

Mechanisms regulating the G1-S transition in mammalian cells

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Abbreviations

ATF4	Activating transcription factor 4
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and rad3 related
CDK	Cyline-dependent kinase
CDC6	Cell division control 6
Cdt1	Chromatin licensing and DNA replication factor 1
Chk1/2	Checkpoint kinase 1/2
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
eIF	Eukaryote initiation factor
GCN2	General control non-derepressible 2
HPG	L-Homopropagylglycine
IR	Ionizing radiation
MCM	Minichromosome maintenance
MMS	Methyl mehtanesulfonate
MRS	methionyl-tRNA synthetase
NOS	Nitric oxid synthase
ORC	Origin recognition complex
pre-RC	Pre-Replicative complex
RB	Retinoblastoma
RNR	Ribonucleotide reductase
<i>S.cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
<i>S.pombe</i>	<i>Schizosaccharomyces pombe</i>
Tor	Target of rapamycin
tRNA	Transfer RNA (ribonucleic acid)
uORF	Upstream open reading frame
UV	Ultraviolet light (200-280nm)
UVB	Medium wave UV (280-315nm)

Introduction

The human body contains approximately 3.75×10^{13} cells (1). Every day the human body and each cell must withstand a range of potential dangers, it being viruses or bacteria wanting to occupy our abode, toxins or irradiation in our environment damaging our cells, or even the cells themselves make mistakes for example when replicating DNA. Nevertheless, most of the time we walk around quite healthy and unaware of the battles taking place within us, thanks to our body's remarkable ability to maintain itself. Despite the great capacity to overcome dangers, nothing is error free. Bacteria or virus mutate, resisting our body's immune system and the drugs we have available. We may be predisposed to diseases through our genetic make-up, or the cells might be exposed to damage so severe that it is not possible to repair it. A large part of the diseases we encounter today, we can combat with the help of knowledge and medicine derived from research. Understanding the smallest details of the cells can help us comprehend how a certain disease develops or how a drug will function. In this thesis molecular mechanisms are addressed that will benefit the understanding of how the cell is regulated.

The eukaryotic cell cycle

The eukaryotic cell goes through four coordinated processes in its cycle; cell growth, DNA replication, distribution of the duplicated chromosomes to daughter cells, and cell division. Mammalian cells in culture spend approximately 24 hours on the cell cycle (Figure 1). In the microscope we can clearly distinguish between Interphase and Mitosis. Interphase is comprised of the cell-cycle phases G1, S, and G2 and about 95% of the cell cycle is spent on these phases. The cells grow steadily during the course of Interphase and usually double in size during this time. Mitosis (M-phase) is the fourth cell-cycle phase and also the most dramatic for the cell. The cells distribute the daughter chromosomes and undergo cytokinesis (cell division) all within an hour. G1 (Gap1)-phase is usually the longest of the cell-cycle phases and is the interval (gap) between mitosis and initiation of replication. G1-phase is followed by S (synthesis)-phase, where the cells replicate their DNA. Before M-phase can

commence the cells go through an additional gap phase, G₂-phase. Here the cells continue to grow and synthesize proteins required for mitosis.

Chromosome replication is strictly confined to S-phase and the replicated chromosomes are distributed to daughter nuclei in an intricate series of events leading up to cell division. The correct order of events in the cell cycle is crucial for the cell to maintain its integrity and is controlled by several molecular mechanisms termed checkpoints.

CDK-Cyclins

Many proteins are involved in regulating the cell cycle, the most important being cyclin-dependent kinases (CDKs). CDKs vary in type and numbers between eukaryotic cells. In mammalian cells there are 4 different CDKs associated with the cell cycle whereas there is only one in yeast. However common for them all is their ability to phosphorylate various protein substrates that are involved in cell-cycle progression. CDKs are regulated by three major mechanisms: cyclin binding, phosphorylation, and CDK inhibitors (2,3). Checkpoints target CDK activity and can arrest the cell cycle by using either major mechanism to regulate CDK activity. As their name indicates, CDKs are dependent on cyclins for their activity. Cyclins do not have any enzymatic activity but activate CDKs by binding to them. Cyclins are synthesized and degraded throughout the cell cycle unlike CDKs that are constitutively expressed, so when a cyclin is degraded, the corresponding CDK becomes inactive (4). Cyclin D is synthesized in G₁-phase and its concentration remains high until mitosis where it is degraded. Cyclin E is only expressed from late G₁-phase until mid S-phase, controlling the G₁-S transition. Cyclin A and B increase more slowly from G₁ to a peak in S-phase and M-phase respectively, before rapid degradation (5). Together with the phosphorylation status of CDKs and the expression of different cyclins, the cyclin-CDK complexes guide the cell through the different cell cycle phases. If the cyclin is not degraded or if the phosphorylation status of CDKs is changed the cells are arrested in the cell cycle.

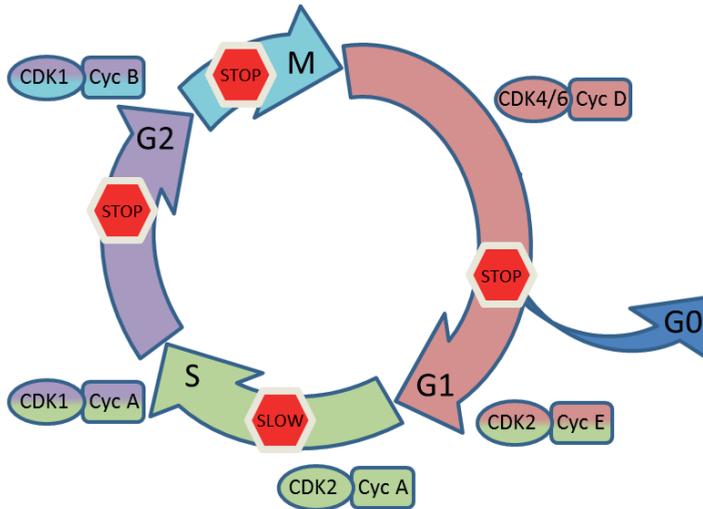


Figure 1: The cell cycle; illustrating the progression through the four phases regulated by different CDK/Cyclin complexes. Checkpoints are indicated in each cell cycle phase.

Checkpoints

Checkpoints serve as halting points throughout the cell cycle, stopping or pausing the cycle in the case of unfavorable conditions for the cell. Four cell-cycle checkpoints ensure proper cell division and genome stability (Figure 1). The G1-S checkpoint and G2-M checkpoint stop entry into S-phase and mitosis, respectively, after DNA damage. The intra-S checkpoint delays S-phase progression in response to DNA damage. These three checkpoints provide the cell with extra time to repair DNA damage before entering the next cell cycle phase (6). In M-phase we find the spindle assembly checkpoint that prevents separation of the duplicated chromosomes until each chromosome is properly attached to the spindle apparatus (7). Furthermore, the Nocut checkpoint prevents cytokinesis unless the chromosomes are properly separated (8). The importance of the checkpoints is underlined by findings that suggest that the checkpoint response upon DNA damage represent the first barrier against cancer in human cells (9,10). The work presented in this thesis revolves around G1-phase and the entry into S-phase and therefore the focus will be on the G1-S checkpoint.

The G1-S checkpoint

The main regulators for checkpoints are ATR and ATM in cooperation with the checkpoint kinases Chk1 and Chk2 (11). This is also true for the G1-S checkpoint. This checkpoint can be activated via two pathways, one that induces a rapid and transient response and one that induces a sustained and slower response. The initial and rapid G1 arrest is triggered by a swift cascade of phosphorylation events, involving ATM-dependent activation of Chk2 after ionizing radiation (IR) and ATR-dependent activation of Chk1 after ultraviolet (UV) light (200-280 nm) (12). These kinases in turn phosphorylate the Cdc25A phosphatase, thereby priming it for ubiquitination and rapid degradation by the proteasome. The absence of Cdc25A phosphatase activity forces CDK2 to remain in its inactive form, resulting in the failure to load Cdc45 onto chromatin, and a rapid blockade of initiation of DNA replication.

A sustained G1-S arrest is a delayed response that requires transcription, translation and protein stabilization of checkpoint transducers and effectors. Phosphorylation of the tumor suppressor p53, by ATM/ATR and Chk1/2, stabilizes the protein by preventing its interaction with the ubiquitin ligase Mdm2. This leads to accumulation of a stable and transcriptionally active p53 in the nucleus. Amongst the p53-induced genes is p21, a CDK inhibitor. When p21 reaches a certain threshold level it binds and inhibits the S-phase promoting cyclinE-CDK2. By inhibiting the CDK2 kinase complexes RB is activated, thereby inactivating the transcription factor E2F, responsible for transcription of several S-phase genes (12). These events secure the maintenance of the G1 arrest.

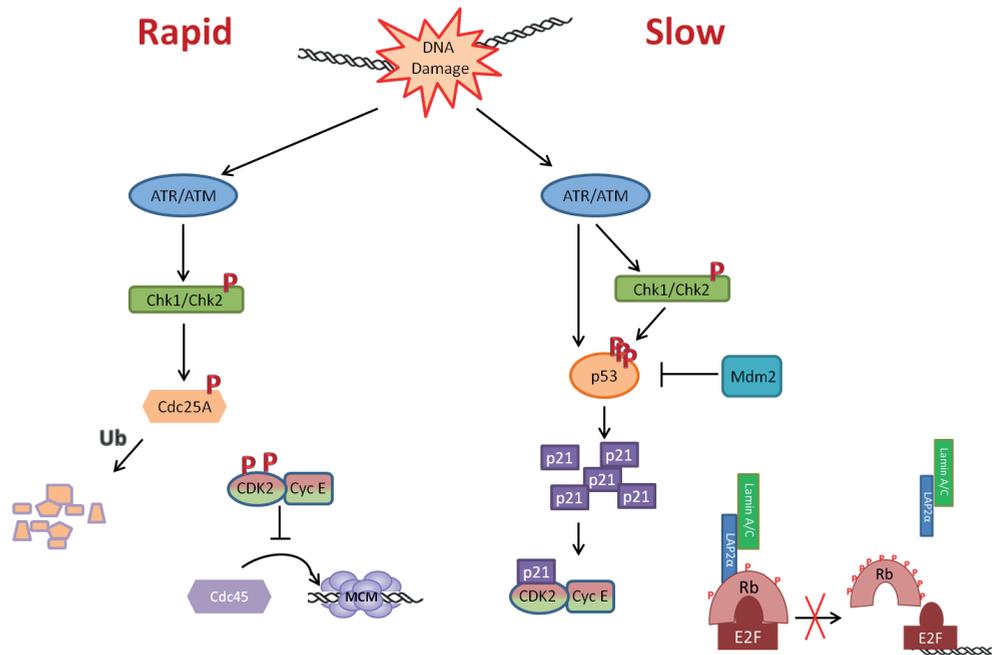


Figure 2: The two pathways of the G1-S checkpoint. The rapid response is due to degradation of Cdc25A via ATR/ATM and Chk1/Chk2, leading to inactive CDK2-cyclin E. In the slow response ATR/ATM and Chk1/Chk2 stabilizes p53 that in turn transcribes the CDK inhibitor p21.

Replication initiation

Chromosome replication takes place in S-phase. It is carefully regulated to make sure that the genome is duplicated completely, and that only one copy of the genome is made. To ensure this, loading and activation of the replicative helicase are separated in time. Preparation for replication begins already in early G1 phase where the origin-bound Origin Recognition Complex (ORC) recruits CDC6 to the DNA (13) (Figure 3). The minichromosome maintenance proteins (MCM2-7) form a complex with Cdt1 before they are recruited by CDC6. The process of loading the MCMs is also known as licensing and Cdt1 is an important licensing factor. The newly formed complex containing ORC, CDC6, Cdt1 and MCM2-7 is what is known as the pre-Replicative Complex (pre-RC). The replicative helicase, MCM 2-7, can only be loaded in G1-phase (14), and the helicase is activated when S-phase has commenced. To ensure this, a series of events must take place before full activation of MCM2-7 is achieved. First, while the cells are still in G1-phase the ATP on both CDC6 and ORC

are hydrolyzed, leading to the release of both CDC6 itself and Cdt1. Cdt1 is degraded and CDC6 is exported to the cytoplasm to guarantee that new pre-RCs cannot form, and thereby no re-replications can occur in S-phase (15,16). Secondly, MCM2-7 becomes phosphorylated and activated in a process that requires both Cell division cycle 7-related (Cdc7) protein kinase and S-phase CDK. These processes lead to the recruitment of several replication factors needed for full helicase activity, including the helicase activating proteins Cdc45 and GINS (17,18). The cells are now in S-phase with a fully active replicative helicase, and no means of priming new origins.

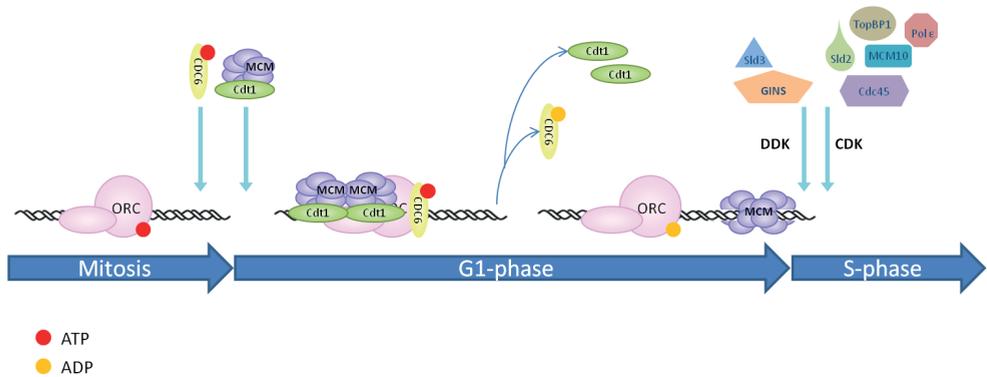


Figure 3: Outline of the events in G1-phase leading up to replication initiation.

Cdt1

Cdt1 plays a major role in ensuring that re-licensing and thereby re-replication cannot occur, and is therefore tightly regulated by several mechanisms. Binding of Geminin to Cdt1 is the first known mechanism by which Cdt1 is inhibited. Geminin is an E2F target and is expressed from S-phase to mitosis. It is thought that Geminin binding to Cdt1 blocks the ability to bind MCMs, and Geminin is also supposed to stabilize and inhibit Cdt1 through G2 and mitosis making sure that formation of pre-RCs can only take place in G1-phase (14). The other means of inhibiting Cdt1 are via proteolytic regulation. First, Cdk2 and Cdk4 phosphorylate Cdt1 (19). These phosphorylations, in turn, result in the binding of Cdt1 to the SCF^{Skp2} ubiquitin ligase complex and the subsequent degradation of Cdt1 (20). Second, Cdt1 can be proteolytically degraded by the Cul4- DDB1^{Cdt2} ubiquitin ligase. The association of PCNA to Cdt1 both during replication and DNA damage promotes Cul4- DDB1^{Cdt2}

directed ubiquitination and degradation of Cdt1 (21). Recently, a novel role for Cdt1 outside of replication initiation has also been proposed, namely that Cdt1 accumulates in G2-phase and has an essential role in the spindle assembly checkpoint (22).

Restriction point

The Restriction point was described as early as in 1974 as a specific point of commitment in G1-phase (23). The cell commits to start a new cell cycle. At the molecular level, the Restriction point is defined by the phosphorylation status of the retinoblastoma protein (RB) (Figure 4). RB was first discovered as a tumor suppressor and is frequently mutated in cancers. RB is known to be phosphorylated by CDK4/6-CyclinD in early G1-phase. This leaves RB in a hypo-phosphorylated state in which it is able to bind and inhibit E2F (24,25). E2F is a family of transcription factors and have a range of target genes involved in promoting S-phase, including genes encoding replication initiation factors such as Cdt1 (26). In its hypo-phosphorylated state RB also binds LAP2 α which, via Lamin A/C, anchor RB to the nuclear membrane (27). As G1-phase progresses RB is further phosphorylated, now by CDK2-Cyclin E, making it hyper-phosphorylated. This hyper-phosphorylation is what most often is used as a marker for the Restriction point (28). RB undergoes a conformational change that releases RB from its anchor via LAP2 α and frees E2F, allowing it to translocate into the nucleus and activate transcription of its target genes. The cells can now start a new round of the cell cycle.

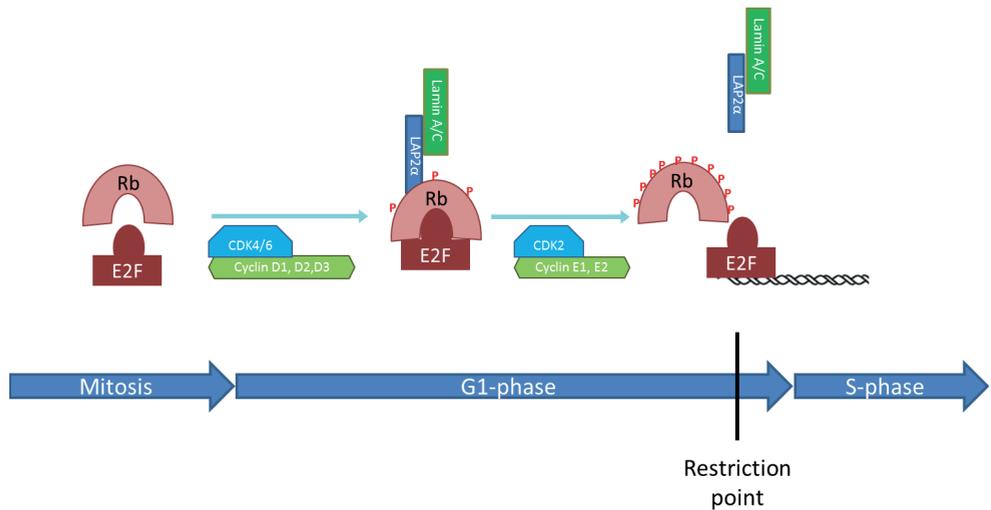


Figure 4: Phosphorylation status of RB through G1-phase. RB activated by CDK4/6-cyclinD allowing the binding to both E2F and its nuclear anchor LAP2 α . Later in G1-phase RB is hyper-phosphorylated by CDK2-cyclinE leading to inactivation.

Model organisms

Model organisms are widely used in research of cell biology. A model system is a simpler system that can be easily manipulated. Therefore, when selecting living organisms as models to work with, certain criteria are used depending upon the experimental purposes. As a result, there is a wide range of characteristics common to model organisms, often including short life cycles, small adult size, ready availability, and easily manipulated (29). Being small, growing rapidly and being readily available are crucial in terms of housing them, given the budget and space limitations of research and teaching laboratories. It is of course also important to choose an organism where the basic biological processes have been conserved through evolution, leaving us with knowledge that can be transferred to human cells. To best address a problem of interest make sure that the chosen organism can be manipulated in the required way, and that the right techniques are available.

Schizosaccharomyces pombe

Schizosaccharomyces pombe (*S.pombe*) is a unicellular eukaryote belonging to the fungus kingdom and shares a common ancestor with the animal kingdom.

S.pombe has not specialized to a great extent, making many of the basic mechanisms of cell biology equivalent to those found in human cells. Taken together with a short generation time and well-developed techniques for genetic manipulation, *S.pombe* is a useful model organism, something that was underlined in 2001 when Paul Nurse received the Nobel Prize in Physiology or Medicine for his work on the regulation of the cell cycle in *S.pombe*.

Human cells

Human cells in culture are considered a model organism and they contribute greatly to the understanding of human biology. There are a variety of techniques available for manipulation of human cells in culture, and the relevance to human disease is self-evident. Another advantage is the diversity in the different cell lines available. A great number of cancer cell lines are available that grow fast and almost infinitely. These cell lines can easily be synchronized with drugs like Nocodazole, enabling us to study the cell cycle in more detail. Normal fibroblast cell lines are also widely used and these cell lines can in addition, be synchronized using contact inhibition. This means that when a cell comes in contact with other cells it ceases to grow and enter G₀-phase. Cells enter G₀, a resting phase, when conditions for cell growth are not favorable, either by confluency or due to starvation. So why do we need other model organisms than human cells to solve biological issues relevant for human diseases? Human cells are complex and it can be difficult to deduce why we get a certain phenotype after manipulation. Using simpler unicellular eukaryote organisms such as yeast makes it easier to understand how the basic mechanisms work. The principles discovered in simpler organisms can then be transferred to human cells and help decipher complex pathways. Combining human cell culture and other model organisms will help us get a better understanding for the fundamentals leading to human diseases.

Novel G1-S checkpoint

The G1-S checkpoint described above has not been observed in *S.pombe*, and together with the very short G1-phase in culture, G1-phase has not been widely studied due to technical reasons. However, synchronizing the cells using a

temperature-sensitive *cdc10* mutant will ease investigation of G1-phase issues. Cdc10 is a transcription factor much like E2F, responsible for the transcription of several genes, including *cdt1* and *cdc18* (CDC6), necessary for the transition from G1-phase to S-phase. The temperature-sensitive mutant has a mutation that hinders the correct protein folding when the cells are grown at 36°C. Leaving the cells at 36°C for 4 hours ensures that all cells have completed a cell cycle and are in early G1-phase, unable to move further until *cdc10* transcripts are available again. Irradiating cells with UV just after release from such a *cdc10* block resulted in a G1-block (30). This delay was independent of Rad3 (ATR), Chk1 and Cds1 (Chk2), and did therefore not display the molecular hallmarks of the known checkpoint response. It was also shown that the UV-irradiation induced a delay in the formation of pre-RC, causing the delay in S-phase entry, and that this was totally dependent on the kinase Gcn2 (31). Unexpectedly, this response to UV early in G1-phase is not the same for all types of DNA damaging agents. Methyl methane sulphonate (MMS) and hydrogen peroxide (H₂O₂) does induce a G1-arrest. Ionizing radiation (IR) and psoralen in combination with UVA (PUVA) on the other hand, does not cause a cell cycle delay.(32), indicating that this is not a general DNA-damage checkpoint. More recent studies have concluded that the G1-S delay is dependent on some form of DNA damage. However, the activating signal does not derive from the initial DNA damage but from one or more repair intermediates (33).

GCN2

GCN2 (General control non-derepressible 2) is a serine/threonine-protein kinase that was first discovered to be part of a pathway that responds to amino acid deprivation and survival in *Saccharomyces cerevisiae* (*S.cerevisiae*) (34). Uncharged tRNAs accumulate in the cell due to amino acid starvation and they bind to a histidyl-tRNA synthetase domain of GCN2, leading to autophosphorylation at specific residues in the protein kinase domain and dimerization (35,36). The active GCN2 dimer can efficiently phosphorylate Serine51 (Ser51) on the α subunit of eukaryotic translation initiation factor 2 (eIF2 α) (37). As the name indicates, eIF2 is important for the initiation of translation. When eIF2 is in a GTP-bound form it delivers the initiator methionyl tRNA (met-tRNA_i) to the ribosome. Once protein synthesis is

initiated the GTP is hydrolysed and eIF2 is released from the ribosome in a GDP-bound form. eIF2 needs to be recycled to a GTP-bound form by eIF2B, a guanine nucleotide exchange factor (GEF). However, the phosphorylation by GCN2 on eIF2 α transforms eIF2 to a competitive inhibitor of eIF2B, thought to lead to global downregulation in protein synthesis, and thereby reducing the utilization of amino acids (Figure 5). Even though global translation is reduced, eIF2 α phosphorylation does also induce increased translation of specific mRNAs containing upstream open reading frames (uORFs), such as GCN4 in *S.cerevisiae* or ATF4 in mammals (37). ATF4 and GCN4 are transcriptional regulators that control hundreds of genes, many of which are involved in amino acid regulation.

Several studies from work with *S.cerevisiae* have led to a model of how GCN2 detects uncharged tRNA after starvation (38-41). A scaffold protein, Gcn1, is found to promote Gcn2 activity when the equivalent charged tRNA is scarce, either by positioning Gcn2 close to the ribosome or more directly, delivering uncharged tRNA from the ribosome, to Gcn2. GCN1 is conserved in mammals and is shown to co-immunoprecipitate with GCN2 (38). An inhibitor of the interaction between GCN2 and GCN1, IMPACT, has been shown to impair GCN2 activation not only after amino acid starvation, but also after UV-irradiation, glucose starvation and proteasome inhibition when overexpressed (42).

GCN2 is not the only kinase to phosphorylate Ser51 on eIF2 α . In mammalian cells there are a total of four kinases that give the same downstream effect of eIF2 α phosphorylation; GCN2, PKR, HRI and PERK, each of which respond to different stimuli (Figure 5). In *S.pombe* three kinases, Gcn2, Hri1 and Hri2, phosphorylate eIF2 α .

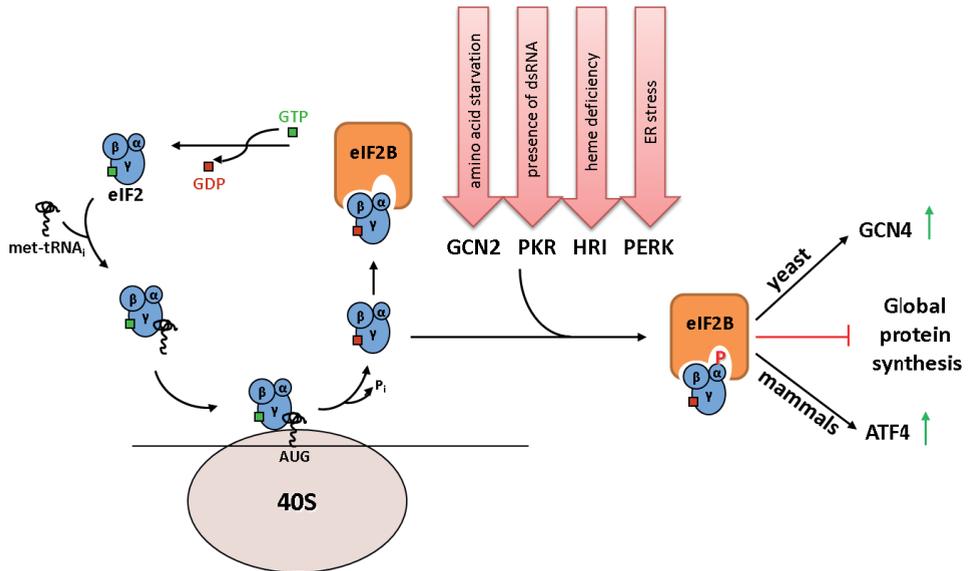


Figure 5: Translational regulation by eIF2. In its GTP-bound form eIF2 delivers met-tRNA_i to the 40S ribosomal subunit to initiate translation. eIF2 need to be recycled back into a GTP-bound form by eIF2B, however if eIF2 is phosphorylated by GCN2, PKR, HRI or PERK the eIF2-eIF2B interaction is stabilized inhibiting global translation, but not translation of specific target genes such as GCN4 in yeast and ATF4 in mammalian cells.

GCN2 has been linked to diseases such as cancer and Alzheimer's, and is reported to be involved in a large array of biological functions that are not directly coupled to amino acid starvation, such as the novel G1-S checkpoint in *S.pombe* (38,43,44). The function of GCN2 is therefore likely to exceed the current knowledge. Like in *S.pombe*, UV-irradiation has been shown to activate GCN2 and downregulate translation in mammals (45). However, there is no indication of UV-irradiation resulting in accumulation of uncharged tRNAs, and it has been suggested that GCN2 activation is due to either UV crosslinking tRNAs to GCN2, causing activation (45) or, in the case of UVB, causes a swift consumption of arginine when nitric oxide is produced from it with the aid of nitric oxide synthetase (46).

In a more recent study GCN2 was reported to phosphorylate methionyl-tRNA synthetase (MRS) after UV-irradiation (47). MRS is part of a multisynthetase complex and catalyzes the attachment of methionine to both the initiator tRNA as well as the tRNA^{met} required for elongation (48). Under normal conditions MRS strongly

associates with the tumor suppressor AIMP3. However, after UV-irradiation the phosphorylated MRS is reported to undergo a conformational change that blocks tRNA binding and causes the dissociation of AIMP3 (47). AIMP3 then supposedly translocates to the nucleus and mediates DNA damage repair in an unknown manner.

Aim of study

Checkpoints are of great importance for the cells to maintain genomic stability and represent the first barrier against cancer. Our group described a novel checkpoint governing the G1-S transition after UV irradiation in *S.pombe*. The mechanism was different from the classic G1-S checkpoint, both considering its mechanism of action and its target. The checkpoint is absolutely dependent on the Gcn2 kinase and it delays entry into S-phase by delaying the formation of the pre-replicative complex, a obligatory step in the preparation for DNA replication.

The overall aim of this study was to investigate the role of GCN2 in G1-phase and the regulation of pre-RC formation in mammalian cells:

Paper 1) The aim of this work was to develop a method that allows us to investigate pre-RC loading in mammalian cells. This method was combined with detection of RB, allowing us to study the timing of both pre-RC loading and the restriction point in single cells, both important events in G1.

Paper 2) The aim of this work was to study the effects of GCN2 in cell-cycle regulation, both after stress and in unstressed cells

Paper 3) The aim of this work was to investigate the mechanism of GCN2 activation after different stresses.

List of papers

List of papers included in this thesis, referred to as papers I-III in the text:

Paper I

A novel method for simultaneous measurement of RB phosphorylation status and MCM loading in single cells

Håland, T.W., Boye, E., Stokke, T., Grallert, B. *, Syljuåsen, R.G.

Manuscript

Paper II

GCN2 affects the formation of pre-Replicative Complex in mammalian cells

Håland, T.W., Syljuåsen, R.G., Boye, E., Grallert, B. *

Manuscript

Paper III

Activation of Gcn2 in response to different stresses

Håland, T.W., Bøe, C.A., Boye, E., Grallert, B. *

Manuscript

Summary of results

Paper I

Loading of the pre-Replicative Complex (pre-RC) and the Restriction point are well studied events in G1-phase. These events are important for the cell to maintain genomic integrity. Hyper-phosphorylation of the tumor suppressor Retinoblastoma protein (RB) is commonly used as a marker for the Restriction point. Hyper-phosphorylation of RB leads to the release of the transcription factor E2F. The relative timing of pre-RC formation and the Restriction point and their interdependence is not clear. Several pre-RC components are listed as E2F targets and it is therefore often suggested that pre-RC formation must come after the Restriction point. We have developed a novel method that allows us to measure the timing of these events in single cells. We take advantage of the fact that minichromosome maintenance (MCM) proteins bind chromatin during pre-RC formation and that RB is anchored to the nucleus before it is hyper-phosphorylated. Extracting all unbound proteins thereby allows us to study the loading of pre-RCs and hyper-phosphorylation of RB as well as the order of these events. We find that the timing of pre-RC loading and the Restriction point varies between cell types and between synchronization methods. In U2OS cells both the Restriction point and pre-RC loading occur earlier in G1-phase than in BJ cells. In both cell lines, when growing exponentially, contain a small fraction of cells that load MCMs before the Restriction point. Furthermore, U2OS cells released from a Nocodazole block are able to load pre-RC prior to the Restriction point. We therefore conclude that there is no strict inter-dependence between pre-RC loading and the Restriction point, and E2F-driven transcription is not essential for pre-RC formation.

Paper II

Our group has previously described a checkpoint governing the G1-S transition in *S.pombe*. Cells exposed to UV-irradiation delay pre-RC formation and therefore entry into S-phase in a GCN2-dependent manner. GCN2 is a protein kinase known to be involved in translational regulation. Its best characterized substrate is eIF2 α , an important factor for initiation of translation. The GCN2 checkpoint regulates the loading of the pre-RC, independently of known checkpoint regulators such as Rad3 (ATR), Chk1 and Cds1 (Chk2). Here we find that GCN2 can regulate pre-RC loading also in mammalian cells. Cells depleted of GCN2 show significantly less delay in the loading of pre-RCs after UV-irradiation. We also find that GCN2 can regulate the level of Cdt1, a component of pre-RC, in unstressed cells. Depletion of the transcription factor ATF4, a well-characterized target of GCN2-eIF2 α , did not have any effect on the level of Cdt1, indicating that the regulation of Cdt1 is not via eIF2 α -ATF4, the best known pathway of GCN2. We also observed that neither Cullin4 nor proteasome inhibition appeared to bring the level of Cdt1 up to the control level, indicating that GCN2 does not regulate Cdt1 via protein degradation. These data suggest that GCN2 has a novel role(s) in mammalian cells, and can regulate pre-RC both in stressed and unstressed cells.

Paper III

The molecular mechanism of GCN2 activation has been extensively studied in *S.cerevisiae*, where the scaffold protein GCN1 is required for GCN2 activation. GCN1 facilitates the delivery of uncharged tRNAs from the ribosome to GCN2. However, accumulation of uncharged tRNAs is not a known consequence of either UV- or H₂O₂-exposure. Both stresses are shown to activate GCN2, leading us to speculate that GCN2 can be activated by other mechanisms than via uncharged tRNAs and GCN1. By deleting *gcn2* in *S.pombe* we find that Gcn1 is required for GCN2 activation after amino acid starvation, but not after exposure to UV-irradiation or H₂O₂. We also find that ongoing translation is not required for UV-induced activation of Gcn2 in *S.pombe*, suggesting that uncharged tRNAs are not necessary for Gcn2 activation after UV-irradiation. Surprisingly, in mammalian cells GCN1 seems to be required for GCN2 activation after UV-irradiation but not after amino acid or serum starvation, indicating that the importance of GCN1 for GCN2 activation may differ from organism to organism. We also find that a mechanism previously described after UVB-irradiation and involving arginine starvation resulting from NOS activation does not contribute to initial activation of GCN2 after UV (UVC)-irradiation in mammalian cells. These results suggest that GCN2 is not activated by uncharged tRNA after UV in mammalian cells either. To test whether GCN2 was activated by any of the kinases mTor, ATR, ATM, DNA-PK and Chk1, we treated U2OS cells with appropriate inhibitors and found that none of these inhibitors reduced the UV-induced eIF2 α phosphorylation. Our findings suggest that GCN2 can be activated by other mechanism than that involving GCN1 and uncharged tRNAs in both *S.pombe* and mammalian cells.

General discussion and further work

We have developed a novel method to investigate the phosphorylation status of RB and chromatin-bound MCMs (Paper I). This method allowed us to study the loading of MCMs after exposure to UV-irradiation. We have found that GCN2 does take part in the regulation of pre-RC formation after UV-irradiation and, in addition, regulates Cdt1 in an unperturbed cell cycle (Paper II). We have also investigated the activation of GCN2 in response to different stresses in both *S.pombe* and mammalian cells, and find that GCN2 can be activated by other means than via GCN1 and uncharged tRNAs. The work in this thesis has broadened our understanding of mechanisms regulating the G1-S transition in mammalian cells. I will now discuss the findings and experimental considerations as well as speculate on future work.

A novel G1-S checkpoint in mammalian cells

There are several mechanisms preventing entry into S-phase when DNA is damaged by UV-irradiation. ATR/ATM can be activated in the classic checkpoint and induce a G1 arrest as described in the Introduction. But there are also two pathways that can be activated earlier in G1. Both these pathways delay pre-RC formation (figure 1). First the cells can degrade Cdt1 in a Cul4-DDB1^{Cdt2} dependent manner. This pathway is conserved from *S.pombe* (49) to mammalian cells (21,50,51). Second, the novel Gcn2-dependent checkpoint also delays the pre-RC formation. The checkpoint we have discovered in *S.pombe* did not appear to be dependent on Cdt1. However, fission yeast also has the Cdt2-dependent Cdt1 degradation pathway and we reason that the role of Gcn2 was possible to reveal by our experimental system. As explained earlier these experiments were done by synchronizing the cells using a Cdc10 block. By using this method the level of Cdt1 is reduced to a minimum. The delay we see in the loading of MCMs and the eIF2 α phosphorylation is therefore Cdt1 independent and Gcn2 dependent. Synchronizing the cells using a mitotic block reveals the Cdt2-dependent Cdt1 degradation, but at the same time it masks the Gcn2-dependent checkpoint. This indicates that the GCN2 dependent delay is overpowered by the delay caused by the degradation of Cdt1. In mammalian cells

Cdt1 was present at the start of all our experiments, implying that any delay in the loading we see after UV-irradiation is at least in part due to degradation of Cdt1. However, we do see a partial effect of GCN2, suggesting that there are two possible pathways that can delay pre-RC loading in G1-phase after UV, one dependent on Cdt1 degradation by Cdt2 and its upstream regulators like ATR and the other dependent on GCN2.

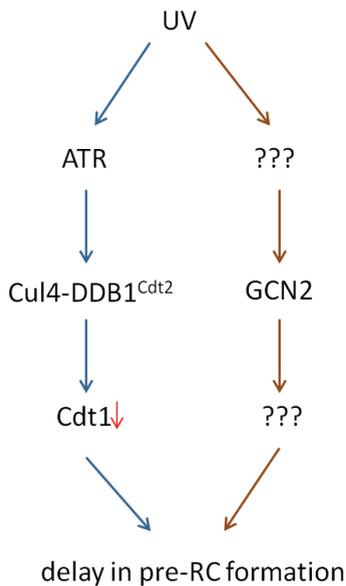


Figure 1: Pathways delaying pre-RC formation after UV-irradiation. Blue arrows indicate Cdt1 degradation. Brown arrows indicate the GCN2-dependent checkpoint.

GCN2 regulates ATF4 in unstressed cells

Activation of GCN2 inhibits the ternary complex consisting of eIF2, GTP and methioninyl-initiator tRNA (Met-tRNA^{met}), by phosphorylating eIF2 α . This inhibits further rounds of translation initiation (52). However, a number of mRNAs containing uORFs (upstream open reading frames) upstream of the protein-coding ORFs are selectively transcribed (34,53). One of the GCN2-eIF2 α P targets is the transcription factor ATF4. The GCN2-eIF2 α P-ATF4 pathway is shown to be critical for maintaining metabolic homeostasis in tumor cells (54). ATF4 is known to upregulate

genes involved in amino acid import, glutathione biosynthesis, and resistance to oxidative stress. Because we find that the downregulation of Cdt1 after GCN2 deletion is not due to protein degradation, one possibility could be a downregulation in transcription. However, we also find that downregulation of Cdt1 is ATF4 independent. Nevertheless, it is possible that ATF4 also has a role in unstressed cells and that this could be regulated by GCN2. That would mean that depleting GCN2 would reduce the ATF4 level and thus reduce transcription of its target genes. We have done preliminary experiments where we deplete ATF4 and GCN2 with siRNA and run whole cell lysates on immunoblot interestingly, we do see that GCN2 regulates ATF4 in unstressed cells, indicating a role for GCN2 beyond stress regulation (figure 2).

GCN2 regulates Cdt1

In Paper II we clearly see that GCN2 can regulate Cdt1, but the transfection efficiency has been varying during the experimental work. We experienced that when depleting GCN2 by siRNA transfection, we only saw a reduction in Cdt1 when the GCN2 level were reduced by 75% or more. We experienced a change in the cell growth conditions when we moved to another laboratory and found it difficult to continue with the seeding density required for the transfection reagent we were using. We therefore changed transfection reagent, allowing more flexibility in the seeding density of the cells. The fact that GCN2 needed to be reduced substantially, indicate that GCN2 is in excess in the cells and that activation of only a proportion of the kinase is enough for a response. Indeed, we did observe some induction of eIF2 α phosphorylation after UV-irradiation even when GCN2 was knocked down (data not shown). It is also likely GCN2 regulates Cdt1 indirectly through other selectively translated genes. It would be interesting to immunoprecipitate GCN2 or Cdt1 to investigate if there could be a direct interaction between the two proteins. Performing mass spectrometric analysis of Cdt1 from cell with or without GCN2 could also provide us information about any GCN2-dependent modifications.

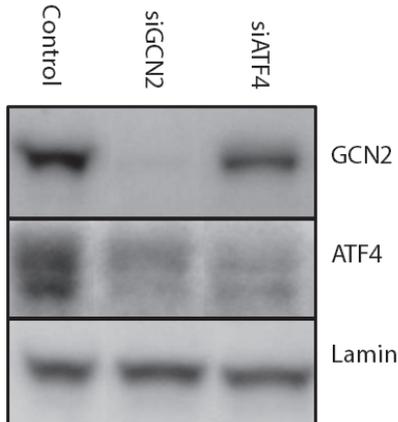


Figure 2: Cdt1 does not appear to be regulated by ATF4. U2OS cells were transfected with siGCN2 or siATF4. Whole cell lysates were analyzed by immunoblots for ATF4 and GCN2. Loading control: Lamin

The effect of Cdt1 reduction

We have shown that the protein level of Cdt1 is reduced when depleting GCN2 (Paper II). We were expecting that a reduction in the level of Cdt1 would induce some form of cell-cycle defect, because Cdt1 is required for the loading of the MCM helicase. A reduction in the Cdt1 level could lead to a prolonged G1-phase due to problems loading the right amount of pre-RCs. The cells could also possibly enter S-phase with too few pre-RCs formed. Fewer licensed origins would lead to a greater distance between origins, meaning that the replication forks would have to travel further without stalling (55). But the greater the distance between forks the more likely it is to encounter fork stalling and subsequently fork collapse. If a reduction in the Cdt1 level does induce fork collapse we would have expected there to be DNA damage in S-phase. We have labelled cells depleted for GCN2 with EdU, a thymidine analog, allowing us to identify cells in S-phase after analysis by flow cytometry. However, we did not observe any significant difference in the S-phase population when compared to control cells (date not shown), and cannot conclude that GCN2 depleted cells spend longer time in S-phase. We have also stained cells with antibodies recognizing γ H2AX or 53BP1, both marker for DNA damage, and found no clear indications of increased DNA damage after GCN2 depletion (data not shown).

However, it does appear that GCN2-depleted cells have more micronuclei than control cells. Micronuclei are small nuclei that are formed when chromosomes or fragments of chromosomes are not properly segregated into one of the daughters during cell division. Depletion of GCN2 could therefore cause defects in mitosis and a recent paper has suggested a novel role for Cdt1 in mitosis (22). In this paper they observed that Cdt1 binds to the Ndc80 complex that links the microtubules to the kinetochore and helps ensure proper segregation of chromosomes during mitosis (56). In the absence of Cdt1 the Ndc80 complex was found to be highly bent, but in the presence of Cdt1 the structure was elongated. Our own observations of microtubule assembly and disassembly might also be related to this chromosome segregation phenotype. In *S.pombe*, leaving the cells on ice for 30 minutes disassemble the microtubules, but rapid reassembly can be seen when the cells are returned to 25°C. A *Gcn2*^{-/-} strain however, appears to struggle to rebuild microtubules after exposed to cold shock (data not shown). These findings are very interesting and it would be exciting to follow microtubule dynamics after a cold shock in mammalian cells with and without GCN2, using live-cell imaging. In addition it would be interesting to deplete cells for Cdt1 and see whether there are phenotypes mimicking those we observe in GCN2 depleted cells. This might reveal a role of GCN2 in mitosis.

A link between GCN2 and ATR

GCN2 is a large protein of around 190 kDa. When the cells have been exposed to UV immunoblots contain an additional, GCN2-specific band above the usual band for GCN2 (figure 2). The appearance of the upper band correlates with activation of GCN2. The upper band also corresponds to the band we observe when using an antibody against the GCN2 autophosphorylation site at threonine 898 (figure 2). Surprisingly, we also observe such a band-shift when the cells are treated with VE-821, an ATR inhibitor. The band-shift we observe after the ATR inhibitor is not as prominent as after UV-irradiation, but it appear together with increased eIF2 α phosphorylation, again suggesting GCN2 activation. ATR is essential in mammalian cells and to investigate whether this activation of GCN2 is due to general stress when ATR is inhibited we treated the cells with the inhibitor for different lengths of time.

We observed that even with an incubation time of 15min GCN2 was activated (figure 2). Preliminary data also show that entry into S-phase is delayed when the ATR inhibitor was added in early G1-phase (Bøe et.al manuscript in preparation). These data indicate that ATR may have a role in G1-phase that involves GCN2. In *S.cerevisiae* the ATR homolog Mec1 regulate Ribonucleotide reductase (RNR) by inhibiting Sml1, an RNR inhibitor (57). It has also been shown that GCN2 is activated by nucleotide starvation in budding yeast (58) as well as in fission yeast (our unpublished results), leading us to speculate that ATR inhibition results in a reduction of nucleotide levels also in mammalian cells, thereby activating GCN2. Interestingly, we have preliminary result showing that in *S.pombe* Cid13, a poly(A)polymerase accountable for increasing the RNR level, is found in the polysome fraction after UV. This indicates that Cid13 is translated and therefore is a potential target of GCN2. An interesting speculative model supported by these observations is that GCN2 is activated either directly by ATR or by the level of nucleotides. The level of nucleotides is tightly regulated through the cell cycle (59), making it more plausible that a reduction in nucleotide levels after ATR inhibition would activate GCN2 after only 15min. The activation of Gcn2 could lead to increased Cid13 translation and thereby *suc22* (an RNR subunit) mRNA stabilization and increased translation to increase nucleotide levels.

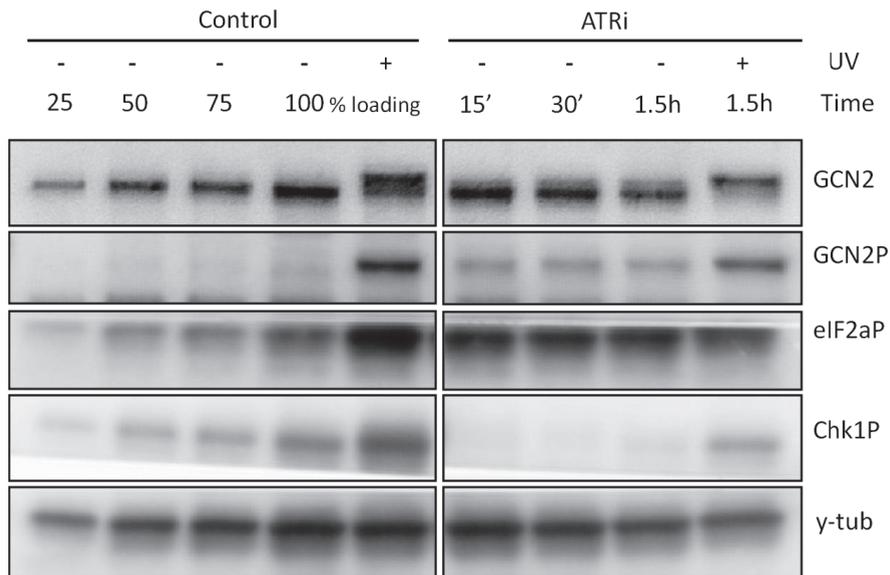


Figure 2: ATR inhibitor activates GCN2. U2OS cells were treated with ATR inhibitor (ATRi) for the times indicated (right). UV-irradiation was given at 60J/m^2 and recovery for 1h. Whole-cell lysates were analyzed by immunoblotting for GCN2, Phospho-GCN2 (Thr898) and eIF2 α -P. Chk1 P317 was used as an indicator on the effect of the ATRi. Loading control: γ -tubulin (γ -tub)

In a recent paper, a novel role of GCN2 has been described. It was shown that GCN2 phosphorylates methionyl-tRNA synthetase (MRS) in response to UV (47). MRS is an aminoacyl-tRNA synthetase (ARS), a group of essential proteins linking amino acids with their correct tRNA (60). MRS is necessary to make sure Met-tRNA^{met} is available to form the ternary complex together with eIF2 α and GTP. Phosphorylation of MRS by GCN2 leads to a reduction in Met-tRNA^{met} and thereby a downregulation of global protein synthesis (47). An additional effect of phosphorylating MRS is that it induces a conformational change that releases the tumor suppressor AIMP3 (p18) from the complex (47). AIMP3 then translocates into the nucleus where it can activate a DNA damage response via ATR/ATM (61-63). It was also demonstrated that MRS is phosphorylated prior to eIF2 α (47). This may indicate that MRS induces the initial downregulation of global translation whereas eIF2 α -P is responsible for selective translation to counter effect the damage/translational downregulation.

The role of GCN1

We find that GCN1 is not required for activation of eIF2 α after serum starvation or amino acid starvation in mammalian cells (Paper III). This is in contrast to findings in *S.cerevisiae*. Furthermore, the requirement for Gcn1 for Gcn2 activation after amino acid starvation is conserved between *S.cerevisiae* and *S.pombe*. UV-irradiation does not require Gcn1 for Gcn2 activation in *S.pombe* but it seems to be required in mammalian cells.

The proposed role of GCN1 for GCN2 activation in budding yeast is to ensure transfer of the uncharged tRNAs arriving at the ribosome A site (41). Accumulation of uncharged tRNAs is not a known consequence of either UV- or H₂O₂-exposure, and in combination with Gcn1 not being required after UV or oxidative stress in *S.pombe*, these findings suggest the existence of a novel pathway of activating GCN2. Serum starvation also activated eIF2 α in the absence of GCN1, which could suggest that serum starvation activated GCN2 in a similar way as UV or that it is rather one of the other eIF2 α kinases that are responsible for this activation. It has been shown that glucose starvation activates GCN2 in *S.cerevisiae* (64), however in mammalian cells it has been shown that PERK is the primary kinase responsible for eIF2 α -P after glucose starvation (65), an eIF2 α kinase not present in either *S.cerevisiae* or *S.pombe*. The interplay between GCN1 and GCN2 has been widely studied in *S.cerevisiae* (38). However, only one paper has reported experimental evidence that GCN2 requires binding to GCN1 for activation in mammalian cells (42), and in this paper they overexpressed a fragment of GCN1 which resulted in impaired function of GCN2. It is on the other hand not unlikely that GCN1 would interact with other yet unknown proteins, since it contains >20 HEAT repeats that are proposed to function as interaction sites (66), and one could imagine that overexpressing parts of a protein could induce unphysiological effects. It is also important to note that in our amino acid starvation experiments in mammalian cells, we starved the cells for glutamine, cysteine and methionine. Most studies in *S.cerevisiae* have been performed with either leucine or histidine starvation. Mammalian cells depleted for ATF4 are particularly sensitive to asparagine starvation and ATF4 regulates

asparagine synthetase (54). It is therefore possible that the effect is not the same for all amino acids.

In order to unambiguously determine the involvement of tRNA binding it would be interesting to mutate the tRNA-binding domain in GCN2 and transfect this version of GCN2 into GCN2^{-/-} cells. This would allow us to study the effect of this domain after several stresses and could give us an indication of whether binding of uncharged tRNAs is required for GCN2 activation under these conditions.

GCN2 and disease

GCN2 has been implicated in several diseases including cancer and Alzheimer's (38). Cancer cells depend on GCN2 for survival and proliferation and this is thought to be because of its role in starvation responses (54). In acute lymphoblastic leukemia the cells are unable to synthesize the amino acid asparagine. Treating these cells with asparaginase hinders the cells in acquiring asparagine from the circulations around the cell, leading to cell death. Interestingly, studies suggest that inhibiting GCN2 may enhance the effect of this drug even further (67). In Alzheimer's disease, suppressing GCN2 in mouse models lead to a decreased eIF2 α phosphorylation, and an alleviation of memory impairments and synaptic failure, but how GCN2 is linked to synaptic failure and memory defects is still unknown (68). GCN2 is to an increasing extent being implicated in biological processes seemingly unrelated to responses to amino acid starvation (reviewed in 38). Our findings of a novel role for GCN2 could give new insight to the biological functions of GCN2. This knowledge can then be exploited in targeting GCN2 for therapy and possibly for combination therapy approaches.

The Restriction point

I find the timing of the Restriction point an interesting topic. It was first reported that the Restriction point occurs 2-3h prior to S-phase entry (69). However, we find, in agreement with Stokke et.al (70), that the Restriction point, as measured by RB hyper-phosphorylation, occurs around 6h prior to S-phase entry. Cancer cells are often deficient in the G1-S checkpoint and we observed that U2OS cells released from a mitotic shake-off, are past the Restriction point already after 15 minutes and

that they enter S-phase 6-7 hours after release (data not shown). Passing the restriction point immediately after the cells enter G1-phase leave them no time to evaluate whether the conditions are right for committing to a new round of the cell cycle. When doing a mitotic shake-off only the cells in mitosis at this moment (1-2% for U2OS cells) are detached by physically shaking the flask. This should disturb the cell cycle minimally, in contrast to a mitotic block-and-release experiment, where the cells are arrested in metaphase by Nocodazole. Upon release from Nocodazole, both the Restriction point and entry into S-phase is delayed by about 3 hours as compared to a mitotic shake-off, but the duration of G1 after the restriction point remains the same. Also normal fibroblast cells (BJ) seem to pass through the Restriction point around 6 h prior to S-phase entry, meaning that the variations we see between different cell lines in cell-cycle length, may very well be due to the variations in time spent in G1-phase prior to the Restriction point. It is interesting that the cells need to spend so long after passing the Restriction point before entering S-phase. There are undeniably several S-phase genes that need to be transcribed. These genes also need to be translated before they can start doing their responsibilities, but if this takes around 6h is unknown.

Ionizing and UV irradiation affect RB and MCM differently

In paper I we showed that ionizing radiation (IR) blocked the loading of MCMs and the phosphorylation of RB. Cyclin D-Cdk4/6 becomes constitutively active after DNA damage induced by doxorubicin, a topoisomerase II inhibitor causing double strand breaks (24). After this treatment RB becomes hypo-phosphorylated, consistent with our findings after IR. However, because we observed that some cells are able to load MCMs prior to RB hyper-phosphorylation we do not believe that the block of RB hyper-phosphorylation after IR is the cause of delayed loading of MCMs. Cdt1 is, however, rapidly degraded after IR (50,71), and the degradation of Cdt1 will block the loading of MCMs and explain the effect we see after IR.

It should be noted that in *S.pombe* we have previously shown that IR does not affect pre-RC loading (32). However, these experiments were done by synchronizing the cells with a *cdc10* block-and-release (as explained in the

introduction). An additional target of Cdc10 is Cdt2, a substrate receptor protein for the E3 ubiquitin-ligase complex responsible for the degradation of Cdt1 (49). This implies that after a Cdc10 block-and-release Cdt2 is not present to be activated by IR and can therefore not degrade the Cdt1 that is produced. IR may therefore not affect the pre-RC loading after a Cdc10 block-and-release.

In addition to the IR (Paper I), we also investigated the relative timing of the restriction point and MCM loading after UV. The flow cytometric analysis of the RB phosphorylation it appeared very different from what we observed after IR (figure 3A). In the IR experiments the cells were all RB-positive (Q3), whereas in the UV experiment the cells all appear RB-negative (Q4). This could indicate that the cells stop at a later stage in the cell cycle (after RB hyper-phosphorylation). However, if the protein level of RB is significantly reduced by the UV-irradiation this would also change the RB-staining pattern as if they stop at a later stage in G1-phase. Immunoblot analysis showed that RB was not hyper-phosphorylated after UV irradiation (RB upper band figure 3B) as we showed for the cells treated with IR (Paper I figure 2A). This leads us to conclude that there is less RB after exposure to UV (figure 3B). An attractive hypothesis is that MDM2 might be responsible for the reduction in RB levels after UVC irradiation. MDM2 is an E3 ligase and it has been shown to promote RB degradation (72). It has also been shown that MDM2 binds selectively to hypo-phosphorylated RB inhibiting the RB-E2F interaction (73), leading us to speculate that MDM2-binding to RB might prevent RB anchoring to the nucleus. Intriguingly, MDM2 is also shown to have transcript variants containing uORF, a known trait of GCN2-eIF2 α -P target genes. It would therefore be interesting to see if GCN2 affects the level of MDM2 and the reduction in RB protein level after UVC-irradiation and to investigate if the uORF of MDM2 has similarities with the uORF of ATF4.

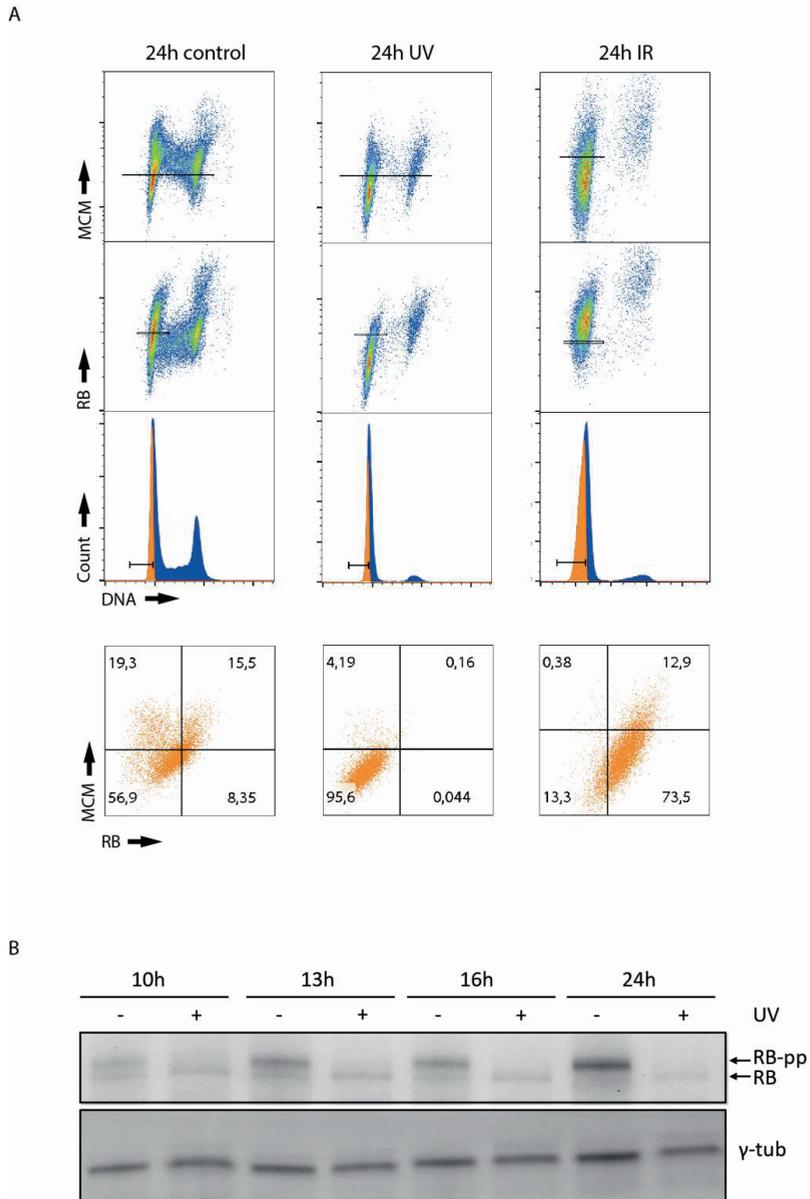


Figure 3: RB appears different in synchronized BJ cells in response to UV and IR. A) BJ cells synchronized by contact inhibition was treated with UV ($60\text{J}/\text{m}^2$) or IR (6Gy) 1h after release and were extracted and fixed 24h after release. The samples were analyzed by flow cytometry. B) BJ cells synchronized by contact inhibition was treated with UV ($60\text{J}/\text{m}^2$) 1h after release and fixed at time points indicated. Whole cell lysates were analyzed by immunoblotting for RB. γ -tub was used as a loading control.

Gating the right population after flow cytometry

Studying the timing of RB hyper-phosphorylation and the loading of MCM is based on whether or not the cells contain MCMs or RB after extraction. This implies that it is important that the gates are set correctly. Gating of the RB-positive cells was quite straightforward. There was a clear drop in RB staining once the cells entered S-phase and there were also two distinct populations in the G1-population, making it easy to separate positive from negative cells. The clear drop in RB staining also indicates that rather than a gradual phosphorylation event, all RB in a single cell becomes hyper-phosphorylated at one time. For the MCMs a clear positive and negative population was not obvious in all experiments, suggesting that there is a continuous loading of MCM proteins throughout G1-phase. Several papers have shown that there are two populations of MCM-positive cells in G1 (74,75) and it has also been shown that MCMs load gradually during the course of G1 phase with maximum loading preceding S phase (76). We therefore decided to use the decline we observe in loaded amounts of MCM when setting the gate for MCM positive cells. We argue that when replication is completed in S-phase the MCM are subsequently off-loaded and the cells in G2/ M are expected to be MCM-negative. It is also important to note that in Paper II we use the median value of MCMs in G1-phase, rather than just the positive cells. This allows us to determine if there is a shift in the overall level of MCMs in extracted cells.

Loading of MCMs in mitosis

It is worth noting that an MCM-positive G2/M population can be observed in exponentially growing BJ cells (paper I, figure 4A). It is therefore likely that some BJ cells start loading MCMs before cytokinesis is completed, in agreement with previous reports of MCM loading occurring in late mitosis (77,78). In contrast, exponentially growing U2OS cells did not show MCM loading in cells with G2/M DNA content (Paper I figure 1A and figure 4A). However, when mitosis was prolonged after Nocodazole treatment of U2OS cells, we consistently observed a fraction of MCM-positive G2/M cells (Paper I figure 5). We cannot separate G2 and M cells based on DNA content. But, histone H3 (phosphor S10) (H3P) antibody is often used as a mitotic marker. This phosphorylation is crucial for chromosome condensation

and progression through mitosis. The phosphorylation remains high until metaphase and in anaphase H3 is gradually dephosphorylated (79), leaving the cells H3P negative at the end of mitosis. We have seen that the H3P-positive population is not included in the MCM-positive population we observe in G2/M (data not shown), suggesting that MCM only start loading once the cells pass anaphase.

Concluding remarks

We have mainly been focusing our work on GCN2 in G1-phase, and it clearly has a role there, but that does not exclude that GCN2 has functions in other cell-cycle phases. We have indication that GCN2 does have other roles than what is currently known. For example, we observed that GCN2-depleted cells grow more slowly than wild-type cells. We also observe that Nocodazole affects GCN2-depleted cells more severely than wild-type cells and that GCN2-depleted cells appear to have more micronuclei suggesting a role in mitosis. These phenotypes are difficult to explain solely based on current knowledge. We are confident that GCN2 will be an interesting subject for future studies, and most likely more substrates will be identified.

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