Regulation of Cdk activity in the cell cycle

Thesis for the degree of Philosophiae Doctor

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Christiane Rothe
Oslo, November 2016

Abbreviations

APC/C	anaphase-promoting complex/cyclosome
BI	binucleate index
CAK	Cdk-activating kinase
Cdk	cyclin-dependent kinase
CPD	cyclobutane pyrimidine dimer
CHX	Cycloheximide
DAPI	4’,6-diamidino-2-phenylindole; fluorescent dye that binds DNA
DIC	differential interference contrast
DNA	deoxyribonucleic acid
DNA-A	DNA-area
DNA-W	DNA-width
dNTP	deoxyribonucleoside triphosphate
DSB	double-strand break
eIF	eukaryotic initiation factor
FSC	forward scatter
G1/G2 phase	gap1/ gap2 phase
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HeLa	immortal cell line derived from cervical cancer
IR	ionizing radiaton
IRES	internal ribosome entry site

M	mitosis
m7G	7-Methylguanosine
MAPK	mitogen-activated protein kinase
Mb	megabase
MCM	minichromosome maintenance complex
Met	methionine
mRNA	messenger RNA
nt	nucleotide
ORC	origin recognition complex
PI3K	phosphatidylinositol 3-kinase
Poly(A)	polyadenylation
Pre-RC	pre-replicative complex
S	Svedberg unit; sedimentation rate
SAC	spindle assembly checkpoint
SPB	spindle pole body
S phase	synthesis phase
SSC	side scatter
ssDNA	single-stranded DNA
RNA	ribonucleic acid
RNR	ribonucleotide reductase
T-loop	Cdk- activation loop; contains a threonine (T) residue which normally blocks the ATP binding site
tRNA	transfer RNA

Tyr	tyrosine
uORF	upstream open reading frame
UTR	untranslated region
UV	ultraviolet

List of publications

This thesis is based on the following publications:

Paper I

Knutsen, JHJ, Rein, ID, **Rothe, C**, Stokke, T, Grallert, B, Boye, E. (2011) „Cell-cycle analysis of fission yeast cells by flow cytometry." PLoS ONE **6**(2): e17175.

Paper II

Anda, S*, **Rothe, C***, Boye, E, Grallert, B. (2016) „Consequences of abnormal Cdk activity in S phase." Cell Cycle **15**(7): 963-973 *these authors contributed equally to this work

Paper III

Rothe, C, Rødland GE, Anda, S, Lopez-Aviles, S, Grallert, B. (2016) "A checkpoint-independent mechanism delays entry into mitosis." Manuscript

The publications are included at the end of the thesis and will be referred to as paper I-III in the text.

Introduction

The human body is made of several millions of cells. New cells are constantly made from pre-existing cells through the process of cell division in order to grow new tissue or substitute existing cells. Cell division is one of the most fundamental processes in all living organisms. The events leading to cell division are coordinated in the cell cycle, which is carefully regulated to ensure that each daughter cell is equipped with a complete set of chromosomes. Consequently, malfunction in cell-cycle regulation might result in inappropriate cell division and abnormal cell growth such as found in malignant tissues. Many aspects of cancer development are attributed to failure in cell-cycle control.

Investigating the basic mechanisms of cell-cycle regulation does not only provide a better understanding of how cells ensure genomic integrity and cell survival; it also enables to tackle complex diseases such as cancer.

We use a simple organism, the unicellular yeast *Schizosaccharomyces pombe,* as a model in our research to investigate the regulatory mechanisms of cell proliferation. By studying the cell cycle in *Schizosaccharomyces pombe* we are able to understand more complex regulation of the cell cycle in higher eukaryotes and human cells because these fundamental processes are highly conserved through evolution.

Below I will describe the mechanisms and interactions that form the background for the present work. It includes an overview on cell-cycle regulation in *S. pombe* with focus on S phase and G2/M transition and the surveillance mechanisms (checkpoints) preventing genome instability. In addition, I will explain the process of protein synthesis and its regulation. I will start with a description of the model organism used in this study.

Schizosaccharomyces pombe

S. pombe was first described by Paul Lindner in 1893 who isolated the non-pathogenic, unicellular yeast from African millet beer. Hence, the species name is *pombe,* which is the Swahili word for “beer” (Hoffman et al. 2015). In the 1940s Urs Leupold was the first to use *S. pombe* as an experimental model to investigate the genetic basis of sexual reproduction in

yeast (Leupold 1949). From there on it emerged as a model organism to study basic molecular principles including cell-cycle regulation, chromosome dynamics, epigenetics and gene expression. Especially recognised was the work on cell-cycle progression by Paul Nurse, who, together with Leland H. Hartwell and Tim Hunt, was awarded the Nobel Prize in Physiology or Medicine in 2001. In 2002 *S. pombe* was the sixth eukaryotic organism to have its genome fully sequenced (Wood et al. 2002). The 13.8 Mb-genome is organised into three chromosomes and codes for ~5054 proteins (Hoffman et al. 2015).

Like many other yeasts *S. pombe* belongs to the phylum Ascomycota, the largest and most diverse group of fungi. *S. pombe* is rod-shaped with a diameter of 3-4 µm and a length of 7-14 µm (Fig. 1A). It has both a vegetative and a meiotic life cycle. During vegetative growth *S. pombe* is haploid. It grows by extending the tips of the cell and divides by medial fission which results in two genetically identical daughter cells of the same size. *S. pombe* is therefore also known by the name "fission yeast". The vegetative cell cycle consists of four phases: G1, S, G2 phase and mitosis (Fig. 1B). Under poor growth conditions, cells enter a meiotic cell cycle to undergo sexual differentiation. If a partner of opposite mating type is present, they conjugate and go through meiosis, which produces four-spored asci.

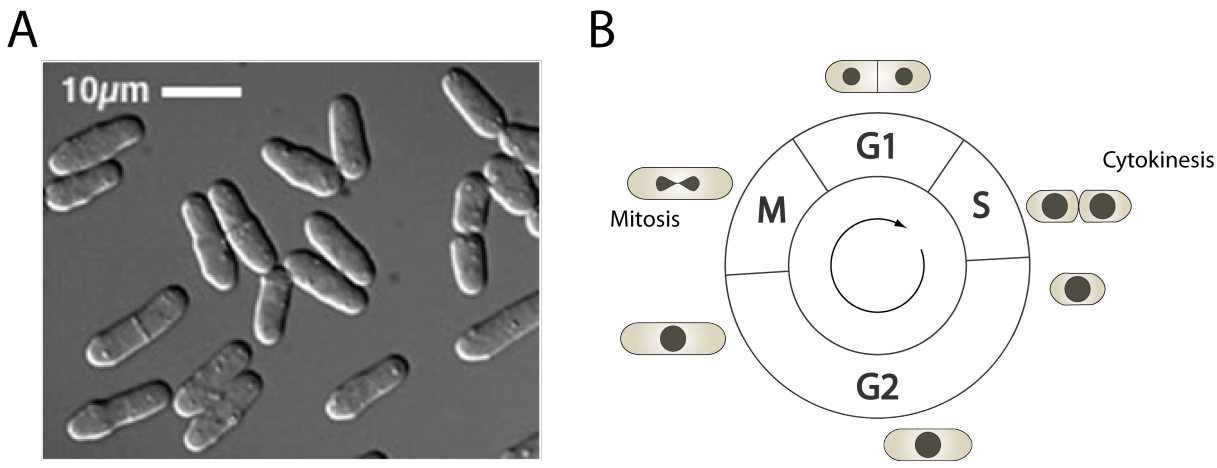

Figure 1. Vegetative growth of *Schizosaccharomyces pombe* (A) wild type cells in DIC microscopic image from http://eishinoguchi.com/pombe.html (B) Mitotic cell cycle of *S. pombe* cells.

The eukaryotic cell cycle

In order to proliferate, a cell has to grow and divide - a complex process that is coordinated in the cell cycle. The cell cycle comprises a series of events leading to the production of two genetically identical daughter cells from a parental cell. This requires the faithful duplication of chromosomes and their segregation into two new cells. The eukaryotic cell cycle is divided into four phases and includes S phase where chromosomes are duplicated by replication, and M phase in which the chromosomes are segregated (mitosis) and the cytoplasm is divided (cytokinesis). In between S and M phase are two additional phases, G1 and G2 (G for gap). In general, these gap phases ensure that the cell grows and increases in mass. However, G1 and G2 also have functions that are more specific. In G1 a cell decides whether environmental conditions are favourable for cell division or whether to enter quiescence (G0 phase). If the cell commits to a new round of the cell cycle, it prepares for DNA replication in G1 phase. In G2 phase cells ensure that they have obtained the right size and that DNA synthesis and repair are complete before entering mitosis.

In *Schizosaccharomyces pombe*, a whole cell cycle takes about 3-4 hours depending on the growth medium. In standard laboratory growth medium G2 phase is longest and takes about 80 % of the cell cycle. This is in contrast to the cell cycle in mammalian cells where a cell spends most time in G1 phase. Another feature that is different from the mammalian cell cycle is that cytokinesis coincides with S phase and not the end of mitosis, because G1 is so short that most cells finish S phase by the time they complete cytokinesis.

The temporal order of S and M phase is crucial for successful proliferation. The sequence of events in the cell cycle is tightly regulated by a family of protein kinases called cyclin-dependent kinases (Cdk-s). Cdk activity itself is controlled through binding to a regulatory subunit, a cyclin. Cyclin expression oscillates during the course of the cell cycle and thereby determines the catalytic activity of the Cdk. In *S. pombe* a single Cdk associates with different cyclins during the cell cycle. Higher eukaryotes, by contrast, express both multiple cyclins and Cdk-s to control passage through the cell cycle. In addition to cyclin availability, Cdk activity is regulated by activating and inhibitory phosphorylations as well as the presence of Cdk inhibitors.

Regulation of the cell cycle in fission yeast

In fission yeast the single Cdk, Cdc2, binds to one of four different cyclins: Cig1, Cig2, Puc1 and Cdc13. The protein level of Cdc2 remains constant throughout the cell cycle, while that of the four cyclins changes.

Upon exit from mitosis, the mitotic cyclin Cdc13 is degraded and consequently Cdc2 activity is low (Fig. 2). Any remaining Cdc2-Cdc13 complex present in G1 phase is inhibited by the Cdc2 inhibitor Rum1 (CorreaBordes et al. 1997). The cyclins Cig1, Cig2 and Puc1 accumulate after mitosis and, in complex with Cdc2, promote progression through G1 phase. Among the three G1 cyclins, Cig2 has the major role in promoting progression through G1 and entry into S phase. The-Cdc2-Cig2 complex is inhibited by Rum1 during G1 phase until the cells have achieved the size needed for S phase (Martin-Castellanos et al. 1996; Mondesert et al. 1996). Cdc2-Cig1 and Cdc2-Puc1, the two other cyclin-Cdk complexes regulating G1 phase, are insensitive to Rum1 regulation (Benito et al. 1998; Martin-Castellanos et al. 2000). Both are able to phosphorylate Rum1, which triggers its degradation and consequently leads to activation of the Cdc2-Cig2 complex that promotes entry into S phase (CorreaBordes et al. 1997; Martin-Castellanos et al. 2000).

During S, G2 and M phase Cdc2 is bound to the mitotic cyclin Cdc13. It is the only cyclin in *S. pombe* which is essential and can, in the absence of the three other cyclins, drive the cell cycle on its own (Fisher and Nurse 1996; Coudreuse and Nurse 2010). At entry into S phase Cdc2 becomes phosphorylated at tyrosine 15 which results in a cyclin-Cdk complex with relatively low activity (Gould and Nurse 1989; Gould et al. 1998). The inhibitory phosphorylation is maintained during S and G2 phase by the two kinases Mik1 and Wee1. When cells are ready to enter mitosis Cdc25, a phosphatase, removes the Tyr15 phosphorylation and renders the Cdc2-Cdc13 complex in an active state that triggers entry into M phase. Upon exit from mitosis Cdc13 is targeted by the anaphase-promoting complex/cyclosome (APC/C) (reviewed in Moser and Russel, 2000). The APC/C is a ubiquitin ligase that triggers polyubiquitination of Cdc13, which in turn promotes destruction of Cdc13 by the proteasome. Degradation of the cyclin subunit leads to the inactivation of Cdc2 at the end of mitosis.

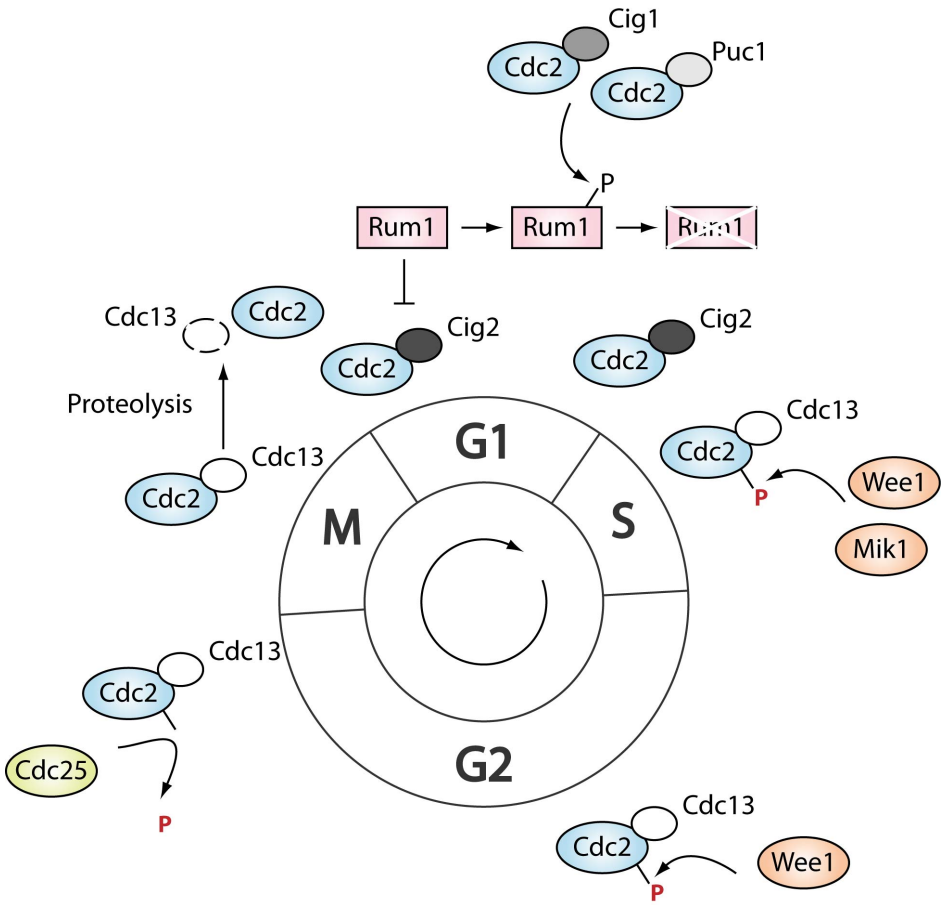

Figure 2. Regulation of the mitotic cell cycle in *S. pombe* through the Cdk, Cdc2. The mitotic cyclin Cdc13 is degraded at the end of mitosis. The Cdk inhibitor Rum1 inhibits the Cdc2-Cig2 complex in early G1. In late G1, Cdc2-Cig1 and Cdc2-Puc1 phosphorylate Rum1, which marks it for degradation. The Cdc2-Cig2 complex is no longer inhibited and promotes entry into and progression through S phase. The mitotic cyclin-Cdk complex, Cdc2-Cdc13, is kept inactive during S and G2 phase by an inhibitory phosphorylation. This phosphorylation is maintained by Wee1 and Mik1. At the end of G2 phase the inhibitory phosphorylaion is removed by Cdc25, which activates the Cdc2-Cd13 complex and triggers entry into mitosis.

In the following chapters, DNA replication in S phase and the G2/M transition will be described in more detail.

Regulation of DNA replication

The genome size of higher eukaryotes is in the range of 107 to >109 base pairs (Kelly and Brown 2000). The large size of the genome brings several challenges: First, DNA replication has to be completed within the relatively short time frame of S phase. This is achieved by

initiating replication at multiple origins along the chromosomes. Furthermore, the genome has to be replicated accurately and only once per cell cycle. In order to avoid firing of the same origin twice within the same S phase, origin activation and initiation of DNA replication are separated in time. Origins are activated in early G1 phase, when Cdk activity is low. Firing of these origins is dependent on high Cdk activity and happens in S phase (Blow and Dutta 2005; Diffley 2010).

Origins are activated in a process called licensing. Licensing includes the assembly of a pre-replicative complex (pre-RC) at each origin of replication (Fig. 3). The pre-RC comprises the origin recognition complex (ORC), Cdc18, Cdt1 and the minichromosome maintenance protein complex (MCM). ORC binds to the origin of replication and recruits Cdc18 and Cdt1. ORC, Cdc18 and Cdt1 together facilitate the association of the MCM with DNA (Bell and Dutta 2002). The MCM is a hexamer containing the proteins Mcm2 through Mcm7 that form a ring-like structure. It has helicase activity and is important for unwinding of double-stranded DNA. With the pre-RC complex assembled in G1 phase, cells are competent to initiate DNA synthesis but are dependent on Cdk activity for the MCM helicase to be activated.

In order to initiate DNA replication and establish replication forks, the activity of two S phase-promoting kinases is required: The Cdk-cyclin complex Cdc2-Cig2 and Hsk1 with its regulatory subunit Dfp1. Both kinases contribute to the recruitment of Cdc45, GINS and additional replication co-factors to origins. This finally activates the MCM helicase and brings about origin firing and consequently DNA replication (Labib 2010).

Cdc45, MCM and GINS move with the replication fork during elongation while the pre-RC components Cdt1 and Cdc18 are phosphorylated by Cdc2 due to its increased S phase activity and become marked for degradation (Jallepalli et al. 1997; Kominami and Toda 1997). The degradation of pre-RC components considerably contributes to prevent the assembly of the pre-RC outside G1 phase and consequently inhibits re-replication (Kelly and Brown 2000; Bell and Dutta 2002; Arias and Walter 2007).

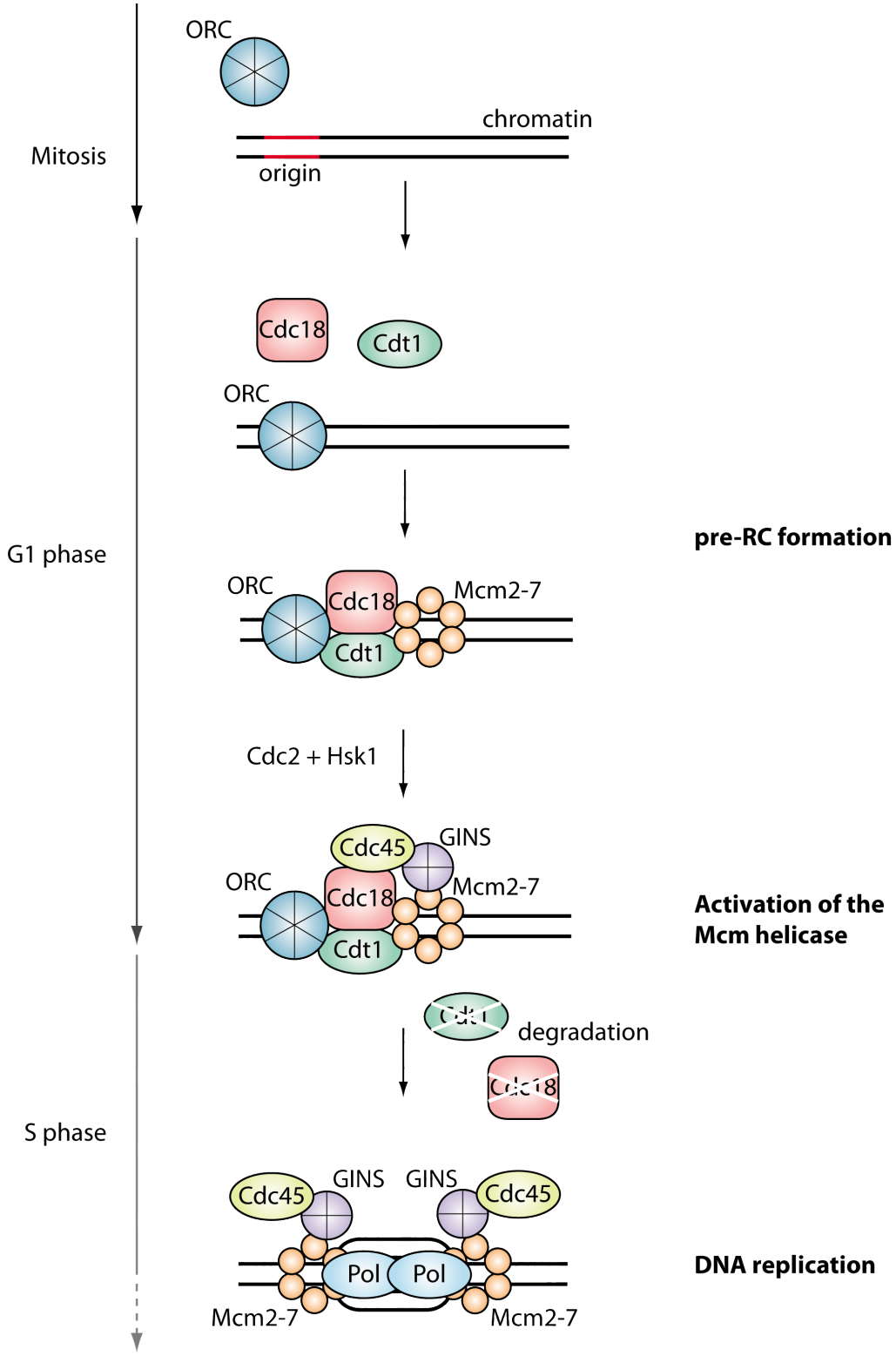

Figure 3. A simplified model of replication initiation. The ORC binds to origins in late mitosis. Followed by recruitment of Cdc18, Cdt1 and Mcm2-7 in G1 phase which together form the pre-RC. Upon entry into S phase Cdc2 and Hsk1 kinase activity promote activation of the Mcm helicase and subsequent DNA replication. Rereplication is prevented by multiple mechanisms including degradation of Cdc18 and Cdt1.

Regulation of the G2/M transition

Entry into mitosis is dependent on the activation of the cyclin-Cdk complex Cdc13-Cdc2. Its activity is regulated by inhibitory and activating phosphorylations and its subcellular localization. In S phase the Cdc13-Cdc2 complex is kept inactive by an inhibitory phosphorylation on Tyr15. Two kinases, Wee1 and Mik1, are responsible for maintaining this phosphorylation. *mik1* expression is dependent on the Cdc10 transcription factor and accumulates during S phase (Christensen, PU et al. 200) while the Wee1 protein level is constant throughout the cell cycle. Wee1 and Mik1 are counteracted by the phosphatase Cdc25 which removes the Tyr15 phosphorylation on Cdc2 when cells are ready to enter mitosis. In addition, Cdc2 activity is regulated by phosphorylation of its T-loop. This specific phosphorylation is mediated by the Cdk-activating kinase (CAK) Mcs6 and brings the T-loop in an active conformation allowing Cdc2 to bind its substrates (Hermand et al. 2001).

Wee1 kinase activity is controlled by the Pom1-Cdr2-Cdr1 signalling pathway (Fig. 4). Pom1 is an inhibitor of Cdr1/Cdr2, which in turn inhibit Wee1. Pom1 is located at the cell tips and forms a negative gradient towards the centre of the yeast cell. Cdr1 and Cdr2 are present together with Wee1 in nodes around the equator of the cell. In spite of the gradient leading to lower amounts of Pom1 in the middle, in small cells there is still enough Pom1 to inhibit Cdr1/Cdr2. Consequently, Wee1 is active and inhibits the cyclin-Cdk complex. As the cell grows, Pom1 is gradually depleted from the cortex in the middle of the cell and can no longer inhibit Cdr1/Cdr2 leading to inactivation of Wee1 (Martin and Berthelot-Grosjean 2009; Moseley et al. 2009). At the same time, the Cdc25 phosphatase accumulates in the cell. Together, the inhibition of Wee1 and activation of Cdc25 lead to the removal of the inhibitory phosphorylation on Cdc2 Tyr 15. Once the cyclin-Cdk complex is active, it promotes a positive feedback loop that further enhances Cdc25 activity and inhibits Wee1 to ensure complete activation of the Cdk (Fig.4). In this feedback loop, Cdc25 is both directly activated by the Cdk and through the mitotic kinases Fin1 and Plo1 (Grallert and Hagan 2002; Grallert et al. 2012). Fin1 is activated by Sid2 and promotes recruitment of Plo1 to the spindle pole body (SPB), the equivalent to the mammalian centrosome. SPB-associated Plo1 activates Cdc25 and inhibits Wee1 resulting in complete activation of the Cdk and commitment to mitosis (Grallert and Hagan 2002). In response to nutrient changes, phosphorylation of Plo1 on Ser402 by Aurora

kinase Ark1 promotes Polo recruitment to the SPB (Petersen, Hagan 2005; Halova, Petersen 2011).

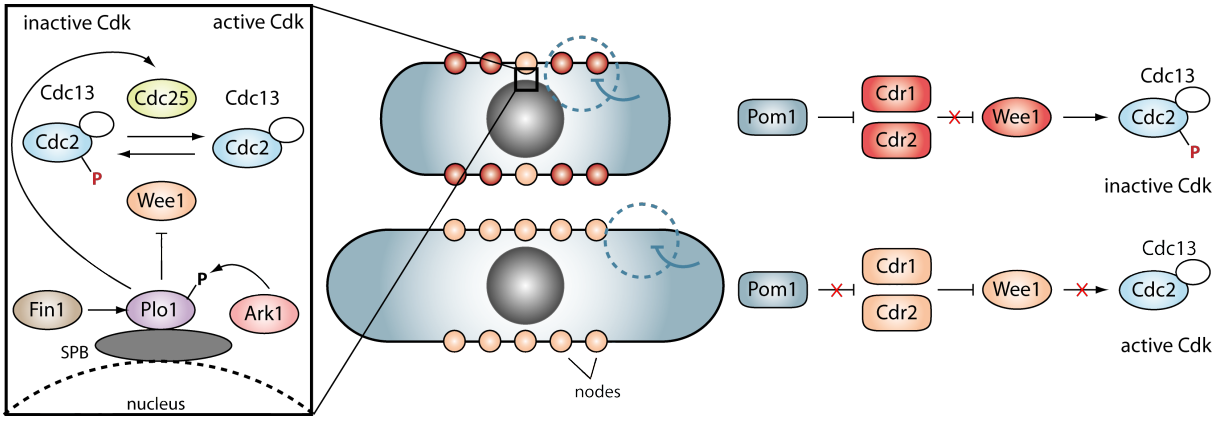

Figure 4: Activation of the Cdc2-Cdc13 complex upon entry into mitosis. The cyclin-Cdk complex is first activated at the SPB. Fin1 recruits Plo1 to the SPB, which promotes a positive feedback loop to activate the Cdk. In small cells the Pom1 gradient inhibits cyclin-Cdk activation throughout the cell (modified after Sawin 2009).
Upon changes to poor nutrients, Ark1 phosphorylates Ser402 on Plo1, which leads to premature recruitment of Plo1 to the SPB and acceleration into mitosis.

Checkpoints

The activity of cyclin Cdk complexes regulates transition from one cell-cycle phase to the next under unperturbed conditions. However, in response to DNA-damage, the cell has evolved mechanisms that can block cell-cycle progression, which allows DNA repair, preventing that damage is inherited by daughter cells. These mechanisms are called checkpoints. Misregulation of checkpoints can have serious consequences and may cause genomic instability, which is a hallmark of cancer.

Specifically, checkpoints include mechanisms to inhibit mitosis when the DNA is damaged (G2/M checkpoint) or when S phase is incomplete as well as a mechanism to inhibit ongoing DNA replication when forks stall or the DNA is damaged (S-phase checkpoint). In addition, the spindle assembly checkpoint (SAC) in metaphase responds to problems regarding spindle assembly and correct attachment to kinetochores.

The G2/M checkpoint

The G2/M checkpoint detects DNA lesions that arise in G2 phase of the cell cycle and inhibits entry into mitosis until damaged DNA is repaired. The checkpoint response consists of an evolutionary conserved signalling cascade comprising sensor, mediator and effector proteins (Fig. 5). Central to the checkpoint pathway is the PI3K-like kinases Rad3 (ATR^{Hs}), which plays an important role in checkpoint signalling. Rad3 belongs to the group of sensor proteins that localize to damaged DNA at the beginning of the signalling cascade. Rad3 is recruited to sites of DNA-damage by RPA, a single-strand DNA (ssDNA) binding protein, and Rad26. Additionally, the checkpoint sliding clamp, Rad9-Rad1-Hus1 (9-1-1 complex), is independently recruited and loaded at the damage site (Parrilla-Castellar et al. 2004). The 9-1-1 complex recruits Cut5/Rad4 which in turn is crucial for Rad3 activation (Furuya et al. 2004). Rad3, in complex with Rad26, activates the effector kinase Chk1 (reviewed in Humphrey et al. 2005). The interaction of Rad3 and Chk1 is facilitated by mediator proteins Cut5 and Crb2, which are necessary for Chk1 recruitment to the damage site (Qu et al. 2012). Active Chk1 targets Cdc25 for phosphorylation. Phosphorylation of Cdc25 inhibits its phosphatase activity but serves also as a binding site for Rad24, which sequesters Cdc25 in the cytoplasm thereby inhibiting its nuclear import (Zeng and Piwnica-Worms 1999; Lopez-Girona et al. 2001). Wee1, usually responsible to restrain Cdk activity during S and G2 phase under unperturbed conditions, is also phosphorylated by Chk1 in response to DNA-damage. Its contribution to the checkpoint response is, however, debated in the literature (O'Connell et al. 1997; Rhind and Russell 2001). Cdc2 is the final target in the checkpoint signalling cascade. Cell-cycle progression is ultimately blocked through sustained phosphorylation of Tyr15 on Cdc2.

Another protein that has been implicated to have a role in the DNA-damage response in G2 phase is the mitogen-activated protein kinase (MAPK) Sty1, which is the fission yeast homolog of p38 (Alao and Sunnerhagen 2008). Although it has not been shown to be part of the checkpoint pathway above it is known to be involved in a wide range of stress responses (Millar et al. 1995; Degols and Russell 1997; Chen et al. 2003).

Sty1 is activated after exposure to DNA-damaging agents like UVC and ionizing radiation (IR) (Degols and Russell 1997; Watson et al. 2004). Interestingly Sty1 is not activated by direct damage to the DNA but rather by the oxidative stress caused by UVC irradiation. After UVC

exposure Sty1 is phosphorylated in a Wis1-dependent manner and activates the Atf1 transcription factor (Degols and Russell 1997). Atf1 is responsible for upregulation of environmental stress-response genes. However, upregulation of Atf1 target genes has very little effect on cell survival, indicating that downstream effectors of Sty1 others than Atf1 are more important.

In response to other types of stress than UVC, such as starvation or changes in nutrient quality, Sty1 activation leads to activation of Cdc25 and acceleration into mitosis mediated by a premature recruitment of Plo1 to the SPB (Kishimoto and Yamashita 2000; Petersen and Hagan 2005). Moreover, Sty1 has been shown to activate the Srk1 kinase in response to osmotic stress. Srk1 phosphorylates Cdc25 and promotes its translocation to the cytoplasm, which leads to a cell-cycle arrest and delayed entry into mitosis (Lopez-Aviles et al. 2005; Lopez-Aviles et al. 2008). Whether Srk1 is also a downstream target of Sty1 after UVC irradiation has not been shown, but since Srk1 mutants are not sensitive to UVC it is unlikely to play an important role in cell survival.

The S-phase checkpoint

There are several terms in the literature describing checkpoint responses during the DNA-synthesis phase. To avoid confusion, I have chosen the term S-phase checkpoint and define it as follows: The S-phase checkpoint is activated in response to stalled replication forks, damaged DNA and/or aberrant DNA structures. This checkpoint has to manage four important things: It has to block entry into mitosis, inhibit further origin firing, stabilize the replication forks to prevent fork collapse and activate deoxynucleoside triphosphate (dNTP) production via regulation of the ribonucleotide reductase (RNR) (Labib and De Piccoli 2011).

The S-phase checkpoint (Fig. 5) shares many proteins with the G2/M checkpoint, such as the Rad3-Rad26 complex and the 9-1-1 complex. However, in the S-phase checkpoint Rad3 exerts its effect through a different effector kinase, namely Cds1. Cds1 localizes to stalled replication forks or DNA lesions in an Mrc1-dependent manner. Mrc1 serves as a docking site for Cds1 and brings Cds1 in close proximity to Rad3 for activation. Cds1 regulates Cdc25 in a similar way as Chk1 in the G2/M checkpoint (Furnari et al. 1999). Cds1 activates Mik1 and to a smaller degree Wee1 (Boddy et al. 1998). The activation of Mik1 results in a sustained

phosphorylation of Tyr15 and inhibition of Cdc2 activity, which consequently slows down progression through S phase and inhibits entry into mitosis.

In order to repair damaged DNA the cell depends on the availability of dNTPs. The production of dNTPs is regulated through the RNR inhibitor Spd1. One mechanism of how Spd1 activates dNTP production after DNA-damage is through re-localisation of the small RNR subunit (Suc22) to the cytoplasm, which leads to RNR complex formation and dNTP synthesis (Nestoras et al. 2010).

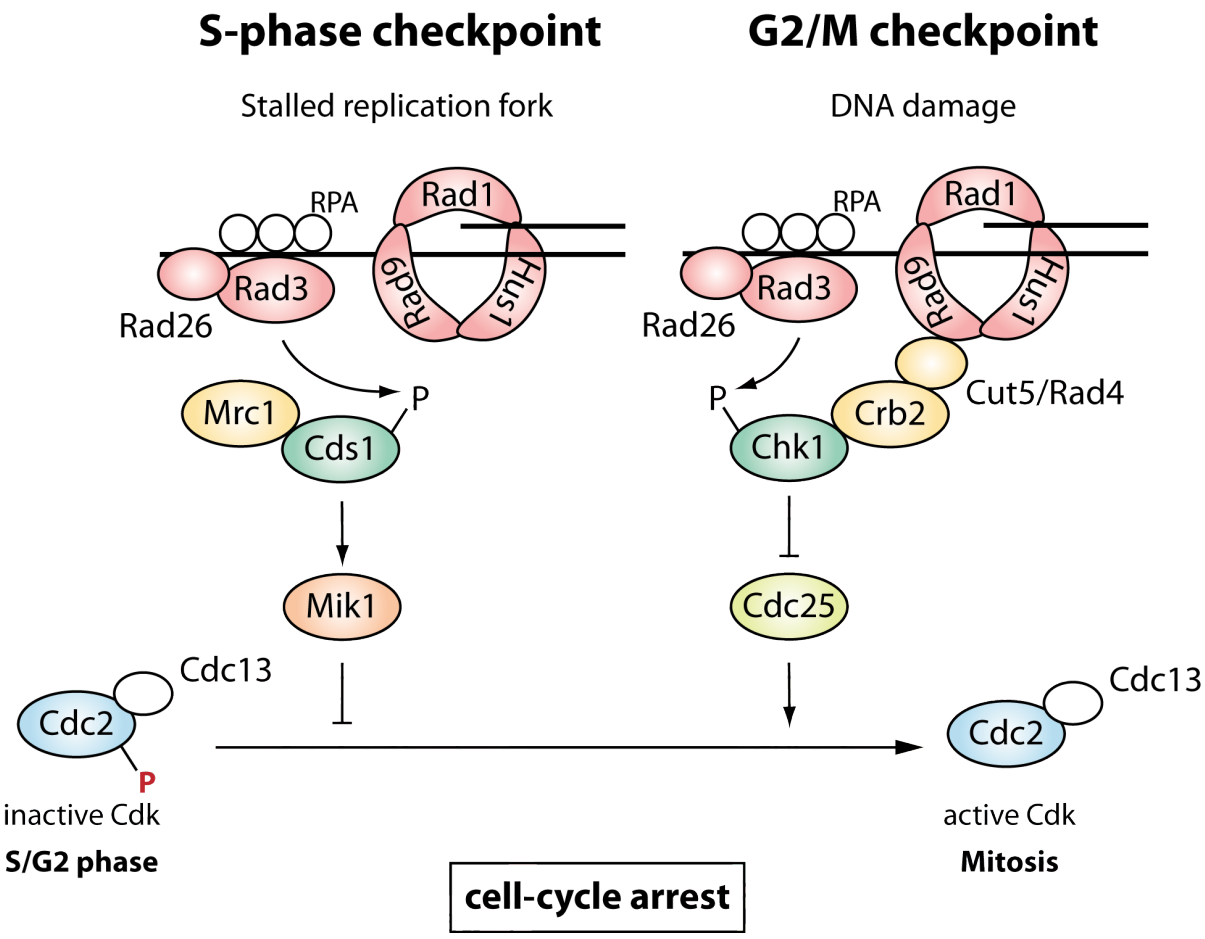

Figure 5: Simplified model of the S- and G2/M checkpoint. In response to replication stress or DNA-damage the sensor complexes Rad3-Rad26 and 9-1-1 complex are recruited to DNA. In the S-phase checkpoint Rad3 phosphorylates and activates Cds1 leading to the activation of Mik1. In the G2/M checkpoint Rad3 phosphorylates and activates Chk1 leading to the inhibition of Cdc25. Both checkpoints result in the sustained phosphorylation of Cdc2 and a cell-cycle arrest. (sensor proteins = red; mediator proteins = yellow; effector proteins = dark green)

The spindle assembly checkpoint

For proper chromosome segregation, it is important that each chromosome is attached to spindle microtubules emanating from opposite poles prior to anaphase. This socalled bi-oriented attachment creates tension between the chromatids. Large protein complexes that assemble on centromeric DNA, called kinetochores, mediate the attachment of microtubules to the chromosome. The spindle assembly checkpoint (SAC) is triggered when kinetochores lack tension or attachment to spindle microtubules (London and Biggins 2014). The checkpoint thereby ensures that the cells remain in metaphase until correct attachment is achieved. SAC activation is dependent on Ark1, the fission yeast homolog of Aurora B. Ark1 is responsible for the recruitment of Mad2, one of the core SAC proteins, to unattached kinetochores (Petersen & Hagan, 2003). Mad2 accumulates at high levels at unattached kinetochores and binds the APC/C-activator Slp1 (Cdc20 in mammalian cells) thereby inhibiting the activity of the APC/C and progression into anaphase (Hauf 2013; Musacchio 2015).

Protein synthesis - translation

Protein synthesis is the final process in gene expression in which the genetic information encoded by a messenger RNA (mRNA) molecule is translated into protein. Translation is performed by ribosomes in the cytoplasm and is divided into three phases: initiation, elongation and termination (Fig.6). Ribosomes consist of a small subunit (40S in eukaryotes) and a large subunit (60S in eukaryotes). At the initiation step, the small ribosomal subunit binds the 5'end of the mRNA and scans along it until it finds the AUG start codon where the joining of the large subunit completes formation of the active 80S ribosome. The ribosome now moves along the mRNA and a polypeptide chain is built according to the mRNA sequence (elongation). Another RNA molecule facilitates delivery of the amino acids to the translation machinery: transfer RNA (tRNA) coupled to an appropriate amino acid. Each tRNA consists of an anticodon-loop with three bases that are complementary to a nucleotide triplet (codon) on the mRNA. Translation is terminated at the stop codon, where the polypeptide chain and the ribosomal subunits are released.

Several other proteins are involved in the process of protein synthesis. They are generally known as translation factors.

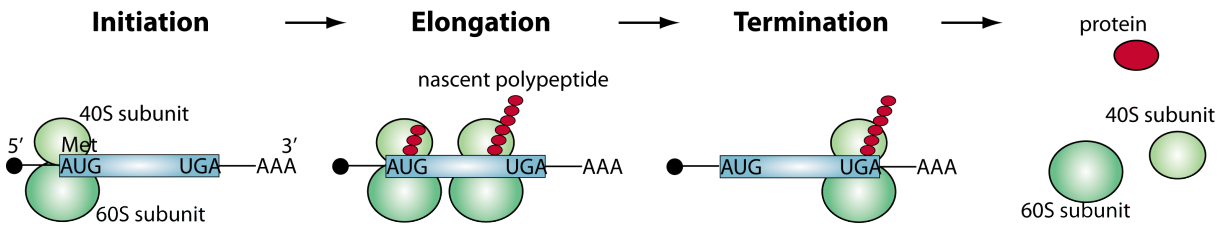

Figure 6: Simplified model of translation (modified after Scheper et al. 2007)

Cells are able to regulate protein synthesis and quickly respond to environmental changes. Most control mechanisms target the initiation step of translation rather than elongation or termination. In order to understand the regulatory mechanism controlling translation, the initiation step of translation (Fig.7) is described in more detail below.

At the beginning of translation initiation the small 40S ribosome and the four eukaryotic initiation factors eIF1, eIF1A, eIF3 and eIF5 associate with the ternary complex consisting of GTP-bound eIF2 and the initiator Met-tRNA to form the 43S pre-initiation complex. Meanwhile the eIF4F complex containing eIF4E, eIF4A and eIF4G associates with the m7G-cap at the 5' terminus of the mRNA (Gebauer and Hentze 2004). Interaction between eIF3 and the eIF4F complex, in particular the eIF4E component, leads to binding of the 43S pre-initiation complex to the 5'end of the mRNA. Next, the 43S pre-initiation complex migrates across the 5' untranslated region (5'UTR) in a 5'→3' direction until the anticodon of initiator Met-tRNA recognises the start codon. This leads to the hydrolysis of the eIF2-bound GTP and release of eIF2-GDP and other eIFs (Sonenberg and Hinnebusch 2009; Jackson et al. 2010). This enables the 60S ribosome to bind and the catalytically active 80S ribosome is now ready for elongation. The described mode of initiation depends on the cap structure at the 5'end of the mRNA and is therefore known as cap-dependent initiation. Although most mRNAs use this mechanism of initiation, some mRNAs contain secondary structures in their 5'UTR that enable the ribosome to bind directly at the start codon. This type of initiation where the start codon is not identified by scanning is called internal ribosome entry site (IRES)-mediated initiation.

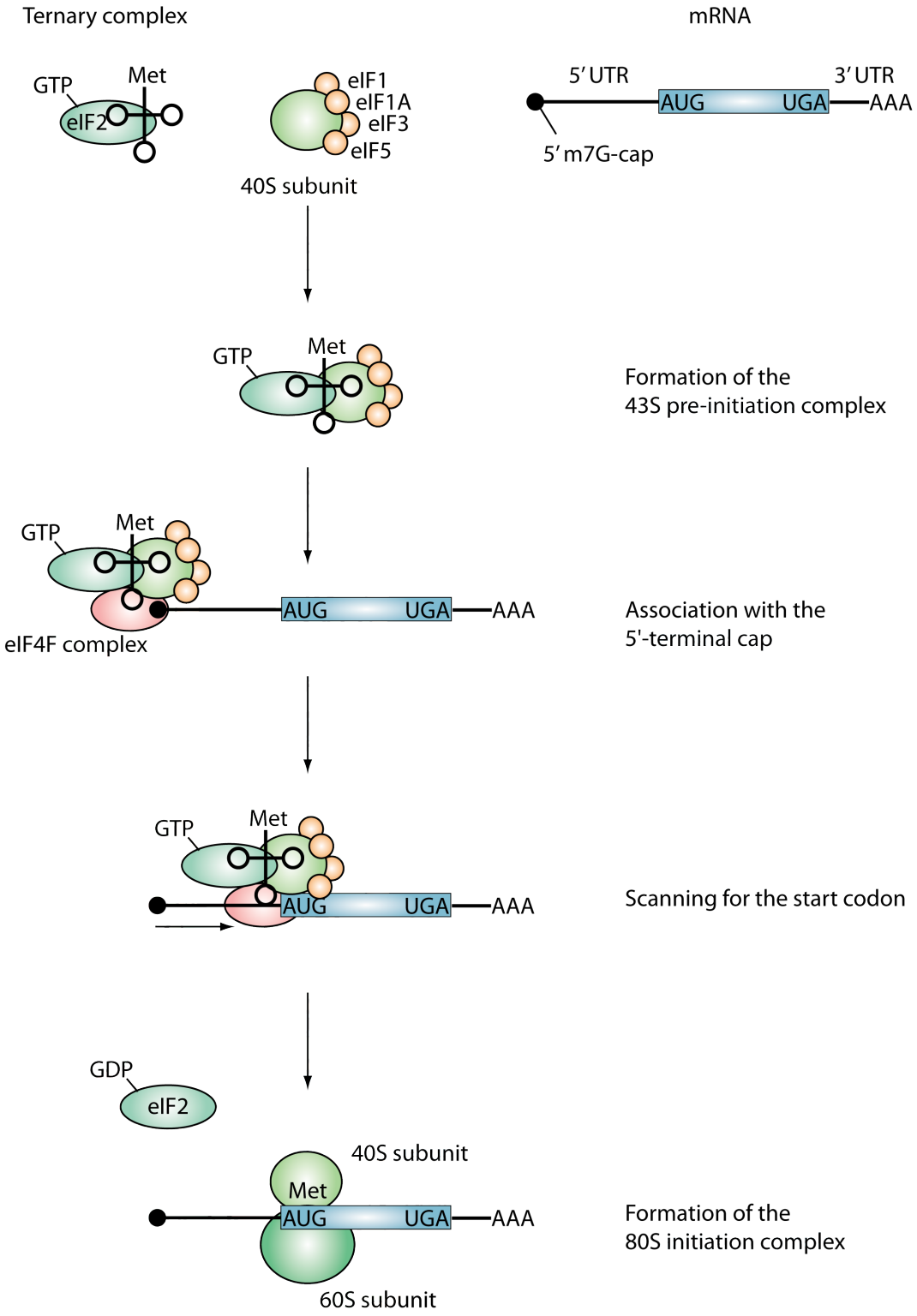

Figure 7: Cap-dependent translation initiation. The 43S pre-initiation complex, comprising the 40S ribosomal subunit with four eIFs and the ternary complex, binds the 5'-terminal cap and scans along the mRNA to find the start codon (AUG). Recognition of the start codon leads to hydrolysis of eIF2-bound GTP and release of eIF2-GDP. Joining of the 60S ribosomal subunit completes formation of the 80S initiation complex (modified after Scheper et al. 2007).

Translational control mechanisms

Regulation of translation is a way to modulate the amount of protein that is being produced. It enables the cell to respond to nutrient availability, cellular energy, stress, hormones and growth-factor stimuli (Ma and Blenis 2009). Commonly one distinguishes between global and selective downregulation of translation. A reduction in global translation affects expression of many transcripts at the same time and is achieved through phosphorylation of one of the initiation factors. The best characterized example is phosphorylation of the alpha subunit of eIF2 (Gebauer and Hentze 2004). As mentioned above eIF2-GTP is part of the ternary complex that binds the 40S ribosome. The GTP is hydrolysed to GDP at a later stage of the initiation process and eIF2-GDP is released from the ribosome. In order for eIF2 to participate in a new round of initiation the GDP has to be exchanged with GTP. This reaction is inhibited when eIF2α is phosphorylated at Ser52 and results in a reduced level of active ternary complex and, consequently, a block of global protein synthesis. The kinases that are known to catalyse phosphorylation at Ser52 of eIF2α in *S. pombe* are Gcn2, Hri1 and Hri2. Other mechanisms that regulate global translation involve the disruption of the eIF4F complex by inhibition of the cap-binding protein eIF4E but are not further explained here.

Selective downregulation of specific mRNAs is usually achieved through binding of proteins to cis-elements in the UTR of the target transcript (Mazumder et al. 2003). These cis-elements involve secondary and tertiary structures and upstream open reading frames (uORFs) in the 5'UTR as well as a polyadenylation (poly(A)) signal in the 3'UTR. Most of these regulatory elements are inhibitory. They prevent efficient scanning or block interaction with the 43S pre-initiation complex and other regulatory proteins. Exceptions are secondary structures that facilitate ribosome binding in IRES-mediated translation initiations. Polyadenylation of the 3' terminus usually has a positive effect on mRNA stability but shortening of the poly(A) tail is a way to downregulate mRNA levels and thus protein amount (Gebauer and Hentze 2004). Thus, the structure of UTR sequences can give information about the translation efficiency of the transcript.

Regulation of cyclins during the cell cycle

In fission yeast the fluctuation of cyclins during the cell cycle is mainly caused by post-translational regulation, namely proteolytic degradation (Glotzer et al. 1991; Yamano et al. 1996; Yamaguchi et al. 1997; Yamano et al. 2000). For example, the periodicity of Cdc13, the only cyclin required for cell viability, is achieved by the APC/C which targets Cdc13 for destruction by the proteasome (Yamano et al. 1998). The APC/C is active from anaphase throughout G1 and accumulation of Cdc13 protein is seen from S phase to mitosis. Out of the four cyclins in *S. pombe*, only accumulation of Cig2 has been shown to be strongly regulated by transcription (Ayte et al. 2001). This is in contrast to mammalian cells and budding yeast where the regulation of cyclins at the transcriptional level plays an important role (Bahler 2005). In addition, expression of both Cdc13 and Cig2 seem to be subject to translational control. Both cyclins have extremly long 5'UTR which makes them dependent on helicase activity from eIF4A and Ded1 (Daga and Jimenez 1999; Grallert et al. 2000). These helicases facilitate ribosome scanning and help to overcome potential secondary structures and uORFs (Jackson et al. 2010).

Aims of the study

The major regulators of cell-cycle progression are cyclin-Cdk complexes. Periodic inactivation and activation of these complexes control the transition from one cell-cycle phase to the next. This work aimed to gain new insight into how cyclin-Cdk complexes are regulated and how stress-response pathways exploit the multiple levels of their regulation. The objectives of the individual projects are presented below.

Paper I

Cell-cycle analysis of fission yeast cells by flow cytometry

Flow cytometry is a powerful method where multiple physical characteristics of single cells are analysed simultaneously. The aim of this paper was to further improve cell-cycle analysis of fission yeast cells by flow cytometry. We wished to develop a method that enables us to easily distinguish cell-cycle phases in order to monitor cell-cycle progression in different mutants or after exposure to stress.

Paper II

Consequences of abnormal CDK activity in S phase

While the regulation of Cdk activity at entry into mitosis is well characterized, much less was known about how Cdk activity is regulated during S phase. We wished to study the consequences of increasing and decreasing Cdk activity in S phase. To this end, we investigated the roles of known Cdk regulators in S phase in order to identify the important players. By using appropriate mutants in these regulators we increased and decreased Cdk activity and investigated the effects on genome stability.

Paper III

A checkpoint-independent mechanism delays entry into mitosis

Cell-cycle checkpoints are crucial for the integrity of the genome. They block cell-cycle progression by inhibiting Cdk activity and thereby provide time to repair DNA-damage or allow completion of a cell cycle phase. The DNA-damage checkpoint in G2 phase ensures that cells enter mitosis without any damage. Checkpoints are often deficient in cancer cells and thus these cells rely on alternative pathways. Here we set out to investigate checkpoint-independent pathways that regulate entry into mitosis after UVC irradiation.

Summary of papers

Paper I

Cell-cycle analysis of fission yeast cells by flow cytometry.

Flow cytometry measures the characteristics of single cells. Analysis of cellular DNA content by flow cytometry is a useful tool to monitor cell-cycle progression. The DNA content of a cell gives information about where it is in the cell cycle. In a eukaryotic cell cycle in a haploid organism, the nucleus of G1 phase cells contains a single copy of the genome (1C). In S phase cells duplicate their genome and consequently the nucleus has a genome size between 1C and 2C. In G2 phase and mitosis cells have a 2C DNA content. However, in fission yeast cell-cycle analysis poses some challenges. First, under standard laboratory growth conditions fission yeast cells complete cytokinesis at the end of S phase. Therefore, G1 cells have two nuclei each with a single complete genome (1C DNA). Hence, the total amount of DNA of a G1 cell is 2C (2x 1C DNA) which is the same DNA amount as a G2 phase cell (2C DNA). For this reason, G1 and G2 phase cells cannot be distinguished by measuring DNA content alone (Fig. 8A). And second, the formation of cell doublets, i.e. individual cells sticking together, interferes with the analysis of single-cell behaviour and cell-cycle kinetics. In this paper, we show that we can solve both problems. We exploit the rod-shaped nature of fission yeast cells, which orients the cell parallel to the laminar flow. Fission yeast cells in G1 phase are binuclear and will therefore give a longer-lasting DNA signal when passing through the excitation focus of the laser beam than G2 phase cells with only one nucleus. Thus, measuring the width of the DNA signal (DNA-W) in addition to the total DNA content (DNA-A) will result in a cytogram where mononuclear G2 cells can be distinguished from the binuclear G1 cells (Fig. 8B). Cell doublets are best discriminated from single cells when measuring forward (FSC) and side scatter (SSC). Single cells show very little light scattering and have a tight distribution close to the origin of the cytogram while cell doublets have a very broad distribution.

Taken together, measuring DNA-W in addition to DNA-A allows discrimination of M/G1 cells and G2 phase cells. Further improvement of cell-cycle analysis is achieved by separation of

single cells from doublets in a FSC/SSC cytogram. This method is technically very simple and allows detailed cell-cycle analysis, e.g. monitoring entry into mitosis of a synchronous G2 culture.

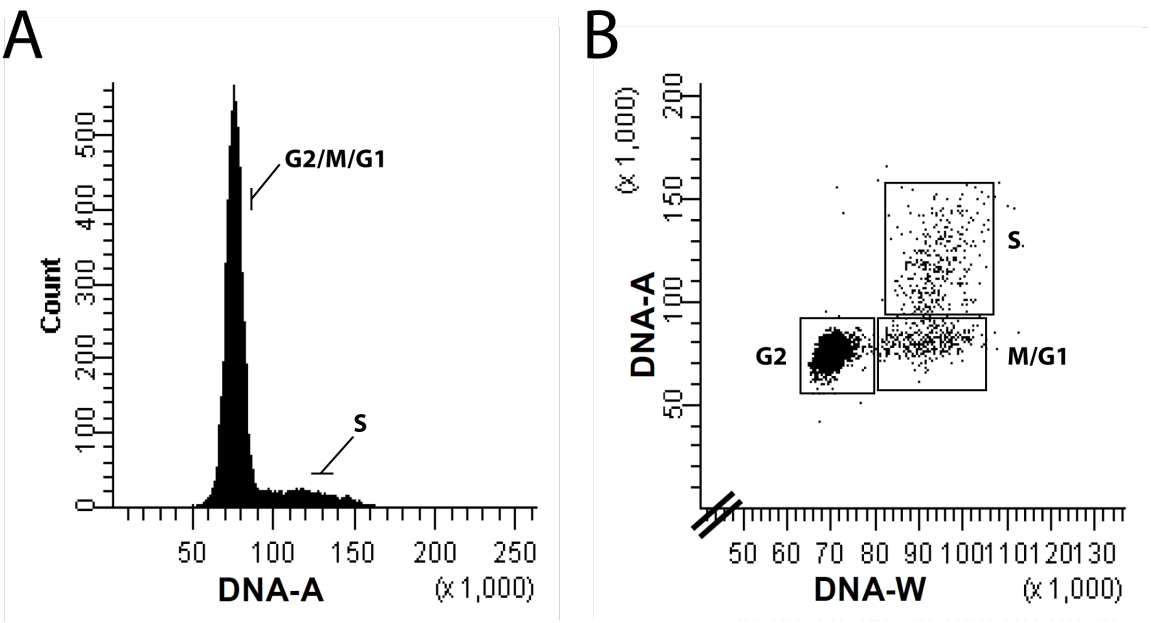

Figure 8. Cell-cycle analysis of exponentially growing *S. pombe* cells by flow cytometry.
(A) The DNA histogram shows two populations: G2/M/G1-phase cells and S-phase cells. (B) The DNA-A/DNA-W cytogram allows discrimination of G2-, M/G1- and S-phase cells (modified from paper I).

Paper II

Consequences of abnormal CDK activity in S phase

Activity of the cyclin-dependent kinase (Cdk) is carefully regulated in the cell cycle and governs the onset of S phase and mitosis. In S phase the cell duplicates its chromosomes by DNA replication and Cdk activity is needed to control the initiation step of DNA replication. We show here that Wee1 is an important regulator of S-phase Cdk activity. Loss of Wee1 leads to increased Cdk activity concomitant with altered regulation of replication. We show that a *wee1*$^{-}$ mutant has increased incorporation of the nucleotide analogue EdU and more Cdc45-positive cells. This suggests that increased Cdk activity in the *wee1*$^{-}$ mutant results in a higher number of active replication forks. We reasoned that if cells have more ongoing replication

they must be more vulnerable to replication stress. Indeed, when *wee1*$^{-}$ mutants are challenged by reduced nucleotide pools they show very poor survival. Furthermore, the increased replication stress in *wee1*$^{-}$ mutants leads to increased DNA-damage, particularly in the absence of checkpoint genes such as the ATR homologue *rad3*. Consequently, *wee1*$^{-}$ cells depend on the S-phase checkpoint for survival. Cdc25 is counteracting the inhibitory activity of Wee1 at entry into mitosis. We demonstrate that Cdc25 is involved in the regulation of Cdk activity in S phase. The lack of Cdc25 and thus decreased Cdk activity results in reduced incorporation of EdU and confers resistance to reduced nucleotide pools.

We conclude that correct regulation of CDK activity by Wee1 and Cdc25 in S phase is important to maintain genomic stability.

Paper III

A checkpoint-independent mechanism delays entry into mitosis

In response to DNA-damage, e.g. caused by UV radiation, cells activate signalling cascades, called checkpoints that stop cell-cycle progression and allow time for DNA repair. The checkpoint operating in G2 phase of the cell cycle prevents entry into mitosis through inhibitory phosphorylation of the Cdk. In this work, we show that the cells are able to delay entry into mitosis after irradiation with UVC independent of the known checkpoint proteins (Rad3, Chk1 and Cds1). We considered other regulators of the G2/M transition, such as the mitotic kinases Fin1, Plo1 and Ark1, and Wee1 as a cause for the arrest, but none of these regulators was responsible for the delay. In addition, we show that the checkpoint-deficient *rad3*$^{-}$ cells arrest with an inactive Cdk. This indicates that the mechanism causing the delay inhibits Cdk activity. Both availability of the cyclin and subsequent formation of the mitotic cyclin-Cdk complex are requirement for an active Cdk and entry into mitosis. We find that there is a distinct reduction in the protein level of the mitotic cyclin Cdc13 after exposure to UVC. To investigate the regulation of the cyclin after UVC irradiation we isolated polysomes from UVC-irradiated and untreated cells and show that less *cdc13* mRNA is found on translating ribosomes after UVC-irradiation. We conclude that the checkpoint-independent mechanism delays entry into mitosis through selective downregulation of cyclin B translation.

Methodological challenges

Flow cytometry as a tool to analyse cell-cycle progression

Flow cytometry is an important tool when studying the cell cycle. This method measures light scattering and fluorescence intensities of single cells. The result is usually presented in a DNA histogram, which shows the DNA-associated fluorescence intensity on the x-axis and cell number on the y-axis. The DNA histogram of exponentially growing *S. pombe* cells shows two populations of cells. First, a population with a low fluorescence signal and hence low DNA content (2C = 2 copies of the genome) which contains G2-, M- and G1-phase cells. Second, a population with higher fluorescence signal and a DNA content greater than 2C which comprises the S-phase cells. In paper I we describe how we can discriminate G2 from M/G1 phase cells by flow cytometry, namely by measuring the width of the DNA-associated fluorescence signal. Cells in M/G1 contain two nuclei, which will give a greater DNA-width signal than the mononuclear G2 cells. Thus, by measuring the DNA-width in addition to the DNA content we can distinguish G2, M/G1 and S-phase cells. This is a great improvement and allows more detailed cell-cycle analyses. However, this method has some limitations and is only applicable when cell morphology is intact. The basis of this method is the cylindrical shape of the cells, which aligns them parallel to the laminar flow and allows them to pass the excitation focus in the same orientation. Mutants such as the *wee1*$^{-}$ mutant are difficult to analyse by this method because they do not have the pronounced rod-shaped appearance. These mutants have problems to align parallel with the laminar flow and measuring DNA-width does not give a reliable result. Alternatively, the difference in the DNA-width signal between mononuclear and binuclear cells might not be great enough in small *wee1*$^{-}$ cells to distinguish them (Fig. 9). Another critical factor is the condition of the DNA/chromosomes. Cell-cycle distributions of mutants that show chromosome fragmentation during mitosis such as the UVC-irradiated *rad3*$^{-}$ mutant are also difficult to analyse (Fig. 9).

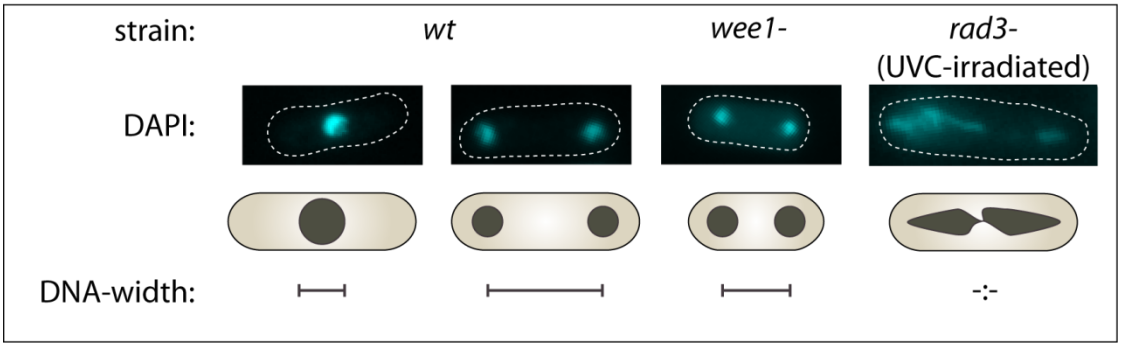

Figure 9: Illustration of challenges in flow-cytometry analysis of *wee1*$^{-}$ and *rad3*$^{-}$ mutant.

Working with conditional mutants - the *wee1-50* strain

In paper II and paper III we used the temperature-sensitive *wee1* mutant, *wee1-50*. In this mutant Wee1 function is maintained at the permissive temperature (25°C) but lost at restrictive temperature (36°C), resulting in premature entry into mitosis and division at a small size (Nurse 1975; Russell and Nurse 1987). Loss of Wee1 is synthetic lethal with checkpoint *rad* mutants (al-Khodairy and Carr 1992). Therefore, we took advantage of this temperature-sensitive *wee1* allele to investigate the consequences of *wee1* loss in checkpoint-deficient cells. Although the *rad wee1-50* double mutant is viable we observe that Wee1 function is compromised already at the permissive temperature. First, *wee1-50* single mutants divide at a smaller size than *wt* cells. Furthermore, the compromised function of Wee1 at 25°C is reflected in experiments measuring incorporation of the nucleotide analogue EdU and Cdc45 loading (paper II). The results for the *wee1-50* mutant at 25°C are more similar to that of the *wee1-50* mutant at 36°C than to the *wt* strain, like one would expect if Wee1 function was normal. In agreement with these results is also the activation of Cds1 (as measured by Mus81 phosphorylation in the *wee1-50* mutant) already at the permissive temperature (paper II).

The compromised activity of *wee1-50* at 25°C has been observed by others as well (Rhind and Russell 2001).

Limitation of synchronization

A prolonged checkpoint-delay in G2 phase, such as the one induced by UVC in *wt* cells, can be observed also in asynchronous cultures. However, shorter delays are apparent only when the cells are synchronized prior to the treatment. In order to study more subtle effects of UVC on cell-cycle progression we synchronised cells in G2 phase. There are two methods of choice: synchronization by size selection or synchronization by block and release of a *cdc25-22* conditional mutant. Both methods have their advantages and drawbacks. Size-selection methods such as lactose gradient centrifugation and elutriation select the smallest cells in a population. The synchrony achieved with this method is highly dependent on experimental skills but also on the size distribution of the strain used. On the other hand, size selection is considered a very gentle synchronization method, which is not interfering much with the subsequent cell-cycle progression.

Cdc25 block and release takes advantage of the temperature-sensitive *cdc25-22* mutant. The Cdc25 phosphatase is inactivated at the restrictive temperature of 36°C and thereby arrests the cells in late G2 phase. Release from the temperature block after four hours of incubation leads to synchronous entry of cells into mitosis. Cdc25 block-and-release is very easy to perform and gives a very good synchrony. But cells already in G2 phase at the start of the temperature block, will remain there for the whole incubation time, grow and accumulate proteins. Therefore, the experimental conditions in a yeast culture synchronised with Cdc25 block and release might be more artificial than after synchronisation based on size.

For our purposes the synchrony achieved by elutriation was not satisfactory and using Cdc25 block and release on its own was not optimal either. Therefore, we used, when possible, a combination of both synchronization methods. First, we synchronised a *cdc25-22* strain using elutriation and then synchrony was improved further by a one-hour block at 36°C.

Choice of reference gene for quantitative real-time PCR

For our purposes an optimal reference gene for mRNA expression studies should have at least two features. First and most importantly, transcription of the reference gene should be stable during the cell cycle and not influenced by UVC irradiation.

We chose *leu1*, which codes for an enzyme in leucine synthesis, and *nda3*, which encodes beta tubulin, as reference genes. To our knowledge, neither of these genes is cell-cycle regulated or regulated at the transcriptional level upon irradiation. Second, the mRNA of the reference gene should have a similar amount of mRNA molecules per cell as the target gene. There are as few as 12 *cdc13* mRNA molecules per cell (www.pombase.org). *leu1* and *nda3* have 31 and 25 mRNA molecules per cell, respectively. For comparison, rRNA, which is frequently used as a reference gene in transcriptional studies, has 38 000 molecules per cell and is therefore not suitable for our purposes.

We also used *leu1* as reference gene when we isolated actively translated mRNAs from polysomes. UVC irradiation leads to global downregulation of translation, which also effects *leu1* translation. However, it is unlikely that translation of the *leu1* mRNA is selectively regulated after UVC irradiation. Furthermore, selective translation is often brought about by long UTRs in the mRNA. The *leu1* message has short untranslated regions (5‘ + 3‘ UTR = 217 nt), consistent with our presumption that it can serve as an appropriate reference also for polysome analysis.

Discussion

Regulation of Cdk activity in S phase

During S phase the genome is replicated completely and accurately. This requires careful regulation of origin firing, which, in turn, is dependent on Cdk-s. Cdk activity is needed for loading of auxiliary factors such as Cdc45 and GINS onto replication origins which subsequently activate the Mcm helicase complex. The activity of the cyclin-Cdk complex is kept at a low level and restrained by inhibitory phosphorylation on Tyr15 (Morla et al. 1989) at the start of the cell cycle. There are two kinases phosphorylating Cdc2 on Tyr15, Wee1 and Mik1 (Lundgren et al. 1991; Gould and Feoktistova 1996). Previously it was thought that Mik1 is regulating Cdk activity in S phase. This assumption is based on the observation that Mik1 expression is cell-cycle regulated in a Cdc10-dependent manner and that it accumulates upon activation of the S-phase checkpoint (Christensen et al. 2000; Rhind and Russell 2001). Wee1 has a major role in G2 phase controlling entry into mitosis. Whether it has any function in other cell-cycle phases had not been studied. However, there were indications in the literature that Wee1 might have some function outside G2 phase. In particular, its sensitivity to the topisomerase inhibitor camptothecin, which leads to breaks at DNA replication forks, and hydroxyurea, an inhibitor of dNTP synthesis (Mahyous Saeyd et al. 2014) indicate a function of Wee1 in S phase.

We have investigated this further and show that Wee1 has an important role in regulating Cdk activity in S phase (paper II) . We have monitored two important S-phase events: Loading of Cdc45, an indicator for active origins, onto chromatin, and incorporation of the nucleotide analogue EdU. We find that in the absence of Wee1, more cells are EdU-positive than in wild-type cells. This suggests that the loss of *wee1* leads either to higher replication rates or that these cells simply spend more time in S phase. Also Cdc45 loading was affected when Wee1 was lost. Cdc45 is a cofactor of the replicative helicase and its loading onto chromatin is dependent on Cdk activity. The *wee1*$^{-}$ mutant shows more cells with chromatin-bound Cdc45 compared to wild-type cells. Both results suggest altered regulation of replication in the absence of Wee1, presumably caused by increased Cdk activity.

Enhanced EdU incorporation, seen as an increase in EdU-positive cells, can be the consequence of either increased origin firing, meaning that replication is initiated at more origins than usual or it can be the result of higher replication fork speed leading to longer stretches of DNA being synthesized in a short time. A third explanation is that there are more EdU-positive cells in the unsynchronized culture because cells delay in S phase so that a larger fraction of cells are in S phase at any one time. An increase in Cdc45-positive cells indicates that more origins are fired, therefore the enhanced EdU incorporation is more likely a result of more origin firing rather than increased replication fork speed. The increase in Cdc45-positive cells could, however, still be the consequence of an S-phase delay.

To address whether *wee1*$^{-}$ cells simply delay S phase one could measure EdU intensity after pulse-labelling. In this case the EdU intensity per cell should be comparable to that in wild-type cells. In contrast, higher EdU intensity per cell would suggest a higher rate of replication rather than a delay in S phase.

Additionally, a DNA-fibre assay could be applied to answer whether origin firing or replication-fork speed is affected through enhanced Cdk activity in the *wee1*$^{-}$ mutant. A DNA-fibre assay is a way to directly visualize the incorporation of nucleotide analogues such as EdU by fluorescence microscopy. In this assay, DNA fibres are spread out on a microscope slide and EdU incorporation is seen as labelled stretches of DNA. This assay has been applied in mammalian cells where it was shown that WEE1 inhibition leads to replication of short stretches of DNA despite an increased EdU incorporation, suggesting increased origin firing (Beck et al. 2012). This finding supports the view that the loss of Wee1 results in aberrant origin firing and that replication fork speed is not increased. Consistent with this model is the finding that the lethality of the *wee1*$^{-}$*rad3*$^{-}$ double mutant is partially rescued when the number of pre-RCs, which are essential for origin firing, is reduced. If pre-RCs are assembled on fewer origins, the number of origins that can be fired at all is lower and thus increasing Cdk activity has a smaller impact.

The inhibitory effect of Wee1 on Cdk activity is counteracted by the tyrosine phosphatase Cdc25 at entry into mitosis. Inhibiting Cdc25 would therefore have the opposite effect to that of Wee1 on the Cdk and consequently cause a reduction in Cdk activity. We measured EdU incorporation when Cdc25 is inhibited and show that there are fewer EdU-positive cells in

mutant than in wild-type cells (paper II). This result indicates that also Cdc25 regulates replication in S phase in fission yeast. In mammalian cells, regulation of CDC25A during S phase is critical for coordinated duplication of the genome. Upstream kinases ATR and CHK1 are activated during replication and inhibit CDC25A, leading to restrained CDK activity. Inhibition of components in the ATR-CHK1-CDC25A-CDK pathway results in increased CDK activity and loss of genome integrity during replication (reviewed in Sorensen and Syljuasen 2012).

How does increased Cdk activity lead to DNA damage?

The aberrant firing of origins as a consequence of increased Cdk activity in S phase leads to a higher consumption of dNTPs. Forks stall because dNTPs and/or replication factors become limiting and the S-phase checkpoint is activated to prevent forks from collapsing. This reasoning is supported by the finding that loss of central proteins of the S-phase checkpoint Rad3, Mik1 or Cds1 is lethal in a *wee1*$^-$ mutant (al-Khodairy and Carr 1992). We also confirmed the activation of checkpoint effector kinase Cds1 in the *wee1*$^-$ mutant by monitoring the phosphorylation of the Cds1 substrate Mus81 (Boddy et al. 2000; Kai et al. 2005). Checkpoint proteins have an important role in promoting replication fork stabilization and repair (reviewed in Branzei and Foiani 2007). Forks that are not protected lead to DNA double-strand breaks and cells die when they enter mitosis with damaged DNA.

The assumption that *wee1*$^-$ cells accumulate DNA damage in the absence of the checkpoint is confirmed in the dramatic increase in Rad22 foci, which arise during repair of double-strand breaks (paper II).

It is possible that additional substrates of Wee1 might contribute to the S-phase-related phenotypes observed in *wee1*$^-$ mutants. In a recent study WEE1 inhibition was reported to promote Cdk-dependent degradation of the ribonucleotide reductase subunit RRM2 in mammalian cells (Pfister et al. 2015). Untimely degradation of RRM2 contributes to the depletion of dNTPs in addition to increased origin firing which results in inhibition of replication, DNA damage and checkpoint activation. Also the endonuclease Mus81 has been suggested as a substrate of Wee1 (Dominguez-Kelly et al. 2011). Mus81 cleaves stalled replication forks and enhanced Mus81 activity might be responsible for the DNA double-strand breaks observed in the *wee1*$^-$ mutant.

The sensitivity of *wee1Δ* to DNA-damaging agents

There has been a debate in the literature whether or not Wee1 is part of a checkpoint response (Rowley et al. 1992; Barbet and Carr 1993; Raleigh and O'Connell 2000; Rhind and Russell 2001). It is known that *wee1Δ* cells are sensitive to DNA-damaging agents like UVC, IR and camptothecin (al-Khodairy and Carr 1992; Rowley et al. 1992; Mahyous Saeyd et al. 2014). We suggest that *wee1*$^{-}$ cells are sensitive to DNA-damaging agents because they already experience replication stress and altered dNTP pools. Stalled forks are particularly vulnerable to DNA-damaging agents. Furthermore, the shortage of dNTPs hinders repair synthesis and makes the cells particularly vulnerable if DNA damage occurs.

What is the function of Mik1/ MYT1?

Mik1 is a redundant kinase that, like Wee1, phosphorylates Tyr15 on Cdc2. The general view that Mik1 regulates Cdk activity during S phase is based on the observation that it is regulated by the Cdc10 transcription factor, which also controls expression of other genes important for entry into S phase, such as *cdt1* and *cdc18* (Ng et al. 2001), and that it is a target of the S-phase checkpoint (Rhind and Russell 2001). So if Wee1 is a main regulator of S-phase Cdk activity what is the function of Mik1? Other studies have shown an involvement of Mik1 in the G2/M checkpoint. Mik1 accumulates in arrested G2-phase cells in response to prolonged checkpoint activation by bleomycin and high doses of gamma radiation (Baber-Furnari et al. 2000) and it has been suggested that it is both involved in checkpoint activation (Rhind and Russell 2001) as well as maintenance of the checkpoint signal (Baber-Furnari et al. 2000). Interestingly, Cdc10-dependent transcription can be induced outside S phase after exposure to IR in G2 phase (Watson et al. 2004). Since Mik1 is a Cdc10 target it can be expressed during G2 phase upon exposure to IR in order to reinforce the checkpoint response. These findings support the role of Mik1 as a checkpoint kinase in fission yeast not only in S phase but also in G2 phase (Rhind and Russell 2001).

Metazoans also possess two kinases, WEE1 and MYT1 that negatively regulate CDK activity. MYT1 is a dual specific kinase phosphorylating both threonine 14 and tyrosine 15, ensuring inhibition of the CDK during G2 phase in a normal cell cycle (Mueller et al. 1995; Booher et al. 1997). In contrast to WEE1 which is mostly present in the nucleus (Mcgowan and Russell 1995),

MYT1 localizes to membranes of the Golgi and endoplasmic reticulum (Mueller et al. 1995; Liu et al. 1997). It seems that compared to WEE1, MYT1 plays a minor role in regulation of the mitotic cell cycle. The checkpoint function of MYT1 has been mostly studied in respect to meiotic progression and oocyte maturation. Studies in *Xenopus laevis* and *Caenorhabditis elegans* have shown that MYT1 plays a critical role in maintaining the G2 arrest of oocytes by inhibiting Cdk activity (Nakajo et al. 2000; Karaiskou et al. 2004; Burrows et al. 2006). Here, MYT1 is the responsible kinase for arrest, because WEE1 is absent in G2-arrested oocytes (Nakajo et al. 2000).

Clinical relevance of Wee1 inhibition

A challenge in cancer treatment is to selectively kill tumour cells and avoid harming normal tissue. Inhibitors of the G2/M checkpoint were considered a promising treatment strategy based on the idea that many cancer cells are defective in the G1/S checkpoint and depend on the G2 checkpoint for survival (Fig. 10). Thus, the prediction is that combining DNA-damaging agents, such as IR, and G2 checkpoint inhibitors would lead to selective cancer-cell killing since cancer cells would enter mitosis despite the presence of damaged DNA leading to mitotic catastrophe and cell death. In contrast, normal cells are proficient in the G1/S checkpoint and can arrest in G1 to repair the damage before entering mitosis. More recent studies, however, find that CHK1 inhibitors by themselves cause DNA damage in S phase of cancer cells (Ferrao et al. 2012; Brooks et al. 2013). Indeed, checkpoint kinases ATR, CHK1 and WEE1 have been found to play a important roles in regulating Cdk activity in S phase. Inhibition of checkpoint kinases leads to aberrant increase of CDK activity, increased origin firing and a shortage of nucleotides and double-strand breaks, commonly known as replication stress (Syljuasen et al. 2005; Beck et al. 2012; Sorensen and Syljuasen 2012). Cancer cells are already exposed to a high level of replication stress through the expression of oncogenes (Bartkova et al. 2005; Gorgoulis et al. 2005). Therefore, inhibition of ATR, CHK1 and WEE1 does not only abolish the G2/M checkpoint but also induces DNA damage in S phase. Both effects may contribute to the cancer-selective cytotoxicity of these checkpoint inhibitors and emphasise their role as promising tools for cancer therapy.

Both the idea of inhibiting the G2 checkpoint in cancer cells deficient in the G1 checkpoint and imposing replication stress in cancer cells already stressed are exploiting weaknesses in the cancer cells and result in synthetic lethality. Both of these strategies are built on our knowledge about DNA damage and cellular responses to them. However, DNA-damaging agents used in the clinic often cause damage to other macromolecules and invoke stress responses in parallel to the DNA-damage repsonse. Targeting such pathways operating in parallel to known checkpoints could also be exploited to achieve synthetic lethality and selective killing of cancer cells.

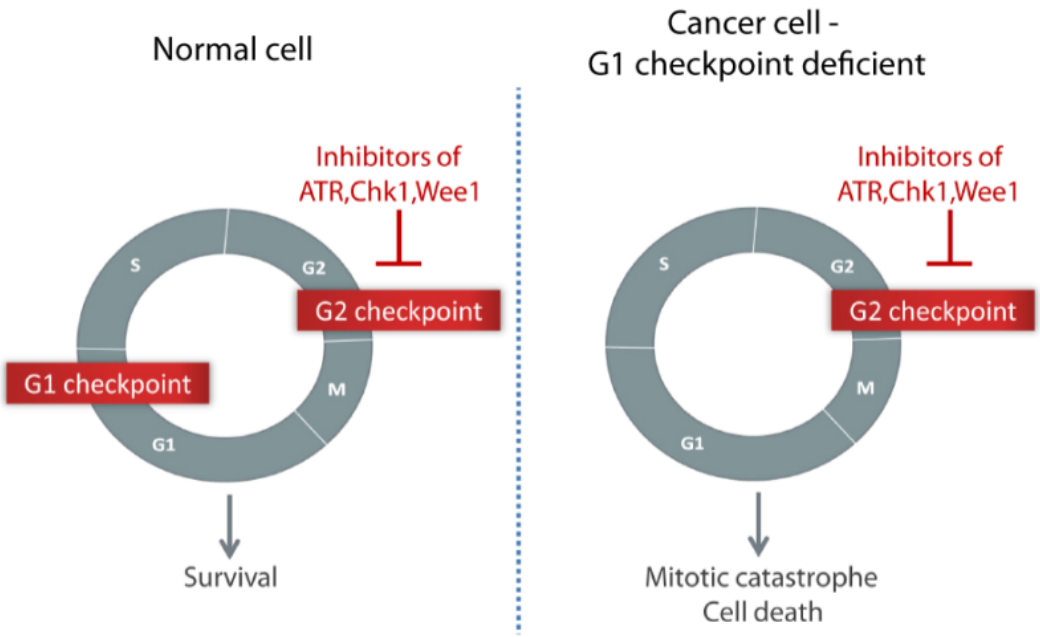

Figure 10: G1/S-checkpoint deficient tumour cells are selectively killed through G2 checkpoint abrogation by inhibitors of ATR, CHK1, or WEE1 (modified from Syljuasen et al. 2015).

Additional stress-response pathways to the classic G2/M checkpoint

In this work we show that cells are able to delay entry into mitosis independent of the classic checkpoint proteins Rad3, Tel1, Chk1 and Cds1 (paper III), indicating that alternative pathways exist that regulate entry into mitosis in response to DNA-damaging agents.

An obvious candidate for regulating such an alternative pathway is the stress response kinase Sty1. In humans, the Sty1 homologue p38 is activated in parallel to the ATR-Chk1 pathway and induces a G2 arrest in response to UV irradiation (Warmerdam et al. 2013). Also in fission

yeast Sty1 has been implicated to play a role in the response to DNA damage (Alao and Sunnerhagen 2008). Furthermore, fission yeast *sty1* mutants are UV sensitive (Degols and Russell 1997) and in response to osmotic stress Sty1 activation leads to a G2 arrest through sequestration of Cdc25 in the cytoplasm (Lopez-Aviles et al. 2005; Lopez-Aviles et al. 2008). Nevertheless, we find that loss of *sty1* in the *rad3*$^{-}$ background did not abolish the delay, suggesting that Sty1 is not responsible for the G2 arrest in response to UVC irradiation (paper III).

Another key target in the mammalian DNA damage checkpoint is the Polo-like kinase Plk1. Plk1 is inhibited in response to DNA damage in G2 phase through premature activation of the APC/C and subsequent Plk1 degradation (Smits et al. 2000; Bassermann et al. 2008). Also, the upstream activator Aurora A is inhibited when double-strand breaks are induced (Krystyniak et al. 2006; Bruinsma et al. 2016). Budding yeast polo kinase Cdc5 has been shown to be involved in the DNA damage checkpoint (Toczyski et al. 1997; Sanchez et al. 1999). When we tested Plo1 and Ark1, fission yeast homologues of Plk1 and Aurora A, respectively, we did not find that they affected the Rad3-independent delay, indicating that Polo and Aurora homologues in fission yeast are not required for the UVC-induced delay.

Regulation of cyclin B has also been proposed to regulate the G2/M checkpoint. Treatment with ionizing radiation decreased the stability of the cyclin B message and resulted in reduced cyclin B mRNA and protein levels in Hela cells (Maity et al. 1996; Kao et al. 1997). A similar effect on cyclin B1 has also been seen after treatment of brain tumour cells with the DNA-damaging agent camptothecin (Janss et al. 2001). Kao et al. (1997) show also that overexpression of cyclin B1 reduced the G2 delay in the irradiated HeLa cells, consistent with a model in which the reduced cyclin levels are responsible for the delay. We have shown that the protein level of the fission yeast homologue of cyclin B, Cdc13, is reduced after exposure to UVC (paper III). In contrast to the transcriptional regulation in mammalian cells, we found that Cdc13 is regulated at translation level in response to UVC.

Regulation of translation in stress-responses

Regulation of protein synthesis is a common response to environmental stress in order to cope with and adapt to diverse stress stimuli such as starvation, oxidative damage, osmotic stress

and DNA damage. Exposure to stress leads to phosphorylation of the translation initiation factor eIF2α as well as a decrease in global protein synthesis and translation of selected mRNAs (reviewed in Wek et al. 2006; Simpson and Ashe 2012; Pakos-Zebrucka et al. 2016). There are indications that translational control might even be involved in checkpoint responses. An example is the G1/S checkpoint in fission which is totally dependent on the kinase Gcn2. Exposure to UVC, MMS or oxidative stress leads to Gcn2-dependent phosphorylation of the translation initiation factor eIF2α and cell-cycle arrest (Nilssen et al. 2003; Tvegard et al. 2007).

In paper III we observe that less *cdc13* mRNA is translated after UVC treatment leading to a decrease in Cdc13 protein level. The decreased availability of Cdc13 in the cyclin-Cdk complex is most likely responsible for the delayed activation of Cdc2 after UVC irradiation. However, this proposal has yet to be proved. In order to prove that downregulation of Cdc13 is the reason for the UVC-induced delay, we need to maintain Cdc13 levels after UVC irradiation in a *rad3*$^-$ background and monitor entry into mitosis. We have attempted to construct a strain where *cdc13* expression is driven by the *nmt41* promoter in *rad3*$^-$ cells but the strain was not viable. In *nmt.cdc13 rad3*$^+$ cells the Cdc13 protein level is 2-3-fold higher than in *cdc13*$^+$ cells (not shown), probably leading to an increase in cyclin-Cdk complexes and increased Cdk activity. We have shown that increased Cdk activity is lethal in the *rad3*$^-$ background (paper II), which is probably the reason why expression of *cdc13* from the *nmt41* promoter failed.

How is translation of the *cdc13* mRNA regulated after UVC? It is known that exposure to UVC results in global downregulation of translation (Iordanov and Magun 1998; Deng et al. 2002; Tvegard et al. 2007). The *cdc13* mRNA has an unusually long 5'UTR containing secondary structures and uORFs and it has been suggested that these regions make *cdc13* particularly sensitive to reduced translation (Daga and Jimenez 1999). Although most of these regulatory elements lie within an intron (www.pombase.org), the spliced 5'UTR is still extremely long and has the potential to form complex structures. The requirement of Ded1 RNA helicase for efficient translation of *cdc13* supports this idea (Grallert et al. 2000). Ded1 helps unwinding long and structured mRNAs and facilitates the ribosome in scanning for the AUG codon (reviewed in Tarn and Chang 2009). Further investigation is needed to elucidate how specific

downregulation of Cdc13 is achieved and whether it involves Ded1 or other proteins regulating translation.

Activation of the spindle assembly checkpoint (SAC)

In the absence of Rad3 irradiated cells clearly enter mitosis after a delay, but they never display a peak of mitotic cells (Fig.1; paper III). Rather, they reach a plateau in the level of binucleate cells that is maintained during the course of the experiment. There are two alternative explanations for this phenotype; either a proportion of the *rad3* cells never enter mitosis while some of those that enter are arrested in mitosis, or the cells lose synchrony upon recovery from the arrest.

In contrast to the *rad3* single mutant the *rad3*$^{-}$*mad2*$^{-}$ and *chk1*$^{-}$*cds1*$^{-}$double mutant (Fig.1 and 2; paper III) show a peak of binucleate cells. The phenotype of *rad3*$^{-}$*mad2*$^{-}$ suggests that the SAC is activated in the *rad3*$^{-}$ single mutant, probably due to inefficient alignment of damaged chromosomes along the metaphase plate. This raises the question what it is that triggers SAC activation upon DNA damage. Is it simply that the absence of DNA repair leads to problems with chromosome alignment? Is chromosome condensation affected by DNA damage, which would make chromosome alignment difficult? Or could there be direct damage to the kinetochores? These alternatives are at present difficult to distinguish. Interestingly, it has been reported that there is cross-talk between the DNA-damage checkpoint and the SAC and it has been proposed that Chk1 plays a role in SAC activation (Kim and Burke 2008). In mammalian cells Chk1 was shown to localize to kinetochores in prometaphase and to be required to maintain mitotic arrest in response to the spindle poison taxol (Zachos et al. 2007). Furthermore, depletion of Chk1 led to decreased Mad2 levels and abrogation of the SAC (Carrassa et al. 2009; Chila et al. 2013; Yang et al. 2014). Also in fission yeast Chk1 was shown to be required for the SAC when cells with damaged DNA enter mitosis (Collura et al. 2005).

Concluding remarks

In this work we have investigated the regulation of cyclin-Cdk complexes both in an unperturbed cell cycle (paper II) and after treatment with UVC irradiation (paper III). Accurate regulation of Cdk activity during S phase is of utmost importance for faithful DNA replication and genome stability. We have shown that the Wee1 kinase inhibits Cdk activity in S phase and in the absence of Wee1 cells suffer replication stress and DNA damage. In response to UVC irradiation CDK activity is inhibited leading to an arrest that enables the cells to repair DNA damage before entry into mitosis. We show that in addition to the classic checkpoint that maintains Tyr15 phosphorylation on Cdc2, Cdk activity is also regulated by the availability of Cdc13. Our results highlight that control of Cdk activity, both in an unperturbed cell cycle and as a checkpoint target, is essential for the maintenance of genome stability.

Abnormal Cdk activity and checkpoint defects result in genomic instability which is a characteristic of cancer cells. It is important understand these basic mechanisms of cell-cycle regulation and how their misregulation can contribute to the development of cancer. Furthermore, a better understanding of how cells respond to stress will provide novel strategies and targets for cancer therapy.

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