Regulation of the G1-S transition in fission yeast

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Introduction

The eukaryotic cell cycle

Cancer

Cancer is a term used for a complex set of diseases, which arise from cells that through a series of genetic changes have lost control of cell growth and division. Uncontrolled cell growth may have different causes including lack of response to growth-inhibitory signals, deregulated control of cell division, self-sufficiency in growth signals etc resulting in continued growth that may lead to tumour formation (Hanahan and Weinberg, 2000). Tumours are classified as either being benign or malignant, depending on their ability to invade other tissues. Only malignant cells can spread and form metastases and this is a common event in the late stages of cancer development. Cancer is a threat for all multicellular organisms and the risk of cancer development is influenced by several factors like genetic pre-disposition, the environment and lifestyle. Increasing our knowledge of the mechanisms involved in the regulation of cell growth and division is important as such studies can provide future targets for cancer therapy.

The cell cycle

Cells that grow and proliferate must go through the cell cycle. A cell cycle is series of stages in which the cell grows and the genetic material is duplicated and separated into daughter cells. A eukaryotic cell can go through two different cell cycles: a mitotic or meiotic cell cycle. The mitotic cell cycle produces two daughter cells that are genetically identical to the parent cell, whilst during meiosis four cells of unique genetic content are produced. The scope of this work has been to study regulatory events during the mitotic cell cycle, and this is further described in detail.

The mitotic cell cycle is divided into distinct cell-cycle phases depending on the cellular events taking place in each phase. During a cell cycle the genome is replicated (S phase) and the duplicated DNA is segregated to the new daughter cells (M phase) (Figure 1). In M phase the chromosomes are condensed, sister chromatids are separated and two distinct nuclei are formed. Later the cell is cleaved into two separate daughter cells (cytokinesis). DNA replication and mitosis are considered the two major events of the cell cycle and they are

separated by two gap phases G1 and G2. In G1 the cell prepares for DNA replication while preparations for mitosis occur in G2. Onset of a cellular event is often dependent on completion of a previous event, and the progression from one phase to the next is tightly regulated. In G1 the cell makes the important decision to enter another mitotic cycle, a meiotic cycle, or cease cycling and enter a quiescent stage (Figure 1). This decision point in G1 depends upon environmental and cellular conditions and is termed "Start" in yeast and the "Restriction point" in mammals (Hartwell, 1974). Progression past Start commits the yeast cell to complete a new round of the mitotic cell cycle.

The length of each cell-cycle phase relative to the complete cycle varies between different organisms, cell types and growth conditions (Figure 1). The lower eukaryote and model organism *Schizosaccharomyces pombe* (fission yeast) uses approximately four hours to complete a mitotic cell cycle under standard laboratory conditions. During this time only 15 minutes are spent in G1 phase. This is different from a typical human cell that can spend over 24 hours to complete a cycle of which many hours in G1.

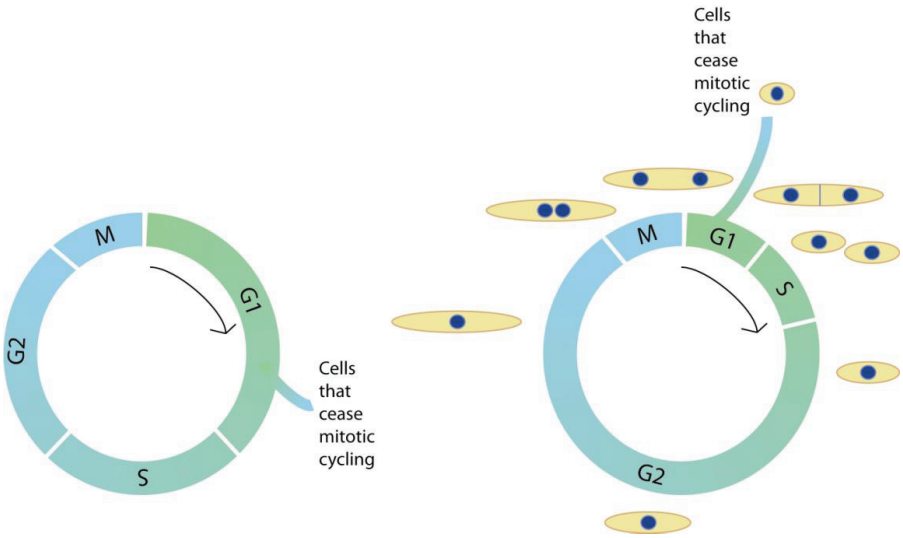

Figure 1. Illustration of the eukaryotic mitotic cell cycle.
Left: Typical cell cycle of a eukaryotic cell. Right: The mitotic cell cycle of fission yeast.

Cell-cycle regulation

It is crucial for a cell to regulate its cell-cycle progression. Genome maintenance, DNA replication and cell division are all processes that need monitoring and regulation for a cell to survive. Cells that fail in these processes may die or acquire mutations eventually leading to cancer in multicellular organisms.

The regulation of the eukaryotic cell cycle is governed by cyclin-dependent kinases (CDKs). Periodical activation and inactivation of the CDK activity occurs throughout the cell cycle and is regulated in different ways (Morgan, 1995). The CDKs require binding to a cyclin partner to be active, and various phosphorylation events and/or the presence of CDK inhibitors (CDIs) regulate the CDK activity. When active, the CDKs phosphorylate numerous substrates on the serine or threonine residue of a CDK target sequence (Moreno and Nurse, 1990). These phosphorylations lead to a response carrying the cell further in the cell cycle. The CDKs are expressed constitutively, but the levels of the different cyclins and CDIs vary during cell-cycle progression. Much of the substrate specificity for the CDK is conveyed by their cyclin partner and different CDK-cyclin pairs thus launch different cellular responses.

The CDKs are conserved through evolution from yeast to humans. Higher eukaryotes express several CDKs and these are responsible for different cell-cycle phase transitions in concert with specific cyclins. However, CDK1 can drive the cell cycle alone in mammalian cells by binding to its cyclin partners (Santamaria *et al.*, 2007).

Cell-cycle regulation in fission yeast

Studies in the two yeasts *Saccharomyces cerevisiae* (budding yeast) and fission yeast in the 1960s through 1980s revealed much of the basic mechanisms concerning cell-cycle regulation. Several of the genes that are required for the cell division cycle were identified during this period and they were termed cell division cycle (*cdc*) genes. Among the *cdc* genes in fission yeast *cdc2* is a key factor in regulation of the cell cycle, since its encoded gene product is the only CDK found in this organism. Cdc2 is a homologue of mammalian CDK1 and CDC28 of budding yeast (Lee and Nurse, 1987). The presence of only one CDK, the conservation of cell-cycle related processes, the short generation time, and the ease of performing genetic modifications make fission yeast a valuable organism to study cell-cycle processes.

The regulation of the cell-cycle progression in fission yeast is executed by Cdc2 and the regulatory cyclins Cig1, Cig2, Puc1 and Cdc13. The levels of these cyclins fluctuate during the cell cycle and thereby alter the Cdc2 kinase activity accordingly. Cig2 is the major G1 cyclin and associates with Cdc2 in G1 (Martin-Castellanos *et al.*, 1996). As Cig2 levels rise in G1, Cdc2-Cig2 activity brings the cells past Start and into S phase (Figure 2A). When the cells enter S phase, Cig2 is degraded (Figure 2B), thereby preventing re-entry into S-phase (Mondesert *et al.*, 1996; Yamano *et al.*, 2000). The level of the mitotic cyclin Cdc13 is low in G1, but starts rising through S and reaches a maximum level in G2, which is maintained through M phase. However, the Cdc2-Cdc13 activity is kept low through S and G2 phase due to an inhibitory phosphorylation by Mik1 or Wee1 on the Cdc2 amino acid residue Tyr15 (Russell and Nurse, 1987; Lundgren *et al.*, 1991). The phosphorylation of Cdc2 on Tyr15 prevents the cell from entering mitosis prematurely, but is removed by the phosphatase Cdc25 acting at the G2-M transition (Fantes, 1979; Russell and Nurse, 1986; Millar *et al.*, 1991). This dephosphorylation causes Cdc2-Cdc13 activity to rise and brings the cells into mitosis (Figure 2A), and then at the end of mitosis Cdc13 is degraded (Yamano *et al.*, 1996).

The CDK activity in G1 is low due to the presence of the CDI Rum1. Rum1 is the only CDI in fission yeast and determines the length of G1 before Start by inhibiting both Cdc2-Cdc13 and Cdc2-Cig2 (Figure 2B). Binding of Rum1 to Cdc2-Cdc13 inhibits the CDK activity directly, and in addition Rum1 promotes degradation of Cdc13 in G1. These actions prevent premature mitosis in G1 (Correa-Bordes and Nurse, 1995; Correa-Bordes *et al.*, 1997). Two other G1 CDK-cyclin pairs, Cdc2-Cig1 and Cdc2-Puc1, can target Rum1 for ubiquitination leading to its degradation (Benito *et al.*, 1998; Martin-Castellanos *et al.*, 2000). The Cdc2-Cig2 activity then increase and the cell progresses from G1 to S phase (Figure 2A). The level of Rum1 is also regulated by the nutritional state of the cell since the stability of the Rum1 mRNA is dependent on nitrogen availability. Under nitrogen-limiting conditions Rum1 mRNA is stabilized, Rum1 levels are sustained and this prolongs G1 (Daga *et al.*, 2003).

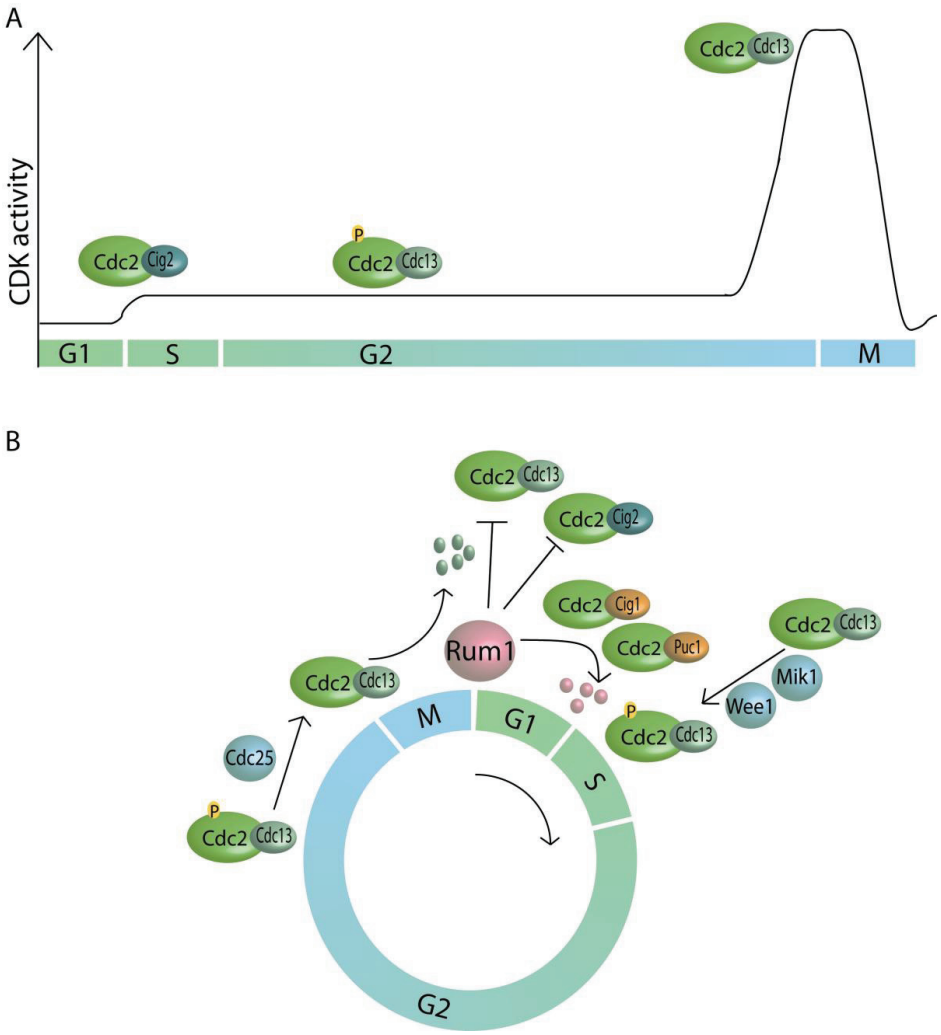

Figure 2. Cell cycle regulation by Cdc2 and cyclin partners in fission yeast.
A) The plot illustrates the CDK activity through the cell cycle. B) The CDI Rum1 promotes degradation of the mitotic cyclin Cdc13 and inhibits Cdc2-Cdc13 in late M and early G1 phase. The G1 cyclins Cig1 and Puc1 target Rum1 for degradation in late G1 and S phase. In S phase the two kinases Mik1 and Wee1 phosphorylate Cdc2 (yellow P) and thereby inhibit Cdc2-Cdc13 activity. The inhibitory phosphorylation is removed by the phosphatase Cdc25 late in G2 allowing progression of the cell into mitosis.

The G1-S transition

In G1 phase, before passing Start, the cell must take into consideration cellular and external signals regarding cell size, nutrient status and cellular fitness. Most of the preparations for DNA replication are also performed in G1. During mitosis the chromosomes are condensed,

and this tight packing must be relieved for the replication machinery to assemble and function. The assembly of the replication machinery starts in late M, goes on through G1 and ends in S phase with active replication (Takeda and Dutta, 2005). Several of the genes required for these cellular events are under periodic regulation, ensuring their expression from late mitosis until initiation of S phase. In addition their gene products are modified in different ways ensuring their activation or degradation at appropriate times.

Transcriptional regulation of the G1-S transition

The main transcriptional regulators required for the G1-S transition share little sequence homology between yeast and humans, but the structure of the regulatory networks are similar. The responsible transcription factors Cdc10 and SWI/SWI6 of fission and budding yeast, respectively, are homologues and parts of larger complexes (DSC/MBF (DNA synthesis control/MCB (Mlu1 cell cycle box)-binding factor)) that bind similar DNA consensus sites (MCB) in the promoter region of their target genes. In mammalian and plant cells the transcription factor E2F, which is unrelated in sequence to Cdc10 and SWI4/SWI6, regulates a similar set of genes (Nurse *et al.*, 1976; Andrews and Herskowitz, 1989; Ogas *et al.*, 1991; Cross *et al.*, 2011). Known target genes of DSC/MBF in fission yeast include *cdt1*, *cdt2*, *cdc18*, *cdc22*, *mik1* and *cig2*. These genes are all important for initiation of DNA replication or cell-cycle progression in G1 or S phase (Nasmyth and Nurse, 1981; Lowndes *et al.*, 1992; Kelly *et al.*, 1993; Hofmann and Beach, 1994; Obara-Ishihara and Okayama, 1994; Ng *et al.*, 2001; Yoshida *et al.*, 2003).

Initiation of DNA replication

Mitotic cycling cells depend on faithful replication and accurate transmission of one copy of the genetic material to each daughter cell during a cell cycle. Regulation of the initiation of DNA replication helps to ensure that each chromosome is replicated completely and only once per cell cycle. The process of DNA replication initiation is a four-step mechanism conserved in bacteria, archaea and eukaryotes (Bell and Dutta, 2002). It starts with origin recognition (Figure 3) followed by assembly of the pre-replicative complex (preRC) onto the origin, helicase activation and finally loading of the replisome. In eukaryotes, DNA replication starts at many origins on each chromosome. With the exception of budding yeast, these origins of replication are not well defined sequences. Instead they seem to combine different features of their DNA sequence (AT-rich, CpG islands), DNA conformation (bending and loops), chromatin structure (epigenetic markers) as well as transcriptional

activity in the area (Stinchcomb *et al.*, 1979; Takeda and Dutta, 2005; Sclafani and Holzen, 2007; Mechali, 2010). The preRC-bound (licensed) origins are not all activated at the same time during S phase and they can thus be classified as "early-" "mid-" or "late-firing". In fission yeast the timing of preRC assembly (during M and G1 phase) and its firing (in S phase) correlate (Wu and Nurse, 2009). Although numerous origins are licensed for DNA replication during G1 phase, many are never fired. It has been suggested that these "dormant" origins might simply be back-ups that can be activated at certain conditions ensuring flexibility in the replication process (Legouras *et al.*, 2006).

Recognition of the origins and assembly of the preRC

In fission yeast the preparations for DNA replication starts late in M phase when the Origin Recognition Complex (ORC) is recruited to the origins (Wu and Nurse, 2009). The ORC consists of six subunits, Orc1-6. ORC binding is followed by a stepwise loading of the factors that constitute the preRC (Figure 3). As the transcriptional program of the DSC/MBF complex commences in late mitosis, the two factors Cdc18 (homologue of mammalian and budding yeast Cdc6/CDC6) and Cdt1 are expressed and associate with the ORCs. In mammalian cells Cdt1 has recently been shown to be involved in the process of chromosome decondensation, a process required to make the DNA accessible for the replication machinery (Wong *et al.*, 2010).

The binding of Cdc18 and Cdt1 is followed by recruitment of the minichromosome maintenance (Mcm) complex, a hexameric ring of subunits Mcm2-7 (Figure 3). Multiple Mcm complexes are loaded and spread beyond the origin point although only two Mcm complexes are required for bidirectional replication from an origin. The reason for the apparent overload of Mcm2-7 onto DNA is yet unclear (Forsburg, 2004; Arias and Walter, 2007). The Mcm complex functions as the replicative helicase and upon activation it is suggested to unwind parental double-stranded (ds) DNA at origins and in front of the replication fork through S phase. Loading of the Mcm2-7 is the final step in the preRC assembly and occurs in G1 phase (Arias and Walter, 2007; Wu and Nurse, 2009).

Activation of the helicase

Formation of active replisomes requires unwinding of the origins by activation of the replicative helicase, stabilization of the single-stranded (ss) DNA exposed and loading of replication factors. Activation of the helicase is initiated at numerous preRCs in a regulated

and timed manner throughout S phase (Takisawa *et al.*, 2000). The mechanism of helicase activation is not fully understood but it involves recruitment of the helicase co-factors Cdc45 and the multimeric complex GINS to form the CMG (Cdc45-Mcm2-7-GINS) helicase complex (Figure 3) (Takeda and Dutta, 2005; Remus and Diffley, 2009). The active helicase unwinds the DNA at the origin and exposed ssDNA immediately attracts the single-strand binding protein RPA. The DNA sliding clamp (PCNA) is loaded onto DNA by the clamp loader RFC1-5 (replication factor C complex 1-5). DNA polymerase α, together with other proteins required for replication, can now be tethered to PCNA and DNA replication can begin.

Regulation of the initiation of DNA replication

Several of the steps in the initial process of DNA replication are regulated to ensure that replication is not initiated at inappropriate times during the cell cycle. Low CDK activity (G1 phase) is required to allow preRC assembly, whilst higher CDK activity is required for converting the preRCs to active replisomes and it also immediately blocks further preRC loading (S phase). As the CDK activity increases it inhibits rereplication via several mechanisms (Arias and Walter, 2007). For instance, Cdc18 is phosphorylated by Cdc2-Cig2 and, targeted for destruction when the replicative helicase is activated (Figure 3) (Jallepalli *et al.*, 1997; Lopez-Girona *et al.*, 1998). Other mechanisms that inhibit rereplication rely on regulation of the localisation and levels of DNA replication components, their access to chromatin, or the activity of the replicative helicase (Arias and Walter, 2007).

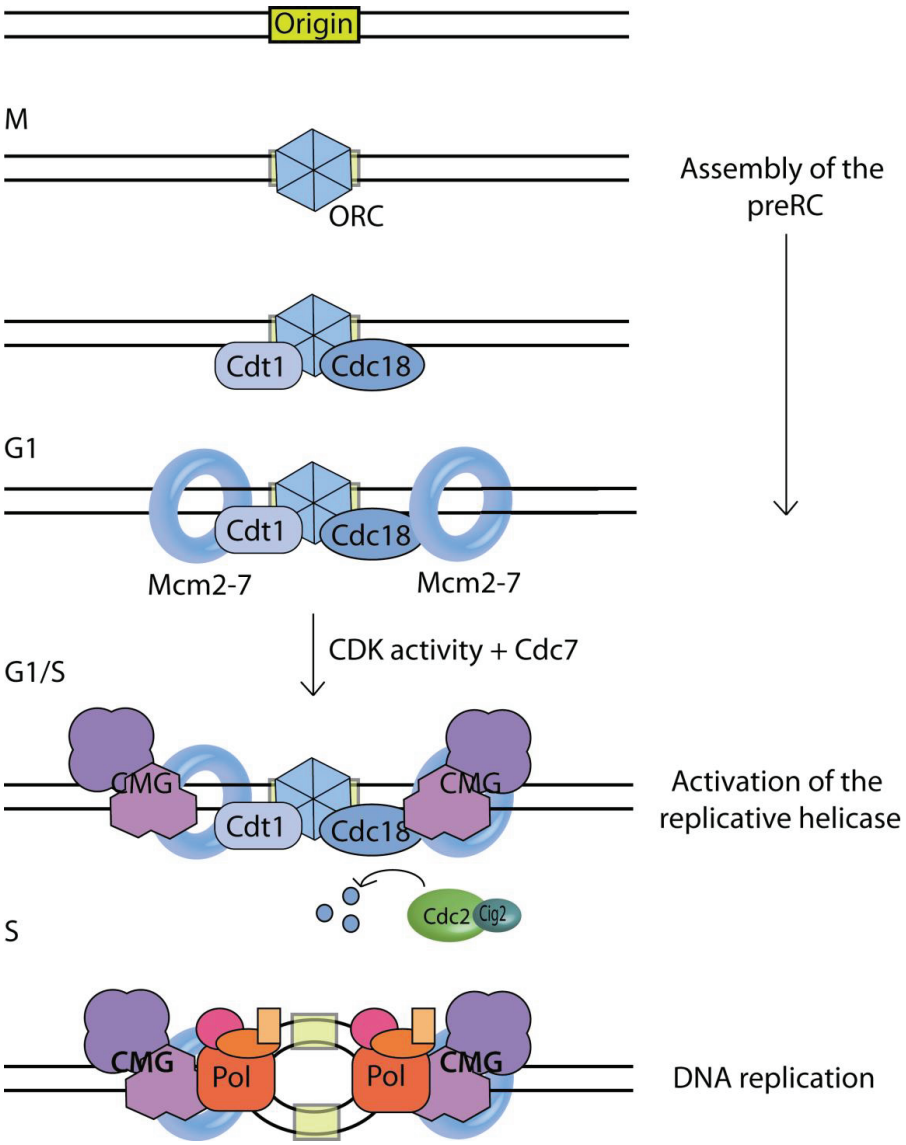

Figure 3 A simplified presentation of DNA replication initiation in fission yeast. The ORC binds to origins (green box) in late M phase. This is followed by recruitment of Cdt1, Cdc18 and Mcm2-7 in G1 phase, forming the preRC. Upon entry into S phase CDK and Cdc7 activity promotes CMG (Cdc45-Mcm2-7-GINS) helicase formation. DNA can then be unwinded, replication factors are recruited and DNA replication begins.

Checkpoints

The activity of different CDK-cyclin pairs carries the cell from one cell-cycle phase to the next if conditions are favourable. However, most cells have also evolved mechanisms to delay these transitions if the DNA is damaged or a cell-cycle phase is not completed properly. Such mechanisms are called checkpoints and they ensure the correct order and timing of cell-cycle events and leads to increased genetic stability and enhanced cell survival (Hartwell and Weinert, 1989). The delay is considered a checkpoint if a mutation or treatment with a drug abolish the delay. Mutations in checkpoint pathway genes or changed expression of their gene products are often found in cancer cells. The sustained cell cycling observed in these cells, regardless cellular fitness or environmental conditions, is partly a result of defective cell-cycle checkpoints (Hanahan and Weinberg, 2000; Shimada and Nakanishi, 2006).

The target of a checkpoint is often the major regulator of cell-cycle progression, the CDK activity, and the result is a cell-cycle delay that allows the cell to recover from the obstacles it has encountered. Other targets include DNA replication and transcriptional programs (Zegerman and Diffley, 2009). Another important property of the checkpoint mechanism is to ensure re-entry into the cell cycle after the obstructions are cleared. In the event that the cell damage is too severe permanent cell-cycle arrest or cell death can be initiated to eliminate the putatively dangerous cell from the multicellular organism (Zhou and Elledge, 2000). Most checkpoints are highly conserved from yeasts to mammalian cells and they govern many different processes in eukaryotic cells. Only the checkpoints that secure the genomic integrity will be discussed in more detail below.

Checkpoints in fission yeast

Fission yeast exhibits several checkpoints that are activated at different times in the cell cycle and by different irregularities in the genome (Figure 4) (Caspari and Carr, 1999; Humphrey, 2000; Musacchio and Salmon, 2007). For instance, entering mitosis with DNA strand breaks or unreplicated DNA can lead to loss of genetic material. Therefore the cell monitors its genomic status before mitosis and if necessary delay in G2 to initiate repair of damaged DNA or complete DNA replication. DNA damage encountered in G2 phase activates the G2-M checkpoint whilst the S-M checkpoint ensures that the replication of the genome is complete. Both checkpoints delay the cell-cycle progression before entry into mitosis. DNA damage encountered in S phase activates the intra-S checkpoint and halts the progression of S phase

securing adequate time for clearance of the perturbation(s). During mitosis the attachment of chromatids to the spindle is monitored by the spindle assembly checkpoint (SAC). The SAC operates through a different checkpoint pathway than the three other more "classic" checkpoints and will not be described further in this thesis.

The classic checkpoints share a conserved set of checkpoint proteins which acts as sensors, transducers and effectors of the checkpoint response (al-Khodairy and Carr, 1992; Enoch *et al.*, 1992; Rowley *et al.*, 1992). Several of the genes encoding these checkpoint proteins were identified in screens for radiation sensitive mutants and consequently named *rad* genes (Phipps *et al.*, 1985). A core of six *rad* genes, *rad1*, *rad3*, *rad9*, *rad17*, *rad26* and *hus1*, are referred to as the *rad* checkpoint genes in fission yeast. The classic checkpoints are all absolutely dependent on the *rad* checkpoint genes, but these genes are not essential for fission yeast and deletion of the genes does not affect cell-cycle progression during normal growth conditions (Humphrey, 2000).

Initiation of classic checkpoints

Mammalian cells rely on two sensor kinases, ATM (Ataxia-Telangiectasia-Mutated) and ATR (ATM- and Rad3-related), for checkpoint initiation. Depending on the genomic lesion ATM or ATR is activated and ensures checkpoint activation. In fission yeast a single sensor kinase, the ATR-homologue Rad3, is required for induction of the classic checkpoints. The sensor mechanism comprises two complexes (Figure 4): Rad3 in association with the regulatory subunit Rad26 (ATRIP in mammalian cells) and the Rad9-Rad1-Hus1 (9-1-1) complex (Wolkow and Enoch, 2002). Similar sensor complexes are formed in mammalian cells and budding yeast. Upon genomic perturbations the two sensor complexes associate with DNA independently of each other, but both complexes are required at the damaged site to induce the checkpoint. The 9-1-1 complex, which resembles PCNA in structure, is suggested to be loaded onto DNA by a checkpoint-specific clamp loader in which RFC1 is substituted with the checkpoint Rad17 protein (Zhou and Elledge, 2000; Melo *et al.*, 2001). All six checkpoint *rad* gene products are thus required for the initial step of the checkpoint response (Figure 4).

Extensive research has been performed using different model organisms to elucidate the early steps of checkpoint initiation and the nature of the molecular structure(s) that are sensed by the two sensor complexes (Garvik *et al.*, 1995; Lee *et al.*, 1998; Melo *et al.*, 2001; Ellison and Stillman, 2003; Zou and Elledge, 2003; Zou *et al.*, 2003; Dart *et al.*, 2004; Byun *et al.*, 2005;

Majka *et al.*, 2006). The 9-1-1 complex is preferentially loaded onto 5' recessed DNA ends by the checkpoint clamp loader in a process dependent on ssDNA and RPA. RPA-covered ssDNA is also required for recruitment of the other sensor complex ATR-ATRIP to the perturbed site. Stretches of ssDNA that are formed both during DNA repair and at stalled replication forks are immediately covered by RPA. A common structure formed through the recovery processes after different DNA perturbations thus seems to be required to induce the classic checkpoints. However, for ATR-dependent checkpoint activation also other factors like the replication initiation factor TopBP1 (Cut5 in fission yeast) are required. The role of TopBP1, which seems to be conserved from yeast to mammalian cells, is to recruit 9-1-1 to the perturbed site and this association stimulates TopBP1-mediated activation of ATR (Parrilla-Castellar and Karnitz, 2003; Kumagai *et al.*, 2006; Delacroix *et al.*, 2007). The most recent suggestion for the identity of the ATR-dependent checkpoint inducer is based on discoveries from studies in *Xenopus laevis*, and is a structure resembling DNA primed for replication (MacDougall *et al.*, 2007; Yan and Michael, 2009; Zegerman and Diffley, 2009). This model is supported by the discovery of a connection between ongoing primer synthesis at stalled forks and checkpoint activation in *X. laevis* (Van *et al.*, 2010).

Classic checkpoint signal transduction and effects

Once activated, Rad3 phosphorylates and activates the checkpoint transducers Cds1 and/or Chk1. This action requires the adaptor proteins Mrc1 and Crb2 (Figure 4) (Caspari and Carr, 1999; Melo and Toczyski, 2002). The association with either adaptor protein is dependent on which cell-cycle phase the cell is in and/or the abnormal structure that was sensed. The expression of Mrc1 is cell-cycle regulated and restricted to S phase. Signalling through the Mrc1-dependent pathway leads to phosphorylation of Cds1, the S-phase specific checkpoint kinase. The second adaptor protein, Crb2, is expressed throughout the cell-cycle and is required in G2 or late S phase for Rad3-mediated phosphorylation and activation of Chk1. Several proteins, including many involved in the cell-cycle and DNA repair machineries, are regulated by the activity of the two transducing checkpoint kinases. The major downstream targets of Chk1 and Cds1 are the phosphatase Cdc25 and the kinases Mik1 and Wee1 (Figure 4). Cdc25 is primarily the target of Chk1, while Cds1 activates Mik1 and to a smaller degree affects Wee1 and Cdc25. The result is inhibition of Cdc2-cyclin activity by maintaining or increasing the inhibitory phosphorylation on Tyr15 (Figure 4). Inhibition of Cdc2-cyclin leads to a pause in the cell-cycle progression which presumably provides time for DNA repair or correct assembly of the cellular structure.

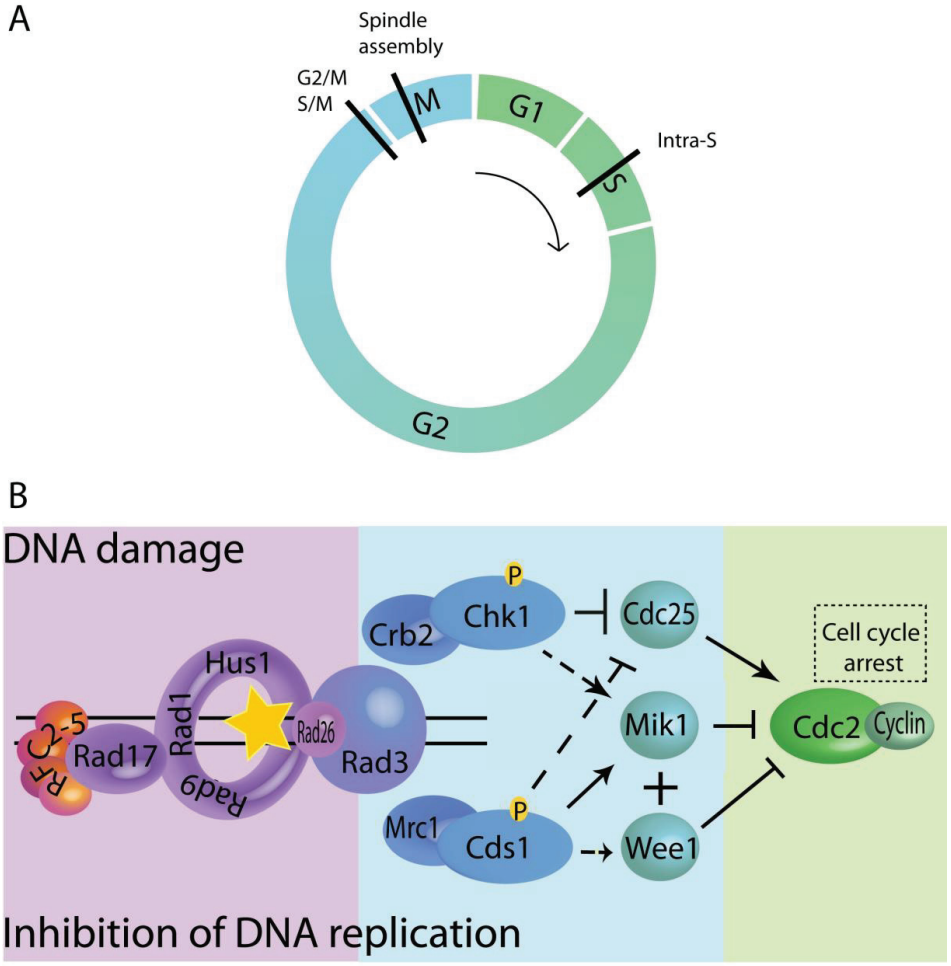

Figure 4. A schematic presentation of the major checkpoints in fission yeast.
(A) The mitotic cell cycle of fission yeast. The major checkpoints are pinpointed. B) An overview of the classic checkpoint response to DNA damage or inhibition of DNA replication in fission yeast. Sensors are shown in purple, signal transducers in blue and the major effector proteins in green. The Rad3-Rad26 and 9-1-1 complexes associate with DNA independently of each other. Activated Rad3 signals through Mrc1-Cds1 (upon inhibition of DNA replication) or Crb2-Chk1 (upon DNA damage). Both checkpoint transducers inhibit Cdc25 and activate Mik1, but to different degrees. Cds1 also activate Wee1. These actions function to phosphorylate or maintain Cdc2 in a phosphorylated state, thus inhibiting cell-cycle progression.

The G1-S checkpoint in fission yeast

The G1-S transition is governed by a checkpoint in many organisms and the necessity for proper regulation of this transition is clarified if considering the following facts. First: in G1 the cells are about to make a major decision of which cellular developmental pathway to take.

Passing Start/the Restriction point prematurely can be detrimental as it commits the cell to complete another round of the cell cycle. Second: error-free replication is critical for the cell. Entering S phase with unrepaired DNA lesions can cause mutations and genetic instability that, in multicellular organisms, can cause cancer. Defective regulation of the G1-S transition is often found in cancer cells (Sherr and McCormick, 2002; Nojima, 2004) and this emphasizes the importance of elucidating the underlying regulatory mechanisms acting at this transition. In eukaryotes ranging from budding yeast to *X. laevis* and mammalian cells it has been shown that induction of DNA damage in G1 is followed by a cell-cycle delay before bulk DNA replication commences (Konig and Baisch, 1980; Painter and Young, 1980; Siede *et al.*, 1993; Costanzo *et al.*, 2000). This G1-S checkpoint follows the conserved classic checkpoint mechanism described earlier for the intra-S, S-M and G2-M checkpoints.

Fission yeast does not possess a classic G1-S checkpoint, but does hold a G1-S checkpoint. This checkpoint was found by irradiation of cells in G1 phase with ultraviolet C (UVC) light (Nilssen *et al.*, 2003). However, the G1-S checkpoint in fission yeast is not a general DNA-damage checkpoint, since it is activated by some, but not all DNA-damaging agents. Treatment of cells in G1 phase with the alkylating agent methyl methanesulfonate (MMS), the oxidative agent hydrogenperoxide (H_2O_2) or UVC activate the checkpoint, whilst other DNA-damaging agents, such as ionising radiation (IR) or psoralen + ultraviolet A light (PUVA treatment) do not (Krohn *et al.*, 2008). The G1-S checkpoint in fission yeast also differs from the classic checkpoints in that deletion of the checkpoint genes *cds1* or *rad3* is not sufficient to abolish the checkpoint delay. The second classic checkpoint effector Chk1 is not required for the G1 delay and Cdc2 does not become phosphorylated (Nilssen *et al.*, 2003). Instead the checkpoint has been shown to depend on the protein kinase Gcn2 (general control non-depressible 2), which is involved in regulation of general translation. This result provides a link between cell growth and checkpoint induction. After treatment of G1-cells with UVC/MMS/H_2O_2, Gcn2 is activated and phosphorylates the translation initiation factor eIF2α (eukaryotic initiation factor 2α) leading to downregulation of global translation. The presence of phosphorylated eIF2α seems to coincide with a delay in cell-cycle progression in G1 (Tvegård *et al.*, 2007; Krohn *et al.*, 2008). The G1-S checkpoint is only partly understood, and the upstream signal(s) for checkpoint induction is largely unknown and was part of the scope of the present study. In addition the contribution of Rad3 to the G1-S checkpoint mechanism was further explored.

DNA repair

A cell acquires vast amounts of DNA damage daily. DNA lesions can form spontaneously, by external (radiation or chemicals) or internal (by-products of cellular metabolism) DNA damaging agents, or during endogenous processes such as DNA replication. DNA damage is thus a continuous danger for all living cells, a threat that has existed since the beginning of life (Taylor and Lehmann, 1998). Faithful maintenance of the genome, by minimizing the number of heritable mutations and clearing genomic defects that can interfere with important cellular processes such as transcription or DNA replication, is crucial for the survival of the organism. If left unrepaired the DNA lesions can lead to cancer, premature aging or diseases causing mental retardation in humans (O'Driscoll and Lehmann, 2010).

Most of the repair pathways are conserved and they include nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), homologous recombinational repair (HR) and non-homologous end-joining (NHEJ). These pathways recognize and repair a variety of lesions ranging from base modifications, base pair mismatches and DNA strand breaks to crosslinks (Fleck and Nielsen, 2004; O'Driscoll and Lehmann, 2010; Rastogi *et al.*, 2010). Upon double strand breaks (DSBs) the choice of repair pathway depends on the presence of a homologous DNA strand as template. In S and G2 phases, where a homologous template is available HR is the preferred repair pathway of DSBs, whilst NHEJ dominates in G1 phase. MMR mainly corrects errors introduced during DNA replication like mismatched, deleted or inserted bases. BER and NER repairs modified bases, but differ in substrate specificity based on the bulkiness of the lesions they correct. Smaller modifications like alkylation, oxidation, deamination and abasic sites are substrates for the BER pathway whereas the NER pathway is more versatile and particularly efficient on bulkier substrates. In addition to these repair pathways some specific base modifications can be repaired through a much simpler process. Several organisms possess single enzymes that can directly reverse DNA lesions. Examples of such enzymes are the O^6-methylguanine-DNA methyltransferase that repair O^6-alkylated bases and the photolyases that repair certain UV-induced DNA damages using the energy obtained from an absorbed photon (photoreactivation).

UV radiation is one of the most potent DNA-damaging agents that organisms are exposed to in nature. It can cause a variety of DNA lesions like cyclobutane pyrimidine dimers (CPDs) and the helix-distorting 6-4 photoproducts (6-4 PPs) in addition to abasic sites and oxidative

damage (Rastogi *et al.*, 2010). Other cellular components like lipids and proteins are also damaged by UV radiation, but such damage does not pose an equal hazard to the cell since these components have a limited lifetime and are replaced upon need.

Nucleotide excision repair

The most versatile of the DNA repair pathways is NER, which is found in most organisms and highly conserved among eukaryotes (Rastogi *et al.*, 2010; Kuper and Kisker, 2012). The substrates of NER vary from bulky lesions, to DNA-intrastrand crosslinks and some forms of oxidative damage. However, the most critical function of NER is the repair of UV-induced lesions such as CPDs and 6-4 PPs. The NER pathway starts with damage recognition followed by DNA unwinding, endonuclease incisions on each side of the damage, removal of the damaged oligonucleotide and finally gap filling and sealing of the resulting nick (Figure 6). NER can be subdivided into two pathways called global genome NER (GG-NER) and transcription-coupled NER (TC-NER). Lesions over the entire genome can be repaired through GG-NER, whereas lesions encountered by the transcriptional machinery, during transcription, can also activate the TC-NER pathway.

The difference in the nomenclature of the NER components between mammalian cells and fission yeast is extensive, even though most of the factors involved are conserved. The mammalian nomenclature is used in the following text and the fission yeast homologues are provided in a box in Figure 6.

DNA lesions repaired by NER are recognized by one of two different complexes (Figure 6). Lesions formed in transcriptionally silent areas are recognized by an XPC-HR23B protein complex and this is specific for GG-NER. In TC-NER it is the RNA polymerase II (RNApolII) that encounters the damage and attracts CSA and CSB to the site. The initial damage recognition is followed by recruitment of the transcription factor IIH (TFIIH) a large multisubunit complex. TFIIH unwind the DNA helix around the damaged site. Additional factors such as RPA, XPA and XPG are then recruited. XPA, RPA and TFIIH form a pre-incision complex. Incisions are made after recruitment of the endonuclease XPF-ERCC1. XPF-ERCC1 and XPG cleave 5’ and 3’ to the damage respectively, but the order of these incisions is unclear. The damage-containing oligomer (24-32 nucleotides) is released from the site by helicase activity and repair is completed by DNA synthesis and ligation. These final

steps are performed by DNA polymerase (DNApol) δ/ε and DNA ligase 1 (LIG1) along with other replication factors like PCNA and RFC (Rastogi *et al.*, 2010).

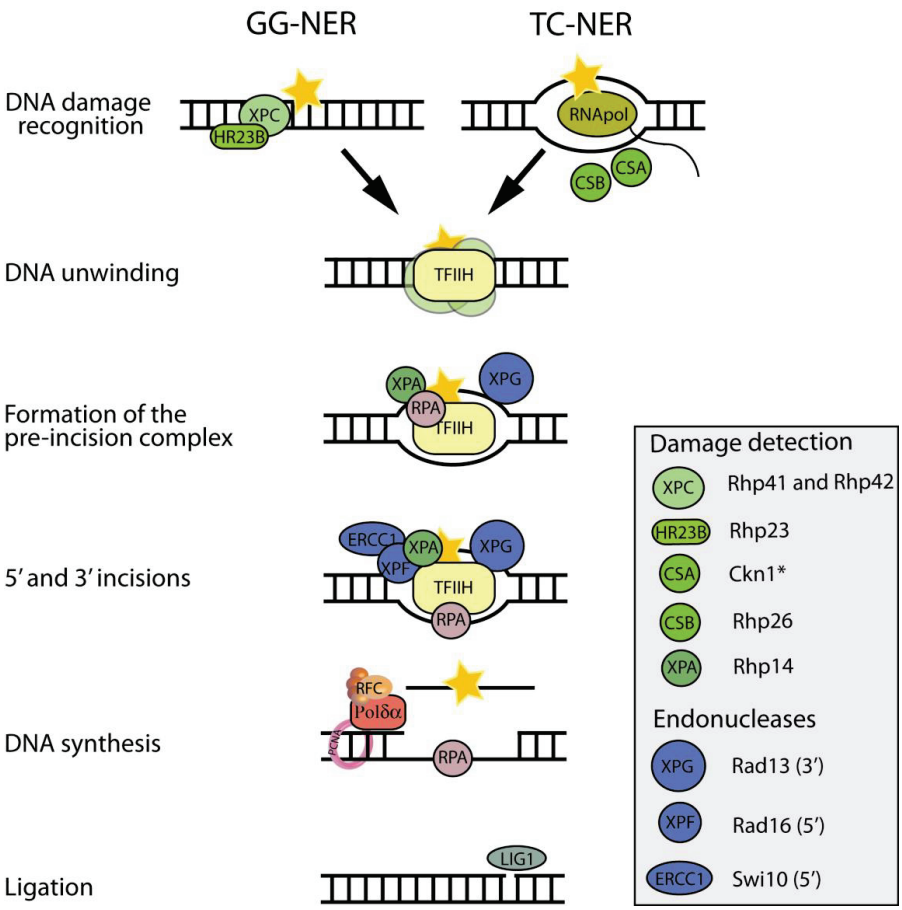

Figure 6. A simplified schematic presentation of NER.
DNA damage is recognized by two different complexes (green) of the GG-NER (left) and TC-NER (right) pathways. TFIIH is recruited to the site followed by TFIIH-mediated DNA unwinding. Recruitment of XPA and RPA completes the formation of a pre-incision complex. Endonucleases (dark blue) make incisions on each side of the damage and the oligomer is removed. The gap is filled by the action of DNA polymerase and other replication factors (pink). Finally the nick is sealed by LIG1. The difference in nomenclature of major NER factors between mammalian cells and fission yeast is presented in the grey box. The asterisk indicates a putative homologue.

UV-damage DNA endonuclease-dependent excision repair

Fission yeast holds an extreme resistance to UV-induced DNA damage, even in the absence of NER. This trait cannot be caused by photolyases as fission yeast has none, but is rather the result of an additional repair pathway capable of removing UV-induced lesions, including

CPDs, 6-4 PPs, abasic sites and to some extent mismatches and certain intrastrand crosslinks (Bowman *et al.*, 1994; Avery *et al.*, 1999; Kanno *et al.*, 1999; McCready *et al.*, 2000). This pathway is called UV-damage endonuclease (UVDE)-dependent excision repair (UVER) and is independent of NER (Yasui and McCready, 1998). UVDE (also designated Uve1) is the key player of UVER being responsible for both damage recognition and the first incision step of the repair pathway (Figure 7). Uve1-homologues have been found in many bacteria and several fungi (Takao *et al.*, 1996; Goosen and Moolenaar, 2008). Upon damage recognition Uve1 cleaves the proximal DNA phosphodiester backbone 5' to the damaged site creating a nick in the damaged strand (Bowman *et al.*, 1994). Two pathways have been proposed for processing nicks introduced by Uve1 (Fig1). One pathway is dependent on different recombination factors, the other on the flap endonuclease Rad2 (Yonemasu *et al.*, 1997; McCready *et al.*, 2000). Only the steps of the Rad2-dependent pathway will be further described. DNApol δ along with other replication factors like PCNA and RFC extends the free 3' end eventually leads to displacement of the damaged strand and creation of a 5' flap structure (Figure 7). The flap structure is the substrate of Rad2 which removes the flap in the next repair step. The remaining nick is sealed by DNA ligase (Alleva *et al.*, 2000).

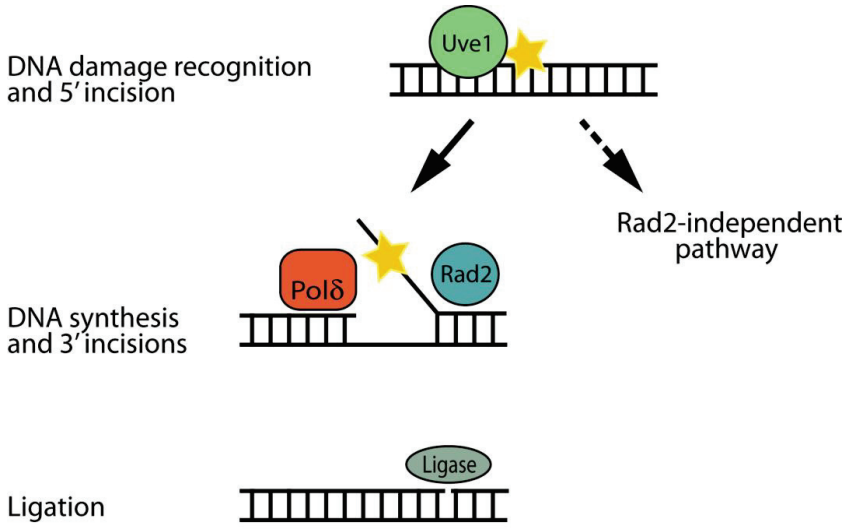

Figure 7. A schematic presentation of UVER.
The DNA damage is recognised and the 5' incision is performed by Uve1. DNA synthesis by DNApol δ causes displacement of the damaged strand. Rad2 removes the flap structure by an incision 3' to the damage. Finally the resulting nick is sealed by a ligase. The proposed Rad2-independent pathway is indicated (dotted arrow).

List of papers

Paper I: Rapid regulation of protein activity in fission yeast.
Bøe CA*, Garcia I*, Pai CC*, Sharom JR*, Skjølberg HC*, Boye E, Kearsey S, Macneill SA, Tyers MD and Grallert B.

BMC Cell Biol. 2008 May 5;9:23

* shared first-authorship

Paper II: Induction of a G1-S checkpoint in fission yeast.
Bøe CA, Krohn M, Rødland GE, Capiaghi C, Maillard O, Thoma F, Boye E and Grallert B.

Proc Natl Acad Sci U S A. 2012 Jun 19;109(25):9911-6. Epub 2012 Jun 4.

Paper III: Hpz1 modulates the G1-S transition in fission yeast.
Bøe CA, Knutsen JHJ, Boye E and Grallert B.

PLoS One. 2012;7(9):e44539. Epub 2012 Sep 6.

Aims of study

The purpose of this work was to shed light on the most upstream events of the Gcn2-dependent G1-S checkpoint pathway in fission yeast and also to investigate the contribution of Rad3 to the regulation of the G1-S transition.

Paper I

Development of a method to quickly and reversibly activate proteins in fission yeast, since no such methods are available. Our plan was to exploit such a method to (i) explore how the cells respond to a DSB in G1 phase with special emphasis on the role of Rad3 and (ii) to determine whether a DSB can activate the Gcn2-dependent checkpoint.

Paper II

Investigations of the inducing signal for the Gcn2-dependent checkpoint. A central question to answer was whether the inducing signal was the DNA damage itself, a DNA repair intermediate or damage to other macromolecules.

Paper III

Characterization of a putative Rad3 binding partner, Hpz1 (Homologue of PARP-type Zn-Finger). Of special importance was the determination of a possible interaction between Hpz1 and Rad3.

Summary of results

Paper I

From earlier work in our group we knew that UVC irradiation induces a Gcn2-dependent G1-S checkpoint that appeared to be independent of Rad3. Determination of the inducing signal for the Gcn2-dependent checkpoint was a main interest of the group, and we also wanted to better understand the signal(s) activating Rad3 to decipher why it is not essential for our checkpoint. To investigate these issues we wanted to exploit and analyze the different responses between cell-cycle phases to a treatment that activates Rad3 in S-G2, but not in G1, as well as to explore which treatments can activate the Gcn2-dependent checkpoint. Therefore we wished to generate a system where we could introduce DSBs in a given cell-cycle phase, and ask whether it activates Rad3 or the Gcn2-dependent checkpoint. To this end, we needed to be able to control the activity of the HO-endonuclease on demand in G1 cells (the HO endonuclease of budding yeast initiates mating-type switching by generating a DSB at the mating-type locus). The available systems for regulation of protein activity in fission yeast were not suitable for this purpose, and therefore we wanted to develop a method to rapidly and efficiently regulate protein activity in fission yeast.

We employed a principle that had been shown to work in other organisms. The protein of interest is fused with the hormone-binding domain (HBD) of the vertebrate estrogen receptor (ER). The HBD binds the Hsp90 complex at normal conditions resulting in an inactive protein, possibly because of steric hindrance. Upon addition of estradiol, a hormone-induced conformational change in the HBD causes the Hsp90 complex to be released and the HBD-fused protein becomes active. We demonstrate that this system can be used to regulate some, but not all proteins in fission yeast. Unfortunately we found that the HO endonuclease retained little activity when fused with the HBD and upon addition of estradiol, therefore our investigations of the requirements for induction of Rad3 in G1 and whether a DSB can activate the Gcn2-dependent checkpoint remained unanswered. However, the method in itself can prove to be a powerful tool for fission yeast researchers by rendering novel experimental approaches possible.

Paper II

In this paper we continued our investigations of the most upstream events of the G1-S checkpoint in fission yeast. We wanted to determine whether the signal that activates the checkpoint is the DNA damage itself, an intermediate formed through repair of the lesion, or damage to some other cellular macromolecule. To explore this we exploited several DNA repair-deficient mutants that show a reduced rate of DNA damage removal, but does not influence the removal of damage to other cellular components. If DNA damage is involved in activation of the checkpoint, we expected that the G1 delay would be prolonged in such mutants compared to wild type cells. If the signal arose from damage to other macromolecules in the cell, the length of the delay would be unaffected by reduced DNA repair. UVC irradiation was used to introduce damage and induce the checkpoint. The length of the G1 delay was compared between wild type cells and different NER- and UVER-defective mutants.

The G1 delay was completely lost in a mutant unable to initiate repair through both NER and UVER. From this we concluded that repair of DNA damage lead to induction of the checkpoint. We also found that a mutant that recognizes DNA damage via the NER pathway, but is defective in all the incision steps, lost the G1 delay, whilst a mutant with partial NER-incision activity and blocked UVER showed a prolonged checkpoint delay. This means that endonuclease activity or their presence at the damaged site is required to produce the inducing signal. We concluded that the Gcn2-dependent G1-S checkpoint is not induced by the DNA damage itself, but by a DNA repair intermediate(s). We also found that Gcn2 was activated in the repair mutants that showed no checkpoint delay. Based on these findings we suggested that activation of Gcn2 does not depend on processed DNA damage and that the G1-S checkpoint in fission yeast is dependent on at least two different inputs.

Paper III

In this paper we describe the protein Hpz1 for the first time. Based on protein sequence information we discovered that several fungal Rad3 homologues contain an extension on their C-terminal. This extension exists as a separate protein in fission yeast (Hpz1). The fact that Rad3 and Hpz1 are fused in some organisms indicates that they might share a joint function. We explored the phenotypes of an *hpz1* deletion mutant and also tried to establish whether Rad3 and Hpz1 interact *in vivo*.

We found that Hpz1 is expressed in a cell-cycle regulated manner restricted to G1 and early S phase, and that it localize to the nucleus. Unsynchronized *hpz1*Δ cells were mildly sensitive to hydroxyurea (HU) and UVC irradiation, however, a dramatic reduction in survival upon UVC irradiation in G1 phase was observed. From this we concluded that Hpz1 has an important cellular function in G1 phase. We were able to detect a weak interaction between Rad3 and Hpz1 in cell extracts from cells in G1 phase and in extracts from cells stopped early in S phase by HU-treatment. Based on these findings we suggested that Rad3 and Hpz1 interact in G1 and early S phase. The most profound phenotype of *hpz1*Δ cells was an advanced entry into S phase when resuming cell-cycle progression from a *cdc10*-block, and earlier restart of DNA replication after HU treatment. We concluded that Hpz1 modulates the G1-S progression, but how it exerts this effect is still unknown.

General discussion and further work

Several mechanisms regulate the G1-S transition in fission yeast. In this work we have contributed to further elucidation of two such mechanisms: The G1-S checkpoint (Paper II) and regulation of initiation of DNA replication (Paper III). The discussion will concern one paper at a time.

Novel insight into the Gcn2-dependent G1-S checkpoint (Paper II)

The G1-S checkpoint in fission yeast was first described by Nilssen *et al*. (2003) and further characterized by Tvegård *et al*. (2007) and Krohn *et al*. (2008). We knew from those studies that the checkpoint is totally dependent on Gcn2 and that it is not a general DNA damage checkpoint. Gcn2 is a kinase and the serine residue at position 52 of the translation initiation factor eIF2α is its sole known substrate. Activation of Gcn2 during the G1-S checkpoint response in fission yeast leads to phosphorylation of this factor and subsequent downregulation of global translation (Tvegård *et al.*, 2007; Krohn *et al.*, 2008).

In the present work we have linked the Gcn2-dependent G1-S checkpoint to DNA repair (Paper II). We show that UVC irradiation activates two different pathways that later converge to bring about the G1 delay. Only one of these pathways is dependent on Gcn2. We have also shown that the signal for induction of the cell-cycle delay stems from processed DNA damage. In addition we have further elucidated the role for Rad3 in the checkpoint mechanism and found that it is required for induction of parts of the cell cycle delay observed in a DNA repair-deficient mutant. These findings answered some of our initial questions, and we have generated a simple working model that encompasses most of our results and knowledge so far (Figure 8). However, several issues remain to be solved to fully understand the mechanism of the G1-S checkpoint in fission yeast:

1. What is the signal for Gcn2 activation?
2. How does Gcn2 activation affect the G1-S checkpoint?
3. What is the structural identity of the checkpoint-inducing molecule(s)?
4. How does the signal from DNA repair delay Mcm2-7 loading?

These questions will be addressed in the following discussion. The possible conservation of the checkpoint in other organisms will also be discussed.

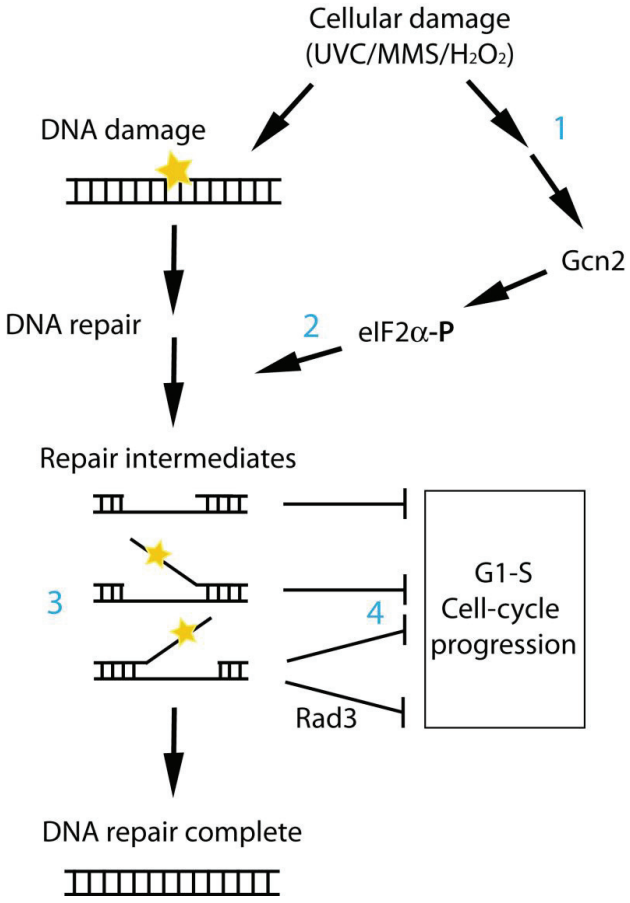

Figure 8. A working model for the G1-S checkpoint in fission yeast. The three repair intermediates indicated are hypothetical intermediates thought to be generated in wild type cells (top, centre), *uve1*Δ*rad13*Δ cells (centre) or *uve1*Δ*rad16*Δ cells (bottom) upon NER. The numbers 1-4 indicate the main topics for the discussion. See text for further details.

1. What is the signal for activation of Gcn2?

The results obtained in this study show that the G1-S checkpoint in fission yeast requires two different inputs for checkpoint activation: (i) a repair intermediate formed by processing DNA damage through excision repair and (ii) activation of Gcn2 by a yet unknown mechanism (Figure 8). We know that Gcn2 is not activated by a NER-intermediate since mutant cells defective in the earliest step of NER still activate Gcn2. Gcn2 might be activated by DNA

damage directly or indirectly, but also other damaged macromolecules are plausible candidates. In budding yeast it has been shown that elevated levels of uncharged tRNAs activate GCN2, and this mechanism for GCN2 activation has later been confirmed to be conserved in other eukaryotes (Wek *et al.*, 1989; Wek *et al.*, 1995; Jefferson and Kimball, 2003). When cells are subjected to nutritional stress, uncharged tRNAs accumulate, GCN2 is activated, and global translation is subsequently downregulated. It has been suggested that uncharged tRNAs could accumulate upon the stresses that are known to induce GCN2/Gcn2 (high salinity, oxidizing treatments and UVC irradiation amongst others) (Hinnebusch, 2005; Krohn *et al.*, 2008). It is not obvious how UVC irradiation would lead to the accumulation of uncharged tRNAs. We have tested whether the ratio of charged to uncharged tRNAs changes, but found no difference for the few tRNA species that were tested (E. Boye, unpublished data). This suggests that UVC irradiation does not cause a global change in the uncharged:charged tRNA ratio. However, the level of a specific uncharged tRNA could account for Gcn2 activation. The fission yeast genome encodes 171 tRNAs (www.pombase.org/status/statistics) and a comprehensive study of the levels of all these gene products has not been performed.

Recent data show that in mammalian cells the activity of nitric oxide synthase (NOS) is increased upon UVB and UVC radiation (Fotiou *et al.*, 2009; Lu *et al.*, 2009). NOS use $_{L}$-Arginine as a substrate to produce nitric oxide (NO), which is an important cellular signalling molecule in mammals. The NOS-catalyzed NO production depletes the levels of $_{L}$-Arginine and thereby activates GCN2 (Lu *et al.*, 2009). A similar mechanism could activate Gcn2 in fission yeast upon UVC irradiation, but a NOS has not been described in fission yeast. However, the presence of NO and a reduction of the NO levels upon removal of known NOS cofactors, or by addition of NOS inhibitors, have been measured in fission yeast extracts, strongly implying that they contain NOS-like activity (Kig and Temizkan, 2009). It would be interesting to measure the levels of charged and uncharged $_{L}$-Arginine-specific tRNAs upon UVC irradiation in G1 phase, or add NOS-inhibitors to the medium and measure the effect on the levels of eIF2α-phosphorylation, to determine whether increased NOS activity and depletion of $_{L}$-Arginine could be the cause of Gcn2 activation upon UVC irradiation in G1 phase.

The treatments that caused Gcn2 activation and checkpoint delay in fission yeast, UVC irradiation, MMS treatment and H_2O_2 (Krohn, Skjolberg et al. 2008), also cause damage to

RNAs, proteins and other cellular macromolecules. An intriguing and speculative thought is that these treatments could damage tRNAs so that they cannot be charged with amino acids. The levels of uncharged tRNAs would then increase and cause activation of Gcn2. To test this hypothesis one could isolate RNA from cells, irradiate it with UVC *in vitro*, deliver it back into cells in G1 phase by electroporation or to cell extract from G1 cells, and examine the change in Gcn2 activity. However, we have not pursued these approaches since Gcn2 is a kinase activated by stress and both alternatives would inflict serious stress to the Gcn2-environment, causing its activation and complicate the interpretation of the results.

An alternative or additional mechanism to increase Gcn2 activity could be a modification of Gcn2 that increases its affinity for uncharged tRNAs. Such regulatory modifications have been found in GCN2 from budding yeast. A phosphorylation of serine residue 577 promoted by the TOR (target of rapamycin) kinases decreased the affinity of GCN2 for uncharged tRNAs. Rapamycin-treatment activated GCN2 by inhibiting TOR kinases thus reducing the inhibitory phosphorylation (Kubota *et al.*, 2003; Hinnebusch, 2005). The G1-S checkpoint-inducing treatments could operate through a similar mechanism, although the S577 residue does not seem to be conserved in the fission yeast GCN2 homologue (http://www.uniprot.org/uniprot/Q9HGN1) and other modified residues have not been reported so far. Also, results from our group show that the TOR kinases of fission yeast do not influence Gcn2 activity upon UVC irradiation in G1 (G. E. Rødland, unpublished data). Whether the affinity of Gcn2 for uncharged tRNAs can be influenced by stress-signalling pathways induced by UVC/MMS/H_2O_2 treatment remains an unanswered question.

2. How does Gcn2 activation affect the G1-S checkpoint?

Our working model for the G1-S checkpoint pathway puts Gcn2 upstream from the repair intermediate(s) required to halt cell-cycle progression (Figure 8). It is still unclear which step in the repair pathway Gcn2 affects. The checkpoint delay is lost in a non-phosphorylatable eIF2α mutant (Tvegård *et al.*, 2007). This means that there is a dependency on phosphorylation of eIF2α for checkpoint induction. The major function of Gcn2 in the checkpoint response is therefore to phosphorylate eIF2α. Whether eIF2α-P influence the repair pathway directly or through its effect on translation is not known, but the latter is more likely given that this is the only function of eIF2α-P reported so far.

It is conceivable that the level of a factor required for production of the inducing signal is changed as a part of the initial checkpoint response. Previous studies of the transcriptional response to UVC irradiation in G1 have shown that level of transcripts are only mildly affected and cannot account for any drastic changes in protein amounts (Skjolberg *et al.*, 2009). Phosphorylation of eIF2α leads to a reduction in global translation, however, during such circumstances the translation of some transcripts is specifically enhanced (Wek *et al.*, 2006). Increasing the levels of a required protein by selective translational upregulation is thus a possible function for eIF2α-P in the G1-S checkpoint mechanism. Preliminary results from our group seem to support this hypothesis. Transcripts encoding several factors required in the first steps of NER show an increased association to polysomes after UVC irradiation, indicating active translation of these factors although global translation is downregulated (J. H. J. Knutsen, unpublished data).

3. What is the structural identity of the checkpoint-inducing molecule(s)?

We have established that a NER intermediate can induce the G1-S checkpoint delay (Paper II). The presence of either endonuclease is a requirement for checkpoint induction, but the exact nature of the inducing structure is not known. Based on our findings, some suggestions on the structural identity of the inducer can be proposed and these will be discussed below.

During NER the TFIIH unwinds DNA at the site of the lesion and RPA is recruited prior to endonuclease incisions. Upon endonuclease activity of solely Rad13 or Rad16 one can imagine a structure being formed with a free 3' end and a 5' primer junction or a free 5' end and a 3' primer junction, respectively, in addition to RPA-covered ssDNA (see Figs 6 and 8). Some of these features are identical to the suggested inducing signal for the classic checkpoints and activation of the ATR-dependent checkpoint pathway (MacDougall *et al.*, 2007). However, the formation of such structures requires that either endonuclease can cut in the absence of the other. In spite of extensive research on this issue, the results are still not conclusive (see Fagbemi *et al.*, 2011 for review). In mammalian cells XPF and XPG have been shown to cut in an orderly fashion *in vivo*, the 5' incision first and the 3' incision last, arguing for a 5' end + 3' primer junction as the only possible structure being made *in vivo*. However, in the presence of either catalytically-deficient endonuclease *in vitro* both structures have been detected. It has also been proposed that XPG could make the first cut if it is in excess *in vivo*. To further complicate the issue there is evidence to support a model where neither endonuclease can make a cut unless the other is also present in the complex. A

sequential order of NER-incisions in fission yeast has not been established. Based on our results, two explanations for activation of the G1-S checkpoint are possible and one of them could provide insight on the order of incisions. The minimal requirement for checkpoint activation could either be (i) the presence of any of the NER-endonucleases close to the damaged site or (ii) a single incision with or without start of repair synthesis. Of these two alternatives the latter is more reasonable since the first model creates two very dissimilar protein-DNA structures. Incision at either side of the lesion creates more similar structures as both would produce a ssDNA "flap" (possibly still bound by earlier NER-factors), a primer junction and probably RPA-covered ssDNA on the undamaged strand. This alternative would also imply that the fission yeast NER-endonucleases are not mutually dependent on each other for making the first incision and that the incisions are not ordered. Further investigations are required to determine which of the two suggested explanations for checkpoint activation is correct. For example one could modify the active site of either endonuclease to establish whether incisions are required or if presence of an endonuclease at the site is sufficient. In the event that incision activity is a requirement for induction of the G1-S checkpoint, our findings of checkpoint induction in both *uve1*Δ*rad16*Δ and the *uve1*Δ*rad13*Δ mutants would support a NER model where incisions are unordered and mutually independent.

If there is no dependency on the presence of the other NER endonuclease before the first incision is made, one would in the specific case of *uve1*Δ*rad16*Δ stop the repair process at a repair intermediate reminiscent of known classic checkpoint inducers: a 5' primer junction and RPA-covered ssDNA (Majka *et al.*, 2006). Experiments should be conducted to further explore the possibility that in the specific situation of *uve1*Δ*rad16*Δ, a classic checkpoint response is induced, and that the contribution of Rad3 is restricted to a response to the repair intermediate formed in this specific mutant. A strategy to look into this would be to create a *uve1*Δ*rad13*Δ*gcn2*Δ mutant and measure the length of the checkpoint delay in this mutant. In the event that the whole checkpoint delay is gone, a different situation than found in *uve1*Δ*rad16*Δ*gcn2*Δ, one would have separated the contribution of Rad3 to a specific repair structure. These results could also explain the prolonged checkpoint delay seen in *uve1*Δ*rad16*Δ, compared to the *uve1*Δ*rad13*Δ, and mean that 2 different pathways lead from the repair intermediate to delay the Mcm2-7 loading (Figure 8).

4. How does the signal from DNA repair delay Mcm2-7 loading?

It is not obvious how the DNA repair intermediate can signal to delay loading of Mcm2-7. Preventing passage past the restriction point by targeting the CDK activity is a strategy employed in the classic G1-S checkpoints of mammalian cells. Inhibition of CDK activity leads to sustained Rb association to the transcription factor E2F and thereby transcriptional repression of factors required for G1-S progression like Cdt1 and Cdc6 (Nojima, 2004). Decreasing the levels of these factors would subsequently reduce the loading of MCM2-7. However, the G1-S checkpoint mechanism of fission yeast does not seem to target the CDK activity since inhibitory phosphorylation at T15 of the sole fission yeast CDK, Cdc2, cannot be detected during checkpoint activation (Nilssen *et al.*, 2003). We also know that in fission yeast the transcription factor Cdc10, which is responsible for transcription of a similar subset of genes as E2F, is probably not involved since the levels of Cdt1 and Cdc18 are not affected after UVC irradiation in G1 phase (Tvegård *et al.*, 2007). The other preRC factors have been reported to be present throughout the mitotic cell-cycle (Kearsey and Labib, 1998; Bell and Dutta, 2002). It is therefore unlikely that the absence or delayed production of a preRC factor can be the cause of the delay we observe in the Mcm2-7 loading. Thus, the G1-S checkpoint response in fission yeast is different from classic G1-S checkpoints although the outcome is similar in that DNA replication is delayed.

The G1-S checkpoint in higher organisms also inhibits loading of Cdc45, a component of the CMG helicase required for initiation of DNA replication, through inhibition of the CDK activity (Bartek and Lukas, 2001; Lukas *et al.*, 2004). Assembly and activation of the CMG is however exerted later than loading of MCM2-7 and cannot be the target of the G1-S checkpoint in fission yeast. It is also not known whether the Mcm2-7 loading is the direct target of the G1-S checkpoint in fission yeast, or if loading of any other previous preRC factor is delayed. Even though Cdt1 and Cdc18 are present, one cannot exclude that their association to chromatin is inhibited. Detection of Cdt1 and Cdc18 association to chromatin, or more specific ORC-bound origins, should be examined and might reveal what is the real target of the G1-S checkpoint in fission yeast. Also proteins that regulate the association of preRC factors to origins are likely targets. Alternatively, the loading of Mcm2-7 could be directly inhibited upon modification of any of its six subunits. Studies in different eukaryotes have revealed that several of the MCM proteins can be phosphorylated by ATR, ATM or Cds1 at different sites upon replication stress or DNA damage in S phase (Ishimi *et al.*, 2003; Cortez *et al.*, 2004; Yoo *et al.*, 2004; Shi *et al.*, 2007; Bailis *et al.*, 2008). Some MCM proteins are

also phosphorylated during normal cell-cycle progression in mammalian cells by CDK activity (Ishimi *et al.*, 2000; Ishimi and Komamura-Kohno, 2001; Masai and Arai, 2002). Introduction of CDK-cyclinA activity in G1 can cause phosphorylation of both MCM2 and MCM4 and this led to partial inhibition of the chromatin loading of MCM2-7 and seemed to completely block DNA replication in mammalian cells (Wheeler *et al.*, 2008). The modification status of Mcm2-7 in G1 before and during checkpoint induction in fission yeast seems like an important clue to pursue in future work, both for the major Rad3-independent and the minor Rad3-dependent response.

Conservation of the Gcn2-dependent G1-S checkpoint mechanism

The inducing signals of the G1-S checkpoint in fission yeast and the classic G1-S checkpoints in mammalian cells and budding yeast are strikingly similar. All three organisms require damage recognition by their NER XPA homologues for signal generation (Giannattasio *et al.*, 2004; Bomgarden *et al.*, 2006; Bøe *et al.*, 2012). In addition, further processing of the damage is required for induction of the checkpoints in both fission (Paper II) and budding yeast (Giannattasio *et al.*, 2004). The inducing signal for G1-S checkpoints thus seems to be conserved. However, the downstream actors mediating signal transduction and effectuating the checkpoint delay are different as Rad3 homologues are essential for the classic checkpoints, but not for the G1-S checkpoint in fission yeast (Humphrey, 2000; Zhou and Elledge, 2000; Nilssen *et al.*, 2003).

A requirement for Gcn2 in the classic checkpoints has not been previously reported. However, there are some reports suggesting that Gcn2 can affect the cell-cycle progression also in other organisms. Treatment of budding yeast with MMS or activation of the unfolded protein response pathway by endoplasmatic reticulum stress in mammalian cells, induce a G1 arrest in a GCN2-dependent manner (Hamanaka *et al.*, 2005; Menacho-Marquez *et al.*, 2007). Furthermore, GCN2 is activated by UVC in budding yeast (Tvegård *et al.*, 2007) and in mammalian cells (Deng *et al.*, 2002; Jiang and Wek, 2005). UVC delays preRC loading also in mammalian cells (T. W. Håland, unpublished data), but it is still unclear whether this depends on GCN2.

These findings strongly suggest that there is a common response in distantly related organisms to UVC irradiation in G1 phase and indicate that the Gcn2-dependent G1-S checkpoint of fission yeast might be conserved. Modern cancer therapy targets the checkpoint

responses to increase the toxicity of radiotherapy or genotoxic agents (Toledo *et al.*, 2011). Factors involved in the Gcn2-dependent G1-S checkpoint can be future cancer therapy targets assuming that the checkpoint is conserved.

Hints of a novel mechanism modulating the initiation of DNA replication (Paper III)

Initiation of DNA replication can be divided into four steps: origin recognition, assembly of the preRC, helicase activation and loading of the replisome. Several of these steps are under regulatory control and connected to changes in CDK activity. In Paper III we have characterized a novel protein, Hpz1, which appears to modulate the initiation of DNA replication both before entry into S phase, and in S phase when resuming DNA replication after release from HU-induced replication arrest. This phenotype of *hpz1*Δ is remarkable and unusual and is an indication of a novel mechanism modulating the initiation of DNA replication in fission yeast. The following discussion will concern the possible function of Hpz1 in such a regulatory mechanism, other components involved, the possible target(s) of such a mechanism and resemblance to other reported cases.

The function of Hpz1 (and Rad3) in pre-replication events

We have shown that the levels of Hpz1 are cell-cycle regulated peaking in G1 phase. Hpz1 localizes in the nucleus where it performs an important function since UV damage in G1 dramatically affected the survival in absence of the protein. However, the most startling phenotype of *hpz1*Δ is its advanced entry into S phase after a *cdc10*-block and that these cells also resume DNA replication earlier than wild type cells after release from replication stress. These findings suggest a function for Hpz1 in modulation of events prior to S phase, and that it also affects fork restart or unused primed origins in S phase. We know that after a *cdc10*-block, the final step in preRC formation, the loading of the Mcm2-7, is advanced in the absence of Hpz1, indicating its requirement prior to or at the step of Mcm2-7 loading. Bulk DNA replication and Mcm loading is also earlier in a *rad3*Δ mutant than in wild type cells after a *cdc10*-block (Figure 9). These results, together with the fact that homologous protein sequences of Hpz1 and Rad3 are fused in some fungi, strongly suggest that Hpz1 and Rad3 participate in the same mechanism. Rad3 is activated by RPA-covered ssDNA, but it is not obvious how such a structure could be formed during a *cdc10* block-and-release experiment.

Our results are the first evidence implying a role for Rad3 in events regulating the initiation of DNA replication in the absence of stress. More detailed studies should be executed to unravel

this novelty. Rad3 is one of the major classic checkpoint proteins and questions of special interest are whether the observed phenotype is due to loss of a checkpoint in G1 (will be referred to as a "DNA replication initiation checkpoint" in the following discussion), whether the modulatory mechanism represents a completely novel function of Rad3 and why *hpz1*Δ cells show decreased tolerance to DNA damage.

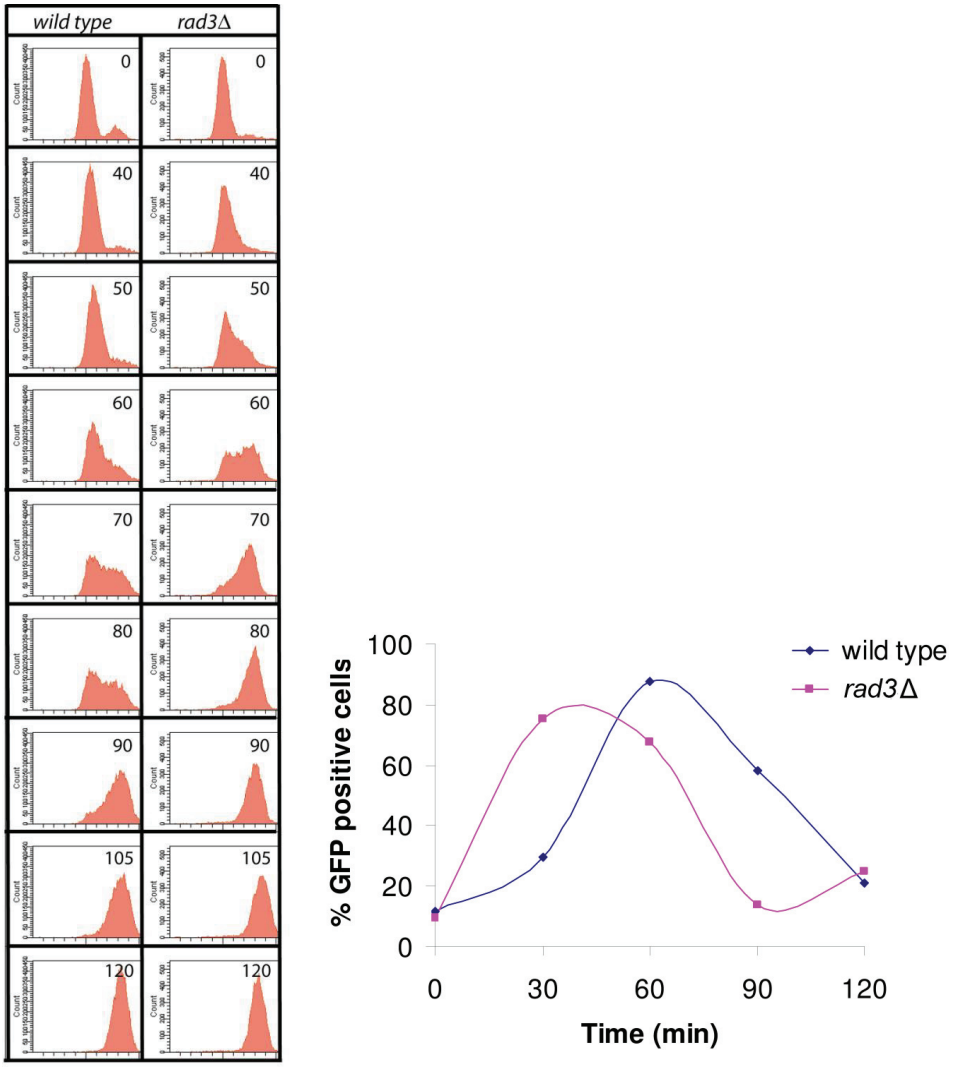

Figure 9. Advanced entry into S phase in *rad3*Δ cells compared to wild type cells. Left: DNA histograms of wild type cells and *rad3*Δ cells released from a *cdc10*-block. Wild type cells increase their DNA content from 60 to 105 minutes, while *rad3*Δ cells increase their DNA content from 40 to 80 minutes. Right: Loading of Mcm-GFP onto chromatin after release from a *cdc10*-block in wild type (blue) and *rad3*Δ (pink) cells. These data are unpublished.

Are Hpz1 and Rad3 part of a DNA replication initiation checkpoint in fission yeast?

Our results could suggest that cells in G1 are delayed by a checkpoint-like mechanism that is activated during G1 after a *cdc10*-block. The definition of a checkpoint is a delay in cell-cycle that is lost due to a mutation or drug treatment. In our case we have several deletion mutants that exhibit earlier S phase entry, i.e. loss of checkpoint. By re-analyzing earlier published data from our group (Nilssen *et al.*, 2003) we find that caffeine-treatment also shortens G1 phase after a *cdc10*-block. Caffeine-treatment is known to abolish classic cell-cycle checkpoints by inhibition of ATR (Sarkaria *et al.*, 1999). It is tempting to suggest that caffeine-treatment could operate through a similar mode. We have preliminary evidence that *rad26*Δ, encoding the Rad3 binding partner Rad26, also displays the early replication phenotype (C. A. Bøe, unpublished data).

The reason for activation of a checkpoint in G1 phase, after release from a *cdc10*-block, is not clear. Whether this mechanism also functions during normal G1 progression should be determined and we have tried to address this issue by studying cell-cycle progression in cells arrested and released from G2 phase. For these experiments, we employed a $cdc25^{ts}$ mutant that arrests the cells before mitosis, when cultured at the restrictive temperature. Then we released the cells from the block and followed them as they progressed from G2 phase. Wild type, *hpz1*Δ and *rad3*Δ cells started to increase their bulk DNA with the same timing, as measured by flow cytometry, and, in addition, did not show a difference in timing of Mcm loading onto chromatin (B. Grallert, unpublished data). These results imply that the modulatory mechanism we observe might be restricted to after release from a *cdc10*-block. Still, we did detect earlier bulk DNA replication after release from an HU-induced arrest in the *hpz1*Δ mutant, when no *cdc10*-block was applied, and this supports our hypothesis, that Hpz1 is involved in a more general mechanism for cells initiating DNA replication. It is not feasible to perform similar HU experiments with the classic checkpoint mutants since these are defective in the intra-S checkpoint and do not arrest in early S phase upon HU treatment, but enter mitosis prematurely instead. So far the results suggest that the early replication phenotype we observe is not connected to the *cdc10*-block, but rather a general modulatory mechanism or checkpoint that occurs prior to initiation or restart of DNA replication.

Disturbed initiation of DNA replication in other mutants/organisms

Altered initiation of DNA replication is reported for several mutants in various organisms. However, most of these reports concern requirements for initiation of DNA replication, or

requirements for firing of late origins versus early origins and thus deflects the order of the replication firing programme, rather than DNA replication initiation in general. There are also reports linking Rad3 to origin firing as it is shown to be required for suppression of late origins after HU-treatment, but not for the firing of early origins (Kim and Huberman, 2001). Still, this cannot explain our observation that *rad3*Δ displays earlier initiation of DNA replication in general in G1 phase after a *cdc10*-block.

The early replication phenotype found in *hpz1*Δ is puzzling, but there a few reports of other mutants exhibiting similar phenotypes. The fission yeast *mrc1* deletion mutant was recently shown to initiate DNA replication earlier than wild type cells (Hayano *et al.*, 2011). DNA replication from early origins was advanced in this mutant, but the association of the Mcm proteins to both early and late origins was unaffected. Instead the chromatin binding of Cdc45, a component of the replicative helicase, is earlier in *mrc1*Δ compared to wild type cells, and therefore the absence of Mrc1 delays the process of DNA replication initiation at a later step than that we observe in the *hpz1*Δ cells.

Cdc18 is considered to be a key protein in the preRC assembly process. Recently it has been reported that during mild hypoxia in mammalian cells ATR mediates degradation of Cdc6, thereby suppressing initiation of DNA replication (Martin *et al.*, 2012). In a mammalian cell-free system, increased levels of Cdc6 introduced to G1 nuclei caused advanced entry into S phase (Stoeber *et al.*, 1998) and overexpression of Cdc18 in fission yeast causes reinitiation of DNA replication throughout the cell-cycle (Nishitani and Nurse, 1995). In light of this it could be interesting to compare Cdc18 levels after a *cdc10*–block between wild type and *hpz1*Δ cells. The absence of Hpz1 would then have to increase Cdc18 levels specifically in G1 and after HU-treatment to cause the effects we observe.

Advanced entry into S phase has also been reported from studies of RPD3, a histone deacetylase from budding yeast (Vogelauer *et al.*, 2002). Deletion of the *RPD3* gene caused cells which had been synchronized and released from G1 phase, to initiate DNA replication earlier. Absence of RPD3 increased the levels of acetylated histones causing the chromatin to be less condensed and presumably facilitate the assembly of the preRC. An advanced loading of CDC45 was observed in *RPD3*Δ compared to wild type cells, but the previous steps of the initiation process were not studied (Vogelauer *et al.*, 2002). It remains to be seen whether Hpz1 exerts a function influencing the condensation status of chromatin.

The function of Hpz1 in light of its protein sequence

Hpz1 is a protein with an N-terminal Zn-finger domain and, at its C-terminal region, a more unfamiliar structure containing several clusters of negatively charged amino acids. The Zn-finger domain is homologous to PARP-type Zn-fingers and these are found in proteins of different functions, like PARPs and DNA ligase 3, conferring DNA binding capacity. The C-terminal negatively charged clusters are seemingly important for the function of Hpz1 given the fact that they were conserved in the Rad3-Hpz1 fungal fusion protein. Such charged clusters are often used to associate to molecules of the opposite charge and facilitate diverse processes like docking, orientation etc. Given that Hpz1 is a protein containing negatively charged clusters a possible "partner", interacting with the C-terminal domain, should possess positively charged regions. Negatively charged DNA or RNA is probably not associated to this part of Hpz1 directly, but one cannot exclude that Hpz1 could interact with the highly positively charged histone tails of nucleosomes. This hypothesis might be far-fetched, but fits with the cell-cycle regulated presence of Hpz1, in late M through G1 phase, since M phase contains the most condensed DNA and thus deacetylated positively charged histones, whilst before DNA replication this must be relieved by acetylation of histone tails. Several reports link chromatin remodeling to initiation of DNA replication. For instance, targeted acetylation of late origins can cause them to fire earlier (Vogelauer *et al.*, 2002). Acetylation of histone tails is also suggested to be a prerequisite for Mcm2-7-loading in various eukaryotes and histone modifiers are found to interact with several players of the preRC like ORC1, MCM2 and Cdt1 (Iizuka and Stillman, 1999; Burke *et al.*, 2001; Miotto and Struhl, 2008). The early replication phenotype was as mentioned earlier, observed in a deletion mutant of a histone deacetylase in budding yeast (Vogelauer *et al.*, 2002). This strengthens our suspicion that Hpz1 can have a function related to the chromatin decondensation process and this is a theory we would like to pursue.

Why are hpz1*Δ cells sensitive to UVC irradiation?*

In G1, *hpz1*Δ showed 50% reduced survival after UVC irradiation compared to wild type cells, indicating a function for Hpz1 in DNA repair or recovery from damage. We would like to consider this possibility in the future by performing an experiment where *hpz1*Δ cells are arrested in G1 phase using a *cdc10*-block, then irradiate the cells and culture them further at the restrictive temperature to allow time for repair before plating out to assay survival. If the *hpz1*Δ cells survive better after this "pause" one can make the assumption that Hpz1 is not involved in repair, but that the cells devoid of Hpz1 die because they enter S phase earlier

than wild type cells with unrepaired lesions. I.e. these cells had less time in G1 phase for DNA repair. A different solution could be that Hpz1 helps to keep chromatin condensed for a longer time (see previous discussion). Wild type cells might acquire less DNA damage than the deletion mutant since condensed chromatin is suggested to protect the DNA from DNA damaging agents (Cann and Dellaire, 2011). This could easily be addressed by determining whether the amount of damage is different in the absence or presence of Hpz1/Rad3.

What is the actual target of the suggested novel mechanism modulating initiation of DNA replication?

The target of the Hpz1- and Rad3-dependent modulatory mechanism has not been determined. We know that Mcm2-7 loading occurs earlier in these mutants implying that the target of the mechanism is prior to or at this step. Further investigations to determine which step in the preRC assembly is the real target, could be performed by measuring the loading of Cdt1 or Cdc18 onto chromatin by chromatin extraction, in a similar fashion to the Mcm proteins, or by chromatin immunoprecipitation (ChIP).

The fact that absence of Hpz1 affects both Mcm2-7 loading after a *cdc10*-block, and also restart of replication forks or usage of primed later origins after HU-induced replication arrest, can have several possible explanations. First: Hpz1 can affect both the preRC and an inherited quality of the fork, the Mcm2-7 complex, so that they restart earlier or *de novo* firing in S phase occurs earlier. Second: Hpz1 affects surrounding chromatin so that in its absence replication can commence or recommence earlier. The first explanation could demand modification of Mcm proteins in a way that makes them (i) load onto chromatin earlier after a *cdc10*-block and (ii) render their replication forks able to resume replication earlier after release from replication stress. Several Mcm proteins are phosphorylated in S phase, but it is not known so far whether they are modified in G1 phase (Ishimi *et al.*, 2003; Cortez *et al.*, 2004; Yoo *et al.*, 2004; Shi *et al.*, 2007; Bailis *et al.*, 2008). The second explanation, that Hpz1 could affect the surrounding chromatin in a way that facilitates earlier DNA replication, can be supported if we are able to prove our earlier mentioned theory: Hpz1 influence the decondensation process.

Concluding remarks

The transition from G1 to S phase is often deregulated in cancer cells allowing cell proliferation under otherwise unfavourable conditions. Better understanding of the underlying

basic mechanisms can provide useful targets for future cancer therapy. In this work we have investigated such basic mechanisms operating in G1 phase and regulating the G1-S transition in the model organism fission yeast. We have further characterized the Gcn2-dependent G1-S checkpoint, which is induced upon certain DNA damaging treatments, and found that DNA repair is required for induction of the G1 delay, but not for activation of Gcn2 (Paper II). We have also discovered and characterised Hpz1, a protein modulating the initiation of DNA replication presumably through a novel mechanism (Paper III). Further we have established that Rad3 can be required for regulatory events in G1 (Paper II and III). A method for rapid protein activation in fission yeast was also developed (Paper I).

Further analysis remains to unravel the complete G1-S checkpoint mechanism, the actual contribution of Hpz1 and Rad3 to initiation of DNA replication, and the possible conservation of these processes in higher eukaryotes.

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Methodology article

Rapid regulation of protein activity in fission yeast

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Abstract

Background: The fission yeast *Schizosaccharomyces pombe* is widely-used as a model organism for the study of a broad range of eukaryotic cellular processes such as cell cycle, genome stability and cell morphology. Despite the availability of extensive set of genetic, molecular biological, biochemical and cell biological tools for analysis of protein function in fission yeast, studies are often hampered by the lack of an effective method allowing for the rapid regulation of protein level or protein activity.

Results: In order to be able to regulate protein function, we have made use of a previous finding that the hormone binding domain of steroid receptors can be used as a regulatory cassette to subject the activity of heterologous proteins to hormonal regulation. The approach is based on fusing the protein of interest to the hormone binding domain (HBD) of the estrogen receptor (ER). The HBD tag will attract the Hsp90 complex, which can render the fusion protein inactive. Upon addition of estradiol the protein is quickly released from the Hsp90 complex and thereby activated. We have tagged and characterised the induction of activity of four different HBD-tagged proteins. Here we show that the tag provided the means to effectively regulate the activity of two of these proteins.

Conclusion: The estradiol-regulatable hormone binding domain provides a means to regulate the function of some, though not all, fission yeast proteins. This system may result in very quick and reversible activation of the protein of interest. Therefore it will be a powerful tool and it will open experimental approaches in fission yeast that have previously not been possible. Since fission yeast is a widely-used model organism, this will be valuable in many areas of research.

Background

Regulating protein function or protein level is often useful in order to investigate diverse biological processes. The fission yeast *Schizosaccharomyces pombe* is a popular model organism. It is genetically tractable and a wide variety of methods have been developed to facilitate molecular genetic manipulations in *S. pombe*.

It is usually more advantageous to regulate the activity of the target protein than the protein level, because this results in faster regulation of the protein's activity at wild type protein levels. The most commonly used approach to regulate the activity of the protein of interest is the isolation of conditional mutants, which have been vital tools in many areas of research. Indeed, one of the many advantages of fission yeast as a model system is that it is haploid, which makes it easier to isolate and work with conditional mutants. Most conditional mutants are temperature sensitive. However, not all genes can be mutated such that the corresponding protein becomes temperature sensitive. Furthermore, a temperature shift in itself might stress the cells. Temperature-sensitive proteins often have considerable residual activity at the restrictive temperature such that they rescue the temperature-sensitive mutant when overexpressed. Another common problem is that many temperature-sensitive proteins are not fully active at the permissive temperature. Therefore, temperature shifts of temperature-sensitive mutants are frequently far from the ideal "on" and "off" states that might be desired when regulating protein function. The reversibility of the inactivation varies greatly from mutant to mutant. Upon shift back to the permissive temperature, some temperature-sensitive proteins regain their activity, thus allowing block-and-release experiments. However, many other temperature sensitive proteins do not regain their activities after a period of temperature shift or are degraded at the restrictive temperature. Temperature-sensitive mutants have been particularly useful to explore the functions of essential proteins. However, it is difficult to identify temperature-sensitive mutants of non-essential genes, unless their function is known so that appropriate screens can be designed.

Regardless of the many advantages associated with the use of conditional mutants, they are not always available or applicable. A commonly used alternative is regulating the level of the protein of interest, either by regulating transcription or by regulating protein degradation (see below).

Numerous plasmids have been designed for regulated expression of genes [1], but there are no good tight and rapidly inducible promoters for use in fission yeast. The nmt1 (*n*o *m*essage in *t*hiamine) promoter was the first regulatable promoter to be described in fission yeast [2] and it remains the most commonly used one. This promoter is strong, but mutated versions with reduced strengths are available [3]. The promoter is repressed by thiamine (vitamin B1). The main drawback with the *nmt* promoter is that induction of protein expression is rather slow and it takes several generations to achieve full activation, presumably because the cellular vitamin pools have to be depleted first. Furthermore, thiamine confers over 100-fold repression of nmt1-driven transcription, but the promoter is still somewhat leaky and many cloned genes are expressed to near wild-type levels even in the presence of thiamine, such that they can complement chromosomal mutations. Shut-off experiments, where expression of the protein of interest is turned off by the addition of thiamine, are particularly inefficient for stable proteins, since not only is the promoter leaky, but the protein of interest also has to be diluted out as the cells grow.

There are several other and less widely used regulatable promoters that to some extent can be used in fission yeast. Although they confer regulated expression, there are also severe drawbacks to their use, as detailed below. The tetracycline regulatable promoter is a derivative of the Cauliflower Mosaic Virus (CaMV) promoter, fused to a tetracycline binding site [4]. The use of this promoter requires not only cloning the gene of interest behind the CaMV promoter but also manipulating the parent strain such that it expresses the Tet repressor. The *fbp1* promoter is repressed by glucose but it can only be used in liquid cultures [5]. The invertase promoter is also repressed by glucose and is activated by sucrose within an hour of medium shift. However, the glucose produced by invertase activity leads to repression of the promoter within a short time, so this promoter can only be used for short periods of expression [6]. Since regulation of the latter two promoters requires changing the carbon source, their use implies dramatically changing the growth conditions during the course of the experiment.

Only recently has a uracil-regulateable promoter been described that allows rapid activation and inactivation of transcription [7]. This system is expected to become a useful tool to regulate protein expression, but it should be noted that it might not always be sufficient to regulate transcription levels to achieve efficient regulation of protein levels.

The above regulatable systems all employ heterologous promoters. The expression levels from these promoters might or might not correspond to that from the native promoter of the gene of interest. The degron method, that circumvents this drawback, is based on regulated degradation of the target protein and has been used successfully in fission yeast [8-10]. However, it depends on a temperature shift to 37°C and the degron tag must be on the N-termi-

nus of the target protein. Depending on the stability of the protein of interest, additional measures might also need to be taken to inactivate the protein. One improvement to the method in fission yeast was to combine the degron with an existing temperature sensitive mutation [8,9]. Another strategy that was employed in budding yeast is overexpression of the ubiquitin ligase Ubr1 [10,11]. This approach however cannot be used in fission yeast to improve degron-directed degradation [10]

In summary, despite having a selection of approaches to regulate protein levels, fission yeast researchers often find it difficult to achieve the desired expression level of their favourite proteins.

Here we describe the application of a system that is based on regulated protein function [12,13] without the need for a temperature shift, as opposed to regulated transcription or protein degradation. We have tested the system on four proteins and were able to regulate the activity of two of them.

Results

The principle

The approach we have used is based on the normal regulatory activity of the hormone binding domain (HBD) of vertebrate steroid receptors. The Hsp90 molecular chaperone binds the HBD in the absence of estrogen hormones. Upon addition of estradiol a hormone-induced conformational change in the HBD results in the dissociation of Hsp90 [14].

The HBD can also confer sensitivity to estradiol to the activity of heterologous proteins [13]. Fusing a heterologous protein of interest with the hormone-binding domain of the estrogen receptor (ER) renders it inactive presumably because it is bound by the Hsp90 (Fig. 1). Within a few minutes of addition of estradiol the hormone-induced conformational change in the HBD results in dissociation of the Hsp90 and activation of the chimeric protein (Fig. 1) [12,13]. The mechanism of inhibition by Hsp90 is thought to be by steric interference [14] but regulation of the intracellular localization of the chimeric protein has also been reported [15].

In the following sections, we shall refer to the fusion protein as "*active*" or "*inactive*" in quotation marks, reflecting the presence or absence of estradiol, respectively. This indicates the protein activity expected based on the model described above and shown in Figure 1, rather than that observed experimentally.

Cdc13-des2-HBD

Cdc13 is the mitotic B-type cyclin in fission yeast. Cdc13 protein levels are stringently regulated through the cell cycle. The protein starts accumulating at the G1-S transition until, in late G2, the high level required for entry into and progression through mitosis is reached [16]. Cdc13 is then degraded via the APC (anaphase promoting complex) at the end of mitosis [17,18]. We wished to be able to regulate the Cdc13 levels independently of the cell cycle stage, i.e. allowing regulation that would be independent of APC activity. Therefore we employed a non-degradable mutant form of Cdc13, Cdc13-des2, which lacks the recognition sequence that targets the protein for ubiquitylation by the APC [18].

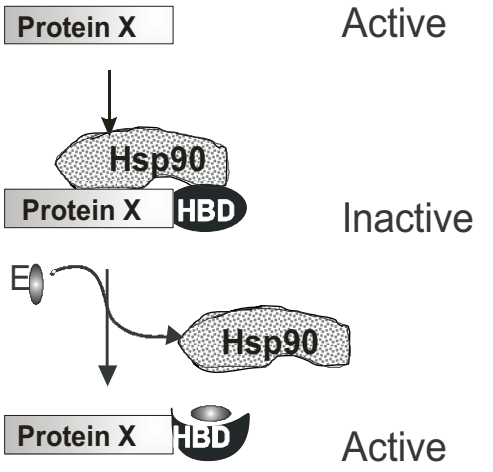

Figure 1
The principle of regulating protein function by estradiol. See text for details.

We fused sequences encoding the ER hormone binding domain to the 3' end of the *cdc13-des2* ORF. It had been previously shown that fission yeast cells expressing Cdc13-des2 from the medium strength nmt41 promoter are inviable when the promoter is induced [18] (Fig. 2A), but the cells are viable when the promoter is repressed. To ensure more physiological levels of Cdc13, we used the weak nmt81 promoter to regulate the expression of the Cdc13-des2-HBD fusion protein.

Expression of Cdc13-des2 or Cdc13-des2-ERHBD from the nmt81 promoter was not lethal even when the promoter was induced (Fig. 2A). However, when estradiol was added (fusion protein "active"), the cells expressing ERHBD-tagged Cdc13-des2 grew very poorly as shown by a spot test of serially diluted cells (Fig. 2A, compare "active" to "inactive"). These observations suggest that the

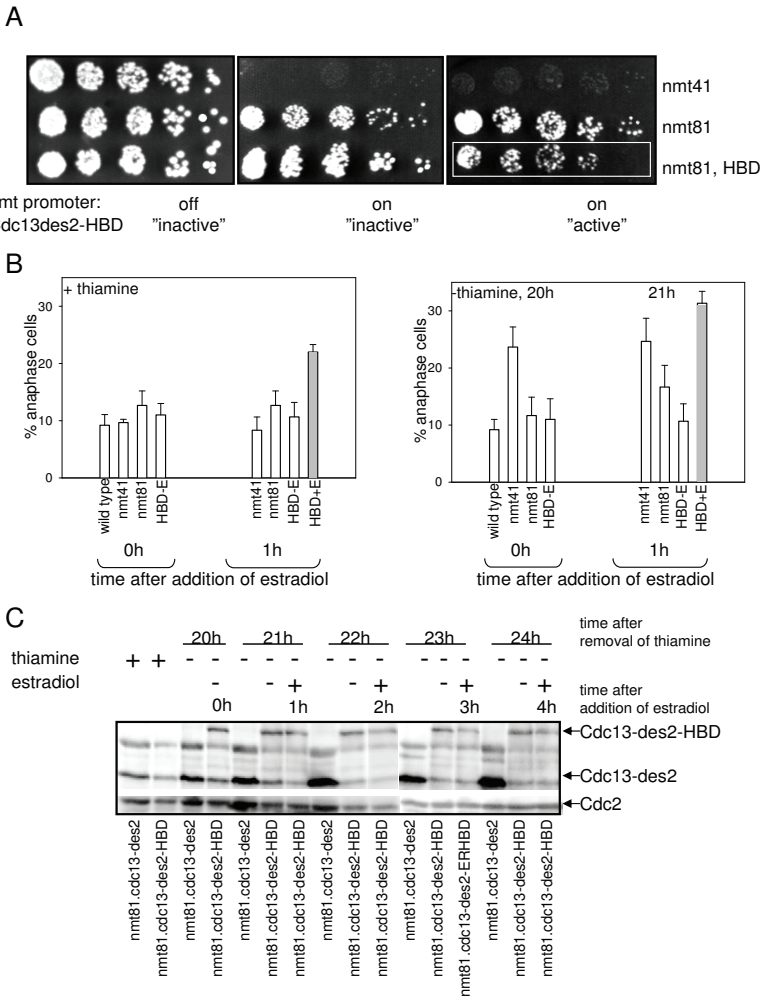

Figure 2

The activity of Cdc13-des2-HBD is regulated by estradiol. **A**, Cells transformed with plasmids carrying the nmt.cdc13-des2 and nmt.cdc13-des2-HBD constructs were serially diluted (4X) and plated onto minimal plates with and without thiamine and estradiol as indicated. The cells carrying the ERHBD tagged cdc13-des2 growing poorly in the presence of estradiol are highlighted with a white rectangle. **B**, Expression of Cdc13-des-HBD results in anaphase delay in the presence of estradiol. The nmt promoter was induced for 20 h before addition of estradiol for 1 h. Anaphase index is shown before and 1 h after addition of estradiol. Bars show anaphase indices in the presence (left panel) and absence (right panel) of thiamine. Anaphase index observed in wild type cells is shown for comparison. The bar representing the tagged construct in the presence of estradiol is shaded. **C**, Cdc13 levels are not increased by the presence of the tag or estradiol. Cells carrying the nmt81.cdc13-des2 and nmt81.cdc13-des2-HBD plasmids were grown in minimal medium in the presence of thiamine, then thiamine was washed out to induce the nmt promoter. Estradiol was added to half of the cultures after 20 h induction. Samples for protein extracts were taken at the indicated times. TCA extracts were made and western blot analysis was performed using the SP4 anti-Cdc13 antibody [40] and the anti-PSTAIRE (Santa Cruz) antibody to detect Cdc2 which serves as loading control.

fusion protein is indeed activated in the presence of estradiol.

Fission yeast cells expressing Cdc13-des2 from the medium strength nmt41 promoter delay at the anaphase-telophase transition [18]. To measure more accurately the activity of the Cdc13-des2-HBD fusion protein, we counted anaphase indices in the presence and absence of estradiol and/or thiamine (Fig. 2B). Expression of Cdc13-des2 from the weak nmt81 promoter leads to a marginal increase of anaphase index, whereas expression from the medium strength nmt41 promoter brings about a pronounced anaphase delay (Fig. 2B, white bars). Interestingly, addition of estradiol to cells expressing Cdc13-des2-HBD (fusion protein "active") (Fig. 2B, shaded bar) results in an anaphase delay comparable to that in cells expressing the protein without the HBD tag from the medium strength nmt41 promoter. The anaphase index significantly increases by an hour after hormone addition, indicating a quick response, and remains high for at least one generation time (data not shown). At later timepoints cut cells were observed both with and without the ERHBD tag (data not shown). These data strongly suggest that estradiol indeed activates the Cdc13-des2-HBD fusion protein.

It is noteworthy that expression of Cdc13-des2-HBD produces a higher anaphase index and, at later timepoints after hormone addition, more cut and septated cells than expression of Cdc13-des2 from the same promoter. One possible explanation is that the expression level of Cdc13 and/or the copy number of the plasmid is affected by the presence of estradiol. However, western blot analysis of Cdc13 levels shows no increase of Cdc13 level by the presence of the hormone, nor does the tag increase the amount of the protein (Fig. 2C). We do not observe an increased amount of the endogenous Cdc13 either (Fig. 2C), which would be expected if the HBD tag was cleaved off. If there is a difference, it is that the tagged protein is present in somewhat lower amounts then the untagged protein. We considered the possibility that the HBD tag itself is responsible for the mitotic defects but we deem this most unlikely. The differences between the effects of expressing Cdc13-des2 with and without the HBD tag are quantitative, not qualitative, indicating that the tag itself does not confer a novel function on the fusion protein. Consistently, in the absence of estradiol the cells carrying the tagged construct grow like wild type cells (Fig. 2B). It is likely that a sudden increase of Cdc13 levels upon hormone addition disturbs the localization and/or function of Cdc13 and thus aggravates the effects of overexpressing a non-degradable Cdc13.

A major limitation with the use of the nmt promoter is the high background expression level even in the presence of thiamine. We wished to evaluate the effectiveness of inhibiting Cdc13-des2-HBD protein function with the HBD tag in the absence of estradiol versus repressing expression of Cdc13-des2-HBD from the nmt81 promoter in pREP82 by addition of thiamine to the growth medium. To this end we compared the anaphase indices of cells where we inhibited Cdc13-des2-HBD protein activity by not adding estradiol (but maintained full expression from the nmt81 promoter) to that of cells where transcription from the nmt81 promoter was repressed by addition of thiamine (while the fusion protein was "active"). In the latter case (transcriptional regulation), repression of the promoter still allowed enough Cdc13-des2-HDB expression to produce an anaphase delay (see shaded bar, left panel on Fig. 2B). In contrast, when the cells expressing the fusion protein were grown in the absence of estradiol (fusion protein "inactive"), the anaphase index corresponds to that of wild type cells that do not carry the *nmt.cdc13-des* construct indicating that the fusion protein is indeed inactive (see "HBD-E", right panel on Fig. 2B). We conclude that negatively regulating Cdc13-des2 protein activity using the HBD tag results in lower background activity than regulating transcription with the nmt promoter.

Psf2-HBD

GINS is a tetrameric complex essential for the initiation and elongation steps of DNA replication [19,20]. The four subunits of GINS are essential for cell viability in budding yeast [19,20] therefore analysis of GINS function requires the isolation of conditional mutant alleles. In fission yeast temperature-sensitive alleles of the Psf2 and Psf3 subunits have been isolated and it was shown that Psf2 and Psf3 are required for DNA replication [21,22]. We explored whether the HBD could confer conditionality on the Psf2 subunit of fission yeast GINS. We fused sequences encoding the ERHDB to the 3' ends of the *psf2*$^+$ gene in the chromosome using the PCR-mediated gene targeting method [23]. Haploid cells expressing Psf2-ERHBD were viable when grown in the presence of estradiol in the growth medium but were inviable on medium lacking estradiol (Fig. 3A).

To determine whether the lethality was indeed due to a defect in DNA replication, a strain expressing Psf2-ERHBD was arrested in G1 by nitrogen starvation and released from the block in the presence or absence of estradiol. Cells released from the starvation block in the absence of estradiol ("inactive") only show some evidence of DNA replication at 5 h (Fig. 3B left panel), consistent with a role of Psf2 in DNA replication. Cells released from the starvation block in the presence of estradiol (fusion protein "active") carry out DNA replication 3–4 h after release (Fig 3B right panel) confirming that the fusion protein is indeed active. These data demonstrate that the

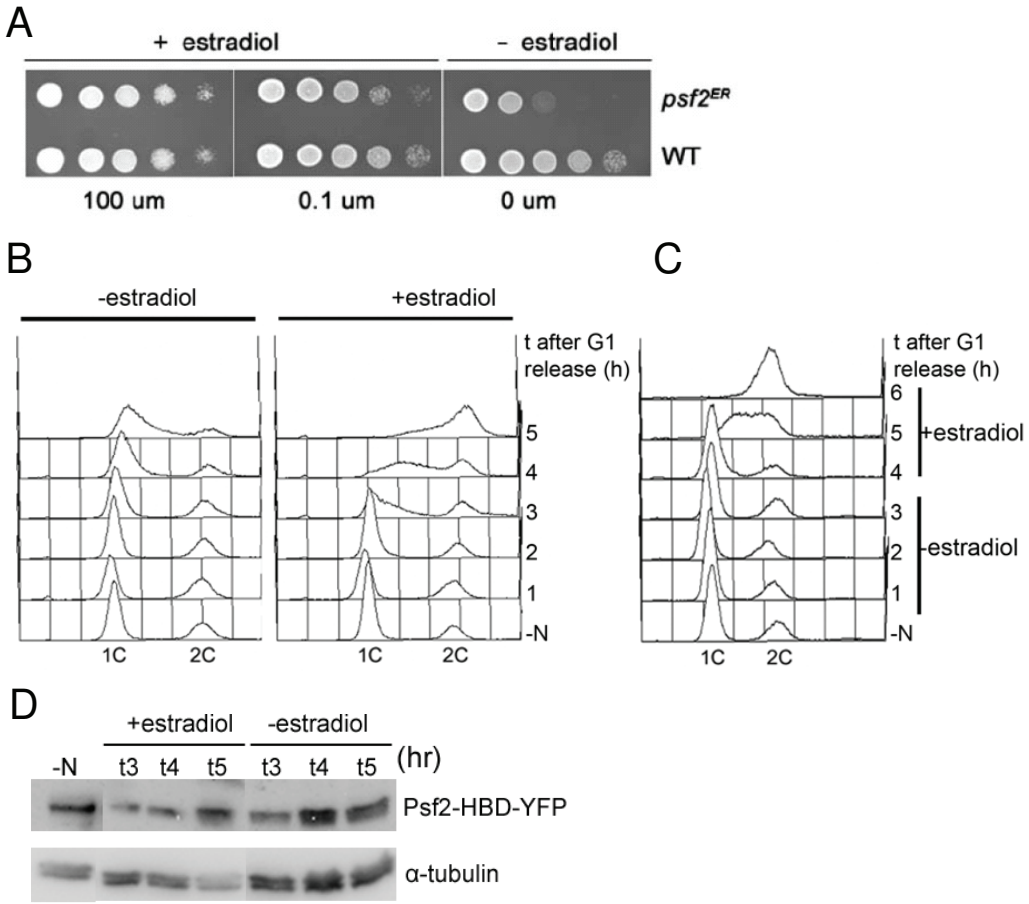

Figure 3

Psf2-HBD is inactivated in the absence of estradiol. **A**, *psf2-HBD* and wild type cells were serially diluted and spotted onto YE plates containing estradiol at the indicated concentrations. **B**, Strain P1520 (*psf2-HBD:kanMX6*) was grown in EMM plus 125 mM estradiol to log phase then shifted to EMM-N (+estradiol) for 16 at 25°C. Cells were released from the block by transferring to EMM+N in the absence (left) or presence (right) of estradiol. **C**, As in A, except that cells were released into EMM+N in the absence of estradiol, and 125 nM estradiol was added at 4 h. **D**, Cell extracts were made using the TCA method from cells incubated with and without estradiol as indicated. The extracts were run on protein gels and Psf2 was detected using an antibody against the YFP tag.

ERHBD tag confers conditionality on Psf2 and the fusion protein can be activated by estradiol. Similar results were obtained with Psf1-ERHBD (data not shown, manuscript in preparation), the activity of which is also regulated by estradiol.

A similar experiment had been performed by Gomez et al [21] using the temperature sensitive *psf2* allele, where the cells were arrested by nitrogen starvation and then released from the block at the restrictive temperature. It is interesting to note that the *psf2-HBD* allele arrests more tightly than the available $psf2^{ts}$ allele (compare fig. 5 in [21] to fig. 3B in the current paper). The mechanism of

leakage at the late time-points in *psf2-HBD* is not known. Possible mechanism include release of some fusion protein from the Hsp90 complex even in the absence of estradiol or the fusion protein might be cleaved such that wild type Psf2 is produced.

We addressed the possibility that the presence of the tag might affect the stability of Psf2 and performed western blot analysis of extracts prepared from cells grown in the presence and absence of estradiol. Neither the N starvation-refeed procedure we used to synchronize the cells, nor the presence or absence of estradiol significantly affect the level of Psf2 (Fig. 3D).

To explore the reversibility of the arrest caused by inactivation of Psf2 -HBDby the absence of estradiol, cells were initially released from the N starvation block for 4 h in the absence of estradiol (fusion protein "inactive"). As shown above, the cells remain arrested with a 1C DNA content during this time (Fig 3C). After the 4-hour incubation in the absence of hormone, estradiol was added to the culture (fusion protein "active"). The cells carry out substantial DNA replication within 1 h, and replication is largely complete by 6 h (Fig. 3C) suggesting that the estradiol block is rapidly reversible.

Limitations

HO-HBD

HO is an endonuclease that initiates mating-type switching by generating a double-strand break in the DNA in budding yeast [24,25]. Since the double-strand break occurs at a specific site, its fate can be conveniently investigated at the molecular level. Therefore, HO activity is often exploited to investigate checkpoint and repair pathways. However, such studies in fission yeast are hampered by the poor regulatability of the expression of HO. Its expression from the nmt promoter leads to a gradual accumulation of double strand breaks, which are processed as they arise. Thus, a mixed population of cells is investigated at any one time during the course of such an experiment, making it difficult to interpret the results. We therefore fused the ERHBD to the C-terminus of the HO endonuclease to test whether regulation of HO protein function by estradiol would provide a better tool to create double strand breaks in a controlled manner. We found that the HO-HBD fusion protein retains only a little endonuclease activity as compared to untagged HO, even in the presence of estradiol ("active") [see Additional files 1 and 2]. Similar result was obtained with an N terminally tagged HBD-HO fusion protein (Yari Fontebasso and Johanne Murray, personal communication).

Wee1-HBD

There are several protocols to synchronise *S. pombe* cells in different parts of the cell cycle. Induced synchronisation is often preferred over selection synchronisation because it is experimentally easier, especially for large cultures, and gives a high level of synchrony. However, induced synchronisation is usually dependent on temperature shifts which are sometimes not desirable. We sought to use Wee1 to generate synchronous cultures without involving a temperature shift. Wee1 is a protein kinase that inhibits entry into mitosis by phosphorylating Cdc2 [26,27]. Overexpressing Wee1 leads to a reversible G2 arrest. However, the currently available expression systems allow too strong expression even when *wee1* is repressed, since fission yeast cells delay in G2 and become elongated even when one extra copy of *wee1* is introduced into the cells. Therefore, long term overexpression can only be achieved if the overproduced Wee1 protein is inactive. We attempted to inactivate Wee1 by fusing it to the HBD. We fused the HBD to the C-terminus of Wee1, where the catalytic domain is located. We found that the Wee1-HBD fusion protein retains its activity in the absence of estradiol ("inactive") [see Additional files 1 and 3].

Discussion

Here we show that the estradiol-regulatable hormone-binding domain provides a means to regulate efficiently and quickly the function of some fission yeast proteins, namely Cdc13-des2 and Psf2. In contrast, the HO-HBD fusion protein retains little activity even in the absence of estradiol ("active"), while the Wee1-HBD fusion protein was active even in the absence of estradiol ("inactive").

The Hsp90 complex was highly conserved through evolution. Therefore we expected that the HBD tag might confer sensitivity to estradiol to proteins in fission yeast. Analysis of each HBD-tagged protein requires an individual assay, therefore a large-scale analysis of the regulatability of fission yeast proteins is not feasible. Since here we show that the activities of some fission yeast proteins fused to the HBD are indeed regulated by estradiol, we speculate that the mechanism of regulation is probably through binding to Hsp90, as it is in other organisms.

In those cases when the ERHBD tag confers regulatability, the rate of activation and the tightness of the "off" state favourably compare with those obtainable with currently available expression systems. Fast activation of the fusion proteins is reflected in the rapid increase of the anaphase index and swift entry into S phase after activation of Cdc13-des2-HBD and Psf2-HBD, respectively. In the absence of estradiol (fusion protein "inactive") a tight inactivation is observed in both cases; cells expressing Cdc13-des2-HBD do not delay in anaphase and *psf2-HBD* cells remain arrested with unreplicated DNA for at least one generation time.

Switch-off experiments require removal of estradiol by extensive washing, which in itself stresses the cells and might be undesirable in a physiological experiment. However, as inactivation is tight and it does not require potentially time-consuming protein degradation once estradiol is removed, we expect that the system will be usable to switch off protein function within the time-scale of one cell cycle.

Since the initial discovery that HBD-tagged heterologous proteins are subject to hormonal regulation [13] a large number of proteins from various organisms has been tagged [28]. It is difficult to predict whether a fusion protein will be regulated by the hormone. In general, the effectiveness of the system may be determined by how the Hsp90 complex is positioned relative to the key functional domains of the tagged protein. We have fused the HBD close to the kinase domain of Wee1, expecting it to be inactivated by such a fusion. Apparently, the kinase domain might not be accessible to the Hsp90 complex, since the fusion protein is active in the absence of estradiol. However, Wee1 binds Hsp90 and this interaction protects it from degradation by the proteasome [29-31]. Although it is not clear which motifs or structural elements in protein kinases are recognized by the Hsp90 chaperone, the kinase domain is a possible candidate site of interaction. Thus, estradiol might not be able to induce a conformational change that is sufficient to override the interaction between Wee1 and Hsp90. Few endogenous Hsp90 substrates are known in fission yeast. Regulating such substrates with the HBD tag will obviously be difficult.

It is noteworthy that the proteins that were regulated by the HBD fusion and estradiol were proteins that depend on complex formation with other proteins for their function. Components of protein complexes might be more sensitive to regulation by steric interference, because complex formation may be affected. This conclusion is in line with the general trend observed in a large number of HBD fusion proteins [28]. It appears that proteins that must interact with other proteins or DNA to carry out their function, such as transcription factors or recombinases, have been successfully regulated by fusion to the HBD and estradiol presumably because their function can be inhibited by steric interference [12,13,28]. Simultaneous regulation of several components of a complex through this approach might be even more effective. On the other hand, enzymes such as β-galactosidase, galactokinase or URA3, that have small molecules as substrates, were not inactivated by steric interference by Hsp90 [13].

The classic model of steroid hormone receptor (SHR) action dictates that SHR-s are sequestered by chaperones in the cytoplasm and are released upon hormone addition. Indeed, Hsp90 is mainly cytoplasmic, but at least in some cell types it is also nuclear, especially after certain stresses [32-34]. In fission yeast Hsp90 is mainly cytoplasmic, but it is not excluded from the nucleus [35]. Localisation signals on the target protein might not be concealed by interaction with the chaperone, so different localisation signals might compete to determine the localisation of the "inactive" fusion protein. Thus, the intracellular localisation of a fusion protein in the absence of hormone is difficult to predict. After hormone addition, the localisation signals on the tagged protein are expected to determine the localisation of the fusion protein.

Conclusion

The estradiol-regulatable hormone-binding domain provides a means to regulate efficiently and quickly the function of at least some fission yeast proteins. In some cases the system provides lower background protein activity and better kinetics of regulation than currently available regulatable expression systems. Since fission yeast is a useful model organism in a number of areas of biological research, this tool will greatly facilitate research in these fields.

Methods

General fission yeast methods

General fission yeast methods and growth media were as described before [36]. Estradiol (Sigma E2758) was made as a 10 mM stock in ethanol and used at a final concentration of 125–500 nM. Nourseothricin (ClonNAT) was obtained from Werner Bioagents. Cells were grown in EMM medium with supplements as required. Thiamine was made as a 10 mg/ml stock in water and used at a final concentration of 5 μg/ml in EMM. To derepress the *nmt* promoter, the cultures were washed three times with water and reinoculated at appropriate cell density in EMM.

Plasmid and strain constructions

The strains used in this work are listed in Table 1.

pFA6-ERHBD-kanMX6

A C terminal tagging vector in the pFA6a-kanMX6 series [23] was constructed by replacing the GFP region with the

Table 1: Strains used in this study

Strain	Carrying the plasmid
ade6-M210 leu1-32 h^-	cdc13-des2-pREP41 cdc13-des2-pREP81
ade6-M210 ura4-D18 h^+	cdc13-des2-ERHBD-pREP81 HO-ERHBD-pREP81 wee1-ERHBD-pREP81
psf2-ERHBD:kanMX6 h^+ psf2-ERHBD:YFP:kanMX6 h^-	

Table 2: Primers used for plasmid construction

	Primers	Template
cdc13-des2-HBD:	TCCTC**CATATG**ACTACCCGT A CAC TAA A**TT AAT T**AA CCA TTC	pREP81-cdc13-des2
wee1-HBD:	GGAATTC**CATATG**AGCTCTTCTTCTAATAC C C**TT AAT TAA**AAC ATT CAC CTG CCA ATC TT	Genomic
HO-HBD:	GGAATTC**CATATG**CTTTCTGAAAACACGAC C C**TT AAT TAA**GCA GAT GCG CGC ACC TGC GT	Genomic

HBD in pFA6a-GFP(S65T)-kanMX6 [23,37]. HBD was amplified from pHCA/GAL4(848).ER (D. Picard) as a PacI-AscI fragment using the following primers:

AAAA TTA ATT AAC TCT GCT GGA GAC ATG AGA GCT GCC

AAAA GG CGC GCC TCA GAC TGT GGC AGG GAA ACC CTC TGC and inserted into PacI AscI digested pFA6a-GFP(S65T)-kanMX6.

Cdc13-des2-HBD, Wee1-HBD and HO-HBD

The *cdc13-des2*, *wee1* and *HO* genes were amplified by PCR with Nde1 site introduced at START and Pac1 site introduced upstream of STOP using the primers shown in Table 2 (the sequences for the introduced sites are underlined): The PCR products were cut with Nde1 and Pac1.

HBD was isolated as a Pac1 – Asc1 fragment from pFA6-ERHBD-kanMX6. The pREP82 plasmid was cut with Sma1 and Asc1 linker was inserted. The Nde1 and Pac1 cut *cdc13-des2*, *wee1* and *HO* PCR products were ligated with the HBD into Nde1 Asc1 cut pREP82-Asc1.

Psf2-HBD

In order to tag the *psf2*$^+$ gene in the chromosome, the PCR-mediated gene targeting method for fission yeast [23] was used with plasmids pFA6a-HBD-kanMX6 and pFA6a-HBD-natMX6 as templates, the latter being constructed by transferring the PacI-AscI HBD fragment from the former into pFA6a-GST-natMX6 [38]. The primers used for amplification are shown below. Sequences with identity to the template plasmid are underlined.

PSF2-CTAG-5 5'-TGGAAATTAACGAAATACGTCCTATATT TCGAGAG GTGATGGACAGAATGCGCAAAATTGTTCAA GTTTCCCAAGAAGAACGGATCCCCGGGTTAATTAA-3'

PSF2-CTAG-3 5'-ATTTCACTACTACAAAGTTGGTATTCAT-AAACACTT CGTAGGATTCATTATCATTATTTTTAAAGTAC ATCATCCACACGGAATTCGAGCTCGTTTAAAC-3'

The resulting PCR products (5–10 μg) were transformed into S. pombe h^{-N} and h^{+S} strains as described and transformants selected with either 100 μg/ml G418 or 100 μg/ml nourseothricin [23,38]. Transformants were then screened by PCR to confirm that the gene was successfully tagged. The sequences of the PCR primers used for this can be obtained from the authors on request.

Flow cytometry

Was performed using SYTOX Green as described previously [8].

Immunoblots

Cell extracts were made by the TCA protein extraction method [39]. Detection was performed using the ECF or ECL kits (Amersham Biosciences).

Authors' contributions

CAB made and characterized the HO-ERHBD construct under the guidance of BG. IG tagged the *psf2* gene in the chromosome and performed the initial analysis of the tagged strain under the direction of SAM. CCP characterized the *psf-ERHBD* strain under the direction of SK. JS constructed the pFA6-ERHBD-kanMX6 plasmid under the guidance of MT. HCS constructed the *nmt82.cdc13-des2-ERHBD* plasmid and performed the initial characterization of the construct under the guidance of BG. EB, SK, SAM and MT contributed to writing the manuscript and designing experiments. BG constructed and characterized the *nmt82.wee1-ERHBD* plasmid, completed the characterization of *nmt82.cdc13-des2-ERHBD* and wrote the manuscript.

Additional material

Additional file 1

Limitations of the system. Two examples where fusing the ERHBD tag to the protein of interest did not lead to regulatability with estradiol are presented.

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[http://www.biomedcentral.com/content/supplementary/1471-2121-9-23-S1.pdf]

Additional file 2

HO-HBD has little HO activity even in the presence of estradiol. The data provided present evidence that the HO-HBD fusion protein retains little endonuclease activity even in the presence of estradiol.
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[http://www.biomedcentral.com/content/supplementary/1471-2121-9-23-S2.pdf]

Additional file 3

Wee1-HBD is active even in the absence of estradiol. The data provided present evidence that the Wee1-HBD fusion protein is active even in the absence of estradiol.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2121-9-23-S3.pdf]

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Additional file 1.

HO-HBD

HO is an endonuclease that initiates mating-type switching by generating a double strand break in the DNA in budding yeast [23,24]. Since the double strand break occurs at a specific site, its fate can be conveniently investigated at the molecular level. Therefore, HO activity is often exploited to investigate checkpoint and repair pathways. However, such studies in fission yeast are hampered by the poor regulatability of the expression of HO. Its expression from the nmt promoter leads to a gradual accumulation of double strand breaks, which are processed as they arise. Thus, a mixed population of cells is investigated at any one time during the course of such an experiment, making it difficult to interpret the results. We therefore fused the *ERHBD* to the C-terminus of the *HO* endonuclease to test whether regulation of HO protein function by estradiol would provide a better tool to create double strand breaks in a controlled manner. Expression of the fusion protein was driven by the nmt81 promoter. In order to test the activity and kinetics of activation of the fusion protein, we employed a strain carrying a minichromosome with a recognition sequence for HO in the *kanR* gene [40]. We followed HO activity by Southern blotting, using the *kanR* gene as a probe [40]. As a positive control, we used a strain carrying a plasmid with the wild type *HO* gene driven by the same promoter. A 6 kb fragment representing the uncut *kanR* gene was detected at all timepoints. Very little cutting was detected when the promoter was repressed or after 27 h of induction (Fig. 4 and not shown). After 30 h of induction the appearance of a 3.5 kb fragment indicated cutting in the control strain expressing wild type HO. However, we detected very little cutting in the strain expressing the HO-HBD fusion protein even in the presence of estradiol (fusion protein “active”) (Supplementary fig. 1). We conclude that the HO-HBD fusion protein retains only a little endonuclease activity as compared to untagged HO, even in the presence of estradiol (“active”).

Wee1-HBD

We sought to use Wee1 to generate synchronous cultures without involving a temperature shift. Wee1 is a protein kinase that inhibits entry into mitosis by phosphorylating Cdc2 [25,26]. Overexpressing Wee1 leads to a reversible G2 arrest. However, the currently available expression systems allow too strong expression even when *wee1* is repressed, since fission yeast cells delay in G2 and become elongated even when one extra copy of *wee1* is introduced into the cells. Therefore, long term overexpression can only be achieved if the overproduced Wee1 protein is inactive. A temperature-sensitive mutant has been successfully employed [41], but cell synchronisation required a temperature shift. We attempted to inactivate Wee1 by fusing it to the HBD. We expected that the fusion protein could be expressed to a high level in the absence of estradiol (fusion protein "inactive") but it would mediate a G2 arrest after addition of estradiol (fusion protein "active"). Upon inactivating Wee1 by removal of estradiol the cells would enter a synchronous mitosis.

We fused the HBD to the C-terminus of Wee1, where the catalytic domain is located. Expression of the fusion protein was driven by the weak nmt promoter. We found that cells carrying the fusion protein grew poorly in the absence of estradiol (fusion protein "inactive"), even with the promoter repressed. They died as elongated cells with the promoter induced (Supplementary fig. 2). When estradiol was added to the cells, no dramatic difference was observed either in cell length or apparent generation time (data not shown). We conclude that the Wee1-HBD fusion protein retains its activity in the absence of estradiol ("inactive").

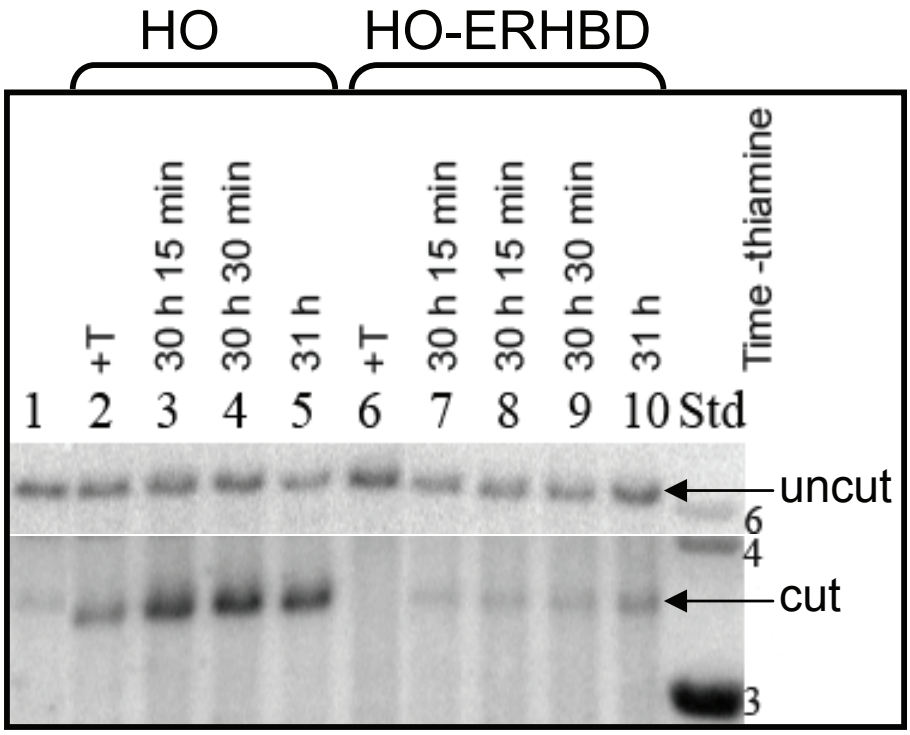
Additional figures

Additional figure 1. HO-HBD has little HO activity even in the presence of estradiol.

Cutting in the *kanR* gene. A control plasmid (lane 1) or a plasmid carrying the wild type *HO* gene (lanes 2-5) or a plasmid carrying *HO-HBD* (lanes 6-10) was introduced into a strain carrying a minichromosome with the HO recognition sequence in the *kanR* gene. Lanes 2 and 6 show cutting in cells grown in the presence of thiamine for 30 h 15 min. The nmt81 promoter was induced for 30 h 15 min (lanes 3, 7, 8); 30 h 30 min (lanes 4, 9) and 31 h (lanes 5, 10). Estradiol was added after 30h (lanes 8, 9, 10; 15, 30 and 60 min with estradiol, respectively). The upper panel shows a southern blot using the *kanR* gene as a probe. The 6 kb band represents the uncut *kanR* gene, the 3.5 kb band represents the cut gene. The graph shows the relative intensity of the cut/uncut bands. The presence or absence of thiamine and estradiol, as well as the length of time in the absenece of thiamine and in the presence of estradiol, are indicated. na: not applicable

Additional figure 2. Wee1-HBD is active even in the absence of estradiol.

Cells were grown in the presence (**A**) and absence (**B**) of thiamine. Inserts show a wild type cell in anaphase for comparison. Bar represents 10μM.


HO
HO-ERHBD
Time -thiamine
1 2 3 4 5 6 7 8 9 10 Std
+T
30 h 15 min
30 h 30 min
31 h
+T
30 h 15 min
30 h 15 min
30 h 30 min
31 h
uncut
cut
6
4
3
na + - - - + - - - - Thiamine
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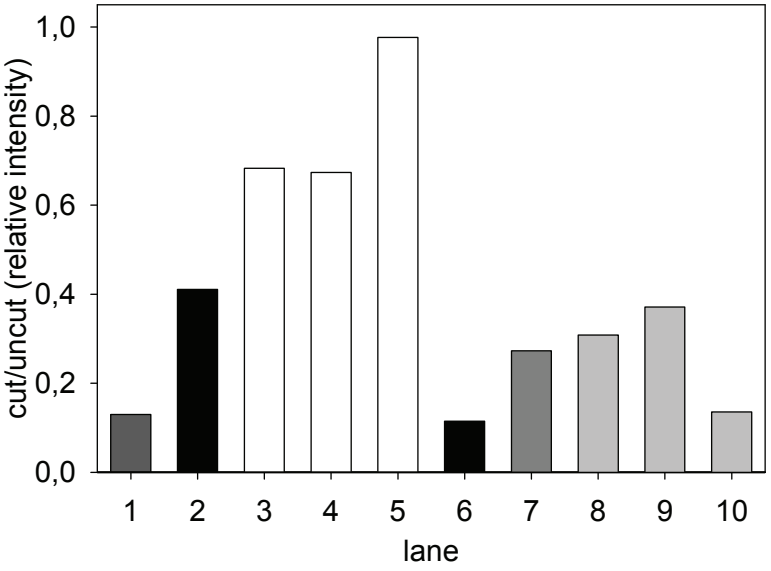
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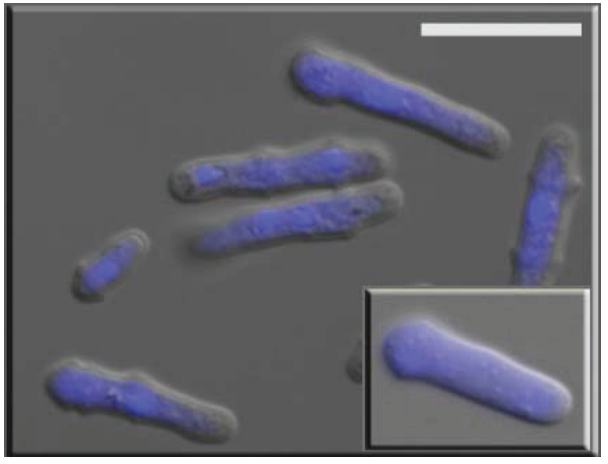

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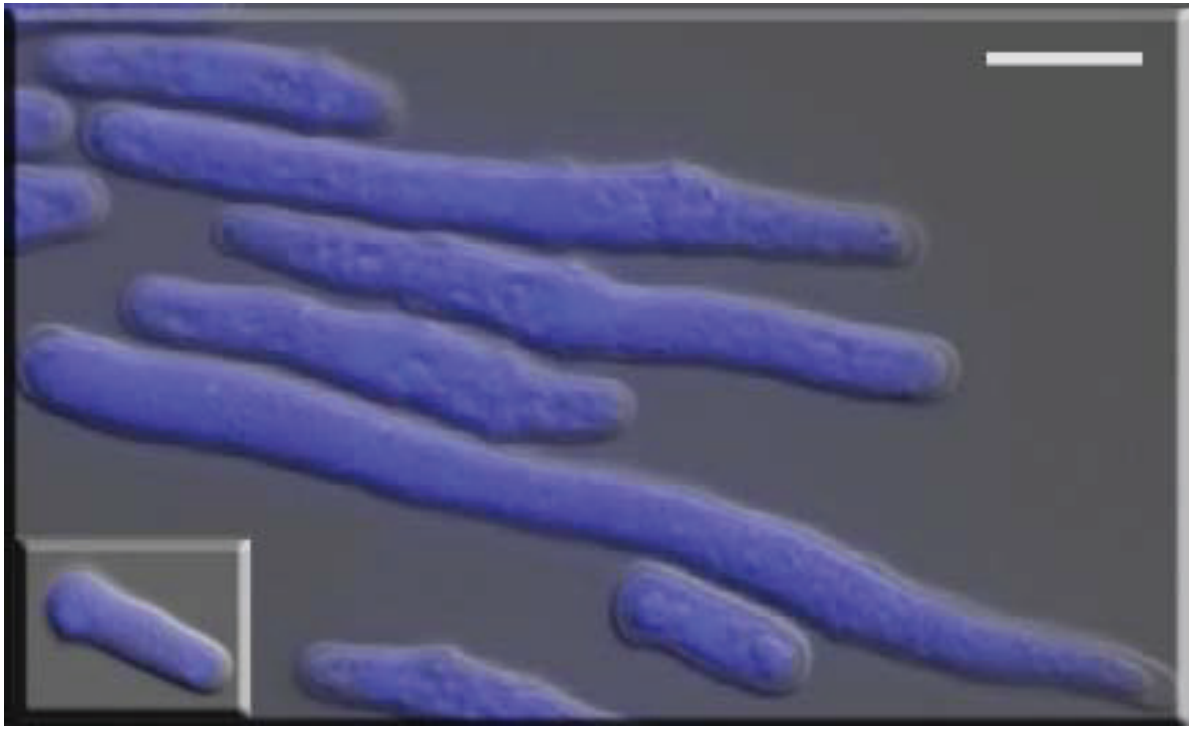

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Hpz1 Modulates the G1-S Transition in Fission Yeast

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Abstract

Here we characterize a novel protein in *S. pombe*. It has a high degree of homology with the Zn-finger domain of the human Poly(ADP-ribose) polymerase (PARP). Surprisingly, the gene for this protein is, in many fungi, fused with and in the same reading frame as that encoding Rad3, the homologue of the human ATR checkpoint protein. We name the protein Hpz1 (Homologue of PARP-type Zn-finger). Hpz1 does not possess PARP activity, but is important for resistance to ultraviolet light in the G1 phase and to treatment with hydroxyurea, a drug that arrests DNA replication forks in the S phase. However, we find no evidence of a checkpoint function of Hpz1. Furthermore, absence of Hpz1 results in an advancement of S-phase entry after a G1 arrest as well as earlier recovery from a hydroxyurea block. The *hpz1* gene is expressed mainly in the G1 phase and Hpz1 is localized to the nucleus. We conclude that Hpz1 regulates the initiation of the S phase and may cooperate with Rad3 in this function.

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Introduction

Cell growth and proliferation involve a series of distinct reaction pathways that are linked together in what is termed the cell cycle [1–3]. Preparation for another round in the cell cycle is made already as the cells exit mitosis, when the Origin Recognition Complex (ORC) is bound at the future origins of DNA replication, to be activated in the following S phase. In late mitosis or G1 phase the replicative helicase, the MCM hexamer, is loaded onto the replication origins marked by ORCs. This event is dependent upon a transcripton factor that activates genes encoding the proteins responsible for MCM loading. In human cells the loading is dependent upon the CDC6 and CDT1 proteins and homologous proteins have similar activities in all other eukaryotes. Thereafter, a series of events, including the activation of an S-phase cyclin-dependent kinase (CDK), leads to initiation of DNA replication at a subset of the replication origins [4–6]. Some origins are initiated early in S phase, others at a later stage. After successful completion of S phase the cell prepares for mitosis and CDK activity is required also for the G2-M transition [7–9]. In mitosis the chromosomes are segregated, the nucleus divides, and the cell can prepare for division.

Regulation of the cell cycle is performed by a number of feedback and feed-forward mechanisms and in addition by external checkpoint mechanisms that arrest the cell cycle if the DNA is damaged or if one phase of the cell cycle has not been properly finished [10]. The central checkpoint proteins in human cells are the ataxia telangiectasia mutated (ATM) and the ATM and RAD3-related (ATR) proteins. Both ATR and ATM are large phosphoinositide 3-kinase-related protein kinases (PIKKs) with multiple substrates.

ATR associates with its obligate partner ATRIP to perform its function. The ATR protein, as well as its homologues in other eukaryotes, contains a C-terminal kinase domain and an N-terminal ATRIP-binding domain, separated by a large α-helical HEAT domain. A similar structure is found for the ATR homologue in fission yeast, Rad3, whose binding partner is Rad26. There are undoubtedly a large number of proteins that the heterodimer Rad3/Rad26 interacts with, but few of them are known.

Human cells are not viable without ATR, but the essential function has not been identified. ATR is involved in the activation of chromosomal replication origins within S phase as well as in the stabilization of stalled replication forks [11–13], but the detailed molecular functions are still poorly understood. ATR phosphorylates a subunit of the replicative helicase, MCM2 [14,15], in a reaction that may regulate S-phase progression [16]. ATR is activated by DNA damage and in particular by single-stranded DNA generated by repair processes and bound by Replication Protein A [17], but the mechanism of activation is not well characterized. Furthermore, ATR phosphorylates proteins involved in recombination [18–21] and nucleotide excision repair [22]. The intracellular activity of PIKK kinases is known to be regulated, at least in part, by their localization [23] and this is likely to be true also for ATR.

In this work we describe a fission yeast protein whose homologue in many fungi is encoded within the same open reading frame as the Rad3 homologue, suggesting that the two proteins are acting together also when they are encoded separately. This protein shows a high degree of homology with the Zn- finger domain of the human Poly(ADP-ribose) polymerase (PARP). We present evidence that the gene is involved in DNA replication control and may interact with Rad3. In particular, absence of the protein conveys some of the same phenotypes that are found for the *rad3* deletion mutant, arguing that the two proteins are acting in some common reaction pathway(s).

Results

Identification of a Potential Functional Partner of Rad3

In fission yeast Rad3 is a major regulator of the response to DNA damage and stalled replication forks. We compared the homologues of Rad3 in a wide range of organisms and found that in several fungi the protein is extended at the C-terminus with an additional motif (Fig. 1 A), that shows extensive homology to the Poly(ADP-ribose) polymerase (PARP)-type Zn-finger (IPR001510) (Fig. 1 B). The C-terminal extension also contains a region enriched in negatively charged residues. The fission yeast genome contains two genes encoding proteins with extensive homology to the PARP-type Zn-finger motif, SPBC2A9.07c and SPAC13F5.07c (Fig. 1 A). Of the two, only SPBC2A9.07c contains the negatively charged clusters conserved in the fungal Rad3 homologues and is therefore the homologue investigated further in this work. We named SPBC2A9.07c Hpz1 for Homologue of PARP-type Zn-finger. No obvious Hpz1 homologue can be identified in *Saccharomyces cerevisiae*. The highest degree of similarity to Hpz1 in the current genome databases was found in the C-terminal end of the Rad3-homologue XP_00122235 in *C. globosum*. The PARP-type Zn-finger motif shows a higher degree of conservation between the fungal homologues and Hpz1 than between Hpz1 and the human PARP1 or DNA ligase 3 (Fig. 1 B). However, this motif is found in several eukaryotes and even in bacteria. For example, there are 15 proteins with this motif in mouse and 13 proteins in the human genome. Of these, there are several small proteins with a PARP-type Zn-finger motif but no other obvious domains, including the negatively charged C-terminal domain. It is unclear whether these proteins share functions with each other and whether they can be considered are functional homologues of Hpz1.

Hpz1 is predicted to contain 246 amino acid residues with a molecular weight of 28.1 kDa. The protein contains a PARP-type Zn-finger domain on the N-terminus and a region enriched in negatively charged amino acid residues on the C-terminus (Fig. 1 A and C). We considered the intriguing possibility that Hpz1 might have PARP activity. However, the homology of Hpz1 to established PARP genes is limited to the Zn-finger domain. In eukaryotes PARPs belong to a protein family catalyzing poly(ADP-ribosyl)ation of DNA-binding proteins. The active site of PARPs is located within a highly conserved 50 amino acid sequence called "the PARP signature" [24,25]. There is no obvious PARP signature in the protein sequence of Hpz1. Consistently, we could not detect poly(ADP-ribosyl)ated proteins in cell extracts from *S. pombe* (data not shown). These results are consistent with previous findings that fission yeast does not contain a PARP homologue [26].

The fusion of Rad3 to Hpz1 homologues in several fungi indicates that the two proteins share function(s) or participate in the same biological process(es). Therefore we decided to explore whether Hpz1 has functions related to those of Rad3.

*hpz1*Δ is Sensitive to Ultraviolet Light in G1 Phase and to HU Treatment

One known function of Rad3 is to induce an appropriate response to DNA damage or stalled replication forks, and *rad3*Δ cells are extremely sensitive to DNA-damaging agents. We found that the *hpz1*Δ mutant was slightly more sensitive to ultraviolet light (UVC) than wild-type cells (Fig. 2 A), but not as sensitive as a checkpoint deficient mutant (*rad26*Δ). We considered the possibility that Hpz1 is only required in a small fraction of the cells in an asynchronous population. The UVC sensitivity in different cell-cycle phases was determined in wild-type and *hpz1*Δ cells synchronized in G1 phase, using a *cdc10* block-and-release (see M&M) followed by UVC-irradiation in G1, S or G2 phase. Wild-type cells were most resistant to UVC in G2 and least in S phase (Fig. S1). The survival of *hpz1*Δ cells irradiated in G1 phase was reduced by 50% compared to a wild-type strain, but no differences were found in the other cell-cycle phases (Fig. 2 B). These results indicate an important function for Hpz1 after UVC irradiation specifically in G1 phase.

The sensitivity to ionizing radiation of *hpz1*Δ mutant cells was no different from that of wild-type cells (Fig. S2) suggesting that Hpz1 does not play an important role in double-strand break repair.

Rad3 is activated when replication forks stall and this leads to checkpoint activation (see Introduction) and cell cycle arrest. Hydroxyurea (HU) inhibits the ribonucleotide reductase leading to depletion of the nucleotide pools and to the stalling of replication forks [27] and to checkpoint induction. HU-treated *rad3*Δ cells do not arrest in the intra-S checkpoint, but rather continue into mitosis and divide with the DNA unevenly distributed between the daughter cells [28], displaying the so-called "cut" phenotype [29,30], which results in poor cell survival. To investigate the requirement for Hpz1 when replication forks stall we determined the tolerance of *hpz1*Δ to HU. The survival of *hpz1*Δ after 4 hours in HU (15 mM) was 10% lower than for wild-type cells (Fig. 2 C), but the *hpz1*Δ cells did not appear cut (Fig. 2 D left) and they arrested with 1C DNA (early S phase) as judged by flow cytometry (data not shown). However, 1 hour after release from HU ~7% of *hpz1*Δ cells displayed the cut phenotype (Fig. 2 D). It is not unlikely that the cutting corresponds to the 10% reduction in survival.

Hpz1 and Rad3 Might Interact

The UV and HU sensitivity of the *hpz1*Δ mutant (above) indicates a role for Hpz1 under these conditions. We therefore chose UVC- and HU-treatments to investigate the interaction between Hpz1 and Rad3. Cells carrying Hpz1-HA and Rad3-myc were synchronized in G1 phase by a *cdc10* block, released into the cell cycle and either UVC-irradiated in G1 or S phase or released into an HU–induced S-phase arrest. Hpz1-HA was immunoprecipitated from the extracts of these cells and the immunoprecipitate was analyzed for the presence of Rad3-myc. Co-immunoprecipitated Rad3-myc could be detected in extracts from untreated G1 cells and HU-treated cells, but not from S-phase cells (Fig. S3). However, this interaction was only detected in two experiments and cannot be considered conclusive. Nonetheless, the data suggest that an interaction between Hpz1 and Rad3 might indeed exist, but that it is indirect or transient.

Initiation of DNA Replication is Advanced in *hpz1*Δ Mutant Cells

The HU sensitivity assay indicated an abnormal response of *hpz1*Δ to stalled replication forks, but not a checkpoint defect similar to that of *rad3*Δ cells. To further explore this response the cellular DNA content of wild-type and *hpz1*Δ cells was monitored after they were released from an HU block. In several repeated experiments the *hpz1*Δ mutant cells invariably increased their DNA content earlier than wild-type cells did (Fig. 3 A). The quantification of cells with a 1C (early S phase) or 2C DNA content (G2) from these experiments showed that the time lag between wild-type and *hpz1*Δ is about 15 min throughout S-phase (Fig. 3 B).

The above results suggest that in the *hpz1*Δ mutant initiation or restart of replication forks are advanced. To determine whether the earlier increase in DNA content also occurs when the cells are synchronized before S phase, we arrested cells in G1 phase in a *cdc10* block, released them from the block and followed their

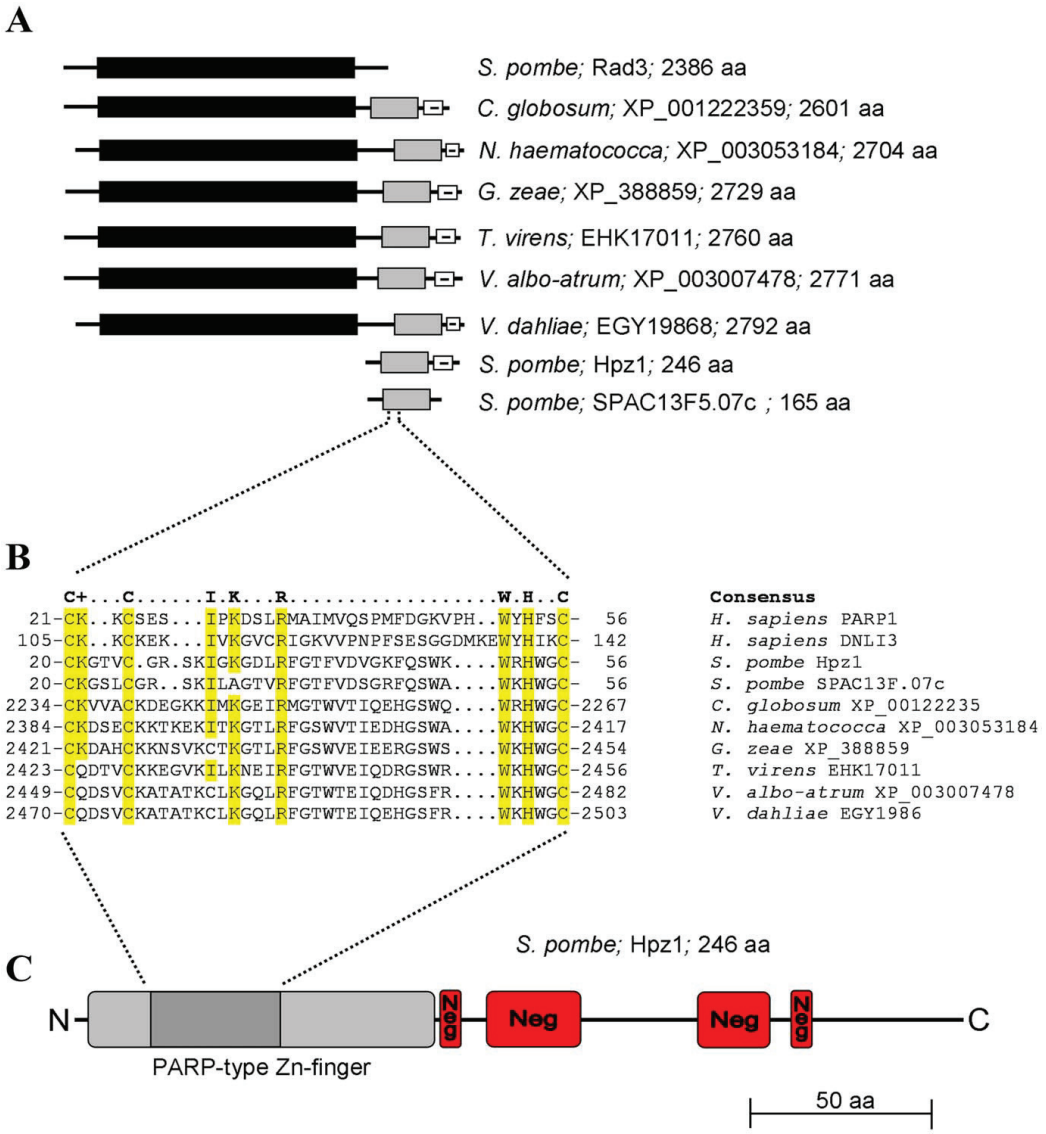

Figure 1. Homology and composition of Hpz1. A. Schematic presentation of Rad3 (black bar) from *S. pombe*, six Rad3-like proteins in different fungi with a PARP-type Zn-finger domain (gray bar) and a negatively charged C-terminal domain (white bar with minus sign), and the two homologues of the PARP-type Zn-finger found in *S. pombe*, Hpz1 and SPAC13F5.07c (not drawn to scale). B. Multiple-sequence alignment showing the consensus sequence of the PARP-type Zn-finger domain (IPR001510, http://www.ebi.ac.uk/interpro/) and aligned sequences below. Conserved residues are highlighted in yellow. The numbers in front of and after the sequence indicate the residue numbers. C. Schematic representation of the Hpz1 protein in *S. pombe* (drawn to scale). Indicated are the PARP-type Zn-finger domain (light gray), the Zn-finger signature sequence used in the multiple-sequence alignment (dark gray) and four regions that show bias towards the negatively charged amino acids glutamate and aspartate (red). doi:10.1371/journal.pone.0044539.g001

progression into and through S-phase (Fig. 3 C and D). Surprisingly, *hpz1*Δ cells seemed to increase their DNA content earlier than wild-type cells did.

To exclude the possibility that *hpz1*Δ cells normally progress faster through S phase and therefore spend shorter time in S phase we analyzed asynchronous populations of cells by flow cytometry

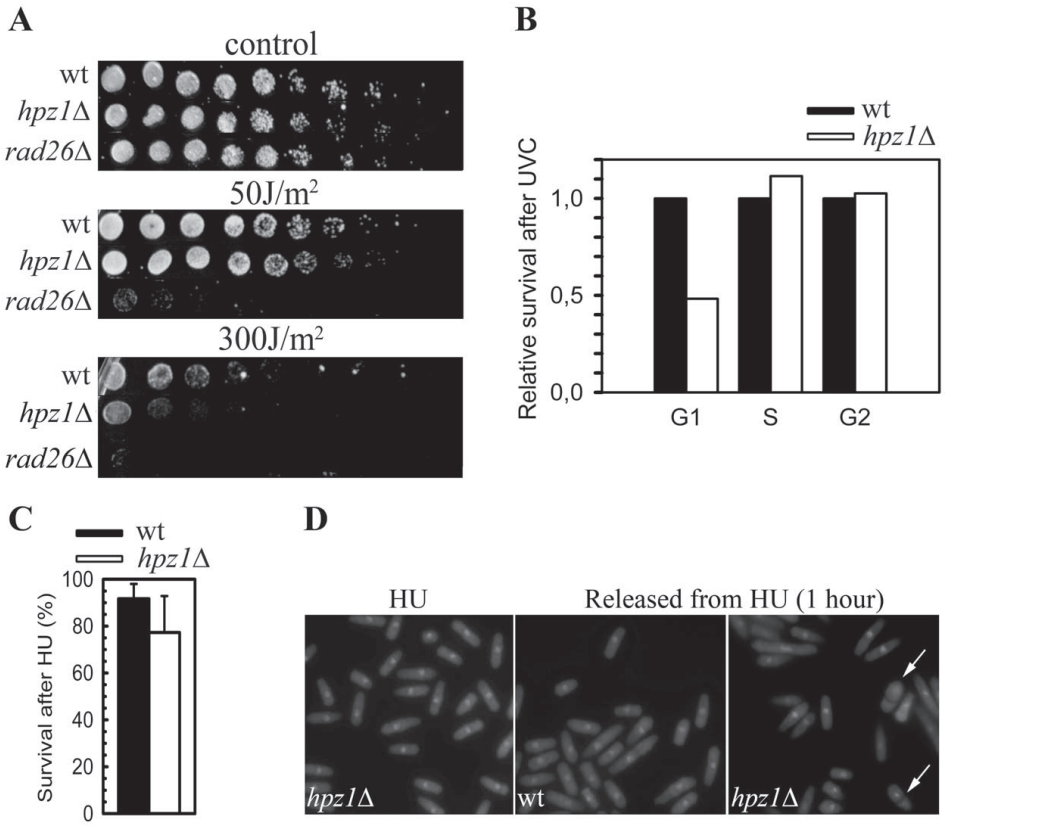

Figure 2. UVC- and HU-sensitivity. A. Spot test for UVC sensitivity of wild-type (wt), *hpz1*Δ and *rad26*Δ cells. Upper: unirradiated cells, center: 50J/m^2, lower: 300J/m^2. B: Survival after UVC irradiation of wild-type or *hpz1*Δ cells in G1, S or G2 phase. The survival of wild-type cells was normalized to 1 in each cell cycle phase (data without normalization are shown in Fig. S1). C. Survival of wild-type or *hpz1*Δ cells after HU-treatment. D. Microscopy images of *hpz1*Δ cells in HU (left) and 1 hour after release from HU (center) and wild-type cells released from HU (right). The DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI). The arrows point to cut cells.
doi:10.1371/journal.pone.0044539.g002

and measured the numbers of cells in the different cell-cycle phases [31]. The results showed no differences in the percentage of wild-type versus *hpz1*Δ cells in S phase, arguing that the time spent in S phase was the same (Fig. S4).

We also measured the timing of MCM loading in G1 phase in the two strains after a *cdc10* block-and-release. The MCM complex is loaded onto future replication origins to form the Pre-replicative complex (PreRC) and this event can be followed in a microscope when employing a fluorescently tagged MCM [32]. We found that maximal loading of MCMs occurred 60 min after the release of wild-type cells (Fig. 3E), in agreement with earlier observations [33]. However, in the *hpz1*Δ cells the maximum consistently occurred about 15 minutes earlier, suggesting that Hpz1 is negatively modulating an event at or before PreRC formation. It should be noted that this phenotype is different from that observed above for cells synchronized inside S phase, and this will be discussed below.

Hpz1 Localizes to the Nucleus and is Expressed in a Cell-cycle-dependent Manner

The Zn-finger domain in Hpz1 indicates that it is capable of DNA binding, and hence suggests a nuclear localization. In a global ORFeome analysis, over-expressed Hpz1 was found to localize to the mitochondria and some nuclear signal was also observed [34]. We have fused a GFP-tag to the C-terminus of Hpz1 and the fusion protein was expressed from its endogenous promoter. GFP localization was determined by fluorescence microscopy of exponentially growing cells. We observed a strong and clear nuclear signal in a significant fraction of the cells and no signal in the other cells (Fig. 4 A). Furthermore, the nuclear signal was apparently dependent upon the cell-cycle stage, since Hpz1-GFP was mainly detectable in cells with two nuclei (M or G1 phase) and in some of the smallest cells (late S – early G2).

To explore the suggested cell-cycle regulated expression further the presence of Hpz1-HA was investigated by immunoblotting of the total extracts of cells synchronized in G2/M by a *cdc25* block-and-release experiment (Fig. 4 B). The frequency of cells in

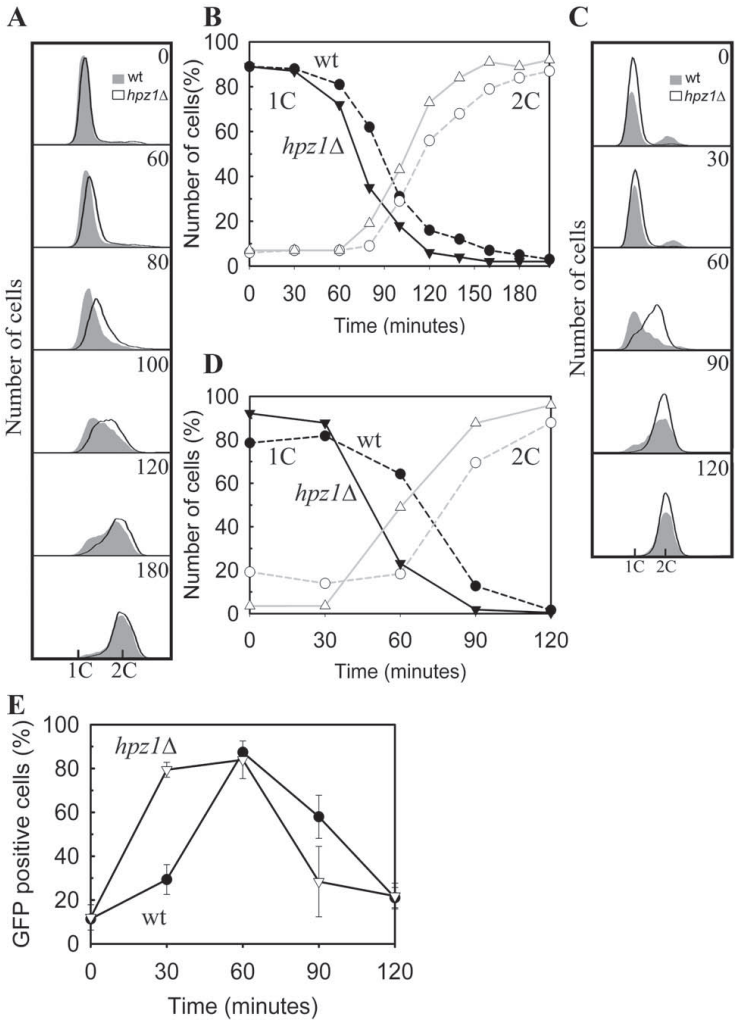

Figure 3. Progression of DNA replication. Analysis of the increase in DNA content in individual wild-type and *hpz1*Δ cells after two different methods for synchronization and release. A. DNA histograms of cells blocked in early S phase by HU-treatment for 4 hours, then washed and released into the cell cycle for the times indicated. B. Quantification of the cells (in A) with a 1C DNA or 2C DNA content after HU-treatment and release into the cell cycle for the times indicated. C. DNA histograms of *cdc10* cells that were synchronized in G1 phase, released into the cell cycle and incubated for the time indicated. D. Quantification of the cells (in panel C) with a 1C DNA or 2C DNA content after a *cdc10* block and release into the cell cycle and incubated for the time indicated. E. PreRC formation in wild-type and *hpz1*Δ cells as a function of time after release from a *cdc10* block.
doi:10.1371/journal.pone.0044539.g003

anaphase and the septation index were determined at different times after release into the cell cycle (Fig. 4 C) as a measure of synchronous progression through the cell cycle. The cellular level of Hpz1 was found to increase in late anaphase, was maximal in G1 phase and declined in S phase (Fig. 4 B and C).

Proteins specifically expressed in G1 are often regulated by the Cdc10 transcription factor [35]. To determine whether the cell-cycle-regulated expression of Hpz1 depends on Cdc10, we monitored the expression of an Hpz1-HA fusion protein after a *cdc10* block-and-release experiment (Fig. 4 D). Hpz1 was present at the time of G1 arrest, but disappeared shortly after release from the *cdc10* block, arguing that *hpz1* is not a Cdc10 target. We conclude that the expression of Hpz1 is limited to the M/G1 phase. The PCB (Pombe Cell-cycle Box)-binding factor (PBF) is a transcription factor responsible for M/G1-specific transcription of its target genes [36]. A search for PCB-motifs, the known binding site of PBF [36], revealed two PCB-motifs upstream of *hpz1* (Fig. S5), suggesting that it is a target of PBF.

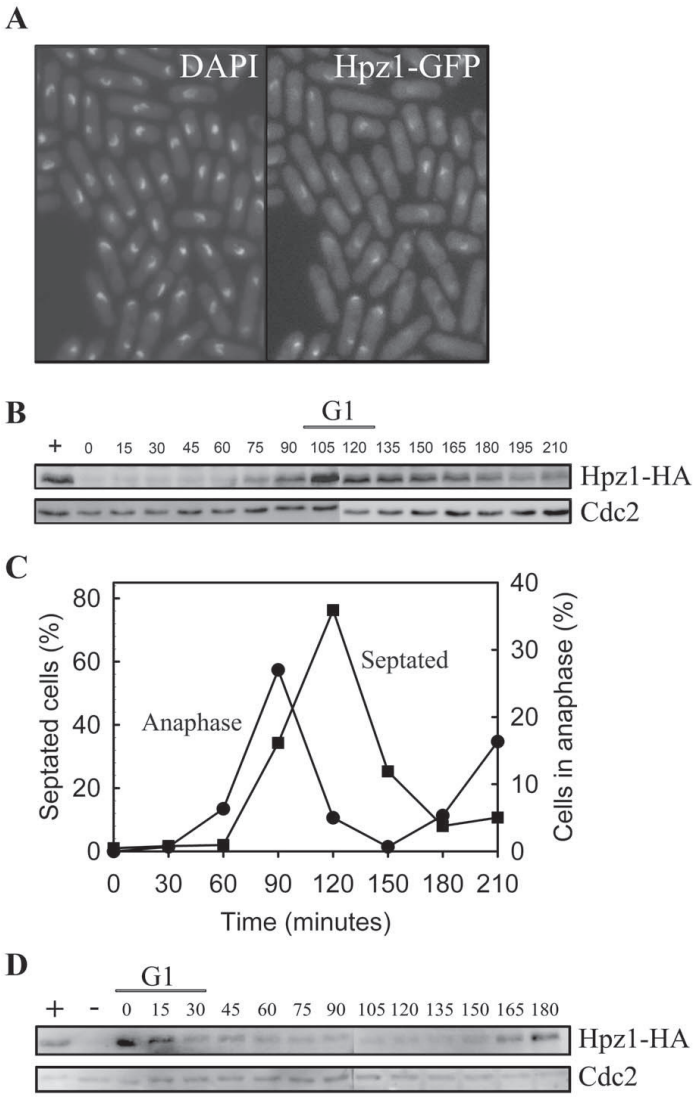

Figure 4. Cell-cycle regulation and localization of Hpz1. Fluorescence microscopy pictures showing all nuclei (DAPI-staining, left) and Hpz1-GFP localization (right) in the same cells. B. Immunoblot showing the expression of Hpz1-HA in total cell extracts taken at the indicated time points after a *cdc25* block-and-release. C. The percentage of cells in anaphase (DAPI-stained cells with 2 nuclei) or septated cells (stained with aniline blue) from the experiment shown in panel B. D. Immunoblot showing the presence of Hpz1-HA in total cell extracts taken at the indicated time points after a *cdc10* block-and-release.
doi:10.1371/journal.pone.0044539.g004

Discussion

We have identified and characterized Hpz1, a novel putative partner for Rad3 in fission yeast. This partnership may give important insights into the functions of Rad3 and of its homologues in the ATR family of proteins since these proteins perform important, and sometimes essential, functions in cell-cycle regulation and in maintenance of the genome.

Functional Clues from Protein Sequence Information

Hpz1 contains a PARP-type Zn-finger domain, but lacks other features necessary for PARP function. There is no evidence for the existence of poly (ADP-ribosyl)ated proteins in cell extracts from

fission yeast ([26] and this work). PARPs play an important role in human cells by detecting and binding to single-stranded DNA breaks and thereby to signal to repair enzymes [37]. We show here that the *hpz1* deletion mutant is more sensitive to UVC than wild-type cells are. However, we reason that such a minor increase in sensitivity is unlikely to be caused by the absence of a major DNA repair function. Together these results point to a different function of Hpz1 than poly (ADP-ribosyl)ation and DNA repair. The PARP-type Zn-finger is a domain found in several human proteins. Some of them have a known function, like PARP-1 and DNA ligase 3, whereas others are yet uncharacterized, but resemble Hpz1 in size and in that they only contain one PARP-type Zn-finger domain. Thus, the available sequence information suggests that Hpz1-like proteins might also be conserved in higher eukaryotes.

Hpz1 and Rad3 Might Act Together in G1 Phase

The fact that homologues of the two very different proteins Hpz1 and Rad3 are fused in several fungi suggests a shared function of the separate proteins in other organisms. We show here that *hpz1*Δ does not share the checkpoint defects known for *rad3*Δ cells, so Hpz1 is not required for the checkpoint functions of Rad3. However, Hpz1 is exerting its function in G1, where no function of Rad3 is yet described. Several Mcms have been shown to be substrates of Rad3 or of Rad3 homologues in other species. But these modifications either occur in S phase [38] or have been shown to be important for the intra-S checkpoint [14], pointing to a role for Rad3 in the cell-cycle progression after PreRC loading. We have been able to produce weak evidence that the two proteins are interacting and specifically in G1 phase, but the interaction is probably transient and not very strong. These observations point to a function also for Rad3 in G1 phase. Indeed, we have shown that the early-replication phenotype of the *hpz1*Δ mutant can also be observed in *rad3*Δ cells after a *cdc10* block-and-release (manuscript in preparation). These observations argue that the two proteins might share at least one function in the regulation of G1-S progression.

Hpz1 Regulates the Start of DNA Replication

Our results from two distinct types of experiments clearly show that Hpz1 has a function in the start of DNA replication. First, when the cells are arrested in early S phase by HU and the drug is washed out, the *hpz1* deletion mutant resumes DNA replication earlier than wild-type cells do. This restart may involve already assembled replication forks that have been stalled by a lack of deoxyribonucleotides during HU treatment. Alternatively, the premature firing of late origins might be promoted in *hpz1*Δ mutant cells after release from the HU block. Second, cells arrested in G1 phase by a *cdc10* block and released into the cell cycle also start DNA replication earlier in the absence of Hpz1. One possible explanation for both of these sets of results is that there are more replication forks active in the absence of Hpz1, yielding faster chromosome replication rates and a shorter S phase. This explanation was ruled out by separate experiments (Fig. 3 and Fig. S4). Furthermore, in the *cdc10*-experiments we could show that an event before initiation of DNA replication, namely the formation of PreRC formation, was delayed by the presence of Hpz1. We conclude that Hpz1 has a negative regulatory role for the start-up of DNA replication, both at the initiation stage in G1 and at restart from an HU-block. The premature restart of DNA replication of the *hpz1*Δ mutant may be the reason for a higher rate of mitotic cells after release from the HU block and the increased sensitivity to HU.

The simplest explanation for these findings would be that the two phenotypes of the *hpz1*Δ mutant stem from the same function of the Hpz1 protein. Expression of Hpz1 appears to be initiated in mitosis, possibly regulated by PCB boxes, and the maximal amount of Hpz1 is found in G1 phase. These results are in agreement with an earlier genome-wide analysis of the cell-cycle dependence of the mRNA levels of numerous genes in *S. pombe* [39] and strongly argues for a function of Hpz1 in a cell-cycle-related process in G1 phase. This timing of Hpz1 expression is consistent with a function in the early phases of DNA replication, since the formation of the PreRCs at the chromosomal origins starts in late M with origin binding [40] and ends in G1 with the loading of the MCM complex [32]. The timing and extent of origin binding, PreRC assembly and origin firing are closely connected [40]. We show here that the PreRCs were assembled earlier when Hpz1 was absent and, furthermore, that DNA replication restart occurred earlier without Hpz1. In the latter case the early replication origins were already initiated, allowing the MCMs to travel some distance before the forks were arrested. This may suggest a function of Hpz1 after the initiation step. However, up to one-third of the origins have not fired in an HU block [41,42] and a possible negative effect of Hpz1 on the firing of late replication origins could affect the kinetics of DNA replication restart. We speculate that the reason for the early resumption of DNA replication in HU-treated *hpz1*Δ mutants is the same as for *cdc10*-synchronized cells: early initiation of replication forks. Alternatively, it is possible that some quality of the MCM complex loaded in G1 phase is different in the presence versus absence of Hpz1, a difference that can affect both the initiation kinetics and the properties of the replication forks.

We conclude that Hpz1 is a novel modulator of the G1-S transition by negatively regulating the initiation of DNA replication.

Materials and Methods

Bioinformatical Methods

The protein seuquence of Rad3 from *S. pombe* was used as a query in a standard protein BLAST against the non-redundant protein sequences from fungi (taxid:4751). Results were analyzed using MyHits Motif scan (http://myhits.isb-sib.ch/). Multiple-sequence alignments were performed using ClustalW [43] with default options. The bias towards negatively charged amino acids was determined using ProBias [44,45].

Yeast Strains, Cell Handling, Staining and Strain Construction

All strains used in this study (Table S1) were derivatives of *Schisosaccharomyces pombe* L972 h-. Media and conditions were as described previously [46]. The cells were grown exponentially in Edinburgh minimal medium to a density of $2–4\times10^6$/ml (OD_{595} nm of 0.1–0.2). Synchrony of cells in G1 or G2 phase was obtained by incubating temperature-sensitive mutants (*cdc10-M17* [47] or *cdc25–22* [48], respectively) at 36°C for 4 hours before they were released into the cell cycle at 25°C. UVC irradiation (254 nm) was performed as described previously [49]. *Hpz1:HA* and *hpz1:GFP*, were constructed using the PCR-mediated gene targeting method for fission yeast [50]. Flow cytometry was performed as described previously [31,51] using Sytox Green to stain DNA. Aniline Blue and DAPI (4',6-diamidino-2-phenylindole) were used to stain the septa and nuclei of cells, respectively [46,52].

Cell Survival Assays

The spot test for survival after UVC irradiation was performed by spotting 5 µl of threefold serially diluted cultures (starting $OD_{595} = 0.5$) on yeast extract agar (YEA) plates. The plates were either untreated or irradiated with UVC doses of 50J/m^2 or 300J/m^2. A checkpoint defective mutant (*rad26*Δ) was included as a UVC-sensitive control strain.

Cell survival assays after UVC irradiation in different cell-cycle phases were performed as described previously [33]. For irradiation in G2 phase, cells were irradiated 2 hours after release from a *cdc10* block.

The cell survival assay after HU treatment was performed by incubating exponentially growing cells in 15 mM HU for 4 hours before plating onto YEA plates. Untreated cells were plated as a control.

Immunoprecipitation

Cells were harvested by centrifugation (3000 *g*) for 5 min at 4°C and washed with STOP buffer (1× PBS, 50 mM NaF, 1 mM NaN3). The pellet was frozen in liquid nitrogen. Total cell extracts were made by adding 250µl glass beads and 200µl cold immunoprecipitation buffer (IPB) (25 mM Tris pH 7.5, 0.1 M NaCl, 10% glycerol, 0.5% NP40, 15 mM $MgCl_2$, 15 mM EDTA, 60 mM glycerophosphate, 15 mM ρ-nitrophenylphosphate, 0.5 mM DTT, 1× Complete Protease Inhibitor (Roche), 1 mM Na-orthovanadate, 0.1 mM NaF) before the cells were broken using a Fast Prep (FP120, Bio 101, Thermo Electron Cooperation) for 7×20 sec at a setting of 6.5. After breakage, additional IPB (600µl) was added, cell debris pelleted and the extract cleaned by an additional centrifugation step for 15 minutes at 4°C. For immunoprecipitation 1.5 mg protein from the supernatant fraction was used. Hpz1-HA was immunoprecipitated from total cell extract with MAb 16B12 α-HA (mouse HA.11, Covance) bound to Protein G-coated Dynabeads (Dynal).

Immunoblots

Total cell extracts for immunoblots were made by TCA protein extraction [53]. Antibodies used in this study were α-HA (1:1000, Covance Mab 16B12), α-PSTAIRE, recognizing a motif in Cdc2 (1:2000, Santa Cruz Biotechnology sc-53), α-c-myc (1:1000, BD Pharmingen). Appropriate ECL kits from Amersham Biosciences were used for detection.

Supporting Information

Figure S1 Survival of *hpz1*Δ cells after UVC irradiation. Survival (with standard errors from three experiments shown), of wild-type or *hpz1*Δ cells, after UVC-irradiation in G1, S or G2 phase.
(TIF)

Figure S2 Survival of *hpz1*Δ cells after ionizing radiation. Threefold serially diluted cultures of the indicated strains were spotted on yeast extract agar (YEA) plates. The plates were either untreated or irradiated with 300 Gy. A checkpoint defective mutant (*rad26*Δ) was included as a radiation-sensitive control strain.
(JPG)

Figure S3 Co-immunoprecipitation of Rad3 with Hpz1. Immunoblot showing Rad3-myc co-immunoprecipitated with Hpz1-HA. A total cell extract from G1-synchronized cells was used as a positive control for Rad3-myc presence (+). Beads without antibody was incubated with a cell extract from G1 cells to serve as a control for exclude Rad3-myc binding to the beads only (BO).
(TIF)

Figure S4 The cell cycle of wild-type and *hpz1*Δ cells. Percentage of wild-type or *hpz1*Δ in the different cell-cycle phases S, G2, or M-G1 in an exponentially growing culture.
(TIF)

Figure S5 PCB boxes in the promoter region of *hpz1*. A schematic display of the localization of putative PCB boxes (green) in the promoter region of *hpz1* relative to its transcription start point and the open reading frame.
(TIF)

Table S1 Strains used in this study.
(DOCX)

Author Contributions

Conceived and designed the experiments: BG. Performed the experiments: CAB JHJK. Analyzed the data: CAB JHJK. Wrote the paper: CAB EB BG.

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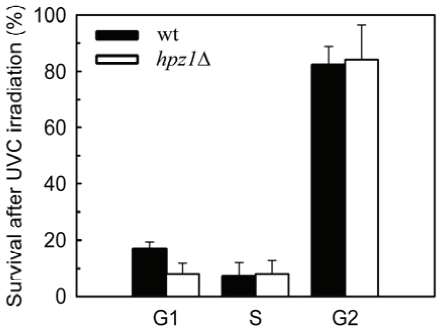
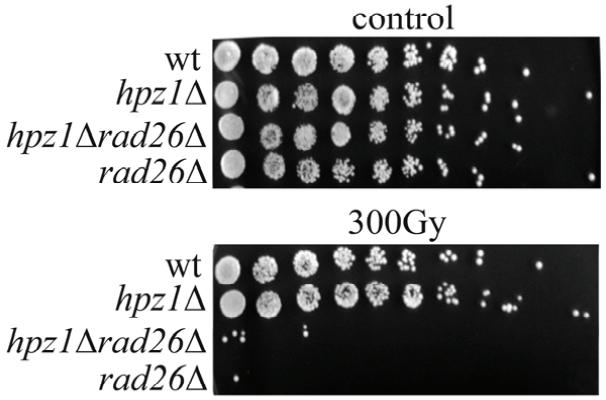
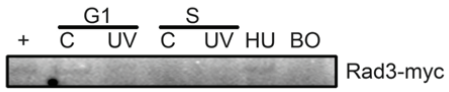
Supplementary Figure 1



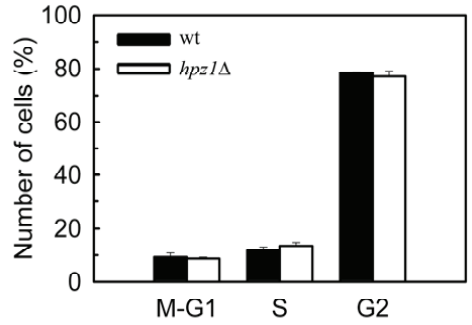
Supplementary Figure 2



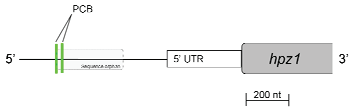
Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5



Supporting Table S1. Strains used in this study

Strain	Genotype	Source
489	cdc10-M17	P. Nurse
1340	hpz1::kanMX4 ura4-D18 h-	Bioneer [54]
1337	cdc10-M17 hpz1::kanMX4 ura4-D18 h+	This work
996	rad26::ura4+ ade1-D25 ura4-D18 h+	Lab collection
1417	cdc10-M17 rad3:myc hpz1:3HA:kanR ade6-704 ura4-D18	This work
983	cdc10-M17 cdc21:GFP h-	Lab collection
1448	hpz1::kanMX4 cdc10-M17 cdc21:GFP:ura4+ ura4-D18	This work
1375	hpz1:GFP:clonnatMX6 cdc10-M17 h-	This work
1418	cdc25-22 hpz1:HA:kanR	This work
1379	cdc10-M17 hpz1:3HA:kanMX6 h-	This work