1: Found using *E. coli* Codon Usage Analysis 2.0 by Morris Maduro (Appendix I)

2: Graphical representation can be found in Appendix III.

3: Rare codons are defined having less than 10 % usage in *E. coli*.

BL21-CodonPlus-RIPL strain from Stratagene contains plasmids with extra copies of the *argU*, *ileY*, *proL*, and *leuW* genes. These genes encode tRNAs that recognise some of the rare codons encountered in the *tif211* gene (see Table 5.1). The expression plasmids pET101/D/*tif211*, pET101/D/*tif211B*, pET101/D/*tif211B-V5-6xHis*, and pET101/D/*lacZ-V5-6xHis* were transformed into this strain and induction of protein expression was carried out as described above. However, no expression of Tif211, Tif211B or Tif211B-V5-6xHis was observed (data not shown).

The failure to express recombinant Tif211 in *E. coli* may have several reasons, most likely a toxic interference between the recombinant protein and important processes in *E. coli*. Another reason might be that there still is a high frequency of rarely used codons in the target sequence, despite the attempt to express Tif211 in a CodonPlus strain.

Recombinant *SUI2*, the gene coding for eIF2 α in budding yeast, has been successfully expressed in *E. coli*. To achieve this, the distal *SUI2*p coding region from amino acid position 76 to 304 was ligated in-frame with the C-terminal end of the *E. coli* *trpE* codon region (Cigan et al., 1983).

Expression of the human analogue of eIF2 α has also been successful in the BL21 Star (DE3) *E. coli* strain (Invitrogen); however the wild-type eIF2 α had very low expression levels (Ito et al., 2004). To counteract this low expression, they optimised the expression in several steps. They synthesised an artificial gene consisting of *E. coli* major codons for all amino acid residues to solve the codon bias problem, while at the same time removing hydrophobic amino acid residues which were located on the protein surface. In addition, both N- and C-terminal trimming was done to improve the expression. This led to an expression ratio at least three times higher than for the wild-type protein (Ito and Wagner, 2004).

The next logical step is to either switch expression vector or expression system. One might believe that Tif211 should be possible to express in greater amount in for example budding yeast or fission yeast. However, due to the time limit imposed, the strategy of expressing recombinant Tif211 in *E. coli* was abandoned.

4.2 Tagging of eIF2 α in *S. pombe*

Having failed to purify the recombinant Tif211 protein, MedProbe was engaged to make antibodies against a small peptide derived from *tif211* (Section 5.3). In the meantime, epitope tagging of the Tif211 protein was carried out. There are several advantages with epitope tagging of Tif211 in fission yeast. First, a tagged Tif211 protein may be used to verify a functional antibody against the protein, since the tagged version will be bigger than the unmodified protein (discussed in Section 5.3). Second, the epitope tag may be used directly for isolation and characterisation of the Tif211 protein with a commercially available antibody against the epitope tag (discussed in Section 5.4 and 5.5). However, a major disadvantage of this approach is that the tag may interfere with the structure or activity of the protein or its interacting partners, and thus the tagged protein may not function as the wild type protein.

4.2.1 PCR-based gene targeting in fission yeast

A modified form of a PCR-based gene targeting method (Bähler et al., 1998) was employed for C-terminal tagging of *tif211* with two different epitope tags; one tag containing three copies of HA, named 3HA, and another tag containing thirteen copies of Myc, named 13Myc (Figure 5.3A). The method is based on the one-step gene replacement approach, in which the cells are transformed with a linear piece of DNA carrying the desired construct, a selectable marker and ends homologous to the target sequence. The linear DNA integrates through homologous recombination. In the PCR-based gene targeting method the transforming DNA is generated by PCR. Several different plasmids have been developed for molecular studies in fission yeast, including vectors for expression, epitope tagging, and integration (reviewed by Siam et al., 2004). A vector containing the epitope tags 3HA and 13Myc and a kanamycin resistance selection marker (kanMX6) was used as a template (Section 3.2.2) with primers that anneal to the vector and also carry 80 nucleotides that are designed to target integration of the amplified tag-kanMX6 cassette to the *tif211* locus. The forward primer includes a sequence homologous to the extreme 3'-end of the *tif211* gene, while the reverse primer includes a sequence homologous to a region downstream of the *tif211* gene. The primers were designed using the Pombe PCR Primer Program for C-terminal tagging (Appendix I) and is listed in Table 3.3. The PCR products were separated and isolated from an agarose gel (Figure 5.3B) using QIAquick Gel Extraction Kit as described in Section 4.1.4 (Figure 5.3C).

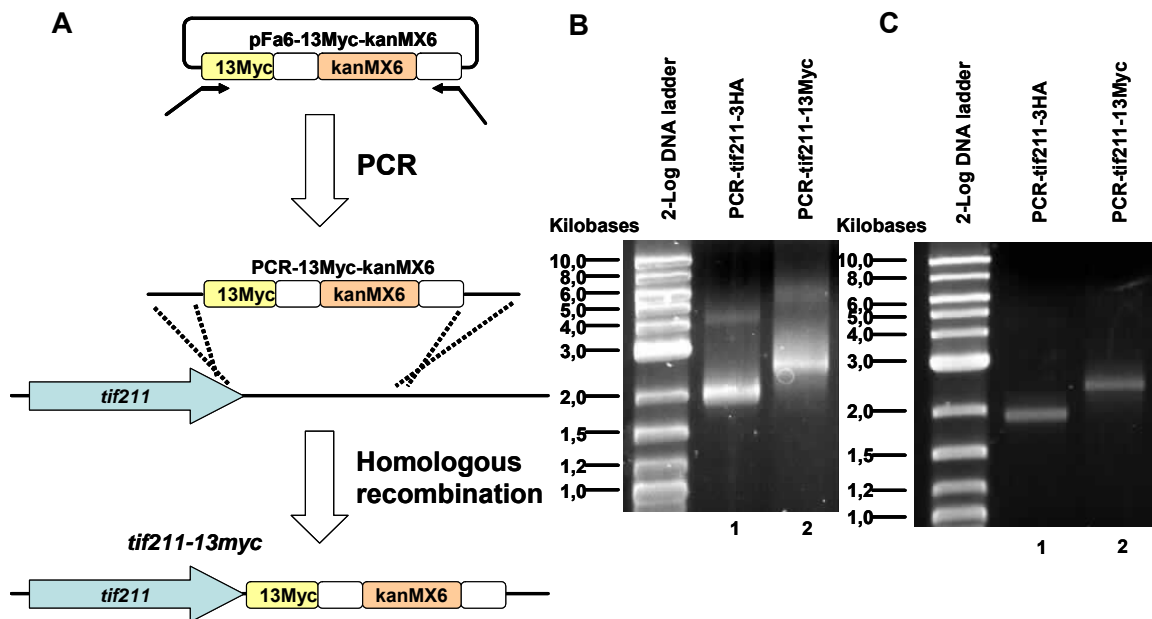


Figure 5.3 PCR-based gene targeting in fission yeast. (A) The pFa6-13Myc-kanMX6 vector carries an epitope tag and the kanMX6 cassette (Bähler et al., 1998). This region was amplified by PCR as described in Section 4.1.1, using 100 bp primers with an 80 bp long homologous region to the extreme C-terminal end of *tif211* and to a region downstream of *tif211*. The amplified product was then transformed into fission yeast and expected to integrate into the correct place in the genome by homologous recombination. The resulting strain carries one copy of the *tif211* gene tagged with an epitope tag and with an adjacent kanamycin resistance marker, at the *tif211* locus. (B) The PCR products were separated on a 0,8 % agarose gel at 120 V for 1 hour and stained with ethidium bromide as described in Section 4.1.3. (C) The PCR products were cut from the gel and purified using QIAquick Gel Extraction Kit as described in Section 4.1.4. The expected size for the PCR products were 2.0 kbp for the PCR-tif211-3HA and 3.0 kbp for the PCR-tif211-13Myc.

4.2.2 Transformation of *S. pombe*

The concentration of the purified PCR products were quantified using spectrophotometry (Section 4.1.7), and 300 ng DNA was used to transform the fission yeast cells directly using a modified form of Bähler's transformation method (Section 4.2.5). A plasmid containing the kanMX6 cassette and an ARS, a Kan2 plasmid, was used as a positive control for transformation and selection. Gene replacement utilizes the cells' own homologous recombination machinery, a mechanism to repair double-stranded breaks in DNA, to integrate the exogenic DNA at the *tif211* locus. However, an alternative mechanism, non-homologous end joining, competes with homologous recombination. Non-homologous end joining may insert the epitope tag and the selective marker at the wrong place. To further enhance

homologous recombination, and thereby increase the probability of integration, the transformation was performed in a *pku70Δ* deletion background. Pku70 is essential in non-homologous end joining (Baumann and Cech, 2000).

The expected transformation efficiency is 10^6 / μg DNA (Okazaki et al., 1990), whereas transformation efficiency is defined as number of colonies / μg DNA. The positive transformation control with the Kan2 plasmid gave an transformation efficiency of $3,0 \times 10^4$ colonies/ μg DNA. For unknown reasons, our lab is known to have lower transformation efficiencies than expected. Transformation with PCR-tif211-3HA and PCR-tif211-13Myc gave only one colony each on YEG plates with kanamycin. However, because these pieces are linear we expect fewer transformants.

4.2.3 Testing the transformants

The colonies transformed with PCR-tif211-3HA and PCR-tif211-13Myc were picked and re-streaked onto YEG+kanamycin plates to confirm kanamycin resistance. Colony PCR (Section 4.1.1) was performed to test the transformants for integration at the correct place. Two primer sets were employed. One primer set was made to amplify a 653 bp region from inside *tif211* to upstream of *tif211*. When a correct insertion tagged *tif211* with either 3HA or 13Myc, this region should expand to 2449 bp and 2898 bp, respectively. The other primer set was made to amplify a region from inside *tif211* to inside the *kanR* gene. No product is expected using a wild type template, while a band at 1517 bp and 1949 bp, is expected in the 3HA-tagged and 13Myc-tagged strain, respectively. The PCR products were separated on a 0.8 % agarose gel and compared against the untagged parent strain (strain 1311). Both the 1311 strain and the strain transformed with 3HA showed bands as expected for wild type cells, while the strain tagged with 13Myc showed a pattern as expected for a correctly myc-tagged *tif211* (Figure 5.4). The new strain carrying *tif211-myc:kanR* was called 1471.

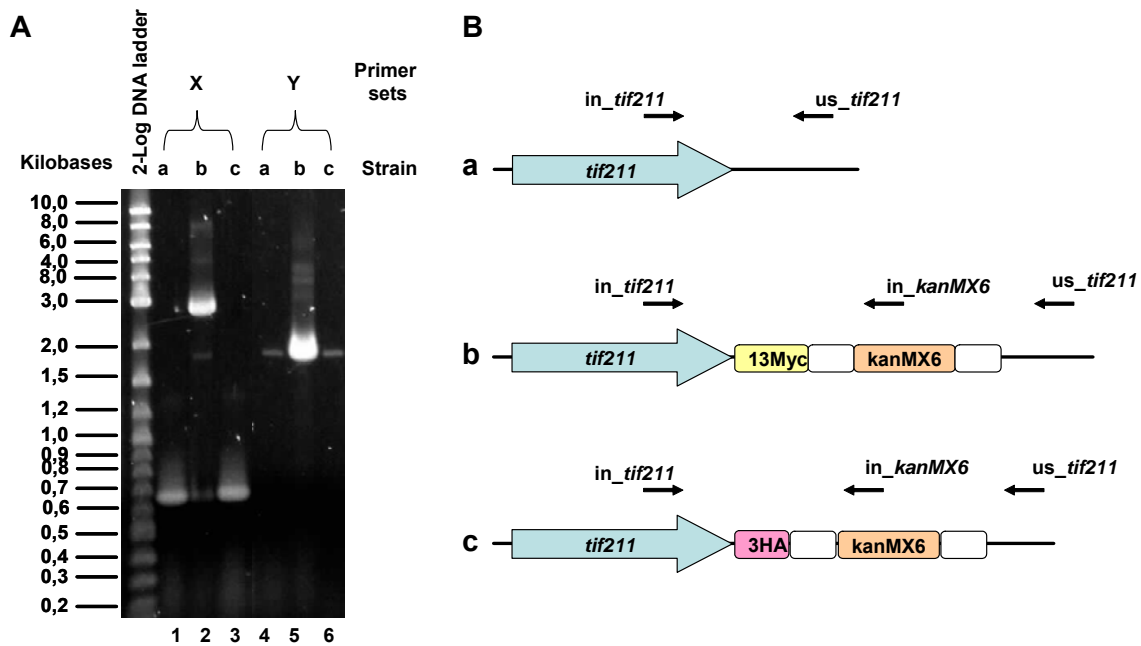


Figure 5.4 Testing transformants with PCR. (A) A picture of an agarose gel showing the products after colony PCR to verify correct integration. Two primer pairs were used: (X) One pair to amplify inside (*in_tif211*) and upstream (*us_tif211*) of *tif211*. (Y) One pair to amplify between *tif211* (*in_tif211*) and *kanMX6* (*in_kanMX6*). The parent strain, strain a, and strain c show a band with primer pair X as expected for wild type cells (653 bp). Strain b, the *tif211-13myc* tagged strain, show the expected band for primer pair X (2898 bp) and Y (1949 bp). (B) Graphical representations of desirable strains and the primers which might be used to verify correct integration.

The *tif211:13myc* construct was further verified by SDS-PAGE and immunoblotting (Section 4.3.5) to confirm the presence of a Myc-tag on the Tif211 protein. The protein samples used were the “wild type” parent strain without the tag and strain 1471 with the tag. Two different antibodies were employed; one phospho-specific antibody that recognises Tif211 phosphorylated on Ser52 and one antibody against Myc. The phospho-specific antibody gave a band at ~35 kDa for the wild type Tif211 and a band at ~55 kDa for the tagged Tif211 (Figure 5.5A). The antibody against Myc did not recognise anything in the total protein sample containing wild type Tif211, but gave a prominent band at ~55 kDa in the sample containing Myc-tagged Tif211 (Figure 5.5B).

Based on the above analyses, I conclude that the myc-sequence is at the correct position in the genome, and that the Tif211-13Myc fusion protein is expressed in the cells.

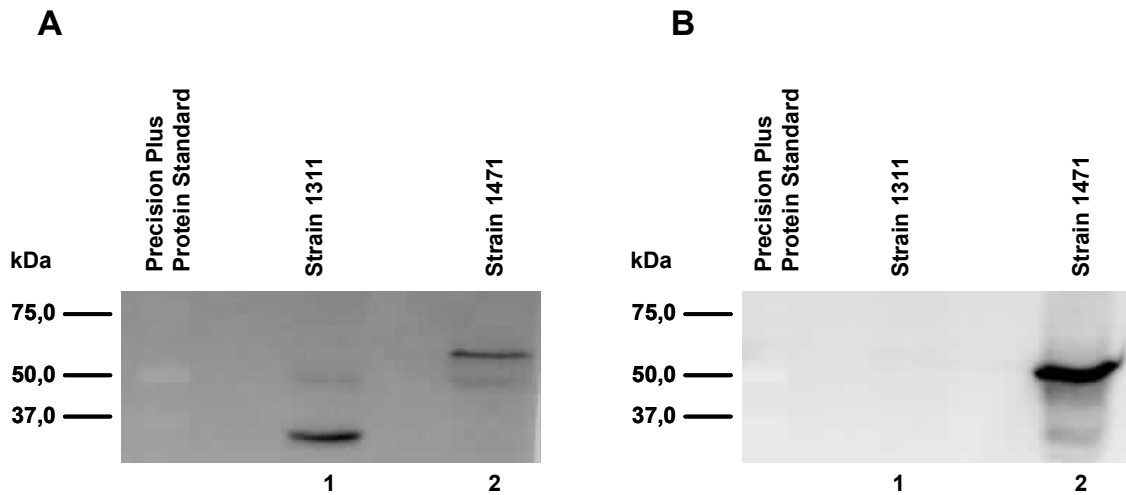


Figure 5.5 Testing transformants with Western blotting. Immunoblotting was done as described (Section 4.3.5) using antibody against (A) eIF2 α phosphorylated on Ser52 and (B) Myc. The phospho-specific antibody recognises wild type eIF2 α (A, lane 1) at ~35 kDa and tagged eIF2 α (A, lane 2) at ~55 kDa. The Myc-specific antibody recognises only Myc-tagged eIF2 α (B, lane 2).

The *tif211:13myc:kanMX6* cassette had been integrated in a *pku70* Δ genetic background (above). To isolate a *pku70*⁺ colony, the 1471 strain was backcrossed as described in Section 4.2.6. The planned physiological experiments involve synchronisation of the cells in the cell cycle. To this end, I also crossed in a temperature sensitive *cdc10-M17* mutation, which makes it possible to synchronise the cells in G1 phase. In the resulting *cdc10-M17 tif211:13myc:kanMX6* strain the presence of *tif211-13myc* was verified based on kanamycin resistance and PCR, while the presence of *cdc10-M17* mutation was confirmed based on temperature sensitivity and flow cytometry (data not shown).

4.3 Making and testing antibody against peptide

Instead of expressing a recombinant protein for immunisation, one can produce a short peptide, 10-16 amino acids long, biochemically. The Tif211 amino acid sequence was sent to MedProbe and examined for several factors, including folding, hydrophobicity, hydrophilicity, and antigenic index. The aim was to select a peptide which is expressed on the protein surface, and is expected to raise a good immunological response in the rabbits (Medprobe, personal communication). The

sequences of the suggested peptides were analyzed to exclude sequences with significant similarity to other proteins. If the peptide and another protein share more than 4 conserved amino acids in a row, it's likely that the antibody against the peptide will cross-react with the other protein. Based on these criteria, the sequence RRIRSVQKHIRVGS (amino acid 54-67 of Tif211) was chosen for production. It is predicted to be presented on the surface; however it has some conserved regions against other fission yeast proteins (data not shown).

The production of the peptide and the immunisation of two rabbits were carried out by MedProbe's SuperSpeedy poly-clonal anti-peptide production service. The two rabbits were injected with the peptide four times and there was taken a pre-immunisation bleed, a large bleed and a final bleed.

4.3.1 Testing the serum

Two strains, a wild type strain and a strain expressing myc-tagged Tif211, were grown up as described in Section 4.2.4 and protein samples were taken from the log phase culture. The samples were separated on a SDS-PAGE gel and blotted onto a PVDF-membrane using western blotting (Section 4.3.5). The primary antibodies used were pre-immune sera, large bleed and final bleed from each of the two immunized rabbits, diluted 1/300 and 1/1000 each. The secondary antibody was an alkaline phosphatase-linked anti-rabbit IgG antibody. A serum containing polyclonal antibodies against Tif211 should give a visible band at 34.5 kDa after developing the membrane. A myc-tagged Tif211 has a theoretical size of 55.1 kDa. There should therefore be a visible band-shift on the membrane if the serum recognizes the correct antigen, which is Tif211. For easier identification of the correct band, it would have been advantageous to include a Tif211-depleted sample. However, it is not possible to make a *tif211*Δ deletion mutant in fission yeast, since this gene is essential for survival.

The pre-immune sera of both rabbits showed extensive cross-binding with the fission yeast's protein sample (data not shown), and probably masking any signal from the

recognition of Tif211 and Tif211-myc in the large and final bleed. The specificity was low. The sera might contain antigens against many fission yeast proteins or other agents that are known to interfere with proteins and make the correct bands difficult to detect. I attempted to remove these agents by isolating IgG from the rabbit sera (see below).

4.3.2 Purification of antibodies and re-testing specificity

Sera from the most promising bleed (rabbit SM989, large bleed), determined by the testing above, was diluted with sample buffer and applied to a HiTrap Protein A column (Section 4.3.7). Protein A is a protein originally found in the cell wall of the bacteria *Staphylococcus aureus* and is known to bind to the Fc region of IgG with a very high specificity. This may be utilized to specifically isolate IgG and wash away other agents in the serum. After washing the column the IgG bound to protein A was eluted with citric acid and the pH was adjusted back to pH ~7. The purification process was analyzed by SDS-PAGE (Figure 5.6).

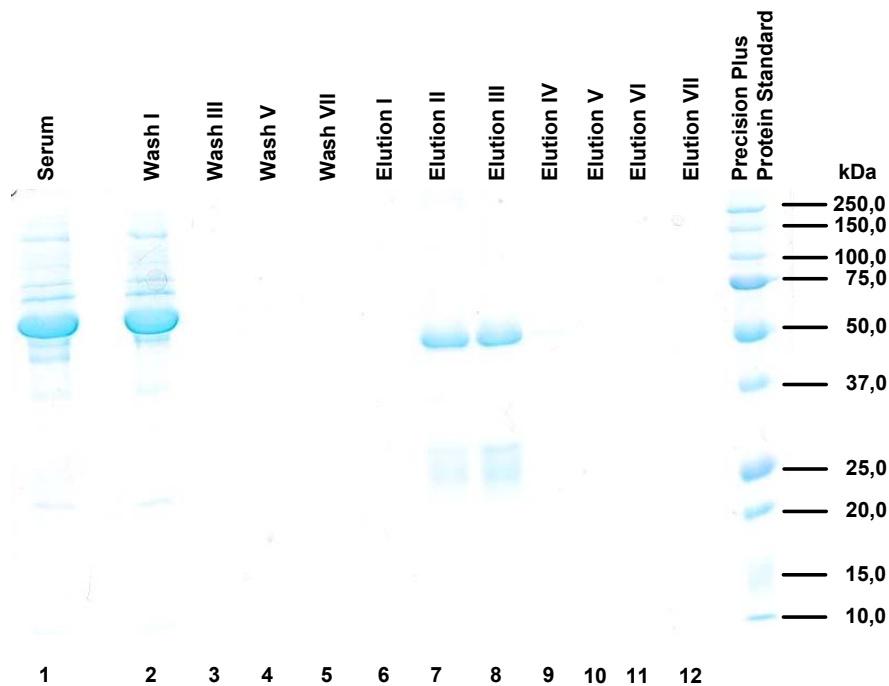


Figure 5.6 Purification of IgG from rabbit serum. Serum from rabbit (lane 1) contains several proteins, which only a small fraction being the IgG. IgG contains of four chains; two identical heavy chains and two identical light chains. The IgG heavy chain has an expected size of ~50 kDa, while the light chain has an expected size of ~25 kDa. Sera was added to the HiTrap Protein A column and washed with seven column volumes of washing buffer (lane 2 – 5). IgG was eluted by the addition of elution buffer and collected in seven elutions (lane 6 – 12). Elution II (lane 7) and elution III (lane 8) contained purified IgG.

The isolated IgGs was re-tested against protein samples containing Tif211 and Tif211-myc, as described above. The western was much cleaner than with the complete sera, but there was still cross-binding to several fission yeast proteins. The rabbits must have had antibodies recognizing some fission yeast proteins, as judged from the high background in the pre-immune serum. Indeed, some cross-binding was expected due to the amount of conserved residues between this peptide and other proteins. However, Tif211 and Tif211-myc could not be detected with the antibodies raised against the peptide (data not shown). Therefore, the approach using antibodies to detect and isolate unmodified eIF2 α in fission yeast was abandoned.

4.4 Detecting isoforms of eIF2 α in *S. pombe*

The *tif211-13myc* strain (Section 5.2) provides the means to isolate and analyse Tif211 further. To investigate whether there are other phosphorylation sites on eIF2 α in addition to Ser52, both bioinformatical and laboratory experiments were employed as described below.

4.4.1 Phosphorylation-site prediction using NetPhosYeast

NetPhosYeast (Appendix 1) was used to predict phosphorylation sites on Tif211 in fission yeast. NetPhosYeast is a bioinformatical program made to predict serine and threonine phosphorylation sites in budding yeast. It uses a neural-network based method to predict phosphorylation sites and has a relatively high specificity and sensitivity compared to other phosphorylation site prediction programs (Ingrell et al., 2007).

Eight serine residues and three threonine residues in Tif211 had a score above the threshold, and were therefore predicted to be phosphorylated (Figure 5.7). The high density of predicted phosphorylation sites at the C-terminal end of the protein was especially interesting, since eIF2 α is shown to be phosphorylated at the C-terminal end in budding yeast, brine shrimp, and wheat (Mehta et al., 1986). It is also worth mentioning that this program did not annotate Ser52 as a probable phosphorylation site, highlighting the limitations of a bioinformatical approach and the need to experimentally confirm the results of such analyses. The results obtained using bioinformatics strengthened our hypothesis, but further experimental characterisation had to be carried out.

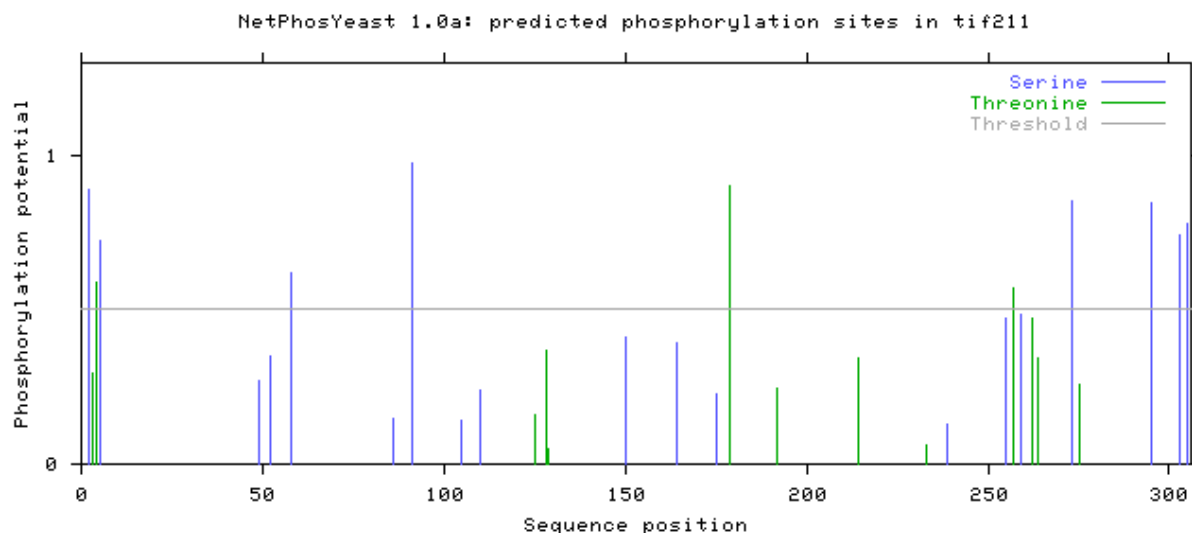


Figure 5.7 Predicted phosphorylation sites in Tif211 using NetPhosYeast. This figure shows predicted serine (blue lines) and threonine (green lines) phosphorylation sites in the Tif211 amino acid sequence. Every residue with a phosphorylation potential above the 0,5 threshold (gray line) is predicted to be phosphorylated. The complete output from NetPhosYeast is shown in Appendix IV.

4.4.2 Immunoprecipitation and mass spectroscopy

To confirm or reject the putative phosphorylation sites predicted by bioinformatical tools, a mass spectroscopy analysis of the protein was employed.

First, immunoprecipitation (Section 4.3.6) was performed to isolate the Tif211 protein from 1 mg of total protein extract (Section 4.3.2) from logarithmically growing fission yeast cells. An anti-myc antibody was used to immunoprecipitate Tif211-13Myc, which was shown to be specific for the Myc-tag in fission yeast in section 5.2.3 (Figure 5.5). Eluted proteins from were separated using SDS-PAGE (Section 4.3.3) and the band of interest was sliced out from the gel (Figure 5.8). Immunoblotting confirmed this to be the band corresponding to Tif211-13Myc (data not shown).

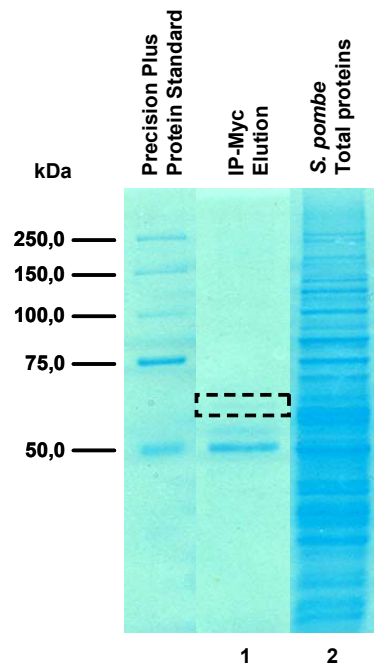


Figure 5.8 Immunoprecipitation of Tif211. Immunoprecipitation was carried out as described on total protein extract from fission yeast (lane 2) with antibodies against Myc. Eluted protein was separated on a SDS-PA gel (lane 1) and a band at size of approximate 55 kDa was cut out (stippled box) and prepared for MS analysis.

The gel piece was trypsinated as described in Section 4.3.9. Trypsin is a serine-protease that specifically hydrolyses peptide bonds at the carboxyl side of lysine. Treatment of a protein with trypsin will produce a set of peptides that serves as a unique fingerprint of a given protein. The masses of the peptides produced were determined by LC-MS/MS (Section 4.3.9) and the spectra obtained analyzed using Data Analysis and Spectrum Mill (Agilent). Since the 13Myc-tag starts with a lysine residue, it was thought to not interfere with the obtained spectra.

Analysis of the spectra obtained gave only one significant hit (Table 5.1); eIF2 α from fission yeast. A full summary of the result from Spectrum Mill is given in Appendix V.

Table 5.1. LC-MS/MS identification of protein band displayed in Figure 5.8

Accession #	Protein name	Distinct peptides	% AA coverage	Summed score	Mean intensity
P56286	Eukaryotic translation initiation factor 2 alpha subunit	5	25	85,14	1,90e+6

Further analysis of the obtained spectra did not reveal any phosphorylated residues in Tif211 (data not shown). This can by no means be taken as evidence for no phosphorylation of Tif211. There are several reasons why a phosphorylated residue might not show up in the MS analysis. First, only one of the putative phosphorylation sites was on the detected peptides. The amino acid sequence of Tif211 is given below, with covered peptides in red and putative phosphorylation sites in blue:

M**STTS**CRMYE NRFPEVDELV VVNV**RQIQEM** GAYVK**LLEYD** **NIEGMVLLSE** **L**SRRRIR**S**VQ
 KHIRVGRNEV VVVL**RVDKEK** GYIDLSKRRV **S**PEDVVKCEE RFNKSKAVHS IMRHIAEKHN
 VPLETMYTTI GWPLYRKYGH AYDAFKLAIS NPDHVFEGLE **PPKSGVINDL** **LAQISRR**L**T**P
 QPIKIRADVE VT**CFGYEGIN** AIKAALKAAE DVHTEEVPIK VKLVAPPLYV LLTNALDKSL
 GLKKLEEAIG AIEK**SIT**ASN GTCTVKMKPK **AVSETDELEL** **ADLMKK**FEKE NAEI**S**GDEED
 DQ**S**GSE

There are several reasons why a peptide and/or phosphorylated residues might not be detected. The lack of positive identification by MS is most often due to low protein amount (personal communication, Anders Øverby). This might be solved by increasing the amount of protein used in the immunoprecipitation step or enrich for phospho-peptides using either immobilized metal affinity chromatography (Corthals et al., 2005) or TiO₂ (Larsen et al., 2005). Phosphorylation might also have been removed by phosphatase activity or the predicted residues might not be phosphorylated at all. The lack of results may be a combination of these sources of error, or may be strongly influenced by one of them. However, the lack of a positive control (for example a synthetic peptide), makes the elimination of errors difficult.

Due to the time limit imposed on the master thesis, MS has only been performed once, and it should therefore be considered a pilot experiment. The result obtained confirms that I am able to isolate Tif211 and detect it by MS analysis. The above potential problems can therefore be tackled one by one. Optimized repeats of the MS run or the use of programs optimized for phospho-peptide detection might also help to reveal the phosphorylation events (personal communication, Anders Øverby).

4.5 Phosphorylation status of eIF2 α after UV irradiation

To investigate whether there is a difference in the phosphorylation status of eIF2 α before and after UV irradiation, another approach was employed. 2D-PAGE (Section 4.3.4) can be used to analyze both the mass and pI of a protein. pI is the pH at which the protein carries no net electrical charge. 2D-PAGE is well suited to detect post-translational modifications of a protein, since these modifications will change the proteins' mass and/or pI. Phosphorylation is one such post-translational modification that may be detected by 2D-PAGE. Phosphorylation will increase the mass of the protein slightly and, due to its negative charge, decrease the pI. A 2D-PAGE with a good separation will therefore show multiple phosphorylated isoforms of a protein as a train of separate spots. However, it's not possible to identify which residue is modified by 2D-PAGE alone.

4.5.1 Cell treatment

Fission yeast cells (Figure 5.9A) of the *cdc10-M17 tif211:13myc:kanMX6* genotype (Section 5.2.3) were synchronised in G1 phase by a four-hour incubation at 36°C (Figure 5.9B). Immediately after release, the cells were UV-irradiated (254 nm, UVC light) by a dose of 1100 J/m². Protein samples for 2D-Western were taken directly before and after the UV irradiation as described in Section 4.3.1. To examine whether newly made *tif211-13myc* strain behaved as cells containing the unmodified *tif211*, both cell survival and checkpoint response were measured.

To measure the cell survival, irradiated and untreated cells were diluted and plated onto YEG plates. Both the dilution series and the plating were done in duplicates. Colonies were counted after five days of incubation at 25°C. The cell survival after 1100 J/m² UV irradiation in G1 phase was 8 % (data not shown). This is close to what was observed for wild type cells, where 10 % survival is expected after UV irradiation in G1 phase (Tvegard et al., 2007).

To measure the checkpoint response, both untreated and irradiated cells were incubated for 20 minutes at 25°C after release from 36°C before flow cytometry samples (Section 4.2.8) were taken (Section 4.2.8). Flow cytometry may be used to measure replication of DNA and thereby, indirectly, the cell cycle progression. As expected, unirradiated cells continued the cell-cycle progression (Figure 5.9C), while irradiated cells delayed replication of the chromosomes (Figure 5.9D). In addition, Tif211-13Myc was shown to be phosphorylated at the Ser52 residue after UV irradiation (data not shown), as previously shown for the untagged protein (Tvegard et al., 2007). Thus, the tagged protein appears to behave similarly to the wild type, untagged protein and can be used to study the phosphorylation status of Tif211 using 2D-Western (see below).

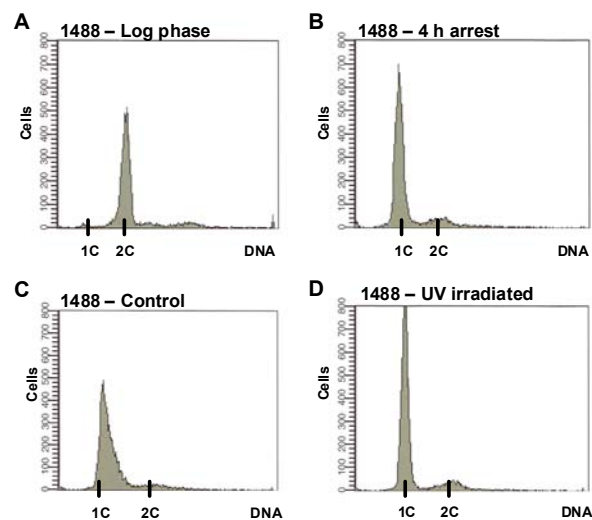


Figure 5.9 Flow cytometry. To be sure that the cells were synchronised in G1 phase before UV irradiation, samples were taken (A) before and (B) after incubation at 36°C for four hours. Logarithmically growing fission yeast cells have 2C DNA content, while cells in G1 have 1C DNA content. To measure the cell cycle progression after UV irradiation, (C) untreated and (D) UV irradiated cells were incubated for 20 min. at 25°C. Cells commencing chromosome replication should have a broad “shoulder” stretching from the 1C peak to the 2C peak approximately 20 min. after the release from 36°C. Sample preparation and flow cytometry was carried out as described in Section 4.2.8.

4.5.2 2D-Western

To study the phosphorylation status of Tif211 after UV irradiation, a 2D-PAGE was run as described in Section 4.3.4. To detect Tif211, immunoblotting (Section 4.3.5) was performed as described using antibodies against Myc and Ser52 phosphorylated eIF2 α . Several spots were observed (Figure 5.10). Tif211-13Myc was predicted to have a pI of 4.59 and a molecular weight of 55 kDa, based on computations using the Compute pI/Mw tool (Appendix I). The spots were close to the predicted pI and molecular weight of Tif211-13Myc. Therefore, the results suggest that all the observed spots on the 2D-Western correspond to different isoforms of Tif211.

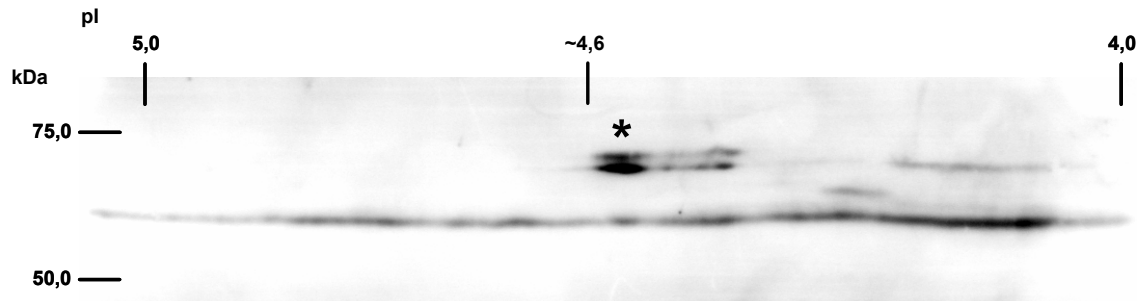
2D-Western of both control and UV-irradiated samples, show a region of several spots stretching from pH 4 to 5 at a molecular weight of ~55 kDa. Immunoblotting using an antibody specific for phosphorylated Ser52 shows they are not phosphorylated on Ser52 (Figure 5.10 C and D). One cannot exclude that these isoforms are due to unspecific modifications from sample preparation or incomplete separation, but these isoforms might also be a result of modifications in the cell which change the charge of the protein, but not the size. Examples of such modification are hydroxylation or oxidation.

The unirradiated sample has in addition to the “stretch of spots” at ~55 kDa discussed above, several isoforms of Tif211 with lower mobility. The most predominant isoform is marked with an asterisk (*) in Figure 5.10A. As expected, none of these isoforms have phosphorylated Ser52 (Figure 5.10C). After UV irradiation, the predominant isoform seen in the control seems to shift to a more acidic form (Figure 5.10B, double asterisk). However, it is unclear what modification caused the shift.

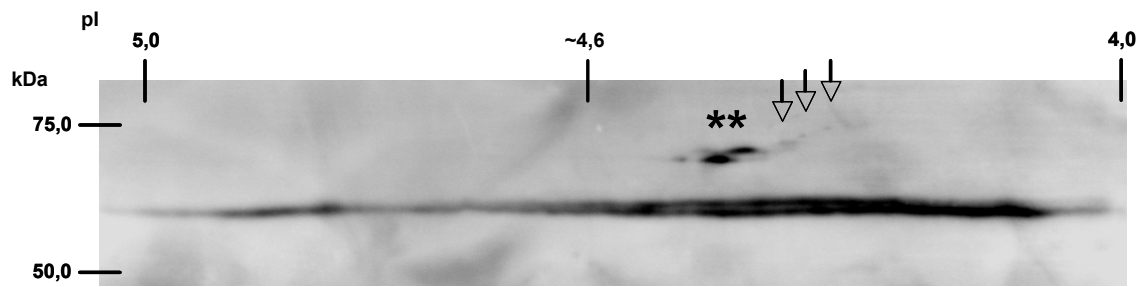
In addition, at least five spots can be clearly seen stretching toward the acidic end, and at the same time their sizes increase. Such “trains of spots” are often seen when a protein is present as several phosphorylated isoforms. Three of the five spots, marked with arrows, are shown to be phosphorylated at the Ser52 residue (Figure 5.10D) using the phospho-Ser52-specific antibody. This result proves that Tif211 in fission

yeast is modified at several residues after UV irradiation and support the prediction that several of the residues are phosphorylated.

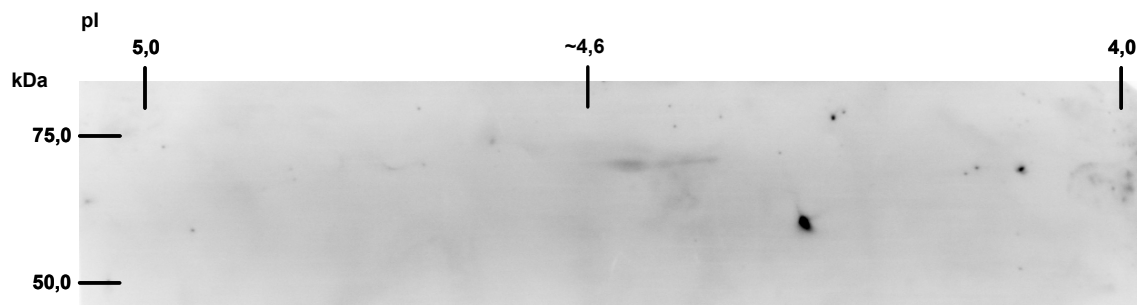
A – Control: Anti-myc antibody



B – UV irradiated: Anti-myc antibody



C – Control: Anti-S52-P antibody



D – UV irradiated: Anti-S52-P antibody

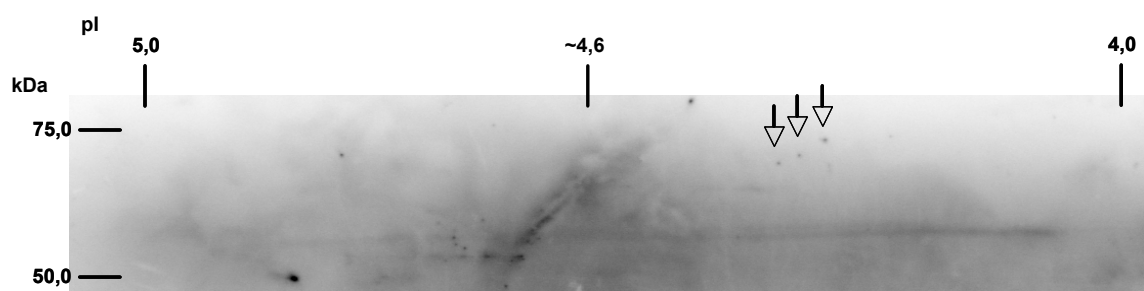


Figure 5.10 Modification of Tif211 after UV irradiation in G1 phase. Total protein extract from fission yeast cells synchronized in G1 and treated with UV irradiation was separated using 2D-PAGE with an 18 cm pH 4-5 IPG strip and a 10 % SDS-PA gel as described in Section 4.3.4. Immunoblotting (Section 4.3.5) was employed to detect either total Tif211-13Myc or Tif211 phosphorylated on Ser52. The predominant isoforms are marked with an asterisk (*) in the control sample and double asterisks (**) in the UV irradiated sample. Protein isoforms phosphorylated at Ser52, as shown in panels B and D after UV irradiation, are marked with arrows.

5. Discussion

The main goal of this study was to characterise the molecular mechanisms involved in down-regulation of translation after UV irradiation. To that end, eIF2 α was tagged with an epitope tag (Section 5.2), which made it possible to isolate the protein. Further analysis of eIF2 α was carried out using bioinformatics (Section 5.4.1), mass spectroscopy (Section 5.4.2) and 2D-PAGE (Section 5.5).

When the work of this master thesis was started, we had reasons to believe that eIF2 α was a target of more post-translational modifications than the well-characterised Ser52-phosphorylation. As speculated in Section 1.4, this could either be another site on eIF2 α or another factor involved in translation.

5.1 Post-translational modifications of eIF2 α

There are several compelling pieces of evidence for more post-translational modification of eIF2 α than the Ser52-phosphorylation. (1) Fission yeast cells grown in a media containing proline as the nitrogen source instead of the easier-to-utilize NH₄Cl, shows a prominent band shift. This band shift, which is observed on regular SDS-PAGE gels, is not due to Ser52-phosphorylation eIF2 α (Tonje Tvegård, unpublished data). (2) eIF2 α is conserved through evolution and post-translational modifications, in addition to Ser52-phosphorylation, of eIF2 α have also been observed in several other organisms. eIF2 α of budding yeast, brine shrimp and wheat germ cells is phosphorylated at several residues by casein kinase II (CK-II). (Mehta et al., 1986). For example, in budding yeast, mutational studies and isoelectric focusing showed that eIF2 α was phosphorylated at the C-terminal end by CK-II (Feng et al., 1994). Therefore, eIF2 α is likely to be phosphorylated on multiple sites, or hyperphosphorylated, in fission yeast as well.

5.1.1 Multiple phosphorylation of eIF2 α in fission yeast

Shortly after the initial bioinformatical study of eIF2 α in fission yeast (Section 5.4.1) had been carried out, a phosphoproteome analysis of fission yeast was published. This study, measuring phosphorylation of the proteome after thiabendazole (TBZ) treatment, found the following residues to be phosphorylated on eIF2 α : Thr179, Ser273, Ser295, Ser303 and Ser305 (Wilson-Grady et al., 2008). If one compares this result with the bioinformatical study (Figure 6.1), five out of the seven residues with the highest scores were phosphorylated after TBZ treatment. Thus, the study supports the prediction that eIF2 α may exist as a hyperphosphorylated protein.

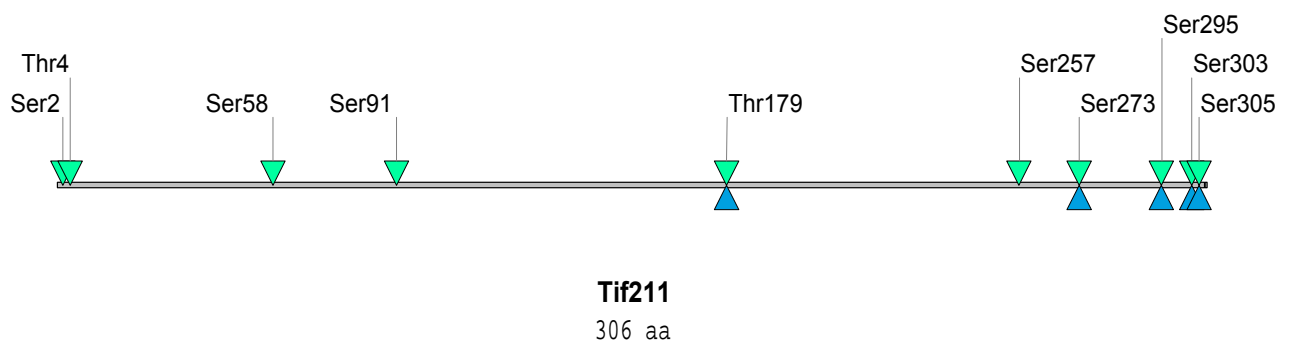


Figure 5.1 Multiple phosphorylation of eIF2 α . Residues predicted to be phosphorylated by NetPhosYeast (Section 5.4.1) are indicated with green triangles above the Tif211 sequence. Positions in the amino acid sequence are also annotated. Residues shown to be phosphorylated after TBZ treatment in fission yeast (Wilson-Grady et al., 2008) are shown by blue triangles below the Tif211 sequence.

Due to the nature of the phosphoproteome study (Wilson-Grady et al., 2008), which lacked a comparison between untreated and TBZ-treated cells, it is not possible to conclude whether phosphorylation of eIF2 α is due to TBZ-treatment or if some of the above-mentioned residues are constitutively phosphorylated in eIF2 α . Two different mechanisms for hyperphosphorylation of eIF2 α are discussed in the literature, and one of those mechanisms might be correct for eIF2 α in fission yeast as well: The C-terminal end of eIF2 α in fission yeast could either be (1) constitutively phosphorylated as in budding yeast (Feng et al., 1994) or (2) undergo progressive phosphorylation as in wheat (Le et al., 1998).

Furthermore, as observed by 2D-PAGE, eIF2 α in fission yeast is extensively modified after UV irradiation in G1 phase (Section 5.5.2). Due to the nature of the observed spots, some are believed to arise due to phosphorylation events. This supports the notion that eIF2 α undergoes increasing phosphorylation as in wheat, but one can not rule out that some of the residues are constitutively phosphorylated.

5.1.2 Physiological effects of the phosphorylation

More research has to be carried out to elucidate the physiological effects of the hyperphosphorylation of eIF2 α in fission yeast. Even if the phosphoproteome analysis by Wilson-Grady et al. (2008) had in fact shown that the hyperphosphorylation was due to addition of TBZ, several difficulties would have arisen in the interpretation of the result. TBZ is a microtubule depolymerising agent that induces a metaphase arrest in fission yeast (Walker, 1982). However, one could not directly have linked the observed phosphorylation with a metaphase arrest. TBZ has to be converted to the active compound carbendazim in the cell to act as a microtubule depolymerising agent. However, TBZ or some intermediate that is generated during the conversion reaction also interferes with other components in the cell, for example the actin cytoskeleton (Iain Hagan, personal communication). Thus, one can only speculate on the physiological significance of hyperphosphorylation based on observations of eIF2 α in other organisms.

In budding yeast, isoelectric focusing detects two isoforms of eIF2 α , where the more acidic form increases in relative abundance in response to amino acid starvation (Feng et al., 1994). In addition, the mutational data obtained suggested that eIF2 α was constitutively phosphorylated at three Ser-residues at the C-terminal end. Mutations at these sites did not cause any defect in growth properties, nor did they suppress a non-AUG start codon at *his4*, or alter the general amino acid control response (Feng et al., 1994). Only in combination with mutated *GCN2* did these strains grow more poorly compared to the wild type strain. This was interpreted as evidence that the constitutively phosphorylation of eIF2 α is required for optimal

function of eIF2 α in budding yeast cells (Feng et al., 1994). This was thought to be a result of conformational change in the protein, which might have led to altered ternary complex formation or GTP exchange, increased phosphorylation of Ser51 (which is homologous to Ser52 in fission yeast), or, most likely, due to instability or increased turnover of eIF2 α (Feng et al., 1994). Later, it was also found that deletion of the 13 amino acid long C-terminal region of budding yeast eIF2 α lead to depression of GCN4 *in vivo* (Heuvel et al., 1995). However, fission yeast does not have a homologues gene to GCN4.

In contrast to the phosphorylation events in budding yeast eIF2 α , none of the phosphorylation sites in wheat eIF2 α is constitutively phosphorylated. Wheat eIF2 α undergoes progressive phosphorylation during seed development and is dephosphorylated during germination (Gallie et al., 1997; Le et al., 1998). The conversion of eIF2 α to a hyperphosphorylated state during seed development occurs in a regulated manner, suggesting either that its phosphorylation occurs in a distributive manner or that it is under temporal control during development (Le et al., 1998). Changes in eIF2 α phosphorylation correlate with substantial changes in translation rates, suggesting a possible role for phosphorylation to regulate eIF2 α activity. Phosphorylation of some residues might promote translation, whereas phosphorylation of other residues might inhibit protein synthesis or alter which mRNAs are selected for translation (Le et al., 1998).

The latter prediction is especially interesting, since induction of the novel G1 checkpoint in fission yeast leads to a global down-regulation of translation, while at the same time translation of factors involved in pre-RC formation is sustained (Tvegard et al., 2007). In human cells and in budding yeast, eIF2 α phosphorylation on Ser51 results in downregulation of translation and selective upregulation of some mRNAs (Hinnebusch, 2005; Proud, 2005; Sonenberg and Hinnebusch, 2009). Therefore it is tempting to speculate that regulation of eIF2 α phosphorylation might be responsible for selective translation also in fission yeast.

5.2 Phosphorylation status of eIF2 α after UV irradiation

eIF2 α is known to be phosphorylated at the Ser52 residue by Gcn2 after UV irradiation of fission yeast (Tvegard et al., 2007). Results presented in this thesis suggest that eIF2 α is not only phosphorylated at the Ser52-residue, but extensively modified at several residues after UV irradiation (Section 5.5.2). At least four of the modifications observed are believed to be the result of phosphorylation, including the known Ser52-phosphorylation. At least two of the residues are modified progressively after Ser52 phosphorylation (Figure 5.9D).

To establish what kind of modification creates the spots observed, one could use chemical reagents together with point-mutations of the putative phosphorylation sites. One such reagent of interest is the Pro-Q Diamond dye, which is a phosphate-specific fluorophore (Bockus and Scofield, 2009) able to detect phosphorylated residues. One could also isolate the protein using immunoprecipitation (Section 4.3.6), separate the protein using 2D-PAGE (Section 4.3.4), stain the gel and excise the spots and analyze them using mass spectroscopy (Section 4.3.9). This would reveal both the kind of modification and where in the protein the modified residue is.

5.3 The role of Gcn2

The last goal was to investigate whether phosphorylation sites on eIF2 α are phosphorylated in a Gcn2-dependent manner. It is known that the Ser52 residue is phosphorylated by Gcn2, and not Hri1 or Hri2, after UV irradiation in G1 phase (Tvegard et al., 2007). The plan was to compare 2D-PAGE results of eIF2 α phosphorylation between UV irradiated wild type and *gcn2 Δ* mutant. A difference of the phosphorylation status of eIF2 α between the wild type and *gcn2 Δ* deletion mutant would have indicated that another protein was responsible for the modifications observed. However, there was sadly not enough time to finish this part of the project.

In addition to the known kinases that phosphorylates Ser51 on eIF2 α (presented in Chapter 1.3.3), Casein kinase II (CK-II) phosphorylates eIF2 α in budding yeast, brine

shrimp and wheat (Laxminarayana et al., 2002; Mehta et al., 1986). CK-II is a serine and threonine protein kinase which is ubiquitously found in eukaryotes. It plays a role in signal transduction events, and has several functions in processes like cell cycle control and DNA repair. CK-II is also known to phosphorylate mammalian translation initiation factors eIF2 β and eIF2B. In fission yeast, CK-II is known to be involved in establishment of cell shape and polarized growth (Roussou and Draetta, 1994; Snell and Nurse, 1994). CK-II phosphorylates both serine and threonine residues located within a negatively charged amino acid sequence with the consensus sequence S/T-X-X-E/D (Pinna, 1990). Three out of the five residues shown to be phosphorylated in eIF2 α after TBZ-treatment (Wilson-Grady et al., 2008) had a motif corresponding to the consensus sequence; Ser273, Ser295, and Ser303. Involvement of Gcn2 and/or CK-II in the phosphorylation of eIF2 α in fission yeast will be a subject of further investigations. However, this may indicate that CK-II, and not Gcn2, phosphorylates eIF2 α on the newly discovered phosphorylation sites in fission yeast.

5.4 CONCLUSION

In the present study, eIF2 α in fission yeast has been investigated. A method to identify and purify eIF2 α was developed. Due to the inability to express recombinant eIF2 α from fission yeast in *E. coli* (Section 5.1), and the difficulties in producing antibodies against a peptide derived from eIF2 α (Section 5.3), eIF2 α was tagged with an epitope tag by homologous recombination (Section 5.2). The strain carrying the epitope-tagged eIF2 α was viable and stable. It was shown that the tagged strain had a survival rate and cell-cycle progression like wild type cells after UV irradiation in G1 phase (Section 5.5.1). Based on these observations, the tagged protein was presumed to behave as the untagged protein for further characterisation of eIF2 α .

The second task was to investigate whether there are other phosphorylation sites on eIF2 α in addition to Ser52. Based on prediction made using bioinformatical tools (Section 5.4.1), and a recently published phosphoproteome study (discussed in

Section 6.1.1), eIF2 α is now known to have several phosphorylation sites. eIF2 α in fission yeast has the potential to be phosphorylated at the following residues: Thr179, Ser273, Ser295, Ser303, and Ser305 (Wilson-Grady et al., 2008). In addition, eIF2 α is also predicted to be phosphorylated at residue Ser2, Thr4, Ser58, and Ser91 (Section 5.4.1). Mass spectroscopy analysis did neither verify nor reject this hypothesis (Section 5.4.2).

The third task was to investigate whether there is a difference in the phosphorylation status of eIF2 α before and after UV irradiation – beyond the phosphorylation of Ser52. Based on 2D-PAGE analysis, eIF2 α was shown to be extensively modified after UV irradiation in G1 phase (Section 5.5.2). The nature of these modifications is still not known. However, at least five of the isoforms observed is believed to have different phosphorylation status.

The last task was to investigate whether the identified phosphorylation sites on eIF2 α is phosphorylated in a Gcn2-dependent manner. A strain carrying the tagged eIF2 α together with a *gcn2 Δ* deletion could not be constructed because of time constraints. Therefore, further study is needed to determine the exact role of Gcn2 in the phosphorylation of additional residues on eIF2 α . However, based on observation of eIF2 α in budding yeast, brine shrimp, and wheat, it seems likely that at least some of the residues on eIF2 α are phosphorylated by casein kinase II (Section 6.2).

This master thesis has characterised several novel aspects of eIF2 α in fission yeast and has established methods to study its modification under different conditions. The results suggest that eIF2 α is indeed phosphorylated on several sites, but the physiological relevance of these events remain to be determined.

6. Further work and perspective

More work has to be carried out to fully understand the mechanisms behind the observed down-regulation of translation after UV irradiation, and the assumed link between translational down-regulation and the cell cycle delay. There are several logical ways to continue this project.

First, mass spectroscopy should be repeated with enrichment of phosphopeptides with either TiO_2 or immobilised metal affinity chromatography. A higher amount of phosphorylated residues will make it more likely to detect them by the use of mass spectroscopy. Another way to improve the mass spectroscopy results is to use optimised programs, as discussed in Chapter 5.4.2. For example, the experimental set-up used for mass spectroscopy by Wilson-Grady (2008) is known to detect several phosphorylated forms of eIF2 α .

Second, 2D-Western should be repeated several times with antibodies against both total eIF2 α and Ser52-phosphorylated eIF2 α . In this process, parallel samples treated with phosphatases should also be run. This treatment will remove all phosphorylations and show the unphosphorylated protein. This will let us identify which spots correspond to phosphorylated isoforms, and would greatly increase our ability to draw conclusions about the different isoforms observed. In addition, as discussed above, 2D-Western results should be obtained from irradiated *gcn2 Δ* cells.

Based on the phosphoproteome data (Wilson-Grady et al., 2008) and the data obtained in the above experiments, a more direct approach could be taken to determine the role of eIF2 α in the checkpoint response. It is possible to exchange the phosphorylatable serine residues with non-phosphorylatable, but structurally similar, alanine residues. This may be done with PCR-based site-directed mutagenesis (Liu and Naismith, 2008). The resulting mutated eIF2 α gene may be transformed back into fission yeast to replace the wild-type gene. The checkpoint response of these mutants

should reveal whether phosphorylation of the specific sites is required for the cell cycle delay and/or down-regulation of translation.

To assess other targets of Gcn2 in the checkpoint response, one could choose a more global approach. One such method to identify all the targets of the Gcn2 kinase, is the “Shokat method”. This approach uses ATP analogs that can distinguish between wild-type and mutated Gcn2 kinase. The use of ATP analogs together with a mutated kinase, allows selective labelling of substrates specific for the Gcn2 (reviewed in Manning and Cantley, 2002).

A more long-term project is to analyze whether and how specific eIF2 α modifications might regulate transcript-specific translation. We have shown that after UV irradiation translation is down-regulated by 80%, but some mRNAs continue to be efficiently translated (Tvegard et al., 2007). It is conceivable that modifications of translation initiation factors, possibly eIF2 α among them, are responsible for selective translation. This can be investigated using the site-specific eIF2 α mutants generated during the above work. The polysome fraction, which contains the actively translated mRNAs, can be isolated from UV-irradiated wild type and eIF2 α mutant strains. The mRNAs can be isolated from these fractions and identified by hybridising them to microarrays. If the modifications are required for regulating transcript-specific translation, a different subset of mRNAs will be translated in the mutants and in wild type cells.

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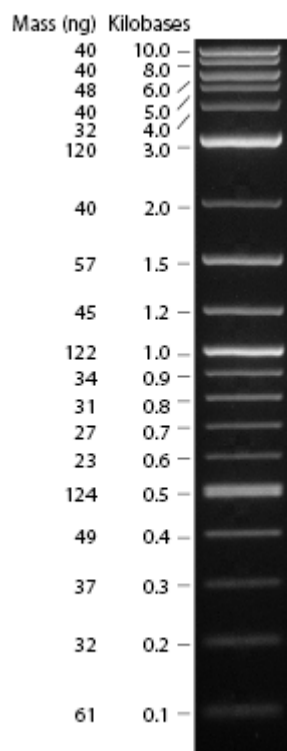
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Appendix I: WWW references

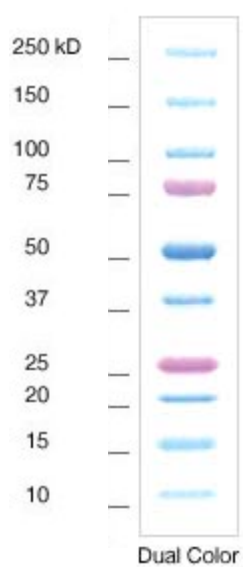
- 1) PombeNet at the Forsburg Lab: <http://www-rcf.usc.edu/~forsburg/>
- 2) Steve's place:
http://www.steve.gb.com/images/science/schizosaccharomyces_pombe.jpg
- 3) Invitrogen: <http://www.invitrogen.com/>
- 4) GeneDB: <http://www.genedb.org/>
- 5) Bioneer: <http://pombe.bioneer.co.kr/>
- 6) Nobelprize.org: <http://nobelprize.org/>
- 7) *E. coli* Codon Usage Analysis 2.0:
- 8) <http://www.faculty.ucr.edu/~mmaduro/codonusage/usage.htm>
- 9) Pombe PCR Primer Program for C-terminal tagging:
http://www.sanger.ac.uk/cgi-bin/PostGenomics/S_pombe/PPPP/pppp_c_term.pl
- 10) NetPhosYeast: <http://www.cbs.dtu.dk/services/NetPhosYeast/>

Appendix II: Molecular weight standards

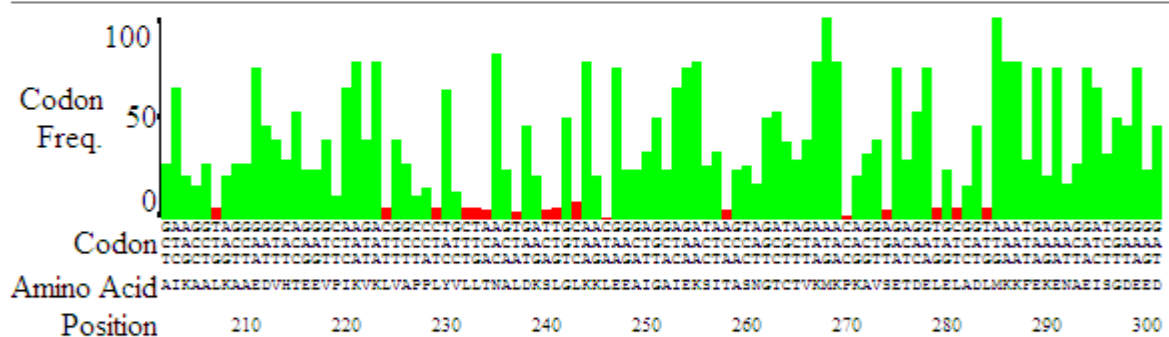
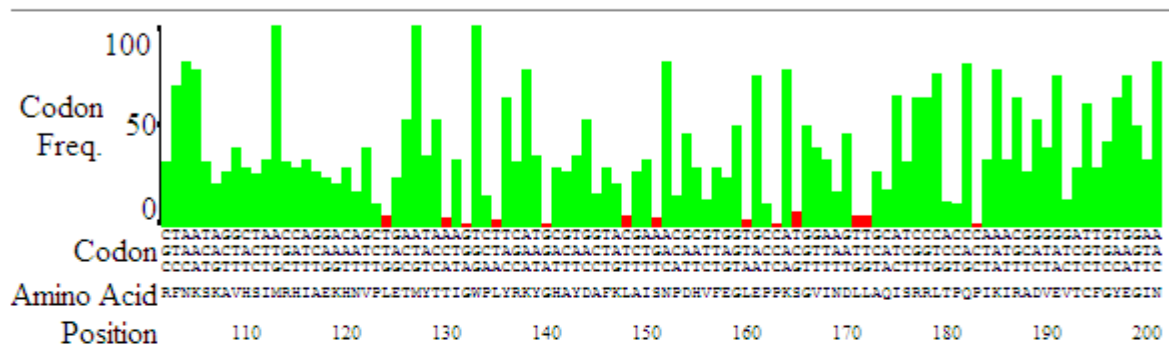
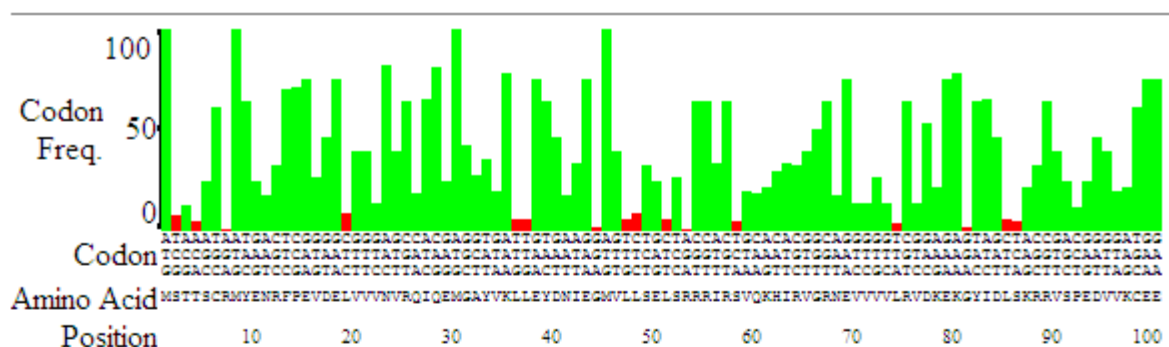
2-Log DNA Ladder (NEB)



Dual Color Precision Plus Protein Standard (Bio-Rad)



Appendix III: Codon Usage in *tif211*



Colors: ■ = less than 10% of codons for same amino acid; ■ = at least 10%

Fraction of sense codons below threshold (=10.00): **46/306 (15%)**

Appendix IV. NetPhosYeast server prediction

```

>Sequence          306 amino acids
#
# netphosyeast-1.0a prediction results
#
# Sequence          # x   Context      Score   Kinase   Answer
# -----
# Sequence          2 S   ---MSTTSC   0.888   main    YES
# Sequence          3 T   --MSTTSCR   0.297   main    .
# Sequence          4 T   -MSTTSCRM   0.587   main    YES
# Sequence          5 S   MSTTSCRM   0.723   main    YES
# Sequence          49 S  MVLLSEL   0.269   main    .
# Sequence          52 S  LSEL   0.352   main    .
# Sequence          58 S  RRIRSVQKH   0.620   main    YES
# Sequence          86 S  YIDLKRRV   0.147   main    .
# Sequence          91 S  KRRVSPEDV   0.976   main    YES
# Sequence          105 S RFNKSKAVH   0.140   main    .
# Sequence          110 S KAVHSIMRH   0.241   main    .
# Sequence          125 T VPLETMYTT   0.159   main    .
# Sequence          128 T ETMYTTIGW   0.368   main    .
# Sequence          129 T TMYTTIGWP   0.047   main    .
# Sequence          150 S KLAISNPDH   0.412   main    .
# Sequence          164 S EPPKSGVIN   0.395   main    .
# Sequence          175 S LAQISRRLT   0.229   main    .
# Sequence          179 T SRRLTPQPI   0.904   main    YES
# Sequence          192 T DVEVTCFGY   0.243   main    .
# Sequence          214 T EDVHTEEV   0.343   main    .
# Sequence          233 T YVLLTNALD   0.061   main    .
# Sequence          239 S ALDKSLGLK   0.130   main    .
# Sequence          255 S AIEKSITAS   0.472   main    .
# Sequence          257 T EKSITASNG   0.572   main    YES
# Sequence          259 S SITASNGTC   0.482   main    .
# Sequence          262 T ASNGTCTVK   0.470   main    .
# Sequence          264 T NGTCTVKMK   0.345   main    .
# Sequence          273 S PKAVSETDE   0.853   main    YES
# Sequence          275 T AVSETDELE   0.256   main    .
# Sequence          295 S NAEISGDEE   0.845   main    YES
# Sequence          303 S EDDQSGSE-   0.743   main    YES
# Sequence          305 S DQSGSE---   0.781   main    YES
#
MSTTSCRM YENRFPEVDELVVVNV RQIQEMGAYVKLL EYDNI EGMVLLSE # 50
LSRRRIRSVQKH IRVGRNEVVV LRV DKEKGYIDL SKRRVSPEDV VKCEE # 100
RFNKSKAVHSIMRH IA EKHNVPLETMYTTIGW PLYRKYGHAYDAFKLAIS # 150
NPDHVFEGLEPPKSGVIN D LLAQISRRLTPQPIKIRADVEVTCFGYEGIN # 200
AIKAALKAAEDVHTEEVPIKVKLVAPPLYVLLTNALDKSLGLK KLEEAIG # 250
AIEKSITASNGTCTVKMKPKAVSETDELELADLMKKFEKENAEISGDEED # 300
DQSGSE # 350
%1 .S.TS..... # 50
%1 .....S.....S..... # 100
%1 ..... # 150
%1 .....T..... # 200
%1 ..... # 250
%1 .....T.....S.....S..... # 300
%1 ..S.S.

```


Appendix V: Spectrum Mill – Peptide Summary

Spectrum Mill - Protein Peptide/Summary

Results Shown Filtered by Validation Category: valid

Data Directory: msdataSMEXTERN/2008/jonhalvor-cellebio/IP-tif211/jhk1_08nov10

hit table read - SpecFeatures read

valid hits read from tagSummary file - Files: 6 Hits: 6

beginning to assemble proteins proteins assembled 0.000645 sec

proteins filtered by unique peptides 0.000202 sec

proteins filtered by score

calculated protein coverage maps 0.001949 sec

beginning to roll up proteins into groups proteins rolled up into groups 0.000154 sec

protein groups ready for display

proteinGroupingMethod: oneSharedPeptide 1 Proteins listed

proteinGroupingMethod: oneSharedPeptide 1 Proteins listed

proteinGroupingMethod: oneSharedPeptide 1 Proteins listed

proteinGroupingMethod: oneSharedPeptide 1 Proteins listed

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proteinGroupingMethod: oneSharedPeptide 1 Proteins listed

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Database Accession #	Protein Name
1	6	5	85.14	25	1.90e+006	P56286	Eukaryotic translation initiation factor 2 alpha subunit (eIF-2-alpha)
Totals:	6	5					

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Database Accession #	Protein Name
1	6	5	85.14	25	1.90e+006	P56286	Eukaryotic translation initiation factor 2 alpha subunit (eIF-2-alpha)

#	Filename	z Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
1	JHK1000002.1071.1071.2	2	19.74	95.9	2.23e+006	(K) LLEYDNIEGMVLISELSR (R)	2094.079
2	JHK1000002.0971.1036.0	2	18.71	95.8	3.61e+006	(K) SGVINDLLAQISR (R)	1385.775
3	JHK1000001.0875.0882.2	2	16.89	91.1	5.10e+005	(K) AVSETDELELADLMK (K)	1663.810
4	JHK1000001.0829.0829.2	2	14.98	92.7	2.63e+005	(R) ADVEVTCFYGEGINAIAK (A)	1885.900
5	JHK1000002.1267.1308.3	3	14.82	76.4	2.51e+006	(K) LVAPPLYVLLTNALDK (S)	1740.031
6	JHK1000002.1281.1281.3	3	11.23	75.8	2.29e+006	(K) LVAPPLYVLLTNALDK (S)	1740.031

Appendix VI: Amino acids

Amino Acids and Their Symbols			Codons
A	Ala	Alanine	GCA GCC GCG GCU
C	Cys	Cysteine	UGC UGU
D	Asp	Aspartic acid	GAC GAU
E	Glu	Glutamic acid	GAA GAG
F	Phe	Phenylalanine	UUC UUU
G	Gly	Glycine	GGA GGC GGG GGU
H	His	Histidine	CAC CAU
I	Ile	Isoleucine	AUA AUC AUU
K	Lys	Lysine	AAA AAG
L	Leu	Leucine	UUA UUG CUA CUC CUG CUU
M	Met	Methionine (Initiation)	AUG
N	Asn	Asparagine	AAC AAU
P	Pro	Proline	CCA CCC CCG CCU
Q	Gln	Glutamine	CAA GAG
R	Arg	Arginine	AGA AGG CGA CGC CGG CGU
S	Ser	Serine	AGC AGU UCA UCC UCG UCU
T	Thr	Threonine	ACA ACC ACG ACU
V	Val	Valine	GUA GUC GUG GUU
W	Trp	Tryptophan	UGG
Y	Tyr	Tyrosine	UAC UAU
Termination			UGA UAA UAG